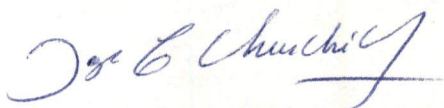


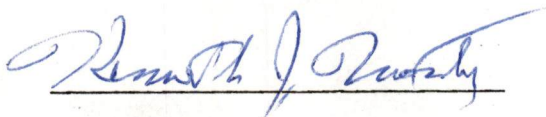
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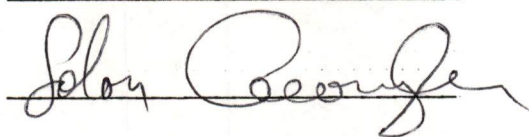


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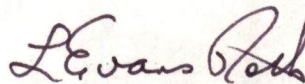
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TWO DEHYDROGENASES: SUCCINIC SEMIALDEHYDE
DEHYDROGENASE AND RETINOL DEHYDROGENASE

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

William Stephen Blaner
December 1979

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ABSTRACT

This work reports studies on two dehydrogenases from mammalian sources. Porcine succinic semialdehyde dehydrogenase was purified to homogeneity from brain while retinol dehydrogenase was partially purified from bovine retina. The purification and characterization of these two enzymes are discussed in detail in this dissertation.

The enzyme succinic semialdehyde dehydrogenase from pig brain was purified 2400-fold by a combination of DEAE-cellulose, hydroxyapatite and AMP-sepharose chromatography. This preparation had a molecular weight of 160,000 and a specific activity of $5.3 \mu\text{mole/min mg}$ at 25°C . The enzyme was shown to be composed of four identical subunits of 41,000 molecular weight, by SDS-polyacrylamide gel electrophoresis and 8M urea isoelectric focusing polyacrylamide gel electrophoresis.

The kinetic properties of this enzyme were examined in detail. The enzyme was found to be irreversible and NAD^+ specific. The K_m 's for succinic semialdehyde and NAD^+ were $15 \mu\text{M}$ and $310 \mu\text{M}$ respectively. Succinic semialdehyde was also found to be a substrate inhibitor with an inhibition constant of $90 \mu\text{M}$. The five carbon semialdehyde, glutaric semialdehyde, was also found to be both a substrate and a substrate inhibitor for succinic semialdehyde dehydrogenase with a Michaelis constant of $3.4 \mu\text{M}$ and an inhibition constant of 3.1mM . The three carbon malonic semialdehyde was neither a substrate nor an inhibitor. With the use of the product inhibitor NADH and dead end inhibitors p-hydroxybenzaldehyde and m-hydroxybenzaldehyde, succinic semialdehyde

dehydrogenase was shown to function via a rapid equilibrium random bi bi mechanism.

Studies of the coupled assay system of 4-aminobutyric acid transaminase and succinic semialdehyde dehydrogenase indicate the two enzymes can function as a valid assay system if the dehydrogenase is in a five fold molar excess of the transaminase. A transient kinetic study of the lag time of the coupled assay as a function of dehydrogenase concentration indicates these two metabolically connected enzymes do not physically interact in solution even with dehydrogenase concentrations as high as $0.6\mu\text{M}$.

The inhibition of succinic semialdehyde dehydrogenase by carbonyl compounds such as P-pyridoxal and o-phthalaldehyde was investigated in detail. The enzyme is reversibly inhibited by preincubation with P-pyridoxal (300:1 mixing molar ratio) at either 25° or 37°C . Reduction with NaBH_4 results in the incorporation of approximately 4 mole of P-pyridoxal residues per mole of enzyme. NAD^+ protects the enzyme against inactivation by P-pyridoxal, whereas the substrate succinic semialdehyde failed to prevent the reaction of P-pyridoxal with lysine residues of the protein. The binding of approximately 10 moles of o-phthalaldehyde per mole of enzyme results in irreversible loss of catalytic activity. The reaction was fast and easily monitored by absorption and fluorescence spectroscopy.

The binding of NADH to succinic semialdehyde dehydrogenase was examined using the fluorescent properties of NADH. The emission spectrum of NADH in the presence of enzyme is found to be enhanced and blue

shifted from that of free NADH indicating the formation of a binary enzyme-NADH complex. Addition of succinic semialdehyde (10^{-4}M) to this binary complex brings about further fluorescence enhancement to the binary complex but no additional spectral shift was observed. Addition of succinate (10^{-3}M) to the binary enzyme-NADH complex had no effect upon the complexes emission.

A titration of the emission anisotropy of NADH upon addition to succinic semialdehyde dehydrogenase indicates two moles of NADH bind per mole of enzyme with an affinity of $4\mu\text{M}$.

A titration of enhancement of NADH fluorescence upon addition of NADH to the enzyme also indicates two NADH binding sites with a dissociation constant of $4\mu\text{M}$, exist per mole of enzyme. Initial velocity studies demonstrate NADH is a competitive inhibitor of NAD^+ with an inhibition constant of $1.5 \times 10^{-4}\text{M}$. These observations coupled with the subunit composition and inability to detect any association-dissociation phenomena suggests succinic semialdehyde dehydrogenase displays extreme negative cooperativity with respect to NADH.

Retinol dehydrogenase was purified 25 fold from the rod outer segments of bovine retinas by a combination of hydroxyapatite and retinyl sepharose chromatography. The enzyme was found to be reversible with an equilibrium constant of $1.1 \times 10^7\text{M}^{-1}$. The Michaelis constants for NADP^+ and NADPH were $6\mu\text{M}$ and $4\mu\text{M}$ respectively, while those of all-trans retinol and all-trans retinal were respectively $9\mu\text{M}$ and $4\mu\text{M}$.

The enzyme could be partially inactivated with the carbonyl reagent P-pyridoxal. However, contrary to previously reported results, the enzyme could not be inactivated by addition of NaBH_4 to a mixture of all-trans retinal and partially purified retinol dehydrogenase.

The phospholipid dependence of this enzyme was investigated using phospholipase A_2 and phospholipase C. Phospholipase A_2 could bring about a 75% inactivation of this enzyme within two minutes of addition. Phospholipase C had little effect on enzymatic activity. Addition of mixed phospholipids to an inactivated preparation of dehydrogenase from which the phospholipase had been removed resulted in a partial restoration of activity.

Retinal dehydrogenase could be inactivated by illumination with visible light when exogenous retinal (10^{-5}M) had been added to the enzyme preparation, while no inactivation was observed in the absence of retinal. The possibility that this inactivation was due to the production of singlet oxygen by the photosensitizer retinal was substantiated by the observations that the singlet oxygen quencher azide protected against inactivation while D_2O , which increases the lifetime of singlet oxygen, enhanced inactivation of retinol dehydrogenase.

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PART ONE

SUCCINIC SEMIALDEHYDE DEHYDROGENASE

CHAPTER I

PURIFICATION, SUBSTRATE SPECIFICITIES AND MOLECULAR WEIGHT

Succinic semialdehyde dehydrogenase (succinate-semialdehyde:NAD⁺ oxidoreductase, EC 1.2.1.24) in nervous tissue is of great interest since it catalyzes the oxidation of succinic semialdehyde to succinate. This catalytic event is the third and final step of the so-called "GABA shunt" (γ -aminobutyric acid shunt) of the Krebs cycle. This pathway, as seen in Figure 1, involves the conversion of glutamate into the inhibitory neurotransmitter 4-aminobutyrate by the enzyme glutamate decarboxylase and the subsequent conversion of 4-aminobutyrate into succinic semialdehyde by 4-aminobutyrate transaminase. Succinic semialdehyde dehydrogenase is the least studied enzyme of this shunt. The enzyme has been purified to homogeneity from bacterial sources (1) and more recently has been isolated from mammalian nervous tissue (2,3).

This study is the first purification to homogeneity and examination of porcine brain succinic semialdehyde dehydrogenase.

I. EXPERIMENTAL

Materials

One pound of brain from freshly killed pigs was obtained from East Tennessee Packing Company, Knoxville, Tennessee. The brains were placed on ice immediately after slaughter and all procedures were started within one hour.

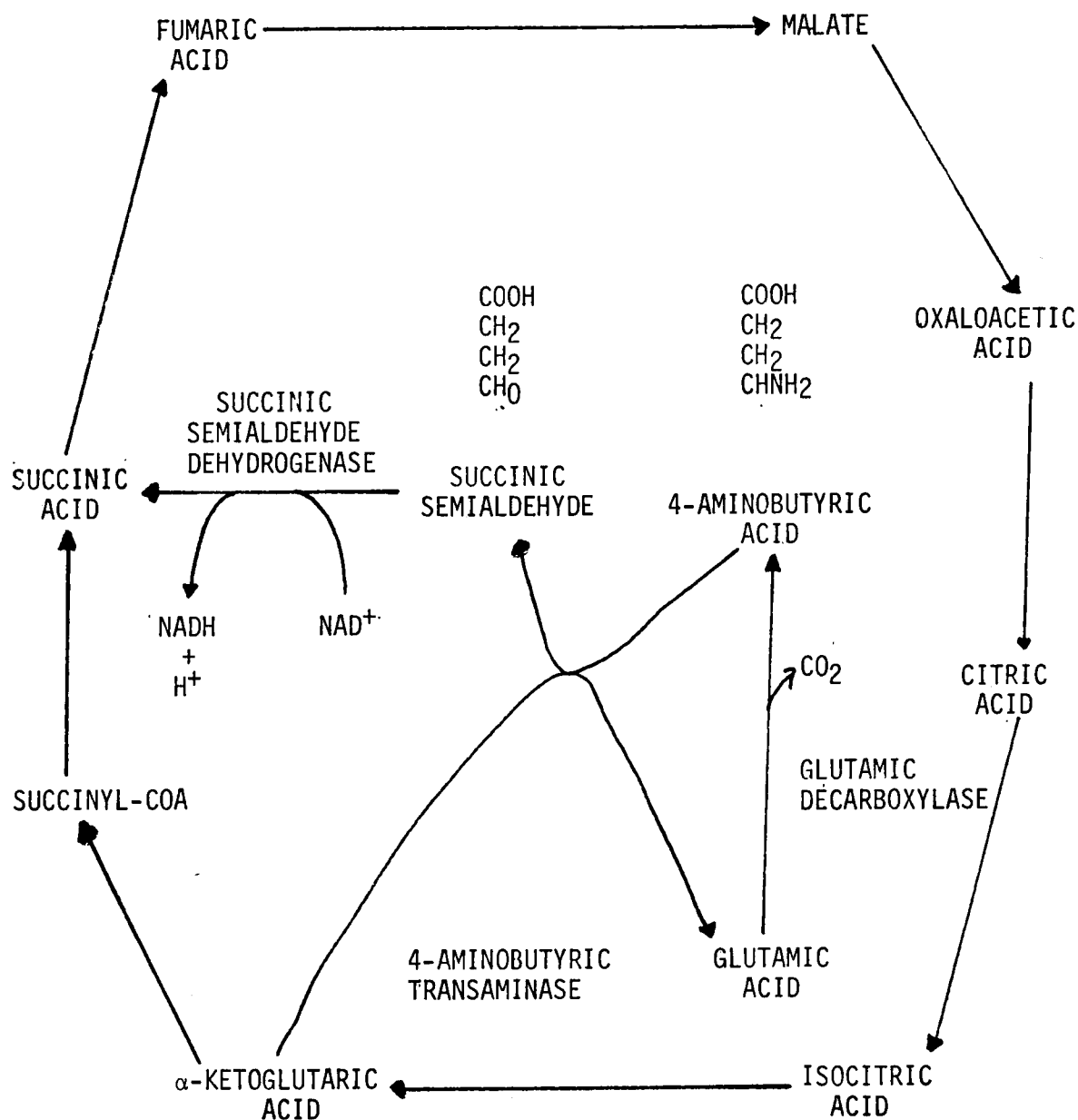


Figure 1. The GABA Shunt

Succinic semialdehyde, 2-mercaptoethanol, EDTA, α -amino adipate, chloramine T and NAD^+ were purchased from Sigma Chemical Company, St. Louis, Missouri. Sephacryl S-200 and AMP-Sepharose were purchased from Pharmacia, Piscataway, New Jersey. Bio-Lyte 3/10 ampholyte was purchased from Bio-Rad Inc. DEAE-cellulose and all other chemicals of reagent grade or better were purchased from Fisher Scientific Company, Atlanta, Georgia.

Methods

Assay of succinic semialdehyde dehydrogenase. The enzyme was assayed spectrophotometrically by following the increase in absorbance at 340nm due to NADH formation at 25°C. A Cary 15 or an Aminco DW-2 recording spectrophotometer was used for all assays. Standard assay mixtures consisted of 0.7ml 0.1M pyrophosphate buffer (pH 8.4), 0.1ml 50mM NAD^+ , 0.1ml 0.5mM succinic semialdehyde and 0.1ml of enzyme preparation.

Purification of succinic semialdehyde dehydrogenase. A Waring Blendor was used to prepare a 25% (w/v) whole brain homogenate in a solution of 2mM phosphate (pH 7.2), containing 1mM 2-mercaptoethanol and 0.5mM EDTA (Buffer I). The homogenate was centrifuged at 10,000 x g for 30 minutes. The precipitate obtained at 30 to 70% saturation was dissolved in Buffer I and dialyzed overnight against several changes of Buffer I.

The preparation was then applied to a column (35 x 2.6cm) of DEAE-cellulose equilibrated with the Buffer I. The column was eluted

with Buffer I; the enzyme is not retained by DEAE-cellulose, whereas contaminating protein remained attached to the supporting material. The active fractions were combined and dialyzed overnight against Buffer I.

Hydroxyapatite was prepared exactly as described by Levin (4) prior to each purification attempt. The enzyme was applied to a column (25 x 2.6cm) of hydroxyapatite equilibrated with Buffer I. The enzyme was eluted using a linear gradient made with the equilibration buffer (250ml) and the same volume of 150mM potassium phosphate (pH 7.2) containing 1mM 2-mercaptoethanol and 0.5mM EDTA.

After hydroxyapatite chromatography, the enzyme was applied to a column (10 x 1cm) of AMP-sepharose equilibrated with Buffer I. The column was then washed with Buffer I followed by a wash with Buffer I containing 100mM KCl. The enzyme was eluted by using increasing concentrations of AMP. A sharp peak containing succinic semialdehyde dehydrogenase was eluted from the AMP-sepharose at a concentration of AMP of approximately 10mM.

Storage of succinic semialdehyde dehydrogenase. The purified enzyme was kept in Buffer I containing 20% (v/v) glycerol at -12°C. Under these conditions, the enzyme remains stable for at least one week without appreciable loss of activity.

Preparation of glutaric semialdehyde. Glutaric semialdehyde was prepared by the procedure of Chang and Adams (5). Two millimoles of D-L α -amino adipate were dissolved in 20ml 0.1N NaOH in a 50ml graduated cylinder. This mixture was placed in a waterbath at 50°C and

bubbled with N_2 vigorously. After ten minutes of bubbling two millimoles of chloramine-T were added and bubbling with N_2 was allowed to continue for fifteen additional minutes. The mixture was then cooled in an ice bucket for one hour. The crystalline material which forms upon cooling is removed by filtration through Whatman No. 1 filter paper. The crystals were washed with ice cold water and the resulting mixture was brought to pH 3.0 with 3.0N HCl.

The newly formed glutaric semialdehyde was then separated from unreacted α -amino-adipic acid by chromatography on Dowex 50-X-8 equilibrated at pH 6.0. The Dowex 50-X-8 had previously been washed with 1.0l 3.0N HCl, 1.0l 3.5N NaOH, 1.0l 3.0N HCl and enough distilled water to bring the pH of the eluent to 6.0. The glutaric semialdehyde was not retained by the Dowex 50-X-8.

The glutaric semialdehyde, when spotted onto filter paper, contained no ninhydrin reactive materials. The 2,4 dinitro-phenylhydrazone derivative of glutaric semialdehyde migrated as one spot on Silica gel thin layer chromatography plates developed for ten hours in isopropanol:H₂O:NH₃ (120:20:10).

The concentration of glutaric semialdehyde was determined by allowing complete oxidation of this substance by succinic semialdehyde dehydrogenase and measuring the NADH formed after completion of the reaction.

Sucrose density gradient centrifugation. Cellulose nitrate centrifuge tubes containing a linear 5-20% sucrose gradient in Buffer I were prepared according to the procedure of Martin and Ames (6).

These gradients were allowed to stand for five hours at 4°C before use. Samples of 0.2ml were applied to the top of each gradient. These samples consisted either of a mixture of marker enzymes (beef heart catalase, beef liver glutamic dehydrogenase, beef heart malate dehydrogenase and egg lysozyme) or of the purified succinic semialdehyde dehydrogenase. The sucrose gradients were centrifuged in a Beckman SW-39 swinging bucket rotor at 37,000 RPM for 16 hours. After centrifugation 0.15ml samples were collected by puncturing the centrifuge tubes from the bottom. These fractions were assayed for either the marker enzymes or succinic semialdehyde dehydrogenase.

Native polyacrylamide gel electrophoresis. The purity of the enzyme preparation was judged by electrophoresis on 7.5% polyacrylamide tube gels. The gels were prepared and run according to the procedure of Davis (7) in 0.01M Tris-glycine buffer (pH 8.3). A current of 4ma per tube was applied for four hours.

Polyacrylamide gradient gels of 4-27% polyacrylamide were purchased from Isolabs, Inc., Canton, Ohio. These gels were run identically to the 7.5% gels previously described except the electrophoresis was allowed to continue for 24 hours. A 0.2% solution of bromophenyl blue was used as tracking dye.

The electrophoresed gels were stained for one hour in a 0.2% solution of Coomassie Brilliant Blue R250 in a 45% (v/v) methanol, 9% (v/v) acetic acid aqueous solution. Destaining was accomplished by diffusion in an aqueous solution containing 10% (v/v) methanol and 7% (v/v) acetic acid.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis. Reducing sodium dodecyl sulfate polyacrylamide gels were prepared according to the procedure of Weber and Osborn (8). Gels containing 7.5% polyacrylamide, 1% 2-mercaptoethanol and 0.1% sodium dodecyl sulfate were electrophoresed for five hours at 8ma per tube. Samples were in 0.01M phosphate (pH 7.2) containing 1% sodium dodecyl sulfate and 1% 2-mercaptoethanol. Molecular weight standards used include bovine serum albumin (MW = 67,000), ovalbumin (MW = 43,000), pepsin (MW = 35,000) and lysozyme (MW = 14,700). A 0.2% solution of bromophenol blue was used as tracking dye.

Staining of the electrophoresed samples was as described in the previous section for native gel staining.

8M urea isoelectric focusing gels. Seven and one half percent polyacrylamide gels were prepared in an aqueous 8M urea and 1% 2-mercaptoethanol solution containing 0.4% ampholyte. Enzyme samples were dialyzed against an 8M urea and 1% 2-mercaptoethanol solution containing 0.4% ampholyte. Samples containing 100 μ g of protein were applied to five gels and electrophoresed for 24 hours at 4°C across a potential difference of 200 volts. The upper electrophoresis chamber contained 0.06N H₂SO₄ and the lower one 0.04N NaOH and 0.02N Ca(OH)₂.

After electrophoresing one gel was checked for pH gradient formation using a BioRad Inc. pH-rofiler. The remaining gels were stained for six hours in 0.2% bromophenol blue made up in isopropanol:acetic acid:H₂O (90:30:30). De-staining in an isopropanol:acetic acid:H₂O

(10:10:80) required from 48 to 72 hours for all dye to be removed from the gels. The stained gels were immediately photographed before the protein stain could fade.

Gel exclusion chromatography. The molecular exclusion gel Sephacryl S-200 was used to determine the approximate size of succinic semialdehyde dehydrogenase. A column (57 x 0.1cm) was equilibrated with Buffer I. The void volume of this column was determined with Blue Dextran 2000. The marker enzymes used to determine the size of succinic semialdehyde dehydrogenase were beef heart catalase, yeast alcohol dehydrogenase, beef heart malate dehydrogenase and rabbit muscle lactate dehydrogenase. A sample of 0.5ml was applied to the column and eluted with Buffer I. Fractions containing 1.0ml of eluent were collected and assayed for enzymatic activity.

Protein assay. Protein was determined by the method of Lowry (9). A 1.0% solution of purified succinic semialdehyde dehydrogenase was found to have an absorbance at 280nm of 7.3 in a 1.0cm cuvette.

II. RESULTS

Purification of Succinic Semialdehyde Dehydrogenase

The purification scheme for succinic semialdehyde dehydrogenase is given in Table I. The enzyme is not retained by the DEAE-cellulose column and passes straight through this column whereas many contaminating proteins are retained by this ion exchange resin. The elution profile from the hydroxyapatite chromatography is given in Figure 2.

TABLE I
PURIFICATION SCHEME OF SUCCINIC SEMIALDEHYDE DEHYDROGENASE

The purification of succinic semialdehyde dehydrogenase from 400g of pig brain tissue, wet weight.

Treatment	Volume ml	Protein mg/ml	Specific Activity at 25°C units/mg	Yield %
Homogenate	2000	22	0.0022	100
Supernatant	1350	15	0.0037	81
Dialysed, 30-70% (NH ₄) ₂ SO ₄ fraction	27	11	0.051	77
DEAE-cellulose	37	2.8	0.12	12
Hydroxyapatite	20	1.2	0.50	11
AMP-Sepharose	2.2	0.3	5.3	1.7

One unit of enzymatic activity is defined as that amount of enzyme which produces 1 μ mole/min of NADH at 25°C.

Figure 2. Hydroxyapatite Chromatography Elution Profile.

Each fraction contained 10ml. The succinic semialdehyde dehydrogenase activity is given in μ moles of NADH formed per hour (----). The ionic strength of the elution buffer is given in terms of millimolar phosphate (~~0-0-0~~).

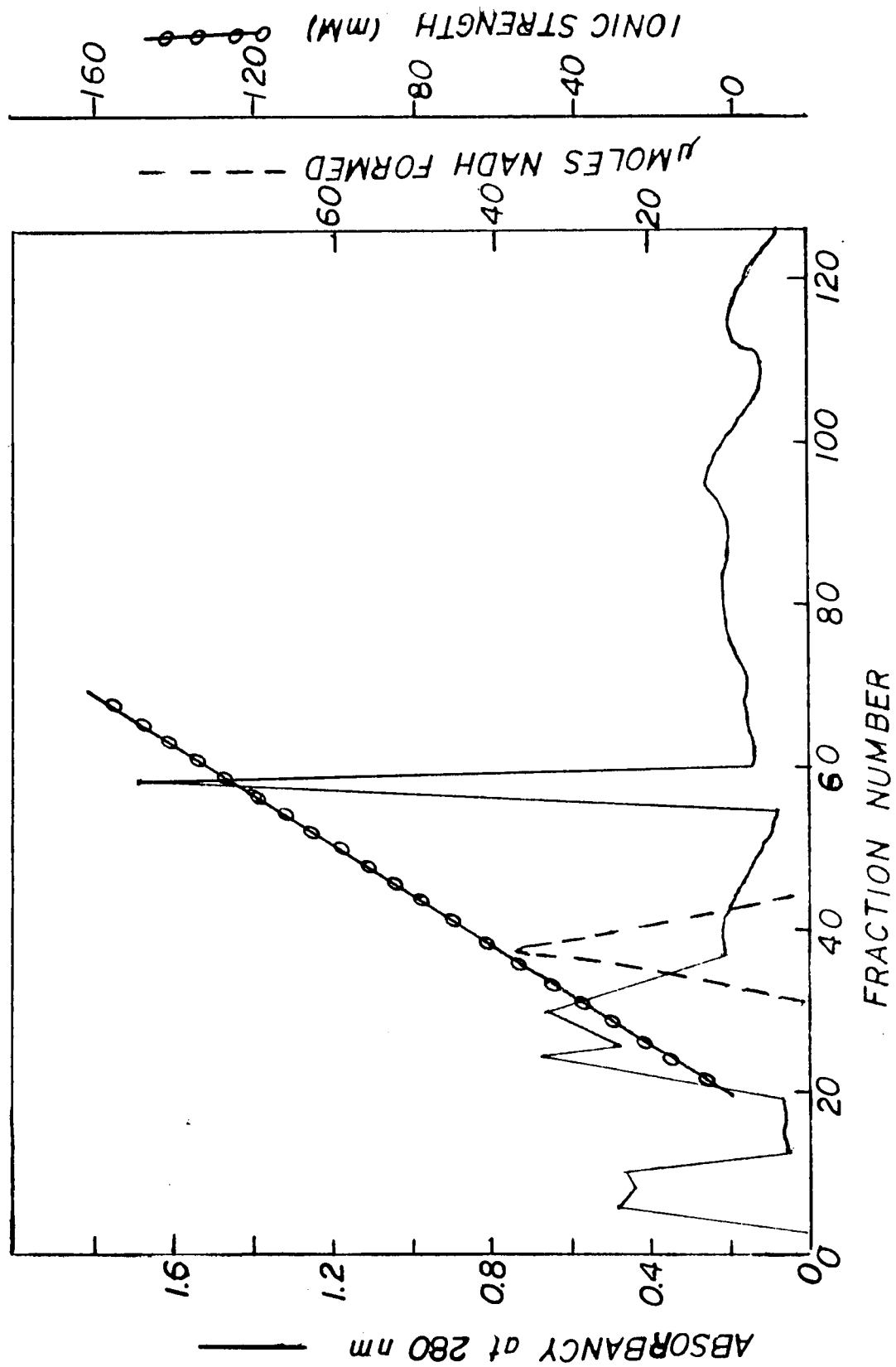


Figure 2

The enzyme is retained on this column and is eluted with 70mM phosphate. After dialysis, the enzyme is applied to an AMP-Sepharose affinity column and is eluted by AMP with an elution profile as shown in Figure 3. The enzyme may be eluted from the AMP-Sepharose column with either 10mM NAD^+ or 0.7M KCl as an alternative to the 10mM AMP. After these purification steps, the enzyme has undergone a purification of approximately 2400 fold, with a recovery of 1.7%; and has a specific activity of 5.3 $\mu\text{moles/min mg}$.

Polyacrylamide Gel Electrophoresis

The enzyme eluted from the AMP-sepharose column migrated as one protein band on native polyacrylamide gels. On the native polyacrylamide gradient tells the enzyme migrates as a single species with a molecular weight of 160,000.

On sodium dodecyl sulfate polyacrylamide gels, the enzyme migrates as a single protein species as can be seen in Figure 4. When the single protein band observed for succinic semialdehyde dehydrogenase was compared with known protein standards in Figure 5, a molecular weight of 41,000 was estimated for this species.

The enzyme also behaved as a single protein species on 8M urea reducing isoelectricfocusing polyacrylamide gels (Figure 6). The protein stopped migrating at its isoelectric pH of 7.1.

Sucrose Density Gradient Centrifugation

Succinic semialdehyde dehydrogenase migrated as a single protein and activity band through a 5-20% sucrose gradient. When compared with

Figure 3. AMP-Sepharose Chromatography Elution Profile

Each fraction contained 1.0ml. The succinic semialdehyde dehydrogenase activity is given in μ moles of NADH formed per hour (----). The concentration of the AMP in the eluting buffer is given in molar terms.

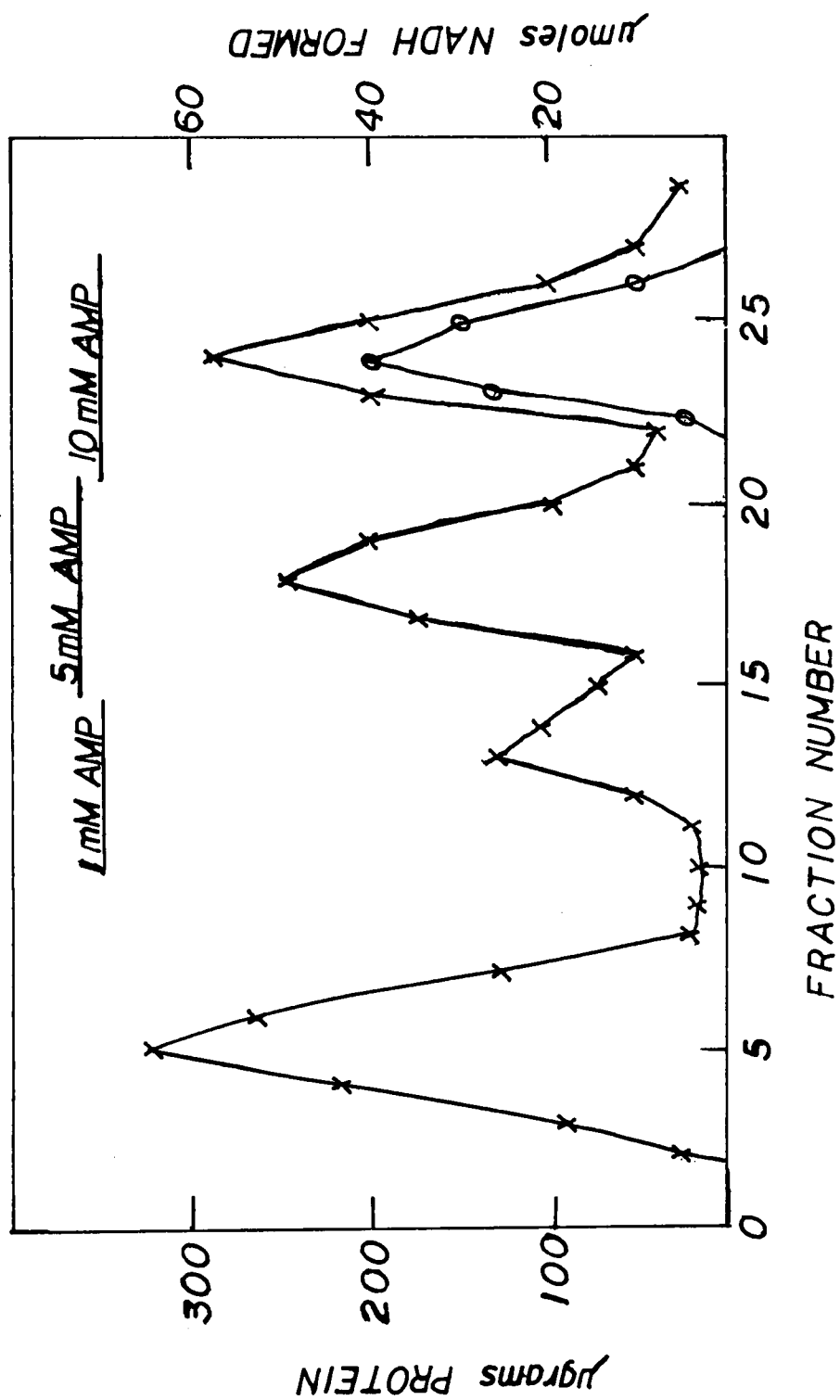


Figure 3

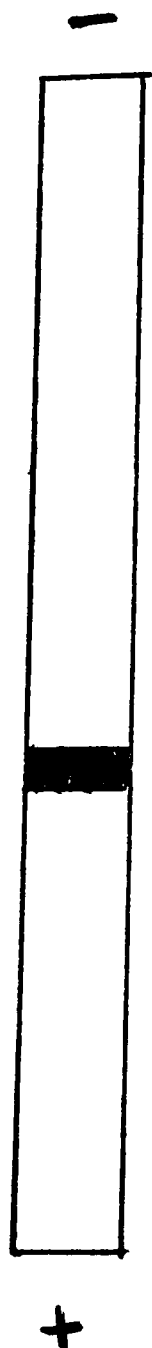


Figure 4. Sodium Dodecyl Sulfate Electrophoresis Gels of Succinic Semialdehyde Dehydrogenase.

Figure 5. Subunit Molecular Weight Determination from Sodium Dodecyl Sulfate Electrophoresis Gels.

The following proteins were used as standards: (1) bovine serum albumin, (2) ovalbumin, (3) pepsin and (4) lysozyme. The relative mobility of succinic semialdehyde dehydrogenase corresponds to a molecular weight of 41,000.

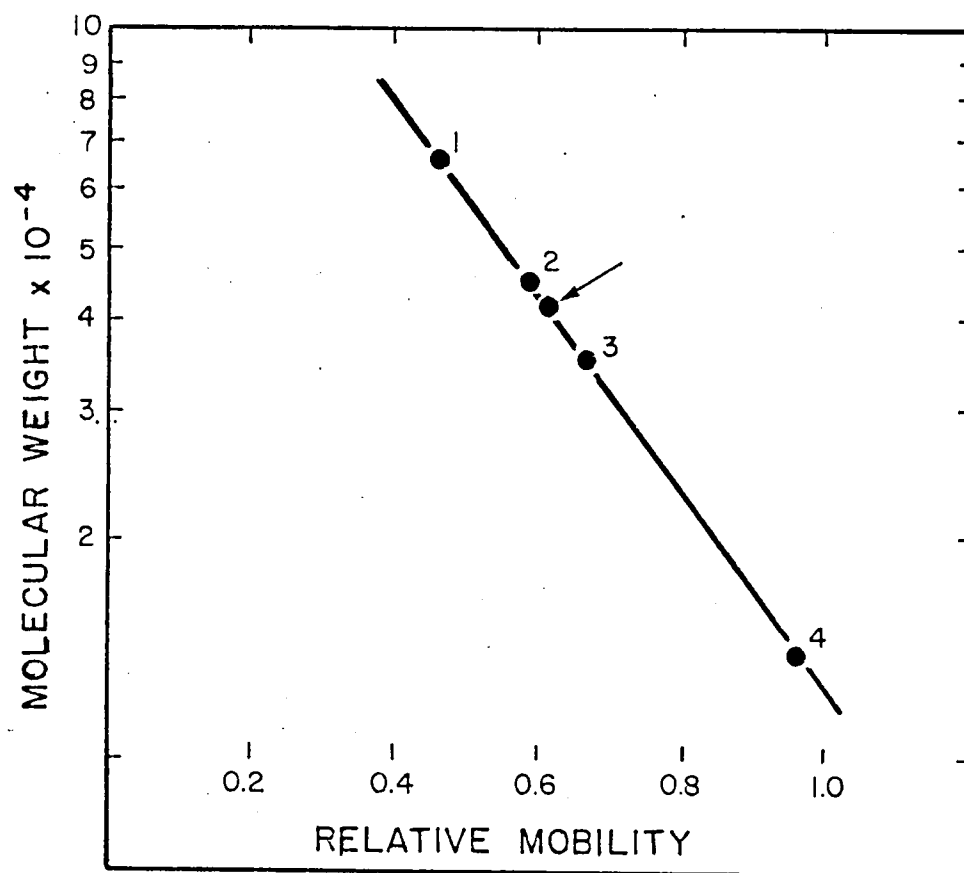
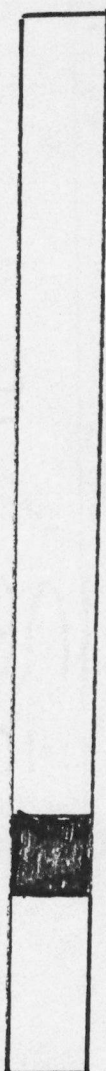


Figure 5

pH 4.0



pH 9.0

Figure 6. Isoelectricfocusing Electrophoresis Gels of Succinic Semialdehyde Dehydrogenase.

standards of known molecular weight as in Figure 7, it was estimated that succinic semialdehyde dehydrogenase had a molecular weight of 166,000.

Gel Exclusion Chromatography

The elution pattern from Sephacryl S-200 of succinic semialdehyde dehydrogenase and several standard enzymes of known molecular size is given in Figure 8. The enzymes migrated as a single protein and activity peak. The molecular weight as determined in Figure 9 of succinic semialdehyde dehydrogenase from this gel exclusion chromatography was 148,000.

Substrate Specificities

Succinic semialdehyde dehydrogenase was found to be NAD^+ specific. Additionally, this enzyme was unable to reduce succinate to succinic semialdehyde or succinic semialdehyde to 4-hydroxybutyrate.

Commercial succinic semialdehyde and synthesized glutaric semialdehyde were both found to be good substrates for this dehydrogenase. Malonic semialdehyde synthesized enzymatically by 4-aminobutyrate transaminase from β -alanine was not a substrate for succinic semialdehyde dehydrogenase. Acetaldehyde and glyceraldehyde-3-phosphate were not substrates for this enzyme.

The kinetic constants for these substrates will be presented and discussed in the next chapter.

The enzyme could not catalyze succinic semialdehyde formation in pyrophosphate buffer (pH 8.4) from 0.1mM NADH and 0.1mM succinate.

Figure 7. Molecular Weight Determination by Sucrose Density Gradient
Centrifugation of Succinic Semialdehyde Dehydrogenase.

All enzymatic activities are expressed relative to the fraction containing the most activity which was considered as 100% activity. Each fraction collected contained 0.15ml.

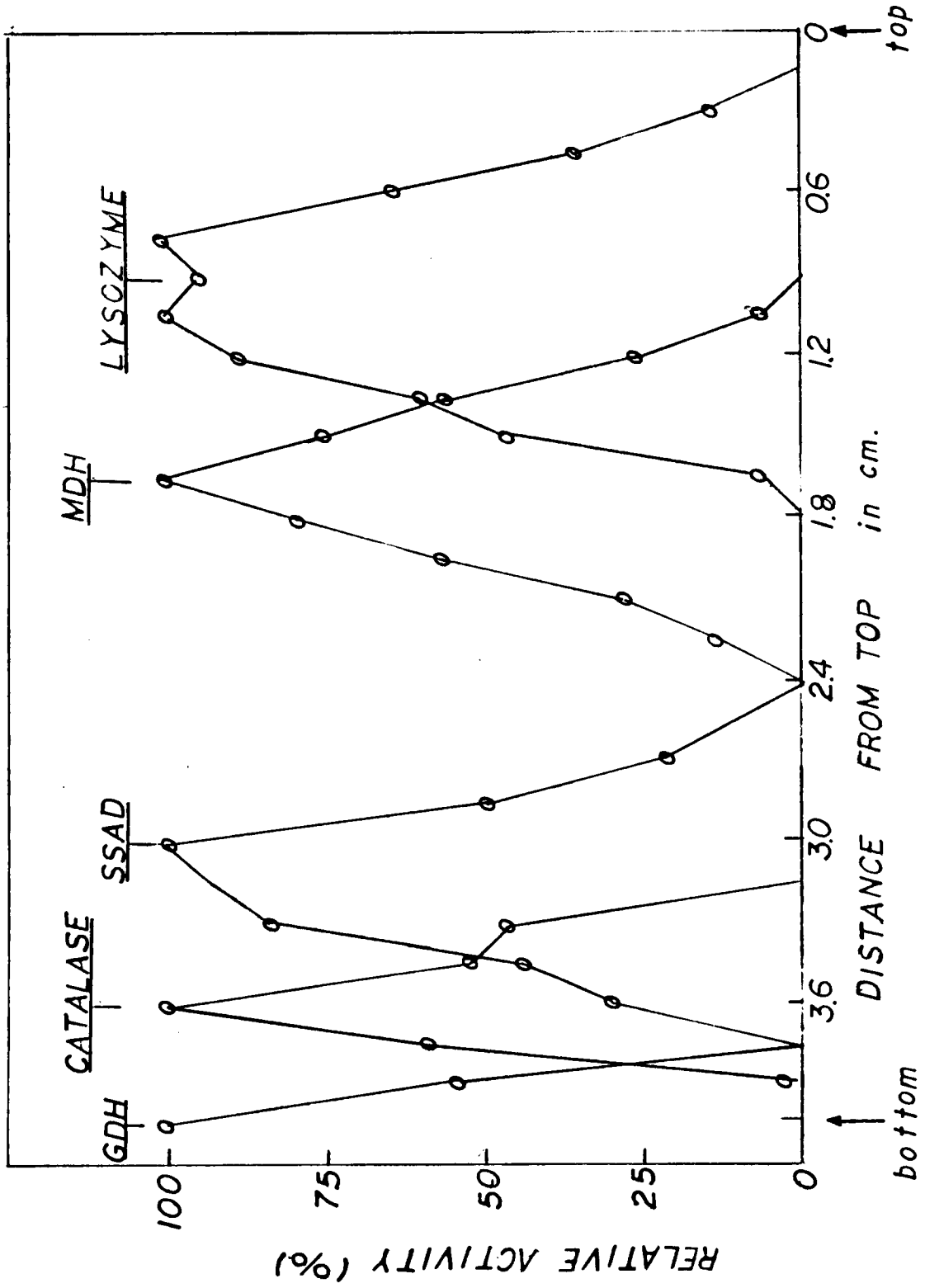


Figure 7

Figure 8. Elution Profile of Succinic Semialdehyde Dehydrogenase and Standards from Sephacryl S-200 Chromatography.

All enzymatic activities are expressed relative to the fraction containing the most activity, which was considered as 100% activity. Each fraction contained 1.0ml and the flow rate of the column was 0.25ml/minute.

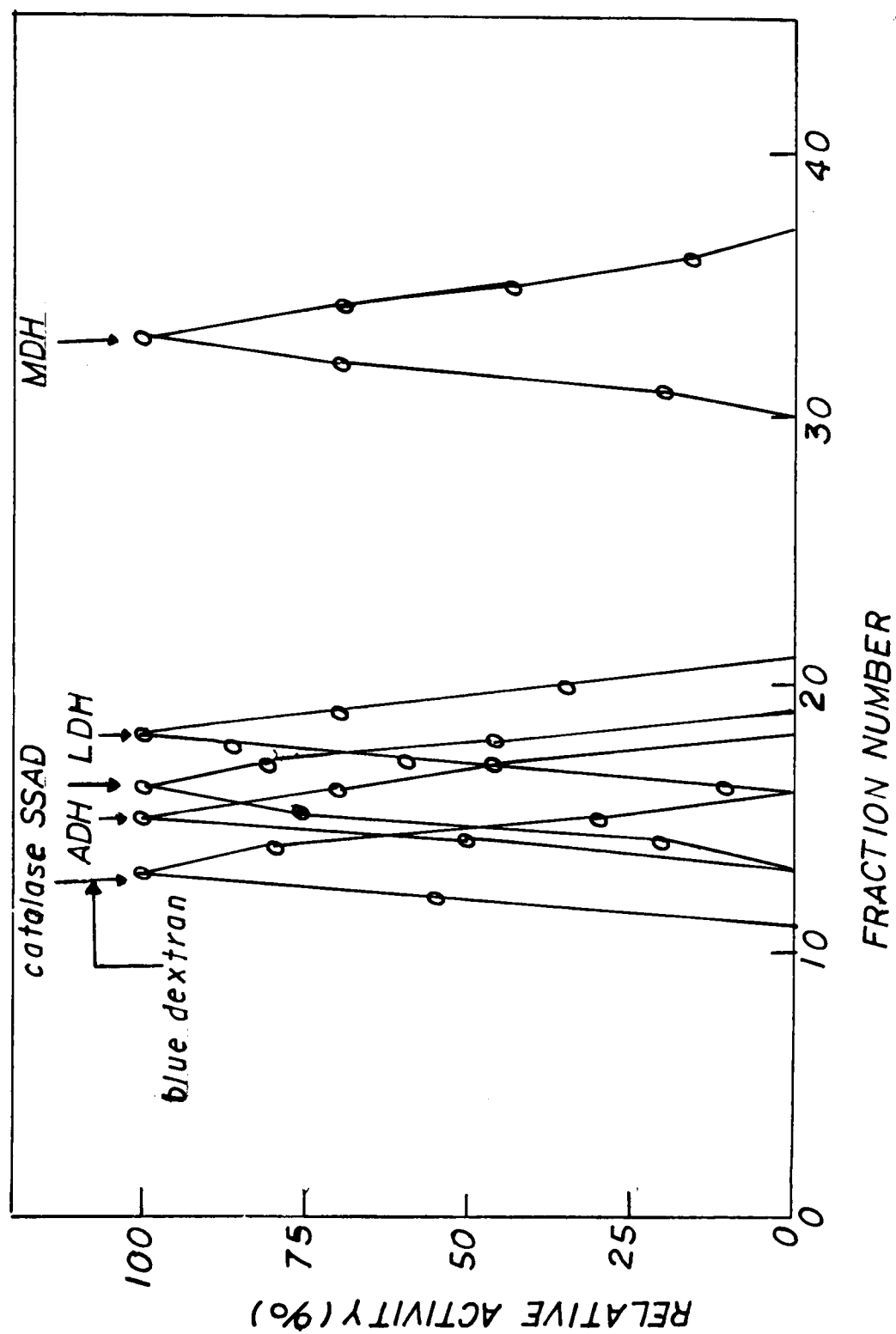


Figure 8

Figure 9. Molecular Weight Determination of Succinic Semialdehyde Dehydrogenase from Sephacryl S-200 Chromatography.

The following proteins were used as standards: (1) yeast alcohol dehydrogenase, (2) rabbit muscle lactate dehydrogenase and (3) pig heart malate dehydrogenase. The elution position of succinic semi-aldehyde dehydrogenase corresponds to a molecular weight of 148,000.

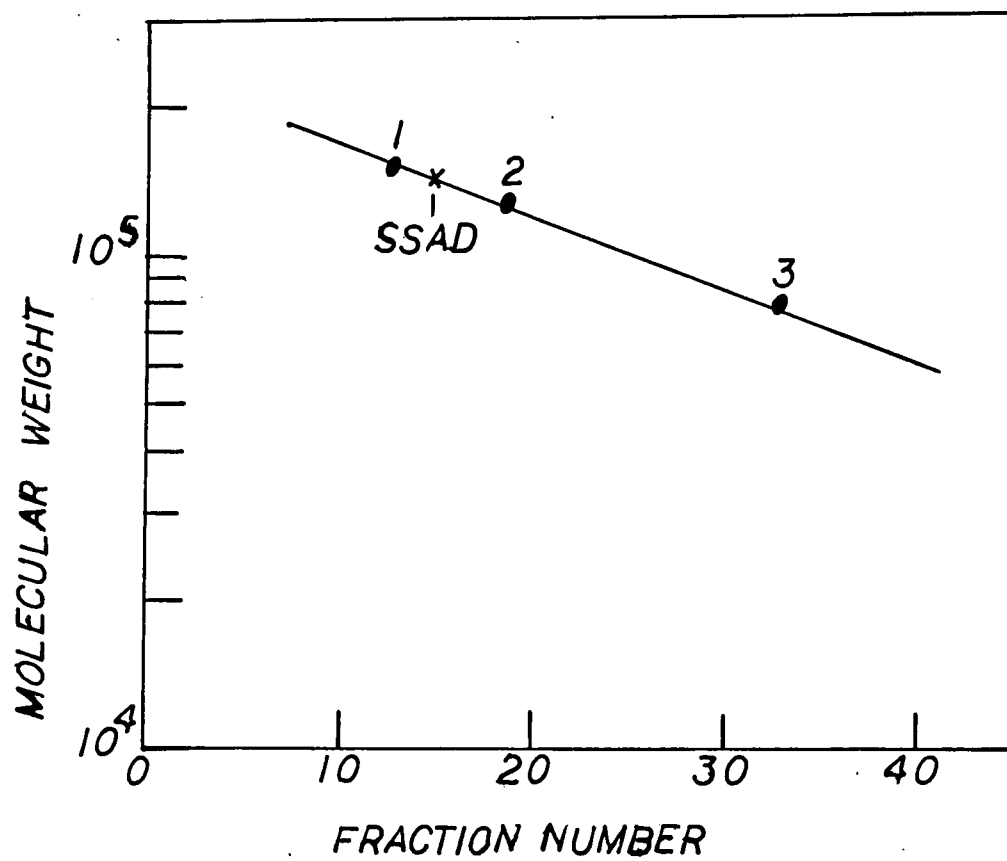


Figure 9

III. DISCUSSION

Succinic semialdehyde dehydrogenase was purified 2400 fold to a specific activity of 5.3 μ moles/min mg. The enzyme was judged to be homogeneous on the basis of native polyacrylamide gel, sodium dodecyl sulfate polyacrylamide gel and 8M urea isoelectricfocusing polyacrylamide gel electrophoresis; and on the observation that the protein migrated as a single species both with respect to protein and activity when chromatographed on Sephacryl S-200 and centrifuged through a sucrose gradient.

The enzyme was found to have a molecular weight of 148,000 by gel exclusion chromatography, 166,000 by sucrose density gradient centrifugation and 160,000 by gradient polyacrylamide gel electrophoresis. The subunit molecular weight was determined to be 41,000 by sodium dodecyl sulfate polyacrylamide gel electrophoresis. These findings indicate succinic semialdehyde dehydrogenase is a tetramer composed of four identical subunits. The 8M urea isoelectricfocusing electrophoresis is consistent with only one type of subunit.

The bacterial enzyme has been reported to be a trimer with a molecular weight of 165,000 (1) while the rat brain enzyme is reported to be a dimer with a molecular weight of 150,000 (2).

Other literature reports indicate the monkey brain succinic semialdehyde dehydrogenase is a tetramer with a subunit molecular weight of 40,000 (11). The findings reported here are consistent with the previously published native molecular weights for this enzyme and in agreement with the report indicating the monkey brain enzyme is a tetramer.

Succinic semialdehyde dehydrogenase was found to have a high degree of substrate specificity. Only the four-carbon succinic semialdehyde and the five-carbon glutaric semialdehyde were able to serve as the aldehyde substrates for this enzyme. Both of these substrates were also found to be substrate inhibitors at substrate concentrations around 0.1mM. This enzymatic reaction was also found to be NAD^+ specific and irreversible. The purified enzyme could not catalyze the conversion of succinic semialdehyde into 4-hydroxybutyrate, contrary to previous suggestions (12,13). The very limited substrate specificity, substrate inhibition and reaction irreversibility may be important in regulating this enzyme. Additionally, the irreversibility of the enzyme catalyzed reaction prevents the build-up of the potentially toxic substances succinic semialdehyde during times at which the citric acid cycle intermediates' concentrations are high.

The finding that glutaric semialdehyde is a substrate for this enzyme is of unknown physiological significance. No role for glutaric semialdehyde or its α -amino precursor, 5-aminovaleric acid, has been reported in mammalian nervous tissue. However, both of these compounds have been used as 4-aminobutyric and succinic semialdehyde analogs in many physiological and pharmacological studies with nervous tissue (14, 15,16). Thus, it seems the roles of glutaric semialdehyde and 5-aminovaleric acid need more study in nervous tissue before conclusions of their importance may be drawn.

CHAPTER II

KINETIC PROPERTIES OF SUCCINIC SEMIALDEHYDE DEHYDROGENASE

The kinetic properties of many dehydrogenases have been studied in great detail (17). These studies on the kinetic properties of dehydrogenases have yielded much information concerning the affinities of various substrates, the effects and properties of various enzymatic inhibitors and, most importantly, the enzymatic reaction mechanism. Kinetic studies on specific enzymes such as horse liver alcohol dehydrogenase have contributed much to the understanding of the actual chemical reaction sequence which occurs when ethanol is converted to acetaldehyde by this enzyme (18). Aldehyde dehydrogenases, a group of enzymes which like succinic semialdehyde dehydrogenase catalyze the oxidation of an aldehyde group to a carboxylic acid group, have been studied from several sources and potentially can serve as a kinetic model for succinic semialdehyde dehydrogenase (19,20).

The simplest kinetic experiment is the determination of the K_m 's for the two substrates of this bimolecular reaction. In the process of determining the Michaelis constants for the two substrates, it is also possible to determine if the reaction mechanism is sequential; in that the two substrates must bind the enzyme before product is released, or if the reaction is a ping-pong reaction where one substrate binds and one product is released prior to the binding of the second substrate.

Using substrate analogs known as dead-end inhibitors (because they bind the enzyme as a substrate would, but are not transformed into product) and the product inhibitor NADH, it is possible to gain additional information about the kinetic mechanism of succinic semialdehyde dehydrogenase. These two types of inhibitors and their inhibitory effects with respect to the various substrates (i.e. competitive, non-competitive or uncompetitive) can be used to determine if a sequential mechanism requires an ordered or random addition of substrates or release of products, or what the order of substrate addition in a ping-pong mechanism is.

Since the semialdehyde substrates of succinic semialdehyde dehydrogenase are also enzyme inhibitors when they are at concentrations of 0.1mM or greater, it is possible to examine this inhibition and demonstrate that this inhibition is consistent with the concept of the substrate being inhibitory because it binds an enzyme molecule already bound to the semialdehyde substrate and prevents the access to the enzyme of the NAD^+ . It is also kinetically possible to determine an inhibition constant, K_I , for the substrate inhibitor and also predict the maximum non-inhibitory concentration of substrate (11).

The coupled assay for 4-aminobutyric acid transaminase which involves this transaminase and succinic semialdehyde dehydrogenase is also a system which may be studied kinetically (21). This coupled assay system is extensively used to study this transaminase (22,23,24) but never has the system been studied using two purified enzymes. With the two purified enzymes, it becomes possible to define the conditions under which the coupled system is valid; additionally, using

the analysis of assay lag times of Hess (25) it is possible to kinetically demonstrate the existence or non-existence of complex formation between these two enzymes.

I. EXPERIMENTAL

Materials

Succinic semialdehyde, NAD^+ , α -ketoglutarate, 4-aminobutyrate, m-hydroxybenzaldehyde, p-hydroxybenzaldehyde, NADH and 2-mercaptoethanol were purchased from Sigma Chemical Co., St. Louis, MO. Purified 4-aminobutyric acid transaminase with a specific activity at 25°C of 6.0 $\mu\text{Moles/min mg}$ was a gift of Mr. Udo Moses. Glutaric semialdehyde was synthesized and purified as described in the previous chapter. Sodium pyrophosphate and all other chemicals used in these experiments were obtained from Fisher Chemical Co., Atlanta, GA.

Methods

All assays were carried out on an Aminco DW-2 or a Cary 15 recording spectrophotometer. The formation of NADH (molar extinction coefficient = $6220 \text{ cm}^{-1}\text{M}^{-1}$) was monitored at 340nm at 25°C for at least 2 minutes. A good straight line was generally obtained. All assay mixtures consisted of 0.7ml 0.1M pyrophosphate buffer (pH 8.4), 0.1ml succinic semialdehyde or variable concentration in pyrophosphate buffer, 0.1ml NAD^+ of variable concentration in pyrophosphate buffer and 0.1ml of enzyme. If an inhibitor was added to the assay mixture, it was added as part of the pyrophosphate buffer.

The coupled assay was measured as were the individual assays for succinic semialdehyde dehydrogenase. The assay mixture consisted of 0.6ml of 0.1M pyrophosphate buffer (pH 8.4), 0.1ml of 10mM 4-aminobutyric acid in pyrophosphate buffer, 0.1ml 5mM α -ketoglutarate in pyrophosphate, 0.1ml 5mM NAD^+ in pyrophosphate, 0.05ml 4-aminobutyric acid transaminase and 0.05ml succinic semialdehyde dehydrogenase. The two enzymes were mixed at various concentration ratios depending upon the experiment.

Theory

Introduction to two substrate reaction mechanisms. Before considering the various mathematical models of multisubstrate reactions, a brief description of the terminology used to describe them is necessary. First, ping-pong describes a mechanism in which one or more products must be released before all substrates can react. Second, there are sequential mechanisms in which all substrates must combine with the enzyme prior to reaction. Sequential mechanisms may be classified further into ordered mechanisms, where the reactants combine with the enzyme and dissociate from it in an obligatory order, and random mechanisms, where the order of combination and release is not obligatory. Kinetic experiments are used first to determine if the reaction proceeds by a ping-pong or sequential mechanism. After this initial segregation, the order of substrate binding, or lack of it, may be determined by additional kinetic analyses utilizing enzyme inhibitors.

The symbols used here and in the following sections to describe the various equilibrium constants are those recommended by the Enzyme Commission. The symbols used are v for the observed initial velocity, K_m^A is the Michaelis constant for substrate A, K_m^B is the Michaelis constant for substrate B, K_S^A is the dissociation constant for A from the enzyme, K_I is the inhibition constant for a given inhibitor and V is the maximum velocity (V_{max}). In ordered reactions A symbolizes the substrate which must first react with the enzyme and B represents the second substrate (if the reaction is not ordered, then A and B are arbitrary designations for the two substrates).

Determination of the type of reaction mechanisms and the Michaelis constants for the substrates. The rate expression for a ping-pong reaction mechanism is given by the equation

$$\frac{v}{V} = 1 + \frac{K_m^A}{[A]} + \frac{K_m^B}{[B]} \quad (1)$$

The rate expression describing a sequential reaction mechanism is

$$\frac{v}{V} = 1 + \frac{K_m^A}{[A]} + \frac{K_m^B}{[B]} + \frac{K_S^A K_m^B}{[A][B]} \quad (2)$$

Since there are two substrates whose concentrations may be varied, there are two possible double reciprocal plots: $1/v$ versus $1/[A]$ at constant concentrations of B, and $1/v$ versus $1/[B]$ at constant concentrations of A. As can be seen from Equation 1, the plots of $1/v$ versus $1/[A]$ at different concentrations of B yield parallel lines; that is, the slopes of all lines are the same and equal to K_m^A/V . The plots of

$1/v$ versus $1/[B]$ at different concentrations of A also yield parallel lines with slopes of K_m^B/V . Parallel lines on such double reciprocal plots are diagnostic for a ping-pong type mechanism.

For a sequential mechanism, a linear plot of $1/v$ versus $1/[A]$ at fixed concentrations of B is obtained when Equation 2 is rearranged as

$$\frac{1}{v} = \frac{1}{[A]} \frac{K_m^A}{V} + \frac{K_s^A K_m^B}{V[B]} + \frac{1}{V} + \frac{K_m^B}{V[B]}. \quad (3)$$

A linear plot of $1/v$ versus $1/[B]$ at fixed concentrations of A obtained when Equation 2 is rearranged to give

$$\frac{1}{v} = \frac{1}{[B]} \frac{K_m^B}{V} + \frac{K_s^A K_m^B}{V[A]} + \frac{1}{V} + \frac{K_m^A}{V[A]}. \quad (4)$$

Several features of these double reciprocal plots should be noted.

Both plots of $1/v$ versus $1/[A]$ at fixed $[B]$ and $1/v$ versus $1/[B]$ at fixed $[A]$ intersect at a point to the left of the vertical axis. This characteristic feature is indicative of a sequential mechanism.

Secondly, the intercepts and slopes of these plots are complex constants which require secondary plots of the data to evaluate kinetic and binding constants. For the plot of $1/v$ versus $1/[A]$ at fixed $[B]$ the vertical intercepts are

$$\text{INTERCEPT} = \frac{1}{V} + \frac{K_m^B}{V[B]}. \quad (5)$$

A secondary plot of the intercept versus $1/[B]$ yields a straight line with a slope of K_m^B/v and a vertical intercept equalling $1/v$. The slopes of the plots of $1/v$ versus $1/[A]$ are

$$\text{SLOPE} = \frac{K_m^A}{V} + \frac{K_S^A K_m^B}{V[B]} \quad (6)$$

A secondary plot of the slope versus $1/[B]$ yields a straight line with a slope of $K_S^A K_m^B/V$ and a vertical intercept of K_m^A/V . The slopes and intercepts of plots of $1/v$ versus $1/[B]$ at fixed $[A]$ may be used in an analogous manner to evaluate these same constants.

Determination of ordered or random substrate addition in a sequential reaction. The three general types of inhibition found with sequential mechanisms are the same as those seen with enzymatic reactions involving a single substrate: These are competitive, noncompetitive and uncompetitive inhibition. Two types of compounds which are commonly used in inhibition studies are product inhibitors and dead-end inhibitors.

Product inhibition is simply inhibition of the enzymatic reaction by a normal product of the reaction; while a dead-end inhibitor is never a normal reaction product, but can combine with the enzyme so that the enzyme-inhibitor complex cannot go on to produce.

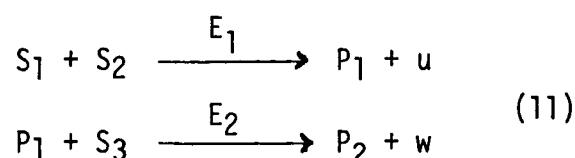
Cleland has formulated a set of rules based on rigorous mathematical analyses for predicting the types of inhibition (competitive, noncompetitive or uncompetitive) to be expected by inspection of a proposed mechanism (26). From the observed inhibition patterns of product and dead-end inhibitors, it is possible to predict, using Cleland's rules, if a sequential mechanism is either random or ordered.

Substrate inhibition. A rather direct means for regulation of enzyme catalyzed rates is provided by inhibition of the reaction by

Thus, the K_I for the substrate inhibitor may be obtained from a plot of $1/v$ versus $[A]$ at high $[A]$.

It is also possible to determine the concentration of A at which maximum reaction velocity will be observed. If one maximizes Equation 8 by setting $dA/dt = 0$, one finds that maximum velocity occurs when $[A] = K_m^A \cdot K_I$.

Transient kinetics of the 4-aminobutyric and succinic semialdehyde dehydrogenase coupled assay. In a coupled assay system like the one studied in this section, it is possible to describe the coupled system by the general scheme



If S_1 , S_2 and S_3 are supplied in concentrations saturating their respective enzymes, the general treatment of this two enzyme system can be simplified to a zero order reaction of E_1 and a first order reaction of E_2 as long as the steady state concentration of P_1 is small compared to the Michaelis constant of E_2 for P_1 . In the 4-aminobutyric acid transaminase-succinic semialdehyde dehydrogenase system, S_1 and S_2 are α -ketoglutarate and 4-aminobutyric acid, S_3 is NAD^+ , P_1 is succinic semialdehyde, P_2 is NADH, U is glutamic acid, W is succinic acid, E_1 is 4-aminobutyric transaminase and E_2 is succinic semialdehyde dehydrogenase. If K_1 represents the rate constant for the 4-aminobutyric acid transaminase reaction and K_2 represents the rate constant for the succinic semialdehyde dehydrogenase reaction, the NADH concentration at any time, t , may be determined using the following equation.

$$[\text{NADH}] = k_1[\text{transaminase}]\left(t + \frac{1}{k_2[\text{dehydrogenase}]}\right) \times e^{-k_2[\text{dehydrogenase}]t},$$

$$- \frac{1}{k_2[\text{dehydrogenase}]} \quad (12)$$

The intersection of the asymptote with the time axis yields the transient or lag time, τ .

$$\tau = \frac{1}{k_2[\text{dehydrogenase}]} \quad (13)$$

τ also has been related by Welch (28) to the diffusion coefficient of P_1 , its molecular radius, the viscosity of the assay medium, the absolute temperature, the Boltzmann constant and the activation energies for each step in the coupled assay.

As is obvious from Equation 13, a plot of $\log\left(\frac{1}{\tau}\right)$ versus $\log[\text{dehydrogenase}]$ at fixed $[\text{transaminase}]$ should yield a straight line if the two enzymes of the coupled system do function independently. However, if interaction occurs between the transaminase and dehydrogenase, the value of τ should show a sudden decrease at the concentration of dehydrogenase where this interaction occurs and the resulting plot of $\log\left(\frac{1}{\tau}\right)$ versus $\log[\text{dehydrogenase}]$ will show a sharp break from linearity. Thus, a plot of $\log\left(\frac{1}{\tau}\right)$ versus $\log[\text{dehydrogenase}]$ can be used to demonstrate the existence of interaction between the two proteins in that concentration range.

Validity of the coupled assay system. As discussed by Bergmeyer (21), a coupled assay is valid if it is zero order with respect to the

first or primer enzyme of the coupled system and first order with respect to the second or indicator enzyme. These conditions will hold for the 4-aminobutyric acid transaminase-succinic semialdehyde dehydrogenase system if NAD^+ , 4-aminobutyric acid and α -ketoglutarate are saturating; and succinic semialdehyde is at a steady state concentration much lower than the K_m of the dehydrogenase for succinic semialdehyde. The effective observed velocity, v_{eff} , of the coupled assay which equals the velocity, V_{prim} , of the primer enzyme can be defined

$$v_{\text{eff}} = V_{\text{prim}} = \frac{V_{\text{SSAD}}}{1 + \frac{K_m^{\text{SSA}}}{[\text{SSA}]_{\text{ss}}} + \frac{K_m^{\text{NAD}}}{[\text{NAD}^+]_{\text{ss}}}} \quad (14)$$

where V_{SSAD} is the maximum velocity of the succinic semialdehyde dehydrogenase if both substrates were saturating and $[\text{SSA}]_{\text{ss}}$ and $[\text{NAD}^+]_{\text{ss}}$ represent the steady state concentrations of succinic semialdehyde and NAD^+ , respectively. In the steady state $[\text{SSA}]_{\text{ss}}$ is a constant as is K_m^{SSA} , thus, it is possible to define a term Q where

$$Q = K_m^{\text{SSA}} / [\text{SSA}]_{\text{ss}} . \quad (15)$$

Since $[\text{NAD}^+]_{\text{ss}}$ is in great excess of $K_m^{\text{NAD}^+}$, the ratio $K_m^{\text{NAD}^+} / [\text{NAD}]_{\text{ss}}$ goes to zero. Therefore, substituting Q for one term in Equation 14 and setting the $K_m^{\text{NAD}^+} / [\text{NAD}^+]_{\text{ss}}$ term to zero we are left with

$$V_{\text{prim}} = v_{\text{eff}} = \frac{V_{\text{ind}}}{(1 + Q)} . \quad (16)$$

Since V_{eff} is the measured coupled assay rate and V_{IND} the maximum rate of reaction which can be catalyzed by the dehydrogenase under saturating condition for both substrates, are known; it is possible to calculate the value of Q . From Q one can determine the steady state concentration of succinic semialdehyde and by comparison to the Michaelis constant for this compound determine the validity of the coupled assay.

II. RESULTS

Kinetic mechanism and kinetic constants. Double reciprocal plots of initial velocity versus succinic semialdehyde concentration at fixed concentrations of NAD^+ and initial velocity versus NAD^+ concentration at fixed succinic semialdehyde concentrations gave rise to a family of lines which intersected to the left of the vertical axis and above the horizontal axis (Figures 10 and 11). Secondary plots of the slopes and intercepts of the double reciprocal initial velocity versus succinic semialdehyde concentration plot, versus reciprocal NAD^+ concentration as given in Figures 12 and 13 were used to determine the Michaelis constants for the various substrates. Secondary plots of the slopes and intercepts of the double reciprocal plot of initial velocity versus NAD^+ concentration, versus reciprocal succinic semialdehyde concentration gave the same Michaelis constants for the substrates as those obtained from Figures 12 and 13. Double reciprocal plots of initial velocity versus substrate concentration when glutaric semialdehyde was used as substrate resulted in the same sort of intersecting lines obtained with succinic semialdehyde as the substrate. Secondary plots

Figure 10. Double Reciprocal Plots of Initial Velocity Versus Succinic Semialdehyde Concentration.

The concentration of succinic semialdehyde was varied while the NAD^+ concentration was held constant at the values: (a) 11mM, (b) 5mM, (c) 3mM and (d) 1mM.

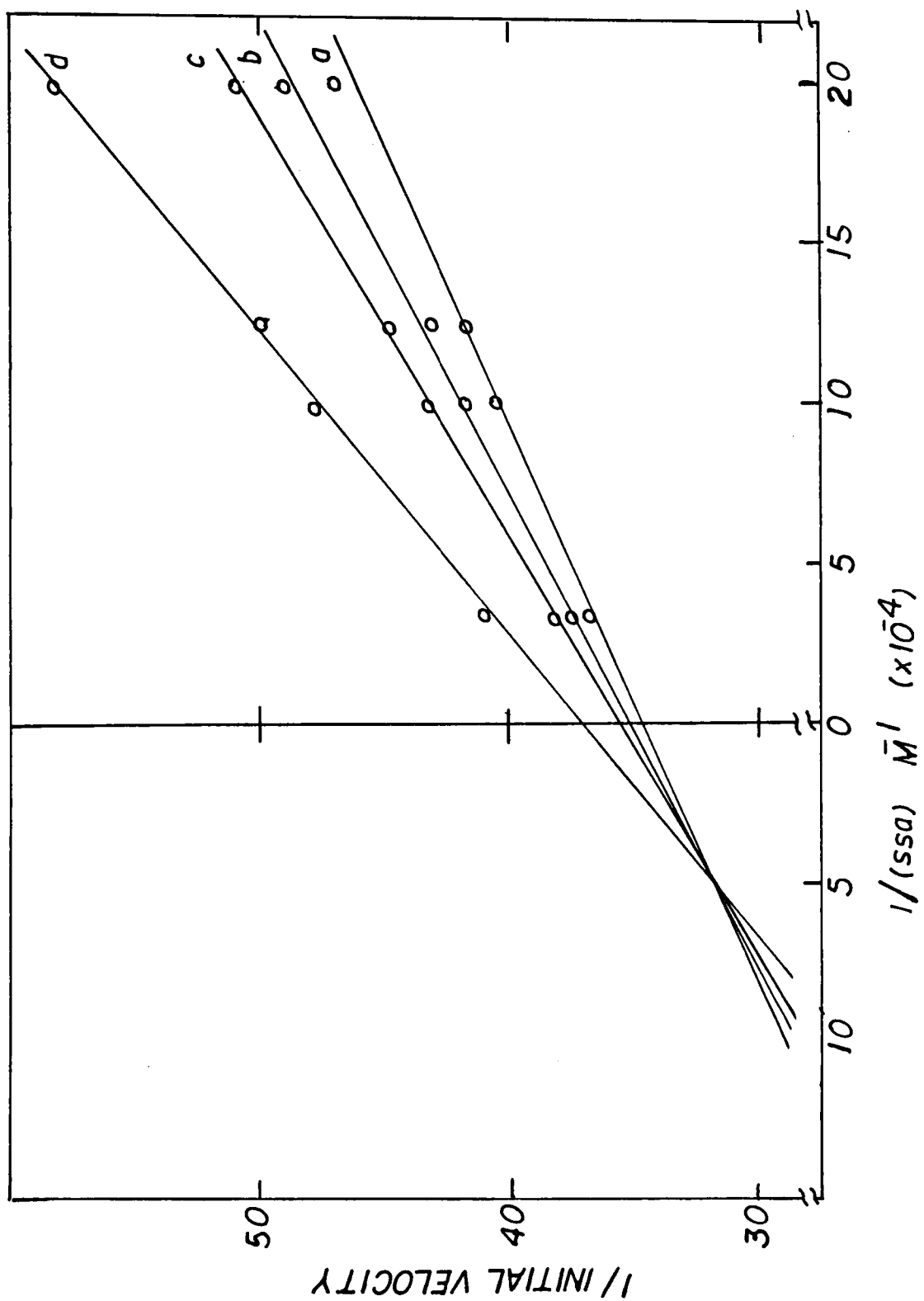


Figure 10

Figure 11. Double Reciprocal Plots of Initial Velocity Versus NAD^+ Concentration.

The concentration of NAD^+ was varied while succinic semialdehyde concentration was held constant at the following values: 5, 8, 10, and $30\mu\text{M}$.

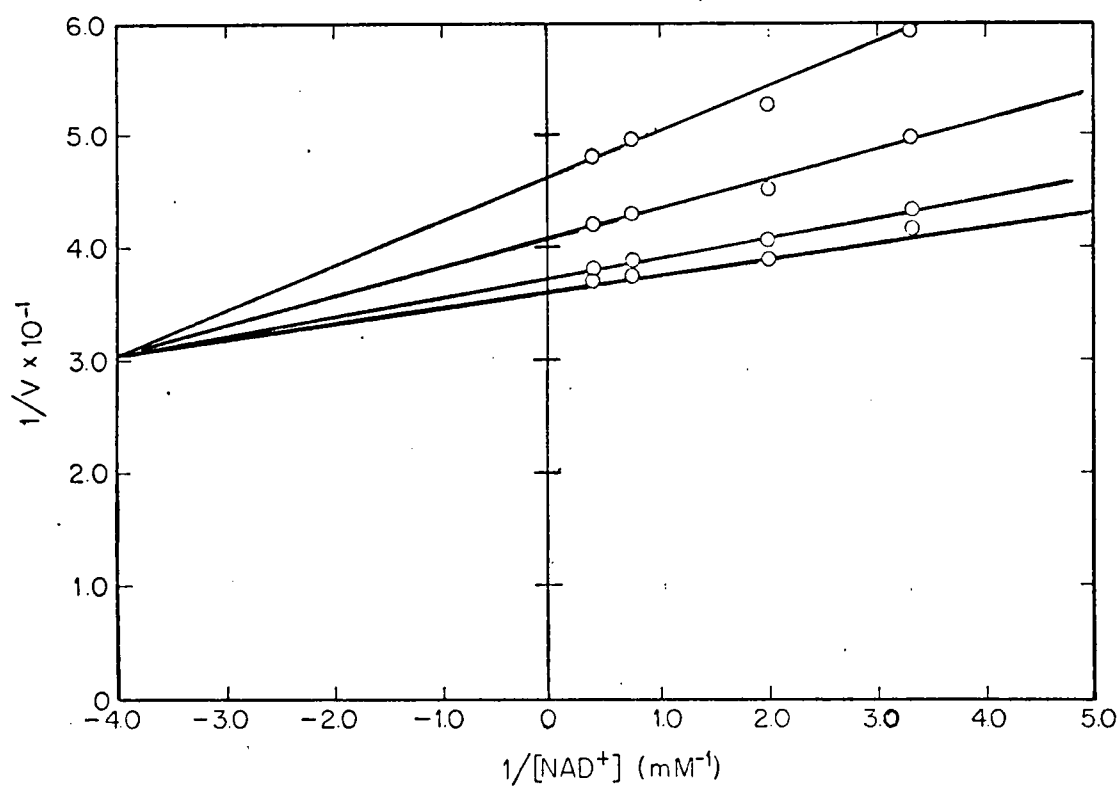


Figure 11

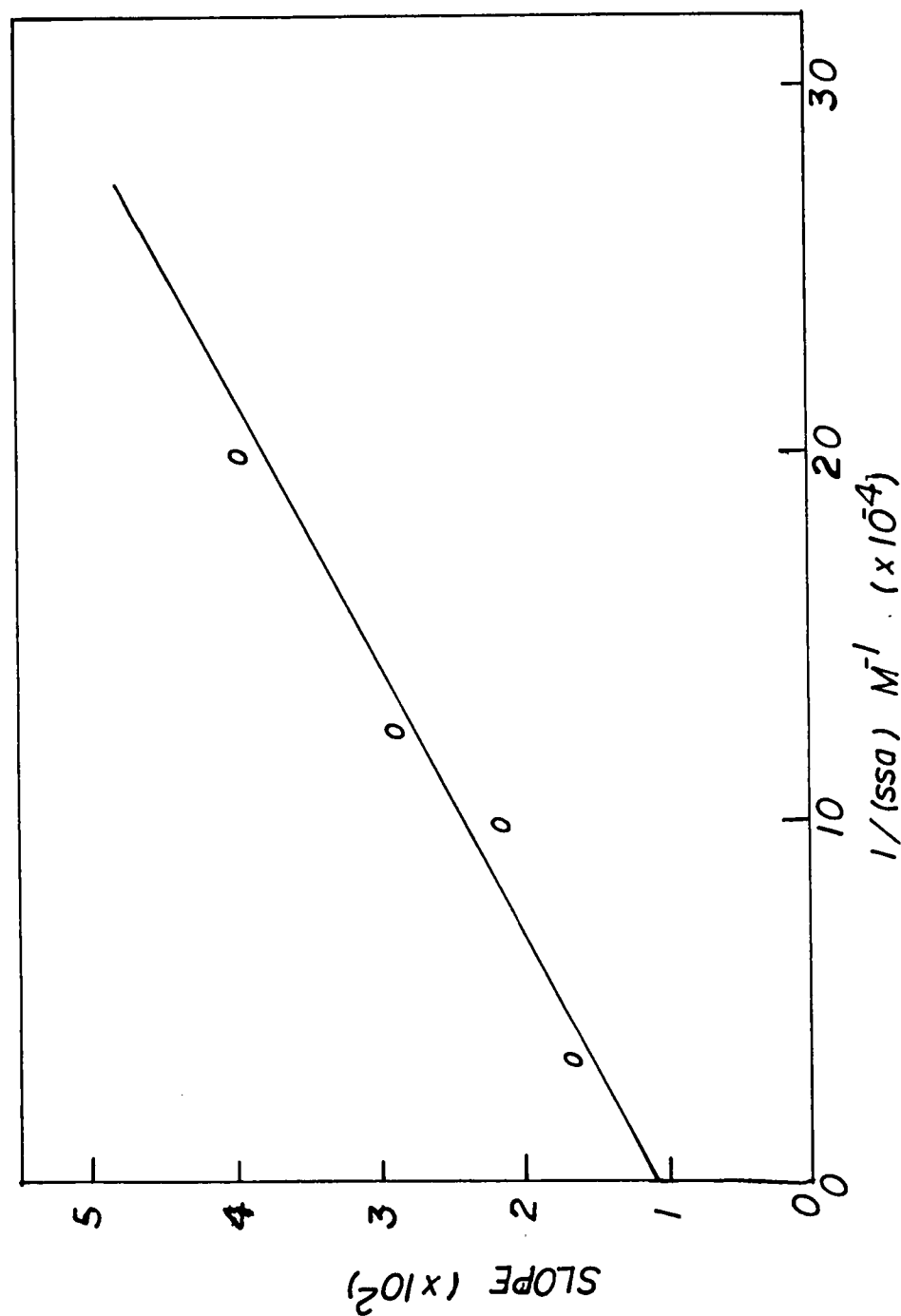


Figure 12. Secondary Plot of Slopes of Double Reciprocal Plots of Initial Velocity Versus NAD^+ Concentration Versus Succinic Semialdehyde Concentration.

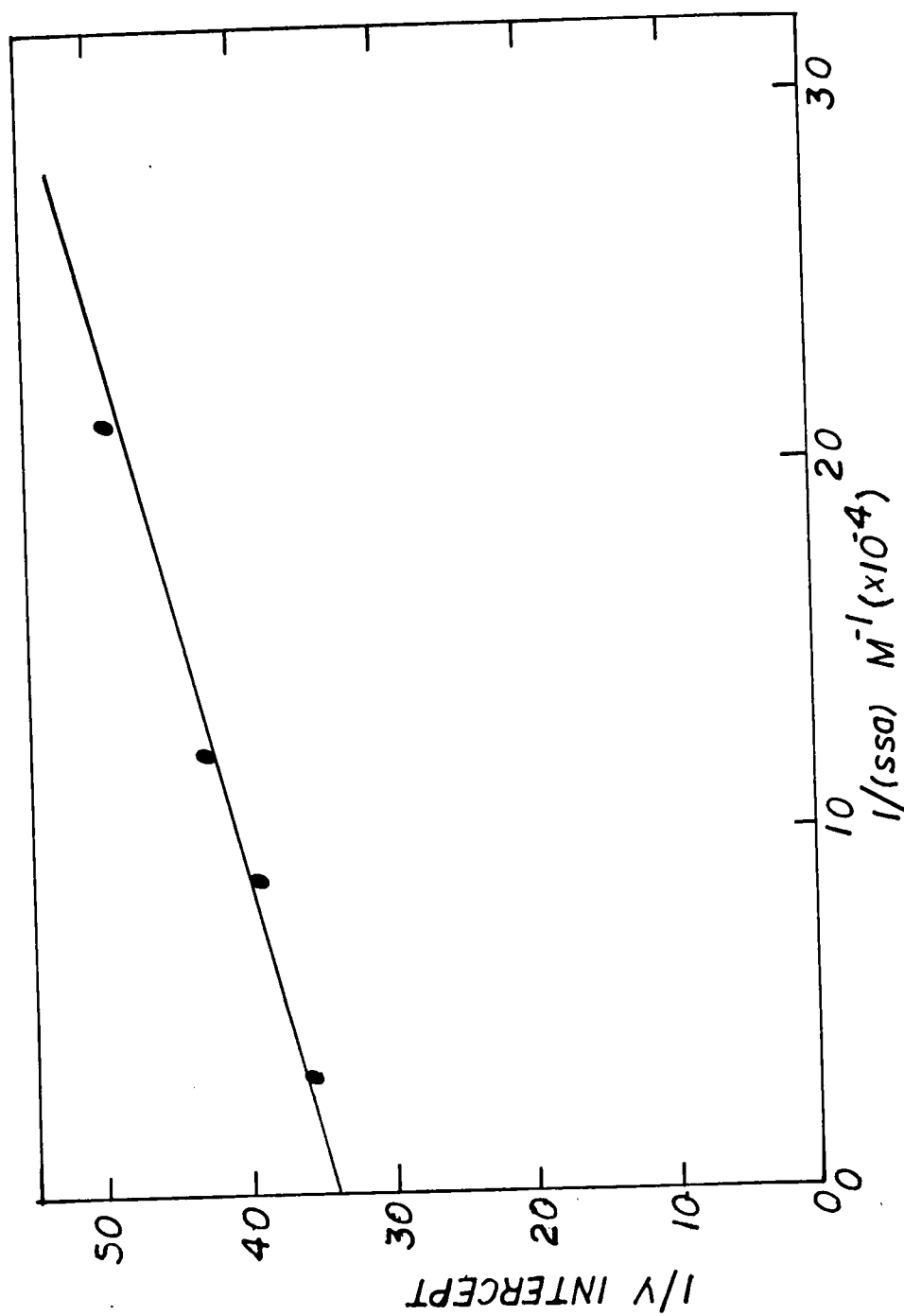


Figure 13. Secondary Plot of Intercept of Double Reciprocal Plots of Initial Velocity Versus NAD^+ Concentration Versus Reciprocal Succinic Semialdehyde Concentration.

of the glutaric semialdehyde data gave straight lines which were used to determine the kinetic constants for this substrate.

The kinetic constants determined for each substrate are given in Table II. In all determinations, the concentration of semialdehyde substrate used was never at a concentration high enough to give substrate inhibition.

The reaction product NADH was found to be an inhibitor of the dehydrogenase reaction. Double reciprocal plots of initial velocity versus substrate concentrations in the presence of various concentrations of NADH are given for both substrates in Figures 14 and 15. These results show NADH is a competitive inhibitor with respect to NAD^+ and a non-competitive inhibitor with respect to succinic semialdehyde. The inhibition constants were 0.15mM and 0.47mM, respectively for NAD^+ and succinic semialdehyde. Succinate showed no inhibitory effect on the reaction.

Para-hydroxybenzaldehyde and m-hydroxybenzaldehyde were both found to be inhibitors of the succinic semialdehyde dehydrogenase reaction. These two compounds were both found to be competitive inhibitors with respect to succinic semialdehyde and non-competitive with respect to NAD^+ . The inhibition constants for these two inhibitors are given in Table III. Neither of these two compounds was a substrate for the dehydrogenase.

Substrate inhibition. Both succinic and glutaric semialdehydes were found to be inhibitors of the succinic semialdehyde dehydrogenase reaction. A double reciprocal plot of initial velocity versus

TABLE II

MICHAELIS CONSTANTS AND SUBSTRATE INHIBITION CONSTANTS FOR
THE SUBSTRATES OF SUCCINIC SEMIALDEHYDE DEHYDROGENASE

Substrate	K_M (μM)	K_I (μM)	$V_{max}^{(1)}$
NAD^+	310	----	----
Succinic Semialdehyde	15	90	100
Glutaric Semialdehyde	3.4	3000	25
Malonic Semialdehyde	----	----	0

(1) V_{max} values as given relative to succinic semialdehyde
as substrate.

Figure 14. Double Reciprocal Plot of Initial Velocity Versus NAD^+ Concentration in the Presence of Inhibiting NADH.

The concentration of succinic semialdehyde is $50\mu\text{M}$, and the concentrations of NADH are 0 (O), $50\mu\text{M}$ (◼), $100\mu\text{M}$ (Δ), and $200\mu\text{M}$ (∇).

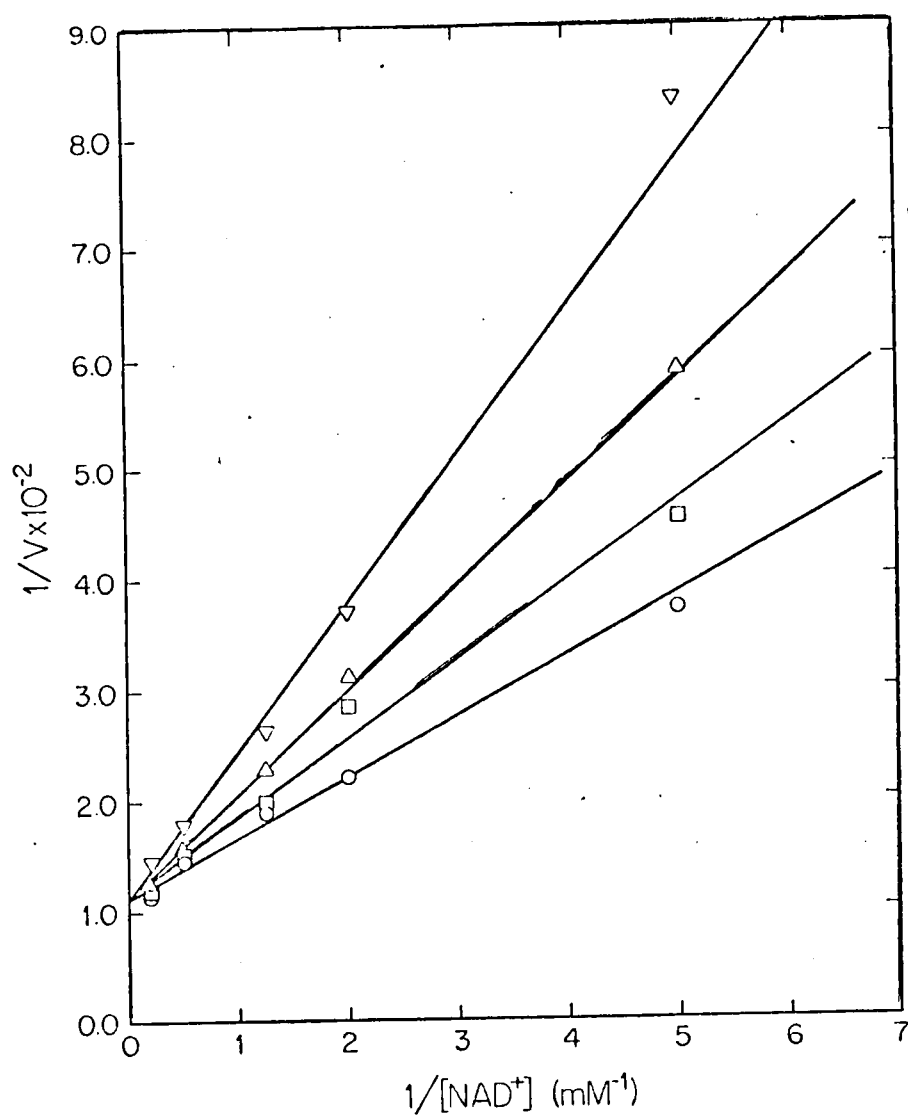


Figure 14

Figure 15. Double Reciprocal Plot of Initial Velocity Versus Succinic Semialdehyde Concentration in the Presence of Inhibitory NADH.

The concentration of NAD^+ is 5mM, and the concentrations of NADH are 0 (O), 50 μM ($\square$), 100 M (Δ) and 200 M (∇).

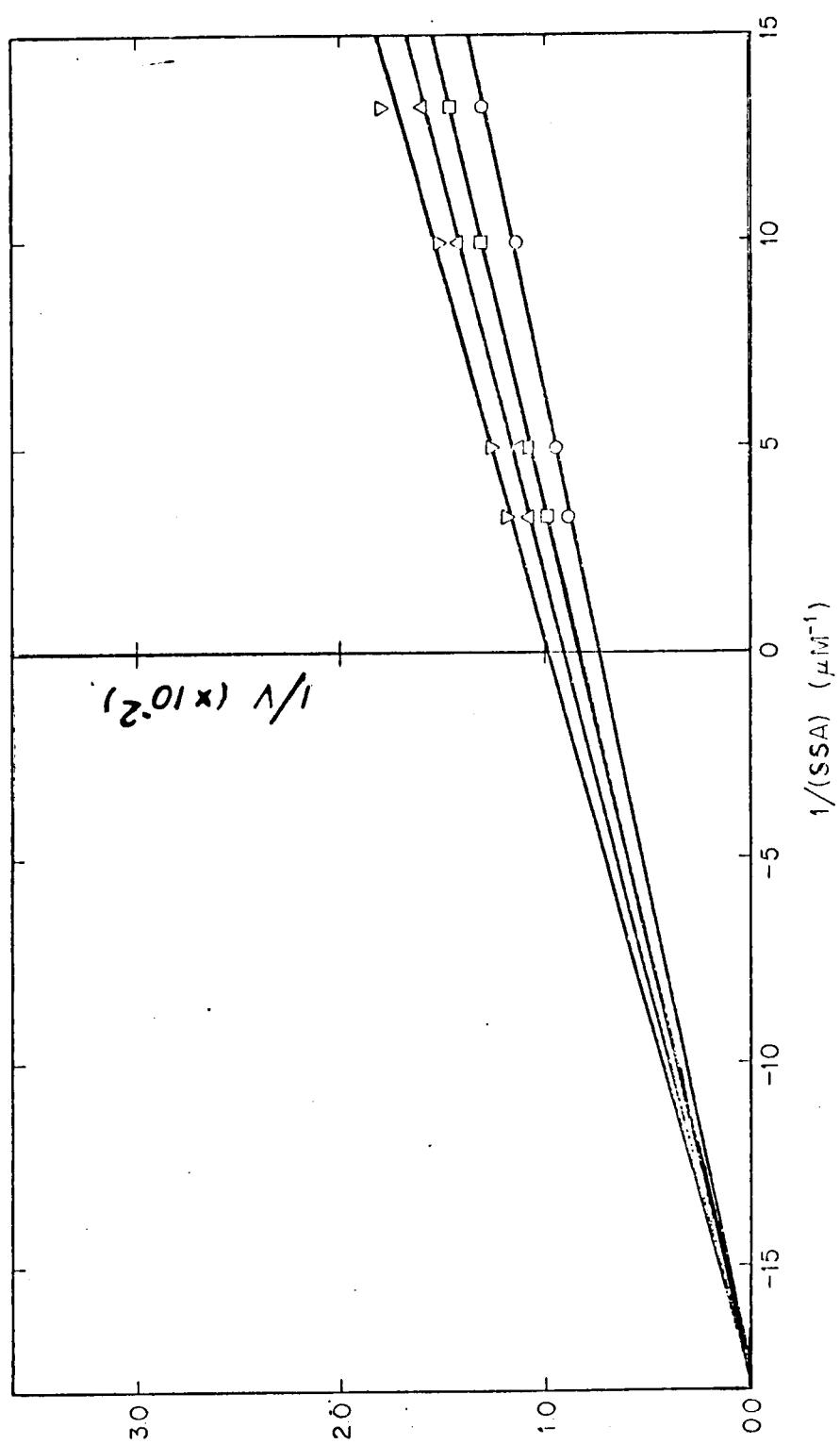


Figure 15

TABLE III

DEAD END INHIBITOR PATTERNS AND CONSTANTS OF p-HYDROXYBENZALDEHYDE AND m-HYDROXYBENZALDEHYDE FOR SUCCINIC SEMIALDEHYDE DEHYDROGENASE

Inhibitor	Varied Substrate	Pattern	KI	
			Slope	Intercept
p-Hydroxybenzaldehyde	NAD ⁺	Noncompetitive	1.4	2.3
	Succinic Semialdehyde	Competitive	11.0	
m-Hydroxybenzaldehyde	NAD ⁺	Noncompetitive	34.2	27.1
	Succinic Semialdehyde	Competitive	50.1	

semialdehyde concentration at fixed NAD^+ concentrations is given in Figure 16, for both semialdehyde substrates, including both inhibitory and non-inhibitory semialdehyde concentrations. Figure 17 is a plot of reciprocal/initial velocity versus semialdehyde from which the substrate inhibition constants may be determined. The inhibition constants for succinic semialdehyde and glutaric semialdehyde were 0.09mM and 3.4mM respectively. Malonic semialdehyde, the three carbon analog of succinic semialdehyde, was not an inhibitor of succinic semialdehyde dehydrogenase.

Coupled assay with 4-aminobutyrate transaminase. The coupled assay system of 4-aminobutyrate transaminase and succinic semialdehyde dehydrogenase behaved as a valid coupled assay system when the concentration of the dehydrogenase was in a three fold excess of the transaminase concentration. The values of Q and the steady state concentrations of succinic semialdehyde are listed in Table IV. From Figure 18, it can be seen that as the molar ratio of dehydrogenase to transaminase approaches three, the reaction velocity becomes independent of this ratio indicating the rate transamination is correctly being measured by the dehydrogenase. With a transaminase to dehydrogenase ratio of 1:3, one finds a Q value of 5.8 and a steady state concentration of succinic semialdehyde of 2.6 μM . Thus, when the steady state concentration of succinic semialdehyde is six times less than the Michaelis constant of the dehydrogenase for succinic semialdehyde, the coupled assay system is measuring the rate of transamination.

Figure 16. Double Reciprocal Plot of Initial Velocity Versus Semialdehyde Concentration with Inhibiting Concentrations of Succinic and Glutaric Semialdehyde.

The concentration of NAD^+ is 5mM. The concentration of glutaric semialdehyde (●—●) and succinic semialdehyde (○—○) were varied from non-inhibitory through inhibitory concentrations.

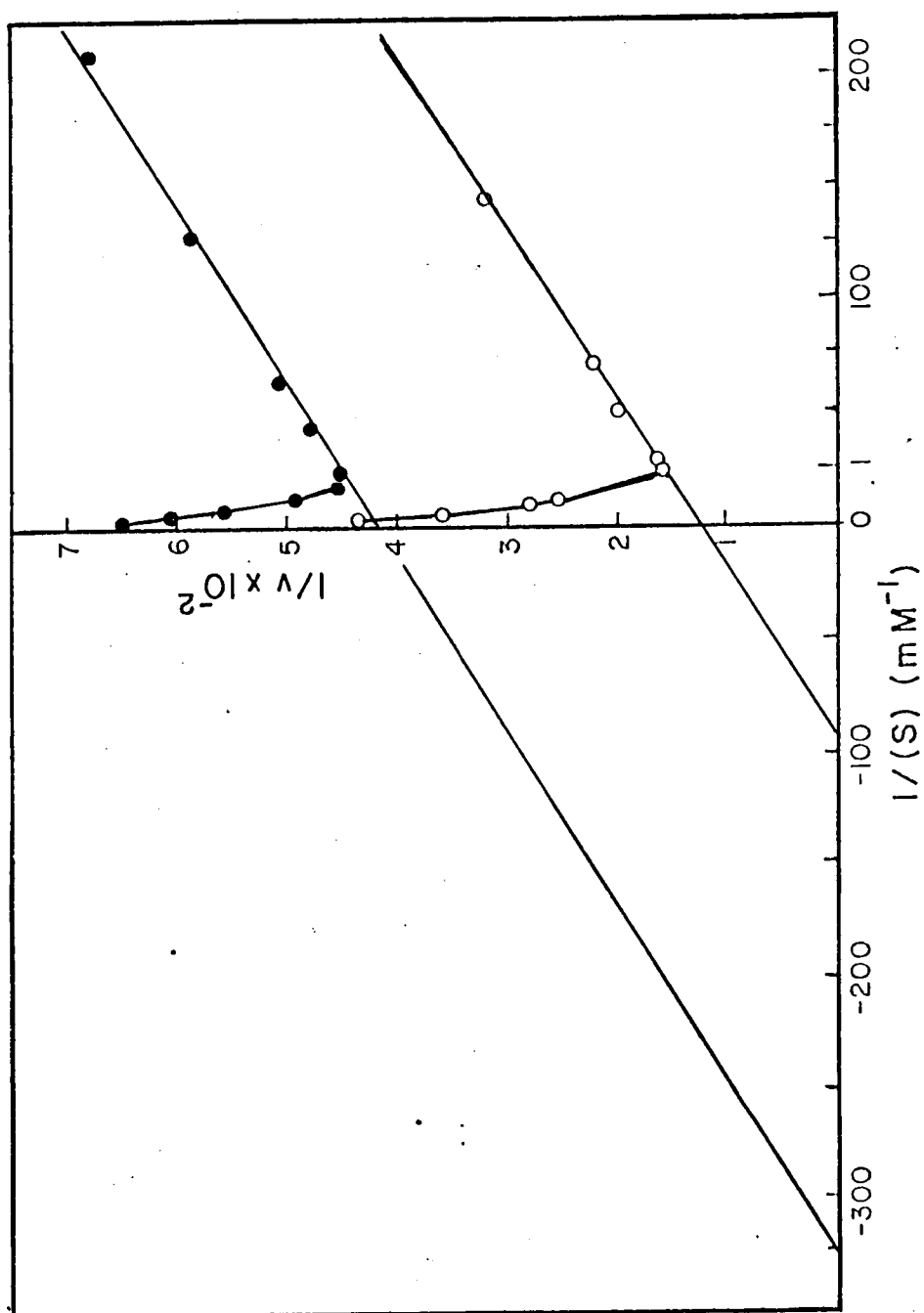


Figure 16

Figure 17. Plot of Reciprocal Velocity Versus Inhibitory Semi-aldehyde Concentration.

Succinic semialdehyde is represented by (●—●) and glutaric semialdehyde by (○—○).

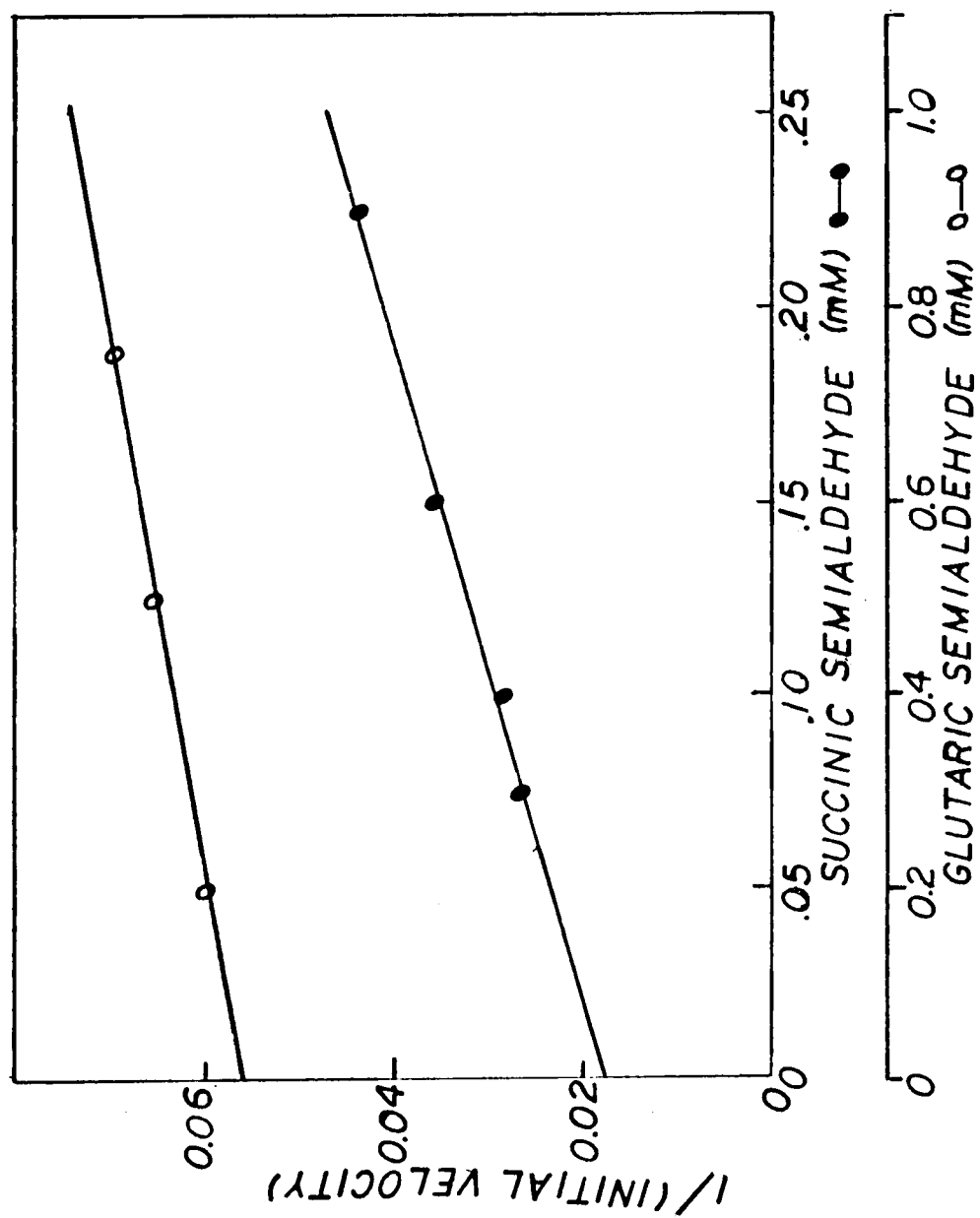


Figure 17

TABLE IV

STEADY STATE CONCENTRATIONS OF SUCCINIC SEMIALDEHYDE
AND "Q" VALUES FOR THE COUPLED ASSAY

Dehydrogenase/Transaminase ⁽¹⁾ (molar ratio)	[SSA] _{ss} (μ M)	Q ⁽²⁾
30	0.17	88
15	0.30	50
7.5	0.55	27
3.0	2.6	5.8
1.0	6.1	2.4
0.5	16.1	0.93
0.25	46.0	0.32

(1) The concentration of 4-aminobutyrate transaminase was kept constant at 3nM.

(2) $Q = K_m^{SSA} / [SSA]_{ss}$

Figure 18. Plot of Log of Initial Velocity Versus Log of the Ratio of Succinic Semialdehyde Dehydrogenase to 4-Aminobutyric Acid Transaminase Concentration.

The concentration of 4-aminobutyric acid transaminase was kept constant at 3nM.

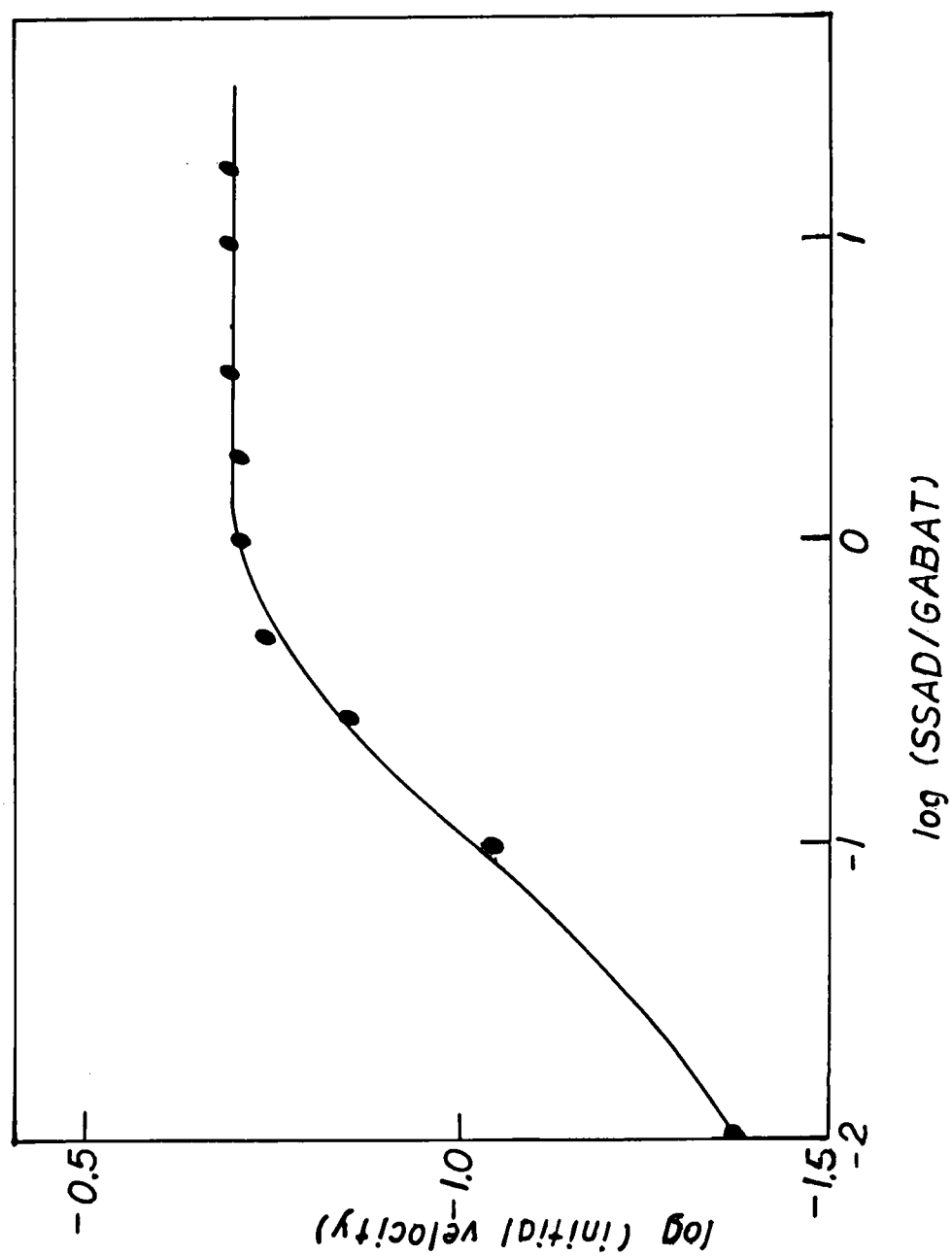


Figure 18

From the lag or transient times of the coupled assay, it is possible to predict physical interaction between the transaminase and dehydrogenase. Log plots of reciprocal transient time versus dehydrogenase concentration give rise to straight lines when both glutaric and succinic semialdehyde are used as substrates (Figure 19). The linearity of these plots argues against physical interaction between the two enzymes.

III. DISCUSSION

The intersecting pattern of lines obtained on the double reciprocal velocity versus substrate concentration plots demonstrates that succinic semialdehyde functions by a sequential kinetic mechanism. This result is not unexpected, since all other dehydrogenases which have been studied exhibit sequential mechanisms (29).

Since the product of the reaction, i.e. succinic acid, does not inhibit the reaction catalyzed by the enzyme, it is virtually impossible to distinguish whether the order of substrate addition is ordered or random on the basis of the inhibition patterns obtained with NADH. However, the inhibition pattern obtained with the dead-end inhibitors m-hydroxybenzaldehyde and p-hydroxybenzaldehyde coupled with the NADH data are consistent with a rapid equilibrium random bi bi mechanism, in which a binary enzyme-aldehyde complex and a binary enzyme-NAD⁺ complex are formed in kinetically significant amounts. Since the reaction is irreversible, it is not possible to use these inhibition studies to determine if product release is ordered or random. Thus, the reaction scheme might be represented as:

Figure 19. Plot of Log of Reciprocal Transient Time Versus Log of Succinic Semialdehyde Dehydrogenase Concentration.

Relation between the reciprocal of transient time (τ) and the molarity of succinic semialdehyde dehydrogenase. The concentration of 4-aminobutyric acid transaminase was kept constant at 3nM; and succinic semialdehyde dehydrogenase was varied. The open circles ($\circ$) were with 4-aminobutyric acid as substrate and the closed circles ($\bullet$) were with 5-aminovaleric acid as substrate.

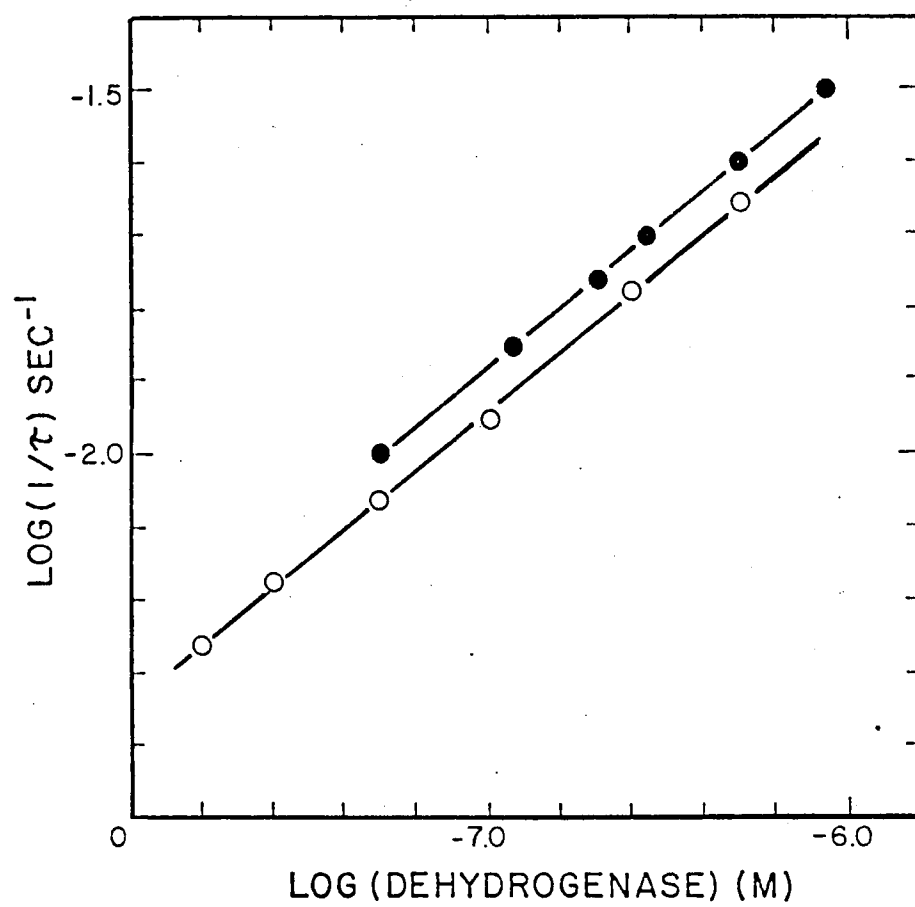
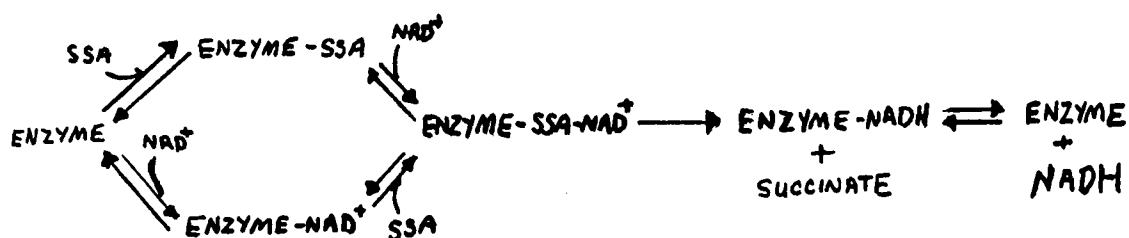


Figure 19



The order of release of products might be either ordered or random, but nonetheless, since the addition of substrates is random, the overall mechanism is considered to be a random mechanism.

Rapid equilibrium Random BiBi mechanisms have been proposed for the aldehyde dehydrogenase isolated from liver (30,31). Thus, succinic semialdehyde dehydrogenase appears to share a common kinetic mechanism with the aldehyde dehydrogenases of other sources. Additionally, these liver aldehyde dehydrogenases are irreversible and NAD^+ specific, so that the similarities of these two enzymes possibly represent an important mechanistic link in the conversions of aldehydes to acids.

Substrate inhibition is not an uncommon property of enzymes. For instance, 4-aminobutyric acid transaminase is inhibited by its substrate α -ketoglutarate ($K_I = 0.03\text{M}$) (32). However, instances where substrate inhibition as potent as succinic semialdehyde on succinic semialdehyde dehydrogenases are rare. The Michaelis constant of the enzyme for succinic semialdehyde is $15\mu\text{M}$, the concentration of substrate which gives maximum velocity is $50\mu\text{M}$ and the concentration of semialdehyde which gives 50% inhibition is $90\mu\text{M}$. Thus, a change of less than an order of magnitude in concentration of substrate can lead to a change from 50% maximal velocity to maximal velocity back to 50%

maximal velocity. The inhibitory effect of glutaric semialdehyde is much less pronounced than that of succinic semialdehyde. The Michaelis constant and inhibition constant for this substrate differ by three orders of magnitude ($3.1\mu\text{M}$ versus 3.4mM).

The differences in inhibitory properties of these two substrates suggests the inhibition by the four carbon succinic semialdehyde is a chemically specific event which requires both a four carbon chain length and an aldehyde group (succinate is not an inhibitor). This inhibition may be of physiological significance, and certainly is of pharmacological interest. Recent reports indicate the anticonvulsant drug di-n-propylacetate exerts its action by inducing substrate inhibition of this dehydrogenase and consequently results in a build-up of the inhibitory neurotransmitter 4-aminobutyric acid (33). Additionally, it is possible that substrate inhibition of this enzyme causes succinic semialdehyde to be reduced by a recently described NADP^+ specific reductase which forms the sleep inducing compound 4-hydroxybutyric acid (34). Thus, this inhibition might induce alternative metabolism of succinic semialdehyde and may be a mechanism for sleep induction.

The coupled assay system of 4-aminobutyric acid transaminase and succinic semialdehyde dehydrogenase has been often used to measure 4-aminobutyric acid transaminase activity and 4-aminobutyric acid concentration since such a system was first proposed by Jakoby (35). However, even though the coupled assay is often used as a measure of 4-aminobutyric acid transaminase activity in extracts of mammalian nervous tissue (36,37), the coupled assay system has never been examined using the two purified enzymes. Indeed, when purified enzymes are

in couple, it is possible to fulfill the requirements that the transaminase display zero order kinetics, the dehydrogenase first order kinetics and the overall reaction proceeds in the desired direction for a detectable period of time. Thus, when the molar ratio of dehydrogenase to transaminase approaches three, we are truly measuring transaminase activity.

It is logical to ask why a molar mixing ratio of three is required to have a coupled system which correctly measures the activity of the transaminase if the two purified enzymes have essentially the same specific activities and approximate molecular weights (160,000 for the dehydrogenase and 100,000 for the transaminase). This question is answered when one considers the lag time of the coupled assay system and the steady state concentration of succinic semialdehyde. As succinic semialdehyde is produced by the transaminase, time must elapse for it to first build up to detectable concentrations and then for it to diffuse to the dehydrogenase. This lag time influences the steady state concentration of succinic semialdehyde. As seen from Figure 18 on page 60 and Table IV on page 59, the steady state semialdehyde concentration must be less than 10 times the Michaelis constant of the dehydrogenase, thus ensuring first order kinetics with respect to the dehydrogenase. If this steady state concentration rises, the dehydrogenase reaction begins to exhibit mixed order kinetics and the validity of the coupled system breaks down. It is therefore important to maintain the proper enzyme mixing ratio to ensure the dehydrogenase shows first order kinetics with respect to succinic semialdehyde.

As mentioned above, the lag or transient time of this coupled assay is dependent upon the time in which the semialdehyde diffuses from the transaminase to dehydrogenase. By following this lag time as a function of enzyme mixing ratio, it is possible to predict physical interaction between the protein if an abrupt change in the lag time occurs. As Figure 19 demonstrates, no such abrupt change in lag time occurs, thus eliminating the possibility of predicting protein-protein interaction. This finding is important, for many metabolically adjoining enzymes have been demonstrated to interact physically (38-40). Additionally, transaminases such as glutamate-oxaloacetate transaminase have been shown to interact with many dehydrogenases (41), and 4-aminobutyric acid transaminase has been shown by Mr. Udo Moses in our laboratory to physically interact with beef liver glutamate dehydrogenase. But, over the concentration range used in this study, these two enzymes appear to function independently (however, interaction at other enzyme concentrations cannot be ruled out) and not show the type of interaction reported for other metabolically related enzymes.

CHAPTER III

REACTIVITY OF LYSYL RESIDUES

Understanding the chemical basis for the inhibition of the dehydrogenase by succinic semialdehyde was the original motivation of this experiment. Suggestions had been made in the early literature on succinic semialdehyde dehydrogenase that the substrate inhibition was due to the reactive aldehyde group of the succinic semialdehyde (11). Presumably, this inhibition would occur when this carbonyl group reacted with an essential amine group on the enzyme. This reaction would most likely involve the formation of a Schiff's base linkage between the carbonyl and an ϵ -amino group of a lysyl residue within the enzyme. If this lysyl residue were in the catalytic center of the enzyme or necessary to maintain a required conformation, then its interaction with succinic semialdehyde would cause loss of enzymatic activity.

Since a lysyl residue could be postulated to be involved in this inhibition, it is possible to chemically modify the lysyl residues of the protein in order to determine their essentiality. The most selective and useful chemical reagent for modification of lysyl residues is pyridoxal-5'-phosphate (PLP) (42). PLP is very specific for free primary amine groups and is easily detected spectrophotometrically when it reacts with these groups. Another reagent which has a high specificity for primary amines, and is easily detectable by its spectral properties upon reaction, is o-phthalaldehyde. This reagent is coming

into wide use as a means of detecting primary amine groups during amino acid analysis (43). Although this reagent has not been previously in chemical modification studies, its high specificity and ease of reactivity would make it a useful reagent in determining the role of lysine in succinic semialdehyde dehydrogenase. Thus, these two reagents were used in the study of lysyl residues of succinic semialdehyde dehydrogenase.

I. EXPERIMENTAL

Materials

Pyridoxal-5'-phosphate and pyridoxal were purchased from Sigma Chemical Co., St. Louis, MO. o-Phthalaldehyde was purchased from Pierce Chemical Co., Rockford, IL. Sodium borohydride was obtained from Metal Hydrides Inc., Beverly, OH. All other chemicals were purchased from those sources disclosed in previous chapters.

Methods

Sodium borohydride reduction of succinic semialdehyde in the presence of succinic semialdehyde dehydrogenase. In order to check the possibility that at inhibitory concentrations succinic semialdehyde might form a Schiff's base with a free primary amine, several grains of NaBH_4 were added to a 1ml solution of 1mM succinic semialdehyde and $1\mu\text{M}$ succinic semialdehyde (pH 8.4). The NaBH_4 was allowed to incubate with the enzyme for one hour at 37°C to insure complete reduction of any Schiff's base formed, NaBH_4 was removed from the reaction mixture by ultrafiltration through an Amicon PM-30 diaflow membrane. Several

volumes of buffer were used to wash away excess NaBH_4 and succinic semialdehyde from the enzyme which is retained by the membrane.

Reaction with pyridoxal-5'-phosphate. The effect of PLP on the enzymatic activity of succinic semialdehyde dehydrogenase was studied by preincubating the enzyme ($2\mu\text{M}$) with PLP in 0.1M sodium pyrophosphate ($\text{pH } 8.4$), containing 1mM 2-mercaptoethanol at 37°C . Aliquots of the preincubation mixtures were removed and transferred to assay mixtures in which initial velocities of NAD^+ reduction were obtained at 25°C .

The amount of pyridoxal-5'-phosphate bound to the enzyme, after NaBH_4 reduction was determined by measuring the increase in absorbance at 325nm using a molar extinction coefficient of $9710\text{ m}^{-1}\text{cm}^{-1}$ for ϵ -pyridoxal-lysine. The number of PLP residues bound per mole of enzyme ($\text{Mw} = 160,000$) was also determined by fluorescence spectroscopy using an excitation wavelength of 325nm and emission wavelength of 390nm .

Reaction with o-phthalaldehyde. The reaction of o-phthalaldehyde with the enzyme was performed at 25°C . The enzyme ($2\mu\text{M}$) was preincubated for varying time intervals with o-phthalaldehyde in 0.1M sodium pyrophosphate ($\text{pH } 8.4$) containing 1mM 2-mercaptoethanol at 25°C . A fresh solution of o-phthalaldehyde (10mM) in 0.1M potassium borate ($\text{pH } 10.4$), containing 1mM 2-mercaptoethanol, was prepared before the reaction with the enzyme. The degree of labeling of the enzyme reacted with o-phthalaldehyde was determined from the absorption spectrum using a molar extinction coefficient of $12,000\text{ M}^{-1}\text{cm}^{-1}$ at 335nm .

II. RESULTS

NaBH₄ treatment of succinic semialdehyde inhibited enzyme. Addition of NaBH₄ to succinic semialdehyde dehydrogenase containing inhibitory concentrations of succinic semialdehyde (1mM) resulted in no detectable change in the activity of the enzyme. Changes in succinic semialdehyde concentration and changes in the length of incubation time with NaBH₄ had no effect on succinic semialdehyde dehydrogenase activity.

Reversible binding of pyridoxal-5'-phosphate. The enzyme succinic semialdehyde dehydrogenase is inactivated by incubation with pyridoxal-5'-phosphate over the pH range from 7.4 to 8.6. An increasing degree of inhibition was observed when the pH of the solution approached the optimum pH of enzyme activity (Figure 20).

The effect of addition of PLP on the activity of succinic semialdehyde dehydrogenase was investigated by preincubating the enzyme with increasing concentrations of PLP at a temperature of 37°C. Aliquots of the preincubation mixtures were removed and assayed for enzymatic activity. The presence of increasing concentrations of PLP in the incubation mixtures has a dramatic effect on the enzymatic activity as demonstrated by the results obtained at pH 8.4 as shown in Figure 21. Thus, the addition of 300 moles of PLP per mole of enzyme at pH 8.4 followed by incubation at 37°C for 30 min, resulted in a substantial loss of the catalytic activity (80%). Complete inactivation of the enzyme is attained when the enzyme is preincubated with an excess of PLP (mixing molar ratio, 5000:1) at pH 8.4.

Figure 20. The pH Dependence of Pyridoxal-5'-Phosphate Inhibition.

Samples of enzyme preincubated with 300-fold molar excess of PLP for 30 min (37°C) at the indicated pH values were assayed for enzymatic activity (■). Samples containing no PLP were preincubated for 30 min (37°C) at the indicated pH values and assayed for enzymatic activity (●). A/A_0 is the ratio of enzymatic activity in the presence and absence of PLP.

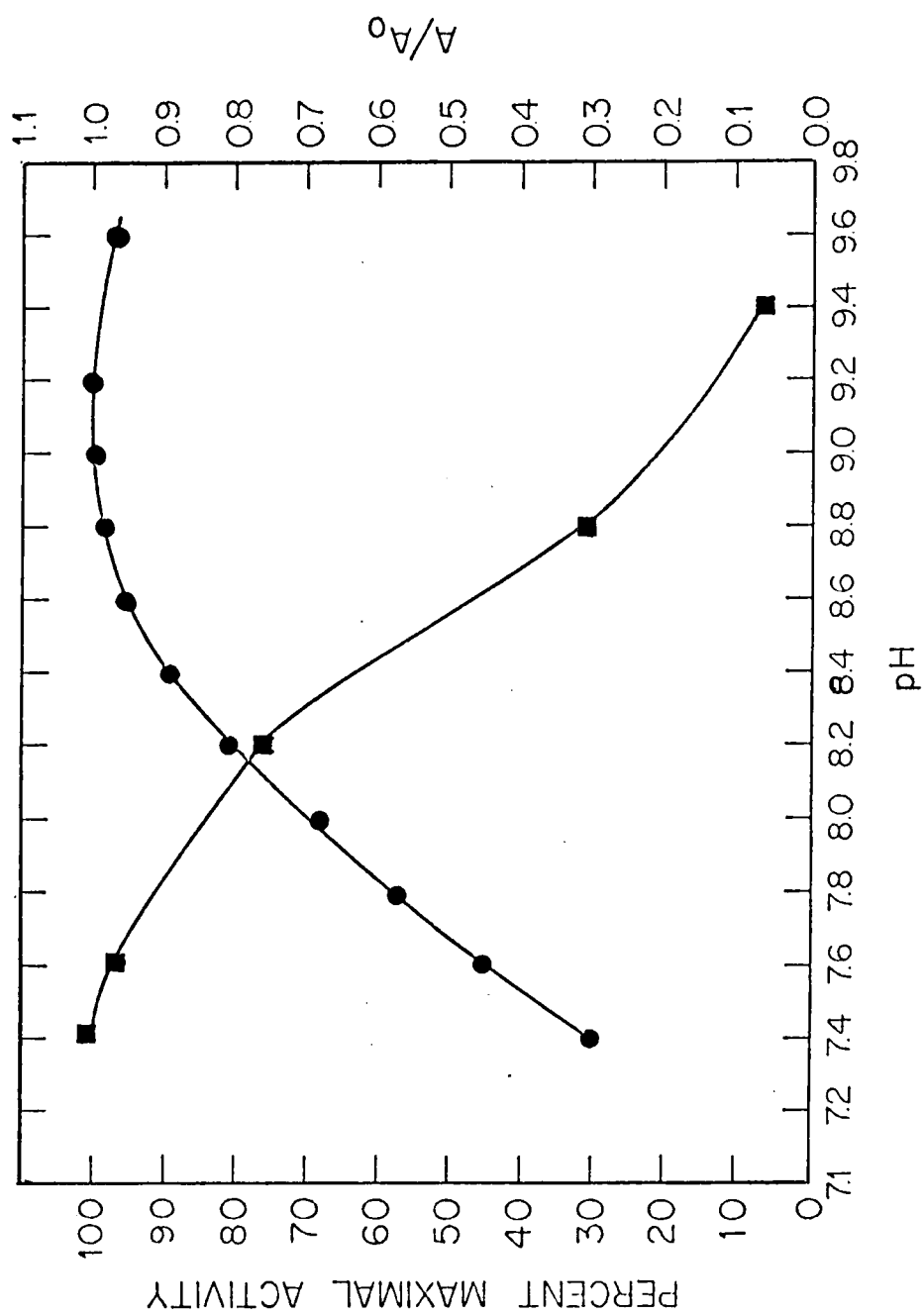


Figure 20

Figure 21. Pyridoxal-5'-Phosphate Inactivation of Succinic Semialdehyde Dehydrogenase as a Function of Pyridoxal-5'-Phosphate Concentration.

The enzyme, at a concentration of $2\mu\text{M}$, was preincubated at pH 8.4 with increasing concentrations of PLP for 30 min at 37°C . Aliquots withdrawn from the incubation mixtures were tested for enzymatic activity. Results obtained when the preincubation mixture contained NAD^+ (5mM) (■) and in the absence of NAD^+ (●).

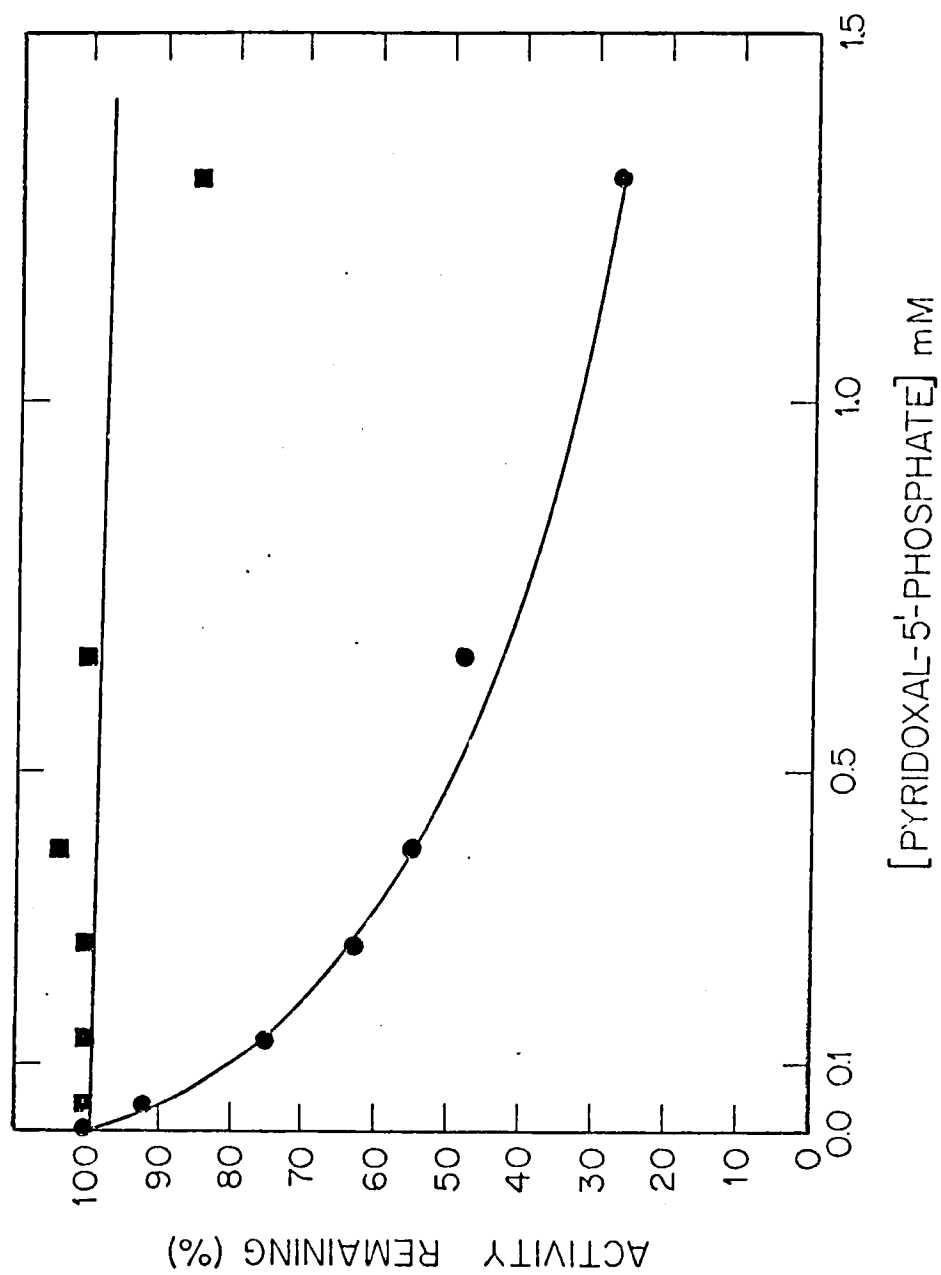


Figure 21

However, the inhibitory effect exerted by pyridoxal-5'-phosphate is reversed by addition of compounds which react with the Schiff base. This is shown by an experiment in which the enzyme (2 μ M) was incubated with 0.25mM PLP at pH 8.4 for 60 min at 37°C. Then, the enzyme inactivated by PLP was allowed to react with aminooxyacetate (1mM) for 30 min at 25°C. As seen in Figure 22, aminooxyacetate reacts with both free and unbound PLP and the enzyme recovers 80% of the original activity. Similar recovery of the enzymatic activity was observed when the enzyme preincubated with PLP was passed through a Sephadex G-25 column, previously equilibrated with 0.1M pyrophosphate buffer (pH 8.4). Since the binding of PLP to the enzyme is reversible, it was possible to determine the affinity of PLP for ϵ -lysine residues of the protein by using the data given in Figure 21. The dissociation constant (K_D) is related to the velocities V_0 and V_∞ of the reaction catalyzed by the enzyme, in the absence and presence of PLP, respectively, by means of the following equation

$$K_D = \frac{[\text{PLP}] \cdot V_\infty}{V_0 - V_\infty}$$

where V_0 is the velocity of the reaction in the absence of inhibitor and V_∞ is the velocity of the reaction in the presence of inhibitor after the PLP binding equilibrium has been reached.

A dissociation constant of 0.1mM was obtained for the binding of PLP to the enzyme succinic semialdehyde dehydrogenase at pH 8.4.

Figure 22. Reversal of Pyridoxal-5'-Phosphate Inhibition by Amino-oxyacetic Acid as a Function of Time.

The dehydrogenase activity is displayed in the lower curve (O) while the formation of the PLP-amino-oxyacetic acid complex is represented by the upper curve (O). The concentration of enzyme was $2\mu\text{M}$ and was inhibited with a 300-fold molar excess of PLP. The sample was at pH 8.4 in 0.1M pyrophosphate (37°C). The enzyme had been preincubated for 60 min at 37°C (pH 8.4) with the PLP prior to reactivation. The concentration of amino-oxyacetic acid was 3mM. The remaining activity represents the percentage of the uninhibited activity remaining.

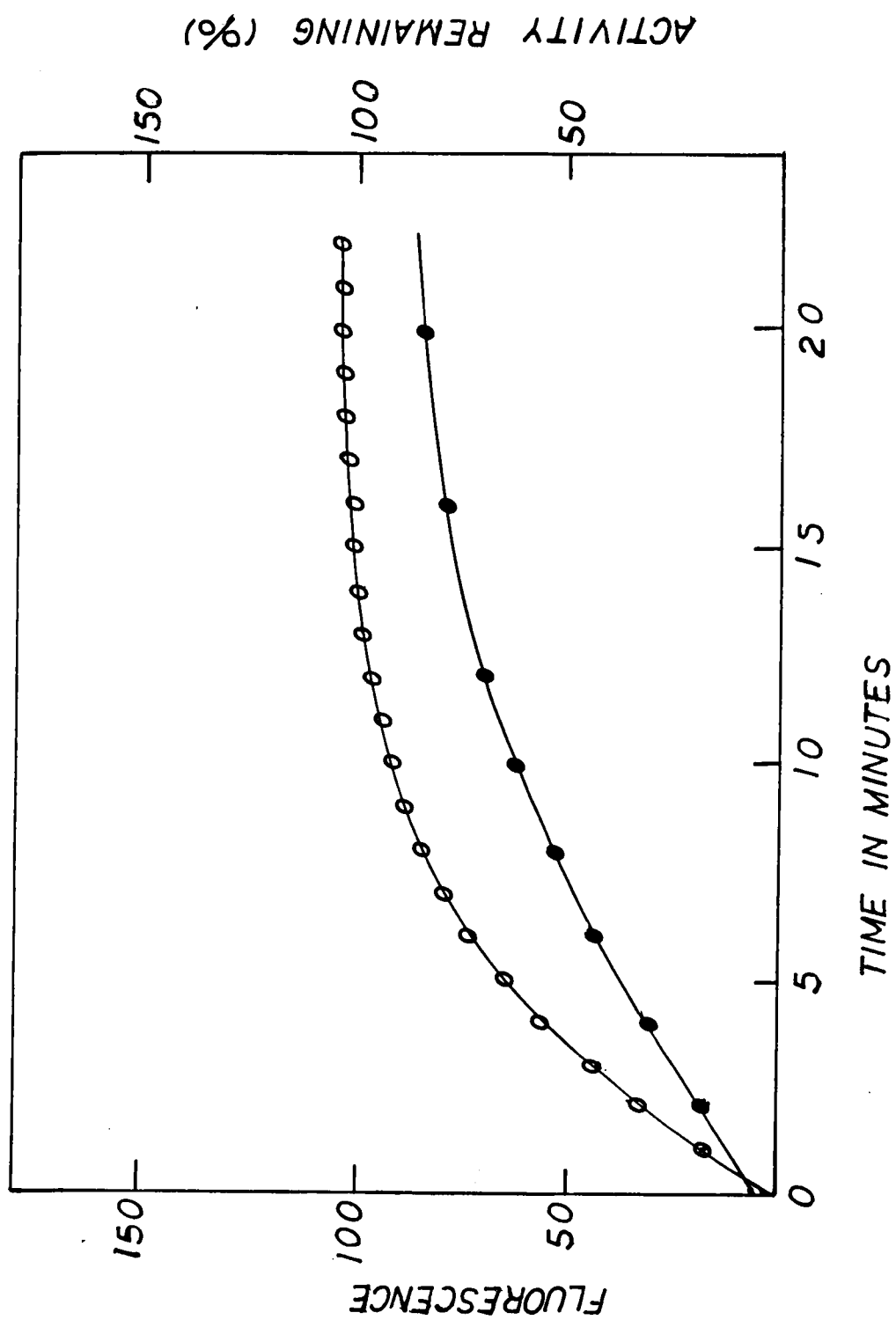


Figure 22

In an effort to demonstrate that pyridoxal-5'-phosphate is covalently bound to ϵ -amino groups of the protein, the enzyme ($2\mu\text{M}$) preincubated with PLP ($0.25\mu\text{M}$) at pH 8.4 for 60 minutes was reduced with NaBH_4 and dialyzed against several changes of 0.1M pyrophosphate buffer (pH 8.4). After dialysis, the reduced enzyme treated with PLP was examined by absorption and fluorescence spectroscopy. The spectrum of the PLP enzyme displayed a maximum of emission at around 395nm , due to the presence of P-pyridoxyl residues, whereas a sample of enzyme treated with NaBH_4 in the absence of PLP did not show any emission at 395nm upon excitation at 330nm . When the intensity of the fluorescence emitted by the PLP enzyme was compared to the fluorescence emitted by known concentrations of PLP- ϵ -lysine it was found that approximately 4 molecules of PLP have reacted per molecule of enzyme at pH 8.4.

The effects of NAD^+ and succinic semialdehyde on the inactivation of succinic semialdehyde dehydrogenase were examined at pH 8.4. The cofactor NAD^+ (5mM) as seen in Figure 23 produced a large decrease in the rate of inactivation, whereas succinic semialdehyde at a concentration of $5 \times 10^{-5}\text{M}$ did not provide significant protection.

Inactivation by o-phthalaldehyde. Inactivation of succinic semialdehyde dehydrogenase is also attained by addition of the reagent o-phthalaldehyde. The extent of the reaction of o-phthalaldehyde with primary amine groups of the enzyme is easily determined by measuring either the increase in absorbance at 335nm or the increase of fluorescence at 450nm . Free o-phthalaldehyde does not absorb at 335nm and

Figure 23. NAD^+ Protection Against Pyridoxal-5'-Phosphate Inactivation.

Succinic semialdehyde dehydrogenase ($2\mu\text{M}$) was incubated with a 300-fold excess of PLP at 37°C , pH 8.4 for varying time intervals. Results obtained in the absence ($\blacktriangle$) and presence ($\bullet$) of 5mM NAD^+ in the preincubation mixture. The decay in enzymatic activity of a sample of enzyme preincubated at 37°C in the absence of PLP and NAD^+ is given in the figure ($\blacksquare$).

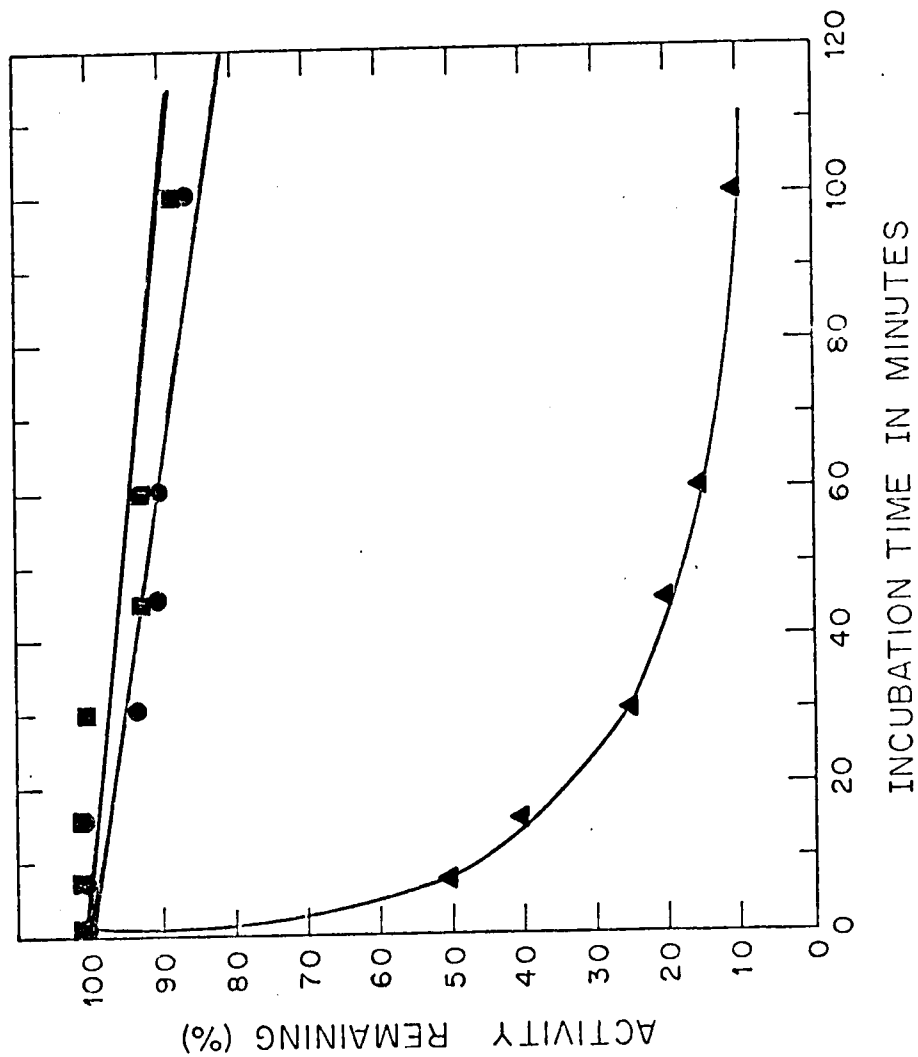


Figure 23

it does not emit any fluorescence over the spectral range of 400 to 500nm. The time course of the reaction of the enzyme with o-phthalaldehyde is given in Figure 24 where it may be seen that the formation of adduct follows monophasic kinetics. Under this set of experimental conditions, the binding of o-phthalaldehyde causes an irreversible loss of catalytic activity. Experiments, designed to correlate changes in enzymatic activity with the number of o-phthalaldehyde molecules reacted, revealed that complete inactivation of succinic semialdehyde dehydrogenase is achieved after the reaction of approximately 10 moles of o-phthalaldehyde per mole of enzyme.

Although the absorption spectrum of the modified enzyme resembles that of α -benzyloxycarbonyl lysine reacted with o-phthalaldehyde, it is difficult to prove that lysyl residues are the only amino acid residues modified, because the fluorescent adduct is easily decomposed at acid pH values, preventing any quantitative identification of the modified amino acids after acid hydrolysis of the protein. Thus, the possibility that the reaction of cysteinyl and arginyl residues with o-phthalaldehyde contributes to inactivation of succinic semialdehyde dehydrogenase cannot be ruled out at the present time.

III. DISCUSSION

An interesting aspect of this research is the finding that some aldehyde derivatives inhibit the enzyme by forming covalent linkages with amino acid residues. Pyridoxal-5'-phosphate at concentrations

Figure 24. o-Phthalaldehyde Inactivation of Succinic Semialdehyde Dehydrogenase.

A sample of enzyme ($2\mu\text{M}$) was incubated with o-phthalaldehyde ($50\mu\text{M}$) at 25°C and the reaction was monitored at 335nm (●). Aliquots withdrawn from the incubation mixture were tested for enzymatic activity (■). PROT, protein.

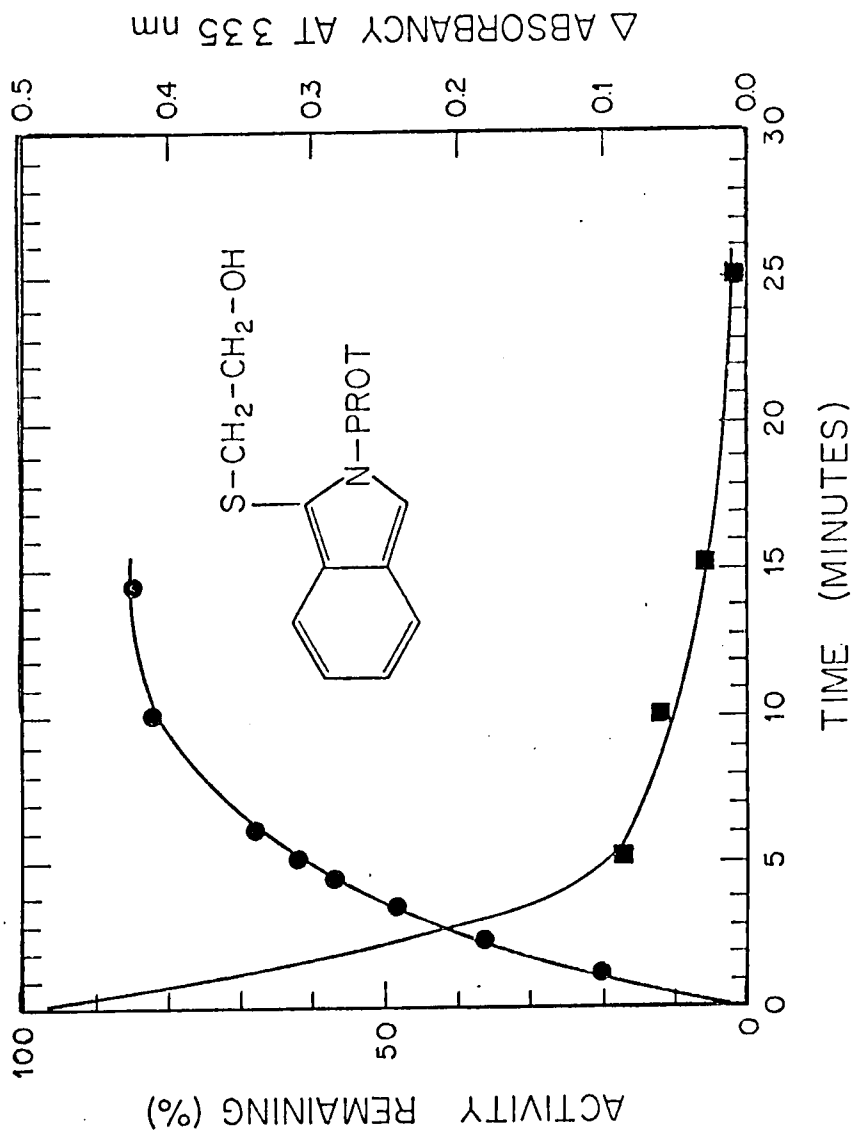


Figure 24

of 0.25mM inhibits the reaction catalyzed by the dehydrogenase. The requirement for phosphate as well as the aldehyde group of the PLP is shown by the fact that neither pyridoxal or pyridoxamine-5'-phosphate are effective inhibitors.

The evidence for Schiff base formation between pyridoxal-5'-phosphate and lysine is provided by the finding that addition of NaBH_4 to the enzyme-PLP complex results in the incorporation of the PLP residues as revealed by spectroscopic methods.

The nature of the inhibitory effect exerted by pyridoxal-5'-phosphate was studied in detail. The possibility that PLP inhibition is the result of the reaction of essential lysine residues critically connected with catalysis was investigated by performing inhibition studies in the presence and absence of NAD^+ . An increasing degree of inhibition was observed when the pH of the solution approached the pH optimum of enzymatic activity. At pH 8.4, the inhibitory effect of PLP was influenced by the cofactor NAD^+ at a concentration of 5mM. The nearly complete protection afforded by NAD^+ strongly suggests that inactivation occurred due to interaction with lysine residues at or near the cofactor binding site. In marked contrast to NAD^+ , succinic semialdehyde did not afford any protection against the inactivation by PLP.

The inactivation of succinic semialdehyde dehydrogenase by O-phthalaldehyde was rapid and irreversible. The adduct was unstable in acidic solution or after storage for 48 hours at 4°C in pyrophosphate buffer (pH 8.4). This instability limited the usefulness of

this reagent in distinguishing the essential residues of this enzyme. Although model compound studies suggest only lysyl residues are modified by o-phthalaldehyde, the possibility that other residues such as cysteinyl or arginyl side chains may be modified cannot be ruled out.

The inability of NaBH_4 to affect the activity of the dehydrogenase in the presence of inhibitory concentrations of succinic semialdehyde suggests the mechanism of substrate inhibition does not involve Schiff base formation between the semialdehyde and an amine group within the enzyme. This conclusion is consistent with the previously described data which shows glutaric semialdehyde is a less potent inhibitor than succinic semialdehyde; suggesting the chain length of the inhibitor is important to the substrate inhibition phenomena. Thus, it seems unlikely that succinic semialdehyde dehydrogenase is inhibited by substrate through a mechanism involving Schiff base formation with the enzyme.

One might ask if the inhibitory effect of pyridoxal-5'-phosphate has any in vivo significance. From a physiological point of view, the storage and regulation of PLP metabolism is dependent upon the content of multiple pyridoxal binding proteins and their individual affinities for PLP. For this coenzyme, it has been shown that in the mitochondrial and cytosol fractions of rat liver, most of the PLP is bound to three enzymes, i.e., alanine aminotransferase, aspartate aminotransferase and phosphorylase (44). Thus, the concentration of free PLP is very low in the cytosol and mitochondrial fractions of liver tissue. From a thermodynamic point of view, it is well established that the enzymes of the GABA shunt, 4-aminobutyric acid

transaminase and glutamate decarboxylase, bind PLP with association constants at least 10^3 greater than the association constant determined for PLP bound to succinic semialdehyde dehydrogenase (45). This implies that the enzymes of the GABA shunt are saturated at concentrations of PLP considerably lower than 0.1mM, the concentration required to inhibit succinic semialdehyde dehydrogenase.

Although it seems unlikely that the inhibition of the dehydrogenase by pyridoxal-5'-phosphate has any regulatory function, the reactivity of the carbonyl group of this coenzyme can be used conveniently to define the role played by lysine residues in the catalytic function of succinic semialdehyde dehydrogenase.

CHAPTER IV

THE BINDING OF NADH

In order to better understand the mechanism of action of succinic semialdehyde dehydrogenase, the binding of NADH to the enzyme was studied in detail. This cofactor is fluorescent and can be used as a probe to study substrate and product binding to the enzyme. Although NADH is not a substrate for the enzyme, it is a competitive inhibitor of NAD^+ as shown in Chapter II. Thus, NADH, a normal product of the succinic semialdehyde dehydrogenase reaction, can bind the enzyme and serve as a tool to study the enzyme properties of succinic semialdehyde dehydrogenase.

I. EXPERIMENTAL

Materials

All chemicals were purchased from sources described in the "Experimental" sections of previous chapters.

Methods

Fluorescence spectra were recorded in a precision spectrofluorimeter equipped with two Bausch and Lomb monochromators. The slits of the monochromators were set to give a band width of 3nm. Titrations of enhancement of NADH fluorescence were performed in the same spectrofluorimeter by adding small aliquots ($2\mu\text{l}$) of NADH to an enzyme solution ($3.0\mu\text{M}$) in 0.1M pyrophosphate buffer (pH 8.4) in a 3.0ml cuvette.

Polarization of fluorescence measurements were performed in an instrument built in the laboratory and described in detail elsewhere (46). Illumination was provided by a xenon lamp (150W) with wavelengths selected by a Quartz prism monochromator. The bandwidth for excitation was 5nm. Polarized emission light was passed through a C-S-3-72 Corning glass filter. The degree of polarization ($\bar{P}$) was measured with a precision of ± 0.02 . The emission anisotropy ($\bar{A}$) was calculated using Equation 1.

$$\bar{A} = \frac{2\bar{P}}{3 - \bar{P}} \quad (1)$$

Binding constants and the number of NADH binding sites were determined according to the technique of Scatchard as described by Montgomery and Swenson (47) for the polarization of fluorescence titration. The method of Hill as described by Gutfreund (48) was used to determine the number of binding sites and binding constants from the enhancement of NADH fluorescence data.

II. RESULTS

The strong binding of NADH to the dehydrogenase can be easily demonstrated by fluorescence emission spectroscopy. At an enzyme concentration of $3\mu\text{M}$ and NADH concentration of $6\mu\text{M}$, the binding of NADH to the dehydrogenase is accompanied by an enhancement of the fluorescence emitted over the spectral range 400 to 500nm (Figure 25). The binding of NADH also brings about a blue shift in the band position

of the emission spectrum. The fluorescence emitted by the binary complex enzyme - NADH is not influenced by the addition of succinic acid up to concentrations of 1mM. However, the addition of succinic semialdehyde to the binary complex at concentrations higher than those required to inhibit the enzyme (0.1mM) results in further fluorescence enhancement of bound NADH (Figure 25).

The interaction of NADH with succinic semialdehyde dehydrogenase is accompanied by an increase in the emission anisotropy. In the presence of excess enzyme (enzyme/NADH = 5) the emission anisotropy of bound NADH (A_B) approaches the limiting value of 0.33, which is significantly higher than the emission anisotropy of free NADH ($A_F = 0.11$).

This change in emission anisotropy values associated with binding of NADH to the dehydrogenase was used to determine the stoichiometry of binding. To this end, the emission anisotropy ($\bar{A}$) of samples containing fixed concentrations of enzyme (1 μ M) and varying concentrations of NADH was measured at constant temperature (10°C) using an excitation wavelength of 340nm. The results of these measurements are presented in Figure 26.

Since the emission anisotropy values at varying concentrations of NADH reflect the relative contribution of free and bound NADH to the fluorescence properties of the system, it is possible to calculate the fraction (α) of bound NADH by resorting to the following equation

$$\alpha = (\bar{A} - A_F) / (A_B - \bar{A})\beta + (\bar{A} - A_F) \quad (2)$$

Figure 25. Fluorescence of NADH in the Absence and Presence of Succinic Semialdehyde Dehydrogenase, Succinic Acid and Succinic Semialdehyde. 92

The fluorescence spectra of NADH ($6\mu\text{M}$) (1), NADH ($6\mu\text{M}$) + enzyme ($3\mu\text{M}$) (2), and NADH ($6\mu\text{M}$) + enzyme ($3\mu\text{M}$) + succinic semialdehyde (0.2mM) (3) at pH 8.4, excitation wavelength 340nm. The addition of 2mM succinic acid to the NADH ($6\mu\text{M}$) + enzyme ($3\mu\text{M}$) complex gave no additional enhancement of fluorescence (2).

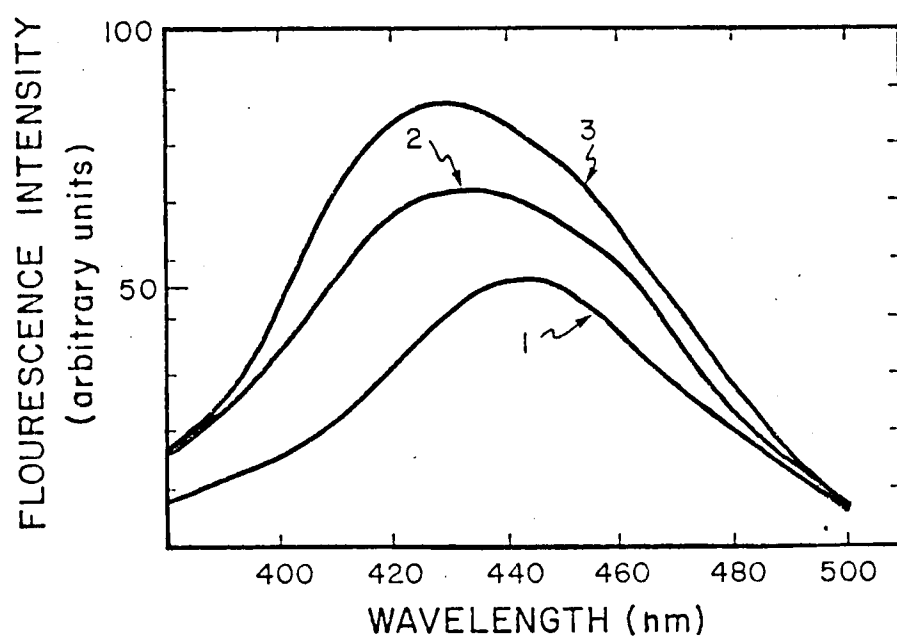


Figure 25

Figure 26. Emission Anisotropy Versus NADH Concentration.

Changes in emission anisotropy ($\bar{A}$) upon addition of NADH to a fixed concentration of enzyme ($1\mu\text{M}$) in 0.1M sodium pyrophosphate (pH 8.4) at 10°C .

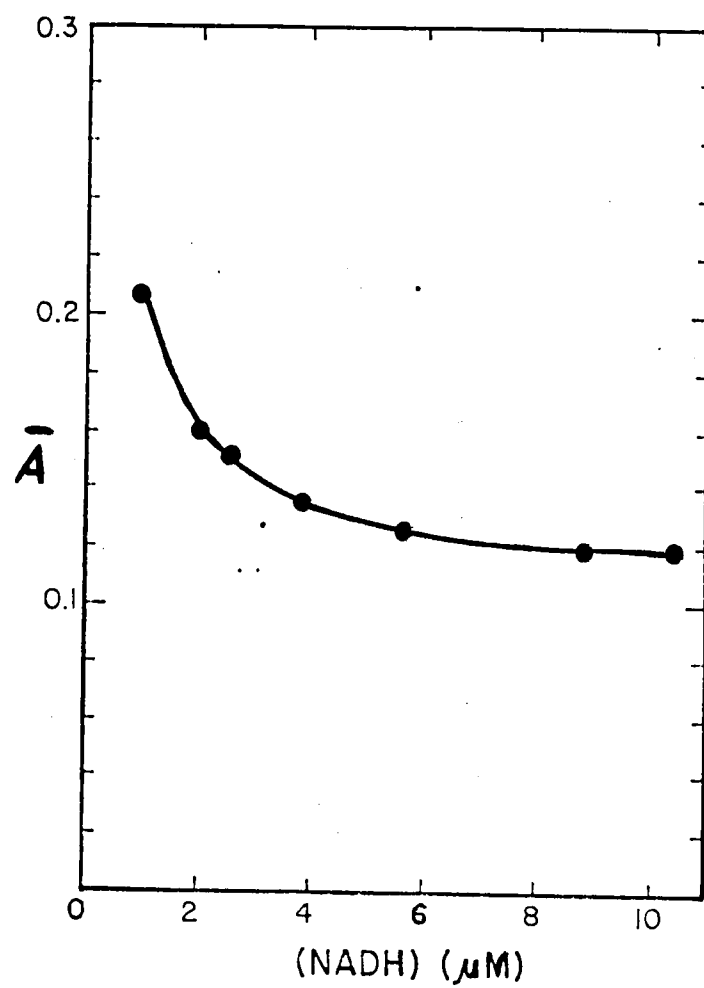


Figure 26

where $\beta = q_B/q_F$ is the ratio of fluorescence yields for bound and free NADH, respectively ($\beta = 3.0$).

The average number of NADH molecules bound ($\bar{v}$) per mole of enzyme was determined by the following equation

$$V = \alpha[L_0]/[P_0]$$

where L_0 represents total ligand and P_0 represents total enzyme concentration. When the titration results were analyzed by plotting $\bar{v}/[L]$ vs. $\bar{v}$, where $[L]$ is the concentration of free NADH, the linear plot depicted in Figure 27 was obtained. The horizontal intercept of this plot gave the number of binding sites per enzyme molecule (1.8) while the dissociation constant of $4.5\mu\text{M}$ was calculated from the vertical intercept.

The fluorescence enhancement that follows the addition of increasing concentrations of NADH to a fixed concentration of enzyme ($1\mu\text{M}$) was also used to determine the stoichiometry of binding (Figure 28). Results of the fluorometric titrations were analyzed by an equation of the form $\bar{V}/n - \bar{v} = K_D[\text{NADH}]$. The best fit of the data was obtained with $K_D = 3.6\mu\text{M}$ and $n = 1.8$ binding sites. Thus, both methods yield essentially the same information, i.e., two out of the four subunits of succinic semialdehyde dehydrogenase displays a strong affinity for NADH. However, the dissociation constant determined by fluorometric titrations is considerably lower than the inhibition constant ($K_I = 1 \times 10^{-4}\text{M}$) determined by enzymatic methods in Chapter II.

Figure 27. Scatchard Plot of Emission Anisotropy of NADH.

Plot of $\bar{V}/[L]$ versus $\bar{V}$. A dissociation constant of $4\mu\text{M}$ for two binding sites per tetramer is obtained.

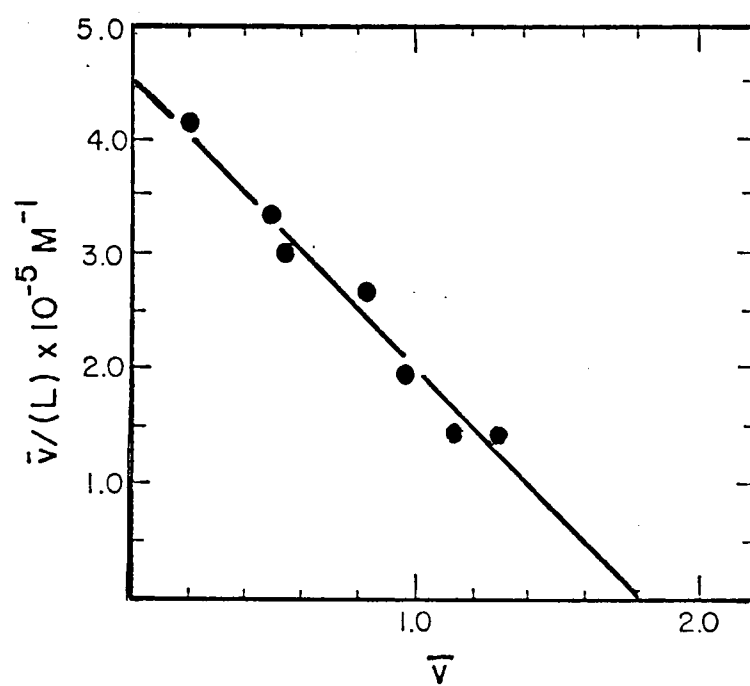


Figure 27

Figure 28. Titration of Fluorescence of NADH Binding to Succinic Semialdehyde Dehydrogenase.

The succinic semialdehyde dehydrogenase concentration was $3.0\mu\text{M}$. NADH was added in 2λ aliquots. The buffer was 0.1M pyrophosphate pH 8.4.

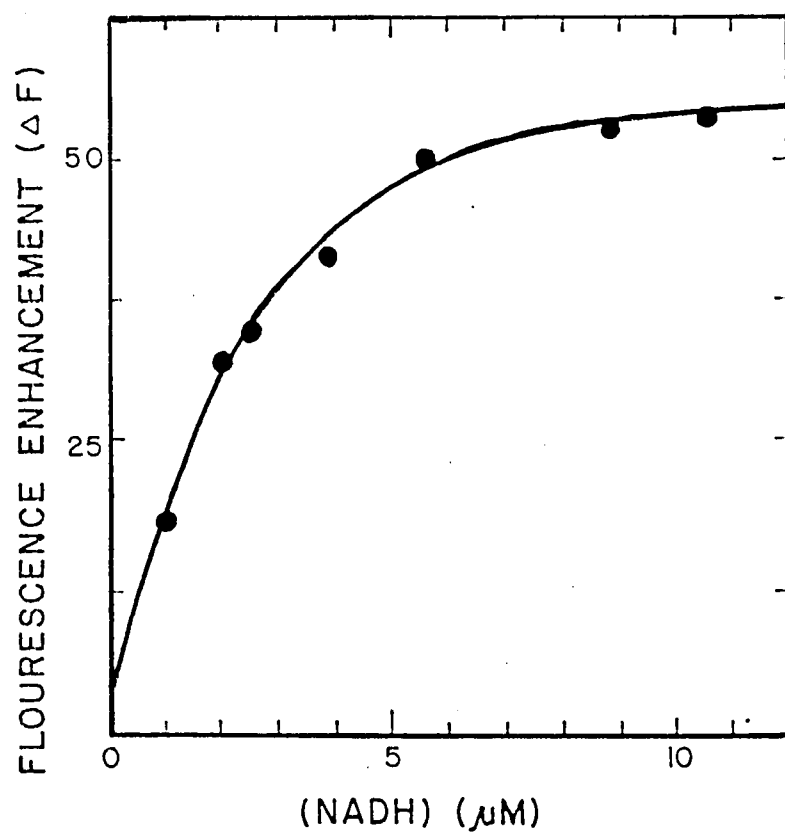


Figure 28

III. DISCUSSION

The strong binding of NADH to succinic semialdehyde dehydrogenase can be demonstrated by fluorescence spectroscopy. The fluorescence emitted by the binary complex, enzyme-NADH, is not influenced by addition of succinic acid at concentrations of 1mM. In marked contrast to succinic acid, succinic semialdehyde at inhibitory concentrations of 0.1mM induces further enhancement of the fluorescence emitted by the binary complex, a finding which is consistent with the concept that succinic semialdehyde exerts its inhibitory effect by binding at the catalytic site of the dehydrogenase.

The equilibrium state of the oligomeric structure of succinic semialdehyde dehydrogenase and the coenzyme NADH was quantitatively investigated by means of emission anisotropy measurements at protein concentrations of around $1\mu\text{M}$. The results of the titration experiments revealed that two molecules of NADH bind with the same dissociation constant ($4\mu\text{M}$) to the enzyme at pH 8.4. This unusual stoichiometry of binding can be explained in terms of two distinct mechanisms; a) a reversible dissociation of the tetrameric structure into a dimeric structure occurs upon addition of the ligand and b) protomer interactions in succinic semialdehyde dehydrogenase brings about extreme negative cooperativity between the binding sites.

A reversible dissociation of the tetrameric structure into two dimers may play an important role in the binding of the coenzyme NADH as well as in the regulation of the total enzyme activity. Examples of dissociation of oligomeric structures have been described in the literature (49).

However, the results of polyacrylamide gradient gel electrophoresis, gel filtration and sucrose density gradient centrifugation conducted at pH 8.4 in sodium pyrophosphate buffer containing 1mM 2-mercaptoethanol indicate that the tetrameric structure remains essentially stable over a wide range of enzyme concentrations (0.1-4 μ M). Furthermore, no hysteresis effect attributable to a slow association or dissociation of oligomeric structures could be detected in the enzymatic assays of succinic semialdehyde dehydrogenase at concentrations of enzyme below 0.005 μ M. In view of the above considerations, it appears unlikely that the asymmetry of NADH binding can be explained on the basis of a dimeric-tetrameric equilibrium, although such an equilibrium might exist under experimental conditions which differ from those chosen in the present studies.

The alternative mechanism, i.e., the binding of two molecules of NADH, induce a conformational change which affects the binding properties of the remaining unoccupied binding sites, could well explain the apparent asymmetry of binding as revealed by the fluorometric titrations.

Direct binding studies by equilibrium dialysis designed to demonstrate if there is a set of binding sites characterized by a low affinity constant for NADH at pH 8.4 are not possible because of the tendency of the enzyme to undergo irreversible activity losses upon dialysis at 4°C. This complication is avoided by measuring the inhibition of dehydrogenase activity by increasing concentrations of NADH. Initial velocity studies performed at pH 8.4

have shown NADH to be a competitive inhibitor with respect to NAD^+ with an apparent K_I of $100\mu\text{M}$, which is 25 fold greater than the dissociation constant determined by fluorometric titrations. Thus, it appears that there are other sites on the enzyme which become saturated at concentrations of NADH above 0.1mM leading to reversible inhibition of succinic semialdehyde dehydrogenase activity.

It should be noted that the reaction catalyzed by succinic semialdehyde dehydrogenase is essentially irreversible, and that any inhibitory effect exerted by NADH would result in accumulation of succinic semialdehyde which would further inhibit enzymatic activity. It is possible that accumulation of succinic semialdehyde in vivo is prevented by two independent enzymatic reactions catalyzed by 4-aminobutyric acid transaminase and by the NADPH linked dehydrogenase which interconverts succinic semialdehyde into 4-hydroxybutyric acid.

CHAPTER V

GENERAL DISCUSSION

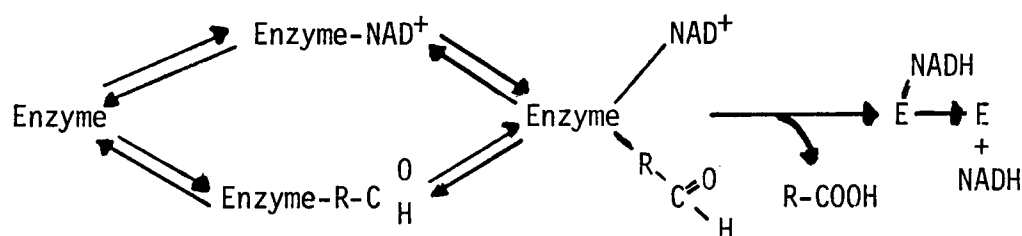
Porcine brain succinic semialdehyde dehydrogenase is a tetramer composed of four identical subunits. The molecular weight of this enzyme is 160,000. The reaction catalyzed by the enzyme is irreversible and NAD^+ specific. Glutaric semialdehyde and succinic semialdehyde are the only known substrates for this enzyme. The enzyme requires no metal cofactor.

The kinetic mechanism of succinic semialdehyde dehydrogenase as deduced from double reciprocal plots of velocity versus substrate concentration, product inhibition and dead end inhibition is a rapid equilibrium random bi bi mechanism. This mechanism predicts a random addition of substrates to the enzyme, an irreversible and rapid conversion of substrates to products immediately upon formation of a ternary enzyme $\cdot \text{NAD}^+$ $\cdot$ succinic semialdehyde complex and either an ordered or random release of product.

The prediction of irreversibility is certainly experimentally valid for succinic semialdehyde dehydrogenase.

The nature of product release might be further clarified from fluorescence data. NADH is able to bind the enzyme as evidenced by an enhancement in NADH fluorescence upon binding. Succinic semialdehyde is also able to bind this enzyme $\cdot$ NADH complex; however, succinic acid has no effect on the fluorescence of the enzyme $\cdot$ NADH complex. Thus, succinic acid is unable to bind a binary enzyme $\cdot$ NADH complex and form a ternary enzyme $\cdot$ products complex. This inability to

form a ternary complex would be consistent with an ordered release of products, with succinic acid released immediately upon formation followed by a later release of NADH. The overall reaction scheme given below would be consistent with the data concerning the succinic semialdehyde dehydrogenase reaction.



Chemically, the inability of succinic acid to bind succinic semialdehyde dehydrogenase could be explained if a negatively charged amino residue such as an aspartyl or glutamyl residues were located in the catalytic center near the site of succinic semialdehyde oxidation. This negatively charged residue would immediately repulse the newly formed negatively charged succinic acid and facilitate its rapid removal from the active center. This postulated amino acid having a negative charge would explain the irreversibility of the reaction, the inability of succinic acid to bind a binary enzyme-NADH complex and support an ordered release of products from the enzyme.

The chemical basis of substrate inhibition is still unclear. Data presented in this thesis indicates succinic semialdehyde does not inactivate the dehydrogenase through a reaction of its aldehyde group with a free primary amine. Additionally, the chain length of the semialdehyde affects the inhibitory properties of the substrate (glutaric semialdehyde $K_I = 3.4\text{mM}$; succinic semialdehyde $K_I = 0.09\text{mM}$).

One model for this substrate inhibition could involve the binding of two semialdehyde molecules to the enzyme; the first to the catalytic site and the second in a manner which either deforms the conformation of the enzyme or directly obscures NAD^+ from its binding site. This hypothesis, however, is difficult to prove.

The role of succinic semialdehyde in regulating this dehydrogenase is an interesting and important phenomena. The "GABA shunt" has been reported to handle up to 10% of the metabolic flux through the Krebs Cycle (50). No regulatory system for the three enzymes of the "GABA shunt" has been postulated, thus this potent substrate inhibition of succinic semialdehyde dehydrogenase may be the primary means of regulating the shunt pathway. The ability of succinic semialdehyde to fully inhibit the "GABA shunt" and possibly cause the formation of 4-hydroxybutyric acid may be of extreme physiological importance. Accumulation of succinic semialdehyde upon inhibition of the dehydrogenase would shift the equilibrium of the 4-aminobutyric acid transaminase reaction towards 4-aminobutyric acid formation; and formation of 4-hydroxybutyric could result in a sedating effect. The metabolism and formation of 4-hydroxybutyric acid is not well understood so the effect that inhibition of the dehydrogenase has on this compound's metabolism is in need of further clarification.

The finding that succinic semialdehyde dehydrogenase possesses binding sites for NADH with two different binding affinities is also of regulatory significance. Fluorescence measurements indicate two high affinity ($K_D = 4\mu\text{M}$) binding sites per enzyme molecule while inhibition studies indicate NADH inhibits the dehydrogenase with an

inhibition constant of $100\mu\text{M}$. The presence of these two types of binding sites suggests negative cooperativity in NADH binding and a regulatory role for NADH. The high affinity NADH binding possibly induces a conformational change in the enzyme which allows for the low affinity inhibitory binding of NADH. Since cellular NADH concentrations can be as high as 0.5mM (51), it is possible that NADH can shut down the succinic semialdehyde dehydrogenase reaction in times of high cellular catabolic activity.

The inhibitory properties of succinic semialdehyde and NADH when considered together could offer a means of fine control over the enzyme. As NADH starts to accumulate, it will begin to inhibit the dehydrogenase, which in turn will cause accumulation of succinic semialdehyde inhibiting the dehydrogenase further. The inhibition will be reversed by oxidation of NADH and conversion of succinic semialdehyde to 4-aminobutyric acid or 4-hydroxybutyric acid. Thus, a combination of substrate and product inhibition of succinic semialdehyde dehydrogenase might serve to modulate the "GABA shunt."

The finding that the purified 4-aminobutyric acid transaminase can be assayed validly in a coupled system with the purified dehydrogenase is useful. From the calculated values of "Q" listed in Table IV, it is possible to check the validity of any purified or partially purified assay system. Since the maximum velocity for the dehydrogenase is experimentally measurable, as is the effective velocity of the dehydrogenase in the presence of a given quantity of transaminase, the value of "Q" may be determined. Comparing this value with the

values of "Q" listed in Table IV on page 59 and demonstrated in Figure 18 on page 60, it is possible to ascertain the validity of the coupled system. Thus, any user of this coupled system can easily determine if he is truly assaying 4-aminobutyric acid transaminase.

The observations from the transient kinetics of the coupled assay indicate that the two purified enzymes, 4-aminobutyric acid transaminase and succinic semialdehyde dehydrogenase do not physically interact. Since these two enzymes are metabolically related and localized together in the mitochondria (52), a physical complex between them might be indicative of their in vivo behavior. This sort of physical complex between two similarly compartmentalized and metabolically related enzymes is not unusual; glutamate dehydrogenase-mitochondrial aspartic aminotransferase (53), aldolase-glyceraldehyde-3-phosphate dehydrogenase (54) and malate dehydrogenase-citrate lyase (55) all have been shown to participate in complex formation. However, succinic semialdehyde dehydrogenase and 4-aminobutyric transaminase do not appear to complex under the experimental conditions used in this study. If a complex formation did exist, it might be detected at higher concentration of both enzymes ($>2\mu\text{M}$); but the transient times of this coupled system at these concentrations are too small to detect accurately without resorting to stopped flow or other more sensitive techniques.

The chemical modification studies of succinic semialdehyde dehydrogenase with the lysine reagent pyridoxal-5'-phosphate demonstrate an essential lysine residue at or near the NAD^+ binding site. The

pH dependence of the inactivation and its overlap with the pH activation of the enzyme may possibly be due to one of two considerations. As the pH approaches the pH optimum, the conformation of the enzyme may open and allow NAD^+ or PLP access to the binding site; or the pH dependence may represent a titration of the removal of a proton from the ϵ -amino group of a lysine. The ϵ -amino groups of lysine residues in various enzymes have been shown to have pK values several units lower than the value of 10.5 normally associated with that of the ϵ -amino group of the free amino acid lysine (56). Since PLP will only react with unprotonated amine groups, the pH dependence of PLP inactivation may demonstrate the deprotonation of an ϵ -amino group of a lysine residue in the NAD^+ binding site and its subsequent reaction with PLP.

If research on this enzyme is to continue, one fruitful approach may be the use of systematic chemical modifications to elucidate the mechanism of catalysis of this enzyme. Preliminary experiments with *n*-bromosuccinimide indicate the enzyme is sensitive to this reagent suggesting an essential tryptophan residue. Furthermore, the enzyme is insensitive to the histidine reagent diethyl formate. Both of these results were not examined in depth and the possibility of other residues reacting with *n*-bromosuccinimide was not explored. The most interesting modification would be an attempt to demonstrate the negatively charged residue postulated earlier to repulse succinic acid from the catalytic site. This modification, however, is complicated by the fact that few reagents exist which react specifically with

glutamyl and aspartyl residues (57). Additionally, the role of cysteine in this dehydrogenase may be important, as judged by the rapid inactivation of the enzyme in the absence of thiol reducing groups.

PART TWO

RETINOL DEHYDROGENASE

CHAPTER I

PARTIAL PURIFICATION AND CHARACTERIZATION OF RETINOL DEHYDROGENASE FROM BOVINE RETINA

Retinol dehydrogenase of bovine retina rod outer segments catalyzes the interconversion of retinol and retinal as demonstrated in Figure 29. The enzyme is a membrane bound enzyme presumed to exist in the disc membrane of the rod outer segments, in close proximity to the visual pigment rhodopsin (Figure 30) (58). The interconversion of two forms of Vitamin A, retinal and retinol, is of critical importance in the visual cycle (Figure 31), in that Vitamin A is delivered to the eye as the alcohol retinol, while the form of Vitamin A in rhodopsin is the aldehyde retinal. Thus, retinol dehydrogenase plays a central role in the process of vision.

The enzyme has never been studied in a soluble system. Literature studies of this enzyme usually deal either with whole rod outer segments or with particulate matter generated from whole rod outer segments by homogenization in aqueous media. These studies indicate that the enzyme prefers NADP^+ over NAD^+ as a cofactor and catalyzes freely reversible reaction.

The substrates retinol and retinal are not water soluble. Experimentally, these two chemicals must be dissolved in organic solvents or detergents, or solubilized on a protein carrier. Thus, this system is complicated by the fact that both the enzyme and one substrate are not soluble in aqueous buffer systems.

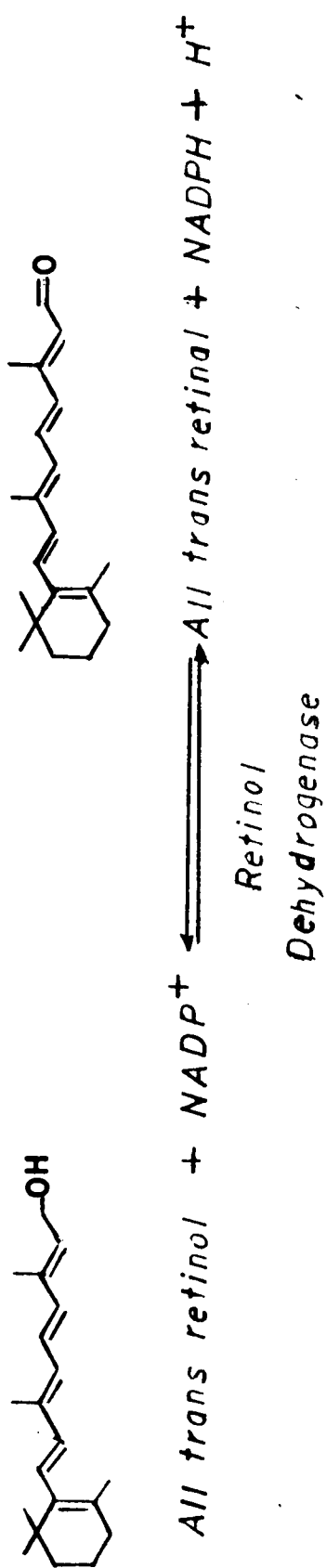


Figure 29. The Retinol Dehydrogenase Reaction

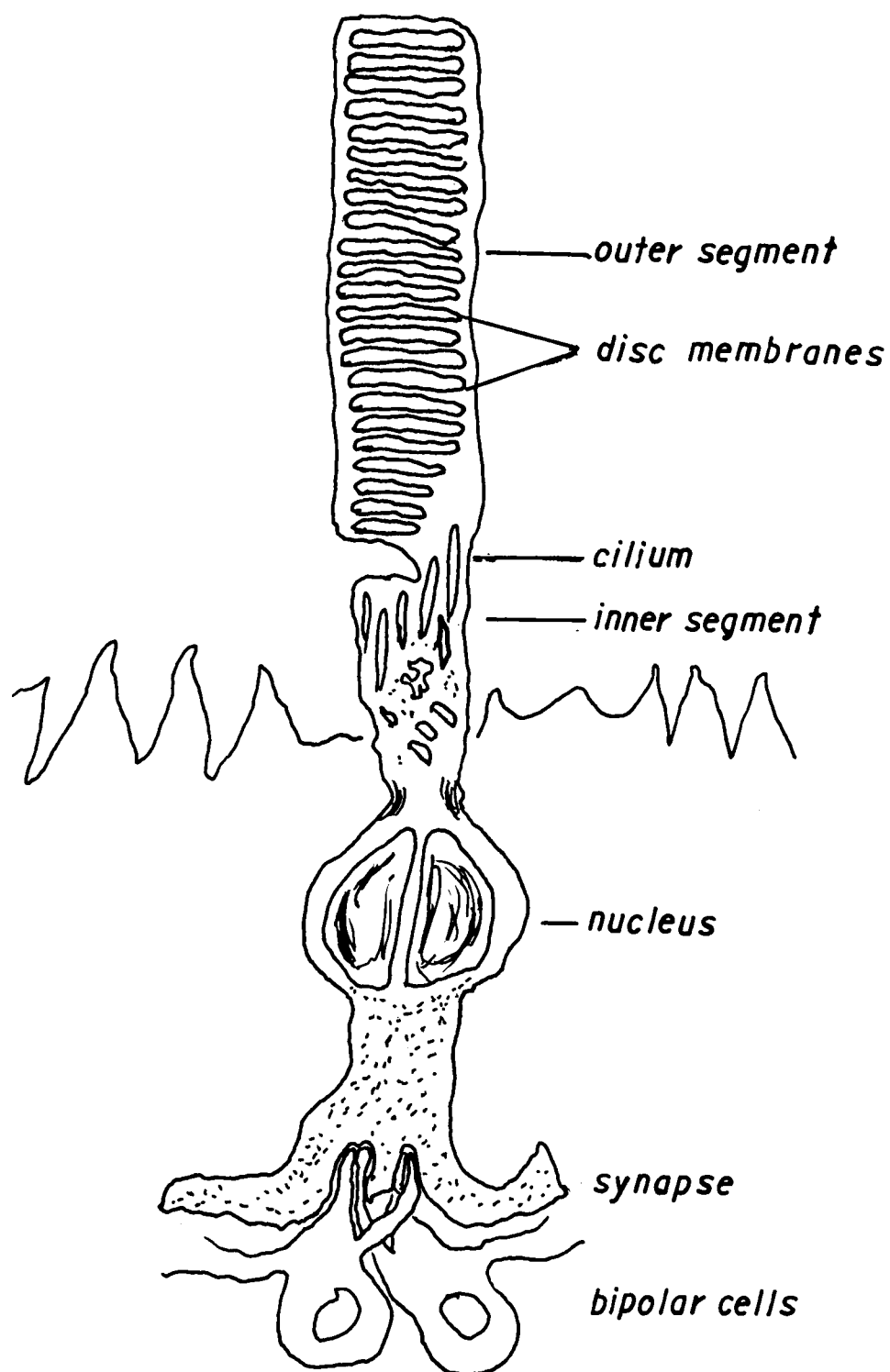


Figure 30. Rod Outer Segment Structure

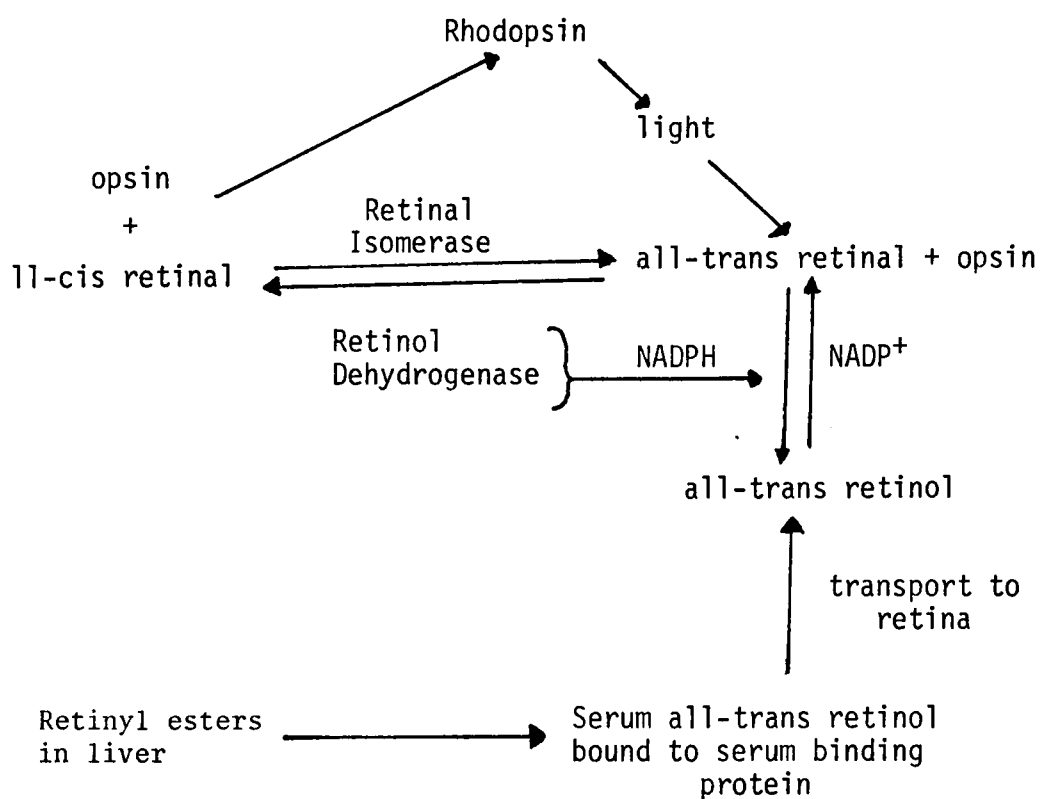


Figure 31. The Visual Cycle.

I. EXPERIMENTAL

Materials

All-trans retinal, all-trans retinol, 13-cis-retinal, NADP⁺, NADPH, NAD⁺, sodium cholate, 2-mercaptoethanol, AH-Sepharose, Pyridoxal-5'-phosphate, bovine serum albumin, Sephadex G-200 were all purchased from Sigma Chemical Co., St. Louis, MO. Monobasic and dibasic sodium phosphate, sodium pyrophosphate, all other inorganic salts used in buffers and all organic liquids were purchased from Fisher Chemical Co., Atlanta, GA. All other chemicals used in these studies were of reagent grade or better and purchased from major chemical distributors.

Methods

Handling of substrates. Stock samples of all-trans retinol, all-trans retinal or 13-cis-retinal were prepared in methanol. Exact concentrations of these solutions were determined spectrophotometrically using the molar extinction coefficients given by Thompson of 52,000, 51,400 and 51,000 M⁻¹cm⁻¹ for all-trans retinal, all-trans retinol and 13-cis-retinal respectively (59).

These stock solutions were then diluted in appropriate fashion to suit experimental requirements. The dilution was either with 0.1M pyrophosphate buffer (pH 8.6), 0.1M pyrophosphate buffer (pH 8.6) containing a pre-determined concentration of sodium cholate or 0.1M pyrophosphate buffer (pH 8.6) containing standardly 5mg/ml of bovine serum albumin (BSA).

All manipulations with the various forms of Vitamin A were done in dim light and all retinol or retinal solutions were wrapped in aluminum foil to protect them from light.

If all-trans retinol is added to a solution of buffer alone, aggregates of retinol are formed. To examine the nature of these aggregates 0.3ml of a 4mm all-trans retinol in methanol solution was added to 2.7ml of 0.1M pyrophosphate buffer (pH 6.8). This suspension immediately became milky in color. The suspension was chromatographed on a Sephadex G-200 column and two peak fractions were obtained, one excluded, the other retained. The molecular weight and aggregate size were obtained using the light scattering technique of Zimm as described by Tanford (60) in which the light scattering properties of a suspension are measured as a function of concentration and angle of scattering. This light scattering experiment was performed in a Brice Phoenix Series 2000 photometer. A discussion of light scattering theory appears in Appendix A.

Upon addition of any Vitamin A species to either a cholate-buffer or a bovine serum albumin (5mg/ml) buffer system, the vitamer was immediately solubilized as judged by the lack of turbidity of the solution. If, however, cholate was added to an all-trans retinol-BSA buffer mixture, the retinol could be displaced from the bovine serum albumin as evidenced by monitoring the fluorescence of bound retinol. Cholate was added in small aliquots (3 μ l) to 3.0ml of a 10 μ M solution of all-trans retinol solubilized in standard BSA-pyrophosphate buffer. The decrease in fluorescence intensity at 465nm was observed on the same fluorimeter described in PART ONE of this thesis. The exciting light was at a wavelength of 340nm.

Assay conditions for retinol dehydrogenase. All assays were performed spectrophotometrically on an Aminco DW-2 spectrophotometer. The 0.1 full-scale absorbance range was used for all assays, and generally an absorbance change of 0.02 to 0.07 absorbance units in five minutes was observed.

If retinol formation was to be assayed, the absorbance decrease at 430nm due to retinal consumption was monitored. The molar extinction coefficient of all-trans retinal in standard BSA-buffer at 430nm is $18,000\text{m}^{-1}\text{cm}^{-1}$. A standard assay mixture consisted of 0.1ml of enzyme in 10mM sodium phosphate buffer (pH 7.0) containing 0.4% (w/v) cholate, 0.8ml of 0.1mM all-trans retinal in standard BSA-phosphate buffer (pH 6.5), and 0.1ml of 1mM NADPH in phosphate buffer (pH 6.5). The reaction was always blanked against the same mixture lacking only the NADPH.

The formation of retinal was the preferred assay used throughout this study and henceforth will be referred to as the standard assay. Retinal formation was monitored at 390nm, at which wavelength it has a molar extinction coefficient in standard BSA-buffer of 33,800. The standard assay mixture consisted of 0.1ml of enzyme in 10mM sodium phosphate (pH 7.0) containing 0.4% (w/v) cholate, 0.8ml of 0.1mM all-trans retinol in standard BSA-pyrophosphate buffer (pH 8.6), and 0.1ml of 1mM NADP^+ . The assay was always blanked against the same mixture of reagents excluding only the NADP^+ . The reaction was always begun by addition of enzyme solution. At the concentrations of NADPH formed in this assay no contribution to the change in absorbancy at 390nm is made by the NADPH.

Determination of the equilibrium constant for retinol dehydrogenase. The equilibrium constant at 25°C was determined for all-trans-retinol formation. The assay used to measure retinol formation, modified only in that the reference cuvette contained no retinol, was allowed to go to completion (in about 2 hours). From the final absorbance readings, the known initial concentrations of substrates and the final pH, it is possible to calculate an equilibrium constant for the reaction. The achievement of equilibrium was demonstrated when addition of more enzyme had no effect on the final absorbance value. Addition of substrates restarted the reaction, indicating the presence of active enzyme.

Purification of rod outer segments. Retinas were isolated within one hour after slaughter from bovine eyes. The retinas were gently removed from the eye sockets with forceps and placed in tubes for storage at -12°C for up to one month.

Rod outer segments (ROS) were removed from a volume of retinas by gentle shaking in an equal volume of 34% (w/v) sucrose in 0.1M phosphate (pH 7.0). After two minutes of shaking, the preparation was centrifuged at 2000 x g for four minutes. The supernatant containing the ROS was removed and saved. The pellet was once again subjected to shaking in the same sucrose solution and was centrifuged as before. This supernatant was added to the first, and two volumes of 0.01M phosphate buffer (pH 7.0) were added to the combined ROS-containing supernatants. This mixture was then centrifuged at 2000 x g for 10 minutes. The pellet after this spin contained the crude ROS. These ROS were resuspended in a small volume of 0.01M phosphate

(pH 7.0) and layered over a discontinuous sucrose gradient in 0.01M phosphate (pH 7.0) of 0.77m, 0.84m, 1.0m and 1.14m sucrose. The gradients were spun in an SW27 rotor at 100,000 x g for one hour at 4°C. The purified ROS were carefully collected with a syringe from the interface of the 0.84 and 1.0M layers. The purified ROS were diluted with two volumes of ice cold distilled water and recentrifuged at 15,000 x g for 30 minutes. The bright yellow pellet of ROS was frozen at -12°C for up to one month.

A flow chart of this purification scheme is given in Figure 32.

Solubilization of retinol dehydrogenase. To the rod outer segments from twenty retinas 3.0ml of cold 10mM phosphate buffer (pH 7.0) containing 1.0% (w/v) sodium cholate and 1mM 2-mercaptoethanol was added. The suspended ROS was then homogenized by 30 strokes in a Dounce homogenizer. This homogenate was then sonicated for 10 minutes in a Bronson W-75 ruby-tipped sonicator at 100 watts power and 70% power cycle. The sample during sonication was in a water-jacketed holder kept at 4°C and flushed continuously with argon to prevent oxidation of the sample.

This sonicate was then centrifuged at 100,000 x g for 30 minutes in a Beckman 50ti rotor. The intense yellow supernatant contained the retinol dehydrogenase activity. Some activity remained with the pellet; however, the specific activity in this fraction was always ten times less than that of the supernatant, and was therefore discarded.

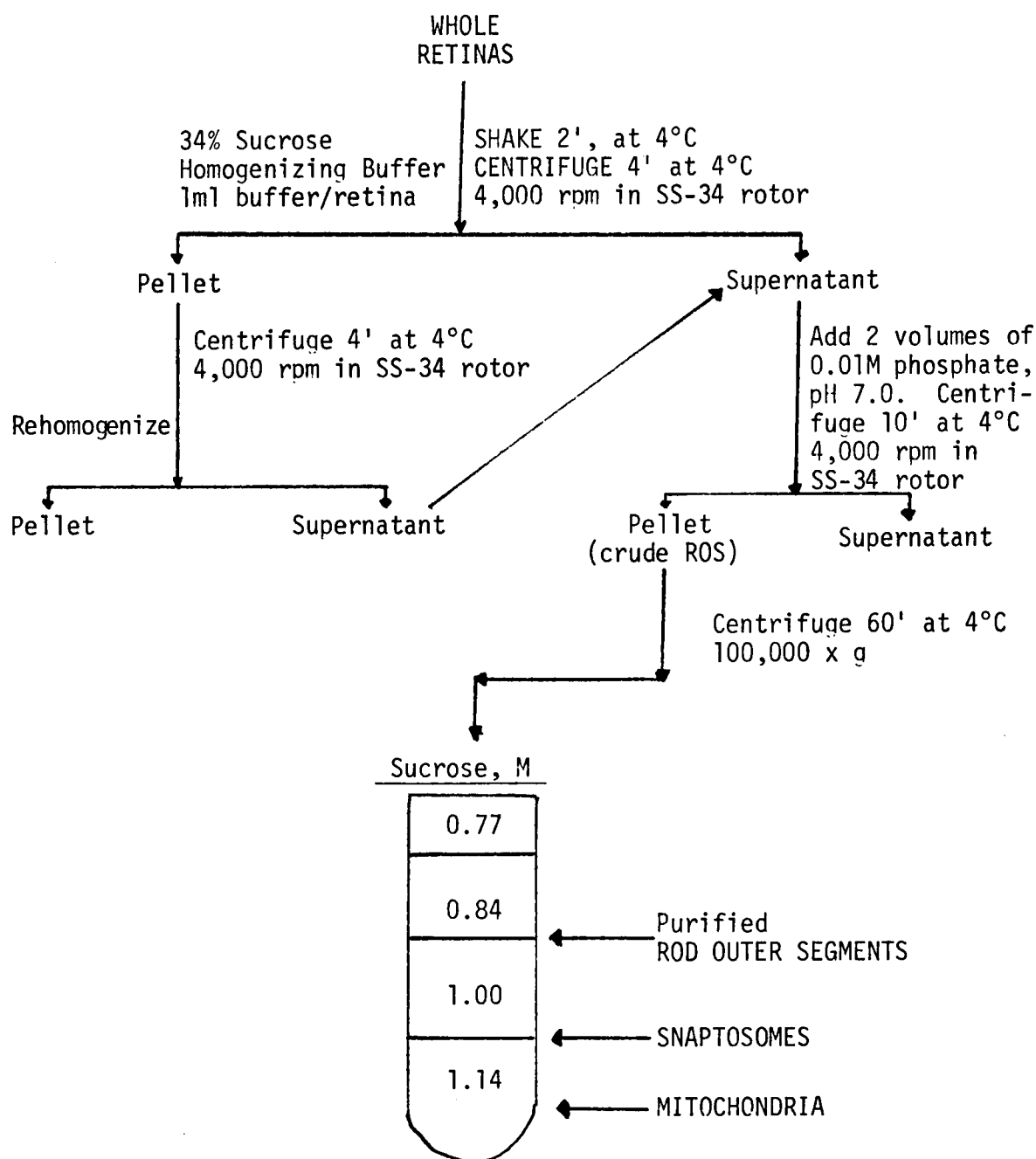


Figure 32. Purification of Rod Outer Segment

Partial purification of retinol dehydrogenase. Hydroxyapatite was prepared by the method of Levin (4), a 1.6 x 10cm column was equilibrated with 200ml of 10mM phosphate (pH 7.0) buffer containing no other additions. The solubilized supernatant was applied directly to the column. Elution of the enzymatic activity from the column was accomplished with a linear phosphate gradient (pH 7.0) from 0.1M - 0.6M. This elution buffer was also 0.4% (w/v) with sodium cholate and 1mM in 2-mercaptoethanol. The enzymatic activity eluted as a broad band between 0.4 - 0.5M phosphate.

This eluted enzyme was then concentrated in an Amicon concentrator using a PM-30 membrane. The concentrated sample was washed with three successive washes of one volume equal to the sample volume of 10mM phosphate (pH 7.0) containing 0.4% (w/v) cholate and 1mM 2-mercaptoethanol. This washing process lowers the ionic strength of the enzyme solution to around 50mM phosphate.

A retinyl-sepharose affinity gel was prepared according to the following procedure. Sepharose AH (1.0gm) was swollen in 10.0ml of distilled water for several hours at room temperature. After swelling, the sample was washed with 250ml of distilled water and 250ml of 0.1M NaHCO_3 containing 0.5M NaCl. To the swollen and washed Sepharose AH suspended in 10ml of 0.1M NaHCO_3 containing 0.5M NaCl was added 1.0ml of a 2mM all-trans retinol in methanol. The reaction vessel was covered with aluminum foil to protect retinal from light and was incubated for 90 minutes at 37°C. The sample was then left at 4°C overnight. Next, 40-50mg of NaBH_4 was added to the sample

and allowed to incubate at 4°C for one hour. The reduced sample was first washed with 250ml of 0.1M NaHCO₃ containing 0.5M NaCl, followed by 250ml of 0.1M sodium acetate (pH 5.0) containing 0.5M NaCl and finally with 250ml of 0.1M NaHCO₃ containing 0.5M NaCl.

This retinyl-sepharose was packed in a 0.9 x 10cm aluminum-foil-wrapped column and washed with 250ml 10mM phosphate (pH 7.0). The reconcentrated and washed enzyme preparation was applied to this column and eluted with a step gradient of NaCl in 10mM phosphate (pH 7.0) containing 0.4% (w/v) cholate and 1mM 2-mercaptoethanol. The activity eluted with 0.7M NaCl.

The retinyl-sepharose was washed with 200ml of 2.0M NaCl and one liter of 10mM phosphate (pH 7.0). However, each successive use of this affinity gel resulted in a 40% loss in the retention capacity of the gel, and only three uses of the gel were possible before the gel was discarded.

Determination of kinetic constants for retinol dehydrogenase.

Michaelis constants for the substrates of this enzyme were determined graphically from double reciprocal plots of initial velocity versus substrate concentration. The two assay systems described earlier were used to determine the Michaelis constants for NADP⁺, NADPH, all-trans-retinal and all-trans retinol.

Covalent binding of all-trans retinal to retinol dehydrogenase.

Enzyme samples were preincubated with 0.1mM all-trans retinal for 15 minutes at 37°C. To these samples several grains of NaBH₄ were

added and allowed to react at 4°C for one hour. After this time, all bubbling stopped, indicating the completion of the reaction. The samples were then assayed for activity to determine if this reduction with NaBH_4 results in activity loss.

Inactivation of retinol dehydrogenase with pyridoxal-5'-phosphate.

Pyridoxal-5'-phosphate (PLP) was incubated with the partially purified enzyme for varying time intervals. The incubations were at 25°C in 0.1M phosphate buffer (pH 6.5) containing 0.4% (w/v) sodium cholate. After a given period of preincubation, the samples were assayed by standard methods.

II. RESULTS

All-trans retinol aggregates formed upon addition to aqueous buffers. All-trans retinol solubilized in methanol forms aggregates immediately upon addition to 0.1M pyrophosphate buffer (pH 8.5). These aggregates can be separated into two classes by Sephadex G-200 gel exclusion chromatography. One class, comprising about 25% of the total applied retinol, passes through the column in the void volume. The other fraction is retained by the column and elutes as a broad band at a position one would find a spherical molecule of 80,000 molecular weight eluting from such a column. An elution profile of these aggregates from the Sephadex G-200 column is given in Figure 33.

The excluded aggregate and the central peak fractions from the included aggregate were subjected to a light scattering determination of their molecular weights. Zimm plots for both aggregates are

Figure 33. Elution Profile of All-Trans Retinol from Sephadex G-200 Chromatography.

The all-trans retinol was eluted in 0.1M pyrophosphate buffer pH 8.4. Fractions of 1.0ml were collected.

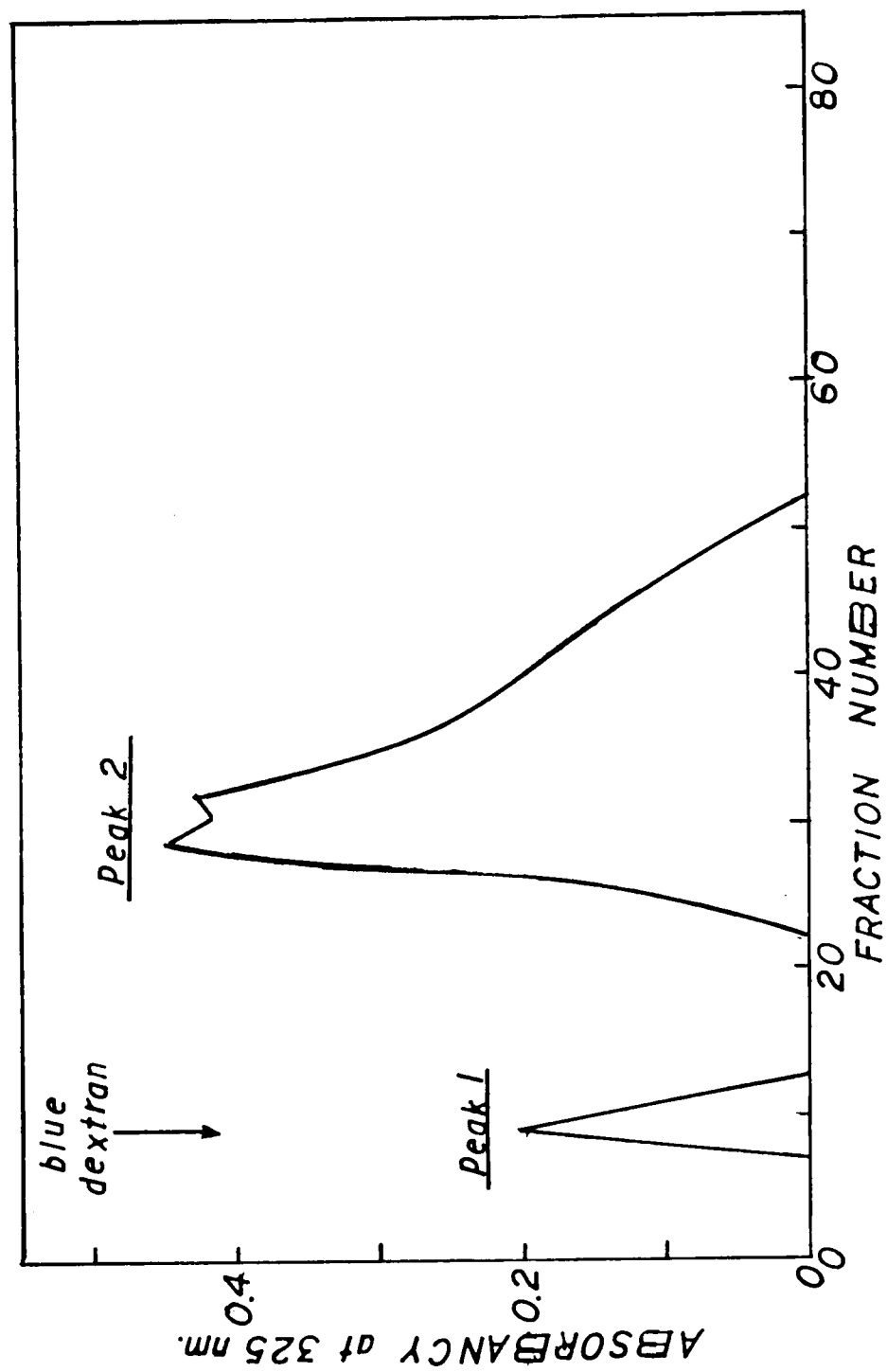


Figure 33

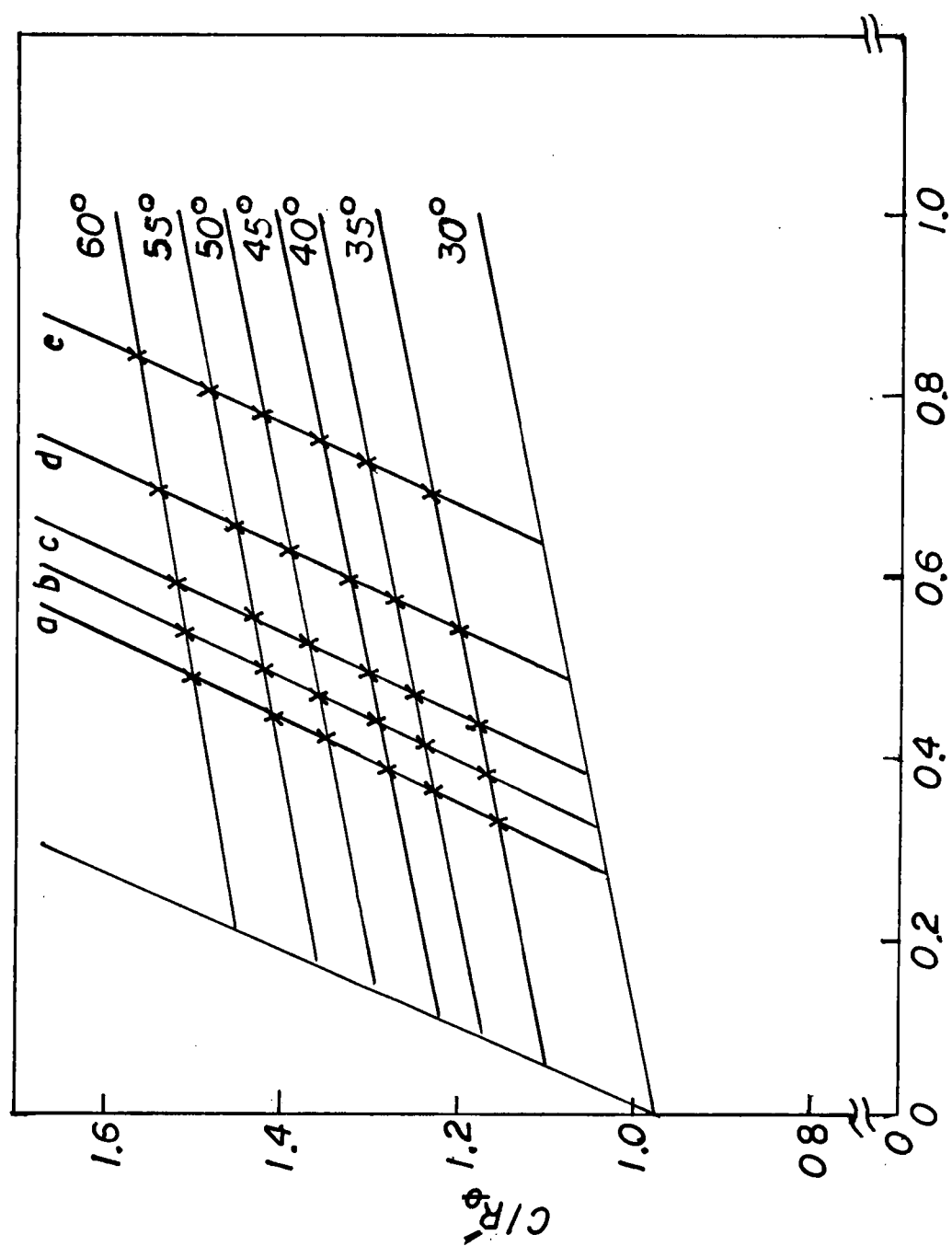
included in Figures 34 and 35. The larger aggregates had a molecular weight of 9,000,000, as determined from the Zimm plots; while the included retinol aggregate showed a molecular weight of 120,000.

Assay conditions for retinol dehydrogenase. The means of solubilizing either all-trans retinal or all-trans retinol for the assay of retinol dehydrogenase is of extreme importance. Table V lists the effects various means of solubilizing the substrate has on the activity of the enzyme. If all-trans retinol (0.1mM) was assayed with the protein carriers bovine serum albumin (5mg/ml) or β -lactalbumin (5mg/ml) maximal activity was observed. The protein carriers ovalbumin (5mg/ml) and γ -globulin (5mg/ml) were less effective carriers for the substrates than were BSA or β -lactalbumin. Solubilization of Vitamin A in various concentrations of the detergents cholate, lubrol WX and digitonin was still less effective than using a protein carrier, in terms of substrate activity for retinol dehydrogenase. Neither the mixed sized aggregates formed from addition of retinol to pyrophosphate buffer nor the separated aggregates from Sephadex G-200 chromatography were substrates for the dehydrogenase. Addition of BSA or detergents to these aggregates did not change the inability of the enzyme to metabolize this form of the substrate.

Effect of the detergent sodium cholate on the reaction catalyzed by retinol dehydrogenase. Figure 36 demonstrates that sodium cholate, which is necessary to solubilize the enzyme, also has an inhibitory

Figure 34. Zimm Plot of Light Scattering Data from Peak 1
All-Trans Retinol Aggregates from Sephadex G-200 Chromatography.

The vertical lines labeled a, b, c, d and e respectively represent 0.27, 0.31, 0.38, 0.48 and 0.64 μ grams of all-trans retinol per milliliter.

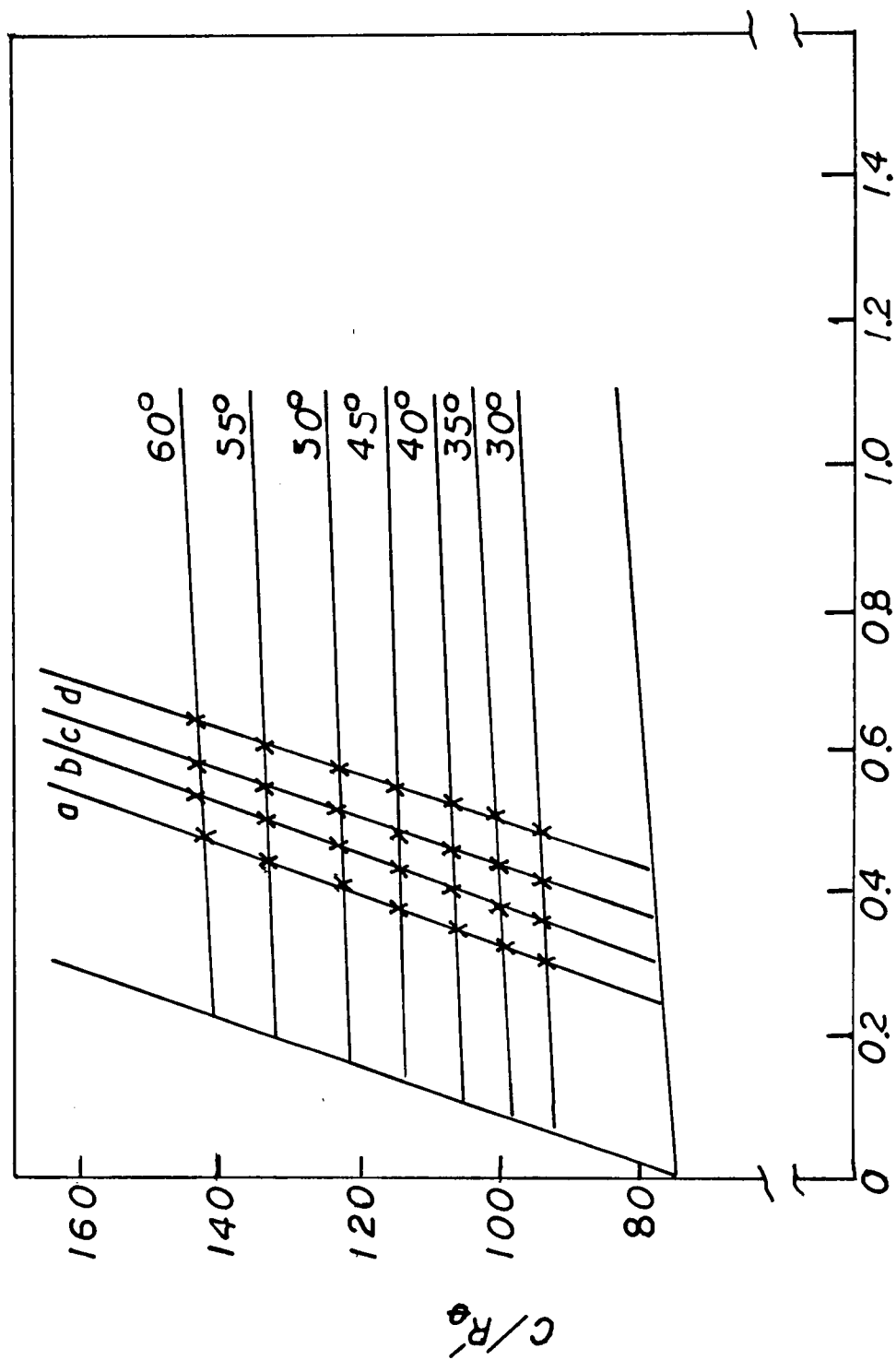


$$\sin^2 \phi / 2 + 10^6 C$$

Figure 34

Figure 35. Zimm Plot of Light Scattering Data from Peak 2 All-Trans Retinol Aggregates from Sephadex G-200 Chromatography.

The vertical lines labeled a, b, c and d respectively represent 2.5, 2.9, 3.5 and 4.4 μ grams of all-trans retinol per milliliter.



$\sin^2 \phi/2 + 10^5 C$
Figure 35

TABLE V

RELATIVE ACTIVITIES OF RETINOL DEHYDROGENASE TOWARDS
ALL-TRANS RETINOL SOLUBILIZED BY VARIOUS TECHNIQUES

Form of Retinol as a Substrate	Relative Maximum Velocity
1. Retinol (10^{-4} M) in buffer only	
A) Peak I aggregates	0%
B) Peak II aggregates	0%
C) Combined aggregates	0%
D) Peak I + 5mg/ml BSA	0%
E) Peak II + 5mg/ml BSA	0%
F) Combined + 5mg/ml BSA	0%
2. Retinol (10^{-4} M) in detergents	
A) Cholate (0.4%)	17%
B) Cholate (0.4%) + 5mg/ml BSA	25%
C) Lubrol (0.1%)	40%
D) Lubrol (1.0%)	17%
E) Digitonin (0.1%)	35%
F) Digitonin (1.0%)	11%
3. Retinol (10^{-4} M) + protein carrier (5mg/ml)	
A) Bovine serum albumin	100%
B) β -lactoglobulin	90%
C) Ovalbumin	25%
D) γ -globulin	17%

(Specific activity of enzyme = 1.0 nmoles/min/mg)

Figure 36. Cholate Inhibition of Retinol Dehydrogenase Activity.

Aliquots of sodium cholate were added from a stock solution of 0.1M sodium cholate. The enzyme was in 0.1M pyrophosphate buffer pH 8.6.

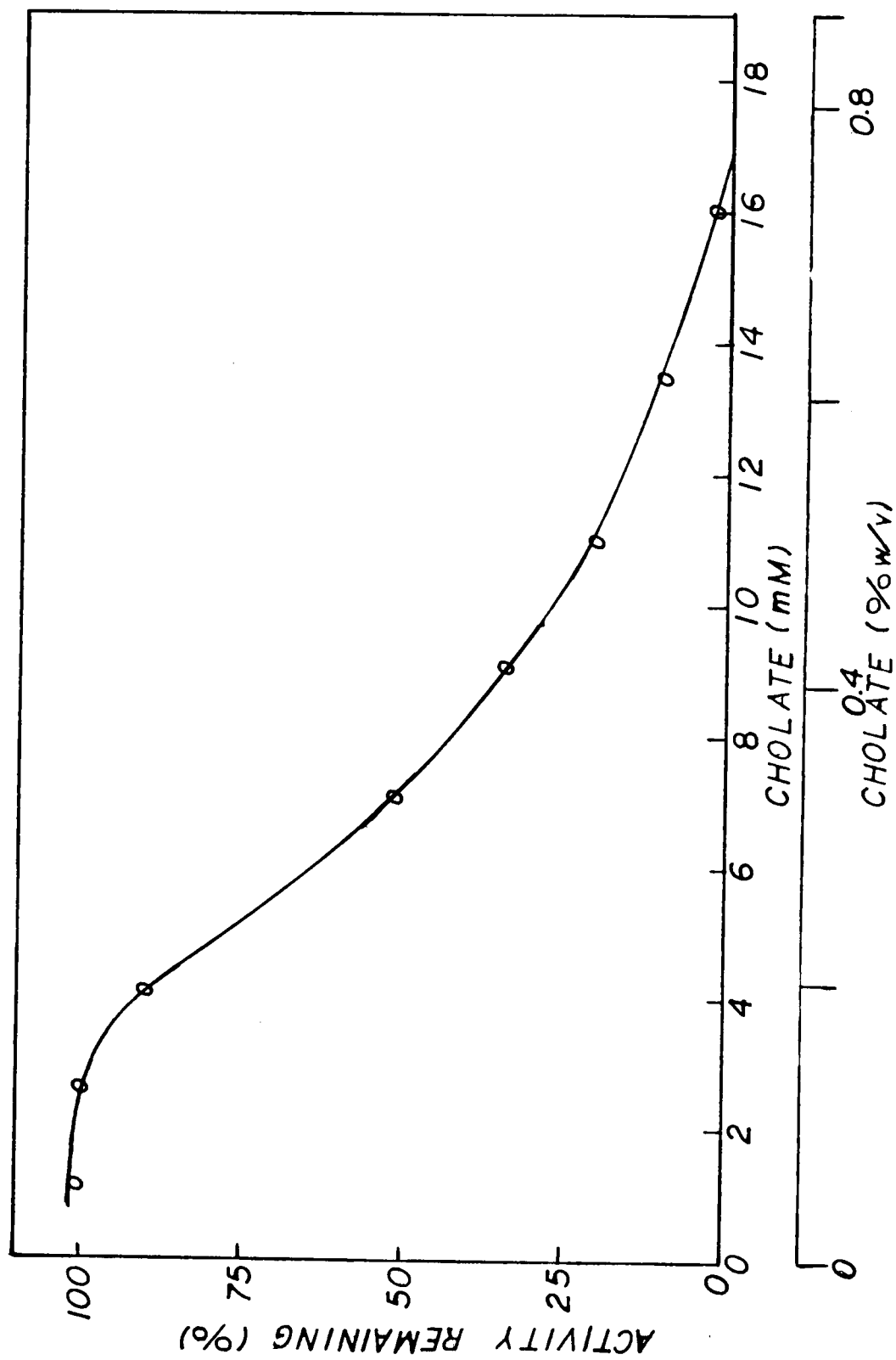


Figure 36

effect on the enzyme at concentrations greater than 0.1% (w/v). Additionally, sodium cholate can displace retinol bound to the protein carrier, BSA, as evidenced by the decrease in the enhancement of retinol fluorescence in Figure 37. The detergents triton X-100 and tween-80 completely inhibit enzymatic activity. The detergents lubrol WX and digitonin inhibit the enzyme in a manner similar to cholate.

Partial purification of retinol dehydrogenase. Table VI gives the partial purification and recovery scheme for retinol dehydrogenase. The enzyme can be purified about twenty fold over the crude homogenate. The initial step of purification, hydroxyapatite chromatography, gives an eight to 10 fold purification and the subsequent retinyl-sepharose chromatography results in an additional two to three fold purification. If these two steps are reversed, the same degree of purification is obtained.

Many different purification steps were attempted to purify this enzyme, but with little success. Ammonium sulfate and ethanol precipitation both inactivate the enzyme. DEAE-sephadex, AMP-sepharose and 2'5'ADP sepharose all failed to effectively retain the retinol dehydrogenase. Phenyl-sepharose bound the enzyme so tightly it could not be eluted by drastic changes in pH or ionic strength, addition of detergents, kaotropic agents or combinations of these changes. Con-cannavalin A-sepharose did not bind retinol dehydrogenase and retained many additional contaminating proteins; however, this step, when coupled with the two successful steps listed above, resulted in no additional purification---presumably concannavalin A and the above steps remove the same proteins.

Figure 37. Displacement of Retinol from the Protein Carrier Bovine Serum Albumin.

The upper curve (~~—O—O—O—~~) represents the decrease in retinol ($10\mu\text{M}$) fluorescence in the presence of bovine serum albumin ($10\mu\text{M}$). The lower curve represents the increase in retinol ($10\mu\text{M}$) fluorescence dispersed in buffer. The buffer was 0.1M pyrophosphate pH 8.6. Excitation wavelength was 340nm and emission was read at 465nm.

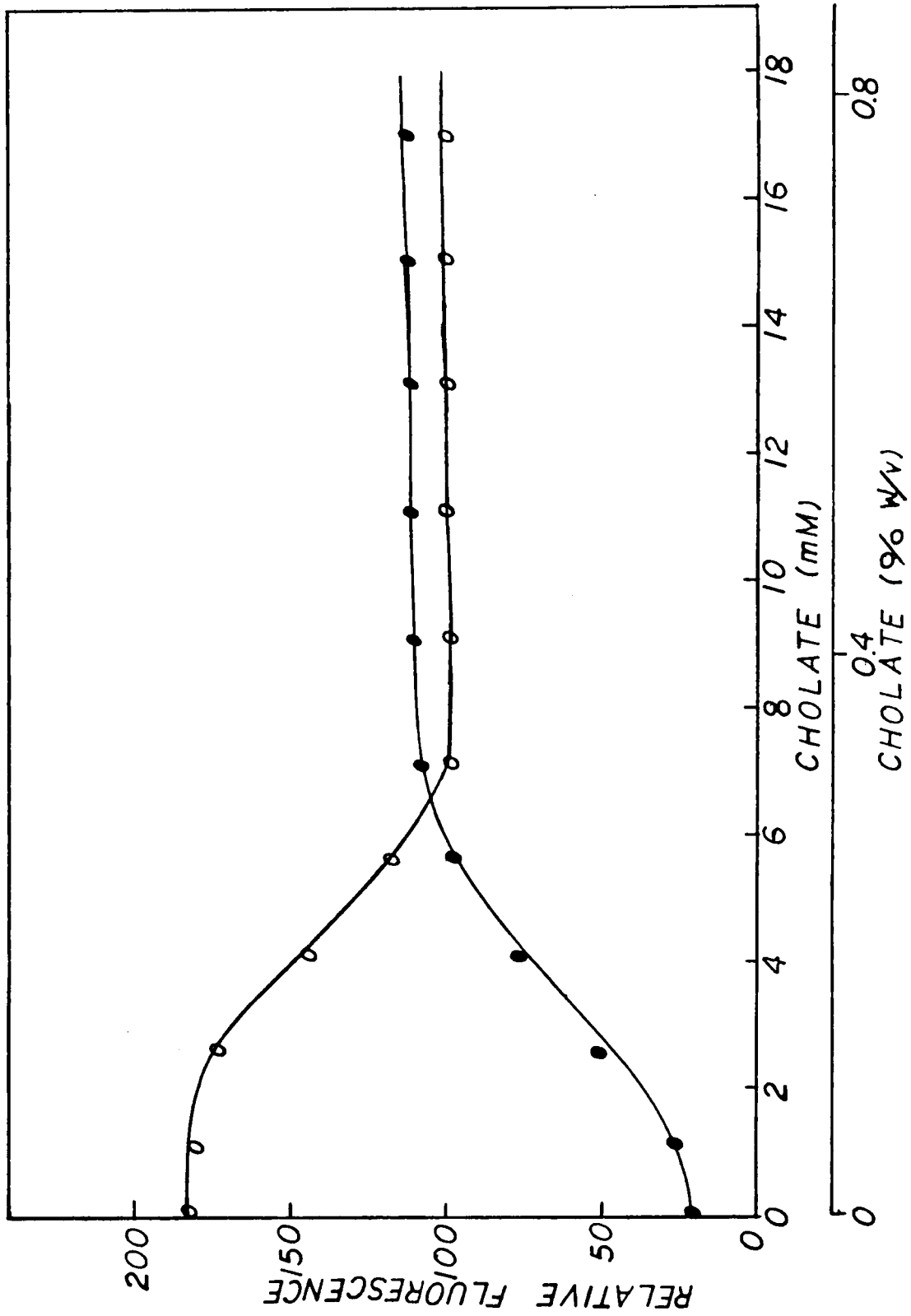


Figure 37

TABLE VI

PARTIAL PURIFICATION SCHEME FOR RETINOL DEHYDROGENASE

Purification Step	Volume ml	Protein mg/ml	Units nmoles/min	Specific Activity units/mg
1) Crude Homogenate Supernatant from ROS	3.5	21.4	5.35	.250
2) Hydroxyapatite Chromatography	3.0	0.80	1.74	2.18
3) Retinyl-Sepharose	2.5	0.16	1.00	5.62

1 unit = 1 nmole Retinal formed per min. at 25°C.

Kinetic constants and mechanism of retinol dehydrogenase. Double reciprocal plots of initial velocity versus NADP^+ , NADPH, all-trans retinol or all-trans retinal concentration with the corresponding second substrate concentration fixed gave rise to a pattern of intersecting straight lines. The intersections, which were to the left of the vertical axis and below the horizontal axis, were diagnostic of a sequential kinetic mechanism. The Michaelis constants for each of the substrates, given in Table VII, were determined from these double reciprocal plots.

NaBH_4 reduction of all-trans retinal in the presence of retinal dehydrogenase. Addition of NaBH_4 to a mixture of retinal and retinol dehydrogenase had no effect on the dehydrogenase. No such inhibition could be induced by alterations of the concentrations of either all-trans retinal or retinol dehydrogenase.

Pyridoxal-5'-phosphate inhibition of retinol dehydrogenase. Pyridoxal-5'-phosphate was found to be a reversible inhibitor of retinol dehydrogenase. Figure 38 gives the time dependence of PLP inhibition on retinol dehydrogenase. The inhibition could be reversed by addition of the PLP complexing reagent aminooxyacetic acid to the enzyme. Additions of concentrations of PLP to 10mM or incubation times as great as two hours still would not entirely abolish the retinol dehydrogenase activity.

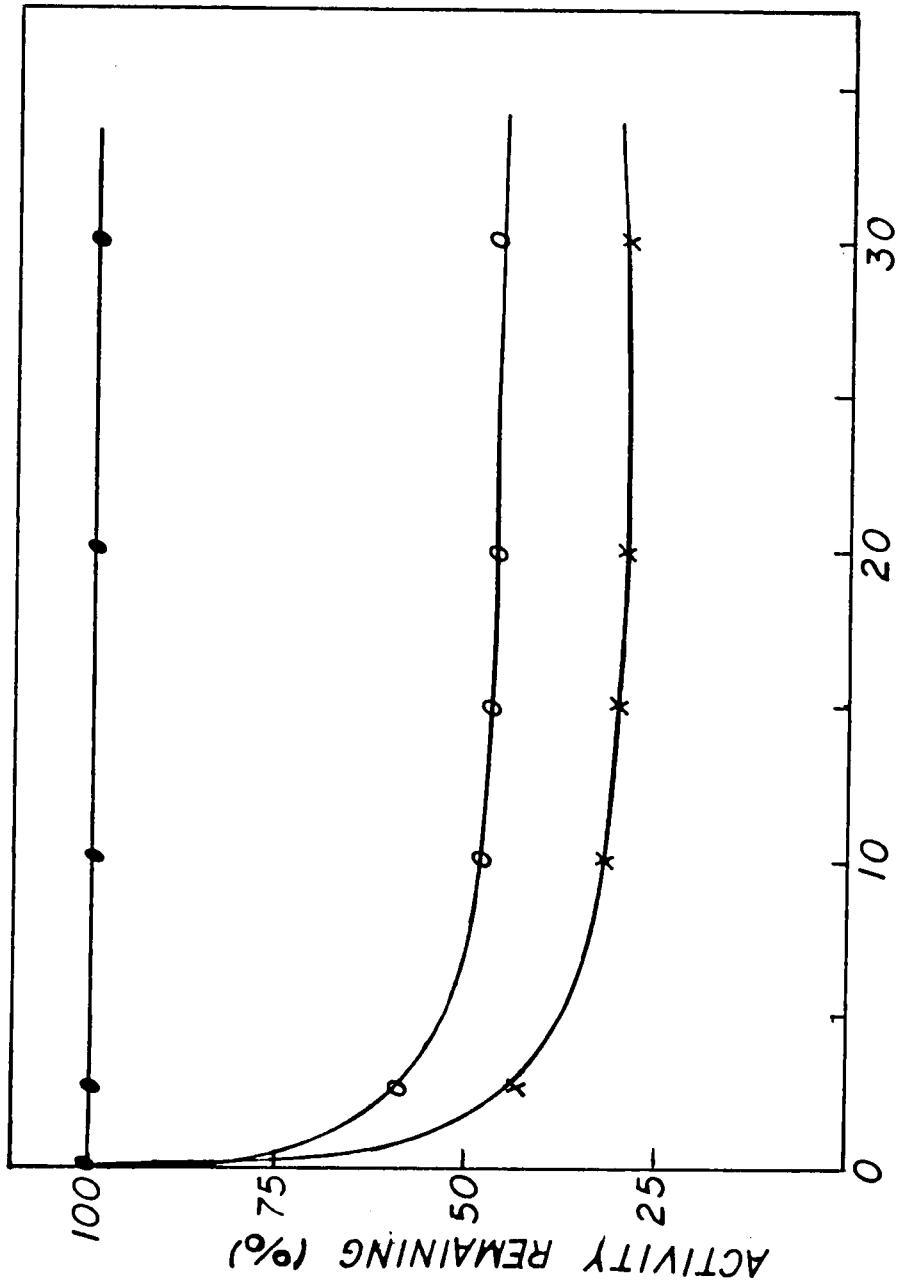
TABLE VII

MICHAELIS CONSTANTS FOR THE SUBSTRATES
OF RETINOL DEHYDROGENASE

Substrate	K _m (μM)
ALL-TRANS Retinol	9.0
ALL-TRANS Retinal	4.0
NADP ⁺	6.0
NADPH	3.5

Figure 38. Time Dependence of Pyridoxal-5'-Phosphate Inhibition of Partially Purified Retinol Dehydrogenase.

The enzyme (0.20mg protein/ml, 4.8 units/mg) was incubated with PLP concentrations of 0 (O), 1mM (O) and 3mM (X). Aliquots were withdrawn at different time intervals and assayed for dehydrogenase activity.



TIME (MINUTES)

Figure 38

III. DISCUSSION

Any study of retinol dehydrogenase is complicated by the fact that both the vitamin A substrates and the enzyme itself are not water soluble. Thus, the properties of the vitamin A substrates must be examined and considered when studying this dehydrogenase. Additionally, the dehydrogenase system becomes complicated by the fact that detergents must be utilized to make the dehydrogenase behave as a soluble rather than particulate entity.

The finding that all-trans retinol forms aggregates or micelles of two distinct sizes in new; but consistent with the reported properties of other lipids such as phosphatidylcholine, which has been demonstrated to produce micelles fitting into distinct size classes when subjected to gel exclusion chromatography (61). The smaller micelle, which has a molecular weight of 120,000 as determined by light scattering measurements, appears to be of homogenous size, as judged by Sephadex G-200 chromatography, containing about 400-600 molecules of all-trans retinol per micelle (molecular weight all-trans retinol = 283). The larger excluded micelle is probably a heterogenous collection of large micelles. Although light scattering data indicates this large micelle has molecular weight of around 9,000,000, it must be remembered that the light scattering molecular weight is a weight average molecular weight and could simply represent the averaged molecular weight of a heterogenous collection of micelles.

The manner in which the vitamin A substrates are solubilized in the enzymatic assay is of critical importance when measuring dehydrogenase activity. The micelles of all-trans retinol, generated when

all-trans retinol is added to buffer alone, are not substrates for retinol dehydrogenase. This inability to serve as a substrate could be due to several possible reasons. Perhaps, the hydroxyl groups of the retinol are not at the surface of the micelle; however, this seems unlikely since this portion of the molecule is the most hydrophilic one and would probably be on the surface of the micelle interfacing the surrounding aqueous environment. More probable explanations are that the large shape of the micelle may prevent access of the substrate to the enzymatic active site or the addition of all-trans retinol to water may induce some chemical change in the retinol molecule which makes it unsuitable as a substrate.

All-trans retinol dissolved in a detergent solution can serve as a substrate for retinol dehydrogenase. The detergents sodium cholate, lubrol WX and digitonin all were effective in solubilizing retinol for substrate purposes. Presumably, since the detergent concentrations used were always near the critical micelle concentrations of the detergents, the retinol was incorporated into detergent micelles (62). These micelles were able to present the retinol to the enzyme in a manner which allowed catalysis to occur. Perhaps these mixed micelles were able to present the substrate to the active site either through direct contact and substrate exchange with the enzyme, or by direct incorporation of the detergent solubilized enzyme onto or into the micelle.

The proteins bovine serum albumin and β -lactalbumin were both able to solubilize all-trans retinol by binding it; and both protein carriers presented retinol to the enzyme in a manner which enabled

the enzyme to perform catalysis at a greater velocity than with detergent solubilized retinol. The proteins ovalbumin and human γ -globulin could both bind retinol; however, neither protein carrier-retinol complex was as good a substrate for the dehydrogenase as were the BSA or β -lactalbumin-retinol complexes.

The finding that a protein-retinol complex is the best substrate for retinol dehydrogenase is one which may represent the true in vivo state of vitamin A. Neither all-trans retinol nor all-trans retinal is ever found as a free molecule in living systems. Rather, these molecules are always complexed to retinol binding proteins. The serum retinol binding protein has been the most studied retinol-protein complex (53-65); however, retinol binding proteins have been reported in the pigment epithelium of the eye (55), the retina (67) and in the cytoplasm of cells in the testes (68). Thus, the ability of protein-retinol complexes to serve as retinol dehydrogenase substrates may simply represent the true physiological requirements of the dehydrogenase.

Detergents are necessary to remove retinol dehydrogenase from the membranes of the rod outer segments. If detergents are not added to rod outer segments which have been sonicated and hand homogenized, the enzyme will appear in the pellet of a high speed centrifugation, indicating the enzyme is not solubilized. The detergents cholate, lubrol wx and n-octylglucoside, at concentrations of 1% (w/v) in the homogenization buffer, all cause retinol dehydrogenase to appear in the supernatant of a high speed centrifugation after homogenization

and sonication. However, after removal of the enzyme from the membrane with 1% (w/v) detergent, the detergent concentration may be reduced by dilution or ultrafiltration to 0.02% (w/v) without making the activity precipitate upon ultracentrifugation.

However, these detergents have adverse effects on the enzymatic activity of this enzyme. Sodium cholate at concentrations greater than 0.1% (w/v), as was demonstrated in Figure 38 on page 141, can displace all-trans retinol from bovine serum albumin. This displacement of retinol makes it a less suitable substrate for the dehydrogenase. Additionally, sodium cholate by itself inhibits the dehydrogenase as evidenced by Figure 37 on page 136. Both wx and n-octylglucoside were similar to cholate in their ability to displace retinol and inhibit the dehydrogenase. Thus, an assay system which balances the inhibitor effects of the detergent and the need to fully solubilize the enzyme has been developed. The assay system used throughout this study is designed so that the cholate concentration inside the cuvette never exceeds 0.04% (w/v); a concentration which is not inhibitory to the dehydrogenase nor able to displace retinol from the protein carrier.

The purification procedure as given in Table VI on page 138 for dehydrogenase consistently results in a 20 to 25 fold purification of the enzyme. The recovery of activity, however, is consistently low at 20% of the original activity in the ROS homogenate. If the two purification steps are reversed, the degree of purification and recovery of activity are unchanged. Other conventional purification techniques used in enzyme purification have not been successful as purification steps for this

membrane bound enzyme. The presence of detergent to solubilize the enzyme, the small quantity of enzyme and the instability of the enzyme all contribute to the difficulty experienced in purification of this enzyme.

The double reciprocal plots of initial velocity versus substrate concentrations indicate retinol dehydrogenase proceeds by a sequential reaction mechanism. Other similar dehydrogenases such as horse liver alcohol dehydrogenase, proceed by ordered sequential mechanisms and it would not be unexpected for retinol dehydrogenase to do so also. However, detailed kinetic studies on this enzyme are complicated by the carrier protein and the association-dissociation of the vitamin A species from this carrier.

The inability of NaBH_4 to reduce all-trans retinal to the partially purified enzyme and inactivate the enzyme, contradicts the work of Bonting (70) who reports inactivation of the enzyme in whole rod outer segments upon illumination of an incubation mixture with added retinal and NaBH_4 . This discrepancy in results may be due to the fact that the partially purified enzyme exists in a different conformation or lipid environment than the native enzyme in the rod outer segment; and as a result, is not susceptible to this inactivation. However, a more likely explanation would be that the severe experimental conditions of high retinal concentration (1mM), and lengthy illumination with visible light modify the membrane environment necessary for enzymatic activity.

Inactivation of retinol dehydrogenase with pyridoxal-5'-phosphate is consistent with Bonting's report (71) which suggests this enzyme

in whole rod outer segments is sensitive to reagents such as dansyl chloride and methylacetimidate, which react with primary amine groups. Additionally, this inactivation is similar to the inactivation of horse liver alcohol dehydrogenase by PLP in that large concentrations of PLP (5mM) never fully inhibit enzymatic activity unless NaBH_4 is added to reduce the Schiff base formed (72). This inhibition by carbonyl reagents has been attributed by Bonting to the modification of an essential lysine residue within the enzyme. However, the PLP used in this study also could conceivably be reacting with the free amine groups of phosphatidylethanolamine molecules in the rod outer segments, in the case of Bonting, or with phosphatidylethanolamine associated with the solubilized enzyme in this study. This reaction with a phospholipid molecule could affect enzymatic activity by modifying the three dimensional conformation of the enzyme and must be considered as a possible alternative to the more conventional explanation involving modification of essential amino acid residues.

CHAPTER II

THE ROLE OF PHOSPHOLIPIDS IN RETINOL DEHYDROGENASE

Phospholipids play an important role in the functioning of many membrane proteins. Monoamine oxidase has been shown to require phospholipids for activity (73), cytochrome oxidase needs phospholipid for activity (74), phospholipids activate β -hydroxybutyric acid dehydrogenase (75) and the HL-A antigen copurifies with specific phospholipids (77). Thus, it seems likely that retinol dehydrogenase, a tightly bound membrane enzyme, may require a phospholipid environment for activity; or, at least, will retain phospholipid upon purification.

The phospholipid composition of rod outer segments has been well-defined in the literature (78). Phosphatidylcholine and phosphatidylethanolamine each compose about 40% of the total membrane lipid. Phosphatidylserine makes up about 10% of the membrane lipid; and the remaining lipid is composed of cardiolipid, cholesterol, triglycerides, and sphingolipids. The fatty acid side chains of the phospholipids are unusual in that 75% of the side chains contain between 22 and 26 carbons and have four to eight double bonds.

The role phospholipids play in retinol dehydrogenase is important for reasons other than just understanding the role lipids play in establishing an environment for the enzymes. These lipids could affect the results of any chemical modification of the enzyme, greatly affect the stability of the enzyme and even affect the ability to purify this enzyme.

I. EXPERIMENTAL

Materials

Bee venom phospholipase A₂ and phospholipase C were purchased from Sigma Chemical Co., St. Louis, MO. Sephacryl S-200 was purchased from Pharmacia Inc., Piscataway, NJ. Asolectin was purchased from Allied Concentrates, Hemstead, NY. All other chemicals and materials were purchased from the manufacturers as listed in previous chapters of this thesis.

Methods

Phospholipase inactivation of retinol dehydrogenase. Bee venom phospholipase A₂ and phospholipase C were dialyzed overnight against 10mM phosphate buffer (pH 7.0) prior to use. No addition of calcium was made prior to use.

The phospholipases were demonstrated to be free of proteolytic enzyme activity by two different techniques. Quantities of phospholipase in excess of those used in the retinol dehydrogenase inactivation study were incubated at 37°C with the enzymes beef heart catalase and horse liver alcohol dehydrogenase. This incubation which was in the same cholate-phosphate buffer system used in the retinol dehydrogenase inactivation, resulted in no loss of either enzymatic activities, even with incubation times of two hours. The second demonstration of no contaminating proteolytic activity involves high voltage electrophoresis to demonstrate peptide fragments produced upon proteolysis. Electrophoresis was conducted for one hour in acetic acid-pyridine buffer (pH 3.5) at 800 V and 4°C. To 1ml of 20mg/ml bovine

serum albumin in 0.05M NaHCO_3 (pH 7.9) was added 0.5mg of phospholipase A_2 and to another similar BSA sample 0.1mg of trypsin was added. The samples were incubated for 1 hour at 37°C and subsequently lyophilised and then redissolved in 0.2ml of electrophoresis buffer. The trypsin treated BSA showed over 10 different ninhydrin positive peptides while the phospholipase treated BSA showed one ninhydrin positive spot which coincided with an untreated BSA band.

To retinol dehydrogenase in 50mM phosphate (pH 7.0) 0.4% cholate was added either phospholipase A_2 or phospholipase C and 5mg/ml of BSA. The enzyme phospholipase mixture was incubated at 37°C. Assays of dehydrogenase activity were started within two minutes after addition of the phospholipase. The quantity of phospholipase added or mixing ratio of phospholipase to dehydrogenase is expressed in terms of milligrams of phospholipase per milligram of protein in the retinol dehydrogenase. The BSA was added to absorb any free fatty acids which were produced by the lipases.

Reconstitution of retinol dehydrogenase with phospholipid. Retinol dehydrogenase, incubated with phospholipase A_2 in a 1 to 200 mixing ratio for 30 minutes at 37°C, was chromatographed on Sephacryl S-200 (0.9 x 20cm column) to remove phospholipase A_2 . This chromatography separated the partially inactivated dehydrogenase, which came several fractions (0.5ml/fraction) after the void volume, from the phospholipase which came over 15 fractions after the dehydrogenase.

The inactivated dehydrogenase was then added to various phospholipid films which had been generated in test tubes by evaporation of

the organic solvents used to solubilize the phospholipids. The phospholipids used were phosphatidylcholine, phosphotidylethanolamine, phosphatidylserine, cardiolipin, a 1:1 molar mixture of phosphatidylcholine and phosphotidylethanolamine and azalecthin, an undefined mixture of soy bean lipids. Phospholipid concentrations were determined assuming an average phospholipid molecular weight of 800. The dehydrogenase phospholipid mixture was then sonicated in a Bronson bath sonicator at 4°C for various time intervals. The enzyme was then assayed and the specific activity of the phospholipid added enzyme was compared to that of phospholipase inactivated enzyme as an indication of reconstitution of enzymatic activity.

II. RESULTS

Phospholipase inactivation of retinol dehydrogenase. As can be seen in Figure 39, phospholipase A₂ at a mixing ratio of 1:200 inactivates 70% of the retinol dehydrogenase activity within five minutes of addition. Phospholipase C at a 1:200 mixing ratio abolishes about 8% of the dehydrogenase activity within five minutes. A mixture of phospholipases A₂ and C at mixing ratios of 1:200 still only inactivated enzymatic activity at the 70% level seen with phospholipase A₂ alone. Further incubation times with these mixtures resulted in no additional loss of enzymatic activity.

Figure 40 shows the mixing ratio dependence of inactivation on phospholipase A₂ concentration. Although the rate of dehydrogenase inactivation is dependent on lipase concentration, the final degree

Figure 39. Inactivation of Partially Purified Retinol Dehydrogenase by Phospholipase A₂ and Phospholipase C.

Retinol dehydrogenase (0.18mg protein/ml, 5.2 units/mg) was incubated with no additions (O), phospholipase C (.005 mg lipase protein/mg enzyme preparation protein) (●), phospholipase A₂ (.005 mg lipase protein/mg enzyme preparation protein) (X), and a phospholipase C-Phospholipase A₂ mixture (0.005mg of each lipase/mg of enzyme protein (▲). Aliquots of enzyme were removed at various time intervals and assayed for dehydrogenase activity.

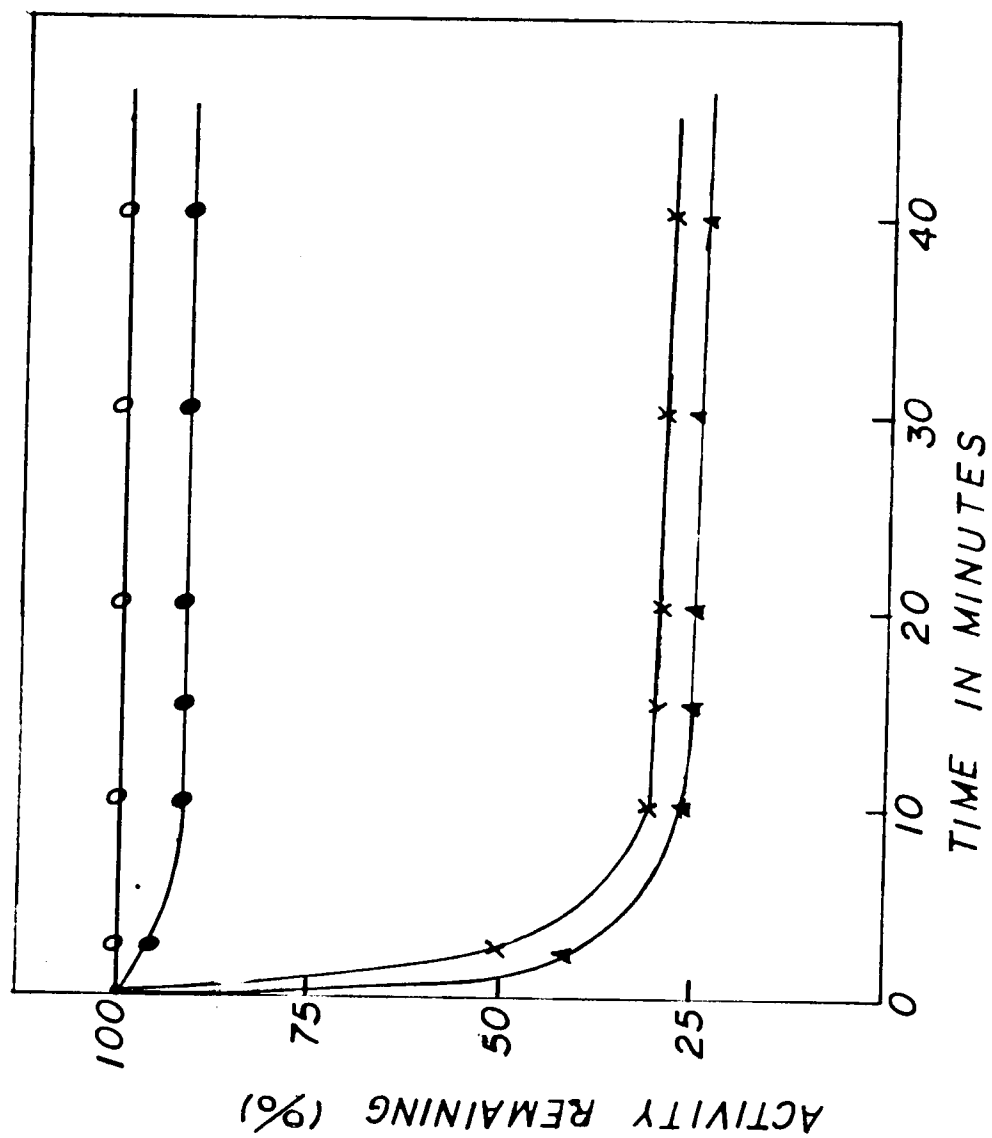
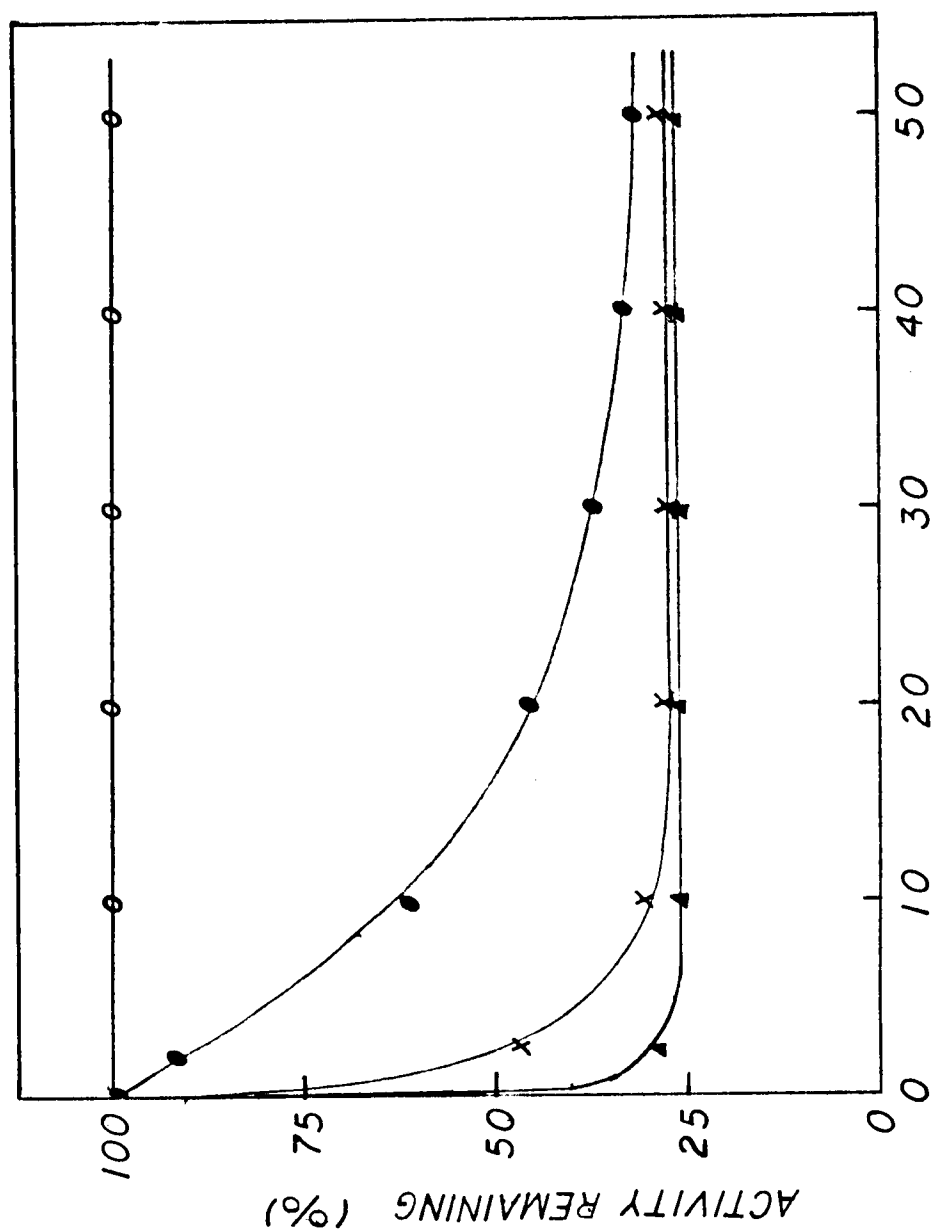


Figure 39

Figure 40. Time Dependence of Phospholipase A₂ Inactivation of Partially Purified Retinol Dehydrogenase.

Retinol dehydrogenase (0.18mg protein/ml, 5.2 units/mg) was incubated with phospholipase A₂ at mixing ratios of 0 (○), 0.0002mg lipase/mg enzyme protein (●), 0.001 mg lipase/mg enzyme protein (X) and 0.050mg lipase/mg enzyme protein (Δ). Aliquots were removed at the specified time intervals and immediately assayed for enzymatic activity.



TIME (MINUTES)

Figure 40

of inhibition remains stationary at around 70%. No greater inhibition of dehydrogenase activity than this 70% inhibition could be observed with prolonged incubation or a phospholipase A₂ to dehydrogenase protein ratio of 1:25.

Addition of palmitic acid (1mM) or lysophosphatidylcholine to the (1mM) active dehydrogenase resulted in no loss of enzymatic activity.

Reconstitution of retinol dehydrogenase. Table VIII gives the reconstitution results for phospholipid activation of phospholipase A₂ inactivated retinol dehydrogenase. Of all lipids used in reconstitution attempts, only the mixture of soy bean lipids, asolectin, resulted in any reconstitution of enzymatic activity. This reconstitution was dependent upon the time of sonication; however, no additional regain of activity occurred after 25 minutes of sonication. If the azalecthin-dehydrogenase mixture was not sonicated, no reactivation of the enzyme occurred. The reconstitution attempts using more defined phospholipids never resulted in regain of any dehydrogenase activity even after 45 minutes of sonication.

The reconstitution of retinol dehydrogenase activity with asolectin never fully reconstituted the dehydrogenase activity. The reconstituted specific activity was twice that of the phospholipase inactivated enzyme; but was still only about 60% that of the untreated enzyme.

III. DISCUSSION

Retinol dehydrogenase is dependent on phospholipid for maintaining

TABLE VIII

RECONSTITUTION OF PHOSPHOLIPASE A₂ INACTIVATED RETINOL DEHYDROGENASE
WITH PHOSPHOLIPIDS

Phospholipid Added	Fold Increase In Inactivated Specific Activity	Percent of Original Untreated Specific Activity
None	1	30
Phosphatidylcholine 25' sonication 4°C	1	30
Phosphatidylethanolamine 25' sonication 4°C	1	30
Phosphatidylserine 25' sonication 4°C	1	30
Cardiolipin 25' sonication 4°C	1	30
Phosphatidylethanolamine, phosphatidylcholine mixture	1	30
Asolectin No sonication	1	30
Asolectin 10' sonication 4°C	1.3	40
Asolectin 25' sonication 4°C	2.0	60

enzymatic activity. Phospholipase A₂, which removes the fatty acyl side chain from the central carbon of the glycerol backbone of a phospholipid, inactivates the enzyme so that only 30% of the original activity remains. Phospholipase C, which removes the polar head groups from phospholipids, has little effect on dehydrogenase activity when added to the dehydrogenase by itself; and no additional effect over that of phospholipase A₂ when used in conjunction with this lipase. Since the phospholipase was dialyzed to remove possible inhibitory small molecules was free of protease activity and the enzyme is not inhibited by fatty acids, it would appear that the fatty acyl side chains of the phospholipids surrounding the dehydrogenase are necessary to maintain enzymatic activity.

Attempts to reconstitute lipase inactivated dehydrogenase were only partially successful. Only asolectin could restore lost activity; but this restoration was only 60% of the original untreated enzymatic activity.

The inability of phosphatidylcholine, phosphotidylethanolamine, phosphatidylserine, cardiolipin or a phosphatidylcholine-phosphotidylethanolamine mixture to reconstitute dehydrogenase activity may be due to the nature of the fatty acyl side chains of these lipids. Only palmitic, oleic and stearic acid side chains are found in the lipids used in these reconstitution attempts. Since rod outer segments contain phospholipids with much longer and more unsaturated side chains, the dehydrogenase may specifically require these long unsaturated fatty acyl chains for activity.

Although the composition of asolectin is variable, it does contain about 70% phosphatidylcholine and phosphatidylethanolamine; and is reported to be high in long unsaturated fatty acyl side chain composition (79). This side chain composition may account for the ability of this mixture to reconstitute enzymatic activity. However, the possibility that a minor lipid component may be responsible for this reconstitution cannot be ruled out.

The role of sonication in reconstitution is probably to stimulate the exchange of the lysophospholipids generated by the lipase with the phospholipids of the asolectin. This sonication was necessary. Omission of sonication brought no reconstitution of the dehydrogenase, presumably because the added phospholipids could not reproduce the proper environment around the protein without added energy to stimulate phospholipid exchange and incorporation onto the protein.

The inability to fully reconstitute the dehydrogenase may be due to the inability of the asolectin to completely displace the lysolipids about the protein. Another explanation might be that the phospholipase digested dehydrogenase may undergo an irreversible conformation change and as a result may never be fully reactivated. Finally, the asolectin may not contain the exact lipid composition found in the rod outer segments and as a result, one does not see full reconstitution because specific lipids are absent.

Obviously, phospholipids do surround partially purified retinol dehydrogenase and play a role in stabilizing the enzyme. The nature of this stabilization is only speculative but phospholipid bound to

the protein in rod outer segment membranes could specify a specific protein conformation which is required for catalysis. This phospholipid also could produce a hydrophobic pocket in the enzyme which is necessary for vitamin A substrate binding.

CHAPTER III

PHOTOINACTIVATION OF RETINOL DEHYDROGENASE

Recently, Damelle has reported all-trans retinol, upon illumination, is a singlet oxygen generator and can be responsible for morphological damage to the retina (80,81). Another group has demonstrated that high light intensity can induce structural changes in the membranes of the rod outer segments. The effect of this photosensitized damage caused by retinol was examined with retinol dehydrogenase.

The use of photosensitizers is well documented as a means of studying enzyme catalysis (82). Additionally, these photosensitizers have been shown to induce hydroperoxide formation in unsaturated fatty acyl side chains of phospholipids; like those found in rod outer segments. Thus, the effect of illumination on retinol dehydrogenase incubated with exogenous retinal, a singlet oxygen generator, was examined in hopes of gaining insight into the protein and phospholipid character of this enzyme.

In addition to the chemical information which these photoinactivation studies may provide, the retinal sensitized inactivation of retinol dehydrogenase may be of physiological importance. Mice, which have been on high vitamin A diets, when exposed to bright light are blinded for a period of several months following which time the animals' sight gradually returns (83). This blindness has been linked to damage of the retina, but no functional changes or molecular basis for the blindness has been demonstrated. The photoinactivation of retinol

dehydrogenase may be one of the functional changes which occurs in the retina during this blindness syndrome.

I. EXPERIMENTAL

Materials

Sodium azide was purchased from Sigma Chemical Co., St. Louis, MO. All other materials mentioned in this chapter were purchased from sources described in earlier chapters or from readily available commercial chemical supply companies.

Methods

Photoinactivation of retinol dehydrogenase. Partially purified retinol dehydrogenase (specific activity of 4.2 units/mg) in 10mM phosphate buffer pH 7.0, containing 0.4% (w/v) sodium cholate and 10 μ M exogenous all-trans retinal, was irradiated with light from a xenon light source (intensity of light of 8×10^5 ergs cm⁻² sec⁻¹). The irradiating light was passed through a Corning CS3-71 filter which allowed light with a wavelength greater than 380nm to pass. The irradiated sample was kept at 4°C with a constant temperature water bath and aliquots were taken at various time intervals for activity determinations.

A sample wrapped in aluminum foil so that no light could reach the enzyme served as the control for each experiment. An additional control, in which all-trans retinal was photoirradiated in the absence of enzyme and subsequently added to the dehydrogenase and measured

for activity, demonstrated that the photodecomposition products of all-trans retinal were not inhibitory to the dehydrogenase.

The same irradiating procedure was used for sodium azide containing samples. Enough solid sodium azide was added to each enzyme preparation to make the sample 20mM with respect to azide. The sodium azide had no effect on enzymatic activity.

Samples irradiated in D₂O were treated the same as the other irradiated samples except the phosphate buffer was made in 99% D₂O. The 10mM phosphate buffer was made by diluting an enzyme sample in 0.1M phosphate buffer in H₂O to the required 10mM concentration. Thus, the final D₂O concentration was around 90%.

II. RESULTS

As demonstrated in Figure 41, retinol dehydrogenase is sensitive to photoirradiation in the presence of the singlet oxygen generator all-trans retinol. The presence of the singlet oxygen quencher sodium azide protects against photoinactivation; and the presence of D₂O, which extends the lifetime of the singlet oxygen, increases the rate of inactivation of retinol dehydrogenase.

The inactivation of the enzyme was never 100%. Increased time of irradiation, addition of more singlet oxygen generator, or the presence of D₂O could not increase the degree of inactivation of retinol dehydrogenase over the 75% inactivation seen under the unaltered inactivating conditions.

The enzyme could not be inactivated by the photo-decomposition products of all-trans retinal or by irradiation in the absence of exogenous all-trans retinol.

Figure 41. All-Trans Retinal Photosensitized Inactivation of Retinol Dehydrogenase.

Retinol dehydrogenase (0.28mg protein/ml, 4.2 units/mg) was irradiated for varying time intervals. To the enzyme sample was added $10\mu\text{M}$ exogenous all-trans retinal. To one sample 0.01M sodium azide was added (O), another had no further additions (X), a third was irradiated in D_2O (v) and the fourth was wrapped in aluminum foil and not exposed to light (O). Aliquots were removed from each sample at the specified time and assayed for enzymatic activity.

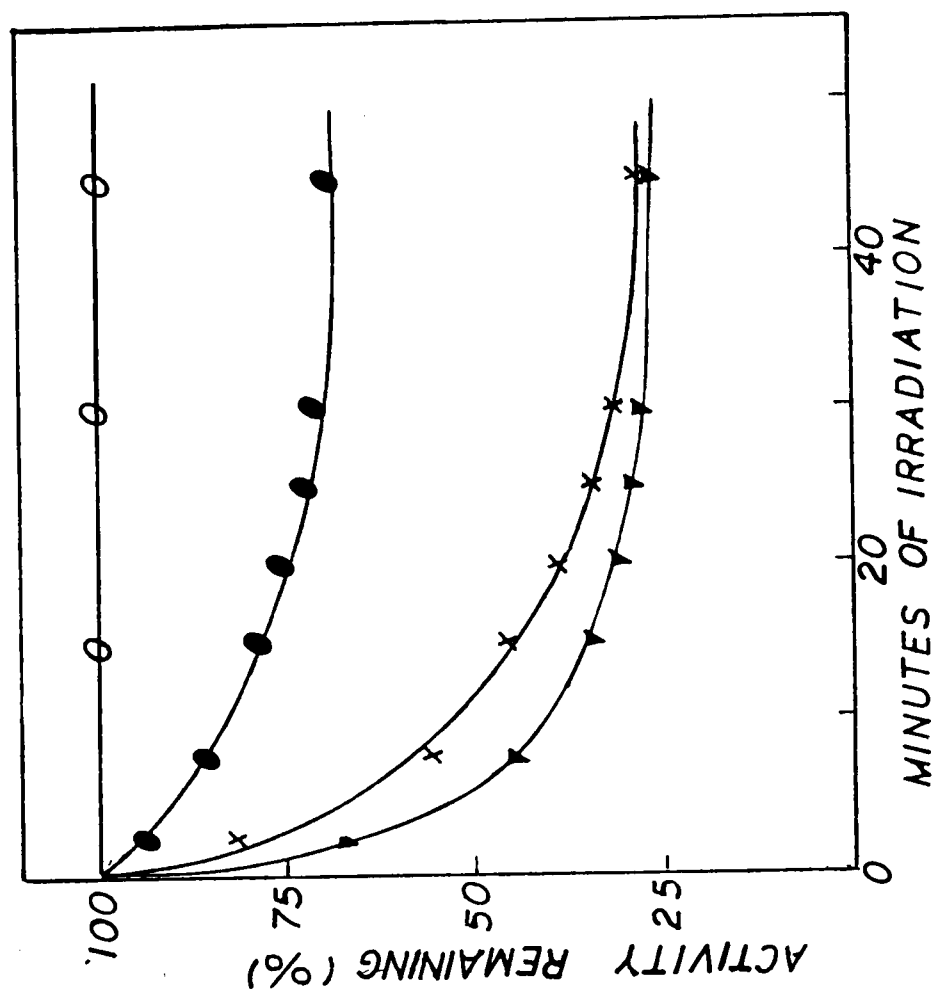


Figure 41

III. DISCUSSION

Retinol dehydrogenase can be inactivated by photoirradiation in the presence of all-trans retinal. The mechanism of inactivation appears to be through the oxidative effects of singlet oxygen. The evidence for this singlet oxygen mechanism is that the singlet oxygen quencher sodium azide protects against photooxidation and that D_2O , which is a known enhancer of singlet oxygen lifetime, increases the rate of inactivation. The possibility that the inactivation of retinal dehydrogenase was due to the formation of inhibitory photodecomposition products of all-trans retinal, was eliminated by incubation of unirradiated enzyme with these photodecomposition products and the subsequent retention of enzymatic activity.

Although singlet oxygen is implicated as the mediator of this enzyme inactivation, the direct changes occurring within the enzyme are less obvious. Singlet oxygen has been demonstrated to oxidize the amino acid residues methionine, histidine, and tryptophan within proteins (84). Additionally, singlet oxygen is a demonstrated oxidizer of unsaturated lipids (85). Thus, the inactivation of retinol dehydrogenase might involve actual destruction of the protein, alteration of the lipids around the protein or possibly both of these events.

The photoinactivation of retinol dehydrogenase never goes to 100% completion, but remains at the 75% inactivation level. This degree of inactivation is similar to the phospholipase A_2 inactivation of retinol dehydrogenase. The possibility that these two different experiments inactivate the enzyme through the same mechanism is distinct. If the

singlet oxygen generated from the illuminated all-trans retinal modifies the unsaturated fatty acyl side chains of the phospholipids surrounding retinal dehydrogenase, the resulting enzyme inactivation may be due to the same effects which bring about phospholipase inactivation. However, it should be noted that phospholipid free yeast alcohol dehydrogenase is also sensitive to retinol sensitized photoinactivation and that this inactivation also is never 100% (86). Thus, although the similarity between retinal sensitized photoinactivation and phospholipase inactivation is strong, it is not conclusive.

The possible physiological implications of these photoinactivation experiments are significant. The loss of retinol dehydrogenase activity by organisms on high vitamin A diets and exposed to high light intensities may be a cause of the blindness mentioned in the Introduction to this chapter. The loss of this enzyme would prevent regeneration of the photopigment rhodopsin and result in blindness. This blindness would be reversible in that, as new disc membranes containing newly synthesized retinal dehydrogenase were made and incorporated into the rod outer segments, the vision of the individual would return. This possibility is further substantiated by the fact that whole rod outer segments photoirradiated without exogenous retinal have 40% less retinal dehydrogenase activity than unirradiated rod outer segments.

CHAPTER IV

GENERAL DISCUSSION

Retinol dehydrogenase from bovine rod outer segments was partially purified by hydroxyapatite and retinyl-sepharose chromatography. The membrane bound enzyme required homogenization and sonication in a detergent before it was liberated from its membrane environment. However, once removed from its membrane environment, the enzyme behaves as a solubilized protein as judged by its chromatographic and ultracentrifugation properties as long as certain minimum detergent concentrations are maintained.

The best form to supply the vitamin A substrate to the enzyme is in a retinol-protein carrier complex. This preference for a protein carried vitamin A molecule suggests the in vivo substrate of the dehydrogenase is a retinol molecule bound to a retinol binding protein. This suggestion is not unfounded, for retinol in living systems is always found complexed to a retinol binding protein. However, no carrier protein specific for all-trans retinol has been demonstrated, in a believable fashion, to exist in the retina. Thus, to verify this hypothesis, one must first find an all-trans retinol binding protein in the eye and demonstrate that it is the true substrate for dehydrogenase.

An alternative explanation for this substrate preference of all-trans retinol bound to a protein carrier would be that, by solubilizing the retinol in detergent, the detergent inactivates the enzyme. This

explanation may in part be correct for the detergents cholate, lubrol ex, digitonin, triton X-100 and Tween 80; all do to a varying degree inhibit retinol dehydrogenase activity. However, if the concentration of detergent used to solubilize the retinol is kept below the concentration found to be inhibitory in the protein-carrier based assay system, the maximal enzymatic velocity is still less than one would observe if the retinol had been solubilized with a protein carrier.

The purification of retinol dehydrogenase is complicated by the fact that the enzyme is membrane bound. The enzyme may be thought of as an aggregate of protein, phospholipids and detergent; and as such an aggregate, possesses the combined properties of these three different chemical entities. These combined properties must be considered in any purification scheme; and it must be recognized that other proteins may be surrounded by the same phospholipids and detergent, and, as such, may be difficult to separate from the dehydrogenase. This composite chemical property may account for the inability of the affinity resins AMP-Sepharose and 2',5'-ADP-Sepharose to bind this dehydrogenase and for Phenyl-Sepharose to release the retained enzymatic activity.

This dehydrogenase-phospholipid-detergent aggregate may limit the ability to purify the dehydrogenase. The soluble rod outer segment enzyme, rhodopsin kinase, is the only enzyme purified from this source to homogeneity (87). This purification which results in a single protein band upon polyacrylamide gel electrophoresis, was purified only 110 fold. If rhodopsin kinase is as abundant as retinol dehydrogenase,

it would not be unreasonable to expect that a similar fold purification would result in a homogeneous retinol dehydrogenase preparation. Thus, an additional purification step of five to six fold may result in pure enzyme. However, developing this remaining purification may be difficult, in light of the number of techniques tried which were unsuccessful and the aggregate nature of the dehydrogenase. The best approach to follow before further purification is attempted would be to try to alter the phospholipid detergent environment around the protein so that the purification can be based more on the protein's properties rather than that of the aggregate. Organic solvent extraction of the lipids, further sonication of the partially purified enzyme, removal of the sodium cholate, addition of other detergents may accomplish this goal of altering the aggregate environment; or even phospholipase digestion followed by subsequent reconstitution with phospholipid may be appropriate.

The inability of all-trans retinal to form a covalent linkage with the enzyme as evidenced by the inability of NaBH_4 to reduce such a linkage refutes the work of Bonting (70). Bonting has postulated that this covalent linkage as a part of the catalytic mechanism of the enzyme; even though a Schiff base intermediate has not been demonstrated with other more extensively studied dehydrogenases which convert terminal aldehydes to alcohols. Considering Bonting's use of whole rod outer segments, high concentrations of all-trans retinal, a high degree of illumination and the extreme sensitivity of the enzyme to its phospholipid environment, it is likely that Bonting's experiments were altering the enzymatic activity through another mechanism than direct modification of the active center.

The chemical inactivation of retinol dehydrogenase with pyridoxal-5'-phosphate suggests primary amine groups are necessary for enzymatic activity. This observation is in agreement with those of Bonting (88). The precise nature of inactivation is not clear. The presence of either NADP^+ or all-trans retinol failed to protect against this inactivation indicating that the modification is not active site directed. The PLP modification may either be of a lysine residue on the protein, which upon modification changes the conformation of the protein, or of a phospholipid molecule which affects the enzymatic activity. The latter explanation seems the least likely since the modification by PLP would be of a phospholipid head group; but head group modification as evidenced by phospholipase C digestion does little to alter enzymatic activity.

The membrane environment about retinol dehydrogenase is important as evidenced by the phospholipase inactivation of the enzyme. The role phospholipids play in the maintenance of enzymatic activity is clearly demonstrated by the phospholipase A_2 sensitivity and the ability of exogenous phospholipids to partially restore this activity. The exact role of phospholipids is still undefined but it is clear that the fatty acyl side chains of the phospholipids are the important structural feature of phospholipids necessary to maintain activity. The importance of these fatty acyl side chains is clear from the susceptibility of the enzyme to phospholipase A_2 ; while the insensitivity of enzymatic activity to phospholipase C suggests the polar head groups of the phospholipids are less important in the retention of a proper membrane environment about the protein.

Other proteins, which have been demonstrated to require phospholipids in order to maintain physiological activity, have been reported to be surrounded by from two to 80 phospholipid molecules (89). Additionally, these protein-phospholipid aggregates incorporate detergent molecules into the aggregate, when detergent is present. When cholate is used to solubilize these protein-phospholipid micelles, four to eight molecules of cholate usually incorporate into the protein-lipid complex, displacing several phospholipid molecules (62). This displacement of phospholipid may be the reason for the inhibition of dehydrogenase activity by cholate.

The structure of the fatty acyl side chain of the phospholipid also appears to be extremely important in maintaining enzymatic activity. In the reconstitution experiment, only the crude mixture of soy bean lipids, which is known to contain many long unsaturated side chains, could partially restore enzymatic activity to phospholipase inactivated enzyme. More defined lipids with saturated side chains containing between 14 and 18 carbons could not restore enzymatic activity. This result suggests that retinol dehydrogenase requires the long (22 to 26 carbons) unsaturated fatty acyl side chains, reported to be abundant in rod outer segments, for activity. However, it must be remembered that the soy bean lipid is crude and the restoration of enzymatic activity may be due to a minor lipid component which has no relation to the lipids of the rod outer segment. The only means of demonstrating conclusively the long unsaturated fatty acyl side chain requirement postulated for retinol dehydrogenase would be through

a systematic attempt to reconstitute enzymatic activity with a series of phospholipid of known long side chain length and degree of saturation.

The photoinactivation experiments demonstrate retinol dehydrogenase is sensitive to photoirradiation both in intact rod outer segments and in the solubilized form. This inactivation appears to proceed through a singlet oxygen mechanism as judged by the azide protection from inactivation and the D₂O induced increase in rate of inactivation. This inactivation may be of physiological significance in syndromes of light induced blindness where the inactivation of the enzyme may be a molecular reason for the temporary blindness of the syndrome.

The degree of photosensitized inactivation of retinol dehydrogenase and the phospholipase inactivation of the enzyme both maximally decrease activity by 75%. This observation might indicate the two inactivations result from the same chemical event: The modification of the lipid environment about the protein. This possibility needs further investigation since the unsaturated phospholipids of the rod outer segments are extremely sensitive to photosensitized hydroperoxide formation. Experiments designed to determine if photoinactivated enzyme may be further inactivated by phospholipase A₂ and if phospholipase A₂ inactivated enzyme is still photosensitive need to be performed. Attempts to reconstitute photoinactivated enzyme with phospholipids should also be tried. These experiments could build a

circumstantial argument for modification of the lipids about the enzyme as a mechanism of photoinactivation or suggest the inactivation is due to modification of the protein itself.

Further work with this enzyme must concentrate on the complete purification of this enzyme. If complete purification is achieved, many research horizons are opened. The exact role of phospholipids in retinol dehydrogenase can be fully examined and characterized. The physical and chemical properties of the enzyme can fully be explored. The existence or nonexistence of the much argued retinyl isomerase can perhaps be demonstrated through experiments to reconstitute rhodopsin from all-trans retinol with added opsin and retinol dehydrogenase. Thus, additional work with this enzyme is justified and might result in the answers to many questions posed by this thesis.

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APPENDIX

APPENDIX

MACROMOLECULAR PROPERTIES AS DETERMINED BY LIGHT SCATTERING

The amount of light scattered by a macromolecular solution is related to the concentration and angle of observation; and to the intrinsic properties of the macromolecule, such as size and shape. In order to understand how these variables influence the amount of light scattered by a solution, one must start with the simplest system, an ideal gas and progress to the more complicated non-ideal macromolecular solutions.

When a gas particle is subjected to an electric field strength, E , an induced dipole moment, P , is produced in the particle. The magnitude of this dipole moment is proportional to the field

$$P = \alpha E \quad (1)$$

and α is the polarizability of the particle; or put in another way, the amount of dipole moment induced by the unit field. The electric field of a plane polarized light wave is

$$E = E_0 \cos 2\pi(vt - x/\lambda) \quad (2)$$

where E_0 is the maximal amplitude, v is the frequency of light, λ its wavelength, t the time and x the location at time t along the line of propagation.

By combining Equations 1 and 2 into

$$P = \alpha E_0 \cos 2\pi(vt - x/\lambda) \quad (3)$$

the value of the oscillating induced dipole moment of the particle may be determined. This oscillating dipole itself is a source of radiation. The new radiation or the scattered beam has a field strength which is proportional to the second derivative of the oscillating dipole, d^2p/dt^2 . Although the scattered radiation is a spherical wave, its field strength depends on the angle of observation, θ .

The law of conservation of energy requires the field strength of the scattered radiation should vary with $1/r$ when r is the distance of the observer from the scattered center. Taking the second derivative of Equation 3 and introducing $\sin\theta/cr$ as the angular and distance dependence, where c is the velocity of light, one obtains

$$E_s = \frac{4\pi^2 V^2 E_0 \sin\theta}{c^2 r} \cos 2\pi(vt - x/\lambda) \quad (4)$$

where E_s is the field strength of the scattered light.

Experimentally, one measures light intensity rather than field strength. The two are related as

$$I \propto E^2. \quad (5)$$

Therefore, Equation 4 may be written as

$$\frac{I_s}{I_0} = \frac{16\pi^4 \alpha^2 \sin^2\theta}{\lambda^4 r^2} \quad (6)$$

where I_s is the intensity of scattered light, I_0 the intensity of incident light and λ has been written in place of c/v .

It is possible to define the term R_θ , the Rayleigh ratio where

$$R_\theta = \frac{I_s r^2}{I_0 \sin^2} = \frac{16\pi^4 \alpha^2}{\lambda^4} \quad (7)$$

The subscript θ indicates the angular dependence of scattering intensity.

The polarizability can be related through the dielectric constant to the refractive index, and Equation 7 can be written for the intensity of scattered beam per unit volume as

$$R_\theta = \frac{4\pi^2 (dn/dc)^2 MC}{N_A \lambda_0^4} \quad (8)$$

where M is the molecular weight of the particle, N_A is Avagadro's number, C is the concentration in grams per cubic centimeter, and dn/dc is the refractive index of the gradient.

Now Equation 8 must be modified to account for real rather than ideal conditions. In the derivation of Equation 8, it was assumed that plane polarized light was used; however, the light scattering done in this thesis used unpolarized light. Since unpolarized light can be regarded as the super-position of two plane polarized light beams, instead of a $\sin^2\theta$ angular dependence, one now has a $(\sin^2\theta_1 + \sin^2\theta_2)$ dependence where θ_1 and θ_2 are the angles made by the line of observation with the Y and Z axes of the Cartesian coordinates.

However,

$$\sin^2 \theta_1 + \sin^2 \theta_2 = 1 + \cos^2 \theta \quad (9)$$

where θ is now defined as the angle between the incident and the scattered beam. Thus, for unpolarized light, Equation 8 becomes

$$R^1_{\theta} = \frac{I_s r^2}{I_0 (1 + \cos^2 \theta)} = \frac{2\pi^2 (dn/dc)^2 MC}{N_A \lambda_0^4} \quad (10)$$

where R^1_{θ} is the Rayleigh ratio for unpolarized light.

We now must consider the scattering of a dilute solution rather than an ideal gas. First, Equation 10 must be corrected to account for the refractive index of the solvent n_0 , thus

$$R^1_{\theta} = \frac{2\pi^2 n_0^2 (dn/dc)^2 MC}{N_A \lambda_0^4} \quad (11)$$

A further correction must be made because solutions of finite concentrations are not ideal systems. This non-ideality demonstrates itself in the dependence of the chemical potential on concentration. Introducing the non-ideal condition into Equation 12 one obtains

$$R^1_{\theta} = \frac{2\pi^2 (dn/dc)^2 C}{N_A \lambda_0^4 \left(\frac{1}{n} + B^*C + C^*C + \dots \right)} \quad (12)$$

where in the additional terms $B^*C + C^*C + \dots$ B^* and C^* are the second and third virial coefficients. As solutions become more and more dilute, B^* and C^* become zero.

The final correction which must be made arises because large macromolecules have more than one scattering center. This means the

total scattering intensity depends not only on the interference of the scattered beams coming from different particles but also on interference of scattered beams coming from the same particle. This interference becomes destructive as the angle of observation increases. It is possible to define a scattering function P_θ where

$$P_\theta = \frac{I_{si}}{I_s} \quad (13)$$

I_{si} is the intensity of scattering with interference and I_s is this intensity without interference. P_θ is a function of the scattering angle and the radius of gyration of the molecule. For example, for a random coil

$$P_\theta = \frac{2}{x^2} [\exp(-x) - (1 - x)] \quad (13)$$

where

$$x = \frac{4\pi^2}{\lambda_0^2} R_G^2 \sin^2 (\theta/2)$$

when R_G represents the radius of gyration of the molecule.

As $\theta \rightarrow 0$, P becomes independent of particle shape and $P_\theta \rightarrow 1$, thus

$$\lim_{\theta \rightarrow 0} \frac{1}{P_\theta} = 1 + \frac{16\pi^2}{3\lambda_0^2} R_G^2 \sin^2 \frac{\theta}{2} \quad (14)$$

Now Equation 12 can be modified to be applicable to macromolecular solutions

$$R_{\theta}^1 = \frac{KC}{(1/m + B^*C^* + C^*C + \dots)P_{\theta}} \quad (15)$$

where K is an optical constant

$$K = 2\pi n_0^2 (dn/dc)^2 / N_A \lambda_0^4 \quad (16)$$

Extrapolation to zero concentration eliminates the viral coefficients

$$\lim_{c \rightarrow 0} \frac{KC}{R_{\theta}^1} = \frac{1}{n(P_{\theta})} = \frac{1}{n} \left(1 + \frac{16}{3\lambda_0^2} R_G^2 \sin^2 \frac{\theta}{2} \right) \quad (17)$$

and extrapolation to zero angle eliminates the radius of gyration dependence

$$\lim_{\theta \rightarrow 0} \frac{KC}{R_{\theta}^1} = \frac{1}{M} + B^*C + C^*C + \dots \quad (18)$$

Using double extrapolation, to zero concentration and to zero angle, through the Zimm plot, it is possible to obtain the molecular weight of the molecule under examination. This Zimm plot, plots C/R_{θ}^1 versus $\sin^2 \theta/2 + k^1 C$ where k^1 is an arbitrary constant to spread the plot and is usually selected so that $k^1 C$ has about the same magnitude as $\sin^2(\theta/2)$.

The molecular weight obtained in light scattering measurements is the weight average molecular weight. This is the case because the heavier molecules contribute more to the scattering intensity than do the light molecules.

In the actual light scattering experiment, a cylindrical cell must be used. The instrument must be calibrated so that the relationship between each coarse and fine sensitivity knob on the photometer is well defined.

The instrument response of the instrument must be determined for each concentration and angle, but first the instrument constant must be calculated. This constant can be calculated from the equation

$$\text{Instrument response} \times \text{instrument constant} = R_{\theta} \quad (19)$$

If benzene is used as a standard, with light of 546nm, and a scattering angle of 90° , $R_{90} = 16.3 \times 10^{-6}$. Thus, the instrument constant may be determined from this known R_{θ} and the instrument response (photometer reading) for benzene. Now the R_{θ} values for each solution at each angle can be obtained by subtracting the instrument response for buffer from the instrument response of the solution and multiplying the result by the instrument constant.

The optical constant, K , may be easily calculated if it is assumed that dn/dc is 10% of the refractive index of the solvent. The double extrapolation should intercept at one point on the Y axis (c/R_{θ}^1 axis). The reciprocal of this intercept is related to the molecular weight according to Equations 17 and 18.

VITA

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Mr. Blaner plans to continue his research into Vitamin A metabolism as a postdoctoral trainee in the laboratory of Dr. Dewitt Goodman, College of Physicians and Surgeons, Columbia University.