PROTOPLASTS FROM *NEUROSPORA CRASSA*[1]

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Weibull (1953) demonstrated that the intact protoplasts are liberated from cells of *Bacillus megaterium* after digestion of the cell wall by the enzyme lysozyme, and that these structures persist and exhibit many of the activities of the whole cell provided they are maintained in solutions of appropriate osmotic pressure. Since that time free protoplasts have been obtained from many other gram-positive bacteria through the action of lysozyme, whereas osmotically sensitive, protoplast-like bodies, probably possessing the remnants of a cell wall, have been obtained from a number of gram-negative bacteria by a variety of methods. The now extensive literature relating to free bacterial protoplasts and similar structures has been reviewed in detail recently by Weibull (1958).

Liberated protoplasts have proved to be of unique value in morphological, biochemical, and genetic studies of a number of the bacteria from which they have been obtained. It seems likely that homologous structures will be of equal value in studies of other groups of microoorganisms. We therefore set out to obtain protoplasts from the filamentous, coenocytic ascomycete, *Neurospora crassa*.

Nêcas (1956) obtained structures resembling fragmented free protoplasts from *Saccharomyces cerevisiae* as a result of (a) mechanical pressure exerted upon the cells and (b) partial autolysis of cultures. He reported the growth and ultimate regeneration of a small proportion of these structures. Eddy and Williamson (1957) liberated intact protoplasts from *S. cerevisiae* and *Saccharomyces carlsbergensis* by digestion of the cell wall with enzymes from the gut of the snail *Helix pomatia*. Emerson and Emerson (1958) have obtained protoplast-like structures from strains of *N. crassa* bearing the mutant gene os (osmotic) by the action of a commercial hemicellulase and of snail enzymes. These structures are relatively stable and will grow and regenerate under appropriate conditions. The fact that they can be obtained only from strains bearing this mutant gene renders them of somewhat limited value, however.

The present paper describes a similar enzymatic method which has consistently given a high yield of protoplasts from all wild type and mutant strains of *N. crassa* so far tested.

MATERIALS AND METHODS

The St. Lawrence wild type strain 74-A was used throughout most of this work. Stock cultures were maintained on slants of a chemically defined medium (Vogel, 1956) plus 2 per cent agar. Macroconidia were obtained by seeding 500 ml of the above solidified medium in a 1-L flask with a heavy suspension of conidia washed from a slant. Incubation was at 28 C for 3 to 4 days. Production of conidia was improved by reducing temperature the last 24 hr of incubation to around 25 C. Conidia were harvested by washing them from the medium and freeing them from the accompanying mycelium by filtration through glass wool. Young hyphae were obtained by inoculating 100 ml of the above liquid medium, contained in a 250-ml flask, with a heavy suspension of conidia washed from a slant culture and incubating on a rotary shaker at 28 C for 16 to 18 hr. The hyphae were harvested by centrifugation. Mutant strains of *N. crassa* were grown and harvested as described above, except that the appropriate growth factors were added to the minimal medium.

The enzyme preparation used was the "Suc digestif d'*Helix pomatia*" obtained from L'Industrie Biologique Francaise, 35 a 49, Quai du Moulin de Cage, Gennevilliers, Seine, France. The ampoules were kept frozen until used.

For light microscopy, a Spencer AO N-17 microscope was used. Photographs were taken with a Zeiss Opton microscope model W, using a phase contrast 40× lens.

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Microscopic observations on living material were made either on wet-mount preparations or in moist chambers prepared in the following manner: a layer of Vaseline was applied to the edge of a cover slip and a thin layer of nutrient agar medium, when desired, was applied to the center. A drop of the material to be examined was placed in the center of the cover slip and the cover slip was immediately inverted on a slide in such a manner that the Vaseline formed a seal, air was trapped in the preparation, and the drop of liquid touched the slide, providing a continuous phase for the light path.

EXPERIMENTAL RESULTS

Formation of protoplasts from conidia. Free protoplasts are obtained when conidia or hyphae are incubated in a solution containing 10 per cent enzyme preparation, 20 per cent sucrose and approximately 0.03 M phosphate buffer at pH 6.0. After about 1 hr of incubation at 28 C, the walls of conidia begin to bulge, assuming at first a lantern shape and later becoming spherical. If the conidium was initially rather oblong in shape, the protoplast may be seen to retract from the ends of the cell. After 2 to 3 hr incubation, the protoplasts begin to emerge from some of the conidia. They appear to be squeezed out through small pores that develop in the walls. As a rule, the entire contents of one conidium are extruded through one pore in the side wall to form one free protoplast. In many cases, the protoplasts do not emerge from the conidia, however, and remain as spherical bodies within the weakened walls. After 5 to 6 hr of treatment, the conidial walls become almost invisible, with only the cross-walls originally between conidia showing up clearly. This makes it difficult to distinguish free protoplasts from those still remaining within weakened walls. For these reasons, conidia do not represent the most satisfactory starting material for obtaining suspensions of free protoplasts.

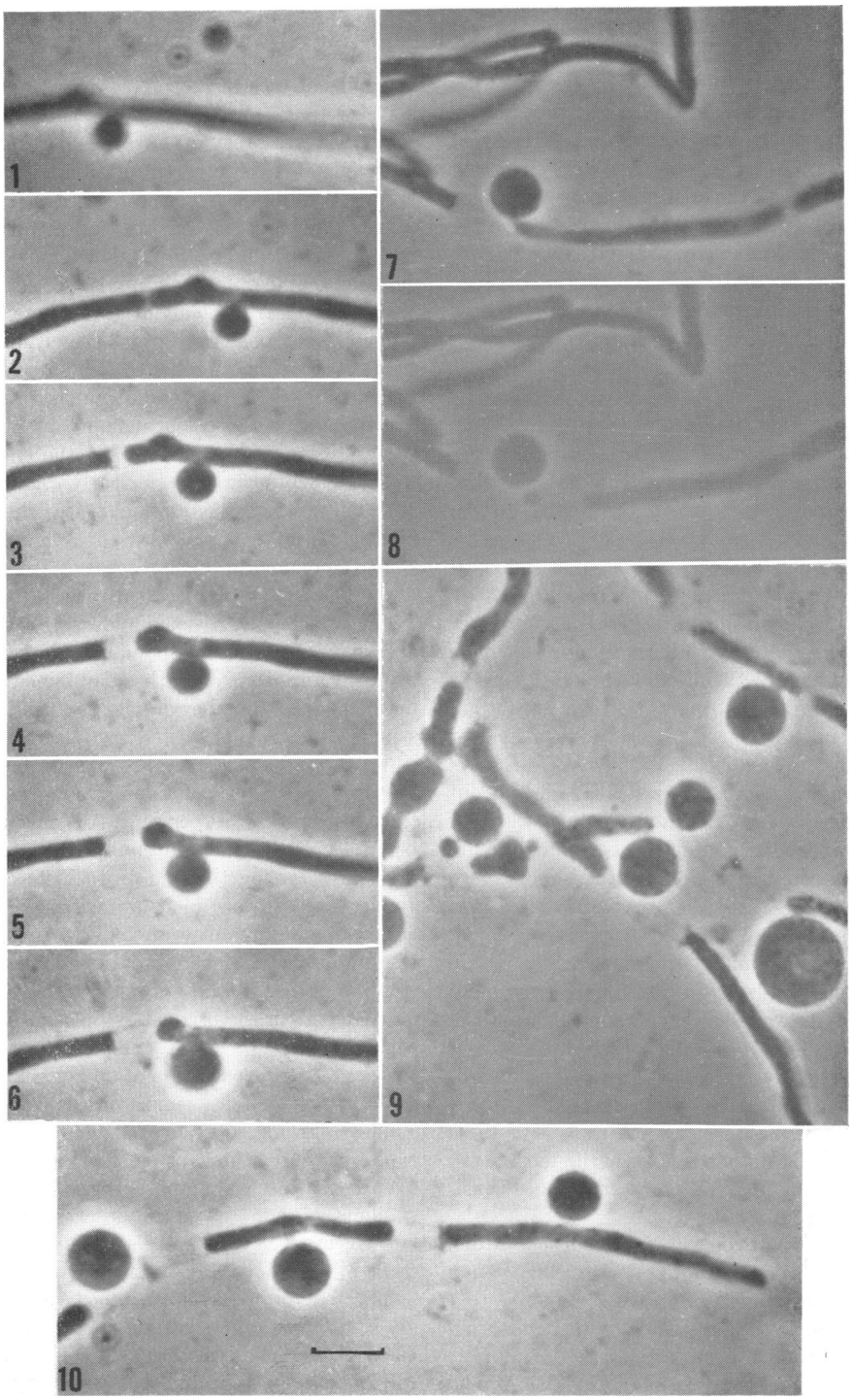
Formation of protoplasts from hyphae. Much better preparations are obtained using young hyphae as starting material. After about ½ to 1 hr incubation in the presence of 10 per cent snail preparation and 20 per cent sucrose, protoplasts begin to emerge through pores in the hyphal walls, which are distorted only slightly, if at all, during the treatment. Frequently, the entire contents of a hyphal compartment, between septa, are extruded as a single, large protoplast. On other occasions, the contents are extruded intermittently through one or several pores, giving rise to a number of protoplasts from one compartment. Most often, some portion of the contents of a compartment remains behind. Figures 1 through 8 consist of series of photographs showing the emergence of protoplasts from hyphae. The protoplasts, examined in the light microscope, appear granular, contain vacuoles, and appear to be about as dense as the contents of the hyphae from which they came. Sometimes the extruded hyphal contents disperse into the medium as diffuse granular material, except for the vacuoles, which remain intact. After the extrusion of the protoplasts, the empty cell walls and septa can be seen clearly (figures 9 and 10). They retain their original size, shape, and high refractility.

The emerging protoplasts measure from 5 to 15 μ in diameter; the young hyphae are usually 4 to 7 μ in width. Older mycelium measures 10 to 12 μ and gives rise to correspondingly larger protoplasts. Protoplasts have been obtained by this method from a number of wild type and mutant strains of *N. crassa* as listed in table 1.

Stability of protoplasts. Protoplasts have been liberated in sucrose solutions ranging in concentration from 5 to 30 per cent. At concentrations below 15 per cent, in addition to structures more nearly resembling the free protoplasts described above, large, watery blebs are extruded through large pores (apparently up to 12 μ in width) in the hyphal walls. All gradations appear from "empty" blebs to those appearing only slightly less dense than the original cytoplasm. At a concentration of 30 per cent, the protoplasts appear to shrink. A concentration of 20 per cent sucrose (0.59 M) was chosen for most of this work.

The following substances at 0.59 M concentration can substitute for sucrose as stabilizing agent: maltose, rhamnose, fructose, sorbose, sodium chloride (listed in order of decreasing effectiveness). Xylose and Carbowax 4000 proved to be ineffective at all concentrations tested.

The free protoplasts are sensitive to osmotic shock and lyse immediately when placed in distilled water. The lysis has been observed under the microscope repeatedly, by running distilled water under the cover slip of a wet mount, and at no time was it possible to observe a membrane of any sort remaining after the explosive disper-



Figures 1–10

TABLE 1

Strains of Neurospora crassa from which protoplasts have been prepared

Strain	Phenotype
St. Lawrence 74A	Wild type
Emerson 5256A	Wild type
Chilton a	Wild type
Lein 8a	Wild type
Abbott 4a	Wild type
S Y 7a	Wild type
Td 71-1-19A	Tryptophan requiring
39113-7	Albino; nicotinic acid requiring
31881-1-9a	Nicotinic acid requiring
Giles F 9 pan 1a	Adenine, pantothenate requiring
Giles F 10 pan 2a	Adenine, pantothenate requiring

sion of the cell contents. The vacuoles, however, remain intact for some time. They are the only recognizable entity surviving lysis.

The protoplasts retain their morphological integrity for days when stored at room temperature in the incubation mixture in which they were formed. They may be centrifuged, washed, and plated provided they are maintained in solutions of the appropriate sucrose concentration.

Regeneration of protoplasts. When transferred to a suitable liquid or solid nutrient medium, some of the protoplasts regenerate to give, ultimately, normal mycelial growth. The regeneration of individual protoplasts on solid defined medium has been followed microscopically in moist chambers (figures 11 to 28). The course of regeneration varies tremendously. Some protoplasts produce convoluted figures which may or may not give rise, eventually, to normal hyphae (figures 16, 17, 27, and 28), whereas others grow out directly into more or less normal hyphae (figure 18). During the course of regeneration a highly refractile layer appears around the protoplast. No growth of the protoplasts as such has been observed. Regeneration in a moist chamber usually requires from 8 to 12 hr before normal growth is established.

Quantitative plating experiments indicate that between 20 and 80 per cent of the protoplasts are capable of regenerating to give visible colonies when they are formed in the presence of 20 per cent sucrose and plated on minimal medium containing 20 per cent sucrose. The results of these experiments are not yet satisfactory due to clumping of the protoplasts and the inability to separate them from fragments of mycelium. The regenerated mycelium resembles in all respects the original strain from which the protoplasts were liberated.

DISCUSSION

The original meaning and current usage of the term "protoplast" has been discussed in an excellent critical note by Brenner *et al.* (1958). They point out that if the term is to be used in its original sense, i. e., to refer to that portion of a cell lying within the cell wall, then it must be established that no portion of the wall still surrounds structures referred to as protoplasts. They provide a set of criteria for establishing the nature of bacterial protoplasts.

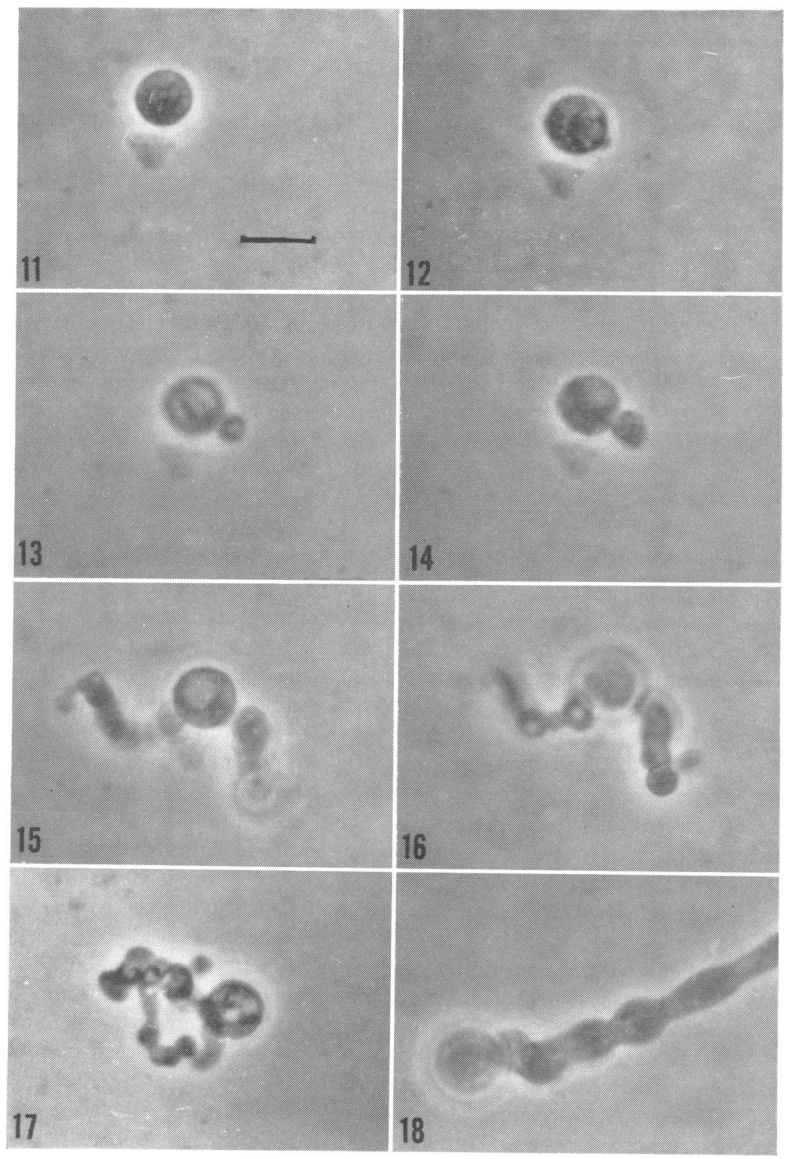
Not all of these criteria are applicable to the present case. The structures arising from Neurospora hyphae are spherical, in contrast to the cylindrical shape of the hyphae. They are sensitive to osmotic shock. The nature of the outer layer has not yet been examined in thin sections. Immunochemical tests relating to wall material in *N. crassa* are not available, nor is the chemistry of the wall material known in detail. Reaction with bacteriophage is not applicable. The enzyme used here obviously does not completely dissolve the wall; it seems possible that it attacks only a minor component.

Figures 1 through 10. The liberation of protoplasts. The scale length is given in figure 10 and represents 10 μ.

Figures 1 to 6. A single protoplast photographed at various times after the addition of snail extract to the reaction mixture: *figure 1*, after 137 min; *figure 2*, after 138 min; *figure 3*, after 141 min; *figure 4*, after 146 min; *figure 5*, after 148 min; and *figure 6*, after 196 min.

Figures 7 and 8. A second protoplast photographed 72 and 85 min after the addition of snail extract.

Figures 9 and 10. Show protoplasts of various sizes and empty cell walls after 82 and 78 min of incubation, respectively.

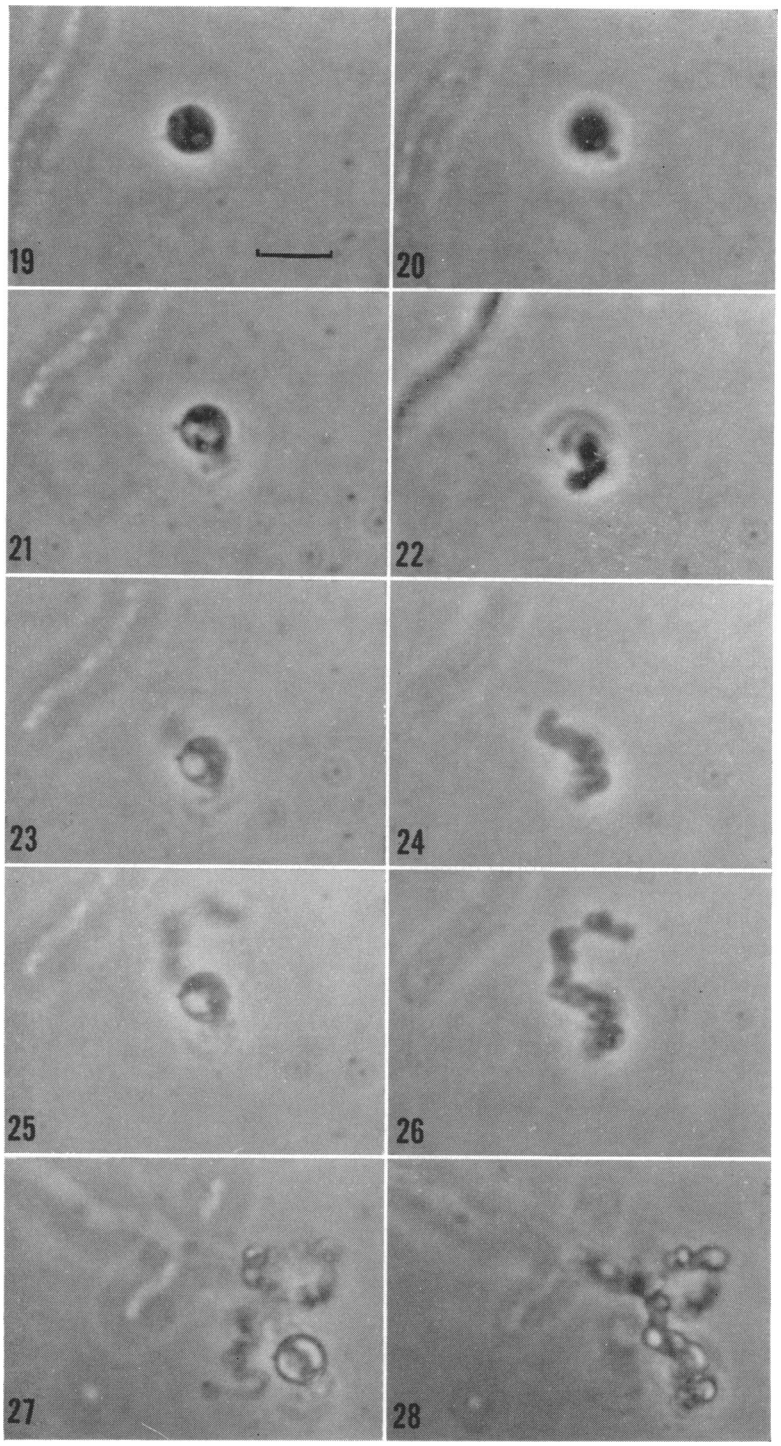


Figures 11 through 18. Regeneration of protoplasts. The scale length is given in figure 11 and represents 10 μ.

Figures 11 to 16. Show the regeneration of a single protoplast photographed at various periods after being placed on minimal medium in a moist chamber: *figure 11*, after 1 hr; *figure 12*, after 7 hr; *figure 13*, after 9 hr; *figure 14*, after 10 hr; and *figures 15 and 16*, same protoplast photographed at two levels after 14 hr.

Figure 17. Another protoplast from the same preparation showing convoluted growth after 24 hr.

Figure 18. Another protoplast from the same preparation showing regeneration into normal mycelium after 24 hr.



Figures 19 through 28. Show the regeneration of a single protoplast photographed at two levels at various times after being placed on minimal medium in a moist chamber. The scale length is given in figure 19 and represents 10 μ: *figures 19 to 20,* after 3 hr; *figures 21 and 22,* after 7 hr; *figures 23 and 24,* after 8 hr; *figures 25 and 26,* after 9 hr; and *figures 27 and 28,* after 24 hr, showing the normal mycelium arising from this protoplast extending across the upper left of the figures.

The fact that the empty wall can be seen after emergence of these spherical structures does argue for the conclusion that they actually represent naked protoplasts. So, too, does the fact that no distinct membrane is seen after lysis. Such structures must be, in a sense, artifacts, when arising from a coenocytic organism such as Neurospora in which the cytoplasm is continuous throughout the filaments. Even if a compartment between septa be regarded as analogous to a single cell of a cellular organism, the protoplasts in many cases represent artifacts, since the contents of one compartment may give rise to more than one protoplast. If the outermost layer of these structures is, indeed, the cytoplasmic membrane, then this membrane is a structure of considerable versatility for it must be broken and reformed in this process and probably becomes stretched or increases in quantity.

The formation of these structures converts the coenocytic mycelium of Neurospora into units which can be handled quantitatively by many of the techniques used for unicellular microorganisms.

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SUMMARY

Spherical, osmotically sensitive, protoplasts are liberated from conidia and hyphae of *Neurospora crassa* by the action of a commercial enzyme preparation from the snail, *Helix pomatia*. These structures are stable when maintained in solutions containing 20 per cent sucrose, and, in a suitable liquid or solid medium, may regenerate to give normal mycelial growth.

This method can be used to obtain protoplasts from all wild type and mutant strains of *N. crassa* so far tested. The protoplasts can be seen to emerge from the rigid conidial or hyphal walls.

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