Properties of *Proteus mirabilis* and Providence Spheroplasts

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SUMMARY

Proteus mirabilis strain 13 and Providence strain NCTC 9211 were converted to osmotically sensitive spheroplasts by growth in the presence of penicillin or glycine in sucrose-supplemented or unsupplemented medium. Lysozyme in the presence of tris and ethylenediaminetetra-acetic acid (EDTA) converted both these strains to spheroplasts. Penicillin spheroplasts could be stabilized with sucrose, streptomycin, spermine or di-valent cations. Electron micrographs of these spheroplasts demonstrated that the outer layer of the bacterial cell wall was retained. Penicillin-induced spheroplasts possessed receptor sites for bacteriophages.

INTRODUCTION

Bacterial protoplasts have been reviewed by McQuillen (1956, 1960), Weibull (1958) and Martin (1963). The term protoplast is limited to a cell devoid of cell wall (McQuillen, 1960). Cells converted to osmotic sensitive spherical bodies which retain non-rigid cell walls are called spheroplasts (Tulasne, Minck, Kirn & Krembel, 1960; McQuillen, 1960). The cell walls of *Bacillus megaterium*, *Sarcina lutea* and *Micrococcus lysodeikticus* consist predominantly of mucopolymer (Salton, 1960). The cell wall of *Escherichia coli* consists of a superficial lipoprotein, an intermediate lipopolysaccharide and an innermost rigid mucopolymer layer (Weidel, Frank & Martin, 1960). Salton & Shafa (1958) emphasized that cell walls of Gram-negative bacteria contain the mucopeptide-substrate of lysozyme as a minor component. In *E. coli* and *Proteus mirabilis* the lipoprotein and lipopolysaccharide layers are anchored to the mucopolymer in an intricate mosaic of functional surface sites (Hofschneider & Martin, 1968). Weidel *et al.* (1960) also showed that the receptor sites for bacteriophages were located in the lipoprotein and lipopolysaccharide layers and that phages did not adsorb to the rigid layer. Kellenberger & Ryter (1958) provided microscopic proof for the multi-layered cell wall in *E. coli*. They demonstrated a cell wall in which two electron dense layers were separated by a less dense layer. Depolymerization of the mucopolymer which provides rigidity to the cell wall is necessary for conversion to spheroplasts. This can be accomplished by lysozyme which acts on β -1,4-*N*-acetylglucosamides (Salton & Ghuysen, 1959) or penicillin (Lederberg, 1956, 1957) which inhibits mucopolymer synthesis (Park & Strominger, 1957). To explain the action of penicillin on synthesized mucopolymer the presence of two enzymes in the cell was suggested by Weidel *et al.* (1960). One splits certain bonds in the mucopolymer while the other resynthesizes mucopolymer in these gaps. Bayer (1967) observed the formation of bag-like protrusions of the soft layers and the simultaneous appearance of gaps in the proteinaceous portion of the rigid layer during penicillin treatment of *E. coli*.

Weibull (1953*a, b*) converted cells of *Bacillus megaterium* suspended in 0.15 to 0.2M-sucrose to protoplasts by treatment with lysozyme. These protoplasts did not adsorb bacteriophages (Weibull, 1953*a*) and were devoid of the cell-wall component diaminopimelic acid (Weibull & Bergström, 1958). Fukuda (1961), however, described the adsorption of bacteriophages to lysozyme-induced protoplasts of several strains of *B. subtilis*, suggesting incomplete removal of cell-wall components. To enhance the effect of lysozyme on Gram-negative bacteria additional procedures were employed. These included freezing and thawing (Kohn, 1960), incubation at alkaline pH (Zinder & Arndt, 1956) and the addition of the chelating agent ethylenediaminetetra-acetic acid (EDTA) in tris buffer (Repaske, 1956, 1958). Repaske (1958) reported differences in susceptibility of Gram-negative bacteria to lysis by the combined effects of lysozyme, EDTA and tris buffer. *Escherichia coli* strains B and H, *Pseudomonas aeruginosa*, *P. fluorescens* and *Azotobacter vinelandii* were rapidly lysed. *Proteus vulgaris* was slightly lysed while *Aerobacter aerogenes* and *Serratia marcescans* were not affected. Gebicki & James (1958) proved *A. aerogenes* to be resistant to lysozyme even in the presence of EDTA. Voss (1964) showed that lysis of *E. coli* and *P. aeruginosa* by lysozyme in the presence of EDTA and tris buffer was not necessarily preceded by spheroplast formation. These organisms were converted to osmotically fragile rods rather than spheres. Jeynes (1957) prepared protoplasts of *Vibrio cholera*, *Salmonella typhi*, *S. typhimurium* and *S. paratyphi* by growth in liquid medium containing glycine.

Tabor (1961) reported that 10^{-3} M-spermine or spermidine prevented lysis of lysozyme-induced protoplasts of *Escherichia coli* strains B and W and *Micrococcus lysodeikticus* in hypotonic media. Stabilization of penicillin spheroplasts of *E. coli* W was less complete. Streptomycin and Ca^{2+} ions were also effective stabilizers, but Mg^{2+} ions as well as mono-valent cations had no protective properties at 10^{-3} M-concentrations with slight protection at higher concentrations. Indge (1968) found that K^+ , Na^+ , Mg^{2+} and spermidine inhibited the effect of chelating agents on lysis of yeast protoplasts.

As a preliminary to the study of transfection (Spizizen, Reilly & Evans, 1966) in *Proteus* and Providence strains the properties of spheroplasts of these organisms were investigated and are reported here.

METHODS

Media. Difco nutrient broth was used. The spheroplasting medium consisted of nutrient broth supplemented with 0.5M-sucrose and 0.2% (w/v) $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$. Tris buffer (0.067M, pH 8.0), lysozyme (Sigma) and ethylenediaminetetra-acetic acid (Merck) were used. Incubation was at 37°.

Bacteria and bacteriophages. Providence strain NCTC9211 (Coetzee, 1963*a*), *Proteus mirabilis* strain 13 (Coetzee & Sacks, 1960) and *Escherichia coli* strain B were used. For phage adsorption studies the temperate Providence bacteriophage PL25 (Coetzee, 1963*a*) and the virulent *P. mirabilis* bacteriophage 13vir (Prozesky, De Klerk & Coetzee, 1965) were used.

Lysozyme + EDTA spheroplasts. The spheroplasts of *Escherichia coli* B were prepared according to Fraser & Mahler (1957) as modified by Fraser, Mahler, Shug & Thomas (1957). Spheroplasts of *Proteus mirabilis* 13 and Providence NCTC9211 were prepared from overnight cultures in nutrient broth. Organisms were harvested by centrifugation of 10 ml. of the cultures at 2000 g for 30 min., washed twice with 0.067M-tris and

resuspended in 1 ml. tris containing 0.5 M-sucrose. To the suspension of the Providence strain 0.2 ml. lysozyme (2 mg./ml.) and after 3 min. 0.05 ml. EDTA (4 %, w/v) were added. For the *P. mirabilis* strain 0.4 ml. lysozyme and 0.1 ml. EDTA were used. The mixtures were then incubated for 8 min. followed by a 1/5 dilution with nutrient broth. The preparations were examined in a phase-contrast microscope.

Glycine spheroplasts. These were obtained by adding 5 ml. of an overnight culture to 100 ml. nutrient broth supplemented with 0.5 M-sucrose, 0.2 % $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and glycine (3 %, w/v). The cultures were aerated. Samples were removed at intervals and examined by phase-contrast microscopy. After incubation for 5 hr the spheroplasts were collected by centrifugation at 2000g for 30 min.

Penicillin spheroplasts. Spheroplasts of *Escherichia coli* B were prepared by the method of Lederberg (1956). Penicillin spheroplasts of Providence and *Proteus mirabilis* strains were obtained by diluting 10 ml. of an overnight culture in 100 ml. spheroplasting medium. Penicillin G (100 to 10,000 u./ml.) was added to the medium which was then incubated with aeration. Samples were removed at intervals, examined microscopically and plated on MacConkey agar to determine colony-forming bacteria. The method was varied by substituting unsupplemented nutrient broth for the spheroplasting medium. Spheroplasts were counted in a Petroff-Hauser chamber with the use of a phase-contrast microscope. Spheroplasts were harvested after 3-hr incubation.

Osmotic sensitivity. Duplicate samples of lysozyme, glycine and penicillin spheroplasts were collected by centrifugation at 2000 g for 30 min. The pellets were resuspended in equal volumes of 0.5 M-sucrose and deionized water respectively. Extinctions at 650 m μ were determined within 10 min. in a Hitachi Elmer-Perkin spectrophotometer. Stability of spheroplasts prepared in unsupplemented medium were determined by resuspending in deionized water and nutrient broth.

Stabilization of penicillin spheroplasts. After incubation for 3 hr the penicillin spheroplasts were collected by centrifugation at 2000 g for 30 min. The pellets were suspended in equal volumes of 0.5 M-sucrose, water, streptomycin sulphate (10^{-3} M), CaCl_2 (10^{-2} , 10^{-3} M), spermine (10^{-3} , 10^{-4} , 10^{-5} M), MgCl_2 (10^{-2} , 10^{-3} M), NaCl (10^{-2} M) and unsupplemented nutrient broth. Extinctions of the suspensions at 650 and 260 m μ were determined within 10 min. after suspending in a Hitachi Elmer-Perkin spectrophotometer. The suspensions were also examined microscopically.

Electron microscopy. Penicillin spheroplasts of Providence and *Proteus mirabilis* were prepared and collected as above. Fixation was done in osmium tetroxide (Kellenberger & Ryter, 1958). Preparations were stained in uranyl acetate before dehydration through a graded acetone series and embedding in Epon-Araldite. Electron microscopy was performed in a Philips EM 200 electron microscope.

Phage adsorption. Penicillin spheroplasts of *Proteus mirabilis* and Providence were collected by centrifugation and resuspended in 0.5 M-sucrose. Bacteriophages PL 25 and 13vir at multiplicities of input of 100 to 300 were added to spheroplasts of Providence strain NCTC9211 and *P. mirabilis* strain 13, respectively. After incubation for 10 min. the interaction was stopped by the addition of osmium tetroxide. Staining and embedding were done as before.

RESULTS

Lysozyme + EDTA spheroplasts

At least 99 % of the Providence organisms were converted to spherical forms by this method. *Proteus mirabilis* 13 was more resistant to lysozyme-EDTA treatment and higher concentrations of lysozyme (0.4 ml.) and EDTA (0.1 ml.) were necessary for optimum yields. For this strain conversion to spherical forms was never more than 80 % and was not improved by further increase in the concentrations of lysozyme or EDTA.

Glycine spheroplasts

Glycine at concentrations of 3 to 5 % (w/v) proved an effective inducer of spheroplasts. In both strains the first small spheroplasts were observed at 40 min. At 4 hr all the cells were spherical and incubation for 12 hr yielded a dense-culture of spheroplasts.

Penicillin spheroplasts

Incubation of the *Proteus mirabilis* and Providence strains in the presence of penicillin caused the rod-shaped cells to develop swellings centrally or terminally. The swellings enlarged to form spheres with remnants of rods protruding. The sequence of forms in both *P. mirabilis* and Providence was similar to those described for *P. vulgaris* and *Escherichia coli* (Liebermeister & Kellenberger, 1956; Hahn & Ciak, 1957). The concentration of penicillin was not critical and 500 to 10,000 u./ml. were equally efficient. Incubation of the *P. mirabilis* and Providence strains in unsupported nutrient broth in the presence of high concentrations of penicillin (500 u./ml. for the *P. mirabilis* and 3000 u./ml. for the Providence strain) also resulted in dense cultures of spheroplasts which were identical to those described above. Lower concentrations of penicillin (2000 and 500 u./ml. respectively) caused the formation of spherical as well as filamentous forms in both the organisms (Fleming, Voureka, Kramer, Hughes 1950). The *E. coli* strain treated similarly underwent complete and rapid lysis. Irrespective of whether the spheroplasts were prepared in unsupported nutrient broth or in spheroplasting medium all spheroplasts lysed in water. While the spheroplasts prepared in spheroplasting medium lysed when resuspended in unsupported nutrient broth those obtained in nutrient broth were stable in the latter medium.

Stability of penicillin spheroplasts

On account of the efficiency and reproducibility of the penicillin method of spheroplast formation these spheroplasts prepared in spheroplasting medium were examined in greater detail. As indicated in Table 1 the spheroplasts were stabilized when suspended in 0.5M-sucrose. Streptomycin sulphate (10^{-3} M), CaCl_2 (10^{-3} M) and MgCl_2 (10^{-3} M) were effective stabilizers while NaCl (10^{-2} M) provided very little support. Spermine at 10^{-3} M was a good stabilizer for penicillin spheroplasts of *Escherichia coli* B. Maximal stabilization of penicillin-induced spheroplasts of *Proteus mirabilis* 13 and Providence NCTC9211 was obtained at a spermine concentration of 10^{-4} M. Higher concentrations caused aggregation of the spheroplasts while low concentrations provided no protection against lysis. Suspension in water caused rapid lysis of the spheroplasts with a drastic decrease in optical density at $650\text{ m}\mu$ and the presence of membranous structures detected by phase-contrast microscopy.

Table 1. *Stability of penicillin induced spheroplasts*

Ten ml. of penicillin spheroplasts prepared in sucrose-supplemented medium were centrifuged at 2000 g for 30 min. and resuspended in equal volumes of the different solutions.

Suspending medium (M)	Providence NCTC 9211	<i>Proteus</i> <i>mirabilis</i> 13	<i>Escherichia</i> <i>coli</i> B
Sucrose, 0.5	0.52*	0.48	0.64
Water	0.16	0.14	0.095
Streptomycin sulphate, 10 ⁻³	0.49	0.32	0.60
CaCl ₂	0.34	0.26	0.36
Spermine, 10 ⁻³	0.29	0.155	0.45
Spermine, 10 ⁻⁴	0.50	0.42	0.16
Spermine, 10 ⁻⁵	0.24	0.13	0.08
MgCl ₂ , 10 ⁻³	0.31	0.24	0.32
NaCl, 10 ⁻²	0.26	0.16	0.27
Nutrient broth	0.29	0.14	0.11
Sucrose, 0.5†	0.38	0.36	—
Nutrient broth†	0.38	0.36	—

* Extinction determined at 650 mμ within 10 min. after suspension.

† Spheroplasts produced in unsupplemented nutrient broth.

Electron microscopy

A section of a penicillin-induced spheroplast of Providence strain NCTC9211 is shown in Pl. 1, fig. 1. The cell contents are surrounded by two superimposed integuments identical in appearance. The innermost layer adheres closely to the cytoplasmic mass and has the triple layer structure of a unit membrane (Robertson, 1959). This layer corresponds to the cytoplasmic membrane in *Escherichia coli* (Kellenberger & Ryter, 1958). The outer triple layer is located in a position corresponding to the cell wall of *E. coli* and *Proteus mirabilis* (Hofschneider & Martin, 1968). Areas where the outer triple membrane is stripped from the inner membrane can be seen. No structural differences were detected between penicillin spheroplasts prepared in sucrose-supplemented or unsupplemented medium. In Pl. 1, fig. 2, bacteriophage PL25 is adsorbed to spheroplasts of the Providence strain. Similar results were obtained in the *P. mirabilis* 13—phage 13^{vir} system.

DISCUSSION

The spherical bodies of Providence and *Proteus mirabilis* described here correspond to spheroplasts on grounds of morphology and osmotic sensitivity. Lysozyme in the presence of the chelating agent EDTA converted cells of Providence NCTC9211 to spheroplasts but *P. mirabilis* 13 was more resistant to the combined action of these reagents. This suggests differences in the cell walls of these closely related species (Coetzee, 1963*b*). Glycine and penicillin were equally efficient in the production of spheroplasts in the Providence and *P. mirabilis* strains. These results are in agreement with the postulate of Park (1958) that both glycine and penicillin inhibit synthesis of cell-wall material in a manner which causes accumulation of uridine-5'-pyrophosphate derivatives. The reason why *Proteus* and Providence spheroplasts can be prepared in nutrient broth unsupported by sucrose is obscure. No morphological differences were detected between spheroplasts prepared in sucrose-supplemented or plain nutrient broth. Preparation in the latter medium may select for a different type of cell which is less fragile. The *P. mirabilis* and Providence strains differ in this respect from *Escherichia*

coli and this behaviour may indicate that cell walls of the former organisms are less affected by penicillin than those of *E. coli*.

The mechanism of stabilization of spheroplasts by spermine is not clear, but Mager (1959) suggested that binding of the negatively charged polyamine molecules is a pre-requisite for its action.

Electron micrographs did not reveal morphological differences in spheroplasts obtained by the lysozyme-EDTA, penicillin or glycine methods. They demonstrated the presence of two triple layers similar in appearance. The outer layer corresponds in position to the cell wall of normal bacteria. Electron micrographs also demonstrate the adsorption of bacteriophages to penicillin spheroplasts. This indicates that these spheroplasts retain receptor sites for bacteriophages and suggests that only the mucoprotein layer is affected during penicillin treatment. The adsorption of phages to penicillin spheroplasts supports the concept that penicillin prevents synthesis of mucopeptide (Duguid, 1946) but does not affect the lipoprotein and lipopolysaccharide which contain specific receptor sites for phages (Burnet, 1934; Jesaites & Goebel, 1952; Oram & Reiter, 1968). Oram & Reiter (1968) have, however, demonstrated the inactivation of phage *ml 3* by the plasma membrane of *Streptococcus lactis* strain ML 3 while the cell wall did not possess inactivating properties.

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EXPLANATION OF PLATE

Fig. 1. Ultrathin section of a penicillin spheroplast of Providence NCTC 9211. *SW*, Spheroplast wall; *CM*, cytoplasmic membrane. The bar represents 1 μ .

Fig. 2. Adsorption of phage PL25 to penicillin spheroplasts of Providence NCTC 9211. The bar represents 1 μ .