TECHNICAL NOTES

Bosl, M., and A. M. SrB. Procedure for preparing individual asci of Neurospora for electron microscopy. The following procedure has been used with repeated success for ultrastructural studies of developing asci of N. crassa and N. tetrospérmum. The procedure provides excellent material for ultrastructural work and permits the investigator to focus efforts on particular asci at a specific developmental stage. The procedure also provides a basis for serial sectioning of identified asci. Perithecial conidia containing asci at the desired stage of development are scraped from the surface of petri plates containing crossing media (Westergaard and Mitchell, 1974, Amer. J. Bot. 34: 573) and transferred to vials containing Kanavsky's (1965, J. Cell Biol. 27: 137) cocomulate-buffered 3% formaldehyde-3% glutaraldehyde - 10% sucrose (w/v) adjusted to pH 6.8. Allow the preparation to stand 2 hr. at room temperature; then using an eyedropper transfer numerous perithecia (about 1/2 eyedoopperful) onto the center of an inverted lid of a plastic Petri dish (30 x 150 mm). The bottom of the Petri dish is then fitted inside the lid so as to form a press. Using pressure, combined with a rotating motion, crush the perithecia, thereby liberating asci. Remove the bottom, hold the lid at an angle and flush the area with an eyedoopperful of fresh fixative. Pick up the resulting material with the eyedoopper and deliver into a double layered cheesecloth filter formed over a 15 ml centrifuge tube kept on ice. Repeat this procedure until all of the perithecia have been transferred. Concentrate the sample by centrifugation, if necessary. The filtrate and consequently the pellet contain some perithecial and hypothal debris, which is, however, easily recognized in the final preparation and does not present problems. Further steps are carried out in the cold (ice bucket or refrigerator) until noted otherwise.

After an additional hour in fixative, pellet the asci by centrifugation at 200g for 5 min. and decant the supernatant. Suspend the asci in a 0.1 M cacodylate buffer pH 6.8 + 10% sucrose; after 20 min., centrifuge as before. Repeat rinsing procedure 4 times more. Decant supernatant after the 5th rinse, suspend the pellet in 2% OsO_4 in 0.1 M cacodylate and refrigerate overnight. Rinse the sample 5x (10 min. each) with 1% cacodylate followed by 2 quick rinses in water. Dehydrate (3 min. each) in a graded acetone series (possible because asci pellet readily once out of 10% sucrose) of 25%, 50%, 75%, 2 changes in 90% (taking to room temperature at the first 90% step) and 3 changes in 100% acetone. After decanting the supernatant at the final 100% acetone step, add 2 drops of epon-araldite mixture (Mollenhauer, 1964, Stain Tech. 39: 111) to the pellet covered by a small amount of acetone that inadvertently remains. Suspend the pellet in the acetone-plastic by stirring gently with an applicator stick. After 1 hr, add freshly prepared epon-araldite (ca. 3 ml is usually sufficient, but the amount depends on size of the sample), resuspend by mixing as before, pick up entire sample with a Pasteur pipet and dispense 2-4 drops each in aluminum weighing dishes. Using a tungsten depressor, gently spread sample over the mid-section of the weighing dish so that a thin sheet results. Take care not to cause marks on the bottom of the dish. Place the sample in a 70°C oven for an additional 3 hr. at room temperature and leave overnight. Remove the coated plastic sheet from the weighing dish by turning the edges of the dish down and gently bending the bottom downward. The sheet will peel off.

Prepare the embedded asci for examination with the phase contrast microscope by first trimming away the edges of the plastic sheet with a sharp razor blade. Then with the razor, cut loose a section (10-15 mm square) and with a drop of clear fingernail polish attach it bottom side up to a glass slide. Because the asci tend to sink in unpolymerized plastic, they are more readily available for observation if mounted bottom side up. Place the slide with the mounted plastic in a 70°C oven for 1 hr. or longer. The asci can now be examined with a phase contrast microscope. The asci lying closest to the plastic surface can be observed with the oil immersion objective. (Cytological observations here are more difficult than with material prepared specifically for conventional microscopy. However, nuclear areas are distinguishable in that they appear smooth in contrast to the granular appearing cytoplasm. In unincutile asci nuclear areas are readily observed, but are increasingly difficult to discern as the nuclear divisions progress. Fortunately, other features aid in determination of a developmental stage, which later can be verified by electron microscopy. Different strains provide different landmarks, which once determined are reliable. E.g., wild type strain N. tetrospérmum (T-220) accumulates vacuoles in the mid-region of its cytoplasm during the interphase following meiosis. When vacuoles of unique appearance are seen in this region the nuclei are in mitotic metaphase.)

Select a desired cell, note its orientation and relocate it with the aid of dissection microscope and stage-lighting. Wipe away the oil and with a razor blade cut out a small block containing the desired cell. The plastic readily snaps free from the slide despite the fingernail polish and care must be taken to avoid disturbing the area of desired plastic is not lost. Loss can be minimized by applying slight pressure with fine forceps (Dumont 2S) on the area to be saved while doing the final trimming. Place the trimmed cell in a small drop of Scotch brand epoxy resin on another glass slide. Using a toothpick apply epoxy resin to the top of a blank mount prepared by polymerizing Spurr's (1969, J. Ultrastr. Res. 26: 31) epon in 00 beem capsules and place the trimmed block atop. Check with the aid of a dissection microscope to assure that the bottom side of the plastic is the same as when mounted to the glass slide and that it is properly centered on the blank. Insert the mounted blank, bottom end first, into an empty beem capsule so that the mounted end is sticking up. Place the "fooded" capsule into a beem capsule holder and place in a 70°C oven overnight.

The specimen is ready for this sectioning by first manually trimming it to as small a block size as possible. Then 1-0.5μm sections are taken on an ultramicrotome using a glass knife. Watch for any subtle irregularities in the plastic that may serve as an indicator that the cell is present. When seen, mount that section on a slide, stain with azure B and ascertain the presence of the cell. When the cell is found, thin section the remainder of the cell with a glass or, preferably, a diamond knife. Pick up sections on formvar-coated grids and stain with uranyl acetate and lead citrate before viewing with a transmission electron microscope of 60KV. This procedure was originally done with Spurr's epon rather than epon-araldite as the embedding plastic, but lack of contrast prevented good imaging at the EM level. (Supported by Grant GM-12953 from the National Institute of General Medical Sciences, U.S.P.H.S.) - - - Section of Botany, Genetics and Development, Cornell University, Ithaca, New York 14853.

Burr, A. G., and A.M. SrB. Perithecial submersion: a method for detecting the effect of compounds on ascus development. In an attempt to study the effect of antimicrobial substances on developing asci, the ability of perithecia to survive submersion was examined. This was necessitated by the observation that transport of many, if not all, molecules from the medium to the developing perithecia ceases approximately 4-5 days after fertilization (Nasrallah and SrB, unpublished). Three lines of evidence suggest that certain molecules can enter the perithecium via submersion: 1) submersion of perithecia in 3M-leuine or 3M-His in incorporation of the label into perithecial protein; Nasrallah and SrB, unpublished; 2) perithecia grown on blatin-deficient media normally produce many indurated asci, but if such perithecia are submerged at an early stage in a blatin solution, only normal spores are produced. Still other perithecia are submerged in a solution containing methylene blue; the dye enters the perithecial cavity within the first hour of submersion. Presumably, sub-
mersion of perithecia results in uptake via the ostiole.

The following is a description of our current procedure. Perithecia are collected from crosses made by spreading a conidial suspension of both parental strains on Westergaard's medium in petri plates. After the onset of ascus formation, but before the beginning of ascus formation, perithecia are collected with the aid of fine forceps and transferred onto 4% agar. After removing any excess Westergaard's agar medium adhering, a flattened inoculating needle is used to transfer the perithecia to 10 x 75 mm test tubes filled with 1.5 ml submersion medium. Usually 10 perithecia are transferred to each of two tubes. The perithecia in one tube are left submerged and the contents checked at appropriate intervals to determine whether further development, as evidenced by the formation of ascocarps, occurs during submersion. The perithecia in the second tube are submerged for a specific length of time (6 hrs, works well), then poured into a filter-lined funnel, washed with about 30 ml distilled water, and transferred to a slant of 2% purified agar. Perithecial contents are checked at intervals (usually 1, 2, and 3 days after transfer) to determine whether ascocarp formation proceeds normally.

The following parameters have been tested for their effect on perithecial development. a) Preliminary handling of perithecia -- when perithecia that have been "cleaned" by quickly removing excess agar and by washing in distilled water both before and after submergence compared to perithecia not cleaned, no differences in ascocarp production are seen. Thus, if care is taken to avoid dessication, the perithecia can be handled without ill effect. b) Perithecial stage -- in general, the older the perithecia, the better it survices submersion. When 2--4 day old material is submerged and subsequently transferred to agar, few mature and form spores, but the majority deteriorate before forming asci. Most 4--5 day old perithecia survive submersion and form ripe spores, but the frequency of ascus abortion is often higher than in unsubmersed perithecia. Perithecia that already contain asci, but no spores, usually survive submersion well. c) Submergence medium -- in most experiments involving several submergence media, distilled water was used as the control, and thus as a standard of comparison for evaluating the other media tested.

Following is a list of submergence media tested. In each case, perithecial contents were examined qualitatively to determine the presence or absence of detrimental effects, such as increased ascus abortion, variation in spore size or shape, or increased frequency of 5-spored asci. -- Best, subsequent perithecial development; 5 units ascorbic acid/1.05% NaCl, Squibb mineral oil; Good: 0.05% NaCl, 50 units ascorbic acid/1.05% NaCl; Average: distilled water, liquid Westergaard's medium containing 0%. 2%, or 4% sucrose, liquid Westergaard's medium diluted 1:1 with distilled water, 5mM caffeine, 10 mM caffeine; Poor: liquid Westergaard's medium containing 8% sucrose, 8% sucrose solution, 0.05 M colchicine; Toxic: 0.1 M colchicine, 1 M phosphate buffer (pH 6.7), 0.3 M acetate buffer (pH 5.2).

Requirements for completion of perithecial development during continuous submergence are stricter than those for survival after temporary submergence. -- Good, comparable to an unsubmerged culture: 5 units ascorbic acid/1.05% NaCl, Squibb mineral oil, 0.05% NaCl; Average: distilled water, 50 units ascorbic acid/1.05% NaCl; Poor, only a few spores form or those spores or asci are abnormal; liquid Westergaard's medium containing no sucrose, 8% sucrose solution, 5mM caffeine; little or no further development; liquid Westergaard's medium containing 2%, 4%, or 8% sucrose, liquid Westergaard's medium (2% sucrose) diluted 1:1 with distilled water, 10 mM caffeine.

Several interesting facts emerge. Although Westergaard's medium is a widely used crossing medium, continuous submergence of developing perithecia in liquid Westergaard's medium inhibits further development, even when 6-day-old perithecia, which already contain young spores, are submersed. Sucrose in the submergence medium also seems detrimental to further development. Also, once perithecia start forming asci, they seem to be self-contained, requiring no obvious source of nutrients (i.e., they will develop in distilled water and little or no external oxygen (good development in mineral oil). Perithecia that have been submersed in distilled water for up to 7 days -- with no further development during submergence -- will, upon transfer to agar, resume development and form normal spores. There does, however, seem to be a correlation between the length of submergence before transfer and the amount of ascus abortion. A six hour submergence seems to provide adequate uptake of the compound of interest and does not usually result in increased ascus abortion. There is also a relationship between the age of the peritheium and the length of submergence tolerated. For example, perithecia which contain only sterile hyphae and crosiers will tolerate a six hour submergence in liquid Westergaard's medium quite well; but, if submerged for 24 hours, approximately 80% of the perithecia degenerate. Older perithecia, which already contain asci, will survive quite well after a 24-hour submergence in the same solution.

No differences in development were detected between perithecia transferred to purified agar and those transferred to agar containing Westergaard's medium. The advantage of purified agar is that subsequent hyphal growth and de novo perithecial formation are kept at a minimum.

Submergence may be a useful procedure for a variety of studies. It can be used as a means for effecting perithecial uptake of nutrients, inhibitors, etc., and would be especially suited for compounds that are too unstable to be added directly to a crossing medium, or, conversely, for compounds that inhibit crossing per se. Results from continuous submergence may provide insight into the nutrients, etc. necessary for in vitro development of isolated asci. (This research was supported by a Predoctoral Training Grant, TI GM-01035, from the National Institute of General Medical Sciences, USPHS, and by Grant GM-12953 to A.M. (to A.M. from the National Institute of General Medical Sciences, USPHS)) -- Section of Botany, Genetics and Development, Cornell University, Ithaca, NY 14853.

Johnson, T. E. A method for obtaining high specific activity radioactive extracts from perithecia.

One way of dissecting a biological process is by isolating mutants which are blocked at different points in that process. There are numerous techniques which allow us to determine which alleles have occurred in the molecular components of that process. Many mutants have been isolated which block perithecial development at either the male or female component of a cross (review Johnson, T. E., (1978) Genetics 88: 27-47). One very general 4 ppm method for understanding the molecular alterations in these mutants is to determine what changes have occurred in the proteins which are synthesized by those mutants. This report presents a reliable way to label perithecial proteins with radioactive amino acids. The proteins can then be displayed by one or two dimension electrophoresis and autoradiography. The desired maternal component is inoculated on crossing medium supplemented with 2% sucrose and appropriate growth requirements, and the male paper plates are so that the whole surface of the plate is covered (VWR grade no. 613 is especially suitable become the paper withstands long periods of wetting without shredding).

Conditions for growth and fertilization have been given (Johnson 1978 Genetics 88: 27-47). At appropriate time after fertilization protoperithecia and perithecia can be isolated by scraping the surface of the paper with a blunt spatula. The mat is pressed dry, weighed, and placed in a filter paper containing water (5 ml per plate harvested) and the desired radioactive label. The culture is then incubated on a rotary shaker for six hours, and harvested by filtration through a 0.45 Millipore filter. The sample is