

MITOCHONDRIAL OXIDASE SYSTEMS IN NEUROSPORA

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ABSTRACT

Mitochondrial oxidase systems of Neurospora crassa were investigated with respect to their mechanisms of electron transport and their relationships to respiratory metabolism.

A mitochondrial oxidase system able to utilize dihydroorotic acid as a primary substrate was found and characterized. Another substrate for this system was isolated from yeast extract and identified as 5 N-methylformamido-L-dihydroorotic acid. This system was found to occur in both wildtype and poky, a respiratory mutant. No linkage to the cytochrome chain or to oxidative phosphorylation could be detected.

A succinate oxidase system and NADH oxidase system independent of cytochromes b, a, and a₃ were found to occur in both wildtype and in the poky mutant. The system was partially characterized using the poky mutant, which is deficient in cytochromes b, a, and a₃. It was found that this oxidase system was a part of respiratory metabolism and was linked to oxidative phosphorylation.

Mechanisms of electron transport in these oxidase systems are discussed and models presented.

Possible biological origins and the biological significance of 5 N-methylformamidodihydroorotic acid are also discussed.

The development of poky and wildtype mitochondria during the growth cycle were studied and compared. Differences in morphology were detected using electron microscopy and differences in cytochrome content were detected by absorption spectroscopy. The relationships between the oxidase systems and mitochondrial development are discussed.

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INTRODUCTION

The tropical bread mold Neurospora crassa has a classical mammalian type cytochrome system consisting of cytochromes a, a₃, b, and c (1). Using electron microscopy it has been shown that Neurospora also contains numerous mitochondria of the classical type found in other organisms (2,3), and association of the cytochromes with isolated mitochondria has been demonstrated (4). Particles (material sedimenting under high speed centrifugation, consisting mainly of mitochondria) from cell free extracts contain citric acid cycle enzyme activities as do mammalian mitochondria (5).

Thus the respiratory system and the mitochondria of Neurospora seem to be very similar to those of mammalian systems, but there are significant differences. Cyanide and azide, poisons which inactivate cytochrome oxidase activity almost completely, can inhibit respiration in Neurospora only by about 80% (6). Also a loss of the cytochrome system by mutation is not lethal. Many mutants with deficient cytochrome systems exist in Neurospora (1,6), suggesting that alternate mechanisms of respiration are operative. Of these respiratory mutants, poky exhibits the most extreme variations in phenotype and has been investigated more extensively than have the others. Because of the diversity of the phenotype of this mutant, it has been very useful in the study of many phenomena, including cytoplasmic inheritance, respiratory deficiency, the regulation of fatty acid metabolism, and the regulation of cytochrome c biosynthesis. From the accumulated knowledge about the biochemistry associated with the poky mutation, it appeared that this would

also be an excellent system for studying the biochemical and morphological development of mitochondria, and the nature of cytochrome oxidase independent respiration in *Neurospora*. The objectives of the work undertaken for this thesis were to investigate the changes in mitochondrial function and biochemistry during development, and to further characterize the mechanisms of respiration.

The Poky Phenotype

The original poky strains were derived from spore pairs of crosses between wildtype strains (7). From genetic analyses it was found that in sexual crosses the mutation was transmitted through the cytoplasm of the protoperithecial parent (7). The genetic determinant being located in the cytoplasm is also a characteristic of the petite system in yeast (8). Other cytoplasmic mutants in Neurospora have subsequently been found (4,9-12) but are female sterile and hence the mutations must be transmitted through heterokaryosis or microinjection. It is interesting to note that all these cytoplasmic mutants are characterized by a pleiotropic phenotype involving altered growth rates and respiratory systems.

Though the poky mutation results in a pleiotropic phenotype, the original strains were detected by their very slow growth rates. These rates were not altered by biochemical supplements (7), but in liquid culture the rates could be increased by using a very heavy inoculum of conidia (6,13). Mitchell and Mitchell (14) found that the growth rate of poky could also be increased by the presence of a suppressor gene, f.

This gene is nuclear and is specific for the poky mutation. When poky is kept in a state of continuous vegetative growth along growth tubes, it is possible to maintain a wildtype growth rate (15).

The poky mutant is notable for its many deficiencies and accumulations. Haskins, et al. described the deficiencies of cytochromes b, a, and a₃ in young cultures of poky and the accumulation of cytochrome c, up to a 16 fold excess in these cultures (6). It has been found that the poky mutant also accumulates a two fold excess of both FAD (flavin adenine dinucleotide) and nicotinamide (16). In addition there is a great accumulation (up to a 20 fold excess) of unesterified fatty acids, more than half of which is linoleic acid with the remainder predominately other unsaturated acids (17,18).

Perhaps the most interesting feature of the poky phenotype is its variation during the growth of a culture. Unlike the static nature of the phenotypes expressed by most nuclear mutations, the poky phenotype is constantly changing throughout the life cycle of a culture. The descriptions of deficiencies and accumulations above apply to young cultures where the phenotype is at its extreme. As a culture ages its phenotype approaches that of wildtype. Cytochromes b, a, and a₃ eventually appear and the excess of cytochrome c is greatly reduced (6). Accompanying this dilution of excess cytochrome c is its conversion from an unbound form (C_{II}) to a chromatographically distinct form (C_I) which can be bound in mitochondria, as shown by Scott (13).

This conversion, involving modification of a lysine residue to an unidentified lysine derivative, also occurred in wildtype. The level of

excess unesterified fatty acids also decreases as a culture ages, but not to the same extent as with cytochrome c (17,18).

Though a poky culture will approach a wildtype phenotype as it ages it never becomes indistinguishable from wildtype and never loses its genetic defect. Thus after going through a conidial or spore stage, the extreme of the phenotype again appears upon germination (6).

The changes in poky during growth are reminiscent of the adaptation of anaerobically growing yeast when suddenly exposed to an aerobic atmosphere. A deficiency of cytochromes c, b, a, and a₃ is progressively overcome implicating an induction of synthesis by oxygen. Furthermore if a culture containing these cytochromes is put back in an anaerobic environment the synthesis of these cytochromes is inhibited and eventually their absorption bands disappear (20). But in this system, regulation of synthesis is a function of an environmental factor rather than a genetic one.

Recent studies by Woodward and Munkres (21) have shown that the primary genetic defect in poky is an amino acid substitution in the mitochondrial structural protein. A tryptophan residue in the wildtype protein is apparently replaced by a cysteine residue in the mutant structural protein, presumably as a result of an alteration in the mitochondrial DNA. Such an alteration could be accomplished by a single base substitution since the only codon for tryptophan is UGG and the only codons for cysteine are UGU and UGC (22). Assuming that mitochondrial DNA is transcribed by the same mechanism as nuclear DNA, this would correspond to a single site substitution of either adenine or

guanine for cytosine in the mutant mitochondrial DNA. This is compatible with the finding of Luck and Reich that there is no difference in the buoyant densities of wildtype and poky mitochondrial DNAs (23).

The mechanism of the altered structural protein affecting the regulation of so many biosyntheses is apparently through its altered binding properties. The mutant structural protein has been shown to have a much lower affinity for binding both NADH and ATP than does the wildtype protein (24). Also malate dehydrogenase when complexed with mutant mitochondrial structural protein has a much lower affinity for binding malate than when complexed with wildtype structural protein (25). The identical amino acid substitution has been found in structural protein isolated from nuclear membrane, from plasma membrane, from the microsomal supernatant, and from an acetone powder of the poky mutant (26). This suggests that all of the cellular membranes may have a common component whose genetic determinant is in the mitochondrial DNA. If this were the case it could account for aspects of the phenotype which are seemingly unrelated to mitochondria such as fatty acid metabolism and cell wall synthesis.

Respiration

Cytochrome abnormalities in poky were described earlier. Corresponding to the deficiencies in cytochromes a and a₃, Haskins, et al. found that young cultures of poky had no cytochrome c oxidase activity (6). They also found that these cultures were deficient in succinate oxidase activity but had a normal level of succinic dehydrogenase activity,

corresponding to the absence of cytochrome b. As with the cytochromes, the oxidase activities appeared and increased during the growth of a culture. The succinic dehydrogenase activity remained near the wildtype level throughout the growth.

Tissieres (27) was able to reconstruct a functional succinate oxidase system by mixing alkaline treated wildtype particles (deficient in succinic dehydrogenase activity) and particles from young poky cultures (deficient in cytochrome oxidase activity), but function was determined from spectroscopic observations. The reduced cytochrome c band disappeared upon mixing poky particles with wildtype particles in the presence of succinate, or even in the absence of succinate. This indicated that the cytochrome c in the poky particles, mostly in the reduced state, can interact with the cytochrome oxidase of wildtype particles in a mixture. However, reconstitution of the succinate oxidase system could not be confirmed by measuring oxygen consumption manometrically. This was attributed to an inhibitor in the poky particles.

Tissieres et al. (16) showed that oxygen uptake of both intact mycelium and cell free extracts from young poky cultures was insensitive to cyanide and azide, but that sensitivity to these poisons increased as a culture aged. Oxygen consumption by poky mycelium was dependent on the oxygen tension while that by wildtype mycelium was not. The uptake of oxygen by crude cell free extracts was attributed to oxidation of endogenous substrates derived from polysaccharides. This endogenous respiration was stimulated somewhat by NAD and to a large extent by NADP. These results led to a postulation of an alternate electron transport

pathway independent of cytochrome oxidase and probably involving a flavoprotein.

The existence of cyanide insensitive electron transport chains independent of cytochrome oxidase in plant tissues has been known for many years and has been extensively reviewed (28-35). Yet there is still much confusion about the nature of these systems. Various terminal oxidases of alternate electron transport pathways which have been postulated include: flavoproteins such as glycolate dehydrogenase in the spinach leaf (36); phenol oxidase in potato tubers (37); ascorbic acid oxidase in barley root tips (38); and an autooxidizable cytochrome b_7 in skunk cabbage (39).

Bacteria also exhibit cyanide resistant respiration. During germination the spores of Bacillus cereus use a system of electron transport which apparently involves dipicolinic acid and flavin mononucleotide as carriers and a flavoprotein as the terminal oxidase (40,41). Azotobacter uses a cytochrome ρ as a terminal oxidase (42). This cytochrome has also been found to be a terminal oxidase in blue-green algae (43,44).

Respiration independent of cytochrome oxidase has also been found in intestinal worms, ascribed to terminal oxidations by flavoproteins (45-48) and by cytochrome ρ (49,50).

The hibernating alfalfa weevil acquires a cyanide resistant succinate oxidase system in which cytochrome b_5 is apparently the terminal oxidase (51).

Cyanide and azide resistant respiration also occurs among fungi. Lindenmayer and Smith have found evidence for an alternate terminal oxidase in yeast (52). Cytochrome c peroxidase has been found in yeast (53), and Chance has proposed that a flavoprotein-cytochrome c peroxidase couple might be a means of electron transport bypassing cytochrome oxidase (54). Recently a cyanide and azide insensitive system of oxygen consumption, presumably related to mitochondria, has been found in Phycomyces blakesleeianus (55). Strains of Neurospora other than poky have been found to contain alternate pathways of electron transport. Haskins et al. could not completely inhibit respiration of wildtype with cyanide or azide (6). Tissieres and Mitchell found that Cl17, a nuclear mutant with deficiencies in cytochromes a, a₃ and c, has a respiratory system which was unaffected by cyanide or azide (1).

Biogenesis of Mitochondria

Morphologically normal and biochemically functional mitochondria have been observed in all stages of growth of wildtype Neurospora, from conidia to old hyphae (56). From autoradiographic studies, and from studies based on mitochondrial density changes, Luck has shown that the mitochondria arise from pre-existing mitochondria by a process of continual addition of components and subsequent division (3,57,58). Hawley and Wagner have found evidence that the division of the mitochondria within a hypha is synchronous (59).

The situation in the poky mutant is not so straight forward. Freese-Bautz reported short, rod-like mitochondria in poky conidia observed under

phase contrast (60). However, electron micrographs of germinating conidia showed a large amount of membrane material but no morphologically distinguishable mitochondria (61).

Yotsuyanagi has found that cytoplasmic respiratory mutants of yeast have a similar cytoplasmic membrane system with no well defined mitochondria (62). Phenocopies can be made from wildtype yeast by subjecting them to anaerobic conditions (63), by growing them in a high concentration of glucose (64,65) or by growing them in the presence of chloramphenicol (66). Mitochondrial formation can be induced in anaerobic cultures by exposure to oxygen, with development starting during the transitional period between exponential growth and the stationary phase (63). During this development there is a progressive recovery of a high respiratory rate and finally a large number of mitochondria with well developed cristae appear. Bartley and Tustanoff showed that the development of respiration in such an adapting culture was not affected by inhibitors of glycolysis, protein synthesis, RNA synthesis or DNA synthesis (67). Thus the formation of functional mitochondria during adaptation to oxygen required little energy and no significant macromolecular synthesis. This strongly supports the concept of "promitochondria" in anaerobic cultures which has been advanced by Gibor and Granick (68). Schatz has also suggested a "promitochondrion" structural precursor in glucose repressed cultures (65).

MATERIALS AND METHODS

Strains of Neurospora

Two strains of Neurospora crassa were used in this investigation. The strain 25a described by Beadle and Tatum (69) was used as a standard wildtype. The poky strain used was one isolated in collaboration with William A. Scott. This strain, designated as po-6a, was obtained as a single ascospore from a cross of po 3627-2a (protoperithecial parent) with the wildtype strain 4A as the conidial parent. The origin of the poky parent is given by Mitchell and Mitchell (7) and that of the wildtype parent is given by Beadle and Tatum (69).

Culture Media and Growth Procedures

Cultures of the strains used were perpetuated in the vegetative state by monthly transfer of conidia onto 5 ml slants of Horowitz complete medium (70) supplemented with 1.5% agar.

Large quantities of conidia were cultured in 1000 ml wide mouth Erlenmeyer flasks on 250 ml of Horowitz complete medium supplemented with 1.5% agar. The walls of the flasks were coated with a thin film of the solidified medium. These flasks were inoculated with conidia from slants and incubated at 35°C for 24 hours. Then the flasks were transferred to 25°C and kept under constant illumination. Wildtype conidia cultures were harvested after five days and poky conidia cultures were harvested after nine days. Conidia were harvested by adding 250 ml of sterile distilled water to each flask, followed by vigorous shaking and filtering the suspension through glass wool.

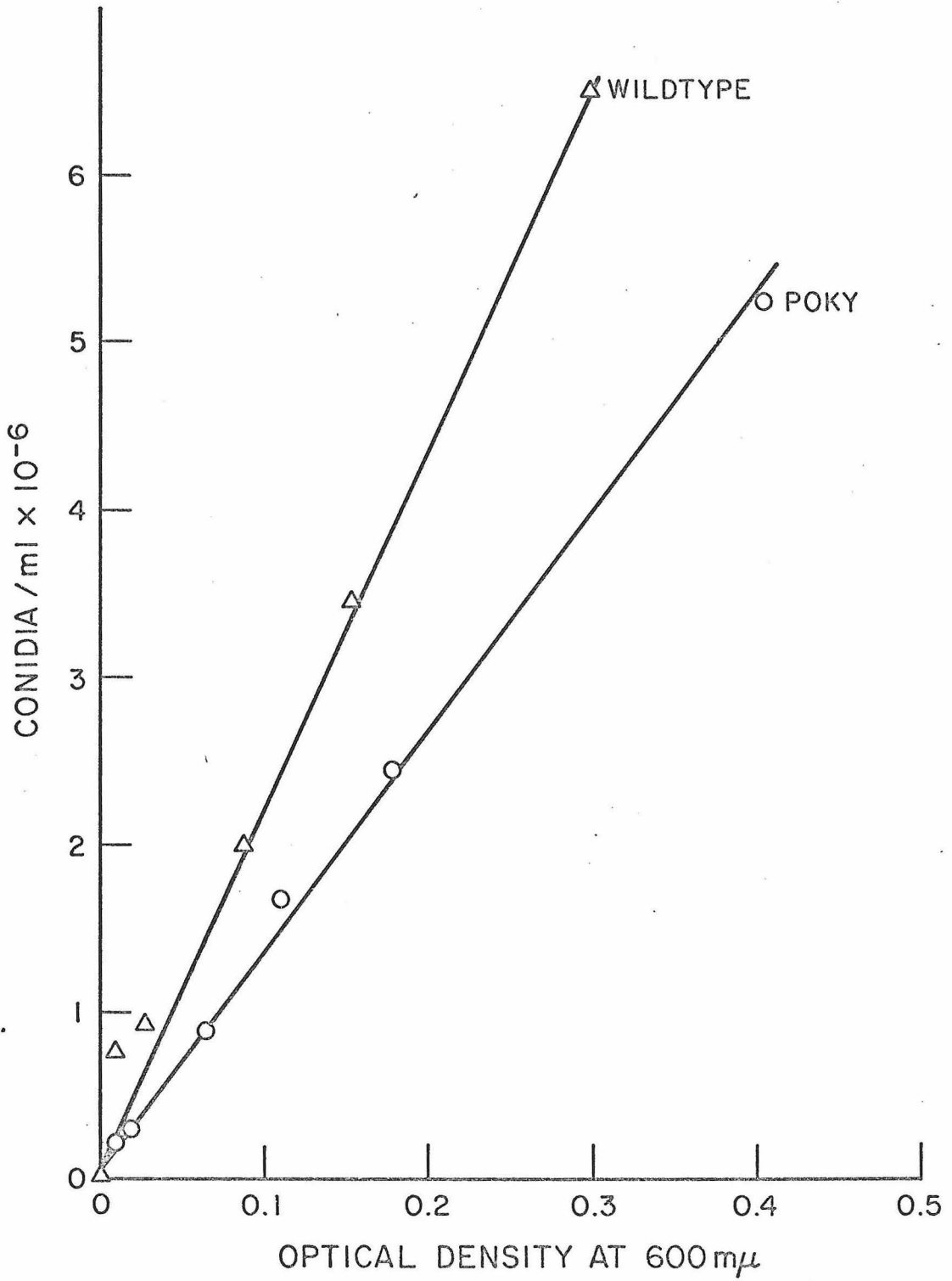
The concentration of conidia suspended in water was determined by measuring the optical density of the suspension at 600 m μ in a Bausch and Lomb Spectronic 20 colorimeter. When necessary, dilutions of a conidial suspension were made with distilled water. Calibration curves for both poky and wildtype conidia were made using a hemocytometer to count the number of conidia in a known volume. Suspensions between 1×10^6 conidia/ml and 6×10^6 conidia/ml gave optical densities which were linear with respect to concentration (Figure 1).

Mycelia were grown at 25°C in 5 gallon Pyrex carboys each containing 16 liters of Vogel's minimal salt solution (71) supplemented with 2% sucrose. Sterile water-saturated air was bubbled through the carboys at a rate of 12 liters/min. Carboys were inoculated with between 10^8 and 10^{10} conidia. The mycelia were harvested by filtering the carboy contents through two layers of Miracloth. If the conidia were harvested before or just after germination, it was necessary to filter through Whatman No. 1 filter paper instead of the Miracloth. After filtration, the mycelia were washed free of growth medium with distilled water.

Isolation of Mitochondria

The method used for isolating mitochondria was based on the procedure described by Luck (3). Washed mycelium was pressed dry with paper towels to determine the fresh weight. If a dry mass was desired, a small amount of mycelium was dried in a vacuum dessicator over P_2O_5 for 3 days.

FIGURE 1. Light Scattering by Poky and Wildtype Conidial Suspensions.



All operations in the preparation of mitochondria were carried out at 5°C. A cell-free extract of the mycelium was obtained by grinding in a mortar and pestle using 1.0 part (v/w) of homogenizing medium and 0.5 part (w/w) of sand. The homogenizing media were sucrose solutions between 0.25M and 0.44M. When present, EDTA (ethylenediaminetetraacetate) was 10^{-3} M; BSA (bovine serum albumin) was 0.15%; and Tris (hydroxymethylaminomethane) or orthophosphate was 0.05M.

When the extract had a very homogeneous texture it was centrifuged at 500 g for 10 minutes. The pellet was resuspended in the original volume of homogenizing medium used and ground a short while longer. This second homogenate was also centrifuged at 500 g for 10 minutes. The supernatants from both centrifugations were then pooled and centrifuged once more at 500 g for 10 minutes. Mitochondria were pelleted from the resulting supernatant by centrifugation at 15000 g for 30 minutes. The crude mitochondria were washed with homogenizing medium and repelleted by centrifuging at 15000 g for 30 minutes when used for assaying column fractions. For analytical work the mitochondria were further purified on an isopycnic sucrose density gradient.

Density gradients were prepared from two solutions:

- (1) 0.58M sucrose, 10^{-3} M EDTA, 0.15% BSA, 0.05 M Tris, pH 7.5.
- (2) 1.9M sucrose, 10^{-3} M EDTA, 0.15% BSA, 0.05 M Tris, pH 7.5.

Two ml of each solution were put in two parallel 1.0 cm diameter cylindrical chambers connected by a small bore at the bottom. The chamber with the more dense solution was equipped with a motor driven stirrer and a capillary tubing outlet at the bottom. Gradients made by this

method were linear and ranged in density from 1.05 g/cm^3 to 1.28 g/cm^3 .

Crude mitochondrial pellets were resuspended in a small volume of homogenizing medium and 1.0 ml aliquots of the suspension were layered on 4 ml density gradients. The density gradients were loaded in an SW39L rotor and centrifuged at 39000 rpm in a Beckman L-2 65 refrigerated ultracentrifuge for 90 minutes. Mitochondrial bands were collected with a Pasteur pipette and diluted with 5 volumes of homogenizing medium. The purified mitochondria were then pelleted by centrifuging at 15000g for 30 minutes and resuspended in a small volume of homogenizing medium.

Electron Microscopy

All materials prepared for thin sectioning were fixed in 2.5 % glutaraldehyde in 0.15M potassium phosphate buffer (pH 7.15) for 18 hours at 4°C . The material was then briefly washed in 0.15M potassium phosphate buffer (pH 7.15) and then post-fixed with a 2% solution of OsO_4 in 0.15M potassium phosphate buffer (pH 7.15) for two hours. After dehydration with a series of alcohol solutions (25%, 50%, 70%, 95%, 100% ethanol) the material was embedded in the epoxy resin araldite using the method of Luft (72). Thin sections were cut on a Porter Bloom ultramicrotome by Dr. S.K. Malhotra, and the sections stained in a saturated aqueous solution of uranyl acetate for one hour followed by staining with lead citrate for 5 minutes using the procedure of Venable and Coggeshall (73).

If the mitochondria were to be negatively stained, a drop of the mitochondrial suspension was diluted with several drops of distilled water cooled to 4°C. A drop of this diluted suspension was placed on a grid coated first with a thin film of Formvar or Parlodion and then with carbon. This was allowed to stay for 1-2 minutes at room temperature and most of the fluid was then removed from the grid by touching a piece of filter paper along the edge of the grid. Without further waiting 2-4 drops of a solution of potassium phosphotungstate (1-4% phosphotungstic acid brought to pH 6.8-7.0 with 10% potassium hydroxide) were applied to the surface of the grid. As much of the fluid as possible was immediately removed by touching the edge of the grid with a piece of filter paper. The grid was then allowed to dry for at least five minutes before it was examined in the electron microscope.

Examination of sectioned and negatively stained material was with a Phillips-200 Electron Microscope.

Assay Procedures

Mitochondrial suspensions were diluted with 200-500 volumes of distilled water to determine the protein content. Aliquots of 1.0 ml were used to assay for protein by the method of Lowry, et al. (74). using BSA as a standard. Appropriate corrections were made if the medium in which the mitochondria were suspended contained BSA.

The cytochrome content of mitochondria was determined from reduced versus oxidized difference spectra obtained by the method of Williams (75). Spectra were recorded by scanning the wavelength range from 650-480 m μ

on a Cary Model 15 recording spectrophotometer equipped with a 0-0.1 absorbance slide wire.

Oxidation of substrates coupled to the reduction of molecular oxygen was determined by recording the rate of oxygen consumption using a Model 53 Biological Oxygen Monitor (Yellow Springs Instrument Company), a polarographic oxygen electrode.

Assays were run at 30°C in a volume of 2.5-3.5 ml. The reaction mixtures were buffered at pH 6.9 by the use of 2.4-2.8 ml of a stock solution containing 44g/l of sucrose, 2.0 g/l of $MgCl_2 \cdot 6H_2O$, 0.37 g/l of EDTA, 4.0 g/l of BSA, and 1.36 g/l of KH_2PO_4 . Endogenous rates of oxygen consumption were determined before the addition of substrate. Substrates or other components were added to the reaction system in a volume of 0.1 ml through a piece of small diameter polyethylene tubing. The oxygen monitor was calibrated with air saturated distilled water at 30°C such that a 100 mv signal giving a full scale galvanometer deflection corresponded to a concentration of 0.21 μ moles of dissolved oxygen per ml.

Oxidative phosphorylation was measured by a procedure modified from the method of Hall and Greenawalt (76). The reaction system consisted of 2.4 ml of the stock solution described above, 0.1 ml of a 30 mg/ml solution of ADP (adenosine 5' diphosphate), 0.1 ml of a 20 mg/ml solution of NAD (β nicotinamide adenine dinucleotide), 0.1 ml of a 20 mg/ml solution of hexokinase (Sigma, Grade III) in 0.3M glucose, 0.1 ml water, and 0.1 ml of a solution of substrate. Reactions were started by adding 0.1 ml of a mitochondrial suspension containing 2-5 mg mitochondrial protein. The reactions were terminated by the addition of 0.1 ml of a

20% sodium deoxycholate solution, immediately followed by the addition of 0.5 ml of 10% trichloroacetic acid. For each reaction, a control using water instead of substrate was allowed to incubate for the same period of time, and endogenous oxygen consumption was monitored. Precipitate in the terminated reaction mixtures was removed by centrifugation and the supernatants were assayed for glucose 6-phosphate using a method based on the procedure described by Hohorst (77). A 0.5 ml aliquot was mixed with 0.01 ml of 2% NADP (β nicotinamide adenine dinucleotide phosphate), 0.01 ml of 10% $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, and 0.48 ml of 0.4M triethanolamine hydrochloride (pH 7.6). The absorbance at 340 m μ was recorded and then the reaction mixture was allowed to incubate at 25°C in the dark for three hours in the presence of 0.01 ml of a 5 mg/ml solution of yeast glucose 6-phosphate dehydrogenase. Afterwards the absorbance at 340 m μ was again recorded, and the amount of NADPH (reduced form of β nicotinamide adenine dinucleotide phosphate), produced was calculated using a micromolar extinction coefficient of 6.3. The amount of NADPH produced was assumed to be equivalent to the amount of glucose 6-phosphate present based on the stoichiometry of the glucose 6-phosphate dehydrogenase reaction.

Endogenous rates of oxygen consumption and ATP (adenosine 5' triphosphate) content were determined from the controls run in the absence of substrate. P/O ratios were determined from the substrate dependent oxygen consumption and phosphate esterification.

Preparation of Chemicals, Stains, and Chromatography Materials

Ferrous (reduced) cytochrome c was prepared from commercial horse-heart cytochrome c (Sigma Chemical Company). The cytochrome c was dissolved in 0.01 M sodium dithionite to a concentration of 100 mg/ml, then desalted on a Biogel P-2 column and lyophilized to dryness.

L-ureidosuccinic acid was prepared from L-aspartic acid and potassium cyanate by the method of Nyc and Mitchell (78).

Commercial nucleotides and enzymes were obtained from Sigma Chemical Company or the California Corporation for Biochemical Research.

Bromocresol green and ninhydrin stains for paper chromatograms were obtained as aerosol sprays from Sigma Chemical Company.

A bismuth iodide stain was prepared by dissolving 0.5 g of $\text{Bi}(\text{NO}_3)_3 \cdot 5\text{H}_2\text{O}$ and 0.4 g of potassium iodide in 50 ml of acetone plus 50 ml of methanol. Insoluble material was filtered off. Chromatograms were stained by irrigating in the bismuth iodide reagent and drying at room temperature.

A periodate-benzidine stain was used to detect α glycols on paper chromatograms. The chromatogram was irrigated in 0.0025 M periodic acid in acetone and then dried. Color development was then obtained by wetting with a 0.01M benzidine solution made up in 1% acetic acid in acetone, followed by drying at room temperature.

A reducing sugar stain was made by dissolving 1.69 g of 2-amino-biphenyl and 0.9 g of oxalic acid in a mixture of 5 ml of glycerol, 10 ml of water, and 84 ml of acetone. Chromatograms were stained by wetting in the reagent, drying at room temperature for 10 minutes,

followed by drying at 110°C for 5 minutes.

Phosphate compounds were detected using a spray reagent made from 25 ml of 4% ammonium molybdate, 5 ml of 60% perchloric acid, 10 ml of 1 M hydrochloric acid, and 60 ml of water. After spraying, the chromatogram was dried in an 80°C oven for one minute and then irradiated with a short wavelength ultraviolet mineral light lamp for 10 minutes at a distance of 10 cm.

Biogel P-2, 100-200 mesh, obtained from the California Corporation for Biochemical Research, was used in gel filtration and in desalting columns. The polyacrylamide spheres were soaked for 2 hours in distilled water and the fines removed before use.

Dowex 1-X8, 200-400 mesh, obtained from the J.T. Baker Chemical Company, was used for anion exchange chromatography. The resin was washed sequentially with 10 ml of 4N HCl/g resin, 20 ml of water/g, 10 ml of 2N NaOH/g, and 20 ml of water/g before use. This left the resin in the OH⁻ form. If the Cl⁻ form of the resin was desired it was subsequently washed with 10 ml of 4N HCl/g and 20 ml of water/g.

Dowex 50W-X4, 200-400 mesh, also obtained from the J.T. Baker Chemical Company, was used for cation exchange chromatography. This resin was washed sequentially with 10 ml of 2N NaOH/g resin, 20 ml of water/g, 10 ml of 4N HCl/g, and 20 ml of water/g, leaving the resin in the H⁺ form.

Whatman #3 filter paper, cut into 30 cm x 20 cm sheets, was used for ascending paper chromatography. Two solvent systems were used for paper chromatography, one basic and one acidic. The basic solvent system

was prepared by mixing 1.0 ml of 30% ammonia solution with 29 ml of water and 60 ml of n-propanol. The acidic solvent system was prepared by mixing 0.5 ml of glacial acetic acid with 49.5 ml of water and 100 ml of n-propanol. Chromatograms were developed in solvent saturated air at room temperature and dried before staining or eluting strips. Strips from dried chromatograms were eluted with water in a water saturated air atmosphere using descending chromatography. Elution volumes were reduced either by evaporation with an air stream or by lyophilization.

R E S U L T S

A. Characterization of the poky and wildtype phenotypes and mitochondria

Growth rates

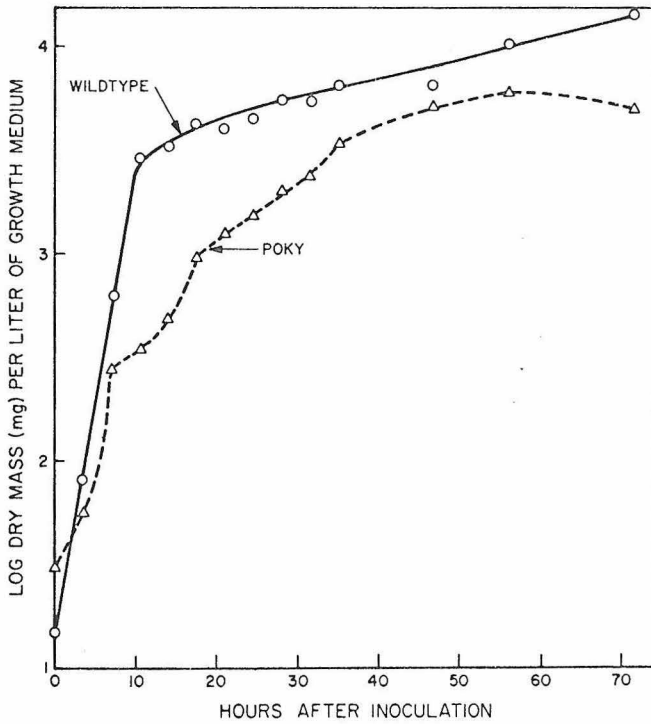
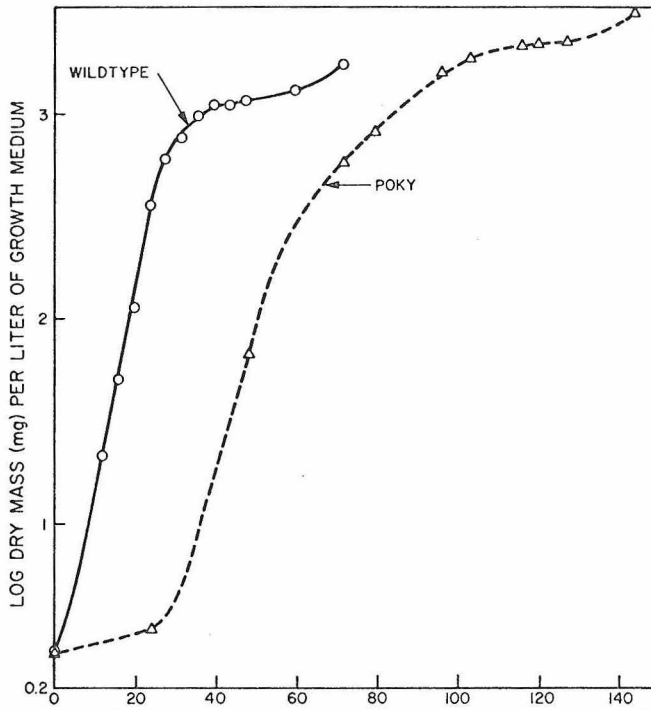
Because of the variations of cytochrome content in different ages of poky (6), it was necessary to standardize growth as much as possible.

Initially poky and wildtype mycelia were grown starting with an inoculation of 10^4 conidia/ml of medium. This gave the patterns of growth shown in Figure 2A. Using a light microscope, it was observed that wildtype conidia germinated about 4 hours after inoculation and that poky conidia germinated about 7 hours after inoculation. After germination the wildtype mycelium went into an exponential phase of growth for about 24 hours. During this time the rate of growth was a linear function of the mass, and the mass doubling time was 3 hours. The growth rate in the period between 28 hours and 40 hours after inoculation became less dependent on the mass. This phase will be referred to as the post-exponential phase. Between 40 hours and 60 hours after inoculation there was a stationary phase where the growth rate became quite independent of the mass and nutrients were probably limiting.

The poky growth curve in Figure 2A is different from that of wildtype in that there is a lag period (pre-exponential phase) after germination and before the exponential phase of growth. From 28 hours to 56 hours after inoculation the poky mycelium was in an exponential phase of growth with a mass doubling time of 5.5 hours. Then there was a post-exponential phase from 56 hours to 104 hours after inoculation, and a stationary phase after 104 hours.

FIGURE 2. Growth Curves for Poky and Wildtype Mycelium.

- A. Inoculum of 10^4 conidia/ml
- B. Inoculum of 10^5 conidia/ml



An inoculation of 10^4 conidia/ml did not yield sufficient quantities of young mycelia to work with, so inoculations of 10^5 conidia/ml were used. The resulting growth patterns for poky and wildtype mycelium are shown in Figure 2B. Germination times were the same as with the smaller inoculations, but the kinetics of growth were quite different. Growth was much faster with the larger inoculations for both poky and wildtype, and the growth rates were not so easily separable into discrete phases.

Since respiration in young poky was reported to be insensitive to azide (16), growth of poky and wildtype mycelia in the presence of 0.003 M NaN_3 was measured. The conidia of both strains germinated but no subsequent hyphal growth occurred.

It has been found that cytochromes a and b are absent in yeast grown under anaerobic conditions (20). However, under a nitrogen atmosphere wildtype Neurospora conidia germinated but did not grow. As far as could be determined with a Zeiss hand spectroscope the germinated conidia had a normal cytochrome spectrum.

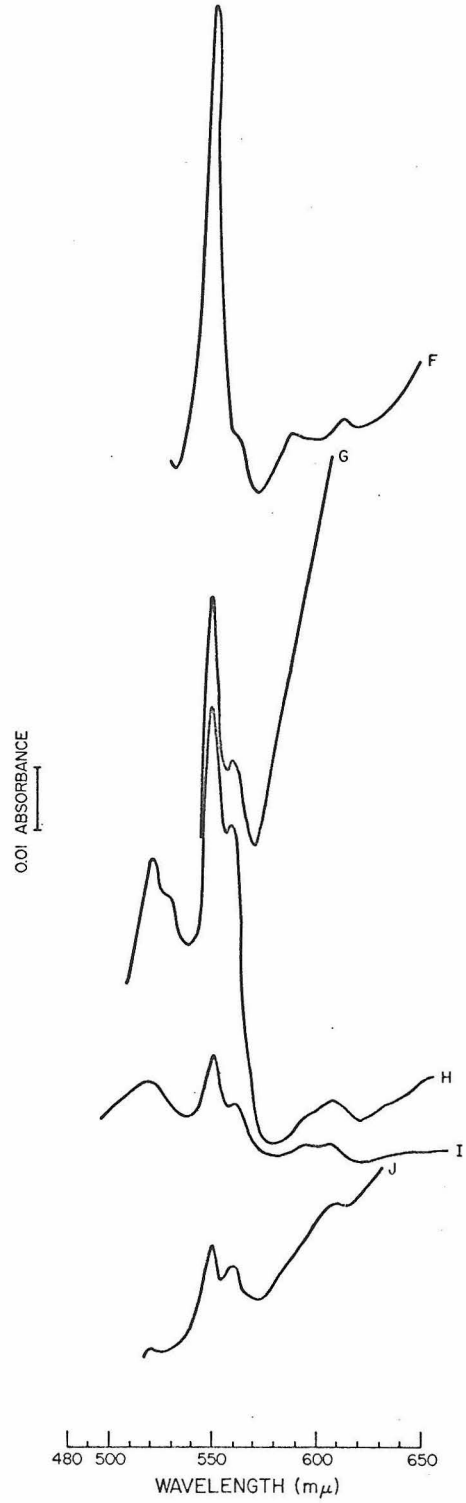
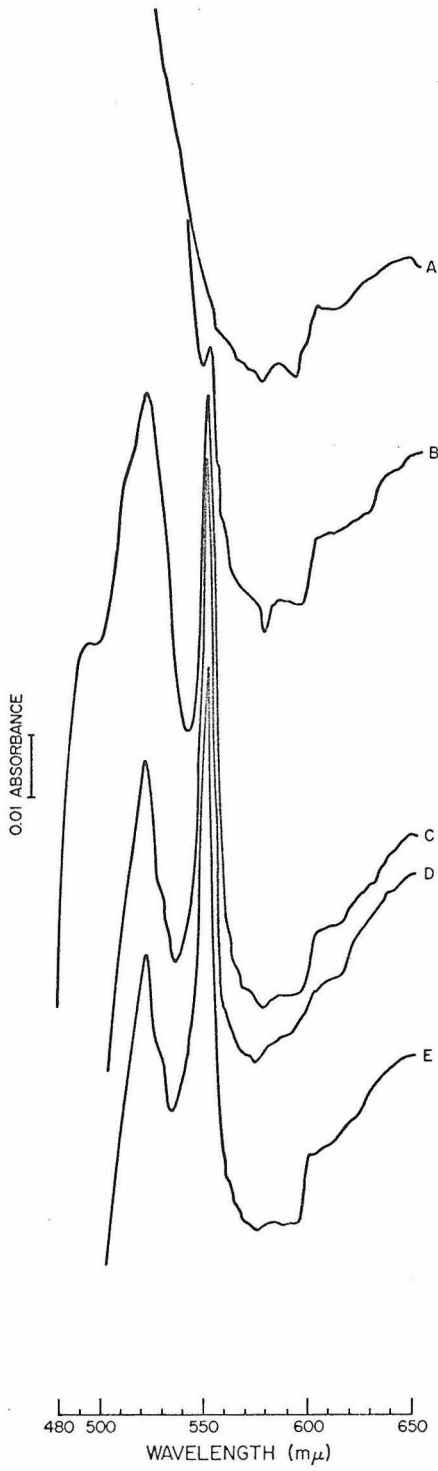
Cytochrome content of mitochondria during growth

Changes in the cytochrome content of poky mitochondria during the growth cycle are shown in Figure 3. In difference spectra of Neurospora mitochondria the α band of cytochrome a appears at 610 $\mu\mu$, the α band of cytochrome b appears at 560 $\mu\mu$, the β band of cytochrome b appears at 530 $\mu\mu$, the α band of cytochrome c appears at 550 $\mu\mu$, and the β band of cytochrome c appears at 520 $\mu\mu$. (4). However, in the spectra of Figure 3

FIGURE 3. Difference Spectra of Mitochondria from Poky Cultures of Various Ages.

Cultures were started with inoculations of 10^5 conidia/ml. Absorbances were recorded on the 0 - 0.1 scale. Equal amounts of mitochondrial protein were in the sample cell (reduced) and in the reference cell (oxidized).

- A. From conidia, 2.5 mg protein/ml
- B. From germinating conidia (7 hours), 10 mg protein/ml
- C. From a 10 hour culture, 10 mg protein/ml
- D. From a 17 hour culture, 10 mg protein/ml
- E. From a 24 hour culture, 10 mg protein/ml
- F. From a 30 hour culture, 10 mg protein/ml
- G. From a 37 hour culture, 5 mg protein/ml
- H. From a 60 hour culture, 10 mg protein/ml
- I. From a 72 hour culture, 1.5 mg protein/ml
- J. From a 120 hour culture, 1.5 mg protein/ml



and Figure 4, the α band of cytochrome a appears at 603 m μ , the α band of cytochrome b appears at 562 m μ , and the β band of cytochrome c appears at 522 m μ .

Isolation of mitochondria from conidia was extremely difficult. The material which was pelleted at 15,000 g had a very sticky and gummy texture and was difficult to resuspend. After centrifugation through a density gradient almost all the material was at the bottom of the tube. However, there was a barely detectable band corresponding to mitochondria. The same phenomenon was observed during isolation of mitochondria from germinating conidia and from very young mycelium, though the yield of mitochondria was much greater. The heavy material at the bottom of the gradients had mitochondrial oxidase activities but could not be solubilized with deoxycholate. Because of this insolubility in deoxycholate, the high density, and the sticky texture, it is likely that the material represents mitochondria or mitochondrial fragments associated with cell wall fragments. During germination and early hyphal growth the mitochondria become more easily dissociable from the cell wall fragments.

The difference spectrum of mitochondria from conidia does not have bands corresponding to cytochromes a, b, or c, although there is a shoulder around 600 m μ which may be due to a very small amount of cytochrome a. Mitochondria from germinating conidia contained a small amount of cytochrome c, but no detectable cytochromes a or b. Pre-exponential phase mitochondria contained a very large quantity of cytochrome c, but no detectable cytochromes a or b. During subsequent growth the amount of mitochondrial cytochrome c relative to total mito-

chondrial protein decreased as the culture aged. Cytochrome b did not begin to appear until the post-exponential phase of growth, 30 hours after inoculation. In older cultures the amount of cytochrome b relative to total mitochondrial protein increased as the culture aged. Although there were distortions around 600 m μ in the difference spectra of mitochondria from younger cultures, the absorption band of cytochrome a did not appear until 60 hours after inoculation.

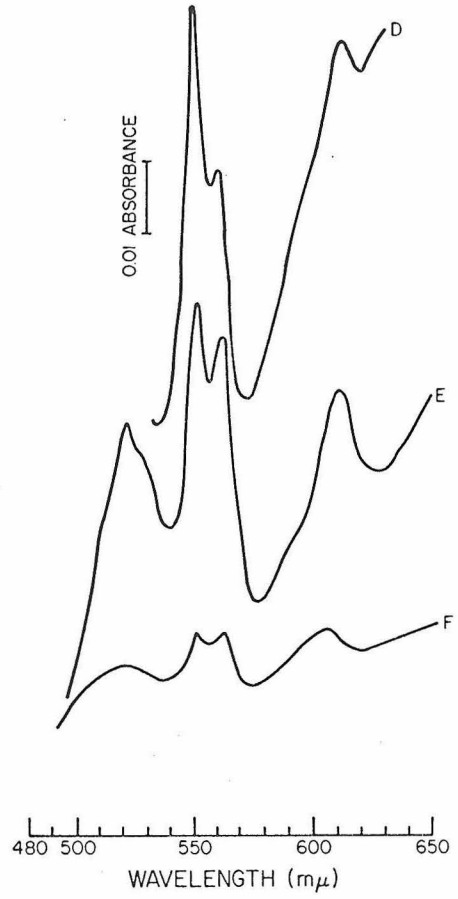
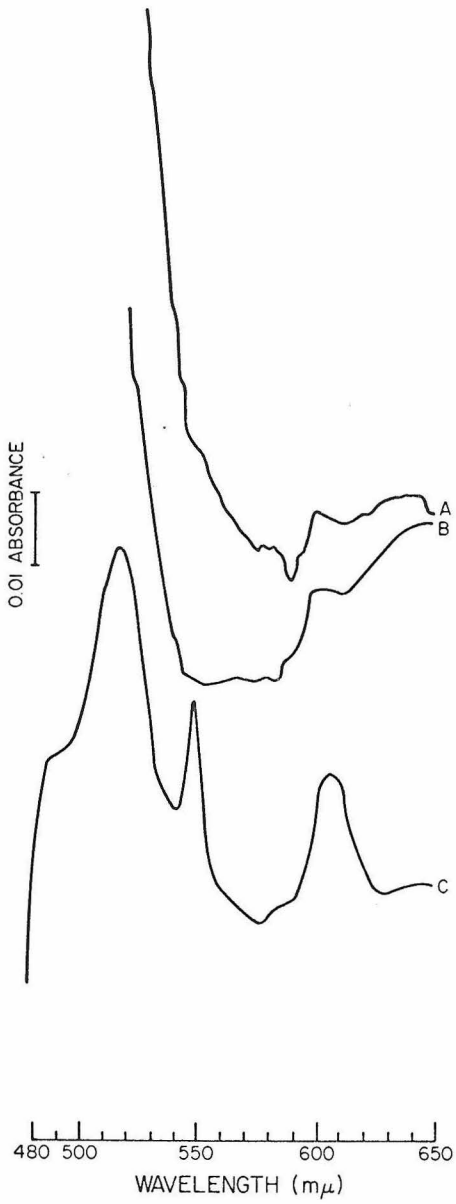
Cytochrome difference spectra of wildtype mitochondria are shown in Figure 4. As with poky, there were problems of association of cell wall fragments with mitochondria from wildtype conidia and germinating conidia. The difference spectra of these looked very similar to the corresponding preparations from poky. No cytochromes b or c were present in mitochondria from conidia or from germinating conidia, but there did appear to be small quantities of cytochrome a in both preparations. Again as with poky, the amounts of mitochondrial cytochrome c in wildtype mitochondria relative to total mitochondrial protein increased during the early part of the growth cycle and then decreased as the culture aged. Also the amount of cytochrome b relative to total mitochondrial protein increased as the culture aged, as was the case with poky.

Buoyant densities of mitochondria from both poky and wildtype were measured for cultures of various ages. Values obtained gravimetrically and from refractive index measurements of gradient fractions varied from 1.18 g/cm³ to 1.20 g/cm³ for both strains, but with no particular relationship to the developmental stage of growth.

FIGURE 4. Difference Spectra of Mitochondria From Wildtype Cultures of Various Ages.

Cultures were started with inoculations of 10^5 conidia/ml. Absorbances were recorded on the 0 - 0.1 scale. Equal amounts of mitochondrial protein were in the sample cell (reduced) and in the reference cell (oxidized).

- A. From conidia, 2.5 mg protein/ml
- B. From germinating conidia (4 hours), 2.5 mg protein/ml
- C. From a 10 hour culture, 10 mg protein/ml
- D. From a 20 hour culture, 6 mg protein/ml
- E. From a 23 hour culture, 10 mg protein/ml
- F. From a 72 hour culture, 1.5 mg protein/ml



Electron microscopy

Electron microscopy was used to look for differences in the physical appearance of mitochondria from cultures in different phases of growth. Cross sections of a conidium, germinating conidium, young hypha, and an old hypha from poky cultures are shown in Figure 5. Corresponding cross sections from wildtype cultures are shown in Figure 6.

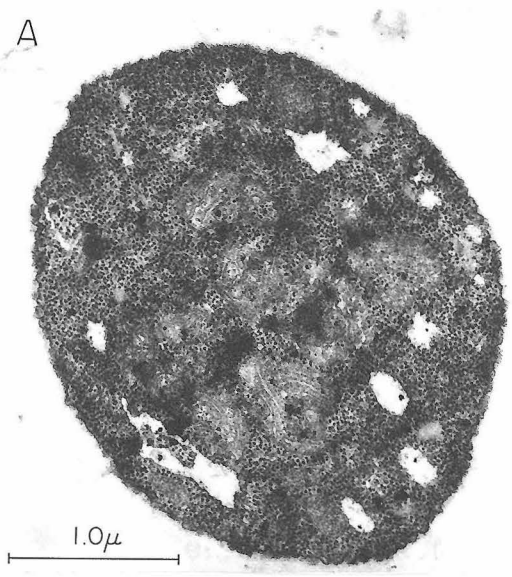
The poky conidium has condensed aggregations of membranes which are apparently mitochondria. Although it was very difficult to separate conidial mitochondria from cell wall fragments, there is no noticeable attachment of the mitochondria to plasma membrane or cell wall. The germinating conidium from poky has distinct mitochondria with very few cristae. The mitochondrial membranes are not nearly in such a condensed state as in the conidium. Mitochondria in the young hypha seem to be about the same as those in the germinating conidium. In the old hypha, the mitochondria do not appear to be abnormal with respect to size, shape, or cristae.

Mitochondria in the wildtype conidium are much more distinct and have a more usual shape than those in the poky conidium, and the membrane material is much less condensed. Again there is no apparent association of mitochondria with the plasma membrane or the cell wall. In the germinating conidium from wildtype, the mitochondria are not much different from those in the dormant conidium though there are more cristae. Compared with the germinating conidium from poky, there is much more condensation of membrane material and there are many more cristae. Mitochondria in the young wildtype hypha are very similar to those in

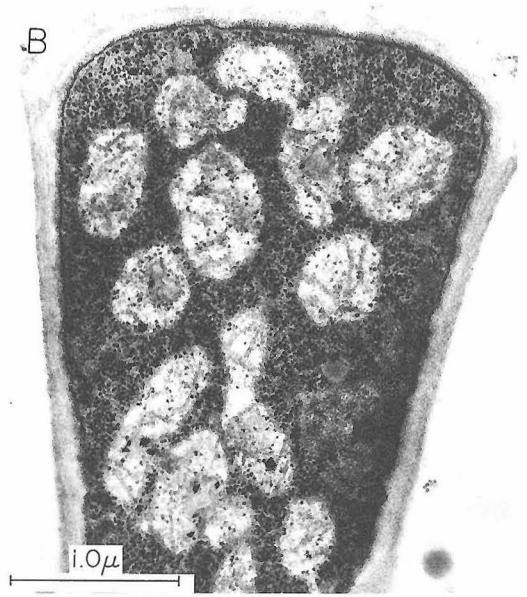
FIGURE 5. Electron Micrographs of Material From Poky Cultures.

All material except the old hypha (D) was fixed with glutaraldehyde and osmium tetroxide, and stained with uranyl acetate and lead citrate. The old hypha (D) was fixed and stained with 2% KMnO_4 .

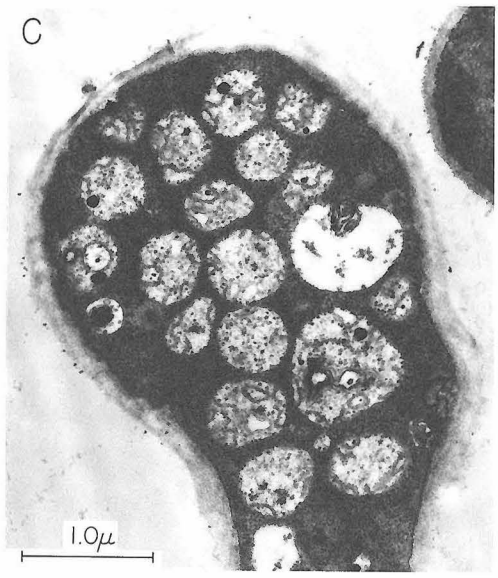
- A. Conidium
- B. Germinating conidium
- C. Young hypha (from a 10 hour culture)
- D. Old hypha (from a 96 hour culture)



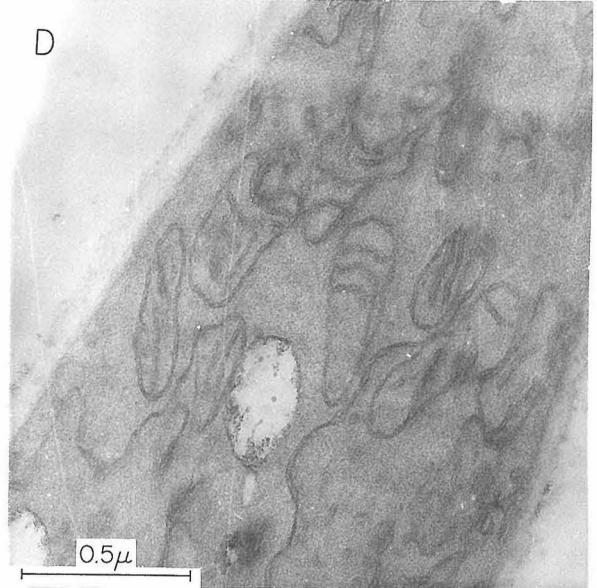
POKY-CONIDIUM



POKY-GERMINATING CONIDIUM



POKY-YOUNG HYPHA

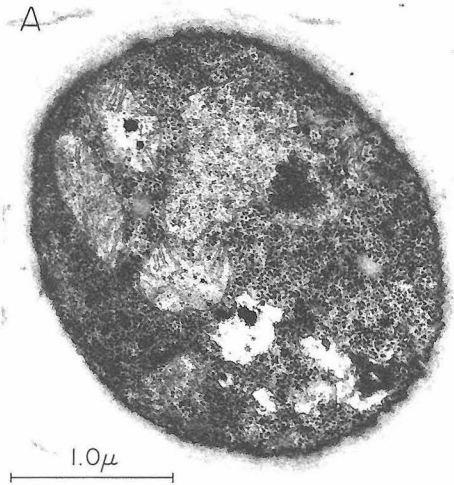


POKY-OLD HYPHA

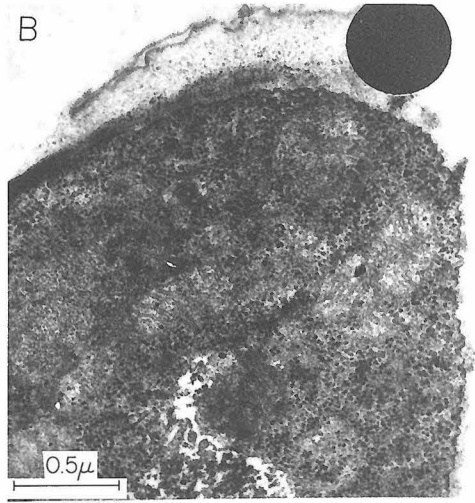
FIGURE 6. Electron Micrographs of Material From Wildtype Cultures.

All material except the old hypha (D) was fixed with glutaraldehyde and osmium tetroxide, and stained with uranyl acetate and lead citrate. The old hypha (D) was fixed and stained with 2% KMnO_4 .

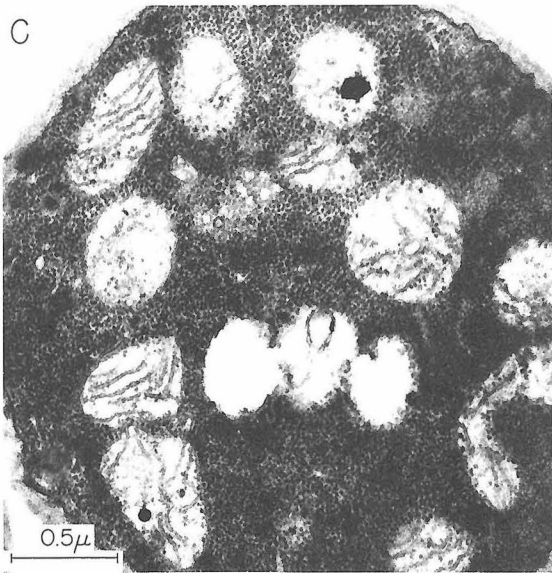
- A. Conidium
- B. Germinating conidium
- C. Young hypha (from a 6 hour culture)
- D. Old hypha (from a 168 hour culture)



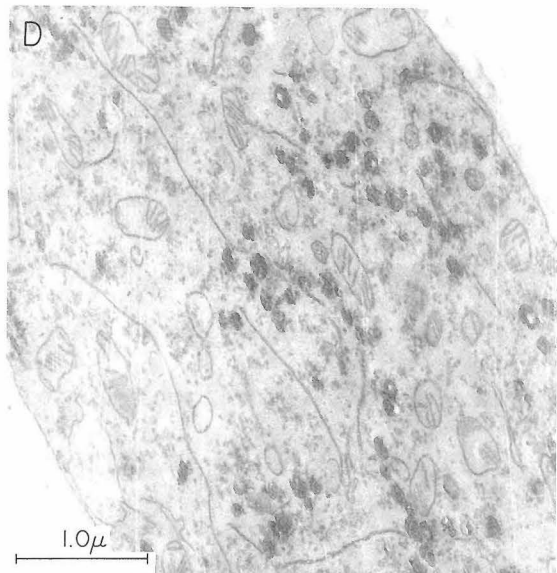
WILDTYPE-CONIDIUM



WILDTYPE-GERMINATING CONIDIUM



WILDTYPE-YOUNG HYPHA



WILDTYPE-OLD HYPHA

the germinating conidium from wildtype, and compared to the mitochondria in the young poky hypha, there is more condensation and there are more cristae. In the old wildtype hypha, the mitochondria are similar in size, shape, and cristae content to both the younger wildtype material and the corresponding old poky hypha.

A peculiar effect on mitochondrial membrane structure caused by the presence of EDTA in the homogenizing medium was studied in collaboration with S.K. Malhotra (79). Wildtype mitochondria were prepared by the method of Hall and Greenawalt (76) using 10^{-3} M EDTA in the homogenizing medium. Under these conditions, the mitochondrial membranes, when observed by negative staining, were completely devoid of the elementary particles described by Fernandez-Moran (80), and found in Neurospora by Stoeckenius (81). Figure 7A is representative of these preparations. There was some indication that the material comprising the elementary particles was still associated with the membrane, as a globular particle either in or on the membrane (Figure 7B). When EDTA was omitted from the homogenizing medium, elementary particles associated with the mitochondrial membrane could easily be demonstrated by negative staining as shown in Figure 7C. Mitochondria isolated in the presence of 10^{-3} M EDTA at 4°C for 12 hours still had elementary particles associated with the mitochondrial membrane (Figure 7E).

Since the elementary particles have been reported to be associated with ATPase activity and oxidative phosphorylation (82), a comparison of the biochemical properties of the two types of mitochondrial preparations (isolated in the presence and in the absence of EDTA) was made. The results are outlined in Table 1.

FIGURE 7. Negatively Stained Isolated Mitochondria.

A. From a wildtype mitochondrial fraction isolated in sucrose with EDTA, showing typical appearance of a fairly well-preserved mitochondrion. Stalked elementary particles are absent.

B. Part of a well-preserved wildtype mitochondrion isolated in sucrose with EDTA, showing possible globular particles in or on the membranes of the cristae.

C. From a wildtype mitochondrial fraction isolated in sucrose without EDTA. Elementary particles are definitely present.

D. From a poky mitochondrial fraction isolated in sucrose with EDTA and stained by spreading onto the surface of an aqueous solution of potassium phosphotungstate. There are elementary particles in close proximity to the membrane, but their attachment to the membrane is not clear.

E. From a wildtype mitochondrial fraction isolated in sucrose without EDTA and then incubated in the presence of 10^{-3} M EDTA for 12 hours at 4°C . Stalked elementary particles are present.

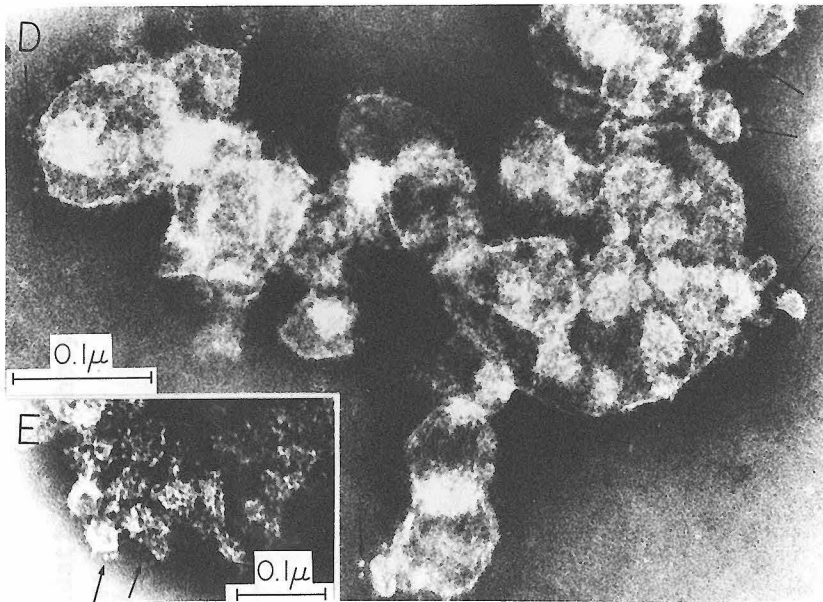
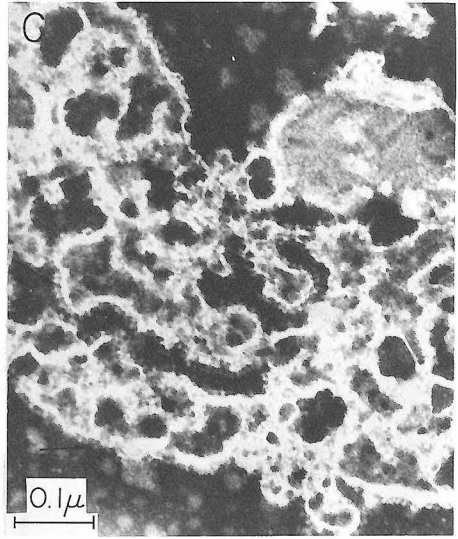
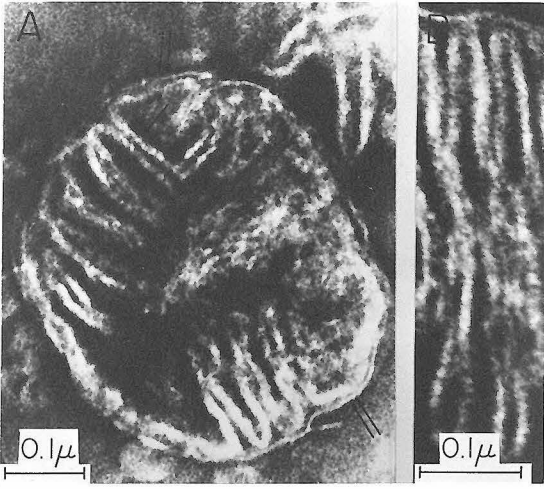


TABLE 1

Oxidative Phosphorylation by Isolated Wildtype Mitochondria Using Succinate as a Substrate

Mitochondrial Preparation	Succinate Concentration (mM)	Exogenous cytochrome $\frac{c}{c}$ Concentration ($\mu\text{g/ml}$)	Rate of O_2 Consumption (μ moles/min)	P : O (μ atom P/ μ atom O)
Without EDTA	8.3	0	0.086	0.57
Without EDTA	16.1	0	0.091	43
Without EDTA	15.6	0.3	0.127	

With 10^{-3}M EDTA	8.3	0	0.101	0.36
With 10^{-3}M EDTA	16.1	0	0.120	
With 10^{-3}M EDTA	15.6	0.3	0.146	

The cytochrome difference spectra of the two types of mitochondrial preparations were identical. Oxidative phosphorylation was not significantly different, the phosphorylation being about the same in both preparations, and the oxygen consumption being a little faster in mitochondria isolated in the presence of EDTA. Thus the mitochondria isolated in the absence of EDTA gave a slightly higher P:O ratio. Doubling the substrate concentration and adding horseheart cytochrome c to the reaction system each stimulated the rate of oxygen consumption in both mitochondrial preparations, but to about the same extent so that the original difference in the oxidation rates of the two mitochondrial preparations still existed.

Elementary particles could not be detected in poky mitochondria isolated in the presence of EDTA if the usual method of negative staining was used. However, if the poky mitochondria were stained by disruption on the surface of an aqueous solution of potassium phosphotungstate (0.5 - 2%, pH 6.8 - 7), elementary particles associated with the mitochondrial membrane could be demonstrated (Figure 7D).

B. Oxygen consumption in poky mitochondria prepared by standard procedures

Initially all mitochondrial preparations were obtained using the standard methods described by Hall and Greenawalt (76). The homogenizing medium in this procedure is not buffered and contains 0.25 M sucrose to maintain sufficient osmotic pressure, 10^{-3} M EDTA to prevent aggregation of mitochondria, and 0.15% bovine serum albumin. BSA is necessary for

preserving the capacity for oxidative phosphorylation. This is probably due to the BSA complexing with free fatty acids which normally would uncouple oxidative phosphorylation (83). Wildtype mitochondria isolated using this medium appeared to be stable and were capable of carrying out oxidative phosphorylation as shown in Table 1.

Since wildtype mitochondria had an active succinate oxidase system and there was a reported absence of a succinate oxidase system in cell-free extracts of poky (16), succinate oxidase activity was to be used as a measure of a functional classical cytochrome electron transport system. In order to detect and measure the activity of an alternate electron transport system it was necessary to find a respiratory substrate whose oxidation was linked to the consumption of molecular oxygen. When used as a substrate, water soluble extracts of both poky and wild-type mycelia caused consumption of oxygen by young poky mitochondria, indicating that there did exist a respiratory substrate for an alternate electron transport chain. The substrate activity was found to be soluble in 50% ethanol and insoluble in 100% ethanol. Failure to detect any substrate activity in a 50% ethanol extract of mitochondria corresponding to 1.25 g of mitochondrial protein ruled out localization of the active component in that organelle.

Compounds and extracts which did not serve as respiratory substrates

Before an isolation of the active compound was attempted, a survey of likely respiratory substrates was made. The citric acid

cycle was apparently non-functional in young poky mitochondria as pyruvate, citrate, cis-aconitate, isocitrate, α -ketoglutarate, succinate, and malate all failed to serve as a respiratory substrate. Also lactate, glycerol, α -glycerol phosphate, and glyceraldehyde 3-phosphate were inactive as substrates, so glycolysis was not the source of the respiratory substrate. Common sugars were tested for activity as a substrate since Tissieres et al. reported that Neurospora contains polysaccharides which may be a reservoir of substrate for endogenous respiration (16). The following sugars had no activity as a respiratory substrate: glucose, fructose, ribose, L-lyxose, L-xylose, D-xylose, L-rhamnose, L-sorbose, D-arabinose, and raffinose. Also, mannitol glucuronic acid lactone, arabinic acid, and levulinic acid showed no activity as a substrate. Other miscellaneous compounds sometimes associated with a specific dehydrogenase or oxidase system were tested for activity as a substrate. Choline, glutamate, β - hydroxybutyrate, glycollate, sarcosine, and xanthine all failed to exhibit any activity as a substrate. Commercially available extracts which had very little or no activity as a substrate included peptone, beef liver extract, and malt extract.

Compounds and extracts which did contain respiratory substrate activity

Originally, the only good respiratory substrate found was NADH. Specific activity of oxygen consumption linked to NADH oxidation was found to be 6 μ moles consumed per minute per mg of mitochondrial protein.

Phosphorylation coupled to this oxidation could be demonstrated, giving a P:O ratio of 0.3. NADPH could also be oxidized by poky mitochondria but with much less efficiency, the specific activity being only 0.7 μ moles O_2 consumed/min/mg of mitochondrial protein.

Using ascorbic acid as a substrate, 2 μ moles O_2 /min were consumed in the presence of 5 mg of poky mitochondrial protein. However, in the absence of mitochondria the ascorbate was autooxidized at a rate of 67 μ moles of oxygen consumed per minute. Thus the presence of mitochondria severely inhibited the autooxidation of ascorbic acid. It is likely that the oxygen consumption in the presence of mitochondria was not enzymatic but residual autooxidation.

Dihydroorotic acid, reported as a respiratory substrate in Pseudomonas (84), was able to serve as a substrate for oxygen consumption in young poky mitochondria. Using L-dihydroorotic acid as a substrate, the mitochondria consumed oxygen at a rate of 4 μ moles/min/mg of protein. The carboxyl group of dihydroorotic acid was necessary for activity as a substrate since dihydrouracil and dihydrothymine were completely inactive.

When testing commercial extracts for activity as a respiratory substrate it was found that casein hydrolysate could be oxidized slowly by young poky mitochondria. The specific activity of oxygen consumption was 2.2 μ moles O_2 /min/mg of protein. The twenty protein amino acids were all tested for activity as a substrate and it was found that many could be very slowly oxidized. Glycine as a substrate resulted in an oxygen consumption rate of 1.4 μ moles O_2 /min/mg of protein; and alanine,

arginine, glutamine, serine, threonine and histidine all caused oxygen to be consumed at rates less than 1.0 μ mole/min/mg of protein. Using cysteine as a substrate, 17.6 μ moles of oxygen were consumed per minute in the presence of 4 mg of mitochondrial protein. But this was meaningless as cysteine was autooxidized at the same rate in the absence of mitochondria. An amino acid mixture, 0.005 M in all 20 protein amino acids except cysteine was prepared and used for a respiratory substrate. This gave an oxygen consumption rate of 0.4 μ moles/min/mg of mitochondrial protein. No phosphorylation coupled to this oxidation could be demonstrated.

Although poky and wildtype mycelia have very different growth rates when grown on sucrose as a carbon source, the growth rates when utilizing casein hydrolysate as a carbon source are virtually identical (Figure 8).

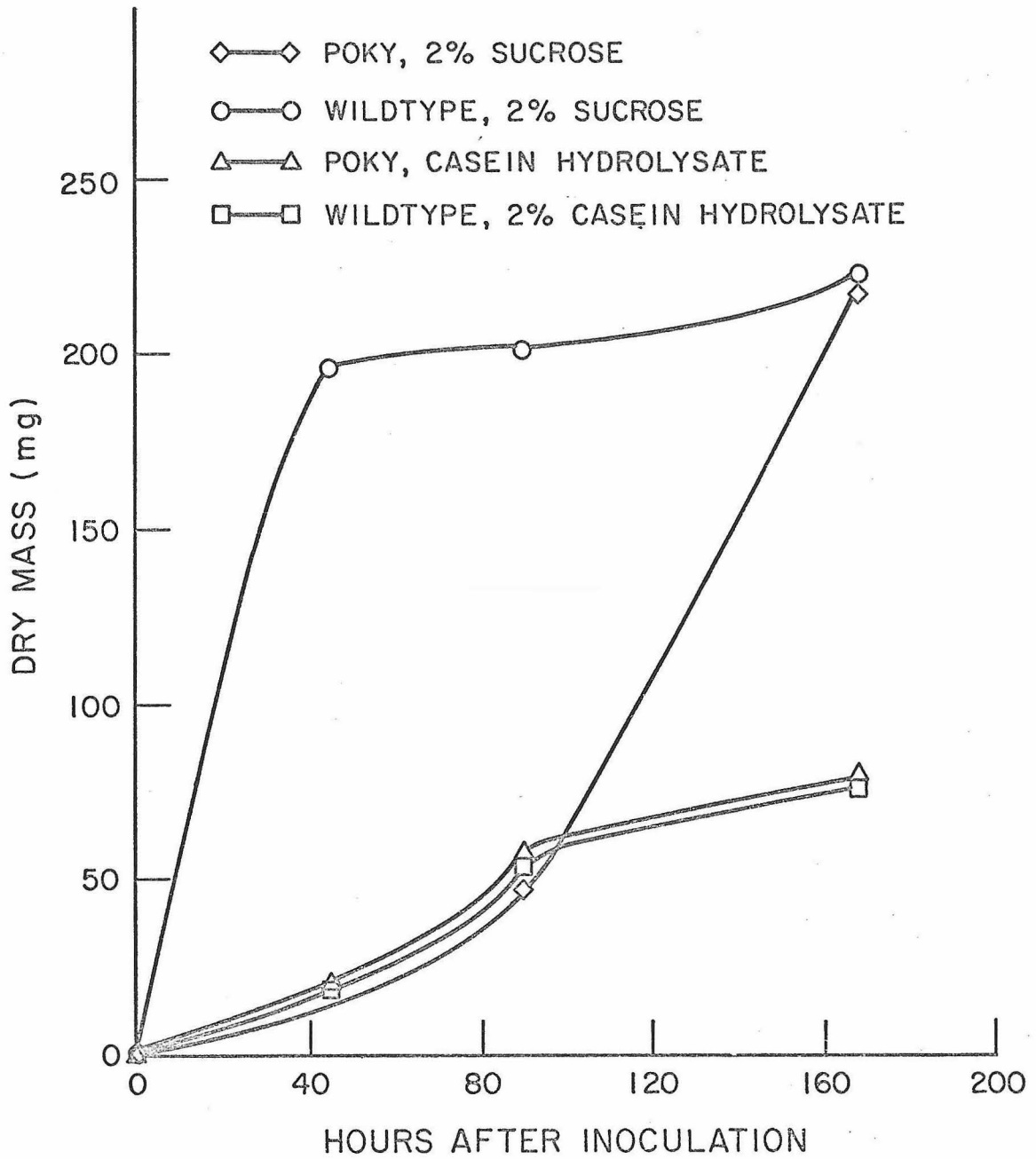
For the first 90 hours the growth rates of poky were about the same on both carbon sources, but there was a great difference in the growth rates of wildtype.

If poky were utilizing amino acids in respiration one might expect an accumulation of α -keto acids in the medium. The media from 90 hour cultures of poky and wildtype grown on 2% sucrose as described in Figure 8 were reacted with 2,4 dinitrophenylhydrazine in 1.0 N HCl. Both media gave 0.03 mg of insoluble 2,4 dinitrophenylhydrazones per mg of dry mycelium. Thus there was not a very large quantity of α -keto acids in the media and no difference between poky and wildtype.

A sample of young poky mitochondria was given to Helen Macleod to test for the presence of the inducible L-amino acid oxidase described by

FIGURE 8. Growth of Poky and Wildtype Mycelia Utilizing 2% Sucrose and 2% Casein Hydrolysate as Carbon Sources.

Cultures were grown in 25 ml of Vogel's minimal medium (71), starting with 4×10^6 conidia. Aeration was on a reciprocal shaker at 25°C.



Bender et al. (85), but she was not able to detect any activity. Therefore it is most likely that the very slow oxygen consumption in the presence of casein hydrolysate and in the presence of amino acids represents some sort of oxidative degradation other than L-amino acid oxidase and is not related to respiration at all.

Commercial yeast extract was found to contain activity as a substrate for oxygen consumption. Using yeast extract as a substrate, young poky mitochondria could consume oxygen at a rate of 2.2 μ moles O_2 /min/mg of protein. The rate of oxygen consumption using yeast extract as a substrate could not be stimulated by adding either NAD or NADP. Also addition of poky mitochondrial supernatant proteins, precipitated with ammonium sulfate, failed to stimulate the rate of oxidation of yeast extract.

C. Isolation and Characterization of the Active Component from Yeast Extract.

Because of its commercial availability yeast extract rather than Neurospora extract was used to isolate an active substrate for oxygen consumption. Young poky mitochondria with no succinate oxidase activity were used to assay for the active component.

General Properties

The active compound in yeast extract was soluble in water and in 50% ethanol. Most of the activity was precipitated by increasing the alcohol concentration from 50% to 75%. No activity was found in the 100% ethanol or 2:1 chloroform-methanol soluble fractions from yeast extract. The activity was not removed from an aqueous solution of yeast extract by treatment with activated charcoal. Treatment with 1.0 N NaOH at 0°C for 5 days did not destroy the activity, and treatment with 1.0 N HCl at 0°C for 5 days resulted in only a slight loss of activity. Also autoclaving a solution of yeast extract did not destroy the activity. On a Biogel P-2 gel filtration column, the activity came off at an elution volume to void volume ratio of 2.25, indicating a molecular weight in the range of 100-200. It was found that the activity in yeast extract could be removed by treatment with the OH⁻ form of Dowex 1-X8. Subsequent washing of the resin with water did not result in elution of activity, but washing with 1.0 M NaCl eluted all the activity. Thus the compound appeared to be an anion.

Isolation of the compound

Twenty grams of commercial yeast extract (Difco Laboratories) were dissolved in 100 ml of water. Then 100 ml of ethanol were added making the solution 50% ethanol by volume. The precipitate was removed by centrifugation at 15,000 g for 10 minutes. The supernatant was treated with 20 g of Dowex 1-X8 in the OH^- form. The resin was then washed with water and poured into a 13.5 cm column, 2.0 cm in diameter. The column was washed with water until the eluant was colorless. By elution with 1.0 M NaCl and collection of 5 ml fractions, the activity was found to come off the column in the eluant volume between 15 ml and 50 ml. The fractions comprising this volume were pooled, lyophilized, and dissolved in 1.0 ml of water. The extract was then run through a Biogel P-2 column (30 cm by 1.5 cm). By elution with water and collection of 2.5 ml fractions, the activity was found in the eluant volume between 10 ml and 32.5 ml. The fractions comprising this volume were pooled, lyophilized and dissolved in 2 ml of water. This solution was then treated with 0.5 g of Dowex 1-X8 (OH^-), and the resin was layered onto a column (20 cm by 1.5 cm) of Dowex 1-X8 (Cl^-). The column was then washed with water until the eluant was colorless. A 200 ml linear gradient from 0 to 1.0 M NaCl was used for elution.

Fractions were collected in 4 ml volumes. The activity was eluted in the volume between 64 ml and 104 ml. Fractions comprising this volume were pooled and reduced in volume to 2 ml on a flash evaporator. From conductivity measurements on adjacent fractions it was determined that the activity was eluted in the range of 0.25 M NaCl to 0.38 M NaCl.

The solution was desalted on a Biogel P-2 column (30 cm by 1.5 cm). Water was used to elute the column and 2 ml fractions were collected. Activity from this column was in the elution volume between 16 ml and 30 ml. The fractions comprising this volume were pooled, lyophilized and dissolved in 1.0 ml of water. Further purification of the compound was by paper chromatography. The solution was chromatographed 20 cm as a strip using the propanol-ammonia solvent. Strips perpendicular to the direction of solvent flow were cut from the chromatogram, eluted with water, and the eluants tested for activity. Activity was found in the region between 1.0 cm and 9.0 cm from the origin. The eluants from this region were pooled, lyophilized and dissolved in 0.2 ml of water. This solution was then chromatographed as a strip for 20 cm using the propanol-ammonia solvent. Strips were again cut, eluted with water, and the eluants tested for activity. This time the activity was confined to the region between 4 cm and 8 cm from the origin. When the edges of the chromatogram were treated with the BiI_3 reagent, this region remained white against a brown background, so the presence of the compound on chromatograms was henceforth detected in this manner rather than by assaying every strip for activity. Final purification of the compound was by additional chromatography on paper, first using the propanol-acetic acid solvent and then the propanol-ammonia solvent. Lyophilization of the eluant from the last chromatogram gave 10 mg of material. Therefore, the amount of the ammonium salt of the anion obtained from yeast extract was 0.5 mg per gram. The ammonium salt was recrystallized from an aqueous solution by adding ethanol until there was

a slight turbidity, and then cooling at 0°C. A small amount of this material was chromatographed as a spot for 20 cm using the propanol-ammonia solvent. The chromatogram was then dried and developed with the propanol-acetic acid solvent for 20 cm in a perpendicular direction. The chromatogram was then dried and reacted with the BiI₃ reagent. Only one spot was detected, indicating that the active material was pure and consisted of only one compound.

To convert the compound into the free acid, it was run through a column (4.5 cm by 1.0 cm) of Dowex 50W-X4 in the H⁺ form, eluting with water. The eluant was evaporated to dryness with an air stream and the residue dissolved in 2 drops of ethanol. Chloroform was added until there was a slight turbidity, then after cooling at 0°C the free acid crystallized out of solution.

Physical and Chemical Properties of the Compound

Chromatographic properties

In the basic propanol-ammonia solvent system the ammonium salt of the anion had an R_f value of 0.30, and in the acidic propanol-acetic acid system the R_f value was 0.49. Thus the compound appeared to be a stronger acid than acetic acid. A comparison of these R_f values with those of known acidic compounds in the same solvent systems is given in Table 2. It can be seen in this table that monocarboxylic acids have higher R_f values in general and that tricarboxylic acids have lower R_f values than the compound obtained from yeast extract. The R_f values of dicarboxylic acids are very similar to those of the compound obtained from yeast extract.

TABLE 2

R_f values for the compound obtained from yeast extract and for known standard compounds in both the basic and the acidic solvent systems.

Compound	R_f in propanol- ammonia	R_f in propanol- acetic acid
Substrate from yeast extract	0.30	0.49
Butyrate	0.70	0.60
β - hydroxybutyrate	0.53	0.48
Glycollate	0.46	0.45
α - ketobutyrate	0.63	0.66
Levulinic acid	0.57	0.88
Succinate	0.32	0.45
Fumarate	0.35	0.38
Malate	0.25	0.39
α - ketoglutarate	0.34	0.55
Glutamate	0.27	0.33
α, β unsaturated adipate	0.39	0.50
Tartrate	0.22	0.28
Citrate	0.23	0.35
Isocitrate	0.17	0.33

The ammonium salt was dissolved in a citrate buffer at pH 3 to assure that it would be mostly in the form of a free acid rather than an anion, and then chromatographed on a Beckman GC-5 gas chromatograph. A 10% diethylene glycol succinate on 60-80 mesh chromabsorb G column (6 ft by 2 mm) was used at a temperature of 125°C. Under these conditions the compound came out in a single peak with a retention time of 1.20 minutes relative to water. Citric acid was retained for 10.90 minutes and levulinic acid was retained for 5.70 minutes under the same conditions. Hence the free acid was quite volatile at 125°C and the compound was very pure.

Electrophoresis

The ammonium salt was subjected to electrophoresis on Whatman No. 3 filterpaper using a solvent system of 1.0 M acetic acid adjusted to pH 4.0 with pyridine. The current was set at 15 m amp which gave a potential of 200 volts. After 3 hours, the compound had migrated a distance of 5.3 cm from the origin. In the same system, oxalate (pK 2.46) migrated 11.5 cm and glycollate (pK 3.83) migrated 7.5 cm. Therefore, the pK of the compound is between 3.8 and 4.0.

Functional groups

To test for functional groups the ammonium salt was chromatographed in the propanol-ammonia solvent system along with a compound known to give a positive result with the particular reagent. Using this method, it was found that the compound was not reactive to ninhydrin, periodate,

2-aminobiphenyl, or ammonium molybdate. This excluded amino acids, α glycols, reducing sugars, and phosphates from consideration. Also, there was no detectable absorption or fluorescence under ultraviolet light, so an aromatic system or an extensive system of conjugated double bonds was unlikely.

The only reagents found to give a positive reaction with the ammonium salt were bromocresol green, which gave a blue spot on a yellow background, and bismuth iodide, which gave a white spot on a brown background. This confirmed that there was a strong anion present but did not give any information on functional groups present.

Neutralization equivalent

To find the neutralization equivalent, 1.2 mg of the free acid were titrated with 0.069 N NaOH. This gave an equivalent weight of 104, or if two protons came off before pH 7, the molecular weight would be about 208.

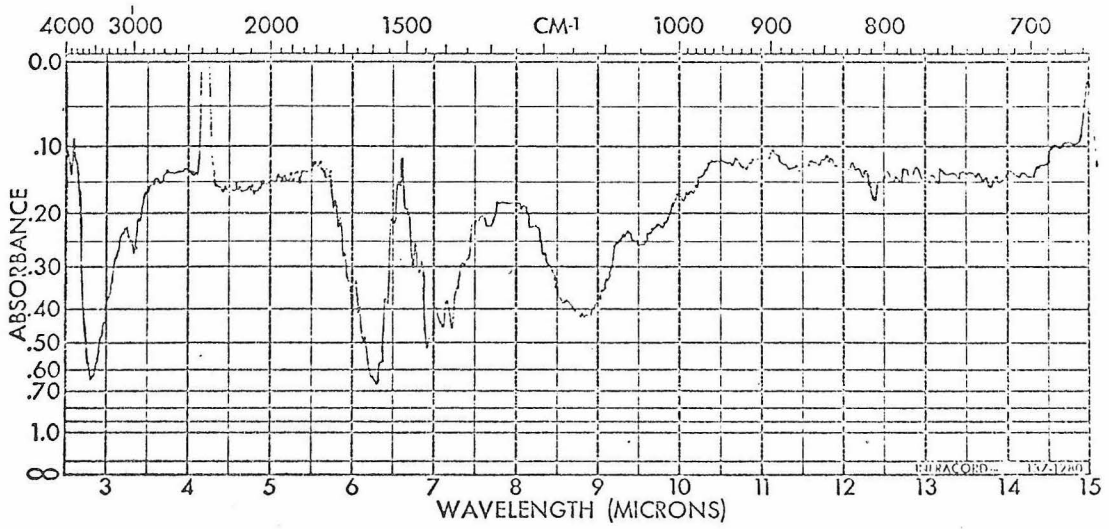
Spectral properties

About half a milligram of the salt was mixed with 300 mg of spectral quality KBr, dried in a vacuum for 15 minutes, and then compressed into a pellet under a total load pressure of 18,000 lbs. for one minute. A KBr pellet with no compound was also made. An infrared absorption spectrum was then recorded on a Perkin-Elmer Infracord Spectrophotometer using the blank KBr pellet in the reference beam. This spectrum is shown in Figure 9A.

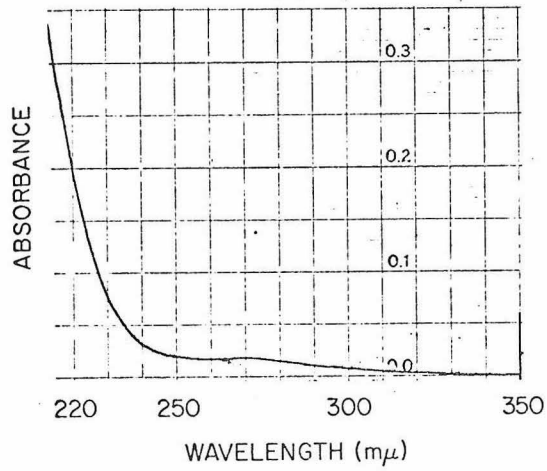
FIGURE 9.

A. Infrared Absorption Spectrum

B. Ultraviolet Absorption Spectrum



A. INFRARED ABSORPTION SPECTRUM



B. ULTRAVIOLET ABSORPTION SPECTRUM

The most prominent features of this spectrum are the peaks at 2.8 μ , 3.35 μ , 6.3 μ , the group around 7.15 μ , and the absence of any strong absorption bands at wavelengths above 11.0 μ . The peak at 2.8 μ corresponds to the stretching of the N-H or the O-H bond (86). The peak at 3.35 μ corresponds to the stretching of the C-H bond (86). Carboxylate anions show a strong band in the region between 6 and 6.4 μ and a weaker band around 7.15 μ , both associated with the stretching of the C-O bond (86). The absence of strong absorption bands at wavelengths above 11 μ indicates a non-aromatic structure (86).

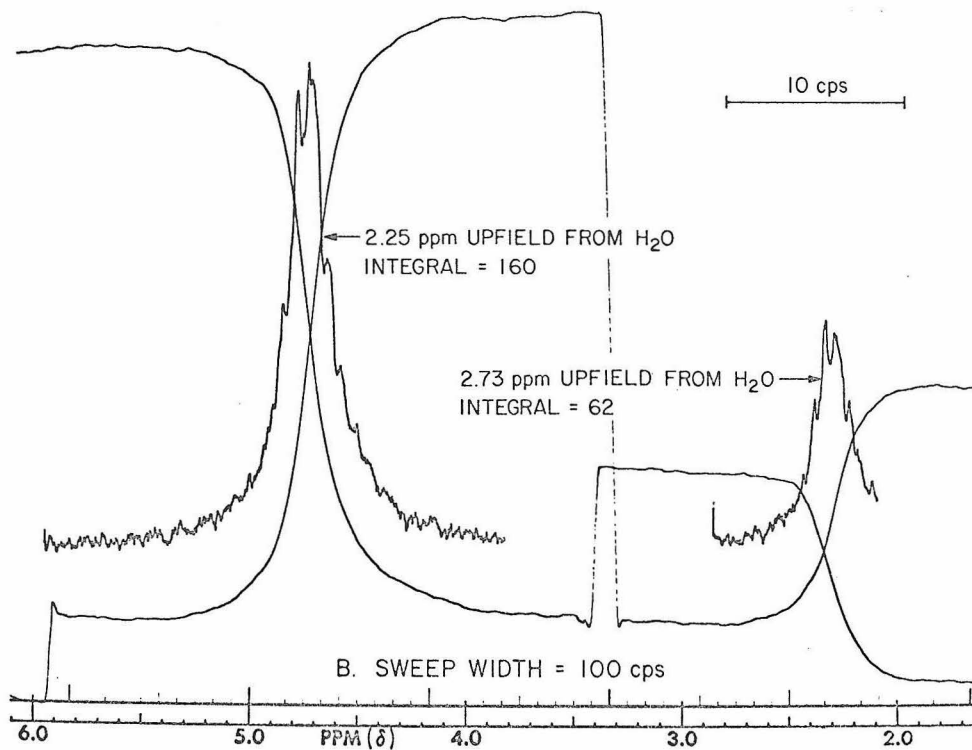
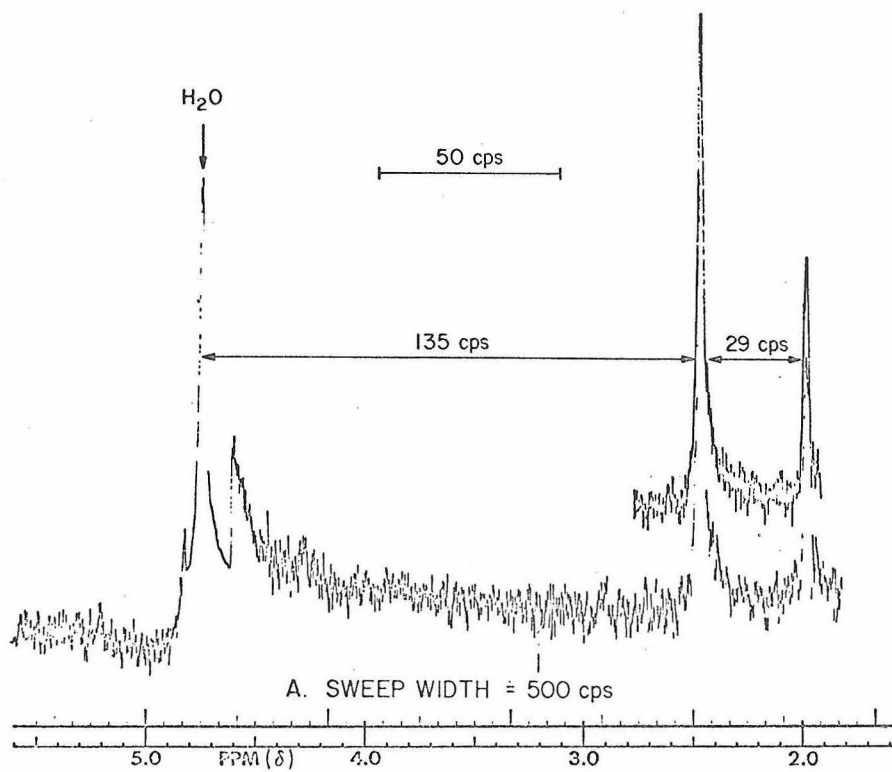
An ultraviolet absorption spectrum of an aqueous solution of the ammonium salt (0.1%) is given in Figure 9B. End absorption starts at about 240 $m\mu$ and at higher wavelengths there is only weak absorption in the range of 260 $m\mu$ - 280 $m\mu$. This also indicates that the compound does not have an aromatic or highly conjugated double bond system.

The nuclear magnetic resonance spectrum of the ammonium salt, shown in Figure 10, was obtained on a 60 megacycle Varian A-60A Analytical NMR Spectrometer. No peaks were observed downfield from the H₂O signal and two singlet peaks were observed upfield from the H₂O signal. One was at 135 cps (cycles per second) upfield (2.25 ppm) and the other at 164 cps (2.73 ppm) upfield with amplitudes in the ratio of 8 to 3. This indicates that there are two types of protons in the molecule which are not exchangeable in D₂O. Since the occurrence of 8 identical protons is unlikely, there are probably three of one type and one of the other. The absence of any splitting of the peaks indicates that they are not coupled through bonding to adjacent atoms.

FIGURE 10. Nuclear magnetic resonance absorption spectrum of the ammonium salt of the anion obtained from yeast extract.

A sample with a concentration of about 0.1 g/ml was used. The solvent was deuterium oxide (99.7%), the temperature was 35°C, the filter band width was 0.4 cps, and the oscillator frequency was 60 megacycles.

- A. Absorption spectrum upfield from H_2O , sweep width = 500 cps
- B. Expanded scale (sweep width = 100 cps) around the regions
135 cps upfield from H_2O and 164 cps upfield from H_2O



A sample of the free acid in ethanol was run on a high resolution mass spectrometer at the Jet Propulsion Laboratory by Dr. Heinz Boettger. Perfluorokerosene was used as a standard, and the injection temperature was 200°C. The data were collected on magnetic tape and analyzed on a computer. The m/e ratio of each peak was determined to the nearest 0.0001 atomic mass unit. Then all combinations of $C \leq 100$, $H \leq 200$, $N \leq 6$, $O \leq 6$ which gave masses within 0.005 atomic mass units of the m/e value were listed as formula assignments. The results of this analysis are summarized in Table 3.

The parent peak, m/e 171, carried a very large portion of the ion current indicating stabilization by a ring structure. Since dihydroorotic acid was active as a substrate, and since the ion fragments with m/e above 85 all were assigned formulas with at least two nitrogens, it is probable that the ring structure is the same as in dihydroorotic acid.

The m/e 171 ion was assigned only 3 oxygen atoms whereas dihydroorotic acid contains 4 oxygen atoms. The carboxyl group on C-4 of dihydroorotic acid was essential for activity so presumably the original free acid had a carboxyl group on C-4. Considering this and the fact that the ion fragment carrying the largest proportion of the ion current (base peak) has the formula CO_2 , it is apparent that the compound was quantitatively decarboxylated in the mass spectrometer. This would not be unusual if the carboxyl group were attached directly to the ring, as is the case with dihydroorotic acid. Adding CO_2 to the parent m/e 171 peak gives a molecular formula of $C_7H_9O_5N_3$ and a molecular weight of 215 for the original compound.

TABLE 3

High resolution mass spectrum analysis of the crystallized acid obtained from yeast extract.

m/e	Formula Assignment	Peak Intensity
171.0619	C ₆ H ₉ O ₃ N ₃	20321
143.0458	C ₅ H ₇ O ₃ N ₂	5759
142.0616	C ₅ H ₈ O ₂ N ₃	1702
113.0341	C ₄ H ₅ O ₂ N ₂	1146
85.0420	C ₃ H ₅ O N ₂	1540
83.0123	C ₄ H ₃ O ₂	2484
69.0353	C ₂ H ₃ N ₃ or C ₄ H ₅ O	1197
58.0287	C ₂ H ₄ O N	22902
57.0209	C ₂ H ₃ O N	11713
56.0118	C ₂ H ₂ O N	12675
55.0540	C ₄ H ₇	1020
48.0462	C H ₆ O N	1896
47.0327	C H ₅ O N	3236
46.0205	H ₂ O N ₂	2775
44.9942	C H O ₂	1221
44.0593	C ₃ H ₈	2453
44.0492	C ₂ H ₆ N	3881
44.0381	C H ₄ N ₂	2495
43.9898	C O ₂	35796
42.0472	C ₃ H ₆	1960
42.0344	C ₂ H ₄ N	1680
41.0390	C ₃ H ₅	3827
40.0310	C ₃ H ₄	1561
39.9949	C ₂ O	7399
33.0225	H ₃ O N	8177
31.0421	C H ₅ N	1802
31.0037	H O N	3275
30.0334	C H ₄ N	2175
29.9987	O N	1970
29.0280	C H ₃ N	2530
28.0181	C H ₂ N	9286
27.0118	C H N	5104
26.0046	C N	1036

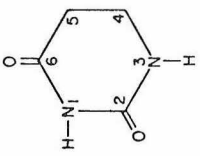
The quantitative decarboxylation suggests that the m/e 171 parent compound is an analog of dihydrouracil (Figure 11A) which has a molecular formula of $C_4H_6O_2N_2$. Side chains on the dihydrouracil ring would replace hydrogens and the bonds attaching them to the ring would be easily cleaved. Therefore, one would expect that cleavage of a side chain at the ring would result in a fragment $C_4H_5O_2N_2$ (m/e 113) if the ring were substituted at only one position. Such a fragment does exist, and furthermore a single side chain would have to have a formula of C_2H_4ON (m/e 58). This corresponds to the most intense peak in the spectrum after CO_2 . It is obvious then that the m/e 171 peak represents dihydrouracil singly substituted with a side chain C_2H_4ON . The peaks at m/e 27 (CHN), m/e 28 (CH_2N), and m/e 29 (CH_3N) indicate that an N-methyl group is present in the side chain.

Selection of a Structural Formula

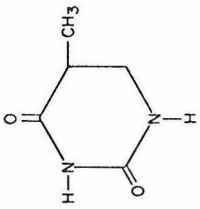
Considering the NMR spectrum, the proton on C-4 can account for one of the peaks, and the other, being three times as intense, must represent a methyl group. The chemical shift of the larger NMR peak is in the right range for an N-methyl group. This leaves two possibilities for the side chain, either an N-methylformamido side chain or a methyl formylamino side chain. The latter can be excluded since the NMR spectrum showed no aldehyde protons present. Three possibilities exist for the site of attachment of the side chain to the ring: at N-1 (Figure 11D), at C-5 (Figure 11E), or at N-3 (Figure 11F).

FIGURE 11

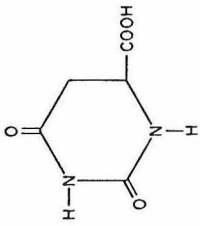
Derivatives of Dihydrouracil



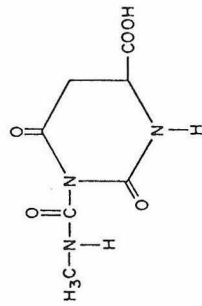
A. DIHYDROURACIL



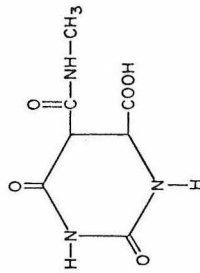
B. DIHYDROTHYMINE



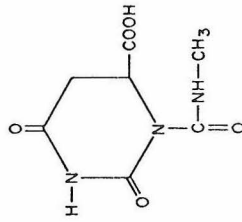
C. DIHYDROOROTIC ACID



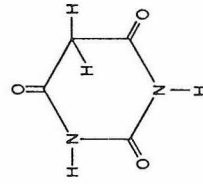
D. 1-N-METHYLFORMAMIDO-DIHYDROOROTIC ACID



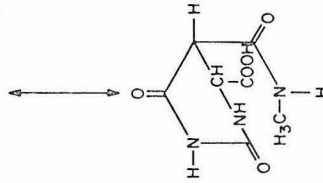
E. 5-N-METHYLFORMAMIDO-DIHYDROOROTIC ACID



F. 3-N-METHYLFORMAMIDO-DIHYDROOROTIC ACID



G. BARBITURIC ACID



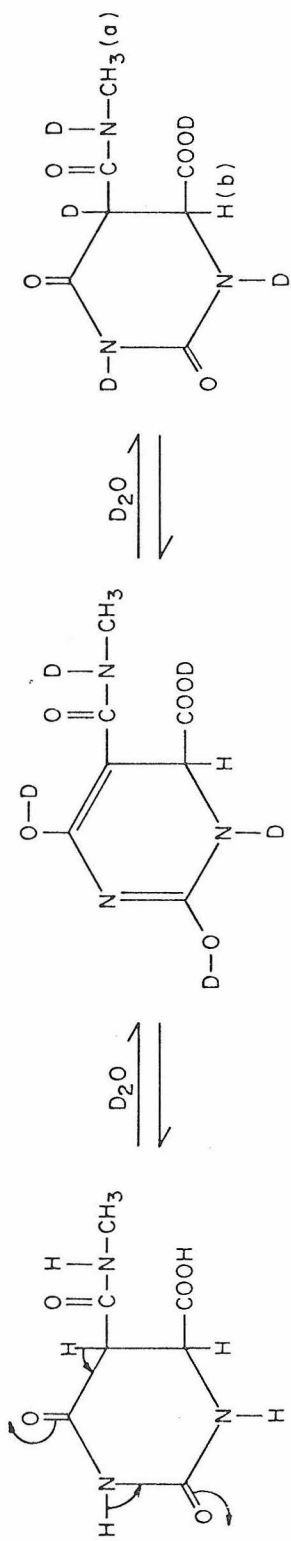
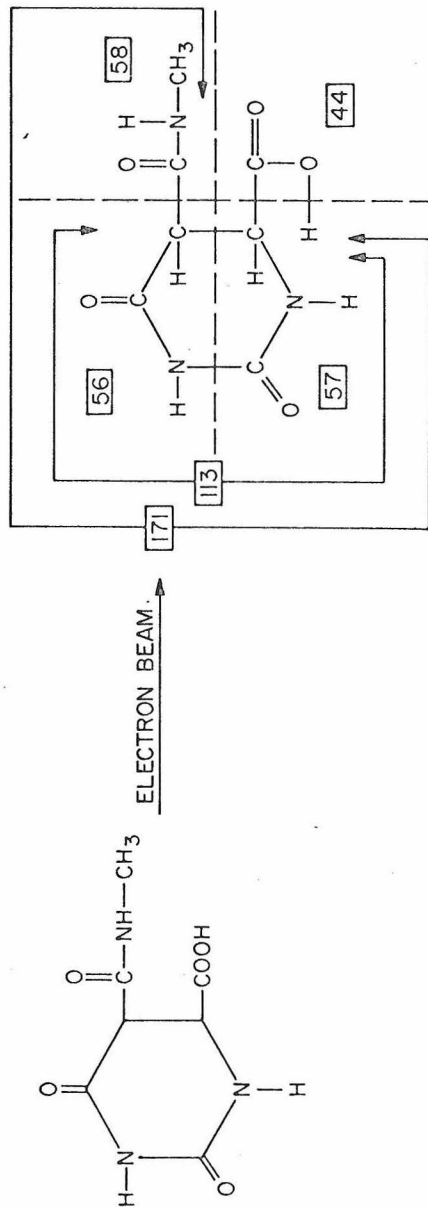
The NMR spectrum tends to exclude N-1 as the attachment site. Since the peak at 2.73 ppm upfield from H_2O is not split, the proton on C-4 is not coupled to any proton on C-5. Therefore all protons on C-5 are exchangeable in D_2O . This can be explained by keto-enol tautomerization, which would be much more energetically favorable if the conjugated system shown in Figure 12 could be formed. Of course this would be impossible if there were substitution at N-1.

Substitution at C-5 is more likely than at N-3 because of the strong acidity of the compound. A molecular weight of 215 with a neutralization equivalent of approximately 104 indicates that there are two acidic protons. One obviously is the C-4 carboxyl proton. The other has to be an enolic proton on C-5. The enolic protons of dihydrouracil (Figure 11A) are not very acidic and there is no reason to believe that the C-5 protons of 3N-methylformamidodihydroorotic acid (Figure 11F) would be either. However, the C-5 substituted dihydroorotic acid has structural similarities to barbituric acid (Figure 11G) which loses a proton at C-5 very readily ($pK = 4$). Therefore it would be reasonable to assume that the C-5 proton of 5 N-methylformamidodihydroorotic acid (Figure 11E) is acidic and readily exchanges in D_2O . The NMR and mass spectra could conceivably be generated from 5 N-methylformamidodihydroorotic acid by the mechanisms shown in Figure 12.

Considering all of the physical, chemical, and biological properties of the compound isolated from yeast extract, it is almost certainly 5 N-methylformamidodihydroorotic acid (Figure 11E). Since this compound has two asymmetric carbon atoms (C-4 and C-5) there exist four diastereomers. No data relating to the stereo-chemistry were collected, but

FIGURE 12. Mechanisms for the generation of the experimental NMR and mass spectra by 5N-methylformamidodihydroorotic acid.

- A. Basis for the NMR spectrum. All protons exchange in D_2O except those labelled (a) which give rise to the peak at 2.25 ppm upfield from the H_2O signal, and the one labelled (b) which gives rise to the peak at 2.73 ppm upfield from the H_2O signal.
- B. Basis for the mass spectrum. This pattern of primary fragmentation can account for all the extremely intense peaks (m/e 171, 58, 57, 56, 44) in the spectrum.

A. PROTON EXCHANGE IN D_2O 

B. ELECTRON BEAM FRAGMENTATION

since L-dihydroorotic acid was active as a substrate and since dihydro-uracil derivatives are synthesized biologically from L-aspartic acid, it is assumed that the configuration at C-4 is in the L form.

D. Degeneration of the Succinate Oxidation System in Poky Mitochondria

By chance it was found that young poky mitochondria could oxidize succinate if the crude mitochondrial pellet from the first 15,000 g centrifugation was used immediately. However, as the crude mitochondrial pellet incubated at 4°C, succinate oxidase activity was rapidly lost. The degeneration of this system with time is shown in Figure 13. In this figure it can be seen that the NADH oxidase system is also unstable, but that the dihydroorotate oxidase system is fairly stable.

If the inactivation of the succinate oxidase system in each mitochondrion were completely independent of other mitochondria in the environment, the kinetics of inactivation of succinate oxidase specific activity should be independent of the concentration of mitochondria in the incubating suspension. This was clearly not the case, as shown in Figure 14. Inactivation was not nearly as rapid in suspensions with a high concentration of mitochondria. As incubating suspensions became more dilute, the rates of inactivation of the succinate oxidase systems became more rapid. Diffusion of some essential component of the succinate oxidase system out of the mitochondrion could account for this phenomenon. In the highly concentrated suspensions the initial diffusion would rapidly decrease the concentration gradient between the inside and the outside of

FIGURE 13. Degradation of poky mitochondrial oxidase systems in unbuffered homogenizing medium. Reactions were run in a volume of 3.0 ml starting with 0.6 μ moles of dissolved oxygen and 5.0 μ moles of substrate. Mitochondria were from a 17 hour culture of poky.

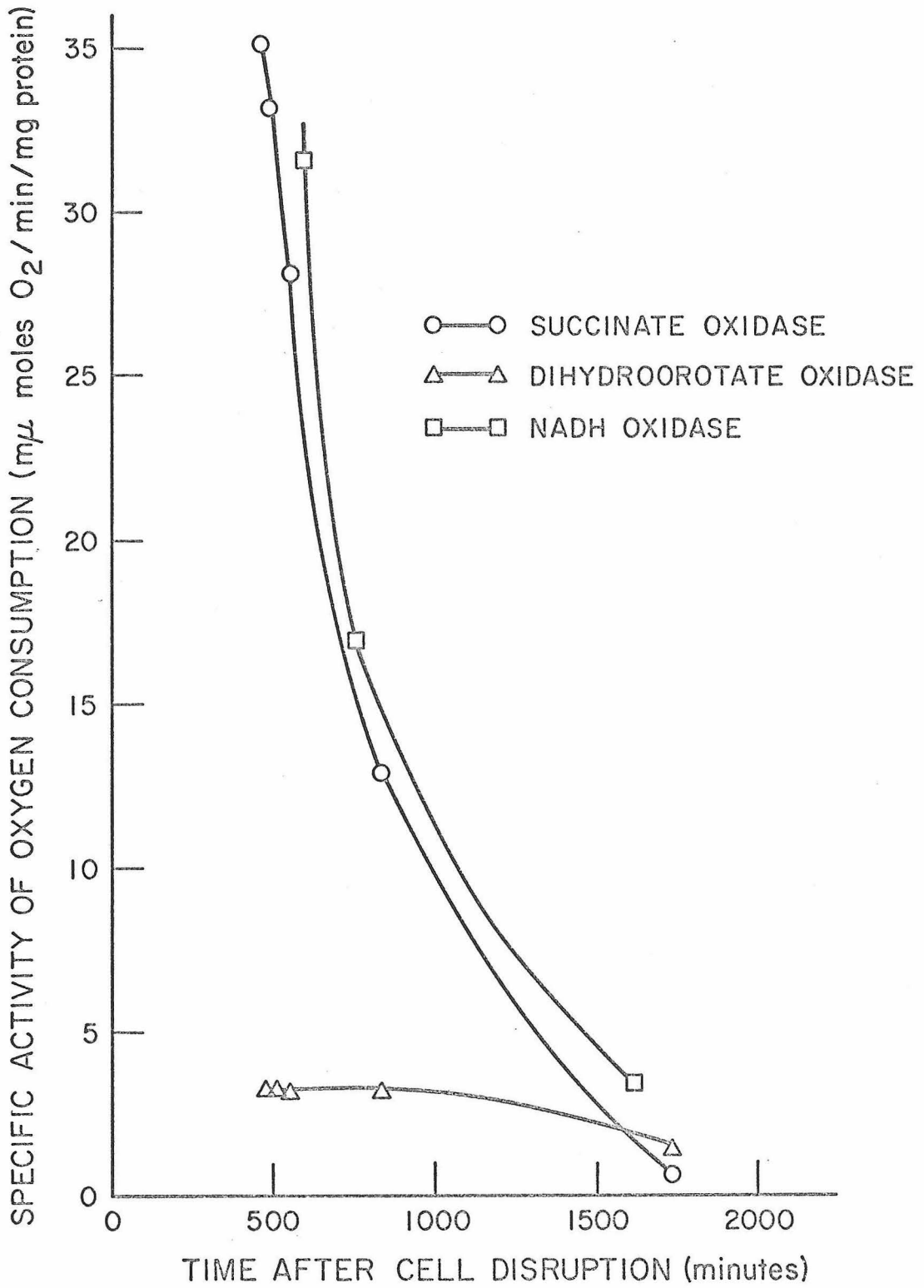
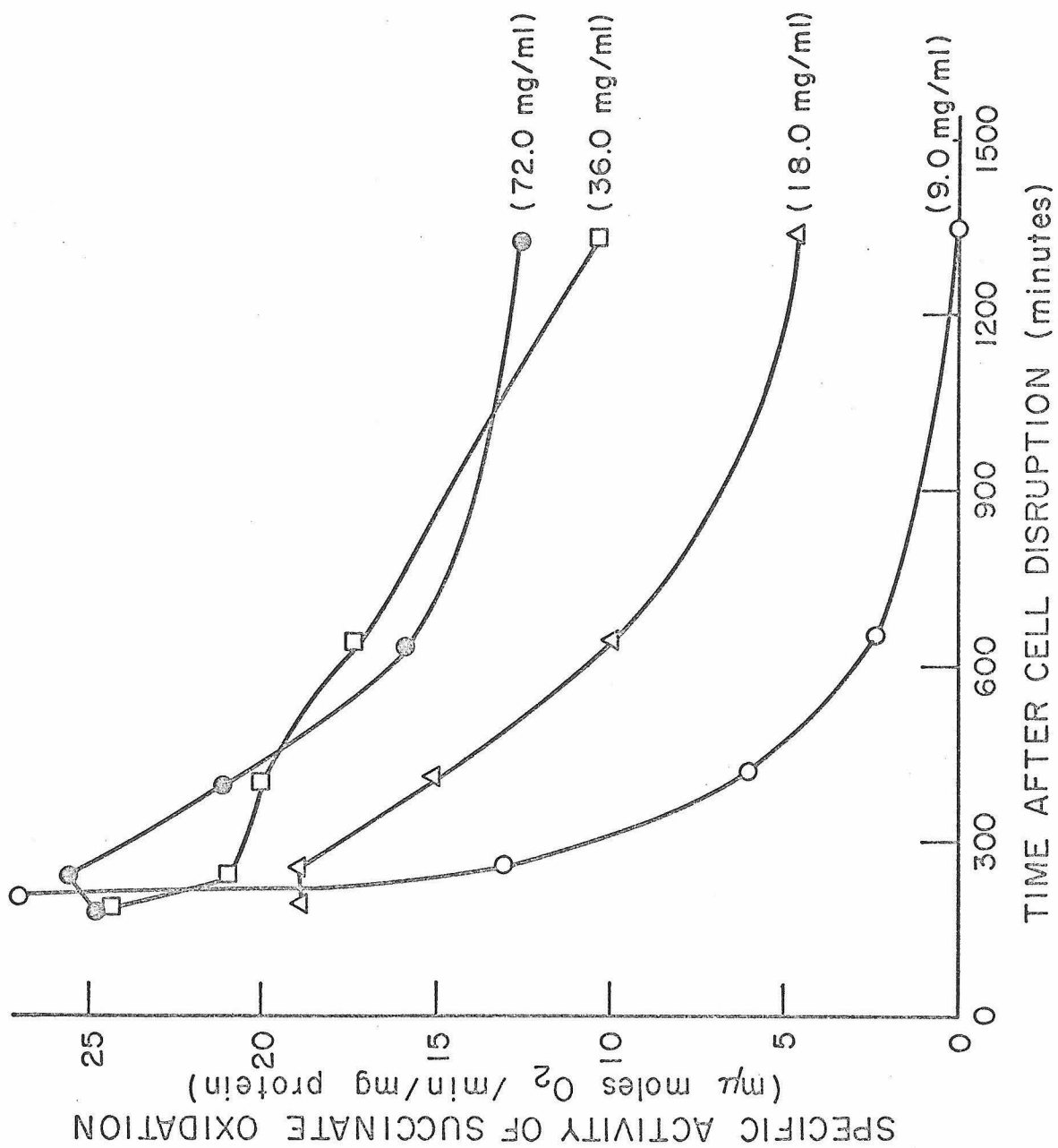


FIGURE 14. Degeneration of succinate oxidase activity for mitochondrial suspensions of various concentrations. Reactions were run in a volume of 3.0 ml starting with 0.6 μ moles of dissolved oxygen and 5.0 μ moles of succinate. Mitochondria were prepared from a 17 hour culture of poky.



the mitochondrion, while in very dilute solutions the concentration gradient would stay at about the same level. Since the rate of diffusion is a function of the concentration gradient, the diffusion in the concentrated suspensions would be retarded much sooner than in the very dilute suspensions.

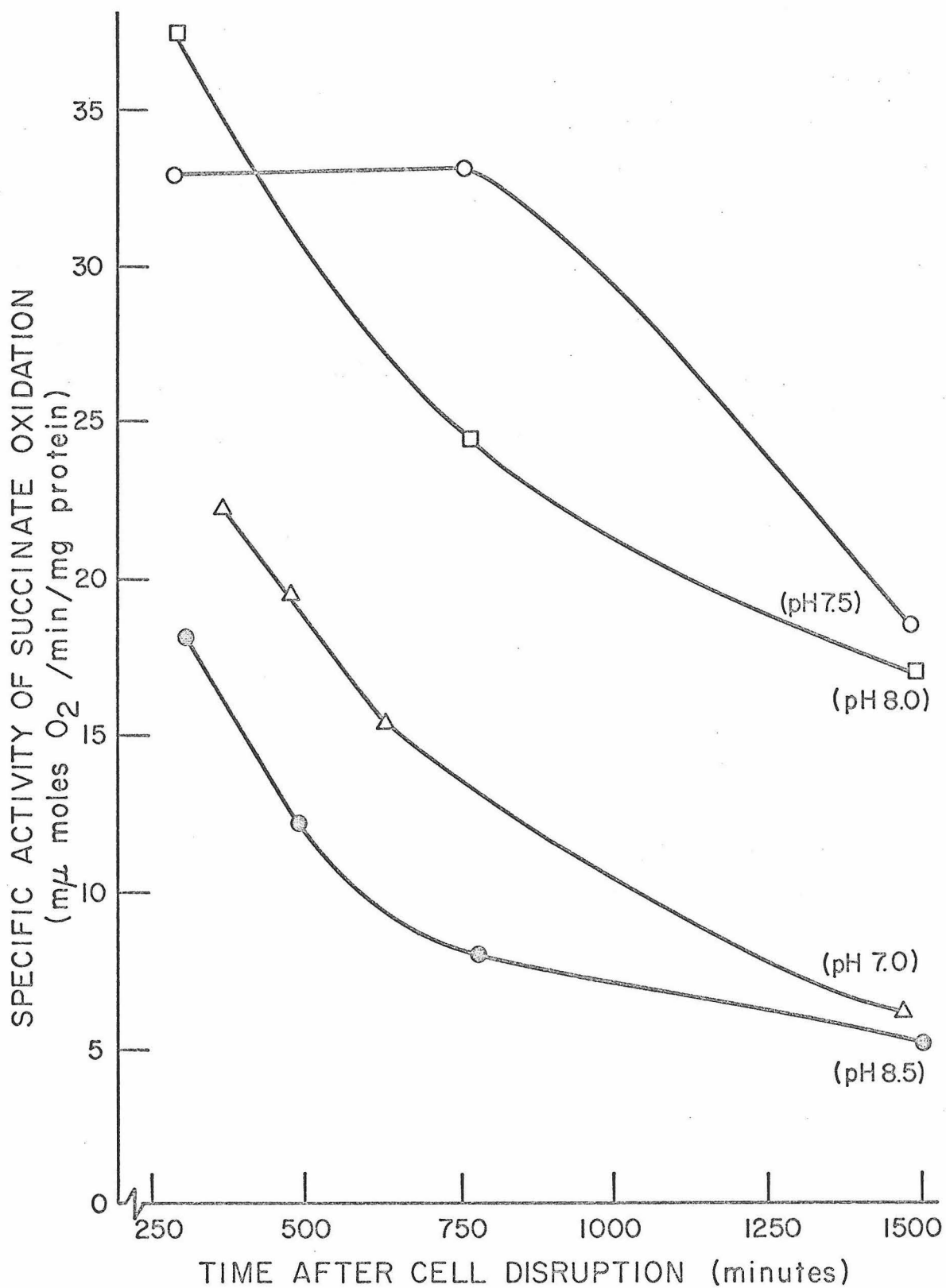
To test the possibility of degeneration by diffusion of an essential component, a sample of young poky mitochondria was allowed to incubate at 0°C overnight. Then the mitochondria were removed from the suspension by centrifuging at 15,000 g. The supernatant, which should have contained any material that had diffused out of the mitochondria, was lyophilized to dryness and then resuspended in one tenth of the original volume. This extract was added to a suspension of young poky mitochondria whose succinate oxidase system had degenerated, but the succinate oxidase activity was not restored.

The succinate oxidase system of young poky mitochondria could be partially stabilized by adequate buffering, as shown in Figure 15. The optimum pH for stabilizing the mitochondria was around 7.5.

A series of different molarities of sucrose were tried in an effort to stabilize the mitochondria to a further extent, but the osmotic pressure was not nearly so critical as the pH. A sucrose concentration of 0.25 M was found to give the best results.

In subsequent experiments investigating the dihydroorotate oxidase system and comparing the properties of poky and wildtype respiratory systems, mitochondria were prepared in 0.25 M sucrose buffered at pH 7.5 with 0.05 M Tris.

FIGURE 15. Degeneration of succinate oxidase activity for mitochondrial suspensions of 20.0 mg/ml at various pHs. Reactions were run in a volume of 3.0 ml starting with 0.6 μ moles of dissolved oxygen and 5.0 μ moles of succinate. Mitochondria were prepared from a 16 hour culture of poky.



E. Characterization of the Dihydroorotate Oxidase System

Substrates

The dihydroorotate oxidase system in young poky mitochondria is able to utilize L-dihydroorotic acid and 5 N-methylformamidodihydroorotic acid as electron donors. Perhaps other analogs of dihydroorotic acid can also be utilized, but none were available. Since L-dihydroorotic acid was commercially available (California Corporation for Biochemical Research) it was used as the electron donor substrate in experiments characterizing the system. Molecular oxygen is utilized as the terminal electron acceptor.

Products

Young poky mitochondria were allowed to oxidize 15 μ moles of L-dihydroorotate. Mitochondria in the reaction mixture were disrupted with deoxycholate and then the reaction mixture was deproteinized with trichloroacetic acid. Precipitate was removed by centrifugation and the supernatant lyophilized. The resulting material was resuspended in 2 ml of water and run through a Biogel P-2 column (17 cm by 1.5 cm), eluting with water. The material from this column which absorbed at 280 m μ was chromatographed in both the basic and acidic solvent systems along with orotic acid. As shown in Table 4, the R_f values for the oxidized dihydroorotic acid and for orotic acid are very similar in both solvent systems. Also the absorption spectrum of the oxidation product of dihydroorotic acid and of orotic acid are the same, as shown in Figure 16. Therefore it can be concluded that the product of dihydroorotic acid oxidation is orotic acid.

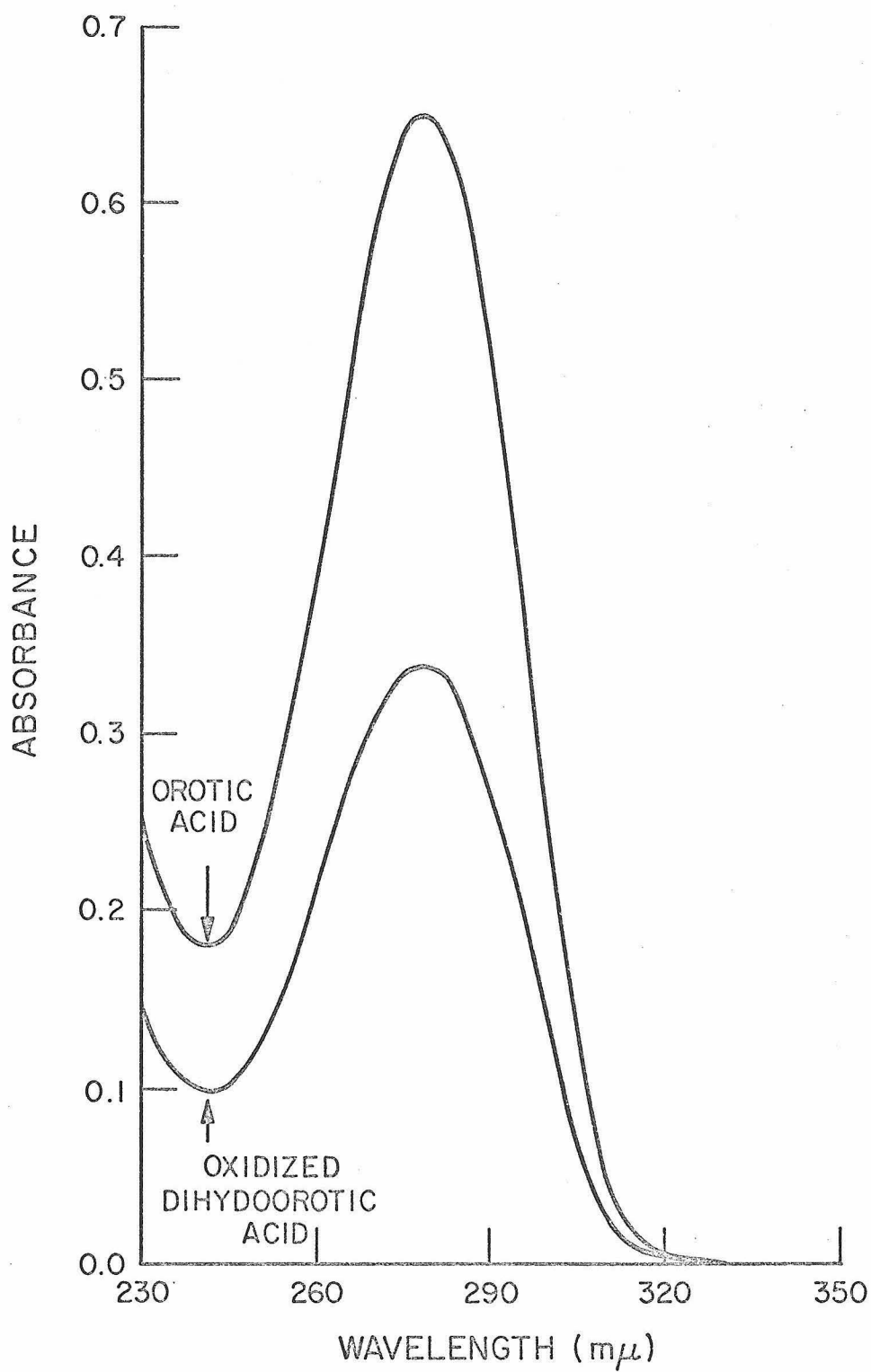
TABLE 4

R_f values for orotic acid and for the product of dihydroorotic acid oxidation in a basic solvent system and in an acidic solvent system.

Solvent System	Orotic Acid	Oxidized Dihydroorotic Acid
Propanol-ammonia	0.5	0.5
Propanol-acetic acid	0.45	0.4

Chromatograms were run for 20 cm on Whatman No. 3 filter paper using the two solvent systems described in the Materials and Methods section. After drying, the chromatograms were cut into ten sections each 2 cm in length. Each section was eluted with 1 ml of water and the absorbance at 280 $m\mu$ was recorded to determine the positions of the compounds.

FIGURE 16. Ultraviolet absorption spectra of orotic acid and the product of dihydroorotic acid oxidation



If the oxygen is not incorporated into the oxidation product, which is apparently the case, then the reduction of molecular oxygen should result in the formation of either water or hydrogen peroxide. The rate of oxygen consumption in the presence of catalase should be about one half the rate in the absence of catalase if peroxide were being formed. This would be caused by the rapid regeneration of half of the oxygen consumed: $O_2 + 2e^- + 2H^+ \rightarrow H_2O_2 \xrightarrow{\text{catalase}} H_2O + \frac{1}{2}O_2$. However, as shown in Table 5, the rate of oxygen consumption is not at all affected by the presence of catalase. Therefore it is probable that the molecular oxygen is incorporated into water during the terminal oxidation reaction.

pH Optimum

The variation in the specific activity of oxygen consumption as a function of the pH of the assay medium was measured. The resulting pH curve, shown in Figure 17, shows an optimum in the range between pH 6.8 and 7.0. Therefore the reaction system used in assaying for dihydroorotate oxidase activity was buffered at pH 6.9.

Kinetics of Oxygen Consumption

Specific activity of oxygen consumption was measured as a function of the initial dihydroorotate concentration, based on the initial reaction velocity. The reaction showed classical Michaelis kinetics, the inverse of the initial reaction velocity being a linear function of the inverse of the initial substrate concentration (Figure 18). The K_m value obtained was 5.32×10^{-5} M, and the V_{\max} for oxygen consumption

TABLE 5

Effect of catalase on the oxidation of substrates by poky and wildtype mitochondria.

Substrate	Specific Activities (μ moles O_2 consumed/min/mg of protein)			
	<u>Poky Mitochondria</u>		<u>Wildtype Mitochondria</u>	
	Control	With catalase	Control	With catalase
Succinate	79	79	22	22
NADH	128	130	69	69
Dihydroorotate	23	23	3	3

Assays were run in a volume of 3.0 ml containing 0.6 μ moles of dissolved oxygen and using 5.0 μ moles of substrate. In the experimental reactions 0.1 mg of catalase with a specific activity of 3000 μ moles H_2O_2 decomposed/min/mg was used. Mitochondria were prepared from a 17 hour culture of poky and from a 24 hour culture of wildtype.

FIGURE 17. pH curves for substrate oxidations. Reactions were run in a volume of 3.0 ml starting with 0.6 μ moles of dissolved oxygen and 5.0 μ moles of substrate. Mitochondria were prepared from a 17 hour culture of poky and from a 20 hour culture of wildtype.

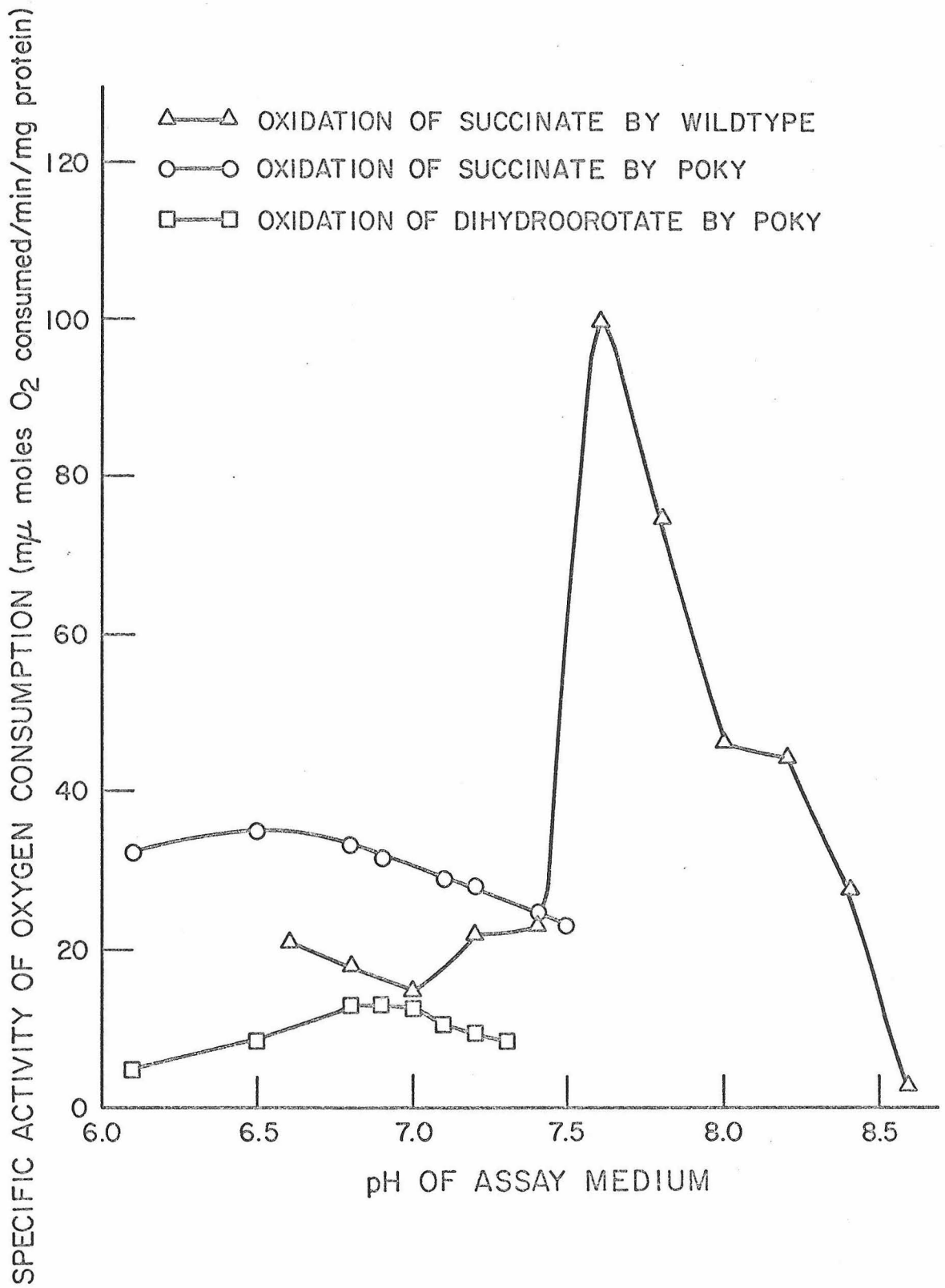
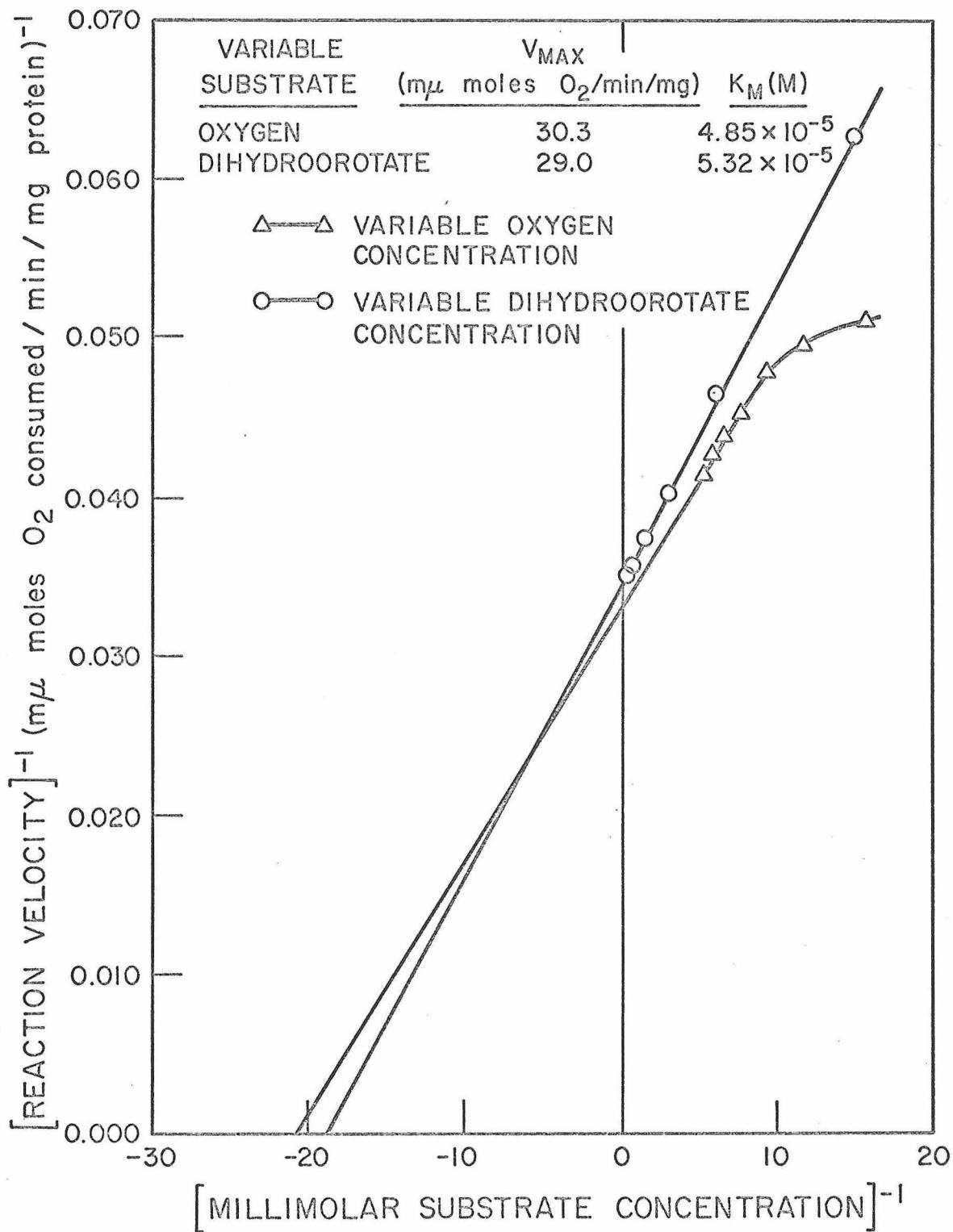


FIGURE 18. Kinetics of dihydroorotate oxidation as a function of substrate concentration. Mitochondria from a 17 hour culture of poky were used.



was 29.0 μ moles/min/mg of protein. However, these values were based on varying the concentration of one substrate while measuring the disappearance of another. Yet the affinity of dihydroorotate for the active site may still be reflected in the K_m value if binding of the dihydroorotate is the rate limiting step and if the rate of oxygen consumption is directly proportional to the rate of dihydroorotate oxidation.

Starting with 5 μ moles of dihydroorotate and 0.63 μ moles of dissolved oxygen in a 3.0 ml volume, the rate of oxygen consumption by young poky mitochondria was measured until all the oxygen was reduced. The recorder tracing was a plot of oxygen concentration vs. time. Determining the slopes at various oxygen concentrations gave reaction velocities as a function of oxygen concentration. By plotting the reciprocal of the reaction velocity as a function of the reciprocal of the oxygen concentration it was found that the system had classical Michaelis kinetics at oxygen concentrations above 10^{-4} M (Figure 18). Extrapolation of this plot gave a K_m value of 4.85×10^{-5} M and a V_{max} value of 30.3 μ moles oxygen consumed/min/mg of protein. In this case the K_m value is directly related to the affinity of oxygen for the active site.

Obtaining similar K_m and V_{max} values by varying the dihydroorotate concentration and by varying the oxygen concentration indicates that the oxidation of dihydroorotate and the reduction of oxygen occur at about the same rate.

Independence from respiration

To test for linkage of the dihydroorotate oxidase system to the respiratory chain, young poky mitochondria were allowed to oxidize dihydroorotate in an oxidative phosphorylation reaction system. The mitochondria had a high level of endogenous ATP which was converted to glucose 6-phosphate over a 30 minute period (Figure 19). The synthesis of glucose 6-phosphate during the oxidation of dihydroorotic acid is not much different from the control (Figure 20). Therefore there seemed to be no synthesis of glucose 6-phosphate that was dependent on the oxidation of dihydroorotate. The P/O ratios for dihydroorotate oxidation by both young poky mitochondria and wildtype mitochondria were 0.0, as shown in Table 6.

Cytochrome c was removed from young poky mitochondria and from wildtype mitochondria by a procedure similar to the method of Jacobs and Sanadi (87). This treatment also removed most of the dihydroorotate oxidase activity from both poky and wildtype mitochondria, but adding back horseheart cytochrome c did not stimulate dihydroorotate dependent oxygen consumption by either mitochondrial preparation (Table 7). Therefore some component of the dihydroorotate oxidase system other than cytochrome c is removed by this washing procedure. This does not eliminate cytochrome c as a possible electron carrier in the oxidase system, but it does differentiate the dihydroorotate oxidation system from the succinate oxidation systems associated with respiration in both poky and wildtype (Table 7).

FIGURE 19. Glucose 6-phosphate synthesis by mitochondria from a 17 hour culture of poky incubated in the presence of glucose and hexokinase.

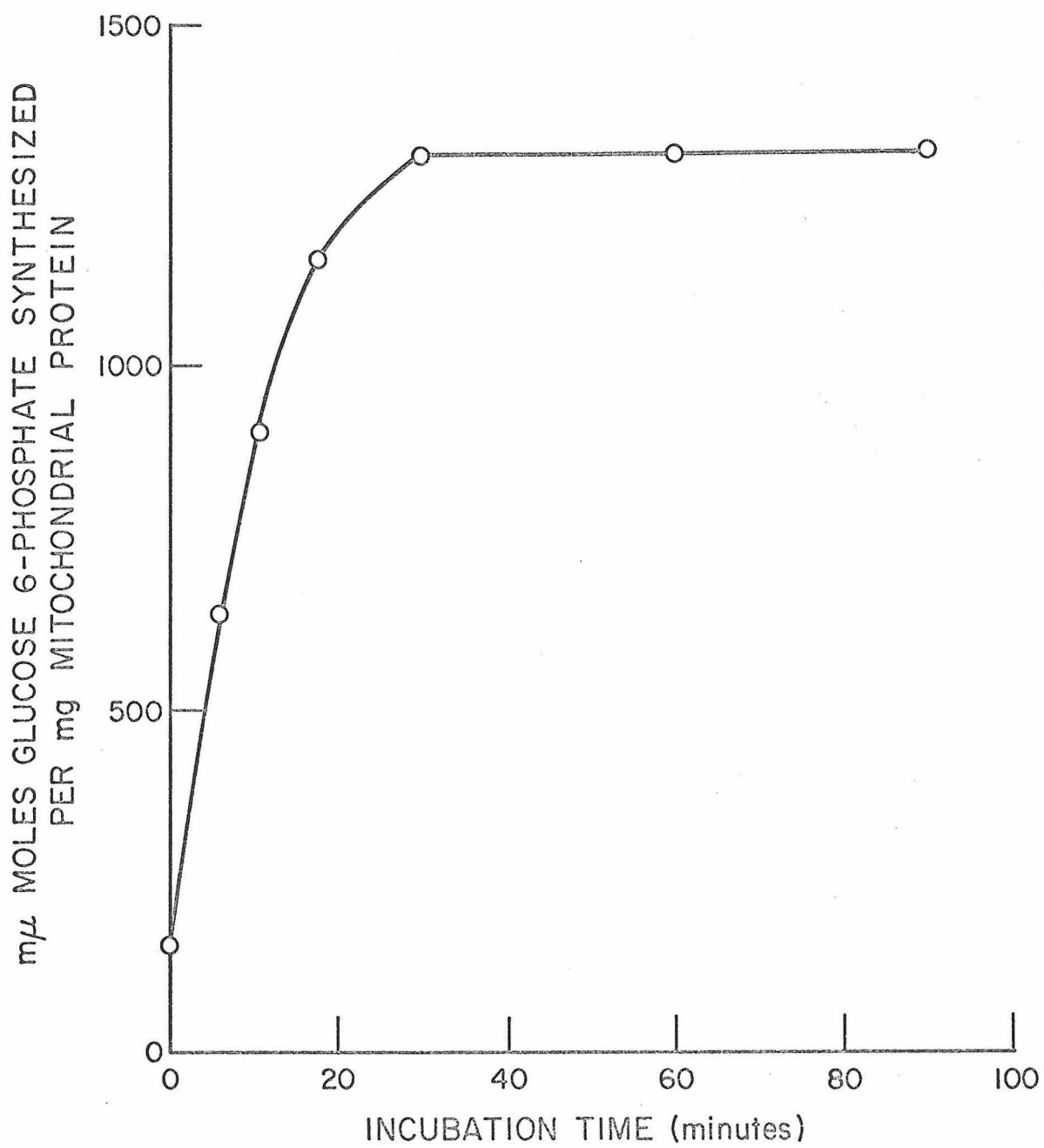


FIGURE 20. Glucose 6-phosphate synthesis by poky mitochondria incubated in an oxidative phosphorylation assay system with various substrates, glucose, and hexokinase. Assays were run in a 3.0 ml volume starting with 5.0 μ moles of substrate. Oxygen was continually supplied to the system by shaking on a reciprocal shaker at 25°C. Mitochondria were prepared from a 17 hour culture of poky.

$\Delta m\mu$ MOLES GLUCOSE 6-PHOSPHATE SYNTHESIZED/mg MITOCHONDRIAL PROTEIN
 [IN THE PRESENCE OF SUBSTRATE - H₂O CONTROL]

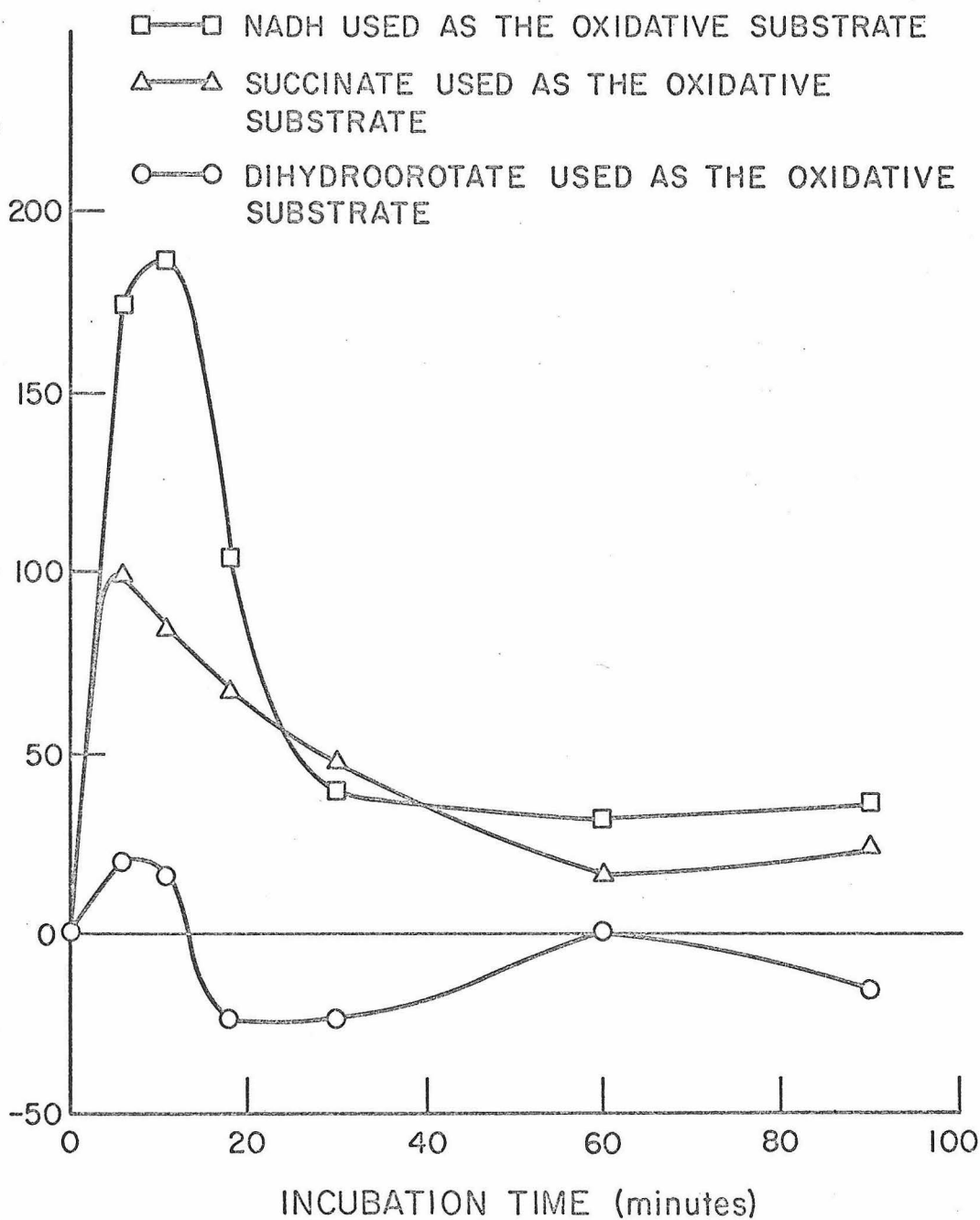


TABLE 6

Oxidative phosphorylation by poky and wildtype mitochondria

Substrate	Uncoupler	P/O ratios	
		<u>Poky</u> Mitochondria	Wildtype Mitochondria
Succinate	None	0.2	0.4
Succinate	Azide	0.05	0.15
Succinate	DNP	0.0	0.0
NADH	None	0.35	0.5
NADH	DNP	0.1	0.0
Dihydroorotate	None	0.0	0.0
Dihydroorotate	DNP	0.0	0.0

Reactions were run in a volume of 3.0 ml containing 0.6 μ moles of dissolved oxygen and using 5.0 μ moles of substrate. When used, the dinitrophenol concentration was 0.003 M. Mitochondria were prepared from an 18 hour poky culture and from a 12 hour wildtype culture.

TABLE 7

Reactions were run in a volume of 3.0 ml containing 0.6 μ moles of dissolved oxygen, using 0.1 ml of mitochondrial suspension and 5.0 μ moles of substrate. Rates were determined initially and then again after the addition of 1 mg of horseheart cytochrome c.

Both the poky and the wildtype mitochondrial suspensions initially had a protein concentration of 30.0 mg/ml. Washing was with ten volumes of distilled water for ten minutes, followed by ten volumes of 0.15 M KCl for 110 minutes, and finally with 0.15 M KCl for twelve hours.

The insoluble material was resuspended in the initial volume, giving a suspension of washed poky mitochondria with a protein concentration of 15.4 mg/ml, and a suspension of washed wildtype mitochondria with a protein concentration of 27.1 mg/ml.

The mitochondria used in the experiment were prepared from a 15 hour culture of poky and from a 24 hour culture of wildtype.

TABLE 7

Reconstitution of poky and wildtype mitochondrial oxidase systems with
horseheart cytochrome c

Mitochondria	Substrate	Rate of oxygen consumption (μ moles O_2 /min)	% control rate
<u>Poky</u> -control	Dihydroorotate	21	100
Washed <u>poky</u>	Dihydroorotate	3	14
Washed <u>poky</u> , cytochrome <u>c</u>	Dihydroorotate	3	14
<u>Poky</u> - control	Succinate	141	100
Washed <u>poky</u>	Succinate	9	6
Washed <u>poky</u> , cytochrome <u>c</u>	Succinate	38	27
Wildtype-control	Dihydroorotate	190	100
Washed wildtype	Dihydroorotate	6	3
Washed wildtype, cytochrome <u>c</u>	Dihydroorotate	7	4
Wildtype-control	Succinate	66	100
Washed wildtype	Succinate	29	44
Washed wildtype, cytochrome <u>c</u>	Succinate	64	97

The differential stability of the dihydroorotate and the succinate oxidase systems in unbuffered suspensions of young poky mitochondria was previously shown (Figure 13).

Mitochondria from poky and wildtype cultures of various ages were prepared over a period of a few weeks and the suspensions were frozen at -20°C from the day of preparation until it was convenient to assay the preparations all at once. At that time the suspensions were thawed and the protein concentration of each was determined. Then all the mitochondrial suspensions were diluted to a mitochondrial protein concentration of 25 mg/ml and assayed for both succinate oxidase activity and for dihydroorotate oxidase activity. As shown in Figure 21, the succinate oxidase activity of both poky and wildtype showed variations during the growth cycle, perhaps as a result of variations in the cytochrome content of the mitochondria (Figures 3 and 4). However the dihydroorotate oxidase activity showed no variation during the growth cycle in either poky or wildtype (Figure 21) and was therefore independent of the mitochondrial cytochrome composition.

Considering these facts it appears that the dihydroorotate oxidase system is not linked to respiration or to ATP synthesis.

Inhibition

Inhibition of the dihydroorotate oxidase system by azide was found both in young poky and wildtype mitochondria. The loss in specific activity with increasing azide concentration is similar in poky and in wildtype mitochondria (Figure 22). The dihydroorotate oxidase system

FIGURE 21. Specific activities of oxidase systems in poky and wild-type mitochondria during growth. Reactions were run in a volume of 3.0 ml starting with 0.6 μ moles of dissolved oxygen and 5.0 μ moles of substrate.

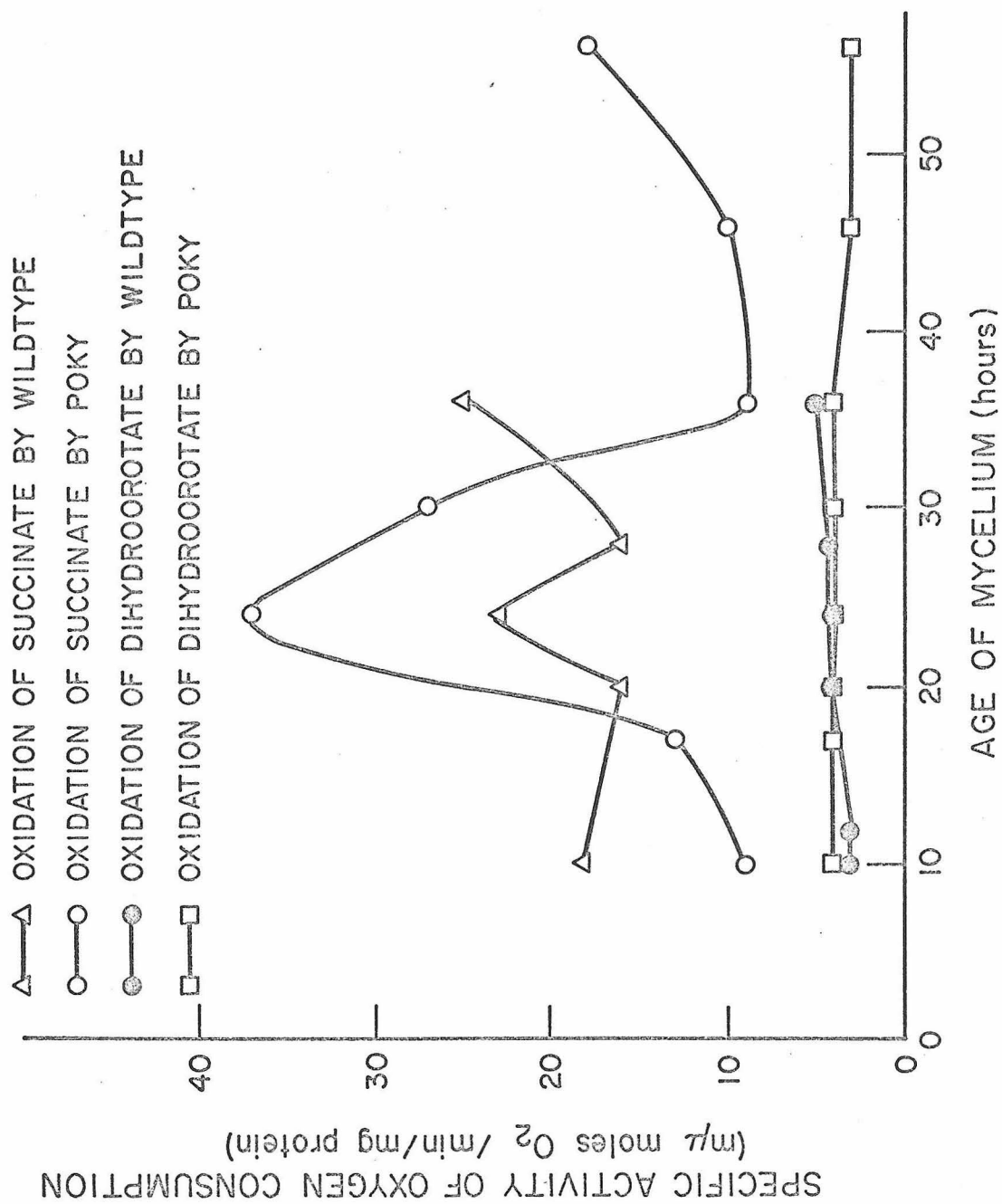
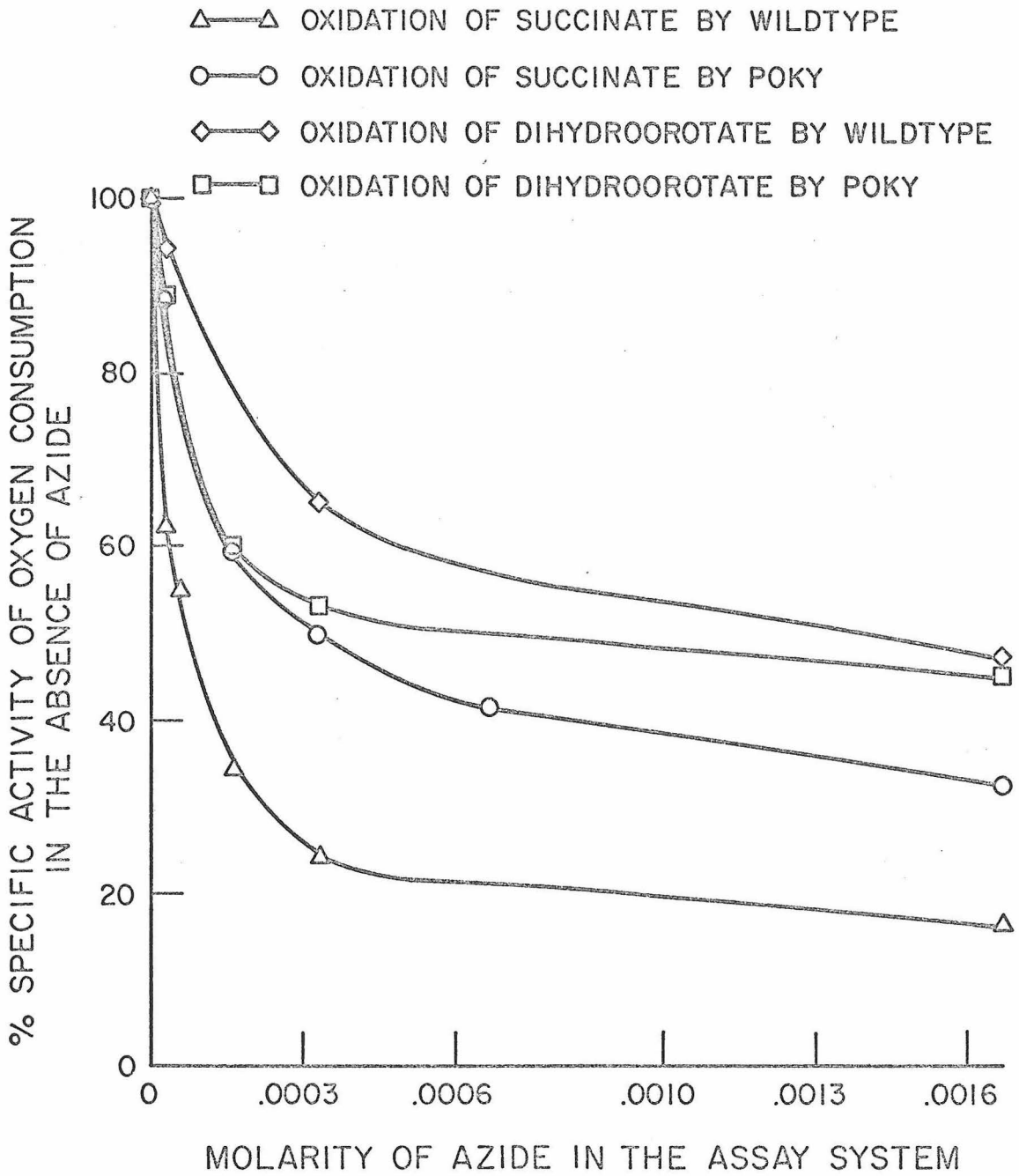


FIGURE 22. Sensitivity of substrate oxidations to azide. Reactions were run in a volume of 3.0 ml containing 0.6 μ moles of dissolved oxygen and using 5.0 μ moles of substrate. Mitochondria were prepared from a 17 hour poky culture and from a 24 hour culture of wildtype.



of poky mitochondria showed more sensitivity to low concentrations of azide than did the dihydroorotate oxidase system of wildtype mitochondria but at higher concentrations both systems were inhibited to about the same extent.

Dinitrophenol at a concentration of 0.003 M inhibited dihydroorotate oxidase activity in both poky and wildtype mitochondria to about the same extent (Table 8).

The effect of o-phenanthroline on the dihydroorotate oxidase systems of poky and wildtype mitochondria is shown in Table 9. Both systems are inhibited similarly at high and at low concentrations. This inhibition indicates that non-heme iron plays an important role in the dihydroorotate oxidase systems, but not necessarily as an intermediate electron carrier.

The similarities in the effects of inhibitors on the poky and the wildtype dihydroorotate oxidase systems, and the evidence that these systems are not linked to respiration indicate that they may be the same.

F. Comparison of the respiratory systems in young poky and wildtype mitochondria.

Substrates

A variety of compounds were tested for activity as a substrate for oxygen consumption in young poky mitochondria and in wildtype mitochondria. The specific activities of oxygen consumption using these substrates are listed in Table 10.

TABLE 8

Effect of dinitrophenol on the rates of oxidation of succinate and dihydroorotate by poky and wildtype mitochondria.

Mitochondria	Substrate	Specific activity of oxygen consumption (μ moles O_2 consumed/min/mg of protein)		
		0M DNP	.003M DNP	% Inhibition by DNP
<u>Poky</u>	Succinate	54	38	30
<u>Poky</u>	Dihydroorotate	18	14	22
Wildtype	Succinate	64	35	45
Wildtype	Dihydroorotate	15	11	26

Assays were run in a volume of 3.0 ml containing 0.6 μ moles of dissolved oxygen and using 5.0 μ moles of substrate. Mitochondria were prepared from a 17 hour culture of poky and from a 12 hour culture of wildtype.

TABLE 9

Effect of o-phenanthroline on the oxidation of substrates by poky and wildtype mitochondria.

Mitochondria	o-phenanthroline	% Inhibition of substrate oxidation	
		Succinate	Dihydroorotate
Wildtype	.0017 M	12	57
<u>Poky</u>	.0017 M	35	68
Wildtype	.0001 M	0	16
<u>Poky</u>	.0001 M	15	18

Reactions were run in a volume of 3.0 ml containing 0.6 μ moles of dissolved oxygen and using 5.0 μ moles of substrate. Mitochondria were prepared from a 17 hour culture of poky and from a 20 hour culture of wildtype.

TABLE 10

Oxidation of substrates by poky and wildtype mitochondria.

Substrate	Specific activity of oxidation (μ moles O_2 consumed/min/mg of protein)	
	<u>Poky</u> mitochondria	Wildtype mitochondria
NADH	82	80
Succinate	68	48
Dihydrooorotate	16	14
NADPH	3	13
Cytochrome <u>c</u> (Fe^{++})	2	1
Citrate	0	3
Isocitrate	5	4
α -Ketoglutarate	0	3
Malate	0	1
Pyruvate	0	0
Pyruvate + Malate	0	1
Aspartate	0	0
Ureidosuccinate	0	0

Assays were run at pH 6.9 in a volume of 3.0 ml containing 0.6 μ moles of dissolved oxygen and using 5.0 μ moles of substrate. Mitochondria were prepared from a 17 hour culture of poky and a 36 hour culture of wildtype.

Citric acid cycle intermediates are oxidized by wildtype mitochondria but succinate was the only one which gave a fast rate. Citric acid cycle enzymes except for succinic dehydrogenase are all very loosely associated with mitochondria (88) and may be lost to a great extent during purification. In the poky mitochondria succinate was rapidly oxidized and isocitrate was slowly oxidized. Other citric acid cycle intermediates were inactive as substrates, but again this may have been a result of the loss of loosely associated enzymes during purification of the mitochondria.

In both the poky and the wildtype mitochondrial preparations NADH was rapidly oxidized. NADPH also could be oxidized but at a much slower rate.

Oxidation of ferrous horseheart cytochrome c could be demonstrated in both mitochondrial preparations, but the rates were very slow, perhaps because a protein cannot diffuse into or be taken up by mitochondria as quickly as can smaller molecules.

Dihydroorotate oxidation in both mitochondrial preparations was not related to the respiratory systems as was shown in the previous section. The biosynthetic precursors of L-dihydroorotate, L-aspartate and L-ureidosuccinate, were inactive as substrates for oxygen consumption. Therefore dihydroorotase activity, responsible for the dehydration and cyclization of L-ureidosuccinate to form L-dihydroorotate is not associated with the mitochondria.

In experiments on the respiratory systems of poky and wildtype mitochondria, succinate was used as a substrate.

pH Optima

The two respiratory systems had different pH optima for succinate oxidase activity (Figure 17). The poky system showed a broad optimum around pH 6.5. The succinate oxidase activity in wildtype had an optimum at pH 7.6.

Oxidative phosphorylation

The poky respiratory system is able to couple phosphate esterification to the oxidation of both NADH and succinate (Figure 20). After 10 to 15 minutes the difference in phosphate esterification from the controls rapidly decreased. This might represent metabolism of glucose 6-phosphate but no corresponding decrease of glucose 6-phosphate content made from endogenous ATP was detected (Figure 19). Another possibility is that the endogenous ATP is converted into glucose 6-phosphate at a faster rate in the presence of succinate and NADH oxidation; or more likely, that phosphorylation linked to the oxidation of endogenous substrates proceeds more rapidly in the presence of succinate and NADH oxidation. If this were the case then the phosphate esterification dependent only on the added substrate would be represented by the difference at a time when the control was no longer making glucose 6-phosphate. In Figures 19 and 20 this time is the period after 30 minutes.

The P/O ratios obtained for succinate and NADH oxidation by both poky and wildtype mitochondria are shown in Table 6. Wildtype gave higher P/O ratios for both substrates than did poky. Oxidative phosphorylation was uncoupled in both systems by dinitrophenol and by azide.

Role of Cytochrome c

Both respiratory systems could be inhibited by washing out cytochrome c and both could be reconstituted by adding back horseheart cytochrome c (Table 7). The wildtype system could be reconstituted almost back to the original activity. Succinate oxidase activity of the poky system was increased significantly by the external horseheart cytochrome c but not to the extent as was the wildtype system.

An interesting phenomenon was observed while experimenting with wildtype mitochondria from which the cytochrome c had been washed out. Addition of horseheart cytochrome c in the absence of substrate resulted in a small amount of oxygen being consumed. Subsequent addition of more cytochrome c to the same reaction mixture had no effect. The same phenomenon occurred in the presence of 10^{-3} M azide. It suggests that some membrane bound component in the reduced form was oxidized by the first addition of cytochrome c. The reduced cytochrome c thus formed was then oxidized by molecular oxygen through a terminal oxidase which was able to operate in an environment of 10^{-3} M azide. This phenomenon did not occur in the young poky mitochondria from which the cytochrome c had been washed.

Terminal Oxidation

The kinetics of oxygen consumption linked to succinate oxidation was measured in the absence and in the presence of catalase. Since catalase did not alter the kinetics of either system (Table 5) the terminal oxidation step of both the poky and the wildtype respiratory systems must

involve the reduction of molecular oxygen to water rather than to peroxide.

Sensitivity to inhibitors

Both respiratory systems were sensitive to azide, though the wild-type system was more sensitive than the poky system at very low azide concentrations (Figure 22). At an azide concentration of 10^{-3} M which should completely inactivate cytochrome oxidase activity the wildtype succinate oxidase system was inhibited by about 80%, while the poky succinate oxidase system was inhibited by about 60%.

Dinitrophenol inhibited succinate oxidase activity in both respiratory systems. Inhibition was slightly greater in the wildtype system than in the poky system (Table 8).

Inhibition by o-phenanthroline was measured at two concentrations, 10^{-4} M and 17×10^{-4} M. The presence of o-phenanthroline in either of these concentrations caused the background endogenous rate of oxygen consumption to increase to about four times the normal level. As shown in Table 9, the low concentration of o-phenanthroline did not inhibit succinate oxidase activity in wildtype mitochondria, but the higher concentration was somewhat inhibitory. In the poky succinate oxidase system both the low and the high concentration of o-phenanthroline gave about the same level of inhibition as the high concentration gave in the wildtype system.

DISCUSSION

A. The Dihydroorotate Oxidase System of Neurospora

From the results of the experiments on the dihydroorotate oxidase system of Neurospora, the following properties have been determined:

- (1) The system is localized in the mitochondria.
- (2) The system is stable in an unbuffered suspension of mitochondria.
- (3) Dihydroorotic acid is oxidized to orotic acid using molecular oxygen as the terminal electron acceptor.
- (4) The reaction exhibits classical Michaelis kinetics and both substrates, dihydroorotate and oxygen, are consumed at about the same rate.
- (5) The pH optimum is about 6.9.
- (6) There is no linkage of electron transport to the cytochrome system or to oxidative phosphorylation.
- (7) The system is sensitive to o-phenanthroline.
- (8) Catalase has no effect on the rate of oxygen consumption.

Using these facts and relating them to the properties of dihydroorotate dehydrogenase systems in other organisms, it is possible to speculate upon the metabolic role of the dihydroorotate oxidase system in Neurospora, the significance of its intracellular localization, and the mechanism of electron transport that is used.

Role in Metabolism

Apparently dihydroorotate oxidase plays no role in respiratory metabolism. Although such a role has been found in Pseudomonas (84), there is no evidence whatsoever that the Neurospora system is linked to the respiratory chain.

Certainly dihydroorotate oxidase does have a use in pyrimidine biosynthesis as the conversion of dihydroorotate to orotate gives the pyrimidine from which all other pyrimidines are derived (89).

Through the production of orotic acid, this system could also regulate de novo purine synthesis. Rajalakshmi and Handschumacher (90) have found that orotic acid does affect de novo purine synthesis in mouse liver by controlling the supply of 5-phosphoribosyl 1-pyrophosphate available for condensing with glutamine. Another possible metabolic role is in the regulation of m-RNA synthesis. Caldarera et al. (91) have obtained evidence that orotic acid stimulates the activity of nuclear DNA dependent RNA polymerase.

Intracellular Localization

Enzymatic mechanisms of dihydroorotate oxidation have long been known but have not as yet been shown to be associated with purified mitochondria. Rat liver homogenates show activity for conversion of ureidosuccinate to orotic acid distributed among the nuclear fraction, the mitochondrial particle fraction, and the microsomal fraction (92). The bacterial dihydroorotate dehydrogenases induced by using orotic acid as a carbon source are in the supernatant fraction of a cell free extract after high speed centrifugation (84, 93). However, a particulate membrane bound dihydroorotate dehydrogenase has recently been found in Pseudomonas (84).

Because of the recent finding that Neurospora mitochondria contain the components necessary for nucleic acid synthesis (94), the mito-

chondrial localization of dihydroorotate oxidase is particularly interesting. Though the synthesis of nucleotides in mitochondria has not been demonstrated, the presence of the dihydroorotate oxidase system would assure that pyrimidines were available. If orotic acid affects m-RNA synthesis from nuclear DNA, perhaps it would also affect m-RNA synthesis from mitochondrial DNA. In such a case, the presence of the dihydroorotate oxidase system in mitochondria might be important in the regulation of m-RNA synthesis in the mitochondria.

Mechanism of Electron Transport

It is convenient to consider the electron transport of the dihydroorotate oxidase system in four parts: (1) substrates which can serve as the original source of electrons; (2) dihydroorotate dehydrogenase; (3) other possible intermediate electron carriers; (4) the terminal step of reducing molecular oxygen.

(1) Substrates

The Neurospora system apparently uses derivatives of dihydroorotic acid exclusively as no dihydropyrimidine with activity as a substrate but without a C-4 carboxyl group was found. An enzyme has been obtained from Clostridium uracilium which can utilize dihydrouracil as a substrate, giving uracil as a product, but it is mainly used in the reductive catabolism of pyrimidines (95).

The C-5 atom is extremely important, as shown by the behavior of orotic and dihydroorotic acid analogs. One might expect that a stabilized carbanion at C-5 as an intermediate would facilitate the transfer

of a hydride ion to and from C-4. If such were the case then the acidity of the proton on C-5 should influence the reactivity, the more acidic the proton the faster the rate of reaction. This is the behavior of all analogs reported so far. 5-fluoroorotate is reduced faster by the reversible dihydroorotate dehydrogenase from Zymobacterium oroticum than is orotate (96). The electronegative fluorine atom on C-5 would be expected to make the proton on C-5 more acidic. 5-methylorotate on the other hand is inactive as a substrate for the same enzyme and in fact inhibits the reduction of orotate (93). The electropositive methyl group on C-5 should make the C-5 proton less acidic and the carbanion less stable. The dihydroorotic acid analog 2,4 dioxohexahydro 1,3,5 triazine 4-carboxylic acid has a nitrogen in place of C-5 thus preventing the possibility of carbanion formation. It is a potent inhibitor of dihydroorotate oxidation by mouse liver homogenate, suggesting that it also cannot be oxidized (97). Though 5 N-methylformamidodihydroorotic acid was never obtained in sufficient quantity to use as a substrate, it appeared from measuring oxidation rates during its purification from yeast extract that it is oxidized at a faster rate than is dihydroorotic acid. Therefore the electronegative N-methylformamido side chain probably enhances the rate of oxidation by making the C-5 proton more acidic.

(2) The Dihydroorotate Dehydrogenase

The dihydroorotate dehydrogenase is most likely a metalloflavoprotein, though the enzyme was not characterized. All other dihydroorotate dehydrogenases which have been characterized are metalloflavo-

proteins. The crystallized enzyme from Zymobacterium oroticum contains FAD, FMN, and non-heme iron in the ratio 1:1:2 (93). If the enzyme in Neurospora is in fact a metalloflavoprotein, then the possibility of intramolecular electron transport exists. Such intramolecular electron transport does exist in metalloflavoproteins. Xanthine oxidase, for example, has an internal electron transport system in which the proposed electron flow is from substrate to molybdenum to flavin to iron to oxygen (98).

There is evidence that iron is a component in the path of electron flow in the dihydroorotate dehydrogenase from Zymobacterium (99). Since the dihydroorotate oxidase activity in Neurospora is inhibited by δ -phenanthroline, it is likely that in this system too there is participation of iron in electron transport. Because the transfer of hydride ion from the substrate involves two electrons it is probable that a flavin is the first electron acceptor. The reduced flavin can then transfer electrons one at a time to the iron since semiquinone free radicals of flavins are stable. The reduced flavin form of the enzyme from other organisms (except for the membrane bound enzyme in Pseudomonas) can be used to reduce NAD or NADP and thus the dehydrogenation of dihydroorotic acid is reversible. Perhaps this can occur in Neurospora too, though NAD and NADP did not stimulate the rate of oxidation of yeast extract. If the only path of electron transfer is to oxygen, then the reaction should be irreversible. The large accumulation of orotic acid by certain pyrimidineless mutants of Neurospora (100) supports this view.

(3) Other Intermediate Electron Carriers

The postulated linkage of the membrane bound dihydroorotate oxidase system of Pseudomonas is through transfer of electrons from dihydroorotate dehydrogenase to ubiquinone and subsequent electron transfers to cytochrome b, cytochrome c, cytochrome oxidase, and oxygen. This does not occur in the Neurospora system, and Friedmann and Vennesland (96) have found that the Zymobacterium system cannot use mammalian cytochrome c as an electron acceptor. Besides the linkage to the respiratory chain, the Pseudomonas system, which is membrane bound, can go through a cyanide insensitive pathway utilizing ferricyanide or oxygen as the electron acceptor.

Lacroute (101) found that the dihydroorotate dehydrogenase of yeast could not use NAD or NADP as electron acceptors, but could use dichlorophenolindophenol as an electron acceptor. Since yeast, like Neurospora, is a fungus, one would expect that the yeast dihydroorotate oxidase system would resemble that of Neurospora more than would the systems from bacteria or mammals. It is likely that in vivo the yeast system uses oxygen as the terminal electron acceptor since dichlorophenolindophenol is used as a substitute for oxygen in assaying other terminal oxidases.

There may be more electron carriers in the Neurospora dihydroorotate oxidase system before the terminal reduction of oxygen, but other flavoprotein oxidases such as xanthine oxidase transfer electrons directly from the substrate dehydrogenase to oxygen (98). Also there is evidence that the oxidation of Zymobacterium dihydroorotate dehydrogenase involves a transfer of electrons from the iron component to molecular oxygen (99).

Therefore the direct electron transfer from dihydroorotate dehydrogenase to oxygen in the Neurospora system is a likely possibility.

(4) Terminal Oxidation

The use of oxygen as a terminal electron acceptor has not been considered as important except in the membrane bound dihydroorotate oxidase system of Pseudomonas (84). However, terminal oxidation by oxygen in dihydroorotate dehydrogenase systems has been observed. The NAD dependent Zymobacterium enzyme was found to give a significant background rate in the absence of NAD, presumably due to the use of oxygen as an electron acceptor (96). Also the soluble dehydrogenase of Pseudomonas, induced by growth on orotate, was found to react with O_2 in the absence of NADP to give H_2O_2 .

The terminal oxidation of the Neurospora dihydroorotate oxidase system apparently results in the formation of water. However, the formation of peroxide is still questionable since it has been reported that poky has a cytochrome c peroxidase (102). Since the cytochrome c in poky is largely in the reduced form (27) it is conceivable that any peroxide formed from reduction of oxygen by dihydroorotate dehydrogenase could be immediately reduced further to water by electrons from cytochrome c via cytochrome c peroxidase. If this were a very closely linked system, the external addition of catalase might not influence oxidation rates even if peroxide were being formed. Such a system would have the interesting property of having two different electron transport chains supply the electrons for the complete reduction of each individual oxygen molecule.

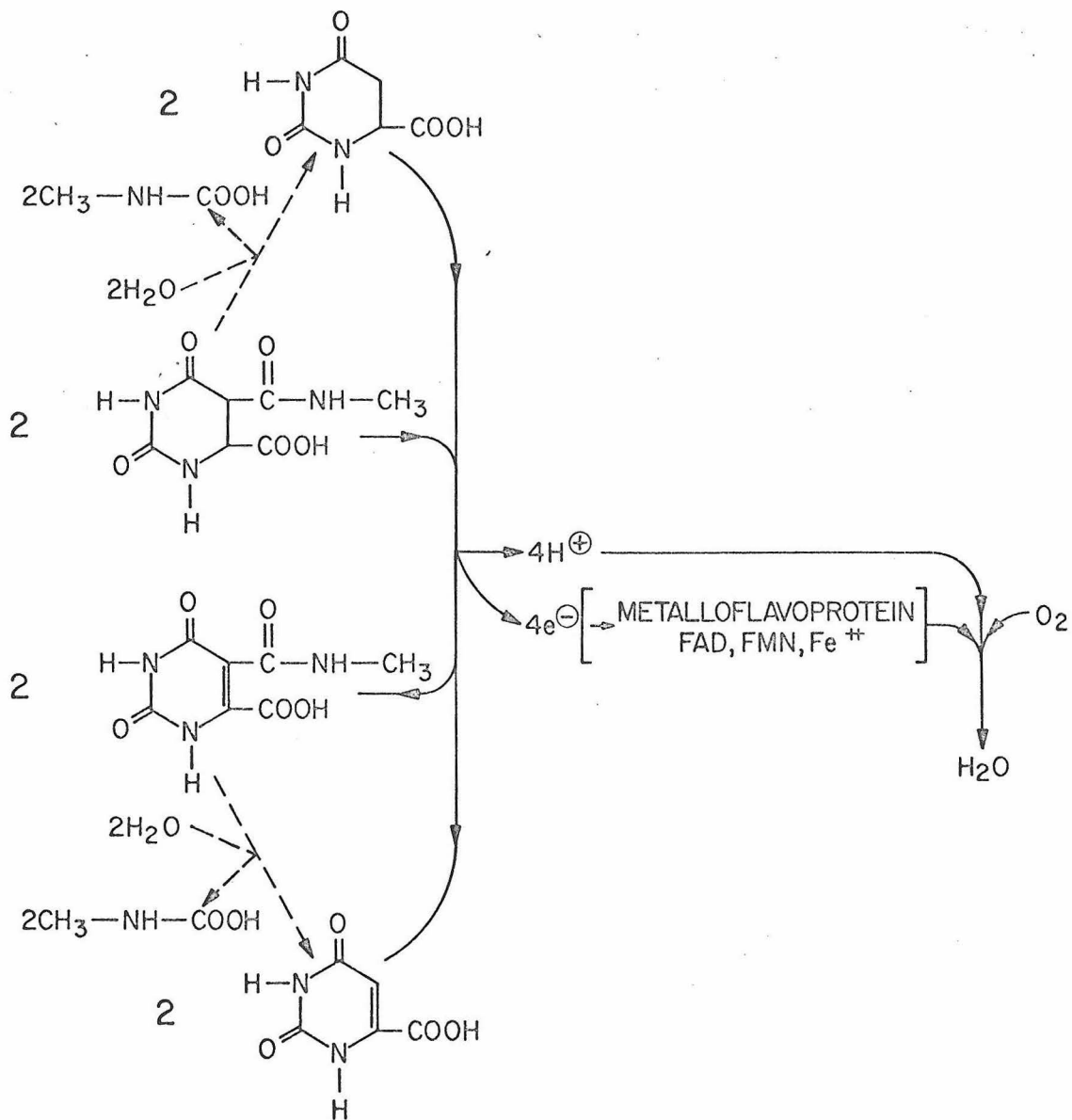
A model for the most likely system of electron transport in the Neurospora dihydroorotate oxidase system is presented in Figure 23. The dihydropyrimidine is oxidized to the pyrimidine with the concurrent formation of hydrogen ions and electrons. Dihydroorotic acid is oxidized to orotic acid and presumably an analogous reaction occurs when 5 N-methylformamidodihydroorotic acid is used as a substrate. It is possible though unlikely that the side chain is removed before oxidation so that dihydroorotic acid is actually the substrate. Or the side chain might be removed during or after the oxidation resulting in a final product of orotic acid.

The electrons are transported along an intramolecular chain within a metalloflavoprotein dehydrogenase. The pathway includes FAD, FMN, or both, and then non heme iron. Electrons are transferred from the non-heme iron directly to molecular oxygen resulting in the formation of water.

B. The Biological Significance of 5 N-methylformamidodihydroorotic Acid

The absolute certainty of structure of the compound isolated from yeast extract depends upon its synthesis, which has not been performed. However, in the absence of proof by synthesis and on the basis of its physical, chemical, and biological properties, the most reasonable structure for the compound is 5 N-methylformamido-L-dihydroorotic acid. The presence of such a compound in yeast extract to the extent of 10 mg per 20 g raises interesting questions as to its origin and its biological function.

FIGURE 23. Model for electron transport in the dihydroorotate oxidase system of Neurospora. The use of 5 N-methylformamidodihydroorotate is also indicated. The dashed arrows indicate possible but unlikely pathways of conversion either to dihydroorotate before oxidation or to orotate after oxidation.



Origin of the Compound

It is hard to conceive of any straightforward mechanism of biosynthesis using well known metabolic pathways, but since the compound is a dihydropyrimidine and an analog of dihydroorotic acid, its biosynthesis is most likely related to the biosynthesis or catabolism of pyrimidines.

It is unlikely that the side chain is attached to C-5 before ring closure. However, calf liver has a dihydropyrimidine hydrolase which catalyzes the reversible hydrolyses of dihydropyrimidines to give the corresponding N-carbamyl β amino acids (103). If such a system in yeast were responsible for the synthesis of 5 N-methylformamidodihydroorotic acid, it would require a prior synthesis of β carboxyl aspartate or its N-methylamide. The only other anabolic process which is reasonable is attachment of the side chain to the ring. Since side chain attachment is more reasonable if there is an aromatic system, it is probable that orotic acid is a precursor of the compound. Perhaps the simplest way to get carbon substitution on C-5 is to use the thymidylate synthetase reaction involving the transfer of a one carbon unit from methylene tetrahydrofolate to deoxyuridylic acid. Synthesis of deoxyorotidine 5'-phosphate could parallel the synthesis of deoxyuridylic acid but with the omission of the decarboxylation step. Williams (104) has shown that Neurospora has a mechanism of catabolizing pyrimidine deoxynucleosides, so an equivalent system may be operative in yeast. First the Neurospora system hydrolyzes off the deoxy sugar. Then in the case of thymidine degradation the 5-methyl side chain of thymine is oxidized to

form a 5-hydroxymethyl side chain. This is further oxidized to a 5-formyl side chain. Though Neurospora apparently removes the 5-formyl side chain to form uracil (104), the formation of 5-carboxy uracil from thymine has been detected in rat liver slices (105). By a series of analogous reactions yeast might be able to convert deoxyorotidine to 5-carboxyorotic acid. Conversion of the 5-carboxyl group to an amide might be accomplished by the mechanism which is used in the similar conversion of nicotinic acid adenine dinucleotide to nicotinamide adenine dinucleotide. This involves an ATP dependent transamidation with glutamine. The next step then would be methylation of the amide. Numerous biological transmethylating agents exist including tetrahydrofolate derivatives, choline, and S-adenosyl methione. Methylation would give the oxidized form of the compound isolated from yeast extract. As a final step in the synthesis, an enzyme similar to the dihydrouracil dehydrogenase of Clostridium uracilium (95) might reduce 5 N-methylformamidoorotic acid to 5 N-methylformamidodihydroorotic acid.

Formation of the compound from catabolic processes seems unlikely. Flavins, purines, and pteridines all have pyrimidine ring structures substituted at C-4 and C-5 but nitrogens rather than carbons are attached to the ring.

In summary, a logical and reasonable mechanism for the biosynthesis of 5 N-methylformamidodihydroorotic acid is to make orotidine 5'-phosphate from known metabolic pathways followed by these conversions: orotidine 5'-phosphate \rightarrow deoxyorotidine 5'-phosphate \rightarrow 5 methyl deoxyorotidine 5'-phosphate \rightarrow 5 methyl deoxyorotidine \rightarrow 5 methylorotic acid \rightarrow

5 hydroxymethylorotic acid \rightarrow 5 formylorotic acid \rightarrow 5 carboxyorotic acid \rightarrow 5 formamidoorotic acid \rightarrow 5 N-methylformamidoorotic acid \rightarrow 5 N-methylformamidodihydroorotic acid.

The Biological Function of 5 N-Methylformamidodihydroorotic Acid

Three possibilities exist for the occurrence of this compound in yeast extract. It can be an intermediate in the anabolism or catabolism of other molecules, it can be accumulated as the end product of some degradative process, or it may have a useful biochemical role.

The structure does not suggest any obvious precursor relationship to known biochemical compounds. Also there is no obvious relationship to possible degradative pathways. Furthermore the quantity of the compound in yeast extract makes it unlikely that it is only an intermediate.

The compound might be the end product of a degradative pathway, but most catabolic degradations are to components which can be reused in metabolism.

The compound may be acting as a coenzyme of some sort. If so the most likely function would be as a hydrogen carrier, similar to the function of NADH. In this respect the structural similarities to nicotinamide are interesting. Both are dihydro derivatives of 6 member nitrogen heterocycles with an amide side chain. The 5 N-methylformamidodihydroorotic acid might also be functional in the form of a nucleotide, either as a coenzyme as just described, or perhaps as a nucleic acid component. Transfer RNAs have unusual pyrimidine components but so far

all seem to be methylated derivatives of uracil and cytosine. There are rare instances of unusual pyrimidines in DNA, but these too are always C-5 derivatives of uracil and cytosine. The incorporation of the compound into DNA or RNA would most likely involve the oxidized form, 5 N-methylformamidoorotic acid. However a dihydropyrimidine (dihydro-uracil) has recently been found in the RNA associated with pea chromatin histone (106).

C. Respiratory Systems in Neurospora

The Mammalian-type Cytochrome System

Experiments with wildtype mitochondria have shown that Neurospora has both an NADH dehydrogenase and a succinate dehydrogenase linked to the cytochrome chain and to an ADP phosphorylating system. Succinate and NADH dehydrogenases are flavoproteins in other organisms, and presumably this is also true in Neurospora. In other systems ubiquinone is implicated as an electron carrier between the dehydrogenases and cytochrome b, but no proof of ubiquinone involvement in the Neurospora system has been reported. However, Lester and Crane (107) have extracted ubiquinone from Neurospora. Cytochromes b, c, and a-a₃ can be detected from difference spectra. Cytochrome c₁ may be present but has not been detected from spectra. Perhaps a liquid nitrogen temperature is needed to resolve its absorption band from that of cytochrome c. However, mitochondria which had been washed free of cytochrome c showed no absorption bands in the region where reduced cytochrome c₁ absorbs.

The citric acid cycle is linked to the cytochrome system in intact mitochondria though citric acid cycle enzyme activity is low in mitochondria purified on density gradients.

The cytochrome system is linked to an ADP phosphorylating system. Oxidation and phosphorylation are operative whether the mitochondria are buffered or not. Results from the work with EDTA indicate that the presence of stalked elementary particles, associated with oxidative phosphorylation (82), may be dependent on the physiological state of the mitochondria, and their absence in electron micrographs may not have any biochemical significance.

The cytochrome system is very sensitive to cyanide and azide. Residual oxygen consumption linked to succinate oxidation in the presence of these poisons is probably due to an alternate respiratory pathway.

The Alternate Respiratory System

Young cultures of poky must depend solely on the non cytochrome system for respiration. Studies using young poky mitochondria have given some information on this system.

Primary substrates are NADH and succinate as with the cytochrome system. This indicates that the same dehydrogenases that are used in the cytochrome system constitute the first part of the alternate respiratory chain.

Reconstitution experiments indicate that the next part of the pathway consists of a soluble electron carrier, a membrane bound component and cytochrome c in that order. Washing wildtype mitochondria with

water gave a membrane system which would not oxidize succinate, but which would consume oxygen in the presence of azide the first time cytochrome c was added. This suggests that cytochrome c is a component and that there is a membrane bound component in the reduced form which can serve as a substrate for oxygen consumption in the presence of cytochrome c until it (the membrane bound component) is completely oxidized. The failure of subsequent addition of succinate to stimulate oxygen consumption indicates that some carrier between the succinate dehydrogenase and the membrane bound component is missing. A likely candidate for this carrier is ubiquinone. The membrane bound component is equivalent to the cytochrome b - cytochrome c₁ complex of mammalian respiratory chains. It may consist of more than one molecule and be similar to cytochromes. However, it does not show any significant absorption between 500 m μ and 650 m μ at room temperature.

The partial reconstitution of succinate oxidase in KCl extracted poky mitochondria after cytochrome c addition further points toward the participation of cytochrome c. Failure to obtain complete reconstitution may have been related to the degeneration observed in unbuffered suspensions of mitochondria, or it may have been the result of something in addition to cytochrome c being partially removed by the extraction procedure. Participation of cytochrome c in electron transfer chains other than the normal cytochrome chain is not unprecedented. Sorger (108) has found that cytochrome c can participate in the electron transport pathway of nitrate reductase in Neurospora.

The pathway between cytochrome c and oxygen is associated with membrane and consists of a terminal oxidase which is less sensitive to azide than is cytochrome a₃. Again there is a possibility of additional membrane bound carriers in the complex but there is no evidence for it. The terminal oxidase uses molecular oxygen as an electron acceptor and water rather than peroxide is formed. Here as in the dihydroorotate oxidase system the possibility of peroxide formation with immediate decomposition by cytochrome c peroxidase exists.

The alternate respiratory pathway is linked to an ADP phosphorylation system as phosphate esterification dependent on succinate oxidation could be detected. It is most likely the same system that is linked to the cytochrome chain since elementary particles associated with poky mitochondrial membranes could be demonstrated.

The rapid degeneration of succinate oxidase activity in poky mitochondria during ageing in vitro is probably related to the defective structural protein of the mitochondrial membrane. The succinate oxidase system linked to the cytochrome system is less labile since activity in older cultures of poky, which have a complete cytochrome chain, is stable. Unfortunately the extreme lability of oxidase activities linked to the alternate respiratory chain in poky makes previous in vitro studies on poky respiration, including part B of the Results section of this thesis, obsolete.

A model for the respiratory systems operating in Neurospora is presented in Figure 24. The first part of the pathways are the same. The poky system then uses unidentified soluble and membrane bound com-

FIGURE 24. Model for the respiratory systems in Neurospora.

Abbreviations used are:

fp_{NADH} = NADH dehydrogenase

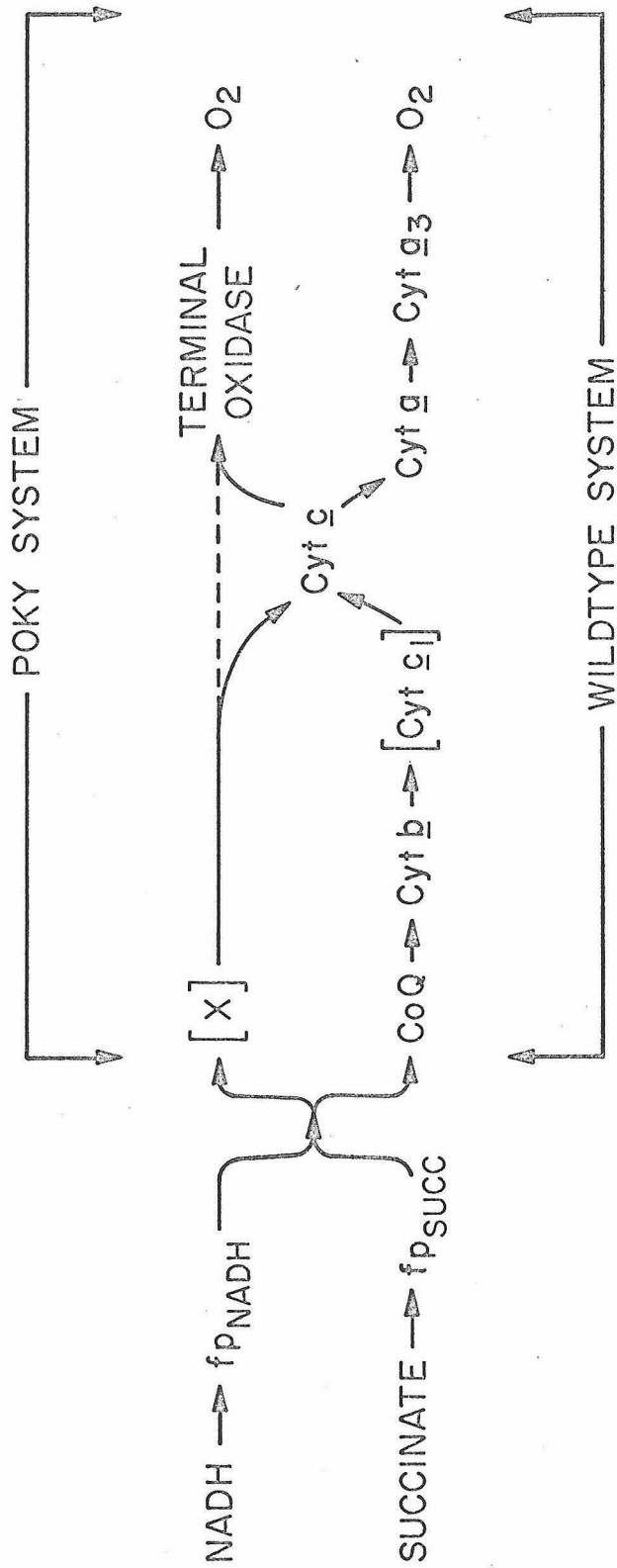
fp_{SUCC} = Succinate dehydrogenase

CoQ = Ubiquinone

X = Unknown components, at least one of which is soluble
and one of which is membrane bound.

The dashed line indicates a possible but unlikely route.

The system labelled POKY is the system present in young cultures of poky but also occurs in wildtype. Conversely, the system labelled WILDTYPE accounts for most of the respiration of wildtype, but also occurs in older cultures of poky.



ponents, cytochrome c, and finally a membrane bound terminal oxidase. It is possible but unlikely that cytochrome c is not part of the chain. But then to explain other data it would have to be assumed that Neurospora cytochrome oxidase can not be completely inhibited by azide, and that it is present in spectroscopically undetectable amounts in young poky. The model for wildtype assumes that the ubiquinone found in Neurospora is used and allows for the possible presence of cytochrome c₁.

D. Development of Mitochondria

The patterns of development occurring in mitochondria during the growth cycle of poky and wildtype are very similar. The main differences are quantitative rather than qualitative, and the time scale is expanded for poky.

Structural Changes

Throughout the life cycle, from conidia to old hyphae, Neurospora has mitochondria. Even in the poky mutant there are always discreet compact bodies corresponding to mitochondria though the internal structure is broken down in conidia. The most striking morphological difference is the change from a state of highly condensed membranes in poky conidia to a state with almost no internal membrane structure during germination. Cristae in the mitochondria become distinct again during hyphal growth.

Biochemical Changes

There was hardly any variation in the activity of the dihydroorotate oxidase system during development of either wildtype or poky mitochondria. This is not surprising since it was shown that dihydroorotate oxidase is unrelated to respiration. However, there were significant changes in succinate oxidase activity in both poky and wildtype mitochondria. This is reflected in the great changes in cytochrome composition during the growth of both poky and wildtype. Cytochrome c was not detected in mitochondria from conidia. But preparation of mitochondria from conidia was very difficult and required extensive grinding. Since cytochrome c is loosely associated with mitochondrial membrane and is extractable with salt solutions, it could very easily have been lost during purification. In poky, turnover and digestion during conidiation apparently remove cytochromes b, a, and a₃. During growth the ratio of cytochrome b to cytochrome c in poky mitochondria goes from zero to about one. A similar change occurs in wildtype though not as extreme as in poky. In the development of poky mitochondria, the order of appearance of cytochromes is cytochrome c, cytochrome b, and finally cytochromes a-a₃. Thus the induction of cytochrome b and cytochrome a syntheses is not coordinate. The sequence is also interesting in relation to the recent proposal of Reilly and Sherman (109) that the synthesis of cytochrome a in yeast may be regulated by other cytochromes.

Changes in the time scale of development with respect to cytochromes corresponding to changes in growth rates can be obtained for both poky and wildtype by altering the conidial concentration during germination.

If the germination and subsequent hyphal development of each conidium were independent of other conidia in the environment, one would expect that by greatly increasing the size of the inoculum the time scale would be shifted but that the mass doubling time would remain the same. However, an increase in inoculation size greatly reduces the mass doubling time for both poky and wildtype mycelium. Perhaps this is caused by a more rapid hydrolysis of sucrose. Metzzenberg (110) has shown that conidia contain an invertase which can partially be removed by washing. Conidia might then be expected to release some invertase in an aerated liquid medium. Increasing the concentration of conidia would then increase the concentration of exogenous invertase thereby increasing the rate of sucrose hydrolysis, which in turn could cause an increase in the growth rate.

Cytochrome c was the only component of the alternate respiratory system that was followed during growth and development. The changes of cytochrome c content did not parallel the changes in succinate oxidase activity, so there are apparently important variations in other components of the alternate respiratory system during mitochondrial development.

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