The metabolism of succinic semialdehyde by a psychrophilic basidiomycete
by Parker Nelson Davies

A thesis submitted to the Graduate Faculty in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE in Microbiology
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Abstract:
It has been established that succinic semialdehyde was oxidized to succinic acid by a coenzyme dependent dehydrogenase found in brain and bacterial extracts, Strobel (196?) recently reported preliminary evidence that the enzyme was present in a psychrophilic basidiomycete (strain W-2, J. B. Lebeau, Lethbridge, Alberta, Canada). This prelimini-ary evidence had not been further investigated. When a mycelial-mat was incubated at 23°C with uniformly labeled succinic semialdehyde (14C), 12.7% of the total 14C administered was recovered as 14CO2 providing evidence that succinic semialdehyde was metabolized by the fungus. Four mycelial mats equal in size were incubated with labeled succinic semialdehyde (14C). Each was extracted at a specific time and these extracts were separated into fractions using Dowex columns. Labeling in the cation, anion, neutral, and cell wall fractions was found to increase with incubation time. Investigation of the most heavily labeled fraction, the 4 hr anion fraction, showed that there were two predominantly labeled organic acid peaks. Paper chromatography and two dimensional autoradiochromatography established that the two compounds were succinic semialdehyde and succinic acid. The labeling in both of these compounds increased as time increased. When labeled succinic semialdehyde (14C) was mixed with an acetone enzyme preparation of the fungus, it was found that after the control had been subtracted 22.1% of the initial or labeled succinic semialdehyde had been converted to succinic acid.

Boiled and frozen extracts of the fungus demonstrated a 100% loss of the activity that a control demonstrated. Using the acetone preparation, a soluble succinic semialdehyde dehydrogenase was characterized as to pH optimum, coenzyme specificity, substrate specificity, and Km. Electrophoresis of an acetone preparation showed that there were three active electrophoretic forms of this enzyme. In summary it was found that succinic semialdehyde was metabolized and directed to all fractions of the fungus. Furthermore, the fungus was found to have a DPN dependent succinic semialdehyde dehydrogenase.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

Abstract

It has been established that succinic semialdehyde was oxidized to succinic acid by a coenzyme dependent dehydrogenase found in brain and bacterial extracts. Strobel (1967) recently reported preliminary evidence that the enzyme was present in a psychrophilic basidiomycete (strain W-2, J. B. Lebeau, Lethbridge, Alberta, Canada). This preliminiary evidence had not been further investigated. When a mycelial mat was incubated at 23°C with uniformly labeled succinic semialdehyde (^{14}C), 12.7% of the total ^{14}C administered was recovered as $^{14}CO_2$ providing evidence that succinic semialdehyde was metabolized by the fungus. Four mycelial mats equal in size were incubated with labeled succinic semialdehyde (^{14}C). Each was extracted at a specific time and these extracts were separated into fractions using Dowex columns. Labeling in the cation, anion, neutral, and cell wall fractions was found to increase with incubation time. Investigation of the most heavily labeled fraction, the 4 hr anion fraction, showed that there were two predominantly labeled organic acid peaks. Paper chromatography and two dimensional autoradiochromatography established that the two compounds were succinic semialdehyde and succinic acid. The labeling in both of these compounds increased as time increased. When labeled succinic semialdehyde (^{14}C) was mixed with an acetone enzyme preparation of the fungus, it was found that after the control had been subtracted 22.1% of the initial or labeled succinic semialdehyde had been converted to succinic acid. Boiled and frozen extracts of the fungus demonstrated a 100% loss of the activity that a control demonstrated. Using the acetone preparation, a soluble succinic semialdehyde dehydrogenase was characterized as to pH optimum, coenzyme specificity, substrate specificity, and K_m. Electrophoresis of an acetone preparation showed that there were three active electrophoretic forms of this enzyme. In summary it was found that succinic semialdehyde was metabolized and directed to all fractions of the fungus. Furthermore, the fungus was found to have a DPN dependent succinic semialdehyde dehydrogenase.

INTRODUCTION

Green (1942) first implicated succinic semialdehyde in intermediary metabolism. He reported that heart extracts were able to catalyze the anaerobic decarboxylation of α-ketoglutarate yielding succinic semialdehyde. Ochoa (1944) also worked on succinic semialdehyde metabolism using heart extracts. He found that aerobic decarboxylation produced a succinyl derivative. He was unable to isolate free succinic semialdehyde and concluded that only the anaerobic decarboxylation of α-ketoglutarate produced free succinic semialdehyde. Shemin and Wittenberg (1951) suggested that free succinic semialdehyde might be a precursor in protoporphyrin synthesis, but no evidence for this hypothesis has been forthcoming. Bessman, et al. (1953) using brain tissue extracts, discovered a -amino butyric acid transaminase yielding succinic semialdehyde and glutamate from γ-amino butyric acid and α-ketoglutarate. The discovery of this transamination reaction led to further work by Albers and Salvador (1958). They reported that rat brain extracts oxidized succinic semialdehyde to succinate. This reaction was dependent on the coenzyme DPN. Using cell free extracts of Pseudomonas, Scott and Jakoby (1958) found a transaminase functionally similar to that of Bessman. They also found a soluble dehydrogenase functionally similar to that of Albers and Salvador. The dehydrogenase was specific for succinic semialdehyde. The enzyme was both DPN and TPN dependent, although the TPN gave eight times more activity than DPN. Nirenberg and Jakoby (1960) characterized a DPN dependent γ-hydroxybutyric acid dehydrogenase from Pseudomonas. They also characterized two different

succinic semialdehyde dehydrogenases. One required DPN, the other TPN. Consequently succinic semialdehyde was shown to be a common product in two different pathways.

The primary results of this early work established that succinic semialdehyde was a free intermediate metabolite in several reactions. The investigators also characterized several enzymes immediately involved in the reactions of succinic semialdehyde. However, their work did not integrate these isolated reactions with functional metabolic pathways. The first attempts to integrate these isolated reactions with a biological scheme were Kretovish et al. (1966) and Strobel (1967). Kretovish, et al. demonstrated that succinic semialdehyde is metabolized in green soybean leaves and roots. This is the first work implicating succinic semialdehyde in plant metabolism. They fed succinic semialdehyde to green soybean leaves and roots and showed a marked increase in glutamine synthesis while the control leaves and roots did not show such an increase. Similar results were observed when the plants were fed γ-amino butyric acid. Marked decreases in free ammonia were noted in plants that had been fed succinic semialdehyde and γ-amino butyric acid. From these data they concluded that succinic semialdehyde and γ-amino butyric acid stimulated glutamine synthesis and are the precursors in this synthesis. Using a psychrophilic basidiomycete Strobel has shown that succinic semialdehyde is a precursor in the biosynthesis of glutamate. With labeled succinic semialdehyde (^{14}C) he found that labeled $H^{13}C^{15}N$ and ammonia react with succinic semialdehyde to form

4-amino-4-cyanobutyric acid. A nitrilase hydrolyzes the nitrile giving glutamate and ammonia. His evidence indicates that glutamate is eventually recycled to succinic semialdehyde by a decarboxylation and a deamination of glutamate. He also pointed out that succinic semialdehyde is oxidized by a DPN dependent dehydrogenase by crude extracts. Certainly one important aspect of these pathways is the oxidation of succinic semialdehyde to succinate. Hence, the purpose of this report is to present evidence for the following: 1) to confirm that succinic semialdehyde is converted to succinate in the psychrophilic basidiomycete, 2) to demonstrate some properties of this succinic semialdehyde dehydrogenase and 3) to implicate succinic semialdehyde in the general metabolism of a lower plant form.

MATERIALS AND METHODS

Culturing

The organism used for the research was a Type B strain of an unidentified psychrophilic basidiomycete supplied by J. B. Lebeau, Research Station, Canada Department of Agriculture, Lethbridge, Alberta. Mycelial mats three weeks old unless otherwise stated were used for both the ^{14}C labeling experiments and the enzyme studies. The stock culture was maintained on Potato Dextrose Agar at 10°C.

Materials

The labeled succinic semialdehyde was prepared from uniformly labeled glutamate (^{14}C) according to the method of Arnoff (1956). The labeled succinic semialdehyde was separated from the other soluble compounds in the mixture by chromatographing on Whatman #1 in n-butanol:acetic acid:water (4:1:5). The compound was then eluted from the paper with distilled water. The eluate was placed on a 1 x 3 cm Dowex 1 Column (formate form), 200-400 mesh, and rinsed with distilled water to remove contaminating cations. The succinic semialdehyde was eluted with 20 ml of 6N formic acid, dried, and stored in a vacuum desiccator. All other chemicals used were reagent grade.

General Methods

Protein was quantitatively determined by the method of Lowry, et al. (1951). All colorimeter measurements were made on a Bausch and Lomb Spectronic 20 Colorimeter.

Radioactivity Determinations

Radioactive samples were counted in a Nuclear Chicago Liquid Scintillation Counter, Model 6804. The solvent used in each counting vial consisted of 1.5 ml methanol and 13.5 ml of toluene containing 4.0 g of 2,5-diphenyloxayole and 100 mg of p-bis-2(5-phenyloxayolyl)-benzene per liter. The radioactive areas on the chromatogram were located by a Packard Radiochromatogram Strip Counter. After location these radioactive areas were cut out, shredded, and placed into a vial and counted. For all cases the counts were converted to dpms by the quench correction method using a standard curve.

Chromatography

Sheets of Whatman #1 and #541 were used for paper chromatography. The following solvent systems were used:

1) n-butanol-acetic acid-H_2O (4:1:5)
2) n-pentanol-5N formic acid (1:1)
3) ethanol-NH_4OH-H_2O (80:4:16)

The organic acid spots were located on the chromatograms by an acid-base indicator (Arnoff, 1956). The sugars were located using ammoniacal silver nitrate (Trevelyan et al., 1950). The amino acids were detected by spraying the chromatogram with 0.3% ninhydrin in 95% ethanol.

Administration of Succinic-Semialdehyde ^{14}C to Cultures

Mycelial mats equal in size were rinsed in sterilized distilled water. These mats were aseptically transferred to a 250 ml Erlenmeyer

flask. Uniformly labeled succinic semialdehyde (0.5 μc) was added to the flask which was sealed with a sterile plug and incubated at room temperature for ½, 1 and 4 hrs. Each mat was ground in a Sorvall Ommimixer at 16,000 rpm for one min. The homogenate was centrifuged at 14,000 x g for ten min to remove the precipitate. An equal volume of ethanol and water (2:1) was added to the supernatant liquid. The precipitate was removed by centrifugation at 20,000 x g for ten min. This supernate was then passed through a column of Dowex 50 (H^+ form, 2 x 3 cm) and then through a column of Dowex 1 (formate form, 2 x 3 cm). Ten ml of 6N HCl and 6N formic acid was added to the Dowex 50 and Dowex 1 columns, respectively, to remove the anion and cation fractions. These two fractions plus the neutral fractions were dried by flash evaporation and stored in an evacuated desiccator.

Conversion of Succinic-Semialdehyde ^{14}C to $^{14}CO_2$

A mycelial mat was rinsed in sterile distilled H_2O and was transferred to an altered 250 ml Erlenmeyer having a center well containing one ml of hyamine hydroxide. Uniformly labeled succinic-semialdehyde ^{14}C, .016 μc, was added to the flask which was sealed with a sterile plug and incubated at 23°C. At specific time intervals the contents of the center well were removed and counted. An equal volume of fresh hyamine hydroxide was added, and the flask was resealed.

Succinic Semialdehyde Dehydrogenase Studies

Assay of Succinic Semialdehyde Dehydrogenase

Since the assay substrate succinic semialdehyde reduces DPN the activity of the enzyme was followed by measuring the increase in absorbance at 340 mμ in a Beckman D.U. Spectrophotometer with a 1 cm light path. The assay system consisted of 45 μmoles phosphate buffer, pH 8.5; 3 μmoles DPN, 3 μmoles mercaptoethanol, 60 μmoles succinic semialdehyde and .2 ml of enzyme preparation. A unit of activity is defined as a mμmole of substrate converted per min. Specific activity is defined as units per mg protein.

Preparation of Succinic Semialdehyde Dehydrogenase

Five mycelial mats were collected, drained, and washed in 0.05M phosphate buffer, pH 7.0 at 4°C. The mats were ground in a prechilled Servall Omnimixer for 30 seconds. The homogenate was centrifuged at 14,000 x g for ten min. The precipitate was discarded and acetone at -15°C was slowly added to the remaining supernate up to an equal volume. The precipitate was removed by centrifugation at 28,000 x g for ten min and was taken up in 5.0 ml of 0.05M phosphate buffer, pH 7.0, that was reduced with mercaptoethanol to protect possible labile sulphide bonds. The precipitate was removed by centrifugation at 25,000 x g for ten min.

Electrophoresis of Succinic Semialdehyde Dehydrogenase

One and six-tenths mg of protein was subjected to disc gel electrophoresis according to the method of Ornstein and Davis (1964). The protein was submitted to electrophoresis at 2.5 milliamps per gel until the initial boundary was one inch into the small pore gel. One gel was treated with a mixture of nitroblue tetrazolium in order to locate succinic semialdehyde dehydrogenase activity. A second gel was stained with aniline blue black to detect protein. These two gels were scanned in a Joyce Chromoscan Densitometer.

EXPERIMENTAL RESULTS

Demonstration of Succinic Semialdehyde Metabolism

Collection of $^{14}CO_2$

Uniformly labeled ^{14}C (35,000 dpms) of succinic semialdehyde was fed to a fungal mat to determine if it is metabolized. CO_2 was collected at specific time intervals and counted. After incubation for 48 hrs at 23°C at least 12.7% of the original ^{14}C in succinic semialdehyde was collected as $^{14}CO_2$. $^{14}CO_2$ production was immediate in that 41.1% of the total $^{14}CO_2$ collected was given off by the 6th hr of incubation (Fig. 1).

Distribution of Labeling in Four Fractions

Uniformly labeled ^{14}C (1.1×10^6 dpms) succinic semialdehyde was fed to 4 fungal mats of equal size in order to determine the distribution of labeling. At specific times each mat was extracted and fractioned as previously described. The cation, anion, neutral, and cell wall fractions of the mycelia were examined for radioactivity. The results show that as incubation time increased, there was a concurrent increase of labeling in all four fractions (Table I).

Examination of the Organic Acid Fraction

The four hour organic acid fraction was separated by paper chromatography in solvent 3 and scanned to locate the radioactivity. Although several peaks of radioactivity were observed, two predominated (Fig. 2).

Figure 1 Conversion of succinic semialdehyde ^{14}C to $^{14}CO_2$.

Uniformly labeled ^{14}C (35,000 dpm) was fed a fungal mat and CO_2 collected in a center well containing 1 ml of hyamine hydroxide.

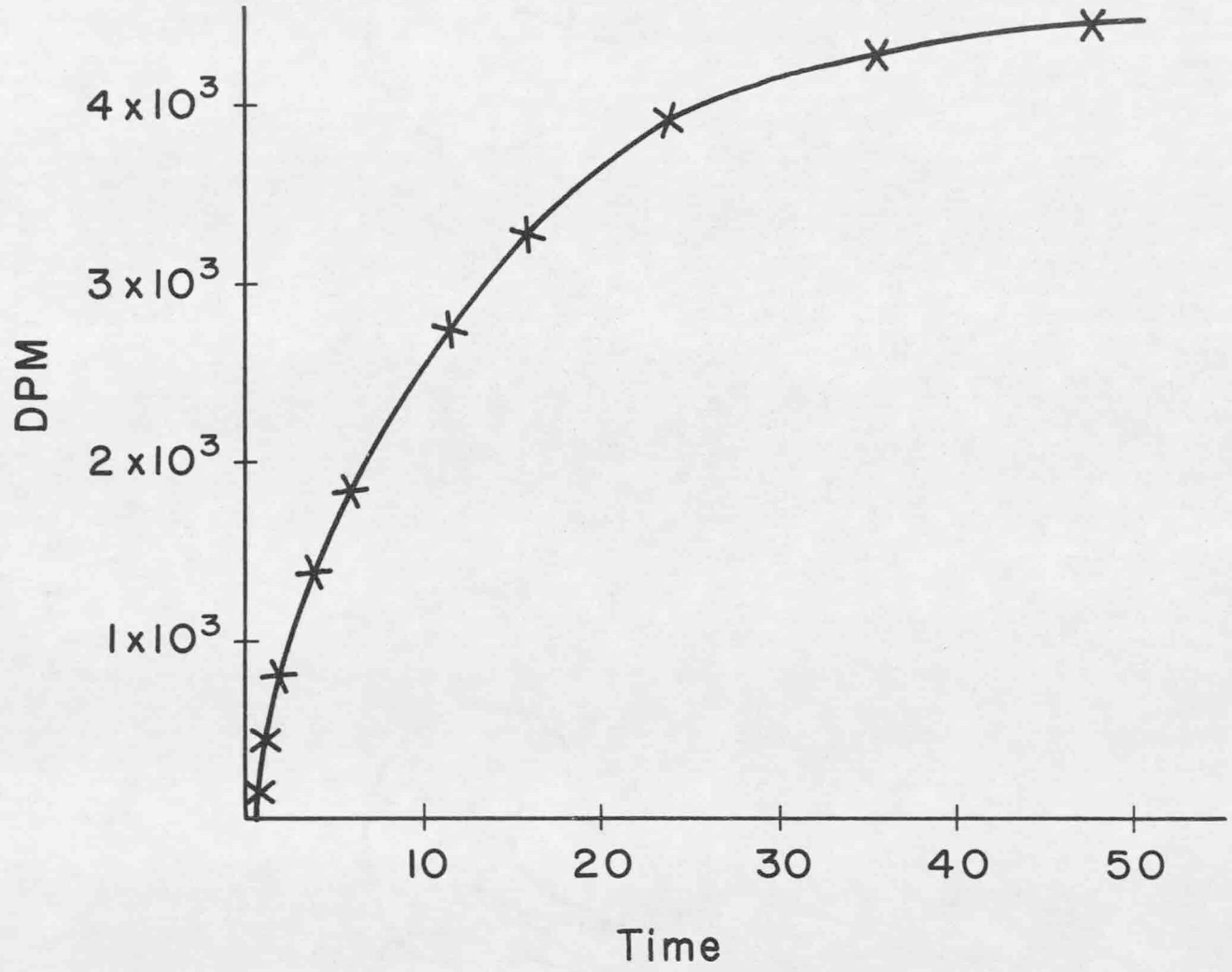
4 x 10^3
3 x 10^3
2 x 10^3
1 x 10^3
DPM
10
20
30
40
50
Time

Table I

Distribution of Labeling in the Basidiomycete

After the Administration of Succinic Semialdehyde (^{14}C).

Time	Cation	Anion	Neutral	Cell Wall
½ hr	12,220	43,700	800	38.4 $\frac{dpm}{mg}$
1 hr	26,400	52,800	6,400	20.4
2 hr	12,600	59,540	12,600	21.5
4 hr	15,340	80,660	34,600	43.0

Figure 2 Radiochromatoscan of the 4 hr anion fraction from the feeding experiment.

Radioactive material from the 4 hr anion fraction (Experimental Results) was chromatographed on Whatman #1 in solvent system 3. The radioactive areas on the paper were located and recorded by a Packard Radiochromatogram Strip Counter.