A DNA-MEMBRANE COMPLEX IN GROUP D STREPTOCOCCI: ISOLATION AND CHARACTERIZATION

JO-ANN LASZCZYCH MALTAIS

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UNIVERSITY OF NEW HAMPSHIRE, PH.D., 1977
A DNA-MEMBRANE COMPLEX IN GROUP D STREPTOCOCCI:
ISOLATION AND CHARACTERIZATION

by

Jo-Ann Laszczych Maltais
B.S., Long Island University, 1969

A THESIS

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# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>List of Tables</td>
<td>vii</td>
</tr>
<tr>
<td>List of Schemes</td>
<td>viii</td>
</tr>
<tr>
<td>List of Figures</td>
<td>ix</td>
</tr>
<tr>
<td>Abstract</td>
<td>x</td>
</tr>
<tr>
<td><strong>I. INTRODUCTION</strong></td>
<td></td>
</tr>
<tr>
<td>A. Group D Streptococci</td>
<td>1</td>
</tr>
<tr>
<td>B. Membrane - chromosome complexes</td>
<td>3</td>
</tr>
<tr>
<td>Morphological evidence</td>
<td>3</td>
</tr>
<tr>
<td>Biochemical investigations</td>
<td>4</td>
</tr>
<tr>
<td>Methods of isolation</td>
<td>5</td>
</tr>
<tr>
<td>C. Lysis</td>
<td>19</td>
</tr>
<tr>
<td>Enzymatic methods</td>
<td>20</td>
</tr>
<tr>
<td>Cell wall and the lytic process</td>
<td>21</td>
</tr>
<tr>
<td>Enzyme specificity</td>
<td>23</td>
</tr>
<tr>
<td>D. Lysis of Gram-negative bacteria</td>
<td>26</td>
</tr>
<tr>
<td>EDTA</td>
<td>28</td>
</tr>
<tr>
<td>E. Lysis of group D streptococci</td>
<td>29</td>
</tr>
<tr>
<td>F. Lipids</td>
<td>37</td>
</tr>
<tr>
<td>Fatty acids</td>
<td>40</td>
</tr>
<tr>
<td>Proposed function of membrane lipids</td>
<td>42</td>
</tr>
<tr>
<td>Factors affecting membrane lipid composition</td>
<td>43</td>
</tr>
<tr>
<td>Phospholipids of group D streptococci</td>
<td>44</td>
</tr>
<tr>
<td><strong>II. MATERIALS AND METHODS</strong></td>
<td></td>
</tr>
<tr>
<td>A. Cultures</td>
<td>51</td>
</tr>
</tbody>
</table>

iv
B. M-bands ....................................... 53
  Technique *E. coli* B/r(w) .................... 53
  Technique *B. megaterium* .................... 54
  Technique *S. faecalis var. liquefaciens* .... 55
  Fractionation of gradients .................... 56
  Radioactivity counting ....................... 57

  *E. coli* B/r(w), *B. megaterium* and *S. faecalis var. liquefaciens* .......... 57

  Enterococci 564 P, 615 M and 9790 .......... 58

  Lytic procedure and M-bands—group D streptococci .......................... 59
  DNA-membrane isolation—Enterococci ............. 61

C. Fluorometric analysis of DNA and RNA content of Lysates and M-band gradient fractions ..... 62

D. Electron microscopy .......................... 66

E. Lipids ........................................ 67
  Extraction ................................... 67
  Storage ...................................... 70
  Thin layer chromatography (TLC) of washed lipid extracts ......................... 71
  Thin layer chromatography of standards .................. 74
  Quantitation of phospholipids .................... 74

III. RESULTS ........................................... 75

  A. M-bands — *E. coli* B/r(w) and *B. megaterium* ............................. 75
  B. Development of a lytic procedure for group D streptococci to use in conjunction with M-band technique: *Streptococcus faecalis var. liquefaciens* .................. 82
  C. Development of a lytic procedure for group D streptococci to use in conjunction with M-band technique: *S. faecalis* (faecium) 9790 and UNH strains .................. 86
<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>Evaluation of the lytic response of <em>S. faecium</em> 9790 to versions of</td>
<td>90</td>
</tr>
<tr>
<td>the procedure of Clewell <em>et al.</em>, 1974.</td>
<td></td>
</tr>
<tr>
<td>D. M-bands of group D streptococcal strains 564 P, 615 M and 9790</td>
<td>93</td>
</tr>
<tr>
<td>Fluorometry—DNA and RNA content analysis of M-band fractions</td>
<td>93</td>
</tr>
<tr>
<td>Electron microscopy—ultrastructural analysis of M-band fractions</td>
<td>99</td>
</tr>
<tr>
<td>E. Phospholipid composition of group D streptococci; distribution in</td>
<td>118</td>
</tr>
<tr>
<td>the DNA-membrane complex</td>
<td></td>
</tr>
<tr>
<td>Qualitative analysis of phospholipids</td>
<td>119</td>
</tr>
<tr>
<td>Quantitative analysis of phospholipids via labelling</td>
<td>128</td>
</tr>
<tr>
<td>IV. DISCUSSION</td>
<td>132</td>
</tr>
<tr>
<td>LITERATURE CITED</td>
<td>156</td>
</tr>
<tr>
<td>APPENDIX</td>
<td>176</td>
</tr>
</tbody>
</table>
LIST OF TABLES

1. Reported lipid composition of selected Gram positive and Gram negative bacteria................ 41
2. Biochemical characteristics of selected group D streptococci...................................... 52
3. Summary of M-band parameters for verification of technique with E. coli B/r(w) and B. megaterium. 78
4. Distribution of methyl-3H thymidine in M-band gradient fractions of E. coli B/r(w)................ 80
5. Distribution of butanol extractable 32P-labelled material in M-band gradient fractions of B. megaterium after 10 and 30 min contact time between Sarkosyl, Mg2+, and sensitized cells........ 81
6. Susceptibility of S. faecalis var. liquefaciens grown in semi-synthetic medium to Sarkosyl lysis following various pretreatments at 37°C.................. 84
7. Effect of growth with reduced phosphate and increasing concentrations of vitamin-free casamino acids on susceptibility of S. faecalis var. liquefaciens to lysozyme and Sarkosyl........ 85
8. Distribution of methyl-3H-thymidine labelled DNA in gradient fractions following M-band analysis of S. faecalis var. liquefaciens.......................... 87
9. Sensitivity of enterococcus strains to the lytic procedure of Clewell et al., 1974.................. 89
10. Effect of lysozyme inclusion upon the response of S. faecalis 9790 to various aspects of the lytic procedure as measured by changes in optical density........................................ 92
11. Comparison of the effects of lytic procedure reagents upon S. faecalis 9790 during lysate formation, in terms of optical density, viability, and cell integrity.................................. 94
12. DNA and RNA distribution in M-band gradient fractions of group D streptococci determined by fluorometry.............................................................. 98
13. Phospholipid distribution in group D streptococci....................................................... 129
## LIST OF SCHEMES

<table>
<thead>
<tr>
<th>Scheme</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Procedure for preparation of M-bands from group D streptococci.</td>
<td>60</td>
</tr>
<tr>
<td>2. Lipid extraction</td>
<td>68</td>
</tr>
<tr>
<td>Figure</td>
<td>Description</td>
</tr>
<tr>
<td>--------</td>
<td>-----------------------------------------------------------------------------</td>
</tr>
<tr>
<td>1.</td>
<td>Typical M-band gradients after centrifugation, test and control types</td>
</tr>
<tr>
<td>2.</td>
<td>Response curves for fluorometric assay of DNA and RNA standards using ethidium bromide as the fluorochrome</td>
</tr>
<tr>
<td>3.</td>
<td>A DNA-membrane complex in the M-band fraction of <em>S. faecalis</em> 9790; rotary shadowed with platinum-palladium</td>
</tr>
<tr>
<td>4.</td>
<td>A DNA-membrane complex in the M-band fraction of strain 615 M</td>
</tr>
<tr>
<td>5.</td>
<td>A DNA-membrane complex in the M-band fraction of strain 564 P</td>
</tr>
<tr>
<td>6.</td>
<td>Pattern type 3, exhibited by M-band fractions of group D streptococci</td>
</tr>
<tr>
<td>7.</td>
<td>Top fraction of 564 P, M-band gradients exhibiting loop pattern</td>
</tr>
<tr>
<td>8.</td>
<td>Top fraction of 564 P, M-band gradients exhibiting &quot;core&quot; and nicked, supercoiled DNA</td>
</tr>
<tr>
<td>9.</td>
<td>Network pattern typical of Top fractions with high RNA content</td>
</tr>
<tr>
<td>10.</td>
<td>Typical crystals of control Top fractions</td>
</tr>
<tr>
<td>11.</td>
<td>Typical crystals of control M-band fractions</td>
</tr>
<tr>
<td>12.</td>
<td>Lysate pattern immediately after addition of Sarkosyl</td>
</tr>
<tr>
<td>13.</td>
<td>Lysate pattern 5 min after addition of Sarkosyl</td>
</tr>
<tr>
<td>14.</td>
<td>Thin layer chromatogram of $^{32}$P labelled whole cells, lysate, and M-band gradient fractions of strain 564 P</td>
</tr>
<tr>
<td>15.</td>
<td>Thin layer chromatogram of $^{32}$P labelled whole cells, lysate, and M-band gradient fractions of strain 615 M</td>
</tr>
<tr>
<td>16.</td>
<td>Thin layer chromatogram of $^{32}$P labelled whole cells, lysate, and M-band gradient fractions of <em>S. faecalis</em> 9790</td>
</tr>
</tbody>
</table>
ABSTRACT

A DNA-MEMBRANE COMPLEX IN GROUP D STREPTOCOCCI: ISOLATION AND CHARACTERIZATION

by

Jo-Ann Laszczych Maltais

The "M" band technique developed by Earhart et al. (1968) has been used for the isolation of DNA-membrane complexes from bacteria. Involvement of such complexes in "in vivo" cellular processes ranging from regulation of DNA replication to segregation and cell division, has been implied. Analysis of the membrane component of isolated complexes revealed a selective enrichment of the phospholipid, phosphatidylethanolamine, in both Escherichia coli and Bacillus megaterium when the M-band lipid composition was compared to that of whole cell extracts of the same organisms.

Members of the group D streptococci, classified as such via Lancefield's group typing system for streptococci, and conforming to Sherman's physiological criteria for enterococci are devoid of phosphatidylethanolamine. If in fact such complexes represent true "in vivo" functional entities in bacteria, one would predict that 1) a DNA-membrane complex similar to those previously isolated from other organisms could be extracted from the group D streptococci by the M-band technique and, 2) if so, some phospholipid constituent should substitute for phosphatidylethanolamine in such a complex.

x
Therefore three enterococcal strains were chosen for examination: UNH 564 P (a pigmented triterpenoid containing strain), UNH 615 M (a motile, non-pigmented strain), and *Streptococcus faecalis* 9790 as its lipid composition as well as other characteristics have been well documented in the literature.

The necessity of utilizing protoplasts or spheroplasts in the M-band technique, combined with the natural resistance of streptococci to the commonly utilized methods of obtaining osmotically fragile cells, necessitated modification of the basic M-band technique. Optimal susceptibility to disruption by the anionic detergent Sarkosyl was achieved via treatment of log phase cells with lysozyme, EDTA, and pronase prior to the addition of Sarkosyl and Mg$^{++}$ as prescribed by the M-band technique.

Formation of a white opalescent band at the appropriate position in discontinuous sucrose gradients subsequent to centrifugation was taken as preliminary evidence of the successful formation of M-bands. Criteria established to verify isolation of the membrane-chromosome complex included: DNA and RNA distribution via fluorometry, ultrastructural similarity via electron microscopy, and membrane content by phospholipid analysis.

Results of the nucleic acid distribution and ultrastructural analyses indicated that a DNA-membrane complex comparable to those obtained from the bacillary organisms by this technique had been isolated from the group D streptococci tested.
Phospholipid analysis of M-bands revealed fractionation of the membrane and altered proportions of phospholipids in each case. However, these differences appear to reflect phospholipid pattern changes induced during lysis, as they parallel proportions observed upon lipid analysis of lysed cells rather than those of whole cells.

The impact of these findings has dramatic import not only in terms of this study, but also for those studies previously reporting phospholipid distributions in group D streptococci, and in terms of conclusions drawn concerning a specific phospholipid association with the DNA-membrane attachment site in *E. coli* and *B. megaterium*.

The critical experiment to eliminate the possibility of artifactual enrichment of PE occurring during the lytic process, i.e., analysis of the phospholipid pattern of cell lysates has not been reported for the bacillary organisms thus question remains as to the validity of the suggested membrane lysed specificity at the DNA-membrane attachment site in bacteria.
INTRODUCTION

A. Group D Streptococci

Group D streptococci were classified as such by Lancefield's serological grouping system (1933), based upon the presence of the carbohydrate containing group D antigen. This antigen is found in all group D streptococci as an intracellular or membrane teichoic acid, glycerol phosphate polymers frequently substituted with D-alanine and glucose residues. The grouping was further subdivided by physiological criteria (Sherman, 1937) into two types: enterococci and "viridans" type.

The former division included S. faecalis and varieties liquefaciens and zymogenes, as well as S. durans. S. faecium, motile and/or pigmented varieties were added in 1957 (Graudal a,b). All exhibited growth at 10 and 45 C, at pH 9.6, in the presence of 6.5 % NaCl or 40 % bile. They reduced 1 % methylene blue milk, and could survive a temperature of 60 C for 30 min. The "viridans" group which included S. bovis and S. equinus exhibited similar properties with the exception that no growth was observed at 10 C or in the presence of 6.5 % NaCl.

The enterococci have been the object of extensive study, due to their widespread occurrence and association with man. They exist as part of the normal flora their chief reservoir being the alimentary canal of animals and humans, and are thought to occupy no characteristic ecological niche.
Their widespread distribution is evidenced by the following list of reported isolations: mammals, birds, reptiles, rodents (in low numbers) /Eaves and Mundt, 1960/7, insects (gypsy moth larvae) /Cosenza and Lewis, 1965/7, the gingival crevice of humans /Gibbons and Van Houte, 1975/7, plants /Mundt et al., 1958, 1962; Sherman, 1937/7 and as a transient resident of the soil /Mundt et al., 1958; Medrek and Litsky, 1960/7, in food products such as milk and frozen foods /Sherman, 1937; Hugh, 1959; Niven, 1964/7, and water /Deibel, 1964/7.

As with many organisms considered to be normal flora disruption of the ecological balance via external mediation, or displacement from areas where normal competition suppresses their proliferation allows the pathogenic potential of the enterococci to be realized. This has resulted in claims of association with such conditions as acute bacterial endocarditis, urinary tract infections, gastroenteritis (food poisoning) /Shattock, 1962; Deibel and Silliker, 1963/7, fissure caries and some cases of root caries (Patterson, 1972). Motile enterococci have also been implicated in peritonitis (Wheeler and Foley, 1943), meningitis and possibly in untreated oral cancer (Liu et al., 1955; Hugh, 1959) and isolated from the orapharyngeal region and urinary tract infections of man. In addition their ability to survive pasteurization, high salt, and relative resistance to freezing makes them excellent candidates for potential food spoilage.

Thus the enterococci lend themselves well to studies along numerous lines and across a variety of disciplines. In
addition, their lack of phosphatidylethanolamine (PE), a phospholipid (p-lipid) found in most other bacteria; a fatty acid composition similar to that of many Gram negative genera; motility and, in the case of the pigmented varieties, an unusual triterpenoid type pigment, makes them of interest for basic research as well.

B. Membrane - Chromosome Complexes

Morphological Evidence

The hypothesis, based on genetic data, that the bacterial genome is associated with the cell membrane was advanced by Jacob, Brenner, and Cuzin (1963) and supported by morphological studies (Ryter and Jacob, 1963; Ryter and Landman, 1964; Ryter, 1968; Ryter, Hirota, and Jacob, 1968).

Although the most frequently observed association pattern seemed to be via mesosomal type structures, at least in Bacillus subtilis, a direct connection between the nucleus and cell membrane was seen in all cases where mesosomes were either absent, extruded or their formation prevented. Mesosomes have also been reported in other Gram positive organisms, including Streptococcus faecalis 9790 (Higgins and Shockman, 1970) invariably closely associated with the nuclear pool, suggesting the possibility of a similar attachment of chromosome and membrane in these organisms. The fact that attachment was not seen in all serial sections (Ryter, 1968) led to further speculation that the actual site of attachment was confined to a specific area of the membrane.
While most Gram negative bacteria do not exhibit well-developed mesosomes, distinct membranous structures termed "intracytoplasmic membranes" (Schnaitman and Greenwalt, 1966; Altenburg and Suit, 1970) or "membrane invaginations" (Ryter, 1968) have been observed in contact with the chromosome under certain conditions of growth and fixation for morphological study (Ryter and Jacob, 1966; Kohijama, et al., 1966). Such observations support the postulation by Jacob et al. (1963) that the bacterial membrane could provide the structural basis for regulation of chromosome replication and the segregation of daughter chromosomes.

Biochemical Investigations.

Characterization of the components of these DNA-membrane complexes has resulted in proposals of their "in vivo" involvement in DNA replication as well as various other related cell activities. Proposed function of these complexes have included conformation (Pettijohn et al., 1973) and stabilization (Smith and Hanawalt, 1967) of the nucleoid, direction and regulation of DNA synthesis (initiation, synthesis, termination, and segregation (Olsen, et al., 1974) transcription and translation, as well as the site of bacteriophage replication.

**Methods of Isolation**

Procedures for isolation of such complexes from bacteria developed along four general lines, dependent upon the mode of excision of the complex from the cell and the method of removing it from the remainder of the cellular components. Such method types include:

- **method A**—collection of rapidly sedimenting fractions after gentle lysis of whole cells speroplasts or protoplasts;
- **method B**—isolation of folded chromosomes either free or membrane-bound via controlled lysis with non-ionic detergents. Separation from other cellular components was based upon differences in sedimentation characteristics;
- **method C**—release from sensitized cells via ionic detergents such as Sarkosyl and isolation via membrane affinity for Sarkosyl-Mg++ crystals;
- **method D**—separation of membrane-bound and free DNA via isopycnic velocity centrifugation (eg. renografin) after cell lysis by lysozyme (5-10 min, 37 C).
All complexes although isolated by these varied methodologies exhibited similarities in structure and functional properties, which is often cited as indicative of their "in vivo" validity. Interpretation of results obtained with each method enables one to gain valuable information concerning the organization and regulation of many cellular processes in the bacterial cell.

Ganesan and Lederberg (1965) reported isolation of a complex enriched in both nascent DNA and RNA from *B. subtilis* via method A. The complex obtained after lysozyme-EDTA, sodium dodecyl sulfate (SDS) treatment also contained membrane, cell wall fragments, and DNA polymerase activity (the latter only in the absence of SDS) and could incorporate all four deoxynucleoside triphosphates. Thus the first evidence for involvement in replication other than by morphological association of the membrane and chromosome was obtained.

Porter and Fraser (1968) concluded that protein was involved in binding of nascent DNA and suggested that such protein was of membrane origina, based upon similar release of nascent DNA by pronase and detergents, the latter known to be a membrane disruptive agent (Woldringh, 1970).

Other treatments of the lysozyme induced lysates prior to sedimentation analysis shed additional light on the composition of this complex and indicated that DNA binding (stabilization) involved more than one type of bonding (possibly hydrophobic and ionic) or more than one type of macromolecule (Smith and Hanawalt, 1967).
An apparent involvement of specific cell envelope/membrane sites in highly stable attachments of DNA, which may be important in chromosome segregation, has been reported in *E. coli* by Olsen et al. (1974). A similar situation may exist in the *S. faecalis*, as electron microscopic studies revealed an association of the nuclear body with the septum (annular, double membrane invagination) between which the cross-wall forms for cell division (Higgins et al., 1970). Thus the cross-wall initiation as well as DNA replication and segregation may be linked to DNA synthesis regulation in this organism.

Fractions isolated via method A by nature of the fairly crude methods employed in their isolation, cannot provide the definitive proof necessary to establish a specific association of DNA and membrane in a functional unit of DNA replication. Therefore the necessity of isolating specific "in vivo" complexes while preserving structural and functional integrity and enzymatic activity, became clear.

Such a necessity led a series of investigators (Godson and Sinsheimer, 1967; Knippers and Strätling, 1970; Stonington and Pettijohn, 1971; Pettijohn et al., 1973; Worcel and Burgi, 1972, 1974) to utilize a more gentle, controlled process to lyse spheroplasts of *E. coli* which result upon lysozyme-EDTA treatment of cells. The method (type B) resulted in isolation of the entire chromosome in a folded, supercoiled state, reminiscent of that found with replicating T4 phage DNA and eukaryotic cells (Huberman, 1968, 1973). Both membrane-bound and free chromosomes have been isolated and as yet the
question of their relation to the situation found in intact cells remains unresolved.

The problem stems from equivocal evidence concerning the origin and functional properties of such complexes. On the one hand Pettijohn et al. (1973) found these folded chromosomes both in the membrane bound and free state to be excellent templates for RNA synthesis, even more efficient than the unfolded genome. Consistent with the principles of polymer chemistry, they would qualify as a functional chromosomal unit of DNA helices stabilized by an RNA core and protein (Pettijohn et al., 1973). They exhibit, in the membrane free state, a high RNA polymerase activity and nascent RNA content, and low DNA polymerase activity (1%) consistent with hypothesized membrane free chromosomes being involved more in transcription and translation (Lark, 1972) or as a pre-initiation state. The fact that such chromosomes are the only type isolatable from amino acid starved cells (presumably having completed their last round of replication) support this contention.

On the other hand, skepticism has arisen with regard to the structural validity and functional properties of these chromosomes. This is based upon reports of condensation of the nucleoid in the presence of high salt, 1-2 M as used in this method, even when the cell wall and outer membrane appear intact (Woldringh and Van Iterson, 1972); the ability of high lysozyme concentrations (800 ug/ml) to increase membrane/envelope association with the chromosome of E. coli isolated
by this method; and Mg\(^{++}\) concentration effecting the membrane pore size of Brij-58 treated cells.

Concurrent with studies on DNA replication complexes isolated via method type B (non-ionic detergent, Brij-58) and spurred by the criticisms that a spurious association of cellular components of similar sedimentation characteristics could account for the observed associations made using either method A or B, a new means of separating DNA-membrane complexes which did not depend primarily upon the sedimentation characteristics of these two cellular components was developed (Earhart et al., 1968; Tremblay et al., 1969).

The technique designated the M-band technique was based upon the high affinity of membrane for the hydrophobic surfaces of crystals which form upon mixing Mg\(^{++}\) with the anionic detergent lauryl sarcosinate (Sarkosyl). Gradient fractionation produced a crystalline band (M-band) near the middle of the gradient, which contained the appropriate complex.

The technique's potential for inducing artifacts was investigated along three lines 1) adventitious attachment of added DNA to membrane 2) affinity of individual cellular components such as DNA for Mg\(^{++}\)-Sarkosyl crystals 3) entrainment of whole or lysed cells in the M-band complex. Entrainment of cellular components was not seen, at least in the M-bands of B. megaterium (Earhart et al., 1968) as neither purified DNA (in the native or denatured state), ribosomes, polyribosomes, nor nucleoprotein could be shown to interact directly with the crystals. Dworsky and Schaechter (1973) did report an association of denatured DNA with the crystals
when such DNA was added in the presence of Sarkosyl to induce cell lysates of *E. coli*. Based upon recent work (Dworsky, 1976; Meyers et al., 1976; Harmon and Taber, 1977) cell envelope association and the protection afforded to the inner membrane by outer membrane observed in *E. coli* upon treatment with Sarkosyl suggests the possible entrainment of such DNA by these components.

Based upon these results and the fact that semi-purified membrane, cell ghosts or purified P-lipids exhibited a high affinity for the crystals (Tremblay et al., 1969), one would predict that the presence of DNA in the M-band fraction, would not occur due to its own inherent affinity for the crystals but rather reflect an association mediated by some hydrophobic component(s) of the cell which does exhibit such high crystal affinity along with DNA-binding capacity.

The M-band technique has been used to study such complexes in a variety of bacteria: *B. megaterium* (Tremblay et al., 1969), *E. coli* (Earhart et al., 1968; Niveleau, 1974), *Pneumococcus* (Firshein, 1972; Greene and Firshein, 1976), *B. subtilis* (Harmon and Taber, 1977). They reportedly contain 80-90% of the DNA of the cells, are rich in newly replicated regions of the DNA molecule, and exhibit DNA polymerase activity. The degree of membrant association is dependent upon conditions of isolation /Sarkosyl/Mg++ ratio, temperature, at least in *E. coli* (McIntosh and Earhart, 1975) and depending upon the organism used, the M-band can contain from 4-90% of the total cell membrane (Ballesta, et al., 1972).
Evidence such as the "in vivo" association and the "in vitro" activity of enzymes known to be associated with DNA replication (Firshein, 1972; Greene and Firshein, 1976) in the M-band fraction have been influential in verifying the authenticity of the complex as the membrane-chromosome replication complex in these bacteria.

A rate of DNA synthesis "in vitro" $\sqrt{2.66 \times 10^5}$ nucleotides/min/cell (Firshein, 1972) close to the "in vivo" rate $\sqrt{3-5 \times 10^5}$ nucleotides/min/cell (Helmstetter et al., 1968; Smith et al., 1970; Wickner, et al., 1972) and incorporation of nucleotides into heavier molecular weight strands (Firshein, 1972), as well as direction of synthesis proceeding distally from membrane established by autoradiography (Niveleau, 1974) is also supportive of a role in replication.

Specific attachment of DNA to membrane as well as evidence of multiple attachments have been postulated based upon autoradiographic (Niveleau, 1974), phospholipid (Ballesta et al., 1972), double labelling (Firshein, 1972), and X-ray sensitivity data (Dworsky and Schaechter, 1973).

However, evidence in support of the extent of attachment is indirect and the physiological significance of this observation is still in question. Niveleau (1974) reported observing loops of DNA associated with a single electron dense structure presumably membrane as well as DNA in a more extended form in shadow cast preparations. The actual sites of attachment could not be discerned by autoradiography however. The number of attachment sites reported, range from 20 (Dworsky and Schaechter, 1973) estimated by X-ray

The complexes isolated via the M-band technique seem to exhibit greater shear sensitivity than reported for complexes isolated by other methods, which may reflect a more extended form of the DNA, or less protection being afforded by the membrane and/or other stabilizing molecules. Harmon and Taber (1977) reasoned that finding less than 5% DNA after shearing indicated multiple attachment sites with membrane affording protection against shear forces, and that finding only .5% DNA after DNase treatment was reflective of origin attachment only.

The question of membrane specificity at the attachment site explored by Ballesta et al. (1972) via analysis of the P-lipid distribution in M-band fractions. They observed a 12-16% enrichment of PE for *E. coli* strains tested and a 36% enrichment of PE with similar fractions of *B. megaterium* KM, when the M-band phospholipid distribution was compared with that of whole cells of these organisms and concluded that membrane specificity did occur at the DNA-membrane attachment site in these organisms. The authors discounted the possibility that PE was simply more resistant to Sarkosyl solubilization by citing no difference in affinity of either purified PE, phosphatidylglycerol (PG) or diphosphatidylglycerol (diPG) for the Mg^{++} Sarkosyl crystals.

Such a P-lipid enrichment, if physiologically valid would be teleologically desirable in DNA-membrane complexes based on the data reported by Stevens (1975). She showed
inhibition of DNA-dependent RNA polymerase in *E. coli* by PG and diPG, even at low concentrations, while PE was not inhibitory. Ten-fold higher concentrations of PE were required to partially overcome inhibition by the other two lipids. In accordance with the model presented by Lark (1972), these phospholipids would prevent synthesis of RNA required to initiate a new round of chromosome replication. Once formed, presumably under conditions of high PE concentration in the membrane, RNA polymerase would be derepressed, RNA made could act as a primer attachment site for DNA polymerase and initiation could occur. A similar effect upon initiation is seen when rifampin, known to bind to RNA polymerase is used. Turnover of phospholipids, altering the ratio of PE/PG + diPG such that a lower PE level existed could once again act to inhibit RNA polymerase and therefore initiation. Under conditions of rapid synthesis in rich media, the ratio may change often enough to allow multiple initiations to occur.

The M-band technique has also been employed for studying the involvement of membrane in other DNA associated cell processes. Earhart et al. (1968) and Linial and Malamy (1970) reported binding of both parental and progeny DNA in *T₄* phage replication, once again implicating membrane attachment in DNA replication; Tremblay et al. (1969) reported an association of RNA synthesis with the chromosome. Rouviere et al. (1969) followed the distribution of m-RNA in *E. coli*. Cundliffe (1970) studied biogenesis and intracellular distribution of polysomes and ribosomal subunits in *B. megaterium* while Broiewer and Planta (1975) studied
the protein composition of ribosomes in DNA-membrane complexes from *B. licheniformis*.

Effects of various enzymes and inhibitors on the structure and functional properties of these complexes have also been investigated such as DNase (Earhart et al., 1968; Tremblay et al., 1969; Niveleau, 1974; Firshein, 1972); RNase (Firshein, 1972; McIntosh and Earhart, 1975); pronase and phospholipase C (Firshein, 1972; Meyers et al., 1976); Triton X-100 (Cundliffe, 1970); actinomycin D (Tremblay et al., 1969; Cundliffe, 1970); rifampin, nalidixic acid, chloramphenicol, and various other drugs (Dworsky and Schaechter, 1973; Harmon and Taber, 1977). Such studies have resulted in a clearer understanding of the now presumed replication complex in other processes such as transcription and translation.

Interest in such involvement of the DNA replication complex in such processes has also been stimulated by the presence of nascent RNA to a high degree in sedimented fractions of lysed *B. subtilis* cells, detected by pulse labelling studies (Ganesan and Lederberg, 1965); the "in vitro" incorporation of nucleosides by crude DNA-membrane fractions isolated from *E. coli* (Rouviere et al., 1969) and pneumococcus (Greene and Firshein, 1976; Firshein, 1972); the association of nascent RNA and RNA polymerase as the major non-membrane protein; as well as the association to varying degrees of ribosomal and soluble RNA with chromosomes isolated in one piece by method type B.

Here the use of the hydrolytic enzymes, inhibitors and detergents have been useful in the elucidation of the
significance of RNA in the M-band fractions and therefore in DNA replication complexes. In 1966, Schaechter and McQuillen reported the bulk of m-RNA associated with ribosomes, and suggested that the most recently made m-RNA is stabilized "in vivo" by association with ribosomes, as seen by Byrne et al. (1964) "in vitro" and suggested by Stent (1966), to form the association possibly before the molecule m-RNA is even completed. This would be of aid in preventing nuclease attack of m-RNA; polyribosomes could then form and translation proceed. Rouviere et al. (1969) provided some additional proof for this mode of interaction between the replication complexes and transcription by recovering m-RNA chains attached to 30s, 70s and multiribosomal complexes from fast sedimenting membrane-DNA fractions of E. coli. Subjecting this fraction to the M-band technique, the most rapidly labelled T₄ phage RNA was found in association with the DNA-membrane complex, highly enriched in DNA.

M-bands isolated from B. megaterium (Tremblay et al., 1969), E. coli (Earhart et al., 1968), pneumococcus (Firshein, 1972), all reportedly contain similar amounts of RNA (30-45 %) and show an extremely high association of nascent RNA (60-100 %) with the DNA-membrane complex after 10-30s pulse labelling. The enrichment could be reduced by continued labelling, and nascent RNA could be released to the same degree as DNA by DNase or shearing (Earhart et al., 1968; Tremblay et al., 1969). These studies in combination suggested that nascent RNA was attached to DNA and that the complex was also involved in RNA synthesis.
RNA association with more than one component of the DNA-membrane complex was suggested by observations of Earhart et al. (1968) that 5-10% of the RNA was still associated with the M-band after more than 99% of the DNA had been released by DNase or shearing, 15-20% of the RNA was released by DNase, the resistance of 25% of the same initial RNA to sodium deoxycholate (Rouviere et al., 1969), or Triton X-100 plus Sarkosyl (Cundliffe, 1970) as well as the release of only about 70% of ribosomes associated with nascent RNA chains by mild RNase treatment (Tremblay et al., 1969).

Cundliffe (1970) provided additional evidence for this showing that 50s and 30s ribosomal subunits were found in the M-band from protoplasts of B. megaterium and could be selectively removed. Treatment with Triton X-100, a membrane affecting reagent, released only 50s particles while the 30s subunits were removed by RNase but not by Triton X-100. Simultaneous treatment with both reagents resulted in quantitative removal of both types of ribosomal subunits from association with the M-band. Thus it was concluded that 50s ribosomes were associated with membrane while the 30s were not.

Additional studies noted that actinomycin D or rifampin treated protoplasts showed selective retention of 50s subunits but not 30s upon M-banding, implicating involvement of 30s subunits with RNA polymerase and DNA, and a possible role as initiators of polyribosome formation.

Although an artifactual association is possible due to the affinity of 50s subunits for some component of lysed actinomycin D treated protoplasts resulting in retention of 50s
subunits in M-bands, such subunits are released by Triton X-100 but not DNase or RNase and therefore are probably membrane associated. A similar association was also noted by Moore and Umbreit (1965) in S. faecalis 9790, under different conditions.

Caution must be exercised in interpreting the effects of inhibitors, detergents, or degradative enzymes on the isolation of, or integrity (post-isolation) of DNA-membrane complexes, as the observed effects may reflect involvement of affected macromolecules in stabilization and folding rather than or in addition to synthesis. RNA involvement in initiation of DNA synthesis and stabilization of the nucleoid has been proposed (Lark, 1972) and supportive evidence obtained when Worcel and Burgi (1974) failed to isolate folded chromosomes if rifampin treated cells were used. However, Harmon and Taber (1977) claimed this might also be due to the increased sensitivity of DNA to shear forces during centrifugation as rifampin treatment tends to increase the viscosity of resulting cell lysates.

Specific involvement of 30s ribosomal subunits in the process of ribosome-polyribosome assembly via initial association of 30s subunit with nascent DNA chains was shown by Cundliffe (1970). This and isolation of the subunit even in minimal M-bands, i.e. 4% membrane, lends credence to the suggestion of Tremblay et al. (1969) that regions on DNA chains containing the bulk of the ribosomes of the M-band fraction might be involved in the process of translation. In addition, presence of nascent protein, 80% reduction in the
presence of puromycin, and shift (after pronase treatment) of partially digested protein to the position of ribonucleoprotein in cesium chloride gradients (Firshein, 1972) suggested an important role in protein synthesis for these complexes.

However, the "in vivo" significance of such findings must be viewed in terms of known effects of the isolation technique parameters upon ribosomes and their subunits. Godson and Sinsheimer (1967) reported that Mg$^{++}$ concentration effected the type and distribution of RNA in isolatable complexes during treatment with nonionic detergent Brij-58. Coleman (1969) showed favored binding of 50s subunits to membrane in the presence of Mg$^{++}$. This process could be inhibited by salts (Schlessinger et al., 1965). Woldringh and Van Iterson (1972) showed an accumulation of electron dense, granular opaque regions, sensitive to RNase. These regions surrounded the nucleoplasm and extended to the cell periphery upon prolonged treatment of *E. coli* with ionic detergents. Cundliffe (1970) reported a concentration effect of Sarkosyl upon both membrane and ribosome content in M-bands of *B. megaterium*, supportive of membrane association of ribosomes reported by Schlessinger *et al.* (1965) to be RNase resistant. Patterson *et al.* (1970) suggested artifactual attachment of ribosomes to membrane by lysozyme. However, the significance of their data has been dampened by that of Cundliffe (1970) showing actinomycin D treated cells exhibited 50s ribosomes associated with M-band in the absence of lysozyme.

Thus the M-band technique has proved extremely useful for the study of RNA synthesis and its association with the
bacterial replicative complex, and indeed provides a useful analytical tool for the study of certain structural and functional interrelationships, allowing maximal efficiency in macromolecular synthesis. In addition, it is the only methodology for isolating such complexes which has been fairly well tested for the possibility of artifactual associations and most likely represents the best current method for isolating a complex most closely resembling the "in vivo" physiologically functional components of replication, transcription and translation.

C. Lysis

Pethica (1958) used the term "lysis" to describe the optical clearing of a bacterial suspension (Salton, 1957a,b; Welsh, 1958), and the leakage of small molecular weight materials without optical clearing. McQuillen (1958) extended the term to include microscopically detected disintegration as well.

Differences in susceptibility to lysis amongst organisms, a reflection of genetic and/or environmental factors (cell wall composition; metabolic activity, growth phase, growth media, pH, temperature, buffer system and ionic strength) determine the degree to which the lytic phenomenon can be expressed. The lytic response reflects the selective removal of all or parts of the cell wall or envelope. Stabilized residual cellular forms have been classified as protoplasts, spheroplasts or L-forms depending upon the amount and type of cell wall components removed.
Lysis as described above may be induced by two methods: exogenous (mechanical, physical, chemical, and biological), resulting in disruption of the structural integrity of some aspect of the cell envelope in Gram negative organisms, or the cell wall/membrane in Gram positive organisms; or endogenous, via direct action of autolysins, or as a result of indirect activation of the cell's own autolytic system (Tomasz and Waks, 1975). A clear cut distinction between the two means is often not possible, as the observed action of an exogenously added "hydrolytic agent" may be due in part to the agent's ability to activate the organism's autolytic system (Welsh, 1958; Pollock et al., 1976).

**Enzymatic methods**

Enzymatic approaches allow somewhat more control of the lytic process than is afforded by physical means (such as osmotic or mechanical stress) due to the increased specificity of various agents and thus have been used extensively for both structural analysis and selective removal of certain cellular components.

Three main types of chemical cell wall lytic agents have been employed:

Glycosidases - either endo-\(N\)-acetylmuramidases or endo-\(N\)-acetylglucosaminidases hydrolyze the \(\text{B}(1\rightarrow4)\) linked \(N\)-acetylmuramyl-\(N\)-acyetylglucosamine linkages to produce fragments with either \(N\)-acetylmuramic acid or \(N\)-acyethylglucosamine at the reducing end. Lysozyme is an example of this type of agent.
Peptidases - hydrolyze peptide linkages either in the interior of the peptide bridges of peptidoglycan or those involving C-terminal D-alanine residues of peptide units.

Amidases - specifically hydrolyze the linkage between the carboxyl of the D-lactyl group of N-acetylmuramic acid and the amino group of the L-alanine residue at the amino terminus of the peptide unit (Ghuysen and Shockman, 1973). For excellent reviews of site and strain specificities as well as their potential value in bacterial investigations see Weibull (1958) and Ghuysen and Shockman (1973).

The remainder of this review will focus upon the current state of knowledge of the effects of the lytic agent lysozyme, ionic conditions, chelating agents, and detergents (alone and in combination) on the lysis of Gram positive organisms. Attention will be focused primarily on the genera Bacillus and Streptococcus, and the Gram negative organism Escherichia coli as this is pertinent to the ensuing thesis. The extensive literature available on these genera alone, makes a complete review of the literature concerning the effects of these agents on all bacteria impractical. For additional information the reader is directed to the following reviews: Pethica (1958), Ghuysen and Shockman (1973) and Rogers (1970).

Cell Wall and the Lytic Process

In order to appreciate the problems involved in the chemical disruption of bacteria, and to attempt to compare results amongst organisms of different genera, some knowledge of the composition of and variations in the cell wall is
necessary. As variations in degree of response, even amongst organisms of the same genus are observed, knowledge of factors affecting the enzyme activity is also of benefit.

Cell walls of Gram positive and Gram negative organisms differ qualitatively and quantitatively on both a structural and molecular level. Salton (1952a,b; 1953) reported that cell walls of Gram positive organisms were composed of mucopolysaccharide containing peptides, amino sugars, and sugars, but no lipid. The wall is essentially comprised of one layer as seen by electron microscopy, however great variation in composition and arrangement of this so-called peptidoglycan does exist even within genera (Schleifer and Kandler, 1972).

The peptidoglycan backbone consisting of N-acetyl-
muramic acid B(1-4) N-acetylglucosamine units is associated with a pentapeptide of either D or L amino acids (L-lysine, D or L alanine, D-glutamic acid, meso or L-DAP). These may be cross-linked into the major network either directly or via an elaborate peptide bridge (such as a penta-glycine), allowing bonding to occur between carboxyl groups as in S. aureus.

Gram negative cell walls on the other hand contain a minimal amount of peptidoglycan, which shows no great variation in composition or structural arrangement among the genera. They do, however, exhibit up to 20% lipid as well as increased protein in an additional outer lipoprotein (LP), lipopolysaccharide (LPS) layer not found in G positive organisms.

Evidence is accumulating suggestive of similar functional properties as those exhibited by the LPS of Gram
negative bacteria by lipoteichoic acid (LTA) in Gram positive organisms (Baddiley, 1968; Wicken and Knox, 1973). The contribution of LTA to lysozyme resistance of some Gram positive organisms (e.g. streptococci) via a similar mechanism to that of LPS, known to influence lysozyme action in Gram negative organisms is still unknown.

As very few species of bacteria have been shown to be directly lysed by lysozyme (Gibbons et al., 1966) numerous studies have been undertaken to pinpoint other factors involved. Resistance and specificity have reportedly been related to the compositional variations in cell wall (Heymann et al., 1964); peptide substitution of peptidoglycan; association with polysaccharide, lipids or proteins; presence of free amino groups (Hayashi et al., 1973), O-acetyl groups (Abrams, 1958); N-acetylation in streptococcal cell wall (Gallis et al., 1976); degree of cross-linkage within the wall; as well as steric hinderance of enzyme-substrate interactions (Heymann et al., 1961, 1964; Glick et al., 1972, 1976), and specificity of the lytic agent itself (Strominger and Ghuysen, 1967).

Enzyme Specificity

The site of hydrolytic action of lysozyme seems to be the cell wall glycopeptide or peptidoglycan (Salton, 1952a,b) as judged by the products released upon treatment of the highly sensitive M. lysodeikticus with egg white derived lysozyme (Strominger and Guysen, 1967). Knowledge of the specificity for B(1-4) linkages in the cell wall resulted from the work of Berger and Weiser (1957) who found that lysozyme hydrolyzed
chitin, a B(1-4) linked polymer of N-acetylglucosamine. Subsequently Sharon et al. (1966) and LeyhBouille et al. (1966) showed that peptidoglycan in the cell wall of bacteria consisted of alternating residues of N-acetylglucosamine and N-acetylmuramic acid in a B(1-4) linkage pattern, thus establishing the substrate site in bacteria. The occurrence of the amino sugars in Gram positive and Gram negative peptidoglycan structures probably accounts for a degree of sensitivity to lysozyme being expressed by both groups.

Current theory favors two modes of lysozyme binding to tetrasaccharide: 1) at subsites A, B, C, D of the lysozyme molecule in a non-productive manner which does not lead to cleavage 2) productive binding at sites C, D, E and F. Binding at E and F can lead to transglycosylation and provides an explanation for the simultaneous release of both high and low molecular weight products with differing electrophoretic patterns so often seen (Salton, 1956a,b; Sharon and Seifter, 1964; Chipman et al., 1968). It is now thought that resistance to lysozyme may reflect involvement either of the non-productive mode of binding by cell surface components (carbohydrates) or differences in acceptor saccharides for transglycosylation reactions (Pollock and Iacono, 1975, unpublished information).

Evidence suggestive of the involvement of other factors in addition to the extent of cross-linking within the cell wall peptidoglycan for action of lysozyme comes from studies comparing the extent of cell wall cross-linking to the sensitivity expressed upon treatment with lysozyme. Although E. coli has been reported to have as "loose" a cell wall
network (Van Heijinoort et al., 1969) as M. lysodeikticus (Ghuysen et al., 1965) with 50% peptide units as uncross-linked monomers, lysozyme will degrade cell walls of the latter whereas the former exhibits only a more elastic boundary while still maintaining cell integrity and rod-like shape. *Staphylococcus aureus*, however, provides the other extreme, where extensive cross-linking appears within the peptidoglycan and contributes to the resistance of this organism to lysozyme action.

Substituent groups associated with peptidoglycan have also been implicated as affecting the hydrolytic activity of lysozyme. Glick et al. (1972) reported group A streptococci, normally resistant to lysozyme, to be sensitive after partial removal of cell wall carbohydrates with formamide or HCL, substitution of free amino groups (associated by Hayashi et al. (1973) with resistance to lysozyme in *Bacillus* species), and saponification of O-acyl groups. No one treatment was completely successful, whereas the combined treatment regime was effective. Hayashi et al. (1973) as well as Gallis et al. (1976) concluded that resistance in this group was related to cell wall composition and carbohydrate.

This was extended to include steric hinderance, as it related to the presence of bound, group specific carbohydrate (Heymann et al., 1961). In addition, artificial lipoprotein has been shown to be capable of masking the action of this enzyme (Romeo and Bernard, 1966).

Additional evidence to suggest the involvement of surface differences in observed variability, stems from
observations of pH optima and sensitivity to ionic strength of isolated cell wall and whole cells to lysozyme action \(M.\) lysodeikticus, Sarcina lutea, B. megaterium (Salton, 1956a,b), Streptococcus faecalis 9790 (Metcalf and Deibel, 1969; Joseph and Shockman, 1971). Variations in the rate and extent of lysozyme binding by serotypes of S. mutans (Pollock et al., 1976); lysis at pH 10.5 (Nakamura effect) observed by Guila and Hartsell (1957a,b) or at pH 9.4 by Chesbro (1961) working with strains of enterococci; necessity of adding salts or detergents to certain lysozyme treated streptococci before a lytic response is detectable, Metcalf and Deibel (1969), Bleiweis et al. (1971), Kruse and Hurst (1972) and Iwamoto et al. (1971), also support surface differences being involved. Most recently in fact Pollock et al. (1976) even suggested that hydrolysis of the cell wall by lysozyme may not be involved in the lytic process in certain species.

D. Lysis of Gram Negative Bacteria

The abundance of reports in the literature utilizing lysozyme to effect disruption of Gram negative bacteria might lead one to conclude that these organisms are more sensitive to the action of lysozyme than are Gram positive bacteria. As a group however, the Gram negatives have fewer members as sensitive to lysozyme as some of the Gram positive species such as Micrococcus lysodeikticus or various members of the genus Bacillus (Salton, 1958). However, it is also apparent that some Gram negative species are at least as sensitive to lysis by lysozyme as the more resistant Gram positive organisms.
The lipid-carbohydrate-protein containing outer membrane, has been shown to act as a barrier to most peptidoglycan hydrolyzing enzymes, such as lysozyme, resulting in only partial dissolution even if isolated cell envelope fractions were used. With whole cells of \textit{E. coli}, only a 4% decrease in optical density after 24 h treatment with 100 \textmu g/ml lysozyme at 37°C was observed. Some effect on the organism's cell wall had occurred however as DAP, alanine, glutamic acid, glucosamine, small amounts of muramic acid and other amino acids, all known components of the cell wall of this organism, were released as soluble, non-dialyzable components following lysozyme treatment (Salton, 1958).

This variable response to lysozyme, due in part to the refractory LPS envelope associated with the cell wall peptidoglycan of the Gram negative organisms has required methods combining the effects of lysozyme with other agents to obtain cell disruption. Such treatments as the addition of alkali after lysozyme (Nakamura effect, 1923); lysozyme at low pH (3.5) and 45°C followed by alkali (pH 10) (Peterson and Hartsell, 1955; Grula and Hartsell, 1957a,b); treatment with acetone or heat prior to lysozyme (Warren \textit{et al.}, 1955); lysozyme-EDTA combinations (Repaske, 1956; Earhart \textit{et al.}, 1968; Dworsky and Schaechter, 1973; Niveleau, 1974; McIntosh and Earhart, 1974; Stonington and Pettijohn, 1971; Worcel and Burgi, 1972; Meyers \textit{et al.}, 1976; Smith and Hanawalt, 1967) have been employed with \textit{E. coli} yielding satisfactory results.

In 1968, 1969, Miura and Mizushima first described the osmotic lysis of \textit{E. coli} after removal of the outer membrane,
and the resulting formation of spheroplasts by lysozyme-EDTA. Spheroplasts produced in such a fashion retained their rod shape and could not be reverted to an osmotically stable state (Asbell and Eagon, 1966a,b; Voss, 1964, 1967). Since then the most widely utilized lysis procedure for Gram negative organisms has become a lysozyme-EDTA combination followed by the addition of detergent.

**EDTA**

The mode of action of EDTA on Gram negative organisms' outer membrane has had fair exploration. The primary role is thought to be as a chelating agent, sequestering divalent cations involved in lipopolysaccharide crosslinkages (Gilleland et al., 1974; Asbell and Eagon, 1966a,b; Roberts et al., 1970). This in turn leads to the release of lipopolysaccharide (30-50%), phospholipids (5%), and certain proteins (e.g. enzymes) of the LPS complex (Lieve, 1965a,b; Rogers and Perkins, 1968; Rogers, 1969) and the cells are rendered osmotically fragile but exhibit no decrease in viability.

Asbell and Eagon (1966) reported that osmotic stability could be restored in the presence of multivalent cations following EDTA treatment, unless cell wall damage had occurred as well. This has ultimately led to the proposal that cations function in the linking of the phospholipids with LPS and LP and in the alignment of components at biological interfaces as previously proposed by Bungenberg de Jong (1936, 1949).

Ultrastructural examination of EDTA treated cells of *Pseudomonas aeruginosa* revealed approximately 50% of cell wall associated material is removed by the EDTA, consistent
with 50% removal of lipopolysaccharide reported from cell envelopes of the same organism by Thota, Ph.D. Thesis, University of Georgia, Athens (1972).

E. Lysis of Group D Streptococci

The report by Salton (1956a, b) of the extreme resistance of *S. faecalis* to the action of lysozyme, led to considerable research activity in an attempt to elucidate the mode of resistance and means of circumventing it.

Abrams (1957) reported that cell walls of *S. faecalis* ATCC 9790 were in fact susceptible to lysozyme when grown in complex media and treated with the enzyme at 35°C in phosphate buffer at pH 6.2 for 5 h. Whole cells of this strain likewise appeared susceptible to lysozyme.

The differences observed between the Salton strain and that of Abrams was thought to be due to the presence of galactose in the cell wall polysaccharide of the former but not the latter strain. It is now accepted that the 9790 strain is in fact a *S. faecium* strain which is more sensitive to lysozyme. In addition, *S. faecalis* strains harbor a peptide bridge of L-lys-L-alal-L-alal-L-alal whereas that of *S. faecium* consists of L-lys-D-asp (Schleifer and Kandler, 1972).

Chesbro (1961) produced osmotically fragile bodies from *S. faecium* HFBAG. They were designated true protoplasts as no rhamnose was detectable in the osmotically fragile forms whereas it was detectable in whole cells. Such fragility was observed upon removal of the hypertonically protective environment after a 65 min lysozyme treatment at 37°C. Twelve other enterococcal strains tested under similar conditions were
not rendered osmotically fragile. In addition, the author showed that even with the susceptible strain, hydrolysis of the cell wall could be inhibited if cells were grown in the presence of 1 M NaCl or to a lesser extent in 1 M KCl. Alkaline pH enhanced development of osmotic fragility among many of the strains and may be related to the Nakamura effect (1923) observed with Gram negative bacteria. In face one S. faecium strain reportedly lysed during growth at this elevated pH 9.4.

Bibb and Straughn (1962) prepared protoplasts of water washed cells of S. faecalis F24 via 2 h treatment with lysozyme (200 μg/ml) at 37 C. If kept under osmotically stabilizing conditions the cells remained intact even after overnight storage at 5 C. Dilution with cold Tris buffer, pH 6.4 was sufficient to lyse these "protoplasts".

Bleiweis and Zimmerman (1961) reported that the highly lysozyme resistant strain S. faecalis var. liquefaciens required pre-sensitization by growth in the presence of penicillin (known to prevent transpeptidation and therefore cross-linking of peptidoglycan units) for lysozyme to effect formation of spheroplasts. Unlike many Gram positive bacteria such as Bacillus and S. faecium which respond to treatment with lysozyme, the liquefaciens strain did not form protoplasts (free of cell wall) or osmotically fragile forms unless treated with a phage associated lysin derived from S. faecalis var. zymogenes.

Elwood and Tempest (1972) showed that lysozyme sensitivity measured as a decrease in optical density (O.D.) over time varied not only with pH but also other factors which
affect the physiological state of the organism (temperature, ionic strength, growth rate, nutrient deprivation, etc.). They associated these effects with changes in the ratio of cell wall components, although their data could also be explained in terms of the effects of these conditions during growth on membrane composition as well (Op den Kamp et al., 1967; Van Iterson and Op den Kamp, 1968; Daneo-Moore et al., 1966).

Similar effects of growth conditions on lysozyme sensitivity of streptococci have been reported. Rapid lysis was observed upon depletion of cell wall amino acids (Toennies and Gallant, 1949).

In addition, the latter investigators found that a distinction could be made between factors which made cells prone to lysis and those which allowed lysis to occur. Depletion of essential cell wall amino acids (e.g. lysine) during growth made cells prone to lysis whereas subsequent suspension media composition (phosphate buffer, pH, sucrose, NaCl, complete but lysine free growth media) seems to affect the degree and rate of lysis allowed for these cells. Log phase cells also exhibited a somewhat similar pattern with the exception that cells suspended in buffered sucrose remained stable unless the tonicity of the milieu was altered by dilution with water.

In addition, the observation that streptococcal cells were more stable in H₂O than in low phosphate buffer (0.01 M), and that lysozyme increased the rate of lysis and osmotic fragility, suggested that more than one type of lytic phenomenon was operational here. Increasing sensitivity to alkali and a
decrease in cell viability observed also led these authors to conclude that cell wall damage had occurred, even though no decrease was seen. Needless to say, the lytic response of this strain is a highly complex phenomenon.

Metcalf and Deibel (1969) expanding upon the observations of Shockman et al. (1961) but using strain S. faecium F24, showed that lysozyme at concentrations greater than 50 ug/ml could cause an increase in turbidity of either log phase or stationary phase cells which could be maintained for as long as 12 h. This effect was evident within 5 min of lysozyme addition, whether the cells were suspended in phosphate buffer, H2O, or either one with NaCl as long as the ionic strengths remained less than (0.18). Higher ionic strengths resulted in a decrease in optical density and clearing of the cell suspension. The authors interpreted this as a dependency of the lytic reaction upon the radius of the anionic species added to induce lysis (e.g. NaCl or detergent). However they did not rule out the potential of lysozyme to aggregate the cells. If the anionic moiety was added prior to lysozyme, no increase in optical density was observed and the lytic response was slow and dependent upon the growth phase at harvest. On the other hand, treatment with lysozyme prior to the addition of the anionic moiety resulted in a rapid increase in optical density after the first addition followed by instantaneous clearing upon addition of the second. This may in part explain the observations of Shockman et al. (1961) of changes in optical density which were inconsistent with cell wall damage, osmotic sensitivity, and viability. Similar
findings were reported by Friedberg and Avigard (1966) in work with *M. lysodeikticus*.

This method was effective regardless of growth phase or strain utilized. *S. faecalis* responded as well as *S. faecium*, with the former requiring slightly longer treatment times in terms of minutes. In contrast with the high halide concentrations (0.13 to 0.22 M) required to clear cell-lysozyme mixture with an avidity series I Br Cl F, detergents such as Tergitol or SLS were effective at far lower concentrations (0.008 M).

An effect of lysozyme on the cells could be demonstrated even in the absence of detectable decrease in optical density both by release of N-acetyl hexosamines and microscopic loss of refractility. Continued incubation with lysozyme resulted in microscopically evident clumping concomitant with an increase in optical density. Friedberg and Avigard (1966) suggested that this was the result of an electrostatic interaction of lysozyme with acidic cell components. Control (non-lysozyme treated cells in H$_2$O), showed some autolytic effects over this period as well. Even with this system which is extremely effective with the enterococcal strains tested, group A streptococci did not respond and *S. faecium var. casseliflavus* showed only partial lytic response.

The authors Metcalf and Deibel (1969) explained their observations by concluding that anionic species in effect competed for lysozyme with negatively charged sites on the cell surface of enterococci while a similar process occurred between the cation portions of the salt and lysozyme for the cell surface. In high concentrations of lysozyme the cells become coated and overt lytic reaction is prevented even
though lysozyme is hydrolytically active. Addition of anions results in the disruption of the protective layer and lysis ensues. Unlike the larger molecule lysozyme, cations of salts added prior to lysozyme can compete with this basic molecule without forming a protective layer around the cell. Due to competition the hydrolytic effect of lysozyme proceeds at a slower rate.

Subsequent studies by these authors (1973) claim loss of viability by two strains of *S. faecium* and *S. faecalis* upon treatment of cells with lysozyme in a low ionic environment (H₂O). Minimal loss of DNA, RNA, and protein but extensive loss of cell wall occurred as judged by release of D-alanine to the supernate. However this study must be viewed with skepticism for several reasons. The authors' previous report of massive clumping, microscopically observable, which accompanied an increase in optical density of lysozyme-treated, H₂O-suspended cells is inconsistent with their earlier interpretation of the results of MPN (most probable number) tests as indicative of loss of cell viability. The test, based upon a dilution scheme, and dependent upon the equal distribution of particles, would not have met this criterion in the manner in which it was reportedly performed. By far the more obvious explanation for the observed decrease in MPN is the NaCl content of their diluent which would have been capable of inducing lysis of pre-sensitized, lysozyme treated cells.

In addition, their conclusion that D-alanine release is reflective of cell wall hydrolysis, does not take into account D-alanine substituted membrane (intracellular) teichoic
acid reported by Toon et al. (1972) in *S. faecalis* and by Joseph and Shockman (1975) to increase in the supernate with increasing culture age of *S. faecalis* 9790. A relationship between D-ala release and amino acyl PG as a result of lysozyme treatment is also possible. Although both D- and L-ala have been associated with such an amino acyl PG in organisms, conclusive evidence in streptococci to indicate which amino acid is thus associated is not available. The inability of Metcalf and Deibel (1973) to detect large amounts of DNA, RNA, and protein in supernates after sedimentation of cell-lysozyme complexes may not reflect cell integrity as they suggest but rather the formation of an artificial barrier by lysozyme, approximating a shell, as they themselves have previously suggested (1969). However their latter contention, that lysozyme treatment may affect the cell membrane of group D streptococci, is in fact supported by their observance of interference with phage reproduction in *S. faecium var. durans*, inhibition of acid production, and cessation of oxygen consumption.

The fact that treatment with other basic substances such as cytochrome C and lysostaphin also shows an increase in optical density suggests the basic protein nature of lysozyme is important in the observed increase in optical density under conditions of low ionic strength. However, neither cytochrome-C nor lysostaphin effect susceptibility to lysis upon addition of NaCl while high molecular weight polymers of lysine and arginine which evidence only a slight increase in optical density and practically no lysis upon addition of salt, results in a 4 log loss in viability.
This brings up an interesting point with regard to the function of amino-acyl phosphatidyl-glycerol. Perhaps due to their basicity a competition is set up between lysyl-PG or arginyl-PG and lysozyme. For example, with lysozyme being less protective than the amino-acyl derivatives, which normally tend to accumulate in response to adverse conditions (e.g. acid pH, high temperature) the lysozyme treated cells would be more prone to disruption of normal membrane functions, possibly leading to their death.

Coleman et al. (1970) also found differences in viability as a result of lysozyme treatment. Utilizing a somewhat better although not perfect system of quantitation, namely, CFU (colony forming units) rather than an MPU system, they found a 96% loss in viability of S. faecalis within 3 min of lysozyme addition.

It is interesting to note that these authors also observed that effects on optical density and viability seen with cells in H2O were also apparent when S. faecalis was treated in Tris buffer with lysozyme. Whether these cells were subsequently treated with NaCl according to method of Metcalf and Deibel (1973) sucrose and detergent SLS (Schaechter et al., 1965), or SLS without sucrose (Coleman et al., 1970), similar results were obtained. The latter investigators also demonstrated that variations in susceptibility to lysozyme which occur among different streptococci (ranging from 0 to 100%) is often overlooked, as most studies have been done on the faecium and faecalis strains.
F. Lipids

As the membrane provides a flexible, permeability barrier to maintain osmotic stability at the cell surface, it must be capable of responding to both hydrophobic and hydrophilic conditions (Cronan and Vagelos, 1972; Mindich, 1973). Lipids, at least those of complex nature, contain both polar and non-polar groups and are thus able to impart such properties to the cell. As such, they are the most characteristic components of biological membranes.

Implicated involvement of the membrane in growth active transport, and maintenance of certain enzymatic activities, as well as its role in preserving structural integrity, makes an understanding of the distribution and properties of the lipids involved relevant to any attempts at elucidating bacterial membrane structure and function.

Comprising 20-30% of the membrane in bacteria, lipid (predominantly phospholipid) can be associated with protein, neutral glycolipid, and fatty acids. Very little lipid has been reported associated with either the cell wall or cytoplasm (Salton and Freer, 1965) especially amongst the Gram positive organisms. Vorbeck and Marinetti (1965a) in fact reported the cell wall of S. faecalis to be devoid of phospholipid. Op den Kamp et al. (1967) noted that 95% of the phospholipids of B. megaterium were associated with protein in the cell membrane. Thus study of membrane in terms of phospholipid composition (in Gram positive organisms at least) becomes greatly simplified.
The picture is more complicated in Gram negative bacteria as their outer membrane as well as inner cytoplasmic membrane contains lipid components including phospholipids. Thus separation of inner and outer membranes prior to lipid analysis becomes necessary if definitive associations of structure and function of the lipid components is to be established. Until such separation was made possible, lipid composition data could only be related to the cell envelope rather than specifically to the cell membrane of these organisms.

Goldfine (1972) determined that Gram positive and Gram negative bacteria could be differentiated on the basis of their lipid composition. Each could then be further subdivided. The Gram positive Eubacteriales form two distinct groupings. One composed of the non-spore forming lactic acid bacteria (streptococci and pneumococci) and Micrococcaceae (micrococci and staphylococci) contain phospholipids of the phosphatidylglycerol family type (PG, diPG and O-aminoacyl-phosphatidylglycerol). In addition, these organisms contain glycosyl diglycerides which comprise from only a few to as much as 35 % by weight of the total lipid.

The second of the Gram positive groupings includes the endospore forming Bacillaceae (Bacillus and Clostridia). Unlike the previous grouping, these organisms synthesize PE as 20-40 % of their phospholipid composition in addition to lipids of the PG family mentioned above.

A more extensive treatment of this subject and information concerning the specific characteristics of the
lipids of other Gram positive organisms such as Corynebacteria or Propionibacteria as well as that of the order Spirochaetales, the reader is directed to the reviews of Asselineau (1966), Goldfine (1972) and Lederer (1967).

The Gram negative bacteria of the orders Pseudomonadales and Eubacteriales are not as easily distinguishable from one another in terms of either quantitative or qualitative differences in phospholipid composition. Members of both groups contain PE, PG, diPG. Pseudomonadales have a somewhat higher combined PG and diPG content (17-44%) as compared with a (20-33%) combined content among the Eubacteriales. Methylated derivations of PE occur at the (55-78%) level in the former compared with (64-79%) in the latter. However, such information hardly provides a definitive delineation among the Gram negative bacteria. Within each order however, organisms can be shown to exhibit various degrees of methylation of PE, suggesting carry over of order variability to the family level (Goldfine, 1972).

Although criticism may be raised concerning this scheme, such as the presence of PE in both Gram positive and Gram negative organisms, in the Gram positive Bacillaceae the average is much lower than that found in the Gram negative species and methylated derivations seem to be absent in the bacilli (Mindich, 1973). Even amongst the Gram negative organisms variations in type, position, and degree of methylation of the fatty acids contribute to qualitatively observable differences.
Table 1 summarizes the reported distributions of phospholipids in strains of enterococci (*S. faecalis* and *S. faecium*), *B. megaterium*, *B. subtilis*, and *E. coli*. This is done in an effort to provide easy reference, as the composition of these organisms will be pertinent to the ensuing discussion of the results of this study. For additional information concerning the lipids of other species and genera the reader is directed to any of the following: Op den Kamp et al., 1969a; Goldfine, 1972; Reaveley and Burge, 1972).

**Fatty Acids**

In general, families tend to exhibit similar fatty acid patterns. Enterobacteriacea (*E. coli*, Enterobacteria, Serratia, Proteus, and Salmonella) favor C14 – C18 unsaturated fatty acids (palmitoleic and vaccenic) usually with only one double bond of variable position (Goldfine, 1972; Op den Kamp et al., 1969a) and straight chain fatty acids such as palmitic (Mindich, 1973; Kates, 1964; Cronan and Vagelos, 1972).

Members of Bacillacea prefer methyl branched-chain fatty acids (iso and anteiso) C15 and C17 as well as lesser amounts of even number and straight chain types (Kaneda, 1963).

Although seldom found in higher organisms, the cyclopropane fatty acids are found widely distributed among both Gram positive and Gram negative bacteria (lactobacilli, streptococci, clostridia, enterobacteria and brucella) (Goldfine, 1972); whereas the 3-hydroxy polar fatty acids are common among the Gram negatives.

The major fatty acids of *S. faecalis* and *S. faecium* have been reported by Girard and Cosenza (1971) and dos Santos Mota et al. (1970) to be 16:0, 18:1, 19:0 (the latter being of
Table 1. Reported Lipid Composition of Select Gram Positive and Gram Negative Bacteria

<table>
<thead>
<tr>
<th>Organism</th>
<th>PA</th>
<th>PG</th>
<th>amino-acyl</th>
<th>PE</th>
<th>PG</th>
<th>cardiolipin</th>
<th>glycolipid</th>
<th>growth phase</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. faecalis major</td>
<td></td>
<td></td>
<td>major</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>stationary</td>
<td>Ikawa, 1963</td>
</tr>
<tr>
<td>S. faecalis 9790</td>
<td>major</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>exponential</td>
<td>Shockman, 1963</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>minor</td>
<td></td>
<td></td>
<td>minor</td>
<td>+</td>
<td>stationary</td>
<td>Vorbeck and Marinetti, 1965a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>major</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>stationary</td>
<td>Vorbeck and Marinetti, 1965b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>increases</td>
<td></td>
<td></td>
<td></td>
<td>+</td>
<td>stationary</td>
<td>dos Santos Mota, 1970</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>54 %</td>
<td>stationary</td>
<td>Ibbott and Abrams, 1964</td>
</tr>
<tr>
<td>E. megaterium</td>
<td>49-69</td>
<td>34-20</td>
<td>10-2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>stationary</td>
<td>Bertsch et al., 1969</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>30-35</td>
<td>stationary</td>
<td>OpdenKamp et al., 1965</td>
</tr>
<tr>
<td></td>
<td>5-10</td>
<td></td>
<td>+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>exponential</td>
<td>Ballesta et al., 1972</td>
</tr>
<tr>
<td></td>
<td>35-45</td>
<td>36-45</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>stationary</td>
<td>Bishop et al., 1967</td>
</tr>
<tr>
<td></td>
<td>41 (6)</td>
<td>34 (31)</td>
<td>25 (22)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>exponential</td>
<td>Ballesta et al., 1972</td>
</tr>
<tr>
<td></td>
<td>13</td>
<td>10</td>
<td>40</td>
<td>38</td>
<td>15</td>
<td>stationary</td>
<td></td>
<td></td>
<td>Cronan and Vagelos, 1972</td>
</tr>
<tr>
<td></td>
<td>0.9</td>
<td>18.3</td>
<td>52.1</td>
<td>28.1</td>
<td></td>
<td>stationary</td>
<td></td>
<td></td>
<td>Randle et al., 1969</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>5-15</td>
<td>70-80 trace</td>
<td>5-15</td>
<td></td>
<td></td>
<td></td>
<td>stationary</td>
<td></td>
</tr>
</tbody>
</table>

* ( ) = M-band %
a cyclic type in *S. faecalis*). Pigmented, motile streptococci closely related physiologically to *S. faecium* contain 16:0, 16:1 and 18:1 as the major fatty acid components. It should be noted that such fatty acid spectra are more likely to be found among Gram negative species, having large amounts of even numbered saturated and unsaturated straight chain acids and odd numbered cyclopropane acids. Gram positive organisms are more likely to have high proportions of odd numbered branched-chain acids and relatively small amounts of straight chain saturated or unsaturated acids. Other members of the family Lactobacteriaceae also resemble the Gram negatives in fatty acid composition (Thorne and Kodicek, 1962; MacLeod and Brown, 1963). However no speculations were made as to the physiological implications of these findings.

**Proposed Functions of Membrane Lipids**

Various functions have been postulated for the cell membrane and in some cases for the individual lipid types as well. These range from a structural role to regulate permeability or as a matrix in which membrane proteins can be embedded and protected (Cronan and Vagelos, 1972), to specific lipid requirements for certain enzymatic activities. The galactosyl transferase of *Salmonella typhimurium* for example, involved in LPS synthesis requires either PG, PE, or dPG, containing monounsaturated or cyclopropane fatty acids to effect a transfer of a galactose molecule to LPS (Rothfield and Romeo, 1971). In *E. coli*, the phosphotransferase system is activated by PG (Kundig and Roseman, 1971) while induction
and maintenance of the β-galactoside transport system seems to be dependent on phospholipid synthesis (Cronan and Vagelos, 1972; Op den Kamp et al., 1969a).

Additional roles in active transport, maintenance of structural integrity, in growth and macromolecular synthesis have also been proposed. Several lines of investigation suggest that unsaturated fatty acids are needed to support growth and cellular integrity in E. coli and that lack of the appropriate unsaturated fatty acids in phospholipids results in cell lysis (Cronan and Vagelos, 1972).

Membrane lipids involvement in other cell processes has also been suggested. They can also provide specific binding sites for intracellular polymer intermediates allowing further enzymatic conversion and additional assembly exterior to the cell.

In addition, the enrichment of PE reported by Ballesta et al. (1972) in the DNA membrane complexes of E. coli and B. megaterium implies specific lipid involvement in bacterial reproduction. The ability of lipoteichoic acid and cardiolipin, but not neutral or glycolipid, nor deacylated LTA or diPG (Cleveland et al., 1976a,b), to inhibit autolysis of intact cells, a process which normally occurs at the cell septum in S. faecalis 9790 (Shockman et al., 1967) allowing cell division to occur, implies specific lipid involvement in cell division as well.

**Factors Affecting Membrane Lipid Composition**

Comparison of the lipid content of log phase cell membranes of S. faecalis (Shockman et al., 1963) with similar
preparations from stationary phase cells show an increase of from 8 to 12 %, depending on whether the cells are derived from amino acid depleted media or not.

**Phospholipids of Group D Streptococci**

Information concerning the lipid composition of group D streptococci has resulted primarily from studies on *S. faecalis* ATCC 9790, as this organism is more sensitive to lytic methods than most of the streptococci. Whole cells, protoplast membranes, and subcellular fractions from exponential and stationary phase cells have been examined under conditions of normal or deficient growth. Rather than clarifying the situation, however, this conglomeration of works has resulted in a somewhat more confused state, since no two studies are comparable in terms of conditions used, which are known to alter the phospholipid composition quantitatively if not qualitatively (Goldfine, 1972; Op den Kamp *et al.*, 1969a; Reaveley and Burge, 1972; Kanfer and Kennedy, 1963; Houtsmuller and Van Deenen, 1964).

As such alterations will be of import in evaluation of the data in this thesis, the various compositions reported by individual investigators, presumably for the same organism, as well as information pertinent to evaluation of their findings will be presented here.

Ikawa (1963) reported that *S. faecalis* as well as other lactic acid bacteria (*Lactobacillus casei*, *L. plantarum*) after poor growth in lipid-free media, exhibited increasing amounts of bound lipid not extractable by the normal Bligh
and Dyer (1959) or Kanfer and Kennedy (1963) chloroform: methanol methods of lipid extraction. In addition, and probably of greater significance, was the observed association of certain amino acids such as L-lysine in *S. faecalis*, and D-alanine in *L. mesenteroides* with a phospholipid, which comprised a major portion of the phospholipid composition elucidated. This was the first indication of such components in these organisms, although MacFarlane (1962) had reported a similar type compound in *Clostridium perfringens*. The components now recognized as aminoacyl derivatives of PG have been reported in a wide variety of Gram positive bacteria (Ambron and Pieringer, 1973).

Lipids of *S. faecalis* were the focus of a study by Vorbeck and Marinetti (1965). They examined both the individual phosphatides and non-phosphatides of the subcellular fractions of exponential phase cells and compared them with those found in stationary phase cells grown under similar conditions in lipid-free synthetic media. Chromatographic behavior, chemical analysis, specific staining properties, radioisotopic data, and identification of the water soluble hydrolysis products of the chloroform:methanol lipid extracts formed the bases for identifying and quantitating the components involved. Their finding that 94% of the total cell lipid was contained in the membrane fraction, with very little in the protoplasm and none detected in the cell wall fraction agrees with previous reports of Kolb et al. (1963), Weibull (1957), Gilby et al. (1958), MacFarlane (1961), Yudkin (1962), for other Gram positive organisms and for *S. faecalis* as well (Shockman et al., 1963).
Washing the membranes with PO\textsubscript{4} buffer and NaCl without Mg\textsuperscript{2+}, they reported PG as the major phosphatide of exponential phase cells, PA and diPG as minor compounds and a higher lipid content in membranes than did Shockman et al. (1963) or Ibbott and Abrams (1964). However, the authors claim this was due to their inclusion of non-phosphatides in their reported value.

In contrast to their findings were those reporting PA as predominant phospholipid (Shockman et al., 1963) or diPG (Ibbott and Abrams, 1964). Vorbeck and Marinetti (1965a) also noted as did Ikawa (1963) a lysine derivative of PG with a small amount of glycine and alanine as well if the lysine derivative was subjected to alkali or acid hydrolysis. This component increased in amount in stationary phase cells. This suggests that amino acyl derivatives of PG may be preferentially formed in stationary phase.

Although various functions have been proposed for this type of phospholipid such as involvement in cell wall synthesis, active transport, or to maintain cell membrane charge, conflicting data exists with respect to each possibility (Ambron and Pieringer, 1973). The most appealing proposal favors a role in membrane stabilization which would be consistent with observations of Vorbeck and Marinetti (1965a) and is supported by data derived from studies with \textit{B. subtilis} (Van Iterson and Op den Kamp, 1969; Ambron and Pieringer, 1973).

Stationary phase cells under similar conditions show an increase in amino-acyl PG, similar to observations made by Houtsmuller and Van Deenen (1964, 1965) with \textit{S. aureus} and \textit{S. faecalis} among others, when either grown or subsequently
treated at a pH lower than 5. This led the authors Houtsmuller and Van Deenen (1965) to further exploration and to test their hypothesis that such compounds were synthesized in response to an increase in environmental acidity.

An even more complex array of phospholipids particularly of the amino acyl variety were reportedly isolated from stationary phase cells of \textit{S. faecalis} (dos Santos Mota et al., 1970). These included an alanyl-PG, arginyl-PG, 2'-lysinyl-, 3'-lysinyl-, and a 2'3'-dilysyl-PG in addition to PG, diPG, and a diglucosyl derivative of PG. These cells, however, had been grown in complex media pH 4.2 at harvest, and subjected to wash and extraction at the same pH, which may account for the complex array seen. In addition, the analysis revealed a high diPG content and a decrease in the total lipid extracted, consistent with observations of Houtsmuller and Van Deenen (1965) as a result of lowered pH.

Initial studies with \textit{S. aureus} (Houtsmuller and Van Deenen, 1965) indicated an increase in lysyl-PG at the expense of PG and in \textit{S. faecalis} an increase in diPG as well, when the pH during growth fell below 5. The reverse was seen to occur at pH 7. Additional studies with \textit{S. aureus} by these same authors however revealed the complexity of the metabolism of these compounds. Altering the pH during growth from 7.0 to 4.0 in a stepwise fashion gave the following altered patterns: pH 7.0-5.6, no change; pH 5.2-4.9, lysyl-PG increased, PG decreased; pH 4.9, large quantities of diPG accumulated (only small amounts were the usual); pH 4.0, composition consisting almost exclusively of lysyl-PG. Overall, the phospholipid
content decreased from pH 7.0 to 4.8; the loss primarily in PG which was not found to be compensated for by increases in lysyl-PG or diPG. It is interesting to note that under normal growth conditions, the pH never fell below 4.8 although the cells remain metabolically active even at pH 4.2. The authors suggested activation of a phospholipase specific for PG or its derivative, amino-acyl PG or diPG, or incorporation of PG into a more complex structure making the lipid non-extractable, as possible explanations for the observed alterations.

The situation is further complicated in *S. faecalis* ATCC 8049 as Kocun (1970) found an initial increase in lysyl-PG followed by a leveling off as the pH of the growth media decreased. Alanyl-PG on the other hand decreased initially followed by a leveling off, the exact opposite of the response of the lysyl derivative, while diPG and PG levels appeared to remain fairly constant.

Other factors such as cell density, Mg$^{++}$ concentration, and turnover rates have also been purported to play some role in the metabolism of these compounds, reminiscent of factors implicated in membrane associated chromosomes isolated from *E. coli* via method type B.

The functional role of these compounds remains obscure and at least two mechanisms for synthesis of such compounds is possible: 1) via aminoacyl-tRNA (Lennarz *et al.*, 1966) 2) via AMP-aminoacyl-enzyme (synthetase) complex, formed via ATP pyrophosphate exchange (Koostra and Smith, 1969). In addition, both D and L aminoacyl derivatives have been reported to be possible (Ito and Strominger, 1962; Okuda *et al.*, 1964),
although Lennarz (1966) claims that only the L-lysyl PG can be found.

Functions such as a role in selective amino acid transport, especially those related to cell wall synthesis and regulation of cell membrane lipid core charge have been proposed for these lipids. The data in all cases is equivocal however (Ambron and Pieringer, 1973). Current leanings are towards a role in stabilizing the membrane in order to counteract disruption of structure and/or function under adverse conditions.

Evidence in support of this type of role for aminoacyl-PG put forth by Van Iterson and Op den Kamp (1969) shows increased resistance of B. subtilis to osmotic lysis and maintenance of rod-like shape with accumulation of lysyl-PG at low pH. Such results are seen even after treatment of cells with lysozyme.

In addition to the aminoacyl-PG derivatives, studies have reported the presence of saccharides complexed with phospholipids (Lefevre et al., 1964). In a recent study of the lipids of Streptococcus hemolyticus D-58, Ishizuka and Yamakawa (1968, 1969) isolated a diglucosyl derivative of phosphatidylglycerol. The glucose molecules were linked via an (1–2) bond and probably via a glycosidic linkage to the 3-OH of the terminal glycerol. Fischer and Seyferth (1968) proposed the same structure for lipids isolated from S. lactis and S. faecalis, as did dos Santos Mota et al. (1970) for a lipid purified from S. faecalis ATCC 9790. However, one could as easily conclude from the data presented that the structure was in fact a glycerophosphate derivative of diglucosyl
diglyceride, a phosphoglycolipid. Such a compound was reportedly found in *E. megaterium* and to comprise from 15% to as much as 80% of the total phospholipids found under acidic culture conditions at pH 5 (Murray and Freeman, 1951; Opdenkamp et al., 1969b).

In addition, the report by Toon et al. (1972) of a lipid-teichoic acid complex in the cytoplasmic membrane of *S. faecalis N.C.I.B. 8191* and the isolation of two phosphatidyl-glycolipids closely resembling those found in association with the teichoic acid may hold a clue to the significance of these compounds. This becomes especially exciting as the teichoic acid, a polyglycerophosphate polymer, is thought to be associated with the membrane via attachment to 2-0-α-D-glucopyranosyl-α-D-glucopyranosyl(kojibiosyl) residues through a phosphodiester linkage and D-alanine ester residues (Wicken and Baddiley, 1963).
CHAPTER II

MATERIALS AND METHODS

A. Cultures

The University of New Hampshire (UNH) culture collection served as the source of the group D streptococcal strains used in this study. Strains 564P and 615M, originally isolated and characterized by S. Weinstein (Ph.D. Thesis, Department of Microbiology, University of New Hampshire, 1969) as well as Streptococcus faecalis var. liquefaciens and S. faecalis 9790 of the culture collection were cloned. The resulting cultures were preserved with 10% dimethylsulfoxide (DMSO) after 12 h growth in T-Soy or Brain Heart Infusion broth. Such preserved cultures were then frozen at -12 C until required. This system was highly successful in viability and previous biochemical characteristics (including generation time) after more than one year of storage. Revitalization procedures commenced with inoculation of broth of the same media used to grow the cultures for preservation with a thawed portion of the preserved culture. Following growth such cultures were streaked onto blood agar and M-enterococcus media and portions Gram-stained to insure purity. Cultures were tested periodically and prior to preservation for compliance with Sherman's physiological criteria for enterococci (Table 2).

The E. coli B/r (w) strain belonged to the collection of M. Schaechter (Department of Molecular Biology and Microbiology, Tufts University School of Medicine) and the
TABLE 2. Biochemical Characteristics of Selected Group D Streptococci

<table>
<thead>
<tr>
<th>Sherman's Criteria (Enterococcal Division)</th>
<th>Enterococcus 564P</th>
<th>Enterococcus 615M</th>
<th>S. faecalis 9790</th>
<th>S. faecium</th>
<th>S. faecalis</th>
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</thead>
<tbody>
<tr>
<td>Growth at 10 C</td>
<td>+</td>
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<td>+</td>
<td>+</td>
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Other

<table>
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</tr>
<tr>
<td>arabinose</td>
<td>+</td>
<td>+</td>
<td>+/-</td>
</tr>
<tr>
<td>0.04 % K tellurite</td>
<td>+</td>
<td>-</td>
<td>-</td>
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</table>

+= growth; +/- = variable growth; - = no growth
B. *megaterium* strain was kindly donated by M. Green (Department of Biochemistry, University of New Hampshire). Work with the former strain was done at Tufts University, and the latter strain was preserved in a similar fashion to that described for the enterococci but with C media plus sucrose serving as growth media (Appendix).

**B. M-bands**

**Technique - E. coli B/r(w)**

The technique, essentially that of Earhart *et al.* (1968) as modified by Schaechter (personal communication) was used. Cultures grown with aeration for 16 h at 37 C in phage growth (Appendix) were used to provide a 0.5 % or 1 % seed to pre-warmed, pre-aerated medium, and the seeded medium reincubated. The culture was harvested via centrifugation (12,000 x g, 15 min) in a Sorvall RC-2 centrifuge at 0 C at an O.D. 0.37 (generation time 22.5 min) determined with a Zeiss spectrophotometer blanked with phage broth. The resulting pellet was resuspended in phage broth to one-fifth the original volume. Five ml of this suspension was added to an equal volume of frozen phage broth containing sucrose, centrifuged (3,000 x g, 0 C) and resuspended in 1.6 ml 10 % sucrose in 0.1 M Tris buffer, pH 8.1. One-tenth ml of a 1:1 mix of 2 mg/ml lysozyme in 0.25 M Tris buffer, pH 8.1 and 2.7 mg/ml disodium EDTA, was added per 0.4 ml of the suspension for each gradient required. In some cases, the resulting mixture was layered directly atop sucrose block gradients 5 ml 15 % sucrose in TMK (10 mM Tris, 10 mM magnesium acetate, 100 mM KCl) pH 7.0 at 0 C/
over 5 ml 50 % sucrose in TMK with subsequent treatments occurring as additions to the layered material on the gradients. In other cases, especially those where shearing of lysate was included prior to M-banding, all additions including Sarkosyl were performed prior to layering atop gradients. After 30 min of lysozyme treatment at 0 C, 0.05 ml 5 % Sarkosyl was added to the mixture to effect lysis. Mg\textsuperscript{2+}-Sarkosyl crystal formation followed by addition of 0.1 ml of 0.1 M MgSO\textsubscript{4}. The layered portion of the gradients was gently mixed with a drawn-out Pasteur pipet so as not to disturb the gradients, which were then centrifuged in a SW27 swinging bucket rotor in a Beckman L3-40 ultracentrifuge at 0 C (15,000 x g, 30 min).

**Technique - B. megaterium**

The technique used was essentially that of Earhart *et al.* (1968), developed for isolation of DNA-membrane complexes from *B. megaterium*. Cells grown in modified C medium (Appendix) with or without 20 % sucrose, aerated overnight at 37 C served to make a 1 % inoculum of the same broth. Cultures resulting upon re-incubation to an O.D. of 0.13, measured with a Klett colorimeter (#64 filter), were chilled by pouring over four-fifths vol of crushed, frozen TMK buffer, pH 7.0 containing 20 % sucrose. In some cases the frozen buffer also contained enough lysozyme to give a final concentration of 250 ug/ml after the addition of cells. In other cases, an equivalent weight of lysozyme was added immediately after frozen buffer treatment. Incubation with lysozyme was carried out for 30 sec at 0 C in the former case, and 1-2 min at 37 C in the latter case. A
sample was then removed for viewing by phase contrast microscopy to determine the extent of lysozyme action. The appearance of spherical forms was interpreted as an indication of protoplast formation. Subsequent steps were carried out at 0 C. The protoplasts were centrifuged (3,000 x g, 10 min) to remove excess lysozyme, washed once with 20% sucrose in TMK buffer and resuspended in 15% sucrose in TMK buffer. One-tenth ml of Sarkosyl was added per 0.5 ml of this suspension either layered atop sucrose gradients (for on-gradient lysis) or in test tubes (for off-gradient lysis). The off-gradient treated samples were then layered onto sucrose block gradients. One-tenth ml of 0.1 M MgSO₄ was layered atop each gradient with gentle mixing of the layered volume. Subsequent operations were as described for E. coli B/r(w).

Technique - S. faecalis var. liquefaciens

An overnight culture in phage broth was used to make a 1% seeding into phage broth supplemented with methyl-³H thymidine (5 uCi/100 ml). Static growth at 37 C was terminated at O.D. 0.3 by centrifugation at 0 C (4,000 x g, 10 min). After two cold distilled water washes, the cells were resuspended in cold distilled water to 15% of their original volume. An equal volume of lysozyme in water was added to give a final concentration of 200 ug/ml. After 10 min at 37 C, either 10% sucrose in Tris-EDTA (0.125 M-1.35 mg/ml) pH 8.1, or 10% sucrose in distilled water (0.25 ml per 1 ml lysozyme treated cells) was added. After an additional 10 min incubation, 0.4 ml of the mixture was layered onto 15/50%
sucrose block gradients kept on ice followed by 0.1 ml 5 % Sarkosyl, and 0.1 ml of 0.1 M MgSO₄ at 10 min intervals. Subsequent treatment was as described for E. coli and B. megaterium, including gradient fractionation and radioactivity counting.

**Fractionation of Gradients**

The gradients were fractionated into three parts utilizing 5 ml plastic (Falcon) pipets (siliconized if radioactive material was involved) and a propipet. Fractions were designated as follows: **Top** fraction—that portion of the gradient above the M-band; **M-band** fraction—the white, opalescent material usually at or slightly above the interface of the two sucrose concentrations used per gradient; **Bottom** fraction—that portion of the gradient fluid remaining. In cases where pellets were detected and/or residual material in centrifuge tubes was to be determined for estimates of efficiency of recovery, 1 ml distilled H₂O was added per gradient tube plus a few glass beads. Following vortexing for a few seconds, fluid was collected with a pasteur pipet and designated the **Pellet** fraction. Fraction volumes were measured and comparable fractions from 3 gradients pooled. The nature of the subsequent analyses of these pooled samples determined further handling and storage of these and remaining Lysate samples.
Radioactivity Counting:

_E. coli B/r(w), B. megaterium, S. faecalis var. liquefaciens_

When radioactively labelled cells were used unless otherwise noted enough 50% cold trichloroacetic acid (TCA) was added to each sample to yield a final concentration of 10% w/v. After 30 min at 0°C, samples were filtered through Whatman GPA (glass fiber) filters (2.4 cm circles) prerinsed with distilled water. The material collected was then rinsed with 5 ml of 5% TCA followed by two (7 ml) rinses with 1% acetic acid to minimize quenching of radioactivity by TCA. The filters, dried in uncapped scintillation vials for 30 min at 60°C, or overnight at 37°C were made transparent by addition of 4 ml toluene liquafluor (NEN #NEP903) (160 ml/gal) and radioactivity determined in a Nuclear Chicago, Beckman L-235, or Packard Tri-Carb liquid scintillation counter. Filters which failed to become transparent upon addition of scintillation cocktail, due to incomplete drying, gave erroneous and variable results and were not counted.

Enterococci 564P, 615M and 9790

Phospholipid content of strains 564P, 615M and 9790 was determined by liquid scintillation counting of $^{32}$P in lysates and M-band fractions prepared from $^{32}$P labelled cells. Thin layer chromatographic development of equivalent chloroform:methanol:water (65:25:4 %) of these samples separated the different phospholipids. Areas of the plate which revealed the presence of lipid by response to iodine vapors were then
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marked, scribed with a scalpel, removed following slight moistening of the silica gel surface with distilled water and moistened silica gel removed was placed in scintillation vials. Aquasol (NEN) was used for the scintillation cocktail to eliminate the necessity of drying samples prior to counting. Radioactivity was determined in a Packard Tri Carb liquid scintillation counter. Phospholipid content was reported as % cpm/total cpm of sample examined.

Lytic Procedure and M-bands—Group D Streptococci:

The method adopted for lysate formation from group D streptococci is essentially that reported by Clewell, et al. (1974) for S. faecalis modified by the addition of EDTA, pH 6.25 rather than pH 8.0. Cultures of each strain were prepared by observing growth turbidimetrically with a Klett-Summerson colorimeter (#64 filter) until the desired O.D. (usually mid log phase) was attained (see results for exact figures). Scheme 1 was then followed.

Upon addition of Sarkosyl, the suspension began to clear and increase in viscosity. The time necessary for complete clearing at 25 C varied with the strain used; in most cases clearing occurred within 5-10 min when EDTA, pH 6.25 was used. The cleared sample was designated the Lysate and used for subsequent lipid extraction, M-banding, and analysis of nucleic acid content.

It is important to note that the final concentration of reagents in proportion to CFU treated should be maintained as given in Scheme 1 for maximum effectiveness of this procedure.
### Scheme 1

**PROCEDURE FOR PREPARATION OF M-bands FROM GROUP D STREPTOCOCCI**

**Growth conditions**

- **Medium**: Brain Heart Infusion Broth (BHI)
- **Inoculum**: 1% from overnight BHI culture
- **Incubation**: 37°C
- **Phase at Harvest**: Log

**Protoplast/Spheroplast Formation**

- Chill 25 ml immediately on ice
- Centrifuge 5 min, 8,000 x g, 0°C
- Pellet appropriately stored (Supernate)
- Resuspend in cold, 25% sucrose in 0.05 M Tris, pH 8 to 1/15 of original volume
- Add at 5 min intervals at 25°C

<table>
<thead>
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<th>Reagent</th>
<th>Vehicle</th>
<th>pH</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.4 ml lysozyme</td>
<td>TES**</td>
<td>7</td>
<td>1 mg/ml</td>
</tr>
<tr>
<td>0.7 ml EDTA</td>
<td>H₂O</td>
<td>6.25</td>
<td>0.06 M</td>
</tr>
<tr>
<td>0.4 ml Predigested Pronase</td>
<td>TES</td>
<td>7.5</td>
<td>0.6 mg/ml</td>
</tr>
</tbody>
</table>

**Lytic Induction**

- 1.9 ml Sarkosyl
  - TES
  - pH 8.1
  - 1%

Final Incubation 5 min, 25°C

**Lysate**


**TES (0.03 M Tris, 0.05 M EDTA, 0.05 M NaCl) pH 8**
DNA-membrane isolation-Enterococci

A method combining those of Tremblay et al. (1969) and Clewell and Helinski (1970) was used for isolation of the DNA-membrane complex.

Discontinuous block gradients containing equal volumes (2 ml) of 15% sucrose in pH 7.1, over 40% sucrose in TEK, pH 7.3, were formed in cold cellulose nitrate tubes, 1/2 in diameter x 2 in (Beckman Instrument Company). Gradients were constructed with the aid of 5 ml syringes and cannulae in tubes pre-rinsed with distilled water, air dried, and chilled just prior to use. Gradients were kept cold during subsequent manipulations.

Two-tenths ml Lysate and 0.04 ml of 1 M MgSO$_4$ or Mg acetate were layered in sequence atop each gradient and mixed gently to insure maximum interaction of the detergent with Mg$^{++}$. Care was taken not to disturb the gradient during this process. White crystals of Mg-Sarkosyl formed under these conditions. Gradients were then held at 0 C under vacuum for 90 min in a Beckman L2-65B ultracentrifuge in an SW65K swinging bucket rotor (for test gradients) or a Spinco L1 ultracentrifuge in an SW39 swinging bucket rotor (for control gradients). Control gradients were formed by layering with 0.2 ml of a mixture which included all reagents used to produce protoplasts/spheroplasts in the absence of material) followed by additional layering of 0.04 ml 1 M Mg$^{++}$. All gradients were then centrifuged at 15,000 rpm for 30 min at 0 C. Differences in g forces exerted upon the gradients in the two centrifuges at this speed were insignificant.
The resulting gradients showed discrete white opalescent bands at the interface of the two sucrose solutions (Fig. 1). Those of the test gradients appeared more globular, while those of control gradients appeared as compact, crystalline, thin bands which were more difficult to fractionate.

C. Fluorometric Analysis of DNA and RNA Content of Lysates and M-band Gradient Fractions

DNA and RNA were determined fluorometrically by the methods of Le Pecq and Paoletti as described by Prasad et al. (1972). It is based upon the ability of ethidium bromide to increase fluorescence upon excitation with ultra violet light (uv) as a result of intercalation of the dye between the bases of the nucleic acid concentration was found with this method which can be 10,000 fold more sensitive than conventional colorimetric or spectrophotometric methods, with sensitivity on the order of ng quantities. Other advantages include: both DNA and RNA can be measured within the same sample; high salt or up to 60 % sucrose does not interfere; a pH range of 4 to 10 with any buffer system can be employed (Van Dyke and Szustkiewicz, 1968; Waring, 1965); and a temperature range of 12 to 25 C, pH 6.0 - 8.8, and a salt concentration of 0.1 to 0.2 M are optimum (Prasad et al., 1972). Under these conditions as little as 0.01 ug/ml nucleic acid could be detected and recoveries of 101 ± 8 % DNA and 96 ± 8 % RNA could be expected.
Fluorescence measurements were determined with a fluorometer (Model III, Turner Associates, Palo Alto, California) equipped with temperature stabilized sample holder and general purpose lamp. An excitation wavelength of 365 nm (7-60 1° filter) and an emission wavelength of 590 nm (25 2° filter) were used. The instrument was prewarmed for at least 1 h and nulled with a reagent blank (assay mixture) which included 0.1 ml TEK at pH 7.3; 1 ml ethidium bromide (E.B.) (5 ug/ml) as the flurochrome; 0.9 ml of 0.1 M Tris - 0.1 M NaCl at pH 7.0. Stock solutions of E.B. (10 ug/ml) in distilled water were made the day before, refrigerated, protected from light, and diluted to 5 ug/ml just prior to use.

Fluorescence of test samples (lysate or gradient fractions) untreated or treated immediately upon fractionation of the gradients with a nuclease (DNase or RNase) was measured by substituting 0.1 ml of sample for 0.1 ml TEK at pH 7.3 in the blanking mixture. Lysate was normally diluted 1:10 with TEK at pH 7.3 buffer just prior to analysis to insure fluorescence measurement within the range of instrument sensitivity selected.

The viscosity of the 9790 strain lysate necessitated sonication for 2 min with an MSE 100 Watt ultrasonic disintegrator equipped with a microtip probe to affect more reproducible transfer of lysate samples for fluorometric analysis. Otherwise all samples were analysed as previously described. Sonication reportedly produces fragments which are biologically inactive but retain the double stranded helical structure necessary for intercalation of EB (Guild
et al., 1957; Doty et al., 1958). Lysate layered atop sucrose gradients however, was not presonicated.

The recommended concentration of E.B. for gradient derived samples based upon estimated nucleic acid content of 0.01 - 1.0 ug was 5 ug/ml. Similarly the recommended concentration for lysate samples was 10 ug/ml. A decision to use the lower concentration was based upon the additional observation of high background readings which interfered with analysis of gradient derived samples.

When samples to be assayed had been subjected to nuclease treatment (DNase or RNase), a blank containing 0.1 ml DNase or RNase to simulate the concentration added to the test sample was substituted for the TEK buffer of the usual blanking mixture. Readings were done at 25 C in the temperature stabilized sample holder with samples and reagents maintained in a water bath at 25 C. Nalgene tubes and plastic disposable pipets were used to avoid DNA association with glass surfaces. This precaution maximized reproducibility of the system.

Fluorescence measurement of untreated lysate and gradient fractions represented combined DNA and RNA contents and was expressed as fluorescence units (FLU) per 2 ml assay mixture. DNA content was determined by residual fluorescence in identical samples having undergone one hour treatment at 50 C with RNase (100 - 200 ug/ml) (the RNase having been heated at 85 C for 15 min prior to use to destroy DNases) or directly by DNase treatment for 30 min at 37 C (10 ug/ml for gradient fraction, 100 ug/ml for lysate samples, Tongur et al., 1968). Corollary experiments indicated that 100 ug RNase (preheated
as described) was sufficient to destroy 96% of 100 \(\mu\)g RNA in TEK buffer after one hour at 50°C. DNase treatment proved similarly effective.

Fluorescence units were subsequently converted to \(\mu\)g DNA or RNA by comparison with standard curves constructed from values obtained on the day of sample analysis. Either salmon sperm DNA (100 \(\mu\)g/ml) or calf thymus DNA diluted in TEK and covering a concentration range of 0 - 5 \(\mu\)g/2 ml assay mixture were used. The concentration of calf thymus DNA stock was determined by 260 absorbance in TEK buffer, pH 7.3; 0.4 units was taken as equivalent to 20 \(\mu\)g/ml DNA. Torula RNA (1 mg/ml) stock diluted serially in TEK covering a concentration range of 0 - 10 \(\mu\)g/2 ml assay mixture constituted the standard for RNA.

The \(\mu\)g of DNA or RNA obtained represented DNA and RNA content per 0.1 ml of sample. The values were multiplied by the appropriate volume factors to yield total DNA or RNA contents of the pooled fractions. To eliminate interference contributed by reagents, each sample tested was accompanied by a control sample from a corresponding control gradient fraction or control lysate, containing all but cellular material and having been processed in the same was as the test samples. Any fluorescence exhibited by these control samples was converted to equivalent \(\mu\)g of appropriate nucleic acid and used to correct fraction contents of corresponding test samples. Distribution of DNA or RNA among gradient fractions was expressed as \(\mu\)g DNA or RNA/total \(\mu\)g DNA or RNA added to the gradients.
D. **Electron Microscopy**

Portions of the M-band gradient fractions and lysates of each strain were diluted 1:10 with cold, saline citrate (0.15 M NaCl, 0.015 M sodium citrate) immediately after fractionation of the gradients and refrigerated overnight. Rationale for such treatment stemmed from the recommended procedure of Marmur (1961) for isolation and maintenance of biologically active DNA in the native state, suitable for transformation. Nalgene tubes minimized adherence of DNA to vessel walls.

Acetone pre-cleaned, formvar and carbon coated, 300 mesh copper grids, were routinely used and prepared as follows: coating was achieved by positioning cleaned grids atop a thin, formvar film floated onto a distilled water surface. Such a film was achieved by dipping a clean, grease-free (via ethanol washing) glass microscope slide (VWR slides were optimal) into a 0.25% solution of formvar resin dissolved in 1,2 dichloroethane (ethylene dichloride—EM grade—E.L. Pullam, Inc.). After allowing for solvent evaporation with slide held in a vertical position, a single-edged (grease free) razor blade was passed with a single, downward stroke along each edge and across the top of the film. This facilitated transfer to the water surface if the slide was positioned at a 45 degree angle of inclination to the water surface.

The film with a fixed grids, positioned with the aid of forceps, was removed from the water with lens paper laid onto the water surface over the grids and film. The coated grids were air dried, followed by carbon-coating (Ca. 150Å)
to increase stability and resolution under vacuum. They were stored in a dessicator until used, normally within one or two days, and never after more than one week. Remaining formvar solution and ethylene dichloride were protected from moisture when not in use via storage in the presence of Drierite (CaSO₄).

For examination of samples for ultrastructural composition, one drop of diluted sample was applied via the wide-bore end of a 1 ml plastic disposable pipet and allowed to settle for one min. Excess sample fluid was removed via filter paper held at the edge of the grid. After air drying, sample grids were rotary shadowed at a low angle (less than 10°) to deposit ca. 25-50 Å of platinum-palladium onto the grid surface. A Philips EM 200 electron microscope at 40 kV and 20 um objective aperture were used to examine the samples thus prepared. Magnification calibration at 40 kV was checked and adjusted with an "image magnification ruler" (E.F. Fullam Inc.) grated in 10,000ths.

Ultrastructural measurements were determined on final prints with a PEAK optical comparator (7x lupe) equipped with a 0.1 mm micrometer scale, and are reported corrected for print magnification unless otherwise specified.

E. Lipids

Extraction

Organic solvents and water were redistilled in a glass still; glassware was acid-washed (see Appendix for more detailed procedures). Lipid extractions were carried out according to the method of Kanfer and Kennedy (Scheme 2).
Scheme 2

LIPID EXTRACTION

Sample

BSA added (final concentration 100 ug/ml)
TCA added (final concentration 5 % w/v)

Centrifuge

TCA Precipitated Fraction

Resuspend in methanol
(8 ml per equivalent 100 ml original cell culture)

Warm 15 min, 55 C in a closed vessel

Cool to ambient temperature

Add CHCl₃ at double the volume of MeOH added

Store overnight at ambient temperature

CHCl₃ : MeOH Extracted Sample

Filter through glass wool

Filtrate

Equilibrate against an equal volume of 2 M KCl

Aqueous Phase (removed)  Organic Phase

Re-equilibrate against 2 M KCl

Wash with distilled H₂O

Lipid Extract
TCA precipitates were collected by centrifugation at 0°C (12,000 x g, 15 min, in a Sorvall RC2 centrifuge). Bovine serum was added prior to TCA to enhance precipitation of lipid. Samples to be extracted, whole cells, lysates, and M-band fractions were then TCA precipitated by addition of sufficient 50% TCA to give a final concentration of 5% (w/v). After chloroform addition the samples were mixed by vortexing for 2 min to facilitate interaction between solvent and lipid material. Care must be taken to maintain a chloroform:methanol ratio of 2:1 and volumes were adjusted by appropriate additions when necessary. The methanol:chloroform extraction mixtures were allowed to stand at room temperature overnight, filtered through a glass wool plug, and the filtrate equilibrated against an equal volume of 2 M KCl following vortexing for 2 min in a tightly stoppered all glass volumetric flask. Upon achievement of an adequate biphasic system, the upper aqueous phase was drawn off with a Pasteur pipet, the organic phase washed twice more with 2 M KCl and finally with distilled water.

In the event of interfacial fluff formation, as occurred with some whole cell fractions after the second or third salt solution wash, samples were quantitatively transferred to a screw-capped test tube, the volumetric flask rinsed with a small volume of chloroform:methanol (2:1 v/v) and the rinse included along with the extract. Centrifugation followed at (1,000 x g, 20 min) in an IEC international Centrifuge (Model UV0). This reduced the interfacial fluff to a thin film, usually attached to the wall of the tube and allowed for easy
removal of the aqueous phase. The organic phase was further subjected to the washing procedure, if necessary.

The washed, chloroform extracts were evaporated in a stream of nitrogen either to dryness, or if traces of water were present resulting in an aqueous emulsion, small amounts of benzene:absolute ethanol were added. The ethanol entraps water droplets which are subsequently evaporated along with the ethanol by a stream of nitrogen; the benzene is then removed by lyophilization. (Wuthier, 1966). Alternatively samples were frozen at -70 C and lyphilized.

Storage

Lyophilized lipid extracts were resuspended in known amounts of chloroform and portions not immediately used were stored in scintillation vial inserts (Rochester Scientific) at -70 C. These inserts were the best available storage vessels as they were not adversely effected by low storage temperature nor were the polyethylene caps distorted after long periods of contact with chloroform. Radioactively labeled extracts, were chromatographed on the same day that the lipid extracts were resuspended. Samples frozen at 0 to -15 C have been reported to survive storage hydrolysis and peroxidation in chloroform:methanol (2:1) for several weeks, while addition of an antioxidant such as butylated hydroxytoluene (BHT) at a concentration of 0.05 % insured storage time for periods of one to two years at -40° C or lower. Within the time span utilized no differences were observed in lipid extracts in the presence or absence of antioxidant.
Thin Layer Chromatography (TLC) of Washed Lipid Extracts

Silica gel 60 precoated TLC plates (E. Merck), 0.25 mm layer thickness without fluorescent indicator were purchased from Brinkmann as Siliplate 22 and were used for chromatographic analyses. Plates activated at 110°C for 1.5 h were stored in a bell jar containing Drierite (W.A. Hammond Drierite Co.). No significant differences in chromatographic patterns were observed whether activated or nonactivated plates were used.

Three solvent systems were used to develop washed lipid extracts. Chloroform:methanol:water (65:25:4, v/v)—a one step development system. Acetone-light petroleum (1:3, v/v), chloroform-methanol-acetic acid-water (80:13:8:0.03, v/v)—a two step development system in one direction. Chloroform-methanol-water (70:25:4, v/v), chloroform-methanol-7 M ammonium hydroxide (60:35:5, v/v) a two step development system in two directions.

Pre-activated plates to be developed in the single solvent system were pre-run in the solvent prior to application of the samples to remove impurities in the plate. One plate from each batch was routinely subjected to such treatment and exposed to iodine vapors to detect any organic compounds below the solvent front. In all plates so tested, no organic impurities remained after pre-run.

Samples of lipid extracts as small spots 2 mm or less in diameter were applied to the silica gel plates with Ziptrol microdispenser tubes (Helena Laboratories, Beaumont, Texas) calibrated to deliver 1-5 µl with an assured accuracy of ±%. Evaporation of solvent was facilitated by a hand-held hair...
drier on the manufacturer's cool setting. When multiple samples were to be simultaneously chromatographed, spots were applied no less than 1.5 cm apart and 2 cm from either edge. In cases of two dimensional development only one sample as a single spot was applied per plate and the plates were allowed to air dry between dimensions.

Plates were developed in one of the above solvent systems in a glass chromatography tank lined with Whatman #1 filter paper on three sides and saturated overnight with at least 100 ml of the appropriate solvent system. The resulting chromatograms were air-dried and the separated components were detected by use of one or more of the following methods:

1) Iodine vapors. Iodine crystals placed in a large dessicator generated iodine vapors. A dry, developed chromatogram was then introduced. After a few minutes, organic compounds appeared as yellow to brown spots, with P-lipids staining more intensely than glycolipids (Christie, 1973). Since iodine does not interfere with most subsequent analyses and is readily sublimed it could be used even if subsequent detection sprays or other analyses were contemplated. However, since the iodine so readily sublimes, care was taken to mark resulting spots immediately after treatment. A modified version utilizing a Pasteur pipet filled with iodine crystals to which positive pressure was applied, served to direct vapors at limited portions of the chromatograms when iodine vapors would interfere with subsequent analyses.

2) Ninhydrin spray. A stock solution of 0.2 % ninhydrin in redistilled acetone was mixed 1:1 with redistilled
water just prior to use. Concentrated acetic acid was added to give a final concentration of 1% (v/v). Chromatograms were uniformly sprayed with a fine mist until the entire surface was slightly moistened. The plate was heated at 100° C in a water saturated oven for 5-10 min and red-violet spots recorded. Ninhydrin reaction detects free amino groups and also ε-amino acids, other types of amino acids and amines (Losden et al., 1939).

3) Hanes reagent. The spray reagent was prepared as follows: 0.5 g of ammonium molybdate was dissolved in 5 ml of water and 1.5 ml of 25% (v/v) HCl and 2.5 ml of 70% (v/v) perchloric acid were added to the solution. After cooling, the solution was made up to 50 ml with acetone and allowed to stand one day before use. Chromatograms were sprayed with the reagent, dried and exposed to UV light. Phosphate esters develop as blue spots. Ninhydrin spray was normally applied immediately after iodine sublimation. Spraying with Hanes reagent after ninhydrin led to an enhanced and more rapid reaction, often giving blue spots before the plate was subjected to UV light.

4) Detection of vicinal-OH groups. Developed plates were sprayed with the following sequence of agents with 5 min of heating at 110° C between each agent:

- 3% sodium metaperiodate in 90% cold acetone
- 0.2% o-tolidine plus 0.6% acetic acid in 95% acetone

Glycolipids appear as yellow spots on a grey-green background; PG also reacts in a similar fashion.
Thin Layer Chromatography of Standards

Chromatographically pure phospholipid standard compounds phosphatidylethanol-amine \( \text{PE} \) (bacterial), phosphatidylglycerol \( \text{PG} \), phosphatidylserine \( \text{PS} \) (ex. bovine brain, A grade), phosphatidic acid \( \text{PA} \), diphosphatidylglycerol \( \text{di PG} \) (cardiolipin) were used. For commercial sources see Appendix.

A composite which included each of the above standards was made just prior to application to the TLC plate at appropriate concentrations, predetermined to give adequate detection and separation in solvent systems previously described, and were included on each plate to be chromatographed unless otherwise specified.

Individual standards were stored either in the crystalline state or dissolved in chloroform or chloroform: methanol (2:1, v/v) in 1/2 dram glass vials with foil lined screw cap tops at -70 C in the presence of dessicant. Each was checked periodically to guard against degradation or transesterification especially those stored in chloroform: methanol (2:1, v/v).

Quantitation of Phospholipids

See Radioactivity Counting:

Enterococcus 564P, 615M, 9790
CHAPTER III

RESULTS

A. M-bands - *E. coli* B/r(w) and *B. megaterium*

Established methodologies applied to *E. coli* B/r(w) and *B. megaterium* to insure successful manipulation of the technique gave comparable complexes in the M-band fraction to those isolated by the original investigation. Three criteria must have been met prior to any further content analysis: 1) appearance of a white, opalescent, crystalline band at the interface of the two sucrose concentrations within the sucrose block gradients (Fig. 1) termed the M-band; 2) absence of any visible pellet after ultra-centrifugation, indicating that lysis had occurred in the presence of the detergent Sarkosyl (Earhart et al., 1968; Schaechter, personal communication); 3) the majority of the DNA and a portion of the membrane found associated with the M-band fraction.

Results (Table 3) of such experiments with cells grown in complex media indicate that criteria 1 and 2 were met by M-band gradients of both organisms. No effect on the first two criteria were observed when slight variations occurred in cell numbers (10^8-10^9 organisms/ml), in lysozyme final concentration at time of addition (200-300 ug/ml for *E. coli*; 89-111 ug/ml for *B. megaterium*), in Sarkosyl concentration (0.2-0.45; 0.08-0.8), Mg++ concentration (10-15 mM), or mode of lysozyme and/or Sarkosyl addition. Compliance with criterion 3 was determined in terms of 1)
Fig. 1. Typical M-band gradients after centrifugation. On the left, a test gradient of S. faecalis 9790; M-band is seen as a white band in the middle of the gradient tubes. On the right, a control gradient run simultaneously is identical in content except for lack of cellular material. Upper white band is meniscus with no crystalline or cellular material evident.
Table 3. Summary of M-band Parameters for Verification of Technique\textsuperscript{a} with \textit{E. coli} B/r(w) and \textit{B. megaterium}

\begin{tabular}{|c|c|c|c|c|c|c|c|}
\hline
Organism & Concentration prior to further additions & & & & & & \\
 & O.D. at harvest & CFU applied\textsuperscript{b} per gradient & lysozyme (\textmu g/ml) & Sarkosyl (\%) & \textsuperscript{Mg\textsuperscript{++}} (mM) & Sarkosyl treatment\textsuperscript{c} & Presence of M-band Pellet \\
\hline
\textit{E. coli} B/r(w) & .28 & $3.3 \times 10^8$ & 200 & .45 & 15 & on & + - \\
 & .37 & $2.5 \times 10^9$ & 333 & .20 & 11 & off & + - \\
\hline
\textit{B. megaterium} & .12 & $2.0 \times 10^9$ & 111 & .08 & 10 & on & + - \\
 & .14 & $4.0 \times 10^8$ & 89 & .80 & 14 & off & + - \\
\hline
\end{tabular}

\textsuperscript{a} In terms of compliance with criteria 1 and 2 (see text)

\textsuperscript{b} Determined by pour-plate technique in duplicate on T-Soy Agar

\textsuperscript{c} Sarkosyl treatment was carried out either on or off (prior to addition of sample) sucrose gradients
the percent of methyl-$^3$H thymidine recovered in the M-band isolated from uniformly labelled cells; 2) the distribution of butanol extractable, $^{32}$P label in the M-band gradient fractions (see Tremblay et al., 1969, Table 1, legend 5).

Table 4 shows that 74% of the methyl-$^3$H thymidine label was recovered in the M-band from *E. coli* prepared by off-gradient lysis and mild shearing (3-4 pipette passages) from cells harvested from phage broth supplemented with 0.125 uCi/ml radioactive label. Additional pipeting reduced the percentage of the label recovered to 49%. This was not unexpected, as McIntosh and Earhart, 1975, observed as much as 82% loss of DNA from M-bands of *E. coli* B upon shearing 4X through a 22 gauge needle.

Distribution of membrane in M-band gradient fractions was determined by the amount of butanol-extractable (lipid) $^{32}$P labelled material in the various fractions of M-band gradients. Such extracts prepared from *E. megaterium* cells, uniformly labelled with $^{32}$P (8 uCi/ml) during growth in modified C media without sucrose (Appendix), indicated that the distribution of membrane was dependent upon the amount of contact time allowed between Sarkosyl-Mg crystals and cellular material. These results were in agreement with those reported by Ballesta et al., 1972. Table 5 shows that 10 min contact time results in the appearance of a slight pellet, indicating incomplete lysis of the cells in the presence of Sarkosyl, and 47% of the butanol extractable $^{32}$P label in the M-band, whereas 30 min contact time resulted in 74% of the extractable labelled material in the M-band and
Table 4. Distribution of methyl-$^3$H thymidine in M-band Gradient Fractions of E. coli B/r(w)

<table>
<thead>
<tr>
<th>M-band gradient fraction$^{a,b}$</th>
<th>Percent of total methyl-$^3$H thymidine recovered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>unsheared</td>
</tr>
<tr>
<td>Top</td>
<td>25.0</td>
</tr>
<tr>
<td>M-band</td>
<td>74.0</td>
</tr>
<tr>
<td>Bottom</td>
<td>0.8</td>
</tr>
</tbody>
</table>

$^a$ Off gradient Sarkosyl treated sample

$^b$ Pellet fraction not determined since no visible pellet was detected

$^c$ Following Sarkosyl addition, samples were sheared by pipeting 3-4 times prior to addition to gradients
Table 5. Distribution of Butanol Extractable, $^{32}\text{P}$-labelled Material in M-band Gradient Fractions of B. megaterium after 10 and 30 min Contact Time Between Sarkosyl, Mg$^{++}$, and Sensitized Cells

<table>
<thead>
<tr>
<th>M-band gradient fraction</th>
<th>Percent of total $^{32}\text{P}$ recovered</th>
<th>10 min</th>
<th>30 min</th>
<th>Percent of total $^{32}\text{P}$ added</th>
<th>10 min</th>
<th>30 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>Top</td>
<td></td>
<td>2.4</td>
<td>21.0</td>
<td>1.0</td>
<td>11.0</td>
<td></td>
</tr>
<tr>
<td>M-band</td>
<td></td>
<td>47.0</td>
<td>74.0</td>
<td>19.0</td>
<td>37.0</td>
<td></td>
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<tr>
<td>Bottom</td>
<td></td>
<td>3.8</td>
<td>2.5</td>
<td>1.6</td>
<td>1.3</td>
<td></td>
</tr>
<tr>
<td>Pellet</td>
<td></td>
<td>46.0$^a$</td>
<td>2.2</td>
<td>19.0</td>
<td>1.0</td>
<td></td>
</tr>
</tbody>
</table>

$^a$Slight pellet seen after centrifugation
no pellet. Comparison on the basis of % of added, butanol extractable, $^{32}$P labelled material indicates that 19 % of the membrane associated with the M-band after 10 min and 37 % after 30 min, was more in line with reported literature values for this organism (Tremblay et al., 1969).

B. Development of a Lytic Procedure for Group D Streptococci to use in Conjunction with the M-band Technique: Streptococcus faecalis var. liquefaciens

Attempts to form M-bands with the group D streptococcal strains by direct application of either the method described for E. coli B/r(w) or B. megaterium was unsuccessful. The E. coli method applied to S. faecalis var. liquefaciens resulted in the formation of pellets as well as M-bands when cells grown in phage broth were subjected to the M-banding process. The formation of pellets, plus evidence that the same cells subjected to the lytic procedure apart from the gradients did not clear upon the addition of Sarkosyl, indicated the inadequacy of the lytic procedure in this case. The 9790 strain of S. faecalis (S. faecium), grown in the media of Metcalf and Deibel (1969) to exponential phase and prepared for M-banding by both the E. coli method and the B. megaterium method showed slightly greater lysis using the B. megaterium method, i.e., fewer counts recovered in and smaller size of the observed pellet, but with the majority of the radioactivity still in the pellet fraction.

Reports by Metcalf and Deibel (1969) and others concerning variation in sensitivity to lysozyme expressed by
enterococci from various growth media, and other treatments led to the testing of various growth media and physiological age on the susceptibility of the resulting cell population to lysozyme and Sarkosyl.

*Streptococcus liquefaciens* was chosen initially since it is generally the most resistant of the enterococci to lysozyme, and if it could be lysed and analyzed, the other species should yield to the same treatment (an erroneous deduction as events subsequently proved).

Cultures grown in semi-synthetic medium (Appendix), compared with those grown in the same medium with lowered phosphate concentration, or the latter with varied concentrations of vitamin-free casamino acid supplements, showed reduced growth yield, with reductions becoming more dramatic as the casamino acid concentration was decreased (data not shown). However, response to Sarkosyl was not affected by any of the above parameters provided lysozyme treatment for at least 10 min at 37 C preceded the addition of the detergent (Tables 6 and 7).

Lysozyme alone did not cause lysis of this strain and no loss of optical density was observed in control cells suspended in water over the time period examined. In addition, sucrose did not seem to significantly effect lysozyme activity providing it was added after lysozyme nor was it capable of inhibiting the disruptive action of Sarkosyl (Table 6).

In all cases where lysozyme was added to water-suspended cells, an increase in optical density as seen by Metcalf and Deibel (1969) for certain *S. faecium* and *S. faecalis* strains
Table 6. Susceptibility of *S. faecalis* var. *liquefaciens* Grown in Semi-synthetic Medium\(^a\) to Sarkosyl Lysis Following Various Pretreatments at 37 C

<table>
<thead>
<tr>
<th>Reagent &amp; order of addition</th>
<th>0 min</th>
<th>10 min</th>
<th>0 min</th>
<th>10 min</th>
<th>0 min</th>
<th>30 min</th>
<th>60 min</th>
</tr>
</thead>
<tbody>
<tr>
<td>lysozyme(^c)</td>
<td>10% sucrose in water</td>
<td>0.75</td>
<td>1.90</td>
<td>2.00</td>
<td>0.64</td>
<td>1.80</td>
<td>1.80</td>
</tr>
<tr>
<td>lysozyme</td>
<td>H(_2)O</td>
<td>Sarkosyl</td>
<td>0.75</td>
<td>1.90</td>
<td>2.00</td>
<td>0.64</td>
<td>1.80</td>
</tr>
<tr>
<td>H(_2)O</td>
<td>10% sucrose in water</td>
<td>Sarkosyl</td>
<td>0.30(^d)</td>
<td>0.30</td>
<td>0.33</td>
<td>0.25</td>
<td>0.24</td>
</tr>
<tr>
<td>H(_2)O</td>
<td>H(_2)O</td>
<td>Sarkosyl</td>
<td>0.75</td>
<td>0.75</td>
<td>0.75</td>
<td>0.64</td>
<td>0.62</td>
</tr>
</tbody>
</table>

\(^a\) See Appendix for media composition

\(^b\) O.D. expected for dilutions immediately upon addition of the appropriate reagent, assuming compliance with Beer's law; O.D. at harvest 0.36, generation time 32 min. Cells washed twice with cold distilled H\(_2\)O, resuspended to O.D. 1.5

\(^c\) Lysozyme final concentration 200 ug/ml; Sarkosyl concentration 0.35%

\(^d\) Cell resuspended to O.D. 0.60
Table 7. Effect of Growth with Reduced Phosphate and Increasing Concentrations of Vitamin-free Casamino Acids on Susceptibility of *S. faecalis* var. *liquefaciens* to Lysozyme and Sarkosyl

<table>
<thead>
<tr>
<th>Reagent &amp; order of addition</th>
<th>A&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th></th>
<th>B&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th>Absorbance (260 nm)&lt;sup&gt;g&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>expected&lt;sup&gt;e&lt;/sup&gt;</td>
<td>observed</td>
<td>expected</td>
<td>observed</td>
<td></td>
<td>expected</td>
<td>observed</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>lysozyme</td>
<td>0 min&lt;sup&gt;c&lt;/sup&gt; 10 min</td>
<td></td>
<td>0 min&lt;sup&gt;d&lt;/sup&gt; 10 min</td>
<td></td>
<td>0 min&lt;sup&gt;c&lt;/sup&gt; 10 min</td>
<td></td>
<td>0 min 10 min</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.28</td>
<td>0.45</td>
<td>0.39</td>
<td>0.02</td>
<td>0.02</td>
<td>0.29</td>
<td>0.44</td>
<td>0.38</td>
<td>0.01</td>
<td>0.01</td>
</tr>
<tr>
<td></td>
<td>0.29</td>
<td>0.30</td>
<td>0.26</td>
<td>0.26</td>
<td>0.26</td>
<td>0.29</td>
<td>0.30</td>
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<td>0.26</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
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<td>0.25</td>
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<td>0.26</td>
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<td>0.30</td>
<td>0.26</td>
<td>0.27</td>
<td>0.26</td>
</tr>
<tr>
<td>H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>Sarkosyl</td>
<td></td>
<td></td>
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<td></td>
<td></td>
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<td></td>
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<td>0.28</td>
<td>0.45</td>
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<td>0.02</td>
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<td>0.26</td>
<td>0.29</td>
<td>0.30</td>
<td>0.27</td>
<td>0.26</td>
<td>0.26</td>
</tr>
<tr>
<td></td>
<td>0.32</td>
<td>0.29</td>
<td>0.25</td>
<td>0.26</td>
<td>0.26</td>
<td>0.30</td>
<td>0.30</td>
<td>0.26</td>
<td>0.27</td>
<td>0.26</td>
</tr>
</tbody>
</table>

<sup>a</sup> See Appendix for media composition; phosphate reduced to 17 μg/ml

<sup>b</sup> A-late log phase cells, B-mid log phase cells; cells grown at 37 °C in semi-synthetic media with reduced phosphate and supplemented with 0.8 % and 1.6 % respectively; O. D. at harvest 0.20, washed 3 x with distilled water, resuspended to 10 ml

<sup>c</sup> Optical density determined immediately upon addition of the appropriate reagent; incubation with reagents at 37 °C

<sup>d</sup> 0 min indicates 0. D. immediately after addition of variable 2; the sample having been pretreated for 10 min with variable 1

<sup>e</sup> Expected values for reagent addition induced dilution at 0 min assuming compliance with Beer's law

<sup>f</sup> Lysozyme final concentration 200 μg/ml; Sarkosyl final concentration 0.35 %

<sup>g</sup> Absorbance determined on supernates obtained from mixtures A and B (see b above) centrifuged 10 min after addition of the last reagent
was observed with the *liquefaciens* strain. Ten minutes after addition of Sarkosyl, however, all lysozyme treated cells regardless of growth conditions or physiological age had achieved better than 97% loss in optical density. Neither lysozyme, nor detergent treatment alone was sufficient to effect a similar lytic response.

Numerous M-banding attempts indicated that sufficient lytic response was achieved to allow formation of M-bands from this organism when Sarkosyl concentration was reduced to 0.1% and Mg++ at 16 mM was employed. Table 8 shows that M-bands formed comply with criterion 3, as the majority of the DNA in the M-band fraction and that no difference in distribution is observed as a result of varied conditions of treatment prior to Sarkosyl addition. It might be argued, however, that maintenance of 0°C temperature after 10 min incubation with lysozyme helps to minimize shearing and/or endonuclease activity (Column 3).

C. Development of a Lytic Procedure for Group D Streptococci to use in Conjunction with the M-band Technique: *S. faecalis* (faecium) 9790 and UNH strains

The method developed for preparation of sensitized cells of *S. faecalis* var. *liquefaciens* failed to work with both stationary and exponential phase cells of *S. faecalis* 9790. Stationary phase cell suspensions showed no loss in optical density, even after extended incubation (up to one hour at 37°C) in the presence of the detergent, Sarkosyl. Exponential phase cells grown under similar conditions failed to form a
Table 8. Distribution of methyl-\(^3\)H thymidine Labelled DNA in Gradient Fractions Following M-band Analysis of *S. faecalis* var. *liquefaciens*.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Radioactivity (CPM fraction/total CPM recovered per gradient)</th>
<th>% methyl-(^3)H thymidine recovered / gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1(^b)</td>
<td>2(^c)</td>
</tr>
<tr>
<td>Top</td>
<td>3191/27241</td>
<td>3514/19386</td>
</tr>
<tr>
<td>M-band</td>
<td>23866/27241</td>
<td>15782/19386</td>
</tr>
<tr>
<td>Bottom</td>
<td>186/27241</td>
<td>90/19386</td>
</tr>
</tbody>
</table>

\(^a\) Cells grown in phage broth

\(^b\) 10 % sucrose in distilled water, 10 min, 37 °C following lysozyme treatment

\(^c\) 10 % sucrose in 0.125 M Tris-1.35 mg/ml EDTA, pH 8.1, 10 min, 37 °C following lysozyme treatment

\(^d\) 10 % sucrose in 0.125 M Tris-1.35 mg/ml EDTA, pH 8.1, 10 min, 0 °C following lysozyme treatment
pellet when centrifuged. This indicated that autolysis had occurred.

Although the above method was acceptable for the formation of M-bands from *S. faecalis* var. *liquefaciens*, its inapplicability to other enterococcus strains necessitated the testing of other methods to facilitate lysis and allow the study of M-bands (DNA-membrane complexes) from several strains of group D streptococci.

The method of Clewell *et al.* (1974) developed to isolate plasmids from *S. faecalis* utilizing Sarkosyl as the lytic inducing agent, was tested for its ability to lyse a series of enterococcus strains and proved successful.

Table 9 indicates that when applied in its original form, the technique resulted in a total lack of response by the *liquefaciens* strain. In direct contrast, the 9790 strain of *S. faecalis* (*faecium*) exhibited an immediate response with increased viscosity and complete clearing. A considerable degree of variation in response was noted with the *fecium* and *fecium*-like (UNH enterococcal culture collection) strains.

Log phase cells of the motile strains tested tended to compare most favorably with the 9790 strain but only after 1 to 4 h of incubation with the detergent. Similar observations were made with the pigmented and pigmented-motile strains. However, strain 564P harvested from stationary phase of growth gave a substantial response in 1 h.

Alteration of the procedure by addition of EDTA, pH 6.25 rather than pH 8.0 reduced the time necessary for complete
<table>
<thead>
<tr>
<th>Strain</th>
<th>O.D. at harvest</th>
<th>Phase of growth</th>
<th>CFU/ml treated</th>
<th>Viscosity</th>
<th>Clearing</th>
<th>Time (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. faecalis var. liquerifaciens</td>
<td>0.28</td>
<td>early stationary</td>
<td>N. D.</td>
<td>—</td>
<td>—</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.27</td>
<td>log</td>
<td>1.7 x 10^10</td>
<td>—</td>
<td>—</td>
<td>1, 4</td>
</tr>
<tr>
<td></td>
<td>0.24</td>
<td>early stationary</td>
<td>N. D.</td>
<td>—</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.25</td>
<td>log</td>
<td>8.9 x 10^9</td>
<td>+</td>
<td>1, 4</td>
<td></td>
</tr>
<tr>
<td>S. faecium</td>
<td>0.20</td>
<td>log</td>
<td>N. D.</td>
<td>++</td>
<td>++</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>0.12</td>
<td>early log</td>
<td>3.6 x 10^9</td>
<td>++</td>
<td>+++</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>0.19</td>
<td>log</td>
<td>N. D.</td>
<td>+</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.08</td>
<td>late log</td>
<td>3.5 x 10^9</td>
<td>++</td>
<td>++</td>
<td>4</td>
</tr>
<tr>
<td>617 M</td>
<td>0.4</td>
<td>stationary</td>
<td>2.2 x 10^10</td>
<td>++</td>
<td>++</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.41</td>
<td>stationary</td>
<td>2.2 x 10^10</td>
<td>++</td>
<td>++</td>
<td>4</td>
</tr>
<tr>
<td>564 P</td>
<td>0.17</td>
<td>log</td>
<td>4.8 x 10^9</td>
<td>—</td>
<td>+</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>0.17</td>
<td>log</td>
<td>4.8 x 10^9</td>
<td>—</td>
<td>++</td>
<td>4</td>
</tr>
<tr>
<td>9790</td>
<td>0.29</td>
<td>late log</td>
<td>2.2 x 10^10</td>
<td>++</td>
<td>+++</td>
<td>0, 1</td>
</tr>
</tbody>
</table>


b Colony forming units/ml suspended in 25% sucrose-0.05 M Tris, pH 8.0

c Designations: — non-viscous; +, low viscosity; ++, viscous; +++ extremely viscous, can be removed as a single unit; determined via degree of fluidity upon release from a 1 ml pipet (Falcon) plastic.

d Designations: — no clearing observed; sl, slight clearing; +, noticeably clearer, less dense but incomplete clearing; ++, almost completely clear, clearer than at 1 h if a 4 h observation is made, still not completely clear; +++ completely clear.

e Not determined.

f Representative of two separate determinations, each monitored at 1 and 4 h.
clearing of all but the *liquefaciens* strains from 1-4 h to 5-10 min.

Strains 564P, 615M and 9790 were chosen for further study since each had a distinguishing characteristic: 1) strain 564P had a triterpenoid pigment and was physiologically most closely related to *S. faecalis* (Table 1); 2) strain 615M exhibited motility; 3) strain 9790 had an extensive data base in the literature. In addition, all three showed maximal response to the modified Clewell procedure for their respective strain type and all were free of the nitrogen containing P-lipids, phosphatidylethanolamine.

**Evaluation of the Lytic Response of *S. faecium* 9790 to Versions of the Procedure of Clewell et al, 1974**

The immediate response of the 9790 strain to this modified procedure and reports of autoplasting ability of this organism (Joseph and Shockman, 1974) led to a more extensive and quantitative investigation of the effect of various aspects of the lytic procedure on this organism.

Twenty-five ml of exponential phase culture were harvested and subjected to the lytic procedure. The same sequence of additions was followed, substituting the reagent solvent for the reagent when the effect of eliminating a specific reagent was being tested. At 5 min intervals after each addition, 0.1 ml sample was removed from one of six duplicate tubes for each reagent combination set to eliminate drastic alterations in the proportion of CFU treated, to reagent added at succeeding steps. A 1:10 with Sorensen's PO₄ buffer
(0.01 M PO₄, 0.15 M NaCl at pH 7.0) ensured optical density readings within the range of sensitivity of the spectrophotometer. The necessity of each reagent addition and the effect of its presence on the osmotic fragility and Sarkosyl susceptibility of the cells was determined by monitoring changes in O.D. (Table 10).

Irrespective of other additions, lysozyme, 20 min after addition, rendered cells 84-98% sensitive to Sarkosyl while effects on osmotic fragility seemed to vary depending upon additions subsequent to lysozyme. Lysozyme treatment alone resulted in 50% osmotic fragility as soon as 10 min after reagent addition (line 6). Cells not treated with lysozyme were found to be resistant to both Sarkosyl and Pronase, and were osmotically stable (data not shown).

EDTA addition diminished osmotic fragility (compare line 6 with 3 and 4 and line 5 with 1 and 2). Pronase addition 10 min after lysozyme resulted in an increase in fragility to 85% as opposed to 54% for lysozyme treatment only. Lysozyme-EDTA treated cells with subsequent pronase addition were also more osmotically fragile (compare lines 1 and 2 with lines 3 and 4).

Although it could be argued based on the optical density data that EDTA or pronase could be eliminated from the lytic procedure, such action resulted in incomplete lysis of both of the other enterococcal strains chosen for this study. This conclusion was based upon incomplete clearing and residual turbidity even after 1 h treatment in the presence of the detergent Sarkosyl.
<table>
<thead>
<tr>
<th>Reagent Combination Set</th>
<th>% O.D.(^c)</th>
<th>% O.D.(^c)</th>
<th>% O.D.(^c)</th>
<th>% O.D.(^c)</th>
<th>% O.D.(^c)</th>
<th>% O.D.(^c)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>sucrose + lysozyme</td>
<td>EDTA</td>
<td>H(_2)O</td>
<td>Pronase</td>
<td>TES</td>
<td>Sarkosyl</td>
</tr>
<tr>
<td>1</td>
<td>aver. -45</td>
<td>-84</td>
<td>-92</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>aver. +5</td>
<td>-98</td>
<td>+6</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>average +11.4</td>
<td>aver. -25</td>
<td>-97</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>aver. -54</td>
<td>-85</td>
<td>-94</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>aver. -54</td>
<td>-85</td>
<td>-94</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>aver. -54</td>
<td>-85</td>
<td>-94</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) See Materials and Methods

\(^b\) Each combination set consists of all reagents where the combination set number is included within the limits of the bars for that reagent addition; bars indicate inclusion of the column designated reagent 5 min after addition of the previous reagent. Thus reagent combination set 1 includes sequential additions at 5 min intervals of sucrose + lysozyme, EDTA, pronase, and Sarkosyl. At points where more than one reagent set was identical in composition to another, % O.D. was reported as an average of all such sets. All sets contained equivalent and identically prepared cells, although not all assays were performed at the same time.

\(^c\) O.D.\(_{o}\) - O.D.\(_{e}\) x 100 O.D.\(_{o}\) = optical density observed

\(^d\) + = increase in O.D. % O.D. = O.D.\(_{o}\) - O.D.\(_{e}\) x 100 O.D.\(_{o}\) = optical density observed in the absence of lytic response

\(^e\) - = decrease in O.D. compared with expected values
In conjunction with the optical density determinations and to test for cell clumping (aggregation), shrinkage, or other morphological changes reportedly capable of prejudicing optical density determinations, samples at each interval of the lytic procedure of lysozyme and non-lysozyme treated cells were examined by phase microscopy. The observations (Table 11) essentially substantiated optical density data, as positive changes in optical density of lysozyme treated cells were seen to be accompanied by clumping, whereas negative changes were accompanied by either shrinkage, decreased clumping or loss of cell integrity.

D. M-bands of Group D Streptococcal Strains 564 P, 615 M, and 9790

Lysates prepared by the modified Clewell procedure were subjected to M-band gradient fractionation (Scheme 1) to determine if this lytic procedure was compatible with the M-band technique. All three strains exhibited visibly detectable M-bands, identical to that shown in Fig. 1. Fluorometric analysis of these M-band fractions was employed to establish through DNA and RNA content that such fractions did in fact contain the DNA-membrane complex sought.

**Fluorometry—DNA and RNA Content Analysis of M-band Fractions**

Figure 2 illustrates response curves for DNA and RNA determinations by the fluorometric method. Each line represents the regression line obtained from 3 determinations. Each one was typical of the types of standards used for
Table 11. Comparison of the Effects of Lytic Procedure Reagents upon S. faecalis 9790 during Lysate Formation in Terms of Optical Density\textsuperscript{a}, Viability\textsuperscript{b}, and Cell Integrity\textsuperscript{c}

<table>
<thead>
<tr>
<th>Treatment\textsuperscript{d}</th>
<th>% change O.D.</th>
<th>CFU</th>
<th>% survivors</th>
<th>Phase Microscopic Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>lysozyme only</td>
<td>+11.4</td>
<td>18</td>
<td></td>
<td>slight loss in refractility</td>
</tr>
<tr>
<td>lysozyme + EDTA</td>
<td>+ 5.0</td>
<td>16</td>
<td></td>
<td>clumping</td>
</tr>
<tr>
<td>lysozyme + pronase</td>
<td>-45.0</td>
<td>12</td>
<td></td>
<td>decrease size, less clumping</td>
</tr>
<tr>
<td>lysozyme + Sarkosyl</td>
<td>-84.0</td>
<td>1</td>
<td></td>
<td>loss of cell integrity</td>
</tr>
<tr>
<td>TES</td>
<td>+12</td>
<td>52</td>
<td></td>
<td></td>
</tr>
<tr>
<td>EDTA</td>
<td>+33</td>
<td>127</td>
<td></td>
<td>no perceptible changes</td>
</tr>
<tr>
<td>pronase</td>
<td>+15</td>
<td>96</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sarkosyl</td>
<td>+25</td>
<td>88</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\textsuperscript{a} Optical density changes were determined as given in legend Table 12

\textsuperscript{b} Viability was measured as \% colony forming units (CFU) surviving

\textsuperscript{c} As observed by phase microscopy

\textsuperscript{d} See Table 11 for experimental conditions
Fig. 2. Response curves for fluorometric assay of DNA and RNA standards using ethidium bromide as the fluorochrome.
determinations of the three enterococcal strain samples and included determinations with both salmon sperm and calf thymus DNA. As reported by Prasad et al. (1972) the fluorescence of standards for both DNA and RNA were proportionally related to concentration. However, the slope of the lines changed slightly from one day to the next. Variance throughout the range tested, however, was never more than ± 7% and fluorescence of DNA was routinely read below the 20 unit fluorescence level to avoid a slight deviation from linearity observed at higher concentrations of DNA. Samples showing fluorescence, levels higher than 20 units as with most lysate samples, were diluted with TEK buffer to fall within the proper range and fluorescence redetermined.

No effect on linearity or variance of response to E.B. was seen whether standard curves were obtained utilizing salmon sperm or calf thymus DNA. Sonication (2 min, 8 u) of highly polymerized varieties of salmon sperm DNA standard in 0.02 M Tris-0.01 M EDTA was found necessary to facilitate solution. Sufficient KCl was then added to render final concentration as found in TEK. Once again, no effect on linearity or variance was observed.

Results (Table 12) indicated that the majority of DNA was associated with the M-band fraction of each strain as expected. Analysis of the 9790 strain showed an additional 9% in the combined Bottom and Pellet fractions, giving a total recovery of 100% of the DNA added. Total recovery of lysate RNA added to the gradients was 98%. Similar analyses performed on samples from strain 615 M, with EB at a final
Table 12. DNA and RNA Distribution in M-band Gradient Fractions of Group D Streptococci, Determined by Fluorometry, Compared with Literature Values for *E. coli* and *B. megaterium*

<table>
<thead>
<tr>
<th>Bacterial strain</th>
<th>% of total DNA/ gradient</th>
<th>% of total RNA/ gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Top Fraction</td>
<td>M-band</td>
</tr>
<tr>
<td><em>Enterococcus</em> 564 P</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>22 (1)</td>
<td>79 (1)</td>
</tr>
<tr>
<td>615 M</td>
<td>13 (4)</td>
<td>80 (4)</td>
</tr>
<tr>
<td><em>S. faecalis</em> 9790</td>
<td>12 (3)</td>
<td>79 (3)</td>
</tr>
<tr>
<td><em>E. coli</em> b</td>
<td>N.R. c</td>
<td>80-98</td>
</tr>
<tr>
<td><em>B. megaterium</em> KMTT  b</td>
<td>1-10</td>
<td>80-98</td>
</tr>
</tbody>
</table>

\( ^a \) Values represent the average of a number of independent experiments (numbers in parentheses) for each strain.

\( ^b \) Values for *E. coli* and *B. megaterium* KMTT are derived from reported values in the literature (Earhart et al., 1968).

\( ^c \) Values not reported by the authors.
concentrations of 5 uM/ml, reduced RNase concentration (100 uM/ml) and calf thymus DNA as a standard, revealed that no more than 8% of the RNA was found in the combined Bottom and Pellet fractions. Recovery of lysate DNA, in this case, was 103 ± 4% of that added to the gradients.

Electron Microscopy—Ultrastructural Analysis of M-band Fractions

As the DNA and RNA distribution patterns of group D streptococcal M-band gradient fractions were closely compatible with those reported by Tremblay et al. (1969) and Niveleau (1974) for similar fractions isolated from B. megaterium and E. coli, respectively. I proceeded to an ultrastructural analysis of the M-band fractions via electron microscopy. M-band fractions thus examined revealed the presence of complexes identical in appearance to those reported for B. megaterium and E. coli and identified by the above authors as DNA-membrane complexes.

Components of the M-band fractions of the group D streptococci were identified in an indirect manner by comparison of dimensions and patterns observed with those reported in the literature, and more directly on the basis of the relative proportions of DNA, RNA, and phospholipids in the individual fractions as determined by chemical and radio-labelling techniques.

Three ultrastructural patterns were routinely observed amongst the group D streptococci in these fractions: 1) linear chains of DNA, often stretching the length of the
mesh width, associated with a large mass of membranous material (9790, Fig. 3; 615 M, Fig. 4; 564 P, Fig. 5); 2) loops, presumably of DNA, found associated either directly with membrane or most often indirectly, mediated by one or more electron dense particles on the membrane surface (9790, Fig. 3; 615 M, Fig. 4; 564 P, Fig. 5); 3) linear regions of DNA differentiated along their length by areas of ribosome containing material (Fig. 6) as reported by Tremblay et al. (1969) in their Fig. 1 (b).

In addition to the above patterns reportedly observed in other systems, some unique, previously unreported ultrastructural pattern observations were made. At a fixed distance from the membrane, along the extended DNA of both the 9790 and 615 M strains, a particle (arrow, Figs. 3 and 4) was evidenced. The dimensions suggest that it may be of ribosomal origina, possibly a series of 70s ribosomes, as their shape agrees with that shown by Lubin (1968) and are identical to that expected for a cluster of three such subunits 420 Å and 436 Å respectively for the two strains. These particles were too large to be RNA polymerase when compared to reported literature values of 75-120 Å (Miller et al., 1970; Slayter and Hall, 1966).

In addition, the DNA located between the membrane and such particles always had a diameter exactly twice that of subsequent lengths of DNA, making the possibility of artefact less tenable, and involvement of these particles in replication more attractive. To the left of such a particle in the 615 M complex (Fig. 4), a strand of m-RNA (m)
Fig. 3. A DNA-membrane complex in the M-band fraction of *S. faecalis* 9790; rotary shadowed with platinum-palladium. Arrow designates particle at fixed distance from membrane.

Fig. 4. A DNA-membrane complex in M-band fraction of strain 615 M. Arrow (see Fig. 3); (m) - strand of m-RNA; P - possible RNA polymerase; L- loops of DNA.
Fig. 5. A DNA-membrane complex in the M-band fraction of strain 564 P. Arrow, particle possibly ribosomal.

Fig. 6. Pattern type 3, exhibited by M-band fractions of group D streptococci.
can be seen attached to the duplexed, double stranded DNA, presumably the newly synthesized portion, via a particle (p) whose dimensions of 100 Å in diameter corresponds closely to those quoted for RNA polymerase (Slayter and Hall, 1966).

Although the duplex dimensionality was also evident for the appropriate distance distal to the membrane in complexes of strain 564 P (Fig. 5, arrow), the particle normally marking termination of the duplex was not found. Rather a particle of similar dimension was seen at the end of a short linear segment perpendicular to the extended, membrane associated DNA.

Another seemingly unique ultrastructural pattern was exhibited by the 9790 strain (Fig. 3). This strain had what appeared to be an additional DNA chain which also seemed to be associated with the membrane. Limited resolution in the area prevented determination of a specific point of attachment to the membrane. However, examination of the area at a seven-fold higher magnification (not shown) revealed that the point of entrance was probably on the opposite side of the membrane than appears in the less magnified print and the double stranded DNA seemed to unwind just prior to membrane attachment.

At intervals along this chain, particles ranging in size from 200 to 400 Å, within the range reported by others for ribosomes (Schaechter, 1967 /E. megaterium/; Miller et al., 1970 and Revel et al., 1968 /E. coli/ appear.

Pattern 2 as shown in Figs. 4, 7, 8 was exhibited by all three enterococcus strains tested. Best represented by Fig. 4 the pattern consists of a series of loops (L) attached
Fig. 7. Top fraction of 564 P, M-band gradients exhibiting loop pattern.

Fig. 8. Top fraction of 564 P, M-band gradients exhibiting "core" and nicked, supercoiled DNA.
to the membrane as expected for multiple attachment sites predicted by Dworsky and Schaechter (1973) and seen in electron micrographs (Niveleau, 1974) for E. coli. Resolution was not sufficient to determine the number of loops thus associated. However, the association with membrane of each loop end seemed to be mediated in most cases by particles with dimensions corresponding to 50s ribosomes (Lubin, 1968) reportedly also associated with membranes of the 9790 strain by Moore and Umbreit (1965).

Pattern 3, representative of that seen in the M-band fractions of enterococci, is shown in Fig. 6. Such linear regions of DNA differentiated along their length into "bare" regions and "ribosome rich" ones were also described in ultrastructural analyses of M-bands of B. megaterium by Tremblay et al. (1969).

In general, top fractions of the streptococcal M-band gradients examined showed the presence of various components of disrupted cells: short segments of DNA, cytoplasmic contents, and deoxyribonucleoprotein as observed by Itzhaki and Rowe (1969).

When fluorometric analysis revealed a Top fraction rich in RNA (70% of the recoverable RNA), lattice-like networks exemplified by that of 564 P Top fraction (Fig. 9) were seen. Beaded cords with particle size ranging from 250–300 Å were detected, seemingly interconnecting short segments of nucleic acid.

The possibility that the particles represented Mg-Sarkosyl crystals was ruled out as the network associated
Fig. 9. Network pattern typical of Top fractions with high RNA content.
particles had dimensions of 250-300 Å (Fig. 10) whereas particles found in control gradient Top fractions, formed in the absence of cellular material were routinely much larger (530 Å, Fig. 11) and only in control M-band fractions are particles of somewhat similar size (350 Å) found.

Another interesting assembly noted in the Top fraction of 564 P was a rosette type structure or series of loops associated with a central particle (Figs. 7, 8), resembling the non-membrane associated supercoiled, folded chromosome. Such structures were reported by Worcel and Burgi (1974), Pettijohn et al. (1973) and Ryder and Smith (1975), to be the chromosome organized around a central RNA core which provides stability to the chromosome. Relaxation loops, probably due to nicks in individual loops without complete unfolding as predicted by Worcel and Burgi (1972) can also be seen (Fig. 8).

Neither the Bottom or Pellet fractions exhibited structures similar to those found in the M-band or Top fraction. Only cell wall fragments (in Pellet fractions) and an occasional protoplast-like structure (in Bottom fractions) could be distinguished.

Observations made of Lysates such as seen in Fig. 12, immediately after detergent treatment and in Fig. 13 after 5 min or more, show protoplasts or spheroplasts at various stages of disruption, with very little if any cell wall in evidence. Residual cross-wall (c) may be in evidence in the 564 P strain lysate (Fig. 13).
Fig. 10. Typical crystals of control Top fractions.

Fig. 11. Typical crystals of control M-band fractions.
Fig. 12. Lysate pattern immediately after addition of Sarkosyl.
Fig. 13. Lysate pattern 5 min after addition of Sarkosyl; c, possible residual cross-wall.
The above results along with the DNA and RNA distribution patterns of the M-band fractions of the group D streptococcal strains tested here support the contention that the M-band technique is effective in isolating DNA complexes comparable to those of \textit{B. megaterium} and \textit{E. coli}.

**E. Phospholipid Composition of Group D Streptococci: Distribution in the DNA - Membrane Complex**

The question remained as to whether a phospholipid enrichment occurred in the M-band fractions of the group D streptococci and if so, to identify the phospholipid which replaced the phosphatidylethanolamine found enriched in similar fractions of \textit{B. megaterium} and \textit{E. coli}.

An analysis of the phospholipid composition of whole cells, lysates, and M-band gradient fractions of the streptococci was undertaken. Log phase cells of each of the enterococcal strains grown in BHI broth and labelled with $^{32}$P were divided into two parts.

One portion was immediately processed through the lipid extraction procedure (Scheme 2). A second portion was subjected to the lytic procedure previously described. After removal of appropriate amounts of the lysate to load atop gradients for the formation of M-bands, the remaining lysate was also extracted for phospholipids. Upon fractionation of the prepared gradients, into Top, M-band and Bottom fractions, similar ones were pooled from 6 gradients per run and subjected to the same extraction regime as above. To evaluate the contribution of lipid by contaminants in the lytic procedure
reagents, control lysates and gradient fractions were similarly extracted.

Qualitative Analysis of Phospholipids

Qualitative patterns appear in Figs. 14, 15, 16 (illustrations of actual chromatograms developed in CHCl₃: MeOH:H₂O (65:25:4 v/v) for each strain). Each whole cell (WC) and lysate (L) fraction chromatographed corresponds to approximately equivalent cell material extracted, while T (top) + M (M-band) + B (bottom) per chromatogram is equivalent to either WC or L. Thus a rough quantitative comparison can be made by spot size differences as well.

Qualitatively all 3 enterococcus strains exhibited the same major whole cell phospholipids (PA, PG, diPG, and amino acyl PG). Identification was made by comparison with simultaneously chromatographed standard compounds, chromatography in three different solvent systems, and response to detection spray reactions for specific molecular species and/or linkages. As no standard for amino acyl PG was commercially available, identification was made by comparison of P⁺, N⁺ spots of 564 P and 615 M strains with those found in the 9790 strain, known to contain such phospholipids.

The three strains also contained what appeared to be glycolipids or possibly phosphoglycolipids in whole cell extracts. This is based upon Rf values, comparison with reports in the literature, and reactions to detection spray reagents. A positive reaction for vicinal-OH groups (PS⁺) indicated probable glycolipid whereas variable reaction to detecting agents for phosphate esters and ³²P label of 3 %
Fig. 14. Thin layer chromatogram of $^{32}$P labelled whole cells (WC), lysate (L), and M-band gradient fractions (T,M,B) of strain 564 P. Spray reagent detection code: $N^+$, ninhydrin positive (contains free amino group); $P^+$, Hanes reagent positive (contains phosphate esters); $PS^+$, vicinal-OH group positive; $\gamma$, $^{32}$P labelled.
SOLVENT FRONT

SPOT #

7

SPOT #

6

5

4

3

2

1

Origin

WC L standards T M B

\[ \text{SPOT} \text{#} \]

\[ \text{SOLVENT \ FRONT} \]

\[ \text{SPOT} \text{#} \]

\[ \text{Origin} \]

\[ \text{WC} \ L \ \text{standards} \ T \ M \ B \]
Fig. 15. Thin layer chromatogram of $^{32}$P labelled whole cells (WC), lysate (L), and M-band gradient fractions (T,M,B) of strain 615 M. For spray reagent detection code refer to Fig. 14.
Fig. 16. Thin layer chromatogram of $^{32}$P labelled whole cells (WC), lysate (L), and M-band gradient fractions (T,M,B) of *S. faecalis* 9790. For spray reagent detection code refer to Fig. 14.
depending upon the strain made identification as phospho-
glycolipids possible but tenuous.

In each strain, three such lipids were observed. The first and most polar, slowest migrating (spot 4 in each strain Figs. 14, 15, 16) was identified as PG since it coincided with chromatographed standards of the same compound and was \( \text{PS}^+, \text{P}^+, \text{P}^{32} \) labelled. Although the chromatogram chosen to illustrate the phospholipid pattern of the 9790 strain was not sprayed for the presence of vicinal-OH groups (\( \text{PS}^+ \)), all other data and literature indicated a similar compound to that of the other two strains.

One and possibly both of the remaining two lipids containing vicinal-OH groups (spot 5 of 564 P, Fig. 14, spot just above 5 of 9790 Fig. 16; spot 7 of 615 M, Fig. 15) and (spot 6 of 564 P and 9790; spot 8 of 615 M) one may be phosphoglycolipids as they appeared to incorporate \( 32\text{P} \). But as it was only at 3% of the total \( 32\text{P} \) incorporated into the extractable phospholipids of these strains and as the response to molybdate spray reagent for detection of \( \text{PO}_4 \) esters was variable, such an identification remains highly speculative.

The less polar, farther migrating of these compounds with \( \text{Rf} \) 5-5.5 in 564 P and 615 M show variable \( 32\text{P} \) labelling and molybdate reaction which may be due to the presence of phosphate at the limiting level of sensitivity of the detection methods, i.e., probably below the 1% level as \( 32\text{P} \) at the 1.5% level can be detected in the 9790 strain.

With respect to the additional presence of variable phosphate (\( \text{P}^+ \)) reaction and extremely low \( 32\text{P} \) labelling in the
more polar vicinal-OH containing spot 5, strain 564 P representing 0.5%; spot just above 5, strain 9790 1% and spot 7 strain 615 M 2% of the total extractable $^{32}$P labelled lipid provides insufficient data for any judgment to be made concerning their identification as phosphoglycolipids and will therefore be considered as non-phosphatide containing glycolipids.

The identification of these lipids was not pursued further for the following reasons: neither the $^{32}$P labelling, phosphate ester detection, nor detection of vicinal-OH groups was maintained in the M-band and the less polar component disappeared as a result of the lytic process.

An additional note in terms of the qualitative investigation of the whole cell extract, chromatographic analysis showed the presence of Hanes reagent positive (P$^+$), extremely non-polar component with Rf 0.98 in strain 564 P. It has been suggested by Dr. Richard Taylor (unpublished information) to be due to the presence of a pigment in this strain (carotenoid/triterpenoid in nature), as they and isoprenoids will react with molybdate, the active component in the Hanes reagent.

The Lysate lipid composition maintained the qualitative pattern seen with whole cells with the exceptions already mentioned of the disappearance of the less polar, possible glyco/phosphoglycolipid from this fraction, and the apparent loss of PG from the 9790 strain with slight increase in the Rf of diPG.
The M-band, in turn, maintained the same pattern observed in the Lysate except for the disappearance of the more polar (PS⁺) spot 5 of 564 P. Thus the M-band phospholipid pattern qualitatively differed from the composition of the whole cells essentially as did that of the Lysate.

Quantitative Analysis of Phospholipids via $^{32}$P Labelling

Quantitatively drastic alterations occurred among the predominant phospholipids of these organisms (PA, PG, diPG, amino acyl PG) as a result of the lytic process.

If one compares M-band phospholipid distributions for each strain directly against those of whole cells (Table 13) one would conclude that an enrichment of amino acyl PG occurred at least in two out of the three strains tested, strains 564 P and 615 M, with the 9790 strain showing a slight but insignificant decrease. In addition, the 615 M strain showed an enrichment of PA and diPG while the 9790 strain showed a slight enrichment in diPG as well.

If on the other hand, one compares the Lysate phospholipid pattern with that of the whole cells in each case the changes observed in the M-bands can be seen to have already occurred at this stage.

Evidence to substantiate this claim is summarized in Table 12. Percentages reported are as compared to equivalent extracted whole cell material. Ratios of PA:PG:amino acyl PG: diPG are used in conveying results to facilitate comparison among the strains unless otherwise specified.
Table 13. Phospholipid Distribution in Group D Streptococci

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Fractions</th>
<th>% total cell $^{32}$P-labelled extractable lipid</th>
<th>Phosphatidic acid</th>
<th>Phosphatidylglycerol</th>
<th>amino-acyl PG</th>
<th>Cardiolipin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>PA</td>
<td>PG</td>
<td></td>
<td>diPG</td>
</tr>
<tr>
<td>Enterococcus 564 P</td>
<td>Whole cells</td>
<td>1</td>
<td>71</td>
<td>17</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Lysate</td>
<td>1</td>
<td>48</td>
<td>44</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>M-band</td>
<td>1</td>
<td>34</td>
<td>36</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td>Enterococcus 615 M</td>
<td>Whole cells</td>
<td>12</td>
<td>49</td>
<td>21</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Lysate</td>
<td>42</td>
<td>30</td>
<td>15</td>
<td>9</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>M-band</td>
<td>36</td>
<td>17</td>
<td>30</td>
<td>6</td>
<td>—</td>
</tr>
<tr>
<td>S. faecalis 9790</td>
<td>Whole cells</td>
<td>52</td>
<td>4</td>
<td>22</td>
<td></td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Lysate</td>
<td>36</td>
<td>—</td>
<td>27</td>
<td>11</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>M-band</td>
<td>34</td>
<td>—</td>
<td>17</td>
<td>13</td>
<td>—</td>
</tr>
</tbody>
</table>
The predominant lipid in the WC extracts was found to be PG in both 564 P (71%) and 615 M (49%), while in the 9790 strain PA predominated (52%). In all three cases, diPG was an extremely minor, if not undetected component while the amino acyl derivatives of PG constituted approximately the same proportion of about 20%. Percent total recovered $^{32P}$ label accounted for by the major phospholipids were 88% from 564 P, 85% from 615 M, and 78% from 9790.

Analysis of the Lysate lipids revealed that the major phospholipids accounted for 92, 96, 73% respectively of the whole cell lipids, suggesting at least in the UNH strains, the possible release of bound lipids as a result of the lytic procedure or in the latter case slight loss in lipid. Shifts in the levels of each lipid found are also seen. The ratio of PG:amino acyl PG changed from 4.2 to 1.1 indicating a conversion (almost quantitative) from PG to amino-acyl PG.

Similarly with strain 615 M, a PG:amino-acyl PG ratio of 2.3 changed in the Lysate to 2.0 indicating a similar conversion of PG to amino-acyl PG along with possible degradation of PG to PA and synthesis of diPG judging by ratio changes of PG:PA of 4.1 to .7 and of PA:diPG of 4.0 to 4.7.

The 9790 strain, exhibiting a high level of PA in the WC pattern as opposed to the other strains, presented a different situation in that alterations of PG to amino-acyl PG ratios although they do occur seemingly in a quantitative fashion, are minor compared to the loss of PA from the system and the increases in diPG. The mechanisms can only be
speculated upon, and will be explored in the discussion section.

The M-band in each case as observed quantitatively seemed to maintain those ratios observed in the Lysate for PG and amino-acyl PG, with the exception of 615 M where PG: amino-acyl PG reverses from 30/15 to 17/30, i.e., a change of 2.0 in the Lysate to .57 in the M-band indicating continued quantitative conversion of PG to amino-acyl PG during the M-banding process of this strain.
CHAPTER IV

DISCUSSION

Discussion of the results of this study can be approached in two ways. One is based upon the assumption that the DNA-membrane complex isolated by the M-band technique is a physiologically valid "in-vivo" association; the other that such an association occurs during cell disruption, or as a result of lytic procedure reagents used, and therefore is an artifactual entity.

While evidence in support of both approaches appear in the literature (Tremblay et al., 1969; Ballesta et al., 1972; Dworsky and Schaechter, 1973; Patterson et al., 1970), certain findings warrant discussion in terms of a valid association. Similar composition and properties exhibited by complexes isolated by a variety of methods (see Introduction, recent work with the M-band technique (Firshein, 1972; Harmon and Taber, 1977) indicating additional components and enzymes in association with the complex, necessary for DNA synthesis and related cellular processes, plus retention of synthetic capabilities in some cases (Greene and Firshein, 1976) support its validity. On the other hand sufficient controversy and equivocal points have also been raised to warrant discussion in that vein as well.

It is not unreasonable, based upon the current concept of lysozyme action (Pollock et al., 1975, 1976; Pollock and Sharon, 1970) and knowledge of structural and physiological differences amongst bacteria, that I found it necessary to
modify the lysozyme technique for each genus studied to induce formation of Sarkosyl-sensitive cells.

In attempts to isolate DNA-membrane complexes by the M-band technique, from E. coli, B. megaterium, and a series of group D streptococci ranging from the reportedly most lysozyme sensitive, S. faecalis 9790 strain to one of the most resistant, S. faecalis var. liquefaciens, all were subjected to lytic procedures which included lysozyme.

As Earhart et al. (1968), using the Godson and Sinsheimer method with E. coli, and Tremblay et al. (1969, using one more suitable for B. megaterium as well as methods other than the M-band technique (see Introduction), had successfully isolated DNA membrane complexes with similar properties each utilizing variations of a lysozyme mediated sensitization, it seems improbable that variations in the sensitization methods drastically altered the DNA-membrane complex isolated or that complexes isolated are artifacts formed during the Lytic process. My attempts to verify the results of the previously mentioned investigators by formation of M-bands from E. coli B/r(w) and a B. megaterium strain KM resulted in isolation of comparable complexes as demonstrated by DNA and membrane content (3H-thymidine labelled) and content of 32P labelled, extractable lipid respectively (compare Tables 4 and 5 with literature values for E. coli and B. megaterium Tables 1 and 15).

Observations of Ballesta et al. (1972) and Tremblay et al. (1969), reporting 10-30% of the membrane associated with the M-band fraction, compared favorably with my values
of 19 and 37% at 10 and 30 min after Sarkosyl addition, both determined by butanol extraction of $^{32}$P labelled material. Such an increase (2 fold) in membrane content with time of interaction sensitized cells with Mg$^{++}$-Sarkosyl, from 10 to 30 min compares well with 1.6 fold increase reported by these authors for the same time period. Such time dependence has been suggested by Tremblay et al. (1969) to be due to an equilibrium being established between the membrane solubilization properties of the detergent and the rate of Mg$^{++}$-Sarkosyl crystal formation.

Sarkosyl concentrations of .25 to 2% did not alter membrane patterns (Filip et al., 1973), nor did my use of .2-.8% seem to affect complex composition as judged by DNA, RNA, and ultrastructural parameters. As reported by Ballesta et al. (1972), affinity of crystals for membrane is more critical than concentration of reagents used. However Mg$^{++}$ concentration appears to be important as it regulates crystal formation and therefore influences the degree of membrane association possible.

Thus in agreement with results of Earhart (1968), McIntosh and Earhart (1975), M-bands were formed from both E. coli and B. megaterium consistent with Sarkosyl, Mg$^{++}$, and lysozyme concentrations used by these authors and containing appropriate distributions of DNA and membrane. The present observations of identical results in terms of DNA and membrane distribution irrespective of whether lysis with Sarkosyl occurred prior to, or after layering of cells on gradients is also consistent with literature reports, as Harmon and Taber
(1977) reported similar findings, with \textit{B. subtilis} as did Cundliffe (1970), with \textit{B. megaterium}. This indicates that there is something unique about the membrane associated with the DNA of the complex to allow its isolation under these varied conditions.

Similar response to shear forces as observed by McIntosh and Earhart (1975) was seen in attempts to recapitulate the original technique with \textit{E. coli} (Table 4). Seventy percent of DNA (\textsuperscript{3}H-thymidine labelled) was recoverable in the M-band of \textit{E. coli} B/r(w) (unsheared) comparing favorably with the 74\% DNA observed in the M-band of an \textit{E. coli} B strain by McIntosh and Earhart (1975). Shearing resulted in the release of 25\% of the DNA to the Top fraction in my attempts with the B/r(w) strain which could not be directly compared with the B strain results as the method of shearing in the latter case was more extensive (theirs: 22 gauge needle, 4x; compared with mine: pipeting 3-4x with a 5 ml pipet) and resulted in their more drastic loss of 82\% of the DNA from the M-band.

Neither the Earhart nor Tremblay methods of sensitization proved successful with the group D streptococci tested (pellets formed when M-banding was attempted). It was apparent from these studies and those in the literature (see Introduction) that the streptococci were more resistant to lysozyme attack, therefore another approach was investigated. This approach which included both lysozyme and Sarkosyl proved to be successful, with the most resistant strain, \textit{liquefaciens}. However, the procedure required use of increased temperature of lysozyme treatment (37 C as opposed to 0 C for \textit{E. coli} and
10 min compared with 30 sec - 5 min of incubation time), to elicit a lytic response, thus increasing the possibility of enzymatic alterations occurring.

In agreement with the literature, lysozyme alone was insufficient even at 37 °C to effect lysis in the liquefaciens strain, although subsequent treatment with Sarkosyl led to a lytic response. This was not unexpected based upon results of Coleman et al. (1970), showing susceptibility of lysozyme treated streptococci, previously thought to be refractory to such treatment, upon addition of detergent. It may also reflect the formulation of a lysis resistant complex around the cell by lysozyme as proposed by Metcalf and Deibel (1969) which can be disrupted by competing anionic (salt or detergent) molecules or via pronase digestion as predicted by Olsen et al. (1974).

As predicted by Metcalf and Deibel (1969), optical density increased after exposure of cells to lysozyme, which was consistent with formation of a lysis resistant lysozyme shell. Such increases were enhanced by growth in media with elevated phosphate and other nutrients. Apparently these compounds could remain loosely affixed to the cells and inhibit the lytic response possibly due to masking of or competition for lysozyme binding sites. A similar observation was made by Shockman et al. (1961) in the presence of growth media. On the other hand, and in accord with the current theory of lysozyme binding to saccharides, N-acetylglucosamine or possibly teichoic-acid (a phosphate containing compound) may
allow aggregation and thus an increase in optical density to occur (Pollock et al., 1976).

Decreased phosphate content of growth media is also known to effect phospholipid synthesis and allow more favorable lysis of group D streptococci to occur. The fact that a suspension of cells in phosphate buffer plus lysozyme increases in optical density (Metcalf and Deibel, 1969) while leakage of phosphate occurs when water washed cells of streptococci such as _S. mutans_ (a known producer of teichoic acid) are suspended in H₂O at 37°C without lysozyme, and that leakage can be inhibited by addition of lysozyme (Pollock et al., 1976), supports teichoic acid involvement.

Absorbance studies (260 nm) of cell supernates after treatment with lysozyme, Sarkosyl, etc. were attempted with the _liquifaciens_ strain to determine if leakage of nucleic acid was occurring despite increase in optical density (Woldringh and Van Iterson, 1972). At first glance one would conclude that lysozyme as well as Sarkosyl effect release of nucleic acid (Tables 8 and 9) and seemingly to the same extent. Caution is advised however in interpreting such results as Sarkosyl has been reported to interfere with 260 nm absorbance (Porter and Fraser, 1968). Pollock _et al._ (1976) have interpreted their results as an indication of lysozyme affecting membrane rather than cell wall. They support this with similar observations in the presence of muramidically inactivated lysozyme. In addition, ultrastructural data on the effect of SLS or SDS on _E. coli_ (Woldringh, 1970; Woldringh and Van Iterson, 1972) does indicate extensive intracellular
reorganization and leakage of nucleic acid after less than ten minutes of treatment with the detergent.

Cell wall damage is incurred under these conditions as Metcalf and Deibel (1969) reportedly could measure N-acetyl-glucosamine release. The latter investigators however monitored release of radioactively labelled D-alanine as an indicator of cell wall degradation, invalid in organisms which produce and excrete teichoic acid (D-alanine substituted) during growth (Joseph and Shockman, 1975).

The lack of differential response of S. faecalis var. liquefaciens cells grown in various growth media to either lysozyme or Sarkosyl or both was unexpected as the literature indicates increases resistance to lysozyme at least with stationary phase cells and those grown under conditions of nutrient depletion (Shockman et al., 1961, among others). This suggests that at least during exponential growth, substrates or accessibility necessary for activity of these agents are not effected.

Substitution of Tris-EDTA for phosphate buffer might also tend to prevent lysis probably as a result of inhibiting lysozyme. However, Imoto et al. (1974) reported such inhibition by 0.2 M Tris at pH 8 whereas our system utilizes a lower concentration 0.01 M Tris pH 8, which may not produce the same effect. Tris may also act as a stimulant for EDTA as reported by Asbell and Eagon (1966), Voss (1967) and Tucker and White (1970) and enhance EDTA mediated aggregation, thus reducing access to disruptive agents and autolytic enzyme activators such as Sarkosyl and pronase. In fact, the lytic
procedure was more comparable to that utilized in studies with *E. coli* as it included the chelating agent EDTA. Control of the lytic reaction was afforded by the addition of agents which apparently inhibit lysozyme, to a certain extent. This could either result from enhancement of or failure to disrupt the lysozyme shell which surrounds cells thus treated. Such agents include EDTA and possibly pronase and sucrose (Shockman *et al.*, 1961; Pollock *et al.*, 1976). This is supported by the observation of increased optical density which is maintained over a longer period, especially if EDTA is added and is usually maintained until Sarkosyl (causing complete disruption) or pronase (causing partial disruption) are added.

As more than one strain of the group D streptococci were to be tested for the presence of DNA-membrane complexes during the exponential phase of growth, and as the 9790 strain was of particular interest because of extensive documentation of its cell wall and lipid composition, several strains were subjected to the lytic procedure developed for the *liquefaciens* strain. However when applied to exponential phase cells, especially the 9790 strain, the procedure resulted in uncontrollable lysis. On the other hand, when the process was applied to these strains in stationary phase, the cells proved too refractory.

Therefore an alternative lytic procedure, capable of eliciting a more controlled lytic response for several group D streptococcal strains and compatible with the M-band technique was developed. A modified version of the Clewell *et al.* (1974) procedure, originally developed for isolation of plasmids from...
S. faecalis, and incorporating Sarkosyl as the disruptive agent fulfilled all the criteria and within 5 min of detergent addition cell lysis was achieved. This was unexpected as Coleman et al. (1970) reported a 2 h lysozyme treatment prior to detergent was required whereas strains in this study, 564 P, 615 M, and 9790, responded after a total of 20 min in the presence of lysozyme.

Recently the observation of loss of viability upon treatment with lysozyme of streptococci has been resurrected (Coleman et al., 1970; Bleiweis et al., 1971; Metcalf and Deibel, 1973; Pollock et al. 1976). Prior to that time other workers, Epstein and Chain (1940), Kern et al. (1951) and Pellegrini and Vertova (1969), had alluded to this phenomenon. Although a similar loss in viability was evident in my studies with the 9790 strain as determined by plate counts, it must be emphasized that normally accepted methods for quantitating viable cells become increasingly inaccurate when streptococcal populations are evaluated and lysozyme and/or EDTA are used. This is primarily due to aggregation induced by the latter agents. Thus CFU or MPN tests as means of evaluation are suspect and even my results while reported as a 72 % loss in viability within 5 min of lysozyme addition, in reality represent less than a 4 fold decrease in the number of viable cells. Five minutes after detergent addition however almost a billion fold decrease in viability is seen. Coleman et al. (1970) reported a similar situation with S. faecalis strain 31—a 96 % loss of viability (CFU) after 60 min of lysozyme treatment with 1 % viability remaining after additional 15
min treatment with the detergent sodium lauryl sulfate. However, this system differed from mine in that it included incubation at 37 °C, the pH was higher (8.2) and sucrose was not included, as well as use of SLS rather than Sarkosyl.

Current theory suggests an effect of lysozyme upon cell membrane (Pollock et al., 1976). Metcalf and Deibel (1973) suggested effects on energy generating mechanisms at the membrane level based upon observed effects of lysozyme on oxygen uptake. However, the question of lysozyme effect on membrane structure or function has not yet been definitively resolved.

Contribution of the autolytic enzyme system was apparently minimal at best in these studies although conditions for activation (pronase addition) were present. No changes in optical density, viability or appearance of cells were seen when non-lysozyme treated or H₂O suspended cells were monitored for expression of autolytic activity. Such apparent lack of expression in the 9790 strain in sucrose is especially puzzling as exponential phase cells were used, a population supposedly possessing a high level of enzymatic activity. However as the increment in susceptibility to detergent treatment was small, the autolytic system must play only a small part in the destruction of these cells under these conditions. Alternatively involvement of the autolytic enzyme system may be at the level of Sarkosyl addition or may be inhibited until addition of detergent. In either situation definitive involvement of the autolytic system could not have been determined in this study.
The major ultrastructural details of the group D streptococcal complexes isolated may be briefly recapitulated. True patterns were seen resembling those reported in the literature: 1) membrane associated linear strands presumably DNA based upon their agreement with 20 Å measurements for DNA in the literature and by DNA content obtained via fluorometry; 2) loops of DNA associated directly or indirectly with electron dense particles whose dimensions and shape identified them as ribosomes; 3) linear DNA covered intermittently with ribosome containing material, possibly representative of additional attachment sites.

Observations of such patterns attest to the validity of these M-band derived complexes as compared to the DNA-membrane complex isolated from E. coli and B. megaterium, and conceptually associated with the replication complex in bacterial cells.

In addition, some patterns were observed unique to the streptococcal M-band complex. The increased dimensions of double stranded DNA for a fixed distance from the membrane, corresponding to 4 double strands with bifurcation occurring immediately prior to membrane association (also observed by Habener et al., 1969) and the strand size decreasing to 1/2 at the membrane-distal end, at or near a particle complex, suggest a possible functional involvement in DNA replication. This process is reported to commence at the membrane (at a fixed origin) (Sueoka and Quinn, 1968; Fielding and Fox, 1970) and to proceed along the DNA chain at a non-membrane bound growing point (Ganesan and Lederberg, 1965; Smith and Hanawalt, 1967).
Alternatively, the particle, based upon shape and size bears sufficient resemblance to 70s ribosomes (Lubin, 1968) to postulate its being a polysome, and the increased DNA dimensions reflective of association with ribonucleic protein, reported to tenaciously adhere to DNA upon extraction (Itzhaki and Rowe, 1969).

However, an additional membrane attachment site cannot be ruled out, nor the possibility of a mulbenzyne RNA polymerase complex as proposed by Firshein (1972), to be associated with such complexes in pneumococcus.

Herein the 564 P strain exhibits the first ultra-structural difference from that of the other two strains. Although the duplex dimension of the DNA strand is evident for the appropriate distance from the membrane, the particle seen in the other two strains is now missing. Instead a short chain is seen attached to the DNA with a particle at the opposite end. Such an entity has been reported by Schaechter and McQuillen (1966) and Krembel (1971) to be RNA with terminal ribosomal subunits providing protection against nuclease action and to possibly be involved in transcription and translation. Such an association of ribosomes with short segments of m-RNA have also been observed by Bremer and Konrad (1964), Cundliffe (1970) and Stent (1966), even before the entire message was read.

Although no definitive proof for bi-directional replication can be provided by this data, the appearance of two strands, both seemingly associated with the same membrane fragment in the 9790 complex could be seen. One exhibited a
sequence of particles irregularly spaced as seen by Hart (1958) which spacings were supportive of Okasaki fragments. Fragment size falls within the range of DNA fragment length of 400 to 2000 nucleotides predicted by Okasaki et al. (1972) to be involved in discontinuous DNA synthesis. The other strand, the previously mentioned duplex dimensionality terminating in an electron dense particle, either reflects discontinuous synthesis in "two" directions with one direction terminating shortly after initiation (Yamaguchi and Yoshikawa, 1973) or an artifact of preparation and fortuitous positioning of the second strand. Consequently such observations are not strongly advanced as evidence of bidirectional chromosome replication in group D streptococci.

Loops associated with membrane fragments in the M-band, seemingly associated with ribosome-like particles, are strong candidates for multiple attachment sites especially as Cundliffe (1970), Tremblay et al. (1969) reported 50s ribosome association at site of membrane attachment. Loops also appear in the Top fractions of 564 P, another unique characteristic of this strain and in high probability are related to the folded chromosomes isolated by Worcel and Burgi (1972). The fact that they are associated with the Top fraction suggests that they are folded but not membrane-bound chromosomes, as Dworsky and Schaechter (1973) reported membrane-bound, folded chromosomes prepared by the methods of Worcel and Burgi (1972) or Stonington and Pettijohn (1971) (method type B) associated with the M-band fraction if cells thus prepared as subjected to M-band gradient fractionation.
Finally, the networks observed in the Top fraction of 564 P, consisting of polyribosomes and DNA and supported by fluorometric determination of high RNA content in this fraction may represent the lattice suggested by Woldringh and Van Iterson (1972) to provide the spatial properties necessary for cytoplasmic synthetic processes to occur. One cannot discount the possibility of the "beaded cords" being mesosomes as seen by Miller et al. (1967) and Joseph and Shockman (1974).

Thus in terms of ultrastructure the group D streptococci at least in the M-band exhibit no outstanding differences in DNA membrane complexes from that of other organisms examined, with the possible exception being the ultrastructural characteristics of the 564 P strain exhibited by the Top fraction are interesting and may be related to the increased RNA content and different phospholipid pattern changes in response to subjection to the M-band methodology. This in turn may reflect greater physiological similarity to S. faecalis than S. faecium a strain more resistant to lysozyme. The overall result may then be response of the 564 P strain to the modified Clewell procedure as if it were a more gentle lytic procedure such as method type B. These characteristics seem to set this system apart from the other two enterococcal strains studied.

Although much work has been done utilizing DNA-membrane complexes to study synthesis and control in bacterial and associated viral systems (see Introduction), specificity at the attachment site (s) is assumed based upon results of rifampin treatment, x-irradiation (Dworsky and Schaechter,
1973), RNase (Cundliffe, 1970), shearing (Rosenberg and Cavalieri, 1968). All these approaches are based upon the association and role of DNA and RNA in the complexes. Two notable exceptions are the works of Ballesta et al (1972, Harmon and Taber (1977), both utilizing the M-band technique for isolation of DNA membrane complexes. They examined the membrane components of the complexes (phospholipids and proteins, respectively) and suggested that specificity of these components in the isolated complexes may hold the key to the regulation of cellular activities normally associated with these complexes.

Similar analogy with the streptococcal strains studied in terms of phospholipid specificity at the membrane-chromosome attachment site cannot be made, as data from lipid analysis of streptococci indicates that enrichment of specific phospholipids in the complexes desired although detected in the M-band fractions actually occur during processes associated with lysis and subsequent extraction. Under these conditions "in vitro" alterations can occur.

Although strictly speaking my data cannot be extrapolated to contest the functional specificities attributed to the phospholipid enrichment found in complexes from the organisms studied by Ballesta et al. (1972), neither has the critical experimental data appeared in the literature, i.e., a comparison of the Lysate phospholipid distribution with that of whole cells and M-bands of these organisms, which would be necessary to definitively dispel the possibility of created artifact.
If one makes a comparison of phospholipid distribution of the whole cells to that of the M-band for each streptococcal strain as was done by the Ballesta group with their organisms, the amino acyl PG of all these strains, 564 P, 615 M, and 9790 would qualify as a replacement for enriched PE in the complexes. This would not be an unattractive outcome as the amino acyl derivatives of PG exhibit many of the characteristics and potential functional qualities of PE. Both are basic molecules capable of balancing lipid core change (Minnikin and Abdolrahimzadeh, 1974); of maintaining membrane stability (Cronan and Vagelos, 1972; Houtsanelller and Van Deenen, 1964; MacFarlane, 1964) and accumulate under adverse conditions such as low pH (Houtsanelller and Van Deenen, 1964, 1965; Lee and Co, 1973). Lysyl-PG artificial membranes have been shown to be impermeable to protons as well (Hopfer et al., 1970), and an analogy between increases in the proportion of PE and amino acid esters of PG at low pH has been suggested (Houtsanelller and Van Deenen, 1964).

However, upon reevaluation of the conclusions of Ballesta et al. (1972) concerning phospholipid enrichment in M-bands, in terms of known proportions of phospholipid in exponential phase cells, effects of agents of the lytic procedure and certain facts derived from whole cell-to-lysate changes in the streptococci, alternative explanations to that of the membrane-chromosome attachment site being specifically enriched in specific phospholipid in the intact cell were sought. The facts derived from the streptococcal strains studied included: 1) that the 564 P strain showed quantitative
conversion of PG to amino-acyl PG; 2) that strain 615 M showed
an enrichment of PA and doubling of diPG as well as enrichment
of amino acyl content in M-bands; 3) that the 9790 strain
showed minimal increase in amino acyl PG, but loss of certain
phospholipids which could not be accounted for by the slight
increases observed in amino acyl PG and diPG.

The enrichment of amino acyl PG at the expense of PG
from whole cell to lysate or whole cell to M-band in 564 P
strain bears a remarkable resemblance to the S. faecalis
pattern changes observed by Houtsmuller and Van Deenen (1965)
upon reducing pH "in vivo" or "in vitro" from pH 7.2 to 4.8.
Similar responses have also been noted in whole cell analysis
of B. subtilis var. niger and in S. aureus as well (Houtsmuller
and Van Deenen, 1964, 1965). In addition, the phospholipid
pattern of this strain resembles that reported by Vorbeck and
Marinetti (1965a) for protoplasts of exponential phase cells
of S. faecalis 9790, with PG and then amino acyl PG occurring
as major components.

The pH at time of harvest was never lower than about
6.3 and the pH of lysate ranges from 5.5 - 6.5 at least for
the 9790 strain. This suggests that neither growth nor lytic
procedure pH could account for the observed increase in amino
acyl PG. In addition, the only study to my knowledge which
followed phospholipid changes over a pH range (Houtsmuller
and Van Deenen, 1965) with S. aureus indicated drastic changes
only occurred below pH 5.3.

However the 10 % TCA added to each sample (whole cells,
lysate, M-band gradient fractions) to terminate activity and
precipitate lipid and protein prior to lipid extraction, might have sufficiently lowered the pH to account for the observed amino acyl PG accumulation.

If this were true, one might expect to see similar distributions amongst all fractions, as all were TCA precipitated. This was not the case. The whole cells were precipitated while suspended in culture medium whereas the lysate and M-bands were suspended in Tris-sucrose plus various other components which may have affected the acylation process in different ways.

The chloroform: methanol extraction and subsequent washings were discounted as causal in promulgating the observed enrichment as one would again expect a similar reaction observed in all samples processed, which was not seen.

Another alternative is that differences in lipid-lipid and lipid-protein associations among the samples (e.g. the lysate having been treated with pronase and Sarkosyl, protein and membrane disruptive agents, respectively) whereas the whole cells would not have been similarly treated would undoubtedly affect solubility properties with respect to the lipid solvent system used. Such a possibility to explain the complex phospholipid patterns observed in B. stearothermophilus was also suggested by Card (1973).

The accumulation of amino acyl PG and PE has also been reported to occur in cells as they transit from exponential to stationary phase. This suggests that conditions employed in my studies and those of the Ballesta group may in fact be
inducing a response mimicking the transition to stationary phase in the same strain. De Siervo (1969) stated, "the major phospholipid classes of E. coli B represent 'dynamic chemical constituents . . . [which] can independently change in relative proportions as well as in actual percentage of the total cell mass" and as Op den Kamp et al. (1965) reported that "in vitro" changes in the immediate environment could effect such altered distributions, this possibility became more and more attractive.

With this in mind, perusal of the phospholipid distributions in stationary phase cells of S. faecalis 9790 (Table 1) shows amino acyl PG (Ikawa, 1963), PA (Shockman et al., 1963), or diPG (Ibbott and Abrams, 1964) each reported as the major phospholipid of stationary phase cells. This suggests that a high degree of variability exists in phospholipid content even within a given strain. Such variations may have been due to differences in growth media, harvesting and/or extraction procedures. However, as all 3 strains we tested were grown in the same media to the same stage of growth (exponential) with the 9790 strain at a slightly later stage of exponential growth than the other two strains, growth phase and media composition difference as contributory can be ruled out. On the other hand, the diversity of opinion concerning the major phospholipid in studies previously mentioned, with supposedly the same strain, attests to the dynamic status of these lipids.

Card (1973) reported "in vitro" phospholipid pattern shifts similar to those observed upon commencement of stationary
phase involving synthesis, degradation, and interconversion amongst the more metabolically active phospholipids, PG, diPG, as well as stability of PE in studies with B. sterothermophilus.

*B. sterothermophilus* phospholipids after transfer of cells during exponential phase to non-growth media of .01 M Tris, CaCl$_2$, 0.15 M NaCl resemble the pattern observed in stationary phase. Since the suspending milieu of the streptococcal lysates contain both 0.01 M Tris and NaCl, a similar shift to stationary phase patterns and/or other conversions suggested might be expected.

This becomes a very attractive possibility when one compares the shifts observed with the bacillus strains to streptococcal data for the 615 M and 9790 strains exhibiting a more complex pattern than the 564 P strain. In addition a similarly complex situation was reported by Houtsmuller and Van Deenen (1965) to occur with *S. aureus* under certain conditions induced subsequent to growth.

In addition, Card (1973) reported that at the transition from exponential to stationary phase, PE concentration nearly doubled by the time growth ceased in *B. sterothermophilus*. A similar but less dramatic increase was seen for *E. coli* by De Siervo (1969). A similar statement was also made by Ballesta et al. (1972) when comparing the enrichment of PE found in *B. megaterium* and *E. coli* as possibly accounting for patterns observed in these strains.

Phospholipase activity as a mechanism for the phospholipid patterns observed in the Lysate and M-bands of the group D streptococci would seem unlikely. Consideration of the
reactions catalyzed by these enzymes:

phospholipase A—conversion of lysyl PG to lyso phosphotidyl glycerol and lysine;
phospholipase C—conversion of lysyl PG to an amino acid ester of glycerophosphate plus a diglyceride;
phospholipase D—lysyl or alanyl PG to PA and amino acyl glycerol.

All convert the amino acyl derivatives to simpler phosphatides capable of being recycled into biosynthetic pathways. However, such activities usually require pH around neutral and phospholipase A is inhibited at pH 6 (Houtsmuller and Van Deenen, 1965). Although amino acyl PG has been shown to be susceptible to phospholipases (Houtsmuller and Van Deenen, 1964) Lysate and M-band phospholipid pattern alterations in the streptococcal strains support accumulation rather than degradation of these amino acyl derivatives.

It is interesting to speculate however that these enzymes are either inhibited upon disruption of the cells during the lytic and M-banding process or, if the exponential cells are responding in non-growth media as stationary phase cells or to decreases in pH during TCA precipitation, that the observed increases in amino acyl PG reflect this inhibition. A similar hypothesis was proposed by MacFarlane (1962) to explain accumulation of lysyl PG as the reaction occurred "in vitro" as well as "in vivo" and therefore appeared to be non-enzymatic in nature.

The possibility of synthesis, metabolism and interconversions proposed in the literature as well as involvement
of phospholipase activity occurring prior to treatment of samples with methanol at 55 °C constitute viable alternatives as well and help explain the patterns observed with the 615 M and 9790 strains. Phospholipase D specific for degradation of diPG PA + PG stimulated by Mg²⁺ (Cole, 1974) in the lysate might also account for observed increase in PA in the 615 M strain. However one would also expect PG to increase and diPG to decrease which does not seem to occur even in the M-band where Mg²⁺ stimulation is possible.

The most plausible explanation for observed pattern changes in the lipids of the 615 M and 9790 strain is probably a complex series of syntheses, degradations and interconversions with diPG synthesize either via condensation of two PG molecules to yield diPG and glycerol, thereby reducing the PG content as shown to occur in B. sterothermophilus (Card, 1973); or via cytidine-5'-diphosphate CDP diglyceride (Staracev et al., 1967). Reduction in the concentration of diPG could then occur via metabolism to PA and PG as seen in E. coli (Hostetler et al., 1972), S. aureus (Short and White, 1972), M. lysodeikticus (De Siervo and Salton, 1971). PG could then be degraded to PA and glycerol or converted to amino acyl PG, while PA would enter the synthetic pathways for synthesis of phospholipids or glycolipids (Krag et al., 1974). The latter molecules would be lost, as non-phosphatides were not quantitated in this study.

Results of this work then with the group D streptococci indicate that membrane chromosome complexes comparable in DNA, RNA, and ultrastructural to those of E. coli and B. megaterium
can be isolated by the M-band technique. To accommodate differences in susceptibility to lysozyme exhibited by the streptococci and to induce adequate cell disruption necessary for release of the complex, the lytic procedure of the original M-band technique as utilized by Ballesta et al. (1972) had to be modified. The resulting procedure included lysozyme, EDTA, pronase and Sarkosyl, with the EDTA and possibly the pronase serving to slow down lysozyme action and thus allow lysis only upon addition of detergent.

In agreement with the literature (Ballesta et al., 1972) effects of such variation did not seem to significantly alter the composition of streptococcal complexes isolated, as judged by the above parameters.

As the DNA membrane complexes of the group D streptococci examined appear mundane, especially those of the 615 M and 9790 strains in terms of DNA, RNA, and ultrastructure, no great advantage is seen in studying complexes of these strains as opposed to those of E. coli and B. megaterium. At the outset it appeared as though their lack of phosphatidyl-ethanolamine as a phospholipid component might prove a significant factor in organization of group D membrane chromosome association. Such does not seem to be the case however. This study has established however that such complexes do exist in group D streptococci supporting the Jacob et al. (1963) model of membrane associated DNA replication.

In addition, and most significantly, results of this study seriously question extrapolated assignments of phospholipid proportions and distributions found in M-bands to that
found in living cells, in the group D streptococci, *E. coli* and *B. megaterium* based upon altered phospholipid patterns observed as a result of analysis of cell lysates.

Questions could now be raised concerning the functioning of such complexes in replication, transcription, translation, etc. as little is known concerning replicative mechanisms of this group. Effects on the DNA membrane complex by growth under specific conditions (e.g. high salt) normally detrimental to *E. coli* and *B. megaterium*, but characteristic of the group D streptococci would also be interesting especially as the 564 P strain exhibits structures which resemble folded chromosomes reported to be induced by high salt (*Lerman et al.*, 1970 abstract) or upon completion of replication (*Worcel and Burgi, 1974*).
LITERATURE CITED


APPENDIX

EDTA disodium ethylenediamine tetraacetate
Fisher Scientific, Fairlawn, New Jersey

Lysozyme chicken egg white
Worthington Biochemical, Freehold, New Jersey

Pronase free from nucleases, B grade
Calbiochem, San Diego, California

Govine Serum Albumin Fraction V (Fisher Scientific)

Sarkosyl sodium lauroyl sarcosinate
Schwarz Mann, Division of Becton, Dickinson and Company, Orangeburg, New York

Sucrose special enzyme grade For work with E. coli
Schwarz Bioresearch B/r(w) and B. megaterium
Orangeburg, New York ultra-pure density gradient
grade sucrose, Schwarz Mann
grade sucrose, Schwarz Mann

Tris (THAM) Tris(hydroxymethyl)aminomethane
Fisher Scientific

TCA trichloroacetic acid

Phage broth 10 g Bactopeptone
5 g Sodium Chloride
3 g Beef Extract
1 g Dextrose (autoclaved separately at 18 lb pressure for 20 min, then added to other sterile ingredients)
1 liter distilled water
Autoclave all but dextrose for 15 min at 121° C,
18 lb pressure
Modified C media (500 ml)

\[
\begin{align*}
\text{NH}_4\text{Cl} & \quad 1.00 \text{ g per } 200 \text{ ml} \\
\text{Na}_2\text{HPO}_4 & \quad 3.00 \text{ distilled } \text{H}_2\text{O} \\
\text{NaCl} & \quad 1.50 \\
\text{MgCl}_2 \cdot 6\text{H}_2\text{O} & \quad 0.04 \\
\text{Na}_2\text{SO}_4 & \quad 0.04 \\
\text{Casamino acids (DIFCO)} & \quad 2.50 \\
\text{glucose} & \quad 0.50 \text{ g per } 100 \text{ ml} \\
\text{sucrose} & \quad 100.00 \text{ g } 200 \text{ ml of solution} \\
\end{align*}
\]

Autoclaved each separate solution designated above for 15 min, 121 C. Sterile solutions were then aseptically combined.

Semi-synthetic media for group D streptococci

<table>
<thead>
<tr>
<th>Salts</th>
<th>g/l</th>
<th>m/stock/l</th>
</tr>
</thead>
<tbody>
<tr>
<td>sodium citrate</td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td>sodium acetate</td>
<td>0.5</td>
<td></td>
</tr>
<tr>
<td>(NH_4)_2SO_4</td>
<td>2.0</td>
<td></td>
</tr>
<tr>
<td>MgSO_4 \cdot 7H_2O</td>
<td>0.2</td>
<td></td>
</tr>
<tr>
<td>MnSO_4 \cdot H_2O</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>FeSO_4 \cdot 7H_2O</td>
<td>0.01</td>
<td></td>
</tr>
</tbody>
</table>
Vitamins

<table>
<thead>
<tr>
<th>Vitamin</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>nicotinic acid</td>
<td>0.01</td>
<td>20 ml ea</td>
</tr>
<tr>
<td>thiamine</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>riboflavin</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Ca pantothenate</td>
<td>0.01</td>
<td></td>
</tr>
<tr>
<td>biotin</td>
<td>0.03</td>
<td></td>
</tr>
<tr>
<td>paraaminobenzoid acid</td>
<td>0.02</td>
<td></td>
</tr>
<tr>
<td>pyridoxal phosphate</td>
<td>0.04</td>
<td></td>
</tr>
<tr>
<td>folic acid</td>
<td>0.02</td>
<td></td>
</tr>
</tbody>
</table>

Each vitamin solution was prepared and autoclaved as a separate solution and 20 ml/liter of media added aseptically. Vitamins must be made fresh each time as they do not store well even when in frozen state.

Casamino Acids

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vitamin-free casamino acids</td>
<td></td>
<td>200 ml</td>
</tr>
<tr>
<td>tryptophan</td>
<td>.2</td>
<td></td>
</tr>
<tr>
<td>cysteine-HCl</td>
<td>.04</td>
<td></td>
</tr>
</tbody>
</table>

PO₄ buffer stock solution m/stock/1

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>K₂HPO₄</td>
<td>0.5 M</td>
<td>combined using 0.125 M solution</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>0.125 M</td>
<td>to adjust 0.5 M solution to pH 7.2</td>
</tr>
</tbody>
</table>

Purines and pyrimidines

<table>
<thead>
<tr>
<th>Component</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>quanine</td>
<td>.1 g dissolved in small amount 4N KOH 25 ml ea</td>
</tr>
<tr>
<td></td>
<td>dilute to 25 ml with distilled water</td>
</tr>
</tbody>
</table>
uracil \(0.1\ g / 25\ ml\) in \(0.01\ \text{NKOH}\)

adenine \(0.04\ g / 25\ ml\) in \(0.01\ \text{NKOH}\)

tymine \(0.04\ g / 25\ ml\) in \(0.01\ \text{NKOH}\)

Each of above solutions were autoclaved separately, combined when warm but not hot.

Glucose \(20\ g / 100\ ml\)

\(H_2O\)

final pH 6.5 - 7.0

Each category autoclaved separately unless otherwise indicated for 15 min at 121° C and combined aseptically while warm but not hot.

TES \(0.03\ \text{M Tris}-0.005\ \text{M EDTA}-0.05\ \text{M NaCl}, \text{pH} 8.0\)

TEK \(0.02\ \text{M Tris}-0.01\ \text{M EDTA}-0.06\ \text{M KCl}, \text{pH} 7.1\)

TMK \(0.01\ \text{M Tris}-0.01\ \text{M EDTA}-0.10\ \text{M KCl}, \text{pH} 7.0, 0^\circ C\)

Cellulose Nitrate Tubes 1/2" x 2" Beckman, Palo Alto, California

Ethidium Bromide 2, 7 diamino-10-ethylphenyl phenanthridium bromide

B grade

Calbiochem

DNA Calf Thymus Mann Research Laboratories, Inc.

New York, New York

RNA Torula (yeast)

Calbiochem

RNAse (free of phosphate)

Worthington Biochemical
Copper grids 300 and 400 mesh size

Ladd Research Industries, Inc., Burlington, Vermont
Pelco, Costa Mesa, California
E. F. Fullham, Schenectady, New York

Formvar E. F. Fullham

Ethylene dichloride EM grade
E. F. Fullham

Solvent Purification

$\text{CHCl}_3$ Chloroform J. T. Baker Chemical Company, Phillipsburg, N. J.

To remove phosgene, formed by photochemical reaction and HCl from chlorinated solvents, solvent was washed with water, dried over anhydrous calcium chloride and distilled in a glass still over calcium chloride. To inhibit further production of phosgene, 1% methanol is added and the solvent stored protected from light (Skipski, 1967; Kates, 1972).

MeOH Methanol Mallinckrodt Chemical Works, St. Louis Missouri

Fisher Scientific

Traces of aldehydes and amines were removed by distillation with potassium hydroxide pellets, followed by storage protected from light, preferably in brown bottles (Christie, 1973).

H$_2$O Water

Redistilled in an all glass still.
Acetone  J. T. Baker Chemical Company
Redistill under anhydrous conditions in the presence of potassium permanganate and potassium carbonate.

Light Petroleum for "fat determination"
Hexane was distilled and the fraction collected at 42-58°C and considered to be light petroleum.

Glassware
For lipid studies and distillation of solvents to be used in lipid procedures glass ware was acid washed with dichromate-sulfuric acid to remove any lipid material and residual detergent. Thorough rinsing with both tap water and distilled, deionized water was followed by heating at 320°F for 1 hr.

For studies involving radioactive labelling of DNA, all glassware was additionally coated with silicone (Siliclad, Plastic disposable pipets were used whenever DNA and ^32P was handled due to adsorptive properties exhibited by them to glass.

Plastic disposable pipets and Nalgene tubes were employed in fluorometric analyses for the same reasons.

Phospholipid Standards
PE phosphatidylethanolamine (bacterial) crystalline Pierce Chemical, Rockford, Illinois
PS phosphatidylserine (ex. bovine brain) crystalline A grade Calbiochem
PG  phosphatidylglycerol  (in CHCl₃:MeOH 2:1 v/v)
    Supelco, Bellefonte, Pennsylvania
PA  phosphatidic acid  (in CHCl₃)
    Supelco
diPG diphosphatidylglycerol
    Cardiolipin  (bovine, in CHCl₃)
    Supelco
    (in absolute ethanol)
    General Biochemicals, Chagrin Falls, Ohio