Bertrand, H. and T. H. Pittenger. An improved method for the determination of mitochondrial cytochromes.

Several methods describing the preparation of mitochondria for the spectrophotometric determination of the cytochromes have been published. The final suspensions generally ore turbid, such that relatively low concentrations of the cytochromes must be scanned by means of a highly sensitive spectrophotometer. These problems con be eliminated by obtaining clear cytochrome solutions.

Neurospora cultures are grown from about lo6 conidia/ml in 2 l culture flasks containing 500 ml of Vogel's medium with 2% sucrose. The flasks are incubated at 25°C to 30°C for about 24 hours with vigorous shaking. Longer periods of incubation are not recommended for normally growing strains, but may be required for slow-growing nuclear and cytoplasmic mutants. The cultures should be harvested when the hyphal mass reaches about 10 g moist weight per flask. The mycelium is collected by suction-filtration on coarse filter paper or discs of a double layer of cheese-cloth, washed twice on the filter with distilled water, and once with sucrose-EDTA (0.44 M sucrose containing 0.005 M EDTA and 0.01 M Tris.HCl, pH 7.4). Ten to forty grams of mycelium may be necessary, depending on the spectrophotometer that is available. All subsequent step are executed at 0-4°C.

The mycelium is dispersed in 30 to 120 ml of sucrose-EDTA by stirring. If desired, about 0.5 g of 320 mesh carborundum for every 30 ml of isolation medium may be stirred into the suspension as a grinding aid. The hyphae ore disrupted in a fairly loose-fitting 40 ml Tenbroeck homogenizer with little pressure on the shaft as it is rotated. The number of strokes required for effective breaking of the hyphae varies between one and ten, depending on the fit of the homogenizer and the condition of the mycelium. The homogenate is centrifuged twice at 1500 x g to remove cell debris, nuclei and carborundum. The mitochondria are sedimented by 30 min centrifugation at 12,000 x g. Any lipid-like material clinging to the walls of the centrifuge tube is wiped off with tissue-paper after decanting the supernatant. The mitochondrio are washed by stirring the pellet into 30-40 ml of sucrose-EDTA and resedimentation at 12,000 x g. The pellet is then dispersed in about 2 ml of sucrose-EDTA, and two ml are transferred to a small plastic tube containing 40 mg of sodium desoxycholate. The mitochondria are disrupted ultrasonically at 0-4°C. Two one-minute treatments, with a one-minute intermission, in a Raytheon DF101 with cup assembly give satisfactory results. A different schedule of treatments must be worked out if another sonicator is used, taking core that the suspension remains cold. After, sonication, the solution is cleared by IO min centrifugation at forces greater than 3000 x g.

The difference spectrum of the cytochromes is obtained at room temperature. Two microcuvettes (0.7 ml capacity, IO mm path) are filled with the cytochrome solution, and the base line (zero difference spectrum) is determined for wavelengths between 700 mμ and 400 mμ. The contents of the sample cuvette ore then reduced by stirring a few small crystals of sodium dithionite into the solution, and the difference spectrum is obtained by scanning the reduced vs. the oxidized sample. Any split- or double-beam spectrophotometer can be used, provided that the preparation contains levels of the cytochromes that are well within the detective capacity of the instrument. Instruments without automatic wavelength drives are operated manually by taking readings at 5 to IO mμ intervals for the base line, and at every 2 mμ for the difference spectrum. It should be noted that all three major cytochromes have absorption peaks between MO mμ and 500 mμ, such that readings within this region of the visible spectrum may be satisfactory in most cores. A Beckmann DK-2A (very insensitive) and a Cary 16 (sensitive) spectrophotometer both have been used in this laboratory.

The critical steps in the preparation of mitochondria for spectrophotometry ore the careful removal of lipid-like substances after the sedimentations at 12,000 x g, the addition of desoxycholate prior to sonication, and the centrifugation after sonication. The concentration of protein in the sucrose and desaxycholate-containing solution can be determined by the Folin method.

Cytochrome	Peek α	Peek β	
a + a3	609 mμ		443 mμ
b	560	530 mμ	428
c	550	520	418

The characteristic absorption maxima for the cytochromes of Neurospora are given in the accompanying table. Typical spectra are found elsewhere in this (Bertrand and Pittenger 1969 Neurospora Newsl. 14: 6) and the previous issues of the Newsletter (Griffiths et al. 1968 Neurospora Newsl. 13: 16). Portions of the above procedure were adapted from methods described by Hall and Baltscheffsky (1967 Neurospora Newsl. 12: 13) and Diacumakos et al. (1965 J. Cell Biol. 26:427). - - -

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