The Preparation and Properties of Spheroplasts of *Aerobacter aerogenes*

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SUMMARY

Cells of *Aerobacter aerogenes* growing in a nutrient medium were lysed by 1000 u. penicillin/ml. When the medium contained penicillin and sucrose, bacterial mass continued to increase on incubation, although no further cell division took place. After growth for 3 hr. in the presence of penicillin, all the rod-shaped bacteria were converted to fragile spheroplasts. The internal osmotic pressure of the spheroplasts was estimated by direct counts of the intact cells in solutions containing various concentrations of sucrose, sodium chloride or mixed phosphates. Measurements of optical densities of suspensions were unsuitable for the estimation of the numbers of spheroplasts. Morphological study suggested the existence of a spheroplast cell wall outside the protoplasmic membrane.

INTRODUCTION

Lysozyme digests the cell walls of several sensitive species of bacteria (Salton, 1953, 1954). Exposure of such organisms to lysozyme in hypertonic medium results in the release of the bacterial protoplast devoid of cell-wall material (Weibull, 1953; Gooder & Maxted, 1958; Dark & Strange, 1957; Brenner *et al.* 1958). These protoplasts are only stable in high osmotic pressure medium.

The main differences between bacteria and their protoplasts appear in the properties depending on the presence of a cell wall. The intact protoplasts are always spherical in shape and fragile to osmotic and mechanical shock (Weibull, 1953). Under very favourable conditions protoplasts have been observed to divide; the process is slower than with normal cells (McQuillen, 1955).

The preparation of spherical bodies of *Escherichia coli* exposed to high concentrations of penicillin in hypertonic media (Lederberg, 1956, 1957; Liebermeister & Kellenberger, 1956; Hahn & Ciak, 1957) gave experimental confirmation of the suggestion (Duguid, 1946) that penicillin interferes with the synthesis of bacterial cell walls while otherwise allowing growth to proceed. Like protoplasts, the penicillin-induced bodies are spherical and only stable in hypertonic media. Certain observations suggest, however, that penicillin does not completely remove the cell walls of sensitive bacteria (Tomesik, 1954; Kandler, Hund & Zehender, 1958; Salton & Shafa, 1958). The process of reversible vacuolation exhibited by penicillin-

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induced 'spheroplasts' (Hurvitz, Reiner & Landau, 1958) of *Aerobacter aerogenes* suggests the existence of an envelope external to the protoplasmic membrane (Gebicki & James, 1958). Unlike protoplasts spheroplasts are capable of exponential growth and division (Lark, 1958). Spheroplasts share the fragility and osmotic properties of protoplasts but differ in their method of preparation (Salton, 1957; Chain, Duthie & Callow, 1945), growth and subsequent formation of normal viable bacteria (Lederberg, 1956).

Growth in the presence of penicillin alters the physical properties of the cell walls, but the nature of the alteration in terms of the chemistry of the structure is still obscure. The reason for the plasticity of the spheroplast envelope may lie in the inability of the cell to incorporate certain cell-wall materials in the presence of penicillin (Park & Strominger, 1957). On the other hand, a more important factor may be the alteration of the cross-linking rather than the amount of the cell-wall polymer.

In view of these uncertainties, it was considered useful to compare the properties of normal cells and spheroplasts of one organism. The surface properties of *Aerobacter aerogenes* have already been studied in this laboratory (James, 1957). The organism is not sensitive to lysozyme, even in the presence of versene (Gebicki & James, 1958; Repaske, 1958) so that comparative studies of its protoplasts were not possible. This paper gives an account of the preparation and properties of penicillin-induced spheroplasts of *Aerobacter aerogenes*. An account of their electrokinetic behaviour is published elsewhere (Gebicki & James, to be published).

METHODS

Organism and culture media. Cultures of *Aerobacter aerogenes* (NCTC, no. 240) were maintained by monthly subculture in Lab-Lemco broth (Oxoid). For the preparation of spheroplasts the medium was supplemented with magnesium sulphate (0.2%) and sucrose (0.5M). The cultures were incubated at 37° and usually aerated by gentle shaking. Cultures in defined media (Lowick & James, 1955) were incubated at 40° and aerated with a stream of sterile air.

Measurement of cell populations. Optical densities of cell suspensions were measured against the suspension medium in a Unicam S.P. 1400 spectrophotometer at 600 m μ . Cell counts were made in a haemocytometer (depth 0.02 mm.) with Thoma rulings.

Microscopy and photomicrography. Cell suspensions in wet films sealed between slide and coverslip were examined with a Reichert Neozet binocular microscope, equipped with a 'Polyphos' condenser. Phase contrast was usually employed. The same system was used for photomicrography.

Reagents. All reagents were of A.R. grade. Crystalline sodium salt of penicillin G (benzylpenicillin) of potency 1670 i.u./mg. (Glaxo Laboratories, Greenford, Middlesex) was stored in the cold before use.

Growth in hypertonic media. Synthetic medium. Rapidly growing cells were suspended in synthetic growth medium and in the same medium supplemented with either glucose (0.7M), sodium chloride (1.0M), magnesium sulphate (1.0M) or mixed sodium and potassium phosphates (0.5M). The suspensions were incubated without aeration and the optical densities measured at frequent intervals.

Nutrient medium. Cultures in nutrient broth supplemented with sucrose (0.7 M) or glucose (0.7 M) were incubated at 37° and aerated by shaking. Controls, in which the high concentrations of sugar were omitted, were also included.

The lytic action of penicillin. Cultures rapidly growing in nutrient medium, supplemented with 0.2% magnesium sulphate, were incubated with various concentrations of penicillin (0–1000 u./ml.). Optical densities of samples removed at intervals were determined.

Growth in the presence of penicillin and sucrose. A young culture containing 7.5×10^8 cells/ml. was diluted fivefold into fresh nutrient medium containing sucrose and penicillin (0.6 M and 1000 u./ml. respectively). Samples were removed during subsequent incubation at 37° and their optical densities and cell counts determined. The cultures were aerated by mild shaking.

Standard method for the preparation of spheroplasts. A young (8 hr.) bacterial culture containing about 10^9 cells/ml. was diluted fivefold into fresh nutrient broth containing magnesium sulphate (0.2%), sucrose (0.5–0.6 M) and penicillin (500–2000 u./ml.). After 3 hr. incubation with gentle shaking at 37° the culture was cooled and the spheroplasts harvested at 0° in the pre-cooled centrifuge. The collected spheroplasts were covered quickly with cold suspension medium and resuspended by gentle agitation. Shaking and warming were avoided. For overnight storage, the spheroplasts were suspended in buffered 1.0 M-sucrose or 0.5 M-sodium chloride and kept at 0°.

Thermal fragility of the spheroplasts and the effect of formalin. The spheroplasts were harvested and resuspended in 0.2 M solutions of sucrose in M/300 mixed phosphate buffer at pH 7.0. One half of the suspension was allowed to stand at room temperature and the other was warmed rapidly to 65°. Changes in the optical densities of the suspensions were followed for 10 min. Samples were examined under phase contrast before and after heating.

Two portions of a similar preparation of spheroplasts in 1.0 M-sucrose at pH 7.0 were diluted into 20 vol. of the buffered sucrose solution at 0°. Formalin was added to one portion (final concentration 0.5%) and both were quickly heated to 60°. Numbers of the intact cells in each sample were determined at intervals. In addition the samples were examined under phase contrast.

Photomicrography of the spheroplasts. Drop samples of bacterial cultures incubated with penicillin in a hypertonic medium were placed between a slide and coverslip and examined under phase contrast. Typical stationary organisms were photographed. No dyes or killing agents were added to the suspensions.

The internal osmotic pressure of the spheroplasts. (a) *Optical densities of suspensions.* Spheroplasts were suspended in solutions containing various concentrations of sodium chloride or sucrose buffered at pH 7.0. Changes in the optical densities of suspensions were followed, frequently over several hours.

(b) *Lysis in sucrose solutions.* 0.5 ml. portions of a thick suspension of spheroplasts in 1.0 M-sucrose were diluted into 9.5 ml. of solutions containing various concentrations of sucrose at pH 7.0. The suspensions were incubated at $25 \pm 0.1^\circ$, samples withdrawn at intervals, treated with formalin, to preserve the spheroplasts, and cell counts determined.

(c) *Lysis in sodium chloride solutions.* Spheroplasts in 0.5 M buffered solution of sodium chloride were diluted with salt solutions at pH 7.0. The final concentrations

of sodium chloride were 0.05–0.5 M. The numbers of intact spheroplasts remaining in suspension after incubation at 25° were determined by direct counting.

(d) *Lysis in mixed phosphate buffer solutions.* In a similar experiment, the spheroplasts were incubated in solutions containing various concentrations of phosphates. These were obtained by mixing and diluting equal volumes of M/3 disodium hydrogen phosphate and potassium dihydrogen phosphate. Intact spheroplasts were counted as previously.

Optical properties of suspensions of spheroplasts. Spheroplasts were suspended in hypertonic solutions and changes in the optical densities of the suspensions with time were measured. The variables investigated were the osmotic pressure, pH and electrolyte contents of the solutions.

RESULTS

Penicillin lyses cells of *Aerobacter aerogenes* growing in a simple synthetic medium (Gebicki, 1959), but the high solute concentrations required to preserve any formed spheroplasts prevented cell division altogether. Since the antibiotic acts only on actively growing bacteria, the synthetic medium could not be used for the preparation of spheroplasts.

On the other hand, the mean generation time of the nutrient broth cultures containing 0.7 M-sucrose were the same as those of control suspensions (30 min.). This was even reduced when glucose was the added solute, but as sucrose is the most commonly used substance for the stabilization of protoplasts, it was used in further experiments.

The lytic action of penicillin on cells growing in the nutrient medium is shown in Fig. 1. The rate of growth is affected by penicillin concentrations above 10 u./ml. At 100 u./ml., normal growth was restored after 4 hr. incubation. The initial increase of the optical densities of suspensions containing the highest concentrations of penicillin used indicates that the mass of cells is approximately doubled before the onset of lysis.

The nutrient medium was suitable for the production of spheroplasts since it did not prevent bacterial growth when supplemented with high concentrations of sucrose and, further, suitable amounts of penicillin lysed the cells growing in it. In Fig. 2 the mass of cells growing in the medium containing sucrose in the presence of 1000 u./ml. of penicillin is compared with the actual number of cells per ml. of suspension. A series of photomicrographs illustrate a typical sequence of changes which the normal cells (Pl. 1, fig. 1) undergo under the influence of penicillin. Characteristic protoplasmic protrusions were formed 30 min. after the exposure to penicillin (Pl. 1, figs. 2, 3). Further incubation led to the increase of the size of the protrusions (Pl. 1, figs. 4, 5), detachment of the rod-shaped fragments and the appearance of characteristic vacuolated spheroplasts (Pl. 2, figs. 6–8). When the external osmotic pressure was carefully lowered by the addition of water, the protoplasm expanded in the initially vacuolated spheroplast and, after breaking through the confining barrier, formed a spherical protrusion outside the spheroplast proper (Pl. 2, fig. 9). The protrusion then expanded at the expense of the spheroplast, the protoplasm gradually leaving the original cell. After a short time (2–3 min.) the new sphere burst leaving no clearly defined structure. A normal 'ghost' was left

by the spheroplast (Pl. 2, fig. 10). Frequently the whole process was so rapid that the change from spheroplast to 'ghost' appeared to proceed directly. However, the controlled lysis was observed on numerous occasions.

The process of vacuolation could be reversed by placing the spheroplasts in media of lower osmotic pressure. On subsequent addition of sucrose or sodium chloride, the vacuoles formed again. This could be repeated several times.

Fig. 1. The lytic effect of penicillin in nutrient media. ○, Control; ●, 10 u. penicillin/ml.; ●, 100 u. penicillin/ml.; ●, 1000 u. penicillin/ml.; ⊗, 2000 u. penicillin/ml.

The lack of correlation between the actual numbers of intact spheroplasts and the optical density of the suspension is shown in Fig. 3. The optical density curve suggests a continuous decrease in the number of cells which does not actually occur. Several other experiments, in which the concentration of the stabilizing solute was the independent variable, emphasized the unsuitability of the method for the determination of the spheroplast populations.

The effectiveness of sucrose and sodium chloride in the preservation of intact spheroplasts at different osmotic pressures is shown in Fig. 4. The figure taken as 100% preservation for the given solute was obtained by averaging the numbers of spheroplasts per unit volume of suspension after 1 min. standing in solution of osmotic pressure above 5 atmospheres. Values above 90% must be regarded as complete preservation in view of the errors involved. Comparative studies for

sodium and potassium phosphates were not made because of the difficulty of obtaining accurate values for the osmotic pressures of their solutions. Some indication of the stabilizing action of the phosphates is seen in Fig. 5. Spheroplasts suspended in M/6 buffer solution were counted before and after storage for 24 hr. at 0° C.; the counts were 279 and 284×10^6 cells/ml. of suspension respectively.

Fig. 2

Fig. 3

Fig. 2. Growth of *Aerobacter aerogenes* in presence of sucrose and penicillin. ○, Optical density, i.e. bacterial mass; ●, no. of cells.

Fig. 3. Spheroplasts suspended in 7% sucrose at pH 6.8 and 20° C. ○, Optical density; ●, no. of cells.

Fig. 4

Fig. 4. Stability of spheroplasts in solutions of sucrose and sodium chloride at 25° C. Sucrose, ○, after 60 min., ●, after 20 hr.; NaCl, ○, after 60 min., ●, after 23 hr.

Fig. 5

Fig. 5. Stability of spheroplasts in mixed phosphate buffer solutions at 25° C. ○, After 5 min., ●, after 30 min., ⊙, after 18 hr.

An attempt to discover the factors responsible for the anomalous optical properties of suspensions of spheroplasts is summarized in Table 1. Changes in the optical densities of the suspensions were followed under identical conditions for 1 hr. All solutions were buffered with mixed phosphates unless otherwise stated. Since it has been demonstrated (Fig. 3) that a decrease in the optical density of 46% can occur without lysis, values less than this figure can be assumed to be due to factors other than destruction of the spheroplasts. This was, in fact, confirmed by microscopic examination of cell suspensions. Some cell damage occurred at pH 7.5, both at 20° and at 37°. Trypsin had no accelerating effect on lysis. A certain regularity of the pattern of changes in the optical densities of suspensions with pH suggests that even at pH 7.5 lysis may be fairly rapid. An experiment in which a wide range of pH values was used suggested that at neutral pH there was no lysis, while below pH 4.8 the optical densities of suspensions increased. Spheroplasts in acid buffers appeared as highly refracting irregular bodies and they did not lyse on resuspension in distilled water. Spheroplasts suspended at pH 8.5 for 1 hr., collected by centrifugation and resuspended at pH 6.7, behaved normally, indicating that the effect of the acid was reversible.

Table 1. *Relationship between lysis and percentage lowering of the optical densities of suspensions of spheroplasts*

Suspension medium	Temp. (° C.)	pH	% lowering of optical density after 1 hr.	Lysis
7 % sucrose	20	6.8	46	—
10 % sucrose	37	7.5	57	(+)
10 % sucrose + trypsin	37	7.5	54	(+)
10 % sucrose	20	7.5	50	(+)
10 % sucrose	20	3.4	—47	—
10 % sucrose + pepsin	20	3.4	—14	—
20 % sucrose + phosphate buffer	20	6.7	32	—
20 % sucrose + citrate buffer	20	6.7	27	—
7 % sucrose + phosphate buffer	20	6.8	41	—
7 % sucrose + citrate buffer	20	6.8	32	—

(+), Slight lysis confirmed by microscopic examination.

DISCUSSION

The term spheroplasts has been suggested to describe abnormal bacterial forms fragile to osmotic and mechanical shock, spherical in shape and released by the action of penicillin on the rapidly growing cells: spheroplasts of *Escherichia coli* lyse rapidly when warmed to 56° (Hurwitz *et al.* 1958). The description of the fragile, spherical forms of *Aerobacter aerogenes* as spheroplasts can be justified on several grounds.

Normal growth precedes lysis of cells exposed to penicillin during a period corresponding to the doubling of the bacterial mass in suspension (Fig. 1). That this is not accompanied by actual cell division is shown by direct cell counts (Fig. 2). Thus, during the initial period of exposure to the inducing agent, the cell-growth mechanism is not damaged, but division ceases. Growth continues, at a diminished rate, for a further 3 hr. During this time the individual cells undergo a series of changes which result in the production of the spherical forms. The synthesis of cell

material is counterbalanced by a process of gradual lysis, the optical density of the culture becoming constant about 5 hr. after the initial exposure to penicillin.

The internal osmotic pressure of the spheroplasts lies between 5 and 6 atmospheres at 25° C. This compares well with the value for *Escherichia coli* estimated by a different method (Mitchell & Moyle, 1956), and suggests that the spheroplast envelopes have decreased tensile strength. The superiority of sucrose in the stabilization of spheroplasts can be explained if it is assumed that the cells possess a mechanism for the active transport of K^+ and PO_4^{---} .

Various workers have confirmed a direct relation between lysis and turbidities of suspensions of protoplasts and bacteria (Mitchell & Moyle, 1956; Salton, 1957; Christian & Ingram, 1959). Turbidity measurements, however, cannot be applied to the estimation of the numbers of spheroplasts remaining in suspension. The most highly organized fragments visible after lysis of bacteria by lysozyme are the cytoplasmic membranes and the associated lipid granules. These 'ghosts' are difficult to observe under the microscope (Weibull, 1955). In contrast, the spheroplast 'ghosts' appear to be more dense optically (Pl. 2, fig. 10) and they frequently flocculate. Extensive vacuolation of the spheroplasts in response to the external osmotic conditions may also explain their unexpected optical properties. Bacteria are difficult to plasmolyse and the extent of shrinkage is usually slight (Weibull, 1956). Volumes of protoplasts vary slightly with the external conditions, the osmotically inert fraction of the total volume being 36 % for protoplasts of *Bacillus megaterium* (Weibull, 1955) and 76 % for those of *Micrococcus lysodeikticus* (Gilby & Few, 1959). The process of vacuolation of the spheroplasts is rather slow; this probably accounts for the continuous changes in the optical densities of their suspensions (Fig. 3).

Lysis of spheroplasts at high temperatures and at values of pH greater than 7.0 must be the result of a rupture in the protoplasmic membrane. In alkaline conditions the membrane constituents are probably slowly denatured, the process resulting in alteration and breakdown of the structure. Such changes do not occur in acid conditions, but it is surprising to note the profound alteration of the physical properties of the protoplasm at pH values below 4.3. Resistance of the acid-treated protoplasts to lysis is probably due to the coagulation of the cell contents. The process is reversible even after prolonged standing at pH 3.5. It is interesting to speculate whether such changes occur in normal bacteria, whose viability may not be affected by acid treatment.

The swellings and protrusions observed in bacteria exposed to penicillin in hypertonic media are first visible after 30 min. incubation, i.e. at the time corresponding to the onset of osmotic fragility. The protrusions appear in the middle of the bacteria, where the cells would be expected to divide. Protrusions continue to grow, until finally the remaining rod-shaped fragments become detached, leaving the large spheres. In 5 hr. the surface area of a normal cell converted to a spheroplast increases by a factor of about 70.

The presence of an envelope around the spheroplasts of *Aerobacter aerogenes* which can be differentiated from the protoplasmic membrane is suggested by the examination of the vacuolated bodies. The protoplasm must at all times be surrounded by a hydrophobic membrane if lysis is not to take place. In hypertonic solution, the membrane becomes detached from the inside of the envelope which

surrounds the spheroplast and which remains clearly visible (Pl. 2, fig. 8). The vacuole then occupies the space between the protoplasmic membrane and the spheroplast cell wall. The two structures can be demonstrated by observing the emergence of a protoplasmic extrusion from within the spheroplast (Pl. 2, fig. 9). Bacterial protoplasts are surrounded by the protoplasmic membranes only and do not develop vacuoles. Although their size varies slightly with the external osmotic pressure, they remain spherical in shape. It is interesting to note that many authors have observed the presence of vacuoles in spheroplasts, without commenting on the implication of the phenomenon (Lederberg, 1956; Liebermeister & Kellenberger, 1956; Hahn & Ciak, 1957; Lark, 1958, McQuillen, 1958).

The results obtained support the hypothesis that bacterial spheroplasts are surrounded by a modified form of cell wall in addition to the protoplasmic membrane. This is further confirmed by a comparison of the electrokinetic properties of the normal cells and spheroplasts (Gebicki & James, to be published).

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EXPLANATION OF PLATES

Anoptal Phase Contrast, 3500

PLATE 1

- Fig. 1. Normal cells of *Aerobacter aerogenes*.
- Figs. 2, 3. After 30 min. incubation with sucrose and penicillin.
- Fig. 4. After 60 min.
- Fig. 5. After 2 hr.

PLATE 2

- Figs. 6-8. Vacuolated spheroplasts.
- Fig. 9. Protoplast emerging from within a spheroplast, only after careful water treatment.
- Fig. 10. Spheroplast ghost, after extreme dilution with water.