AN INVESTIGATION INTO THE MEMBRANE COMPOSITION OF A PLANOCOCCUS SPECIES

Mark Summerfield

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at the
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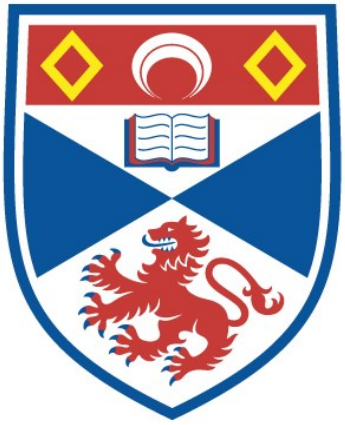

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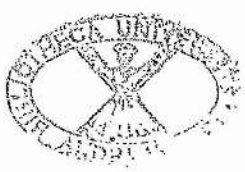


ABSTRACT

Planococcus C.C.M. 316, a gram-positive facultative marine halophile, was studied with respect to growth and membrane composition of cells grown in media containing 0.5%, 3% and 10% sea salt. Membranes were prepared from cells grown in the three sea salt concentrations and analysed to determine any changes which may have been caused by the increasing concentrations of salt in the growth media. The three membrane preparations were found to have similar compositions to those reported for other gram-positive cocci. Cells grown in the 3% sea salt concentration contained membranes with a higher protein:lipid ratio and RNA content than the membranes from cells grown in the 0.5% and 10% concentrations. Amino acid analysis of the membrane proteins showed that the composition remained virtually unchanged in the three membrane preparations. The ratio of acidic: basic amino acid residues was nearer to the figures reported for non-halophiles than for those of the extreme halophiles. Examination of the lipids showed that phospholipids predominated to the extent of about 70% of the total lipids. Cardiolipin and lysocardiolipin were the major phospholipids, with phosphatidyl ethanolamine, phosphatidyl glycerol and phosphatidyl serine present as minor components. Glycolipids were found to decrease with increasing sea salt concentration in the medium, and in all three membrane preparations constituted only a very small proportion of the total lipids. Neutral lipids contained long chain alcohols, mono-, di- and tri-glycerides, as well as relatively large amounts of the isoprenoid compound squalene. The major fatty acid associated with the lipids was a branched saturated C_{15} acid which constituted 50 - 70% of the total fatty acids in most fractions. Although increasing salt in the medium produced changes within the proteins and lipids in the membranes, these changes were

not such that they could be interpreted as an increase in the halophilic nature of the membrane. The carotenoids were shown to be derived from β carotene and to consist mainly of 3'hydroxy 4'oxo compounds, although the extent of polar substitutions was dependent on both culture age and the concentration of salt in the medium.

DECLARATION

I hereby declare that the following thesis is based on 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.

The research was conducted in the Department of Biochemistry, United College of St. Salvator and St. Leonard, University of St. Andrews, under the direction of Dr D. Thirkell.

CERTIFICATE

I hereby certify that Mark Summerfield has spent nine terms engaged in research work under my direction and that he has fulfilled the conditions of the General Ordinance No. 12 (Resolution of the University Court No. 1, 1967) and that he is qualified to submit the accompanying thesis for the degree of Doctor of Philosophy.

ACADEMIC RECORD

I first matriculated at the University of St Andrews in October 1967, and graduated with the degree of Bachelor of Science, Second Class Honours (Division I) in Biochemistry in June 1972. I matriculated as a research student in the Department of Biochemistry, University of St Andrews, in October 1972.

ACKNOWLEDGEMENTS

I should like to thank Dr D. Thirkell for his continuing advice and encouragement during the past three years. I should also like to thank Dr M.I.S. Hunter for much helpful discussion, and my wife for typing this manuscript.

Aims of Thesis

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

1. To grow Planococcus C.C.M. 316 at various sea salt concentrations and determine growth parameters.
2. To grow up large batch cultures of Planococcus in 0.5%, 3% and 10% sea salt concentrations and to prepare membranes from these cells.
3. To carry out quantitative and qualitative comparisons of the chemical composition of the membranes from the cells grown in the three different sea salt concentrations.
4. To analyse in detail and to compare the lipids, proteins and carbohydrates from the three membrane types.
5. To analyse in detail the fatty acid composition of the individual lipid classes.
6. To extract and analyse the carotenoids from the three membrane types.
7. To observe changes in pigmentation which occur in the cells grown in different sea salt concentrations.
8. To investigate the morphology of the cells in different salt concentrations by light microscopy and electron microscopy.
9. To obtain some information regarding the cations associated with the membranes and the whole cells.

CONTENTS

INTRODUCTION

Organisms which survive and flourish in environments which are at the extremes of biological tolerance will always be of interest to man. Bacteria are perhaps the most successful inhabitants of this planet with regard to adaptation to a wide variety of different environments and thus they can often be found under conditions where no other organism could survive. Halophilic bacteria are no exception; these microorganisms are able to live in concentrations of salt in which other cells would rapidly be crenated or destroyed by the extremely high osmotic pressure drawing water out of the cells. Only those organisms with a specially adapted protective mechanism can prevent this from occurring. Halophilic bacteria are particularly unusual in that not only do they survive in high salt environments, but many of them actually require large amounts of salt in their growth medium in order to reproduce and carry out normal cell functions. Not all halophiles exhibit this remarkable feature however, and this forms the basis for a distinction between organisms which are salt dependent and those which are merely salt tolerant.

Obligate extremely halophilic bacteria are generally classed as those organisms which grow best at sodium chloride (NaCl) concentrations of around 25% and which require a minimum concentration of about 15%, below which no growth occurs. Some of these organisms will grow in salt concentrations of over 30% (1), a figure which is approaching the solubility limit of NaCl in water. Mild or moderate halophiles are classed as those organisms which can tolerate NaCl concentrations up to 15 - 20% but which generally have an optimum growth concentration of between 5% and 10% NaCl. These organisms also have a lower limit below which no growth occurs, and this may be only 1% or 2% NaCl (2,3).

The third type of halophiles are classed as facultative halophiles and are not halophilic (salt loving) in the true sense of the word.

These organisms are able to adapt to salt concentrations which may be up to 15% NaCl, but have no lower limit as do the two classes previously mentioned. This means that they may be cultured equally well in media which contains salt and in that which does not.

Extreme halophiles are generally found in and around salt marshes, salt flats and shallow salty pools where solar heat has caused the build up of high concentrations of salt by evaporation. They may also be found on animal hides, or meats such as bacon which have been treated with brine, or between fish scales, where they cause discoloration of the material since they are often brightly coloured due to the presence of carotenoid pigments (2,4). Mild and facultative strains can be isolated from marine or estuarine water samples.

An analysis of strains of extreme halophiles has shown that there is much species synonymy and only two genera are thought to be substantiated (1). These are (a) halobacteria, which are rod-like gram-negative organisms, and (b) halococci, which are spherical gram-variable or gram-positive aerobic organisms. When examined under the light microscope, halococci tend to be about 1.5 μ in diameter. Depending on the species, they can be seen as single cells, as pairs of cells, as tetrads, as "packets of eight" formations usually ascribed to most of the Sarcinae species (5), or as irregular clusters as seen with Sarcina marina. The halobacteria have been more extensively studied than the halococci, partly due to the fact that the osmotic and mechanical fragility of the halobacteria makes their cell components more accessible. Mild and facultative halophiles have received only scant attention.

Halophilic rods are usually fairly large and differ from the halococci in that their cell walls tend to be much thinner than those of non-halophilic microorganisms when thin sections are viewed under the electron microscope (6,7,8). The halobacteria are classified in the genus Pseudomonadaceae, according to Bergey's Manual (1957).

A major difference between the rods (halobacteria) and the cocci (halococci) of these extremely halophilic species is the fact that the halococci are much more resistant to a hypotonic environment. The halococci do not lyse when the salt concentration in their environment is reduced, and they generally have a lower limit of salt concentration in which growth will occur. This lower limit for the halococci is 5 - 10% NaCl as opposed to 10 - 15% NaCl for the halobacteria.

The taxonomic status of the halococci has been clarified, mainly by the work of Kocur and Bohacek (9,10,11). The extreme halococci were originally grouped with the genus Micrococcus, but on the basis of percentage guanine + cytosine (% GC) content of the DNA, Kocur suggested that they be placed in a separate genus, namely "Halococcus". The % GC of extremely halophilic cocci at 57 - 61% is considerably lower than that of the Micrococci, in which most strains range from 66 - 75 % GC. (see Fig. 1).

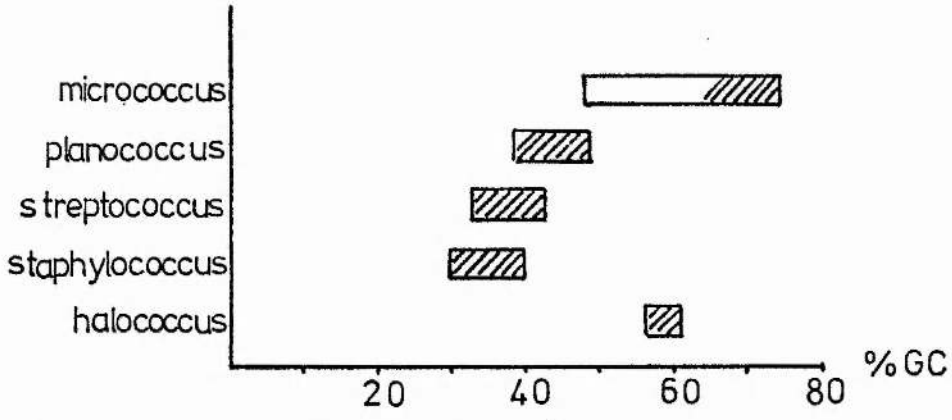


Fig. 1: % GC content of the Halococcus and Planococcus species in relation to other cocci.

Kocur also suggested that the genus Planococcus be made separate from the genus Micrococcus (12). He pointed out that flagellated cocci had a % GC considerably lower than the rest of the Micrococci and on this basis he proposed that flagellated cocci with a % GC ranging from 39 - 51% be allocated to the genus Planococcus Migula. Members of this

genus, which includes the organism on which the work in this thesis was performed, are described as flagellated marine facultative halophilic cocci.

The physiological, biochemical and morphological bases for salt tolerance and salt requirement have stimulated the main research interest with respect to halophiles in general. Extreme halophiles differ from their non-halophilic counterparts in a number of ways apart from the lower % GC content already mentioned. They have a much slower growth rate; the doubling times for halobacteria range from a minimum of seven hours whereas the doubling times for halococci are even longer, some species having a minimum doubling time of 15 hours (2).

When thin sections are viewed on the electron microscope, to a larger extent with halobacteria and to a lesser extent with halococci, the thickness of the cell wall material appears to be less than that found in non-halophilic microorganisms. This wall material in both cases does not have the usual chemical composition (13), being made up of polysaccharide, peptides and protein, but lacking muramic acid and diaminopimelic acid which are usual cell wall "building blocks" in their non-halophilic counterparts. Another unusual feature of these microorganisms is their high internal salt concentration which is usually at least equal to that found in the external growth medium, and in some cases is greater (14). Furthermore, although the overall cation concentration within the halophilic cell approximates that of the growth medium in which they normally grow, the concentrations of the individual ions vary quite considerably. Thus, the growth medium usually contains Na^+ at several hundred times the concentration of K^+, but the cells manage to concentrate K^+ to over 2M in some cases, making the internal K^+ concentration several times the Na^+ concentration (14). This means that the cell membrane is able to distinguish between two similar

monovalent cations and to selectively concentrate one of them enormously at the expense of the other. In H.salinarium, Christian and Waltho (14) found that the concentration of KCl within the cell was close to the limit of its solubility. They also found that the sum of the internal $K^+ + Na^+$ concentrations in extreme halophiles was greater than that of the medium, while in the mild halophiles it was less. Their findings are summarised in Table 1.

	Staph. aureus	M.halo-denitri-ficans	Vibrio costi-colus	Sarc. morr-huae	H. sali-narium	
NaCl in medium	0.150	1.000	1.000	4.000	4.000	moles
KCl in medium	0.025	0.004	0.004	0.032	0.032	
Na^+ in cells	0.098	0.311	0.684	3.170	1.370	moles/ mg cell water
K^+ in cells	0.680	0.474	0.221	2.030	4.570	
Cl^- in cells	0.008	0.055	0.139	3.660	3.610	

Table 1: A comparison of the internal and external ion concentrations in some extreme, mild and non-halophilic species.

Internal Cl^- concentrations were shown to be higher in extreme halophiles than in mild and non-halophilic species, and Christian and Waltho (14) suggested that in non-halophiles, the free anions are predominantly organic in nature. This means that the internal solutes of extreme halophiles are mainly NaCl and KCl, whereas in the non-halophiles, Na^+ and K^+ probably occur largely associated with organic anions such as amino acids. Mild halophiles would seem to occupy a mid-way position with both organic and inorganic anions associated with the Na^+ and K^+ ions. This high internal salt concentration raises the question of how the cellular enzymes can operate in this type of environment. When examined in vitro, many of these enzymes were found to exhibit maximum efficiency at salt concentrations at or around those thought to be existing within the cell and in most cases activity

was lost on removal of the salt (15,16,17). This fact perhaps accounts for cessation of growth of whole cells in a medium of low ionic strength. However, since not all enzymes from extreme halophiles exhibit their optimum activity at the salt concentrations known to exist within the cell, this may suggest that certain enzymes cannot operate maximally under the normal ionic conditions within the cell.

In Table 2 is a list of enzymes from H.cutirubrum which showed little or no activity below NaCl concentrations of 0.5% and which showed their maximum activity in solutions of between 10% and 25% salt.

Table 2: Obligate halophilic enzymes (24)

Aspartate transcarbamylase

NADH: Menadione reductase

Cytochrome oxidase

Polynucleotide phosphorylase

Citrate synthetase

Malic enzyme

The above can perhaps be classed as extremely halophilic enzymes. Enzymes showing maximum activity at intermediate NaCl concentrations (3 - 10%) have been isolated from H.salinarium and are shown in Table 3.

Table 3: Salt tolerant enzymes from halophiles (24)

Malic dehydrogenase

Ornithine transcarbamylase

Arginine desimidase

Isocitric dehydrogenase

Many of the above enzymes are inhibited at higher salt concentrations. There are, in addition, a few enzymes which have been isolated from extreme halophiles which do not require salt or which are inhibited even at low salt concentrations (Table 4).

Table 4: Halophilic enzymes requiring no salt for activity (24)

Fatty acid synthetase RNA dependent RNA polymerase	from H.cutirubrum
Amylase	from H.halobium

Inhibition of fatty acid synthetase by high salt concentration is consistent with the findings that the membrane lipids of these micro-organisms contain almost exclusively ether-linked analogues of acidic phospholipids (see later) and that fatty acids are only present in small quantities, if at all (18). It is a curious feature of these microorganisms that they have an enzyme which is inhibited under normal circumstances, but which is synthesised in reasonable quantities within the cell to no apparent end. The side chains in ether-linked lipids in plants and animals are derived from long chain alcohols which are added to dihydroxyacetone phosphate by means of an exchange reaction with an acyl grouping. The alcohol is derived from a fatty acid by means of a reduction reaction. The low rate of fatty acid synthesis in extreme halophiles makes this pathway less likely and the side chains in this case are probably derived from isoprenoid compounds which are known to be present in the neutral lipids of such microorganisms.

The inhibition of amylase in H.halobium may be linked with the observation that most halophiles do not grow well in media which contain carbohydrate as the sole carbon source. Amino acids are the carbon sources which seem to be utilised and metabolised most readily (3). However, it has been shown (19) that Sarcina morrhuae can utilise glucose normally, but here amylase would not be involved.

The explanation for the diversity in salt requirement by these enzymes may lie in the complex relationship that exists between proteins and lipids. The activity of an enzyme may differ enormously after it has been removed from its normal situation in the cell, and reproducing these internal conditions in vitro is very difficult to achieve.

Another interesting aspect of the salt dependance of halophilic enzymes is that although many of them are inactivated by the removal of the high salt environment, adding back the salt does not result in reactivation of some enzymes unless the salt is added slowly, over a number of hours. This indicates that a salt-dependent reorientation of the macromolecule is occurring and that time is required for a number of intermediates to form before the final active conformation is achieved (20).

The inactive conformation of these enzymes after removal of salt is probably extensively unfolded. This view is supported by evidence from Holmes and Halvorson (21) who found a marked decrease in sedimentation constants after denaturation of proteins in this manner.

Furthermore, the enzymes of halophilic organisms show a marked specificity for certain cations. In H.salinarium, Baxter and Gibbons (16) found that KCl was twice as effective as NaCl in the activation of some enzymes. This is not a totally unexpected observation in view of the fact that the internal K^+ concentration is two to three times that of the Na^+ concentration in many species (14, 22, 23).

The ability of the enzymes within the extremely halophilic cell to withstand high salt concentrations allows these microorganisms to balance their internal salt concentration with the external concentration thus eliminating the enormous osmotic pressures which would have to be withstood by the cells if their enzymes could not function in a high salt environment.

An examination of the proteins in extreme halophiles has shown that there is a marked preponderance of acidic amino acids relative to the basic amino acids, particularly in the cell envelope and in the ribosomal proteins (24). In general therefore, halophilic proteins are rich in glutamic and aspartic acids and poor in lysine and arginine

when compared with their non-halophilic counterparts (24,25,26). This can be seen from the data presented in Table 5.

		Moles % excess of acidic amino acids over basic amino acids	After correction for amide
Rods	H.salinarium	17.1	10.5
	Pseudomonas fluorescens	7.2	-1.6 (non-halophile)
Cocci	Halophilic cocci strain 24	18.0	10.8
	Halophilic cocci strain 46	16.4	10.0
	Sarcina lutea	8.6	1.9 (non-halophile)

Table 5: A comparison of the excess acidic and basic amino acid residues in the total proteins of some extreme halophiles and their non-halophilic counterparts (25).

After correction for the amount of glutamic and aspartic acid present in the amide form, it appears that the extreme halophiles have a 10 mole % excess of acidic amino acids in their total proteins, whereas non-halophiles have values for their proteins which approximate nearer to net charge neutrality.

It has been suggested that the high salt concentration plays a part in allowing the proteins to assume their active conformations. Cations from the medium or from the cytoplasm, depending upon the location of the protein, may be able to neutralise excess negative charges on the molecule and thus prevent charge repulsion which may cause unfolding and consequent loss of activity (27).

Further evidence linking high activity in proteins with halophilic nature comes from work carried out by Brown (28). He showed that increased halophilic character could be produced in a species of marine pseudomonad by the introduction of extra carboxyl residues on to membrane

proteins. This was achieved by succinylation of the ϵ-amino groups of lysyl residues and it resulted in an increase in the acid : base ratio from 5 to 15 mole %. No alteration to membrane structure was observed under the electron microscope. The membranes before succinylation were found to disaggregate at concentrations of NaCl below 1 -2%. However, after succinylation, concentrations of NaCl around 5% were found to be sufficient to cause disaggregation. This was interpreted as an increase in the halophilic nature of the cell envelope. Brown (28) draws the analogy with the protein haemerythrin, which on succinylation of amino groups, to increase the number of negative charges, disaggregates into eight sub-units, owing to the increased electrostatic repulsions.

The "charge screening effect" however, cannot entirely explain the salt dependance of halophilic proteins. Charge screening by salt should be achieved at much lower salt concentrations than the several molar quantities apparently required. Also, a specificity for various ions has been observed (29,30), and neither of these observations are in keeping with a simple charge shielding theory. Although charge shielding cannot explain the phenomenon entirely, it is very likely that this is in some way involved. Norberg, Kaplan and Kushner (31) found that the high concentration of monovalent cations required for the stabilisation and activity of certain enzymes could be partially replaced with lower concentrations of divalent or polyvalent cations such as spermine. Polyvalent cations have a much greater charge density, and they may be expected to reduce the electrostatic free energy of proteins at lower concentrations than monovalent ions. Furthermore, enzymes from extreme halophiles may also require high salt concentrations for their activity because they are stabilised in an active conformation by the formation of intramolecular hydrophobic bonds which are only

thermodynamically favourable under such conditions (24).

The cell membrane in extreme halophiles is also unusual in that the polar lipids are extremely acidic and contain almost exclusively ether-linked analogues of acidic phospholipids, particularly phosphatidyl glycerol and phosphatidyl glycerophosphate. Kates' group have been most active in elucidating this aspect, with reference to H.cutirubrum (18, 32,33,34,35,36), but other workers have also been involved, particularly with studies on H.halobium and H.salinarium (37,38).

The neutral, non-polar lipids in H.cutirubrum constitute about 10% of the total membrane lipid and consist mainly of carotenoid pigments and the isoprenoid compounds, squalene, dihydro- and tetrahydro-squalene. Menaquinone 8 has also been found in all the extreme halophiles studied (39). The combination of polar and non-polar lipids with acidic proteins and cations forms the structure known as the cell envelope. In the halobacteria, the association of cations with this membrane has been found to be of great importance. When the cation concentration around the membrane is reduced, the membrane disintegrates into small lipoprotein sub-units and a number of inactivated enzymes are released (40, 41). If the salt is removed slowly from around whole cells, they can be seen to distort, and their shape alters from rods to irregular spheres, and finally to perfect spheres which lyse when the salt concentration is reduced to 5 - 10% (42,43). The cell lysis is not solely accounted for by osmotic pressure changes alone but is thought to be due to the fact that the cell envelope requires a high salt environment for the maintenance of its integrity. Plachy (44) noted during studies on dispersions of the polar lipids isolated from H.cutirubrum that in the absence of added salts ($MgCl_2$ and NaCl), lipid fluidity increased. This supports the theory that salt has a stabilising effect on the lipid components. Investigations into the interrelationships between the

membrane lipid components of H.cutirubrum have revealed that squalene may play an important part in maintaining the stability within the lipid bilayer. Lanyi, Plachy and Kates (45) 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 Ca^{++} and Mg^{++}, as do lipids from other bacteria, unless squalene was also present in the bilayers at or above 6 - 8% of the total lipid. By using fluorescent probes, these workers concluded that squalene was orientated perpendicular to the plane of the membrane (parallel to the phytanyl chains). Their results were consistent with the theory that aggregation is dependent on the ability of Ca^{++} and Mg^{++} 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 groups 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. Lanyi et al. (45) suggested that it would not be unreasonable to assume the existence of a packing geometry which favours interaction between the phytanyl chains and squalene. Cations are thought to reduce the net anionic charge density of the lipid head groups and allow closer packing of the lipid molecules (46,47). A membrane containing phosphatidyl glycerophosphate is thought to have two layers of negative charges (48,49) (Fig. 2); one layer is exposed on the outer surface of the membrane and the other buried in closer proximity to the hydrophobic regions (45).

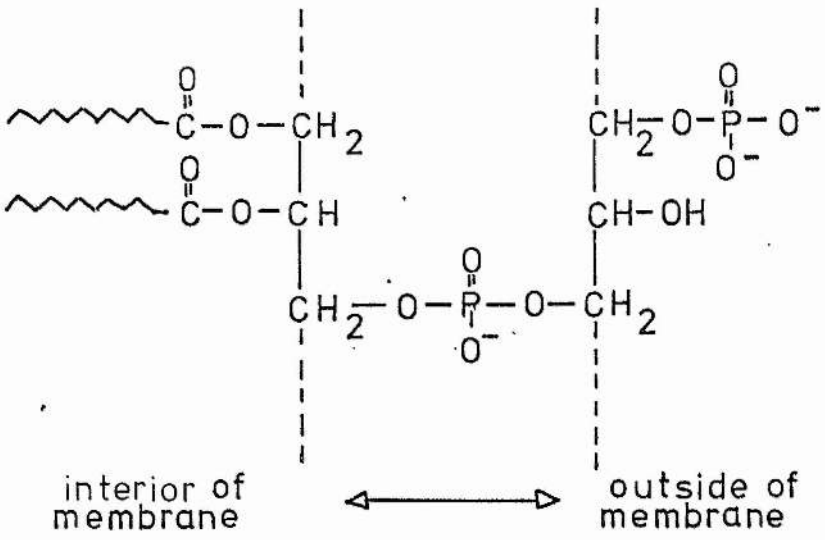


Fig. 2: Proposed orientation of phosphatidyl glycerophosphate in the membrane (45).

Squalene is thought to space the lipids so that cations can reach the inner phosphate groups, screening their negative charges and allowing the formation of "bridge" complexes involving the external phosphate groups and the divalent cations. The concentration of squalene required to permit flocculation is not very different from that found in the cell envelope (49). The binding of certain proteins to phospholipids, involving Ca^{++} and Mg^{++} has been suggested by other workers (50,51), and the rearrangement of the polar lipid anionic head groups at lowered NaCl concentrations may explain the release of protein from the membrane observed under these conditions.

The extremely halophilic cocci also appear to contain a high proportion of very polar ether-linked lipids (18). Very little work has been carried out as it is difficult to obtain membrane preparations which are free from cell wall material, and because of their extended doubling times.

Mild and facultative halophiles appear to exhibit some of the unusual features of extreme halophiles, but other features appear to

be unique to the latter group. The membranes of the mild halophiles studied so far, in contrast to those of the extreme halophiles, do not predominantly contain the unusual alkyl diether analogues of acidic phospholipids (52,53,54), even when they are grown in salt concentrations comparable with the natural habitat of the extreme halophiles (i.e. in 3M NaCl) (1) (Table 6).

		% Non-saponifiable material	% Fatty Acid
Extreme	H.cutirubrum	70.4	0.6
	H.halobium (M)	73.6	0.4
	H.halobium (P)	68.7	0.3
	H.salinarium	66.5	0.7
	S.littoralis	64.8	2.3
Mild	M.halodenitrificans (48 h)	0.5	62.6
	M.halodenitrificans (20 h)	9.1	68.3
	V.costicolus	5.7	60.5
Non	S.lutea	13.7	68.3
	S.flava	13.5	59.6

Table 6: A comparison of the amounts of non-saponifiable material and fatty acids obtained from some extremely halophilic, mildly halophilic and non-halophilic microorganisms (18). Values are expressed as a percentage of the total lipid.

M.halodenitrificans is a mildly halophilic gram-positive non-flagellate coccus. This organism was found (53) to contain less phospholipid (approximately 50% of the total lipid) than was found in H.cutirubrum (approximately 90% of the total lipid). Phosphatidyl glycerol and phosphatidyl ethanolamine were identified in the mild halophile. Examination of the lipids of other mild halophiles has shown that they consist of the more usual ester-linked membrane

phospholipids (52) found in non-halophilic species (cardiolipin, phosphatidyl glycerol, phosphatidyl ethanolamine and phosphatidyl serine (18).

Some of the mild halophiles have been found to contain squalene and other isoprenoid compounds in their neutral lipids (39). This is an interesting observation in view of the role of squalene in the membrane of extreme halophiles discussed above.

A comparison of the amounts of lipid present in halophiles and non-halophiles shows that the extreme halophiles have a much lower total lipid content than other species (Table 7) (18).

		Lipid as a % of salt free dry weight of cells	Phosphorus content as a % of total lipid
H.cutirubrum	extreme	3.5	4.25
H.halobium (M)	extreme	4.1	4.32
H.halobium (P)	extreme	2.6	3.83
H.salinarium	extreme	3.6	3.37
S.littoralis	extreme	0.7	3.70
M.halodenitrificans	mild	6.0	3.74
V.costicolus	mild	9.4	3.76
S.lutea	non	7.2	2.89
S.flava	non	5.9	3.28

Table 7: A comparison of the percentage lipid and lipid phosphorus in some extremely halophilic, mildly halophilic and non-halophilic organisms.

Lipid phosphorus is high in the extreme halophiles because of the large amounts of phosphatidyl glycerophosphate (% phosphorus 6.6) present in the lipids.

Thus, to summarise, mild halophiles do possess the preponderance of acidic amino acids in their proteins (40), although this imbalance is not so marked as that found in the extremely halophilic species. Mild and facultative halophiles also have increased internal salt concentrations (14) and the ability to selectively concentrate a particular cation against a gradient to a greater degree than non-halophiles. Their cell wall structure resembles non-halophiles in that it is lysozyme-sensitive (12), indicating the presence of muramic acid.

The membranes of extreme halophiles would seem to be unique to this species. Mild halophiles, although similar to extreme halophiles in a number of aspects, appear not to have the unusual ether-linked highly acidic lipids, although they do contain acidic proteins and the membrane does become unstable under conditions of low salt. The membranes of mild halophiles are generally of the type normally associated with non-halophilic organisms, but they have evolved through minor changes to allow the cells to function normally. Because of the high acidity of the lipids found in extreme halophiles it is not unreasonable to expect high acidity in the ester-linked lipids 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. Stern, Peleg and Tietz (52,55,56) have shown the presence of glycolipids and glucosyl-phosphatidylglycerol in a moderate halophile, and Kates (18), as previously mentioned, has shown the presence of cardiolipin, phosphatidyl glycerol and its amino acyl derivatives in M.halodenitrificans. These lipids are of course present in non-halophilic membranes also, and it remains to be shown conclusively whether or not halophilic organisms contain these lipids in a higher proportion than their non-halophilic counterparts. The phosphorus content of the lipids of at least two mild halophiles (Table 7) has already been shown to be high.

In general, the polar membrane lipids of non-halophilic species contain a mixture of acidic and neutral polar lipids. Neutral polar lipids, such as phosphatidyl ethanolamine, commonly found in gram-negative bacteria, and glycosyl diglycerides, commonly found in gram-positive bacteria (57,58) must also have an important function within the membrane, although they may not be directly associated with ion binding. It has been shown (59) that acidic phospholipids are capable of discriminating between different univalent cations whereas phospholipids such as phosphatidyl ethanolamine and phosphatidyl choline are not. Recent work on erythrocyte membranes (60) has shown that different species of phospholipid are located on different sides of the membrane bilayer, i.e. the internal or external sides of the bilayer. This asymmetric arrangement of the lipids is almost certainly due to their different functions, for example the acidic ion binding phospholipids are located predominantly on the inside of the bilayer; this may or may not be the case in bacterial membranes.

The ratio of acidic to neutral polar lipids is obviously important to the permeability properties of the membrane. Minnikin, Abdolrahimzadeh and Baddiley (61) have shown with B.cereus that under growth conditions which limit phosphate, diglycosyldiglyceride can partially replace phosphatidyl ethanolamine (both neutral polar lipids) and an acidic glycolipid can partially replace the acidic phospholipids. This shows an interesting adaptability of the cells, and implies the importance to the cell of maintaining the correct acidic to neutral polar lipid ratio even when some of the usual membrane building materials are restricted.

The neutral lipids of extreme halophiles have not been found to comprise ether-linked mono-, di- or tri-glycerides as might have been expected. Phytanyl side chains appear only in polar lipids.

Work by Kates (62) with labelled mevalonate has shown that the biosynthetic pathway probably proceeds through mevalonate → isopentanyl pyrophosphate → dimethylallyl pyrophosphate → phytanyl pyrophosphate. Along with the lack of fatty acids this results in the neutral lipids containing almost exclusively menaquinone 8, and isoprenoid compounds ranging from squalene to C_{50} carotenoids.

The carotenoids of halophilic organisms are often red in colour and are almost certainly located in the membrane, as is invariably the case in non-halophiles. The main pigment found in the extreme halophiles so far studied is bacterioruberin, a tetra-hydroxy C_{50} carotenoid with no ring structures (63). The structure is shown below.

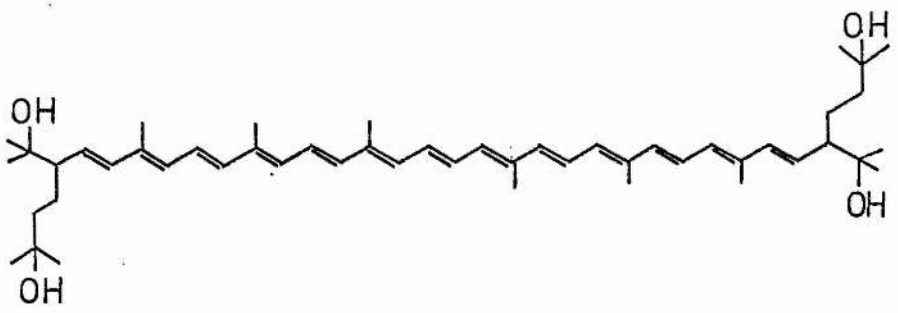


Fig. 3: Bacterioruberin.

Thirteen conjugated double bonds are responsible for the chromophore, giving λ_{max} absorption at 468 - 498 - 532 nm in acetone. Other carotenoids are produced by the halobacteria but, for example, bacterioruberin accounts for approximately 85% of the carotenoids of H.salinarium when grown in media containing 25% NaCl. Kushawa, Pugh, Kramer and Kates (64) reported the occurrence of the C_{40} carotenes, β carotene, neo α carotene and neo β carotene in H.cutirubrum. They also presented evidence for the presence of other members of the Porter-Lincoln series, cis and transphytoene and phytofluene. They suggested that H.cutirubrum utilises the same biosynthetic pathway for C_{40} carotenoids as that found in higher plants. Kelly, Norgard and

Liaaen-Jensen (63) suggested that the pathway for the biosynthesis of the bacterioruberins involves the addition of two C_5 isoprenoid units on to a C_{40} linear carotene molecule as follows:

$$\text{mevalonate} \xrightarrow{(a)} C_{40} \text{ carotenes} \xrightarrow{(b)} \text{bacterioruberin}$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad 2\ C_5 \text{ units}$$

Gochnauer, Kushawa, Kates and Kushner have shown (65) that the addition of small amounts of glycerol (0.1%)to a defined salt medium almost completely inhibited the formation of bacterioruberins in H.cutirubrum and H.halobium, although growth was stimulated. Carotenoid production was diverted towards the synthesis of retinal, and the proportion of C_{40} carotenoids (mainly β carotenes) within the membrane increased. Gochnauer et al. (65) suggested that glycerol inhibited step (b) in the above scheme, and stimulated the production of retinal from the C_{40} carotenes. Steps (a) and (b) were found to be stimulated by the addition of glucose and only small amounts of retinal were produced in the absence of glycerol.

The function of the carotenoids in the membrane appears to be to protect components of the cell against photochemical damage which may result from the intense solar radiation which normally occurs in the natural habitat of extreme halophiles. Dundas and Larsen (66) suggested that carotenoids act by quenching electronic excitations impressed by light upon other pigment systems within the envelope. Their conclusions were based on the fact that colourless mutants of extreme halophiles grow more slowly than the pigmented wild type when grown in the same medium and exposed to strong light (66). This was in contrast to the findings of Mathews and Sistrom (67,68) who showed that a colourless mutant of S.lutea (a non-halophile) was rapidly killed when aerated suspensions were exposed to bright sunlight.

The carotenoid content of halophiles is thought to remain constant with variation of the salt concentration in the medium, but is thought

to increase with culture age (38) and then decline fairly rapidly (65).

The pigments of mild halophiles have received little attention, but it seems that the C_{40} as opposed to the C_{50} carotenoids predominate (63,64).

C_{50} carotenoids have also been found in nature in other non-halobacteria. Flavobacterium dehydrogenans (69,70) and Sarcina flava (71,72) are known to contain carotenoids of this type. These carotenoids do not apparently impart any halophilic characteristics to the cells and are perhaps only present in a protective capacity. The reasons why C_{50} carotenoids are preferentially biosynthesised in these organisms must remain a matter for conjecture at present.

MATERIALS AND METHODS

GROWTH

Maintenance of bacterial cultures on solid media

A culture of Planococcus species C.C.M. 316 (also known as Planococcus Citreus Migula) was obtained as a gift from Dr M. Kocur, University J.E. Purkyne, Brno, Czechoslovakia. The culture was maintained on solid media at 30° and subcultured on to fresh media at weekly intervals. The composition of the maintenance medium was as follows (the percentages are % w/v in distilled water): 1% glucose; 1% Beef Extract (Oxoid Ltd., Lablemco Powder); 1% Bacteriological Peptone (Oxoid Ltd.) 2% Agar No. 3 (Oxoid Ltd.); 3% sea salt.

Liquid culture

150 - 500 ml cultures of the Planococcus were grown in a New Brunswick G25 orbital incubator-shaker at 30° with an agitation speed of 300 r.p.m. To a basic medium, known as beef extract peptone glucose medium (BEPG) (1% glucose, 1% beef extract, 1% bacteriological peptone - all w/v in distilled water), sea salt was added to give media of various final sea salt concentrations. The pH of the media was then adjusted to pH 7.0 with either HCl or NaOH. The media were sterilised by autoclaving at 15 lb/sq in pressure for 20 min. These cultures were used for all of the growth experiments described below, and as inoculation cultures for the large scale batch culture of cells.

Production of sea salt

A salt slurry was produced by evaporation of filtered sea water at 60° under reduced pressure on a rotary evaporator. This slurry was dried at 100° in an oven and the salt stored in an airtight container. Evaporation of known volumes of sea water in this way allowed the salinity of sea water to be determined.

Bacterial growth measurements

The degree of bacterial growth in a culture at any given time was determined by one or more of the following methods.

1. Absorbance measurements (turbidimetry)

Absorbance at 600 nm measured the proportion of the incident light which did not reach the photocell due to the presence of micro-organisms in suspension. The values obtained were related to the dry weight of microorganisms as follows.

Duplicate aliquots of cell suspension were removed and dried to constant weight in an oven at 100°. Aliquots of the same cell suspension were diluted with known volumes of sterile medium and their absorbance read at 600nm on a Pye Unicam SP 600 spectrophotometer. Calculation of the weight of cells in the diluted samples allowed a calibration curve of weight of cells against absorbance at 600 nm to be drawn.

For experimental work, samples were diluted to give an absorbance (0.1 - 0.8) which was on the linear portion of the calibration curve. The readings were taken on an SP 600 spectrophotometer using glass cells with a 1 cm light path, against a blank of fresh medium.

2. Trichloroacetic acid (TCA) precipitation (73)

With bacteria which tend to aggregate, turbidimetric determinations can be inaccurate. This method can be used successfully with such bacteria.

2 - 5 ml aliquots of bacterial culture were centrifuged and the pellet resuspended in a volume of distilled water equal to the original sample volume. The resulting suspension, free from media, was subjected to ultrasonic disintegration for 2 min. 1 ml of the disrupted preparation was diluted with 1 ml of distilled water and 2 ml 10% (w/v) TCA. After mixing, the absorbance of the resulting suspension was determined at 600 nm against a distilled water blank.

3. Viable cell counting

This method was used to ensure that the results obtained by both

the turbidimetric estimation and the TCA precipitation method were related to the number of viable cells within the culture.

Serial 1/10 dilutions of a sample of liquid culture were made to a point at which 0.1 ml of the final dilution, when spread across the surface of an Agar plate, gave between 20 and 200 colonies after incubation. This number of colonies can be accurately counted on a Gallenkamp colony counter without appreciable error. All procedures were carried out aseptically and the number of dilutions required in each instance was found by trial and error. Determinations were always carried out in duplicate and the number of cells per ml was calculated. The method uses a lot of sterile glassware and is very time consuming and consequently was not adopted as a routine means of measuring bacterial growth.

Investigation of the salt tolerance of Planococcus 316

1. Solid media

Preliminary experiments were carried out on solid BEPG + 2% Agar media containing NaCl in increasing concentrations (0 - 20% w/v). The cultured plates were examined at 24 h intervals over two weeks for signs of growth.

2. Liquid media

Duplicate flasks containing 150 ml of sterile BEPG media and various concentrations of NaCl were inoculated with identical volumes of a homogeneous culture of Planococcus 316.

Samples were removed at intervals from the cultures and the absorbance at 600 nm measured on an SP 600 spectrophotometer against a blank of sterile medium. Growth curves were plotted for the different salt concentrations.

Similar experiments were carried out using sea salt at different final concentrations. If, in any culture, no increase in absorbance

was detected within 15 days, the salt concentration was recorded as being beyond the salt tolerance of the microorganisms.

Cultures of Planococcus were also grown in BEPG media in the presence of KCl and NaCl/KCl mixtures to determine whether the presence of KCl had any effect on the limits of salt tolerance. Cultures were monitored by turbidimetry.

Determination of the optimum pH for growth

The optimum pH for growth of Planococcus 316 was determined in BEPG media with three different final concentrations of sea salt, namely: 0.5%, 3% and 10% (w/v). A number of flasks of BEPG media and sea salt were prepared and the final pH was adjusted with either NaOH or HCl to give a range of pH values between 5.0 and 9.0 in the different flasks. After autoclaving, the pHs were checked and the flasks then inoculated with identical volumes of a homogeneous suspension of a logarithmic culture of cells. Growth in each flask was monitored by means of the turbidimetric and TCA precipitation methods.

Large scale culture of Planococcus 316

12 litre batch cultures were grown in a New Brunswick MF 114 fermenter at 30° with an aeration rate of 15 l/min (1.25 l air/min/ l culture) and an agitation rate of 300 r.p.m. The medium used was the basic BEPG medium to which sea salt was added to give a final sea salt concentration of 0.5%, 3% or 10% (w/v). After adjusting the pH in each case to 7.0, the media were autoclaved in the usual manner. Each batch culture was inoculated with 300 ml of a logarithmic phase culture grown in the New Brunswick G25 orbital incubator-shaker in an identical medium. The progress of each batch culture was monitored by removing samples and determining the absorbance at 600 nm.

Harvesting large scale cultures

Harvesting was carried out when the culture had reached very early

stationary phase, using either a continuous-flow head on a MSE Super 18 centrifuge or, alternatively, on a Sharples centrifuge. The rate of flow through either centrifuge was adjusted to give the fastest flow compatible with a clear effluent.

Storage of cells

Cells from each batch culture were stored in the frozen state in sealed plastic containers at -15°.

MEMBRANES

Membrane preparation

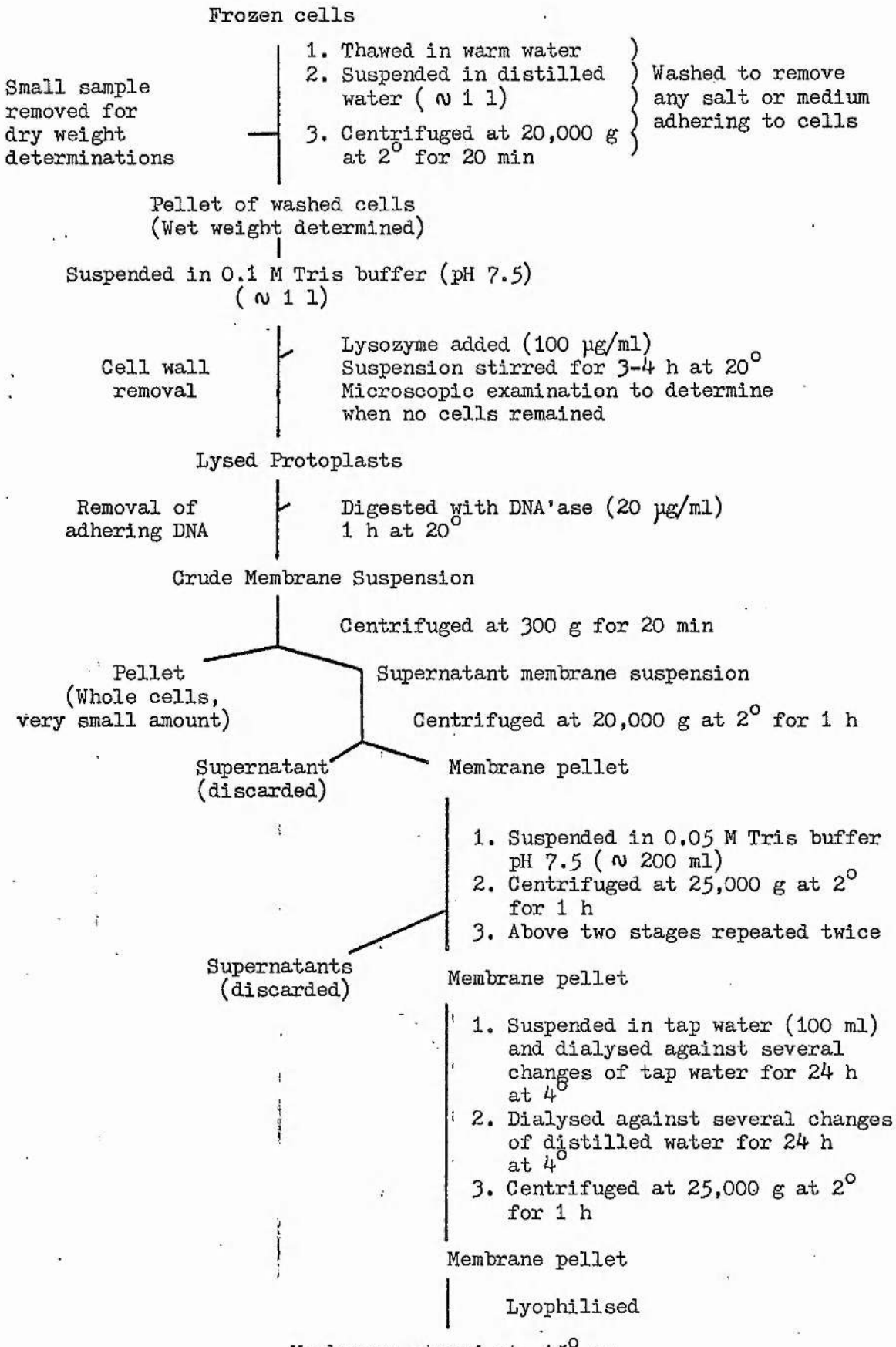
Membranes were prepared from cells grown in the three different sea salt concentrations. The method of Salton and Freer (74) was used. Sufficient membranes were prepared to enable all the composition analysis and lipid work to be carried out on the material obtained from a single batch preparation.

Materials

The following materials were used: Egg white lysozyme (Sigma Ltd.); DNA'ase (Sigma Ltd.); 1 l Tris buffer 0.1 M pH 7.5; 1 l Tris buffer 0.05 M pH 7.5.

The sequence of procedures employed is shown in Fig. M1.

Fig. M1: Flow diagram for membrane preparation.



After lyophilisation, the membranes appeared as orange flakes which were lightly ground to a fine powder to ensure a uniform distribution of material. This was stored in bulk at -15°.

Determination of the membranes as a percentage of total cell dry weight

A small aliquot of wet cells (approximately 1 g) was weighed in a pre-weighed crucible and then dried in an oven at 100° to constant weight. From this, the dry weight of the total amount of cells was calculated and the dry weight of membranes produced was related to it later.

Membrane composition

Membranes are known to consist of protein, lipid, RNA, carbohydrate, water and ash. A quantitative determination for each of these components was carried out using the methods described below.

Moisture content

Duplicate samples were weighed in a pre-weighed glass weighing bottle and dried to a constant weight at 100°. Water content was calculated from the difference in weights.

Ash content

Duplicate known weights of membranes (approximately 5 g) were heated for 3 h in pre-weighed crucibles in a furnace at 500°. After cooling, 1 drop of concentrated sulphuric acid was added and the crucibles and contents heated for a further 5 h at 700°. Finally the crucibles and contents were weighed after cooling in a desiccator, to determine the weight of ash.

Protein determination (Moore and Stein) (75)

Hydrolysis of the proteins to release amino acids is necessary before quantitative analysis by this technique. Duplicate 25 mg samples of lyophilised membranes were weighed into glass hydrolysis tubes. 10 ml of 6 N. HCl were added, the tubes sealed in an oxygen flame and then heated in an oven at 110° for 18 h. After being cooled, the tubes were

opened and the contents washed out through a filter to remove the "humin". The aqueous solution was reduced in volume on a rotary evaporator and the HCl removed by the addition of multiple aliquots of distilled water. The hydrolysates were taken up in 10 ml of distilled water for the Moore and Stein determination (75). Similar hydrolysates were taken up in 25 ml distilled water for the amino acid analysis. Using the 10 ml solution, 0.2 ml was removed, made up to 1 ml with distilled water and 0.1 ml of this solution was used in the actual determination (Moore and Stein).

Reagents

The following reagents were used: 50% ethanol (by vol) in distilled water; ninhydrin reagent (1 g ninhydrin, 150 mg hydrantanin in 37.5 ml methoxy ethanol, 2.5 ml sodium acetate buffer); sodium acetate buffer (5.4 g sodium acetate dissolved in 4 ml warm water, 1 ml glacial acetic acid added and the solution made up to 100 ml with distilled water); standard nor-leucine (1 μM/ml) in distilled water.

Duplicate hydrolysed samples and standard amounts of nor-leucine ranging from 0.1 - 1 μM were pipetted into separate test tubes and distilled water was added to make all volumes up to 1 ml. 1 ml of ninhydrin reagent was added and the tubes mixed vigourously for several minutes on a "rotamixer" before being placed in a water bath at 100°. After 15 min, 5 ml of 50% ethanol was added and the intensity of the purple colour was determined spectrophotometrically at 570 nm against a reagent blank.

The μM of amino acid in the samples was read directly from a calibration plot of the nor-leucine standards against absorbance.

Amino acid analysis

0.1 ml samples from the hydrolysates, in 25 ml of distilled water, were analysed on a Locarte automatic amino acid analyser. The amino acid composition of the membrane proteins was determined from the resulting traces.

RNA determination (76)

The following reagents were used: chloroform/methanol (2:1 by vol); 10% TCA; 5% TCA; orcinol reagent (1 g orcinol dissolved in 100 ml concentrated sulphuric acid containing 0.4 g ferric chloride); standard ribose (100 µg/ml).

The membranes were defatted before the RNA determination. 500 mg of membranes were soaked in a small volume of distilled water until just moist, and then were stirred in 100 ml of chloroform/methanol (2:1 by vol) for 1 h. The pellet was recovered by centrifugation and re-extracted with another 100 ml of the chloroform/methanol. The supernatants were evaporated to dryness on a rotary evaporator under reduced pressure and weighed. The defatted membranes were lyophilised and weighed.

RNA extraction

100 mg of defatted membranes were suspended in 2.5 ml 10% TCA and heated at 90° with occasional stirring for 15 min. The pellet collected by centrifugation was re-extracted as above. The combined supernatants contained DNA in solution (if present) and were discarded. The pellet containing RNA was extracted twice with 2.5 ml 5% TCA at room temperature and the two supernatants which contained the total RNA were pooled.

Colorimetric determination

Ribose was assayed using the orcinol reaction. The 5 ml (2 x 2.5 ml 5% TCA) extract was diluted to 50 ml with distilled water and duplicate 0.5 ml samples removed for the determination. The samples and volumes of standard ribose containing between 5 and 50 µg pentose were pipetted into separate test tubes and made up to 1.5 ml with distilled water. 1.5 ml orcinol reagent were added, the tubes were mixed and placed in a boiling water bath for 20 min, and after rapid cooling,

the intensity of the green colour was determined spectrophotometrically at 660 nm against a reagent blank. The concentration of ribose in the samples was read directly from a calibration graph prepared from the standards. The percentage RNA was calculated from the ribose estimation by estimating the average molecular weight of a nucleotide, assuming equal amounts of purine and pyrimidine bases.

moles of ribose = moles of nucleotide

moles of nucleotide x estimated molecular weight = weight of RNA

Carbohydrate determination

Membrane carbohydrate was determined using the phenol sulphuric acid method (77).

Hydrolysis of the membranes was carried out for various periods to determine the length of time required to give maximum release of sugars. 25 mg membrane samples were carefully weighed into glass hydrolysis tubes and hydrolysed in 10 ml 1M HCl at 105° for various lengths of time between 0.25 and 10 h. The hydrolysates were analysed for sugar as described below.

Samples of membrane hydrolysed for the optimum time were washed through a filter paper and taken to dryness on a rotary evaporator. The sugar residue was taken up in a known volume of distilled water (usually 10 ml) and assayed.

Reagents

The following reagents were used: 5% w/v phenol in distilled water; 96% sulphuric acid; standard glucose (100 µg/ml).

Duplicate 1 ml samples of the hydrolysate were pipetted into test tubes. Standard glucose solutions containing 10 - 100 µg in

1 ml were also prepared. 1 ml 5% phenol solution was added to each tube and 5 ml 96% H_2SO_4 mixed in rapidly from a fast flowing pipette. After 10 min at room temperature, the tubes were mixed and placed in a water bath at 30° for 20 min. The intensity of the yellow/green colour was determined spectrophotometrically at 490 nm against a reagent blank. The concentration of sugar in the samples was read directly from a calibration plot prepared from the standard glucose readings.

Qualitative carbohydrate analysis of membranes

Membrane samples were hydrolysed as described above for the quantitative sugar determination and the hydrolysates concentrated on a rotary evaporator. The hydrolysed sugar samples were spotted on to a sheet of Whatman No. 1 chromatography paper along with some authentic standard sugars (glucose, galactose, mannose, arabinose, ribose, rhamnose, glucuronic acid, N.acetyl-glucosamine and galactosamine hydrochloride). The chromatogram was developed in a butanol/pyridine/water (6:4:3 by vol) solvent system overnight, in a descending manner. After drying, spots were detected with anisidine phthalate spray and the sugars identified by comparison of their R_fs with those of the standard sugars. (See Appendix I.)

Phosphorus determination

Total membrane phosphorus was determined using the method of Allen (78).

Reagents

The following reagents were used: Amidol reagent (prepared fresh) (1 g amidol (2'4' diaminophenyl hydrochloride) dissolved in 100 ml 20% (w/v) aqueous sodium metabisulphite solution); ammonium molybdate reagent (8.3% solution in distilled water); 10 N. sulphuric acid; standard potassium-dihydrogen-phosphate (100 µg phosphorus/ml).

Triplicate samples of 6 mg of membrane were carefully weighed into Kjeldahl tubes and 1.2 ml 10 N. H_2SO_4 was added. The membranes were digested until colourless. A few drops of hydrogen peroxide were added after $\frac{1}{2}$ h to accelerate decolourization. After cooling, the following additions were made to each sample, and to a range of standard solutions containing from 10 - 100 µg phosphorus and 1.2 ml H_2SO_4: 6.4 ml distilled water, 2 ml amidol reagent, 1 ml ammonium molybdate and, finally, 15 ml distilled water. After 10 min at room temperature the intensity of the blue colour which developed was determined spectrophotometrically at 640 nm against a reagent blank. The amount of phosphorus in the samples was read directly from a calibration plot prepared from the standard phosphorus readings.

Lipid content of membrane

Lipid content of the membrane was determined by extracting the lipid from a known weight of membranes as described below and then weighing it.

LIPIDS

Preparation of solvents

All solvents, unless stated otherwise, were dried and redistilled before use.

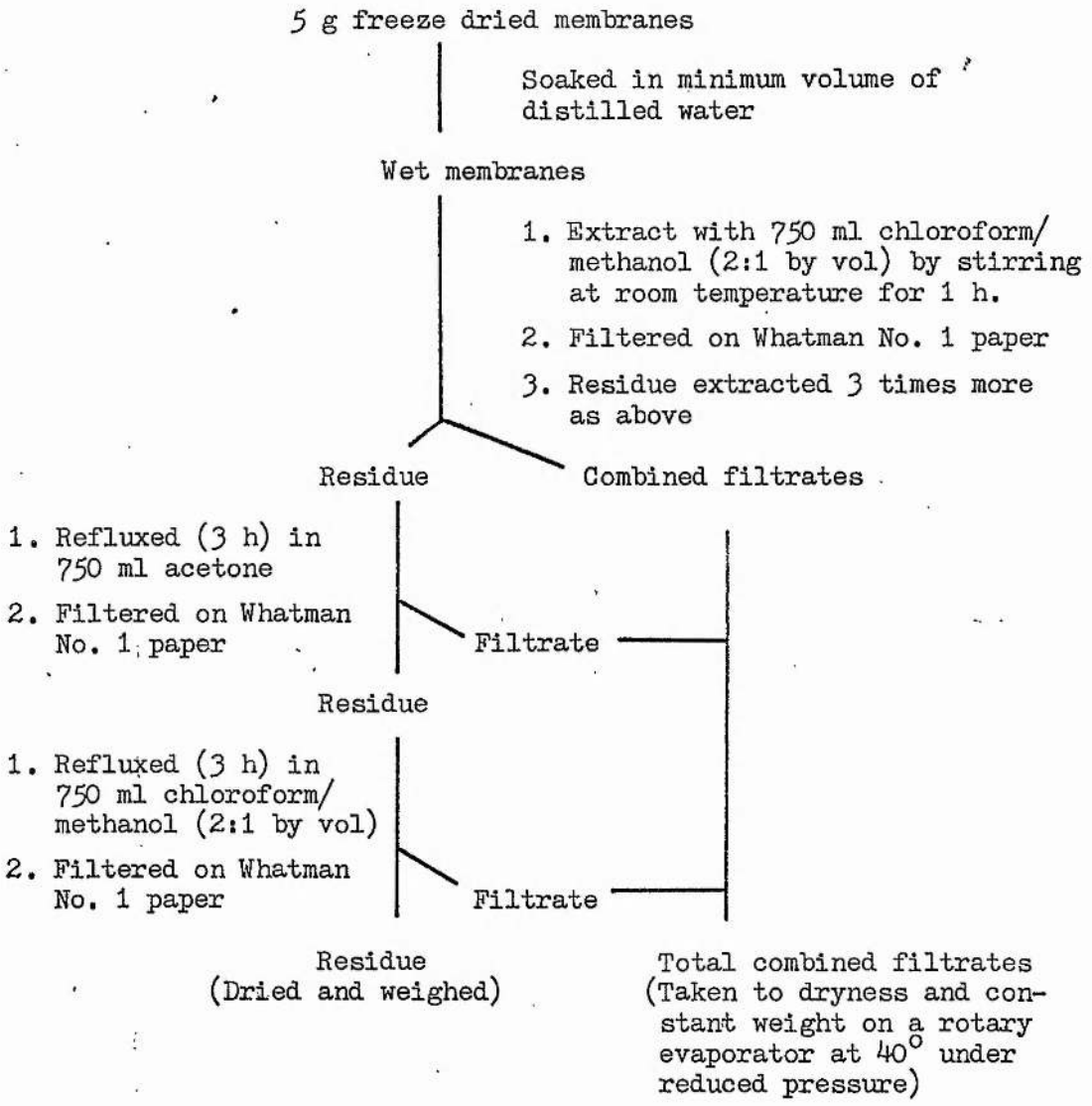
Anhydrous sodium sulphate was added to Winchester bottles of solvent which were then shaken and left for several hours before being redistilled. Commercial chloroform contains 2% ethanol (by vol) which was removed by washing with water. The chloroform was then dried over anhydrous sodium sulphate and redistilled. Pyridine was redistilled and stored over anhydrous potassium hydroxide pellets. All solvents were stored at room temperature in dark glass bottles.

Lipid extraction

Lipid was extracted from 5 g of dry membranes, previously soaked in a minimum volume of distilled water to increase the yield (79), by

the method shown in Fig. M2. The weights of both the residue and the extracted lipid were determined so that losses could be accounted for.

Fig. M2: Extraction of lipid (79).



Weighing of lipid samples

After removal of volatile organic solvents on the rotary evaporator at 40°, any water present in the samples was removed by azeotroping with ethanol and/or benzene. Large samples dried in this manner in pre-weighed round-bottomed flasks were finally taken to constant weight in vacuo, whereas small samples were reduced in volume in pre-weighed glass vials (2.5 ml) with a nitrogen stream and finally dried to constant weight in vacuo.

Storage of lipid material

Lipids were stored at - 15° in small glass vials, as a solution in chloroform/methanol (2 - 3 ml), gassed out with nitrogen.

Purification of crude lipid extracts

Non-lipid material was removed from the extract obtained as in Fig M2, by the method of Wells and Dittmer (80). For approximately 1 g of crude lipid, 20 g of fines-reduced Sephadex G25 was suspended in chloroform/methanol/water (60:30:4.5 by vol), allowed to swell, and packed into a glass column of 1.5 cm internal diameter. The lipid, dissolved in a maximum of 300 ml chloroform/methanol/water (60:30:4.5 by vol) was passed through the column and the column then eluted with chloroform/methanol (2:1 by vol); 400 ml of column effluent were collected, leaving non-lipid material on the column. The purified lipid was dried on a rotary evaporator and weighed. Non-lipid material remaining on the column was removed by washing the Sephadex with large volumes of methanol/water (1:1 by vol). This was most easily carried out by removing the Sephadex from the column and washing it on a Buchner funnel.

Separation of the purified lipid into classes

The lipids were separated into phospholipids, glycolipids and neutral lipids. Phospholipid was separated by acetone precipitation and the neutral and glycolipids separated from each other by silicic acid column chromatography.

Acetone precipitation (81)

The procedure makes use of the fact that most phospholipids are insoluble in cold acetone and can be separated from the more soluble neutral and glycolipids on this basis.

Reagents

The following reagents were used: cold acetone; 10% magnesium chloride ($MgCl_2.6H_2O$) (w/v) in methanol.

Approximately 1 g of total lipid dissolved in chloroform/methanol (2:1 by vol) was evaporated to about 1 ml with a jet of nitrogen. 50 ml of cold acetone and 1 ml of 10% $MgCl_2$ solution were added. The suspension was mixed thoroughly on a 'rotamixer' and cooled on ice for 1 h. A pellet of precipitated material was recovered by centrifugation and this was re-extracted with 10 ml of cold acetone and cooled as before. Several extractions were necessary to obtain all of the phospholipid. The procedure was most easily carried out using a small Potter homogenizer to resuspend the pellet each time after centrifugation. The acetone supernatants which contained the neutral and glycolipids were pooled and dried to constant weight. The phospholipid fraction was also dried to constant weight and an allowance was made in the calculations for the $MgCl_2$ which was added.

Separation of the neutral lipids from the glycolipids

80 g of fines-reduced Mallinckrodt silicic acid (100 mesh) were activated by heating at 110° for 12 h. After cooling in a desiccator, the silicic acid was packed into a glass column of 3 cm internal diameter, as a slurry in heptane. The column was washed through with 480 ml of diethyl ether followed by 480 ml of chloroform. The lipid sample, dissolved in a small volume of chloroform, was loaded on to the column and washed in with 10 ml chloroform. The column was then eluted with

the following solvents at a rate of 1.5 ml/min:

480 ml chloroform to elute neutral lipids;

400 ml chloroform/acetone (1:1 by vol) followed by

400 ml acetone to elute glycolipids;

400 ml methanol to elute any remaining phospholipids.

The fractions obtained from the column were evaporated to dryness and constant weight.

Separation of neutral lipids by column chromatography

Separations were attempted in columns using (a) Mallinckrodt silicic acid (100 mesh), and (b) Florisil.

(a) Silicic acid

5 g activated Mallinckrodt silicic acid, as a slurry in hexane, were packed into a glass column of internal diameter 1 cm. The column was washed with the following solvents:

30 ml diethyl ether;

30 ml 15% benzene in hexane;

30 ml hexane.

Up to 75 mg of neutral lipid, dissolved in hexane, were applied to the column and washed in with a few ml hexane. The column was then eluted as follows:

25 ml hexane to elute hydrocarbons;

50 ml 15% benzene in hexane to elute sterol esters;

100 ml 5% diethyl ether in hexane to elute triglycerides and free fatty acids if present;

100 ml 15% diethyl ether in hexane to elute sterols;

50 ml 30% diethyl ether in hexane to elute diglycerides;

50 ml diethyl ether to elute monoglycerides.

The fractions were collected and reduced in volume.

(b) Florisil

7% hydrated Florisil was prepared by shaking 100 g of dry Florisil (60 - 100 mesh) with 7 ml of distilled water overnight. 24 g of 7% hydrated Florisil were packed, as a slurry in hexane, into a glass column of internal diameter 1 cm. The lipid sample, containing up to 300 mg of lipid dissolved in a few ml of hexane, was loaded on to the column and washed in with hexane. The column was then eluted with the following solvents at a rate of 2 - 3 ml/min:

80 ml hexane to elute hydrocarbons;

180 ml 95% hexane in diethyl ether to elute sterol esters;

300 ml 85% hexane in diethyl ether to elute triglycerides;

200 ml 75% hexane in diethyl ether to elute sterols;

200 ml 50% hexane in diethyl ether to elute diglycerides;

270 ml 98% diethyl ether in methanol to elute monoglycerides;

100 ml 4% acetic acid in diethyl ether to elute free fatty acids.

The fractions were collected and reduced in volume. Effluent from this and from the silicic acid column was monitored using the microscope-slide thin-layer chromatography (TLC) technique.

Microscope-slide TLC

A thin layer of silica gel H. (Merck) was spread manually on to clean microscope slides as evenly as possible. The slides were dried in an oven at 100° and, after cooling, small samples of column effluent were spotted on to the plates along with authentic standards. Development of the chromatograms in a suitable solvent enabled the neutral lipids to be identified and the efficiency of the column separation to be checked.

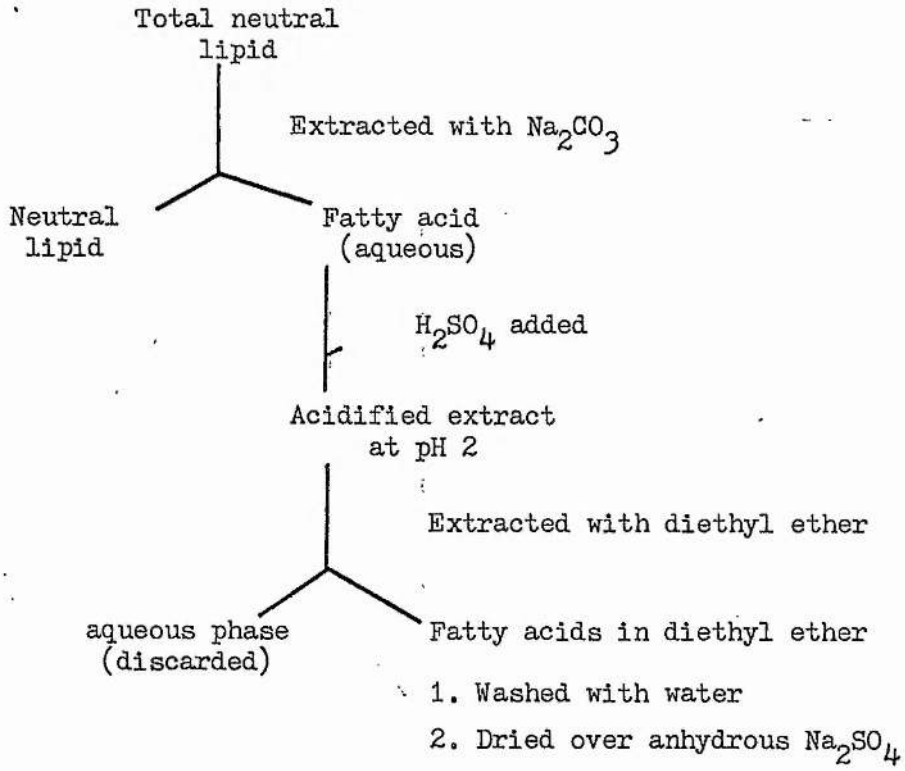
Separation of free fatty acids by washing with carbonate

Free fatty acids may be removed from the neutral lipids prior to chromatographic separation by making them water soluble.

Reagents

The following reagents were used: diethyl ether; 4% (w/v) sodium carbonate in distilled water; 1.5 N. sulphuric acid.

The neutral lipids were dissolved in 3 ml diethyl ether and extracted four times in a 10 ml separating funnel with 1 ml 4% sodium carbonate. The aqueous phase (lower) was washed with 2 ml diethyl ether which was added back to the ether phase. The aqueous phase was acidified to pH 2.0 with 1.5 N. H_2SO_4 and re-extracted once with 2 ml and twice with 1 ml of diethyl ether. The diethyl ether containing the fatty acids was washed three times with 1 ml of water and dried over anhydrous sodium sulphate. The procedure is summarised below:



The solvent was removed on a rotary evaporator at 20° and the fatty acids dried to constant weight.

Thin-layer chromatographic (TLC) separation of neutral lipids

Preparation of plates

Five 20 x 20 cm glass plates were coated with a 0.25 mm layer of silica gel H. (Merck) using a Desaga adjustable applicator (Camlab. Glass Ltd.). Plates were heated in an oven at 105° for 2 h and then cooled in a dèsiccator where they were stored until required.

Chromatography

Neutral lipid samples, with or without the free fatty acids removed, were concentrated to a small volume in a jet of nitrogen. Small volumes were applied to the surface of the silica gel by means of a capillary tube and the chromatogram developed using one of the following solvent systems.

Solvent systems

Solvents were placed at the bottom of the chromatography tank to a depth of approximately 1 cm and filter paper positioned around the inside of the tank to aid equilibration.

(a) Two-step system:

First solvent: di-isopropylether/acetic acid (96:4 by vol);

Second solvent: light petroleum (B.P. 60°-80°)/diethyl ether/ acetic acid (90:10:1 by vol).

The first solvent was allowed to advance 5 - 6 cm up the plate, after which the plate was removed from the tank and dried at room temperature for 1 h. The second solvent was then allowed to run the full distance up the plate.

(b) Single-step systems:

(i) light petroleum (B.P. 60°-80°)/diethyl ether/acetic acid 70:30:1 by vol, or 70:30:2 by vol).

This single step system was used when rapid development was more important than complete separation.

(ii) heptane/benzene (9:1 by vol).

This system was used only to separate hydrocarbons from other lipids.

Detection of neutral lipids

This was achieved by means of various sprays and vapours which were either general reagents for staining lipids or which produced a particular reaction with certain lipids. The general lipid detection reagents used were: iodine vapour; 50% sulphuric acid; rhodamine 6G (0.005% aqueous). The specific stains used were: 2'7' dichlorofluorescein for all neutral lipids; antimony trichloride and sulphuric acid/acetic acid for sterols (see Appendix I).

Recording of thin-layer chromatograms

A permanent record of TLC separations was made by tracing the suitably detected spots on to a glass or perspex plate which was placed over the silica layer. This could then be transferred to tracing paper and kept in a file.

Identification of neutral lipids

Individual neutral lipid components were identified on the basis of their R_fs in two solvent systems, as compared to those of authentic standards. The following standards were employed: squalene, octacosane, cholesterol stearate, methyl oleate/palmitate, triolein, oleic/palmitic acid, stearyl alcohol, cholesterol, glyceryl 1'2' dipalmitin, glyceryl 1' monostearin.

Preparative TLC

Plates were prepared as before except that the silica was applied as a layer 0.5 or 0.75 mm thick. Plates were pre-run to the top in a polar solvent (chloroform/methanol, 1:1 by vol) to remove any contaminating material present in the silica gel. The top 2 cm of silica were scraped off the plate and discarded. The pre-run plates were then activated in an oven at 105° for 2 h before use.

Neutral lipids were applied to the plates either as multiple spots, or by means of a Desaga automatic strip loader (Camlab. Ltd.), and the chromatograms developed in the two-step solvent system. Individual bands were identified by staining a thin strip down each side of the chromatogram with rhodamine 6G and viewing under UV light. The areas of silica corresponding to the spots already identified by analytical separations were removed and the lipids eluted from the silica with chloroform/methanol/diethyl ether (1:1:1 by vol). Silica was removed by centrifugation and the separate lipid fractions were taken to constant weight.

Preparative TLC of the hydrocarbon fractions was carried out in the heptane/benzene (9:1 by vol) system in a similar manner.

Sterol investigations (108)

Bands thought to contain sterol were subjected to digitonide precipitation to try and isolate pure sterol.

Reagents

The following reagents were used: 0.5% Digitonin (Sigma Ltd.) in 50% aqueous ethanol; diethyl ether; 10% methanolic potassium hydroxide; light petroleum (B.P. 60°-80°).

Sterol esters were converted to free sterol and free fatty acid by saponification overnight in 10% methanolic KOH and the sterols extracted into diethyl ether. Free sterol was dissolved in 1 ml light petroleum and 1 ml of digitonin solution was added. The solution was mixed and left at 37° for 16 h. An insoluble digitonide precipitates almost immediately if 3 β hydroxy sterols are present, reaching a maximum after 16 h.

Free sterol can be recovered from the insoluble precipitate by refluxing with pyridine for 20 min and extracting into diethyl ether after the addition of two volumes of water.

Gas liquid chromatographic (GLC) separation of neutral lipid components

Lipid fractions which had been obtained from preparative TLC were further separated by GLC to determine the distribution of carbon chain lengths and the degree of unsaturation and branching.

Gas chromatographic separations were carried out on a twin column Pye model 104 gas chromatograph, fitted with flame ionisation detector and using nitrogen as a carrier gas. Peaks were recorded on a Pye P8000 chart recorder.

Samples were injected manually on to the top of one of the columns through a gas-tight rubber septum by means of calibrated microlitre syringes with capacities of either 1 µl or 5 µl. The nitrogen flow rate was calibrated using a bubble flow meter. Volatile compounds (hydrocarbons, long chain alcohols and sterols) were chromatographed without prior modification, whereas less volatile compounds were modified to increase their volatility. Free fatty acids were methylated and the fatty acids from mono-, di- and tri-glycerides and from sterol esters were transesterified before chromatographing.

Separation of hydrocarbons

The hydrocarbons prepared by preparative TLC were dissolved in small volumes of diethyl ether, reduced in volume to approximately 50 - 100 µl in a stream of nitrogen and analysed under the following conditions:

1. Alkanes

column	5 ft glass
column packing	3% SE 30 on 100 - 120 mesh celite
injection volume	1 - 2 µl
column oven temperature	200° or 250° isothermal
detector oven temperature	300°
injection port heater temperature	230°

attenuation	5×10^2
N_2 carrier flow	45 ml/min
H_2 flow to detector	45 ml/min
air flow to detector	600 ml/min
chart speed	5 mm/min

2. Isoprenoids

Isoprenoid compounds were analysed under the same conditions except that the column oven temperature was 230° isothermal and the attenuation was 50×10^2.

A standard mixture of straight chain saturated hydrocarbons was injected under identical conditions to that of the hydrocarbon samples. The standard mixture contained the alkanes with an even number of carbon atoms from C_8 to C_{36}. Standard squalene and perhydro squalene were also injected.

Long-chain alcohols

These were separated under the same conditions as for the hydrocarbons except that the column oven temperature was lowered to 153° and the attenuation required was 5×10^2.

A standard mixture of C_{14}, C_{16} and C_{18} straight chain saturated alcohols was injected under the same conditions.

Sterol

Sterol obtained by digitonide precipitation can be analysed by GLC under the same conditions as those required to separate hydrocarbons.

Glycerides and fatty acid esters

Fatty acids esterified to glycerol or other molecules were converted to methyl esters to increase volatility and to achieve a uniformity in the group esterified to the acid.

Transesterification (81)

Reagents

The following reagents were used: 5% (w/v) hydrogen chloride in methanol (prepared by cooling 50 ml of dry methanol to -5° and adding 5 ml acetyl chloride slowly with constant stirring); 2% sodium bicarbonate (w/v) in distilled water; 5% sodium chloride (w/v) in distilled water; pentane. The methylating agent was stored in the dark at 4° and made up fresh every four weeks.

Up to 50 mg of lipid to be methylated were transferred to a glass hydrolysis tube with a Teflon sealed screw cap. The solvent was removed in a stream of nitrogen and 1 ml of benzene or dichloromethane* was added. After the addition of 2 ml of methanolic HCl the mixture was heated at 65° for 2 h in the sealed tube. If free fatty acids alone were to be esterified no solvent was required and only 20 min at 65° was necessary. After cooling, 5 ml of 5% NaCl were added and the esters extracted into pentane (2 x 5 ml). The pentane layer was washed with 2% $NaHCO_3$ (4 ml) and dried over anhydrous sodium sulphate. The solvent was evaporated to a small volume prior to injection on to the GLC.

* Polar lipids such as phospholipids dissolved well in the methanolic HCl and no solvent was required.

Methylation using Boron trifluoride (BF_3)-methanol complex (BDH Ltd.)

BF_3-methanol was used initially as a methylating reagent. Dry lipid samples were refluxed with 10 ml BF_3-methanol complex for 20 min. Methyl esters were extracted into diethyl ether after the addition of two volumes of distilled water.

GLC of methylated fatty acids

The methylated fatty acids were reduced in volume to approximately 50 µl in a nitrogen stream and injected on to the column under the following conditions:

column	5 ft glass
column packing	FFAP (Phase Sep. Ltd.)
injection volume	1 µl
column oven temperature	185° isothermal
detector oven temperature	250°
injection port heater temperature	230°
attenuation	$5 \times 10^2 - 50 \times 10^2$
N_2 flow	45 ml/min
H_2 flow to detector	45 ml/min
air flow to detector	600 ml/min
chart speed	5 or 10 mm/min

Standard methylated fatty acids (C_{10}, C_{12}, C_{14}, C_{16}, C_{18} and mono-unsaturated C_{18}) were injected under identical conditions.

Estimation of carbon number corresponding to GLC peaks (109)

The carbon chain lengths of the standard compounds were plotted against the logarithm of the retention times of the peaks obtained from the chart recorder (James plot). The logarithms of the retention times of the unknown peaks gave the apparent carbon number when referred to the James plot. Carbon numbers were not always whole numbers, the fractions being due to peaks derived from either branched or unsaturated components.

Determination of peak areas

The traces obtained from the GLC were also used to determine the percentage of each fatty acid as a proportion of the total fatty acids. Peak areas were calculated by multiplying the peak height by the width of the peak at half the height. The area of each individual peak was then expressed as a percentage of the total area under all the peaks. These calculations were in fact performed by a computer program which estimated the carbon number and expressed the areas of the peaks as

a percentage of the total area. Appendix II gives details of the computer program.

Hydrogenation of fatty acid methyl esters

The methyl ester samples were hydrogenated and analysed by GLC to determine which peaks were due to esters of unsaturated fatty acids. The sample to be hydrogenated was dissolved in 5 ml methanol 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. 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.

Hydrogenation of hydrocarbons

Hydrocarbons were hydrogenated by dissolving them in butanol (3 ml) and shaking for 1 h in the presence of a few mg of 5% palladium on charcoal catalyst (BDH Ltd.) under an atmosphere of hydrogen at room temperature. Identification of unsaturated compounds was again by their disappearance on rechromatographing and the increase in the area of the peak corresponding to the saturated compound.

Glycolipids

The glycolipid material was obtained from a silicic acid column as described previously. The chloroform/acetone (1:1 by vol), the acetone and the methanol fractions were all analysed by TLC to identify their components and to check the efficiency of resolution achieved on the column.

TLC of glycolipids

Separation was carried out on 0.25 mm layers of silica gel H. (Merck) prepared in the same manner as described in the neutral lipid

section (p. 41). Chromatograms were developed in a chloroform/methanol (9:1 by vol) solvent system. Detection of the glycolipid components was by means of spray reagents (Appendix I). These were diphenylamine and Schiffs periodate (carbohydrate detection), rhodamine 6G and 50% H_2SO_4 (general lipid detection) and a copper sulphate spray (sulphur glycolipid detection).

Carbohydrate determination

Quantitative carbohydrate estimations on glycolipid-containing fractions were carried out using the 'phenol sulphuric acid' method as described in the membrane section (p. 32).

Phosphorus determination

Analysis of the phosphorus content of the glycolipid fractions was carried out using the method of Bartlett (82) as described in the phospholipid section (p. 52).

Identification of the sugar residues associated with the glycolipids

The identities of the sugars associated with the glycolipids were determined by the two following methods.

(a) Paper chromatography

The glycolipids were first hydrolysed to release the sugars as follows. After removal of the solvent, the glycolipids were heated in sealed hydrolysis tubes with 2 ml 1 N. HCl at 105° for the previously determined optimum period of 2 h. After cooling, the hydrolysate was filtered and washed with chloroform to remove lipid constituents and the aqueous layer reduced to a small volume on a rotary evaporator at 40°. Samples of concentrated sugar solution were spotted on to a sheet of Whatman No. 1 chromatography paper, along with standard sugars (glucose, galactose, mannose, ribose, rhamnose, arabinose, glucuronic acid, N. acetyl glucosamine and galactosamine HCl). The chromatogram was developed in a descending manner in a solvent system of butanol/

pyridine/water (6:4:3 by vol); the solvent was allowed to run to the end of the paper (55 cm) overnight. The spots were detected with anisidine phthalate spray (see Appendix I) and the R_fs of the sample spots compared to those of the standard sugars.

(b) GLC analysis of sugars

The glycolipids were first hydrolysed as described for the paper chromatography, and the aqueous hydrolysate washed with chloroform and passed through a small column of Dowex 50 H^+ ion exchange resin. A small column was prepared in a Pasteur pipette and the aqueous hydrolysates passed through in about 20 ml of distilled water. Charged molecules such as amino acids and hexosamines remained on the column; the neutral sugars passed through in the effluent. The Dowex 50 resin was reactivated by washing in 1 N. HCl. The purified sugars were then converted to trimethylsilyl (TMS) derivatives to increase their volatility.

Preparation of Trimethylsilyl (TMS) derivatives (83)

Reagents

The following reagents were used: pyridine; hexamethyldisilazane (HMDS) (BDH Ltd.); trimethylchlorosilane (TMCS) (BDH Ltd.).

Standard solutions (1 mg/ml) of the following sugars were prepared: glucose, galactose, mannose, rhamnose, ribose, arabinose, fucose and xylose. The glycolipid hydrolysates and 1 ml of each standard sugar solution were dried in vacuo at 50° and then to each sample was added: 0.7 ml pyridine, 0.2 ml HMDS and 0.1 ml TMCS. It was found convenient to dissolve the sugars in the pyridine and to add the silylating reagents as a mixture. 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 at 40° and finally dissolved in a small volume of dichloromethane. The white precipitate present was a mixture of ammonium and pyridinium chlorides

and does not interfere with the GLC separation. The TMS derivatives of the sugars were separated by GLC under the following conditions:

column	5 ft glass
column packing	3% SE 30 on 100 - 120 mesh celite
injection volume	1 µl
column oven temperature	180° isothermal (or temperature programme as below)
detector over temperature	300°
injection port heater temperature	230°
attenuation	10×10^2
N_2 flow	45 ml/min
H_2 flow to detector	45 ml/min
air flow to detector	600 ml/min
chart speed	5 mm or 10 mm/min
Temperature programme	
Starting temperature	140°
holding time	4 min
increment	5°/min
finishing temperature	190°

Conditions were otherwise identical to those used above. Standard sugars were run under identical conditions to enable the sugars in the glycolipid hydrolysates to be identified.

Preparative TLC of glycolipids

Glycolipids were separated on 0.5 mm layers of silica gel H. (Merck) in the chloroform/methanol (9:1 by vol) solvent system. Lipid was eluted from the silica with chloroform/methanol (1:1 by vol) after detection of the bands with rhodamine 6G using the technique described for the preparative separation of the neutral lipids. The fractions obtained were subjected to:

(a) Sugar analysis which was performed by GLC, the individual glycolipids being subjected to the same procedures as those already described, and,

(b) Fatty acid analysis. Glycolipid fractions were transesterified using the method (methanolic HCl) described in the neutral lipid section (p. 46) and the methylated fatty acids analysed by GLC under the same conditions as those shown on p. 46.

Phospholipids

Phosphorus determination

A comparison of the amount of phosphorus in the glycolipid, the methanol effluent from the silica column and acetone-insoluble material was made using the method of Bartlett (82).

Reagents

The following reagents were used: standard phosphorus solution (0.5 mM potassium dihydrogen phosphate); ammonium molybdate solution (dissolve 4.4 g ammonium molybdate in 200 - 300 ml of water, add 14 ml concentrated sulphuric acid and make up to 1 l with water); Fiske and Subba Row reducing agent (dilute 1:12 before use) (grind 30 g sodium bisulphite, 6 g sodium sulphite and 0.5 g 1,2,4, aminonaphthol sulphonic acid in a mortar and pestle. Dissolve in distilled water and make up to 250 ml, allow to stand in the dark at room temperature for 3 h and filter); perchloric acid (70 - 72%).

Small samples of lipid were placed in pyrex test tubes and the solvent removed in a stream of nitrogen. 0.4 ml of perchloric acid was added and the tubes heated so that the acid boiled and condensed on the sides of the tubes. When decolourised, the samples were removed from the heat and cooled. Standard phosphorus solutions containing between 1 and 10 µg of phosphorus were prepared and made up to 1 ml. 0.4 ml acid was added but no heating was required. Lipid samples were

made up to 1 ml with distilled water and the following reagents added to both standards and samples: 2.4 ml ammonium molybdate solution and 2.4 ml Fiske and Subba Row reducing reagent (diluted 1:12). The tubes were mixed and heated in a boiling water bath for 10 min. After cooling, the intensity of the resulting blue colour was determined spectrophotometrically at 830 nm against a reagent blank. The phosphorus content of the samples was read directly from a calibration plot prepared from the values obtained for the standards.

Chromatographic separation and identification of phospholipid components

Separation on Whatman SG-81 silica-gel-loaded chromatography paper

A sample of acetone-insoluble material was spotted on to a strip of SG-81 30 cm in length and the chromatogram was developed in an ascending manner in the following solvent system: chloroform/methanol/di-isobutylketone/acetic acid/water (45:15:30:20:4 by vol). After drying the chromatogram, the spots were detected by spraying with a phosphorus detecting spray reagent (see Appendix I).

TLC separation

Thin layer separations were carried out on 0.25 mm layers of silica gel H. (Merck). Plates were prepared as described on p.41, except that the silica gel was applied to the glass as a slurry in 0.01% sodium carbonate instead of water. Acetone-insoluble material was applied to the silica by means of a capillary tube and the chromatograms developed in either

(a) chloroform/methanol/water (65:25:4 by vol),

(b) chloroform/methanol/water/ammonia solution (65:25:3:1 by vol),

(c) chloroform/acetic acid/methanol/water (80:18:12:5 by vol), or

(d) chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1 by vol).

A two-dimensional system was also used. A spot of lipid was placed on one corner of the chromatogram which was then developed in solvent

system (a). After drying, the plate was rotated through 90° and re-developed in solvent system (c). Phospholipid spots were detected with a phosphorus detecting spray reagent (see Appendix I). Group specific spray reagents were used along with authentic phospholipid standards(diphosphatidyl glycerol, phosphatidyl ethanolamine, phosphatidyl inositol, lysophosphatidyl ethanolamine, phosphatidyl serine and N.N.* dimethyl phosphatidyl ethanolamine) to identify the components of the acetone-insoluble material. The detection reagents used were iodine vapour, 50% H_2SO_4 and rhodamine 6G (as general lipid detectors), the phosphorus detecting spray, ninhydrin (to detect amino groups), Schiffs-periodate (to detect α-glycols), diphenylamine (to detect reducing sugars) and the copper sulphate spray (to detect sulphur glycolipids). (See Appendix I.)

Preparative TLC

Phospholipids were separated on 0.5 or 0.75 mm layers of silica gel H. (Merck) in the chloroform/methanol/water (65:25:4 by vol) solvent system. Bands were detected by exposing only the edges of the plate to iodine vapour and removing the rest of the bands from the plate. The phospholipids were then eluted from the silica with methanol. Quantitative estimations of the proportions of each component were carried out by (a) phosphorus determination, and (b) weight determination.

(a) Phosphorus determination

The bands from a preparative plate were scraped directly into Kjeldahl tubes and the amounts of phosphorus in each was determined by the method of Allen (78) as already described (p.33). Silica was removed by centrifugation before determining the absorbance and an area of silica which did not contain lipid was carried through the procedure as a blank.

* Sigma Ltd.

(b) Weight determination

The lipid components were recovered from the silica gel after preparative separation by elution with methanol and the fractions dried to constant weight.

Fatty acid analysis

Phospholipids were subjected to the methylation procedure described on p. 46 and the methylated fatty acids analysed by GLC under the same conditions as those used on p. 46 in the neutral lipid section.

Saponification

A sample of acetone-insoluble material was saponified overnight at room temperature in 10% methanolic KOH. Non-saponifiable material was extracted into diethyl ether by the addition of two volumes of saturated sodium chloride and, after drying over anhydrous sodium sulphate, the diethyl ether layer was concentrated and applied to a TLC plate of 0.25 mm silica gel H. (Merck). The chromatogram was developed in chloroform/methanol/water(65:25:4 by vol) and sprayed with a phosphorus detecting spray (see Appendix I). Any ether-linked lipids present would not be split by saponification and would remain in the non-saponifiable material.

Confirmation of the identity of phospholipids by determination of the molar ratios of the components

The identity of some phospholipids can be confirmed by an analysis of the molar ratio of: glycerol:fatty acid:phosphorus. The phospholipid was obtained by preparative TLC as described previously and checked for purity by rechromatographing a small sample. The pure phospholipid was dissolved in a few ml of methanol and known volumes removed for quantitative analysis.

Phosphorus

Duplicate samples were analysed by the method of Bartlett (82) in the manner already described on p. 52 .

Glycerol

Glycerol was determined by the chromotropic acid method described by Kates (81).

Reagents

The following reagents were used: 2M hydrochloric acid; 5M and 12M sulphuric acid; 0.1M sodium periodate solution; 10% aqueous sodium bisulphite; 0.18% chromotropic acid (dissolve 100 mg 1,8-dihydroxy-naphthalene 3,6-disulphonic acid in 10 ml water and dilute with 45 ml 12M sulphuric acid); 0.25 mM standard glycerol solution (Analar)).

Triplicate samples of phospholipid were placed in glass hydrolysis tubes and the solvent removed in a stream of nitrogen. 3 ml of 2N. HCl were added and the sealed tubes heated in an oven at 125° for 48 h. After cooling, the contents of the tubes were removed with 2 ml of water washings and these were washed with 2 ml of chloroform. 2 ml were pipetted from the aqueous layer and the following reagents added to this and to standard glycerol solutions containing 0.1 - 0.5 µM glycerol in 2 ml water: 0.1 ml 5M H_2SO_4 and 0.5 ml 0.1M sodium periodate. The tubes were mixed and left at room temperature for 5 min after which time 0.5 ml 10% sodium bisulphite was added and the tubes again mixed. 0.5 ml aliquots of this solution were mixed with 5 ml of chromotropic acid reagent and the stoppered tubes heated in a boiling water bath for 135 min. After cooling for 30 min, the intensity of the green colour was determined spectrophotometrically at 570 nm against a reagent blank. The concentration of glycerol in the samples was determined by reference to a calibration plot prepared from the values obtained for the standards.

Fatty acid analysis

Duplicate samples of the phospholipid were methylated and dissolved in a known volume of methanol. 1 µl samples were injected on to the

GLC and the areas under the peaks calculated. A standard solution of methyl palmitate of known concentration was injected under identical conditions and the area under the peak obtained was calculated. The areas under the peaks were then related to the weight of the fatty acid injected. A calculation of the average chain length of the fatty acids in the sample allowed the number of moles of fatty acid to be estimated.

Analysis of peptido-lipids

Peptido-lipid material was obtained by preparative TLC of the acetone-insoluble material. Polar amino acid-containing material was analysed to determine its amino acid and lipid composition. Cleavage of lipid material from the amino acids was attempted using (a) carboxy peptidase, and (b) crude protease (Sigma type vi, ex Streptomyces griseus). Peptido-lipids were dissolved in 5 ml 0.1M phosphate buffer (pH 7.0), 1 ml of enzyme solution (1 mg/ml) added and, after mixing, the solution was incubated at 37° for 1 h. The lipid components were isolated by washing the reaction mixture with 3 x 1 ml aliquots of chloroform. This material, along with standard lipids, was applied to TLC plates (0.25 mm silica gel H. (Merck)) and chromatograms developed in either:

(a) light petroleum (B.P. 60°-80°)/diethyl ether/acetic acid 70:30:1 by vol),

(b) chloroform/methanol (9:1 by vol), or

(c) chloroform/methanol/water (65:25:4 by vol).

The spots were detected using either rhodamine 6G, 50% H_2SO_4, or ninhydrin sprays (see Appendix I).

Amino acid analysis

Peptido-lipids were hydrolysed in 6N. HCl and analysed on a Locarte automatic amino acid analyser, using the method described in the membrane section on pp. 29 and 30.

Separation in a polar solvent

The peptido-lipids were also separated on 0.25 mm layers of silica gel H. (Merck) in the solvent system chloroform/methanol/water (50:30:5 by vol) to determine how many different compounds were present.

CAROTENOIDS

Large scale cultures of Planococcus 316 were grown in the New Brunswick MF114 fermenter in the BEPG medium containing 3% sea salt at 30° and with an agitation rate of 250 r.p.m. and an aeration rate of 15 l/min. Cells were harvested after 48 h when the culture was in stationary phase.

Carotenoid extraction

Carotenoid material was extracted in batches from approximately 230 g wet weight of cells, suspended in methanol, in a Ballatini ball mill (Edmund Bühler). The cells, in 60 - 70 g wet weight aliquots, were disrupted with 0.25 mm diameter glass beads for several 2 min periods, with 1 min cooling intervals. Cooling was achieved by cold water circulating around the extraction thimble. The disrupted cells were centrifuged and the pellets re-extracted until no more pigment was removed and the cell remnants were white. The supernatants were pooled and stored, gassed out with nitrogen, in the dark at 4°.

Storage of carotenoids

Because of their unstable nature, solutions of carotenoid material were always stored in the dark, gassed out with nitrogen, and at 4°. During the extraction procedures and purification steps, whenever possible fractions were kept away from light and on ice.

Purification and separation of carotenoids

Carotenoids in methanol were reduced in volume on a rotary evaporator at 30° and split into two fractions: 2/3 were saponified and extracted into diethyl ether and 1/3 was extracted into diethyl ether without saponification (see Fig. M3).

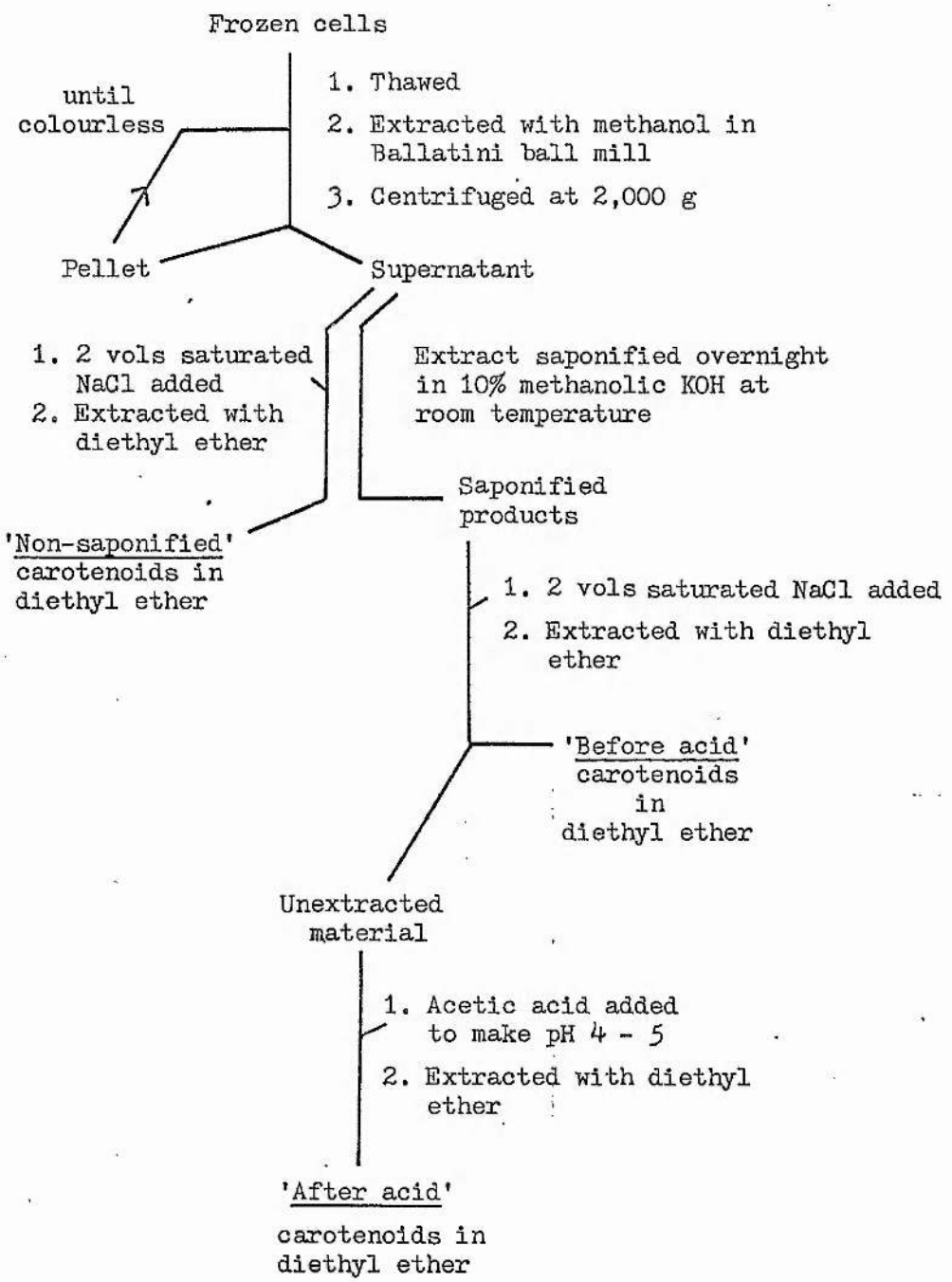


Fig. M3: Flow diagram for carotenoid preparation.

Saponification (84)

Saponification is an easy way of removing unwanted lipids which are extracted with the carotenoids. Some carotenoids, however, are labile to strong alkali. KOH pellets were added, with vigorous stirring, to the methanolic extract to make it 10% with respect to KOH. Saponification proceeded overnight in the dark, gassed out with nitrogen and at room temperature. Some carotenoid was extracted into diethyl ether by the addition of two volumes of saturated sodium chloride solution and the pigmented aqueous phase was then acidified to pH 5 with glacial acetic acid and re-extracted with diethyl ether. Both the diethyl ether extracts were reduced in volume and dried over anhydrous sodium sulphate.

Spectra

Absorption in the visible region was measured in various solvents against solvent blanks on a Pye Unicam SP 800 recording spectrophotometer scanning between 375 and 700 nm.

Infra-red spectra

Infra-red spectra were recorded using a Pye Unicam SP 200G spectrophotometer scanning the wavelengths between 650 and 4000 cm^{-1}. Carotenoids were dissolved in carbon tetrachloride or ethylene tetrachloride and held in a F. 05 liquid cell with a 0.25 mm teflon spacer and sodium chloride windows.

TLC separation of the carotenoid fractions

Analytical separations were carried out on layers of 0.25 mm silica gel H. (Merck) in a chloroform/methanol (9:1 by vol) solvent system. Preparative separations were carried out on 0.5 mm layers of silica gel H(Merck) in the same solvent. Samples were applied to the silica surface with a Desaga automatic strip loader (Camlab. Glass Ltd.), and, after development, individual carotenoid fractions were eluted from the silica with ethanol. The individual carotenoids obtained by

preparative work were subjected to a number of chemical tests to determine their identity.

Acetylation (85,86)

Acetylation of primary or secondary hydroxyl groups on the carotenoid molecule was detected by an alteration in chromatographic mobility. Samples for acetylation were dissolved in 1 ml of dry pyridine and 0.1 - 0.2 ml acetic anhydride added. The reaction was allowed to proceed in the dark at room temperature and was followed by the periodic removal of samples which were compared chromatographically with the original compound. Chromatography was carried out on microscope slides covered with a thin layer of silica gel H. (Merck) in the chloroform/methanol (9:1 by vol) solvent system. The reaction products and intermediate compounds were identified by the decrease in polarity which accompanies acetylation of the hydroxyl groups. The number of intermediates observed over several hours gave an indication of the number of primary or secondary hydroxyl groups present. Mono-hydroxy compounds gave only two spots, the original and the acetylated product. Di-hydroxy compounds gave a maximum of three spots which corresponded to the original, the intermediate and the final di-acetate products. Tri-hydroxy compounds gave a maximum of four spots which corresponded to the original material, the two possible intermediates and the final product. Absorption spectra were recorded before and after acetylation.

Trimethylsilylation (87)

Primary, secondary and tertiary hydroxyl groups may be silylated and thus the presence of a tertiary hydroxyl group can be indicated by silylation of the acetylation products. Samples were dried in vacuo and dissolved in 0.5 ml of dry pyridine; 0.2 ml hexamethyldisilazane and 0.1 ml trimethylchlorosilane were added and the reaction allowed to proceed for 1 h at room temperature. The reaction was stopped by the addition of excess carbon tetrachloride and the silylated products

concentrated on a rotary evaporator. Examination of the products by TLC shows the presence of tertiary hydroxyl groups by a decrease in polarity compared to the original material.

Reduction (88)

Reduction with lithium aluminium hydride ($LiAlH_4$) converts carotenoid aldehydes, ketones, acids and esters to the corresponding carotenols. The carotenoid sample was dissolved in 10 ml dry diethyl ether and a few mg of $LiAlH_4$ added. The reaction was allowed to proceed in the dark at room temperature for 1 h, after which it was terminated by the cautious addition of water. The reduced products were extracted into diethyl ether, helped by the addition of a small quantity of ethanol which assists in releasing polar carotenoids which may become associated with inorganic material. Chromatographic comparison of the products with the original compound on silica gel H. (Merck) in the chloroform/methanol (9:1 by vol) solvent system showed that reduction had occurred when a spot more polar than the original was observed. Intermediate compounds were observed if two carbonyl groups were present, and the reaction was stopped after 15 min.

Examination of the spectra of reduced products showed a shift in the absorption λ_{max} of approximately 4 nm per reduced carbonyl group towards lower wavelengths.

Oxidation (89)

This reaction is specific for hydroxyl groups allylic to the polyene chain or isolated double bonds. The carotenoid was dissolved in diethyl ether, a few mg nickel peroxide added and the reaction allowed to proceed at room temperature and in the dark for 1 - 2 h. Reaction products were identified by a decrease in polarity on chromatographing, and by a change in absorption spectra. However, many carotenoids are destroyed by this reagent.

Epoxide and Aldehyde test (90)

5'6' epoxides and aldehydes produce a stable blue colour on shaking with inorganic acids in diethyl ether. The carotenoid, dissolved in 5 ml diethyl ether, was shaken with 2 ml of 20% aqueous HCl. The appearance of a blue colour can be indicative of one or more epoxide or aldehyde groups.

Partition ratio (91)

This simple procedure give a rough idea of the extent of polar substitution on the carotenoid molecule. Equal volumes of hexane and 95% methanol were equilibrated by shaking together in a separating funnel. The carotenoid fraction was dissolved in 10 ml of one or other of the phases and the absorbance at the λ_{max} recorded on an SP 600 spectrophotometer. The addition of an equal volume of the other phase partitioned the carotenoid between the two solvents, and, by again determining the absorbance at the λ_{max}, the distribution of the carotenoid between the two phases was calculated.

Variation in pigmentation with culture age

A quantitative determination of carotenoid production was carried out to determine at what stage carotenoid production reached a maximum and to show how pigmentation varied with the amount of salt in the medium. 500 ml cultures were grown in the New Brunswick G25 orbital incubator-shaker at 30° with an agitation rate of 250 r.p.m. The BEPG medium was used with final sea salt concentrations of 0.5, 3 and 10%. After inoculation, samples were removed aseptically (10 or 25 ml) at intervals and the amount of pigment per unit cell dry weight was determined as follows.

The absorbance of a known volume of culture medium was determined at 600 nm of a SP 600 spectrophotometer against a fresh medium blank. The cells were collected by centrifugation and washed with water to remove adhering salt or media. The cells were suspended in 5 ml methanol

and disrupted by ultrasound, in an ice bath for 2 x 3 min periods with a 1 min cooling interval. The suspension was centrifuged and the pellet resonicated in another 5 ml of methanol. After centrifuging, the supernatants were pooled, the volume measured and the absorbance of the solution at the λ_{max} measured against a solvent blank. The pellet was suspended in 1% acetic acid in methanol and sonicated for 3 min. After centrifugation, the volume of the supernatant was measured and the absorbance of the solution measured at the λ_{max}. This procedure relates the total amount of pigment to the weight of cells from which it was extracted. Pigment per mg dry weight of cells was calculated as below:

$$\text{Absorbance at } \lambda_{max} \text{ of pigment} \times \text{volume} = \text{total pigment}$$

$$\text{Absorbance at 600 nm} = \text{mg dry weight of cells per ml (from Fig. 1, Results)}$$

$$\frac{\text{total pigment}}{\text{mg dry weight of cells}} = \text{pigment per mg dry weight of cells}$$

The spectra of the extracted pigment was recorded each time and the pigment separated on 0.25 mm layers of silica gel H. (Merck) in a chloroform/methanol (9:1 by vol) solvent system to detect any changes in the overall composition.

CELL DIMENSIONS

Determination of the size of Planococcus 316

150 ml cultures were grown on the New Brunswick G25 orbital incubator-shaker at 30° with an agitation rate of 250 r.p.m., in BEPG medium at sea salt concentrations of 0.5, 3 and 10%. Samples were removed at intervals as the culture aged and stained with carbol fuchsin dye. Stained slides were observed through a microscope fitted with a 'Watson image splitting eye piece' calibrated in μ. Cell diameters were measured and an average of 75 - 100 readings was recorded.

Electron microscopy

Preparation of blocks for section cutting was performed as shown in Fig. M4. Thin sections were cut with a glass knife on an LKB I Microtome and post-stained with either 2% uranyl acetate or with 2% uranyl acetate and 0.4% lead citrate. Sections, on copper grids, were examined on an AEI 6B electron microscope.

Fig. M4: Materials and method for preparation of specimens for electron microscopy.

Solution A: Veronal-acetate buffer: 2.94 g sodium barbitone + 1.94 g sodium acetate $3H_2O$ + 3.40 g NaCl made up to 100 ml with distilled water.

Solution B: 'Kellenberger buffer': 5.0 ml solution (A) + 13.0 ml distilled water + 7.0 ml 0.1 N HCl + 0.25 ml 1 M $CaCl_2$, pH adjusted to 6.0 with HCl. Prepared fresh as required.

Fixative solution: 0.1 g OsO_4 dissolved in 10 ml solution (B) (osmic acid).

Washing fluid: 0.5 g uranyl acetate in 100 ml solution (B).

Tryptone medium: 1.0 tryptone + 0.5 g NaCl in 100 ml distilled water. Prepared fresh as required.

Embedding medium

A : Araldite epoxy resin (CY 212)

B : Araldite hardener (HY 964)

C : Araldite accelerator (DY 064)

D : Dibutyl phthalate.

(a) Mix 10 g A + 10 g B by inversion overnight = (A + B)

(b) Mix 10 g C + 10g D by inversion for 2 h, store at 4° (C + D)

(c) Mix 19 g (A + B) + 1 g (C + D) by inversion for 6 h (ABCD).

Cells in liquid culture

20 ml removed, 2 ml fixative solution added, centrifuged.

Pellet resuspended in 5 ml fixative solution + 0.5 ml tryptone medium.

Fixation proceeded overnight at room temperature.

Diluted with 8 ml solution (B), centrifuged, and pellet mixed with molten Agar No. 3 at 50°

After cooling, Agar cut into 1 mm cubes and placed in washing solution for 2 h.

Fixed cells in Agar cubes

Cells in Agar cubes dehydrated:-

20% ethanol in water for 10 min
50% ethanol in water for 10 min
70% ethanol in water for 10 min
95% ethanol in water for 15 min
100% ethanol in water for 30 min
100% ethanol in water for 30 min.

Agar cubes suspended in 100% epoxy propane for 30 min x 2.

Dehydrated fixed cells

Cells suspended in 50% ABCD 50% epoxy propane (v/v) overnight.

Cells suspended in 100% ABCD for 1 h.

Cells suspended in 100% ABCD in Beem capsules and polymerised at 60° for 48 h.

Cells embedded in Araldite in Beem capsules

CATION ANALYSIS

Analysis of the major cations associated with the membrane

Ca^{++}, Mg^{++}, K^{+} and Na^{+} ions associated with the membrane were measured with a Varian Techtron Model AA1 atomic absorption spectrophotometer. Membrane samples for analysis were dried in an oven at 100° for 24 h, weighed and suspended in 10 ml 5% analar HCl. The suspensions were sonicated for 10 min to disperse the membrane particles

and then made up to 50 ml with 5% analar HCl. Homogeneous suspensions were aspirated into the flame and the average of 15 readings recorded for each sample. Standard solutions of 'Spec pure' calcium carbonate, magnesium oxide, sodium carbonate and potassium carbonate were made up in 5% analar HCl at concentrations of 25, 10, 5, 2.5 and 1 µg/ml and analysed in the same manner. The standard concentrations chosen were those which had their absorbances as near as possible to those of the sample. The µg/ml for each ion was calculated as shown below:

$$\mu g/ml \text{ sample} = \frac{\text{average absorbance of the sample x (}\mu g/ml \text{ of the higher standard + } \mu g/ml \text{ of lower standard}}{\text{sum of the absorbances of the standards}}$$

Analysis of the major cations associated with whole cells

Cells grown in BEPG medium containing either 0.5%, 3% or 10% sea salt were harvested by centrifugation and washed rapidly with distilled water. The washed pellet was suspended in 20 ml 5% analar HCl and duplicate 2 ml samples removed and dried to constant weight in an oven at 100°. A 10 ml sample from the washed pellet suspension was sonicated for 10 min to form a colloidal suspension and 1 ml of this was made up to 50 ml with 5% analar HCl. This homogeneous suspension was aspirated into the flame in the atomic absorption spectrophotometer and analysed for Ca^{++}, Mg^{++}, K^{+} and Na^{+} ions. The average of 15 readings was recorded for each sample.

A solution of sea salt (4 µg/ml) was also analysed in the same manner. The same standards were used as in the previous experiment and the ion concentrations calculated as described above.

Ash content of whole cells

The ash content of whole cells was determined by subjecting known weights of dry cells to the procedure described on page 29 for the determination of the ash content of membranes.

Appendix I

1. Anisidine phthalate spray reagent for carbohydrates (110)

1.23 g para-anisidine and 1.66 phthalic acid were dissolved in 100 ml of methanol. Chromatograms were sprayed with the reagent and heated to 60°-70° to develop the colours. This spray gives various colour reactions with different sugars, for example: hexoses - green; pentoses - pink; methyl pentoses - yellow; uronic acids - brown.

2. Antimony trichloride spray reagent for sterols (110)

A saturated solution of antimony trichloride in chloroform was prepared. Chromatograms were sprayed with this solution and then heated in an oven at 120° for 10 min. Sterols and sterol esters appear as red/violet spots on a white background. Highly unsaturated lipids may give a similar reaction.

3. Copper sulphate spray for sulphur-containing glycolipids (111)

A 10% cupric sulphate solution in water was prepared; chromatograms were sprayed and then heated in an oven for 20 min at 120°. Sulphur-containing glycosides are detected as brown spots on a green background.

4. Diphenylamine spray for glycolipids (110)

20 ml of 10% diphenylamine solution in ethanol were mixed with 100 ml conc. HCl and 80 ml of glacial acetic acid. Chromatograms were sprayed with the reagent and heated for 30 - 60 min in an oven at 105° with another glass plate placed directly on the gel surface. Sugar-containing lipids appear as grey/blue spots on a white background.

5. 2'7' dichlorofluorescein spray for neutral lipids (81)

A 0.2% solution of 2'7' dichlorofluorescein in 95% ethanol was prepared; chromatograms were sprayed with the reagent and viewed under UV light while still wet. Neutral lipids appear as yellow or green spots on a purple background. Polar lipids (glycolipids and phospholipids) do not stain well with this reagent.

6. Iodine vapour (81)

Crystals of sublimed iodine were placed in crucibles at the bottom of a chromatography tank and chromatograms were placed in the tank for 2 - 3 min. All lipids appear as yellow spots on a white background. Fully saturated lipids and some glycolipids do not stain well. The iodine vapour can be removed by heating or leaving at room temperature for a number of hours. Individual spots may be detected by blowing through a Pasteur pipette plugged with glass wool and containing crystals of iodine.

7. Ninhydrin spray for amino groups (81)

A solution of 0.2% ninhydrin in butanol saturated with water was prepared and chromatograms were sprayed and heated in an oven at 105° for 5 - 10 min. Lipids containing free amino groups appear as pink or purple spots on a white background.

8. Phosphorus detecting spray (81)

Reagent (a): 8 g of ammonium molybdate were dissolved in 60 ml water. Reagent (b): 5 ml of mercury were added to 20 ml conc. HCl and 40 ml of solution (a). The mixture was stirred for 30 min and filtered. Spray reagent: 100 ml of conc. H_2SO_4 and all of solution (b) were added to the remainder of solution (a) (20ml). The mixture was cooled and diluted to 500 ml with water, resulting in a brown/green clear liquid. Chromatograms were sprayed with the reagent. Phosphorus-containing lipids appear very rapidly as blue spots on a white background without heat being required.

9. Rhodamine 6G (81)

Rhodamine 6G was prepared as 0.01% or 0.005% aqueous solution from a stock solution of 0.12% rhodamine, which is stable indefinitely in the dark. Chromatograms were sprayed and viewed under UV light while still wet. Acidic phosphatides and other lipids appear as blue or

purple fluorescent spots. Neutral lipids and other phosphatides give yellow or orange spots.

10. Sulphuric acid/acetic acid spray for sterols (81)

A 1:1 (by vol) solution of conc. H_2SO_4 and acetic acid was prepared; chromatograms were sprayed and heated in an oven at 90° for 15 min. Sterols and sterol esters appear as red spots on a white background. Unsaturated lipids may give pink/brown spots, and other lipids will char with prolonged heating.

11. Sulphuric acid spray (81)

50% aqueous sulphuric acid was prepared; chromatograms were sprayed lightly with the reagent and heated in an oven at 160°-180° for 30 - 60 min. All lipids show up as dark spots on a white background, but volatile hydrocarbons and fatty acids may evaporate before charring occurs. Unsaturated lipids tend to produce more intense spots than saturated compounds. Charring can be accelerated to reduce the loss of volatile components by spraying with a saturated solution of potassium dichromate in 50% H_2SO_4.

12. Schiffs-periodate stain for vicinal hydroxyl groups (81)

100 ml of 1% sodium periodate solution was made up in water. 100 ml of 1% para-rosaniline hydrochloride was made up in water and SO_2 gas bubbled through from a cylinder until the solution became colourless (Schiffs reagent). The chromatogram was sprayed with sodium periodate until saturated and then left for 5 - 10 min (oxidation step). It was then placed in a chromatography tank and the tank filled with SO_2 gas. After a few minutes, the plate was removed and sprayed lightly with the Schiffs reagent. Spots appear after a few mins at room temperature and can be identified by their colour and order of appearance. Phosphatidyl glycerol spots appear quickly and are purple in colour. Glycosyl diglycerides and other glycolipids appear more

slowly and their spots are dark blue. Phosphatidyl inositol appears as a yellow spot due to the formation of a malondialdehyde and is easily identified.

Appendix II

The program in FORTRAN IV was used to calculate percentage peak areas and estimate chain lengths from information obtained from the GLC traces, on an IBM 360/44 computer.

```
      DIMENSION HT(100),W(100),HTW(100),PP(100),ECL(100),RT(100)
      COMMON INT,GRAD
      REAL INT,LRT(100)
      REAL*8 AA,AS,AD,AF,AG,AH,AJ,AK,AL,AZ
      READ(5,12) NT
   12 FORMAT(12)
      DO 13 K=1,NT
      CALL LESQ
      READ(5,30) AA,AS,AD,AF,AG,AH,AJ,AK,AL,AZ
   30 FORMAT(10A8)
      READ(5,1) ND
    1 FORMAT(12)
      DO 2 J=1,ND
      READ(5,4) HT(J),W(J),RT(J)
    4 FORMAT(3F6.2)
      LRT(J)=ALOG(RT(J))
      HTW(J)=HT(J)*W(J)
    2 CONTINUE
      SHTW=0.
      DO 6 JJ=1,ND
      SHTW=SHTW+HTW(JJ)
    6 CONTINUE
      DO 7 JK=1,ND
      PP(JK)=(HTW(JK)/SHTW)*100
    7 CONTINUE
      DO 20 JH=1,ND
   20 ECL(JH)=((LRT(JH)-INT)/GRAD)
      PRINT 31,AA,AS,AD,AF,AG,AH,AJ,AK,AL,AZ
   31 FORMAT('1',10A8//' ')
      PRINT 8
    8 FORMAT('0','PEAK NO.      HEIGHT      WIDTH      HTW      RT      %AREA      ECL')
      DO 10 JL=1,ND
      IF(PP(JL).LT.1.0) GO TO 21
      PRINT 18,JL,HT(JL),W(JL),HTW(JL), RT(JL),PP(JL),ECL(JL)
   18 FORMAT('0',I5,F13.2,F11.2,F9.2,F9.3,F8.1,F9.2)
      GO TO 10
   21 PRINT 11,JL,HT(JL),W(JL),HTW(JL), RT(JL),ECL(JL)
   11 FORMAT('0',I5,F13.2,F11.2,F9.2,F9.3,3X,'TRACE',F9.2)
```

RESULTS

GROWTH

Solid media cultures

Planococcus 316 grew well at 30° on solid media containing 3% sea salt and round, convex, smooth and glistening orange colonies, 2 - 3 mm in diameter, could be observed after 48 h. Cells remained viable for at least one month.

Estimation of sea-water salinity

Sea water obtained from St. Andrews bay was found to contain 3.4% salt.

Determination of the relationship between dry weight and absorbance at 600 nm

From Fig. R1 it is seen that the relationship is linear between optical densities of 0.1 and 0.75.

Comparison of turbidimetric and viable cell counting techniques for the study of cell growth

Fig. R2 shows that the growth curves obtained using the two techniques were closely parallel. An absorbance of 4.0 was found to correspond to approximately 6×10^7 cells per ml.

Determination of the salt tolerance of Planococcus 316

The results obtained from growth on solid media containing sodium chloride are shown in Table R1.

Table R1: Salt tolerance of Planococcus 316 on solid media.

NaCl concentration in BEPG media	Observations
0%	No growth observed
3%	Good growth and pigmentation after 24 h
7%	Good growth and pigmentation after 24 h
11%	Reasonable growth after 36 h, but less pigment than 3% and 7% concentrations
14%	Scanty growth and pigmentation
20%	No growth observed

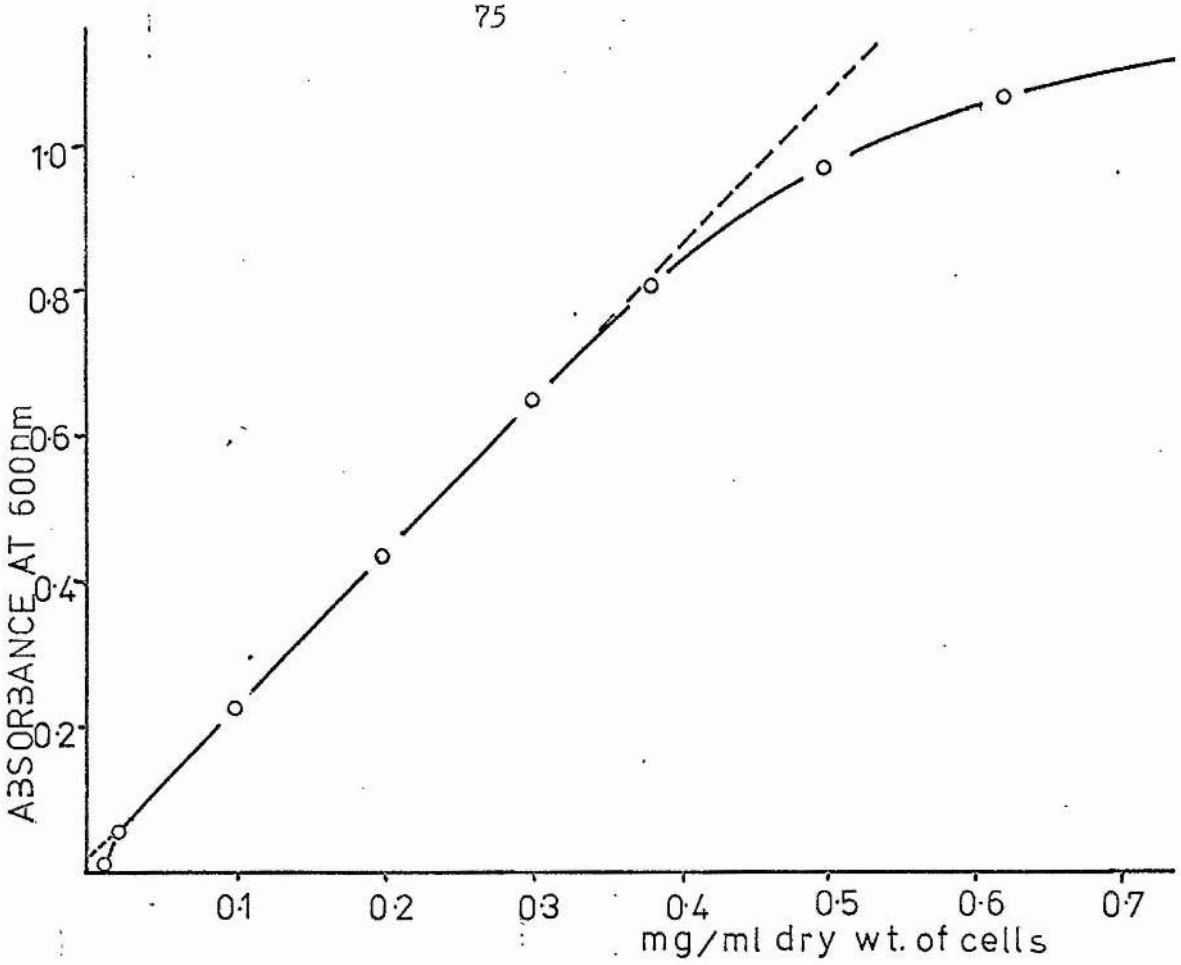


Fig. R1: The relationship of absorbance at 600 nm to dry weight for a suspension of microorganisms

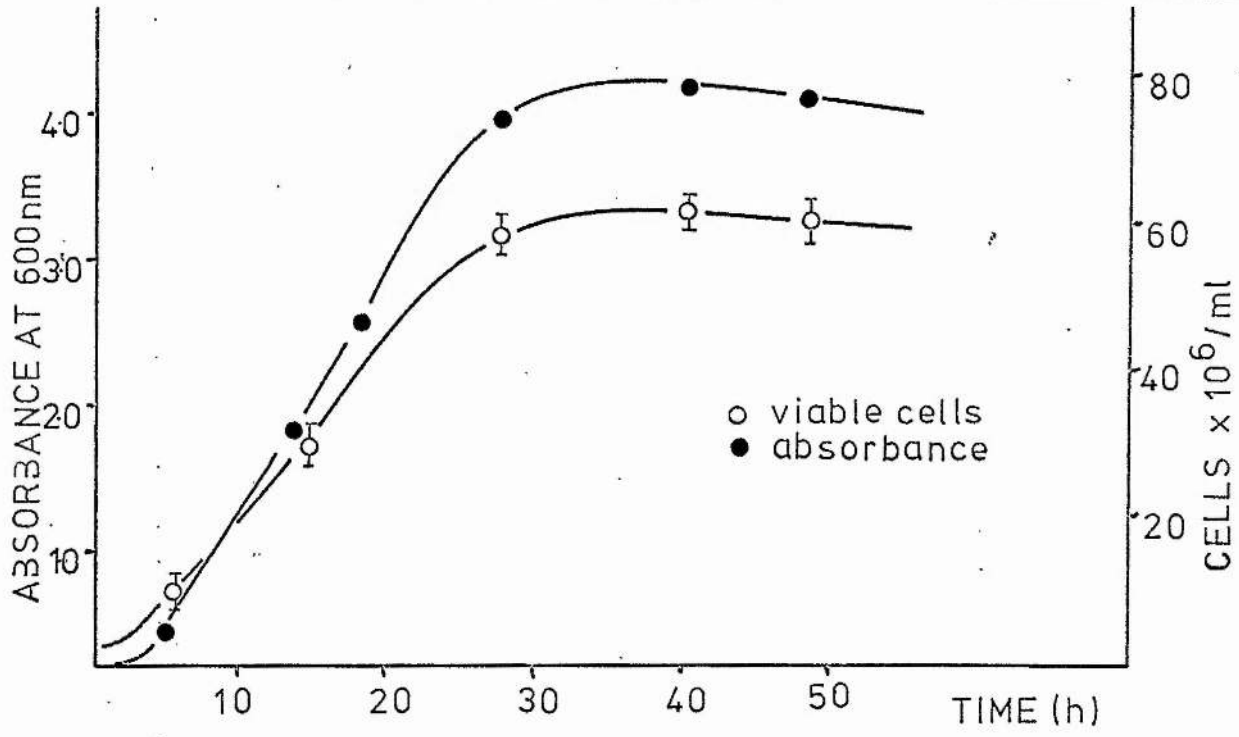


Fig. R2: A comparison of the results obtained from the turbidimetric and viable cell counting techniques for the study of growth of Planococcus 316 in BEPG medium at 30° C

Small quantities of NaCl present in the media were ignored. The results indicate that the lower level of salt tolerance is below 3% and the upper level between 14% and 20%

Liquid media

Figs. R3, R4 and R5 show the absorbances reached and the time required to reach stationary phase for cultures of Planococcus grown in the presence of sea salt, NaCl, KCl and mixtures of NaCl and KCl.

Table R2: Summary of growth information obtained from Figs. R3, R4 and R5.

Sea salt concentration in BEPG medium	Length of lag phase h	Time until stationary phase h	Doubling time in log phase h	Max absorbance
0%	-	-	-	-
0.5%	2	25	5	4.0
3.0%	5	28	5	3.7
10.0%	8	30	5	3.0
NaCl concentration in BEPG medium				
0-0.25%	-	-	-	-
0.5%	10	50	10	3.1
3.0%	14	50	8	3.5
10.0%	20	70	16	2.5
15.0%	-	-	-	-
KCl or NaCl/KCl concentration in BEPG medium				
0.25% KCl	-	-	-	0.00
0.5% KCl	80	-	-	0.01
3.0% KCl	8	30	8	3.00
10.0% KCl	18	80	20	2.40
0.25% KCl + 0.25% NaCl	-	-	-	-
5.0% KCl + 10.0% NaCl	40	85	20	1.00
10.0% NaCl	18	70	16	2.70

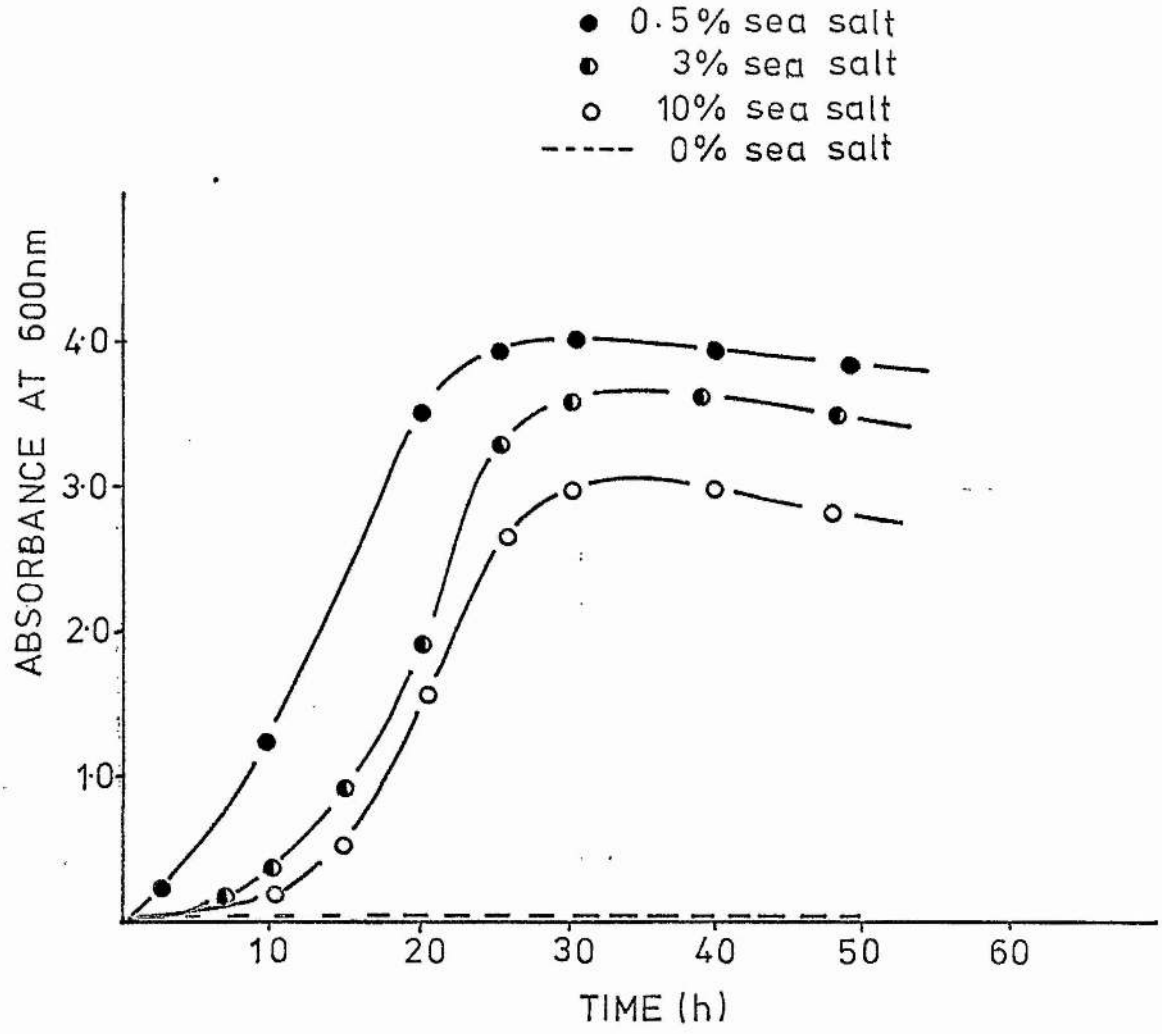


Fig. R3: Growth curves showing the tolerance of Planococcus 316 to various concentrations of sea salt when grown in BEPG medium at 30°

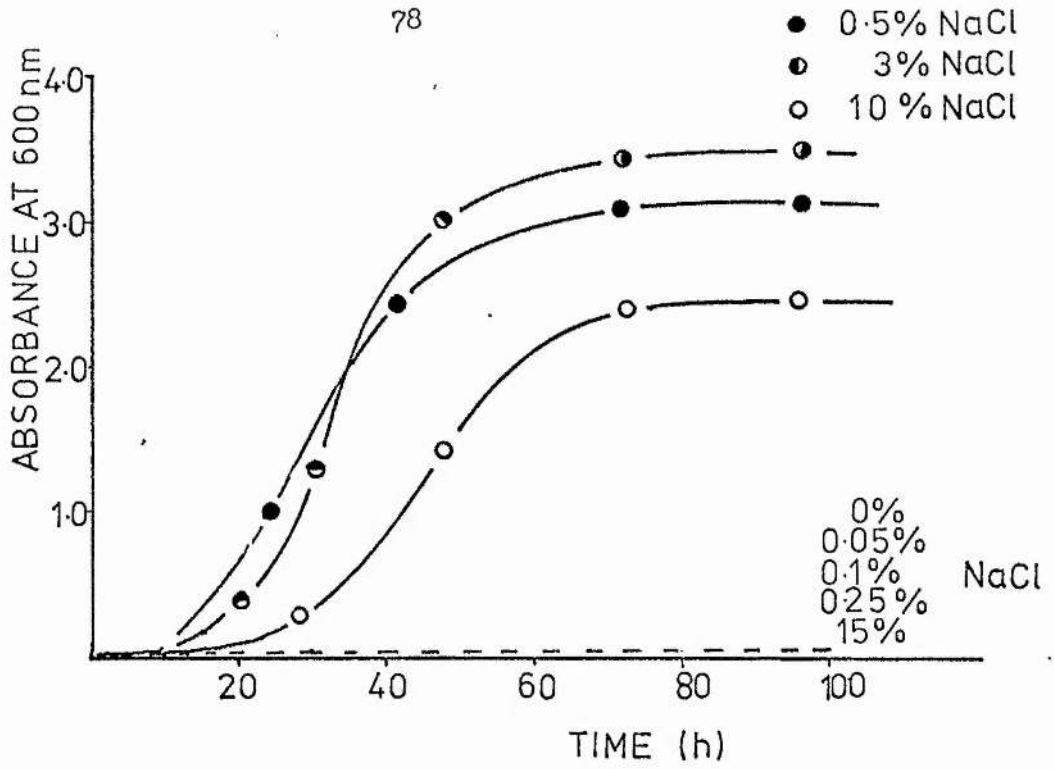


Fig. R4: Growth curves showing the tolerance of Planococcus 316 to various concentrations of NaCl when grown in BEPG medium at 30°

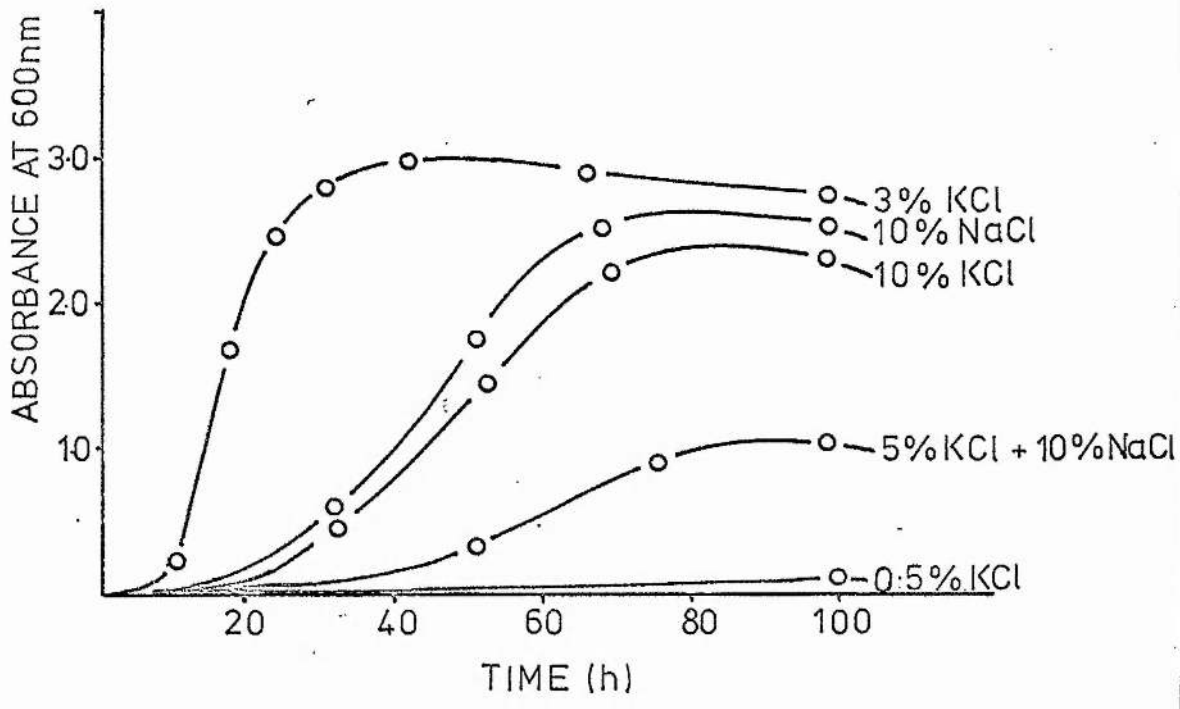


Fig. R5: Growth curves showing the tolerance of Planococcus 316 to KCl and NaCl/KCl mixtures when grown in BEPG medium at 30°

The differences in doubling times and the final absorbances achieved at the same concentrations of sea salt and NaCl may be due to the presence of more potassium, magnesium and calcium ions in the sea water. The lower salt limit in liquid culture appears to be just less than 0.5% and the upper limit between 10% and 15%.

Determination of the optimum pH for growth of Planococcus 316 in BEPG medium

Figs. R6 and R7 were obtained by plotting maximum absorbance achieved against pH of the growth medium. Planococcus 316 showed good growth at all three salt concentrations and from approximately pH 7.0 - pH 9.0, with better acid tolerance in the higher salt concentrations. An unusual 'dip' was observed at pH 7.5 which was reproducible and detected by both turbidimetric and TCA precipitation techniques.

Large scale culture of Planococcus 316

Cells were harvested after 24 - 26 h when the cultures were entering stationary phase. With 0.5% sea salt in the medium, a yield of 9 - 10g/l was achieved; with 3% sea salt, 8.5 - 9.5 g/l; with 10% sea salt, 6 - 7 g/l.

MEMBRANES

Yield of membranes

An average of two membrane preparations gave the results shown in Table R3.

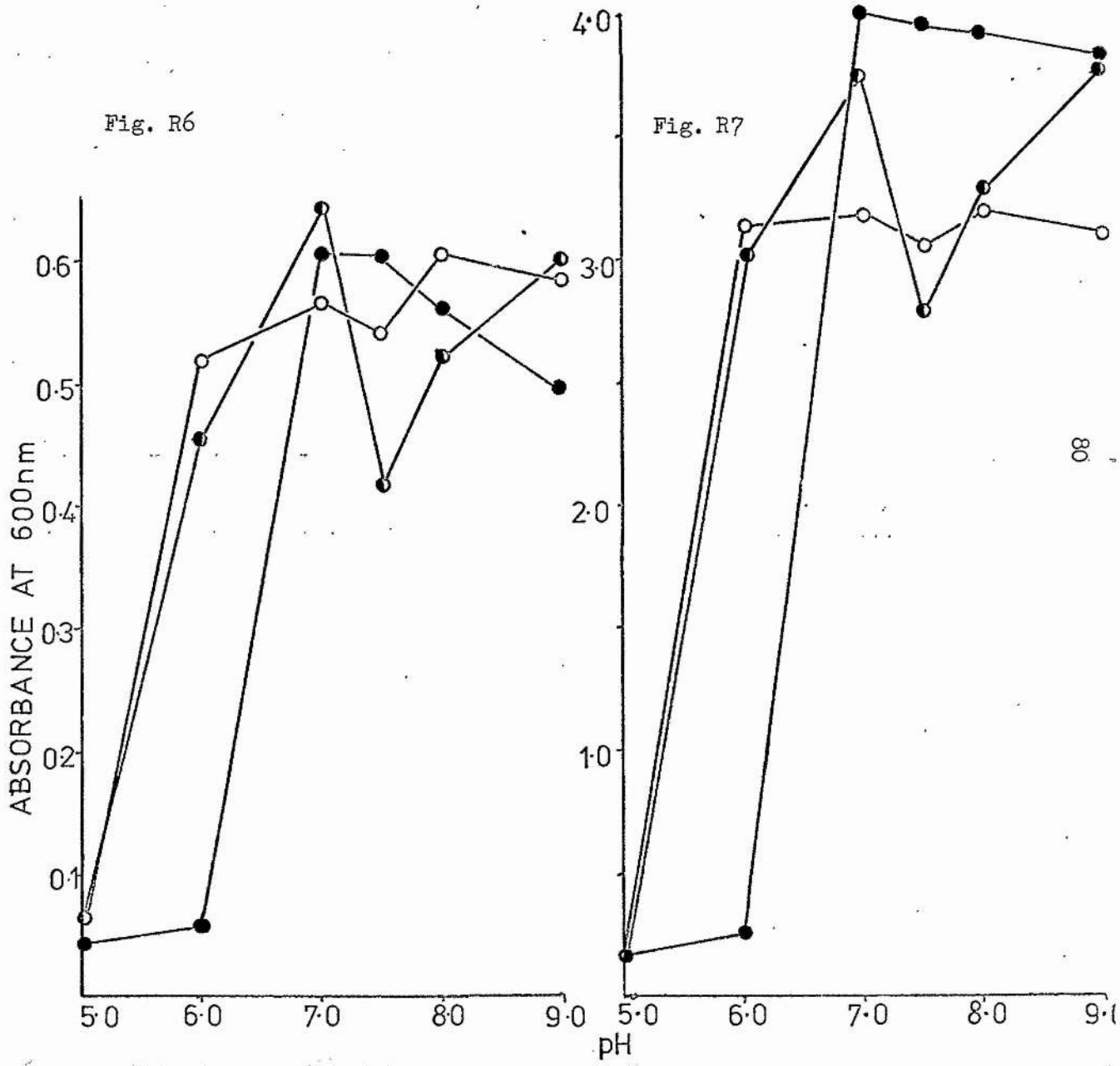
Table R3: Membrane material as a percentage of dry cell weight on an ash free basis.

% sea salt in media	%
0.5	22.9
3.0	34.5
10.0	17.4

● 0·5% sea salt
◐ 3% sea salt
○ 10% sea salt

Fig. R6: A plot of the maximum growth attained by Planococcus 316 in three sea salt concentrations at various pH values as determined by the TCA precipitation technique

Fig. R7: A plot of the maximum growth attained by Planococcus 316 in three sea salt concentrations at various pH values as determined by the turbidimetric technique



Membrane composition

In these results, membrane type 0.5%, 3% or 10% refers to the percentage sea salt in the media in which the original cultures were grown and from which the membranes were prepared. The results are presented in the following tables and each is the average of at least two determinations. With the exception of moisture, ash and phosphorus, all other results of chemical analysis are expressed as a percentage of the total organic material.

Table R4: Membrane ash content.

Membrane type	%
0.5%	4.0
3.0%	8.9
10.0%	9.7

Table R5: Membrane moisture content.

Membrane type	%
0.5%	3.6
3.0%	3.0
10.0%	9.2

Table R6: Membrane carbohydrate content.

Membrane type	%
0.5%	3.0
3.0%	7.1
10.0%	6.1

Table R7: Membrane RNA content.

Membrane type	%
0.5%	1.7
3.0%	11.7
10.0%	5.7

Fig. R8 shows a plot of carbohydrate release against hydrolysis time. Optimum time for hydrolysis of the membranes was found to be $\frac{1}{2}$ h in 1 N. HCl at 105°.

Table R8: Membrane protein content.

Membrane type	%
0.5%	78.9
3.0%	67.9
10.0%	74.6

Table R9: Membrane lipid content.

Membrane type	%
0.5%	21.7
3.0%	13.6
10.0%	20.8

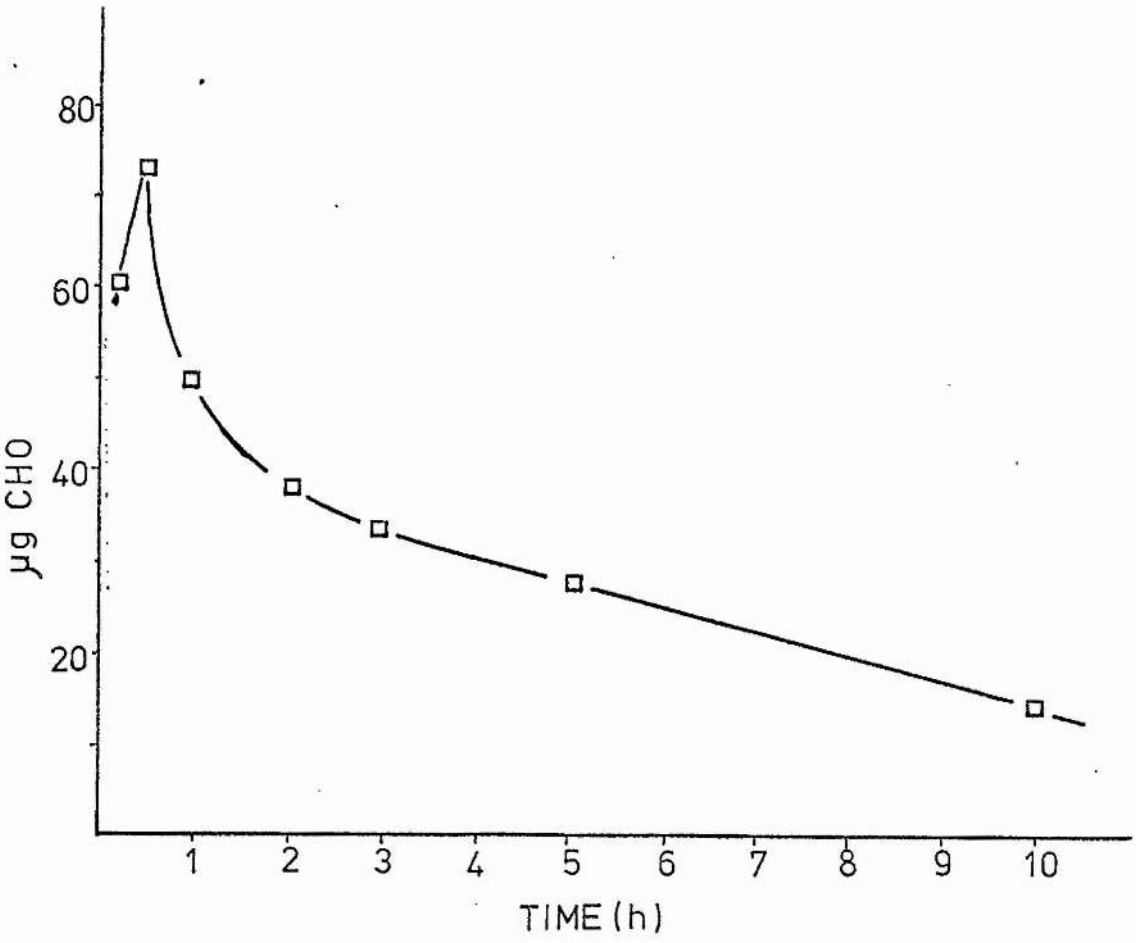


Fig. R8: Carbohydrate released from Planococcus 316 membranes as determined by the phenol-sulphuric acid method, after hydrolysis in 1 N. HCl at 105° for various periods of time

Table R10: Membrane protein:lipid ratio.

Membrane type	ratio protein : lipid
0.5%	3.6 : 1
3.0%	5.0 : 1
10.0%	3.6 : 1

Table R11: Membrane phosphorus content.

Membrane type	%
0.5%	1.26
3.0%	4.39
10.0%	4.15

Fig. R9 summarises the membrane compositions.

Amino acid composition of membrane proteins

The results are expressed in two ways: Table R12 gives the average amino acid composition from two determinations on the three membrane types where the amount of each amino acid is expressed in moles/100 moles; Table R13 shows the distribution of acid, basic and aliphatic amino acids in the three membrane types. Again in Table R13, the amino acids are expressed in moles/100 moles. Comparisons with published results (25) of the amino acid compositions of extreme halophiles and non-halophiles are made in both tables.

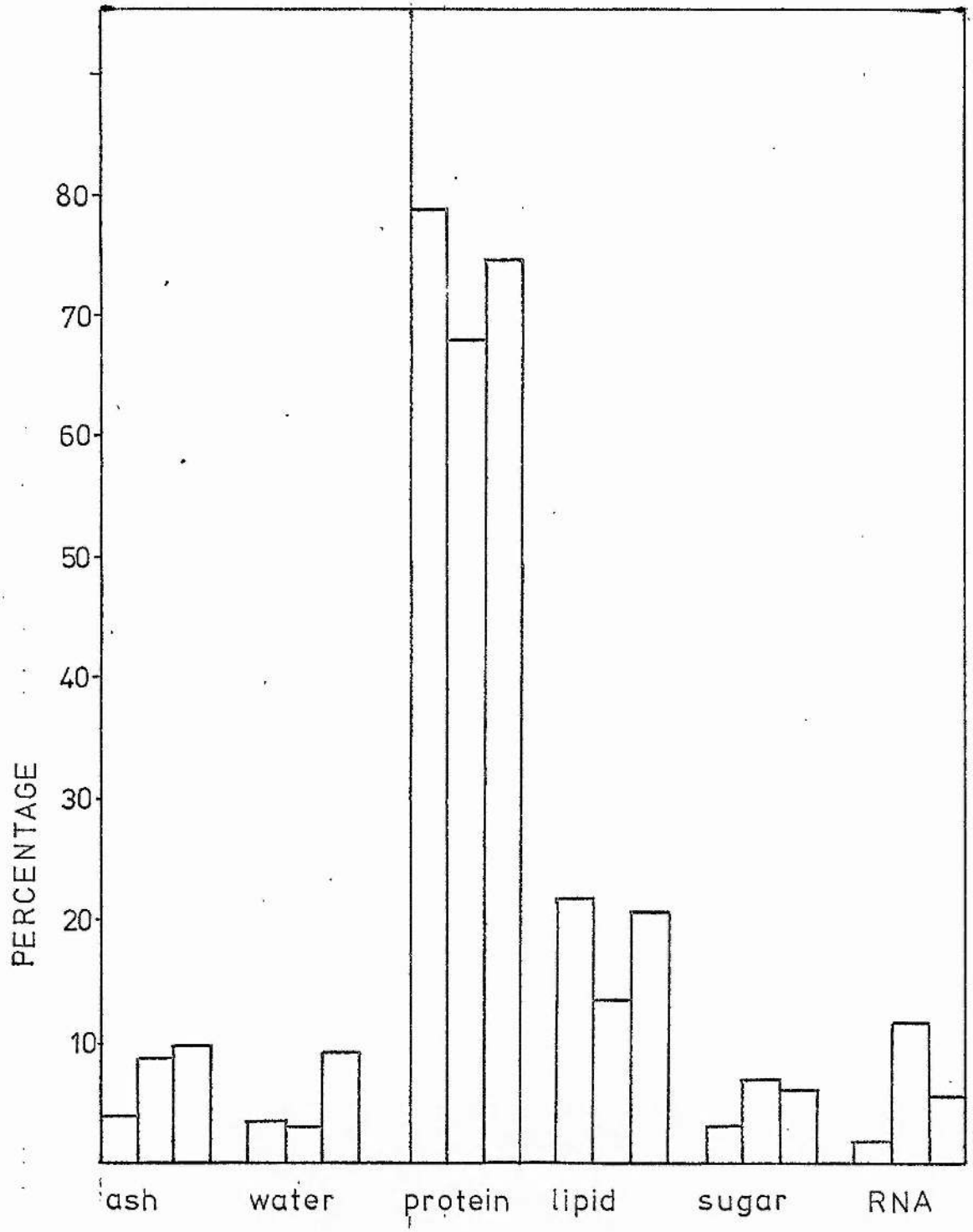


Fig. R9: A summary of the composition of the three membrane types

Each group of three columns, from left to right, corresponds to the 0.5%, 3% and 10% membrane types respectively

Table R12: Amino acid composition of membrane proteins.

Amino acid	Membrane type 0.5%	3.0%	10.0%	Halococcus strain 46	S.lutea
Cysteic acid	0.62	0.5	0.7		
Aspartic acid	11.2	10.7	11.4	12.9	9.4
Threonine	6.1	5.5	5.6	6.0	5.8
Serine	4.5	5.6	5.5	4.4	4.1
Glutamic acid	11.0	12.7	12.4	13.9	11.8
Proline	3.9	4.1	3.4	4.3	4.9
Glycine	8.5	9.6	8.4	8.8	9.3
Alanine	8.7	8.8	8.9	10.6	12.6
Valine	7.8	6.7	6.5	8.8	8.7
Methionine	0.3	0.5	0.7	1.9	1.9
Iso-leucine	6.9	5.6	6.4	4.2	4.1
Leucine	10.3	8.2	8.8	7.7	8.3
Tyrosine	1.6	1.7	2.1	2.5	2.2
Phenyl alanine	4.4	3.6	4.0	3.0	3.0
Lysine	6.8	7.9	7.8	2.4	4.1
Histidine	2.3	2.5	1.9	2.2	2.1
Arginine	5.0	5.8	5.3	5.7	6.4

In the proteins from the 0.5% and 3% membrane types, 1 small peak was observed between valine and methionine; 3 small peaks were observed between phenyl alanine and histidine and 2 after arginine. In the proteins from the 10% membrane type, 2 small peaks were observed between alanine and valine; 3 between valine and methionine and 3 between phenyl alanine and histidine.

Table R13: Distribution of amino acid types in the three membrane preparations.

	Membrane type			Halococcus strain 46	S.lutea
	0.5%	3.0%	10.0%		
Acidic amino acids	22.2	23.4	23.8	26.8	21.2
Basic amino acids	14.1	16.2	15.0	10.3	12.6
Excess of acidic over basic	10.4	9.7	10.7	16.5	8.6
Aliphatic amino acids	42.2	38.9	39.0	40.1	43.0

Although there is an excess of acidic amino acids over basic amino acids, this does not vary with increasing salt in the medium. Aliphatic amino acids at 40% of the total moles constitute the largest group. Aromatic and sulphur-containing amino acids are present only in small amounts in all three membrane types.

Qualitative carbohydrate analysis(Fig. R10)

For both standards and the membrane components, $R_{glucose}$ values were calculated. On this basis, spots (a) could not be identified; spots (b) were tentatively identified as galactose; spots (c) were tentatively identified as glucose and spots (d) were tentatively identified as ribose. On staining, spots (a) were green/brown; spots (b) and (c), glucose, galactose, mannose, N-acetyl glucosamine and galactosamine hydrochloride were green; spots (d) ribose and arabinose were pink, rhamnose was yellow/green and glucuronic acid was brown.

LIPIDS

Lipid extraction

After purification of the crude extracted lipid from the three membrane types on Sephadex G25, the percentages recovered as 'pure' lipid were 93.3%, 86.8% and 82.5% respectively from the 0.5%, 3% and

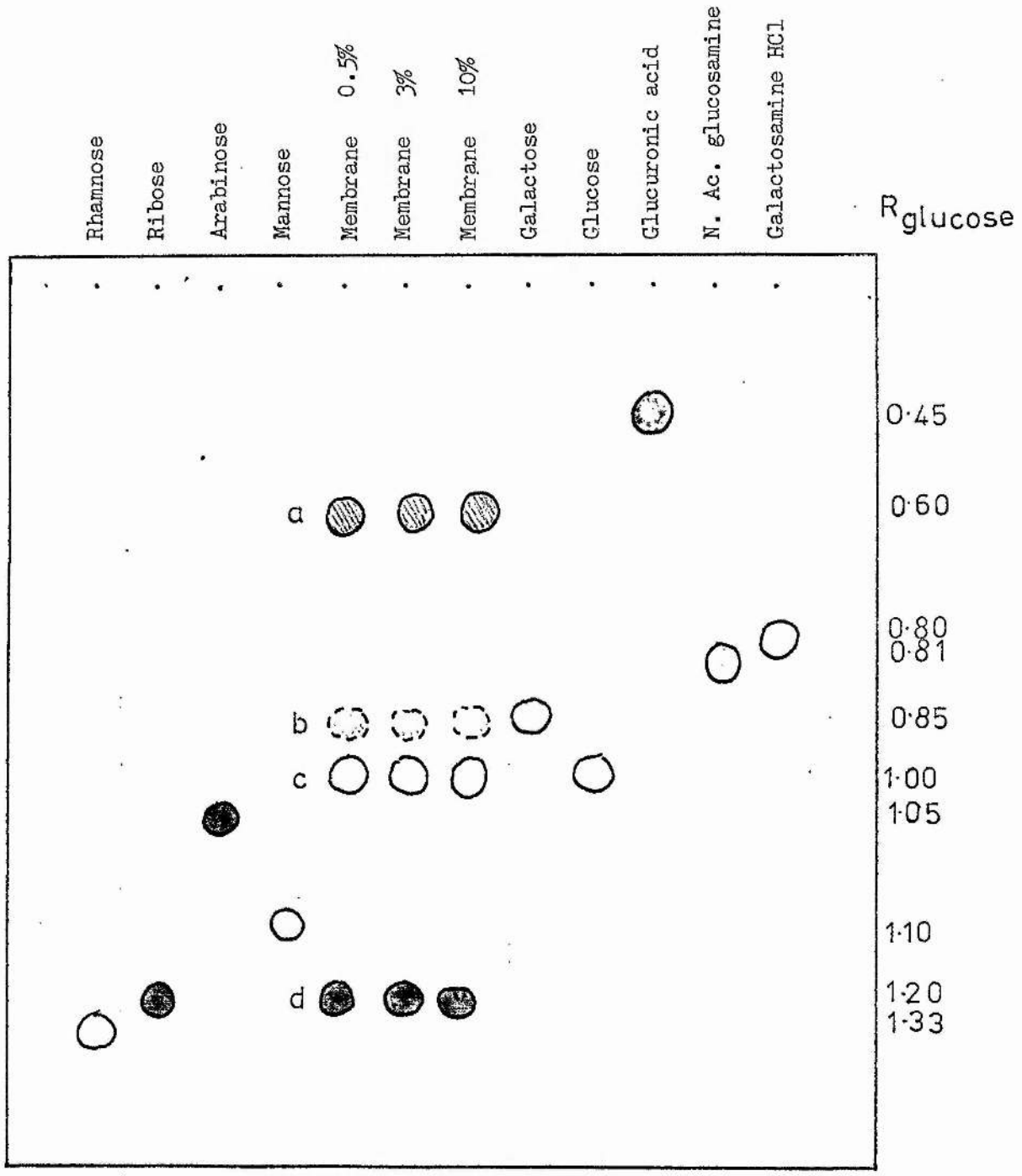


Fig. R10: Qualitative carbohydrate analysis of the three membrane types by paper chromatography

Solvent: butanol/pyridine/water (6:4:3 by vol)

Detection: anisidine phthalate spray

◌ : faint

10% membrane types.

Separation into lipid classes

Acetone precipitation

Table R14 shows the amounts of material which were precipitated in cold acetone and which are expressed as a percentage weight of the total 'pure' lipid.

Table R14: Acetone precipitable material in the 'pure' lipids from the three membrane types.

Membrane type	% acetone insoluble (phospholipids)	% acetone-soluble	Ratio acetone-insoluble : acetone-soluble
0.5%	76.9	23.1	3.3 : 1
3.0%	71.3	28.7	2.5 : 1
10.0%	70.3	29.7	2.3 : 1

Phospholipids constitute a very large proportion of the total lipids in this microorganism but the proportion decreases slightly in relation to other lipids when the salt in the growth medium is increased.

Silicic acid solumn fractionation of the acetone-soluble lipids

Table R15 shows the amounts of neutral and glycolipid fractions obtained from the column, expressed as a percentage weight of the total 'pure' lipid. The methanol fraction was thought to contain phospholipids which had not been acetone-precipitated and glycolipids which had not been eluted by acetone.

Table R15: Lipid classes separated by column chromatography.

Membrane type	% Neutral lipid	% Glycolipid	% Methanol fraction
0.5%	20.08	2.36	0.66
3.0%	17.98	1.54	9.18
10.0%	23.63	2.31	3.76

The glycolipids are composed of the sum of the weights of lipid eluted in the chloroform/acetone (1:1 by vol) and acetone fractions. Fig. R11 summarises the proportions of phospholipid, glycolipid and neutral lipid in the membrane types.

Neutral lipid separations

Separation of free fatty acids by sodium carbonate washing

Table R16 shows the free fatty acids as a percentage of the total neutral lipids by weight. Results are an average of two determinations.

Table R16: Free fatty acid content of neutral lipid fraction.

Membrane type	%
0.5%	25.4
3.0%	18.4
10.0%	28.7

Separation of neutral lipid components by silicic acid column chromatography

Results from this separation were disappointing. Free fatty acids were removed prior to loading the lipid on to the column, but only the hydrocarbons were eluted as a discrete fraction. Other fractions all contained more than one component.

Separation of neutral lipid components by 7% hydrated florisil column chromatography

This system was more efficient than the silicic acid, but did not achieve a good enough separation to isolate all the individual components. Hydrocarbons separated well and the majority of the triglyceride was obtained pure. The free fatty acids were removed on their own at the end by eluting with diethyl ether/acetic acid. Other fractions generally contained at least one other component.

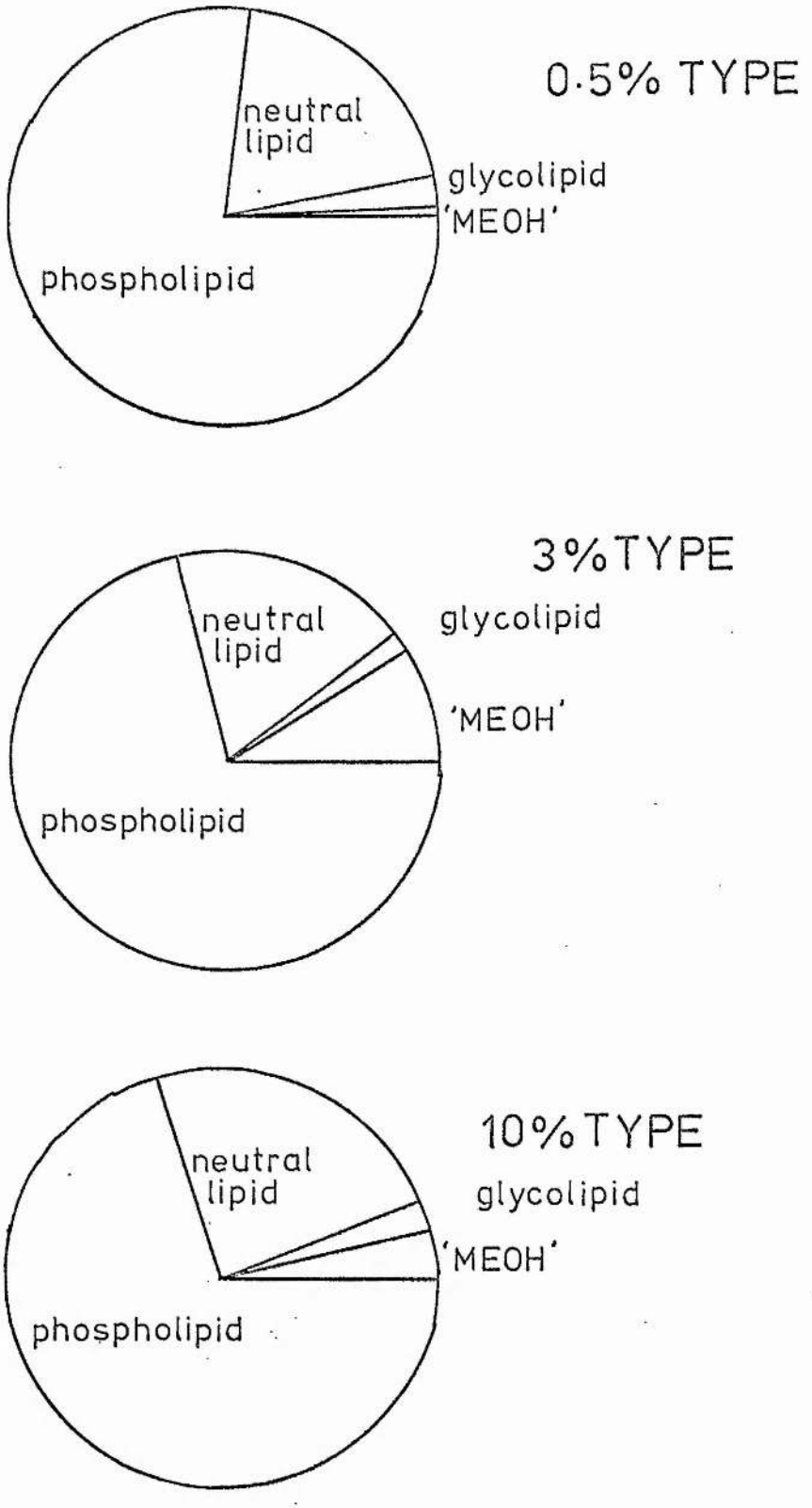


Fig. R11: Diagrammatic representation of the proportions of Phospholipid, Glycolipid and Neutral lipid in the three membrane types

Thin-layer chromatographic separation of neutral lipids

The two-step solvent system gave excellent separation of all the main neutral lipid components. The single-step system (light petroleum B.P. 60°-80°/diethyl ether/acetic acid, 70:30:1 by vol) was quicker but the separation was slightly inferior. Increasing the proportion of acetic acid in the single step system improved the separation among the more polar compounds. Fig. R12 shows a typical separation of the neutral lipids. All three membrane types contained the same components although in different proportions. Comparison of the R_fs of the spots with authentic standards allowed the following classes of compounds to be tentatively identified: hydrocarbons, sterol esters (?), fatty acid esters, triglycerides, free fatty acids, long chain alcohols, sterols (?), diglycerides, monoglycerides. Neither sugar nor phosphorus was detected at the origin or anywhere else on the plate.

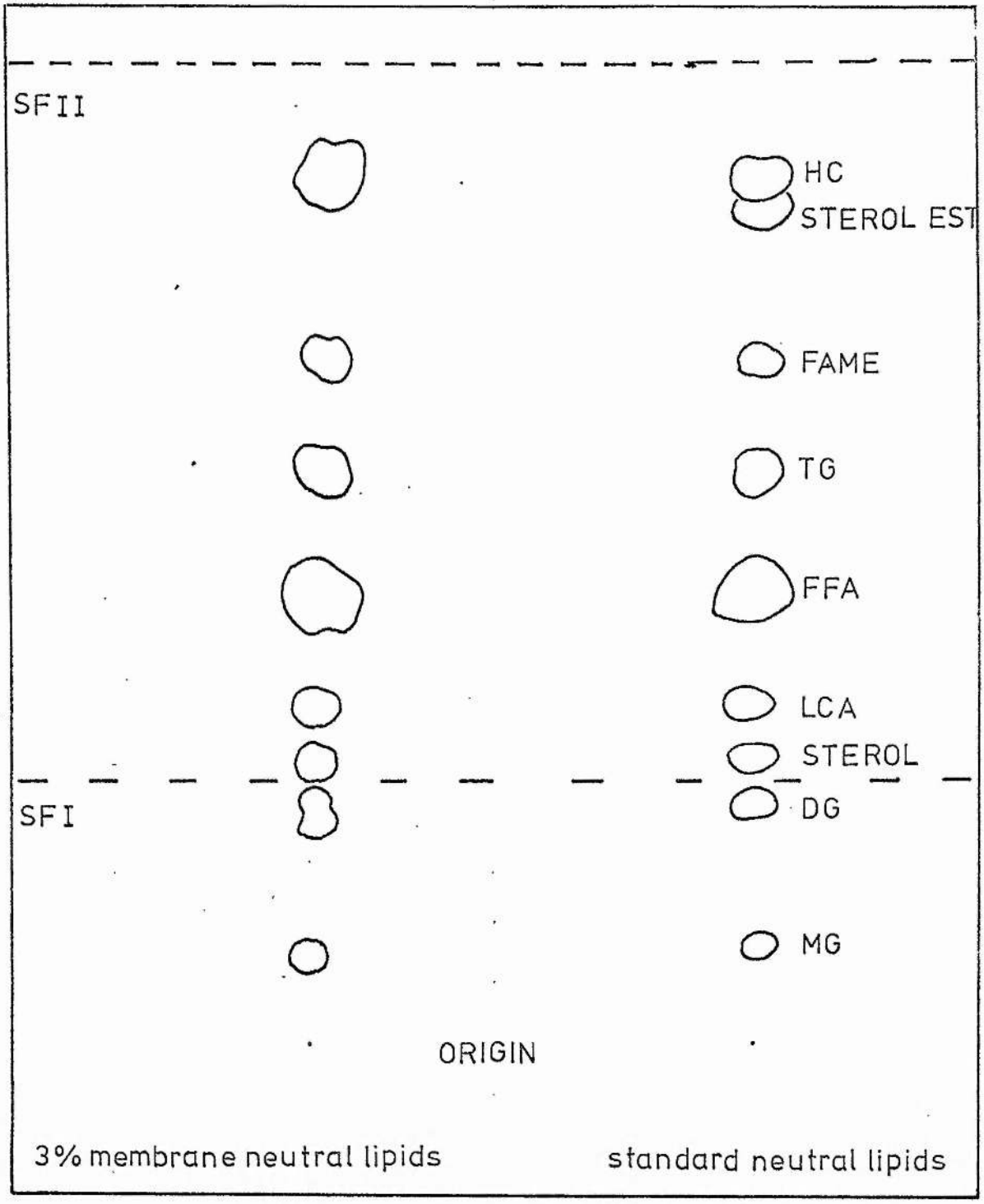
Separation of the hydrocarbon fraction and identification of isoprenoid compounds

Hydrocarbons were separated well from the rest of the neutral lipids in the heptane/benzene (9:1 by vol) system. Alkanes and alkenes formed a spot with an R_f of 0.7 and isoprenoid compounds had an R_f of 0.45. All other components remained around the origin with an R_f of less than 0.1. Alkanes and alkenes were present in the membrane neutral lipids in very small quantities and were not detected with iodine vapour but appeared as a faint grey spot after charring with 50% H_2SO_4. An isoprenoid compound with an R_f identical to that of squalene was present in large quantities.

Preparative separation and quantitative analysis of neutral lipid components

Nine bands were removed from the TLC plate after separation in the 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

Fig. R12: TLC separation of neutral lipids from the 3% membrane type



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 vol).
Detection: 50% H_2SO_4 spray.
SF I: First solvent front; SF II: Second solvent front.
HC: hydrocarbons; STEROL EST: sterol esters; FAME: fatty acid methyl esters;
TG: triglycerides; FFA: free fatty acid; LCA: long chain alcohols;
STEROL: sterols; DG: diglycerides; MG: monoglycerides.

(90:10:1 by vol). The lipid fractions were weighed and Table R17 shows the proportions of each present, expressed as a percentage of the total neutral lipid by weight.

Table R17: Distribution of neutral lipids from the three membrane types

Membrane type	Mono-glycerides	Di-glycerides	Sterol	Long chain alcohol	Free fatty acid	Tri-glycerides	Fatty acid ester	Sterol ester	Hydrocarbon
0.5%	11.9	10.3	<5	10.6	23.2	13.1	<5	<5	18.5
3.0%	11.7	6.3	<5	12.2	17.8	14.5	<5	<5	24.5
10.0%	6.0	14.8	<5	14.0	29.8	16.3	<5	<5	11.2

Any percentage recorded as < 5% indicates that the error in weighing the quantities involved was too great to give a more accurate figure.

Preparative separation of the hydrocarbons

Quantitative estimations of the amounts of non-isoprenoid hydrocarbons proved fruitless due to the small amount of material. The isoprenoid fraction probably accounted for over 90% of the hydrocarbon fraction in all three lipid types.

Sterol determination

Neutral lipid separations in heptane/benzene (9:1 by vol) and in light petroleum B.P. 60°-80°/diethyl ether/acetic acid (70:30:1 by vol) were carried out and when chromatograms were sprayed with antimony trichloride or sulphuric/acetic acid reagents, sterol was not always detected. A positive reaction was obtained on several occasions in the area where sterol esters would be expected to be found; however, no positive reaction was recorded among the more polar compounds, which indicated the absence of free sterol. Sterol sprays sometimes give positive results with branched unsaturated lipids and it is probable that the isoprenoid compounds in the hydrocarbons were mistaken for sterol esters.

Digitonide precipitation

This procedure was carried out on the possible sterol and sterol ester fractions obtained by preparative TLC as well as a sample of total neutral lipid. The sterol ester fraction was first saponified to release any free sterol. No precipitate was observed with any of the samples, indicating the absence of 3 β hydroxy sterols in the neutral lipids. It is therefore unlikely that sterols are present.

GLC analysis

All the major neutral lipid fractions obtained by preparative TLC were analysed by GLC with or without prior modification (see Methods). Fatty acids from the glycerides and free fatty acids were methylated and the fatty acid compositions for each of these components are shown in Figs. R13 - R24. The major fatty acid in the mono- and di-glycerides was shown to be a branched C_{15} saturated acid with an apparent carbon number of 14.7. This usually constituted between 45% and 70% of the total acids. Other major acids present were C_{16}, mono-unsaturated C_{16}, C_{18} and mono-unsaturated C_{18}. The triglycerides in all three lipid types (0.5%, 3% and 10%) differed from the mono- and di-glycerides as they have a fatty acid profile without a single predominant acid. The free fatty acids have a composition similar to that of the mono- and di-glycerides.

Use of Boron tri-fluoride-methanol complex as a methylating agent

Boron trifluoride was used initially as a methylating agent for fatty acids. A control esterification run involving reagents only, revealed a large number of peaks when analysed by GLC. These peaks were artifacts and were indistinguishable from normal fatty acid peaks. They occupied a region where fatty acids in the range C_{10} - C_{17} usually occur. The artifact peaks were probably due to the use of reagent which was old and which was used in quantities in excess of those required.

Notes for fatty acid histograms

Figures R13 - R24, R27 - R33 and R35 - R49 show the amount of each fatty acid as a percentage of the total fatty acids.

Numbers along the bottom indicate the carbon chain length obtained from a 'James plot'.

:1 and 'br' after the carbon chain number denote monounsaturated and branched acids respectively.

* denotes acids thought to be both branched and unsaturated.

0% values denote acids which were considered to be present in trace quantities, i.e. less than 1% of the total fatty acids.

Figs. R13, R14, and R15: Fatty acid composition of the monoglyceride fractions obtained from the 0.5%, 3% and 10% membrane types respectively

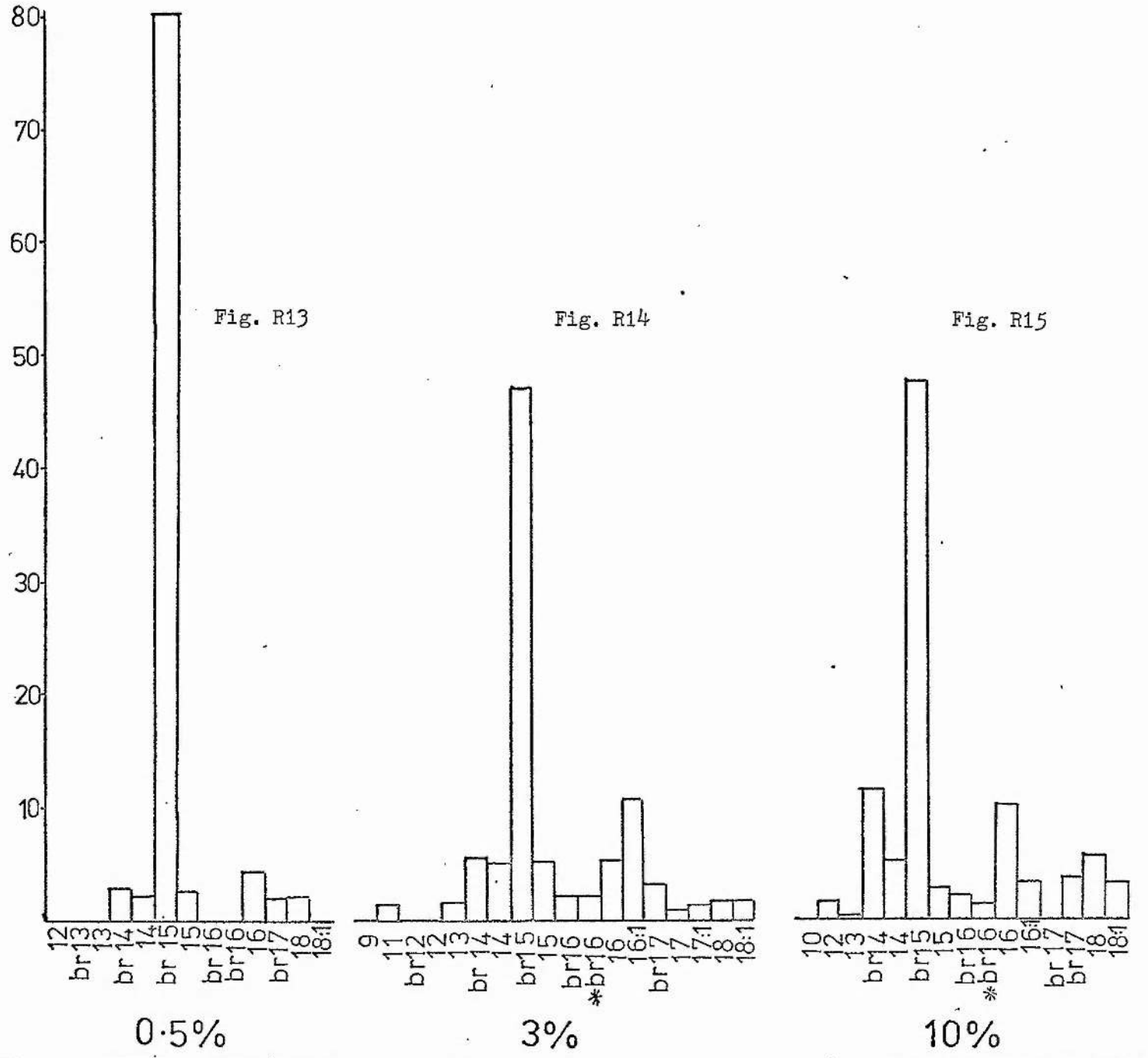
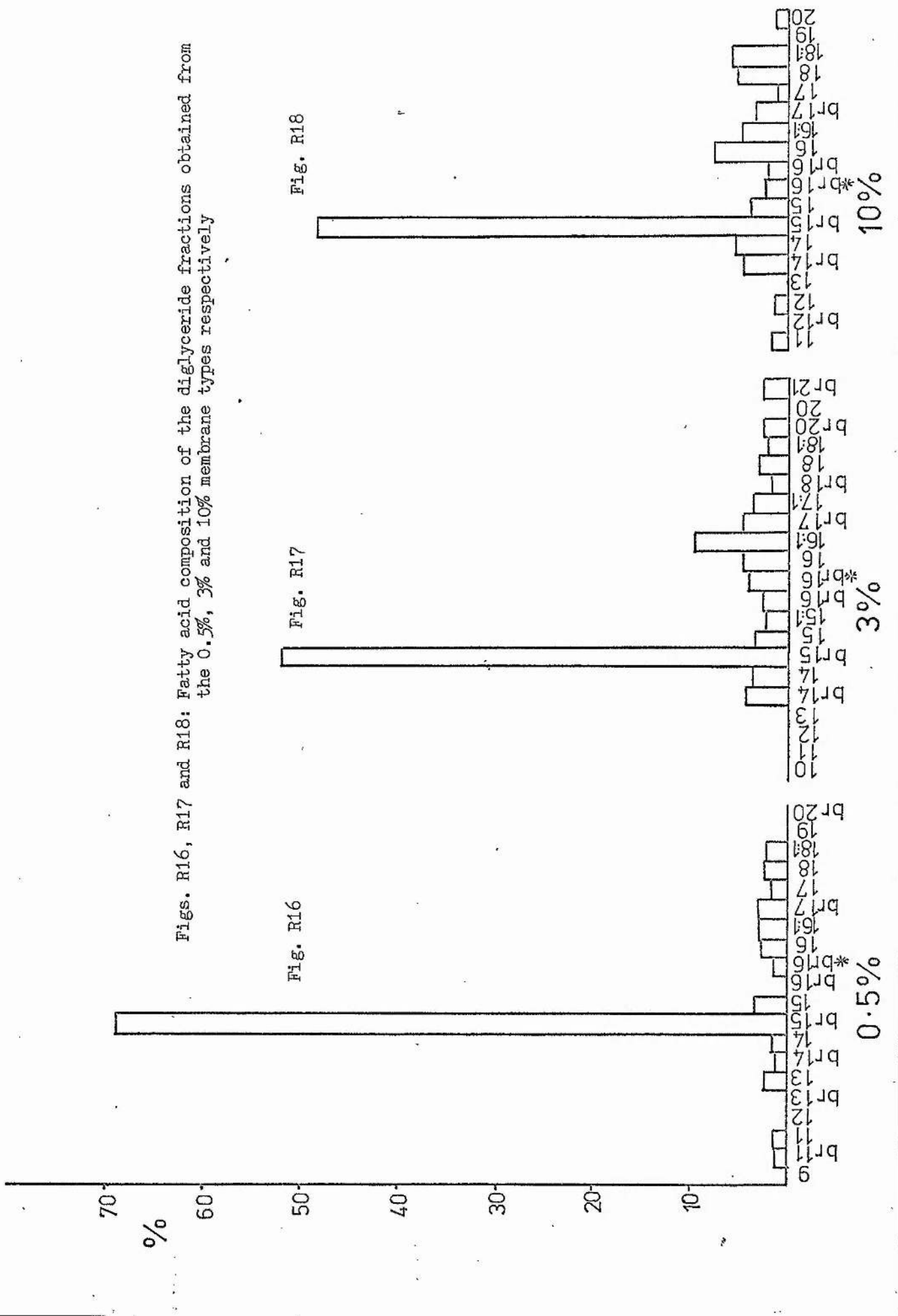


Fig. R13

Fig. R14

Fig. R15



Figs. R16, R17 and R18: Fatty acid composition of the diglyceride fractions obtained from the 0.5%, 3% and 10% membrane types respectively

Figs. R19, R20 and R21: Fatty acid composition of the triglyceride fractions obtained from the 0.5%, 3% and 10% membrane types respectively

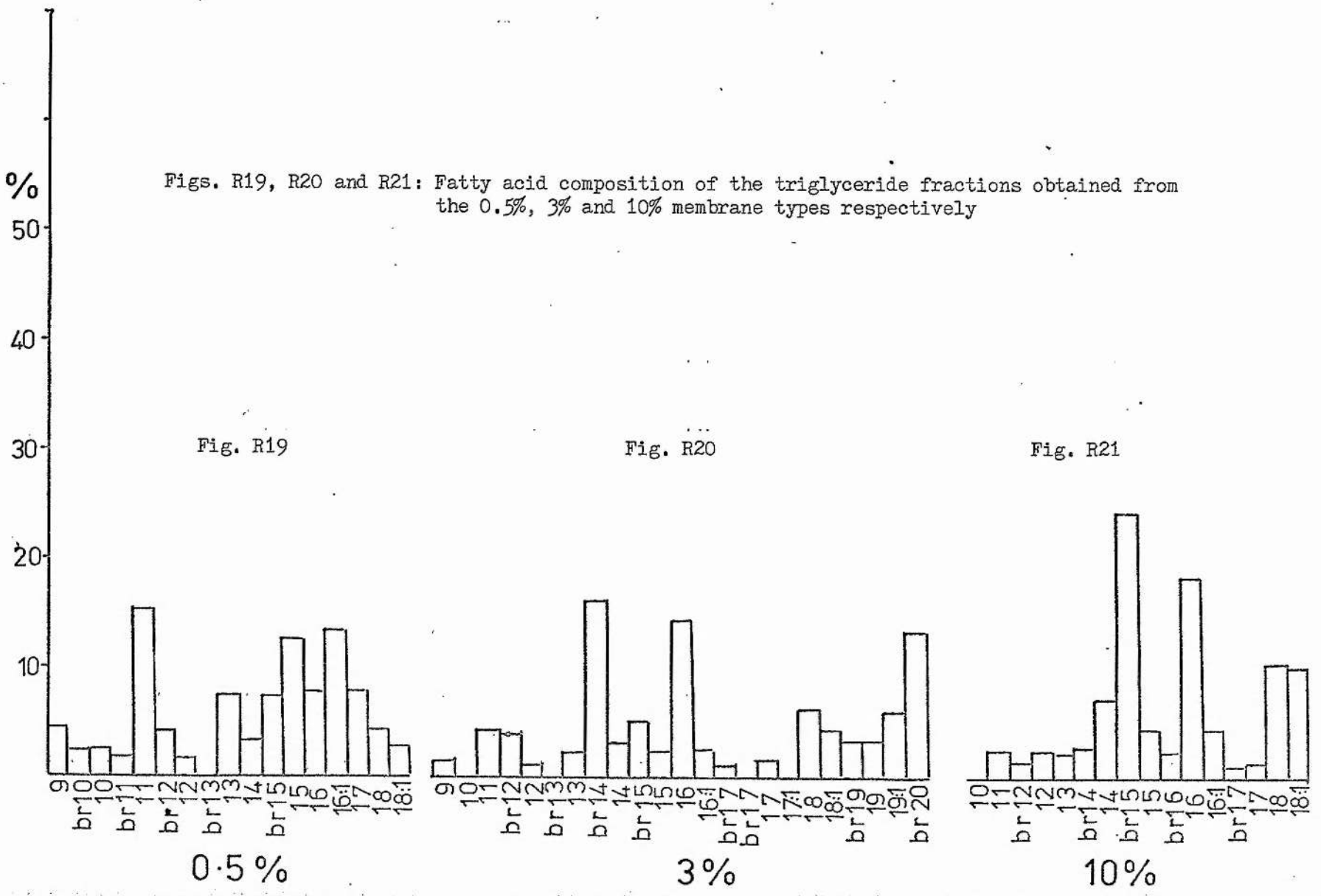
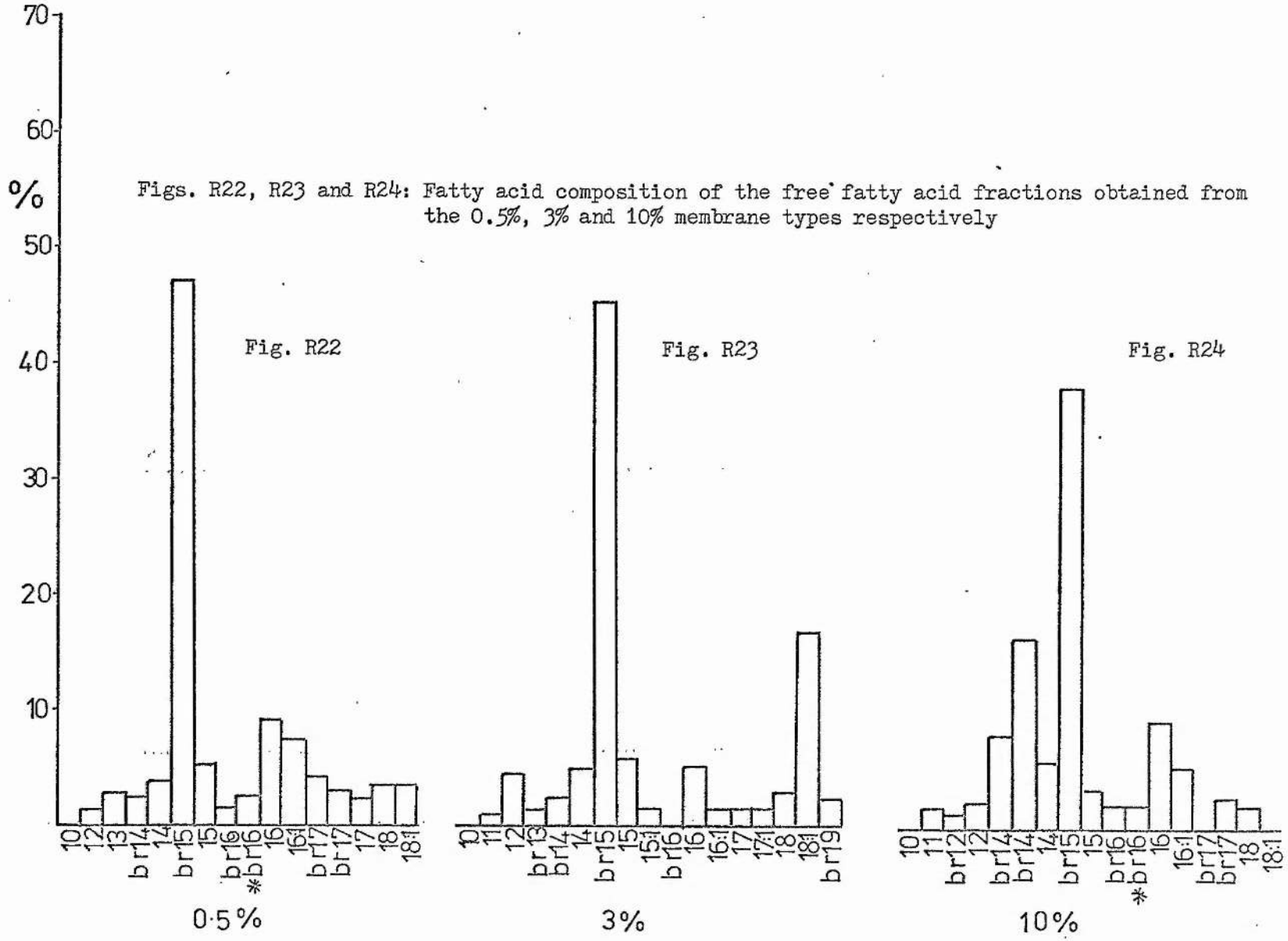


Fig. R19

Fig. R20

Fig. R21

Figs. R22, R23 and R24: Fatty acid composition of the free fatty acid fractions obtained from the 0.5%, 3% and 10% membrane types respectively



GLC analysis of non-isoprenoid hydrocarbons

Table R18 shows the apparent carbon numbers and percentage peak areas for the non-isoprenoid hydrocarbons. On hydrogenation, only peak 14 disappeared and peak 12 increased in size indicating peak 14 was unsaturated.

Table R18: GLC analysis of non-isoprenoid hydrocarbons

Peak No..	Apparent Carbon No.	% of total peak area Membrane type 0.5%	3.0%	10.0%
1	17.50	tr	tr	tr
2	18.50	4.1	tr	tr
3	19.00	16.2	20.0	19.4
4	19.25	2.6	tr	tr
5	20.75	tr	2.3	3.8
6	21.25	4.4	2.6	5.4
7	21.75	tr	3.0	tr
8	22.75	5.2	3.3	3.1
9	23.75	4.8	6.1	7.2
10	25.00	12.4	10.0	13.4
11	25.50	2.0	3.0	4.2
12	26.00	19.2	16.4	18.7
13	27.00	8.0	11.3	7.4
14	28.00	20.1	21.0	17.2

tr denotes a percentage less than 1%.

Peak 14 was found to co-chromatograph with authentic squalene (see below) and on hydrogenation was converted to a product having the same retention time as peak 12 which co-chromatographed with perhydro squalene (squalane), the fully saturated parent compound.

The presence of squalene in small amounts in the non-isoprenoid fraction may be due to an association of squalene with alkane material. Cross contamination is unlikely to have taken place during preparative TLC because the bands were so well separated.

GLC analysis of isoprenoid compounds

GLC separation of the isoprenoid fraction showed only one peak. This peak co-chromatographed with authentic squalene on a 3% SE30 column at both 180° and 230° and, after catalytic hydrogenation, the products co-chromatographed with authentic perhydro squalene. Partial hydrogenation of the fraction yielded the five possible intermediates as well as the original and final compounds, indicating partial saturation of the double bonds. It seems almost certain therefore that the major component of the hydrocarbons is squalene, but small quantities of other hydrocarbons are also present. Comparison with straight chain saturated hydrocarbon standards showed that squalene and perhydro squalene have apparent carbon numbers of 28.0 and 26.0 respectively.

GLC analysis of long-chain alcohols

Table R19 shows the distribution of apparent carbon chain lengths within the long-chain alcohol fractions. The two main alcohols appear to be hexadecanol and one with an apparent carbon number of 12.2. Increasing salt in the medium causes an increase in the amounts of the $C_{12.2}$ component at the expense of the hexadecanol. The long-chain alcohol fraction was not hydrogenated so the unsaturated compounds were not distinguished from the saturated.

Table R19: GLC analysis of the long-chain alcohols.

Peak No.	Apparent Carbon No.	% of total peak area Membrane type 0.5%	3.0%	10.0%
1	7.7	tr	tr	tr
2	9.0	tr	tr	tr
3	9.6	1.6	tr	tr
4	10.0	1.4	tr	tr
5	10.75	3.6	3.4	tr
6	11.7	tr	tr	tr
7	12.2	11.1	21.3	39.0
8	12.7	tr	tr	tr
9	13.2	tr	6.2	3.5
10	13.9	tr	5.0	4.0
11	14.3	4.8	8.0	8.4
12	15.1	7.9	8.2	8.4
13	15.4	tr	tr	5.1
14	15.6	6.3	7.9	15.3
15	16.0	43.8	28.7	10.2
16	16.2	19.1	10.4	5.1

tr denotes percentage of less than 1%.

Glycolipids

The glycolipid fractions from the silicic acid column were pooled. The methanol fraction was kept separate because, although it contained some glycolipids, phospholipids were also present.

Quantitative comparison of the total carbohydrate content of the glycolipid-containing fractions

The results are seen in Table R20.

Table R20: Percentage carbohydrate in glycolipid-containing fractions.

	Glycolipid fraction	% sugar
Membrane type	0.5%	8.5
	3.0%	5.7
	10.0%	3.7
	Methanol fraction	
Membrane type	0.5%	4.1
	3.0%	2.5
	10.0%	3.0

The percentage of sugar in the glycolipids decreased with increasing salt in the culture medium.

Quantitative comparison of the carbohydrate content of total and defatted membranes

The results are shown in Table R21.

Table R21: Percentage of carbohydrate in whole and defatted membranes.

	Total Membrane	% sugar expressed as % of total membrane
Membrane type	0.5%	2.8
	3.0%	6.3
	10.0%	5.0
	Defatted Membranes	
Membrane type	0.5%	2.1
	3.0%	6.1
	10.0%	4.9

By expressing the sugar in the defatted membranes as a percentage of the total membrane, it is evident that the majority of membrane sugar is not present as extractable glycolipid, and is perhaps associated with proteins.

Comparison of the phosphorus content of the lipid fractions

The results in Table R22 express phosphorus as a percentage of the total lipid phosphorus.

Table R22: Distribution of phosphorus in the membrane lipid fractions.

Membrane type		%
0.5%	Glycolipid	0.8
	Methanol	0.27
	Acetone-insoluble	98.93
3.0%	Glycolipid	0.32
	Methanol	1.38
	Acetone-insoluble	98.3
10.0%	Glycolipid	0.27
	Methanol	0.90
	Acetone-insoluble	98.83

Since the neutral lipid contains no phosphorus and the glycolipid and methanol fractions contain very little, this indicates the efficiency of the acetone precipitation procedure.

Thin-layer chromatographic separation of glycolipids

Separations were carried out in chloroform/methanol (9:1 by vol) and Fig. R25 shows the typical separation achieved. The methanol fractions gave identical separations and the same number of spots when sprayed with diphenylamine. Spots (a), (b) and (c) were phospholipid contaminants and (b) and (c) had similar R_fs to lysocardiolipin and cardiolipin respectively. All glycolipid spots showed up with both diphenylamine and Schiffs-periodate sprays for vicinal hydroxyl groups. Spot 1 was much the strongest; all other spots except 2 and 3 were faint. Spot 3 was unusual in that it turned purple/red with diphenylamine instead of the usual blue/grey. When sprayed with copper sulphate spray for sulphur-containing glycolipids, it gave a positive reaction.

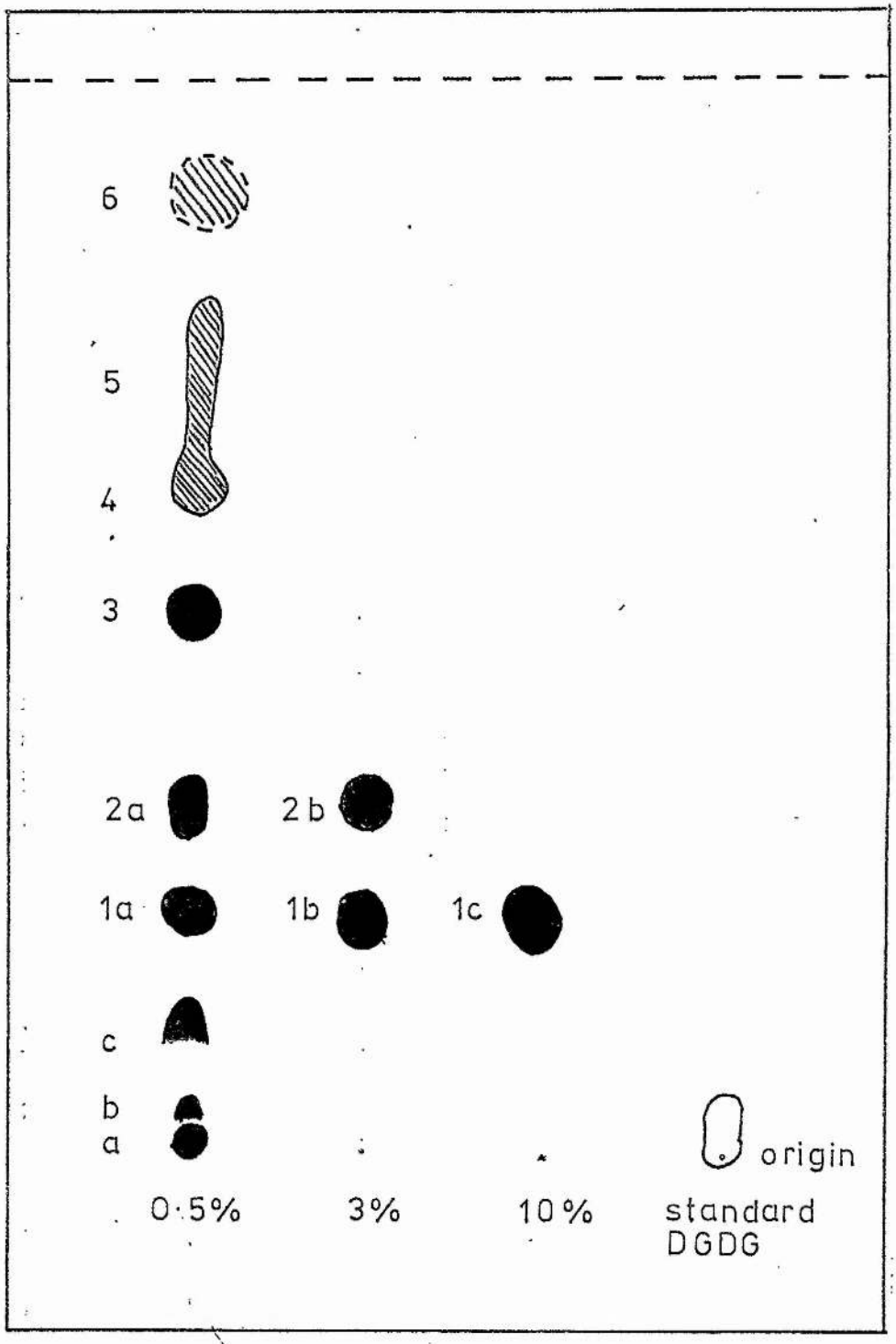


Fig. R25: TLC separation of the glycolipids from the three membrane types

Solvent: chloroform/methanol (9:1 by vol)

Detection: diphenylamine and phosphorus detecting sprays

SF: solvent front

DGDG: diglucosyl diglyceride

◌ : faint

However, in view of its unusual staining with diphenylamine and its chromatographic relationship to other lipids, no definite conclusions as to its identity were drawn.

The sugar present in spot 6 may be associated with carotenoid material and perhaps is a carotenoid glycoside complex.

Standard diglucosyl diglyceride was more polar than any of these glycolipid spots and it was concluded that only monoglycosyl diglycerides were present. No monoglycosyl diglyceride standard was available for comparison.

Fig. R25 shows an obvious decrease in the number of glycolipid types with increasing sea salt in the growth medium. This is consistent with the reduction in the overall lipid carbohydrate content already mentioned. It was observed in these TLC separations that as the amount of glycolipid decreased with increasing salt, there was a corresponding rise in the amount of carotenoid in the glycolipid fractions. Separation of the glycolipid fractions in a more polar solvent system (chloroform/methanol/water, 65:25:4 by vol) showed no evidence of any sugar-containing lipids which were more polar than those already described.

Paper chromatographic analysis of glycolipid sugars

Descending paper chromatography of water-soluble glycolipid hydrolysis products gave the separation depicted in Fig. R26. After comparison of unknown spots with those of authentic sugars, the chromatogram showed that glucose was present in all three lipid types. The 0.5% type contained a pentose with a chromatographic mobility similar to that of arabinose. All three lipid types contained an unidentified polar spot which had an $R_{glucose}$ between that of glucuronic acid and N. acetyl glucosamine.

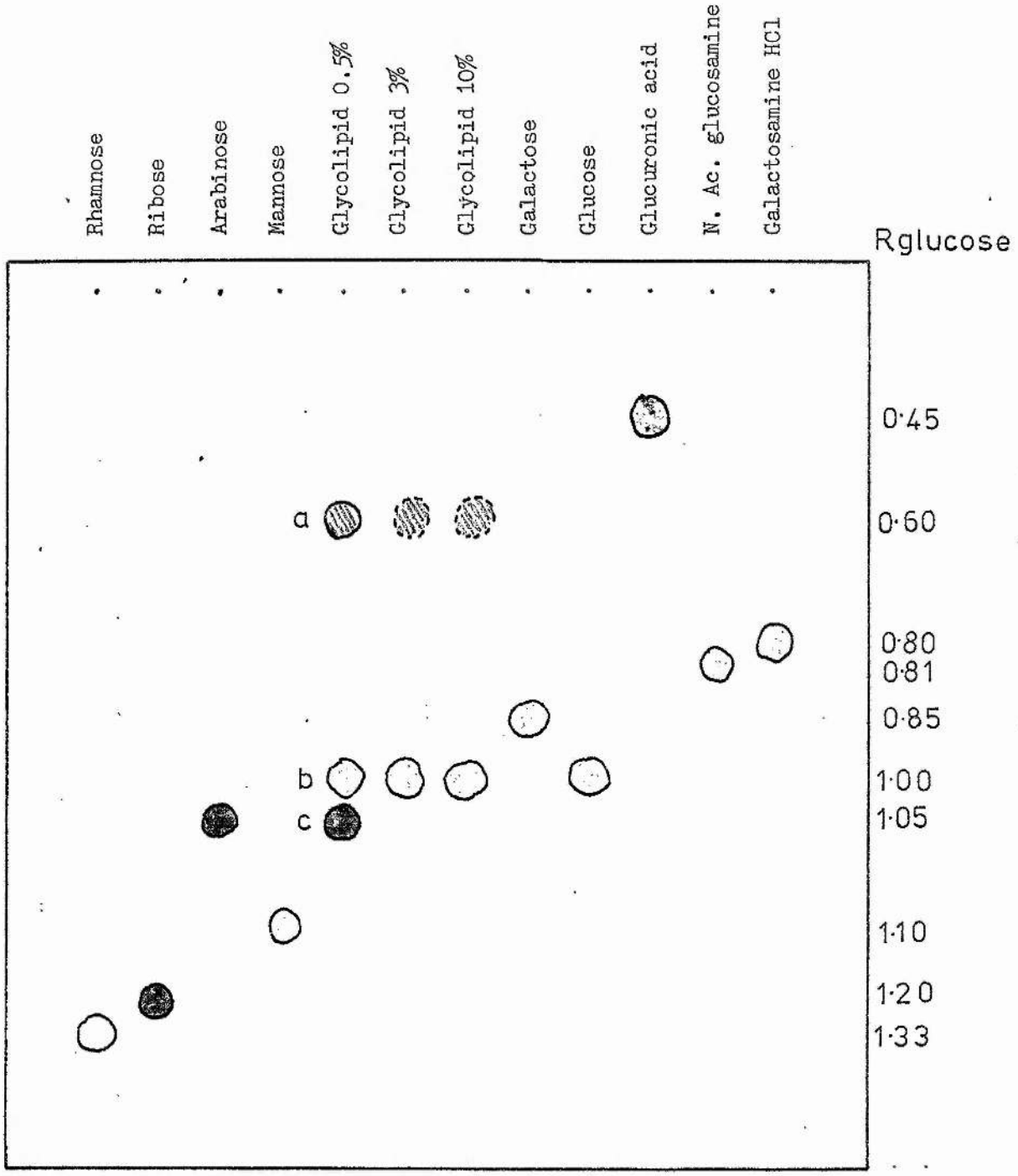


Fig. R26: Qualitative analysis by paper chromatography of the sugars obtained after hydrolysis of the glycolipids from the three membrane types

Solvent: butanol/pyridine/water (6:4:3 by vol)

Detection: anisidine phthalate spray

◌ : faint

Preparative TLC of glycolipids

Glycolipids were separated in chloroform/methanol (9:1 by vol) and the silica removed from the areas corresponding to spots 1(a), (b) and (c), 2(a) and (b), 3 and 6 on Fig. R25. Spots 4 and 5 were present in quantities too small for analysis.

Sugar analysis by GLC of individual glycolipid fractions

Interpretation of the traces obtained on GLC analysis of the silylated sugars derived from the glycolipids was difficult due to the small amounts of material available. The pentose spot (spot c, Fig. R26), which chromatographed with arabinose on paper, did not co-chromatograph with authentic arabinose on the GLC. Galactose was identified as a minor component although not detected previously on the paper chromatogram.

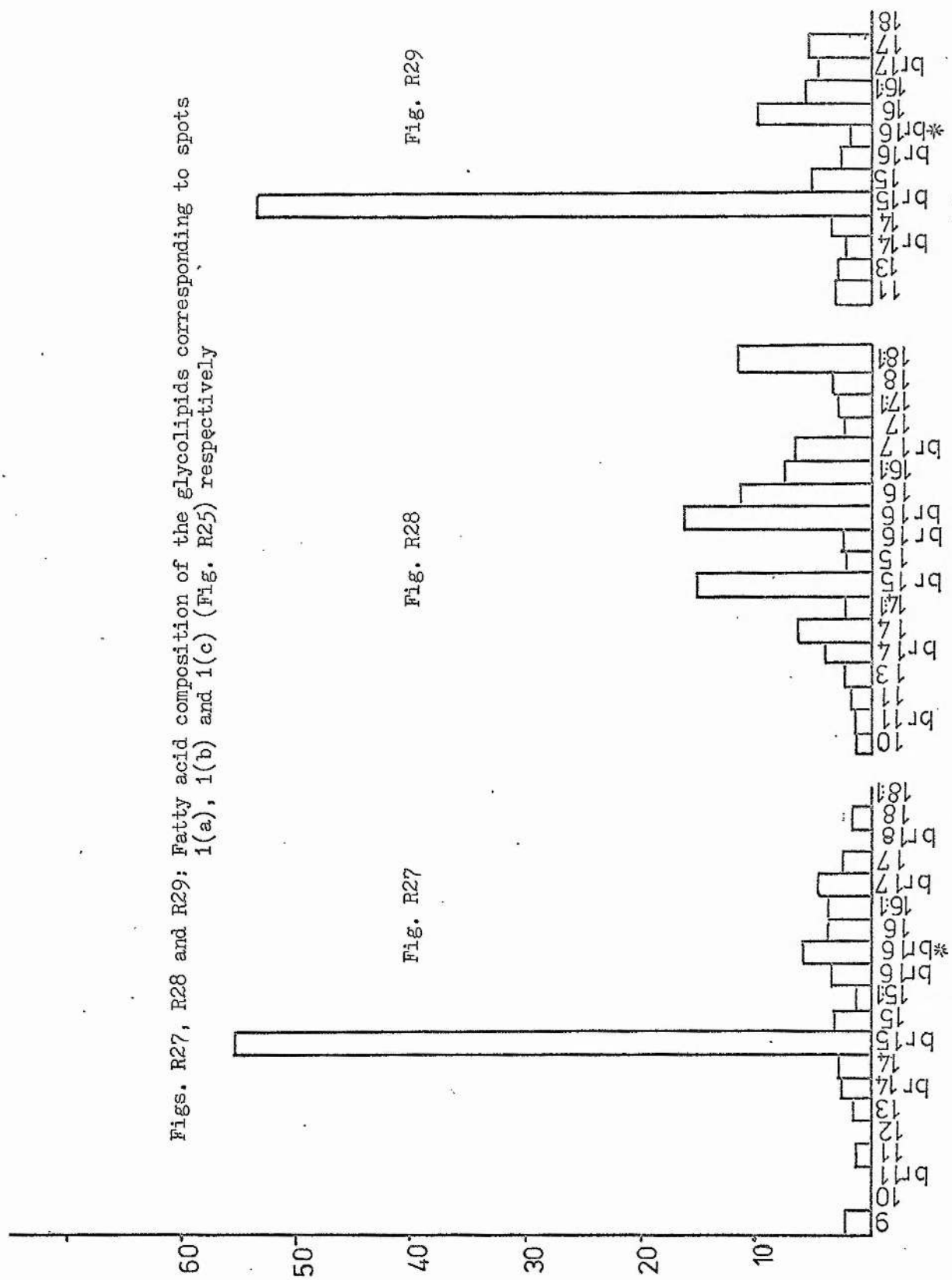
A summary of the sugars thought to be associated with the glycolipid spots is shown in Table R23.

Table R23: Proposed sugar components in glycolipids.

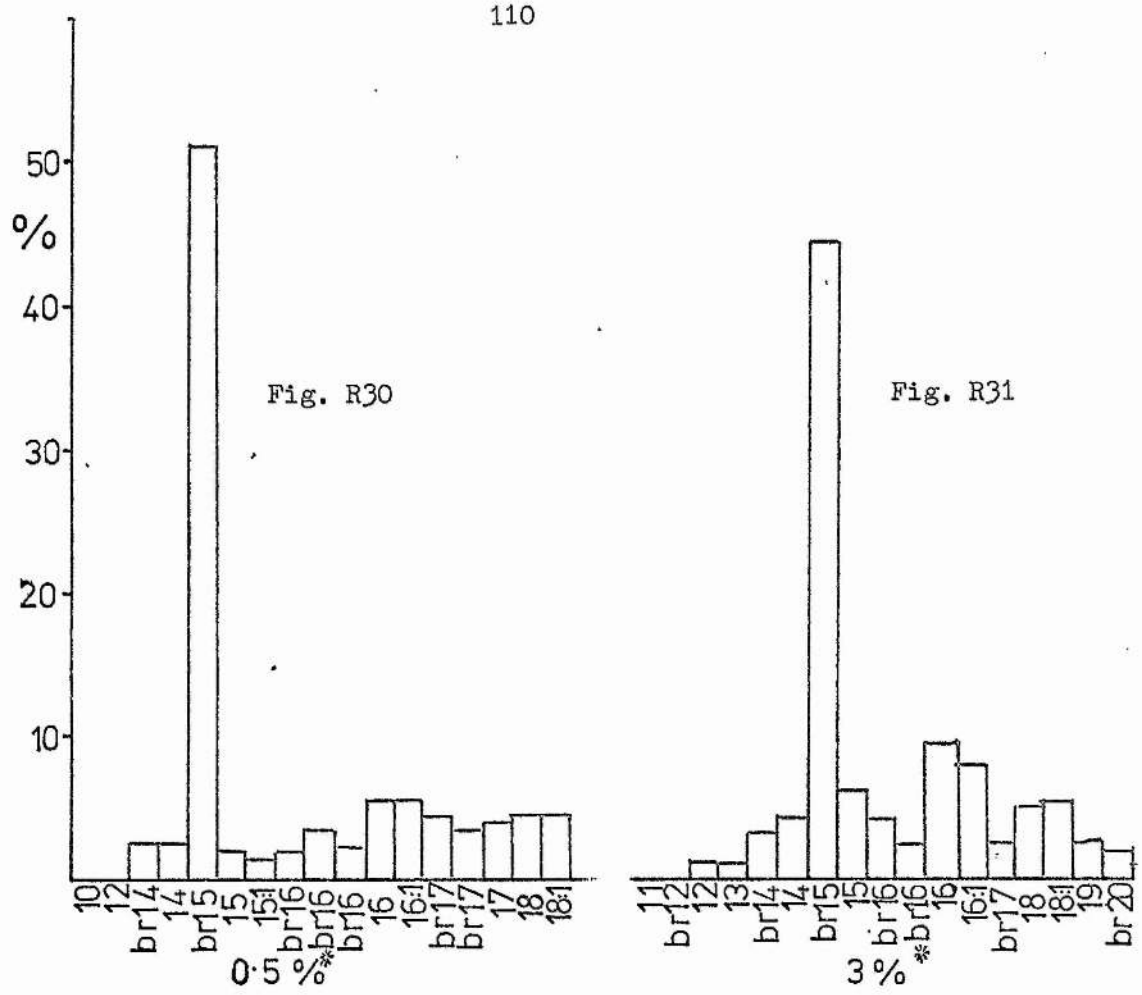
Spot on Fig. R25	Proposed sugar identity
1(a),(b) & (c)	glucose
2(a) & (b)	glucose
3	pentose
6	glucose/galactose

Fatty acid analysis

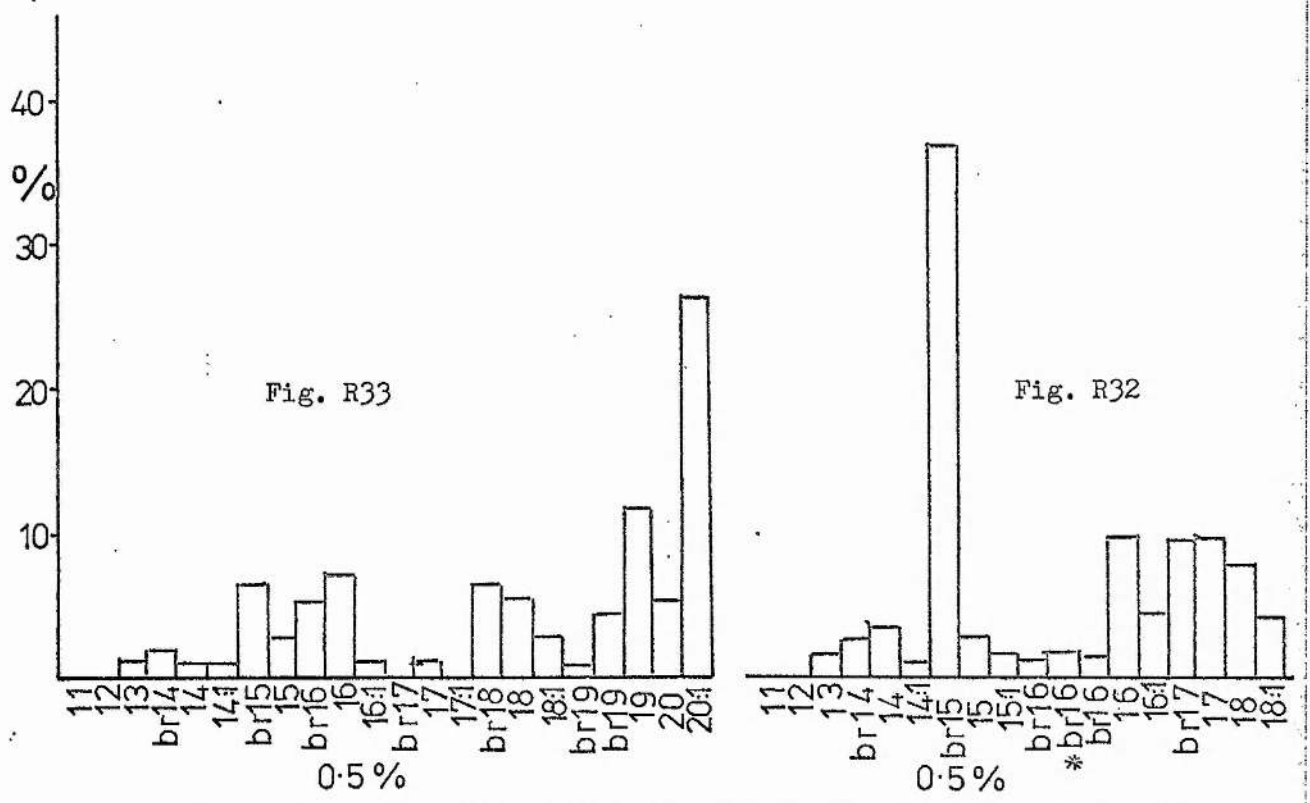
The fatty acids associated with spots 1(a),(b) and (c), 2(a) and (b), 3 and 6 were examined by GLC after methylation. The fatty acid composition of the various fractions are shown in Figs. R27 - R33. The fatty acid composition is similar to that already found for the



Figs. R27, R28 and R29: Fatty acid composition of the glycolipids corresponding to spots 1(a), 1(b) and 1(c) (Fig. R25) respectively



Figs. R30 and R31: Fatty acid composition of the glycolipids corresponding to spots 2(a) and 2(b) (Fig. R25) respectively



Figs. R32 and R33: Fatty acid composition of the glycolipids corresponding to spots 3 and 6 (Fig. R25) respectively

mono- and di-glycerides in the neutral lipid fractions. The major acid is a saturated branched C_{15} in most cases. The glycolipid spot 6, present only in the 0.5% salt type, had an unusual distribution in that the major fatty acids were of much greater chain length; C_{19} and unsaturated C_{20} were the major acids as opposed to the branched C_{15} found in most of the other fractions. The 'purple' spot (spot 3, Fig. R25) had the branched C_{15} as the major acid but the other main acids were of longer chain length.

Phospholipids

Preliminary separation of the phospholipids on SG 81 silica-loaded chromatography paper was not very efficient. The major spots were not well separated. Separation by TLC in chloroform/methanol/water (65:25:4 by vol), or with the addition of a small amount of ammonia, gave a good separation of the components which were easily stained with iodine vapour, rhodamine or phosphorus detecting reagent. A typical separation is shown in Fig. R34. Seven phosphorus-containing spots were observed in the phospholipids from each membrane type. Chromatography in another solvent system, chloroform/acetone/methanol/acetic acid/water (6:8:2:2:1 by vol) and in a two-dimensional system, (a) chloroform/methanol/water (65:25:4 by vol) and (b) chloroform/acetic acid/methanol/water (80:18:12:5 by vol), did not show the presence of any other phospholipids in addition to those already observed. As well as chromatographic comparisons with authentic standards, the phospholipids were identified by spraying with various reagents.

(a) Phosphorus spray

All spots except those around the origin (spots 1, Fig. R34) stained with phosphorus detecting reagent; spots 2, 3 and 4 were faint.

(b) Ninhydrin

Spot 6, which had an R_f similar to that of phosphatidyl ethanolamine, gave a positive reaction with this reagent. Spot 2, a minor component,

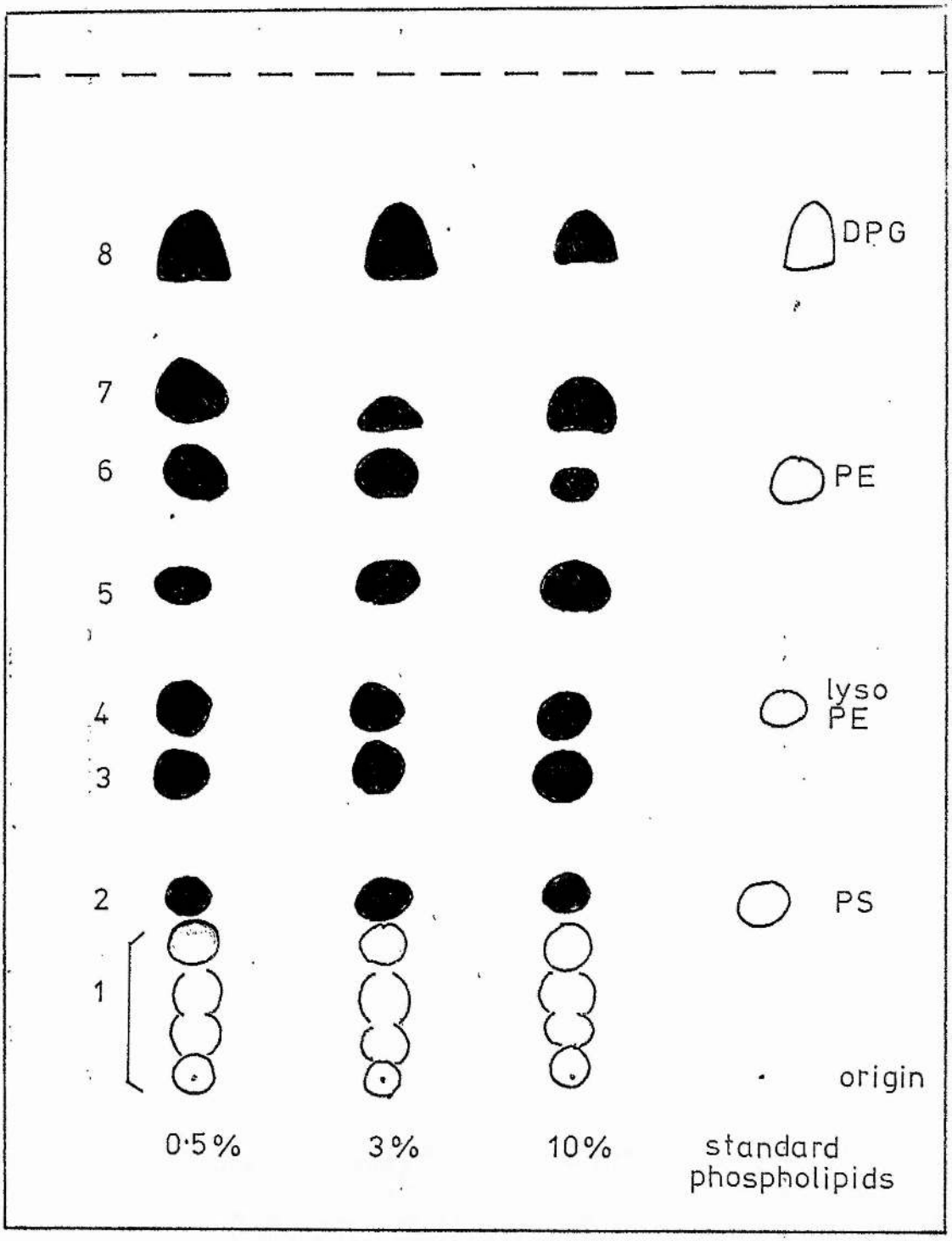


Fig. R34: TLC separation of the phospholipids from the three membrane types

Solvent: chloroform/methanol/water (65:25:4 by vol)

Detection: phosphorus detecting spray and iodine vapour

DPG: cardiolipin; PE: phosphatidyl ethanolamine; lyso PE: lyso phosphatidyl ethanolamine; PS phosphatidyl serine

SF: solvent front

also gave a positive reaction with ninhydrin and it had an R_f similar to that of phosphatidyl serine. Several spots which did not contain phosphorus but which were very polar (Spots 1) gave strong ninhydrin-positive results after spraying.

(c) Schiffs-periodate

This reagent revealed a spot with an R_f comparable to that of phosphatidyl glycerol. After spraying with Schiffs reagent, this spot appeared very quickly (as does phosphatidyl glycerol) and was purple in colour. Although no standard was available for co-chromatography, the R_f of the spot was similar to that reported in the literature for phosphatidyl glycerol (81). One other spot gave a positive reaction with Schiffs-periodate reagent. This was a minor component (spot 4, Fig. R34) which had an R_f similar to that of standard lyso-phosphatidyl ethanolamine (R_f = 0.35), which is normally Schiffs-periodate negative.

(d) Diphenylamine

This spray reagent detected only the minor phospholipid component (spot 4, Fig. R34) which reacted with Schiffs-periodate spray. Diphenylamine detects reducing sugars so it may be possible that a sugar-containing phospholipid is present in the acetone-insoluble material as a minor component.

A summary of the staining and chromatographic properties of the acetone-insoluble lipids from the membrane types is shown in Table R24.

Table R24: Characteristics and possible identity of the phospholipid components from the three membrane types.

Spot No. Fig. R34	R_f*	Phos-phorus	NH_2	Vicinal OH	Sugar	I_2 vapour	Tentative identity
1	0-0.13	-	+	-	-	-	Peptido lipids
2	0.18	+	+	-	-	+	Phosphatidyl serine
3	0.30	+	-	-	-	+	?
4	0.36	+	-	+	+	+	Sugar/ phospholipid
5	0.48	+	-	+	-	+	Phosphatidyl glycerol
6	0.60	+	+	-	-	+	Phosphatidyl ethanolamine
7	0.66	+	-	-	-	+	Lysocardiolipin
8	0.83	+	-	-	-	+	Cardiolipin

* in chloroform/methanol/water, 65:25:4 by vol.

Detection of ether-linked lipids

Chromatography of the non-saponifiable fraction in the acetone-insoluble fraction, by TLC in chloroform/methanol/water (65:25:4 by vol) and spraying with phosphorus-detecting reagent showed no spots, and it was assumed that no ether-linked material was present.

Quantitative analysis of acetone-insoluble components

Quantitative analysis was carried out as follows:

1. Phosphorus determination

The results shown in Table R25 are the average of two separate estimations carried out using the method of Allen (78) and are expressed as a percentage of the total lipid phosphorus.

Table R25: Distribution of total phosphorus among the membrane phospholipids.

Lipid	% Phosphorus Membrane type 0.5%	3.0%	10.0%
Spots 1 - 4, Fig. R34	20.0	9.1	10.5
Phosphatidyl glycerol	9.4	15.2	7.0
Phosphatidyl ethanolamine	16.3	9.3	12.6
Lysocardiolipin	23.8	7.5	41.1
Cardiolipin	30.4	58.9	28.8

The minor components were pooled (spots 1 - 4, Fig. R34) as it would have been impractical to have determined their phosphorus contents individually.

The moles % of each phospholipid is shown in Table R26 and was calculated assuming only cardiolipin and lysocardiolipin to have two phosphorus atoms per molecule.

Table R26: The moles % of each phospholipid in the total phospholipid from the three membrane types (phosphorus determination).

	% moles Membrane type		
Lipid	0.5%	3.0%	10.0%
Spots 1 - 4, Fig. R34	27.4	13.6	16.1
Phosphatidyl glycerol	13.0	22.7	10.7
Phosphatidyl ethanolamine	22.3	13.9	19.3
Lysocardiolipin	16.3	5.6	31.5
Cardiolipin	20.8	44.0	22.1
% moles of cardiolipins in acetone-insoluble material	37.1	49.6	53.6

Peptido-lipids are not included in these calculations because, although present, they make no contribution to the phosphorus content. The table shows an overall increase in the amount of cardiolipins (lyso-cardiolipin + cardiolipin) as the salt type increases from 0.5% to 10%. There is some evidence that lysocardiolipin does not exist within the membrane. Fatty acids may be enzymatically cleaved from cardiolipin during storage of the cells after harvesting. It is interesting to note that the 3% salt type lipids have the lowest amount of lyso-cardiolipin, the highest amount of cardiolipin and the lowest amount of free fatty acids in the neutral lipid fractions when compared with the 0.5% and 10% salt types.

2. Quantitative analysis by weight

The results, an average of two separations and weighings, are expressed as a percentage of total weight, in Table R27.

Table R27: The percentage by weight of each lipid in the acetone-insoluble material from the three membrane types.

Lipid	% by weight Membrane type 0.5%	3.0%	10.0%
Peptido-lipids	3.85	5.9	6.5
Spots 2 - 4, Fig. R34	10.8	5.8	9.9
Phosphatidyl glycerol	9.95	9.1	8.4
Phosphatidyl ethanolamine	16.8	12.5	12.8
Lysocardiolipin	25.7	5.3	36.7
Cardiolipin	32.7	61.2	25.4
% cardiolipins	58.4	66.5	62.1

The moles % of each phospholipid were estimated by assuming that cardiolipin and lysocardiolipin were twice the molecular weight of the other phospholipids. Results are shown in Table R28 and are similar to those obtained by the phosphorus determination.

Table R28: The moles % of each phospholipid in the total phospholipid from the three membrane types (weight determination).

Lipid	% moles Membrane type 0.5%	3.0%	10.0%
Spots 2 - 4, Fig. R34	16.2	9.5	15.9
Phosphatidyl glycerol	14.8	15.0	13.5
Phosphatidyl ethanolamine	25.2	20.6	20.6
Lysocardiolipin	19.2	4.2	29.4
Cardiolipin	24.4	50.4	20.4
% Cardiolipins	43.6	54.6	49.8

Confirmation of the identity of spot 7 (Fig. R34) by quantitative analysis of the molar ratios of phosphorus, glycerol and fatty acids

Spot 7 was a major component in the 0.5% and 10% type phospholipids; its R_f was in the region of that expected for authentic lysocardiolipin, and its negative reaction with other spray reagents, except that of phosphorus, left its identity unconfirmed. A sufficient quantity of spot 7 was obtained by preparative TLC and a sample re-chromatographed to ensure homogeneity. Quantitative estimations of the amount of phosphorus, glycerol and fatty acids were performed. Molar ratios were obtained for spot 7 and for a sample of authentic cardiolipins as a comparison (no standard lysocardiolipin was available) and these ratios are shown in Table R29.

Table R29: Molar ratios of phosphorus, fatty acid and glycerol in spot 7 and cardiolipin.

	Cardiolipin	Spot 7	Expected for lysocardiolipin
Phosphorus	2	2	2
Fatty acid	4	3	3
Glycerol	3	3	3

The results confirm that spot 7 is lysocardiolipin.

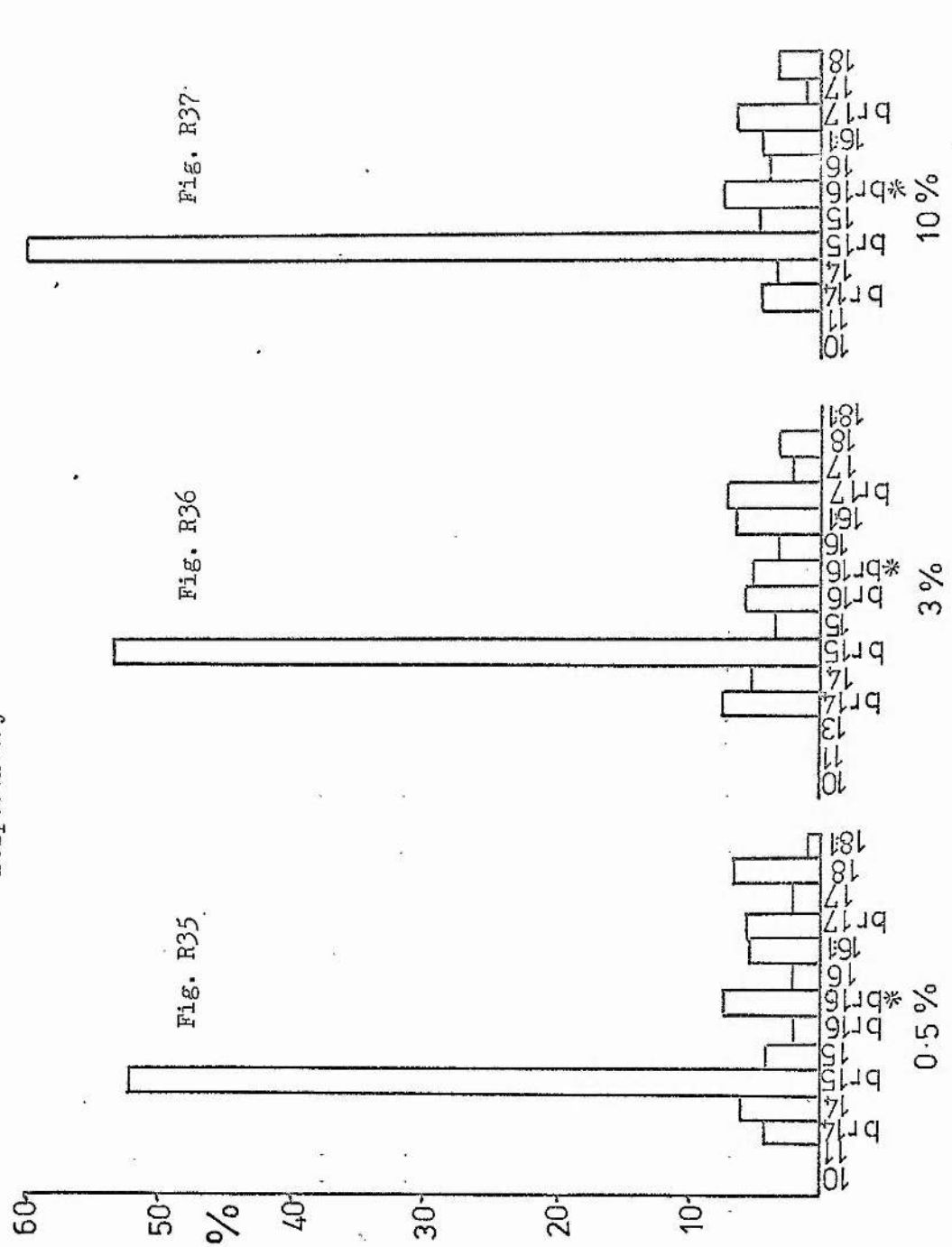
GLC analysis of phospholipid fatty acids

Phospholipids in a pure state were obtained by preparative TLC. After methylation, the fatty acids were analysed by GLC. The fatty acid compositions of the various phospholipids are shown in Figs. R35 - R49. The major fatty acid was again shown to be a branched C_{15} saturated acid in all fractions and the overall pattern was similar to that found in the mono- and di-glycerides (neutral lipid section).

Table R30 shows the variation in the percentage of unsaturated and branched acids in the phospholipids obtained from the 0.5%, 3% and 10% type membranes.

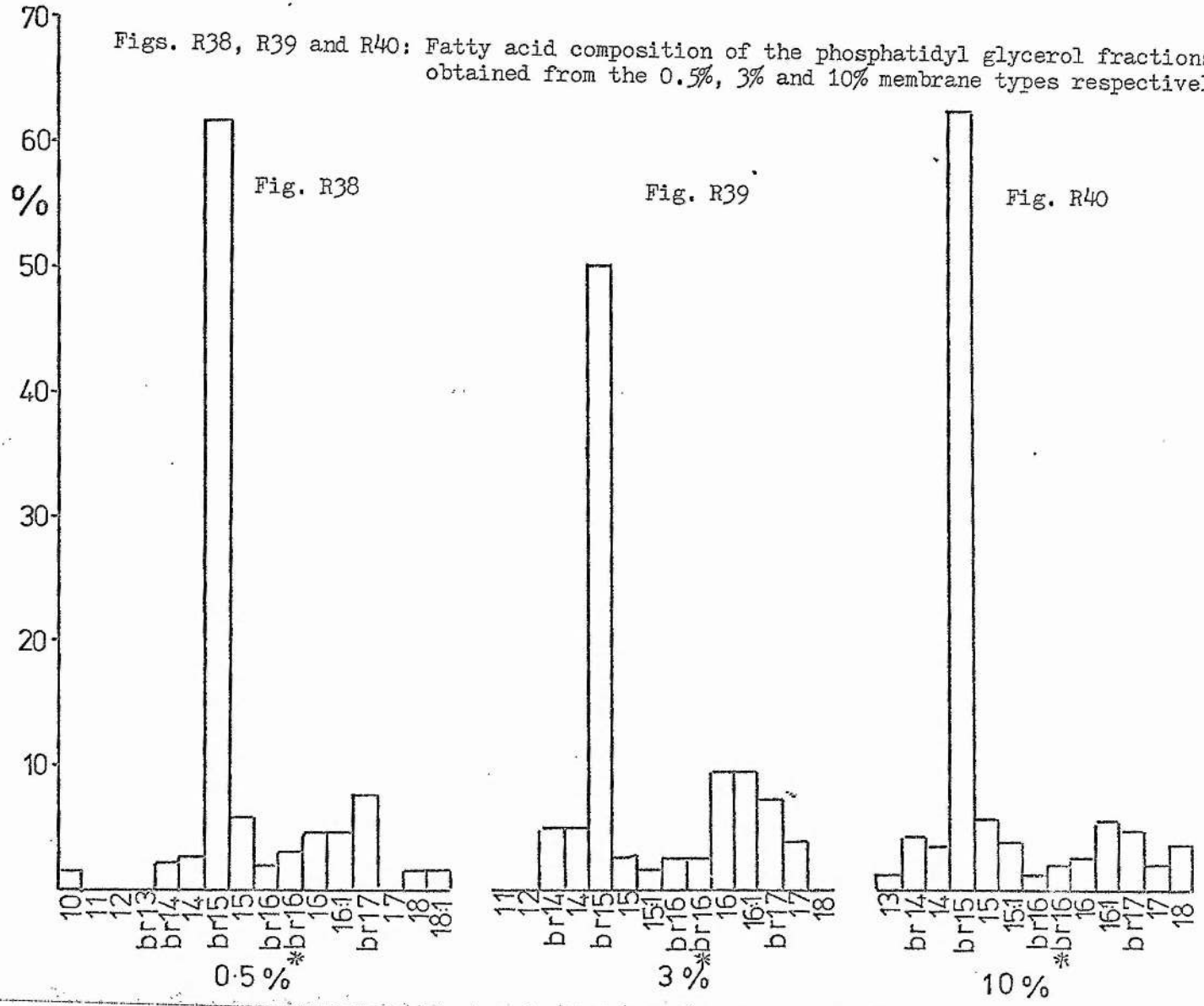
Table R30: Percentage of unsaturated and branched acids in the phospholipids.

	% Unsaturated acids Membrane type			% Branched acids Membrane type		
Lipid	0.5%	3.0%	10.0%	0.5%	3.0%	10.0%
Cardiolipin	8.7	16.1	9.9	79.8	63.6	77.9
Lyso-cardiolipin	11.2	19.2	13.1	85.8	67.1	78.6
Phosphatidyl ethanolamine	11.2	12.3	12.0	75.5	67.8	68.9
Phosphatidyl glycerol	8.8	14.0	11.1	76.0	67.6	74.7
Spots 2 - 4 Fig. R34	13.9	11.0	11.8	71.1	77.0	77.7

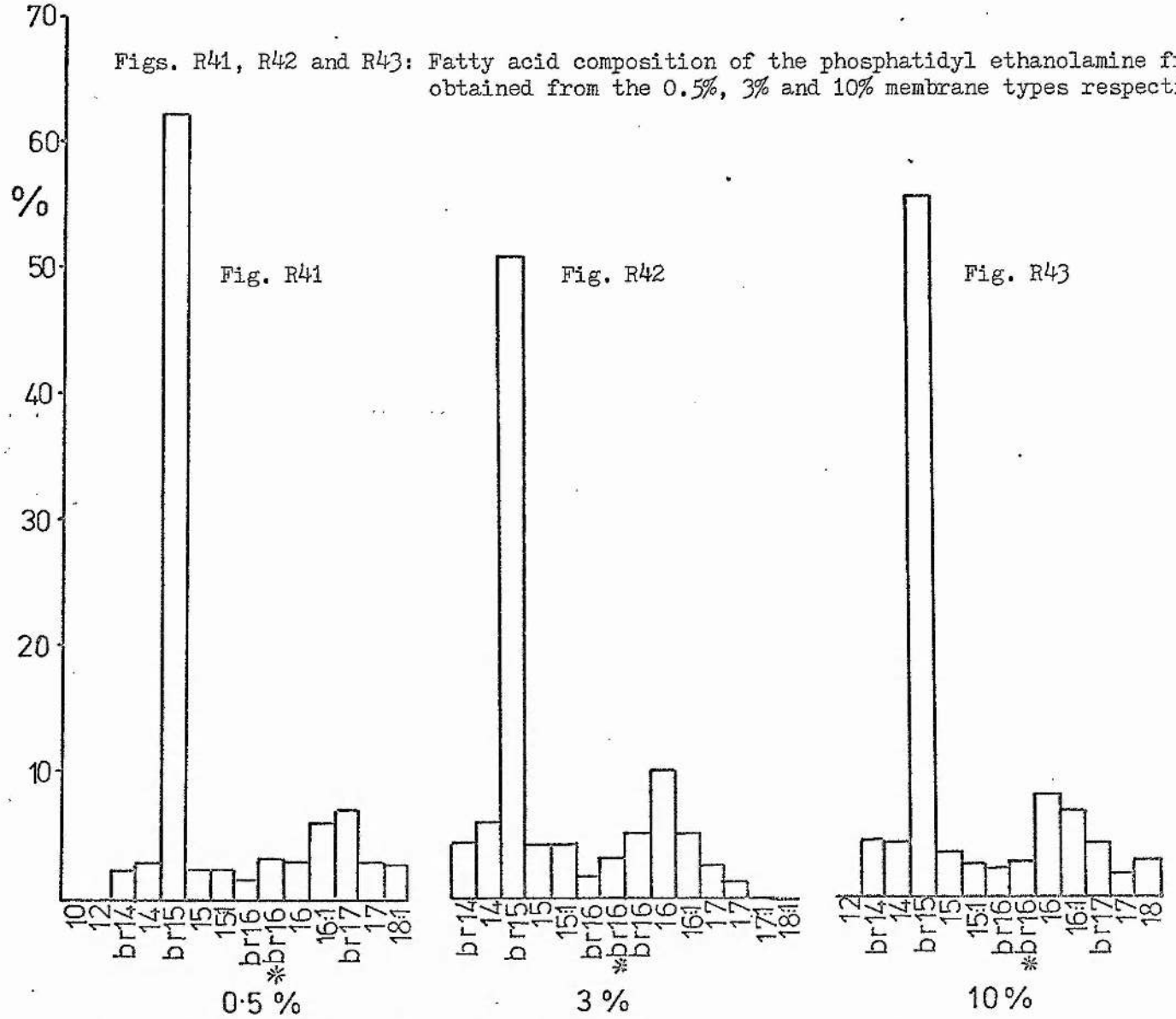


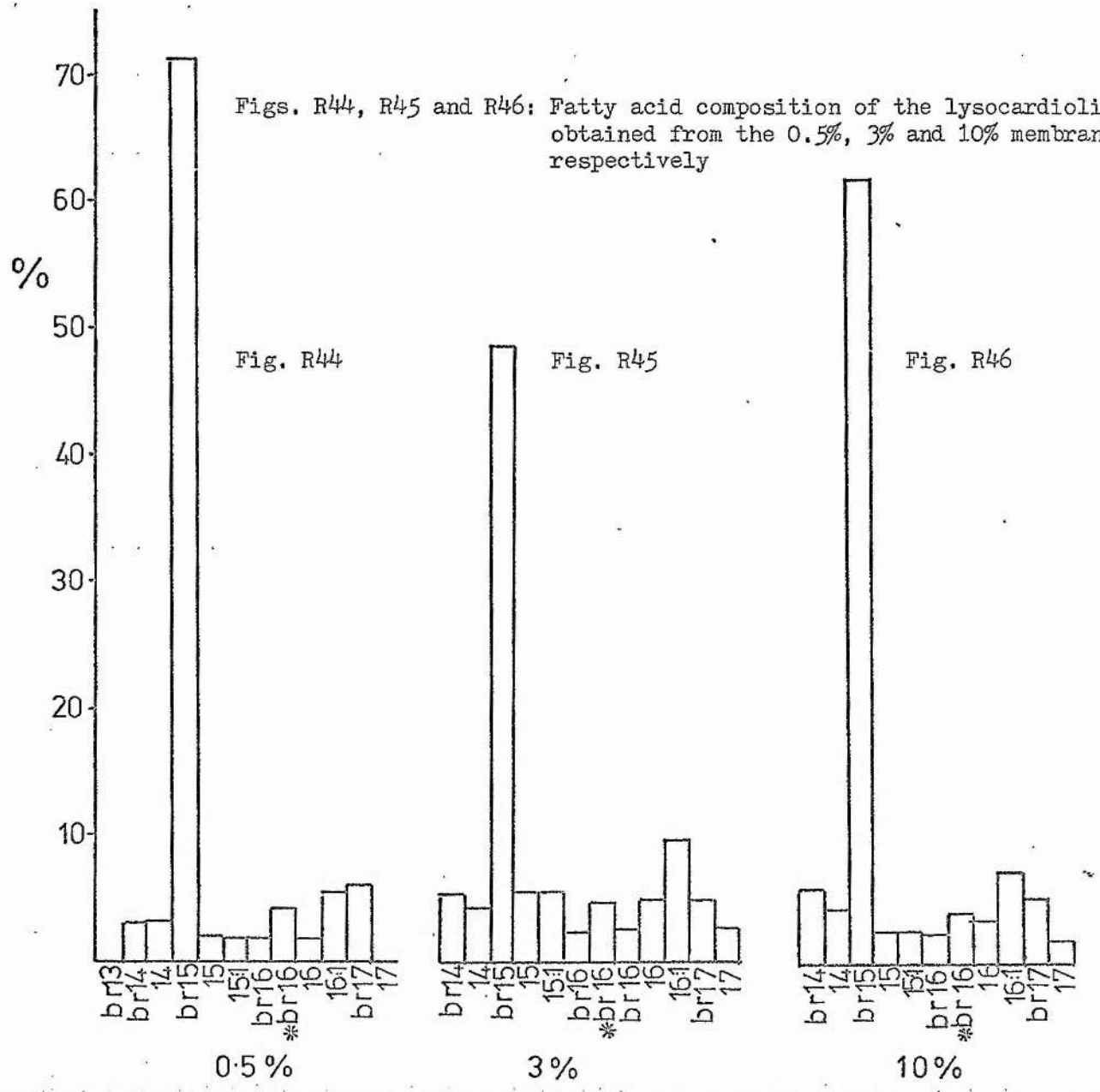
Figs. R35, R36 and R37: Fatty acid composition of the phospholipids corresponding to spots 2 - 4 (Fig. R34) from the 0.5%, 3% and 10% membrane types respectively

Figs. R38, R39 and R40: Fatty acid composition of the phosphatidyl glycerol fractions obtained from the 0.5%, 3% and 10% membrane types respectively



Figs. R41, R42 and R43: Fatty acid composition of the phosphatidyl ethanolamine fractions obtained from the 0.5%, 3% and 10% membrane types respectively





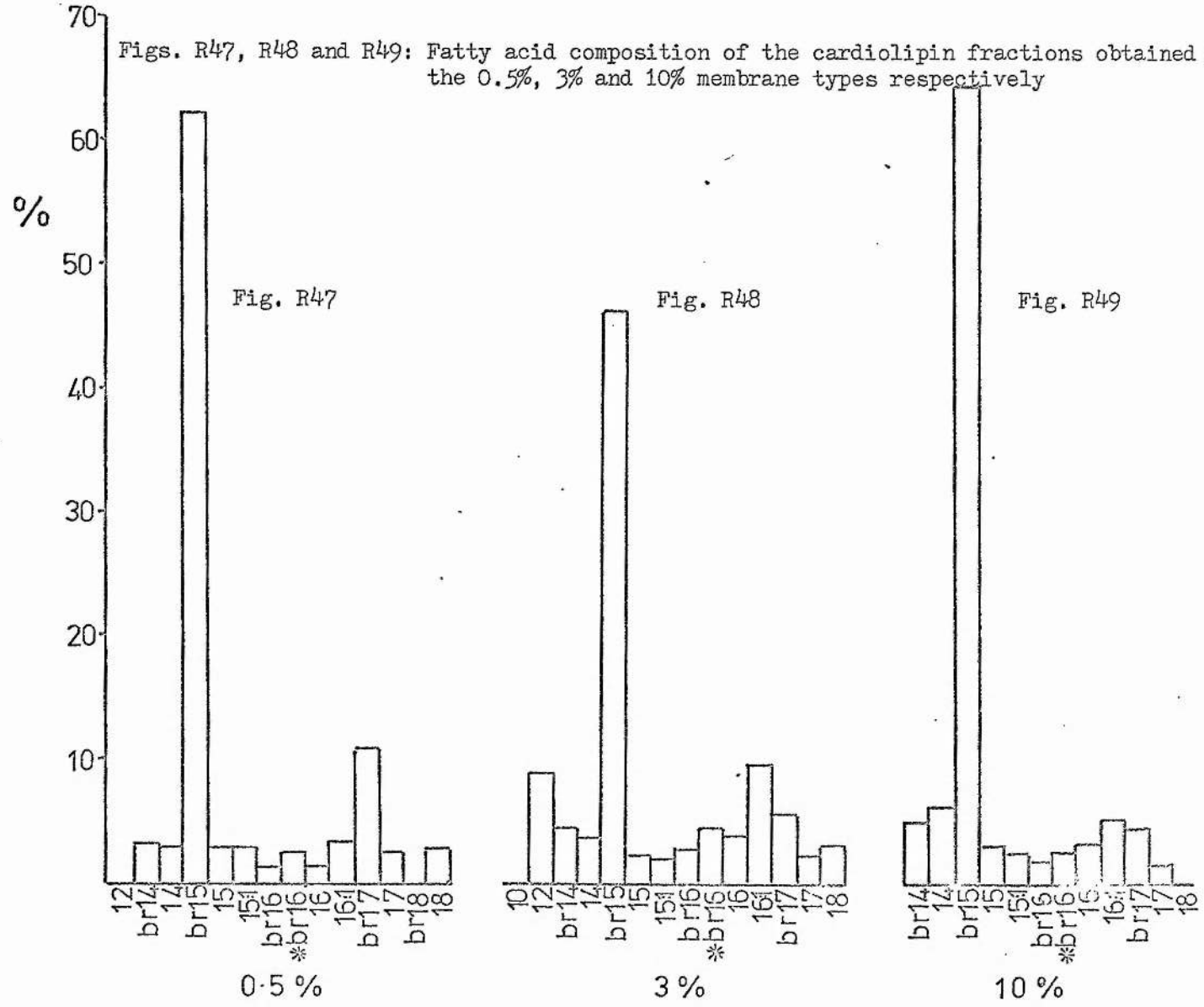
Figs. R44, R45 and R46: Fatty acid composition of the lysocardiolipin fractions obtained from the 0.5%, 3% and 10% membrane types respectively

Fig. R44

Fig. R45

Fig. R46

Figs. R47, R48 and R49: Fatty acid composition of the cardiolipin fractions obtained from the 0.5%, 3% and 10% membrane types respectively



Analysis of the peptido-lipids

These lipids were very polar, ninhydrin-positive, phosphorus-negative compounds which remained close to the origin on thin-layer separation of the acetone-insoluble material. Analysis of their components was carried out on the basis that they had a lipid molecule(s) attached to short peptides. Peptido-lipid material was obtained by preparative TLC and checked for contaminating phospholipids by rechromatographing and spraying with phosphorus-detecting reagent.

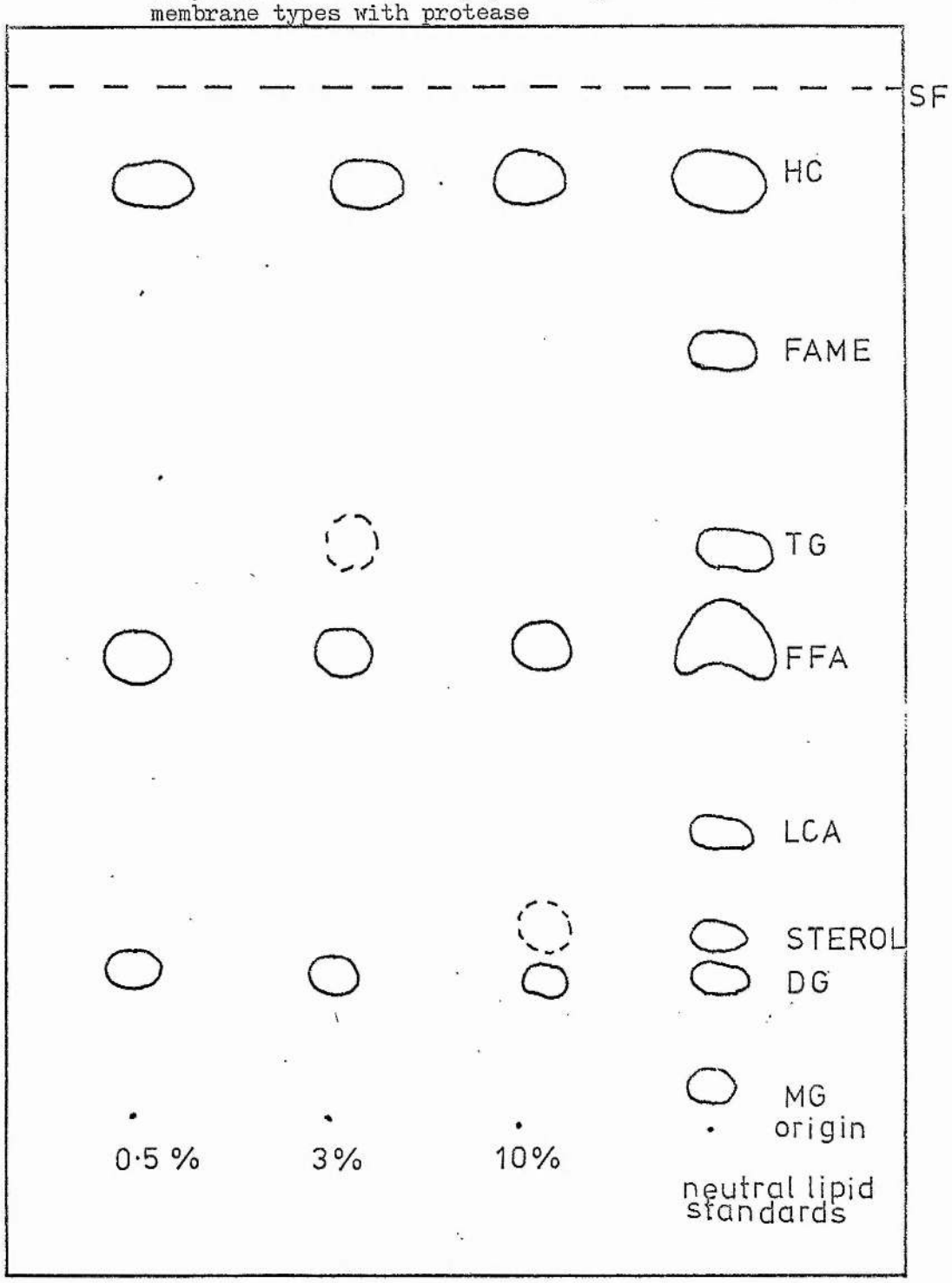
Lipid analysis

Lipid material was cleaved from the amino acid material enzymatically. Carboxypeptidase was tried unsuccessfully, but a crude protease preparation released lipid components which were extracted with chloroform and examined chromatographically. Fig. R50 shows a typical separation and comparison of the spots with authentic neutral lipid standards. Hydrocarbons and fatty acids appeared to be the major lipids released. Minor components detected were spots with R_fs similar to triglyceride and diglyceride. Extraction of the enzyme solution with chloroform in the absence of peptido-lipids showed no spots when chromatographed and sprayed with 50% H_2SO_4. No ninhydrin-positive material was observed, and no spots more polar than those shown in Fig. R50 were observed when chromatographed in more polar solvents.

Amino acid analysis

Analysis of hydrolysed peptido-lipid amino acids gave the results shown in Table R31. The composition of these peptides is markedly different from the pattern obtained for the total membrane proteins. Aliphatic amino acids constituted 70 - 80% of the total amino acids; acidic amino acids constituted about 5 - 6% and no basic acids were present at all. The 0.5% type peptido-lipids did not contain enough material to give an accurate analysis of the amino acid composition.

Fig. R50: TLC separation of the chloroform-soluble products after enzymatic treatment of peptide lipids from the three membrane types with protease



Solvent: light petroleum B.P. 60°-80°/diethyl ether/acetic acid (70:30:2 by vol)

Detection: 50% H_2SO_4

(◌): faint spots

SF: solvent front

HC: hydrocarbons; FAME: fatty acid esters; TG: triglycerides; FFA: free fatty acids; LCA: long chain alcohols; STEROL: sterols; DG: diglycerides; MG: monoglycerides

Table R31(a): Moles/100 moles of each amino acid present in the peptido-lipids.

Amino acid	3%	10%
Aspartic acid	2.0	3.9
Threonine	5.8	1.14
Serine	9.6	2.95
Glutamic acid	4.0	1.05
Proline	-	1.63
Glycine	10.2	3.1
Alanine	8.34	2.28
Valine	11.9	2.9
Iso-leucine	17.7	25.4
Leucine	26.8	51.0
Tyrosine	3.4	4.5

Table R31(b): Distribution of amino acid types in the peptido-lipids, expressed as moles/100 moles.

	3%	10%
% acidic	6.0	4.95
% basic	0	0
% aliphatic	74.9	84.6
% aromatic	3.4	4.5

The peptido-lipids were separated into at least five ninhydrin-positive spots in a polar solvent. Fig. R51 shows a typical separation in chloroform/methanol/water (50:30:5 by vol). No attempt was made to characterise individual spots.

CAROTENOIDS

The carotenoids were extracted efficiently into methanol by the Ballatini ball mill. Six extractions were required to obtain all the coloured material; the cell debris remaining was white in colour. The first extracts contained only a small amount of carotenoid because

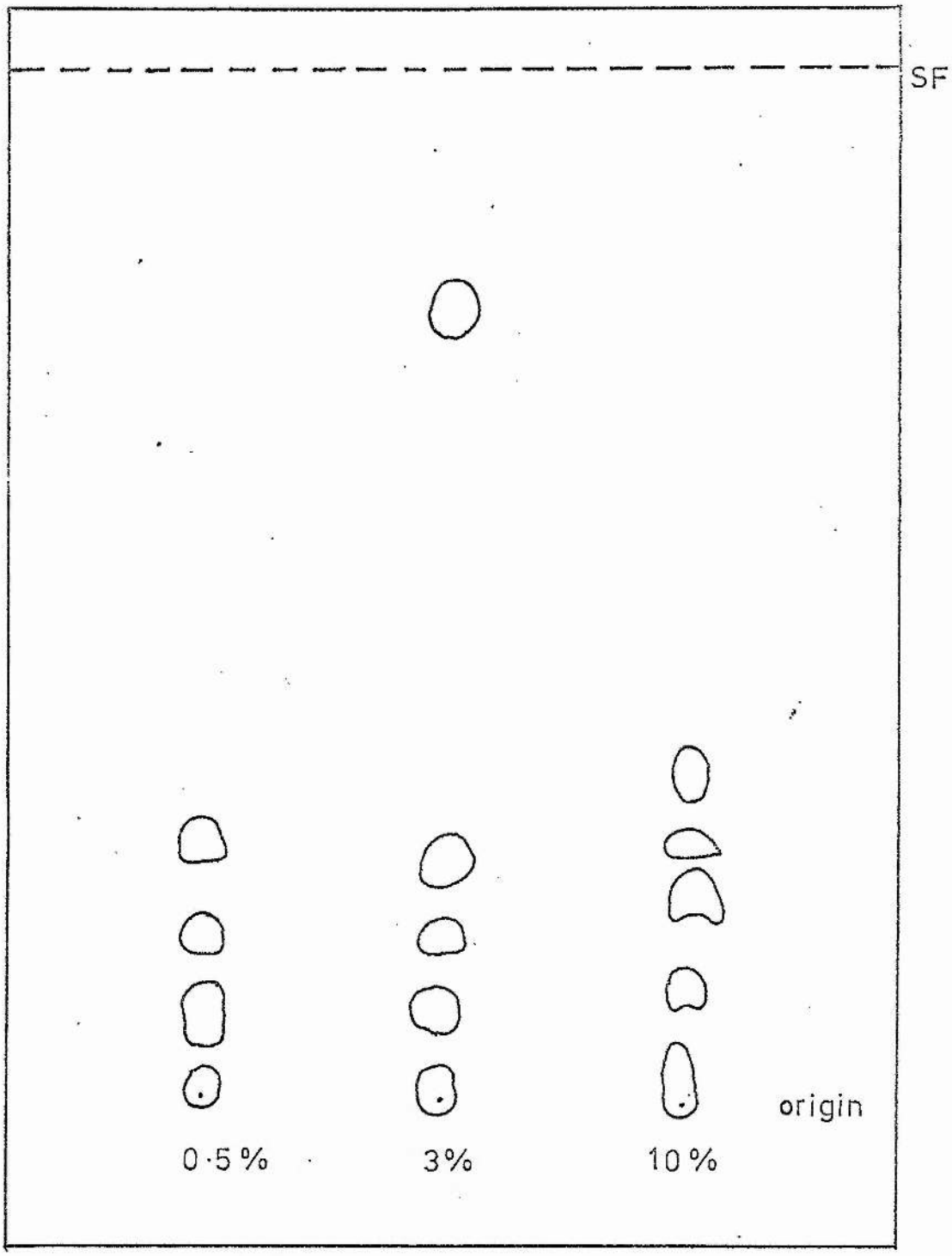


Fig. R51: TLC separation of the peptido-lipids from the three membrane types

Solvent: chloroform/methanol/water (50:30:5 by vol)

Detection: ninhydrin spray

SF: solvent front

the water present in the cells decreased the efficiency of extraction. Subsequent extractions were bright orange in colour. Fig. M3 (Methods) shows the method of isolation of the various fractions analysed. Pigment was first separated into two portions: one was saponified before analysis in an attempt to remove contaminating saponifiable lipid; the other was not saponified in case the carotenoid material was alkali-labile.

Extraction into diethyl ether after saponification

Several extractions were required to remove the bulk of the material which could be extracted with diethyl ether; thereafter only a small amount was extracted each time. The point was never reached where no material was extracted into the diethyl ether, and acidification was carried out when it became apparent that the bulk of the extractable material had been removed.

Extraction after acidification

Only a few extractions were necessary for complete extraction of the pigment into diethyl ether after acidification. No coloured material remained in the aqueous phase. Insoluble material which was dark red and collected at the interface was found to dissolve in methanol and could be extracted into diethyl ether separately after the addition of a small amount of water and acetic acid.

Non-saponified material

The non-saponified material was extracted easily into the diethyl ether phase, no coloured material remaining in the aqueous phase.

The three fractions obtained were named: (a) 'before acid', (b) 'after acid', and (c)'non-saponified'. The visible light absorption spectra of the three fractions were measured in various solvents (see Figs. R52 - R54).

Fig. R52

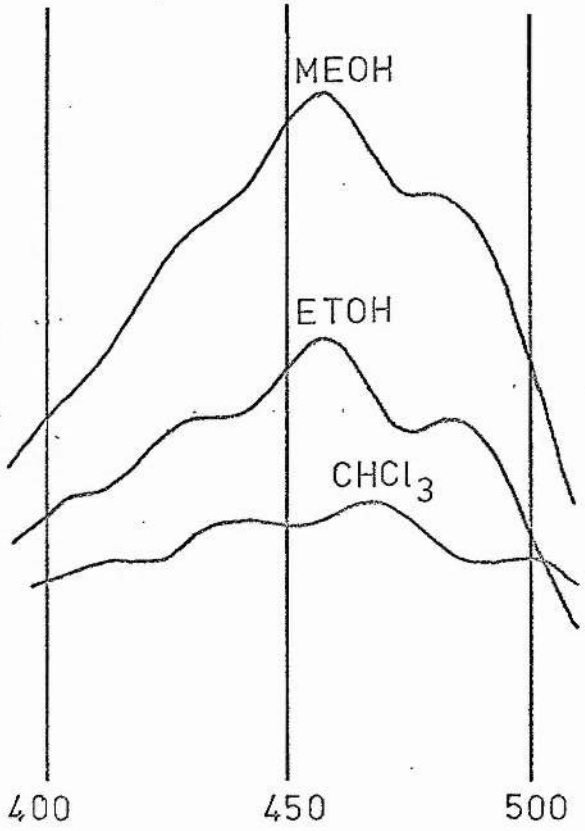


Fig. R53

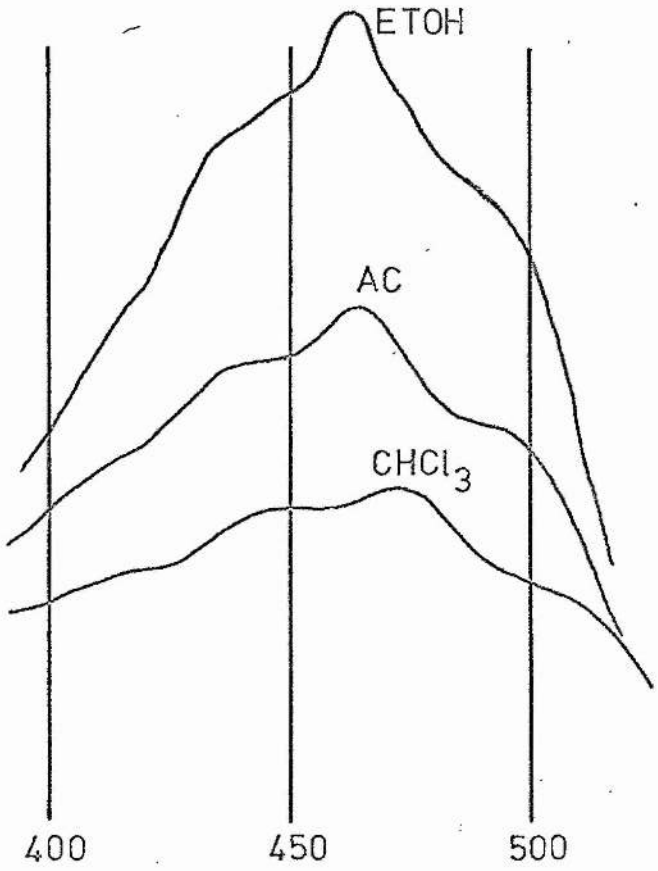
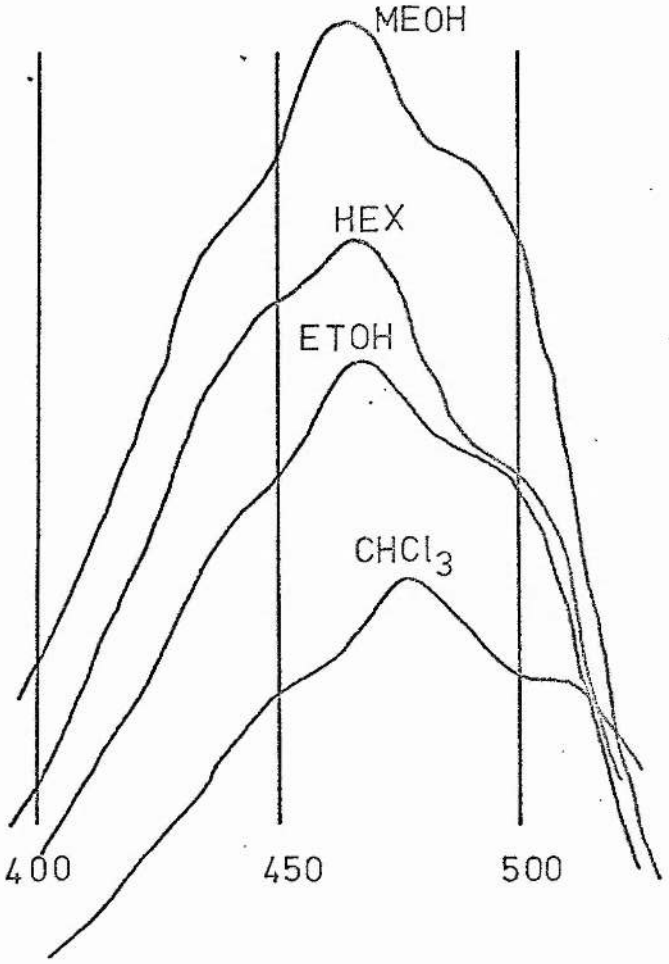


Fig. R54



Figs. R52, R53 and R54: Visible absorption spectra of the 'before acid', 'after acid' and 'non-saponified' fractions in various solvents

Determination of the proportions of pigment in the 'before' and 'after' acid fractions

The absorbance of samples from each fraction was determined at the λ_{max} in methanol. Determination of the volume of each fraction allowed an approximation of the amount of carotenoid distributed in each fraction to be made (Table R32).

Table R32: The λ_{max} (methanol) and the percentage of total carotenoid in the 'before' and 'after' acid carotenoid extracts.

	λ_{max} in methanol	Approximate % of total carotenoid
'Before' acid	458	81
'After' acid	462	19

Absorbance at λ_{max} x vol = quantity of pigment

Since the λ_{max} in methanol was not identical for the two fractions the percentages are only approximate.

TLC separation of fractions

1. Non-saponified

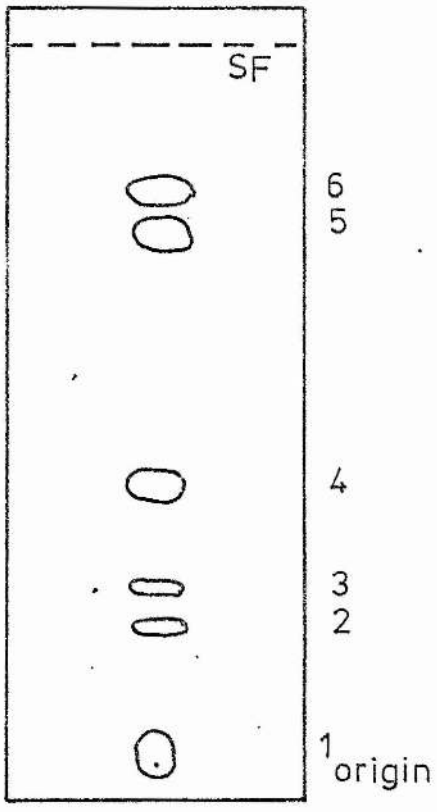
Separation was carried out in chloroform/methanol (9:1 by vol) and Fig. R55 shows the six spots which were observed. Spot 4 was the major spot. Spots 2 and 3 were minor spots.

2. Before acid

Separation was carried out in chloroform/methanol (9:1 by vol) and Fig. R56 shows the five spots which were observed. Spots 1 and 3 were the major spots; spot 2 was very diffuse and contained two components.

3. After acid

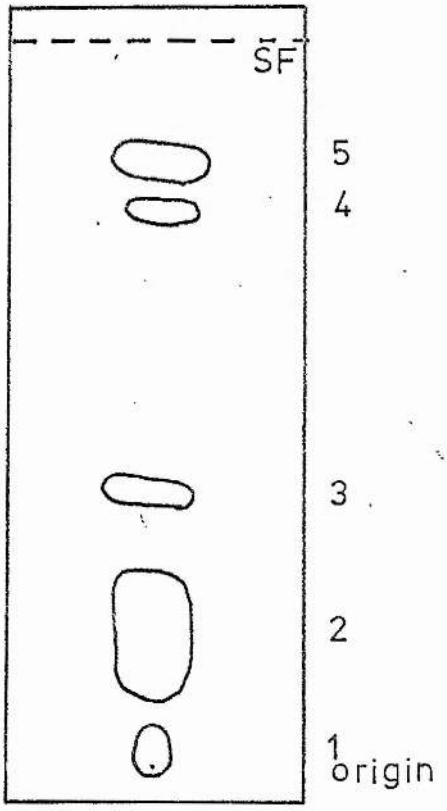
This fraction was separated into four components as shown in Fig. R57. Spot 4 was the major component and was bright orange in colour.



Fraction	R_f
1	0 - 0.1
2	0.2
3	0.25
4	0.4
5	0.75
6	0.8

SF: solvent front

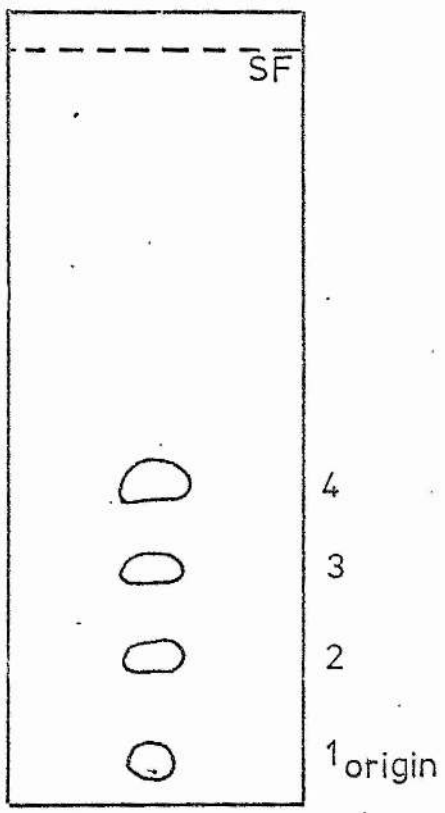
Fig. R55: TLC separation of the 'non-saponified' carotenoid fraction in chloroform/methanol (9:1 by vol)



Fraction	R_f
1	0.0
2	~0.2
3	0.4
4	0.15
5	0.8

SF: solvent front

Fig. R56: TLC separation of the 'before acid' carotenoid fraction in chloroform/methanol (9:1 by vol)



Fraction	R_f
1	0.0
2	0.15
3	0.3
4	0.4

SF: solvent front

Fig. R57: TLC separation of the 'after acid' carotenoid fraction in chloroform/methanol (9:1 by vol)

Preparative TLC and spectral characteristics of the fractions

Preparative TLC was carried out and the fractions eluted from the silica into ethanol. Absorption in the visible region of the spectrum was measured for each fraction immediately after separation and elution from the silica. Each individual fraction was subjected to chemical analysis to determine the nature of any functional groups present. Tables R32, R33 and R34 summarise the information from chemical tests and absorption spectra obtained for each of the individual carotenoid fractions. (The following abbreviations relate to the three tables:

P/R - partition ratio

M - methanol

H - hexane

E - ethanol

C - chloroform

L/P - light petroleum B.P. 60°-80°

D/E - diethyl ether

C=O - carbonyl

OH - hydroxyl

1^{ry} - primary

2^{ry} - secondary)

Table R32: Information obtained for the individual fractions from the non-saponified fraction.

Fraction identified in Fig. R55	P/R 95% M:H	Visible light absorption maxima (nm)	No. of C=O groups	No. of 1ry or 2ry OH groups	R_f in C/M 9:1	Comments
1	*	E - 420 -	*	*	0 - 0.1	Origin material, very polar, minor component
2	*	E - 440 -	*	*	0.2	Minor component
3	98:2	E - 450 -	2	2	0.25	Di-keto, di-hydroxy compound
4	30:70	E - 468 (490)	1	1	0.4	Mono-keto, mono-hydroxy compound, major fraction
		M - 464 (490)				
		C - 476 (508)				
		**H 440 462 492				
		**L/P 442 463 493				
		D/E - 464 494				
5	0:100	E 400 426 452 487	0	0	0.75	Non-polar, minor components
6	0:100	E 402 428 454 (480) 516	0	0	0.8	Probably hydrocarbons

* Insufficient material was available for analysis to be carried out.

** The fraction was only partially soluble in this solvent.

After reduction, spectral examination of the products showed a shift in the absorption λ_{max} towards shorter wavelengths, corresponding to 4 nm for each carbonyl group detected. Silylation of acetylated products did not show the presence of tertiary hydroxyl groups in any of the fractions. Oxidation with nickel peroxide resulted in all fractions being destroyed within 15 min.

The infra-red spectrum for fraction 3 showed absorption in the following regions:

(a) Absorption at 1025 cm^{-1}. This is characteristic of an allylic secondary hydroxyl group in a β ring.

(b) Absorption at 1260 and 1742 cm^{-1}. This pair of absorptions is characteristic of a carbonyl function.

In the carotenoids examined in Table R33, silylation of acetylated products did not reveal the presence of tertiary hydroxyl groups in any of the fractions. All fractions were destroyed by nickel peroxide within 15 min.

Table R33: Information obtained for the individual fractions from the 'before acid' fraction.

Fraction identified in Fig. R56	P/R 95% M:H	Visible light absorption maxima (nm)				No. of C=O groups	No. of 1^{ry} or 2^{ry} OH groups	R_f in C/M 9:1	Comments
1	95:5	E	(432)	457	486	0	3+	0 - 0.05	Origin material, very polar
2A	90:10	E	428	448	480	0	2	0.2	Separated by re-chromatography of fraction 2 in C/M 9:1 B > A and less polar. Probably isomers
2B	90:10	E	424	450	478	0	2	0.24	
		C	432	462	493				
3	19:81	E	424	448	480	0	1	0.4	Major fraction, mono-hydroxy
		C	434	463	493				
		H	425	452	478				
4	0:100	E	428	448	472	0	0	0.75	Similar to fractions 5 and 6 in non-saponified fraction
5	0:100	E	425	449	474	0	0	0.8	

Table R34: Information obtained for the individual fractions from the 'after acid' fraction.

Fraction identified in Fig. R57	P/R 95% M:H	Visible light absorption maxima (nm)			No. of C=O groups	No. of 1^{ry} or 2^{ry} OH groups	R_f in C/M 9:1	Comments
1	100:0	E 438	460	-	1	3+	0.05	Origin material, very polar
2	100:0	E (440)	464	-	2	2	0.15	Di-keto, di-hydroxy
3	100:0	E -	462	-	1	2	0.3	Mono-keto, di-hydroxy
4	100:0	E -	462	-	1	1	0.4	Major fraction, mono-keto, mono-hydroxy

Silylation of the acetylated products did not reveal the presence of tertiary hydroxyl groups in any of the fractions. After reduction, examination of the visible absorption spectra of the products showed a shift in the λ_{max} towards shorter wavelengths corresponding to 4 nm for each carbonyl group detected. All fractions were destroyed by nickel peroxide within 15 min.

A comparison of the visible absorption spectra of several fractions with those published in the literature (92) for βcarotene, zeaxanthin and cryptoxanthin is shown below in Table R35.

Table R35: A comparison of the visible light absorption spectra of some carotenoids.

	Visible light absorption maxima from Planococcus carotenoids		Visible light absorption maxima from published data	
Non-saponified fractions 5 & 6 (Fig. R55)	Ethanol	426 452 487	425 450 478	βcarotene
Before acid fractions 4 & 5 (Fig. R56)	Ethanol	428 448 472		
Before acid fraction 2(a) (Fig. R56)	Ethanol	428 448 480		
2(b)	Ethanol	424 450 478	425 451 482	zeaxanthin
	Chloroform	432 462 493	429 462 494	
Before acid fraction 3 (Fig. R56)	Ethanol	424 448 480	424 452 486	
	Chloroform	434 463 493	434 464 495	cryptoxanthin
	Hexane	425 452 478	425 452 480	

A tentative identification of some of the fractions and a proposed biosynthetic pathway are dealt with in the Discussion.

Examination of the variation in pigmentation with culture conditions

The quantitative and qualitative variation in pigmentation with

different stages of growth were investigated at different salt concentrations.

Quantitative estimation of pigment production

Pigment was extracted using two solvents which allowed the pigment to be divided approximately into two types, depending on polarity: (a) pigment which could be extracted into methanol ('methanol extractable'), and (b) pigment which required a small amount of acetic acid (1%) in the methanol in order to be extracted ('methanol/HAC extractable').

The absorbance at the λ_{max} for the pigment was related to the absorbance at 600 nm of the cell suspension from which the pigment was extracted (equivalent to dry weight of cells), in order to determine the weight of cells from which the pigment was derived. Figs. R 58 - R60 show how the two types of pigment varied with time. Pigmentation reached a peak at the onset of stationary phase and then began a rapid decline. Salt had an inhibitory effect on pigmentation as a whole, but also appeared to encourage the production of the more polar carotenoids (methanol/HAC extractable) rather than the less polar ones (methanol extractable). The more polar carotenoids predominated in the 10% type cells after 50 h and perhaps in the 0.5% and 3% types after a longer period of time.

Variation in type of pigment

Absorption in the visible region was measured at various stages of growth of the microorganisms to determine the λ_{max} of the pigments. Fig. R61 shows how the absorption λ_{max} changed with culture age. Addition of acetic acid (1%) to the methanol had no effect on the absorption λ_{max}. Total pigment extracted in methanol/acetic acid showed a slow shift of the λ_{max} from 450 nm at 10 h to 464 nm at 50 h.

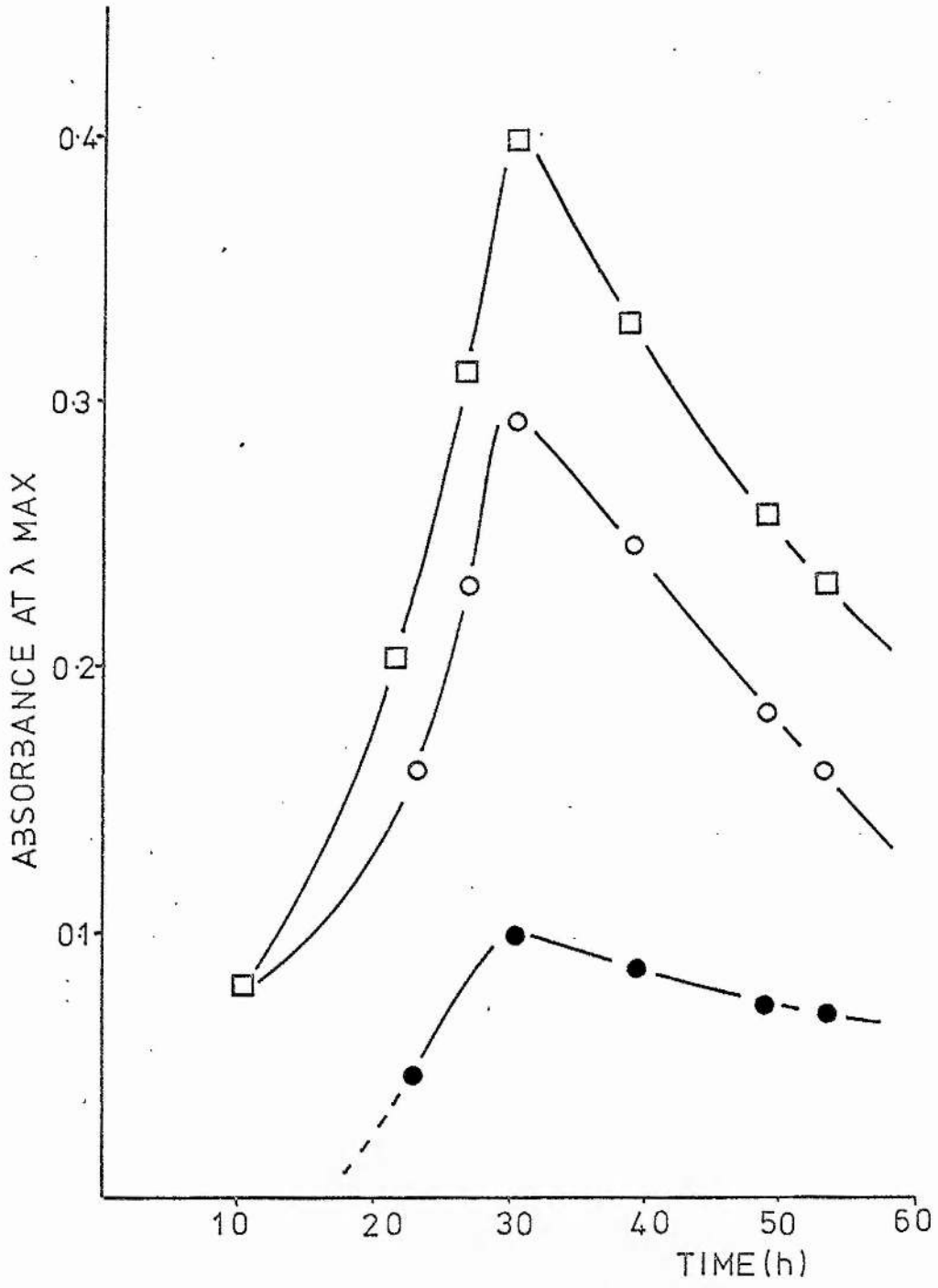


Fig. R58: Variation in the amounts of total pigment, 'methanol extractable' pigment and 'methanol/HAC extractable' pigment per unit weight of cells, obtained at various stages of growth from a culture of Planococcus 316 grown in 0.5% sea salt BERG medium

□ Total pigment

○ Methanol extractable pigment

● Methanol/HAC extractable pigment

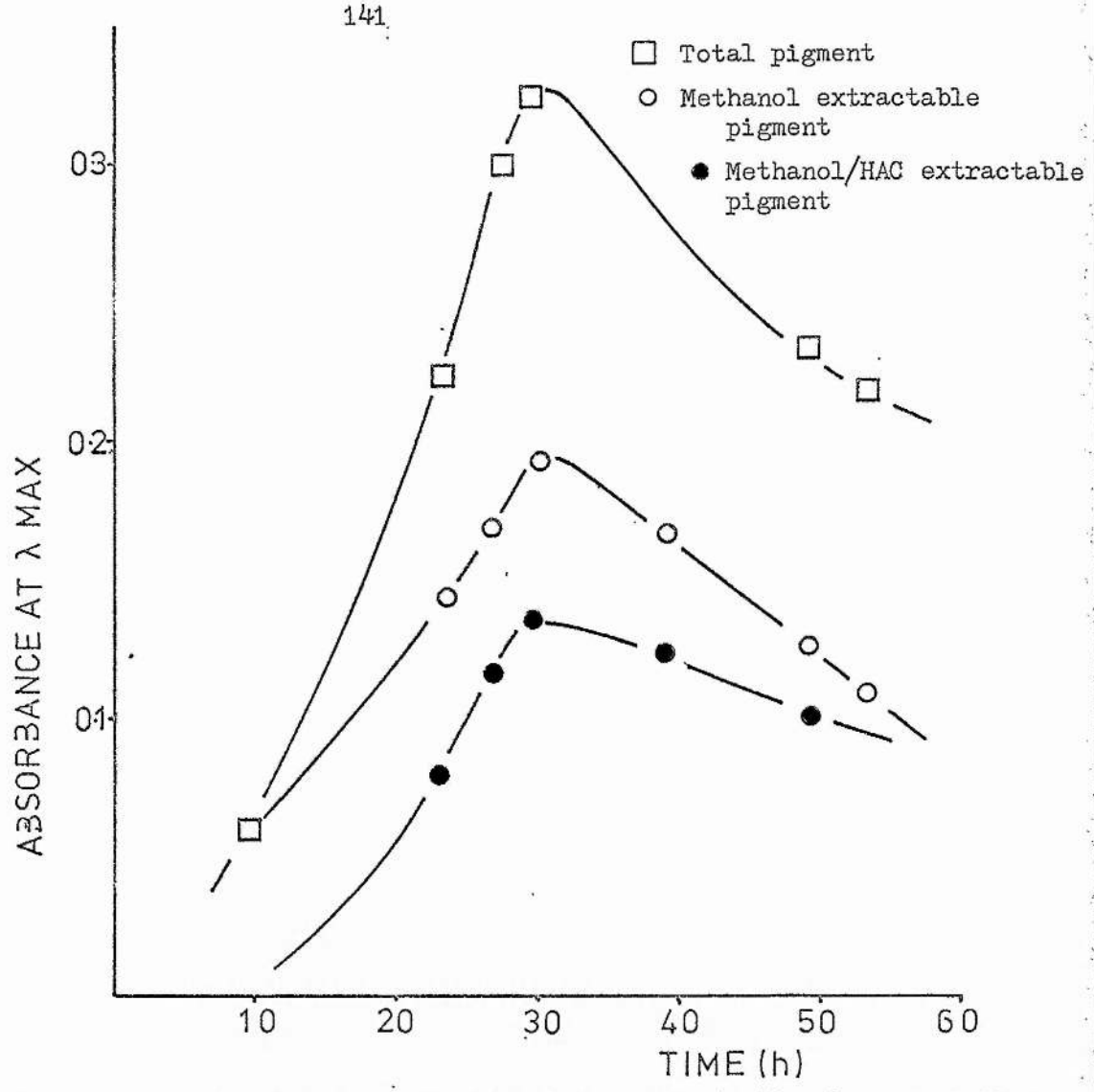


Fig. R59: Variation in the amounts of total pigment, 'methanol extractable' pigment and 'methanol/HAC extractable' pigment per unit weight of cells, obtained at various stages of growth from a culture of Planococcus 316 grown in 3% sea salt BEPG medium

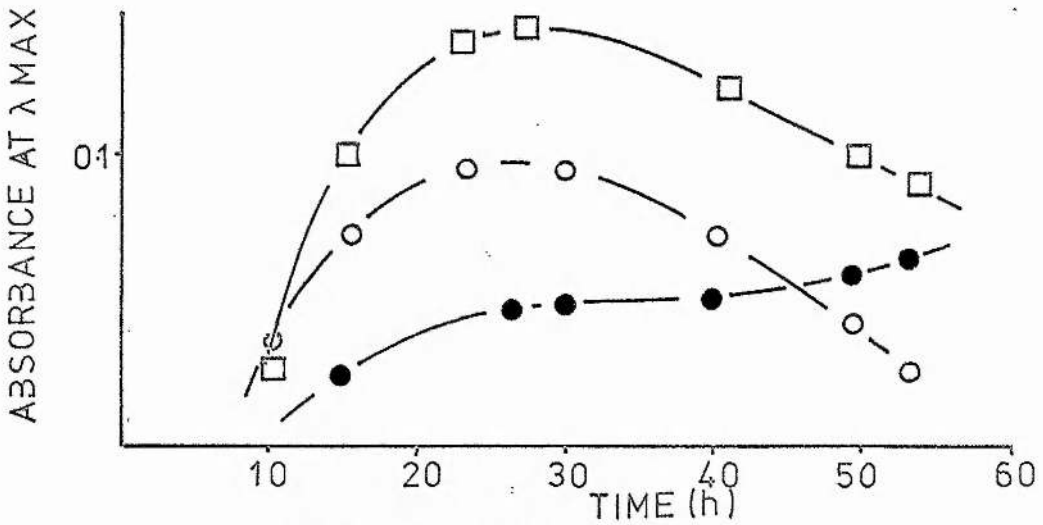


Fig. R60: Variation in the amounts of total pigment, 'methanol extractable' pigment and 'methanol/HAC extractable' pigment per unit weight of cells, obtained at various stages of growth from a culture of Planococcus 316 grown in 10% sea salt BEPG medium

□ Total pigment ○ Methanol extractable pigment

● Methanol/HAC extractable pigment

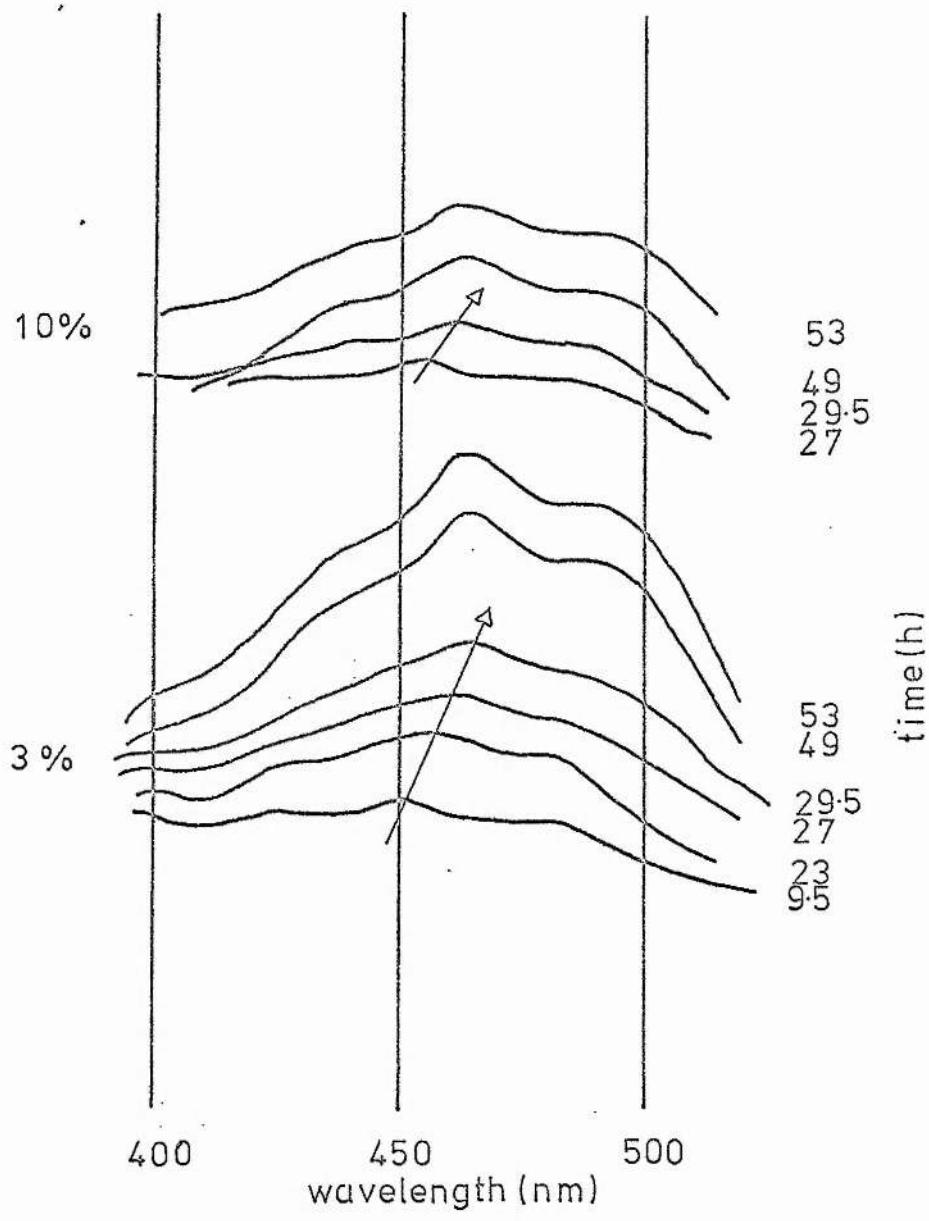


Fig. R61: Spectral changes observed in the total pigments of Planococcus 316 with increasing culture age

Solvent: 1% acetic acid in methanol

In view of the above observation on the increase in polar material with time, it was thought that the more polar pigments would have an absorption λ_{max} of 464 nm in methanol/acetic acid (1%).

Chromatographic investigation of pigment

The pigments from various stages of growth of cells grown in 3% salt were examined chromatographically in chloroform/methanol (9:1 by vol) and the result is shown in Fig. R62. The spot with an R_f of 0.8 decreased with increasing age of the culture; conversely, the spot with an R_f of 0.4 increased with increasing age of the culture and was the major spot after 40 h. A minor spot with an R_f of 0.2 appeared after 49 h. The spots with R_fs of 0.4 and 0.8 were eluted from silica into ethanol. The spot with an R_f of 0.4 had a λ_{max} of 464 nm, while that with an R_f of 0.8 had a λ_{max} of 450 in ethanol. These spots corresponded to purified fractions already investigated and the change in absorption λ_{max} reflected an increase in polarity due to the substitution of hydroxyl or oxo groups on to the carotenoid molecule.

Variation in size of the microorganisms with increasing sea salt concentration in the BEPG medium

Determination of the dimensions of the microorganisms showed that cultures grown in the presence of 10% sea salt contained cells which were generally larger than those grown in the 3% concentration, and in turn these cells were larger than those grown in the presence of 0.5% sea salt. 3% and 10% salt-grown cells also contained unusually large cells (named 'elephant cells') which appeared to be misshapen. The variation in size as determined with the Watson eye piece micrometer is shown in Table R36.

Table R36: Average diameter of cells grown in the presence of different concentrations of sea salt.

Cell type	0.5%	3.0%	10.0%
Diameter (μ)	3.45	3.78	4.65

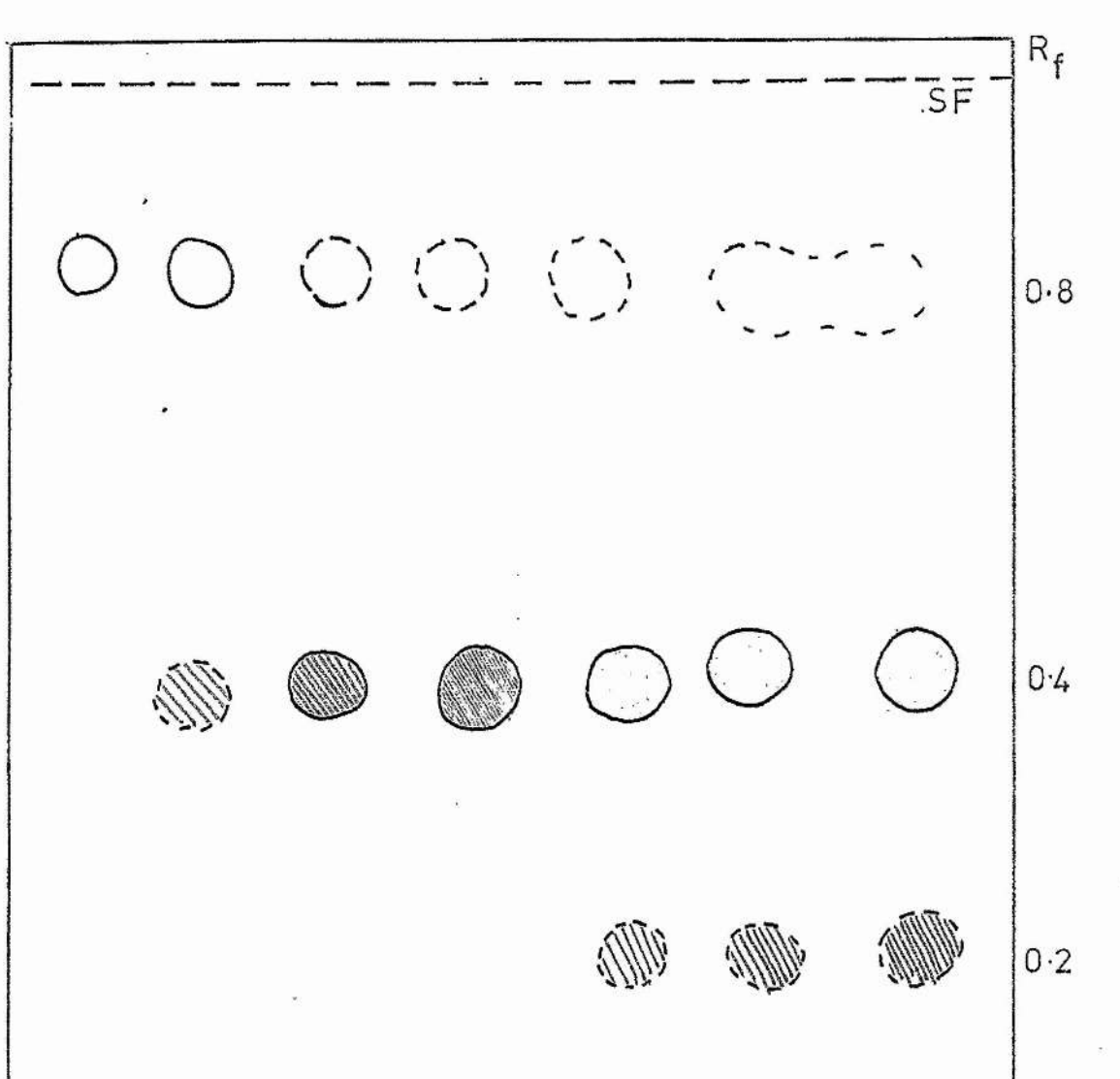


Fig. R62: TLC separation in chloroform/methanol (9:1 by vol) of the carotenoids extracted from a culture of Planococcus 316 at various intervals of time after inoculation

The amount of shading on any spot related to the intensity of the original carotenoid spot

◌ : faint

The 'elephant cells' measured between 5 - 8 μ in diameter. Examination of these cells under the electron microscope showed two types of large cell to be present: (a) very large single cells, and (b) large conglomerations of cells which had multiple septa. The cells with multiple septa appeared to form cross-walls but did not break away from each other so that clumps of bacteria containing 6 - 8 divided cells resulted. Difficulty was experienced with embedding the cells for section cutting, probably due to salt which can interfere with this process. Photo 1 shows a large single cell with at least 5 septa beginning to form (Mg. x 55,000).

Examination of the cations associated with the membrane

A determination of the relative proportions of Ca^{++}, Mg^{++}, K^{+} and Na^{+} ions associated with the membranes was made using the atomic absorption spectrophotometer. Since only the four main cations were investigated, results are expressed in Table R37 as a percentage of the total of these four.

Table R37: Proportions of cations associated with the three membrane types.

Membrane type	$\%Ca^{++}$	$\%Mg^{++}$	$\%K^{+}$	$\%Na^{+}$	% Divalent ions	% Monovalent ions
0.5%	45.0	19.2	15.6	20.2	64.2	35.8
3.0%	29.4	38.2	5.5	26.9	67.6	32.4
10.0%	49.8	18.3	7.0	24.9	68.1	31.9
Sea salt	1.7	10.3	2.3	85.6	12.0	88.0

The membranes have an obvious preference for divalent cations as shown by the approximate 2 : 1 ratio of divalent to monovalent ions. This only increases very slightly with increasing salt concentration. Ca^{++} ions are associated preferentially with the 0.5% and 10% membranes

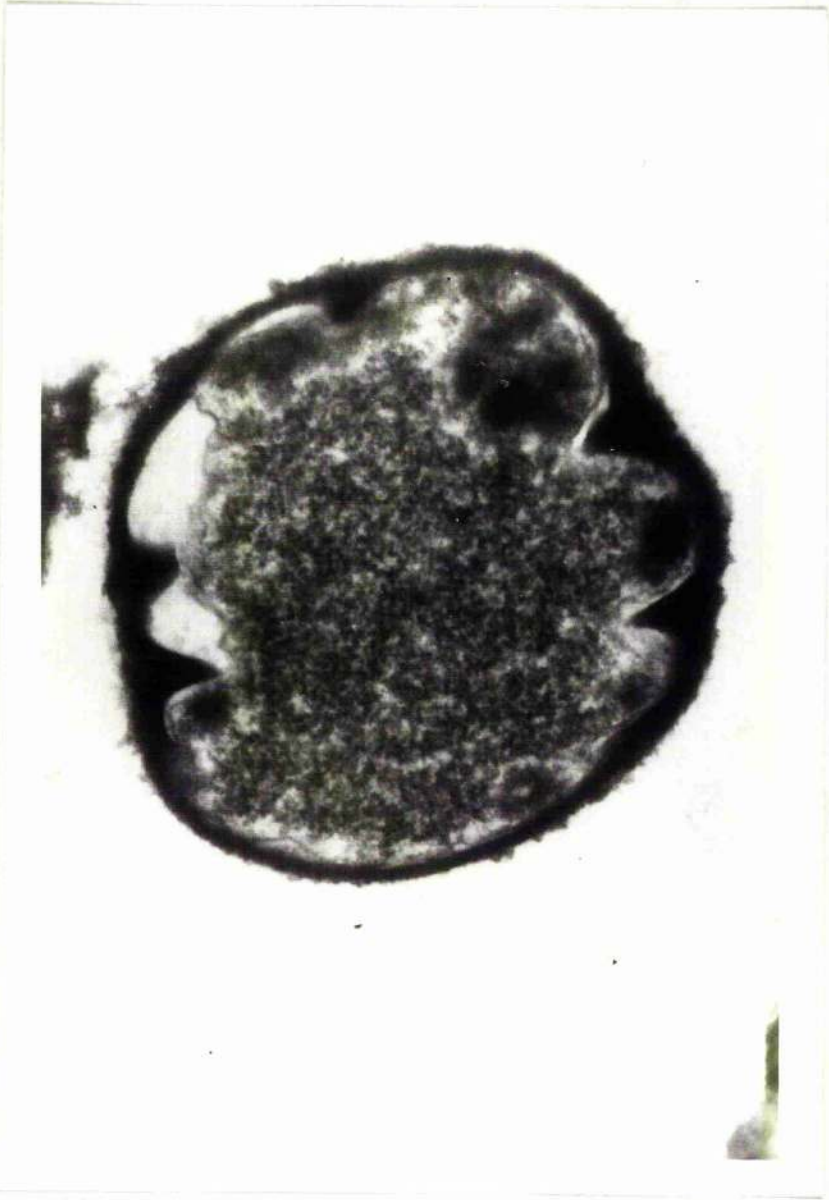


Photo 1: A large single Planococcus cell from a 10% sea salt culture, showing multiple septa

whereas the Mg^{++} ions are favoured by the 3% type.

Ash content of whole cells

The ash content of washed whole cells was shown to increase with increasing sea salt in the medium as shown in Table R38.

Table R38: Whole cell ash content.

Cell type	% Ash
0.5%	17.6
3.0%	19.2
10.0%	27.6

The results are expressed as a percentage of the dry weight of the cells.

Atomic absorption analysis of Ca^{++}, Mg^{++}, K^{+} and Na^{+} in whole cells

The cations associated with whole cells were analysed by atomic absorption spectrophotometry as for the membranes. The results are shown in Table R39 and are expressed as a percentage total Ca^{++}, Mg^{++}, K^{+} and Na^{+} ions.

Table R39: Proportions of cations associated with whole cells.

Cell type	% Ca^{++}	% Mg^{++}	% K^{+}	% Na^{+}	% Divalent ions	% Monovalent ions
0.5%	9.7	34.6	20.2	35.5	44.3	55.7
3.0%	7.9	28.4	25.5	38.2	36.3	63.7
10.0%	10.3	30.3	17.7	41.7	40.6	59.4
Sea salt	1.7	10.3	2.3	85.6	12.0	88.0

These results show the reverse of that found in the membranes. Divalent ions are present in smaller quantities than monovalent ions, with Na^{+} the dominant ion. K^{+} is present in a much greater proportion, in relation to the other cations, in the cells, than in sea water.

DISCUSSION

The salt concentrations in the different media used for the growth of Planococcus 316 were chosen so that the microorganism could be studied under conditions similar to those found in its normal habitat (3% sea salt) and at concentrations which approached the extreme limits of its salt tolerance. 10% sea salt was the practical upper limit, because this was the solubility limit of sea salt in water; it also approached the maximum NaCl concentration in which the cells would grow normally. Likewise, 0.5% salt was the minimum concentration which would support growth. However, this figure excludes any salt which may be in the BEPG media, but this is only in the region of 0.06%. The observation that this organism would not grow on BEPG medium without added salt is in contradiction to the findings of Kocur et al. (12), who reported that good growth was observed in sea water media as well as in media without added salt. The concentration of salt required for growth could also be dependent on the organic constituents in the media, as Kocur et al. grew the cells on different media from that used in these experiments. This raises the question of whether this microorganism should be considered salt dependent and not merely salt tolerant.

Growth in 5% KCl + 10% NaCl, in the BEPG medium was slow, but as the cells would not grow in the presence of 15% NaCl, the KCl seems to increase the upper limit of salt tolerance in liquid media. The cells grew well in 3% and 10% KCl + BEPG, although not quite as well as in similar concentrations of NaCl. This is unusual as most halophiles, and particularly extreme halophiles exhibit a specific requirement for NaCl (3). The effects on growth of increasing amounts of salt in the medium were not as marked as had been expected. The cells appear to have precise upper and lower limits of salt tolerance between which growth curves closely parallel each other, and beyond which no growth occurs. The main effects of increasing concentrations of sea salt in

the medium on the growth of Planococcus were to lengthen the lag phase and to decrease the maximum cell numbers attained. Very little difference was noted in the doubling times. Cultures of Staphylococcus aureus (a salt tolerant microorganism) were found by Kanemasa, Yoshioka and Hoyashi (93) to react in a similar manner to increasing salt concentration in the medium. These workers noted a small increase in the doubling time and a drop in the final cell numbers as the concentrations of NaCl in the media were raised from 0.05% to 5% to 10%.

The observed lengthening of the lag phase may represent the extended time required for cells to adapt to the increased osmotic pressure in the medium. The fact that doubling times are not altered implies that the enzymes are not impaired by higher salt concentrations, and the decrease in final absorbance points to inhibition by some limiting factor in the medium. The decrease in the solubility of oxygen in solutions containing salt may be one of the factors involved; however, decreased efficiency in the respiratory chain due to interference from salt, or energy being diverted from anabolism towards ion pumping mechanisms may also explain the decrease in final absorbance of cultures grown in high salt concentrations.

The three methods used for monitoring growth gave similar results. The TCA precipitation method was particularly useful for overcoming difficulties which arose during experiments to determine the optimum pH for growth, because salt precipitating from the medium at alkaline pH made turbidimetric readings unreliable. The viable cell counting technique, although time consuming and requiring large amounts of glassware, is the only technique which actually measures the number of viable cells in a culture, as opposed to dead cells and/or cell debris which would be recorded as well as the viable cells when using turbidimetric methods. This method is also the only way

of determining the number of cells which remain viable once stationary phase has been reached. Experiments to study the optimum pH for growth of Planococcus in the BEPG medium at the three different salt concentrations showed that the microorganism has a wide degree of pH tolerance on the alkaline side of neutrality and exhibits two pH optima. Maximum growth was recorded at around pH 7.0 and pH 8.0 with a decrease in growth between these values. The decrease was most apparent in the 3% type cells but only very small in the 0.5% type. Cells grown in the presence of 3% and 10% sea salt were more acid tolerant than the 0.5% type which did not grow well at pH 6.0. Thierry and Cooney (94) reported a dual pH optima of 6.8 and 7.5 in M.roseus. The pH optimum at 8.0 for Planococcus is not unexpected as sea water has approximately this pH. It is interesting to speculate whether the optimum pH of 7.0 is an indication that the microorgansim was originally land- or fresh water-based, and has adapted to marine conditions, while still retaining some of its previous characteristics.

The membranes of gram-positive microorganisms are generally reported to constitute between 9% and 49% of the dry weight of the cells (79). The yields of membranes from Planococcus, prepared from cells grown in the presence of the three concentrations of sea salt, were within this range. The mechanism by which salt affects the amount of membrane is not completely clear. Salt concentrations above and below the normal (3%) concentration apparently reduce the amount of membrane, when this is expressed as a percentage of the salt-free dry weight of cells. The low value obtained for the cells grown in the presence of 10% salt may be due to the observed increase in size of these cells, which would result in a decreased surface area to volume ratio. The presence of mesosomes or vesicles within the cells may also influence the amount of membrane isolated.

With increasing salt in the medium, the ash content of the membranes increases. Ash consists mainly of phosphate derived from RNA and phospholipid and also small ions which are associated with the membrane. Apart from some metal ions which are associated with particular proteins and are specifically required for enzymic activity, membranes are thought to contain divalent ions which act as bridges between molecules and hold the various components in a regular structure. Mg^{++} ions are necessary for the artificial reassociation of membrane components into sheets and ionic interactions appear to have an important role in the arrangement of membrane components into structured aggregates. The results obtained from the atomic absorption analysis of the membranes with respect to Ca^{++}, Mg^{++}, K^{+} and Na^{+} ions show that divalent cations were present in a 2:1 ratio with respect to monovalent cations. Since membrane preparations were exhaustively washed and dialysed, these ions are not in loose association with the membrane components. These findings support the theory that Ca^{++} and Mg^{++} ions are involved in lipid-lipid and lipid-protein interactions. The presence of monovalent ions (with Na^{+} in greater concentration than K^{+}) suggests that these too are strongly associated with the membrane and are perhaps in the process of transfer across the membrane or are associated with proteins and involved in maintaining their active conformations. Membrane ash content more than doubles with an increase of salt concentration in the medium from 0.5% to 3%. The figure for the 3% membrane type may be slightly elevated by the increased RNA content which would contribute via the phosphorus atoms

The RNA figures obtained for the 0.5% and 10% membrane types compare well with those reported for other gram-positive cocci (95). The value obtained for the 3% salt type membrane was higher than

expected and nearer the values obtained for some of the gram-positive bacilli (e.g. Bacillus megaterium). The RNA found in membranes is reported (96) to be firmly bound to the membrane and only removed by RNA'ase treatment; thus it is unlikely to be merely a cytoplasmic contaminant but more probably due to membrane-bound ribosomes involved in the production of membrane proteins 'in situ'. It is possible that the number of membrane bound ribosomes is related to the internal concentration of salt and that the maximum number of ribosomes are associated with the membrane under conditions similar to those found in the natural habitat (i.e. 3% sea salt). The high level of RNA in the 3% salt type membranes increases the apparent percentage of carbohydrate in these membranes due to the pentose content of the nucleic acids. The carbohydrate content of the 3% and 10% salt type membranes is double that obtained for the 0.5% type. Salton (95) reports large variations in membrane carbohydrate both between species and by different workers for the same species. Carbohydrate values between 0.8% and 20% were found in membranes of gram-positive cocci with an average of around 4 - 9%. The phenol-sulphuric method for carbohydrate analysis detects both hexoses and pentoses and is not subject to interference from proteins. Analysis of the carbohydrate present as extractable glycolipid in Planococcus showed this to be a very small proportion of the total carbohydrate. As the concentration of sea salt in the medium is increased, the total amount of carbohydrate in the membranes increases; however, the total amount of extractable glycolipid decreases. This suggests that either, (a) there is an increase in the amount of glycolipid with increasing salt concentration but that much of this glycolipid becomes bound so that it is no longer extractable, or, (b) that there is an increase in the utilisation of carbohydrate for the formation of glycoproteins

and a decrease in glycolipid synthesis, or (c) that a combination of both factors is operating. In Staphylococcus aureus, it was shown (97) that glycolipids accounted for all the carbohydrate present in the membrane. In Planococcus, sugars were released more easily on hydrolysis from the total membrane preparation than from glycolipids; maximum release from the membrane occurred after $\frac{1}{2}$ h under the conditions used, but 2 h under the same conditions were required for maximum release of sugar from the glycolipids. This fact points to differences in bond strength, due to either the different moieties involved in the bond or to the type of linkage in glycolipids and in the sugars which remain in the defatted membranes. Qualitative analysis of the membrane carbohydrate revealed the presence of glucose, galactose and ribose, as well as one unidentified sugar of low R_f. The ribose presumably was derived from the RNA. Glucose is found almost universally in bacterial membranes and galactose is perhaps the next most common sugar.

Proteins and lipids make up the bulk of the membrane in Planococcus as in other gram-positive bacteria. Protein to lipid ratios in the membrane show a marked increase in cells grown in the presence of salt concentrations both above and below the normal 3% concentration, (3:1 for 0.5% and 10%, as opposed to 5:1 for 3%). Although the 3% salt type membranes apparently have a lower protein content than the 0.5% or 10% types (Fig. R 9), this is probably due to the elevated RNA level, resulting an an anomalously low level of protein. Calculation of the percentage protein, ignoring the RNA, shows the 3% salt type membrane to contain more protein than the others. The lower lipid value also contributes towards the 5:1 protein to lipid ratio, and again the question arises of whether this reflects a greater amount of 'bound' lipid or merely a reduction in the total amount of lipid actually present in the membrane. Salton quotes 'average'

bacterial membranes as having a 3:1 protein to lipid ratio (98), but also indicates that the quantity of lipid is often dependent on the composition of the medium and the age of the culture (74). Since all three types of membrane were prepared from cells in very early stationary phase, it is likely that the salt content of the medium influences the protein : lipid ratio. However, when cells are grown in the presence of sea salt concentrations which are above or below the normal habitat level of 3%, the proportion of 'bound' lipid may increase, thus giving an elevated protein : lipid ratio in the cells grown in the presence of the 3% sea salt. This could indicate that salt concentration in the medium influences the type of associations which occur within the membrane between protein and lipid. The protein values for the three membrane types are generally higher by a few percent than those quoted for other gram-positive microorganisms (98). Since proteins are involved in the active transport of ions across membranes, a high proportion of protein in the membrane may well be an advantage in halophiles as a means of selectively maintaining the correct balance of ions within the cells while at the same time providing an efficient barrier to unwanted ions. The Moore and Stein method for protein determination is subject to interference from other non-protein membrane components (such as ethanolamine from the lipids), but was found by Gray (79) to give the most consistent results with Sarcina aurantiaca membranes. Methods dependent on the presence of aromatic amino acids (Folin-Lowry) are of limited use with bacterial membranes which are usually low in tyrosine, tryptophan and phenylalanine residues. The amino acid composition of the membrane compares well with published data for bacterial membranes, having a high proportion of alanine, glycine, leucine, isoleucine and aspartic and glutamic acid residues and a low proportion of sulphur-containing, aromatic and basic amino acids. Increasing

salt in the medium does not result in large changes in the amino acid composition of the membrane proteins. Small trends are noticeable however, indicating that minor alterations to the amounts of individual proteins occur. Increases in the proportions of glutamic and aspartic acids, tyrosine, lysine, methionine, alanine, arginine and serine, and decreases in threonine, valine, iso-leucine and leucine were observed with increasing salt in the growth medium. Comparison with the amino acid composition of the total proteins of the extreme halophile, Halococcus strain 46, and the non-halophile S.lutea, show that the membrane proteins of Planococcus are not as acidic as those of the extreme halophile but are more acidic than those of S.lutea. The proportion of aliphatic amino acids in Planococcus remains fairly constant with increasing salt and compares with the values for both Halococcus strain 46 and S.lutea. No correction was made for amide, but it was thought that overall the membrane proteins were slightly more acidic than those of the non-halophile. It seems unlikely in the case of Planococcus that new proteins in any substantial amount are synthesised during growth in high salt medium, but more likely that the existing proteins are able to cope with the high ionic strength. The small changes in amino acid composition may be due to salt influencing the rate of synthesis of some of the membrane proteins and this is reflected as minor changes in the amino acid composition of the total membrane proteins.

Because of the large variation in the levels of RNA, phospholipid and total lipid between the three membrane types, it is difficult to draw any valid conclusions from the analysis of the total membrane phosphorus.

Total recoveries of organic material from the 0.5% 3% and 10% membrane types were 105.3%, 100.3% and 107.2% respectively. The

recoveries are greater than 100% because carbohydrate values include pentose from the RNA and the protein values may be slightly elevated due to interference from non-protein material present in the membrane.

The proportion of the total lipid constituted by the phospho-, glyco- and neutral lipids varies only slightly with an increase in salt concentration in the medium. The ratio of acetone-insoluble material to acetone-soluble material decreases with increasing salt, indicating a smaller proportion of phospholipids in the higher salt membrane types. On the basis of carbohydrate determinations, glycolipid material decreases with increasing salt concentration in the growth medium. The proportion of neutral lipids increases in salt concentrations above and below the 3% value, and is greatest in the 10% membrane type.

Within the neutral lipids, the effects of increasing salt in the growth medium does not cause any major alteration to the proportions which each class constitutes of the whole. Long chain alcohols and triglycerides increase with increasing salt in the medium; free fatty acids and hydrocarbons increase and decrease respectively with variation above and below the normal 3% value. Squalene is not normally found in the membranes of bacteria. Mammalian membranes contain large amounts of cholesterol of which squalene is a precursor. However, the absence of sterols from bacterial membranes (although there are exceptions (99)), raises questions as to the purpose of this molecule which can constitute up to 25% of the neutral lipids in Planococcus. The role of squalene in extreme halophiles has been discussed in the Introduction. However, the fact that both squalene and perhydro squalene have been found in this microorganism and that significant amounts of squalene and tetrahydro squalene have been reported in a number of other mild halophiles (39) would seem to indicate that squalene may be associated with adaptation to halophilic

environments in some microorganisms. In so far as squalene is not a precursor for sterols in this microorganism, its function may be as a structural component within the membrane. Since it constitutes only 3 - 4% of the total lipid, it is obviously not a major component. Squalene and carotenoids have a common biosynthetic pathway to farnesyl pyrophosphate and the amount of squalene produced may be influenced by the efficiency of conversion of farnesyl pyrophosphate to carotenoids. Since the microorganism produces less squalene and less carotenoids when grown in high salt concentrations, this may indicate that salt inhibits the pathway before the branch point at farnesyl pyrophosphate.

The membrane glycolipids were assumed to be monoglycosyl diglycerides on the basis of their chromatographic mobility as compared to that of standard diglucosyl diglyceride. The relatively high number of sugar-containing components in the 0.5% lipid type is unusual, particularly since the number of components is in excess of the number of individual sugars detected in these fractions, and may represent substitutions on some of the sugar hydroxyl groups, thus decreasing the polarity and giving rise to a number of compounds. The unusual 'purple' spot (spot 3, Fig. R25) found only in the 0.5% lipid type may be a sulphur-containing glycolipid, as shown by the positive reaction with the copper sulphate spray reagent. However, if this is the case, the R_f is less polar than would be expected. The unidentified sugar (spot (a), Fig. R26) was not detected by GLC and this may be due to either an extended retention time, making it difficult to detect, or to its being a hexosamine which was removed by the ion exchange resin during the preparation procedures. The detection of galactose by GLC and the failure to do so with sugar sprays on paper chromatograms is indicative of the greater sensitivity of the former method, although interpretation of the traces may be difficult due

to the sugar anomers which give rise to several peaks for each sugar.

The phospholipid components were measured quantitatively by both phosphorus and gravimetric determinations. Phosphorus determinations were probably more accurate since losses would not be incurred through material remaining on the silica, and only phospholipids would be detected. The phospholipids from the three membrane types contain large amounts of cardiolipins (cardiolipin + lysocardiolipin) which constitute 54%, 66% and 69% of the total lipid phosphorus in the 0.5%, 3% and 10% membrane types respectively. Phosphatidyl ethanolamine was also identified, although this phospholipid is more usually found in gram-negative microorganisms. In view of the very small amounts of glycosyl diglycerides in this microorganism and the proposed interchangeability of phosphatidyl ethanolamine and glycosyl diglyceride in bacterial membranes (100) it could be suggested that phosphatidyl ethanolamine is compensating for the reduced amount of glycolipid. Phosphatidyl glycerol, the main phospholipid in many microorganisms, is certainly not the major component in Planococcus membranes. 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 (93). The percentage cardiolipins in Planococcus membranes, although high at low salt concentrations, does increase with increasing salt in the medium (Table R26). It is not clear whether the presence of lysocardiolipin in the phospholipids is due to cleavage of fatty acids from the cardiolipin molecules during isolation procedures, or whether lysocardiolipin is actually present in the membrane and has a particular function. It is interesting to note that the 3% type lipids contain the lowest amount of lysocardiolipin, but also have the highest amount of free fatty acid in the neutral lipid fraction. This may indicate that fatty acids were in

fact cleaved from cardiolipin during the membrane preparation and isolation procedures. If lysocardiolipin is present in the membranes in vivo in the proportions indicated by the quantitative determinations, then concentrations of salt above and below the normal 3% value greatly increase the amount of this phospholipid within the membrane, mainly at the expense of cardiolipin. Papahadjopoulos (59) showed that ion permeability in model membrane systems was determined by the charge on the polar head group of the lipids used. Only membranes constructed from acidic phospholipids (phosphatidyl glycerol and cardiolipin, or phosphatidyl glycerol and phosphatidyl serine) were found to be highly cation selective. Large amounts of these acidic phospholipids in the membrane would probably be advantageous to halophiles which have an internal ion composition very different from that of the external medium. The absence of ether-linked lipid material in the phospholipids was not unexpected. Kates et al. (18) had already confirmed the lack of large amounts of non-saponifiable material in other mild halophiles. Presumably the much increased stability which the ether-linked lipids confer on the membranes of extreme halophiles is not necessary in the mild halophiles which have been able to adapt the normal bacterial membrane to cope with increased ionic strength.

The peptido-lipids were composed of amino acids attached to glycerides and fatty acids. Analysis of the amino acid composition of these lipids showed that aliphatic amino acids predominate to the extent of 78 - 85% of the total residues. The association of lipids and 'integral' proteins in the membranes of bacteria is thought to be mainly through the 'burying' of fatty acid chains in the hydrophobic areas of globular proteins, which are an integral part of the membrane. Hydrophobic areas of proteins are thought to be sequestered from the hydrophilic areas of the membrane and to consist of aliphatic amino acid residues. Bonding consists mainly of hydrophobic interactions

between aliphatic amino acid side chains and fatty acids. Peptido-lipids may represent bridge molecules which are capable of joining lipid and protein together, thus helping to maintain the overall structure of the membrane. Alternatively, they may be specific transport molecules involved in the transport of ions across the membrane. This type of molecule must have a hydrophobic 'outside' to enable it to pass through the membrane, but must also have hydrophilic areas with which ions may be associated. The observed increase in the amount of peptido-lipid with increasing salt concentration in the medium may reflect an increase in the number of these molecules which are required to deal with the larger number of ions present.

The fatty acid composition does not vary much between lipid classes; the major fatty acid is a branched saturated C_{15} acid. A branched C_{15} saturated acid has been reported as the main fatty acid in a number of gram-positive non-halophilic microorganisms (101,102,103). In Planococcus, only the triglycerides and certain of the glycolipid fractions do not contain this as a major fatty acid. The triglycerides of all three lipid types contain the branched C_{15} acid, but as a minor component. This is unusual in that the mono- and di-glycerides from which the triglycerides are presumably derived contain large amounts of the branched C_{15} acid. This may be due to a specificity in the triglyceride acyl-transferase, or to some modification to the triglycerides through a de-acylation - re-acylation reaction. The major fatty acids found in other halophiles studied are not the branched C_{15} type. Kates et al. (18) have shown that the major acids in M.halodenitrificans and V.costicolus are C_{16}, monounsaturated C_{16} and monounsaturated C_{18}, and Stern and Tietz (52), working with a mildly halophilic rod, showed C_{16}, monounsaturated C_{18}, and C_{19} to be the major acids. Kanemasa et al., working with S.aureus (93) grown in various salt concentrations, have shown that increasing salt in the medium (0.05 - 10%) causes an increase in the

amount of branched fatty acid in cardiolipin, but a decrease in the amount of branched fatty acid in phosphatidyl glycerol (the major phospholipid) and lyosphosphatidyl glycerol. Coupled with the fact that the amount of cardiolipin also increased with increasing salt, Kanemasa et al. (93) suggested that branched fatty acids and cardiolipin may act as a barrier against high ionic strength, or enhance active transport. Planococcus does not exhibit a direct increase in the amount of branching with increasing salt concentration in the medium, but there appears to be an interesting relationship between the amount of branching and the extent of desaturation within the fatty acids. In salt concentrations above and below the optimum (3%), similar trends in the proportions of unsaturated and branched acids were observed. An examination of Fig. D1 shows that for the fatty acids from the major, and many of the minor, phospholipid components, an increase in the proportion of unsaturated fatty acids is balanced by a decrease in the proportion of branched acids and vice versa. Since branched and unsaturated fatty acids have similar effects on the packing of the membrane lipids and hence on the fluidity, this probably reflects an attempt by the cells to maintain a constant level of fluidity within the membrane under conditions where one of the enzymes which normally controls these processes is influenced by the changing salt concentration; i.e. if synthesis and/or incorporation of unsaturated fatty acids is inhibited by salt, then the amount of branched fatty acid incorporated in the membrane is increased to compensate. In most cases the proportion of branched fatty acids increases at salt concentrations above and below the 3% value, and it may be that an increase in branched acids at the expense of unsaturated acids allows the membrane to function normally under adverse conditions.

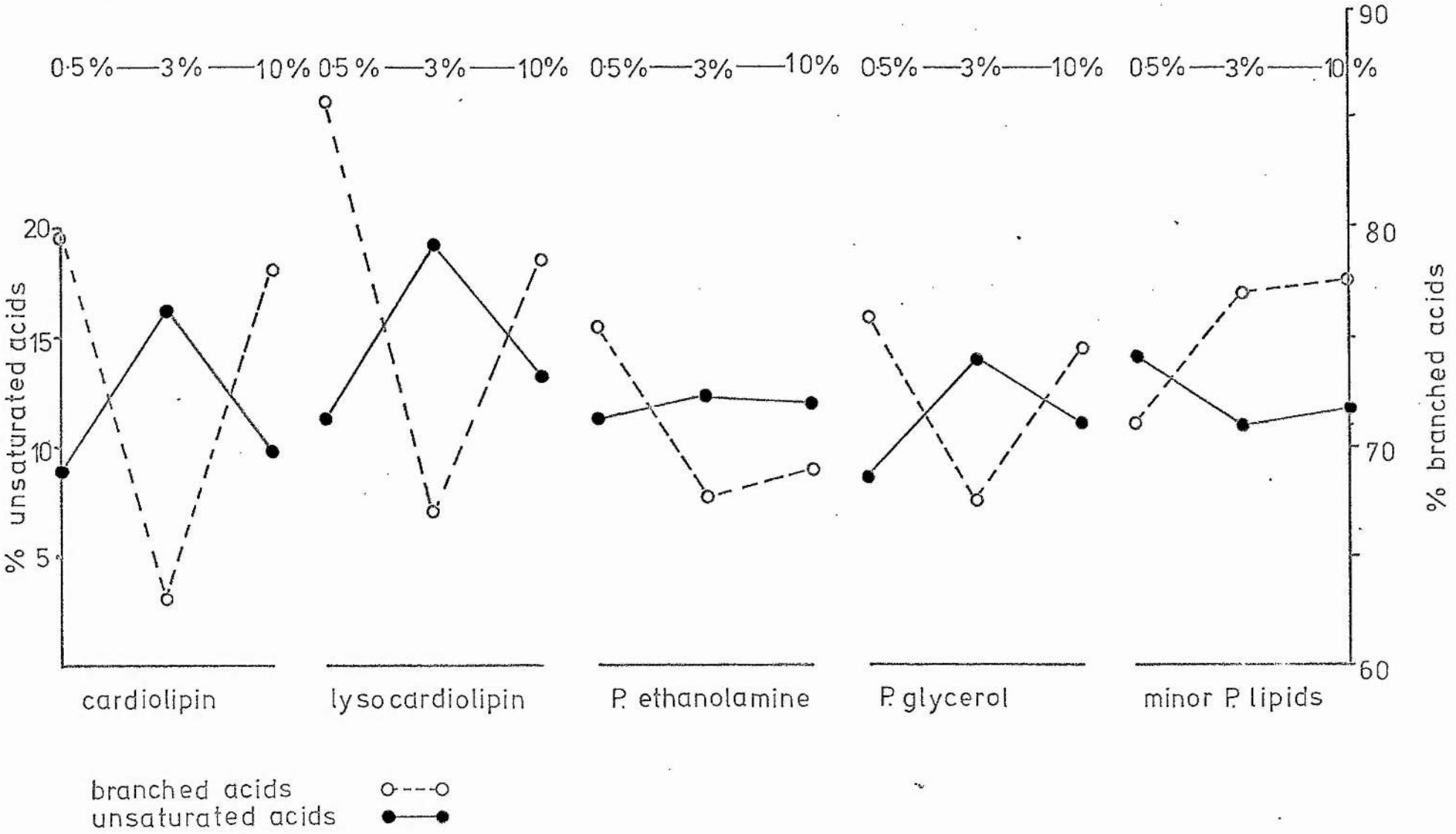
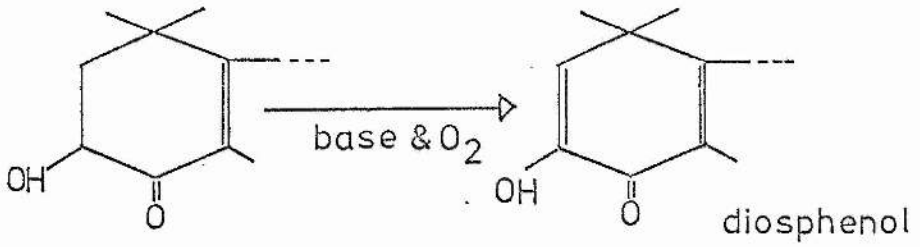


Fig. D1: A comparison of the percentages of branched and unsaturated fatty acids associated with the individual phospholipids from the three membrane types

The degree of carotenoid biosynthesis in Planococcus was influenced by the salt concentration in the medium. Increasing salt decreased the total amount of carotenoid synthesised, but also increased the number of oxygen functions on the carotenoid which was produced. Carotenoid production increased with age and reached a peak at the same time as maximum cell numbers were achieved in the culture. Other workers report similar findings with other microorganisms (94,104). At all three salt concentrations, after stationary phase was reached, the total amount of carotenoid declined rapidly, although this was mainly due to a decrease in the less polar components. However, in cells grown in the presence of 10% sea salt, polar pigments increased after stationary phase had been reached (Fig.R60). An accumulation of polar carotenoid material during stationary phase has been observed by other workers, although the polar material was carotenoid glycoside (105,106). The carotenoids of Planococcus were extracted from a 48 h culture and consequently contained only small amounts of non-polar material (Fig.R62). β carotene was thought to be the parent hydrocarbon. Difficulty was experienced during the purification, as the majority of the material would not extract into diethyl ether after saponification. Infra-red and visible absorption spectra had indicated the presence of carbonyl groups. Jensen reported (107) that the presence of material which cannot be extracted into diethyl ether after saponification may be indicative of diosphenols in the aqueous methanol phase and that these compounds can be extracted into diethyl ether after the pH has been lowered to pH 4 - 5 with acetic acid. Diosphenols are formed from carotenoids having hydroxyl and oxo substitutions in the 3 and 4 positions on the same ring as shown below.



diosphenol

Base and oxygen, both present during saponification, provide the conditions for the reaction to take place. Chemical tests revealed that these compounds were in fact present. Complete identification of all the carotenoids present was not possible due to the extreme lability of some of the non-polar fractions, and the fact that only tentative identification can be made without the aid of mass spectrometry and n.m.r. facilities which were not available. Identification of most of the components was made on the basis of the information gained from chemical tests, chromatographic mobilities and infra-red and visible absorption spectral characteristics. On this basis, a tentative biosynthetic route (with a possible variation) for all of these compounds has been drawn and this is shown in Fig. D2. Those compounds enclosed in brackets were not detected but this could be due to the fact that they are present merely as transient intermediates. Diosphenols are probably not naturally occurring compounds, but are artifacts produced on saponification from their relatively base/oxygen-unstable 3'hydroxy 4'oxo precursors. This seems to be borne out by the results from this microorganism, in that diosphenols were not detected unless the carotenoids had been saponified, but the diosphenol precursors were identified. Furthermore, saponification of individual diosphenol-precursor fraction (e.g. 'non-saponified' fractions 3 and 4) showed that the products of this procedure were only ether-extractable after acidification.

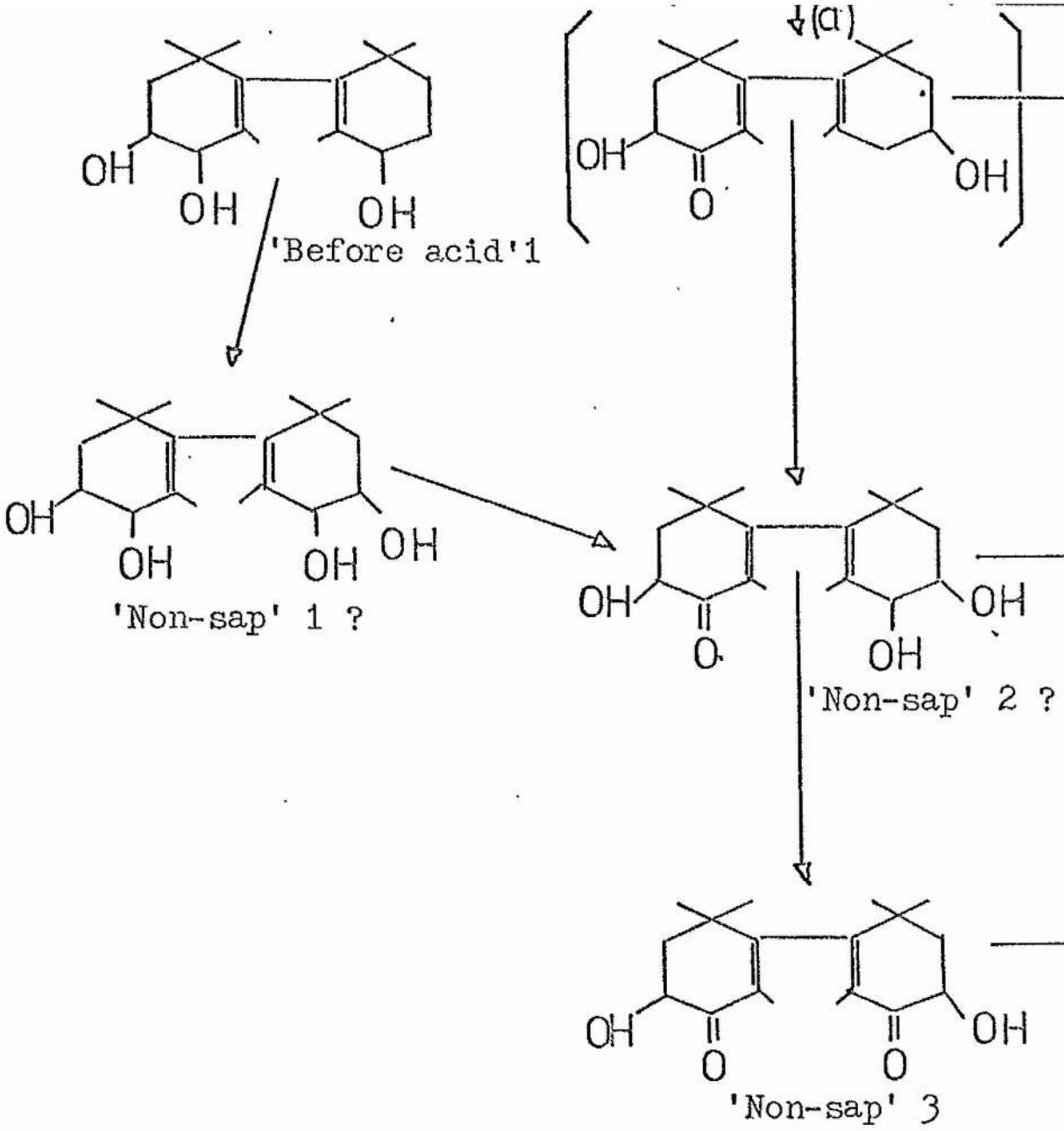
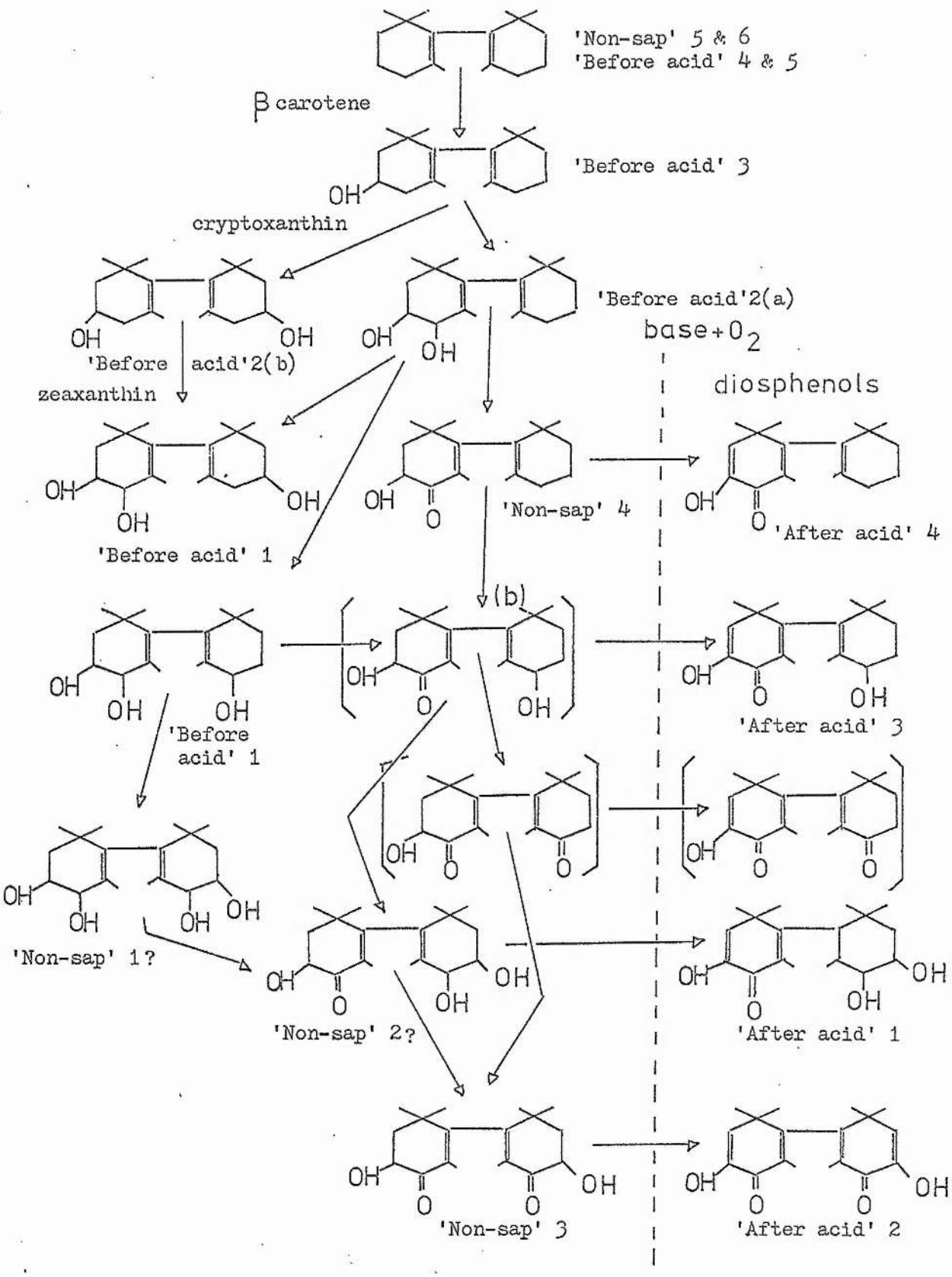


Fig. D2: Proposed pathway of biosynthesis of caroten

Brackets indicate that these compounds were
chemical analysis.

Pathway (a) would seem to be the most likel
of the components were accounted for in the


'Non-sap' 5 & 6
'Before acid' 4 & 5
β carotene
'Before acid' 3
cryptoxanthin
'Before acid'2(a)
base+O2
diosphenols
'Before acid'2(b)
zeaxanthin
'Non-sap' 4
'After acid' 4
'Before acid' 1
(b)
'After acid' 3
'Before acid' 1
'Non-sap' 1?
'Non-sap' 2?
'After acid' 1
'Non-sap' 3
'After acid' 2

The atomic absorption results showing the proportions of Ca^{++}, Mg^{++}, K^{+} and Na^{+} ions in whole cells grown in the three different salt concentrations show that Na^{+} ions are the predominant cation within the cell. Christian and Waltho (14) found Na^{+} ion concentrations higher than K^{+} ion concentrations within the cell in two of the six microorganisms they examined. The increase in ash content of whole cells indicates that the internal salt concentration probably compares with that in the external medium, as was found by Christian and Waltho (14) in the microorganisms they examined. Although internal ion concentrations in Planococcus were not determined, it appears that K^{+} ions are concentrated within the cells as they constitute a much greater proportion of the total ions within the cell that they do in sea salt. The results also showed that monovalent ions were in the majority in the whole cell, whereas in membranes, divalent ions predominated.

The variation in size observed in the different salt concentrations remains unexplained. 'Elephant cells' were observed in the 3% and 10% type cultures, with more in the 10% than in the 3% type. During lag phase all the cells in the 3% and 10% cultures appeared very large. An increase in the size of many bacteria has been observed during lag phase and this represents the cells increasing in size before dividing. However, this does not account for the overall increase in the size of the average single cells in Planococcus as the concentration of salt in the growth medium was increased. The appearance of 'elephant' cells and clumps of 6 - 8 cells which had divided but not separated indicates that salt may be influencing the division and separation processes in such a way that the septa form across some cells but separation does not take place.

To summarise, Planococcus 316 cells grew well in sea salt concentrations between 0.5% and 10%, at pH values between 7.0 and 9.0, and exhibited dual pH optima. Membranes prepared from cells grown in the three concentrations of sea salt (0.5%, 3% and 10%) had similar chemical compositions to those reported for other gram-positive cocci. Cells grown in the 3% sea salt concentration contained membranes with a higher protein : lipid ratio and RNA content than the membranes from cells grown in the 0.5% and 10% concentrations. Amino acid analysis of the membrane proteins showed that the composition remained virtually unchanged in the three membrane types. The ratio of acid : basic amino acid residues was nearer to the figures reported for non-halophiles than for those of the extreme halophiles. Examination of the lipids showed that phospholipids predominated to the extent of about 70% of the total lipids. Cardiolipin and lysocardiolipin were the major phospholipids with phosphatidyl ethanolamine, phosphatidyl glycerol, phosphatidyl serine, and two unidentified phospholipids present in smaller quantities. Glycolipids were found to constitute only a very small proportion of the total lipids in all three membrane types. A decrease in the number of glycolipid types as well as in the total amounts of glycolipid present was observed with increasing salt in the growth medium. The neutral lipids contained long chain alcohols, mono-, di- and tri-glycerides, as well as relatively large amounts of squalene. The major fatty acid associated with the lipids was found to be a branched saturated C_{15} acid which constituted 50 - 70% of the total fatty acids in most fractions. Although increasing salt in the medium produced changes within the proteins and lipids in the membranes, these changes were not such that they could be interpreted as an increase in the halophilic nature of the membrane. The carotenoids were shown to be derived from β carotene and to consist

mainly of 3'hydroxy 4'oxo compounds, although the extent of polar substitution was shown to be dependent on both culture age and the concentration of salt in the medium. Analysis of the cations associated with the membrane and whole cells showed that in the former, divalent cations predominated over monovalent, whereas in the latter the reverse was true, with Na^+ as the major cation. The observed increase in the size of cells grown in high salt concentrations remains unexplained, but may be due to salt interfering with the division and separation processes occurring within the cells.

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