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A study of succinic dehydrogenase, indole acetic acid oxidase, nitrate reductase and cytochrome c reductase in Hygrophorus Conicus

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A STUDY OF SUCCINIC DEHYDROGENASE, INDOLE ACETIC ACID OXIDASE, NITRATE REDUCTASE AND CYTOCHROME c REDUCTASE IN *HYGROPHORUS CONICUS*

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A STUDY OF SUCCINIC DEHYDROGENASE, INDOLE ACETIC ACID OXIDASE, NITRATE REDUCTASE AND CYTOCHROME c REDUCTASE IN *HYGROPHORUS CONICUS*

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ABSTRACT

The activity of the enzyme succinic dehydrogenase (succinate : phenazine methosulfate oxidoreductase, EC 1.3.99.1) of *Hygrophorus conicus*, a member of a class of fungi known as basidiomycetes, was found to be stimulated by nitrate. Two substances were tested as electron acceptors in the measurement of succinic dehydrogenase activity. Of the two substances tried, phenazine methosulfate and brilliant cresyl blue, the former was found to be the best. Nicotinamide adenine dinucleotide (NAD^+) was a necessary cofactor for the enzyme and could be readily dissociated from the enzyme. The enzyme has the properties of a high molecular weight nucleo- or lipoprotein.

The presence of two kinds of succinic dehydrogenase in *H.conicus* was demonstrated. One was precipitated at 10% ammonium sulfate saturation and the other at 40%.

The succinic dehydrogenase which precipitated at 10% ammonium sulfate saturation was purified 13 fold. It showed a pH optimum of 7 to 7.1 at 30°, it had a Michaelis constant for succinate of 24 mM at pH 7.6 and 30°. The enzyme was inhibited by malonate and o-phenanthroline

but not by pyrophosphate. The enzyme was comparatively labile, losing about 70% activity after about one month at -15°.

Attempts to purify succinic dehydrogenase which precipitated at 40% ammonium sulfate saturation were unsuccessful. This enzyme showed a pH optimum of 6.7.

Nitrate reductase (NADPH : nitrate oxidoreductase, EC 1.6.6.3) of H.conicus was found to be an inducible enzyme. The enzyme was specific for NADPH. The activity of nitrate reductase was lost after a period of one year. This loss is attributed either to genetic changes in the organism or to the inhibition of the enzyme by nitrite. It is believed that the nitrate reductase of H.conicus is not involved in the utilization of nitrate as a nitrogen source such as the case in Neurospora crassa but resembles more the nitrate reductase observed in Escherichia coli grown anaerobically. Nitrate is used strictly as a terminal electron acceptor in the oxidation of substrate in the latter case.

The enzyme cytochrome c reductase (EC 1.6.99.3) of H.conicus was also found to be induced by nitrate.

Attempts to make an active cell-free preparation of the enzyme indole acetic acid (IAA) oxidase of H.conicus were unsuccessful. On these grounds it is believed that the enzyme is not a simple oxygenase but may require certain, as yet unknown, cofactors for its optimum activity.

The oxidation of IAA by the enzyme IAA oxidase of H.conicus produces at least two more products besides oxindole acetic acid. Attempts to separate and characterize these products were unsuccessful. It is suggested that one of the products has a close structural relationship with o-formaminobenzoyl acetic acid while the other may be 5-hydroxy oxindole acetic acid.

It was concluded that H.conicus has a great facility for detoxification of toxic substances present in the medium by either stimulating or inducing oxidative enzymes.

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LIST OF ABBREVIATIONS

Abbreviations used in this investigation are as follow.

BCB	Brilliant cresyl blue
DNA	Deoxyribonucleic acid
FAD	Flavin-adenine dinucleotide
$FADH_2$	Reduced form of flavin-adenine dinucleotide
IAA	Indole acetic acid
NAD^+	Nicotinamide-adenine dinucleotide
NADH	Reduced form of nicotinamide-adenine dinucleotide
NADPH	Reduced form of nicotinamide-adenine dinucleotide phosphate
OIAA	Oxindole acetic acid
PMS	Phenazine methosulfate
RNA	Ribonucleic acid
Tris	Tris (hydroxymethyl) amino methane

I. INTRODUCTION

The Krebs cycle is widely distributed in nature and occurs in the respiring tissues of animals, higher plants, amd microorganisms. In living organisms this cycle has considerable significance from the standpoint of energy production. The enzymes which catalyze the reactions of the Krebs cycle are for the most part associated with the mitochondria.

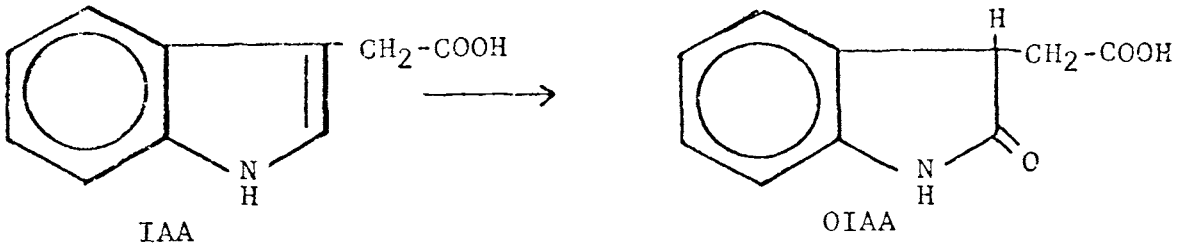
Succinic dehydrogenase (succinate : phenazine methosulfate oxidoreductase, EC 1.3.99.1), an enzyme of the Krebs cycle has been found in many plants and fungi. It catalyzes the dehydrogenation of succinic acid to fumaric acid. Succinic dehydrogenase is thought to be closely associated with the mitochondria and is difficult to solubilize. As a member of the Krebs cycle, it participates in terminal oxidations and serves as one of the points of linkage between the electron transport and oxidative phosphorylation systems in aerobic metabolism (Singer and Kearney, 1963).

In a previous study with Hygrophorus conicus, a member of the class of fungi known as basidiomycetes, the enzyme succinic dehydrogenase was found to be present in the mitochondria as well as in the 24,000 x g supernatant of a cell-free preparation. It was observed that the production of the enzyme was stimulated by growing H.conicus in a medium containing nitrate.

The enzyme nitrate reductase (NADPH : nitrate oxidoreductase, EC 1.6.6.3) had been found in plants and microorganisms which utilize inorganic nitrate. The enzyme is inducible. A correlation between nitrate reductase and cytochrome c reductase (EC 1.6.99.3) has been found in Neurospora crassa by Kinsky and McElroy (1958).

Nitrate has been reported to be utilized as a terminal electron acceptor in certain anaerobic or partially anaerobic organisms. Although the growth of H.conicus was poor on the nitrate medium, the succinic dehydrogenase activity of nitrate grown cells was greater than cells grown on other media. If one assumes that nitrate is toxic for H.conicus one approach to the detoxification of nitrate by the organism would be the reduction to nitrite. The reduction would require a source of electrons and a rapid method of supplying these electrons would be the oxidation of succinic acid by succinic dehydrogenase. A possible correlation between succinic dehydrogenase and nitrate reductase in H.conicus was therefore sought.

The IAA oxidase of H.conicus is an inducible, intracellular enzyme. The product of the oxidation of IAA by H.conicus is oxindole acetic acid (OIAA).



Patterson (1965) found that the concentration of OIAA increases in the culture medium for 48 hours and then decreases indicating further degradation by the microorganism. The nature of these further degradation products of IAA was of interest especially in light of the observations of Ray and Thimann (1955) and Hinman and Lang (1965) that methyloxindoles were the end-products of IAA metabolism.

The objectives of this investigation were as follows:

To isolate and partially purify the succinic dehydrogenase and nitrate reductase from H.conicus and study their properties; to determine if the stimulation of succinic dehydrogenase activity by nitrate in basidiomycetes was a common occurrence, and to isolate and characterize additional metabolic products of IAA in H.conicus.

II. LITERATURE REVIEW

A. KREBS CYCLE

Krebs in 1937 proposed that the oxidation of pyruvic acid to carbon dioxide and water was a cyclic reaction. This reaction was named the Krebs cycle in honor of him. It is also known as the tricarboxylic acid 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 living organisms this cycle has considerable significance from the standpoint of energy production. The enzymes which catalyze the reactions in this cycle are the condensing enzyme, aconitase, isocitrate dehydrogenase, α-ketoglutarate dehydrogenase, succinic thiokinase, succinic dehydrogenase, fumarase, and malate 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 (Singer and Kearney, 1963). It has been shown that the various enzymes of the cycle are present in the mitochondria of different cells in different amounts but in nearly constant proportion to each other. Although all the enzymes are probably mitochondrial, they are not integrated into the mitochondrial ultrastructure to the same extent. Three groups can be distinguished in order of difficulty of detachment: (a) succinic dehydrogenase; (b) α-ketoglutarate dehydrogenase; (c) all the remainder; with group c being the most easily detached (Mahler and Cordes, 1966a).

Until a few years ago the existance of a functional Krebs cycle in fungi was in serious doubt, but now it has been shown that this cycle

does occur in fungi. Particulate matter containing various enzymes of the Krebs cycle have been isolated from Allomyces macrogynus, Candida albicans, Fusarium lini, Aspergillus cryzae, Myrothecium verrucaria, Neurospora crassa and other fungi (Niederpruem, 1965). Various workers (Barron and Ghiretti, 1953; Staples, 1957; Wessels, 1959; Niederpruem and Hackett, 1961; Newcomb and Jennison, 1962) have shown the presence of some or all of the individual enzymes of the Krebs cycle in the following basidiomycete species: Merulius niveus, Merulius tremellosus, Polyporus palustris, Puccinia graminis and Schizophyllum commune.

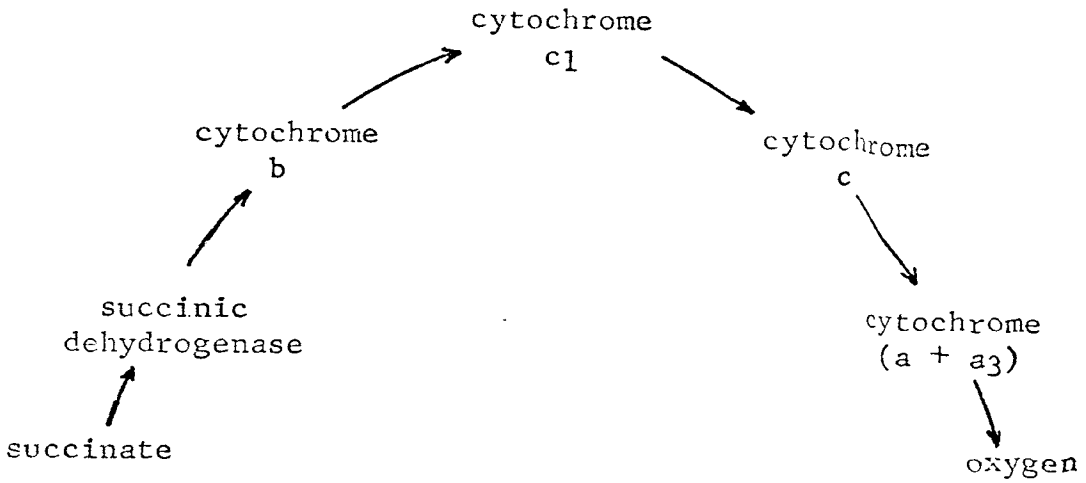
B. SUCCINIC DEHYDROGENASE

In the early studies which preceded the identification of the cyclic nature of the oxidation of pyruvate by Krebs, it was recognized that succinate was rapidly converted to fumarate by suspensions of minced muscle. The enzyme catalyzing this reaction was termed succinic dehydrogenase and was subsequently shown to be an important member of the Krebs cycle enzymes.

$$\text{succinic acid} + \text{FAD} \underset{\text{succinic dehydrogenase}}{\rightleftharpoons} \text{fumaric acid} + \text{FADH}_2$$

Succinic dehydrogenase catalyzes the anaerobic oxidation of succinate. It requires an electron and a hydrogen acceptor (Singer and Kearney, 1963). The molecular weight of succinic dehydrogenase is about 200,000.

The succinoxidase system is the system which catalyzes aerobic oxidation of succinate in the presence of cytochromes b, c, a, a_3 and succinic dehydrogenase (a flavin system). The pathway of electrons from succinate to oxygen may be represented as:



Keilin and Hartree 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" (Dixon and Webb, 1964).

Slater (1949) observed that the reduction of cytochrome c by cytochrome b in succinoxidase system is readily inhibited by 2,3-dimercaptopropanol, diethyldithiocarbamate and cysteine. These observations led Slater to postulate the existence of an intermediary carrier between cytochromes b and c. This is known as the Slater factor. He also reported that the same factor acts between diaphorase and cytochrome c. Investigations carried out in the last few years (Lehninger, 1964a) proved that coenzyme Q is an intermediary between cytochromes b and c.

The activity of succinic dehydrogenase has been detected in every aerobic organism in which it has been sought. As mentioned earlier, the enzyme is generally found in the mitochondria, however, there are reports in the literature of soluble forms of succinic dehydrogenase (McDonald et al, 1963; Singer et al, 1956 and 1957).

In the assay of succinic dehydrogenase numerous electron acceptors have been used (Singer and Kearney, 1963) and it has been observed that succinic dehydrogenases from various sources react with these electron acceptors with varying degrees of efficiency.

McDonald et al (1963) studied the succinic dehydrogenase from the fungus Claviceps purpurea. They found that their preparation was a soluble form of the enzyme and it reacted with PMS but not with other electron acceptors such as methylene blue, triphenyl tetrazolium chloride, 2,6-dichlorophenolindophenol, ferricyanide and mammalian cytochrome c. The succinic dehydrogenase preparations from beef heart and from yeast (Singer et al, 1956) also catalyzed electron transfer from succinate to PMS, but not to several other dyes (such as methylene blue), or to cytochrome c. It was suggested that methylene blue and PMS react at different points in the enzyme system (Singer and Kearney, 1963).

Succinate oxidation activity of a mitochondrial preparation from a basidiomycete S.commune was reported by Niederpruem and Hackett (1961). They used cytochrome c as an electron acceptor. No succinate oxidation activity was found in the particulate fraction obtained by hand grinding and centrifugation at 10,000 x g for 20 minutes. Wessels (1959) reported the presence of succinic dehydrogenase in a cell-free preparation of S.commune. This preparation also could utilize cytochrome c as an electron acceptor.

Shepherd (1951) showed the presence of succinic dehydrogenase in a cell-free preparation of Neurospora and found that the enzyme could utilize cytochrome c efficiently as a hydrogen carrier. The enzyme had a double pH optima of 6.9 and 8.1.

The cell-free preparation of H.conicus succinic dehydrogenase could not utilize mammalian cytochrome c efficiently as an electron acceptor (Mehta, 1966).

The succinic dehydrogenase of Aspergillus niger was found to be attached to particulate matter and had a pH optimum of 7.3. The enzyme was quite labile, loosing 53 to 93% of its activity in 22 hours at -40° (Martin, 1954).

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.

In order to study an enzyme such as succinic dehydrogenase it is desirable to free it from the cells of the organism. With an intracellular enzyme this involves the rupturing of the cell-walls. The methods which have proven effective in liberating enzymes from microbial cells have been largely mechanical rupture of the cell-wall and membrane. The method of choice depends on several factors including the quantity of cells, and the sensitivity and the localization of the enzyme within cell (Cunsalus, 1955).

The following procedures are generally used for the rupturing of cells. A mechanical pressure method consists of forcing a liquid suspension at high pressure through a very fine aperture, which causes the disruption. One fortunate aspect of this method is that it does not require the addition of abrasives to the suspension. However, this method has not been employed extensively because it is not applicable to large quantities of cells.

The cell suspension can also be treated with sonic waves either alone or with finely powdered abrasives in a specially designed vessel. Generally the treatment time varies between 10 and 30 minutes depending on the cells to be ruptured. This method has proven successful in breaking ordinarily difficult-to-rupture cells.

A technique in which the pressure on the cell suspension is released suddenly after compressing a water soluble gas (nitrous oxide) also results in the cell rupture. This technique is known as "pressure release". It can be applied to small volumes, 5 to 10 ml, and to dilute cell suspensions, 10^8 cells per ml. The method is applicable to heavier cell suspensions, but the percentage of cells ruptured decreases. The procedure has been especially useful in releasing particulate cellular components.

Another method involves the mechanical shaking (300 to 3,000 oscillations per minute) of the cell suspension with regular small particles (50 to 500μ size). Important variables in this method are the size and uniformity of the particles, the speed of shaking, and the nature and density of the cell suspension. The Waring blender, ball mill, and other homogenizer can be used in this method.

Either manual or mechanical grinding is also employed with cell pastes, frozen cell pastes, or dried cells. Abrasives are generally used in this procedure. The abrasives used are powdered glass, sand, alumina, and glass beads. One criterion for effective grinding is an abrasive of uniform particle size and particles of a size approximately that of the cells to be ruptured.

Regardless of the method used to rupture the cell-wall, a cardinal rule for handling all enzyme extracts, is to keep the material at low temperatures, 0 to 4°, during all manipulations.

After rupturing the cell-wall, it is necessary to separate the cell-wall, the nucleus, the mitochondria, the microsomes, and the endoplasmic reticulum from one another and the soluble fraction. With advances in high speed centrifugation, it is possible to fractionate the cell particles released during the rupture of the cell. The nuclei and cell-walls can be removed from a cell-free preparation at centrifugal speeds of 1,000 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). After this separation one can measure the enzymic activity in the desired fraction.

It had been mentioned earlier that the enzyme succinic dehydrogenase is generally associated with the mitochondria. The mitochondria or large granules as these are called, are smaller than the nucleus, yet visible under an ordinary high power microscope. Although the mitochodria may vary considerably in number, size, and shape in different cells, their ultrastructure is sufficiently similar and distinct from that of other organelles so as to make their unambiguous identification easy in most cases (Mahler and Cordes, 1966b).

The most salient feature of mitochondrial structure is the membrane system. It consists of a relatively smooth outer membrane, and intermembranous space, chamber, or compartment, and a particularly high structured inner membrane. These features are intricately involved in the control and channeling of enzymic pathways within the mitochondrion and its metabolic interactions with the surrounding cytoplasmic matrix (Lehninger, 1964b).

The mitochondria contain a significant amount of lipid and phospholipid. It has been found (West et al, 1966) that rat liver mitochondria contained 3.5 to 4% protein, 25 to 30% lipid, 16.5 to 19.8% phospholipid, 11.7% and 19 to 46% of total cell nucleic acid as DNA and RNA respectively. Besides these the mitochondria also contained vitamins (B_2, B_6, B_{12}), FAD, and coenzyme A (Neilands and Stumpf, 1958).

The mitochondria are the sites of intracellular energy production and transduction. Most of the enzymes of the Krebs cycle and certain accessory oxidative enzymes, such as the pyruvic dehydrogenase complex and the β-oxidation system for fatty acids appear to be localized on the outer membrane, which also controls mitochondrial permeability. The inner membrane appears to be the site of the enzymes of the electron-transport chains. Here electrons from NADH and succinate are transported to molecular oxygen, and oxidative phosphorylation takes place. It is not yet possible to specify completely the nature of all the electron carriers and catalytic proteins involved in the electron-transport chain. However, there is considerable evidence that the sequence of electron transfer involves a flavin system and cytochromes b, c, a and a_3. The pathway of electrons from succinate to oxygen is described on page 6 (Mahler and Cordes, 1966c).

C. NITRATE REDUCTASE

Besides the more common sequence of electron transport described previously, nitrate may be used by certain organisms as a terminal electron acceptor in place of oxygen. Usually this takes place under anaerobic or partially anaerobic conditions. Because of its physiological and enzymological similarity to aerobic respiration, this process is usually spoken as nitrate respiration or dissimilatory nitrate reduction.

Since many aerobic microorganisms utilize nitrate as their sole nitrogen source a second type of process which converts nitrate to ammonia or amino acid also exists. This process is called nitrate assimilation or assimilatory nitrate reduction (Fewson and Nicholas, 1961a).

Nicholas and Nason (1954) have shown that FAD and molybdenum function as electron carriers in Neurospora nitrate reductase as follow.

NADPH ⟶ FAD ⟶ Mo ⟶ nitrate

Since succinic dehydrogenase is a FAD enzyme and since FAD is an electron carrier in the case of nitrate reductase, the study of the nitrate reductase along with succinic dehydrogenase is interesting.

Workers in a number of laboratories have identified nitrate reductase in fungi as well as in bacteria and plants. The enzyme is found in both a soluble and in a particulate form (Nason, 1963) and it requires either NADH, NADPH or in some cases both as cofactors. An interrelationship between the enzymes cytochrome c reductase and nitrate reductase has been observed.

The nitrate reductase of Escherichia coli K-12 was found in a particulate fraction of the cell (Showe and DeMoss, 1968). They reported that the enzyme synthesis was depressed by aeration in the presence or absence of nitrate. However, under anaerobic conditions the rate of the enzyme synthesis depended on the amount of nitrate present in the medium.

Cove (1966) studied the nitrate reductase in the fungus A.nidulans and found that the enzyme was induced by nitrate but repressed by ammonium ions. The rate of induction of the enzyme was not directly proportional to mycelial mass. He also reported the presence of two cytochrome c reductases, one was associated with nitrate reductase activity while the other was not.

A parallel induction of assimilatory type nitrate reductase and cytochrome c reductase was observed in N.crassa by Kinsky and McElroy (1958). The simultaneous induction of both of these enzymes in N.crassa was also reported by Sorger (1965). He concluded on the basis of his observations that: "Nitrate reductase is an aggregate of two polypeptides. One transports electrons from NADPH to FAD and from there to cytochrome c (cytochrome c reductase) and another accepts electrons from the reduced FAD in the first polypeptide, passes them to molybdate, and from there to nitrate (nitrate reductase)".

The nitrate reductase of Nitrobacter agilis (Straat and Nason, 1965) used reduced cytochrome c as an electron donor. The enzyme contained cytochromes c, b, a and a_1 as determined spectrophotometrically.

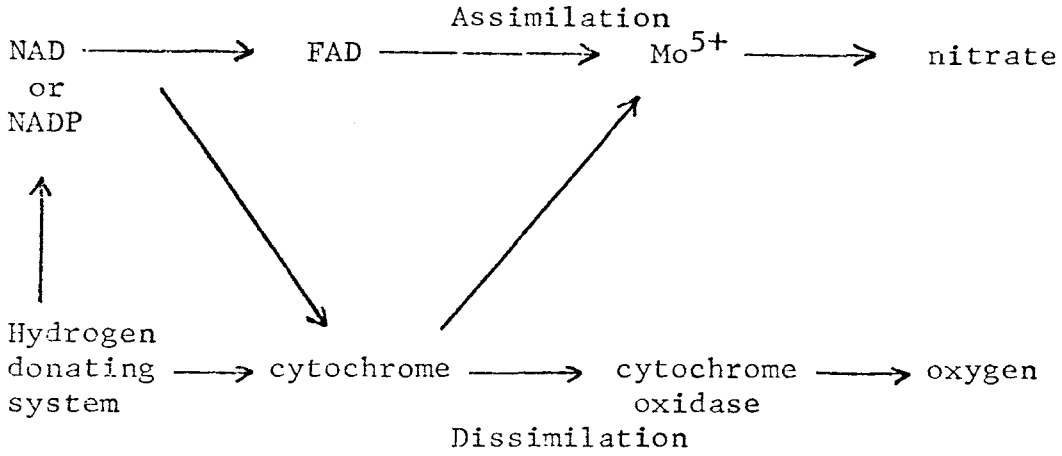
Nitrate reductase has also been found in apple roots (Grasmanis and Nicholas, 1967).

Sato and Niwa (1952) studied the nitrate reductase in E.coli and emphasized the iron-protein nature of the enzyme. They reported that cytochrome b is closely associated with the nitrate reducing mechanism.

Several attempts have been made to purify nitrate reductase with varying degrees of success. Cove and Coddington (1965) purified the nitrate reductase and cytochrome c reductase of A.nidulans 300 fold using ammonium sulfate, DEAE-cellulose and DEAE-Sephadex.

The respiratory type nitrate reductase of Pseudomonas aeruginosa was purified about 115 fold using ammonium sulfate, calcium phosphate gel, and electrophoresis (Fewson and Nicholas, 1961b). The enzyme contained FAD, cytochrome c, and molybdenum and was NADH dependent. They proposed that the assimilatory type nitrate reductase is composed of

flavin and molybdenum while that of dissimilatory type contains cytochrome. Their hypothesis is summarized as follows:



They also suggested that nitrate reductase freed from the hydrogen or electron transfer system contains molybdenum only and is devoid of flavin.

About a 10 fold purification was obtained in the case of a soluble respiratory type nitrate reductase of E.coli (Itagaki and Taniguchi, 1959). The enzyme was completely inactive with NADH, had a pH optimum of 6.4 and a K_m of 7.5×10^{-5}M. The enzyme was stable only when frozen in the presence of 10^{-4}M cystine.

Kinsky and McElroy (1958) studied nitrate reductase activity in N.crassa. They were able to purify the enzyme over 70 fold using ammonium sulfate fractionation. They found that the purified nitrate reductase preparation contained an active NADPH-cytochrome c reductase.

Taniguchi and Itagaki (1960) compared the nitrate reductases of E.coli and N.crassa. They concluded that the physiological function of nitrate reductase of E.coli was nitrate respiration while that of N.crassa was nitrate assimilation.

D. INDOLE ACETIC ACID OXIDASE

Since enzymes are commonly named for the substance on which they act, a class of enzymes which brings above the inactivation of indole-3-acetic acid (IAA) by oxidation are referred to collectively as IAA oxidases. These enzymes are widely distributed in plants (Hare, 1964). The metabolic products obtained by the oxidation of IAA vary from species to species. Some of the products have been characterized, but still there are a few which remain unidentified (Ray and Thimann, 1956; Stutz, 1958; Siehr, 1961).

Goldschmidt et al (1967) described IAA oxidase activity in citrus plants. They studied the interrelationship between 2,4-dichlorophenol, Mn^{2+} and the enzyme activity. 2,4-Dichlorophenol was found to inhibit the enzyme at the concentration of 10^{-3}M, however, no inhibitory effect was observed in the presence of Mn^{2+}.

Hinman and Lang (1965) studied the oxidation of IAA catalyzed by peroxidase. The auxin properties of IAA can be destroyed by treatment with hydrogen peroxide and peroxidase (Hare, 1964). This has led to the belief that IAA is inactivated in plants by a non-specific peroxidase. They found that the oxidation of IAA was concentration dependent. At substrate concentrations of 2×10^{-4}M and below 3-methyleneoxindole was the major product, however, at higher concentrations a neutral indole was found to be the principal product.

Still et al (1965) found that extracts of pea seedlings oxidize IAA to 3-hydroxymethyloxindole. At physiological pH this compound is dehydrated to 3-methyleneoxindole which in turn is reduced enzymatically to 3-methyloxindole.

King et al (1963) studied indole metabolism in the rat. They found 6-hydroxyoxindole as the product in which oxindole was an intermediate.

The oxidation of IAA by crystalline Japanese radish peroxidase a and c in the presence of H_2O_2 was studied by Morita et al (1962). The enzymes functioned optimally at pH 3.6 and 3-4.8 respectively. Spectrophotometric evidence was obtained for methyldioxindole and indole carboxaldehyde as metabolic products of IAA.

Ray and co-workers (1962, 1960, 1956, 1955) studied the IAA oxidase of Omphalia flavida. The enzyme has a pH optimum of 3.5, was moderately stimulated by Mn^{2+} but there was no stimulation by 2,4-dichlorophenol. The reaction product of IAA oxidation was a mixture of at least four components, one of which was identified as 3-methyldioxindole. This group also studied the kinetics of the IAA oxidation and they proposed a free radical mechanism.

Tonhazy and Pelczar (1954) studied the IAA oxidase of Polyporus versicolor. The enzyme did not require Mn^{2+} or monophenols for activity. The enzyme was specific for IAA, and had a pH optimum of 4.5. The end-product of the reaction had not been identified but a possibility of indole-3-aldehyde was suggested as an end-product.

Siehr (1961) isolated oxindole acetic acid as a product of IAA oxidation by an enzyme in the basidiomycete, H.conicus.

Stutz (1958) found that indole-3-carboxaldehyde was a major reaction product of IAA oxidation in Lupinus albus provided the system is coupled to the cytochrome-cytochrome oxidase system. He also suggested the presence of anthranilic acid and an aliphatic compound in the reaction mixture. From the data he concluded that the ring system, not the side chain, is the primary site of oxidation in the case of indoles.

Partially purified enzyme preparation of wheat leaves destroyed physiological activity of IAA oxidatively in the presence of Mn^{2+} and a monohydroxyphenol or resorcinol (Waygood et al, 1956). They found that maleic hydrazide and a naturally occurring ether soluble factor from wheat leaves can substitute for the active phenols, while catechol, hydroquinone, pyrogallol, scopoletin, and riboflavin competitively inhibit the oxidation.

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, yeast extract, agar	Difco Chemical Company Detroit, Michigan
Bovine serum albumin, glucose, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, glutathione, nicotinamide-adenine dinucleoxide, phenazine methosulfate, N-naphthyl ethylene diamine dihydrochloride	Sigma Chemical Company St. Louis, Missouri
p-dimethyl amino benzaldehyde, Folin phenol reagent	Fisher Scientific Company St. Louis, Missouri
X-ray film	General Electric Company St. Louis, Missouri
$^{14}C_1$- and $^{14}C_2$-indole acetic acid (0.1 mc per 10 ml)	New England Nuclear Corporation Boston, Massachusetts

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 Hygrophorus conicus was obtained from the Abbott Laboratories, North Chicago, Illinois, and the cultures of Coprinus lagopus were obtained from Dr. P. R. Day, The Connecticut Agricultural Experiment Station, New Haven, Connecticut.

B. APPARATUS

Centrifuges: International Equipment Company, Boston, Massachusetts. Model CL.

Lourdes Instrument Corporation, Brooklyn, New York. Model LCA-1.

Colorimeter: Bausch and Lomb Incorporated, Spectronic 20, Rochester, New York.

French Press Compression Chamber (No. 4-3398): American Instrument Company, Incorporated, Silver Springs, Maryland.

Hydraulic Press: Wabash Metal Products Company, Wabash, Indiana. Model 12-105.

pH Meter: Leeds and Northrup Company, Philadelphia, Pennsylvania. Model 7664.

Rotary Evaporator: Rinco Instrument Company, Greenville, Illinois.

Radiochromatogram Scanner: Packard Instrument Company, Downers Grove, Illinois. Model 7201.

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

Sonifier: Branson Instruments Incorporated, Danbury, Connecticut. Model S-75.

Spectrophotometers: Beckman Instruments, Incorporated, Fullerton, California. Model DK-2A, automatic scanning.

Perkin Elmer Corporation, New York, New York. Model 139, manual.

Sterilizer, Steam Heat: American Sterilizer Company, Erie, Pennsylvania.

Ultra Violet Light: Ultra Violet Products Incorporated, South Pasadena, California. Model SL-2537 - Short wave length.

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

C. MEDIA FOR HYGROPHORUS CONICUS

The following media were used.

Malt extract medium: 4% malt extract in deionized water.

Nitrate medium: 30 g glucose; 3 g $NaNO_3$; 1 g K_2HPO_4; 0.5 g $MgSO_4.7H_2O$; 0.5 g KCl; 0.01 g $FeSO_4$; 100 μM thiamine in 1 liter of deionized water.

0.5% Yeast extract medium: 30 g glucose; 5 g yeast extract; 1 g K_2HPO_4; 0.5 g $MgSO_4.7H_2O$; 0.5 g KCl; 0.01 g $FeSO_4$; 100 μM thiamine in 1 liter of deionized water.

Yeast extract medium: 30 g glucose; 8.85 g yeast extract; 1 g K_2HPO_4; 0.5 g $MgSO_4.7H_2O$; 0.5 g KCl; 0.01 g $FeSO_4$; 100 μM thiamine in 1 liter of deionized water.

Neurospora medium: 20 g sucrose; 5 g ammonium tartrate; 1 g NH_4NO_3; 1 g KH_2PO_4; 0.5 g $MgSO_4.7H_2O$; 0.1 g NaCl; 0.1 g $CaCl_2$; 8.8 x 10^{-5} g ammonium molybdate; 9.6 x 10^{-4} g $FeCl_3.6H_2O$; 8.8 x 10^{-3} g $ZnSO4.7H_2O$; 2.7 x 10^{-4} g $CuCl_2$; 7.2 x 10^{-5} g $MnCl_2.4H_2O$; 5 x 10^{-6} g biotin in 1 liter of deionized water.

D. MEDIA FOR COPRINUS LAGOPUS

The following media were used.

Minimal medium: 20 g glucose; 2 g asparagine; 25 ml salt solution; 1 ml thiamine solution; 40 ml trace element solution, deionized water to 1 liter.

Complete medium: 20 g glucose; 2 g asparagine; 25 ml salt solution; 1 ml thiamine solution; 0.75 g yeast extract; 0.75 g hydrolyzed casein; 0.60 g malt extract; 1.25 ml hydrolyzed nucleic acid, deionized water to 1 liter.

Minimal-nitrate medium: Asparagine and ammonium tartrate were replaced with an equivalent amount of sodium nitrate. All other salts are same as shown in minimal medium.

Salt solution: 10 g ammonium tartrate; 20 g KH_2PO_4; 45 g K_2HPO_4; 5.6 g Na_2SO_4, deionized water to 500 ml.

Trace element solution: 1 g $CaCl_2$; 4.1 g $MgCl_2$; 0.531 g ferric citrate; 0.531 g citric acid; 0.443 g $MgSO_4.7H_2O$; 0.405 g $ZnSO_4.7H_2O$, deionized water to 400 ml.

Thiamine solution: 10 g thiamine hydrochloride, deionized water to 100 ml.

Hydrolyzed nucleic acid: One gram deoxyribonucleic acid and 1 g ribonucleic acid was suspended in 15 ml 1N sodium hydroxide and 1 g of each nucleic acid in 15 ml 1N hydrochloric acid. The two mixtures were autoclaved at 15 psi for 10 minutes, the hydrolysates mixed, the pH adjusted to 6.0, filtered, made up to 40 ml with deionized water and stored at 4° with 1 ml of chloroform.

E. METHODS AND RESULTS

1. Cultivation of *Hygrophorus conicus*

Three hundred milliliter erlenmeyer flasks containing 60 ml of a particular medium, or 500 ml erlenmeyer flasks containing 100 ml of medium were autoclaved at 120° and 15 psi for 20 minutes. After cooling to room temperature the flasks were kept in a cold room until used.

Stock cultures of *H.conicus* were grown at 25° in one of the previously mentioned media (see page 21) for four days. These cultures of *H.conicus* were prepared once a month by inoculation of the autoclaved medium from a culture grown on a malt-agar slant, and were maintained by transferring 3 ml of mycelium suspension every three days to 60 ml of fresh medium. Immediately after inoculation the stock culture flasks were placed on a rotary shaker (240 rpm) where they remained for three days before serving as inoculum for fresh medium.

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% malt extract solution to which 2% agar was added. The medium was prepared by adding the agar to the malt extract solution and heating the mixture until the agar was dissolved. The hot medium was poured into culture tubes and autoclaved. Following sterilization, liquefied medium was poured into sterile petri dishes, or allowed to cool and solidify in the tubes as 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. Growth of Hygrophorus conicus

Several 300 ml erlenmeyer flasks containing 60 ml of the various media previously described (see page 21) were prepared and autoclaved. The flasks were inoculated with 3 ml of a three day old stock culture of H.conicus. The flasks were harvested at different times by centrifuging the mycelial suspensions.

The cells obtained by centrifugation were transferred to gooch crucibles which had been previously dried to a constant weight in an oven at 105°. The cells and gooch crucibles were dried at 105° until they reached constant weight (about 20 hours) and the increase in weight was determined. The average dried mycelium weights from four or more individual flasks are tabulated in Table 1; Figure 1 is the growth curve of H.conicus in various media.

3. Growth of Coprinus lagopus

The media for C.lagopus are described on page 22. The organism was grown either on surface culture or in shake flasks. For surface growth the organism was transferred from a slant using a platinum wire loop to 500 ml erlenmeyer flasks containing 100 ml medium. The flasks were incubated at 37° in an incubator for approximately 5 days.

The method for submerged growth was the same as described for H.conicus.

4. Disintegration of the cell-wall and Preparation of Cell-Free Extracts of Hygrophorus conicus

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

a. Sonication: The submerged mycelium from eight or nine 300 ml flasks was combined and harvested by centrifugation at 1,000 x g

TABLE 1

GROWTH OF HYGROPHORUS CONICUS

IN DIFFERENT MEDIA

Medium	Weight of dried mycelium after 48 hrs. of growth mg per 60 ml medium*
Nitrate	118
Nitrate + 0.5% yeast extract	937
0.5% yeast extract	652
0.885% yeast extract	1157
4% malt extract	215
Neurospora	600

*Average of 4 individual flasks.

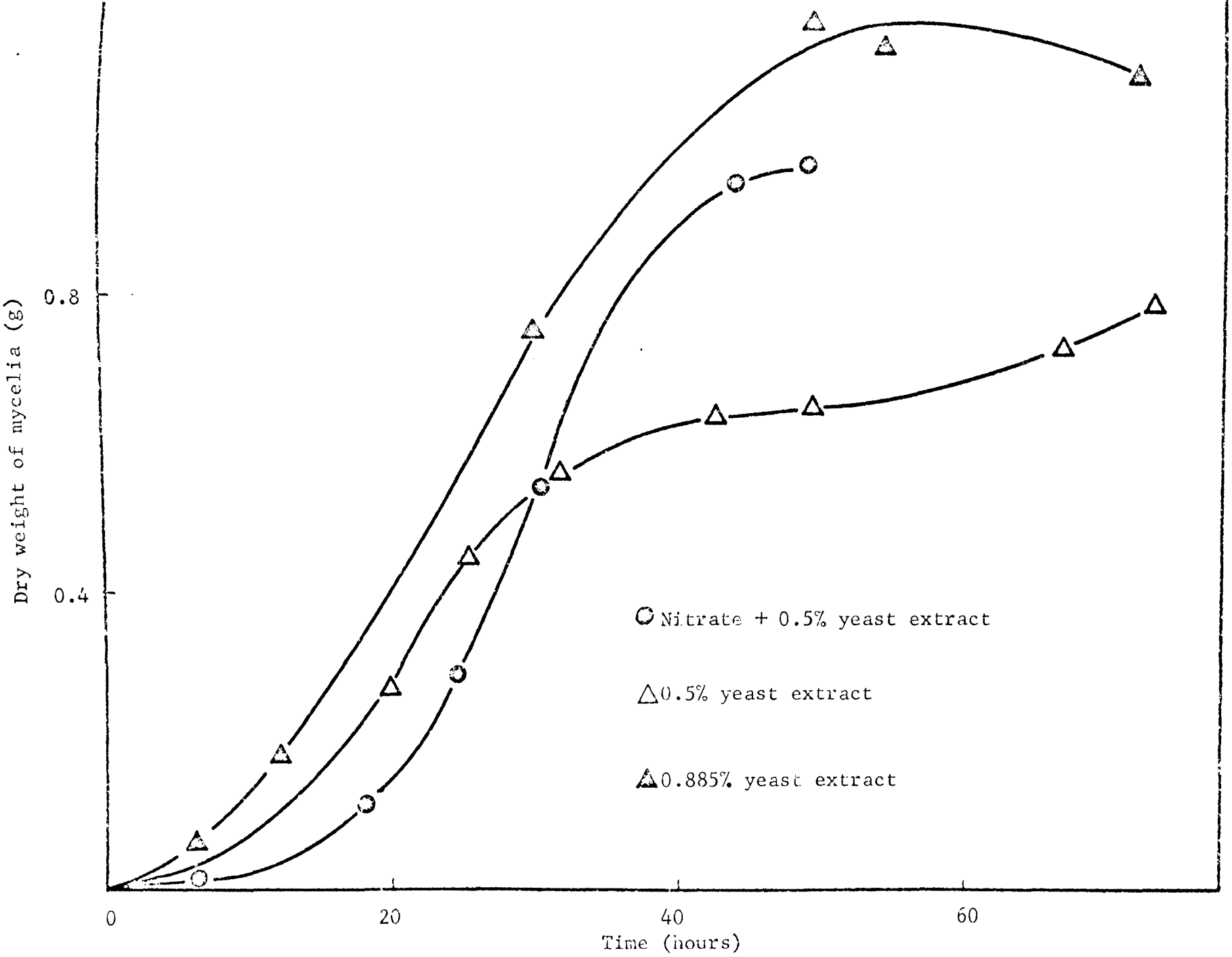


Figure 1. Growth of Hygrophorus conicus in Different Media

for 20 minutes and washed twice with deionized water. The cells were suspended in 0.01M phosphate buffer, pH 7 and the suspension was exposed to the maximum output from a Branson sonicator for 10 minutes. The Rosett cell (Branson Instruments, 1963) was kept in ice cold water bath during this operation. The homogenate was centrifuged at 1,000 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 primarily mitochondria was resuspended in 3-4 ml 0.01M phosphate buffer, pH 7.

b. French Press Technique: The submerged mycelium from eight or nine 300 ml flasks containing 60 ml of medium was combined, and washed twice with 0.01M phosphate buffer, pH 7. The cells were suspended in about 50 ml 0.01M phosphate buffer, pH 7 and the suspension was transferred to the compression chamber which was kept in the cold room (4-6°) overnight. The compression chamber was placed on a hydraulic press and subjected to a pressure of 10,000 psi. The homogenate was forced out from the compression chamber through the control valve and simultaneously pressure was applied on the compression chamber in such a way that the pressure was maintained at about 10,000 psi. The homogenate was collected in a beaker surrounded with ice and it was centrifuged at 1,000 x g for 20 minutes to remove the unbroken cells. The cell-free supernatant was centrifuged at either 20,000 x g or 24,000 x g for 20 minutes. The residue obtained after 24,000 x g centrifugation (assumed to be mitochondria) was suspended in 3-4 ml of 0.01M phosphate buffer, pH 7.

The 20,000 x g supernatant was used for the measurement of either nitrate reductase activity when the mycelia were grown in the nitrate medium or IAA oxidase activity when the mycelia were grown in 4% malt extract medium in the presence of Na-IAA. The 24,000 x g supernatant and the mitochondrial suspension were used for the measurement of succinic dehydrogenase activity.

5. Preparation of Cell-Free Extracts of Coprinus lagopus

a. From Surface Culture: The surface mycelium from 3-4 flasks was recovered by draining the medium. The mycelium was washed twice with deionized water and transferred to a cold Waring blender. It was blended at high speed for 10 seconds with about 20 ml 0.01M phosphate buffer, pH 7. The suspension was then transferred to a cold compression chamber and the extract was prepared using the French press technique as described on page 27 .

b. From Submerged Culture: The mycelium from 3-4 flasks recovered by centrifugation at 1,000 x g for 20 minutes, was washed twice with deionized water, and the cell-free preparation was made as described in the previous paragraph.

6. 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% 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 the case of the mitochondrial preparation) or 1.5 ml deionized water. The enzyme preparation was made as described on page 27. To the side arms of each vessel were added 0.3 ml succinate, 0.1 ml PMS, and 0.3 ml KCN solution. Each vessel was immediately connected to its manometer and the stopcock was closed in order to prevent escape of HCN gas. Excess HCN gas was absorbed by the KOH solution which was in the inner well of the vessel. The pressure was released by momentarily opening the stopcock. One vessel which contained 3.2 ml deionized water in the main compartment and 0.2 ml of 20% KOH solution 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° 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. 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).

Oxygen uptake in $\mu l = h \times k$

where:

h = The observed change in the manometer reading in mm

k = Flask constant

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

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) minus 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°).

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

d = Solubility of oxygen in reaction liquid.

7. The Activity of Succinic Dehydrogenase in Hygrophorus conicus

The mycelium of H.conicus was harvested by centrifugation and the enzyme preparations were made as described on pages 24 and 27. 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 comparison regardless of the growth of the organism, the protein concentration in each enzyme preparation was determined using Folin Phenol Reagent method described by Lowry et al (1951). This method is described on page 69.

The systems studied are described in Tables 2 to 7 and the results obtained are given in Appendix where specific succinic dehydrogenase activities in $\mu l O_2$ per mg protein are reported for different time intervals. The specific activity data are also plotted in Figures 2 to 7. The $\mu l O_2$ taken up during a run was calculated as described by Umbreit et al (1964).

Q_{O_2} (N) values for the various enzyme preparations are tabulated in Table 8. The Q_{O_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 Q_{O_2}(N) values, 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 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 Q_{O_2}(N) value which is defined as the $\mu l O_2$ taken up per hour per mg protein.

The relative amount of protein and the Q_{O_2}(N) values for succinic dehydrogenase of H.conicus for various preparations are given in Table 9. Almost all protein and the enzyme activity was found in the 24,000 x g supernatant.

TABLE 2

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

paratus: Warburg respirometer

dia for organism growth:

(a) Nitrate

(b) Nitrate +0.5% yeast extract.

bstrate: Sodium succinate, 0.2M, pH 7.6.

zyme: Cell-free preparation (0.5 ml).

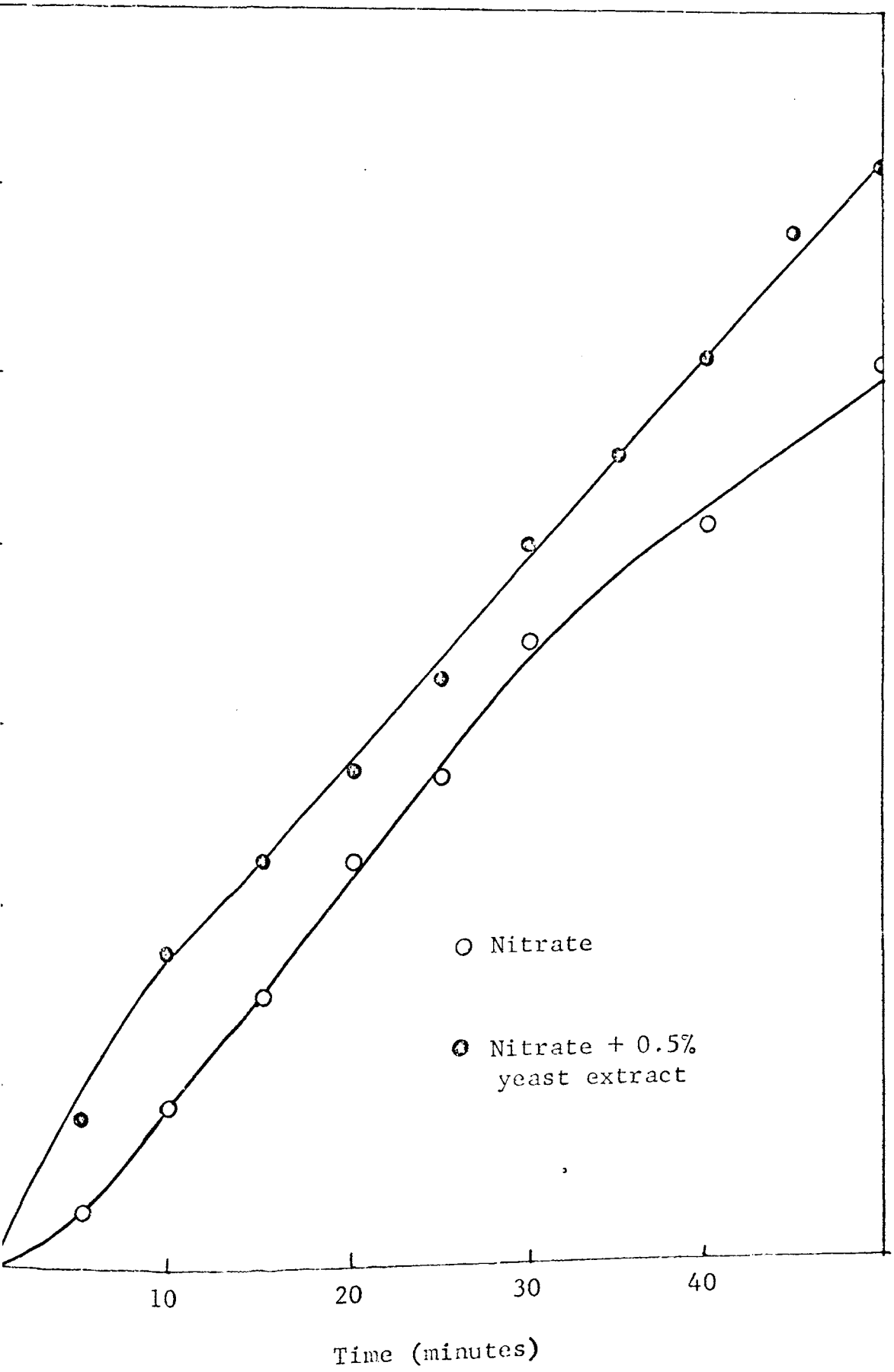
ectron acceptor: PMS, 1% in deionized water.

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

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

tal liquid volume: 3.4 ml.

lysis: Succinic dehydrogenase by the Phenazine Methosulfate assay method.



igure 2. Succinic Dehydrogenase Activity of the Cell-Free

TABLE 3

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

Apparatus: Warburg respirometer.

Media for organism growth:

(a) 4% malt extract

(b) 0.5% yeast extract

(c) 0.885% yeast extract.

Substrate: Sodium succinate, 0.2M, pH 7.6.

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.

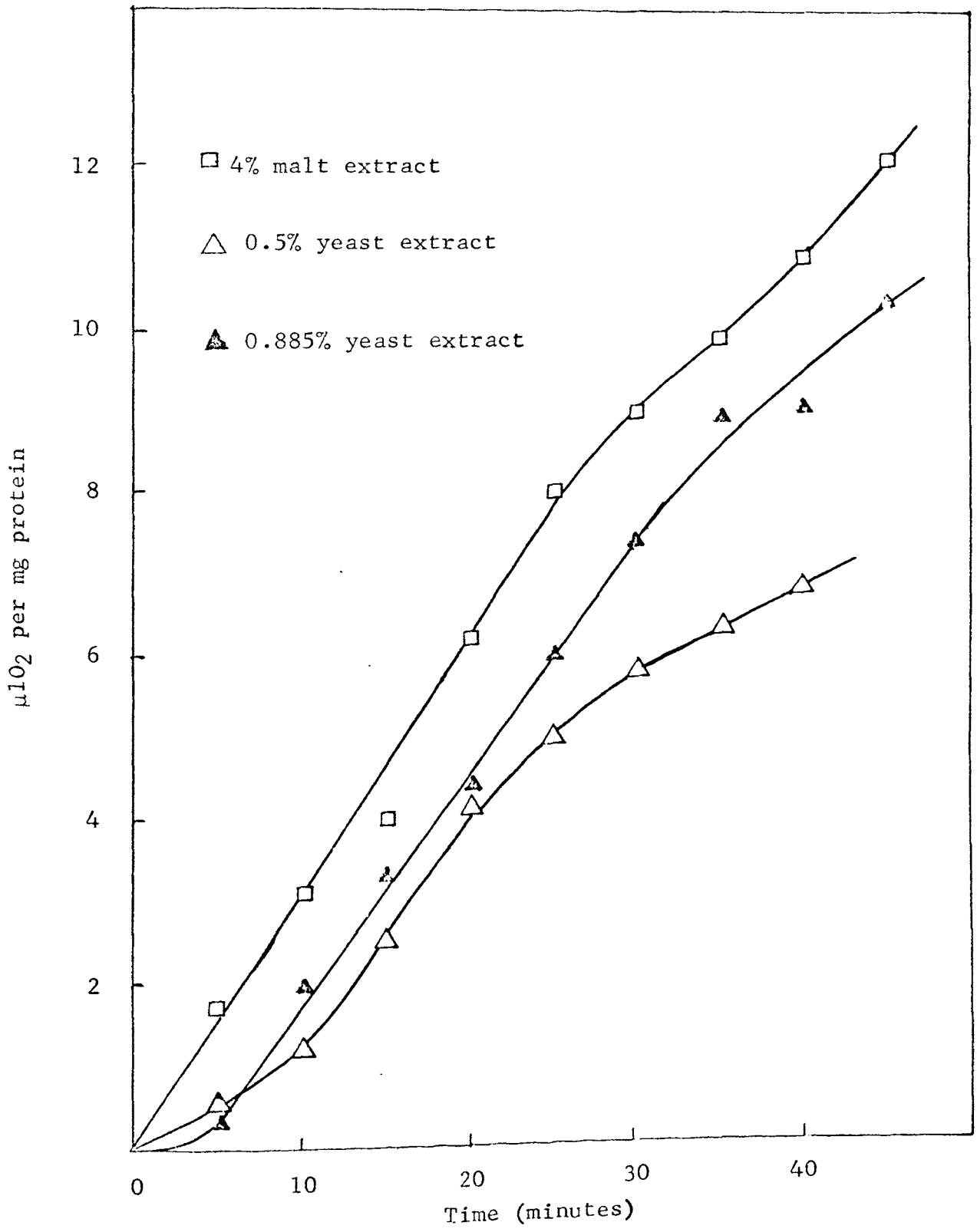


Figure 3. Succinic Dehydrogenase Activity of the Cell-Free Preparations as a Function of the Culture Medium

TABLE 4

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

Apparatus: Warburg respirometer.

Media for organism growth:

(a) Nitrate

(b) Nitrate + 0.5% yeast extract.

Substrate: Sodium succinate, 0.2M, pH 7.6.

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 Metholsulfate assay method.

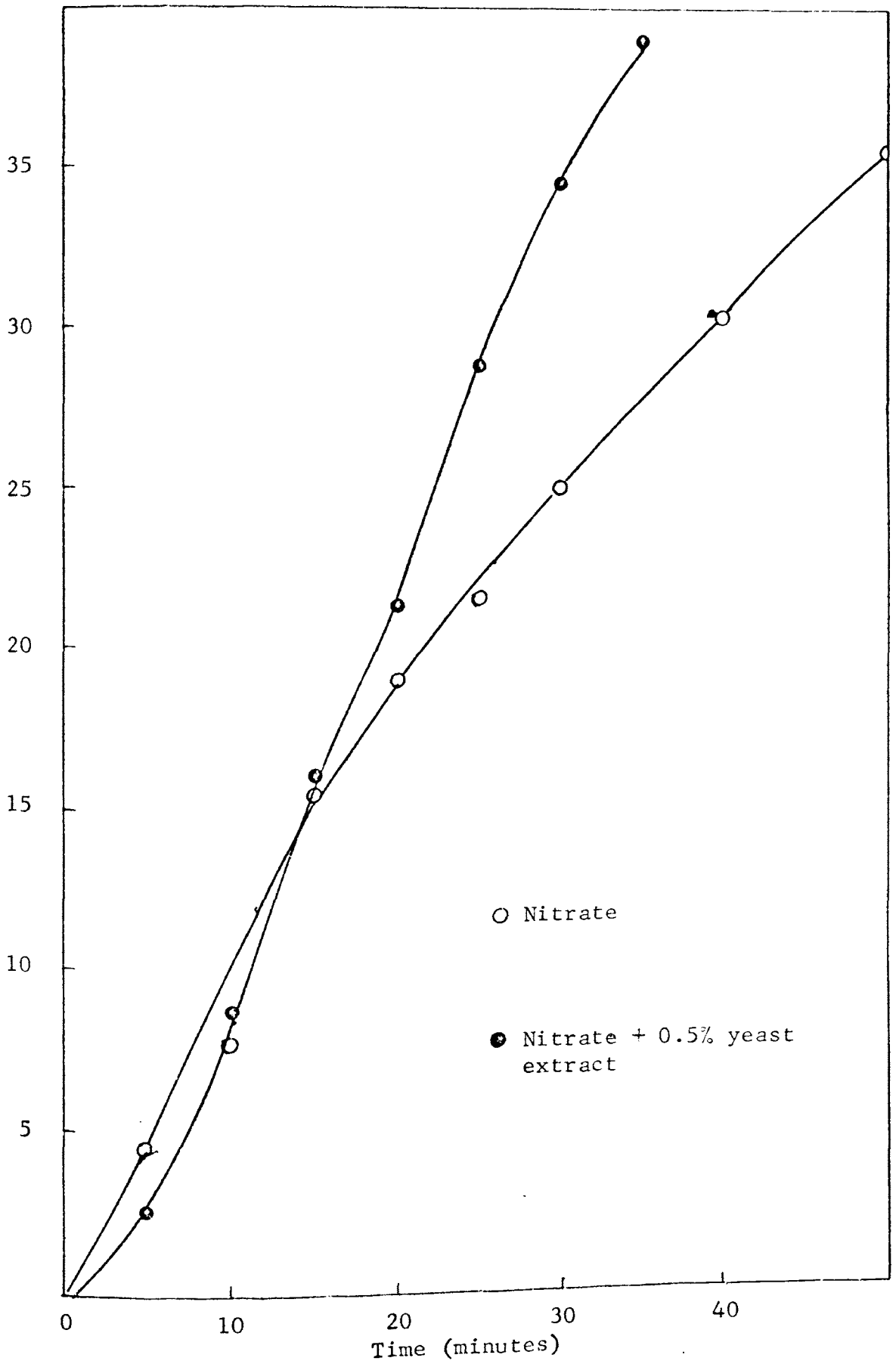


Figure 4. Succinic Dehydrogenase Activity of the Mitochondrial Preparations as a Function of the Culture Medium

TABLE 5

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

Apparatus: Warburg respirometer.

Media for organism growth:

(a) 4% malt extract

(b) 0.5% yeast extract

(c) 0.885% yeast extract.

Substrate: Sodium succinate, 0.2M, pH 7.6.

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.

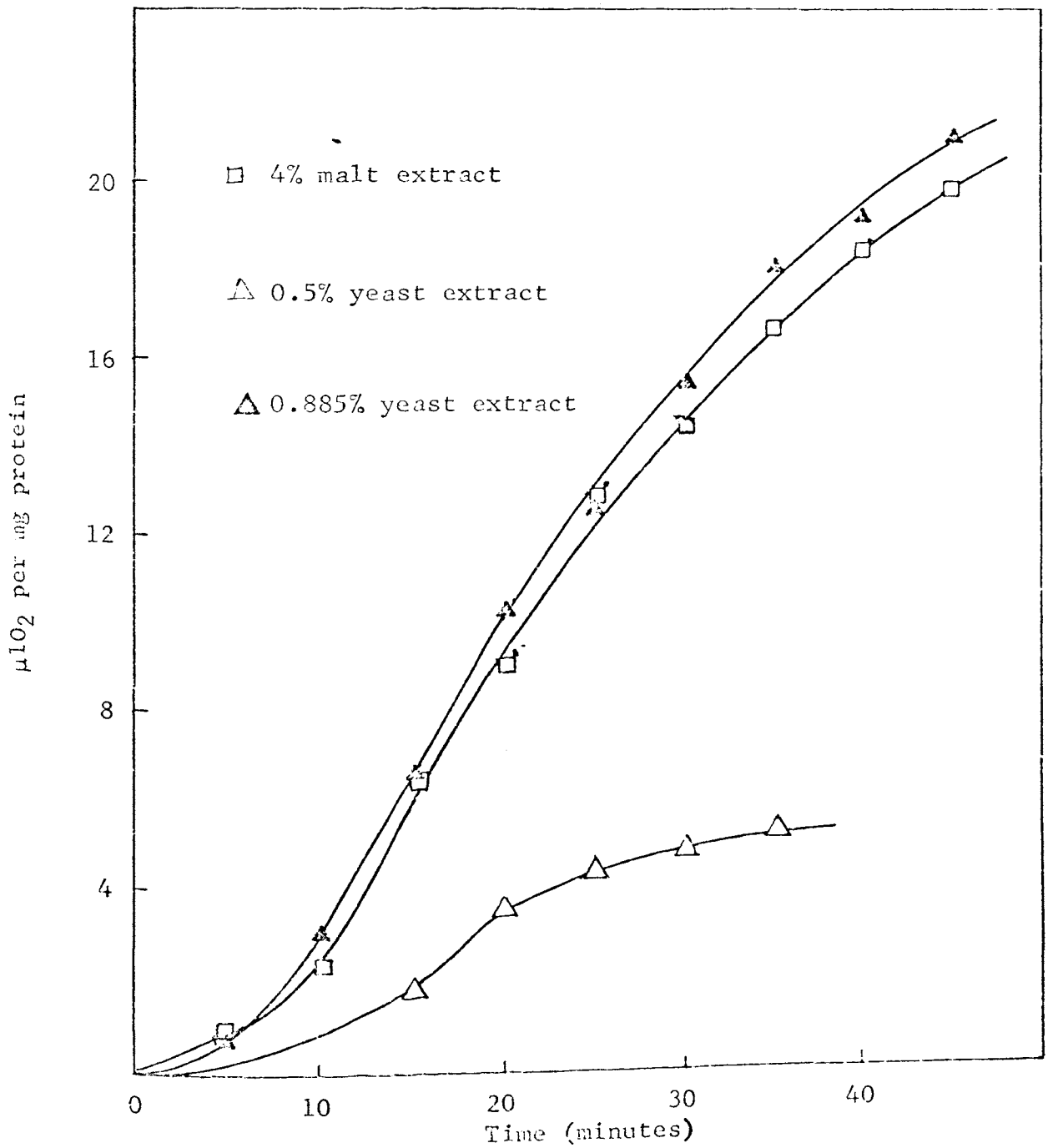


Figure 5. Succinic Dehydrogenase Activity of the Mitochondrial Preparations as a Function of the Culture Medium

TABLE 6

SUCCINIC DEHYDROGENASE ACTIVITY OF THE

24,000 x g SUPERNATANT AS A

FUNCTION OF THE CULTURE MEDIUM

Apparatus: Warburg respirometer.

Media for organism growth:

(a) Nitrate

(b) Nitrate + 0.5% yeast extract.

Substrate: Sodium succinate, 0.2M, pH 7.6.

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.

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.

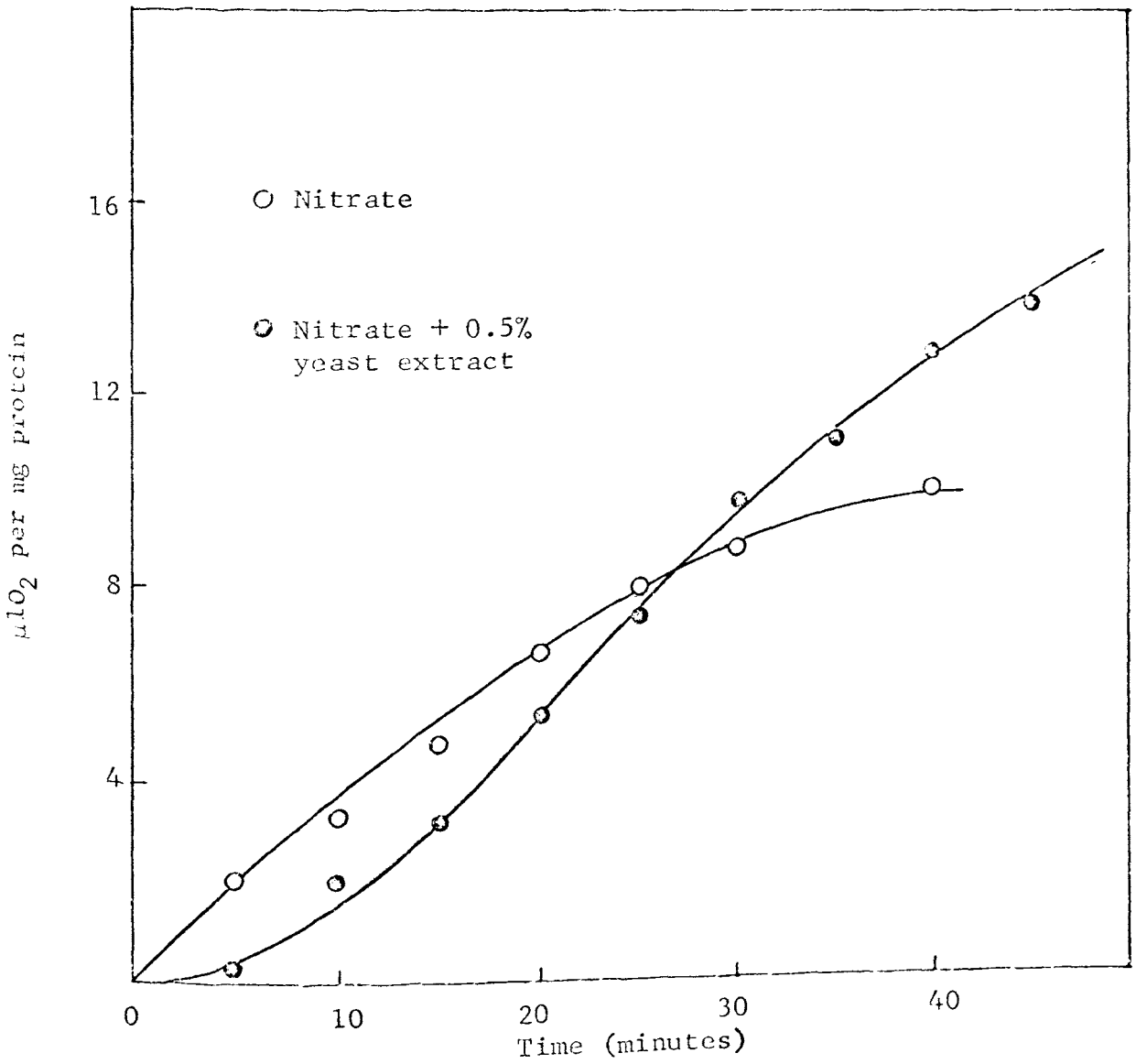


Figure 6. Succinic Dehydrogenase Activity of the 24,000 x g Supernatant as a Function of the Culture Medium

TABLE 7

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

Apparatus: Warburg respirometer.

Media for organism growth:

(a) 4% malt extract

(b) 0.5% yeast extract

(c) 0.885% yeast extract.

Substrate: Sodium succinate, 0.2M, pH 7.6.

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.

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.

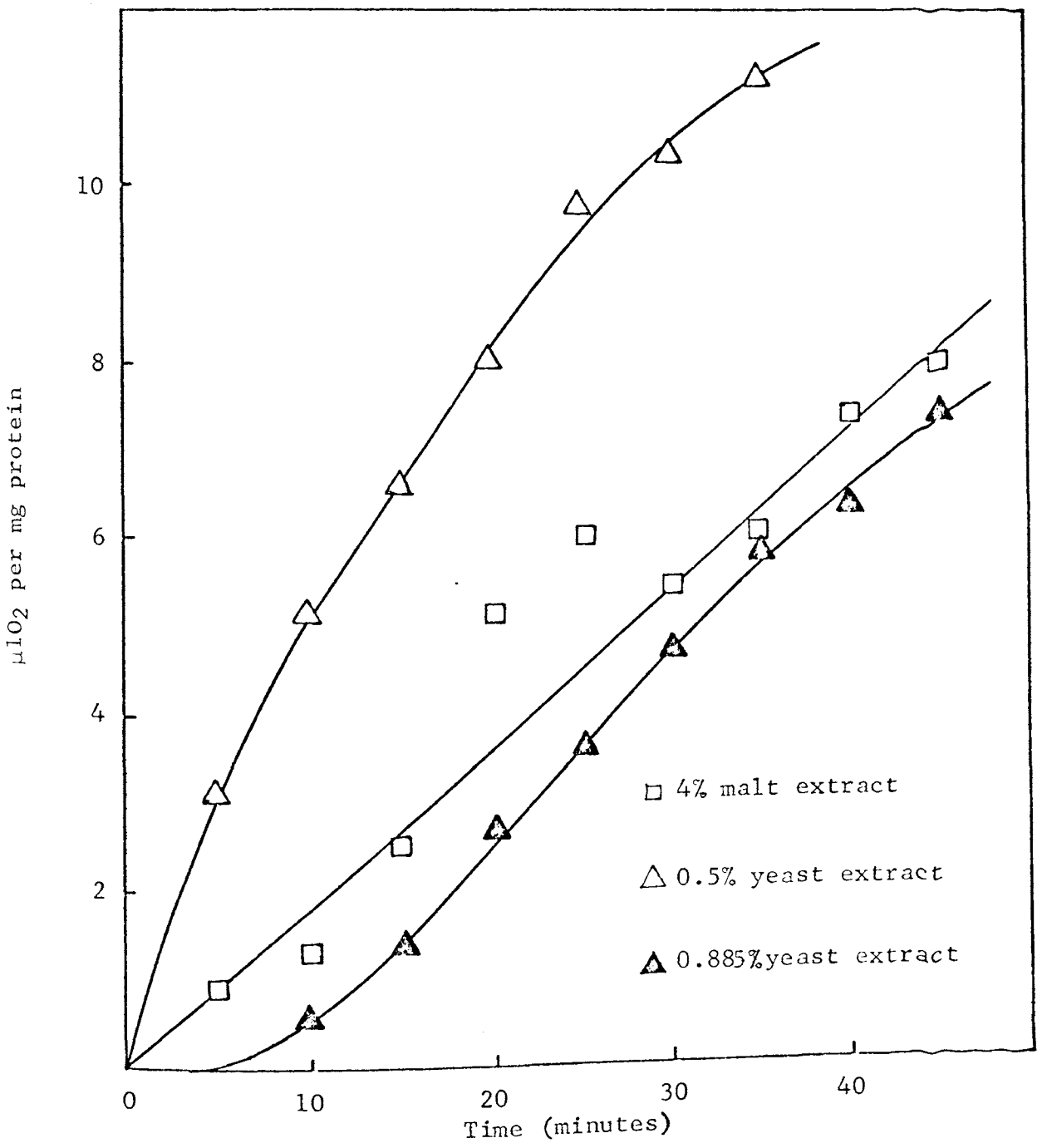


Figure 7. Succinic Dehydrogenase Activity of the 24,000 x g Supernatant as a Function of the Culture Medium

TABLE 8

$Q_{O_2}(N)$* VALUES FOR SUCCINIC DEHYDROGENASE OF

HYGROPHORUS CONICUS IN DIFFERENT MEDIA

Enzyme preparation	4% Malt extract	Nitrate	Nitrate + 0.5% yeast extract	0.5% yeast extract	0.885% yeast extract
Cell-free	18	26	37	14	18
Mitochondrial	34	41	81	38	35
24,000 x g supernatant	15	26	33	15	13

*$\mu l O_2$ per hour per mg protein.

TABLE 9

RELATIVE AMOUNTS OF PROTEIN AND THE SUCCINIC DEHYDROGENASE ACTIVITY IN THE ENZYME PREPARATIONS OF HYGROPHORUS CONICUS

Medium	Enzyme preparation	Total protein mg	$-Q_{O_2(N)}$ $\mu l O_2/hr/mg$ protein	Total $Q_{O_2(N)}$
Nitrate	Cell-free	262	26	6810
Nitrate	Mitochondrial	18	41	738
Nitrate	24,000 x g supernatant	228	26	5940

a. Effect of Niacin and Riboflavin on Succinic Dehydrogenase Activity of Hygrophorus conicus

H. Conicus was grown in nitrate medium to which 6 μM of either niacin or riboflavin was added per 60 ml of medium. The mycelium was harvested by centrifugation and the enzyme preparations were made using the French press. The PMS method was used to measure succinic dehydrogenase activity. The results are tabulated as $Q_{O_2}(N)$ values in Table 10 and are compared with the results of nitrate medium alone.

b. Comparison of Phenazine Methoaulfate and Brilliant Cresyl Blue as an Electron Acceptor for the Enzyme Succinic Dehydrogenase in Hygrophorus conicus

H.conicus was grown in either nitrate or nitrate containing 0.5% yeast extract medium. The cells were harvested by centrifugation and the enzyme preparations were made using the French press. The activity of succinic dehydrogenase was measured as described in the PMS assay method using either PMS or BCB as an electron acceptor. The $Q_{O_2}(N)$ values for all the systems studied are summarized in Table 11.

c. Comparison of Two Cell Rupturing Methods for Succinic Dehydrogenase in Hygrophorus conicus

Two methods were employed for rupturing the cells of H.conicus. They were (a) ultrasonic oscillations using a Branson sonifier, and (b) high pressure extrusion using the French press. Both of these methods are described on pages 24 and 27. The enzyme preparations were made as described previously. The succinic dehydrogenase activity was measured using PMS as an electron acceptor. The results of

TABLE 10

EFFECT OF NIACIN AND RIBOFLAVIN ON

SUCCINIC DEHYDROGENASE ACTIVITY OF HYGROPHORUS CONICUS

Enzyme preparation	$Q_{O_2}(N)$* Nitrate medium	Nitrate medium + niacin**	Nitrate medium + riboflavin**
Cell-free	26.4	23.8	29.15
Mitochondrial	41.4	38.9	43.6
24,000 x g supernatant	25.7	20.3	26

*$\mu l O_2$ per hour per mg protein.
**6μM per 60 ml medium.

TABLE 11

COMPARISON OF ELECTRON ACCEPTORS FOR

SUCCINIC DEHYDROGENASE ACTIVITY IN HYGROPHORUS CONICUS

Medium	Enzyme preparation	$Q_{O_2}(N)$* PMS	BCB
Nitrate	Cell-free	26.4	3.5
Nitrate	Mitochondrial	41.4	8.6
Nitrate	24,000 x g supernatant	25.7	8.6
Nitrate + 0.5% yeast extract	cell-free	37.2	5.5
Nitrate + 0.5% yeast extract	Mitochondrial	81.0	19.2
Nitrate + 0.5% yeast extract	24,000 x g supernatant	33.3	9.1

*$\mu l O_2$ per hour per mg protein.

experiments are tabulated in Table 12.

8. The Activity of Succinic Dehydrogenase in Coprinus lagopus

Two different compatible monokaryon cultures, H_1 and H_9, and the dikaryon H_1 x H_9 of C.lagopus were used for succinic dehydrogenase measurement. The organism was grown in different media as both surface and submerged cultures. The enzyme preparations were made as described on page 28. During this period the Lourdes centrifuge was not available to the author due to its being repaired and so in these experiments with C.lagopus the International desk model cnetrifuge was used through out and the enzyme activity was measured only in the cell-free preparations using the PMS method. The results are tabulated in Tables 13 and 14 as $Q_{O_2}(N)$. Protein was determined by the Folin phenol reagent method as described on page 69.

9. Purification of Succinic Dehydrogenase of Hygrophorus conicus

H.conicus was grown in the nitrate plus 0.5% yeast extract medium. The cells were harvested by centrifugation, washed twice with deionized water and a cell-free preparation was made using the sonifier. The cell-free preparation was centrifuged at 24,000 x g for 20 minutes and the supernatant (570 ml) was used as the crude extract. To the extract 63.3 ml of saturated ammonium sulfate solution, pH 7, was gradually added with constant stirring to give a 10% saturated ammonium sulfate solution. After 30 minutes the precipitate was collected by centrifuging the suspension at 10,000 x g for 20 minutes. The precipitate was resuspended in 0.01M phosphate buffer, pH 7, and stored in the freezer (-15°) for about a week.

To the supernatant (615 ml) from the above precipitation was added 316.7 ml of saturated ammonium sulfate solution, pH 7, with constant

TABLE 12

COMPARISON OF SUCCINIC DEHYDROGENASE ACTIVITY IN THE ENZYME PREPARATIONS OBTAINED BY TWO DIFFERENT METHODS

Medium	Enzyme preparation	$Q_{O_2}(N)$* Sonification	$Q_{O_2}(N)$* French Press
Nitrate + 0.5% yeast extract	Cell-free	36	37
Nitrate + 0.5% yeast extract	Mitochondrial	77	81
Nitrate + 0.5% yeast extract	24,000 x g supernatant	22	33

*$\mu l O_2$ per hour per mg protein.

TABLE 13

SUCCINIC DEHYDROGENASE ACTIVITY OF CELL-FREE PREPARATIONS OF *COPRINUS LAGOPUS* GROWN IN SUBMERGED CULTURE

Organism	Medium	$Q_{O_2}(N)$*
H_1	Complete	20.7
H_1	Minimal	11.5
H_1	Minimal-nitrate	12
H_9	Complete	13.7
H_9	Minimal	12.5
H_9	Minimal-nitrate	15.5

*$\mu l O_2$ per hour per mg protein.

TABLE 14

SUCCINIC DEHYDROGENASE ACTIVITY OF CELL-FREE PREPARATIONS OF *COPRINUS LAGOPUS* GROWN ON SURFACE CULTURE

Organism	Medium	$Q_{O_2}(N)$*
H_1	Complete	24.5
H_1	Minimal	28.2
H_1	Minimal-nitrate	24
H_9	Complete	12
H_9	Minimal	22.7
H_9	Minimal-nitrate	21.3
$H_1 \times H_9$	Minimal	30.5
$H_1 \times H_9$	Minimal-nitrate	25.4

*$\mu l O_2$ per hour per mg protein.

stirring to obtain 40% saturated ammonium sulfate solution. After 30 minutes the suspension was centrifuged at 10,000 x g for 20 minutes. The precipitate was resuspended in 0.01M phosphate buffer, pH 7, and stored in the freezer (-15°) for about a week.

The precipitates obtained at 10% saturated ammonium sulfate and at 40% saturated ammonium sulfate were used for further purification. The method used in both cases was exactly the same.

The supernatant (60ml) upon thawing of the suspension of 10% saturated ammonium sulfate precipitate was recovered after 20 minutes of centrifugation at 10,000 x g. The supernatant was a clear, yellowish solution. To this solution 10 mg dry weight equivalent of calcium phosphate gel per mg of protein was added and the mixture was allowed to stand for about 12 hours. The mixture was centrifuged at 10,000 x g for 20 minutes and the supernatant was rejected because it contained no significant succinic dehydrogenase activity. About 20 ml of 0.05M phosphate buffer, pH 7.2 was then added to the gel, shaken well for about 5 minutes, and after 12 hours the suspension was centrifuged at 10,000 x g for 20 minutes. The supernatant (15 ml) is subsequently referred to as "0.05M gel eluate". Again about 20 ml of 1M phosphate buffer, pH 7.2 was added to the gel, shaken well for about 5 minutes, and after 12 hours the suspension was centrifuged at 10,000 x g for 20 minutes. The supernatant (15 ml) will be referred to as "1M gel eluate". The "1M gel eluate" contained the highest specific succinic dehydrogenase activity and was used in all other experiments, i.e. K_m measurement, optimum pH measurement, and effects of inhibitors.

Protamine sulfate precipitation was tried on the 10% saturated ammonium sulfate precipitate solution but it always resulted in the complete loss of activity from the solution.

The results of purifications are summarized in Tables 15, 16, and 17.

a. Optimum pH Determination

All the buffers used were 0.3M. Citrate buffer was used at pH 4.5 to 5.5, phosphate between pH 6 to 7, tris at pH 7.6 and 8, and alanine at 8.8. Each flask contained 0.2 ml 20% KOH in the inner well; 1.5 ml buffer and 1 ml enzyme solution in the main compartment; 0.3 ml succinate, 0.1 ml PMS, and 0.3 ml KCN in the side arm. The assay was carried out as described on page 28.

The data are plotted in Figures 8 and 9. The optimum pH for the enzyme succinic dehydrogenase of H.conicus precipitated at 10% ammonium sulfate saturation was found to be between 7 and 7.1, while the optimum pH for the enzyme precipitated at 40% ammonium sulfate saturation was found to be 6.7.

b. Michaelis Constant (K_m) Measurement

Different aliquots of 0.2M succinate, i.e. 0.3 ml, 0.4 ml, 0.6 ml, and 0.8 ml were used. Each flask contained 0.2 ml 20% KOH in the inner well; 0.5 ml 0.3M tris buffer, pH 7.6, and 1 ml enzyme solution in the main compartment; 0.1 ml PMS, 0.3 ml KCN and a specific amount of succinate in the side arm. Water was added to the main compartment to make a total volume of 3.4 ml. The assay method was the same as described on page 28.

Figure 10 is the Lineweaver-Burk plot drawn from the results obtained in this experiment. The K_m value was found to be 2.4×10^{-2}M.

TABLE 15

PURIFICATION OF SUCCINIC DEHYDROGENASE FROM HYGROPHORUS CONICUS

Enzyme	Total volume ml	Protein mg per ml	Protein Total mg	$Q_{O_2}(N)$ $\mu l O_2$ per hour per mg protein	Total $Q_{O_2}(N)$	Purification	Yield %
Cell-free	---	7.85	---	24.8	---	---	---
24,000 x g supernatant	570	7.25	4130	28.5	118,000	---	100
Supernatant after 10% $(NH_4)_2SO_4$ precipitation	615	4.05	2490	13.3	35,000	---	29.6
10% $(NH_4)_2SO_4$ precipitate	65	19.8	1285	22.2	28,500	---	24.2
Supernatant after 40% $(NH_4)_2SO_4$ precipitation	860	0.655	563	35.1	19,400	1.23	16.4
40% $(NH_4)_2SO_4$ precipitate	33	12.8	423	21.4	9,050	---	7.65

TABLE 16

PURIFICATION OF 10% SATURATED AMMONIUM SULFATE PRECIPITATE

Enzyme	Total volume ml	Protein		$Q_{O_2}(N)$ $\mu l O_2$ per hour per mg protein	Total $Q_{O_2}(N)$	Purification	Yield %
		mg per ml	Total mg				
10% $(NH_4)_2SO_4$ precipitate	65	19.8	1285	22.2	28,500	---	24.2
Supernatant upon first thawing	60	3.65	219	37	8,100	1.3	6.7
0.05M gel eluate	15	0.510	7.65	116.5	890	4.1	0.75
1M gel eluate	15	0.083	1.25	374	467	13.1	0.4

TABLE 17

PURIFICATION OF 40% SATURATED AMMONIUM SULFATE PRECIPITATE

Enzyme	Total volume ml	Protein		$Q_{O_2}(N)$ $\mu l O_2$ per hour per mg protein	Total $Q_{O_2}(N)$	Purification	Yield %
		mg per ml	Total mg				
40% $(NH_4)_2SO_4$ precipitate	33	12.8	423	21.4	9,050	---	7.65
Supernatant upon first thawing	30	9.2	276	10.5	2,900	---	2.46
0.05M gel eluate	20	0.725	14.5	24	348	---	0.295
1M gel eluate	20	1.88	37.6	30	112.5	1.1	0.01

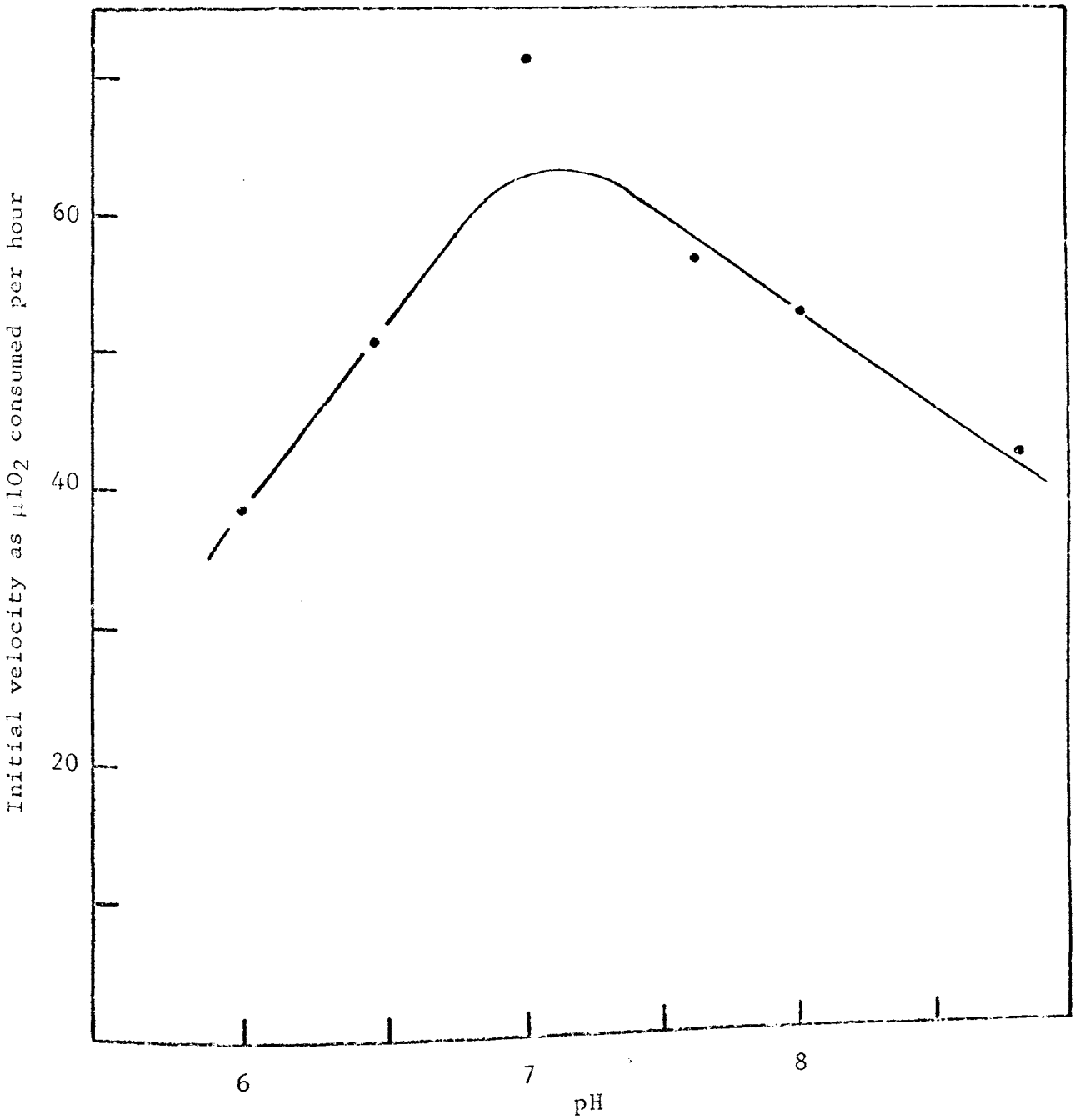


Figure 8. Variation in Reaction Rate with Change in pH in the Case of 1M Gel Eluate from 10% Ammonium Sulfate Precipitate

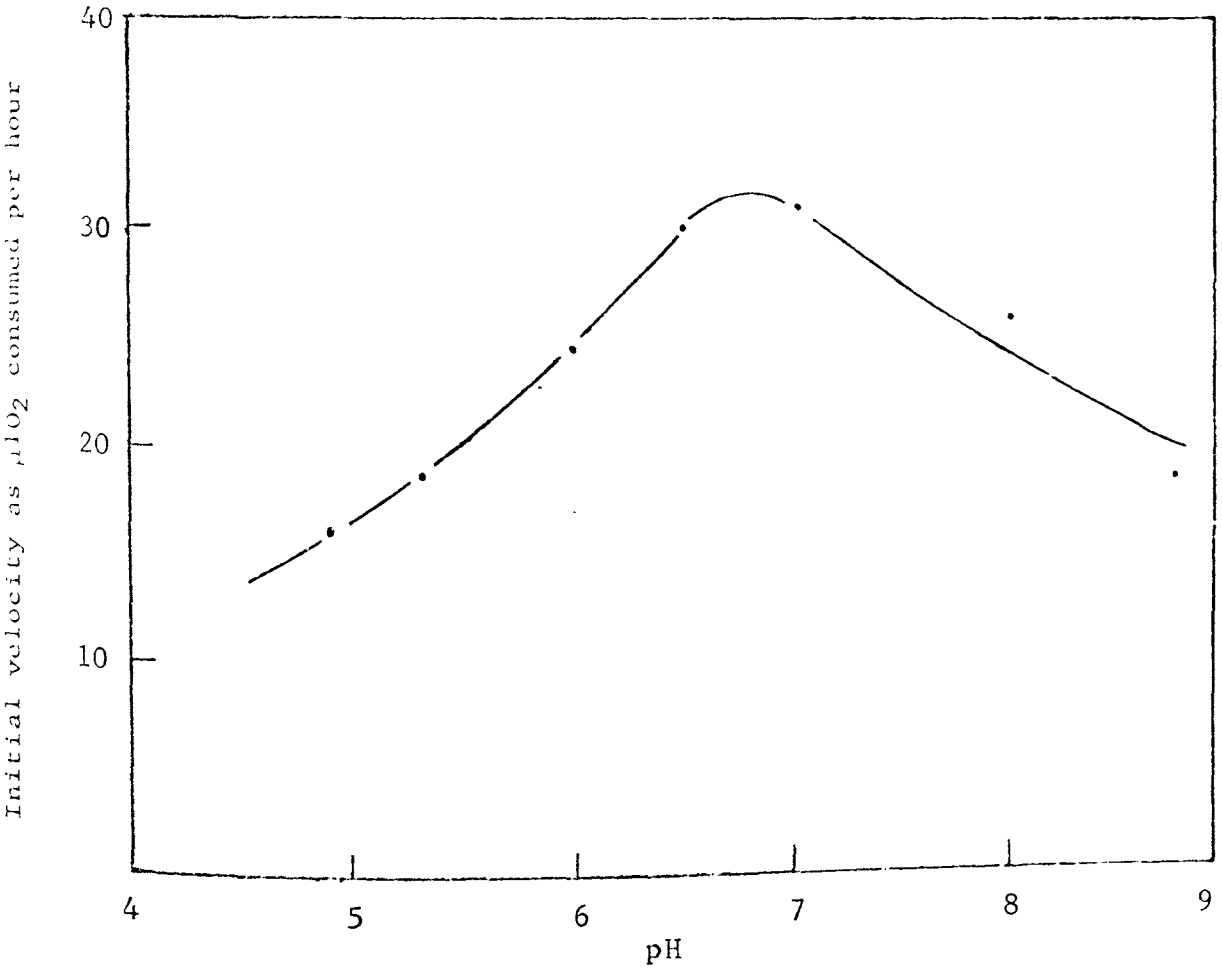


Figure 9. Variation in Reaction Rate with Change in pH in the Case of 1M Gel Eluate from 40% Ammonium Sulfate Precipitate

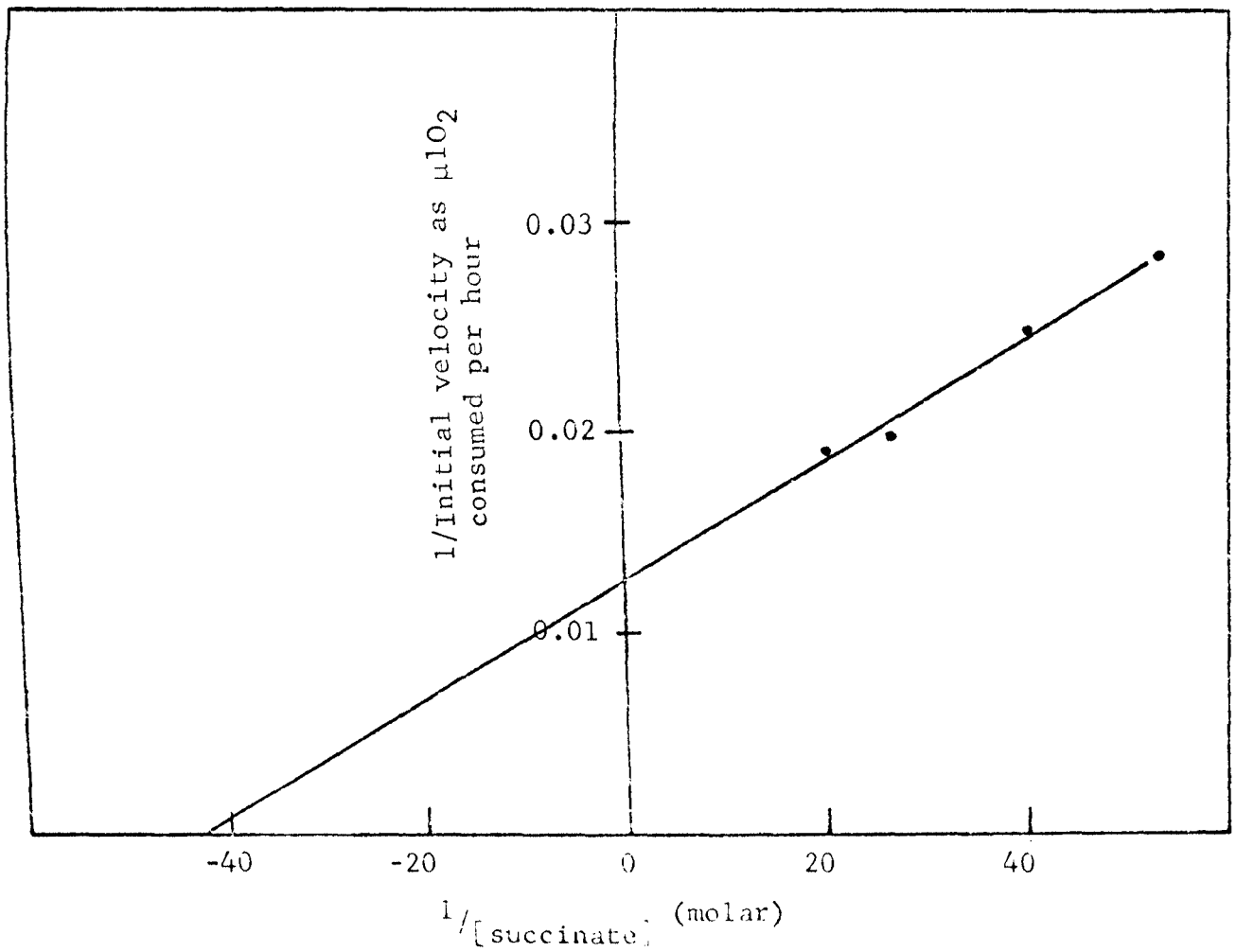


Figure 10. Lineweaver-Burk Plot in the Case of 1M Gel Eluate from 10% Ammonium Sulfate Precipitate

c. Effect of Inhibitors

Malonate, o-phenanthroline and sodium pyrophosphate were tested as inhibitors of succinic dehydrogenase. Each flask contained 0.2 ml 20% KOH in the inner well; either 0.1 ml of 0.2M malonate, 0.3 ml of 0.02M o-phenanthroline or 0.3 ml of 0.02M pyrophosphate solution was placed in the main compartment along with 0.5 ml of 0.3M tris buffer, pH 7.6 and 1 ml enzyme solution; 0.3 ml succinate, 0.1 ml PMS, and 0.3 ml KCN was in the side arm. Water was added to the main compartment to make the total volume 3.4 ml. The assay was carried out as described on page 28.

The data are presented in Table 18. It was found that malonate and o-phenanthroline inhibited the succinic dehydrogenase of H.conicus while there was no significant inhibition by pyrophosphate.

d. Stability

The stability of H.conicus succinic dehydrogenase was measured after storing the enzyme preparation for about 1 month at -15°. The assay method was the same as described on page 28. The results, tabulated in Table 19, suggest that about 70% of the enzyme activity was lost on storing for a month at -15°.

10. The Activity of Nitrate Reductase in Hygrophorus conicus

a. Quantitative Measurement

H.conicus was grown in either nitrate medium or in nitrate containing 0.5% yeast extract medium. The cells were harvested by centrifugation and the enzyme preparations were made using the French press.

The enzyme activity was measured by the method of Sorger (1965). The following substances were added to a test tube so that their

TABLE 18

EFFECT OF INHIBITORS ON SUCCINIC DEHYDROGENASE

FROM HYGROPHORUS CONICUS

Inhibitor	Initial velocity $\mu l O_2$ consumed per hour	Inhibition %
None	35.2	---
Malonate (5.9mM)	8.7	75.3
o-Phenanthroline (1.8mM)	6.8	80.7
Pyrophosphate (1.8mM)	36.4	0

TABLE 19

STABILITY OF SUCCINIC DEHYDROGENASE FROM HYGROPHORUS CONICUS

Enzyme	$Q_{O_2}(N)$ $\mu l O_2$ per hour per mg protein	Loss of Activity %
Original	330	---
After one month storage at -15^{o}	100	70

final concentrations were as shown: FAD, 8.3×10^{-7}M; sodium pyrophosphate buffer, pH 7.0, 6.7×10^{-2}M; KNO_3, 1.67×10^{-2}M; and cell-free preparation, 0.1 ml. NADPH, 1.67×10^{-4}M, was added to each tube except the control at zero time. The total volume in each tube was 0.6 ml. The mixture was incubated at room temperature for 15 minutes and the reaction was stopped by the addition of 0.1 ml of 25.5% barium acetate followed by 2.5 ml of ethanol (96-97%). The resulting suspension was centrifuged in an International desk model centrifuge at high speed for 5 minutes. The supernatant was transferred to another test tube to which 1 ml each of 1% sulfanilamide in 3.2M HCl, and 0.02% N-(naphthyl) ethylenediamine dihydrocloride were added. The mixture was allowed to stand for 30 minutes, and absorbancy was read at 540mμ in a Perkin-Elmer model 139 spectrophotometer against a control which contained no NADPH.

Figure 11 is the standard curve for nitrite which was made in the same manner using KNO_2 as a standard.

The specific activity of the enzyme is defined as a unit of enzyme which produces 10^{-3}μM of nitrite per mg protein under the above mentioned conditions. The specific activity of the enzyme in two different media is given in Table 20.

b. Qualitative Measurement

H.conicus was grown in the nitrate medium. The cells were harvested by centrifugation, washed twice with deionized water and the cell-free preparation made by using the sonifier.

Reagents: (1) 0.2M Succinate

(2) 0.01M KCN

(3) 1% KNO_3

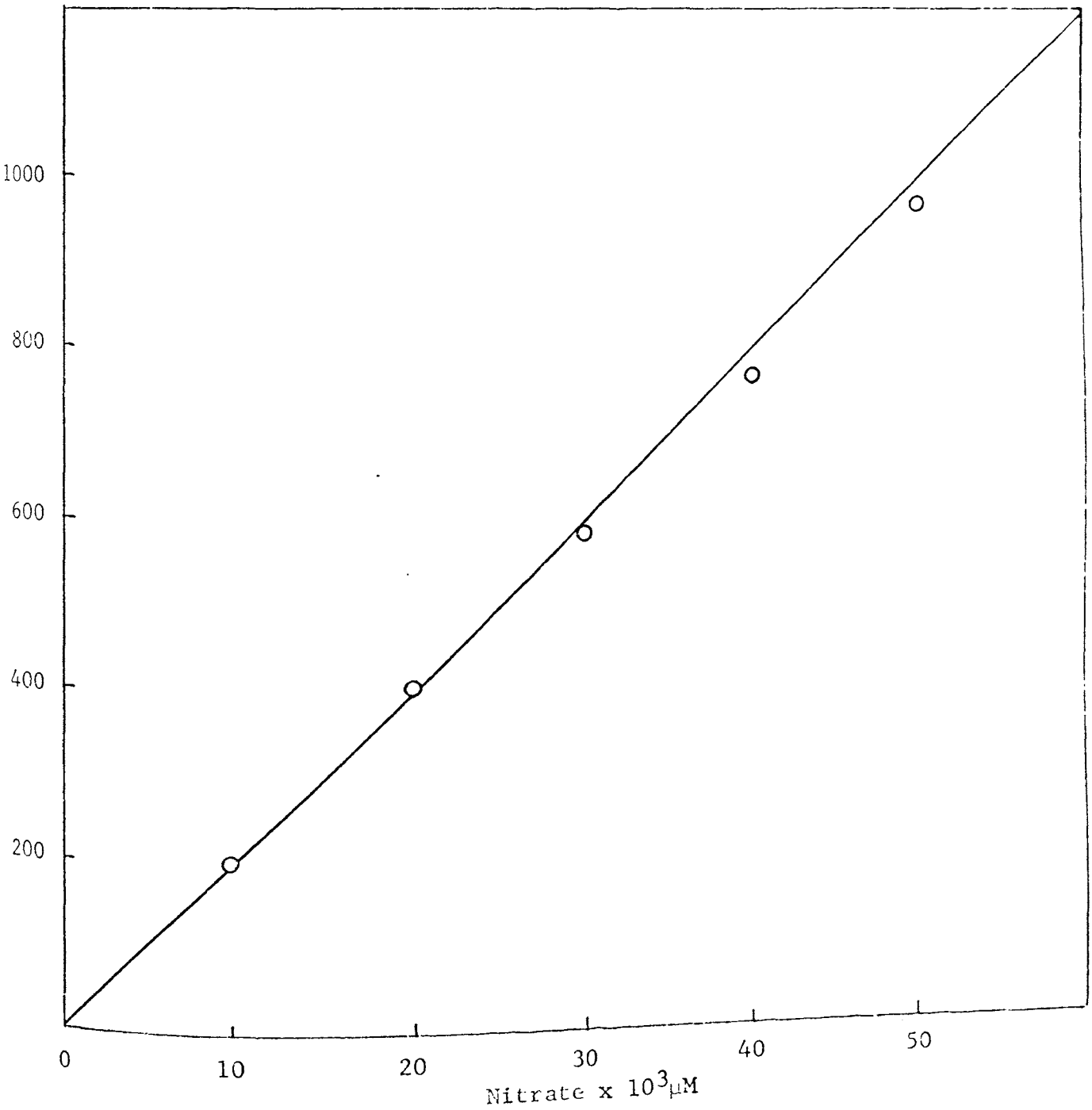


Figure 11. Standard Curve for Nitrite Determination

TABLE 20

NITRATE REDUCTASE ACTIVITY IN THE CELL-FREE PREPARATIONS OF HYGROPHORUS CONICUS

Medium	Specific activity units per mg protein
Nitrate	55.8
Nitrate + 0.5% yeast extract	63.5

(4) 0.05% Methylene blue

(5) 1% PMS

(6) 0.2M Malonate

Procedure: The experiment was carried out in Thunberg tubes by the method described by Sato and Niwa (1952). The following reagents were added to the main compartment of the tubes: succinate, 0.5 ml, KCN, 0.5 ml, methylene blue, 0.2 ml and the enzyme preparation 0.5 ml. To the side arm was added either 0.5 ml KNO_3 in the case of the sample or 0.5 ml water in the case of the control. The side arm was fitted on the main part of the tube and the outlet was jointed to a water aspirator. The tubes were evacuated for 30 minutes using the same aspirator. During evacuation period about 50% of the methylene blue color disappeared in both the sample and control tube. The side arms were turned to close the opening and seal the contents of the tubes from contact with the air. The tubes were then placed in a constant temperature (30°) water bath for 5 minutes and the contents of the side arm tipped into the main compartment. The rate of disappearance of methylene blue color was observed. The color disappeared very quickly on the addition of nitrate. It took about one hour for the color in the control tube to disappear. The tubes were removed from the water bath (30°) and kept at room temperature. After about 36 hours, color reappeared in the sample tube but not in the control tube.

Exclusion of KCN from the reaction mixture also showed the disappearance and reappearance of methylene blue color.

In another experiment 0.2 ml malonate was added along with nitrate from the side arm. Under these conditions the color disappeared in neither the sample nor the control tubes.

When methylene blue was replaced with PMS, the color of PMS disappeared in both tubes but did not reappear even after standing for 36 hours.

11. The Cytochrome c Reductase Activity in Hygrophorus conicus

The cell-free preparations were made as described in the preceding section.

Reagents: (1) 2% Cytochrome c solution

(2) 1×10^{-4}M FAD

(3) 0.1M Potassium phosphate buffer, pH 7.4

(4) 8×10^{-3}M NADPH

Procedure: The enzyme activity was measured as described by Kinsky and McElroy (1958). The following substances were added to cuvettes with a light path of 1 cm: 0.1 ml of cytochrome c solution, 0.06 ml of FAD solution, 0.1 ml of the enzyme solution and either 1.74 ml of buffer solution in the case of the control cuvette or 1.7 ml of buffer solution in case of the sample cuvette. The reaction was started by the addition of 0.04 ml of NADPH in the sample cuvette and the increase in the absorbancy was measured at 550 mμ every 15 seconds for 2 minutes against a control in a Perkin-Elmer model 139 spectrophotometer.

The unit of enzyme activity is defined as that amount of enzyme which produces an optical density change of 0.001 between 15 and 75 seconds under the above mentioned conditions of assay. The specific activity is defined as units per mg protein. Protein was determined by Folin phenol reagent method.

The specific activity of the enzyme cytochrome c reductase measured in the cell-free preparations of H.conicus grown in different media is given in Table 21.

12. Protein Measurement

Folin Phenol Reagent Method

The protein measurement was carried out as described by Lowry et al (1951).

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: 1N Folin phenol reagent

Procedure: The sample was diluted so that an approximate protein concentration of 100 to 200 gamma per ml was obtained, and 0.2 to 1 ml aliquots of the diluted sample were placed in test tubes. Each sample was diluted to 2 ml with deionized water; the control tube contained 2 ml of deionized water. Ten ml of reagent C was added to each tube and the mixture was allowed to stand. After 10 minutes 1 ml of reagent D was added very rapidly and mixed within a second or two. After 30 minutes the sample was read at 500mμ in a Spectronic 20 colorimeter against a control.

Figure 12 is a standard protein curve where bovine albumin was used as a standard protein.

13. Endogenous and Exogenous Respiration of Hygrophorus conicus

The method described by Umbreit et al (1964) was used for respiration studies of H.conicus. The anaerobic measurements were made in a nitrogen atmosphere while aerobic measurements were made in an atmosphere of air.

TABLE 21

CYTOCHROME c REDUCTASE ACTIVITY IN THE CELL-FREE PREPARATIONS OF HYGROPHORUS CONICUS

Medium	Specific activity units per mg protein
4% malt extract	36.4
Nitrate	103
Nitrate + 0.5% yeast extract	103

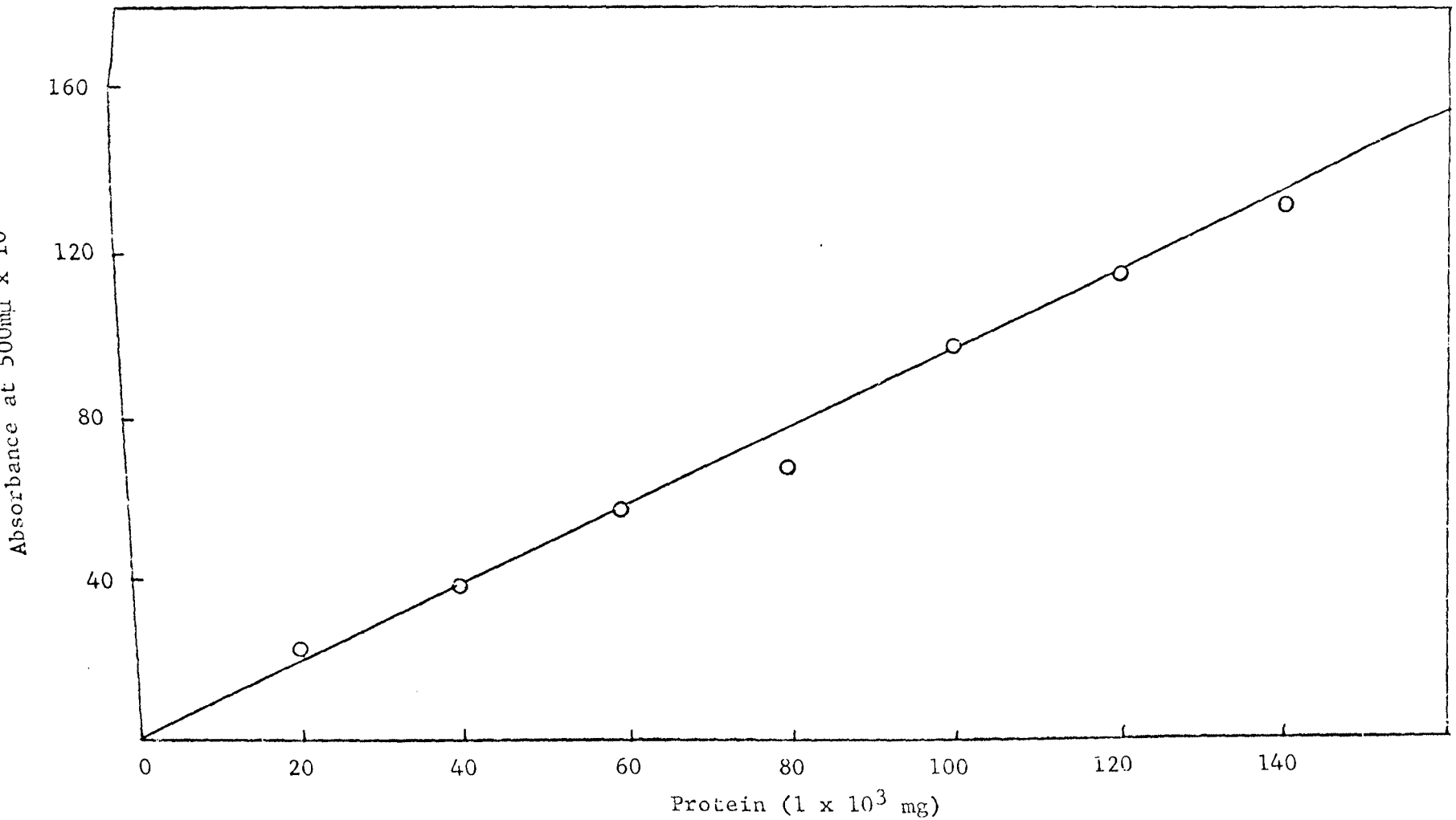


Figure 12. Standard Curve for Protein Determination by Folin Phenol Reagent Method

H.conicus was grown either in nitrate or nitrate plus 0.5% yeast extract medium. The mycelium was collected by centrifugation, washed twice with deionized water and was suspended in 0.01M phosphate buffer, pH 7 using a glass rod. This suspension was used for the respiratory measurements. In the aerobic experiments each Warburg vessel contained 0.2 ml 20% KOH in the inner well and the cell suspension in the main compartment. Either water or 0.01M glucose (0.2 ml) was placed in the side arm. The anaerobic measurements were the same except KOH was omitted.

An aliquot of cell suspension was dried in a Gooch crucible at 105-110° for about 20 hours, and the dry weight was determined.

The results are tabulated in Table 22 as either $\mu l O_2$ taken up per mg dry weight or $\mu l CO_2$ produced per mg dry weight.

14. Metabolism of Indole Acetic Acid and Oxindole Acetic Acid by whole Hygrophorus conicus cells

In order to identify the metabolic product(s) of IAA by cells of H.conicus, the cells were grown in 60 ml of 4% malt extract medium in the presence of 28.2 mg Na-IAA for 48 hours. The cells were harvested by centrifugation at 1,000 x g for 20 minutes at 4-5°. They were washed twice with deionized water to remove all traces of the malt extract medium, IAA, and products(s) and were then suspended in 50 ml of deionized water. One ml of a 2.8 mg per ml solution of Na-IAA with radioactive ^{14}C in either C_1 or C_2 position was added and the cell suspension was incubated in an erlenmeyer flask on the shaker for various times at 25°.

When OIAA was used as the substrate, the cells obtained from six to seven 500 ml erlenmeyer flasks each containing 100 ml of medium were

TABLE 22

ENDOGENOUS AND EXOGENOUS RESPIRATION OF HYGROPHORUS CONICUS

Medium	Aerobic condition $\mu l O_2$ per mg dry weight		Anerobic condition $\mu l O_2$ per mg dry weight	
	Endogenous	Exogenous (glucose 2μM)	Endogenous	Exogenous (glucose 2μM)
Nitrate	16.7	17.3	2.5	3.1
Nitrate + 0.5% yeast extract	6.1	6.0	1.6	2.1

edge of the paper. The chromatogram was removed from the glass cylinder, and was dried in a hood. The chromatogram was again formed into a cylinder and the two edges which were formerly the top and bottom were stitched. The chromatogram was placed in a glass cylinder containing one of the following solvents.

B.n-butanol: acetic acid: water (180: 45: 75);

C.benzene: propionic acid: water (100: 70: 5);

D.20% KCl

The solvent was allowed to rise to within a few centimeters of the top edge. The chromatogram was removed from the cylinder and dried. The dried chromatogram was examined under UV light and the spots were marked. Usually one blue-green fluorescence spot (OIAA) and two or more absorbing spots were observed. A drawing of a typical chromatogram is shown on page 77. The R_f values of these compounds are given in Table 23.

The chromatogram containing radioactive material was used for radioautography (see page 79).

The chromatograms which did not contain radioactive material were used as a source for the recovery of the unknown (see page 79) or were developed with different locating reagents. The reagents used for the chromatogram color development and the observed results are outlined in Table 24.

It was found that all of the added IAA (2.8 mg) was converted to the product(s) by the mycelium when it was incubated with cells of H.conicus for 4 or more hours.

TABLE 23

R_f VALUES OF UNKNOWN PRODUCTS

Unknown	R_f x 100 Isopropanol: ammonia: water	R_f x 100 Benzene: propionic acid: water
A_L*	39	35
A_R*	38	81
A_B*	9	74

*See Figure 13

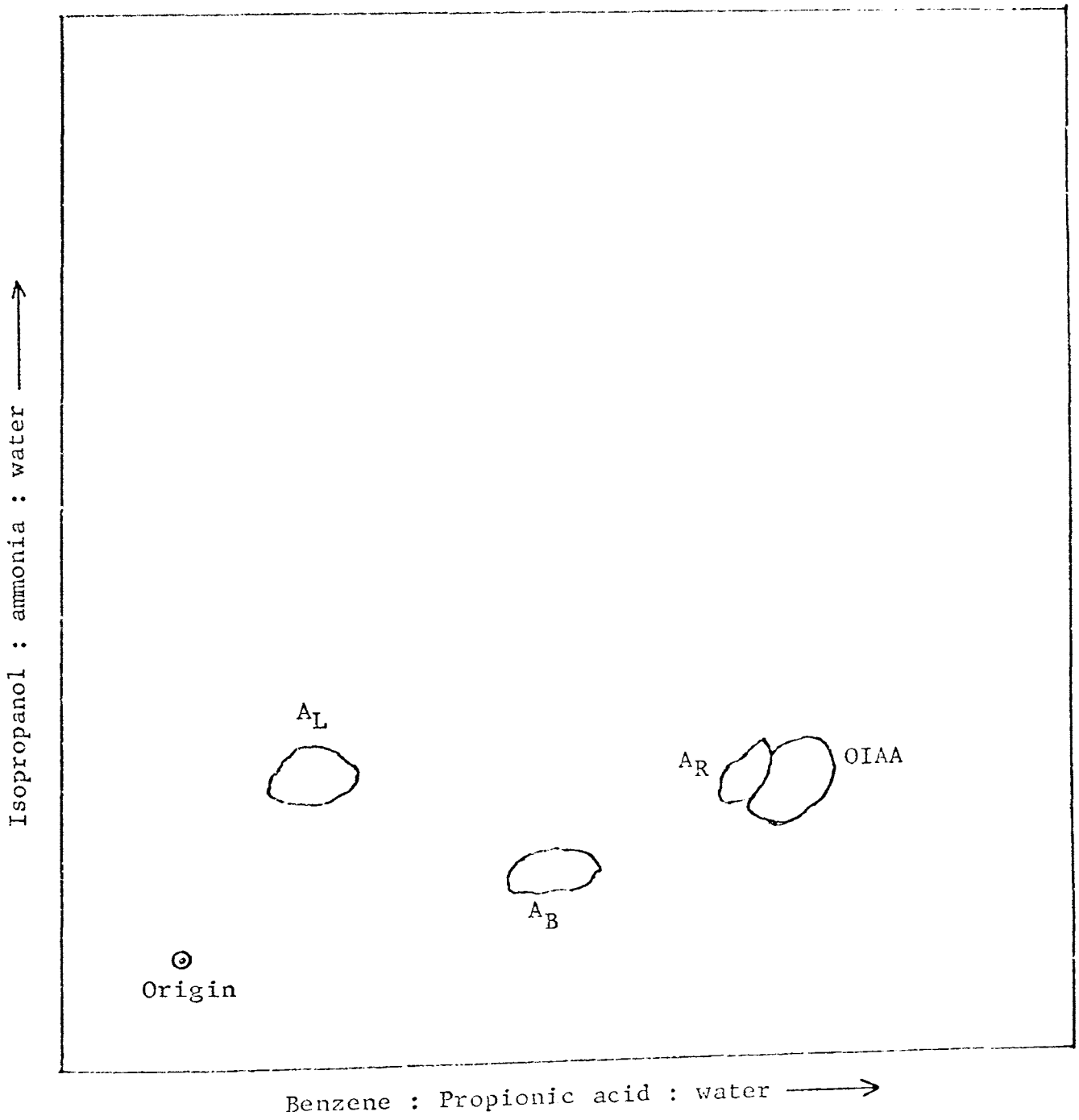


Figure 13. A Typical Chromatogram of Metabolic Products of Indole Acetic Acid

TABLE 24

REAGENTS USED FOR THE CHROMATOGRAM DEVELOPMENT

Reagent	Observation	
	Unknown A_L	Unknown A_R
Ehrlich's	No color	Pink after about 3 days
Benzidine	No color	No color
2,4-dinitro-phenylhydrazine	No color	No color
Formaldehyde-Hcl	No color	No color
$AgNO_3$-NH_3	No color	No color
$FeCl_3$-$K_3Fe(CN)_6$	No color	Blue on drying
$FeCl_3$	No color	No color
Diazotized Sulfanilic acid	No color	On drying first yellow, then brown, red and finally violet
Folin	No color	Blue

b. Elution of the Unknowns From the Chromatogram

The chromatogram was developed as described (see page 74). It was examined in UV light and the spots were marked either as absorbing or fluorescence. There were two absorbing spots and one fluorescent spot. The section containing one or the other of the absorbing spots was cut from the chromatogram in a strip. The strip was about 1 inch wide and 2 inches long. The desired material was eluted from the paper with ethanol using the set up pictured in Figure 14. The eluant collected in the beaker was suitably diluted and the UV spectrum was run using the Beckman DK 2A spectrophotometer.

The unknown A_L* showed two absorbing peaks in the UV, one at 253 mμ and another at 287 mμ, the former being the greater.

The unknown A_R* showed absorbance at 250 mμ in the UV. This spectrum was very similar to that for OIAA.

c. Radioautography

The chromatogram developed in two different solvents was marked with radioactive ink in all but the one corner where the radioactive material was spotted.

The following steps were performed in the dark room. The chromatogram was placed against an x-ray film (12 x 12 inches) and all four corners were taped using 3M cello-tape to insure maximum contact. The film and papergram sandwich was kept in the light tight film folder. After five days the chromatogram was removed from the x-ray film and the latter was mounted on a tension hanger. The hanger was

*See Figure 13.

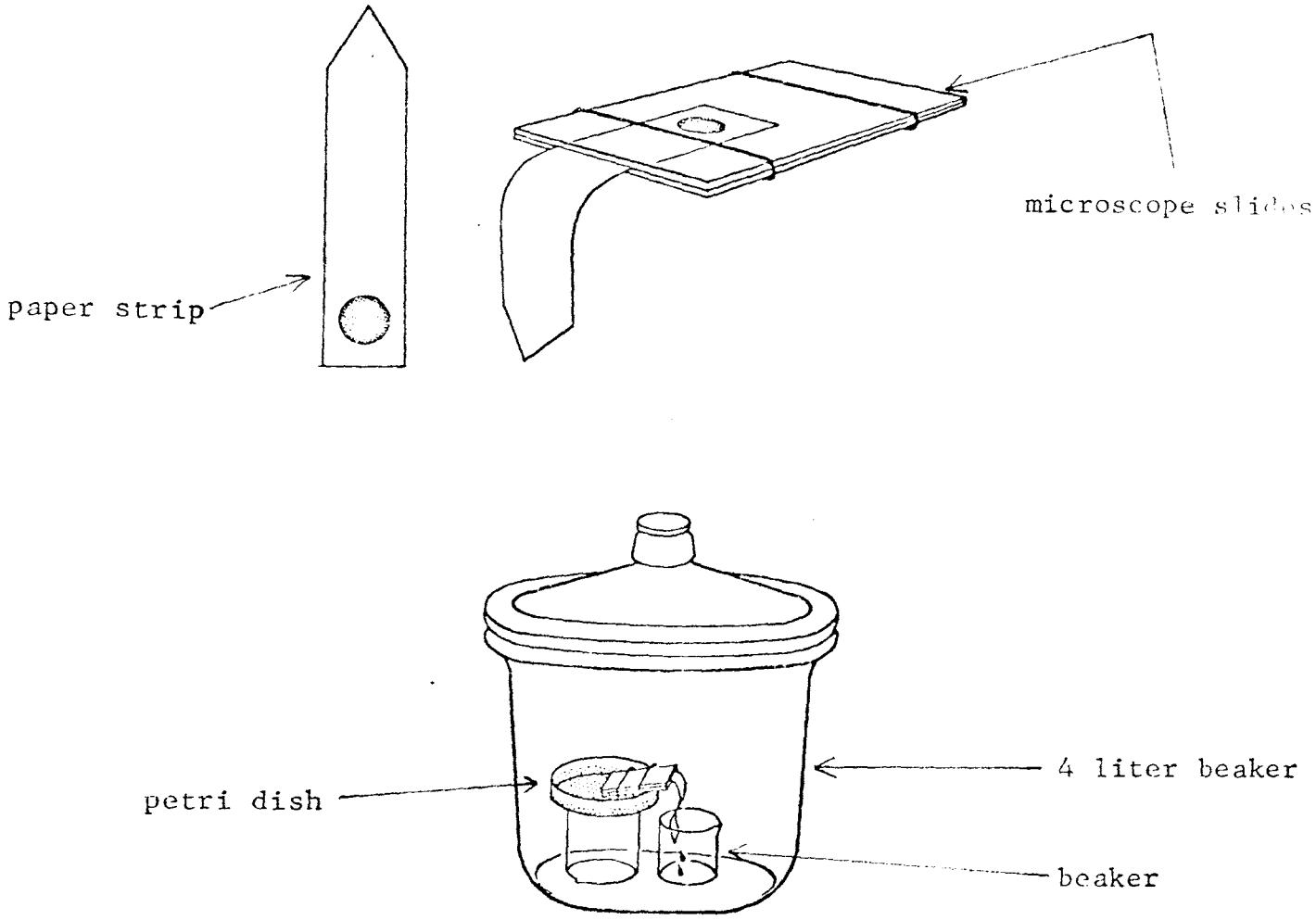


Figure 14. Diagram of the Elution Apparatus.

This figure is taken from "Microbial Physiology and Biochemistry" by Seaman, G. R., Burgess Publishing Co., Minneapolis (1963).

placed vertically in developer solution, developed for five minutes and washed with water. Then it was fixed for seven minutes, washed with water and dried in an oven at 40°.

The film was examined for areas of darkening. In each case two or more black spots were observed on the film. Figures 15 and 16 are the drawings of a typical radioautograph.

The chromatogram was examined under UV light. There were more than two absorbing and one fluorescent spots on the chromatogram. The chromatogram was sprayed with Ehrlich reagent. The fluorescent spot showed the characteristic blue green color of OIAA on drying. No violet spot for IAA was observed on the chromatogram.

d. Radiochromatogram Scanning

The radiochromatogram was developed as described on page 74. It was then cut into 1.5 inch wide strips. Adjacent strips were joined at the end to make a continuous strip. A blank paper strip was taped on each end of the chromatographic strip as a leader and a trailer. The leader and a trailer were necessary to position the chromatographic strip at the detector area.

After a warming up period of about thirty minutes the scanner was ready to use. The high voltage was set at 1000 volts and the gas (mixture of 0.95% isobutane and 00.05% helium) flow was adjusted to a rate of 300 cc per minute. The chromatographic strip was inserted between the paper guide pins, between the detectors, and between the drive capstan and pressure roller. The front paper guide was mounted on paper guide pins so that the strip edges touched the front and rear paper guides. After the necessary instrumental adjustments the radioactivity on the strip was recorded

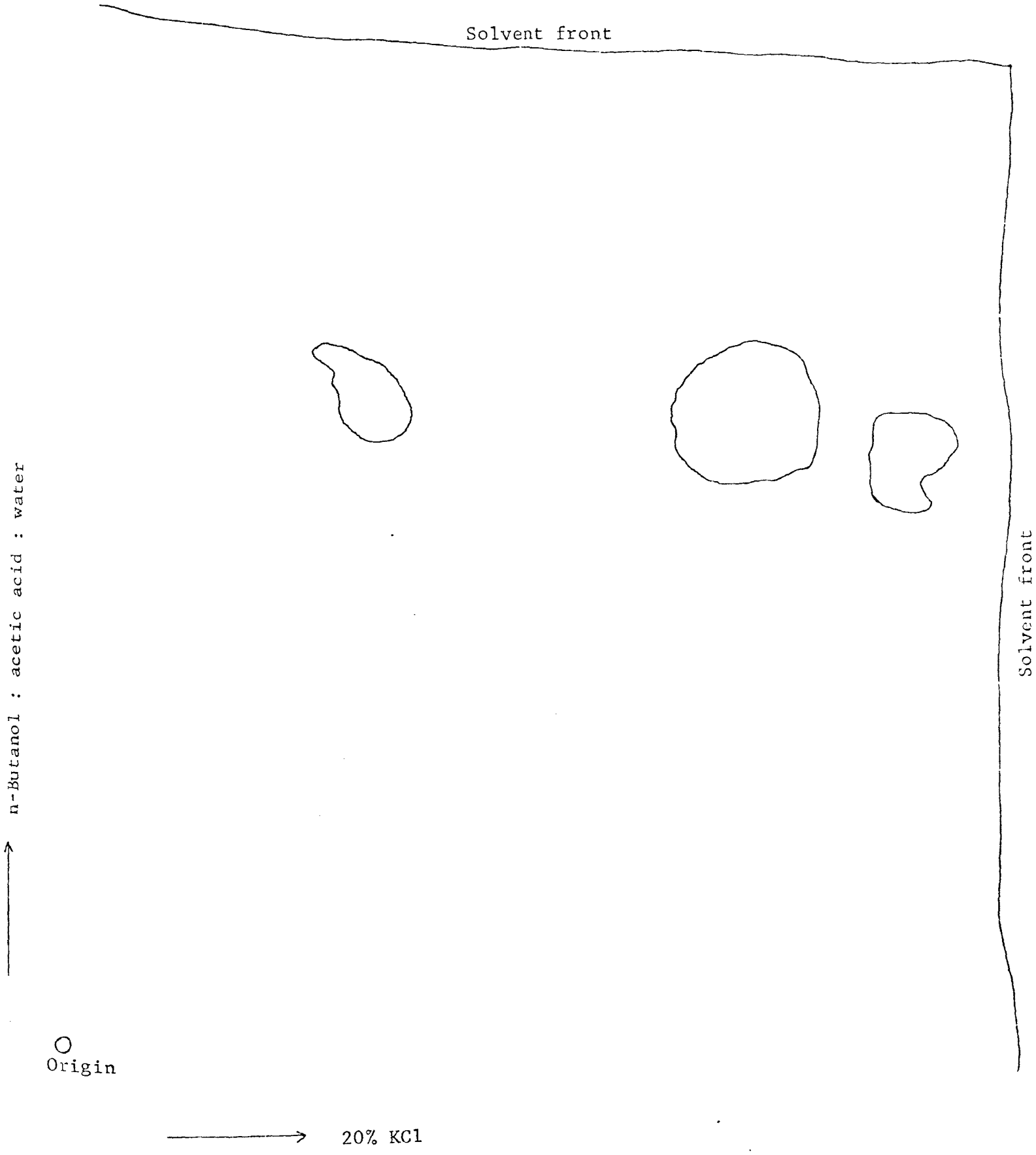


Figure 15. Drawing of a Typical Radioautograph

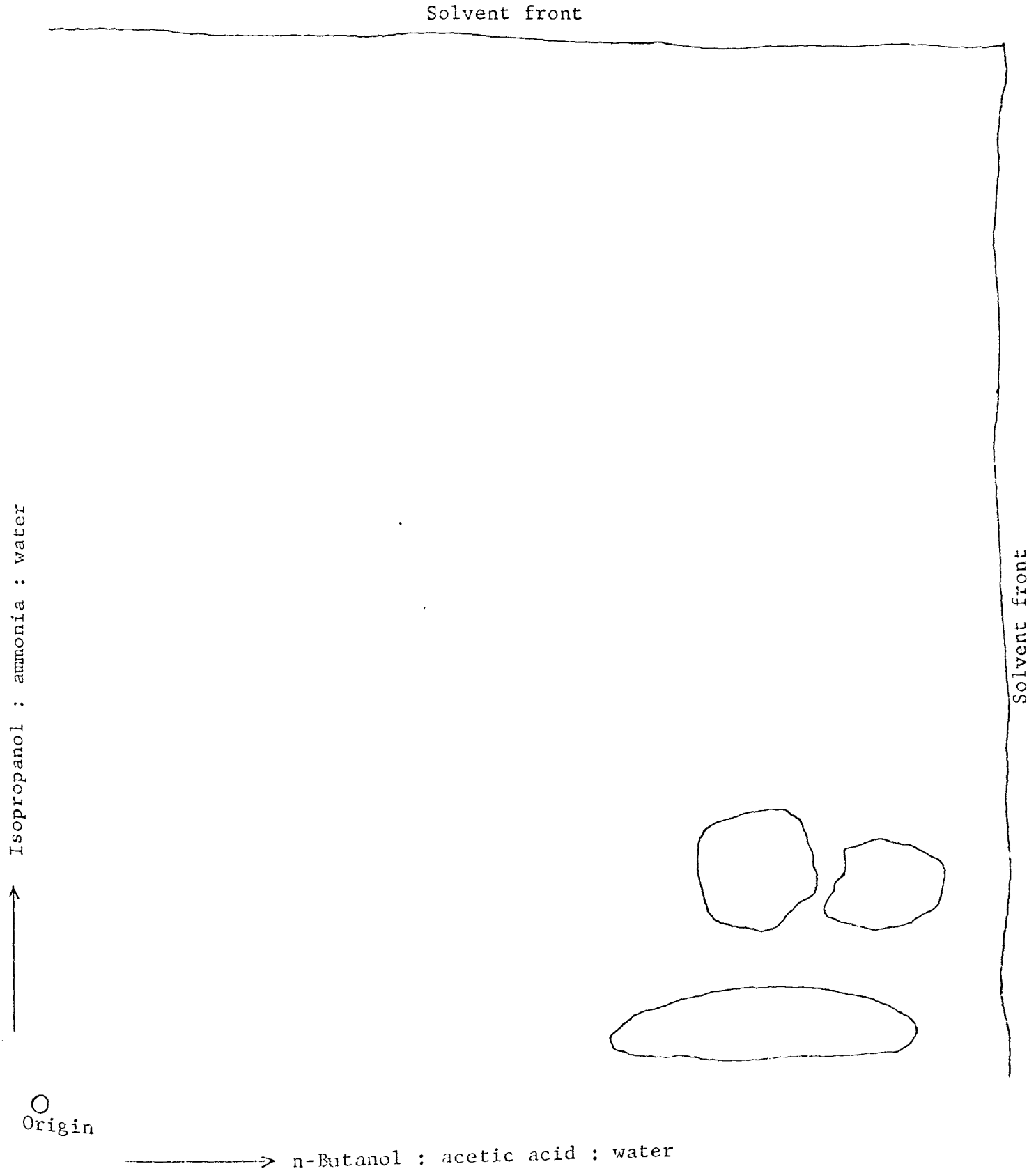


Figure 16. Drawing of a Typical Radioautograph

quantitatively on a chart paper using different speeds, i.e. 0.2 and 1 cm per minute, and 2 cm per hour.

Radioactivity was found in all three of the major UV light absorbing spots.

The same strip was dipped in Ehrlich's reagent. The result showed the presence of OIAA but there was no indication that IAA was present.

15. Attempts to Observe Indole Acetic Acid Oxidase Activity in Cell-Free Preparations from Hygrophorus conicus

a. Hydroxylase Activity

The cell-free preparation was made using the French press. The suspension was centrifuged at 20,000 x g for 10 minutes and the supernatant was used as the enzyme solution.

Reagents: (1) NADPH, 200 mμM per ml

(2) FAD, 0.1mM per ml

(3) Na-IAA, 200 μM per 0.1 ml

(4) Phosphate buffer, 0.01M, pH 7

The following solutions were added in cuvettes, Na-IAA, 0.1 ml; FAD, 0.1 ml; buffer, 0.9 ml and enzyme solution 0.5 ml. Two milliliters of deionized water was added to a control cuvette. In a sample cuvette 2 ml of NADPH was added at zero time and the decrease in absorbancy was measured against control at 340 mμ in a Perkin-Elmer spectrophotometer using 1 cm light path.

No decrease in the absorbancy was observed indicating the absence of IAA oxidase activity.

b. Measurement of Activity by Substrate Loss.

The cell-free preparation was made using the French press and was centrifuged at 20,000 x g for 20 minutes. Both the cell-free preparation and 20,000 x g supernatant were used as the enzyme source.

Reagents: (1) Salkowski reagent: 3 ml 1.5M $FeCl_3$; 60 ml H_2SO_4; 100 ml H_2O

(2) 1×10^{-3}M 2,4-dichlorophenol

(3) 0.2 M acetate buffer, pH 5

(4) Na-IAA, 200 μM per 0.1 ml

Two milliliters of enzyme solution, 1 ml dichlorophenol and 5.5 ml buffer solution were added to test tubes. At zero time 1.5 ml Na-IAA solution was added in all tubes but the control and the tubes placed in a water bath at 30°. One milliliter aliquots were taken from the sample tube after 5, 10, 20 and 40 minutes and were added to a tube containing 2.5 ml Salkowski reagent. From the control tube a 0.85 ml aliquot was taken at the same time intervals and was added to a tube containing 2.5 ml Salkowski reagent to which 0.15 ml Na-IAA was then added.

After 20 minutes the contents of the tubes were diluted with 10 ml of water and the absorbancy at 530 mμ was measured in a Spectronic 20 colorimeter against a blank. The blank tube contained 2.5 ml Salkowski reagent, 10.8 ml water and 0.2 ml enzyme solution.

The results are tabulated in Table 25. From the results it can be said that no IAA oxidase activity is present in the enzyme preparations.

TABLE 25

ASSAY OF INDOLE ACETIC ACID OXIDASE BY SALKOWSKI REAGENT

Time Minutes	Absorbancy at 530 mμ		
	Sample		Control
	cell-free	20,000 x g supernatant	
5	0.330	0.325	0.355
10	0.310	0.328	0.340
20	0.310	0.320	0.330
40	0.315	0.340	0.330

IV. DISCUSSION

A. GROWTH OF HYGROPHORUS CONICUS

Both sodium nitrate and yeast extract were used as nitrogen sources in the study of the growth of H.conicus. Poor growth of H.conicus was observed on the nitrate medium (Table 1) as compared to its growth on the yeast extract medium. The fact that nitrate was a poor nitrogen source for H.conicus was consistent with the results of other workers with basidiomycetes (Robbins, 1950; Jennison et al, 1955). The probable reason for the poor growth in the nitrate medium was that the organism was unable to utilize nitrate efficiently. Since the yeast extract is a complex mixture of vitamins, purines, pyrimidines and amino acids plus certain growth factors (Robbins et al, 1963) the yeast extract undoubtedly provides all the nitrogen compounds needed by the organism.

The production of mycelium by H.conicus in 0.5% yeast extract medium was lower than that in the nitrate plus 0.5% yeast extract medium. However, the growth of H.conicus was higher in 0.885% yeast extract medium than that in the nitrate plus 0.5% yeast extract medium even though the nitrogen content of the two media was equivalent. Since the mycelium production in 0.885% yeast extract medium was greater than that in 0.5% yeast extract medium, perhaps the 0.5% yeast extract medium is limiting in some essential growth factor for H.conicus other than nitrogen although nitrogen may be limiting.

B. SUCCINIC DEHYDROGENASE

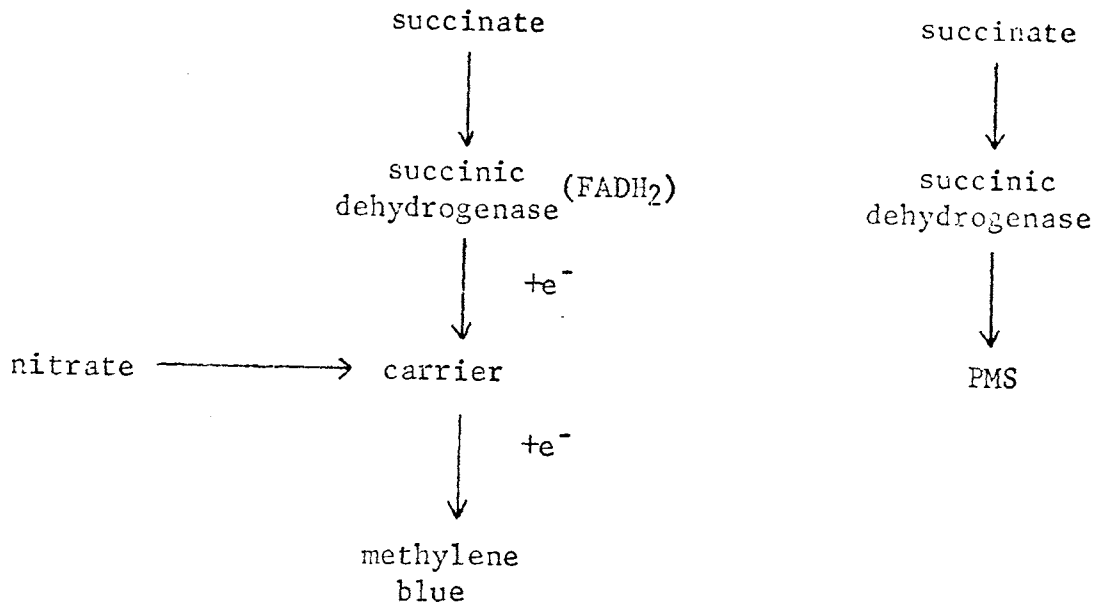
Succinic dehydrogenase activity in H.conicus was noticeably stimulated by growing the organism on a medium containing nitrate (Table 8).

Succinic dehydrogenase activity in the nitrate plus 0.5% yeast extract medium was the sum of the activity in the nitrate medium and the 0.5% yeast extract medium (Table 8). In order to check the possibility that the succinic dehydrogenase activity was related to the increased growth of the organism rather than the presence of nitrate, a medium was prepared containing 0.885% yeast extract. This medium as mentioned earlier was equivalent in nitrogen to the nitrate plus 0.5% yeast extract medium. The production of mycelium on 0.885% yeast extract medium was excellent (Table 1) however the specific activity of succinic dehydrogenase was about half of that in the nitrate plus 0.5% yeast extract medium (Table 8). These results indicate that the increased succinic dehydrogenase activity in cell-free extracts of H.conicus was primarily due to the presence of nitrate in the growth medium.

An additional question which arose during this study was "Is the stimulation of succinic dehydrogenase activity by nitrate a common occurrence in basidiomycetes?". To answer this question mycelium of two compatible monokaryons (H_1 and H_9) and of a dikaryon ($H_1 \times H_9$) of C.lagopus were used. These were grown in media both with and without nitrate on surface and in submerged cultures. (C.lagopus grows on a nitrate medium at about 10% of that on a complete medium with asparagine as a nitrogen source.) Succinic dehydrogenase activity was measured in cell-free preparations made from these cells. No stimulation of succinic dehydrogenase activity by nitrate was observed in any case with C.lagopus. Apparently the stimulation of succinic dehydrogenase activity by nitrate is not a common occurrence in basidiomycetes.

During the qualitative measurement of nitrate reductase activity using methylene blue as an electron acceptor under anaerobic conditions, it was observed that the color of methylene blue disappeared rapidly when nitrate was added to the reaction mixture. This effect of nitrate was not observed when malonate an inhibitor of succinic dehydrogenase was present in the reaction mixture. This would suggest the direct stimulation of succinic dehydrogenase activity by nitrate. This effect of nitrate was not observed when methylene blue was replaced with PMS in the reaction mixture under anaerobic conditions and during oxygen uptake studies when PMS was used as an electron acceptor.

Evidence obtained during the present investigation suggests that there may be two types of succinic dehydrogenase enzymes present in H.conicus when the organism is grown on nitrate. One is a normal succinic dehydrogenase and the other is induced by nitrate and dependent upon the presence of nitrate for its activity. On the basis of the effect of nitrate on the reduction of methylene blue by succinate referred to above, it is postulated that succinic dehydrogenase requires an electron carrier for the transfer of electrons from substrate to methylene blue and so methylene blue is slowly reduced. The carrier is not necessary with PMS so that "the rate determining step" (transfer of electrons to the carrier) of the methylene blue reduction is not seen under these conditions. The fact that nitrate stimulates the rate of methylene blue reduction by succinate requires that the postulated electron carrier is activated by nitrate. It is possible that the carrier is an allosteric type protein.



When mammalian cytochrome c was used as an electron acceptor, very little succinic dehydrogenase activity was observed in a preparation made by sonication (Mehta, 1966). It is possible that disruption of the mitochondria occurred during sonication since changes in the properties of enzymes are common, particularly in the case of NADH dehydrogenases, during mechanical disruption of cells (Mahler and Cordes, 1966d). In addition the fact that the enzyme was precipitated at 10% ammonium sulfate saturation during its isolation and that there was a total loss of activity when protamine sulfate precipitation was tried that further indicated an appreciable amount of succinic dehydrogenase in H.conicus was either a nucleo or lipoprotein or bound to such a protein.

Because of the high succinic dehydrogenase activity in the 24,000 x g supernatant obtained from cells disrupted by sonication, succinic dehydrogenase activity in the mitochondrial and the soluble portion of a cell-free preparation obtained by an alternate procedure, a French press, was measured. Regardless of which technique was used to rupture

the cells, succinic dehydrogenase activity was high in the 24,000 x g supernatant indicating a soluble or easily solubilized form of the enzyme (Tables 9 and 12). McDonald et al (1963) have shown that a large percentage of the succinic dehydrogenase activity in cell-free preparations obtained by sonication of C.purpurea cells was soluble.

Since yeast extract contains a significant amount of niacin and riboflavin, the effects of these substances on succinic dehydrogenase obtained from H.conicus was determined. These substances had no effect on the enzyme activity and apparently they do not play a part in the succinic dehydrogenase reaction (Table 10).

The measurement of succinic dehydrogenase activity depends upon the availability of an electron acceptor which can pick up the electrons liberated in the oxidation of succinate. Singer et al (1956) working with succinic dehydrogenase of bakers' yeast found the relative activity of various redox dyes used as electron acceptors as follows: PMS, 100; methylene blue, 40; BCB, 30; ferricyanide, 28. The results obtained with succinic dehydrogenase of H.conicus (Table 11) were comparable with the results of Singer et al (1956) so far as PMS and BCB were concerned. McDonald et al (1963) found PMS much more effective than any other acceptors with their soluble succinic dehydrogenase, however, they did not test BCB.

The purification of the enzyme succinic dehydrogenase is a difficult task because of the lability of the enzyme. Bernath and Singer (1962) purified the succinic dehydrogenase of beef heart about 20 fold, while McDonald et al (1963) purified their preparation from C.purpurea about 4 fold. The succinic dehydrogenase of H.conicus has been purified about 13 fold (Table 16).

It was found that some soluble microbial preparations of succinic dehydrogenase can be sedimented at 144,000 x g for 1 hour, or precipitated by ammonium sulfate at 20 to 30% of saturation (McDonald et al, 1963). The preparation of H.conicus succinic dehydrogenase was precipitated at two different ammonium sulfate saturations, i.e. 10% and 40%. This suggests the presence of two types of succinic dehydrogenase in H.conicus. The enzyme which was precipitated at 10% ammonium sulfate saturation appeared to be a normal succinic dehydrogenase and particulate bound. The succinic dehydrogenase which was precipitated at 40% ammonium sulfate saturation would appear to be a soluble form.

When a cell-free preparation of H.conicus was treated with protamine sulfate all the succinic dehydrogeanse activity was lost in the precipitate. Forty percent of the activity was lost in the soluble succinic dehydrogenase preparation of McDonald et al (1963) when protamine sulfate was added. The succinic dehydrogenase of H.conicus which was precipitated at 10% ammonium sulfate saturation behaved as a high molecular weight protein and could be adsorped on a lipoprotein since the mitochondria are composed primarily of lipoprotein. The occurrence of the enzymic activity in the 24,000 x g supernatant can be explained if there was considerable degradation of mitochondria during the cell disruption. If this was true then the disruption of H.conicus cells either by sonication or rapid decompression in the French press degrade the mitochondria similarly. Lipid extraction, centrifugation of the cell-free preparation at 144,000 x g to precipitate particulate matter, or electron micrographs of the mitochondrial preparation could possibly answer this question of mitochondrial degradation and succinic dehydrogenase adsorption.

The K_m value for the succinic dehydrogenase of H.conicus which was precipitated at 10% ammonium sulfate saturation was found to be 24 mM at pH 7.6 and 30°. This value is higher than the K_m values for the mammalian and yeast enzymes. McDonald et al (1963) reported the K_m value for the succinic dehydrogenase of C.purpurea to be 3mM at pH 7.7 and 35°. The K_m values for heart and M.lactilyticus succinic dehydrogenase are 1.3mM and 5mM respectively (Bernath and Singer, 1962). The highest K_m value reported in the literature was that for the succinic dehydrogenase from Brucella melitensis. It varied from 16 to 86 mM (Dranovskaya, 1966).

The optimum pH for the two enzyme preparations from H.conicus was 7 to 7.1 for the 10% saturated ammonium sulfate precipitate and 6.7 for the 40% saturated ammonium sulfate precipitate (Figures 8 and 9). These values compare well with the optimum pH values for succinic dehydrogenases from A.niger and N.crassa which are 7.3 and 6.9 respectively (Martin, 1954; Shepherd, 1951).

Malonate and pyrophosphate are competitive inhibitors for succinate (McDonald et al, 1963). As expected malonate inhibited H.conicus succinic dehydrogenase by about 75% at 5.9mM (Table 18). However, no inhibition was observed with pyrophosphate. Since only 1.8mM pyrophosphate was present in the reaction mixture, it is possible that the concentration of pyrophosphate was too low. Ortho-Phenanthroline inhibited about 80% of the activity of H.conicus succinic dehydrogenase at 1.8mM. Since o-phenanthroline is a metal chelating agent for iron and zinc, the succinic dehydrogenase of H.conicus could be a metallo-enzyme containing either iron or zinc. The succinic dehydrogenases from beef

heart, M.lactilyticus and C.purpurea were reported to contain iron as a prosthetic group (Bernath and Singer, 1962; McDonald et al, 1963).

The succinic dehydrogenase of H.conicus was found to be comparatively labile. About 70% of the activity was lost on storing the enzyme at -15° for a month. The enzyme from C.purpurea (McDonald et al, 1963) lost only 10% of its activity on storing at -15° for a month. However, the enzyme from beef heart could be stored for only several days at this temperature (Bernath and Singer, 1962).

C. NITRATE REDUCTASE

The enzyme nitrate reductase catalyzes the reduction of nitrate to nitrite. The enzyme has been found in several fungi. Nason and Evans (1953) reported the presence of nitrate reductase in N.crassa and Cove (1966) described its presence in A.nidulans. The enzyme has now also been found in H.conicus. Its specific activity in H.conicus was 55.8 and 63.5 in the nitrate and the nitrate plus 0.5% yeast extract media respectively (Table 20). The specific activity of A.nidulans nitrate reductase was 52 while the specific activity of N.crassa nitrate reductase was 60 (Nason and Evans, 1953).

The fact that the nitrate reductase activity was the same in a nitrate containing media regardless of whether the growth of the organism was optimum or not, indicates that nitrate alone and not growth was essential for the production of nitrate reductase by H.conicus. Pateman et al (1967) reported that nitrate was essential for nitrate reductase activity in A.nidulans. The specific activity of the enzyme varied between 4 and 52 when the various amounts of nitrate were present in the

medium. Sorger (1965) also established the fact that nitrate was an absolute requirement for the nitrate reductase activity in N.crassa. The specific activity of the enzyme was increased to a maximum of 35 as the concentration of nitrate present in the medium increased.

In general nitrate reductase uses either NADH, NADPH or both as a cofactor. The enzymes from A.nidulans and N.crassa were specific for NADPH (Cove, 1966; Sorger, 1965) while the enzyme from E.coli was specific for NADH (Taniguchi and Itagaki, 1960). In the measurement of H.conicus nitrate reductase activity NADPH was used as a cofactor but not NADH. Hence, the nitrate reductase of H.conicus was specific for NADPH.

For a period of about one year, no attempts were made to measure H.conicus nitrate reductase activity because of involvement with another project. After that period, it was decided to find a possible correlation, if there was one, between the enzymes nitrate reductase and succinic dehydrogenase. Enzyme preparations were made either by a sonifier or by the French press technique. The H.conicus nitrate reductase activity measurements were made on preparations centrifuged at various speeds from 1,000 x g to 24,000 x g. The enzyme activity was measured in both the supernatant and the sediment. No significant activity was observed in any preparation. However, in each preparation a very high concentration of nitrite was found. The presence of high concentrations of nitrite in the preparation could be due to the inhibition of the enzymes which are responsible for the further reduction of nitrite or it could be that the organism had lost its ability to synthesize the enzymes which further utilize nitrite.

Since the organism reduced nitrate to nitrite during its growth, there can not be any doubt about the presence of nitrate reductase in the organism. However, because the enzyme activity was not measurable, it is possible that the high concentration of nitrite in the enzyme preparations inhibited the nitrate reductase.

Methylene blue was used in a qualitative measurement of H.conicus nitrate reductase activity. In the presence of succinate, succinic dehydrogenase under anaerobic conditions can reduce methylene blue (oxidized form) to leucomethylene blue (reduced form). The leucomethylene blue can be again converted to the oxidized form under anaerobic conditions if the enzyme nitrate reductase and nitrate is present in the reaction mixture. In the experiments with the H.conicus preparation, the color of methylene blue disappeared and then reappeared after 36 hours in the sample tube containing nitrate. The control tube with no nitrate did not show the reappearance of methylene blue color. This experiment suggests that nitrate reductase was present but of extremely low activity since it took so long for the color to reappear. Since there was a large amount of nitrite present in the preparation, it is possible that there was a significant inhibition of nitrate reductase by nitrite.

It is believed that the nitrate reductase of H.conicus is not involved in the utilization of nitrate as a nitrogen source as was the case in N.crassa but nitrate reductase of H.conicus resembles more the nitrate reductase observed in E.coli grown anaerobically where nitrate is used strictly as a terminal electron acceptor in the oxidation of

substrate. At present the experimental evidence which supports this statement is, the poor growth of H.conicus on the nitrate medium, a significant growth of H.conicus on the nitrate medium under anaerobic conditions, the fact that leucomethylene blue was oxidized in the presence of nitrate under anaerobic conditions, and the stimulation of succinic dehydrogenase activity by nitrate under anaerobic conditions.

H.conicus does not have the great anaerobic potential as does the facultative aerobe E.coli. Under anaerobic conditions E.coli will grow very well on nitrate medium and the enzyme nitrate reductase will be induced. The rate of synthesis of this enzyme has been shown to be dependent upon the amount of nitrate present in the medium (Showe and DeMoss, 1968). The enzyme induction was inhibited by oxygen. E.coli nitrate reductase acts as a terminal electron acceptor in place of oxygen, requires NADH as a supplier of electrons and no relation has been observed between it and cytochrome c. Contrary to the behavior of E.coli, H.conicus grows poorly under anaerobic conditions even in the presence of nitrate and the induction of nitrate reductase will occur even under aerobic conditions in H.conicus. Because of this latter observation it is assumed that the nitrate reduction in H.conicus is not a normal metabolic pathway as it is in the anaerobic growth of E.coli but would appear more likely that nitrate is toxic for H.conicus and that its reduction to nitrite is a detoxification mechanism.

The reduction of nitrate to nitrite requires electrons. A source of electrons is the oxidation of succinate by the enzyme succinic dehydrogenase. Nitrate in the medium stimulates the succinic dehydrogenase activity in H.conicus cells. This indicates that the organism may be

utilizing succinate oxidation for the reduction of nitrate to nitrite.

H.conicus also induces the oxidative enzyme IAA oxidase in the presence of IAA (see page 72). Perhaps IAA is toxic for H.conicus and to overcome IAA toxicity the organism induces the enzyme IAA oxidase which inactivates IAA by an oxidative mechanism.

Since the toxic effects of nitrate and IAA are destroyed by oxidative mechanisms, it appears that H.conicus has a great facility for detoxification of toxic substances present in the medium by either stimulating or inducing oxidative enzymes.

D. CYTOCHROME c REDUCTASE

Kinsky and McElroy (1958) reported that the enzyme cytochrome c reductase of N.crassa is induced by nitrate along with the enzyme nitrate reductase. Since nitrate reductase was found to be induced in H.conicus, it was interesting to know whether or not cytochrome c reductase was also stimulated by nitrate in H.conicus. The activity of H.conicus cytochrome c reductase in mycelia grown in three different media was measured. The specific activity of cytochrome c reductase in 4% malt extract medium was 36.4, and in the nitrate, and the nitrate plus 0.5% yeast extract medium it was 103 (Table 21). The large increase in the specific activity of cytochrome c reductase when nitrate was present in the medium suggests the induction of the enzyme by nitrate. The results also indicate that there is no effect of yeast extract on the activity of H.conicus cytochrome c reductase.

E. ENDOGENOUS AND EXOGENOUS RESPIRATION OF HYGROPHORUS CONICUS

In the past it was observed that mechanical treatment of fungal

mycelium to make a homogenous suspension may injure the cells and reduce the respiratory rate (Newcomb and Jennison, 1962; DeFiebre and Knight, 1953). Because of this possibility the cell suspension of H.conicus used for the respiratory measurements was carefully made using a glass rod.

The respiration rates of H.conicus under aerobic conditions was found to be 17.3 with glucose and 16.7 without glucose, and under anaerobic conditions the rate was 3.1 with glucose and 2.5 without glucose (Table 22). The results indicated that exogenous glucose has no significant effect on the respiration rate of H.conicus. Since H.conicus grows in a medium containing glucose as a sole carbon source, there is no doubt about its utilizing glucose. It is well known (Cochrane, 1958) that endogenous respiration is very high in fungi and H.conicus appears to be no exception.

F. INDOLE ACETIC ACID OXIDASE

Earlier work with H.conicus had shown that the organism forms an enzyme which catalyzes the conversion of indole-3-acetic acid (IAA) to oxindole-3-acetic acid (OIAA) (Siehr, 1961). The conversion was readily carried out with the intact cells but as yet it has not been possible to obtain an active cell-free preparation of the enzyme.

The attempts to make an active cell-free preparation of IAA oxidase by rupturing the cells either with a Branson sonifier or in the French press have proved to be unsuccessful. Both processes are known to effectively rupture cell-walls (Gunsalus, 1955). There are a number of possibilities which might account for this lack of success. The enzyme may contain sensitive -SH groups which on exposure to high oxygen tension

of the atmosphere are readily oxidized thereby inactivating the enzyme. To exclude this possibility reduced glutathione was added to the cell-free preparation but to no avail.

It is also possible that the enzyme responsible for oxidizing IAA is tightly bound to cell membranes and that the enzyme is a so called "insoluble" enzyme. This fact is true in the case of the enzymes ascorbic acid oxidase, acid phosphatase, and invertase (Kivilaan et al, 1961). This possibility was checked by using the cell debris in an assay. Again this approach was not successful.

A more probable reason for the inability to observe IAA oxidase activity in the cell-free preparations is that certain cofactors are required for the oxidation. The case of oxidations by mixed function oxidases (hydroxylases or monooxygenases) has been most extensively studied. This enzyme (mixed function oxidase) functions by incorporating one atom of molecular oxygen into the substrate molecule and reducing the other atom to water. An electron donor (a reductant) is a requirement for these enzymes to carry out the reaction (Hayaishi, 1962). Hosokawa and Stanier (1966) reported that a preparation of p-hydroxybenzoate hydroxylase from P.putida requires NADPH as the hydrogen donor. Yamamoto et al (1965) found FAD as a specific cofactor for the enzyme salicylate hydroxylase of Pseudomonad. Since one atom of oxygen is incorporated into the substrate molecule in the conversion of IAA to OIAA, it was thought H.conicus IAA oxidase might be a mixed function oxidase and that a cofactor was required for the IAA oxidase activity. NADPH and FAD were tried as cofactors for the IAA oxidase of H.conicus but no enzyme activity was observed in the presence of these

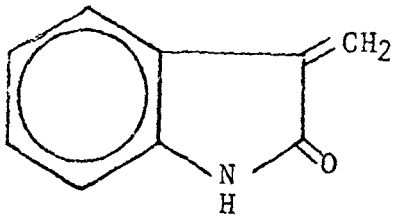
cofactors. From these results it was concluded that the enzyme IAA oxidase of H.conicus is not a simple oxygenase but that it is quite likely that it requires additional unknown cofactors.

In all the above mentioned experiments the measurement of IAA oxidase activity was based on the formation of OIAA in the reaction mixture. The possibility always exists that the compound on which the assay technique is based is not formed in the cell-free condition although such a compound accumulates in the whole cell technique. Because of this a technique based upon the disappearance of substrate IAA rather than the appearance of product was tried. Due to its sensitivity in detecting IAA, the Salkowski reagent was used to measure the rate of disappearance of IAA. The cell-free preparation of IAA oxidase was made by the French press and the Salkowski reagent method was used to measure the enzyme activity. No significant amount of IAA disappeared.

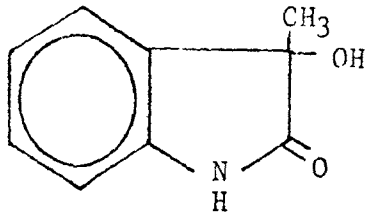
The presence of 2,4-dichlorophenol and manganese ions was found to stimulate IAA oxidation by the enzyme peroxidase (Hinman and Lang, 1965). Both of these substances were added to the reaction mixture in the measurement of H.conicus IAA oxidase activity by the Salkowski reagent method. This approach was unsuccessful.

Patterson (1965) found that OIAA, the product of IAA oxidation, also induced an enzyme in H.conicus when H.conicus cells were incubated with IAA. The concentration of OIAA increased during the first 48 hours, but after that time the OIAA concentration decreased. This suggested the induction of an enzyme which carried out the breakdown of OIAA. In the past, several substances have been reported as the end product of

the enzymic oxidation of IAA. Hinman and Lang (1965) reported 3-methyleneoxindole (I) while Ray and Thimann (1955) reported 3-methyldioxindole (II).



I



II

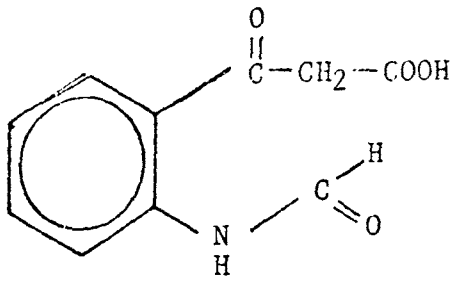
The nature of the further degradation products of IAA in H.conicus was of interest especially in light of the observations of Hinman and Lang (1965) and Ray and Thimann (1955) that the methyl oxindoles were the end products of IAA metabolism. Paperchromatography, radioautography, and UV spectroscopy were used to attempt to characterize the metabolic product(s) of OIAA.

If the carbon of the carboxyl (-COOH) group of OIAA is lost during its degradation then it is possible that methyl oxindole is a product of IAA metabolism in H.conicus. When either $^{14}C_1$ or $^{14}C_2$ IAA was added to a culture of H.conicus, and chromatograms were run of extracts of the culture medium three radioactive spots were observed on a radioautograph of the developed chromatogram. The presence of radioactivity in the three spots was also confirmed by scanning the chromatogram strip on a Packard radiochromatogram scanner. Two of these spots absorbed UV light while the third fluoresced in the UV. The fluorescence spot corresponded to OIAA while the two UV absorbing spots were unknowns

A_L and A_R. The R_f x 100 values for A_L and A_R in isopropanol : ammonia : water system were 39 and 38 respectively, while in benzene : propionic acid : water they were 35 and 81 respectively (Table 2). The presence of radioactivity in both of these unknowns when either the carboxyl or methylene carbon of IAA was labelled suggest that the carbon of carboxyl group of IAA was not lost and the side chain of IAA remained intact during the enzymic reaction. Thus the possibility of A_R or A_L being either I or II is ruled out. Since no other UV absorbing spots were detected on papergrams of extracts of the medium it is unlikely that any I or II is formed during the subsequent decomposition of OIAA by H.conicus.

None of the unknown spots was IAA since none of them showed a violet color with Ehrlich reagent, and no peak was observed at 280 mμ in a UV spectrum of the materials eluted from papergrams.

The unknown A_L was eluted with ethanol (95%) from a papergram. The UV spectrum of the eluate showed two peaks, one at 253 mμ and another at 287 mμ (ratio, approximately 4:1). This spectrum had a very close resemblance to that of o-formaminobenzoyl acetic acid (III) (Jones and Stevens, 1953).



III

Schopf (1967) reported that the compound III was quite unstable. Since III contains a carbonyl (-CO-) group, it was expected that it

would react with 2,4-dinitrophenyl hydrazine and silver nitrate-ammonia solution. The unknown A_L gave negative results when the chromatogram was sprayed with either of these solutions. Due to the negative results with the color reagents and due to its reported instability, the possibility of III being a metabolic product in H.conicus has been ruled out. However, it is believed that the unknown A_L has a very close structural relationship to compound III.

Oxindole acetic acid is readily converted to 3,4-dihydroquinolone - 4-carboxylic acid in acidic condition (Julian et al, 1953). Because of this facile reaction the possibility that the unknowns were quinoline derivatives was tested. 3,4-dihydroquinolone-4-carboxylic acid, 2,8-quinolinediol and 2,4-quinolinediol were available and solutions of all of these substances were used as standards. The UV spectrum of these substances were quite different from those of either unknown. The R_f value of 2,4-quinolinediol was almost the same as the unknown A_R in the isopropanol : ammonia : water and in the benzene : propionic acid : water systems. However, 2,4-quinolinediol did not show a characteristic phenol color with Folin reagent while unknown A_R did. These results ruled out the possibility either unknown being a quinoline derivative.

The unknown A_R showed a peak at 250 mμ in the UV spectrum. The spectrum was very similar to the one for OIAA. It is possible that the unknown A_R was mixed with OIAA. The R_f value of unknown A_R in almost all the solvent systems was also very close to that of OIAA.

5-Hydroxy indole acetic acid was one of the metabolic products of IAA in H.conicus only when the reaction was allowed to proceed for 2 hours or less. The absence of 5-hydroxy indole acetic acid in the

reaction mixture obtained in a reaction period of 4 hours or more, the R_f value of unknown A_R which was very close to OIAA, and its positive reaction with Folin reagent suggested that the unknown A_R could be 5-hydroxy oxindole acetic acid.

An attempt was made to prepare the unknown A_R by incubating 5-hydroxy indole acetic acid with the whole cells of H.conicus. When extracts of the culture medium were made and chromatographed, no spot corresponding to A_R was observed. Hence 5-hydroxy indole acetic acid is not a direct precursor of A_R. It is never-the-less possible that A_R is 5-hydroxy oxindole acetic acid.

An authentic sample of 5-hydroxy oxindole acetic acid was not available so its chromatographic properties and its ultraviolet absorption spectrum could not be compared with A_R.

V. CONCLUSIONS

From the results obtained in this investigation the following conclusions were made:

(1) The disruption of H.conicus cells by sonication or under high pressure resulted in the degradation of mitochondria.

(2) Phenazine methosulfate was found to be a more efficient electron acceptor than brilliant cresyl blue for the enzyme succinic dehydrogenase of H.conicus.

(3) NAD^+ was found to be a necessary cofactor for H.conicus succinic dehydrogenase and it could be readily dissociated from the enzyme.

(4) The enzymic activity of succinic dehydrogenase from H.conicus was found to be stimulated by nitrate. No such stimulation was observed in the case of C.lagopus.

(5) It is postulated that H.conicus succinic dehydrogenase requires an electron carrier for the transfer of electrons from succinate to methylene blue and that the carrier is activated by nitrate.

(6) H.conicus contains two types of succinic dehydrogenase, one is precipitated at 10% ammonium sulfate saturation and another at 40%. It is concluded that the former is a normal enzyme associated with the mitochondria while the latter is either a partly or completely soluble enzyme.

(7) The enzyme succinic dehydrogenase of H.conicus precipitated at 10% ammonium sulfate saturation showed a pH optimum of 7 to 7.1. The Michaelis constant K_m was determined to be 24mM at pH 7.6 and 30°.

(8) The enzyme succinic dehydrogenase of H.conicus precipitated at 40% ammonium sulfate saturation showed a pH optimum of 6.7.

(9) The enzyme nitrate reductase of H.conicus was found to be an inducible enzyme. The enzyme is specific for NADPH. After a time it was not possible to prepare an active cell-free preparation. The loss of activity was attributed either to genetic chenges in the organism or to the inhibition of the enzyme by nitrite. It is believed that the nitrate reductase of H.conicus is not involved in the utilization of nitrate as a nitrogen source but it is involved where nitrate is used strictly as a terminal electron acceptor in the oxidation of substrate.

(10) The enzyme cytochrome c reductase of H.conicus was found to be induced by nitrate.

(11) H.conicus could not utilize exogenous glucose directly.

(12) No satisfactory method has been found for obtaining an active cell-free preparation of H.conicus IAA oxidase. On the basis of these failures, it is assumed that the enzyme is not a simple oxygenase but may require certain, as yet unknown, cofactors for its activity.

(13) There are at least two other oxidation products of IAA besides OIAA in H.conicus. None of these has been identified, however, some of the properties of these compounds were studied. It is believed that the breakdown of the five membered ring of indole occurs during the further enzymic oxidation. There is a possibility that one of the unknown compounds is 5-hydroxy oxindole acetic acid.

(14) It appears that nitrate and IAA are toxic for H.conicus, and the organism has a great facility for detoxification of these substances by either stimulating or inducing oxidative enzymes.

VI. RECOMMENDATIONS

(1) Mitochondrial preparations of H.conicus should be made with a Branson sonifier and a French press and the integrity of mitochondria should be observed with an electron microscope.

(2) The cell-free preparation of H.conicus should be centrifuged at 144,000 x g and the succinic dehydrogenase activity in the supernatant determined to see whether or not the enzyme is in a soluble form.

(3) Purified H.conicus succinic dehydrogenases should be examined by electrophoresis to see if they are pure.

(4) Check the effect of protamine sulfate precipitation on the purified H.conicus succinic dehydrogenase.

(5) Attempt to separate lipid or nucleic acid from the purified preparation of H.conicus succinic dehydrogenase to establish whether or not the enzyme is attached to a lipoprotein or a nucleoprotein.

(6) Try to concentrate the purified H.conicus succinic dehydrogenase by ammonium sulfate precipitation.

(7) Grow H.conicus with various amounts of nitrate in the medium under anaerobic conditions and determine what effect this has on the enzyme nitrate reductase.

(8) Use ^{14}C-glucose to determine the utilization of glucose by H.conicus.

(9) Grow H.conicus on the medium with and without nitrate and study the relative amounts of protein present in the cells.

(10) Collect cell walls of H.conicus and attempt to locate IAA oxidase activity in them.

(11) Synthesize 5-hydroxy oxindole acetic acid and compare its properties with the metabolic product A_R.

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VIII. APPENDIX

SPECIFIC ENZYME ACTIVITY DATA

Medium: Nitrate and nitrate +0.5% yeast extract

Enzyme preparation: Cell-free extract

Assay: Succinic dehydrogenase

Method: Phenazine methosulfate

Time in minutes	μlO_2 per mg protein in the nitrate medium	μlO_2 per mg protein in the nitrate +0.5% yeast extract medium
0	-	-
5	1.21	3.42
10	3.62	6.83
15	6.01	9.27
20	9.05	10.9
25	10.85	12.9
30	13.8	16.1
35	-	18.3
40	16.3	20.2
45	-	23.0
50	19.9	24.4

For complete description of the system see Table 2; these data are plotted in Figure 2.

Medium: Nitrate and nitrate + 0.5% yeast extract

Enzyme preparation: Mitochondrial

Assay: Succinic dehydrogenase

Method: Phenazine methosulfate

Time in minutes	$\mu l O_2$ per mg protein in the nitrate medium	$\mu l O_2$ per mg protein in the nitrate +0.5% yeast extract medium
0	-	-
5	4.54	2.64
10	9.84	9.86
15	15.5	16.0
20	18.9	22.3
25	21.6	28.8
30	25	34.5
35	-	39
40	30.3	43.5
45	-	47.9
50	35.6	-

For complete description of the system see Table 4; these data are plotted in Figure 4.

Medium: Nitrate and nitrate +0.5% yeast extract

Enzyme preparation: 24,000 x g supernatant

Assay: Succinic dehydrogenase

Method: Phenazine methosulfate.

Time in minutes	μlO_2 per mg protein in the nitrate medium	μlO_2 per mg protein in the nitrate +0.5% yeast extract medium
0	-	-
5	2.0	0.229
10	3.35	2.06
15	4.68	3.44
20	6.68	5.5
25	8.0	7.34
30	8.7	9.86
35	-	11
40	10	12.8
45	-	13.8
50	-	14.9

For complete description of the system see Table 6; these data are plotted in Figure 6.

Medium: 0.5% yeast extract

Assay: Succinic dehydrogenase

Method: Phenazine methosulfate

Time in minutes	Cell-free extract $\mu l O_2$ per mg protein	Mitochondrial $\mu l O_2$ per mg protein	24,000 x g supernatant $\mu l O_2$ per mg protein
0	-	-	-
5	0.55	-	3.14
10	1.37	-	5.15
15	3.02	1.72	6.58
20	4.11	3.44	8.02
25	4.95	4.29	9.7
30	5.78	4.72	10.3
35	6.3	5.15	11.4
40	6.87	6.86	12.45

For complete description of the system see Tables 3, 5, and 7; these data are plotted in Figures 3, 5, and 7.

Medium: 0.885% yeast extract

Assay: Succinic dehydrogenase

Method: Phenazine methosulfate

Time in minutes	Cell-free extract $\mu l O_2$ per mg protein	Mitochondrial $\mu l O_2$ per mg protein	24,000 x g supernatant $\mu l O_2$ per mg protein
0	-	-	-
5	0.274	0.6	-
10	1.92	3.0	0.55
15	3.3	6.6	1.38
20	4.37	10.2	2.75
25	6.03	12.6	3.57
30	7.42	15.3	4.68
35	8.78	18.0	5.75
40	9.05	19.2	6.32
45	10.25	21.0	7.42

For complete description of the system see Tables 3, 5, and 7; these data are plotted in Figures 3, 5, and 7.

Medium: 4% malt extract

Assay: Succinic dehydrogenase

Method: Phenazine methosulfate

Time in minutes	Cell-free extract $\mu l O_2$ per mg protein	Mitochondrial $\mu l O_2$ per mg protein	24,000 x g supernatant $\mu l O_2$ per mg protein
0	-	-	-
5	1.86	0.356	0.95
10	3.1	3.2	1.27
15	4.02	6.75	2.54
20	6.2	9.6	5.06
25	8.05	12.8	6.04
30	8.97	14.2	5.39
35	9.94	17.1	6.04
40	10.8	18.85	7.3
45	12.1	19.9	7.9

For complete description of the system see Talbes 3, 5, and 7; these data are plotted in Figures 3, 5, and 7.

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