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Fine Structure of Protoplasts and L-Forms of *Clostridium botulinum* Types A and E[1]

GEORGE W. BROWN, JR., J. L. PATE, AND H. SUGIYAMA

Department of Bacteriology and the Food Research Institute, University of Wisconsin, Madison, Wisconsin 53706

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Spherical bodies, that are obtained by adding penicillin and lysozyme to *Clostridium botulinum* types E and A cultures which are growing in an osmotically stabilized medium, are shown to be protoplasts by electron microscopy. The L-forms of these two culture types have morphologically different inclusion bodies.

Cultures of *Clostridium botulinum* types E and A are changed into suspensions of protoplast-like spheres when penicillin and lysozyme are added to cultures which are growing logarithmically in a broth with polyethylene glycol osmotic stabilizer. The converted forms can be propagated as L-form cultures (1). The present communication describes the electron microscopic observations during the morphological transformation of type E cultures and considers the ultrastructure of the derived spheres and L-forms.

The procedures for protoplasting and obtaining L-forms have been described previously (1). The spheres were induced with 1,250 units of penicillin G and 100 μg of lysozyme per ml of medium. The stable L-forms were grown in 40 ml of biphasic medium; the type E culture had been transferred 100 times and type A 75 times at 3- to 4-day intervals.

Type E cultures at different stages of conversion treatment were centrifuged for 5 min at 3,000 $\times$ *g*, and the sedimented cells were treated with osmium tetroxide (RK fixation; 4). Alternatively, double fixation was used: the pellet of cells, while chilled on cracked ice, was fixed for 2 hr (mixture of 1 ml of 0.2 M, *p*H 7.2, phosphate buffer; 0.5 ml of 5% osmium tetroxide; and 1.5 ml of 2.5% glutaraldehyde) and then washed with 0.66 M phosphate buffer (*p*H 7.2). Only the RK fixation was used on the 20-hr-old L-form cultures.

The fixed cells were mixed with molten agar on a glass slide. After the agars solidified, they were cut into cubes. Those with the RK-fixed specimens were washed with uranyl acetate for 2 hr at 25 C. The cubes were dehydrated and embedded (5). Thin sections, obtained with a Porter-Blum Sorvall MT-2 microtome, were mounted on 200- or 300-mesh copper grids which had been coated with carbon and parlodion. Sections of specimens given double fixation were stained 10 min with uranyl acetate and lead citrate; those of RK fixation were stained 10 min with lead citrate. Examinations were made with a Zeiss EM 9A electron microscope at original magnifications of 1,700 to 25,000.

Normal vegetative cells have a cell wall which appears trilaminated by the double-fixation procedure (dark-light-dark staining), and many contain inclusion bodies (Fig. 1). During the first 30 min after the addition of the converting agents, many cells show a break in the cell wall through which the intact cytoplasmic mass escapes (Fig. 2). This corresponds to the phase-contrast microscopic observation in which the rods develop a swelling or bulge and gain the appearance of "rabbit ears" (1). The number of cells which undergo this change reaches a maximum during the next 30 min. The conversion process is completed by about 3 hr, at which time intact rods are seldom found (Fig. 3). That all the original rods are not changed into spherical forms is indicated by the lysis that is evident.

Absence of cell wall constituents as shown by procedures such as chemical analyses is needed for final proof, but the intact spheres (Fig. 4) obtained are considered on morphological grounds to be protoplasts. Cell walls which are left behind by the osmotically fragile units usually have the three layers seen in the walls of the parent form (Fig. 1, 2). The complete disappearance of such wall casings upon continued incubation indicates that cell wall remnants, if present on the escaping spheres, would be dissolved subsequently. Additionally, suspensions of these spheres can be cultured directly into stable L-form cultures. The observations on the cell wall of spheres present in type A cultures after the 8-hr treatment required for maximum conversion were similar.

Approximately 50% of the intact cell units

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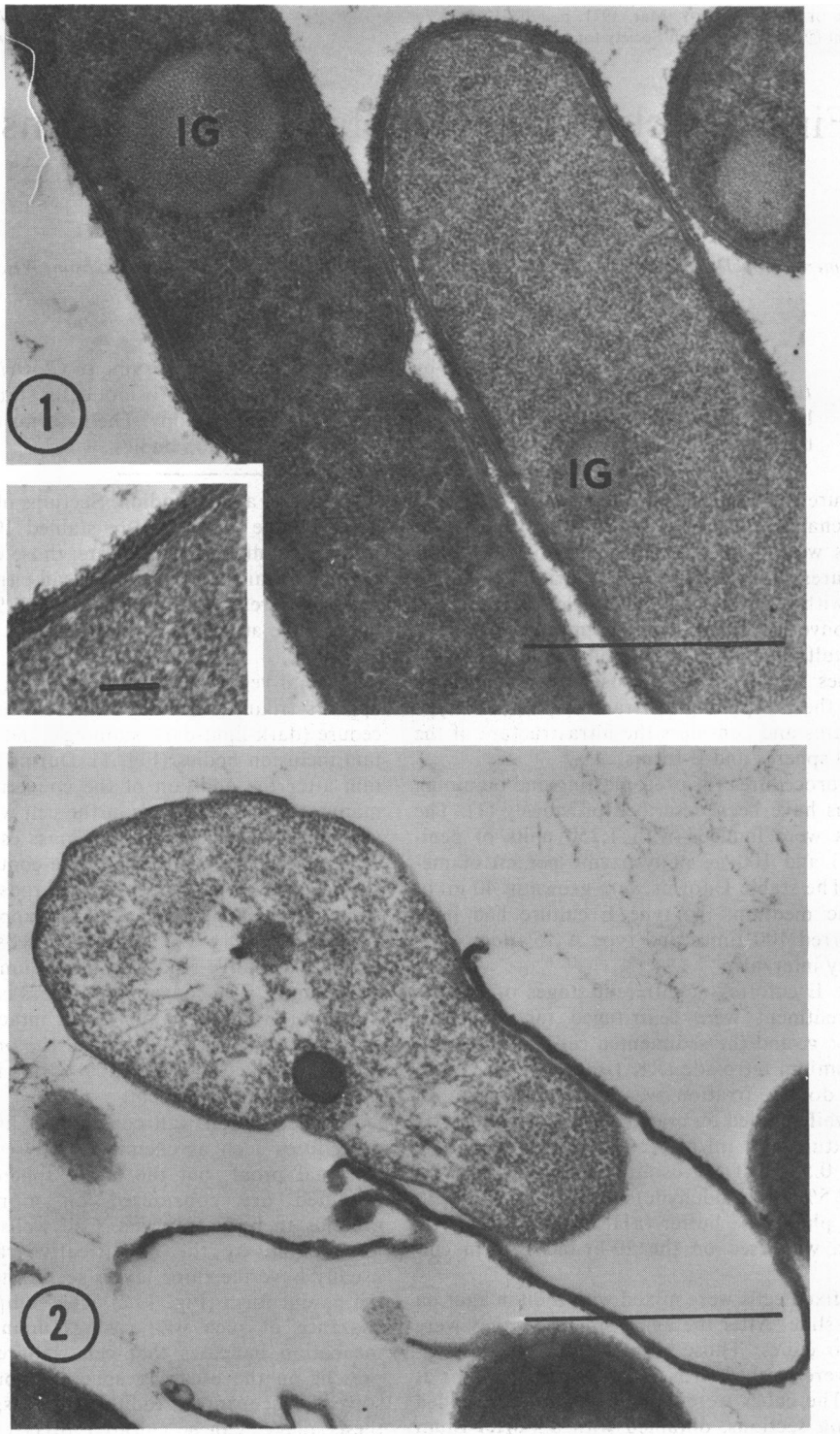

FIG. 1. *Thin section of normal cells of C. botulinum type E in an 18-hr-old culture showing inclusion granules (IG) and the three layers of the cell wall (inset). Bar for inset, 0.1 μm; others, 1 μm.*

FIG. 2. *Protoplast of type E escaping through the ruptured cell wall 30 to 60 min after addition of penicillin and lysozyme. Bar, 1 μm.*

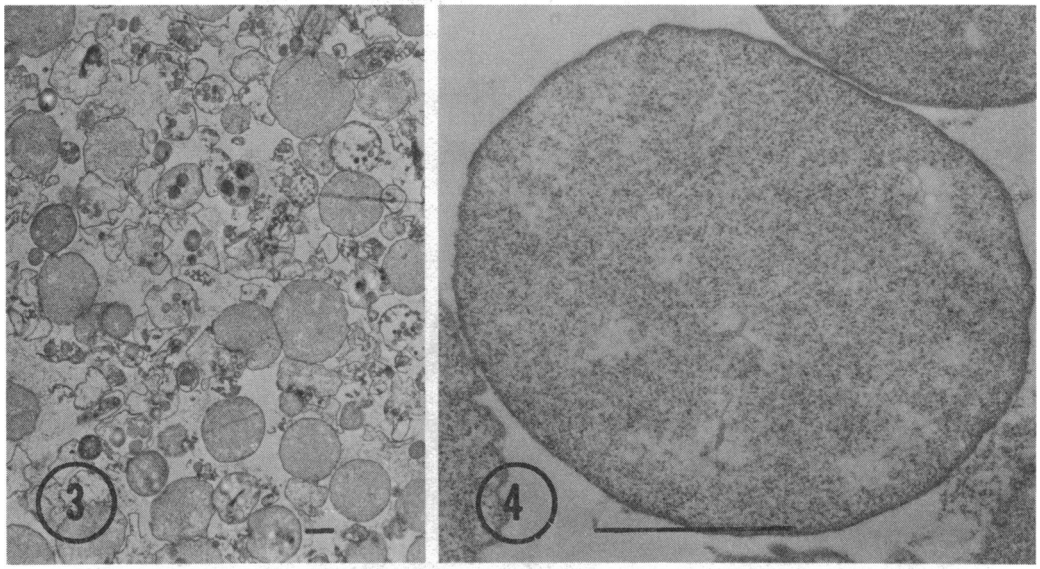

FIG. 3. *Thin section of type E culture after 2.5 to 3 hr of conversion treatment. Numerous inclusion granules as well as lysis of some protoplasts are seen. Bar, 1 μm.*

FIG. 4. *Intact protoplast obtained after 90-min conversion treatment. Bar, 1 μm.*

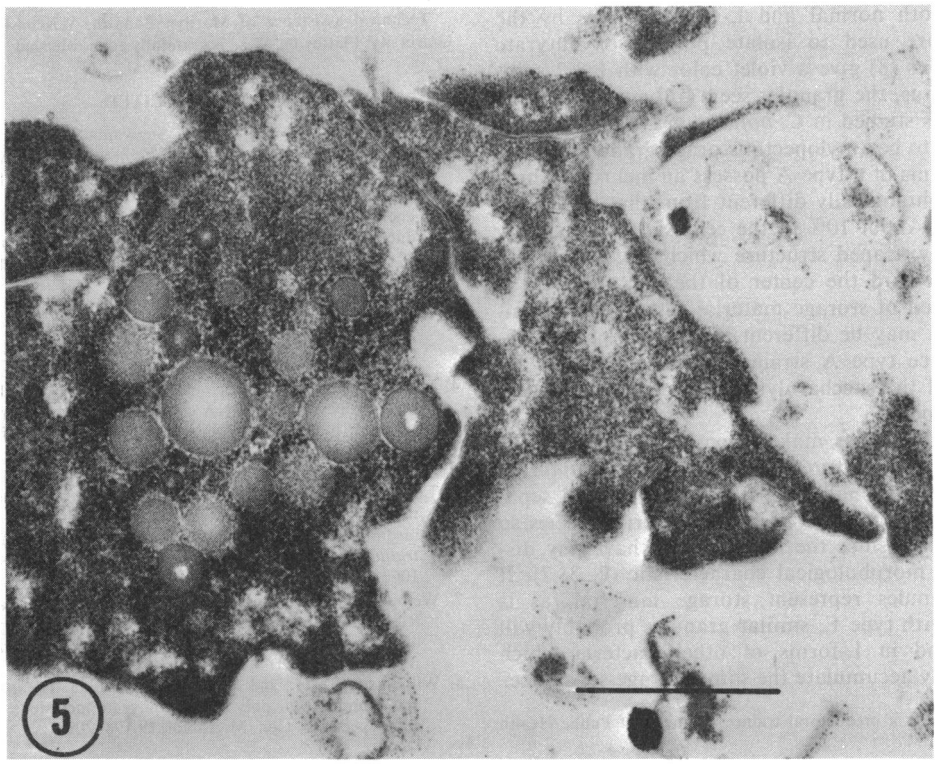

FIG. 5. *Lobular shaped L-form of type E with numerous inclusion granules. Bar, 1 μm.*

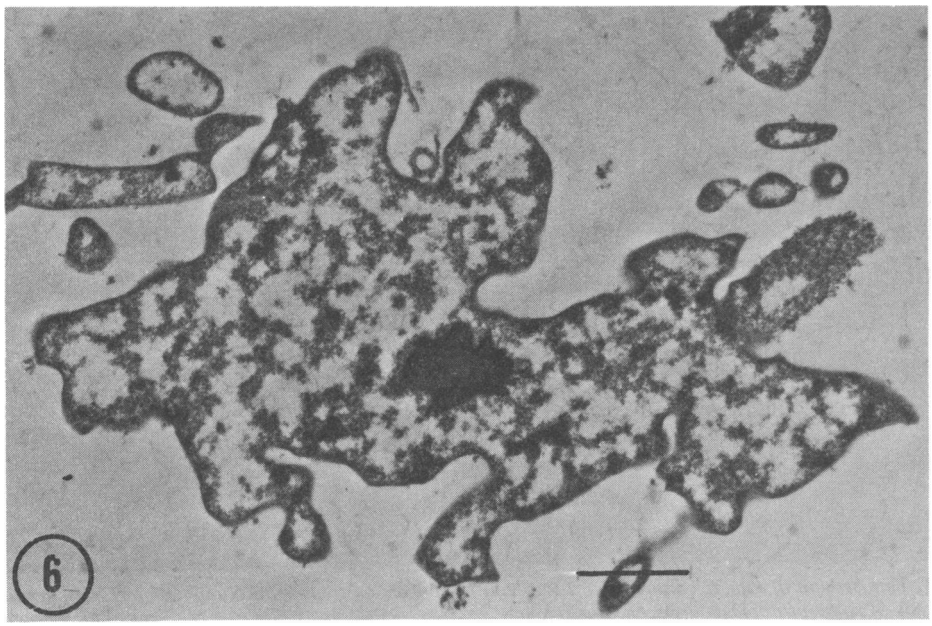

FIG. 6. *L-form of type A having an unidentified, dark-staining structure near the center. Bar, 1 μm.*

found in 20-hr-old L-form cultures of type E have single to several granules (Fig. 5) which are morphologically indistinguishable from those in normal vegetative cells. The granules obtained from both normal and L-form cultures by the procedure used to isolate polyhydroxybutyrate inclusions (8) give a violet color with I_2-KI solution. Thus, the granules seem to be the iodiphilic granules studied in *C. botulinum* type E and considered to be amylopectin storage granules (6).

L-forms of a type A possess an inclusion which is morphologically different from that present in type E. About 10% of the cells show a single, irregularly shaped structure which is generally located toward the center of the cell (Fig. 6). If composed of storage material, its chemical composition may be different from the type E granules since type A strains have a proteolytic instead of the saccharolytic metabolism characterizing type E.

The inclusions make it possible to distinguish the L-form cultures of the two *C. botulinum* types. Additionally, these L-forms can be separated from those of all other bacterial species so far studied since the latter do not have any distinctive morphological characteristic (2, 3, 7). If the granules represent storage material, as is likely with type E, similar granules probably will be found in L-forms of other bacteria which normally accumulate the same storage substance.

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