Protoplast and Cytoplasmic Membrane Preparations from *Streptococcus sanguis* and *Streptococcus mutans*

By MARIE SCHÖLLER,* J. PAUL KLEIN, PASCAL SOMMER AND
ROBERT FRANK

Unité de Recherches, INSERM U157, Faculté de Chirurgie Dentaire, 67000 Strasbourg, France

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Protoplasts were prepared from *Streptococcus sanguis* and some *S. mutans* serotypes by use of lysozyme (EC 3.2.1.17) under particular conditions: cells had to be grown in DL-threonine (20 mM) and harvested in early exponential phase. The efficiency of protoplast formation was enhanced by two additional steps: plasmolysis (in 12% PEG), prior to addition of lysozyme, and a swirling phase, after the enzymic action. This procedure allowed us to obtain clean protoplasts, with only 0.5% contamination by bacterial cell walls. Up to 90% protoplast lysis was obtained in 0.5 M-NaCl. Cytoplasmic membrane purification was achieved by centrifugation on a glycerol cushion.

INTRODUCTION

Streptococcus sanguis and *Streptococcus mutans* are commonly associated with dental caries (Guggenheim, 1968). The latter species appears to be very heterogeneous, and has been divided into five biotypes (Shklair & Keene, 1976), four genetic groups (Coykendall, 1974) and eight (*a* to *h*) serological groups (Bratthall, 1970; Perch *et al.*, 1974; Beighton *et al.*, 1981). Since purified serotype antigens were proposed as possible vaccines against dental caries (Taubman, 1973) much attention has been given to those characterized as wall-associated polysaccharides containing, in some cases, lipoteichoic acid (Silvestri *et al.*, 1978; Vaught & Bleiweis, 1974). Because of the poor cross-reactivity between the different serotypes, more recent studies have dealt with the characterization of extracellular or wall-associated proteins (Russell & Lehner, 1978; Russell, 1979; Schöller *et al.*, 1981). The difficulty in obtaining pure plasma membranes, free of cell wall components, from *S. mutans* and *S. sanguis* stems from the lack of efficient methods of protoplast preparation. Hence their cytoplasmic membranes are poorly characterized. Spheroplast induction with phage-associated lysins has been described for streptococci of Lancefield groups A, C, D and H (Krause, 1972; Bleiweis & Zimmermann, 1961; Calandra *et al.*, 1975), but not for oral streptococci. Except for those belonging to Lancefield group D, most streptococci are very resistant to lysozyme lysis (Smith, 1973).

Lysozyme (*N*-acetylmuramidase, EC 3.2.1.17) exerts its lytic effect through hydrolysis of specific glycosidic linkages in its substrate, the peptidoglycan of the bacterial cell wall. Streptococci, and especially strains of *S. mutans* and *S. sanguis*, are considered refractory to lysozyme action (Gibbons *et al.*, 1966) and few studies have shown lysis of *S. mutans* cell walls with the enzyme alone (Coleman *et al.*, 1970). Bacterial resistance to lysozyme may stem from several factors including the three-dimensional structure of peptidoglycan (Chiu *et al.*, 1974; Formanek *et al.*, 1974), the presence of teichoic acids covalently bound to peptidoglycan (Joseph & Shockman, 1975; Markham *et al.*, 1975), the presence of unacetylated glucosamine residues in peptidoglycan (Hayashi *et al.*, 1973) and surface adsorption of macromolecules from growth media (Chassy, 1976). Procedures used to enhance sensitivity to lysozyme include use of

Abbreviations: DAP, diaminopimelic acid; PEG, polyethylene glycol.

appropriate culture media and age of culture, and the addition of high concentrations of sucrose, detergents and salts (Coleman *et al.*, 1970; Eisenberg & Lillmars, 1975; Chassy, 1976; Pollock *et al.*, 1976; Shockman *et al.*, 1979; Goodman *et al.*, 1981). Thus, lysozyme can reach and degrade the murein layer after acetylation of cell wall muramic acid residues in exponential phase cultures of streptococci (Eisenberg & Lillmars, 1975), or by inclusion of DL-threonine in culture media, which interferes with cell wall cross-linking in *S. mutans* by a penicillin-like inhibition of lysine incorporation (Chassy, 1976). Destabilization of cell wall can also be achieved by resuspending cells in dilute Tris/HCl buffer supplemented with high concentrations of sucrose or polyethylene glycol (PEG), and by adding lysozyme in considerable excess relative to total cell peptidoglycan (Witholt & Boekhout, 1978; Owen & Freer, 1972; Johnston & Gotschlich, 1974). Although all these procedures were effective in the lysis of *S. sanguis* and *S. mutans* cells, no satisfactory method for conversion of these bacteria into stable protoplasts with lysozyme has yet been described.

Owen & Freer (1972) showed the efficiency of whole-cell plasmolysis, before removal of cell wall with lysozyme, for the preparation of mesosomal and cytoplasmic membranes from *Micrococcus lysodeikticus*. We tested the effect of such treatment for the preparation of stable protoplasts from *S. sanguis* and *S. mutans* strains representative of each of Bratthall's serological groups. The present paper describes a method for preparation of cytoplasmic membranes from protoplasts of *S. sanguis* OMZ9 and different *S. mutans* serotypes.

METHODS

Bacteria and growth conditions. *Streptococcus sanguis* OMZ9 was used as a reference strain throughout this study. The following *S. mutans* serotypes were used: OMZ51 (*b*), OMZ70 (*c*), OMZ176 (*d*), B-2 (*e*), OMZ175 (*f*). The origin and maintenance of all strains were described by Schöller *et al.* (1981).

Cells of *S. sanguis* used for conversion to protoplasts were grown in Brain Heart Infusion Broth (BHI, Difco) supplemented with 20 mM-DL-threonine at 37 °C (Chassy, 1976). The bacteria were harvested by centrifugation (5000 *g*, 20 min, 4 °C) in mid-exponential phase ($OD_{540} = 1.0$) and washed twice in 10 mM-Tris/HCl buffer pH 7.2 (referred to as 'Tris-buffer' throughout this paper) before use as described below.

The efficiency of protoplast formation was monitored by measuring the cell-associated peptidoglycan and DNA at each step of the preparation. *meso*-Diamino[G-³H]pimelic acid ([³H]DAP) was employed to label peptidoglycan *in vivo*. Cells of *S. sanguis* OMZ9, grown as previously described, were transferred into 100 ml BHI containing [³H]DAP (25 mM; 4 Ci mmol⁻¹; 148 GBq mmol⁻¹), and allowed to grow for three mean generation times. DNA was labelled with [³H]thymidine by growing bacteria as above, in 100 ml BHI supplemented with DL-threonine (20 mM), deoxyadenosine (20 µM) and [*methyl*-³H]thymidine (0.5 µM; 2 Ci mmol⁻¹; 74 GBq mmol⁻¹). Labelled cells were harvested in mid-exponential phase and used for protoplast formation.

Protoplast formation. Washed cells were resuspended to one-tenth of the culture volume in Tris-buffer containing 6% (w/v) PEG 4000. Lysozyme was added to final concentrations between 125 and 1000 µg per ml of initial culture volume, and the suspensions were incubated for 1 h at 30 °C.

Optimal PEG concentration for protoplast stability was determined by suspending protoplasts for 24 h in Tris-buffer containing various PEG concentrations.

In the procedure involving plasmolysis and swirling, washed cells were allowed to equilibrate for 2 h at 30 °C in Tris-buffer containing 12% PEG. After lysozyme treatment, the suspension was made 6% with respect to PEG, and subjected to swirling (100 r.p.m.) at 30 °C for 1 h.

Preparation of membranes. Protoplasts were sedimented from the suspending medium by centrifugation (8000 *g*, 20 min, 4 °C), washed twice with Tris-buffer containing 6% PEG and resuspended (2 h, 30 °C) in 10 ml Tris-buffer with or without 15% PEG. In both cases lysis was performed in one of four ways: (i) sonication, (ii) freeze-thawing procedures, (iii) osmotic shock (addition of 0.5 M-NaCl to Tris-buffer or resuspension of protoplasts in 1 M-phosphate buffer pH 7.2), and (iv) treatment with SDS (2%, w/v).

Lysates were centrifuged three times at 6000 *g* for 20 min at 4 °C to remove any residual protoplasts or bacteria. The 6000 *g* supernatant, after 15 min treatment with DNAase (10 µg ml⁻¹) and RNAase (10 µg ml⁻¹), was centrifuged at 200000 *g* for 30 min at 4 °C. The membrane pellet was washed twice, resuspended in Tris-buffer and layered on a glycerol cushion. Centrifugation was carried out in an SW-25 rotor at 40000 *g* for 60 min at 4 °C. The upper aqueous phase was carefully removed and centrifuged (200000 *g*, 30 min, 4 °C), and the sedimented membranes were washed three times with Tris-buffer and stored as a thick suspension at -20 °C.

Monitoring procedures. The efficiency of protoplast formation was assessed by monitoring the extent of [³H]DAP labelling of cells at each stage of the procedure. Samples (1 ml) were collected on membrane filters (pore size 0.22 µm, Millipore) and immediately precipitated with 10% (w/v) cold TCA. The dried filters were

transferred to glass vials containing toluene supplemented with PPO (0.5%, w/v) and POPOP (0.01%, w/v) before counting in an SL-32 liquid scintillation counter (Intertechnique).

Protoplast integrity was assessed by measuring non-sedimentable TCA-precipitable [^3H]thymidine radioactivity of incubation mixtures. Samples (10 to 30 μl) were spotted on 3MM Whatman filters, precipitated with 10% TCA and counted as previously described. Protoplast leakage was determined by measuring TCA-precipitable [^3H]thymidine radioactivity released into the supernatant fraction of sedimented incubation mixtures. Purity of membrane preparations was evaluated by counting residual TCA-precipitable [^3H]DAP and [^3H]thymidine label present in membrane suspensions.

The different steps in protoplast and membrane preparations were also followed by electron microscopy. Protoplast pellets were fixed for 2 h in a mixture of 2% (v/v) paraformaldehyde and 2% (v/v) glutaraldehyde in 0.1 M-cacodylate buffer (pH 7.4), washed with cacodylate buffer and postfixed in 1% (w/v) osmium tetroxide for 1 h, dehydrated with ethanol and embedded in Epon. Membrane preparations after osmium tetroxide postfixation were first embedded in 1% (w/v) agar, before dehydration and embedding in Epon.

Analytical procedures. Protein was determined by a modified Lowry method (Dulley & Grieve, 1975), with bovine serum albumin as standard, in the presence of 2% (w/v) SDS. Total hexose was estimated with the anthrone reagent, with glucose as standard (Morris, 1948). DNA was determined by the fluorimetric method of Thomas & Farquhar (1978), with calf thymus DNA as standard. Total lipid was extracted according to Osborn *et al.* (1972) and the phosphorus concentration measured according to Baginsky & Zak (1960).

Enzyme assays. Adenosine-5'-triphosphatase (ATPase, EC 3.6.1.3) was analysed by the method of Abrams (1965) on freshly isolated membranes. The released inorganic phosphate from ATP was measured by the procedure of Baginsky & Zak (1960). The reaction mixture (1 ml) contained 50 mM-Tris/HCl buffer, pH 7.2, 5 mM-MgCl₂, 5 mM-ATP and the fraction to be tested. Glucose-6-phosphate dehydrogenase (EC 1.1.1.49) activity was measured by following the increase in absorption at 340 nm resulting from the glucose 6-phosphate dependent reduction of NADP. The assay (1 ml) contained 100 mM-Tris/HCl buffer pH 7.5, 2 mM-disodium glucose 6-phosphate, 0.25 mM-NADP and the fraction to be tested.

RESULTS

Protoplast formation. The susceptibility of *S. sanguis* OMZ9 to different lysozyme concentrations, under various conditions, was assayed by measuring the residual [^3H]DAP contained in protoplasts. Incubation of *S. sanguis* suspensions with 500 to 1000 μg lysozyme ml^{-1} for 1 h resulted in a loss of 85% of the [^3H]DAP label (Fig. 1). Plasmolysis for 2 h in Tris-buffer containing 12% PEG before lysozyme treatment allowed an additional loss of [^3H]DAP of 10% (Fig. 1). When a swirling step (1 h) in Tris-buffer containing 6% PEG followed plasmolysis and lysozyme treatment, only 1.45% of the [^3H]DAP remained sedimentable (Fig. 1, Table 1).

PEG concentration was chosen after assessing the effect upon protoplast stability of various concentrations (4 to 12%) in Tris-buffer, after 24 h incubation of cell suspensions obtained by a 1 h treatment with lysozyme (500 μg ml^{-1}). A 6% PEG concentration gave minimal lysis and was employed during enzymic digestion and swirling, resulting in negligible lysis (0.48 to 1.4%) throughout protoplast preparation (Table 1).

Protoplast lysis and membrane preparation. The efficiency of protoplast disruption was investigated by four different lysis procedures (osmotic and detergent shocks, sonic oscillation, freeze-thawing) in either hypotonic (without PEG) or hypertonic (15% PEG) Tris-buffer (Table 2). The lysates were centrifuged at 200 000 g for 30 min at 4 °C and the percentage of lysis was calculated from the amount of [^3H]thymidine radioactivity found in the supernatant fraction compared to that found in untreated protoplasts. Addition of 2% SDS or 0.5 M-NaCl to hypotonic Tris-buffer resulted in maximal lysis and a 90% release of [^3H]thymidine from the cells (Table 2). However, SDS is known to solubilize most membranes (Moldow *et al.*, 1972; Helenius & Simons, 1975) and hence NaCl (0.5 M) in hypotonic buffer was the method of choice.

After DNAase and RNAase treatment of the lysates, membranes were collected by centrifugation at 200 000 g (30 min at 4 °C), and purified on a glycerol cushion.

Analysis of membranes for purity and biological activity. Each membrane preparation from *S. sanguis* was analysed for contamination by either cell wall or cytoplasmic constituents. Radioactive DAP represented only 0.5% of the original whole-cell radioactivity (Table 1). The nucleic acid content of the membranes, determined either by thymidine-incorporated radioactivity or by fluorimetry, represented 1% and 0.17% of total cell DNA respectively. The total membrane

Fig. 1. Effect of various amounts of lysozyme, plasmolysis and swirling on lysozyme-induced protoplast formation. Remaining cell wall associated [³H]DAP radioactivity was measured in the sedimented cells after incubation of bacteria for 1 h at 30 °C with various amounts of lysozyme in Tris-buffer supplemented with 6% PEG, without plasmolysis (●), with plasmolysis (■) or with plasmolysis and swirling (▲).

Table 1. *Degree of lysis and cell wall contamination during preparation of protoplasts and membranes from S. sanguis*

Successive treatments	Percentage (w/v) of PEG in Tris-buffer	Lysis* (%)	Residual cell wall DAP† (%)
Plasmolysis (2 h)	12	0.48	100
Incubation with lysozyme (1 h)	6	1.40	5.1
Swirling (1 h)	6	1.37	1.45
Lysis (0.5 M-NaCl)	0	92	—
Cytoplasmic membrane preparations	0	—	0.52

* The percentage of lysis was calculated as the fraction of total [³H]thymidine radioactivity found in the supernatant of samples at the end of each treatment per OD₅₄₀ unit.

† The percentage of cell wall contamination was measured as the fraction of total [³H]DAP radioactivity found in the pellet of samples at the end of each treatment per OD₅₄₀ unit.

Table 2. *Efficiency of different methods of protoplast lysis in either hypotonic or hypertonic medium*

Method of lysis	Lysis* of protoplasts (%)	
	In hypotonic medium (Tris-buffer)	In hypertonic medium (Tris-buffer + 15% PEG)
SDS (2%, w/v)	91	99
NaCl (0.5 M)	92	50
Freeze-thawing	6	8
Sonic oscillation	6	7
Resuspension in 1 M-phosphate buffer pH 7.2	33	33

* The percentage of lysis is calculated as the fraction of total [³H]thymidine radioactivity found in the supernatants.

fraction also contained less than 1% of the glucose-6-phosphate dehydrogenase, a cytoplasmic marker enzyme (Osborn *et al.*, 1972) present in intact protoplasts.

The activity of ATPase, a membrane enzyme (Salton, 1974), was measured to determine whether the procedure used in membrane purification released membrane-associated enzymes. No ATPase activity was found in the different supernatants during protoplast formation. The *S. sanguis* membrane fractions contained 1.7 units of ATPase activity [66.6% of total activity,

Fig. 2. Electron micrographs of thin sections of *S. sanguis* OMZ9 after different treatments. (a) Control bacteria; (b) cells after treatment for 1 h with lysozyme ($500 \mu\text{g ml}^{-1}$); (c) cells prior to lysozyme treatment but after plasmolysis (2 h at 30°C in presence of 12% PEG); (d) cells after plasmolysis, lysozyme treatment and swirling (1 h at 30°C in presence of 6% PEG). Progressive solubilization of the cell wall occurred during the above mentioned treatments and a typical protoplast apparently devoid of cell wall material can be seen in (d). The bar markers represent $0.1 \mu\text{m}$.

specific activity $0.2 \text{ units (mg protein)}^{-1}$] whereas $0.6 \text{ units (23.8\%, } 0.0012 \text{ units mg}^{-1}\text{)}$ was found in the cytoplasmic fraction, and $0.24 \text{ units (9.5\%)}$ in the different membrane washes, indicating that rather low release of enzyme occurred during this procedure.

Chemical analysis of the membranes. The results of the gross chemical analysis of *S. sanguis* cytoplasmic membranes are expressed as percentages of the total dry weight of membranes. Total protein accounted for 62% of the membrane preparation. Total lipids comprised 20% of the dry weight, with a phosphorus/lipid ratio of 0.045. Total hexose content of the membrane was relatively low, representing only 4.2%, which agrees with the work of others (Owen & Freer, 1972; Van de Rijn & Kessler, 1979).

Electron microscopy. All steps in protoplast and membrane preparations were further followed by electron microscopy to confirm the effectiveness of the method. Figure 2 illustrates the different stages in protoplast formation after 1 h incubation in the reaction mixture with lysozyme alone (Fig. 2b), with preliminary plasmolysis (Fig. 2c), and with additional swirling (Fig. 2d). While initially the streptococci had a typical cell wall (Fig. 2a), progressive elimination of wall material was seen after the different treatments, and wall contamination was

Fig. 3. Electron micrograph of a purified membrane fraction. Thin sections were examined after staining with uranyl acetate and lead citrate. The bar marker represents 0.1 μm .

minimal after plasmolysis and swirling (Fig. 2*d*). Thin sections of cytoplasmic membranes after centrifugation on a glycerol cushion appeared to be completely devoid of cell wall contamination (Fig. 3).

Application of the method to other serotypes. The results described above showed that cytoplasmic membrane preparations could be obtained from *S. sanguis* OMZ9. The same procedure, outlined in Fig. 4, was successfully used to obtain cytoplasmic membranes from different *S. mutans* serotypes.

DISCUSSION

With the exception of Siegel *et al.* (1981), who used mutanolysin to induce spheroplasts, no suitable method has been described for preparation of plasma membranes from *S. sanguis* or *S. mutans*. We report a method for producing membranes from these species by a preliminary conversion of bacteria into stable protoplasts with lysozyme. Acetylation of bacterial cells according to Eisenberg & Lillmars (1975) did not provide any advantage in protoplast formation (data not shown). In order to enhance lysozyme sensitivity, bacteria from exponential phase cultures grown in the presence of DL-threonine (Chassy, 1976) were diluted in Tris/HCl buffer (Witholt & Boekhout, 1978) supplemented with PEG (Owen & Freer, 1972). After determination of optimal concentrations of lysozyme (500 $\mu\text{g ml}^{-1}$) and PEG (6%), we tested the effect of plasmolysis and swirling on the efficiency of protoplast formation. Exposure of cells to 12% PEG increased their sensitivity to lysozyme and increased the yield of protoplasts. This was further improved by a swirling step after PEG dilution. Chemical and ultrastructural studies indicated the almost complete lack of cell wall material in the protoplast preparations. Addition of 0.5 M-NaCl to osmotically sensitive protoplasts suspended in hypotonic Tris-buffer resulted in up to 90% lysis, and membranes prepared in this way contained only a small amount of the peptidoglycan DAP. This method allowed us to prepare cytoplasmic membranes from the different *S. mutans* serotypes.

The gross chemical composition of *S. sanguis* cytoplasmic membranes appeared to be in broad agreement with that reported by Kessler & Van de Rijn (1979). Contamination of the membranes by either extracellular or intracellular components was less than 1%, as estimated by the peptidoglycan or DNA content, and the protein/phospholipid ratio was in accord with those generally found for Gram-positive bacteria (Owen & Freer, 1972; Van de Rijn & Kessler, 1979).

It should be pointed out that the above results are drawn from membranes with minor cytoplasmic or wall contamination. The enzyme treatment and washing steps do not appear to affect the integrity of the membranes, which can be compared to membranes obtained by other methods (Siegel *et al.*, 1981).

Fig. 4. Procedure used to obtain cytoplasmic membrane preparations from protoplast suspensions.

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REFERENCES

- ABRAMS, A. (1965). The release of bound adenosine triphosphatase from isolated bacterial membranes and the properties of the solubilized enzyme. *Journal of Biological Chemistry* **240**, 3675-3681.
- BAGINSKI, E. S. & ZAK, B. (1960). Microdetermination of serum phosphate and phospholipids. *Clinica chimica acta* **5**, 834-838.
- BEIGHTON, D., RUSSELL, R. R. B. & HAYDAY, H.

- (1981). The isolation and characterization of *Streptococcus mutans* serotype *h* from dental plaque of monkeys (*Macaca fascicularis*). *Journal of General Microbiology* **124**, 271–279.
- BLEIWEIS, A. S. & ZIMMERMANN, L. B. (1961). Formation of two types of osmotically fragile bodies from *Streptococcus faecalis* var. *liquefaciens*. *Canadian Journal of Microbiology* **7**, 363–373.
- BRATTHALL, D. (1970). Demonstration of five serological groups of streptococcal strains resembling *Streptococcus mutans*. *Odontologisk revy* **21**, 143–152.
- CALANDRA, G. B., NUGENT, K. M. & COLE, R. M. (1975). Preparation of protoplasts of group H streptococci (*Streptococcus sanguis*). *Applied Microbiology* **29**, 90–93.
- CHASSY, B. M. (1976). A gentle method for lysis of oral streptococci. *Biochemical and Biophysical Research Communications* **68**, 603–608.
- CHIU, T. H., EMDUR, L. I. & PLATT, D. (1974). Lipoteichoic acids from *Streptococcus sanguis*. *Journal of Bacteriology* **118**, 471–479.
- COLEMAN, S. E., VAN DE RIJN, I. & BLEIWEIS, A. S. (1970). Lysis of grouped and ungrouped streptococci by lysozyme. *Infection and Immunity* **2**, 563–569.
- COYKENDALL, A. L. (1974). Four types of *Streptococcus mutans* based on their genetic, antigenic and biochemical characteristics. *Journal of General Microbiology* **83**, 327–338.
- DULLEY, J. R. & GRIEVE, P. A. (1975). A simple technique for eliminating interference by detergents in the Lowry method of protein determination. *Analytical Biochemistry* **64**, 136–141.
- EISENBERG, R. J. & LILLMARS, K. (1975). A method for gentle lysis of *Streptococcus mutans*. *Biochemical and Biophysical Research Communications* **65**, 378–384.
- FORMANEK, H., FORMANEK, S. & WAWRA, H. (1974). A three dimensional atomic model of the murein layer of bacteria. *European Journal of Biochemistry* **46**, 279–294.
- GIBBONS, R. J., DE STOPPELAAR, J. D. & HARDEN, L. (1966). Lysozyme insensitivity of bacteria indigenous to the oral cavity of man. *Journal of Dental Research* **45**, 877–881.
- GOODMAN, H., POLLOCK, J. J., KATONA, L. I., IACONO, V. J., CHO, M. I. & THOMAS, E. (1981). Lysis of *Streptococcus mutans* by hen egg white lysozyme and inorganic sodium salts. *Journal of Bacteriology* **146**, 764–774.
- GUGGENHEIM, B. (1968). Streptococci of dental plaque. *Caries Research* **2**, 147–163.
- HAYASHI, H., ARAKI, Y. & ITO, E. (1973). Occurrence of glucosamine residues with free amino groups in cell wall peptidoglycan from bacilli as a factor responsible for resistance to lysozyme. *Journal of Bacteriology* **113**, 592–598.
- HELENIUS, A. & SIMONS, K. (1975). Solubilization of membranes by detergents. *Biochimica et biophysica acta* **415**, 29–79.
- JOHNSTON, K. H. & GOTSCHLICH, E. C. (1974). Isolation and characterization of the outer membrane of *Neisseria gonorrhoeae*. *Journal of Bacteriology* **119**, 250–257.
- JOSEPH, R. & SHOCKMAN, G. D. (1975). Cellular localization of lipoteichoic acid in *Streptococcus faecalis*. *Journal of Bacteriology* **122**, 1375–1386.
- KESSLER, R. E. & VAN DE RIJN, I. (1979). Quantitative immunoelectrophoretic analysis of *Streptococcus pyogenes* membrane. *Infection and Immunity* **26**, 892–902.
- KRAUSE, R. M. (1972). The antigens of group D streptococci. In *Streptococci and Streptococcal Diseases*, pp. 67–74. Edited by L. W. Wannamaker & J. M. Matsen. New York: Academic Press.
- MARKHAM, J. L., KNOX, K. W., WICKEN, A. J. & HEWETT, M. J. (1975). Formation of extracellular lipoteichoic acid by oral streptococci and lactobacilli. *Infection and Immunity* **12**, 378–386.
- MOLDOW, C., ROBERTSON, J. & ROTHFIELD, L. (1972). Purification of bacterial membrane proteins. The use of guanidium thiocyanate and urea. *Journal of Membrane Biology* **10**, 137–152.
- MORRIS, D. L. (1948). Quantitative determination of carbohydrates with Dreywood's anthrone reagent. *Science* **107**, 254–256.
- OSBORN, M. J., GANDER, J. E., PARISI, E. & CARSON J. (1972). Mechanism of assembly of the outer membrane of *Salmonella typhimurium*. *Journal of Biological Chemistry* **247**, 3962–3972.
- OWEN, P. & FREER, J. H. (1972). Isolation and properties of mesosomal membrane fractions from *Micrococcus lysodeikticus*. *Biochemical Journal* **129**, 907–917.
- PERCH, B., KJEMS, E. & RAVN, T. (1974). Biochemical and serological properties of *Streptococcus mutans* from various human and animal sources. *Acta pathologica et microbiologica scandinavica B*, **82**, 357–370.
- POLLOCK, J. J., IACONO, V. J., GOODMAN BICKER, H., MACKAY, B. J., KATONA, L. I., TAICHMAN, L. B. & THOMAS, E. (1976). The binding, aggregation and lytic properties of lysozyme. In *Proceedings, Microbial Aspects of Dental Caries*, vol. 2, pp. 325–352. Edited by H. M. Stiles, W. J. Loesche & T. C. O'Brien. Special supplement to *Microbiology Abstracts*. Washington, D.C.: Information Retrieval.
- RUSSELL, M. W. & LEHNER, T. (1978). Characterization of antigens extracted from cells and culture fluids of *Streptococcus mutans* serotype *c*. *Archives of Oral Biology* **23**, 7–15.
- RUSSELL, R. R. B. (1979). Wall-associated protein antigens of *Streptococcus mutans*. *Journal of General Microbiology* **114**, 109–115.
- SALTON, M. R. J. (1974). Membrane associated enzymes in bacteria. *Advances in Microbial Physiology* **11**, 213–282.
- SCHÖLLER, M., KLEIN, J. P. & FRANK, R. M. (1981). Common antigens of streptococcal and non-streptococcal oral bacteria: immunochemical studies of extracellular and cell-wall associated antigens from *Streptococcus sanguis*, *Streptococcus mutans*, *Lactobacillus salivarius* and *Actinomyces viscosus*. *Infection and Immunity* **31**, 52–60.
- SHKLAIR, I. L. & KEENE, H. J. (1976). Biochemical characterization and distribution of *Streptococcus mutans* in three diverse populations. In *Proceedings, Microbial Aspects of Dental Caries*, vol. 1, pp. 201–210. Edited by H. M. Stiles, W. J. Loesche & T. C. O'Brien. Special Supplement to *Microbiology Abstracts*. Washington, D.C.: Information Retrieval.
- SHOCKMAN, G. D., DANEOMORE, L., CORNETT, J. B. & MYCHAILONKA, M. (1979). Does penicillin kill bacteria? *Reviews of Infectious Diseases* **1**, 787–796.

- SIEGEL, J. L., HURST, S. F., LIBERMAN, E. S., COLEMAN, S. E. & BLEIWEIS, A. S. (1981). Mutanolysin-induced spheroplasts of *Streptococcus mutans* are true protoplasts. *Infection and Immunity* **31**, 808–815.
- SILVESTRI, L. J., CRAIG, R. A., INGRAM, L. O., HOFFMANN, E. M. & BLEIWEIS, A. S. (1978). Purification of lipoteichoic acid by using phosphatidyl choline vesicles. *Infection and Immunity* **22**, 107–118.
- SMITH, D. G. (1973). The lysozyme sensitivity of *Streptococcus equinus*. *Microbios* **8**, 199–207.
- TAUBMAN, M. A. (1973). Role of immunization in dental disease. In *Comparative Immunology of the Oral Cavity*, pp. 138–158. Edited by S. E. Mergenhagen & H. W. Sherr. Washington, D.C.: U.S. Government Printing Office.
- THOMAS, P. S. & FARQUHAR, M. N. (1978). Specific measurement of DNA in nuclei and nucleic acids using diaminobenzoic acid. *Analytical Biochemistry* **89**, 35–44.
- VAN DE RIJN, I. & KESSLER, R. E. (1979). Chemical analysis of changes in membrane composition during growth of *Streptococcus pyogenes*. *Infection and Immunity* **26**, 883–891.
- VAUGHT, R. M. & BLEIWEIS, A. S. (1974). Antigens of *Streptococcus mutans*. II. Characterization of an antigen resembling a glycerol teichoic acid in walls of strain BHT. *Infection and Immunity* **9**, 60–67.
- WITHOLT, B. & BOEKHOUT, M. (1978). The effect of osmotic shock on the accessibility of the murein layer of exponentially growing *Escherichia coli* to lysozyme. *Biochimica et biophysica acta* **508**, 296–305.