[24] L. Michaelis, in J. B. Sumner and K. Myrbäck, *The Enzymes*, Vol. 2, Part 1 (New York: Academic Press, 1951).

[25] H. Theorell, in R. Stoope, *Les Protéines* (Brussels: Solvay Institute, 1953).

[26] R. S. Mulliken, *J. Am. Chem. Soc.*, **74**, 811, 1952.

[27] R. S. Mulliken, *J. Phys. Chem.*, **56**, 801, 1952.

[28] E. M. Kosower, *J. Am. Chem. Soc.*, **78**, 3497, 1956.

[29] T. Förster, *Fluorescenz organischer Verbindungen* (Göttingen: Vandenhoeck & Ruprecht, 1951).

[30] F. W. J. Teale and G. Weber, *Biochem. J.*, **65**, 476, 1957.

[31] G. Karreman and R. H. Steele, *Biochem. et Biophys. Acta*, **25**, 280, 1957.

[32] G. Karreman, R. H. Steele, and A. Szent-Györgyi, these PROCEEDINGS, **44**, 140, 1958.

PRODUCTION, REPRODUCTION, AND REVERSION OF PROTOPLAST-LIKE STRUCTURES IN THE OSMOTIC STRAIN OF NEUROSPORA CRASSA

BY STERLING EMERSON AND MARY R. EMERSON

CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

Communicated by G. W. Beadle, May 16, 1958

Protoplasts devoid of cell walls have been produced in *Bacillus megaterium* and certain other Gram-positive species of bacteria.[1] Structures resembling protoplasts but not completely devoid of cell walls have also been produced in *Escherichia coli* and some other Gram-negative bacteria.[2] Those from Gram-positive and Gram-negative bacteria are alike in that they are spherical in shape and are lysed by osmotic shock. Bacterial protoplasts have already proved to have many useful applications—among others, in the extraction of cellular constituents, in studies of the biosynthesis of enzymes and other macromolecular substances, and in host-parasite interrelations.[3]

Protoplasts, or protoplast-like structures, have also been produced in one of the higher fungi, the unicellular Ascomycete, *Saccharomyces cerevisiae*.[4] The present paper reports the production and reproduction of coenocytic protoplast-like structures in one strain of the filamentous Ascomycete, *Neurospora crassa*.

Strain Specificity.—Under the conditions employed, protoplasts have consistently been formed by all tested strains carrying the osmotic mutant gene, *os*.[5] An exhaustive survey has not been made, but all tested strains carrying the wild-type allele of *os* have either been completely refractory or have responded poorly. The only non-osmotic strains which have so far yielded protoplasts are two with maternally inherited cytochrome abnormalities, *poky*[6] and *mi-3*,[7] which appear to require higher enzyme concentrations than strains carrying *os* and, even then, are erratic in response. Descriptions to follow refer to *os* strains.

Treatment.—Culture media in which protoplasts have been produced consist of the standard *Neurospora* salt mixture and biotin,[8] sugars, and either a commercial hemicellulase preparation[9] or, in a few tests, a crude preparation of snail hepatic juice.

While the concentrations of enzymes, salts, and sugars do influence the ease with which protoplasts are produced and maintained, no clear end-point for dilution

has been observed in either their production or their maintenance. Protoplasts have been produced in media containing as little as 0.5 per cent (*w/v*) hemicellulase, 1 per cent sucrose, and the standard salt mixture. In most experiments, however, media have contained 2 or 3 per cent hemicellulase, 2 per cent sucrose, 5–10 per cent rhamnose or sorbose, and two to four times the standard strength of the salt mixture. Protoplasts are more readily maintained at the higher concentrations of sugars and enzymes and are more uniformly lysed by osmotic shock than those cultured at lower concentrations.

Origin, Growth, and Reproduction of Protoplasts.—When hyphae or hyphal fragments are transferred to hemicellulase media, protoplasts are extruded through pores in the side or end walls of the hyphal cells. Monilioid conidia (macroconidia)

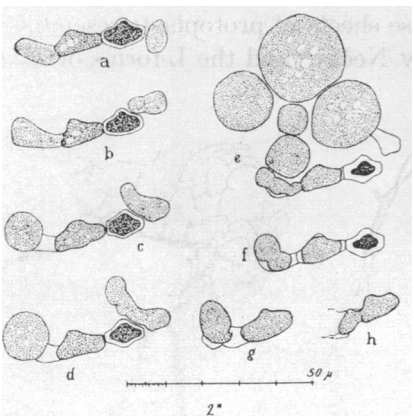

FIG. 1.—Camera lucida drawings of successive stages in protoplast development from two of three conidia; the conidium in the middle is degenerating. Conidia of osmotic strain Em 11200a in 10 per cent rhamnose, 2 per cent sucrose, 3 per cent hemicellulase concentrate, 3.2× standard salt mixture, in a small drop under oil at room temperature. Elapsed times in hours and minutes: inoculation to *a*, 4:15; *a* to *b*, 0:25; *b* to *c*, 1:30; *c* to *d*, 1:25; *d* to *e*, 13:30; *e* to *f*, 0:13; *f* to *g*, 0:30; *g* to *h*, 0:20.

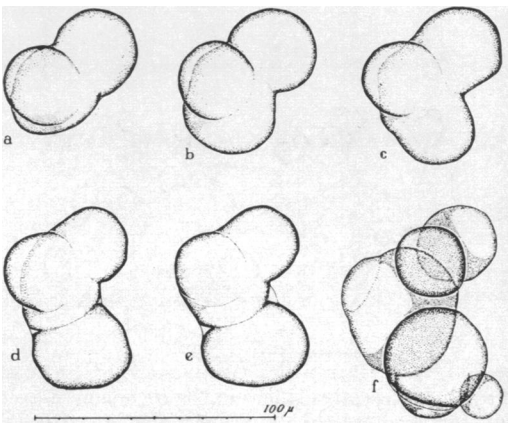

FIG. 2.—Growth of a protoplast subcultured in 10 per cent sorbose, 2 per cent sucrose, 2 per cent hemicellulase concentrate, 4× standard salt mixture, in a small drop under oil at room temperature. Elapsed times: inoculation to *a*, 20:05; *a* to *b*, 1:37; *b* to *c*, 1:48; *c* to *d*, 1:05; *d* to *e*, 0:50; *e* to *f*, 21:15.

germinate directly into protoplasts (Fig. 1). Protoplasts produced directly from conidia seem to be less uniform than those arising from hyphae, especially at low concentrations of sugars and enzymes, and are more likely to revert to hyphal-type growth in the media in which they were produced.

Newly formed protoplasts are spherical, average about 10 μ in diameter, and contain several nuclei. Most protoplasts increase in diameter to about 50 μ, some to 100 μ, and contain a hundred or more nuclei. Upon lysis in distilled water, the cell contents disperse rapidly, leaving delicate membranes, or cell walls, the constitution of which has not been determined. Protoplasts produced by snail enzymes are also readily lysed by osmotic shock, but much more prominent walls remain.

In older cultures many protoplasts become highly vacuolate. When these are

lysed, the discharged vacuoles remain visible and increase in diameter to as much as 50 μ without bursting. Membranes of discharged vacuoles show remarkable elasticity when poked with fine glass needles. Upon transfer to fresh media, highly vacuolated protoplasts continue growth and division.

Division of protoplasts commonly occurs as a simple pinching in two by a furrowing process (Fig. 2) or sometimes by a process resembling the budding of yeasts (similar to that illustrated in Fig. 3). Daughter protoplasts are at first contained within the membrane of the parental cell, but they separate readily, either spontaneously or upon gentle agitation.

When protoplasts are grown on an agar surface or on glass, they often become greatly flattened, irregularly shaped, and closely packed into a thin sheet (similar to the nonreverted cells in Fig. 4). Division of protoplasts under these conditions continues by rather irregular furrowing. These sheets of protoplasts resemble the multinucleate protoplasts of yeasts figured by Nečas,[4] and the L-forms of *E. coli* described by Lederberg.[10]

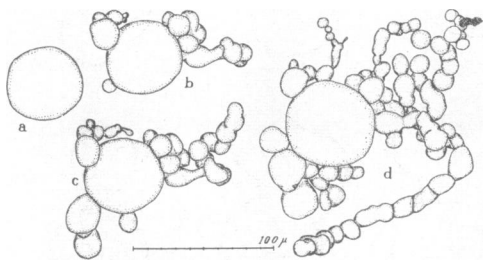

FIG. 3.—Successive stages in the reversion of a protoplast to hyphal-type growth after transfer from hemicellulase medium to 10 per cent sorbose, 2 per cent sucrose, 4× standard salt mixture (osmotic concentration 30.7 atm.), in 0.2 per cent agar under oil at room temperature. Elapsed times: inoculation to *a*, 0:15; *a* to *b*, 6:23; *b* to *c*, 2:22; *c* to *d*, 11:50.

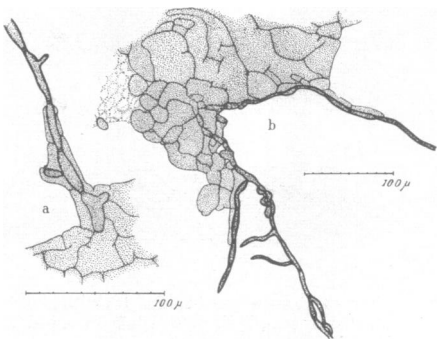

FIG. 4.—Portions of a flat pseudo-parenchymatous colony developed from protoplasts on an inverted cover-glass mount, showing transition from parenchymatous to hyphal growth, about 48 hours after transfer from a hemicellulase medium to one containing 5 per cent sucrose and the standard salt mixture (osmotic concentration 7.5 atm.).

Reversion to Hyphal-Type Growth.—Upon transfer of protoplasts to media lacking enzymes, they revert to typical mycelial growth, though the time necessary for complete reversion varies from a few hours to several days. The type of mycelial growth produced by *os* strains depends upon the osmotic strength of the medium.[5] In media of less than 5 atm. osmotic pressure, long, thin, sparsely branched hyphae are produced (as in Fig. 4). At higher osmotic concentrations, there result progressively more contorted and more branched hyphae, with short, broad cells (Fig. 3). Individual protoplasts sometimes produce one or more hyphae as direct outgrowths of the mother cell. In other instances (as in Figs. 3 and 4) a considerable amount of parenchymatous tissue is first formed, from which hyphae differentiate. Variations in response to the absence of enzymes, in respect to both the time required for reversion and the growth forms produced, may reflect the degree of somatic differentiation of individual protoplasts from hyphal-type growth, as well as differences in the cultural conditions under which they had developed.

Appearances of regeneration from naked masses of protoplasm have sometimes been noted, but never in examples in which it had been established that there were no small, fully formed cells from which the growth might have arisen. It is, of course, possible that cultural conditions will be found under which masses of naked protoplasm will revert to cellular growth, such as has been reported by Nečas[4] in *Saccharomyces cerevisiae* and by Pease[11] in *Proteus vulgaris*.

[1] C. Weibull, *Symposium Soc. Gen. Microbiol.*, **6**, 111, 1956.

[2] J. Lederberg, these PROCEEDINGS, **42**, 574, 1956; K. McQuillen, *Biochim. et Biophys. Acta*, **27**, 410, 1958.

[3] K. McQuillen, *Symposium Soc. Gen. Microbiol*, **6**, 127, 1956; S. Spiegelman, in *The Chemical Basis of Heredity*, ed. W. D. McElroy and B. Glass (Baltimore: Johns Hopkins Press, 1957), pp. 232–267; J. Spizizen, these PROCEEDINGS, **43**, 694, 1957; D. Fraser, H. R. Mahler, A. L. Shug, and C. A. Thomas, Jr., these PROCEEDINGS, **43**, 939, 1957; E. Chargaff, H. M. Schulman, and H. S. Shapiro, *Nature*, **180**, 151, 1957.

[4] O. Nečas, *Biol. Zentr.*, **75**, 268, 1956; A. A. Eddy and D. H. Williamson, *Nature*, **179**, 1252, 1957.

[5] Mary R. Emerson, in manuscript. Our mutant strain, Em 11200, has been shown to be allelic to a number of others of independent origin: B 135 and M 16 obtained from Dr. D. D. Perkins, Stanford University; "wooly," from Dr. H. L. K. Whitehouse, Cambridge University; and "ginger" and an unnamed mutant from Dr. J. R. S. Fincham, University of Leicester. From published descriptions it is likely that these are also allelic to the "cut" mutant of Dr. H. Kuwana (*Cytologia*, **18**, 235, 1953).

[6] Mary B. Mitchell and H. K. Mitchell, these PROCEEDINGS, **38**, 442, 1952.

[7] Mary B. Mitchell, H. K. Mitchell, and A. Tissieres, these PROCEEDINGS, **39**, 606, 1953.

[8] G. W. Beadle and E. L. Tatum, these PROCEEDINGS, **27**, 499, 1941.

[9] The hemicellulase preparation was obtained from the Nutritional Biochemical Corporation, Cleveland 28, Ohio, who inform us that it is a concentrate of microbiological origin, containing cellulase, gumase, and maltase in addition to hemicellulase. A single test in our laboratory indicated strong chitinase activity.

[10] J. Lederberg and Jacqueline St. Clair, *J. Bacteriol.*, **75**, 143, 1958.

[11] Phyllis Pease, *J. Gen. Microbiol.*, **17**, 64, 1957.

---

*THE REPLICATION OF DNA IN ESCHERICHIA COLI**

BY MATTHEW MESELSON AND FRANKLIN W. STAHL

GATES AND CRELLIN LABORATORIES OF CHEMISTRY,† AND NORMAN W. CHURCH LABORATORY OF CHEMICAL BIOLOGY, CALIFORNIA INSTITUTE OF TECHNOLOGY, PASADENA, CALIFORNIA

Communicated by Max Delbrück, May 14, 1958

Introduction.—Studies of bacterial transformation and bacteriaphage infection[1–5] strongly indicate that deoxyribonucleic acid (DNA) can carry and transmit hereditary information and can direct its own replication. Hypotheses for the mechanism of DNA replication differ in the predictions they make concerning the distribution among progeny molecules of atoms derived from parental molecules.[6]

Radioisotopic labels have been employed in experiments bearing on the distribution of parental atoms among progeny molecules in several organisms.[6–9] We anticipated that a label which imparts to the DNA molecule an increased density might permit an analysis of this distribution by sedimentation techniques. To this end, a method was developed for the detection of small density differences among