EVIDENCE FOR CYTOPLASMIC EXCHANGE IN MATINGS OF SCHIZOPHYLLUM COMMUNE

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ABSTRACT

EVIDENCE FOR CYTOPLASMIC EXCHANGE IN MATINGS OF *SCHIZOPHYLLUM COMMUNE*

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The objective of the present study was to determine if cytoplasmic exchange occurs in compatible and incompatible matings of the tetrapolar Basidiomycete *Schizophyllum commune*. Methods were developed to differentially label partners in a mating based on the selective uptake of cobalt by mitochondria. Mitochondria so labeled were distinguishable cytologically and ultrastructurally, and could be separated by isopycnic sucrose density gradient centrifugation. Matings were analyzed by three methods: 1) direct microscopic examination of individual anastomoses to see if cobalt-stained mitochondria were transferred from the strain grown in the presence of cobalt to the strain grown in the absence of cobalt, 2) microscopic analysis of mycelial plugs of a resident grown in the absence of cobalt to see if cobalt-stained donor mitochondria were present, 3) density gradient analysis of non-labeled residents to determine the presence of mitochondria which were more dense because of cobalt labeling. Mitochondrial transfer was detectable in fully compatible, common-A, and common-AB matings, but not in common-B matings. Possible inter-relationships between the observed phenomenon of mitochondrial transfer with the time of nuclear migration and mode of action of the incompatibility factors are discussed.

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By

Lidia Sicari Watrud

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DEDICATION

To Lee, for his love and understanding

To Buck, for being

To Mom and Dad, *con affetto*

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INTRODUCTION

The objectives of the present study were to determine if cytoplasmic transfer occurs in the various classes of matings of the tetrapolar Basidiomycete *Schizophyllum commune*, and to assess the role of the cytoplasm with respect to the time and mode of action of the genetic incompatibility factors. Evidence for nuclear transfer and migration can be confirmed morphologically by the development of clamp connections (Buller, 1931), or by the use of appropriately marked biochemical mutants and subsequent tests for complementation on minimal medium (Snider and Raper, 1958; Ellingboe, 1964). It generally has been assumed that transfer of cytoplasm is concommitant with nuclear transfer. However no direct test for this has been available.

Earlier studies of hyphal anastomosis (Sicari and Ellingboe, 1967), indicate: a) anastomoses occur in all classes of matings, compatible and incompatible; b) there is no immediate incompatibility reaction such as plug formation, protein precipitation or lysis; however within

2-8 hours, one of the hyphal tips involved in the anastomosis or a cell adjacent to the anastomosed cell may develop a lysed appearance; and c) the frequency of such lysed hyphal tips can generally be correlated with the degree of incompatibility of the mating; i.e., the lowest frequency is found in fully compatible matings. The suggestion was made that the action of the incompatibility factors was of an inducible rather than constitutive nature.

Modifier mutations of S. commune which do not directly affect the incompatibility factors, but disrupt the morphogenetic sequence associated with each, are believed to act via or in the cytoplasm (Raper, 1966). Hyphal tips isolated from doubly modified heterokaryons in which each mate carries a modifier mutation retain the ability to produce pseudoclamps, a property which neither parent has, upon hundreds of subsequent isolations (Raper and Raper, 1964). Much earlier (Harder, 1927), it was reported that subcultures of uninucleate subterminal cells from dikaryotic hyphae of S. commune isolated by micrurgical procedures had the ability to produce pseudoclamps, again suggesting the activation of normally suppressed morphogenetic sequences, or perhaps the activation of a self-replicating cytoplasmic factor (Raper, 1966). The behavior of unilateral maters has also been suggested to be related to the state of the cytoplasm (Papazian, 1956). Other instances of persistent cytoplasmic information can be found in the transfer of senescence factors in *Podospora anserina* (Jinks, 1964), in the spread of vegetative death in *Aspergillus glaucus* (Jinks, 1959), in the apparent "transformation" of wild type mitochondria to abnormal ones in *Neurospora crassa* (Diacumakos, et al., 1965), and most strikingly in the alga *Acetabularia*, where information for specific cap type formation persists for many days after enucleation of the young plant (Hammerling, 1953; Harris, 1968). Nucleo-cytoplasmic interactions have also been studied in animal and bacterial systems, where the analogues to fungal heterokaryons would be cultures of somatic cell hybrids (Harris and Watkins, 1965), and heterogenotes (Morse, et al., 1956). Thus it can be seen that the role of the cytoplasm in cellular control mechanisms and morphogenesis is of basic biological significance.

The immediate objective of the present study was to determine if cytoplasmic transfer occurs following hyphal fusion in the various classes of matings. A specific organelle of the cytoplasm, the mitochondrion, was chosen to be monitored, since it had been reported that cobalt uptake resulted in its *in vivo* staining (Lindegren and BeMiller, 1969). Further development of the technique to differentially label mates in a diffusion-controlled system could then be used to visually determine, by direct phase contrast examination, if mitochondria were transferred at individual sites of anastomosis. In addition, if it could be shown that such vitally stained mitochondria differed in density from non-stained mitochondria, then analyses based on the detection of donor density-labeled mitochondria in non-labeled residents could be used. Information on whether or not transfer occurs in the various classes of matings would help to assess the role of the cytoplasm, and perhaps that of one of its components, on the times and mode of action of the incompatibility factors.

LITERATURE REVIEW

The wood rotting Basidiomycete *Schizophyllum commune*, variously placed in the Agaricales and Polyporales, is characterized morphologically by a fan shaped basidiocarp having split gills on the underside (Buller, 1941; Raper, 1953, 1966). Haploid basidiospores germinate to produce elongate cylindrical hyphae having simple septa perforated by circular openings, and/or dolipore septa characterized by flange shaped domes which project into the cytoplasm of adjoining cells (Moore and McAlear, 1962; Giesy and Day, 1965).

The term tetrapolar Basidiomycete applied to *S. commune* follows from the independent segregation of the *A* and *B* factors at meiosis, which results in production of basidiospores of four different mating types (Kniep, 1918, 1920). Thus if we term the mating type factors in one strain *A1B1* and those in its fully compatible mate *A2B2*, we would expect to find, following completion of the sexual cycle and basidiospore production, the two parental types *A1B1* and *A2B2*, and the recombinant types *A1B2* and

A2B1 in 1:1:1:1 frequencies, if there were no linkage between the A and B factors.

The total number of different A factors is estimated at 450, the total number of different B factors at 93 (Raper and Raper, 1968). The A factor is made up of two linked subunits, perhaps representing cistrons, termed the alpha and beta (Raper et al., 1958; Raper et al., 1960). The B factor likewise is made up of two and possibly three such subunits (Koltin et al., 1967). A difference at only one of the subunits is sufficient to confer compatibility with regard to a single mating type factor.

If hyphae which have germinated from A1B1 and A2B2 basidiospores come into contact and anastomose, effecting a fully compatible mating, we would expect reciprocal exchange and migration of nuclei to occur, followed by the association of nuclei in pairs. Conjugate, synchronized nuclear divisions of the two paired, unfused nuclei occurs in the dikaryon (Kniep, 1918, 1920; Bensaude, 1918). The dikaryon, with its buckle-like clamp connections at the septa, is the form one is most likely to encounter in nature. During fructification two nuclei in a dikaryotic tip cell fuse to form a short-lived diploid nucleus which migrates into the base of the developing basidium. Subsequent meiosis produces four haploid nuclei which migrate into the developing basidiospores. Thus the sexual cycle is completed.

One may speak also of common-A, common-B and common-AB matings in which, respectively, the mates share in common the A factor, i.e., A1B1 x A1B2; the B factor, A1B1 x A2B1; and both factors, A1B1 x A1B1. In *S. commune* these different types of matings can be distinguished morphologically (Papazian, 1950a, 1950b). The common-A mating results in the formation of a heterokaryon characterized by a "Flat," or appressed appearance; the common-B interaction by a region of low hyphal density between the mates (Barrage phenomenon), and by the presence of pseudoclamps, i.e., clamps that have failed to fuse with the adjacent cell; and the common-AB interaction in growth essentially like that of the unmated homokaryons.

The phenomenon of nuclear migration (Buller, 1931) can be quantitatively estimated and shown to be greater than the rate of intrusive hyphal growth by the use of appropriate morphological mutants, or by tests for complementation between auxotrophic mutants (Snider, 1963; Snider and Raper, 1958; Ellingboe, 1964). Nuclear migration is generally associated with fully compatible and common-A matings; it is limited in common-B and common-AB interactions. Thus control of nuclear migration is inferred to reside in paired unlike B factors. Control of the subsequent process leading to formation of clamp connections requires the presence of paired unlike A factors (Raper, 1966).

Mutations at the incompatibility factor loci lead to loss of discrimination normally due to those factors (Parag, 1962; Raper and Raper, 1964; Raper, et al., 1965). Thus a strain with a mutated A mimics a common-B heterokaryon; one with a mutated B mimics a common-A heterokaryon; and an A mut B mut homokaryon mimics a dikaryon. Modifier mutations which do not affect the incompatibility factors directly or their primary regulatory functions, affect all stages subsequent to nuclear migration (Raper and Raper, 1966, 1968). Such mutations are not normally expressed phenotypically in the homokaryon and are believed to be involved in sequential stages of dikaryosis, following their release or induction by paired or mutated incompatibility factors (Raper, 1966). They are viewed as mutations of structural genes that are under the direct or indirect control of the incompatibility factors. A dominant gene, dik$^+$, has been identified (Koltin and Raper, 1968) that is required for stable and persistent dikaryosis; the presence of dik$^+$ is required in one or both partners; in the presence of dik the nuclei fuse to form stable diploid nuclei. Normally, the diploid phase is restricted to a single nuclear generation in the basidia.

Various models have been proposed to explain compatibility in the tetrapolar Basidiomycetes, among them differences in charge or complementation between the mating type factors. The former model has been rejected since the number of incompatibility factor alleles is greatly in excess of two, the latter also since alleles at each locus are numerous and functionally equivalent (Prévost, 1962). Two models presented as alternatives by Prévost would require anti-repressor substances to release suppression of metabolic processes; in one case these would be induced, in the other, be constitutive.

In a complementary mode of action to explain sterility in Angiosperms, growth of the pollen tube can be attributed to recognition of unlike S alleles to produce a substance required for pollen tube growth. In an oppositional model interaction of like S alleles would inhibit growth of the pollen tube (Lewis, 1954). Each of these modes, complementary and oppositional, is rejected for Schizophyllum. The complementary model is rejected since disomic A and disomic B strains are completely compatible with normal homokaryons carrying a factor in common with the disomic. The oppositional model is rejected, since individual alleles at each locus are known to be distinct but equivalent in function and no overlapping specificities occur (Raper, 1966).

Serological evidence has been presented for specific pollen, style, and pollen-style proteins (Lewis, 1960, 1964). A unique type of RNA is found in styles pollinated with incompatible pollen; its production is thought to be induced, and its function to code for inhibition of growth of the pollen tube. A dimer made up of two polypeptide chains, alpha and beta, is proposed to be coded for by S and Z genes, specific dimers then resulting from the interaction of specific S and Z alleles (Lewis, 1964). In Schizophyllum, differences between protein spectra in acrylamide gels and serological differences between homokaryons and dikaryons have been reported (Dick, 1965; Wang and Raper, 1970; Esser and Raper, 1961); however, these are not interpreted to be the primary products of the incompatibility factors. With regard to postulated control mechanisms in bacteria (Monod and Jacob, 1961), a model has been proposed (Dick, 1965; Raper, 1966), in which the _A_ and _B_ factors are considered "master switches," with the subunits of each, alpha and beta, considered as dual regulator genes. It has been suggested that the products of such subunits may be protein dimers which act to suppress the dikaryotic sequence. In compatible matings, the combination of two such dimers would produce a tetramer ineffective as a repressor, allowing the dikaryotic morphogenetic sequence to proceed.

Cell fusion appears to be indiscriminate in _S. commune_ and _Coprinus lagopus_ (Sicari and Ellingboe, 1967), and is not accompanied by an immediate incompatibility reaction. In _Neurospora crassa_, however, there is superimposed upon the bipolar mating system a cytoplasmic compatibility controlled by the C, D, and E loci (Wilson and Garnjobst, 1966). Fusion of cells having only one gene difference, e.g., CDE x CDe, results in rapid death of the fusion cell. Artificial mixing of cytoplasms by micrurgical techniques, although admittedly drastic, also results in plug formation in septal pores (Wilson, 1961; Wilson, et al., 1961). These results suggest a constitutive nature to cytoplasmic incompatibility. By contrast, in cultures of interspecific somatic hybrids of various vertebrates, there appear to be no intracellular mechanisms for recognition and destruction of tissue, as one might normally expect in tissue grafts (Harris, 1968). Cultures of heterokaryotic vertebrate cells produced with a killed virus pretreatment can transcribe from both genomes, and may even form common mitotic spindles (Harris and Watkins, 1965). In the slime mold *Physarum*, in which four loci controlling fusion have been identified, strains which do not have identical alleles at all four loci will not fuse, but only collide (Collins and Haskins, 1972). Extreme incompatibility reactions may be seen in the protozoan *Paramecium*, where extended conjugation occurs between kappa-containing and sensitive non-kappa-containing mates (Sonneborn, 1943, 1947). In higher animals we see manifestations of incompatibility in clumping or lytic reactions of red blood cells, based on the production of specific antibodies to specific antigens present on the membrane of the erythrocytes (Race and Sanger, 1968).

Thus it becomes apparent that whether incompatibility is expressed immediately or its expression is delayed, the roles of both nucleus and cytoplasm should be considered. In the present study an organelle of the cytoplasm, the mitochondrion, was chosen for "tagging" to determine if cytoplasm was transferred following anastomosis. A brief review of mitochondrial structure and related functions will follow.

The term mitochondrion, coined by Benda, refers to the thread-like and grain-like appearances these organelles can assume (in Lehninger, 1964). Composed primarily of phospholipid and protein, mitochondria house the enzymes of the Krebs cycle for cellular aerobic respiration of pyruvate to CO_2 and H_2O, with the concommitant production of energy in the form of ATP by interactions with the electron transport system, and also enzymes for the oxidation of fatty acids (Lehninger, 1964). Mitochondria vary greatly in size, principally in the dimension of the long axis, and occasional reports have been made of granular forms coalescing to form the filamentous form and vice-versa (Chèvremont, 1963). The number of mitochondria per cell may be numerous, or just one, as has been reported for the water mold _Blastocladiella emersonii_, in which a single large mitochondrion is found in close proximity to the flagellum of the motile spore (Cantino, et al., 1963). Histochemically, mitochondria are distinguished by their affinity for Janus Green B, a dye which can serve as an oxidation-reduction indicator. Biochemically, cytochrome oxidase and succinic dehydrogenase activity can be used as criteria for mitochondrial activity. On the ultrastructural level, they are distinguished by double unit measures (Robertson, 1958), the inner one possessing characteristic projections called the cristae (Palade, 1952b). The unit membrane (Davson and Danielli, 1952) is conceived as a protein lipid bilayer sandwich. Protein is on the outside, and polar groups of the fatty acids are oriented toward the protein coats; the hydrocarbon chains are oriented inwards. The mitochondrial membrane, especially the inner membrane, is thought to have repeating tripartite units having a base, stalk and knob-like head (Fernández-Morán, et al., 1964). There may be a functional division of labor between these sections; i.e., with respect to oxidation and phosphorylation mechanisms, energy transfer, and movement of ions. Exact mechanisms and locations for these vital processes are far from settled. Some workers suggest that reactions of the electron transport system occur in the basepiece (Green and Young, 1971), and that ATP synthesis occurs in the head piece (Kagawa and Racker, 1966), the stalk serving as a link between the two systems (MacLennan and Asai, 1968). Postulated conformational changes of the tripartite repeating units may be related to trapping of free energy released by the electron transfer process (Green and Young, 1971). Conformational energy would then drive ATP synthesis or active transport. Some ultrastructural support for this model is visualized in the energized, non-energized and energy-twisted forms of mitochondria (Green, et al., 1968). Mitochondria have been demonstrated to exhibit changes of volume (Lehninger, 1964); this has been related to uptake of certain anions, especially phosphate. A contractile protein in the mitochondrial membrane exhibits characteristics quite similar to myosin, the contractile protein of muscle tissue (King, et al., 1965). The concept of a "proton pump" has been advanced, in which two protons would traverse the mitochondrial membrane for two electrons going the opposite way; this method of uptake would be directly related to the electron transport system (Mitchell and Moyle, 1965).

It has been shown that mitochondria accumulate Ca^{++}, Mg^{++}, and Mn^{++} by active transport mechanisms, and that an anion requirement for this exists (Lehninger, 1964; Chance, 1967). During _in vitro_ Ca^{++} uptake in the presence of phosphate, it is believed that an insoluble phosphate, Ca-hydroxyapatite, precipitates in the intra-mitochondrial space (in Lehninger, 1964). Mitochondria so loaded with Ca^{++} show an increased density when purified by sucrose density gradient centrifugation (Lehninger, 1964). Differences in the bouyant densities of mitochondria isolated from a choline mutant of Neurospora crassa grown for different periods of time in choline have also been reported (Luck, 1965).

It has been reported that a slow in vivo uptake of Co^{++} for 72 hours by Saccharomyces from a growth medium containing $CoCl_2 \cdot 6H_2O$ results in a visible darkening of the mitochondria as viewed by phase contrast microscopy (Lindegren and BeMiller, 1969).

Cobalt, atomic number 27, atomic weight 58.94, is a first transition series element, as are its neighbors iron and nickel, and differs in the number of electrons in the 3 d shell (Hodgman, et al., 1961). Solutions of cobalt chloride hexahydrate are pink at room temperature, but become blue when treated with concentrated sulfuric acid or dehydrated with heat, as when it is used for invisible ink (Piatnitski, 1969; Hodgman, et al., 1961). Changes of color by cobalt solutions may be due to solvation or dehydration of the cobalt ions, the state of coordination of cobalt atoms, the presence of complexes, or simply electron transfers between quantum levels (Young, 1960).

The tolerance for cobalt by different organisms varies widely, and may vary between parts of the same organism. Thus the concentration of cobalt in root nodules of legumes may greatly exceed concentrations in leaves (Bertrand and DeWolf, 1955). Cobalt concentrations in stigma, style and pollen may greatly exceed those found in petals (Yamada, 1958). Microorganisms generally appear to have a greater tolerance for cobalt than do higher plants. Actinomycetes, Saccharomyces, Aspergillus, barley, and wheat may tolerate, respectively, 10,000, 750, 1500, 29.5, and 7 ppm (Young, 1960; Perlman and O'Brien, 1954). It is interesting to note that much of the cobalt appears to be chemically combined, not merely adsorbed to protein, and that the protein content of organisms grown in the presence of cobalt may increase (Young, 1960; Ballentine and Stephens, 1951).

Cobalt has been reported to stimulate certain enzymes, e.g., pyruvate decarboxylase, malate dehydrogenase, peptidases, and to inhibit esterase activity (Dixon and

Webb, 1964). It forms an essential part of vitamin B_{12} (cobalmin), and carbamide enzymes; it is chelated in vitamin B_{12} by a porphyrin system, much as is iron in hemoproteins and magnesium in cholorophyll (Dwyer and Mellor, 1964).

MATERIALS AND METHODS

Maintenance of Cultures

All strains of *Schizophyllum commune* were highly isogenic except for mating type (Ellingboe and Raper, 1962). Cultures were maintained on 2% agar Migration Complete medium (Snider and Raper, 1958), or on Migration Complete medium (MC) modified by the addition of disodium ethylene diamine tetraacetic acid (EDTA) and cobalt chloride hexahydrate in equimolar quantities, prior to addition of other components of the medium. At high concentrations of cobalt, it was necessary to increase the agar to 3.5%. In matings made on Y-plates as described below, the concentrations of EDTA and $CoCl_2 \cdot 6H_2O$ used were 0.39 and 0.25 g/liter, respectively. This medium will henceforth be referred to as Co-250 ppm. In the analysis of matings based on the detection of density differences of mitochondria, the concentrations of EDTA and cobalt chloride were 1.56 and 1.0 g/liter, respectively. This medium will henceforth be referred to as Co-1000 ppm.

Determination of Mitochondrial Transfer at Points of Anastomosis

One well of a Petri dish containing three compartments separated by plastic divisions (Falcon Plastics, Y-plate), was filled with approximately six ml of Co-250 ppm medium, the remaining two with MC medium. The volume was such as to ensure that the plastic dividing walls were plainly visible above the levels of the agar media. Strips of dialysis membrane 5 cm x 2.5 cm, previously sterilized in distilled water, were freed of obvious moisture by blotting with sterile filter paper and transferred with sterile forceps to the Y-plate such that each of two strips straddled the divisions between Co-250 ppm and MC media. Inocula consisted of mycelia grown on Co-250 ppm or MC agar media for 72-96 hours. Blocks of agar (approximately 4 cm x 0.5 cm) containing mycelia of the appropriate mating type were positioned parallel to each other and to the division between them. Each inoculum block was approximately 1.5 cm distant from the appropriate divider. The supporting medium in the well of the Petri plate corresponded to the medium used for the inoculum block. Thus two sets of matings of strains maintained on Co-250 ppm medium x strains maintained on MC medium could be done per Y-plate (Figure 1a). After 48 hours incubation at 32 C, the hyphae had generally grown out far enough from the inocula to establish anastomoses at points of contact in the vicinity of the plastic divider. This central area of the dialysis membrane, not in direct contact with either supporting medium, was cut out, mounted in water and examined by phase contrast microscopy (Zeiss Standard WL), using a green filter. A scan at low power was performed to find points of anastomosis, followed by examination at 800 X, to determine if dark staining cobalt mitochondria could be seen on only one or both sides of the anastomosis.

Determination of Long-Distance Transfer of Mitochondria

A resident mycelium (Snider and Raper, 1958) was prepared by pour-plating molten MC agar with two ml of hyphal fragments in suspension. The latter was prepared by macerating a colony approximately 5 cm in diameter with 50 ml of MC broth for 30 seconds in a Waring Blendor. The resident was incubated 72 hours at 32 C prior to application of a cobalt donor mycelium. A modification of the "racetrack" technique (Ellingboe, 1964) was used, in which transfer and migration are allowed to proceed essentially unidirectionally by cutting out all agar of the resident plate except for a narrow 3 cm strip down the center of the plate (Figure 1b). Donor mycelium was applied without any adhering cobalt agar. This was accomplished by growing macerated hyphal fragments on sterile squares of dialysis membrane on plates of Co-250 ppm medium. A narrow strip (3 cm x 1 cm) of dialysis membrane with mycelium growing on it could then be cut with a scalpel, peeled off with forceps, and be applied mycelial side down at one end of the resident mycelium. After 48 hours at 32 C, small plugs 2 mm in diameter (Figure 1b) were cut out from the resident with a metal tube. The mycelium in the upper part of the plug was examined by phase contrast microscopy to see if any cobalt-stained mitochondria were present.

Isolation of Mitochondria

Methods used for the isolation and purification of mitochondria were similar to those employed for

Neurospora crassa (Luck, 1963a, b; Kuntzel and Noll, 1967), and differed primarily in methods of growing and harvesting mycelium. Cultures were grown on squares of dialysis membrane on either MC or Co-1000 ppm media. Inoculum consisted of approximately six ml of a suspension hyphal fragments prepared by macerating a 5 cm diameter colony on agar medium with 50 ml of the appropriate broth for 30 seconds. The plates were incubated at 32 C for 72-96 hours prior to harvesting mycelium. Dialysis membranes with adherent mycelia from a total of six plates were peeled off and macerated 20 seconds in cold 0.25 M sucrose containing 0.01 M Tris-HCl and 0.001 M EDTA, buffered at pH 7.0, in prechilled Waring Blendor cups. The homogenate was centrifuged at 4 C at 3,000 x g for 20 minutes to pellet nuclei, cell walls, and fragments of dialysis membrane. The supernatant was filtered through 400 mesh nylon prior to a second centrifugation at 4 C for 20 minutes at 10,000 x g to obtain the crude mitochondrial pellet. The pellet so obtained was resuspended in 1.0 ml of 0.25 M sucrose buffer described above, using a glass rod in a test tube or several strokes in a Toenbroeck tissue homogenizer. It was then carefully pipetted onto a previously chilled linear sucrose gradient ranging in molarity from 0.9 M to 2.0 M, buffered at pH 7.0 (0.001 M Tris-HCl), and containing EDTA (0.001 M). The gradient was prepared according to the method of Britten and Roberts (1960). One ml of sample was layered on top of the gradient and centrifuged at 35,000 rpm (SW 50L rotor in Beckman L2-65 B Ultracentrifuge) at 4 C for 4-5 hours. The gradient was pumped through a Uvicord II flowcell to obtain the profile of absorbancy at 254 nm. Fractions were collected with an LKB Ultrorac 7000. Five drop fractions were collected starting from the top of the tube by pumping a 2.0 M sucrose solution containing neutral red dye through a bottom puncture of the tube at constant rate. Addition of the dye served to indicate the beginning and end of the sample. Fractions so collected could then be tested for enzymatic activity.

Succinic Dehydrogenase Assay

A histochemical method (Gomori, 1957) was modified for routine and rapid estimation of localization of activity in the sucrose gradients following centrifugation and banding of mitochondria. The reaction mixture consisted of 0.25 M sodium succinate, 0.1 M phosphate buffer pH 7.0, 0.1% w/v nitro blue tetrazolium chloride, and 0.1 M NaCN in a ratio of 3:3:3:1. Three-tenths ml was added to 0.1 ml samples of each fraction collected and the mixture was incubated at 32 C for one hour. One ml of water was added to each sample and the absorbancy at 620 nm was determined with a Beckman DU spectrophotometer. A profile of succinic dehydrogenase activity was thus obtained for each sucrose gradient. The control mixture contained the same components minus the succinate substrate.

Cytochrome Oxidase Assay

Cytochrome oxidase activity was monitored by following the rate of decrease in absorbancy at 550 nm of reduced cytochrome c (Nielsen and Lehninger, 1955; Smith, 1955). The cytochrome c had been reduced with sodium borohydride and dialyzed overnight at 4 C against 0.1 M phosphate buffer (pH 7.4) containing EDTA, to remove the excess reducing agent (Martin, et al., 1957). This assay is based on the oxidation of iron in the ferrous state to the ferric state, thus one had to be certain that a significant portion of the iron in the cytochrome c was indeed in the reduced state prior to running the assay. The cytochrome c reduced as above was checked for its absorbancy at 550 nm and 565 nm. If the ratio was greater than six, then the cytochrome was considered to be in a form suitable for the assay (Smith, 1955). Change in absorbancy at 550 nm with time following addition of the mitochondrial suspension was then monitored for each fraction to determine which one had the greatest activity. The reaction mixture in a 3.0 ml cuvette consisted of 2.4 ml of phosphate buffer (0.1 M, pH 7.4), 0.5 ml of 2.0×10^{-4}M reduced cytochrome c, and at zero time, 0.1 ml of the fraction being tested. The change in absorbancy at 550 nm was monitored with a Beckman DU spectrophotometer connected to a recorder with a chart speed of one inch per minute. The reaction was followed 2-3 minutes at room temperature, and the oxidation of ferrocytochrome c brought to completion by the addition of 0.04 ml of saturated potassium ferricyanide. The rate of reaction could be estimated by determining the rate of change of absorbancy at 550 nm with time (i.e., the slope). The reaction is considered to be first order for the time interval studied (Smith, 1955).

Electron Microscopy

Inocula were grown on squares of dialysis membrane on MC or Co-1000 ppm medium for 96 hours. Small pieces of dialysis membrane covered with mycelium were prefixed overnight at room temperature in 2.5% glutaraldehyde (Sabatini, et al., 1963), and rinsed three times with 0.1 M phosphate buffer pH 7.4 at 20 minute intervals prior to fixation in buffered (pH 7.4, 0.1 M PO_4) 2% OsO_4 for 1-1/2 hours (Palade, 1952a). Dehydration was in ethanol (25, 50, 70, 95%, 10 minutes each; absolute ethanol, two 15 minute rinses). The samples were then placed in two changes of propylene oxide for 30 minutes each, followed by infiltration with a 1:1 mixture of Epon 812 (7A:3B) and propylene oxide. Final embedding was in Epon complete mix (Luft, 1961), in a 00 gelatin capsule at 60 C. The embedded samples were sectioned on a Blum Ultramicrotome with a diamond knife. Sections were floated onto 400 mesh copper grids and stained 30 minutes with a saturated solution of uranyl acetate. This was followed by a water rinse, and a 5 minute staining in saturated lead citrate; a rinse in 0.02 M NaOH preceded a final water rinse prior to viewing in a Philips 300 electron microscope. All electron micrographs were made at 32,000 X magnification.

Mitochondria rich fractions were pooled, prefixed in 2-1/2% glutaraldehyde, and pelleted by centrifugation at 10,000 x g. Three rinses with phosphate buffer as described above preceded fixation in OsO_4; subsequent steps were as described for mycelia above.

Fixation of mitochondrial fractions with 2% aqueous $KMnO_4$ was preceded by an overnight pre-fixation in glutaraldehyde, pelleting at 10,000 x g, and phosphate buffer rinses as above. Fixation was in 2% $KMnO_4$ for 24 hours at room temperature followed by several rinses in phosphate buffer and dehydration in ethanol. Subsequent steps for embedding and sectioning were as described in the preceding section with the elimination of uranyl acetate and lead citrate stains.

Extraction of Mitochondria from Matings

The MC resident mycelium was prepared by pouring a suspension of hyphal fragments onto sterile squares of dialysis membrane on MC plates. The suspension was prepared by macerating a colony 5 cm in diameter with 50 ml of MC broth for 30 seconds in a Waring Blendor. Approximately 6 ml were poured on each plate. The plates were incubated 72-96 hours at 32 C. The cobalt-labeled donor to be used for the mating was similarly prepared using Co-1000 ppm broth and plates. Strips of dialysis membrane (2.5 x 8 cm), containing cobalt-labeled donor mycelium, free of supporting medium, were cut, removed from the Co-1000 ppm medium with forceps, and applied mycelial side down onto the MC resident. Two such strips were placed on a plate for a mating (Figure 1c). After incubation for 24 hours at 32 C, the entire area covered by the cobalt-labeled implant, as well as the implant, was discarded. Mycelium on the dialysis membrane which previously surrounded the implants was then extracted as described above for isolation and purification of mitochondria by isopycnic sucrose density gradient centrifugation. Analysis of absorbancy profiles at 254 nm, along with profiles of succinic dehyrogenase activity was then made to determine if denser cobalt-labeled mitochondria had been transferred in the various classes of matings. A minimum of four replicate experiments was performed for each class of matings. This included the use of at least two different combinations of strains and/or the alternation of the mate which was labeled with cobalt.

Fig. 1.--Methods of differential labeling in matings and determination of tolerance for cobalt.

1a. Triplate mating. Dialysis membrane strips are straddled between the MC and Co-250 ppm wells. Dialysis membrane in the region of the plastic divider was later cut out for microscopic examination of individual anastomoses.

1b. Racetrack method for determination of long distance transfer of mitochondria. Donor (d) placed at one end of resident (r) prior to removal of plugs for microscopic examination 48 hours later.

1c. Agar-free cobalt donor implants (d) on MC resident (r) plates. Both donor and resident mycelium are contained on dialysis membranes. Mitochondria from resident areas surrounding the donor implants were extracted and purified; donor and resident immediately below it were discarded.

1d. Tests for mating type on Co-1000 ppm medium. Clockwise, C: fully compatible mating, ring of fruit body initials is visible. B: common-B mating; Barrage area between the mates is evident. A: common-A mating demonstrates "Flat," appressed mycelium. AB: common-AB mating; mates remain essentially as homokaryons.

1e. Tolerance test for cobalt; no inhibition apparent to 900 ppm Co^{++}.

1f. Tolerance test for cobalt; complete inhibition at 2500 ppm Co^{++}.

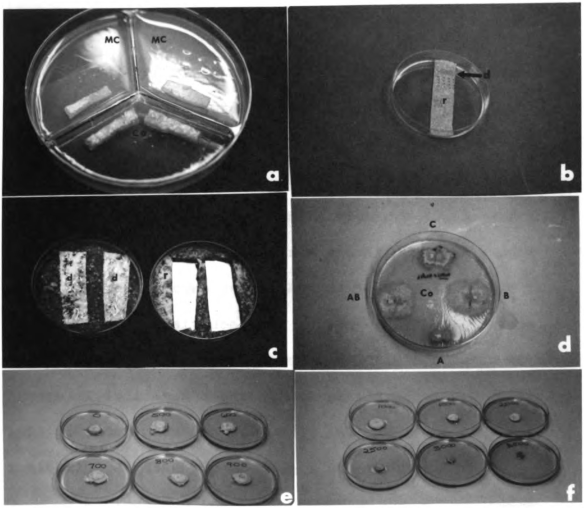

Figure 1

RESULTS

Determination of Tolerance for Cobalt

Plugs of mycelium grown on MC medium were cut out with a sterile cork borer at equal distances from the initial inoculum on plates which had been previously incubated for 96 hours at 32 C. The plugs were then transferred to Petri plates containing MC broth supplemented with cobalt chloride hexahydrate and disodium EDTA in a range of equimolar amounts. As can be seen in Figure 1e, up to 900 ppm cobalt does not appear to inhibit growth; growth was possibly somewhat enhanced. Examination of Figure 1f indicates a slight inhibition of growth at 1000 ppm, definite inhibition at 1500 ppm, and complete inhibition at 2500 ppm of cobalt added.

Mating type tests performed on the cobalt media proceeded normally as could be determined by gross macroscopic as well as microscopic morphology (Figure 1d). In the fully compatible matings, both partners formed clamp connections, and normal looking fruiting bodies were formed. Common-A matings showed the characteristic "Flat" morphology. Common-B matings developed the expected "Barrage" reaction. The common-AB interaction also appeared to be as normal; i.e., the homokaryons retained the morphology of homokaryons.

Kinetics of Visual Uptake of Cobalt

Small plugs of mycelium grown on MC agar were placed in Co-250 ppm broth and samples of the mycelia were removed after 24, 48, and 72 hours at 32 C and examined by phase contrast microscopy. At zero time, hyphae from MC media have indistinct mitochondria and walls that are thick (Figure 2b). A histochemical stain for mitochondria based on cytochrome oxidase activity (Gomori, 1957) showed that mitochondria were numerous in the cells. However, the procedure results in an apparent swelling of both hyphae and mitochondria (Figure 2a). Mycelia 24 hours after transfer to Co-250 ppm broth had cell walls that were obviously darkened, but the mitochondria were normal in appearance (Figure 2c). By 48 hours, darkening was not particularly apparent in the walls, but a general darkening of the cytoplasm was visible (Figure 2e). After

72 hours in Co-250 ppm broth, mycelia had mitochondria that were darker and somewhat smaller in size (Figure 2g). Normal appearing mitochondria were sometimes also present. In Co-1000 ppm broth, the walls were also visibly darker by 24 hours (Figure 2d); by 48 hours a few mitochondria occasionally were seen that were darker (Figure 2f), and by 72 hours virtually all the mitochondria were darker and somewhat smaller (Figure 2h).

Checks on Cobalt Diffusion on Y-Plates

Cobalt-5000 ppm water-agar blocks were positioned as if for inoculum on dialysis membranes straddling the divisions between wells of cobalt and MC media in Y-plates. There was no visual or spectrophotometric (510 nm absorption peak for aqueous cobalt chloride) evidence of diffusion on the dialysis membrane in the vicinity of the divider after 48 hours incubation at 32 C. Some diffusion to the supporting medium below the cobalt block was evident visually however.

Fig. 2.--Staining of mitochondria; kinetics of visual uptake of cobalt.

2a. Histochemical test for cytochrome oxidase; mitochondria stained dark bluish-purple, some are quite swollen in appearance.

2b. Normal appearing hyphae and mitochondria grown on MC medium. Mitochondria are relatively indistinct; several seen near arrow along wall.

2c. Hyphae maintained in Co-250 ppm medium for 24 hours; the walls are darker than those of the controls.

2d. Hyphae maintained in Co-1000 ppm medium for 24 hours; the walls are much darker than those of the controls.

2e. Hyphae maintained in Co-250 ppm medium for 48 hours; neither the walls nor the mitochondria are particularly dark.

2f. Hyphae maintained in Co-1000 ppm medium for 48 hours; a few mitochondria may be somewhat darker.

2g. Hyphae maintained in Co-250 ppm medium for 72 hours; mitochondria are darker.

2h. Hyphae maintained in Co-1000 ppm medium for 72 hours; mitochondria appear darker and also smaller in size than those in controls.

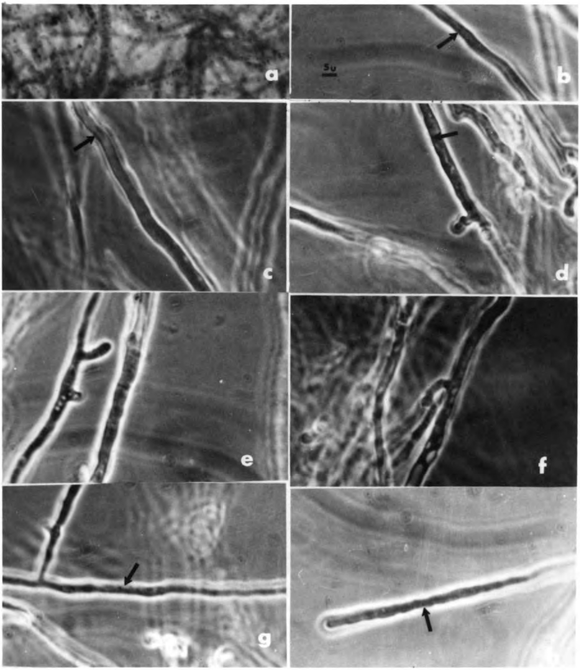

Figure 2

Direct Observations of Individual Anastomoses

All observations were made on anastomoses already established at the time of microscopic examination. Only those in which both partners could be clearly traced back to their respective origins on opposite sides of the slide were scored for transfer of mitochondria. If the darker (bluish) mitochondria were present on both sides of the anastomosis, it was scored as positive evidence for transfer. If the dark mitochondria were visible on only one side of the anastomosis, it was scored as having no transfer of mitochondria. All anastomoses examined in common-A (Table 1, Figure 3a), and common-AB (Table 1, Figure 3b) matings showed positive evidence for transfer for at least two and often to four cells beyond the point of anastomosis. It was difficult to trace hyphae involved further than that since the hyphal density became too great to trace single hyphae by microscopic examination. In common-B matings, on the other hand, there was no evidence for transfer in any of the more than 120 anastomoses observed (Figures 3c and 3d, Table 1). In fully compatible matings, either of haploid x haploid, or haploid x diploid, all anastomoses showed evidence for mitochondrial

Fig. 3.--Determination of transfer of mitochondria in individual anastomoses.

3a. Common-A mating; dark mitochondria are visible (arrows) on both sides of the anastomosis.

3b. Common-AB mating; dark mitochondria are visible in both mates.

3c. Common-B mating; dark mitochondria are visible in only one of the mates; nucleus (n) is visible near the anastomosis.

3d. Common-B mating; dark mitochondria are visible in only one mate; possible developing pseudoclamp (pc) visible; one of mates has branch with lysed appearance to cytoplasm (lt).

3e. Fully compatible mating; dark mitochondria are visible in each mate; dense cytoplasm is present in the anastomosis. A clamp connection is visible in one of the mates.

3f. Same anastomosis as in Figure 3e photographed two minutes later; a mass movement of cytoplasm out of area of anastomosis has occurred, leaving a clear zone in the region of the anastomosis.

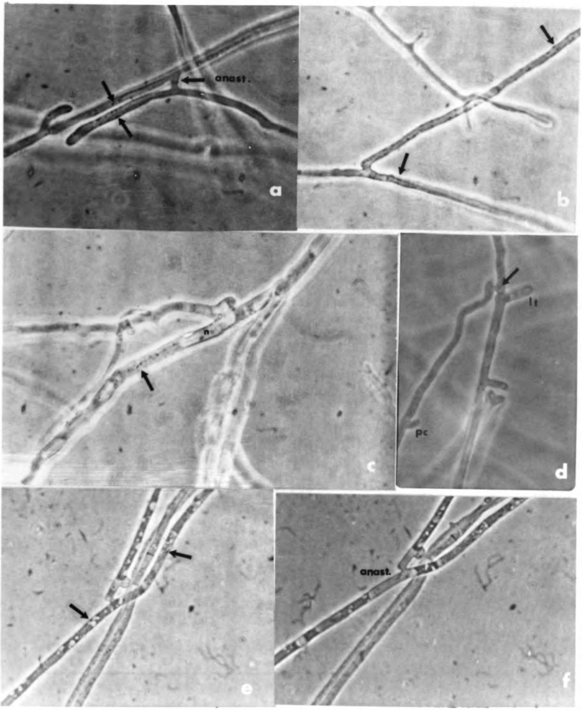

Figure 3

TABLE 1.--Relationship of class of mating to detection of cobalt-labeled mitochondria on both sides of the anastomosis.

Class of Mating	Mating Type Cobalt Strain	Mating Type Unlabeled Strain	Number of Anastomoses Scored	Number Having Dark Mitochondria on both sides of the Anastomosis
Fully Compatible n x n	A41 B41	A42 B42	42	42
	A42 B42	A41 B41	24	24
	A42 B41	A41 B42	29	29
	A41 B42	A42 B41	3	3
Class totals			98	98
Fully Compatible 2n x n	A41 B42 / A41 B42	A42 B41	60	60
	A41 B41 / A41 B41	A42 B42	95	95
Class totals			155	155
Common-A	A42 B42	A42 B41	22	22
	A42 B41	A42 B42	2	2
	A41 B41	A41 B42	17	17
Class totals			41	41
Common-B	A42 B42	A41 B42	19	0
	A41 B42	A42 B42	25	0
	A41 B41	A42 B41	74	0
	A42 B41	A41 B41	7	0
Class totals			125	0
Common-AB	A41 B41	A41 B41	22	22
	A42 B42	A42 B42	10	10
	A41 B42	A41 B42	23	23
	A42 B41	A42 B41	44	44
Class totals			99	99

transfer (Figures 3e and 3f, Table 1). That the anastomoses were indeed functional (i.e., nuclei had been transferred), was evidenced by the presence of clamp connections in one or both mates in fully compatible matings, and by the production of pseudoclamps in the growing hyphal tips of common-B matings. Thus in all classes of matings examined except the common-B, there was direct visual evidence for transfer of mitochondria via functional anastomoses.

Determination of Long-Distance Transfer

Resident racetracks (Figure 1b), and cobalt-labeled donor mycelia were prepared as described in the materials and methods and incubated 48 hours at 32 C. Plugs 2 mm in diameter were removed from the resident for microscopic examination to determine if the darker staining cobalt-labeled mitochondria could be detected when examined at high magnification (800 X) with the phase contrast microscope. Plugs from up to 1.2 cm from the cobalt-labeled donor were examined. The darker mitochondria were detected in plugs up to 6 mm from the cobalt-labeled donor. As in the direct observation of individual anastomoses, there was evidence for transfer in fully compatible, common-A, and common-AB matings, but none in the common-B matings (Table 2). No attempt was made to count the number or proportion of darker mitochondria in these samples, only the presence or absence of darkly stained mitochondria was scored.

TABLE 2.--Determination of long-distance transfer of mitochondria.

Class of Meeting	Mating Type Cobalt-labeled strain	Mating Type Unlabeled strain	Number Plugs Scored	Number Plugs Having Dark Mitochondria
Common-A	A41 B41	A41 B42	48	26
	A42 B41	A42 B42	96	46
Class totals			144	72
Common-B	A41 B42	A42 B42	32	0
	A41 B41	A42 B41	48	0
	A42 B41	A41 B41	16	0
Class totals			96	0
Common-AB	A41 B42	A41 B42	32	14
	A42 B42	A42 B42	48	26
Class totals			80	40
Fully compatible	A42 B41	A41 B42	32	24
	A41 B41	A42 B42	32	20
Class totals			64	44

Characterization of
Mitochondria from Mycelia
Grown on Co-1000 ppm and
MC Media

Mitochondria purified by isopycnic sucrose density gradient centrifugation banded at characteristic regions of the gradient after 5 hours at 35,000 rpm. Macroscopic observations of the mitochondrial bands indicate that mitochondria isolated from mycelium grown on Co-1000 ppm medium are denser, i.e., band is lower in the tube, than mitochondria isolated from mycelium grown on MC medium (Figure 4e). A size difference between MC and Co mitochondria is apparent in living cells, as examination of Figures 4a and 4b and Figures 2b and 2h indicates. Samples taken from the respective fractions of greatest absorbancy at 254 nm show a difference in size and color of isolated mitochondria (Figures 4c and 4d). The cobalt-labeled mitochondria banded in a lower position in the centrifuge tube (Figure 4e) as viewed macroscopically. The absorbancy at 254 nm for each centrifuge tube was obtained by passage of the gradient through a Uvicord II flowcell as described in the materials and methods. The maximum absorbance at 254 nm for purified mitochondria extracted from mycelium maintained on MC medium was in a

Fig. 4.--Characterization of mitochondria in living cells and *in vitro*.

4a. Hypha grown on Co-1000 ppm medium; mitochondria are darker and smaller than MC counterparts. Several are visible near the arrow tip.

4b. Hypha grown on MC medium; mitochondria are relatively indistinct. Several are visible near the wall in the region indicated by the arrow.

4c. Mitochondria extracted from mycelium grown on Co-1000 ppm medium and purified by isopycnic sucrose density gradient centrifugation; sample taken from fraction having maximal absorbancy at 254 mu (see Figure 6). Size and color of mitochondria comparable to those seen *in situ* in Figure 4a.

4d. Mitochondria extracted from mycelium grown on MC medium and purified by isopycnic sucrose density gradient centrifugation; sample taken from fraction having maximal absorbancy at 254 mu (see Figure 5). Mitochondria are not as dark as those labeled by cobalt.

4e. Comparison of banding patterns of non-labeled and cobalt-labeled mitochondria. Cobalt-labeled mitochondria sediment lower in tube.

4f. Mitochondria extracted from Common-*B* (Figure 9), and common-*AB* (Figure 11), matings; band of cobalt-dense mitochondria is evident in latter, as well as the band of unlabeled mitochondria.

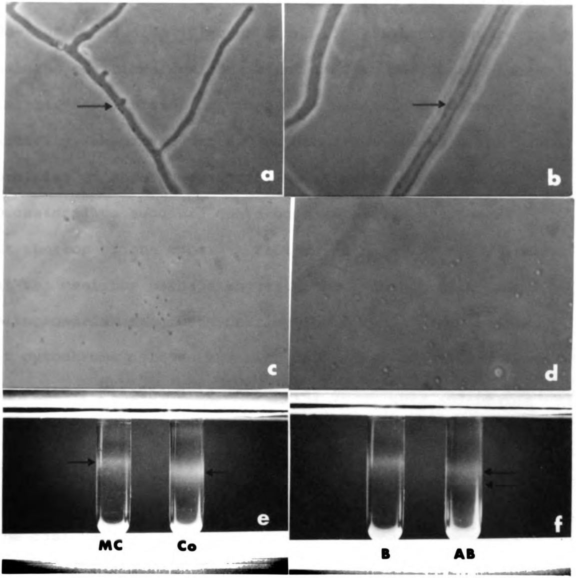

Figure 4

different position in the centrifuge tube than for mitochondria similarly extracted from mycelium maintained on Co-1000 ppm medium (Figures 5 and 6). This difference in banding positions was consistent in five replicate experiments. Analysis of fractions for succinic dehydrogenase activity (absorbancy at 620 nm) corresponded with the profiles of absorbancy at 254 nm (Figures 5 and 6). Occasionally, succinic dehydrogenase activity appeared at the top of the tube. A failure of material to sediment at the meniscus perhaps suggests the presence of broken mitochondrial fragments bearing the enzyme. The activity of cytochrome oxidase was also determined as another means to monitor mitochondria in density gradients. As is evident in Figure 7, the mitochondria grown on MC media show the greatest activity at fraction 8, which corresponds well with the maximum absorbancy at 254 nm (Figure 5). The cobalt-labeled mitochondria have maximal absorbancy at 254 nm and maximum cytochrome oxidase activity at fraction 10 (Figures 6, 7).

Fig. 5.--Absorbancy profiles at 254 nm and 620 nm for mitochondria extracted from mycelia grown on MC medium: Absorbance at 254 nm (——) represents a constant monitoring of effluent from the top of the gradient tube. Maximal absorbance at 254 nm for unlabeled purified mitochondria occurs in the region of fraction 8. Measurement of succinic dehydrogenase activity in aliquots from five drop fractions collected starting from the top of the tube was made by determining absorbancy at 620 nm (o----o) of reduced nitro blue tetrazolium chloride. The maximum activity occurs at fraction 8.

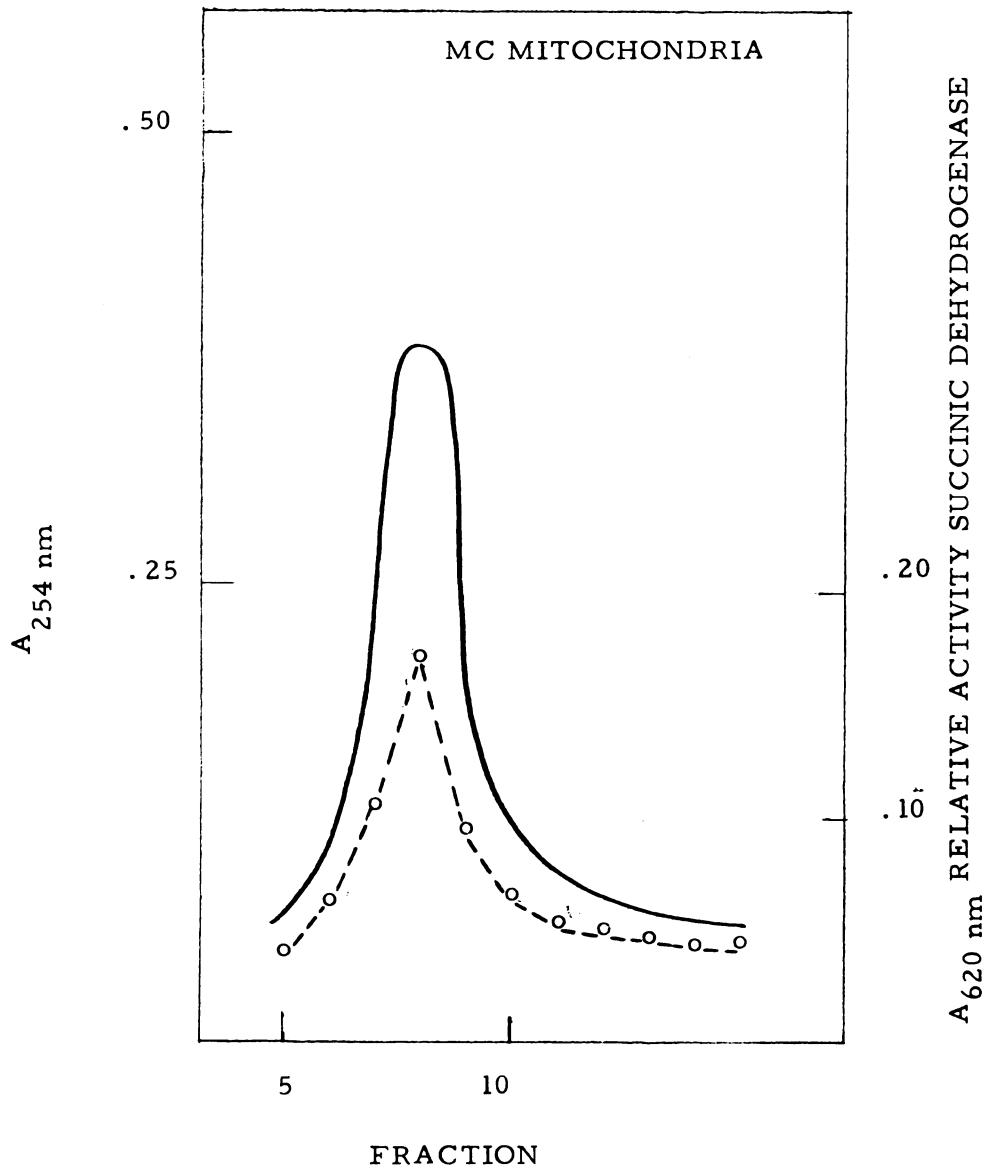
MC MITOCHONDRIA
.50
.25
$A_{254\ nm}$
.20
.10
$A_{620\ nm}$ RELATIVE ACTIVITY SUCCINIC DEHYDROGENASE
5
10
FRACTION

FIGURE 5

Fig. 6.--Absorbancy profiles at 254 nm and 620 nm for mitochondria extracted from mycelia grown on Co-1000 ppm medium: Absorbance at 254 nm (——) represents a constant monitoring of the effluent from the top of the tube. Maximal absorbance at 254 nm for cobalt-labeled mitochondria occurs in the region of fraction 10. Measurement of succinic dehydrogenase activity in aliquots of five drop fractions collected starting from the top of the tube was made by determining the absorbancy at 620 nm (o----o) of reduced nitro blue tetrazolium chloride. Maximal activity occurs at fraction 10.

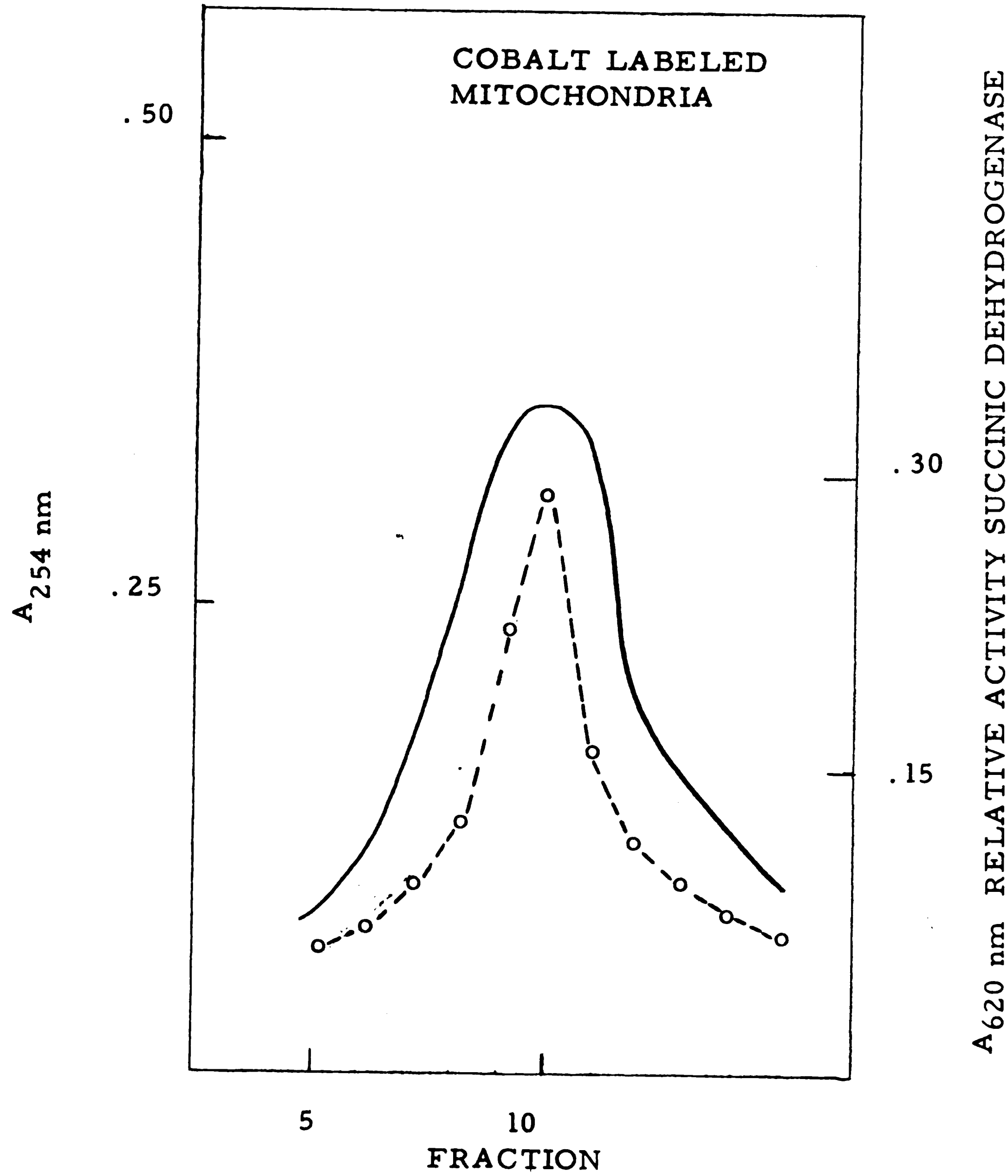
COBALT LABELED
MITOCHONDRIA
.50
.25
A_{254} nm
.30
.15
A_{620} nm RELATIVE ACTIVITY SUCCINIC DEHYDROGENASE
5
10
FRACTION

FIGURE 6

Fig. 7.--Cytochrome oxidase profiles for MC (o——o) and cobalt-labeled (+——+) mitochondria: Cytochrome oxidase activity in 0.1 ml aliquots of five drop fractions collected starting from the tops of the tubes was determined by monitoring the change in absorbancy at 550 nm/minute. Maximum activity for mitochondria extracted from mycelium grown on MC medium occurs at fraction 8. Maximum activity for mitochondria extracted from mycelium grown on Co-1000 ppm medium occurs at fraction 10.

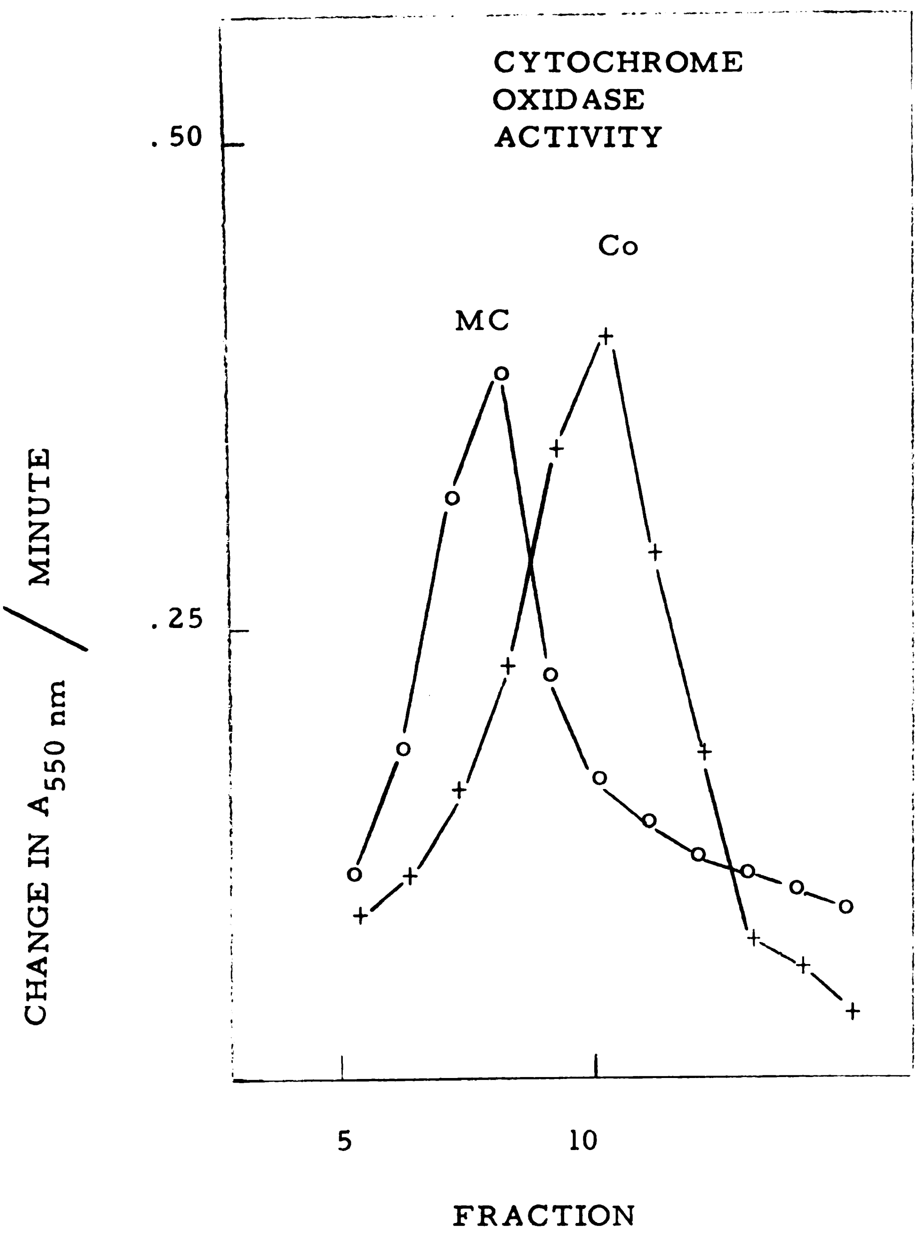
CYTOCHROME
OXIDASE
ACTIVITY
.50
.25
Co
MC
CHANGE IN A550 nm / MINUTE
5
10
FRACTION

FIGURE 7

Electron Microscopy
of Mitochondria

Examination of mitochondria *in situ* in cells grown on MC medium exhibit classical mitochondrial structure of double membranes, the inner one bearing numerous characteristic projections, the cristae (Figure 8a). These mitochondria occur in both filamentous and spherical configurations. In several sections, they were in close proximity to the nuclear membrane.

Examination of mitochondria *in situ* from mycelia grown on Co-1000 ppm medium for three days at 32 C revealed stark differences in size of the mitochondria; cristae were also reduced in size and number (Figure 8b) as compared to those of mitochondria in mycelium grown on MC medium.

Sections through fixed and pelleted (10,000 x g) bands of sucrose gradient-purified material confirmed that the main component of the bands were mitochondria (Figures 8c-8f). The mitochondria were swollen, which is probably indicative of hypotonicity of the extracting medium, and their reaction to dilution in glutaraldehyde and continued respiration in the absence of ATP (Lehninger, 1964). Significantly, even the mitochondria isolated from mycelium maintained on Co-1000 ppm medium exhibited

Fig. 8.--Ultrastructural characterization of mitochondria *in situ* and *in vitro*.

8a. A hypha from mycelium grown on MC medium. A thick wall (w) is evident; cristae (c) of mitochondria (m), are readily visible. Some mitochondria appear to have close association with the nuclear membrane (nm) of the nucleus (n).

8b. A hypha grown on Co-1000 ppm medium for three days. Size of mitochondria appears to be reduced; cristae are reduced in size and number.

8c. Section through pellet of purified mitochondria extracted from mycelium grown on MC medium. Fixation was in $KMnO_4$. Non-energized, orthodox (o) and aggregated (ag), energized (en), and energy twisted (et) conformations (Green et al., 1968) are visible.

8d. Section through pellet of purified mitochondria extracted from mycelium grown on MC medium and fixed in $KMnO_4$ showing energy-twisted mitochondrion.

8e. Section through pellet of purified mitochondria extracted from mycelium maintained on Co-1000 ppm medium and fixed in $KMnO_4$. Orthodox and energy-twisted forms are visible.

8f. Section through pellet of purified mitochondria extracted from mycelium grown on Co-1000 ppm medium and fixed in $KMnO_4$. Non-energized and energy-twisted conformations are visible. Cristae are more readily visible than they were *in situ*.

Note: All electronmicrographs taken at 32,000 X

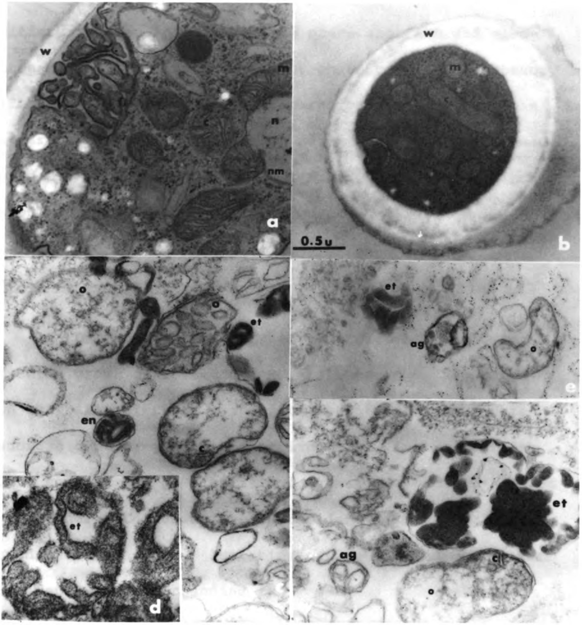

Figure 8

definite cristae. Perhaps the cristae in the mitochondria in the cells were merely contracted. The apparent size difference seen *in situ* by both phase contrast and electron microscopy, and *in vitro* by phase contrast microscopy (Figures 4a-4d) of unfixed preparations, is not quite as apparent in sections of mitochondria purified by sucrose gradient centrifugation and fixed and pelleted for electron microscopic processing (Figures 8c-8f).

Analysis of Matings

Once it was established that mitochondria from mycelia grown on cobalt and MC media could be separated on the basis of their respective densities by isopycnic sucrose density gradient centrifugation, it became feasible to test the possibility of transfer of cobalt-labeled mitochondria to the unlabeled resident mycelium by density gradient techniques. Isolation of dense mitochondria from resident areas surrounding the agar-free cobalt-labeled donor implants should provide additional evidence for transfer and migration of mitochondria. The time of incubation for these matings was 24 hours to minimize expected loss of the density label due to mitochondrial replication while on a non-cobalt medium. The first class of matings to be analyzed by this method was the common-B, since previous evidence from use of Y-plates and "racetracks" indicated a lack of transfer of mitochondria in common-B matings. The common-B mating should serve as the control for diffusion of cobalt. Thus if a lack of evidence for transfer of mitochondria could be demonstrated, it should be feasible to analyze fully compatible, common-A and common-AB matings by this method. The results of a common-B mating are presented in Figure 9. Maximal absorbancy at 254 nm and maximal succinic dehydrogenase activity (absorbancy at 620 nm), correspond to the position expected of mitochondria extracted from mycelia grown on MC medium. No evidence of denser cobalt-labeled mitochondria was found. One would have expected to detect cobalt-labeled mitochondria approximately two fractions later (i.e., fraction 10), if they were present in the mycelia surrounding the cobalt donor implants.

The results of a common-A mating are presented in Figure 10. A continual monitoring of the effluent starting from the top of the centrifuge tube at 254 nm indicates a bimodal distribution of mitochondria. The maximum at fraction 8 is interpreted to represent mitochondria

Fig. 9.--Absorbancy profiles at 254 nm and 620 nm for a common-B mating: Mitochondria were extracted from areas of the unlabeled resident mycelium (A41 B41) surrounding cobalt donor implants (A42 B41). Absorbance at 254 nm (——) represents a constant monitoring of the effluent from the top of gradient tube. A single absorbtion maximum is apparent in the region where one would expect unlabeled mitochondria to sediment (fraction 8). Measurement of succinic dehydrogenase activity in aliquots of five drop fractions collected starting from the top of the tube was made by determining absorbancy at 620 nm (o----o) of reduced nitro blue tetrazolium chloride. The maximum occurs at fraction 8.

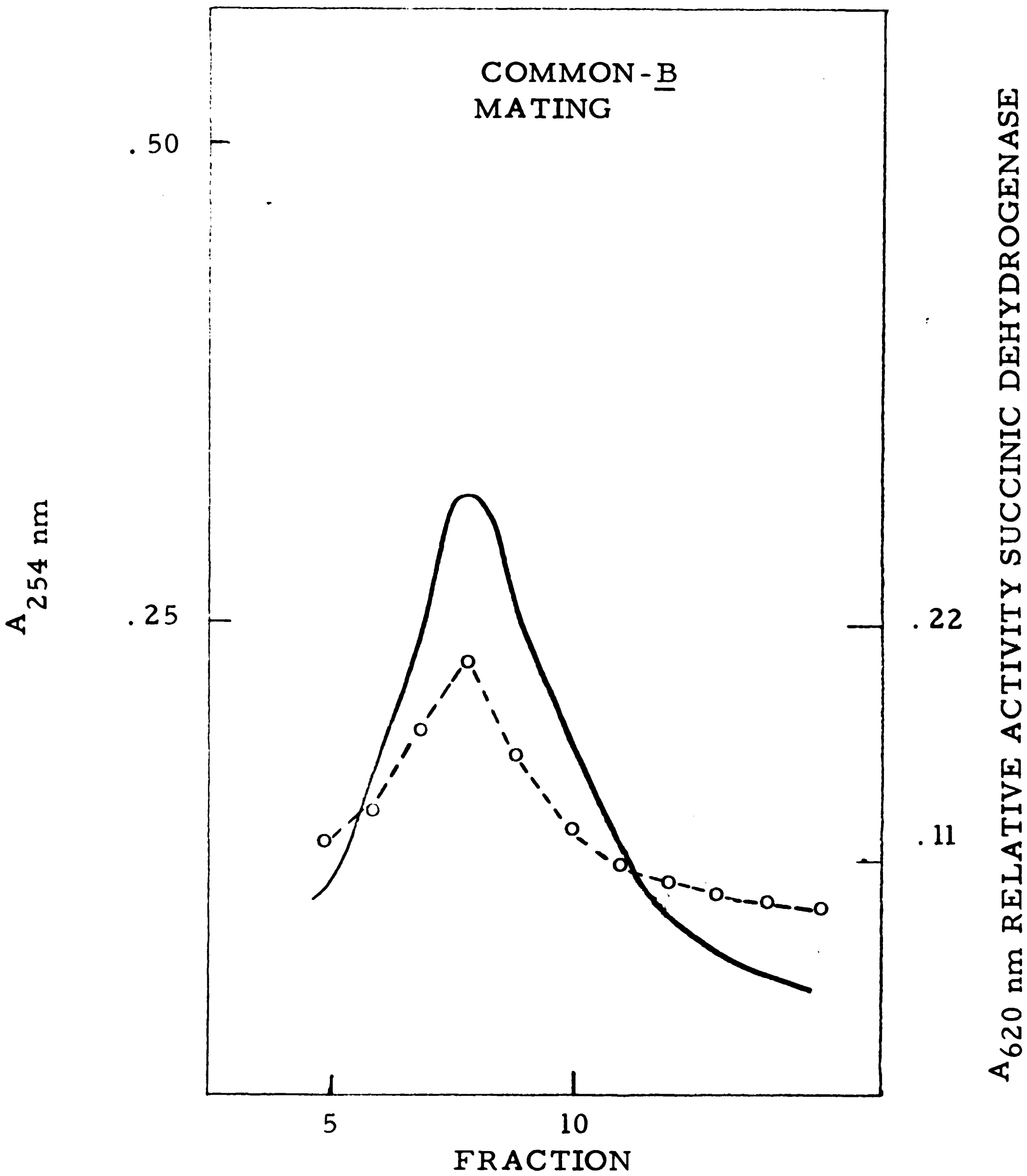
COMMON-B
MATING
.50
.25
A254 nm
.22
.11
A620 nm RELATIVE ACTIVITY SUCCINIC DEHYDROGENASE
5
10
FRACTION

FIGURE 9

Fig. 10.--Absorbancy profiles at 254 nm and 620 nm for a common-A mating: Mitochondria were extracted from areas of the unlabeled resident mycelium (A41 B41) surrounding cobalt donor implants (A41 B42). Constant monitoring of the effluent from the top of the tube at 254 nm (——), indicates a maximum at fraction 8, a shoulder at approximately fraction 10. Succinic dehydrogenase activity in aliquots of five drop fractions collected starting from the top of the tube was made by determining the absorbancy at 620 nm (o----o) of reduced nitro blue tetrazolium chloride. Maximum activity occurs in the region where one would expect the unlabeled resident mitochondria to sediment (fraction 8); a second peak occurs at fraction 10. The latter is where one would expect the denser cobalt donor mitochondria to sediment.

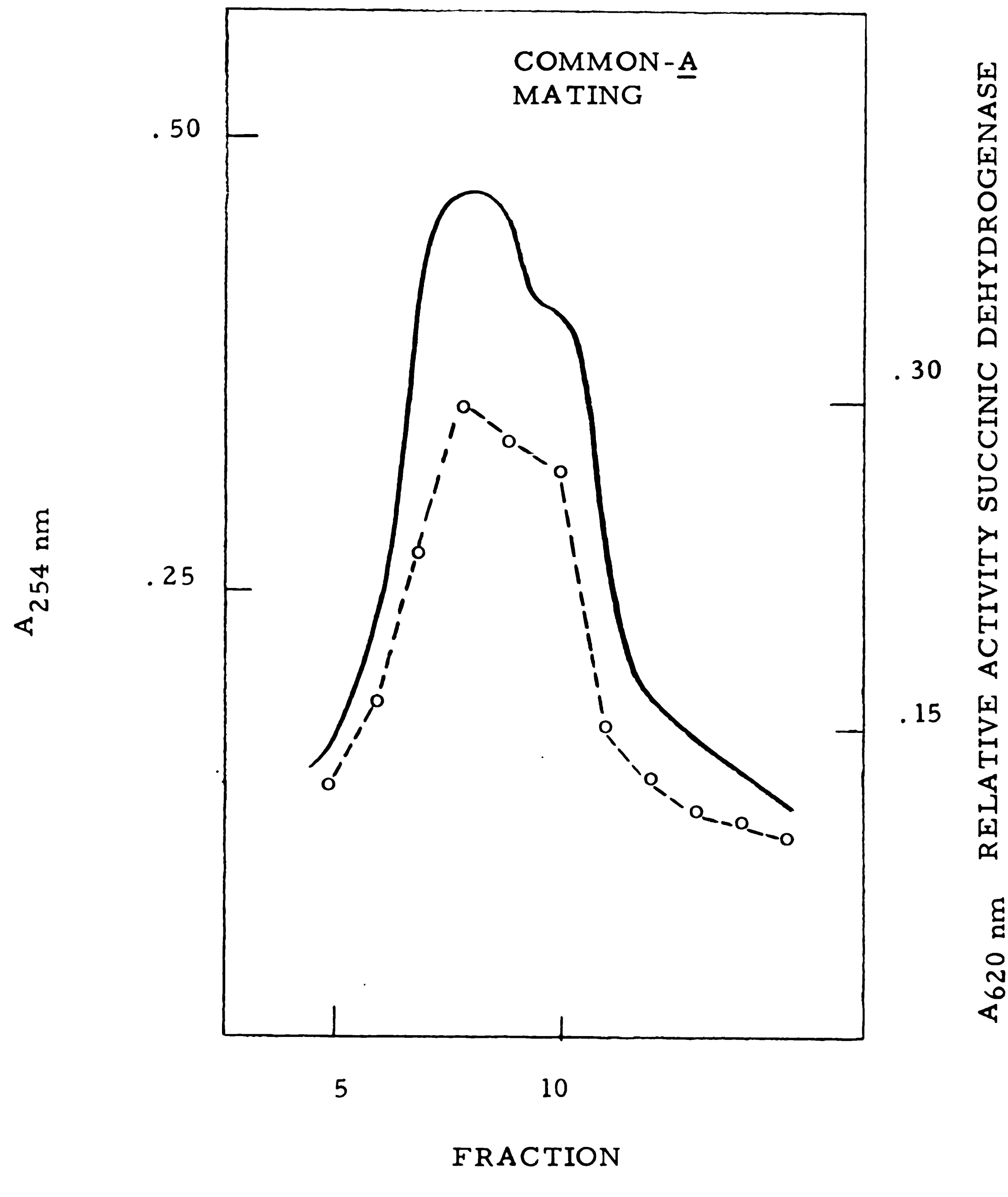
COMMON-A
MATING
.50
.25
.30
.15
A_{254} nm
A_{620} nm RELATIVE ACTIVITY SUCCINIC DEHYDROGENASE
5
10
FRACTION

FIGURE 10

from the unlabeled resident mycelium, the shoulder, the presence of denser cobalt-labeled mitochondria. Analysis of fractions for succinic dehydrogenase activity (Abs. at 620 nm of reduced tetrazolium dye), indicates a bimodal distribution, with a maximum at fraction 8 and a distinct shoulder at fraction 10.

The results of a common-_AB_ mating (Figure 11) indicate a bimodal asymmetric distribution for absorbancy at 254 nm and 620 nm. The maximum at fraction 8 and second peak at fraction 10 are interpreted to represent resident and transferred cobalt-labeled donor mitochondria respectively.

The results from a fully compatible mating (Figure 12) also suggest that transfer of denser cobalt-labeled mitochondria has occurred. The absorbance at 254 nm indicates an asymmetric, bimodal distribution, as does the absorbancy at 620 nm. The observed maximum at fraction 8 is characteristic of mitochondria extracted from mycelium grown on MC medium, the second peak at fraction 10 is characteristic of cobalt-labeled mitochondria.

Thus transfer of cobalt-labeled mitochondria to a non-labeled resident mycelium is evident in common-_A_,

Fig. 11.--Absorbancy profiles at 254 nm and 620 nm for a common-AB mating: Mitochondria were extracted from areas of unlabeled resident mycelium (A41 B41) surrounding cobalt donor implants (A41 B41). Constant monitoring of the effluent from the top of the gradient tube at 254 nm (——), indicates a maximum in the region of fraction 8, a shoulder approximately two fractions later. Maximal succinic dehydrogenase activity, as measured by absorbancy at 620 nm (o----o) of reduced nitro blue tetrazolium chloride in aliquots of five drop fractions collected from the top of the tube, occurs at fraction 8; a second peak occurs at fraction 10. The maximum at fraction 8 is interpreted to represent unlabeled mitochondria from the resident, the second peak those from the cobalt donor.

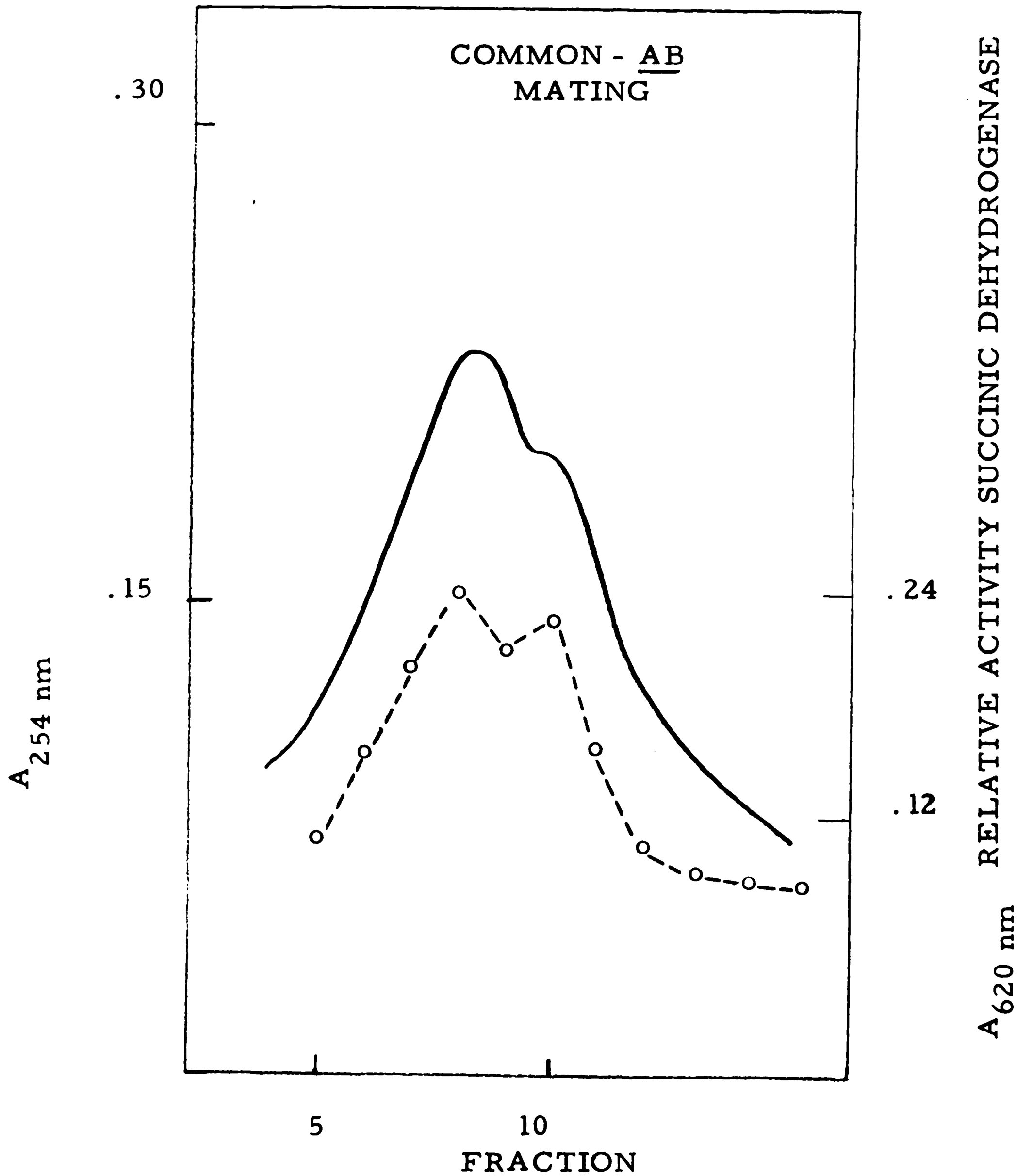
COMMON - AB
MATING
.30
.15
A_{254} nm
.24
.12
RELATIVE ACTIVITY SUCCINIC DEHYDROGENASE
A_{620} nm
5
10
FRACTION

FIGURE 11

Fig. 12.--Absorbancy profiles at 254 nm and 620 nm for a fully compatible mating: Mitochondria were extracted from the unlabeled resident mycelium (_A41_ _B41_) surrounding cobalt donor implants (_A42_ _B42_). Constant monitoring of the effluent from the top of the tube at 254 nm (——), indicates maximal absorbancy at fraction 8; a second peak occurs some two fractions later. Maximum succinic dehydrogenase activity, as measured by absorbancy at 620 nm (o----o), of aliquots of five drop fractions collected from the top of the tube and allowed to react with succinate in the presence of nitro blue tetrazolium chloride, occurs at fraction 8; a second peak occurs at fraction 10. Maxima at fractions 8 and 10 are interpreted to represent mitochondria from resident and cobalt donor mycelia, respectively.

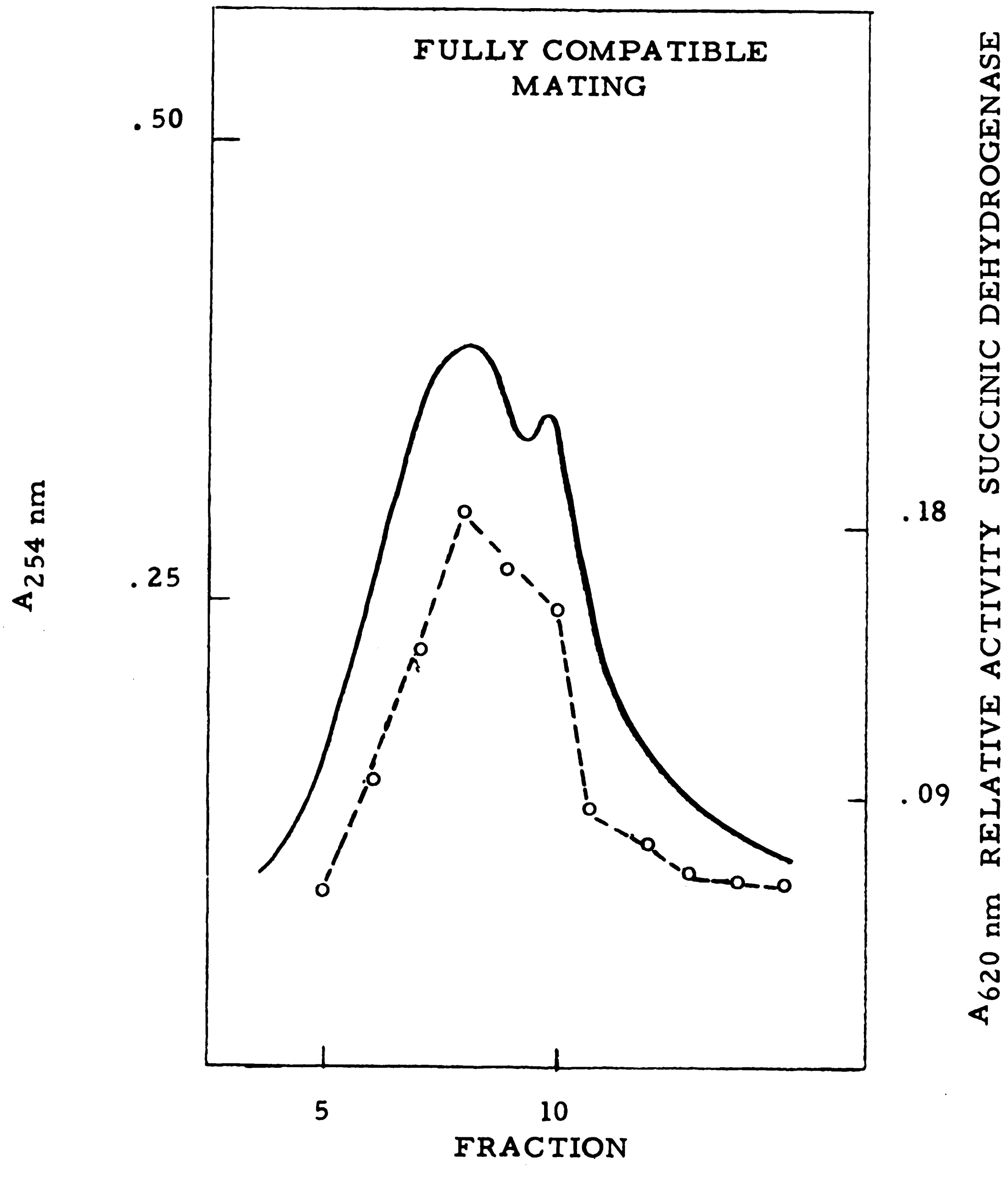
FULLY COMPATIBLE
MATING
.50
.25
A_{254} nm
.18
.09
A_{620} nm RELATIVE ACTIVITY SUCCINIC DEHYDROGENASE
5
10
FRACTION

FIGURE 12

common-AB, and in fully compatible matings. Transfer is not detectable in common-B matings. The results of the four classes of matings are summarized in Table 3. The possible significance of these findings will be discussed below.

TABLE 3.--Density gradient analysis of matings.

Type of Mating	Cobalt donor	MC resident	Replicates	Bimodal pattern of A_{254} nm
Common-B	A42 B41	A41 B41	2	-
	A41 B42	A42 B42	1	-
	A42 B42	A41 B42	2	-
Common-A	A41 B42	A41 B41	4	+
	A42 B42	A42 B41	1	+
Common-AB	A42 B42	A42 B42	1	+
	A41 B41	A41 B41	2	+
	A42 B41	A42 B41	1	+
Fully Compatible	A42 B42	A41 B41	4	+
	A41 B41	A42 B42	1	+
	A42 B41	A41 B42	1	+
	A41 B42	A42 B41	1	+

DISCUSSION

Several methods of analysis were used to determine if transfer of mitochondria occurs after hyphal fusion. These include (1) direct observation by phase contrast microscopy, (2) density gradient analysis, (3) electron microscopy, and (4) enzyme assays. Each method used slightly different techniques to differentiate donor from recipient organelles in matings. The first two have been used to demonstrate migration of mitochondria, the latter two to demonstrate that the organelles in question were, in fact, mitochondria.

The data suggest that the transfer of mitochondria is detectable in fully compatible, common-_A_, and common-_AB_ matings, but not in common-_B_ matings. Transfer is not restricted to any one strain, since alternating the mate which was labeled with cobalt did not alter the observed patterns of transfer (Tables 1, 2, 3). The importance of controls to determine if cobalt is transferred due to diffusion is essential to the types of analysis used. In the first method, where Y-plates were used, even at a concentration of cobalt 20 X higher than that normally used, no cobalt was detected in the immediate region of the plastic divider where anastomoses were normally observed. Blotting of the dialysis membrane to remove obvious moisture must have acted to decrease any diffusion from the cobalt medium to the MC medium via the dialysis membrane. It is significant to note that the dialysis membrane in the region of the divider was not in direct contact with either medium, and was dry and rather brittle by the time anastomoses developed there. In addition, even if cobalt could diffuse to the central observation region, the 48 hour incubation would probably have been insufficient to allow the incorporation of enough cobalt to stain the mitochondria. (This argument is based on the results from the rate of uptake in liquid Co-250 ppm broth, which indicate that 72 hours is the usual time before staining is readily apparent.) The interpretation of these data is that, where transfer of mitochondria was evident, it took place via anastomoses.

The controls for diffusion in the "racetracks" and matings on plates are more indirect ones. In each of these cases, the chances for diffusion from the cobalt donor mycelium were minimized by using donor free of any obvious adhering cobalt agar medium. Discarding the cobalt donor implant and the resident immediately below it minimized the inclusion of contaminating donor hyphal fragments. The common-B mating was also considered to be a control since no transfer of mitochondria was observed in such matings.

That the organelles observed cytologically were indeed mitochondria was based on comparison of these organelles in this and other organisms (Papazian, 1950b; Palade, 1952b; Zalokar, 1960; Lehninger, 1964). Histochemical evidence of cytochrome oxidase activity indicated their cellular location and abundance.

Other than the method of harvesting mycelium, procedures used for the isolation and purification of mitochondria were standard and quite similar to those employed for Neurospora crassa (Luck, 1965; Kuntzel and Noll, 1967). The visible band of suspected mitochondria in sucrose gradients corresponded with regions of maximal succinic dehydrogenase and cytochrome oxidase activity. Electron microscopic examination of fixed and pelleted bands indicated the presence of mitochondria in abundance. Although swollen, the various appearances of the isolated mitochondria compare favorably with several conformations they may assume, termed non-energized (aggregated and orthodox), energized, and energy-twisted (Figures 8c-8f; Green, et al., 1968; Green and Young, 1971). Perhaps the most significant difference *in situ* between mitochondria from mycelia grown on Co-1000 ppm medium and those from mycelia grown on MC medium is a reduction in size and number of visible cristae. A similar "disappearance" of the cristae was noted in mitochondria from *Saccharomyces* grown on a cobalt medium (Lindegren and BeMiller, 1969). The observed contraction of the cristae or failure to expand from the inner membrane may be due to the increased osmotic potential of the surrounding medium or intramitochondrial fluid associated with the uptake of cobalt. A chemical bonding between the cobalt and nitrogen atoms of the proteins of the mitochondrial membranes may also play a role in the apparent contraction. Shrinkage of mitochondria during phosphate-enhanced Mn^{++} uptake has been correlated with the production of an intramitochondrial precipitate of $Mn_3(PO_4)_2$ (in Racker, 1970). No clear evidence of a precipitate is available with cobalt, however. Whatever the cause of the contraction, the latter is probably sufficient to explain both the phenomena of the mitochondria being more visible, i.e.,

stained by cobalt, and, if there is no loss of mass of the mitochondrial membranes, why they exhibit a greater density following accumulation of cobalt. The possibility of cobalt entering as the chelate also exists. In a naturally occurring form, it is as the chelate that cobalt is present in vitamin B 12 (Cobalmin) (Dwyer and Mellor, 1964). Although here it is as the unnatural chelate of EDTA that cobalt is introduced to the medium, it may well enter the cell and then the mitochondrion as the chelate. Cobalt reacts in a 1:1 relationship with EDTA, forming a stable octahedral configuration (Dwyer and Mellor, 1964). It may not be unreasonable to expect that cobalt penetrated the mitochondrial membrane as the chelate, with perhaps a methyl substitution to increase its lipophilic character. Such a substitution has been proposed for the 1,10-phenanthroline chelate of cobalt to explain its penetrance of brain mitochondria (Koch and Gallagher, 1959). Whatever form cobalt takes in mitochondria, the data suggest that cobalt does not appear to be readily lost from mitochondria.

As to the basis of physical mechanisms by which mitochondria may be transferred through anastomoses, i.e., whether by their own activities and/or mass flow of cytoplasm, one can only speculate at this date. Observations of living hyphae by phase contrast microscopy indicate that both means may be available, but the former is favored based on past experience with nuclear migration in S. commune. In Figures 3e and 3f, no visible breaks of the hyphae or evidence of cytoplasmic extrusion was apparent, and photographs taken at an interval of two minutes indicate a mass movement of cytoplasm through and out of the indicated anastomosis. Much more commonly, mitochondria in spherical form can be seen darting the length of a cell in several rapid steps; they may stop, retreat, or proceed into the adjacent cell. As to how they move, one can only speculate here. Mitochondria have a ready source of ATP and have been shown to contain a contractile protein similar to myosin. Ion uptake and release has also been correlated with contraction and swelling cycles. On an ultrastructural level, energy and ion transduction mechanisms have been suggested to be related to changes of conformation of the repeating tripartite units of the mitochondrial membranes (Green, et al., 1968; Green and Young, 1971). It is tempting, then, to suggest that lateral mitochondrial movement might also ultimately be related to energy transfer and internal changes of conformation.

It is interesting to note that mitochondria are often seen in close association with nuclei (Figure 8a; Chèvremont, 1963), particularly when in their filamentous form. Whether the close association of an ATP-ase permeable nuclear membrane with mitochondria could result in alterations of available levels of ATP, and thereby play a role in nuclear migration, poses an interesting possibility.

Under normal conditions, exchange and migration of nuclei is extensive in fully compatible and common-A matings, but severely limited in common-AB and common-B matings. With the notable exception of the common-AB matings, the observed pattern of mitochondrial transfer parallels that found for cases where one normally expects nuclear migration to occur. Previous studies (Sicari and Ellingboe, 1967) indicate that hyphal anastomoses occur regardless of whether matings are compatible or incompatible, indicating that discrimination by the incompatibility factors must occur after the anastomosis has been effected. That the incompatibility factors are nuclear genes is conclusively demonstrated by their segregation at meiosis. That they would exert their influence in the cytoplasm, perhaps by the production of specific protein products, is to be expected. However the exact mode of regulation, which may perhaps involve cellular repressor or inducer substances, is not known. There is no known specific "incompatibility protein," or inducer or repressor of such, in _S. commune_. Thus any models to explain the mode of action of the incompatibility factors on a molecular basis are necessarily of a speculative nature.

Based primarily on the length of time elapsed between the initial observation of an anastomosis and subsequent production of lysed hyphal tips or branches in the immediate vicinity several hours later, the suggestion was made that some molecular changes had to be induced, i.e., produced, whether actually induced or derepressed, and that the observed cytoplasmic incompatibility reaction was not based on constitutive (prior) differences (Sicari and Ellingboe, 1967). Results of the present analyses, based on the detection of mitochondrial transfer, suggest a dual regulation of mitochondrial transfer by both the _A_ and _B_ factors. The common-_AB_ interaction, in which nuclear migration is limited and mitochondrial transfer appears not to be, suggests that the two events are, at least initially, independent in time. Observations of anastomoses in fully compatible matings in which mitochondrial transfer was already evident prior to completion of the development of clamp connections would then support the view that transfer of mitochondria could occur prior to migration of nuclei. Thus the discrimination of the incompatibility factors, more specifically the B factors, as to whether nuclear migration will occur is not immediately expressed, but may involve mediation (induction or derepression), of one or more enzymes. Conceivably one of these intermediate steps may take place in or on the mitochondria, perhaps affecting their ability, and that of nuclei too, to migrate.

Mitochondria are self-replicating, and contain their own DNA, RNA, and ribosomes (Luck and Reich, 1964; Kuntzel and Noll, 1967; Attardi and Attardi, 1967; Sager, 1972). Nuclear genes are thought to code for the assembly of the mitochondrial membrane (Munkres, et al., 1965; Woodward and Munkres, 1966). The concept that information could travel the reverse route to the nucleus has already been suggested (Attardi and Attardi, 1967).

Although it is firmly established that the mating type factors are nuclear genes, it would be of interest to test the possibility of a cytoplasmic expression of the incompatibility factors in protein and isozyme spectra of mitochondria from isogenic homokaryons, and to see if any differences are related to specific incompatibility factors. We might then expect to find either mosaic or hybrid mitochondrial types, particularly in common-A heterokaryons and dikaryons, if we assume a biogenesis of mitochondria from pre-existing units (Luck, 1963a, 1963b, 1965). Induction of mitochondrial mutants would also be useful in studies of cytoplasmic transfer, and to serve as mapping tools in genetic analysis.

SUMMARY

Methods were developed to determine if transfer of cytoplasm occurs following hyphal fusion in compatible and incompatible matings of *Schizophyllum commune*, a tetrapolar Basidiomycete. Analysis was by two major methods: 1) Direct observation of individual anastomoses in which one mate was differentially labeled with cobalt, a vital stain for mitochondria, and 2) Density gradient analysis of non-cobalt residents for detection of transfer of denser cobalt-labeled mitochondria. In addition, electron microscopy and enzymatic tests were used to determine if the organelles in question were indeed mitochondria. Results of the two types of analysis suggest that mitochondrial transfer via anastomoses occurs in fully compatible, common-*A* and common-*AB* matings, but not in common-*B* matings. Possible inter-relationships of the observed phenomenon of mitochondrial transfer with nuclear migration and the time of action of the incompatibility factors are discussed.

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