A Comparative Study of Certain Lipids of *Micrococcus marepuniceus* and *Micrococcus infimis*

by

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DECLARATION

I hereby declare that this thesis is a record of the work carried out by me, that the thesis is my own composition, and that no part of it has been presented previously for a higher degree.

This research was carried out in the Department of Biochemistry in the University of St. Andrews under the supervision of Dr. D. Thirkell.

(S. S. Baznboor)

CAREER

I first matriculated at the University of Baghdad in October, 1964, and graduated with a degree of Bachelor of Science in Zoology and Microbiology in June, 1968

I worked for one year as a science teacher in Mukalla Secondary School and then took up employment in the Health Service in the People's Democratic Republic of Yemen as a clinical microbiology laboratory technician.

In December, 1973, I was awarded a Fellowship by the World Health Organisation to study first in the Western General Hospital microbiology laboratories in Edinburgh and then to undertake a research degree in St. Andrews University. I first matriculated as a research student under Ordinance 51 in the Faculty of Science (Department of Biochemistry), St. Andrews University in October, 1975 to work under the supervision of Dr. D. Thirkell.

CERTIFICATE

I hereby certify that Saleh Salim Baznboor has spent seven terms engaged in research work under my direction and that he has fulfilled the conditions of the Resolution of the University Court, 1974, No.2, and that he is qualified to submit the accompanying thesis for the Degree of Master of Science.

(Dr. D. Thirkell)

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CONTENTS

AIMS OF THE THESIS

The main lines of investigation in this work were as follows:-

1. To determine the best growth medium for *Micrococcus marepuniceus* and for *Micrococcus infimus*.
2. To investigate the feasability of membrane preparation from the two above bacteria.
3. To grow up batch cultures of cells of the two bacteria from which to extract lipids.
4. To separate the total lipid extracts from the two bacteria into neutral lipids, glycolipids and phospholipids and to determine the distribution of these lipid classes in the two bacteria.
5. To carry out qualitative and quantitative comparisons of the neutral lipid and phospholipid fractions from the two bacteria.
6. To analyse in detail, the fatty acid composition of the relevant lipid fractions.
7. To analyse the hydrocarbon, sterol ester and long chain alcohol fractions from the two bacteria.

INTRODUCTION

Living bacteria have been found in virtually all of the different environments on this earth. Each type has adapted with special mechanismsand devices to overcome any handicap that it might face. Some bacteria live in unusual environments such as hot springs and salt marshes, and the latter have received a great deal of attention in the last few years. These bacteria are called halophiles (salt-loving bacteria) and are found wherever sodium chloride and other ions occur in adequate concentrations to support their growth, e.g. the lake of Utah in the U.S.A., in the Dead Sea of Palestine (from which two types of halophiles are known - M. morrhuae and M. litoralis) and salterns, and they remain alive even in salt crystals (Rheinheimer, 1971). In salt lakes with a high concentration of salt, the majority of bacteria are extreme halophiles; the proportion of salt tolerants as a rule is small (ZoBell, 1946). The optimum salt concentration for extreme halophiles ranges from 20-30% (w/v) but they do not grow at all at concentrations of sodium chloride under 10%. The sodium, chloride and magnesium ions are required to maintain cell structure and rigidity. As the concentration of these ions is reduced, the cell wall "dissolves" and the cell membrane breaks up into tiny fragments (Larsen, 1973). A requirement for magnesium ion concentrations of 0.1 - 0.5 M in any medium is necessary to support good growth and this is 1 - 2 orders of magnitude higher than for bacteria in general (Brown and Gibbons, 1955). On the other hand, their optimum requirement for potassium ions (K^+) is comparatively modest, about 25 mM (Gochnauer and Kushner, 1969). Experimental evidence has shown that the internal salt concentration of halo-

philes is just as high, or possibly even somewhat higher than that of the environment. The dominant anion is Cl^- as might be expected, but the cation concentrations show greater variability (Christian and Waltho, 1962). Frequently, the dominant internal cation is K^+ which may reach a concentration in the cells which is close to the solubility limit in water for this ion, and it has been claimed by both Rheinheimer (1971) and by Larsen (1973) that potassium ions are required to maintain the structural integrity of the ribosomes, for protein synthesis, and to maintain the intracellular enzymic activities. It has been shown that the enzymes of the extremely halophilic bacteria are extremely salt tolerant and in most cases, even strikingly halophilic themselves (Baxter and Gibbons, 1956). Thirty different enzymes have been tested from halophilic bacteria and in each case, they are found to function at sodium chloride concentrations approaching saturation (Larsen, 1973). Interestingly, these enzymes are generally even more strongly activated by potassium chloride than by sodium chloride which fits nicely with the finding that potassium is a dominating component of the salts inside the cells. It could thus be postulated that potassium is an activator of the enzymes in the intact cells. In the mild halophile, Planococcus citreus Migula, Thirkell and Summerfield (1977a) however, showed that the membranes of this microorganism preferentially concentrated the divalent cations, Mg^{++} and Ca^{++} from the surrounding medium.

As a rule, the halophilic bacteria are pigmented due to the presence of carotenoids (Anderson, 1954; Larsen, 1967). It has been shown experimentally that these carotenoids provide pro-

tection for the bacteria against sunlight. The phenomenon is just another example in support of the old thesis "survival of the fittest" (Larsen, 1973).

Extreme halophilism is a character which is neither acquired nor lost. It is found only among bacteria and is confined to two genera (Larsen, 1962; 1967). These genera are distantly related but are selected for extreme halophilism by their adaptation to their environment. The two genera of halophilic bacteria were assigned to the orders Pseudomonadales, family Pseudomonadaceae, genus Pseudomonas, and Eubacterials, family Micrococcaceae, genus Micrococcus (Breed, Murray and Smith, 1957). But recently, and according to Buchanan and Gibbons (1975), halophilic bacteria are classified, as proposed initially by Schoop (1935) into one family, Halobacteriaceae with two genera, Halobacterium (the salt bacteria) and Halococcus (the salt coccus).

Halobacteria are rods which require more than 12% (w/v) sodium chloride for growth, which grow optimally in the presence of 25 - 30% (w/v) salt, and which grow well even in saturated salt solutions (Larsen, 1962). They have a thin and fragile cell envelope composed of an outer proteinaceous coat and an inner membrane structure and these two layers may be broken down by removal of salt from their growth media (Steensland and Larsen, 1969; 1971; Larsen, 1973).

Non-halophilic microorganisms depend on their cell wall for support and rigidity and to prevent swelling of the cells and rupture of the membranes in hypotonic solutions. In gram-positive bacteria, the peptidoglycan (mucopeptide) constitutes the major component of the dry weight of the cell wall

(50 - 90%). The rest of the wall is made up of proteins, polysaccharides and teichoic acids. Peptidoglycan, common to both gram-positive and gram-negative cells, is normally a polymeric structure composed of strands of amino sugars in one dimension, and cross-linked through branched polypeptides to other sheets of glycan strands. The backbone of the glycan polymer consists of alternating sub-units of N-acetyl glucosamine and N-acetyl muramic acid connected by β-1-4 linkages. The peptides are bound through their N-termini to the carbonyl group of muramic acid, and usually contain four alternating L- and D- amino acids. L-alanine is bound to the carbonyl group of muramic acid, followed by for example, D-isoglutamine, L-lysine and D-alanine. The carbonyl group of D-alanine and the ε-amino group of lysine are now available for cross-linking either directly to one another as in E. coli, or through an interpeptide bridge, as in Staph. aureus (Schleifer and Kandler, 1972). The chemical composition of the cell wall of extreme halophiles, and particularly in halobacteria, is different in that it does not contain muramic acid or diamino-pimelic acid which are the usual cell wall "building blocks" in non-halophiles. For example, these compounds were shown to be absent from the cell walls of Sar. litoralis and of Sar. morrhuae by Brown and Cho (1970), from several halobacteria by Brown and Shorey (1963) and from nine strains of halococci by Reistad (1975). He also found that the total amount of amino acids and hexosamines constituted 7-15% of the dry weight of the cell walls which is far below the amount of peptidoglycan in the cell walls of common non-halophilic

micrococci. It is likely however, that another polymer with the functions of peptidoglycan is present in these halophilic cells.

Furthermore, it is known that penicillin inhibits cross-linking of peptidoglycan, while lysozyme (muramidase) brings about the solubilisation of this layer as a consequence of the hydrolysis of the -1-4 glycosidic linkages between carbon 1 of muramic acid and carbon 4 of N-acetyl glucosamine. In both mechanisms (antibiotic action and enzymic action) the end result is that bacteria lose their protective wall coating and rigid shape and the protoplasts/spheroplasts formed become roughly spherical. Due to the differences in the chemical composition of the peptidoglycan or equivalent layer between halophilic and non-halophilic bacteria, Brown et al. (1970), for example, noted that lysozyme had no action on extremely halophilic cocci.

The major fraction of the halobacterial cell wall appears to be lipoprotein with a protein to lipid ratio of 3:1. Particularly the protein moiety of this, is highly acidic (the amount of acidic amino acids is high and the amount of basic amino acids is low), and it is claimed that the excess charge must be neutralised by cations of the salts in order to preserve the necessary corformational state for it to perform its functions. Thus, if salt is removed, as stated earlier, repulsiv forces will denature the proteins, particularly the structural proteins, and these may then even go into solution. In addition, very little amino sugar of any kind is present in the cell envelope (0.5 - 1.5%) (Larsen, 1973).

Larsen (1973) reported that the genus Halococcus are typical micrococci with a minimum growth requirement of 2 M sodium chloride and an optimum requirement of 4.5 M sodium chloride. The halococci retain their shape in lowered concentrations of salt and appear to be more resistant to osmotic change than halobacteria even though their cell walls may be similar in not containing muramic acid or diamino-pimelic acid. Brown et al. (1970) and Steensland et al. (1971) claim that this is due to the fact that the halococci (micrococcus type) possess a relatively thick wall composed of a polysaccharide which successfully resists forces which might start to act once salt is removed.

In halococci, the guanine (G) + cytosine (C) content of the DNA is 67 moles% for the major component (97% of total DNA) and 59 moles % for the minor component (3% of total DNA). In halobacteria, the G + C content of the DNA is 66-68 moles % for the major component and 57-60 moles % for the minor component (Moore and McCarthy, 1969). It was according to this G + C ratio that the taxonomic status of the halococci has been clarified, mainly by the work of Kocur, Bergan and Mortensen (1971) and of Kocur and Bohacek (1972). On this basis, Kocur suggested that they be placed in a separate genus, the Halococcus, instead of the genus Micrococcus as originally grouped.

Among the two genera some major differences exist (Larsen, 1967). It has been mentioned that halobacteria readily lyse in hypotonic solutions whereas halococci do not. Before they lyse, halobacteria become sticky and this is claimed to be due to DNA adhering to the outer surface of the cells, having previously

leaked out of the cell. Perhaps this implies a role of salt in the integrity of the membranes of these bacteria. The generation time for halobacteria is often quoted as not less than seven hours whereas for halococci, it is often quoted as not less than twelve hours. Halobacteria are gram-negative rods, sometimes motile, whereas halococci are gram-variable, non-motile cocci. Neither genera produce spores.

It has been previously mentioned that the enzymes of the halophilic bacteria display optimal efficiency at salt concentrations at or around those in the cells, and in most cases, activity is lost in the absence of salt. But not all enzymes exhibit optimal activity at the salt concentration within the cell suggesting that certain cellular enzymes cannot operate maximally under normal conditons. It has been suggested that in extreme halophiles there are three classes of enzymes:

(i) obligate halophilic enzymes, e.g. aspartate transcarbamylase, cytochrome oxidase and citrate synthetase.

(ii) salt tolerant enzymes, e.g. malic dehydrogenase and isocitric dehydrogenase.

(iii) enzymes requiring no salt for activity, e.g. RNA-dependent RNA polymerase, fatty acid synthetase and amylase (Lanyi, 1974).

As the fatty acid synthetase is not salt dependent, its activity may be suppressed at high salt concentrations. This would be consistent with the findings that the membrane lipids of halophilic bacteria contain almost exclusively ether-linked analogues of phospholipids, and in fact, in some species, fatty acids have not been found at all (Kates, Palameta, Joo, Kushner and Gibbons, 1966). The inhibition of amylase shown in

H. halobium may be linked with the observation that many halophiles do not grow well in media which contain carbohydrates as the sole carbon source, and in such cases, amino acids are the usual carbon source supplied. Some of these amino acids may be essential, e.g. valine, methionine, leucine and iso-leucine in the case of H. salinarium, whereas the other amino acids merely stimulate growth (Larsen, 1967). Aitken and Brown (1969), working with H. salinarium, demonstrated both citrate and glyoxylate cycles. The citric acid cycle would be expected in microorganisms utilising amino acids, but this is one of the few halophiles in which the glyoxylate cycle has been demonstrated. Sar. morrhuae can utilise glucose normally, but here amylase would not be involved (Hunter, 1976).

Mild or moderate halphiles are classed as those microorganisms which can tolerate sodium chloride concentrations of between 5-10%. They have a lower limit below which no growth occurs, and this may be only 1% or 2% sodium chloride (Larsen, 1967; Gibbons, 1969).

A third type of halophile exists and these are facultative halophiles but since they will grow, not necessarily well, in the complete absence of added salts, they are not completely halophilic in nature. Both M. marepuniceus and M. infimus are gram-positive facultative mild halophilic cocci which will produce orange to pink pigments in/on suitable media within 2-3 days at room temperature.

It is now accepted that lipids and proteins can associate together to form membranes. From the Davson-Danielli/Robertson/Singer models, the lipid-globular protein mosaic model for membranes was proposed. It stated that the membrane structure

comprises a lipid bilayer with the polar groups of the lipids exposed at the surface and the hydrophobic fatty acid chains occupying the interior. Some protein penetrates into the hydrophobic interior of the bilayer, some polypeptides may traverse the bilayer, and other proteins may be associated with the polar surface of the bilayer. The proteins, lipids and carbohydrates are asymmetrically distributed in the structure and together form a mobile, flexible yet cohesive sheet (Robinson, 1975). The present membrane structural models also apply to bacteria.

As a generalisation, lipids constitute between 20-30% of the membrane dry weight but the nature of the lipid varies according to bacterial species. One interesting feature is that diphosphatidyl glycerol, found in the membranes of microorganisms, also appears in mitochondrial inner membranes and possibly in lysosomal membranes, but not in other membranes from mammalian cells (Lockwood, 1974; Robinson, 1975). Cholesterol as such is not usually found in microorganisms. However, it must be noted that where present, cholesterol influences the packing of phospholipids in membranes and its presence in high concentration in some membranes may be physiologically significant (Lockwood, 1974; Robinson, 1975).

Virtually all the lipids in bacteria are associated with their membranes. Part of the evidence on which the above is based is that if such cells are lysed, the cytosol from these cells contains no lipid (Salton and Freer, 1965). Thus lipids obtained from whole cell homogenates will consist of only those lipids which are associated with the membranes of the cell. The other alternative location for lipid is inside bacterial cells as storage lipid globules in the form of poly-β-hydroxy butyric acid (Baird-Parker and

Woodroffe, 1967). This polymeric material is soluble in chloroform, diethyl ether and petroleum ether, but is resistant to potassium hypochlorite solution. Its production is stimulated in certain species when in the presence of nitrogen and phosphorus, protein synthesis and growth are inhibited, or when the cells have reached the stationary phase of growth. This has been confirmed by many workers (Lemoigne, 1926; Lemoigne and Girard, 1943; Schlegel, Gottschalk and Von Bartha, 1961).

Until 1967, steroids had been encountered only in eukaryotic organisms and had not been detected in prokaryotic organisms (the bacteria and blue-green algae). In recent years sterols, sterol esters and the sterol precursor, squalene, have been detected in several bacteria. Thirkell and Summerfield (1977b) reported the presence of both sterols and sterol esters in Planococcus citreus Migula. They also showed that the hydrocarbon fraction of the membrane lipids of this microorganism, grown in the presence of different sea salt concentrations, was very rich in squalene, and that squalane (perhydrosqualene) was also present. The amount of squalene decreased with increasing salt concentration in the medium and they suggested that increasing concentration of salt may suppress terpenoid biosynthesis since they also reported that the production of carotenoids was similarly suppressed. Squalene has also been reported in several classes of microorganisms, for example methane utilisers such as Methylococcus capsulatus, extreme halophiles such as H. cutirubrum, certain streptomyces such as Streptomyces olivaceus and so on (Bird, Lynch, Pirt, Reid, Brooks and Middleditch, 1971). Further work on the terpenoid hydrocarbons of H. cutirubrum was reported by Kramer,

Kushwaha and Kates (1972).

Plachy, Lanyi and Kates (1974) noted during studies on dispersions of the polar lipids isolated from H. cutirubrum that in the absence of added magnesium and sodium chlorides, lipid fluidity increased. This supports the theory that salt has a stabilising effect on the lipid components. Investigations into the inter-relationships between the membrane lipid components of H. cutirubrum have revealed that squalene may play an important role in maintaining the stability of the lipid layer. Lanyi, Plachy and Kates (1974) showed that the polar lipid of H.cutirubrum (mainly di-O-phytanyl phosphatidyl glycerophosphate and a glycolipid sulphate) would not show aggregation and flocculation with calcium and magnesium, as do lipids from other bacteria, unless squalene was also present in the bilayers at or above 6-8% of the total lipid. This is consistent with the theory that aggregation is dependent on the ability of magnesium ions to penetrate the head group regions of the polar lipids, and that the role of squalene is to space the molecules sufficiently apart to permit entry of the divalent cations to the inner charged phosphate group of the phosphatidyl glycerophosphate. Squalene is a linear molecule, 28-29 Å in contour length, and if extended, would almost span the hydrophobic region of the bilayer.

Another terpenoid, menaquinone 8 has also been found in all the extreme halophiles in which its presence has been looked for (Brown et al. 1963; Kushwaha, Cochnauer, Kushner and Kates, 1974).

Aliphatic hydrocarbons were studied in some marine microorganisms by Oro, Tornabene, Nooner and Gelpi (1967). They claimed that the hydrocarbon

patterns were relatively simple and that the predominant hydrocarbons tended to be in the C17 region.

Little work has been published on the long chain alcohols from mild halophiles, but Thirkell et al (1977b) stated that the predominant components in Planococcus citreus Migula had apparent carbon numbers of 12.2 and 16.0.

Although the glycolipids of the two bacteria under investigation in the work to be reported in this thesis were not investigated, such compounds in bacteria have been reviewed by Shaw (1970), and Minnikin, Abdolrahimzadeh and Baddiley (1971) have discussed their inter-relationship with phospholipids with respect to membrane function.

A comparison of the amounts of lipid present in halophiles and in non-halophiles has shown that extreme halophiles have a much lower total lipid content than do other species. Values of less than 1% lipid as a percentage of the salt-free dry weight of extremely halophilic cells have been reported. The phosphorus content however, in extreme halophiles is high because of the large amounts of phosphatidyl glycerophosphate in their lipids (Kates et al., 1966). It was also claimed by Kates, Sehgal and Gibbons (1961) that some mild halophiles contain less phospholipid than do extreme halophiles.

Phosphatidyl glycerol and phosphatidyl ethanolamine were reported in the mild halophilic bacterium, M. halodenitrificans (Kates et al. 1961), and cardiolipin, lysocardiolipin, phosphatidyl glycerol and phosphatidyl ethanolamine were reported in Planococcus citreus Migula (Thirkell et al., 1977b). Examination of the phospholipids of other mild halophiles has shown that they are the more usual ester-linked types found in non-halophilic bacteria (Stern and Tietz, 1973) even when the bacteria are grown in salt concentrations comparable with the natural habitat of extreme halophiles. By contrast, the phospholipids in extreme halophiles are predominantly

unusual alkyl diether analogues of acidic phospholipids (Kates et al., 1961: Peleg and Tietz, 1971; Oliver and Colwell, 1973) as previously mentioned. Thus a comparison of the amounts of non-saponifiable material and fatty acids obtained from some extremely halophilic (e.g. H. cutirubrum, H. halobium(M), H. salinarium and Sar. litoralis), mild halophilic (e.g. M. halodenitrificans and V. costicolus)and non-halophilic bacteria (e.g. Sar. lutea and Sar. flava) showed that the amount of fatty acid is less than 1% in most of the extreme halophiles while the percentage of non-saponifiable material is high, ranging from 64-74%. The situation in both mild halophiles and non-halophilic bacteria so far as the fatty acids and non-saponifiable material is concerned, is the other way round, i.e. virtually all contain high percentages of fatty acids and low percentages of non-saponifiable material (Kates et al. 1966).

Although mild halophiles are similar to extreme halophiles in some aspects, for example, they contain acidic proteins and the membrane does become unstable under conditions of low salt concentration, the membranes of mild halophiles are generally of the type normally associated with non-halophilic bacteria. These membranes must have evolved through minor changes to allow the cells to function normally in their natural habitat. Because of the high acidity of the phospholipids found in extreme halophiles, it is not unreasonable to expect high acidity in the ester-linked phospholipids of mild halophiles.

The most usual way for cells to increase the acidity of their membrane lipids is to produce large amounts of acidic phospholipids, namely cardiolipin, phosphatidyl glycerol and phosphatidyl serine. Peleg et al., (1971) and Stern et al., (1973) have reported the

presence of glycolipids and glucosyl phosphatidyl glycerol in a moderate halophile, and Kates (1966) has reported the presence of cardiolipin, phosphatidyl glycerol and its amino acyl derivatives in M. halodenitrificans. These lipids are of course present in non-halophilic bacteria also, and it remains to be shown conclusively whether or not halophilic bacteria contain these lipids in higher proportions than their non-halophilic counterparts.

Evidence from differential scanning, calorimetry, X-ray diffraction and spin-label studies confirms the bilayer fluid arrangement of bacterial membrane lipids (Robinson, 1975). The chemical nature and composition of these bacterial lipids may vary according to a number of factors and let us first consider the effect of available substrate.

Numerous examples of lipid variation according to substrate availability (carbon source) are documented, particularly with respect to fatty acid composition even though the bacteria have similar pathways for fatty acid synthesis (Kates, 1964; Salton, 1967; Kaneda, 1967; 1968; Daron, 1970). Weerkamp and Heinen (1972a) found that the fatty acid patterns of B.caldolyticus were drastically altered if the B.H.I. complex (brain-heart infusion) was omitted from the medium, resulting in an increase of the branched-even, straight-even, straight-odd and ante-iso compounds, accompanied by a sharp decrease of the branched-odd fatty acids. Leucine, isoleucine or valine were found to act as precursors for the corresponding fatty acids with final concentrations as low as 0.05 M, leading to the predominance of either iso-odd or iso-even compounds. Greshnykh, Grigoryan, Dikanskaya, Dyatloritskaya and Bergelson (1968) demonstrated that there is a decrease in phos-

phoslipid and a concomitant increase in triglycerides in the alkane-utilising Yeast Candida tropicalis if nitrate replaces ammonium ions as the nitrogen source during growth. Razin (1967) showed that the fatty acid components of the phospholipids and neutral lipids of mycoplasmal membranes could be changed considerably by altering the fatty acid composition of the growth medium. It has also been shown that the total amount of lipid produced can be dependent upon the substrate in the medium (Mizuno, Shimojima, Iguchi, Takeda and Sench, 1966; Vestal and Perry, 1971). Comparison of the lipids of Thiobacillus neapolitanus grown in the presence of thiosulphate and in a sulphur-deficient medium, shows differences. The major alteration seen is with respect to the amounts of the individual phospholipids present rather than to a change in which phospholipids are produced. (Agate and Vishniac, 1973). They also showed that the amount of phospholipids present increased to a maximum during the stationary phase and then decreased. Similar results were shown with two different strains of a hydrogen-utilising bacterium (Thiele, Dreysel and Hermann, 1972). The amounts of lipids were shown to vary with the growth phase. Along with the accumulation of poly-B-hydroxy butyric acid as the major storage material, the amounts of other lipids were shown to increase 1.8 fold at the storage phase to decrease at the reutilisation stage. It was suggested that the 1.8 fold increase of the lipids during the storage phase is due to the synthesis of membrane lipids that are required for the formation of membranes surrounding the intracellular granules of accumulating poly-B-hydroxy butyric acid. Changes in the amounts of lipid present according to bacterial growth phases have been reported/

by other workers (Veerkamp, 1971; Hunter and Thirkell, 1971; Thirkell and Hunter, 1972; Thrikell and Gray, 1974).

Temperature is one of the most important factors affecting the growth of microorganisms and it can reflect qualitative and quantitative changes in their fatty acids (Weerkamp and Heinen, 1972b; Wilkinson, 1972). There is a minimum temperature below which growth will not occur. Above this temperature the rate of growth increases in accordance with normal laws governing the effect of temperature on the chemical reactions, mainly enzyme-catalysed, that make up growth. However, a point is reached where there is always a very rapid increase in the rate of inactivation of heat-sensitive cell components such as enzymes, ribosomes and DNA. This is the optimum temperature above which heat denaturation occurs so rapidly that there is a corresponding rapid drop in the rate of growth (Wilkinson, 1972). Marr and Ingraham (1962) and Gad and Hassan (1964) observed that the proportion of unsaturated fatty acids produced can decrease with increasing temperature. Richard (1972) proposed that this effect was directly related to an increase in the bacterial growth rate, and Thirkell, Strang and Carstairs (1965) also showed that temperature can govern the growth rate. Weerkamp et al. (1972b) also showed that temperature can affect fatty acid composition when working with B. caldloticus and B. caldotanex. They found that as the temperature was increased, the amount of iso-C_{15} and iso-C_{16} fatty acids was decreased and that the proportions of iso-C_{17} and n-C_{16} fatty acids were increased. Ray, White and Brock (1971) found that the total

lipid content of Thermus aquaticus was proportional to temperature. As the growth temperature was increased from 50° to 75°, the phospholipid content increased two-fold, the carotenoids 1.8 fold and the glycolipids four-fold. The amounts of phosphatidyl glycerol, cardiolipin, phosphatidyl ethanolamine and the fatty acid composition of Ps. fluorescens are affected not only by the temperature and rate of growth, but also by growth-limiting factors (Gill, 1975).

Most pigment producing non-photosynthetic bacteria tend to produce more carotenoids at a lower temperature than the optimum for growth (Goodwin, 1963) and this was shown to be the case in Sar. flava. It grows optimally at 34° and produces pigment optimally at 25° (Thirkell et al., 1965). However, in Sar. aurantiaca it was found that the optimum temperature for both growth and pigment production was the same (30°) (Thirkell and Gray, 1971). Below 30°, the production of pigment was substantially delayed.

According to their temperature requirements, microorganisms are classified as psychrophilic, mesophilic and thermophilic. Most microorganisms are killed above their maximum growth temperature, but not necessarily at low temperatures. Indeed, the ability of most bacteria to survive even at the temperature of liquid nitrogen, is the basis of what is becoming the most popular method for the preservation of microorganisms.

Whereas fatty acids with more than one double bond are rare in bacteria, unsaturated acids as a whole are not very common, and this is compensated for by an abundance of branched chain species which have similar packing arrangement in the membrane (Gurr and

James, 1971). This is important in considerations of membrane fluidity. However, studying the effect of age on bacterial fatty acid composition of Pyrenochaeta terrestris, Gunasekaran, Hess and Weber (1973) reported that as ageing progresses, the amount of unsaturation in the free fatty acids, the total fatty acids and the fatty acids of the di- and triglycerides increased. Hunter et al., (1971) using Sar. flava, showed that the major fatty acid, a saturated branched C_{15} component, decreased in concentration as growth proceeded. Their results also suggested either a very rapid turnover of fatty acids during the late exponential phase and early stationary phase or a considerable modification of existing fatty acids within the membranes. The saturated branched C_{15} fatty acid has been reported as the major fatty acid in several gram-positive bacteria (Akashi and Saito, 1960; Albro and Dittmer, 1964; Cho and Salton, 1966; Morrison, Tornabene and Kloos, 1971). The amounts of phospholipids have also been shown to reflect the age of the bacterial culture from which they were extracted. Stern et al., (1973) reported that the amounts of glucosyl phosphatidyl glycerol and phosphatidyl ethanolamine did not change as the cultures grow older, but in contrast to these two phospholipids, the relative amount of phosphatidyl glycerol decreased and that of cardiolipin increased as the cultures grew older. This reciprocal relationship between phosphatidyl glycerol and cardiolipin has been reported by others (Cronan, 1968; Randle, Albro and Dittmer, 1969; Short and White, 1972).

pH of the medium is another of the factors that have been shown to affect the chemical composition of bacterial lipids. It is known that there is an optimal pH which microorganisms

will prefer and at which they will multiply normally. At this pH the enzymatic activity within the microorganisms is at its best since enzymatic proteins have their usual conformation and their active sites are not affected. On the other hand, there is a pH range which microorganisms can tolerate. Below and above this range, microorganisms do not grow and may die. Such pH ranges are often species specific.

A pH tolerance study using Sar. aurantiaca showed a sharp cut-off in growth on either side of the pH range 6.2 - 7.5, and with an optimum for growth slightly on the acidic side of neutrality (Thirkell et al., 1971). The relative proportions of the individual polar lipids may also alter with pH variations. In B. subtilus, the concentration of lysylphosphatidyl glycerol has been shown to be high at neutral pH (Minnikin and Abdolrahimzadeh, 1974). Elevating the pH resulted in the disappearance of both lysylphosphatidyl glycerol and of phosphatidyl ethanolamine, but both these phospholipids can be detected if the pH is lowered. Similar results illustrating the pH effect on bacterial lipids have been reported by Haest, de Gier, Op den Kamp, Bartels and van Deenen (1972). It was suggested that the positively charged phospholipids could play a role in inhibiting protons from entering the cell from acidic media. Furthermore, Gould and Lennarz (1970) have shown that a decrease in phosphatidyl glycerol was due to a decrease in the total synthesis and to an increase in the catabolism of the phosphatidyl glycerol at pH of about 5.0.

It is obvious that to study the lipid profiles of microorganisms, and particularly where more than one microorganism is being investigated, so far as is possible, all growth and harvesting parameters must be kept constant. Even then, variab-

ility of results is not necessarily eliminated since it is equally important to standardise on a lipid extraction procedure and on all other experimental details. The effects of these factors are discussed in the"Discussion" section of this thesis.

MATERIALS AND METHODS

MATERIALS AND METHODS

Maintenance of bacterial cultures on solid media.

Cultures of Micrococcus marepuniceus (CCM 2180) and Micrococcus infimus (CCM 2179) were gifted by Dr. M. Kocur, University J.E. Purkyne, Brno, Czechoslovakia. They were both maintained on solid media at 30° and sub-cultured onto fresh media at weekly intervals. The composition of the maintenance medium was as follows: 1% (w/v) glucose, 1% (w/v) beef extract (Oxoid Ltd., Lab-Lemco powder), 1% (w/v) bacteriological peptone (Oxoid Ltd.,), 2% (w/v) Agar No.3 (Oxoid Ltd.), made up in 75% matured and filtered sea water and 25% tap water. The pH was adjusted to 7.4 and the medium was autoclaved at 15 lbs/sq" steam pressure (121°) for 20 min. The medium is a glucose-supplemented sea water agar recommended by Kocur, Pacova, Sovadina, Pleva and Marvanova (1975). The common use of matured rather than fresh sea water seems to be historical rather than being based on scientific grounds. Since bacterial numbers tend to decline logarithmically in sea water, it may be an attempt to use a reasonably sterile matured fluid as against a fresh one which may be polluted with a variety of microorganisms.

Liquid culture.

Qualitative investigation of the best medium for the growth of M. marepuniceus and M. infimus.

The following media were prepared:

Medium A : 1.3% (w/v) 'Oxoid' nutrient broth in distilled water.

B : 1.3% (w/v) 'Oxoid' nutrient broth + 1% (w/v) glucose in distilled water.

C : 0.5% (w/v) 'Oxoid' bacteriological peptone + 0.1% (w/v) 'Oxoid' yeast extract + 0.01% (w/v) ferric ortho-phosphate in sea water.

D : 0.5% (w/v) 'Oxoid' bacteriological peptone + 0.1% (w/v)

'Oxoid' yeast extract + 0.01% (w/v) ferric orthophosphate + 1% (w/v) glucose in sea water.

E : 1% (w/v) 'Oxoid' Lab-Lemco powder + 1% (w/v) 'Oxoid' bacteriological peptone in 75% (v/v) sea water and 25% (v/v) distilled water.

F : 1% (w/v) 'Oxoid' Lab-Lemco powder + 1% (w/v) 'Oxoid' bacteriological peptone in sea water.

G : 1% (w/v) 'Oxoid' Lab-Lemco powder + 1% (w/v) 'Oxoid' bacteriological peptone + 1% (w/v) glucose in 75% (v/v) sea water.

Each medium was prepared so that after autoclaving at 15 lbs/sq." (121°) for 20 min, the pH was 7.2 $\pm$ 0.1. Duplicate sets of "Universal" bottles of media were inoculated with equal volumes of a log. phase culture of either M. marepuniceus or M. infimus grown in the identical medium. The bottles were incubated at 30° with regular shaking and after 24, 48, and 72 h, it was obvious that the greatest density of cells was present when the microorganisms were grown in medium G. Growth did occur in all media. This result was not surprising since the broth was a glucose-supplemented recommended medium. This medium was therefore chosen for all subsequent liquid culture work with both bacteria.

Bacterial growth curves.

Duplicate flasks containing 250 ml medium G (pH 7.2) were inoculated with 20 ml of a log. phase culture of either M. marepuniceus or M. infimus grown in the same medium at 30°. The flasks were incubated in a New Brunswick G 25 orbital incubator-shaker at 30° with an agitation speed of 300 r.p.m. Samples were withdrawn at regular time intervals (4 hourly) from each

flask and the degree of growth measured by either turbidimetry or by the trichloroacetic acid (T.C.A.) precipitation technique as follows:

(a) Turbidity.

Absorbance at 610 nm measured the incident light which did not reach the photocell due to bacteria in suspension. The wavelength was chosen so that the pink cellular pigmentation could in no way interfere with the results obtained. Samples were withdrawn at regular time intervals and diluted with sterile medium to give an absorbance of less than 0.4 when read on a Unicam SP 600 spectrophotometer using glass cells with a 1 cm light path and against a blank of fresh sterile medium.

(b) T.C.A. precipitation technique (Stormonth and Coleman, 1972).

Since turbidity can give anomalous results if there is any tendency for a microorganism to clump, the growth curves were checked using this technique since it can be used with such bacteria. At regular time intervals, 2-5 ml (known volume) of bacterial culture were withdrawn and centrifuged in a bench centrifuge. The pellet was resuspended in a volume of distilled water equal to the original sample volume. The suspension, free from media, was subjected to ultrasonic disintegration for 4 x ½ min with ½ min cooling periods, and with the suspension surrounded with ice. 1 ml of the disrupted material was diluted with 1 ml of distilled water and 2 ml 10% (w/v) T.C.A. After thorough mixing, the absorbance was read at 610 nm on a Unicam SP 600 spectrophotometer using glass cells with a 1 cm light path and against a blank of distilled water.

Use of medium G for liquid cultures.

150 - 500 ml cultures of each microorganism were grown in a New Brunswick G25 orbital incubator-shaker at 30° with an agitation speed of 300 r.p.m. Such cultures were used both for individual experiments and as inocula for large scale batch cultures.

Large scale batch culture of M. marepuniceus and of M. infimus

12 litre batch cultures of the bacteria were grown in a New Brunswick MF 114 fermenter at 30° with an aeration rate of 12 litres/min (1 litre air/min/litre culture) and an agitation rate of 300 r.p.m. The medium used was medium G, MS Antifoam A was added to control foaming during growth, and the pH in each case was adjusted to 7.4 before autoclaving at 15 lbs/sq." steam pressure for 45 min. The sterilising time was chosen to allow a sufficient "heating-up" period. Each batch culture was inoculated with 300 ml of a log. phase culture grown as described above. The pH of each run was maintained at pH 7.0 by using an E.I.L. pH controller model 91B, coupled to a Delta pump charged with 5M NaOH. The progress of each batch culture was monitored by removing small aliquots at set time intervals and determining their absorbance at 610 nm. The cultures were harvested as soon as maximum cell numbers were reached (top of the log. phase/entry into stationary phase.)

Harvesting of large scale cultures.

Each culture was harvested on an air-turbine Sharples centrifuge. The rate of flow through the centrifuge was adjusted to give the fastest flow rate compatible with a clear effluent. After centrifugation, the solid cell paste was weighed and then stored at -20° until used.

Effect of lysozyme on bacterial cells.

In order to determine whether the lipids should be extracted from whole cells of the two bacteria, or whether to produce a membrane fraction from which to extract the lipids, the effect of lysozyme on the two bacteria was investigated. If lysozyme was effective in producing protoplasts, membranes could readily be prepared for this work, and if not, whole cells would have to be used.

Duplicate flasks containing 250 ml of medium G and lysozyme

at a concentration of 250 µg/ml were inoculated with either a log. phase culture of M. marepuniceus or with a log. phase culture of M. infimus (grown in the same medium at 30°) to give an initial optical density of about 0.5. As a control, duplicate flasks containing 150 ml nutrient broth + 1% (w/v) glucose and lysozyme at a concentration of 250 µg/ml were similarly inoculated with a log. phase culture of Micrococcus lysodeikticus. The flasks were then incubated at 30° in the New Brunswick G 25 orbital incubator-shaker. At hourly intervals, aliquots were withdrawn from each flask and the absorbance read at 610 nm against a distilled water blank. If the lysozyme is effective, a gradual drop in the optical density is to be expected, if the lysozyme has no action, the culture would be expected to follow the normal growth pattern.

Determination of cell moisture content of both microorganisms.

Six crucibles and lids were chromic acid-washed and dried to constant weight in an oven at 100° (cooled in a desiccator). Triplicate cell paste samples of known weight (about 1g) of each microorganism were weighed into the crucibles and each taken to dryness and constant weight in the oven at 100°. From the results, the dry weight of the cells was determined and by difference, the moisture content was recorded.

Extraction of lipids.

Preparation of solvents.

All solvents were dried and freshly distilled before use. The chloroform which is stabilised with 2% (v/v) ethanol was first washed with distilled water to remove the ethanol, then dried over anhydrous sodium sulphate and redistilled. Pyridine was re-distilled and stored over anhydrous sodium hydroxide pellets. Al of the solvents were then stored at room temperature in dark glass bottles in a light-proof cupboard.

Lipid extraction procedures.

Two different methods were used and these were as follows:

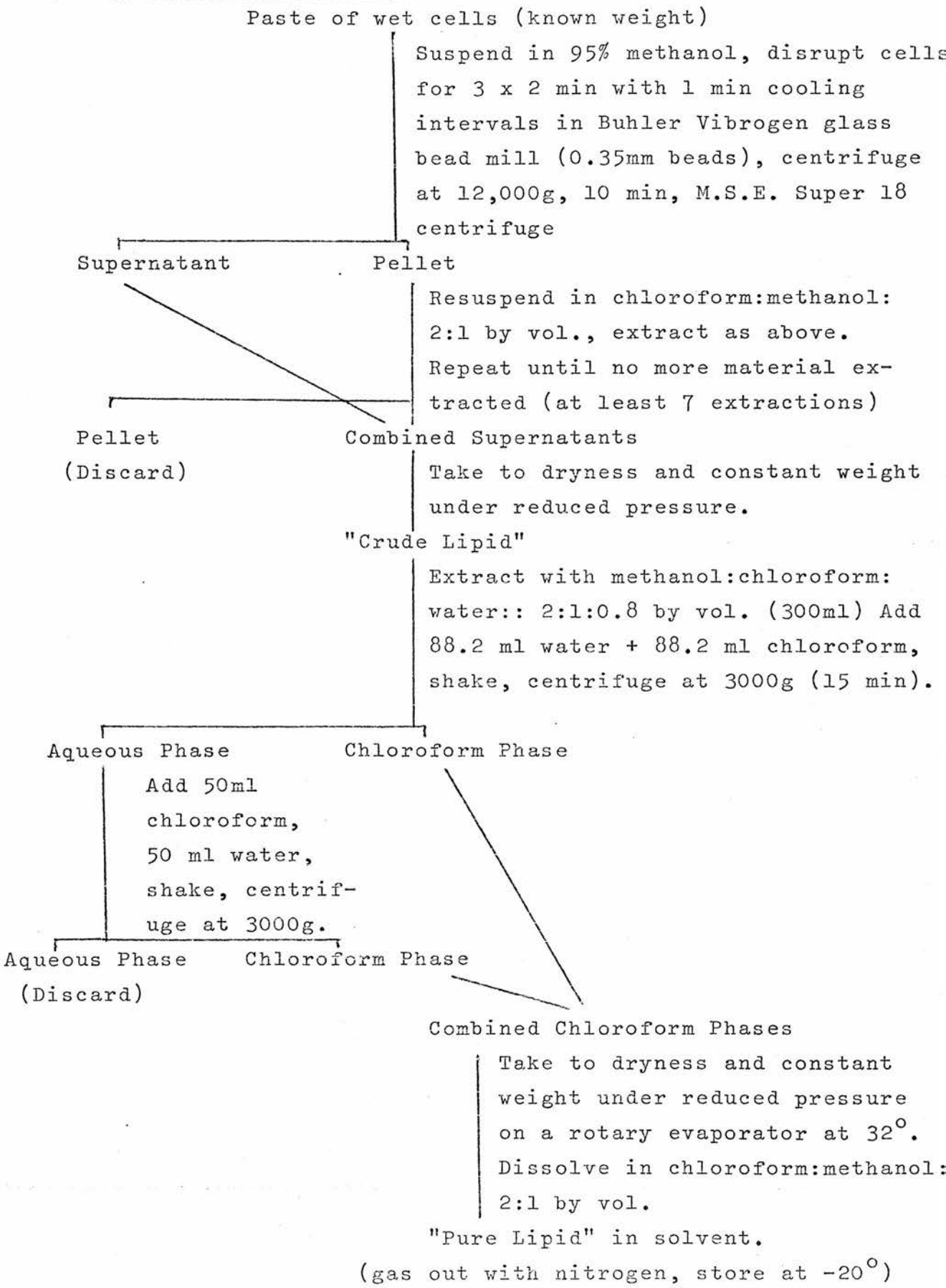
Method A.

This method is shown as a flow diagram in Fig. M1.

Method B.

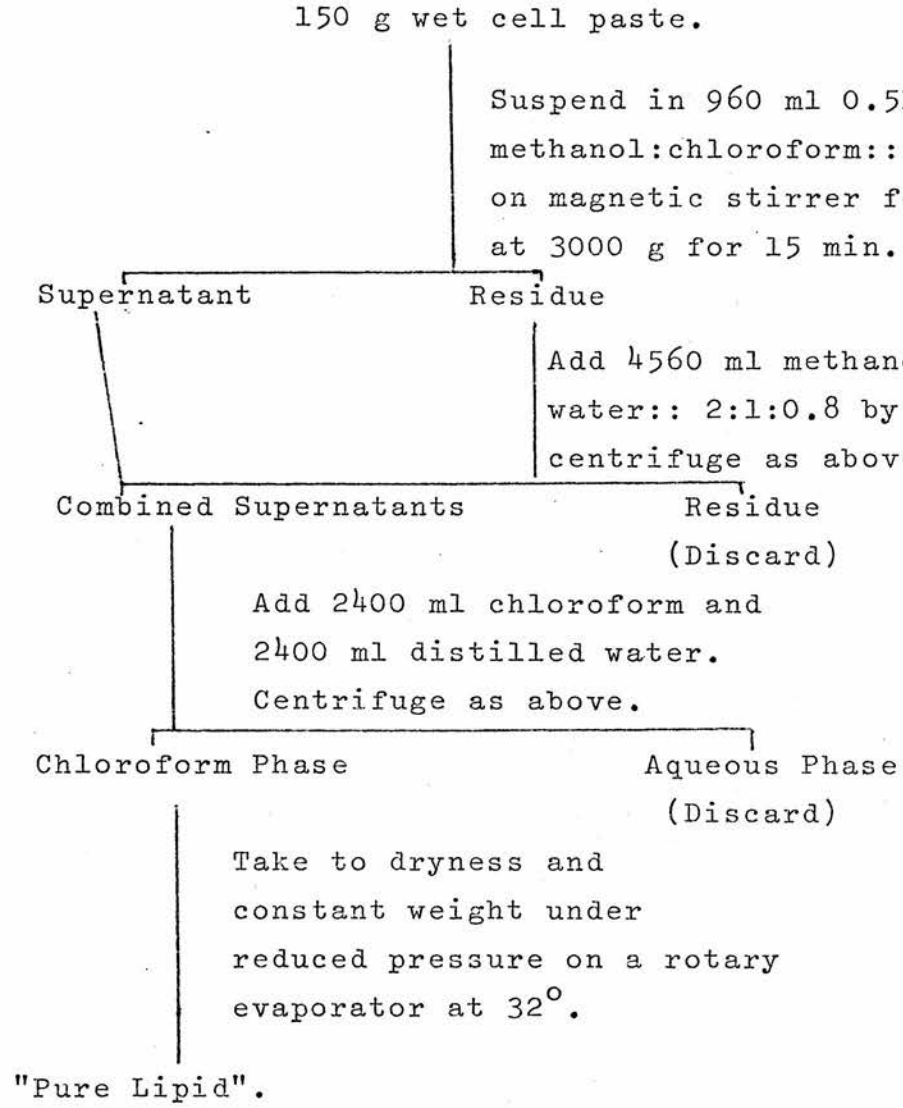
This method is shown as a flow diagram in Fig. M2.

Fig.M1: Method A for lipid extraction (Ballotini mill method)



NB. If one uses chloroform:methanol for the first extraction, centrifugation produces a biphasic system as a result of the moisture in the cell paste.

Fig.M2: Method B for lipid extraction (after Bligh and Dyer,1959)



Separation of the "pure" lipid into lipid classes.

The total extracted "pure" lipid from each microorganism and from each of the extraction procedures was separated into neutral lipids, glycolipids and phospholipids. In the first instance, almost all of the phospholipids were removed by cold acetone precipitation (Kates, 1972), and the acetone-soluble material, containing neutral lipids, glycolipids and residual phospholipids was separated by silicic acid column chromatography.

Cold acetone precipitation of phospholipids (Kates, 1972).

Since most phospholipids are insoluble in ice-cold acetone, the procedure can be used to separate most of the phospholipids from the more soluble neutral lipids and glycolipids. The removal of most of the phospholipids at this stage also aids the separation of the acetone-soluble material on subsequent column chromatography.

The reagents used were ice-cold acetone and 10% magnesium chloride ($MgCl_2.6H_2O$)(w/v) in methanol. The total lipid was dissolved in chloroform:methanol:: 2:1 by volume, and evaporated to about 2 ml with a jet of nitrogen gas. 50 ml of ice-cold acetone and 1 ml 10% $MgCl_2$ solution were added. The suspension was mixed thoroughly and cooled on ice for at least one hour. The pellet of precipitated phospholipid was recovered by centrifugation at 12,000g on the M.S.E. Super 18 centrifuge (10 min and this was re-extracted with 10 ml ice-cold acetone and cooled as before. Several extractions (at least eight) were necessary to obtain almost all of the phospholipids. The procedure was most easily carried out in a small Potter homogeniser to resuspend the pellet each time after centrifugation. The acetone supernatants, which contained the neutral lipids, glycolipids and small

amounts of phospholipids which had not been precipitated, were pooled and taken to dryness and constant weight under reduced pressure on a rotary evaporator at 30°. The phospholipid fraction was also dried to constant weight in a similar manner and then stored in an atmosphere of nitrogen at -20° until used.

Column chromatography to separate neutral lipids, glycolipids and residual phospholipids.

80 g of "fines-reduced" Mallinckrodt silicic acid (100 mesh) were activated by heating for 12 h at 110°. After cooling in a desiccator, the silicic acid, as a slurry in chloroform, was packed into a glass column of 3 cm internal diameter. All tubing connections between the solvent reservoir and the top of the column were of Teflon. The column was washed through with 480 ml chloroform. Each lipid sample, in a small volume of chloroform, was loaded onto a column and washed in with 10 ml chloroform. The column was then eluted with the following solvents (in order) and the flow rate was adjusted to 1.5 ml/min.:

480 ml chloroform to elute neutral lipids

400 ml acetone to elute glycolipids

400 ml methanol to elute any residual phospholipids.

Each fraction was taken to dryness and constant weight under reduced pressure on a rotary evaporator at 30°. The phospholipid material was added to the appropriate acetone-precipitated material.

Fractionation of neutral lipids

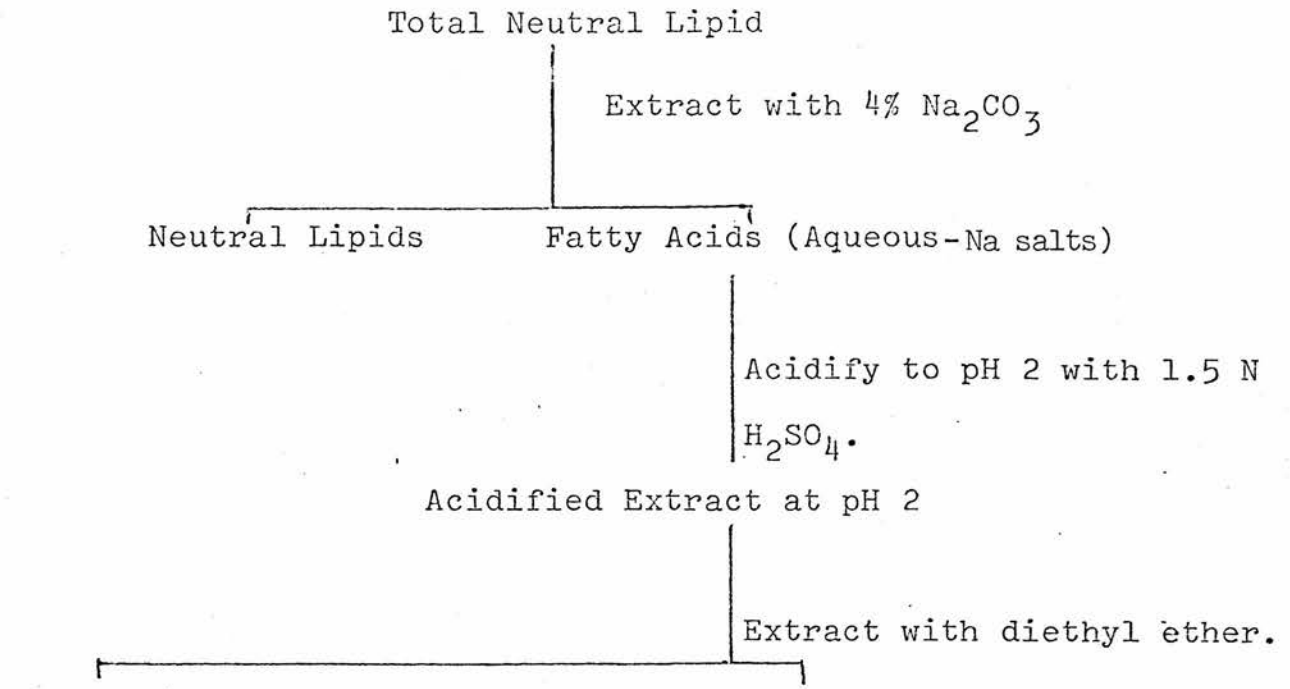
1. Investigation for the presence of poly-β-hydroxy butyric acid.

The method of Schlegel et al., (1961) was used. Isolation of poly-β-hydroxy butyric acid is dependent upon the insolubility of this compound in the presence of ice-cold diethyl ether. The neutral lipid fractions were each dissolved in 10 ml chloroform and cooled over ice to 0°. 12 ml ice-cold diethyl ether were

added to give a chloroform:diethyl ether ratio of 1: 1.2 by volume which the authors claim gives a 100% precipitation of the poly-β-hydroxy butyric acid. If a precipitate forms, this is recovered by filtration.

2. Removal of free fatty acids by carbonate wash (Dittmer and Wells, 1969).

Virtually all of the free fatty acids were removed from the neutral lipid fractions before they were subjected to chromatographic separation, by making the free fatty acids water-soluble. The neutral lipid fractions were taken up into 3 ml diethyl ether and these solutions were extracted four times with 1 ml 4% (w/v) sodium carbonate solution in a 10 ml glass separating funnel. The aqueous phase (lower) was washed with a further 2 ml diethyl ether which was added back to the etherial phase (upper). The aqueous phase was acidified to pH 2 by the careful addition of 1.5 N H_2SO_4 and re-extracted once with 2 ml diethyl ether and twice with 1 ml diethyl ether. This etherial phase which contains the free fatty acids, was washed three times with 1 ml distilled water and then dried over anhydrous sodium sulphate. The dried fraction was finally taken to dryness and constant weight under reduced pressure on a rotary evaporator at 30°. The method is best followed by reference to the following flow diagram:



The fractions were then stored under nitrogen at -20° until further examined.

3. Separation of the neutral lipids minus free fatty acids.

Two different procedures were investigated for this purpose. These were as follows:

A. Thin-layer chromatography (T.L.C.)

(a) Preparation of plates.

20 x 20 cm glass plates were thoroughly washed and dried and these were coated, in batches of five, with 0.25 mm layers of silica gel H (Merck) using the Desaga adjustable spreader (Camlab Glass Ltd.). After coating, the plates were dried and activated in an oven at 105° for 2 h before being cooled and stored in a desiccator until used.

(b) Chromatography.

The neutral lipids from M. infimus (extracted by method A), after removal of the free fatty acids, were concentrated to a few ml under a jet of nitrogen and resolution was attempted by applying a small aliquot to the surface of the silica with a micropipette. The chromatograms were developed in one of the following solvent systems. (All plates were pre-run in the solvent system to be used and reactivated before use).

(i) Single step solvent system: (Kates, 1972)

Solvent : Light petroleum (b.p. 60-80°): diethyl ether: acetic acid :: 70: 30: 1 or 70: 30: 2 by volume.

(ii) Two step development system (Kates, 1972)

Solvent A : Di-isopropyl ether: acetic acid:: 96: 4 by volume.

B : Light petroleum (b.p. 60-80°): diethyl ether: acetic acid:: 90: 10: 1 by volume.

Solvent A was allowed to proceed approximately 6 cm. up the plate at which time the plate was removed from the tank and dried at room temperature for at least 1 h. The plate was then introduced into solvent B which was allowed to run the entire length of the plate before the plate was again removed and dried at room temperature.

(c) Detection of the neutral lipid spots.

This was achieved by one of the following two systems which were:

(i) Iodine vapour (Kates, 1972)

The developed plate was placed in a sealed tank containing

several small crucibles of sublimed iodine. Yellow spots were rapidly seen against a white or pale yellow background. The iodine vapourised from the plates fairly quickly once they were removed from the tank.

(ii) Sulphuric acid-dichromate spray (Kates, 1972)

The spray reagent was 55% (by weight) sulphuric acid containing 1.2% (w/v) potassium dichromate. The reagent was sprayed onto the plate using an all-glass atomiser operating off a compressed air pump. The plate was then heated at 170° for about 30 min, and charred spots were seen against a white background.

B. Column chromatography (Kates, 1972)

For this, a column of 7% hydrated Florisil (B.D.H. Ltd.) was used. The material was prepared by shaking 100 g dry Florisil (60-100 mesh) with 7 ml distilled water on a wrist-action shaker overnight. 24 g of the 7% hydrated Florisil, as a slurry in hexane, were packed into a glass column of internal diameter 1 cm. The lipid sample, in a small volume of hexane, was applied to the top of the column and washed in with a small volume of hexane. The column was then eluted with the following solvents (in order) with a flow rate adjusted to about 3 ml/min.:

80 ml hexane to elute hydrocarbons

180 ml 95% hexane in diethyl ether (by volume) to elute esters (sterol esters and fatty acid esters)

300 ml 85% hexane in diethyl ether (by volume) to elute triglycerides

200 ml 75% hexane in diethyl ether (by volume) to elute sterols

200 ml 50% hexane in diethyl ether (by volume) to elute diglycerides

270 ml 98% diethyl ether in methanol (by volume) to elute monoglycerides

100 ml 4% acetic acid in diethyl ether (by volume) to elute any residual fatty acids.

All of the fractions were collected and reduced in volume and then checked for purity. This was done by T.L.C. on 0.25 mm layers of silica gel H (Merck) using the two-step development system by co-chromatography with authentic standards. In almost all cases, the individual fractions were not pure. Such fractions were then purified using preparative T.L.C. with 0.5 mm layers of silica gel H with the same two-step system. Material was "strip-loaded" onto the plates using the Desaga automatic applicator, and after chromatographic separation, the bands were visualised using iodine vapour. After the iodine had vapourised from the plates, the bands were removed and the individual fractions were recovered from the silica into chloroform: methanol:: 2:1 by volume or, less regularly, chloroform: methanol: formic acid:: 20: 10: 1 by volume. Once the fractions were shown to be chromatographically pure and once they had been identified by co-chromatography of a very small aliquot with the appropriate standard, they were taken to dryness and constant weight under reduced pressure on a rotary evaporator at 30°.

4. Resolution of the hydrocarbon fractions. (Material recovered from lipid extraction method B only).

In order to determine whether the hydrocarbon fractions consisted only of alkanes/alkenes or whether any isoprenoid hydrocarbons were also present, T.L.C. can be used with heptane: benzene:: 9: 1 by volume as solvent. In this system, alkanes have

an R_f of about 0.7 and isoprenoid hydrocarbons such as squalene, have an R_f of about 0.4. Apart from sterol esters which just and no more, leave the origin, no other neutral lipid moves in this solvent system.

Initially, analytical T.L.C. was carried out on the hydrocarbon fractions using 0.25 mm layers of silica gel H (Merck) and later, preparative T.L.C. using 0.5 mm layers of the silica was used to resolve these fractions. Visualisation of the spots (or bands) was by iodine vapour as previously described. Since benzene is potentially hazardous, these separations were carried out with the T.L.C. tank in a fume cupbaord, and all plates were dried in a fume cupboard prior to visualisation.

In this and in all T.L.C. work, the solvent was put into the base of the tank to a depth of about 1 cm and the tank was lined with chromatography paper to within 1 cm of the top to ensure better and rapid equilibration of the atmosphere within the tank.

Examination of the sterol ester fraction from M. marepuniceus (material recovered from lipid extraction method B only)

This was approached in three ways:

(i) Analytical T.L.C.

The sterol ester fraction, purified from the Florisil column, was chromatographed on 0.25 mm layers of silica gel H (Merck) with hexane: diethyl ether: acetic acid:: 70: 30: 1 by volume as solvent. The fraction and authentic cholesterol stearate were applied to the plates both separately and together. After development, the spots were visualised using the sulphuric

acid-dichromate spray followed by charring as described earlier.

(ii) Preparation of the free sterol.

The sterol ester fraction was converted into the free sterol and free fatty acid(s) by saponification overnight in 10% (w/v) methanolic KOH at room temperature, in the dark and in an atmosphere of nitrogen. The unsaponifiable material was recovered by phasing into diethyl ether after the addition of 1-2 volumes of distilled water to the saponification mixture. The pooled extracts were back-washed several times with distilled water until neutral, dried over anhydrous sodium sulphate and reduced to a small volume. A sample of cholesterol stearate (as a standard) was similarly treated. The product from the sterol ester sample and from the standard were then chromatographed by T.L.C. as described above.

(iii) Gas-liquid chromatographic (G.L.C.) analysis of the sterol.

The G.L.C. analyses were carried out as described for the long chain alcohols (later) except that the oven temperature was 220°. The recovered sterol, the recovered cholesterol (from the standard) and standard cholesterol were each applied to the column dissolved in diethyl ether.

NB. Preparation of digitonides (reasonably specific for 3βhydroxlated sterols) from the recovered sterol was not attempted since it was thought that not enough material was available.

Gas-liquid chromatographic (G.L.C.) analysis of the neutral lipid components.

The individual neutral lipid fractions which had been shown

to be chromatographically pure by T.L.C. were subjected to G.L.C.analysis. The G.L.C. was performed on a Pye Model 104 gas chromatograph fitted with a flame ionisation detector and using nitrogen as the carrier gas. The individual peaks were recorded on a Phillips P 8000 chart recorder. Samples were injected on to the top of the appropriate column through a gas-tigh septum using a calibrated microlitre syringe of either 1µl or 5µl capacity. The nitrogen flow rate was calibrated using a bubble flow meter. Hydrocarbons and sterols were chromatographed without prior modification, whereas less volatile compounds were modified to increase their volatility. Free fatty acids, fatty acid esters and the fatty acids from the glyceride fractions were methylated or transesterified to methyl esters and the long chain alcohols were converted into silyl esters before chromatographing.

1. Hydrocarbons. (Only those recovered from lipid extraction method B analysed).

The non-isoprenoid hydrocarbons recovered from preparative T.L.C. of the hydrocarbon fractions, were dissolved in diethyl ether, reduced in volume to approximately 50-100 µl in a jet of nitrogen and analysed under the following conditions:

Column	: 5 ft glass
Column packing	: 3% S.E. 30 on 100-200 mes celite.
Injection volume	: 1 µl
Column oven temperature	: 220° isothermal
Detector oven temperature	: 300°
Injection port heater temp.	: 230°
Attenuation	: 50 x 10^2

N_2 carrier flow	: 45 ml/min
H_2 flow to detector	: 45 ml/min
Air flow to detector	: 600 ml/min
Chart speed	: 25 mm/min.

A standard mixture of straight chain alkanes with even numbers of carbon atoms in their skeletons (C_{18}-C_{24}), were also injected into the column under identical conditions and these were used as standards.

The isoprenoid hydrocarbons recovered by preparative T.L.C. of the hydrocarbon fractions were prepared for analysis as above. The G.L.C. conditions used were similar except that the column oven temperature was 230° isothermal. For standards, samples of both squalene and squalane (perhydrosqualene) were also injected into the column under identical conditions.

The more polar hydrocarbons were analysed as described for the isoprenoid hydrocarbons. No other standards were used in this case.

2. Long chain alcohols. (Only those recovered from lipid extraction method B analysed).

These fractions were first converted into trimethylsilyl (TMS) derivatives by the method of Holligan (1971). Each sample was dried in vacuo at 55° and to each was added 0.7 ml pyridine, 0.2 ml hexamethyldisilazane (HMDS - BDH Ltd.) and 0.1 ml trimethylchlorosilane (TMCS - BDH Ltd.). The reaction was allowed to proceed at room temperature for 1 h in a desiccator, after which time, the reaction products were taken to dryness under reduced pressure on a rotary evaporator at 30° and re-

dissolved in a small volume of dichloromethane. Derivatives were similarly prepared from standard long chain alcohols (C_{14}, C_{16}, C_{18}) and both these and the samples were injected into the top of the column and the material analysed under the following conditions:

Column	: 5 ft. glass
Column packing	: 3% SE 30 on 100-200 mesh celite
Injection volume	: 1 µl
Column oven temperature	: 188° isothermal
Detector oven temperature	: 300°
Injection port heater temp.	: 230°
Attenuation	: 20 x 10^2
N_2 carrier flow	: 45 ml/min
H_2 flow to detector	: 45 ml/min
Air flow to detector	: 600 ml/min
Chart speed	: 25 mm/min

3. Glycerides and fatty acid esters.

Free fatty acids and fatty acids esterified to other molecules were converted into methyl esters to increase volatility and to achieve uniformity in the group esterified to the acid. This was achieved by the transesterification procedure outlined by Kates, (1972). The reagents were prepared as follows:

(a) 5% (w/v) hydrogen chloride in methanol. 50 ml of dry methanol were colled to -5° and 5 ml acetyl chloride were added with constant stirring.

(b) 2% (w/v) sodium bicarbonate solution in distilled water.

(c) 5% (w/v) sodium chloride solution in distilled water.

(d) pentane.

The sample to be methylated was transferred to a glass hydrolysis tube with a screw cap containing a Teflon liner. The

solvent was evaporated off in a jet of nitrogen and 1 ml benzene and then 2 ml methanolic HCl were added. The mixture was heated at 65° for 2 h in a sealed tube in a water bath. If free fatty acids alone were to be esterified, no solvent (benzene) was required and the sealed tube was heated at 65° for 20 min. After cooling, 5 ml 5% NaCl were added and the fatty acid methyl esters extracted into pentane (2 x 5 ml). The pentane layer was washed with 2% $NaHCO_3$ and then dried over anhydrous sodium sulphate. The solvent was evaporated to a small volume in a jet of nitrogen before injection on to the G.L.C. column under the following conditions:

Column	: 5 ft. glass
Column packing	: FFAP (Phase Sep Ltd.
Injection volume	: 1µl
Column oven temperature	: 188° isothermal
Detector oven temperature	: 250°
Injection port heater temp.	: 230°
Attenuation	: 10/20/50 x 10^2
N_2 carrier flow	: 45 ml/min
H_2 flow to detector	: 45 ml/min
Air flow to detector	: 600 ml/min
Chart speed	: 10 mm/min

Methyl esters were prepared in the same manner from the following standard fatty acids - C_{10}, C_{12}, C_{14}, C_{16} and C_{18} and from monoenoic C_{18}, and injected into the G.L.C. column under identical conditions.

Hydrogenation of fatty acid methyl esters.

The methyl ester samples were hydrogenated and again

analysed by G.L.C. to determine and/or confirm which peaks in the original G.L.C. separations were due to esters of unsaturated fatty acids.

The sample to be hydrogenated was dissolved in 5 ml abs. ethanol in a round-bottomed flask. A few mg of 5% palladium on charcoal catalyst (BDH Ltd.) was added and the mixture shaken under an atmosphere of hydrogen for 20 min at room temperature. The catalyst was removed by filtration and the hydrogenated esters recovered into pentane by the addition of two volumes of water and one volume of pentane. Esters of originally unsaturated fatty acids were identified by their absence and by the increase in the size of the peak of the corresponding saturated ester.

Estimation of apparent carbon numbers for G.L.C. peaks.

In each case, the carbon chain lengths of the standard compounds used in analysis were plotted against the logarithm of the retention times of the peaks obtained on the chart recorder to produce James plots. The logarithm of retention times of the unknown peaks gave the apparent carbon number when referred to the appropriate James plot. Apparent carbon numbers are not always whole numbers since fractions are obtained from peaks derived from material with a branched chain structure or from peaks derived from material which is unsaturated. Branching tends to reduce the retention time when compared with the equivalent fully saturated straight chain compound, and unsaturation tends to slightly increase the retention time when compared with the equivalent fully saturated straight chain compound on a polar stationary phase. Additionally, the retention times obtained were compared with those previously reported by Burchfield and Storrs (1962).

Determination of peak areas.

The traces obtained on the chart recorder from the G.L.C. were also used to determine the percentage of each component as a proportion of the total components in any fraction. Individual peak areas were calculated by multiplying the peak height (in mm) by the width of the same peak at half the height (in mm). The area of each peak was then expressed as a percentage of the total area under all of the peaks.

Identification and separation of the components of the phospholipid fractions. (Only the phospholipids recovered from lipid extraction method B were examined).

A sample of the total phospholipid from each microorganism was dissolved in chloroform: methanol:: 2: 1 by volume. Use was then made of analytical T.L.C. in two different solvent systems, differential staining techniques and co-chromatography with authentic standards to establish the number and identity of the various components present.

Analytical T.L.C.

Either 0.25 or 0.5 mm layers of silica gel H (Merck), prepared as described previously, were used after being "pre-run" and reactivated. The phospholipid samples and the standards were applied with a micropipette and the chromatograms were developed in one of the following two solvent systems:

(a) chloroform: methanol: water:: 65: 25: 4 by volume

(b) chloroform: di-isobutyl ketone: acetic acid: methanol: water:: 45: 30: 20: 15: 4 by volume

After the various plates were developed and dried, the

positions of the components were shown by the use of one of the following systems:

(a) Ninhydrin spray

The plate was sprayed with 0.5% ninhydrin in butanol and then heated in an oven at 100° for about 10 min. Purple spots were indicative of free amino groups present in a particular compound.

(b) Iodine vapour. (Kates, 1972)

The plate was placed for 2-3 min in a sealed tank containing crucibles of sublimed iodine. All lipids show up as yellow spots against a white or pale yellow background.

(c) Phosphorus-detecting spray. (Kates, 1972)

The spray reagent was prepared as follows:

Reagent A : 8 g of ammonium molybdate were dissolved in 60 ml of distilled water.

Reagent B : 5 ml of mercury were added to 20 ml conc. HCl and to 40 ml of reagent A. This mixture was stirred for 30 min and then filtered.

Spray reagent: 100 ml conc. H_2SO_4 and all of reagent B were added to the remainder of reagent A (20ml). This mixture was cooled and diluted to 500 ml with distilled water to give a clear brown/green liquid.

The plate was sprayed with the reagent using an all-glass system pressurised by a compressed air pump. The phosphorus-containing lipids appeared almost at once as blue spots on a white background. No heating was required.

(d) Schiff's-periodate stain. (Kates, 1972)

This stain is specific for vicinal hydroxy groups and was

prepared and used as follows:

100 ml of 1% (w/v) sodium periodate solution was made up in distilled water. 100 ml of 1% (w/v) p-rosaniline hydrochloride was made up in distilled water and sulphur dioxide gas bubbled through from a cylinder until the solution became colourless (Schiff's reagent). The plate was sprayed with sodium periodate until well saturated and then left for oxidation to occur for 5-10 min. It was then placed in a closed chromatography tank which had been filled with sulphur dioxide gas. After a few minutes, the plate was removed and sprayed lightly with the Schiff's reagent. Spots appeared after a few minutes at room temperature and can be identified by their colour and order of appearance. Phosphatidyl glycerol spots appeared quickly and were purple in colour. This colour compares with dark blue for glycolipids and yellow for phosphatidyl inositol.

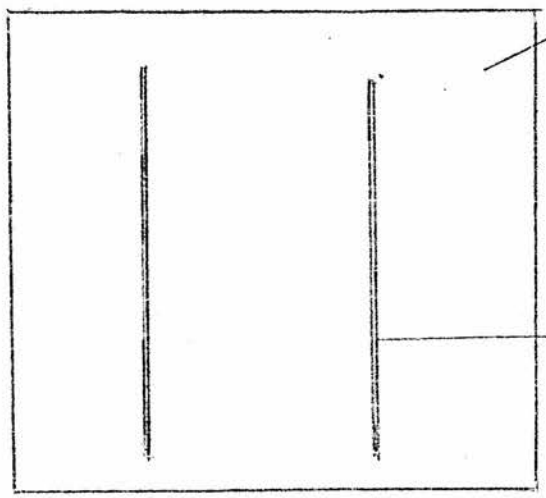
Phospholipid standards for T.L.C. work.

The following authentic phospholipids, at a concentration of 10 mg/ml, were used in the above T.L.C. work :
Phosphatidyl serine, phosphatidyl inositol, phosphatidyl ethanolamine, phosphatidyl chloine, phosphatidyl glycerol, diphosphatidyl glycerol (cardiolipin), N.N. dimethyl phosphatidyl ethanolamine, lysophosphatidyl ethanolamine and lysophosphatidyl glycerol.

Preparative T.L.C. of phospholipids.

Once the identity of the individual phospholipids from the two microorganisms had been established, the individual phospholipids were obtained in pure form by preparative T.L.C. This

was performed on 0.5 mm layers of silica gel H on 20 x 20 cm glass plates with chloroform: methanol: water:: 65: 25: 4 by volume as solvent. A known weight of total phospholipid material was strip-loaded on to "pre-run" plates using the Desaga automatic applicator (Camlab Ltd.). After the plates were developed and dried, the position of the individual bands was determined using an overlay and spraying only two narrow bands down the plate with the phosphorus-detecting spray:



Card overlay placed over the surface of a developed and dried plate.

Slots cut in overlay allowing staining of narrow bands with the phosphorus-detecting spray. No heat treatment required.

The individual bands (minus the small stained areas) were then scraped off and the fractions eluted from the silica with chloroform: methanol: formic acid:: 20: 10: 1 by volume, or chloroform: methanol:: 2: 1 by volume. The fractions were checked for purity and any impure fractions were re-chromatographed in the same system. All like fractions were pooled and the identity of each fraction confirmed by co-chromatographing small aliquots with the appropriate standards. All of the pure fractions were then taken to dryness and constant weight under reduced pressure on a rotary evaporator at 30° and then stored under an atmosphere of nitrogen at -20° until used.

Fatty acids associated with individual phospholipid fractions.

Each individual phospholipid fraction was transesterified by the method previously described and the fatty acid methyl esters recovered into pentane. The fatty acid profiles were then established by G.L.C. using the conditions outlined for the fatty acid methyl esters derived from the three neutral lipid glyceride fractions.

RESULTS

Bacterial growth in liquid culture.

1. Growth curves.

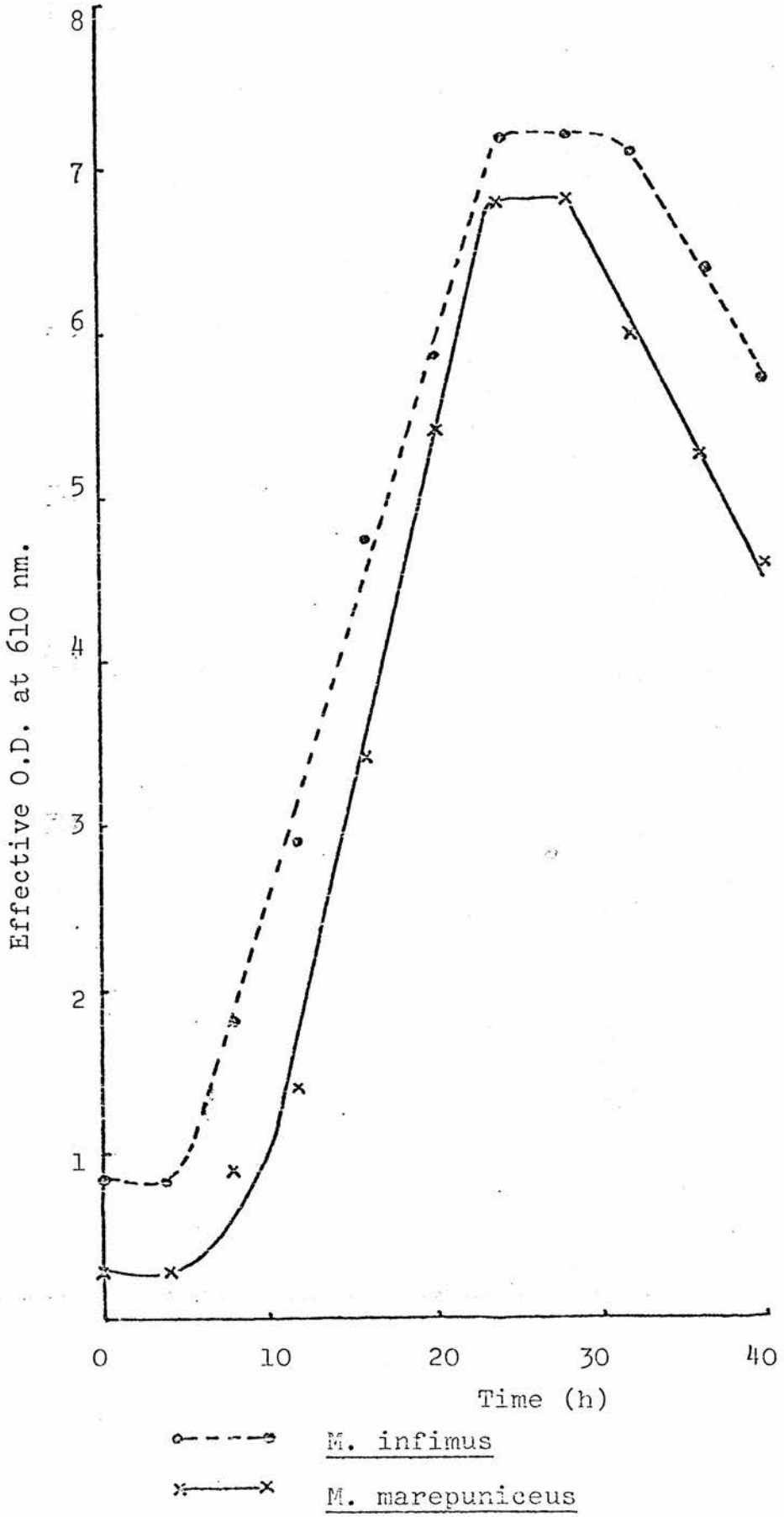
The growth of both bacteria was monitored by the two methods described in the "Methods" section and both produced similar results. Typical results obtained by turbidimetry are seen in Fig. R1. In both cases there was a slight drop in turbidity during the early part of the lag phase which in itself, was relatively short (approximately 4 h). This was followed by a normal log. phase of growth and maximum cell numbers were reached after about 24 h. The stationary phases were short lived before the onset of quite steep decline phases. The initial pH was 7.2 and at the termination of the experiments, the pH values had fallen. After 40 h, the medium in which M. marepuniceus has grown was at pH 5.2 and that in which M. infimus had grown was at pH 6.2. The lowering of the pH during growth may be a contributory factor in determining the end of the log. phase in the relatively rich medium and may also contribute to the short stationary phase and sharp decline phase.

Such results made it obvious that for large scale batch culture, it would be preferable to maintain the pH at around pH 7.0.

2. Batch culture.

Growth of both bacteria was better in batch culture than in the above experiments, and at maximum cell numbers, the effective O.D. was usually between 12 and 14. The stabilisation of pH referred to above may have been responsible for this. Harvesting of the batch cultures on the Sharples centrifuge normally produced between 200 and 240 g of a solid cell paste.

Fig.R1 : Growth curves for M. marepuniceus and M.infimus in medium G at 30° monitored by turbidimetry.



3. The effect of lysozyme on bacterial cells.

Over a six hour period, there was a steady decline in the absorbance of the M. lysodeikticus culture relating to the removal of cell wall material and subsequent lysis of the cells. With both M. marepuniceus and M. infimus, there was no such gradual drop in absorbance, but the readings obtained followed similar patterns to those of their normal growth curves (see Fig. R1). As a result of these findings, it was decided to extract the lipids from whole cells rather than to attempt to isolate membrane fractions for this purpose.

Determination of Cellular Lipids in M. marepuniceus and M.infimus.

Determination of moisture contents of cell pastes.

Before either of the two lipid extraction procedures was employed, the moisture content of the cell pastes was determined so that the subsequent lipid extraction results could be expressed on a dry cell weight basis. The results are shown in Table 1.

Table 1 : Estimation of cell paste moisture contents.

	M. marepuniceus		M. infimus	
	1.	2.	3.	4.
Average moisture content (%)	67.26 ± 0.5	67.99 ± 0.4	66.05 ± 0.3	64.80 ± 0.4

Notes. : 1 and 3 were accumulated batches of cell pastes used for lipid extraction by the Ballotini mill method. (Method A)

2 and 4 were accumulated batches of cell pastes used for lipid extraction by the Bligh and Dyer method. (Method B).

LIPID EXTRACTION PROCEDURES.

A. Ballotini mill method. (Method A)

Weight of lipid recovered.

This was the first extraction method used and the results obtained are shown in Table 2.

Table 2 : Extraction of the lipids from M. marepuniceus and from M. infimus using the Ballotini mill method.

	M. marepuniceus	M. infimus
Wet weight (g) of cells used	223.9885	203.0593
Dry weight (g) of cells used	73.3338	68.9386
Weight (g) of "crude" lipid extracted	27.5200	27.9884
Weight (g) of "pure" lipid extracted	2.0056	2.3049
"Crude" lipid as % dry cell weight	37.53	40.60
"Pure" lipid as % dry cell weight	2.73	3.34

With both microorganisms, at lease eight extractions were made before no more material was being extracted as shown by the absence of solid when the final extract was taken to dryness. The weights of "crude" lipid were excessive. This may have been due to an over-efficient disintegration of the cells resulting in fine suspended matter being recovered in the centrifuge supernatant, or to an excessive amount of lipoprotein complexes being recovered in the supernatants. As a result, purification of the "crude" lipid using Sephadex G 25 was not used. Nevertheless, the weights of "pure" lipid subsequently recovered were within published values. Since a large proportion of the "crude" lipid was insoluble material, some "pure" lipid may have been sequestered in/on the insoluble material and/or if large amounts of lipoprotein had been extracted, this material was subsequently insoluble in the solvent used for purification. Such a great difference between the "crude" and "pure" lipid weights obtained for both bacteria cast doubt on the use of this technique for the extraction of the lipids from these two microorganisms.

Resolution of total lipid into lipid classes.

However, the "pure" lipid obtained from each microorganism by this extraction method was first subjected to acetone precipitation to remove the bulk of the phospholipids, and the acetone-soluble material was fractionated into neutral lipids, glycolipids and residual phospholipids (see "Methods section"). The latter were added to the acetone-precipitated material and the weight distribution of the lipid classes obtained is shown in Tables 3 and 4.

Table 3 : Recovery of lipid after separation into neutral lipid, glycolipid and phospholipid.

	M. marepuniceus	M. infimus
Total weight of lipid fractionated (g)	2.0056	2.3049
Weight recovered (column + precipitation) (g)	1.9675	2.1816
% weight recovery	98.10	94.65

Table 4 : Weights and percentages of lipid classes in recovered lipid.

	M. marepuniceus		M. infimus	
	Wt.(g)	% recovered material	Wt.(g)	% recovered material
Neutral lipid	0.3980	20.23	0.3049	13.98
Glycolipid	0.1219	6.20	0.1449	6.64
Phospholipid	1.4476	73.57	1.7318	79.38

The distribution of lipid classes within the two micro-organisms gives fairly similar results. There appears to be slightly more neutral lipid and slightly less phospholipid from M. marepuniceus.

When each of the neutral lipid fractions was investigated for the presence of poly-β-hydroxy butyric acid, that compound was shown to be absent in that no precipitation was observed.

In each case, the free fatty acids were first removed from the neutral lipids by carbonate wash. Thereafter, fractionation of the remaining neutral lipids was attempted by either -

(a) preparative T.L.C., or

(b) column chromatography using Florisil.

T.L.C. of neutral lipids. (M. infimus).

Preparative T.L.C. was used with the neutral lipids from M. infimus but it was quickly apparent that even although the conditions were varied, good resolution could not be achieved and as a result, the use of the method was discontinued.

Separation of neutral lipids by column chromatography (M. marepuniceus).

The Florisil column was used to resolve the neutral lipids minus the free fatty acids from M. marepuniceus. The individual fractions from the column were checked for purity by T.L.C. Those containing more than one fraction were resolved by preparative T.L.C. and all like fractions were pooled. The purity and identity of these "pure" fractions was checked by T.L.C. using authentic standards. The fractions were reduced to dryness and constant weight and the overall results obtained from the total fractionation of the neutral lipids from M. marepuniceus are shown in Table 5.

Table 5 : Fractionation of the neutral lipids from M. marepuniceus, total lipid extracted by method A.

	Weight recovered g	% recovered material
Hydrocarbons	0.1808	66.51
Sterol esters	0.0005	0.18
Fatty acid esters	0.0109	4.0
Triglyceride	0.0058	2.13
Long chain alcohols	0.0262	9.63
Diglyceride	0.0130	4.78
Monoglyceride	0.0046	1.69
Free fatty acids	0.0300	11.09
Total weight recovered	0.2718	
% weight recovery	60.75	

From these results, two observations can be made -

(a) The losses were far greater than could be accounted for in the procedures used. The great loss was incurred at the carbonate wash stage, and although all phases were checked, the disappearanc of material could not be accounted for. A similar result was found when the free fatty acids were removed from the neutral lipids of M. infimus prior to the preparative T.L.C. work.

(b) The figures obtained for both free fatty acids and for long chain alcohols seemed very high.

Although the free fatty acid and glyceride fractions were kept so that their fatty acid profiles could be determined (see later), the losses incurred here and the previously mentioned great differences between the "crude" and "pure" lipid weights, suggested that an alternative method of lipid extraction should be employed. This was the Bligh and Dyer method (Method B).

B. Bligh and Dyer method. (Method B).

Weight of lipid recovered.

The results obtained from the extraction of lipid from the two microorganisms by this extraction technique are shown in Table 6.

Table 6 : Extraction of lipids from M. marepuniceus and M. infimus using the Bligh and Dyer method.

	M. marepuniceus	M. infimus
Wet weight (g) of cells used	150.0000	150.0000
Dry weight (g) of cells used	48.0072	52.7951
Weight (g) of lipid recovered	1.6978	2.0073
Lipid as % dry cell weight	3.54	3.80

From each microorganism, the lipid extracted as a percentage of the cellular dry weight by this method, was greater than with the previous method. Since it cannot be claimed that either method achieves a total lipid extraction, the percentage of individual lipids extracted by the two methods may be variable. In addition, the excessive "crude" lipid weights of the previous method, as stated, may involve a higher release of lipid as lipoprotein.

Resolution of total lipid into lipid classes.

The total lipids from each microorganism were first subjected to acetone precipitation to remove the bulk of the phospholipids and the acetone-soluble material was fractionated into neutral lipids, glycolipids and residual phospholipids. The latter were added to the acetone-precipitated material and the distribution of the lipid classes from the two bacteria which were obtained

are shown in Tables 7 and 8.

Table 7 : Recovery of lipid after separation into neutral lipid, glycolipid and phospholipid.

	M. marepuniceus	M. infimus
Total weight (g) lipid fractionated.	1.6978	2.0073
Weight (g) recovered (column + precipitation).	1.6548	2.0028
% weight recovery	97.47	99.78

Table 8 : Weights and percentages of lipid classes in recovered lipid.

	M. marepuniceus		M. infimus	
	Wt. (g)	%	Wt. (g)	%
Neutral lipid	0.1832	11.07	0.2310	11.53
Glycolipid	0.1286	7.77	0.1081	5.40
Phospholipid	1.3430	81.16	1.6637	83.07

These results are also shown in histogram form for comparison (Fig. R2A). Again it is seen that the distribution of lipid classes obtained here differs from that obtained with the lipids extracted by method A (Table 4). There were more phospholipids and less neutral lipids. However, the distribution in the two bacteria are fairly similar. When each of the neutral lipid fractions was investigated for the presence of poly-β-hydroxy butyric acid, that compound was shown to be absent in them all in that no precipitation was observed.

T.L.C. of neutral lipids.

In each case, the free fatty acids were removed from the

neutral lipids by carbonate wash and taken to dryness and constant weight. A very small aliquot of the neutral lipids from each bacterium from which the free fatty acids had been removed, was examined by T.L.C. on 0.25 mm layers of silica and both gave similar results with the two solvent system. A typical result, obtained with M. infimus is shown in Fig. R3. It was seen that not all of the free fatty acids had been removed by carbonate wash although very little remained, that very little material resembling sterol esters was present, that the relative density of staining of the long chain alcohols and the glyceride fractions was greater in M. infimus and that if anything, the relative density of staining of the hydrocarbon fraction was greater in the sample from M. marepuniceus.

Separation of neutral lipids by column chromatography.

These two neutral lipid fractions from which the free fatty acids had been removed were fractionated on Florisil columns. The individual fractions were checked for purity by T.L.C. Those containing more than one fraction were resolved by preparative T.L.C. and all like fractions were pooled. The purity and identity of these pure fractions was then checked by T.L.C. using authentic standards. The fractions were reduced to dryness and constant weight and the overall results obtained from the total fractionation of the neutral lipids from both M. marepuniceus and M. infimus are shown in Table 9.

Fig.R2(A): Lipid classes in the total lipid from M.marepuniceus and from M. infimus

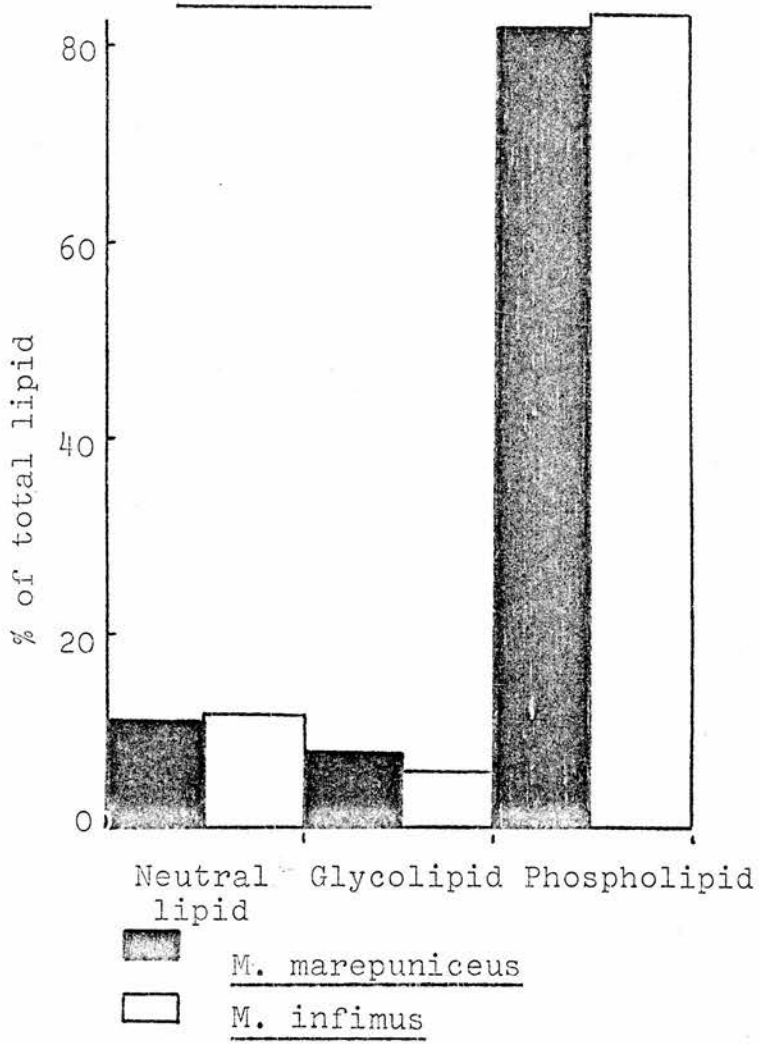


Fig.R2(B): Distribution of neutral lipids in M. marepuniceus and in M. infimus

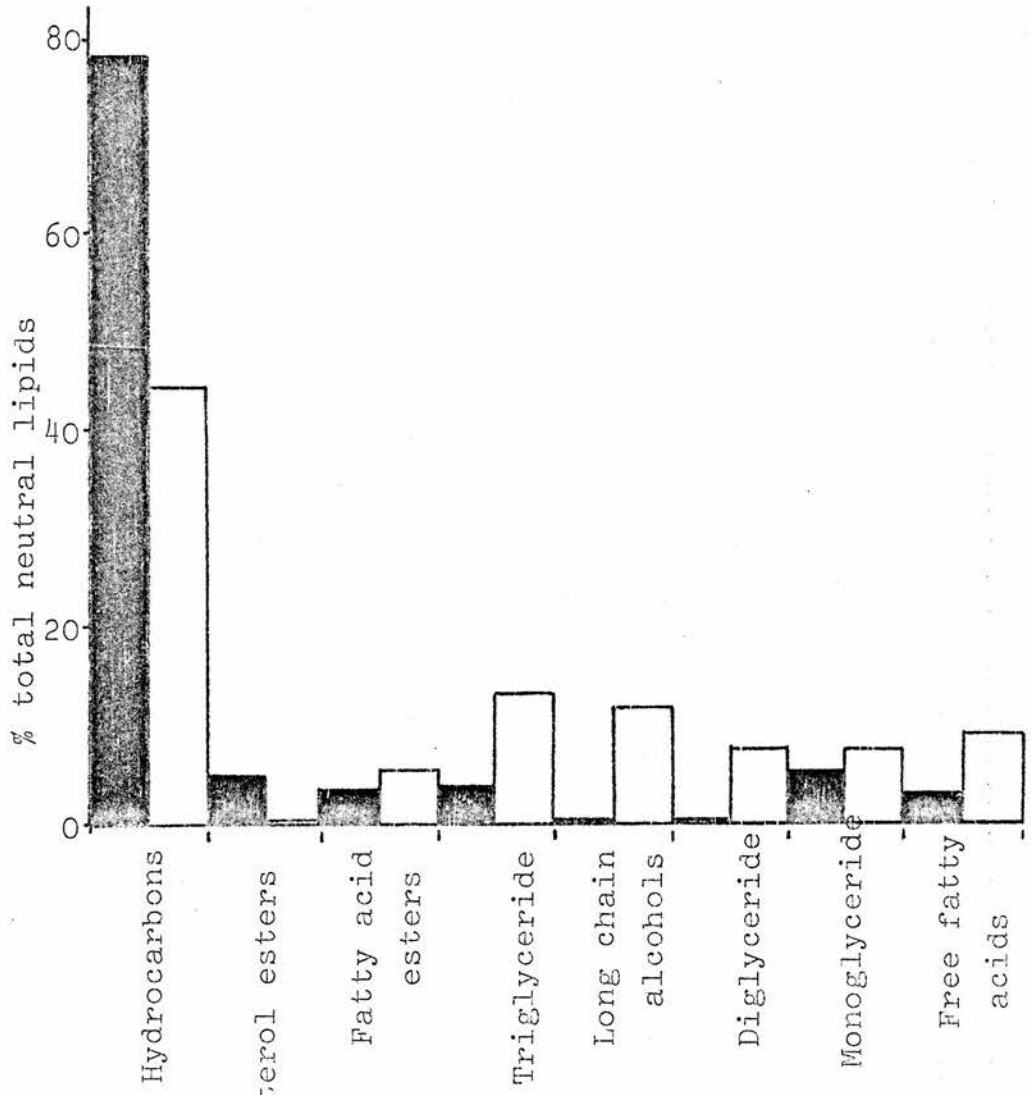
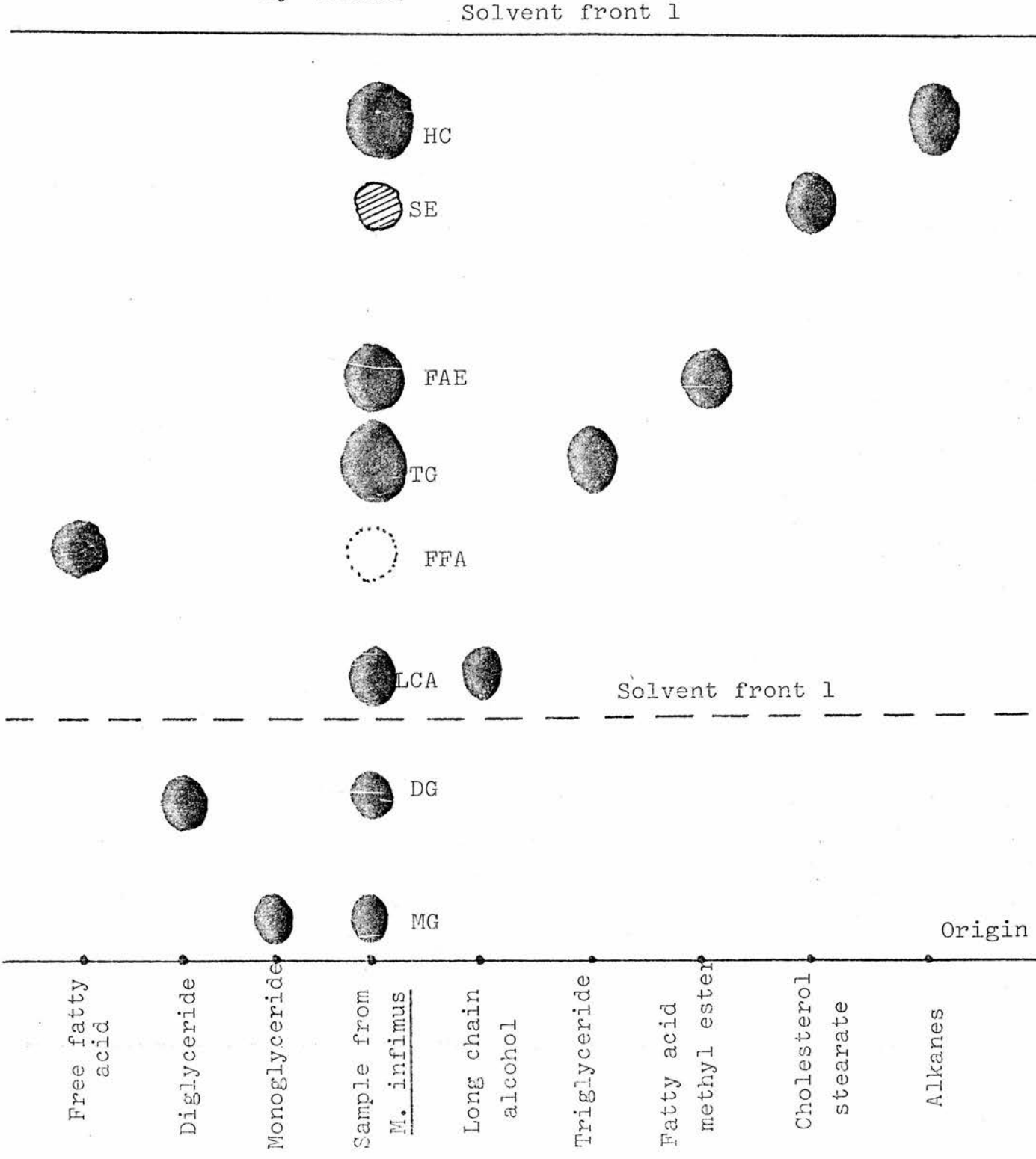


Fig.R3: T.L.C. separation of the neutral lipids from M. infimus.

Two-step solvent system: A - di-isopropyl ether: acetic acid:: 96: 4 by vol.; B - Light petroleum (b. p. 60-80°): diethyl ether: acetic acid:: 90:10:1 by volume.



Detection: Sulphuric acid spray and charring

Table 9 : Fractionation of the neutral lipids from M. marepuniceus and from M. infimus

	M. marepuniceus		M. infimus	
	Wt. (g) recovered	% total neutral lipid	Wt.(g) recovered	% total neutral lipid
Hydrocarbons	0.1398	77.88	0.1005	44.35
Sterol esters	0.0102	5.68	0.0001	0.04
Fatty acid esters	0.0067	3.73	0.0124	5.47
Triglyceride	0.0067	3.73	0.0305	13.46
Long chain alcohols	0.0002	0.11	0.0271	11.96
Diglyceride	0.0004	0.22	0.0169	7.46
Monoglyceride	0.0101	5.63	0.0179	7.90
Free fatty acids	0.0054	3.01	0.0212	9.36
Total weight recovered	0.1795		0.2266	
% weight recovery	97.98		98.10	

A comparison of the distribution of the neutral lipid fractions in the two microorganisms can readily be seen when the results are displayed in histogram form (Fig.R2B).

Again, the pattern seen here for M. marepuniceus differs from that seen in Table 5 from extraction method A. The major differences are with respect to the sterol esters, long chain alcohol and the mono- and diglycerides. There is a reasonable similarity only with respect to hydrocarbons, fatty acid esters and triglycerides. There are also obvious differences in the distribution of the neutral lipids between the two microorganisms. The most obvious difference is with respect to the hydrocarbons - there is

1.76 times as much in M. marepuniceus as in M. infimus. Other major differences are in the long chain alcohols, di- and triglycerides and free fatty acids.

Resolution of hydrocarbon fractions.

The two hydrocarbon fractions were further investigated using T.L.C. with heptane:benzene as the solvent system. Both showed the presence of three different spots, one of which appeared to co-chromatograph with alkanes, another of which appeared to co-chromatograph with squalene and third of which had a much lower R_f value and was of unknown identity. The two hydrocarbon fractions were then resolved by preparative T.L.C., the individual fractions taken to dryness and constant weight and the results obtained were as shown in Table 10. Each of the three fractions was later analysed by G.L.C. and the results of these analyses are given later.

Table 10 : Fractionation of the hydrocarbon fractions from M. marepuniceus and from M. infimus.

	M. marepuniceus		M. infimus	
	Wt.(g)	% recovered material	Wt.(g)	%recovere material
Fraction 1 - co-chromatographed with alkanes	0.1355	97.57	0.0966	96.12
Fraction 2 - co-chromatographed with squalene	0.0004	0.35	0.0002	0.20
Fraction 3 - Unknown - low R_f.	0.0029	2.08	0.0037	3.67
Total recovery	0.1388	99.28	0.1005	100.00

From the table it is obvious that almost all of the hydrocarbon fraction in each case, is made up of alkanes/ alkenes.

Investigation of the sterol ester fraction from M. marepuniceus.

The chromatographic mobility of this fraction was compared with cholesterol stearate and found to be similar. After saponification and recovery into diethyl ether, the material now co-chromatographed with authentic cholesterol. However, G.L.C. investigation using cholesterol as standard showed that the material was not cholesterol and that it has a longer retention time.

Gas-Liquid Chromatographic analysis of neutral lipid fractions from both bacteria.

All of the major neutral lipid fractions obtained by column chromatography and T.L.C. from the Bligh and Dyer extraction method B were analysed by G.L.C. with or without prior modification (see "Methods" section). Some of the fractions from M. marepuniceus recovered from the Ballotini mill extraction method A were similarly analysed for comparison.

Free fatty acids, fatty acid esters and glycerides.

The fatty acids from the glycerides, the fatty acid esters and the free fatty acids were methylated and the fatty acid profiles of these fractions are displayed in histogram form which allows for better and easier comparison of the results. In each of the fatty acid histograms, the amount of each fatty acid is shown as a percentage of the total fatty acids. Data along the bottom indicates carbon chain lengths obtained from

the appropriate James plot; : 1 and br- denote unsaturated and branched chain acids respectively. The histograms are as follows:

1. Fig. R4 displays the fatty acid profiles of the glycerides and of the free fatty acids of M. marepuniceus where the lipids were extracted by method A. The results are extremely variable, but the saturated branched C_{15} acid predominates in the diglycerides and the same fatty acid and the saturated branched C_{16} acid predominates in the free fatty acids. The saturated 16:0, br 18:0 and 18:0 acids predominate in the monoglycerides, and in the triglycerides, several acids each constitute around 10% of the total acids (16:0, br 18:0, 19:0, br 20:0).
2. Fig. R5 displays the fatty acid profiles of the free fatty acids and fatty acid esters from the two bacteria where the lipid was extracted using method B. Not only are the profiles different for the two bacteria, but the profile of the free fatty acids of M. maripuniceus also differs from that shown in Fig. R4 for the same fraction derived from lipids extracted by a different method, For the free fatty acids, the predominant acids are 16:0, br 18:0, br 19:0, br 20:0 and br 21:0 for M. marepuniceus, and 14:0, br 15:0, 16:0 and 18:0 for M. infimus. For the fatty acid esters, the predominant fatty acids are br 22:0 and br 23:0 for M. marepuniceus and br 14:0, br 15:0, 16:0 and br 22:0 for M. infimus.

From the results shown in Fig. R5, the percentage of the total fatty acids occurring in any one fraction as saturated branched chain acids, saturated straight chain acids and

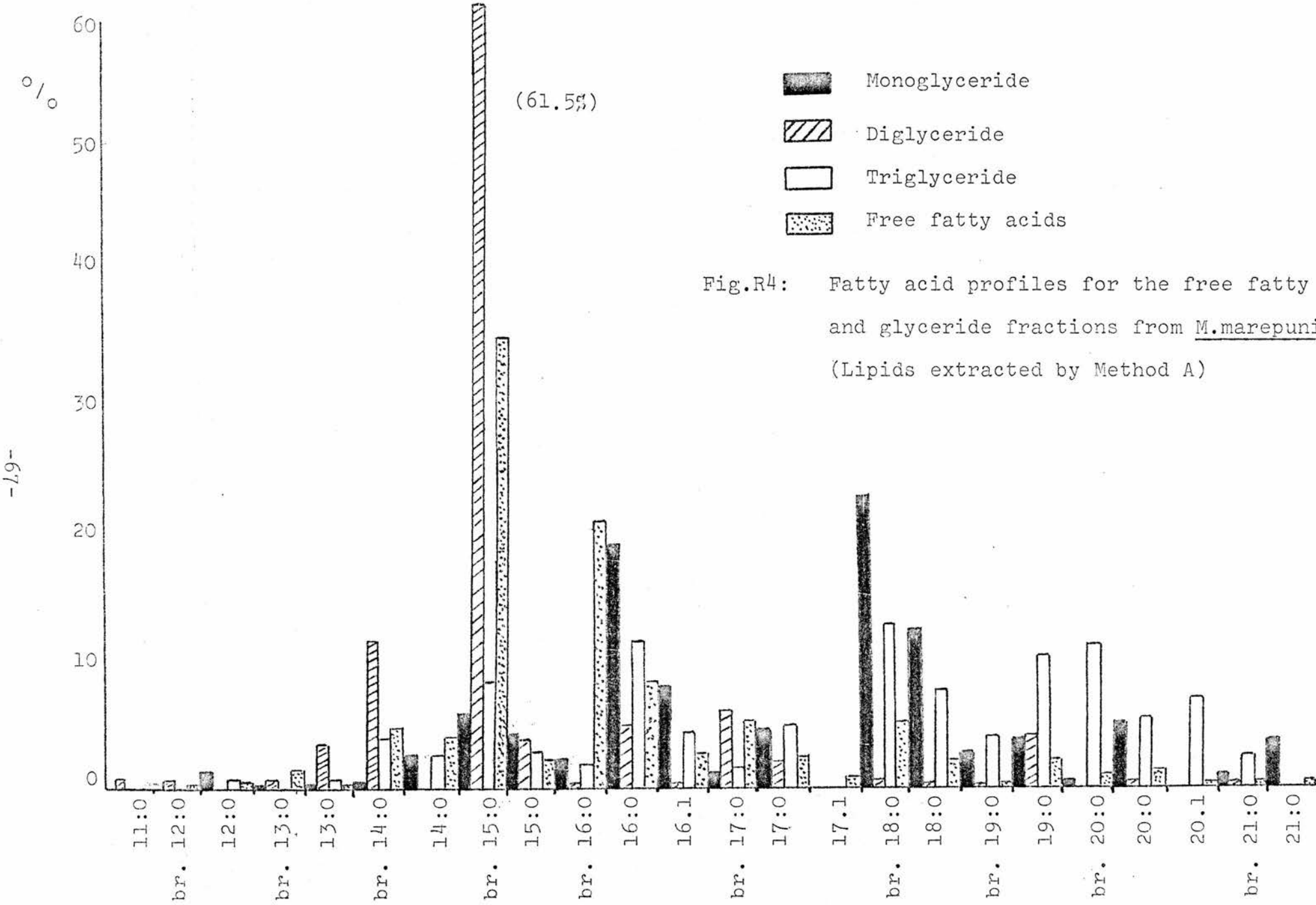
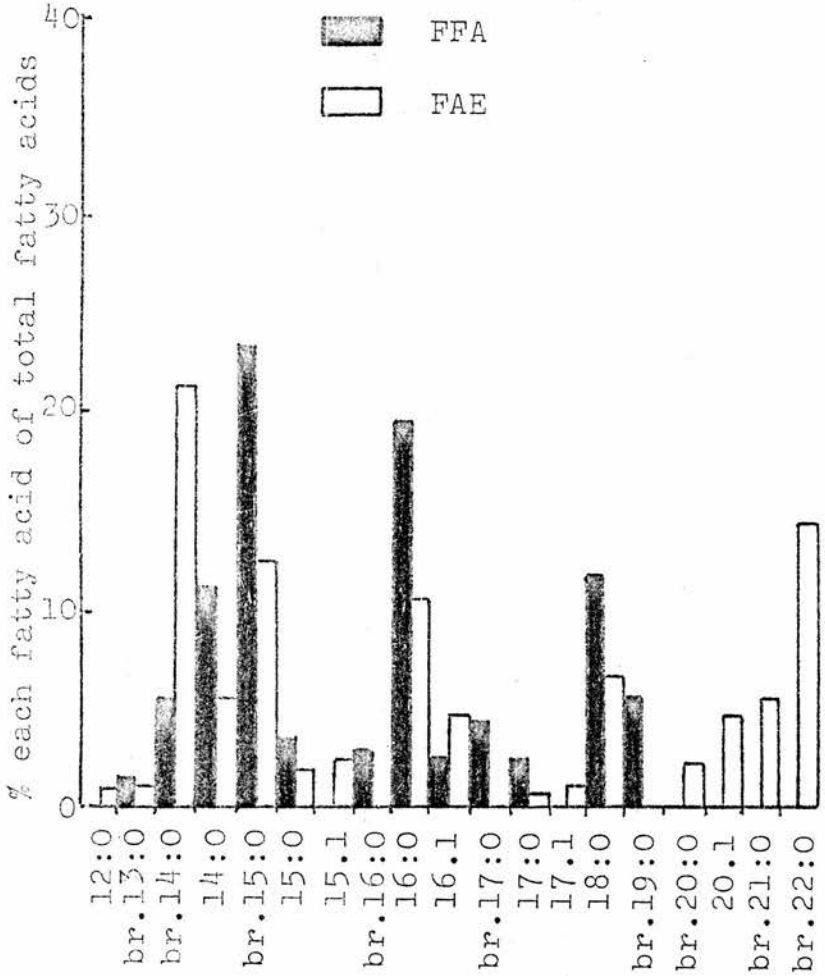


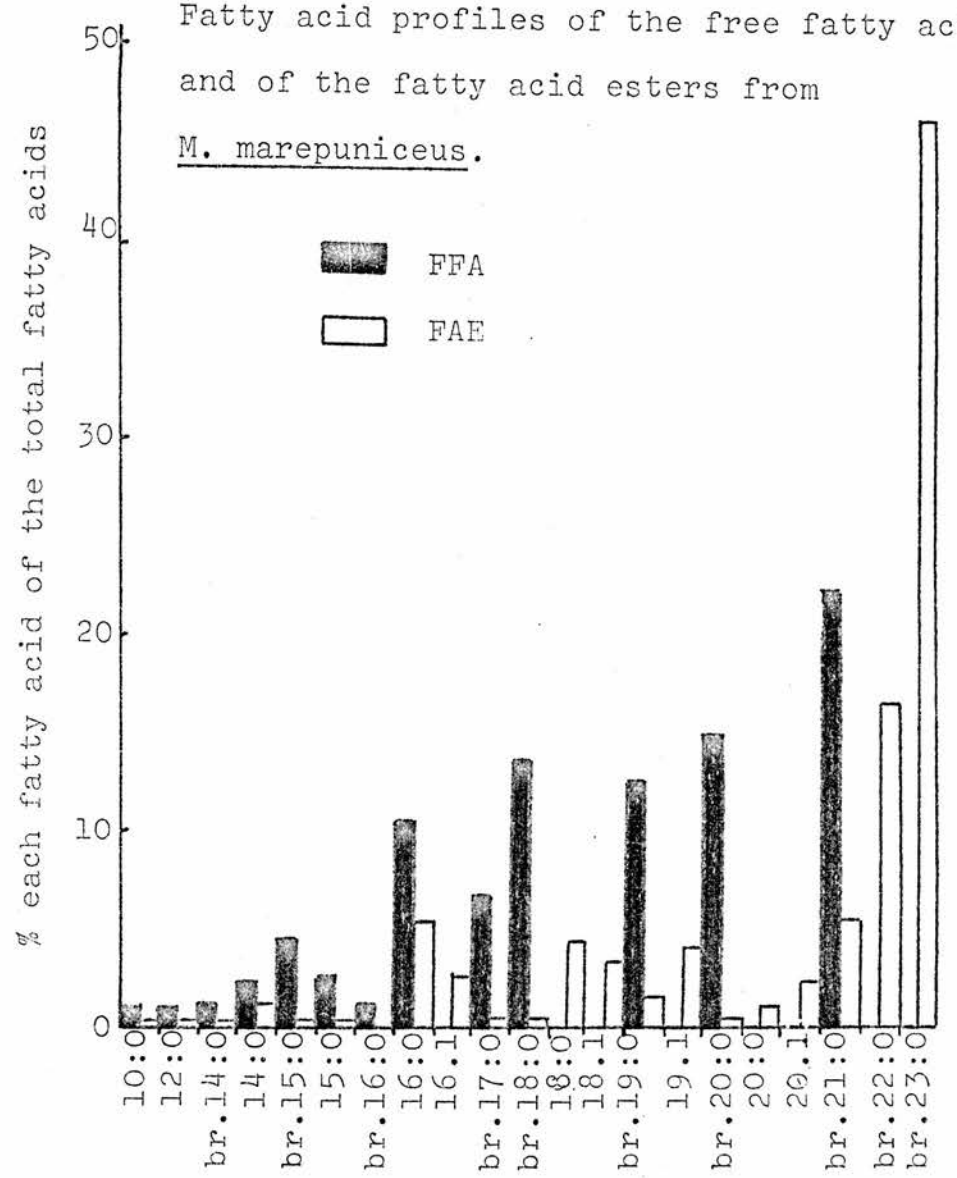
Fig.R4: Fatty acid profiles for the free fatty acids and glyceride fractions from M.marepuniceus (Lipids extracted by Method A)

Fig.R5

Fatty acid profiles of the free fatty acids and of the fatty acid esters from M. infimus.



Fatty acid profiles of the free fatty acids and of the fatty acid esters from M. marepuniceus.



unsaturated acids has also been determined. For comparison, the results are shown in Fig. R6. This again reveals the differences found between the two bacteria, in particular, M. marepuniceus contains a higher proportion of branched acids in these two fractions and a markedly lower content of saturated straight chain acids.

3. Fig. R7 displays the fatty acid profiles of the glyceride fractions from M. marepuniceus (extraction method B) and the distribution of fatty acid types within the three glycerides. Some very obvious differences can be seen, viz.:

(a) whereas the br 15:0 is a major component of the monoglycerides, it is not a major component of the di- and triglycerides.

(b) whereas the br 16:0 and 18:0 acids are major components in the di- and triglycerides, they are present only in trace amounts (less than 1%) in the monoglycerides.

(c) generally speaking (with the exception of the br 15:0 of the monoglycerides mentioned above), fatty acids with 16 or 18 carbon skeletons appear to predominate.

(d) the profiles bear little comparison with those displayed in Fig. R4 for the glycerides from M. marepuniceus obtained using extraction method A.

The distribution of fatty acid types does not show marked variation between the three glycerides except for the relatively high level of unsaturated fatty acids in the diglyceride fraction.

4. Fig. R8 displays the fatty acid profiles of the glyceride fractions from M. infimus (extraction method B) and the

Fig.R6: Distribution of fatty acid types in free fatty acids and fatty acid esters from (A) M.marepuniceus and (B) M. infimus.

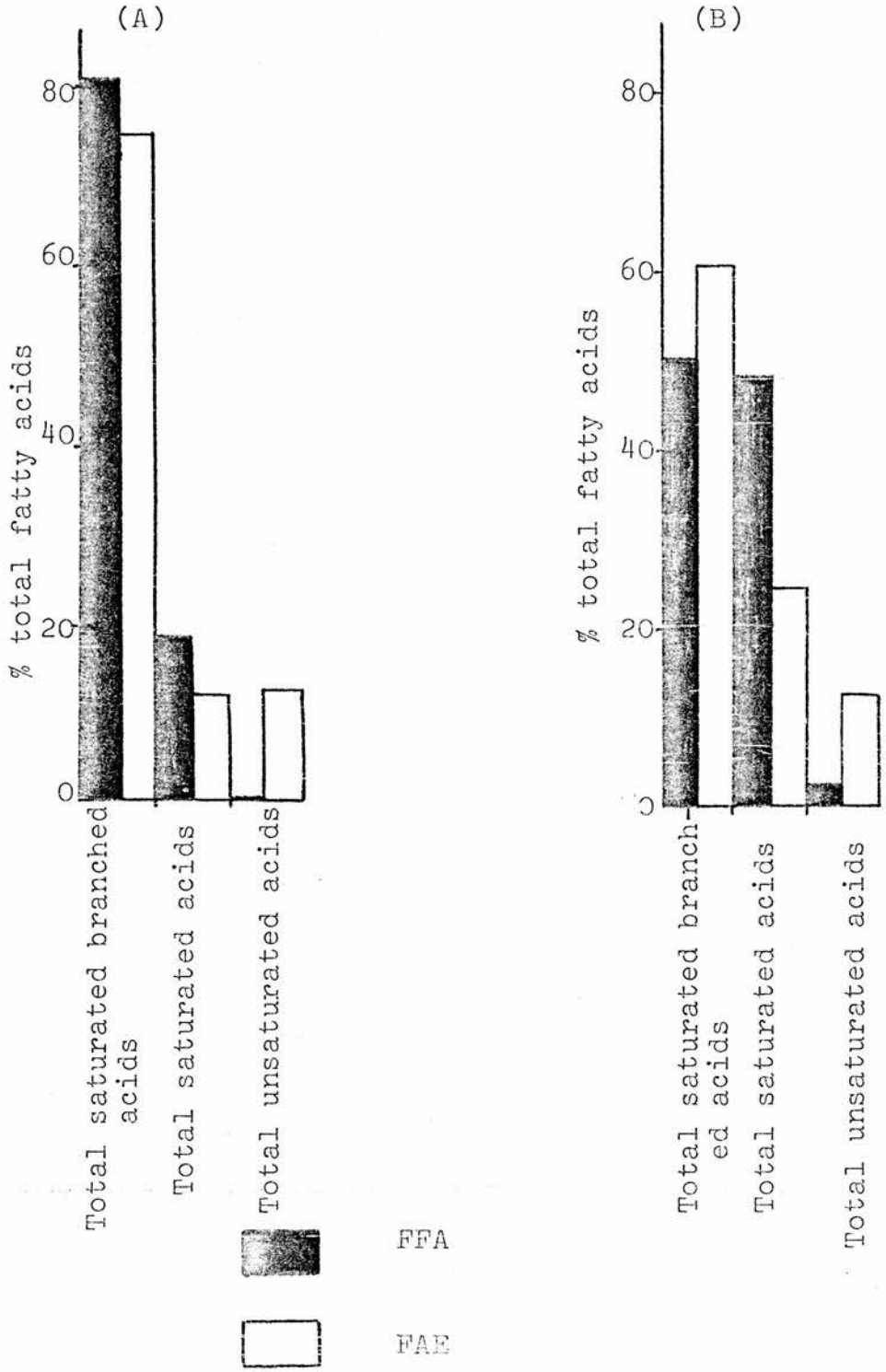
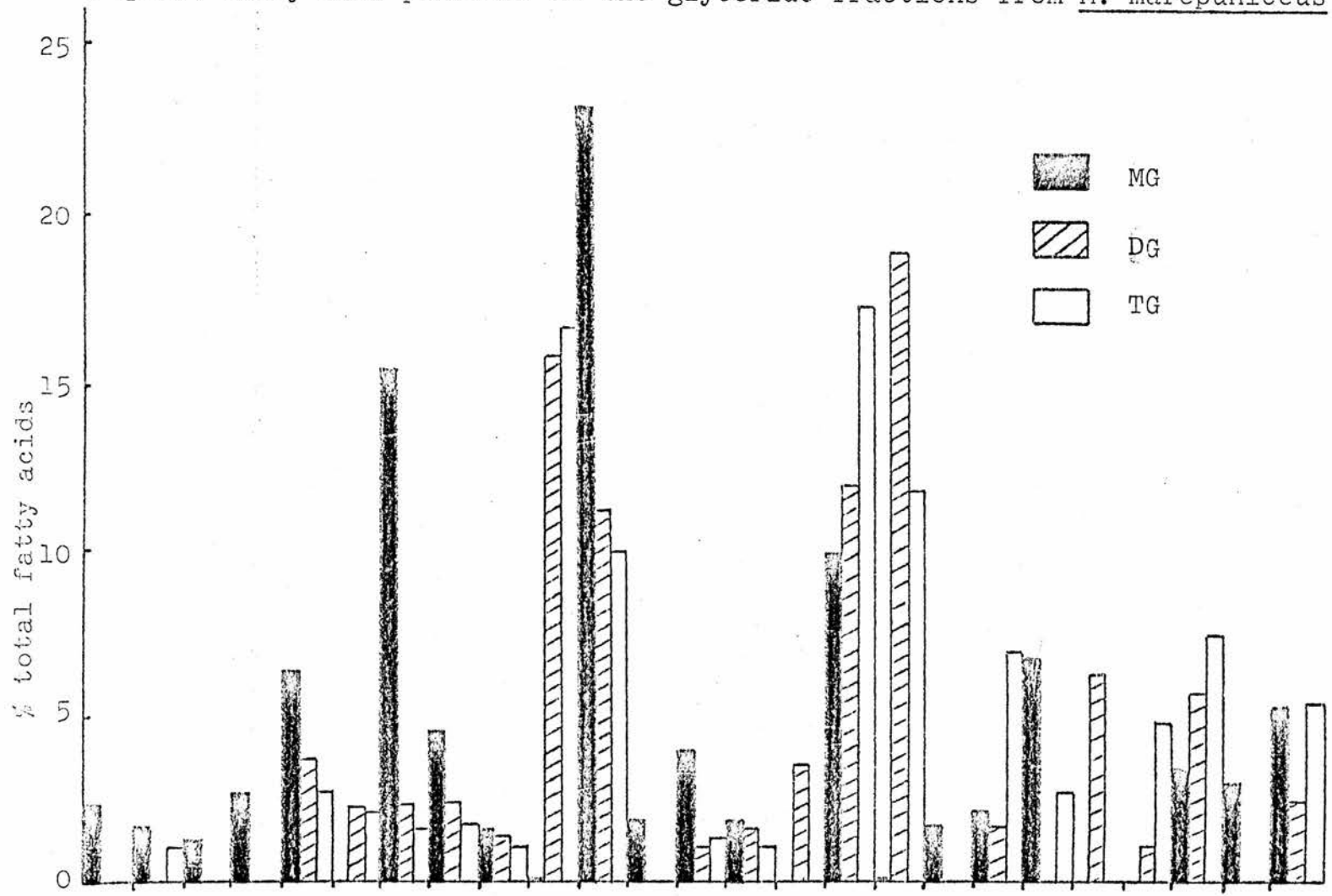


Fig.R7: Fatty acid profiles in the glyceride fractions from M. marepuniceus



Distribution of fatty acid types in the glyceride fractions from M. marepuniceus.

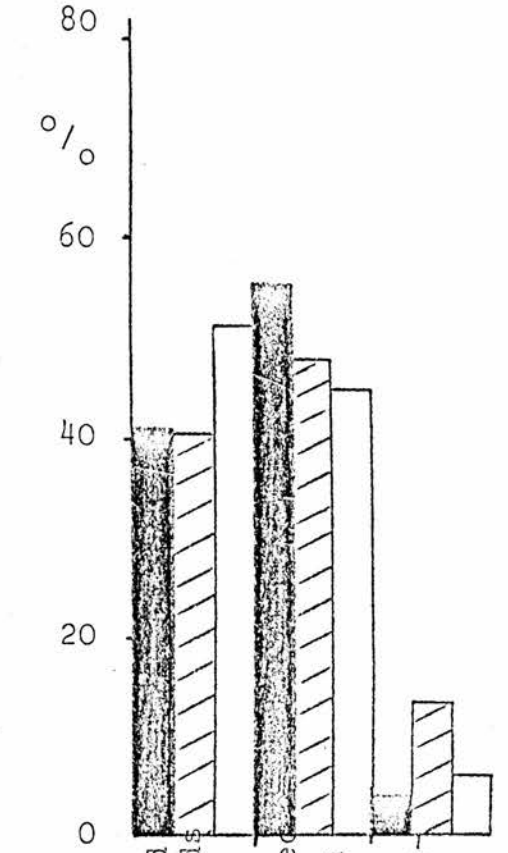
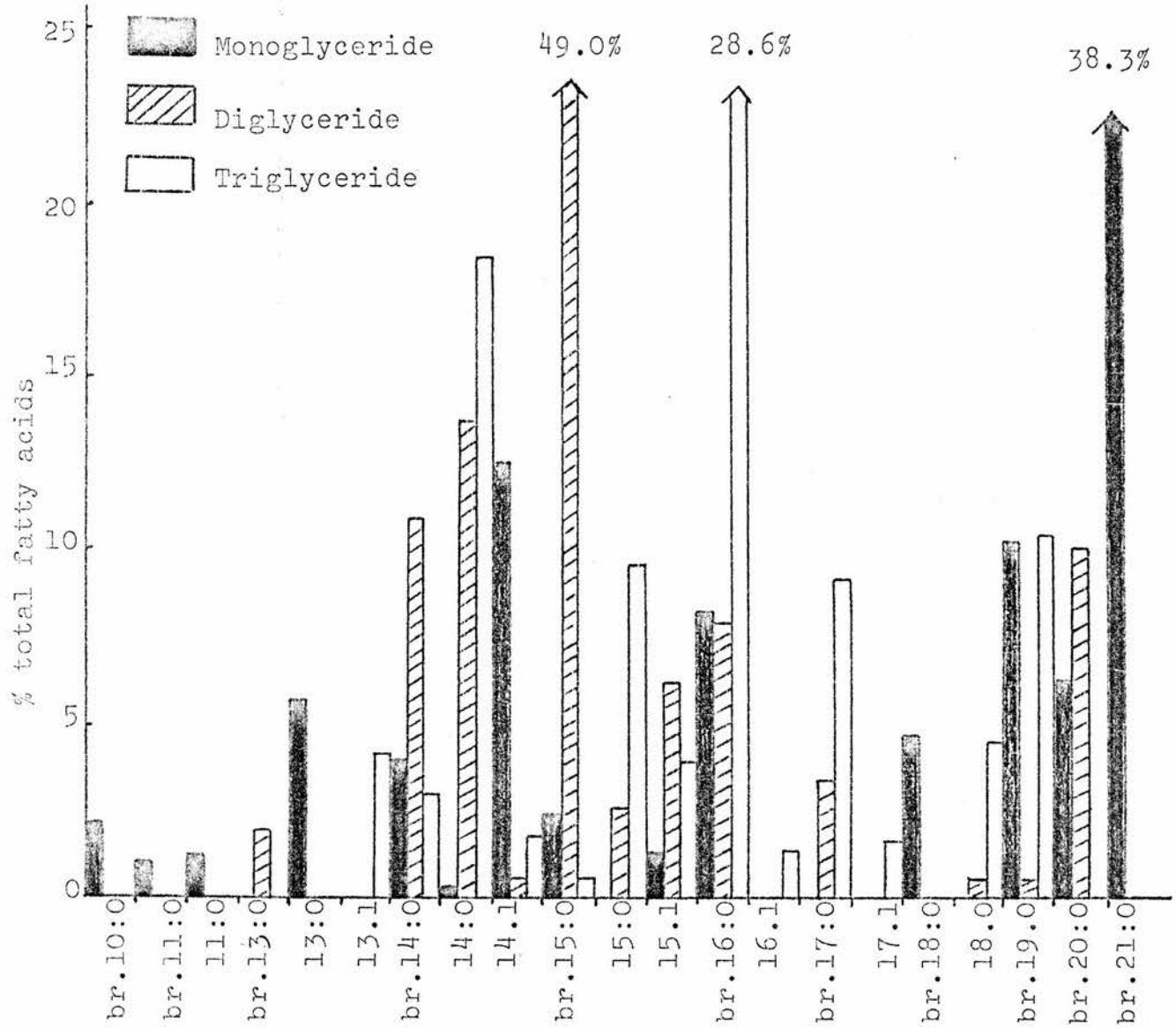
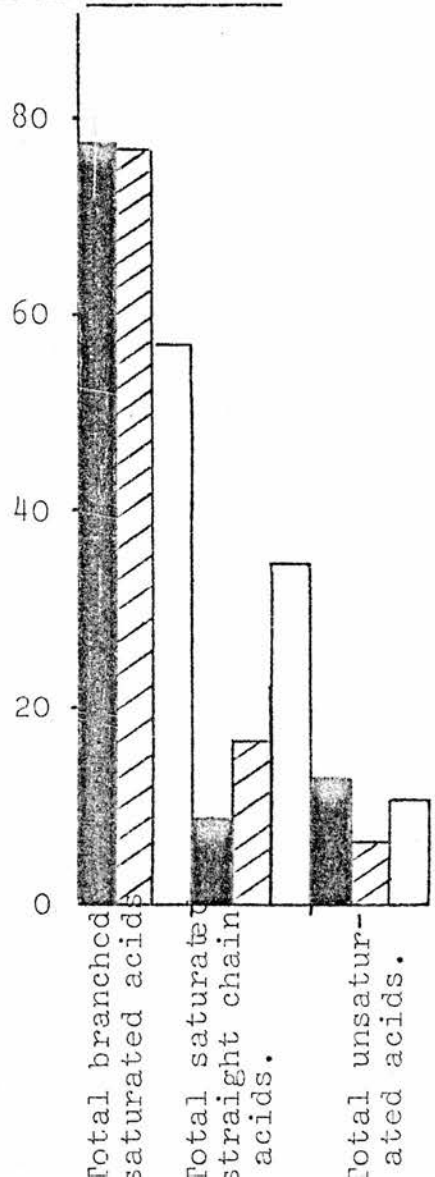


Fig.R8: Fatty acid profiles in the glyceride from M. infimus.



Distribution of fatty acid types in the glyceride fractions from M. infimus



distribution of the fatty acid types within these three glycerides. The profiles differ markedly from those obtained with similar fractions from M. marepuniceus (Fig.R7) and from one another, e.g.:

(a) the predominant fatty acid in the monoglycerides is a br 21:0.

(b) the predominant fatty acid in the diglycerides is a br 15:0.

(c) the predominant fatty acid in the triglycerides is a br 16:0.

(d) the average fatty acid chain length is longer in the monoglycerides than in the other two glyceride fractions.

(e) other major fatty acid components are (i) for monoglycerides, 14:1, br 16:0 and br 19:0; (ii) for diglycerides, br 14:0, 14:0, br 16:0 and br 20:0 and (iii) for triglycerides, 14:0, 15:0, br 17:0 and br 19:0.

The distribution of fatty acid types, again very different to those shown in Fig. R7 for M. marepuniceus, suggests that as one goes from mono- to di- to triglycerides, the amount of saturated branched chain acids decreases with a concomitant rise in saturated straight chain acids although this is less noticeable with the diglyceride fraction in that although the saturated straight chain fatty acids increase, there is only a marginal decrease in the amount of saturated branched chain acids.

G.L.C. analysis of non-isoprenoid hydrocarbons.

Table 11 shows the apparent carbon numbers and percentage peak areas for the non-isoprenoid hydrocarbons. Only those peaks constituting more than 1% of the total hydrocarbons are

shown. In each case, the remaining 4-5% was made up of a number of small peaks with carbon numbers between 16 and 22.

Table 11 : G.L.C. analysis of the non-isoprenoid hydrocarbons from M. marepuniceus and M. infimus.

Apparent carbon No.	M. marepuniceus	M. infimus
22.0	4.25	-
22.50	3.25	-
22.80	1.93	-
23.00	16.10	2.62
23.50	13.47	3.35
24.00	45.08	8.02
25.00	4.84	-
25.50	4.25	1.79
26.00	2.41	3.18
26.30	-	2.88
26.80	-	1.84
27.00	-	10.88
27.50	-	17.25
28.00	-	39.82
29.00	-	1.21
29.30	-	2.99

Again, different profiles are revealed with the two bacteria, With M. marepuniceus, approximately 75% of the total hydrocarbons have an apparent carbon number of 23 or 24, whereas with M. infimus approximately 68% of the total hydrocarbons have an apparent carbon number of 27 or 28. Although the apparent carbon

number obtained with hydrocarbons is not necessarily a true carbon skeleton number, the fact that all of the apparent carbon numbers were either whole numbers (x), x.50 or x.80 suggests that homologous series were being examined. The fractions were not hydrogenated so the presence of unsaturated compounds was not confirmed.

G.L.C. analysis of the other hydrocarbon fractions.

The material from the band with an R_f of about 0.4 after T.L.C. separation and classed as isoprenoid hydrocarbons, was analysed separately and with standards. It was found to contain one major peak and one very minor component of relatively short retention time. The major peak was identified as squalene by co-chromatography. The peak from the sample and from squalene had an apparent carbon number of 24.7 when alkanes were used as standards.

The more polar material, which was expected to give one or more components with retention time(s) greater than that of squalene, contained six main components, but the retention time of all of them was shorter than that of squalene. Using alkanes as standards, the apparent carbon numbers of these components were 17.8, 18.8, 19.3, 20.0, 21.0, 22.8.

G.L.C. analysis of long chain alcohols.

Table 12 shows the distribution of apparent carbon numbers within the long chain alcohol fractions from the two bacteria. The long chain alcohol fractions were not hydrogenated so the presence of unsaturated compounds was not confirmed.

Table 12 : G.L.C. analysis of the long chain alcohols.

Apparent carbon No.	M. marepuniceus	M. infimus
10.0	-	3.94
11.5	4.41	1.09
12.0	7.30	Tr.
12.5	-	3.18
13.0	-	2.25
13.2	4.68	-
13.5	Tr	5.98
14.2	Tr	1.58
14.5	Tr	4.38
15.2	2.03	13.04
15.7	Tr	-
16.0	8.14	1.18
16.5	7.70	8.96
16.7	1.29	1.28
17.2	1.40	Tr
17.7	-	Tr
18.2	Tr	-
18.5	1.99	-
19.0	2.38	2.36
19.2	3.74	-
19.5	37.00	11.17
19.7	-	2.76
20.0	Tr	7.15
20.5	14.33	4.20
21.2	Tr	23.91

The figures are the percentage of each component in the total long chain alcohols and Tr denotes a percentage of less than 1%.

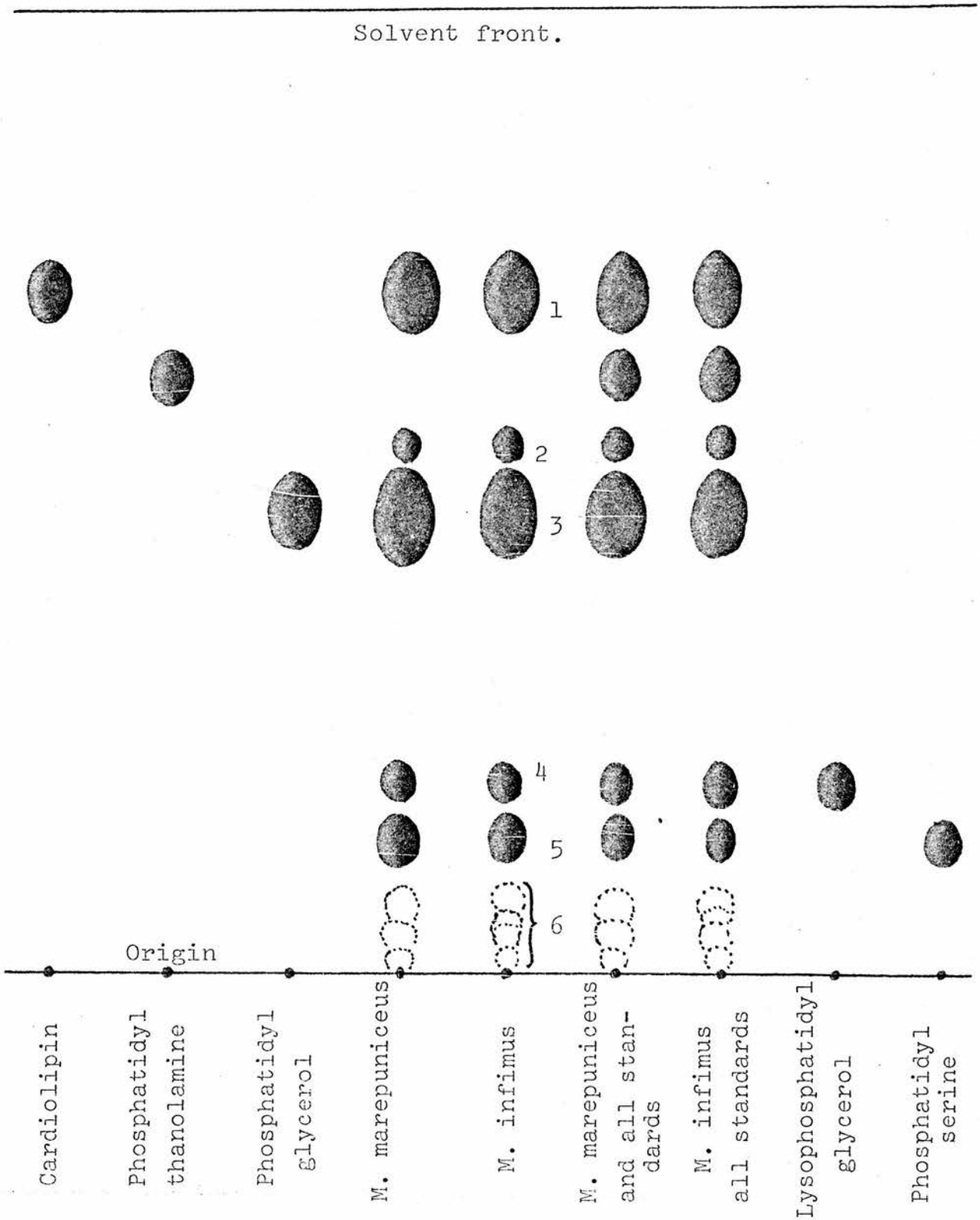
The two bacteria display individual profile patterns. The long chain alcohols constituting more than 10% of the total fraction are those with apparent carbon numbers of 19.5 and 20.5 for M. marepuniceus and 15.2, 19.5 and 21.2 for M. infimus. Although, as with the non-isoprenoid hydrocarbons, the carbon number may not reflect the true carbon skeleton, all of the components display apparent carbon numbers which are whole numbers (x), x.2, x.5, or x.7 which suggests that homologous series of compounds were present. Furthermore, the above data suggests that perhaps different homologous series predominate in the two bacteria.

Examination of the phospholipids.

Identification of individual phospholipids present.

A small sample of the phospholipid material from each micro-organism was dissolved in chloroform:methanol:: 2:1 by volume, and reduced to a few ml for chromatographic investigation. Resolution of the phospholipids from the two microorganisms using chloroform: methanol:water:: 65:25:4 by volume as solvent, gave similar results. Several chromatograms were stained with individual non-specific or group-specific sprays. Fig. R9 shows a typical separation obtained for the phospholipids from the two bacteria and the position of the authentic standards on the chromatograms. The separation shown is that obtained after spraying with the phosphorus-detecting spray, but in addition and for completeness, spots 6, which are ninhydrin-positive and phosphorus-negative have been included.

Fig.R9: T.L.C. Separation of the phospholipids of M. marepuniceus and M. infimus



Solvent : Chloroform: methanol: water :: 65:25:4 by vol.

Stain: Phosphorus detecting spray (O)

(Spots 5 and 6 Ninhydrin positive on duplicate chromatograms)

The use of the various means of visualisation enabled the characteristics and possible identity of the components present to be tentatively identified. These results are shown in Table 13.

Table 13 : Characteristics and possible identity of the phospholipid components from M. marepuniceus and from M. infimus.

Spot No. (Fig.R9)	R_f [1]	R_f [2]	P	$-NH_2$	Vic.-OH	I_2	H_2SO_4/ dichrom.	Tentative identity
1.	0.70	0.71	+	-	-	+	+	Cardiolipin
2.	0.55	N/D	+	-	-	+	+	Lysocardiolipin
3.	0.48	0.48	+	-	+	+	+	Phosphatidyl glycerol
4.	0.20	N/D	+	-	+	+	+	Lysophosphatidyl glycerol
5.	0.14	0.15	+	+	-	+	+	Phosphatidyl serine
6.	0-0.08	N/D	-	+	-	?	+	Peptido-lipids [3]

R_f [1] Solvent : chloroform:methanol:water:: 65:25:4 by volume.

R_f [2] : Quoted by Kates (1972) for the same solvent.

[3] : Based on staining and correlation with similar material reported by Summerfield (1975) to be present in Planococcus citreus.

N/D : No data available.

When resolution of the phospholipid samples was attempted by T.L.C. using chloroform:di-isobutyl ketone:acetic acid: methanol:water:: 45:30:20:15:4 by volume as solvent, although

good separation was achieved, only four spots could be detected. The missing fraction was the lysocardiolipin and this was found to run with the front of the phosphatidyl glycerol.

Using chloroform:methanol:water:: 65:25:4 as solvent, two further procedures were followed -

(a) a series of spots of different loading were resolved by T.L.C. on 0.5 mm layers of silica and the plates, after development, were treated with the sulphuric acid-dichromate spray and charred. The separations derived from plates with the correct loading were then scanned on a Vitatron TLD 100 densitometer and the relative amounts of some of the phospholipids determined.

Preparative T.L.C. of phospholipids.

(b) known weights of phospholipid from each microorganism were separated by preparative T.L.C. on 0.5 mm layers of silica. The bands were visualised as described in the "Methods" section and the individual fractions were removed and eluted as described in the "Methods" section. Each band was checked chromatographically for purity and where necessary, re-chromatography was carried out. Finally, all like fractions were pooled and their purity and identity checked by co-chromatography with authentic standards. The individual phospholipids were taken to dryness and constant weight. The results obtained and the figures obtained by densitometry are seen in Table 14.

Phospholipid weight profiles.

Table 14 : Phospholipid weight profiles from M. marepuniceus and M. infimus.

	M. marepuniceus			M. infimus		
	Wt(g).	% total weight	Equiv. wt.[1] by densitometry	Wt(g).	% total weight	Equiv. wt.[1] by densitometry
Cardiolipin (C)	0.1820	68.30	68.30	0.1551	65.58	65.58
Lysocardiolipin (LC)	0.0032	1.20	1.82	0.0167	7.06	4.61
Phosphatidyl glycerol (PG)	0.0601	22.55	26.99	0.0477	20.17	20.17
Lysophosphatidyl glycerol (LPG)	0.0150	5.62	*	0.0070	2.96	*
Phosphatidyl serine (PS)	0.0062	2.30	*	0.0100	4.23	*
Weight recovered	0.2665			0.2365		
Weight separated	0.2800			0.2500		
% recovery	95.18			94.60		

[1]: It was assumed that since cardiolipin constituted at least 65% by weight of the total phospholipids, the weights of this compound obtained were high enough to be considered highly accurate. The areas on the densitometry traces were assumed to be equivalent to these weights and the equivalent weights for the LC and PG fractions from densitometry were calculated on a relative area to weight basis.

* : The resolution of these peaks on the densitometry traces was never fine enough to allow accurate measurements to be made.

G.L.C. analysis of phospholipid fatty acids

Phospholipids in a pure state were obtained by preparative T.L.C. and subsequent re-chromatography. After methylation, the individual fatty acid profiles were obtained by G.L.C. and the compositions are shown in Figs. R10 and R11. In all cases, the major fatty acid, accounting for at least 45% of the total fatty acids, was a saturated branched C_{15} fatty acid. Table 15 shows the variation in the percentage of unsaturated, saturated and saturated branched chain acids from the various phospholipids from the two bacteria.

Table 15 : Percentage of fatty acid types in the phospholipids from M. marepuniceus and from M. infimus.

	C		LC		PG		LPG		PS	
Fatty acids	M.m	M.i	M.m	M.i	M.m	M.i	M.m	M.i	M.m	M.i
Unsaturated	3.72	6.62	8.13	6.29	1.97	5.20	2.55	6.01	2.31	13.82
Branched	88.98	78.06	65.43	82.49	91.60	85.53	85.54	68.99	67.80	58.00
Saturated	7.30	15.32	26.44	11.42	6.43	9.27	11.91	25.00	29.89	28.18

It is noticeable that -

(i) saturated branched chain fatty acids constitute a very high percentage of the total fatty acids present in both cardiolipin and phosphatidyl glycerol from both bacteria and in lysocardiolipin from M. infimus and lysophosphatidyl glycerol from M. marepuniceus.

(ii) Lysocardiolipin from M. marepuniceus and lysophosphatidyl glycerol from M. infimus have lower amounts of saturated branched chain fatty acids and higher amounts of saturated straight chain

Fig.R10: Fatty acid profiles from the phospholipids of M. marepuniceus.

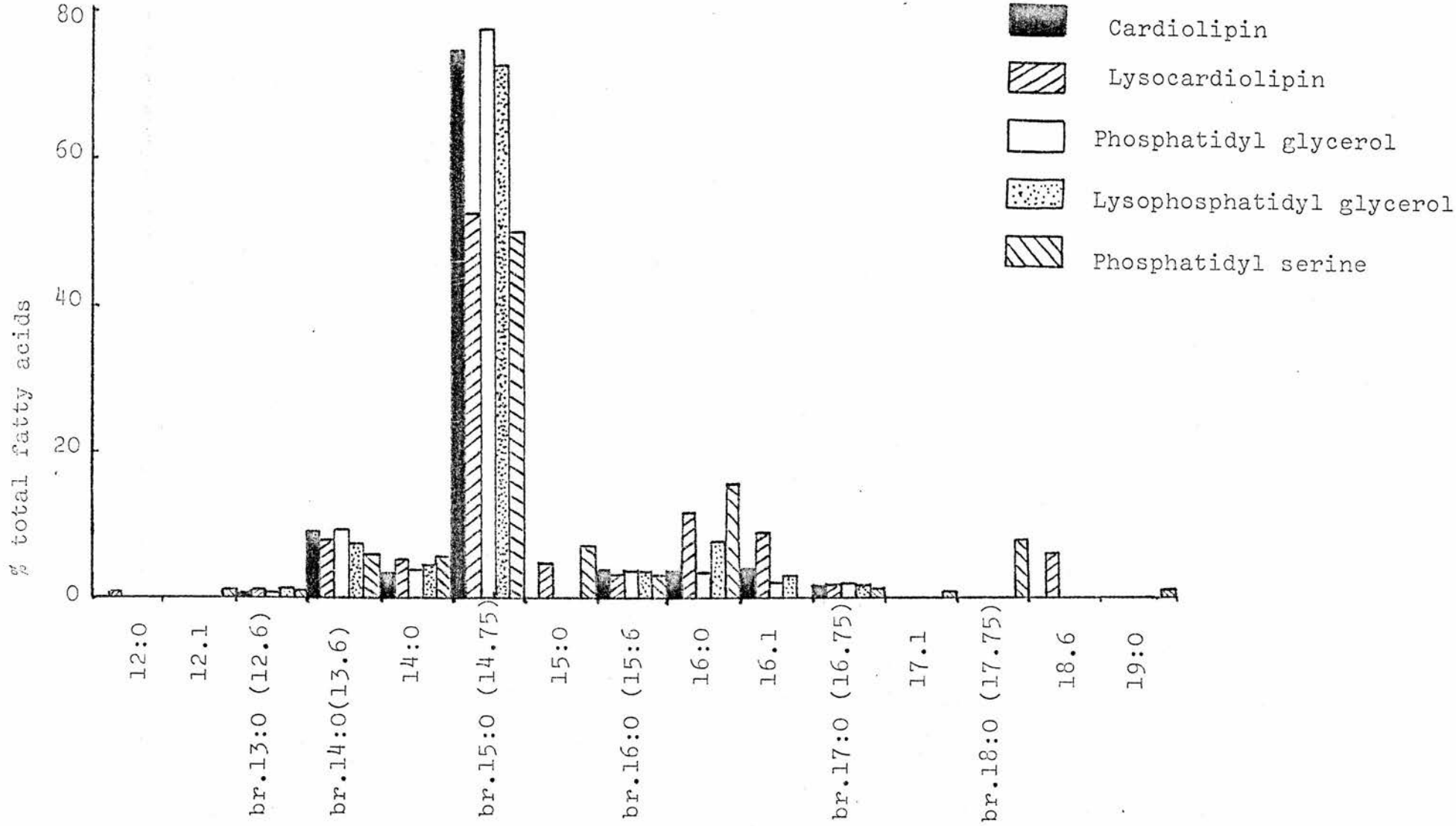
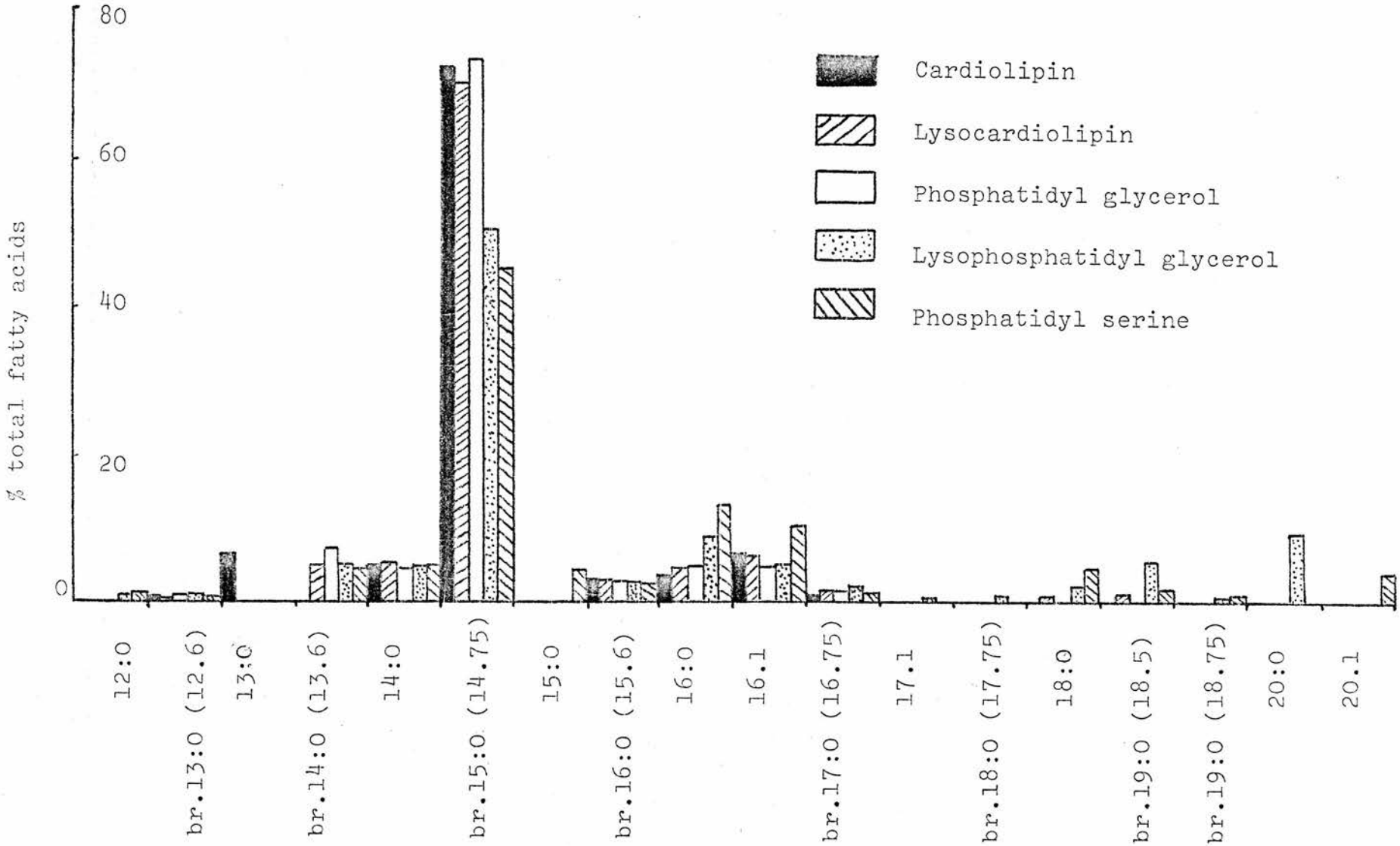


Fig. R11: Fatty acid profiles from the phospholipids of M. infimus.



fatty acids.

(iii) In the phosphatidyl serine from both bacteria, there is a lower content of saturated branched chain fatty acids and a higher content of saturated straight chain fatty acids than in any of the other phospholipids from either microorganism.

(iv) With the exception of the phosphatidyl serine from M. infimus, the amounts of unsaturated fatty acids are generally low.

DISCUSSION

From the results obtained with the qualitative experiments on the growth of both bacteria in different media, it would appear that both M. marepuniceus and M. infimus are facultative mild halophiles capable of growing well in a sea salt medium, but also capable of growth in media containing no added sea salts. This differs from the findings of Summerfield (1975) with Planococcus citreus Migula. The latter microorganism was claimed by Kocur, Pacova, Hodgkiss and Martinec (1970) to be a facultative mild halophile, but Summerfield showed that growth did not take place in this instance unless 0.5% (w/v) sea salt was added to the medium.

The finding that medium G supported growth better than the other media investigated was not surprising. The recommended medium for these microorganisms is a sea salt broth (agar) and medium G was simply this medium supplemented with 1 % (w/v) glucose. It has regularly been shown by Hunter (1976) that the addition of glucose increases both the generation time and the bacterial numbers obtained with extreme halophiles, even though it had been previously claimed that such microorganisms had only poorly developed metabolic machinery for the utilisation of carbohydrates, and that as a consequence, their major carbon sources should be provided by peptides and/or amino acids (Larsen, 1967). There was reason to hope that glucose may have a similar stimulatory effect on the growth of mild halophiles.

In liquid culture, neither of the two microorganisms displayed a tendency to clump during growth and this was borne

out by the fact that similar results were obtained for their growth curves by either turbidimetry or the TCA precipitation technique. Again, this was contrary to the work of Summerfield (1975) with Planococcus citreus Migula. An even more accurate growth curve may have been obtained in each case had viable cell counting been used. The latter will only record the viable cell population whereas inaccuracies may be introduced into the two methods used as a result of the presence of dead cells and/or cell debris. Both microorganisms, on inoculation into liquid culture, displayed a small drop in absorbance before the onset of the logarithmic phase. The reason(s) for this is not easy to find when one considers that logarithmic phase cells were used as an inoculum and that an identical medium was used for the inocula. One possibility is that as it was subsequently shown that the pH of the medium fell during growth (more so with M. marepuniceus than with M. infimus), a change in pH may have been a contributory factor. Acid production resulting in this fall in pH, as stated in the "Results" section, may also have contributed to the control of the end of the logarithmic phase and also to the fact that there was a very short stationary phase with both bacteria. These findings suggested that during batch culture, the best time to harvest would be just as the cells of each microorganism were entering stationary phase from the logarithmic phase. This was achieved by regular monitoring of the cell population in the batch cultures around this time. Knowing that the pH fell in both cases, the batch cultures were maintained at around pH 7.0 using pH control and it was noticeable that a higher cell density was produced and that good yields were obtained on

harvesting with the Sharples centrifuge. Other factors could also have contributed to these higher cell populations, for example, the achievement of a higher oxygen tension in solution in the medium or a more thorough stirring of the medium. At the termination of the batch cultures, they were allowed to stand for 5 - 10 min before harvesting and this allowed almost all of the added antifoam to rise to the surface. This was removed before harvesting was started. This is a wise precaution as most of the antifoams are organic solvent-soluble and can give rise to problems in later lipid extraction and separation procedures.

Work previously carried out on the lipids of Micrococci or Sarcinae in this laboratory (Thirkell et al., 1971; Thirkell et al., 1974 and Thirkell et al., 1977a/b) has involved the examination of membrane lipids. Work with these two microorganisms showed that either they were resistant to attack by lysozyme (as are extreme halophiles), or that the enzyme had only marginal activity in that neither microorganism was readily lysed by treatment with the enzyme whereas the control microorganism used, Micrococcus lysodeikticus, was lysed quite quickly. It could also be postulated as a result of these findings, that the cell walls of these two microorganisms do not contain muramic acid. As a result it was decided to extract the lipids from whole cells in this work. As stated in the "Introduction", it is generally recognised that virtually all, if not the entire lipid in bacterial cells is localised in the membrane and so one could assume that the results presented in this thesis are in actual fact, with respect to the membranes. The major exception to this situation is when a microorganism lays down lipid as storage globules. This

material is normally poly-β-hydroxy butyric acid and it was shown that neither of these two bacteria produced this compound in that it could not be detected when the appropriate lipid fractions were examined for its presence.

The chemical composition of bacterial membranes has been claimed to vary with the methods used for their isolation, purification and assay as well as with the age of the culture (Salton et al., 1965; Salton, 1967; Bishop, Rutberg and Samuelsson, 1967; Ward and Perkins, 1968 and Brown and Stevenson, 1975) and it is likely therefore that the composition of the lipids extracted from whole cells may well also vary with similar parameters. Consequently, the medium was kept constant, the growth conditions were constant and the age of the culture on harvesting was kept constant. However, two different lipid extraction procedures were used. One of them, the method of Bligh et al., (1959) (method B) was a standard well-tried procedure, whilst the other method (method A), involving the use of the Buhler ball mill was not. The immense variation in the results reported would certainly support the argument that different results may be obtained for the lipid composition which are at least to some extent, dependent upon the method used for the isolation of these lipids.

Previous work had shown that the extraction method of Bligh et al., (1959) does not extract the total lipid from bacterial membranes (Thirkell et al., 1974) in that further lipid could be removed from the membrane residues by acid and/or alkali treatment. Furthermore, the membranes investigated in this department have always been pigmented with carotenoids, them-

selves unsaponifiable lipids, and it is not usually possible to achieve a total pigment extraction. It was therefore thought that perhaps a more rigorous lipid extraction procedure would achieve a higher degree of lipid extraction, particularly from the whole cells, and so the Buhler ball mill was used in the first instance. Various difficulties became apparent when this extraction method was used. In the first instance, the initial extraction had to be carried out using 95% (by volume) methanol or otherwise, if chloroform:methanol was used as the extracting solvent, a biphasic system was produced on centrifugation as a result of the extraction of water from the moist cell pastes. The method achieved a high degree of disintegration of cellular material and when the initial extracts ("crude lipid") were taken to dryness, the "crude lipid" accounted for about 40% of the dry cell weight, a value obviously high and obviously incorrect. The two most likely explanations for this were that fine suspended material was being recovered in the centrifuge supernatants, and/or that a large amount of lipoprotein was being extracted, and that the protein content was significantly elevating the "crude" lipid weight. Although no solid material was present when the final extraction was taken to dryness, it was also noticeable that a total lipid extraction was still not being achieved since the bacterial remnants were pale pink in colour. It was originally intended to purify the "crude lipid" from this procedure on Sephadex G 25 by the method of Wells and Dittmer, (1963) which removes non-lipid contaminants. The bulk of the "crude lipid" was found to be insoluble in chloroform:methanol:water in the proportions re-

commended and so the above purification step could not be followed. Only that material which was soluble was worked on further. The presence of this insoluble material again suggested that either particulate matter or a high proportion of lipoprotein had been recovered in the "crude lipid". By taking only that material which was soluble for further investigation, this suggested that only the "free" and readily extractable material was being examined. It is not even possible to say that all of such material was present in the soluble lipids or that what was present was a representative sample of it. The possibility of occlusion of free lipid in the insoluble material cannot be ignored. After separation into neutral lipid, glycolipid and phospholipid, the distribution of the lipid classes was in agreement with many published values, but showed a higher content of phospholipid and glycolipid and a lower content of neutral lipid than previously reported from another mild halophile (Summerfield, 1975). During the next stage of isolation, the removal of free fatty acids by carbonate wash from the neutral lipids, there was about a 35 - 40% weight loss and although every phase was closely examined, this could not be accounted for. A double check on the weight of material taken for the procedure was made by repeating the extraction and the results obtained were reproducible. The fact that poly-β-hydroxybutyric acid was not shown to be present, removed one possible explanation for such losses. Although some further work was done on some of the neutral lipid material resulting from this extraction procedure, it was decided that since too many odd results seemed to be appearing, an alternative method

of lipid extraction should be attempted. The method of Bligh et al (1959) seemed to be an obvious choice.

As with method A, the method of Bligh et al (1959) did not extract all of the lipid present in the cells. Further investigation of the total lipids extracted by this procedure therefore, should not assume that the extracted lipid is a representative sample of that present in the cells. It is not even true to say that all of the 'free' extractable lipid was removed for if the remnants were left standing under chloroform:methanol at 4^{o}, pigment, and presumably other lipid, slowly leached out.

The lipid content as a percentage of the cellular dry weight from both extraction procedures was within normal limits. However, extraction method B appeared to produce more "pure lipid" from both of the bacteria, again suggesting the possible occlusion of material in the insoluble material from extraction method A. Resolution of the total lipid from both extraction procedures produced fairly similar amounts of neutral lipid. One might expect this if method A extracts a lot of lipoprotein which would not go into solution at a later stage, for lipoprotein would more likely involve polar rather than non-polar lipids.

Further work on the neutral lipids from M. marepuniceus extracted by both lipid extraction procedures revealed many variations. Fractionation of the neutral lipids showed that although hydrocarbons account for about 75% of the total neutral

lipids in each case, the weight distribution of the other neutral lipids was dependent upon the original extraction procedure used and showed few similarities. Such differences become more obvious when the fatty acid profiles of the glycerides and free fatty acids are compared and this will be discussed later.

Let us now consider the lipids extracted by method B. The neutral lipids from both bacteria did not show weight losses when the free fatty acids were removed by carbonate wash (cf. earlier work from method A). Both neutral lipid fractions showed the presence of the same components when they were resolved by TLC. TLC also revealed that removal of free fatty acids by carbonate wash is not absolute.

Whereas resolution of the hydrocarbons in Planococcus citreus Migula showed that the major component was squalene, for both of these bacteria, at least 96% of the total hydrocarbon fractions co-chromatographed with standard alkanes. The remaining 3-4% was resolved into two further bands representing isoprenoid hydrocarbons and some more polar material. The isoprenoid hydrocarbon band contained only one minor peak and squalene. It has been suggested that squalene itself plays an important role in maintaining the stability of the lipid bilayer in Halobacterium cutirubrum (Lanyi et al., 1974). It is a linear molecule 28-29 Å in contour length and if extended, would almost span the hydrophobic region of the bilayer. It may therefore be required for a similar purpose in mild halophiles but in variable concentration. The larger amount by comparison, found in Planococcus citreus Migula may suggest that there is a greater requirement for squalene in obligate rather than in facultative mild halophiles. Squalene has also been reported in other extremely and moderately halophilic

bacteria by Kushwaha et al. (1974). The more polar hydrocarbon material is of unknown identity. One component could be a precursor related to the C_{55} isoprenoid alcohol used as a membrane carrier in cell wall biosynthesis, but the relatively short retention times of the components would appear to rule this out.

There was too little sterol ester material from M. infimus for further invesitgation and so only that from M. marepuniceus was examined. Whereas mammalian membranes contain large amounts of cholesterol of which squalene is the precursor, with few exceptions such as Mycoplasmas and Streptococcal L-forms (Hunter, 1971), it is not normally found in bacteria. Sterol and sterol ester fractions have been isolated respectively from the membranes of Sarcina flava (Hunter, 1971) and Planococcus citreus Migula (Summerfield, 1975) in this laboratory. As with previous material, this sterol ester fraction co-chromatographed with authentic cholesterol stearate, and after saponification, the free sterol co-chromatographed with standard cholesterol. However, GLC analysis with the recovered sterol from the sterol esters of M. marepuniceus showed that under the conditions used, the free sterol was not cholesterol and that on GLC it had a longer retention time than did cholesterol. This result was also in agreement with those obtained with the sterols recovered from S. flava and from the Planococcus. The exact identity of the material has yet to be shown. If a sufficient quantity could be prepared, GLC coupled to mass spectrometry may give the answer itself, or in addition, nuclear magnetic resonance (n.m.r.) studies could be carried out.

The non-isoprenoid hydrocarbons from the two bacteria (Table 11 - "Results") are seen to be different. The average chain length in M. marepuniceus (approximately 75% have apparent carbon number of 24-25) is shorter than in M. infimus (approximately 68% have apparent carbon number of 27-28). The major component in M. marepuniceus has an apparent carbon number of 24.0 (45.08%) whereas in M. infimus, it is 28.0 (39.82%). These major components compare with the following; the main hydrocarbon reported present in a Micrococcus species (ATCC 146) had an apparent carbon number of 25 (Morrison et al. 1971) and in Sarcina lutea the main hydrocarbon is reported as an unsaturated branched C29 compound by Tornabene, Morrison and Kloos (1971) and as having an equivalent carbon number of 28.0 by Albro and Huston (1964). It is thus obvious that apparent carbon numbers of hydrocarbons, either whole numbers or fractions, are not necessarily indicative of straight chain saturated compounds, unsaturated and branched derivatives or of the actual carbon skeleton as a high degree of structural variability is possible. Nevertheless, the fact that the apparent carbon numbers were whole numbers (x), x.3, x.5 or x.8 suggested that homologous series of compounds were being examined.

Analysis of the long chain alcohols again shows differences between the two bacteria. The major component in M. marepuniceus has an apparent carbon number of 19.5 (37%) and in M. infimus, the major component had an apparent carbon number of 21.2 (24%). With both bacteria, more than 50% of the total long chain alcohols have an apparent carbon number of 19.0 or more. From both bacteria, the apparent carbon numbers of most of these long chain alcohols were greater than those reported for the other mild halophile, Planococcus citreus Migula where the major long chain alcohols had apparent carbon numbers of 12.2 and 16.0 (Thirkell

et al, 1977).

It is in the fatty acid profiles of the glycerides, free fatty acids and fatty acid ester fractions that the greatest differences are seen, not only from one bacterium to another, but with respect to M. marepuniceus according to the method used initially for the total lipid extraction. Analysis to produce fatty acid profiles from similar fractions in several other gram-positive species has shown that generally a branched saturated C15 fatty acid is the major component (Akashi et al., 1960; Cho et al., 1966; Girard, 1971; Hunter et al., 1971; Morrison et al., 1971). In Planococcus citreus Migula, it was the predominant fatty acid in all of the above fractions with the exception of the triglycerides where the content of it was realtively small (Thirkell et al., 1977b). In the work reported in this thesis, this branched saturated C15 fatty acid was only the predominant fatty acid in the diglyceride and free fatty acids from M. marepuniceus when the total lipid was extracted by method A, and in the free fatty acids and diglycerides of M. infimus when the total lipid was extracted by method B. The full list of predominant fatty acids (more than 10% of the total fatty acids in the fraction) in the various fractions is as shown below. They are given in order of relative concentration so that the major fatty acid is shown first.

M. marepuniceus (lipid extracted by method A)

Monoglycerides	: br. 18:0, 16:0, 18:0.
Diglycerides	: br. 15:0, br. 14:0
Triglycerides	: br. 18:0, 16:0, br. 20:0, 19:0.
Free fatty acids	: br. 15:0, br. 16:0

M. marepuniceus (lipid extracted by method B)

Monoglycerides	: 16:0, br. 15:0, br. 18:0
Diglycerides	: 18:0, br. 16:0, br. 18:0, 16:0.
Triglycerides	: br. 18:0, br. 16:0, 18:0, 16:0.
Free fatty acids	: br. 21:0, br. 20:0, br.18:0, br. 19:0, 16:0.
Fatty acid esters	: br. 23:0, br. 22:0.

M. infimus. (lipid extracted by method B)

Monoglycerides	: br. 21:0, 14.1, br. 19:0
Diglycerides	: br.15:0, 14:0, br.14:0, br.22:0.
Triglycerides	: br. 16:0, 14:0, br.19:0.
Free fatty acids	: br. 15:0, 16:0, 18:0, 14:0.
Fatty acid esters	: br. 14:0, br. 22:0, br. 15:0, 16:0.

Pools of all of the predominant fatty acids in the glyceride fractions, with the exception of the unsaturated C_{14} acid in the monoglycerides of M. infimus, are available in either the free fatty acids or fatty acid ester pools of the neutral lipids. All other predominant acids are in the free fatty acid pools alone with the exception of the saturated 18:0 acid (di- and triglycerides of M. marepuniceus), the br-21:0 acid (monoglycerides of M. infimus) and the br-22:0 acid (diglycerides of M. infimus) which are only in the fatty acid ester pools. These acids are presumably available for incorporation into the glycerides during synthesis. One must assume that with respect to M. infimus, since the 14.1 acid appears in all three glyceride fractions, that a pool does exist which is too small to detect on analysis, and that once it is incorporated into

the monoglyceride, little or none is lost by subsequent deacylation/reacylation reactions. Some of the monoglycerides containing this fatty acid must then be specifically further acylated to produce those di- and triglycerides which contain this fatty acid.

Looking at the results from M. marepuniceus, the br.18:0 and 16:0 fatty acids predominate in the monoglycerides from both extraction procedures, and the br.18:0 and 16:0 fatty acids similarly predominate in the two triglyceride fractions from this microorganism. Otherwise, there is little similarity in the fatty acid profiles within these fractions as a result of the two different lipid extraction procedures. Possible explanations of these differences are that whilst it is acknowledged that neither extraction procedure achieves a 100% extraction of lipid, different proportions of the individual neutral lipid fractions may be extracted by the two methods or that different areas of the cell membranes are extracted by the two methods, or that the growth and harvesting parameters were not as constant as was thought. The latter is the least probable explanation but at the same time, it is difficult to justify the first two explanations.

It is also seen that the fatty acid profiles for individual fractions from the two bacteria differ even when the same lipid extraction procedure has been used. This simply follows the pattern seen when other neutral lipid fractions from the two bacteria are compared (e.g. hydrocarbons and long chain alcohols) The variability in the glyceride fatty acid profiles in any of

the sets of results seems to indicate that a pool of different mono- and diglycerides exist in both bacteria and that there may be a high degree of selectivity of specific monoglycerides as precursors of diglycerides and in turn, of diglycerides as precursors of triglycerides, i.e. specificity shown by the di- and triglyceride acyl transferases. There may also be a degree of deacylation/reacylation occurring too, particularly in the di- and triglyceride fractions which would help to explain the differences in the fatty acid profiles in the glyceride series from the individual bacteria.

The saturated C16 fatty acid which is a predominant fatty acid particularly in the glycerides of M. marepuniceus, has been shown to be a major fatty acid in the glycerides of the extreme halophile Micrococcus halodenitrificans (Kates et al., 1966) and in a mildly halophilic rod (Stern et al., 1973).

Comparing the fatty acid profiles of the free fatty acids and fatty acid ester fractions from both bacteria where method B has been used for the extraction of the lipid, again reveals marked differences. (see also Fig. R5). The average chain length of the fatty acids in both fractions from M. marepuniceus is longer than in the same fractions from M. infimus. It is noticeable that a high percentage of the total fatty acids in the fatty acid ester fraction from both bacteria constitutes saturated branched chain fatty acids of carbon chain length C20 and above, and that this is more pronounced in the fraction from M. marepuniceus.

Not only do the fatty acid profiles differ from fraction to fraction in each bacterium and between the two bacteria, but the ratio of saturated straight chain fatty acids to saturated

branched chain fatty acids to unsaturated fatty acids also shows marked differences. The data previously expressed in histograms can also be shown collectively as in the following table where the figures are expressed as percentages of the total fatty acids.

Table D 1 : Ratio of fatty acid types in the free fatty acids, fatty acid esters and in the glycerides from the two bacteria.

	M. marepuniceus			M. infimus		
	Sat.	Sat-br.	Unsat.	Sat	Sat-br.	Unsat.
Free fatty acids	18.12	81.88	0	48.55	49.08	2.37
Fatty acid esters	12.07	75.36	12.57	25.33	61.24	13.43
Mono-glycerides	55.81	40.56	3.63	8.05	77.96	13.99
Di-glycerides	46.37	40.20	13.43	16.39	77.29	6.32
Tri-glycerides	44.10	50.79	5.11	32.53	56.53	10.94

In M. marepuniceus, whereas at least 75% of the total fatty acids in the free fatty acids or fatty acid esters are saturated branched chain acids, the ratio of saturated branched chain fatty acids to saturated straight chain acids is approximately 1:1 in the three glyceride fractions. Except in the fatty acid ester and diglyceride fractions, there is only a small amount of unsaturated fatty acids.

In M. infimus, the profiles are very different. Here there is an approximate 1:1 ratio of saturated branched chain fatty acids to saturated straight chain acids in the free fatty acid

material, but in all of the other fractions, saturated branched chain fatty acids predominate, more so in the mono- and diglyceride fractions and less so in the fatty acid ester and triglyceride fractions. In general the fractions from this bacterium also appear to contain more unsaturated fatty acids. Thus in the glycerides of this microorganism, the saturated branched + unsaturated fatty acids account for between 67-92% of the total acids, whereas in the same fractions from M. marepuniceus, these two fatty acid types together account for only between 44-56% of the total acids. Since branched chain fatty acids and unsaturated fatty acids have similar effects on the packing of membrane lipids and hence on fluidity, the fluidity of the membrane lipids in the two bacteria may be different.

Whereas the response of bacteria to a wide variety of metabolic reactions and to gram-staining, or the guanine+cytosine/adenine + thymine ratio have been used as tools in bacterial classification, it has been suggested by some workers that fatty acid profiles could similarly be used (Cho et al., 1966; Moss, Kellogg, Farshy, Lambert and Thayer, 1970; Auran and Schmidt, 1972). From the results presented in this work it is obvious that a comparison of two mildly halophilic Micrococci has shown not only marked differences in fatty acid profiles within the neutral lipid fractions, but also marked differences in the profiles of the long chain alcohols and hydrocarbons thus apparently ruling out such analyses as a possible means of classification. The results have also shown that the distributic of lipids found and the profiles of the components within the different neutral lipid fractions may well be very dependent upon the lipid extraction procedure employed, particularly if

whole cells are used.

The phospholipid components were measured quantitatively by gravimetric determination. Had time permitted, phosphorus determinations may have given an even more accurate picture. It is seen that in both microorganisms, large amounts of cardiolipins (cardiolipin + lysocardiolipin) are present (72.64% of the total phospholipids in M. infimus and 69.50% in M. marepuniceus). It is not clear whether the presence of the lyso- derivatives is due to cleavage of fatty acid(s) from cardiolipin and phosphatidyl glycerol molecules during isolation procedures or whether they are naturally occurring components, presumably of the bacterial membranes. The latter is almost certainly the case for no procedure used would seem strong enough to release fatty acid(s). Although several molecular species of lysocardiolipins, with respect to fatty acids, are undoubtedly present, the chromatographic mobility suggests that all molecules possess the same number of fatty acids., viz. 1, 2 or 3. Although phosphatidyl glycerol accounted for around 20% of the total phospholipids, it was not the major phospholipid as it is in many microorganisms, e.g. it is the only glycerophospholipid in Acholeplasma laidlawii where at least some of it interacts with another membrane component which may be an integral protein (Bevers, Singal, Op den Kamp and van Deenen, 1974). They also showed that at least some of this phosphatidyl glycerol is capable of translocation from the inner layer of the membrane to the outside by a flip-flop mechanism. Experiments with Staphylococcus aureus showed that the main effects on the phospholipids of increasing NaCl concentrations in the growth

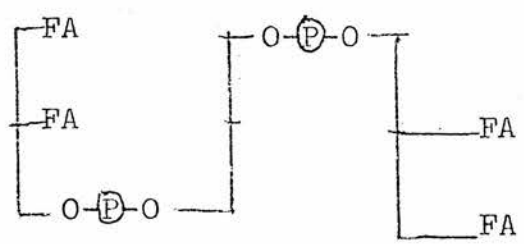
medium (0.05→10%) was an increase in cardiolipin at the expense of phosphatidyl glycerol (Kanemasa, Yoshioka and Hoyashi, 1972). This may well be a natural phenomenon in this type of mild halophilic microorganism. The presence of lysophosphatidyl glycerol was unexpected since it is not a common phospholipid, and it was of interest to show that the only other phospholipid present in the two bacteria was phosphatidyl serine. Neither lysophosphatidyl glycerol nor phosphatidyl serine was detected in the Planococcus by Summerfield (1975), but that microorganism contained phosphatidyl ethanolamine. Since it has been reported that phosphatidyl ethanolamine may be interchangeable with glycosyl diglycerides (Minnikin et al., 1971), the absence of phosphatidyl ethanolamine in these bacteria may be explained by the higher levels of glycolipids found when compared with Planococcus citreus Migula. Papahadjopoulos (1971) reported that ion permeability in model membrane systems was determined by the charge on the polar head groups of the lipids which he used. Only membranes formed from acidic phospholipids (cardiolipin and phosphatidyl glycerol, or phosphatidyl glycerol and phosphatidyl serine) were found to be highly cation selective. All three of these phospholipids are present in these bacteria and large amounts of them may well be an advantage to mild halophiles which generally have an internal ion composition very different from that of the external medium .

The major fatty acid associated with all of the phospholipid fractions is the saturated branched chain C15 acid which accounts for between 45.17% (phosphatidyl serine from M. infimus)

and 77.54% (phosphatidyl glycerol from M. marepuniceus) of the total fatty acids in a single phospholipid component. The only other fatty acids constituting more than 10% of the total fatty acids of any single phospholipid component are 16:0 (13.71%) and 16.1 (10.63%) in the phosphatidyl serine from M. infimus, 16:0 (16.75%) in the phosphatidyl serine and 16:0 (10.94%) in the lysocardiolipin from M. marepuniceus.

With one exception, the saturated straight chain C13 fatty acid in cardiolipin from M. infimus, the fatty acid components of the two phosphatidyl glycerol fractions are similar to those of the respective cardiolipin fractions. This can be expected since phosphatidyl glycerol is known to be the biosynthetic precursor of cardiolipin (Short et al., 1972).

With respect to the phospholipids of M. marepuniceus it is of interest to look at the fatty acid profiles of the two pairs, cardiolipin and lysocardiolipin, and phosphatidyl glycerol and lysophosphatidyl glycerol. Diagramatically, cardiolipin can be represented as shown. There are two fatty acids esterified to each of two of the three glycerol skeletons and it would appear that on average, three of these fatty acids are the saturated branched C15 acid. From the other fatty acids detected, it is obvious that a series of cardiolipins are present where on average, the fourth fatty acid is variable. The lysocardiolipins were chromatographically pure and so the greatest possibility is that one fatty acid has been cleaved from the cardiolipin. Since the lysocardiolipins on average, have 50% of their fatty acids as



the saturated branched C15 acid, it could be assumed that initially there is a preferential loss of one such residue from the cardiolipin. However, there are changes in the ratios of the other fatty acids present in the lysocardiolipin as compared with the original phospholipid. One possible explanation of this is that a further deacylation/reacylation stage follows the production of the lysocardiolipin and that the second fatty acid lost is not a C15 fatty acid and that specific reacylation occurs with fatty acids other than the C15 acid in order to produce the fatty acid profile recorded. Since the lysocardiolipin was chromatographically pure, as previously stated, it can only be assumed that any such subsequent deacylation/reacylation is a rapid process which does not allow for the build up of the doubly-deacylated intermediate.

In the phosphatidyl glycerols, two fatty acids are esterified to the single glycerol skeleton and in the lysophosphatidyl glycerols, one of these fatty acids is lost. In both cases, about 75% of the total fatty acids are the saturated branched chain C15 fatty acid. A possible explanation of this is that random cleavage occurs and again, a degree of rapid deacylation/reacylation could occur to account for the variations in the percentages of the other fatty acids present.

It is seen that the same fatty acid predominates in the phosphatidyl serines (45%) suggesting that on average, one of the two fatty acids esterified is the C15 acid and the other fatty acid is variable to produce a series of these phospholipids. The other fatty acids present in this material show significant differences from those present in the other phospholipids.

With respect to M. infimus, a different situation is seen in the various fatty acid profiles of the individual phospholipids. In both the cardiolipins and lysocardiolipins, the C15 fatty acid accounts for about 70% of the total fatty acids. This suggests, as before, that three of the four esterified fatty acids in cardiolipin may be this compound. Here the production of lysocardiolipins would seem to be by random cleavage of a fatty acid but there is also evidence to suggest subsequent rapid deacylation/reacylation of the lysocardiolipins to account for the variation of percentages of the individual fatty acids present as compared with the cardiolipins.

In the phosphatidyl glycerols, approximately 75% of the total fatty acids are the saturated branched C15 acid, but this acid accounts for only 50% of the total fatty acids in the lysophosphatidyl glycerols . For two possible explanations of this, consider first that two populations of phosphatidyl glycerols exist as shown diagramatically below:

If the cleavage was due to a phospholipase A_2 which showed specificity for cleavage of the C15 fatty acid from carbon 2 of the glycerol backbone, lysophosphatidyl glycerols would be produced where on average, 50% of the total remaining fatty acids would be the C15 acid. Alternately, phospholipases A_1 and A_2 could be operative in cleaving fatty acid residues from carbons

1 and 2 respectively, and that the profiles seen are the result of differences in the net activity of these two enzymes.

The fatty acid profiles for the phosphatidyl serines again suggest that on average, one of the two esterified fatty acids is the same C15 compound and that again, the other fatty acid component is variable and shows significant differences when compared with the other fatty acids associated with the other phosphilipid fractions.

Nevertheless, comparing the two bacteria, the weight ratios of the individual phospholipids and their fatty acid profiles show much less species variation than was seen in the neutral lipid fractions.

Because of a lack of time, neither the glycolipid fractions nor the peptido-lipids associated with the phospholipid fractions were further investigated. The peptido-lipids, also reported by Summerfield (1975) may well represent an association of lipid material with some of the integral proteins of the cell membrane. They could be the result of the "burying" of fatty acid chains in the hydrophobic areas of globular proteins. Another possibility is that they may represent specific carrier molecules in the membrane, maybe associated with ion transfer mechanisms.

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