MEMBRANE STABILITY IN *MICROCOCCUS LYSODEIKTICUS* AND ITS PROTOPLASTS

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Summary

The response of *Micrococcus lysodeikticus* and its protoplasts to storage in 1M sucrose has been studied by spectrophotometry and electron-microscopy. During several days, changes in membrane permeability properties result in the leakage of intracellular components absorbing at 260 mμ without loss of the stabilizing effect of sucrose against osmotic explosion. Under some conditions of temperature and pH, autolytic conversion of bacteria to osmotically sensitive "protoplasts" can be almost complete. Fresh protoplasts in 0·05M NaCl plus 1M sucrose are stable within the range of pH 5·5–8·0. Exposure of protoplasts to pH conditions outside these limits causes lysis with disruption of the protoplast membrane.

I. Introduction

The well-known lytic effect of lysozyme on *Micrococcus lysodeikticus* (Fleming 1922) by depolymerizing its cell wall (Salton 1952, 1956) has led to a number of attempts to prepare protoplasts from this organism by adapting the methods used successfully by Weibull (1953) with *Bacillus megaterium*. Due to the much higher osmotic pressures necessary in the stabilizing medium during the controlled action with lysozyme, some of these attempts were only partially successful with *M. lysodeikticus*. However, McQuillen (1955*a*) and Mitchell and Moyle (1956*a*, 1956*b*) have now described the liberation by lysozyme of protoplasts of *M. lysodeikticus* and have studied some of their properties. In other publications (Few, Fraser, and Gilby 1957; Gilby and Few 1957*a*, 1957*b*) the results of experiments with stable preparations in sucrose of protoplasts from *M. lysodeikticus* have been reported.

Our work on the preparation of protoplasts of *M. lysodeikticus* (Gilby 1957) has confirmed that of Mitchell and Moyle referred to above. Protoplasts were prepared by treatment with lysozyme in media containing sucrose at different concentrations. Measurements are made of the optical density at 500 mμ of protoplast suspensions and of the ultraviolet absorption spectra of the supernatants from suspensions which had been centrifuged with precautions to avoid mechanical damage (Weibull 1953). Both types of measurement indicated that 95 per cent. of protoplasts were stabilized when prepared in 1M sucrose and no improvement was achieved at higher concentrations.

Thus, provided lysozyme treatment is carried out in the presence of 1M sucrose, there appears to be insignificant damage to the permeability barriers of

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M. lysodeikticus. This paper describes subsequent changes occurring on storage of protoplasts and of bacteria and the stability of the protoplast membrane to pH.

II. Methods

(*a*) *Growth of Bacteria and Preparation of Protoplasts*

M. lysodeikticus (N.C.T.C. 2665) was grown in surface culture on a beef tryptic digest medium at pH 7·2 contained in 20-oz bottles which were plugged with cotton wool. Each bottle was inoculated with *c.* 2 ml of a 48-hr broth culture. After incubation for 20 hr at 37°C the organisms were harvested, passed through a coarse glass-wool filter, and washed three times by centrifugation with distilled water. Finally they were resuspended in distilled water at a concentration of *c.* 30 mg dry wt./ml. To prepare protoplasts, bacteria at a concentration of 10 mg dry wt./ml were treated with lysozyme (concn. 100 μg/ml) in a medium containing 1M sucrose plus 0·05M NaCl. As indicated by spectrophotometric measurements at 500 mμ, depolymerization of the cell walls was complete after 30 min at 20°C. After this time, the protoplast suspension was centrifuged at *c.* 1200 *g* for 30 min and the supernatant reaction medium, containing 10–15 per cent. of the protoplasts still unsedimented, discarded. The sedimented pellet was rinsed with the sucrose medium and the protoplasts were gently resuspended in 1M sucrose plus 0·05M NaCl. In this way the complex mixture of reaction products was removed without the protoplasts undergoing any destruction detectable by phase- or electron-microscopy or by spectrophotometry.

(*b*) *Senescence of Bacteria and Protoplasts*

Stock suspensions of bacteria and of protoplasts were prepared at 5 mg bacterial dry wt./ml. Two types of suspension medium were used, each containing 1M sucrose, but one was buffered at pH 6·5 and the other at pH 5·5 with 0·05M phosphate buffer. The organisms were stored at 15°C and at 0°C. At intervals over 6 days, 1-ml samples were withdrawn and each diluted with 4 ml of the appropriate sucrose medium. The optical density at 500 mμ of each of the diluted suspensions was determined before centrifugation. Centrifugation was carried out in four stages to minimize mechanical damage to abnormally sensitive organisms. These stages consisted in two centrifugations at 400–500 *g* for 10 min followed by another at 1500 *g* for 30 min, each at 2°C, and finally centrifugation at 3000 *g* for 30 min at room temperature. The optical density at 260 mμ was then measured on the clear supernatants. Spectrophotometric determinations were made using a Unicam SP500 spectrophotometer.

Observations with the electron microscope were made on bacteria and protoplasts before and after aging. The organisms, at a concentration of 2 mg dry wt./ml, were fixed by treatment for 30 min at room temperature with 2 per cent. formalin in 1M sucrose. Osmic acid treatment was not successful as this caused lysis of protoplasts. The formalin-hardened preparations were centrifuged and washed twice in distilled water. The specimens were dried under vacuum after transfer to nitrocellulose-filmed grids and shadowed with gold–palladium before examination in a Siemens electron microscope, generally at a direct magnification of 8000–12,000.

(c) *pH Stability of Protoplasts*

It was found impossible to centrifuge protoplasts which had been exposed to unfavourable pH conditions without additional uncontrolled mechanical damage. Calibration curves were constructed relating the optical density at 500 and 260 mμ to the composition of artificially prepared mixtures of protoplasts and protoplasts

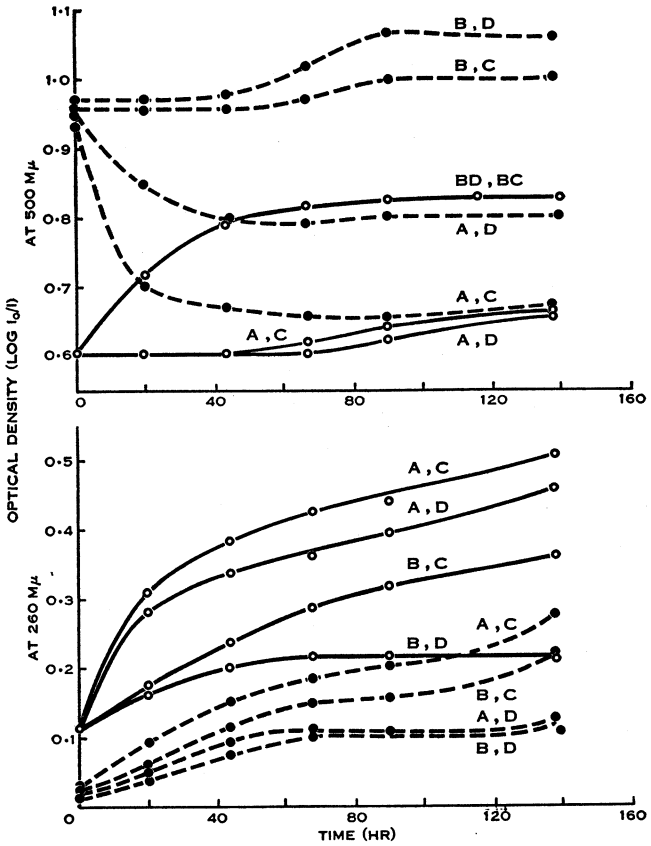

Fig. 1.—Storage of *M. lysodeikticus* (broken lines) and protoplasts at a concentration of 1 mg dry wt./ml in 1M sucrose. Optical densities at 500 mμ measured on suspensions and at 260 mμ on centrifuged supernatants. Conditions of storage indicated on curves: *A*, pH 6·5; *B*, pH 5·5; *C*, 15°C; *D*, 0°C.

lysed by osmotic explosion. These mixtures corresponded to a total dry weight of 0·25 mg/ml and were made up in 1M sucrose plus 0·05M NaCl, and ranged from 100 per cent. protoplasts to 100 per cent. lysate.

To determine their pH sensitivity, protoplasts at a concentration of 2·5 mg dry wt./ml were suspended in 1M sucrose buffered to cover the range of pH 4–11·5. The buffers were used at an approximate ionic strength of 0·05 and consisted of mixtures of citric acid and Na_2HPO_4, NaH_2PO_4 and Na_2HPO_4, $Na_2B_4O_7$ and NaOH.

Samples were taken after 0·5 and 2·5 hr at the test pH and returned to pH 6·5 by a dilution of 1/10 into 1M sucrose buffered at this pH. This was to avoid the complication caused by precipitation at low pH of the cell contents of lysed protoplasts. The optical density at 500 and 260 mμ were then determined for each of the diluted suspensions.

III. Results

(a) *Effects of Storage*

The results of measurements of optical density at 500 and 260 mμ on suspensions of bacteria and protoplasts stored under various conditions are illustrated in Figure 1. Appreciable changes occur with time in each case. The 260 mμ

Table 1

Optical densities of 1/10 dilutions of bacteria stored in 1M sucrose for 6 days

Final concentrations 0·5 mg dry wt./ml, 0·1M sucrose

Conditions of Storage		Optical Density at 500 mμ		Intact Bacteria (%)
pH	Temperature (°C)	In 0·1M Sucrose	In Water (calc.)	
6·5	15	0·082	0·086	6
6·5	0	0·276	0·288	20
5·5	15	0·146	0·156	11
5·5	0	0·452	0·475	34

absorption measurements indicate that there is greater leakage of material absorbing at this wavelength at the higher temperature and pH. However, even after 6 days, none of the preparations show greater than 20 per cent. of the absorption observed on complete lysis. Since 260 mμ absorbing material is likely to be lost by chemical reaction and adsorption, quantitative deductions are unreliable.

Protoplasts show an increase in optical density at 500 mμ which is immediate at pH 5·5 and delayed at pH 6·5. Bacteria at pH 5·5 exhibit a trend similar to protoplasts at pH 6·5. Of particular interest are the bacteria stored at pH 6·5 and 15°C which exhibit a decrease in light scattering and approach the optical density reached by protoplasts stored under the same conditions. This observation suggested that at this stage the two preparations may be essentially identical and the bacteria spontaneously converted to protoplasts. In Table 1 the optical density at 500 mμ is given for each of the four aged bacterial suspensions diluted to 0·1M sucrose concentration which is sufficient to lyse any protoplasts which may have

formed. By allowing for the known effect of sucrose on the established curve calibrating optical density and bacterial concentration, the percentage of bacteria not lysed was calculated. These values, also shown in Table 1, indicate substantial conversions of bacteria to "protoplasts". It has been assumed that aged bacteria have similar light-scattering properties to fresh bacteria. Since the results in Figure 1 suggest that, at pH 5·5 at least, the scattering of light increases on aging, the bacteria calculated as surviving unchanged will tend to have been overestimated.

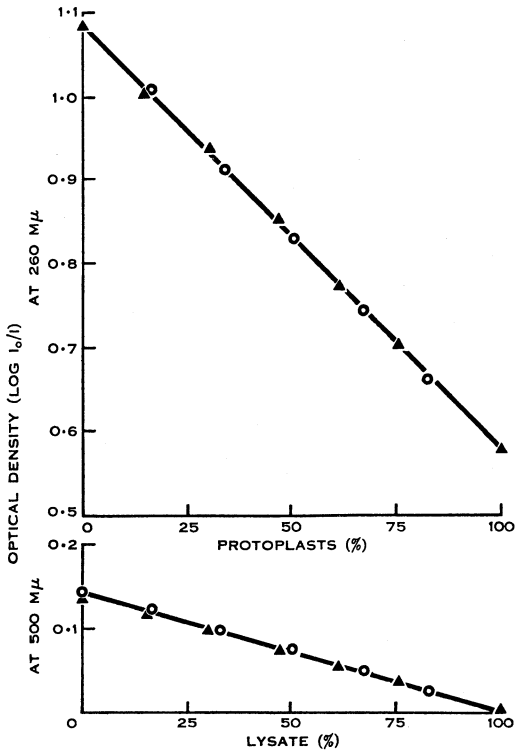

Fig. 2.—Optical properties of artificial mixtures of protoplasts and lysates of *M. lysodeikticus* in 1M sucrose plus 0·05M NaCl. Total concentrations 0·25 mg dry wt./ml. Two independent experiments indicated.

Viewed in the electron microscope, fresh protoplasts appear as discrete spheres, 0·7–0·8 μ in diameter. In this respect they differ from the bacteria from which they were derived, which invariably form clumps. On the other hand, aged bacteria believed to have changed spontaneously to "protoplasts" appear to be no longer aggregated. These latter preparations, however, no longer consist wholly of spheres, but show a very large proportion of dumb-bell shapes. Lysozyme-induced protoplasts which have aged in sucrose medium retain their spherical form and only occasional dumb-bell shapes have been observed.

(*b*) *Sensitivity of Protoplasts to pH*

The optical properties of artificial protoplasts–lysate mixtures are illustrated in Figure 2. Because of the linearity exhibited, the contributions to the optical density at 260 mμ due to specific absorption in protoplast suspensions and to non-specific scattering are additive. Thus the percentage of intact protoplasts can be calculated from the measurements at 500 mμ and the contribution to the measured optical density at 260 mμ due to these protoplasts may then be computed. The specific absorption at 260 mμ is obtained by difference.

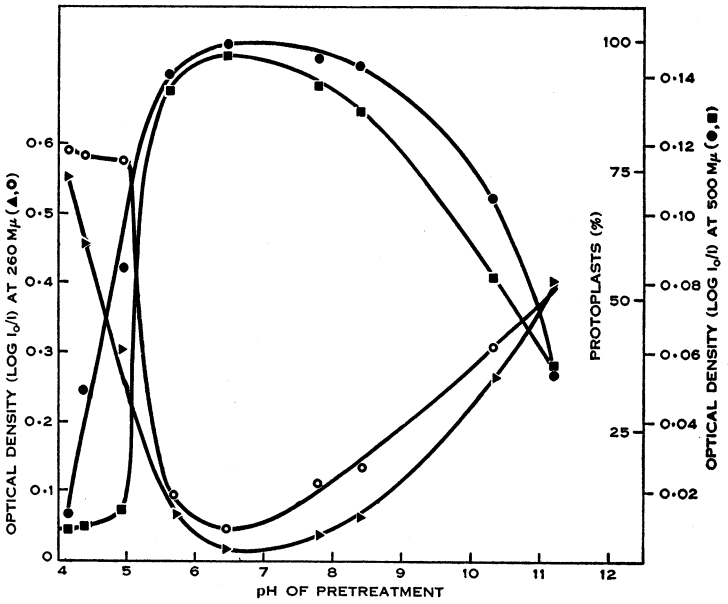

Fig. 3.—Stability to pH of protoplasts of *M. lysodeikticus*. Protoplast suspensions returned to pH 6·5 by 1/10 dilution with 1M sucrose. Conditions of pretreatment: protoplasts 2·5 mg/ml in 1M sucrose; 20°C. Incubation for 0·5 or 2·5 hr. Optical density measured at 500 mμ: ● 0·5 hr pretreatment; ■ 2·5 hr pretreatment. Optical density calculated for specific absorption at 260 mμ: ▲ 0·5 hr pretreatment; ○ 2·5 hr pretreatment.

The results of the optical density measurements at 500 mμ and the calculated specific absorption at 260 mμ are plotted against pH of treatment in Figure 3. A high degree of damage to the protoplasts is associated with a low optical density at 500 mμ and a high 260 mμ absorption. The action on the protoplast membrane is time dependent and incomplete in 30 min. A much sharper delineation of the range of stability is observed after 2·5 hr exposure. For this exposure the curves at 500 and 260 mμ both indicate that the onset of damage under acidic conditions occurs quite suddenly in a narrow range of pH slightly above 5. Over the range of pH 5·5–8·0 the protoplasts are relatively stable, but as the pH is raised they again become unstable. The onset of alkali damage is more gradual than that by acid.

IV. Discussion

During storage of bacteria and protoplasts, it is evident that considerable changes occur in the membrane systems of the organisms. However, the majority of protoplasts are not disrupted by osmotic explosion during storage, indicating that the leakage of 260 mμ absorbing compounds which occurs is not accompanied by breakdown in the impermeability to sucrose of the osmotic barrier. Nevertheless, the use of aged preparations for biophysical investigations appears inadvisable. This has been confirmed in other work (Gilby and Few 1957*a*, 1957*b*) in which the rate of lysis by detergents was observed to increase rapidly with protoplasts older than 8 hr. In view of the composition of the storage medium, it is not likely that growth of protoplasts would be supported. Indeed, although Jeynes (1957) has reported growth of protoplasts produced from bacteria by other methods, growth of lysozyme-induced protoplasts has not yet been observed even when nutrients are present. With aged protoplasts, however, some few dumb-bell shapes similar to those observed by McQuillen (1955*b*) have been noted here in electron-micrographs of formalin-fixed preparations. The two main factors believed to contribute to the observed increase in turbidity of protoplast suspensions are (1) shrinkage of protoplasts due to leakage of intracellular material, and (2) changes in the refractive index difference at the cell/medium interface altering the light-scattering properties.

With lysozyme rigorously excluded, the autolytic conversion of *M. lysodeikticus* to "protoplasts" can, under favourable conditions, be almost complete although apparently slower than with *Staphylococcus aureus* (Mitchell and Moyle 1957). Most of the *M. lysodeikticus* protoplasts resulting from aging are observed by electron-microscopy to exhibit a dumb-bell shape, the proportion being many times greater than in protoplasts aged after lysozyme treatment. It seems probable, therefore, that the steps towards division in the aging bacteria may have occurred before the attainment of the "protoplast" condition. In considering autolysis, Welsch (1958) has pointed out the difficulties in deciding between the presence of a truly bacteriolytic agent or an inducer of autolysis through secondary causes. The presence in the *M. lysodeikticus* cell sheath of an autolytic enzyme system cannot be excluded (Mitchell and Moyle 1956*b*). In the present experiments, the bacteria are grossly deprived of nutrient and do not divide to form the osmotically fragile naked cells observed by Meadow, Hoare, and Work (1957) and Rhuland (1957) when *Escherichia coli* is deprived only of a single agent believed essential to the synthesis of cell wall material. The fact that aged *M. lysodeikticus* bacteria are almost all dumb-bell shaped could mean, on the other hand, that the processes involved in the conversion to protoplasts are intimately linked with those of cell wall production and division. The latter processes may take place at a definite stage during growth, which here cannot proceed past a certain point. The observed loss of 260 mμ absorbing material indicates disorganization of the cytoplasmic membrane, a structure almost certainly involved in the formation of the cell wall. Whether the primary effect of interference with the cell nutrient supply during senescence is to inhibit the process of cell wall synthesis or to induce autolysis by breakdown of internal organization, it is therefore not possible to decide. Failure to observe cell wall fragments when osmotically sensitive, aged bacteria are lysed by dilution of the protective medium could favour the latter alternative.

Freshly prepared *M. lysodeikticus* protoplasts are stable in the range pH 5·5–8·0. Outside these limits, the trends shown by the measurements of optical density at 500 mμ and the calculated specific absorption at 260 mμ are closely parallel. If the optical density at 260 mμ is calculated only on the basis of protoplasts lysed as indicated by measurements at 500 mμ, agreement with the results in Figure 3 is within 5 per cent. This suggests that there is very little leakage of 260 mμ absorbing intracellular material without an accompanying loss of structural integrity and disruption of the protoplasts. Thus, rather than the initial action being to induce leakage of intracellular components, the primary breakdown of the protoplasts as a response to pH appears to be due to the destruction of the impermeability of the protoplast membrane to the stabilizing sucrose with consequent osmotic explosion. In previous work (Few, Fraser, and Gilby 1957) it has been shown that the onset of the leakage of 260 mμ absorbing material from intact *M. lysodeikticus* occurs at pH 4·4, which may be compared with the stability threshold of pH 5·5 for protoplasts. It is generally agreed that it is the protoplast membrane which maintains the osmotic function of bacteria (Mitchell and Moyle 1956*b*). With bacteria, the presence of the negatively charged cell wall will affect both the access of hydrogen and other ions to the protoplast membrane and the escape of intracellular constituents once this membrane is damaged. The observations support the view that the onset of damage to the protoplast membranes resulting in their becoming permeable to intracellular material occurs only under more drastic conditions than those sufficient to disrupt protoplasts by osmotic explosion. Furthermore, when *M. lysodeikticus* is titrated with hydrochloric acid, the barriers to the entry of hydrogen ions are only completely broken down at pH 3 (Gilby and Few 1958).

With the information at present available on the composition and structure of the membranes of *M. lysodeikticus* it is not possible to reach precise conclusions concerning the nature of the changes which produce the effects on cell permeability described. Chemical analysis (Gilby, Few, and McQuillen 1958) indicates the presence of protein, lipid, and polysaccharide material in the protoplast membrane. Unpublished work by Few, Gilby, and Seaman using micro-electrophoresis supports the "protein–lipid sandwich" theory as the basis of protoplast membrane structure, with the added possibility of the carbohydrate contributing to a triplex formation. It is unlikely that such a complex structure would respond in any simple way to changes in its environment.

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