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Penicillin-Lysozyme Conversion of *Clostridium botulinum* Types A and E into Protoplasts and Their Stabilization as L-Form Cultures[1]

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When logarithmically growing cultures of *Clostridium botulinum* types A and E are treated with penicillin in a liquid medium containing 8% polyethylene glycol, protoplast-like spherical bodies are formed. The penicillin effect shows a dose-response relationship; the largest yield of converted forms is obtained with 10,000 units/ml, but the treatment leaves many intact bacilli. Lower antibiotic concentrations produce smaller numbers of spherical bodies, but lysis of bacilli results in suspensions that are relatively free of rods. Cells grown under the same conditions and treated with 250 μg of lysozyme/ml do not form spherical bodies. However, a combination of 1,250 to 2,500 units of penicillin and 100 μg of lysozyme/ml yields suspensions which have sphere counts in excess of 1.0×10^8/ml and only a few intact rods. The state of the culture at the time of addition of the antibiotic and enzyme is critical. Suspensions of these protoplasts can be adapted to grow as stable L-form cultures producing the same toxin type as the parent cultures.

Bacterial protoplasts and spheroplasts are osmotically sensitive forms which lack rigid cell walls. Two approaches have been used in their preparation (1). The formed cell wall may be completely or partially removed by enzymes, such as lysozyme, which attack cell wall components to give protoplasts and spheroplasts, respectively. Alternatively, cell wall synthesis may be inhibited by the use of antibiotics such as penicillin. These methods have been employed with success on a variety of aerobic bacteria, but anaerobic spore-formers have been largely ignored. *Clostridium butyricum*, cultured in the presence of penicillin changed into spheroidal and polyhedral bodies which apparently lacked cell rigidity (6). *C. botulinum* type A cells when treated with lysozyme gave endospore-containing "spheroplasts" (2) and when treated with an autolysin became osmotically sensitive forms believed to be spheroplasts (7).

L-forms, the pleomorphic forms capable of multiplying in spite of their cell wall defects (1), of *C. botulinum* have not been described.

This communication describes the conversion of actively growing *C. botulinum* type A and E cells into protoplast-like bodies with a combination of penicillin and lysozyme. The adaptation of these spheroidal cells to multiply as L-forms is described.

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MATERIALS AND METHODS

Organisms and media. *C. botulinum* type E, strain Alaska E43, was used for most of the study, but five other type E strains (Iwanai, VH, Detroit, 3561, and 4521) and the nontoxigenic S9 strain (5), related to type E, were also tested. Strain 109 was taken as representative of *C. botulinum* type A. Incubation was at 30 C for type E and 37 C for type A.

The basic medium was TPGY (5% Trypticase, 0.5% peptone, 0.5% glucose, 0.1% yeast extract, 0.2% sodium thioglycolate, *p*H 7.2). The medium for converting type E was TPGY with 8% polyethylene glycol (PEG), molecular weight 4,000 (Ruger Chemical Co., Irvington-on-Hudson, N.Y.). Conversion of type A was done in the M1 medium (5% Trypticase, 4% proteose-peptone, 2% yeast extract, 1% glucose, 0.1% sodium thioglycolate) used for the preparation of boticin E (5; Kautter, *personal communication*). The osmotic stabilizer was also 8% PEG. Media over 5 days old were not used because they tended to give inconsistent results.

L-forms were grown in one of two media. A biphasic medium consisted of a slant made with 4 ml of TPGY-salt agar (TPGY containing 1% each of NaCl and Ionagar No. 2, Consolidated Lab., Chicago Heights, Ill.) covered with 6 ml of TPGY containing 1% NaCl. A semisolid medium was TPGY with 1% NaCl and 0.5% agar.

Stock solution of penicillin G (E.R. Squibb and Sons, New York, N.Y.) was 100,000 units/ml in

0.85% NaCl. Lysozyme chloride, three times crystallized (Nutritional Biochemicals, Cleveland, Ohio), dissolved in 0.85% saline at 5 mg/ml was sterilized by membrane filtration (Millipore Corp., Bedford, Mass.)

Protoplasting procedure. Cultures to be converted were bubbled with gas during incubation. Erlenmeyer flasks of 50 ml capacity were tightly fitted with a rubber stopper having two glass tubings. The end of one tube was drawn out to a capillary (Pasteur disposable pipettes, Fisher Scientific Co., Pittsburgh, Pa.) and reached the bottom of the flask; the opposite end was connected to a tank of 95% N_2-5% CO_2. The second short tube served as a gas escape. Both tubes were plugged with cotton at appropriate positions to prevent contamination. By connecting to a manifold, a series of flasks could be gassed simultaneously. The gas flow per flask was about 0.7 liter/hr.

Cultures were activated by taking a culture incubated in TPGY overnight and serially transferring it three times at 6- to 8-hr intervals (2 ml into 10 ml of fresh broth). The last culture was incubated for 8 hr, and 3 ml was inoculated into the 27 ml of TPGY-8% PEG in the flasks. The flasks were then incubated for 4.5 to 5 hr with continuous gassing. The protoplasting agents were added to these logarithmically growing cultures, and the final volume was made to 40 ml by additions of 6 ml of double-strength protoplasting broth and the required amount of single-strength TPGY-8% PEG. The cultures were gassed for 5 additional minutes and were then incubated without agitation. The yields of protoplast-like bodies were counted in a Petroff-Hausser chamber with the use of a phase-contrast microscope.

Experiments with larger volumes of culture in an apparatus similar to that of Willingham and Oppenheimer (14) permitted frequent measurements of optical density. The test culture was obtained by adding a 10% inoculum of the serially transferred culture into the selected volume of protoplasting medium. The changes in turbidity were determined in 0.5-inch (1.27 cm) diameter curvettes at 600 nm in a Spectronic 20 spectrophotometer (Bausch & Lomb, Rochester, N.Y.). Dilution of the PEG by the volumes of penicillin and lysozyme was compensated with double-strength broth.

RESULTS

Penicillin alone. Initial experiments with sucrose as the osmotic stabilizer were unproductive. Type E grew well in TPGY-8% PEG, but no significant conversion of rods into spheres followed the additions of a few units of penicillin to still cultures which were in the logarithmic growth phase. High concentrations of 5,000 to 10,000 units/ml gave rise to some protoplast-like bodies.

When the experiment was repeated with the more synchronous cultures obtainable by gassing, appreciably higher numbers of spheres were produced with the high penicillin levels. It also became evident that the age of the culture at the time of antibiotic addition was important. When treated with 5,000 or 10,000 units of penicillin/ml, extensive lysis of the bacilli and developing spheres occurred in cultures younger than 4.5 hr; if older than 5.0 hr, larger numbers of rods showing no apparent change persisted. All later experiments were performed on cultures prepared for protoplasting by 4.5 to 5.0 hr of incubation with gassing. More recent experiments indicate that best conversion occurs when the culture attains an optical density of 0.50 to 0.55 at 5 hr of incubation.

Penicillin concentration effects were evident (Fig. 1). Maximum sphere counts were produced with 10,000 units/ml, but the suspensions had many intact bacilli, whereas suspensions obtained with 5,000 units/ml produced fewer spheres but had proportionately fewer rods. As in the conversion experiments with cultures obtained without agitation, widespread lysis occurred in cultures treated with penicillin levels of 2,500 units or lower per milliliter, so that intact cells of all forms were often absent after 3 hr.

Lysozyme alone. Less than 1% of the washed cells of types A and E were changed into spherical bodies when treated with lysozyme by the procedure used to obtain *Bacillus megaterium* protoplasts (13). When rapidly growing type E cultures were treated for 3 to 5 hr with up to 250 μg of lysozyme/ml, no morphological changes observable by phase-contrast microscopy occurred. Type A culture showed some lysis but did not give rise to spherical bodies.

Penicillin-lysozyme combination. The possibility that the cell wall formed in the presence of penicillin might be susceptible to lysozyme was tested and confirmed by the simultaneous addition of the two agents to logarithmically growing cultures. The numbers of spheres produced were higher for a given penicillin concentration when lysozyme was present, and, at the antibiotic concentrations giving maximum counts, the concurrent action of lysozyme gave the higher figures (Fig. 2 compared to Fig. 1). An experiment at two penicillin concentrations showed that the maximum lysozyme effect was obtained at 100 μg/ml or higher (Fig. 3). Even when lysozyme was present, 10,000 penicillin units/ml produced suspensions with large numbers of bacilli, although the final sphere yields were highest.

The method selected for routine protoplasting of the Alaska E43 culture was the addition of 1,250 to 2,500 penicillin units and 100 μg of lysozyme per ml of cultures developed by 4.5 to 5 hr of incubation with gassing. Maximum conversion into spheres occurs within 3.5 hr. With the fresh protoplasting medium, the procedure yields suspensions of several hundred million

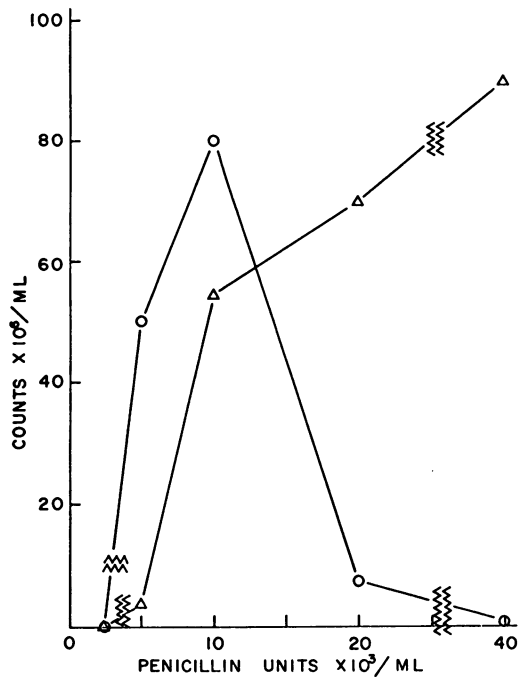

FIG. 1. *Effect of penicillin concentrations, in absence of lysozyme, on yields of protoplast-like spheres and persistence of bacilli. Mean counts from triplicate cultures after 3.5 hr of conversion treatment. Points for 2.5 × 10³ units of penicillin indicate almost complete absence of any intact morphological units. Sphere counts, ○; intact bacilli, △.*

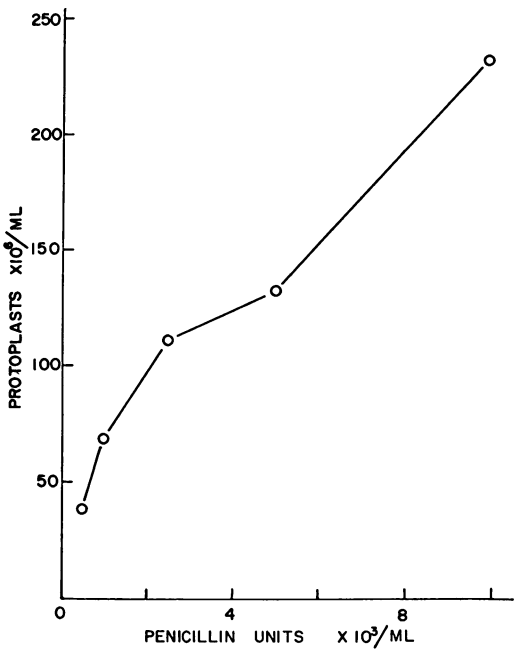

FIG. 2. *Yields of protoplast-like spherical bodies with different concentrations of penicillin when 100 μg of lysozyme/ml is present.*

protoplasts per milliliter which are stable for 4 to 5 hr and which have only occasional bacilli. The procedure gave comparable results with the five other type E strains and the one type E-related strain. The type A strain could also be converted by using M1 medium and 37 C incubation, although 8 to 10 hr of treatment was required to get maximum yields of protoplasts.

Microscopic changes during conversion. The metamorphosis of the Alaska E43 rods was followed by phase-contrast microscopy. Within 30 min of adding the converting agents, many cells showed a single rounded swelling. The swelling rapidly became an extrusion of the cytoplasm of the entire cell through a cell wall break which occurred at random sites along the length of the cells. Other rods degenerated without showing bulging, or immediately thereafter. The attached bulges increased in size, and by 30 to 90 min had become free spheres. After 2 to 3 hr, the cell wall shells and fragments had disappeared. Many spheroidal bodies also lysed but intact ones of varying sizes and densities were more numerous. Intracytoplasmic granules, visible in the parent

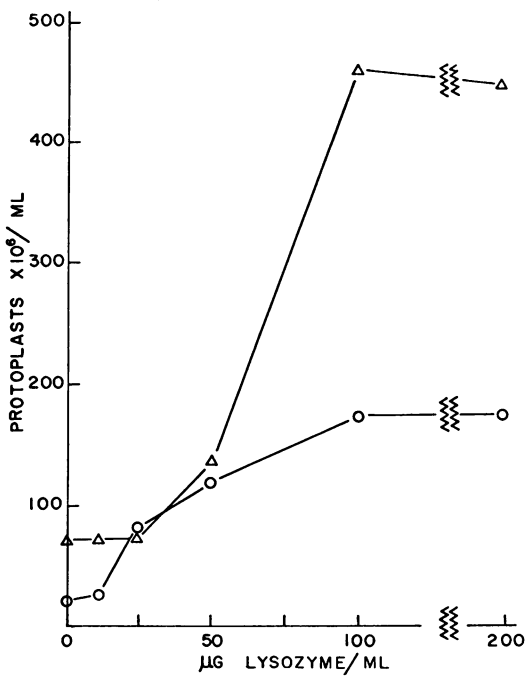

FIG. 3. *Yields of protoplasts with different concentrations of lysozyme in presence of 250 (○) and 1,250 (△) units of penicillin/ml of culture.*

cell, became more apparent as the protoplasts grew larger and less compact. Electron microscopic studies show the freed units to be true protoplasts.

Type A-converted cells had a tendency to cluster and, at the time of release, were smaller than those of type E. Figures 4a and b show the protoplast suspensions of the two types.

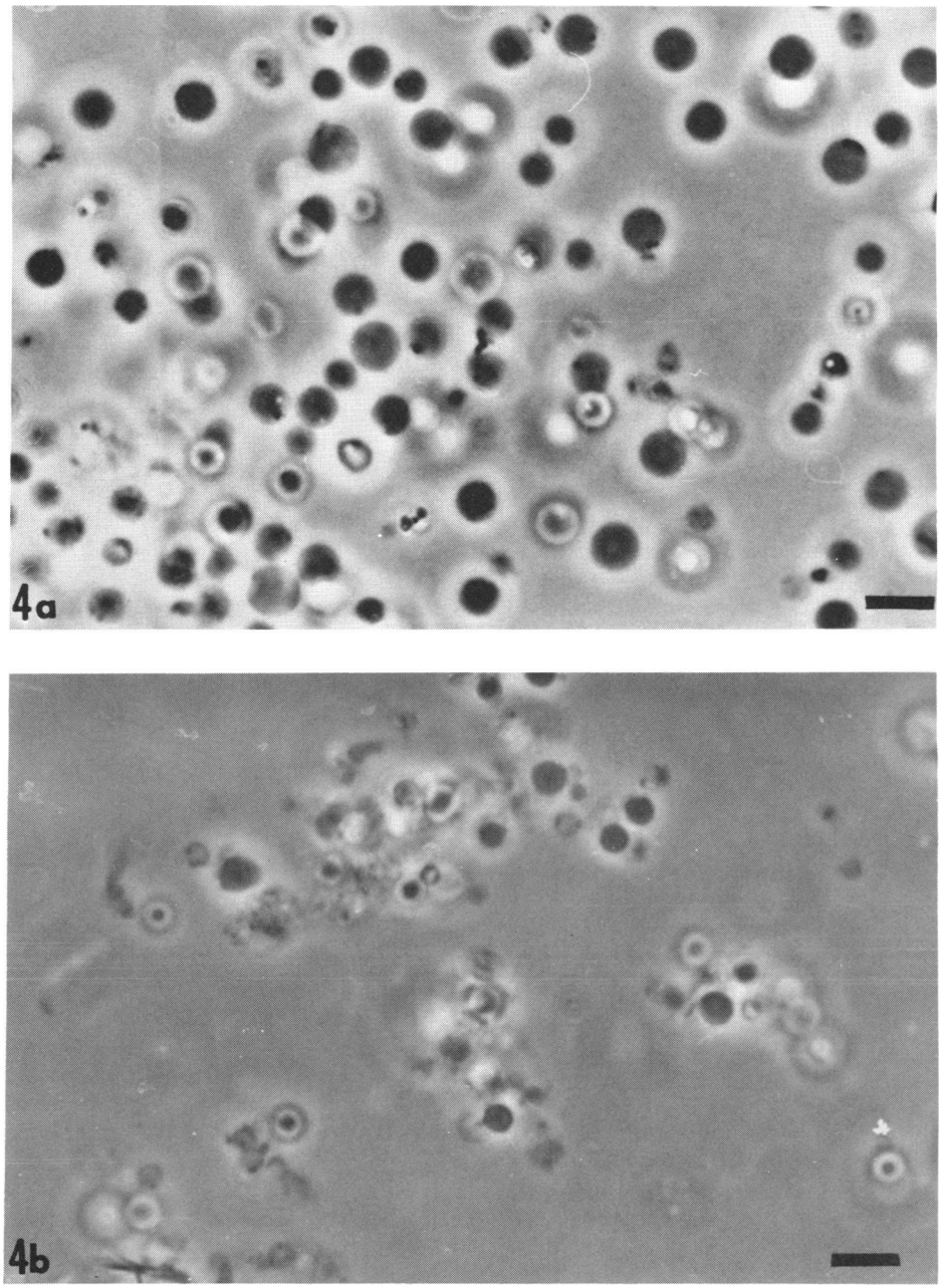

FIG. 4. *a, Phase-contrast microscopy of type E culture treated for 3 hr with 1,250 units of penicillin and 100 μg of lysozyme/ml of culture. Dark granules are visible in some protoplasts. b, Type A culture treated with same concentrations of agents but after the 8 hr treatment needed for maximum conversion. Marker bar denotes 5 μm.*

The penicillin dose response, also evident in the presence of lysozyme, was related to the time of appearance of protoplasts and loss by lysis. At low penicillin concentrations (100 to 500 units/ml), spheres emerged within 10 to 15 min, enlarged rapidly, and lysed soon thereafter. The disintegration of emerging spheres and of bacilli which did not give rise to spheres accounted for the low final yields of protoplasts. At 1,250 to 2,500 units/ml, appearance of significant numbers of free spheres took 30 to 60 min, but most remained intact and grew larger with time. With 10,000 units/ml, spheres took up to 3 hr to appear, and their expansion was at a much slower rate; many bacilli remained unchanged.

As the culture was incubated beyond the actual conversion stage, voluminous evolution of gas for up to 36 hr indicated active metabolism and growth. The accumulation of membranous materials at 24 hr showed that lysis had occurred; the survivors had, however, enlarged 10 or more diameters. Optical density changes of a culture treated with the antibiotic and enzyme (Fig. 5) reflect the changes of optical properties during and after the conversion. Upon further incubation, the still fewer survivors showed bizarre shapes suggestive of L-forms.

L-form cultures. L-form cultures were obtained by incubating a newly converted suspension for 16 to 20 hr and transferring 1 ml of the spontaneously sedimented material to one of the L-form media. An alternate procedure was to centrifuge, at 3,000 × *g* for 5 min, a culture immediately after its conversion, remove 2 ml of the bottom layer directly with a pipette, and inoculate into L-form medium. When growth was evident after 3 to 7 days, a subculture was made into fresh medium. The medium for the second and third subcultures contained 250 penicillin units/ml to eliminate any surviving bacilli or spores.

After the adaption of the first few transfers, L-form cultures grew rapidly so that subcultures were necessary at 3- to 4-day intervals to prevent loss of the culture. One line of each botulinal type has been transferred over 150 times without reversion to bacillary forms. Less frequent transfers are needed when the cultures are refrigerated. When semisolid medium is inoculated and incubated for 24 hr, and the culture is held at 4 C, successful subcultures are obtained after 3 weeks of storage.

L-form cultures (Fig. 6a and b) produce the same type of botulinal toxin as the parent form. A type E L-form which had been transferred over 100 times was inoculated into the biphasic medium and incubated for 10 days. The supernatant fluid had 40 mouse intraperitoneal 50% lethal doses (LD_{50}) before and 3,200 LD_{50} of type E toxin per ml after tryptic activation (3). A similarly stabilized L-form culture of type A grown in a similar manner had 500,000 LD_{50} of type A toxin per ml of supernatant fluid.

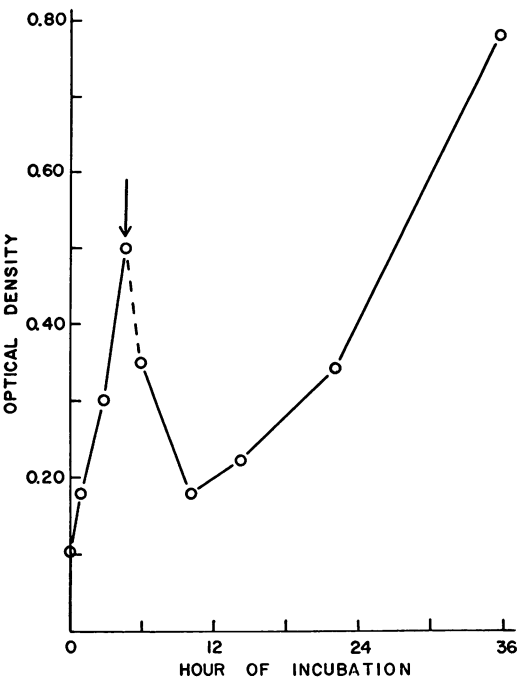

FIG. 5. *Changes in optical density of type E culture during and after protoplasting. Arrow, time when converting agents were added; dotted line, nonspecific change caused by dilution of culture.*

DISCUSSION

Lysozyme, which hydrolyzes the β-1,4 linkage of the cell wall mucopeptide (12), does not noticeably affect growing cells of *C. botulinum* type E and has only minor effects on type A. Penicillin, which blocks the cross-linking of the linear peptidoglycan (10), gives rise to protoplast-like spheres, but the yields are poor. The more quantitative conversion of the cells by the simultaneous action of the antibiotic and enzyme indicates that penicillin acts on the cell in a manner which makes them more susceptible to lysozyme. The presence of lysozyme could contribute to better protoplasting by making possible an earlier weakening of the defective cell wall being synthesized. The delicate protoplasts could then escape the confines of the cell wall before the internal pressure becomes disruptive.

The procedure described here is similar in some

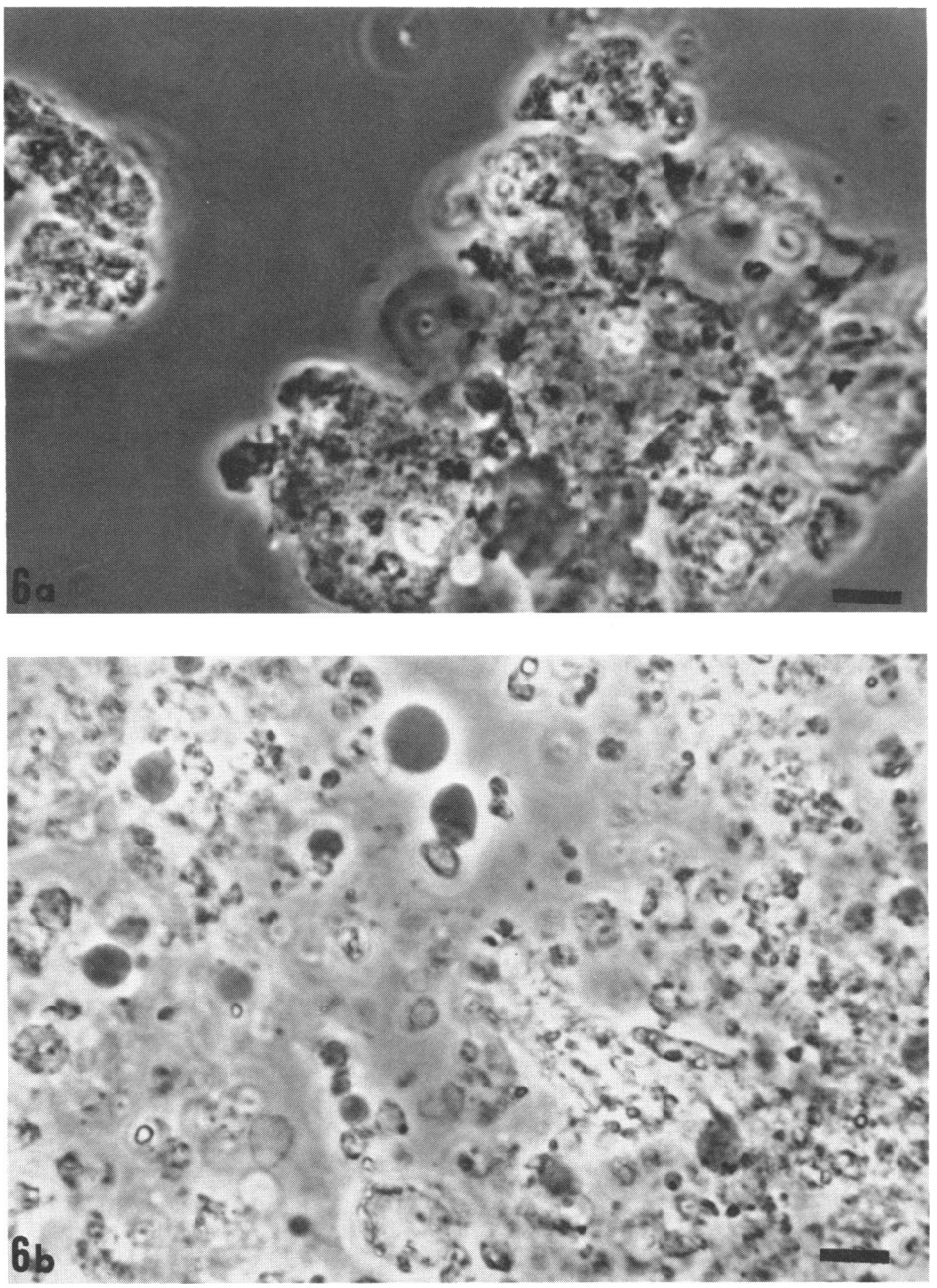

FIG. 6. *Twenty-four-hour-old L-form cultures. a, Type E; b, Type A. Marker bar denotes 5 μm.*

respects to treatment of resting cells to predispose them to lysozyme action (4, 11, 15). The present method differs in that the susceptibility to the enzyme is being induced by forcing growing cells to synthesize defective cell walls.

The high penicillin requirement for good conversion corresponds to the better yields of L-forms with high penicillin concentrations (8, 9) and suggests that the influence of penicillin is not limited to cell wall synthesis. The better conversion with the levels of penicillin chosen for routine protoplasting seems to reflect a slowing

of the growth process so that the increase in cytoplasmic mass is in better balance with the synthesis of the unit membrane. With such an equilibrium, rupture of the nascent protoplasts by the buildup of internal pressure would be less likely. Beyond the optimum level, further penicillin increments slow the growth of organisms to the point at which the appearance of spheres is markedly delayed and the number of unconverted bacilli is increased.

The stable L-form cultures developed from protoplasts produce the type of botulinum toxin characteristic of the parent culture. Since L-forms do not sporulate, the observation would contradict the suggestion (2) that synthesis of botulinal toxin may be related to sporogenesis.

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