To the Graduate Council:

I am submitting herewith a dissertation written by William G. Hearl entitled "Structural and Functional Relationships of the Enzymes of the 4-Aminobutyrate Shunt: 4-Aminobutyrate Aminotransferase, Succinic Semialdehyde Dehydrogenase and Succinic Semialdehyde Reductase." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Biochemistry.

Jorge E. Churchich, Major Professor

We have read this dissertation
and recommend its acceptance:

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Accepted for the Council:

The Graduate School

STRUCTURAL AND FUNCTIONAL RELATIONSHIPS OF THE ENZYMES OF THE 4-AMINOBUTYRATE SHUNT: 4-AMINOBUTYRATE AMINOTRANSFERASE, SUCCINIC SEMIALDEHYDE DEHYDROGENASE AND SUCCINIC SEMIALDEHYDE REDUCTASE

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ABSTRACT

This work reports studies on the interrelationship of three mitochondrial enzymes from pig brain involved in the metabolism of the neurotransmitter 4-aminobutyrate: 4-aminobutyrate aminotransferase (E.C. 2.6.1.19), succinic semialdehyde dehydrogenase (E.C. 1.2.1.24) and succinic semialdehyde reductase (E.C. 1.1.1.2).

4-Aminobutyrate aminotransferase is a dimeric protein with a molecular weight of 100,000 made up of two subunits of identical size. The aminotransferase is dependent on the presence of the cofactor, pyridoxal-5-phosphate (PLP), for enzymatic activity. The holoenzyme contains 1 mol of PLP/mol of dimer with a dissociation constant for the cofactor of 1 nM. Upon addition of PLP, a second molecule of cofactor is bound to the holoenzyme with a dissociation constant of 3 μM. However, the enzyme has the same K_{cat} and K_m for 4-aminobutyrate before and after the binding of the second cofactor molecule.

The enzyme is capable of converting ω-amino acids into the corresponding semialdehyde by transamination with 2-oxoglutarate. Although the enzyme is generally recognized for its ability to metabolize 4-aminobutyrate, it can also utilize β-alanine and δ-aminovalerate as substrates.

Succinic semialdehyde is the product of the aminotransferase reaction with 4-aminobutyrate as the substrate. The main pathway for this intermediate in the brain is oxidation to succinate catalyzed by succinic semialdehyde dehydrogenase. This enzyme has been shown to be a tetramer, composed of subunits of equal molecular weight of 40,000. The dehydrogenase has a very

low K_m for succinic semialdehyde (10 μ M) and is sensitive to substrate inhibition by the aldehyde substrate (K_i = 90 μM).

A physical interaction between these two mitochondrial enzymes was investigated using affinity chromatography. Pyridoxal-5-phosphate-Sepharose was prepared and allowed to interact with 4-aminobutyrate aminotransferase. The enzyme was strongly retained by the modified Sepharose when the aminotransferase had a PLP binding site available. 4-Aminobutyrate aminotransferase which had PLP covalently attached at both active sites failed to be retained by PLP-Sepharose.

The immobilized aminotransferase was found to be catalytically competent and was fully characterized kinetically. K_m values of 4.1 mM and 2.0 mM were found for 4-aminobutyrate and 2-oxoglutarate respectively. Both values are several fold higher than the same constants obtained for the enzyme in solution.

The immobilized aminotransferase showed a linear temperature dependence over a range 4 to 40°C, indicating that diffusion does not significantly contribute to the increased extrinsic kinetic parameters seen for the immobilized enzyme. The altered kinetic constants are more likely due to steric effects.

The well defined aminotransferase-Sepharose system was used as an affinity column to detect a possible interaction with succinic semialdehyde dehydrogenase. The dehydrogenase was found to be retained by aminotransferase-Sepharose column at phosphate concentrations below 0.1 M. To preclude the possibility that succinic semialdehyde dehydrogenase was binding to a free PLP moiety by a Schiff's base linkage, the aminotransferase-Sepharose column was reduced by treatment with sodium borohydride. Following the

chemical modification, it was found that retained succinic semialdehyde dehydrogenase could be eluted from the column by a linear phosphate gradient at concentrations above 0.1 M.

To reaffirm the presence of a specific aminotransferase-dehydrogenase enzyme complex, antibody was raised against purified 4-aminobutyrate aminotransferase for use in immunoaffinity chromatography. The antibody isolated from immune rabbits was found to be monospecific for aminotransferase. Cross-reactivity with succinic semialdehyde dehydrogenase was not observed by immunodiffusion or by immunoblotting techniques.

Anti-aminotransferase bound to protein A-Sepharose retained both the aminotransferase and succinic semialdehyde dehydrogenase. Elution of the dehydrogenase could be accomplished by treatment of the column with buffers containing greater than 0.2 M sodium chloride. The column retained approximately 1 mol of aminotransferase/1 mol of dehydrogenase.

The immunoaffinity column was also able to retain the enzyme complex from a preparation of mitochondrial proteins. In addition to the detection of succinic semialdehyde dehydrogenase activity on the anti-aminotransferase column, several other unidentified proteins were found to be retained as well.

The interaction between the two mitochondrial enzymes was quantitated using polarization of fluorescence. The polarization of iodoacetamide fluorescein labeled 4-aminobutyrate aminotransferase was shown to increase from 0.121 to 0.219 in the presence of succinic semialdehyde dehydrogenase. A titration of the fluorescein-labeled aminotransferase with the dehydrogenase was performed and a dissociation constant of 0.1 μM was calculated for the enzyme complex.

Gel filtration on Sephacryl S-300 was used to identify a molecular weight for the aminotransferase-dehydrogenase complex. A mixture of the two enzymes eluted in a single fraction at a molecular weight of 300,000.

It is postulated that 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase will maintain the enzyme complex in vivo where the protein concentrations clearly favor the formation of an enzyme cluster.

Alternatively, succinic semialdehyde can be reduced to form 4-hydroxybutyrate by succinic semialdehyde reductase. Although the exact relationship of this compound to brain chemistry is not well understood, 4-hydroxybutyrate is currently believed to act as a neurotransmitter. Several aldehyde reductases have been reported in the literature to function in vitro as succinic semialdehyde reductases, however, it is not at all clear which if any of these enzymes function as the primary enzyme involved in the metabolism of 4-hydroxybutyrate.

The mitochondria represents a logical subcellular location for an enzyme involved in the metabolism of succinic semialdehyde. Thus, succinic semialdehyde reductase was purified from pig brain mitochondria 4000 fold by a combination of affinity chromatography on ADP- and blue-Sepharose and ion-exchange chromatography on CM-Sephadex and DE-52. The enzyme was determined to have a molecular weight of 110,000 as judged by gel filtration and gradient gel electrophoresis. The enzyme migrated as a single band of 55,000 under denaturing conditions indicating that the enzyme is composed of two identical molecular weight subunits.

The enzyme was determined to have an isoelectric point of 5.9 and was readily detectable after polyacrylamide gel electrophoresis by an activity stain.

The enzyme was found to be specific for succinic semialdehyde and malonic semialdehyde, both natural metabolites of 4-aminobutyrate aminotransferase. Although NADPH was the preferred cofactor, the enzyme was able to function with NADH.

The reaction was found to be reversible and showed an equilibrium constant of 4.1×10^{11} for the formation of 4-hydroxybutyrate at pH 7.2. The ΔG^{o} for this reaction was calculated to be -15.8 Kcal/mol.

Extensive steady state kinetics revealed the enzyme to function by a sequential Ordered mechanism with NADPH binding before the aldehyde substrate. Product inhibition studies identified 4-hydroxybutyrate to exit from the enzyme prior to the release of $NADP^{+}$.

Complete kinetic constants were obtained for the reductase. The K_m for NADPH and succinic semialdehyde were found to be 6 μ M and 23 μ M respectively. A V_{max} of 144 nmoles/min/mg was found for the enzyme and substitution of the kinetic constants into a Haldane's equation yielded an approximate equilibrium constant in good agreement with the experimentally obtained value.

The binding of NADPH was studied using tryptophan fluorescence. Titration of the enzyme with NADPH revealed two NADPH binding sites/mol of reductase with an apparent dissociation constant of 1.0×10^{-7}M.

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INTRODUCTION

4-Aminobutyrate, a normal constituent of the mammalian central nervous system, functions as an inhibitory neurotransmitter (1-4). This amino acid is found primarily in the brain and appears to have profound physiological significance. Abnormal 4-aminobutyrate levels have been associated with a variety of neurological disorders including Huntington's disease (5), epilepsy (6) and Parkinsonism (7).

The metabolism of 4-aminobutyrate involves three primary enzymes which constitute the 4-aminobutyrate shunt: glutamate decarboxylase, 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase. 4-aminobutyrate is formed as a result of -decarboxylation of L-glutamate by glutamate decarboxylase (E.C. 4.1.1.15). The catabolism of 4-aminobutyrate results in its entry into the TCA cycle as succinate following transamination with 2-oxoglutarate catalyzed by 4-aminobutyrate aminotransferase (E.C. 2.6.1.19). The reaction product, succinic semialdehyde, is subsequently oxidized by the NAD^+-dependent succinic semialdehyde dehydrogeanse (E.C. 1.2.1.24). The net reaction for this pathway,

2-oxoglutarate + NAD^+ ⟶ succinate + NADH + CO_2

results in a bypass of the 2-oxoglutarate dehydrogenase complex and succinyl thiokinase and accounts for 10% of the total flux through the TCA cycle in the brain (8). The relationship of the metabolism of 4-aminobutyrate with that of the oxidative metabolism of carbohydrates is shown in Figure 1.

The relative importance of 4-aminobutyrate and 4-aminobutyrate metabolism in the brain has resulted in intensive study of the enzymes involved in the synthesis and degradation of this amino acid. 4-Aminobutyrate aminotransferase, a pyridoxal-5-phosphate dependent enzyme, has been purified

Figure 1. The interrelationship between 4-aminobutyrate and carbohydrate metabolism. The enzymes involved are glutamate decarboxylase (E1), 4-aminobutyrate aminotransferase (E2), succinic semialdehyde dehydrogenase (E3) and succinic semialdehyde reductase (E4).

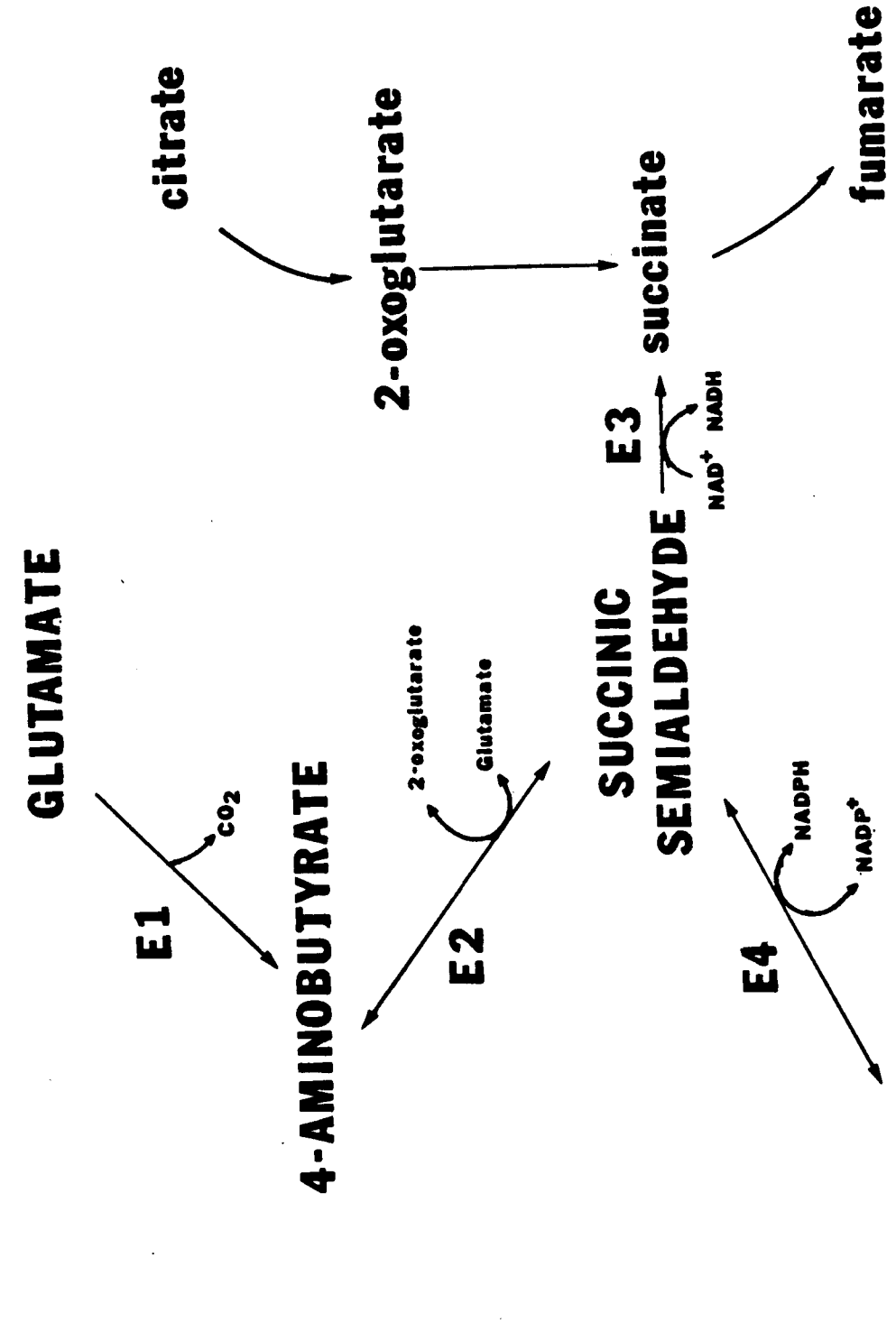

citrate
2-oxoglutarate
fumarate
succinate
E3
NAD+
NADH
GLUTAMATE
SUCCINIC
SEMIALDEHYDE
E1
CO2
4-AMINOBUTYRATE
2-oxoglutarate
Glutamate
E2
NADPH
NADP+
E4
4-HYDROXYBUTYRATE

Figure 1

to homogeneity from the brain tissues of different mammals including rat, mouse, pig, rabbit and human (9-13). The mammalian aminotransferases have been found to have a molecular weight of approximately 100,000 and to consist of two equal molecular weight subunits (14), although there is considerable variability found for the kinetic parameters (i.e. Km, Vm).

The pig brain form of the enzyme has been well characterized in this laboratory. Pig brain aminotransferase has a molecular weight of 100,000 and has been shown to possess two non-equivalent catalytic sites based on differing affinities for the cofactor (11). Churchich and Moses found that the low affinity catalytic site only became functional after the pyridoxal-5-phosphate at the high affinity site had been altered by chemical modification (15).

Brain 4-aminobutyrate aminotransferase is known to be associated with the mitochondria (16-19). Based upon detergent solubilization studies, the enzyme has been localized in the mitochondrial matrix (17) and may be associated with the inner membrane of this organelle (20).

The conversion of succinic semialdehyde to succinate by succinic semialdehyde dehydrogenase is the third and final step of the 4-aminobutyrate shunt. This enzyme, isolated from the brain tissues of rat, pig and human (21-23), has been shown to be highly specific for the substrate (24). The enzyme has a low K_m for succinic semialdehyde (approximately 10^{-6}M) and is subject to substrate inhibition by succinic semialdehyde at concentrations above 100 μM. The reaction is essentially irreversible and appears to function by a rapid equilibrium Random Bi Bi mechanism (22).

The succinic semialdehyde dehydrogenase isolated from pig brain is a tetramer composed of subunits of identical molecular weight of 41,000. It is capable of binding 2 mol of NADH per mol of dehydrogenase showing extreme

negative cooperativity (35). NADH was also found to be a potent inhibitor of the enzyme at concentrations above 0.1 mM.

Succinic semialdehyde dehydrogenase is known to be strictly associated with the mitochondria (26). However, the location of the dehydrogenase in the various regions of the brain does not necessarily coincide with the regional distribution of 4-aminobutyrate aminotransferase (27). This information combined with the isolation of multiple forms of the dehydrogenase in rat and human brain (23) suggests that the enzyme may have some additional function such as oxidizing succinic semialdehyde from another source (27).

An alternate fate of succinic semialdehyde is the reductive pathway leading to the production of 4-hydroxybutyrate. Recent evidence strongly suggests a neurotransmitter role for this compound in the brain. 4-Hydroxybutyrate has been found to be unevenly distributed in the brain (28) and was recently shown to be released by depolarization of rat brain slices (29), satisfying two important criteria for a neurotransmitter.

4-Aminobutyrate has been shown to be the direct precursor of 4-hydroxybutyrate via succinic semialdehyde (8,30). Radiolabeled 4-aminobutyrate applied to brain slices results in the rapid formation of radiolabeled 4-hydroxybutyrate. Further, when the enzymes of the 4-aminobutyrate shunt were treated with metabolic inhibitors, the levels of 4-hydroxybutyrate were altered in a manner consistent with the hypothesis that 4-hydroxybutyrate is synthesized as a result of 4-aminobutyrate catabolism (28).

4-Hydroxybutyrate is formed by an NADPH-dependent aldehyde reductase (E.C. 1.1.1.2) which has been isolated from rat, hamster, ox and human brain (31-36). Multiple forms of the aldehyde reductase were purified from the human brain by Cash et al. (36). This group found the reductases could easily

be distinguished on the basis of substrate specificity. The non-specific succinic semialdehyde reductase was of low molecular weight (48,000) and was inhibited by barbituates, an observation commonly made with other aldehyde reductases. The succinic semialdehyde-specific aldehyde reductase was considerably larger (82,000) and was insensitive to the barbituate inhibitors.

Two forms of succinic semialdehyde reductases were also purified from rat brain. The rat enzymes were found to closely resemble the enzymes isolated from the human brain (31). Rumigny et al. used data obtained from rat brain slices and compared this information with that found for the purified enzymes to show that the succinic semialdehyde-specific aldehyde reductase was responsible for the production of 4-hydroxybutyrate in the rat brain in vivo (31).

Similar data was obtained by Rivett et al. (37) with rat brain reductases. They isolated two forms of the aldehyde reductases which they termed high-K_m and low-K_m aldehyde reductase. The high-K_m aldehyde reductase appears to correspond to the non-specific aldehyde reductase isolated by Rumigny et al. (31). It appears that in the rat brain it is the low-K_m (succinic semialdehyde-specific) aldehyde reductase which is responsible for the production of 4-hydroxybutyrate in vivo (37).

The succinic semialdehyde reductase isolated from the liver and the brain of the hamster appears to be capable of the reverse reaction (i.e., formation of succinic semialdehyde from 4-hydroxybutyrate), although it was clear the reaction was energetically unfavorable. Thus, the significance of the ability of the reductase to operate in the reverse direction remains in dispute.

Succinic semialdehyde reductase has been reported to be a cytoplasmic enzyme (34). The apparent localization of this enzyme activity to the cytosol

has prompted Mandel to propose that succinic semialdehyde is in some way transported out of the mitochondria where it is then metabolized by a cytoplasmic succinic semialdehyde reductase (38). This is not consistent with observation that the endogenous levels of succinic semialdehyde in the brain are either very low (0.1 μM) (39) or non-existent (3).

PART I

AN INTERACTION BETWEEN MITOCHONDRIAL ENZYMES: 4-AMINOBUTYRATE AMINOTRANSFERASE AND SUCCINIC SEMIALDEHYDE DEHYDROGENASE

CHAPTER I

PROPERTIES OF 4-AMINOBUTYRATE AMINOTRANSFERASE IMMOBILIZED ON PYRIDOXAL-5-PHOSPHATE DERIVATIZED SEPHAROSE

Introduction

Applications for enzymes immobilized within a matrix has gained in importance in recent years. In a cell, many of the enzymes are functionally immobilized; they can be fixed in membranes, they can be part of a multienzyme complex or they can be part of a macromolecular structure (e.g. ribosome). Enzymes immobilized within a matrix provide an experimental approach to study problems relating to proteins functionally immobilized *in vivo*. Immobilized enzymes also provide new approaches for studies on the structure and function of enzymes (40,41) as well as serving as theoretical models for enzymes in living systems (42).

A common way of immobilizing enzymes has been to adsorb them onto insoluble cofactor analogs. Pyridoxal-5-phosphate (PLP)-dependent enzymes have been immobilized on matrices developed by Fukui *et al.* (43). These workers immobilized tryptophanase and tyrosinase onto a variety of PLP-derivatized Sepharose. The enzymes were catalytically functional after immobilization although their kinetic properties were somewhat altered (43).

The pyridoxal-5-phosphate dependent enzyme, 4-aminobutyrate aminotransferase from pig brain has been well characterized (44-47). The holoenzyme of 4-aminobutyrate aminotransferase is a dimer with two catalytic sites which have differing affinities for the cofactor (1 nM and 1 μM) (15). The high affinity site is occupied with PLP after purification; the vacant, low affinity site, which is fully capable of binding cofactor at PLP concentrations

above 10^{-5} M, provides a means of attaching the enzyme to a PLP affinity matrix.

This report considers the properties of 4-aminobutyrate aminotransferase immobilized on PLP-Sepharose. The nature of the protein-matrix ineraction will be investigated and the kinetic parameters of the bound enzyme enzyme will be discussed.

Experimental Procedures

Purification of 4-Aminobutyrate Aminotransferase

Unless otherwise specified, the purification steps were performed at 4°C and all buffers contained 0.1 mM EDTA and 1 mm 2-mercaptoethanol. The purification of 4-aminobutyrate aminotransferase was performed as previously described (15). Pig brains were obtained from Lays Meat Company shortly after slaughter and immediately placed on ice; the preparation was begun within 1 hour. A Waring Blender was used to prepare a 25% homogenate (w/v) in a solution of 10 mM potassium phosphate (pH 7.0) containing 1 mM 2-oxoglutarate. The resulting homogenate was adjusted to pH 5.5 by the slow addition of 1 M acetic acid. The homogenate was brought to 50°C in a hot water bath and after cooling to below 10°C, centrifuged at 10,000 g for 40 min and the pellet discarded. The supernatant was treated with ammonium sulfate. The pellet obtained at 40 to 65% saturation was dissolved in 0.01 M sodium acetate (pH 5.7) and dialyzed overnight against several changes of the same buffer (Buffer I). The dialysate was centrifuged at 10,000 g for 30 min and the pellet discarded. The supernatant was applied to a CM-Sephadex column (5 x 80 cm) equilibrated in Buffer I. The column was washed with 500 ml of the equilibration buffer and the enzyme was eluted with a linear gradient

made with buffer I (500 ml) and an equal volume of 0.3 M sodium acetate (pH 5.7). At the conclusion of the gradient, the column was washed with an additional 250 ml of 0.3 M sodium acetate to ensure complete elution of the aminotransferase. The enzyme eluted at an acetate concentration of 0.28 M.

The active fractions were pooled and then protein solution dialyzed extensively against 0.01 M potassium phosphate (pH 7.4). The enzyme was applied to a column (2.6 x 30 cm) of DEAE-Sephadex equilibrated in the same buffer (Buffer II). The column was washed with 50 ml of Buffer II and the aminotransferase was eluted by using a linear gradient made with Buffer II (50 ml) and an equal volume of 0.35 M potassium phosphate (pH 7.0). The enzyme eluted at a phosphate concentration of 0.05 M.

The active fractions were pooled and then dialyzed against 0.01 M potassium phosphate (pH 7.0) (EDTA was omitted) and applied to a hydroxyapatite column (2.6 x 20 cm) equilibrated in the same buffer (Buffer III). The column was washed with 50 ml of Buffer III and the aminotransferase was eluted by using a linear gradient made with Buffer III (50 ml) and an equal volume of 0.35 M potassium phosphate (pH 7.0). The enzyme was eluted at a phosphate concentration of 0.28 M.

Fractions which contained aminotransferase activity were subjected to polyacrylamide gel electrophoresis. Those fractions judged to be electrophoretically pure were pooled and dialyzed against 0.1 M potassium phosphate (pH 7.4). Impure fractions were pooled and dialyzed against Buffer III and subjected to a second hydroxyapatite chromatography. A scheme of the purification is included in Table I.

The enzyme has a specific activity of 20 units/mg of protein and a molecular weight of 100,000. A unit of enzyme activity is defined as the

TABLE I

PURIFICATION OF 4-AMINOBUTYRATE AMINOTRANSFERASE FROM PIG BRAIN

Treatment	Volume	Protein	Specific Activity at 25°C	Yield
	ml	mg/ml	units/ml	%
Homogenate	12000	40	0.005	100
Supernatant after 50°C	6000	6.2	0.022	34
$(NH_4)_2SO_4$ (45-75% satn)	800	10.4	0.035	12
CM-Sephadex fraction (0.01-0.3 M acetate, pH 5.7)	200	9.9	0.094	7.8
DEAE-Sephadex fraction (0.01-0.2 M phosphate, pH 7.4)	50	3.4	0.8	5.7
Hydroxyapatite (0.01-0.35 M phosphate, pH 7)	5	1.0	20	4.2

amount of enzyme needed to produce 1 μmol of succinic semialdehyde per min at 25°C.

Purification of Succinic Semialdehyde Dehydrogenase

Succinic semialdehyde dehydrogenase was prepared as previously described (22). Pig brains were obtained from Lays Meat Company and immediately placed on ice; the preparation was begun within 1 hour. All steps were performed at 4°C and all buffers contained 1 mM 2-mercaptoethanol and 0.5 mM EDTA unless stated otherwise.

Brain tissue (0.5 kg wet weight) was used to prepare a 25% (w/v) homogenate in a solution of 2 mM potassium phosphate (pH 7.2) in a Waring Blender at high speed (3 min). The homogenate was centrifuged at 10,000 g for 40 min and the pellet discarded. The supernatant was then treated with ammonium sulfate. The precipitate obtained at 30 to 70% saturation was dialyzed overnight versus Buffer I with several changes.

The dialysate was centrifuged at 10,000 g for 30 min to remove insoluble material and the supernatant was applied to a column (40 x 2.6 cm) of DEAE-Sephacel equilibrated in Buffer I. Succinic semialdehyde dehydrogenase was eluted with Buffer I. The clear wash fractions (red) with dehydrogenase activity were combined and dialyzed against Buffer I (EDTA was omitted) with several changes.

The protein solution was then applied to a hydroxyapatite column (25 x 2.6 cm) equilibrated in the same buffer. The column was washed with 100 ml of the equilibration buffer and the enzyme eluted by using a linear gradient of 100 ml of the equilibration buffer and an equal volume of 0.15 M potassium phosphate (pH 72.). Succinic semialdehyde dehydrogenase elutes at a phosphate concentration of 0.075 M.

The active fractions were pooled and dialyzed against Buffer I with several changes. The protein was applied to a column (10 x 1 cm) of AMP-Sepharose equilibrated in Buffer I. The column was washed with 25 ml of Buffer I containing 0.1 M KCl. The enzyme was removed from the column with a linear gradient of 30 ml buffer I and an equal volume of buffer I containing 10^{-3} M NAD^+. The enzyme elutes at an NAD^+ concentration of 10^{-4} M. The active fractions were combined and dialyzed against 0.1 M potassium phosphate. The protein was stored in 20% glycerol (v/v) at -20°C. The purified enzyme has a specific activity of 5 units/mg at 25°C. A unit of activity is defined as the amount of protein which will form 1 μmol of NADH per min at 25°C. A scheme of the purification is included in Table II.

Preparation of PLP-Sepharose

Pyridoxal-5-phosphate derivatized Sepharose was prepared using a modification of a published method (43). AH-Sepharose (2 g, dry weight), in 0.2 M sodium borate (pH 9.3), 40% dimethylformamide (v/v), was treated with 0.07 M p-nitrobenzoyl azide for 1 hour at 25°C. The p-nitrobenzamidohexyl-Sepharose was washed extensively with 50% dimethylformamide and then reduced by reaction with 0.1 M sodium dithionite in 0.5 M sodium carbonate (pH 8.5). The derivatized Sepharose was washed with distilled water and was then diazotized by treating with 0.1 M sodium nitrite in 0.5 M HCl for 7 min at 4°C. The diazonium-Sepharose was collected on a glass sintered funnel and washed with distilled water. PLP was linked to the Sephrose by incubation of the gel with 40 mg of PLP dissolved in 10 ml of 0.1 M sodium borate (pH 8.0) for 18 h at 4°C. The PLP-Sepharose thus obtained was thoroughly washed with water and then heated to 60°C for 30 min to destroy unreacted diazonium

TABLE II

PURIFICATION OF SUCCINIC SEMIALDEHYDE DEHYDROGENASE FROM PIG BRAIN

Treatment	Volume	Protein	Specific Activity at 25°C	Yield
	ml	mg/ml	units/mg	%
Homogenate	2000	22	0.0022	100
Supernatant	1000	15	0.0037	81
Dialyzed, 30-70% $(NH_4)_2SO_4$ fraction	400	21	0.051	77
DEAE-cellulose	37	2.8	0.12	12
Hydroxyapatite (2-150 mM potassium phosphate (pH 7.2))	20	1.2	0.50	11
AMP-Sepharose	2.2	0.3	5.3	1.7

sites. PLP-Sepharose was stored at -20°C in 50% glycerol and was stable for several months. The preparation scheme is seen in Figure 2.

Enzymatic Assays

Enzymes in solution were assayed as previously described (48). 4-Aminobutyrate aminotransferase activity was measured by the coupled assay method using succinic semialdehyde dehydrogenase as the second enzyme. Activity was measured by monitoring the change in absorbance at 340 nm for at least 2 min.

Immobilized 4-aminobutyrate aminotransferase was assayed by pumping the substrate mixture consisting of 30 mM 4-aminobutyrate, 10 mM 2-oxoglutarate in 0.1 M potassium phosphate buffer (pH 8.4) through the column with a peristaltic pump at a flow rate of 0.7 ml/min. Aliquots (0.5 ml) were assayed for succinic semialdehyde content using the fluorimetric method (48). The enzyme assay is based on fluorimetric measurements of the condensation product of cyclohexane 1-3-dione with succinic semialdehyde. The reagent solution contained 0.25 g of cyclohexane 1-3-dione, 10 g ammonium acetate, and 5 ml of glacial acetic acid in 100 ml of water. The effluent from the column was collected in 1 min fractions and the volume adjusted to 2 ml. The fraction was assayed in triplicate by adding a 0.5 ml aliquot to 0.4 ml of the reagent solution, heating for 15 min at 60°C and then diluting each sample to a final volume of 3 ml by the addition of water. The fluorescence intensity was recorded at 460 nm with an excitation wavelength of 365 nm. A standard curve of succinic semialdehyde was run in parallel to convert the data into the form μmol/min.

The columns used in the enzymatic assays were approximately 0.2 ml in volume packed in disposable plastic syringe. One hundred μg of

Figure 2: Preparation of 4-aminobutyrate aminotransferase immobilized on PLP-Sepharose. (*) indicates the formation of an irreversible linkage as a result of $NaBH_4$ reduction.

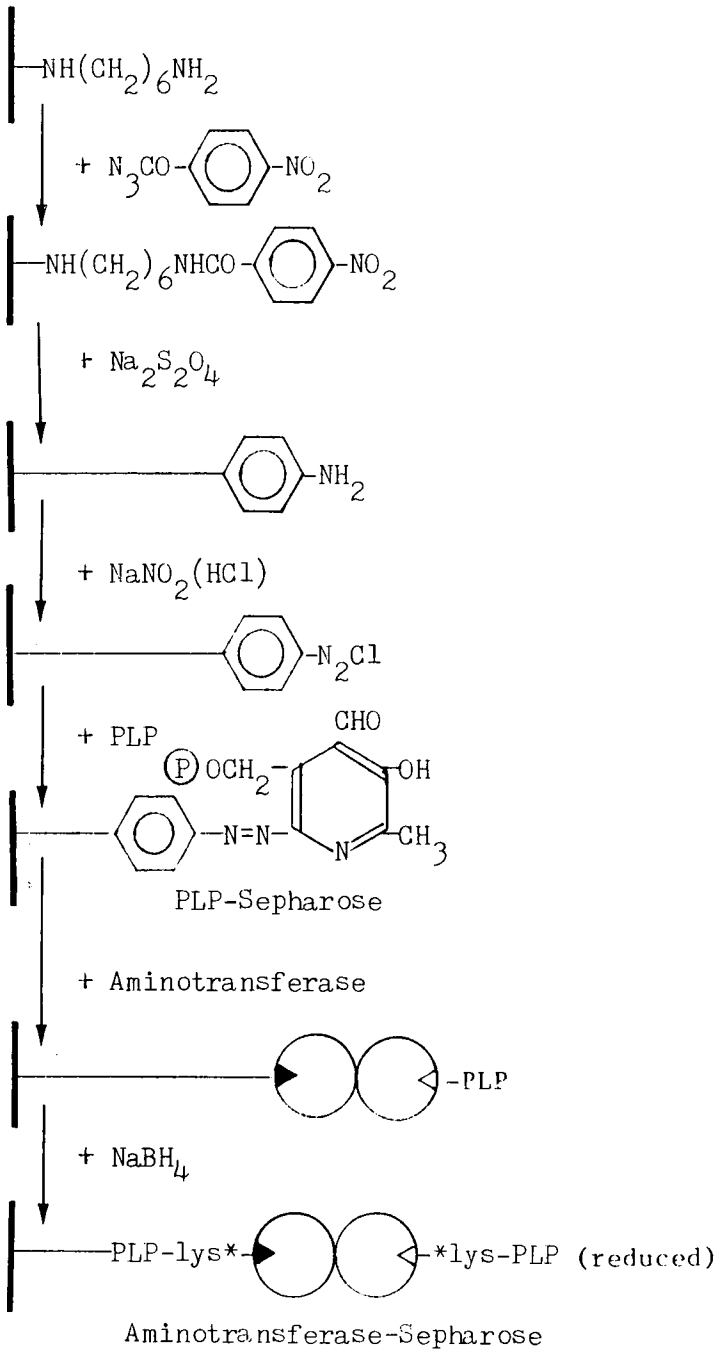

NH(CH2)6NH2
+ N3CO-
-NO2
NH(CH2)6NHCO-
-NO2
+ Na2S2O4
-NH2
+ NaNO2(HCl)
-N2Cl
+ PLP
CHO
P OCH2-
OH
-N=N-
N
CH3
PLP-Sepharose
+ Aminotransferase
-PLP
+ NaBH4
PLP-lys*-
*lys-PLP (reduced)
Aminotransferase-Sepharose

Figure 2

aminotransferase was applied to the column (> 99% retained) and a single column used for a full set of kinetic data.

Preparation of Modified 4-Aminobutyrate Aminotransferase

4-Aminobutyrate aminotransferase as it is isolated from pig brain contains 1 mol of PLP/mol of enzyme. The holoenzyme can be chemically modified with sodium borohydride; this treatment reduces the Schiff's base linkage between the PLP and an active site lysine residue irreversibly attaching the PLP to the enzyme. One mg of $NaBH_4$ is added to 1 mg of aminotransferase and allowed to react for 30 min at 0°C. Excess $NaBH_4$ is removed by dialysis against 10 mM potassium phosphate (pH 7.2). The chemically modified enzyme is catalytically inactive. This procedure can be used to prepare radiolabeled aminotransferase by using $[^3H]$ -$NaBH_4$ (1 mCi/mg $NaBH_4$).

Enzyme activity can be recovered by treating reduced holoenzyme with 10^{-5} M PLP. Reconstitution of enzyme activity is completed after incubation for 2 hours at 4°C. The aminotransferase in this form has a reduced PLP moiety at one active site and a Schiff's base-bound PLP at the second active site (reduced-reconstituted aminotransferase).

When the reconstituted enzyme is treated with $NaBH_4$ for a second time, the two catalytic sites are both irreversibly occupied with reduced PLP. This form of the aminotransferase (twice-reduced aminotransferase) is irreversibly inactivated.

Apo-aminotransferase was prepared by incubating 4-aminobutyrate aminotransferase (holoenzyme) with 20 mM 4-aminobutyrate at pH 7.4 in the presence of 2-mercaptoethanol. The dissociation of the cofactor pyridoxamine phosphate is achieved by the addition of 0.5 M H_2KPO_4. After an incubation

for 1 h at 25°C the sample is dialyzed against 0.1 M potassium phosphate (pH 7.5).

Materials

Succinic semialdehyde, 4-aminobutyrate, 2-oxoglutarate, NAD^+ and pyridoxal-5-phosphate were obtained from Sigma Chemical Company. Sodium boro [^3H] hydride was purchased from Research Products International Corp. Cyclohexane 1-3 dione was purchased from Aldrich Chemical Company. Extracti-gel was obtained from Pierce Chemical Company. CM-Sephadex, DEAE-Sephadex, Sephadex G-25, DEAE-Sephacel, AH-Sepharose and AMP-Sepharose were purchased from Pharmacia. All other chemicals of reagent grade or better were purchased from Fisher Scientific Company.

Results

Binding of 4-Aminobutyrate Aminotransferase to PLP-Sepharose

Native and resolved 4-aminobutyrate aminotransferase bind to pyridoxal-5-phosphate derivatized Sepharose, and remain firmly attached to the matrix as revealed by the absence of enzyme in the fractions eluted from the Sepharose column by increasing concentrations of phosphate buffers at pH 7.

Enzymatically inactive 4-aminobutyrate aminotransferase, prepared by saturating the holoenzyme with excess pyridoxal-5-phosphate followed by reduction with $NaBH_4$, failed to bind to PLP-Sepharose. Since the catalytic sites of the inactive species are blocked by reduction with $NaBH_4$, it appears that empty cofactor binding sites are needed for recognition of the pyridoxal-5-phosphate molecules coupled to the Sepharose matrix. A summary of the efficiency of the enzyme binding to the PLP-Sepharose is shown in Table III.

TABLE III

BINDING OF 4-AMINOBUTYRATE AMINOTRANSFERASE TO PLP-SEPHAROSE

Enzyme form[a]	Retention[b]
Holoenzyme	99%
Reduced Holoenzyme	95%
Reduced-Reconstituted Holoenzyme	92%
Reduced-Saturated Holoenzyme	0%

[a]Enzyme form corresponds to those described in "Experimental Procedures."

[b]Percent retained was measured by the amount of enzyme activity recovered in the wash (Holoenzyme) or by the absorbance at 280 nm of the column wash (Reduced Holoenzyme, Reduced-Reconstituted Holoenzyme, and Reduced-Saturated Holoenzyme).

Functionality of Immobilized 4-Aminobutyrate Aminotransferase

4-Aminobutyrate aminotransferase bound to PLP-Sepharose retains full catalytic activity as determined by direct assay of the column using the fluorimetric assay procedure described in "Experimental Procedures." The immobilized enzyme was found to be stable over a period of several hours at 25°C (Figure 3). The stability of the column allows steady state-type kinetics to be performed using a single column for a complete set of kinetic data.

Effect of Immobilization of the pH Activity Curve

Immobilization of an enzyme has been shown to effect the pH activity curve of other PLP-immobilized enzymes (42). The effects of immobiliztion on the activity of 4-aminobutyrate aminotransferase with respect to pH is shown in Figure 4. The pH optimum of immobilized aminotransferase was approximately the same as that seen for the soluble enzyme, although the range of maximal activity is significantly reduced.

Steady State Kinetics of Immobilized 4-Aminobutyrate Aminotransferase

The kinetic properties of the Sepharose bound aminotransferase were examined in comparison with those of the native soluble enzyme. The native enzyme is known to function via a ping-pong mechanism (11) and to be subject to substrate inhibition by 2-oxoglutarate (D.S. Kim, unpublished result). The steady state kinetic data for the immobilized enzyme with 4-aminobutyrate as the varied substrate and with 2-oxoglutarate fixed at 10 mM gave rise to a set of parallel lines indicative of a Ping-Pong mechanism (Figure 5). The appropriate replot of the data (Figure 5, inset) yielded the kinetic constants seen in Table IV.

Figure 3. Stability of 4-aminobutyrate aminotransferase immobilized on PLP-Sepharose. A 0.2 ml column of PLP-Sepharose containing 100 μg of aminotransferase was maintained at 25°C for 8 h. At two hour intervals, the column was assayed by the fluorimetric method. After each assay, the column was equilibrated and then maintained in 10 mM potassium phosphate (pH 7.2).

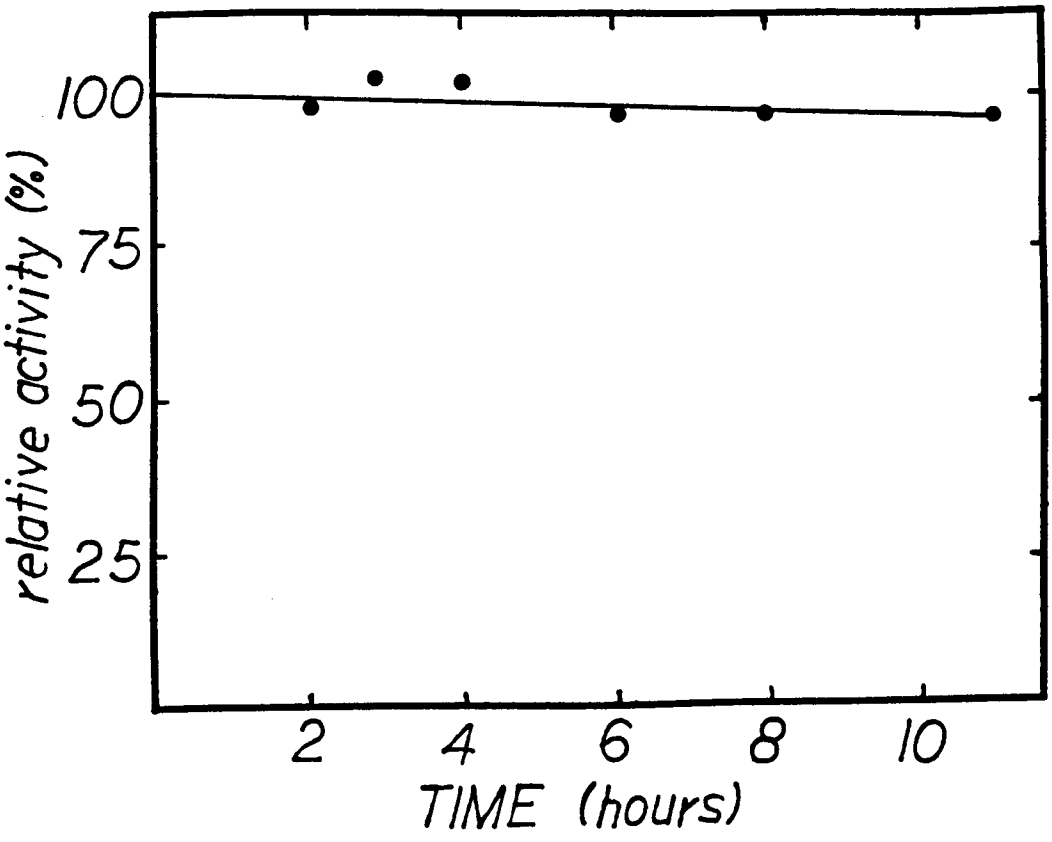


Figure 3

Figure 4. pH activity profile of immobilized 4-aminobutyrate aminotransferase. Enzyme assays were conducted with 30 mM 4-aminobutyrate and 10 mM 2-oxoglutarate in a 0.1 M phosphate buffer at various pH. 10 μg of enzyme was used to assay the protein in solution. The fluorimetric assay was used to detect the presence of succinic semialdehyde.

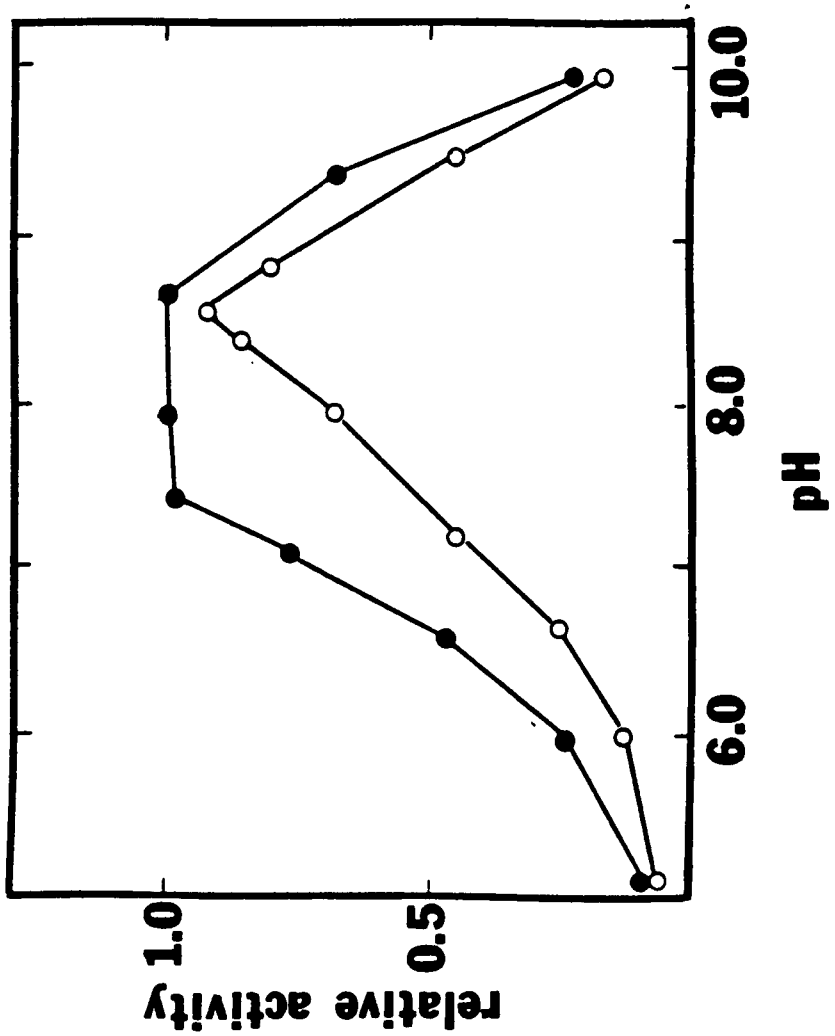


Figure 4

Figure 5. Reciprocal plots of the steady state kinetic data for immobilized 4-aminobutyrate aminotransferase. 4-Aminobutyrate (GABA) was varied at several fixed concentrations of 2-oxoglutarate (α-KG): a, 5 mM; b, 10 mM; c, 15 mM. The inset contains the secondary plot of the 1/v intercept versus the 2-oxoglutarate concentration.

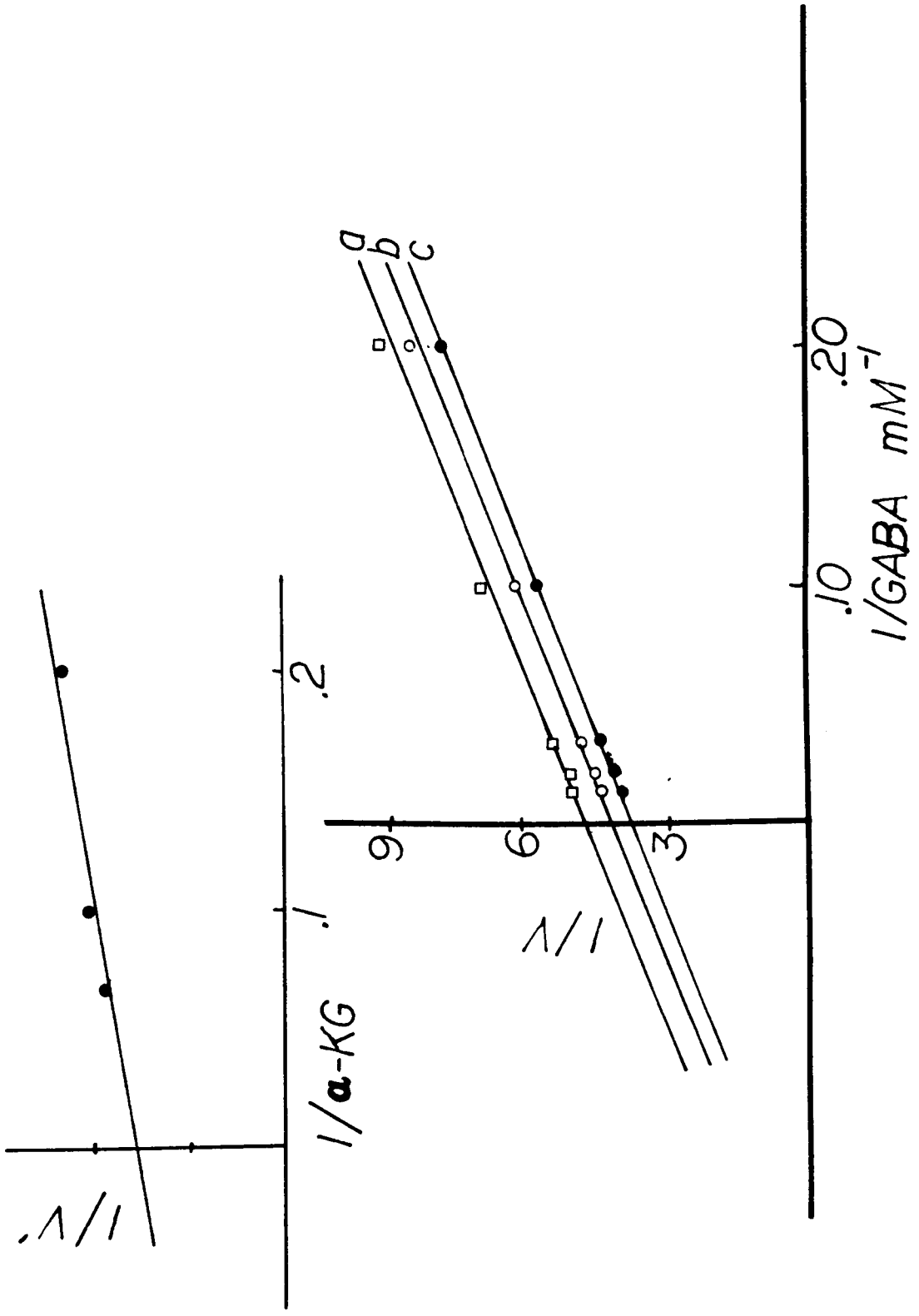


Figure 5

TABLE IV

KINETIC PARAMETERS OF IMMOBILIZED AMINOTRANSFERASE

	K_m^a	K_m^b	V_{max}^c	K_{ia}
Free	0.1 mM	1.0 mM	.31	8.0 mM
Immobilized	2.0 mM	4.1 mM	.28	33.0 mM

[a]Kinetic constants for 2-oxoglutarate.

[b]Kinetic constants for 4-aminobutyrate.

[c]Expressed in μmoles·min-1·mg^{-1}.

The value for the substrate inhibition constant (K_{ia}) for 2-oxoglutarate can be obtained by plotting the data according to the following equation:

$$\frac{1}{v} = \frac{1}{V_m} + \left(\frac{1}{(V_m K_{ia})}\right) [A] \qquad (1)$$

where [A] is the concentration of 2-oxoglutarate. As seen in Figure 6, the soluble enzyme is more sensitive to substrate inhibition than the immobilized aminotransferase. The kinetic constants obtained from the plot of 1/v versus A (Figure 6, B) are contained in Table IV.

Diffusional Effects on the Intrinsic Kinetic Parameters

The kinetic parameters of the immobilized enzymes are affected by factors such as diffusion and steric hindrance. The relative contribution of diffusional effects on the kinetic behavior of an immobilized enzyme system can be assessed from an Arrhenius plot (49). In the case of immobilized 4-aminobutyrate aminotransferase, the plot of log v versus 1/T yielded a straight line over the temperature range 4-40°C, indicating that diffusional effects do not play a significant role in the reaction catalyzed by the immobilized enzyme (Figure 7).

Discussion

Pyridoxal-5-phosphate derivatized Sepharose has been shown to firmly retain 4-aminobutyrate aminotransferase. The immobilized enzyme is catalytically competent and was found to be stable over a period of several hours at 25°C.

Figure 6. Substrate inhibition by 2-oxoglutarate. Soluble and Sepharose bound 4-aminobutyrate aminotransferase were assayed in increasing amounts of 2-oxoglutarate with 4-aminobutyrate held constant at 20 mM. (panel A). Succinic semialdehyde concentrations were determined by the fluorimetric method. Panel B contains the data plotted according to equation (1) (see text).

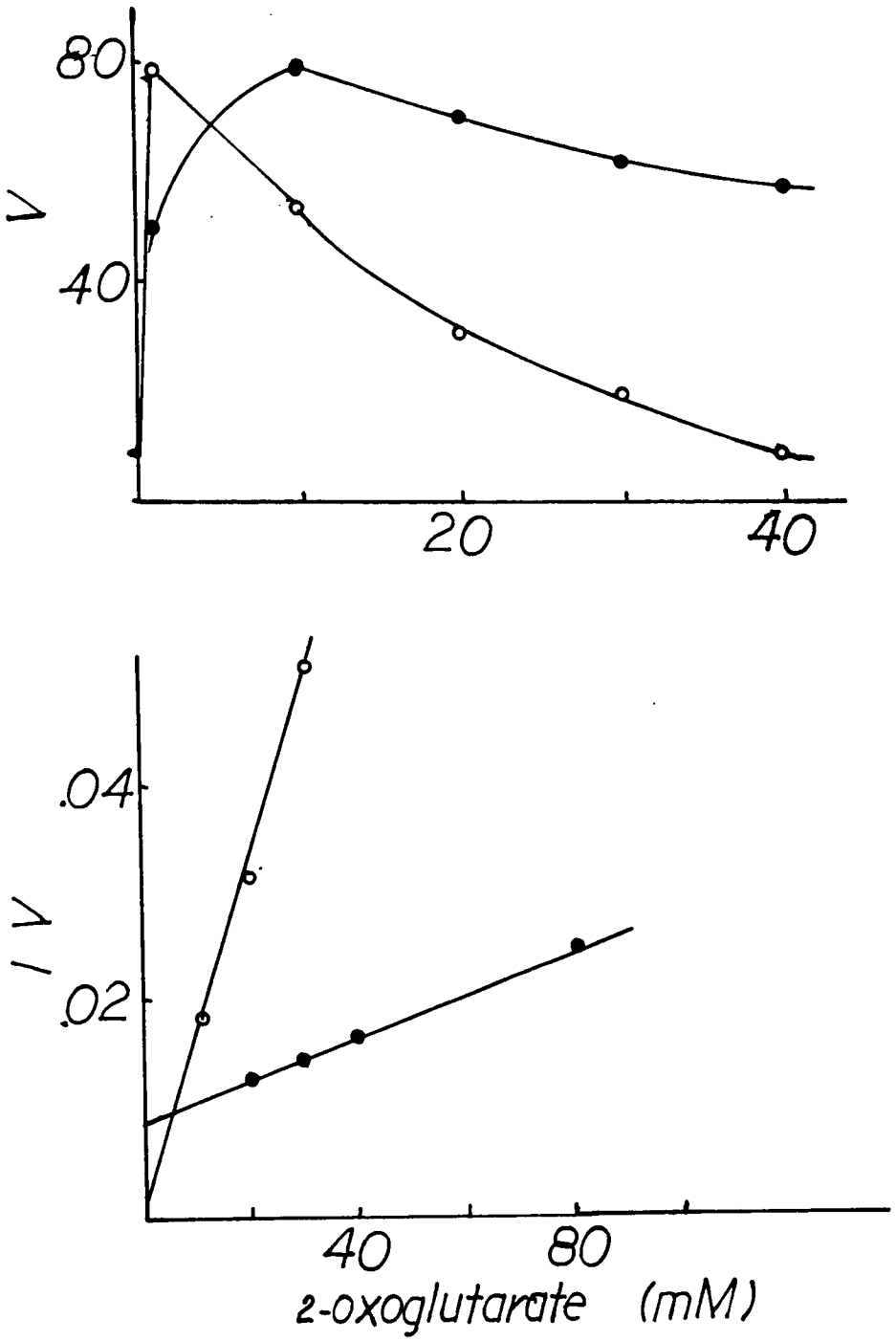


Figure 6

Figure 7. The effect of temperature on the enzymatic activity of immobilized 4-aminobutyrate aminotransferase. A column was prepared as described in Figure 1. The assay buffer of 10 mM 2-oxoglutarate, 10 mM 4-aminobutyrate in 01. M potassium phosphate (pH 8.4) was heated to several different temperatures and applied to the column.

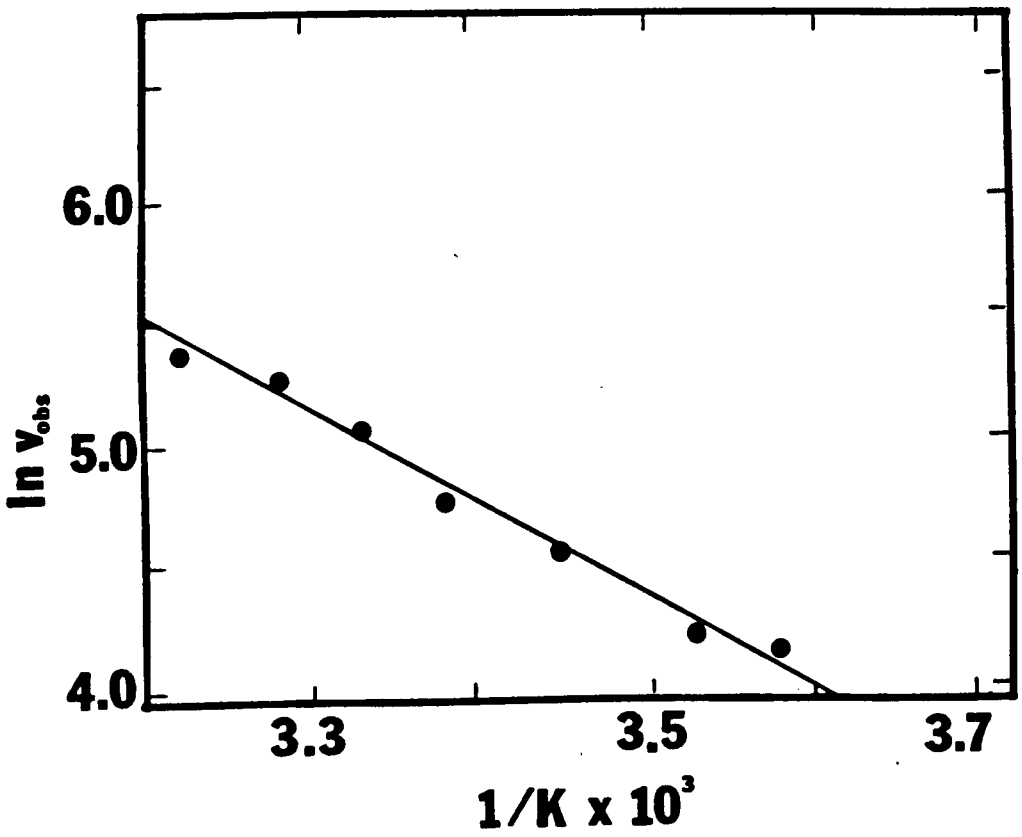


Figure 7

The kinetic constants obtained for the immobilized enzyme are significantly larger than those found for the soluble holoenzyme. The intrinsic kinetic constants are affected by partitioning, diffusion, conformational changes and steric effects that generally result in extrinsic values which are artifically high (49).

The role of diffusion was shown to be a minimal factor in the elevated K_m values observed for the immobilized enzyme. If the orientation of the enzyme while immobilized approximates the configuration shown in Figure 2, then it is also likely that partitioning will have no effect on the kinetic constants.

The binding of the enzyme to the matrix may induce a conformational change which could alter the kinetic properties. Since 4-aminobutyrate aminotransferase binds to the derivatized Sepharose via the vacant PLP binding site, any conformational change which might result in altered kinetic properties should also be observed with PLP-saturated aminotransferase in free solution. However, this form of the soluble enzyme has been shown to have properties identical to the native enzyme (15), suggesting that the binding of a second molecule of PLP should have little or no effect on the immobilized enzyme activity.

Steric hindrance remains the most likely cause of the increased kinetic constants. In the case of most immobilized systems, this refers to the masking of the active site as a result of protein binding. Steric effects which might arise in the aminotransferase-Sepharose system would be more likely to be attributed to the high protein concentration found within the matrix. The concentration of the aminotransferase on a standard column (100 μg bound to 0.1 ml PLP-Sepharose) is about 2×10^{-5} M assuming the excluded volume is

approximately 50% of the total bed volume (Pharmacia). The relatively high concentration found in the matrix compares favorably with the value found for the enzyme in the mitochondria of 2 μ M (Moses, UT thesis) (50). When the enzyme is assayed in solution, the concentration of the aminotransferase is about 1 nM. Thus, the conditions of the column are more closely related to the in vivo situation and the value found for the kinetic parameters for the immobilized enzyme may in fact be a better approximation of in vivo enzyme performance.

The immobilized enzyme system described in this study is well defined. It has physical and chemical properties which are very similar to those of the aminotransferase in free solution but has the added advantages of being at relatively high concentrations and of being attached to a solid support. This immobilized enzyme system favorably reflects the native enzyme and is qualified to serve as a 4-aminobutyrate aminotransferase affinity column.

CHAPTER II

DETECTION OF AN INTERACTION BETWEEN 4-AMINOBUTYRATE AMINOTRANSFERASE AND SUCCINIC SEMIALDEHYDE DEHYDROGENASE BY AFFINITY CHROMATOGRAPHY

Introduction

4-Aminobutyrate aminotransferase, together with succinic semialdehyde dehydrogenase and glutamate decarboxylase, are the main components of the 4-aminobutyyrate shunt. They participate in the metabolism of the neurotransmitter, 4-aminobutyrate.

Accumulation of succinic semialdehyde, a compound highly toxic to the cell, is prevented by 4-aminobutyrate aminotransferase which catalyzes the reversible reaction with succinic semialdehyde and glutamate. Since both the aminotransferase and the dehydrogenase are in the mitochondiral matrix participating in successive reactions in the same metabolic pathway, it seems reasonable to consider the possibility that they form part of a multienzyme complex. Such "enzyme clusters" could provide a suitable device in the control of metabolic crossroads by increasing the efficiency of the sequential catalytic steps throught the decrease of transit time for the passage of metabolites between the enzymes. In addition, "enzyme clusters" could prevent the leakage of intermediate metabolites which are harmful to the cell, i.e., succinic semialdehyde.

It is the purpose of this research to investigate the interaction between 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase and to define the experimental conditions under which such interactions may take place.

Experimental Procedures

Preparation of 4-Aminobutyrate Aminotransferase-Sepharose

PLP-Sepharose was prepared as described in Chapter I. Columns were routinely packed in a disposable plastic syringe with a total bed volume of 0.2 ml. The column was saturated with the addition of 300 μg of 4-aminobutyrate aminotransferase/0.1 ml gel and the protein was covalently linked to the Sepharose by treatment with 5 mg of $NaBH_4$ per 0.1 ml of Sepharose. The reaction was allowed to continue for 30 min at 4°C. The aminotransferase-Sepharose was washed extensively with 10 mM potassium phosphate (pH 7.2) and used immediately.

Preparation of Antiserum

A rabbit monospecific antiserum against porcine 4-aminobutyrate aminotransferase was prepared as described by Isemura *et al.* (51). A New Zealand White rabbit received subcutaneous injections at two week intervals of an emulsified mixture (1.5 ml) of 4-aminobutyrate aminotransferase (1 mg/ml) and Freund's complete adjuvant. Blood was drawn from the ear vein 3 weeks after the final injection and thereafter. The presence of anti-aminotransferase antibody was detected by immunodiffusion using the method of Ouchterlony and Nilsson (52).

Purification of Immunoglobin G

Serum collected from an immunized rabbit was adjusted to 40% saturation in ammonium sulfate. The precipitate was dissolved in 50 mM potassium phosphate containing 0.15 M NaCl (PBS). The ammonium sulfate precipitation was repeated two times and the final precipitate dialyzed against PBS. The dialyate (250 mg/5 ml) was loaded onto a protein A-Sepharose column (5 ml

bed volume) equilibrated in PBS and the column washed with 10 bed volumes of the starting buffer. The IgG fraction was eluted with 0.1 M glycine/HCl buffer (pH 2.8) directly into tubes containing enough 1 M potassium phosphate (pH 7.5) to bring the pH to 7. Protein-containing fractions were combined and dialyzed against PBS. The final IgG preparation was stored at -20°C prior to use.

Western Blots

Protein was transferred from polyacrylamide gels to nitrocellulose filters electrophoretically using a modification of the procedure of Bittner et al. (53). Nitrocellulose filters were prepared for use by soaking for at least 1 h in a buffer containing 0.05 M Tris-HCl, 0.4 M glycine and 20% methanol (transfer buffer). Following polyacrylamide gel electrophoresis (in the presence or absence of SDS), the wet nitrocellulose was layered over the gel and the bubbles firmly pressed out. The gel-nitrocellulose filter sandwich was inserted into the electrophoresis tank containing enough transfer buffer to completely cover the sandwich; the nitrocellulose is oriented in the tank facing the cathode. The proteins are transferred to the nitrocellulose by electrophoresis at a constant voltage of 100 V for 2.5 h at 4°C.

The nitrocellulose was prepared for staining by first saturating the vacant protein binding sites with bovine serum albumin (3%) in 0.01 M Tris-HCl (pH 7.4) containing 0.9% NaCl, for 1 hour with gentle rocking. The nitrocellulose is rinsed with distilled water and then probed with anti-aminotransferase IgG (200 µl) in 10 ml of 0.05 M Tris-HCl (pH 7.4) containing 0.12 M NaCl, 5 mM EDTA, 0.05% gelatin (type III) and 0.5% Nonidet P-50 (NETG). The incubation was allowed to proceed for 2 h with gentle rocking. Following a rinse with water, the blot was stained with [^{125}I] -labeled goat anti-rabbit IgG (10^7 cpm)

in 10 ml of NETG for 1 hour with rocking. The nitrocellulose is then rinsed with 200 ml of 0.05 M Tris-HCl (pH 7.4) containing 0.12 M NaCl, 5 mM EDTA, 0.05% gelatin (type III), 1% SDS, 0.5% Triton. The nitrocellulose is given a final water rinse and allowed to air dry.

The stained nitrocellulose was visualized by exposure to Kodak X-ray film for 24 h at -80°C.

Isolation of Mitochondrial Protein

Mitochondria were isolated from whole pig brain using a procedure adapted from Schnaitman and Greenawalt (54). Pig brain (125 g, wet weight) was homogenized (25%, w/v) in a buffer of 220 mM D-mannitol, 70 mM sucrose, 2 mM HEPES (pH 7.4), 0.5 mM EGTA containing 0.5 mg/ml of bovine serum albumin. The homogenate was centrifuged at 560 g for 15 min to remove large debris, whole cells and nuclei; the pellet was discarded. The supernatant fraction was centrifuged at 7000 g for 15 min to pellet mitochondria. The mitochondrial pellet was washed three times in homogenization buffer, and was resuspended in 25 ml of 0.25 M sucrose. The suspension was layered onto 28 ml sucrose gradient (20-55%) on a 5 ml cushion of 60% sucrose and centrifuged at 30,000 g for 35 min. The fluffy mitochondrial band was removed by aspiration and diluted to 0.25 M in sucrose by the addition of water. The mitochondria were pelleted by centrifugation at 9,000 g for 20 min and then resuspended in a buffer of 10 mM potassium phosphate (pH 7.2). The mitochondria were disrupted with several passes of a Dounce homogenizer. The homogenate was centrifuged for 30 min at 100,000 g to remove membranes. Mitochondrial protein was stored at -20°C prior to use.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed according to the procedure of Davis (55). Discontinuous sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out at 25°C as described by Laemmli (56). The gel (7.5%) contained 0.1% SDS.

Protein bands were detected by staining with Coomassie blue dye for 1 h unless stated otherwise. Gels were destained in a solution containing 5% methanol and 7.5% acetic acid in water.

Silver Staining

Gels were stained with silver using the procedure of Morrissey (57) when the sample contained small amounts of protein. After polyacrylamide gel electrophoresis, the gel was rinsed in 50% methanol, 7% acetic acid for 30 min followed by treatment with 5% methanol, 10% acetic acid for 30 min with gentle shaking. The protein was then fixed with 10% gluteraldehyde for 30 min. After the gel was washed for 2 h under running water, it was soaked for 30 min in water containing 5.0 μg/ml of dithiothreitol. The gel was then placed into a 0.1% solution of $AgNO_3$ for 1 hour with gentle rocking. The stain was developed by treating the gel with 50 μl of 30% formaldehyde in 50 ml 3% sodium carbonate. The reaction was stopped by the rapid addition of 5 ml of 2.3 M sodium citrate. Stained gels were stored wet in 0.1% sodium carbonate.

Materials

Protein A-Sepharose was purchased from Pharmacia. Freund's adjuvant was purchased from Grand Island Biological Company. The rabbit was a gift

from Dr. J. Joshi and the ^{125}I-labeled goat anti-rabbit IgG and the nitrocellulose were gifts from Dr. J. Koontz.

Results

Retention of Succinic Semialdehyde Dehydrogenase on Aminotransferase-Sepharose

The well behaved immobilized enzyme system described in Chapter I was used to detect specific interactions with succinic semialdehyde dehydrogenase. To this end, a sample of succinic semialdehyde dehydrogenase (1 mg) was allowed to equilibrate with immobilized aminotransferase at 25°C for 15 minutes, and the PLP-Sepharose column (0.2 ml) eluted with phosphate buffers (pH 7.2) of increasing ionic strengths. Enzymatic assays of the effluent revealed full retention of the dehydrogenase at phosphate concentrations of 0.1 M at neutral pH.

In order to demonstrate that the retention of succinic semialdehyde dehydrogenase is not due to binding of this enzyme to pyridoxal-5-phosphate covalently attached to the Sepharose matrix, immobilized aminotransferase reduced with $NaBH_4$ (see Figure 2, page 17) was used for the affinity chromatographic experiments.

When purified succinic semialdehyde dehydrogenase was applied to reduced immobilized aminotransferase, it was found that elution of succinic semialdehyde dehydrogenase from the column occurs only at phosphate concentrations higher than 0.1 M as shown by the results included in Figure 8.

Other proteins, i.e. bovine serum albumin, ovalbumin and glutamate dehydrogenase, are not retained by immobilized aminotransferase when the concentration of eluting buffer is 0.01 M at pH 7.2.

Figure 8. Elution of succinic semialdehyde dehydrogenase from aminotransferase-Sepharose. The aminotransferase-Sepharose was prepared as described in the legend of Figure 2 (page 17) and equilibrated in 10 mM potassium phosphate (pH 7.2) containing 1mM 2-mercaptoethanol. 500 µg of succinic semialdehyde dehydrogenase was applied to the column and the column washed extensively with the same buffer. The protein was eluted with increasing concentrations of potassium phosphate (pH 7.2). Fractions were assayed for dehydrogenase activity.

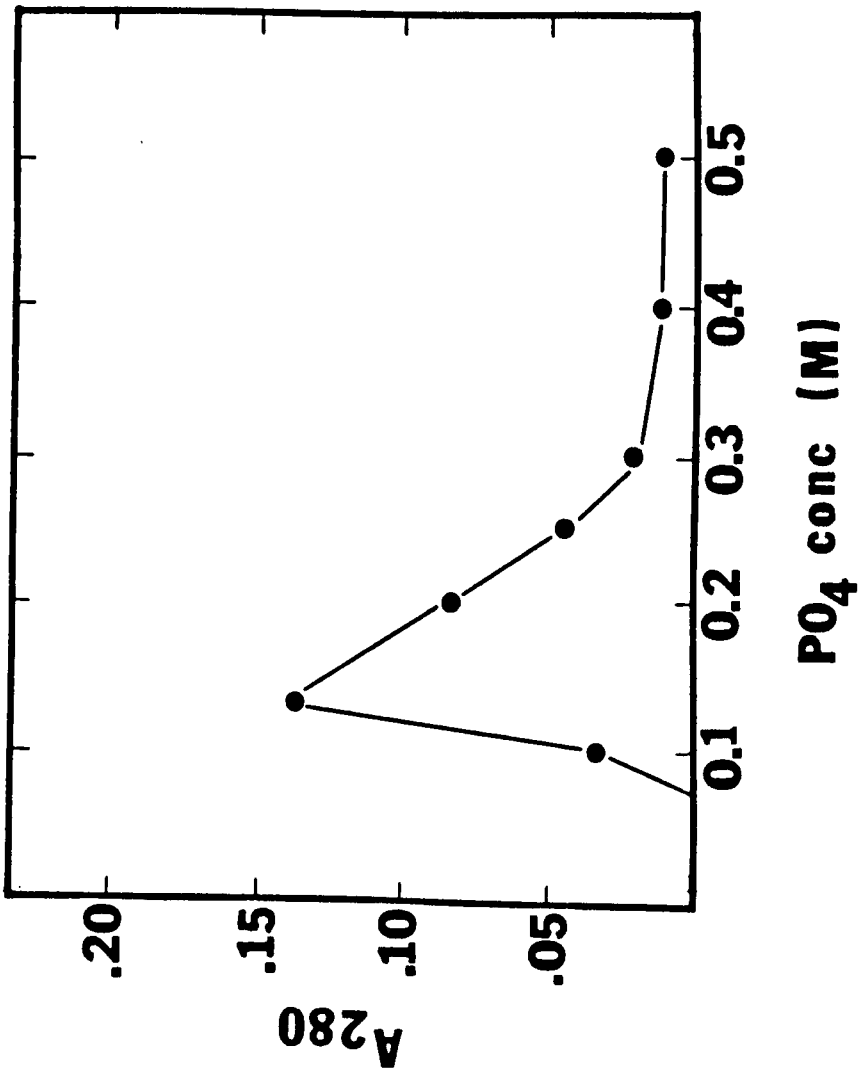


Figure 8

The results lend strong support to the contention that retention of succinic semialdehyde dehydrogenase by immobilized aminotransferase is not due to Schiff's base formation between pyridoxal-5-phosphate coupled to Sepharose and the dehydrogenase.

Specificity of Anti-aminotransferase IgG

The immunoglobin isolated from antisera gave strong precipitation lines on Ouchterlony plates (Figure 9) indicating the presence of anti-aminotransferase antibody in the preparation. In order to quantitate the sensitivity of the antibody-antigen interaction, 4-aminobutyrate aminotransferase was applied directly to nitrocellulose filters using the dot blotting technique. Figure 10 reveals that a 1:25 dilution of the antibody is capable of detecting as little as 0.3 μg of enzyme bound on the nitrocellulose.

When the enzyme was subjected to anionic polyacrylamide gel electrophoresis followed by immunoblotting, the anti-aminotransferase IgG specifically recognized 4-aminobutyrate aminotransferase; there was no cross-reactivity with the succinic semialdehyde dehydrogenase run in the adjoining lane (Figure 11). Also, when a preparation of mitochondrial proteins was probed with anti-aminotransferase IgG, the stained nitrocellulose showed only a band which corresponded to purified 4-aminobutyrate aminotransferase (data not shown).

The immunoblot procedure was also attempted with proteins after SDS-PAGE. Under these conditions the antibody failed to recognize any protein, including the aminotransferase (Figure 12). Although this result is somewhat unusual for polyclonal antibody, it is consistent with the observation that 4-aminobutyrate aminotransferase fails to regain native conformation after denaturation by a variety of agents (e.g. SDS, 4 M guanidine HCl, heat) (data not shown).

Figure 9. Detection of the presence of anti-aminotransferase antibody by immunodiffusion. Ammonium sulfate fractionated antisera was placed in the center well (4 µl) and purified aminotransferase placed in the surrounding wells (1, 4 µg; 2, 2 µg; 3, 1 µg; 4, 0.5 µg). Well 5 contained 4 µg of succinic semialdehyde dehydrogenase.

Figure 9

Figure 10. Dot blot of 4-aminobutyrate aminotransferase probed with anti-aminotransferase IgG. 4-aminobutyrate aminotransferase was transferred to nitrocellulose using the dot blot technique. Various quantities of protein were placed in each well and then transferred directly to the nitrocellulose by gentle suction. The nitrocellulose was then handled in a manner identical to that used for the staining of nitrocellulose from Western blots (see Experimental Procedures). Lanes a-c were probed with increasing dilutions of antibody: lane a, 1:25; lane b, 1:50; lane c, 1:500. The amount of aminotransferase applied to the nitrocellulose is seen on the right. (*) indicates the fraction was impure aminotransferase.

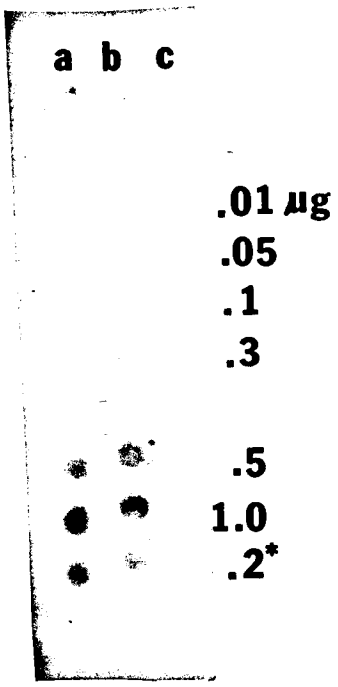


Figure 10

Figure 11. Determination of the specificity of antisera raised against 4-aminobutyrate aminotransferase. 15 μg of 4-aminobutyrate aminotransferase (lanes b and d) and 20 μg of partially purified succinic semialdehyde dehydrogenase were subjected to polyacrylamide gel electrophoresis (9% gel) and transferred to nitrocellulose as described in "Experimental Procedures." The nitrocellulose bound proteins were probed with a 1:50 dilution of anti-aminotransferase IgG and stained with [^{125}I] -labeled goat-anti-rabbit IgG. Lanes c and d are autoradiographs of the stained nitrocellulose and lanes a and b correspond to the Coomassie blue stained gel.

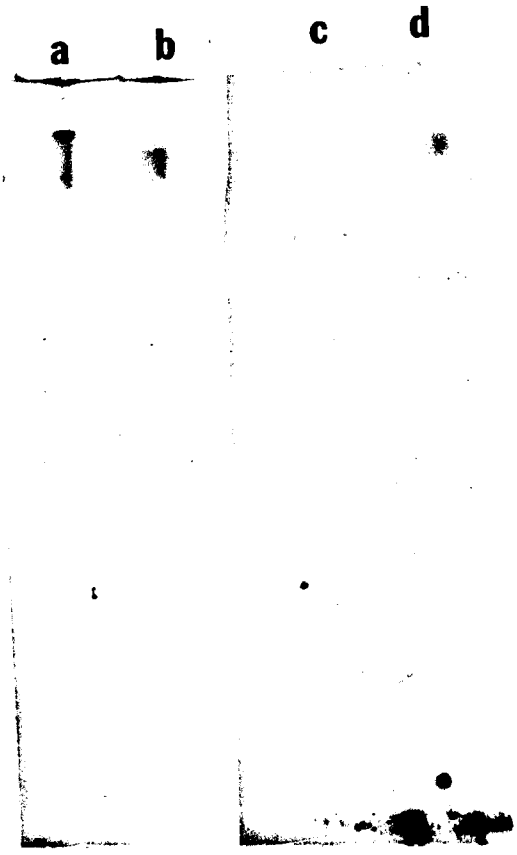


Figure 11

Figure 12. Immunoblot of 4-aminobutyrate aminotransferase after SDS-polyacrylamide gel electrophoresis. Proteins were transferred electrophoretically onto nitrocellulose following electrophoresis as described in "Experimental Procedures." The autoradiograph of the nitrocellulose is contained in lanes a-d. Proteins stained with silver are shown in lanes e-h. Each lane contained approximately 20 μg of protein: partially purified succinic semialdehyde dehydrogenase (a & e); 4-aminobutyrate aminotransferase (b & f); mitochondrial protein (c & g); NaCl wash from anti-aminotransferase-protein A-Sepharose (d & h).

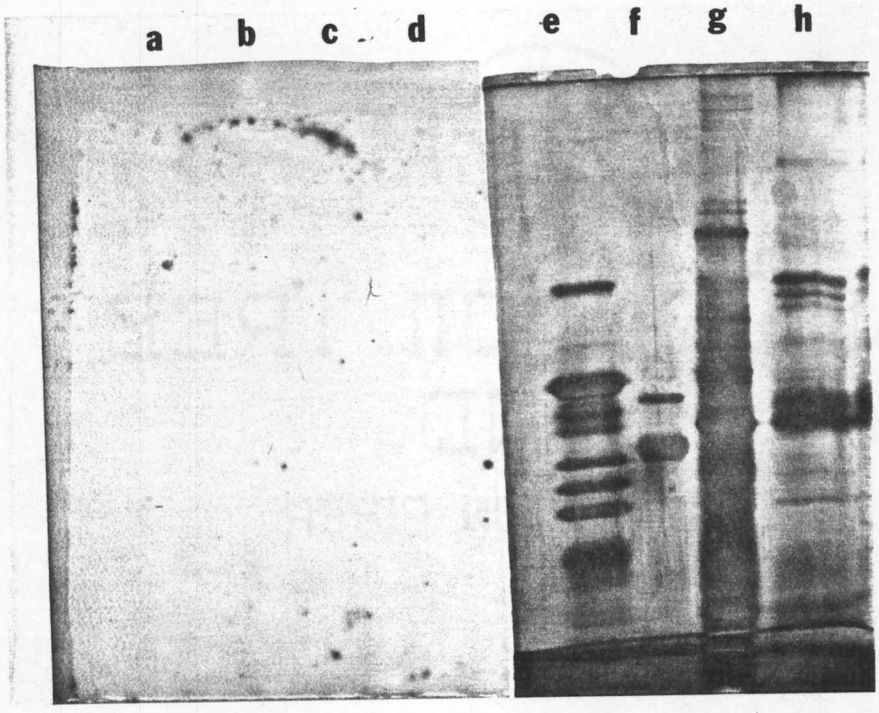


Figure 12

Detection of a Protein Complex by Immunoaffinity Chromatography

Further support for specific interaction between 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase was derived from affinity chromatography experiments using aminotransferase-specific antibodies bound to protein A-Sepharose.

It is known that the binding of IgG molecules to protein A occurs via the Fc portion of immunoglobin, thus leaving the Fab antigen site readily available for the interaction with antigen (58). This scheme is outlined in Figure 13. The obvious advantage of such a system lies in the fact that this indirect and gentle method of immobilization excludes any physical modification of the enzyme as a result of its immobilization. Protein A-Sepharose loaded with purified anti-4-aminobutyrate aminotransferase was washed with increasing concentrations of saline buffer (I = 0.2) and then equilibrated with 10 mM potassium phosphate (pH 7.4).

An enzyme mixture (1 mg each aminotransferase and dehydrogenase) was applied to a small column (2 x 1 cm) packed with anti-aminotransferase bound to protein A-Sepharose and eluted with a linear gradient of NaCl in 10 mM potassium phosphate buffer (pH 7.4). Fractions collected from the column were assayed for catalytic activity. As shown in Figure 14, succinic semialdehyde dehydrogenase is eluted at NaCl concentrations higher than 0.2 M, whereas the 4-aminobutyrate aminotransferase remains firmly bound to the column under similar experimental conditions.

The amount of protein retained by anti-aminotransferase protein A-Sepharose was quantitated by measuring the amount of total enzyme applied to the column and comparing this with the total enzymatic activity eluted in the wash of the column. As seen in Table V, the column retains approximately

Figure 13. Preparation of anti-aminotransferase-protein A-Sepharose.

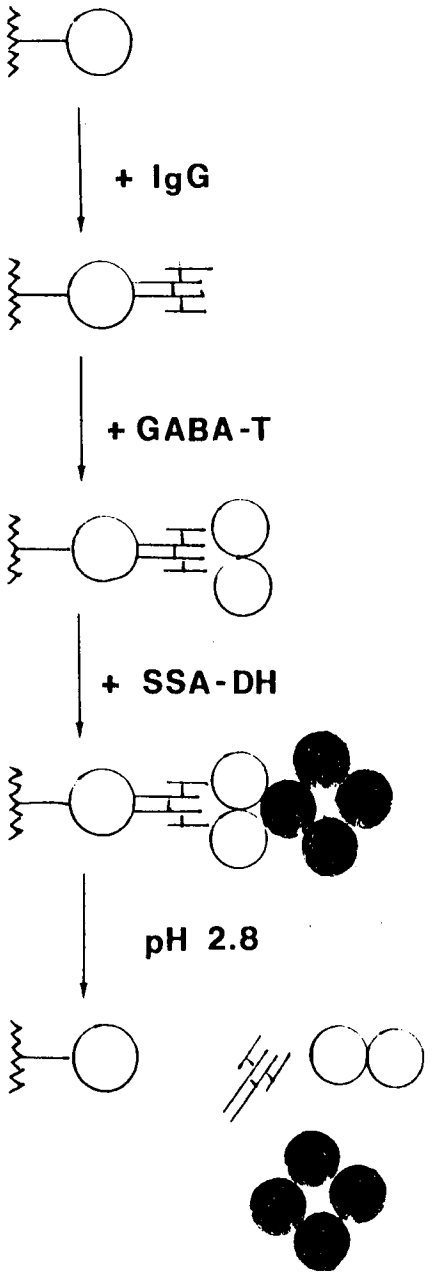


Figure 13

Figure 14. Elution of succinic semialdehyde dehydrogenase from anti-aminotransferase-protein A-Sepharose. A protein A-Sepharose column (2 ml) was loaded with purified antibody against 4-aminobutyrate aminotransferase and washed with phosphate buffer (pH 7.4) containing 0.2 M NaCl. Then the column was equilibrated iwth 10 mM potassium buffer (pH 7.4). A mixture of 4-aminobutyrate aminotransferase (1 mg) and succinic semialdehyde dehydrogenase (1 mg) in 0.5 ml of phophate buffer (pH 7.4) was applied to the column and washed with 10 ml of the same buffer. A linear NaCl gradient made up of equal volumes (15 ml) of 0 and 2 M NaCl in 10 mM phosphate buffer (pH 7.4) was applied to the column. Fractions collected from the column were assayed for succinic semialdehyde dehydrogenase activity.

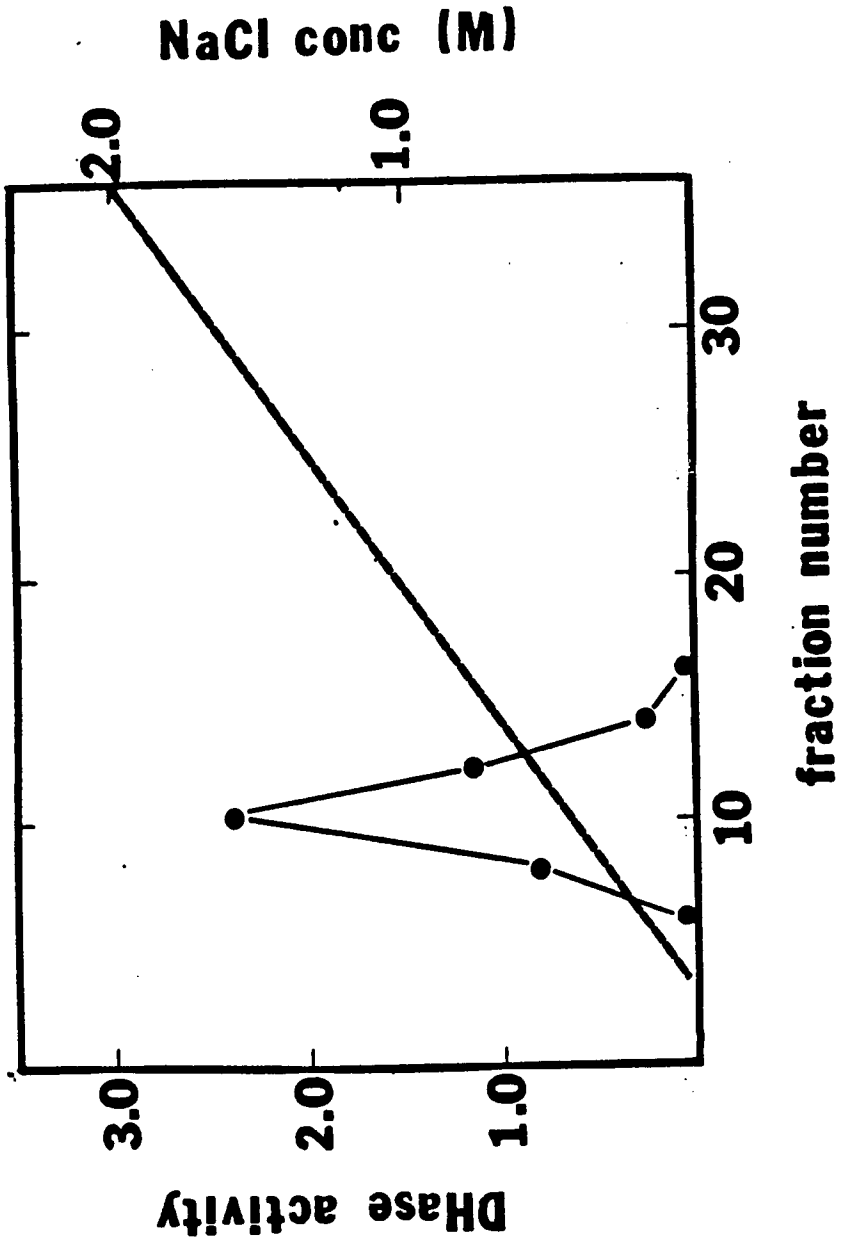

NaCl conc (M)
2.0
1.0
30
20
10
fraction number
3.0
2.0
1.0
DNase activity

Figure 14

TABLE V

RETENTION OF ENZYMES BY ANTI-AMINOTRANSFERASE PROTEIN A-SEPHAROSE

Enzyme	μg applied	μg retained	μ Moles retained
GABA-T	40	11	11×10^{-8}
SSA-DH	25	15	9×10^{-8}

One hundred μg of 4-aminobutyrate aminotransferase was applied to a column (1.0 ml bed volume) of anti-aminotransferase-protein A-Sepharose and washed with 10 ml of 2 mM potassium phosphate (pH 7.2). Succinic semialdehyde dehydrogenase was applied (100 μg) and the column washed with 10 ml of the same buffer. Each wash was assayed for total enzyme content and compared with the enzyme applied.

equal molar amounts of succinic semialdehyde dehydrogenase and 4-aminobutyrate aminotransferase, suggesting a 1:1 stoichiometric ratio for the enzyme complex.

In control experiments, protein A-Sepharose carrying anti-aminotransferase IgG (4-aminobutyrate aminotransferase omitted) failed to retain succinic semialdehyde dehydrogenase at phosphate concentrations of 0.01 M (pH 7.4).

Thus both experiments, i.e. affinity chromatography with 4-aminobutyrate aminotransferase immobilized directly and indirectly to a Sepharose matrix, reveal specific interactions of this enzyme with succinic semialdehyde dehydrogenase.

Investigation of the Enzyme Complex under in vivo-like Conditions

4-Aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase are both known to be located in the mitochondrion. If the interaction observed with the purified enzymes is occurring within the mitochondrion, one would predict that the aminotransferase-dehydrogenase complex could be isolated by the use of anti-aminotransferase-protein A-Sepharose from a preparation of mitochondrial proteins.

To that end, mitochondrial protein was obtained from pig brain and allowed to interact with the anti-aminotransferase-protein A-Sepharose. Following an extensive wash of the column with equilibration buffer (30 ml), the column was assayed directly using a substrate buffer of 20 mM 4-aminobutyrate and 10 mM 2-oxoglutarate containing 1 mM NAD^+. A large increase in the absorbance at 340 nm was observed when compared with a control protein A-Sepharose column containing only anti-aminotransferase IgG (Figure 15).

Figure 15. Detection of succinic semialdehyde dehydrogenase activity on anti-aminotransferase protein A-Sepharose. A sample (1 ml) of mitochondrial protein was applied to an anti-aminotransferase-protein A-Sepharose column (2 ml) equilibrated in 10 mM phosphate buffer (pH 7.4) and the column washed with 20 ml of the same buffer. The column was assayed directly by passing 20 mM 4-aminobutyrate, 10 mM 2-oxoglutarate containing 1 mM NAD^+ in a phosphate buffer (pH 8.4) over the column and collecting fractions. Measuring the change in absorbance at 340 nm in the elutant (-●-). The control column did not contain mitochondrial protein (-o-).

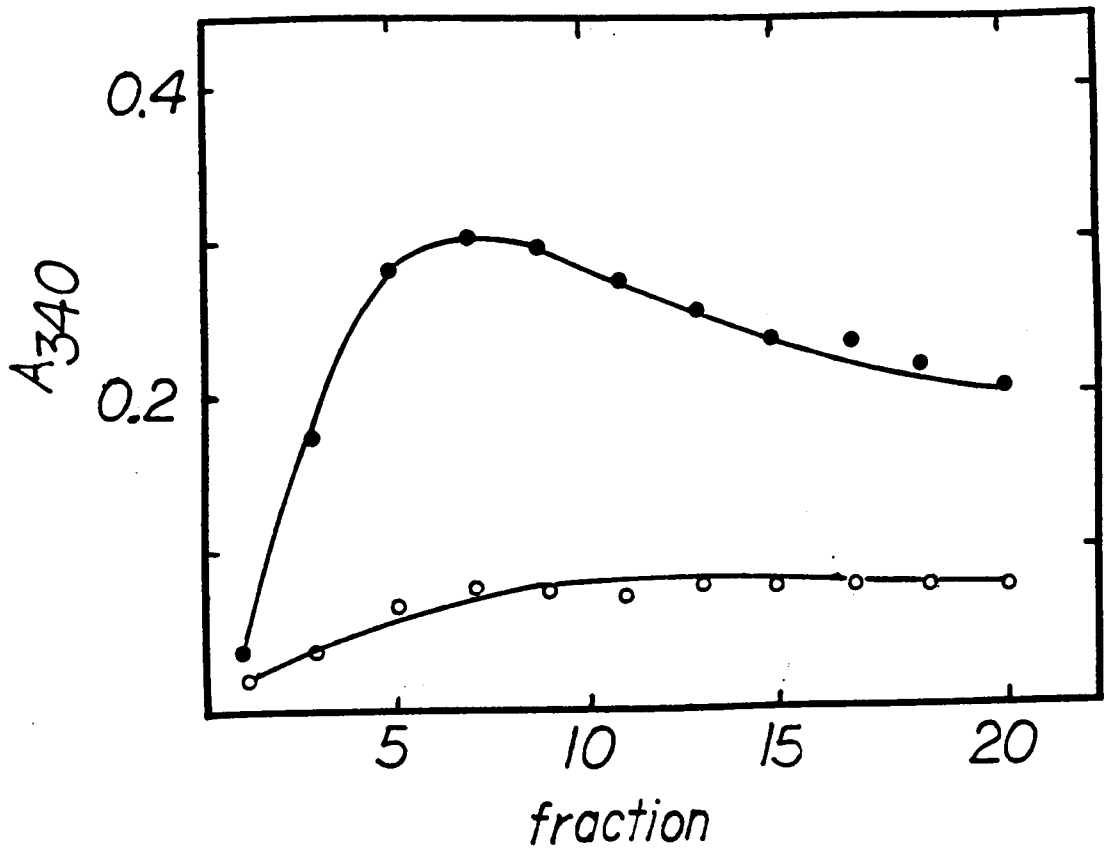


Figure 15

The total protein was removed from the column by treatment with 1.0 M NaCl and subjected to SDS-PAGE (Figure 16). In addition to the bands corresponding to IgG, several protein bands which also appear in the mitochondrial lane are observed in the salt wash from the anti-aminotransferase column. Protein eluted from a column which contained normal rabbit IgG contain only IgG related bands. Thus, there are several unidentified proteins which are retained by the aminotransferase-specific affinity column suggesting that the protein cluster in the mitochondria include proteins other than succinic semialdehyde dehydrogenase.

Discussion

In the present studies we have demonstrated the existence of physical interactions between mitochondrial enzymes involved in the catabolism of the neurotransmitter 4-aminobutyrate.

Using directly immobilized 4-aminobutyrate aminotransferase in chromatographic studies, it was possible to show that the aminotransferase recognizes succinic semialdehyde dehydrogenase at neutral pH.

Interactions between the two enzymes were also detected by means of antibodies bound to protein A coupled to a Sepharose matrix. Instead of covalently linking the aminotransferase to pyridoxal-5-phosphate Sepharose, the mitochondrial enzymes were immobilized indirectly via anti-aminotransferase. This indirect method of immobilization excludes any chemical modification of the aminotransferase brought about by its direct attachment to the Sepharose matrix.

When 4-aminobutyrate aminotransferase was immobilized via antibodies, it was found that about 1 mol of succinic semialdehyde dehydrogenase was

Figure 16. SDS-polyacrylamide gel electrophoresis of the mitochondrial proteins retained by anti-aminotransferase-protein A-Sepharose. A mitochondrial protein preparation was applied to an anti-aminotransferase column as described in the legend of Figure 15. The protein was eluted from the column with 0.1 M NaCl after an extensive wash with 10 mM potassium phosphate (pH 7.4). Lane a contains bovine serum albumin (67,000), ovalbumin (42,000) and trypsin inhibitor (21,000) as standards. A control column containing normal rabbit antiserum was also treated with the mitochondrial protein as a control (lane c). Lane d contains a sample of the total mitochondrial protein.

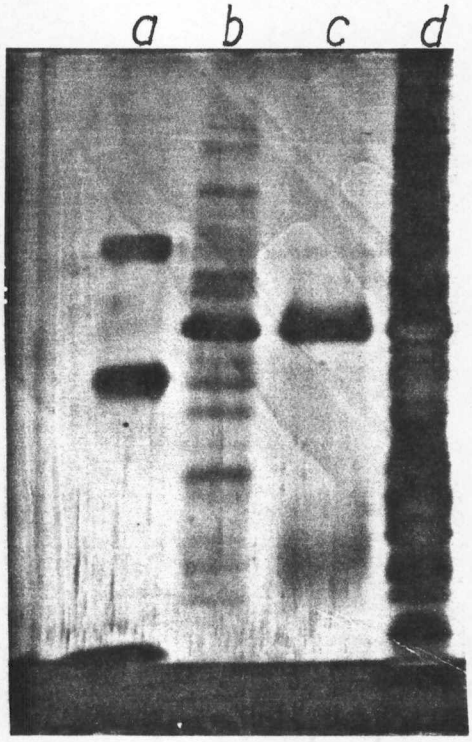


Figure 16

bound per mol of immobilized aminotransferase at NaCl concentrations lower than 0.2 M. At increasing ioninc strength, the interaction between 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase is considerably reduced, indicating the involvement of electrostatic forces in the recognition process.

The interaction was also detected in a preparation of mitochondrial proteins. Rather than using enzymes purified to homogeneity, the protein A-Sepharose bound anti-aminotransferse antibody was able to recognize and retain 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase from a mitochondrial extract. In addition, the anti-aminotransferase column was able to isolate several other proteins as detected by SDS-polyacrylamide gel electrophoresis, suggesting perhaps other enzymes may participate in the cluster. Although no other enzyme activities were successfully identified from the preparation, it is conceivable that the unidentified proteins could be part of a closely related metabolic pathway (e.g. TCA cycle enzyme) or be an important structural protein.

CHAPTER III

CHARACTERIZATION OF THE 4-AMINOBUTYRATE AMINOTRANSFERASE-SUCCINIC SEMIALDEHYDE DEHYDROGENASE ENZYME COMPLEX

Introduction

Detection of a protein-protein interaction by affinity chromatography yields only qualitative data. The results described in the preceding chapter gave substantial support to the premise that a specific protein interaction is occurring between the two mitochondrial enzymes, 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase, without providing details of the nature of the interaction.

The work described here quantitates the enzyme complex formed by the aminotransferase and the dehydrogenase with regard to the molecular weight and to the dissociation constant using gel filtration chromatography and polarization of fluorescence studies.

Experimental Procedures

Preparation of Iodoacetamide Fluorescein Labeled 4-Aminobutyrate Aminotransferase

Iodoacetamide fluorescein labeled 4-aminobutyrate aminotransferase (IAF-aminotransferase) was prepared according to a previously published procedure (59). 4-Aminobutyrate aminotransferase (2 mg/ml) was incubated with 6-iodoacetamide fluorescein (Molecular Probes, Inc.) in 0.1 M potassium phosphate containing 1 mM 2-oxoglutarate. The reaction was allowed to proceed for 3 h at 4°C. Free dye was removed by exhaustive dialysis against 10 mM potassium

phosphate (pH 7.2). Immediately prior to use, IAF-aminotransferase was subjected to Sephadex G-25 chromatography to remove any residual free dye. The degree of labeling was determined spectrophotometrically using an extinction coefficient of 3.4×10^4 $M^{-1}cm^{-1}$ at 490 nm. The incorporation of 0.92 mol of dye/mol of enzyme does not affect the catalytic activity.

Spectroscopy

Polarization of fluorescence measurements were performed in a SLM 4800 spectrofluorimeter. The excitation was set at 440 nm and the fluorescence polarized light emitted by the sample was passed through a Corning Glass filter C.S. 3-69.

UV and visual measurements were performed in a Cary Model 15 spectrophotometer. Fluorescence measurements were recorded on a spectrofluorimeter equipped with two Bausch and Lomb monochromators.

Results

Gel Filtration

The molecular weight of the aminotransferase-dehydrogenase complex was determined by gel filtration using a Sephacryl S-300 column (1 x 15 cm). The column was equilibrated in 10 mM potassium phosphate at pH 7.2 and was calibrated using horse ferritin, human IgG and bovine serum albumin as molecular weight markers and blue Dextran as a marker of the excluded volume. A 1 ml sample containing 1 mg of 4-aminobutyrate aminotransferase, 1 mg of succinic semialdehyde dehydrogenase and blue Dextran was applied to the column and eluted with the same buffer. Fractions were assayed for the presence of aminotransferase and dehydrogenase activities. The enzymes co-eluted at an estimated molecular weight of 300,000 (Figure 17). The second

Figure 17. Gel filtration on Sephacryl S-300. A sample containing 1 mg of 4-aminobutyrate aminotransferase and 1 mg of succinic semialdehyde dehydrogenase was applied to a Sephacryl S-300 column (15 x 1 cm) equilibrated with 10 mM potassium phosphate (pH 7.2). The column was eluted with the same buffer and fractions (0.4 ml) were assayed for succinic semialdehyde dehydrogenase (-•-) and 4-aminobutyrate aminotransferase (-o-) activities. The column was calibrated with the following standards: ferritin (440,000), human IgG (160,000), bovine serum albumin (68,000) and 4-aminobutyrate aminotransferase (GABA-T; 100,000). In the inset the elution volumes are plotted against the log of the molecular weight of the standard proteins.

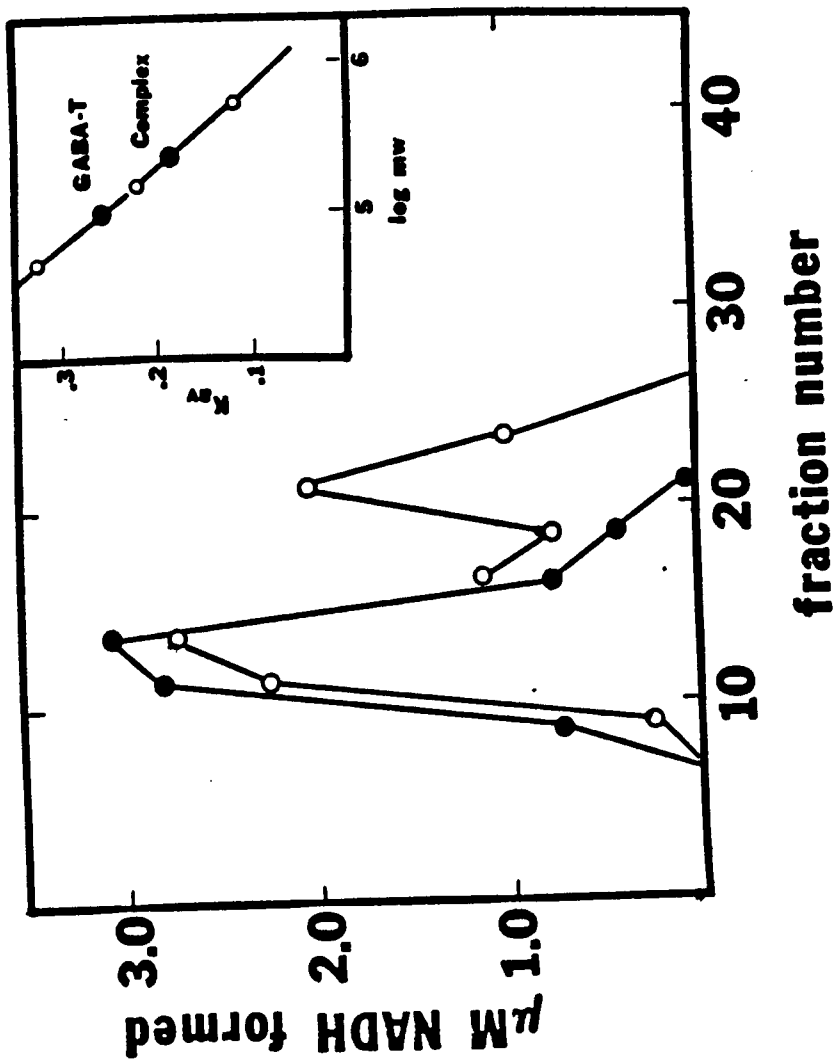

μM NADH formed
3.0
2.0
1.0
10
20
30
40
fraction number
GABA-T
Complex
5
6
log mw
.1
.2
.3

Figure 17

aminotransferase peak, at a molecular weight 100,000, reflects the molar excess of 4-aminobutyrate aminotransferase in the applied sample (1.6 mol aminotransferase: 1.0 mol dehydrogenase). Identical results were obtained when fluorescein-labeled aminotransferase was substituted for the native enzyme in the sample and the elution of 4-aminobutyrate aminotransferase was monitored by fluorescence at 510 nm (excitation at 495 nm).

Polarization of Fluorescence

If the interpretation of the affinity chromatography data from the previous chapter and of the gel filtration results above are correct, then one should be able to detect the association of the interacting enzymes by resorting to physical methods which are sensitive to changes in the hydrodynamic properties of the macromolecules in solution.

Polarization of fluorescence is suitable to study systems where one of the components is tagged with a fluorescent probe. Specific labeling of one component of the system makes it possible to operate at concentrations well below those at which association takes place (60). Iodoacetamide fluorescein-labelled aminotransferase was incubated with partially purified succinic semialdehyde dehydrogenase and the polariztion of fluorescence observed. As shown in Table VI, succinic semialdehyde dehydrogenase caused a large change in the polarization values while glutamate dehydrogenase caused a less significant alteration in the polarization. No effect was observed upon the addition of bovine serum albumin. The observed change in polarization is somewhat larger than what was found in the titration experiment (discussed below). This was probably due to the lack of purity in the dehydrogenase preparation and the increased viscosity of the sample buffer.

TABLE VI

CHANGES IN THE POLARIZATION OF FLUORESCENCE OF IAF-AMINOTRANSFERASE

Addition to 0.1 μm IAF-Aminotransferase	Polarization
None	.128
Succinic Semialdehyde Dehydrogenase (1 μ M)	.219
Glutamate Dehydrogenase (1 μM)	.160
Bovine serum albumin (1 μM)	.130

Samples were 2 ml in volume and contained 20% glycerol. Polarization measurements were carried out at 4°C as described under "Experimental Procedures."

The nature of the protein-protein interaction was further investigated by allowing a sample of IAF-aminotransferase at a concentration of 0.1 μM to interact with increasing concentrations of succinic semialdehyde dehydrogenase at pH 7 in 10 mM phosphate buffer. As seen in Figure 18, the addition of dehydrogenase causes an increase in the polarization value.

The absolute values of the polarization of fluorescence (P) are related to the emission anisotropy (A) by equation (1)

$$A = 2P/3 - P \qquad (1)$$

The fraction of ligand bound (α)

$$\alpha = \frac{PL}{L_o} \qquad (2)$$

is the ratio of bound fluorescein-aminotransferase (PL) to the total concentration of free fluorescein-aminotransferase (L_o). It is related to the emission anisotropy by means of equation (3).

$$\alpha = (A - A_f) / (A_b - A_f) \qquad (3)$$

A is the emission anisotropy value observed when free and bound fluorescein-aminotransferase are in equilibrium. From a plot of α versus concentration of succinic semialdehyde dehydrogenase (Figure 19), one can infer an apparent dissociation constant of around 0.1 μM for a one to one complex formed as a result of binding of labeled aminotransferase to succinic semialdehyde dehydrogenase.

Discussion

Additional support for specific interactions between the two mitochondrial enzymes, succinic semialdehyde dehydrogenase and 4-aminobutyrate aminotransferase, was derived from polarization of fluorescence measurements conducted

Figure 18. Titration of IAF-aminotransferase with succinic semialdehyde dehydrogenase. The polarization of a sample containing 0.1 M of IAF-aminotransferase was measured as described in "Experimental Procedures." Purified succinic semialdehyde dehydrogenase (10 μM) was added in 10 μl aliquots and the change in the fluorescence polarization measured.

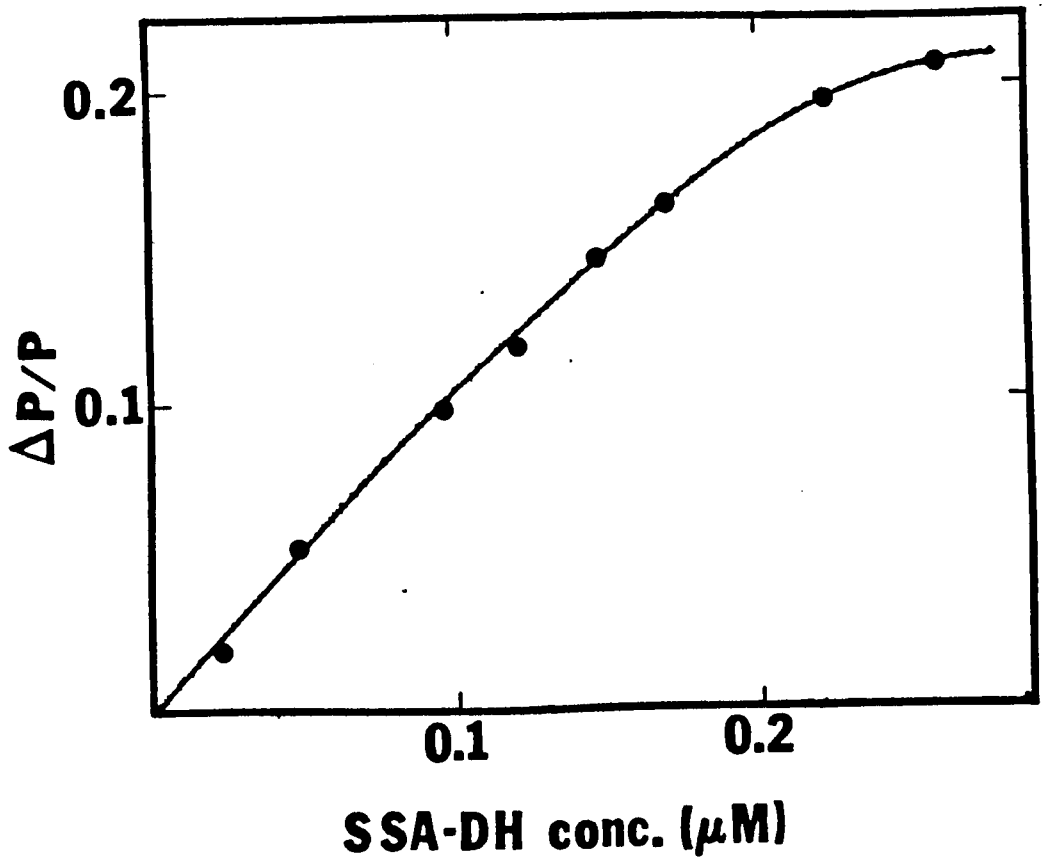


Figure 18

Figure 19. Plot of α versus the concentration of free succinic semialdehyde dehydrogenase. α , the fraction of bound fluorescein-labeled aminotransferase, was determined using equation 3 after conversion of the measured polarization values to anisotropy values according to equation (1).

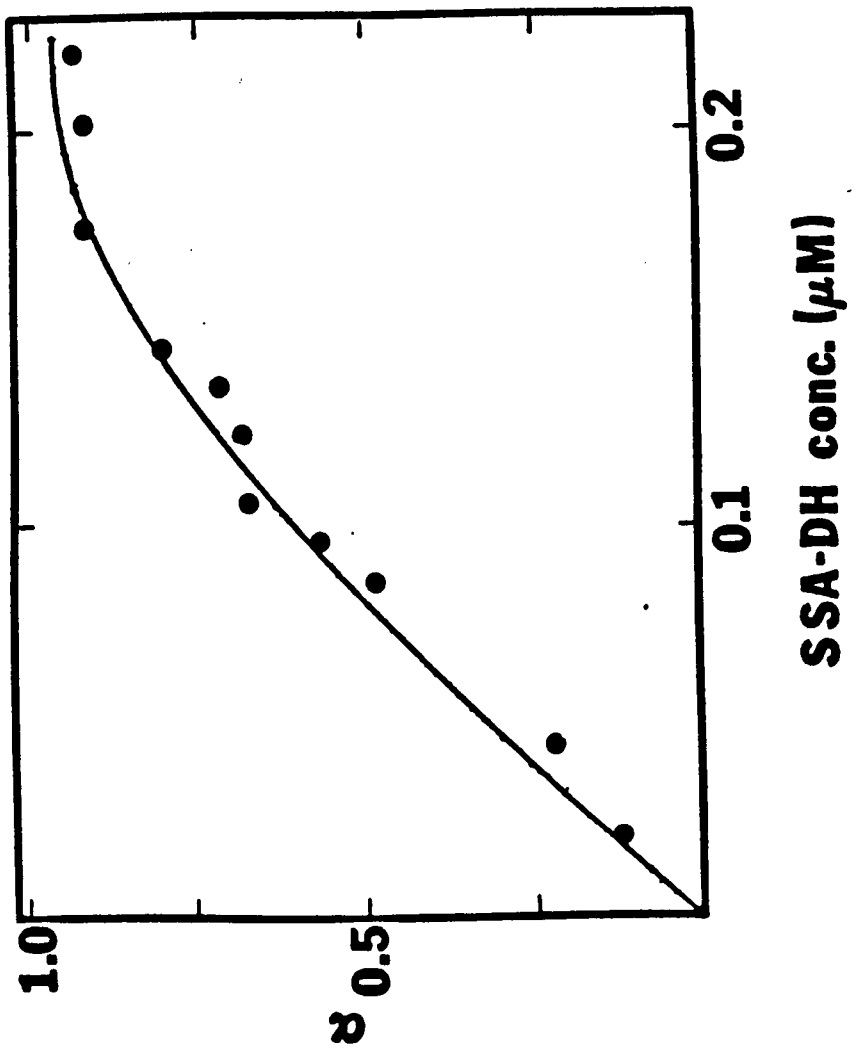


Figure 19

on fluorescein-aminotransferase mixed with increasing concentrations of succinic semialdehyde dehydrogeanse. The results of these experiments clearly established the formation of stable enzyme complexes characterized by a dissociation constant of approximately 0.1 μ M. Since the estimated concentration of 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase in mitochondria is approximately 2 μM each (50), the results obtained in vitro strongly suggest that the aminotransferase is saturated with succinic semialdehyde dehydrogenase under in vivo condition.

The protein concentration in the mitochondrial matrix has been estimated to be about 500 mg/ml (61) and it is evident that high local concentrations of proteins exert several effects on the properties of the medium in which the enzyme act. Thus, the medium becomes more viscous and the rate of macromolecular diffusion may be reduced by an order of magnitude.

An average protein concentration of 500 mg/ml means that individual enzyme molecules will be in close proximity to one another and this will tend to promote interaction between enzymes within the mitochondrial matrix which could not occur in dilute solutions. Indeed, several publications have appeared lately indicating the existence of physical interactions between enzymes of the aspartate-malate shuttle and the citric acid cycle (62-65). The organization of the citric acid cycle enzymes as a complex would facilitate the creation of a special microenvironment around the cycle: the cell would acquire the property of maintaining high flux of substrates through the cycle with a moderate number of intermediate molecules.

It is well established that the brain differs from other organs in having an alternative route of oxidation through α-ketoglutarate; i.e. 4-aminobutyrate bypath. The reactions catalyzed by enzymes of the 4-aminobutyrate, i.e.

glutamate decarboxylase, 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase, lead to the formation of succinate, CO_2 and NADH.

$$\alpha\text{-ketoglutarate} + NAD^+ \longrightarrow \text{succinate} + CO_2 + NADH + H^+$$

Thus, the combined action of three enzymes allows succinate to reenter the tricarboxylic acid cycle. The quantitative contribution of the 4-aminobutyrate bypath to glucose oxidation in the brain has been estimated to be 8-10% of the total flux through the tricarboxylic acid cycle (8).

In the case of 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase, two components of the 4-aminobutyrate shunt, it can be envisaged that close association between both enzymes will facilitate direct transfer of intermediates among catalytic centers, thereby preventing release of free succinic semialdehyde which is completely converted to succinic acid.

Although the present studies have failed to detect any specific interaction between 4-aminobutyrate aminotransferase and glutamate dehydrogenase, it is conceivable that succinic semialdehyde dehydrogenase interacts with the enzymes of the citric acid cycle, such as flavoprotein succinate dehydrogenase, which catalyzes the dehydrogenation of succinate to fumarate.

APPENDIX

APPENDIX

ATTEMPT TO SEPARATE THE SUBUNITS OF 4-AMINOBUTYRATE AMINOTRANSFERASE

Multimeric proteins have been separated into subunits by affinity chromatography (41). Since 4-aminobutyrate aminotransferase shows different affinities for the cofactor binding, an attempt was made to separate the subunits of the aminotransferase in order to determine whether the non-cooperative effect was due to an interaction between identical subunits or due to a different amino acid sequence in each subunit.

The scheme for subunit separation involved binding [^{3}H]-aminotransferase to PLP-Sepharose and then covalently attaching the protein by treatment with sodium borohydride (see Figure 2, page 17) column by reduction with sodium borohydride (Figure 2, B). Following a thorough wash with 10 mM potassium phosphate (pH 7.2), the column was treated with 1% SDS sodium dodecyl sulfate (SDS) in the same buffer. The SDS-Sepharose slurry was collected on a sintered glass funnel and the filtrate recovered. The SDS was removed from the filtrate by passing the solution over a column of Extracti-gel and the elutant from this column was assayed for protein and tritium content.

The SDS-treated aminotransferase-Sepharose was washed with several column volumes of the equilibration buffer to remove the SDS. The column was then allowed to react with 0.1 M sodium dithionite in 0.2 M sodium borate (pH 8.9) for 30 min at 25°C. This treatment disrupts the diazo-linkage between the benzene and the pyridoxal ring (see Figure 2, page 17) releasing all bound PLP and therefore, all bound protein. The Sepharose was collected on a sintered glass funnel and the filtrate dialyzed against 10 mM potassium

phosphate (pH 7.2) to remove the cleavage reagents. The sample was quantitated for protein concentration and for radioactivity.

The data obtained from the attempted subunit separation is shown in Table VII. Although radioactivity was separated into $^{3}H(+)$ and $^{3}H(-)$ pools, recovery of both protein and radioactivity was very poor. It is tempting to interpret the data as suggesting that the subunits were indeed separated into individual protein fractions. However, until the poor recovery of radiolabel and of protein can be fully explained, this will remain speculation.

TABLE VII

RECOVERY OF RADIOACTIVITY FROM [^{3}H] -AMINOTRANSFERASE SEPHAROSE

Fraction	A_{280}	cpm/ml	Specific Activity[a]
[^{3}H] -aminotransferase	0.41	21.9	43.9
SDS wash	0.13	7.2	55.0
NaS_2O_4 treatment	0.05	0.01	0.2

[a]Specific activity is in cpm/mg.

PART II

SUCCINIC SEMIALDEHYDE REDUCTASE

CHAPTER I

THE PURIFICATION, MOLECULAR WEIGHT AND ISOELECTRIC POINT OF SUCCINIC SEMIALDEHYDE REDUCTASE

Introduction

4-Hydroxybutyrate is a normal brain constituent (66) which now appears to play a neurotransmitter or neuromodulator role in the brain (29). It is formed through the catabolism of 4-aminobutyrate. 4-Aminobutyrate aminotransferase produces the intermediary metabolite, succinic semialdehyde, which can then either be oxidized to succinate by succinic semialdehyde dehydrogenase or reduced to 4-hydroxybutyrate by succinic semialdehyde reductase.

Succinic semialdehyde specific and non-specific aldehyde reductases have been isolated from human and rat brain (31, 35). While both enzyme forms are fully capable of 4-hydroxybutyrate production, it appears that the specific reductase is responsible for succinic semialdehyde reduction in vivo (37).

The succinic semialdehyde reductase isolated from hamster brain was shown to be reversible, i.e. produce succinic semialdehyde from 4-hydroxybutyrate (34). The possibility that the enzyme functions in the reverse direction as the main degradative enzyme for 4-hydroxybutyrate seems unlikely based upon the relatively minor dehydrogenase activity shown by this enzyme. However, in vivo it appears that the catabolism of 4-hydroxybutyrate proceeds through succinic semialdehyde and that 4-hydroxybutyrate ultimately enters the tricarboxylic acid cycle as succinate (67).

The subcellular location of the enzymes involved in the metabolism of 4-hydroxybutyrate have been reported to be in the cytosol (34,68). However,

a cytoplasmic location for succinic semialdehyde reductase would leave the reductase without access to succinic semialdehyde since the aldehyde is produced in the mitochondria by 4-aminobutyrate aminotransferase. A value of 0.1 μM has been reported for free succinic semialdehyde in rat brain (39). If this value is correct, the enzyme will be faced with operating at about 1% of its V_m.

Since the other enzymes related to succinic semialdehyde metabolism are found in the mitochondria (e.g. 4-aminobutyrate aminotransferase, succinic semialdehyde dehydrogenase), it seemed reasonable to propose that a mitochondrial enzyme could exist which would be involved in 4-hydroxybutyrate metabolism. In this report, we describe and characterize a succinic semialdehyde reductase isolated from the mitochondria of pig brain.

Experimental Procedures

Preparation of Enzyme

Pig brains were obtained from Lays Meat Company and were immediately placed on ice. Prior to use, the brains were rinsed with cold homogenization buffer and the external membranous tissue removed by hand. The brains were homogenized in a Waring Blender for 3 min at high speed in a buffer (25% w/v) of 2 mM potassium phosphate (pH 7.2) containing 1 mM 2-mercaptoethanol and 0.5 mM EDTA (buffer I). The homogenate was centrifuged at 10,000 g for 30 min. The pellet was discarded and the supernatant treated with $(NH_4)_2SO_4$. The precipitate obtained at 30 to 65% saturation was dissolved in buffer I and dialyzed overnight against the same buffer with several changes. The dialysate was applied to a column (80 x 5 cm) of CM-Sephadex equilibrated in buffer I. The column was eluted with buffer I; succinic semialdehyde reductase was

eluted from the column in the equilibration buffer wash. The active fractions were pooled and then applied to a column (10 x 2.6 cm) of Blue-Sepharose equilibrated in the same buffer. The column was washed with buffer I containing 0.1 M KCl (100 ml) and the enzyme was eluted by using a linear gradient made with the wash buffer (100 ml) and buffer I containing 0.8 M KCl (100 ml). The enzyme eluted at a concentration of 0.4 M.

Following chromatography on Blue-Sepharose, the enzyme was dialyzed against buffer I and then applied to a column (10 x 1 cm) of ADP-Sepharose equilibrated in the same buffer. The column was washed with 25 ml of the equilibration buffer and the enzyme eluted with buffer I containing 0.1 M KCl (25 ml). The active fractions were pooled and dialyzed versus buffer I and then applied to a column (10 x 2.6 cm) of Blue-Sepharose. The column was washed with 50 ml of buffer I containing 5 x 10^{-6}M NADPH and 0.1 M KCl followed by 50 ml of 0.2 M KCl in buffer I. The enzyme was eluted with a linear gradient of 0.2 M to 0.8 M KCl in buffer I (100 ml).

The enzyme was dialyzed against buffer I and applied to a DE-52 column (20 x 2.6 cm) equilibrated in the same buffer. The column was washed with 50 ml of buffer I and eluted with a linear gradient of 0 to 0.3 M KCl in buffer I (100 ml). The final preparation was concentrated to a final volume of 2 ml by centrifugation with Centricon 10 filters.

All enzyme purification steps were performed at 4°C. The purified enzyme was stored in 50 mM potassium phosphate (pH 7.2) containing 1 mM 2-mercaptoethanol and 10% glycerol at -20°C. Under these conditions the enzyme was stable for four weeks without appreciable loss of enzymatic activity. The final preparation had a specific activity of 320 units per mg.

A unit of activity is defined as the amount of enzyme required to oxidize 1 nmole of NADPH per min at 25°C.

Subcellular Fractionation

Mitochondria were isolated from brain tissue using the procedure described in Part I (page 40). Isolated mitochondria were ruptured osmotically in 10 mM potassium phosphate (pH 7.2) containing 1 mM 2-mercaptoethanol with several passes of a Douce homogenizer. The mitochondrial homogenate was centrifuged at 100,000 g for 30 min to remove membrane and the final protein preparation stored at -20°C prior to use.

Microsomes, synaptosomes and cytosol were isolated as previously described (69). The supernatant from the first 7000 g centrifugation was carefully removed and transferred to an ultracentrifuge tube. The solution was centrifuged for 30 min at 100,000 g. The microsomal pellet was resuspended in a small volume of 10 mM potassium phosphate (pH 7.2) and assayed immediately for enzyme activity. The supernatant, containing the soluble proteins, was carefully decanted and assayed immediately for enzyme activity.

Synaptosomes were isolated from the mitochondrial pellet (first spin conducted at 9000 g) after sucrose density centrifugation. Synaptosomes migrated as a fluffy white band near the top of the gradient and were assayed for enzyme activity immediately after isolation.

Enzyme Assays

Succinic semialdehyde reductase was assayed by measuring the change in absorbance at 340 nm due to the oxidation of NADPH. The assay buffer contained 120 μM succinic semialdehyde and 50 μM NADPH in 0.1 M

potassium phosphate (pH 7.2). The reaction was initiated by the rapid addition of 0.1 ml of enzyme to 0.9 ml of assay buffer at 25°C.

Succinic semialdehyde dehydrogenase (22), glutamate dehydrogenase (71), and lactate dehydrogenase (72) were assayed by published methods. All assays were carried out in a Cary Model 15 spectrophotometer.

Polyacrylamide Gel Electrophoresis

Polyacrylamide gel electrophoresis was performed according to the procedure of Davis (55). Discontinuous sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis was carried out as described by Laemmli (56). The gel (10%) contained 0.1% SDS.

Polyacrylamide gradient gel electrophoresis (4%-30%) was performed as described by Manwell (70). A PAA 4/30 gradient gel was equilibrated in 0.10 M Tris, 0.4 M glycine (pH 8.3) by electrophoresis at 125 V for 15 min. The gel was then subjected to pre-electrophoresis at 70 V for 20 min. Protein samples were applied and the gel run at 300 V for 4 hours at 10°C.

Protein bands were stained with Coomassium blue R-250 dye for 1 h and subsequently destained in a solution containing 5% methanol and 7.5% acetic acid in water.

Enzyme activity was detected after polyacrylamide gel electrophoresis using a modification of the procedure of Hallock and Yamada (73). The gel to be stained was rinsed in a small volume of 0.5 M potassium phosphate (pH 7.0) and then immersed in 20 ml of 0.05 M potassium phosphate containing 5 mg nitroblue tetrazolium, 0.5 mg phenazine methosulfate, 0.1 M 4-hydroxybutyrate and 0.1 mM $NADP^+$ for 2 h at 37°C. Enzymatic activity results in the formation of an insoluble dye.

Isoelectric Focusing

Flat plate polyacrylamide isoelectric focusing was performed with LKB pagplates containing 2.4% ampholytes, pH 3.5-9.5 on a Pharmacia flat bed electrophoresis system at 4°C. The anode buffer was 1 M NaOH and the cathode buffer was 1 M H_3PO_4. Samples of 40 µl were applied to the gel by absorbing the protein on two 5 x 10 mm pieces of filter paper and placing them at different locations in the same gel lane. The gel was run at a constant voltage of 1250 V for 1.5 h. At the end of the run, a 2 cm strip was cut horizontally from the end of the plate to be used in determination of the pH gradient. The remainder of the gel was stained for activity as described above. The pH gradient was determined by slicing the 2 cm gel strip into 5 mm segments and placing each piece into a tube containing 1 ml of distilled water. After 1 hour, the pH of each tube was measured with a pH meter.

Materials

Blue-Sepharose, AMP-Sepharose and PAA 4/30 gradient gels were purchased from Pharmacia. Centricon 10 filters were obtained from Amicon Corp. NADPH, $NADP^+$, 4-hydroxybutyrate, phenazine methosulfate and nitro blue tetrazolium were purchased from Sigma Chemical Co.

Results

Purification of Succinic Semialdehyde Reductase

The purification of succinic semialdehyde reductase from pig brain was accomplished by using ion-exchange and affinity chromatography. The enzyme is firmly retained by Blue-Sepharose and can be eluted from the column by a linear salt gradient (Figure 20). The active fractions from the Blue-Sepharose

Figure 20. Elution of succinic semialdehyde reductase from Blue-Sepharose I. The active fractions from chromatography on CM-Sephadex were applied to a column (10 x 2.6 cm) of Blue-Sepharose equilibrated in 10 mM potassium phosphate (pH 7.2) containing 1 mM 2-mercaptoethanol. Succinic semialdehyde reductase activity was detected by enzymatic assay (-▲-). (-o-) denotes the KCl gradient.

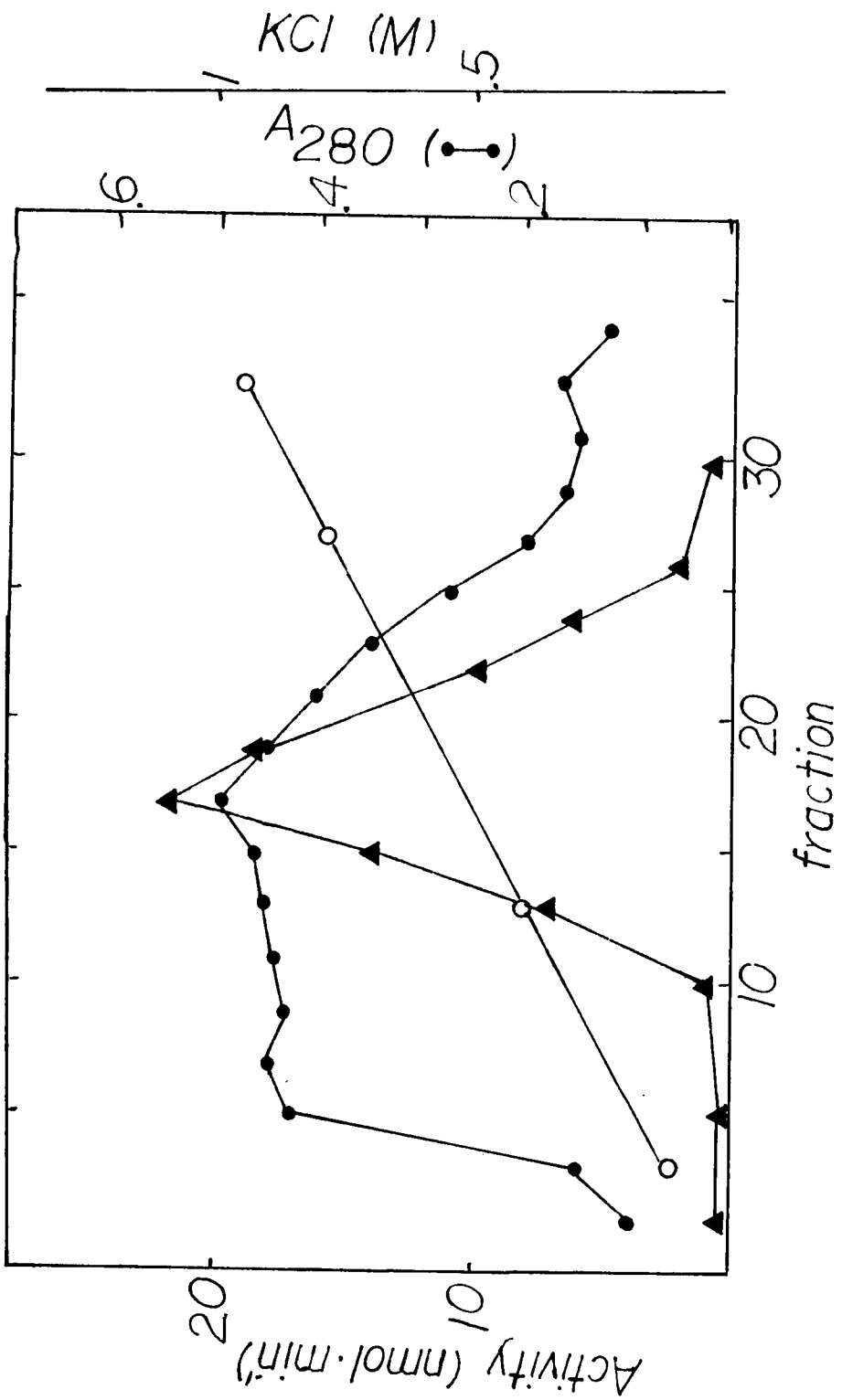


Figure 20

chromatography were pooled and subjected to ADP-Sepharose chromatography, which binds NADP(H)-dependent enzymes. The enzyme binds less tightly to this affinity gel. It was removed from the column at a very low concentration of KCl (Figure 21). The final purification step was chromatography on a column of DE-52. The retained succinic semialdehyde reductase was removed from the column by a linear KCl gradient (Figure 22). It should be noted that the enzyme failed to bind to DEAE-Sephadex, DEAE-Sephacel, CM-Sephadex and hydroxyapatite under a variety of conditions. The purification steps are summarized in Table VIII.

Subcellular Location of Succinic Semialdehyde Reductase

The mitochondrial enzymes 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase have been implicated in the metabolism of 4-hydroxybutyrate. It seemed reasonable to suggest that since the enzymes involved in the metabolism of the necessary intermediate (i.e. succinic semialdehyde) were found in the mitochondria, that the enzyme succinic semialdehyde reductase would also be found in the mitochondria.

To this end, a single pig brain was homogenized and the various subcellular fractions obtained by differential centrifugation and by sucrose density centrifugation. The data shown in Table IX reveals that the NADPH-dependent succinic semialdehyde reductase is found primarily in the mitochondria of the pig brain. Lactate dehydrogenase, a cytosolic enzyme and glutamate dehydrogenase, a mitochondrial enzyme were assayed to assess the quality of the fractionation.

In order to confirm that the purified form of the enzyme was mitochondrial, the enzyme purification was conducted using sucrose gradient purified mitochondria as the starting material; all subsequent steps were

Figure 21. Elution from ADP-Sepharose. The active fractions from Blue-Sepharose I were applied to a column (10 x 1 cm) of ADP-Sepharose. The enzyme was removed from the column by treatment with 0.1 M KCl in phosphate buffer.

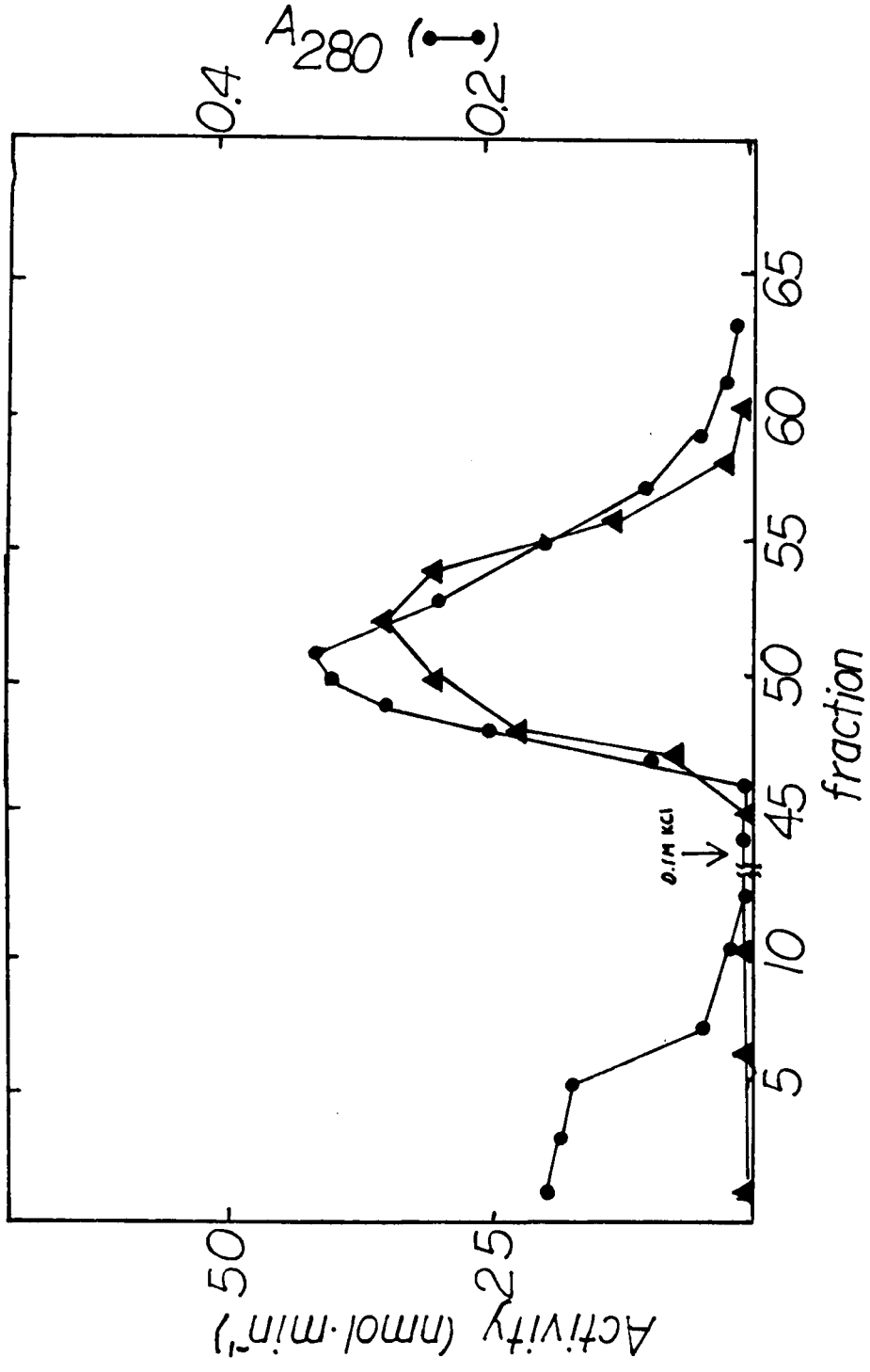

A280 (•—•)
0.4
0.2
Activity (nmol·min⁻¹)
50
25
0.1M KCl
5
10
45
50
55
60
65
fraction

Figure 21

Figure 22. Elution from DE-52. The active fractions from the final Blue-Sepharose column were applied to a column (20 x 2.6 cm) of DE-52. Protein was eluted from the column by a linear KCl gradient (-o-). Succinic semialdehyde reductase was detected by enzymatic assay (-▲-).

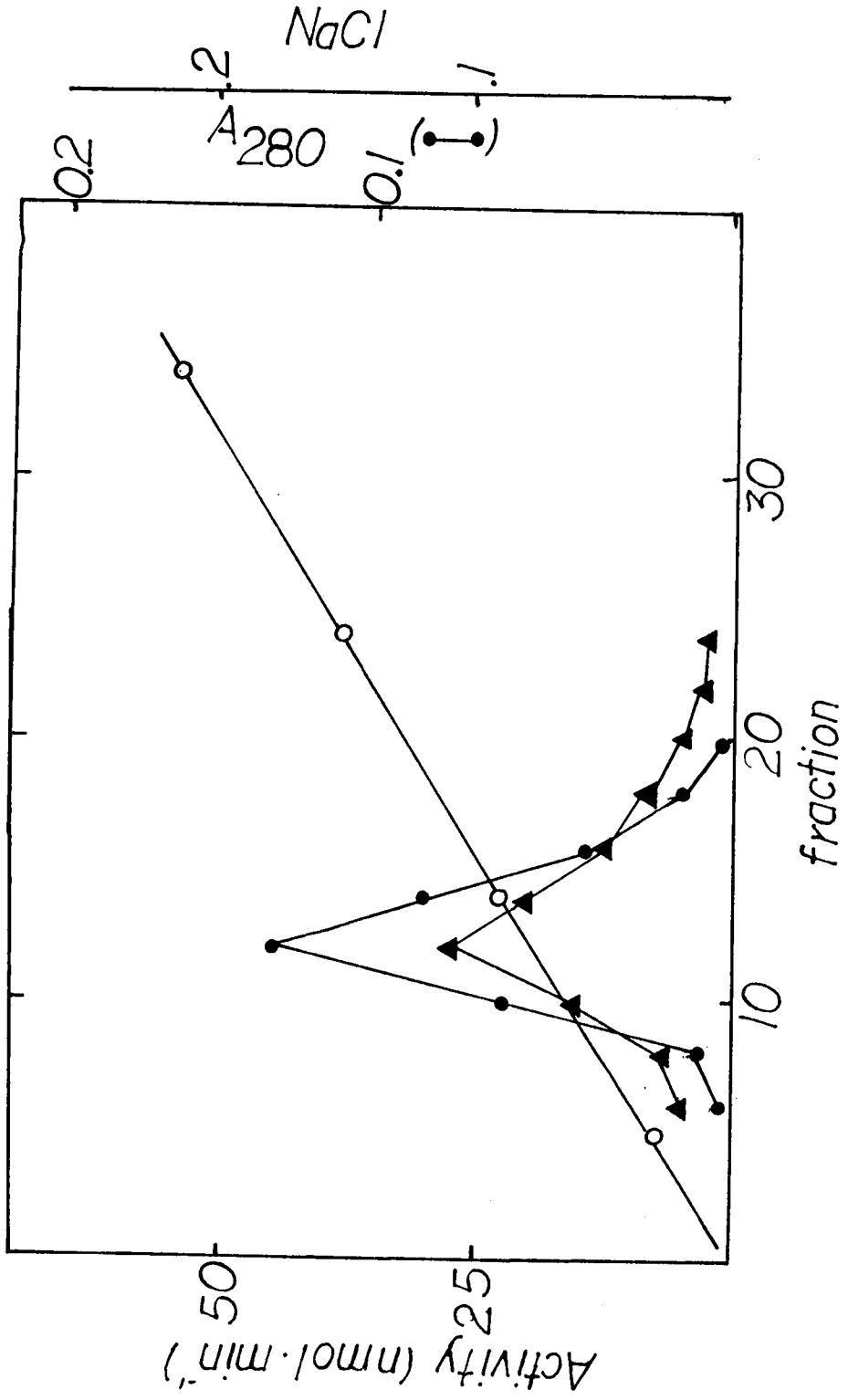


Figure 22

TABLE VIII

PURIFICATION OF SUCCINIC SEMIALDEHYDE REDUCTASE FROM PIG BRAIN

Treatment	Volume ml	Protein mg/ml	Specific Activity[a] units/mg	Yield %
Supernatant[b]	3000	18	.415	100
Dialysed, 30-65% $(NH_4)_2SO_4$ fraction	400	19	1.40	47
CM-Sephadex	200	1.2	6.31	7
Blue-Sepharose I	35	0.15	113.0	3
ADP-Sepharose	8	0.35	161.0	2
Blue-Sepharose II	11	0.14	230.0	1.7
DE-52	1	0.72	320.0	1.1

[a]One unit of enzyme activity is defined as that amount of enzyme that oxidizes 1 nmole/min of NADPH at 25°C.

[b]The specific activity of the whole brain homogenate was 0.073; the specific activity of the homogenate from mitochondria was 4.6.

TABLE IX

SUBCELLULAR DISTRIBUTION OF SUCCINIC SEMIALDEHYDE REDUCTASE

Fraction	SSA-DH	GDH	LDH	SSA-R
Mitochondria	267	85	10	4.8
Microsome	12	n.d.	50	0.9
Synaptosome	36	n.d.	11	0.3
Nuclear	20	38	100	n.d.
Cytosol	2	5	168	0.5

Results are expressed as nmole NAD(H) or NADP(H) utilized per min per mg of protein at 25°C. Abbreviations used are: SSA-DH, succinic semialdehyde dehydrogenase; GDH, glutamate dehydrogenase; LDH, lactate dehydrogenase; SSA-R, Succinic semialdehyde reductase; n.d., not detected.

identical to the whole brain isolation except the column sizes were somewhat smaller. As seen in Figure 23, the protein isolated from mitochondria has an identical electrophoretic mobility in anionic polyacrylamide gel electrophoresis when compared with that seen for the whole brain protein. The kinetic data obtained from the mitochondrial enzyme was indistinguishable from the results obtained for enzyme isolated from whole brain. Based on these observations, the protein obtained from the whole brain is referred to as mitochondrial succinic semialdehyde reductase.

Determination of the Molecular Weight

The molecular weight of native succinic semialdehyde reductase was determined by polyacrylamide gradient gel electrophoresis and by gel filtration. Fifteen μg of succinic semialdehyde reductase were applied at a polyacrylamide gradient (4%-30%) gel in the absence of denaturing agents and compared with standards of known molecular weight. As seen in Figure 24, succinic semialdehyde reductase migrates in between the standard proteins catalase and bovine serum albumin. When the migration distance obtained for the standard proteins is plotted as described by Manwell (70) as $(m.w.)^{1/3}$ versus 1/migration distance, a linear plot is obtained. Extrapolating the succinic semialdehyde reductase migration distance from the plot yields a molecular weight of 110,000.

The molecular weight obtained by gradient gel electrophoresis was confirmed by gel filtration with Sephacryl S-200. Purified reductase was applied to a column previously calibrated with several proteins of known molecular weight. The elution volume of the protein was determined by detection of reductase activity. The protein eluted at an estimated molecular weight of 112,000 (Figure 25).

Figure 23. Polyacrylamide gel electrophoresis of succinic semialdehyde reductase from mitochondria. Succinic semialdehyde reductase isolated from whole brain (lane a) and from mitochondria (lanes b-d) were applied to a polyacrylamide gel (7.5%). Lanes b-d were obtained from the peak fractions of the Blue-Sepharose II column.

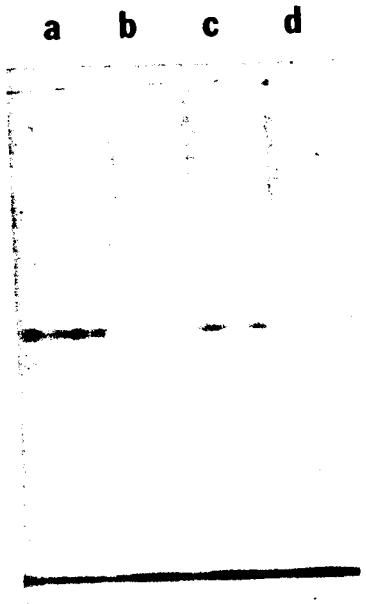
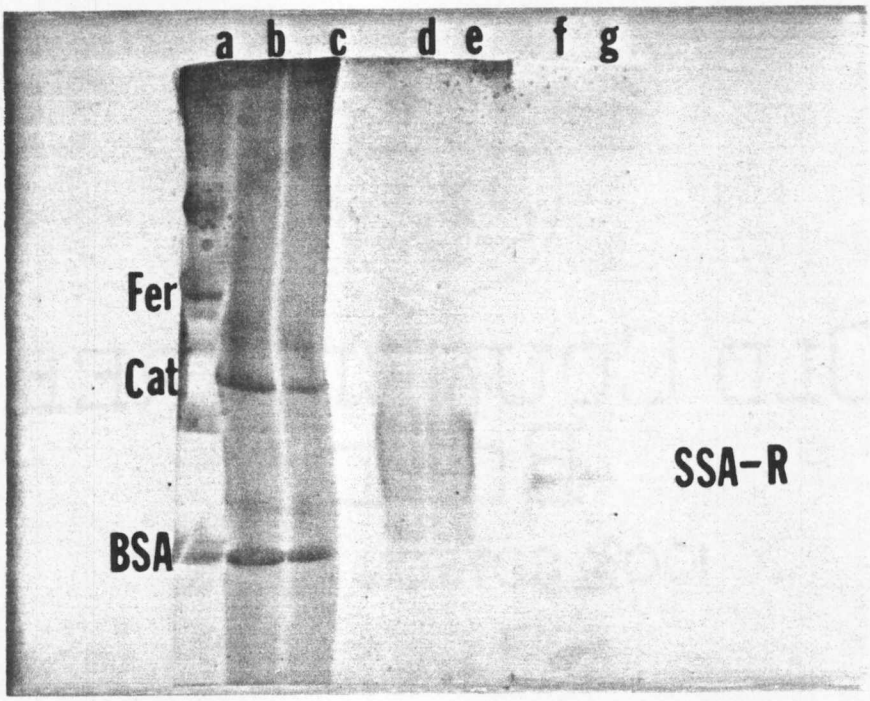


Figure 23

Figure 24. Determination of molecular weight by polyacrylamide gradient gel electrophoresis. Succinic semialdehyde reductase (lanes d-g) was subjected to electrophoresis on PAA 4/30 gradient gels. Lanes a-e were stained for protein with Coomassie blue and lanes f and g stained for activity as described in "Experimental Procedures." Lanes a-c contained the following standard proteins: ferritin (lane a), catalase (lane b) and bovine serum albumin (lane c). The migration distances were within 5% of the predetermined values provided with the gel kit (Pharmacia). Panel B contains a plot of the data as described by Manwell (70) where XL is the migration distance in mm.



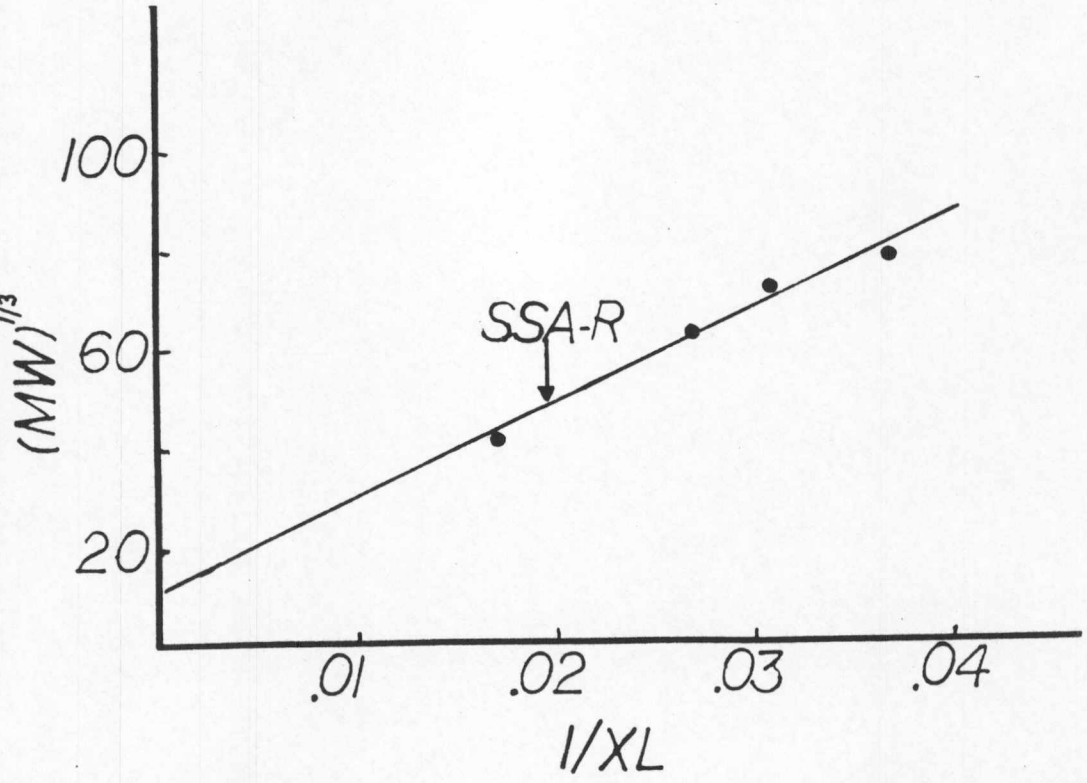


Figure 24

Figure 25. Determination of molecular weight by gel filtration. A 55 x 1 cm Sephacryl S-200 column equilibrated in 10 mM potassium phosphate (pH 7.2) was calibrated with the following proteins: (1) catalase, (2) aldolase, (3) alcohol dehydrogenase, (4) bovine serum albumin, (5) chymotrypsinogen and (6) cytochrome c. Blue dextran (b.d.) was used as a marker of the excluded volume. A sample of succinic semialdehyde reductase containing blue dextran was applied to the column and the elution volume detected by following the elution of reductase activity.

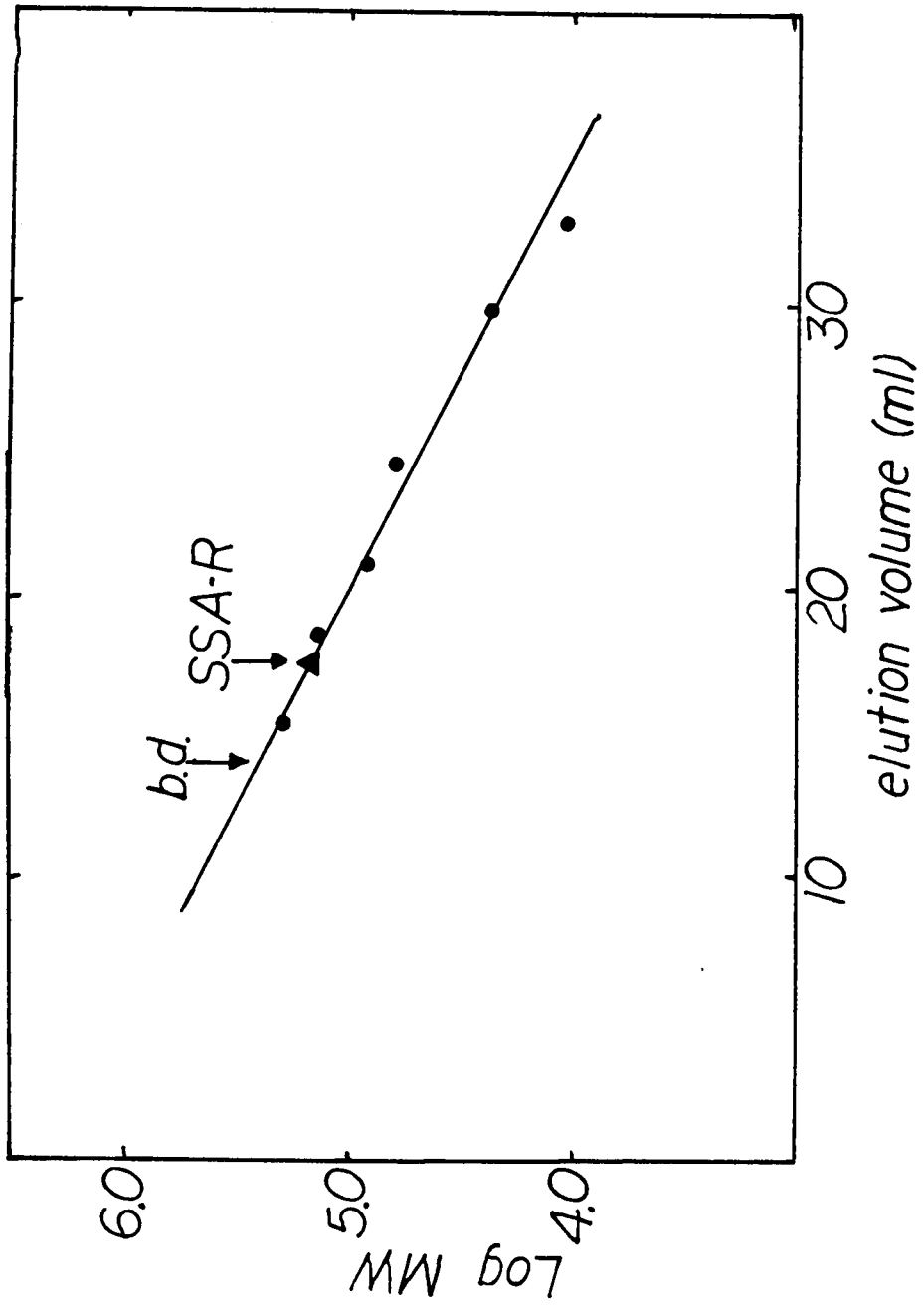


Figure 25

The molecular weight of the enzyme under denaturing conditions was determined with SDS-polyacrylamide gel electrophoresis. Twenty μg of succinic semialdehyde reductase were subjected to electrophoresis in 0.1% SDS. A band corresponding to a molecular weight of 56,000 was observed (Figure 26) suggesting that the protein was a dimer composed of subunits of identical molecular weight.

Determination of An Isoelectric Point

The isoelectric point of succinic semialdehyde reductase was determined using flat-bed isoelectric focusing. The enzyme was applied to an electrofocusing gel which would generate a pH gradient of 3.5 to 9.5 pH units. As shown in Figure 27, a single band of enzymatic activity is observed at a pH of 5.9. Since this method is frequently used to separate isozymes and other proteins which are difficult to separate, the fact that a single band is seen gives strong support to the contention that the enzyme preparation contains a single succinic semialdehyde reductase activity.

Discussion

Succinic semialdehyde reductase was purified 4300-fold from pig brain by affinity and ion-exchange chromatography. The final preparation had a specific activity of 320 nmol/min/mg as detected by the oxidation of NADPH.

The succinic semialdehyde reductase activity was found to be primarily in the mitochondria. The enzyme isolated from purified mitochondria showed identical electrophoretic mobility and kinetic properties with the protein isolated from a whole brain preparation.

The molecular weight of the enzyme was determined to be 110,000 based upon gradient gel electrophoresis and gel filtration chromatography studies.

Figure 26. Determination of the subunit molecular weight . 20 μg of succinic semialdehyde reductase was subjected to SDS-polyacrylamide gel electrophoresis (lane b). The gel (10%) contained 0.1% SDS. Lane a contains the following molecular weight markers: bovine serum albumin (67,000), ovalbumin (43,000) and trypsin inhibitor (21,000).

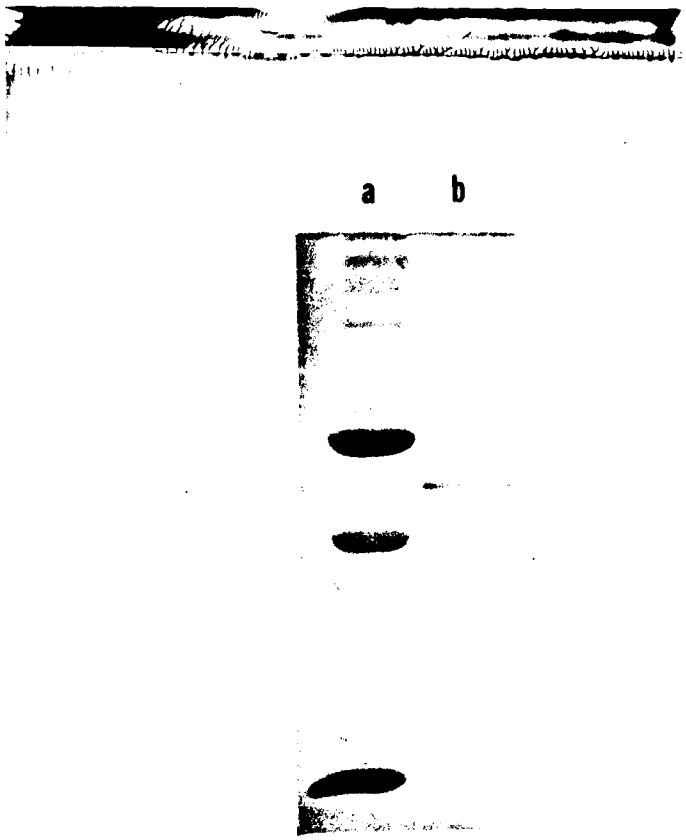
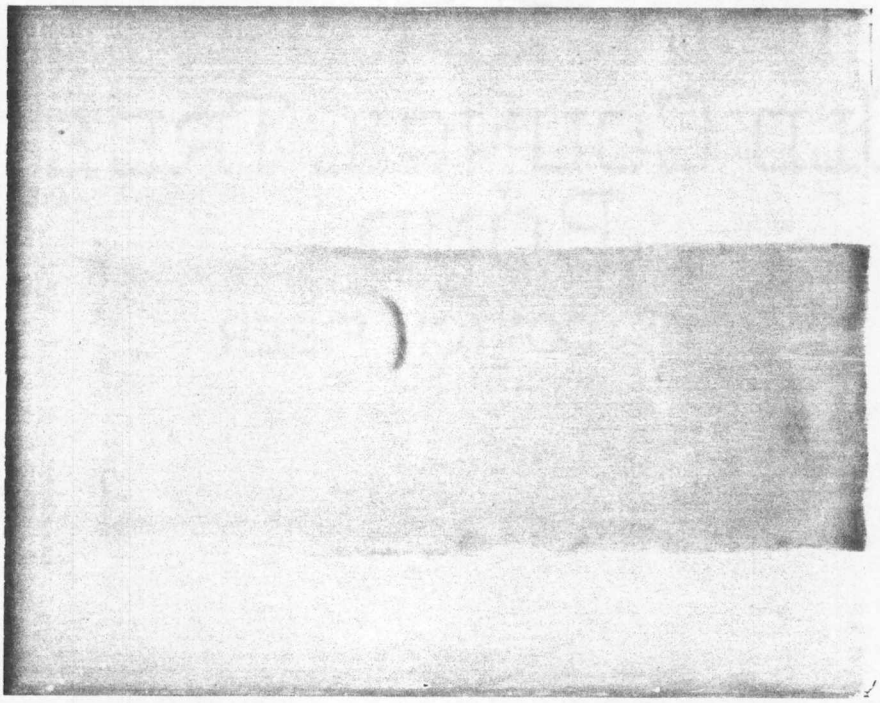


Figure 26

Figure 27. Determination of the pI for succinic semialdehyde reductase. The pI was determined by electrophoresis on LKB pagplates (panel A). 40 μg of enzyme was applied to the gel and electrophoresis was conducted at 1250 V for 1.5 h at 4°C. At the completion of the run, a portion of the gel was cut off and used to determine the pH gradient (panel B). The remainder of the gel was stained for enzyme activity as described in "Experimental Procedures."



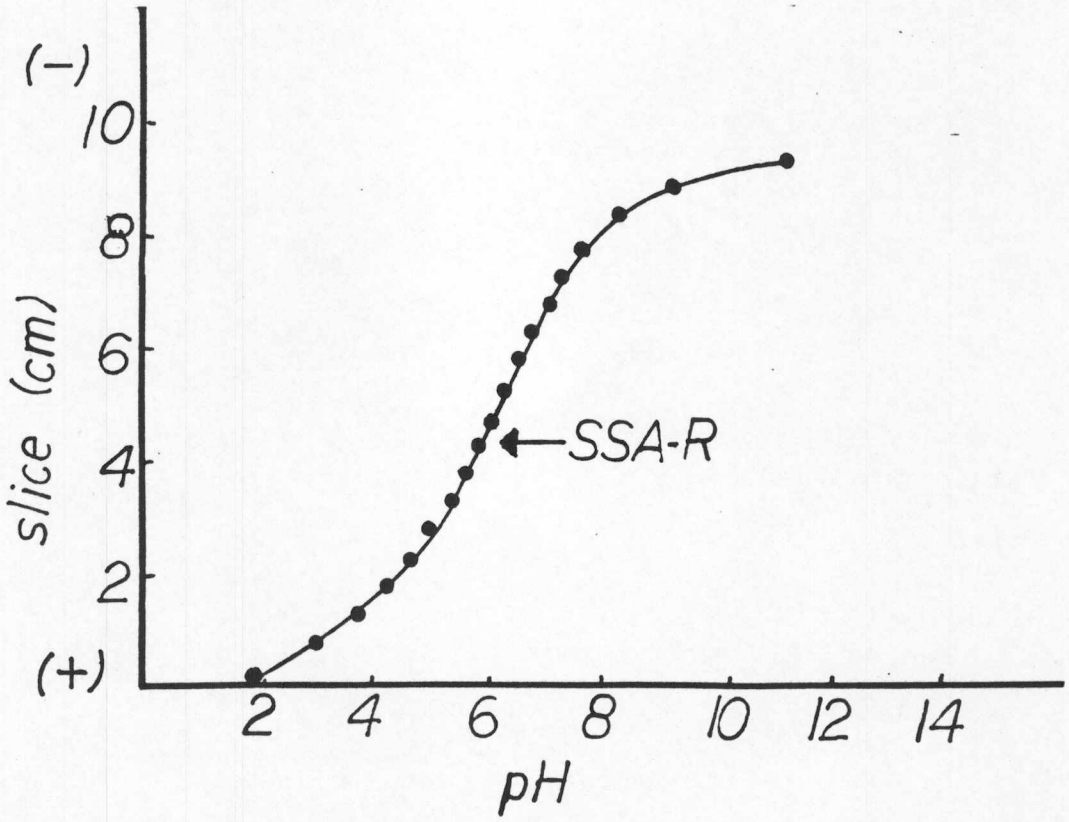


Figure 27

Succinic semialdehyde reductase migrated as a single band on SDS-PAGE at a molecular weight of 55,000 indicating that the holoenzyme is composed of two subunits of equal molecular weight.

The data presented in this work indicates that in the pig brain a mitochondrial form of aldehyde reductase exists, which is capable of utilizing succinic semialdehyde as a substrate.

The finding that the reductase is mitochondrial is in agreement with the subcellular location of 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase, enzymes believed to be intimately involved in the metabolism of 4-hydroxybutyrate. The compartmentation of succinic semialdehyde reductase in the mitochondria eliminates the need for transport of succinic semialdehyde out of the mitochondria to a cytosolic enzyme.

CHAPTER II

KINETIC PROPERTIES OF SUCCINIC SEMIALDEHYDE REDUCTASE

Introduction

Detailed kinetic and substrate specificity studies provide useful information concerning the role of an enzyme in the cell. These data will reflect the likely contributions of the enzyme to the various metabolic pathways and provide insight into the kinetic mechanism.

Several groups have studied the kinetic behavior of the high Km aldehyde reductase. Data obtained from the ox brain enzyme suggested the enzyme followed a Random mechanism (74) while more recent studies have been consistent with a compulsory Ordered mechanism with NADPH binding before the aldehyde substrate (75,76). However, data is lacking with regard to the kinetic mechanism of the specific succinic semialdehyde reductase.

This report investigates the kinetic mechanism and the substrate specificity of the succinic semialdehyde reductase from pig brain. The inhibition patterns for the dead-end inhibitors p-hydroxybenzaldehyde and pyridine-4-aldoxime will also be discussed.

Experimental Procedures

Enzyme Assays

All assays were carried out on a Cary Model 15 recording spectrophotometer. The oxidation of NADPH (molar extinction coefficient = 6225 $M^{-1}cm^{-1}$) was monitored at 340 nm at 25°C for at least 3 min. A good straight line was generally obtained. All assay mixtures contained succinic semialdehyde and NADPH at varied concentrations in 0.1 M potassium phosphate

buffer (pH 7.2) in a total volume of 0.9 ml. The reaction was initiated by the rapid addition of 0.1 ml of succinic semialdehyde reductase. If an inhibitor was to be included in the assay, it was added as part of the phosphate buffer.

Spectroscopy

UV and visible spectra were recorded on a Cary model 15 spectrophotometer.

Kinetic Theory

A bireactant enzyme system can be described by:

$$A + B \rightleftharpoons P + Q$$

and can proceed by several different mechanisms.

A "sequential" mechanism will be one where all substrates must be present on the enzyme before any products may leave. A sequential mechanism will be called "Ordered" if the substrates add in an obligatory order and the products leave similarly. A sequential mechanism where there is not an obligatory order of addition of substrates and release of products will be called "Random." Both mechanisms involve the formation of a ternary complex (i.e. an enzyme form in which both substrates are bound to the enzyme).

A sequential mechanism not involving a ternary complex was proposed by Theorell and Chance (77). This mechanism was proposed for alcohol dehydrogenase and suggested that the first product is released from the enzyme immediately following the addition of the second substrate. A Theorell-Chance mechanism may be either Ordered or Random.

If one or more of the products are released before all the substrates have been added to the enzyme, the mechanism will be called "Ping Pong." Ping Pong mechanisms include two or more stable forms of the free enzyme.

Non-random mechanisms with two substrates give one of the following initial velocity equations:

$$v = \frac{V_1 A B}{K_b A + K_a B + A B} \quad (1)$$

$$v = \frac{V_1 A B}{K_{ia} K_b + K_b A + K_a B + A B} \quad (2)$$

Equation (1) describes a Ping Pong mechanism and the second equation describes a Sequential mechanism.

To determine experimentally which rate equation applies, the concentration of one substrate is held constant at several levels while that of the other substrate is varied. Reciprocal plots of the initial velocity versus the varied substrate are made for each of the non-varied (fixed) substrate concentrations. Equations (1) and (2) can be transformed to:

$$\frac{1}{v} = \left(\frac{K_a}{V_1}\right)\frac{1}{A} + \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right) \quad (3)$$

$$\frac{1}{v} = \frac{K_a}{V_1}\left(1 + \frac{K_{ia} K_b}{K_a B}\right)\frac{1}{A} + \frac{1}{V_1}\left(1 + \frac{K_b}{B}\right) \quad (4)$$

where A is varied and B is fixed. A family of parallel lines is obtained if equation (3) holds and the mechanism is Ping Pong. If the data result in a set of lines which intersect to the left of the vertical axis, then equation (4) holds and the mechanism is Sequential.

The Michaelis constants and maximal velocities are best determined from reciprocal plots by replotting the vertical intercepts and the slopes as a function

of the fixed substrate concentration. The secondary plots for a Sequential mechanism are shown in equations (5) and (6).

$$\text{intercept} = \frac{1}{V_1} + \left(\frac{K_b}{V_1}\right)\frac{1}{B} \qquad (5)$$

$$\text{slope} = \frac{K_a}{V_1} + \left(\frac{K_{ia}K_b}{Ka}\right)\frac{1}{B} \qquad (6)$$

Initial velocity data when applied to the equations described above are capable of determing the mechanism type, but it is impossible to distinguish substrate A from substrate B in a sequential mechanism. Product inhibition studies are necessary to make this distinction.

Three general types of inhibition can be observed. When 1/v versus 1/A plots are made as a function of various fixed inhibitor concentrations, the reciprocal plots usually remain straight lines but either the slope, the intercept or both may vary. If only the slope varies, the lines intersect at the 1/v intercept; this is competitive inhibition. If only the intercepts vary such that parallel lines are obtained from the data, this is an example of uncompetitive inhibition. If both the slopes and the intercepts are affected, the inhibition is described as non-competitive or mixed-type inhibition.

Data obtained from inhibition studies can be interpreted based upon Cleland's rules for evaluating inhibition patterns (77):

Rule 1: The intercept of a reciprocal plot is changed by an inhibitor that associates with the enzyme form other than the one with which the varied substate combines (uncompetitive inhibition).

Rule 2: A slope effect occurs when the inhibitor combines with the same form of the enzyme or is reversibly connected to the same form of the enzyme with which the varied substrate combines (competitive inhibition).

Rule 3: Non-competitive inhibition results when both rules 1 and 2 apply.

Rule 4: An inhibitor cannot bind to an enzyme form that has a steady state concentration of zero.

Rule 5: No intercept effects occur when rapid equilibrium conditions prevail.

The presence of a dead-end inhibitor affects the denominator of the uninhibited rate equations by $(1 + I/K_i)$. The terms multiplied by the factor are those representing the enzyme form combining with the inhibitor. The K_i is the constant for the dissociation of I from the enzyme-inhibitor complex. In a sequential Ordered mechanism, if I reacts with EA, then equation (2) becomes:

$$v = \frac{V_1 A B}{K_a B + K_{ia}K_b(I + \frac{I}{K_i}) + A B + K_b A (K + \frac{I}{K_i})} \quad (7)$$

With B as the varied substrate I will be a competitive inhibitor and K_i can be evaluated from a replot of the data (e.g. slope versus I).

Results

Determination of the pH Optimum

The pH activity curve for succinic semialdehyde reductase was determined using an assay buffer containing 120 μM succinic semialdehyde and 50 μM NADPH in 0.1 M phosphate buffer at various pH. The data reflected in Figure

28 shows the maximal activity is observed at pH 7.2 with a very gradual decrease in activity with increasing pH. The optimum for the reverse reaction (e.g., the formation of succinic semialdehyde) was slightly shifted toward the alkaline with the maximal activity observed at 7.5 (not shown).

Substrate Specificity of Succinic Semialdehyde Reductase

The mitochondrial enzyme was tested for its ability to utilize a variety of aldehydes as substrates. Maximal activity was observed with succinic semialdehyde and malonic semialdehyde (Table X). These aldehydes can be formed by 4-aminobutyrate aminotransferase from 4-aminobutyrate and β-alanine respectively. The only synthetic compound which was a substrate for the enzyme was p-nitrobenzaldehyde, which has been found to be the classical synthetic substrate for many aldehyde reductases. NADH was also suitable as an alternate cofactor although less efficiently than NADPH. The many other aldehydes which are metabolized by non-specific NADPH-dependent aldehyde reductases were not substrates for this enzyme. p-Hydroxybenzaldehyde and pyridine-4-aldoxime were found to inhibit succinic semialdehyde reductase.

Calculation of the Equilibrium Constant and ΔG^{o}

The equilibrium constant (K_{eq}) was calculated by incubating 70 μM succinic semialdehyde and 70 μM NADPH with 15 μg of succinic semialdehyde reductase at 25°C at pH 7.3 and observing the change in absorbance at 340 nm over a period of several hours. The absorbance reached a constant value after about 3 hours and remained constant for 18 h. The equilibrium in a buffered system can be expressed as:

$$K_{eq} = \frac{GHB \quad NADP^{+}}{SSA \quad NADPH \quad H^{+}} \qquad (8)$$

Figure 28. The pH activity profile of succinic semialdehyde reductase. Enzyme assays were carried out as described in the "Experimental Procedures" except that the buffer above pH 7.4 was 0.1 M potassium pyrophosphate.

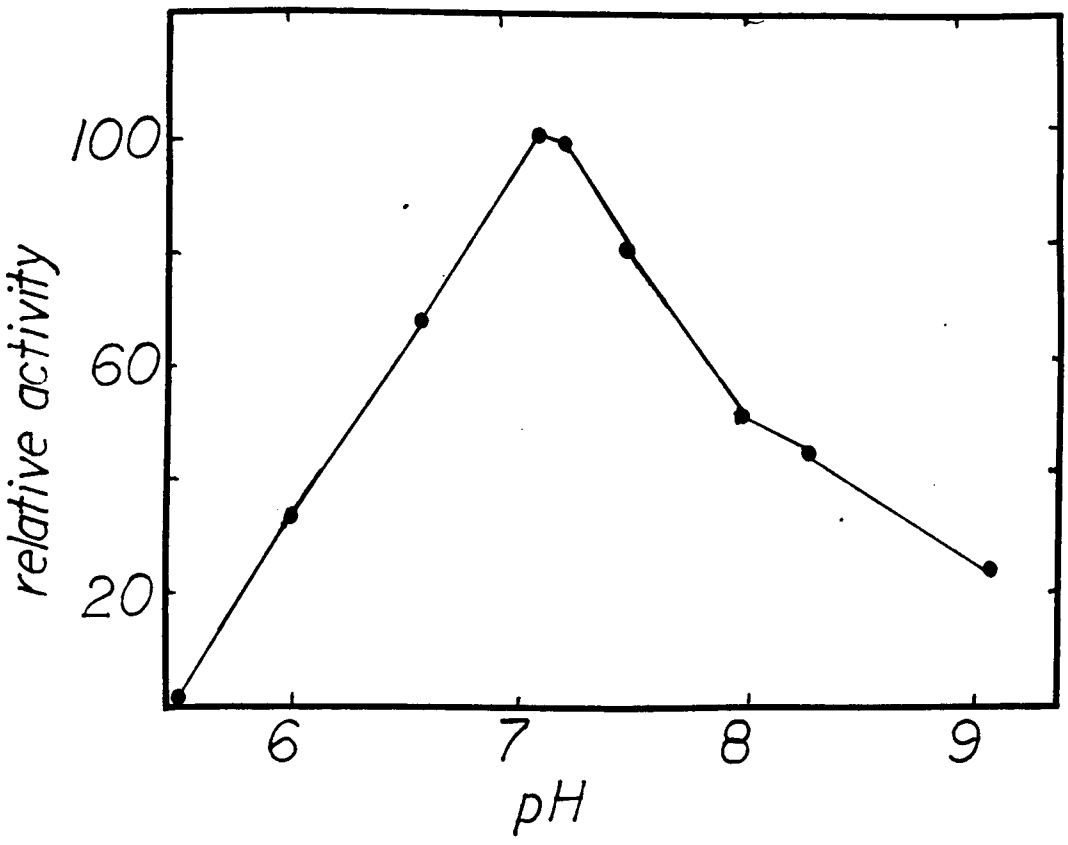


Figure 28

TABLE X

SUBSTRATE SPECIFICITIES OF MITOCHONDRIAL SUCCINIC SEMIALDEHYDE REDUCTASE

Substrate	Relative Activity %	Inhibition
Succinic semialdehyde[a]	100	-
Malonic semialdehyde	98	-
Glutaric semialdehyde	n.d.	-
p-Nitrobenzaldehyde	65	-
Propionaldehyde	n.d.	n.d.
Hydroxybenzyl aldehyde	n.d.	n.d.
Glyceraldehyde	n.d.	n.d.
Glycoaldehyde	n.d.	n.d.
Pyridine-2-aldehyde	n.d.	n.d.
o-Aminobenzaldehyde	n.d.	n.d.
p-Hydroxybenzaldehyde	n.d.	+
Pyridine-4-aldoxime	n.d.	+
Succinic semialdehyde/NADH	70	-

[a]All assays were carried out at a substrate concentration of 10^{-4} M at pH 7.2 with the exception of glutaric semialdehyde which was produced enzymatically by 4-aminobutyrate aminotransferase using 5-aminovalerate as the substrate. n.d. = not detected.

The reaction has been previously shown to produce 1 mol of 4-hydroxybutyrate (GHB) per mol of $NADP^+$ (34). Since the final NADPH concentration can be calculated spectrophotometrically (ε = 6225 $M^{-1}cm^{-1}$) then,

$$\Delta \text{ NADPH} = \text{NADP}^+ = \text{GHB} \qquad (9)$$

and

$$SSA_f = SSA_i - \Delta NADPH \qquad (10)$$

The data obtained from five experiments is shown in Table XI. Similar results were obtained when the formation of SSA and NADPH were followed with 4-hydroxybutyrate and $NADP^+$ provided as substrates.

The ΔG^o can be obtained from the equilibrium constant:

$$\Delta G^o = -RT \ln K_{eq} \qquad (11)$$

The value included in Table XI clearly shows that the reaction strongly favors the formation of 4-hydroxybutyrate.

The question of whether this enzyme can function <u>in vivo</u> as the degradative enzyme for 4-hydroxybutyrate has been the subject of some debate (24). The finding that this enzyme is located in the mitochondria is significant not only because of the presence of 4-aminobutyrate aminotransferase; succinic semialdehyde dehydrogenase is also located exclusively within the mitochondria. The possibility that a coupled reaction between the dehydrogenase and succinic semialdehyde reductase might solve the energetics problem of the reverse reaction was considered. When succinic semialdehyde dehydrogenase and NAD^+ were introduced into the equilibrium reaction going in the direction of formation of succinic semialdehyde, the reaction was driven to completion with succinate, NADPH and NADH resulting as the final products (Figure 29). Thus, the

TABLE XI

EQUILIBRIUM CONSTANTS FOR SUCCINIC SEMIALDEHYDE REDUCTASE AT PH 7.25

Reaction	K_{eq}	$\Delta G^{o'}$
SSA ⟶ GHB	4.1×10^{11} M	-15.8 Kcal/mol
GHB ⟶ SSA	5.3×10^{-12}	+15.4 Kcal/mol

Figure 29. Effect of succinic semialdehyde dehydrogenase on the equilibrium of succinic semialdehyde reductase. To a reaction mixture containing 4 x 10^{-5} M $NADP^+$ and 50 mM 4-hydroxybutyrate, was added 15 μ g of succinic semialdehyde dehydrogenase and NAD^+ to a final concentration of 0.1 mM and the reaction allowed to proceed for 6 hours at 25°C (1). Reactions in the absence of the dehydrogenase (2) and in the absence of the reductase (3) were run as controls.

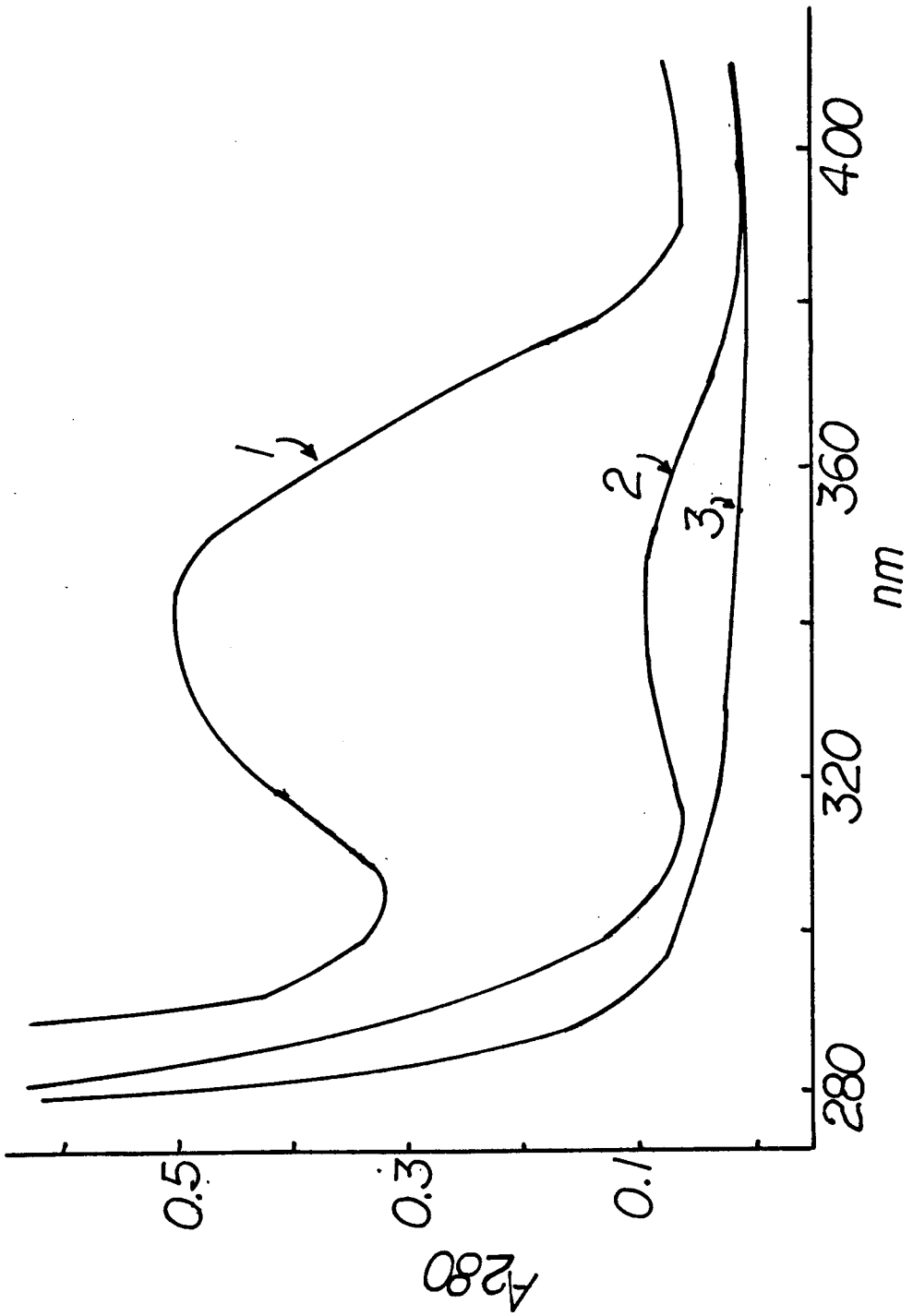


Figure 29

addition of a second enzyme, succinic semialdehyde dehydrogenase, permits the degradation of 4-hydroxybutyrate.

Steady State Kinetics

The steady state NADPH-dependent reduction of succinic semialdehyde to 4-hydroxybutyrate by succinic semialdehyde reductase gave rise to an intersecting pattern of straight lines which are consistent with a sequential Ordered or a rapid equilibrium Random mechanism for the addition of substrates (Figure 30). Secondary plots of the 1/v intercepts and the slopes versus the fixed substrate concentration (insets, Figure 30) yielded the kinetic parameters seen in Table XII.

Product Inhibition

A comprehensive examination of the product inhibition patterns was performed to determine the order of addition of the substrates and the order of release of the products. With $NADP^+$ as the inhibitor and NADPH as the varied substrate, a family of lines intersecting the 1/v axis was observed (Figure 31), indicating that both the inhibitor and the varied substrate are combining with the same form of the enzyme.

If 4-hydroxybutyrate is used as an inhibitor and NADPH is the varied substrate, the reciprocal plot of the data gives a set of parallel lines (Figure 32); thus, the inhibitor interacts with a different form of the enzyme and is not reversibly connected to the varied substrate.

Mixed-type inhibition patterns were seen when either 4-hydroxybutyrate or $NADP^+$ were used as the inhibitor and SSA was the varied substrate (Figures 33 and 34). However, the inhibition by $NADP^+$ versus succinic semialdehyde could be overcome by using a saturating concentration of NADPH (50 μM),

Figure 30. Steady state kinetics of succinic semialdehyde reductase. Kinetic data were obtained for various succinic semialdehyde concentrations with NADPH fixed at: a, 10 μM; b, 25 μM; c, 50 μM; d, 100 μM. The insets contain secondary plots of the 1/v intercept (A) and the slope (B).

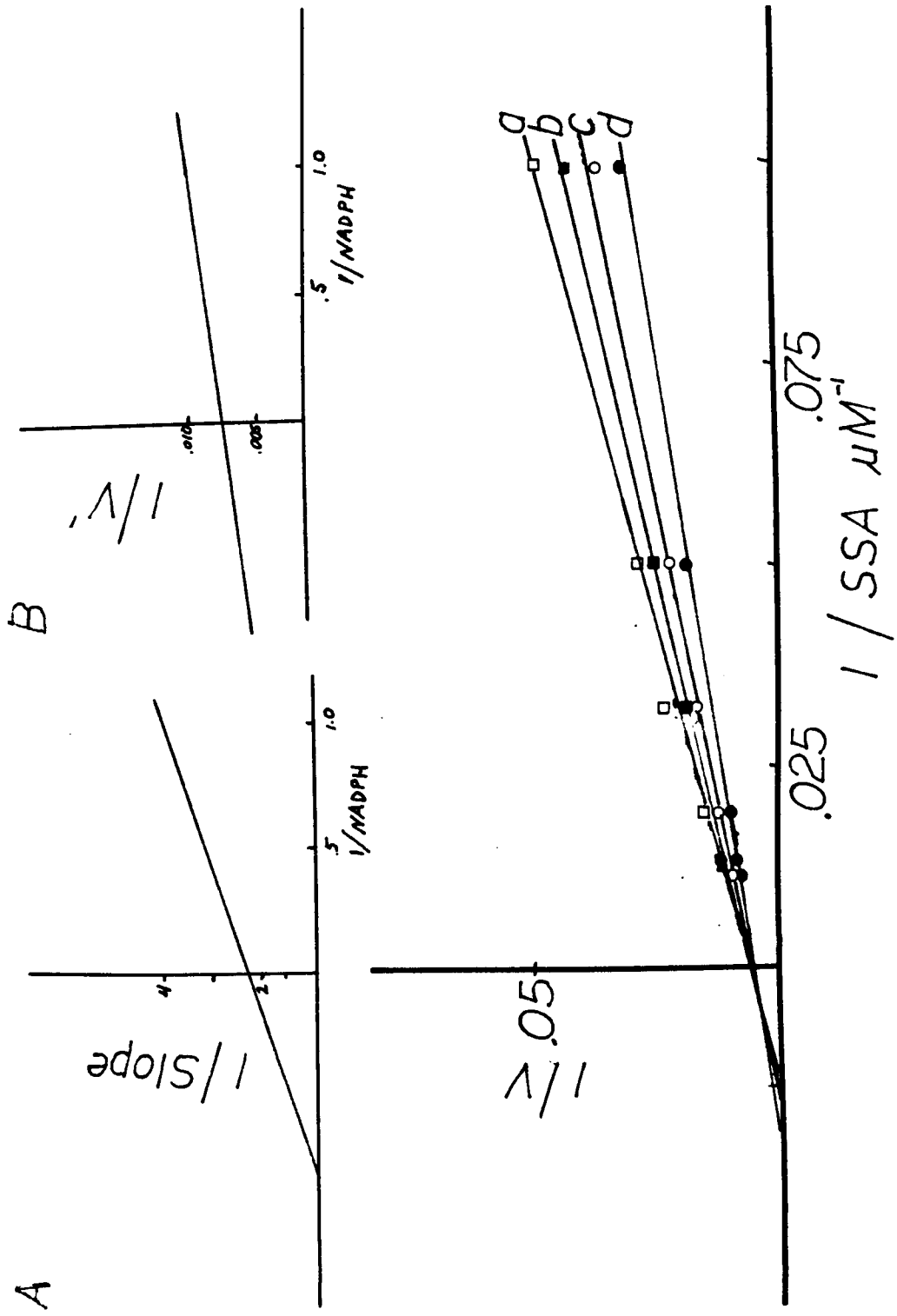

A
1/Slope
4
2
1.0
.5
1/NADPH
B
1/V'
.010
.005
1.0
.5
1/NADPH
a
b
c
d
1/V
.05
.025
.075
1 / SSA μM⁻¹

Figure 30

TABLE XII

STEADY STATE KINETIC PARAMETERS FOR SUCCINIC SEMIALDEHYDE REDUCTASE

Substrate	K_m	K_i	V_m^a
NADPH	5.8×10^{-6} M	5.2×10^{-5} M	144
Succinic semialdehyde	2.3×10^{-5} M		

[a] V_m = nmoles $\cdot min^{-1} \cdot mg^{-1}$.

Figure 31. Product inhibition by $NADP^+$ versus NADPH. Assays were conducted at various NADPH concentrations containing different fixed concentrations of $NADP^+$ (a, 100 μM; b, 60 μM; c, 30 μM; d, 0) and 120 μM succinic semialdehyde. The inset contains a secondary plot of the slopes.

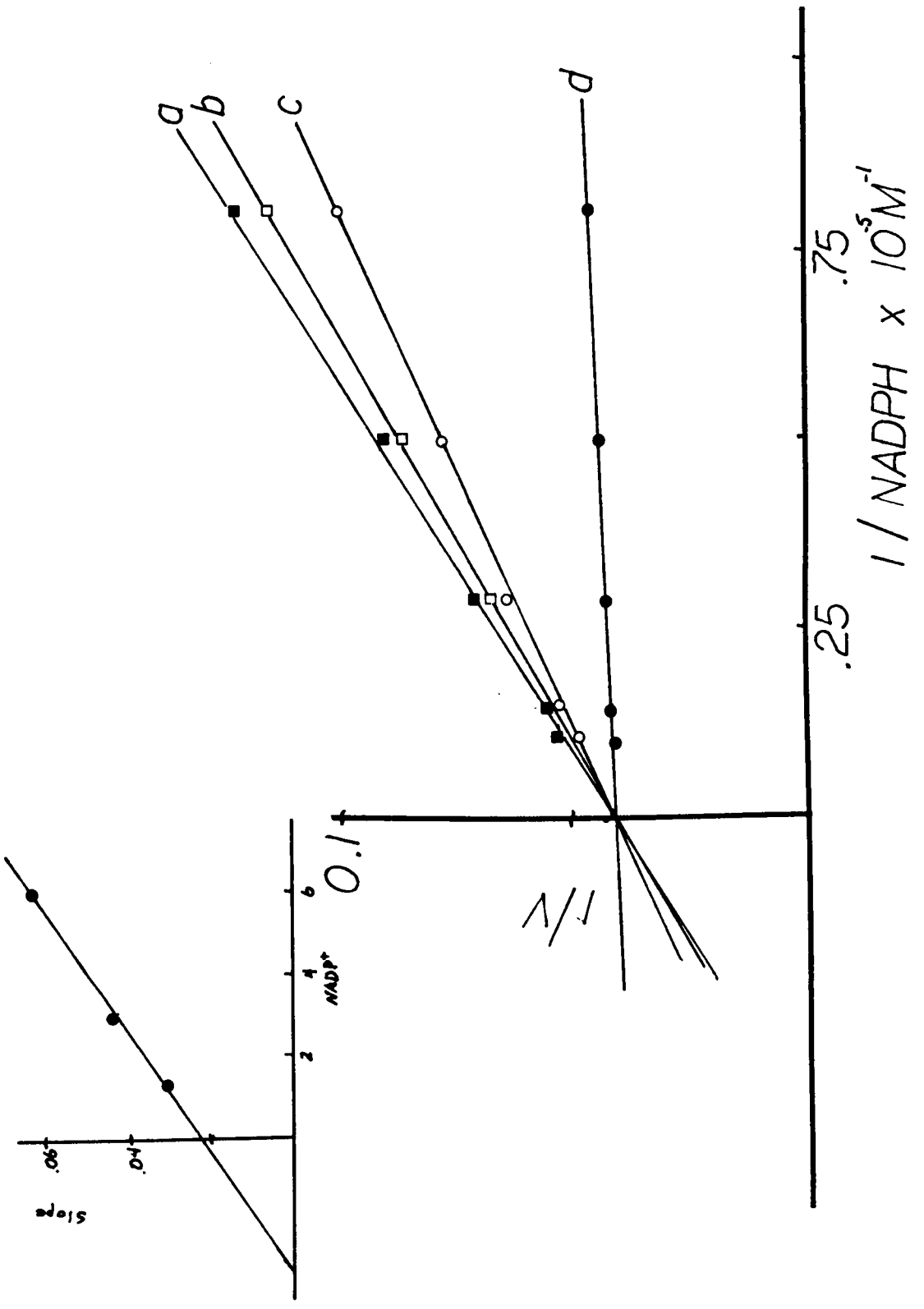


Figure 31

Figure 32. Product inhibition by 4-hydroxybutyrate versus NADPH. Buffer containing varied fixed amounts of 4-hydroxybutyrate (a, 35 mM; b, 20 mM; c, 10 mM; d, 0) and 120 μM succinic semialdehyde were assayed at different concentrations of NADPH. The inset contains a replot of the 1/v intercept.

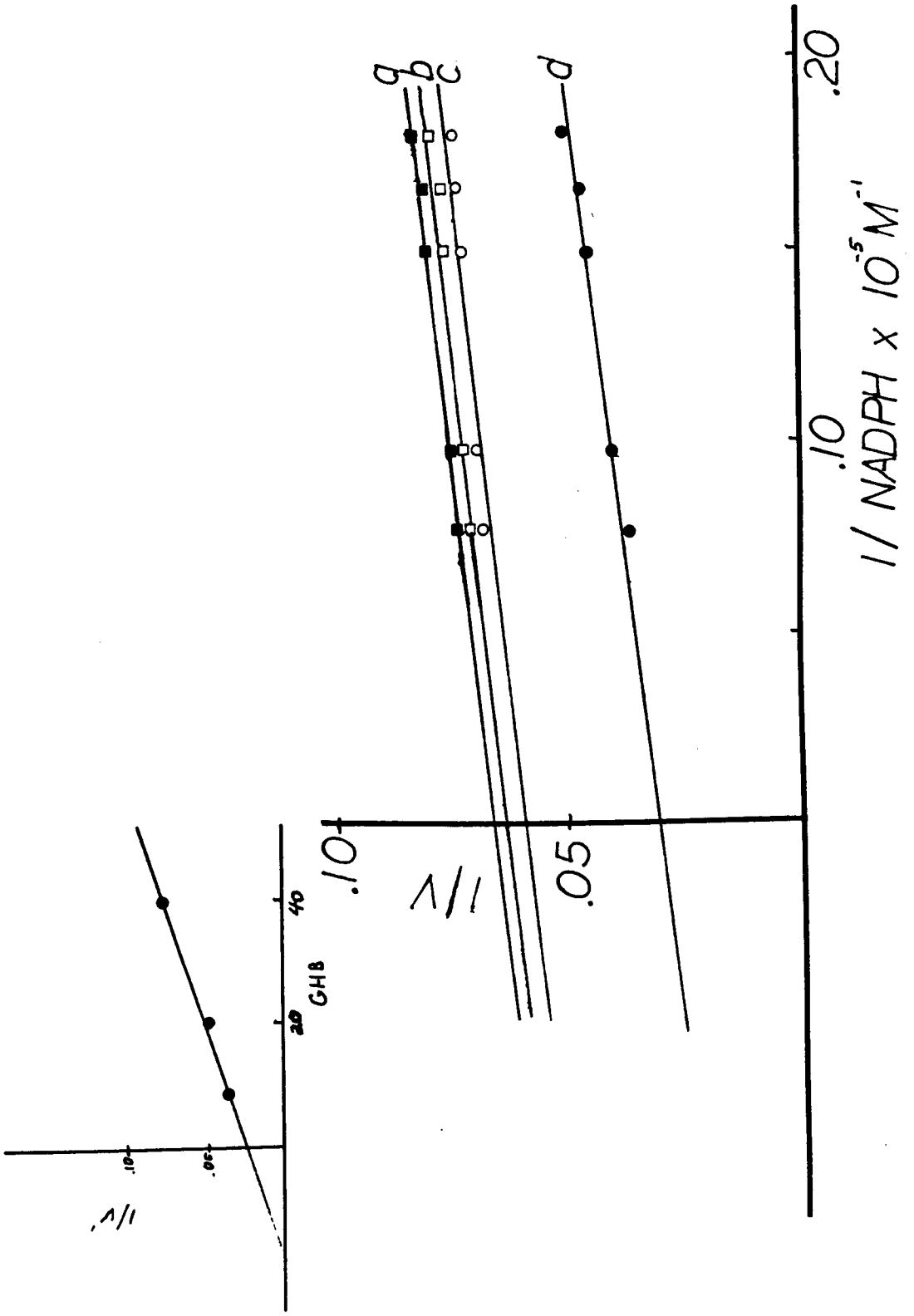


Figure 32

Figure 33. Product inhibition by 4-hydroxybutyrate versus succinic semialdehyde. 4-Hydroxybutyrate was added to the assay buffer at various fixed concentrations (a, 50 mM; b, 25 mM; c, 10 mM; d, 1 mM; e, 0). The assay mixture contained 50 μM NADPH and varied amounts of succinic semialdehyde. Inset A contains the secondary plot of the intercepts and inset B a replot of the slopes.

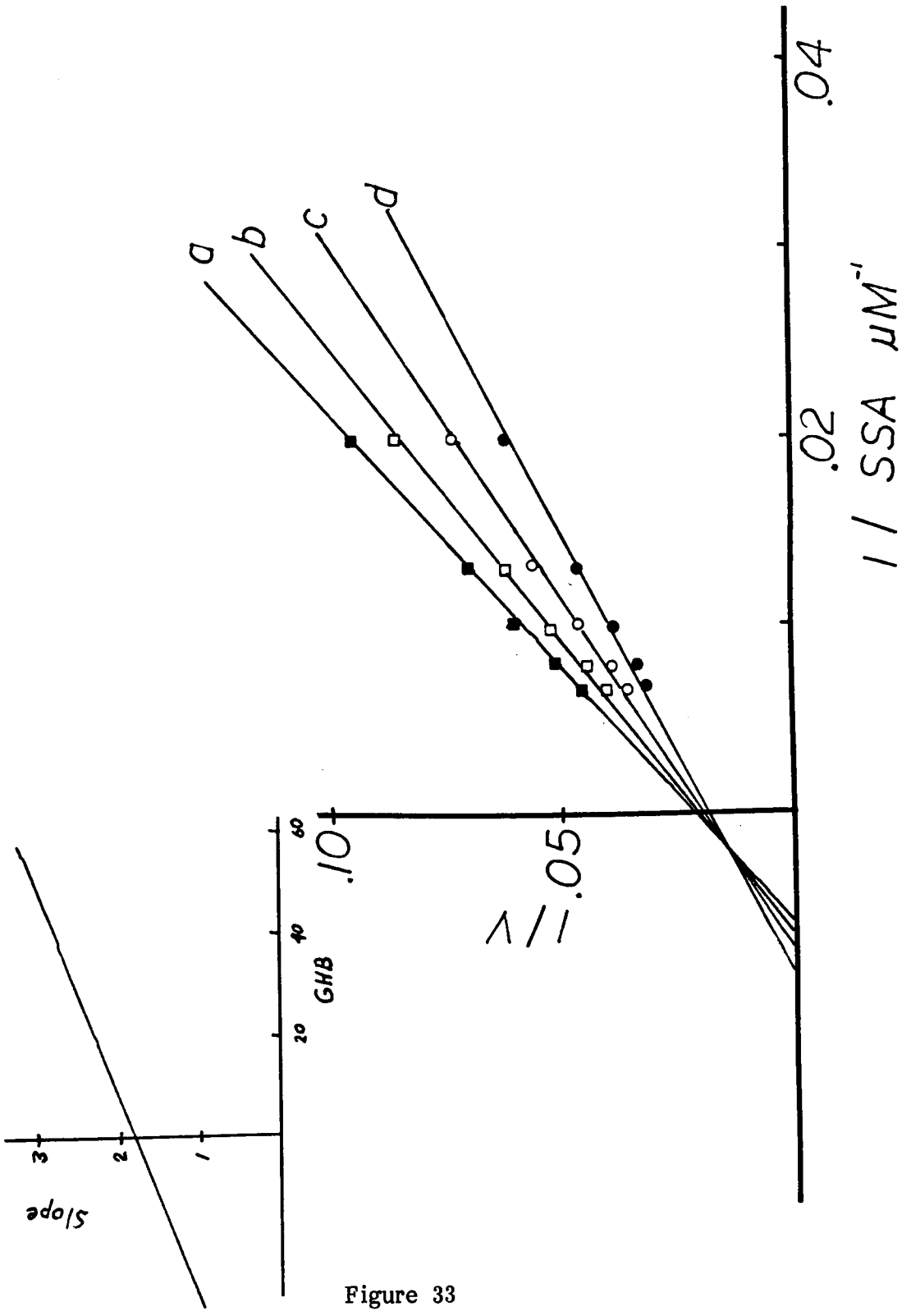


Figure 33

Figure 34. Product inhibition by $NADP^+$ versus succinic semialdehyde. Assay buffer containing varied fixed amounts of $NADP^+$ (a, 150 μM; b, 90 μM; c, 37 μM; d, 0) and 10 μM NADPH were assayed at different concentrations of succinic semialdehyde. The inset contains the secondary plot of the slopes.

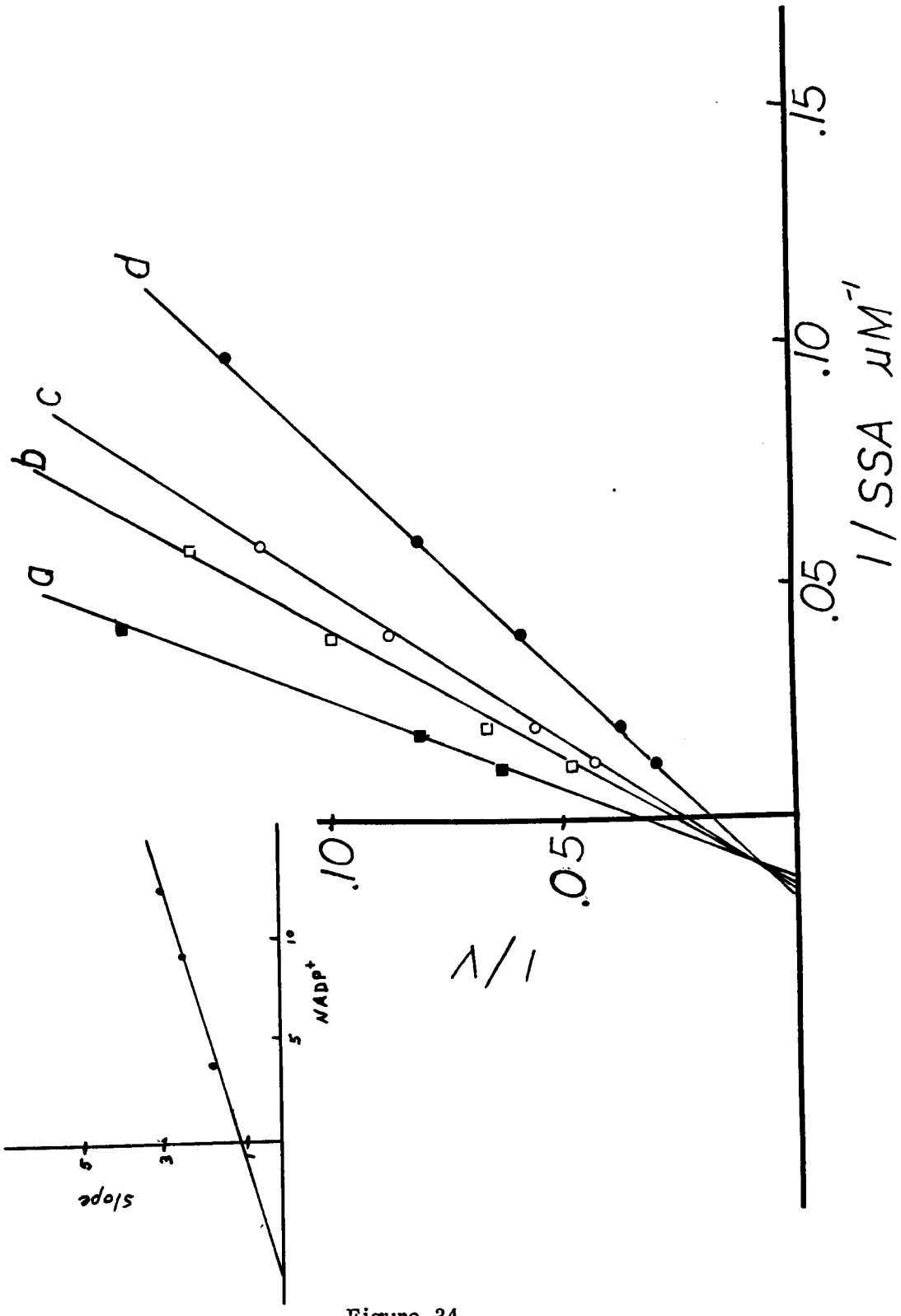


Figure 34

which can be explained by inhibition rule 4. Since $NADP^+$ and NADPH bind to the same form of the enzyme, saturating with NADPH will reduce the concentration of this enzyme form to zero. The kinetic data obtained from the appropriate replots of each reciprocal plot (insets, each figure) was used to obtain the kinetic constants in Table XIII. The inhibition patterns are summarized in Table XIV.

Dead-End Inhibition

Compounds which can specifically bind to an enzyme but are not converted into product are termed dead-end inhibitors, and they are a useful tool for probing enzyme mechanisms. Two compounds, p-hydroxybenzaldehyde and pyridine-4-aldoxime, were found to be reversible inhibitors of succinic semialdehyde reductase. These compounds were used to further investigate the kinetic mechanism of succinic semialdehyde reductase.

When p-hydroxybenzaldehyde is added to the assay buffer with succinic semialdehyde present as the varied substrate, a family of lines intersecting at the vertical axis is observed (Figure 35), which is consistent with competitive inhibition. From a secondary plot of the data, a K_i of 2.8×10^{-4} is calculated for p-hydroxybenzaldehyde. If this inhibitor is tested with NADPH as the varied substrate, parallel lines result from a plot of the initial velocity data, indicative of uncompetitive inhibition (Figure 36).

Similar results were obtained with pyridine-4-aldoxime. This inhibitor was also competitive with respect to succinic semialdehyde and uncompetitive with respect to NADPH. A kinetic constant inhibition constant of 2.7×10^{-4} was derived from a Dixon plot of the data (Figure 37).

TABLE XIII

SUMMARY OF THE KINETIC CONSTANTS OBTAINED FROM PRODUCT INHIBITION DATA

Substrate	K_m (M)	K_i (M)
NADPH	5.8×10^{-6}	5.2×10^{-5}
Succinic semialdehyde	2.3×10^{-5}	
$NADP^+$	1.1×10^{-5}	2.1×10^{-5}
4-hydroxybutyrate	2.4×10^{-2}	12.1×10^{-2}

TABLE XIV

SUMMARY OF THE INHIBITION PATTERNS

Inhibitor[a]	Varied Substrate	Fixed[b] Substrate	Pattern
$NADP^+$	NADPH	SSA	Competitive
$NADP^+$	SSA	NADPH*	Mixed
$NADP^+$	SSA	NADPH	None
GHB	NADPH	SSA	Uncompetitive
GHB	SSA	NADPH	Mixed

[a]SSA, succinic semialdehyde; GHB, 4-hydroxybutyrate.

[b]Fixed substrate levels were at saturating levels of 50 μM for NADPH and 120 μM for succinic semialdehyde except for (*) which had an NADPH concentration of 10 μM.

Figure 35. Inhibition by p-hydroxybenzaldehyde versus succinic semialdehyde. Assay buffer containing various fixed amounts of p-hydroxybenzaldehyde (a, 200 μM; b, 80 μM; c, 40 μM; d, 0) and 50 μM NADPH were assayed at various succinic semialdehyde concentrations. The inset contains a secondary plot of the slopes.

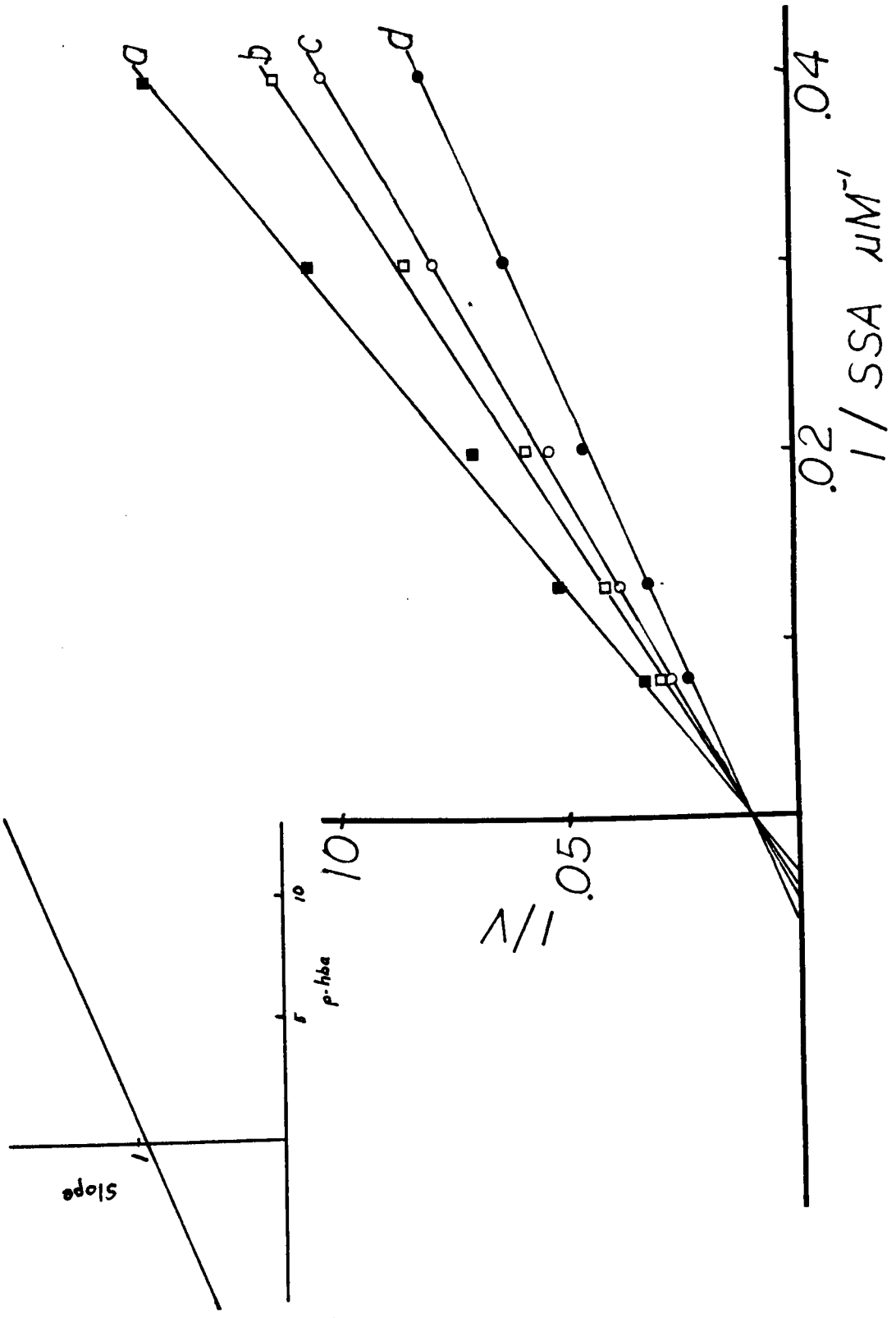


Figure 35

Figure 36. Inhibition by p-hydroxybenzaldehyde versus NADPH. Buffer containing various fixed amounts of p-hydroxybenzaldehyde (a, 200 μM; b, 80 μM; c, 40 μM; d, 0) and 120 μM succinic semialdehyde were assayed at various concentrations of NADPH.

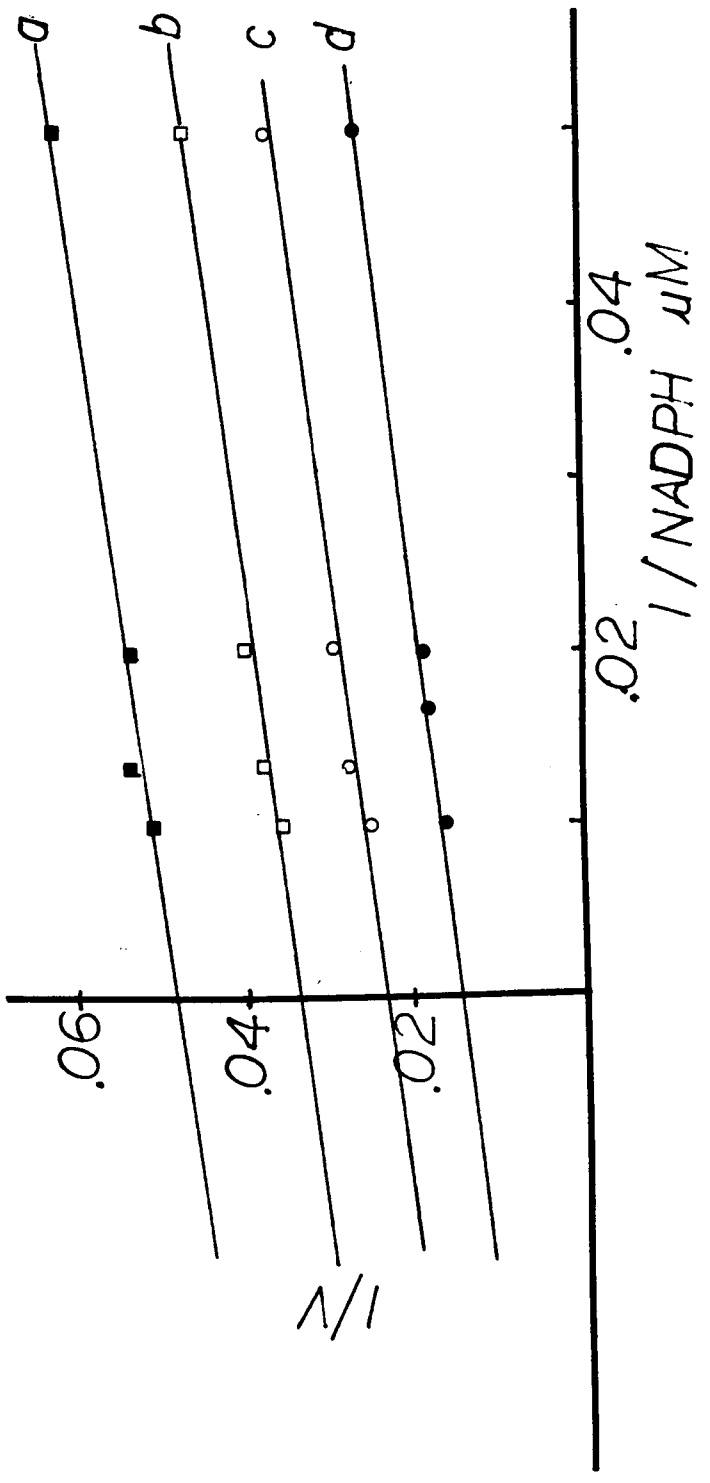


Figure 36

Figure 37. Inhibition by pyridine-4-aldoxime. Buffer containing pyridine-4-aldoxime (a, 170 μ M; b, 68 μ M; c, 34 μM; d, 0) and 50 μM NADPH were assayed at various succinic semialdehyde concentrations. The secondary plot of the slopes is shown in the inset.

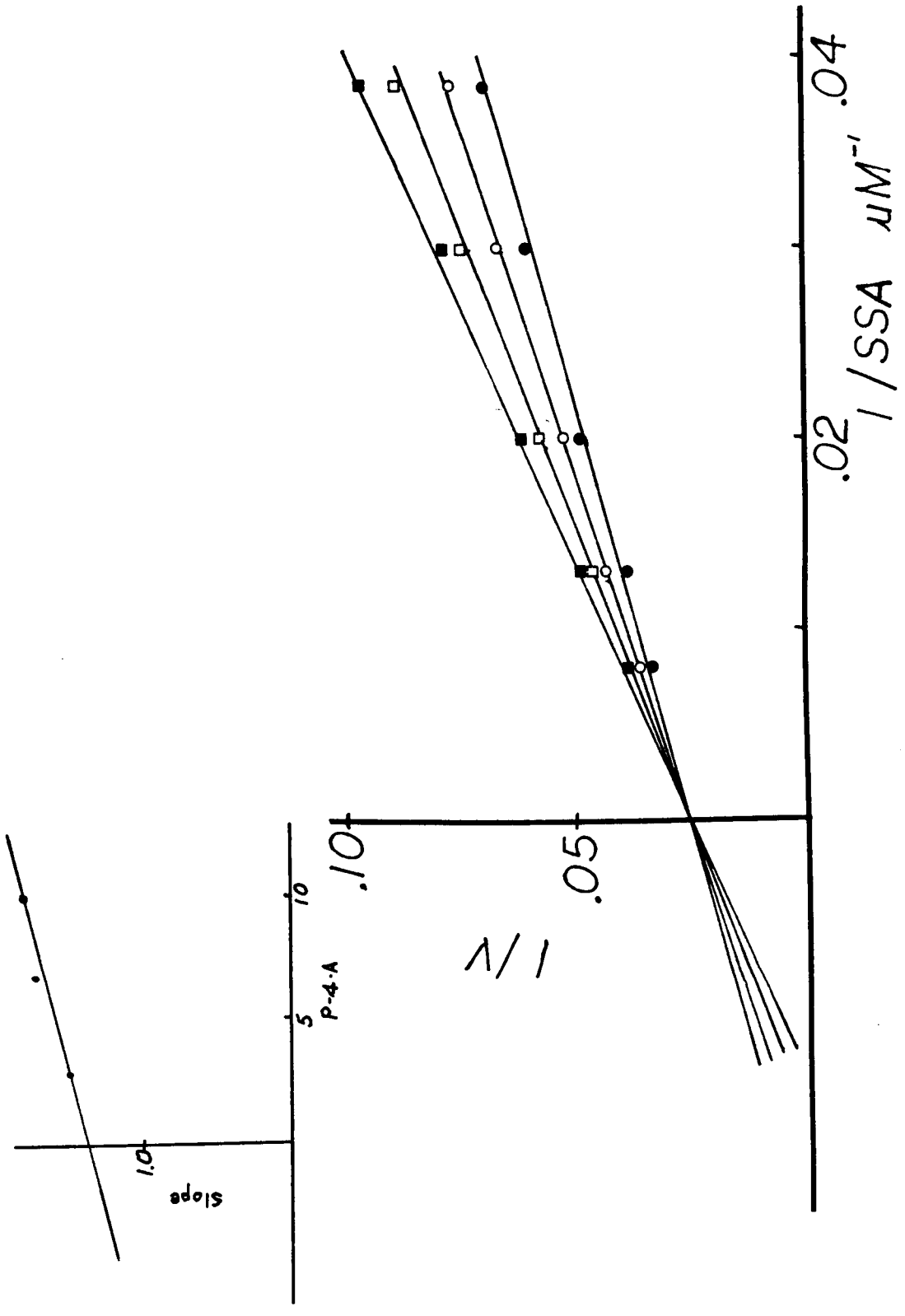


Figure 37

Discussion

The catalytic properties of succinic semialdehyde reductase show this enzyme to be specific for succinic semialdehyde and the closely related metabolite, malonic semialdehyde. The synthetic p-nitrobenzaldehyde was also found to be a substrate. NADH was found to be accepted by the enzyme as cofactor.

The equilibrium constant for the reaction catalyzed by the reductase was determined in the forward and in the reverse directions. The values obtained by either method showed that the formation of 4-hydroxybutyrate is strongly favored.

The observation that the presence of succinic semialdehyde dehydrogenase can drive the reverse reaction to completion is the first good evidence that suggests that this enzyme could function as the degradative enzyme *in vivo*.

Extensive steady state kinetic studies established kinetic constants for all parameters described by Cleland for a sequential bireactant mechanism (79). The quality of the kinetic values can be evaluated by use of the Haldane equation:

$$K_{eq} = \frac{V_1 \, K_p \, K_{iq}}{V_2 \, K_b \, K_{ia}} \qquad (12)$$

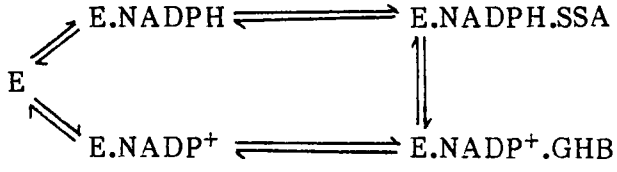
and comparing the value obtained by equation (12) with that obtained experimentally. Using a value of 48 for V_1/V_2 (data for V_2 not shown) and substituting the appropriate kinetic constants into the Haldane equation, a value of 5.3×10^4 is obtained which compares favorably with the values seen in Table XI (p. 123) for the experimentally derived K_{eq}.

The product and dead-end inhibition studies provided sufficient data to propose a sequential Ordered mechanism for succinic semialdehyde reductase.

$NADP^+$ is competitive with NADPH when succinic semialdehyde is at saturating concentrations. This can only occur in a Theorell-Chance or an Ordered mechanism. The uncompetitive pattern seen with 4-hydroxybutyrate as the inhibitor and NADPH varied is consistent only with the Ordered mechanism. The order of addition can be inferred by these inhibition data. The competitive pattern strongly suggests that NADPH adds to the enzyme first and $NADP^+$ leaves as a product last. This order of addition and departure was confirmed by the inhibition patterns found for 4-hydroxybutyrate versus succinic semialdehyde and NADPH.

The dead-end inhibitors p-hydroxybenzaldehyde and pyridine-4-aldoxime gave identical inhibition patterns with respect to NADPH and succinic semialdehyde. The uncompetitive inhibition pattern seen with NADPH as the varied substrate will occur only if the inhibitor adds to the enzyme after the addition of the varied substrate confirming the order of addition determined by products inhibition experiments.

Thus, an ordered sequential mechanism is supported by these data:



The nicotinamide cofactor adds to the enzyme before the aldehyde substrate. The order of release predicted by the product inhibition data is the departure of 4-hydroxybutyrate first followed by the release of $NADP^+$.

CHAPTER III

FLUORESCENCE PROPERTIES OF SUCCINIC SEMIALDEHYDE REDUCTASE

Introduction

In order to better understand the reaction mechanism of succinic semialdehyde reductase, the binding of NADPH to the enzyme was investigated by measuring protein tryptophan fluorescence. Comparison of the fluorescence of the tryptophan residues of the enzyme with free tryptophan provides considerable information about the topography of these amino acid residues within the enzyme, permits the investigation of the binding of NADPH to the protein, and allows the detection of tryptophan residues located near the cofactor binding site in the presence of NADPH.

Experimental Procedures

Fluorescence Spectroscopy

Fluorescence spectra were recorded in a precision spectrofluorimeter equipped with two Bausch and Lomb monochromators.

Results

Emission Spectra of Succinic Semialdehyde Reductase

The strong fluorescence exhibited by tryptophan allows for reliable fluorescence readings at low protein concentrations. A sample of purified reductase with an $A_{280} = 0.10$ was subjected to fluorescence spectroscopy and compared with a solution of free tryptophan of the same optical density. The excitation wavelength was at 290 nm to eliminate fluorescence which could

be attributed to tyrosine. As shown in Figure 38, a blue shift is detected in the emission spectra for the enzyme with a fluorescence intensity peak (λ_{max}) at 336 nm. This type of spectra is indicative of tryptophan residues which are located in hydrophobic regions of the protein.

The role of tyrosine fluorescence was evaluated by conducting the emission spectra at an excitation wavelength of 280 nm. The spectra at this excitation wavelength was not significantly different from the spectra at 290 nm, suggesting that any contribution of tyrosine to the fluorescence is minimal.

The quantum yield (Q) of the reductase was compared with that of free tryptophan by weighing the area under each spectra. The value found for the enzyme of 0.15 was somewhat smaller than the quantum yield obtained for free tryptophan (Q = 0.16).

Effect of NADPH on the Tryptophan Fluorescence

The interaction of NADPH with succinic semialdehyde reductase results in a decrease in the tryptophan fluorescence. In the presence of a 5-fold excess of NADPH, the enzyme showed a maximal decrease in fluorescence intensity of 20%.

The decrease in the fluorescence as a result of NADPH binding to the reductase can be used to determine the stoichiometry of NADPH binding. To this end, the fluorescence intensity (F) of samples containing a fixed concentration of enzyme (8×10^{-7} M) and adding increasing amounts of NADPH was measured at 25°C at an excitation wavelength of 290 nm and an emission wavelength of 336 nm. The results are presented in Figure 39.

Since the decrease in fluorescence can be attributed to the binding of NADPH at various concentrations of the cofactor to the enzyme, it is possible

Figure 38. Fluorescence spectra of succinic semialdehyde reductase. The fluorescence of succinic semialdehyde reductase was compared with free tryptophan (1) at an excitation wavelength at 290 nm (2) and at 280 nm (3).

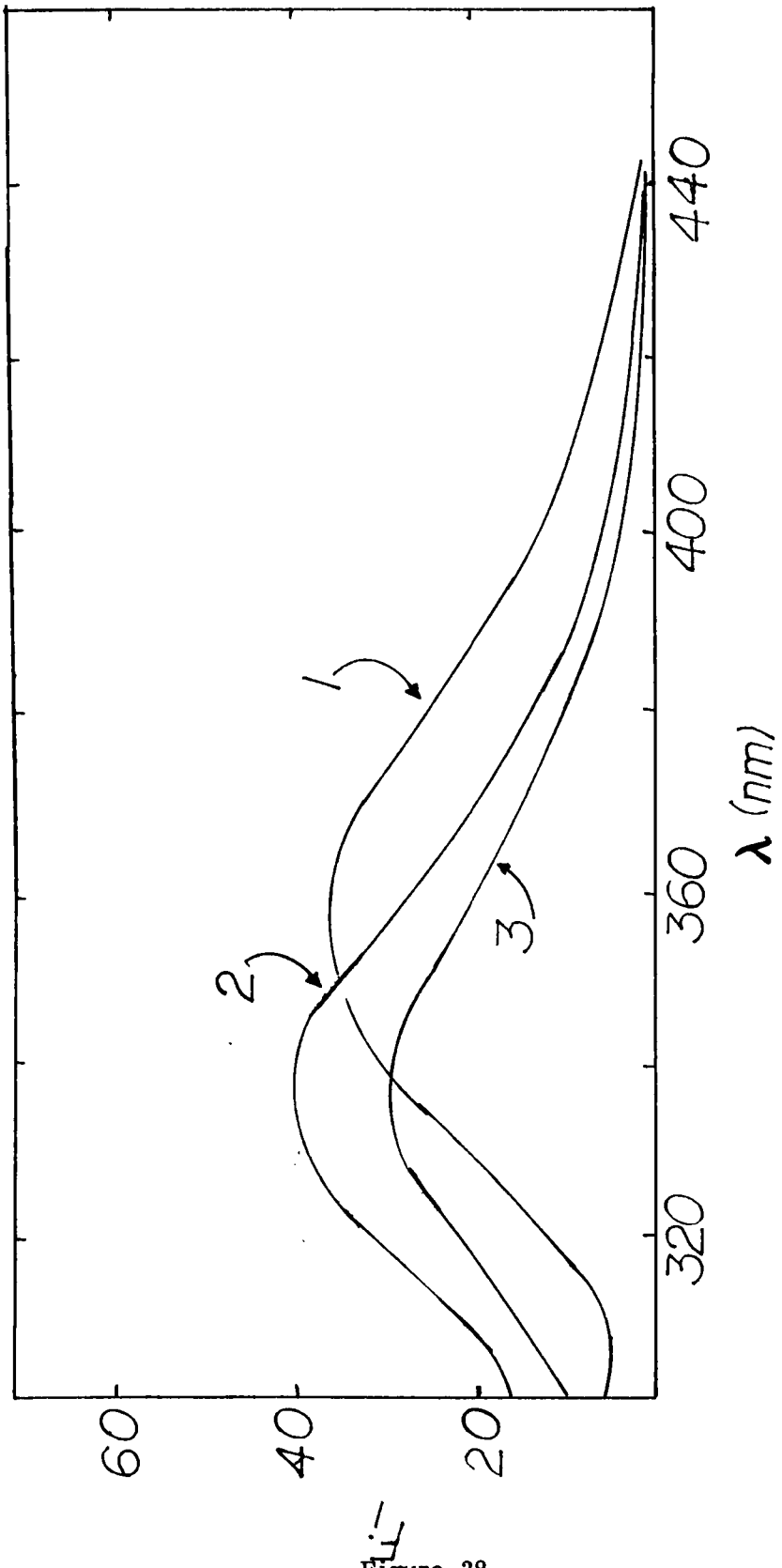


Figure 38

Figure 39. Titration of tryptophan fluorescence by NADPH. Small aliquots of NADPH were added to succinic semialdehyde reductase (8×10^{-7} M) and the fluorescence intensity measured at 336 nm (excit. at 290 nm).

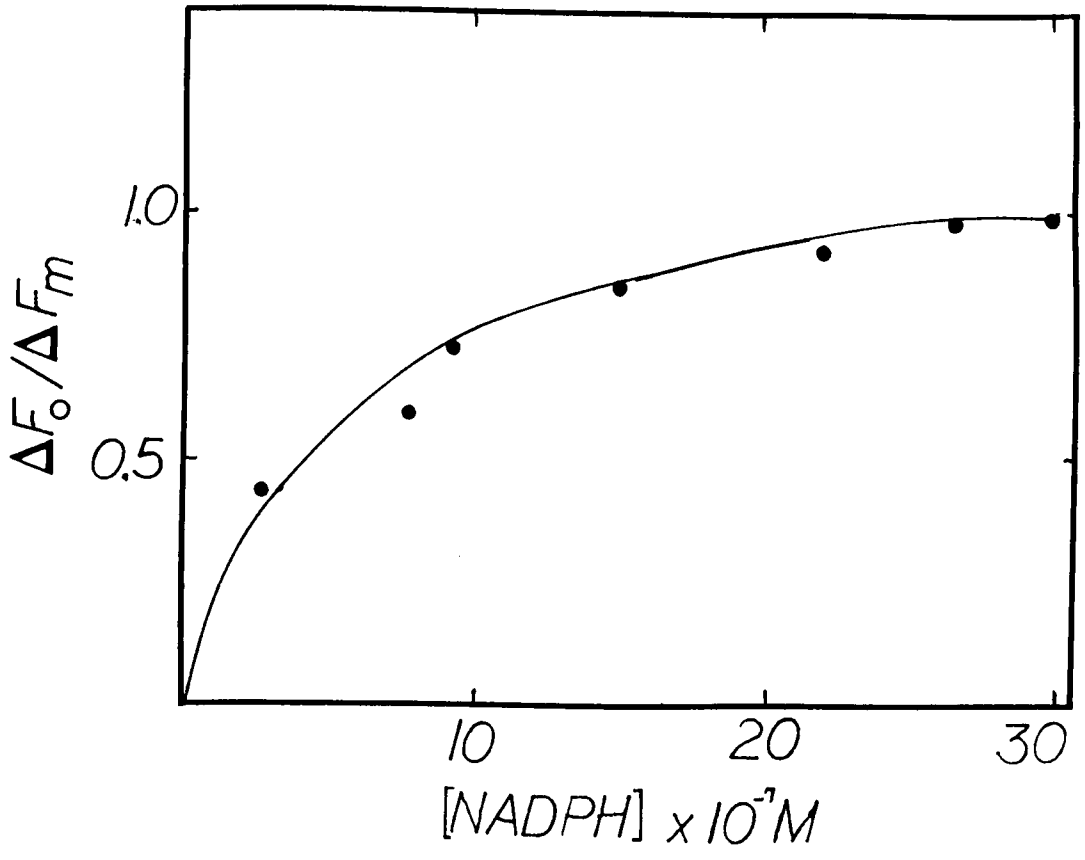


Figure 39

to calculate the fractional saturation (Θ) of the enzyme using the following equation:

$$\Theta = \frac{F_o - F}{F_o - F_m} = \frac{\Delta F}{\Delta F_{max}} \qquad (1)$$

where F_o is the fluorescence in the absence of NADPH and F_m is the fluorescence of the protein at saturating concentrations of NADPH.

The results of the protein titration with NADPH were analyzed using the Hill equation (equ. 2):

$$\frac{\Theta}{1 - \Theta} = K_d[L]^n \qquad (2)$$

where n is the number of binding sites. The data was plotted as log (Θ/1 - Θ) versus log (L) assuming a value of n = 2. A line with a slope of 0.91 is generated (Figure 40). The vertical intercept yields a K_d of 1.1 x 10^{-7} M.

The effect of NADPH binding on the fluorescence spectra of succinic semialdehyde reductase is shown in Figure 41. As noted in the titration experiments, the binding of NADPH to the enzyme causes a decrease in the fluorescence quantum yield but it does not result in a shift in max. A change in Q due to cofactor binding is usually attributed to a conformational change in the enzyme.

The possibility that tryptophan resudies may be at the active site was determined by fluorescence energy transfer. Since NADPH requires light at 340 nm in order to emit fluorescence at 440 nm, excitation of the enzyme at 290 nm in the presence of NADPH will only result in fluorescence at 440 nm when a tryptophan residue is very close to the cofactor such that energy transfer occurs. Although a higher degree of sensitivity was required to detect

Figure 40. Hill plot of NADPH titration data.

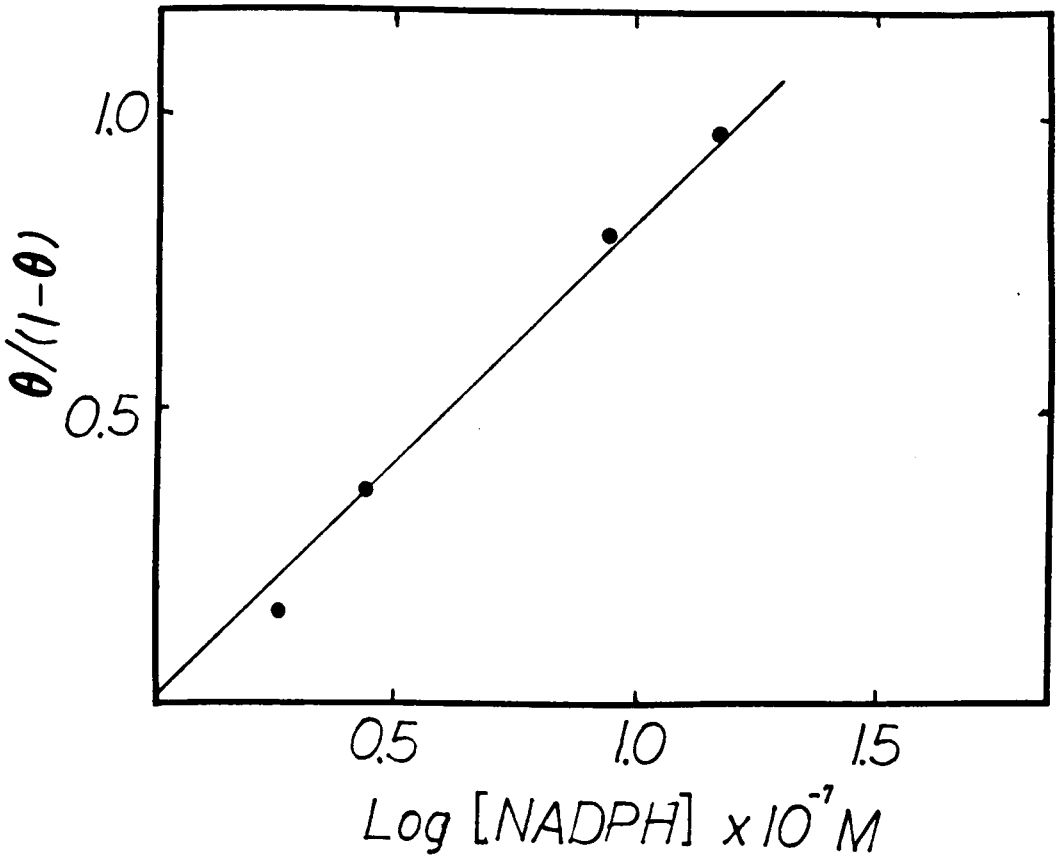


Figure 40

Figure 41. Effect of NADPH binding on the fluorescence of succinic semialdehyde reductase. The fluorescence of NADPH (1), succinic semialdehyde reductase (2) and reductase in the presence of NADPH (3) were carried out at an excitation wavelength of 290 nm.

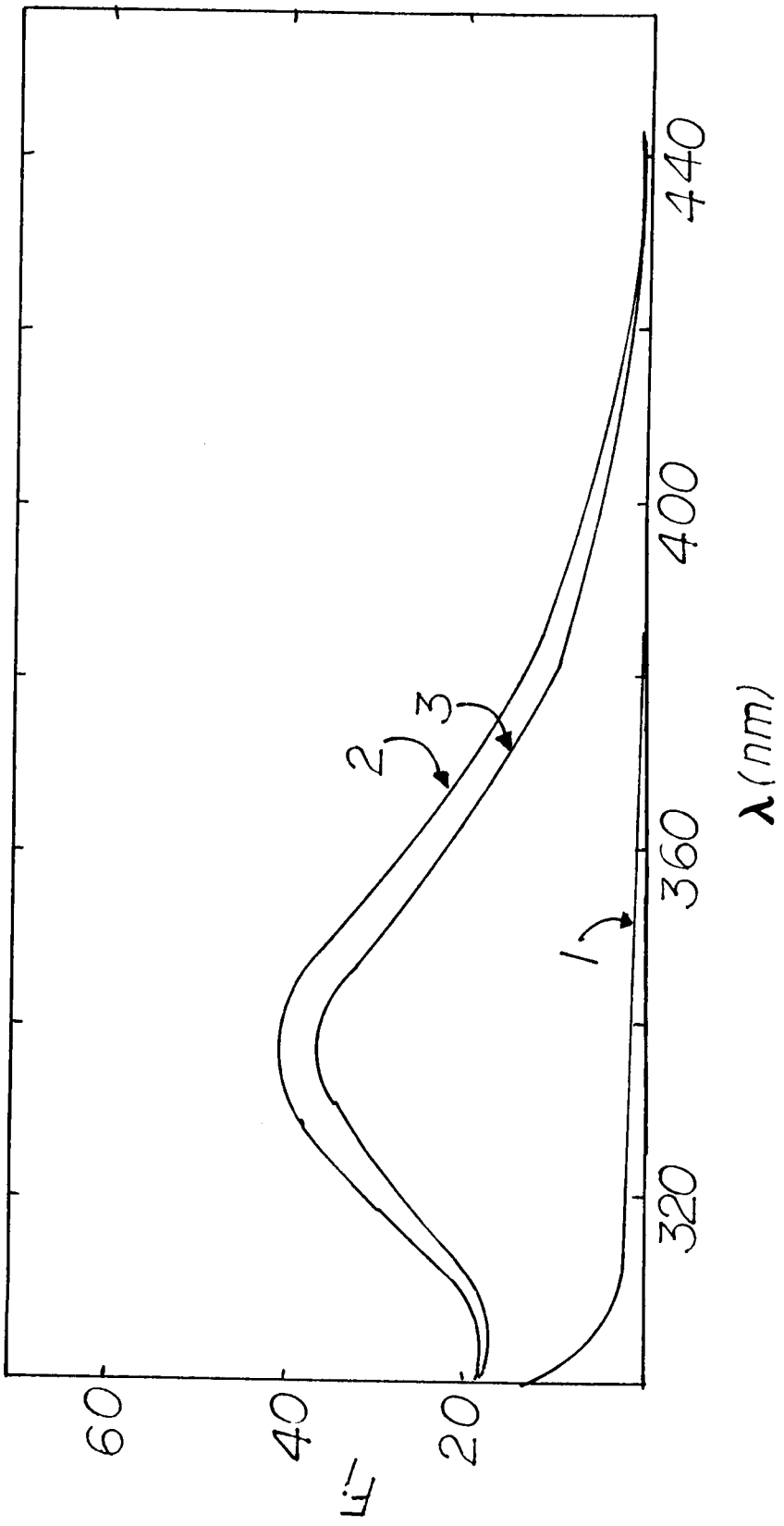


Figure 41

the signal, fluorescence was observed at 440 nm in the presence of cofactor, indicating the presence of a tryptophan residue at or near the active site.

Discussion

Fluorescence studies were used to investigate the binding of NADPH to succinic semialdehyde reductase. The binding of the cofactor resulted in a shift of λ_{max} to 336 nm and a small decrease in the quantum yield.

Titration of the enzyme with NADPH revealed the presence of two cofactor binding sites with equal affinities for NADPH of 1.0×10^{-7} M. Cooperativity does not appear to play a role in the binding of the cofactor since the slope of the Hill plot is close to unity.

The presence of energy transfer at 440 nm indicates that succinic semialdehyde reductase contains a tryptophan residue at or near the active site. However, considering the weak signal seen for the energy transfer and the magnitude of the blue shift in the spectra, it is likely that most of these residues are located in a hydrophobic region of the protein.

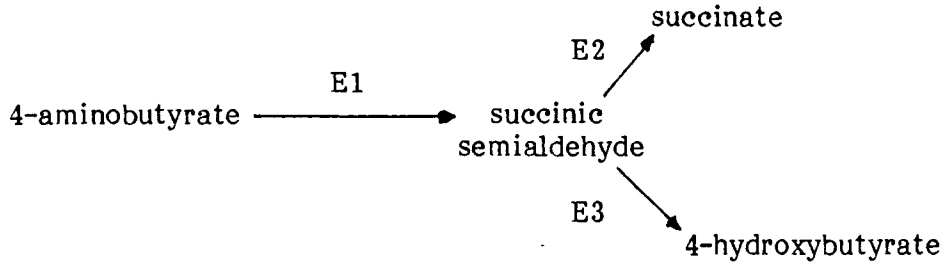
The work presented in this report demonstrates the existence of a physical interaction between two mitochondrial enzymes involved in the metabolism of 4-aminobutyrate: 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase. It also establishes the presence of a third mitochondrial enzyme related to 4-aminobutyrate metabolism, succinic semialdehyde reductase.

The role of succinic semialdehyde reductase in the mitochondria may be two-fold. The enzyme clearly can function as a 4-hydroxybutyrate synthesizing enzyme as shown by a K_{eq} of 10^5. If the enzyme exists as part of an enzyme complex which includes succinic semialdehyde dehydrogenase, then it may also act as the catabolic dehydrogenase for 4-hydroxybutyrate.

The possibility that succinic semialdehyde reductase exists as part of the 4-aminobutyrate aminotransferase - succinic semialdehyde dehydrogenase complex cannot be overlooked. The demonstration of the existence of the aminotransferase - dehydrogenase complex increases the probability that the concentration of free succinic semialdehyde is close to zero. To have the aldehyde substrate readily available for catalysis, the reductase would need to have its active center in close proximity to the active site of 4-aminobutyrate aminotransferase so that direct transfer of the intermediate between the two enzymes could occur.

Using a specific activity of 4.6 for succinic semialdehyde reductase in a mitochondrial preparation, a value of 10^{-7} M can be calculated for the concentration of the reductase in the mitochondria. Since the concentration of 4-aminobutyrate aminotransferase is about 2 μM in the mitochondria, there would be approximately 1 molecule of reductase for every 20 molecules of aminotransferase. Interestingly, this ratio is in close agreement with the observation that the flux of 4-aminobutyrate in 4-hydroxybutyrate represents about 5% of the total flow through the 4-aminobutyrate bypath (68).

The close association of the three enzymes could allow for the fine regulation of the mitochondrial pathway as shown below:



where E1 is 4-aminobutyrate aminotransferase, E2 is succinic semialdehyde dehydrogenase and E3 is succinic semialdehyde reductase. Under conditions of metabolic stress, the absence of NADPH and NADH could neatly draw from

the pools of 4-aminobutyrate and 4-hydroxybutyrate proportionately to supply the TCA cycle with succinate. If energy conditions are favorable, the pathway can be shifted toward synthesis, and the system should be sensitive enough to maintain appropriate steady state levels of the neurotransmitters. A further means of regulation of the bypath based upon the relative availability of NAD^+ and NADPH is possible. The advantage of this would be to eliminate direct competition for the same cofactor (NAD(H) and NADP(H)) and to allow important regulatory enzymes such as pyridine nucleotide transhydrogenase to participate in the control of the pathway.

The ability of enzymes in a complex to effectively lower the K_m values would add an important dimension to a mitochondrial 4-aminobutyrate shunt complex. The K_m for 4-hydroxybutyrate is on the order of 10^{-2} M, which is about 10^3 - 10^4 times the endogenous levels of 4-hydroxybutyrate in the brain. Such a complex could conceivably make the conditions of the reaction more favorable and therefore lower the K_m to a level of biological significance.

The ability of succinic semialdehyde reductase to metabolize malonic semialdehyde gives further evidence that the reductase may be closely related to 4-aminobutyrate aminotransferase. The aminotransferase can use β -alanine as a substrate almost as well as 4-aminobutyrate, but malonic semialdehyde is not a substrate for succinic semialdehyde dehydrogenase. Thus, the reductive route for malonic semialdehyde may be of some importance since the β -alanine concentration in the brain is about 10^{-5} M (4). The reduction of malonic semialdehyde yields 4-hydroxypropionate; the role of this compound in the brain is not currently known (80).

The succinic semialdehyde reductase isolated from mitochondria is likely to be the true enzyme involved in 4-hydroxybutyrate formation based upon

the coincident subcellular location of 4-aminobutyrate aminotransferase and succinic semialdehyde dehydrogenase. Cytosolic forms of the aldehyde reductase may well exist to metabolize any potentially toxic aldehydes (e.g. succinic semialdehyde) which may leak out of the mitochondria.

This research suggests a framework for the organization of the mitochondrial portion of the enzymes of the 4-aminobutyrate shunt. The primary metabolic flux through succinic semialdehyde dehydrogenase is facilitated by the formation of a multienzyme complex involving the dehydrogenase and 4-aminobutyrate aminotransferase. In addition, succinic semialdehyde reductase was shown to be an alternate path for succinic semialdehyde metabolism in the mitochondria and to offer a possible means for 4-hydroxybutyrate entry into the TCA cycle as succinate.

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