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*BACTERIAL PROTOPLASTS INDUCED BY PENICILLIN**

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Bacterial protoplasts are believed to be cellular units that have been deprived of their rigid cell wall.[1] Accordingly, they are distinguished by their spherical shape (in bacilliform species) and their sensitivity to cytolysis in hypotonic media. Several authors[1–3] have suggested that protoplasts might be useful material for the study of biosynthesis of viruses and enzymes in a system simpler or more accessible to external modification than the intact cell. So far, only gram-positive bacteria, which are susceptible to lysozyme, have been used for such studies. A method has now been found for the efficient production of protoplasts from enteric bacteria such as *Escherichia coli* and *Salmonella typhimurium*, which are already familiar physiological and genetic subjects. The technique consists essentially of the exposure of growing cells to a medium containing penicillin, sucrose, and Mg^{++}.

Preparation of Protoplasts.—Escherichia coli strain K-12 and a variety of its mutant substrains are used in most of the experiments. The use of penicillin and sucrose was suggested by the possible analogy between penicillin and lysozyme as lytic agents and by the finding that hypertonic sucrose would interrupt bacteriolysis of *Bacillus megaterium* by lysozyme.[1] In addition, spherical bodies had been casually noted in other applications of penicillin,[4] and many authors have emphasized its use in the production of *L*-forms.[5] The following procedure was adopted after empirical trials and can doubtless be further improved.

The bacteria were grown overnight in tubes with 10 ml. of broth (Difco penassay medium) at 37° C., on a rotator. Samples of 3 ml. of the grown culture (about 2×10^9 cells/ml) were added directly to 10 ml. of broth supplemented with penicillin, 1,000 u/ml, sucrose 20 per cent, and magnesium sulfate 0.2 per cent. In 2–3 hours the cells were quantitatively converted into spheres. During this interval the optical density (measured at 650 mμ in a Coleman 14 spectrophotometer) increased about 50 per cent, but the total count (spheres or rods, estimated in a Petroff-Hausser chamber) remained constant. The spheres promptly lysed when the suspension was diluted in distilled water, and they are therefore regarded as "protoplasts."

The indicated supplements are in substantial excess, and nearly optimal yields of protoplasts can be obtained with 5 per cent sucrose, 100 u. penicillin, and 0.1 per cent $MgSO_4$. The high magnesium requirement may depend partly on binding with the sodium citrate used in the compounding of the penicillin preparation

(Squibb penicillin G, potassium, buffered). The Mg^{++} can be replaced by Ca^{++} but was preferred, to minimize precipitation of phosphate. In the absence of Mg^{++}, protoplasts are formed but are less well preserved, and a third or less of the initial rods are recovered. In the absence of sucrose or comparable stabilizer, the cells are almost totally lysed, leaving only debris. In the absence of penicillin, the rods grow at nearly the normal rate, i.e., are not appreciably inhibited by 20 per cent sucrose.

Protoplasts were also formed in minimal medium, but the transformation has been irregular and incomplete in the trials to date. Cells exposed to penicillin under conditions not supporting growth, e.g., in sucrose buffer, were not transformed into spheres.

Microscopic Observations and Viability.—The transformation of rods into spheres was observed in small droplets each containing ten to twenty cells, immersed in an oil chamber.[6] Each rod gave rise to a single sphere; even cells about to divide were inhibited in further growth and division. The progression of stages as observed with dark phase contrast at 645× is illustrated in Figure 1. A swelling first appears, either subterminally or (in incipient division stages) centrally, and progressively enlarges. The remainder of the rod then withers away and disappears.

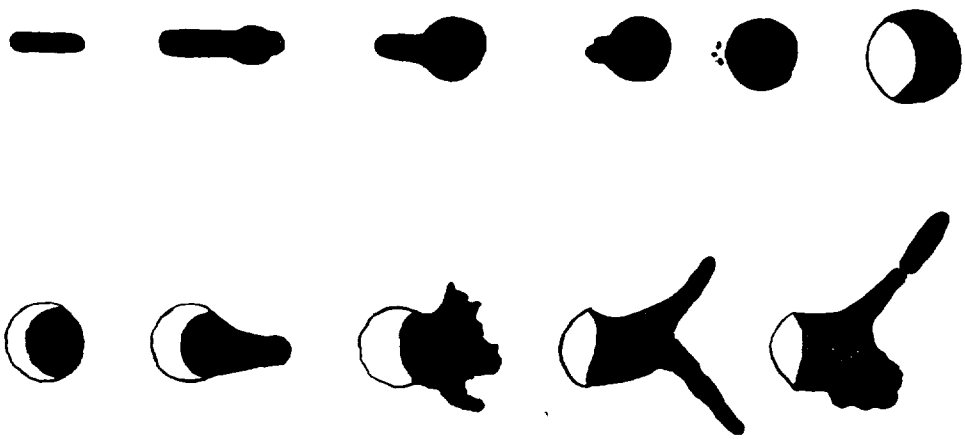

FIG. 1.—*Above:* Transformation of bacterial rod to spherical protoplast in the presence of penicillin. *Below:* reversion of protoplast to rod in absence of penicillin. Observed under dark phase contrast.

The reversion of spherical protoplasts to rods, in the absence of penicillin, has been followed by a similar technique. The spheres first enlarge, then develop amoeboid, finally rhizoid, outgrowths which segment to give typical rods. Spheres incubated in the penicillin medium continue to enlarge but vacuolate and eventually lyse over an interval of 24 hours. The optical density of the suspensions shows a parallel cycle. The enlarging spheres are highly reminiscent of published figures of *L*-forms,[5] but no evidence for multiplication of spherical elements has been seen so far in these experiments or in trials in which 10 per cent bovine or equine serum was furnished. The protoplast suspensions remain intact at least for several days at 4° C. After spontaneous lysis, or upon cytolysis in water, a residual ghost is seen

which retains its spherical form, although deprived of phase-dense material. The lysates are quite viscous, presumably from the release of desoxyribonucleic acid.

About half the protoplasts are capable of reverting to rods and engendering typical bacillary colonies in the absence of penicillin. This was verified by comparisons of total with viable counts on EMB lactose agar plus 20 per cent sucrose. The viability of the protoplasts is completely lost by cytolysis in water. On the other hand, normal rods are unimpaired when suspensions in sucrose solution are similarly diluted.

Enzyme Synthesis and Other Observations.—Protoplasts were tested for their ability to synthesize β-D-galactosidase: suspensions were sedimented, washed with 20 per cent sucrose, and resuspended in lactose medium (casein digest broth plus sucrose plus $MgSO_4$ plus penicillin plus 1 per cent lactose). Over an interval of several hours, samples were assayed with *o*-nitrophenyl galactoside.[7] In 210 minutes the enzyme activity increased from an initial value of 0.38 to 73 units per 10^9 spheres, a two-hundred-fold increase, while the optical density increased about twofold. (One unit corresponds to the splitting of 10^{-8} moles of substrate per minute. The peak activity found in spheres from lactose-grown cells is about 150 units. According to Rotman and Spiegelman,[8] each of these units corresponds to 5×10^{-9} gm. nitrogen, or $\sim 2 \times 10^{10}$ molecules of purified enzyme protein.) Most of the induced enzyme activity sedimented with the protoplasts but was released to the supernate when the protoplasts were cytolysed in water.

Protoplasts were produced in a similar fashion in *E. coli* strain B and in *S. typhimurium* strain TM2. The cells of the latter swam very actively, but the protoplasts were immotile, except for an occasional sphere on which a bacillary protuberance persisted (or had regenerated). Similarly, the protoplasts failed to agglutinate in homologous antiflagellar serum. However, electron-microscopic studies will be needed to establish the relationship of flagella to cell walls in this species. Protoplast suspensions gave a faint agglutination in homologous somatic antiserum.

The protoplasts of *E. coli* K-12 at first appeared to be readily stainable with methylene blue but were later found to be very sensitive to photodynamic effects which result either in lysis or stainability. Boiled protoplasts retain their form and are evidently fixed as they become much more opaque, stain readily, and do not cytolyse in water. Stained protoplasts often show a denser inclusion about one-half their diameter, a poorly staining cortex, and a deeper-staining periphery. The protoplasts reduce triphenyl-tetrazolium chloride, as well as methylene blue, very readily. With the former, a typical formazan granule is produced, usually single and near the periphery of the protoplast, much as in the rods.[9] No corresponding structure was seen in the untreated spheres.

In view of reports on the fusion of protoplasts,[10] and the possibility of DNA-mediated transduction, preparations have been made with a variety of appropriate genotypic mixtures of *E. coli* K-12. No evidence of hitherto unrecognized modes of genetic recombination has been uncovered in preliminary trials. Protoplast suspensions of appropriate compatibility types did, however, retain their ability to mate.

Discussion.—Most of the findings in this preliminary survey warrant more intensive and exact quantitative study. The areas of the interest already expressed

in bacterial protoplasts[1] are already beyond the scope of any one laboratory, and a ready method for their production in enteric bacteria may encourage further attention. In addition to their use in the extraction of intracellular constituents by cytolysis and their availability for biosynthetic studies (enzymes, viruses, cell walls), they may also be useful in cytological, antigenic, and physiological analysis. Their possible genetic applications are currently being studied in this laboratory.

The preservation of viable protoplasts in sucrose solution supports the hypothesis that the *primary* action of penicillin is on the synthesis or maintenance of a component of the cell wall,[11] while the activity and synthesis of other enzymes and over-all protoplasmic mass are unaffected.

The protoplasts of *E. coli* show some instructive differences from those of *B. megaterium*. The latter are rapidly produced by the enzymatic dissolution of the existing wall, and each rod produces not one but several spheres. The latter difference presumably corresponds to the septate structure of gram-positive rods, each compartment containing a single protoplast.[12] Gram-negative bacteria, on the other hand, are believed to be true multinucleate coenocytes. The most striking difference may vanish with technical improvements—namely, that the protoplasts of *B. megaterium* are reportedly inviable, while those of *E. coli* revert to normal rods, an obvious advantage for genetic research.

Summary.—Growing cells of *E. coli* or *S. typhimurium* are quantitatively transformed into spherical "protoplasts" in the presence of penicillin, magnesium, and high concentrations of sucrose. The protoplasts are quickly cytolysed in water but revert to viable rods in the absence of penicillin. The protoplasts have not been observed to divide as such but have been shown to enlarge and to synthesize an inducible enzyme (β-D-galactosidase) in appropriate media. The possible applications of these protoplasts in physiological and genetic research are cited.

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