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To Anne,

my wife

ACKNOWLEDGEMENTS

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SUMMARY

Part One of this thesis describes the structural elucidation of a sulfated tetraglycosyl diether (GL-1), a tetraglycosyl diether (GL-2) and a triglycosyl diether (GL-3) found in the membrane lipids of the extreme halophile Halobacterium cutirubrum. The ammonium salt of the sulfated tetraglycosyl diether was found to have the molecular formula $C_{67}H_{127}O_{26}S \cdot NH_4$, and on strong acid hydrolysis it yielded galactose, mannose and glucose in mole ratio 2:1:1, as well as 2,3-di-O-phytanyl-sn-glycerol. The infrared spectrum indicated the presence of a sulfate group; mild acid methanolysis resulted in the formation of the desulfated tetraglycosyl diether. The sequence and linkage positions of the sugars and the position of the sulfate group were determined by permethylation analysis of the sulfated tetraglycosyl diether and of its partial methanolysis products. The configuration of the glycosidic linkages was established by CrO_3 oxidation studies and optical rotation measurements. The results obtained established the structure of the sulfated tetraglycosyl diether (GL-1) as 2,3-di-O-phytanyl-1-O-[Galp-(3'SO_4^-)β1'→6'Manp(3'←1'αGalf)α1'→2'Glcpα]-sn-glycerol. The structure of the naturally occurring tetraglycosyl diether (GL-2) was shown to be 2,3-di-O-phytanyl-1-O-[Galpβ1'→6'Manp(3'←1'αGalf)α1'→2'Glcpα]-sn-glycerol and that of the triglycosyl diether (GL-3) was shown to be 2,3-di-O-phytanyl-1-O-[Galpβ1'→6'Manpα1'→2'Glcpα]-sn-glycerol. The latter glycolipid (GL-3) is thus derived from the major sulfated triglycosyl diether (GLS) with structure 2,3-di-O-phytanyl-1-O-[Galp-(3'-SO_4^-)β1'→6'Manpα1'→2'Glcpα]-sn-glycerol. The structural relationship between GL-1 and GLS suggests that the sulfated tetraglycosyl diether is derived biosynthetically by transfer of a galactofuranosyl residue to the mannosyl residue of GLS.

Part Two presents the results of further investigations of the lipid composition of H. cutirubrum red and purple membranes. Two-dimensional thin layer chromatography of the polar lipids showed that both membranes contained the 2,3-di-O-phytanyl-sn-glycerol ether analogues of phosphatidylglycerophosphate (PGP), phosphatidylglycerosulfate (PGS) and phosphatidylglycerol (PG), the sulfated triglycosyl diether (GLS), the triglycosyl diether (GL-3) and an unidentified glycolipid sulfate (GL-4). The presence of the latter compound in the lipids of H. cutirubrum has not been previously reported. In contrast, Kushwaha et al., (1975a) observed that the sulfated lipid components, namely GLS and PGS, were present in the purple membrane but not in red membrane. Radioisotopic studies suggested that the apparent absence of GLS in red membrane may have resulted from the occurrence of desulfation during membrane isolation, most likely during the dialysis procedure.

Part Three is a study of the physical properties of the lipids and membranes of extremely halophilic bacteria. The ^{31}P-NMR powder patterns observed for purple and red membranes, purple membrane lipids, total polar lipids, PGP and a PGP + GLS (2:1; w/w) mixture indicated that in each case the lipids had a bilayer arrangement. Freeze-fracture electron microscopy of aqueous dispersions of GLS, GL-3, total polar lipids and a PGP + GLS (2:1; w/w) mixture revealed the presence of multilamellar liposomes, in agreement with the present ^{31}P-NMR studies and previous osmometry and negative staining electron microscopy studies (Chen et al., 1974). In contrast, freeze-fracture electron microscopy of PGP in aqueous dispersion showed the presence of multilamellar structures which were not homogeneous with respect to morphology. The ^{31}P-NMR spectra and freeze-fracture elec-

tron micrographs of PG, a minor phospholipid component, indicated a vesicle type of structure. The nature of the structures observed for both PGP and PG would explain the inability of these phospholipids to behave as ideal osmometers in aqueous dispersion, as reported by Chen et al., (1974).

Temperature dependence studies of the ^{31}P-NMR spectra of PGP dispersions and purple membrane suspensions showed that in each system bilayer structure was preserved up to 60°C. In agreement with previous studies (Chen et al., 1974; Jackson and Sturtevant, 1978a) but in contrast with others (Degani et al., 1978) there was no evidence for a lipid phase transition over the range 5°-60°C. These studies also indicated that in purple membrane interaction of PGP with bacteriorhodopsin and neutral lipids results in a reduced overall motion of the phospholipid.

RESUME

La première partie de cette thèse décrit la détermination des structures chimiques d'un tétraholoside sulfaté (GL-1), d'un tétraholoside (GL-2) et d'un triholoside qui sont trouvés parmi les lipides membranaires d'un halophile extrême, Halobacterium cutirubrum. Nous avons trouvé que le sel d'ammonium du tétraholoside sulfaté (GL-1) a la formule brute $C_{67}H_{127}O_{26}S.NH_4$, et après méthanolyse il se forme en milieu fortement acide, un mélange de galactose mannose et glucose dans la proportion 2:1:1; aussi que du 2,3-di-O- phytanyle-sn-glycérol. Le spectre infrarouge indique la présence d'un groupement sulfate; par méthanolyse en milieu faiblement acide. Nous avons observé la formation du tétraholoside désulfaté. Nous avons déterminé la séquence et la position des liaisons des sucres et celle du groupement sulfate par analyse de méthylation complète du tétraholoside sulfaté et de ses produits de méthanolyse en milieu faiblement acidique. La configuration des liaisons glucidiques a été établie par étude de l'oxydation par CrO_3 et par mesure du pouvoir rotatoire. Ces résultats ont établi pour le tétraholoside sulfaté la structure suivante: 2,3-di-O-phytanyle-1-O-[Galp-(3'SO_4)β1→6'Manp(3'←(αGalf)α1'-2'GLcpα]-sn-glycérol. Nous avons aussi trouvé que le tétraholoside nature (GL-2) a la structure 2,3-di-O-phytanyle-1-O-[Galpβ1'→6'Manp(3'←1'αGalf)α1'-2'Glcpα]-sn-glycérol et le triholoside (GL-3) a la structure 2,3'-di-O-phytanyle-1-O-[Galpβ1'→6'Manpα1'→2'Glcpα]-sn-glycérol. Donc, le triholoside (GL-3) est dérivé du triholoside sulfaté majeur (GLS) qui a la structure 2,3-di-O-phytanyle-1-O-[Galp-(3'-SO_4)β1'→6'Manpα1'→2' Glcpα]-sn-glycérol. La relation structurale entre GL-1 et GLS suggère que

le tétraholoside sulfaté est formé par transfert du groupement galacto-furanosyle au groupement mannosyle du GLS.

La deuxième partie présente les résultats des examens additionnels des compositions lipidiques des membranes poupre et rouge du Halobacterium cutirubrum. La chromatographie sur couche mince bidimensionnelle des lipides polaires a démontré que les deux membranes contiennent les dérivés du 2,3-di-O-phytanyl-sn-glycérol de phosphatidylglycérophosphate (PGP), de phosphatidylglycérosulfate (PGS) et de phosphatidylglycérol (PG), le triholoside sulfaté (GLS), le triholoside (GL-3) et un glycolipide sulfaté inconnu (GL-4). La présence de GL-4 parmi les lipides de H. cutirubrum n'a jamais été rapportée. Par contraste, Kushwaha et al., (1975a) ont observé que les lipides sulfatés, à savoir GLS et PGS sont trouvés dans la membrane poupre mais ils sont absents de la membrane rouge. Les études radioisotopiques ont suggeré que l'absence évidente de GLS dans la membrane rouge peut résulter d'une réaction de désulfatation pendant la préparation des membranes, probablement pendant la procédure de dialyse.

La troisième partie comprend une étude des propriétés physiques des lipides et des membranes des bactéries halophiles extrêmes. Les spectres de la R.M.N. du ^{31}P de la membrane poupre, de la membrane rouge, des lipides de la membrane poupre, des lipides polaires, de PGP et d'un mélange de PGP + GLS (2:1; w/w) ont montré que les lipides sont arrangés en dicouche. La microscopie électronique à cryodécapage des dispersions dans l'eau de GLS, de GL-3, des lipides polaires et d'un mélange de PGP + GLS (2:1; w/w) a révélé la présence de liposomes multilamellaires, résultat compatible avec ceux, obtenus par R.M.N. du ^{31}P, par osmométrie et en microscopie électronique par coloration négative (Chen et al., 1974).

Par opposition la microscopie électronique à cryodécapage des dispersions de PGP dans l'eau a indiqué la présence de structures multilamellaires morphologiquement non homogènes. Les spectres en R.M.N. du ^{31}P et la microscopie électronique à cryodécapage du PG, un phospholipide mineur, ont indiqué la présence de vésicules. Le type de structure que nous avons observé pour PGP et PG expliquerait le fait que ces phospholipides n'ont pas les propriété des osmomètres fermés dans l'eau, comme il l'a été publié (Chen et al., 1974). Les études de la température des spectres en R.M.N. du ^{31}P pour PGP et la membrane poupre dans l'eau ont montré que dans chaque système, la structure bilamellaire est préservée jusqu'à 60°C. Conformément avec les études précédentes (Chen et al., 1974; Jackson et Sturtevant, 1978a) mais par opposition avec d'autres travaux (Degani et al., 1978), il n'y a pas de preuve d'une transition de phase sur l'étendue 5°-60°C. Ces études ont également indiqué que dans la membrane poupre la réduction du mouvement des phospholipides résulte de l'interaction du PGP avec la bactériorhodopsine et les lipides non polaires.

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PART ONE

STRUCTURAL STUDIES ON THE GLYCOLIPIDS OF H. CUTIRUBRUM: SULFATED TETRAGLYCOSYL DIETHER (GL-1), TETRAGLYCOSYL DIETHER (GL-2), AND TRIGLYCOSYL DIETHER (GL-3)

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LIST OF ABBREVIATIONS

ATP	Adenosine-5'-triphosphate
DNA	Deoxyribonucleic acid
ESR	Electron spin resonance
Galf	Galactofuranosyl
Galp	Galactopyranosyl
GC-MS	Gas chromatography-mass spectrometry
GlcNac	N-acetylglucosamine
Glcp	Glucopyranosyl
GL-1*	Sulfated tetraglycosyl diether
GL-2*	Tetraglycosyl diether
GL-3*	Triglycosyl diether
GLS*	Sulfated triglycosyl diether
Manp	Mannopyranosyl
NMR	Nuclear magnetic resonance
PG*	Phosphatidylglycerol
PGP*	Phosphatidylglycerophosphate
PGS*	Phosphatidylglycerosulfate
rRNA	Ribosomal ribonucleic acid
tRNA	Transfer ribonucleic acid
TLC	Thin layer chromatography

*Derivative of 2,3-di-O-phytanyl-sn-glycerol

GENERAL INTRODUCTION

Extremely halophilic bacteria, methanogenic bacteria and certain thermoacidophilic bacteria have been designated as "archaebacteria" and represent a line of early evolutionary divergence from eukaryotes and prokaryotes (Woese et al., 1978). These organisms can exist under conditions that are believed to represent those of primitive earth: i.e., the methanogens are strict anaerobes, the thermoacidophiles occupy a "hot acid niche" while the extreme halophiles require a high salt environment.

Archaebacteria differ from both prokaryotes and eukaryotes in several aspects of their molecular biology, for example: the presence of membrane lipids with isoprenoid side chains linked to glycerol by ether linkage rather than ester-linked fatty acids; the absence of the peptidoglycan-type of cell wall found in most bacteria and the presence of certain characteristic tRNA and rRNA sequences (Woese et al., 1978). Furthermore, some species of extreme halophiles contain bacteriorhodopsin, a retinal-protein complex analogous to the visual pigment rhodopsin but functioning as a light-energy transducer (Stoeckenius et al., 1979).

During the last fifteen years, a large portion of the work in this laboratory has dealt with the structure and function of the lipids of extremely halophilic bacteria. The polar lipids were shown to be derivatives of 2,3-di-O-phytanyl-sn-glycerol and the complete structures of the phospholipids and a major glycolipid sulfate have been determined (Kates, 1978). Also, physical studies of the polar lipids indicated that the major glycolipid sulfate is required for the formation of stable lipid bilayers (Chen et al., 1974). This thesis is concerned with further studies

of the chemical structure and polymorphism of lipids of halophilic bacteria and with an investigation of the lipid composition of red and purple membranes. Before proceeding with the experimental section, the characteristics of extremely halophilic bacteria and the properties of their membranes and lipids will be reviewed.

I. Microbiology of Halophilic Bacteria

1. Classification

Halophilic bacteria, as the name implies, are bacteria that require sodium chloride, in various concentrations, for survival and growth. They may be divided into two classes (Baxter and Gibbons, 1956):

(i) moderately halophilic bacteria (moderate halophiles) which grow in media having 0.5 - 3.5 M NaCl;

(ii) extremely halophilic bacteria (extreme halophiles) which require 4M to 5M NaCl for optimal growth.

The extreme halophiles are subdivided into two genera, namely Halobacteria and Halococci.

The genus Halobacterium consists of Gram-negative bacilli which grow optimally in 25% NaCl (4 M) and require at least 15% NaCl (2.5 M) for growth. The organisms can be non-motile or motile by means of a tuft of polar flagellae. Halobacteria have long been described as being obligate aerobes, but recently H. halobium has been shown to be capable of growth in anaerobic conditions either by fermentation of arginine to ornithine or by illumination provided that the cells contain sufficient purple membrane (Hartmann et al., 1980). Some disagreement exists concerning the number of species in the genus Halobacterium. Only two species are listed in Bergey's Manual (Eighth Edition, 1974) namely

H. salinarium and H. halobium. However, the previous edition (Seventh Edition, 1957) listed five species: H. cutirubrum, H. halobium, H. salinarium, H. marismortui and H. trapanicum. It has been suggested that the apparent homogeneity may result from the isolation methods, and that major revision is required concerning the taxonomy of the genus Halobacterium (Rodriquez-Valera et al., 1980).

Halococci are Gram-negative, non-motile, non-spore-forming obligate aerobes which grow optimally in about 20% NaCl (3.2 M) and require at least 12% NaCl (2 M) for growth; in comparison with Halobacteria they seem to be more resistant to osmotic damage. These organisms are classified in the genera Halococcus (Bergey, 1974).

Extreme halophiles grow very slowly, and generation times of about 7 h and 15 h have been reported for Halobacteria and Halococci, respectively (Larsen, 1967).

2. Salt Requirements

Progressive dilution of the saline environment of Halobacteria from 4 M to 1.5 M results in a change from a rod shape to a spherical one; further dilution below 1-1.5 M results in complete lysis. The factors responsible for this lysis include the negative charges of the carboxyl groups of aspartic and glutamic acids present in the membrane proteins and the negative charges of the acidic lipids. Replacement of sodium ion by potassium or ammonium ions results in loss of rod structure, which suggests that sodium ion is essential for maintenance of the rod-shape in a manner which is not purely osmotic (Kushner et al., 1964). The protective effect of monovalent cations on the morphological integrity

of H. halobium was found to decrease in the same order as their hydration volumes ($Li^+ > Na^+ > K^+ > NH_4^+$), which suggests that the effect of salt concentration results from both electrostatic and osmotic factors (Soo-Hoo and Brown, 1967). Consistent with this effect is the observation that 0.5 M magnesium ion was found to be more effective than 1M sodium ion in preventing lysis of H. cutirubrum cells (Kushner, 1964).

In addition to 4M NaCl, growth media for Halobacteria usually contain 0.1 - 0.5M $MgSO_4$ and approximately 0.03M KCl.

II. Membranes of Extremely Halophilic Bacteria

1. Fractionation of the Cell Envelope

Dialysis of H. halobium suspensions against distilled water, followed by centrifugation of the dialyzate, gave the following four cell fractions (Stoeckenius and Rowen, 1967; Stoeckenius and Kunau, 1968):

(i) a soluble, colourless, lipid-free fraction which contained protein and hexosamines and was most likely cell wall material;

(ii) a red fraction which consisted of 40% lipid and 60% protein

(iii) a purple fraction of similar composition to (ii), which consisted of large membrane sheets;

(iv) a fraction of collapsed gas vacuoles, largely protein in nature

Fraction (i) and (ii) contained high proportion of aspartic and glutamic acid residues which was consistent with the theory that cell disruption at low salt concentration is due to the presence of a high charge density in the cell envelope. Fraction (ii) was later called

red membrane, the colour being due to the presence of the red pigments, bacterioruberins (C_{50} carotenoids). Disc gel electrophoresis of the red membrane showed the presence of many separate polypeptides (Kushwaha et al., 1975a), and this membrane probably carries out the usual membrane functions. The red membrane forms the largest portion of the membrane in cells grown under high aeration, but the purple membrane (fraction (iii)) can account for up to 50% of the cell membrane in illuminated, oxygen-starved cells. Further studies of purple membrane structure and function proved to be very exciting (Stoeckenius et al.,1979).

2. Purple Membrane

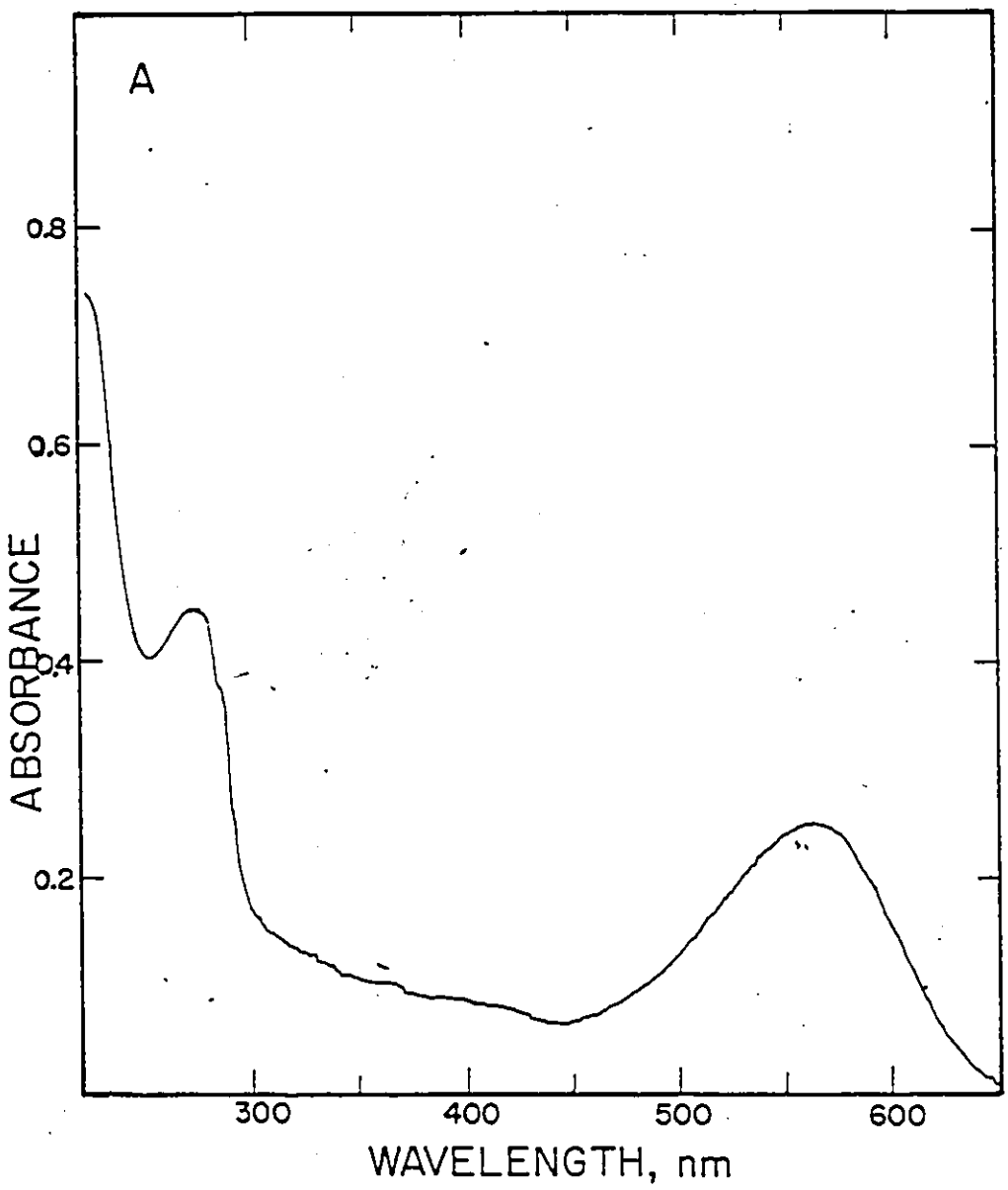
(a) Structure

A suspension of purple membrane shows a broad band at 560 nm and a stronger peak at 280 nm in the UV-visible spectrum (Figure 1). The purple colour has been shown to be due to retinal complexed with a single polypeptide (bacteriopsin) in a 1:1 molar ratio (see General Introduction, III, 3); because of its similarity to mammalian rhodopsin it was called "bacteriorhodopsin" (Oesterhelt and Stoeckenius, 1971). The available evidence suggests that the retinal is complexed to a lysine residue via a Schiff base (Oesterhelt and Stoeckenius, 1971; Mendelsohn et al., 1974).

Freeze-fracture electron microscopy of intact cells revealed that the purple membrane exists as a differentiated but continuous part of the cell membrane (Blaurock and Stoeckenius, 1971). X-ray diffraction data indicated that the protein molecules are arranged as trimers in a two-dimensional hexagonal lattice in a plane parallel to the membrane surface. Other features present in the diffraction pattern suggested

Figure 1

Ultraviolet-visible absorption spectrum of a purple membrane suspension in water (Kushwaha et al., 1975a).


A
0.8
0.6
0.4
0.2
ABSORBANCE
300
400
500
600
WAVELENGTH, nm

that the protein consisted mainly of α-helical segments (Blaurock and Stoeckenius, 1971) and that the lipids are present in a bilayer arrangement (Blaurock, 1975). Further analysis of electron diffraction patterns and electron images revealed that each protein has seven α-helices about 40 Å long and 10 Å apart traversing the plane of the membrane (Figure 2) (Henderson and Unwin, 1975). Studies of the dichroism induced by bleaching of the chromophore with polarized light indicated that protein rotational motion is very slow compared to the mobility of rhodopsin in rod outer segments (Razi Naqvi et al., 1973). Recently, the amino acid sequence of bacteriorhodopsin has been established, and the protein consists of a single polypeptide chain with a molecular weight of 26,000 and possessing 248 amino acid residues (Khorana et al., 1979).

(b) Light-Driven Proton Pumping

Oesterhelt and Stoeckenius (1973) postulated that purple membrane functions as a light-energy transducer. This was based on the observation that protons were released from both cells and purple membrane fragments upon illumination. Later experiments showed that light could induce ATP synthesis and that light driven proton gradients occurred across both intact cell membranes (Danon and Stoeckenius, 1974; Michel and Oesterhelt, 1976) and lecithin vesicle preparations containing purple membrane and $(Na^+ + K^+)$-ATPase (Racker and Stoeckenius, 1974). Also, it was shown that the action spectra for proton pumping and ATP synthesis in whole cells followed the absorption spectrum of purple membrane (Danon and Stoeckenius, 1974). These experiments demonstrated that bacteriorhodopsin can convert light energy into a proton gradient which can be used to drive ATP synthesis in accordance with the Mitchell hypothesis (Figure 3) (Stoeckenius et al., 1979).

Figure 2

Drawing showing the arrangement of α-helices in one protein molecule of the purple membrane, derived from electron microscopy. Lipids have been drawn to occupy their likely but not yet proven position (Henderson, 1977).

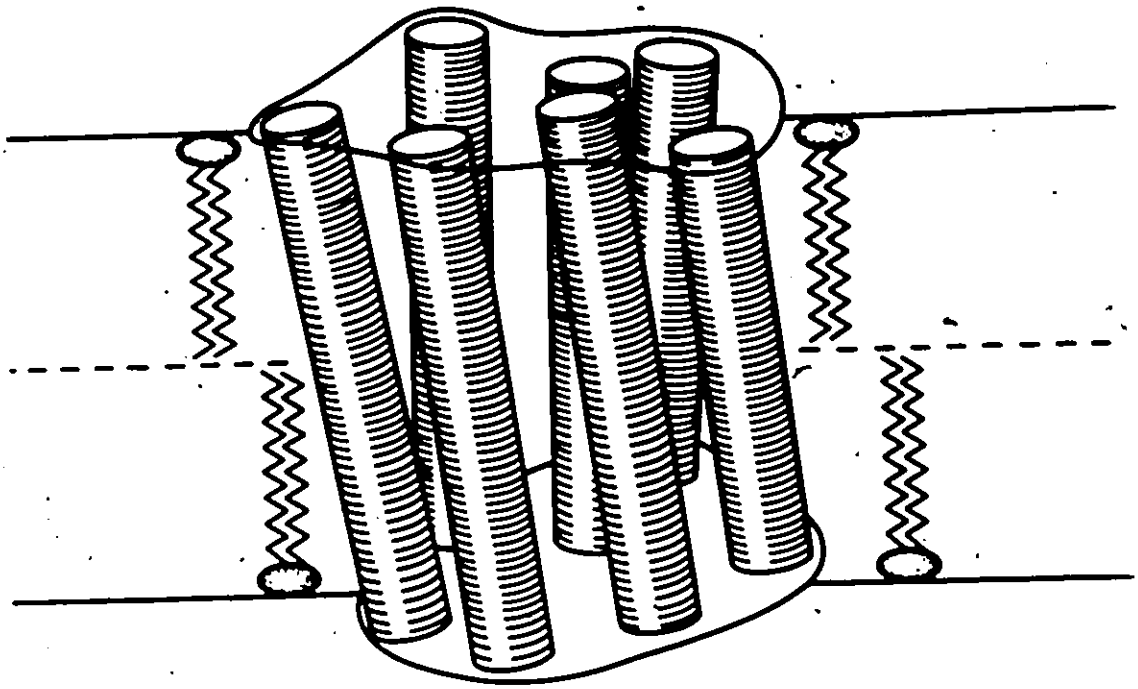
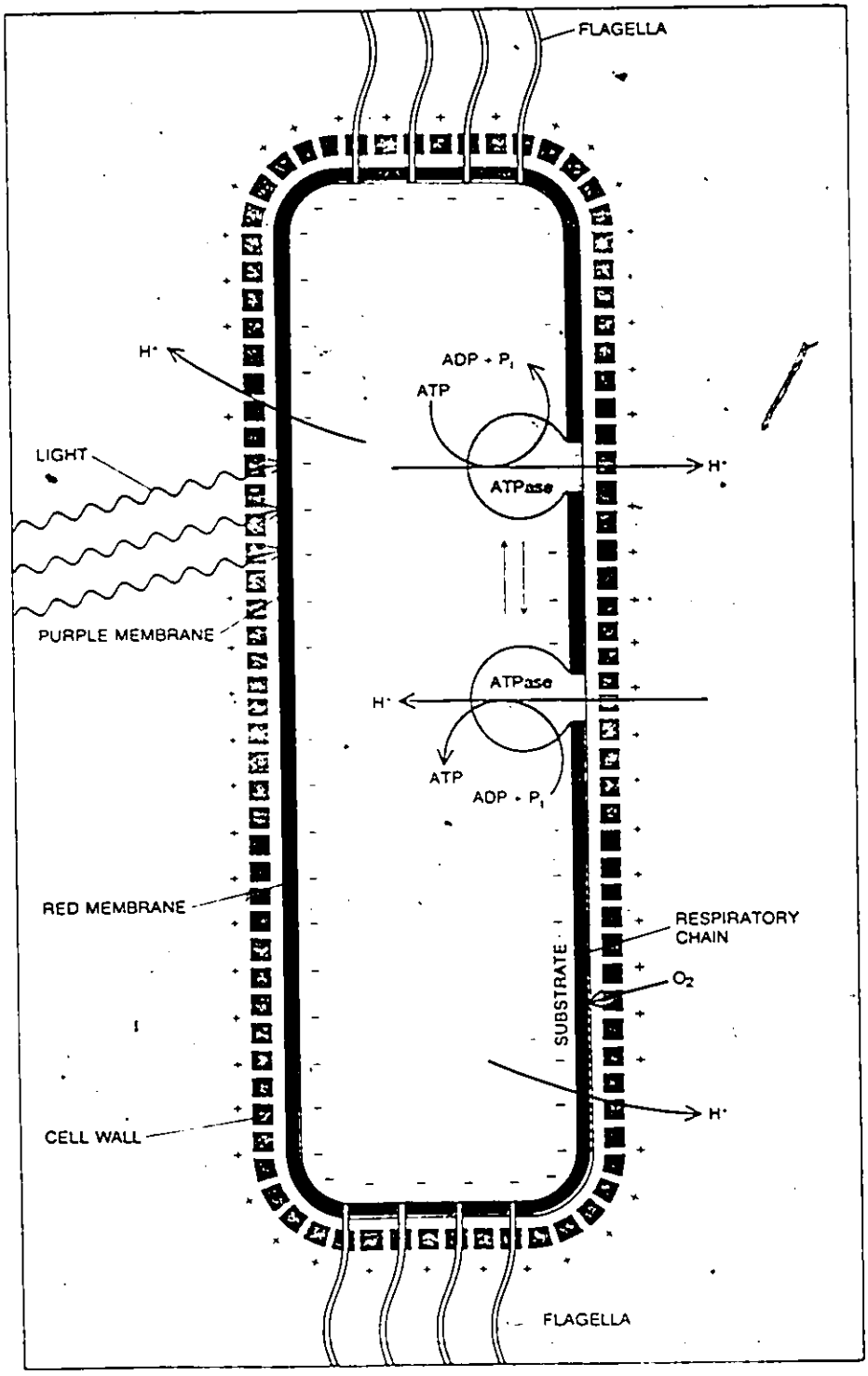


Figure 3

Diagram showing the relationship between proton transport and energy metabolism in a halobacterium cell. The cell membrane contains patches of purple membrane and also respiratory-chain pigments; either light or oxygen can promote the ejection of protons. The light-powered process is mediated by the purple membrane and the oxidative process by the respiratory chain provided that an oxidizable substrate is available. The resulting proton gradient drives a backflow of protons through the ATPase which synthesises ATP from ADP and inorganic phosphate (Stoeckenius, 1976).


FLAGELLA
H⁺
ADP + P$_i$
ATP
LIGHT
H⁺
ATPase
PURPLE MEMBRANE
ATPase
H⁺
ATP
ADP + P$_i$
RED MEMBRANE
RESPIRATORY CHAIN
SUBSTRATE
O$_2$
H⁺
CELL WALL
FLAGELLA

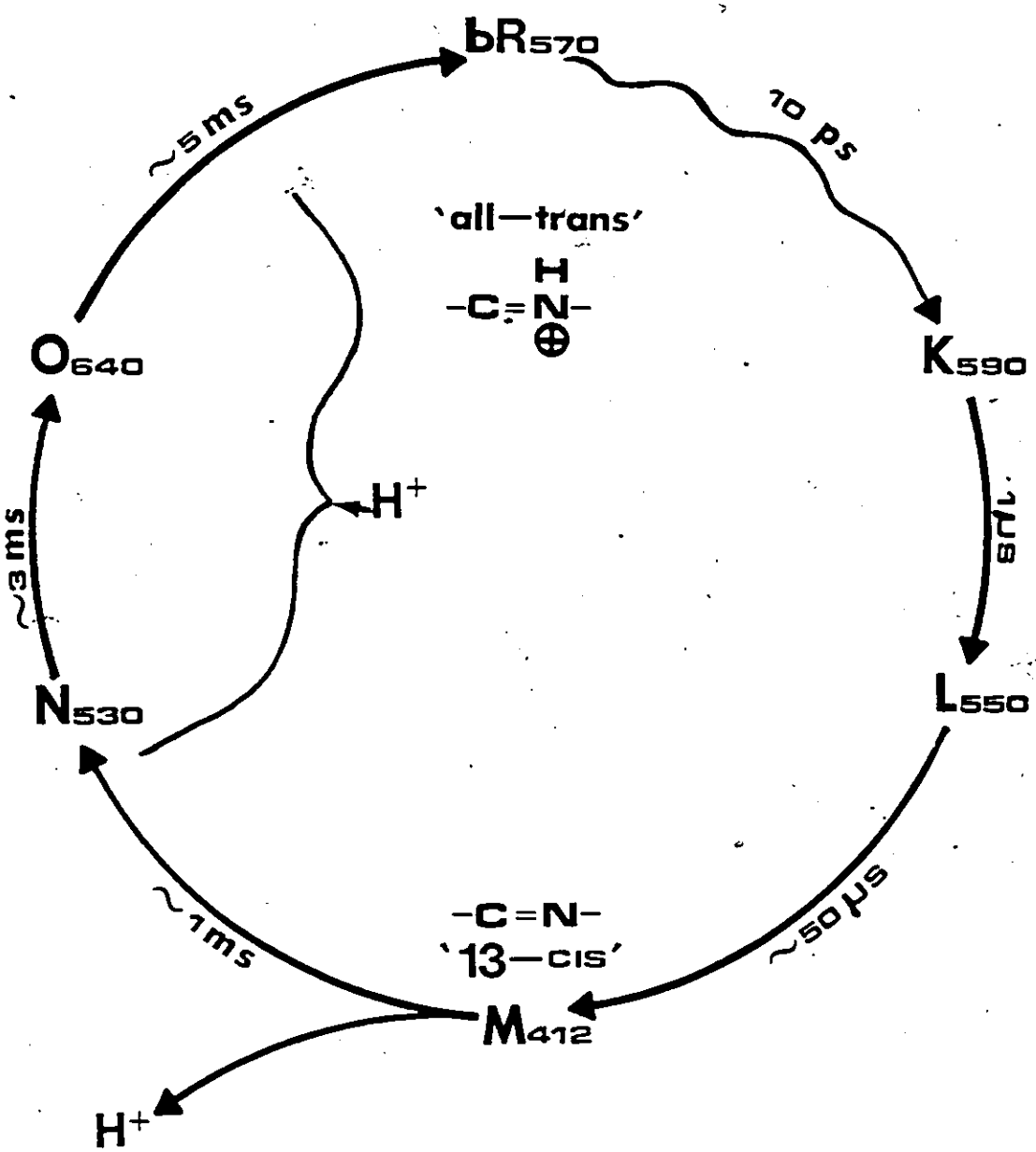
The function of bacteriorhodopsin is similar to the multicomponent electron transport chains of respiration and photosynthesis. However, electron transport is absent and proton translocation is accomplished by the comparatively simple protein-retinal complex mentioned above. Experimental evidence shows that the molecule undergoes a photochemical cycle involving several intermediates (Figure 4). The intermediate having the longest life is the 412 nm chromophore, and upon its appearance a proton is released into the medium; regeneration of the purple complex is associated with the uptake of a proton from the cytoplasm (Oesterhelt and Hess, 1973). It has been suggested that proton translocation results from conformational changes in bacteriorhodopsin which affect the pKa values of side chains arranged as a hydrogen bonded bridge (Stoekenius et al., 1979). Despite the concentrated efforts of several research groups, at present there is insufficient experimental proof for a detailed mechanism of bacteriorhodopsin action. Recently a second retinal-protein complex, called halorhodopsin, has been shown to function as a light-driven sodium pump in H. halobium (Greene and Lanyi, 1979; Weber and Bogomolni, 1981; Matsuno-Yagi and Mukohata, 1980).

(c) Biosynthesis

The purple membrane presents an excellent system for the study of membrane biosynthesis because its biogenesis is inducible and only one protein and two major polar lipid components are present. ^{32}P and ^{35}S labelling experiments indicated that the lipids of purple membrane are formed long before the start of retinal and bacterioopsin synthesis (Sumper et al., 1976). Using nicotine to inhibit retinal synthesis, the precursor of purple membrane has been shown to be a brown membrane

Figure 4

Photochemical cycle of light-adapted bacteriorhodopsin. Wavy lines indicate the light reaction. "All-trans" and "13-cis" indicate the retinal isomer extracted under conditions in which most of the pigment is present as bR_{570} and M_{412}, respectively (Stoeckenius, 1979).


bR570
~5 ms
10 ps
'all—trans'
H
–C=N–
⊕
O640
K590
H+
3 ms
~
3 µs
N530
L550
~1 ms
–C=N–
'13—cis'
~50 µs
M412
H+

containing bacterioopsin and a cytochrome-b type protein (Sumper et al., 1976). This brown membrane reacts with retinal and then crystallizes to form purple membrane in an ATP-requiring process. The synthesis of both retinal and bacterioopsin are separately induced by low oxygen tension, but the molecular mechanism of this is unknown.

III. Chemistry of the Lipids of Extremely Halophilic Bacteria

The total lipids of the extreme halophiles account for about 2.5-4% of the cell dry weight (Sehgal et al., 1962; Kates et al., 1965; Joo et al., 1968). The addition of acetone to a chloroform solution of the total lipids yields a "polar" lipid fraction as an insoluble precipitate (90% w/w of total lipids) and an acetone-soluble "non-polar" lipid plus bacterioruberins fraction (10% w/w of total lipids).

1. Polar Lipids

The acetone insoluble lipids were recognized as being atypical of Gram negative bacteria in four respects (Sehgal et al., 1962):

(i) the low nitrogen content (0.18%) indicated that nitrogenous lipids were present in only trace amounts;

(ii) an unusually high lipid phosphorus content (P = 4.3-4.6%) was present;

(iii) fatty acids were not released by mild alkaline hydrolysis (Dawson, 1954), which showed that the lipids were not derivatives of diacylglycerol;

(iv) Hydrolysis of the total polar lipids yielded 65-76% of a non-saponifiable material which was shown

later to be 2,3-di-O-[3',7',11',15'-tetramethyl-hexadecyl]-sn-glycerol (Kates et al., 1965; Joo et al., 1968; Faure et al., 1963). Each of the three asymmetric centres of the 3,7,11,15-tetramethyl-hexadecyl (phytanyl) groups had the R-absolute configuration (Kates et al., 1967a).

Chromatography of the polar lipids revealed the presence of two major components and several minor components (Figure 5) (Sehgal et al., 1962). All of the polar lipids characterized were shown to be derivatives of 2,3-di-O-phytanyl-sn-glycerol.

The major component (Spot 6, Figure 5) was an acidic phospholipid and accounted for about 60% (w/w) of the polar lipids. Elemental analysis and studies by degradative hydrolysis (Kates et al., 1965) indicated that the phospholipid had a phosphatidylglycerophosphate structure in which ether-linked phytanyl groups replaced the acyl groups. Chemical synthesis (Joo and Kates, 1969) yielded a lipid identical with the natural substance and established that its structure and configuration was 2,3-di-O-phytanyl-sn-glycerol-1-phosphoryl-3'-sn-glycero-1'-phosphate (PGP; Structure II, Figure 6). The configuration of both glycerol moieties is opposite to that of diacyl-PGP found in other bacteria (Slotboom and Bonsen, 1970). Although only two acid groups per molecule could be detected by NaOH titration with phenolpthalein indicator, NMR studies of permethylated PGP and PGP trimethyl ester and elemental analyses of the various salt forms of PGP have established that there are three ionizable P-O-groups per molecule (Hancock, 1972; Kates and Hancock, 1971).

One of the minor components (Spot 8, Figure 5) was a

Figure 5

Tracing of a chromatogram of the total lipids of H. cutirubrum cells and envelopes. The chromatogram was stained with Rhodamine 6G and viewed under ultraviolet light. The fluorescent colors are indicated by the following abbreviations: B, blue; G, grey; Y, yellow; O, orange. Broken lines indicate minor components (Kushner et al., 1964).

Identification of components:

Spot 1	sulfated tetraglycosyl diether (GL-1)*
Spot 2	sulfated triglycosyl diether
Spot 3	unidentified phosphatide
Spot 4	triglycosyl diether (GL-3)*
Spot 5	phosphatidyl glycerosulfate (PGS)
Spot 6&7	phosphatidyl glycerophosphate (PGP)
Spot 8	phosphatidyl glycerol (PG)
Spot 9	neutral lipids (including pigments)

*See Part One of this thesis

Solvent System:

Diisobutylketone-acetic acid-water (45:25:5; v/v/v) on silicic acid impregnated paper.

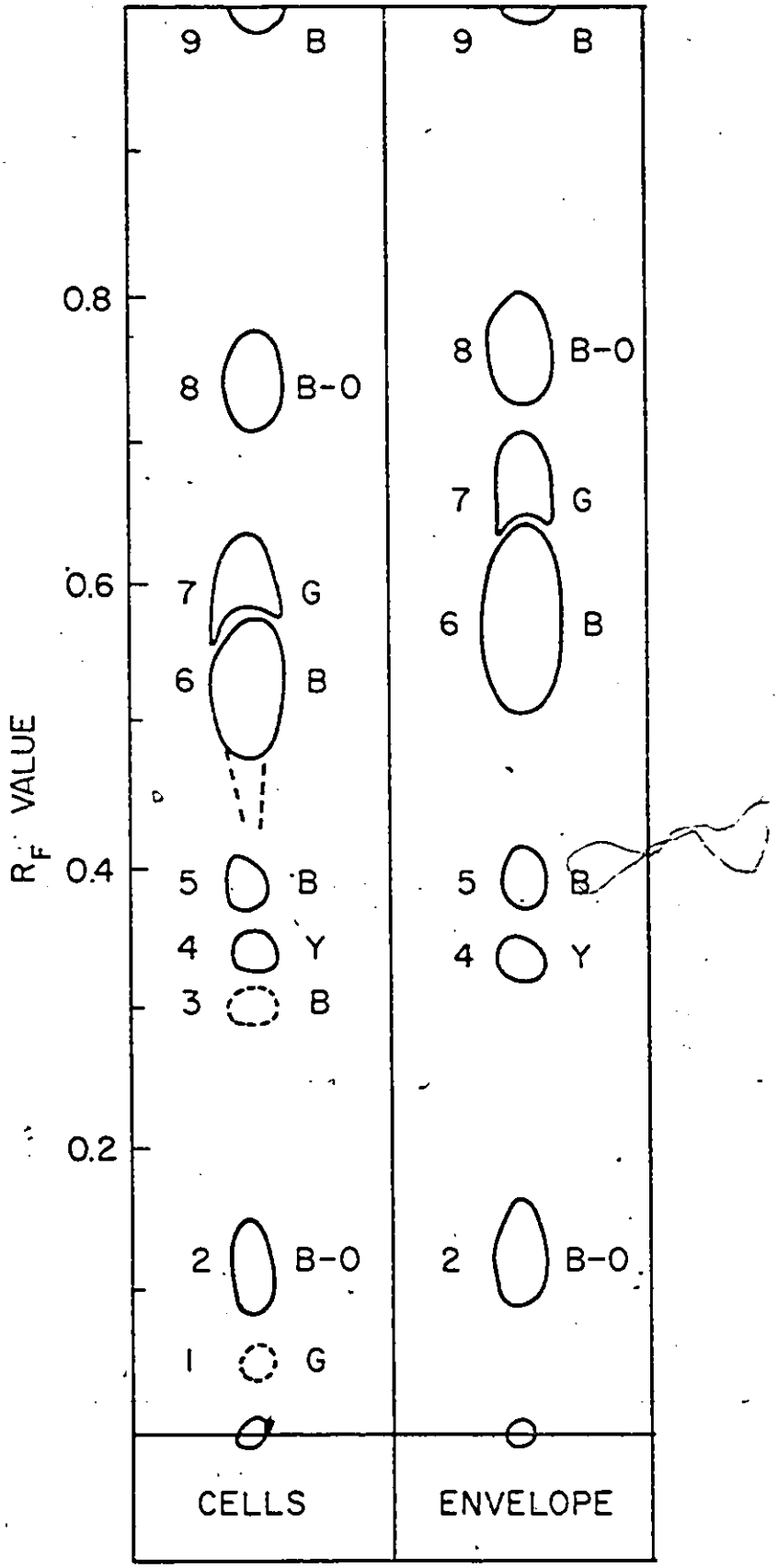

9 B
9 B
0.8
8 B-O
8 B-O
7 G
0.6
7 G
6 B
6 B
R_F VALUE
0.4
5 B
5 B
4 Y
4 Y
3 B
0.2
2 B-O
2 B-O
1 G
CELLS
ENVELOPE

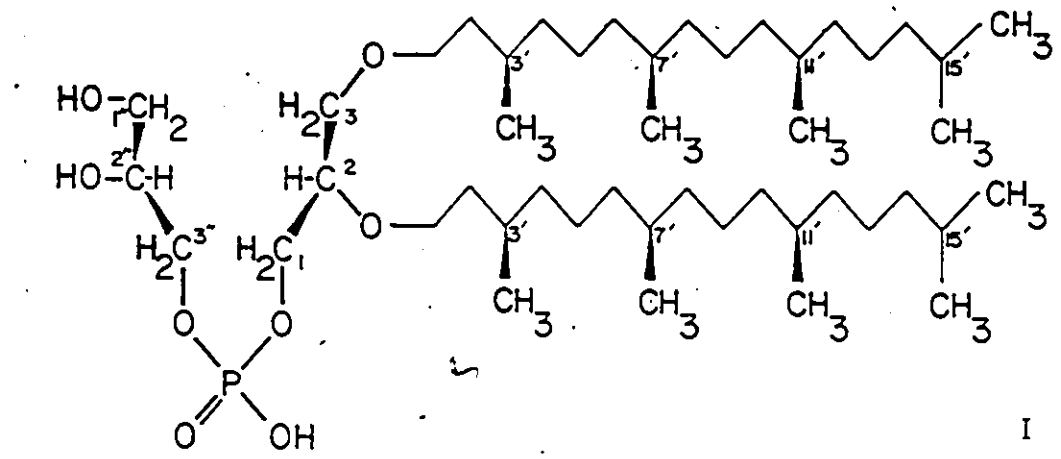
Figure 16

Projection formulae of:

I. 2,3-di-O-(3'R,7'R,11'R,15'-tetramethylhexadecyl)-sn-glycerol-1-phosphoryl-3'-sn-glycerol (PG).

II. 2,3-di-O-(3'R,7'R,11,R,15'-tetramethylhexadecyl)-sn-glycero-1-phosphoryl-3"-sn-glycero-1"-phosphoric acid (PGP).

III. 2,3-di-O-(3'R,7'R,11'R,15,-tetramethylhexadecyl)-sn-glycero-1-phosphoryl-3"-sn-glycero-1"-sulfuric acid (PGS).



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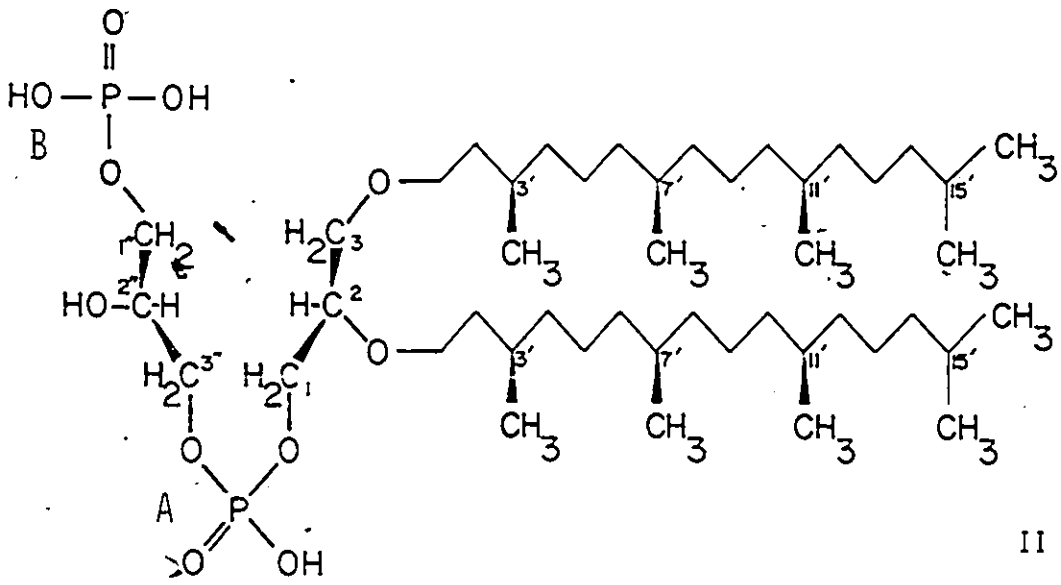
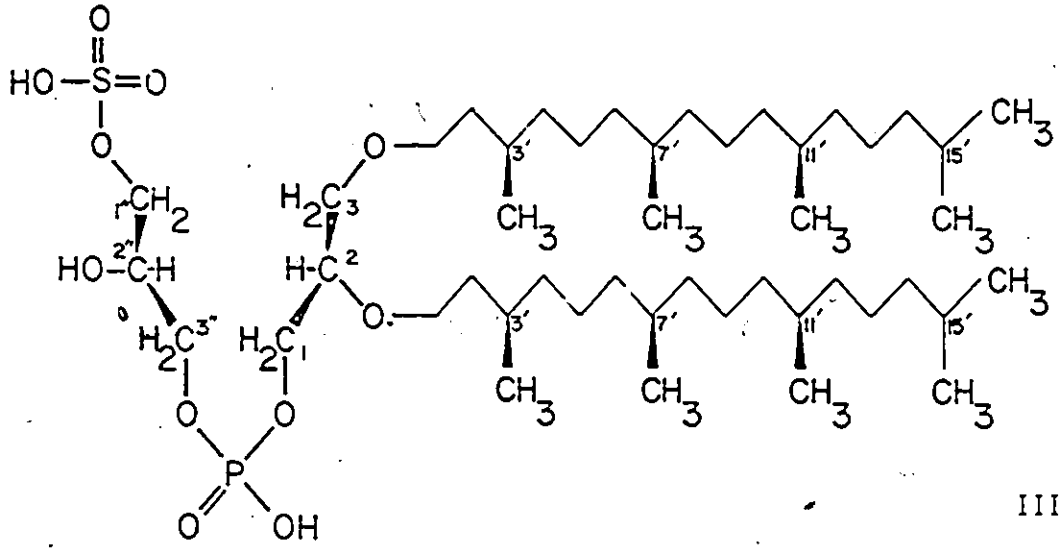


II



III

phospholipid accounting for ca. 4% of the polar lipids, and its structure was shown to be 2,3-di-O-phytanyl-sn-glycero-1-phosphoryl-3'-sn-glycerol (PG; Structure I, Figure 6) (Faure et al., 1964; Kates et al., 1966; Joo and Kates, 1968). A second minor phosphatide (Spot 5, Figure 5) accounted for ca. 4% of the polar lipids and contained sulfur in addition to phosphorus. Degradative studies and chemical synthesis showed its structure to be 2,3-di-O-phytanyl-sn-glycero-1-phosphoryl-3'-sn-glycero-1'-sulfate (PGS; Structure III, Figure 6) (Hancock and Kates, 1972, 1973). As in PGP, these minor phospholipids have glycerol moieties with configurations opposite to those found in other bacteria. Preliminary evidence suggested that a minor phospholipid found in H. halobium purple membrane is the diphytanyl ether analogue of cardiolipin with the structure bis(di-O-phytanyl-glycerophosphoryl)-glycerol (Ushakov et al., 1978). Further investigation is however necessary to establish this structure with certainty.

At least four glycolipids have been found among the polar lipids of H. cutirubrum and H. halobium. The second major polar lipid component was a sulfated glycolipid (GLS; Spot 2, Figure 5) accounting for 20-25% w/w of the polar lipids. Preliminary studies (Kates et al., 1967b) had shown that the compound contained sulfate, diphytanyl glycerol, glucose, galactose and mannose in equimolar amounts. Permethylation analysis indicated that the glucose was linked at position 2, the mannose at position 6, and the terminal galactose residue was sulfated at position 3. Because of the high positive optical rotation of the compound, it was concluded that all glycosidic linkages had the α-configuration. Later studies (Deroo, 1974; Kates and Deroo, 1973) established the sugar sequence and confirmed the sugar linkages given above. However, molecular rotation

data in comparison with calculated values indicated that the galactose residue had the β-configuration rather than the α-configuration. This was confirmed by the action of specific glycosidases on the terminal galman disaccharide released from the glycolipid by mild acid hydrolysis. The anomeric configurations of the sugar residues in the sulfated triglycosyl diether have been confirmed by ^{1}H-NMR of the permethylated compound (Falk et al., 1980). The complete structure of the glycolipid sulfate in H. cutirubrum is thus established as 2,3-di-O-phytanyl-1-O-[Galp-(3-SO4^{-})β1→6Manpα1→2Glcpα]-sn-glycerol (GLS; Structure IV, Figure 7).

A second glycolipid (GL-1; Spot 1, Figure 5) also contains sulfur and can be labelled by cells grown in the presence of [^{35}S] sulfate (Kates et al., 1968). However, it is much more polar than the sulfated triglycosyl diether, suggesting the presence of an additional carbohydrate or sulfate group. Investigation of the structure of GL-1 presented in this thesis showed it to be a sulfated tetraglycosyl diether. Another glycolipid (GL-2) co-chromatographs with the desulfated GL-1 and a third (GL-3) co-chromatographs with the desulfated GLS (Kates and Deroo, 1973). The complete structural elucidation of these three minor glycolipids (GL-1, GL-2 and GL-3) forms a major part of this thesis.

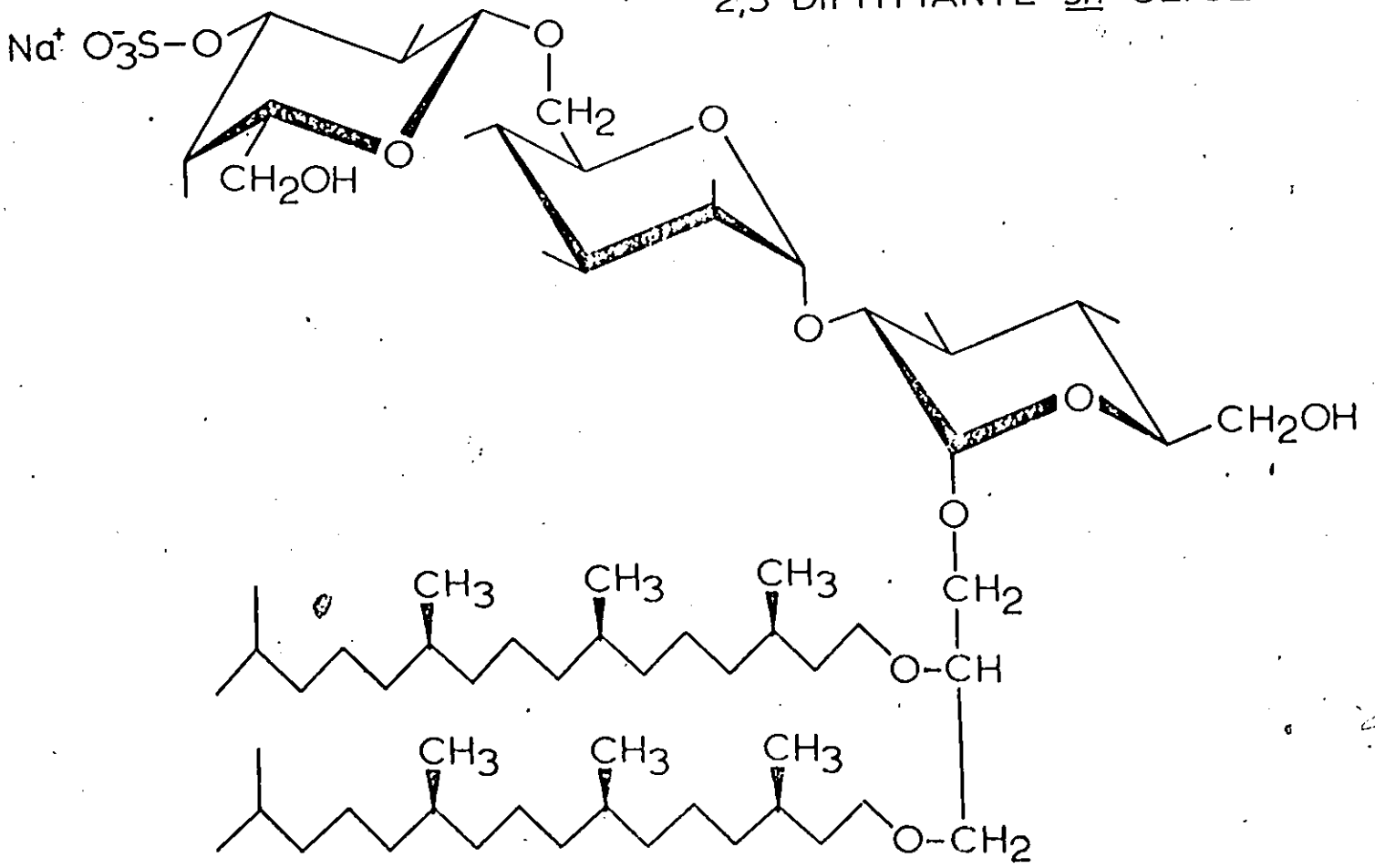
The major glycolipid of H. marismortui is similar in structure to the desulfated GLS of H. cutirubrum, except the terminal sugar is a β-linked glucose residue rather than a β-linked galactose residue (Structure V, Figure 8) (Evans et al., 1980). Recently, a sulfated diglycosyl diether present in a strain of halophilic bacteria from Spanish salt ponds has been identified as 2,3-di-O-phytanyl-1-O-[Manp-(6-SO$_4^{-}$)α1→2Glcpα]-sn-glycerol (Structure VI, Figure 8) (Kushwaha et al., in press).

Figure 7

Structure of the glycolipid sulfate (GLS) of Halobacterium cutirubrum: 2,3-di-O-phytanyl-1-O-[Galp-(3'-SO_4')β1'→6'Manpα1'→2'Glcpα]-sn-glycerol (Kates and Deroo, 1973).

$Na^+O_3^-SO$-3-GALp-β-(1→6)-MANp-α-(1→2)-GLUp-α-(1→1)-

2,3-DIPHYTANYL-sn-GLYCEROL



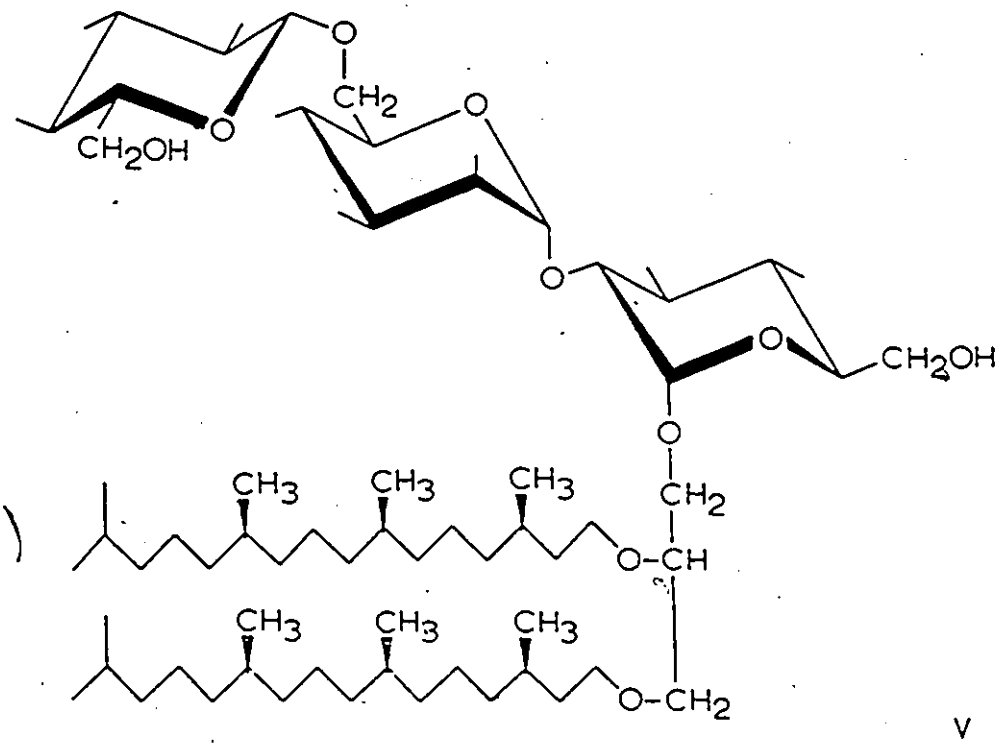
IV

Figure 8

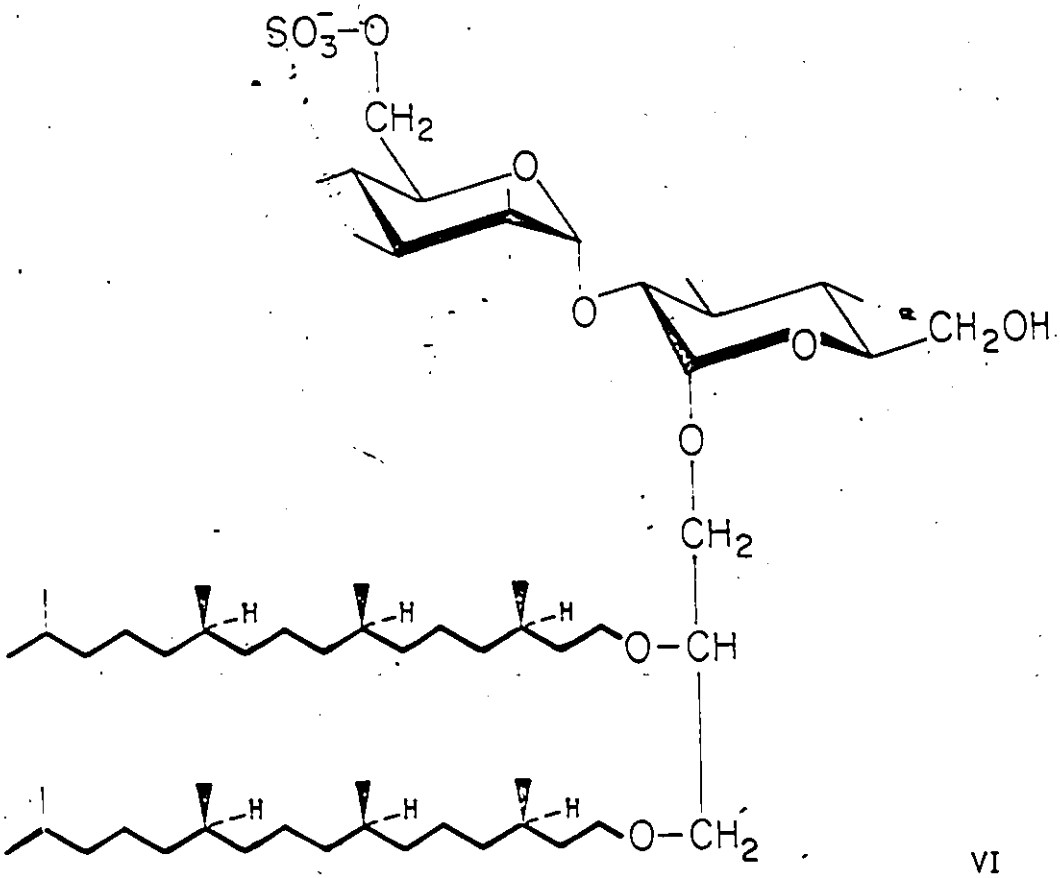
Structures of the major glycolipid components of Halobacterium marismortui (Structure V) and extremely halophilic bacterial strain (R-4) (Structure VI).

V. 2,3-di-O-phytanyl-1-O-[Glcpβ1'→6'Manpα1'→2'Glcpα]-sn-glycerol (Evans et al., 1980)

VI. 2,3-di-O-phytanyl-1-O-[Manp-(6'-SO_4)α1'→2'Glcpα]-sn-glycerol (Kushwaha et al., in press; ref. 178)



V



VI

2. Neutral Lipids

The acetone-soluble lipids consist almost exclusively of isoprenoid-derived compounds, and may be grouped into four classes on the basis of chain length: C_{20}-isoprenoids (geranylgeraniol), C_{30}-isoprenoids (squalenes), C_{40}-isoprenoids (carotenes) and C_{50}-hydroxylated isoprenoids (bacterioruberins).

Geranylgeraniol is the main C_{20}-isoprenoid, and has been found in several species of halobacteria and halococci. Di-O-phytanyl glycerol was found to be present in the free form as ca. 8% of the neutral lipids (Kushwaha et al., 1975b). Retinal is a part of the bacteriorhodopsin molecule present in the purple membrane of H. cutirubrum and H. halobium (Kushwaha et al., 1975a).

Squalenes account for about 36% of H. cutirubrum neutral lipids, and their structures have been established as all-trans-tetrahydrosqualene and dehydrosqualene (Kushwaha et al., 1972). Carotenoids are present in low concentrations (1%) which suggests that they may be precursors for the synthesis of retinal and/or bacterioruberins. Vitamin MK-8 or menaquinone-8 has been found in relatively large amounts in all halophiles which have been examined (Kushwaha et al., 1974). The bright red and pink colours of the extreme halophiles are due to the presence of the red bacterioruberin pigments (Petter, 1935; Kushwaha et al., 1975b). It has been suggested that these pigments protect the DNA of the bacteria from damage caused by ultraviolet light.

3. Lipid Composition of Red and Purple Membranes

A comparison of the lipid composition of red and purple

membranes from H. cutirubrum (Kushwaha et al., 1975a) showed several similarities and differences (Table 1). The total lipid content of purple membrane (20%) was found to be much lower than that of red membrane (37%). Both membranes contained approximately equal amounts of squalenes, carotene and vitamin MK-8. However, retinal was present exclusively in the purple membrane and the bacterioruberins were found only in red membrane. PGP and PG were present in both membranes, but the sulfated lipid components, namely GLS and PGS, appeared to be present exclusively in the purple membrane. Kushwaha et al (1976) have shown by direct comparison that the purple membranes of H. cutirubrum and H. halobium have qualitatively the same lipid composition. Also Wildenauer and Khorana (1977) found that H. halobium purple membrane has essentially the same polar lipid composition reported by Kushwaha et al (1975a) for H. cutirubrum purple membrane (Table 1).

The absence of GLS and PGS in red membrane seems anomolous when it is considered that H. cutirubrum synthesizes both GLS and PGS in the presence of high or low aeration and under light or dark conditions (Deroo, 1974) even though low aeration and illumination are essential for purple membrane synthesis. Furthermore, the ^{32}P and ^{35}S labelling experiments of Sumper et al (1976) indicated that the lipids of purple membrane are formed long before the start of retinal and bacterioopsin synthesis; since red membrane predominates before purple membrane appears, it follows that red and purple membranes should have at least qualitatively the same type of lipids. When compared with intact cells, some preparations of purple membrane from H. cutirubrum were found to contain lower amounts of GLS and higher amounts of GL-3, and this led to the suspicion that the

Table 1. Lipid Composition of Purple and Red Membranes from H. cutirubrum*

Lipid Component	% by weight of Total Lipids	
	Purple Membrane	Red Membrane
Neutral Lipids		
Squalene, dihydrosqualene, tetrahydrosqualene	4.5	5
β-Carotene	Trace	Traces
MK-8	1.6	1.2
Retinal	2.5	N.D.+
C_{50} Red Pigments	N.D.	0.4
Phospholipids		
Phosphatidylglycerol	4.5	2.7
Phosphatidylglycerophosphate	52.0	52.3
Phosphatidylglycerosulfate	4.8	N.D.
Glycolipids		
X_1	Traces	**
Triglycosyl diether	19.3	Traces
X_2	Traces	**
Glycolipid sulfate	10.3	Traces

*Kushwaha et al., 1975a.

**The amount of X_1 + X_2 together was about 32-35% by weight.

+N.D.: Not detected.

apparent lack of GLS in red membrane was the result of the action of a glycolipid sulfatase or alternatively of a non-enzymatic desulfation (Kates, 1978). Therefore further studies were undertaken on the lipid composition of red and purple membranes and on the desulfation of GLS.

The retinal:protein ratio for bacteriorhodopsin was at first found to be approximately one (Oesterhelt and Stoeckenius, 1971). However, Kushwaha et al., (1975a; 1976) reported a retinal:protein mole ratio of 0.6*, based on the extraction of retinal with cetyltrimethylammonium bromide in the presence of hydroxylamine and determination of protein by the Lowry (1951) method. Papadopoulos et al., (1978) later found a retinal:protein ratio of 1.05*, based on direct analysis of retinal extracted with ethanol; protein was determined by the Kjeldahl procedure, which required an assumed nitrogen conversion factor. In order to confirm a 1:1 stoichiometry an independent procedure is presented in this thesis, in which retinal was quantitated by the thiobarbituric acid procedure (Futterman and Saslaw, 1961) and protein was determined by amino acid analysis.

IV General Review of Glycolipids**

1. Glycosyl Diacylglycerols

In Gram-positive bacteria, the most common type of glycolipids are the diglycosyl diacylglycerols (diglycerides), which are

---

*Recalculated on the basis of M.W. = 26,000

**Glycosphingolipids will not be discussed in detail in this review because they are beyond the scope of this thesis.

Figure 9

Structures of the five major types of diglycosyl diglycerides isolated from Gram-positive bacteria (Shaw, 1970).

(a) Galpα1→2Glcpα1→3'(1',2'-diacyl-sn-glycerol)

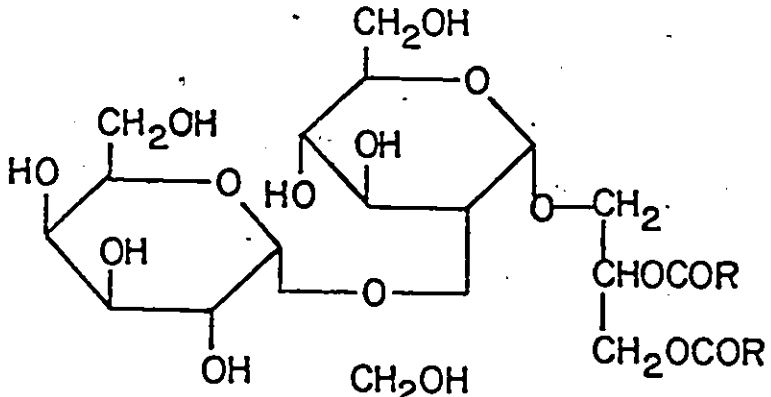
(b) Manpα1→3Manpα1→3'(1',2'-diacyl-sn-glycerol)

(c) Glcpα1→2Glcpα1→3'(1',2'-diacyl-sn-glycerol)

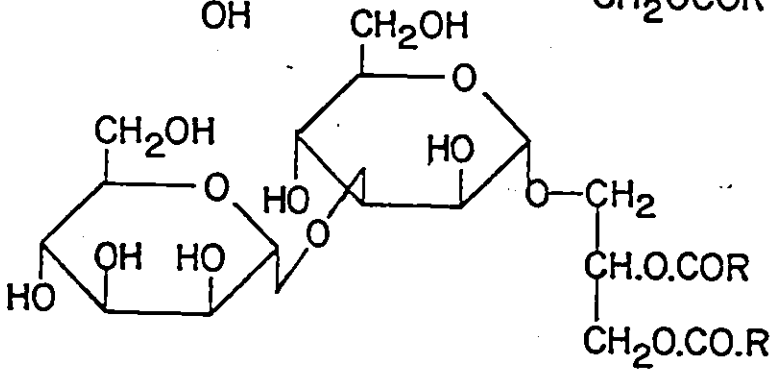
(d) Glcpβ1→6Glcpβ1→3'(1',2'-diacyl-sn-glycerol)

(e) Galpβ1→6Galpβ1→3'(1',2'-diacyl-sn-glycerol)

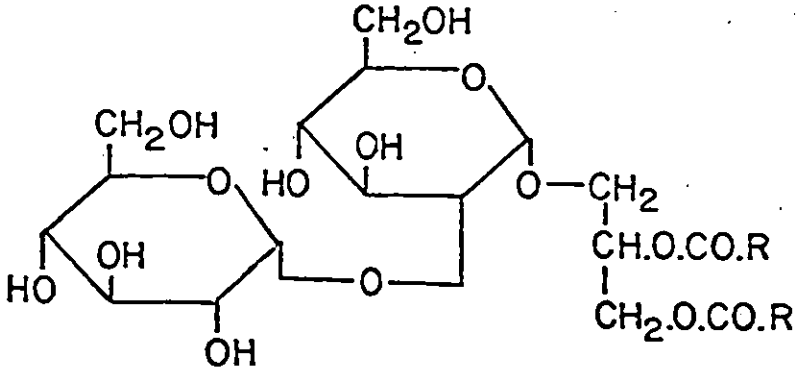
(a)



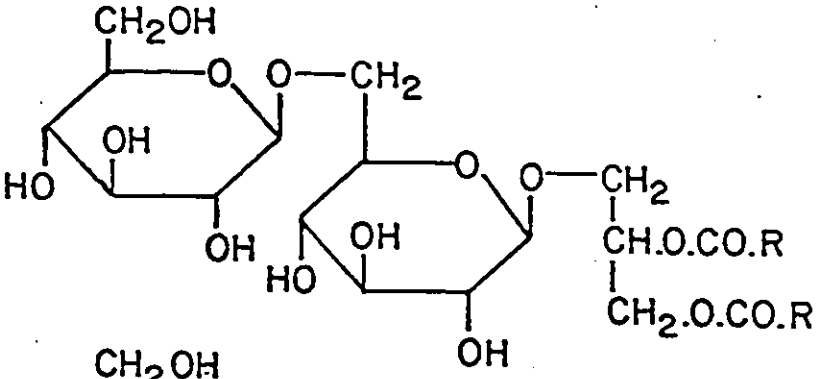
(b)



(c)



(d)



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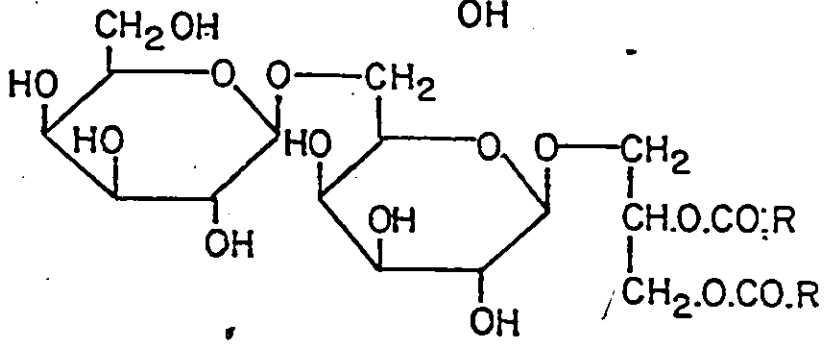


Table 2. Distribution of Glycosyl Diglycerides in Bacteria*

Organisms	Glycosyl groups linked to diglyceride	References
Gram-Negative		
Pseudomonas diminuta	1) α-monoglucosyl- 2) α-monoglucuronosyl- 3) β-glucosyl-(1→4)-α-glucuronosyl-	Wilkinson (1968)
Halobacterium cutirubrum	Sulfated β-galactosyl-(1→6)-α-mannosyl-(1→2)-α-glucosyl**	Kates and Deroo (1973)
Chromatium strain D	1) monoglucosyl 2) mannosyl, glucosyl 3) dimannosyl glucosyl	Steiner et al. (1968)
Mycoplasma neurolyticum	1) β-glucosyl 2) β-glucosyl (1→6)-β-glucosyl	Smith (1972)
Gram-Positive		
Staphylococcus aureus	β-glucosyl-(1→6)-β-glucosyl β-glucosyl-(1→3)-β-glucosyl	Polonovsky et al. (1962)
Streptococcus faecalis	α-glucosyl-(1→2)-α-glucosyl	Brundish et al. (1966)
Lactobacillus casei	α-glucosyl-(1→2)-α-glucosyl	Shaw et al. (1968)
Listeria monocytogenes	α-galactosyl-(1→2)-α-glucosyl	Deroo (1969)
Arthrobacter crystallopoietes	1) β-galactosyl 2) α-mannosyl-(1→3)-α-mannosyl 3) β-galactosyl-(1→6)-β-galactosyl	Shaw and Stead (1971)

* See also review paper by Shaw (1970).

** Linked to 2,3-diphytanyl-sn-glycerol.

predominantly diglucosyl and digalactosyl diglycerides (Figure 9; Table Table 2) (Sastry, 1974; Shaw, 1975). Mono-, tri- and tetraglycosyl diglycerides are not as common, but some species produce them in significant quantities. For example, Lactobacillus casei contains a series of glycosyl diglycerides with the following structures (Nakano and Fischer, 1977):

Glcpα1→3'diglyceride

Galpα1→2Glcpα1→3'diglyceride

Glcpβ1→6Galpα1→2Glcpα1→3'diglyceride

Glcpβ1→6Glcpβ1→6Galpα1→2Glcpα1→3'diglyceride

The homology of this series and the similarity in their fatty acid composition suggest a biosynthetic sequence.

The distribution of glycosyl diglycerides in bacteria seems to have some taxonomic significance. Thus, members of the same genus usually contain the same type of glycosyl diglyceride (Shaw, 1975). As an illustration, lactobacilli contain Galpα1→2Glcpα1→diglyceride whereas streptococci contain Glcpα1→2Glcpα1→diglyceride.

Photosynthetic tissues of plants characteristically have high contents of glycolipids, which consist mainly of monogalactosyl and digalactosyl diglycerides and sulfoquinovosyl diglyceride (Figure 10) (Kates, 1970; Quinn and Williams, 1978). Trigalactosyl diglycerides have been identified in potato tubers (Galliard, 1969), pumpkin fruit (Ito and Fujino, 1975) and rice bran (Fujino and Miyazawa, 1979) and a tetraglycosyl diglyceride has been identified in the latter. These glycolipids were shown to be homologues of the plant mono- and digalactosyl diglycerides with the following structures:

Galpα1→6Galpα1→6Galpβ1→3'diglyceride

Galpα1→6Galpα1→6Galpα1→6Galpβ1→3'diglyceride

Figure 10

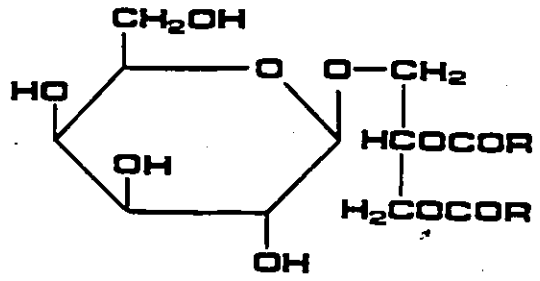
Structure of the major plant glycolipids.

(a) Galpβ1→3'(1',2'-diacyl-sn-glycerol)

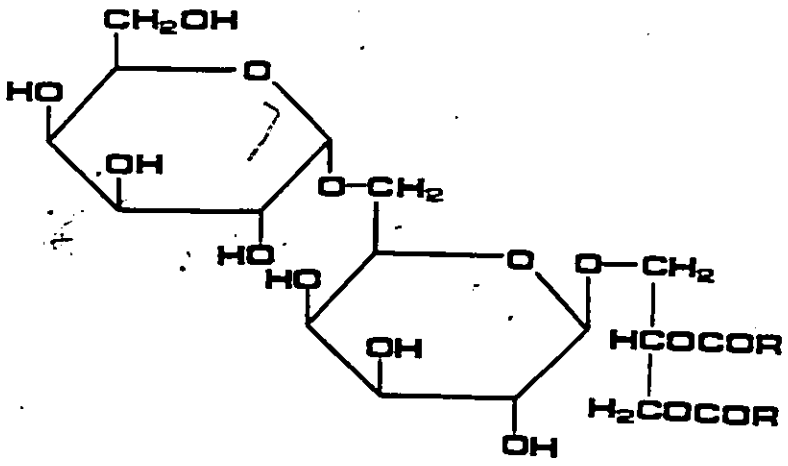
(b) Galpα1→6Galpβ1→3'(1',2'-diacyl-sn-glycerol)

(c) 6-sulfoquinovosylα1-3'(1',2'-diacyl-sn-glycerol)

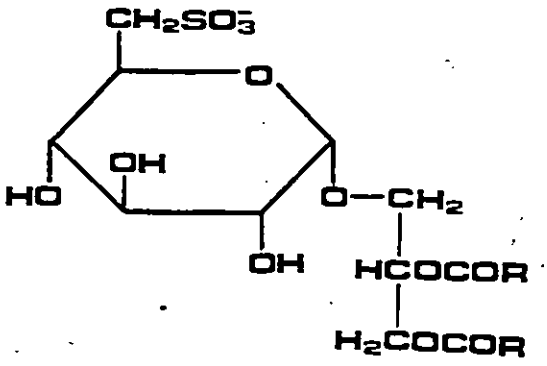
(a)


CH2OH
O
O—CH2
HO
HCOCOR
OH
H2COCOR
OH

(b)


CH2OH
O
HO
OH
O—CH2
HO
HO
O
O—CH2
HCOCOR
OH
H2COCOR
OH

(c)


CH2SO3
O
OH
HO
O—CH2
HCOCOR
OH
H2COCOR

The tri- and tetraglycosyl diglycerides have also been detected in spinach chloroplasts (Webster and Chang, 1969).

Animal nerve tissue contains significant amounts of a monogalactosyl diglyceride which, except for its fatty acid composition, is identical to the plant monogalactolipid (Steim, 1967). A monoalkyl ether analog accounting for 8-12% of the total galactosyl diglyceride has been isolated from brain (Norton and Brotz, 1963; Rumbsby and Rossiter, 1968), and this compound has been shown to be present in mammalian testes in both sulfated and non-sulfated forms (Murray et al., 1976).

2. Sulfoglycolipids

(a) Structure and Occurrence

Thudicum in 1874 was the first to discover a glycolipid sulfate (sulfatide) present in brain tissue. Nearly a century later, its structure was established as ceramide galactosyl-3-sulfate (cerebroside sulfate) (Stoffyn and Stoffyn, 1963):

Galp-(3-SO_4^-)β1→3'ceramide (Structure a, Figure 11)

An analogous glycolipid sulfate, ceramide lactosyl sulfate, has been isolated from human kidney by Martensson (1963) and was shown to have the following structure (Martensson, 1966):

Galp-(3-SO_4^-)β1→4Glcpl→3'ceramide

A more complex glycolipid sulfate has been isolated recently from rat kidney (Tadano and Ishizuka, 1980) and on the basis of permethylation analysis was tentatively assigned the structure:

GlcNAcl→4Galp(3←SO_4^-)1→4Glcpl→3'ceramide

Figure 11

Structures of cerebroside sulfate (a) and seminolipid (b).

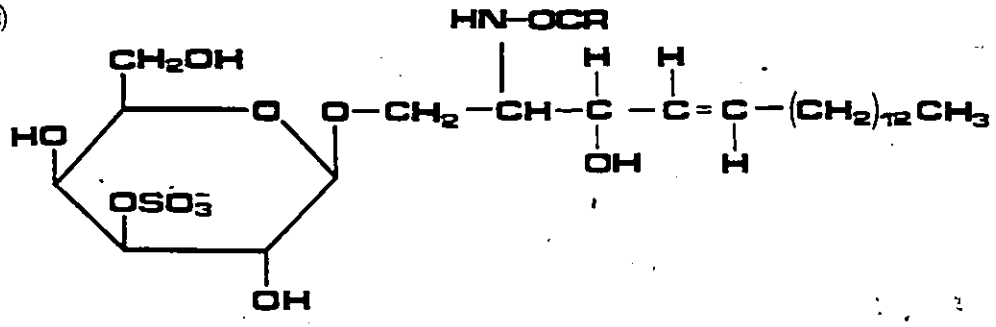
(a) Gal*p*-(3-SO_4^-)β1→3'ceramide

(Stoffyn and Stoffyn, 1963)

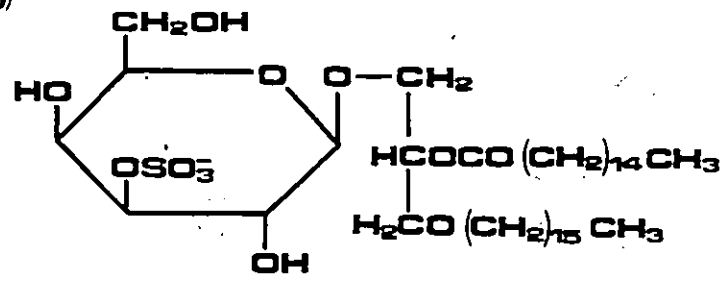
(b) Gal*p*-(3-SO_4^-)β1→3'(1'-*O*-alkyl-2'-*O*-acyl)*sn*-glycerol

(Ishizuka *et al.*, 1973)

(a)


HN–OCR
CH2OH
H
H
O
O–CH2–CH–C–C=C–(CH2)12CH3
HO
OH
H
OSO3−
OH

(b)


CH2OH
O
O–CH2
HO
HCOCO(CH2)14CH3
OSO3−
H2CO(CH2)15CH3
OH

A different class of glycolipid sulfates is represented by "seminolipid" which was isolated from boar testis and spermatozoa and characterized by Ishizuka et al., (1973). The complete structure of seminolipid was shown to be:

Galp(3-SO_4^-)β1→3'-(1'-O-alkyl-2'-O-acyl)sn-glycerol
(Structure b, Figure 11)

Seminolipid was later shown to be widespread in mammals (Murray et al., 1976).

Other complex glycolipid sulfate derivatives of monoalkyl monoacyl glycerols have been detected in gastrointestinal tract and in lung by Slomiany and coworkers. Human gastric secretion was found to contain a glycolipid sulfate having the following triglycosyl sulfate structure (Slomiany et al., 1977):

Glcp-(6-SO_4^-)α1→6Glcpα1→6Glcpα1→3'-(1'-O-alkyl-2-O-acyl)sn-glycerol

Slomiany et al (1979) have also characterized the glycolipid sulfate of rabbit pulmonary surfactant, as a tetraglycosyl-containing compound with the structure:

Glcp-(6-SO_4^-)α1→6Glcpα1→6Glcpα1→6Glcpα1→3'-(1'-O-alkyl-2'-O-acyl)sn-glycerol

In addition to the seminolipid-type of glycolipid sulfate, two ceramide glycolipid sulfates were isolated from hog gastric mucosa and shown to have the following structure (Slomiany and Slomiany, 1978; Slomiany et al., 1980):

Galpβ1→4GlcNAc(6←SO_4^-)β1→3Galpβ1→4Glcp1→1'ceramide

GlcNAc-(6-SO_4^-)β1→3Galpβ1→4Glc1→1'ceramide

It has been suggested (Slomiany and Slomiany, 1978) that the sulfated glycolipids present in the stomach may protect the gastric mucosa from autodigestion by acid and pepsin. Preliminary evidence suggests that the intestinal brush border of the rabbit small intestine contains a sulfated glycolipid in which the sugars are glucose, galactose and glucosamine in mole ratio 1:2:2 (Cooper and Kent, 1978).

In plants, Benson (1963) first reported the presence of a sulfonolipid with the structure of a 6-sulfonate of quinovosyl diglyceride (Structure c, Figure 10). This compound differs from the glycolipid sulfates in that the sugar is present as a sulfonate with a covalent C-S bond. Sulfoquinovosyl diglyceride occurs mainly in the chloroplasts of photosynthetic plants, algae and bacteria (Quinn and Williams, 1978).

Apart from the glycolipid sulfates in Halobacteria (General Introduction, III, 1), a different class of bacterial sulfolipid has been found in a virulent strain of Mycobacterium tuberculosis with the structure of an acylated α, α-trehalose having a sulfate group attached at one of the 2-positions (Figure 12) (Goren et al., 1976).

(b) Function

Experimental evidence strongly suggests that cerebroside sulfate plays an important role in $(Na^+ + K^+)$-ATPase activity and ion transport (Karlsson, 1976). A quantitative relationship between $(Na^+ + K^+)$-ATPase activity and cerebroside sulfate concentration has been shown in tissues which have increased ion transport such as the kidney (Karlsson et al., 1973), the electric organ of Torpedo marmorata (Hansson et al.,

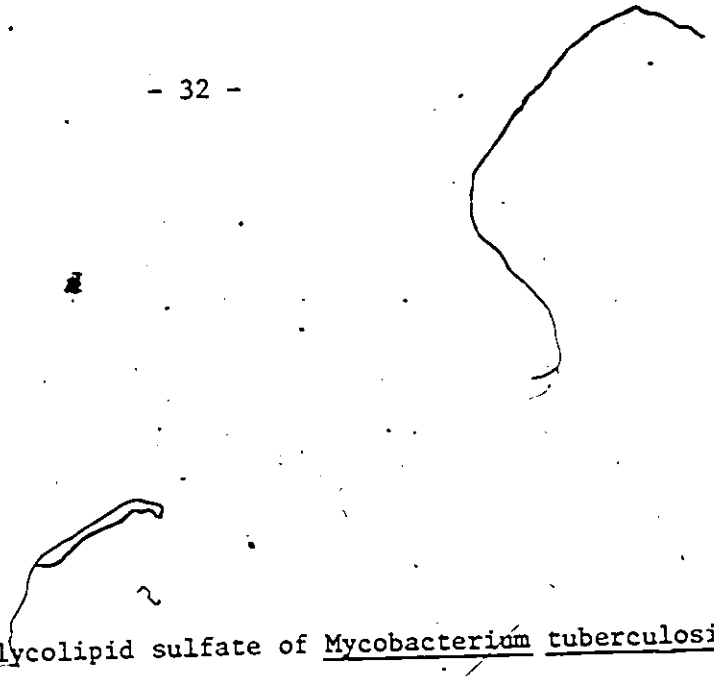
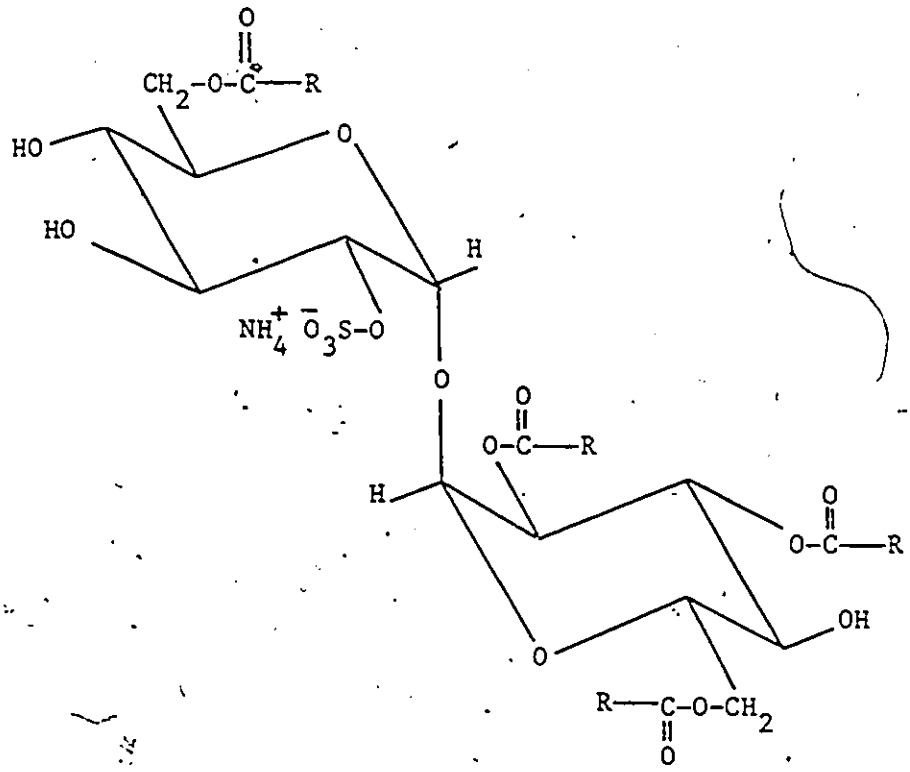


Figure 12

Structure of the glycolipid sulfate of Mycobacterium tuberculosis.

2,3,6,6'-tetraacyl-α,α'-D-trehalose-2'-sulfate

(Goren et al., 1976)


O
CH2–O–C–R
HO
O
HO
H
NH4+ O3S–O
O
O–C–R
H
O–C–R
OH
O
R–C–O–CH2
O

1979), the rectal gland of dogfish (Karlsson et al., 1974a) and the avian salt gland (Karlsson et al., 1974b). During development of the Chilean frog, Calyptocepalella caudiverbera, parallel increases were observed between $(Na^+ + K^+)$-ATPase activity, cerebroside sulfate concentration and sodium flux through the abdominal skin, whereas a parallel decrease was observed in the gill (Gonzalez et al., 1979). The authors also observed that treatment of cell fractions with arylsulfatase resulted in loss of ATPase activity with a concurrent breakdown of the sulfatide.

The degree of association between a cation and a polyanion has been shown to depend upon the charge, radius and hydration of the cation and the polarizability of the anionic group relative to that of water (Teunissen and de Jong, 1938; Bregman, 1953). Because phosphate groups are more polarizable than water, smaller cations prefer the anionic sites before water and therefore sodium ions are bound in preference to postassium ions. However, sulfate and sulfonate groups are less polarizable than water and small cations prefer to remain hydrated; potassium ions are bound preferentially because they are less hydrated than sodium ions (Teunissen and de Jong, 1938; Abramson et al., 1967). Glycolipid sulfates would therefore be expected to have a higher affinity for potassium ion than for sodium ion, and this has been verified experimentally for cerebroside sulfate (Abramson et al., 1967). Because of this property, Karlsson (1976) proposed that the function of cerebroside sulfate is to donate potassium ion to the translocation site of (Na^+K^+)-ATPase.

In plants, adaptation to a saline environment appears to be dependent upon the concentration of sulfoquinovosyl diglyceride in the roots (Kuiper, 1980). Reconstitution studies have suggested that this sulfolipid is required for plant $(Na^+ + K^+)$-ATPase activity (Kuiper, 1972).

V. Physical Aspects of Biomembranes

1. Lipid Polymorphism and the Dynamic Shapes of Lipids

The currently accepted structure for the cell membrane is the fluid mosaic model (Figure 13) (Singer and Nicholson, 1972) in which proteins are imbedded in a lipid bilayer. However, it has been suggested that cell membrane phenomena such as cell fusion, exo- and endocytosis, facilitated transport and lipid transbilayer movement require that some of the lipids must assume a non-bilayer state (Lucy, 1970; Cullis and de Kruijff, 1979). X-ray diffraction studies have shown that in addition to bilayer structures, hexagonal, inverse hexagonal, micellar, inverse micellar, cubic and rhombic phases can be adopted by hydrated lipids (Luzzatti et al., 1968; Reiss-Husson, 1967; Rand et al., 1971). ^{31}P-NMR spectra showing the chemical shift anisotropy of phospholipid dispersions have been correlated with X-ray observations (Cullis and de Kruijff, 1978), and the former technique is applicable to more complex systems by extrapolation.

Chemical shift anisotropy can occur if the electronic shielding of a nucleus varies with the direction of the magnetic field (Figure 14) and if molecular motion is slow enough to allow observation of the inequivalent orientations. In liquids, a time averaged isotropic value is usually obtained, but the chemical shift observed for a single crystal is often dependent upon the rotation angle. The ^{31}P-NMR spectra of phospholipids in aqueous dispersion show a powder pattern with a relatively large chemical shift anisotropy ($\Delta\sigma$)(Figure 15), (Seelig, 1978). In a multilamellar system, lateral diffusion is slow on the NMR time scale, and therefore motion consists primarily of molecular rotation about the long axis and only partial chemical shift averaging is observed (Cullis and de Kruijff, 1976). Proton decoupled spectra are characteristically broad

Figure 13

The fluid mosaic model of the biological membrane. The polar head groups of the lipids are represented by spheres and the hydrocarbon chains by wavy lines. The small circles indicate the annulus of phospholipids surrounding the protein molecules (Singer and Nicholson, 1972).

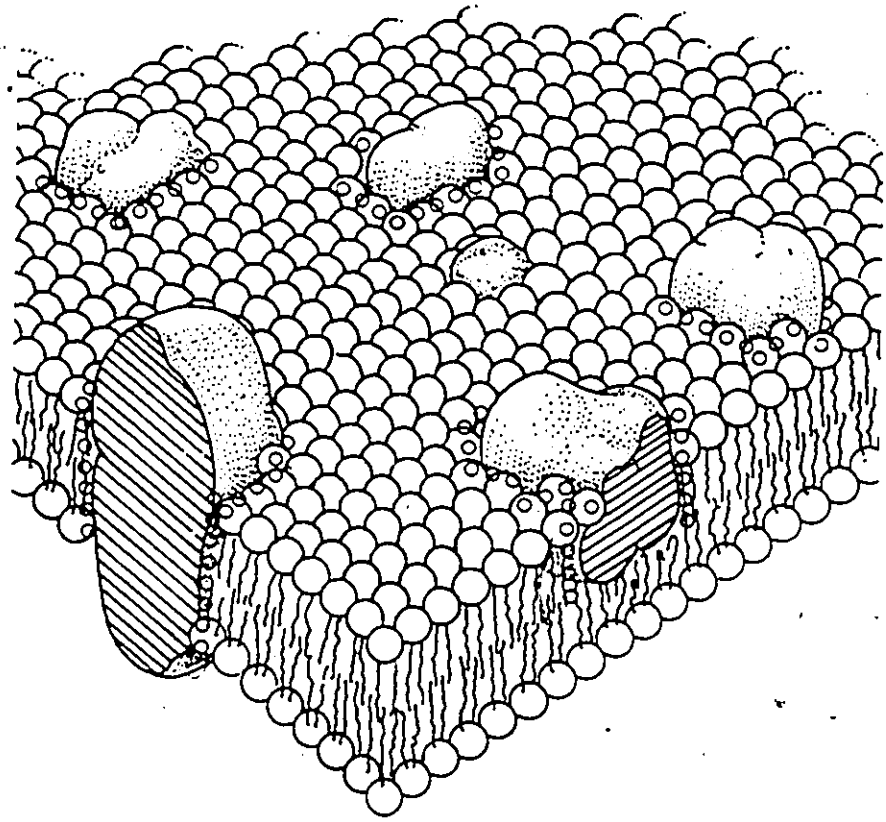
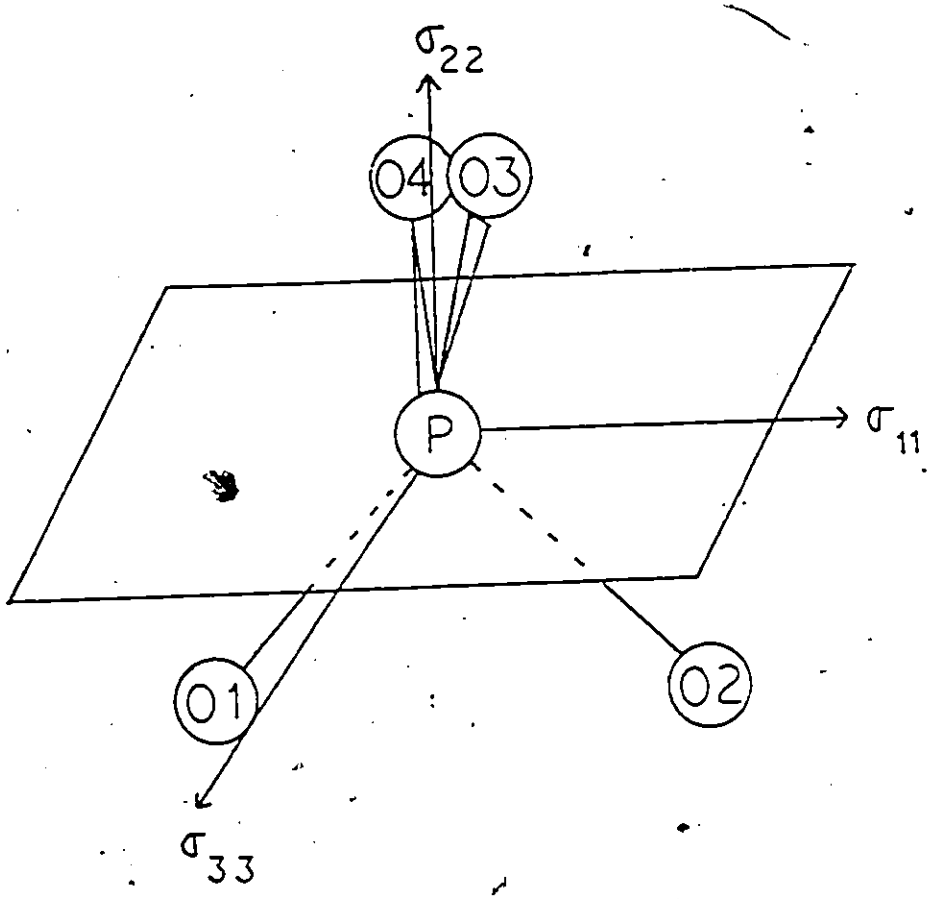


Figure 14

Orientation of the ^{31}P chemical shift tensors σ_{ii} for the phosphate group. O(3) and O(4) are the two nonesterified oxygens. O(1) and O(2) connect the phosphate group to the glycerol backbone and the head group residue, respectively, of the phospholipid molecule. (Seelig, 1978).


σ22
O4
O3
P
σ11
O1
O2
σ33

with a low field shoulder and a high field peak (Figure 15). In the hexagonal phase, lateral diffusion around the tube-like structure occurs rapidly, and therefore additional motional averaging occurs (Cullis and de Kruijff, 1976). This results in lineshapes which, compared to multilamellar systems, have one-half the chemical shift anisotropy and reversed asymmetry ($-\frac{1}{2}\Delta\sigma$) (Figure 16). In small sonicated vesicles, micelles and inverted micelles, tumbling and lateral diffusion result in complete averaging, and a narrow, symmetric peak is observed (McLaughlin et al., 1975).

^{31}P-NMR lineshapes indicate that saturated and unsaturated phosphatidylcholines prefer a bilayer arrangement, whereas unsaturated phosphatidylethanolamines exhibit a bilayer to hexagonal phase transition at a temperature greater than that of the gel-liquid crystalline transition temperature (Cullis and de Kruijff, 1976). Acidic phospholipids such as cardiolipin form bilayers in the absence of Ca^{2+}, however the addition of Ca^{2+} induces the formation of the hexagonal phase. The polymorphic phase behaviour of a lipid species seems to be dependent on its molecular shape, and this allows certain predictions to be made (Figure 17). Cone shaped lipids, such as phosphatidylethanolamine, in which the cross-sectional area of the headgroup is less than that of the acyl chains would be compatible with the hexagonal (H_{II}) phase. Cylindrically shaped lipids such as phosphatidylcholine would be expected to adopt a bilayer arrangement. Lipids in which the headgroup has a cross-sectional area greater than that of the hydrocarbon region, such as lysophosphatidylcholine would likely adopt a micellar phase. The ability of Ca^{2+} to induce a bilayer hexagonal phase transition in acidic lipids suggests that charge repulsion increases the effective area of the polar head (Cullis and de Kruijff, 1979).

Figure 15

Theoretical ^{31}P NMR powder pattern for a bilayer type of dispersion. The two chemical shift tensors $\sigma_{\parallel}$ and $\sigma_{\perp}$ correspond to the points of inflection for the low field shoulder and the high field shoulder, respectively (Seelig, 1978). The apparent chemical shift anisotropy, $\Delta\sigma_{app}$, is the distance between $\sigma_{\parallel}$ and $\sigma_{\perp}$.

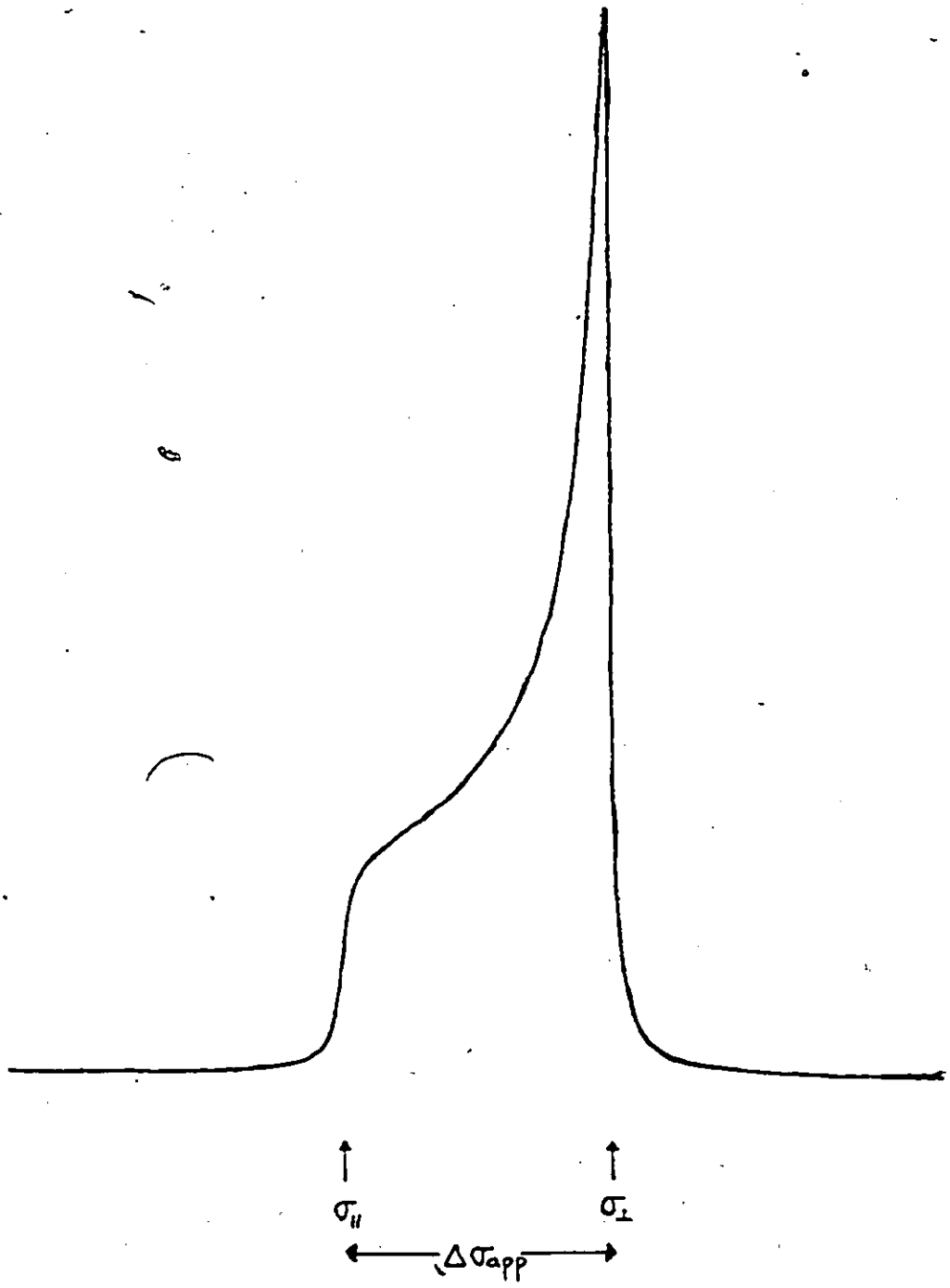

$\sigma_{\parallel}$
$\sigma_{\perp}$
$\Delta\sigma_{app}$

Figure 16

Polymorphic phases available to hydrated liquid crystalline phospholipids and corresponding ^{31}P-NMR spectra. The spectra were obtained at 30 °C in the presence of broad band proton decoupling (from Cullis and Hope, 1978).

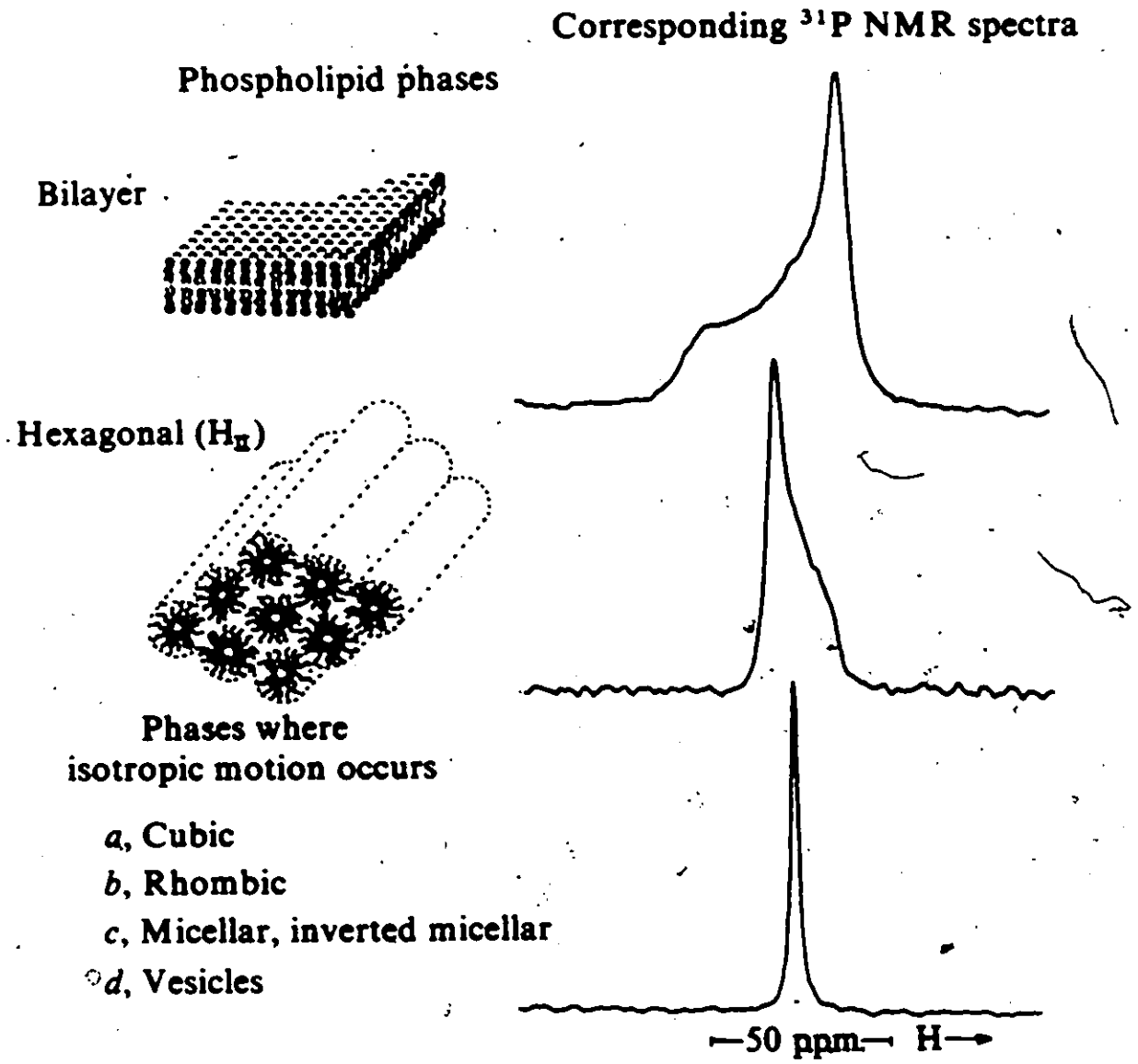
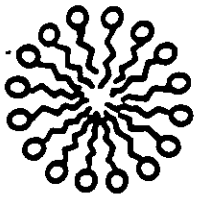
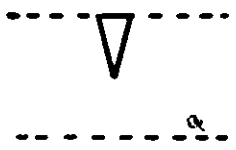
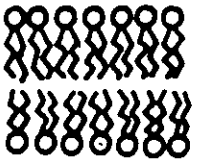
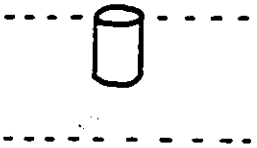
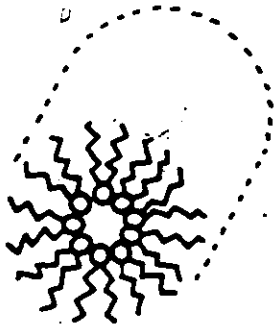

Corresponding ^{31}P NMR spectra
Phospholipid phases
Bilayer
Hexagonal (H_{II})
Phases where
isotropic motion occurs
a, Cubic
b, Rhombic
c, Micellar, inverted micellar
d, Vesicles
—50 ppm— H—

Figure 17

Polymorphic phases and corresponding dynamic molecular shapes of component lipids (from Cullis and de Kruijff, 1979).

Lipid	Phase	Molecular Shape
Lysophospholipids Detergents	 Micellar	 Inverted Cone
Phosphatidylcholine Sphingomyelin Phosphatidylserine Phosphatidylglycerol	 Bilayer	 Cylindrical
Phosphatidylethanol-amine (unsaturated) Cardiolipin – Ca^{2+} Phosphatidic acid – Ca^{2+}	 Hexagonal (H_{II})	 Cone

Oleic acid induces fusion of erythrocytes and causes the formation of hexagonal (H_{II}) phase in regions of isolated erythrocyte membranes (Cullis and Hope, 1978). Also, Ca^{2+} is required for *in vivo* cell fusion and has been shown to induce fusion in cardiolipin-phosphatidylcholine vesicles. These observations support current models of cell fusion which require that some of the lipid must assume a non-bilayer arrangement (Cullis and de Kruijff, 1979). There is some evidence that non-bilayer phases are involved in transbilayer movement of lipids, for example the sarcoplasmic reticulum shows both transbilayer movement and the presence of some non-bilayer structure (Cullis and de Kruijff, 1979).

2. Membrane Fluidity

Many types of phospholipid bilayers, such as those of phosphatidylcholine, can undergo a thermotropic gel to liquid crystalline phase transition in which the bilayer remains intact. The transition temperature observed increases with the length of the acyl chain, decreases with unsaturation and is also dependent upon the nature of the polar head group (Chapman, 1968). The interpretation that this transition results from an increase in trans-gauche conformational changes in the acyl chains is supported by ^{2}H-NMR studies which indicated that in the gel state the chains are highly ordered whereas in the liquid crystalline state they are disordered (Smith, 1979). Disorder in the liquid crystal results in a greater cross-sectional area of the phospholipids and thus above the transition temperature the bilayer is loosely packed, fluid and more permeable (McElhaney, 1976).

Fluidity is defined as the inverse of viscosity, where viscosity is a measure of the resistance to flow (Hildebrand, 1972; Lands, 1980); membrane fluidity therefore implies that membrane components are able to

flow in the plane of the bilayer. ESR experiments have suggested that membrane lipids can undergo rapid lateral diffusion (ca. 10^{-8} cm^2 sec^{-1}) (Deveaux and McConnel, 1972). Membrane proteins are also capable of this type of motion, and recent observations suggest a functional relationship exists between lateral diffusion and electron transport in mitochondria (Hackenbrock, 1981).

It is currently held that in all organisms the membrane lipids are in a fluid state at the growth temperature (Cullis and de Kruijff, 1979). This is supported by findings that in many microorganisms, plants and animals the fatty acids present in the membrane lipids undergo changes in response to variations in environmental temperatures (McElhaney, 1976). For example, it was found that Candida lipolytica grown at 10°C contained more linoleic acid (18:2) and less oleic acid (18:1) when compared with the same yeast grown at 25°C (Kates and Baxter, 1962). Also, organisms such as A. laidlawii and fatty acid auxotrophs of E. coli can incorporate exogenous fatty acids without alteration, and this metabolic feature has facilitated studies of the relationships between membrane fluidity and cellular function. The results of these studies indicate that growth of these organisms ceases at temperatures in which the membrane lipids are in a gel state (McElhaney, 1976). For some membrane-bound enzymes, Arrhenius plots of activity as an inverse function of temperature show breaks which correspond to membrane transition temperatures, and this suggests that membrane fluidity can play a role in modulation of enzyme catalytic properties (Jain, 1980).

II. Review of Physical Studies of Halophile Lipids and Membranes

Lipids containing ether-linked phytanyl chains and large, negatively charged polar head groups would be expected to have physical

properties which differ from those of zwitterionic diacylglycerol lipids, such as phosphatidylcholine. Chen et al (1974) observed that dispersions of total polar lipids of H. cutirubrum in dilute KCl or NaCl solutions (0-0.2 M) behaved as ideal osmometers and showed multilamellar structure in the electron microscope. However, at higher salt concentrations properties of closed liposome structures were not observed because of precipitation of the phospholipids at the higher ionic strengths. The individual phospholipids, namely PGP, PGS and PG, did not behave as ideal osmometers in dilute salt solutions. Because of this observation, the authors concluded that the small polar head groups of these phospholipids compared to the cross-sectional area of the two phytanyl chains must favour the formation of open, non-bilayer structures. In contrast, GLS alone or in various mixtures with phospholipids was capable of forming closed liposomes because the size of its large carbohydrate polar head group would be comparable to the cross-sectional area of the phytanyl groups. To gain further information on the polymorphism of the halophile lipids, studies were carried out using ^{31}P-NMR and freeze-fracture electron microscopy and the results will be presented in this thesis.

Chen et al (1974) also investigated the phase transitions of the lipid dispersions by differential thermal analysis and found the presence of a broad transition at ca.-50 °C in all components. By comparison, the melting point of neat dihydrophytol was found to be in the range of -50° to -60 °C (Plachy et al, 1974). Differential scanning calorimetry studies (Jackson and Sturtevant, 1978b) showed that the transitions observed for mixtures of halophile membrane lipids with dipalmitoylphosphatidylcholine became lower in temperature and broader as the fraction of halophile lipid

increased, and halophile lipids alone showed no transition above 0°C. Furthermore, differential thermal analysis of 1,2-diphytanoyl-sn-glycero-3-phosphocholine, a synthetic analogue of halophile lipids, showed an absence of transitions over the range of -120°C to 120°C (Lindsey et al., 1979). Although these thermal studies of halophile lipids indicated that transitions above 0°C are absent, studies using other techniques have been contradictory. For example, electron spin resonance studies at temperatures above 0°C have shown evidence for a 23°C transition occuring in the polar head group of PGP (Plachy et al., 1974). Also ^{1}H- and ^{13}C-NMR studies of the total lipids showed a discontinuity at 30°C in the plots of peak widths and relaxation rate ($1/T_1$), respectively, as a function of temprature (Degani et al., 1978, 1980) and this transition was also observed in the purple membrane (Degani et al., 1978).

The occurrence of a 30°C transition in purple membrane was suggested earlier by electron spin resonance studies of spin labelled membrane (Chignell and Chignell, 1975). Differential scanning calorimetry (Jackson and Sturtevant, 1978a) showed the presence of a large 100°C transition associated with an irreversible change in colour to orange which was believed to be due to irreversible denaturation of bacteriorhodopsin. A smaller transition at 80°C was eliminated when purple membrane was incorporated into dipalmitoylphosphatidylcholine vesicles and was therefore attributed to a restructuring of the crystal lattice. Because differential scanning calorimetry, a more direct method based on thermodynamics, showed no transition at 30°C, the transitions seen by ESR and ^{1}H-NMR were either artifactual or a change of very low enthalphy occurred. To gain further information concerning the presence or absence of a 30°C transition in halophile lipid dispersions and purple membrane, studies were carried out on the temperature dependence of their ^{31}P-NMR spectra.

Aims of the Research

This thesis is divided into three parts. Part One will describe the structural determination of three minor unidentified glycolipid components, namely GL-1, GL-2 and GL-3. These glycolipids were characterized by elemental analysis, mobilities on thin layer chromatography and optical rotation; their structures were established by chemical degradation, permethylation analysis and spectrometric procedures.

Part Two of this thesis will present the results of further investigations of the lipid composition of red and purple membranes to resolve the question whether GLS is present in both membranes. The apparent lack of GLS in the red membrane may have been due to the occurrence of desulfation during membrane isolation, and therefore studies of GLS desulfation will be described.

Part three of this thesis is concerned with the physical properties of the halophile membranes and lipids. Their phase transitions were studied by ^{31}P-NMR and their lipid polymorphism was investigated by ^{31}P-NMR and freeze-fracture electron microscopy.

GENERAL MATERIALS AND METHODS

I. Materials

1. Solvents and Reagents

All solvents used were glass distilled; a 10% forerun was discarded. Pyridine was refluxed over barium monoxide for 1 h distilled and stored over barium monoxide to remove water.

Deuterochloroform - Merck, Sharp and Dohme "Silanor C", containing 1% tetramethylsilane as an internal reference standard.

Deuteromethanol (d_4) - Merck, Sharp and Dohme

Trimethylchlorosilane ($(CH_3)_3SiCl$) - Pierce Chemical Co.

Hexamethyldisilazane ($(CH_3)_3Si_2NH$) - Pierce Chemical Co.

Silver Oxide - Prepared according to Hirst and Percival (1963) and dried over P_2O_5.

Methyl Iodide - dried and distilled over calcium chloride, then stored in the presence of a drop of metallic mercury.

2. Standards

Potassium Phosphate, Monobasic (KH_2PO_4) - Primary Standard Grade, Fisher Scientific Co.

D - Glucose - Anydrous, ACS Reagent Grade, Anachemia Chemicals Ltd.

D - Galactose - Anhydrous, Mann Research Laboratories, Inc.

D - Mannose - ACS Reagent Grade, Fisher Scientific Co.

Methyl glycoside standards of glucose, mannose and galactose were prepared by mixing 10 mg of each sugar with 1 ml of 2.5% met-

hanolic-HCl and heating at 70°C for 1 h. The samples were brought to dryness under a stream of N_2 then desiccated overnight in vacuo over KOH.

3. Radioactive Labels

[1-^{14}C] Sodium Acetate (55.7mCi/mmole), New England Nuclear Corporation.

[^{35}S] Sulfate, as the acid dissolved in hydrochloric acid, New England Nuclear Corporation. The solution was neutralized to pH 8 with 1.0 N NaOH before use.

II. Culture and Growth of the Organisms

1. Organisms

Halobacterium cutirubrum NRCC strain 34001 was used as a source of glycolipids for structural studies and as a source of lipids and membranes for physical studies. The bacterium was originally isolated, screened and purified by Dr. A.G. Lochhead at the Animal Research Institute of Agriculture Canada.

Halobacterium halobium was used as a source of purple membrane for physical studies. The culture used was obtained from Dr. J. Weber of the Department of Bacteriology and Immunology, University of California (Berkley).

2. Culture Medium

(a) Standard Medium

The standard liquid medium of Sehgal and Gibbons (1960) was used with a few modifications, as follows:

The medium contained per litre: Oxoid Bacteriological

Peptone, 10.0 g; potassium chloride, 2.0 g; trisodium citrate, 3.0 g; magnesium sulfate heptahydrate, 20.0 g; sodium chloride, 250 g; and 1 ml of a 1% Fe^{2+} solution (0.5 g $FeSO_4.7H_2O$ + 10 ml H_2O +0.1 ml 1N HCl). The medium components were made to the appropriate volume (usually a 12 l batch was prepared each time), the pH was adjusted to 6.7, and the medium was autoclaved for 15 min at 121°C before the peptone was added. Higher yields of cells and purple membrane were obtained if the peptone was added after autoclaving.

(b) Low Sulfate Medium

This medium was prepared in the same manner as standard medium, except the magnesium sulfate was replaced by an equimolar amount of magnesium chloride.

3. Growth of the Organisms

Stock cultures were maintained on agar slants at 4°C. Cells were grown on a Psychrotherm rotary shaker (New Brunswick Scientific Co.), and growth was followed by turbidity measurements at 660 nm using culture medium without bacteria as a blank.

Primary inocula were prepared by transferring cells from a stock agar culture into 50 ml of culture medium in a 250 ml side arm flask and incubating at 39°C and 190 rpm in the dark for 48 h (late log phase). Secondary inocula were prepared by adding 25 ml of the primary inoculum to 1.2 litres of medium in a 4 litre flask, and incubating under the same conditions for 72 h (stationary phase).

Liquid cultures were prepared by adding 400 ml of secondary inoculum to a 4 l flask containing 1.6 litres of culture medium. This was incubated at 39°C and 190 rpm in the light for 72 h, then the rate of

shaking was reduced to 100 rpm. When the cells were well into stationary phase (120-145h), they were harvested by centrifugation at 8,000 xg for 10 min using a Sorvall RC2-B automatic refrigerated centrifuge. The cell pellet was washed twice with basal salt solution containing per litre: sodium chloride, 250 g; potassium chloride, 2 g; and anhydrous magnesium sulfate, 9.8 g.

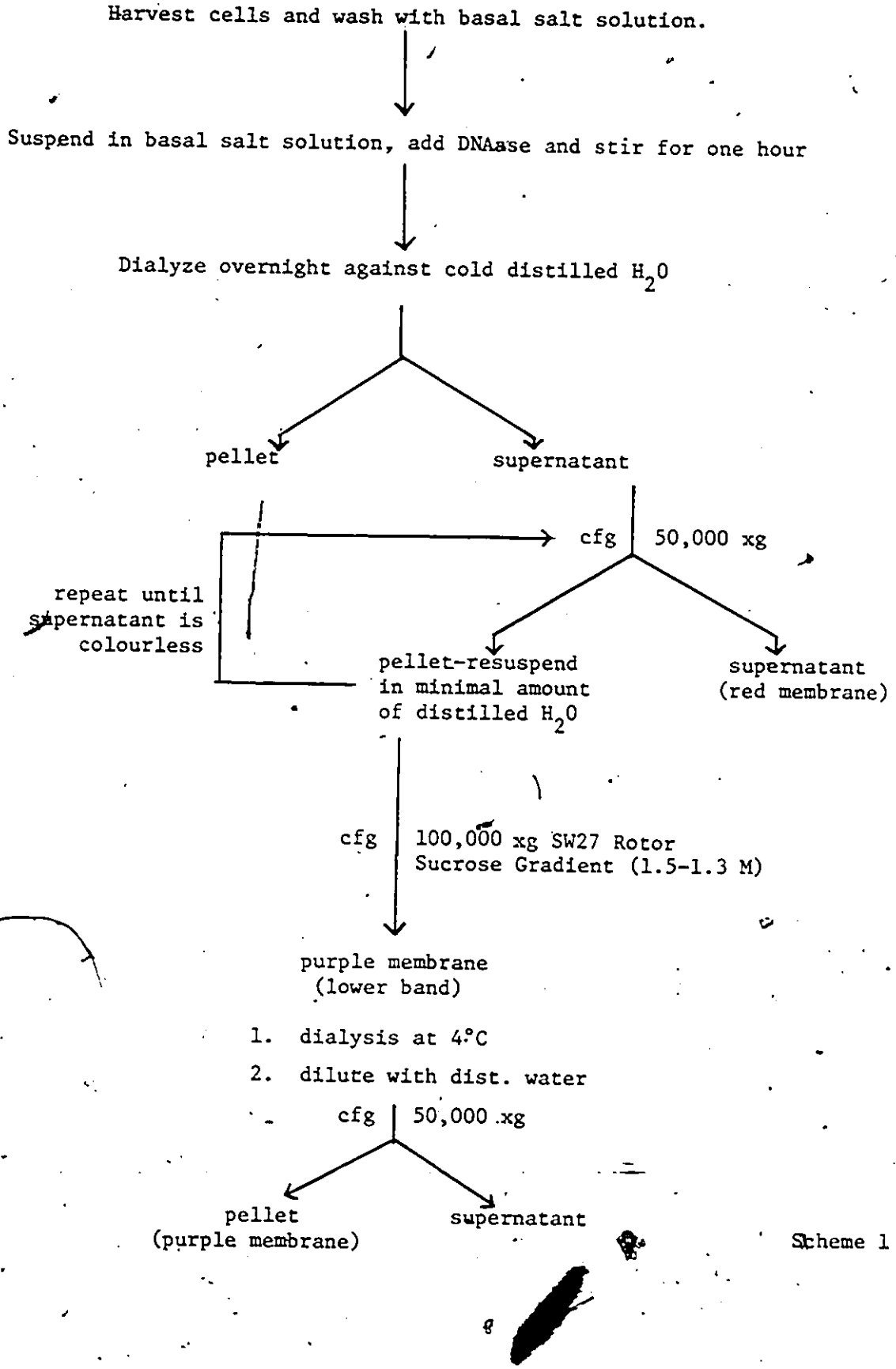
III. Preparation of Purple and Red Membranes (Scheme 1)

H. cutirubrum or H. halobium cells from a 12 litre culture grown under conditions of low aeration were resuspended in 200 ml of basal salt solution containing 10 mg of deoxyribonuclease by magnetic stirring for approximately 2 h at room temperature. The suspension was dialyzed overnight against distilled water at 4°C, centrifuged at 10,000 x g for 20 minutes and the supernatant was decanted and centrifuged at 50,000 x g for 1 h. The pellet obtained was resuspended in cold distilled water, and centrifuged at 50,000 x g for 1 h; this process was repeated until the supernatant did not visibly contain any red membrane.

1. Purple Membrane

The 50,000 x g pellet from a 12 l culture was resuspended in a minimum volume of distilled water and layered onto six discontinuous sucrose gradients prepared by layering 20 ml of 1.3M sucrose onto 10 ml of 1.5M sucrose in 40 ml cellulose nitrate tubes. The gradients were centrifuged at 100,000 x g for 48 h at 15°C using a Beckman SW-27 rotor, then the purple membrane, which appeared at the interface of the 1.3 and 1.5M sucrose layers, was collected by gravity flow. Sucrose was removed from the preparation by dialysis against distilled water at 4°C and the

Isolation of Purple Membrane



Scheme 1

dialyzate was diluted to 400 ml with distilled water and centrifuged at 50,000 x g for 1 h. The purple membrane pellets were resuspended in a minimum volume (20 ml) of 1 mM NaN_3 solution and stored at 4°C.

2. Red Membrane

The 50,000 x g supernatant (400 ml) was recentrifuged at 50,000 x g to remove any residual purple membrane, followed by centrifugation at 200,000 x g for 2 h at 4°C using a Beckman Ti-60 rotor. The pellet was resuspended in a minimum volume (20 ml) of 1 mM NaN_3 solution and stored at 4°C.

IV. Lipid Extraction and Fractionation Procedures

1. Bligh and Dyer (1959) Extraction

The cell pellet obtained from a 12 l culture (25-30 g wet wgt) was completely suspended in 200 ml of 25% sodium chloride solution by magnetic stirring. The cell suspension was transferred to a 4 litre flask, then 750 ml of methanol-chloroform (2:1, v/v) was added to give a one phase solvent system having a chloroform-methanol-water ratio of 1:2:0.8 v/v/v. The mixture was shaken well, left to stand in the dark at room temperature for several hours (the flask was shaken intermittently during this period) and filtered through glass wool on a Buchner funnel; the residue was washed with chloroform-methanol-water (1:2:0.8; v/v/v) until the red pigments were completely extracted.

The one phase system (volume ca. 1080 ml) was transferred to a 2 litre separatory funnel, then ca. 280 ml (total volume of 1 phase system divided by 3.8) each of chloroform and distilled water were added. The biphasic system was shaken well and the phases were allowed to separate

overnight. The lower chloroform phase was withdrawn and the upper phase was washed three times with 100 ml of chloroform. The combined chloroform extracts were diluted with an equal volume of benzene and taken to dryness on a rotary evaporator at 35-40°C. The residue was dissolved in chloroform-benzene (1:1, v/v), cleared by centrifugation and stored at 4°C. Yield: ca. 275 mg total lipids per 12 litre culture.

2. Acetone Precipitation of Polar Lipids

Total lipids were dissolved in a minimum volume of chloroform (usually 1.5 ml per 275 mg of lipid), diluted with ten volumes of dry acetone, mixed well and allowed to stand overnight at 0°C. The tan coloured precipitate of polar lipids was washed twice with small portions of acetone, dried in vacuo over KOH and weighed. Yield: ca. 245 mg per 12 litre culture (ca. 90% of total lipid).

V. Chromatography of Lipids

1. Thin Layer Chromatography (TLC)

(a) Analytical TLC

For purity determinations and for monitoring of reactions, thin layer chromatograms were run on 7.5 cm x 2.5 cm microscope slides or 20 cm x 20 cm plates coated with silica gel G (0.5 mm thickness). Pure lipids were assayed for contaminants by running the sample on two separate plates and spraying each with the phosphate detecting reagent and the α-naphthol reagent for sugars (see below).

(b) Preparative TLC

Preparative thin layer chromatography was performed using 20 cm x 20 cm glass plates coated with silica gel G (1.0 or 0.75 mm thick-

ness). Plates were activated for at least four hours before use.

(c) Detection and Staining of Lipids

The following stains were used to locate lipids on preparative TLC plates, monitoring purity, and for the detection of functional groups.

(i) Rhodamine 6G (Marinetti and Stotz, 1956)

An aqueous stock solution (0.1%) of Rhodamine 6G (colour index number 752, Hartman-Leddon Company, Philadelphia, Pennsylvania) was prepared, stored in a brown bottle and diluted(1:100 v/v) with distilled water immediately before use. Preparative TLC plates were sprayed with a minimal amount of the dye solution, then the bands corresponding to the lipids were located by viewing the wet plate under ultraviolet light (366 mm). It was found that plates developed with chloroform-90% acetic acid-methanol (CAM) (30:20:4, v/v/v) had to be air dried for at least two hours before spraying. Rhodamine could be removed from the eluted lipids by a) repeated precipitation from acetone or b) TLC and location of the lipid by spraying with distilled water.

(ii) 50% Ethanolic Sulfuric Acid

The reagent was prepared by slowly adding 50 ml concentrated H_2SO_4 (J.T. Baker, Phillipsburg, N.J.; reagent grade) to 50 ml of 95% ethanol cooled on ice. Lipids were visualized by spraying with an all-glass atomizer operated from a compressed air pump. The TLC plate was then heated on a hot plate for 15-30 min; all lipids and any other organic compounds appeared as black spots.

(iii) Naphthol Stain for Glycolipids (Siakotos and Rouser, 1965).

The reagent was prepared by dissolving 0.5 g of α-naphthol in 100 ml of methanol-water (1:1). The plate was sprayed with the α-naphthol solution until damp, allowed to dry for about five minutes, then sprayed lightly with sulfuric acid solution. Colour development of the spots was observed during heating; glycolipids gave blue-purple spots whereas phospholipids gave yellow spots.

(iv) Phosphate Stain (Dittmer and Lester (1964) procedure -modified by Vaskovsky and Kosketsky, 1968)

The TLC plate was sprayed with the phosphate reagent*, the phosphatides appeared as blue spots on a white background after a few minutes at room temperature. The reagent is specific for phospholipids.

VI. Gas-Liquid Chromatography of Carbohydrate Derivatives

1. Trimethylsilyl Derivatives and Alditol Acetates

Methyl glycosides as the trimethylsilyl ethers were chromatographed on a 6 ft glass column of 5% SE-30 at 180°C with 1 Kg/cm^2 of nitrogen carrier gas. Alditol acetates were chromatographed on a 6 ft glass column of OV-225 or SP-2330 at 190°C with 1 Kg/cm^2 of nitrogen carrier gas.

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*Phosphate Reagent: Solution A: 16 g ammonium molybdate in 120 ml H_2O.

Solution B: add 10 ml of mercury and 80 ml of solution A to 40 ml conc. HCl; shake for 30 min. and filter.

Spray Agent: add 200 ml of conc. H_2SO_4 and all of solution B to remainder of solution A: cool and make to 1 litre with H_2O.

A Carbo Erba Fractovap fitted with a flame ionization detector (FID) was used for the analyses. Peaks were identified by their relative retention times in comparison to those of authentic standards and their areas were measured by the procedure of Carroll (1961):

$$\% A_i = \frac{d_i h_i}{\Sigma d_i h_i} \times 100$$

where A = peak area

d = distance (mm)

h = peak height (mm)

2. Gas Chromatography-Mass Spectrometry of Partially Methylated Alditol Acetates

Alditol acetates of partially methylated sugars were analyzed using a Perkin-Elmer gas chromatograph interfaced with a Finnigan 1020 Automated GC/MS quadropole mass spectrometer. Samples were chromatographed on a glass column of 3% OV-225 programmed to run isothermally at 180°C for 23 minutes followed by a 5°C/min increase to 220°C. The mass spectrometer was programmed to scan from 35 to 300 atomic mass units at 2.0 second intervals.

Mass spectrograms were interpreted by comparison with published spectra (Jansson et al., 1976), a computer search through 25,000 spectra in the National Bureau of Standards (USA) library, and correlation with fragmentation patterns (see Appendix I).

VII. Analytical Procedures

1. Phenol-Sulfuric Method for Total Sugars (Dubois et al., 1954)

An aliquot of lipid solution or hydrolyzate, containing 10-80 ug of sugar, was transferred to a 25 ml Lewis-Benedict sugar tube, then the sample was brought to dryness under a stream of N_2. The residue was dissolved in 2.0 ml of water, then 1.0 ml of 5% aqueous phenol was added. The solution was immediately "vortexed" while 5.0 ml of concentrated sulfuric acid was added, then it was allowed to cool for 30 min. The absorbance at 490 nm was read against a reagent blank; duplicate standards containing 20, 40 and 80 ug of hexose (a mixed sugar standard of glucose:galactose:mannose, 1:1:1,w/w/w) were run simultaneously. The standard curve was found to be linear in the range 20-100 ug hexose.

2. Phosphorus Determination (Bartlett, 1959)

The lipid sample, containing 0.5 to 10 ug. P was digested by heating with 0.4 ml of 72% perchloric acid. Distilled water (4.2 ml), amidol reagent (0.2 ml)* and ammonium molybdate solution (0.2 ml)** were added successively with "vortexing". The tube was covered with a small beaker, heated in a boiling water bath for 7 min. then cooled at room temperature. The absorbance of the blue colour was read at 780 nm in 12 mm round cuvettes against a reagent blank. Standards in the range of 1.0-8.0 ug P were prepared and carried simultaneously through the procedure; Beer's law was obeyed over this range.

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* Amidol Reagent: 0.5 g 2,4-diaminophenol dihydrochloride (Amidol) and 10 g sodium bisulfite dissolved in 50 ml distilled water and filtered.

** Ammonium molybdate solution: 5.0 g ammonium molybdate dissolved in 100 ml distilled water and filtered.

VIII. Physical Methods Used for Structure Determination

1. Nuclear Magnetic Resonance Spectra

^{1}H-NMR spectra were obtained on 15-25 mg samples dissolved in deuterochloroform-deuteromethanol (2:1; v/v; 0.5 ml) using a Varian HA-100 NMR spectrometer; tetramethylsilane was used as an internal reference and chemical shifts were reported as ppm (δ) relative to TMS.

2. Infrared Spectra

Infrared spectra were obtained from thin films between NaCl plates or from KBr pellets containing ca. 2% w/w lipid. All samples were dried in vacuo over KOH. A Beckman IR-20 double beam spectrophotometer was used for all spectra.

3. Optical Rotation

Optical rotations were measured at ambient temperature on chloroform, chloroform-methanol (95:5; v/v) or chloroform-methanol (1:1; v/v) solutions, using a Perkin-Elmer polarimeter, model 141. Silicic acid in samples was completely removed by centrifugation. The values reported represent the mean of at least three independent readings.

IX. Radioisotopic Procedures

1. Radioisotope Counting

An aliquot of the solution to be counted was transferred to a plastic counting vial and the solvent was removed under nitrogen. Radioactive spots from TLC plates were scraped directly into vials. Each sample was diluted with 10 ml of scintillation fluid, which contained: 2,5-diphenyl oxazole (PPO), 5 g/l; 2,3 -p-phenylene-bis (5-phenyl ozazole)

(POPOP), 0.3 g/l; Beckman Bio-Solv Solubilizer (Formula BBS-3), 100 ml; methanol, 130 ml; toluene was added to make the volume up to 1.0 litre.

Samples were counted in a Beckman LS-150 scintillation counter having an external standard and an automatic quench control. A vial containing scintillation fluid only was included in every set of samples in order to obtain a background reading. Both ^{35}S and ^{14}C were counted in the ^{3}H + ^{14}C window.

2. Autoradiography

^{14}C and ^{35}S-labelled compounds on TLC plates were detected by placing a 20 x 20 cm sheet of Kodak no-screen X-ray film onto the silica gel and exposing the film in a light-proof folder. The period of exposure depended upon the activity of the sample (Kates, 1972).

PART ONE

STRUCTURAL STUDIES ON THE GLYCOLIPIDS OF H. CUTIRUBRUM: SULFATED TETRAGLYCOSYL DIETHER (GL-1), TETRAGLYCOSYL DIETHER (GL-2), AND TRIGLYCOSYL DIETHER (GL-3)

EXPERIMENTAL PROCEDURES

I. Isolation of the Glycolipids (Scheme 2)

1. Barium Chloride Fractionation (Kates et al., 1965)

A solution of the acetone precipitated total lipids (see General Materials and Methods, Section IV-2), (975 mg in 4 ml of chloroform) was diluted with methanol (30 ml), mixed well, and allowed to stand overnight at 0°C. The precipitate was centrifuged and washed twice with cold methanol (4 ml). A solution of 20% aqueous barium chloride was added dropwise to the pooled supernatants until no further precipitation occured, then the suspension was allowed to stand overnight at 0°C.

The precipitate (PGP plus some GLS) was centrifuged, washed three times with cold methanol (4 ml) and used for the preparation of PGP (Part Three, Experimental Procedures, I). The supernatant fractions were pooled with the methanol precipitate and designated "glycolipid-enriched fraction", which contained GL-1, GL-2, GLS, GL-3 and PG.

2. Preparative Thin Layer Chromatography of "Glycolipid-Enriched Fraction"

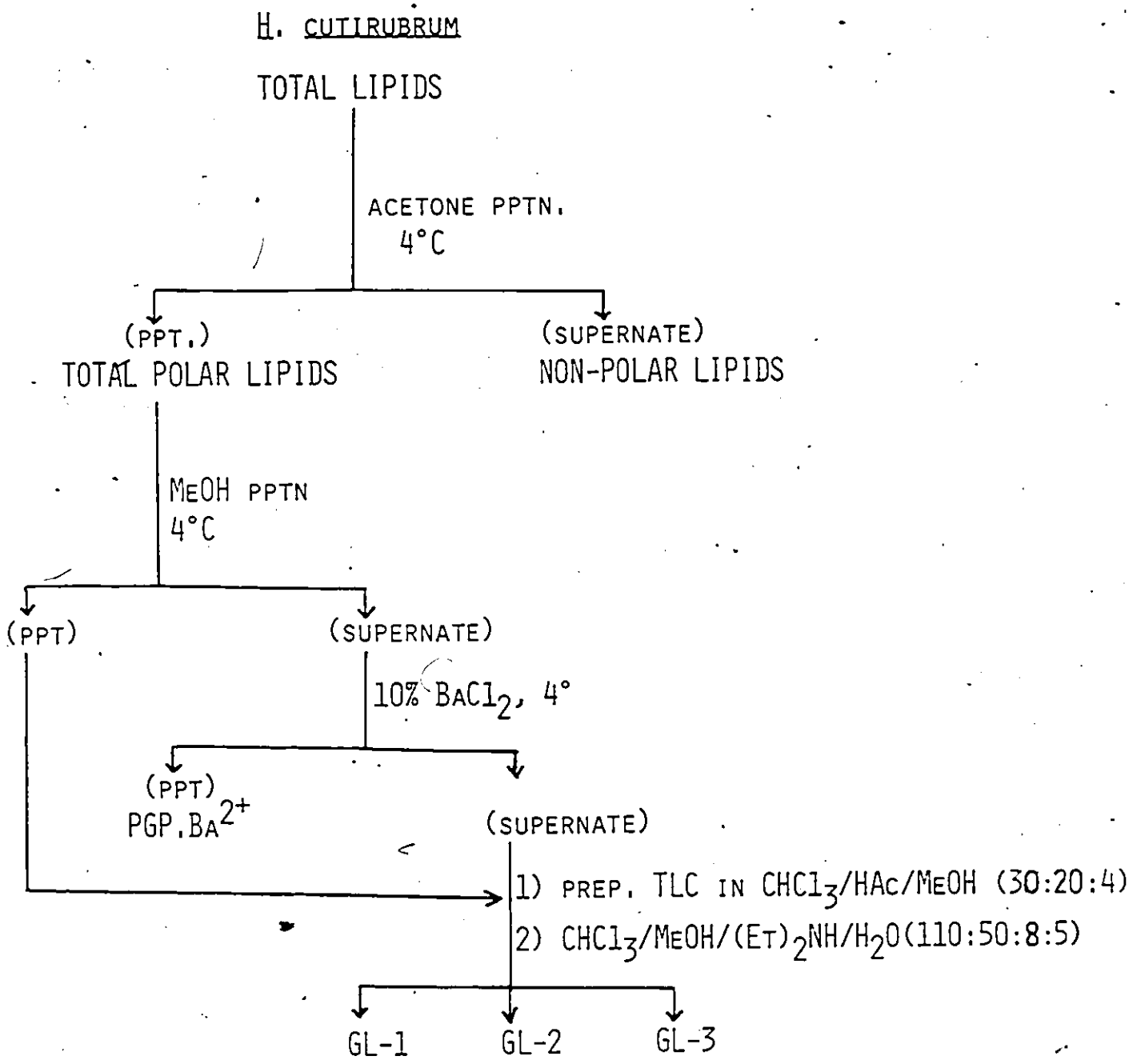
The "glycolipid enriched fraction" was brought to dryness by rotary evaporation, dissolved in 25 ml of chloroform-methanol (1:1; v/v) and the system was made biphasic by the addition of 11.25 ml of 0.1N HCl. The upper phase (methanol-H_2O) was washed twice with lower phase ($CHCl_3$

equilibrated with Bligh-Dyer upper phase) and the pooled chloroform phases were diluted with benzene and brought to dryness on a rotary evaporator.

The crude lipid mixture was fractionated by preparative TLC in chloroform - 90% acetic acid-methanol (30:20:4; v/v/v) on silica gel G (0.75 mm thick layers). The bands corresponding to GL-1 + GLS + GL-2, GL-3 + PGS, PGP, PG and an unidentified phospholipid were eluted from the silicic acid on a sintered glass funnel using chloroform-methanol-water (1:1:0.2; v/v/v). Each extract was diluted with 99% ethanol and brought to dryness under reduced pressure, then benzene was added to remove last traces of water.

The mixture containing GLS, GL-1 and GL-2 was fractionated by preparative TLC in chloroform-methanol-diethylamine-water (110:50:8:5; v/v/v/v) and the mixture of GL-3 and PGS by chromatography in chloroform-methanol-concentrated ammonium hydroxide (65:35:5; v/v/v). Silicic acid and water-soluble contaminants were removed from the preparations by dissolving the material in 10 ml of chloroform-methanol (1:1; v/v) then adding 4.5 ml of 0.2M KCl (aqueous). The chloroform phase was removed, then the upper phase was washed twice with chloroform. The chloroform extracts were pooled, diluted with benzene and brought to dryness by rotary evaporation at 35-40°C. The material was cleared by centrifugation in chloroform-benzene (1:1; v/v), dissolved in a minimal volume of chloroform-methanol (1:1; v/v), precipitated with ten volumes of acetone, and allowed to stand overnight at 0°C. Rhodamine was completely removed by repeated precipitation from acetone. The glycolipids were found to be chromatographically pure (Figure 18) and stained negatively for phosphate.

ISOLATION OF MINOR GLYCOLIPIDS



SCHEME 2

3. Preparation of the Ammonium Salt Forms of GL-1 and GLS

GL-1 and GLS were converted to their free acid forms by passing a solution of 5-10 mg in ca. 1ml of chloroform-methanol-water (1:1:0.05, v/v) through a column of Rexyn 101 (H^+ form, 1 ml) and eluting with 20 ml of chloroform-methanol (1:1, v/v). The eluate was diluted with an equal volume of benzene, rapidly concentrated to 10-15 ml on a rotary evaporator below 25°C, and the free acid was converted to the ammonium salt by titration with 0.2N methanolic NH_4OH. The ammonium salt form was precipitated from a small volume of chloroform with 10 volumes of acetone in the cold, collected by centrifugation and dried in vacuo.

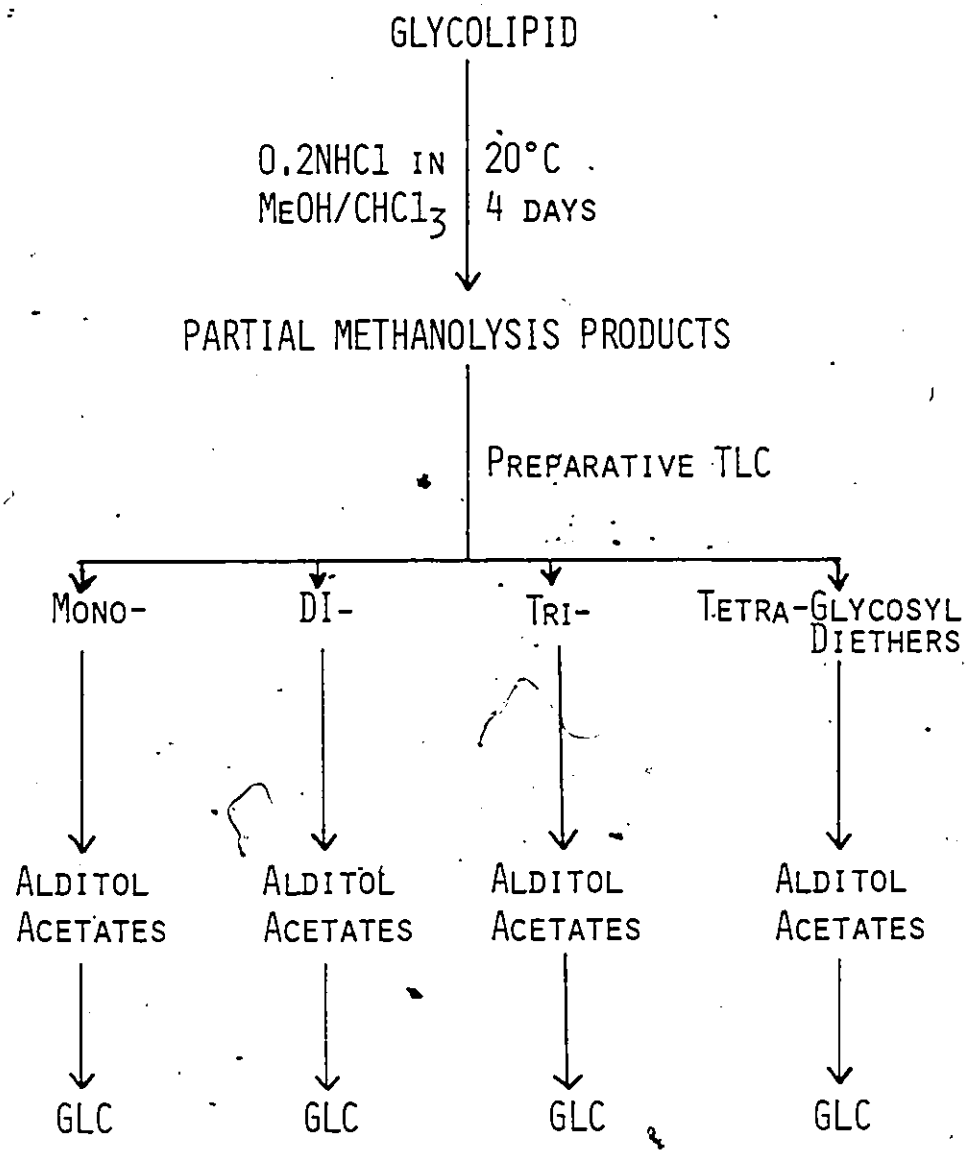
II. Procedures Used for Structure Elucidation

1. Partial Acid Methanolysis of the Glycolipids

(a) Methanolysis Procedure (Scheme 3)

Samples containing 12 mg GL-1, 12 mg GL-2 and 21 mg GL-3 each in 1.5 ml chloroform were mixed with 2.0 ml of 0.25 N methanolic-HCl (prepared by bubbling HCl gas into anhydrous methanol), kept at room temperature, and the reactions were monitored by microslide TLC in chloroform -90% acetic acid-methanol (30:20:4; v/v/v). When each reaction was found to yield approximately equimolar amounts of each glycolipid fragment (4-5 days), it was stopped by making a biphasic system through the addition of 0.5 ml chloroform and 1.8 mL distilled water. The chloroform phase was washed with methanol-water (10:9, v/v), diluted with 99% ethanol and brought to dryness under a stream of nitrogen. Yield of partially methanolyzed glycolipids from GL-1, 6.7 mg; GL-2, 8.6 mg; GL-3, 17.9 mg.

DETERMINATION OF GLYCOLIPID SEQUENCES



SCHEME 3

(b) Isolation of the Chloroform-Soluble Products

The glycolipid mixtures were separated by preparative TLC in chloroform-90% acetic acid-methanol (30:20:4; v/v/v); the separated band were visualized with Rhodamine and eluted four times with 5 ml of chloroform-methanol-water (1:1:0.2; v/v/v). To the pooled extracts, 8.0 ml of 0.2 M KCl was added to made a biphasic system, then the chloroform phases were evaporated. The glycosyl diethers obtained were found to be chromatographically pure.

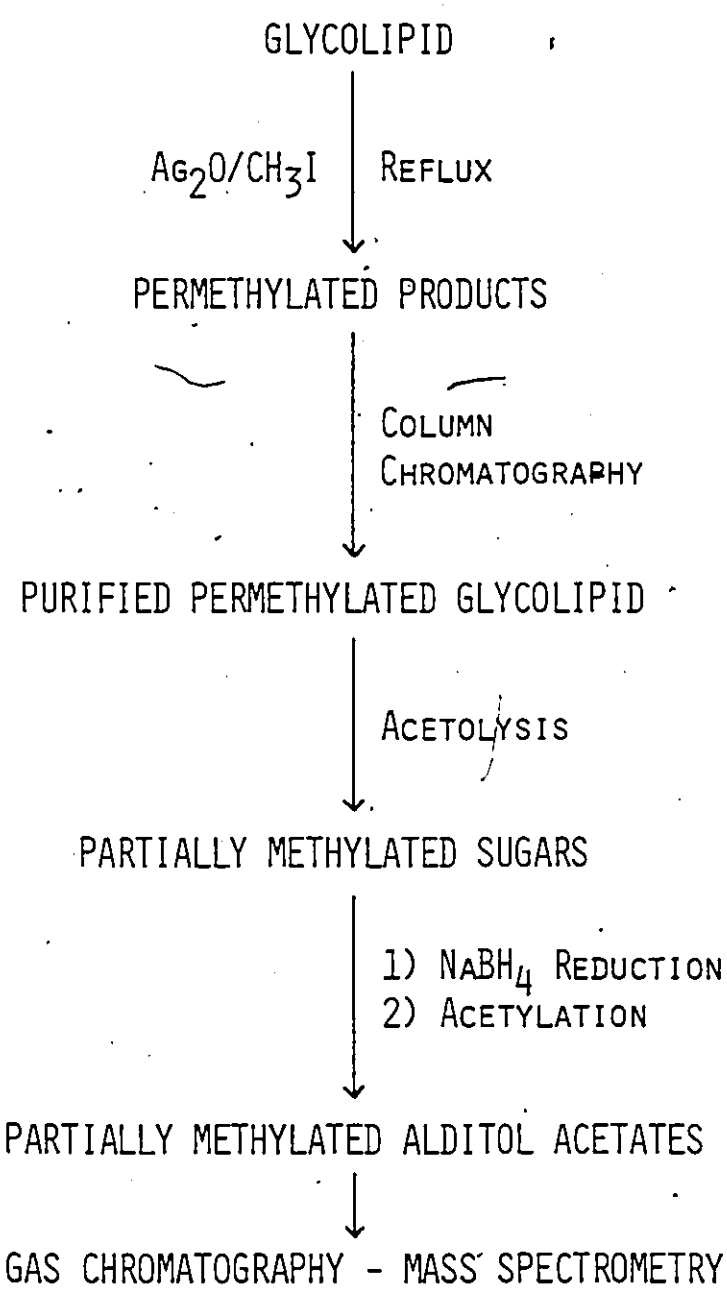
2. Permethylation Procedures for Glycolipids (Scheme 4)

(a) Methylation (Kates and Deroo, 1973)

Sulfated glycolipids were converted to the free acid form prior to methylation by dissolving each sample in 3.8 ml of chloroform-methanol-0.2N HCl (2:1:0; v/v/v) and adding 1.0 ml each of chloroform and water to make a biphasic system; the chloroform phase was washed with 1.0 ml of methanol-water (10:9; v/v), diluted with benzene and evaporated to dryness under nitrogen. Because the high polarity of GL-1 caused problems during Bligh and Dyer partitioning, the compound was first partially methylated as the ammonium salt, then the partially methylated GL-1 was converted to the free acid.

The glycolipid sample (5-10 mg; dried over P_2O_5 under high vacuum for several hours) was dissolved in 5 ml of distilled methyl iodide containing 1% methanol and fresh silver oxide (ca 10 mg) was added every 30 min. The reaction was monitored by microslide TLC using ethyl ether-methanol (99:1). When the reaction was complete, the mixture was diluted with an equal volume of ethyl ether, centrifuged, and the silver

PERMETHYLATION ANALYSIS



Scheme 4

salts were washed twice with 5 ml of benzene-chloroform (1:1; v/v). The supernatants were combined and brought to dryness in vacuo or under a stream of nitrogen.

(b) Purification of Permethylated Glycolipids

(i) Permethylated Glycolipid Sulfates (GLS and GL-1)

The crude methylation mixture was fractionated on a column of silicic acid (1 g; Bio-Sil A, activated at 120°C; made up with benzene) with the following elution sequence: benzene, 10 ml; benzene-ether (95:5), 10 ml; benzene-ether (90:10), 15 ml; benzene-ether (80:20), 15 ml; benzene-ether (70:30),15 ml; benzene-ether (50:50), 10 ml; benzene-ether (25:75), 10 ml; methanol, 10 ml. The fractions were brought to dryness under a stream of nitrogen and analyzed by microslide TLC. Permethylated GLS and GL-1 were eluted in the benzene-ether (80:20) and benzene-ether (70:30) fractions, respectively.

(ii) Permethylated Glycolipids (GL-2 and GL-3)

The crude reaction mixture was separated by preparative TLC using ethyl ether or ethyl ether-methanol (99:1) as the solvent. The bands were located with rhodamine 6G spray and eluted with chloroform-methanol (1:1; v/v). After purification, the permethylated glycolipids showed no OH absorption in their infrared spectra, indicating that methylation had been complete.

3. Degradation of Glycolipids and Permethylated Glycolipids

(a) Methanolysis (Kates, 1972)

Complete methanolysis of the glycolipids (5-10 mg) was carried out in side arm flasks (Kates, 1964) containing 4.5 ml of 2.5% methanolic-HCl under reflux for 4 h. After addition of 0.5 ml of H_2O,

the methanolyzates were extracted with low boiling petroleum ether (5 x 1 ml). The methanol-water phases were transferred to centrifuge tubes, benzene was added, and the samples were brought to dryness under a nitrogen stream and desiccated in vacuo over KOH.

(b) Acetolysis (Stellner et al., 1973)

The sample (0.2 - 2 mg) was digested in 0.5 ml of 0.5 N H_2SO_4 in 95% acetic acid (5 ml of 10 N H_2SO_4 plus 95 ml of glacial acetic acid) for 16 h at 80°C. After cooling, 0.5 ml of distilled water was added, followed by further heating for 5 h at 80°C.

4. Preparation of Carbohydrate Derivatives

(a) Trimethylsilyl Derivatives

The sugar methyl glycosides obtained by methanolysis (Part One, Experimental Procedures, II, 3 (a)) and methyl glycoside standards were made to volume with methanol, then aliquots containing 0.5 mg were transferred to 15 ml stoppered centrifuge tubes and blown dry under nitrogen. To each sample, 50 ul of dry pyridine was added, followed by 20 ul hexamethyldisilazane and 10 ul trimethylsilyl chloride. The reaction was allowed to proceed for 15 min. at room temperature, then the mixture was cleared by centrifugation. The trimethylsilyl derivatives were analyzed by gas liquid chromatography as described above (General Materials and Methods, VI, 1).

(b) Alditol Acetates (Yang and Hakomori, 1971)

The acetolyzed samples (Part One, Experimental Procedures, II, 3 (b)) were evaporated under nitrogen to a volume of 0.2 ml, then solid $BaCO_3$ was added and the samples were allowed to stand at room tem-

perature for several hours. When the pH was found to be neutral, 99% ethanol was added, the mixture was centrifuged, and the precipitate was washed three times with 99% ethanol. The pooled supernatants were brought to dryness by rotary evaporation, then the residues were dissolved in 0.5 ml of distilled water and reduced to alditols with excess sodium borohydride (ca. 20 mg) at room temperature for 1 h. Glacial acetic acid was added dropwise to destroy excess borohydride, then the mixture was taken to dryness with 4 x 10 ml additions of methanol to remove boric acid as the volatile trimethyl ester.

The samples were dried in vacuo over KOH, then 3 ml of redistilled acetic anhydride was added. The reaction mixture was heated at 100°C for 4 h, then brought to dryness on a rotary evaporator with several additions of toluene. The residual alditol acetates were dissolved in chloroform and analyzed by gas liquid chromatography as described above (General Materials and Methods, VI, 1).

5. Determination of Anomeric Configuration by CrO_3 Oxidation

(Scheme 5) (Laine and Renkonen, 1975)

(a) Acetylation of Glycolipids

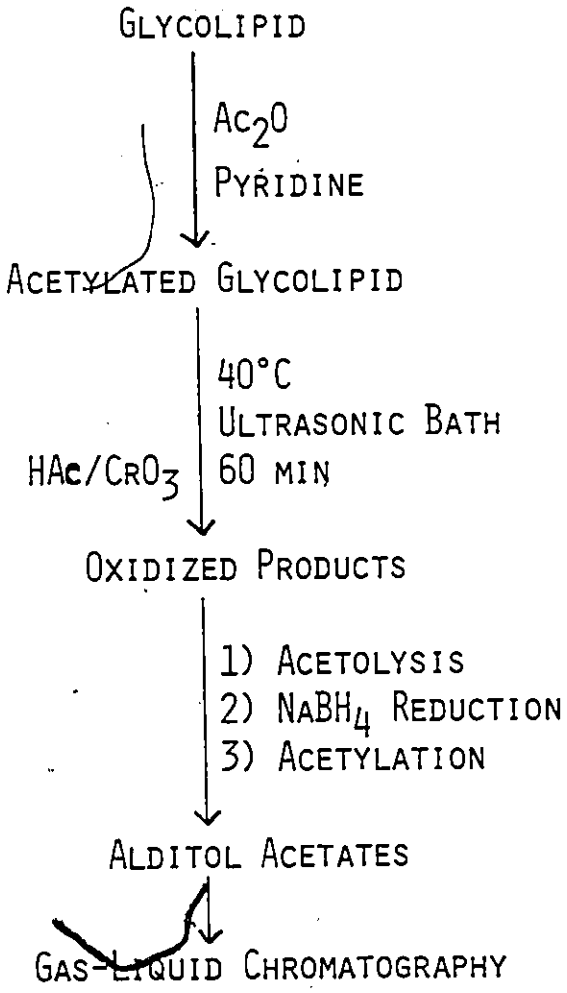
The lipid sample (ca. 1 mg) was acetylated overnight at room temperature with 1 ml of pyridine-acetic anhydride (1:1; v/v), then finally heated at 80°C for 30 min. Acetylated glycolipids were analyzed on microslide TLC using n-butylacetate as the solvent, and acetylated glycolipid sulfates by using chloroform-methanol (9:1; v/v).

(b) CrO_3 Oxidation

An aliquot (ca. 0.5 mg) of the acetylated lipid was diluted with toluene and evaporated to dryness under a nitrogen stream;

DETERMINATION OF ANOMERIC CONFIGURATION

PRINCIPLE: UNDER CONTROLLED CONDITIONS, CrO_3 ATTACKS β-GLYCOSIDIC LINKAGES OF PYRANOSES BUT NOT α-GLYCOSIDIC LINKAGES



SCHEME 5

1.00 ml of acetic acid and 100 mg of CrO_3 were added, and the mixture was treated for 30-60 min. (depending on the number of sugars present) at 40°C in an ultrasonic cleaning bath. The reaction was stopped by the addition of 3 ml of water and the mixture was extracted three times with 3 ml of chloroform. The pooled chloroform phases were taken to dryness in vacuo.

(c) Analysis of Oxidation Products

The acetylated monosaccharides which survived the oxidation were present in the chloroform extracts. The oxidation products and the original acetylation mixture were acetolyzed and the alditol acetates were prepared and analyzed as described above (Part One, Experimental Procedures II, 3 and 4).

6. Desulfation of Glycolipid Sulfates (Ishizuka et al., 1973

A solution of a glycolipid sulfate in 0.05 N methanolic HCl (final concentration: 0.5 mg glycolipid sulfate/ml) was stirred at room temperature for 5 h. The reaction mixture was neutralized with 0.2 M methanolic NH_4OH, dried by rotary evaporation, and ammonium chloride was removed via a Bligh and Dyer partitioning. The mixture of glycolipids was separated by preparative TLC using chloroform-90% acetic acid-methanol (30:20:4; v/v/v) and the individual components were purified as described above (Part One, Experimental Procedures, Section II, 1 (b)).

RESULTS

I. Characterization of GL-1, GL-2 and GL-3

Thin layer chromatography of these glycolipids showed that they were chromatographically pure and well resolved in chloroform-90% acetic acid-methanol (30:20:4, v/v/v) and chloroform-methanol-diethylamine-water (110:50:8:5; v/v/v/v) solvent systems (Figure 18). Note that GL-2 and GL-3 had the same R_f values as desulfated GL-1 and desulfated GLS, respectively (Table 3).

The ammonium salt form of GL-1 is soluble in chloroform-benzene (1:1; v/v) and in chloroform-methanol mixtures rich in chloroform (e.g., 4:1, 3:1, 2:1; v/v) but is only slightly soluble in pure chloroform and in chloroform-methanol (1:1; v/v), methanol and acetone. GL-1 partitions into the upper phase (methanol-water, 10:9; v/v) of the Bligh-Dyer biphasic solvent system unless it is made 0.1 M in KCl. GL-2 and GL-3 are soluble in chloroform-methanol mixtures. They form gels in chloroform and are only slightly soluble in acetone.

The specific and molecular optical rotations of GL-1 and GL-3 (Table 4) are large and positive. These values were used to determine the anomeric configurations of the sugar residues (Part One, Results II, 1, (d)).

II. Structural Identification of GL-1, GL-2 and GL-3

1. Sulfated Tetraglycosyl Diether (GL-1)

(a) Analytical Data

Acid methanolysis of GL-1 yielded a petroleum ether soluble product which had an infrared spectrum (Figure 19) and optical

Figure 18

Thin layer chromatograms of the purified glycolipids and total polar lipids of Halobacterium cutirubrum:

Identification of components:

Lane 1 sulfated tetraglycosyl diether (GL-1)

Lane 2 desulfated tetraglycosyl diether

Lane 3 tetraglycosyl diether (GL-2)

Lane 4 sulfated triglycosyl diether (GLS)

Lane 5 desulfated triglycosyl diether

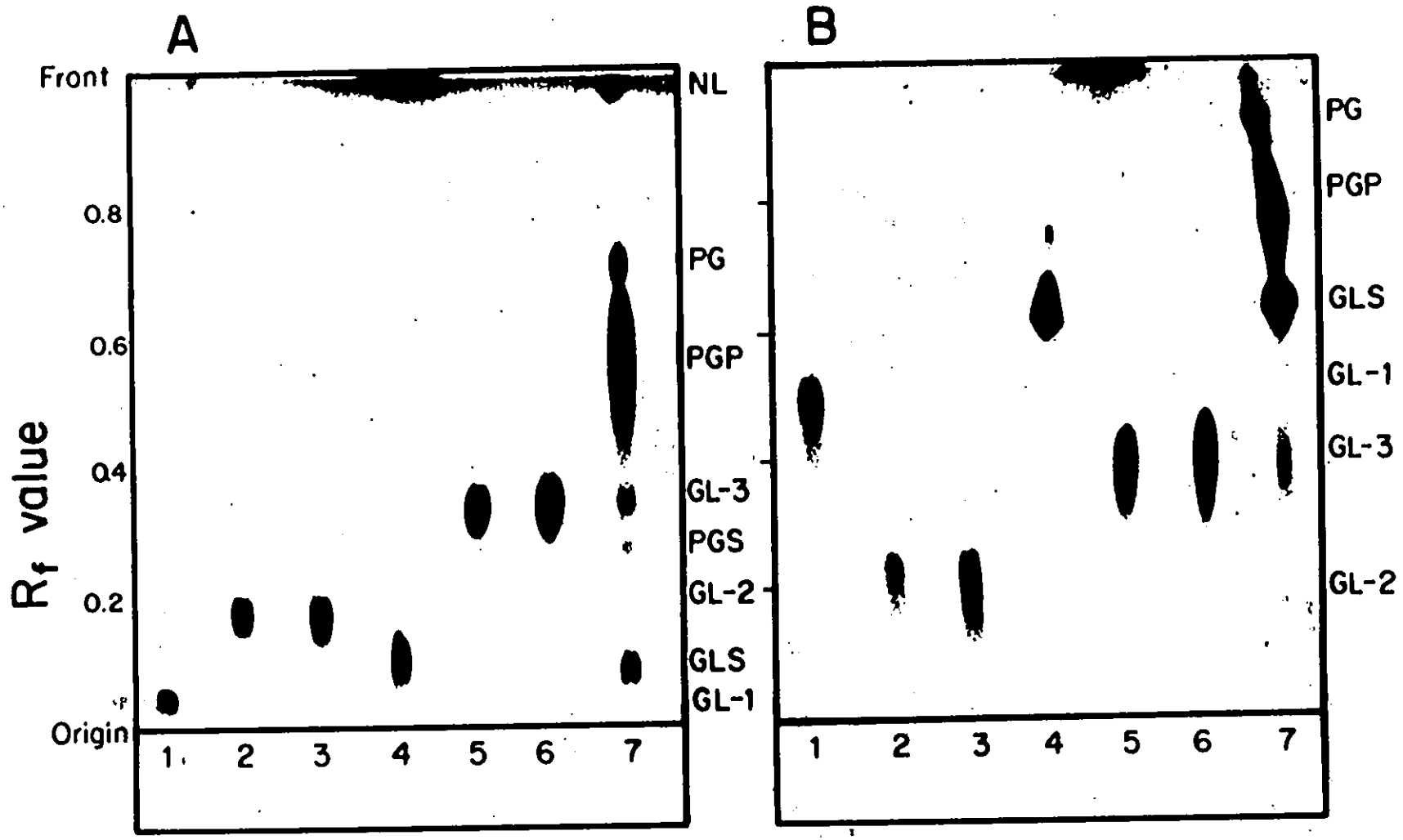
Lane 6 triglycosyl diether (GL-3)

Lane 7 total polar lipids

Solvent Systems:

A - Chloroform-90% acetic acid-methanol
(30:20:4; v/v/v)

B - Chloroform-methanol-diethylamine-water
(110:50:8:5; v/v/v/v)


A
B
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1
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6
7

Table 3. R_f values of GL-1, GL-2, GL-3 and GLS in acidic and basic solvent systems

Glycolipid	Solvent System* A	Solvent System* B
GL-1 (NH_4^+-salt)	0.05	0.46
Desulfated GL-1**	0.17	0.19
GL-2	0.17	0.19
GLS (NH_4^+-salt)	0.11	0.62
Desulfated GLS**	0.33	0.37
GL-3	0.34	0.38

*TLC on silica gel G in solvent systems: A, chloroform-90% acetic acid-methanol (30:20:4; v/v/v) and B, chloroform-methanol-diethylamine-water (110:50:8:5; v/v/v/v).

**Desulfated by solvolysis in 0.05N methanolic-HCl at room temperature.

rotation ($[\alpha]_D$ + 8.7) identical to those of 2,3-di-O-phytanyl-sn-glycerol (Kates, M; 1978). Elemental analyses of GL-1 (Table 4) were consistent with the values calculated for the ammonium salt of a monosulfated tetraglycosyl diphytanyl glycerol ether. The hexose/diether and sulfur/diether mole ratios were 4.0:1 and 1.3:1 respectively, indicating that the molecule contained four carbohydrate residues, one sulfate group and one diphytanyl glycerol moiety. Gas chromatographic analysis of the alditol acetates obtained after acetolysis of GL-1 identified the sugar residues as galactose, mannose and glucose present in mole ratio 2:1:1 (Table 5).

(b) Spectroscopic Data

The infrared spectrum of GL-1 as the ammonium salt (Figure 20) showed strong OH absorption at 3400 cm^{-1}, CH_2 and CH_3 absorption at 2920 and 1450 cm^{-1}, an isopropyl doublet at 1380 cm^{-1}, C-O-C ether absorption at 1100 cm^{-1} as a shoulder on the alcohol C-O band at 1060 cm^{-1}, a sulfate S=O band at 1245 cm^{-1} and a weak S-O-C band at 815 cm^{-1}. Carboxyl or ester carbonyl bands were not present, eliminating the possibility of acyl groups on either the glycerol or sugar hydroxyl groups. The spectrum was similar to that of the major glycolipid sulfate (GLS) (Kates and Deroo, 1973).

The ^{1}H-NMR spectrum of GL-1 (Figure 21) was also similar to that of GLS (Figure 22) and showed the following: a sharp methyl doublet centred at δ0.88 ppm integrating for 30 protons (30 expected); a broad methylene plus methine envelope (phytanyl chains) centred at δ1.33 ppm and integrating for 50 protons (48 expected); several multiplets from δ3.2-4.2 ppm integrating for 32 protons (34 expected) and assigned to carbohydrate and glycerol C<u>H</u> and C<u>H</u>$_2$, to C-1' phytanyl protons and to

Table 4. Analytical Data for GL-1 and GL-3

	GL-1		GL-3	
	Found	Calc.	Found	Calc.
Mol. wt.	-	1398.8[a]	-	1139.6[b]
C (%)	57.21	57.53	62.28	64.29
H (%)	9.33	9.44	9.75	10.44
S (%)	2.8	2.29	-	-
N (%)	0.89	1.00	-	-
Total Hexose (% by wt)	49.1	51.5	47.6	47.4
Diether (% by wt)	44	46.7	54	57.3
Hexose/Diether, mole ratio	4.0	4.0	3.2	3.0
S/Diether, mole ratio	1.3	1.0	-	-
$[\alpha]_D$ (in degrees)	+52.4	-	+46.8	+47.0[e]
M_D (in degrees)	+733	+720[c]	+533	+518[f]
		+301[d]		+899[g]

[a] Calculated for $C_{67}H_{127}O_{26}S.NH_4$

[b] Calculated for $C_{61}H_{118}O_{18}$

[c] Calculated for Galpβ→Manp(←Galfα)α→Glcpα→diether (see Appendix II)

[d] Calculated for Galfα→Manp(←Galfβ)α→Glcpα→diether

[e] $[\alpha]_D$ of desulfated GLS (Kates and Deroo, 1973)

[f] Calculated for Galpβ→Manpα→Glcpα→diether

[g] Calculated for Galpα→Manpα→Glcpα→diether

Table 5. Carbohydrate Composition of Glycolipids Derived from GL-1, GL-2 and GL-3 by Partial Methanolysis

Glycolipid	Sugar Mole Ratios*								
	GL-1			GL-2			GL-3		
	Glc	Man	Gal	Glc	Man	Gal	Glc	Man	Gal
Sulfated Tetraglycosyl Diether**	1.0	1.0	1.9	-	-	-	-	-	-
Tetraglycosyl Diether	1.0	0.8	2.0	1.0	0.8	1.8	-	-	-
Triglycosyl Diether	1.0	0.8	0.8	1.0	0.8	0.8	1.0	1.0	1.1
Diglycosyl Diether	1.0	0.9	-	1.0	0.8	-	1.0	1.0	-
Monoglycosyl Diether	+	-	-	+	-	-	+	-	-

*Carbohydrates were determined as alditol acetates except for diglycosyl diether and monoglycosyl diether in which carbohydrates were determined as trimethylsilyl derivatives.

**Starting Material

Figure 19

Infrared spectrum of the petroleum ether soluble product of methanolyzed GL-1.

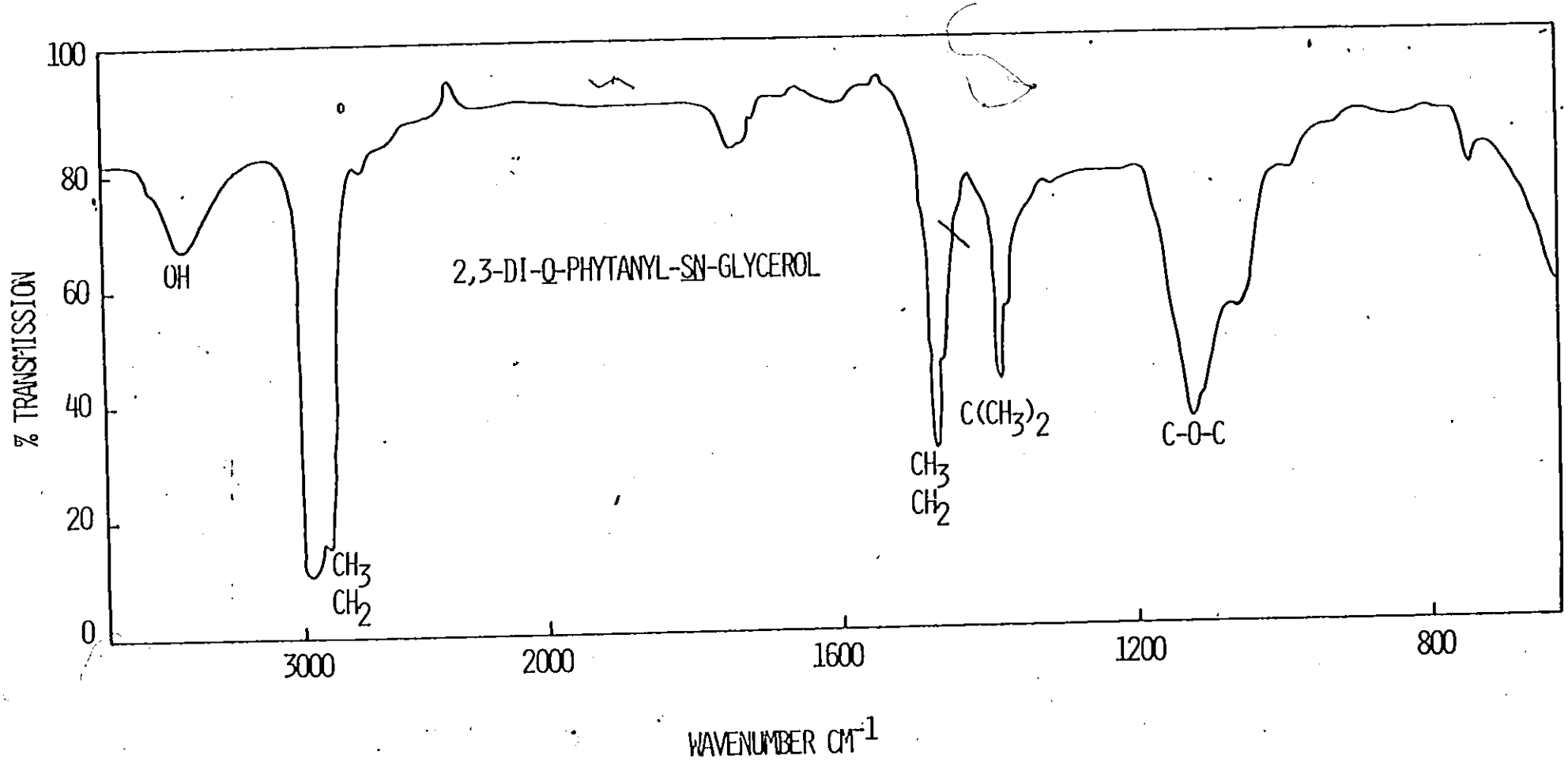

100
80
60
40
20
0
% TRANSMISSION
OH
CH3
CH2
2,3-DI-O-PHYTANYL-SN-GLYCEROL
CH3
CH2
C(CH3)2
C-O-C
3000
2000
1600
1200
800
WAVENUMBER CM-1

Figure 20

Infrared spectrum of the ammonium salt of the sulfated tetraglycosyl diether (GL-1) in a KBr pellet.

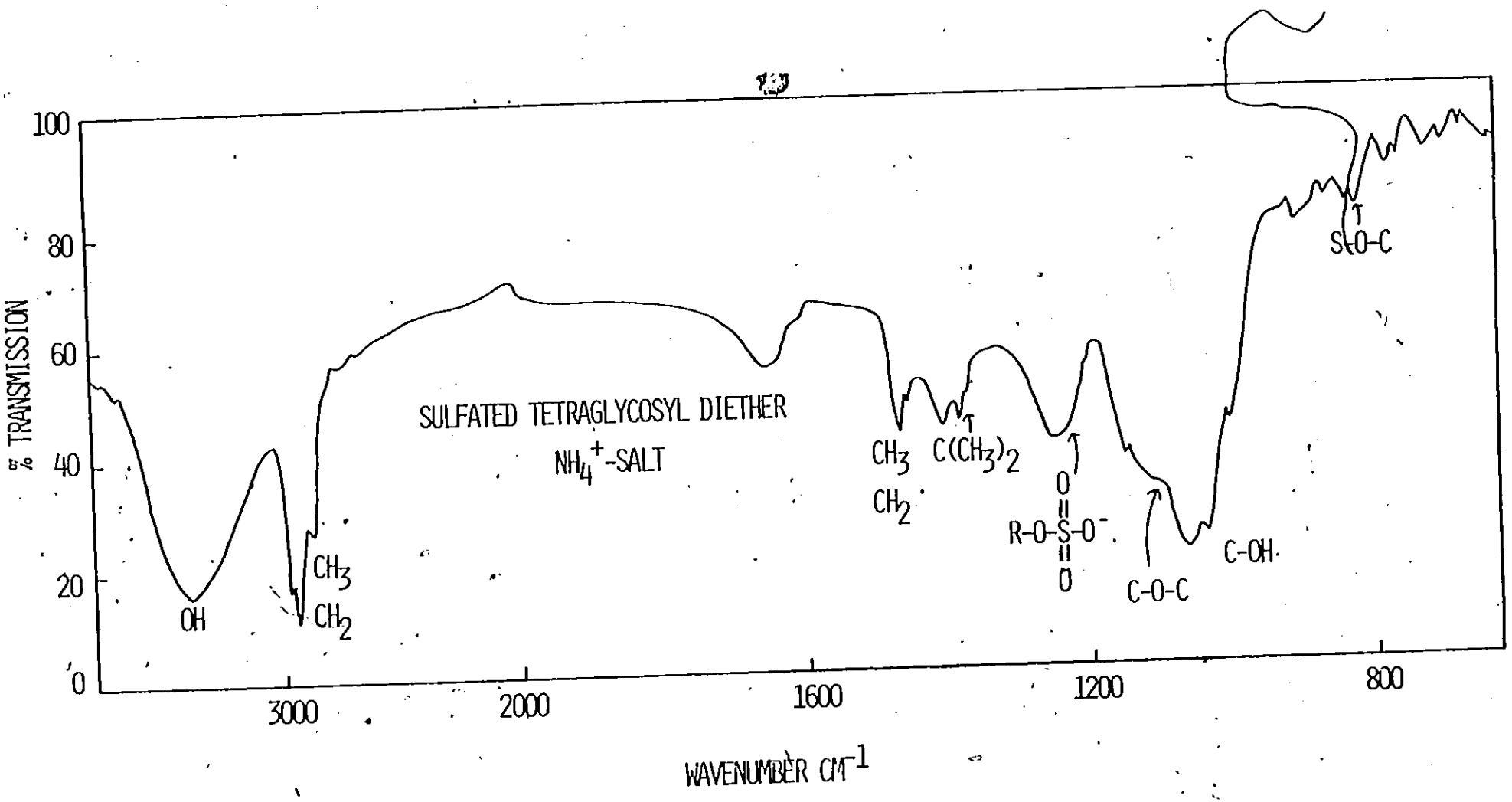

100
80
60
40
20
0
% TRANSMISSION
SULFATED TETRAGLYCOSYL DIETHER
NH_4^+-SALT
OH
CH_3
CH_2
CH_3
CH_2
$C(CH_3)_2$
R-O-S-O⁻
O
O
C-O-C
C-OH
S-O-C
3000
2000
1600
1200
800
WAVENUMBER CM^{-1}

Figure 21

100 MHz ^{1}H-NMR spectrum of the sulfated tetraglycosyl diether (GL-1) in deuterochloroform-deuteromethanol (2:1; v/v) using an internal standard of tetramethylsilane.

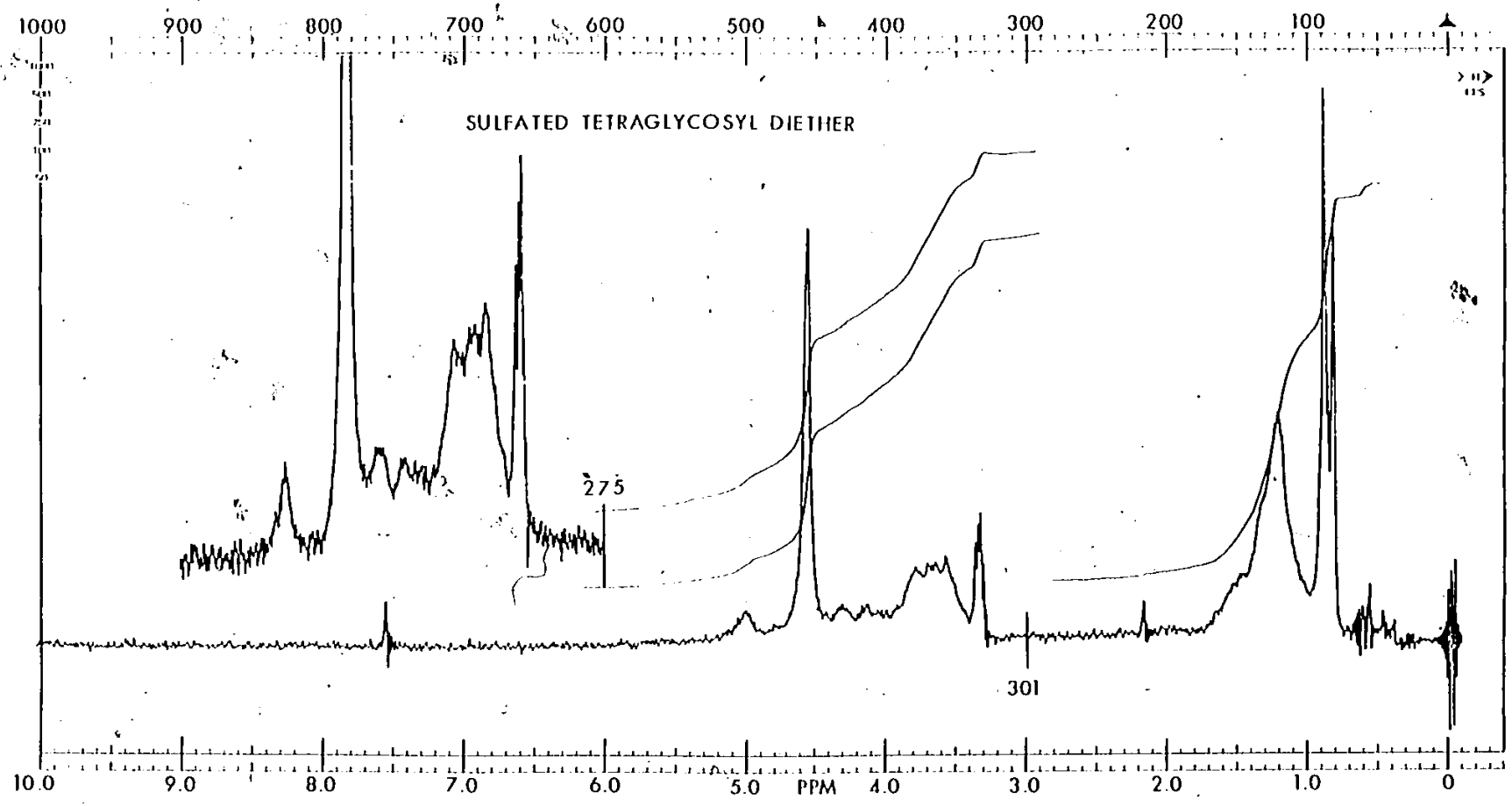
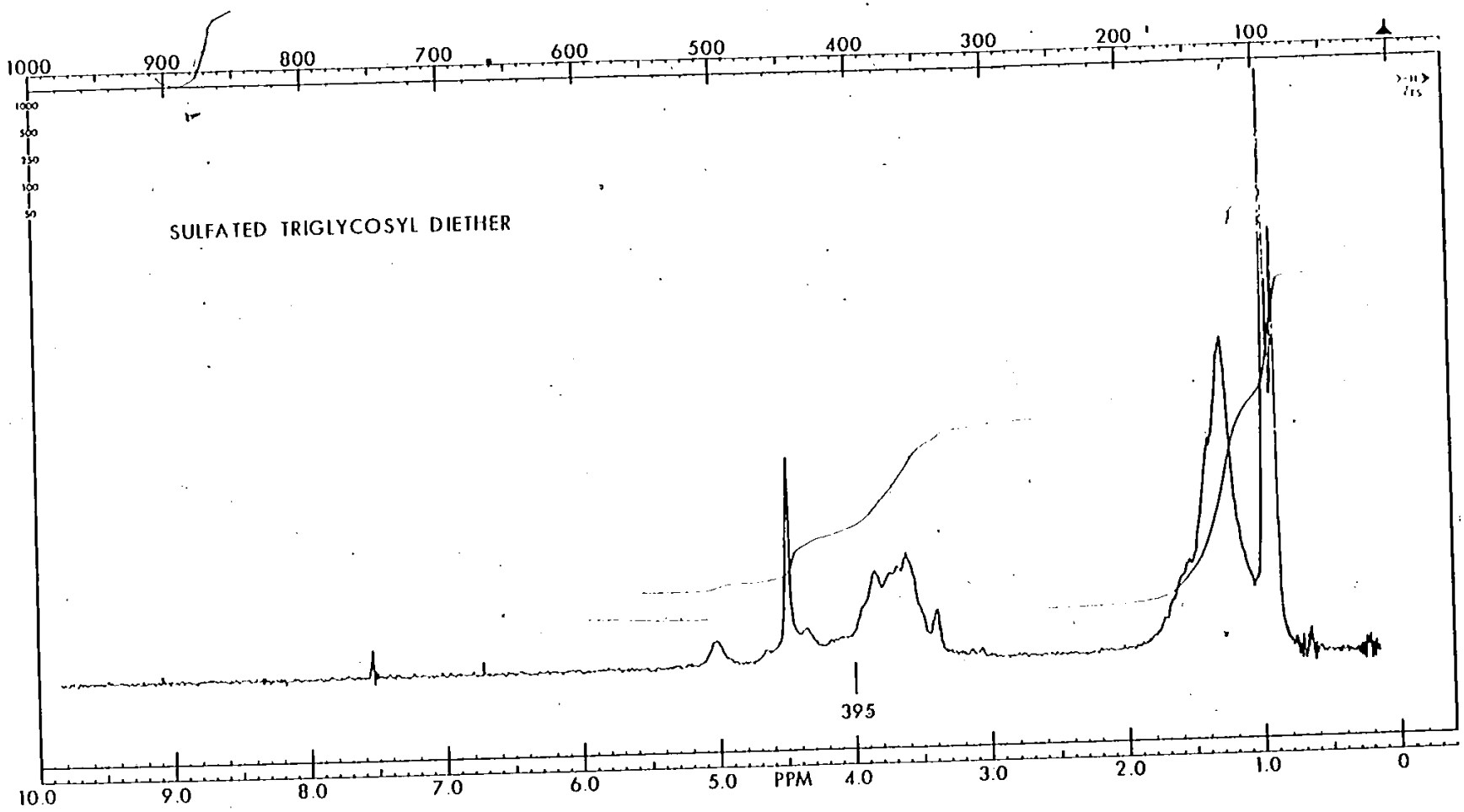

SULFATED TETRAGLYCOSYL DIETHER
1000
900
800
700
600
500
400
300
200
100
275
301
10.0
9.0
8.0
7.0
6.0
5.0
PPM
4.0
3.0
2.0
1.0
0

Figure 22

100 MHz ^{1}H-NMR spectrum of the sulfated triglycosyl diether (GLS) in deuterochloroform-deuteromethanol (2:1, v/v) using an internal standard of tetramethylsilane.


SULFATED TRIGLYCOSYL DIETHER
1000
900
800
700
600
500
400
300
200
100
395
10.0
9.0
8.0
7.0
6.0
5.0
PPM
4.0
3.0
2.0
1.0
0

the anomeric proton of a β-galactopyranose residue; and a broad peak centred at δ4.9 (3 protons) which was later assigned to the anomeric protons of the α-glucopyranose, α-mannopyranose and α-galactofuranose residues (Evans et al., 1980; Capon and Thacker, 1964). The carbohydrate resonances showed broadening and low resolution; these effects are indicative of nuclei having short relaxation times and were most likely due to the formation of inverse micelles in the $CDCL_3$-CD_3OD solvent, which would result in a decrease in relaxation times of carbohydrate protons (Johns et al., 1977).

(c) Sugar Sequence and Linkages

Partial acid methanolysis (Kates and Deroo, 1973) of GL-1 in chloroform - 0.25N methanolic HCl (3:4; v/v) at room temperature for four days yielded a mixture consisting of four new glycolipids (mono-, di-, tri- and tetraglycosyl diethers) and glycerol diphytanyl ether. The desulfated GL-1 (tetraglycosyl diether) was obtained in small quantities by solvolysis of GL-1 (6 mg) in 0.05N methanolic-HCl at room temperature for 5 h.

The carbohydrate compositions of the glycolipids derived from GL-1 are shown in Table 5. Both GL-1 and the tetraglycosyl diether contained galactose, mannose and glucose in mole ratio 2:1:1. The triglycosyl diether contained equimolar amounts of galactose, mannose and glucose; the diglycosyl diether contained mannose and glucose and the monoglycosyl diether contained only glucose. The following carbohydrate sequences for both GL-2 and desulfated GL-1 are consistent with the composition of the fragments obtained by partial acid methanolysis:

1 Gal→Gal→Man→Glu→diether

2 Gal→Man(←Gal)Glu→diether

At this stage, it was not possible to decide between the linear sequence (1) and the branched sequence (2). The final sequence and linkage positions between the sugars were subsequently established by permethylation analysis.

GC-MS* analysis of the alditol acetates of the partially methylated sugars derived from permethylated GL-1 showed the presence of 2,3,5,6-tetramethyl galactose, 3,4,6-trimethyl glucose, 2,4,6-trimethyl galactose and 2,4-dimethyl mannose (Table 6; Figure 23). The presence of 2,4-dimethyl mannose showed that the sugar sequence was branched, the mannose being substituted at positions 3 and 6, and therefore the sequence (1) could be eliminated. The presence of 2,3,5,6-tetramethyl galactose indicated that one of the two galactose residues was a terminal furanoside. After permethylation of the desulfated GL-1, the 2,4,6-trimethyl galactose was replaced by 2,3,4,6-tetramethyl galactose, indicating that the sulfate group was on C-3 of a terminal galactopyranose (Table 6; Figure 24). This also confirmed that GL-1 was monosulfated.

The results so far suggested that one of the following two sequences of sugars (3 or 4) was present in desulfated GL-1:

3 Galp1→6Manp(3←1Galf)1→2Glcp→diether

4 Galp1→3Manp(6←1Galf)1→2Glcp→diether

Furanosides are about 100 times more acid labile than pyranosides (Lindberg, 1972), therefore to decide which sequence is cor-

---

*Mass spectrograms and their interpretations are shown in Appendix I.

Table 6. Gas-Liquid Chromatography of Partially Methylated Alditol Acetates from Permethylated Glycolipids

Permethylated Glycolipid	Peak	Rel. Ret. Time	Peak Area Ratio
GL-1	A	0.62	0.5
	B	1.00	1.0
	C	1.08	0.8
	D**	2.29	0.8
Desulfated GL-1	A	0.62	0.6
	E	0.66	1.0
	B	1.00	1.0
	D**	2.29	0.8
Triglycosyl diether from GL-1	E	0.66	0.8
	B	1.00	1.0
	F	1.18	1.0
GL-2	A	0.62	0.5
	E	0.66	0.8
	B	1.00	1.0
	D**	2.29	0.8
GL-3	E	0.66	0.7
	B	1.00	1.0
	F	1.18	1.1
Standards*			
2,3,5,6-Tetramethylgalactose	A	0.62	-
2,3,4,6-Tetramethylgalactose	E	0.67	-
3,4,6-Trimethylglucose	B	1.00	-
2,4,6-Trimethylgalactose	C	1.08	-
2,3,4-Trimethylmannose	F	1.17	-

* 2,3,5,6-tetramethylgalactose was obtained by permethylation of phosphoglycolipid (PGL-IV) of M. hungatei (Kushwaha et al., 1981). (Galfβ1→6Galpα→Tetraether→glycerol phosphate).

All other standards were obtained by permethylation of glycolipid sulfate and its desulfated derivative from H. cutirubrum.

**Identified by GC-MS as 2,4-dimethylmannose.

Figure 23

Gas chromatogram (selected ion record; m/e = 43) of partially methylated alditol acetates derived from permthylated GL-1.

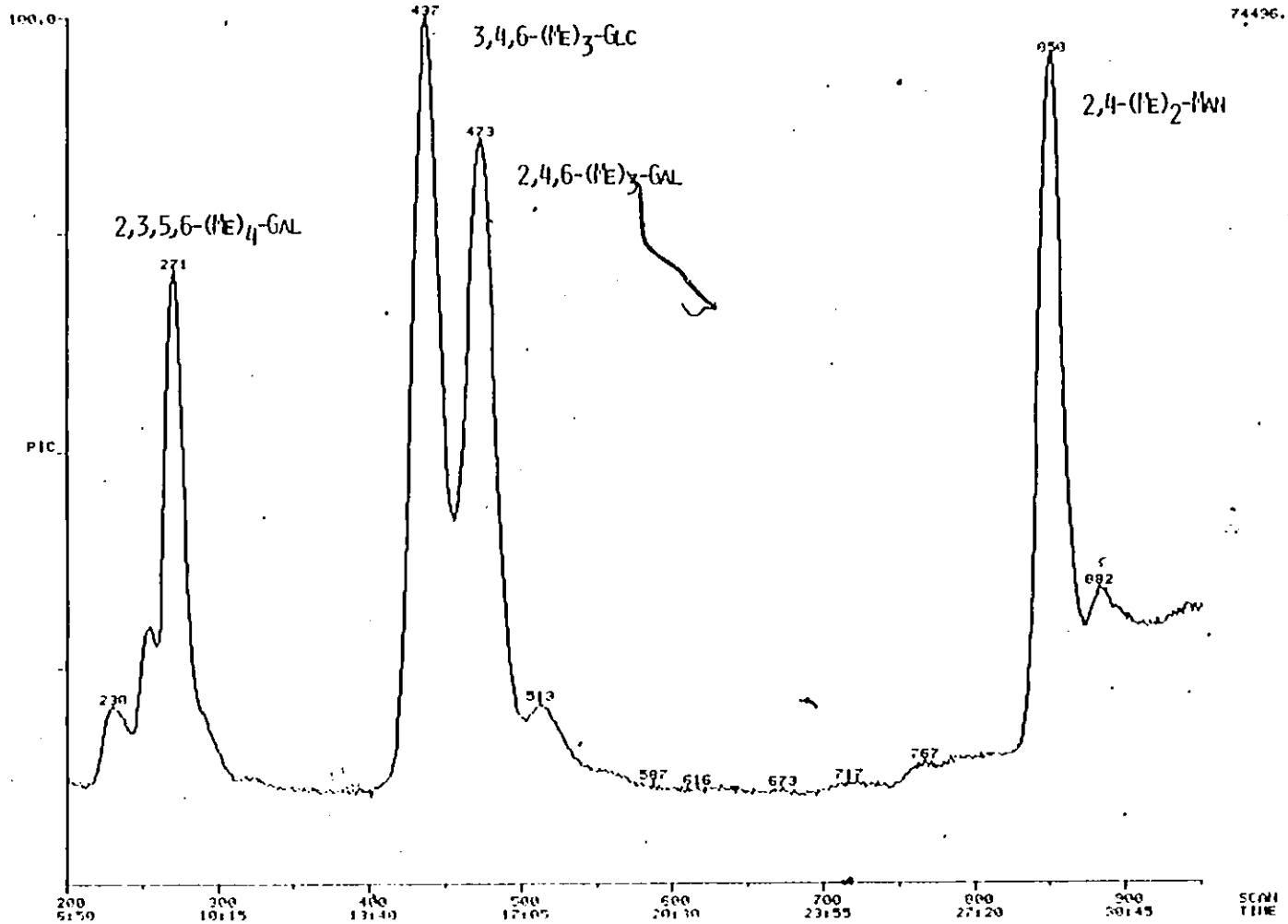
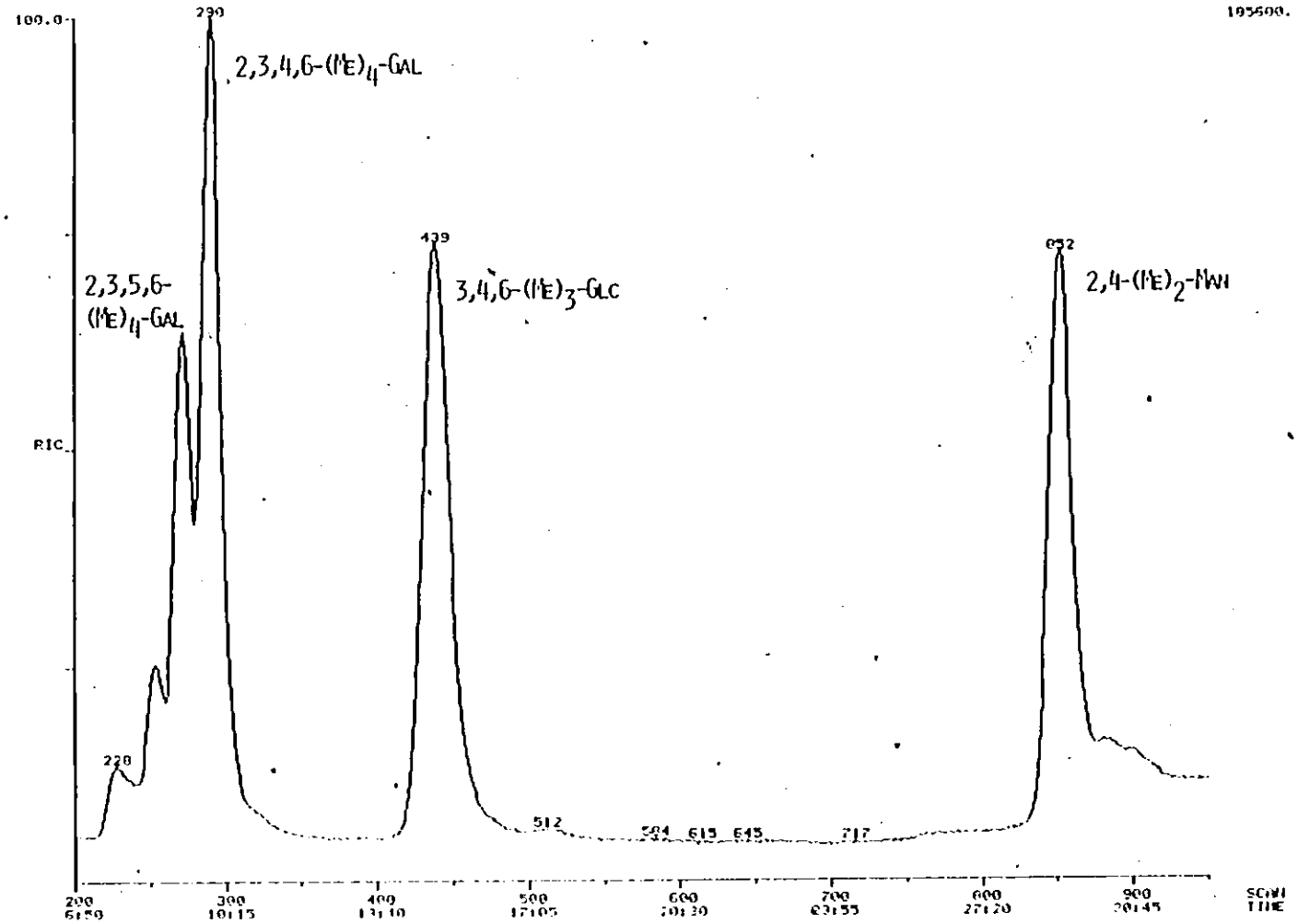

100.0
PIC
74436.
2,3,5,6-(ME)4-GAL
271
230
3,4,6-(ME)3-GLC
437
473
513
2,4,6-(ME)3-GAL
587
616
673
717
767
2,4-(ME)2-MAN
850
882
200
5150
300
10115
400
13140
500
17105
600
20130
700
23155
800
27120
900
30145
SCAN
TIME

Figure 24

Gas chromatogram (selected ion record; m/e = 43) of partially methylated alditol acetates derived from desulfated and permethylated GL-1.


100.0
RIC
105600.
290
2,3,4,6-(ME)4-GAL
2,3,5,6-
(ME)4-GAL
439
3,4,6-(ME)3-GLC
652
2,4-(ME)2-MAN
220
512
584
615
645
717
200
6150
300
10115
400
13110
500
17105
600
20130
700
23155
800
27120
900
30145
SCAN
TIME

rect, the galactofuranose residue was cleaved by partial acid methanolysis; the isolated triglycosyl diether formed was permethylated. Analysis of the alditol acetates of the partially methylated sugars showed the presence of 2,3,4-trimethyl mannose, indicating that the galactofuranose residue had been cleaved (Table 6); the other partially methylated sugars were 2,3,4-trimethyl galactose and 3,4,6-trimethyl glucose, identical to those formed from desulfated GLS, which contains the sugar linkages Galp1→6Manp1→2Glcp. Therefore, the sequence and linkage positions of sugars in GL-1 must be as shown in structure 3.

(d) Anomeric Configuration

Oxidation of acetylated glycolipids with CrO_3 in acetic acid attacks pyranosidic glucose, galactose, mannose, N-acetylglucosamine and N-acetylgalactosamine residues having β-glycosidic linkages, whereas α-glycosidically linked units react much slower (Laine and Renkonen, 1975). The method is not applicable to furanosidically linked sugars because both α and β anomers are oxidized under these conditions (Angyal and James, 1970).

Chromium trioxide oxidation of acetylated GLS resulted in the loss of the galactose residue, showing that the mannose and glucose were both α-linked and that the galactose was β-linked (Table 7). This experimental result was consistent with the reported anomeric configuration of GLS (Deroo, 1974; Kates and Deroo, 1973) and therefore served as a positive control. When desulfated and acetylated GL-1 was oxidized with CrO_3, both galactose residues were lost (Table 7) and this showed that as in GLS, the mannose and glucose residues were α-linked and the galactopyranose residue was β-linked. However, the configuration of the second

Table 7. Determination of Anomeric Configurations by CrO_3 Oxidation

Acetylated Glycolipids	Sugar Mole Ratio*		
	Glc	Man	Gal
GLS			
Before Oxidation	1.0	0.9	1.1
After Oxidation	1.0	0.9	0
Desulfated GL-1			
Before Oxidation	1.0	0.8	2.0
After Oxidation	1.0	0.8	0.01
GL-2			
Before Oxidation	1.0	0.7	1.9
After Oxidation	1.0	0.8	0.1
GL-3			
Before Oxidation	1.0	1.1	1.2
After Oxidation	1.0	0.9	0.03

*Carbohydrates were determined as alditol acetates.

galactose, which was furanosidically linked, could not be determined by this method since both anomers of furanosides react with CrO_3. The galactofuranose residue must have the α-configuration since the molecular rotation of GL-1 (Table 4) (Appendix II) was found to be very close to that calculated for a tetraglycosyl structure containing α-glucopyranose, α-mannopyranose, β-galactopyranose and α-galactofuranose residues. For a tetraglycosyl structure containing a β-galactofuranose residue the molecular rotation calculated was less than half the observed value (Table 4). The complete structure of GL-1 is therefore:

2,3-di-O-phytanyl-1-O-[Galp(3'-SO_4^-)β1'→6'Manp(3'←1'αGalf)α1'→2'Glcpα]-sn-glycerol (see Figure 25)

2. Tetraglycosyl Diether (GL-2)

GL-2 had the same carbohydrate composition as GL-1 and desulfated GL-1. It had the same R_f values as desulfated GL-1 (Figure 18 and Table 3) and gave essentially the same partial acid methanolysis products, partially methylated sugars derived from permethylation and CrO_3 oxidation results (Tables 5, 6 and 7) as was observed for desulfated GL-1.

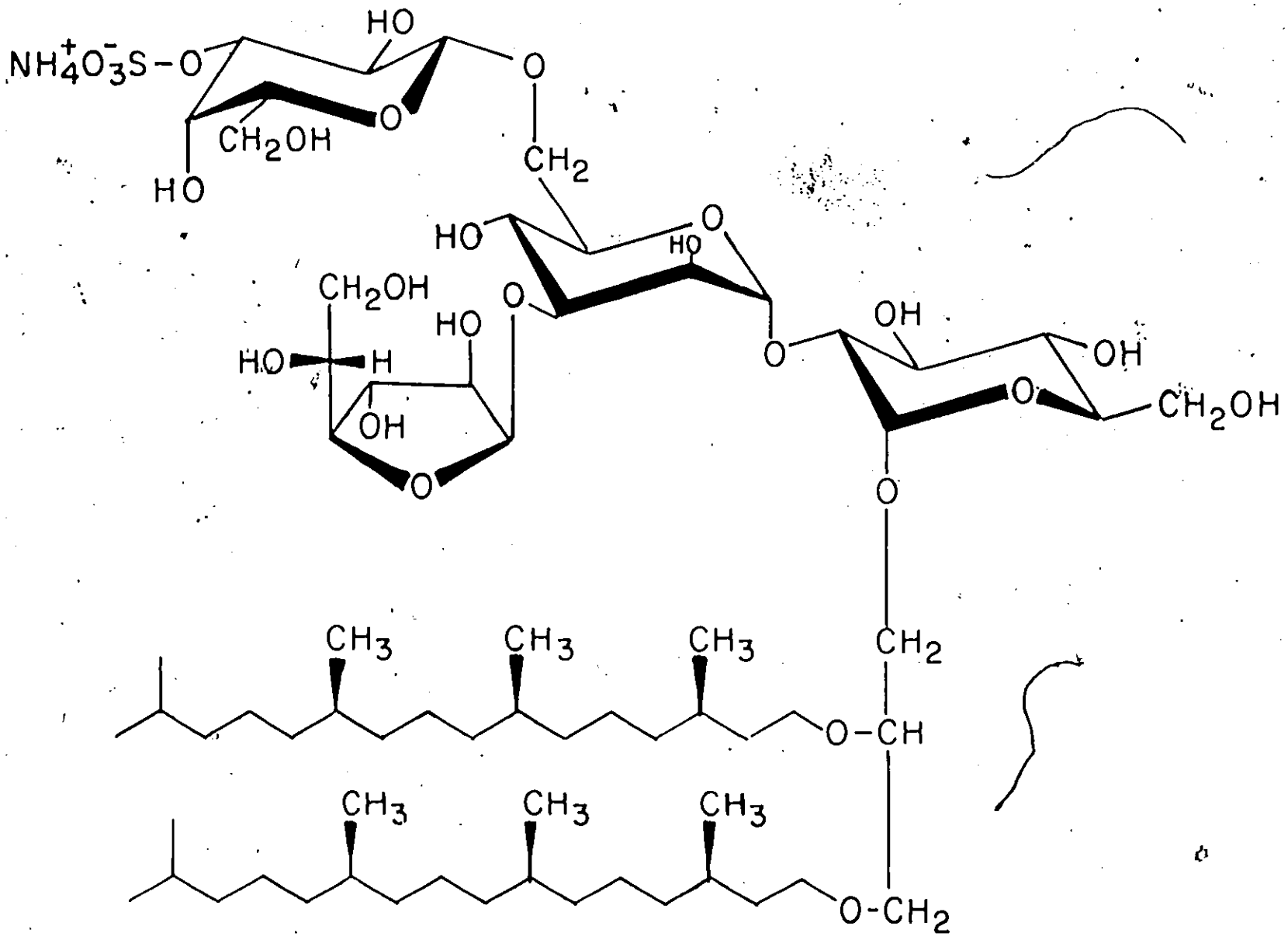
Partial acid methanolysis of GL-2 yielded a mixture consisting of the starting material, three glycolipids (mono-, di- and triglycosyl diethers) and glycerol diphytanyl ether. The triglycosyl diether contained equimolar amounts of galactose, mannose and glucose; the diglycosyl diether contained mannose and glucose and the monoglycosyl diether contained only glucose (Table 5). The four methylated sugars derived from permethylated GL-2 were identified as 2,3,5,6-tetramethyl galactose, 2,3,4,6-tetramethyl galactose, 3,4,6-trimethyl glucose and 2,4-dimethyl-

Figure 25

Structure of the sulfated tetraglycosyl diether (GL-1) of Halobacterium cutirubrum.

2,3-di-O-phytanyl-1-O[Galp-(3'-SO_4^-)β1'→6'Manp(3'←1'Galf)α1'→2'Glcpα]-sn-glycerol

GL-1


NH4O3S-O
HO
CH2OH
HO
O
CH2
HO
HO
O
CH2OH
HO
HO
H
OH
O
O
OH
OH
O
CH2OH
O
CH3
CH3
CH3
CH2
O-CH
CH3
CH3
CH3
O-CH2

mannose (Table 6). GL-2 is thus the naturally occurring desulfated GL-1, with the following structure:

2,3-di-O-phytanyl-1-O-[Galpβ1'→6'Manp(3'←1'αGalf)α1'→2'Glcpα]sn-glycerol

(see Figure 26)

3. Triglycosyl Diether (GL-3)

(a) Analytical Data

The lipid portion of GL-3 was shown to be 2,3-di-O-phytanyl-sn-glycerol. Elemental analysis and the hexose/diether mole ratio of 3.2:1 (Table 4) indicated that GL-3 was a triglycosyl diphytanyl glycerol ether. Gas chromatographic analysis of the alditol acetates obtained after acetolysis of GL-3 showed that galactose, mannose and glucose residues were present in equimolar amounts (Table 5).

(b) Spectroscopic Data

The infrared spectrum of GL-3 (Figure 27) was identical to that of desulfated GLS (Deroo, 1974) and showed strong OH absorption centred at 3400 cm^{-1}, CH_2 and CH_3 absorption at 2950 and 1470 cm^{-1}, a doublet characteristic of isopropyl absorption at 1380 cm^{-1}, C-O-C ether absorption at 1130 cm^{-1} and a strong band at 1070 cm^{-1} which showed the presence of secondary hydroxyl groups. As in GL-1, the hydrocarbon region of the ^{1}H-NMR spectrum (Figure 28) was identical to that of 2,3-di-O-phytanyl-sn-glycerol (Kushwaha et al., 1975b).

(c) Sugar Sequence and Linkages

GL-3 contained equimolar amounts of galactose, mannose and glucose. Partial acid methanolysis of GL-3 yielded a diglycosyl diether with equimolar amounts of mannose and glucose; and a monoglycosyl diether having only glucose (Table 5). Permethylation analysis of GL-3

Figure 26

Structure of the tetraglycosyl diether (GL-2) of Halobacterium cutirubrum.

2,3-di-O-phytanyl-1-O-[Galpβ1'→6'Manp(3'←1'Galf)α1'→2'Glcpα]-sn-glycerol

GL-2

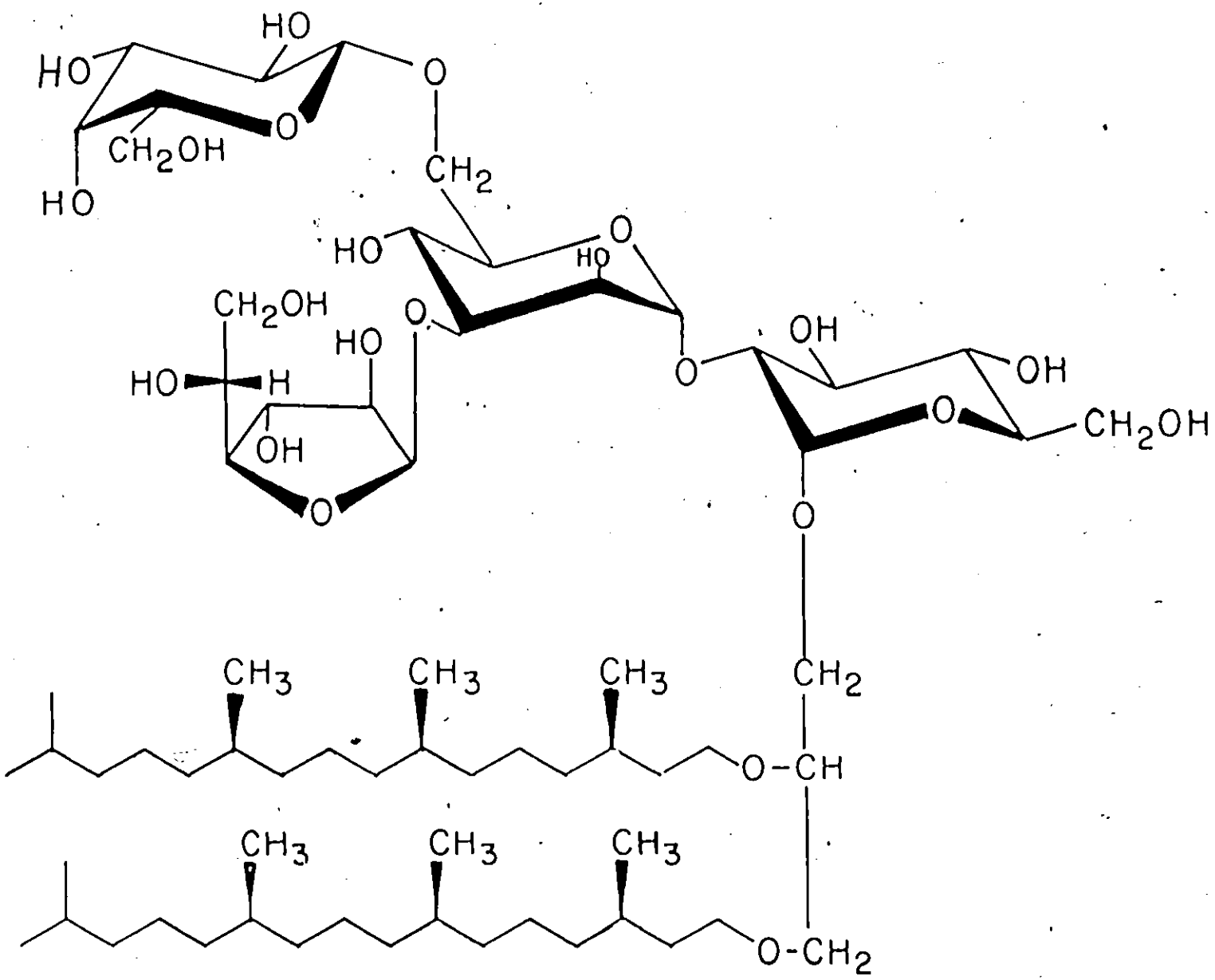

HO
HO
O
CH2OH
HO
CH2
HO
O
HO
CH2OH
O
HO
HO
H
OH
O
OH
O
OH
CH2OH
O
CH3
CH3
CH3
CH2
O-CH
CH3
CH3
CH3
O-CH2

Figure 27

Infrared spectrum of the triglycosyl diether (GL-3) in a KBr pellet.

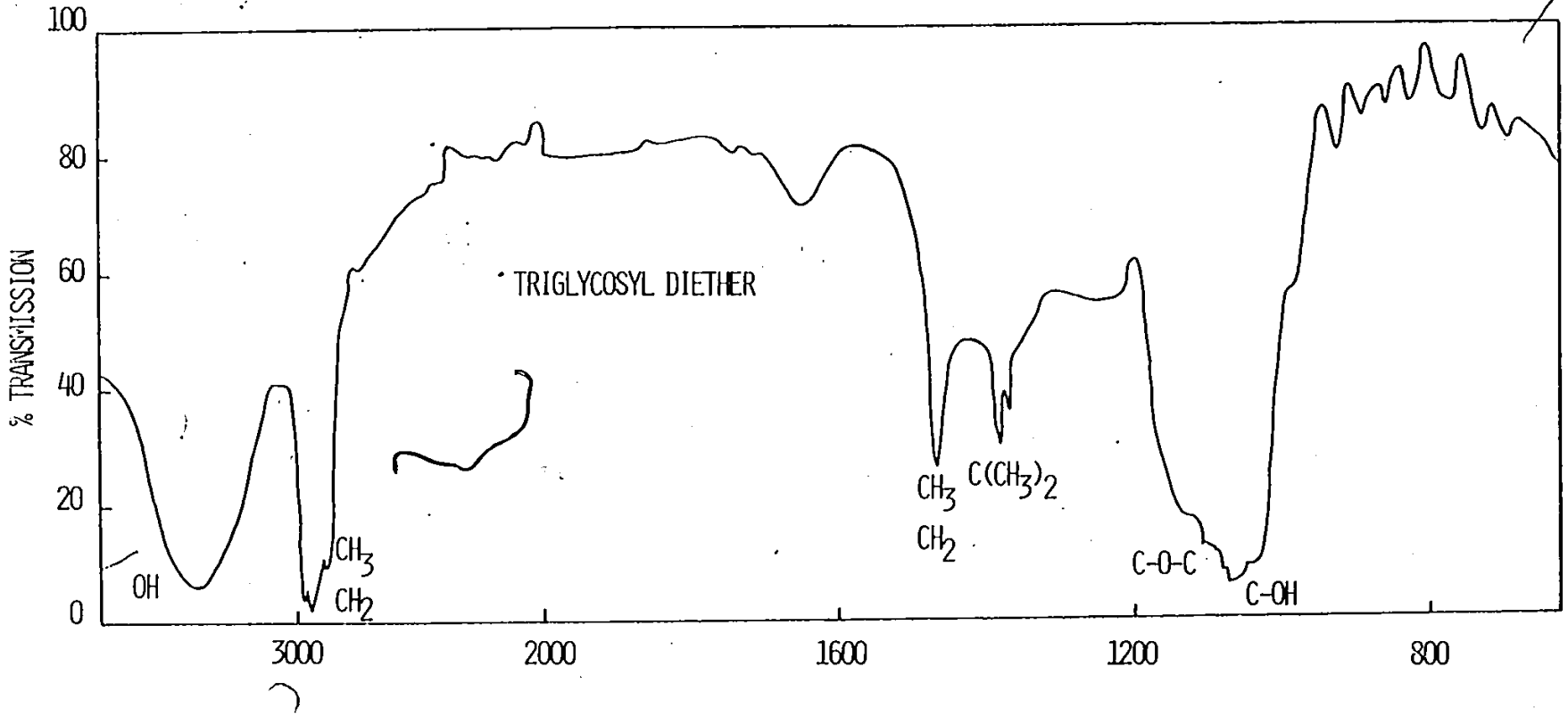
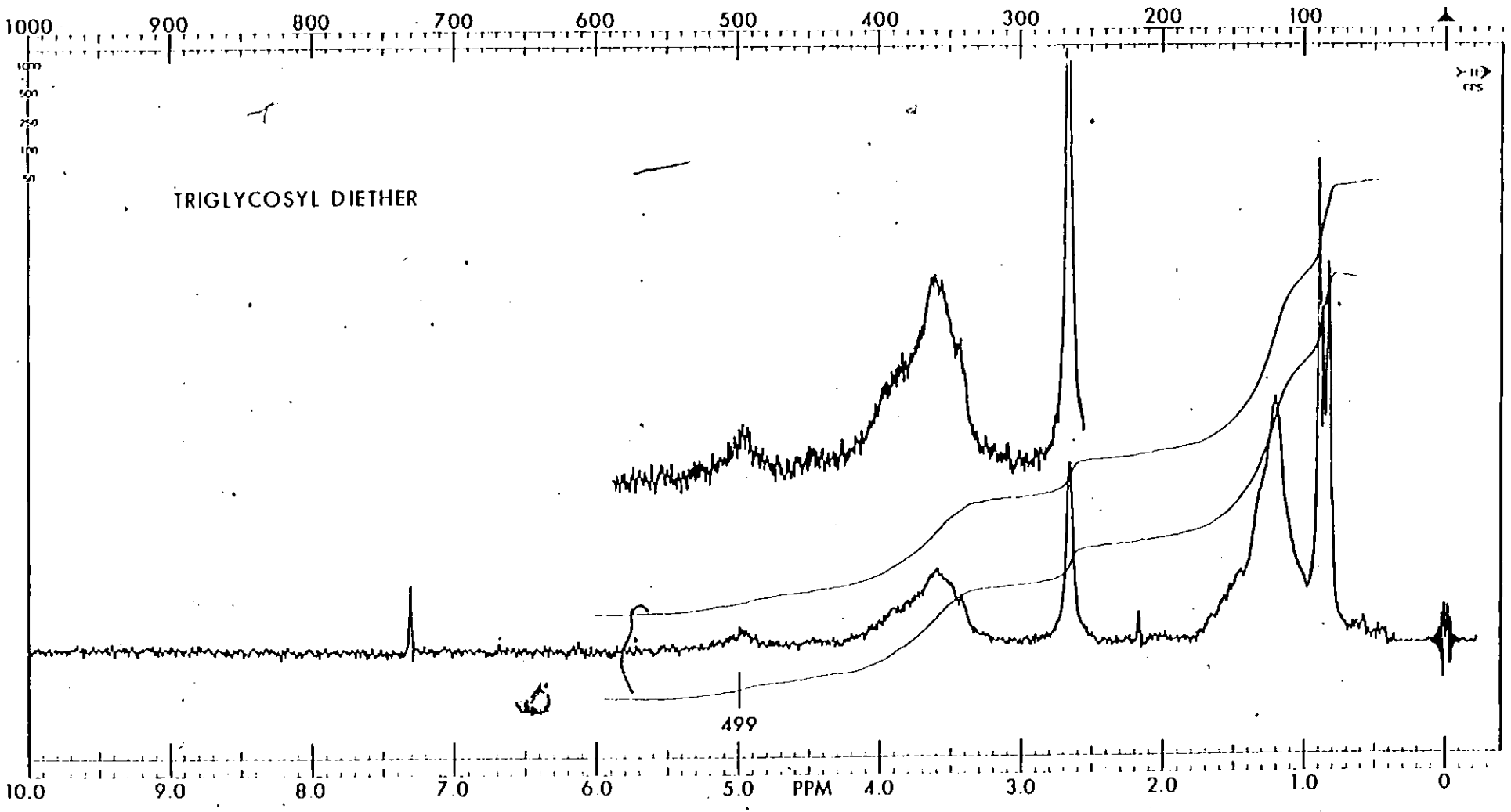

100
80
60
40
20
0
% TRANSMISSION
TRIGLYCOSYL DIETHER
OH
CH3
CH2
CH3
CH2
C(CH3)2
C-O-C
C-OH
3000
2000
1600
1200
800

Figure 28

100 MHz ^{1}H-NMR spectrum of the triglycosyl diether (GL-3) in deuterochloroform-deuteromethanol (2:1; v/v) using an internal standard of tetramethylsilane.


TRIGLYCOSYL DIETHER
1000
900
800
700
600
500
400
300
200
100
499
10.0
9.0
8.0
7.0
6.0
5.0
PPM
4.0
3.0
2.0
1.0
0

showed the presence of 2,3,4,6-tetramethylgalactose, 2,3,4-trimethylmannose and 3,4,6-trimethylglucose (Table 6). GL-3 therefore has the following partial structure:

Galp1→6Manp1→2Glcp→diether

(d) Anomeric Configuration

Oxidation of acetylated GL-3 with CrO_3 resulted in the loss only of galactose (Table 7) which showed that as in GLS, the mannose and glucose were both α-linked and the galactose was β-linked. This result was consistent with the observation that the optical rotation of GL-3 ($[\alpha]_D$ + 46.8) was the same as that of desulfated GLS ($[\alpha]_D$ + 47.0) (Kates and Deroo, 1973). GL-3 is thus identical with the triglycosyl diether derived from GLS (Kates and Deroo, 1973) and has the following structure: 2,3-di-O-phytanyl-1-O-[Galpβ1'→6'Manpα1'→2'Glcpα]-sn-glycerol

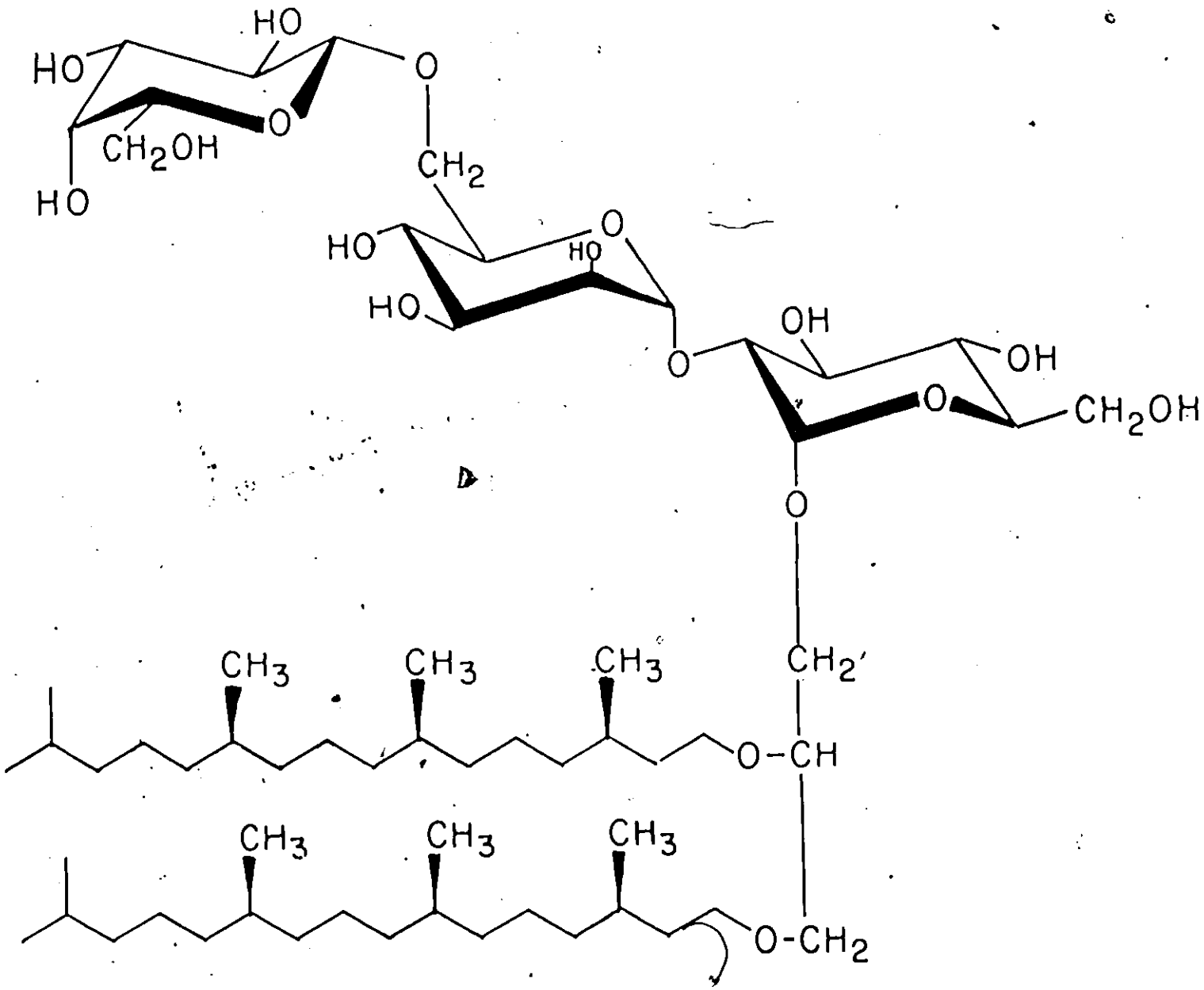
(See Figure 29)

Figure 29

Structure of the triglycosyl diether (GL-3) of Halobacterium cutirubrum.

2,3-di-O-phytanyl-1-O-[Galpβ1'→6'Manpα1'→2'Glcpα]-sn-glycerol

GL-3


HO
HO
O
CH2OH
HO
CH2
HO
O
HO
HO
O
OH
OH
O
CH2OH
O
CH3
CH3
CH3
CH2
O-CH
CH3
CH3
CH3
O-CH2

DISCUSSION

I. Structures of GL-1, GL-2 and GL-3

1. Sulfated Tetraglycosyl Diether (GL-1) and Tetraglycosyl Diether (GL-2)

The evidence presented above established the complete structure of the sulfated tetraglycosyl diether (GL-1) in Halobacterium cutirubrum as shown in Figure 25. GL-2 is the desulfated product of GL-1, and has the structure shown in Figure 26. The furanosidic nature of one of the galactose residues in both GL-1 and GL-2 was revealed by the presence of 2,3,5,6-tetramethylgalactose in the acetolysis products of the permethylated glycolipids. The occurrence of a galactofuranose residue is uncommon in glycolipids but not exceptional since it has been found in glycolipids of several bacteria, namely Mycoplasma mycoides (Plackett, 1967), Bifidobacterium bifidum (Veerkamp, 1972), Thermus thermophilus (Oshima and Ariga, 1976) and Methanospirillum hungatei (Kushwaha et al., 1981).

The branched sugar sequence was shown by the presence of 2,4-dimethyl mannose, the mannose being substituted at positions 3 and 6. An ambiguity concerning the linkage positions of the galactose residues was resolved by mild acid cleavage of the galactofuranose residue followed by permethylation of the resulting triglycosyl diether. The presence of 2,3,4-trimethyl mannose in the permethylated triglycosyl diether showed that the galactofuranose residue is attached to C-3 of the mannose residue. The non-linear tetraglycosyl sequence in GL-1 and GL-2 is unusual, as such a branched structure has not been previously reported for bacterial glyco-

lipids. Linear tetraglycosyl diglycerides occur in some bacteria, for example, in the extreme thermophile, Thermus thermophilus (Oshima and Ariga, 1976), in Lactobacillus acidophilum (Shaw, 1975) and in Lactobacillus casei (Nakano and Fischer, 1977) (see General Introduction, IV, 1). Branched sugar sequences are common in gangliosides and some fucolipids, which are glycolipids occurring in mammalian cells. The presence of a branched sugar sequence in glycolipids of an extreme halophile would suggest that this may be another characteristic held in common with eukaryotes. These similarities to eukaryotes include the glycoprotein cell wall structure, the initiation of protein biosynthesis by nonformylated methionine, the presence of a plant-like ferredoxin and tRNA sequence homology (Bayley and Morton, 1978). Also, the structures of the sulfated glycolipids of H. cutirubrum are similar to the galactose-sulfate containing glycolipids found in animal tissues (e.g. seminolipid, brain sulfatide) in that they all contain a β-linked galactose residue which is sulfated at position 3 (see General Introduction, IV, 1).

The question arises as to the biosynthesis of GL-1. Because of the structural relationship between GLS and GL-1, it is probable that GL-1 is derived biosynthetically from GLS by transfer of a galactofuranosyl group to the mannose residue. This hypothesis is supported by studies of the incorporation of [^{35}S] sulfate into the cellular lipids (Deroo, 1974), which showed that GL-1 was labelled after GLS.

2. Triglycosyl Diether (GL-3)

Because GL-3 co-chromatographs with the desulfated glycolipid sulfate (Kates and Deroo, 1973), it was suspected to have the same carbohydrate structure as the latter. The results obtained

by permethylation analysis, partial acid methanolysis, CrO_3 oxidation and optical rotation measurements confirm that the complete structure of the triglycosyl diether in H. cutirubrum is as shown in Figure 29.

The naturally occurring triglycosyl diether found in H. cutirubrum was present in small and variable amounts (1-5%). This glycolipid may be an intermediate in glycolipid sulfate biosynthesis, as was shown in preliminary pulse-labelled studies (Deroo, 1974) or may be a product of glycolipid sulfate hydrolysis (see Part Two, Results and Discussion, I). Alternatively, it could be an integral component of the membrane, as was found for the triglycosyl diether in H. marismortui (Evans et al., 1980). The latter glycolipid occurred in much larger amounts (11%) and has a somewhat different structure in which the terminal sugar is glucose instead of galactose. H. marismortui does not contain any glycolipid sulfate, and it appears that the lack of a sulfated glycolipid is compensated for by higher contents of phosphatidylglycerosulfate and triglycosyl diether (Evans et al., 1980).

II. Function of Halophile Glycolipids

GL-1 is similar in structure to GLS in that both glycolipids have a sulfate group and a large polar head, and therefore GL-1 may have a function similar to that of GLS. Because GLS is a major membrane lipid component in an organism having a high requirement for cation transport, it has been suggested that this glycolipid participates in such a function (Kates, 1972; Falk et al., 1980). Karlsson (1976) has proposed that GLS plays a role in potassium ion transport, on the basis of the known preference of sulfate groups for potassium ions (see General Introduction, IV, 2, (b)) and on the structural similarity of GLS to cerebro-

side sulfate, namely the presence of a β-galactopyranosyl-3-sulfate. However, recent findings suggest that this structural analogy may be merely coincidental and not necessarily related to function. For example, H. marismortui completely lacks GLS but has a higher content of PGS and has a triglycosyl diether which contains a terminal glucose rather than galactose (Evans et al., 1980). Also, a strain of halophilic bacterium from Spanish salt ponds has been shown to contain a sulfated diglycosyl diether with a terminal α-mannopyranose-6-sulfate residue (Kushwaha et al., in press), which suggests that in extremely halophilic bacteria the sulfate group of a glycolipid does not necessarily have to be esterified to the 3-position of a galactose residue.

In contrast with the postulated role of GLS in potassium ion transport, there is evidence for its role in the maintenance of membrane integrity. Chen et al., (1974) found that GLS was essential for the formation of stable bilayers of H. cutirubrum polar lipids and concluded that this property was related to the presence of the large carbohydrate polar head group (see General Introduction, VI). This finding was confirmed by the freeze-fracture electron microscopy data on GLS and PGP plus GLS mixtures which will be discussed later in this thesis (see Part Three, Results and Discussion, II). Triglycosyl diethers might also play a membrane-stabilizing role, since in H. marismortui, GLS is entirely replaced by a triglycosyl diether (Evans et al., 1980).

The minor glycolipids of H. cutirubrum, namely GL-1, GL-2 and GL-3, have large carbohydrate polar heads and may also participate in bilayer stabilization. However, these glycolipids are present in small and variable amounts and may possibly have another function. In other

organisms, glycolipids such as gangliosides and cord factor are known to have antigenic properties and GL-1 and GL-2, having a branched sugar chain structure and an uncommon galactofuranose residue, could possibly be antigenic determinants and/or receptor sites. It is of interest that qualitative and quantitative differences occur with respect to glycolipid composition among several species of extremely halophilic bacteria (Figure 30). However, immunological studies of the pure glycolipids would be required to establish their antigenic properties.

Figure 30

Thin-layer chromatogram on precoated silica gel G of polar lipids from the following strains:

Lane 1,15	*H. cutirubrum*	Lane 8	*H. marismortui*
Lane 2	R-4	Lane 9	M-2.5
Lane 3	Gla-2.2	Lane 10	Ma-2.38
Lane 4	Y-27	Lane 11	*H. saccharovorum*
Lane 5	Gaa-3	Lane 12	Gca-19
Lane 6	Gaa-6	Lane 13	*H. salinarium*
Lane 7	*H. vallismortis*	Lane 14	*H. halobium*

Abbreviations:

DGD: diglycosyl diether

TGD-1: triglycosyl diether (galactosyl-mannosyl-glucosyl diether)

TGD-2: triglycosyl diether (glucosyl-mannosyl-glucosyl diether)

S-DGD: sulfated diglycosyl diether

S-TGD-1: sulfated triglycosyl diether

S-TeGD: sulfated tetraglycosyl diether

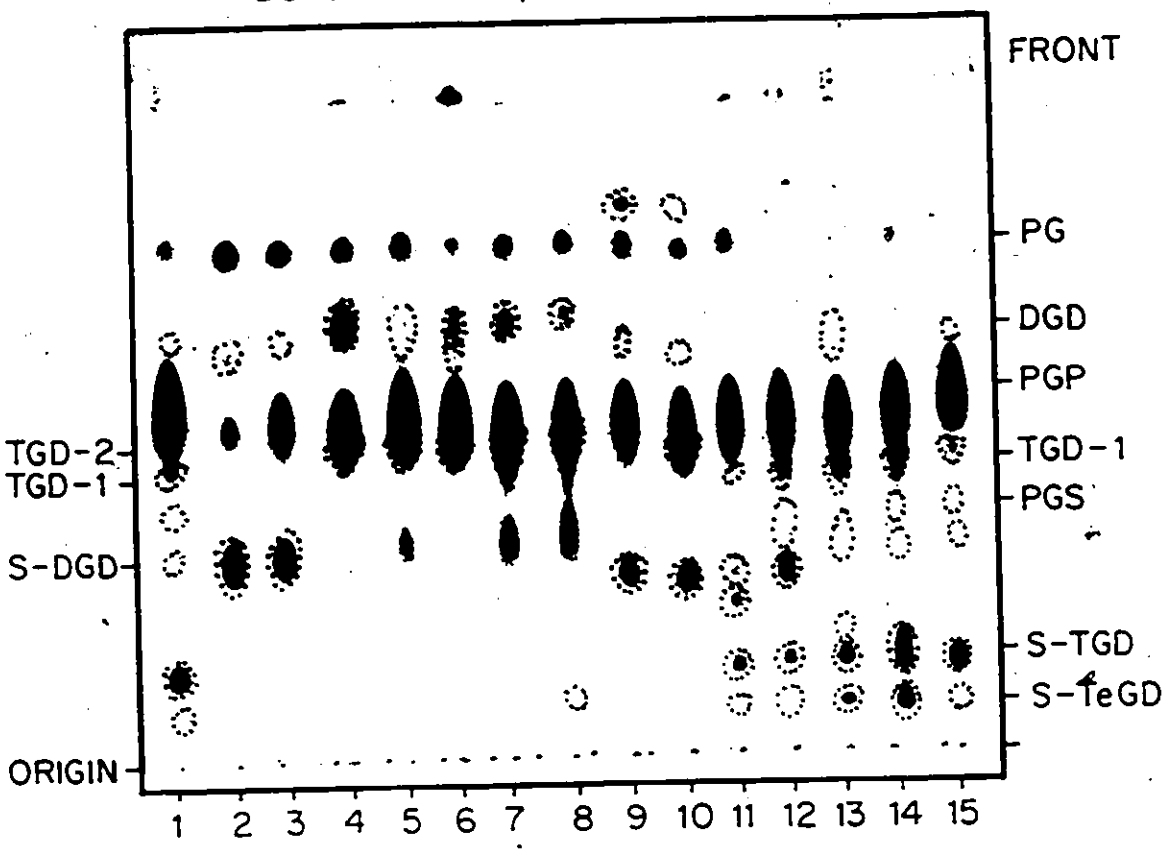
dotted spots are sugar positive

Solvent System:

chloroform-methanol-acetic acid-water (85:22.5:10:4, v/v), double development (Kushwaha *et al.*, submitted to J. Bacteriol; ref. 179)

[$CHCl_3$-MeOH-CH_3COOH-H_2O = 85 : 22.5 : 10 : 4, V/V;]

Double Development



PART TWO

LIPID COMPOSITION OF RED AND PURPLE MEMBRANES

EXPERIMENTAL PROCEDURES

I. Studies on the Desulfation of GLS During Membrane Isolation

(a) ^{14}C-Labelled Cells

The inoculum was prepared by adding 5 ml of preinoculum to 100 ml of standard medium containing 0.1 millicuries of [1-^{14}C] acetate and incubating aerobically in the dark for 70 h. This was transferred to 1.5 l of standard medium containing 1.5 millicuries of [1-^{14}C] acetate and incubated at 39°C in the dark at a shaking rate of 190 rpm for 12 h, then the rate of shaking was reduced to 100 rpm and the cultures were illuminated; the cells were harvested after 120 h.

As described in General Materials and Methods, III, the suspension of cells in basal salt solution was dialyzed overnight against distilled water at 4°C and the membranes were isolated. Aliquots taken from the undialyzed cells, the dialyzate and membrane suspensions were mixed with the appropriate volumes of chloroform, methanol and water to make 3.8 ml of a chloroform-methanol-water (1:2:0.8; v/v/v) system. After several hours, each mixture was made biphasic by the addition of 1 ml each of chloroform and water. The upper phase of each was re-extracted twice with chloroform and the pooled chloroform phases were diluted with benzene and evaporated to dryness under nitrogen. An aliquot from each lipid sample containing ca. 20 x 10^3 cpm was separated by TLC in chloroform-90% acetic acid-methanol (30:20:4; v/v/v), an autoradiogram was made,

and radioactive spots were scraped from the plates and counted as described in General Materials and Methods, IX.

(b) ^{35}S-Labelled Cells

A 20 ml suspension of exponentially growing cells were centrifuged at 10,000 xg for 10 min. and washed twice with 25% NaCl. The pellet was resuspended and transferred to 200 ml of low sulfate medium (Material and Methods, Section II, 2 (b)). This culture was incubated at 39°C at a shaking rate of 190 rpm in the dark for 76 h, then it was transferred to a 2 litre flask containing 800 ml of low sulfate medium to which 3 millicuries of $^{35}SO_4^{2-}$ had been added. The culture was incubated as above for 17 h, a 100 ml sample was taken from the high aeration culture then the rate of shaking was reduced to 100 rpm and the cultures were illuminated.

After 120 h, a 100 ml sample was taken from the low aeration culture; the remainder of the culture was harvested and used for membrane isolation (General Materials and Methods, III). The two 100 ml samples were harvested, made to 6 ml with 25% NaCl and mixed with 15 ml methanol and 7.5 ml chloroform. The mixtures were centrifuged after several hours, the precipitates were washed with chloroform-methanol-water (1:2:0.8; v/v/v) and the pooled supernatants were made biphasic by the addition of the appropriate amounts of chloroform and water. The upper phase from each was removed and the lower phase was washed twice with 2.5 ml of methanol-12.5% NaCl (10:9; v/v); the lower chloroform phase was diluted with 99% ethanol and dried by rotary evaporation. Samples taken from the dialyzate and both membranes were extracted in the same manner except on a smaller scale. As described in the previous section, each lipid sample was chromatographed and the distribution of radioactivity was measured.

II. Determination of the Polar Lipid Composition of Red and Purple Membranes

Total lipids were extracted from the red and purple membranes as follows. A membrane suspension containing ca. 30 mg protein was made to 24 ml with distilled water, diluted with 60 ml methanol, stirred at room temperature for 45 minutes, then 30 ml chloroform was added and the mixture was stirred for a further 30 minutes. The suspension was centrifuged, the pellet was re-extracted with 38 ml of chloroform-methanol-water (1:2:0.8; v/v/v) and the pooled supernatant was made biphasic by the addition of 40 ml each of chloroform and 0.2 M KCl. The chloroform phase was then treated as described in General Materials and Methods, IV,1.

The lipid composition of each membrane preparation was determined by the following procedure. A ca. 30 mg sample of the total lipid extract was separated by two-dimensional TLC; the first dimension was developed in chloroform-methanol-diethylamine-water (110:35:8:5:-v/v/v/v) and the second dimension was developed in chloroform-methanol-concentrated ammonium hydroxide (65:25:5; v/v/v) followed by chloroform-methanol-concentrated ammonium hydroxide (65:35:5;. v/v/v) (Evans et al., 1980). Lipids were visualized with Rhodamine and the spots were scraped then eluted three times with 1.5 ml of chloroform-methanol-water (1:2:0.8;-v/v/v); the extracts were each made to 5.0 ml and made biphasic by the addition of 1.3 ml each of chloroform and 0.2 M KCl. Each upper phase was washed twice with 1 ml of chloroform and the pooled chloroform phases were transferred to Lewis-Benedict sugar tubes. The nature of each spot was determined by staining a parallel set of TLC plates with glycolipid (α-napthol) and phosphorus (molybdate) stains (General Materials and

Methods, V, 1 (c)) before sugar or phosphate analyses. The compounds were identified on the basis of their chromatographic mobilities in the solvents used by Evans et al., (1980) and by their staining properties.

III. Determination of the Retinal:Protein Ratio of Bacteriorhodopsin

(a) Retinal Analysis (Futterman and Saslaw, 1961)

The sample of purple membrane (ca 0.5 mg protein) was suspended in 1.5 ml of acetone-water (9:1; v/v) and allowed to stir for 30 min at room temperature. To this, 0.5 ml each of thiourea* and thiobarbituric acid** reagents were added and the sample was allowed to stand at room temperature in the dark for 30 min. The sample was centrifuged clear and the ultraviolet absorbance spectrum was obtained from 380-680 nm against a reagent blank (λmax = 530 nm).

A standard curve was prepared using aliquots of all-trans retinal in the range of 0.5-5.0 ug.

(b) Determination of Protein by Amino Acid Analysis

A sample of purple membrane containing 100-150 ug protein and 25 nmole norleucine (internal standard) was hydrolyzed for 48 h at 110°C in 0.5 ml of 6 N HCl in a sealed, evacuated tube. Lipids were removed from the hydrolyzate by extraction with 2 x 1 ml of chloroform, and the aqueous phase was dried in vacuo over KOH. The residue was dissolved in citrate buffer and the amino acid composition was determined using a Technicon TSM-1 autoanalyzer. Calculation of the amount of protein was

*Thiourea reagent: 4.0 g thiourea dissolved in 100 ml of glacial acetic acid and filtered.

**Thiobarbituric acid reagent: 600 mg 2-thiobarbituric acid in 100 ml of absolute ethanol, filtered and stored at 4°C.

based on the number of glycine, alanine, valine, isoleucine, leucine, tyrosine and phenylalanine residues present in the sequence of bacteriorhodopsin (Khorana et al., 1979) using the following equation:

$$\text{Amount of Protein (nmole)} = \left(\frac{Bnor}{Anor}\right)\left(\frac{I}{N}\sum_{i=1}^{n}\frac{Ai}{Ci \cdot Ri}\right)$$

Where: Ai = Area of amino acid peak (i=gly, ala, val, etc).

Anor = Area of norleucine peak

Bnor = nmoles of norleucine added

Ci = Colour constant

Ri = Number of residues of amino acid in bacteriorhodopsin

RESULTS AND DISCUSSION

I. Studies on the Desulfation of GLS During Membrane Isolation

During preparation of the membrane from [^{35}S] sulfate labelled cells, it was found that after dialysis of the cells a 36% decrease in the proportion of ^{35}S occurred in GLS in the dialysate (Table 8). Similarly, in the ^{14}C-labelling experiment, a decrease of 16% was observed in the proportion of ^{14}C in GLS (Table 8). The latter was associated with an increase in the proportion of ^{14}C in triglycosyl diether (GL-2). The distribution of the ^{14}C label in the other lipids (Table 8) showed that the proportions of GL-1, PGP, PGS and PG remained fairly constant.

The results obtained would be consistent with the hypothesis that upon dialysis, either enzymatic or spontaneous desulfation of GLS had occurred. In this connection, a cell free system prepared from H. cutirubrum (Kushwaha and Kates, 1976) was assayed for sulfatase activity towards [^{14}C] GLS over pH range 4-8 in the presence and absence of Triton X-100 or sodium deoxycholate as substrate emulsifier and no significant activity was detected either before or after heating in a boiling water bath (data not shown). Perhaps the attempts to demonstrate sulfatase activity in a cell free system were unsuccessful because a specific "protein activator" may be required for the cell-free system, as has been found for the in vitro hydrolysis of cerebroside sulfate, psychosine sulfate or seminolipid by cerebroside sulfatase from human liver (Fischer et al., 1978). However, the possibility of a non-enzymatic breakdown cannot be entirely eliminated at this point. Goren (1971) has reported that the ammonium salt form of the sulfolipid found in M. tuberculosis

Table 8. Distribution of Radioactivity from [^{14}C] Acetate and [^{35}S] Sulfate Among the Polar Lipids of Membrane Fractions (Percent of recovered c.p.m. in polar lipids).

Lipid Component	Total Cells		Dialyzate*		Red Membrane		Purple Membrane	
	^{14}C	^{35}S	^{14}C	^{35}S	^{14}C	^{35}S	^{14}C	^{35}S
GL-1	13.0	1.0	9.7	2.3	12.4	0.8	10.6	1.5
GLS	32.2	77.5	27.3	49.2	25.7	47.9	40.0	57.8
PGS + GL-2	9.1	-	9.8	-	9.4	-	6.3	-
PGS + GL-4	-	21.5	-	48.5	-	51.2	-	45.7
GL-3	0.7	-	5.5	-	6.2	-	3.7	-
PGP	37.0	-	38.8	-	37.9	-	32.9	-
PG	6.2	-	6.5	-	6.4	-	5.6	-
Unidentified	2.0	-	2.5	-	2.0	-	0.8	-

*A suspension of cells in basal salt solution was dialyzed overnight against distilled water at 4°C.

undergoes spontaneous desulfation under various conditions, such as in anhydrous diethyl ether. It is worth mentioning that addition of exogenous [^{14}C] GLS to the dialyzate did not result in any breakdown of the substrate (data not shown). Further experimentation is therefore required to establish the presence or absence of a sulfatase.

II. Lipid Composition of Red and Purple Membranes

Two dimensional autoradiograms of lipids extracted from red and purple membranes labelled with [^{35}S] sulfate showed the presence of PGS, GLS and an unidentified glycolipid sulfate (GL-4) (Figure 31). The latter component had R_f values greater than those of a sulfated diglycosyl diether recently identified in a strain of halophilic bacteria from Spanish salt ponds (Kushwaha et al., in press), and might be a sulfated monoglycosyl diether. The presence of a third sulfated glycosyl diether (GL-4) in the lipids of H. cutirubrum has not been previously reported. During the isolation of GL-1, GL-2, and GL-3 (Part One, Experimental Procudures, I) in 1978-1979, GL-4 was not found in any of the glycolipid fractions. A possible explanation for the appearance of GL-4 might be that the more recent cultures of H. cutirubrum (1980-81) were mutants with a different glycolipid composition. There is little evidence to prove that a mutation has occurred other than the fact that this organism is known to have a high spontaneous mutation rate (Pfeifer et al., 1981).

On the basis of recovered radioactivity, the purple membrane contained 21% more GLS compared to red membrane (Table 8). Charring of the TLC plates with 50% ethanolic sulfuric acid also revealed the pre-

Figure 31.

Autoradiograms showing the distribution of ^{35}S among the sulfolipids of purple and red membranes isolated from cells grown in the presence of [^{35}S] sulfate with illumination and low oxygen tension.

Solvent Systems:

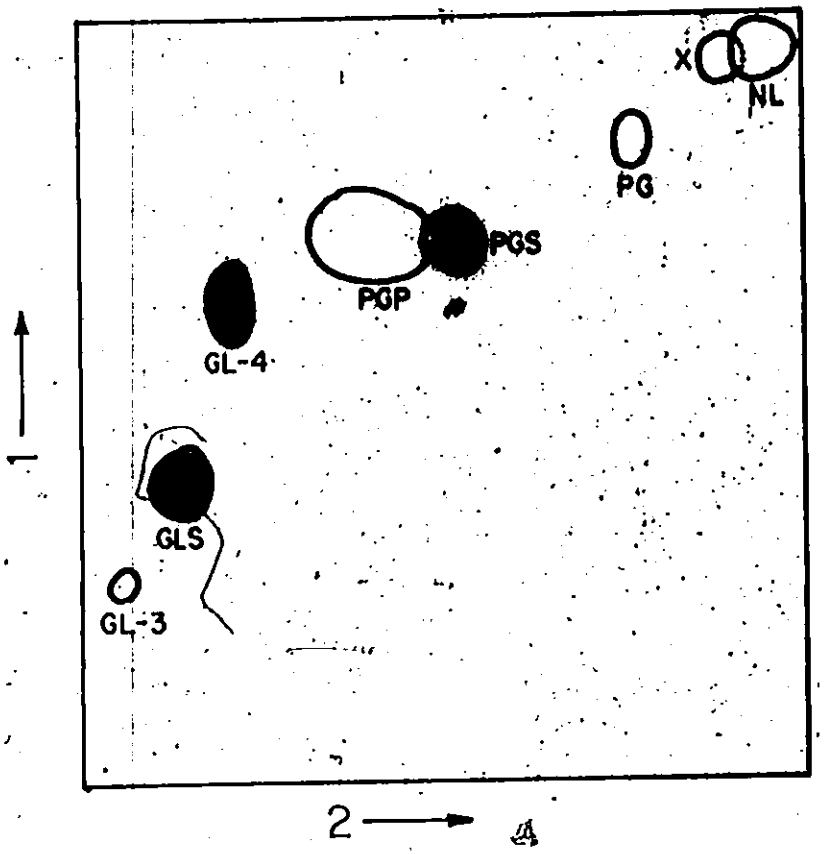
1. Chloroform-methanol-diethylamine-water
   (110:35:8:5; v/v/v/v)

2(a) Chloroform-methanol-conc. ammonium hydroxide
   (65:25:5; v/v/v)

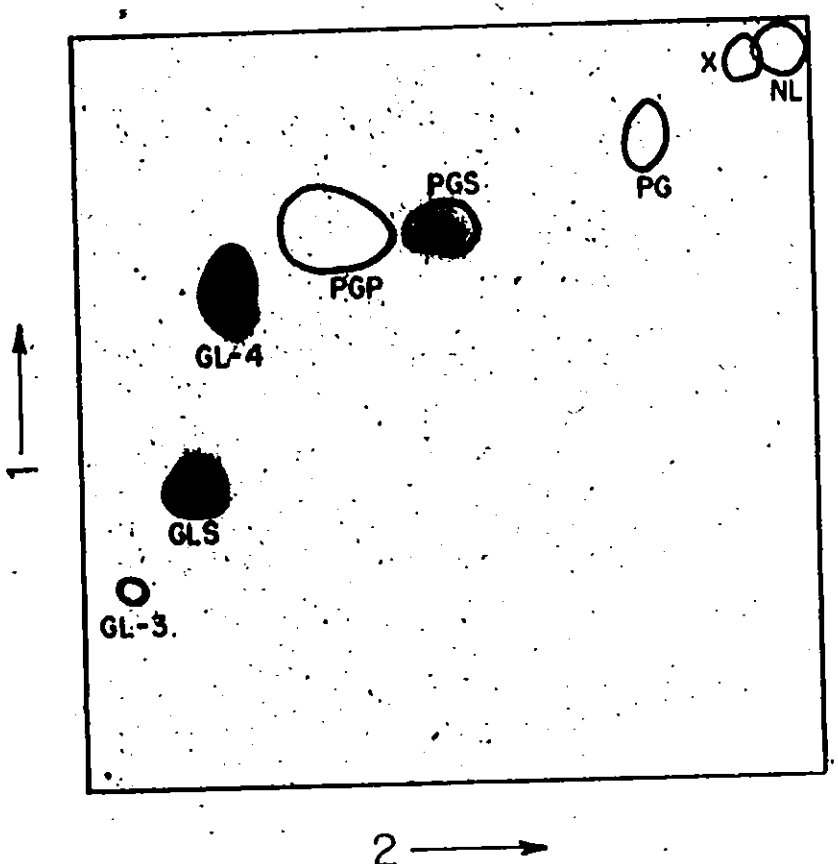
followed by

(b) Chloroform-methanol-conc. ammonium hydroxide
   (65:35:5; v/v/v)

PURPLE MEMBRANE



RED MEMBRANE



sence of GL-3, PGP, PG and an unidentified phospholipid in both membranes (Figure 31). GL-1 was present in both membranes in only trace amounts (Table 9) and the apparent absence of this compound from the two dimensional chromatograms probably resulted from diffusion of the material to a concentration below the limit of detection.

The polar lipid composition, as determined by phosphorus and sugar analyses of unlabelled lipids separated by two-dimensional TLC, was found to be similar for both red and purple membranes (Table 9) and only small differences between the two membranes in the proportions of GL-4, PGP and PG were observed. These differences are probably within experimental error and it can be concluded that both membranes have essentially the same polar lipid composition. The unlabelled membranes had the same proportion of GLS but the [^{35}S] sulfate labelled purple membrane contained 21% more GLS than labelled red membrane. This seems to suggest that the proportions of GLS can vary between preparations.

In contrast with the results presented in this thesis, Kushwaha et al (1975a) previously reported that the sulfated lipid components were present only in purple membrane. The apparent absence of GLS and PGS in red membrane observed by Kushwaha et al (1975a) may have resulted from enzymatic or solvolytic hydrolysis of the sulfate ester during membrane isolation (see Part Two, Results and Discussion, I). The presence of GLS and PGS in both membranes would be consistent with the observation that these lipids are found in the total lipid extract when cells are grown under conditions which do not favour purple membrane synthesis (Deroo, 1974) (see General Introduction, III, 3). The similarity in the polar lipid composition of the two membranes suggests that

Table 9. Polar Lipid Composition of H. cutirubrum Membranes Isolated from Cells Grown Under Illumination and Low Oxygen Tension.

Lipid Component	Mole Percent of Recovered Polar Lipids	
	Purple Membrane	Red Membrane
Sulfated Tetraglycosyl Diether (GL-1)	N.D.*	N.D.
Sulfated Triglycosyl Diether (GLS)	14	14
Tetraglycosyl Diether (GL-2)	N.D.	N.D.
Phosphatidylglycerosulfate (PGS)	5	6
Triglycosyl Diether (GL-3)	2	1
Unidentified Sulfated Glycolipid** (GL-4)	26	33
Phosphatidylglycerophosphate (PGP)	51	42
Phosphatidylglycerol (PG)	2	5
Unidentified phospholipid (X)	<1	<1

*N.D.: Not detected

**Calculation was based on the assumption that the compound was a sulfated monoglycosyl diether.

during the biosynthesis of purple membrane, the bacterioopsin is inserted into a pre-existing lipid matrix with a polar lipid composition similar to that of red membrane.

Extraction of retinal with 90% acetone followed by quantitation by the thiobarbituric acid procedure (Futterman and Saslaw, 1961) combined with protein determination by amino acid analysis gave retinal:protein mole ratios ranging from 0.85 to 0.98 ($\pm$ 0.05) for various purple membrane preparations. These results are close to a 1:1 stoichiometry for retinal and protein in bacteriorhodopsin, and confirm the results reported by Oesterhelt and Stoeckenius (1971) and Papadopoulos et al., (1978).

PART THREE

PHYSICAL STUDIES OF HALOPHILE LIPIDS AND MEMBRANES

EXPERIMENTAL PROCEDURES

I. Preparation of the Diammonium Salt Form of Phosphatidylglycerophosphate (PGP) (Kates et al., 1965)

The barium chloride precipitated lipids (600 mg of a yellow, gelatinous material; see Part One, Experimental Procedures, I, 1) were dissolved in 30 ml of chloroform and diluted with 30 ml of methanol. The mixture was acidified with 1.5 ml of 1.0 N HCl, shaken until the precipitated barium salt dissolved (usually about 10 minutes), then 25.5 ml of distilled water was added and the mixture was shaken and centrifuged. The lower chloroform phase was removed and the upper phase washed once with chloroform; the pooled chloroform extracts were neutralized with 0.2 M methanolic ammonium hydroxide. Benzene was added to the solution, and it was taken to dryness on a rotary evaporator at 35-40°C. The residual lipids were dissolved in a small volume of chloroform, precipitated by the addition of 7.5 volumes of methanol, then kept at 4°C overnight. The precipitate was centrifuged, washed twice with cold methanol and dried in vacuo; at this point the PGP was chromatographically pure. Precipitates which contained traces of GLS were further purified by preparative TLC in chloroform-90% acetic acid-methanol 30:20:4; v/v/v).

Anal.: Calc. for $(NH_4)_2[C_{46}H_{94}O_{11}P_2].H_2O$:
C, 58.88; H, 11.07; N, 2.98; P, 6.59

Found: C, 60.28: H, 10.51; N, 2.27; P, 6.65

II. Purification of Phosphatidylglycerol (PG)

1. Isolation by Thin Layer Chromatography

This phospholipid was chromatographically pure after fractionation of the "glycolipid-enriched fraction" by preparative TLC in chloroform-90% acetic-methanol (30:20:4; v/v/v) (Part One, Experimental Procedures, I, 2). The compound was eluted from the silicic acid on a sintered glass funnel using chloroform-methanol-water (1:1:0:1; v/v/v).

2. Preparation of Ammonium Salt Form

Purified PG (ca. 50 mg) was dissolved in 19 ml of chloroform-methanol-0.2N HCl (1:2:0.8, v/v/v) and mixed with 5 ml each of chloroform and water. After centrifugation, the lower chloroform phase was removed and the upper phase was washed with 2 ml of chloroform. The chloroform phases were neutralized with 0.2N methanolic NH_4OH, diluted with benzene and brought to dryness by rotary evaporation. The residue was dissolved in 0.4 ml of chloroform-methanol (1:1; v/v) and pure PG was precipitated by the addition of 4 ml of acetone; repeated precipitation from acetone was necessary for complete removal of rhodamine. The precipitate was finally dried in vacuo over KOH at room temperature.

Anal: Calc. for $NH_4(C_{46}H_{94}O_8P).H_2O$:
C, 65.59; H, 11.97; N, 1.66; P, 3.68

Found: P, 3.78

III. Purification of Sulfated Triglycosyl Diether (GLS)

GLS was chromatographically pure after TLC fractionation as described above (Part One, Experimental Procedures, Section I, 2). The material was dissolved in chloroform-methanol-0.1N HCl (1:2:0.8; v/v/v/) and the mixture was made biphasic by the addition of one part each of chloroform and 0.2M aqueous NH_4Cl. The upper phase was washed twice with chloroform, and the pooled chloroform phases were neutralized with 0.2M methanolic ammonium hydroxide, diluted with benzene and brought to dryness by rotary evaporation. The ammonium salt of GLS was cleared by centrifugation in chloroform-benzene (1:1; v/v), evaporated to dryness and dissolved in a minimum volume of chloroform-methanol (1:1; v/v). Purified GLS was precipitated by addition of ten volumes of acetone and cooling overnight at 0°C. Rhodamine was completely removed by repeated precipitation from acetone.

Anal: Calc. for $C_{61}H_{117}O_{21}S \cdot NH_4$:

C, 59.24; H, 9.86; N, 1.06; S, 2.59

Found: C, 59.42; H, 9.69; N, 1.22; S, —.

$[\alpha]_D^{24.5} = +46.4°$

Literature Value: $[\alpha]_D +46.79°$

(Kates and Deroo, 1973)

IV. ^{31}P-NMR Studies of Lipids and Membranes

1. Apparatus

^{31}P-NMR spectra were obtained on a Bruker CXP-300 spectrometer operated at 121.5 MHz, using a solenoid detection coil and proton

decoupling. The decoupler was pulsed to avoid sample heating. Chemical shifts were reported with respect to that of 70% phosphoric acid in a sealed tube. Computer simulations of the powder spectra were made according to the formulation of Seelig (1978), with a variable angular-dependent linewidth. The apparent chemical shift anisotropy, $\Delta\sigma_{app.}$, was measured as the distance between the $\sigma_{\parallel}$ and $\sigma_{\perp}$ tensors (Figure 15). The $\sigma_{\parallel}$ tensor is the point of inflection for the low field shoulder and the $\sigma_{\perp}$ tensor is the point of inflection for the high field peak (Seelig, 1978). The NMR spectra and computer simulations were obtained by Drs. D. Marsh, I. Ekiel and I.C.P. Smith of the Division of Biological Sciences, National Research Council of Canada.

2. Sample Preparation

A chloroform solution of pure phospholipid or membrane lipids (50-200 mg in 5 ml of $CHCl_3$) was transferred to a 10 mm O.D. NMR tube. The solvent was removed under a stream of nitrogen, and the material was spread as a thin film inside the tube and dried overnight in vacuo (0.1 mm Hg) over KOH, The dry sample was hydrated in 1.2 ml of H_2O with vortexing, and the suspension was freeze-thawed several times and allowed to equilibrate at room temperature for several hours. Purple membrane suspensions were prepared for NMR studies by centrifuging the sample (ca. 100 mg in 0.1 mM NaN_3) at 200,000 x g for 1 h at 4°C; the packed pellet was transferred directly to the NMR tube.

Freeze-Fracture Electron Microscopy of Lipid Dispersions

1. Apparatus

Freeze-fracture was carried out with a Polaron Freeze-Etch Device, Model E7500. Replicas were observed with a Phillips EM300 Transmission Electron microscope operated at 60 kilovolts.

2. Preparation of Lipid Dispersions

A sample of acetone precipitated lipid (5-10 mg) was weighed into a 2 ml conical centrifuge tube and distilled water was added to make a 5% solution. The tube was vortexed repeatedly and the suspension was allowed to equilibrate at room temperature for approximately one hour before processing for freeze-fracture analysis.

3. Freeze-Fracture Techniques

The tube fracture technique was used for hydrated samples of GLS and GL-3. The foot and top of the specimen tube were filled with the liposome solution, the top was balanced on the foot and the assembly was plunged into freezing Freon 22 (ca. -150°C). The top of the specimen tube was knocked off *in vacuo* at -135°C and the fracture surface was shadowed with platinum and a carbon backing was applied. The knife fracture technique was used for hydrated samples of PGP, PG, total polar lipids and PGP + GLS (2:1). A 1-2 µl sample was applied to the specimen holder and frozen at ca. -150°C as described above. The frozen sample was cleaved with a razor blade *in vacuo* at -135°C and replicated as described above. Each replica was washed with distilled water, followed by washing with 95% ethanol, chloroform-95% ethanol (1:1; v/v) and finally petroleum ether before it was mounted on a grid.

RESULTS AND DISCUSSION

I. ^{31}P-NMR Studies of the Lipids and Membranes

The ^{31}P-NMR spectrum of a suspension of H. cutirubrum purple membrane at 15°C is shown in Figure 32 (upper trace); the spectrum of H. halobium purple membrane was identical. At a low signal to noise ratio, the peculiar lineshape could be incorrectly attributed to the presence of hexagonal phase lipid. However, PGP, the major phospholipid, contains both a phosphomonoester and a phosphodiester and therefore the spectrum could consist of two superimposed bilayer powder patterns having different chemical shift anisotropies. The latter interpretation was verified by computer simulation of the spectrum using chemical shift anistropy values of -18.5 ppm and -61 ppm for the phosphomonoester and phosphodiester species, respectively, in a bilayer arrangement (Figure 32).

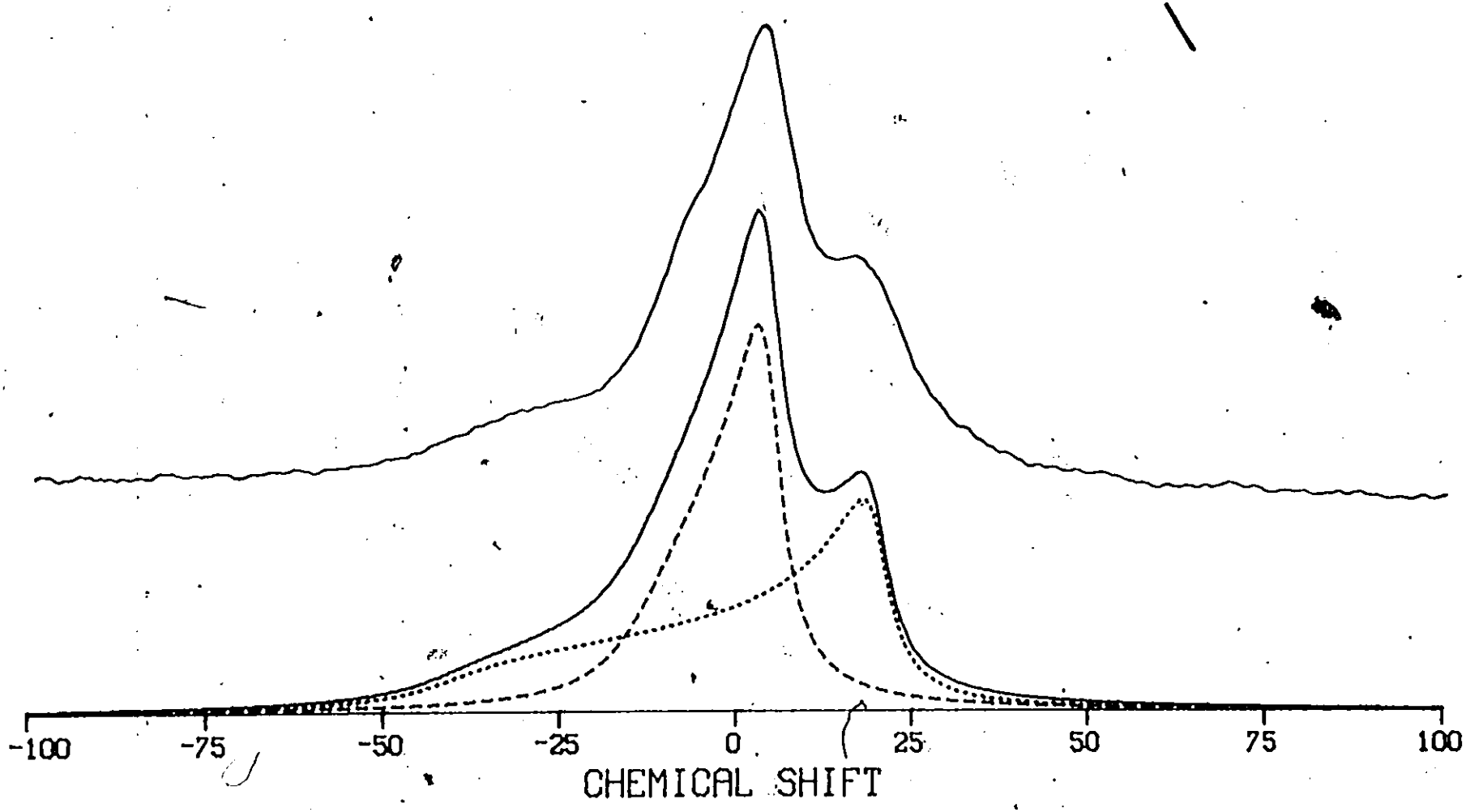
The broader component of the powder pattern was assigned to the phosphodiester group (Structure II, Figure 6; phosphate A) because it had a chemical shift anisotropy similar to those values (-40 to -60 ppm) found for other phospholipids such as phosphatidylcholine (Seelig, 1978). Phosphomonoesters have smaller chemical shift anisotropy values (-18 to -20 ppm) (Seelig, 1978) and therefore the component with the narrower powder pattern was assigned to the terminal phosphate group (Structure II, Figure 6; phosphate B). Also, motional averaging of the PGP headgroup would be expected to be of a greater amplitude at the terminal phosphate since it is located at the periphery of the headgroup region, and this would result in a narrower powder pattern.

The powder patterns observed for total polar lipids, purified PGP, purple membrane lipids and purple membrane indicated that

Figure 32

(Upper) Proton-decoupled ^{31}P-NMR spectrum (121.5 MHz) of the purple membrane from Halobacterium cutirubrum at 15°C; spectral width 125 kHz, acquisition time 6 ms, recycle time 1 s, pulse width 45°, decoupler on 4 μs before acquisition and during acquisition but off during remainder of cycle, Fourier transform of 16 K data points after zero filling. Chemical shifts are expressed with respect to that of 70% phosphoric acid in a sealed capillary.

(Lower) Computer simulation of the above in terms of two axially-symmetric powder patterns characterized by effective chemical shift anisotropies of -61 and -18.5 ppm; the angular-independent and -dependent linewidths were 300 and 200 Hz, and 350 and 250 Hz, for the phosphomonoester and phosphodiester, respectively. The broken curves are the powder patterns for the two types of phosphate ester present.


-100
-75
-50
-25
0
25
50
75
100
CHEMICAL SHIFT

in each case the lipids had a bilayer arrangement (Figure 33). Similar patterns were observed for red membrane and a PGP plus GLS (2:1; w/w) mixture (data not shown). Temperature-dependence studies (Figure 34) showed that in each system bilayer structure was preserved up to 60°C. The bilayer structure of PGP dispersions was observed in salt concentrations up to 0.3 M NaCl and over the pH range 6-9. Conditions outside of these limits were not tested. PG, a minor phospholipid component, contains a single phosphate having a diester linkage, and at 10°C the observed powder pattern indicated the presence of a bilayer arrangement (Figure 35). However, at higher temperatures an isotropic component (-3.3 ppm) appeared, which was superimposed on the bilayer pattern (Figure 35). The appearance of the isotropic peak was not reversed when the sample was cooled to a lower temperature. It would therefore appear that the isotropic component resulted from the formation of small vesicles (McLaughlin et al., 1975).

The temperature-dependence profiles of the chemical shift anisotropy values for the phosphate groups of both PGP and purple membrane (Figure 36) showed a small decrease in $\Delta\sigma_{app.}$ with temperature. However, there was no evidence for a phase transition over the range 5° - 60°C for either the membrane or the dispersions, in agreement with previous calorimetric studies by Chen et al. (1974) and Jackson and Sturtevant (1978), but in disagreement with the calorimetric and ^{1}H-NMR studies of Degani et al. (1978) (See General Introduction VI). Lanyi (1976) predicted earlier that if hydrocarbon chain motion in diphytanyl lipids is dominated by bending at the four tertiary carbons, the bilayer should be a highly cooperative structure with no phase transition. It may be

Figure 33

Proton-decoupled, ^{31}P-NMR spectra (121.5 MHz) of aqueous dispersions of H. cutirubrum lipids and membranes at 5°C. a) Total polar lipids from total membrane. b) Purified phosphatidylglycerophosphate. c) Purified phosphatidylglycerol. d) Total lipids from purple membrane. e) Purple membrane.

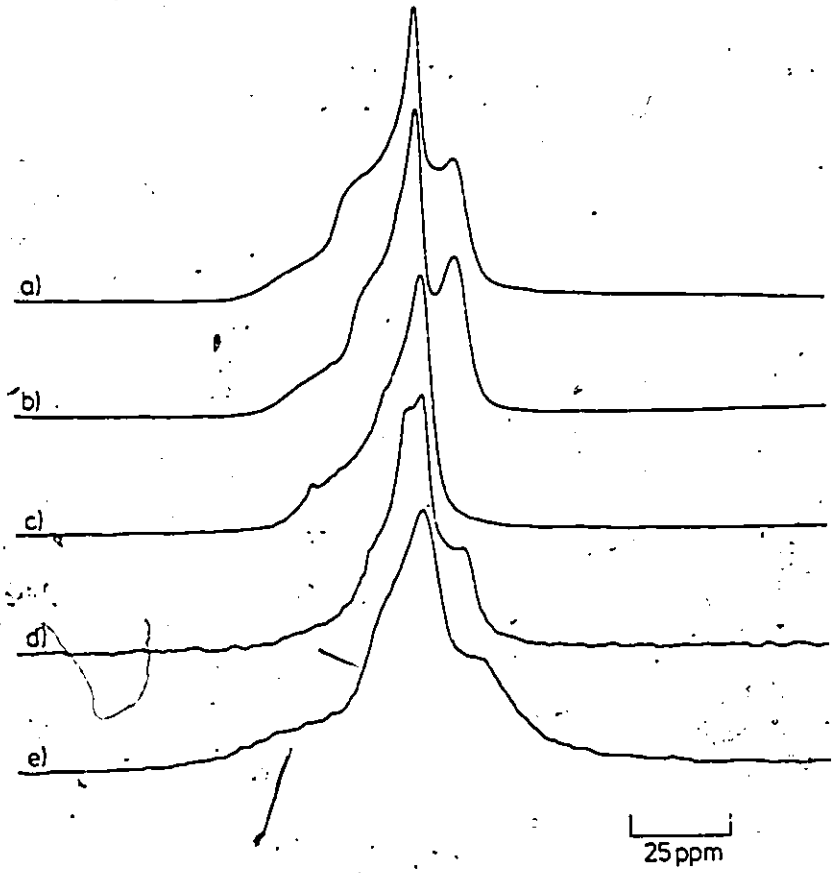

a)
b)
c)
d)
e)
25 ppm

Figure 34

Proton-decoupled ^{31}P-NMR spectra (121.5 MHz) of phosphatidylglycero-phosphate in aqueous dispersion, at the indicated temperatures.

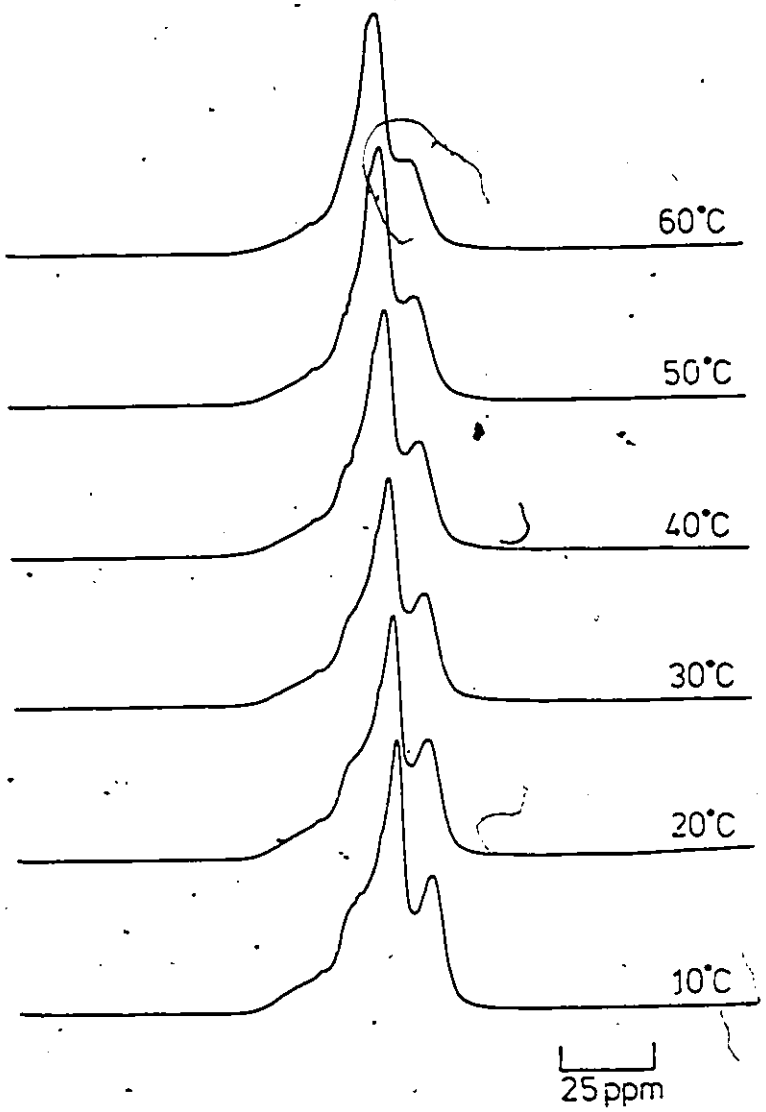

60°C
50°C
40°C
30°C
20°C
10°C
25 ppm

Figure 35

Temperature variation of the proton-decoupled 121.5 MHz ^{31}P-NMR spectra of an aqueous dispersion of phosphatidylglycerol.

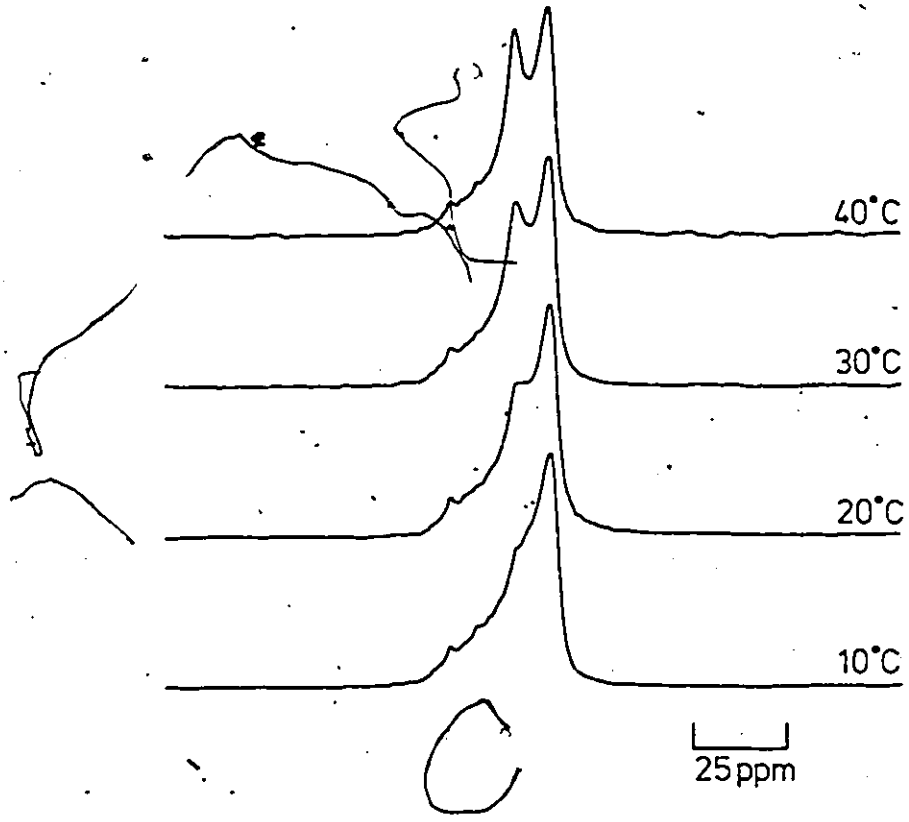
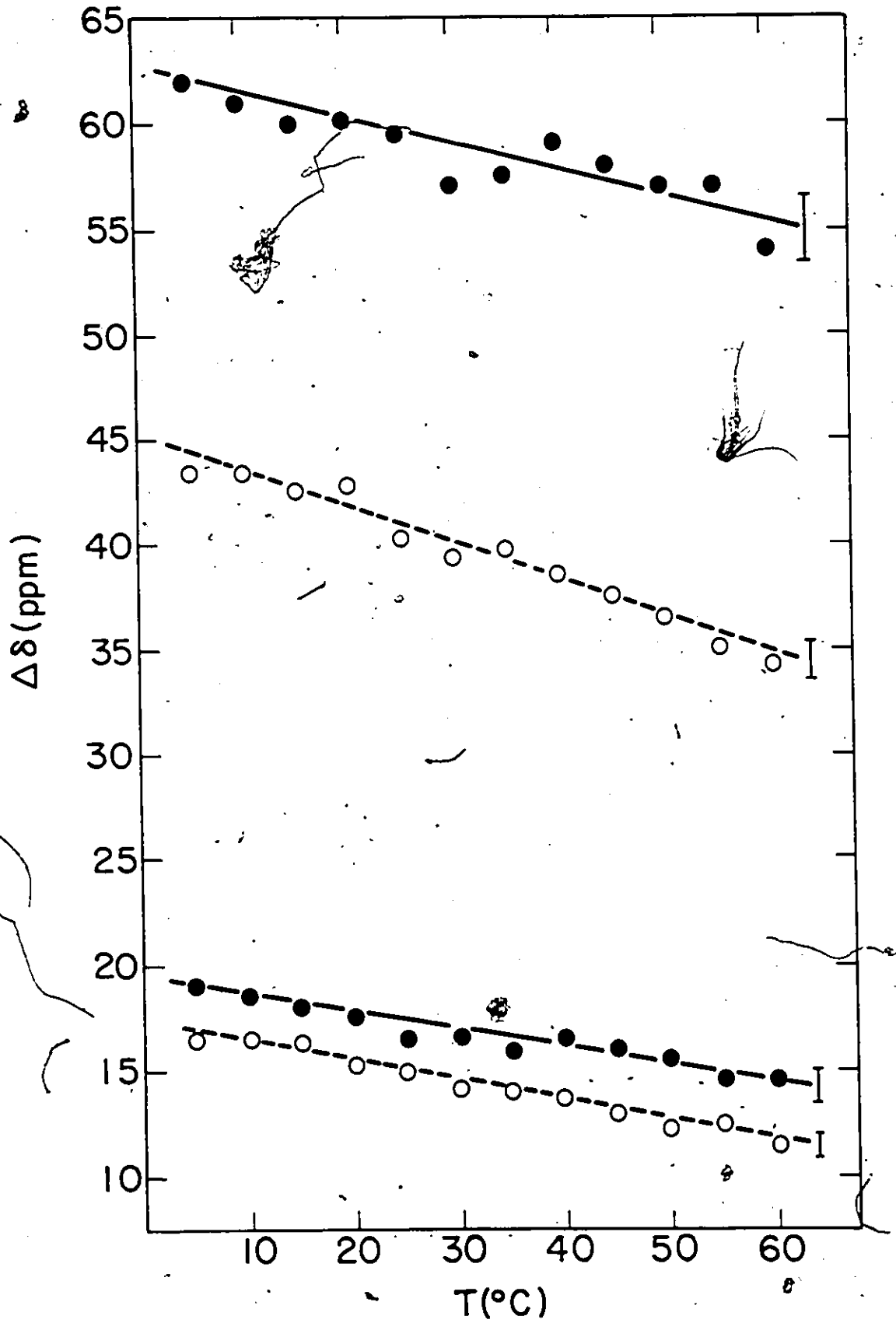

40°C
30°C
20°C
10°C
25 ppm

Figure 36

Temperature dependence of the effective ^{31}P chemical shift anisotropies ($\Delta\sigma$) for the phosphodiester (upper data) and phosphomonoester (lower data) groups in the purple membrane (solid circles) and phosphatidylglycerophosphate (open circles) in aqueous dispersion. The anisotropies were taken from computer simulations of the spectra. The bars on the right sides of the curves are estimates of the precision of the measurements.


65
60
55
50
45
40
35
30
25
20
15
10
Δδ(ppm)
10
20
30
40
50
60
T(°C)

significant that ^{13}C-NMR studies have indicated that in halophile lipid dispersions motion of the tertiary carbons is more hindered in comparison with the rest of the chain (Degani et al., 1980).

The data obtained also give some information concerning the relative motional state of the phospholipids in purple membrane. The chemical shift anisotropy values observed for purple membrane were significantly larger than those of PGP dispersions with the differences being greater for the phosphodiester than for the monoester (Figure 36) indicating that in purple membrane the phosphate groups have a smaller amplitude of motional averaging than in PGP dispersions. Also, the order of linewidth values was generally found to be PGP < total polar lipids < purple membrane lipids << purple membrane (Figure 33). This suggests that overall motion of the phospholipid molecules in purple membrane is slower compared to that in PGP dispersions primarily because of interaction of PGP with bacteriorhodopsin and to a lesser extent because of interaction with neutral lipids.

II. Freeze-Fracture Electron Microscopy of Lipid Dispersions

1. Sulfated Triglycosyl Diether (GLS) and Triglycosyl Diether (GL-3)

Freeze-fracture electron microscopy of GLS dispersions in H_2O revealed the presence of large multilamellar liposomes embedded in a matrix of apparently single bilayer vesicles (Plate 1). The outer layers of the liposomes contained small oval-shaped structures in the plane of the bilayer and both the outer and inner layers contained striations (Plate 2). These structures may have resulted from stresses that occurred during freeze-fracturing; however, they could be related to the presence

of the charged sulfate group, because liposomes formed by GL-3 were much smoother in appearance (Plate 3).

GL-3 formed large multilamellar liposomes in which the bilayers were more closely packed together than in those of GLS (Plates 3, 4 and 5). Because GL-3 is the desulfation product of GLS, the larger interbilayer space of GLS liposomes may be due to repulsion of the negative charges on the sulfate groups or to the more bulky polar head of the sulfated triglycosyl diether.

2. Phosphatidylglycerophosphate (PGP) and Phosphatidylglycerol (PG)

Freeze-fracture electron microscopy of aqueous dispersions of PGP showed the presence of multilamellar structures (Plates 6, 7 and 9). These structures were not homogeneous with respect to shape, for example Plate 7 shows a large multilamellar liposome which appears spherical and closed, whereas Plate 6 shows a multilamellar structure which is non-spherical and appears to be an open structure. Also, the multilamellar structures are surrounded by smaller liposomes which appear to be unilamellar (Plates 6 and 8). The dispersions of PG showed some multilamellar structures, which, in comparison with those of PGP, GLS and GL-3, were much smaller in size (Plates 10 and 11). A much larger proportion of the material seemed to be present in the form of single bilayer vesicles. The structures formed by both PGP and PG contained particles suggesting the presence of a non-bilayer phase. Similar particles have been shown to appear in the fracture faces of cardiolipin-phosphatidylcholine bilayers prepared in the presence of Ca^{2+}; these structures are associated with the appearance of an isotropic peak in the ^{31}P-NMR spectrum (de Kruijff et al.,

1979). Such particles have been interpreted as being inverted phospholipid micelles sandwiched between the two monolayers of the lipid bilayer (Verkleij et al., 1979).

Negative staining electron microscopy using phosphotungstic acid as fixative showed some lamellar structure in PGP dispersions and amorphous structure in PG dispersions (Chen et al., 1974). However, phosphotungstate is a polyvalent ion and when added to dispersions of negatively charged lipids could compete for available cations. During the partial drying process required in the procedure, concentration of phosphotungstate ion occurs and the negative charge induced in the bilayer could possibly result in structural changes (Bangham and Horne, 1964). It has been observed that negative staining with sodium phosphotungstate can cause distortion of bilayer structure in natural membranes such as erythrocyte ghosts (Haggis, 1969).

3. Lipid Mixtures

Freeze-fracture electron microscopy of aqueous dispersions of both total polar lipids and a PGP plus GLS mixture (2:1, w/w) showed the presence of multilamellar liposomes (Plates 12, 13, 14 and 15). These electron micrographs resembled those of GLS dispersions (Plates 1 and 2) with respect to interbilayer distance and the presence of oval-shaped and channel-like structures. Compared with the structures formed by PGP (Plates 6, 7 and 8), both total polar lipids and PGP plus GLS (2:1; w/w) showed liposomes which were closed and more regular in shape.

Plates 1 and 2

Freeze-fracture electron micrographs of GLS in aqueous dispersion.

1: 10,600X

2: 25,000X

Plates 3, 4 and 5

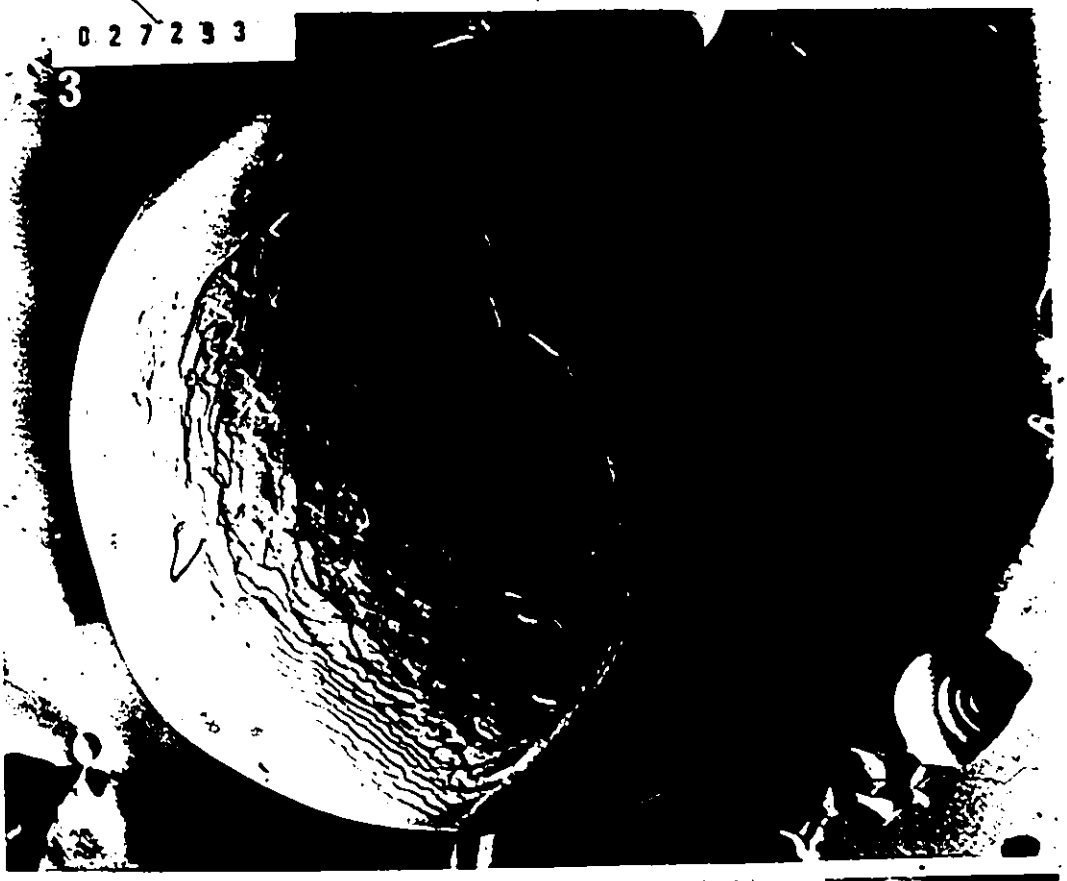
Freeze-fracture electron micrographs of GL-3 in aqueous dispersion.

3: 19,000X

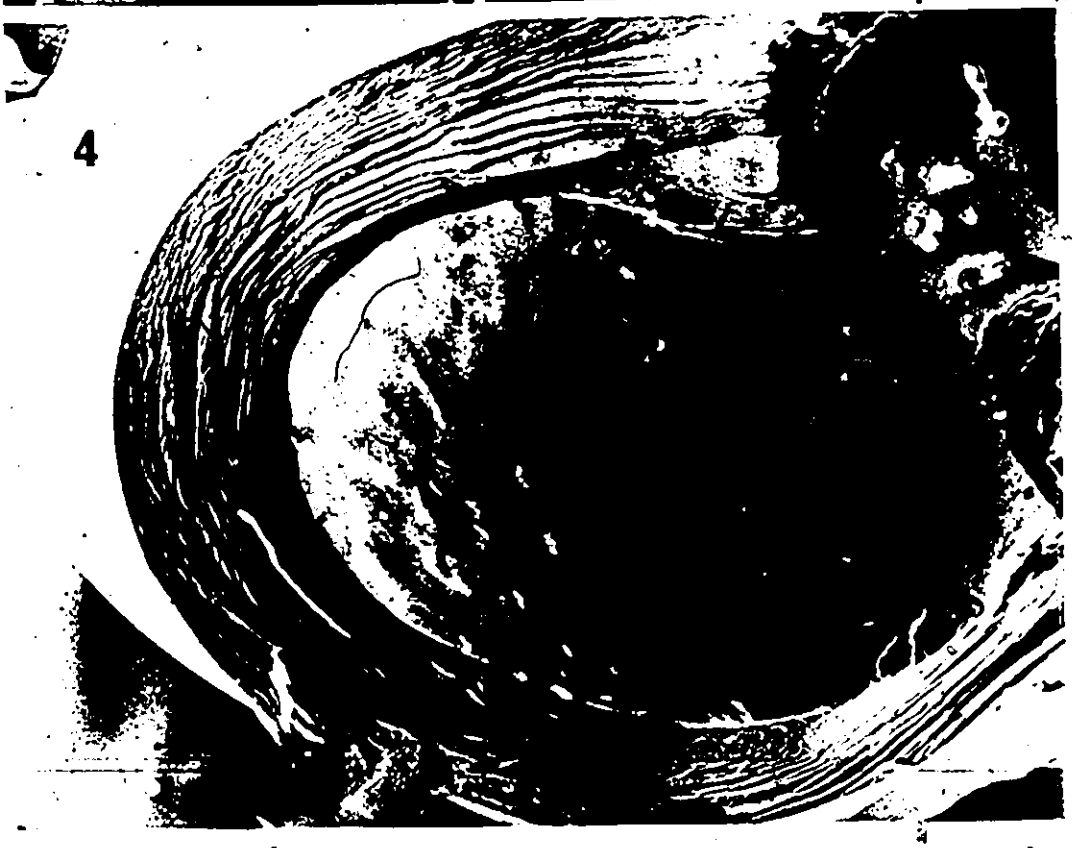
4: 29,500X

5: 93,800X

3



4



5

27

Plates 6-9

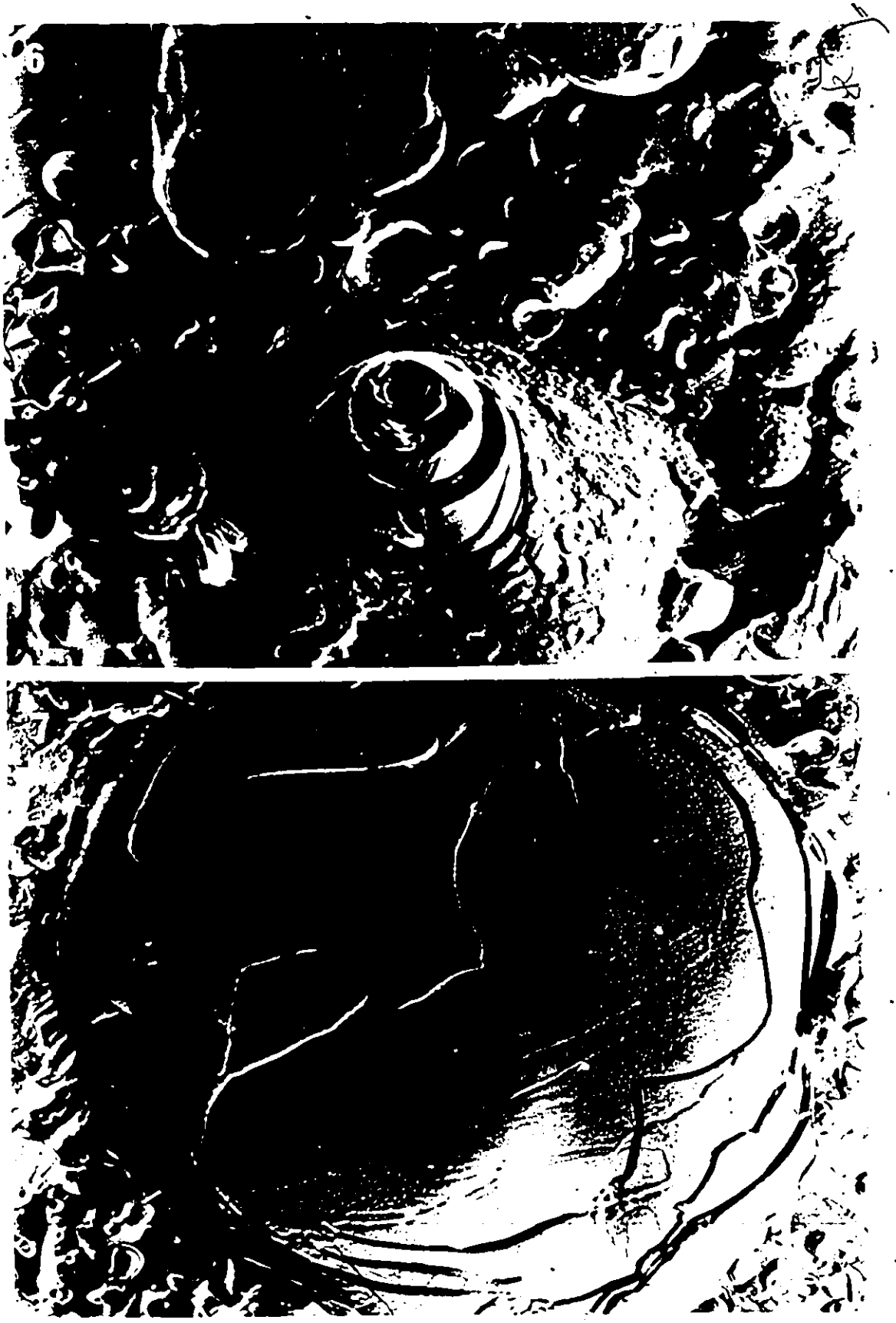
Freeze-fracture electron micrographs of PGP in aqueous dispersion.

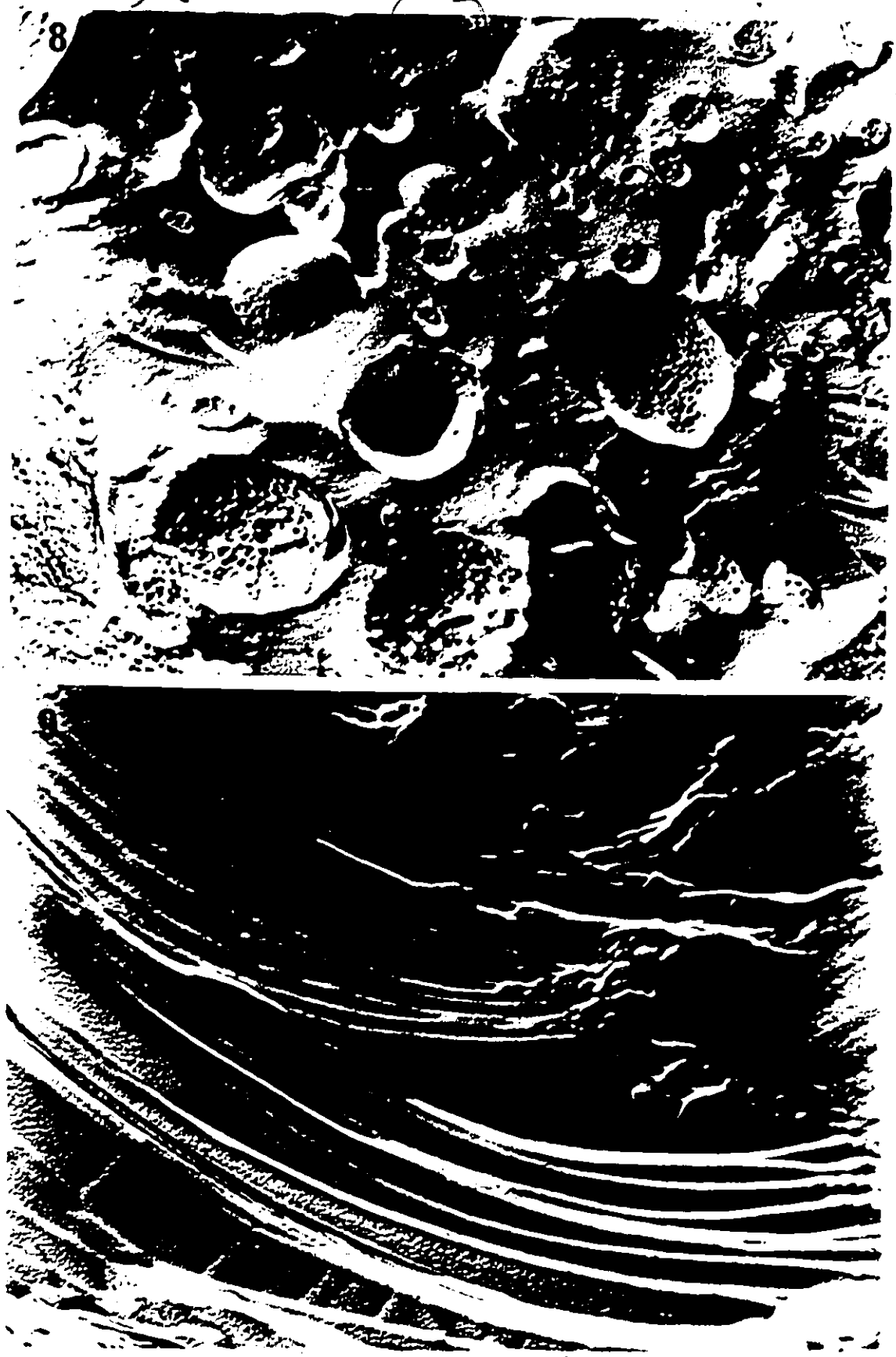
6: 19,800X

7: 10,800X

8: 30,800X

9: 64,700X



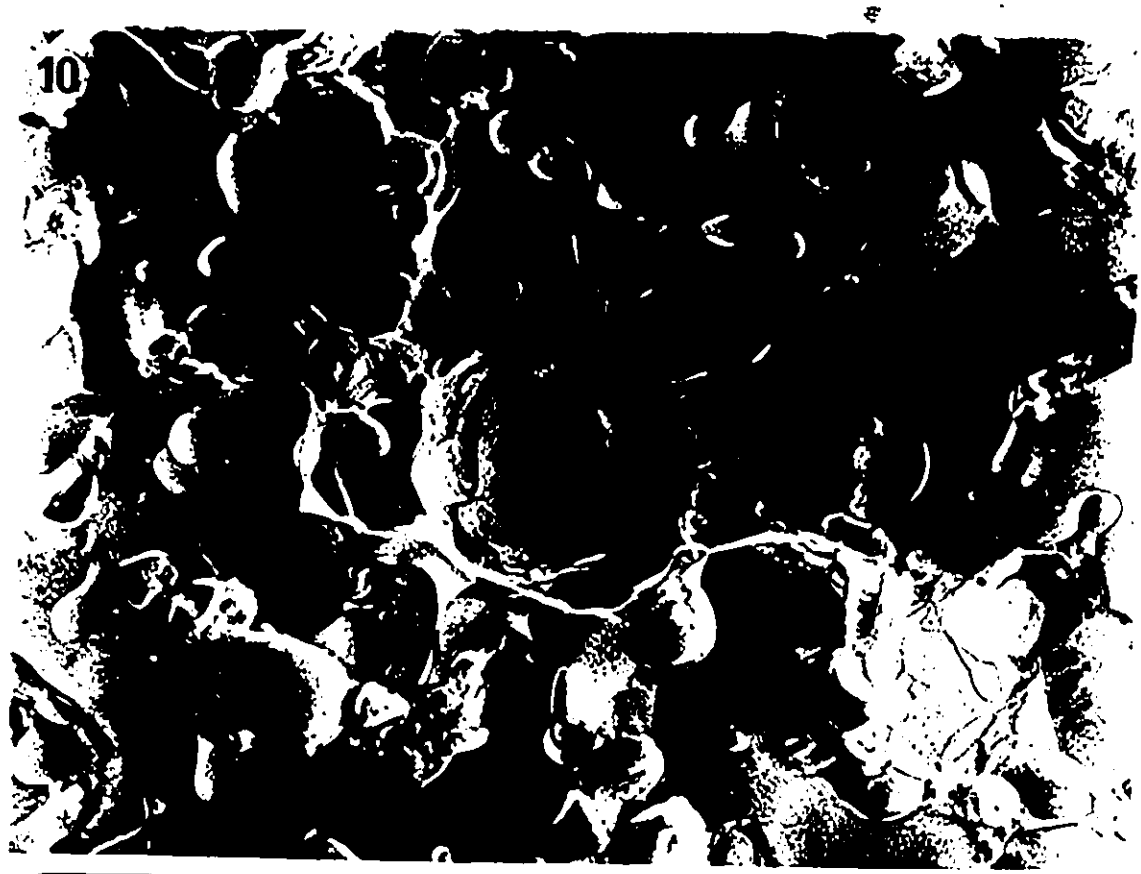


Plates 10 and 11

Freeze-fracture electron micrographs of PG in aqueous dispersion.

10: 25,000X

11: 26,700X


10

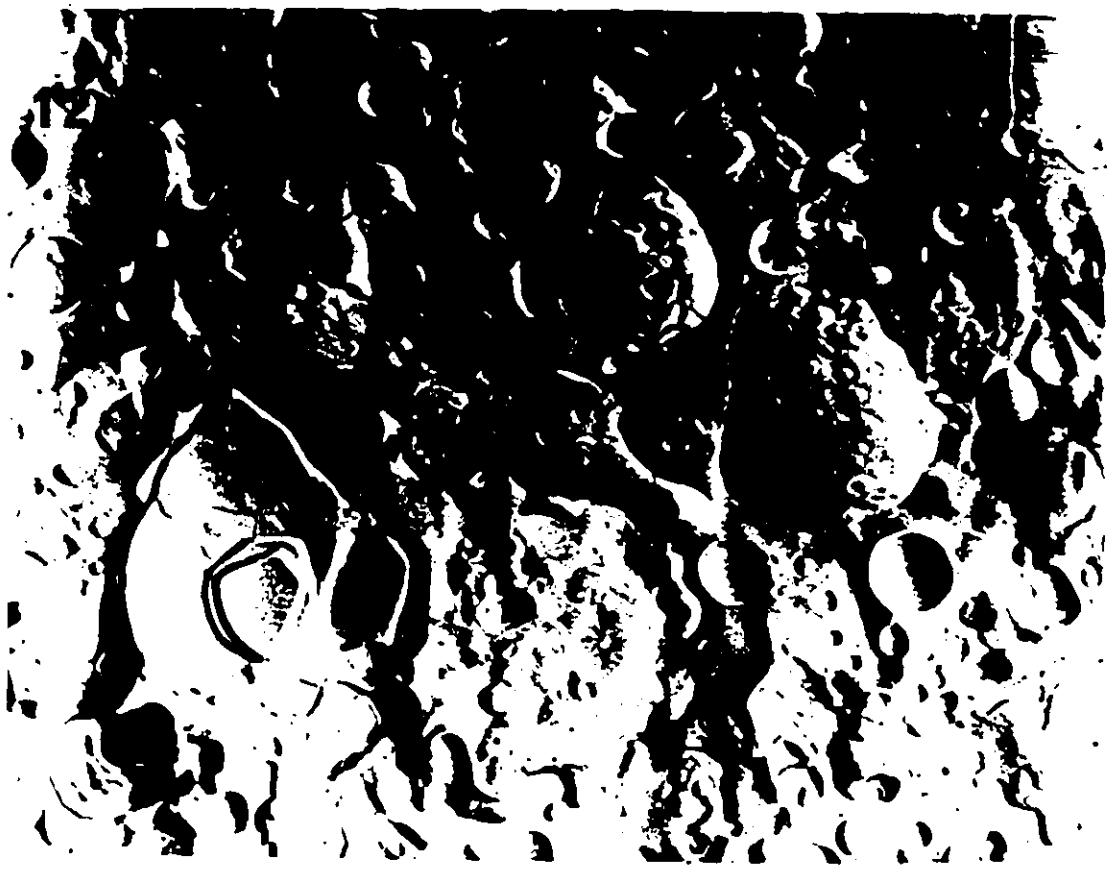

11

Plates 12 and 13

Freeze-fracture electron micrographs of total polar lipids in aqueous dispersion.

12: 16,300X

13: 40,200X




13

Plates 14 and 15

Freeze-fracture electron micrographs of PGP plus GLS mixture (2:1; w/w) in aqueous dispersion.

14: 11,000X

15: 30,700X


14


15

III. Polymorphism of Halophile Lipids - General Conclusions

The ^{31}P-NMR spectra of PGP in aqueous dispersion indicated the presence of lipid bilayers; freeze-fracture electron microscopy revealed structures which were multilamellar but were irregular with respect to size and shape in comparison with liposomes of GLS, GL-3, total polar lipids and the PGP plus GLS mixture. These results together with the inability of PGP dispersions to show osmometric behaviour (Chen et al., 1974) indicate that although PGP is capable of bilayer formation, the resulting structures are unstable and leaky. Also, the structures contained particles which could be interpreted as being lipids in an inverted micellar or non-bilayer phase; the associated isotropic peak could possibly be obscured by the complex spectrum. It is of interest that cardiolipin-phosphatidylcholine bilayers containing lipidic particles have been shown to be permeable to Mn^{2+} (de Kruijff et al., 1979). Freeze fracture electron microscopy of both GLS and GL-3 in aqueous dispersion revealed that these glycolipids formed large, multilamellar liposomes; similar structures were observed for GLS dispersions in the negative staining electron microscopy studies of Chen et al., (1974). As shown by ^{31}P-NMR and freeze-fracture electron microscopy studies, both total polar lipids and a PGP plus GLS mixture (2:1; w/w) formed closed bilayers in aqueous dispersions, in agreement with the osmometric and negative staining electron microscopy studies of Chen et al., (1974). Therefore, as concluded by the latter investigators, the presence of GLS in PGP dispersions appears to result in liposome stabilization, probably because the large carbohydrate polar head of GLS increases the distance between the highly negative PGP molecules in the bilayer, and such an effect would probably lower the amount of intermolecular repulsion. Compressibility studies on lipid monolayers of PGP, GLS and appropriate

mixtures would be required in the testing of the latter hypothesis (Sears and Stark, 1973).

At temperatures above 10°C, the ^{31}P-NMR spectra of PG in aqueous dispersion showed the presence of bilayer structures, as well as an isotropic component. These results were consistent with the freeze-fracture electron microscopy studies of PG dispersions, which revealed a large proportion of apparently single bilayer vesicles, which would be expected to undergo isotropic motion. Chapman (1980) has stated that large, multilamellar liposomes behave as osmometers, whereas small, single bilayer vesicles do not. The observation that PG formed single bilayer vesicles would explain the inability of PG dispersions to show ideal osmometric behaviour (Chen et al., 1974). Also, the presence of particles in the fracture faces suggests that the bilayers may contain lipids in an inverted micellar or other non-bilayer phase and such structures could possibly increase the permeability of the vesicles (see above).

The characteristic striated appearance of the hexagonal (H_{II}) phase (Cullis et al., 1978) was not observed in any of the freeze-fracture electron micrographs of lipids dispersed in distilled water, but the possiblity that hexagonal phase lipids can occur at high salt concentrations cannot be excluded. The physical properties of halophile lipids were not studied at physiological salt concentrations (ca. 4M NaCl) because precipitation of these lipids occurs under such conditions (Chen et al., 1974), and freeze-fracture electron micrographs of lipid dispersions prepared in the presence of salt were found to be obscurred by salt crystals. Consequently, the physiological significance of these studies is uncertain. Bilayer structure of PGP dispersions was observed in salt concentrations up to 0.3 M NaCl but further studies with reconstituted lipid-protein complexes in high salt must be done in order to understand the physical properties of halophile lipids under physiological conditions.

APPENDIX I

Mass Spectrograms of Partially Methylated Alditol Acetates

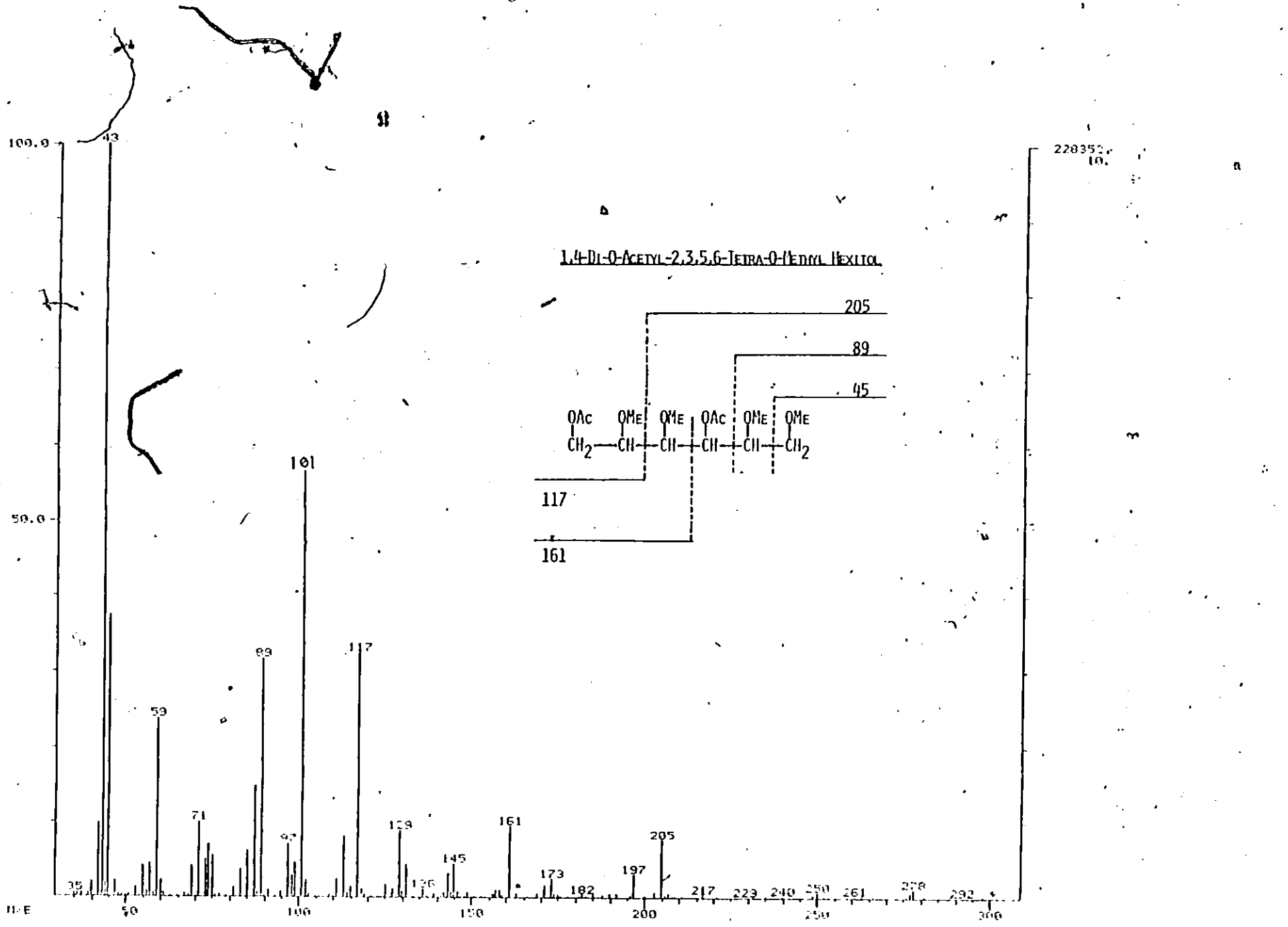
The fragmentation patterns of partially methylated alditol acetates shown in Figures 37 to 42 were based on the following generalizations derived from systematic studies (Lindberg, B., 1972):

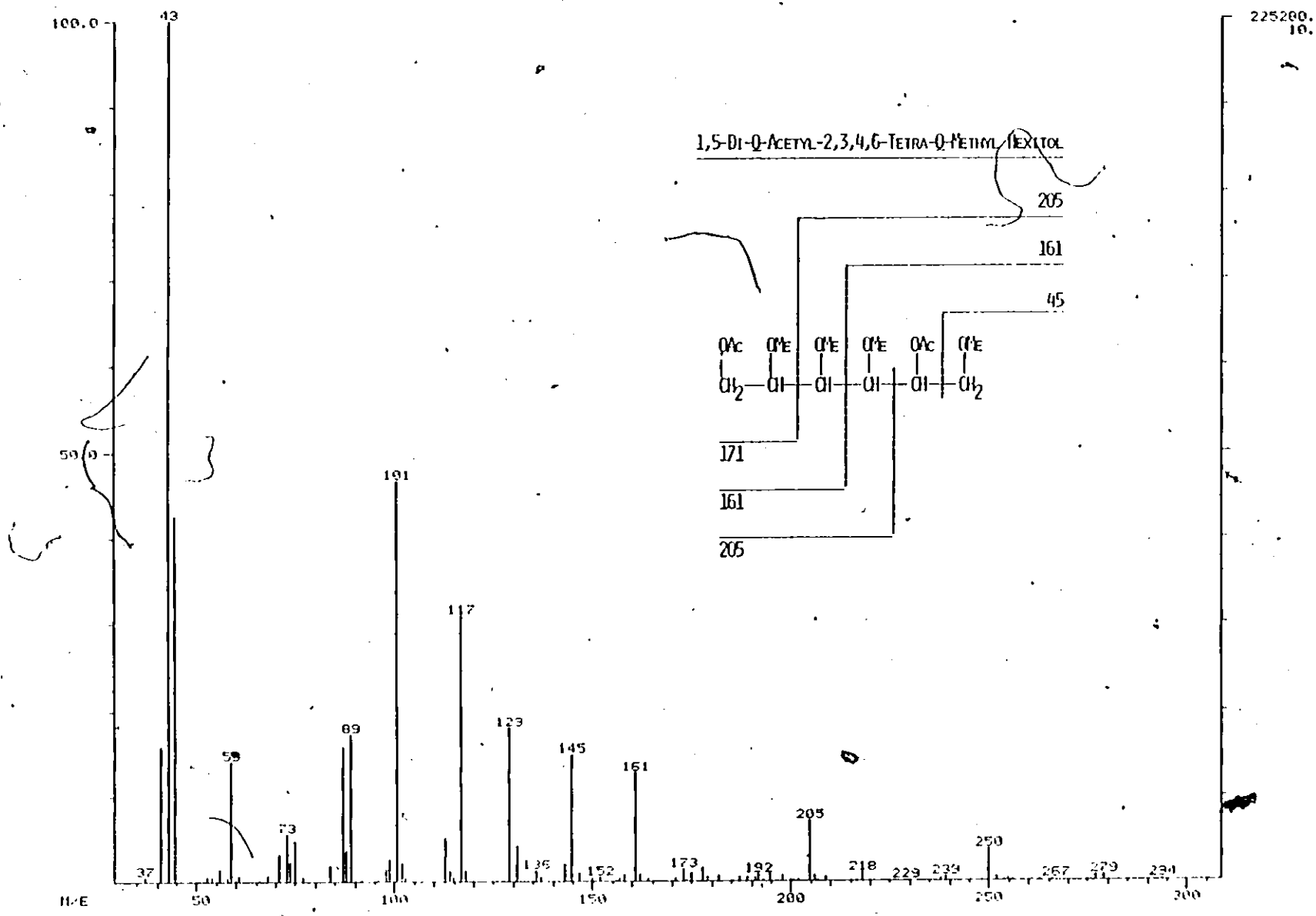
1. Similar mass spectra are observed for derivatives having the same substitution pattern, i.e., epimers and stereoisomers are not differentiated.

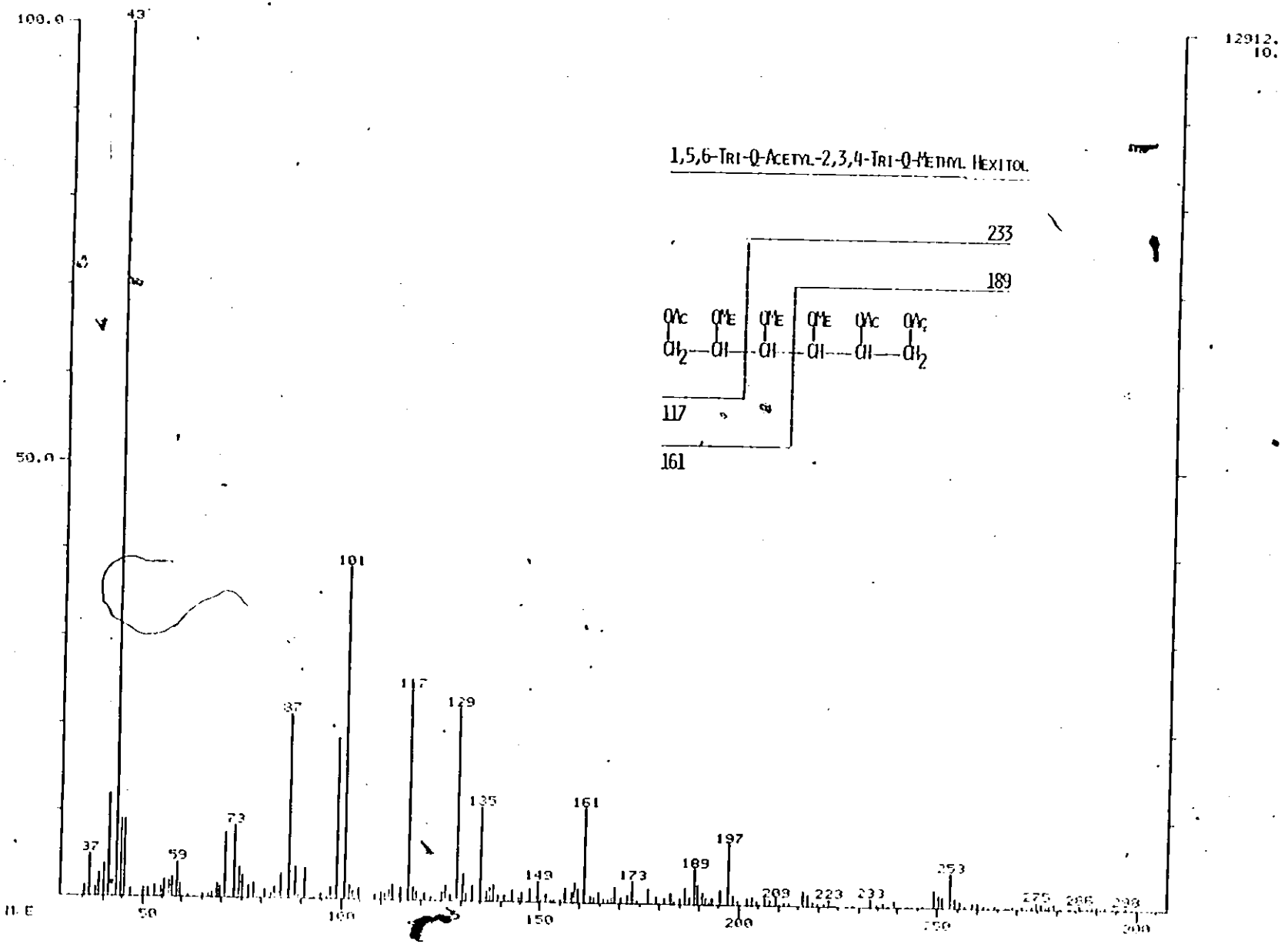
2. Primary fragments are formed by fission between two chain carbon atoms; the fragment with the methoxyl group gives the positive ion. Fission between two methoxylated carbons is preferred over fission between an acetoxylated carbon and a methoxylated carbon, which is in turn preferred over fission between two acetoxylated carbons. An exception to this rule is observed with compounds having two terminal methoxyl groups such as 1,4-di-O-acetyl-2,3,5,6-tetra-O-methyl-D-galactitol, which give M/e 89 as a strong peak (see Figure 37).

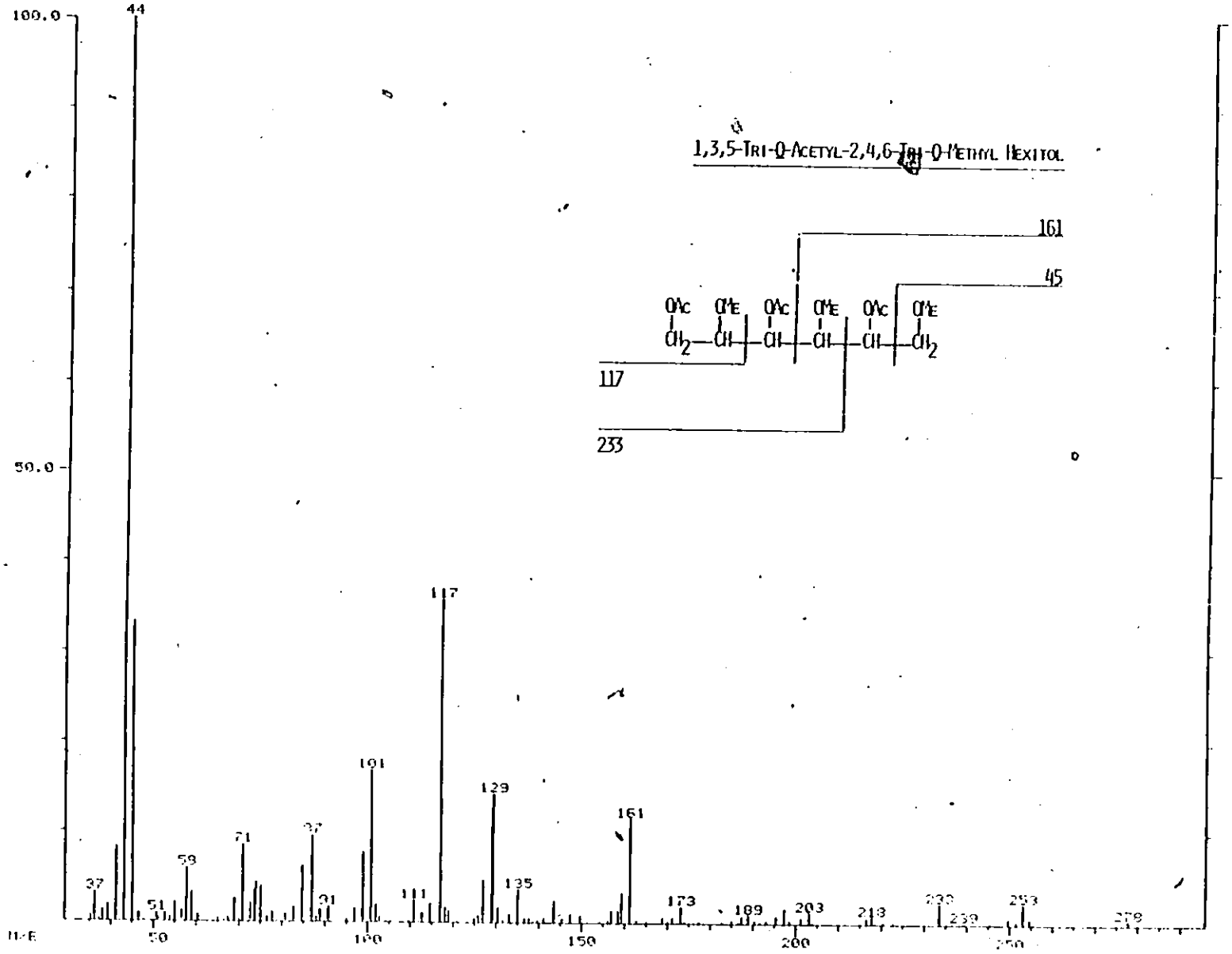
Figures 37 to 42

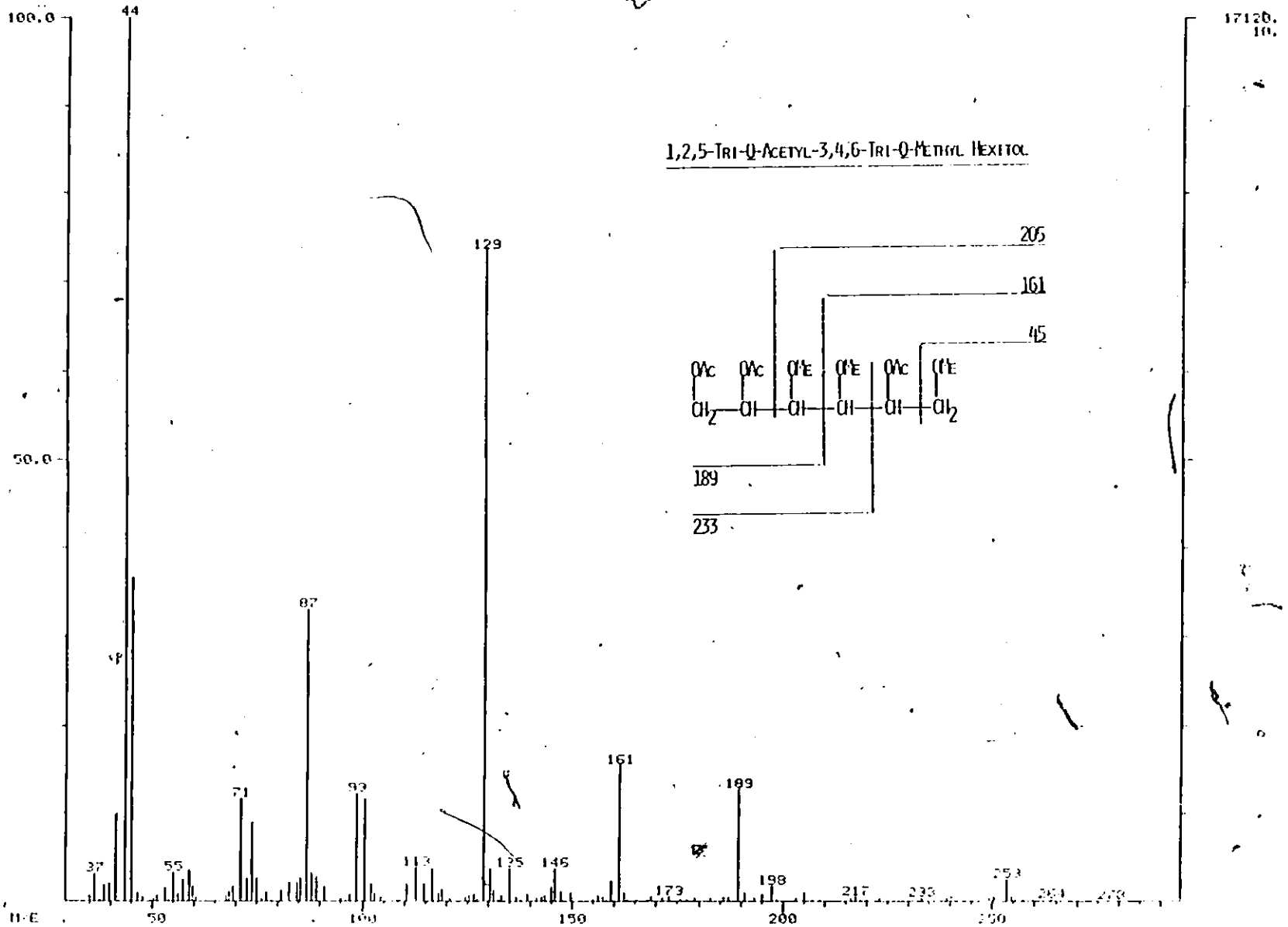
Mass spectrograms of partially methylated alditol acetates derived from permethylated glycolipids.


1,4-Di-O-Acetyl-2,3,5,6-Tetra-O-Methyl Hexitol
205
89
45
OAc OMe OMe OAc OMe OMe
CH2—CH—CH—CH—CH—CH2
117
161
100.0
50.0
43
101
89
117
59
71
97
129
145
161
173
182
197
205
217
223
240
250
261
278
292
35
120
M/E
50
100
150
200
250
300
228352
10.


100.0
50.0
43
191
117
123
145
161
89
58
73
205
250
37
136
152
173
192
218
229
239
267
279
290
300
M/E
50
100
150
200
250
225280.
10.
1,5-Di-O-Acetyl-2,3,4,6-Tetra-O-Metiyl Hexitol
205
161
45
OAc
OMe
OMe
OMe
OAc
OMe
171
161
205


1,5,6-Tri-O-Acetyl-2,3,4-Tri-O-Methyl Hexitol
233
189
117
161
100.0
50.0
12912.
10.
43
101
117
129
37
135
161
73
37
59
189
197
173
143
253
223
233
209
275
286
298
50
100
150
200
250
300


100.0
50.0
44
37
51
59
71
87
91
101
111
117
129
135
161
173
189
203
213
233
253
279
17184.
10.
50
100
150
200
250
1,3,5-Tri-O-Acetyl-2,4,6-Tri-O-Methyl Hexitol
161
45
117
233


100.0
50.0
44
129
87
71
93
161
189
37
55
113
125
145
173
198
217
233
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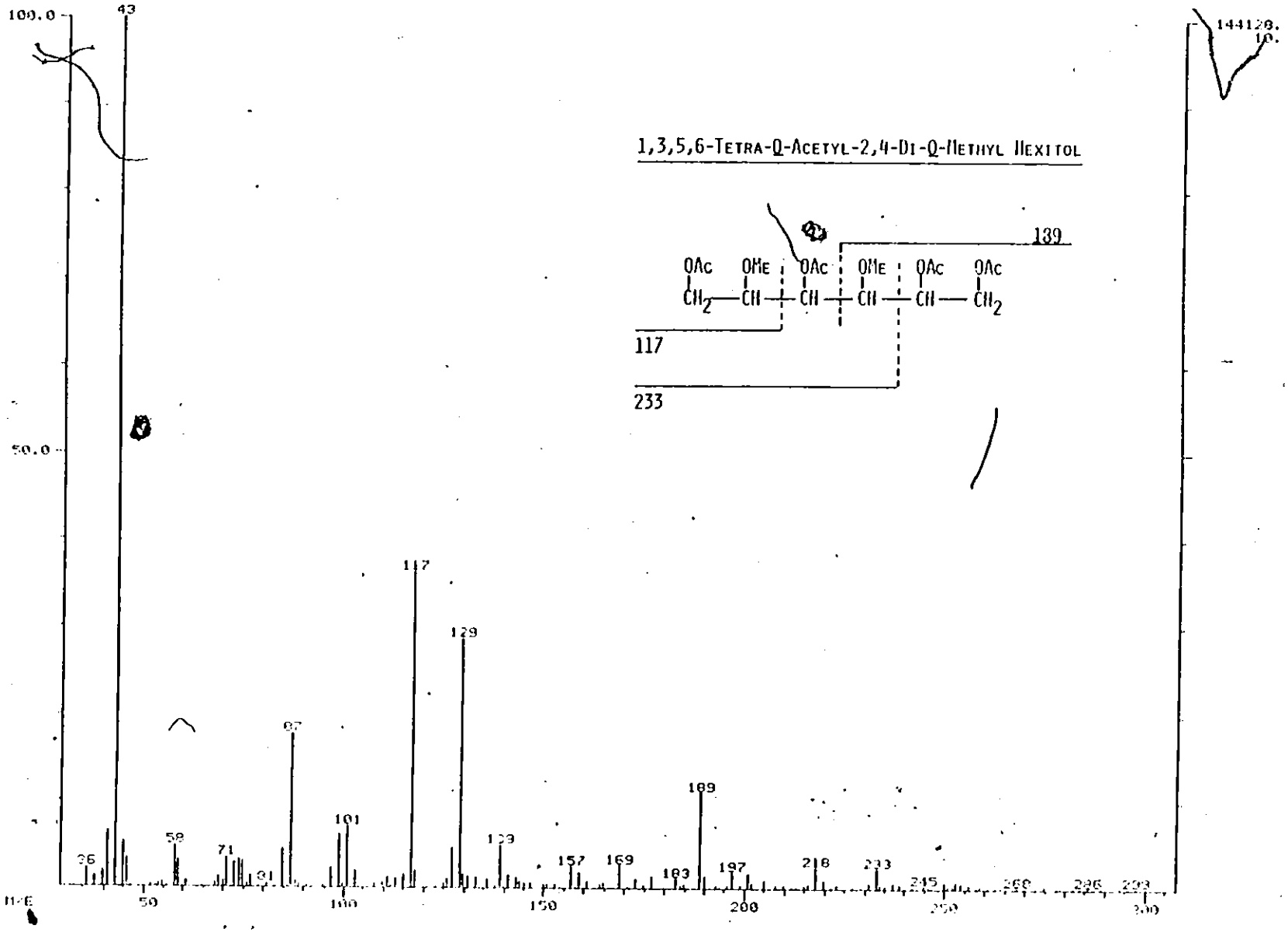
200
1,2,5-Tri-O-Acetyl-3,4,6-Tri-O-Methyl Hexitol
205
161
45
189
233



1,3,5,6-Tetra-O-Acetyl-2,4-Di-O-Methyl Hexitol

Table 10. Computer Correlation of Mass Spectrograms

Partially Methylated Alditol Acetate	% Fit of Spectrum of Partially Methylated Alditol Acetate derived from Glycolipid[a]		
	GL-1	GL-2	GL-3
2,3,5,6-tetramethyl hexitol diacetate	92.5	86.0	-
2,3,4,6-tetramethyl hexitol diacetate	-	96.0	+
2,3,4-trimethyl hexitol triacetate	-	-	91.9
2,4,6-trimethyl hexitol triacetate	81.3	-	-
3,4,6-trimethyl hexitol triacetate	96.6	97.1	91.5
2,4-dimethyl hexitol tetraacetate	94.6	98.7	-

+ present in chromatogram but not searched.

- absent from chromatogram or present only in trace amounts

[a] positional correlation of the sixteen largest mass peaks in the unknown >80% is considered to be a good fit.

APPENDIX II

Calculation of Theoretical Molecular Rotations of GL-1

The theoretical values of molecular rotations of GL-1 were calculated according to Hudson's rule of optical rotation (Hudson, 1909), which states "the molecular rotation of an optical active compound containing more than one asymmetric centre can be calculated from the arithmetic sum of the molecular rotations of their optically active constituents." Chromium trioxide oxidation showed that the mannopyranose and glucopyranose residues were both α-linked, and that the galactopyranose residue was β-linked, and therefore calculations are shown for structure having α- and β-linked galactofuranose residues. The values of molecular rotations used were those reported in the literature (Stanek et al., 1963; Green, 1966) and the molecular rotations of GL-1 were calculated as follows:

(a) Assuming the presence of an α-linked galactofuranose:

$$M_D(\text{GL-1}) = M_D[\text{2,3-di-O-phytanyl-sn-glycerol}] + M_D[\text{α-methylglucopyranoside}] + M_D[\text{α-methylmannopyranoside}] + M_D[\text{β-methylgalactopyranoside}] + M_D[\text{α-methylgalactofuranoside}] = 54.9 + 309 + 154 + 0 + 201.8 = 720$$

(b) Assuming the presence of a β-linked galactofuranose:

$$M_D(\text{GL-1}) = M_D[\text{2,3-di-O-phytanyl-sn-glycerol}] + M_D[\text{α-methylglucopyranoside}]$$

$$+ M_D[\alpha\text{-methylmannopyranoside}]$$

$$+ M_D[\beta\text{-methylgalactopyranoside}]$$

$$+ M_D[\beta\text{-methylgalactofuranoside}]$$

$$= 54.9 + 309 + 154 + 0 - 217.3$$

$$= 301$$

The theoretical values of molecular rotations of GL-3 were calculated in a similar manner.

CLAIMS TO ORIGINAL RESEARCH

1. The structures of two novel glycolipids isolated from Halobacterium cutirubrum have been established as 2,3-di-O-phytanyl-1-O-[Galp-(3'SO_4^-)β1'→6'Manp(3'←1'αGalf)α1'→2'Glcpα]-sn-glycerol and 2,3-di-O-phytanyl-1-O-[Galpβ1'→6'Manp(3'←1'αGalf)α1'→2'Glcp]-sn-glycerol. The structure of the naturally occurring triglycosyl diether has been confirmed as being 2,3-di-O-phytanyl-1-O-[Galpβ1'→6'Manpα1'→2'-Glcpα]-sn-glycerol.

2. The red and purple membranes isolated from Halobacterium cutirubrum were shown to have essentially the same polar lipid composition.

3. ^{31}P-NMR and freeze-fracture electron microscopy studies of the sulfated triglycosyl diether, triglycosyl diether and total polar lipids showed that they formed multilamellar liposomes in aqueous dispersion; phosphatidylglycerol formed vesicles whereas phosphatidylglycerophosphate formed multilamellar structures which appeared to be unstable.

4. ^{31}P-NMR studies of purple membrane indicated a bilayer structure and no lipid phase transition was observed over the range 5°-60°C.

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December 14, 1981

Your file Votre référence

Our file Notre référence

Dr. R. Henderson
Medical Research Council of Molecular Biology
Hills Road
Cambridge, ENGLAND
CB2 2QH

Dear Dr. Henderson:

I am submitting a Ph.D. thesis entitled "Chemical and Physical Studies of Polar Lipids in Extremely Halophilic Bacteria" to the School of Graduate Studies of the University of Ottawa. This thesis has been prepared under the supervision of Professor M. Kates.

I request your permission to use Figure 4 from the paper(s) entitled:

"The Purple Membrane from Halobacterium Halobium"
Ann. Rev. Biophys. Bioeng. 6, 87-109 (1977)

If you agree, please sign below and return this letter to me. Many thanks for your cooperation.

Yours sincerely,

B. W. Smallbone

Barry W. Smallbone

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Date 31/12/81

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Direction de la recherche

December 14, 1981

Your file Votre référence

Our file Notre référence

Dr. S.J. Singer
Department of Biology, P.O. Box 109
University of California at San Diego
La Jolla, California 92037
U.S.A.

Dear Dr. Singer:

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Science, 175, 720-731 (1972)

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B.W. Smallbone

Barry W. Smallbone

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Signed S.J. Singer

Date Dec. 30, 1981

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Institut de recherches sur les aliments
Direction de la recherche

December 14, 1981

Your file Votre référence

Our file Notre référence

Dr. W. Stoeckenius
Department of Biochemistry and Biophysics
University of California, San Francisco
San Francisco, California 94143
U.S.A.

Dear Dr. W. Stoeckenius:

I am submitting a Ph.D. thesis entitled "Chemical and Physical Studies of Polar Lipids in Extremely Halophilic Bacteria" to the School of Graduate Studies of the University of Ottawa. This thesis has been prepared under the supervision of Professor M. Kates.

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Scientific American, 234, 38-46 (1976)
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Biochim. Biophys. Acta, 505, 215-278 (1979).

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Date 12/22/81

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Institut de recherches sur les aliments
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December 14, 1981

Your file / Votre référence

Our file / Notre référence

Dr. J. Seelig
Department of Biophysical Chemistry
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Klingelbergstasse 70
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SWITZERLAND

Dear Dr. Seelig:

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Yours sincerely,

B. W. Smallbone

Barry W. Smallbone

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Signed J. Seelig

Date Dec. 21, 1981

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Institut de recherches sur les aliments
Direction de la recherche

December 14, 1981

Your file Votre référence

Our file Notre référence

Dr. P.R. Cullis
Department of Biochemistry
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V6T 1W5

Dear Dr. Cullis:

I am submitting a Ph.D. thesis entitled "Chemical and Physical Studies of Polar Lipids in Extremely Halophilic Bacteria" to the School of Graduate Studies of the University of Ottawa. This thesis has been prepared under the supervision of Professor M. Kates.

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Biochim. Biophys. Acta, 559, 399-420 (1979)

If you agree, please sign below and return this letter to me. Many thanks for your cooperation.

Yours sincerely,

B. W. Smallbone

Barry W. Smallbone

.....

I agree to permit B.W. Smallbone to reproduce the above-mentioned material in his Ph.D. thesis entitled: "Chemical and Physical Studies of Polar Lipids in Extremely Halophilic Bacteria"

Signed _____ (P.R. Cullis)

Date Dec. 30/81

Agriculture
Canada
Food Research Institute
Research Branch
OTTAWA, Ontario K1A 0C6

Institut de recherches sur les aliments
Direction de la recherche

December 14, 1981

Your file Votre référence

Our file Notre référence

Dr. N. Shaw
Department of Organic Chemistry
University of Newcastle upon Tyne
Newcastle, ENGLAND

Dear Dr. Shaw:

I am submitting a Ph.D. thesis entitled "Chemical and Physical Studies of Polar Lipids in Extremely Halophilic Bacteria" to the School of Graduate Studies of the University of Ottawa. This thesis has been prepared under the supervision of Professor M. Kates.

I request your permission to use Figure 1 from the paper(s) entitled:

"Bacterial Glycolipids"
Bacteriological Reviews, 34, 365-377 (1970)

If you agree, please sign below and return this letter to me. Many thanks for your cooperation.

Yours sincerely,

B. W. Smallbone

Barry W. Smallbone

.....

I agree to permit B.W. Smallbone to reproduce the above-mentioned material * in his Ph.D. thesis entitled: "Chemical and Physical Studies of Polar Lipids in Extremely Halophilic Bacteria"

Signed _____

Date 6/1/82

* and any other relevant material from other of my publications