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# The activity of succinic dehydrogenase and indole acetic acid oxidase in cell-free preparations of Hygrophorous conicus

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Department:

# THE ACTIVITY OF SUCCINIC DEHYDROGENASE AND INDOLE ACETIC ACID OXIDASE IN CELL-FREE PREPARATIONS OF HYGROPHOROUS CONICUS

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## ABSTRACT

The enzyme indole acetic acid oxidase is produced by the fungus Hygrophorous conicus when it is grown in the presence of indole acetic acid (IAA) or tryptamine. The IAA acts both as an inducer and a substrate for this intracellular enzyme. The product of the oxidation catalyzed by the IAA oxidase is oxindole acetic acid (OIAA).

The rate of growth and the rate of formation of the enzyme IAA oxidase by H.conicus were studied in different media. Of these, a nitrate medium containing 0.5 per cent Yeast extract (Y.E.) was found to be the best for the production of H.conicus mycelium. A maltose medium was better than the other media tested so far as the growth of H.conicus and the formation of the enzyme IAA oxidase were concerned. The addition of 0.5 per cent Y.E. to any of the media tested suppressed the formation of the enzyme IAA oxidase of H.conicus.

The Krebs cycle enzyme succinic dehydrogenase was found in the particulate fraction, sedimented at 24,000 x g for 20 minutes from a cell-free preparation of H.conicus.

The use of sonic oscillations for 10 minutes at 4-5°C was found to solubilize, to some extent, the enzyme succinic dehydrogenase of H.conicus.

The phenazine methosulfate method for the assay of succinic dehydrogenase activity gave higher $QO_2(N)$ values than the cytochrome c method.

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# LIST OF ABBREVIATIONS

Abbreviations used are:

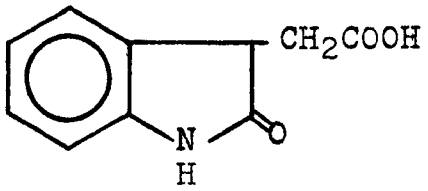
| | |
|---|---|
| cyt. c | Cytochrome c (from horse) |
| IAA | Indole acetic acid |
| NAD | Nicotinamide adenine dinucleotide |
| NADH | Reduced form of nicotinamide adenine dinucleotide |
| OIAA | Oxindole acetic acid |
| PMS | Phenazine methosulfate |
| Tris | Tris (hydroxymethyl) aminomethane |
| Y.E. | Yeast extract |

# I. INTRODUCTION

The Krebs cycle is widely distributed in nature and occurs in the respiring tissues of animals, higher plants, and microorganisms. In living organisms this cycle has considerable significance from the standpoint of energy production.

Succinic dehydrogenase, an important member of the Krebs cycle enzymes, has been found in many plants and fungi. The product of the dehydrogenation of succinic acid is fumaric acid. The enzyme succinic dehydrogenase is thought to be present mainly in the mitochondria.

The indole acetic acid oxidase of Hygrophorous conicus, a member of a class of fungi known as basidiomycetes, is an inducible, intracellular enzyme. The product of the oxidation of indole acetic acid by H.conicus is oxindole acetic acid.




Oxindole Acetic Acid

The objectives of this investigation were as follows:

(a) to ascertain the presence of a Krebs cycle enzyme, in cells of H.conicus;

(b) to localize this enzyme in a subcellular structure of the cell, namely the mitochondria;

(c) to obtain a functional mitochondrial preparation from H.conicus;

(d) to study the activity of the inducible, intracellular enzyme indole acetic acid oxidase in the cells of H.conicus;

(e) to study sonification as a method of rupturing cells in order to obtain intact mitochondria.

# II. LITERATURE REVIEW

## A. PREPARATION OF A CELL-FREE ENZYME

### 1. Rupturing of Cells.

In order to study an enzyme it is desirable to free it from the cells of the organism. With an intracellular enzyme this involves the rupturing of the cell walls. Several methods of liberating enzymes from organisms have been used, and all facilitate the removal of the enzyme by alteration of the cell wall or the cell membrane (Seaman, 1963).

Although several mechanical methods of cell rupturing have been devised, two procedures have been widely used (Salton, 1964); grinding the cells with either powdered glass, alumina, or acid washed sand, and breaking the cell structure by ultrasonic oscillations. Sonic treatment of the cells has proven successful in breaking ordinarily difficult-to-rupture-cells.

Quick drying of the cells from the frozen state can also result in sufficient alteration of the cell membrane to enable the easy extraction of the enzymes (Seaman, 1963). However, an oxygen sensitive enzyme can be inactivated during freeze-drying. For example, the viability of the organism Escherichia coli was lost when it was freeze-dried from distilled water (Lion and Bergmann, 1961). The harmful effect of air could be reduced if certain protective substances were added before the freeze-drying.

Three groups of protective substances were found to be effective. These were thiourea and some of its derivatives, sugars, and some simple inorganic salts.

A cardinal rule for handling all enzyme extracts, no matter which method of preparation is used, is to keep the material at low temperatures, 0-4°C, during all manipulations (Seaman, 1963).

2. Separation of Cellular Components.

The fungal cell contains the nucleus, the mitochondria or large granules, the microsomes, and the endoplasmic reticula. With advances in high speed centrifugation, it had been found that the cell fractions can be separated readily by centrifugation; the nuclei and cell debris at centrifugal speeds of 1000 x g or less; the mitochondria at 5,000 to 20,000 x g; and the microsomes at 25,000 to 50,000 x g (Cheldelin, 1961).

Cheldelin (1961) reported that the size of the cellular components in liver and the conditions for their separation are as follows:

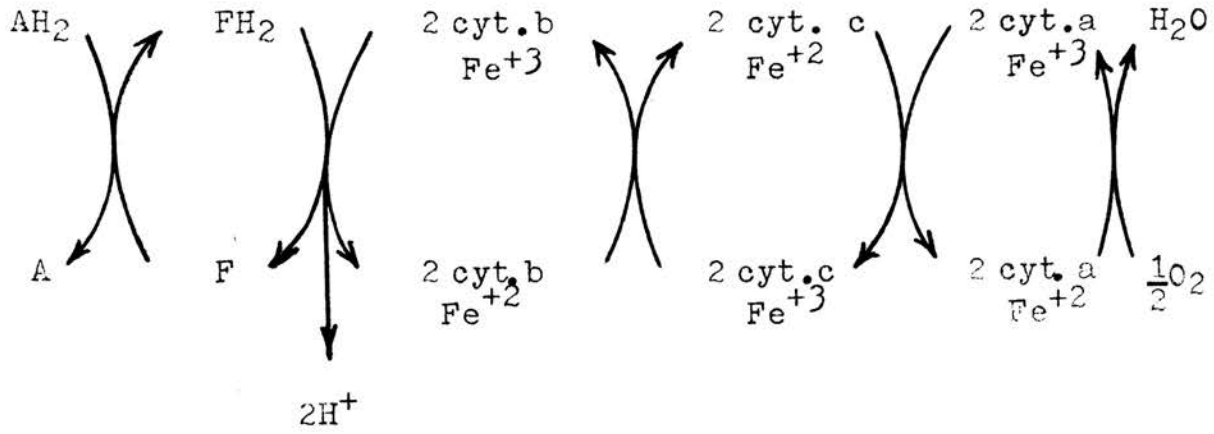
| | | Sedimentation | |
|---|---|---|---|
| Fraction | Diameter μ | Time minutes | Acceleration |
| nuclei | 50-100 | 10 | 600 x g |
| mitochondria | 1-3 | 20 | 24,000 x g |
| microsomes | 0.06- 0.15 | 120 | 41,000 x g |

B. MITOCHONDRIA

The mitochondria or large granules as these are called, are smaller than the nucleus, yet visible under an ordinary high power microscope; they can be separated from ruptured cells by centrifugation at 5,000-20,000 x g (Cheldelin, 1961).

The Krebs cycle enzymes are located in the mitochondria and are closely associated with the electron transport system and oxidative phosphorylation (Conn and Stumpf, 1963a).

It is not yet possible to specify completely the nature of the electron carriers and catalytic proteins involved in the aerobic oxidation of succinic acid. It has been assumed that the sequence of electron transfer involves a flavin system and cytochromes b, c, a, and $a_3$. The pathway of electrons from a succinate ($AH_2$) to $O_2$ may be represented as:




where the oxidized flavin is denoted as F (Fruton and Simmonds, 1961).

C. KREBS CYCLE

Krebs in 1937 (Conn and Stumpf, 1963b) proposed a cycle for the oxidation of pyruvic acid to $CO_2$ and $H_2O$ which is known as Krebs cycle, TCA cycle, or the citric acid cycle. The Krebs cycle is widely distributed in nature and occurs in the respiring tissues of animals, higher plants, and microorganisms. In the living organism this cycle has considerable significance from the standpoint of energy production. The enzymes which are present in this cycle are the condensing enzyme, aconitase, isocitric dehydrogenase, $\alpha$-ketoglutaric oxidase, succinic thiokinase, succinic dehydrogenase, fumarase, and malic dehydrogenase.

As a result of experiments with isolated mitochondria, the concept has arisen that the enzymes of the Krebs cycle are localized mainly in mitochondria, and are closely associated with the electron transport system and with oxidative phosphorylation (Conn and Stumpf, 1963b).

The events in the operation of the Krebs cycle in fungi are similar to those in other biological systems (Schneider and Hogeboom, 1956).

Various workers (McDonald et al, 1963; Wessels, 1959) have shown the presence of some or all of the individual enzymes of the Krebs cycle in fungi, but relatively little work has been done on the isolation of functionally active subcellular particles from fungi. The operation of the cycle in fungi is still the subject of much investigation.

Wessels (1959) has demonstrated the operation of the Krebs cycle in the breakdown of carbohydrate by Schizophyllum commune, a member of the class of higher fungi known as basidiomycetes.

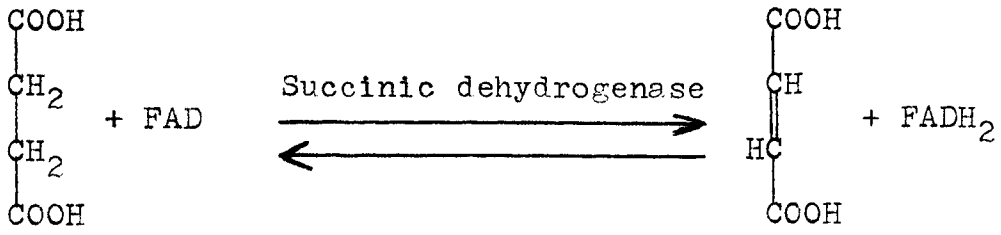
Niederpruem and Hackett (1961) studied the respiratory system in Schizophyllum commune. They found that particles isolated from S.commune contained a typical respiratory chain which could transfer electrons from NADH to molecular oxygen via flavoprotein and the cytochrome system. Experiments carried out with both the intact vegetative mycelium and cell-free extracts indicated that respiration was mediated by a classical cytochrome-cytochrome oxidase system (Fruton and Simmonds, 1961).

## D. SUCCINIC DEHYDROGENASE

### 1. General.

In the early studies on the dehydrogenation of metabolites by tissue preparations, it was recognized that succinate was rapidly converted to fumarate by suspensions of minced muscle; the enzyme responsible for this effect was termed succinic dehydrogenase and was considered an important member of the Krebs cycle enzymes.

The expression "succinic dehydrogenase system" refers to the system which catalyzes the anaerobic oxidation of succinate and which probably includes cyt. c in addition to succinic dehydrogenase and the hydrogen acceptor (Lehninger, 1964).




Succinic acid Fumaric acid

The molecular weight of this enzyme is about 200,000 (Lehninger, 1964).

Because of the difficulties encountered in demonstrating succinic dehydrogenase activity in aqueous extracts of animal tissues, the literature on this subject was for many years unclear and often contradictory.

Keilin and Hartree (Dixon and Webb, 1964a) referring to the succinic dehydrogenase-cytochrome system stated that: "The activity of this system depends not only on the properties of the individual components but also on those of the colloidal protein particles to which they are more or less intimately bound. It is conceivable that each of the colloidal particles acts as a support for the complete system and thus assures the mutual accessibility of its components."

A succinic dehydrogenase preparation which was isolated from a defatted mitochondrial fraction of beef heart and from yeast was found to contain flavin and non-heme iron at a 1:4 ratio (Conn and Stumpf, 1963c). These preparations catalyzed electron transfer from succinate to the oxidation-reduction indicator PMS, but not to several other dyes (such as methyleme blue), or to cyt. c (Lehninger, 1964).

Singer, et al (1956) have shown that the tightly bound flavin of succinic dehydrogenase cannot be liberated even partially and still maintain an active protein.

Hopkins (Dixon and Webb, 1964b) has shown that succinate, or the powerful competitive inhibitor malonate, protect succinic dehydrogenase against inactivation by thiol reagents.

Succinic dehydrogenase is slowly and irreversibly inactivated by cyanide. The rate of inactivation is independent of pH between the values of 6.5 and 8.0, and apparently involves hydrocyanic acid and not cyanide ion. Protection from inactivation is obtained with succinate and sodium dithionate (Bonner, 1959). Tsou (1951) has presented evidence showing that succinic dehydrogenase is the immediate electron acceptor from succinate and that only the oxidized form of the enzyme is susceptible to attack by cyanide.

Many narcotics inhibit succinic dehydrogenase, urethan and phenylurethan being most frequently used (Bonner, 1959).

2. Assay Methods.

No simple successful method for the separation of succinic dehydrogenase from cellular components has been devised to date; hence, any assay for this enzyme has to be carried out in the presence of some or all the components

of the succinic dehydrogenase-cytochrome system. Cytochrome oxidase may be inactivated by the use of cyanide, or by carrying out the measurement anaerobically. Under either of these conditions, and with the addition of a hydrogen acceptor, the activity of the "succinic dehydrogenase system" may be estimated readily by one of the two methods described in the following pages. The choice of method depends on the relative purity and activity of the enzyme preparations (Bonner, 1959).

a. Manometric Method.

Slater described an assay of the succinic dehydrogenase system by a manometric method which depends on the measurement of oxygen consumption during succinate oxidation in the presence of cyanide and methylene blue (Bonner, 1959).

Singer, et al (1956) compared various assay methods for succinic dehydrogenase in intact baker's yeast mitochondria using dyes as hydrogen acceptors. They found that phenazine methosulfate was the most effective and reliable dye. The values for succinic oxidation with other acceptors, taking the activity with phenazine methosulfate as 100, were : methylene blue, 40; brilliant cresyl blue, 30; ferricyanide, 28.

b. Spectrophotometric Method.

Slater and Bonner (Bonner, 1959) described a method which depends upon measuring the rate of potassium ferricyanide reduction in the presence of sufficient potassium cyanide to inhibit cytochrome oxidase. It is not known what components of the succinic oxidase system are involved in the reduction of ferricyanide, although succinic dehydrogenase is probably the most important. This method is particularly useful for partially purified and highly active preparations. It is also a reliable assay for succinic dehydrogenase from plants.

3. Occurrence.

Succinic dehydrogenase activity has been detected in every aerobic organism in which it has been sought (Cochrane, 1958). Its activity in fungi is of interest to this study and hence its occurrence in fungi will be described here.

Hilton and Smith (1959) studied the succinoxidase system in the fungus Myrothecium verrucaria. They found that the oxidation of Krebs cycle intermediates, including succinate, by mitochondria was highly sensitive to washing and observed variations in the ability to oxidise pyruvate, ketoglutarate, fumarate, citrate, and malate even after the addition of known soluble cofactors.

Shepherd (1951) showed the presence of succinic dehydrogenase in a cell-free state of Neurospora which had double pH optima, 6.9 and 8.1. Cytochrome c acted as an efficient hydrogen carrier for the enzyme.

Martin (1954) demonstrated that the succinoxidase of Aspergillus niger differs from that of avian and mammalian tissues and has a single pH optimum at 7.3.

McDonald, et al (1963) studied the succinic dehydrogenase from the fungus Claviceps purpurea with respect to the ease of solubilization, solubility and stability, freedom from cytochrome contamination, electron acceptor specificity, and the nature of inhibition by iron chelators. The enzyme reacted with phenazine methosulfate, while other electron acceptors such as triphenyl tetrazolium chloride, methylene blue, 2-6 dichlorophenolindophenol, ferricyanide, and mammalian cyt. c were ineffective.

Wessels (1959) reported succinic dehydrogenase activity in his cell-free preparation obtained from the basidiomycete Schizophyllum commune. This preparation contained microscopically visible particles. He used cyt. c as an electron acceptor.

Niederpruem and Hackett (1961) reported succinate oxidation activity in a mitochondrial preparation obtained from the basidiomycete Schzophyllum commune. They used cyt. c as an electron acceptor. There was no succinate

oxidase activity in the particulate fraction obtained by hand grinding and centrifugation at 10,000 x g for 20 minutes.

## E. TRANSFORMATION OF INDOLE ACETIC ACID (IAA) TO OXINDOLE ACETIC ACID (OIAA)

Hygrophorous conicus produces the enzyme indole acetic acid oxidase (IAA oxidase) if the mycelia are grown in the presence of IAA or tryptamine. IAA acts both as an inducer and a substrate for the IAA oxidase, and the enzyme is intracellular (Patel, 1964). The product of the oxidation of IAA by the H.conicus enzyme has been isolated and shown to be OIAA (Siehr, 1961).

Since IAA and OIAA absorb maximally at two different wave lengths, 280 mμ and 250 mμ respectively, it is possible to determine the enzyme activity quantitatively either by the disappearance of IAA or the appearance of OIAA (Patterson, 1965).

Since IAA produces a blue-violet spot with Ehrlich reagent (Block, et al, 1958), and OIAA a blue-green spot (Siehr, 1961), paper chromatography can be a useful tool for the qualitative determination of the indole acetic acid oxidase activity in H.conicus.

# III. EXPERIMENTAL

## A. MATERIALS

The chemicals used in this investigation and the companies from which they were obtained are listed below.

| Chemicals | Company |
|---|---|
| Malt extract, agar, casamino acids, and yeast extract. | Difco Chemical Co. Detroit, Michigan. |
| p-dimethylamino benzaldehyde, sodium succinate. | Matheson Chemical Co. Kansas city, Missouri. |
| ATP Batch No. 112B-749<br>Cyt. c Batch No. 104B-7475<br>NAD Batch No. 104B-7551<br>PMS Batch No. 15B-5310<br>Glucose Batch No. 24B-0470 | Sigma Chemical Co. St. Louis, Missouri. |
| Folin Phenol Reagent (2N) Lot No. 742418 | Fisher Scientific Co. Fair Lawn, New Jersey. |

All of these chemicals were used without further purification.

All inorganic salts and solvents were reagent grade and were used without further purification.

The culture of Hygrophorous conicus was obtained from the Abbott Laboratories, North Chicago, Illinois.

B. APPARATUS

Centrifuge, High Speed: Lourdes Instrument Corporation, Brooklyn, N.Y. Model LCA-1.

Colorimeter, Spectronic 20: Bausch and Lomb Incorporated, Rochester, N.Y.

pH meter (No. 7664): Leeds and Northrup Company, 4901 Station Ave. Philadelphia 44, Pa.

Pressure Filtration Funnel (No. 4240) with a Gelman 2 Micron Multipore Filter: Gelman Instrument Company, Ann Arbor, Mich.

Rotary Evaporator: Rinco Instrument Company, Greenville, Ill.

Shaker, Rotary: New Brunswick Scientific Company, New Brunswick, N.J. Model CS-62630.

Sonifier: Branson Sonic Power, Division of Branson Instruments Incorporated, Danbury, Conn. Model S 75.

Spectrophotometers: Beckman DK-2A, Automatic Scanning. Scientific and Process Instruments Division, Beckman Instruments, Incorporated, 2500 Harbor Blvd. Fullerton, Calif., and Hitachi-Perkin Elmer Model 139, Manual. Perkin Elmer Corporation, Church Street Station, New York 8, N.Y.

Sterilizer, Rectangular type, 24 in. x 36 in. x 48 in. (steam heat): American Sterilizer Company, Erie, Pa.

Ultra Violet Light: Short Wave Ultra Violet Lamp, Model SL 2537, Ultra-Violet Products, Incorporated, South Pasadena, Calif.

Warburg Respirometer Model RWB 320: Gilson Medical Electronics, Middleton, Wis.

C. MEDIA

The following media were used:

No. 1: 4% malt extract in deionized water.

No. 2: 10 gm glucose; 2 gm asparagine; 0.5 gm $KH_2PO_4$; 0.5 gm $Na_2HPO_4.2H_2O$; 1 gm $MgSO_4.7H_2O$; 100 µM thiamine (vitamin $B_1$) in 1 liter of deionized water. (Code: Asparagine medium).

No. 3: 30 gm glucose; 3 gm $NaNO_3$; 1 gm $K_2HPO_4$; 0.5 gm $MgSO_4.7H_2O$; 0.5 gm KCl; 0.01 gm $FeSO_4$; 100 µM thiamine in 1 liter of deionized water. (Code: Nitrate medium).

No. 4: 20 gm maltose; 2 gm casamino acids; 1 gm $K_2HPO_4$; 0.5 gm $MgSO_4.7H_2O$; 0.5 gm KCl; 0.01 gm $FeSO_4$; 100 µM thiamine in 1 liter of deionized water. (Code: Maltose medium).

No. 5: 20 gm maltose; 2 gm $NaNO_3$; 1 gm $K_2HPO_4$; 0.5 gm $MgSO_4.7H_2O$; 0.5 gm KCl; 0.01 gm $FeSO_4$; 100 µM thiamine in 1 liter of deionized water. (Code: Mal medium).

No. 6: 20 gm glucose; 2 gm casamino acids; 1 gm $K_2HPO_4$; 0.5 gm $MgSO_4.7H_2O$; 0.5 gm KCl; 0.01 gm $FeSO_4$; 100 µM thiamine in 1 liter of deionized water. (Code: Cas medium).

## D. METHODS AND RESULTS

### 1. Cultivation of Hygrophorous conicus

Three hundred ml erlenmeyer flasks containing 60 ml of a particular medium, or five hundred ml erlenmeyer flasks containing 100 ml were autoclaved at 120°C and 15 psi for thirty minutes. After cooling to room temperature the flasks were kept in a cold room (4-6°C) until used.

Stock culture of H.conicus were grown at 25°C in one of the previously mentioned media (see page 17) for four days. These cultures of H.conicus were prepared once a month by inoculation of the autoclaved medium with a culture from a malt-agar slant, and were maintained by transferring every four days 3 ml of mycelium suspension formed to 60 ml of fresh medium. Immediately after inoculation the stock culture flasks were placed on a rotary shaker (240 RPM) where they remained during the entire period of growth.

The induction of indole acetic acid oxidase (IAA oxidase) was accomplished by adding 28.2 mg of inducer, the sodium salt of indole acetic acid, to 60 ml of the particular medium at the time of inoculation.

H.conicus was transferred once a month from stock cultures to slants or plates to check for possible contamination of the culture. The solid medium used for the slants and plates was a 4 per cent malt extract solution to which 2 per cent agar was added. The medium

was prepared by adding the agar to the malt solution and heating the mixture until the agar was dissolved. The hot medium was poured into culture tubes and autoclaved. Following sterilization, liquified medium was poured into sterile petridishes (plates), or allowed to cool and solidify in the tubes (slants). A platinum wire loop was used to inoculate the slants or the plates with H.conicus from the stock culture. The slant and plate cultures were allowed to grow for several days at room temperature and then examined for contamination. The uncontaminated slants were stored in the refrigerator and were later used to prepare stock cultures. The contaminated slants and the plates were autoclaved and discarded.

2. Preparation of Two-Dimensional Paper Chromatograms.

Paper chromatograms were used for the separation and qualitative determination of IAA and OIAA. The spent medium from which the mycelia had been removed by centrifugation was used in the preparation of the chromatograms.

The medium was acidified with 5 per cent hydrochloric acid to a pH of about 3 and was then extracted three times with 30 ml of ethyl acetate. The ethyl acetate extracts were combined and dried over anhydrous sodium sulfate. The sodium sulfate was filtered out, and the extract was evaporated to dryness on a Rinco evaporator at about 45°C. The solid material obtained was dissolved in ethyl acetate

so that an approximate concentration of 10 mg/ml was obtained. Five lambda ($5 \times 10^{-3}$ ml) of the solution was spotted in a corner located 2.5 cm from both edges of an 11 inch square sheet of Whatman No. 1 filter paper. After the spot was dried with a hand hair dryer the two edges of the chromatogram were stitched together with thread to form a cylinder. The chromatogram was placed in a glass cylinder which contained Solvent A (300 ml isopropanol, 15 ml concentrated ammonia, and 30 ml of water), the cylinder was covered and sealed, and the solvent was allowed to rise to within a few centimeters below the top edge of the paper (this usually required 10 to 11 hours). The chromatogram was removed from the glass cylinder, the threads were cut, and the paper was hung to dry. The chromatogram was again formed into a cylinder and the two edges which were formerly the top and bottom were attached. The chromatogram was placed in a glass cylinder containing Solvent B (180 ml n-butanol, 45 ml glacial acetic acid, and 75 ml water) and the solvent was allowed to rise about 8 to 9 inches over the bottom edge (usually in 6 to 7 hours). The chromatogram was removed from the cylinder and allowed to dry. The dried chromatogram was examined under ultra violet light and the spots seen were marked. The OIAA spot fluoresced blue, while the IAA spot absorbed in the ultra violet light.

To verify these observations, the chromatogram was then dipped in Ehrlich reagent (2.0 gm p-dimethylamino-benzaldehyde, 160 ml acetone, and 20 ml concentrated hydrochloric acid). A violet spot appeared almost immediately at the location as the UV absorbing spot (IAA). After five or ten minutes a yellow-green spot appeared at the same location as the blue fluorescent spot (OIAA). This spot became blue-green after a few more minutes and intensly blue-green after standing for several hours.

3. Growth of H.conicus and Formation of the Enzyme IAA Oxidase.

Several 300 ml erlenmeyer flasks containing 60 ml of the various media previously described (see page 17) were prepared and autoclaved. An insoluble material was formed in all the media during autoclaving but was not removed. The flasks were inoculated with 3 ml of a four day old stock culture of H.conicus. In order to induce the formation of IAA oxidase, 28.2 mg of Na-IAA was added. The flasks were harvested at 24, 48 and 72 hours and the suspensions obtained were centrifuged.

The cells obtained by centrifugation of the various samples were transferred to gooch crucibles which had been previously dried to a constant weight in an oven at 105°C. The cells and gooch crucibles were dried at 105°C until they reached constant weight (about 20 hours) and the increase

in weight was computed. The average dried mycelium weights from four or more individual flasks are tabulated in Table 1, while the average dried mycelium weights from two individual flasks are shown in Appendix A.

The formation of the enzyme IAA oxidase was determined qualitatively and quantitatively using two-dimensional paper chromatography and spectrophotometry, respectively. In the qualitative evaluation, 100 per cent conversion of IAA to OIAA yielded a spot of pronounced blue-green color but no violet-colored spot. Incomplete conversion gave a violet-colored spot in addition to the OIAA spot. The results are tabulated in Table 1 and Appendix A. In the quantitative measurement, the amount of OIAA formed was used as a measure of IAA oxidase activity. The assay method used and the results obtained are discussed in the next section.

4. IAA Oxidase Assay.

Since OIAA and IAA absorb strongly in the UV light range at different wave lengths, 250 mμ and 280 mμ respectively, a simultaneous spectrophotometric analysis of the two substances was possible (Patterson, 1965).

The spectrophotometric analysis was conducted using a Beckman DK-2A recording spectrophotometer. The solutions were usually scanned in the range of 230 to 340 mμ. Before scanning the sample, the matched quartz cuvettes were

TABLE 1

GROWTH OF H.CONICUS AND FORMATION

OF IAA OXIDASE

| Carbon source | Nitrogen source | Y.E. added % | Visual estimated conversion of IAA to OIAA % | Weight of dried mycelium* gm/60 ml medium |
|---|---|---|---|---|
| Glucose | Asparagine | 0.5 | 35-50 | 0.4044 |
| Glucose | Asparagine | --- | 10 | 0.0906 |
| Glucose | $NaNO_3$ | 0.5 | 10 | 0.9369 |
| Glucose | $NaNO_3$ | --- | 0 | 0.1176 |
| 4% malt extract | | 0.5 | 10 | 0.8217 |
| 4% malt extract | | --- | 100 | 0.2149 |
| Maltose | Casamino acids | --- | 100 | 0.4500 |
| Glucose | Casamino acids | --- | 100 | 0.2731 |
| Maltose | $NaNO_3$ | --- | 80 | 0.4186 |
| Glucose | $NaNO_3$ | --- | 0 | 0.1176 |

* Average of 4 individual flasks

cleaned with a 50 per cent ethanol solution containing 10 per cent ammonia. The cleanliness of the sample and reference cuvettes was checked by filling them with deionized water and comparing them in the wave length span used for the sample determination. During scanning the reference cuvette was filled with deionized water which was also used in the preparation of the sample.

In order to assay IAA oxidase activity, the H.conicus cells were grown in 60 ml of medium in the presence of an inducer, 28.2 mg Na-IAA, for 48 hours. The cells were harvested by centrifugation at 6000 r.p.m. for 12 minutes at 4-5°C. They were washed twice with deionized water to remove all traces of the malt medium, inducer, and product and were then suspended in 40 ml of deionized water. Ten ml of a 0.282 mg/ml solution of Na-IAA was added and the cell suspension was incubated with the substrate (Na-IAA) in an erlenmeyer flask on the rotary shaker for one hour at 25°C.

After incubation, the cells were removed from the suspension by centrifugation and were washed twice with deionized water. The supernatant was filtered through a Gelman 2 Micron multipore filter to remove any turbidity present and was diluted to 100 ml. The absorbancy of this solution was measured at 250 mμ and 280 mμ on the Beckman DK-2A recording spectrophotometer. The concentrations of IAA and OIAA were calculated as shown in Appendix B and the results are tabulated in Table 2.

TABLE 2

SPECTROPHOTOMETRIC ASSAY OF IAA AND OIAA

| Medium* No. | Description | IAA in sample mmole x $10^4$ | OIAA in sample mmole x $10^4$ | Conversion of IAA to OIAA** % |
|---|---|---|---|---|
| 1 | 4% malt | 54.60 | 61.5 | 47.3 |
| 4 | Maltose | 9.32 | 93.2 | 71.7 |
| 6 | Cas | 46.20 | 46.6 | 36.0 |
| - | Reference | 130.00 | -- | -- |

*10 ml of 0.282 mg per ml Na-IAA solution was added to sample and reference flasks.

**Based on the 13 μmole IAA in the reference sample (e.g. $\frac{6.15}{13.0} \times 100 = 47.3\%$).

5. Disintegration and Preparation of Cell-Free Extracts.

All the operations were carried out at 4-6°C unless otherwise stated. Two procedures were employed and were as follows:

Procedure I: The submerged mycelium from eight or nine 300 ml flasks containing 60 ml of medium was combined and harvested by centrifugation at 1000 x g for 20 minutes and washed twice with 0.01M phosphate buffer (pH 7). The cells were suspended in either one of the following solutions:

(a) 0.8M glucose, 0.05M $KH_2PO_4$, 0.02M niacin, adjusted to pH 7 with NaOH solution;

(b) 0.01M phosphate buffer, pH 7

and the suspension was exposed to the maximum output from a Branson S 75 sonicator for 10 minutes. The homogenate was centrifuged at 1000 x g for 20 minutes to remove the unbroken cells. The cell-free supernatant was centrifuged at 24,000 x g for 20 minutes and the residue (which was assumed to be mostly mitochondria) was resuspended in 3 to 4 ml of either solution a or b.

Procedure II: The submerged mycelium was harvested as described in procedure I. Instead of sonication, however, the mycelium was ground in a mortar with either acid washed sand or powdered glass for 10 minutes. After filtering through cheese cloth, the homogenate was centrifuged at

18,000 x g for 20 minutes and the residue (which was assumed to be mitochondria) was suspended in 3 to 4 ml of either solution a or b, as described in procedure I. Both the cell-free and the mitochondrial preparations were used for the measurement of succinic dehydrogenase activity.

6. Protein Estimation.

a. Biuret Method.

Aliquotes of the sample and standard (bovine albumin) were pipetted into 10 ml calibrated centrifuge tubes; 2.5 ml of 50 per cent trichloro acetic acid (TCA) was added to each tube to precipitate the protein. The suspensions were centrifuged for 15 minutes, and the supernatants were carefully removed with a pipette. Eight ml of the biuret reagent (1.5 gm cupric sulfate and 6.0 gm sodium potassium tartrate were dissolved in about 500 ml of deionized water; this solution was added to 300 ml of freshly prepared 10 per cent sodium hydroxide, and the total volume was made to 1 liter with deionized water) was added to the solids in the centrifuge tubes, and the precipitates were dissolved in the reagent with careful stirring. The solutions were diluted to 10 ml with deionized water, and the color was allowed to develop for 30 minutes before reading it at 540 mμ on the Spectronic 20 colorimeter against a blank containing 8 ml of biuret reagent and 2 ml of deionized water.

b. Folin Phenol Reagent Method.

Reagent A: 2% $Na_2CO_3$ in 0.1N NaOH.

Reagent B: 0.5% $CuSO_4.5H_2O$ in 1% sodium potassium tartrate.

Reagent C: Alkaline copper solution; 50 ml of reagent A plus 1 ml of reagent B. (freshly prepared).

Reagent D: Same as reagent C except for omission of NaOH in reagent A.

Reagent E: 1N Folin Phenol reagent.

The sample was diluted so that an approximate protein concentration of 100 gamma per ml was obtained, and 0.1 to 0.5 ml aliquots of the diluted sample were added to calibrated centrifuge tubes. One tenth of a ml of 1N NaOH solution was added to each tube. The tubes were placed in a boiling water bath for 20 minutes. After cooling deionized water was added to each tube to make up its volume to 5 ml followed with 5 ml of reagent D. The tubes were allowed to stand for 10 minutes at room temperature. One-half ml of reagent E was added very rapidly and mixed within a second or two. After 30 minutes the sample was read at 500 mμ in a Spectronic 20 colorimeter against a blank containing deionized water in place of the sample, and was treated similarly as sample as mentioned above.

7. Measurements of Respiration

a. Phenazine Methosulfate Assay Method for Succinic Dehydrogenase

Reagents: (1) 0.3M Tris buffer, pH 7.6.

(2) 0.2M Sodium succinate, pH 7.6.

(3) 0.01M Potassium cyanide, neutralized, pH 7.0 to 7.8.

(4) 1% PMS in deionized water.

(5) 0.1M Calcium chloride.

Procedure: To the inner well of all the Warburg vessels used, 0.2 ml 20 per cent KOH solution was added. To the main compartments of the vessels were added 0.5 ml tris buffer, 0.5 ml enzyme preparation, and either 0.03 ml $CaCl_2$ solution plus 1.47 ml deionized water (in case of mitochondrial preparation) or 1.5 ml deionized water. The enzyme preparation was made as described in part D of this section. To the side arm of each vessel were added 0.3 ml succinate, 0.1 ml PMS, and 0.3 ml potassium cyanide solution. Each vessel was immediately connected to its corresponding calibrated manometer and the stopcock was closed in order to prevent escape of HCN gas. The enzyme cytochrome oxidase is inhibited by HCN gas. Excess HCN gas is absorbed by the KOH solution which is in the inner well of the vessel. The pressure was released by momentarily opening the stopcock. One vessel which contained simply 3.2 ml deionized water

in the main compartment and 0.2 ml 20 per cent KOH in the inner well was used as the thermobarometer. The manometers and vessels were transferred to the Warburg apparatus with the vessels placed in the 30°C water bath and with both legs of the manometer open. The vessels were allowed to come to temperature equilibrium for 7 minutes. For each manometer, the index was then set at 150 mm on the right arm of the manometer, the stopcocks were closed, the contents of the side arms were tipped into the main compartment, and the apparatus was allowed to oscillate for 5 minutes. At the end of 5 minutes the closed leg (right arm) of the manometer was adjusted to the 150 mm index and the open leg (left arm) of the manometer was read. This procedure was repeated for all vessels. Readings were repeated at regular intervals of 5 minutes.

The oxygen uptake was calculated using the following equation (Umbreit, et al, 1964).

$$\text{Oxygen uptake in } \mu l = h\ k$$

Where: h = The observed change in the manometer reading in mm.

k = Flask constant.

$$\text{and} \quad k = \frac{V_g \frac{273}{T} + V_f\ d}{P_o}$$

Where: $V_g$= Volume of gas phase in flask including connecting tubes down to the reference point (150 mm on closed arm of manometer) in µl; so $V_g$ = Total volume (V) − Volume of fluid ($V_f$).

V = Total volume of the flask including connecting tubes down to the reference point (150 mm on closed arm of manometer) in µl. This volume was determined by filling with mercury and weighing the mercury.

$V_f$= Volume of fluid in flask in µl.

$P_o$= Standard pressure, which is 760 mm Hg or 10,000 mm of Krebs or Brodie's fluid (Density = 1.033 at 20°C).

T = Temperature of bath in absolute degrees ( = 273 + temperature in °C).

d = Solubility of oxygen in reaction liquid.

b. Results of the Phenazine Methosulfate Assay for Succinic Dehydrogenase.

The mycelium of H.conicus was harvested by centrifugation. The cells were subjected to sonication and the cell-free and mitochondrial preparations were made as described in part 5 of this section. The succinic dehydrogenase activity in these preparations was measured

by means of the Warburg respirometer using the PMS assay method described in the preceding section.

In order to obtain measurements which would have a basis of comparision regardless of the extent of growth of the organism, the protein concentration in each enzyme preparation was determined. Either the Biuret method or the Folin phenol reagent method was used for the protein determination. These methods are described in part 6 of this section.

The systems studied are described in Tables 3, 4, and 5 and the results obtained are given in Appendix C where oxygen uptake data, in $\mu l O_2$, and specific succinic dehydrogenase activities, in $\mu l O_2$ per mg protein, are reported for different time intervals. The specific succinic dehydrogenase activity data are also plotted in Figures 1, 2, and 3, respectively. The method used for the computation of the $\mu l O_2$ taken up during a run is outlined in Appendix D.

$QO_2(N)$ values for the various enzyme preparations are tabulated in Table 6. The $QO_2(N)$ value is the $\mu l O_2$ taken up per hour per mg protein. These values are customarily used for comparing enzymatic activity of Krebs cycle enzymes in various species of organisms. In order to obtain $QO_2(N)$ value, the $\mu l O_2$ taken up per mg protein was plotted versus time. The slope of the straight line portion of this plot was determined. This

TABLE 3

SUCCINIC DEHYDROGENASE ACTIVITY OF THE CELL - FREE PREPARATION AS A FUNCTION OF THE CULTURE MEDIUM

Media for organism growth:

(a) No. 1 (4% malt extract).

(b) No. 4 (maltose).

(c) No. 3 (nitrate) + 0.5% Y.E.

Substrate: Sodium succinate, 0.2M, pH 7.6.

Apparatus: Warburg respirometer.

Enzyme: Cell-free preparation (0.5 ml).

Electron acceptor: PMS, 1% in deionized water.

Cofactors: 0.3M Tris buffer, pH 7.6; 0.01M KCN, neutralized, pH 7.0-7.8.

Inner well of vessel: 0.2 ml 20% KOH solution.

Total liquid volume: 3.4 ml.

Analysis: Succinic dehydrogenase by the Phenazine Methosulfate assay method.

Data: Data are tabulated in Appendix C, pp. 77, 78, and 79 and plotted in Figure 1.

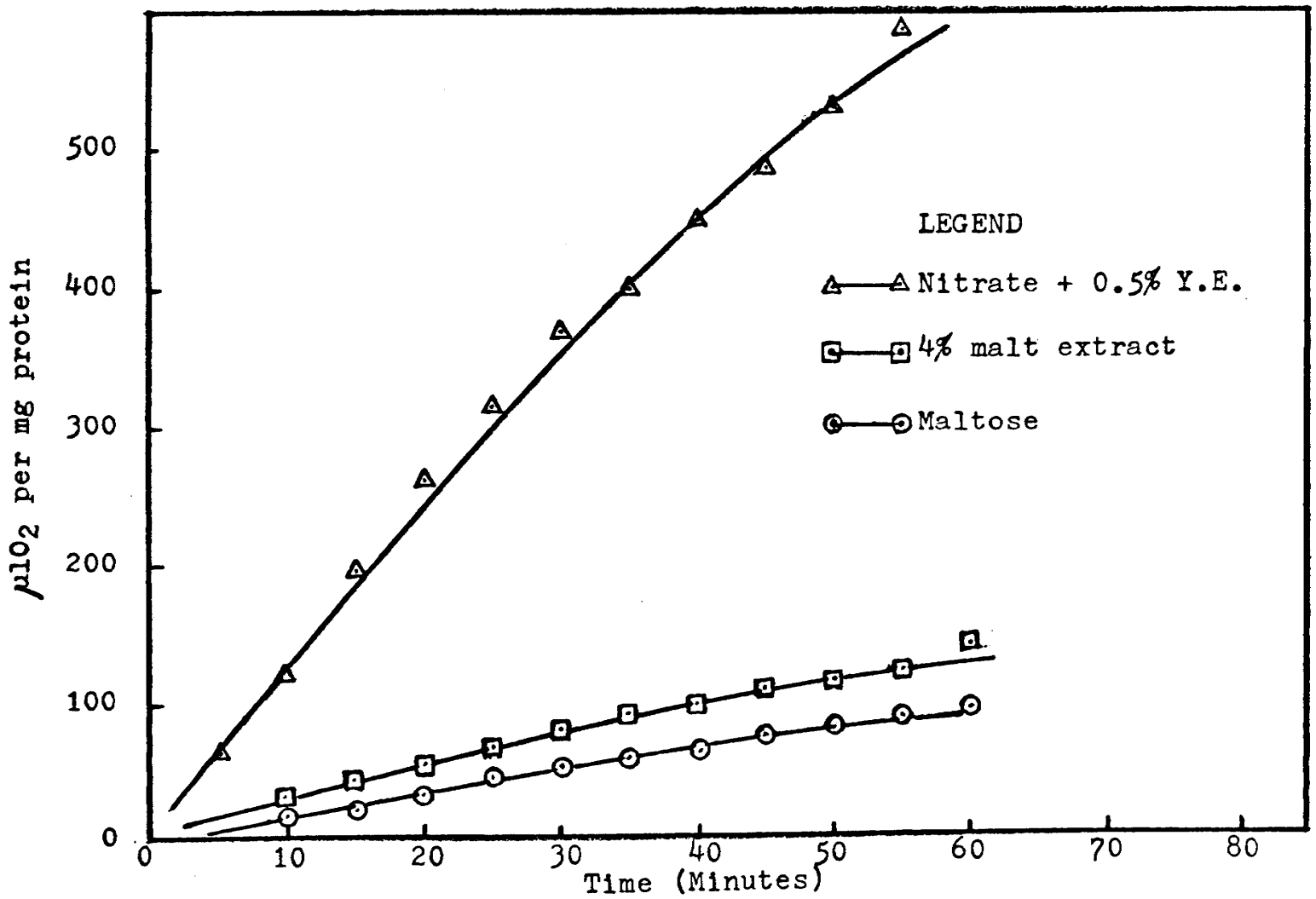



FIGURE 1. SUCCINIC DEHYDROGENASE ACTIVITY OF THE CELL-FREE PREPARATION AS A FUNCTION OF THE CULTURE MEDIUM

TABLE 4

SUCCINIC DEHYDROGENASE ACTIVITY OF THE MITOCHONDRIAL PREPARATION AS A FUNCTION OF THE CULTURE MEDIUM

Media for organism growth:

(a) No. 1 (4% malt extract).

(b) No. 4 (maltose).

(c) No. 3 (nitrate) + 0.5% Y.E.

Substrate: Sodium succinate, 0.2M, pH 7.6.

Apparatus: Warburg respirometer.

Enzyme: Mitochondrial preparation (0.5 ml).

Electron acceptor: PMS, 1% in deionized water.

Cofactors: 0.3M Tris buffer, pH 7.6; 0.01M KCN, neutralized, pH 7.0-7.8; 0.1M $CaCl_2$.

Inner well of vessel: 0.2 ml 20% KOH solution.

Total liquid volume: 3.4 ml.

Analysis: Succinic dehydrogenase by the Phenazine Methosulfate assay method.

Data: Data are tabulated in Appendix C, pp. 80, 81, and 82 and plotted in Figure 2.

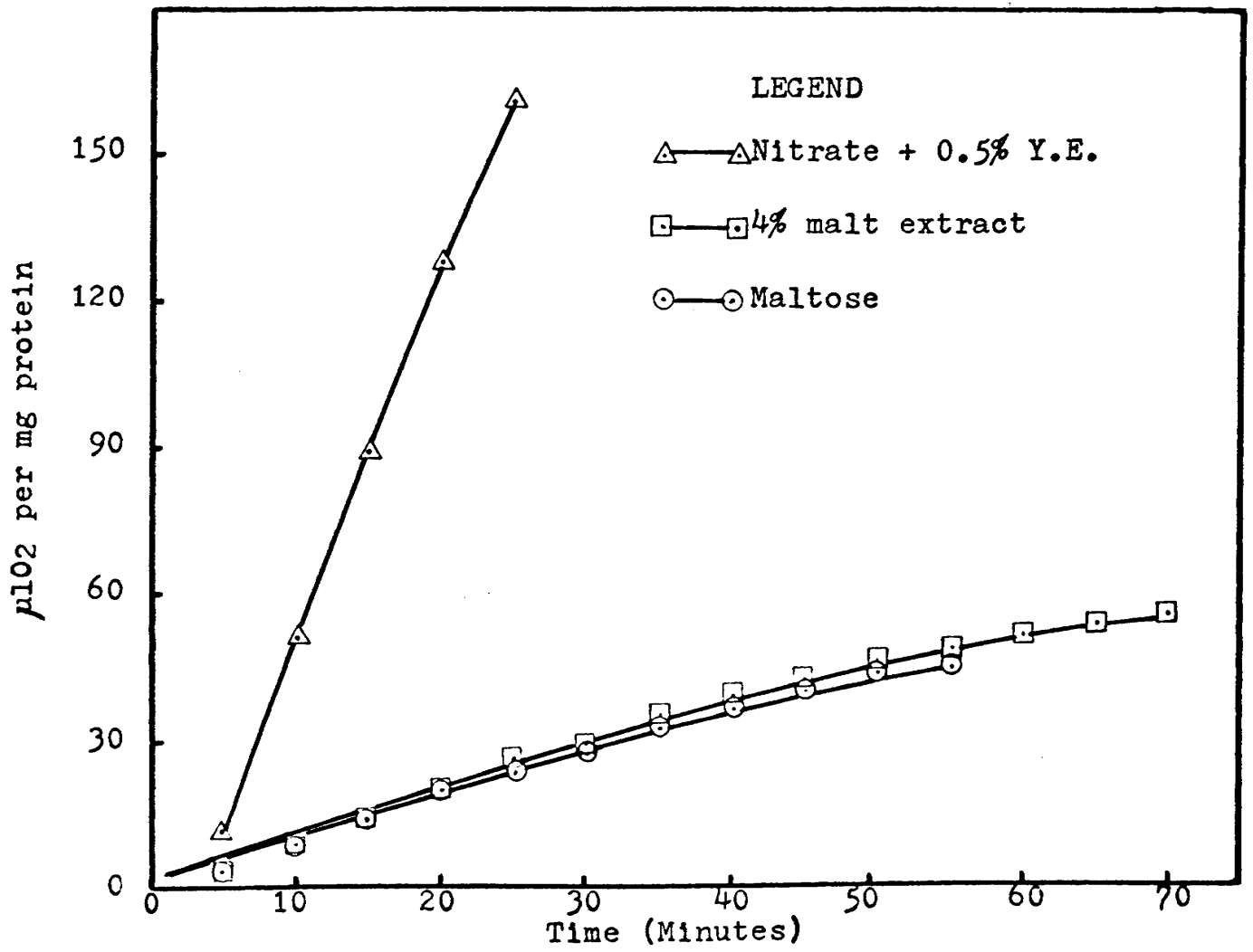



FIGURE 2. SUCCINIC DEHYDROGENASE ACTIVITY OF THE MITOCHONDRIAL PREPARATION AS A FUNCTION OF THE CULTURE MEDIUM

TABLE 5

SUCCINIC DEHYDROGENASE ACTIVITY OF THE 24,000 x g SUPERNATANT AS A FUNCTION OF THE CULTURE MEDIUM

Media for organism growth:

(a) No. 1 (4% malt extract).

(b) NO. 3 (nitrate) + 0.5% Y.E.

Substrate: Sodium succinate, 0.2M, pH 7.6.

Apparatus: Warburg respirometer.

Enzyme: The 24,000 x g supernatant (0.5 ml).

Electron acceptor: PMS, 1% in deionized water.

Cofactors: 0.3M Tris buffer, pH 7.6; 0.01M KCN, neutralized, pH 7.0-7.8; 0.1M $CaCl_2$.

Inner well of vessel: 0.2 ml 20% KOH solution.

Total liquid volume: 3.4 ml.

Analysis: Succinic dehydrogenase by the Phenazine Methosulfate assay method.

Data: Data are tabulated in Appendix C, pp. 83 and 84 and plotted in Figure 3.

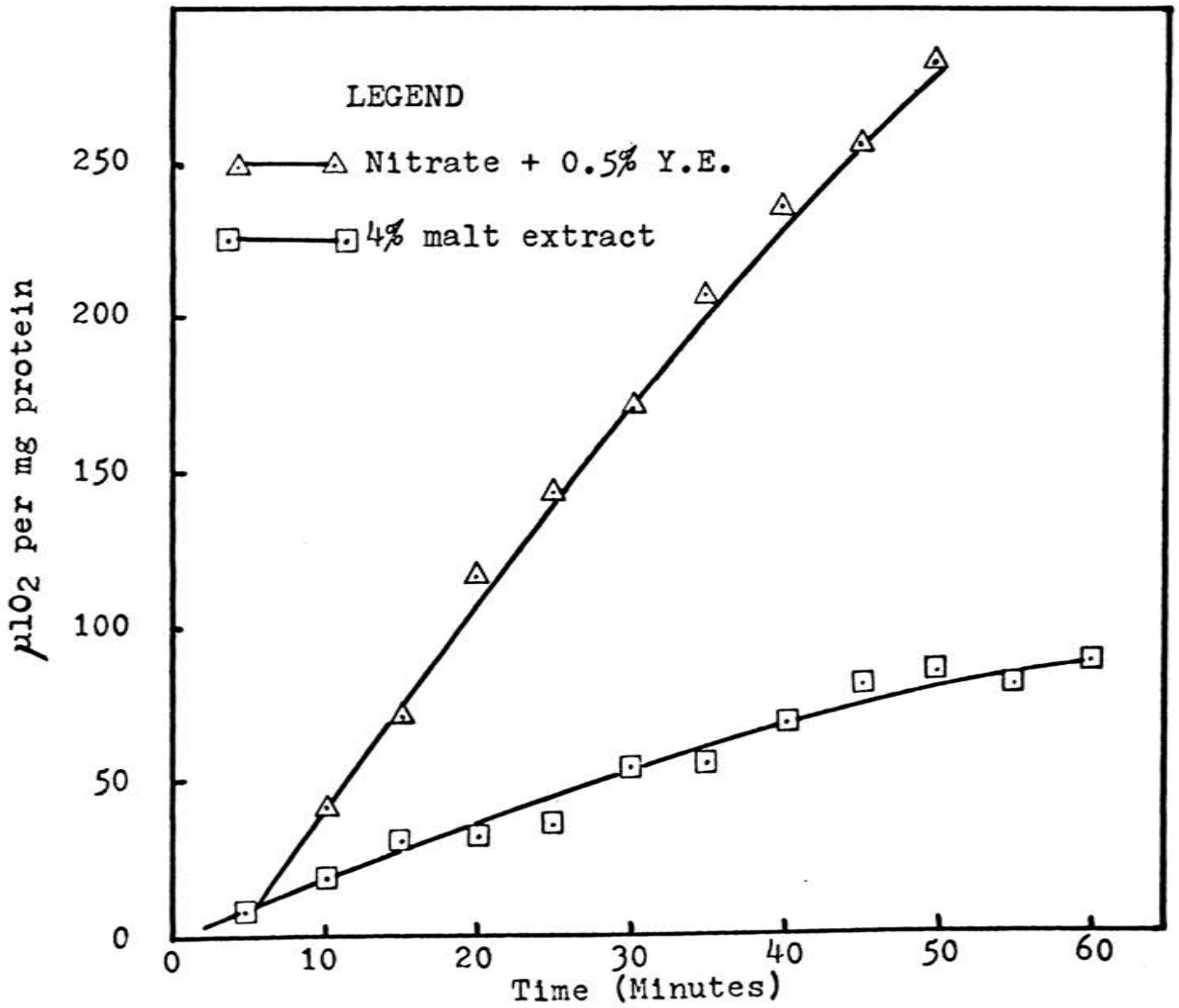



FIGURE 3. SUCCINIC DEHYDROGENASE ACTIVITY OF THE 24,000 x g-SUPERNATANT AS A FUNCTION OF THE CULTURE MEDIUM

TABLE 6

$QO_2(N)$ VALUES FOR VARIOUS ENZYME PREPARATIONS DETERMINED USING THE PHENAZINE METHOSULFATE ASSAY METHOD

| Medium No. | Medium Description | Enzyme preparation | Assay | Rate of reaction* $\mu lO_2$/minute/mg protein | $QO_2(N)$ |
|---|---|---|---|---|---|
| 1 | 4% malt extract | Mitochondrial | Succinic dehydrogenase | 1.2 | 72.0 |
| 4 | Maltose | " | " | 1.2 | 72.0 |
| 3 | Nitrate + 0.5% Y.E. | " | " | 8.4 | 500.0 |
| 1 | 4% malt extract | Cell-free | " | 2.57 | 154.0 |
| 4 | Maltose | " | " | 1.62 | 97.2 |
| 3 | Nitrate + 0.5% Y.E. | " | " | 11.54 | 690.0 |
| 3 | Nitrate + 0.5% Y.E. | 24,000 x g-supernatant | " | 6.67 | 400.2 |
| 1 | 4% malt extract | " | " | 2.27 | 136.2 |

*These are the slopes of the straight line portion of the plots of $\mu lO_2$ per mg protein versus time (see Figures 1, 2, and 3)

slope gave the initial rate which was defined as the $\mu l O_2$ taken up per minute per mg protein. This value was multiplied by 60 to obtain a $QO_2(N)$ value which is defined as the $\mu l O_2$ taken up per hour per mg protein.

c. Cytochrome c Assay Method for Succinic Dehydrogenase.

Reagents: Solution A: 0.8M glucose, 0.05M $KH_2PO_4$, 0.02M niacin, adjusted to pH 7.0 with NaOH solution.

Solution B: 0.005M $MgCl_2$, 0.003M ATP, 16 x $10^{-6}$M cyt. c.

Solution C: 3 mg $NAD^+$ per ml.

Solution D: 0.025M succinic acid.

Procedure: To the inner well of all the Warburg vessels used, 0.4 ml 20 per cent KOH solution was added. To the main compartments of the vessels were added 1.0 ml solution B, 1.0 ml solution C and either 0.5 ml solution D or 0.5 ml deionized water in the case of the blank. The preparation of the enzyme was made in solution A as described in part 5 of this section. To the side arm of each vessel, 0.5 ml of the enzyme preparation in solution A was added. The remainder of the procedure was as previously described in part a of this section.

d. Results of the Cytochrome c Assay for Succinic Dehydrogenase.

The mycelium of H.conicus was harvested by centrifugation. The cells were subjected to sonication and the cell-free and mitochondrial preparations were made as described in part 5 of this section. The succinic dehydrogenase activity in these preparations was measured by means of the Warburg respirometer using the cyt. c method described in the preceding section.

The protein concentration in each enzyme preparation was again determined so that the results could be compared regardless of the extent of growth of the organism. The systems studied are described in Tables 7 and 8 and the results obtained are given in Appendix C. The specific succinic dehydrogenase activities present in the enzyme preparations at different time intervals are presented in Figures 4 and 5. $QO_2(N)$ values for the various preparations are tabulated in Table 9. $QO_2(N)$ value is defined as the $\mu l O_2$ taken up per hour per mg of protein.

TABLE 7

SUCCINIC DEHYDROGENASE ACTIVITY OF

CELLS INDUCED WITH IAA

Medium for organism growth: No. 1 (4% malt extract) + 28.2 mg Na-IAA as an inducer.

Substrate: Sodium succinate, 0.025M, pH 7.2.

Apparatus: Warburg respirometer.

Enzyme: (1) Cell-free preparation (0.5 ml).
(2) Mitochondrial preparation (0.5 ml).

Cofactors: 0.8M glucose; 0.05M $KH_2PO_4$; 0.005M $MgCl_2$; 0.01M niacin; 16 x $10^{-6}$M cyt. c; 0.003M ATP; 3 mg NAD per vessel.

Inner well of vessel: 0.4 ml 20% KOH solution.

Total liquid volume: 3.4 ml.

Analysis: Succinic dehydrogenase by the Cytochrome c assay method.

Data: Data are tabulated in Appendix C, pp. 85 and 86 and plotted in Figure 4.

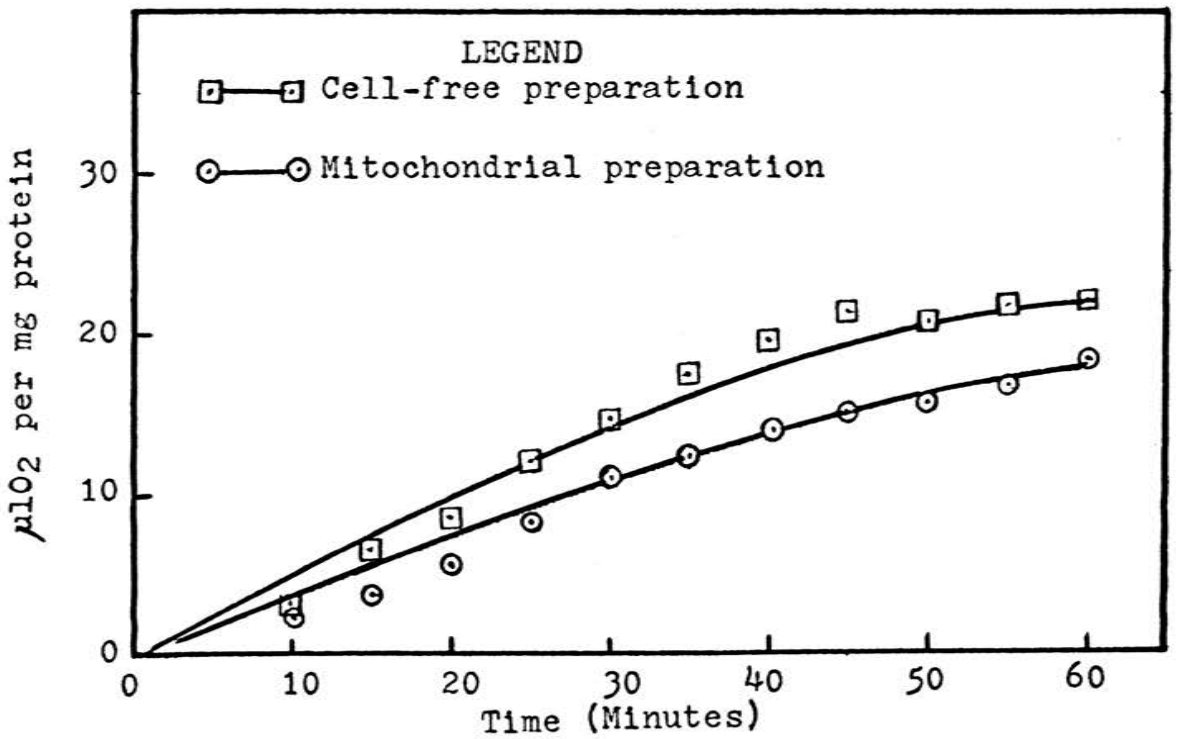



FIGURE 4. SUCCINIC DEHYDROGENASE ACTIVITY OF CELLS INDUCED WITH IAA

TABLE 8

IAA OXIDASE ACTIVITY IN CELLS INDUCED WITH IAA

Medium for organism growth: No. 1 (4% malt extract) + 28.2 mg Na-IAA as an inducer.

Substrate: Na-IAA, 0.025M.

Apparatus: Warburg respirometer.

Enzyme: (1) Cell-free preparation (0.5 ml).
(2) Mitochondrial preparation (0.5 ml).

Cofactors: 0.8M glucose; 0.05M $KH_2PO_4$; 0.005M $MgCl_2$; 0.01M niacin; 16 x $10^{-6}$M cyt. c; 0.003M ATP; 3 mg NAD per vessel.

Inner well of vessel: 0.4 ml 20% KOH solution.

Total liquid volume: 3.4 ml.

Analysis: IAA oxidase by the Cytochrome c assay method.

Data: Data are tabulated in Appendix C, p. 86 and plotted in Figure 5.

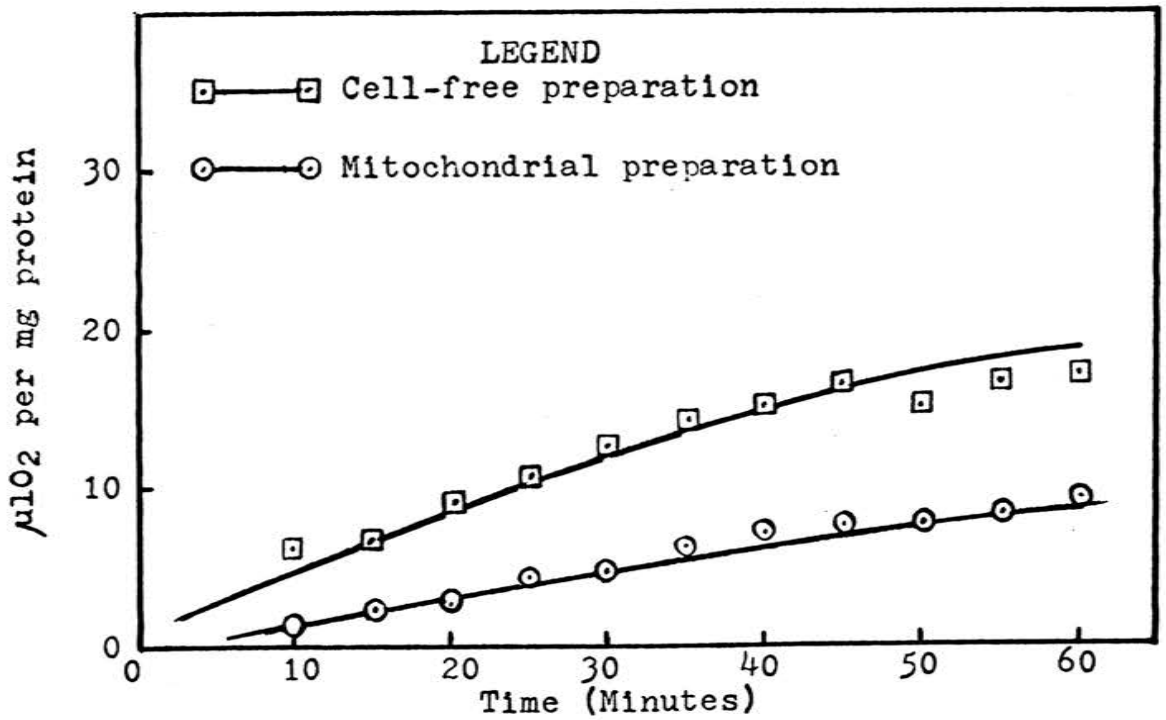



FIGURE 5. IAA OXIDASE ACTIVITY IN CELLS INDUCED WITH IAA

TABLE 9

$QO_2(N)$ VALUES FOR VARIOUS ENZYME PREPARATIONS DETERMINED USING THE CYTOCHROME c ASSAY METHOD

| Medium | Enzyme preparation | Assay | Rate of reaction* $\mu lO_2$/minute/mg protein | $QO_2(N)$ |
|---|---|---|---|---|
| 4% malt extract + 28.2 mg Na-IAA | Cell-free | IAA oxidase | 0.4 | 24.0 |
| " | Mitochondrial | " | 0.2 | 12.0 |
| " | Cell-free | Succinic dehydrogenase | 0.476 | 28.0 |
| " | Mitochondrial | " | 0.391 | 23.5 |

*These are the slopes of the straight line portion of the plots of $\mu lO_2$ per mg protein versus time (see Figures 4 and 5)

# IV. DISCUSSION

## A. GROWTH OF THE ORGANISM

### 1. Carbon Source.

The carbon source in the medium is an important factor for the growth of an organism. In work with fungi, glucose is most often used as the carbon source (Shepherd, 1951; Wessels, 1959).

Both glucose and maltose were used as carbon sources in the present study of the growth of H.conicus. These sugars were used with several different nitrogen sources. The growth of H.conicus obtained with the various combinations of sugars and nitrogen sources is presented in Table 10 in terms of the weights of dried mycelium formed. The medium containing glucose and casamino acids produced 0.273 gm dried mycelium per 60 ml of medium, while the medium containing maltose and the same amount of casamino acids gave 0.450 gm dried mycelium per 60 ml of medium. The growth of the organism in 60 ml of the 4 per cent malt extract medium was 0.215 gm. From these results it can be seen that maltose gave better growth than glucose when it was used as a carbon source for H.conicus. What effect hydrogen ion concentration, buffer action, structural difference, redox potential, etc. played in this is not known because no further experiments were performed to study these variables.

TABLE 10

THE EFFECT OF THE CARBON SOURCE

ON THE GROWTH OF H. CONICUS

| Carbon source | Nitrogen source | Period of growth Hours | Weight of dried mycelium* gm/60 ml medium |
|---|---|---|---|
| Glucose | Casamino acids | 48 | 0.273 |
| Maltose | Casamino acids | 48 | 0.450 |
| Glucose | $NaNO_3$ | 48 | 0.118 |
| Maltose | $NaNO_3$ | 48 | 0.419 |
| 4% malt extract | | 48 | 0.215 |

*Average of 4 individual flasks.

2. Nitrogen Source.

Like the carbon source, the nitrogen source is also an important factor in the growth of an organism (Robbins and Hervey, 1958).

Sodium nitrate, asparagine, and casamino acids were used as nitrogen sources in this study of the growth of H.conicus. In addition, nitrate and asparagine were tested in media supplemented with yeast extract. The results obtained in the study of the various nitrogen sources are given in Appendix A. The average dried mycelium weights presented in Appendix A are the average weights of two individual flasks.

The growth of H.conicus in a 48 hours period was 0.0906 gm and 0.1178 gm in the asparagine and nitrate medium per 60 ml of medium, respectively. It had been assumed that amino nitrogen would be more readily utilized than nitrate nitrogen. Apparently with this organism this was not the case and the difference in growth may be attributed to a poor nitrogen utilization in the case of asparagine.

The asparagine medium contained 2.322 mg nitrogen per 60 ml of medium and the nitrate medium contained 2.970 mg nitrogen per 60 ml of medium. Since the ratio of nitrogen in the two media is very close to the ratio of the dry weights of the mycelium, it is possible that nitrogen was limiting. However, the good growth of H.conicus on the same

quantity of nitrate with the addition of yeast extract would tend to suggest that some factor other than nitrogen was limiting the growth.

The difference between the asparagine and nitrate nitrogen sources in the growth of H.conicus are in good agreement with those observed by Robbins (1950) who worked with several basidiomycetes and found that asparagine was a poor nitrogen source for some of them. However, the work of Jennison, et al (1955) with still other basidiomycetes indicated that asparagine was better than nitrate except in the case of Trametes serialis. Apparently the effectiveness of a nitrogen source depends upon the kind of organism being studied.

When the growth of H.conicus was studied in the asparagine medium containing 0.5 per cent Y.E. and in the nitrate medium containing 0.5 per cent Y.E., 0.4044 gm and 0.9369 gm of mycelium per 60 ml medium were obtained, respectively, for an incubation period of 72 hours. Also the growth of H.conicus in 4 per cent malt extract and in 4 per cent malt extract plus 0.5 per cent Y.E. for a 48 hour period was 0.2149 gm and 0.8217 gm per 60 ml of medium, respectively. These results are tabulated in Table 1. Each time yeast extract was added to the various media there was a marked increase in growth. Since yeast extract is a rich source of the vitamin B complex, purines, and pyrimidines, it is impossible to tell which of these

substances in the yeast extract stimulated the growth of H.conicus. Additional experiments should be performed using individual members of the B complex and individual purines and pyrimidines. If the agent which causes the increase in growth is not found in one of these experiments with known components, then it would seem that some unknown factor is present in yeast extract which causes the stimulation of the growth of H.conicus.

When the casamino acids were used as a nitrogen source, the growth was better than with any other medium, except for those with added 0.5 per cent Y.E. Robbins (1950) described a few species of basidiomycetes which grew poorly on a medium supplemented with vitamins but grew quite well on the basal medium to which casein hydrolysate had been added. For these species asparagine alone was found to be a poor nitrogen source and the constituents of casein hydrolysate were good nitrogen sources. The results obtained in these experiments indicated that the combined amino acids of casamino acids can effectively function as the nitrogen source for H.conicus, while the amino acid asparagine alone cannot. However, the casamino acids do not produce as good growth of H.conicus as nitrate plus 0.5 per cent Y.E.

B. FORMATION OF THE ENZYME IAA OXIDASE

1. Qualitative Estimation.

Two-dimensional paper chromatography was used for the qualitative estimation of the IAA oxidase activity. The amount of OIAA formed was used as a measure of IAA oxidase activity. The 100 per cent conversion of IAA to OIAA yielded only a single spot of pronounced blue-green color and no violet-colored spot. Incomplete conversion gave a violet-colored spot due to some unreacted IAA, as well as the typical OIAA spot. The results from the paper chromatography experiments are tabulated in Table 1 and Appendix A.

As with growth, the conversion of IAA to OIAA was studied in media containing different carbon and nitrogen sources. From the results presented in Table 1, it can be seen that casamino acids were a better nitrogen source, compared to either $NaNO_3$ or asparagine, for the induction of IAA oxidase. When maltose and glucose were compared as carbon sources with casamino acids as the sole nitrogen source, both showed qualitatively the same conversion of IAA to OIAA (100 per cent). However, maltose was better so far as the increase in weight of mycelium was concerned. (cf. Table 10).

From Table 1, it can be seen that the addition of 0.5 per cent Y.E. to the medium stimulated the growth of H.conicus, but the formation of the enzyme IAA oxidase was

significantly reduced. This would indicate the possibility that the presence of yeast extract supressed the formation of IAA oxidase.

During the course of this work it was found that at the end of 24 hours there was no significant conversion of IAA to OIAA in either 4 per cent malt extract or in 4 per cent malt extract containing 0.5 per cent Y.E.; however, significant conversion was noted at the end of 48 hours. This would indicate that the synthesis of IAA oxidase took place between 24 and 48 hours, and substantiates the work of Patel (1964) and Patterson (1965).

2. Quantitative Estimation.

The quantitative estimation of the IAA oxidase activity was carried out spectrophotometrically using three different media; 4 per cent malt extract, maltose, and cas. The amount of OIAA formed was used as a measure of the IAA oxidase activity. The results of the spectrophotometric analyses are tabulated in Table 2.

The formation of OIAA was greater in the maltose medium (93.2 mmole) than in the 4 per cent malt extract (61.5 mmole) or the cas (46.6 mmole) medium. The only difference between the maltose and the cas media was that glucose was the carbon source in the latter; the nitrogen source was the same in both. The disappearance of IAA in the cas medium was greater than that in the 4 per cent malt extract

medium. Since malt extract is a complex mixture of substances, any interpretation of results using this medium is difficult.

As discussed previously (see page 47) the growth of H.conicus was better in the maltose plus casamino acids medium than in the glucose plus casamino acids medium. The difference in growth paralleled the difference in enzyme activity. Thus even though the growth was better with maltose as the carbon source the synthesis of IAA oxidase was not effected. This is in contrast to the results obtained with media supplemented with yeast extract. In these cases the growth of the organism was stimulated but the synthesis of the IAA oxidase was repressed.

The qualitative estimation of the conversion of IAA to OIAA showed that both the maltose medium and the cas medium gave 100 per cent conversion of IAA. The quantitative results support this observation but indicate that it would take only half as long to convert all of the added IAA in the maltose medium as it would in the cas medium.

## C. IAA OXIDASE ACTIVITY IN CELL-FREE AND MITOCHONDRIAL PREPARATIONS

An attempt to use both the PMS and cyt. c methods for the study of the IAA oxidase activity in cell-free preparations of H.conicus was made. When the PMS solution

was added to the side arm of the Warburg vessel containing Na-IAA solution, a violet color was observed. This indicated a reaction between PMS and Na-IAA and so this method was not used.

When the cyt. c method was used, both the unfractioned cell-free and the mitochondrial preparations showed a small, but noticeable oxygen uptake. Figure 5 is the plot of the specific enzyme activity, i.e. $\mu l O_2$ per hour per mg protein, present in the enzyme preparations. The nature of the products formed from the oxidation of IAA under these conditions has not been definitely established, but it is assumed to be OIAA.

If the product formed under these conditions were OIAA then the $\mu$moles OIAA formed per minute would be:

$$\frac{\mu l O_2 \text{ taken up per minute per mg protein}}{22.4/2\ \mu l O_2 \text{ per } \mu\text{mole OIAA formed}}$$

because the oxidation of one mole IAA to OIAA requires one-half mole of oxygen.

Hence, the $\mu$moles OIAA formed in the cell-free preparation would be equal to 0.4/11.2 or 0.0357 per minute per mg protein, while the $\mu$moles of OIAA formed in mitochondrial preparation would be equal to 0.2/11.2 or 0.0179 per minute per mg protein (cf. Table 9).

An approximate comparision of the conversion of IAA to OIAA for the cell-free preparation and for the whole cells can then be made on the basis of the following considerations.

About 50 ml of the cell-free preparation was obtained from nine shake-flasks, consequently each flask contained 5.55 ml of the cell-free preparation. Five-tenths of a ml of this preparation was used in the IAA oxidase assay carried out in the Warburg respirometer. The protein content of this preparation as estimated by the Folin phenol reagent method was 1.88 mg per ml. As discussed above the amount of oxygen taken up per minute per mg protein in the unfractioned cell-free preparation was equivalent to 0.0357 μmoles OIAA. The mg of OIAA formed per flask per hour can be calculated as shown below:

$$\left[\frac{0.0357\ \mu\text{mole OIAA}}{\text{minute mg protein}}\right] \times \left[\frac{5.55\ \text{ml}}{\text{flask}}\right] \times \left[\frac{60\ \text{minutes}}{\text{hour}}\right] \times \left[\frac{1.88\ \text{mg protein}}{\text{ml}}\right] \times \left[\frac{0.191\ \text{mg OIAA}}{\mu\text{mole OIAA}}\right] = 4.28\ \text{mg OIAA per hour per flask*}$$

*Mycelium grown in 60 ml medium.

Similarly the amount of OIAA formed per flask per hour with the whole cells was 1.172 mg.

The considerable increase in yield of OIAA in the cell-free preparation could be due to several things. The rate of transport of IAA across the cell wall might have limited the rate of conversion of IAA to OIAA. Also, in the cell-free preparation OIAA may not be the only product of IAA oxidation. Additional work is required on this point to determine the factors influencing the difference in IAA oxidase activity of the whole cells and the cell-free preparation.

D. SUCCINIC DEHYDROGENASE ACTIVITY

Preparations of bacterial or fungal succinate dehydrogenase have resulted in "soluble" succinoxidase-bound preparations, or in a cytochrome b-linked enzyme (McDonald, et al, 1963). Repaske (1954), and Wilson and Wilson (1955) prepared a succinoxidase system from Azotobacter venelandii in "soluble" form by sonic disruption of the cells and the enzymatically active particles. The "soluble" preparation designation of these workers meant that after centrifugation at 145,000 x g for 30 minutes, 50 to 75 per cent of the succinic dehydrogenase remained in the supernatant fluid.

The supernatant obtained from the cell-free preparation in the present study after centrifugation at 24,000 x g for 20 minutes (see part 6 of section III-D for the preparation) showed high succinic dehydrogenase activity. The results obtained with these preparations are plotted in Figure 3. If the mitochondrial preparations were intact, then no succinic dehydrogenase activity would be expected in the supernatant, unless succinic dehydrogenase had been present in the cells in a soluble form. Unfortunately the lack of an ultra-centrifuge did not allow the determination of the reason for the activity found in the supernatant. However, from the discussion on page 59 and from the results mentioned above it can be surmised that the mitochondrial preparation was probably

not ideal and the disruption of mitochondria into smaller particles was responsible for the presence of succinic dehydrogenase activity in the supernatant.

Greater succinic dehydrogenase activity was found in the cell-free preparations than the mitochondrial preparations of H.conicus. One explanation for this fact might be that some material which stabilizes the mitochondria was present in the cell contents. When a preparation of mitochondria was made, the mitochondria were removed from this stabilizing substance. Other reasons might be that there actually was some soluble succinic dehydrogenase activity in this organism, or that the mitochondria were disrupted as discussed above.

The succinic dehydrogenase activity of H.conicus grown in three different media was compared. The results are plotted in Figures 1 to 4. The succinic dehydrogenase activity was found to be much greater in the nitrate medium containing 0.5 per cent Y.E. than in the 4 per cent malt extract medium or the maltose medium. The growth of H.conicus in the nitrate medium containing 0.5 per cent Y.E. was about four times greater than in the 4 per cent malt extract medium and about two times greater than in the maltose medium (see Table 1). If it is assumed that the protein is the major factor involved in the succinic dehydrogenase activity, then the different activities in three different media might be due to

stimulation of synthesis of succinic dehydrogenase protein by 0.5 per cent Y.E. in the nitrate medium; another explanation would be that the malt extract and maltose media inhibited the production of succinic dehydrogenase protein. Still another possibility is that the latter two media contained an inhibitor which blocked the succinic dehydrogenase activity.

Unfortunately the experiments performed in this investigation have produced more questions than answers. In media which have been shown to stimulate the production of IAA oxidase and given from poor to good growth of mycelium, the activity of succinic dehydrogenase was much poorer than in a medium which gave excellent growth of mycelium and repressed the formation of IAA oxidase. What causes the stimulation of succinic dehydrogenase activity? Is it the nitrate or the yeast extract? Additional experiments have to be performed to clarify these points.

Several experiments for the measurement of succinic dehydrogenase activity were carried out using the cyt. c method. In these, practically nil or only a very small oxygen uptake was observed either with cell-free or mitochondrial preparations. In contrast to these results the PMS method always showed rapid oxygen uptake with both cell-free and mitochondrial preparations. If the mitochondria are intact and succinic dehydrogenase activity is the rate determining step in oxygen uptake, then the

rate of reaction with either cyt. c or with PMS would be expected to be nearly equal. Since this was not the case, it is possible that there may be some degradation of mitochondria during their preparation.

However, the PMS method measures only the succinic dehydrogenase activity and not necessarily the functioning of the mitochondria. Since the electron transport cofactors are by-passed in the PMS method, it is possible that the electron transport is the rate determining step in the cyt. c measurement. It is believed that the electron transport is the rate limiting step because the $QO_2(N)$ values for H.conicus succinic dehydrogenase preparation are in fairly good agreement with the literature values, as discussed in the following section. The aim of the succinic dehydrogenase work was to find a criterion for mitochondrial homogeneity. The great difference in succinic dehydrogenase activity measured by the two methods raises the question just how homogeneous and intact were the mitochondria obtained in this study. One way of determining the homogeneity of the mitochondrial preparation is by viewing a photomicrography of the mitochondria made with an electron microscope. Also a measure of the oxidative phosphorylation capacity of the mitochondria can be made. Instruments for either of these determinations are not presently available to this investigator.

## E. COMPARISON OF THE SUCCINIC DEHYDROGENASE ACTIVITY OF H.CONICUS AND OTHER ORGANISMS

Wessels (1959) reported that the $QO_2(N)$ value for the basidiomycete Schizophyllum commune using cyt. c as an electron acceptor was 49.3.

Niederpruem and Hackett (1961) found succinate oxidation activity in a mitochondrial preparation obtained from the basidiomycete Schzophyllum commune. They used cyt. c as an electron acceptor and determined the $QO_2(N)$ value to be 15. These workers found no succinate oxidase activity in the particulate fraction obtained by hand grinding and centrifugation at 10,000 x g for 20 minutes. This is in agreement with the results obtained in this study for the preparation of H.conicus made by hand grinding and centrifugation at 18,000 x g for 20 minutes using cyt. c as the electron acceptor.

Willims and Ledingham (1964) described succinate oxidation in the mitochondrial preparation of the basidiomycete Puccinia graminis using cyt. c as an electron acceptor. The $QO_2(N)$ value was 40 to 60.

Hilton and Smith (1959) studied the succinate oxidation in the fungus Myrothecium verrucaria which is not a basidiomycete. For the oxidation of succinate by the particles obtained by sedimentation at 20,000 x g for 30 minutes of this organism, they reported a $QO_2(N)$ value of 580. Cytochrome c was used as an electron acceptor.

The $QO_2(N)$ values obtained for the cell-free and the mitochondrial preparations of H.conicus using the cyt. c method were 28.0 and 23.5, respectively. These $QO_2(N)$ values for H.conicus are about intermediate between the lowest and the highest values reported in the literature for the succinic dehydrogenase activity in basidiomycetes. The extraordinary value of 580 found in Myrothecium verrucaria is unusual for fungi in general. Because of the intermediate value for the $QO_2(N)$ for succinic dehydrogenase from H.conicus, it is risky to use this value as a measure of functionality of the mitochondria; therefore, the need for measurements made with an electron microscope is evident.

The $QO_2(N)$ values using the PMS method for the cell-free preparation (the supernatant plus the mitochondria) of H.conicus are shown in Tables 6 and 9. $QO_2(N)$ values of 690, 154, and 97.2 were obtained in the nitrate medium containing 0.5 per cent Y.E., the 4 per cent malt extract medium, and the maltose medium, respectively. The $QO_2(N)$ values for the mitochondrial preparation obtained after centrifugation at 24,000 x g for 20 minutes were 500 in the nitrate medium containing 0.5 per cent Y.E., and 72 in the 4 per cent malt extract medium and the maltose medium. These values are all greater than those obtained with the cyt. c method. Obviously the nitrate medium containing 0.5 per cent Y.E. has a profound influence on succinic dehydrogenase activity of H.conicus.

# V. CONCLUSIONS

From the results obtained in this investigation the following conclusions can be made:

(1) The maltose medium was better than the other media tested in so far as the growth of H.conicus and the formation of the enzyme IAA oxidase were concerned.

(2) The nitrate medium containing 0.5 per cent Y.E. was the best of the media studied for the production of H.conicus mycelium.

(3) One half per cent yeast extract in the medium suppressed the formation of the enzyme IAA oxidase.

(4) The activity of succinic dehydrogenase was greatest in the nitrate medium containing 0.5 per cent Y.E.

(5) The PMS method for the assay of succinic dehydrogenase activity gave higher values than the cyt. c method.

(6) Sonification at the maximum output from a Branson S-75 sonifier for 10 minutes at 4-5°C solubilized H.conicus succinic dehydrogenase to some extent.

(7) The $QO_2(N)$ values of H.conicus using succinate as a substrate with PMS as an electron acceptor were greater than the $QO_2(N)$ values obtained using cyt. c as the electron acceptor.

(8) The hand grinding of the cells resulted in inactivation of succinic dehydrogenase.

(9) H.conicus contained at least one of the Krebs cycle enzymes namely succinic dehydrogenase.

(10) A large portion, if not all, of the succinic dehydrogenase activity of H.conicus was associated with the particulate material in the cell.

# VI. RECOMMENDATIONS

The following is a list of suggestions for the further study of H.conicus enzymes.

Indole acetic acid oxidase:

(1) Determine the reason for the suppression of IAA-oxidase by yeast extract.

(2) Determine the reason for the stimulation of H.conicus growth by yeast extract.

(3) Determine the effect of temperature on the enzyme activity.

(4) Determine the H.conicus growth rate and the rate of enzyme production using tomato extract medium.

(5) Determine with the aid of $O^{18}$ the source of the oxygen in the oxindole acetic acid formed.

Krebs cycle enzymes:

(1) Determine the factor responsible for the increased activity of succinic dehydrogenase when H.conicus is grown in nitrate medium containing yeast extract.

(2) Study the succinic dehydrogenase activity in the cell-free and mitochondrial preparations of H.conicus obtained after centrifugation at 50,000 x g or higher.

(3) Use the hydraulic press for the rupture of cells of H.conicus and study the succinic dehydrogenase activity.

(4) Study the succinic dehydrogenase activity of H.conicus grown in maltose medium.

(5) Determine the pH optimum for the succinic dehydrogenase in H.conicus.

(6) Determine the effect of azide, antimycin and other inhibitors on the succinic dehydrogenase activity.

(7) Use an electron microscope, if available, to study the homogeneity of the mitochondrial fraction of H.conicus.

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# APPENDICES

# APPENDIX A

## GROWTH OF H.CONICUS AND FORMATION OF IAA OXIDASE

| Medium No. | Description* | Period of growth Hours | Visual estimated conversion of IAA to OIAA % | Weight of dried mycelium** gm/60 ml medium |
|---|---|---|---|---|
| 1 | 4% malt extract | 72 | 100 | 0.2288 |
| | " | " | 100 | 0.2224 |
| | " | " | 100 | 0.2113 |
| | " | " | 100 | 0.2787 |
| | " | " | 100 | 0.2568 |
| | " | " | 100 | 0.2116 |
| | " | " | 100 | 0.2227 |
| 2 | Asparagine + 0.5% Y.E. | " | 50 | 0.4164 |
| | " | " | 35 | 0.4068 |
| | " | " | 35 | 0.3900 |
| 3 | Nitrate + 0.5% Y.E. | " | 10 | 0.9489 |
| | " | " | 10 | 0.8719 |
| | " | " | 10 | 0.9542 |
| | " | " | 10 | 0.9727 |
| 1 | 4% malt extract | 48 | 100 | 0.2208 |

*All media contained 28.2 mg Na-IAA.
**Average of two individual flasks.

GROWTH OF H.CONICUS AND FORMATION

OF IAA OXIDASE (Continued)

| Medium No. | Description* | Period of growth Hours | Visual estimated conversion of IAA to OIAA % | Weight of dried mycelium** gm/60 ml medium |
|---|---|---|---|---|
| 1 | 4% malt extract + 0.5% Y.E. | 48 | 10 | 0.8217 |
| 1 | 4% malt extract | " | 100 | 0.2187 |
| | " | " | 100 | 0.2104 |
| 2 | Asparagine | " | 10 | 0.0918 |
| | " | " | 10 | 0.0894 |
| 3 | Nitrate | " | 0 | 0.1198 |
| | " | " | 0 | 0.1154 |
| 4 | Maltose | " | 100 | 0.4630 |
| | " | " | 100 | 0.4370 |
| 5 | Maltose + $NaNO_3$ | " | 80 | 0.4376 |
| | " | " | 80 | 0.3997 |
| 6 | Casamino acids + glucose | " | 100 | 0.2702 |
| | " | " | 100 | 0.2760 |

*All media contained 28.2 mg Na-IAA.

**Average of two individual flasks.

# APPENDIX B

## SAMPLE CALCULATION OF $C_{IAA}$ AND $C_{OIAA}$

The procedure recommended by Patterson (1965) was employed in these computations. On the basis of the following set of spectrophotometric readings

| | Blank | | Sample | | (Sample-Blank) | |
|---|---|---|---|---|---|---|
| | $A_{280}$ | $A_{250}$ | $A_{280}$ | $A_{250}$ | $A_{280}$ | $A_{250}$ |
| 4% malt medium | 0.115 | 0.295 | 0.515 | 0.895 | 0.400 | 0.600 |
| IAA reference | --- | --- | 0.695 | 0.305 | --- | --- |

The concentration of indole acetic acid ($C_{IAA}$) in the reference sample was:

$$C_{IAA} = [3.43 \times 10^{-2} \times A_{280}] - [6.91 \times 10^{-3} \times A_{250}]$$
$$= [3.43 \times 10^{-2} \times 0.695] - [6.91 \times 10^{-3} \times 0.305]$$
$$= [2.48 \times 10^{-2}] - [2.11 \times 10^{-3}]$$
$$= 2.269 \times 10^{-2} \text{ mg/ml}$$

and since the samples had been diluted to 100 ml prior to reading their absorbancies, the total $C_{IAA}$ in the sample = 2.269 mg

Similarly the concentrations of indole acetic acid ($C_{IAA}$) and oxindole acetic acid ($C_{OIAA}$) in the 4% malt medium were

$$C_{IAA} = [3.43 \times 10^{-2} \times A_{280}] - [6.91 \times 10^{-3} \times A_{250}]$$
$$= [3.43 \times 10^{-2} \times 0.400] - [6.91 \times 10^{-3} \times 0.600]$$
$$= [1.372 \times 10^{-2}] - [4.146 \times 10^{-3}]$$
$$= 0.9574 \times 10^{-2} \text{ mg/ml}$$

and the total $C_{IAA}$ in the sample = 0.957 mg

$$C_{OIAA} = [\, 2.57 \times 10^{-2} \times A_{250} \,] - [\, 9.24 \times 10^{-3} \times A_{280} \,]$$

$$= [\, 2.57 \times 10^{-2} \times 0.600 \,] - [\, 9.24 \times 10^{-3} \times 0.400 \,]$$

$$= [\, 1.542 \times 10^{-2} \,] - [\, 3.696 \times 10^{-3} \,]$$

$$= 1.172 \times 10^{-2} \text{ mg/ml}$$

and the total $C_{OIAA}$ in the sample = 1.172 mg

# APPENDIX C

## WARBURG RESPIROMETER OXYGEN UPTAKE DATA AND SPECIFIC ENZYME ACTIVITIES

The following two methods were used for the measurement of the enzyme activity in the preparations made from *H.conicus* cells.

a. Phenazine methosulfate method (For details of this method see page 29); and

b. Cytochrome c method (For details of this method see page 40).

Oxygen Uptake and Specific Enzyme Activity Data*

Medium: 4% malt extract.

Assay: Succinic dehydrogenase.

Enzyme: Cell-free preparation (Mitochondria + supernatant).

Method: Phenazine methosulfate.

| Time in minutes | Run 1 | | Run 2 | |
|---|---|---|---|---|
| | $\mu l O_2$ | $\mu l O_2$ per mg protein | $\mu l O_2$ | $\mu l O_2$ per mg protein |
| 10 | 11.8 | 23.6 | 9.55 | 19.1 |
| 15 | 19.0 | 38.0 | 17.6 | 35.2 |
| 20 | 26.2 | 52.4 | 23.5 | 47.0 |
| 25 | 31.4 | 62.8 | 29.4 | 58.8 |
| 30 | 38.0 | 76.0 | 36.0 | 72.0 |
| 35 | 43.2 | 86.4 | 40.4 | 80.8 |
| 40 | 47.7 | 95.4 | 45.5 | 91.0 |
| 45 | 51.8 | 103.6 | 48.5 | 97.0 |
| 50 | 55.6 | 111.2 | 53.5 | 107.0 |
| 55 | 61.0 | 122.0 | 58.8 | 117.6 |
| 60 | 64.2 | 128.4 | 61.8 | 123.6 |
| 65 | 69.5 | 139.0 | 68.4 | 136.8 |
| 70 | 70.8 | 141.6 | 69.8 | 139.6 |
| 75 | 72.0 | 144.0 | 72.0 | 144.0 |

*For complete description of the system see Table 3; enzyme activity data are plotted in Figure 1.

Oxygen Uptake and Specific Enzyme Activity Data*

Medium: Maltose (No. 4).

Assay: Succinic dehydrogenase.

Enzyme: Cell-free preparation (mitochondria + supernatant).

Method: Phenazine methosulfate.

| Time in minutes | Run 1 | | Run 2 | |
|---|---|---|---|---|
| | $\mu l O_2$ | $\mu l O_2$ per mg protein | $\mu l O_2$ | $\mu l O_2$ per mg protein |
| 5 | 9.2 | 13.1 | 11.1 | 15.9 |
| 10 | 13.8 | 19.8 | 13.95 | 20.0 |
| 15 | 21.1 | 30.2 | 21.21 | 30.4 |
| 20 | 28.8 | 41.2 | 30.08 | 42.8 |
| 25 | 34.2 | 49.0 | 35.2 | 50.4 |
| 30 | 38.19 | 54.8 | 38.82 | 55.5 |
| 35 | 43.4 | 62.0 | 44.8 | 64.2 |
| 40 | 49.4 | 70.5 | 49.99 | 71.5 |
| 45 | 53.2 | 76.0 | 54.5 | 78.0 |
| 50 | 56.4 | 80.5 | 59.5 | 85.0 |
| 55 | 59.6 | 85.0 | 63.2 | 90.5 |

*For complete description of the system see Table 3; enzyme activity data are plotted in Figure 1.

Oxygen Uptake and Specific Enzyme Activity Data*

Medium: Nitrate (No. 3) + 0.5% Y.E.

Assay: Succinic dehydrogenase.

Enzyme: Cell-free preparation (mitochondria + supernatant).

Method: Phenazine methosulfate.

| Time in minutes | Run 1 | | Run 2 | |
|---|---|---|---|---|
| | $\mu l O_2$ | $\mu l O_2$ per mg protein | $\mu l O_2$ | $\mu l O_2$ per mg protein |
| 5 | 11.22 | 65.5 | 2.1 | 11.0 |
| 10 | 23.3 | 121.0 | 13.3 | 70.0 |
| 15 | 37.2 | 195.0 | 25.9 | 137.0 |
| 20 | 49.3 | 259.0 | 35.7 | 188.0 |
| 25 | 59.6 | 314.0 | 44.1 | 232.0 |
| 30 | 69.2 | 364.0 | 50.4 | 266.0 |
| 35 | 76.0 | 400.0 | 56.7 | 297.0 |
| 40 | 84.6 | 445.0 | 63.0 | 332.0 |
| 45 | 91.6 | 483.0 | 68.0 | 357.0 |
| 50 | 100.2 | 528.0 | 73.5 | 388.0 |
| 55 | 106.2 | 588.0 | 78.5 | 413.0 |

*For complete description of the system see Table 3; enzyme activity data are plotted in Figure 1.

Oxygen Uptake and Specific Enzyme Activity Data*

Medium: Nitrate (No. 3) + 0.5% Y.E.

Assay: Succinic dehydrogenase.

Enzyme: Mitochondrial preparation.

Method: Phenazine methosulfate.

| Time in minutes | Run 1 | | Run 2 | |
|---|---|---|---|---|
| | $\mu l O_2$ | $\mu l O_2$ per mg protein | $\mu l O_2$ | $\mu l O_2$ per mg protein |
| 5 | 10.85 | 10.85 | 8.25 | 8.25 |
| 10 | 51.6 | 51.6 | 39.9 | 39.9 |
| 15 | 88.2 | 88.2 | 79.2 | 79.2 |
| 20 | 127.5 | 127.5 | 114.5 | 114.5 |
| 25 | 161.5 | 161.5 | 144.5 | 144.5 |
| 30 | 197.0 | 197.0 | 175.0 | 175.0 |
| 35 | 218.0 | 218.0 | 202.0 | 202.0 |

*For complete description of the system see Table 4; enzyme activity data are plotted in Figure 2.

Oxygen Uptake and Specific Enzyme Activity Data*

Medium: 4% malt extract (No. 1).

Assay: Succinic dehydrogenase.

Enzyme: Mitochondrial preparation.

Method: Phenazine methosulfate.

| Time in minutes | Run 1 | | Run 2 | |
|---|---|---|---|---|
| | $\mu l O_2$ | $\mu l O_2$ per mg protein | $\mu l O_2$ | $\mu l O_2$ per mg protein |
| 5 | 2.75 | 1.53 | 0.7 | 0.388 |
| 10 | 11.7 | 6.5 | 11.2 | 6.2 |
| 15 | 24.7 | 13.7 | 20.3 | 11.3 |
| 20 | 34.4 | 19.1 | 30.1 | 16.7 |
| 25 | 44.0 | 24.4 | 37.1 | 20.6 |
| 30 | 53.6 | 29.6 | 44.1 | 24.5 |
| 35 | 61.2 | 34.0 | 50.4 | 28.2 |
| 40 | 68.8 | 38.2 | 56.6 | 31.5 |
| 45 | 75.0 | 41.7 | 61.6 | 34.2 |
| 50 | 80.5 | 44.6 | 67.2 | 37.4 |
| 55 | 86.0 | 47.8 | 74.2 | 41.1 |
| 60 | 90.0 | 50.0 | 77.0 | 42.7 |
| 65 | 95.5 | 53.0 | 81.2 | 45.0 |
| 70 | 99.0 | 55.0 | 82.6 | 46.0 |

*For complete description of the system see Table 4; enzyme activity data are plotted in Figure 2.

Oxygen Uptake and Specific Enzyme Activity Data*

Medium: Maltose (No. 4).

Assay: Succinic dehydrogenase.

Enzyme: Mitochondrial preparation.

Method: Phenazine methosulfate.

| Time in minutes | Run 1 | | Run 2 | |
|---|---|---|---|---|
| | $\mu l O_2$ | $\mu l O_2$ per mg protein | $\mu l O_2$ | $\mu l O_2$ per mg protein |
| 5 | 10.61 | 3.85 | 6.9 | 2.51 |
| 10 | 21.8 | 7.95 | 16.75 | 6.08 |
| 15 | 37.8 | 13.7 | 27.8 | 10.1 |
| 20 | 53.5 | 19.4 | 40.05 | 14.5 |
| 25 | 63.7 | 23.2 | 47.5 | 17.2 |
| 30 | 74.1 | 26.9 | 55.8 | 20.3 |
| 35 | 83.6 | 30.4 | 64.2 | 23.3 |
| 40 | 94.99 | 34.5 | 72.0 | 26.2 |
| 45 | 102.0 | 37.2 | 79.8 | 29.1 |
| 50 | 114.0 | 41.5 | 88.0 | 32.0 |
| 55 | 121.0 | 44.0 | 93.0 | 33.8 |

*For complete description of the system see Table 4; enzyme activity data are plotted in Figure 2.

Oxygen Uptake and Specific Enzyme Activity Data*

Medium: 4% malt extract (No. 1).

Assay: Succinic dehydrogenase.

Enzyme: The supernatant obtained after centrifugation at 24,000 x g for 20 minutes.

Method: Phenazine methosulfate.

| Time in minutes | Run 1 | | Run 2 | |
|---|---|---|---|---|
| | $\mu l O_2$ | $\mu l O_2$ per mg protein | $\mu l O_2$ | $\mu l O_2$ per mg protein |
| 5 | 2.59 | 11.4 | --- | --- |
| 10 | 4.32 | 19.0 | --- | --- |
| 15 | 6.56 | 28.8 | --- | --- |
| 20 | 6.92 | 30.4 | --- | --- |
| 25 | 7.78 | 34.0 | 0.7 | 3.07 |
| 30 | 12.1 | 53.0 | 3.5 | 15.4 |
| 35 | 12.1 | 53.0 | 5.6 | 24.5 |
| 40 | 15.55 | 68.0 | 6.3 | 27.6 |
| 45 | 18.15 | 79.5 | 7.7 | 33.8 |
| 50 | 19.0 | 83.5 | 8.4 | 36.8 |
| 55 | 18.15 | 79.5 | 7.7 | 33.8 |
| 60 | 19.9 | 87.5 | 9.1 | 39.8 |

*For complete description of the system see Table 5; enzyme activity data are plotted in Figure 3.

Oxygen Uptake and Specific Enzyme Activity Data*

Medium: Nitrate (No. 3) + 0.5% Y.E.

Assay: Succinic dehydrogenase.

Enzyme: The supernatant obtained after centrifugation at 24,000 x g for 20 minutes.

Method: Phenazine methosulfate.

| Time in minutes | Run 1 | | Run 2 | |
|---|---|---|---|---|
| | $\mu l O_2$ | $\mu l O_2$ per mg protein | $\mu l O_2$ | $\mu l O_2$ per mg protein |
| 5 | --- | --- | 1.47 | 7.75 |
| 10 | 5.26 | 27.7 | 8.08 | 42.5 |
| 15 | 13.16 | 69.0 | 13.95 | 73.5 |
| 20 | 20.4 | 107.0 | 22.0 | 116.0 |
| 25 | 27.7 | 146.0 | 27.2 | 143.0 |
| 30 | 34.2 | 180.0 | 32.3 | 170.0 |
| 35 | 39.5 | 208.0 | 38.9 | 205.0 |
| 40 | 45.4 | 238.0 | 44.8 | 236.0 |
| 45 | 50.0 | 263.0 | 48.5 | 255.0 |
| 50 | 54.0 | 284.0 | 52.0 | 278.0 |
| 55 | 58.6 | 307.0 | 57.4 | 302.0 |

*For complete description of the system see Table 5; enzyme activity data are plotted in Figure 3.

Oxygen Uptake and Specific Enzyme Activity Data*

Medium: 4% malt extract (No. 1) + 28.2 mg Na-IAA.

Assay: Succinic dehydrogenase.

Enzyme: Mitochondrial preparation.

Methods: (a) Run 1:- Cytochrome c.

(b) Run 2:- Phenazine methosulfate.

| Time in minutes | Run 1 | | Run 2 | |
|---|---|---|---|---|
| | $\mu l O_2$ | $\mu l O_2$ per mg protein | $\mu l O_2$ | $\mu l O_2$ per mg protein |
| 5 | --- | --- | 4.13 | 1.76 |
| 10 | 4.87 | 2.07 | 13.2 | 5.6 |
| 15 | 8.35 | 3.55 | 21.3 | 9.05 |
| 20 | 12.5 | 5.3 | 32.4 | 13.8 |
| 25 | 18.8 | 8.0 | 44.6 | 19.0 |
| 30 | 24.4 | 10.4 | 55.7 | 23.7 |
| 35 | 28.5 | 12.1 | 69.5 | 29.5 |
| 40 | 32.0 | 13.6 | 82.0 | 34.8 |
| 45 | 34.8 | 14.8 | 95.0 | 40.5 |
| 50 | 36.2 | 15.4 | 104.5 | 44.6 |
| 55 | 38.3 | 16.3 | 114.2 | 48.5 |
| 60 | 41.7 | 17.8 | 126.5 | 54.0 |

*For complete description of the system see Table 6; enzyme activity data are plotted in Figure 4.

Oxygen Uptake and Specific Enzyme Activity Data*

Medium: 4% malt extract (No. 1) + 28.2 mg Na-IAA.

Assay: Succinic dehydrogenase and IAA oxidase.

Enzyme: Cell-free preparation (mitochondria + supernatant).

Method: Cytochrome c.

Run 1: Enzyme + 0.5 ml 0.025M sodium succinate.

Run 2: Enzyme + 0.5 ml 0.025M sodium salt of indole acetic acid.

| Time in minutes | Run 1 | | Run 2 | |
|---|---|---|---|---|
| | $\mu l O_2$ | $\mu l O_2$ per mg protein | $\mu l O_2$ | $\mu l O_2$ per mg protein |
| 10 | 2.59 | 2.76 | 5.6 | 5.95 |
| 15 | 6.05 | 6.45 | 6.3 | 6.7 |
| 20 | 7.77 | 8.3 | 8.4 | 8.95 |
| 25 | 11.2 | 11.9 | 9.8 | 10.4 |
| 30 | 13.8 | 14.7 | 11.9 | 12.7 |
| 35 | 16.4 | 17.5 | 13.3 | 14.1 |
| 40 | 18.15 | 19.3 | 14.0 | 14.9 |
| 45 | 19.9 | 21.2 | 15.4 | 16.4 |
| 50 | 19.0 | 20.2 | 14.0 | 14.9 |
| 55 | 19.9 | 21.2 | 15.4 | 16.4 |
| 60 | 20.6 | 21.9 | 16.1 | 17.1 |

*For complete description of the system see Tables 6 and 7; enzyme activity data are plotted in Figures 4 and 5.

## APPENDIX D

## MATHEMATICAL TREATMENT OF THE DATA FROM A TYPICAL WARBURG EXPERIMENT

Conditions:

The inner wells of the vessels contained 0.4 ml 20 per cent KOH solution.

The final liquid volume was 3.4 ml.

| | Sample | | Blank | | Thermobarometer | |
|---|---|---|---|---|---|---|
| Vessel No. | 10 | | 13 | | 15 | |
| Contents | Succinic acid + Enzyme +PMS | | Water + Enzyme + PMS | | 3.0 ml water | |
| Time in minutes | Reading mm | Change mm | Reading mm | Change mm | Reading mm | Change mm |
| 0 | 152.0 | ---- | 150.5 | ---- | 149.5 | ---- |
| 30 | 123.0 | -29.0 | 148.0 | -2.5 | 159.5 | +10.0 |
| 60 | 97.0 | -55.0 | 142.0 | -8.5 | 160.0 | +10.5 |

True change in sample vessel at 30 minutes =

[(mm change in sample reading) - (mm change in thermobarometer reading)]

- [(mm change in blank reading) - (mm change in thermobarometer reading)]

$= ( -29.0 - 10.0 ) - ( -2.5 - 10.0 )$

$= -29.0 - 10.0 + 2.5 + 10.0$

$= -29.0 + 2.5$

$= -26.5$ mm

The negative sign indicates oxygen uptake in the sample vessel. Therefore at 30 minutes the oxygen taken up in the sample vessel is:

$\mu l O_2$ = (Constant for vessel No. 10) x (True change in mm)

$= 1.375 \times 26.5$

$= 36.4$

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# VITA

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