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Regeneration of uninucleate protoplasts: a potential method for determining nuclear ratios

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Abstract
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Protoplasts of Neurospora crassa (Bachmann and Bonner 1959 J. Bacteriol. 78:550) prepared as described by Trevithick and Metzenberg (1964 Biochem. Biophys. Res. Commun. 16:319) appeared to be predominantly uninucleate under the phase contrast microscope. This observation was confirmed by staining the protoplasts with acetocarmine and counting the number of nuclei in each protoplast. The stain was prepared by adding excess acetocarmine dye to boiling 45% acetic acid, cooling, and filtering. A drop of the stain was smeared on a glass slide and allowed to dry. A drop of a protoplast suspension was placed on the slide and covered with a coverslip. Of 270 protoplasts observed, 220 contained one nucleus, 40 contained 2 nuclei, and 10 contained 3 nuclei.

Conditions have been developed in our laboratory for regeneration of mycelial growth by the protoplasts and for subsequent colonization of the growth centers. Plating of protoplasts derived from heterocaryotic cultures onto appropriately supplemented plates should provide a convenient method for the estimation of nuclear ratios in heterocaryons.

Conidia were inoculated onto Fries minimal salts medium supplemented with 0.044 M sucrose, solidified with 1.5% agar, and overlaid with sterile cellophane squares before the agar had hardened. Cultures were allowed to grow at 24°C for 24-48 hours. The young mycelium was stripped off with sterile forceps. This method was found to be a convenient way to obtain mycelium free from hypotonic medium from a large number of strains.

Each sample of mycelium was placed in a sterile, capped 18 x 150 mm Pyrex tube containing 0.5 ml of 20% snail digestive juice and 0.5 ml of double strength Fries salts supplemented with 1.18 M D-mannitol and containing, in addition, lysozyme, streptomycin, and penicillin. After the mixture had shaken at 120 cycles/min. for 6 hours at 30°C, 8 ml of Fries salts with 0.59 M mannitol were added, and the mixture was filtered through a 19 x 80 mm. column of loosely packed glass wool. The filtrate was centrifuged at 300 x g. for 20 min. and the residue was washed and resuspended in Fries salts with 0.59 M mannitol. The concentrations of protoplasts and mycelial fragments were determined by direct counting in a chamber

with a calibrated grid with the aid of a phase contrast microscope. In a typical preparation, 85-90% of the observed particles behaved as protoplasts, lysing immediately when distilled water was admitted under the coverslip, and 10-15% were non-lysable nucleated mycelial fragments surrounded by refractile cell wall. The suspension of protoplasts was diluted with Fries salts + 0.59 M mannitol to contain approximately [illegible]00 protoplasts/ml. Two milliliters of this solution were pipetted with minimum shear into 18 ml of Fries minimal salts supplemented with 0.59 M mannitol and 0.08 M glucose to promote cell wall regeneration, and 2 ml were pipetted into 18 ml of Fries minimal salts supplemented with 0.08 M glucose alone. The flasks were shaken at 40 cycles/min. at 30°C for 4 hours. Observation of the protoplasts at this time under the phase contrast microscope showed that most of the protoplasts had acquired a refractile outer layer and that many of them had sprouted 1 or 2 hyphal projections. Failure to allow this regeneration to occur prior to stressing the cells at an air-liquid interface resulted in a very low final efficiency of plating. Samples (1 ml) were pipetted onto a Millipore filter (47 mm filter, pore size 0.22μ) attached to a water aspirator. The filter was slowly and gently rinsed with 10 ml of Fries salts + 0.59 M mannitol to insure deposition of all protoplasts onto the millipore and to remove all traces of the previous medium. The filters were then transferred to plates containing Fries salts supplemented with 0.59 M mannitol, 0.08 M glucose, and 1.5% agar, and incubated at 24°C for 10 hours to avoid subsequent killing by sorbose. The filters were then transferred to plates containing Fries salts supplemented with 0.25 M sorbose, 0.004 M sucrose, and 1.5% agar and incubated for 10 hours at 24°C. At this time the colonies were from 1 to 2 mm in diameter and could be counted readily. 60-90% of the particles plated onto the filter form colonies: however, 10-15% of the particles are not true protoplasts, since they are able to survive and regenerate in the hypotonic Fries glucose. Until it is possible to prepare protoplasts free of mycelial fragments, it will be necessary to perform a control with hypotonic medium. However, the method should be much less laborious than cutting hyphal tips when large numbers of cultures are desired. - - - Department of Physiological Chemistry, University of Wisconsin, Madison, Wisconsin.