Permeabilization of conidia with phenethyl alcohol

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Abstract
Permeabilization of conidia with phenethyl alcohol
Isolation and purification of mitochondria from *N. crassa.*

The following describes two methods, used in this laboratory, for the preparation of mycelial homogenates from which *Neurospora* mitochondria may be isolated and purified.

*Neurospora crassa* strain KJT 1960 is grown in shaker flasks (Kiritani et al., 1965 Biochim. Biophys. Acta 100:432). The mycelium is harvested after 16 hours of growth by filtration through a double layer of cheesecloth, resuspended in 0.1 M sucrose in 0.1 M Tris, pH 7.8 and filtered again. When the wet weight of mycelium exceeds 100 g, it is disrupted with an Eppendorf Micro Mill by the method of Greenaway et al. (Methods in Enzymol. 10:142). Smaller quantities of mycelium are homogenized by grinding in a prechilled porcelain mortar and pestle with twice the mycelial wet weight of acid-washed sand. The mycelium is first ground to a coarse paste with sand alone, after which 0.24 M sucrose containing 0.15% BSA is added with continual grinding until a smooth paste is obtained. The final volume in ml of sucrose-BSA added need not exceed twice the wet weight of mycelium.

The crude mitochondrial pellet is obtained by differential centrifugation of the mycelial homogenate obtained by either of the above methods. The homogenate is centrifuged at 1500 x g for 10 minutes, and the supernatant, thus obtained, centrifuged again at 1500 x g for 15 minutes. This process removes sand, unbroken mycelium, nuclei and other large cell fragments. The supernatant is then centrifuged at 37,000 x g for 30 minutes, and the supernatant decanted. The residue consists in large part of crude mitochondrial pellet which is transferred to a glass homogenizer and resuspended with three strokes of a teflon pestle in a minimum of 0.25 M sucrose, 0.15% BSA. An aliquot of the mitochondrial suspension, containing no more than 40 mg of mitochondrial protein, is layered on an 8.0 ml linear sucrose gradient (0.58 - 1.9 M; 20-65%, w/v). The gradients are then centrifuged at 50,000 rpm for 90-120 minutes in a Spinco 50 rotor, after which the bottoms of the gradient tubes are punctured and the mitochondrial band collected as a single fraction. Such mitochondria are relatively free of microsomes, arc capable of synthesizing certain amino acids and can be used in polarographic studies to determine oxygen uptake. - - - Department of Zoology, University of Texas, Austin, Texas 78712.


Enhanced intermediate pool sizes have been obtained by using *neurosporaxanthin-less* or yellow “albino” strains; e.g., ylo-1 or ylo-b, ALS-4, ALS-23. There strains have 55-75% of the total carotenoid fraction yields of the wild type strains, mostly in the form of the early precursor pools (phytofluene, 4-carotene, neurosporene, etc.).

Huang (1964 Genetics 49:453) and Harding (1968 *Neurospora News* I. 13: 8) reported yield improvements by culturing in the dark in liquid medium for 5 days and then draining off the medium and exposing the spread-out mycelial mat to intense fluorescent light for 1 to 24 hours. Cold treatments (6 hrs. at 7°C) do seem to improve yield?, but as yet no quantitative data are available.

Using the above techniques, it has been possible to obtain a yield of 1.8% (total carotenoid fraction/dry weight of mycelium) and isolate short-lived intermediates. 8-zeacarotene has already been identified as a component of the *Neurospora* carotenoid fraction using this technique, which was developed in conjunction with a genetic study attempting to define the specific biosynthetic lesions caused by the “albino” gene cluster alleles. - - - Laboratory of General Microbiology, University of Geneva, Geneva, Switzerland.


by treatment with one of several agents. In addition, to acid inactivation of the enzyme which has been previously shown to acid inaccessible, substrate-accessible compartment represents enzyme imbedded in the plasma membrane. The acid inaccessible to both substrate and acid (cryptic compartment) corresponds to truly intracellular arylsulfatase.

It was previously reported (Scott and Metzenberg 1967 *Neurospora News* I. 11: 8) that *Neurospora* conidia contain a cryptic compartment of aryalsulfatase which can not be detected in intact conidia but can be observed after the conidia have been permeabilized. Although direct evidence is lacking, it is useful to think of these compartments as follows: The enzyme accessible to both substrate and acid represents enzyme in the periplasmic space. The acid inaccessible, substrate-accessible compartment represents enzyme imbedded in the plasma membrane. The enzyme inaccessible to both substrate and acid (cryptic compartment) corresponds to truly intracellular arylsulfatase.
This communication describes conditions under which phenethyl alcohol will reveal the cryptic compartment and will render all of the enzyme susceptible to acid inactivation. Phenethyl alcohol has been previously shown (Lester 1965 J. Bacteriol., 90: 29) to inhibit uptake of various amino acids and glucose in germinated conidia. In addition, 0.3% phenethyl alcohol prevented germination of Neurospora conidia for 8.5 hrs. at 30°C without loss of viability.

Conidia from the strain eth-l (r), cys-5 (85518) A were grown under conditions for derepression for aryl sulfatase synthesis as previously described (Scott and Metzenberg 1967 Neurospora News1. 11: 8). Conidia were harvested, filtered two times differing, through glass wool, washed twice with 0.1 M Na-acetate/acetic acid buffer, pH 5.0 and treated with HCl at a pH of 1.3 at 4°C for 15 minutes; then the pH was readjusted to 4.8 with NaOH. Conidia so treated were centrifuged and resuspended in 0.1 M Na-acetate/acetic acid buffer, pH 5.0, containing 0.1 mM cycloheximide and were incubated with various concentrations of phenethyl alcohol at 37°C for 30 minutes. Conidia were kept in suspension by adding a glass bead and agitating on a shaker. During the incubation, the conidial concentration, measured by turbidity or a suitably diluted sample, was OD 0.5 cm = 24.2. This corresponds to 2.8 mg protein per ml by the method of Lowry et al. (1951 J. Biol. Chem. 193: 265), modified by incubating the sample of conidia in the Lowry alkali reagent at least 12 hours before adding the copper reagent. This modification gives reproducible values for protein but probably does not measure all of the protein present in the conidia.

At the end of the thirty minute incubation, an aliquot from the incubation mixture was treated with HCl at pH 1.3 and 4°C for 2 minutes; then the mixture was readjusted to pH 4.8 with NaOH. Another aliquot was diluted with NaCl as control. Samples from the acid-treated and control tubes were collected by vacuum filtration onto filter paper discs and were washed on the paper with cold 0.1 M Na-acetate/acetic acid buffer, pH 5.0. Additional samples were collected and washed as above and further washed with ice-cold chloroform. Eberhart and Tatum (1961 Am. J. Botany 48: 702) reported on analogous technique using acetone. All of these samples were assayed for aryl sulfatase by shaking the filter disc and conidia under the previously established assay conditions (Metzenberg and Parson 1966 Proc. Natl. Acad. Sci. U.S. 55: 629) with the addition of 0.1 mM cycloheximide. The results are shown in the accompanying figure.

It can be seen that, after incubation in the absence of phenethyl alcohol, 34% of the enzyme is not detected in the assay unless the conidio have been treated with chloroform (cryptic compartment). In addition, about 12% of the enzyme has become vulnerable to acid inactivation during the incubation. Low concentrations (0.25% and 0.50%) of phenethyl alcohol reveal the cryptic compartment almost completely, but a large part of the enzyme is still protected from acid inactivation. After incubation with 0.75% or 1.00% phenethyl alcohol, all of the cryptic compartment become accessible to substrate and all of the enzyme has become susceptible to acid inactivation. The conidia are still able to retain enzyme molecules, however. Even after incubation of the conidia at the highest phenethyl alcohol concentration, washing the conidia removed no more than 10% of the enzyme.

Repetition of this experiment with the same conidial suspension on the same day gave results differing, at most, by 10%. If conidia collected on different days were used, qualitatively the same results were obtained, but there was some variation in the concentration of phenethyl alcohol required to permeabilize the conidia in a fixed period of time. **Department of Physiological Chemistry, University of Wisconsin, School of Medicine, Madison, Wisconsin 53706.**

**Metzenberg, R.L. and S.K. Ahlgren. Hybrid strains useful in transferring genes from one species of Neurospora to another.**

The investigation of natural variation in the genus Neurospora has been limited by the absolute or relative infertility of interspecific crosses. Especially where it is desirable to move moderately deleterious genes, such as auxotrophic markers, from one species to another. A number of workers have done this successfully, but we have found it difficult to circumvent this difficulty, we have developed a "transfer kit" - a