The Enzymes of Carbohydrate Metabolism in *Neurospora*

1. SUCCINIC DEHYDROGENASE

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As a preliminary to combined genetical and biochemical investigations of carbohydrate metabolism in *Neurospora*, it was deemed essential to isolate and study the enzyme systems involved.

There is no information regarding the mechanisms involved in carbohydrate oxidation in *Neurospora*, though there is some indirect evidence for the occurrence of certain enzymic reactions. Ryan, Tatum & Giese (1944) have shown that succinic, fumaric and malic acids can all reverse the inhibition of growth and respiration of the mould caused by iodoacetic acid. Succinic acid could not be used as an energy source, for the mould would not grow on it in the absence of sugar. They concluded that there is a strong suggestion that the action of the C_4 acids is catalytic. Lewis (1948) described *Neurospora* mutants with a genetic block occurring in the carbohydrate metabolism of the mould, the block being postulated to occur in the tricarboxylic acid cycle.

In other fungi, Tausson (1941) concludes that the C_4 dicarboxylic acids play an important part in the synthetic processes of the cell, and Foster & Davies (1949) showed that air-dried mycelium of *Rhizopus nigricans* can oxidize succinate readily at neutral pH values.

The enzyme succinic dehydrogenase is widely distributed in nature, having been found in bacteria, moulds, insects and most vertebrate tissues (Elliott, 1941). In the present paper it is shown that it occurs in *Neurospora* and some of the properties of the enzyme are described.

EXPERIMENTAL

Organisms and medium. The only wild type strain of *Neurospora crassa* used was Beadle 25a. In addition, the 'succiniless' mutants 55901, 39311, 47807, 66702 and 35402, which have been described by Lewis (1948), have also been investigated for succinic dehydrogenase.

The medium used was: 2.0 g. KNO_3 , 1.0 g. KH_2PO_4 , 0.5 g. $MgSO_4 \cdot 7H_2O$, 10.0 g. glucose, 4 μ g. D-biotin, 10 μ g. boron, 100 μ g. copper, 200 μ g. iron, 20 μ g. manganese, 30 μ g. molybdenum, 2 mg. zinc, 1000 ml. water. The pH was adjusted to 5.8 with KOH before autoclaving. Stock material for inoculations was grown on slopes of the above

medium solidified with 1.5% agar. The medium for the mutants had, in addition, 1.0 g. sodium succinate/l.

In all cases, incubation was for 72 hr. at 25°.

Chemicals. All reagents used were 'Analar' grade. Cytochrome c was prepared by the method of Keilin & Hartree (1945) and standardized spectrophotometrically. Sodium succinate (British Drug Houses Ltd.) was purified by the method of Potter & Schneider (1942).

Measurement of enzyme activity. Enzyme activity was measured by the standard Warburg and Thunberg techniques, at 37° unless otherwise stated, and expressed as Q_{O_2} (μ l. O_2 uptake/mg. dry wt./hr.). The total reaction mixture volume used was always 3.2 ml. In the Warburg technique KOH was present in the centre cup and succinic acid, as the neutral Na salt, was added after equilibration.

Enzyme preparations. For experiments on intact mycelium, the mould was grown in a deep aerated culture apparatus, in which the mould produced very numerous short hyphae, instead of a mycelial mat. The culture could then be used as a cell suspension, analogous to a yeast or bacterial suspension.

For cell-free enzyme preparations, the mycelium was grown in Roux bottles, 120 ml. medium being used in each Roux bottle. An inoculum of about 10^6 conidia suspended in 1.0 ml. water was used for each Roux bottle. Cell-free preparations were obtained using a modification of the method of Umbreit, Wood & Gunsalus (1946). The mycelium was filtered from the culture medium and washed with 25 ml. water/g. wet weight of mycelium. It was then pressed to remove surplus moisture, frozen in celluloid tubes in liquid air for 30 min. and finally homogenized in 0.1 M-phosphate buffer (pH 7.4) for 2.5 min., at 0°, in a Waring blender. For each gram of mycelium, 10 ml. buffer was used. The solid fraction of the blend was then spun down by centrifugation at 2400 g for 10 min., washed twice with glass-distilled water and then resuspended in 0.1 M-phosphate buffer, pH 6.74.

Effect of variables during the enzyme preparation. It was found that the method described above gave the most active enzyme preparations. Omission of the preliminary washing of the mycelium led to inactive preparations, presumably due to traces of heavy metals derived from the culture medium being present. Delay between washing and blending the cell-free preparation also led to inactivity. Blending between pH values of 7 and 8 gave the most active preparations, higher and lower pH values resulting in inactivity.

Freezing in liquid air (or in solid CO_2 and acetone) was found essential for optimum activity, yielding preparations with a Q_{O_2} of 10–15. Blending at 0° gave preparations with a Q_{O_2} of only 4–8, while blending at any higher temperature gave totally inactive preparations.

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The most active preparations were obtained from growing cultures, the activity falling off rapidly as soon as autolysis of the mycelium starts. The age of the conidial inoculum was found to have no effect on the enzymic activity of the mycelium produced.

Air-dried and freeze-dried mycelia had negligible succinic dehydrogenase activity. Fresh, intact mycelium had negligible enzyme activity, but a very large respiration blank Q_{O_2} of 5–15. With cell-free enzyme preparations, two washings in distilled water were found adequate to reduce the blank Q_{O_2} to a zero value.

Fig. 1. Effect of pH on enzyme stability. A, pH 7.5; B, pH 6.8; C, pH 8.3. Main flask contained: 0.4 ml. 4×10^{-4} M-cytochrome *c*, 2.5 ml. enzyme suspension in phosphate buffer of appropriate pH; 0.2 ml. sodium succinate solution (final concn. 0.005M) in the side bulb; 0.1 ml. 20% (w/v) NaOH in the centre well. For the Thunberg experiments, 2.0 ml. enzyme in buffer of the appropriate pH, 0.2 ml. of 0.1M-sodium succinate, and 1.0 ml. of 0.001% methylene blue.

All the enzymic activity was found to be associated with the insoluble cellular constituents and, although numerous attempts were made, it was found impossible to increase the activity of the enzyme any further.

The pH of the suspending buffer was shown to have considerable effect on the enzyme stability. The results of this study are shown in Fig. 1.

Enzyme preparations were stored at 0° until they were required for use; they were then used immediately.

RESULTS

Dependence of activity on pH. The activity of succinic dehydrogenase and succinoxidase was

measured at different pH values and in each case there was a double optimum at 6.9 and 8.1 (Fig. 2).

Phosphate buffer was used for the range pH 6–8 and glycine buffer for the range 8.2–10.1. Borate and veronal buffers were found to be inhibitory. All the buffers were used in 0.1M-concentration and the pH was checked at the termination of each experiment, using a Cambridge glass-electrode pH meter.

The optimum activity of the enzyme present in muscle and bacteria occurs according to Ohlsson (1921) and Cook & Alcock (1931) at about pH 9.0, while the optimum for succinoxidase activity in rat liver is reported to be at pH 7.5–7.7 (Schneider & Potter, 1943).

Fig. 2. Effect of pH on enzyme activity. A, Q_{O_2} of succinic dehydrogenase + carrier system; B, reduction of methylene blue by succinic dehydrogenase preparation. Experimental details as for Fig. 1.

Substrate concentration and the quantitative nature of the oxidation. The effect of concentration of succinic acid from 0 to 0.012M was investigated. The Michaelis constant was determined as 0.0015M (Fig. 3).

The oxidation was found to be quantitative over the range of concentrations tested (Fig. 4).

Activators and hydrogen carriers. It has been reported that the succinoxidase system is activated by calcium ions (Axelrod, Swingle & Elvehjem, 1941) and also by rare earths (Horecker, Stotz & Hogness, 1939). It was found that there was no activation of the succinoxidase system of *Neurospora* with chromium, calcium, aluminium, molybdenum, manganese or magnesium ions in a concentration of 5×10^{-4} M.

A cytochrome system has not yet been demonstrated to occur in *Neurospora*, but the effect of various hydrogen carriers on the oxidation of succinate (Table 1) showed that cytochrome *c* was by far the most efficient of the hydrogen carriers tried.

Inhibitors. The results of the inhibitor experiments are shown in Table 2. It was shown by the method of Lineweaver & Burke (1934) that the inhibition by malonic acid was competitive.

It was found that 0.002M-hydrocyanic acid completely inhibited the succinoxidase system.

Isolation of the reaction product. The reaction product of succinate oxidation was expected to be fumarate. It was successfully isolated by a method based on those of Hahn & Haarmann (1927) and of Stotz (1937).

Fig. 3. Effect of substrate concentration. Warburg flasks contained: 2.5 ml. enzyme in 0.1M-phosphate buffer, pH 6.7; 0.4 ml. 4×10^{-4} M-cytochrome c; 0.2 ml. sodium succinate solution of the appropriate molarity in the side bulb, and 0.1 ml. 20% (w/v) NaOH in the centre well. The Michaelis constant (K_m) was calculated to be 0.0015M.

Fig. 4. Quantitative nature of the oxidation of succinic acid by the succinic dehydrogenase + carrier system of *N. crassa*. Warburg technique. The main flask contained 2.5 ml. enzyme suspended in 0.1M-phosphate buffer (pH 6.9), 0.4 ml. of 4×10^{-4} M-cytochrome c. The centre well contained 0.1 ml. 20% (w/v) NaOH and the side bulb contained various amounts of 0.1M-sodium succinate solution.

About 40 g. of wet mycelium were frozen, blended, washed twice with water and suspended in 30 ml. of 0.1M-phosphate buffer (pH 6.9) in a 250 ml. Erlenmeyer flask, 30 ml. of 0.2M-succinic acid (neutralized to pH 6.9 with sodium hydroxide), together with 5.0 ml. of 4×10^{-4} M-cytochrome c were added and the whole incubated at 37° for 2 hr. The solid fraction was removed on a sintered-glass funnel. The filtrate was deproteinized by adding 10 ml. of 25% (w/v) trichloroacetic acid and heating to 100° for 10 min. The precipitate was spun down and washed

with 10 ml. warm ether, the washings being added to the supernatant liquid. One drop of methyl red indicator was added and boiling saturated barium hydroxide, until a permanent yellow colour resulted.

Table 1. *The effect of hydrogen carriers on a washed succinic dehydrogenase preparation*

(Warburg flasks contained 2.5 ml. enzyme in 0.1M-phosphate buffer, pH 6.9; 0.2 ml. 0.075M-succinate; 0.1 ml. 20% (w/v) NaOH in centre well; 0.4 ml. carrier. Run at 37°. Equilibrated 10 min. before tipping substrate from side arm.)

Carrier	Final concn.	Percentage increase in Q_{O_2} over control
None	—	0
Brilliant cresyl blue	0.03%	4
Methylene blue	0.04%	10
Cytochrome c	1.28×10^{-4} M	28
Cytochrome c	1.34×10^{-4} M	26
Cytochrome c	2.57×10^{-4} M	39
Cytochrome c	4.14×10^{-4} M	62

Table 2. *Effect of various inhibitors on the succinic dehydrogenase of Neurospora*

(Thunberg technique: 2.0 ml. enzyme in 0.1M-phosphate buffer, pH 6.9; 0.2 ml. 0.1M-sodium succinate; 1.0 ml. 0.1% methylene blue, 0.5 ml. inhibitor. 25°. Tube evacuated, filled with N_2 and re-evacuated.)

Inhibitor	Molar concn. of inhibitor	Inhibition (%)
HCN	0.002	0
Iodoacetic acid	0.01	22
Sodium pyrophosphate	0.02	78
Malonic acid	0.0025	100
Malonic acid	0.00125	69
Sodium fluoride	0.01	0
Sodium fluoride	0.06	40
Oxaloacetic acid	0.01	100
Copper (Cu^{++})	1×10^{-6}	100
Sodium borate	0.01	100
Oxidized glutathione	0.01	100

Then 20 ml. of ethanol were added and the solution left for 30 min. The precipitate was centrifuged down and dissolved in 12 ml. of 0.55N-nitric acid. This was heated on a water bath to 85° and 10 ml. of a mixture of 10% mercurous nitrate in 0.55N-nitric acid was added. The solution was cooled, kept at 5° for 3 hr. and finally centrifuged. The precipitate was stirred with 5 ml. of 1.65N-nitric acid and heated to 100° for 5 min. on a water bath. 10 ml. water and 2.5 ml. mercurous nitrate-nitric acid reagent were added, the solution cooled, and stored overnight at 5°. The precipitate was centrifuged off and washed in turn with 12 ml. of 1% mercurous nitrate in 5% nitric acid and two 10 ml. portions of 0.2N-nitric acid. The product was dried in a vacuum desiccator over sulphuric acid. The yield was 0.51 g. = 74% theory mercurous fumarate.

The product, assumed to be mercurous fumarate, was suspended in 10 ml. warm water and a fast stream of hydrogen sulphide bubbled through it. The mercuric sulphide was centrifuged off, the supernatant liquid filtered to remove traces of mercuric sulphide, and evaporated to dryness. The residue was recrystallized from hot water. The yield was 0.107 g. = 57 % theory fumaric acid. The melting point (closed tube) of the product was 285°, that of fumaric acid being 287°. A mixed melting point (closed tube) was 285°.

The product (0.100 g.) was then refluxed for 30 min. with 1.0 ml. of thionyl chloride. Then 1.5 ml. aniline and 25 ml. benzene were added and the mixture refluxed gently for a further 15 min. The benzene was distilled off and the residue dissolved in 4.0 ml. ethanol and 2.0 ml. water with the aid of heat and left at 5° to crystallize. The crystals were filtered off and recrystallized from 5.0 ml. hot ethanol, 4.0 ml. water were added to the hot solution, which was left at 5° to crystallize. The yield of product was 0.212 g. = 82.5 % theory fumaryl bis-anilide. The melting point of the product was 310°, that of fumaryl bis-anilide being 314°. A mixed melting point was 309°.

From the yield of fumaric acid obtained, it was concluded that the preparation did not contain appreciable fumarase activity. However, it has been shown by subsequent work to be published by the author, that the mould does contain an active fumarase, as well as malic dehydrogenase, aspartase and aconitase.

Occurrence of the enzyme in the 'succinicless' mutants. Succinic dehydrogenase activity was found in the mutants, 55901, 39311, 47807, 66702 and 35402, described by Lewis (1948), the activity of the enzyme from the mutants being comparable with that from the wild type.

DISCUSSION

The enzyme succinic dehydrogenase has now been shown to occur in *Neurospora crassa* (Beadle 25a)

and in five 'succinicless' mutants. The enzyme is similar in properties to the succinic dehydrogenase obtained from vertebrates, except in its double pH optimum and its greater liability to inactivation during preparation. It is also somewhat more sensitive to inhibition by various agents, but the inhibitions are of the same magnitude as with the vertebrate enzyme (Table 2).

Although cytochrome *c* has been shown to act as an efficient hydrogen carrier for the cell-free enzyme, there is as yet no evidence that the cytochrome system acts as the natural carrier system in the mould.

The activity of the cell-free preparations is low, but it is comparable with that of brain and lung (Elliott & Greig, 1938). The intact mycelium has negligible succinoxidase activity; this has also been shown in the case of *Rhizopus nigricans* by Foster & Davies (1949) and is attributed by them to the fact that succinic acid will not enter the cell as the dissociated molecule.

SUMMARY

1. Succinic dehydrogenase is present in the mycelium of *Neurospora crassa* (Beadle 25a) and in the 'succinicless' mutants 55901, 39311, 47807, 66702 and 35402.

2. The enzyme has been prepared in a cell-free state.

3. The enzyme has pH optima at 6.9 and 8.1 and a Michaelis constant of 0.0015M. It is inhibited by malonic acid, oxaloacetic acid, copper ions, borate, oxidized glutathione and sodium pyrophosphate.

4. Cytochrome *c* acts as an efficient hydrogen carrier for the enzyme.

5. The reaction product, fumaric acid, has been isolated and characterized.

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