STUDIES ON THE STRUCTURE AND METABOLISM OF THE GLYCOLIPIDS AND GLYCOLIPID SULFATE IN THE EXTREME HALOPHILE *HALOBACTERIUM CUTIRUBRUM*

by

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To the memory

of my parents

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SUMMARY

Part One of this thesis reports studies on the chemical structure of a major membrane lipid component of the extreme halophile Halobacterium cutirubrum. This sulfur-containing glycolipid, accounted for ca. 25% of the total polar lipids. The ammonium salt of the lipid was found to have the molecular formula $C_{61}H_{117}O_{21}S{\cdot}NH_4$, and on strong acid hydrolysis it yielded 2,3-di-O-phytanyl-sn-glycerol, glucose, mannose, galactose and sulfate in equimolar proportions. Infrared and NMR spectra indicated the presence of a secondary sulfate group. Solvolysis of the lipid in 0.004 M HCl in tetrahydrofuran resulted in rapid release of inorganic sulfate and formation of galactosyl-mannosyl-glucosyl diphytanyl glycerol ether. With higher acid concentration (0.25 M methanolic HCl), stepwise hydrolysis of monosaccharide units occurred, giving mannosyl-glucosyl diphytanyl glycerol ether and glucosyl diphytanyl glycerol ether. The position of attachment of the sugars and of the sulfate group was determined by methylation of the free acid form of the glycolipid sulfate, followed by acid hydrolysis and gas-liquid chromatographic analysis of the partially methylated sugars as the alditol acetates. The configurations of the glycosidic linkages were established both by optical rotation measurements and by specific enzymatic hydrolysis. The results obtained established the structure as 2,3-di-O-phytanyl-1-O-[β-D-galactopyranosyl-3'-sulfate-(1'→6')-O-α-D-mannopyranosyl-(1'→2')-O-α-D-glucopyranosyl]-sn-glycerol.

Part Two of this thesis describes in vivo studies on the metabolism of the glycolipids and other lipid components of H. cutirubrum

utilizing [^{35}S]sulfate, [^{14}C]glycerol and [^{14}C]glucose as labelled precursors. These studies showed that the glycolipid sulfate is probably formed by the stepwise addition of monosaccharide residues to preformed 2,3-di-O-phytanyl-sn-glycerol. The resulting triglycosyl diphytanyl glycerol is then probably sulfated by an enzymatic reaction involving 3'-phosphoadenosine-5'-phosphosulfate ("active sulfate"). Also, turnover studies were carried out on cells in which all lipid moieties had previously been labelled with ^{14}C or ^{35}S. These studies indicated that no turnover of the major polar lipid components (phosphatidyl glycerophosphate, glycolipid sulfate) or of the minor components (phosphatidyl glycerol, phosphatidyl glycerosulfate) occurs in Halobacterium cutirubrum. This finding rules out the possibility 1) of any enzymatic hydrolysis of sulfate esters, glycosyl residues or glycerophosphoryl residues; 2) of enzymatic transfer of any of these residues from one lipid to another; 3) of enzymatic cleavage of the ether bonds and oxidative degradation of the alkyl (phytanyl) chains released. Generally, the results indicate that any lipids required by the cell during the growth cycle must be synthesized de novo rather than by the metabolism of lipids already existing within the cell.

RESUME

La première partie de cette thèse est consacrée à l'étude de la structure chimique d'un composé lipidique majeur de la membrane d'un halophile extrême, Halobacterium cutirubrum. Ce glycolipide sulfuré représente environ 25% du total des lipides polaires. Nous avons trouvé que le sel d'ammonium du lipide avait la formule brute $C_{61}H_{117}O_{21}S \cdot NH_4$ et que, par hydrolyse en milieu fortement acide, il produisait du 2,3-di-O-phytanyle-sn-glycérol, du glucose, du mannose, du galactose et du sulfate en proportions équimoléculaires. Les spectres de r.m.n. et infrarouge ont confirmé la présence d'un groupement sulfate secondaire. Par solvolyse du lipide en milieu tétrahydrofurane 0,004 M en HCl nous avons constaté la libération de sulfate et la formation de l'éther 2,3-di-O-phytanyle du glycérol portant un diholoside galactosyl-mannosyl-glucosyl en position 1. En utilisant une plus forte concentration d'acide (0.25 M dans le méthanol), nous avons observé une hydrolyse progressive des unités de monoholosides avec formation de l'éther 2,3-di-O-phytanyle du glycérol portant un diholoside mannosyl-glucosyl en position 1, et de l'éther 2,3-di-O-phytanyle du glycérol portant un monoholoside glucosyl en position 1. Nous avons determiné les positions de liaison des sucres et du groupement sulfate par méthylation de la forme acide du sulfate du glycolipide suivie d'une hydrolyse en milieu acide et d'une analyse par chromatographie en phase gazeuze des sucres partiellement méthylés sous la forme d'acétates d'alditols. Les configurations des liaisons glucidiques ont été établies par des mesures de pouvoir rotatoires, et des études d'hydrolyse enzymatique spécifiques.

Ces résultats ont établi la structure suivante: 2,3-di-O-phytanyle-1-O-[β-D-galactopyranosyl-3'-sulfate-(1'→6')-O-α-D-mannopyranosyl-(1'→2')-O-α-D-glucopyranosyl]-sn-glycérol.

La deuxième partie de cette thèse traite des études in vivo du métabolisme des glycolipides et autre composés lipidique d'Halobacterium cutirubrum en utilisant du sulfate[^{35}S], du glycérol [^{14}C] et du glucose[^{14}C] comme précurseurs marqués. Ces études ont montré que le sulfate de glycolipide est probablement formé par l'addition successive d'unités de monoholoside au 2,3-di-O-phytanyle-sn-glycérol. Ce triglycosyle diphytanyle glycérol est en suite probablement sulfaté par une réaction enzymatique qui met en jeu le 3'-phosphoadénosine-5'-phosphosulfate. Des études de turnover ont été faites sur des cellules dans lesquelles tous les constituants lipidiques avaient préalablement été marqués au ^{14}C ou ^{35}S. Ces études ont montré qu'il n'y avait pas de turnover des composés lipidiques majeurs polaires (le sulfate de glycolipide et le phosphatidylglycérophosphate) ou des composés mineurs (le phosphatidylglycérol et le phosphatidylglycérosulfate) d'Halobacterium cutirubrum. Ce résultat exclut les possibilités suivantes:

- une hydrolyse enzymatique des esters sulfates des unités de glucides, ou des unités glycérophosphorylées
- un transfert enzymatique d'une de ces unités d'un lipide à un autre lipide
- une coupure enzymatique des liaisons éther suivie d'une dégradation par oxydation des chaînes d'alkyle (phytanyle) qui sont libérées.

En général les résultats indiquent que tout lipide requis par la cellule au cours de son cycle de croissance doit être synthetisé de novo plutôt que par le métabolisme des lipides qui sont déjà présents dans la cellule.

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PART ONE

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LIST OF ABBREVIATIONS

ADP	Adenosine-5'-diphosphate
AMP	Adenosine-5'-monophosphate
APS	Adenosine-5'-phosphosulfate
ATP	Adenosine-5'-triphosphate
CDP	Cytidine-5'-diphosphate
CoA	Coenzyme A
CTP	Cytidine-5'-triphosphate
DGD*	Diglycosyl diphytanyl glycerol
DHAP	Dihydroxyacetone phosphate
ECNSS	Ethylene glycol succinate silicone
GDP	Guanosine diphosphate
GLC	Gas liquid chromatography
GLS*	Glycolipid sulfate
MGD*	Monoglycosyl diphytanyl glycerol
NMR	Nuclear magnetic resonance
PA*	Phosphatidic acid
PAP	3'-Phosphoadenosine-5'-phosphate
PAPS	3'-Phosphoadenosine-5'-phosphosulfate
PG*	Phosphatidyl glycerol
PGP*	Phosphatidyl glycerophosphate
PGS*	Phosphatidyl glycerosulfate
POPOP	2,2'-p-Phenylene-bis(5-phenyloxazole)
PPO	2,5-Diphenyloxazole
TGD*	Triglycosyl diphytanyl glycerol
TLC	Thin layer chromatography
UDP	Urldine diphosphate

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

GENERAL INTRODUCTION

The study of organisms capable of thriving in extreme environments has on many occasions presented an insight into the functions of the various cellular components. Since the chemical structures of these components can be determined, it is possible to correlate a cell's chemical composition with its ability to adapt to variations in environmental temperature, pH, light intensity, salinity, pressure, or combinations thereof. The ability of extremely halophilic bacteria, e.g. Halobacterium cutirubrum to grow on salted fish and to flourish in natural salt concentrates such as the "Dead Sea" and Owen's Lake in California makes it an extremely interesting species to study.

Work in this laboratory has led to the discovery of lipid components having structural features unique to extreme halophiles. H. cutirubrum contains a large proportion of very polar phospholipids and sulfolipids but no nitrogenous lipids. These polar lipids contain long, branched-chain alkyl groups linked to glycerol via ether linkages as opposed to the ester-linked acyl groups found in most other organisms. The polar lipids have been shown to be derivaties of 2,3-di-O-phytanyl-sn-glycerol rather than of the 1,2-diacyl-sn-glycerol found in most other organisms.

Structural determinations of the components of H. cutirubrum have allowed the determination of those properties necessary for the organism to thrive in media containing 25% sodium chloride. In addition to the acidity of the polar lipids, the proteins of this organism are unusually acidic due to their containing high mole ratios of aspartic

and glutamic acid (Kushner et al., 1964). At neutral pH, the main envelope components, lipid and protein, are negatively charged. The dependence of cells and envelopes on cations for stability is probably due to the presence of these negatively charged groups; the sodium and potassium ions presumably act as counterions to shield the mutually repulsive negative charges of the membrane constituents (Kushner et al., 1964). The characteristics of halophilic bacteria and the chemistry of the H. cutirubrum polar lipids will be discussed in more detail in the next section.

I. Halophilic Bacteria: A Review

1. Classification

Halophilic bacteria are arbitrarily defined as those requiring a minimum of 0.5 M sodium chloride for survival and growth. They may be divided into two classes (Baxter and Gibbons, 1956):

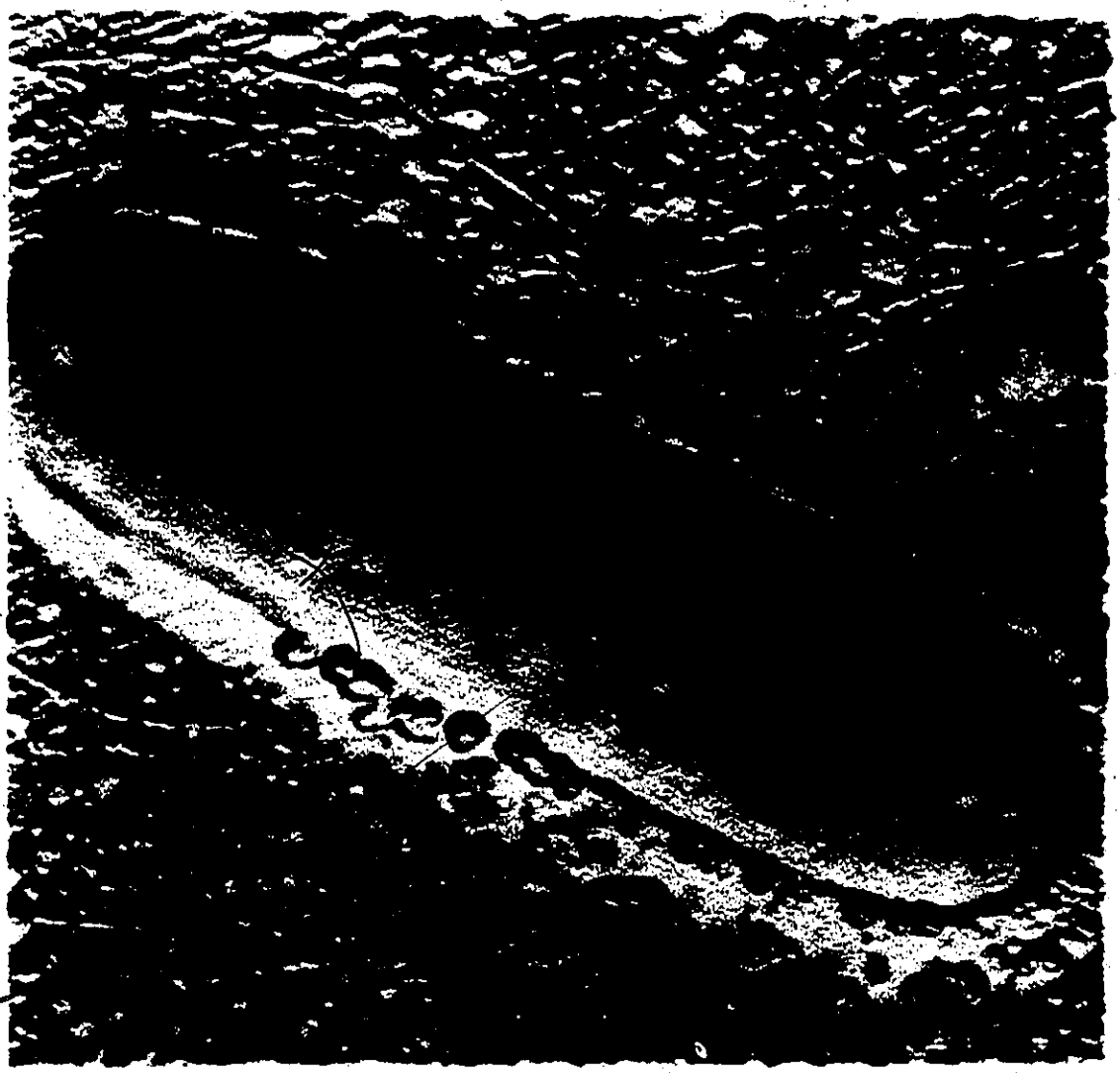
(i) moderate halophiles which grow in media containing 0.5 - 3.5 M sodium chloride

(ii) extreme halophiles which require at least 2.5 M sodium chloride and which grow optimally in ca. 4 M sodium chloride (i.e. a nearly saturated solution).

The genus Halobacterium contains five species, namely H. cutirubrum, H. halobium, H. salinarium, H. marismortui and H. trapanicum (Bergey's Manual, 1957). These bacilli are all Gram-negative, non-spore-forming obligate aerobes which when motile are liphotrichously flagellated (Figure 1). Optimal growth occurs in media containing about 25% sodium chloride; the minimum sodium chloride concentration required for growth is about 15% (2.5 M).

Figure 1

Electron micrograph of the extreme halophile, Halobacterium cutirubrum (x51,000). The replica was prepared by J. Y. D'Aoust, who kindly supplied the photograph.



In addition to the Halobacteria there also exist coccoid halophiles requiring at least 12% sodium chloride (2 M) for growth; their optimum growth is at a concentration of about 20% sodium chloride (3.2 M). Three such species have been assigned to the genera Micrococcus and Sarcina of the family Micrococcaceae: M. morrhuae, S. littoralis and S. morrhuae. These organisms are non-motile, non-spore forming obligate aerobes.

A characteristic of all extreme halophiles is their slow growth, even under optimum conditions. Larsen (1967) reported a generation time for Halobacteria of about 7 h, and for Halococci about 15 h. The complex medium of Sehgal and Gibbons (1960) supports good growth and Onishi et al. (1965) have developed a chemically-defined medium.

2. Salt Requirements

When their saline environment is progressively diluted, extreme halophiles (bacilli) change their rod-like form to a spherical structure and finally lyze completely and irreversibly in 1 - 1.5 M salt (Abram and Gibbons, 1961). The requirements of the rod-form for sodium ion appears to be specific. Replacement of sodium ion by either potassium or ammonium ions results in loss of the rod structure (Kushner et al., 1964). However, sodium, potassium or lithium ions are equally effective in maintaining the integrity of mechanically prepared envelopes, whose dissolution begins at 3 M sodium chloride and is almost complete at 1 M salt. Thus the specific requirement of the intact cell for sodium ion suggests that the role of this ion is not solely osmotic.

Soo-Hoo and Brown (1967) showed that the protective effect of monovalent cations on the morphological integrity of H. halobium decreased in the same order as their hydrated volumes (lithium > sodium > potassium > ammonium) and concluded that the salt concentration effect is a combination of electrostatic and osmotic factors. In accord with these observations, Kushner et al. (1964) and Soo-Hoo and Brown (1967) found 0.5 M magnesium ion to be more effective than 1 M sodium ion in preventing lysis of H. cutirubrum cells.

3. The Cell Envelope Structure

Brown and Shorey (1963) suggested that cells of H. halobium were bounded only by a triple layered plasma membrane; however, later work showed that the cell envelope consisted of a wall and a plasma membrane (Cho et al., 1967; Stoeckenius and Rowen, 1967). The cell envelope of the extremely halophilic bacteria differs distinctly from that of other Gram negative bacteria, in not possessing muramic acid and diaminopimelic acid. Analysis showed that the halophile envelope was largely lipoprotein containing more than 93% of the lipids of the whole cell (Kushner et al., 1964).

The cell envelope requires a high concentration of monovalent ions (or a lower concentration of divalent ions) to prevent electrostatic disaggregation (Brown, 1963; Kushner et al., 1964). The charges responsible for this disaggregation include the negative charges of the carboxyl groups of aspartic and glutamic acids which are present in unusually high proportions in the membrane proteins, as well as the negative charges of the phosphate and sulfate groups of the acidic lipid components.

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

(i) a soluble colorless, lipid-free fraction containing protein and hexosamines, which was considered to be the cell wall;

(ii) a red fraction containing 40% lipid and 60% protein, presumably formed from cell membrane disaggregation;

(iii) a purple fraction consisting of large membrane sheets of similar composition to (ii);

(iv) "intracytoplasmic membrane fraction" (collapsed gas vacuoles), largely protein in nature.

These workers found that fractions (i) and (ii) contained a high proportion of dicarboxylic amino acids in agreement with the view that disruption of the cell envelope upon removal of salt is due to high charge density at the envelope surface.

The color of fraction (iii) has been shown to be due to a complex of retinal and an opsin-like protein (Oesterhelt and Stoeckenius, 1971; Blaurock and Stoeckenius, 1971); the similarity of this complex to the visual pigment-containing membranes of the vertebrate eye suggested a possible photo-receptor function (Danon and Stoeckenius, 1973; Oesterhelt and Stoeckenius, 1973). The protein-retinal complex was appropriately named bacteriorhodopsin; the possible function of this complex will be discussed later in this thesis (Part One, Results and Discussion, VIII. 3; Part Two, Results and Discussion, III).

II. Chemistry of the Membrane Lipids

1. Total Cellular Lipids of Halobacterium cutirubrum

The total lipids (polar lipids plus pigments) in H. cutirubrum account for about 2.5 - 4% of the cell dry weight (Sehgal et al., 1962; Kates et al., 1965; Joo et al., 1968). Acetone precipitation of the total lipids yields an insoluble "polar" lipid fraction (90% by weight) and an acetone-soluble "non-polar" lipid fraction (10% by weight).

The non-polar lipid components are numerous (Kushwaha, Gochnauer, Kushner and Kates, 1974); they include the major pigment bacterioruberin, a red-colored tetrahydroxy C_{50} linear carotenoid (Kelly et al., 1970), as well as traces of the C_{40} carotenoids phytoene, cis- and trans-phytofluenes, neo-α-carotene, β-carotene and neo-β-carotene (Kushwaha et al., 1972) and the colorless hydrocarbons squalene, dihydrosqualene, and tetrahydrosqualene (Tornabene et al., 1969; Kramer et al., 1972).

2. The Polar Lipids of Halobacterium cutirubrum

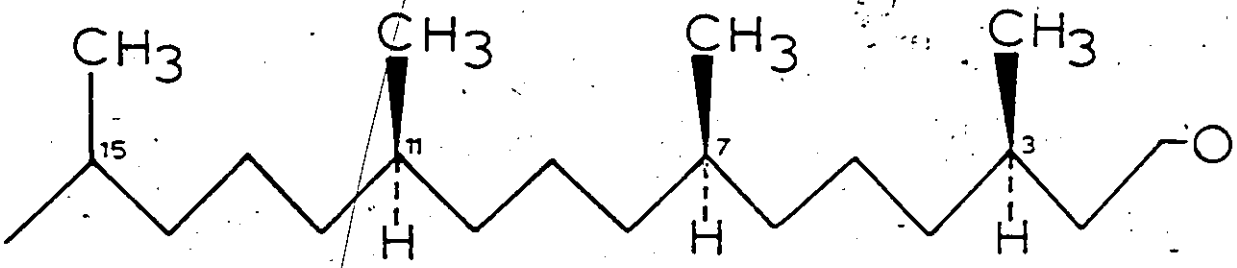
The polar (acetone-insoluble) lipids were recognized as atypical of Gram negative bacteria in three respects:

(i) the low nitrogen content (0.18%, N/P atomic ratio 0.09; Sehgal et al., 1962) indicated the presence of only traces of nitrogenous lipids;

(ii) analysis for phosphorus (P = 4.3 - 4.6%) indicated an unusually high lipid P content;

(iii) neither mild alkaline hydrolysis (Dawson, 1954) nor drastic hydrolysis of the lipids, released fatty acids,

showing that the lipids were not derivatives of diacyl glycerols typically found in bacteria. Instead, hydrolysis gave 65 - 75% of a non-saponifiable material which was shown to be an asymmetrically substituted di-O-alkyl glycerol (Kates et al., 1965; Joo et al., 1968; Faure et al., 1963). Both alkyl groups were found to be 3,7,11, 15-tetramethylhexadecyl- (or phytanyl) groups (Kates et al., 1965). Each of the three asymmetric centers of the phytanyl groups (C-3, C-7 and C-11) was shown to have the R-absolute configuration (Kates et al., 1967). Thus, the bacterial phytanyl group has the 3R,7R,11R,15-tetramethylhexadecyl structure and configuration:



As will be described below, the dialkyl glycerol proved to be 2,3-di-O-phytanyl-sn-glycerol.

Chromatography of the polar lipids revealed the presence of two major components and four or five minor components (Figures 2 and 3)(Sehgal et al., 1962; Kates et al., 1966). All of the components characterized were found to be derivatives of 2,3-di-O-phytanyl-sn-glycerol.

The major component (Spot 6, Figure 2) was an acidic phosphatide which accounted for about 85% of the lipid phosphorus and about 60% of the weight of the polar lipids. Elemental analysis and degradative hydrolysis studies on the lipid indicated a phosphatidyl glycerophosphate structure in which ester-linked acyl groups were replaced by ether-linked branched chain (phytanyl) groups (Structure I, Figure 4). However, the data were not entirely unambiguous; the sodium and potassium salts isolated had a cation to phosphorus atomic ratio of 1:1, and only one ionizable acid group per atom of phosphorus (i.e. two acid groups per molecule of PGP) could be detected by NaOH titration with phenolphthalein indicator, although three acid groups per molecule of PGP were required. It was for this reason that Faure et al. (1963), who had also arrived essentially at a PGP structure for this lipid, concluded that the phosphatide was a dimer in which two molecules of PGP were condensed to give a pyrophosphate structure (Structure II, Figure 4). The chemical synthesis of the monomeric Structure I yielded a lipid identical with the natural substance (Joo and Kates, 1969). Furthermore, its stereochemical structure was shown by this synthesis to be 1-sn-phosphatidyl-3'-sn-glycero-1'-phosphate (Structure IV, Figure 5), in which the configuration of both glycerol moieties is opposite to that of diacyl-PGP found in other bacteria (Slotboom and Bonsen, 1970).

Figure 2

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.

Identification of components:

Spot 1	unidentified sulfoglycolipid
Spot 2	glycolipid sulfate (GLS)*
Spot 3	unidentified phosphatide
Spot 4	glycolipid (TGD)*
Spot 5	phosphatidyl glycerosulfate (PGS)
Spots 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.

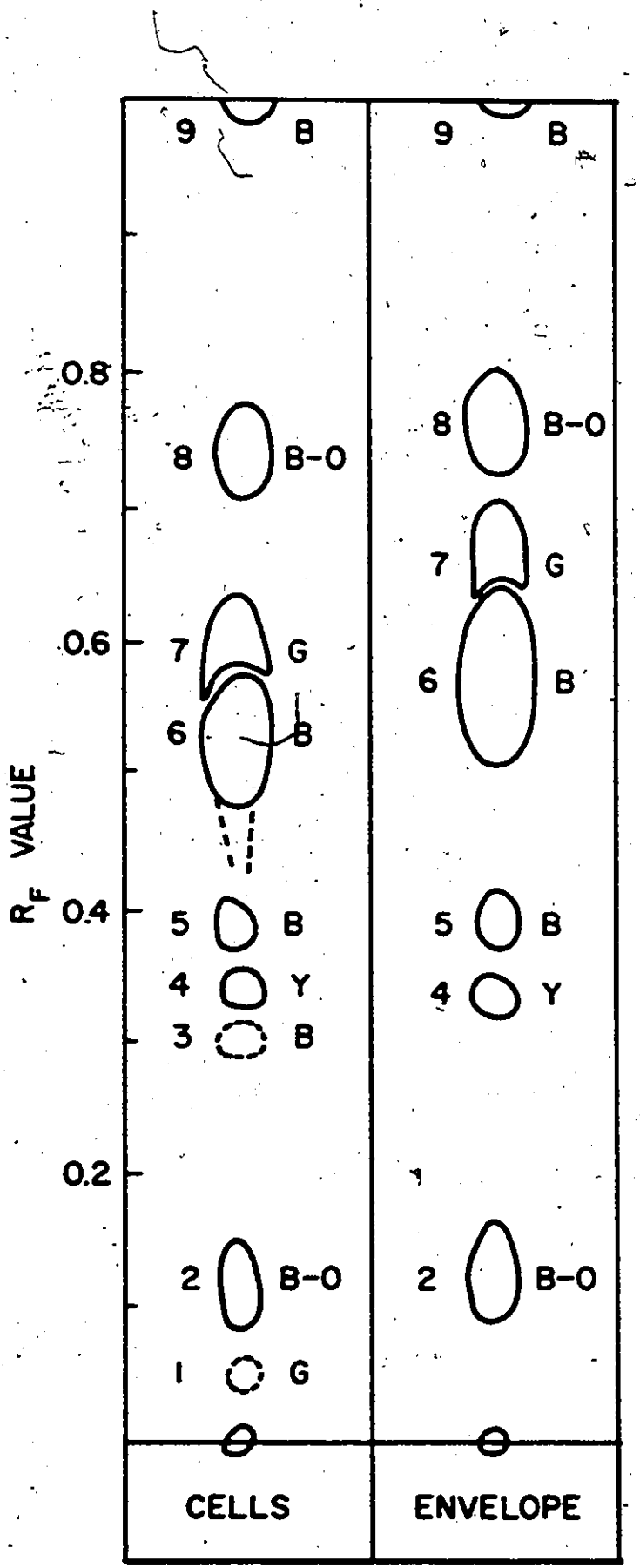

9 B
8 B-O
7 G
6 B
5 B
4 Y
3 B
2 B-O
1 G
0.8
0.6
0.4
0.2
R_F VALUE
CELLS
ENVELOPE

Figure 3

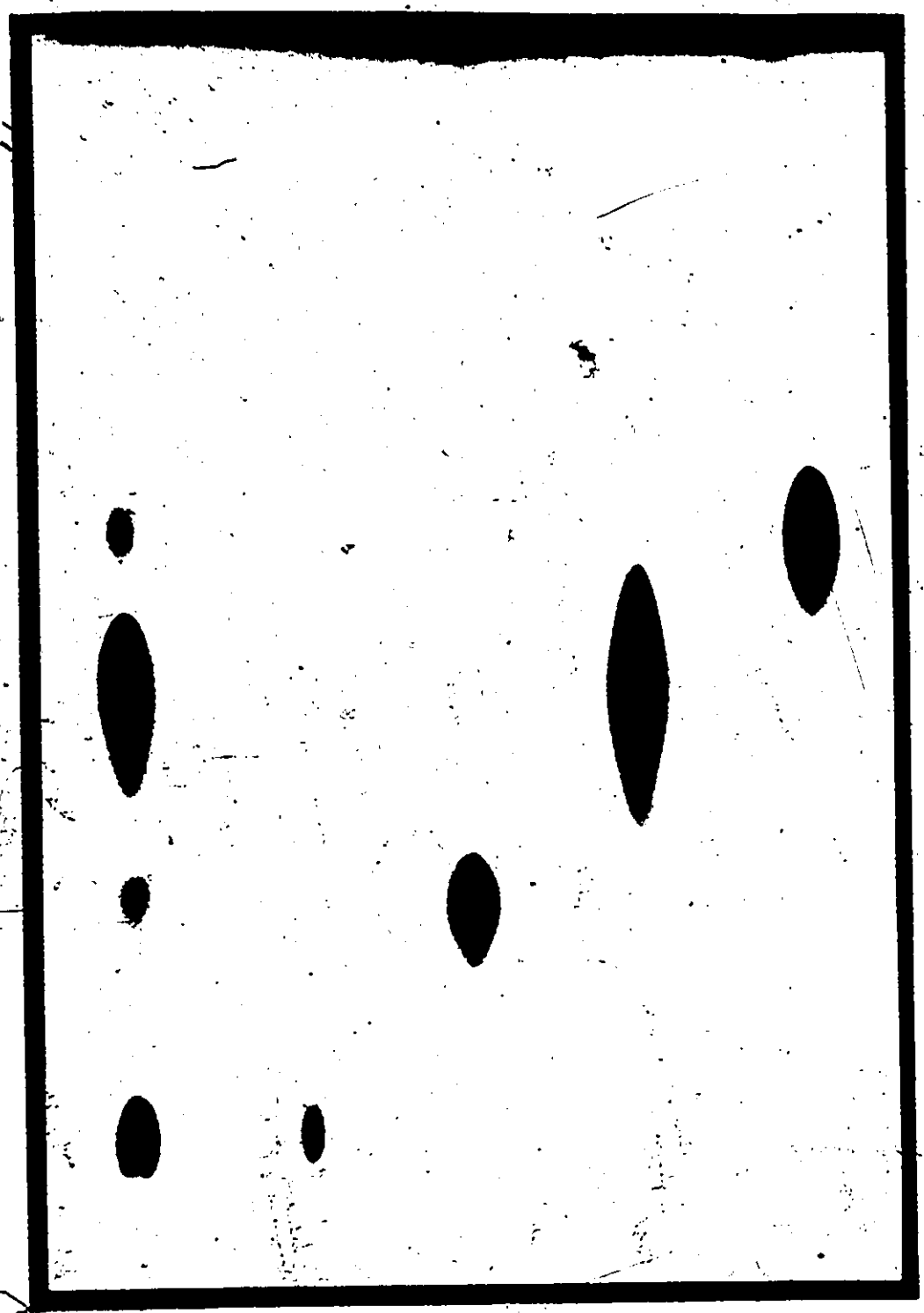
Thin layer chromatogram of the total polar (acetone-insoluble) lipids and purified lipid components from H. cutirubrum.

Identification of components:

TL	total lipids
1	glycolipid sulfate (GLS)
2	phosphatidyl glycerosulfate (PGS)
3	phosphatidyl glycerophosphate (PGP)
4	phosphatidyl glycerol (PG)

Solvent System:

Chloroform-90% acetic acid-methanol (30:20:4, v/v/v). After development, the plate was sprayed with 40% sulfuric acid and charred.



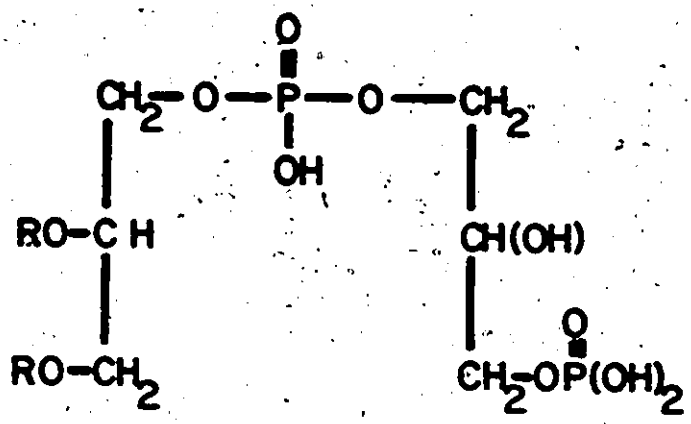
TL 1 2 3 4

Figure 4

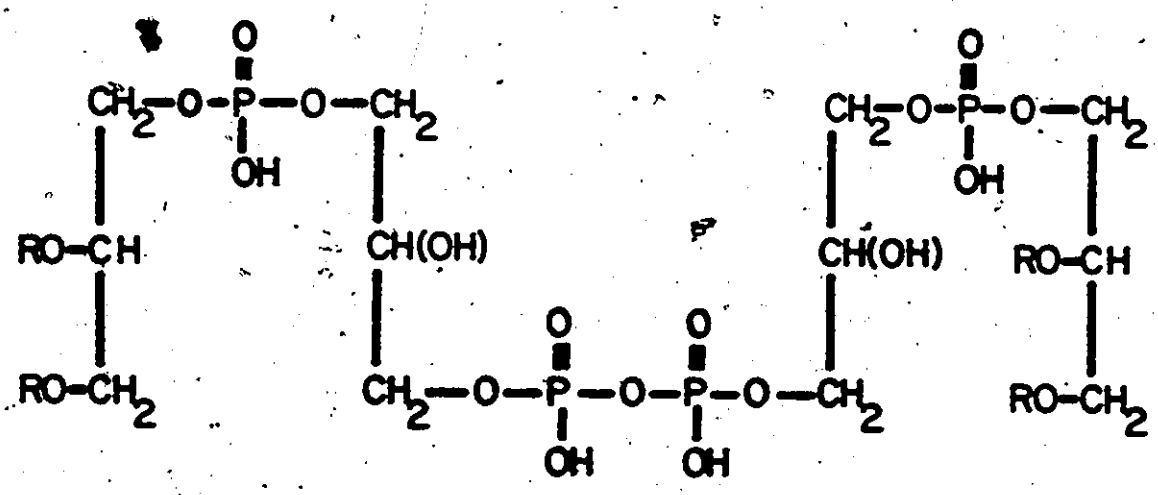
Structures proposed for the phosphatidyl glycerophosphate component in H. cutirubrum:

I - Monomeric structure proposed by Kates et al. (1963)

II - Dimeric pyrophosphate structure proposed by Faure et al. (1963).



Structure I



Structure II

Recent NMR studies (Hancock, 1972; Kates and Hancock, 1971) of permethylated PGP and PGP trimethyl ester and studies on the various salt forms of PGP have established conclusively that there are three ionizable P-O-groups for every two phosphorus atoms and therefore that the structure is monomeric (1-sn-phosphatidyl-3'-sn-glycerol-1'-phosphate).

The minor components include three phospholipids (Spots 3, 5 and 8, Figure 2). One of the phospholipids (Spot 8, Figure 2), accounting for ca. 5% of the lipid phosphorus, has been isolated and identified as the diphytanyl ether analog of phosphatidyl glycerol (Faure et al., 1964; Kates et al., 1966). Joo and Kates (1968, 1969) confirmed this structure and established the stereochemical structure as 1-sn-phosphatidyl-3'-sn-glycerol (Structure III, Figure 5) by chemical synthesis. Thus, as in PGP, the two glycerol moieties in this phospholipid have configurations opposite to those of the glycerol moieties in the diacyl-PG in other bacteria and in plants (Slotboom and Bonsen, 1970).

A second minor phosphatide (Spot 5, Figure 2) was found to account for ca. 6% of the lipid phosphorus. Preliminary studies suggested the presence of both phosphorus and sulfur in this lipid (Kates et al., 1968). Through both degradative studies and chemical synthesis the structure of this compound was shown to be the sulfate analog of PGP, namely 1-sn-phosphatidyl-3'-sn-glycerol-1'-sulfate (Hancock, 1972; Hancock and Kates, 1972, 1973). The novel structure of this phosphosulfolipid led Hancock (1972) to speculate that this lipid might be an intermediate in the biosynthesis of the glycolipid sulfate.

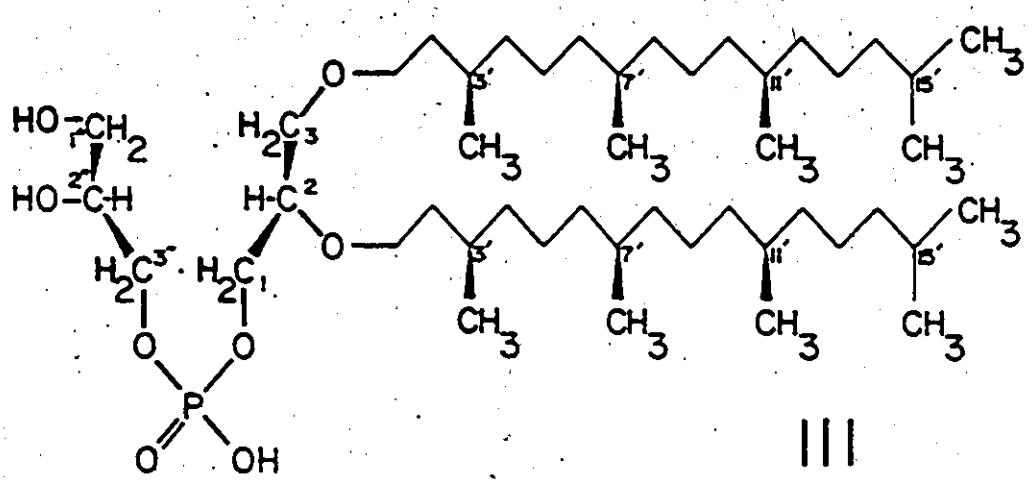
Figure 5

Projection formulae (using stereospecific numbering system according to Hirschmann, 1960) of:

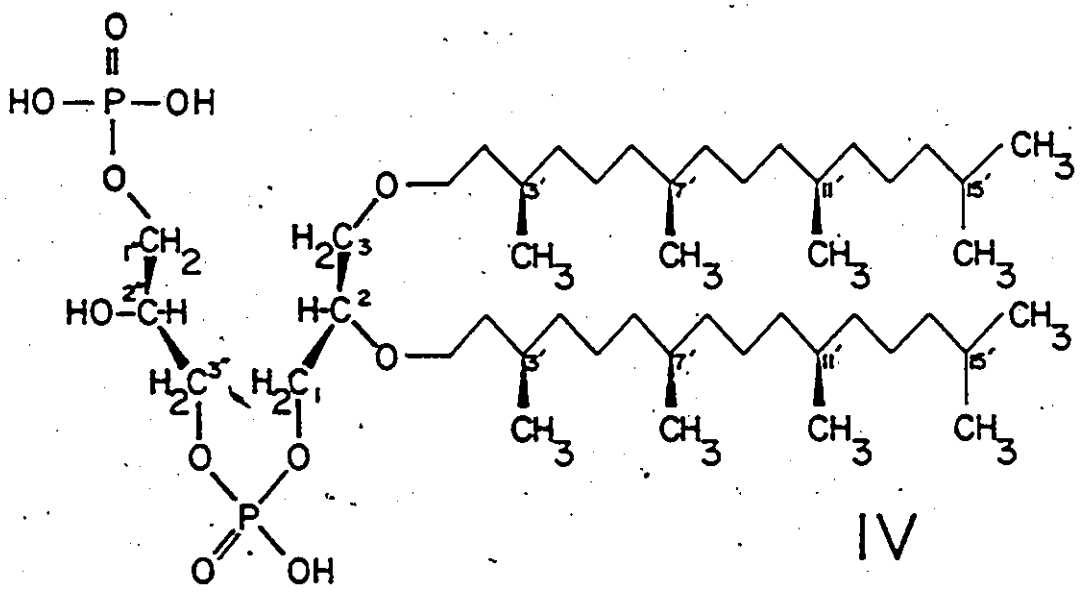
III - 2,3,-Di-O-(3'R,7'R,11'R,15'-tetramethylhexadecyl)-sn-glycero-1-phosphoryl-3"-sn-glycerol[1-sn-phosphatidyl-sn-3'-glycerol; PG]

IV - 2,3-Di-O-(3'R,7'R,11'R,15'-tetramethylhexadecyl)-sn-glycero-1-phosphoryl-3"-sn-glycero-1"-phosphoric acid[1-sn-phosphatidyl-sn-3'-glycero-1'-phosphoric acid; PGP]

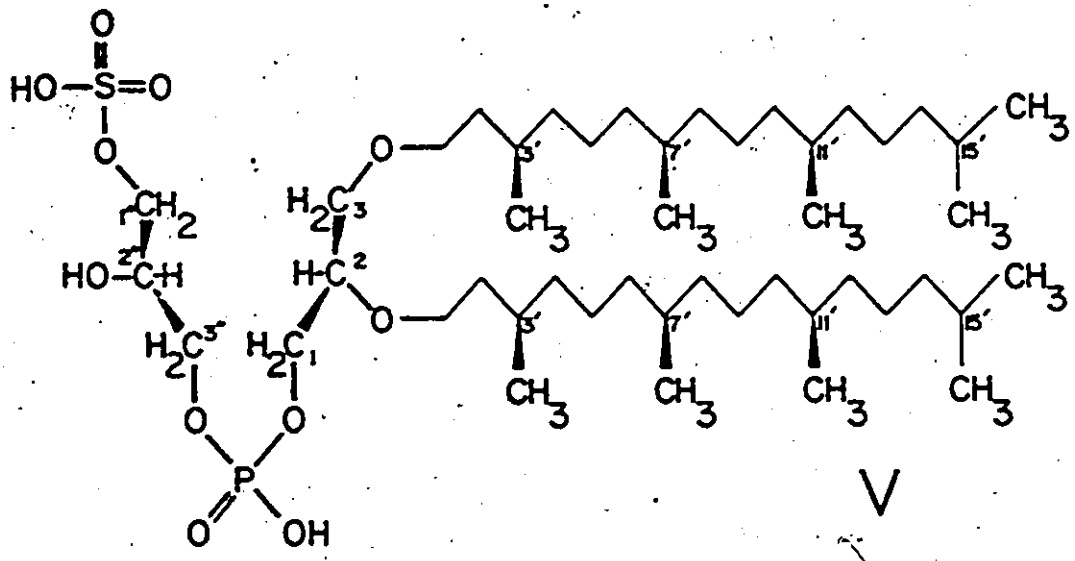
V - 2,3-Di-O-(3'R,7'R,11'R,15'-tetramethylhexadecyl)-sn-glycero-1-phosphoryl-3"-sn-glycero-1"-sulfuric acid[1-sn-phosphatidyl-sn-3'-glycero-1'-sulfuric acid; PGS].



III



IV



V

This point will be discussed in more detail in a later section (Part Two, Results and Discussion, II).

A third minor phospholipid (Spot 3, Figure 2) accounting for less than 1% of the lipid phosphorus has not yet been isolated in sufficient quantities for structural studies.

Three glycolipids (Spots 1, 2 and 4, Figure 2) are found among the total lipids of H. cutirubrum. The main carbohydrate-containing lipid (Spot 2, Figure 2) accounts for ca. 25% by weight of the total acetone-insoluble lipids. Preliminary studies (Kates et al., 1967) had shown that the compound probably contained one mole each of sulfate, diphytanyl glycerol, glucose, galactose and mannose. From the high positive optical rotation of the compound, the authors concluded that the glycosidic linkages were all in the α-configuration. The complete structure determination of this glycolipid sulfate forms a major part of this thesis.

Of the remaining glycolipids, one of them (Spot 4, Figure 2) proved to be the desulfated glycolipid sulfate, the structure elucidation of which also forms part of this thesis. The other (Spot 1, Figure 2) contains both sugar and sulfate but has not yet been isolated in sufficient quantities for further study.

The above-described major diphytanyl ether components have been found in all extremely halophilic bacteria studied, but not in the moderate, or non-halophilic bacteria examined (Kates et al., 1966; Marshall and Brown, 1968).

III. Biosynthesis of Membrane Ether Lipids in Halobacterium cutirubrum

1. In Vivo Incorporation of Labelled Precursors

Because of the unusual structures of the lipids of H. cutirubrum, the pathways for the biosynthesis of these lipids were of considerable interest. Features to be considered were 1) the biosynthesis of the phytanyl chains; 2) the biosynthesis of the phytanyl ether linkages with glycerol with the formation of 2,3-di-O-phytanyl-sn-glycerol; 3) the biosynthesis of phospholipid and glycolipid derivatives of the diphytanyl glycerol.

Early studies by Kates et al. (1968) showed that H. cutirubrum cells readily incorporated several radioisotopically-labelled precursors. [^{14}C]Mevalonate showed the highest degree of incorporation (ca. 8% of the label added) followed by [1(3)-^{14}C]glycerol (2.0%) and [1-^{14}C]acetate (1.9%). Degradative studies of the labelled lipids showed that the label derived from acetate and mevalonate was almost entirely associated with the phytanyl groups, but that derived from glycerol was associated with water-soluble groups as well as phytanyl groups.

These findings suggested that the predominant biosynthetic route for the phytanyl chains was the mevalonate pathway, although the individual steps in the pathway have yet to be elucidated with cell-free or purified enzyme systems. In particular, the reduction steps by which the fully saturated phytanyl chain is formed from the unsaturated dimethylallyl pyrophosphate intermediate remain to be established. Certainly, the reduction mechanism must be stereospecific in order to

generate the D configuration (R absolute) at the C-3, C-7 and C-11 asymmetric centers of the phytanyl chain (Kates et al., 1967). A tentative pathway for the biosynthesis of the phytanyl chain is given in Scheme 1.

The fact that the sugar and glycerol moieties of the lipids are labelled by [1(3)-^{14}C]glycerol suggests that glycerol is able to enter the glycolysis cycle (Kates, 1972). Wassef et al. (1970) showed the existence of a glycerol kinase in H. cutirubrum cell-free homogenates which catalyzes the formation of sn-3-glycerophosphate from glycerol and ATP and also demonstrated specific sn-3-glycerophosphate dehydrogenase activity in cell-free homogenates. These results suggest that glycerol is incorporated into the phytanyl groups and the water-soluble moieties by the pathway shown in Scheme 2.

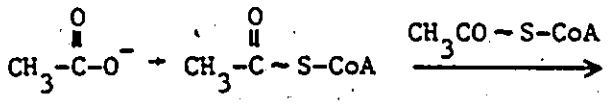
Studies utilizing ^{3}H-^{14}C-labelled glycerol provided further evidence for this pathway (Wassef et al., 1970). Incorporation experiments were performed using both [1(3)-^{3}H]glycerol and [2-^{3}H]glycerol, mixed with known proportions of [1(3)-^{14}C]glycerol. ^{3}H/^{14}C ratios in both the phytanyl groups and sugar moieties agreed well with those calculated for these moities formed by the mevalonate pathway and the glycolysis cycle respectively.

Information concerning the origin of the glycerol moiety in the diphytanyl ether was also obtained in these experiments. The glycerol moiety in the synthesized lipid retained 100% of ^{3}H from [1(3)-^{3}H] glycerol but complete loss of ^{3}H occurred with [2-^{3}H]glycerol. Thus, dehydrogenation at C-2 but not at C-1 (or C-3) must occur in the glycerol moiety. This eliminates involvement of aldo-keto isomerizations such as

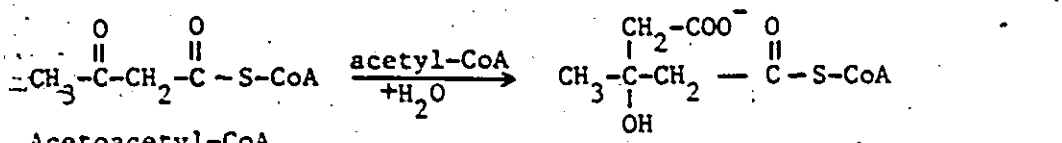
Scheme 1

A tentative pathway for the biosynthesis of the phytanyl chain

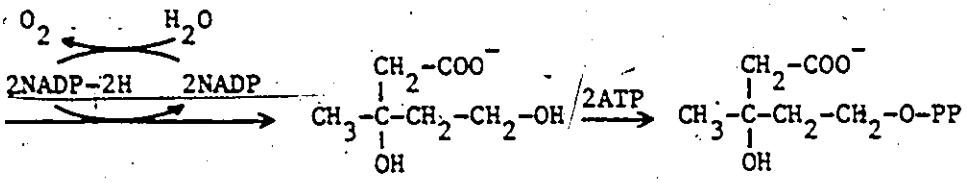
(Wassef, 1969)



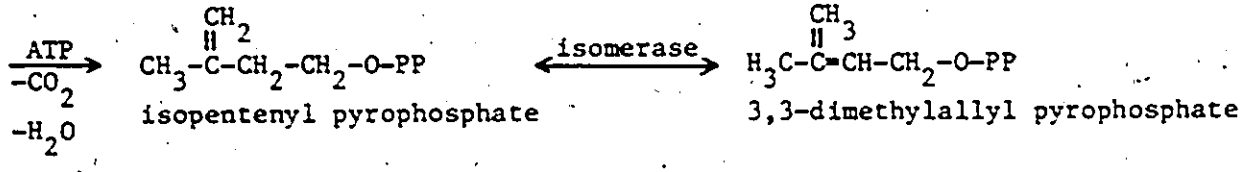
Acetate Acetyl-CoA



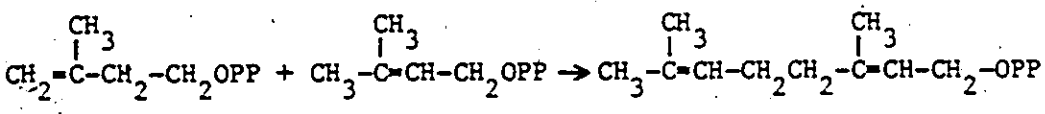
Acetoacetyl-CoA β-hydroxy-β-methyl-glutaryl-CoA



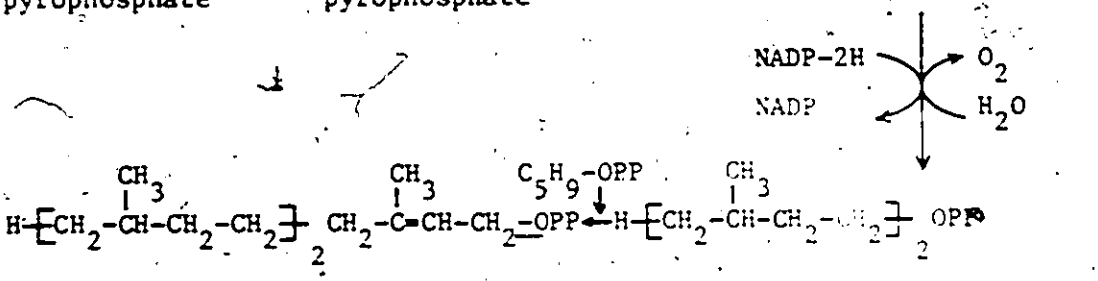
mevalonate 5-pyrophosphomevalonate



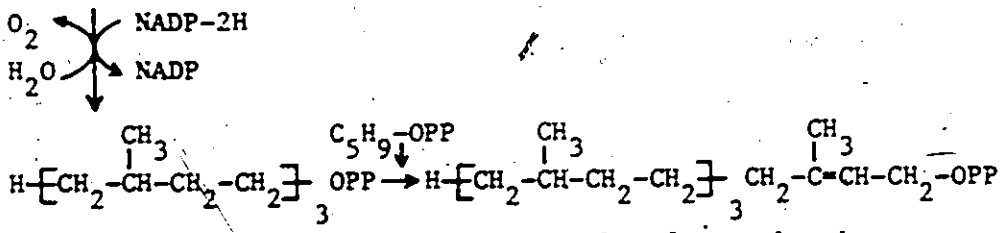
isopentenyl pyrophosphate 3,3-dimethylallyl pyrophosphate



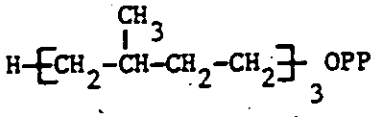
isopentenyl pyrophosphate dimethylallyl pyrophosphate geranyl pyrophosphate



tetrahydrofarnesyl pyrophosphate tetrahydrogeranylpyrophosphate



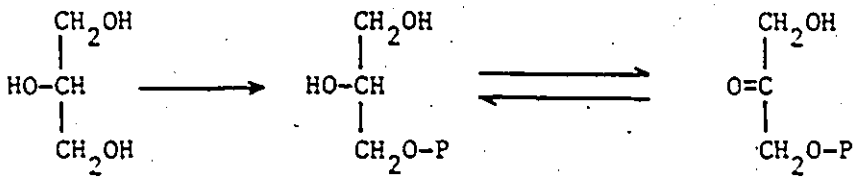
hexahydrofarnesyl pyrophosphate phytyl pyrophosphate



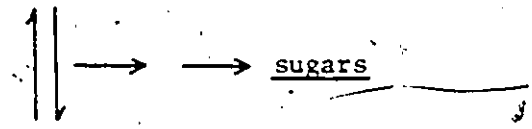
phytanyl pyrophosphate

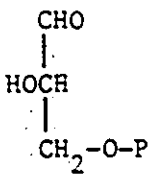
Scheme 2

A tentative pathway for the incorporation of glycerol into the phytanyl groups and into the water soluble moieties of H. cutirubrum lipids (Wassef, 1969; Wassef et al., 1970; Kates, 1972)

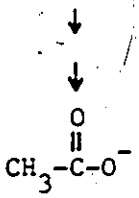


Glycerol sn-3-glycerophosphate dihydroxyacetone phosphate





glyceraldehyde phosphate



acetate ← ← phytanyl groups

that between dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate. Unless DHAP exists in a physically separate pool where it cannot equilibrate with glyceraldehyde-3-phosphate, it does not appear to act as precursor of the glycerol moiety of the glycerol ether, as it does for the monoalkyl glycerol ethers in other organisms (Snyder et al., 1970). A novel biosynthetic pathway must therefore exist for the glycerol diphytanyl ether; this pathway must also explain the fact that the configuration at C-2 in the glycerol moiety is opposite to that in natural diglycerides.

With regard to the phosphate and sulfate moieties of the polar lipids, studies with [^{32}P]orthophosphate and [^{35}S]sulfate have shown that a ready incorporation of the label occurs provided the concentration of unlabelled phosphate and sulfate in the medium is reduced (Kates et al., 1968). For cells harvested in the early stationary phase, the incorporation of ^{32}P largely paralleled the distribution of phosphorus among the lipid components, i.e. PGP accounted for 77% of the total ^{32}P incorporated, PG 8% and PGS 11%. When [^{35}S]sulfate was used as precursor, 80% of the isotope incorporated into the lipids appeared in the glycolipid sulfate component and about 15% was associated with the unidentified glycolipid sulfate component. The remaining 5% of radioisotope was associated with phosphatidyl glycerosulfate.

2. Existence of the Malonyl-CoA Pathway for Fatty Acid Biosynthesis in H. cutirubrum

Although [^{14}C]acetate is incorporated almost entirely into the phytanyl groups, low incorporation into fatty acids (<0.3% of the

total incorporation) was also observed (Kates et al., 1968). The presence of a fatty acid synthetase system could be demonstrated in a cell-free preparation from H. cutirubrum cells (Pugh et al., 1971) but its activity was considerably inhibited at the high salt concentration (4 M) of the normal growth medium. The role of the fatty acids produced in such low amounts has yet to be determined.

IV. A Review of Naturally Occurring Sulfolipids

Since the main carbohydrate-containing lipid of H. cutirubrum has been shown to contain sugar residues as well as sulfur, it is of interest to consider other naturally occurring compounds of similar structure. Haines (1971, 1973) has written comprehensive reviews on the chemistry of sulfolipids.

Of the sulfur-containing glycolipids characterized to date, all except one, the plant sulfonolipid (Benson, 1963), have been found to contain the sulfur as a sulfate residue. A second feature, common to four of these sulfoglycolipids, is that the sulfate is esterified to the C-3 hydroxyl of a β-linked galactose even though these lipids are found in very different species (e.g. bacteria, mammals, etc.). Some structural features of these molecules are summarized in Table 1.

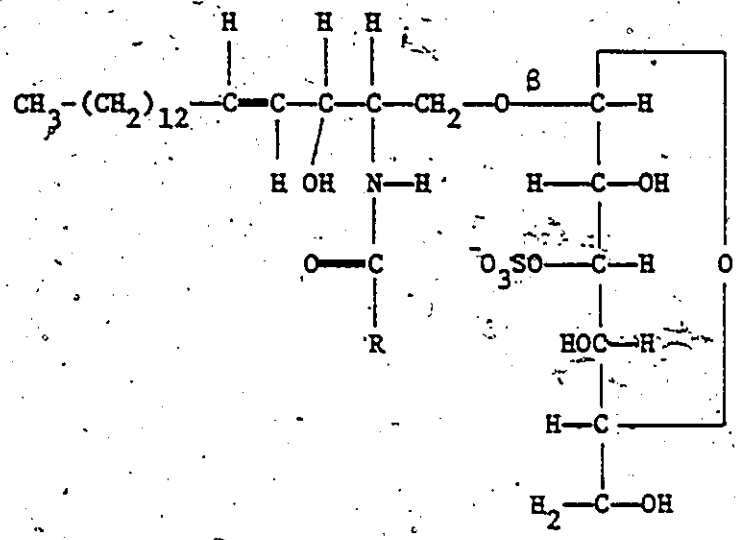
1. Cerebroside Sulfate

This compound was probably the first glycolipid sulfate detected, dating back to the work of Thudicum (1874) who found the compound in brain tissue. It was not until nearly a century later that the structure was established as a ceramide galactosyl-3-sulfate (Stoffyn and Stoffyn, 1963).

Table 1. Sulfated sugars, linkage configurations and sulfation positions of naturally occurring glycolipid sulfates*

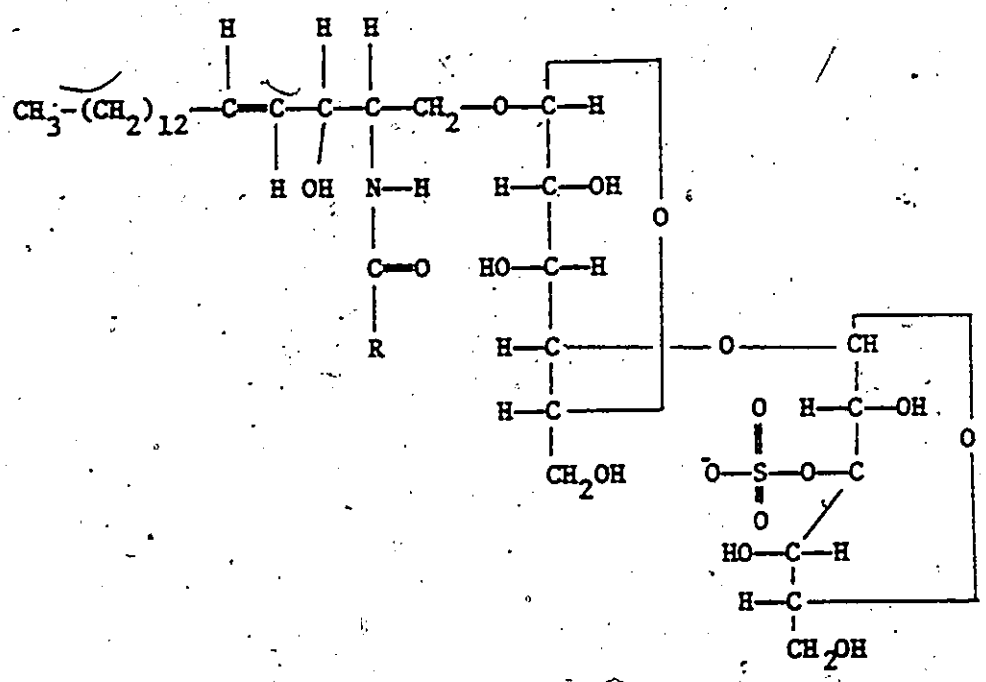
Lipid	Source	Sulfated Sugar	Linkage Configuration	Sulfation Position	Reference
Glycolipid sulfate	Halobacterium cutirubrum	Galactose	β	3	Kates and Deroo (1973)
Cerebroside sulfate	Human brain	Galactose	β	3	Stoffyn (1966)
Lactosyl ceramide sulfate	Human kidney	Galactose	β	3	Martensson (1966)
Seminolipid	Boar testes	Galactose	β	3	Ishizuka et al. (1973)
Acylated trehalose sulfate	Mycobacterium tuberculosis	Glucose	α	2	Goren (1971)
Plant sulfonolipid	Plants	Quinovose	α	6	Benson (1963)

*Note: The last lipid in the table is a sulfonate rather than a sulfate.



2. Lactosyl Ceramide Sulfate

Lactosyl ceramide sulfate was first isolated from human kidney by Martensson in 1963. Several years later (1966) the same worker established that its structure was analogous to that of cerebroside sulfate, differing only from the latter in that it has an extra sugar moiety (glucose) between the β-linked terminal galactose-3-sulfate and the ceramide portion of the molecule.

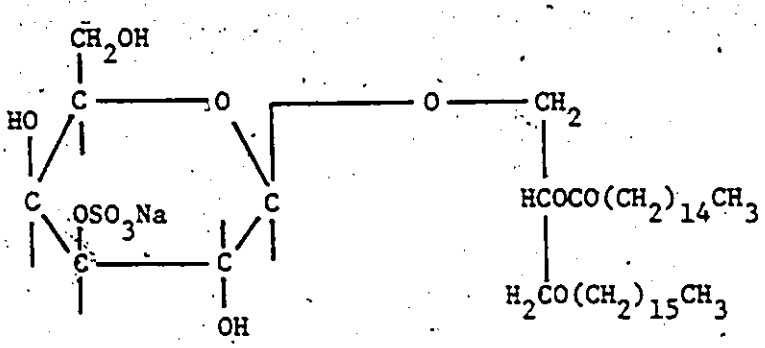


3. Ganglioside Sulfate

In 1967, a new complex sulfated glycolipid was reported to have been found in human epidermis (Nieminen et al., 1967). The same compound was later found to be present in the walls of horses' hooves where it accounted for ca. 50% of the total polar lipids (Leikola et al., 1969). Acid hydrolysis of the sulfatide yielded equimolar amounts of ceramide, galactosamine, galactose, sialic acid and sulfate. The presence of sialic acid led the authors to suggest that the sulfatide was a sulfated ganglioside. However, it has not been shown conclusively that the lipid is not a mixture of cerebroside sulfate and gangliosides. Hydrolysis of such a mixture would in fact yield the same hydrolysis products reported above.

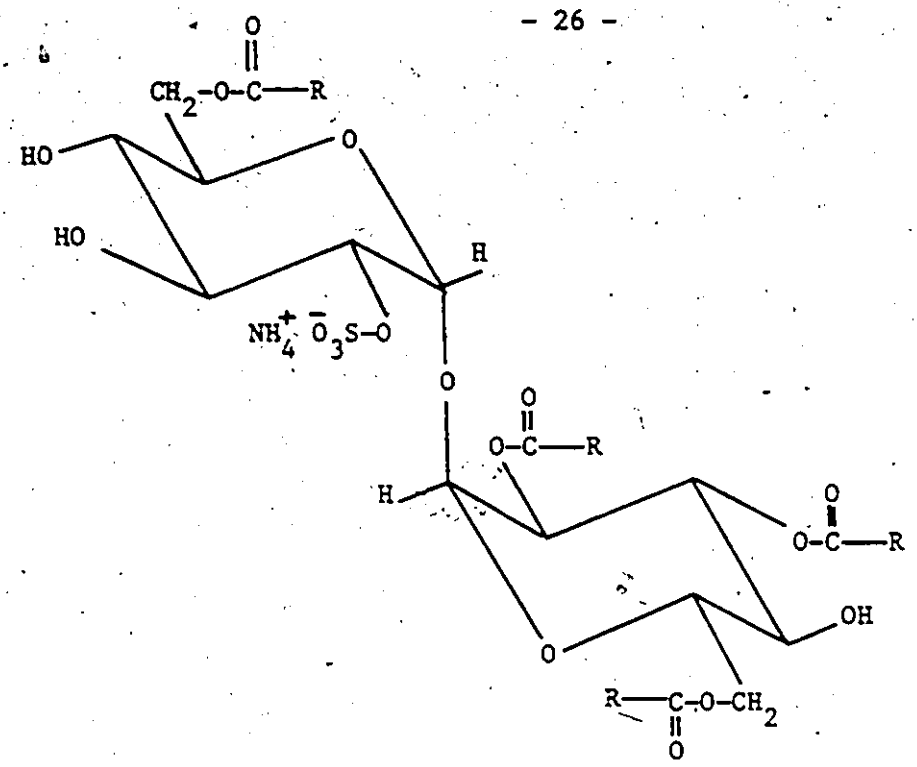
4. Seminolipid

Recently, Ishizuka, Suzuki and Yamakawa (1973) described the isolation and characteristion of a novel sulfoglycolipid from boar testis and spermatozoa. This compound, appropriately designated "seminolipid", accounted for ca. 3% of the total lipids. It was found to contain equimolar amounts of fatty acid, galactose, glycerol and sulfate. The molecule was also found to contain a hexadecyl group linked via an ether bond to the glycerol of the molecule. The final structure of the compound is given below. This lipid and the glycolipid sulfate described in this thesis are the only two ether-containing sulfoglycolipids characterized to date.



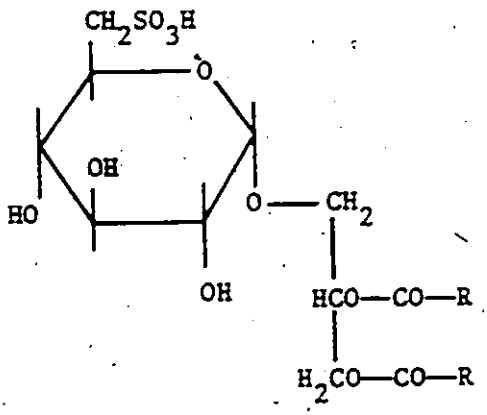
5. Acylated Trehalose Sulfate

Goren (1970a, b) isolated a homologous series of sulfoglycolipids from a virulent human strain of Mycobacterium tuberculosis, $H_{37}Rv$. The main component of the series had a molecular weight of ca. 2400 and contained two moles of glucose, one mole of sulfate and four acyl groups. The carbohydrate moiety of the lipid was shown to be α,α-trehalose. The sulfate group was shown to be esterified at a single 2-position of the trehalose. The acyl groups have been identified as palmitic acid, a multi-methyl-branched acid of mean molecular weight 550 and a hydroxylated derivative of the methyl-branched acid. The acyl groups appear to be esterified to the 3', 3', 6' and 6 hydroxyls of the trehalose. However, the specific point of attachment of any one type of acyl group is not yet established. The proposed structure for the compound is given below.



6. Plant Sulfonolipid

To date, only one sulfolipid has been characterrzed in which the sulfur is present as a sulfonate rather than as a sulfate group. This lipid was first reported to be present in algae by Benson (1959). Several years later the lipid was shown to be the 6-sulfonate of quinovosyl diglyceride Benson (1963). The structure is given below.



7. Alkanediol Sulfates

Characterization of the sulfolipids isolated from algae led to the discovery of a class of sulfolipids heretofore unknown in biological systems. This class is composed of sulfate esters of long chain alcohols (Haines, 1971, 1973). Mayers and Haines (1967) characterized 1-(S)-14-docosane-diol-1,14-disulfate from the phytoflagellate Ochromonas danica. In addition, polyhaloalkyl disulfates containing up to six chlorine atoms per molecule have been detected (Elovson and Vagelos, 1970).

8. Biosynthetic Pathways for Glycolipids and Glycolipid Sulfates

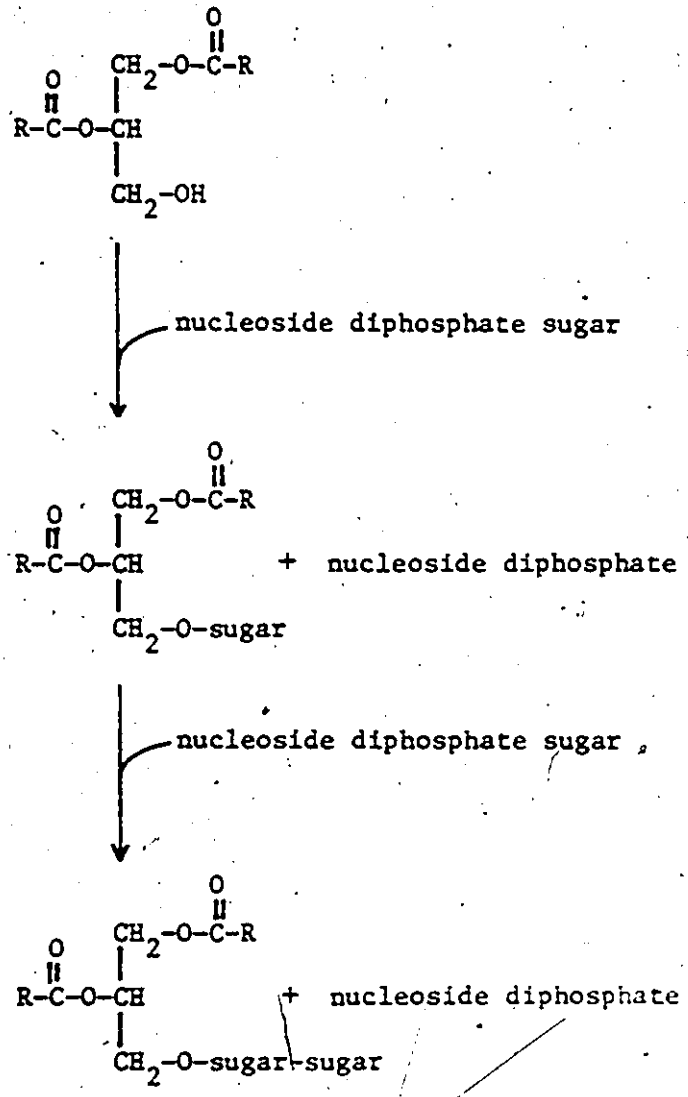
Structurally, the glycolipids and glycolipid sulfate from Halobacterium cutirubrum resemble that class of glycolipids known as glycosyl diglycerides. To be sure, there is a difference, namely the presence of 2,3-di-O-phytanyl-sn-glycerol versus the 1,2-diacyl-sn-glycerol found in glycosyl diglycerides. However, the overall similarity of the H. cutirubrum glycolipids to the glycosyl diglycerides warrants the following brief review of the biosynthesis of latter group of compounds.

(a) Biosynthesis of Glycosyl Diglycerides

The biosynthesis of glycosyl diglycerides has been studied for a number of years and consequently, the reactions involved have been reasonably well elucidated (Scheme 3). Generally, an activated sugar (nuclecside diphosphate sugar) reacts with a hydroxyl group of a receptor molecule such as a diacyl glycerol (Kaufman et al., 1965; Pieringer, 1968); the reaction is catalyzed by a specific glycosyl

Scheme 3

Pathway for the biosynthesis of glycosyl diglycerides (Pieringer, 1968)


nucleoside diphosphate sugar
+ nucleoside diphosphate
nucleoside diphosphate sugar
+ nucleoside diphosphate

transferase. The products of the reaction are a glycosyl diglyceride and a nucleoside diphosphate. If necessary, one of the sugar hydroxyl groups can then act as a acceptor for a second sugar residue (donated by a second nucleoside diphosphate sugar which may be either identical to or different from the first) to form a diglycosyl diglyceride. Although the reaction usually terminates here, triglycosyl diglycerides are also known and have been isolated from some species (Shaw and Baddiley, 1968).

As might be expected, the glycosyl transferases are highly specific with respect to both the substrates utilized and the product formed. The enzyme will accept only a specific nucleoside diphosphate sugar and is also specific for a particular stereoisomer of the lipid (diglyceride) to be glycosylated (Pieringer, 1968). Likewise, the product of the reaction is specific with respect to both the stereochemistry of the glycerol portion of the molecule and the configuration of the glycosidic linkage produced. Similarily, if a second sugar is added to form a diglycosyl diglyceride, the enzyme involved is specific for the sugar to be added, the position of attachment to the first sugar and the configuration of the glycosidic linkage formed.

It might also be noted that an organism is not necessarily limited to the direct utilization of sugars available to the cell for glycolipid biosynthesis. Many species have been found to contain enzymes capable of interconverting nucleoside diphosphate sugars. This occurs through the action of an epimerase which inverts the configuration of a specific hydroxyl group on the sugar ring of the nucleoside diphosphate sugar. In this way, UDP-glucose may be converted to UDP-galactose by a UDP-glucose-4-epimerase which inverts the configuration

of the hydroxyl group on carbon 4 of the sugar ring. The modified nucleoside diphosphate sugar may then be utilized for the biosynthesis of a glycolipid requiring the new sugar (Pieringer, 1968).

(b) Sulfation Pathways

In those systems so far studied (Austin et al., 1969), sulfation of a hydroxyl group occurs via a reaction between this group and 3'-phosphoadenosine-5'-phosphosulfate ("active sulfate", PAPS) catalyzed by sulfotransferase; in the case of glycolipid sulfates, such enzymes would catalyze specifically the transfer of sulfate specifically to one hydroxyl group in the sugar ring (Scheme 4) (Austin et al., 1969).

9. Degradative Pathways for Glycolipids

As much as has been published about the degradation of glycolipids as about their biosynthesis. While many of the studies have been on the in vivo degradation, many of the same pathways have also been demonstrated in vitro. There are several distinct routes by which glycolipids of the glycosyl diglyceride type may be metabolized. These include removal of the acyl group(s) by lipases, cleavage of the glycosidic linkage(s) by glycosidases or even replacement of the acyl group(s) by acyl hydrolase-associated acyl transferases. These three pathways will be discussed briefly in the following sections.

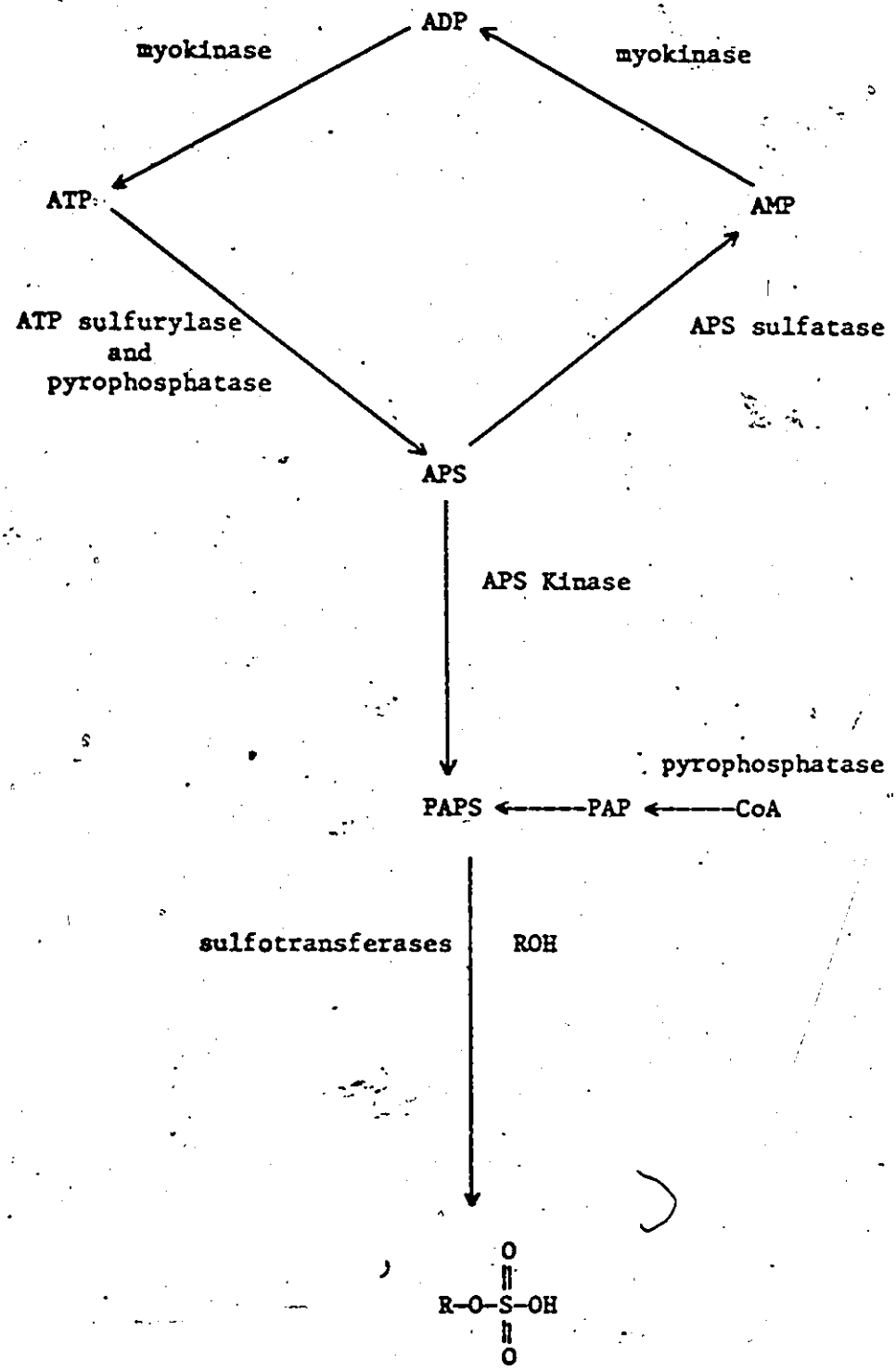
(a) Lipases

Galactolipase activity has been observed in the leaves of the runner bean (Phaseolus multiflorus) by Sastry and Kates (1964, 1969).

Scheme 4

Pathway for the bio-sulfation of hydroxyl groups

(Austin et al., 1969)


ADP
myokinase
myokinase
ATP
AMP
ATP sulfurylase
and
pyrophosphatase
APS sulfatase
APS
APS Kinase
pyrophosphatase
PAPS
PAP
CoA
sulfotransferases
ROH
O
R–O–S–OH
O

On the basis of their data the authors concluded that the monogalactosyl diglyceride and digalactosyl diglyceride were hydrolyzed by two distinct enzymes as shown below:

monogalactosyl diglyceride → monogalactosyl monoglyceride →
+ free fatty acid

monogalactosyl glycerol
+ free fatty acid

digalactosyl diglyceride → digalactosyl monoglyceride →
+ free fatty acid

digalactosyl glycerol
+ free fatty acid

Helmsing (1967) has obtained similar results with enzyme preparations from spinach leaves.

(b) Glycosidases

Further hydrolysis of the galactosyl glycerols formed in the above reactions has been shown to be catalyzed by α- and β-galactosidases (Sastry and Kates, 1964).

digalactosyl glycerol $\xrightarrow{\alpha\text{-galactosidase}}$ monogalactosyl glycerol + galactose

$\xrightarrow{\beta\text{-galactosidase}}$ glycerol + galactose

More recent studies by Short and White (1970) showed that there was a rapid loss of [^{14}C]glucose from the mono- and diglucosyl diglycerides of Staphylococcus aureus when pre-labelled cells were resuspended in "cold" medium. However, there was no loss of ^{14}C from

the fatty acids of these lipids during the same chase experiment. This would seem to indicate that enzymes (glycosidases) of S. aureus are capable of removing sugar residues from glycosyl diglycerides without the prior removal of the acyl groups.

(c) Acyl Transferases

Galliard (1970, 1971) observed galactolipid-acyl hydrolase activity in a particle-free supernatant fraction from potato tubers; this hydrolase activity was associated with acyl transferase activity. Monogalactosyl diglyceride was particularly susceptible to hydrolysis and monogalactosyl monoglyceride was detected as an intermediate product.

Recently, Heinz et al. (1974) showed that spinach leaves contain enzymes capable of forming 6-O-acyl mono- and digalactosyl diglycerides. They demonstrated that digalactosyl diglyceride serves not only as an acyl donor for acyl transfer to monogalactosyl diglyceride, but can itself function as an acyl acceptor.

V. Aims of the Research

Lipids play a major role in the structural integrity and function of biological membranes (Chapman , 1968). Cells dependent on extreme environments for their existence would be expected to have unique membranes and therefore unique membrane components. In the extreme halophile, H. cutirubrum, the membranes contain only two major polar lipids, phosphatidyl glycerol phosphate and the glycolipid sulfate, accounting for ca. 60% and 25%, respectively, of the total polar lipids. The glycolipid sulfate was therefore considered to be an important membrane constituent, either structurally or metabolically, and it was of great interest to elucidate its structure. Analytical and degradative studies were therefore carried out on the intact glycolipid sulfate as well as on the products obtained by mild acid hydrolysis of this lipid viz., triglycosyl-, diglycosyl- and monoglycosyl glycerol diethers. Part One of this thesis deals with these structural studies and establishment of the glycolipid sulfate structure.

While the structure of a membrane component is in itself interesting, it is important to elucidate the pathways by which this component may be synthesized and broken down in the cell. In order to determine the metabolic pathways for the glycolipid sulfate and the other glycolipids, in vivo incorporation experiments and "chase" studies were carried out using radioisotopically-labelled precursors, e.g., [^{35}S]sulfate, [^{14}C]glucose and [^{14}C]glycerol. Part Two of this thesis deals with these metabolic studies.

MATERIALS AND METHODS

I. Materials

1. Solvents and Reagents

All solvents were distilled in glass apparatus, a 10% forerun being discarded.

Anhydrous solvents were prepared as follows:

Chloroform was washed with an equal volume of water to remove ethanol, allowed to stand over anhydrous calcium chloride and distilled from phosphorus pentoxide. The anhydrous chloroform was used immediately after preparation.

Benzene was refluxed over sodium metal for 1 h and distilled.

Methanol was dried by the magnesium ethoxide procedure as described by Vogel (1956).

Pyridine was refluxed over barium monoxide for 1 h and then distilled.

Tetrahydrofuran and diethyl ether were each refluxed over lithium aluminum hydride for 1 h, distilled and used immediately.

Nitromethane was dried over calcium chloride and distilled.

Methyl iodide was washed with aqueous sodium thiosulfate, dried over calcium chloride, distilled and used immediately.

Solvents for spectroscopic measurements were as follows:

Chloroform - Fisher Certified A.C.S. Spectranalyzed.

Carbon tetrachloride - Baker "Instra-analyzed" GC Spectrophotometric Quality.

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

1,8-Dihydroxynaphthalene-3,6-disulfonic acid, disodium salt (Chromotropic acid) - Aldrich Chemical Company.

Barium chloranilate - Aldrich Chemical Company.

Silver oxide - Fisher Scientific Company.

2,5-Diphenyloxazole (PPO) - Liquid Scintillation Counting Grade. J. T. Baker Chemical Co.

2,2'-p-Phenylene-bis(5-phenyloxazole) (POPOP) - Liquid Scintillation Counting Grade. J. T. Baker Chemical Co.

Bio-Solv™ Solubilizer (Formula BBS-3) - Beckman Instruments, Inc.

Silica gel H, silica gel G and kieselguhr G were obtained from Brinkmann Instruments (Canada) Ltd.

Unisil silicic acid (200-325 mesh) was supplied by the Clarkson Chemical Company.

2. Standards, Substrates and Enzymes

Inorganic compounds used as standards for chemical analysis (KH_2PO_4, K_2SO_4) and flame photometry (KCl, NaCl) were all Primary Standard Grade (Fisher Scientific Co.).

D-Glucose - A.C.S. Reagent Grade, Fisher Scientific Co.

D-Galactose - Mann Research Laboratories, Inc.

D-Mannose - Fisher Scientific Co.

The following disaccharides, glycosides and sugar nucleotides were obtained from the Sigma Chemical Company: cellobiose, lactose,

maltose, melibiose, o-nitrophenyl-β-D-galactopyranoside, p-nitrophenyl-α-D-galactopyranoside, GDP-mannose, UDP-galactose, UDP-glucose.

Authentic reference standards of the alditol acetates of 3,4,6-tri-O-methyl-D-glucose, 2,4,6-tri-O-methyl-D-galactose, 2,3,4-tri-O-methyl-D-mannose and 2,3,4,6-tetra-O-methyl-D-glucose were obtained from Dr. G. A. Adams of the National Research Council of Canada.

β-Galactosidase (a lactase preparation from Escherichia coli) was obtained from Worthington Biochemical Corp.

3. Radioactive Precursors

[^{35}S]Sulfate and [^{32}P]phosphate were obtained as the acids dissolved in hydrochloric acid. Both were obtained from Atomic Energy of Canada, Ltd. Before use, the solutions were neutralized to pH 8 with sodium hydroxide and diluted with distilled water to a known concentration. Aliquots were counted to determine the activity.

[U-^{14}C]D-Glucose - Amersham/Searle. Obtained as a sterile aqueous solution containing 3% ethanol. Specific activity 285 mCi/mmol.

[1(3)-^{14}C]Glycerol - Amersham/Searle. Specific activity 32.5 mCi/mmol.

[1(3)-^{3}H]Glycerol - Amersham/Searle. Obtained as an ethanol solution. Specific activity 2.5 Ci/mmol.

Guanosine diphospho[U-^{14}C]D-mannose, ammonium salt - Amersham/Searle. Obtained as an aqueous solution containing 2% ethanol. Specific activity 83 mCi/mmol.

Uridine diphospho[U-^{14}C]D-galactose, ammonium salt - Amersham/Searle. Obtained as an aqueous solution containing 2% ethanol. Specific activity 210 mCi/mmol.

Uridine diphospho[U-^{14}C]D-glucose, ammonium salt - Amersham/Searle. Obtained as an aqueous solution containing 2% ethanol. Specific activity 260 mCi/mmol.

3'-Phosphoadenosine-5'-phospho[^{35}S]sulfate, tetrasodium salt - New England Nuclear. Obtained as a solution in ethanol-water (1:1, v/v). Specific activity 1493 mCi/mmol.

The solutions of the compounds were diluted with distilled water to a known concentration. Aliquots were counted to determine the activity.

II. Culture and Growth of Organism

1. Organism

The organism used throughout this study was Halobacterium cutirubrum NRCC strain 34001, a red-pigmented, extremely halophilic, obligate aerobe. The bacterium was originally isolated, screened and purified by Dr. A. G. Lochhead at the Animal Research Institute, Research Branch, Canada Department of Agriculture, Ottawa.

2. Growth of Organism

Stock cultures were maintained on agar slants at 37°C by adding 2% agar to the standard growth medium (see below).

Broth cultures of H. cutirubrum were grown at 37°C on a Psycrotherm rotary shaker (New Brunswick Scientific Co.) at an oscillation frequency of 120 cycles per minute.

Growth of the cells in broth culture was monitored by turbidity measurements (optical density) at 660 nm on a Coleman Junior Spectrophotometer using the uninoculated medium as a blank.

3. Media

(a) Standard Medium

This is essentially the standard liquid medium described by Sehgal and Gibbons (1960).

The medium contained (g/l): casamino acids (Difco), 7.5; yeast extract (Difco), 10.0; trisodium citrate, 3.0; potassium chloride, 2.0; magnesium sulfate heptahydrate, 20.0; sodium chloride, 250; and, ferrous sulfate heptahydrate, 0.05. The above ingredients were dissolved in 1 liter of distilled water, the pH was adjusted to 6.7 and the medium was autoclaved for 15 min at 121°C (final pH 6.5). The medium was then filtered through a sterile Millipore filter to remove any precipitate. Sehgal and Gibbons (1960) have reported that this precipitate may contain up to 20% of the magnesium and some phosphate; however, a sufficient amount of both ions remains in the medium for optimal growth.

(b) Low Phosphate Medium

This medium differed from the standard medium only with respect to the following: yeast extract, 5 g/l; casamino acids, 3.7 g/l. The concentrations of the other components were the same as in the standard medium.

The low phosphate medium was used for the *in vivo* incorporation experiments so that the $^{32}PO_4^{3-}$ would not be too diluted out by 'cold' PO_4^{3-}.

(c) Low Phosphate, Low Sulfate Medium

This medium was identical to the low phosphate medium except that the magnesium sulfate was replaced by an equimolar amount of magnesium chloride.

This medium was used for the in vivo incorporation of $^{35}SO_4^{2-}$ to avoid dilution of the tracer with 'cold' sulfate.

III. Lipid Extraction Procedures

Cells were harvested in stationary phase by centrifugation at 8000xg in a Sorvall RC2-B centrifuge at 0°C. The cells were washed twice with a solution containing 4 M NaCl, 0.03 M KCl, and 0.1 M $MgSO_4$ (hereafter referred to as "basal salt solution"). After the final centrifugation the cells were resuspended in basal salt solution to a concentration of 20-60 mg of cells (dry weight) per ml.

1. Bligh and Dyer Extraction

The extraction procedure which was used throughout most of the work was based on the method of Bligh and Dyer (1959) as modified by Kates et al. (1965, 1966). To 200 ml of the cell suspension was added 750 ml of methanol-chloroform (2:1, v/v/v) to give a one phase solvent system of chloroform-methanol-water of 1:2:0.8, v/v/v. The suspension was vigorously shaken and left in the dark at room temperature for several hours. After filtration of the suspension through glass wool the solid residue was extracted with 475 ml of a mixture of chloroform-methanol-water (1:2:0.8, v/v/v). The two extracts were combined and diluted with 375 ml of chloroform and 375 ml of water to give a two phase system (final solvent ratio: chloroform-methanol-water, 1.0:1.0:0.9; v/v/v). The mixture was swirled and allowed to stand until the two phases had completely separated (usually overnight). The chloroform phase was drained off and the upper phase was washed with 200 ml chloroform. The combined chloroform extracts were diluted with benzene and taken to

dryness on a rotary evaporator at 30°C. The residue was dissolved in chloroform-benzene (2:1, v/v) and the solution of lipids was cleared by centrifugation and stored at -20°C.

2. Isopropanol Extraction (Kates and Eberhardt, 1957)

Hot isopropanol is known to inactivate lipid degrading enzymes, so that lipids obtained by this method should be free of lipid degradation products. The procedure was carried out as a control to determine whether any enzymatic breakdown of lipids occurred during the Bligh and Dyer extraction.

Wet cells (20 g) were suspended in 20 ml of the basal salt solution. This suspension was poured into 40 ml of boiling isopropanol with stirring. After 1-2 min, the hot homogenate was centrifuged and the cellular residue was washed twice with 20 ml of hot isopropanol and then extracted with 20 ml of chloroform-isopropanol (1:1, v/v); the extracts were combined. The cellular residue was re-extracted with 19 ml of chloroform-methanol-water (1.0:2.0:0.8, v/v/v). This extract was diluted with 5 ml chloroform and 5 ml water, mixed on a Vortex mixer and centrifuged. The chloroform phase was removed, and combined with the isopropanol extracts. The combined extracts were concentrated on a rotary evaporator and the lipid residue was taken up in chloroform. The chloroform solution was washed twice with methanol-water (1.0:0.9, v/v) to remove water-soluble compounds, and then diluted with benzene and taken to dryness in vacuo (30°C). The lipid residue was dissolved in chloroform-benzene (2:1, v/v); the solution was cleared by centrifugation and stored at -20°C.

IV. Chromatography of Lipids

1. Silicic Acid Impregnated Paper Chromatography

The polar lipids of Halobacterium cutirubrum were chromatographed on silicic acid-impregnated Whatman 3MM paper by the method of Marinetti (1962, 1964), using diisobutyl ketone-glacial acetic acid-water (40:25:5, v/v/v) as solvent. The preparation of the impregnated paper and details of the development procedure have been described by Kates (1967, 1972).

The following reagents were used for the detection of lipids on Marinetti chromatograms.

Rhodamine 6G: The stain was introduced by Marinetti and Stotz (1956) and Marinetti (1962, 1964) has described the staining procedure in detail. An aqueous stock solution (0.12%) of Rhodamine 6G (color index 752, National Aniline Division, Allied Chemical Dye Corp., New York), was prepared by dissolving 1.2 g/l of distilled water. In the absence of light the solution was stable indefinitely; it was diluted 1:100 (v/v) with distilled water immediately before use. The developed chromatogram was air-dried for about 30 min and dipped into the stain solution until the spots were evident (usually about 1-3 min). Excess dye was rinsed from the chromatogram with distilled water and the spots were viewed at once under ultraviolet light (366 nm). On the wet chromatogram acidic lipids gave blue or purple fluorescent spots, and neutral species gave yellow or orange spots, whereas on the dried chromatogram all components gave yellow fluorescent spots. The stained areas were outlined in pencil, their color noted and the R_f values of the spots were measured.

Ninhydrin: The reagent, specific for free amino groups, was made by dissolving 250 mg ninhydrin in 100 ml acetone-lutidine (9:1, v/v); it was sprayed on the dry, developed chromatogram which was then kept at room temperature, or heated at 110° in an oven, until the mauve spots appeared. The remaining (ninhydrin-negative) lipid components were located by staining the chromatogram with Rhodamine 6G.

Periodate-Schiff Reagent: This stain was used to detect vicinal diol groups by means of the fuchsin color reaction given by aldehydo-lipids derived from cleavage of the lipid diol. The procedure used was an adaptation to silicic acid-impregnated paper (Sastry and Kates, 1964) of the method devised by Baddiley et al. (1956) for unimpregnated paper. The developed, dried chromatogram was dipped in a 0.25% aqueous solution of sodium metaperiodate, hung for 15-20 min at room temperature and then passed through a 1% aqueous sodium metabisulfite solution until the liberated iodine was completely reduced. A dip in Schiff reagent* revealed lipid components containing vicinal diol groups within minutes as pink-mauve spots on a white background. Glycerol diol lipids (PG) responded considerably more quickly to the periodate-Schiff test than did the glycolipids.

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*The Schiff reagent was prepared as follows: 1 g of basic fuchsin (p-rosaniline) and 10 g of sodium metabisulfite were dissolved in 10 ml of conc. HCl and 100 ml of water; the solution was warmed (60°) with animal charcoal for 1 h, filtered and the colorless filtrate was made up to 500 ml with air-free distilled water. This stock solution was stable for months when kept in a well-stoppered bottle. Before use, it was diluted with one part 1% sodium metabisulfite solution and one part distilled water.

Chlorine-Benzidine Reagent: (Reindel and Hoppe, 1954). This reagent detects those nitrogenous components which can be converted into chloramines. The air-dried chromatogram was exposed for 15 min in a closed jar to an atmosphere of chlorine gas generated by the action of 1.5% potassium permanganate solution on 10% HCl. Excess chlorine was removed from the chromatogram by ventilation in the fume hood (12 h). Immersion of the paper in tolidine reagent* revealed N-chlorinated derivatives (formed from secondary amides) as blue spots on a white background.

2. Thin Layer Chromatography

(a) Analytical TLC

For purity determination 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 H (0.25 mm thickness). Spots were visualized by: (i) spraying with 40% sulfuric acid in ethanol followed by charring, or (ii) spraying with phosphate-detecting reagent of Dittmer and Lester (1964), or (iii) spraying with α-naphthol reagent** for sugars.

(b) Preparative TLC

Preparative thin layer chromatography was carried out on 20 cm x 20 cm plates coated with silica gel H (1 mm thickness). The

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* Tolidine Reagent. 160 mg o-tolidine dissolved in 30 ml acetic acid, the solution diluted with 470 ml water and 1.0 g KI added.

** 0.5% α-naphthol was dissolved in 50 ml methanol and diluted to 100 ml with water. The TLC plate was sprayed with this reagent and then sprayed with conc. sulfuric acid followed by slow heating. Carbohydrate-positive compounds appeared as reddish-purple spots on a white background.

coated plates were air dried, washed once by ascending chromatography in chloroform-methanol (1:1, v/v), air dried and then activated at 110° for 12 h. Solvent systems used for the separation of neutral and polar lipids are given in the appropriate experimental sections (see also Materials and Methods, IV.2.(c)).

For separation of total polar lipids of H. cutirubrum, the plates were streaked with a solution of the lipids (ca. 40 mg/plate) in chloroform-methanol (95:5, v/v) using a "Pelick Streaker" (Applied Science Laboratories, State College, Pa.). The plates were developed twice in pre-equilibrated rectangular jars lined with Whatman 3 MM paper, using chloroform-90% acetic acid-methanol (30:20:4, v/v/v) as solvent. The lipid bands were visualized by spraying the air-dried plates with Rhodamine 6G solution (0.01%) and immediately viewing them under ultraviolet light (366 nm). The elution of the components is described in Part One, Experimental Procedures, I.2.

(c) Solvent Systems for Thin Layer Chromatography of Lipids

The following solvent systems were used for both analytical and preparative TLC of lipids. Hereafter, the systems will be referred to by letter.

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

B, Chloroform-methanol-water (65:25:4, v/v/v)

C, Chloroform-methanol-conc. ammonium hydroxide (65:35:5, v/v/v).

V. Chromatography of Carbohydrates and Their Derivatives.

1. Paper Chromatography

An aliquot containing 100-200 µg of hexose was taken to dryness under a stream of nitrogen. The residue was redissolved in a drop of water, applied to a strip of Whatman No. 1 paper and chromatographed in a descending solvent system of pyridine-ethyl acetate-water (2:5:5, v/v/v; upper phase) for 20 h.

Sugar and polyols were visualized by the alkaline silver nitrate stain of Trevelyan et al. (1950). The chromatogram was first dipped in a solution of silver nitrate* and was then allowed to dry for 5 min. The chromatogram was dipped in a solution of sodium hydroxide** and again allowed to dry. Carbohydrates and polyols (e.g., glycerol) appeared as brownish-grey areas on a light brown background. After color development was complete, the chromatogram was stabilized by dipping it into a 10% solution of sodium thiosulfate. This treatment removed most of the background staining and left greyish-black spots on a light grey background.

2. Thin Layer Chromatography

Hexoses, methyl hexosides, and disaccharides were also chromatographed on thin layer plates coated with kieselguhr G - silica gel G (4:1, w/w). The plates were developed in ethyl acetate-methanol-water (68:23:9, v/v/v) and the carbohydrates were visualized with the α-naphthol reagent (see Materials and Methods, IV.2.(a)).

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* Silver nitrate reagent: 0.7 g silver nitrate was dissolved in 5 ml water and diluted to 200 ml with acetone.

** Sodium hydroxide reagent: 0.8 g sodium hydroxide was dissolved in 10 ml water and the solution was diluted to 200 ml with 95% ethanol.

3. Column Chromatography

Column chromatography of organic solvent-soluble carbohydrate derivatives was carried out on Unisil silicic acid activated at 120° for 12 h using at least a 50:1 weight ratio of silicic acid to material applied.

4. Gas Liquid Chromatography

Gas liquid partition chromatography of methylated sugar derivatives was carried out on a 4-ft glass column (4 mm i.d.) of 3% ECNSS-M on Gas-Chrom Q at 170° and 37 p.s.i. of helium carrier gas using a Hewlett Packard Model 402 Gas Chromatograph with a flame ionization detector.

Peaks were identified by their retention times relative to authentic standards. Quantitative analyses were obtained by measurement of peak areas by the procedure of Carroll (1961):

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

where A = peak area

d = distance (mm)

h = peak height (mm)

VI. Analytical Procedures

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

An aliquot of lipid solution or hydrolyzate, containing 10-80 µg of sugar (as hexose) was pipetted into a 25 ml Lewis-Benedict sugar tube and the solvent was evaporated under a stream of nitrogen.

To the residue was added 2.0 ml of water and 1.0 ml of 5% aqueous phenol solution with mixing, followed by 5.0 ml of concentrated sulfuric acid. The solution was immediately "Vortexed" and then allowed to cool for 30 min. The absorbance was read at 490 nm, against a reagent blank. For calibration, standards containing 20, 40 and 80 µg of hexose (a mixed sugar standard of glucose:galactose:mannose, 1:1:1, w/w/w) were run simultaneously. The standard curve was linear in the range 20-100 µg hexose.

2. Phosphorus Determination (Bartlett, 1959)

The lipid sample was digested with 0.4 ml 72% perchloric acid. Distilled water (4.2 ml), amidol solution (0.2 ml)* and ammonium molybdate (0.2 ml)** were successively added with mixing on a Vortex mixer. Each tube was covered with a small beaker, heated in a boiling water bath for 7 min and then cooled in a cold water bath. After 15 min the absorbance of the stable blue color was read at 800 nm in 12 mm round cuvettes in a Coleman Junior II Spectrophotometer against standards (2 and 4 µg P)† and a reagent blank. Beer's Law was obeyed in the range 1-10 µg P.

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*Amidol reagent: 1.0 g 2,4-diaminophenol dihydrochloride (Amidol) and 20 g sodium bisulfite dissolved in 100 ml distilled water and filtered.

**Ammonium molybdate solution: 8.3 g ammonium molybdate dissolved in 100 ml distilled water.

†Standard phosphorus solution: 1.097 g of KH_2PO_4 (Primary Standard, Fisher Scientific Co.) dissolved in 250 ml of distilled water; this solution was diluted 1 to 100 to give a working solution containing 1 µg P/ml.

3. Ester Sulfate Analysis (Spencer, 1960)

The sulfate ester (glycolipid sulfate) was cleaved by mild acid-catalyzed solvolysis in tetrahydrofuran (Mayers et al., 1969; Goren, 1971) (See Part One, Experimental Procedures, II.1). The liberated inorganic sulfate was determined by a barium chloranilate assay as follows. The methanol-water phase of the solvolysis reaction mixture was made up to 10.0 ml giving a solution containing ca. 1.6 µmol per ml. Aliquots (1.0 ml, containing ca. 50 µg S) were transferred to centrifuge tubes and diluted with 4.0 ml of ethanol and 0.5 ml of 0.5 M acetate buffer (pH 4.0) containing 72.5 µg K_2SO_4 per ml. After "Vortexing" the solution, approximately 10 mg of solid barium chloranilate was added to each tube and the suspension was vigorously "Vortexed" at 1 min intervals during 10 min. The tubes were centrifuged at 3000xg for 5 min and each supernatant was transferred to a 12 mm round cuvette with a Pasteur pipette. The absorption was read at 530 nm against a reagent blank and standard K_2SO_4 solutions (25-100 µg S). The standard curve was linear in the range 25-100 µg S).

4. Cation Analysis by Flame Photometry

Ratios of cation/hexose were determined for the sodium and potassium salts of the glycolipid sulfate by sugar analysis (see Materials and Methods, VI.1) and cation analysis (Na,K) by flame photometry using a Unicam SP-90A Atomic Absorption Spectrophotometer.

A chloroform solution of the lipid (ca. 1 mM) was partitioned between chloroform and methanol-water (10:9, v/v) by a modification of the method of Bligh and Dyer (1964): 1.0 ml of the chloroform solution

was diluted with 2.0 ml methanol and 0.8 ml of 0.5 N aqueous HCl. Chloroform (1.0 ml) and water (1.0 ml) were immediately added to give a biphasic system (chloroform-methanol-water, 1.0:1.0:0.9, v/v/v). The mixture was centrifuged, the upper methanol-water phase was removed by Pasteur pipette, the chloroform phase was washed with ca. 3 ml of methanol-water (10:9, v/v) and the mixture was again centrifuged. The washing was repeated; the methanol-water phases were combined and made up to 10 ml with methanol. Aliquots of this solution (2.0 ml; ca. 0.1 to 0.2 mM in cation) were evaporated to dryness under a nitrogen stream at 60°C and the residues were dissolved in 2.0 ml of methanol-water (10:9, v/v). This treatment removed traces of chloroform present in the mixture, which strongly suppressed spectral emission of the cations. The solutions were analyzed for cation, using solutions of NaCl and KCl in methanol-water (10:9, v/v) as reference standards. The standard curves were linear with cation concentrations of 0.1 - 1.0 mM.

VII. Physical Methods

1. Infrared Spectroscopy

Infrared spectra were measured on solutions (ca. 1%) of the substances in chloroform or carbon tetrachloride in 0.5 mm cells using a Beckman IR-20 double-beam spectrophotometer.

2. Nuclear Magnetic Resonance Spectroscopy

NMR spectra were measured on [^{2}H]chloroform solutions containing ca. 10% sample by weight. In all cases, tetramethylsilane was used as an internal reference and chemical shifts were reported as

p.p.m. (δ) relative to TMS taken as 0 p.p.m. Spectra were recorded on either a Varian T-60 or a Varian HA-100 NMR Spectrometer.

3. Optical Rotations

Optical rotations were measured at 22°C at 589 nm on chloroform, chloroform-methanol (95:5, v/v) or aqueous solutions of the compounds, using a Perkin-Elmer polarimeter, model 141 (digital readout).

VIII: Radioisotopic Procedures

1. Radioisotopic Counting

Aliquots of solutions to be counted were transferred to plastic counting vials and the solvent removed under nitrogen. To each sample was added 10 ml of scintillation fluid which consisted of the following mixture: 2,5-diphenyl oxazole (PPO), 5 g/l; 2,2'-p-phenylene-bis(5-phenyl oxazole) (POPOP), 0.3 g/l; Beckman Bio-Solv™ Solubilizer (Formula BBS-3), 100 ml; methanol, 130 ml; toluene to make volume up to 1.0 liter.

After autoradiography of TLC plates, radioactive spots were scraped directly into counting vials, and 10 ml of the scintillation fluid described above was added. Samples were counted in a Beckman LS-150 scintillation counter equipped with an external standard and Automatic Quench Control (AQC), in general to 1% statistical error. Since no chloroform was present, solvent quench correction was not required. With each set of samples was included a vial containing only scintillation fluid in order to obtain a "background" count. ^{35}S was counted in the $^{3}H + ^{14}C$ window, ^{14}C was counted in the $^{3}H + ^{14}C$ window and ^{32}P was

counted in the ^{32}P window. ^{35}S and ^{32}P counts were corrected for decay. All counts were corrected for efficiency of counting and are given as d.p.m.

2. Autoradiography

^{14}C, ^{35}S and ^{32}P-labelled compounds on TLC plates were detected by placing a 20 x 20 cm sheet of Kodak no-screen X-ray film on the silica and exposing it in a light-proof folder for a suitable period, dependent on the isotope and the activity of the sample. When both ^{14}C and ^{32}P were present, two sheets of film were placed on the plate, one on top of the other. The sheet adjacent to the silica was exposed by both the ^{32}P and ^{14}C while the second sheet was only exposed by the ^{32}P.

PART ONE

STRUCTURAL STUDIES ON THE GLYCOLIPID SULFATE

EXPERIMENTAL PROCEDURES

I. Isolation and Purification of the Glycolipid Sulfate

1. Acetone Precipitation of the Polar Lipids

The chloroform solution of total lipids (Materials and Methods, III.1) was diluted with 10 volumes of acetone and left at 4°C overnight. The tan-colored precipitate of phospholipids and sulfolipids obtained was removed by centrifugation, washed several times with small portions of cold acetone, dried *in vacuo*, weighed and finally dissolved in chloroform to a known concentration. The combined acetone supernatants containing the neutral lipid fraction (hydrocarbons, pigments, etc.) was evaporated *in vacuo* to a small volume and dissolved in acetone to a known volume. In general, the acetone-insoluble lipids amounted to 91-95% by weight of the total lipids.

2. Preparative Thin Layer Chromatography of Polar Lipids

The mixture of polar lipids was separated by preparative TLC (50 mg per 20 x 20 cm plate; 1 mm thick layer of silica gel H) in the solvent system chloroform-90% acetic acid-methanol (30:20:4, v/v/v). The plates were allowed to dry overnight to remove most of the acetic acid, at which time the glycolipid sulfate could be seen as a white band (R_f 0.15) which was eluted as rapidly as possible, at the centrifuge, with chloroform-methanol-0.1 N HCl 1.0:2.0:0.8 (v/v/v) (twice with 38 ml

solvent per 8 plates). The combined extracts (made up to 76 ml with the eluting solvent, if necessary) were immediately converted to a two-phase system by the addition of 20 ml of chloroform and 20 ml of water. The mixture was centrifuged, the chloroform layer was removed and the aqueous phase was washed twice with 10 ml of chloroform. The combined chloroform extracts were immediately neutralized with 0.2 N methanolic ammonium hydroxide, then diluted with benzene and taken to dryness under reduced pressure. The residual ammonium salt of the sulfolipid (NH_4-GLS) represented ca. 24% by weight of the total acetone-insoluble lipids (Table 2); it showed only one spot on TLC in several solvent systems A, B, or C (Figure 6, Table 3), and on chromatography on silicic acid-impregnated paper (Table 3). The ammonium salt was stable in the dry form and in chloroform solution at room temperature for several years. It served as the starting material for the preparation of other derivatives and salt forms. Analytical data and optical rotations for NH_4-GLS are given in Table 4.

3. Preparation of Free Acid Form of the Glycolipid Sulfate

A solution of NH_4-GLS (177 mg) in 10 ml of chloroform-methanol (1:1, v/v) was acidified with 4.5 ml of 0.1 N HCl and the two-phase system was centrifuged briefly. The chloroform phase containing the free acid form of GLS was washed with an equal portion of methanol-water (10:9, v/v). When the free acid as such was required (e.g., for permethylation), the chloroform solution was immediately brought to dryness in vacuo and dried in a desiccator over potassium hydroxide.

4. Preparation of the Sodium and Potassium Salt Forms

To prepare the various salt forms, the chloroform solution of the free acid form of the glycolipid sulfate obtained above was immediately neutralized (to pH 8) by the addition of 0.2 N methanolic sodium or potassium hydroxide to form the sodium or potassium salt, respectively. The chloroform solution was then diluted with benzene and taken to dryness under a stream of nitrogen. A solution of the residual GLS-salt in chloroform (1 ml), cleared by centrifugation, was diluted with methanol (2 ml) and then with acetone (10 ml). After several hours at 4°C the GLS-salt precipitate was centrifuged down and reprecipitated from chloroform solution as described above. The GLS-salt was finally washed with cold acetone (1 ml) and dried in vacuo at room temperature; before analysis the white powder was further dried under vacuum at 80°C for 12 h. Analytical data and optical rotations for the various salt forms are given in Table 4; R_f values were the same for all salt forms (Table 3). The infrared spectrum of the glycolipid sulfate (potassium salt) is given in Figure 7.

II. Procedures for Structure Determination

1. Solvolytic Desulfation of the Glycolipid Sulfate

To a solution of NH_4-GLS (2.5 mg; 2 μmol) in anhydrous tetrahydrofuran (1 ml) was added 1 ml of anhydrous 0.004 N HCl in tetrahydrofuran (made by bubbling anhydrous HCl gas into lithium aluminum hydride-dried tetrahydrofuran and determining the normality by titration), and the mixture was kept at room temperature. The course of the solvolysis was monitored by TLC in solvent A of samples taken at 10 min intervals.

Desulfation was rapid and virtually complete after 90 min (Figure 9). The desulfated product was isolated by preparative TLC in solvent A, B, and C, and found to have chromatographic mobilities identical with those of the triglycosyl diether (Table 3).

2. Partial Acid Hydrolysis of the Glycolipid Sulfate

(a) Hydrolysis Procedure

A solution of 355 mg NH_4-GLS in 4.5 ml chloroform and 6 ml 0.25 N methanolic-HCl (made by bubbling HCl gas into anhydrous methanol) was kept at room temperature. The course of the hydrolysis was monitored by TLC in solvent system A. After 4 days, 1.5 ml chloroform and 5.4 ml water were added and the biphasic mixture was centrifuged; the chloroform phase was washed with methanol-water (10:9, v/v) and taken to dryness on a rotary evaporator with the addition of benzene to aid in removal of traces of water. The residue (154 mg) was found by TLC in solvent systems A, B, or C to contain about equal amounts of mono-, di-, and triglycosyl diphytanyl glycerol ethers.

(b) Isolation of the Chloroform-Soluble Products

The above mixture was separated by preparative TLC using solvent system A. The separated glycosyl glycerol diethers were eluted with chloroform-methanol-water (1.0:2.0:0.8, v/v/v) and the eluates converted to biphasicsystems as described for the NH_4-GLS. Each of the glycosyl diethers obtained on evaporation of the chloroform phases (MGD, 52 mg; DGD, 46 mg; TGD, 42 mg) was analytically pure and chromatographically homogeneous. R_f values and analytical data are given in Tables 3 and 5;

respectively. The infrared spectra of the glycosyl diethers are given in Figure 10.

(c) Isolation of the Water-Soluble Products

The methanol-water phase of the partial acid hydrolyzate of the glycolipid sulfate, when subjected to TLC on kieselguhr G - silica gel G (4:1, w/w) in ethyl acetate-methanol-water (68:23:9, v/v/v), showed two main spots corresponding to a disaccharide (R_f 0.28) and its methyl glycoside (R_f 0.44), and minor spots corresponding to the methyl glycosides of the monosaccharides glucose (R_f 0.76), galactose (R_f 0.81), and mannose (R_f 0.82). The disaccharide (ca. 2 mg) and its methyl glycoside (ca. 1.5 mg) were isolated as amorphous compounds by preparative TLC on kieselguhr G - silica gel G in the above solvent. After acid hydrolysis of the dissacharide or its methyl glycoside the only sugars detected were galactose and mannose. The configuration of the glycosidic linkage in the disaccharide was determined by enzymatic hydrolysis as described below (Part One, Experimental Procedure, II.5(b)).

3. Total Acid Hydrolysis of the Glycolipid Sulfate and Glycolipids

(a) Methanolic-HCl Hydrolysis

Hydrolysis of the glycolipid sulfate salts and the three glycosyl glycerol diethers (25-30 mg each) was carried out in side-arm flasks (Kates, 1964) in 2.5% methanolic-HCl (4.5 ml) under reflux for 3 h. After addition of water (0.5 ml), each hydrolyzate was extracted with low boiling petroleum ether (3 x 5 ml), the extracts were brought to dryness and the residual lipid was weighed to determine the diphytanyl

glycerol ether content (Tables 4 and 5). TLC of the pooled glycerol diether residues in chloroform-ether (9:1, v/v) showed a single spot corresponding to authentic (Joo et al., 1968) 2,3-di-O-phytanyl-sn-glycerol (R_f 0.65). After preparative TLC in the same solvent, the diether had $[\alpha]_D = +8.2^\circ$ reported (Joo et al., 1968) $[\alpha]_D = +8.4^\circ$. The infrared spectrum was identical to that of authentic diphytanyl glycerol ether (Kates et al., 1966).

(b) Aqueous-HCl Hydrolysis

The methanol-water phase of each hydrolyzate was taken to dryness under a stream of nitrogen, and the residue was heated in 1 ml of 1 N aqueous HCl at 100°C (boiling water bath) for 3 h to hydrolyze sugar methyl glycosides. The solution was taken to dryness under nitrogen, and the residue was dried in vacuo over KOH for 24 h to remove HCl; it was then examined by descending paper chromatography in pyridine-ethyl acetate-water (2:5:5, v/v/v, upper phase) to identify the sugar moieties.

4. Methylation Analysis of the Glycolipid Sulfate

(a) Methylation Procedure

The ammonium salt of the sulfolipid (143 mg) was converted to the free acid form as described above (Part One, Experimental Procedures, I.3). A solution of the free acid (115 mg; dried over KOH pellets under high vacuum for 1 h) in 20 ml of freshly distilled methyl iodide was heated under reflux with magnetic stirring in the presence of silver oxide (ca. 250 mg) for 48 h; more silver oxide and methyl iodide were added at 16 h intervals. The reaction mixture was diluted with an equal

volume of ethyl ether and the silver salts were removed by centrifugation and washed twice with 10 ml of benzene-chloroform (1:1, v/v). The combined supernatants were brought to dryness and the residue dried under vacuum over KOH pellets; yield, 134 mg of colorless oil.

(b) Purification of the Permethylated Glycolipid Sulfate

The crude product, containing the permethylated GLS as the major component and three minor partially methylated components was fractionated on a column of silicic acid (20 g, made up in benzene) using the following elution sequence: benzene, 100 ml; benzene-ether (90:10), 100 ml; benzene-ether (75:25), 400 ml; benzene-ether (50:50), 200 ml; benzene-ether (25:75), 200 ml; and ethyl ether, 200 ml (all solvent mixtures are v/v). The permethylated GLS appeared in the benzene-ether (75:25) eluate; it was recovered on evaporation of the solvent and dried under high vacuum; yield, 85 mg of TLC-pure permethylated GLS; R_f 0.65 in ethyl ether on silica gel H; $[\alpha]_D = +57.4^\circ$ (c, 1.28 g/dl in chloroform). The analytical data for the permethylated glycolipid sulfate are given in Table 6.

(c) Hydrolysis of the Permethylated Glycolipid Sulfate

The permethylated glycolipid sulfate (17 mg) was methanolyzed in 2.5% methanolic-HCl (4.5 ml) under reflux for 2 h (Kates, 1964). After the addition of 0.5 ml of water, the hydrolyzate was extracted with low boiling petroleum ether (3 x 5 ml) to remove the diphytanyl glycerol ether; only traces of methylated methyl glycosides were extracted. The methanol-water phase was taken to dryness and the residue was heated in

1 ml of 1 N aqueous HCl in a boiling water bath for 3 h. HCl was removed by repeated concentration in vacuo on a rotary evaporator (bath temperature 30°).

(d) Reduction of the Partially Methylated Monosaccharides

The residual methylated sugars were dissolved in 1 ml of water and reduced to the corresponding alditols with excess sodium borohydride (ca. 10 mg) at room temperature for 1 h. Glacial acetic acid was added dropwise to destroy the excess borohydride and the mixture was repeatedly taken to dryness on a rotary evaporator with additions of methanol to remove boric acid as the volatile trimethyl ester.

(e) Acetylation of the Partially Methylated Alditols

The partially methylated alditols were dissolved in pyridine (2 ml) and acetic anhydride (2 ml) was added. The reaction mixture was kept at 100°C for 3 h, and then brought to dryness on a rotary evaporator.

(f) Identification of Partially Methylated Alditol Acetates

The residual methylated alditol acetates were dissolved in chloroform and analyzed by gas liquid chromatography as described above (Materials and Methods, V.4).

Authentic reference standards of the alditol acetates of 3,4,6-trimethyl glucose, 2,4,6-trimethyl galactose and 2,3,4-trimethyl mannose were used to identify the sugars in the glycolipid sulfate (Table 7). Retention times were calculated relative to 1,5-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol which had a retention time of 8.5 min. under the GLC conditions used (see Materials and Methods, V.4).

5. Enzymatic Hydrolysis Studies on the Configuration of the Terminal Glycosidic Linkage

In order to establish conclusively the configuration of the terminal galactosidic linkage, the small amount of galactosyl-mannose produced during the weak acid hydrolysis of the sulfolipid was isolated by TLC (Part One, Experimental Procedures, II.2.(c)) and its properties compared with those of the synthetic O-β-D-galactopyranosyl-D-mannopyranose, synthesized as described below.

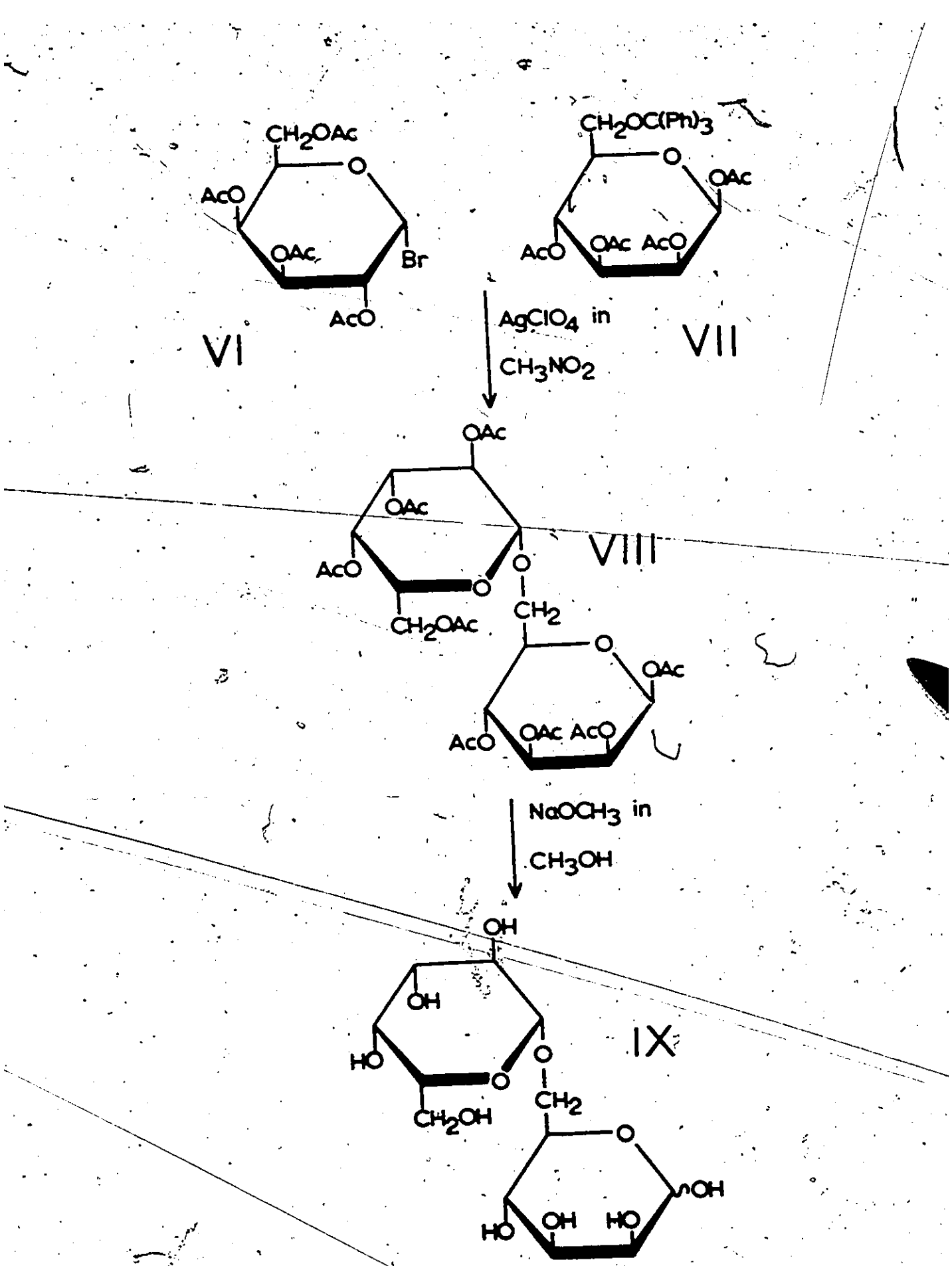
(a) Synthesis of O-β-D-Galactopyranosyl-(1→6)-D-Mannopyranose (see Scheme 5)

Acetobromogalactose (Barczai-Martos and Korosy, 1950) (VI; 411 mg, 1 mmol) was added to an ice-cold mixture of silver perchlorate (207 mg, 1 mmol), 1,2,3,4-tetraacetyl-6-trityl-D-mannose (VII; 577 mg, 1 mmol) and Drierite (1 g) in 20 ml of nitromethane. The mixture was shaken vigorously and allowed to warm up to room temperature. After 5 min the orange precipitate of silver bromide and trityl perchlorate was removed by centrifugation and washed with 10 ml of nitromethane. The combined nitromethane solutions were washed with a cold, saturated solution of sodium bicarbonate followed by water. The solution was then cleared by centrifugation, diluted with an equal volume of chloroform, dried over sodium sulfate and taken to dryness on a rotary evaporator.

The residue was shown by TLC on silica gel H in benzene-methanol (96:4, v/v) to contain the peracetylated disaccharide (VIII) (R_f 0.21) as the major product together with 2,3,4,6-tetraacetyl galactose (R_f 0.17) and some fast moving unreacted starting materials. To remove

Scheme 5

Chemical synthesis of O-β-D-galactopyranosyl-(1→6)-D-mannopyranose


CH2OAc
AcO
OAc
Br
AcO
VI
CH2OC(Ph)3
OAc
AcO
OAc
AcO
VII
AgClO4 in
CH3NO2
OAc
OAc
AcO
CH2OAc
O
CH2
VIII
OAc
AcO
OAc
AcO
NaOCH3 in
CH3OH
OH
OH
HO
CH2OH
O
CH2
IX
OH
HO
OH
HO

the tetraacetyl galactose the residue was treated with 20 ml of pyridine-acetic anhydride (1:1, v/v) for 18 h to convert the tetraacetyl monosaccharide to the faster-running pentaacetate (R_f 0.46). The peracetylated disaccharide was then readily purified by preparative TLC in the above solvent system; yield, 366 mg (54%) of colorless oil; $[\alpha]_D$ = +36.6° (c, 1.05 g/dl in chloroform).

The peracetylated disaccharide (170 mg, 0.25 mmol) was deacetylated in 10 ml of 25 mM methanolic sodium methoxide for 6 h at room temperature. The solution was then deionized on a column of Amberlite MB-2 (mixed-bed) ion-exchange resin, and taken to dryness on a rotary evaporator. The residual β-D-galactopyranosyl-(1→6)-D-mannopyranose (IX) was dried in vacuo over KOH at 60° for 24 h; yield, 82 mg (96%) of colorless gum; $[\alpha]_D$ = +52.8 (c, 0.71 g/dl in H_2O); R_{Glu} values are given in Table 8. The product, although free of other sugars, contained traces of the α-linked disaccharide as determined by enzymatic hydrolysis.

Analysis: $C_{12}H_{22}O_{11}H_2O$ (360.3).

Calculated: C, 40.00; H, 6.71.

Found: C, 39.49; H, 6.62.

(b) Enzymatic Hydrolysis Procedure

To a solution (1 mg in 0.05 ml water) of the galactosyl-mannose disaccharide or its methyl glycoside derived from GLS, or the synthetic disaccharide β-D-galactopyranosyl-(1→6)-D-mannopyranose, was added 0.05 ml of an unbuffered aqueous solution of E. coli β-galactosidase (1 mg/ml), and the mixture was left at room temperature for 6 h. The

hydrolysis products were then examined by chromatography on Whatman No. 1 paper in pyridine-ethyl acetate-water (2:5:5, v/v/v, upper phase) and by TLC on kieselguhr G - silica gel G in ethyl acetate-methanol-water (68:23:9, v/v/v); authentic hexose and methyl hexoside standards were included (Table 8). The β-galactosidase specificity of the enzyme was verified by its positive action on lactose, o-nitrophenyl-β-D-galactopyranoside, and the synthetic β-galactosyl mannose, and its inability to hydrolyze cellobiose, maltose, melibiose and p-nitrophenyl-α-D-galactopyranoside.

RESULTS AND DISCUSSION

I. Glycolipid Sulfate Content of Halobacterium cutirubrum

Treatment of the cells of Halobacterium cutirubrum by the procedure of Bligh and Dyer results in quantitative extraction of the total cellular lipids which account for 2.5-4% of the cell dry weight (Seghal et al., 1962; Kates et al., 1965; Joo et al., 1968). Acetone precipitation of the total lipids yields an insoluble "polar lipid" fraction (90% by weight) and an acetone-soluble "non-polar" lipid fraction (10%).

Preparative thin layer chromatography of the acetone-insoluble polar lipids afforded pure preparations of each of the four major lipids. From the data in Table 2, it can be seen that the glycolipid sulfate comprised about 23% by weight of the polar lipids.

Cells extracted with hot isopropanol yielded amounts of total polar lipids identical to cells extracted by the Bligh and Dyer technique. Also, isopropanol extraction gave the same proportions of individual polar lipids as did the Bligh and Dyer extraction. Therefore, it may be concluded that enzymatic breakdown of cellular lipids does not occur during the Bligh and Dyer extraction procedure.

II. Salt Forms of the Glycolipid Sulfate

1. Physical Properties

Titration of the free acid form of the glycolipid sulfate with ammonium hydroxide, sodium hydroxide, or potassium hydroxide yielded the ammonium, sodium and potassium salts respectively. All three salt forms had the same R_f values on thin layer chromatography (see Table 3 and Figure 6). All three salt

Table 2. Phospholipid and glycolipid composition of the total polar lipids of Halobacterium cutirubrum[a]

Component	Calculated Molecular Weight (sodium salt)	Composition	
		% by weight[b]	moles %[c]
Glycolipid sulfate (GLS)	1241.6	23	20
Phosphatidyl glycerophosphate (PGP)	953.2	63	71
Phosphatidyl glycerosulfate (PGS)	931.3	3	3
Phosphatidyl glycerol (PG)	829.2	4	5
		Recovery = 93%	

[a]All components are derivatives of 2,3-di-O-phytanyl-sn-glycerol. They were isolated by preparative chromatography of the total polar lipids (40 mg/plate on silica gel H in chloroform-90% acetic acid-methanol (30:20:4, v/v/v), and eluted with chloroform-methanol-diethyl ether (1:1:1, v/v/v) in a glass column; complete recovery of the glycolipid sulfate was obtained by further extraction of the silica with acid Bligh and Dyer solvent.

[b]As a % of the weight of the total polar lipids applied to the plates.

[c]Based on the total weight of lipid recovered from the plates.

Table 3. R_f values of the glycolipid sulfate and derived glycolipids in various solvent systems

Compound	Solvent System[a]			
	A	B	C	D
Glycolipid sulfate (NH_4-salt)[b]	0.15	0.25	0.11	0.33
Monoglycosyl diether	0.98	0.96	0.86	0.83
Diglycosyl diether	0.77	0.75	0.42	0.56
Triglycosyl diether	0.41	0.46	0.08	0.41

[a] TLC on silica gel H in solvent systems: A, chloroform–90% acetic acid–methanol (30:20:4); B, chloroform–methanol–water (65:25:4); C, chloroform–methanol–conc. ammonium hydroxide (65:35:5). Chromatography on silica-impregnated Whatman 3MM paper in solvent; D, diisobutyl ketone–acetic–acid–water (40:25:5).

[b] Sodium and potassium salts of SL had the same R_f values.

Figure 6

Thin layer chromatograms of the glycolipid sulfate and derived glycolipids in acidic, neutral and alkaline solvent systems.

Identification of components:

Lane 1 glycolipid sulfate (GLS)

Lane 2 triglycosyl diphytanyl glycerol (TGD)

Lane 3 diglycosyl diphytanyl glycerol (DGD)

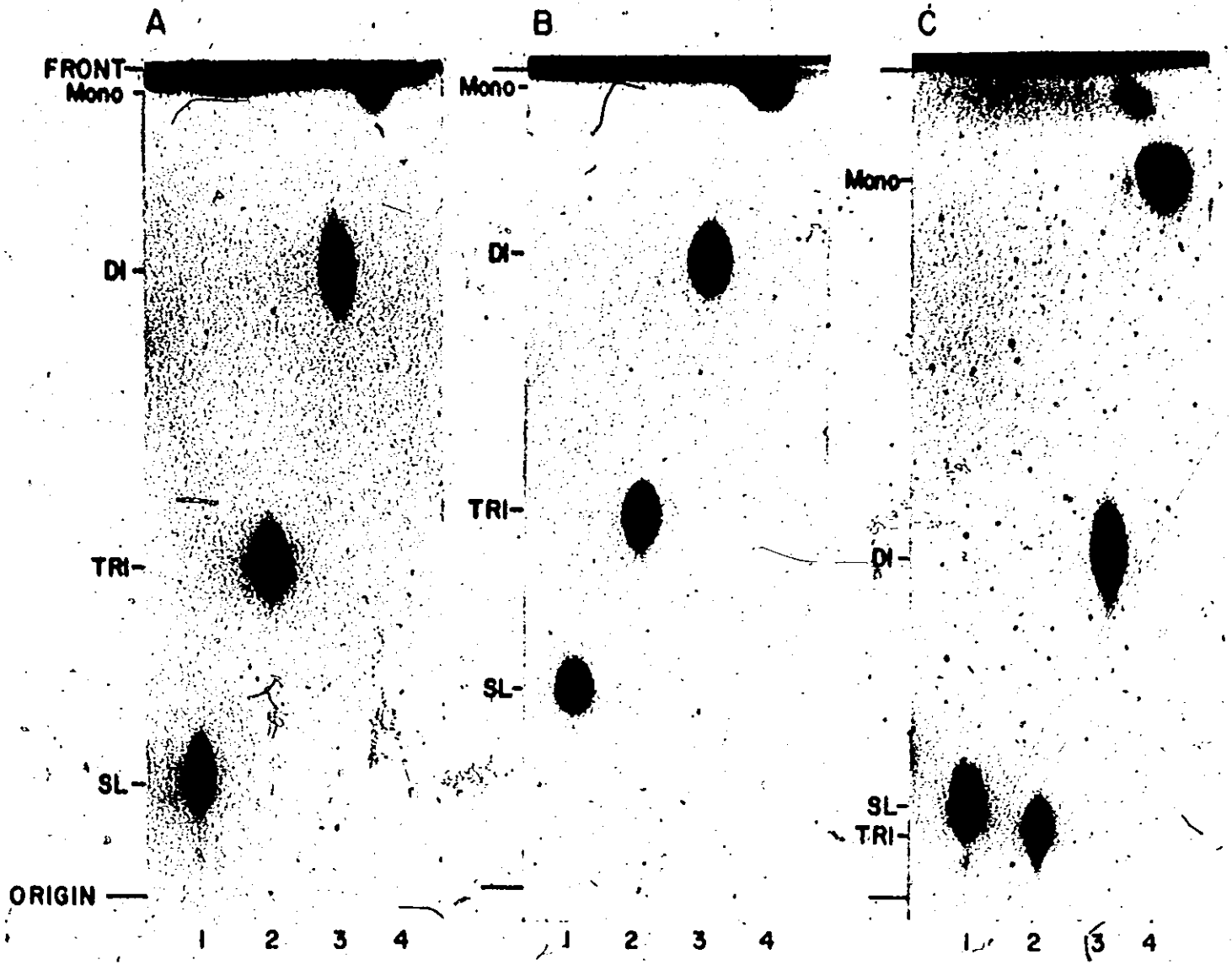
Lane 4 monoglycosyl diphytanyl glycerol (MGD)

Solvent Systems:

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

B - Chloroform-methanol-water
(65:25:4, v/v/v)

C - Chloroform-methanol-conc. ammonium hydroxide
(65:35:5, v/v/v)


A
B
C
FRONT
Mono
DI
TRI
SL
ORIGIN
1
2
3
4

forms are very soluble in chloroform and benzene and extremely soluble in tetrahydrofuran; they are almost insoluble in methanol and acetone. They were stable for several years in the dry state at room temperature and showed only slight degradation in chloroform solution at room temperature and almost no degradation in benzene solution.

The specific and molar optical rotations (Table 4) for the three salt forms are very similar, hence it would seem that the nature of the cation present has no effect on the optical activity of the sulfolipid. The high positive rotation of the sulfolipid sheds some light on the configuration of the glycosidic linkages (see Part One, Results and Discussion, VI).

2. Analytical Data

The analytical data for the ammonium, sodium and potassium salt forms are given in Table 4. The elemental analyses are consistent with those calculated for the respective salts of a sulfated triglycosyl diphytanyl glycerol. The carbon and hydrogen analyses of the sodium salt were consistently low, presumably due to the formation of sodium carbide during the combustion process. The hexose to diether (2,3-di-O-phytanyl-sn-glycerol) ratios and the sulfur to diether mole ratios are what would be expected if the molecule contained one sulfate group, three sugar residues and one diphytanyl glycerol moiety.

Titration of the free acid form of the sulfolipid consumed one equivalent of base which suggested the presence of one acidic group per molecule. Elemental analysis for the cation present (Table 4) confirmed a 1:1 ratio of cation equivalent to mole of diphytanyl glycerol.

Table 4. Analytical data for salt forms of the glycolipid sulfate

	$C_{61}H_{117}O_{21}S.NH_4$		$C_{61}H_{117}O_{21}S.Na$		$C_{61}H_{117}O_{21}S.K$	
	Calculated	Found	Calculated	Found	Calculated	Found
Mol. wt.	1236.7	-	1241.6	-	1257.8	-
C, %	59.24	59.25	59.00	-[a]	58.25	58.08
H, %	9.86	9.46	9.49	-[a]	9.37	9.08
S, %	2.59	2.56	1.58	2.44	2.54	2.84
Total Hexose, %	43.70	45.41	43.53	43.65	42.97	43.57
Diether, %	52.81	51.42	52.60	51.89	51.93	51.28
Hexose/Diether, mole ratio	3.00	3.13	3.00	3.01	3.00	3.04
S/Diether, mole ratio	1.00	1.00	1.00	1.00	1.00	1.13
Cation, %	1.46	1.43	1.85	2.24	3.11	3.58
Equiv. cation/mole diether	1.00	0.98	1.00	1.21	1.00	1.15
$[\alpha]_D$	-	$+46.79^o$	-	$+46.16^o$	-	$+45.15^o$
M_D	-	578.7	-	573.1	-	567.9

[a] C and H analyses for the Na salt were consistently low because of incomplete combustion.

3. Spectroscopic Data

The infrared spectrum of the potassium salt of the glycolipid sulfate (Figure 7) showed strong OH absorption at 3400 cm^{-1}, strong CH_2 and CH_3 absorption at 2900 and 1455 cm^{-1} and isopropyl absorption at 1370 cm^{-1} (doublet). These absorptions are consistent with the molecule having several hydroxyl groups (sugar rings) and long-chain branched alkyl groups (phytanyl moieties). Absorptions attributable to the sulfate group include that at 1265 cm^{-1} (S=O) as well as a typically weak sulfate S-O-C band at 830 cm^{-1}. The presence of a sulfate group was revealed more clearly by the infrared spectrum of the permethylated glycolipid sulfate (Part One, Results and Discussion, V).

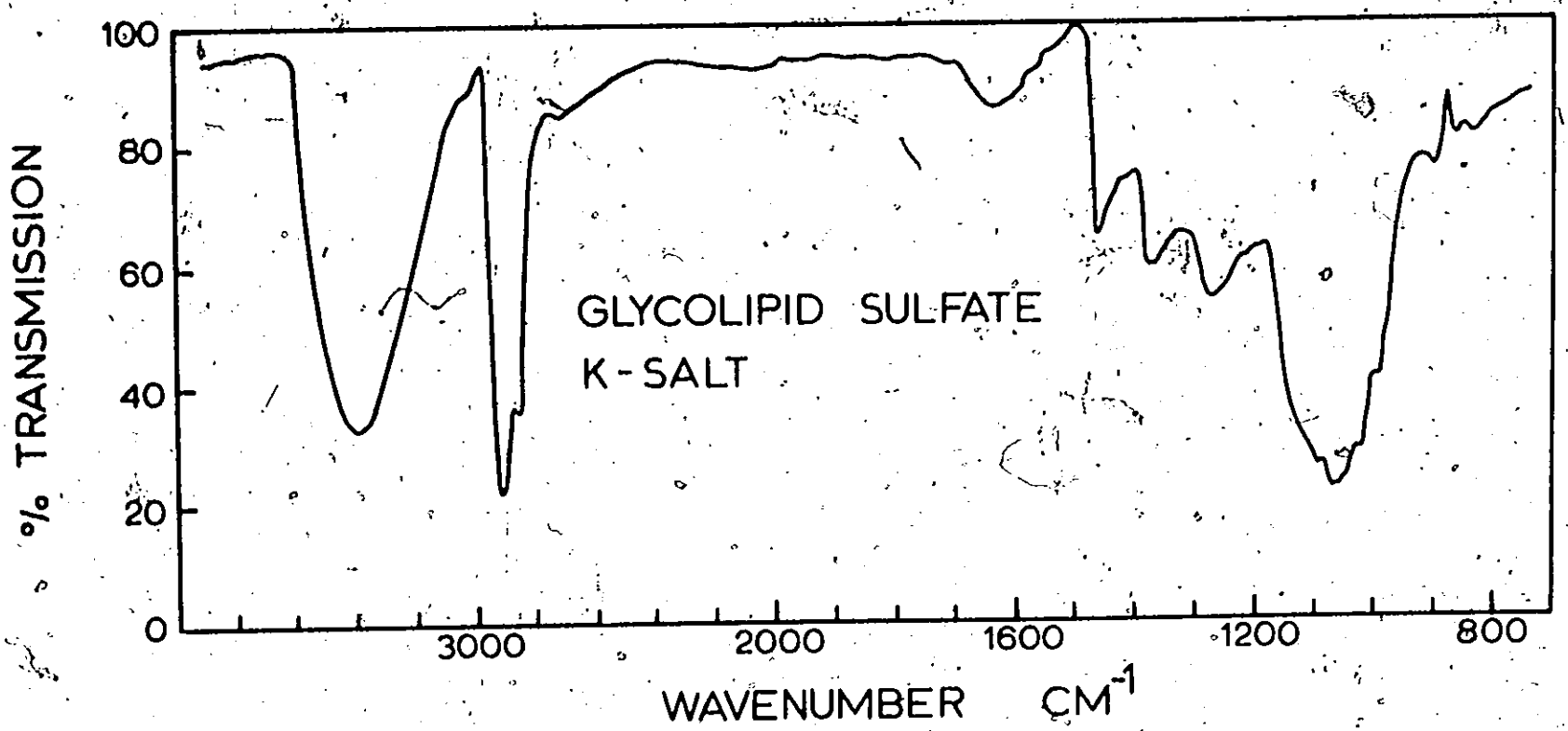
No carboxyl or ester carbonyl bands were present thereby eliminating the possibility of acyl groups on either the glycerol or sugar hydroxyl groups. A C-O-C ether absorption (phytanyl-glycerol linkage) may be discerned at ca. 1100 cm^{-1} as a shoulder on the strong alcohol C-O band at 1060 cm^{-1} (sugar hydroxyl groups). It should be mentioned that there were no peaks attributable to a sulfonate group thereby eliminating the possibility that the compound contained a sulfonic acid group rather than a sulfate group. This was borne out by the "solvolysis" studies described below (Part One, Results and Discussion, III).

4. "Natural" Salt Form of the Glycolipid Sulfate

Silicic acid column chromatography of the total polar lipids of Halobacterium cutirubrum allows a separation of these lipids not only on the basis of their structures but also according to the nature of the

Figure 7

Infrared spectrum of the potassium salt of the glycolipid sulfate in chloroform.


100
80
60
40
20
0
% TRANSMISSION
GLYCOLIPID SULFATE
K-SALT
3000
2000
1600
1200
800
WAVENUMBER CM-1

cation bound to the lipid (Hancock, 1972). Elution of the column with chloroform-methanol (94:6, v/v) removed the magnesium salts of PGS and PGP. No magnesium salt of the glycolipid sulfate was found. An increase in the polarity of the eluting solvent (chloroform-methanol, 80:20, v/v) resulted in the elution of most of the glycolipid sulfate as the sodium salt together with the sodium salts of PG, and PGS. An even more polar solvent mixture (chloroform-methanol, 60:40, v/v) removed mainly the sodium salt of PGP with a trace of glycolipid sulfate present. While most of the lipid eluted with the last solvent was present as the sodium salt, there was a small amount of potassium ion present. From these results it appears that most of the glycolipid sulfate is present as the sodium salt, along with a small amount of the potassium salt. Alternatively, the glycolipid sulfate may have exchanged its natural cation for a sodium ion during the Bligh and Dyer extraction.

III. Solvolysis Studies on the Glycolipid Sulfate

The solvolysis of lipid sulfate esters in the presence of oxonium ion - forming anhydrous solvents (dioxane, ethyl ether, etc.) has recently been discussed (Haines, 1971; Goren, 1971). Haines studied the desulfation of potassium 2-octanol-2-sulfate in refluxing dioxane while Goren worked on the ammonium salts of a series of 2,3,6,6'-tetraacyl-α,α'-trehalose esters which contained a sulfate group on the 2' position of the trehalose. In both, the essential feature is the formation of a labile sulfate-solvent oxonium ion intermediate complex. Their proposed mechanisms for the solvolyses are shown in Figure 8.

Direct chemical evidence for the presence of a sulfate ester

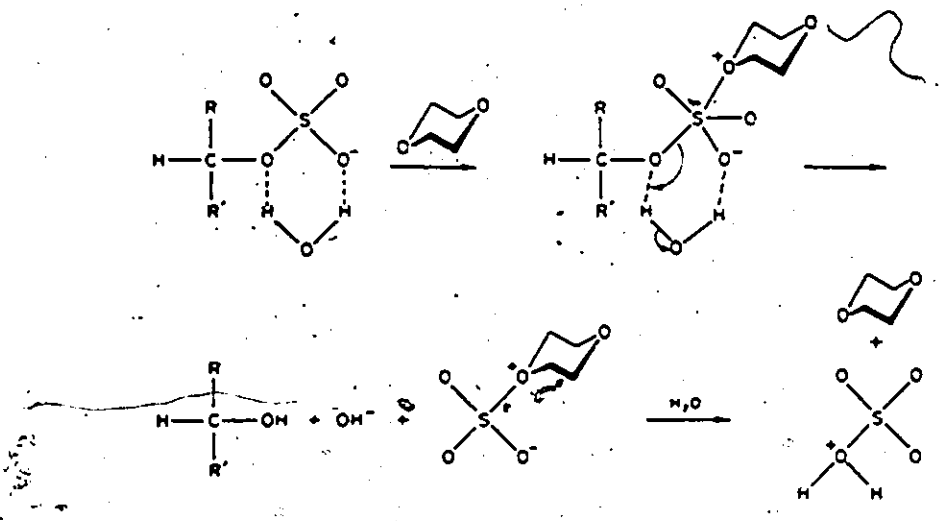
Figure 8

Solvolysis of sulfate esters:

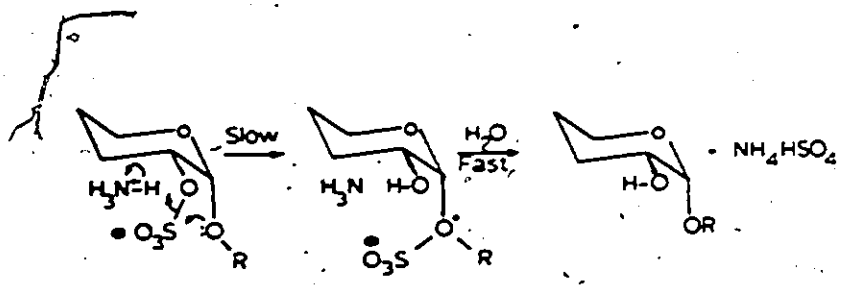
A - Mechanism of solvolysis of alkyl sulfate esters (Haines, 1971)

B - Proposed mechanism for the desulfation of sugar sulfates (Goren, 1971).

A



B



in GLS was obtained by subjecting the ammonium salts of the glycolipid sulfate to mild acid-catalyzed solvolysis in anhydrous tetrahydrofuran-HCl (0.004 N) at room temperature. The ammonium salt of the glycolipid sulfate was rapidly desulfated, the reaction being complete in about 90 min (see Figure 9). The lipid product of the solvolysis was chromatographically identical with the triglycosyl diphytanyl glycerol obtained by acid hydrolysis of the glycolipid sulfate (see Part One, Experimental Procedures, II.2.(b)). The relative ease with which the sulfur-containing moiety of the glycolipid sulfate was lost upon mild solvolysis is a very good indication that the sulfur is indeed present as a sulfate group and not as a sulfonate; the latter would be resistant to either solvolysis or hydrolysis.

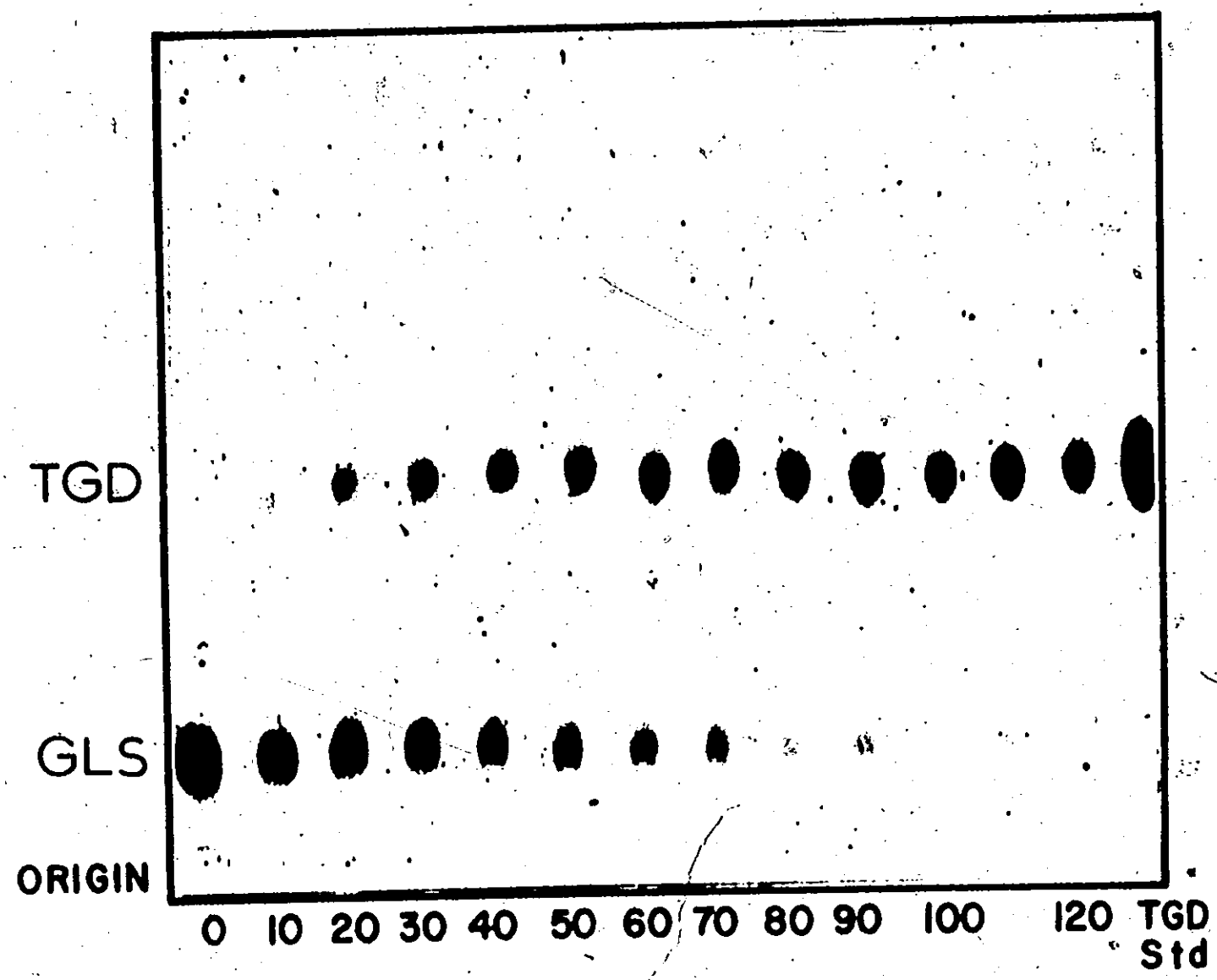
In contrast to the finding of Goren (1971) that the sulfolipid from Mycobacterium tuberculosis is spontaneously desulfated in ether solution at room temperature, the glycolipid sulfate of Halobacterium cutirubrum was quite stable in either tetrahydrofuran or diethyl ether unless a small amount of hydrogen ion (0.004 N) was present. As with the glycolipid sulfate, the phosphatidyl glycerosulfate (Figure 5) from Halobacterium cutirubrum was only solvolyzed by tetrahydrofuran in the presence of acid (Hancock, 1972). Goren has postulated that the spontaneous desulfation of the M. tuberculosis sulfolipid may be due to the proximity of the 2'-sulfate group to the α-anomeric oxygen of the trehalose (Figure 8). In GLS, however, the sulfate is much further removed from the β-anomeric oxygen (see Figure 14) and this might account for its greater stability.

Figure 9

Time course for the solvolysis of the glycolipid sulfate (GLS) in 4 mM HCl in tetrahydrofuran to produce the triglycosyl diphytanyl glycerol (TGD).

Solvent System:

Chloroform-90% acetic acid-methanol (30:20:4, v/v/v).


TGD
GLS
ORIGIN
0 10 20 30 40 50 60 70 80 90 100 120 TGD Std
HYDROLYSIS TIME, MINUTES

IV. The Glycolipids Obtained by Partial Acid Hydrolysis of the Glycolipid Sulfate

The mild acid hydrolysis of the glycolipid sulfate in chloroform-methanolic-HCl (0.25 N)(3:4, v/v) at room temperature for 4 days was monitored by TLC which revealed the following sequence of reactions: first, removal of the sulfate group, yielding the triglycosyl diether (TGD); then, removal of the terminal hexose residue, yielding the diglycosyl diether (DGD); then, cleavage of either the terminal hexose unit from the diglycosyl compound or the terminal disaccharide residue from the triglycosyl compound produced the monoglycosyl diether (MGD). Some further cleavage of the monoglycosyl diphytanyl glycerol gave a water soluble hexose plus the chloroform-soluble diphytanyl glycerol. It was found that approximately equal amounts of each glycolipid had been formed after 96 h. Thus, sufficient amounts of each compound for further analysis could be isolated using preparative TLC (see Materials and Methods, II.2.(b)).

The chromatographic purity and R_f values of the three isolated glycolipids are shown in Figure 6 and Table 3, respectively.

1. Physical Properties of the Glycolipids

The isolated glycolipids were colorless gums, readily soluble in chloroform or benzene; however, these solutions soon became gels, presumably because of the high degree of hydrogen bond formation that is possible between these molecules. The addition of a small amount (5% by volume) of methanol to the solutions of the glycolipids completely prevented these gels from forming.

The optical rotations of all three glycolipids (Table 5)

were high and positive; the significance of these rotations will be discussed in a later section (see Part One, Results and Discussion, VI).

2. Analytical Data

The hexose to diether mole ratios (Table 5) of each of the three glycolipids showed that the slowest moving compound (TGD) (Figure 6) contained three moles of hexose per mole of diether; the elemental analysis of TGD was consistent with the compound being a triglycosyl diphytanyl glycerol. The glycolipid with the second lowest R_f value (DGD) (Figure 6) was shown to contain two moles of hexose per mole of diether; the elemental analysis of DGD was consistent with the compound being a diglycosyl diphytanyl glycerol. The compound with the highest R_f value (MGD) (Figure 6) was found to contain one mole of hexose per mole of diether; the elemental analysis of MGD was consistent with its being a monoglycosyl diphytanyl glycerol.

3. Spectroscopic Study on the Glycosyl Diphytanyl Glycerols

The infrared spectra of the mono-, di-, and triglycosyl diphytanyl glycerols (Figure 10) were similar and showed strong OH absorption at 3400 cm^{-1} increasing in intensity with respect to the number of sugar moieties; all three spectra showed strong CH_2 and CH_3 absorption at 2900 and 1455 cm^{-1} as well as a pronounced isopropyl absorption (doublet) at 1370 cm^{-1}. A C-O-C ether band was seen at 1110 cm^{-1} in all spectra.

Table 5. Analytical data for the glycolipids derived from the glycolipid sulfate

	Monoglycosyl Diether (MGD)		Diglycosyl Diether (DGD)		Triglycosyl Diether (TGD)	
	Calculated	Found	Calculated	Found	Calculated	Found
Mol. wt.	815.3	-	977.5	-	1139.6	-
C, %	72.19	71.78	67.58	67.19	64.29	64.14
H, %	12.12	11.93	11.14	10.79	10.44	10.07
Total Hexose, %	22.09	21.93	36.86	37.06	47.42	47.62
Diether, %	80.11	79.42	66.82	65.18	57.32	56.71
Hexose/Diether, mole ratio	1.00	1.00	2.00	2.06	3.00	3.03
Sugars Present[a]		glucose	glucose mannose			glucose mannose galactose
$[\alpha]_D$	-	+41.36°	-	+59.43°	-	+47.02°
M_D	363[b]	337	517[c]	581	518[d] 899[e]	536

[a]Identified by paper chromatography (see Materials and Methods, V.1).

[b]Calculated for α-Glup-diether ($C_{49}H_{98}O_8$).

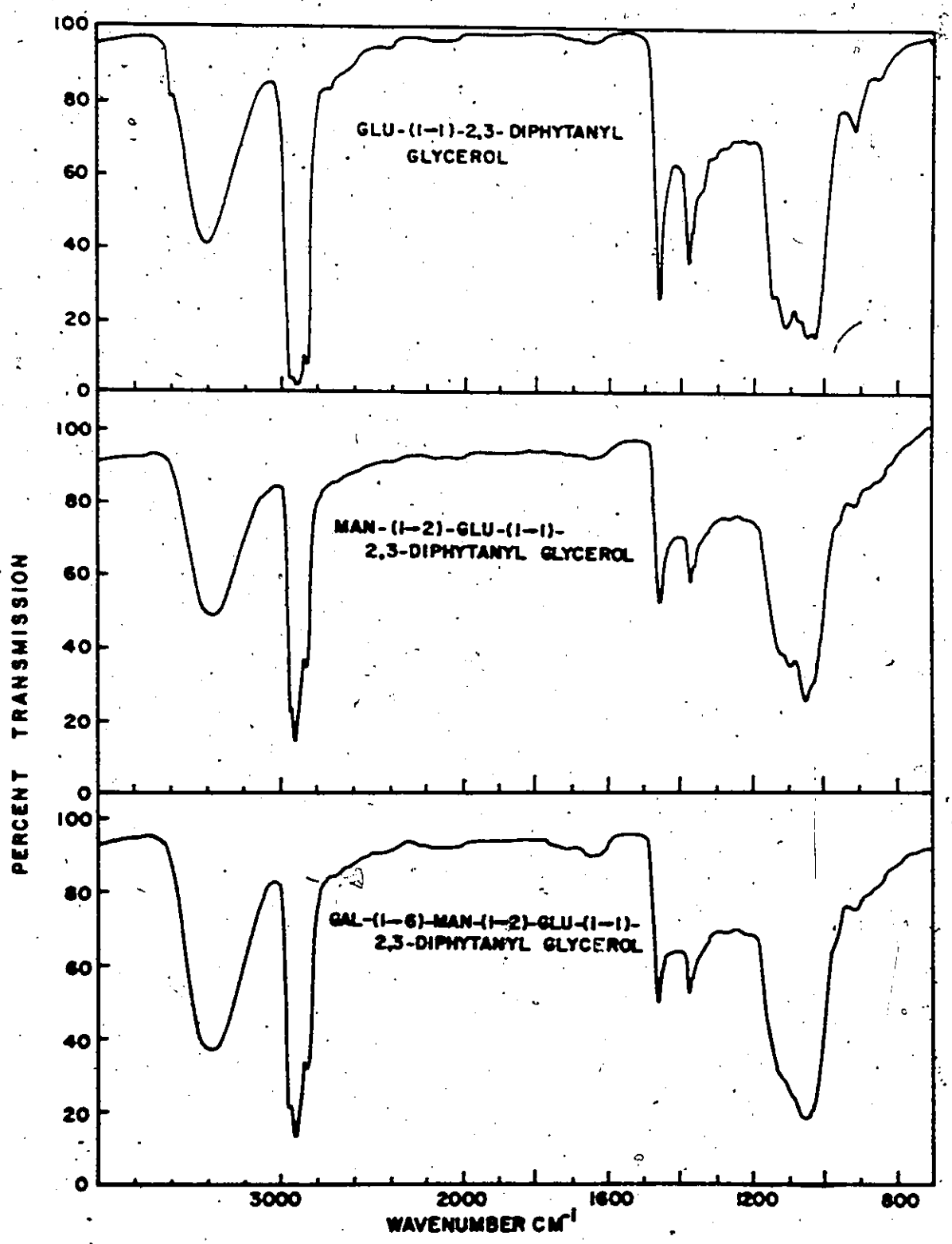
[c]Calculated for α-Manp-α-Glup-diether ($C_{55}H_{108}O_{13}$).

[d]Calculated for β-Galp-α-Manp-α-Glup-diether ($C_{61}H_{118}O_{18}$).

[e]Calculated for α-Galp-α-Manp-α-Glup-diether

Figure 10

Infrared spectra of the monoglycosyl diphytanyl glycerol (MGD), diglycosyl diphytanyl glycerol (DGD), and triglycosyl diphytanyl glycerol (TGD) in carbon tetrachloride


GLU-(1–1)-2,3-DIPHYTANYL GLYCEROL
MAN-(1–2)-GLU-(1–1)-2,3-DIPHYTANYL GLYCEROL
GAL-(1–6)-MAN-(1–2)-GLU-(1–1)-2,3-DIPHYTANYL GLYCEROL
PERCENT TRANSMISSION
WAVENUMBER CM⁻¹
100
80
60
40
20
0
3000
2000
1600
1200
800

4. Sugar Sequence of the Glycolipids and Glycolipid Sulfate

In order to determine the sugars present in the glycolipids, each compound was subjected to strong acid hydrolysis. Paper chromatography of the water-soluble products of each hydrolysis (Figure 11) revealed the following. The only hexose released from the MGD was glucose; therefore, MGD is a glucosyl diphytanyl glycerol. The hexoses released from DGD were glucose and mannose; therefore, DGD is a mannosylglucosyl diphytanyl glycerol. The hexoses released from TGD included galactose, mannose, and glucose; therefore, TGD is a galactosyl mannosyl glucosyl diphytanyl glycerol. Thus, the sequence of the sugar in the parent molecule (i.e., the glycolipid sulfate) was established as being galactose-mannose-glucose-diether.

V. Methylation Analysis and the Glycosidic Linkage Positions

1. Characterization of the Permethylated Glycolipid Sulfate

Treatment of the free acid form of the glycolipid sulfate with methyl iodide and silver oxide resulted in the formation of the permethylated derivative in fair yield.

The permethylated glycolipid sulfate was isolated by column chromatography as a colorless oil that was readily soluble in chloroform, benzene or methanol. The neat compound was stable for several months at -20°C while a benzene solution of the compound was stable at room temperature for the same length of time. In chloroform solution at room temperature, the compound showed some decomposition after a few days.

The high optical rotation of the compound ($[\alpha]_D = +57.4^\circ$) (Table 6) was again indicative of largely α-glycosidic linkage configurations (see Part One, Results and Discussion, VI).

Figure 11

Tracing of a paper chromatogram of the water-soluble hydrolysis products of the monoglycosyl diphytanyl glycerol (MGD), diglycosyl diphytanyl glycerol (DGD), triglycosyl diphytanyl glycerol (TGD) and the glycolipid sulfate (GLS). The lane on the left shows a mixture of standard hexoses, mannose (R_{Glu} 1.18), glucose (R_{Glu} 1.00) and galactose (R_{Glu} 0.85).

The chromatogram was developed in a descending solvent system of pyridine–ethyl acetate–water (2:5:5, v/v/v, upper phase).

Lane 1 GLS

Lane 2 MGD

Lane 3 DGD

Lane 4 TGD

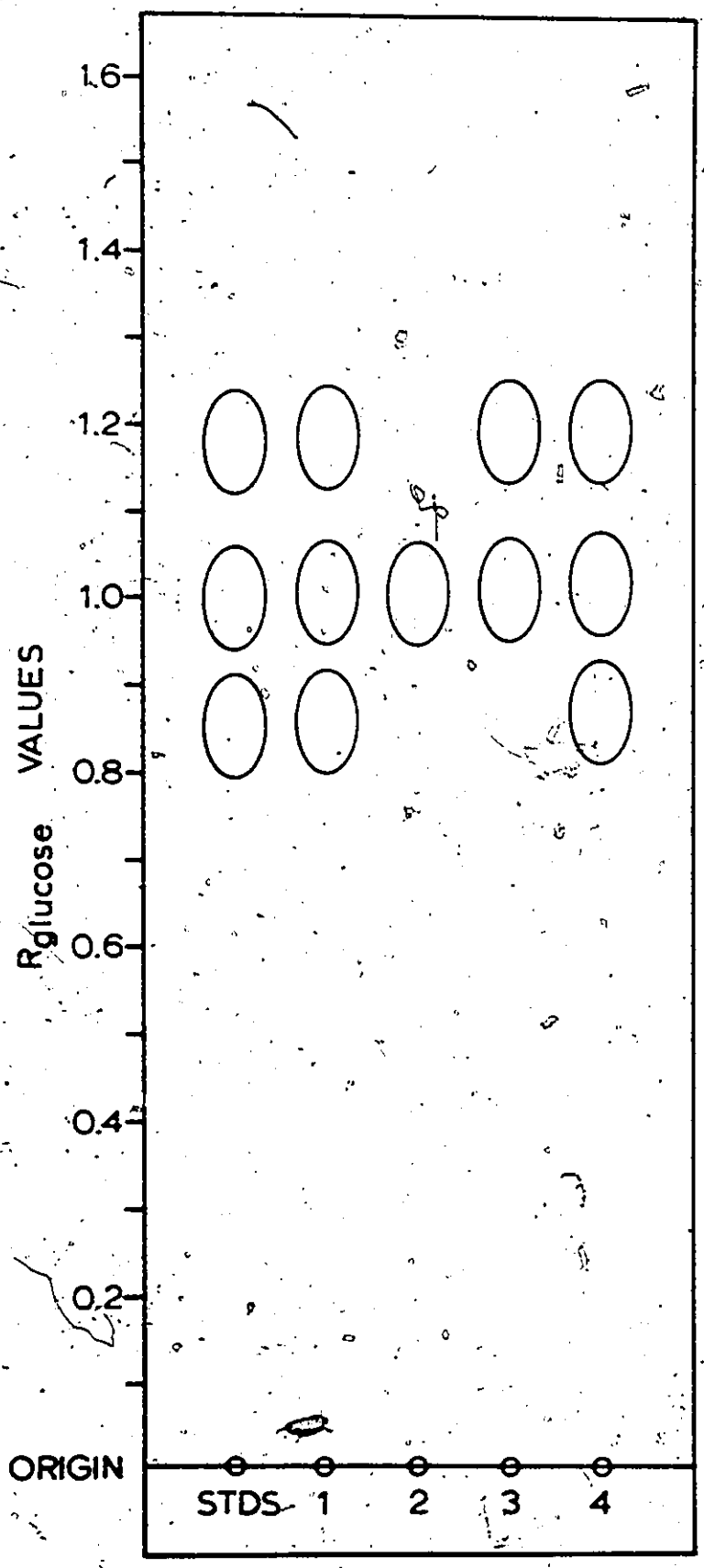

1.6
1.4
1.2
1.0
0.8
0.6
0.4
0.2
R glucose VALUES
ORIGIN
STDS 1 2 3 4

Table 6. Analytical data for the permethylated glycolipid sulfate ($C_{71}H_{138}O_{21}S$)

	Calculated	Found
Mol. wt.	1359.9	-
C, %	62.71	62.62
H, %	10.23	10.28
S, %	2.36	2.79
OCH_3, %	25.10	24.38[a]
Total Hexose, %	39.74[b]	38.14[b]
Diether, %	48.03	47.81
Hexose/Diether mole ratio	3.00	2.89
S/Diether mole ratio	1.00	1.14
$[\alpha]_D$	-	+57.4°
M_D	-	+780.6°

[a] This value has been corrected to include the propyl iodide formed from the glycerol moiety of the lipid during the analysis (assuming one equivalent propyl iodide per molecule of lipid.

[b] As free hexose.

Analytical data for the permethylated glycolipid sulfate are given in Table 6. These data are consistent with the molecular formula $C_{17}H_{138}O_{21}S$ calculated for this compound. The percent OCH_3 is consistent with the compound containing 10 methoxy groups per molecule (nine on the sugar hydroxyl groups and one on the sulfate group).

The infrared spectrum (Figure 12) of compound showed no OH absorption indicating that the molecule had been permethylated. The spectrum showed the same strong CH_2 and CH_3 absorption as in that of GLS but exhibited strong and more discrete sulfate bands: $-O-SO_2-O-$ at 1405 and 1195 cm^{-1}, S-O-C at 855 cm^{-1}, and secondary sulfate S-O-C at 915 cm^{-1}. A C-O-C ether band centered at 1100 cm^{-1} was now prominent, and no alcohol C-O band was present.

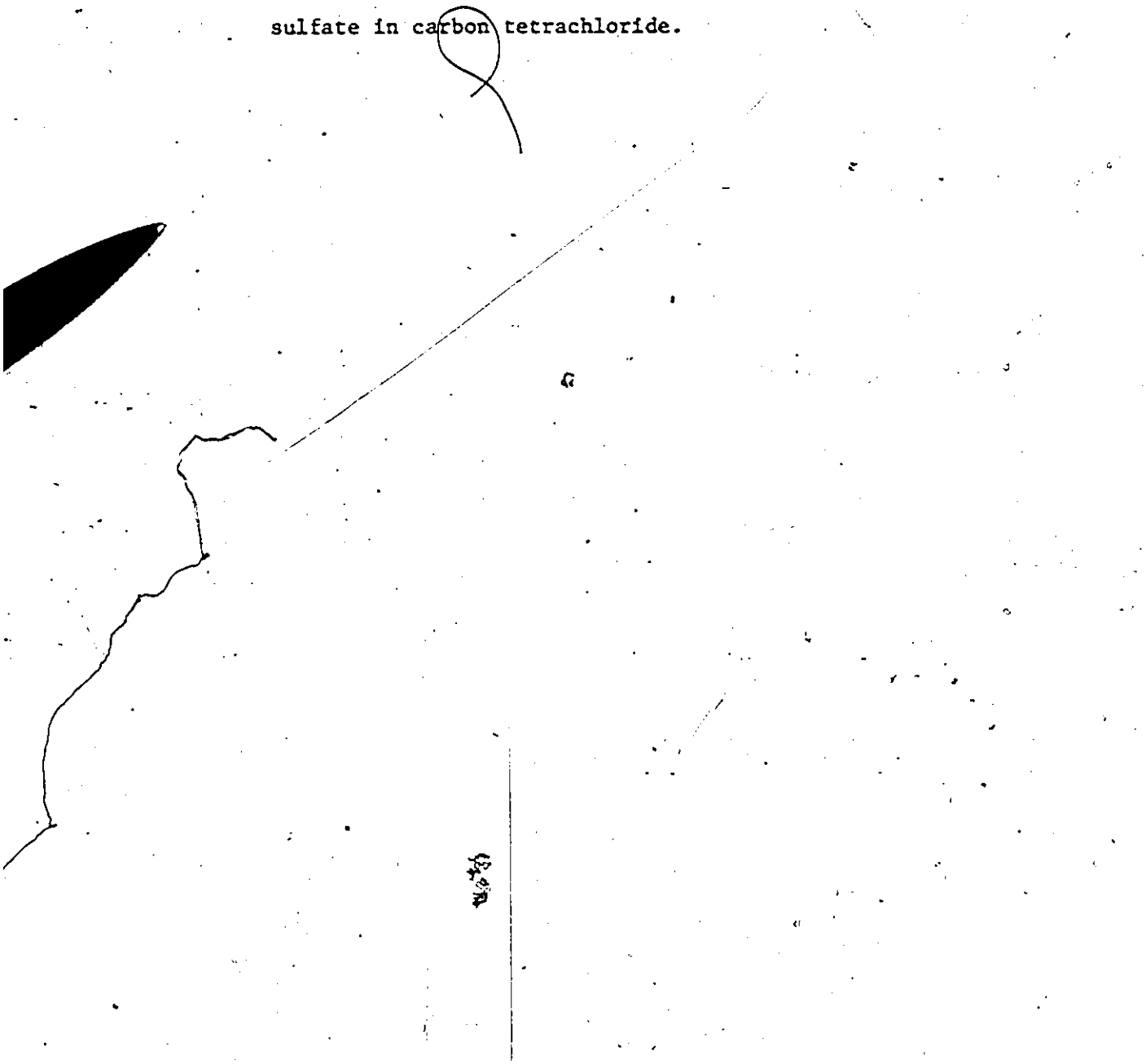
The NMR spectrum of the permethylated glycolipid sulfate (Figure 13) showed sharp methyl signals at 0.75-0.95 p.p.m. integrating for 30 protons (30 expected). Centered at 1.33 p.p.m. is a broad methylene envelope (phytanyl chains) (0.95-1.70 p.p.m.) integrating for 52 protons (52 expected). Several sharp methoxy signals ($-O-CH_3$ on sugar rings) are found between 3.25 and 3.75 p.p.m. A sharp singlet at 4.0 p.p.m. (3 protons) is attributable to the secondary $S-O-CH_3$ group.

2. Linkage Positions of the Glycolipid Sulfate

The linkage between the sugars was established by GLC of the alditol acetates of the partially methylated sugars obtained by acid hydrolysis of the permethylated glycolipid sulfate. The three methylated products correspond to 3,4,6-trimethyl glucose, 2,4,6-trimethylgalactose and 2,3,4-trimethyl mannose in molar properties 0.6:1.0:1.0, respectively

Figure 12

Infrared spectrum of the permethylated glycolipid sulfate in carbon tetrachloride.



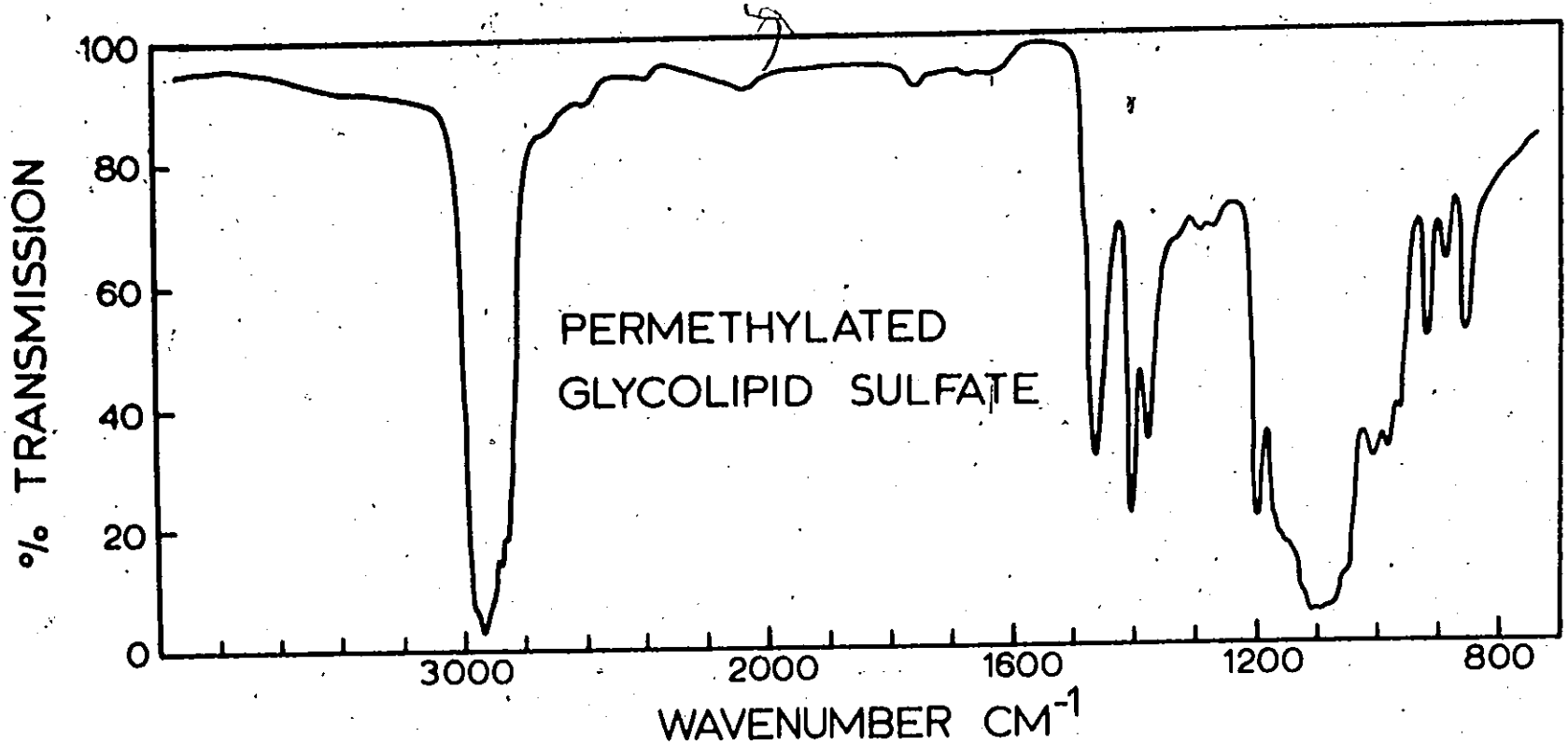
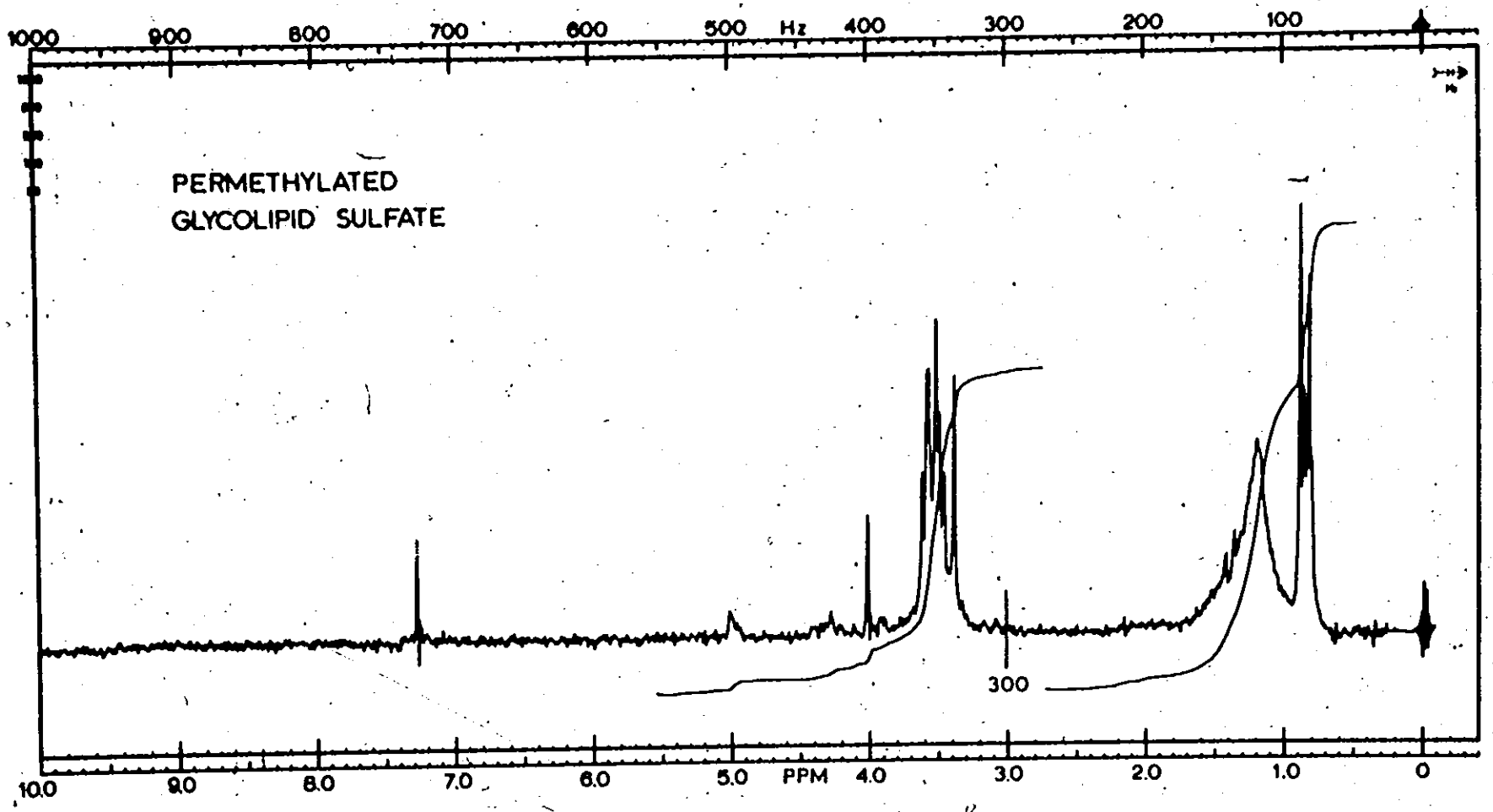

100
80
60
40
20
0
% TRANSMISSION
PERMETHYLATED
GLYCOLIPID SULFATE
3000
2000
1600
1200
800
WAVENUMBER CM-1

Figure 13

100 MHz NMR spectrum of the permethylated glycolipid sulfate in [^{2}H]chloroform using an internal standard of tetramethylsilane.


1000
900
800
700
600
500 Hz
400
300
200
100
PERMETHYLATED
GLYCOLIPID SULFATE
300
10.0
9.0
8.0
7.0
6.0
5.0 PPM
4.0
3.0
2.0
1.0
0

(Table 7). The formation of 3,4,6-trimethyl glucose indicated that glucose was linked at position 2, and formation of 2,3,4-trimethyl mannose indicated that mannose was linked at position 6. The presence of 2,4,6-trimethyl galactose showed that the sulfate group must be esterified at position 3 of the terminal galactose. The assignment of these positions is in agreement with those determined previously by GLC of the partially methylated methyl glycosides (Kates et al., 1967). The low molar proportion of 3,4,6-trimethyl glucose found in the present study may be explained on the basis that the partially methylated glucose is more easily decomposed under the hydrolysis conditions or during the subsequent workup than the other methylated sugars.*

VI. Configuration of the Glycosidic Linkages

On the basis of the high dextrorotatory optical activity of the glycolipid sulfate, Kates et al. (1967) concluded that the glycosidic linkages probably had the α-configuration. However, comparison of the observed molecular rotations of the three glycosyl glycerol diethers with their theoretical molecular rotations (Table 5) calculated from the rotations of known glycosides and the diphytanyl glycerol ether indicated that, although the mannosidic and glucosidic linkages had the α-configuration, the galactosidic linkage was probably in the β-configuration.

In order to establish conclusively the configuration of the terminal galactosidic linkage, the small amount of galactosyl-mannose produced during the weak acid hydrolysis of the sulfolipid was isolated

*Adams, G. A., Personal communication.

Table 7. Hydrolysis products of the permethylated glycolipid sulfate

Methylated Sugars[a]	Relative Retention Times[b]		Molar Ratio
	Unknowns	Standards	
3,4,6-Trimethyl Glucose	2.02	2.00	0.6
2,4,6-Trimethyl Galactose	2.34	2.33	1.0
2,3,4-Trimethyl Mannose	2.56	2.55	1.0

[a] Analyzed by GLC as the partially methylated alditol acetates on 3% ECNSS-M (Gas-Chrom Q) at 170°C.

[b] Relative to 1,5-di-O-acetyl-2,3,4,6-tetra-O-methyl-D-glucitol; retention time, 8.5 min.

by TLC and found to have the same R_f values as the synthetic β-D-galactopyranosyl-D-mannopyranose (Table 8). Both the "natural" and the synthetic disaccharides were readily hydrolyzed by a β-galactosidase preparation known to be completely specific for β-galactosides (see also Part One, Experimental Procedures, II.5.(b)) thereby establishing the β-configuration of the terminal galactosidic linkage.

VII. Structures of the Glycolipid Sulfate and Derived Glycolipids

The evidence presented above thus established the complete structure of the glycolipid sulfate in Halobacterium cutirubrum as 1-O-(O-β-D-galactopyranosyl-3'-sulfate-(1'→6')-O-α-D-mannopyranosyl-(1'→2')-O-α-D-glucopyranosyl)-2,3-di-O-phytanyl-sn-glycerol (Figure 14). The complete structures of the GLS derived triglycosyl diphytanyl glycerol (TGD) diglycosyl diphytanyl glycerol (DGD) and monoglycosyl diphytanyl glycerol (MGD) are therefore as follows: 1-O-[O-β-D-galactopyranosyl-(1'→6')-O-α-D-mannopyranosyl-(1'→2')-O-α-D-glucopyranosyl]-2,3-di-O-phytanyl-sn-glycerol; 1-O-[O-α-D-mannopyranosyl-(1'→2')-O-α-D-glucopyranosyl]-2,3-di-O-phytanyl-sn-glycerol; 1-O-[O-α-D-glucopyranosyl]-2,3-di-O-phytanyl-sn-glycerol (Figure 15). The structures have been arbitrarily drawn with the sugar rings in the C 1 rather than the 1 C conformation, since the hydroxymethyl groups and the majority of the hydroxyl groups are in the thermodynamically more stable equatorial orientation in the C 1 conformation.

It is interesting that while the glucosidic and mannosidic linkages in GLS are in the α-configuration, the galactose is linked in the β-configuration, as has been found for the galactose residue in all

Table 8. Enzymatic hydrolysis of various glycosides by a specific β-galactosidase[a]

Substrate	R_{Glu}[b]		Hydrolysis Products (R_{Glu} Values)[b]					
			Galactose		Mannose		Methyl Mannoside	
	TLC	Paper	TLC	Paper	TLC	Paper	TLC	Paper
"Natural" Gal-(1→6)-Man	0.38	0.34	0.91	0.86	1.06	1.18	-	-
"Natural" Gal-(1→6)-Man methyl glycoside	0.66	0.63	0.90	0.85	-	-	1.25	N.D.[c]
Synthetic Gal-β-(1→6)-Man	0.37	0.32	0.91	0.85	1.06	1.17	-	-
Synthetic Gal-β-(1→6)-Man methyl glycoside	0.66	0.64	0.91	0.86	-	-	1.26	N.D.

[a] A lactase preparation from E. coli, found to be absolutely specific for β-galactosides.

[b] Separated by (a) TLC on kieselguhr G-silica gel G (4:1) in ethyl acetate-methanol-water (68:23:9, v/v), and (b) chromatography on Whatman No. 1 paper in pyridine-ethyl acetate-water (2:5:5, v/v; upper phase).

[c] Not detected due to insensitivity of detecting reagent.

Figure 14

Structure of the glycolipid sulfate (GLS) of Halobacterium cutirubrum:

~ 1-O-[O-β-D-galactopyranosyl-3'-sulfate-(1'→6')-O-α-D-mannopyranosyl-(1'→2')-O-α-D-glucopyranosyl]-2,3-di-O-phytanyl-sn-glycerol.

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

2,3-DIPHYTANYL-sn-GLYCEROL

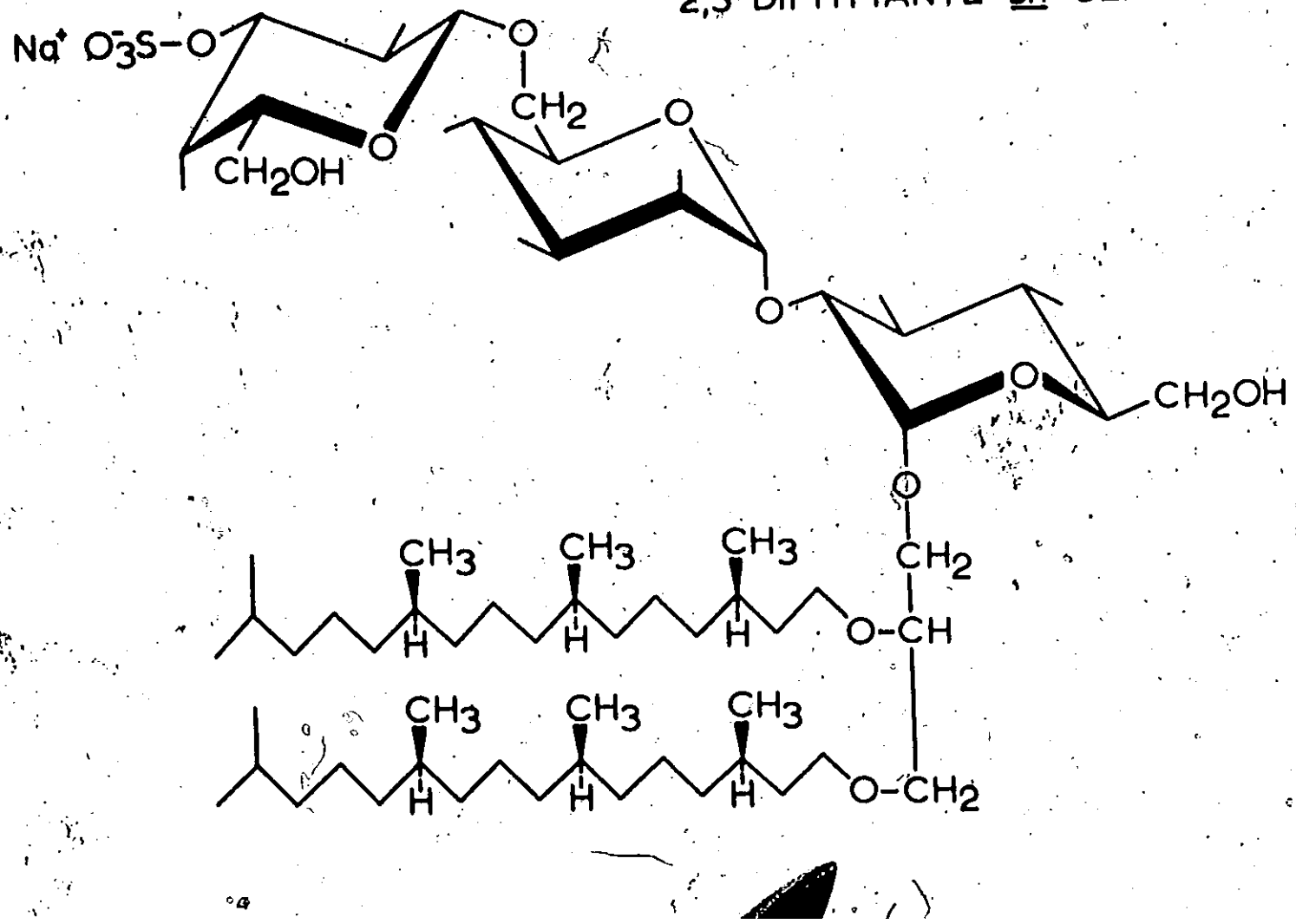


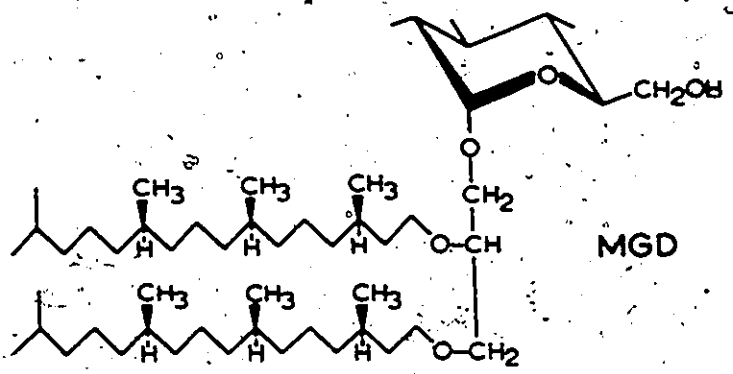
Figure 15

Structures of the glycolipids derived by partial acid hydrolysis of the glycolipid sulfate:

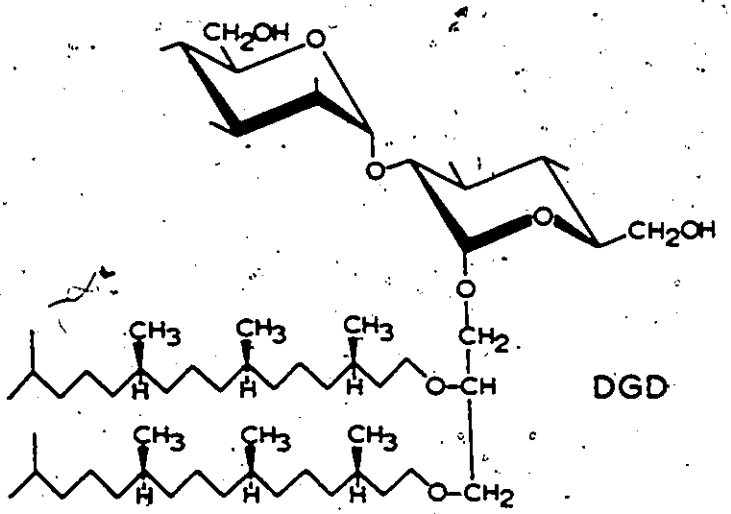
MGD 1-O-[O-α-D-glucopyranosyl]-2,3-di-O-phytanyl-sn-glycerol

DGD 1-O-[O-α-D-mannopyranosyl-(1'→2')-O-α-D-glucopyranosyl]-2,3-di-O-phytanyl-sn-glycerol

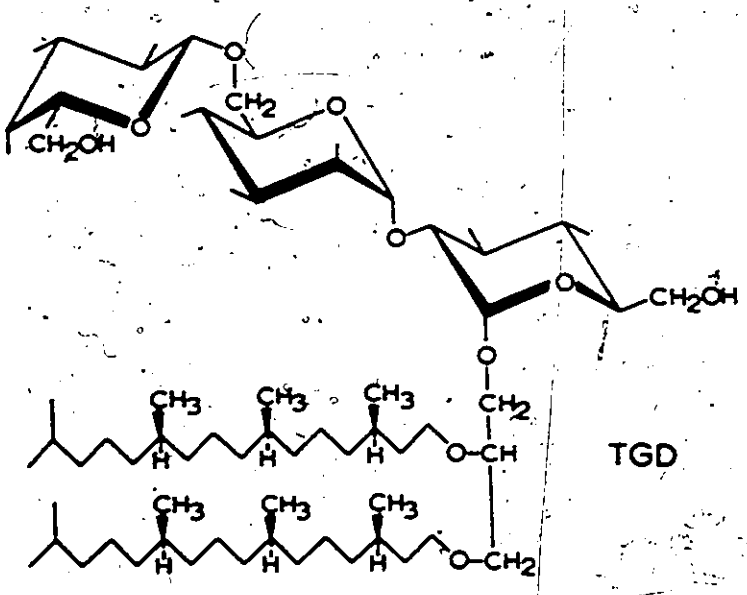
TGD 1-O-[O-ß-D-galactopyranosyl-(1'→6')-O-α-D-mannopyranosyl-(1'→2')-O-α-D-glucopyranosyl]-2,3-di-O-phytanyl-sn-glycerol.


CH2OH
O
CH3
CH3
CH3
CH2
H
O-CH
O-CH2

MGD


CH2OH
O
CH2OH
CH3
CH3
CH3
CH2
H
O-CH
O-CH2

DGD


CH2
O
CH2OH
CH2OH
CH3
CH3
CH3
CH2
H
O-CH
O-CH2

TGD

galactose-sulfate containing glycolipids so far studied (Table 1). Furthermore, galactose-containing glycolipid sulfates generally have the sulfate on the 3-position of the galactose (Table 1). Clearly the enzymes involved in the sulfation of glycolipids are highly specific both for the sugar and for the position that is sulfated.

VIII. Possible Roles of the Glycolipid Sulfate in Membrane Structure and Function

Since the glycolipid sulfate is a major lipid component comprising about 23% by weight (20 mole %) of the total polar lipids of Halobacterium cutirubrum, it would seem reasonable that this lipid is a major structural component of the cell membrane. The results of the pulse-chase experiments described in Part Two of this thesis are consistent with the concept that the glycolipid sulfate is a structural component of the cell membrane rather than a biosynthetic intermediate, for example, in the biosynthesis of the cell wall. The possible roles of the glycolipid sulfate in membrane structure will now be described.

1. Effect of the Glycolipid Sulfate on the Structure and Osmometric Properties of Artificial Bilayers

Recent work by Chen et al. (1974) has shown that when the total membrane lipids of Halobacterium cutirubrum are dispersed in water at room temperature they probably exist in a lamellar liquid-crystalline state. Electron microscopy studies on these lipid dispersions revealed the existence of multibilayer structures. These structures were closed liposomal systems since they behaved as ideal osmometers in the range 0.005 M to 0.2 M sodium chloride.

In attempts to make liposomes with the individual polar lipids or artificial mixtures of these components, only the glycolipid sulfate alone or mixtures of the glycolipid sulfate with the other polar lipids formed liposomes exhibiting ideal osmometric behavior. Neither PGP, PGS nor PG alone showed multibilayer liposomal structures. The authors suggested that the polar head groups of PG, PGS and PGP "may be too small relative to the effective cross-sectional area of the phytanyl chains for stable bilayer formation and that the glycolipid sulfate with its relatively large polar head may be needed for proper packing of these polar lipids into a stable bilayer structure."

2. Localization of the Glycolipid Sulfate within the Cellular Membrane

Recently, Kushwaha and Kates (1973, 1974) isolated two membrane fractions from water dialyzed cells of Halobacterium cutirubrum using essentially the procedure of Stoeckenius and Rowen (1967). Treatment of the lysate with deoxyribonuclease, followed by discontinuous sucrose density gradient centrifugation (18 h, 260,000xg, 5°C), yielded 1) a band of red membrane near the top of the 1.3 M (1.206 g/ml); and 2) a band of purple membrane (bacteriorhodopsin) at the interface of the 1.3 M and 1.5 M (1.244 g/ml) bands. The red and purple membranes were isolated from the gradient, purified by recentrifugation and finally dialyzed against water to remove sucrose.

Examination of the total lipids extracted from each of these membrane fractions by the Bligh and Dyer procedure using two dimensional TLC showed that the glycolipid sulfate, triglycosyl diphytanyl glycerol

and phosphatidyl glycerol sulfate are localized exclusively in the purple membrane. Hence GLS (and PGS) may be involved in a photophosphorylation function of the purple membrane.

3. Function of the Glycolipid Sulfate in the Purple Membrane

Oesterhelt and Stoeckenius (1973) reported that when cells of Halobacterium halobium are grown anaerobically in the presence of light there is a dramatic increase in the amount of purple membrane produced in the cells (this has been confirmed with H. cutirubrum (Kushwaha and Kates, 1974; Can. J. Biochem. In press)). These workers showed that strong illumination of either the intact cells or the isolated purple membrane induced a reversible conformational change in the protein (bacteriorhodopsin) of the purple membrane. This conformational change was accompanied by a decrease in the pH of the medium; removal of the light source was followed by a rise in pH. The authors proposed that the light-generated proton gradient across the cell membrane arose from the uptake and release of protons by the asymmetrically oriented bacteriorhodopsin rapidly oscillating between its conformational forms in the purple membrane. This electrochemical gradient was considered to play an important role in ATP formation in H. halobium in the light as in Mitchell's chemiosmotic theory (1966, 1972).

Danon and Stoeckenius (1973) indeed have shown that ATP levels in cells with and without purple membrane were depleted under anaerobic conditions in the dark but were restored only in the cells with purple membranes when exposed to light.

It is possible that the role of the glycolipid sulfate in the purple membrane of Halobacterium cutirubrum will be directly related to the structural features of the molecule. In addition to the exceptionally large size of the polar head group (i.e., the trisaccharide moiety) one must consider the properties that the sulfate group bestows upon the molecule. The extremely low pK of both the sulfate and the sulfonate group make these moieties unique in biological systems; it is probable that these groups will be ionized under most if not all biological conditions and that a counterion will be present under nearly all circumstances. The principal role of the sulfolipids may be the transport of the counterion. Systems such as the stomach, in which the extremely low pH is maintained, are likely to contain a sulfate ester or sulfonate as the proton carrier, since few other organic groups (if any) can maintain such a low pH (Haines, 1973).

Recently, Racker and Stoeckenius (1974) incorporated the purple membrane from Halobacterium halobium into artificial phospholipid vesicles. Contrary to the finding with intact cells, these reconstituted vesicles took up protons when illuminated and subsequently released them in the dark. Vesicles formed from purple membrane, soybean phospholipids and bovine heart mitochondrial ATPase were able to catalyze light-dependent ATP formation: The authors concluded that "these vesicles represent a simple model system for a biological proton pump capable of generating ATP from ADP and P_i." These results however, are not definitive since the system used was highly artificial; e.g., ATPase is not present in halophiles and soybean phospholipids differ considerably from halophile lipids both with respect to hydrocarbon chains and polar head groups.

PART TWO

STUDIES ON THE METABOLISM OF THE LIPID COMPONENTS OF HALOBACTERIUM CUTIRUBRUM

EXPERIMENTAL PROCEDURES

1. In Vivo Incorporation of [^{35}S]Sulfate; [1(3)-^{14}C]Glycerol and [U-^{14}C]Glucose

Cells of Halobacterium cutirubrum were grown to late log phase in 100 ml of standard medium (see Materials and Methods, II.3.(a)) in a 500 ml side-arm Erlenmeyer flask. The cells were harvested at 800xg in an RC2-B centrifuge at 10°C and washed twice with 50 ml of basal salt solution (Materials and Methods, III). The washed cells were resuspended in 2 ml basal salt solution and added to 100 ml of either low phosphate or low phosphate - low sulfate medium in a 500 ml side-arm Erlenmeyer flask to give an optical density of ca. 0.4 at 660 nm. The flask was then pre-incubated in a Psycrotherm rotary shaker at 37°C and 120 oscillations per min at "low" light intensity. The optical density of the culture was monitored every 30 min. When a definite increase in optical density was observed (usually after 4 h) a solution of the radioactive tracer (usually 1.0 ml) in distilled water was added and the culture was immediately returned to the shaker at 37°C. Aliquots of the culture (5.0 ml for 1-30 min samples; 3.0 ml for the remainder) were removed at 1 min, 3 min, 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 16 h, 20 h and 24 h after addition of the tracer. The optical density of the culture was measured immediately prior to the removal of each aliquot.

Each aliquot of culture was immediately added to the appropriate volume of chloroform-methanol (2:1, v/v) to give a solvent ratio of chloroform-methanol-water of 1.0:2.0:0.8 (v/v/v); after several hours, appropriate amounts of chloroform and water were added to each extract to give two phases with a final solvent ratio of chloroform-methanol-water of 1.0:1.0:0.9 (v/v/v). After centrifugation at 1500 r.p.m. for 5 min the lower chloroform phase was removed with a Pasteur pipette, and the upper methanol-water phase was re-extracted twice with 10 ml of chloroform. The combined chloroform phase and washings were blown down under a stream of nitrogen to a volume of 10 ml and washed twice with 1 ml of methanol-water (10:9, v/v). The chloroform extract was diluted with benzene and taken to dryness under a nitrogen stream and the residue re-dissolved in 2.0 ml of chloroform-methanol (2:1, v/v). Suitable aliquots were transferred to scintillation vials, the solvent was removed under nitrogen, scintillation fluid was added and the samples were counted (see Materials and Methods, VIII.1). Suitable aliquots of the labelled total lipids were subjected to thin layer chromatography. The early time samples contained relatively few counts, late time samples contained a large number of counts and therefore only part of the sample was used, the size of the aliquot depending on the total number of counts present.

^{14}C-labelled total lipids were subjected to two-dimensional TLC, the plates being developed first in chloroform-methanol-conc. ammonium hydroxide (65:35:5, v/v/v), then, after drying in the fume hood for about 30 min to remove most of the ammonia, in chloroform-90% acetic acid-methanol (30:20:4, v/v/v). ^{35}S-labelled lipids were subjected

to one-dimensional TLC in the latter solvent. The plates were then allowed to dry overnight to remove most of the acetic acid from the silica and then exposed to X-ray film for a period of time dependent on the total number of counts applied to each plate. Radioactive spots were scraped from the plates and counted as described in Materials and Methods, VIII.

II. Chase Studies with Labelled Cells

After removal of the 24 h sample in the in vivo incorporation experiment (see previous section), the remaining cells (46 ml of culture) were harvested at 8000xg at 10°C, washed once with "cold" medium and re-centrifuged. The washed cells were then suspended to an $O.D._{660}$ of 0.4 in 100 ml of "cold" medium in a 500 ml side-arm flask. An aliquot was immediately removed and used for a zero time sample. Aliquots of this culture were removed at 1 min, 3 min, 5 min, 10 min, 15 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12 h, 16 h, 20 h and 24 h and extracted as described in the preceding section. Suitable aliquots of the total lipids were counted. Aliquots of the remaining total lipids were chromatographed on TLC plates, the radioactive spots detected by autoradiography and counted, as described in the previous section.

III. Effect of Light on [^{35}S]Sulfate Incorporation into the Lipids of Halobacterium cutirubrum

An experiment was designed to determine whether or not light affects the amount of [^{35}S]sulfate incorporated into the lipids of Halobacterium cutirubrum. A culture was grown under low aeration and high light intensity in order to increase the amount of purple membrane

formation. When the cells reached stationary phase, they were harvested, resuspended in 200 ml of low sulfate medium in a 500 ml side-arm flask and incubated at 37°C under low aeration and high light intensity. The $O.D._{660}$ of the culture was monitored to determine when the cells started growing. When the cells entered the log phase of growth, 1 mCi of [^{35}S] sulfate was added and a 3.0 ml sample was removed at zero time. The culture was then immediately divided into two equal volumes which were placed in identical 500 ml side-arm flasks. One of the flasks had previously been wrapped in several layers of dark green plastic in order to exclude light from the flask. The cultures were incubated in the Psycrotherm incubator at 80 oscillations per min and 37°C in the light (bank of eight 20-in. "cool white" fluorescent lights). The $O.D._{660}$ of the cultures was monitored and 3.0 ml aliquots of the cultures were removed at suitable time intervals, extracted and worked up as described in the previous section.

RESULTS AND DISCUSSION

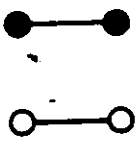
I. In Vivo Incorporation of [^{35}S]Sulfate into the Sulfolipids of Halobacterium cutirubrum

Figure 16 shows the growth curve for the cells during the incorporation experiment under aerobic conditions and low light intensity. The [^{35}S]sulfate was added when the culture was in early log phase (taken as zero time) after a short lag period, and ^{35}S incorporation into the lipids was followed during the logarithmic phase (0 to 6 h) and into the early (6 to 20 h) and late (20 to 24 h) stationary phase of growth. The [^{35}S]sulfate incorporation into the cellular lipids (Figure 16) is expressed in terms of d.p.m. per 100 ml of culture. During logarithmic growth (0 to 6 h) incorporation of the label was rapid but decreased gradually, as the rate of cellular growth decreased, to a level of 800 x 10^3 d.p.m. per 100 ml of culture late in the stationary phase (24 h).

The distribution of the incorporated [^{35}S]sulfate among the individual lipids during the growth cycle is shown in the autoradiogram presented in Figure 17; the results are also given quantitatively in Table 9. Up to the first 30 min, all of the ^{35}S remained at the origin and was presumably due to traces of [^{35}S]sulfate carried over during the extraction. The first compound labelled with ^{35}S was the glycolipid sulfate, followed by phosphatidyl glycerosulfate and finally by the very polar unidentified sulfoglycolipid. On a percentage basis (Figure 18 and Table 9) the ^{35}S in the glycolipid sulfate up to 1 h accounted for 100% of the [^{35}S]sulfate incorporated into the lipids; thereafter, the

Figure 16

Incorporation of [^{35}S]sulfate into the total lipids of *H. cutirubrum* cells during aerobic growth under "low" intensity light:



● growth curve for the cells during the incorporation experiment

○ activity of the total lipids

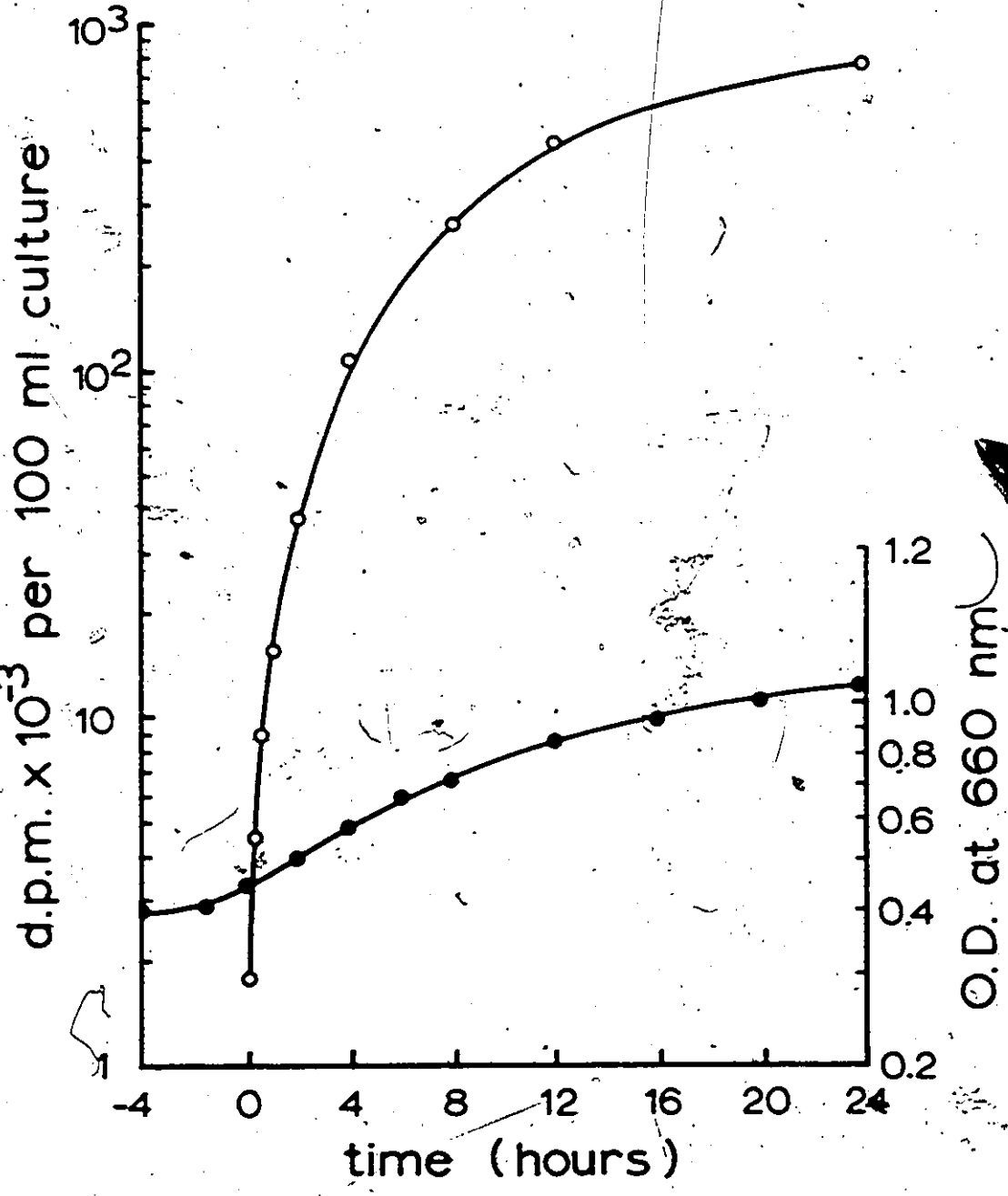

10^3
10^2
10
1
d.p.m. x 10^{-3} per 100 ml culture
1.2
1.0
0.8
0.6
0.4
0.2
O.D. at 660 nm
-4
0
4
8
12
16
20
24
time (hours)

Figure 17

Autoradiogram showing the distribution of the incorporated [^{35}S]sulfate among the sulfolipids of H. cutirubrum.

Solvent System:

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

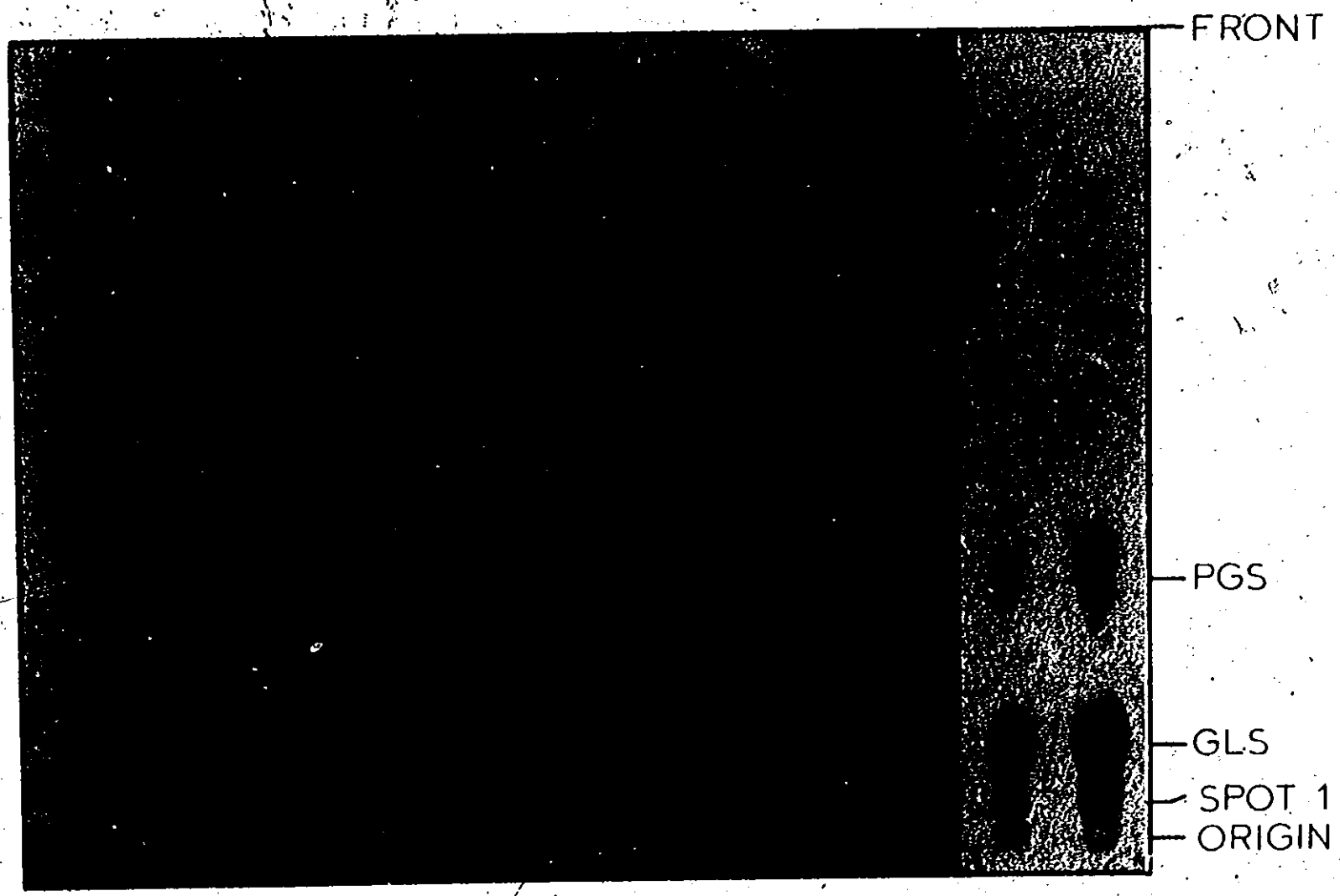

FRONT
PGS
GLS
SPOT 1
ORIGIN
1m 3m 5m 10m 15m 30m 1h 2h 4h 8h 12h 24h
time

Table 9. Distribution of ^{35}S activity among lipid components of *Halobacterium cutirubrum* cells grown under aerobic conditions and "low" intensity light in the presence of [^{35}S]sulfate

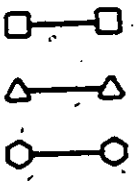
Time	Activity (d.p.m. x 10^{-3} per 100 ml of culture)		
	Glycolipid sulfate	Phosphatidyl glycero-sulfate	Unidentified sulfoglycolipid
15 min[a]	0	0	0
30 min	0.7 (100%)[b]	0	0
1 h	7.1 (100%)	0	0
2 h	27.7 (96.9%)	0.9 (3.1%)	0
4 h	93.6 (95.9%)	4.0 (4.1%)	0
8 h	245.0 (92.8%)	12.5 (4.7%)	6.6 (2.5%)
12 h	415.8 (90.9%)	25.4 (5.6%)	16.2 (3.5%)
24 h	719.7 (88.2%)	53.7 (6.6%)	42.6 (5.2%)

[a] No counts were in any of the lipid components at earlier times.

[b] The values in the brackets are the radioactivities of the lipid components expressed as percentages of the total ^{35}S found in the lipids recovered from the plate.

Figure 18

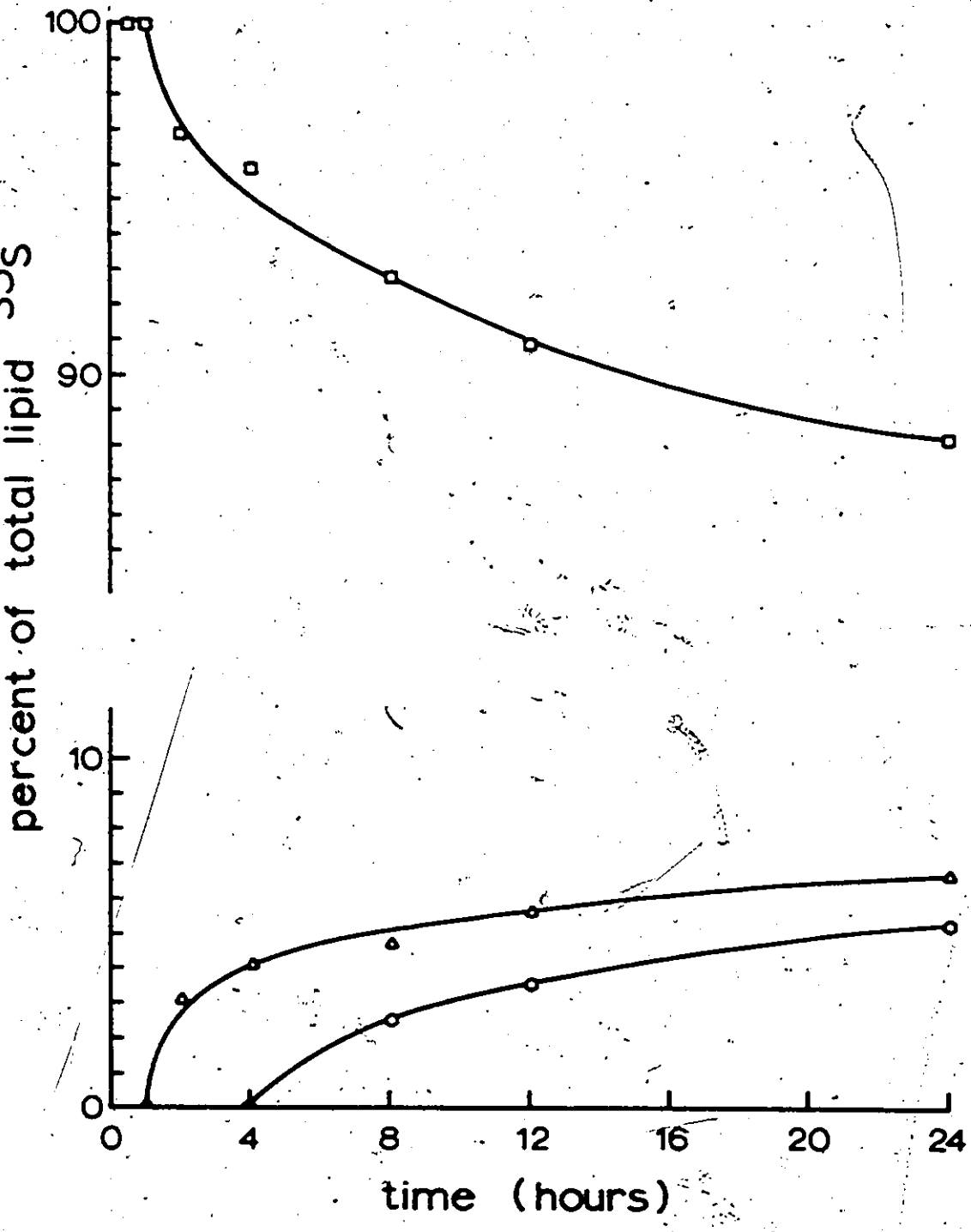
Percent of the total lipid-^{35}S found in each of the sulfolipids during the incorporation of [^{35}S]sulfate by H. cutirubrum cells growing aerobically under "low" intensity light:



glycolipid sulfate

phosphatidyl glycerosulfate

unidentified sulfoglycolipid (Spot 1 in the autoradiograms)


100
90
10
0
percent of total lipid ^{35}S
0
4
8
12
16
20
24
time (hours)

percentage of label in GLS decreased gradually to a level of ca. 88% at 24 h. The percentage of the label in the phosphatidyl glycerosulfate increased gradually after 1 h up to about 7% of the total counts at 24 h. The unidentified sulfoglycolipid was first labelled between 4 and 8 h (i.e. when the cells were in late log phase) and eventually accumulated about 5% of the total [^{35}S]sulfate incorporated into the lipids.

It should be pointed out that the decrease in the proportion of ^{35}S in GLS with concomitant increase of activity in PGS and the unknown sulfoglycolipid does not necessarily indicate a product-precursor relationship. Biosynthesis of each component may simply be following independent pathways. This will be discussed further in the next section.

II. Chase Experiment with [^{35}S]Sulfate-Labelled Cells

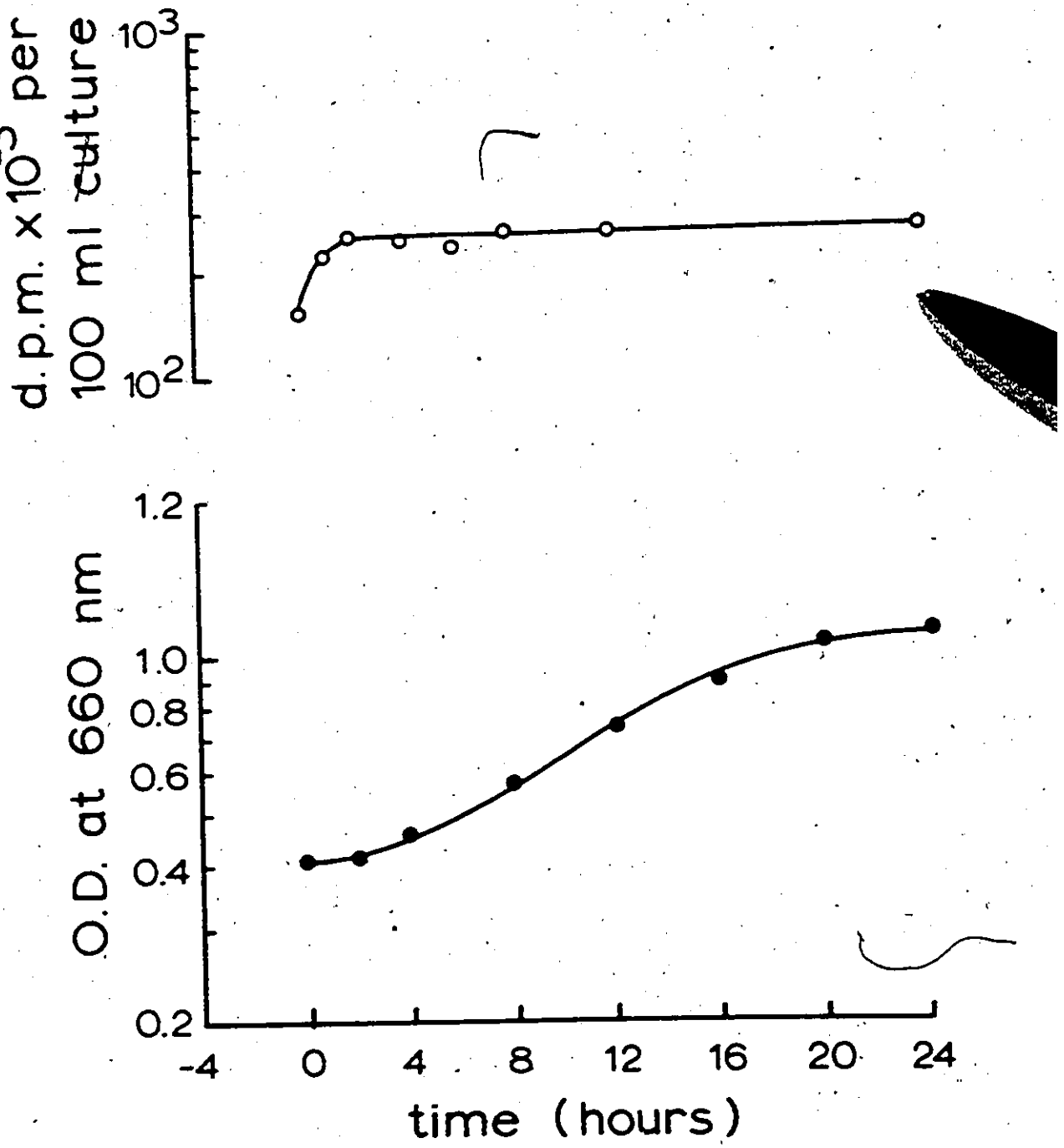
Cells labelled with [^{35}S]sulfate were resuspended in "cold" medium and the ^{35}S content of the lipids was followed with time. Rapid increase in the total lipid [^{35}S]sulfate occurred during the lag phase in the first two hours of the chase experiment, after which the ^{35}S activity remained constant for the next 22 h (Figure 19). The initial increase was probably due to [^{35}S]sulfate still adhering to the cells or to the intracellular presence of either free [^{35}S]sulfate or [^{35}S] PAPS. The fact that no decrease in the total counts was observed during logarithmic growth (2 to 16 h) or during the subsequent stationary phase (16 to 24 h) indicates that there is no transfer of lipid-^{35}S to non-lipid components of the cell or breakdown to water-soluble products. The distribution of [^{35}S]sulfate activity in each of the sulfolipid components

Figure 19

Activity of ^{35}S remaining in the total lipids when [^{35}S] sulfate-labelled cells were grown in "cold" medium:

●——● growth curve for the cells during the chase experiment

○——○ activity of the total lipids


d.p.m. x10^-3 per 100 ml culture
10^3
10^2
O.D. at 660 nm
1.2
1.0
0.8
0.6
0.4
0.2
-4
0
4
8
12
16
20
24
time (hours)

during the "chase" experiment is shown qualitatively in the autoradiogram presented in Figure 20, and quantitatively in Figure 21 and Table 10. The percentage of ^{35}S in each component remained constant throughout the chase: 85% in GLS, 8% in PGS and 7% in the unidentified sulfoglycolipid. These results clearly indicate that there is no turnover of sulfate among the lipids, i.e., none of the sulfolipids is a metabolic precursor or sulfate-transferring agent for any of the other lipids, and none undergoes hydrolysis of its sulfate group.

Hancock (1972) speculated that phosphatidyl glycerosulfate might be an intermediate in the biosynthesis of phosphatidyl glycerol and phosphatidyl glycerophosphate (see Scheme 6). Desulfation of PGS could account for the biosynthesis of phosphatidyl glycerol while subsequent phosphorylation of PG would yield phosphatidyl glycerophosphate with the correct stereochemistry. If PGS is a precursor of PG and PGP by the pathway shown in Scheme 6, [^{35}S]sulfate should be lost from PGS during the "chase" experiment. The absence of turnover of the ^{35}S in the PGS would seem to rule out the possibility of the existence of this pathway.

The lack of turnover of ^{35}S-labelled lipids would seem to indicate that H. cutirubrum contains neither sulfatases or glycosidases capable of hydrolyzing the sulfate group or glycosidic groups, respectively, of the glycolipid sulfate. However, this possibility should be tested by direct attempts to demonstrate the presence of sulfate or glycosidase activity in cell-free extracts.

Figure 20

Autoradiogram showing the distribution of ^{35}S among the sulfolipids of H. cutirubrum during the chase experiment.

Solvent System:

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

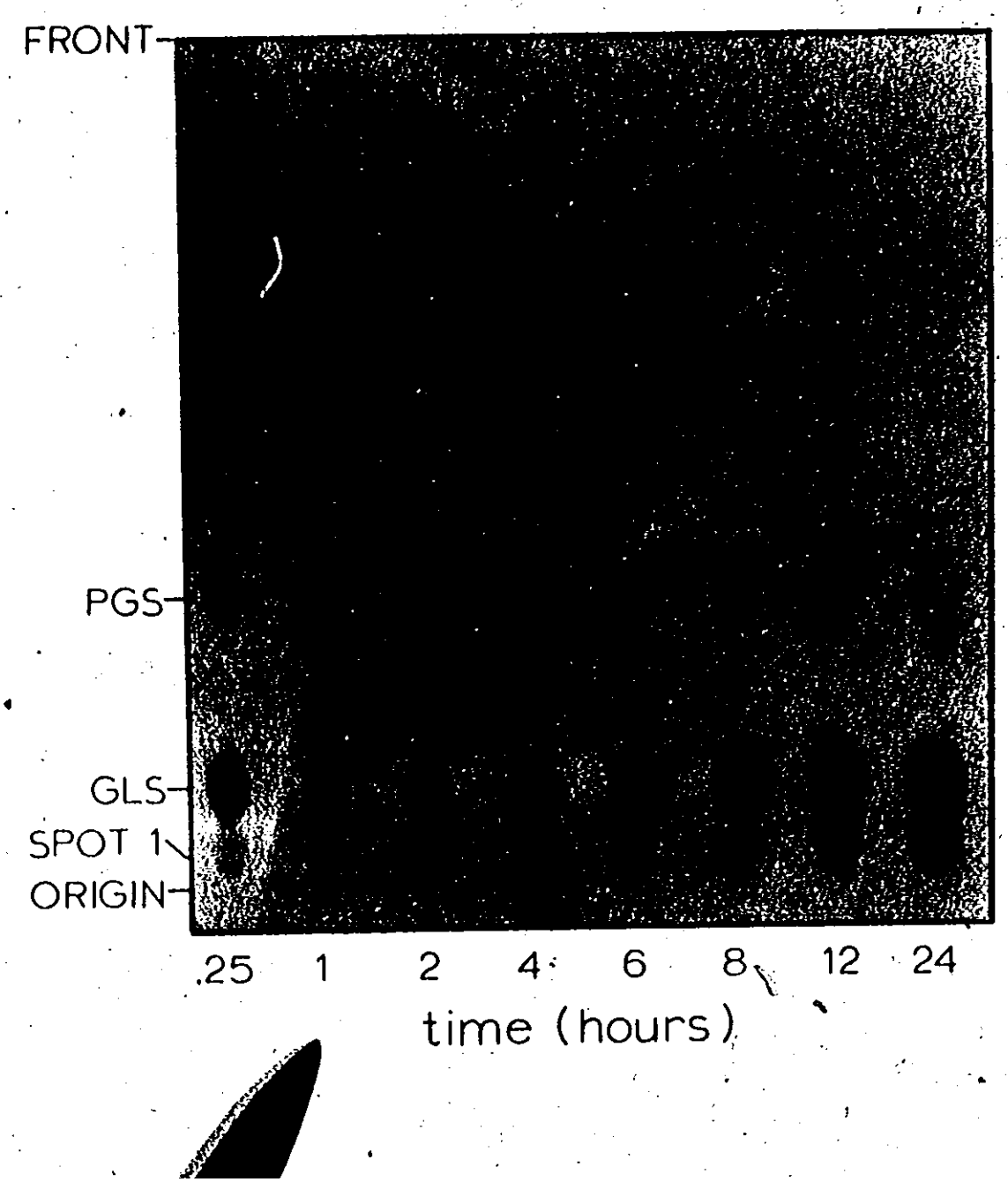

FRONT
PGS
GLS
SPOT 1
ORIGIN
.25
1
2
4
6
8
12
24
time (hours)

Figure 21

Percent of the total lipid-^{35}S remaining in each of the sulfolipids during the chase experiment:


GLS


PGS


unidentified sulfoglycolipid (Spot 1 in the autoradiograms)

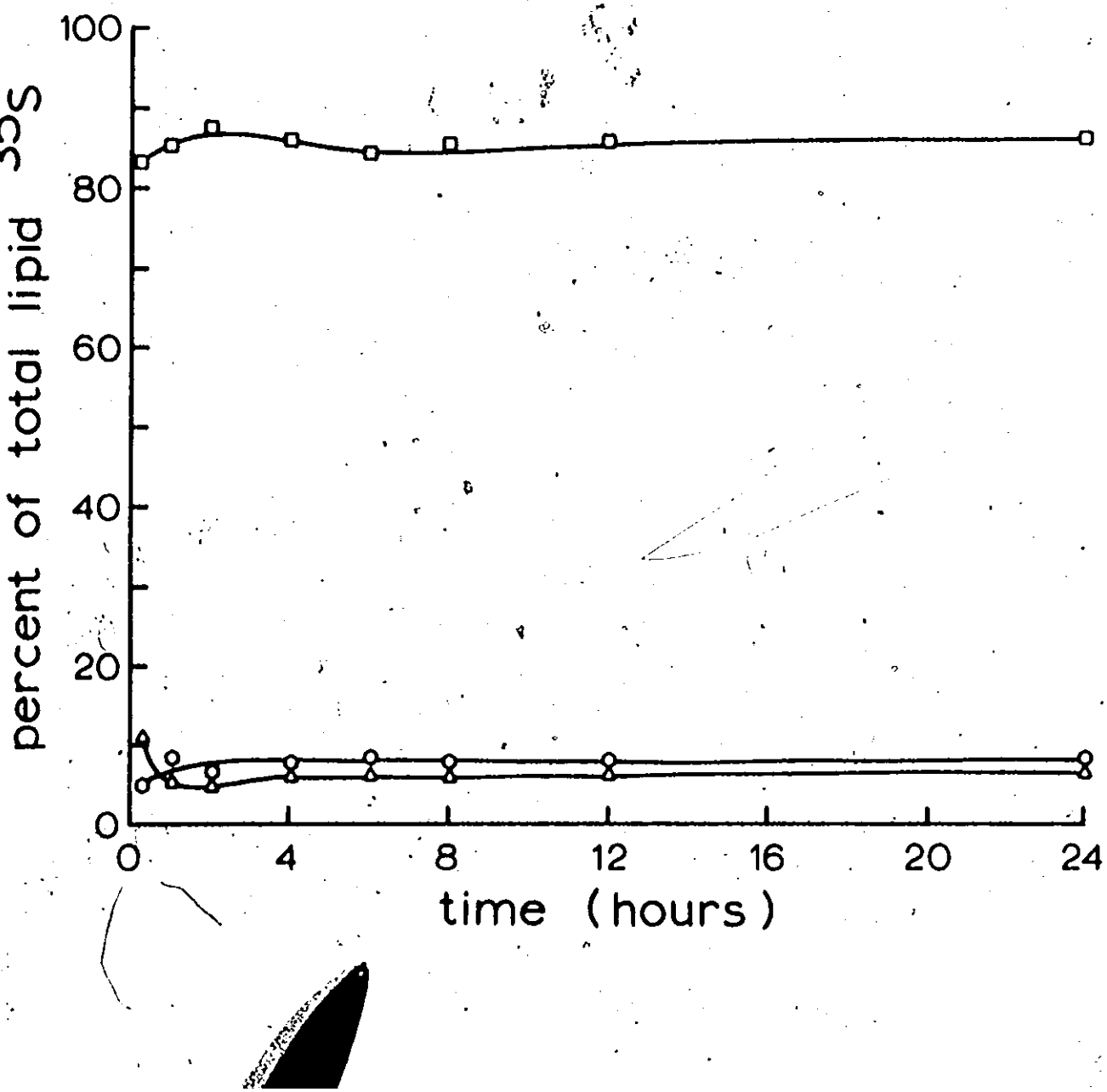

100
80
60
40
20
0
percent of total lipid ^{35}S
0
4
8
12
16
20
24
time (hours)

Table 10. Distribution of ^{35}S activity among lipid components of *Halobacterium cutirubrum* when ^{35}S-labelled cells were grown in "cold" medium

Time	Activity (d.p.m. x 10^{-3} per 100 ml culture)		
	Glycolipid sulfate	Phosphatidyl glycerol sulfate	Unidentified sulfoglycolipid
15 min	127.3 (83.8%)[a]	16.6 (10.9%)	8.0 (5.3%)
1 h	191.1 (85.7%)	12.5 (5.6%)	19.3 (8.7%)
2 h	224.4 (88.1%)	13.6 (5.3%)	16.6 (6.5%)
4 h	212.6 (86.0%)	15.5 (6.3%)	19.0 (7.7%)
6 h	200.0 (84.7%)	15.1 (6.4%)	21.1 (8.9%)
8 h	225.6 (85.6%)	16.3 (6.2%)	21.6 (8.2%)
12 h	230.6 (85.7%)	17.0 (6.3%)	21.3 (7.9%)
24 h	237.2 (85.1%)	18.3 (6.6%)	23.3 (8.4%)

[a] The values in brackets are the percentages of the total ^{35}S found in the lipids.

Scheme 6

Proposed pathways for the metabolism of phosphatidyl glycerosulfate

A Proposed pathway for the biosynthesis of phosphatidyl glycerol sulfate (PGS) (Hancock, 1972)

B Proposed pathway for the biosynthesis of PG and PGP from PGS (Hancock, 1972).

A

(1) sn-3-GP $\xrightarrow{\text{PAPS}}$ sn-glycerol-1-sulfate-3-phosphate $\xrightarrow{-P_i}$ sn-1-GS

(2) sn-1-GS + CDP-2,3-diphytanyl-sn-glycerol → 1-sn-phosphatidyl-3'-sn-glycerol-1'-sulfate (PGS)

B

(3) 1-sn-phosphatidyl-sn-3'-glycero-1'-sulfate $\xrightarrow{-SO_4^{2-}}$ 1-sn-phosphatidyl-sn-3'-glycerol (PG)

(4) 1-sn-phosphatidyl-sn-3'-glycerol $\xrightarrow{\text{ATP}}$ 1-sn-phosphatidyl-sn-3'-glycero-1'-phosphate (PGP)

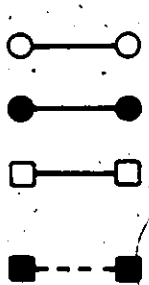
III. Effect of Light on [^{35}S]Sulfate Incorporation into the Lipids of Halobacterium cutirubrum

As discussed in Part One, Results and Discussion, VIII.3, cells of H. cutirubrum grown under conditions of low aeration and high light intensity contain quantitatively much more purple membrane than cells grown under high aeration and low light intensity. Since the sulfolipids were found to be localized in the purple membrane (see Part One, Results and Discussion, VIII.2) it seemed reasonable that cells growing under conditions of low aeration and high light intensity might synthesize the sulfolipids at a higher rate than cells growing under low light intensity and should therefore have a higher rate of incorporation of [^{35}S]sulfate into sulfolipids. In an experiment designed to test this hypothesis (see Part Two, Experimental Procedures, III), cells containing a high proportion of purple membrane were incubated with [^{35}S]sulfate in the light and the dark under low aeration. The growth curve for the cells and the total [^{35}S]sulfate incorporated into the lipids are shown in Figure 22. The growth rate of the cells was the same both in the light and in the dark and was considerably lower than in the previous experiments where high aeration was used (compare with Figure 16). There was no appreciable difference in the amount of [^{35}S]sulfate incorporated by cells grown in the light versus cells grown in the dark (Figure 22). Furthermore, the proportions of [^{35}S]sulfate incorporated into each of the sulfolipids (Figure 23 and Table 11) were not significantly different.

These observations seem to indicate that H. cutirubrum is capable of making the glycolipid sulfate both in the light and in the

Figure 22

Incorporation of [^{35}S]sulfate into the total lipids of *H. cutirubrum* cells during growth both in the light and in the dark under "anaerobic" conditions:



○——○ growth curve for light-grown cells

●——● growth curve for dark-grown cells

□——□ activity of the total lipids of the light-grown cells

■- - -■ activity of the total lipids of the dark-grown cells

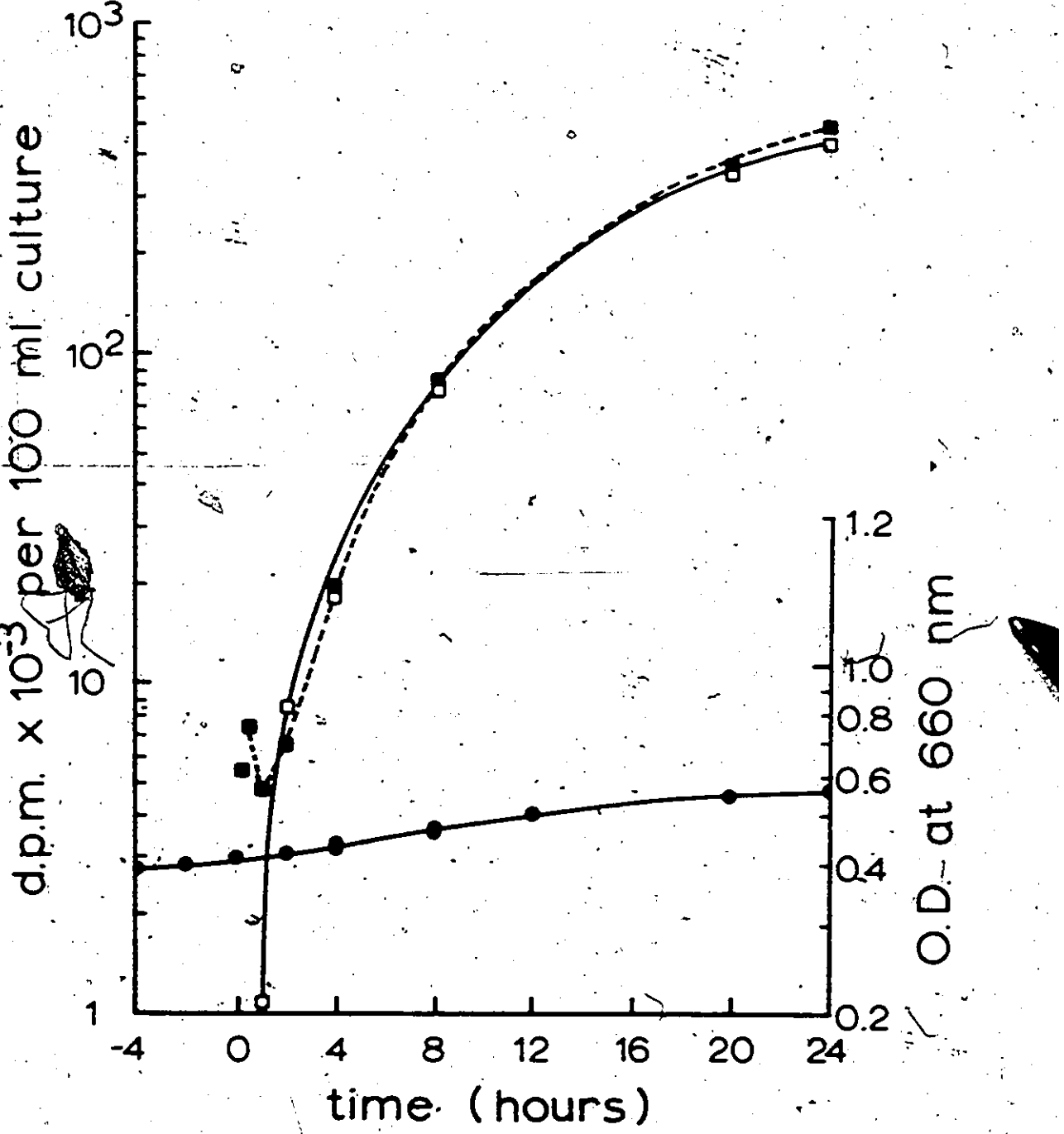
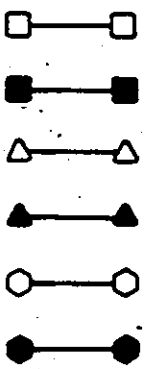

10^3
10^2
10
1
d.p.m. x 10^{-3} per 100 ml culture
1.2
1.0
0.8
0.6
0.4
0.2
O.D. at 660 nm
-4
0
4
8
12
16
20
24
time (hours)

Figure 23

Percent of the total lipid-^{35}S found in each of the sulfolipids during the incorporation of [^{35}S] sulfate by H. cutirubrum cells grown both in the light and in the dark under "anaerobic" conditions:



GLS in the light-grown cells

GLS in the dark-grown cells

PGS in the light-grown cells

PGS in the dark-grown cells

unidentified sulfoglycolipid in the light-grown cells

unidentified sulfoglycolipid in the dark-grown cells

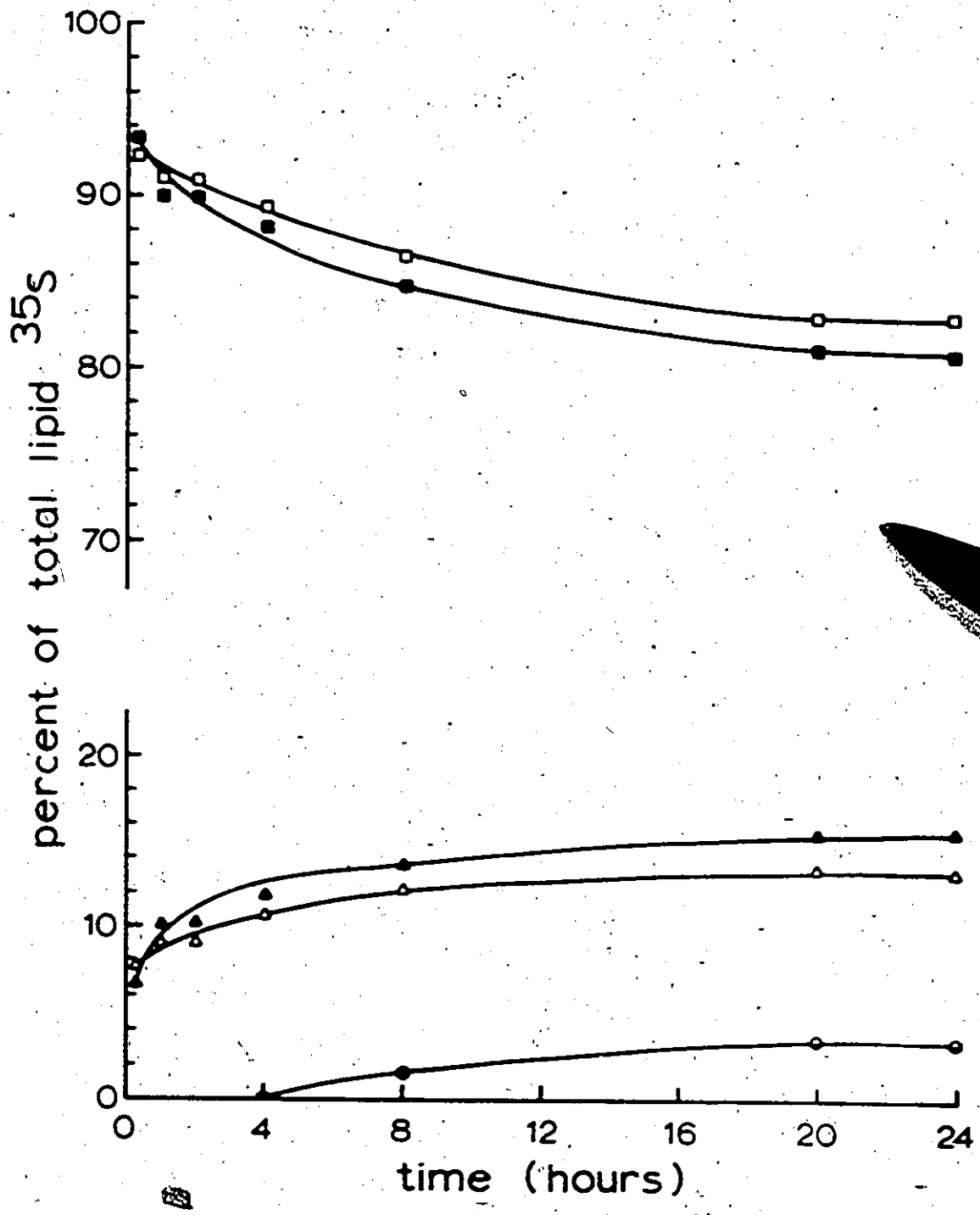

100
90
80
70
20
10
0
0
4
8
12
16
20
24
percent of total lipid ^{35}S
time (hours)

Table 11. Distribution of ^{35}S activity among the sulfolipids of Halobacterium cutirubrum cells grown "anaerobically" both in the dark and in the light in the presence of [^{35}S]sulfate (values in brackets are the percentages of the total ^{35}S found in the total lipids)

Time	Activity (d.p.m. x 10^{-3} per 100 ml culture)					
	Glycolipid sulfate		Phosphatidyl glycerosulfate		Unidentified sulfoglycolipid	
	Light	Dark	Light	Dark	Light	Dark
15 min	0.56 (92.3)	5.1 (93.3)	0.039 (7.7)	0.37 (6.7)	0	0
30 min	0.46 (91.2)	6.9 (91.8)	0.044 (8.8)	0.62 (8.2)	0	0
1 h	1.00 (91.0)	4.3 (90.0)	0.099 (9.0)	0.48 (10.0)	0	0
2 h	7.73 (90.9)	5.9 (89.9)	0.77 (9.1)	0.67 (10.1)	0	0
4 h	16.7 (89.4)	17.6 (88.2)	2.0 (10.6)	2.4 (11.8)	0	0
8 h	69.9 (86.6)	75.1 (84.9)	9.9 (12.2)	11.9 (13.5)	0.97 (1.2)	1.4 (1.6)
20 h	302.3 (83.2)	311.1 (81.4)	48.3 (13.3)	58.9 (15.4)	12.7 (3.5)	12.2 (3.2)
24 h	372.3 (83.3)	402.7 (81.1)	59.4 (13.3)	77.5 (15.6)	15.2 (3.5)	16.4 (3.3)

dark. However, it may be that the cells specifically incorporate the glycolipid sulfate into the purple membrane rather than into the red membrane. It was stated earlier (Part One, Results and Discussion, VIII) that the purple membrane is composed of lipid, retinal and a single species of protein. This protein might specifically interact with cellular lipid components such as the glycolipid sulfate. Such a specific interaction might account for the localization of the glycolipid sulfate in the purple membrane.

IV. In Vivo Incorporation of [1(3)-^{14}C]Glycerol into the Lipids of Halobacterium cutirubrum

Figure 24 shows the growth curve for the cells during the incorporation experiment under aerobic conditions and "low" light intensity. The [1(3)-^{14}C]glycerol was added when the culture was in early log phase. Figure 24 also shows the [^{14}C]glycerol incorporated into the cellular lipids in terms of d.p.m. per 100 ml culture. During early logarithmic growth (0-4 h) labelled glycerol was rapidly incorporated up to a level of ca. 1600 x 10^3 d.p.m. per 100 ml culture. Thereafter, the level of ^{14}C in the total lipids remained relatively constant even though the O.D.$_{660}$ of the culture continued to increase.

The distribution of the incorporated [^{14}C]glycerol among the individual lipids at 10 min and at 8 h after the addition of the label is shown in the autoradiograms presented in Figure 25; the results are also given quantitatively in Figure 26 and Table 12. The earliest-labelled compound was that designated Spot 6*which accounted for a maximum of ca. 47% of the total labelled lipids at 5 min after the

*Possibly an acylated PG on the basis of R_f values

Figure 24

Incorporation of [^{14}C]glycerol into the total lipids of *H. cutirubrum* cells during aerobic growth under "low" intensity light:

●——● growth curve for the cells during the incorporation experiment

○——○ activity of the total lipids

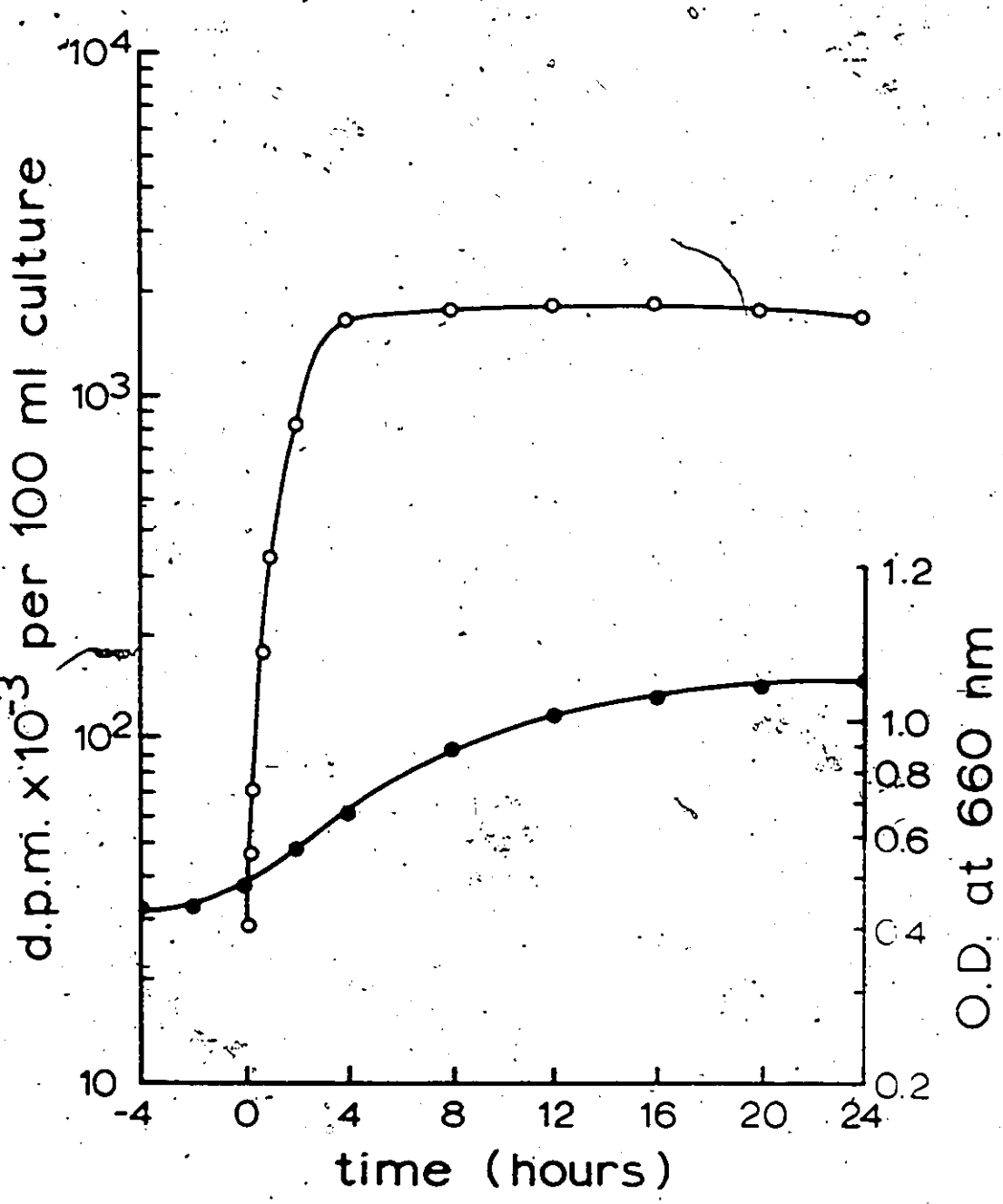

10^4
10^3
10^2
10
d.p.m. $\times 10^{-3}$ per 100 ml culture
1.2
1.0
0.8
0.6
0.4
0.2
O.D. at 660 hm
-4
0
4
8
12
16
20
24
time (hours)

Figure 25

Autoradiograms showing the distribution of ^{14}C among the total lipids of H. cutirubrum cells grown in the presence of [1(3)-^{14}C]glycerol:

A - 10 min sample

B - 8 h sample

Solvent Systems:

1 - Chloroform-methanol-conc. ammonium hydroxide (65:35:5, v/v/v)

2 - Chloroform-90% acetic acid-methanol (30:20:4, v/v/v)

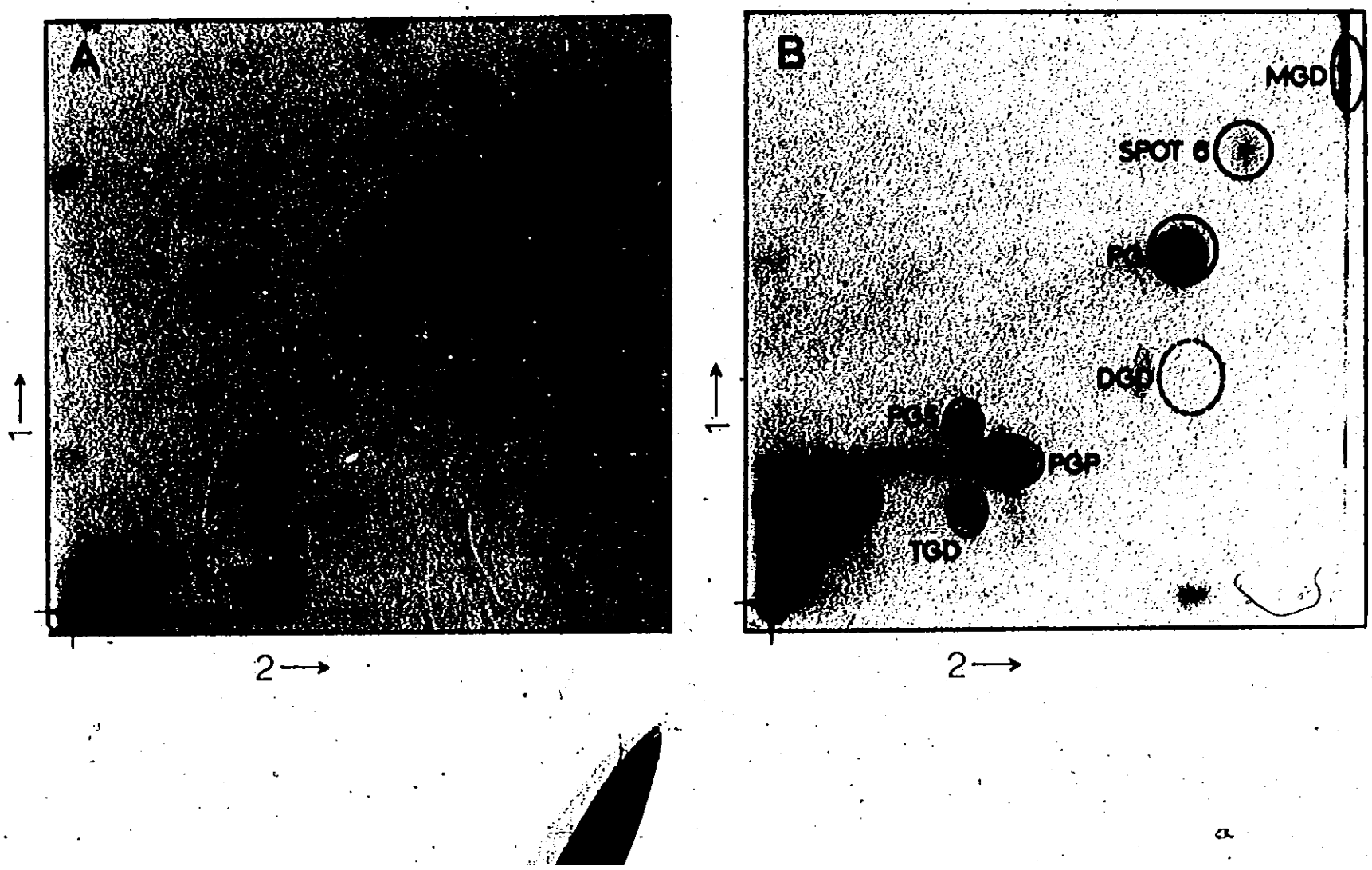
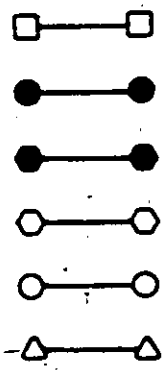

A
1
2
B
MGD
SPOT 6
PC
DGD
PGL
PGP
TGD
1
2

Figure 26

Percent of the total lipid-^{14}C found in the lipids of *H. cutirubrum* during the incorporation of [1(3)-^{14}C]glycerol by cells growing aerobically under "low" intensity light:



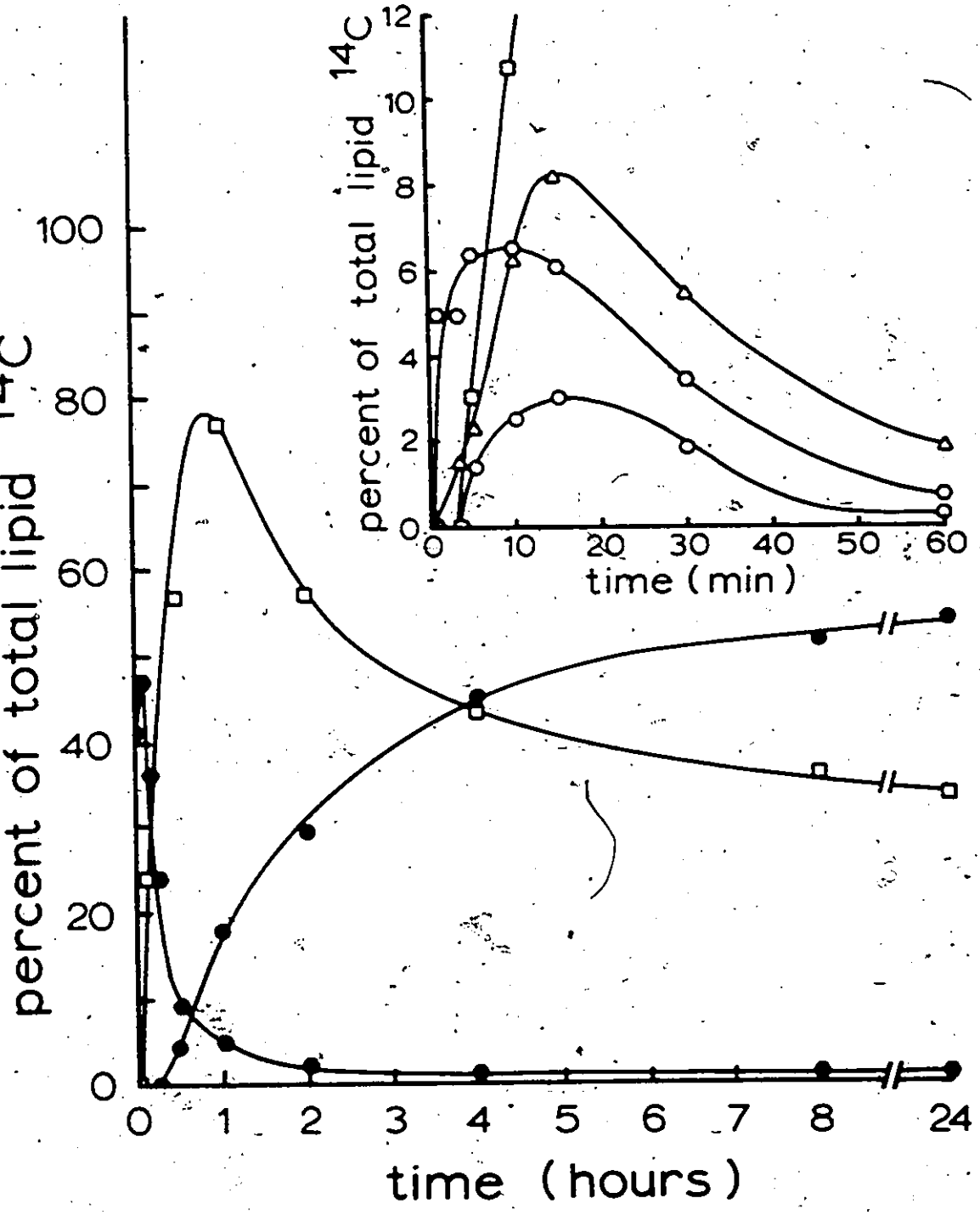

percent of total lipid ^{14}C
100
80
60
40
20
0
0 1 2 3 4 5 6 7 8 24
time (hours)
percent of total lipid ^{14}C
12
10
8
6
4
2
0
0 10 20 30 40 50 60
time (min)

Table 12. Distribution of ^{14}C activity among the lipids of Halobacterium cutirubrum cells grown under aerobic conditions and "low" intensity light in the presence of [1(3)-^{14}C]glycerol (values in brackets are the percentages of the total ^{14}C found in the total lipids)

Time	Activity (d.p.m. x 10^{-3} per 100 ml culture)							
	MGD	DGD	TGD	GLS	PG	PGS	PGP	Spot 6
1 min	4.9 (5.0)	0	0	0	6.8 (6.9)	26.7 (27.2)	0	40.3 (41.1)
3 min	8.3 (4.9)	0	2.6 (1.5)	0	12.6 (7.4)	36.2 (21.3)	0	70.0 (41.2)
5 min	18.5 (6.5)	3.7 (1.3)	6.6 (2.3)	8.8 (3.1)	21.4 (7.5)	33.9 (11.9)	0	133.1 (46.7)
10 min	30.4 (6.6)	11.5 (2.5)	28.5 (6.2)	49.7 (10.8)	25.8 (5.6)	61.2 (13.3)	0	163.3 (35.5)
15 min	42.7 (6.1)	21.0 (3.0)	57.4 (8.2)	170.1 (24.3)	32.2 (4.6)	104.3 (14.9)	0	165.2 (23.6)
30 min	60.5 (3.4)	32.0 (1.8)	97.9 (5.5)	1012.8 (56.9)	30.2 (1.7)	83.7 (4.7)	78.3 (4.4)	160.2 (9.0)
1 h	23.2 (0.7)	6.6 (0.2)	66.4 (2.0)	2573.0 (77.5)	46.5 (1.4)	99.6 (3.0)	597.6(18.0)	162.7 (4.9)
2 h	16.2 (0.2)	0	48.6 (0.6)	4689.9 (57.9)	105.3 (1.3)	251.1 (3.1)	2413.8(29.8)	162.0 (2.0)
4 h	0	0	16.1 (0.1)	6987.4 (43.4)	177.1 (1.1)	418.6 (2.6)	7261.1(45.1)	128.8 (0.8)
8 h	0	0	0	6295.2 (36.6)	154.8 (0.9)	309.6 (1.8)	8995.6(52.3)	120.4 (0.7)

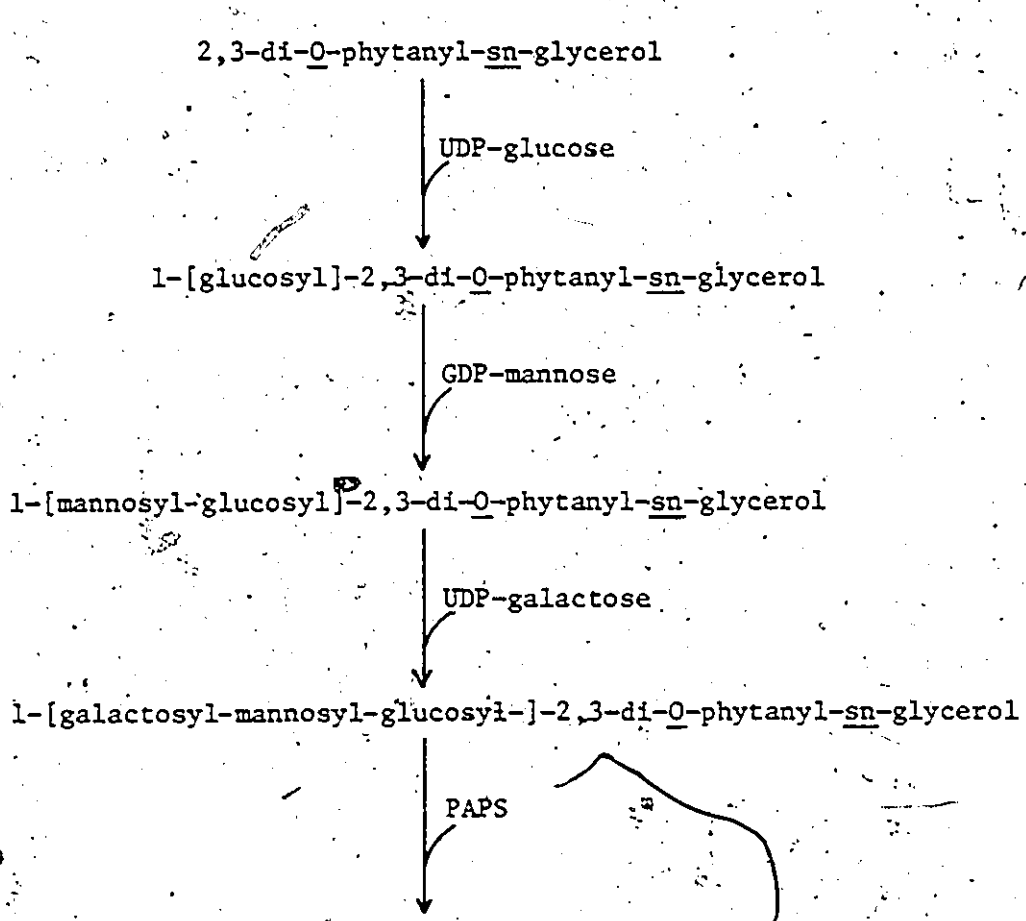
addition of the label. The second most rapidly labelled compound was phosphatidyl glycerol sulfate accounting for ca. 27% of the total label at 1 min. The glycolipid sulfate began to be labelled at around 5 min, the labelling increasing very rapidly to a level of ca. 77% of the total at 1 h. At this time, phosphatidyl glycerophosphate was rapidly labelled with a concomitant decrease (as a % of the total) in the proportion of label in the glycolipid sulfate. After 24 h about 55% of the total incorporated label was found in PGP and about 35% was found in GLS; the remaining lipids accounted for about 10% of the total label.

The incorporation of [^{14}C]glycerol into the mono-, di- and triglycosyl diphytanyl glycerols is shown in the inset in Figure 26. It can be seen that the monoglycosyl diphytanyl glycerol (MGD) is the first glycolipid to be labelled and is maximally labelled (as a percent of the total label) at about 5-7 min after the addition of the [^{14}C] glycerol. The labelling of the diglycosyl diphytanyl glycerol (DGD) reaches a maximum (percent of the total) at about 15 min, and that of the triglycosyl diphytanyl glycerol (TGD) begins at about 1 min and reaches a peak at about 15 min after the addition of the label. As was stated above, the glycolipid sulfate is labelled at a much slower initial rate, beginning at about 5 min and then increasing very rapidly to a maximum of ca. 77% of the total label at 1 h after the addition of the label.

The results obtained are consistent with the existence of a product-precursor relationship for these glycolipids based on the proposed biosynthetic pathway for the glycolipid sulfate shown in Scheme 7. The free 2,3-di-O-phytanyl-sn-glycerol required at the

Scheme 7

Proposed pathway for the biosynthesis of the glycolipid sulfate


2,3-di-O-phytanyl-sn-glycerol
UDP-glucose
1-[glucosyl]-2,3-di-O-phytanyl-sn-glycerol
GDP-mannose
1-[mannosyl-glucosyl]-2,3-di-O-phytanyl-sn-glycerol
UDP-galactose
1-[galactosyl-mannosyl-glucosyl-]-2,3-di-O-phytanyl-sn-glycerol
PAPS
glycolipid sulfate

beginning of the pathway has been shown to be present in H. cutirubrum (Hancock, 1972).

V. In Vivo Incorporation of ^{14}C from [U-^{14}C]Glucose into the Lipids of Halobacterium cutirubrum

Figure 27 shows the growth curve for the cells during the incorporation experiment under aerobic conditions and "low" light intensity. The [U-^{14}C]glucose was added when the culture was in early log phase (taken as zero time) after a short lag period, and ^{14}C incorporation into the lipids was followed during the logarithmic phase (0 to 8 h) and into the early (8 to 16 h) and late (16 to 24 h) stationary phase of growth. Incorporation of ^{14}C from [^{14}C]glucose into the total cellular lipids (Figure 27) is expressed in terms of d.p.m. per 100 ml of culture. During the logarithmic and early stationary phases of growth (0 to 16 h) incorporation of the label proceeded at a moderate rate (compared with the incorporation of [^{14}C] glycerol shown in Figure 24) up to a level of ca. 4000 d.p.m. per 100 ml of culture. Thereafter, the level of ^{14}C in the total lipids remained relatively constant through the stationary phase.

The distribution of the incorporated ^{14}C from [^{14}C]glucose among the individual lipids at 1 h and at 8 h after the addition of the label is shown in the autoradiograms presented in Figure 28; the results are also given quantitatively in Figure 29 and Table 13.

Compounds labelled with ^{14}C from [^{14}C]glucose included the glycolipids (MGD, DGD and TGD) and the glycolipid sulfate as well as PGP and an unknown compound (designated Spot 5 in the autoradiograms in

Figure 27

Incorporation of ^{14}C into the total lipids of H. cutirubrum cells grown aerobically in the presence of [U-^{14}C]glucose under "low" intensity light:

●——● growth curve for the cells

○——○ activity of the total lipids

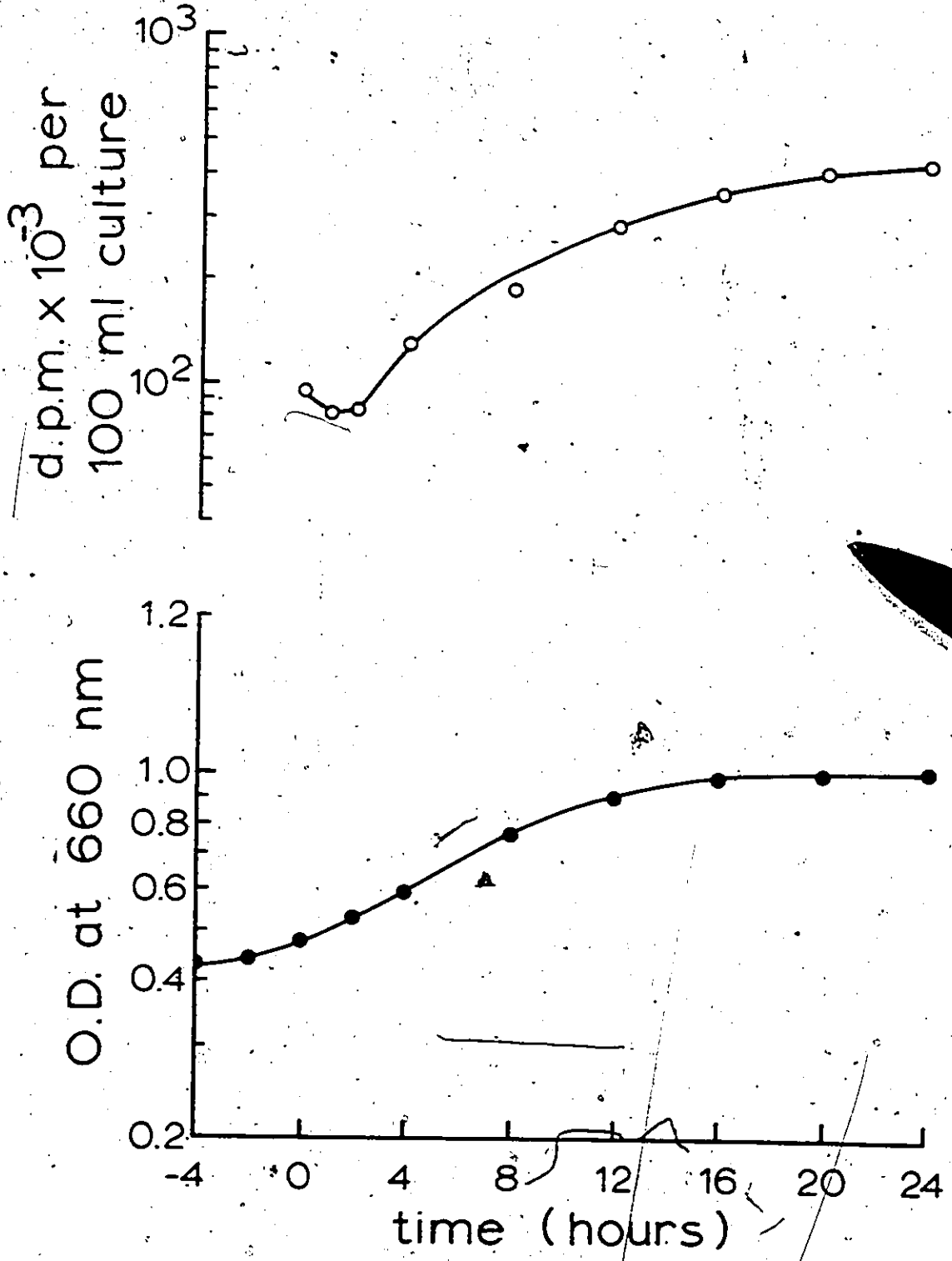

10^3
10^2
d.p.m. x 10^{-3} per 100 ml culture
1.2
1.0
0.8
0.6
0.4
0.2
O.D. at 660 nm
-4
0
4
8
12
16
20
24
time (hours)

Figure 28

Autoradiograms showing the distribution of ^{14}C among the total lipids of H. cutirubrum cells grown in the presence of [U-^{14}C]glucose:

A - 1 h sample

B - 8 h sample

Solvent Systems:

1 - Chloroform-methanol-conc. ammonium hydroxide (65:35:5, v/v/v)

2 - Chloroform-90% acetic acid-methanol (30:20:4, v/v/v)

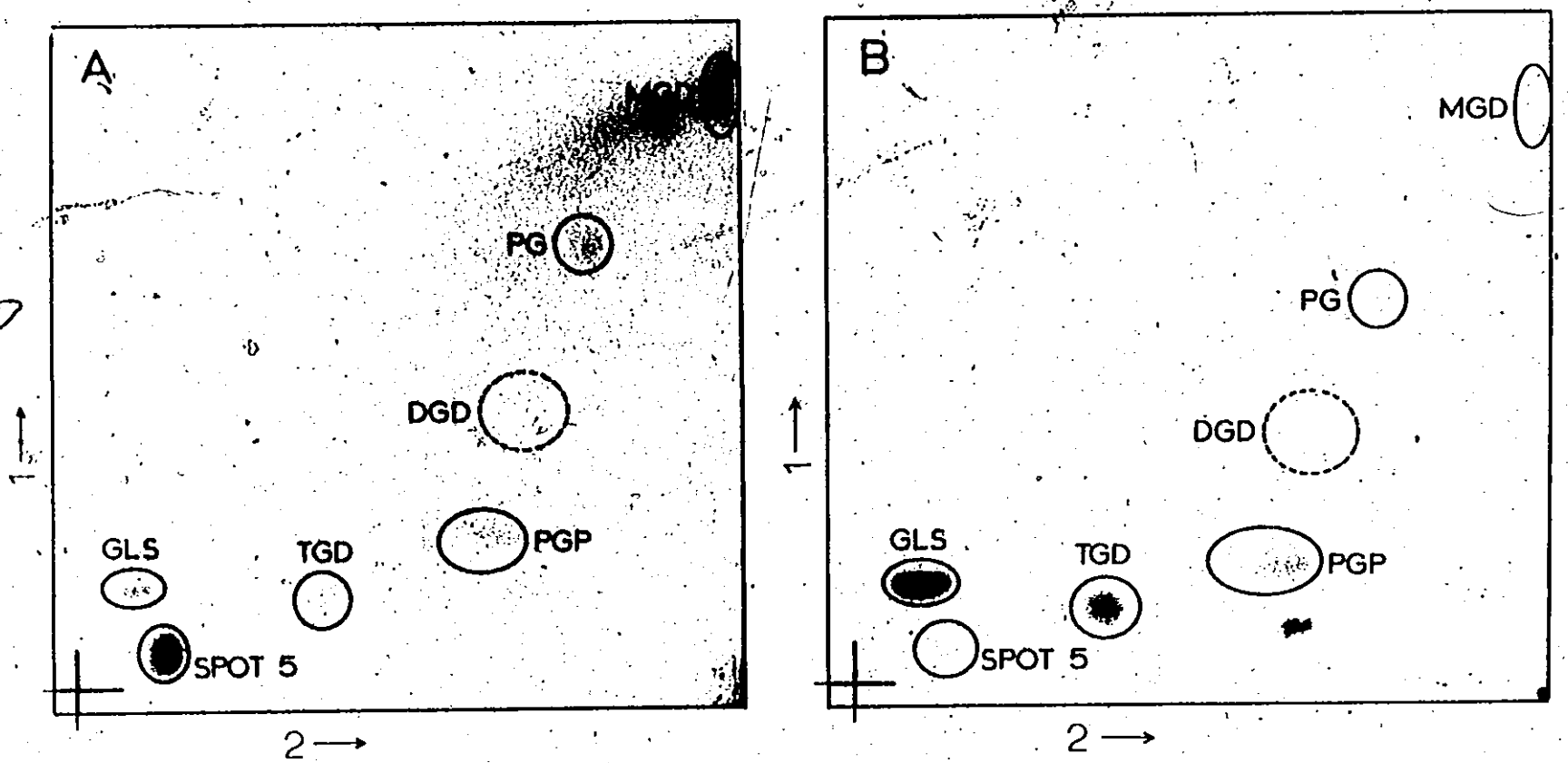
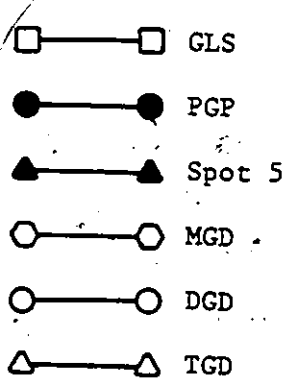

A
PG
DGD
GLS
TGD
PGP
SPOT 5
1
2
B
MGD
PG
DGD
GLS
TGD
PGP
SPOT 5
1
2

Figure 29

Percent of the total lipid-^{14}C found in the lipids of H. cutirubrum during growth of the cells in the presence of [U-^{14}C]glucose under aerobic conditions and "low" light intensity:



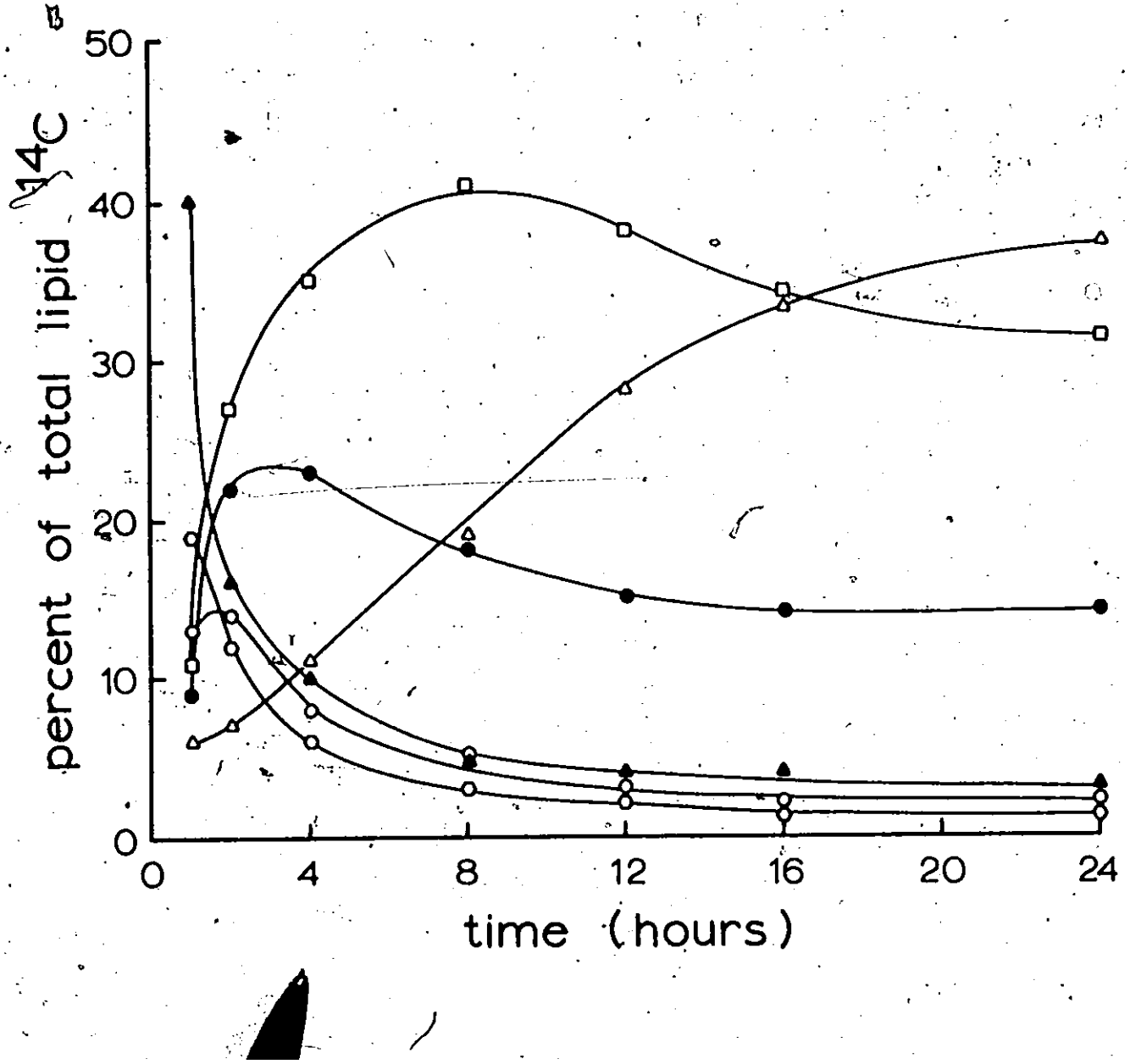

percent of total lipid ^{14}C
50
40
30
20
10
0
0
4
8
12
16
20
24
time (hours)

Table 13. Distribution of ^{14}C activity among the lipids of Halobacterium cutirubrum cells grown under aerobic conditions and "low" light intensity in the presence of [U-^{14}C]glucose (values in brackets are the percentages of the total ^{14}C found in the total lipids)

Time	Activity (d.p.m. x 10^{-3} per 100 ml culture)					
	MGD	DGD	TGD	GLS	PGP	Spot 5
1 h	15.7 (19.1)	10.6 (12.9)	5.0 (6.1)	9.0 (11.0)	7.5 (9.2)	32.6 (39.7)
2 h	9.9 (11.8)	11.9 (14.2)	5.8 (6.9)	23.0 (27.4)	18.6 (22.1)	13.5 (16.1)
4 h	8.1 (6.2)	10.4 (8.0)	14.4 (11.1)	45.4 (34.9)	29.8 (22.9)	13.3 (10.2)
8 h	5.9 (3.1)	9.7 (5.1)	36.5 (19.2)	77.9 (41.0)	34.0 (17.9)	9.3 (4.9)
12 h	5.3 (2.0)	7.7 (2.9)	74.2 (28.0)	101.2 (38.2)	39.8 (15.0)	10.9 (4.1)
16 h	3.3 (0.9)	6.9 (1.9)	120.8 (33.1)	124.1 (34.0)	51.5 (14.1)	14.2 (3.9)
24 h	4.4 (0.9)	9.8 (2.0)	182.8 (37.3)	153.4 (31.3)	68.6 (14.0)	14.2 (2.9)

Figure 28). The earliest compound labelled was that designated Spot 5. At 1 h, this unidentified component accounted for ca. 40% of the ^{14}C-label in the total lipids. Thereafter, it dropped sharply to a level of ca. 3% of the total label at 24 h.

The label in the monoglycosyl diphytanyl glycerol (MGD) was at a maximum of ca. 19% of the total label at about 1 h after the addition of the label. Thereafter, the proportion of the activity dropped sharply until at 24 h it accounted for only about 1% of the total label. The label in diglycosyl diphytanyl glycerol (DGD) reached a maximum of ca. 15% of the total label at about 1.5 h, and that in the triglycosyl diphytanyl glycerol (TGD) steadily increased from a level of ca. 6% at 1 h to ca. 37% of the total label at 24 h. This is in contrast with the finding that TGD labelled with [^{14}C]glycerol rapidly reached a maximum at 15 min and then gradually declined (see Figure 26, inset). The label in the glycolipid sulfate increased rapidly to a maximum of 41% of the total at 8 h after the addition of the label and then gradually decreased to about 31% at the end of 24 h with the concomitant increase in TGD.

The label in PGP (as a percent of the total label) rapidly increased to a maximum of about 23% at about 3 h after the addition of the [^{14}C]glucose; thereafter it gradually decreased and finally levelled off at ca. 14% of the total label at 24 h.

The very high accumulation of activity found in the triglycosyl diphytanyl glycerol remains to be explained. One conceivable explanation might be that a sulfatase cleaved the glycolipid sulfate to produce the TGD. However, this seems to have been ruled out by the

results obtained in the [^{35}S]sulfate chase experiment (see Part Two, Results and Discussion, III) where it was shown that there was no loss of sulfate from either the glycolipid sulfate or from any of the other sulfolipids. Another possible, but unlikely, explanation is that the cells may have been inadvertently exposed to a medium differing in some way from the medium used in the [^{14}C]glycerol experiment.

It has been noted from time to time (unpublished observations) that cultures of H. cutirubrum tend to produce more TGD and even other glycolipids not usually present. It may be that TGD is not only a precursor of GLS but is a bona fide membrane component and indeed has been demonstrated to exist in the purple membrane (Kushwaha and Kates, 1974).

VI. Chase Experiment with ^{14}C-Labelled Cells

The portion of the culture remaining at the end of the [^{14}C]glucose incorporation experiment was harvested and the cells resuspended in "cold" medium (see Part Two, Experimental Procedures, II). The growth curve for the cells and the total lipid ^{14}C activity for the cells (expressed in terms of d.p.m. per 100 ml of culture) are shown in Figure 30. Clearly, there was no loss of ^{14}C from the total lipids during the chase experiment, i.e. none of the lipids or portions of the lipids were metabolized into non-lipid components. Figure 31 shows the percentage of the total lipid ^{14}C remaining in each of the three major labelled compounds (i.e. TGD, GLS and PGP) during the chase experiment. All three compounds retained a constant proportion of the total label. This again indicates that, once synthesized, major membrane lipids are not further metabolized and that any newly required membrane lipids are synthesized de novo.

Figure 30

Activity of the ^{14}C remaining in the total lipids when cells pre-labelled with [U-^{14}C]glucose were grown in "cold" medium:

●——● growth curve for the cells

○——○ activity of the total lipids

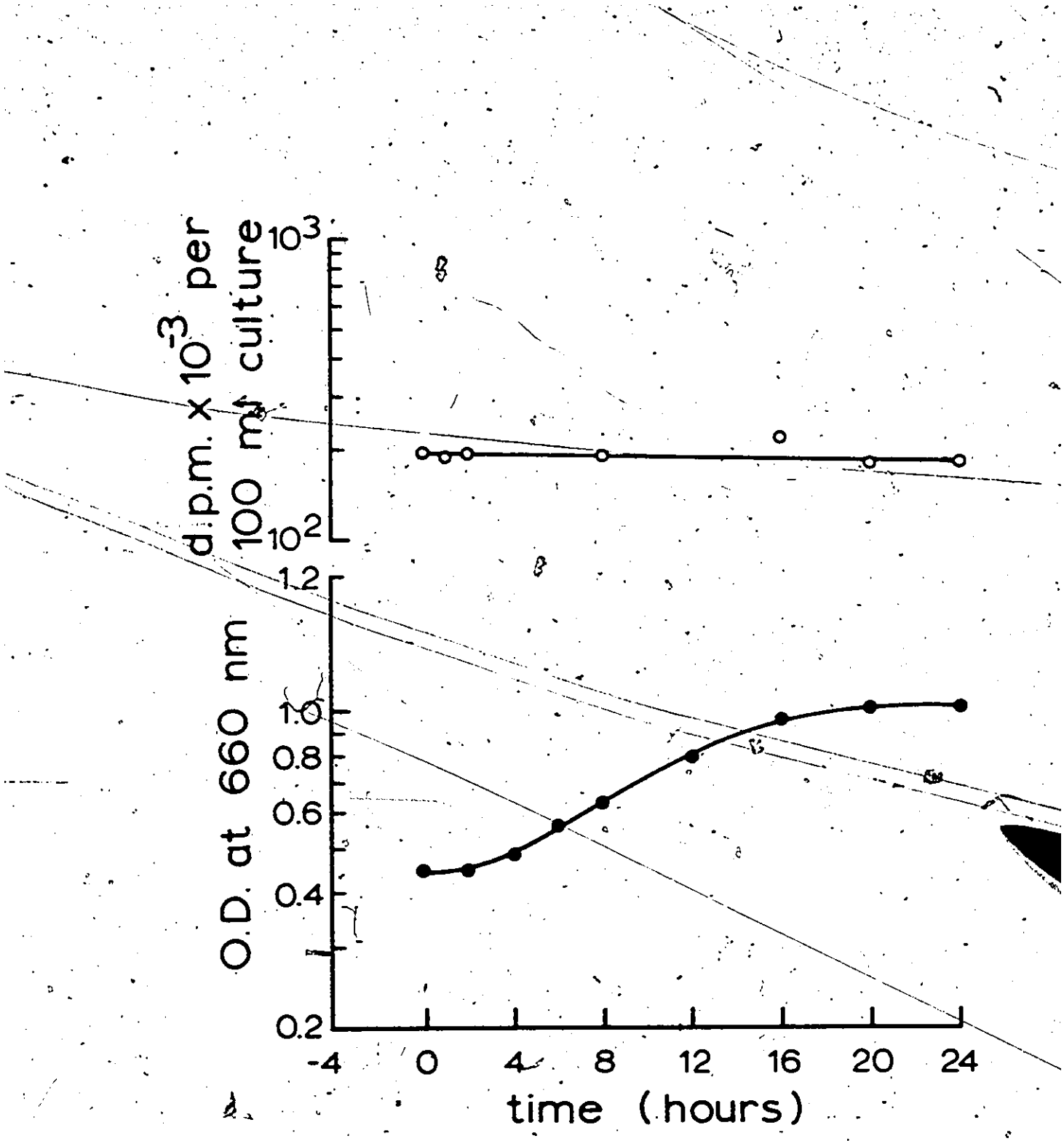
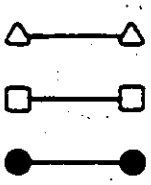
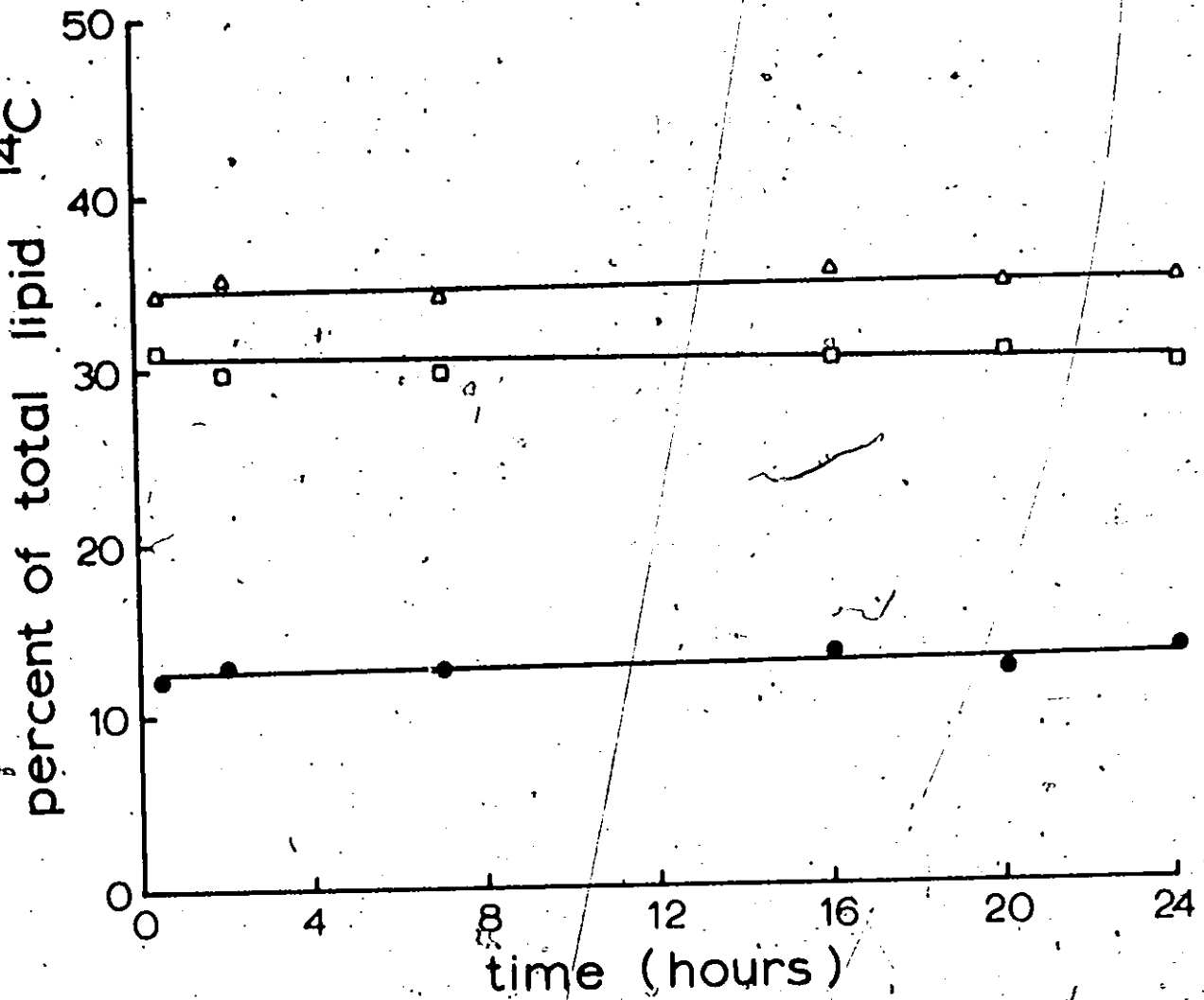

d.p.m. x 10^{-3} per 100 ml culture
10^3
10^2
O.D. at 660 nm
1.2
1.0
0.8
0.6
0.4
0.2
-4
0
4
8
12
16
20
24
time (hours)

Figure 31

Percent of the total lipid-^{14}C remaining in the three main labelled lipids during the chase experiment:




50
40
30
20
10
0
percent of total lipid ^{14}C
0
4
8
12
16
20
24
time (hours)

CLAIMS TO ORIGINAL RESEARCH

1. A novel glycolipid sulfate has been isolated from Halobacterium cutirubrum; its chemical structure has been established as 2,3-di-O-(3,7,11,15-tetramethylhexadecyl-1-O-[β-D-galactopyranosyl-3'-sulfate-(1'→6')-O-α-D-mannopyranosyl-(1'→2')-O-α-D-glucopyranosyl]-sn-glycerol.

2. A novel disaccharide, O-β-D-galactopyranosyl-(1→6)-D-mannopyranose, has been synthesized.

3. The incorporation of [^{14}C]glycerol, [^{14}C]glucose and [^{35}S]sulfate into the lipids of Halobacterium cutirubrum has been studied; these experiments have shown that once the major lipid components are synthesized by the cell, they are not further metabolized, i.e., there is no turnover of these cellular lipids.

REFERENCES

1. Abram, D. and Gibbons, N.E. (1961). Can. J. Microbiol. 6: 535
2. Austin, J., Armstrong, D., Stumpf, D., Luttenegger, T. and Dragoo, M. (1969). Biochim. Biophys. Acta, 192: 29
3. Baddiley, J., Buchanan, J.G., Handschumacher, R.E. and Prescott, J.F. (1956). J. Chem. Soc. 2818
4. Barczai-Martos, M. and Korosy, F. (1950). Nature, 165: 369
5. Bartlett, G.R. (1959). J. Biol. Chem. 234: 466
6. Baxter, R.M. and Gibbons, N.E. (1956). Can. J. Microbiol. 2: 599
7. Benson, A.A. (1963). In "Advances in Lipid Research." Edited by R. Paoletti and D. Kritchevsky. Academic Press, New York. 387
8. Benson, A.A., Daniel, H. and Wiser, R. (1959). Proc. Nat. Acad. Sci., U.S.A. 45: 1582
9. Bergey's Manual of Determinative Bacteriology. (1957). 7th ed. Edited by R.S. Breed, E.G.D. Murray and N.R. Smith. Williams and Wilkins, Baltimore
10. Blaurock, A.E. and Stoeckenius, W. (1971). Nature (New Biology), 233: 152
11. Bligh, E.G. and Dyer, W.J. (1959). Can. J. Biochem. and Physiol. 37: 911
12. Brown, A.D. (1963). Biochim. Biophys. Acta, 75: 425

13. Brown, A.D. and Shorey, C.D. (1963). J. Cell Biol. 18: 681

14. Carroll, K.K. (1961). Nature, 191: 377

15. Chapman, D. (1968). Biological Membranes, Physical Fact and Function. Academic Press, New York

16. Chen, J.S., Barton, P.G., Brown, D. and Kates, M. (1974). Biochim. Biophys. Acta, 352: 202

17. Cho, K.Y., Doy, C.H. and Mercer, E.H. (1967). J. Bacteriol. 94: 196

18. Danon, A. and Stoeckenius, W. (1972). N.A.S.A. Symposium on Extreme Environments: Mechanisms of Microbial Adaption. Ames Research Center, Moffett Field, California. 25

19. Dawson, R. (1954). Biochim. Biophys. Acta, 14: 374

20. Dittmer, J.C. and Lester, R.L. (1964). J. Lipid Res. 5: 126

21. Dubois, M., Gilles, M.K., Hamilton, J.K., Rebers, P.A. and Smith, F. (1956). Anal. Chem. 28: 350

22. Elovson, J. and Vagelos, P.R. (1970). Biochem. 9: 3110

23. Faure, M., Marechal, J. and Troestler, J. (1963). Compt. Rend. 257: 2187

24. Faure, M., Marechal, J. and Troestler, J. (1964). Compt. Rend. 259: 941

25. Galliard, T. (1970). Phytochem. 9: 1725

26. Galliard, T. (1971). Biochem. J. 121: 379

27. Goren, M.B. (1970a). Biochim. Biophys. Acta, 210: 116

28. Goren, M.B. (1970b). Biochim. Biophys. Acta, 210: 127

29. Goren, M.B. (1971). Lipids, 6: 40

30. Haines, T.H. (1971). Progr. Chem. Fats Other Lipids, 11: 299

31. Haines, T.H. (1973). In "Lipids and Biomembranes of Eukaryotic Microorganisms." Edited by J.E. Erwin. Academic Press, New York. 197

32. Hancock, A.J. (1972). Structural Studies on Phytanyl Ether Membrane Lipids in Halobacterium cutirubrum. Ph.D. Thesis. University of Ottawa

33. Hancock, A.J. and Kates, M. (1972). Chem. Phys. Lipids, 8: 87

34. Hancock, A.J. and Kates, M. (1973). J. Lipid Res. 14: 422

35. Heinz, E., Ruukotter, J. and Budzikiewicz, H. (1974). Hoppe-Seyler's Z. Physiol. Chem. 355: 612

36. Helmsing, P.J. (1967). Biochim. Biophys. Acta, 144: 470

37. Hirshmann, H. (1960). J. Biol. Chem. 235: 2762

38. Ishizuka, I., Suzuki, M. and Yamakawa, T. (1973). J. Biochem. 73: 77

39. Joo, C.N. and Kates, M. (1968). Biochim. Biophys. Acta, 152: 800

40. Joo, C.N. and Kates, M. (1969). Biochim. Biophys, Acta, 176: 278

41. Joo, C.N., Shier, T. and Kates, M. (1968). J. Lipid Res. 9: 782

42. Kates, M. (1964). J. Lipid Res. 5: 132

43. Kates, M. (1964). In "Advances in Lipid Research." Vol. 2. Edited by R. Paoletti and D. Kritchevsky. Academic Press, New York. 17

44. Kates, M. (1967). In "Lipid Chromatographic Analysis." Vol. 1. Edited by G.V. Marinetti. Marcel Dekker, New York. 1

45. Kates, M. (1972). In "The Ether Bond in Lipids." Edited by F. Snyder. Academic Press, New York. 351

46. Kates, M. (1972). In "Ether Lipids, Chemistry and Biology." Academic Press, New York. 351

47. Kates, M., Chan, T.H. and Stanacev, N. (1963). Biochem. 2: 394

48. Kates, M. and Deroo, P.W. (1973). J. Lipid Res. 14: 438

49. Kates, M. and Eberhardt, F.M. (1957). Can. J. Botany, 35: 895

50. Kates, M. and Hancock, A.J. (1971). Biochim. Biophys. Acta, 248: 254

51. Kates, M., Joo, C.N., Palameta, B. and Shier, T. (1967). Biochem. 6: 3329

52. Kates, M., Palameta, B., Joo, C.N., Kushner, D.J. and Gibbons, N.E. (1966). Biochem. 5: 4092

53. Kates, M., Palameta, B., Perry, M.P. and Adams, G.A. (1967). Biochim. Biophys. Acta, 137: 213

54. Kates, M., Palameta, B. and Yengoyan, L.S. (1965). Biochem. 4: 1595

55. Kates, M., Wassef, M. and Kushner, D.J. (1968). Can. J. Biochem. 46: 971

56. Kates, M., Wassef, M. and Kushner, D.J. (1968). In "Membrane Models and the Formation of Biological Membranes." Edited by L. Bolis and B.A. Pethica. North-Holland Publishing Co., Amsterdam. 105

57. Kates, M., Yengoyan, L.S. and Sastry, P.S. (1965). Biochim. Biophys. Acta, 98: 252

58. Kaufman, B., Kundig, F., Distler, J. and Roseman, S. (1965). Biochem. Biophys. Res. Commun. 18: 312

59. Kelly, M., Norgard, S. and Liaaen-Jensen, S. (1970). Acta Chim. Scand. 24: 2169

60. Kramer, J., Kushwaha, S. and Kates, M. (1972). Biochim. Biophys. Acta, 270: 103

61. Kushner, D.J., Bayley, S.T., Boring, J., Kates, M. and Gibbons, N.E. (1964). Can. J. Microbiol. 10: 483

62. Kushwaha, S., Gochnauer, M., Kushner, D.J. and Kates, M. (1974). Can. J. Microbiol. 20: 241

63. Kushwaha, S. and Kates, M. (1973). Biochim. Biophys. Acta, 316: 235

64. Kushwaha, S. and Kates, M. (1974). Can. J. Biochem. In press.

65. Kushwaha, S., Pugh, E.L., Kramer, J. and Kates, M. (1972). Biochim. Biophys. Acta, 260: 492

66. Larsen, H. (1967). Adv. Microbiol. Physiol. 1: 97

67. Leikola, E., Nieminen, E. and Teppo, A. (1969). J. Lipid Res. 10: 440

68. Marinetti, G.V. (1962). J. Lipid Res. 3: 1

69. Marinetti, G.V. (1964). New Biochemical Separations. Van Nostrand, New Jersey.

70. Marinetti, G.V. and Stotz, E. (1956). Biochim. Biophys. Acta, 21: 168

71. Marshall, C.L. and Brown, A.D. (1968). Biochem. J. 110: 441

72. Martensson, E. (1963). Acta Chim. Scand. 17: 1174

73. Martensson, E. (1966). Biochim. Biophys. Acta, 116: 521

74. Mayers, G.L. and Haines, T.H. (1967). Biochem. 6: 1665

75. Mayers, G.L., Pousada, M. and Haines, T.H. (1969). Biochem. 8: 2981

76. Mitchell, P. (1966). Biol. Rev. (Cambridge), 41: 445

77. Mitchell, P. (1972). J. Bioenergetics, 3: 5

78. Nieminen, E., Leikola, E., Koljonen, M., Kiistala, U. and Mustakallio, K.K. (1967). Acta Dermato-Venereol. 47: 327

79. Onishi, H., McCance, M.E. and Gibbons, N.E. (1965). Can. J. Microbiol. 11: 365

80. Oesterhelt, D. and Stoeckenius, W. (1971). Nature (New Biology), 233: 149

81. Oesterhelt, D. and Stoeckenius, W. (1973). Proc. Nat. Acad. Sci., U.S.A. 70: 2853

82. Pieringer, R.A. (1968). J. Biol. Chem. 243: 4894

83. Pugh, E.L., Wassef, M. and Kates, M. (1971). Can. J. Biochem. 49: 953

84. Racker, E. and Stoeckenius, W. (1974). J. Biol. Chem. 249: 662

85. Reindel, F. and Hoppe, W. (1954). Ber. 87: 1103

86. Sastry, P.S. and Kates, M. (1964). Biochem. 3: 1271; 1280

87. Sastry, P.S. and Kates, M. (1969). In "Methods in Enzymology." Vol. XIV. Edited by S.P. Colowick and N.O. Kaplan. Academic Press, New York. 204

88. Sehgal, S.N. and Gibbons, N.E. (1960). Can. J. Microbiol. 6: 165

89. Sehgal, S.N., Kates, M. and Gibbons, N.E. (1962). Can. J. Biochem. and Physiol. 40: 69

90. Shaw, N. and Baddiley, J. (1968). Nature, 217: 142

91. Short, S. and White, D.C. (1970). J. Bacteriol. 104: 126

92. Slotboom, A.J. and Bonsen, P. (1970). Chem. Phys. Lipids, 5: 1

93. Snyder, F., Malone, B. and Blank, M.L. (1970). J. Biol. Chem. 245: 1790

94. Soo-Hoo, T.S. and Brown, A.D. (1967). Biochim. Biophys. Acta, 135: 164

95. Spencer, B. (1960). Biochem. J. 75: 435

96. Stoeckenius, W. and Kunau, W.H. (1968). J. Cell Biol. 38: 337

97. Stoeckenius, W. and Rowen, R. (1967). J. Cell Biol. 34: 365

98. Stoffyn, P.J. (1966). J. Amer. Oil Chem. Soc. 43: 69

99. Stoffyn, P.J. and Stoffyn, A. (1963). Biochim. Biophys. Acta, 70: 218

100. Thudicum, J.L.W. (1874). "Researches on the Chemical Constitution of the Brain." Report of the Medical Officer of the Privy Council, London. N.S. No. 3, Append. 5. 113

101. Tornabene, T.G., Kates, M., Gelpi, E. and Oro, J. (1969). J. Lipid Res. 10: 294

102. Trevelyan, W.E., Proctor, D.P. and Harrison, J.S. (1950). Nature, 166: 444

103. Vogel, A.I. (1956). "A Textbook of Practical Organic Chemistry." 3rd ed. Longmans, Green and Co., London.

104. Wassef, M. (1969). Studies on the Lipid Metabolism in the Extremely Halophilic Bacterium, Halobacterium cutirubrum. Ph.D. Thesis. University of Ottawa

105. Wassef, M., Sarner, J. and Kates, M. (1970). Can. J. Biochem. 48: 69