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 53 μmol/min·mg at 25°C. The inhibition of succinic semialdehyde dehydrogenase by carbonyl compounds, i.e. P-pyridoxal and o-pthalaldehyde 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-pthalaldehyde/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 α-amino butyrate, we have decided to study the reactivity of the enzyme with carbonyl compounds, i.e. P-pyridoxal and o-pthalaldehyde, 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 × 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 × 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 × 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 × 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 and 20% (v/v) glycerol at -12°C. 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 polyacrylamide gel electrophoresis. The enzyme has a specific activity of 5.3 units/mg of protein and a molecular weight of 160,000 as determined 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 mM 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.

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

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
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 (units/mg)</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%</td>
<td>127</td>
<td>11</td>
<td>0.051</td>
<td>77</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ fraction</td>
<td>15</td>
<td>1</td>
<td>0.003</td>
<td>22</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>37</td>
<td>2.8</td>
<td>0.12</td>
<td>12</td>
</tr>
<tr>
<td>Hydroxyapatite</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>0.53</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 were examined by polyacrylamide gel electrophoresis at pH 8.3 according to 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 were examined by polyacrylamide gel electrophoresis at pH 8.3 according to 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).

Results

With succinic semialdehyde as substrate (5 × 10⁻³ 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 NAD (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 (10⁻³ 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

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Kᵢ</th>
<th>Kᵢ × 10⁻⁵ M</th>
</tr>
</thead>
<tbody>
<tr>
<td>A = NAD⁺</td>
<td>31</td>
<td>1.1</td>
</tr>
<tr>
<td>B = succinic semialdehyde</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
activity (90%) (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

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Varied substrate</th>
<th>Pattern</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$-Hydroxybenzaldehyde</td>
<td>NAD$^+$</td>
<td>Noncompetitive</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Succinic semialdehyde</td>
<td>Competitive</td>
<td>11.0</td>
</tr>
<tr>
<td>$m$-Hydroxybenzaldehyde</td>
<td>NAD$^+$</td>
<td>Noncompetitive</td>
<td>34.2</td>
</tr>
<tr>
<td></td>
<td>Succinic semialdehyde</td>
<td>Competitive</td>
<td>50.1</td>
</tr>
</tbody>
</table>

**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 (90%) (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

**Table III**

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Varied substrate</th>
<th>Pattern</th>
<th>$K_i$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$p$-Hydroxybenzaldehyde</td>
<td>NAD$^+$</td>
<td>Noncompetitive</td>
<td>1.4</td>
</tr>
<tr>
<td></td>
<td>Succinic semialdehyde</td>
<td>Competitive</td>
<td>11.0</td>
</tr>
<tr>
<td>$m$-Hydroxybenzaldehyde</td>
<td>NAD$^+$</td>
<td>Noncompetitive</td>
<td>34.2</td>
</tr>
<tr>
<td></td>
<td>Succinic semialdehyde</td>
<td>Competitive</td>
<td>50.1</td>
</tr>
</tbody>
</table>
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 for 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 M pyrophosphate buffer (pH 8.4). 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.

![Figure 6](image6.png)

**Figure 6.** Succinic semialdehyde dehydrogenase (2 μM) was incubated with 300-fold molar excess of P-pyridoxal at 37°C (pH 8.4). Results obtained in the absence (A) and presence (B) of NAD⁺ (5 mM) 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 (B).

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-
rium Random Bi Bi. In an Ordered Bi Bi mechanism, the cofactor NAD should be the leading substrate.

Since the product of the reaction, i.e. succinic acid, does not inhibit the reaction catalyzed by the enzyme, it is virtually impossible to distinguish between the two mechanisms on the basis of the inhibition patterns obtained with NADH. However, the inhibition results obtained with m-benzaldehyde and p-benzaldehyde are consistent with a rapid equilibrium Random Bi Bi mechanism. In an Ordered Bi Bi mechanism, the dehydrogenase. The requirement for phosphate, as well as some carbonyl derivatives inhibit the enzyme by forming covalent linkages with amino acid residues. P-pyridoxal at concentrations of 0.25 mM inhibits the reaction catalyzed by succinic semialdehyde dehydrogenase by P-pyridoxal has any regulatory function, the reactivity of the carbonyl group of this coenzyme can be used conveniently to define the role played by lysine residues in the catalytic function of succinic semialdehyde dehydrogenase.

An interesting aspect of this research is the finding that some carbonyl derivatives inhibit the enzyme by forming covalent linkages with amino acid residues. P-pyridoxal at concentrations of 0.25 mM inhibits the reaction catalyzed by the dehydrogenase. The requirement for phosphate, as well as the aldehyde group of P-pyridoxal, inhibition to occur is clearly shown by the fact that neither pyridoxal nor P-pyridoxamine are effective inhibitors.

The evidence for Schiff base formation between P-pyridoxal and lysine is provided by the finding that addition of NaBH4 to the enzyme. P-pyridoxal complex results in the incorporation of P-pyridoxal residues as revealed by spectroscopic measurements. The nearly complete protection afforded by NAD+ strongly suggests that inactivation occurred due to interaction with lysine residues located at or near the cofactor binding site. In marked contrast to NAD+.

![Diagram](http://example.com/diagram.png)

**Fig. 8.** Effect of pH on the reaction catalyzed by succinic semialdehyde dehydrogenase. The irreversible release of the acid product succinic semialdehyde did not afford any protection against the inactivation by P-pyridoxal.

One might ask if the inhibitory effect of P-pyridoxal has any in vivo significance. From a physiological point of view, the storage and regulation of P-pyridoxal metabolism is dependent upon the content of multiple pyridoxal binding proteins and their individual affinities. For this coenzyme, it has been shown that in the cytosol and mitochondrial fractions of rat liver, most of P-pyridoxal is bound to three enzymes, i.e. alanine aminotransferase, aspartate aminotransferase, and phosphatase. Thus, the concentration of free P-pyridoxal is very low in the cytosol and mitochondrial fractions of liver tissues. From a thermodynamic point of view, it is well established that the enzymes of the GABA shunt, GABA transaminase and glutamate decarboxylase, bind P-pyridoxal with association constants at least 10^7 greater (11) than the association constant determined for P-pyridoxal bound to succinic semialdehyde dehydrogenase. It implies that the enzymes of the GABA shunt are saturated at concentrations of free P-pyridoxal considerably lower than 0.1 mM, the concentration required to inhibit succinic semialdehyde dehydrogenase.

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

**REFERENCES**


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


Access the most updated version of this article at http://www.jbc.org/content/254/6/1794

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/254/6/1794.full.html#ref-list-1