The enzyme succinic semialdehyde dehydrogenase from pig brain has been 2000-fold purified by a combination of DEAE-cellulose, hydroxyapatite, and AMP-Sepharose chromatography. This preparation has a molecular weight of 160,000 and a specific activity of 5.3 μmol/min•mg at 25°C. The inhibition of succinic semialdehyde dehydrogenase by carbonyl compounds, i.e. P-pyridoxal and o-phthalaldehyde was investigated in detail.

The enzyme is reversible, inhibited by preincubation with P-pyridoxal (mixing molar ratio, 300:1) at either 25° or 37°C. Reduction with NaBH₄ results in the incorporation of approximately 4 mol of P-pyridoxyl residues/mol of enzyme. NAD⁺ protects the enzyme against inactivation by P-pyridoxal, whereas the substrate succinic semialdehyde failed to prevent the reaction of P-pyridoxal with lysine residues of the protein. The binding of approximately 10 mol of o-phthalaldehyde/mol of enzyme results in irreversible loss of catalytic activity. The reaction is fast and easily monitored by absorption and fluorescence spectroscopy.

In recent years, the enzyme succinic semialdehyde dehydrogenase from bacteria has been isolated, and preliminary steady state studies have provided information on the mechanism of action of this enzyme (1). More recently, a convincing report on the purification to homogeneity of succinic semialdehyde dehydrogenase from rat brain has appeared (2).

In view of the importance of succinic semialdehyde dehydrogenase in the metabolism of 4-aminobutyrate, we have decided to study the reactivity of the enzyme with carbonyl compounds, i.e. P-pyridoxal and o-phthalaldehyde, in an effort to elucidate the functional role of lysine residues located at or near the active site. Since little is known about the order of substrate addition during the reaction catalyzed by succinic semialdehyde dehydrogenase, we have also undertaken a steady state kinetic investigation in order to determine the steady state parameters. All of these studies have been performed with a purified preparation of succinic semialdehyde dehydrogenase isolated from pig brain.

**EXPERIMENTAL PROCEDURES**

Succinic semialdehyde dehydrogenase from pig brain was purified according to a procedure developed by Cash et al. (2) for the purification of the enzyme from rat brain.

Pig brains were obtained from East Tennessee Packing Co. The brains were placed in ice as quickly as possible after slaughter, and preparation was begun within 1 h. Enzyme preparation procedures were carried out at 4°C.

A Waring Blender was used to prepare a 25% (w/v) homogenate in a solution of 2 mM potassium phosphate (pH 7.2), containing 1 mM β-mercaptoethanol and 0.5 mM EDTA (Buffer 1). The homogenate was centrifuged at 10,000 x g for 30 min. The precipitate was discarded and the supernatant was treated with (NH₄)₂SO₄. The precipitate obtained at 30 to 70% saturation was dissolved in Buffer 1 and dialyzed overnight against several changes of Buffer 1. It was then applied to a column (35 x 2.6 cm) of DEAE-cellulose equilibrated with the same buffer. The column was eluted with Buffer 1; the enzyme is not retained by DEAE-cellulose, whereas contaminating protein remained attached to the supporting material. The active fractions were combined and dialyzed against 2 mM potassium phosphate (pH 7.2) containing 1 mM β-mercaptoethanol. The enzyme was then applied to a column (25 x 2.6 cm) of hydroxyapatite equilibrated with the same buffer. The enzyme was eluted by using a linear gradient made with the equilibration buffer (200 ml) and the same volume of 150 mM potassium phosphate (pH 7.2) containing 1 mM β-mercaptoethanol. The enzyme is eluted at a phosphate concentration of 70 mM.

After hydroxyapatite chromatography, the enzyme was applied to a column (10 x 1 cm) of AMP-Sepharose equilibrated with 10 mM potassium phosphate (pH 7.2) containing 1 mM β-mercaptoethanol. The column was then washed with buffer and the proteins eluted by using increasing concentrations of AMP. A sharp peak containing succinic semialdehyde dehydrogenase was eluted from AMP-Sepharose at a concentration of AMP of approximately 10 mM. The purified enzyme was kept in 10 mM potassium phosphate (pH 7.2) containing 1 mM β-mercaptoethanol. Under these conditions, the enzyme remains stable for at least 1 week without appreciable loss in activity. In the absence of glycerol, the enzyme loses a substantial amount of activity after 1 day storage at -12°C. A scheme of the purification is included in Table I. The fraction of succinic semialdehyde dehydrogenase obtained after AMP-Sepharose chromatography was found to be homogeneous, as only one protein band with enzymatic activity was detected by "Gradipore" electrophoresis (3), gel filtration through Sephadex G-200 (4), and sucrose density gradient centrifugations (5).

**Enzymatic Assays**—For precise kinetic data, the formation of NADH was measured by following the increase in absorbance at 340 nm at which NADH is known to have a molar absorption coefficient of 6.22 cm²/mM. All assays were performed in duplicate and the initial velocity data were correlated with a standard assay mixture containing 50 μM succinic semialdehyde and 5 mM NAD⁺ in 0.1 M sodium pyrophosphate (pH 8.4) at 25°C.

Initial rate measurements were carried out by monitoring the change in absorbance at 340 nm for at least 2 min, good straight lines being generally obtained. The initial velocity data were fitted by a least-squares method to the Lineweaver-Burk transformation of Equation 1,

\[ v = \frac{V [S]}{K + [S]} \] (1)

where [S] represents the concentration of the varied substrate, the other substrates being present in fixed amounts. Inhibition data corresponding to linear competitive, linear uncompetitive, or linear noncompetitive inhibition were fitted to the appropriate forms of
Purification of succinic semialdehyde dehydrogenase from pig brain

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

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Volume</th>
<th>Protein</th>
<th>Specific activity at 25°C</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td>2000</td>
<td>22</td>
<td>0.0022</td>
<td>100</td>
</tr>
<tr>
<td>Supernatant</td>
<td>1350</td>
<td>15</td>
<td>0.0037</td>
<td>81</td>
</tr>
<tr>
<td>Dialyzed, 30–70% (NH₄)₂SO₄ fraction</td>
<td>127</td>
<td>11</td>
<td>0.0511</td>
<td>77</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>37</td>
<td>2.8</td>
<td>0.12</td>
<td>12</td>
</tr>
<tr>
<td>Hydroxypapatite</td>
<td>20</td>
<td>1.2</td>
<td>0.50</td>
<td>11</td>
</tr>
<tr>
<td>AMP-Sepharose</td>
<td>2.2</td>
<td>0.3</td>
<td>5.3</td>
<td>1.7</td>
</tr>
</tbody>
</table>

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

Equation 1. The nomenclature used in the analysis of kinetic data is that of Cleland (6).

Polyacrylamide Gel Electrophoresis—The enzyme preparations were examined by polyacrylamide gel electrophoresis at pH 8.3 according to the original procedure of Davis (7). Electrophoresis was conducted at 25°C in a Buchler analytical disc electrophoresis apparatus regulated at 5 mA/tube. Protein bands were detected by staining with Amido schwarz and destaining with 7% acetic acid. Electrophoresis in commercially prepared polyacrylamide gradient gels, Gradi-pores, was performed according to the procedure of Manwell (3). The five proteins used as standards in the determination of molecular weight, i.e., bovine serum albumin, ovalbumin, rabbit muscle aldolase, horse liver alcohol dehydrogenase, and beef liver catalase yield a linear relationship between a hypothetically limiting pore size and the cube root of the molecular weight.

Reaction with Carbonyl Reagents—The effect of P-pyridoxal on the enzymatic activity of succinic semialdehyde dehydrogenase was studied by preincubating the enzyme (2 μM) with P-pyridoxal in 0.01 M sodium pyrophosphate (pH 8.4), containing 1 mM β-mercaptoethanol at 37°C. Aliquots of the preincubation mixtures were removed and transferred to assay mixtures in which initial velocities of NAD⁺ reduction were obtained at 25°C. The amount of P-pyridoxyl residues bound to the enzyme, after NaBH₄ reduction, was determined by measuring the increase in absorbance at 325 nm using a molar extinction coefficient of 9710 for p-pyridoxyllysine. The number of P-pyridoxyl residues bound per mol of enzyme (160,000) was also determined by fluorescence spectroscopy using an excitation wavelength of 395 nm and emission wavelength of 390 nm.

The concentration of NAD⁺ was varied while succinic semialdehyde was held constant at the following values: 5, 8, 10, and 30 μM.

RESULTS

With succinic semialdehyde as substrate (5 × 10⁻⁵ M), the expected stoichiometric amount of NADH was produced in the presence of excess NAD⁺ (5 × 10⁻³ M), corresponding to complete oxidation of the substrate. The reaction catalyzed by succinic semialdehyde dehydrogenase is irreversible since succinic acid was unable to oxidize NADH (3 × 10⁻⁵ M) even after long periods of time at 37°C. This irreversibility of the reaction catalyzed by succinic semialdehyde dehydrogenase has been previously reported on the enzyme isolated from bacteria (1).

The steady state rate of succinic semialdehyde oxidation is sensitive to pH, and maximum initial velocity values were observed over the pH range 8 to 9.5. Therefore, most of the initial velocity studies were performed at pH 8.4, using 0.1 M pyrophosphate buffer. Double reciprocal plots of initial velocity versus NAD⁺ or succinic semialdehyde concentrations gave rise to an intersecting pattern of straight lines which are consistent either with a sequential ordered or a rapid equilibrium random mechanism for the addition of the substrates (Fig. 1).

Secondary plots of the results included in Fig. 1 yielded the various kinetic parameters included in Table II.

Product Inhibition—NADH is a strong competitive inhibitor with respect to NAD⁺ at the aldehyde concentrations tested and a noncompetitive inhibitor with respect to succinic semialdehyde at NAD⁺ concentrations of 5 × 10⁻³ M (Figs. 2 and 3).

The strong binding of NADH to the enzyme can also be demonstrated by fluorescence measurements conducted at pH 8.4 at an enzyme concentration of 3 μM. As shown in Fig. 4, the addition of NADH (3 μM) to the enzyme is accompanied by an enhancement of the fluorescence emitted by NADH over the spectral range 400 to 500 nm. In addition, a shift in the emission spectrum of bound NADH as compared to free NADH in solution is easily detected at an enzyme concentration of 3 μM.

The fluorescence emitted by the binary complex enzyme-NADH is not influenced by the addition of either succinic acid (10⁻⁴ M) or succinic semialdehyde (10⁻¹ M) to the incubation mixture. Furthermore, the addition of NAD (10⁻¹ M) to the binary complex enzyme-NADH brings about a decrease in the fluorescence emitted by NADH, a finding which is consistent with the concept that NAD binds to the enzyme at the NADH site.

Dead End Inhibitors—The compounds p-hydroxybenzaldehyde and m-hydroxybenzaldehyde are reversible inhibitors of the reaction catalyzed by succinic semialdehyde dehydrogenase. As shown in Table III, both p-hydroxybenzaldehyde and m-hydroxybenzaldehyde had been previously reported on the enzyme isolated from bacteria (1).

The concentration of NAD⁺ was varied while succinic semialdehyde concentration was held constant at the following values: 5, 8, 10, and 30 μM.

![Fig. 1. Double reciprocal plot of initial velocity with respect to NAD⁺ at fixed concentrations of succinic semialdehyde at pH 8.4. The concentration of NAD⁺ was varied while succinic semialdehyde concentration was held constant at the following values: 5, 8, 10, and 30 μM.](http://www.jbc.org/)

<table>
<thead>
<tr>
<th>Table II</th>
<th>Kinetic constants for succinic semialdehyde dehydrogenase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Substrate</td>
<td>Kᵣ</td>
</tr>
<tr>
<td>A = NAD⁺</td>
<td>31</td>
</tr>
<tr>
<td>B = succinic semialdehyde</td>
<td></td>
</tr>
</tbody>
</table>
activity (80%) (Fig. 6). Complete inactivation of the enzyme is attained when the enzyme is preincubated with excess of P-pyridoxal (mixing molar ratio, 500:1) at pH 8.2.

However, the inhibitory effect exerted by P-pyridoxal is reversed by addition of compounds which react with the Schiff

![Image](https://example.com/image1.png)

**FIG. 2.** Double reciprocal plot with NADH as the inhibitor and NAD⁺ as the variable substrate at pH 8.4. The concentration of succinic semialdehyde (SSA) is 50 µM, and the concentrations of NADH are 0 ( ○ ), 50 µM ( □ ), 100 µM ( △ ), and 200 µM ( ▽ ).

![Image](https://example.com/image2.png)

**FIG. 3.** Double reciprocal plot with NADH as the inhibitor and succinic semialdehyde as the variable substrate at pH 8.4. The concentration of NAD⁺ is 5 mM, and the concentrations of NADH are 0 ( ○ ), 50 µM ( □ ), 100 µM ( △ ), and 200 µM ( ▽ ).

and m-hydroxybenzaldehyde are competitive inhibitors with respect to the substrate succinic semialdehyde, whereas they act as noncompetitive inhibitors with respect to NAD⁺ when the concentration of succinic semialdehyde is kept constant.

**Reversible Binding of P-pyridoxal—** The enzyme succinic semialdehyde dehydrogenase is inactivated by incubation with P-pyridoxal over the pH range from 7.4 to 8.6.

The effect of the addition of P-pyridoxal on the activity of succinic semialdehyde dehydrogenase was investigated by preincubating the enzyme with increasing concentrations of P-pyridoxal at a temperature of 37°C. Aliquots of the preincubation mixtures were removed and assayed for enzymatic activity. The presence of increasing concentrations of P-pyridoxal in the preincubation mixtures have a dramatic effect on the enzymatic activity as demonstrated by the results obtained at pH 8.4 (Fig. 5). Thus, the addition of 300 mol of P-pyridoxal/mol of enzyme at pH 8.2, followed by incubation at 37°C for 30 min, resulted in a substantial loss of the catalytic activity (80%) (Fig. 6). Complete inactivation of the enzyme is attained when the enzyme is preincubated with excess of P-pyridoxal (mixing molar ratio, 500:1) at pH 8.2.

However, the inhibitory effect exerted by P-pyridoxal is reversed by addition of compounds which react with the Schiff

![Image](https://example.com/image3.png)

**FIG. 4.** Fluorescence spectra of NADH (3 µM) (1), NADH (3 µM) + enzyme (3 µM) (2), and NADH (3 µM) + enzyme (3 µM) + succinate (0.1 mM) (3), at pH 8.4, excitation wavelength 340 nm.

**TABLE III**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Varied substrate</th>
<th>Pattern</th>
<th>Kᵢ (µ M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-Hydroxybenzaldehyde</td>
<td>NAD⁺</td>
<td>Noncompetitive</td>
<td>1.4 2.3</td>
</tr>
<tr>
<td>Succinic semialdehyde</td>
<td></td>
<td>Competitive</td>
<td>11.0</td>
</tr>
<tr>
<td>m-Hydroxybenzaldehyde</td>
<td>NAD⁺</td>
<td>Noncompetitive</td>
<td>34.2 27.1</td>
</tr>
<tr>
<td>Succinic semialdehyde</td>
<td></td>
<td>Competitive</td>
<td>50.1</td>
</tr>
</tbody>
</table>

![Image](https://example.com/image4.png)

**FIG. 5.** Inhibition of succinic semialdehyde dehydrogenase by P-pyridoxal. The enzyme at a concentration of 2 µM was preincubated with increasing concentrations of P-pyridoxal in 0.1 M sodium pyrophosphate (pH 8.4) containing 1 mM β-mercaptoethanol at 37°C for 30 min. Aliquots withdrawn from the incubation mixtures were tested for enzymatic activity. Results obtained when the preincubation mixture contained NAD⁺ (5 mM) ( ■ ) and in the absence of NAD⁺ ( ● ).
base. This is shown by an experiment in which the enzyme buffer (pH 8.4). Since the binding of P-pyridoxal to the enzyme was allowed to react with aminooxyacetate 60 min at 37°C. Then, the enzyme inactivated by P-pyridoxal inhibitor, and enzymatic activity was observed when the enzyme preincubated with P-pyridoxal was passed through a Sephadex G-25 column, previously equilibrated with 0.1

Results obtained in the absence (A) and presence (0) of NAD + (5 X 10^-3 M) in the reaction mixture. The decay in enzymatic activity of a sample of enzyme preincubated at 37°C in the absence of P-pyridoxal and NAD + is given in the figure (■).

A dissociation constant of 0.1 M sodium pyrophosphate (pH 8.4) did not provide significant protection (Figs. 5 and 6).

Inactivation by o-Phthalaldehyde—Inactivation of succinic semialdehyde dehydrogenase is also attained by addition of the reagent o-phthalaldehyde. The extent of the reaction of o-phthalaldehyde with primary amino groups of the enzyme is easily determined by measuring either the increase in absorbance at 335 nm or the increase in fluorescence at 450 nm. Free o-phthalaldehyde does not absorb at 335 nm and it does not exhibit any fluorescence over the spectral range 400 to 500 nm. The time course of the reaction of the enzyme with o-phthalaldehyde is given in Fig. 7, where it may be seen that the formation of the adduct (Fig. 7) follows monophasic kinetics. Under this set of experimental conditions, the binding of o-phthalaldehyde causes an irreversible loss of catalytic activity. Experiments, designed to correlate changes in enzymatic activity with the number of o-phthalaldehyde molecules reacted, revealed that complete inactivation of succinic semialdehyde dehydrogenase is achieved after the reaction of approximately 10 mol of o-phthalaldehyde/mol of enzyme.

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

**Discussion**

The product inhibition patterns of NADH are consistent with either an Ordered Bi Bi mechanism or a rapid equilib-
Succinic Semialdehyde Dehydrogenase

The irreversible release of the acid product

**REFERENCES**


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The abbreviation used is: GABA, γ-aminobutyric acid.
Succinic semialdehyde dehydrogenase. Reactivity of lysyl residues.
W S Blaner and J Churchich


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