TO

MY FAMILY

ACKNOWLEDGMENTS

The author gratefully acknowledges the invaluable guidance, encouragement and patience of Professor Morris Kates during the course of these investigations and preparation of this thesis.

Appreciations with thanks are expressed to the following: Drs. J. Harwig, C. E. Park, and M. B. Gochnauer for isolation and identification of the microorganisms studied; Mr. Pierre Pilon for his excellent technical assistance; Drs. A. J. Hancock, P. W. Deroo and J. A. Oliver for their helpful suggestions and discussions; Dr. M. Perry, N.R.C., Ottawa, for donating methylated sugar derivatives as GLC standards; Dr. N. Shaw for providing the standard deacylated glucophospholipid; Mr. R. Capoor for measurement of NMR spectra; Mr. Ben-Tchavtchavadze for his excellent photography; Mrs. E. Szabo for drafting the figures; and Mrs. W. Storto for her efficient typing.

Sincere gratitude is expressed to the Canadian International Development Agency (CIDA) who provided the author with the opportunity of studying in Canada by their generous financial support during her graduate studies, and to the Thai Government for permission to undertake this work and the granting of educational leave.

SUMMARY

Studies were carried out on the lipid composition of three microorganisms isolated in pure culture from a contaminant found in a growth medium for extreme halophiles which contains 25% sodium chloride. These three microorganisms were identified as: (1) Staphylococcus epidermidis (a bacterium), (2) Wallemia sebi (a fungus), and (3) a Nocardia species (a bacterium).

The lipid composition of W. sebi and the Nocardia sp. were identified only partially by chromatographic methods in order to compare their lipids with that of the original-mixed culture. The polar lipids of W. sebi consisted of five phospholipids, namely phosphatidylserine, phosphatidylcholine, phosphatidylethanolamine, two unidentified phospholipids and three unidentified glycolipids; the total fatty acids contained linoleic acid (60%), oleic acid (13%) and palmitic acid (23%). The polar lipid composition of a Nocardia sp. consisted of five phospholipids and two unidentified glycolipids. The phospholipids were tentatively identified as phosphatidylcholine, lyso phosphatidylglycerol, phosphatidylglycerol and two unidentified phospholipids. The total fatty acids of the Nocardia sp. contained mostly branched chain fatty acids, namely iso 15:0 (21%), iso 16:0 (31%), iso 17:0 (19%) and anteiso 17:0 (22%).

Most of the work presented in this thesis is concerned with the structural determination of the cellular lipids of *S. epidermidis*. The methods used for structural determinations of these lipids include analyses of constituents (phosphorus, sugar, glycerol, and fatty acid) on the pure isolated lipid components; degradation studies using acid, alkaline and specific enzymatic hydrolyses; and physical methods using IR, NMR and optical rotation measurement. From these studies the structure of four polar lipid components of *S. epidermidis* were elucidated, namely: (1) glycerophosphoryl diglucosyl diglyceride of the structure 3-O-[-*sn*-glycero-1-phosphoryl-6'-O-(β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranosyl)]-1,2-diacyl-*sn*-glycerol; (2) diglucosyl diglyceride of the structure 3-O-[β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranosyl]-1,2-diacyl-*sn*-glycerol; (3) monoglucosyl diglyceride having the structure 3-O-[β-D-glucopyranosyl]-1,2-diacyl-*sn*-glycerol and (4) phosphatidylglycerol with the structure 1,2-diacyl-*sn*-glycero-3-phosphoryl-1'-*sn*-glycerol. Phosphatidic acid, cardiolipin and two unidentified phospholipids were also present in small amounts but detailed structural studies were not performed.

Each lipid component studied had essentially the same fatty acid composition namely, *anteiso* 15:0 (60-75%), *anteiso* 17:0 (18-24%), *iso* 17:0 (8-20%), and small amounts of palmitic and stearic acids (2-5%). The fatty acids were found to be non-randomly distributed, the shorter chain

anteiso 15:0 fatty acid being exclusively esterified to the 2-position and the longer-chain anteiso and iso 17:0 fatty acids (as well as some branched 15:0 and other fatty acids) are esterified at the 1-position of the glycerol moiety of phosphatidylglycerol.

Studies on the effect of sodium chloride concentration in the culture medium on growth and lipid composition of S. epidermidis indicated that S. epidermidis is a halotolerant bacterium which grows best in the absence of added sodium chloride, but can tolerate salt in the medium even up to 25% concentration. The fatty acid composition was not affected by increasing sodium chloride content in the media in the range 0-15% but was affected when the salt concentration was increased to 25%. However, the proportions of polar charged lipids such as phosphatidylglycerol, cardiolipin and glyceryl-phosphoryl diglucosyl diglyceride were affected when sodium chloride in the medium was increased from 5 to 25%. Thus, the percentage of phosphatidylglycerol decreased while that of cardiolipin and glycerophosphoryl diglucosyl diglyceride increased. The levels of neutral polar glycolipids (diglucosyl diglyceride and monoglucosyl diglyceride) were not affected by increasing sodium chloride concentration in the medium.

The metabolism of membrane lipids was studied by growing S. epidermidis cells in the presence of [^{32}P]ortho-

phosphate and [1-^{14}C]glycerol. The results from pulse labelling and pulse-chase experiments suggested that the biosynthetic pathways for phospholipids and glycolipids of S. epidermidis may be the same as those found in other bacteria (e.g. E. coli). Phosphatidylglycerol, cardiolipin and glycerophosphoryl diglucosyl diglyceride of S. epidermidis were found to undergo rapid turnover whereas diglucosyl diglyceride and neutral lipids were metabolically stable during the "chase" experiment.

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ABBREVIATIONS

ATP	Adenosine triphosphate
CDP	Cytidine diphosphate
CTP	Cytidine triphosphate
CL	Cardiolipin
DG	Diglyceride
DGD	Diglucosyl diglyceride
DGG	Diglucosyl glycerol
GLC	Gas liquid chromatography
GPDGD	Glycerophosphoryl diglucosyldiglyceride
GPDGG	Glycerophosphoryl diglucosylglycerol
GP	Glycerophosphate
GPG	Glycerophosphoryl glycerol
IR	Infrared spectroscopy
MGD	Monoglucosyl diglyceride
MGG	Monoglucosyl glycerol
NAD	Nicotinamide adenine dinucleotide
NL	Neutral lipid
PA	Phosphatidic acid
PE	Phosphatidylethanolamine
PC	Phosphatidylcholine
PG	Phosphatidylglycerol
PGAA	Aminoacylphosphatidylglycerol
PI	Phosphatidylinositol
PS	Phosphatidylserine
TLC	Thin layer chromatography
TMS	Tetramethylsilane

INTRODUCTION

During routine culturing of the extreme halophile, Halobacterium cutirubrum in our laboratory, it was observed that a flask, containing unsterilized halophile growth medium left at room temperature for 50 days, had become contaminated with moldlike colonies on the surface of the medium. Three species of microorganisms were isolated from the contaminated medium and identified as (1) Staphylococcus epidermidis, a bacterium; (2) Wallemia sebi, a fungus; and (3) a Nocardia species, a moldlike bacterium; (see Appendix I for identification of these organisms). The medium in which these microorganisms were found was the complex medium of Sehgal and Gibbons (1960) containing 25% sodium chloride. It was known that H. cutirubrum contains a novel class of lipids in which fatty acyl groups are replaced by dihydrophytol chains linked to the glycerol backbone by ether bonds (Kates, 1972) and the glycerol moiety has the opposite stereochemical configuration to that found in the corresponding diester forms (Joo et al., 1968). Therefore, it was of interest to see whether or not the contaminating microorganisms isolated from the high salt containing halophile growth medium would have the same type of lipids as H. cutirubrum and other extreme halophiles. This was of particular interest since the related types of the above organisms grown in normal medium were known to contain normal fatty

acid ester-type lipids. A detailed study was then undertaken on the lipids of the contaminant organisms, and in the case of S. epidermidis, also on the effect of increased salt concentration on the composition of the cellular lipids.

First, however, a general review covering the microbiology and membrane lipids of the genera to which the above organisms belong will be given.

I. Microbiology of the Three Isolated Genera

A. Staphylococci

The genus Staphyloccus is classified in the family Micrococcaceae of the order Eubacteriales (Bergey's Manual, Breed et al., 1957). Staphylococci are Gram-positive, non-motile, catalase-positive cocci which grow in clusters and can be isolated from air, dust, water, and human and animal sources. They are facultative anaerobes growing best in the presence of air. For aerobic growth, they require a medium containing amino acids and two or more vitamins; for anaerobic growth they also require uracil and a fermentable carbohydrate (Bergey's Manual, Breed et al., 1957; Cruickshank, 1960). They are typical mesophiles, growing at temperatures between 6.5 and 46°C (optimum 35-40°C) and at pH values between 4.5 and 9.3 (optimum 7.0-7.5). Most strains while growing best in absence of added salt will grow in the presence of up to 15% sodium chloride or 40% bile (Baird-Parker, 1972).

The metabolism of staphylococci is both respiratory and fermentative. Fermentation of glucose under anaerobic conditions results in the production of mainly lactic acid, whereas in the presence of oxygen, mainly acetic acid and small amounts of carbon dioxide are produced. Most strains produce acetoin from glucose and ammonia from arginine; they reduce nitrates and ferment a variety of carbohydrates (Bergey's Manual, 1957; Baird-Parker, 1972).

At present, only two species are recognized, these are Staphylococcus aureus and Staphylococcus epidermidis (Bergey's Manual, Breed et al., 1957; Baird-Parker, 1971). The main criteria distinguishing these two species are summarized in Table 1. It will be observed that the species are clearly distinguished by a number of characteristics. The recent observation by Nielsen (1970) that the DNA from S. aureus will not hybridize with the DNA from S. epidermidis further differentiates these two species.

Staphylococci classified as Staphylococcus epidermidis produce white colonies while those of S. aureus are usually golden-yellow. S. epidermidis is found on the normal skin, and usually is of low pathogenicity, even when deliberately injected into human beings or rabbits. On the other hand, S. aureus is found in pus, and produces a variety of toxins. It is thus potentially pathogenic and may cause food poisoning.

TABLE 1

Differential Characteristics of Species of *Staphylococcus*[a]

Characteristic	*aureus*	*epidermidis*
Coagulases[b]	+	-
Mannitol		
Acid aerobically[b]	+	d
Acid anaerobically[c]	+	-
Alpha toxin[d]	+	-
Heat-resistant endonuclease[c]	+	-
Biotin for growth[e]	-	+
Cell wall		
Ribitol[f]	+	-
Glycerol[f]	-	+
Protein A[g]	+	-

a. + = most (90% or more) strains positive;
- = most (90% or more) strains negative;
d = some strains positive, some negative.

b. Standard methods proposed by the ICNB Subcommittee on the Taxonomy of Staphylococci and Micrococci (see Baird-Parker, 1971).

c. Lachica and Deibel (1969).

d. Elek (1959); Bernheimer (1968).

e. Jones, Deibel, and Niven (1963).

f. Davison and Baddiley (1963).

g. Oeding (1965).

(From Baird-Parker, 1972)

B. Wallemia

Microorganisms in this genus are Eumycetes (true fungi), in the group Fungi Imperfecti (Hyphomycetes), order Moniliales and family Dematiaceae (Gilman, 1957). These ubiquitous fungi, also named Sporendonema, Hemispora or Torula (Wilson et al., 1969), have been isolated from tallow, dried fish, sweet condensed milk, and other osmotically arid substrates, as well as from soil and pathologic processes of man and animals (Wilson et al., 1969). It is familiar to most mycologists as the small, hemispherical, chocolate-coloured colonies that appear as contaminants on drying agar plates.

A typical characteristic of fungi in the Fungi Imperfecti is that they bear nonmotile spores at the tips of specialized hyphae called conidiophores; such spores are named conidiospores or conidia. The conidiophores of the genus Wallemia are hyaline to subhyaline, more or less erect in a dense stand, cylindrical and terminate in a single phialide-like structure with a dark cup. Each phialide produces a filamentous, sporogenous hypha which separate basipetally to form a simple chain of arthrospores. These arthrospores are rather cube-shaped at first and then round off to produce spherical, pigmented, non-septate spores (Barron, 1968). Arthrospores which develop from a sporogenous hypha originating from a phialide-like structure are very unusual and are distinctive features of the genus

Wallemia. The Wallemia sebi studied in this work produce arthrospores which are cubical and become globose with brown colours when grown in media containing malt and 20% sucrose, or malt and 25% sodium chloride (see Appendix I).

C. Nocardia

Microorganisms of this genus are moldlike bacteria of the order Actinomycetales, family Actinomycetaceae (Bergey's Manual, Breed et al., 1957). They are aerobic actinomycetes occurring naturally in the soil. Some of them, such as Nocardia asteroides, the causative agent of nocardiosis, are found all over the world and are partly acid-fast. They are non-photosynthetic organisms which, during the early stages of growth, produce a well defined mycelium, less than 2μ in width, which later fragments completely into branched or unbranched bacillary elements. When aerial mycelia are produced, it also fragments into rods and coccal elements but does not produce differentiated conidia as are found among the Streptomycetaceae (Skerman, 1967). However, Gordon and Mihm (1958), and Bradley (1959) reported that several nocardiae, including N. asteroides, formed conidia similar to those of streptomycetes. This morphological dead-end was circumvented with the advent of cell wall analysis (Becker et al., 1964), a technique that permits a clear-cut separation between the two genera.

Other characteristics of the Nocardia are: endospores not formed; aerobic; Gram-positive; heterotrophic; nonmotile (Skerman, 1967). The organisms grow well on laboratory media. Colonies are usually opaque, and pigmented white, yellow, orange, red, green, or black. They may be smooth or wrinkled and doughlike in consistency. The intensity of pigmentation varies with the medium employed (Skerman, 1967).

II. Lipids of Microorganisms (Bacteria and Fungi)

The lipids of the relatively simple microorganisms (prokaryotes), frequently differ both quantitatively and qualitatively from those found in more complex forms of life (eukaryotes). The most striking difference is in the fatty acyl groups which in eukaryotes contain polyunsaturated fatty acids but little or no cyclopropane and branched chain fatty acids, the reverse being true of prokaryotes (Asselineau, 1966; Shaw, 1966; Goldfine, 1972). Sterols are also absent from bacteria, except the Mycoplasma, wherein certain strains contain cholesterol and require cholesterol for growth (Rottem et al., 1973). Other isoprenoid-derived compounds are present in bacteria, however. For example, the so-called "bactoprenol" (C_{55}-isoprenoid alcohol) has been found in Lactobacilli (Thorne and Kodicek, 1966), and an undecaprenol, consisting entirely of unsaturated isoprene units, has been isolated from L. plantarum (Gouch et al., 1970), Micrococcus lysodeikticus (Scher et al., 1968), Staphylococcus aureus (Higashi et al., 1970) and Listeria monocytogenes (Vilim et al., 1973).

A brief review of extractable lipids of bacteria and fungi will now be given.

A. Bacterial Lipids

Distribution of phospholipids, glycolipids and fatty acids among various classes of bacteria were reviewed

extensively by Kates (1964), Asselineau (1966), Lennarz (1966), Ikawa (1967), Shaw (1970) and Goldfine (1972). In the last few years, information on the lipid composition of various bacteria has increased greatly. The more recent data available for each type of lipid (phospholipids, glycolipids and fatty acids) have been grouped in Tables 2-4 according to the Gram stain behaviour of the bacteria, and subdivided within each group on the basis of the family and genus to which they belong (Bergey's Manual, Breed et al., 1957).

1. Phospholipids

From the information available on phospholipid composition of various bacteria (see Table 2, Kates, 1964; Ikawa, 1967; and Goldfine, 1972) the following conclusions can be drawn:

i) Phosphatidylcholine, the most abundant of the phosphatides in tissues of higher plants and animals, is generally absent in the bacteria studied except in the genus Brucella (Thiele and Schwinn, 1973), Micrococcus denitrificans (Wilkinson et al., 1972), Lactobacillus casei (Thorne, 1964), Agrobacterium (Goldfine and Ellis, 1964; Oliver and Colwell, 1973), Rhizobium japonicum (Bunn and Elkan, 1971), Treponema pallidum, T. zuelerae (Johnson et al., 1970, Meyer and Meyer, 1971), Nocardia coeliaca (Yano et al., 1969), Rhodospirillum rubrum (Benson et al., 1959), Hyphomicrobium vulgare

TABLE 2

Phospholipid Composition in Various Bacteria[a]

Organisms	CL	PA	PG	PGAA	PI	PS	PE	Lyso PE	PC	Other	References
Gram Negative											
Pseudomonadales											
Pseudomonadaceae											
Pseudomona perfectomarinus	2	-	10	-	-	-	86	3	-		Oliver & Colwell (1973)
P. aeruginosa	+	-	+	-	-	-	+	-	-		Wilkinson (1970)
Halobacterium cutirubrum[b]	-	tr	5	-	-	-	-	-	-	92[c]	Kates (1972)
Spirillaceae											
Vibrio algosus	2	-	15	-	-	-	64	11	-		
V. alginolyticus	2	-	22	-	-	-	68	8	-		Oliver & Colwell
V. parahaemolyticus	1	-	18	-	-	-	80	1	-		(1973)
Eubacteriales											
Enterobacteriaceae											
Escherichia coli	5-15	tr	5-15	-	+	tr	70-80	tr	-	+[d]	Cronan & Vagelos (1972)
Salmonella typhimurum	4	-	11	-	-	-	83	-	-	2[e]	Olsen & Ballou(1971)
Achromobacteraceae											
Achromobacter aquamarinus	2	-	37	-	-	-	36	7	-	-	Oliver & Colwell(1973)
Brucellaceae											
Brucella melitensis	20	-	9	-	-	tr	33	-	38	tr[f]	
B. abortus	7	-	23	-	-	5	28	-	35	14[f]	Thiele & Schwinn(1973)
Bordetella pertussis	24	-	tr	-	-	20	46	-	-	10[f]	
Yersinia pestis	+	-	-	-	tr	-	-	-	-	-	
Yersinia pseudotuberculosis	+	+	+	-	tr	+	+[g]	+	-		Tornabene (1973)
Micrococcaceae											
Micrococcus denitrificans	3	tr	52	-	-	1	6	-	31	-	Wilkinson et al(1972)

Table 2 continued

Organisms	CL	PA	PG	PGAA	PI	PS	PE	Lyso PE	PC	Other	References
Mycoplasmatales											
Mycoplasmataceae											Shaw et al. (1968)
Mycoplasma laidlawii	–	–		+	+	–	–	–	–	+[h]	Shaw et al. (1972)
M. neurolyticum	2	34	60	2	–	–	–	–	–	1[i]	Smith (1972)
T-Strain of Mycoplasma	20	70	5	–	–	–	5	–	–	–	Romano et al. (1971)
Thermoplasma acidophilum[b]	---------- unidentified ----------										Langworthy et al (1972)
Gram Positive											
Eubacteriales											
Micrococcaceae											Macfarlane (1961)
Micrococcus lysodeikticus	+	+	+	+	+	–	–	–	–	–	DeSiervo & Salton (1973)
Staphylococcus aureus	15	+	63	19	–	–	+	–	–	+[h]	Short & White (1970)
Lactobacillaceae											
Lactobacillus casei	+	–	–	–	–	–	+	–	+	–	Thorne (1964)
Streptococcus faecalis	+	+	+	+	–	–	–	–	–	+[h]	dosSantos et al (1970)
											Fischer et al. (1973)
Corynebacteriaceae											
Listeria monocytogenes	45–50	–	40–45	–	–	–	–	–	–	13–17[j]	Kosarić & Carroll (1971)
Microbacterium lacticum	+	–	+	–	–	–	–	–	–	–	Shaw (1968a)
Arthrobacter crystallo-poites	+	–	+	–	+	–	–	–	–		
A. pasceus	+	–	+	–	+	–	–	–	–		Shaw & Stead (1971)
A. globiformis	+	–	+	–	+	–	–	–	–		
Bacillaceae											
Bacillus cereus	+	+	35	8	–	–	46	–	–		Houtsmuller & Van Deenen (1963)
B. stearothermophilus	49	–	13	–	–	–	37	–	–		Card (1973)
Clostridium butyricum[k]	–	–	26	–	–	–	14	–	–	78[k]	Baumann et al. (1965)
Actinomycetales											
Actinomycetaceae											
Nocardia polychromogenes	55	–	–	–	25[l]	–	20	–	–		Kataoka & Nojima (1967)
Nocardia coeliaca	7–15	–	–	–	11–14	–	25–30	–	25–40	–	Yano et al. (1969)
Nocardia leishmanii	+	–	+	–	+[m]	–	+	–	–	–	Yano et al. (1970)
Streptomycetaceae											
Streptomyces griseus	33	–	–	–	6[l]	–	49	–	–		Kataoka & Nojima (1967)
Micromonospora	27	–	–	–	40[l]	–	14	10	–		Tabaud (1971)

Footnotes (Table 2)

a. See also Kates (1964) and Ikawa (1967). Values are given as percentage of total phospholipids unless specified; -, absent; +, component present but amount unknown; tr, trace; for other abbreviations, see list of abbreviations.

b. Diether analogue of phospholipids.

c. Composed of 86% of phosphatidyl glycerophosphate and 6% of phosphatidyl glycerosulfate (Hancock and Kates, 1973).

d. Phosphatidyl glycerophosphate (DeSiervo, 1969; Randle, 1969).

e. Acyl phosphatidyl glycerol.

f. Unidentified component.

g. Mixture of phosphatidyl monomethylamine-dimethylamine and PE, the first one was present in major proportion.

h. Phosphoglucolipid.

i. Peracylated cardiolipin.

j. Composed of 8-10% of bis-phosphatidyl glyceryl phosphate and 5-7% of unidentified phospholipids.

k. A total of 55% of the PE, 9% of PG and 78% of the N-monomethylethanolamine phosphatides was present in plasmalogen form.

l. PI-monomannoside.

m. Mixture of PI + PI-monomannoside.

(Goldfine and Hagen, 1968), Rhodomicrobium vannielii (Park and Berger, 1967), Rhodopseudomonas spheroides (Gorchein, 1968), and Thiobacilli (Barridge and Shively, 1968). In these bacteria, phosphatidylcholine is present as a major phospholipid (20-45%).

ii) Phosphatidylethanolamine, which occurs widely in the plant and animal worlds, is also a major phosphatide in most of the bacteria studied, particularly in Gram-negative bacteria. Derivatives of phosphatidylethanolamine such as phosphatidyl-N-methylethanolamine and phosphatidyl-dimethylethanolamine are also found in Gram-negative bacteria which are capable of carrying out a stepwise methylation of phosphatidylethanolamine. These two derivatives of phosphatidylethanolamine sometimes were found together with phosphatidylcholine such as in Hyphomicrobium vulgare (Hagen et al., 1966), Agrobacterium tumefaciens (Law et al., 1963), Azotobacter agilis (Randle et al., 1969), Thiobacillus novellus (Barridge and Shively, 1968) and Ferrobacillus ferro-oxidans (Short et al., 1969). In some organisms lacking phosphatidylcholine, N-methyl PE has been found, for example, in Clostridium butyricum (Baumann et al., 1965), several species of Thiobacillus (Barridge and Shively, 1968) and in Proteus vulgaris (Goldfine and Ellis, 1964; Randle et al., 1969).

iii) Phosphatidylglycerol which is a major component in plants and is also present in small amounts in animal tissues appears to be present ubiquitously in most bacteria along with cardiolipin. Gram-positive bacteria have a higher proportion of phosphatidylglycerol (or the phosphatides derived from it, namely cardiolipin and amino-acyl phosphatidylglycerol) than Gram-negative organisms in which phosphatidylethanolamine is predominant.

iv) Cardiolipin or diphosphatidylglycerol, is found in both Gram-positive and Gram-negative bacteria along with phosphatidylglycerol but in smaller amounts (1-20%). Animal mitochondria contain more cardiolipin (16% of total phospholipids; Fleischer et al., 1961) than phosphatidylglycerol, whereas phosphatidylglycerol predominates in plant subcellular organelles (Benson, 1964; Kates, 1970).

v) Amino-acyl phosphatidylglycerol derivatives are found widely in Gram-positive bacteria. The most common amino acid components are lysine, alanine and ornithine. The structure of this type of phospholipid was elucidated by Macfarlane (1962) who found that the amino acids were esterified to one of the free hydroxyl groups of glycerol in phosphatidylglycerol. Molotkovsky and Bergelson (1968) synthesized lysyl-phosphatidylglycerol with the amino acid on the 3'-hydroxyl group of phosphatidylglycerol and showed it to be identical to the natural compound.

Amino-acyl phosphatidylglycerol in which lysine is an amino acid constituent was found in Staphylococcus aureus (Macfarlane, 1962), Bacillus subtilis (Bishop et al., 1967; Op den Kamp et al., 1969), B. megaterium MK10D (Op den Kamp et al., 1965) and Streptococcus faecalis (Vorbeck and Marinetti, 1965; Kocun, 1970). Alanyl-phosphatidyl-glycerol has been found in S. faecalis, C. welchii, Bifido-bacterium bifidum (Exterkate and Veerkamp, 1969) and B. cereus (Lang and Lundgren, 1970). The ornithine derivative has been reported in B. cereus and tentatively identified in B. natto (Urakami and Umetani, 1968). There are very few well-documented reports of the occurrence of this type of phospho-lipid in Gram-negative bacteria.

vi) Phosphatidlyserine is found in small amounts, or is absent from bacteria generally, except for Bordetella pertussia, in which it accounts for 20% of the total phospho-lipids (Thiele and Schwinn, 1973). The low levels of phosphatidylserine could be due to decarboxylation of phospha-tidylserine to form phosphatidylethanolamine, as has been shown in E. coli (Kanfer and Kennedy, 1964).

vii) Phosphatidic acid, an active biosynthetic precursor of various bacterial phospholipids, is present in small amounts in all bacteria studied except in Mycoplasma neurolyticum and the T-strain of Mycoplasma (see Table 2).

viii) Phosphatidylinositol is not a common phospholipid in bacteria as it is in higher organisms, and it appears to be absent from Gram-negative bacteria. Among Gram-positive bacteria, phosphatidylinositol is found only in Mycobacteria, Actinomycetes and Corynebacteria. Phosphatidylinositol found in Mycobacteria such as in Mycobacterium phlei and M. tuberculosis occurs largely in the form of mannoside derivatives in which a multiplicity of related structures, ranging from the monomannoside through the hexamannoside, have been reported (Lee and Ballou, 1965). However, only the monomannoside derivative of phosphatidylinositol was reported in most species of Actinomycetes (Kataoka and Nojima, 1967; Yano et al., 1970) and dimannoside derivative was found in Corynebacteria such as in Corynebacterium diphtheriae, C. xeosis, C. equi and C. ovis (Brennan and Lehane, 1971).

2. Glycolipids

Glycolipids are defined as lipids which are composed of carbohydrates in combination with long chain aliphatic acids or alcohols and which are readily extracted from bacteria into organic solvents without the prior use of hydrolytic procedures.

The bacterial glycolipids may be divided into two categories: (i) glycosyl diglycerides, (ii) acylated sugar derivatives. The glycosyl diglycerides are structurally

analogous to the phosphoglycerides as they are composed of carbohydrate residues glycosidically bound to the 3-position of sn-1,2-diglyceride (see Fig. 1). The term acylated sugar is used to describe those glycolipids which do not contain glycerol, but which have fatty acid residues bound directly to the carbohydrate residue (see Fig. 2).

i) Glycosyl Diglyceride

Glycolipids of this type were first found in the eubacteria in 1961, in a study of the lipids of Micrococcus lysodeikticus (Macfarlane, 1961). The mannolipid detected was first believed to be a mannosyl diglyceride, but later studies showed that the compound contained two moles of mannose per mole of glycerol, and that the complete structure was α-D-mannosyl-(1 → 3)-α-D-mannosyl-(1 → 3)-diglyceride (Lennarz and Talamo, 1966). A variety of other glycosyl diglycerides containing either glucose and/or galactose in the form of mono- or disaccharides linked to the diglyceride moiety have been reported in Gram-positive bacteria (Shaw, 1970). The major glycolipids found in most bacteria are diglycosyl diglycerides; mono- and triglycosyl diglycerides, if present at all, are usually only minor compounds (Shaw, 1970). The main types of glycolipids found in some bacteria are given in Table 3 and the structures of the diglycosyl diglycerides that have been completely characterized are shown in Figure 1. From these structures, it can be seen

FIGURE 1

Structures of the five major types of diglycosyl diglyceride isolated from Gram-positive bacteria.

(a) 3-[O-α-D-galactopyranosyl-(1→2)-O-α-D-glucopyranosyl]-1,2-diacyl-sn-glycerol

(b) 3-[O-α-D-mannopyranosyl-(1→3)-O-α-D-mannopyranosyl]-1,2-diacyl-sn-glycerol

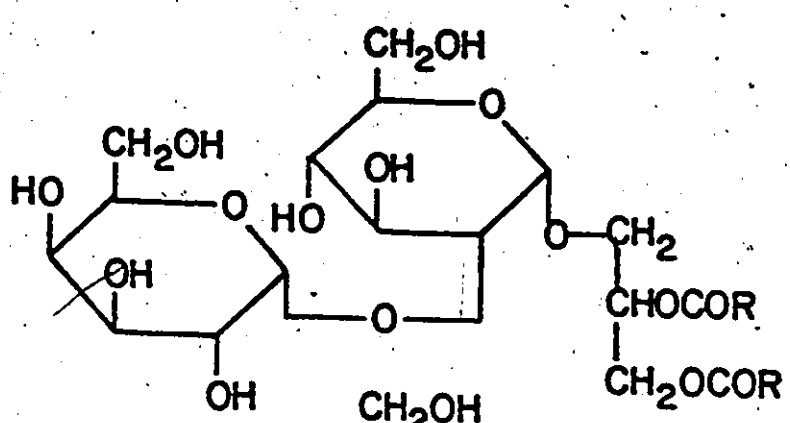
(c) 3-[O-α-D-glucopyranosyl-(1→2)-O-α-D-glucopyranosyl]-1,2-diacyl-sn-glycerol

(d) 3-[O-β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranosyl]-1,2-diacyl-sn-glycerol

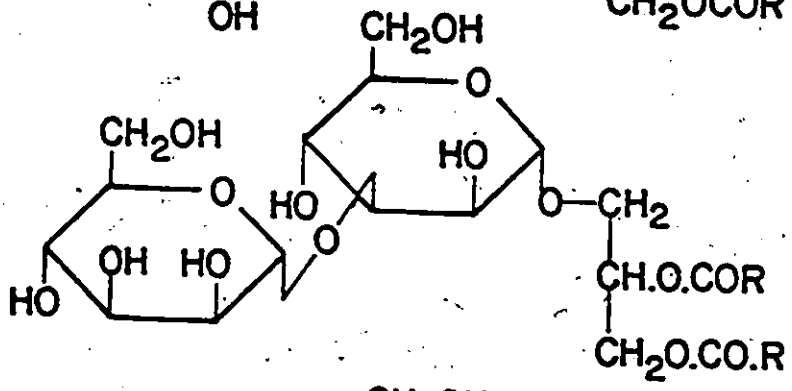
(e) 3-[O-β-D-galactopyranosyl-(1→6)-O-β-D-galactopyranosyl]-1,2-diacyl-sn-glycerol

(From Shaw, 1970)

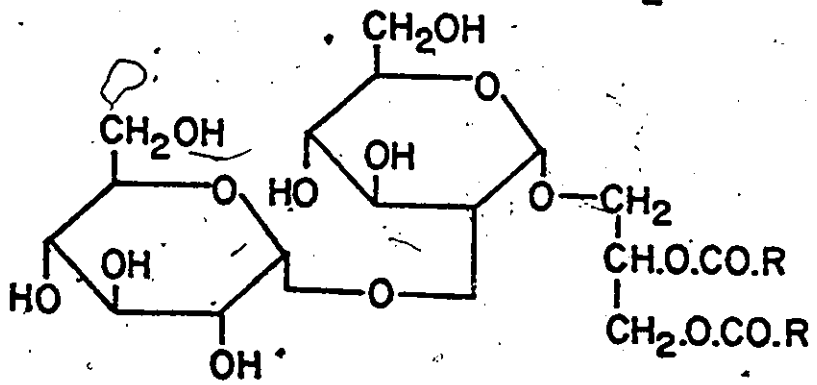
(a)


CH2OH
O
CH2OH
HO
O
HO
OH
O
CH2
OH
CHOCOR
O
CH2OCOR
OH
OH

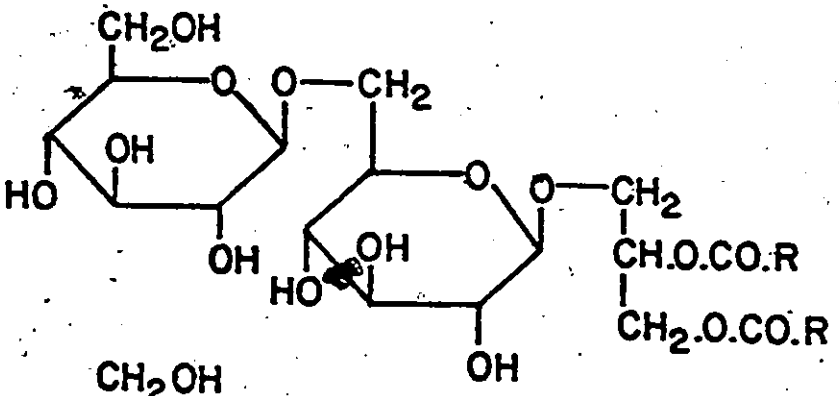
(b)


CH2OH
O
CH2OH
HO
O
HO
O
CH2
OH
HO
CH.O.COR
HO
CH2O.CO.R

(c)


CH2OH
O
CH2OH
OH
HO
O
CH2
OH
CH.O.CO.R
HO
O
CH2.O.CO.R
OH

(d)


CH2OH
O
O
CH2
OH
HO
O
O
CH2
CH.O.CO.R
OH
HO
CH2.O.CO.R
OH

(e)

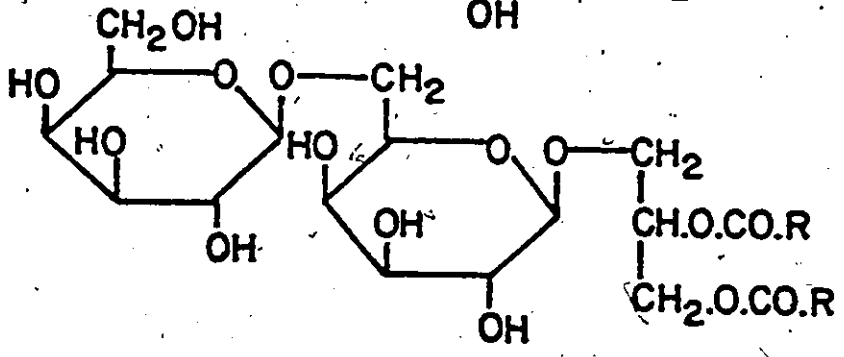

CH2OH
HO
O
O
CH2
HO
HO
O
O
CH2
OH
CH.O.CO.R
OH
CH2.O.CO.R
OH

TABLE 3

Distribution of Glycosyl Diglycerides in Bacteria[a]

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

a. See also review paper by Shaw (1970).
b. Linked to 2,3-diphytanyl-sn-glycerol.

FIGURE 2

Structures of acylated glycolipids found in bacteria.

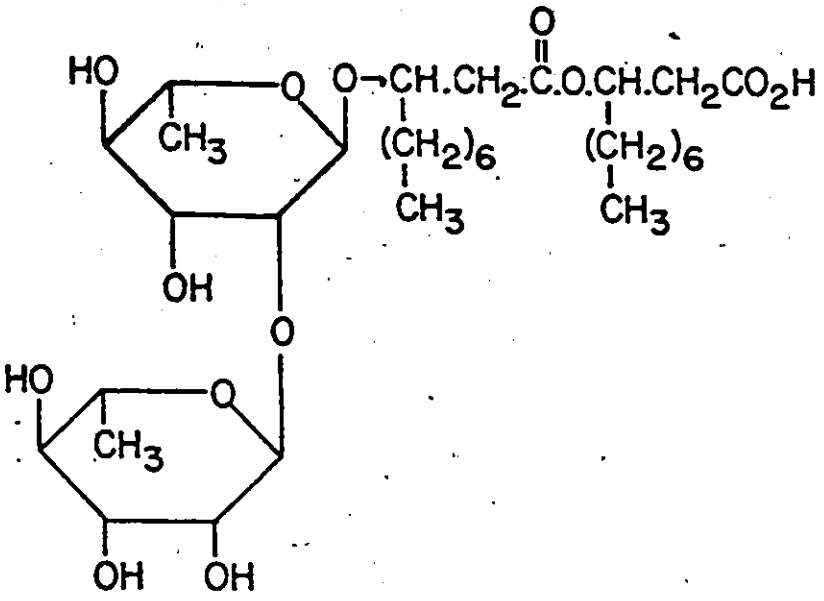
(a) Rhamnolipid from *Pseudomonas aeruginosa* (Edwards and Hayashi, 1965).

(b) Tetraacylglucose found in *Streptococcus faecalis* (Welsh *et al.*, 1968).

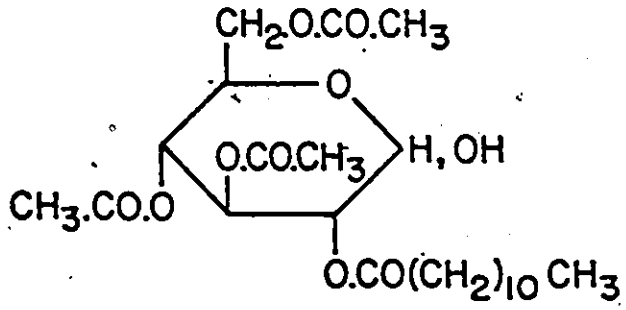
(c) Triacylglucose found in *Mycoplasma* strain J (Smith and Mayberry, 1968).

(d) "Cord factor"; 6,6'-dimycolate of trehalose (Lederer, 1967), found in *Mycobacterium tuberculosis*.

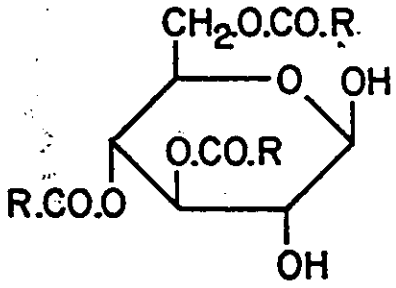
(a)


HO
O
O→CH.CH2.C.O.CH.CH2CO2H
O
CH3
(CH2)6
CH3
(CH2)6
CH3
OH
O
HO
O
CH3
OH
OH

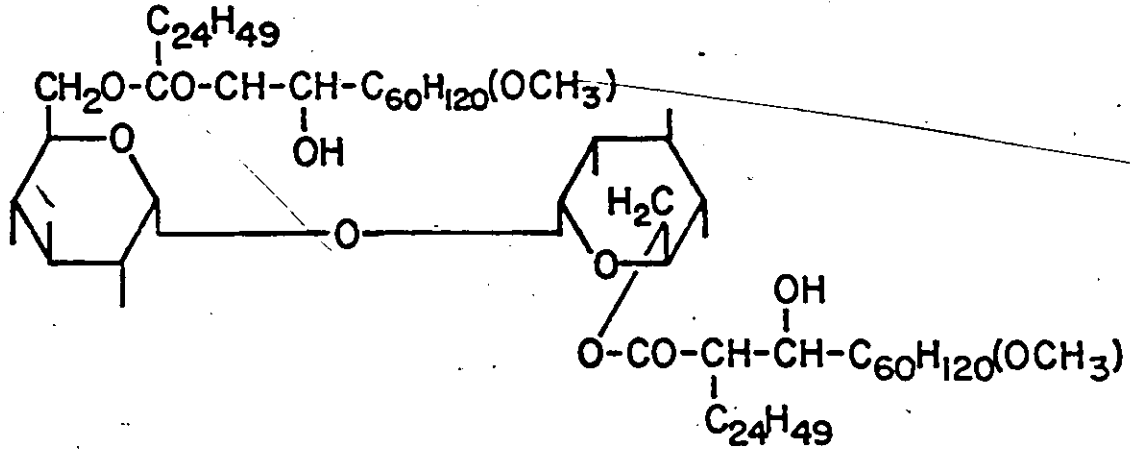
(b)


CH2.O.CO.CH3
O
O.CO.CH3
H, OH
CH3.CO.O
O.CO(CH2)10 CH3

(c)


CH2O.CO.R
O
OH
O.CO.R
R.CO.O
OH

(d)


C24H49
CH2O-CO-CH-CH-C60H120(OCH3)
OH
O
O
H2C
O
OH
O-CO-CH-CH-C60H120(OCH3)
C24H49

that both anomeric protons in the disaccharide moiety have the same configuration (i.e., both α- or both β-), and that the α-linked disaccharides, containing glucose or galactose, have a 1 → 2 linkage between the two sugars, while the β-linked disaccharides have a 1 → 6 linkage.

An examination of the distribution of glycosyl diglycerides (Shaw, 1970) shows that they are most widespread in Gram-positive bacteria. However, glucosyl diglyceride and related lipids containing uronic acids have been detected in some species of Pseudomonas (Wilkinson, 1968, 1969). Certain species of Mycoplasma also contain glycolipids of the glycosyl diglyceride type (Shaw et al., 1968; Smith, 1972).

A lipid related to the glycosyl diglycerides of the Gram-positive bacteria has been found in the Gram-negative, halophilic bacterium, Halobacterium cutirubrum. It is an ether-containing lipid and was identified 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 (Kates and Deroo, 1973). An ether-containing glycolipid was also found in the lipids of Thermoplasma acidophilum, but the structure has not yet been determined (Langworthy et al., 1972).

Shaw and Baddiley (1968) observed that bacteria of the same genus contain identical glycolipids. For instance, all bacteria in the genus Streptococcus so far examined contain a major glycolipid of the structure: glucosyl (1→ 2)-α-

glucosyl-sn-1,2-diglyceride (Fig. 1c), whereas most Lactobacilli contain a glycolipid of the structure: galactosyl (1 → 2)-α-glucosyl-sn-1,2-diglyceride (Fig. 1a). Therefore, it is possible that certain taxonomic relationships might be established from glycolipid compositions as is possible from phospholipid and fatty acid compositions (Kates, 1964; Ikawa, 1967; Abel et al., 1963). Further information on glycolipids would be required for this purpose.

ii) Acylated Sugar Derivatives

The first glycolipid of this type is the rhamnolipid isolated from Pseudomonas aeruginosa. The major structural features were outlined by Jarvis and Johnson (1949), and the complete structure (Fig. 2a) was elucidated by Edwards and Hayashi (1965). Another acylated glycolipid was found in Streptococcus faecalis and a tetraacylglucose structure was proposed on the basis of mass spectrometric studies (Fig. 2b) (Welsh et al., 1968). Similar glycolipids have been found in Aerobacter aerogenes, Pseudomonas fluorescens and E. coli (Welsh et al., 1968). A triacylglucose constitutes the major glycolipid of Mycoplasma strain J, and the full structure (Fig. 2c) has been elucidated (Smith and Mayberry, 1968). Brennan, Flynn and Griffin, (1970) confirmed the presence of acylglucoses in E. coli and separated from them into two components, a tetraacylglucose and another acylated glucose probably having fewer acyl residues.

A complex glycolipid, the so-called "cord factor" isolated from Mycobacterium tuberculosis is an acylated glycolipid in which the acyl moieties are mycolic acid and are directly esterified to the carbohydrate moiety (trehalose) (Fig. 2d; Lederer, 1967). A lipid with a similar structure to the "cord factor" has also been isolated from Corynebacterium diphtheriae (Ioneda et al., 1963). The "cord factor" is a toxic glycolipid and is known to be associated with the degree of virulence of bacteria in which it is found (Lederer, 1967).

3. Glycophospholipid

Glycophospholipids, i.e., lipids containing both phosphate and carbohydrate residues (Shaw and Stead, 1972), have been recently isolated from several bacteria; prior to this, the only known lipids of this type were the family of phosphatidylinositol mannosides (PI-mannosides) present in mycobacteria (Lee and Ballou, 1965) and related organisms (Brennan and Lehane, 1971), and the glucosaminylphosphatidyl glycerols present in Bacillus megaterium and Pseudomonas ovalis (Phizackerley et al., 1966; Op den Kamp et al., 1969a; and MacDougall and Phizackerley, 1969). In both instances, carbohydrate residues are bound glycosidically to known phospholipids (PI and PG, respectively).

Four different glucophospholipids have been isolated from a wide variety of bacteria and the established

structures are summarized in Fig. 3. The first glucophospholipid was isolated by Smith and Henrikson (1965) from Acholeplasma laidlawii B and was identified as phosphatidylglucose. However, recent studies showed that this lipid has a glycerylphosphoryldiglucosyl diglyceride structure (Fig. 3a) (Shaw et al., 1972). A similar glucophospholipid was also found in Streptococcus faecalis and S. hemolyticus (Fischer et al., 1973). An analogous lipid containing four acyl residues was isolated from S. faecalis and S. lactis by Fischer et al. (1970,1973a), (Fig. 3b). Phosphatidylglucosyl diglyceride, a tetraacyl derivative containing only one glucose residue, was isolated from Pseudomonas diminuta by Wilkinson and Bell, 1971; Fig. 3c). A monoglucosyl derivative of phosphatidylglycerol (Fig. 3d) was found in an unidentified Gram-negative, halotolerant bacterium (Pelleg and Tietz, 1973). The glucophospholipid present in Staphylococcus aureus has been studied by Short and White (1970) who proposed the compound to be a glucosyl derivative of phosphatidic acid (Fig. 3e). This proposed structure will be discussed further in the Discussion section.

4. The Fatty Acids of Bacterial Lipids

Typical examples of fatty acids of both Gram-negative and Gram-positive bacteria are shown in Table 4. In general, the chain lengths of the various types of fatty acids are in the range of C_{12} to C_{20}, with C_{16} to C_{18}

FIGURE 3

Structures of glucophospholipids isolated from bacteria.

(a) 3-O-[sn-glycero-1-phosphoryl-6'-O-(α-D-glucopyranosyl-(1→2)-O-α-D-glucopyranosyl)]-1,2-diacyl-sn-glycerol; found in Streptococcus faecalis and S. hemolyticus or 3-O-[sn-glycero-3-phosphoryl-6'-O-(α-D-glucopyranosyl-(1→2)-O-α-D-glucopyranosyl)]-1,2-diacyl-sn-glycerol; in Acholeplasma laidlawii.

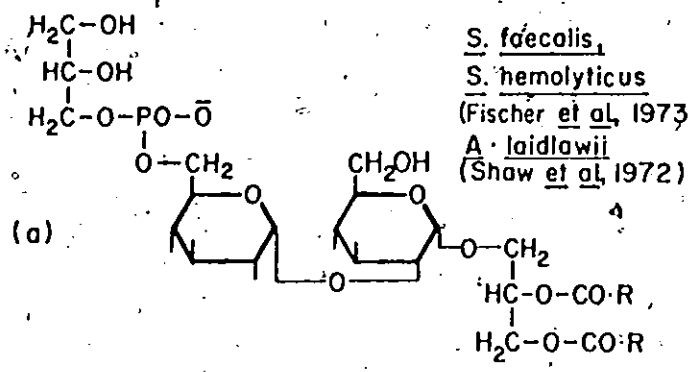
(b) 3-O-[6-(1,2-diacyl-sn-glycero-3-phosphoryl)-2-O-(α-D-glucopyranosyl)-α-D-glucopyranosyl]-1,2-diacyl-sn-glycerol.

(c) 3-O-[6-(1,2-diacyl-sn-glycero-3-phosphoryl)-O-α-D-glucopyranosyl]-1,2-diacyl-sn-glycerol.

(d) 1-O-(1,2-diacyl-sn-glycero-3-phosphoryl)-2-O-(α-D-glucopyranosyl)-sn-glycerol.

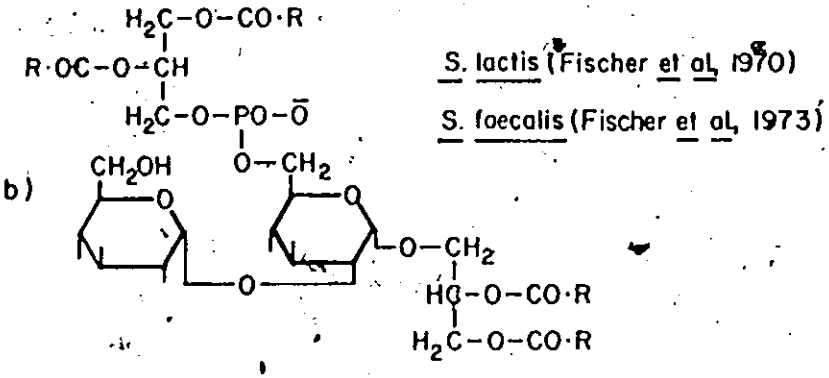
(e) 1,2-diacyl-sn-glycero-3-phosphorylglucose.

(a)



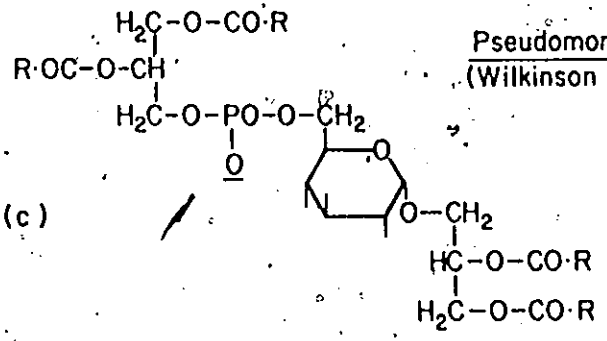
S. faecalis,
S. hemolyticus
(Fischer *et al*, 1973)
A. laidlawii
(Shaw *et al*, 1972)

(b)



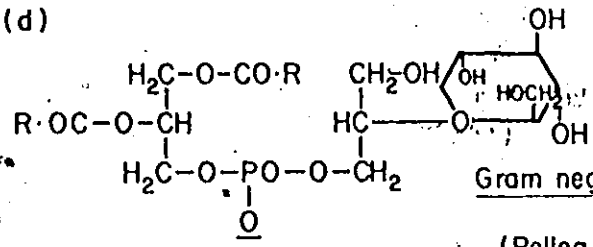
S. lactis (Fischer *et al*, 1970)
S. faecalis (Fischer *et al*, 1973)

(c)



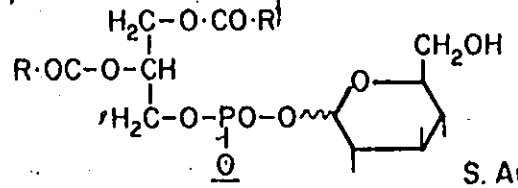
Pseudomonas diminuta
(Wilkinson & Bell, 1971)

(d)



Gram negative, halotolerant bacterium
(Pelleg & Tietz, 1973)

(e)



S. Aureus (Short & White, 1970)

TABLE 4

Major Fatty Acids of Various Bacteria[a]

Organism	Fatty Acids											References
	14:0	16:0	18:0	>18:0	16:1	18:1	17:cy	19:cy	13:br	15:br	17:br	
Gram Negative												
Pseudomonadales												
Pseudomonadaceae												Oliver & Colwell (1973a)
Pseudomonas perfectomarinus	1	22	3	-	24	49	-	-	-	-	-	Wilkinson (1970)
Pseudomonas (12 sp.)	0.2-1	25-5.4	0.4-2	-	12-39	12-43	-	-	-	-	-	
Spirillaceae												
Vibrio algosus	3	31	3	-	38	20	-	-	-	-	-	Oliver & Colwell (1973a)
V. alginolyticus	1	13	1	-	50	15	-	-	-	-	-	
V. parahaemolyticus	2	11	1	-	40	38	-	-	-	-	-	
Eubacteriales												
Enterobacteriaceae												Kanemasa (1967)
Escherichia coli	2	27	-	-	15	-	9	3	-	-	-	Olsen & Ballou (1971)
Salmonella typhimurum	4	38	tr	-	25	24	-	-	-	-	-	
Achromobacteraceae												Oliver & Colwell (1973a)
Achromobacter aquamarinus	tr	12	tr		21	65	-	-	-	-	-	
Brucellaceae												
Brucella melitensis	tr	30	12		2	18	-	29	-	-	-	Thiele & Schwinn (1973)
B. abortus	1	11	30		2	9	-	27	-	-	-	
Bordetella pertussis	2	47	9		29	2	-	0	-	-	-	
Yersinia pestis	3	23	2	2	13	12	20	4	-	-	-	Tornabene (1973)
Y. pseudotuberculosis	3	27	1	tr	27	12	21	2	-	-	-	
Mycoplasmatales												
Mycoplasmataceae												Romijn et al. (1972)
Mycoplasma laidlawii B[b]	25	59	3	-	+	6[c]	-	-	-	-	-	Smith (1972)
M. neurolyticum	3	32	87	-	1	36[c]	-	-	-	tr	-	Romano et al. (1971)
T strain of Mycoplasma	-	30	5	-	7	40[c]	-	-	-	2	2	

Table 4 continued

Organism	Fatty Acids											References
	14:0	16:0	18:0	>18:0	16:1	18:1	17:cy	19:cy	13:br	15:br	17:br	
Gram Positive												
Eubacteriales												
Micrococcaceae										80-90	–	Macfarlane (1961)
Micrococcus lysodeikticus	–	–	–	–	–	–	–	–	–	–	–	Wilkinson (1972)
M. denitrificans	tr	8	4	–	1	86	–	–	–	57	5	Haest *et al.* (1972)
Staphylococcus aureus	1	7	17	5	1	1	–					
Lactobacillaceae												Thorne & Kodicek (1962)
Lactobacillus casei	1	9	1	–	10	26	0	49	–	–	–	dosSantos Mota *et al.* (1970)
Streptococcus faecalis	5	30	–	–	7	37	–	20	–	–	–	Uchida & Hoji (1973)
Pediococcus cerevisiae	4	53	1	–	1	2	10	28	–	–	–	
Corynebacteriaceae										55	35[d]	Kosaric & Carroll (1971)
Listeria monocysogens	3	6	–	–	–	–	–	–	–	67	29	Shaw & Stead (1971)
Arthrobacter crystallopoites	–	2	–	–	–	–	–	–	–	83	12	
A. paseeus	–	4	–	–	–	–	–	–	–			
Bacillaceae										30	–	Saito & Mukoyama (1971)
Bacillus cereus	24	14	tr	–	5	–	–	–	26	50	15	Saito (1960)
B. subtilis	+	+	–	–	–	–	–	–	–	–	–	Goldfine & Bloch (1961)
Clostridium butyricum	2	49	6	–	17	8	9	5	–			
Actinomycetates											[e]	Kataoka & Nojima (1967)
Actinomycetaceae										–		
Nocardia polychromogenes	–	+	–	–	+	–	–	–	–			Kataoka & Nojima (1967)
Streptomycelaceae										+	+	Tabaud (1971)
Streptomyces griseus	+	+	–	–	+	–	–	–	–	26	16[g]	
Micromonospora[f]	13	2	15	–	–	–	15	13	–			

a. See also Kates (1964). Values are given as percentage of total fatty acids. Abbreviations: –, not detected, +, present but amount unknown; tr, trace. Abbreviations for the fatty acids are as follows: the first number indicates chain length, the second indicates the number of double bonds; cy, cyclopropane ring; br, branched.

Footnotes (Table 4)

a. See also Kates (1964)
Values are given as percentage of total fatty acids.
Abbreviations: -, not detected; +, present but amount unknown; tr, trace.
Abbreviations for the fatty acids are as follows: the first number indicates chain length, the second indicates the number of double bonds; cy, cyclopropane ring; br, branched.

b. Fatty acid composition of this organism vary markedly with the composition of the growth medium. Values reported here obtained from growth in the presence of 12:0 fatty acids.

c. Contain small amount of 18:2.

d. Composed of 30% anteiso-C_{17} and 5% iso-C_{16}.

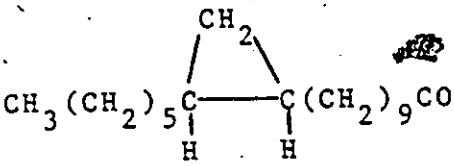
e. iso-C_{19} fatty acid.

f. Fatty acid composition of cardiolipin, one of the lipid component of this bacteria.

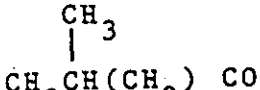
g. iso-C_{16} fatty acid.

predominating. There are mainly four chain types: straight-chain saturated, straight-chain mono-unsaturated, cyclopropane, and branched chains, predominantly iso and anteiso fatty acids. The general formulae of these fatty acids are as follows:

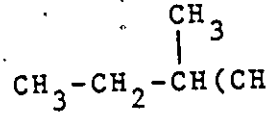
$CH_3(CH_2)_5CH{=}CH(CH_2)_9COOH$ cis-vaccenic acid



lactobacillic acid



iso fatty acid



anteiso fatty acid

The long chain polyunsaturated fatty acids typical of the lipids of higher organisms are absent in bacteria. The most common unsaturated fatty acids in bacteria are cis-vaccenic acid, 18:1, Δ^{11} (rather than oleic acid, 18:1, Δ^9) and palmitoleic acid, 16:1, Δ^9 (Goldfine, 1972). The biosynthetic precursors of the cyclopropane fatty acids are the corresponding unsaturated fatty acids (see Kates, 1964), and as a consequence, the most commonly occurring cyclopropane fatty acids are cis-9,10-methylenehexadecanoic acid and cis-11,12-methyleneoctadecanoic acid (lactobacillic acid), but cis-9,10-methyleneoctadecanoic acid (dihydrosterculic acid)

has also been found (Law, 1971). The most commonly encountered branched-chain fatty acids are anteiso C_{15} and anteiso C_{17}, although iso C_{15}, iso C_{16} and iso C_{19} acids are also found. Hydroxy fatty acids are not generally found in "extractable lipids", but frequently occur as a component of the lipopolysaccharides in cell walls (Osborn, 1969; Key et al., 1970, Hancock et al., 1970).

There appears to be a fairly clear cut distinction between the fatty acids of Gram-positive and Gram-negative bacteria. Virtually most of the Gram-positive organisms thus far studied contain branched chain fatty acids; often these acids make up the major proportion of the total fatty acid. For instance, in M. lysodeikticus, 80% of the total fatty acids are branched C_{15} acids (Macfarlane, 1961), and in Arthrobacter pasceus, 83% of the total fatty acids are anteiso-C_{15} (Shaw and Stead, 1971). In contrast to Gram-positive bacteria, little or no branched chain acids have been found in Gram-negative organisms, which typically contain saturated, unsaturated, and cyclopropane fatty acids (see Kates, 1964, and Table 4). One major exception to the correlation between Gram stain and fatty acid composition is the Gram-positive Lactobacillaceae. The organisms of this family contain saturated, unsaturated, and cyclopropane fatty acids (see Table 4) that are typical of Gram-negative bacteria.

Abel et al. (1963) have suggested that the fatty acid composition of a particular microorganism might be a

tool to aid in the classification of the organism. Examination of the fatty acid profiles of various bacteria (Kates, 1964 and Table 4) indicates that members of the same family generally have qualitatively similar fatty acid patterns. For instance, Wilkinson (1970) examined twelve pseudomonads and found they have the same fatty acid profiles. Oliver and Colwell (1973a) studied the fatty acid composition of thirteen species of Vibrio and found that all of them had the same qualitative fatty acid composition. The Vibrio patterns were also qualitatively similar to those of the pseudomonads examined in that study. Uchida and Mogi (1972) examined the fatty acid composition of thirty-eight strains (representing six species) of Pediococcus and obtained a fatty acid composition profile which was similar to those of Lactobacillus and Streptococcus. The latter two genera are in the same family as Pediococcus (i.e., Lactobacillaceae). However, similarity in fatty acid profile in different families of bacteria was also observed among Gram-positive or Gram-negative bacteria (see Table 4). It is likely that a certain fatty acid profile cannot specify the family of bacteria, but differences in fatty acid profiles of bacteria classified in the same family may suggest that the classification of these bacteria should be reconsidered.

While qualitative similarities appear to be the rule for fatty acids from microorganisms of the same family, quantitative variations in fatty acid compositions are

observed with changes in medium composition, temperature or culture age of the bacteria (Kates, 1964; Uchida and Mogi, 1972; Oliver and Colwell, 1973a).

5. Lipids of Nocardia

Lipids of Nocardia were first studied by Michel (1958) who subsequently identified a series of β-hydroxy-α-branched unsaturated acids (C_{46} to C_{58}), named nocardic acids in Nocardia asteroides (Michel et al., 1960; Bordet and Michel, 1963). These nocardic acids were found associated with the "bound lipid" of the cell walls and were liberated after alkaline hydrolysis (Asselineau, 1966). The free lipids of Nocardia asteroides contain a series of corynomycolic acids (similar in structure to that of nocardic acid) ranging from C_{28} to C_{36} and a "cord factor" which contains only corynomycolic acids ranging from C_{32} to C_{36} (Ioneda and Lederer, 1970). The free lipids of Nocardia rhodochrous also contain a "cord factor" with nocardic acids ranging from C_{38} to C_{46} (Ioneda and Lederer, 1970).

Cells of Nocardia sp. have a high content of lipids, 37% (on a dry weight basis) extractable with alcohol-ether and an additional 6% extractable by chloroform (Guinand et al., 1958; Michel, 1958). The alcohol-ether extract contains a mixture of peptidolipids, consisting of peptides bound to fatty acids, amounting to 5% of the total lipid (Michel, 1958). The peptide moieties contain threonine, alanine, valine,

isoleucine, leucine, and proline; the fatty acids consist of at least four acids ranging from C_{16} to C_{32} (Guinand et al., 1958). These peptidolipids appear to be similar to the peptidolipid, fortuitine, isolated from M. fortuitum (Vilkas et al., 1963), except that the former contain only D-amino acids (Ikawa and Snell, 1962) whereas the latter contains only L-amino acids (Vilkas et al., 1963).

Phospholipids of Nocardia polychromogens consisted of cardiolipin (55%), PE (20%) and PI-mannoside (25%), no PG or PC was detected (Kataoka and Nojima, 1967). In N. leishmanii, Yano et al. (1970) found two species of PE (PE-1 and PE-2) containing different fatty acids, together with cardiolipin, PI and PI-mannoside; PG was a minor component. In contrast, phospholipids of N. coelica contains PC as the major component, accounting for 25 to 40% of the total phospholipids, together with PE (25-30%), PI (11-14%) and cardiolipin (7-15%) (Yano et al., 1969). The presence of PC in N. coelica is unique in the Actinomycetales. PI-mannosides, of the dimannosyl type, are barely detectable in N. polychromogens but were reported by Khuller and Brennan (1972) to be the most predominant phospholipids in N. coelica.

Besides the "cord factor", other glycolipids found in N. polychromogenes and N. coelica include an acylated glucose, diglucosyl diglyceride and a glucophospholipid (Khuller and Brennan, 1972). The latter was suggested to be the same type as the glucophospholipid found in Pseudomonas diminuta (Wilkinson and Bell, 1971).

The fatty acids of several Nocardia species, apart from the high molecular weight (C_{46} to C_{58}) nocardic acids (Michel et al., 1960), include normal saturated acids from C_{14} to C_{19} (palmitic acid predominating), branched chain saturated acids (iso- and anteiso-C_{15}, -C_{16} and -C_{17}; iso-C_{18} and 10-methyl stearic acids), and unsaturated acids (9-hexadecenoic, 10-hexadecenoic, 9-heptadecenoic and oleic acids) (Bordet and Michel, 1963; Kataoka and Nojima, 1967; and Yano et al., 1970). Yano et al. (1970) reported the presence of α-hydroxy fatty acids of: iso-C_{14} (17%), anteiso-C_{15} (65%), C_{15} (4%), iso-C_{16} (12%) and anteiso-C_{17} (2%) in N. leishmanii. These α-hydroxy acids were located exclusively at the 2-position of the PE-2 of N. leishmanii (Yano et al., 1970). The physiological function of the α-hydroxy fatty acid-containing phospholipids remains unknown.

6. Lipid Components of Staphylococci

Macfarlane (1962a) investigated the lipids of S. aureus and found 2.5% of the cell dry weight to be free lipids, of which 16% was neutral lipids (about half being diglycerides), the remainder consisting of phosphatides and a glycolipid. The phosphatides contained mostly phosphatidylglycerol mixed with an unknown nitrogenous component, and smaller amounts of phosphatidic acid and cardiolipin; inositol, glucosamine, and N-acetylglucosamine were absent. In a subsequent investigation, Macfarlane (1962) found that

60% of the phosphatide fraction contained amino acid esters of phosphatidylglycerol, accounting for the "nitrogenous component" reported earlier (Macfarlane, 1962a). The glycolipid component was presumed to be a β-glycosyl glyceride on the basis of its optical rotation ($[\alpha]_D$-19°, in chloroform), and the liberation of glucose after acid hydrolysis and reducing sugar after treatment with emulsin. The fatty acids in the neutral lipid, phosphatide, and glycolipid fractions were similar in composition and consisted mainly of branched chain saturated acids, including anteiso-C_{15} (45%), iso-C_{15} (13%); anteiso- and iso-C_{17} (12%), and straight chain fatty acids, including palmitic (9%), stearic (9%), and C_{20} (3%) acids. More recently, Haest et al. (1972), reported a qualitatively similar fatty acid pattern for S. aureus but with somewhat different percentages of the major fatty acids, thus: iso-C_{15}, anteiso-C_{15} and straight chain-C_{15} (60%), anteiso- and iso-C_{17} (4%), anteiso-C_{19} (2%), stearic (18%), palmitic (7%) and C_{20} (6%) acids. The quantitative differences in fatty acid composition are probably due to culture conditions, since Macfarlane (1962a) cultured S. aureus on Marmite agar while Haest et al. (1972) used a liquid medium. The effect of culture conditions of bacteria on lipid composition has been discussed in more detail elsewhere (Kates, 1964).

Polonovski et al. (1962) also reported that the lipids of S. aureus contained 30% neutral lipids, the

remainder being phosphatides and glycolipids. The phosphatides were mainly phosphatidic acid and phosphatidylglycerol, but traces of nitrogenous phosphatides were detected (cf. Macfarlane, 1962a). The glycolipid fraction appeared to be the same as that found by Macfarlane (1962), and the structure was shown to be a mixture of two compounds: glucosyl-β-(1 → 6)-glucosyl-β-(1 → 1)-diglyceride and glucosyl-β-(1 → 3)-glucosyl-β-(1 → 1) diglyceride (Polonovski et al., 1965; Brundish et al., 1966).

Short and White (1970) reported the presence of phosphatidylethanolamine and glucophospholipid as minor lipid components (2%) together with the other phospholipids and glycolipids found before by Macfarlane (1962a) and Polonovski et al. (1962). These polar lipid components, as well as vitamin MK_2 and carotenoids, were found in the same membrane fraction as cytochrome oxidase and cytochromes a, and b_1, suggesting that these lipids are part of the same membranes as the respiratory pigments (White and Frerman, 1967).

The membrane composition of staphylococcal protoplasts was examined by Mitchell and Moyle (1951), who found 41% protein and 23% lipid in the "small particle" (membrane) fraction from S. aureus Duncan. Ward and Perkins (1968) examined the protoplast membranes of S. aureus and found them to contain a similar content of lipids (22-25%), whereas the L-form membrane contained higher amounts (25-36%).

The lipid content of subcellular fractions prepared from S. aureus and their induced L-forms has been reported by Ward and Perkin (1968), who found 89-94% of the lipid in the membrane fraction and the remaining lipid in the cytoplasmic fraction, none being detected in the cell wall. The major phospholipid component of the protoplast membranes was phosphatidylglycerol, together with its amino-acyl derivatives, whereas the L-form membrane contained cardiolipin as the major component. The L-forms contained much more glycolipid (diglucosyl diglyceride) than the parent bacteria.

Bergh, Webb and McArthur (1964), in their studies on the relationship between bacterial lipids and the Gram staining reaction, reported the fatty acids of S. epidermidis to consist mainly of branched chain fatty acids including branch-C_{15} (37%), branch-C_{17} (12%), branch-C_{19} (20%), branch-C_{21} (11%), and normal fatty acids including palmitic (2%), stearic (6%) and C_{20} (7%); neither cyclopropane nor unsaturated fatty acids were detected. Investigations of the phospholipid and glycolipid content of S. epidermidis have so far not been reported.

B. Lipids of the True Fungi (Eumycetes)

The true fungi are plantlike, chlorophyll-free organisms. Included are the varieties generally called the yeasts and the molds, as well as another large group, known as the Fungi Imperfecti.

Shaw (1966), and Erwin (1973) have reviewed the total fatty acid composition of various fungal spores or mycelia. Generally, fungi contain high amounts of unsaturated fatty acids, including palmitoleic, oleic, linoleic and linolenic acids and straight chain fatty acids, including mainly palmitic and stearic acids. Branched chain and cyclopropane fatty acids are generally absent in fungi.

Spores from three species of fungi, Rhizopus nigricans, Alternaria oleracea and Neurospora setophile, contain similar phospholipid patterns, with PC, PE, PS and an unidentified phosphatide as major components (Jack and Laredo, 1968). However, the fatty acid compositions of the phospholipids of the three species were found to differ considerably with respect to the contents of C_{16}, C_{18} and C_{20} unsaturated acids.

Domer and Hamilton (1971) studied the lipid composition of the cell wall and cell sap (lyophilized whole cells minus cell wall, containing mitochondrial and microsomal fractions) of both yeast and mycelial phases of Histoplasma capsulatum and Blastomyces dermatitidis. Cell wall and cell sap of both mycelial and yeast phases had similar lipid components which consisted of PC, PE, PS, triglycerides, diglycerides, sterols, sterol esters and free fatty acids. Triglycerides were the greatest single component in the extracts; PC and PE were the major phospholipids. The main differences between cell walls and homologous cell

sap and between yeast phase and mycelial phase, involved primarily the quantities of oleic and linoleic acids in the total lipids; these two acids accounted for 75-80% of the total fatty acids in both cases. In general, oleic acid predominated in yeast phase extracts and linoleic acid was either equal or greater in concentration in the mycelial phase extracts. Cell walls usually contained more oleic acid and less linoleic acid than homologous cell sap (Domer and Hamilton, 1971).

Mumma et al. (1970, 1971a) and Bruszewski et al., (1972) examined the lipid composition of nine thermophilic and nine mesophilic species of seven genera of fungi. Most fungi studied contained 8-18% lipids. The predominant fatty acids were found to be palmitic, oleic and linoleic acids. Lesser amounts of arachidic, linolenic, palmitoleic, pentadecanoic, myristic and lauric acids were found. The mesophiles contained 2-20% linolenic acid, while the thermophiles did not contain any appreciable amounts of this acid (<0.5%). The fatty acids of the thermophilic fungi were more saturated than the corresponding mesophilic species. The mesophiles, Mucor globosus, and Humicola grisea contain γ-linolenic acid, (Mumma et al. 1970). The γ-linolenic acid was also found in lipids of Dactylaria ampulliforme (Fungi Imperfecti)(Sumner, 1970) and in Chaetomium cochliodes (Ascomycete)(Safe and Brewer, 1973).

The yeasts, Saccharomyces cerevisiae (Baker's

yeast) and Candida lipolytica, a mesophile, contain similar patterns of fatty acids as the other fungi, that is, they contain high percentages of unsaturated fatty acids. However, Candida lipolytica contained linoleic acid (35-55%), together with oleic acid (17-34%) and palmitic acid (7-9%)(Kates and Paradis, 1973), whereas in baker's yeast, linoleic acid was absent, or present in trace amounts, and palmitoleic acid (42-50%) and oleic acid (22-29%) were the major unsaturated fatty acids (Suomalainen and Nurminen, 1970; Hunter and Rose, 1972). In both species, palmitic and stearic acids were the major saturated fatty acids.

Relatively little quantitative data on the phospholipids of fungi are available. Generally, phospholipids found in fungi are different from those of bacterial phospholipids in that PC is ubiquitous in fungi and always present as a major component; PE, PI, PS and PA are also found. For example, Tricholma nudum contained PC (59%), PE (20%), PS (8%) and PA (7%) (Leegwater et al., 1962), and Pythium ultimum, grown at 30°C, contained PC (34%), PE (24%), PG (12%), PI (11%), lyso PC (6%) and cardiolipin (2%)(Bowman and Mumma, 1967). Growth of the latter organism at a lower temperature (20°C) resulted in higher amounts of PC (41%) and PG (15%), and lower amounts of PA (6%) and lyso PC (3%)(Bowman and Mumma, 1967). Phospholipids of the mesophile Candida lipolytica also showed little quantitative differences in relative amounts of major phospholipid components when grown at 10°

and 25° (Kates and Baxter, 1962). The percentage composition at 25°C and 10°C, respectively, were PC, 41% and 36%; PE, 24% and 29%; and PS + PI, 33% and 31%. Phospholipids of Agaricus bisporus were composed mainly of PC (48%) and PE (50%); PI and PS were present only in small amounts (<1%) (Holtz and Schisler, 1971). Mumma et al. (1971) studied the phospholipids of a thermophilic fungus, Humicola grisea (grown at 45°C), and found an unusually high content of PA, (32%), together with PC (34%), PE (16%), PI (13%), cardiolipin (4%) and PS (2%). The high content of PA in H. grisea was suggested as a characteristic of fungi grown at elevated temperature (Mumma et al., 1971). However, phospholipids of the fungus Blakeslea trispora grown at 29°C also contained high amounts of PA (34%), together with cardiolipin (34%) and PG (22%), neither PC nor PE were present (Chenouda, 1972). It should be noted that a high content of PA may simply be due to the action of phospholipase and not necessary a characteristic of thermophiles. Changes in fatty acids composition of phospholipid components rather than in phospholipid composition are likely to occur when organisms are grown at different temperatures. This phenomenon was observed when Candida lipolytica was grown at 10°C and 25°C: fatty acids of all phospholipid components were more unsaturated (higher linoleic acid content) at 10°C than 25°C (Kates and Paradis, 1973).

Neutral lipids of fungi consist of sterols, sterol glycosides, sterol esters, triglycerides, diglycerides and free

fatty acids (Mumma et al., 1971a,; Chenouda, 1972). The major sterol in fungi is ergosterol (Chenouda, 1972, Safe and Brewer, 1973).

The lipids of fungi in the genus Wallemia have not been studied previously. However, from the available information on fungal lipids, one would expect that members of the genus Wallemia should have a lipid composition similar to that of fungi in the group Fungi Imperfecti (e.g. Blastomyces dermatitidis and Histoplasma capsulatum).

III. Subcellular Distribution of Lipids

A. General Characterization of Bacterial Membranes

Cell membranes, identifiable in the electron microscope by the characteristic profile seen in thin sections, are ubiquitous in Nature as the "unit membrane", that is, alternate electron-dense, electron-transparent layering with overall thickness of about 75 Å (Robertson, 1959). Bacterial cells are much simpler than plant and animal cells in that they contain only a few membrane-bounded organelles, such as mesosomes (Fitz-James, 1964; Reusch and Burger, 1973), and in this respect offer a simple system for investigations of membrane structure and function. The simplest bacterial membrane systems appear to be those of protoplasts obtained from Gram-positive bacteria, and those of the Mycoplasma group of organisms. In both cases, the cells are bounded by a single membrane, with little evidence of internal membranes as seen in those bacteria which possess an outer rigid cell wall (Anderson and Barile, 1965; Ryter and Landman, 1968). More complex than the Mycoplasma and protoplasts are Gram-positive bacteria with walls and plasma and mesosomal membranes, and Gram-negative bacteria, with their multilayered envelope structures and, in some species, mesosomes. The cell envelopes of these two types of bacteria will be described briefly.

Electron microscopy of thin sections of Gram-positive bacteria clearly established the relationship between

cell walls and membranes. The wall is seen as a thick (about 200 to 800 Å), rather amorphous structure with an underlying cytoplasmic membrane of "unit membrane" appearance (Fitz-James, 1964; Weibull, 1965). The cell wall of Gram-positive bacteria can be removed by exposure of resting cells to enzymes such as lysozymes or by culturing replicating cells in the presence of a specific inhibitor of cell wall synthesis, such as penicillin (Gooder, 1968). The cell wall-less structures of bacteria were termed protoplasts, and can be maintained in a viable condition by placing them in a suitable stabilizing medium, such as in 0.01 M tris-chloride buffer, pH 7, containing either 0.6 M sucrose or 8% polyethyleneglycol (Gooder, 1968).

Bacterial cell walls contain a mucopeptide (or peptidoglycan), composed of repeating units of hexosamine-muramic acid, and a small peptide which forms the rigid "backbone" of the wall (Martin, 1966). Walls of probably all Gram-positive bacteria contain teichoic acid (polymeric glycerol or ribitol phosphate) associated with mucopeptides (Archibald et al., 1968; Knox and Wicken, 1973); these teichoic acids apparently are not synthesized by any Gram-negative bacteria (Knox and Wicken, 1973).

The cell envelope of Gram-negative bacteria are more complex; electron microscopy has revealed two membranes, each with a double-track structure (Birch-Anderson et al., 1953; Kellenberger and Ryter, 1958). The outer double-track

layer (outer membrane) contains both the lipopolysaccharide and lipoproteins of the cell envelope (Hofshneider and Martin, 1968; Forsberg et al., 1970a), while the inner cytoplasmic membrane is composed of phospholipids and proteins (Forsberg et al., 1970 ; Martin and Macleod, 1971). In the space between the two membranes a thin layer of peptidoglycan has been observed in certain bacteria by staining with lead or lanthanum salts (Murray et al., 1965). Structured layers exterior to the outer membrane have been revealed in several Gram-negative bacteria (Pate and Ordal, 1967; Forsberg et al., 1970a) and were shown to consist primarily of amino and non-amino sugars, with significant amounts of protein also present (Forsberg et al., 1970 ; Martin and Mcleod, 1971).

Attempts to remove completely the cell wall of Gram-negative bacteria have generally been unsuccessful, yielding structures termed spheroplasts, which still contain fragments of the complex wall. Recently, Forsberg et al. (1970a) demonstrated that in a marine pseudomonad B-16, the outer double-track layer can be separated from the cytoplasmic membrane of the cell envelope by repeated suspension of cells in 0.5 M sucrose in the presence of EDTA. By this procedure, these workers were able to obtain protoplasts of Gram-negative bacteria free of cell wall material.

B. Subcellular Distribution of Lipids

1. In Gram-positive Bacteria

Because of the relative ease with which membranes of Gram-positive organisms can be obtained by numerous techniques (see Salton, 1967; Guze, 1968 and Kaback, 1971), considerable information on the subcellular localization of lipids is available for Gram-positive bacteria. In general, cytoplasmic membranes isolated from various Gram-positive bacteria contain 15-30% lipids, 40-80% protein, and in some cases, 1-20% carbohydrate and 1-10% RNA (see Salton, 1967).

Vorbeck and Marinetti (1965) studied the subcellular distribution of lipids in Streptococcus faecalis and found that 94% of the cellular lipid is found in the membrane fraction, 5.9% in the "protoplasm", and none in the cell wall fraction. The cell wall accounted for 31.2% of the dry weight of the bacteria, whereas the membrane and "protoplasm" accounted for 8.9 and 57.4%, respectively. Therefore, approximately 30% of the dry weight of the membrane was lipid, whereas only 0.3% of the protoplasm was lipid. By way of comparison, the value found for membranes of Bacillus megaterium strain M, is 15-20% (Weibull, 1957), and that of Staphylococcus aureus is 23% (Mitchell and Moyle, 1951). The major lipid components of the membrane are identical to those in the cytoplasm of S. faecalis and consist of PG, aminoacyl PG and glycosyldiglyceride (Vorbeck and Marinetti, 1965). Ward and Perkins (1968) also examined

the distribution of lipids among subcellular fractions of Staphylococcus aureus and obtained results similar to those observed in S. faecalis, namely, 90-94% of total lipids were in the membrane fraction, 6-10% in the protoplasm, and none in the cell wall.

In contrast to other Gram-positive bacteria, the cell walls of Mycobacterium sp. also contained phospholipids in an amount equal to half that of the membrane. Cardiolipin was the predominant phospholipid in the membrane, whereas phosphatidylinositol mannosides were the principal cell wall lipids (Akamatsu et al., 1966; Motomiya et al., 1969). Kotani et al. (1959) viewed phosphatidylinositol mannosides of mycobacterial cell walls as a "cementing substance" for the cell wall skeleton.

Thus, in Gram-positive bacteria, except for Mycobacterium, most of the lipids are located in the cytoplasmic membrane and the composition of total cellular lipids (extracts of whole-cells) will reflect the membrane lipid composition.

2. In Gram-negative Bacteria

Recently, suitable procedures for separation of the outer and inner membranes of Gram-negative bacteria have been developed (Schnaitman, 1970; Fox et al., 1970; Forsberg et al., 1970a, and Osborn et al., 1972). Before the new techniques of separations were available, most of the work

on the distribution of subcellular lipids has been done on the cell envelope. Robrish and Marr (1962), studying fragments of the cell envelope of Azotobacter agilis prepared by osmotic shock or by sonication, found that over 90% of the phospholipid was found in the particulate cell fraction. Similar results have been found by Lennarz (1966) in a study of the lipid distribution in E. coli ruptured by sonication and by extrusion. In any event, it is likely that a sizable proportion of the cellular lipid of Gram-negative organisms is located in the cell envelope.

By using the technique described by Schnaitman (1970) for separation of cell walls and cytoplasmic membranes from cell envelopes of E. coli, White et al. (1972) were able to examine the lipid composition of cell walls and cell membranes separately. They found that both cell wall and cell membrane fractions contained a similar lipid composition (PE, PG, cardiolipin and lyso PE, in decreasing order) except that lyso PE was greater in the wall (9.4%) than in the membrane (<1%). Fatty acid analysis of these phospholipids revealed that the wall phospholipids contained a greater proportion of palmitic acid. Coenzyme Q was almost exclusively localized in the cell membrane (White et al., 1972). Forsberg et al. (1970a) and Martin and MacLeod (1971) studied the composition of the various layers of the cell envelope of the marine pseudomonad B-16 and found an outer double-track layer (outer membrane) composed of lipid (26.1%), with almost

equal amounts of carbohydrate (20.8%), together with protein (41.5%). The cytoplasmic layer, which has recently been separated from the remaining layers (Forsberg et al., 1970), was composed of large amounts of lipid (30.5%), of which 78% was phospholipid, along with protein (62.8%) and trace amounts of carbohydrate (2%) (Martin and MacLeod, 1971). The predominant phospholipid present was PE, with a lesser amount of cardiolipin, and traces of an unidentified component. The underlying layer between the two membranes contained amino acids and amino carbohydrates (39.1%), with large amounts of protein (25.3%), and small amounts of lipid (8.8%)(Martin and MacLeod, 1971).

It is evident, therefore, that in Gram-negative bacteria, a major part of the lipid is present in the cell wall in addition to that in the cytoplasmic membrane, and is present mostly as phospholipid.

IV. Function of Lipids in Bacteria

The role of lipids in bacterial membranes has been the topic of a number of reviews and papers (Rothfield and Finkelstein, 1968; Shaw, 1970; Cronan and Vagelos, 1972; Machtiger and Fox, 1973). The functions of two classes of membrane lipids, phospholipids and glycolipids, will be discussed here.

A. Phospholipids

Phospholipids are believed to have at least two distinct roles in biological membranes: (i) as a structural component, necessary for membrane function (e.g. permeability), as well as providing a matrix in which membrane proteins are imbedded, and (ii) as cofactors for the function of certain membrane-bound enzymes (Cronan and Vagelos, 1972).

The fact that the major component of bacterial lipids, the phospholipids, are localized in the cell envelope supports the idea of lipids being primarily structural components of the cell. In E. coli, there is evidence suggesting that unsaturated fatty acids of phospholipids are needed to support growth and cellular integrity. The supplementation of fatty acid auxotroph of E. coli with unusual positional or geometric isomers of unsaturated fatty acids causes defective growth, followed by lysis and cell death (Silbert et al., 1968; Henning et al., 1969 and Esfahani et al., 1971). These findings suggest that lysis is due to the production

of phospholipids lacking an appropriate unsaturated fatty acid, and that such phospholipids are not sufficient for cell integrity (Esfahani et al., 1971).

It is evident that phospholipids are involved in various permeability processes of cell membranes. Studies, such as those of Tarlov and Kennedy (1965) and Hsu and Fox (1970), demonstrate that the functioning of the β-galactoside permease system or induction of the lactose transport system in E. coli are accompanied by de novo synthesis of lipid. Recently, Haest et al. (1972) studied permeability of erythritol and $^{86}Rb^{+}$ using liposomes prepared from lysyl PG and PG isolated from S. aureus, and found that permeability of erythritol is high and $^{86}Rb^{+}$ low in lysyl PG liposomes, whereas the reverse is true in PG liposomes. A similar observation was observed in the intact cell membrane of S. aureus in which the proportion of lysyl PG to PG can be varied by growth conditions (Houtsmuller and Van Deenen, 1965). Haest et al. suggested that the chemical nature of membrane phospholipids can determine to a great extent the properties of the permeability barrier. The general question as to whether the membrane lipids play an active role in controlling the entrance and exit of compounds from the bacterial cell is of great interest and surely warrants further study.

The function of phospholipids as a cofactor of enzymes have been reviewed by Rothfield and Finkelstein (1968); Cronan and Vagelos (1972), Machtiger and Fox (1973). These

are membrane-bound enzymes that may be solubilized, or at least delipidated to the point where a lipid requirement is demonstrable. One of the systems best studied is the galactosyl transferase of Salmonella typhimurium which is involved in lipopolysaccharide synthesis. The delipidated enzyme was shown to require PG, PE or cardiolipin (but not PC) containing fatty acids with at least one double bond or cyclopropane ring for activity (Rothfield and Romeo, 1971). The second phospholipid-requiring membrane enzyme, purified from S. typhimurium by Muller et al. (1972), is glucosyl transferase, which is also involved in lipopolysaccharide synthesis and requires the same phospholipids as the galactosyl transferase. For both enzymes PE was the most effective phospholipid. The authors have suggested that PE interacts primarily with the lipopolysaccharide acceptor, providing a mixed lipid matrix into which the transferase protein is inserted (Rothfield and Romeo, 1971). A primary interaction of phospholipid with lipopolysaccharide was further suggested by the finding that the amount of PE required for optimal activity was related to the amount of lipopolysaccharide in the reaction mixture, rather than to the amount of enzyme (Muller et al., 1972). The isolation of the binary complex (lipopolysaccharide-PE), and the subsequent isolation of a ternary complex (enzyme-lipopolysaccharide-PE) which was fully active when UDP-[^{14}C]-glucose was added, supports the hypothesis of a primary binding of PE with lipopolysaccharide (Muller et al., 1972).

Another example of a membrane-bound enzyme which has a phospholipid requirement after it is solubilized, is Enzyme II of the phosphotransferase system of E. coli (Kundig and Roseman, 1971). The purified enzyme required crude E. coli lipids for activity, and PG was found to be the lipid factor which reactivated the enzyme. The phosphotransferase system is of particular significance since there is evidence (Roseman, 1969) suggests that it is involved in the active transport of carbohydrates into bacterial cells.

The E. coli pyruvate oxidase which binds both thiamine pyrophosphate and FAD as prosthetic groups and catalyzes the oxidative decarboxylation of pyruvate to acetate and CO_2 also requires phospholipids for activity (Cunningham and Hager (1971a, 1971b). The crystalline pyruvate oxidase activity is stimulated 15-100 fold in the presence of phospholipids or long-chain fatty acids. The authors suggesred that phospholipids might act as allosteric activators of this enzyme in vivo (Cunningham and Hager, 1971b).

Phospholipids have also been suggested to be involved in growth and cell division (Ballesta and Schaechter, 1971), resistance to antibiotics (Birmingham et al., 1971) and in the pathogenicity of Mycobacterium (Asselineau, 1966; Goren, 1972).

B. Function of Glycolipids

Little is known about the functions of glycolipids in bacterial envelopes. However, it is worth considering the functions which have been proposed and the reasoning behind them.

Glycolipids as structural components have been observed in L-forms of Streptococcus pyogenes (Cohen and Panos, 1966) and in Staphylococcus aureus (Ward and Perkins, 1968) in which the L-forms contain twice as high a glycolipid content as that of normal cells. The importance of phospholipids in maintaining the structural integrity of the membrane has long been recognized and the glycolipids may fulfill a similar function in the bacterial cell envelope. Brundish, Shaw and Baddily (1967), by using a molecular model of diglycosyl glyceride, suggested that the bacterial glycolipids of this type can assume a conformation in which all of the sugar hydroxyl groups lie on one side of the molecule, and the lipophilic components (i.e., fatty acids, ring oxygen of sugars and glycosidic oxygens) lie on the other side. The hydrophilic regions of several molecules could come together to form pores in the membrane through which small molecules may pass. The presence in some organisms of large tri- and tetraglycosyl diglycerides may represent an attempt to regulate the size of these pores (Shaw, 1970).

Glycolipids seem to play a role in pathogenicity since they are found associated with antigenic substances

isolated from many pathogenic bacteria. Cross-reactivity was observed between antisera to Mycoplasma pneumonia and purified glycolipids from other sources, including the diglucosyl diglycerides of a streptococcus (Plackett and Shaw, 1967). Veld and Willers (1973) found glycolipids (monoglucosyl diglyceride and diglucosyl diglyceride) associated with type III antigen from cell walls of group F and group Z_3 streptococci. Factors associated with the quality of virulence in Clostridium diphtheriae, C. tetani, C. botulinum and Mycobacterium tuberculosis, were glycolipids containing trehalose as a disaccharide. These glycolipids include "cord factor" (6,6'-dimycolyltrehalose) and sulfolipids (acylated trehalose-2-sulfates) which were first found in Mycobacterium tuberculosis (see Goren, 1972).

The discovery of glycosyl diglycerides during investigations on pneumococcal polysaccharide biosynthesis led to suggestions that they might be involved in transfer of sugar residues to polysaccharide chains (Distler and Roseman, 1964). This suggestion was based on the observations that in many bacteria, such as the type XIV pneumococci, Micrococcus lysodeikticus, Staphylococcus aureus and Streptococcus faecalis, the sugar components of polysaccharides were similar to those found in the respective membrane glycosyl diglycerides. Therefore, transfer of the complete disaccharide residue from glycolipid to polysaccharide chain could have taken place. However, this hypothesis must be discarded since lipid

intermediates have now been isolated in which the sugar residues are bound through a phosphodiester or pyrophosphate linkage to a C_{55} isoprenoid alcohol (Lennarz and Scher, 1972).

V. Effect of Salt on Microorganisms

A. Definition of Halophilic and Halotolerant Organisms

Microorganisms requiring high salt concentrations for growth are termed "halophilic", that is, salt-loving bacteria. According to the nomenclature of Baxter and Gibbons (1956), the term "extreme" halophile was used for those bacterial species (such as Halobacterium species) which require a concentration of NaCl in the growth medium of at least 15%, and up to a maximum of 31% (w/v). The term "moderate" halophile refers to those species that require NaCl concentration in the growth medium ranging from 1 or 2% to about 20%. These include, for example, the marine bacteria. "Halotolerant" bacteria, however, grow in absence of salt, but may grow (though more slowly) in up to 15-20% NaCl (Kushner, 1968); examples of these are Bacillus species and staphylococci. Since complex media contain low amounts of salt as contaminants, a synthetic medium may be needed to demonstrate the requirement for a small amount of salts (MacLeod, 1965).

Based on evidence provided in the Results section, the Nocardia species included in the present study is a "moderate" halophile, whereas W. sebi and S. epidermidis are "halotolerant" fungus and bacterium, respectively.

B. Effect of Salt on Cell Structure and Mechanisms of Adaptation to High Salt Concentrations

Salts are absolutely required by halophiles, primarily to preserve the structure of their cell wall and membranes and to maintain growth. The striking effect of progressive dilution of the saline environment of extreme halophiles results in a change of their rod-like form to a spherical one (in the case of the halobacteria) which finally lyses completely and irreversibly in 1-1.5 M salt (Abram and Gibbons, 1961). The requirement for high salt concentration in extreme halophiles was suggested by Kushner and Onishi (1966) to prevent mutual repulsion between negatively charged groups on proteins rather than to prevent repulsion between negatively charged phosphate groups on the lipids; the latter are associated with Mg^{++} which is also required in high concentrations by growing cells. The Mg^{++}-bound phosphate groups of lipids are necessary to support envelope structure since magnesium salts are observed to be more effective than sodium salts in preventing cell lysis (Kushner, 1968).

Costerton et al. (1967) observed that cells of marine pseudomonads lyse when suspended in water, while addition of salts to the suspending solution prevents lysis. The effect of salts is not in balancing the internal osmotic pressure of the cells but by interacting with components of the wall (Buckmire and MacLeod, 1965). Sucrose added to a

salt-free suspending medium at an appropriate concentration can also prevent lysis of cells, but cannot hold the cell wall and cell membrane together, since suspension of cells in 0.5 M sucrose caused the outer double track layer of the cells to balloon out from the cell membrane (Forsberg et al. 1970a). The protective action of sucrose against lysis has been found to be due to its inability to penetrate the osmotic barrier of the cells (Buckmire and MacLeod, 1965). From these observations, it is likely that in both extreme and moderate halophiles, salts seem to have a dual role: to maintain envelope structure, and to maintain osmotic pressure (Kushner, 1968). However, besides these roles, a high external Na^+ concentration is also required for growth and metabolism, as in active transport of α-amino isobutyric acid in a marine pseudomonad (MacLeod, 1965), and in glutamate uptake by Halobacterium salinarium (Stevenson, 1966).

Two possible explanations for adaptation to high salt medium of microorganisms (both halophilic and halotolerant bacteria) have been suggested by Kushner (1964), namely, (i) that resistant organisms may contain a system to exclude excess ions so that they can maintain a low intracellular solute concentration, optimum for the cell's enzymes or (ii) the intracellular solute concentration is also high and that the cell's enzymes are able to function in such an environment.

Experiments measuring the freezing point depression of the external medium and of the intracellular fluid of both halophile and halotolerant bacteria grown in different salt concentrations favoured the second possibility (Christian and Ingram, 1959). Although the overall internal salt concentration in halophiles or halotolerants is similar to that of the external medium, the ionic composition is quite different (Christian and Waltho, 1962). For instance, S. aureus (a halotolerant bacterium) grown in a medium containing 0.15 M NaCl and 0.025 M KCl, contains intracellular Na^+, K^+ and Cl^- concentrations of 0.098, 0.680 and 0.008 molal respectively; Halobacterium salinarum (an extreme halophile) grown in a medium containing 4 M NaCl and 0.032 M KCl, contains Na^+, K^+ and Cl^- concentrations in cells of 1.37, 4.57 and 3.61 molal, respectively (Christian and Waltho, 1962). This suggests that halophilic and halotolerant bacteria must contain a system to concentrate K^+ and to exclude Na^+, especially in extreme halophiles. Moreover, Christian and Waltho (1961) also found that in halotolerant bacteria, there was a correlation between internal K^+ content and salt tolerance.

Recently, Hurst et al. (1973) observed that S. aureus cells lost their salt tolerance and 30% of their membrane lipids when subjected to sublethal heating (52°C for 15 min in 100 mM potassium phosphate buffer pH 7.2) and regained their salt tolerance, but not membrane transport

functions, after incubation in complex growth medium. They suggested that salt tolerance might not be a membrane-associated function since it was recovered before the membrane was completely repaired. Alternatively, salt tolerance might be associated with certain membrane site(s) which are repaired at a fast rate after sublethal heating damage. The mechanism of salt tolerance in halotolerant bacteria is still not clear, and will require further study.

The effect of salts on fungi have not been studied extensively, probably because of the long growth cycle of these organisms. Vaisey (1954) and Ormerod (1967) studied the nutritional requirements of Sporendonema epizoum and found that this fungus could grow in a medium containing NaCl between 0.8-4.5 M, but that glucose could replace NaCl. Therefore, the requirement of salts in this organism is only to maintain the osmotic pressure since salts can be replaced by glucose. S. epizoum, therefore, should be designated as an "osmophilic" (rather than "halophilic") fungus, analogous to the "osmophilic" yeast.

VI. Metabolism of Bacterial Lipids

A. Biosynthesis

1. Phospholipids

The general scheme of phospholipid biosynthesis in bacteria is given in Scheme 1.

a) Phosphatidic Acid (PA)

Two pathways are known for the biosynthesis of PA in bacteria:

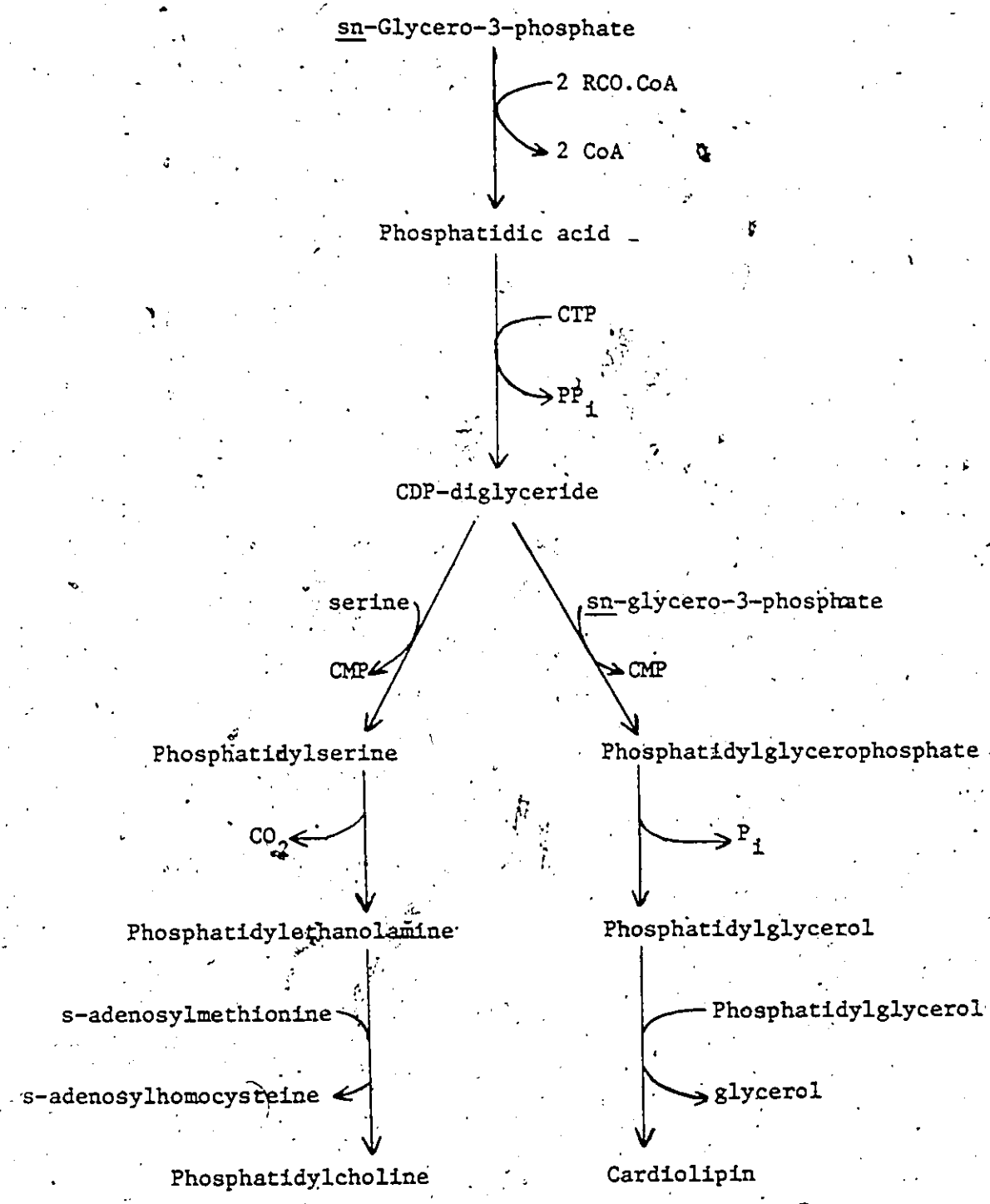
(i) stepwise acylation of sn-glycero-3-phosphate as follows:

$$\alpha GP + \text{acyl CoA} \rightarrow \text{lyso PA} + \text{CoA}$$

$$\text{Lyso PA} + \text{acyl CoA} \rightarrow \text{PA} + \text{CoA}$$

The evidence for this pathway was obtained from the *in vitro* incorporation of sn-glycero-3-phosphate into PA and into small amounts of lyso PA in the presence of acyl CoA and enzymes from the cell envelope fraction of *E. coli* (Ray *et al.*, 1970; Van den Bosch and Vagelos, 1970; and Sinensky, 1971). The reactions were catalyzed by at least two acyl transferases (Hechemy and Goldfine, 1971), which show a great specificity in selecting saturated or unsaturated acyl CoA. The saturated acyl CoA would be added to the 1-position of sn-glycero-3-phosphate, whereas the unsaturated acyl CoA would be added to the 2-position (Van den Bosch and Vagelos, 1970; Ray *et al.*, 1970; and Sinensky, 1971) in the formation of lyso PA or PA.

SCHEME 1 General pathways for the biosynthesis of phospholipids in bacteria.



(ii) Phosphorylation of monoglyceride or diglyceride with ATP to form lyso PA or PA. The former was followed by acylation with acyl CoA to form PA. The glyceride kinase catalyzing this pathway was first described by Pieringer and Kunes (1965). The enzyme has been shown to have an absolute specificity for 1,2-diacyl-sn-glycerol (Lands et al., 1966; Chang and Kennedy, 1967) and has been purified by Horvath and Pieringer (1970) from cell-free extracts of E. coli. However, this enzyme is not assigned a role in the biosynthesis pathway, as Chang and Kennedy (1967) have shown that no rapidly metabolized pool of diglyceride is found in growing cell of E. coli such as would be expected for a biosynthetic intermediate.

b) CDP-diglyceride

The only known pathway for the biosynthesis of CDP-diglyceride is that catalyzed by CTP: phosphatidic acid cytidyltransferase:

$$CTP + PA \rightarrow CDP\text{-diglyceride} + PP_i$$

This evidence is based upon the presence of an enzyme system in the particulate fraction of E. coli which catalyzes the synthesis of CDP-diglyceride from CTP and PA (Carter, 1968). CDP-diglyceride is regarded as an activated phosphatidic acid (Cronan and Vagelos, 1972) and is a key intermediate in the synthesis of bacterial phospholipids. However, there had been no real proof of the natural

occurrence of CDP-diglyceride in Nature until Raetz and Kennedy (1973) were able to isolate small amounts of ^{32}P-labelled CDP-diglyceride from E. coli cells grown in sn-glycerol-3-[^{32}P] phosphate. The very low level of labelled cytosine liponucleotide relative to labelled PA led Raetz and Kennedy (1973) to suggest that the conversion of PA to liponucleotide may be the rate determining step in the overall process of phospholipid biosynthesis in E. coli.

c) Synthesis of Phosphatidyl Glycerol (PG)

The pathway for the biosynthesis of PG in bacteria involves the intermediate formation of phosphatidylglycerophosphate (PGP) from CDP-diglyceride and sn-glycero-3-phosphate (GP) followed by dephosphorylation to PG:

$$GP + CDP\text{-diglyceride} \rightarrow PGP + CMP$$

$$PGP \rightarrow PG + P_i$$

This pathway was first demonstrated in E. coli by Kanfer and Kennedy (1964), based on the evidence of incorporation of [^{14}C] GP into PG and [^{32}P]GP into PGP in the presence of CDP-diglyceride and an enzyme preparation from the particulate fraction of E. coli. PG synthesis by this pathway was also demonstrated in a Bacillus megaterium mutant (Patterson and Lennarz, 1971) and in Salmonella typhimurium (Bell et al., 1971).

d) Synthesis of Cardiolipin

Two pathways are at present known for the biosynthesis of cardiolipin in bacteria:

(1) PG + CDP-diglyceride → cardiolipin + CMP

(2) 2 PG → cardiolipin + glycerol.

The first pathway was proposed by Stanacev, Chang and Kennedy (1967) based on evidence of the formation of [^{14}C]cardiolipin from [^{14}C] PG in an E. coli particulate fraction containing high concentrations of exogenous, unlabelled CDP-diglyceride. The second pathway was shown to be functioning in bacteria by DeSiervo and Salton (1973), with an almost quantitative conversion of PG to cardiolipin by a particulate fraction from Micrococcus lysodeikticus in the absence of CDP-diglyceride. Re-examination of the biosynthesis of cardiolipin in E. coli by Hirschberg and Kennedy (1972) indicated that CDP-diglyceride was not functioning as a phosphatidyl donor by the finding that ^{14}C from [^{14}C]PG, but not ^{32}P from [^{32}P]CDP-diglyceride was incorporated into cardiolipin. Hostetler, Van den Bosch and Van Deenen (1972) have made similar observations in E. coli, and the work of Rampini et al. (1970) strongly suggested that the same CDP-diglyceride independent pathway (reaction 2) operates in Staphylococcus aureus.

e) O-amino Acid Esters of PG

The pathway for biosynthesis of the amino acyl derivatives of PG was first reported by Lennarz et al. (1966), as follows

L-lysyl-tRNA + PG → L-lysyl-PG + tRNA

The evidence is based on labelling of L-lysyl-PG by [^{14}C] lysine or [^{14}C] lysyl-tRNA in the presence of crude extracts of S. aureus. The reaction was found to proceed in two steps; first, formation of lysyl-tRNA by a soluble enzyme requiring Mg^{++} and ATP, and second, transfer of the lysyl moiety from tRNA to endogenous PG by a particulate enzyme. This appears to be the first known involvement of amino acyl-tRNA in the synthesis of O-acyl esters of amino acids. In addition to S. aureus, the pathway has also been demonstrated in Bacillus megaterium, B. cereus and Clostridium welchii (Lennarz, 1966).

f) Phosphatidyl Serine (PS) and Phosphatidyl Ethanolamine (PE)

The biosynthesis of PS is catalyzed by CDP-diglyceride: L-serine phosphatidyl transferase (PS synthetase) as follows:

CDP-diglyceride + L-serine → PS + CMP

The reaction was first described by Kanfer and Kennedy (1964), who found that serine incorporation into PS

by an E. coli cell free extract was completely dependent on exogenous CDP-diglyceride. The enzyme shows maximal activity in a solution of relatively high ionic strength containing a neutral surfactant such as octanol. The above pathway has also been demonstrated in a mutant of Bacillus megaterium by Patterson and Lennarz (1971) and in Salmonella typhimurium by Bell et al. (1971).

That biosynthesis of PE involves decarboxylation of PS was suggested by Kanfer and Kennedy (1963) after their observation that PE was heavily labelled but PS only slightly labelled with ^{32}P in E. coli grown in ^{32}P-containing medium. These authors subsequently demonstrated the presence of a PS decarboxylase in cell free extracts from E. coli and showed it to be much more active than PS synthetase (Kanfer and Kennedy, 1964). PS decarboxylase has recently been isolated from E. coli and purified to homogeneity by Wickner and Kennedy (1971).

PS synthetase, together with PS decarboxylase, has also been detected in a Bacillus megaterium mutant (Patterson and Lennarz, 1971) and in Salmonella typhimurium (Bell et al., 1971).

g) Phosphatidyl Choline (PC)

Although PE is widely distributed among Gram-negative bacteria, a survey of a variety of bacteria (Table 2) has revealed that only a few genera contain PC and partially

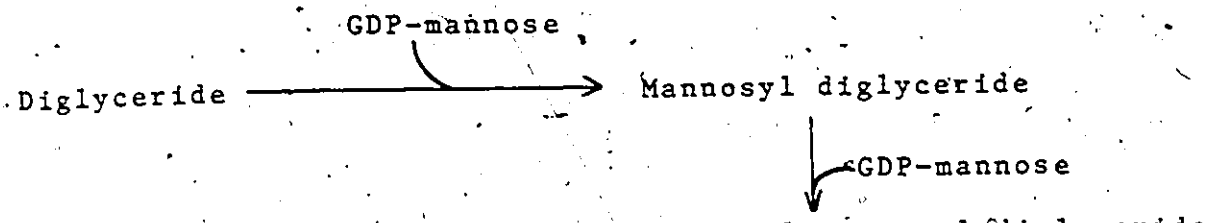
methylated forms of PE. The formation of the partially and completely methylated ethanolamine lipids has been studied in vivo and at the enzymatic level. The reaction has been shown to proceed in a stepwise fashion by addition of methyl groups derived from S-adenosyl methionine to PE in the presence of cell free extracts from Agrobacterium tumefacians (Kaneshiro and Law, 1964), Hyphomicrobium vulgare (Goldfine and Hagen, 1968) and in the photosynthetic bacterium Rhodopseudomonas spheroides (Gorchein , 1968). The presence of N-methyl PE in Clostridium butyricum and Proteus vulgaris suggests that a stepwise methylation of PE is catalyzed by separate enzymes (Goldfine and Ellis, 1964).

h) Phosphatidyl Inositol (PI)

Relatively little is known about the biosynthesis of PI in the few species of bacteria in which it is found. In animal tissues it is formed by a reaction analogous to the bacterial pathways leading to PS and PG, i.e., reaction of free inositol with CDP-diglyceride (Paulus and Kennedy, 1960).

2. Glycolipids

Studies to elucidate the biosynthesis of glycosyl diglyceride with cell free extracts were first reported by Lennarz (1964). The following pathway was proposed for biosynthesis of the mannosyl mannosyl diglyceride found in Micrococcus lysodeikticus:



This pathway was based on evidence that crude extracts of M. lysodeikticus, when incubated with GDP-mannose, catalyzed the formation of mannosyl mannosyl diglyceride and two other mannose containing glycolipids. One was identified as monomannosyl diglyceride and the other was unidentified. The first reaction was catalyzed by a particulate enzyme preparation which showed a high specificity for a diglyceride containing branched-chain fatty acids, similar to those found in M. lysodeikticus, and also required Mg^{++} ions and a cationic detergent (Lennarz and Talamo, 1966). The second reaction was catalyzed by a soluble enzyme also requiring Mg^{++} but no detergent (Lennarz and Talamo, 1966).

A similar pathway has been demonstrated for the biosynthesis of galactosyl glucosyl diglyceride in Pneumococcus type XIV (Distler and Roseman, 1964), diglucosyl diglyceride in Streptococcus faecalis (Pieringer, 1968), diglucosyl diglyceride in Mycoplasma laidlawii (Smith, 1969) and diglycosyl diglyceride in Mycobacterium smegmatis (Schultz and Elbein, 1974). In all examples, glucose or galactose is transferred from UDP-glucose or UDP-galactose, respectively, to a diglyceride. The transferases catalyzing glycosyl diglyceride

biosynthesis are present in a particulate fraction (30,000 xg) of the crude extracts.

Biosynthesis of the glucuronosyl diglyceride found in Pseudomonas diminuta and P. rubescens (Wilkinson, 1968) is similar to that of glycosyl diglyceride in which the glucuronosyl moiety is transferred from UDP-glucuronic acid. The reaction is catalyzed by a transferase from the particulate fraction (Pieringer, 1971). The same pathway also functions in biosynthesis of the glucuronosyl diglyceride found in a Gram-negative, halotolerant bacteria reported by Stern and Tiez (1973).

Biosynthesis of a new type of glycolipid, "glucophospholipid," has been observed by Pieringer (1968) during his studies in the biosynthesis of diglucosyl diglyceride in S. faecalis. The glucophospholipid was found to form in vitro from diglucosyl diglyceride and the particulate enzyme preparations of S. faecalis (Ambron and Pieringer, 1971). Subsequent studies indicate that both phosphatidylglycerol and cardiolipin can function as phosphatidyl donors in the synthesis of the phosphatidyldiglucosyl diglyceride found in S. faecalis (Pieringer, 1972). This appears to be similar to the synthesis of cardiolipin in E. coli (Hirschberg and Kennedy, 1972), where a preformed phospholipid rather than a nucleotide intermediate is the preferred phosphatidyl-group donor.

Biosynthesis of the phosphatidylinositol mannosides (PI-mannosides) found in Mycobacteria (Lee and Ballou, 1965) has been studied by Hill and Ballou (1966) and Brennan and Ballou (1968). The reaction is catalyzed by a particulate enzyme fraction (100,000 xg) which transfers mannose from GDP-mannose to PI, yielding first PI-monomannoside and dimannosides. However, it was found that PI-monomannoside does not serve as an acceptor for a second mole of mannose and it was suggested that "perhaps the monomannoside has to be modified" before the second mannose is transferred (Takayama and Goldman, 1969).

B. Degradation of Polar Lipids

Most of the information available on turnover of membrane lipids has been obtained from in vivo experiments using the pulse-chase technique. By this technique, PE is reported to be rather metabolically stable in E. coli (Kanfer and Kennedy, 1963; Kanemasa, 1967; Bright-Gaertner and Proulx, 1972), Haemophilus parainfluenzae (White and Tucker, 1969), Staphylococcus aureus (Short and White, 1970), and in Micrococcus denitrificans (Wilkinson et al., 1972). However, in H. parainfluenzae, analysis of each moiety of PE indicated that, although diacylated glycerol and fatty acids moieties did not turn over, the phosphate and ethanolamine moieties turn over at the same rate (half-life of about 3 generation times) (White and Tucker, 1969).

In contrast to PE; PG and cardiolipin (CL) are reported to be metabolically active in E. coli (Kanemasa et al., 1967; Bright-Gaertner and Proulx, 1972), S. aureus (Short and White, 1971) and in H. parainfluenzae (White and Tucker, 1969; Tucker and White, 1971). In growing cells of H. parainfluenzae, the free glycerol and phosphate portions of PG turn over at the same rate (half-life 1.3 generation times), which is 2.7 times that of the acylated glycerol; turnover of fatty acid chains is 2.3 times that of the free glycerol or phosphate moiety. Furthermore, the 2-linked fatty acids have a much more active metabolism than the 1-linked fatty acid (White and Tucker, 1969). Similar finding has been reported for PG in S. aureus (Short and White, 1971).

Cardiolipin has a very active phosphate turnover in Mycobacteria (Akamatsu et al., 1967) and in H. parainfluenzae (White and Tucker, 1969; Tucker and White, 1971; Ono and White, 1971). In H. parainfluenzae, the middle, unacylated glycerol and phosphate groups of cardiolipin turn over at the same rate, which is about three times more rapid than the acylated glycerols (White and Tucker, 1969). Furthermore, in S. aureus, the acylated glycerol and the middle glycerol from the PG end of the cardiolipin have a metabolism essentially identical to that of the total cellular PG (Short and White, 1971). Different turnover rates for different portions of the same phospholipid molecule suggest that

partial hydrolysis followed by resynthesis of the lipid *in situ* in the membrane could be an important part of membrane metabolism (Short and White, 1972).

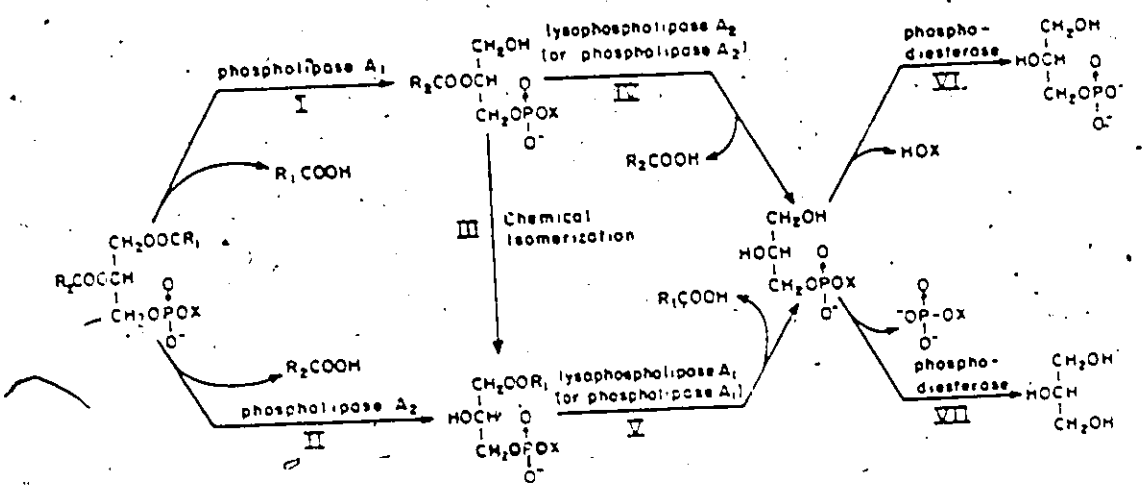
The turnover of monoglucosyl diglyceride, diglucosyldiglyceride and glucophospholipid found in *S. aureus* and in *Mycoplasma laidlawii* has been studied (Short and White, 1970; McElhaney and Tourtellotte, 1970). In *S. aureus*, the mono- and diglucosyl diglycerides showed a rapid loss of ^{14}C glucose (half-life of about 1 generation time) during growth in nonradioactive medium, but no loss of ^{14}C from the fatty acids of these lipids. The glucophospholipid of *S. aureus*, however, behaved in the same way as PE, with no loss of ^{32}P in a pulse-chase experiment; instead ^{32}P and ^{14}C accumulated (Short and White, 1970).

The metabolism of glucophospholipid, mono- and diglucosyl diglyceride of *S. aureus* contrasts sharply with their metabolism in *M. laidlawii* (McElhaney and Tourtellotte, 1970) where these lipids were reported to be metabolically stable (no turnover could be observed). These authors suggested that membrane function in this organism may not require turnover of membrane lipids.

The information available from the apparent turnover of these membrane lipids, however, does not give a clear picture of the intermediate steps during the degradative process. Van Golde, Schulman and Kennedy (1973) suggested that radioactive components disappearing from total lipid

fractions might be used by the cells for the biosynthesis of some non-lipid, water-soluble product which cannot be detected in lipid extracts. They found that during the chase period of E. coli, loss of radioactivity from PG and cardiolipin was accompanied by increases in the radioactivity of the water-soluble fraction of the cell extracts. The radioactivity of this fraction was found to be associated with several oligosaccharides, each containing glycerol and phosphoric acid in equimolar proportions, and glucose as the sole detectable sugar. Some of these oligosaccharides contain succinic acid in ester linkage (Van Golde et al., 1973). The authors suggested that these oligosaccharides are derived from PG or cardiolipin directly or indirectly during the turnover of these lipids.

A general pathway for the catabolism of phospholipids has been proposed by Albright et al. (1973). The pathway involves the action of phospholipase A and lysophospholipase A, which would remove the apolar hydrophobic fatty acyl groups. Subsequent action by a phosphodiesterase on the resultant water soluble glycerophosphate would complete the degradative process (Scheme 2)



Scheme 2. Possible reactions involved in the catabolism of phospholipids in bacteria (From Albright et al., 1973).

The authors have demonstrated the presence of all the enzymes involved in this pathway in three subcellular fractions of E. coli (wall, inner membrane and cytosol) by using radioactive PE as a model substrate. One of the enzymes, phospholipase A_1 (Reaction I, Scheme 2) is associated with the cell wall and was found to be the same as the enzyme purified from crude extracts of E. coli by Scandella and Kornberg (1971). The wall-associated enzyme also showed lysophospholipase A_1 activity, suggesting that it can act either as phospholipase A_1 or lysophospholipase A_1. Because of the high specific activity of this enzyme (relative to the other lipases detected) and its ability to act on both intact PE and the 1-acyl lyso compound, it was suggested that,

if isomerization were sufficiently rapid, it could serve as the major catabolic enzyme to convert PE to glycerophosphoryl ethanolamine (Reactions I, III and V, Scheme 2) (Albright et al., 1973). Phospholipase A_1, phospholipase A_2, lysophospholipases and phosphodiesterases have been characterized before in several strains of E. coli (Fung and Proulx, 1969; Okuyama and Nojima, 1969; Proulx and Fung, 1969; Bernard et al., 1972). Scandella and Kornberg (1971) found that purified phospholipase A_1 from E. coli can act on PG and cardiolipin as well as PE. However, the specificity of the other enzymes in the scheme is not yet known. More information is required to explain the fact that in E. coli, under normal growth conditions, the phosphate moiety of PE is metabolically stable but that of PG and cardiolipin is in a rapid state of turnover.

An alternative pathway for the catabolism of phospholipids in bacteria might involve cleavage of the polar, hydrophilic moiety by action of phospho-diesterases such as phospholipase C or phospholipase D, followed by the action of lipases which catalyze the removal of the fatty acyl chains from the glyceryl moiety (Albright et al., 1973). So far, phospholipase D has only been found in plant tissues (see Kates, 1960). Phospholipase C was found in Clostridium welchii (Phillips and Batty, 1963; Ikezawa et al., 1964), in Bacillus cereus (Stein and Logan, 1963; Haverkate and van Deenen, 1964), and in yeast (Harrison and Trevelyan, 1963),

but was absent in most strains of E. coli (Bright-Gaertner and Proulx, 1972). Lipases are widespread in nature, occurring in animals, plants, insects, bacteria and molds (see Kates, 1960).

Thus, it is likely that either pathway could be functioning in the catabolism of phospholipids in bacteria. The exact function of these enzymes, as well as the mechanism of their regulation, is unknown. Clearly, a great deal is yet to be learned about phospholipid catabolism in bacteria.

VII. Aims of the Research

1. To determine the composition of the cellular lipids of the three microorganisms, Wallemia sebi, a Nocardia species and Staphylococcus epidermidis contaminating the high salt growth medium of Sehgal and Gibbon (1960).

2. To carry out complete structural determinations on the polar lipids of S. epidermidis, namely: (i) a novel polar lipid of the "glucophospholipid" class, (ii) two glycolipids, diglucosyl diglyceride and monoglucosyl diglyceride and (iii) the main phospholipid, phosphatidylglycerol.

3. To study the effect of sodium chloride concentration in the growth medium on the lipid composition of S. epidermidis.

4. To study the turnover of individual lipids in S. epidermidis by pulse-labelling and chase technique using [^{32}P]orthophosphate and [1-^{14}C]glycerol. Results of such studies may shed light on the lipid metabolism in this bacterium.

MATERIALS AND METHODS

I. Materials

A. Microorganisms

The microorganisms studied were isolated from a growth found in unsterilized culture medium for extremely halophilic bacteria (Sehgal and Gibbons, 1960) containing essentially yeast extract and casamino acids in 25% sodium chloride. The medium had been left at room temperature for several weeks. The growth found in this medium consisted of several microorganisms. One species was isolated and purified by Dr. M. B. Gochnauer, Biology Department, University of Ottawa and tentatively identified as a Nocardia species. Two other microorganisms were isolated in pure culture by Dr. J. Harwig, Department of National Health and Welfare, Ottawa as: (1) a fungus identified as Wallemia sebi by Dr. A. C. Stolk, Centraalbureau voor, Baarn, Netherland; (2) a bacterium identified as Staphylococcus epidermidis by Dr. C. E. Park, Department of Health and Welfare of Canada; (see Appendix I).

The lipid and fatty acid compositions were examined in all three groups of microorganisms as well as in the original unpurified culture. The lipid and fatty acid compositions of microorganism in the Staphylococcus epidermidis were similar to those obtained from the original

unpurified culture and not in the other two groups. This indicated that the major microorganism present in the original culture was the Staphylococcus species. Thus, most of the work presented here was done on the Staphylococcus sp. rather than on the other two microorganisms.

B. Chemicals

Unless otherwise stated, all chemicals used in the experiments described were of "Reagent Grade" quality. The sources of the more common chemicals have not been given but suppliers have been given for those chemicals not readily available from most supply houses.

1. Solvents. All solvents used were distilled over glass, a 10% forerun being discarded. Solvents for spectroscopic measurement were "spectral reagent grade."

2. Silica gel. Silica gel H, silical gel G were obtained from Brinkman Instruments (Canada) Ltd., Rexdale, Ontario. Unisil silicic acid (200-325 mesh) was supplied by Clarkson Chemical Co., Williamport, Pennsylvania.

3. Standard compounds

a) Lipids: Cardiolipin (beef heart, Sylvana Chemical Co., Orange, N.J.); phosphatidylglycerol (Serdary Research Lab., London, Ont.); phosphatidylethanolamine, phosphatidylserine, phosphatidylinositol (Pierce Chemical Co., Rockford, Ill.); phosphatidylcholine (isolated from egg lecithin); monogalactosyl diglyceride (MGD)(unsaturated)

from Applied Science Lab., State College, Pa., and also saturated MGD isolated from runner bean and hydrogenated (Sastry and Kates, 1964); digalactosyl diglyceride (hydrogenated) from runner bean (Sastry and Kates, 1964); α,β-dipalmitin (synthesized by the method of Baer and Kates, 1950; fatty acid methyl esters, 14:0 to 18:0 and 16:1, 18:1, 18:2 and 18:3 (Nu Chek Prep., Elysian, Minnesota), iso 14:0, 15:0, 17:0 and anteiso 15:0, 17:0 (Applied Science Lab.).

b) Glycosides: α- and β-methyl-D-glucopyranoside (Mann Research Lab., New York), monogalactosyl glycerol and digalactosyl glycerol prepared by deacylation of the corresponding runner bean glycolipids (Sastry and Kates, 1964).

c) Standard alditol acetates and methyl glucosides were gifts from Dr. M. Perry, N.R.C., Ottawa; barium-sn-glycero-3-phosphate prepared by the method of Baer and Kates (1950).

4. Labelled compounds: [1-^{14}C]glycerol was purchased from Amersham/Searle, Arlington Height, Ill. and [^{32}P]ortho phosphate in dil. hydrochloric acid was from Atomic Energy of Canada.

C. Enzymes

α-Glucosidase (yeast), substantially free of β-glucosidase, α-galactosidase and β-galactosidase, Type I; Sigma Chemical Comp., St. Louis, Mo.

β-Glucosidase (emulsin), α-glucosidase free, cryst in 2.8M ammonium sulfate; Calbiochem, San Diego, Calif.

α-Glycerophosphate dehydrogenase (rabbit muscle), cryst., in 2.0M ammonium sulfate, A grade; Calbiochem., San Diego, Calif.

Glucostat, for the enzymatic determination of glucose; Worthington Biochemical Corp., Freehold, N.J.

Phospholipase A (snake venom); Sigma Chem., St. Louis, Mo.

Phospholipase C (C. welchii); Calbiochem., LaJolla, Calif.

Phospholipase D (cabbage); Calbiochem., LaJolla, Calif.

II. Methods

A. Growth of Microorganisms

1) Culture and Growth of Organisms

a) Staphylococcus epidermidis

The lipids of S. epidermidis studied in all experiments were obtained from cells grown in the medium of Sehgal and Gibbons (1960) containing 10% sodium chloride instead of 25% sodium chloride.

The composition of the growth medium was (g per litre): casamino acids (Difco), 7.5; yeast extract (Difco), 10.0; tri-sodium citrate, 3.0; potassium chloride, 2.0; magnesium sulfate heptahydrate, 20.0; sodium chloride, 100 and ferrous sulfate heptahydrate, 0.05; the pH was adjusted to 6.5 with 1N sodium hydroxide and the medium was autoclaved for 15 minutes at 120°C (15 p.s.i.).

Bacteria were kept on agar slants (Sehgal and Gibbons medium in 10% sodium chloride and 1.5% bacto-agar) and were transferred to new agar slants every three months. Bacteria on slants were incubated at 37°C until full growth was obtained (approx. 10 days). The culture was then covered with sterile mineral oil and kept in a refrigerator (4°C). Microscopic examinations of bacteria was performed regularly to confirm the purity of the culture.

To prepare a starter culture, the bacteria were transferred from the slant to 50 ml of liquid growth medium

and grown for 24 hrs at 37°C on a rotary shaker. Five millilitres of this culture was then innoculated to a 4 litre flask containing 1 litre of medium. Cells were incubated at 37°C in a controlled Environment Incubator Shaker (New Brunswick Co. Inc.) at a shaking rate of 120 rpm. Cells were harvested in the late stationary phase (≈60 hr) by centrifugation at 8000xg, at 4°C for 15 min. The cells were washed twice with 10% sodium chloride solution and kept in the freezer (-10°C) until ready for lipid extraction. Freezing of the cells had no discernible effect on their lipid composition.

b) Wallemia sebi

The media used to grow W. sebi were as follows: (1) the medium of Sehgal and Gibbons (1960) containing 25% sodium chloride as described before and (2) the medium of Harrold (1950) which composed of (g per litre): sucrose 400, malt extract 20, and yeast extract 5. The pH of the medium was adjusted to 6.2, and autoclaved for 15 min at 120°C (15 p.s.i.).

The starting innoculum was obtained by transferring a single spore of W. sebi to a slant of Harrold's medium contain 2% agar. The slant was placed at room temperature in the dark for 2-3 weeks until the appearance of brown spores. The slant was then washed with 10 ml of saline solution (0.85% sodium chloride containing 0.05% Tween 80),

and shaken to release spores from the agar. The spore suspension was poured off and centrifuged at 2,500xg for 10 min. The spores were washed once with 10 ml of the saline solution and resuspended in 5 ml of 0.85% sodium chloride. Aliquots were taken and the number of spores counted using a hemacytometer. The suspension was adjusted to contain $\approx 10^6$ spores per ml. This suspension was used as a starter innoculum. One ml was innoculated to 200 ml of Harrold's liquid medium in a 2.8 liter Fernback flask. The culture was then kept in a cupboard at room temperature until brown spores appeared on the surface of the medium (≈5 days). The spores were harvested by filtering with suction, washed twice with 0.85% sodium chloride, and subjected to lipid extraction described below.

c) Nocardia species

The medium used to grow the microorganism identified as a Nocardia sp. was similar to that described for Staphylococcus epidermidis, that is, that of Sehgal and Gibbons (1960) with 15% sodium chloride instead of 25% sodium chloride.

The organism was kept on agar slants of the Sehgal and Gibbons (1960) liquid medium in 15% sodium chloride containing 1.5% bacto-agar. Cells were grown in the Sehgal and Gibbons (1960) liquid medium (15% sodium chloride) on a rotary shaker (120 rpm) at 37°C. Stationary growth was obtained within 4 days.

The process of preparing the starter culture and harvesting of cells was the same as described for *S. epidermidis*.

2) Growth Curves

Growth curves of cells in liquid media were determined by optical density measurement, as follows:

a) 100 ml cultures were incubated in 500 ml Erlenmeyer flasks with sealed-on side-arm tube (18 x 150 mm). The optical density of the culture was measured at 660 nm in a Coleman junior spectrophotometer by tipping the flask so as to fill the side-arm tube with the culture. The instrument was adjusted to 100%T using uninnoculated culture medium as a blank contained in 500 ml Erlenmeyer flask with an optical matched sealed-on side-arm tube.

b) For cultures of larger volume, 7 ml aliquots were removed periodically and their optical density measured in 19 mm round cuvette against a blank of uninnoculated culture medium.

The growth curve was obtained by plotting optical density against time on semi-log graph paper.

The exponential growth rate (R), which was defined as the number of generations per unit time is calculated from the growth curve using the following formula:

$$R = \frac{\log N_2 - \log N_1}{0.301\ (t_2 - t_1)}$$ (Sokatch, 1969)

N_1, N_2 are the concentration of cells (number of cells per unit volume of culture medium) at times t_1 and t_2 (min) respectively.

Since O.D. α N

so, log O.D. α log N

$$\therefore \quad R(min^{-1}) = \frac{\log O.D_2 - \log O.D_1}{0.301\ (t_2 - t_1)}$$

The generation time G (min) is then defined as

$$G = \frac{1}{R}$$

c) A calibration curve for S. epidermidis (see Fig. 6) relating the cell dry weight per ml of culture volume to the optical density of the culture media was determined as follows:

Cells were harvested from aliquot of culture (100 ml aliquot, total volume 1000 ml) grown to a known optical density and washed twice with distilled water. Cells were dried to constant weight in vacuo in a desiccator over potassium hydroxide.

B. Lipid Extraction Procedure

1. Method of Bligh and Dyer

Lipids were extracted from the organisms investigated here by the method of Bligh and Dyer (1959) as modified by Kates (1972). The washed cells were suspended in 10% sodium chloride solution to a concentration of 20-60 mg

of cells (dry weight) per ml. To 200 ml of the cell suspension were added 750 ml of methanol-chloroform (2:1, v/v); the mixture was shaken and left at room temperature for several hours with intermittent swirling. The suspension was filtered through glass wool with gentle suction to remove most of the cell debris and the filter was washed with a mixture of 125 ml chloroform, 100 ml water and 250 ml methanol. The combined filtrate was diluted with 375 ml of chloroform and 375 ml of water (final solvent ratio, chloroform-methanol-water, 1:1:0.9, v/v) and the mixture swirled and allowed to stand overnight in separating funnel. The lower chloroform phase containing the total lipids was drained off and the upper phase was washed with 200 ml of chloroform. The combined chloroform extracts were diluted with benzene and brought to dryness on the rotary evaporator at 30-35°C. The lipid residue was redissolved in chloroform-methanol (9:1, v/v) and made up to a volume and aliquots were taken for dry weight, sugar and phosphorus-determinations. The lipid solution was stored at -10°C.

2. Isopropanol Extraction (Kates & Eberhardt, 1957)

Hot isopropanol is known to inactivate phospholipid degrading enzymes, so that lipids obtained by this method are considered to be free of lipid degradation products.

In this extraction procedure, 20 g of wet cells were suspended in 10% sodium chloride to a final volume of

20 ml; 40 ml of hot isopropanol was added and the mixture was kept hot for 1 to 2 min with mixing. The hot homogenate was filtered with suction, and the filter residue was washed twice with hot isopropanol and reextracted with 20 ml of chloroform-isopropanol (1:1, v/v); the filtrates were combined. The residue was again extracted with 19 ml of chloroform-methanol-water (1:2:0.8, v/v; one phase Bligh and Dyer mixture). The precipitate was removed by centrifugation. The supernatant was diluted with 5 ml chloroform and 5 ml water, mixed and centrifuged. The chloroform phase was withdrawn and combined with the previous isopropanol extracts. The combined extracts were concentrated *in vacuo* and the residue was taken up in chloroform. The chloroform solution was then washed twice with methanol-water (1:0.9, v/v) to remove water soluble compounds. The chloroform phase was then diluted with benzene and concentrated to dryness *in vacuo* (30-35°C). The lipid residue was immediately dissolved in chloroform and the solution cleared by centrifugation and stored at -10°C.

C. General Chromatographic Methods

1. Column Chromatography

The total lipid extract was first fractionated on a column of "Unisil" silicic acid. The silica was activated for 12 hr at 110° before use, made in a slurry

with solvent and poured into a glass column equipped with a Teflon stopcock above which a glass-wool plug had been introduced. The absorbent was packed down by passing about five column volumes of solvent through the column and a 0.5 cm layer of washed sea sand was added on the top of the silica surface. A solution of the lipid (0.5-1 g in 5-10 ml chloroform for 50 g silicic acid) was applied to the column at the top of the sand layer. The solution was allowed to run into column and 1-2 ml chloroform was used to wash down the sides of the column to ensure quantitative transfer of lipid mixture to the column. The solvent for elution was placed in a 500 ml separatory funnel fitted on top of the column and the elution rate was adjusted to about 5 ml per min by means of the stopcock.

2. Thin Layer Chromatography (TLC)

a) Analytical TLC

For examination of lipid components and for monitoring of column chromatography, 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 spraying with: (i) 40% sulfuric acid in ethanol followed by charring, or (ii) α-napthol-sulfuric acid followed by charring for sugars (Siakotos and Rouser, 1965), (iii) phosphate-detecting reagent (Vaskovsky and Kostetsky, 1968), (iv) ninhydrin

solution for amino group containing lipid (Marinetti, 1964) and (v) periodate-Schiff reagent for vicinal diol and glycolipid (Shaw, 1968).

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 coated plates were washed once by ascending chromatography in chloroform-methanol (1:1, v/v), air dried and then activated at 110° overnight. The lipid solution (ca. 40 mg/ml), in chloroform-methanol (95:5, v/v) was applied to the plate (40 mg/plate), using a "Pelick Streaker" (Applied Science Laboratories, State College, Pa.). Plates were developed in pre-equilibrated rectangular jars lined with Whatman 3MM paper, using a suitable solvent system. The lipid bands were located by exposing the air-dried plate to iodine vapor. After leaving the plate in the fume hood overnight to remove iodine, each lipid band was scraped off into a sintered glass filter, and eluted with the mixture of chloroform-methanol-ethyl ether (1:1:1, v/v). Each extract was diluted with benzene and brought to dryness on the rotary evapprator. The residues were dissolved in about 5 ml of chloroform-benzene (1:1, v/v) and centrifuged. The silica free supernatants were concentrated in a nitrogen stream, and the lipid residues were dried in vacuo, weighed, and dissolved in chloroform-methanol (9:1, v/v). The purity

of each component was determined by analytical TLC or on microslides. TLC in at least two solvent systems.

c) Solvent Systems for TLC

The following solvent systems were used to examine lipid components and the purity of each lipid under investigation.

Solvent System	Composition (v/v)	Ref.
(i) For Polar Lipids		
$CHCl_3$-MeOH-28% NH_3	65:35:5	Davidson and Stanacev (1970)
$CHCl_3$-MeOH-90% Acetic acid	30:4:20	Hancock and Kates (1973)
$CHCl_3$-MeOH-H_2O	65:35:8	Fischer (1970)
$CHCl_3$-Acetone-MeOH-glacial acetic acid-H_2O	6:8:2:2:1	Rouser et al. (1970)
$CHCl_3$-MeOH-H_2O	65:25:4	Lepage (1964)
(ii) For Neutral Lipids		
Pet. ether (b.p. 60-70°C)-ethyl ether-glacial acetic acid	80:20:1	Mangold (1969)
Pet. ether (b.p. 60-70°C)-ethyl ether-glacial acetic acid	90:10:1	Mangold and Malins (1960)

3. Silicic Acid Impregnated Paper Chromatography

a) Solvent System

Total lipids were chromatographed on silicic acid-impregnated Whatman 3MM paper according to the procedure of

Marinetti (1962 and 1964), using diisobutyl ketone-glacial acetic acid-water (40:25:5, v/v), as solvent. The preparation of the impregnated paper and details of the development procedure have been described by Kates (1967).

b) Detection and Staining of the Lipids

The following stains were employed to monitor the purity of isolated lipids and to detect the presence of specific groups during structure elucidation studies.

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 (colour index 752, National Aniline Division, Allied Chemical Dye Corp., New York), was prepared by dissolving 1.2 g in 1 litre 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 colour noted and the Rf 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 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 in natural and synthetic phosphatides by means of the fuchsin colour reaction given by aldehydo-lipids derived from cleavage of the lipid diol. The procedure used was an adaptation to silcic 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 vicinal-diol containing

*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 charcoal for 1 hr, filtered and the colourless filtrate was made up to 500 ml with 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.

lipid components 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 lipid-sugar derivatives (glycolipids).

4. Chromatography of Water-Soluble Compounds

The water-soluble products obtained from acid or alkaline hydrolysis of lipid components were examined for the following substances by paper chromatography.

a) Phosphate Esters

Aliquots containing 10-15 µg P were evaporated to dryness under a stream of nitrogen, dissolved in about 10 µl of water and applied to a strip of Whatman No. 1 paper (19 x 57 cm). The esters were chromatographed for 16-18 hr in the following solvent systems:

(i) n-butanol-acetic acid-water (5:3:1, v/v), ascending technique ;

(ii) phenol-water (5:2, w/w, ascending technique);

(iii) propanol-10N ammonium hydroxide (3:2, v/v), ascending technique..

The developed chromatograms of water-soluble components were stained with sulfosalicylic acid-ferric chloride reagent (Vorbeck and Marinetti, 1965). The dry chromatograms were dipped in an acetone solution of ferric chloride (1.5 g $FeCl_3.6H_2O$ in 30 ml of 0.3N HCl diluted to 1 litre with

acetone), dried again for 10 min and then dipped in a 1.25% solution of sulfosalicylic acid in acetone. The spots appeared white on a violet background.

b) Reducing Sugars and Glycerol

Aliquots containing 100-200 µg of sugar or glycerol were concentrated under a nitrogen stream to near dryness. The residues were dissolved in a drop of water and chromatographed on a strip of Whatman No. 1 paper (19 x 57 cm) in solvent systems: pyridine-ethyl acetate-water (2:5:5, v/v); upper phase, descending technique.

Sugars and polyols were detected by the following stains:

(i) silver nitrate-sodium hydroxide dip reagent. This is essentially the method of Partridge (1946) as modified by Trevelyan, Proctor and Harrison (1950). The dried chromatogram was dipped through silver nitrate solution (0.7 g silver nitrate in 3 ml water was diluted to 200 ml with acetone). The chromatogram was dried in the fume hood for 5 min and then dipped in a solution of sodium hydroxide (0.8 g sodium hydroxide in 10 ml water was diluted to 200 ml with 95% ethanol). The chromatograms were preserved by dipping in a 10% sodium thiosulfate solution and washed immediately with water. Compounds appeared as dark brown spots on a light brown background. Over the period of a few days the colours changed to give dark gray spots on an almost white background.

(ii) Periodate-Tolidine Stain for Vicinal Hydroxyl Groups (White and Frerman, 1967)

The dried chromatogram was dipped in sodium metaperiodate solution (5 ml of 0.25M sodium periodate diluted with 95 ml acetone, allowed to hang for 15 min and then dipped in the O-tolidine reagent (0.4 g O-tolidine, 1.2 ml glacial acetic acid, 9.8 ml water and diluted to 200 ml with acetone). Components appeared as yellow spots on a blue background. Since the spots produced by this method were usually stable for 10-15 min, the spots should be circled by pencil as soon as they appeared.

(iii) Periodate-Schiff Stain for Terminal-Vicinal Hydroxyl Group (Baddiley et al., 1956)

The dried chromatogram was sprayed with 1% aqueous sodium metaperiodate and left for 15 min at room temperature, then decolorized by treatment with sulfur dioxide gas in a closed jar. The treated chromatogram was then sprayed with the Schiff's reagent (see footnote, page 95). The water-soluble compound containing terminal-vicinal hydroxyl group (e.g., deacylated lipid or free glycerol) gives pink colour.

5. Gas-Liquid Chromatography (GLC)

GLC was used to analyze fatty acid methyl esters, methylated sugar derivatives and the acetylated derivatives of the polyol compound.

a) Fatty Acid Methyl Esters

Gas chromatography was carried out on a Carlo Erba Fractovap unit equipped with a flame-ionization detector system. The columns used for the separation of fatty acid methyl esters were: (i) 3% SE-30 ultraphase on Chromosorb W(HP) 80/100 mesh in a 90 cm x 4 mm (ID) glass column or (ii) 10% butanediol succinate polyester (BDS) on Gas Chromosorb W 80/60 mesh in a 180 cm x 4 mm (ID) glass column. Column oven temperature was 175°C, injector port temperature was 225°C and detector block temperature was 235°C; nitrogen carrier gas inlet pressure was 0.70 kg/cm^2.

Preparation of Fatty Acid Methyl Esters by Methanolysis

Methanolysis is defined as the conversion of both free and esterified fatty acids to their corresponding methyl esters, by treatment of the lipids with a solution of hydrogen chloride in anhydrous methanol.

A 2.5% (W/V) solution of hydrogen chloride in methanol was obtained by bubbling hydrogen chloride gas from a cylinder into a known volume of anhydrous methanol. A solution of total lipid (2-10 mg) in chloroform was brought to dryness under a stream of nitrogen in a 50 ml side-arm flask (Kates, 1964a). To the residue was added 4.5 ml of 2.5% methanolic-HCl and the mixture was refluxed for approximately one hour. The solution was allowed to cool, diluted with 0.5 ml of distilled water and extracted three times

with 5 ml of petroleum ether (b.p. 60-70°C), (the petroleum ether being easily decanted off while the aqueous methanol phase remained in the side-arm tube). The petroleum ether extracts containing the fatty acid methyl esters were collected and brought to dryness under a stream of nitrogen; the residue of methyl esters was taken up in a small volume of chloroform (to give approximately a 10% W/V solution) for injection into the GLC. Usually 0.5-1.0 µl were injected by a 10 µl Hamilton syringe through the rubber diaphragm of the injector port. Samples were injected repeatedly to check reproducibility of retention times and peak heights.

A solution of standard fatty acid methyl esters (14:0, 16:0, 16:1, 18:0, 18:1, 18:2, ant 15:0 and 17:0, iso 14:0, 15:0 and 17:0; 19:0:cy) in chloroform was prepared in a suitable concentration (1-10%, W/V). Standards (0.5-1.0 µl) were analyzed along with the unknown samples. Peaks were identified by their retention times relative to authentic standards on two different liquid phases. Quantitative analyses were obtained by measurement of peak areas by the procedure of Carroll (1961):

$$\% \text{ Ai} = \frac{d_i h_i}{\Sigma\, d_i h_i} \times 100$$

where A = peak area

d = distance (mm)

h = peak height (mm).

b) Methylated Sugar Derivatives

GLC of methylated sugar derivatives was carried out on: (i) a glass column (180 cm x 4 mm.ID) packed with 10% BDS on Gas-Chrom W; nitrogen inlet pressure, 0.75 kg/cm^2, 180°C for methyl glycoside derivative and 200°C for alditol acetate derivative; in the Carlo Erba Fractovap Gas Chromatograph, or (ii) on a glass column (120 cm x 4 mm.ID) packed with 3% ECNSS-M on Gas Chrom Q; helium inlet pressure, 3 psi, 170°C for methyl glycoside and 190°C for alditol acetate derivative, using a Hewlett-Packard model 402 gas chromatograph with a flame ionization detector.

The preparation of methyl glucosides and alditol acetate derivatives of permethylated DGG and GPDGD were described in the Experimental Procedure Section II.F. Solutions (2 µl aliquots) of the methyl glucoside or alditol acetate derivatives in chloroform-methanol (1:1, v/v) (20 mg/ml) were injected with a 10 µl Hamilton micro-syringe. Authentic standards, methyl glucosides or alditol acetate derivatives of 2,3,4,6-O-tetramethyl glucose, 2,4,6-O-trimethyl glucose, 2,3,6-O-trimethyl glucose, 2,3,4-O-trimethyl glucose, 3,4,6-O-trimethyl glucose, 1,2-O-dimethyl glycerol and glycerol were used to identify the peaks.

c) Analysis of "Smith Degradation" Products

"Smith degradation" products were polyol compounds (e.g., glycerol, ethylene glycol, erythritol, etc.) which

were analyzed on GLC as their acetylated derivatives. The process of "Smith degradation" is described in the Experimental Procedure Section II.E.2. The GLC analysis was carried out on a glass column (180 cm x 4 mm.ID) packed with 10% BDS on Gas Chrom. W, nitrogen inlet pressure, 0.75 kg/cm^2, 170°C. Solution (2 µl) of the acetylated derivative in chloroform-methanol (1:1, v/v) (20 mg/ml) was injected. Authentic standards (erythritol tetra-acetate, glycerol triacetate and ethylene glycol diacetate were injected for reference purposes.

D. Physical Methods

1. Infrared Spectroscopy

Infrared spectra were recorded on a Unicam SP1000 Infrared Spectrophotometer. Samples were dissolved in "Spectra-analyzed" chloroform or carbontetrachloride (ca. 1% solution) and placed in a 0.1 mm sodium chloride cell.

2. Nuclear Magnetic Resonance (NMR) Spectroscopy

NMR spectra were run at ambient temperature on a Varian Associates A-60 (60 Mc) Proton NMR spectrometer and a Varian HA-100 (100 Mc) Spectrometer. All samples were run at a concentration of 10-20% (w/v). Water soluble samples were run in deuterium oxide (D_2O) with acetone as an internal standard. Chloroform-soluble samples were run in $CDCl_3$ with an internal standard of TMS. Data for individual spectra are given in the corresponding figure legends.

3. Optical Rotations

All optical rotations were measured at room temperature at the D line (589 mμ) of the sodium spectrum in a 1 dm cell using a Perkin-Elmer polarimeter, Model 141, with digital readout.

Optical rotation of the pure, water-soluble compounds were done on aqueous solutions (ca. 30-60 mg/ml). The rotations of the pure lipids were done on chloroform solutions (ca. 40-80 mg/ml).

E. Analytical Procedures

Chloroform solutions of total lipids or pure components were made to known volume in a volumetric flask to about a 1% concentration. An accurate determination of the concentration was then made as described below and aliquots of the stock solution were taken for the various analyses given below.

1. Dry Weight of Lipid

A suitable aliquot of the lipid solution, containing 15-20 mg was pipetted accurately into a 25 ml wide-mouthed weighing flask with ground glass stopper. The solvent was evaporated in a stream of nitrogen at 30°C and the flask was evacuated in a desiccator over fresh potassium hydroxide pellets on a high vacuum pump to a pressure of 0.1 mm Hg. Nitrogen was introduced into the desiccator and the flask

was stoppered and weighed on an analytical balance to 0.05 mg. Drying of the sample in the desiccator *in vacuo* was then repeated as described until the weight was constant to within 0.05 mg.

2. Phosphorus Determination

Phosphorus in the intact lipids or in water-soluble phosphate was determined by modifications of the methods of Allen (1940), or Bartlett (1959);

(a) Allen's method

The sample solution, containing 20-80 µg P (less than 2 mg lipid), in a straight-walled "Lewis-Benedict sugar tube" calibrated at 12.5 and 25 ml, was evaporated to dryness under a nitrogen stream. The residue was digested with 2.0 ml of 72% perchloric acid (Analar, BDH Chemical) at reflux temperature for 4-5 min; after cooling to room temperature, the digest was diluted to 12.5 ml with distilled water and mixed on a vortex mixer. Amidol reagent* (2 ml, freshly prepared) and 8.3% ammonium molybdate solution (1 ml) were added by pipette, with thorough mixing after each addition. The colour was allowed to develop for 20 min; the solution was then diluted to the 25 ml graduation and mixed by inversion. The percent transmission at 680 nm of the sample

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

and standard* solutions was determined in 19 mm round cuvettes against a reagent blank, using a Coleman Junior II Spectrophotometer and converted to optical density (absorbance) from tables. Beer's law was obeyed in the range 10-100 µg P.

(b) Bartlett's method

The lipid sample, containing 0.5 to 10 µg P, was digested with 0.4 ml of 72% perchloric acid; 4.2 ml of distilled water, 0.2 ml of amidol solution and 0.2 ml of 5% ammonium molybdate solution were successively added with vortex mixing. Each tube was covered with a beaker, heated in a boiling water bath for 7 min and cooled by immersion in a cold water bath. After 15 min the absorbance of the stable blue colour was read at 800 nm in 12 mm round cuvettes in a Coleman Junior II Spectrophotometer, against standards* and a reagent blank. Beer's law was obeyed in the range 1-10 µg P.

(c) For Quantitative Analysis of Phosphatides on TLC (Rouser et al., 1966)

The TLC plate was sprayed with sulfuric-dichromate solution (0.6% $K_2Cr_2O_7$ in 55% H_2SO_4) and charred on a hot

*Standard 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 10 to give a working solution containing 10 µg P/ml (Allen) or 1 to 100 to give 1 µg P/ml (Bartlett).

plate. The lipid spots were circled, and appropriate blank spots, corresponding to the various spot sizes, were marked off in the upper right hand corner of the plate. Each spot was scraped into 30 ml Kjeldahl flasks containing 0.9 ml of 72% perchloric acid. The contents of the flask was digested on a Kjeldahl rack with gentle refluxing so that digestion is complete in 20 min with a minimum loss of perchloric acid. The sides of the flask was rinsed down with 5 ml of water and 1 ml of 2.5% ammonium molybdate solution was added and mixed, followed by the addition of 1 ml of 10% ascorbic acid solution (prepared fresh) and 2 ml of water. The mixture was transferred to a 15 ml centrifuge tube with a Pasteur pipet and was heated in a boiling water bath for 5 min, cooled and the mixture was cleared by centrifugation. The clear supernatant was transferred to a 12 mm round cuvette, and the absorbance read at 790 nm, using Coleman Junior II Spectrophotometer, against a reagent blank. The readings were corrected by subtracting the absorbance values of the blank area corresponding in size to that of the sample. The absorbance values were converted to µg P by means of a factor derived from values obtained with known amounts of KH_2PO_4 standard; the method is suitable in the range 0.5-10 µg P.

3. Glycerol Determination (Renkonen, 1962)

An aliquot of phospholipid or glycolipid (containing 0.2-1 µmole glycerol) was placed in a Pyrex glass tube (1.0 x 15 cm) sealed at one end and constricted at the other. Solvent was evaporated in a stream of nitrogen, and 3.0 ml of 2N HCl was added. The tube was sealed under slight vacuum and was kept in an oven at 125°C for 48 hr; cooled and the hydrolysate was transferred to a 15 ml centrifuge tube. The pyrex glass tube was washed with 2 ml water and the combined aqueous hydrolysate was shaken with 2 ml of chloroform and centrifuged briefly to remove free fatty acids. Two ml of the aqueous layer was pipetted to a 15 ml glass-stoppered tube and 0.1 ml of 10N H_2SO_4, 0.5 ml of 0.1M $NaIO_4$ were added with mixing. The mixture was left at room temperature for 5 min and 0.5 ml of 10% $NaHSO_3$ was added with mixing. To a 0.5 ml aliquot in a 15 ml glass-stoppered tube was added 5 ml of chromotropic acid reagent* and the mixture was heated in boiling water bath for 135 min; after cooling to room temperature for 30 min, the absorbance was read at 570 nm in Coleman Spectrophotometer against a reagent blank. Standard amounts of glycerol (0.1 to 0.5 µmoles) were carried through the periodic-chromotropic acid procedure (0.5 µmole glycerol gives absorbance 0.44 in 12 mm round cuvette).

*Chromotropic acid reagent, dissolve 100 mg of 1,8-dihydroxy-naphthalene-3,6-disulfonic acid in 10 ml of water and dilute with 45 ml of 24N H_2SO_4.

4. Vicinal-glycol Determination

a) Lipids with Terminal Glycol Groups

(Ansell and Spanner, 1963)

This method determines the formaldehyde liberated by action of periodate on lipids having terminal glycol groups, as in phosphatidyl glycerol and glycerophosphoryl diglucosyl diglyceride.

The sample, containing 0.2-1 µmole glycerol was dissolved in 0.5 ml 95% ethanol in a 15 ml glass-stoppered centrifuge tube; 0.5 ml of 1% periodic acid solution was added with mixing and left at room temperature for 1 hr. The reaction mixture was then diluted with 1.5 ml of water, and 0.75 ml of 1.0N HCl, 0.25 ml of 1.2N sodium arsenite were added. The final volume was brought to 5.0 ml with water and 0.5 ml aliquot was added to 5.0 ml of chromotropic acid (see footnote p.107). The mixture was heated in a boiling water bath for 30 min. The absorbance was read against a reagent blank at 570 nm in 12 mm cuvette in a Coleman Junior Spectrophotometer. The procedure was standardized with 0.2-1 µmole of pure batyl alcohol, or 0.1-0.5 µmole glycerol and the calibration curves was used to determine the amount of terminal glycol (µmoles) in the sample.

b) Total Vicinal Hydroxyl Groups

(Cheronis and Ma, 1964)

Sample, containing 10-15 µmole total vicinal glycol was dissolved in 1.0 ml of 95% ethanol and 1.0 ml of 0.1 M periodic acid was added and diluted to 2.5 ml with 95% ethanol. The mixture was kept in the dark at room temperature for 30 min; 2 ml aliquot was added to 2 ml of saturated sodium bicarbonate with vigorous stirring and then 4.0 ml of 0.03 M sodium arsenite*, 0.4 ml of 20% potassium iodide and 0.8 g of anhydrous solid sodium bicarbonate were added. The mixture was allowed to stand at room temperature for 15 min and 0.1 ml of starch indicator** was added and titrated with 0.01N iodine solution*** to a faint blue end point. The reagent blank was performed without substrate through the whole procedure.

5. Total Sugars

a) Phenol-sulfuric acid method (Dubois et al.,1956)

Simple sugars, oligosaccharides, polysaccharides and glycolipids, including the methyl esters with free or

*Standard sodium arsenite solution (0.03 M): dissolve 1.484 g of analytical grade As_2O_3 in 30 ml of warm 1.0N NaOH; add 50 ml of water, neutralize to phenolphthalein with 3.0N HCl, and add 2 drops of acid in excess; dilute to 500 ml.

**Starch indicator solution (1%), 1 g soluble starch in 100 ml of 13% KCl; bring to a boil and cool.

***Standard iodine solution (0.01N): dissolve 1.269 g of resublimed iodine and 3.3 g of KI in ca. 350 ml of water with shaking, and dilute to 1 litre; standardize against 0.03 M sodium arsenite.

potentially free reducing groups, give an orange yellow colour when treated with phenol and concentrated sulfuric acid. The reaction is sensitive and the colour is stable. The method has been developed to determine submicroamounts of sugars and related substance (10-80 µg sugars).

An aliquot of lipid solution or hydrolysate, containing 10-80 µg of sugar (as hexose) was pipetted into a 25 ml Lewis-Benedict sugar tube, the solvent was evaporated to dryness in a stream of nitrogen. To the residue was added 2 ml of water and 1.0 ml of 5% phenol solution (freshly prepared) with mixing, followed by 5.0 ml of conc. sulfuric acid. The solution was immediately mixed and 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 glucose were analyzed simultaneously. Beer's law holds up to 80 µg of glucose; 10 µg glucose gave an absorbance of ca. 0.063 in 12 mm round cuvette in a Coleman Junior Spectrophotometer.

b) Enzymatic Determination of Glucose

The presence of glucose in hydrolysis mixtures was detemined quantitatively by the "Glucostat" reagent. The method is based on the oxidation of glucose by glucose oxidase. Hydrogen peroxide produced in the reaction in turn oxidizes a "chromogen" in its reduced form (colourless) to the oxidized form (coloured). The colour produced is then measured spectrophotometrically.

A 1.0 ml aliquot of the hydrolysate or standard solution containing 0.05-0.3 mg glucose was added to 9.0 ml of Glucostat reagent*. A reagent blank and at least two standards were performed simultaneously with each set of unknowns. The mixture was left at room temperature for 10 min and 1 drop of 4 M hydrochloric acid was added to stop the reaction and to stabilize the colour. The reaction time for all tubes should be identical. The tubes were left at room temperature for 5 min after stopping the reaction, and absorbance can be read at any time. The absorbance was read at 400 nm, against a reagent blank, (0.05 mg glucose give absorbance 0.95 in 19 mm round cuvette).

6. Ester Determination (Renkonen, 1961)

The principle of this analysis is based on the reaction of fatty acid esters with alkaline hydroxylamine to form a hydroxamic acid. The latter forms a complex with acid ferric perchlorate to give a brown to purple colour which can be measured spectrophotometrically.

An aliquot of lipid solution (0.2-2 µmoles) was pipetted into a 15 ml glass-stoppered tube. Solvent was evaporated under nitrogen and 1.0 ml of alkaline hydroxylamine solution** was added to the residue. The tube was

*Glucostat Reagent: 60 ml of chromogen solution and 30 ml of glucostat solution in distilled water.

**Alkaline hydroxylamine solution (prepared fresh), mix equal volumes of 4% ethanolic hydroxylamine hydrochloride (2 g in 2.5 ml of water diluted to 50 ml with absolute ethanol) and 8% ethanolic sodium hydroxide (4 g in 2.5 ml of water diluted to 50 ml with absolute alcohol); centrifuge and use clear supernatant. Standard ester solution, 29.8 mg methyl stearate in 100 ml of chloroform (1 µmole/ml).

stoppered and the mixture heated in a water bath at 65°C for 2 min. After cooling for 5 min; 3.0 ml of dilute ferric perchlorate solution* was added with mixing and the colour allowed to develop for 30 min. The absorbance of the mauve colour was read at 530 nm against a reagent blank. Standards containing 0.5, 1.0, and 2.0 µmoles methyl stearate were run simultaneously. Beer's law is valid up to 4 µmoles; 1 µmole ester gives an absorbance of ca. 0.24 in 12 mm round cuvette (Coleman Junior Spectrophotometer).

7. α- and β-Glycerophosphate Analysis

A modified method of Burmaster (1946) was employed to determine the amount of α- and β-glycerophosphate obtained from aqueous alkaline hydrolysis of phospholipids. Essentially, the method is based on the conversion of α-glycerophosphate to glycolic aldehyde phosphate by reaction with sodium periodate at room temperature; the excess of periodate and the iodate formed are destroyed with sodium sulfite, and the glycolic aldehyde phosphate is hydrolyzed with hot acid. The resulting orthophosphate is measured by a modification of the standard colorimetric procedure (Allen, 1940). When corrected for any inorganic phosphate produced by hydrolysis of phospho-

*Stock ferric perchlorate solution, 5 g of $Fe(ClO_4)_3.6\ H_2O$ in 10 ml of 70% perchloric acid and 10 ml of water; dilute to 100 ml with cold absolute ethanol; solution is stable at 4°C for several months. Dilute ferric perchlorate solution (freshly prepared), 4 ml of stock ferric perchlorate solution and 3 ml of 70% perchloric acid diluted to 100 ml with absolute alcohol.

lipid, the orthophosphate is a measure of the α-glycero-phosphate isomer. Total glycerophosphate (α+β) was measured on another portion of the sample by converting β-glycero-phosphate to α-form by acid-catalyzed migration of phosphate and then to orthophosphate by treating it with sodium periodate in hot acid solution as above. The β-glycerphos-phate was found by subtracting the α-phosphorus value from the total (α+β) glycerophosphate phosphorus. Total phosphorus in the sample was determined by a procedure of Allen (1940).

a) Total Glycerophosphate (α+β)

To an aliquot of the hydrolysate or standard solution (not more than 5 ml, containing 30-60 µg P) in a 25 ml Benedict-Lewis sugar tube was added 1.0 ml of 72% perchloric acid (11.8N) and 1.0 ml 0.08 M sodium periodate and water to a total volume of 7 ml; the mixture was heated in a boiling water bath for 1 hr, cooled and diluted with 1.5 ml of 4% sodium sulfite and 1.0 ml of 72% perchloric acid and water to 12.5 ml:2 ml of amidol solution (see p.104) were added with mixing on vortex followed by 1.0 ml of 8.3% ammonium molybdate. After 20 min, the solution was diluted to the 25 ml mark with water and the absorbance was read at 680 nm.

b) α-Glycerophosphate

To the same aliquot as in (a), was added water to 4.0 ml, then 1.5 ml of 0.12N perchloric acid and 1.0 ml

0.08N sodium periodate; the mixture was kept for 10 min at room temperature, then diluted with 1.5 ml of 4% sodium sulfite and 1 ml 75% perchloric acid and heated for 1 hr in boiling water. Thereafter, 1 ml of 72% perchloric acid was added and the volume made to 12.5 ml. The colour was developed and the absorbance measured as above.

c) Control

To the same aliquot of sample solution as in (a) and (b) was added water to 5.0 ml plus 1.0 ml 72% perchloric acid and 1.5 ml of 4% sodium sulfite and 1.0 ml 0.08 M sodium periodate. The mixture was left at room temperature for 1 hr, 1.0 ml 72% perchloric acid was added and the volume was made to 12.5 ml and the colour was developed as usual. The phosphorus obtained in the control is the free inorganic phosphate in the sample which has to be subtracted from the total glycerophosphate and the α-glycerophosphate.

d) β-Glycerophosphate

This value was obtained by calculation as follows: μmoles β-glycerophosphate = μmoles total glycerophosphate - μmoles α-glycerophosphate.

8. sn-Glycero-3-phosphate Analysis (Kennedy, 1962)

The assay of sn-glycero-3-phosphate is carried out spectrophotometrically and is based on the formation of NADH in a reaction catalyzed by the enzyme glycerophosphate dehydrogenase as follows:

$$\underline{sn}\text{-3-GP} + NAD \rightleftharpoons DHAP + NADH + H^+$$

The enzyme is specific for sn-glycero-3-phosphate and does not attack sn-glycero-1-phosphate. The NADH is measured, by its characteristic absorption at 340 nm. Since the equilibrium of the reaction lies well toward the left at neutral pH, it is necessary to add a trapping agent for the dihydroxyacetone phosphosphate (DHAP) and to run the reaction at an alkaline pH in the presence of hydrazine which functions both as an efficient trapping agent and as a buffer.

Into a cuvette (1.0 cm light path) are pipetted 1.8 ml of the hydrazine-glycine-magnesium chloride buffer*, 0.05 ml of 0.02 M NAD, 0.05 ml of 0.075 M ATP, 0.02-0.08 µmole of glycerophosphate and 0.02 ml glycerophosphate dehydrogenase (5 mg protein/ml) in a final volume of 2.0 ml. The enzymatic reaction was followed by measuring absorbance at 340 nm in a Perkin-Elmer DB spectrophotometer. The reaction was complete in 1 hr. Pure sn-glycero-3-phosphate was used as standard for preparation of a calibration curve (1 O.D unit = 0.8 µmole sn-glycero-3-phosphate).

F. Radioisotopic Procedure

1. Radioisotopic Counting

Labelled lipids (^{32}P and ^{14}C) were counted in Beckman Scintillation counter (LS-133, Beckman Instruments Inc.).

*5 g of 100% hydrazine hydrate (d, 1.03), 1.5 g glycine, 0.2 ml of 1M $MgCl_2$, a few drops of 10N KOH to bring the pH to 9.8, in total volume of 100 ml in water.

The scintillation fluid composed of 0.5% 2,5-diphenyloxazole (PPO); 0.05% 1,4-bis-2-(5-phenyloxazolyl)-benzene (POPOP); 130 ml methanol and 100 ml of solubilizer (Beckman Bio-solv, formula BBS-3) in 1 litre of toluene.

Counts obtained from doubly labelled sample (^{14}C and ^{32}P) was corrected for spillover of ^{32}P into the ^{14}C window by standardization with known amount of ^{32}P. The efficiency for ^{14}C counting was 90-95% and for ^{32}P was 95-100%. The counts were corrected for quenching by external standard ratios method.

Radioactive spots on thin layer chromatograms were counted as follows: the radioactive spots located by auto-radiography were scraped directly into 10 ml of the "scintillation cocktail", mixed well and counted as described above. The counts in each spot was expressed as a percentage of the total counts recovered from the TLC plate.

2. Autoradiography

Detection of radioactive lipids on TLC is readily and conveniently achieved by autoradiography using X-ray film. This method is sufficiently sensitive to detect ^{14}C spots with activity as low as 100 cpm above background within a week's exposure, and ^{32}P-labelled spots within 2-3 days. The TLC plate was placed in a light-proof cardboard holder (Eastman Kodak) 21 x 25 cm. A pre-cut sheet of X-ray film (Kodak blue band, medical X-ray film) was placed

directly on to the silica surface and exposed in the folder.

The exposure time will depend on the amount of radioactivity applied to the spots and the energy of the radiation; for ^{14}C-labelled spots the following formula may be used:

$$\text{exposure time (hr)} = \frac{10^6}{\text{cpm applied}} \times \frac{1}{60} \quad \text{(Kates, 1972a)}$$

The exposure time should be halved for ^{32}P-labelled spots. The exposed X-ray film was developed by standard photographic technique.

EXPERIMENTAL PROCEDURES

I. Isolation and Purification of Lipid Components

A. Preliminary Column Chromatography

(Vorbeck and Marinetti, 1965a)

A chloroform solution of the total lipid of S. epidermidis (700 mg in 7 ml chloroform) was applied to a column of 50 g silicic acid prepared in chloroform (column dimensions, 2.8 x 38 cm). The column was eluted with the following solvents: chloroform (300 ml, Fr. 1); chloroform-acetone (1:1, v/v; 400 ml, Fr. 2); acetone (400 ml, Fr. 3); chloroform-methanol (1:1, v/v; 500 ml, Fr. 4) and methanol (500 ml, Fr. 5). The solvent from each fraction was removed on the rotary evaporator to dryness; each lipid fraction was weighed and redissolved in chloroform-methanol (9:1, v/v). Phosphorus and sugar analyses were carried out on the fractions, which were then further purified by preparative TLC.

B. Preparative TLC

Polar lipids obtained from column chromatography were fractionated by preparative TLC as described in Materials and Methods, II.C.2(b). Lipid solution from Fr. 2-5 in chloroform-methanol (9:1, v/v) (40 mg/ml) was applied on a preparative TLC plate (1 ml/plate) and developed in chloroform-methanol-water (65:25:4, v/v). The bands corresponding

to GPDGD, PG, DGD, or MGD (see Table 15 for R_f value) were scraped off and eluted with the mixture of chloroform-methanol-diethyl ether (1:1:1, v/v) (100 ml for 40 mg lipid applied) as described in Materials and Methods, II.C.2(b).

The GPDGD obtained was contaminated with an unidentified component (spot #3, Fig. 9) and was further purified by repeated preparative TLC in the solvent system chloroform-methanol-water (65:35:6, v/v). PG obtained from the first fractionation on TLC was further purified by repeated preparative TLC using the solvent system chloroform-methanol-90% acetic acid (30:4 :20, v/v). DGD and MGD were obtained in a fairly pure state after the first fractionation on TLC. All lipid components (GPDGD, PG, DGD and MGD) after repeated purification on TLC gave only one spot in at least two different solvent systems. The R_f values of these lipids in various solvent systems are given in Table 15. The phospholipid components obtained were in their "natural" salt forms and were converted to their ammonium salt forms, as described below.

C. Preparation of Free Acid and Ammonium Salt of Phospholipids and Acetone Precipitation

A solution of the TLC purified "natural" salt form of PG or GPDGD (60 mg) in 10 ml of chloroform-methanol (1:1, v/v) was acidified with 4.5 ml of 0.2 N aqueous HCl, and the biphasic mixture was centrifuged briefly for 1-2 min. The

chloroform phase, containing the acid form of the lipid was neutralized immediately to pH 7-8 with 1.5 N NH_4OH in methanol and diluted with benzene. The mixture was evaporated to dryness under reduced pressure and the residual ammonium salt was weighed and redissolved in chloroform.

To the chloroform solution of ammonium salt of phospholipids (50 mg in 1.0 ml) was slowly added 5 ml of acetone with mixing on a vortex mixer. After cooling on ice for 1 hr, the precipitate was centrifuged and washed twice by suspending it in 2 ml of cold acetone and recentrifuged. The acetone supernatant was removed and discarded. The precipitated ammonium salt of the phosphatide were freed of excess solvent in a stream of nitrogen and dried *in vacuo* in a desiccator over potassium hydroxide, the dried residue was weighed and dissolved to a known concentration in chloroform.

The phospholipids in the solution were quantitatively (95-100%) precipitated by cold acetone with no change in their salt form; contaminants, such as neutral lipids remained in solution. Therefore acetone precipitation was used as the final purification step for the phospholipids prior to analysis of their lipid constituents (e.g., sugar, phosphorus, ester and glycerol).

Glycolipids such as monoglucosyl diglyceride and diglucosyl diglyceride were partially soluble in acetone and could not be used for final purification. Repeated preparative TLC was therefore used to purify these two glycolipids before their analysis.

II. Procedures for Structural Determination

A. Mild Alkaline Deacylation (Dawson, 1967; Kates, 1972a)

An aliquot of lipid solution (containing 25 mg of pure lipid component) in chloroform was pipetted into 15 ml glass-stoppered tube, and the solvent was removed under a stream of nitrogen. To a solution of the residue in 0.4 ml of chloroform and 0.6 ml of methanol was added 1.0 ml of 0.2 N methanolic-sodium hydroxide (freshly prepared). The mixture was kept at room temperature for 15 min and then diluted with 0.4 ml of methanol, 1.6 ml of chloroform and 1.8 ml of water with mixing. The biphasic mixture was centrifuged for 1 min at 600xg and the upper phase (methanol-water) was immediately removed and neutralized or slightly acidified (ca. pH 5-6) with 1-2 ml of Rexyn RG-50 (H^+). The resin was removed by centrifugation and the supernatant was transferred to another tube. The chloroform phase was washed twice with 1 ml of methanol-water (10:9, v/v) and the washings were also used to wash the resin. The combined methanol-water solutions were then neutralized with a few drops of 1.5 N methanolic-ammonium hydroxide to a slightly alkaline pH and concentrated under a stream of nitrogen at 40°C and the residue was dried in the desiccator over potassium hydroxide in vacuo. The residue was weighed and redissolved in a known amount of methanol-water (10:9, v/v). Aliquots were taken for paper chromatography (50-75 µg per spot) and for analysis of lipid

constituents (P, sugar and glycerol) as described in Materials and Methods II.E.

B. Strong Alkaline Hydrolysis

To a sample of pure lipid component (10 mg) or its deacylated compound (5 mg) in a 15 ml centrifuge tube was added 2.0 ml of a 1.0 N aqueous solution of sodium hydroxide with mixing. Another 15 ml centrifuge tube containing cold water was put on the top of the hydrolysis tube to serve as a stopper and condenser. The tube was heated in a boiling water bath for 2 hr until all turbidity had disappeared. The solution was cooled to room temperature and acidified with cationic exchange resin (Rexyn RG-50, H^+). The supernatant was removed and the resin was washed twice with 2 ml of methanol-water (10:9, v/v). The combined supernatant was extracted with petroleum ether to remove free fatty acid (only in the case of hydrolysis of lipid), and the petroleum ether extracts were combined, concentrated under nitrogen and kept for future analysis. The aqueous phase containing water soluble products was neutralized to pH 7-8 with I.5 N ammonium hydroxide in methanol and concentrated almost to dryness under a stream of nitrogen at 30-40°C. The residue was dissolved in a known amount of methanol-water (10:9, v/v) and were taken for quantitative P or sugar analysis and for qualitative analysis on paper chromatography (50-75 µg deacylated material per spot).

Isolation of water-soluble product (Diglucosyl glycerol)

Mild deacylation product of GPDGD (20 mg) was subjected to alkaline hydrolysis (4 ml of 1 N NaOH, 100°C, 2 hr) as described above. The concentrated and neutralized methanol-water solution (2 ml) was passed through the column of Dowex-2 ($CO_3^=$) (10 ml) to remove glycerophosphate. The column was eluted with methanol-water (10:9) (100 ml) and the eluate was concentrated to dryness under rotary evaporator (30-40°C). The residue was dissolved in distilled water to a known volume and sugar analysis was performed. Yield of chromatographically pure diglucosyl glycerol obtained was 13 mg.

C. Acid Hydrolysis

1. In Glycolipids or Deacylated Glycolipids

Acid hydrolysis (0.5 N HCl, 100°C) was carried out to hydrolyze glycosidic linkages as well as ester linkages since alkaline hydrolysis cleaves only ester linkages and does not attack glycosidic linkages.

To a sample of glycolipid (5-10 mg) or glycoside (1-2 mg) in 15 ml centrifuge tube was added 1.0 ml of 0.5 N HCl, and the tube was heated in a boiling water bath for 1 hr. The hydrolysis tube was stoppered with another 15 ml centrifuge tube filled with cold water to prevent the evaporation of HCl during hydrolysis. In the case of glycolipids, free fatty acids released were removed by extraction

with petroleum ether. The aqueous solution containing free sugars and glycerol was concentrated under a stream of nitrogen (30-40°) to a small volume and was dried over potassium hydroxide in a desiccator *in vacuo* overnight to remove the last traces of HCl. Care was taken not to bring the solution down to complete dryness because glycerol is rather volatile and may be lost during concentration of the aqueous solution. The residue was dissolved in 0.1 ml distilled water and was chromatographed on Whatman No. 1 paper along with authentic standards (glucose, galactose, mannose and glycerol) in the solvent system pyridine-ethyl acetate-water (2:5:5, v/v, upper phase, descending technique); 30-50 µg of each component gives a good sized spot.

2. In Phospholipids

A solution of pure phospholipid, containing 1-2 µmoles P in chloroform was transferred to a pyrex glass tube (1.0 x 15 cm) sealed at one end. The solvent was evaporated under nitrogen and pumped under vacuum for 1 hr to remove traces of chloroform. To the residue was added 1.0 ml of 2 N HCl and the tube was sealed under slight vacuum and kept in an oven at 125°C for 48 hr. In this condition, all phosphate ester, acyl ester and glycosidic bonds will be cleaved with the release of inorganic phosphate, glycerol, fatty acids, and sugars.

After the hydrolysis was complete, the hydrolysate was extracted with petroleum ether to remove free fatty acid as described before in this section. The aqueous solution containing inorganic phosphate and other water soluble products (e.g., sugar, glycerol, etc.) was concentrated under a stream of nitrogen (30-40°C) and the residue dried in a desiccator over potassium hydroxide to remove traces of hydrochloric acid. The residue was redissolved in distilled water and examined by paper chromatography as described for the glycolipids.

The R_f value of authentic standards which may obtain from acid hydrolysis of various lipids under investigation are shown below in Table 17.

D. Degradation with Hydrofluoric Acid (HF)

Hydrofluoric acid is known to hydrolyze only phosphate bonds without attacking any glycosidic or ester linkages (Archibald et al., 1968).

To a sample of phospholipids (5 mg GPDGD or 20 mg PG) in a 15 ml polyethylene centrifuge tube fitted with a stopper was added 0.5 ml of HF (48-51%, w/v, d 1.15-1.18) with mixing. The reaction tube was kept at 0°C for 24 hr, with occasional mixing and the mixture was first neutralized with 2.4 N lithium hydroxide (3.5 ml) and was brought to pH 6.5-7 with 0.2 N lithium carbonate. The neutralized aqueous solution was transferred to a 50 ml centrifuge tube

and the polyethylene tube was washed several times with distilled water to make the final volume 9.0 ml, followed by washing with 10 ml of chloroform and 10 ml of methanol (5 ml each time) to remove water-insoluble degradation products. The washings were combined and added to the aqueous solution, mixed and centrifuged. The chloroform phase was removed, concentrated under nitrogen and the products were examined on TLC developed in petroleum ether-ethyl ether-glacial acetic acid (80:20:1, v/v) for diglyceride and in chloroform-methanol-28% ammonia (65:35:5, v/v) for the diglucosyl diglyceride; authentic standards, 1,2-diglyceride and diglucosyl diglyceride (from S. epidermidis) were also run. The methanol-water phase was concentrated under nitrogen to a small volume (3 ml), deionized with Rexyn RG-50 (H^+), and examined by paper chromatography (solvents, phenol-water, 5:2, w/w; and pyridine-ethyl acetate-water, 2:5:5, v/v, upper phase). Authentic standards of glucose, glycerol and diglucosyl glycerol (from S. epidermidis) were also run.

E. Periodate Oxidation Studies

1. Spectrophotometric Analysis of Total Vicinal-Hydroxyl Groups (Dixon and Lipkin, 1954)

The consumption of periodate by vicinal glycol groups may be followed spectrophotometrically by using the absorption band of metaperiodate which has a maximum at about 220 to 240 nm.

To a solution of diglucosyl glycerol (containing 3.6 µmole glucose) from DGD and glycerophosphoryl diglucosyl glycerol (1.1 µmole P) from GPDGD in 2.0 ml water were added 0.4 ml of o.5 M sodium periodate. The oxidation was carried out at room temperature in the dark, and samples (0.1 ml) were withdrawn at zero time, 24, 48, 96 and 120 hr and diluted to 10 ml with water. The optical density was followed at 223 nm in a Perkin-Elmer DB Spectrophotometer; β-methyl-glucoside (4.3 µmole) was oxidized under identical condition as a control, (0.46 µmole, sodium periodate/10 ml gave O.D 0.455).. Sodium periodate solution was standardized according to the method of Jackson (Jackson, 1944).

For determination of formaldehyde, an aliquot (0.2 ml) from the 120 hr sample (oxidation complete) was mixed consecutively with a 10% solution of sodium metabisulfite (0.2 ml) and chromotropic acid reagent* (5.0 ml) in 15 ml stoppered centrifuge tube. The mixture was heated in a boiling water bath for 135 min and cooled for 30 min. The absorbance was read at 570 nm in Beckman DB spectrophotometer against a reagent blank.

Oxidation of known amounts of glycerol (0.2-0.5 µmole in 2 ml solution) was carried out for calibration purposes; 0.12 µmole formaldehyde equivalent to 0.06 µmole glycerol gave O.D 0.352).

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*The composition of this reagent was described in Materials and Methods, II.E.3.

2. Periodate Oxidation, Borohydride Reduction and Acid Hydrolysis ("Smith Degradation")

(Abdel-Akher et al., 1952)

Diglucosyl glycerol (containing 12 µmole glucose) from DGD, glycerophosphoryl diglucosyl glycerol (5 µmole P) from GPDGD or cellobiose (9 µmole) were oxidized in 18 mM sodium periodate solution (3 ml) at room temperature for 120 hr. Sodium borohydride (15 mg) was added and the mixture was left overnight at 4°C. A few drops of glacial acetic acid were added to destroy excess of sodium borohydride and the solution was passed through a column (10 ml) of Dowex-50 (H^+) resin and evaporated to dryness. The boric acid was removed by evaporation with three successive portions (5 ml each) of methanol. To the dried residue of the reduced oxidation products obtained from diglucosyl glycerol and cellobiose were added 1 ml of 0.5 N HCl and hydrolysis was carried out at 100°C for 1-2 hr as described in the Experimental Procedures II.C.1. Acid hydrolysis of the phosphorylated reduced product obtained from glycerophosphoryl diglucosyl glycerol was performed in a sealed tube at 125°C with 1 ml of 1 N HCl for 48 hr as described in the Experimental Procedures II.C.2.

Each hydrolysate was concentrated under a stream of nitrogen to a small volume (ca. 0.3 ml) and passed through a column of Dowex-2 ($CO_3^=$ form, 4 ml). The column was eluted

with distilled water (15 ml) and the eluate was concentrated to 2 ml. An aliquot (0.1-0.2 ml) was taken for spotting on Whatman No. 1 paper chromatograms using the solvent systems pyridine-ethyl acetate-water (2:5:5, v/v, upper phase, descending technique) and phenol-water (5:2, w/w, ascending technique) along with authentic standards (glucose, glycerol, erythritol and ethylene glycol).

The rest of the solution was evaporated to dryness under a stream of nitrogen, and kept in a desiccator over potassium hydroxide overnight under vacuum. The residual polyhydroxy compounds were then acetylated with 1 ml of the acetylating reagent (dry pyridine-acetic anhydride; 1:1, v/v) in a stoppered tube. The mixture was shaken on a vortex mixer and heated for 3 hr at 100°C in an oil bath. The excess acetylating reagent was removed under nitrogen and the polyol acetates were analyzed by GLC as described in Materials and Methods II.C.5(c). The authentic reference standards, ethylene glycol diacetate, glycerol triacetate and erythritol tetraacetate were prepared by the acetylation method described above.

F. Methylation of Glycolipids

1. Preparation of Permethylated Glycoside

(Brundish et al., 1967)

Diglucosyl glycerol obtained from DGD (26 mg) was dissolved in N,N-dimethylformamide (1.0 ml), silver oxide

(ca. 250 mg) and freshly distilled methyl iodide (5 ml) were added. The mixture was heated under reflux with magnetic stirring at 40°C for 36 hr; more silver oxide and methyl iodide were added at 12 hr intervals. The mixture was shaken for a further 60 hr. The mixture of chloroform-benzene (1:1, v/v) (5 ml) was added and the silver salts were removed by centrifugation and washed twice with 10 ml of the same solvent mixture. The combined supernatants were brought to dryness by rotary evaporation in vacuo over sulfuric acid. The residue was dissolved in chloroform (2 ml) and examined by IR for the presence of free hydroxyl groups. The reaction was complete when no free hydroxyl peak could be detected. Yield of crude permethylated diglucosyl glycerol 24 mg (colourless compound).

The crude product was fractionated on a column of silica gel (E. Merckag Darmstadt, Germany) (15 g, made up in benzene) using the following elution sequence: benzene (50 ml), benzene-ethanol (99:1, v/v, 50 ml), benzene-ethanol (97:3, v/v, 100 ml) and benzene-ethanol (95:5, v/v, 100 ml). The permethylated compound appeared in the benzene-ethanol (97:3) eluate; it was recovered on evaporation of the solvent and dried under high vacuum. Examination of the material on a TLC with solvent system benzene-methanol (9:1, v/v) gave a single spot with R_f 0.23. Yield, 16 mg.

2. Preparation of Permethylated GPDGD

The ammonium salt of GPDGD (33 mg) was converted to the free acid form by the method of Bligh and Dyer (see Experimental Procedures I.C). The free acid form obtained was dried over potassium hydroxide pellets under high vacuum for 1 hr, and 5 ml of freshly distilled methyl iodide and 250 mg silver oxide were added. The conditions were the same as described for methylation of diglucosyl glycerol. The reaction was complete in 72 hr, as revealed by the absence of free OH absorption in the IR spectrum; yield, of crude material 44 mg.

The crude product was purified on a column of silicic acid (15 g made up in benzene). Material was eluted with successive portions of the following solvents: benzene (100 ml), benzene-ether (90:10, v/v, 100 ml) benzene-ether (75:25, v/v, 200 ml), benzene-ether (50:50, v/v, 100 ml), benzene-ether (25:75, v/v, 100 ml) and ethyl ether 400 ml. The permethylated GPDGD appeared in the ether fraction (16 mg). The product was examined by NMR and IR; on TLC in the solvent system benzene-methanol (9:1, v/v), it gave a single spot with R_f 0.36.

3. Methylation Analysis

a) Preparation of Methyl Glucoside and Alditol Acetate Derivatives of Permethylated Diglucosyl Glycerol

The permethylated diglucosyl glycerol (10 mg) in 50 ml pear-shaped flask was methanolyzed in 2.5% methanolic-HCl (2 ml) under reflux at 100°C in an oil bath for 2 hr. The solution was evaporated to dryness on a rotary evaporator and the last trace of HCl was removed in vacuo over KOH. The partially methylated methyl glucosides were examined by GLC on 10% BDS column or on ECNSS-M column as described in Materials and Methods II.C.5(b).

The rest of the O-methylated methyl glycoside was evaporated to dryness to remove methanol, and the residue was heated in 1 ml of 1 N aqueous HCl in a boiling water bath for 3 hr to hydrolyze methyl glycoside linkage. Hydrochloric acid was removed by repeated concentration in vacuo on a rotary evaporator (bath temperature 30°C), and 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 3 hr. Glacial acetic acid was added dropwise to destroy the excess sodium borohydride and the mixture was taken to dryness repeatedly on a rotary evaporator with additions of methanol to remove boric acid as the volatile trimethyl ester. Methanol was added in three 5 ml portions, in order to remove borate ion

quantitatively. The presence of borate ions would interfere with the acetylation of alditols in the next step. (Gee and Walker, 1962). The partially methylated alditols were acetylated with acetic anhydride (2 ml) at 100°C for 3 hr. Sodium acetate present in the mixture served as a catalyst. The acetylation mixture was brought to dryness on a rotary evaporator to remove excess acetic anhydride and redissolved in chloroform (4-10 mg/ml). The alditol acetates were analyzed by GLC on 10% BDS column, as described in Materials and Methods II.C.5(b).

b) Preparation of Alditol Acetate Derivative of Permethylated GPDGD

Permethylated GPDGD (6 mg) was subjected to drastic acid hydrolysis (1 ml of 1 N HCl) in a sealed tube at 125°C for 12 hr as described in Experimental Procedures II.C.2. After the reaction was complete, the hydrolysate was transferred to 15 ml centrifuge tube and the hydrolysis tube was washed three times with 1 ml portion each of 90% methanol in water followed by petroleum ether (2x2 ml). The washings were combined and the petroleum ether extract containing free fatty acids was removed. The methanol-water phase was evaporated to a small volume (ca. 0.1 ml) and the last traces of HCl were removed *in vacuo* over KOH. The residue was redissolved in 2.0 ml water and an aliquot (0.2 ml) was taken for free P_i analysis by the Bartlett method (see

Materials and Methods II.E.2(b). The rest of the solution was deionized by passing through a column of Dowex-2 ($CO_3^=$ form, 3 ml) to remove free P_i. The column was washed twice with 90% methanol in water (10 ml), and the eluate was concentrated to a small volume (2.0 ml). The partially methylated compounds were then reduced with sodium borohydride and acetylated with acetic anhydride as described for permethylated diglucosyl glycerol. The alditol acetate derivative obtained was analyzed by GLC as described in Materials and Methods II.C.5(b).

G. Enzymatic Hydrolysis

Phospholipids and glycolipids can be identified by the products of hydrolysis with highly specific enzymes (e.g., phospholipases, glycolipid hydrolases). The stereospecificity of these enzymes also allows the stereochemical assignment of the lipid to be established. The following enzymes were used in the present structural studies:

1. Phospholipase A (E.C.3.1.1.4) (Haverkate and Van Deenen, 1965; Wells and Hanahan, 1969).

This enzyme specifically hydrolyzes the fatty acid ester linkage in the 2-position of a 1,2-diacyl-3-sn-glycerophosphatide, including plasmalogens, to liberate the 2-linked fatty acids and the corresponding lysophosphatide (see Scheme 4).

To a solution of PG (22 mg) in 10 ml ethyl ether-methanol (98:2, v/v) in a 50 ml stoppered round bottom flask was added 4 mg of phospholipase A (snake venom) in 2.0 ml of 0.1 M borate buffer (pH 7.0-7.5) containing 6.4 mg of calcium acetate. The mixture was stirred continuously with a magnetic stirrer for 3 hr at room temperature. The solvent was removed on a rotary evaporator (adding benzene to aid in removal of water). The residue was dried in vacuo and re-dissolved in chloroform-methanol (1:1). The mixture was centrifuged to remove salts and the clear solution was concentrated under nitrogen and chromatographed on TLC in solvent chloroform-methanol-90% acetic acid (30:4:20, v/v) for separation of free fatty acid and lyso PG. The separated fractions were eluted from the silica (see Materials and Methods II.C.2(b)) and methanolyzed with 2.5% methanolic-HCl; the fatty acid methyl esters were analyzed on GLC as described before (see Materials and Methods II.C.5(a)).

2. Phospholipase C (E.C.3.1.4.3)(Hanahan and Vercamer, 1954; Ottolenghi, 1969)

This enzyme catalyzes the hydrolysis of the diglyceride-phosphate linkage in phospholipids, to liberate the diglyceride and the phosphorylated water-soluble moiety (see Scheme 4)

To a solution of GPDGD (1.2 µmole P), PG (2µmole P) or PC (from egg yolk, 6 µmole P) in 1 ml of ethyl ether-

ethanol (98:2, v/v) was added 0.2 ml of 0.1 mM $ZnCl_2$, 0.4 ml of 0.1 M Tris-buffer pH 7.2 and 0.3 ml of the enzyme preparation (1 mg protein/ml) from C. welchii in 0.1 M Tris-buffer pH 7.2. The mixture was mixed, incubated at 25°C for 3 hr, and brought to dryness in a stream of nitrogen.

The residue was dissolved in 2.0 ml of methanol, 2.0 ml of chloroform and 1.8 ml of water, the biphasic mixture was centrifuged, and the chloroform phase was chromatographed on TLC in n-hexane-ethyl ether-glacial acetic acid (70:30:1) to isolate and identify 1,2-diglyceride. The methanol-water phase containing the water-soluble products were examined by paper chromatography in phenol-water (5:2, w/w).

3. Phospholipase D (E.C.3.1.4.4)(Kates and Sastry,1969)

This enzyme catalyzes the hydrolysis of phospholipids to phosphatidic acid and the respective water-soluble product (see Scheme 4).

To a suspension of GPDGD (1.2 μmole P), PG (2 μmole P) or PC (6 μmole P) in 0.5 ml of 0.2 M acetate buffer pH 5.6 was added 0.1 ml of 1 M $CaCl_2$, 0.4 ml of enzyme solution (from cabbage leaves, 20 mg/ml) and 0.4 ml of ethyl ether. The mixture was shaken vigorously and incubated at 25°C for 4 hr. The ether was removed in a stream of nitrogen and 1.0 ml each of methanol and chloroform were added. The

mixture was centrifuged and the chloroform phase was chromatographed on TLC in chloroform-methanol-90% acetic acid (30:4:20, v/v) and in chloroform-methanol-28% ammonia (65:35:5, v/v) to identify the phosphatidic acid. The methanol-water phase contained the water-soluble product was identified by paper chromatography in phenol-water (5:2, w/w).

4. α-Glucosidase (yeast glucosidase) and β-Glucosidase (almond emulsin)

These two enzymes catalyze the hydrolysis of water soluble glycosides containing the α- or β-glycosidic linkage respectively, on unsubstituted glucose residues with the liberation of free glucose.

The enzymes, α-glucosidase (2 mg protein in 1.0 ml water) and β-glucosidase (1 mg protein in 1.0 ml of 0.3 M ammonium sulfate were dialyzed against 2 litres of deionized water at 4°C for 48 hr before use. The final volume of both enzymes was adjusted to 2 ml with distilled water. Substrates employed in this experiment were the following: DGG from DGD, DGG from strong alkaline hydrolyses of GPDGD, MGG from MGD, maltose and cellobiose. The concentration of each substrate was approximately 100 µg in 0.05 ml distilled water.

The reaction mixture (1.0 ml) contained: 0.05 ml of substrate solution, 0.3 ml of enzyme solution and 0.65 ml

of 0.1 M phosphate buffer pH 6.8; incubation at 30°C for 18 hr. The reaction was stopped by heating in a boiling water bath for 1 min and the solution was deionized with Amberlite MB-2 (mixed cation-anion exchange resin) and the resin was washed twice with distilled water. The supernatant and the washings were combined and concentrated to a small volume in a stream of nitrogen. The enzymatic hydrolysate was examined on paper chromatography in pyridine-ethyl acetate-water (2:5:5, v/v, upper phase, descending technique). Authentic standards (glucose, glycerol and unhydrolyzed substrates) were also chromatographed simultaneously.

The β-glucosidase was also used to determine the location of glycerophosphate on the glucose moieties of GPDGD. The product of mild deacylating (in 1.6 ml distilled water) of GPDGD (2.5 μmole P) or of DGD (4.6 μmole glucose) was incubated with 0.5 ml of β-glucosidase solution (0.3 mg protein) in water and 1.1 ml of 0.1 M phosphate buffer pH 6.8. The reaction mixture was incubated at 30°C and aliquots (0.2 ml) were withdrawn at zero time, 6, 12, 24 and 30 hr for enzymatic determination of glucose liberation by glucostat reagent (see Materials and Methods II.E.5(b)).

III. Effect of Salt on Growth and Lipid Composition of S. epidermidis

A. Preparation of Growth Curve

The ingredients of Sehgal and Gibbons medium (see Materials and Methods II.A.1(a)) except sodium chloride were mixed in 100 ml volumetric flask; 0, 5, 10, 15 or 25 g of sodium chloride were then added and the mixtures were dissolved in distilled water, the pH was adjusted to 6.5 and the final volume made to 100 ml. The solutions were filtered through a millipore filter (0.45 μpore size) instead of autoclaving in order to prevent precipitation of salt which occurs during autoclaving. Each growth medium was transferred to sterilized 500 ml side arm flasks ready for innoculation.

The started innoculum was obtained by growing S. epidermidis in 100 ml of liquid medium of Sehgal and Gibbons (1960) containing 10% sodium chloride at 37°C with rotary shaker to O.D. ≈1.0. Bacteria were harvested and washed twice to remove salt from the medium and suspended in 1.0 ml of sterile distilled water; 0.15 ml of this suspension was used for innoculation of the media containing different salt concentrations. The O.D. of each culture was followed at 660 nm and growth curves were prepared as described before (see Materials and Methods II.A.2).

B. Analysis of Lipid Composition

For this purpose, S. epidermidis were grown in 1.0 litre of Sehgal and Gibbons media containing 0, 5, 10, 15 or 25% sodium chloride to maximum growth. Bacteria were harvested and lipids were extracted by the Bligh and Dyer method (see Materials and Methods II.B.1) and were made up to 10 ml in chloroform-methanol (9:1, v/v). Total phosphorus and sugar analyses of total lipids from each culture were analyzed and aliquots containing 1-2 µmole P were chromatographed on two dimensional TLC, first in chloroform-methanol-28% ammonia (65:35:5, v/v) and in the second direction in chloroform-methanol-90% acetic acid (30:4:20, v/v). Spots corresponding to GPDGD, PG and cardiolipin were scraped off after spraying the plate with dichromate-sulfuric acid solution and charring (see Materials and Methods II.E.2(c)). P-analysis was performed on the silica gel containing PG, cardiolipin or GPDGD by the method of Rouser (see Materials and Methods II.E.2(c)). For analysis of sugar content in GPDGD, DGD and MGD spots, a duplicate two-dimensional TLC was run and the plate was visualized with iodine vapor, spots corresponding to GPDGD, DGD and MGD were scraped off and sugar analyses were performed by phenol-sulfuric acid method (see Materials and Methods II.E.5(a)).

Fatty acid methyl esters were prepared from total lipids (≈4 mg) of each culture and were analyzed on GLC as described before (see Materials and Methods II.C.5(a)).

IV. Studies on Metabolism of Lipid Components of S. epidermidis

A. Incorporation of [^{32}P]orthophosphate and [1-^{14}C] glycerol in S. epidermidis

Cells of S. epidermidis were grown to early stationary phase (O.D. ≈0.8) in 50 ml of complex medium of Sehgal and Gibbons (1960) containing 10% NaCl (see Materials and Methods II.A.1(a)), referred to as the "phosphate-rich" medium. Cells were harvested by centrifugation at 8000xg, washed with 10% NaCl solution and suspended to 1.0 ml in a "low phosphate" medium, essentially the same as the "phosphate rich" medium except that the amounts of yeast extract and casamino acids were decreased by half to reduce the content of phosphate in the medium. The cell suspension (0.5 ml) was innoculated into 48.5 ml of the "low phosphate" medium in a 500 ml side-arm flask and was incubated at 37°C in a rotary shaker (120 rpm); growth was followed by measuring O.D. at 660 nm when cell growth reached early exponential phase, 1.0 ml of a solution containing 1 mCi of [^{32}P] orthophosphate (neutralized to pH 7 with NaOH) and 50 µCi of [1-^{14}C] glycerol were added to the culture and incubation was continued at 37°C with shaking. Aliquots of 5 ml were withdrawn at zero time, 10, 20, 30, 40, 60, 90, 120 and 180 min and immediately added to 18.75 ml of chloroform-methanol (1:2, v/v). After several hours 6.25 ml each of chloroform

and water was added, mixed and centrifuged. The chloroform layer was removed and the methanol-water layer was washed twice with chloroform and the chloroform solutions were combined, diluted with benzene and blown down to dryness under nitrogen. The residue was made up to 1.0 ml in chloroform and a suitable aliquot was taken for radioactive counting (ca. 5000 cpm ^{14}C or 2500 cpm ^{32}P) and for chromatography (ca. 20,000 cpm ^{14}C or 10,000 cpm ^{32}P) by two dimensional TLC in the solvent system chloroform-methanol-28% ammonia (65:35:5, v/v) and chloroform-methanol-90% acetic acid (30:4:20, v/v), or for optimal separation of glycolipids in chloroform-methanol-water (65:25:4, v/v) and acetone-glacial acetic acid-water (100:2:1, v/v). The radioactive components were located by autoradiography and scraped into counting vials for radioactive counting (see Materials and Methods II.F).

B. Chase Studies with ^{32}P- and ^{14}C-labelled Cells

S. epidermidis cells were grown at 37°C with shaking in 100 ml of "low phosphate" Sehgal and Gibbons medium (see above) containing 50 µCi of [1-^{14}C]glycerol and 1 mCi [^{32}P]orthophosphate as described above to early stationary phase (O.D. ≈0.7). The labelled cells were then centrifuged, washed with non-radioactive medium and resuspended in 450 ml of non-radioactive "phosphate rich" medium (Sehgal and Gibbons (1960) containing 10% NaCl) and incubated

at 37°C with shaking. Samples (50 ml) were taken every hour from zero time to eight hours, the O.D. was measured at 660 nm, and the cells were harvested by centrifugation at 8000xg for 5 min at 4°C. Cells were resuspended in 10% NaCl (3 ml) and the lipids were extracted by Bligh and Dyer procedure (see above). The lipids obtained were counted and chromatographed as described above.

RESULTS

I. Growth of Individual Organism and Yield of Lipid

A. Wallemia sebi

Good surface growth of W. sebi was observed after 10 days incubation in the 25% NaCl-containing medium (Sehgal and Gibbons medium, 1960) but after only 5 days in salt free medium (Harrold's medium, 1950). Growth appeared as a dark brown layer of spores covering the surface of the media and amounted to about 1 g and 2 g of fresh spores for 200 ml of the Sehgal and Gibbons and the Harrold's media, respectively. Yield of total lipid obtained was 1.5 g per 100 g of fresh spores, corresponding to an estimated lipid content of 7.5% on a dry weight basis (Table 5). Approximately the same yield of lipids was obtained for cells grown in the two different media. The total lipid was calculated to contain 58% phospholipid and 20% glycolipid (Table 5).

B. Nocardia species

Growth of isolated Nocardia was observed qualitatively in shake culture in the liquid medium of Sehgal and Gibbons containing different concentrations of NaCl (Table 6). Growth was observed in the media containing 15, 20 and 25% NaCl after one day and maximum turbidity was attained after 4 days in the 15% and 20% NaCl containing media but after 7

TABLE 5

Yield of Cells and Lipid Content[a]

Analyses	Organism		
	W.sebi[b]	Nocardia sp.[c]	S.epidermidis[d]
Yield of dry cells, gm/l culture	1.0±0.2	1.8	1.3±0.4
Total lipid, gm lipid/100 gm dry cells	7.5±1.5	4.0	3.1±0.5
Lipid P, % total lipid	2.3±0.3	1.8	3.0±0.2
Lipid sugar, % total lipid	8.0±0.2	4.7	4.7±0.3
% Phospholipid[e]	57.5	45.0	75.0
% Glycolipid[f]	20.0	11.8	11.8
% Neutral lipid[g]	22.5	43.2	13.2

a. Bligh and Dyer (1959) procedure for lipid extraction.

b. Grown in medium of Sehgal and Gibbons (1960), 25% NaCl. Results are averages ± S.D. for 3 separate batch cultures.

c. Same as "b" but containing 15% NaCl. Results are for a single batch culture.

d. Same as "b" but containing 10% NaCl. Results are averages ± S.E. for 3 separate batch cultures.

e. Calculated as follows: % Phospholipid = % P x 25; based on the assumption that the average M.W. of phospholipid is approximately 800.

f. Calculated as follows: % Glycolipid = % hexose x 2.5; based on the assumption that the average M.W. of glycolipids is approximately 900 and contains 2 moles of hexose per mole glycolipid.

g. Calculated by difference.

TABLE 6

Observation of Growth of Nocardia sp. in shake liquid medium of Sehgal and Gibbons (1960) containing various salt concentrations at 37°C

Incubation time (days)	NaCl Concentration, %					
	0	5	10	15	20	25
1	NG	NG	NG	+	+	+
2	NG	NG	NG	++	++	+
3	NG	NG	NG	+++	+++	+
4	NG	NG	NG	++++	++++	++
5	NG	NG	NG	++++	++++	+++
6	NG	NG	NG	++++	++++	+++
7	NG	NG	NG	++++	++++	++++
8	NG	NG	NG	++++	++++	++++
9	NG	NG	NG	++++	++++	++++

Abbreviations: NG = No growth observed

\+ = Slight turbidity

++ = Moderate growth

+++ = Heavy growth

++++ = Very heavy growth

days in 25% NaCl. No growth appeared in media containing 0%, 5% and 10% NaCl after 7 days.

The Nocardia sp. formed white, cartilaginous colonies on agar media in a petri dish which adhered closely to the agar. When the organism was grown on the agar medium of Sehgal and Gibbons containing 10, 12.5 and 15% NaCl, it formed white colonies within 4 days while it took 7 days to form colonies on 20 and 25% NaCl. No growth appeared on agar media containing 0% or 5% NaCl after 7 days.

It can be seen that this species of Nocardia required salt for growth and grows best in the range of 15-20% NaCl. Therefore, this organism may be considered to be a "moderately halophilic" bacterium. In all subsequent studies, the Nocardia sp. was grown in medium containing 15% NaCl. Yield of total lipid obtained from the organism was 4.0% and was calculated to contain 45% phospholipids and 12% glycolipids (Table 5).

C. Staphylococcus epidermidis

Growth of the S. epidermidis isolate in the Sehgal and Gibbons medium containing various salt concentrations (0, 5, 10, 15 and 25% NaCl) is shown in Fig. 4. The exponential growth rate (R) calculated from the above growth curves (see Materials and Methods, II.A.2) as well as the maximum growth (measured as O.D. at 660 nm) decreased linearly with increasing concentration of NaCl in the media, as shown in

FIGURE 4

Growth curves of Staphylococcus epidermidis grown in Sehgal and Gibbons (1960) medium containing various sodium chloride concentrations. Bacteria were grown in 100 ml medium in 500 ml Erlenmeyer flasks with sealed-on side-arm tube (18 x 150 mm) and incubated at 37°C in a shaking incubator (120 rpm); growth was followed by measuring optical density at 660 nm.

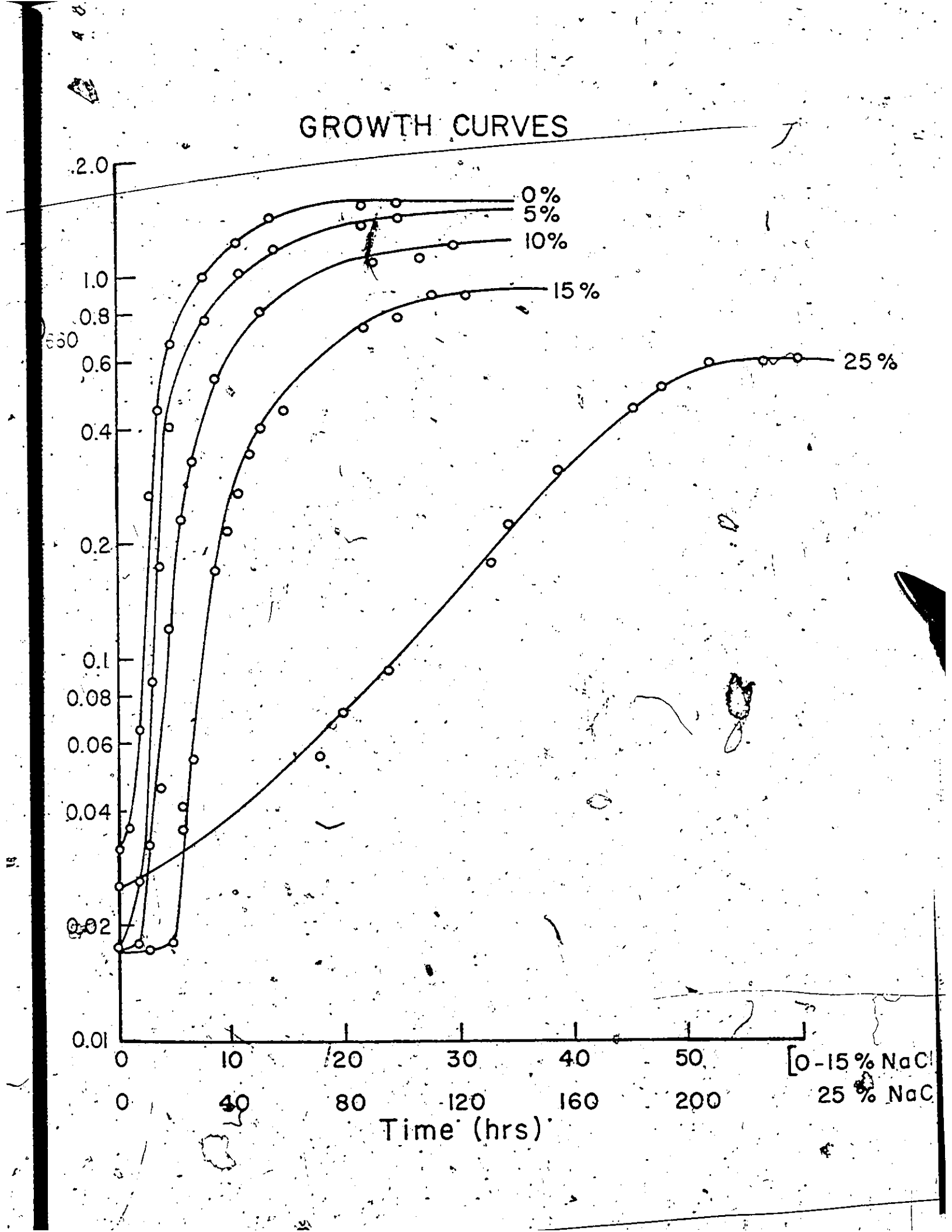

GROWTH CURVES
2.0
1.0
0.8
0.6
0.4
0.2
0.1
0.08
0.06
0.04
0.02
0.01
660
0%
5%
10%
15%
25%
0
10
20
30
40
50
0
40
80
120
160
200
Time (hrs)
[0-15% NaCl
25% NaCl

Fig. 5. The generation time (G = 1/R), calculated from the R values, were 30 min, 35 min, 1 hr, 1.3 hr and 40 hr for growth in 0, 5, 10, 15 and 25% NaCl respectively. From these results, it can be seen that S. epidermidis is a "halotolerant" bacterium because although it can grow in media containing NaCl it grows best in the absence of added NaCl. In subsequent studies S. epidermidis was routinely grown in medium containing 10% NaCl.

The yield of total lipid obtained by the Bligh and Dyer extraction (see Materials and Methods II.B.1) of cells grown in 10% NaCl containing medium was 3.1% of cell dry weight (Table 5), which was not significantly different from cells grown at lower NaCl concentration (0% and 5%) or at higher NaCl concentration (15% and 25%, Table 7). However, the phospholipid content of cells grown in 10% NaCl was slightly higher than that of cells grown in extremely low NaCl or high NaCl concentration. Furthermore, the glycolipid content appeared to increase with increasing NaCl concentration in the medium.

Extraction of lipids from cells grown in the 10% NaCl medium with hot isopropanol (see Materials and Methods II.B.2) followed by Bligh and Dyer extraction gave slightly higher yields of total lipids (4.4%).

The yield of cells grown in 10% NaCl medium (mg dry wt. per ml culture) was essentially linear with the optical density of the culture (Fig. 6). The yield of cells per ml per unit O.D. was 0.80 mg.

FIGURE 5

A. Plot of exponential growth rate (R, min^{-1}), calculated from growth curves of S. epidermidis in Fig. 4 (see calculation in Materials and Methods II.A.2) as a function of sodium chloride concentration in the growth medium.

B. Plot of maximum cell yield (obtained from O.D. values at stationary phase, Fig. 4) as a function of sodium chloride concentration in the growth medium.

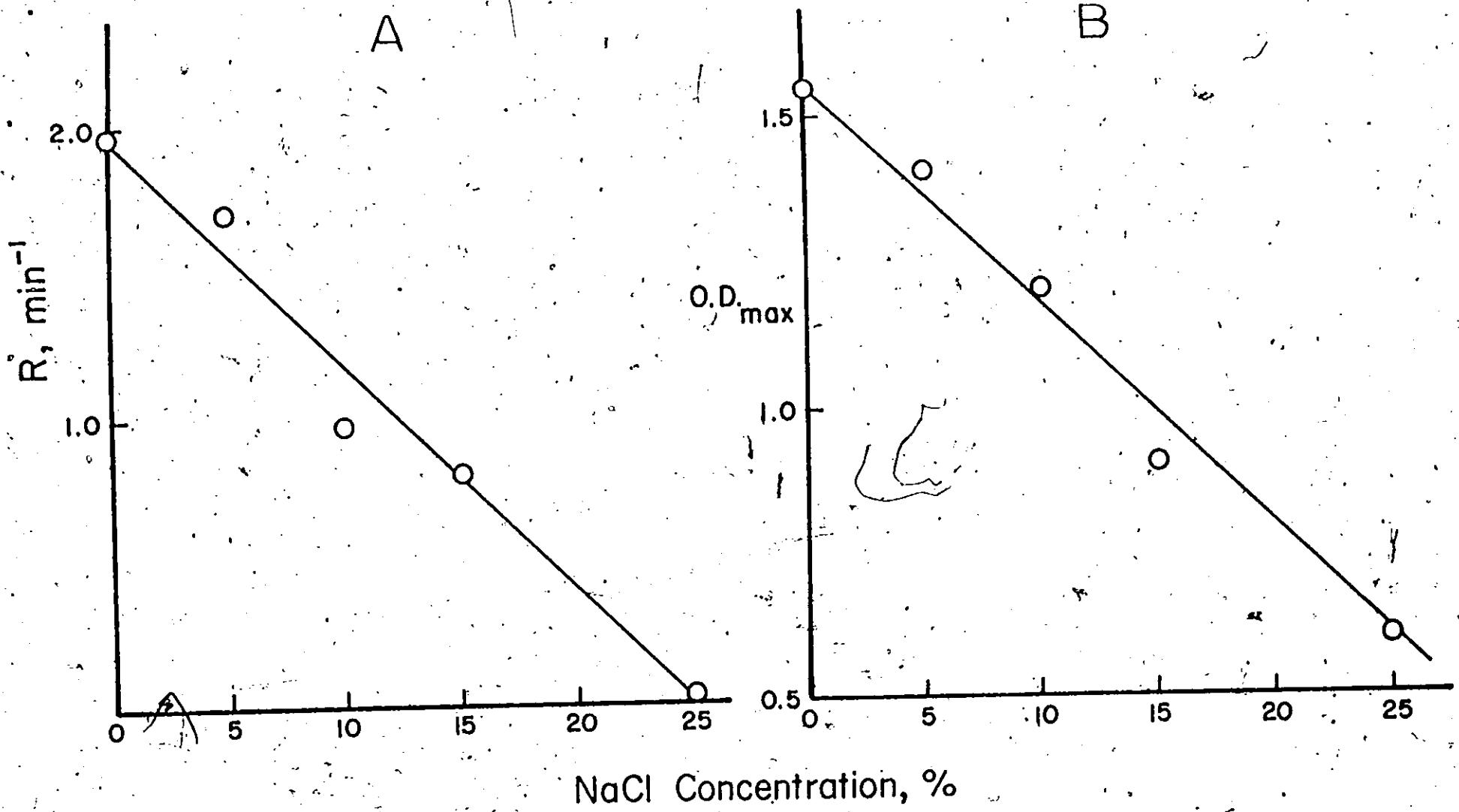

A
B
2.0
1.0
R, min⁻¹
1.5
1.0
0.5
O.D.max
0
5
10
15
20
25
NaCl Concentration, %

TABLE 7

Lipid Content of Cells of S.epidermidis Grown in Various Salt Concentrations

NaCl conc.* % w/v	Total lipid,** gm lipid per 100 gm dry cells	Lipid P, % total	% Phospho-lipid+	Lipid-hexose, % total lipid	% Glycolipid†
0	2.7	2.4	60.0	3.6	9.0
5	2.3	3.0	75.0	4.0	10.0
10	3.1	3.0	75.0	4.7	11.8
15	3.1	2.6	65.0	5.0	12.5
25	3.3	2.8	70.0	5.2	13.0

*in medium of Sehgal and Gibbons (1960)

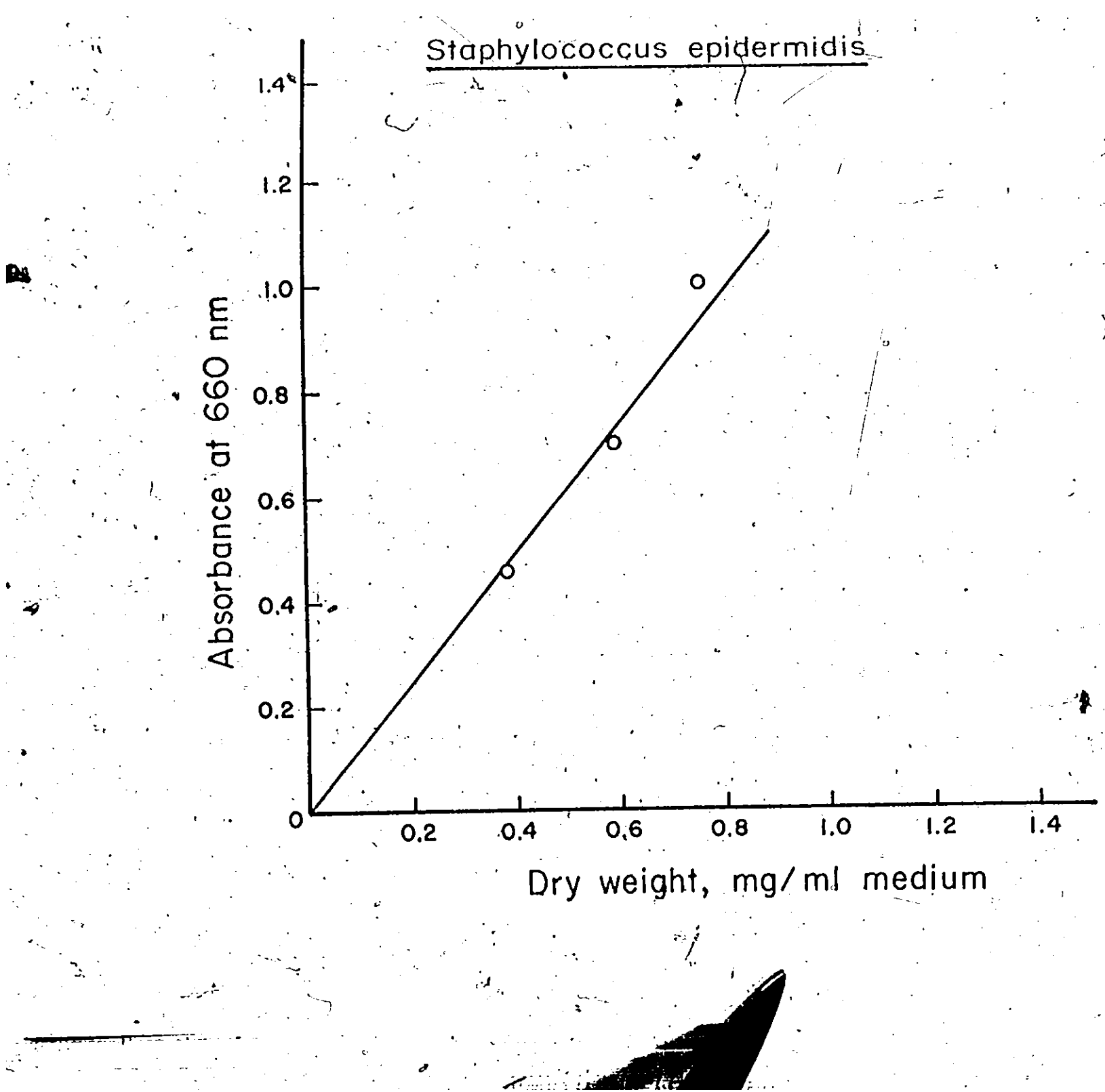
**by Bligh and Dyer (1959) procedure

+see Footnote "e" Table 5

†see Footnote "f" Table 5

FIGURE 6

The relationship between O.D. at 660 nm and dry weight (mg dry cells per ml culture medium of S. epidermidis grown in Sehgal and Gibbons medium (1960) containing 10% NaCl.


Staphylococcus epidermidis
Absorbance at 660 nm
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
Dry weight, mg/ml medium

II. Preliminary Examination of Total Lipids of Each Microorganism

A. Paper Chromatography

Total lipids were first chromatographed on silicic acid impregnated paper developed in the solvent system di-isobutylketones-glacial acetic acid-water (8:5:1, v/v) and stained with specific reagents (see Materials and Methods II.C.3). The tracings of chromatogram of total lipids obtained from a pure culture of each of the three micro-organisms under investigation and from the original-mixed culture are shown in Fig. 7, and the staining behaviour is presented in Tables 8-9. The following components were detected and/or identified.

1. Original-mixed culture

Six major components (spots 2, 3, 4, 5, 6 and 8) and two minor components (spot 1 and 7) were revealed by Rhodamine 6G staining (Table 8, Fig. 7). Spot 5, the major phospholipid detected was tentatively identified as phosphatidylglycerol (PG), on the basis of its blue fluorescence with Rhodamine 6G (Kates, 1960), its positive pink stain with periodate-Schiff reagent (Baddily et al., 1956) and its R_f value close to that of the PG isolated from E. coli (R_f 0.54). Spots 4 and 6 were identified as glycolipids by their yellow fluorescence with Rhodamine 6G, their R_f values close to those of standard digalactosyl diglyceride and monogalactosyl

FIGURE 7

Tracing of silicic acid impregnated paper chromatogram of total lipids of:

A. Original-mixed culture

B. Staphylococcus epidermidis

C. a Nocardia sp.

D. Wallemia sebi

Identity of components are given in Tables 8-9.

The chromatogram was stained with Rhodamine 6G and viewed under ultraviolet light. The fluorescent colours are indicated by the following abbreviations: B, blue; G, grey; Y, yellow, O, orange.

Abbreviations of standards: MGD, monogalactosyl diglyceride; DGD, digalactosyl diglyceride; for others, see list of abbreviations.

positive periodate-Schiff test

positive ninhydrin test

minor component

Solvent system: diisobutylketone-acetic acid-water (40:25:5, v/v)

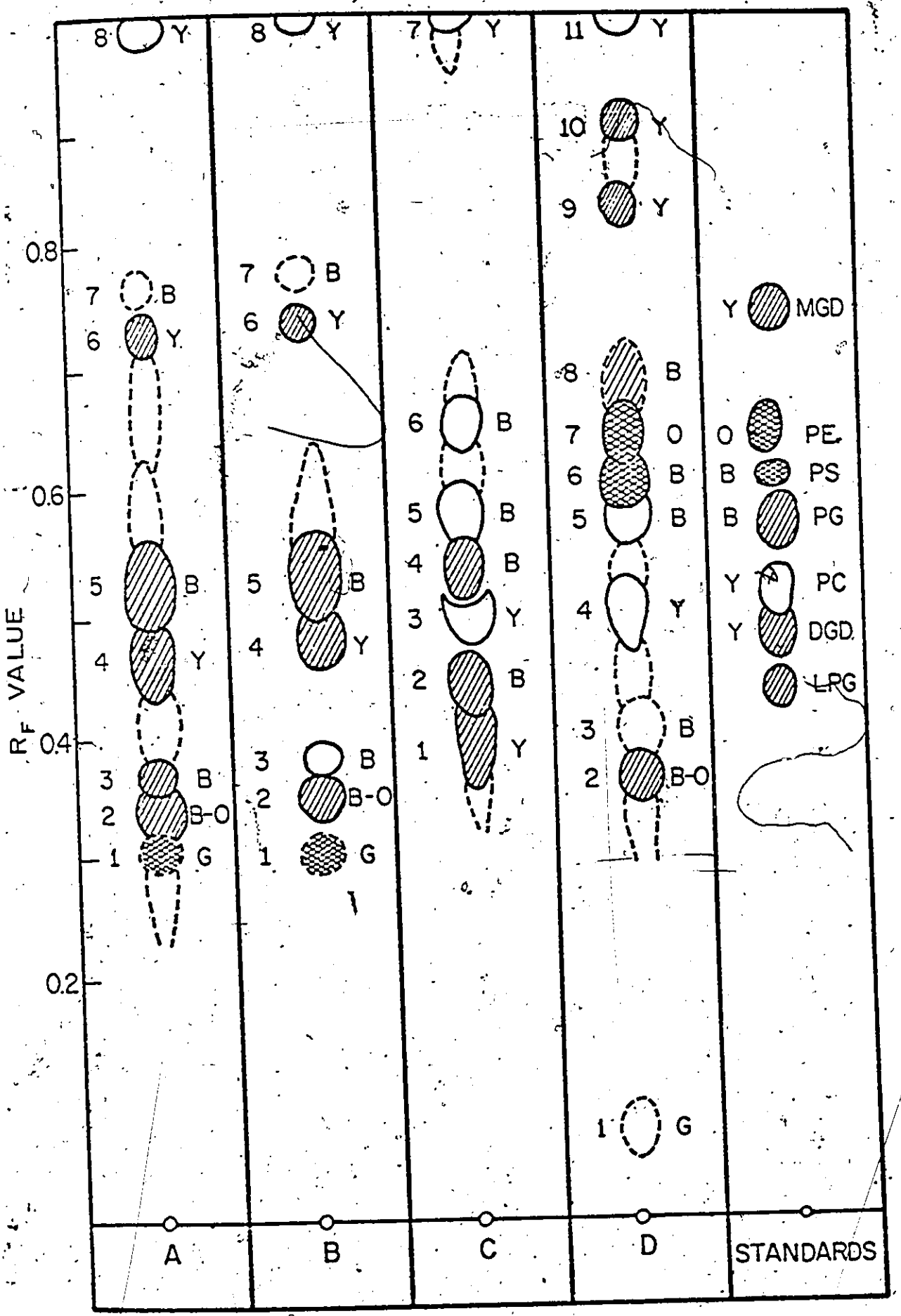

R_F VALUE
0.8
0.6
0.4
0.2
8 Y
7 B
6 Y
5 B
4 Y
3 B
2 B-O
1 G
8 Y
7 B
6 Y
5 B
4 Y
3 B
2 B-O
1 G
7 Y
6 B
5 B
4 B
3 Y
2 B
1 Y
11 Y
10 Y
9 Y
8 B
7 O
6 B
5 B
4 Y
3 B
2 B-O
1 G
Y MGD
O PE
B PS
B PG
Y PC
Y DGD
LPG
A
B
C
D
STANDARDS

TABLE 8

Paper Chromatographic Analysis of Total Lipids

Organism	Spot No.*	R_f	Rhodamine 6G stain**	Ninhydrin stain	Periodate Schiff+ stain	Tentative Identity†
"Mixed Culture"	1	0.30	G	+	–	amino phosphatide (X_1)
	2	0.34	B-O	–	+ (pink)	phosphoglycolipid
	3	0.36	B	+ (weak)	+ (pink)	amino phosphatide (X_2)
	4	0.47	Y	–	+ (purple)	glycolipid
	5	0.53	B	–	+ (pink)	PG
	6	0.73	Y	–	+ (purple)	glycolipid
	7	0.79	B	–	–	phosphatidic acid
	8	0.98	Y	–	–	neutral lipid
S. epidermidis	1	0.30	G	+ (weak)	–	X_1
	2	0.34	B-O	–	+ (pink)	GPDGD
	3	0.39	B	+ (weak)	+ (pink)	X_2
	4	0.47	Y	–	+ (purple)	glycolipid (DGD)
	5	0.54	B	–	+ (pink)	PG
	6	0.74	Y	–	+ (purple)	glycolipid (MGD)
	7	0.77	B	–	–	phosphatidic acid
	8	0.96	Y	–	–	neutral lipid

*See Fig. 7. The chromatogram was run on silicic acid impregnated paper in diisobutyl ketone-acetic acid-water (40:25:5, v/v) and stained with reagents indicated (see Materials and Methods, Section II.C.3.b).

**Y, yellow; B, blue; O, orange; G, grey.

+Purple color of glycolipid developed slowly.

†See list of abbreviations.

TABLE 9

Paper Chromatographic Analysis of Total Lipids (contd.)*

Organism	Spot No.	R_f	Rhodamine 6G stain	Ninhydrin stain	Periodate Schiff stain	Tentative Identity
Nocardia sp.	1	0.39	Y	–	+ (purple)	glycolipid
	2	0.44	B	–	+ (pink)	lyso PG
	3	0.50	Y	–	–	PC ?
	4	0.54	B	–	+ (pink)	PG
	5	0.60	B	–	–	unidentified
	6	0.67	B	–	–	unidentified
	7	0.95	Y	–	–	neutral lipid
W. sebi	1	0.07	G	+ (weak)	–	unidentified
	2	0.37	B-O	–	+ (purple)	phosphoglycolipid
	3	0.41	B	–	–	unidentified
	4	0.50	Y	–	–	PC
	5	0.58	B	–	–	unidentified
	6	0.61	B	+	–	PS
	7	0.66	Y	+	–	PE
	8	0.70	B	–	+ (purple)	glycolipid
	9	0.[illegible]	Y	–	+ (purple)	glycolipid
	10	0.91	Y	–	+ (purple)	glycolipid
	11	0.98	Y	–	–	neutral lipid

*See Fig. 7 and Footnotes of Table 8.

diglyceride (from runner bean), negative stain with ninhydrin and purple stain developing slowly with periodate-Schiff reagent. Spots 1 and 3 stained faintly with ninhydrin and were designated as amino acid containing phosphatides (X_1 and X_2 respectively). Spot 2 is a most interesting component, showing a blue-yellow or orange fluorescence with Rhodamine 6G, a positive stain (pink to purple) with periodate-Schiff reagent and a negative stain with ninhydrin reagent, indicating the presence of acidic groups, sugars and terminal-vicinal OH-groups, and the absence of amino group in the lipid. This compound was designated as a glycophospholipid. Spot 7 was a minor component, gave blue fluorescence with Rhodamine 6G indicated acidic property and was tentatively identified as phosphatidic acid according to its R_f value similar to that of the standard phosphatidic acid (PA). Spot 8 was a mixture of neutral lipids.

2. Staphylococcus epidermidis

The pattern of lipid components of S. epidermidis was similar to that of the original-mixed culture (Fig. 7, Table 8). Spots 2, 4, 5 and 6 were studied in detail concerning their structural formula and were identified as glycerophosphoryl diglucosyl diglyceride (GPDGD), diglucosyl diglyceride (DGD), phosphatidylglycerol (PG) and monoglucosyl diglyceride (MGD) respectively (see below).

3. Nocardia sp.

Seven components were detected (Fig. 7, Table 9), of which spots 1, 2, 3, 4, 5 and 6 were the major components. Spot 1, fluoresced yellow with Rhodamine 6G and gave a slow positive periodate-Schiff stain indicating that it is a glycolipid. Spot 2 was acidic (blue fluorescence) and gave a positive stain (pink) with periodate-Schiff reagent; it had an R_f value close to that of standard lysophosphatidylglycerol (lyso PG). Spot 3 was neutral (yellow fluoresence) and had R_f value the same as standard phosphatidylcholine (PC).

Unlike S. epidermidis, the Nocardia sp. contains relatively the same amount of phosphatidylglycerol (PG) (spot 4, Fig. 7) as the other phospholipids. Spots 5 and 6 were not identified and spot 7 was neutral lipid which was not further investigated. No ninhydrin positive lipids were detected in the Nocardia sp.

4. Wallemia sebi

W. sebi contained higher amounts of glycolipid (20%) than that of S. epidermidis and Nocardia sp. (Table 5). Thus, the four periodate-Schiff positive (purple) spots (2, 8, 9 and 10, Fig. 7) detected on chromatogram were probably glycolipids. In contrast with the other two organisms, no phosphatidylglycerol (PG) was detected in W. sebi; phosphatidylcholine (PC), phosphatidylserine (PS) and phosphatidylethanolamine (PE), (spots 4, 6 and 7 respectively) were

tentatively identified by their staining behaviour and R_f values (Table 9).

Comparison of the lipid pattern of the original mixed culture with that of the isolated pure cultures of the three microorganisms (Fig. 7, Tables 8-9) showed that the lipid pattern of S. epidermidis was predominant in the pattern of the original culture, indicating that S. epidermidis must be the major organism present in the original culture and the Nocardia and W. sebi organisms were only minor contaminants. For this reason the lipid components of S. epidermidis were chosen for further detailed structural study.

B. Thin-Layer Chromatography (TLC)

Preliminary identification of the individual lipids of the three microorganisms was made on the basis of relative mobilities on TLC in a variety of solvent systems (see Materials and Methods II.C.2.c) and by reaction with a number of specific reagents, to supplement results obtained by paper chromatography (Table 8-9). The characteristics of the major lipids observed for the three pure cultures and the original-mixed culture are summarized in Tables 10-11 and a composite chromatogram is shown in Fig. 8.

1. Original-mixed culture

The same six major components observed on paper chromatogram (Fig. 7) were also observed on TLC plates charred

FIGURE 8

Tracing of thin-layer chromatogram of total lipids of:

A. Original-mixed culture

B. S. epidermidis

C. a Nocardia sp.

D. W. sebi

Identity of components are given in Tables 10-11.

Abbreviations of standard, see Fig. 7.

Solvent system: chloroform-methanol-28% ammonia (65:35:5, v/v)

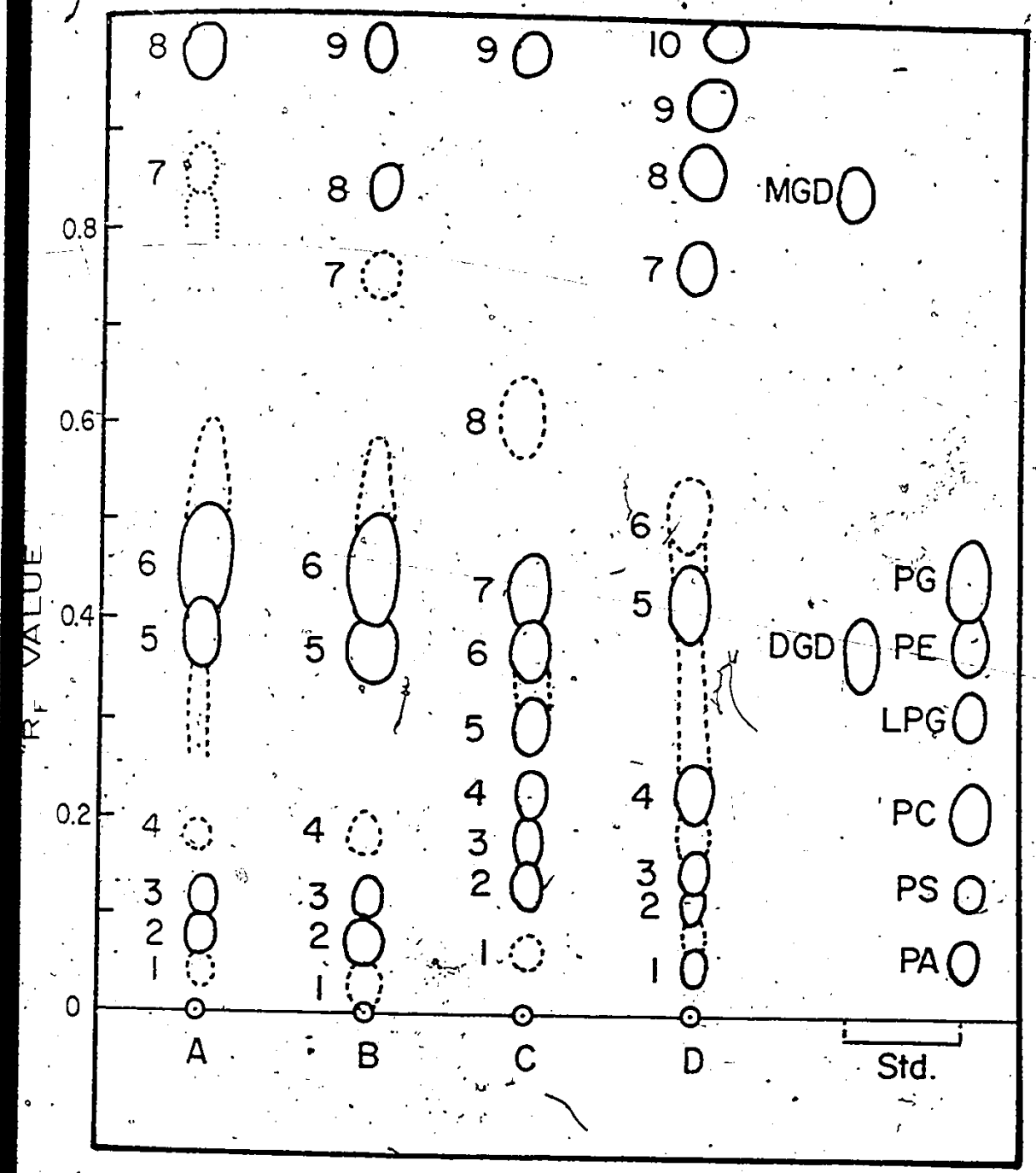

R_F VALUE
0
0.2
0.4
0.6
0.8
A
B
C
D
Std.
MGD
DGD
PG
PE
LPG
PC
PS
PA

TABLE 10

Chromatographic Analysis of Total Lipids on TLC

Organism	Spot No*	R_f	Ninhydrin stain	Periodate Schiff stain	PO_4 stain	Sugar stain	Tentative Identity+
"Mixed Culture"	1	0.04	–	–	+	–	PA
	2	0.07	–	+ (pink)	+	+	phosphoglycolipid
	3	0.11	+ (weak)	+ (pink)	+	–	amino phosphatide (X_1)
	4	0.15	+ (weak)	–	+ (weak)	–	amino phosphatide (X_2)
	5	0.37	–	+ (blue)	–	+	glycolipid
	6	0.45	–	+ (pink)	+	–	PG + Cl
	7	0.84	–	+ (blue)	–	+	glycolipid
	8	0.96	–	–	–	–	neutral lipid
S.epidermidis	1	0.04	–	–	+	–	PA
	2	0.07	–	+ (pink)	+	+	GPDGD
	3	0.11	+ (weak)	+ (pink)	+	–	X_1
	4	0.16	+ (weak)	–	+ (weak)	–	X_2
	5	0.36	–	+ (blue)	–	+	glycolipid (DGD)
	6	0.44	–	+ (pink)	+	–	PG + Cl
	7	0.73	–	–	+ (weak)	–	unidentified
	8	0.82	–	+ (blue)	–	+	glycolipid (MGD)
	9	0.95	–	–	–	–	neutral lipid

*See Fig. 8. The chromatogram was run on a silica gel H plate in chloroform-methanol-28% ammonia (65:35:5, v/v) and stained with the reagents indicated (see Material and Methods, Section II.C.2.a.).

+See list of abbreviations.

TABLE 11

Chromatographic Analysis of Total Lipids on TLC* (cont.)

Organism	Spot	R_f	Ninhydrin stain	Periodate Schiff stain	PO_4 stain	Sugar stain	Tentative Identity
Nocardia sp.	1	0.05	-	-	+(weak)	-	unidentified
	2	0.13	-	-	+	-	unidentified
	3	0.17	-	+(blue)	-	+	glycolipid
	4	0.21	-	-	+	-	PC
	5	0.28	-	+(pink)	+	-	lyso PG
	6	0.36	-	+(blue)	-	+	glycolipid
	7	0.45	-	+(pink)	+	-	PG
	8	0.60	-	-	+	-	unidentified
	9	0.96	-	-	-	-	neutral lipid
W.sebi	1	0.05	-	+(pink)	+	+	phosphoglycolipid
	2	0.10	+	-	+	-	PS
	3	0.13	-	-	+	-	unidentified
	4	0.25	-	-	+	-	PC
	5	0.40	+	-	+	-	PE
	6	0.48	-	-	-	-	unidentified
	7	0.75	-	+(purple)	-	+	glycolipid
	8	0.85	-	+(purple)	-	+	glycolipid
	9	0.93	-	+(purple)	-	+	glycolipid
	10	0.98	-	-	-	-	neutral lipid

*See Fig. 8 and Footnote of Table 10.

with H_2SO_4. Spot 6 (Fig. 8) was the main phospholipid and had R_f values in chloroform-methanol-28% ammonia (65:35:5, v/v) and staining properties similar to those of the standard PG (Table 10). However, when the total lipids were subjected to TLC in an acidic or neutral solvent system (see Materials and Methods II.C.2.c), spot 6 was resolved into two phospholipid components, one having the properties of PG and the other the characteristics of cardiolipin. Spot 2 gave a positive stain for lipid P and sugar and also showed the presence of free terminal vicinal-OH groups (Table 10); it is thus probably identical with the glycophospholipid seen on paper chromatograms (spot 2, Fig. 7). Two glycolipids (spots 5 and 7, Fig. 8) were detected, one of which (spot 5) was the major glycolipid with R_f value similar to that of the standard digalactosyl diglyceride and the other (spot 7) was a minor glycolipid having similar R_f value as the standard monogalactosyl diglyceride. Spot 1, a minor phospholipid component was probably the same as spot 7 seen on paper chromatogram (Fig. 7, Table 8) and was identified as phosphatidic acid (PA) according to its staining properties and R_f values close to the standard PA in several solvent systems. Spots 3 and 4 were weakly stained with ninhydrin the same as observed in spots 1 and 2 on paper chromatogram (Fig. 7, Table 8) and were also designated as amino phosphatides (X_1 and X_2, respectively).

The neutral lipid components (spot 8) when analyzed on TLC in solvent system petroleum ether-ethyl ether-acetic acid (80:20:1, v/v) were found to contain 1,2 and 1,3-diglyceride, free fatty acid and small amounts of fast-moving components (probably hydrocarbons); no cholesterol was detected.

2. Staphylococcus epidermidis

The pattern of phospholipids and glycolipids in S. epidermidis was again found to be the same as in the original mixed culture when analyzed on TLC (Figs. 8 and 9, Table 10). Spots 2, 5, 6 and 8 on TLC plate (Figs. 8 and 9, Table 10) corresponded to spots 2, 4, 5 and 6 on the paper chromatogram (Fig. 7, Table 8) and were identified by detailed structural studies (see below) as glycerophosphoryl diglucosyl diglyceride (GPDGD), diglucosyl diglyceride (DGD), phosphatidylglycerol (PG) and monoglucosyl diglyceride (MGD), respectively.

Two-dimensional TLC of the total lipid (Fig. 10) showed the presence of cardiolipin and phosphatidic acid which could not be seen clearly on one-dimensional TLC (Fig. 9). These two phospholipids were identified by their chromatographic mobilities in basic, acidic and neutral solvent systems (see Materials and Methods II.C.2.c) and relative to authentic standards and also their staining behaviour (positive stain with phosphate-reagent, negative

FIGURE 9

Thin-layer chromatogram of total lipids of S. epidermidis grown in Sehgal and Gibbons (1960) medium containing 10% sodium chloride.

Identity of components (see Table 10 for staining behaviour):

1. phosphatidic acid
2. glycerophosphoryldiglucosyl diglyceride
3. aminophosphatide (X_1)
4. aminophosphatide (X_2)
5. diglucosyl diglyceride
6. mixture of phosphatidylglycerol and cardiolipin
7. unidentified lipid (X)
8. monoglucosyl diglyceride
9. neutral lipids

Solvent system: chloroform-methanol-28% ammonia (65:35:5, v/v)

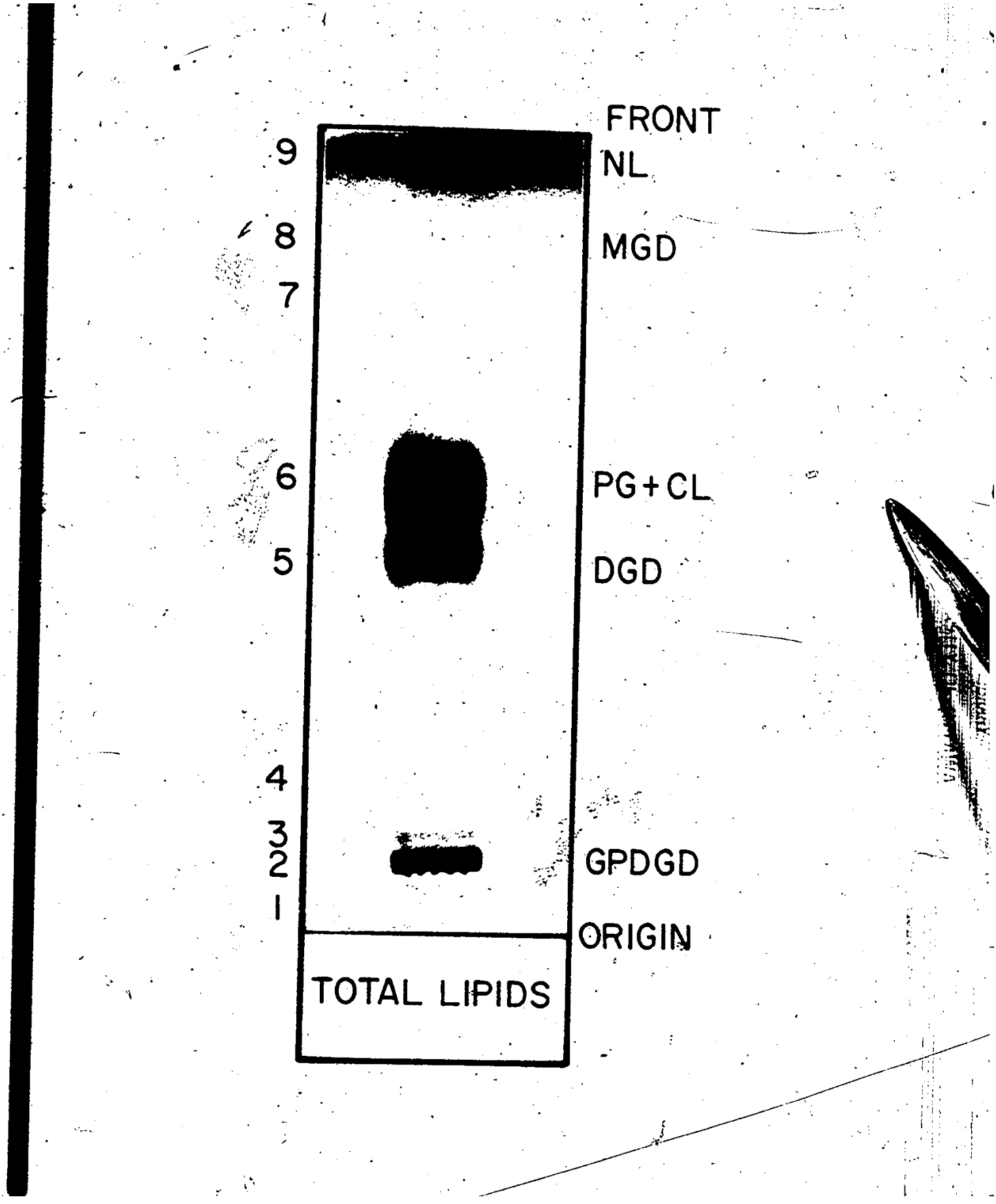
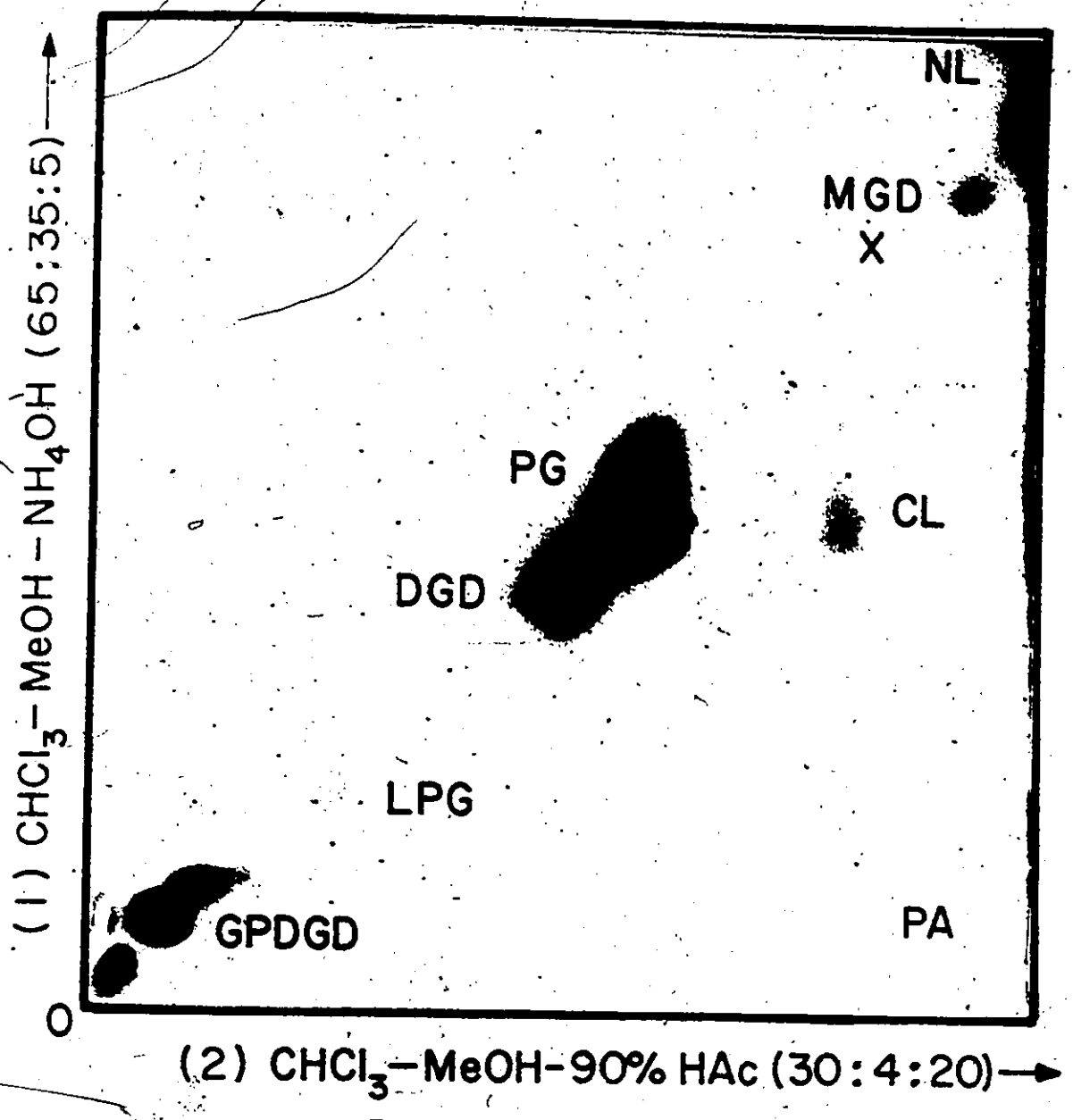

FRONT
9
NL
8
MGD
7
6
PG+CL
5
DGD
4
3
2
GPDGD
1
ORIGIN
TOTAL LIPIDS

FIGURE 10

Two dimensional thin-layer chromatogram of total lipids of S. epidermidis grown in the same medium as in Fig. 9. Identity of components as in Fig. 9 (see List of Abbreviations).

Solvent systems: First direction; chloroform-methanol-28% ammonia (65:35:5, v/v); second direction; chloroform-methanol-90% acetic acid (30:4:20, v/v).


NL
MGD
X
PG
CL
DGD
LPG
GPDGD
PA
0
(1) $CHCl_3$–MeOH–NH_4OH (65:35:5)
(2) $CHCl_3$–MeOH–90% HAc (30:4:20)

with ninhydrin and sugar reagents). No degradation studies were carried out because of insufficient amounts of these compounds.

3. Nocardia sp.

Five phospholipids (spots 2, 4, 5, 7 and 8) and two glycolipids (spots 3 and 6, Fig. 8) were observed on TLC of total lipids of isolated Nocardia. Of these, spots 4, 5 and 7 had staining behaviour (Table 11) and R_f values corresponding to authentic standards of PC, lysq PG and PG in several solvent systems (see Materials and Methods II.C.2.c); these TLC spots also corresponded to spots 2, 3 and 4 on paper chromatograms (Fig. 7) respectively. PG was not the major phospholipid as it was in the original-mixed culture and in the S. epidermidis but was present in the same relative amount as the other phospholipids. Two glycolipids (spots 3 and 6) were revealed on TLC with α-napthol-sulfuric acid reagent, although only one glycolipid could be detected on paper chromatograms (spot 1, Fig. 7). The major, faster moving glycolipid (spot 6, Fig. 8) had the same R_f as digalactosyl diglyceride while the minor, slower one (spot 3) has half the R_f values in neutral, basic and acidic solvent systems (see Materials and Methods II.C.2.c) and contained no terminal vicinal-OH; spot 3 is thus likely to be triglycosyl diglyceride.

No further structural studies of the lipid components

of this organism were done except for total fatty acid analysis (see Table 12 and Fig. 11A) for comparison with that of the original-mixed culture.

In summary, the major lipid components of the Nocardia sp. were tentatively identified as PC, lyso PG, PG, diglycosyl diglyceride and triglycosyl diglyceride.

4. Wallemia sebi

Five phospholipids (spots 1-5) and three glycolipids (spots 7-9) were observed in W. sebi (Fig. 8, Table 11) on TLC, the same as on paper chromatograms (Fig. 7, Table 9). Glycophospholipid (spot 1 on TLC, Fig. 8 corresponded to spot 2 on paper chromatogram, Fig. 7) was also detected in this organism. PG was not detected on TLC nor on paper chromatograms. The remaing phospholipids, spots 2, 4 and 5 (Fig. 8), corresponding to spots 6, 4 and 7 (Fig. 7) respectively were identified as PS, PC and PE respectively according to their chromatographic mobilities relative to authentic standards and their staining properties (Table [illegible]). Spot 3 and 6 (Fig. 8) were unidentified lipids. Three glycolipids (spots 7-9, Fig. 8) moved faster than the standard digalactosyl diglyceride but close to monogalactosyl diglyceride as also observed on paper chromatograms (Fig. 7). Therefore, these three glycolipids might represent different monoglycosyl containing lipids or acylated mono- or diglycosyl diglyceride derivatives.

Further identification of the lipid components of this organism was not carried out except for the total fatty acid analysis (see Table 12, Fig. 11C).

In summary, the lipid components of W. sebi were tentatively identified as: PC, PS, PE, glycophospholipid and three fast moving glycolipids.

III. Fatty Acid Composition

Identification of fatty acids in the three organisms studied was made on the basis of GLC relative retention data on the two liquid phases, 10% BDS and 3% SE-30 as given in Table 12. The SE-30 liquid phase is not efficient in separation of iso- from anteiso-fatty acids and monoenoic from dienoic acids, while the 10% BDS liquid phase showed the separation of peaks between the latter two fatty acids and gave a shoulder of iso-fatty acid on the anteiso peak (Fig. 11). However, when one of these branched fatty acids was present in much higher amounts than the other, the minor component was not detectable (see Fig. 26).

The total lipids of the three organisms studied contained different fatty acids (Table 12, Fig. 11) as follows:

S. epidermidis contained a 15:0 (64%), i 17:0 (7%), a 17:0 (18%) and 18:0 (4%), (Fig. 11B, Table 12) as the main fatty acid components. As mentioned before, the a 15:0 fatty acid peak probably contains the minor component i 15:0 fatty acid, since in the case of PG, the main lipid component of S. epidermidis, i 15:0 fatty acid peak was detected when the major a 15:0 fatty acid was removed by phospholipase A treatment (see Fig. 26). Other fatty acids present in small amounts were: a 13:0, i 14:0, i 16:0, i 18:0, 14:0 and 16:0. Neither unsaturated nor cyclopropane fatty acid were detected (Table 12, Fig. 11B).

Nocardia sp. also contained largely branched chain acids but the components were: i 15:0 (21%), i 16:0 (31%), i 17:0 (19%) and a 17:0 (22%) (Fig. 11A, Table 12). Again, the i 15:0 fatty acid probably contained a small amount of a 15:0 as can be seen by the slight shoulder on the i 15:0 peak (Fig. 11A). A small amount of 18:1 (0.8%) was also detected but there was no cyclopropane fatty acid present in this Nocardia sp.

In contrast with the other organisms, W. sebi contained high amounts of unsaturated fatty acids, linoleic acid (60%), oleic acid (13%) and palmitic acid (23%) (Fig. 11C, Table 12), a pattern characteristic of yeast and fungi.

Comparison of the total fatty acid composition of the isolated pure cultures with that of the original-mixed culture (Fig. 12, Table 12) showed that the latter had a pattern similar to that of S. epidermidis which confirmed the presence of S. epidermidis as a predominant organism in the original-mixed culture.

TABLE 12

Fatty acid composition of lipids from the original-mixed culture, *S. epidermidis*, *Nocardia* sp. and *W. sebi*.

Expressed as percentages of total area under the fatty acid peaks*

Fatty acid**	Retention relative to methyl myristate		Organisms			
	BDS	SE-30	Mixed Culture	*S.epidermidis*	*Nocardia* sp.	*W.sebi*
12:0	0.35	0.46	0.1	-	-	-
a 13:0	0.60	0.59	4.3	0.1	-	-
i 14:0	0.86	0.87	0.5	0.3	1.3	-
14:0	1,00	1.00	1.6	0.3	0.4	0.1
i 15:0	1.20	1.24			21.2+	-
a 15:0	1.30	1.28	72.0+	63.7+	-	-
15:0	1.40	1.47	-	-	-	0.5
i 16:0	1.71	1.85	1.1	1.0	31.2	-
16:0	1.95	2.16	2.0	2.1	2.6	23.2
i 17:0	2.33	2.93	0.7	7.4	19.0	0.5
a 17:0	2.50		12.8	18.2	21.6	-
17:0	2.73	3.18	-	-	1.3	1.5
i 18:0	3.37	3.95	-	2.6	-	0.4
18:0	3.86	4.70	0.9	4.3	0.6	1.4
18:1	4.29	4.10	-	-	0.8	13.4
18.2	5.25		0.9	-	-	59.5
20:0	7.64	10.2	2.8	-	-	-

* GLC on 10% butanediol succinate polyester (BDS) at 175°C and on 3% SE-30 at 180°C with N_2 as carrier gas (0.75 Kg/cm^2).

** Analyzed as methyl ester. Abbreviations: the first number indicates chain length, the second, number of double bonds; a, anteiso, i, iso. A dash (-) indicates that no peak was detected on the chromatogram.

+ Mixture of iso 15:0 and a 15:0 fatty acids.

FIGURE 11

GLC chromatograms of total fatty acids (analyzed as methyl esters) from:

A. Nocardia sp.

B. Staphylococcus epidermidis

C. Wallemia sebi

Column conditions:

Liquid phase: 10% butanediol succinate polyester on Gas-Chrom W

Temperature: 175°C

Carrier gas: Nitrogen, pressure 0.7 kg/cm^2

Abbreviations: the first number indicates chain length, the second number, number of double bond; a, anteiso, i, iso.

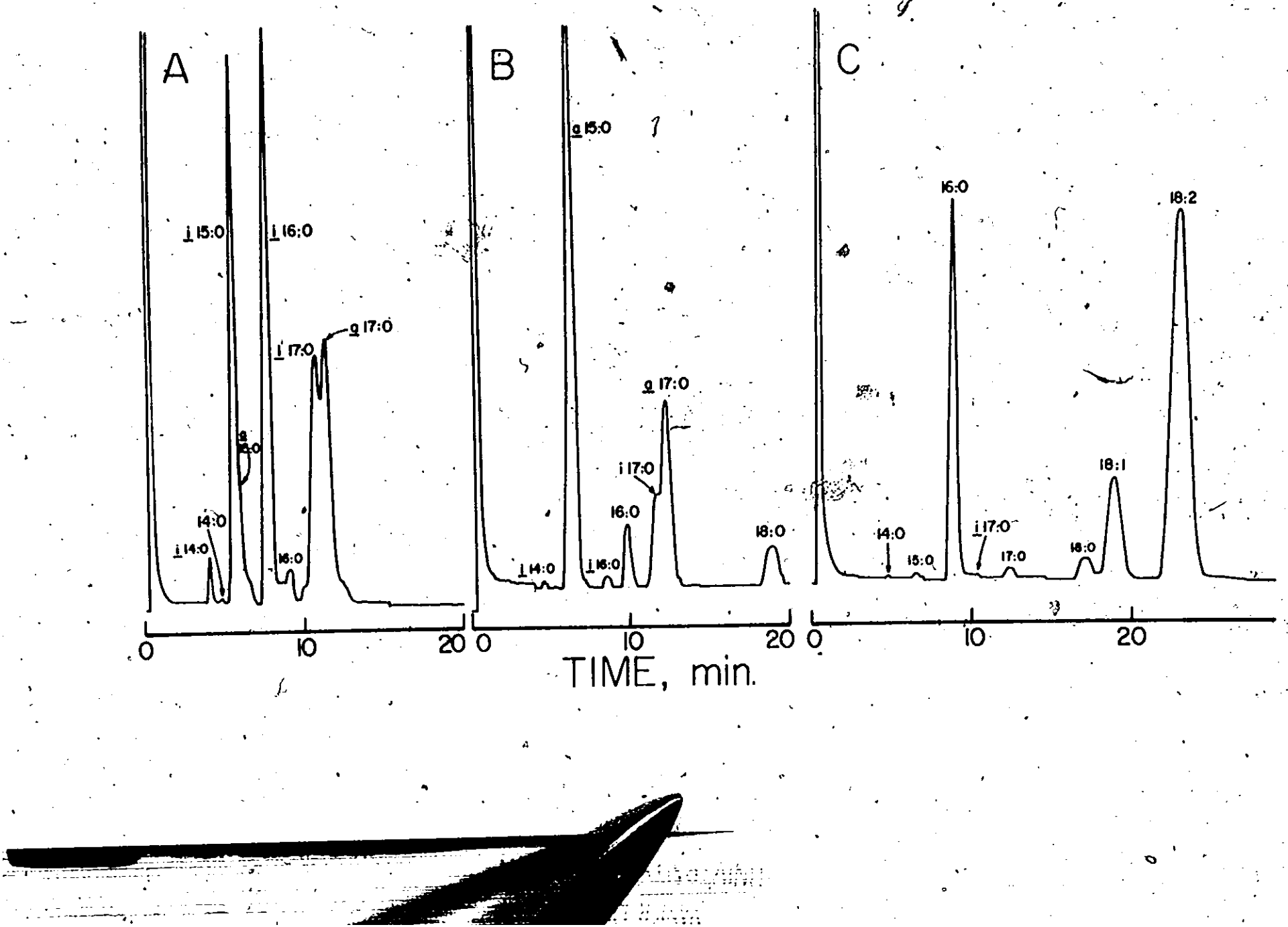
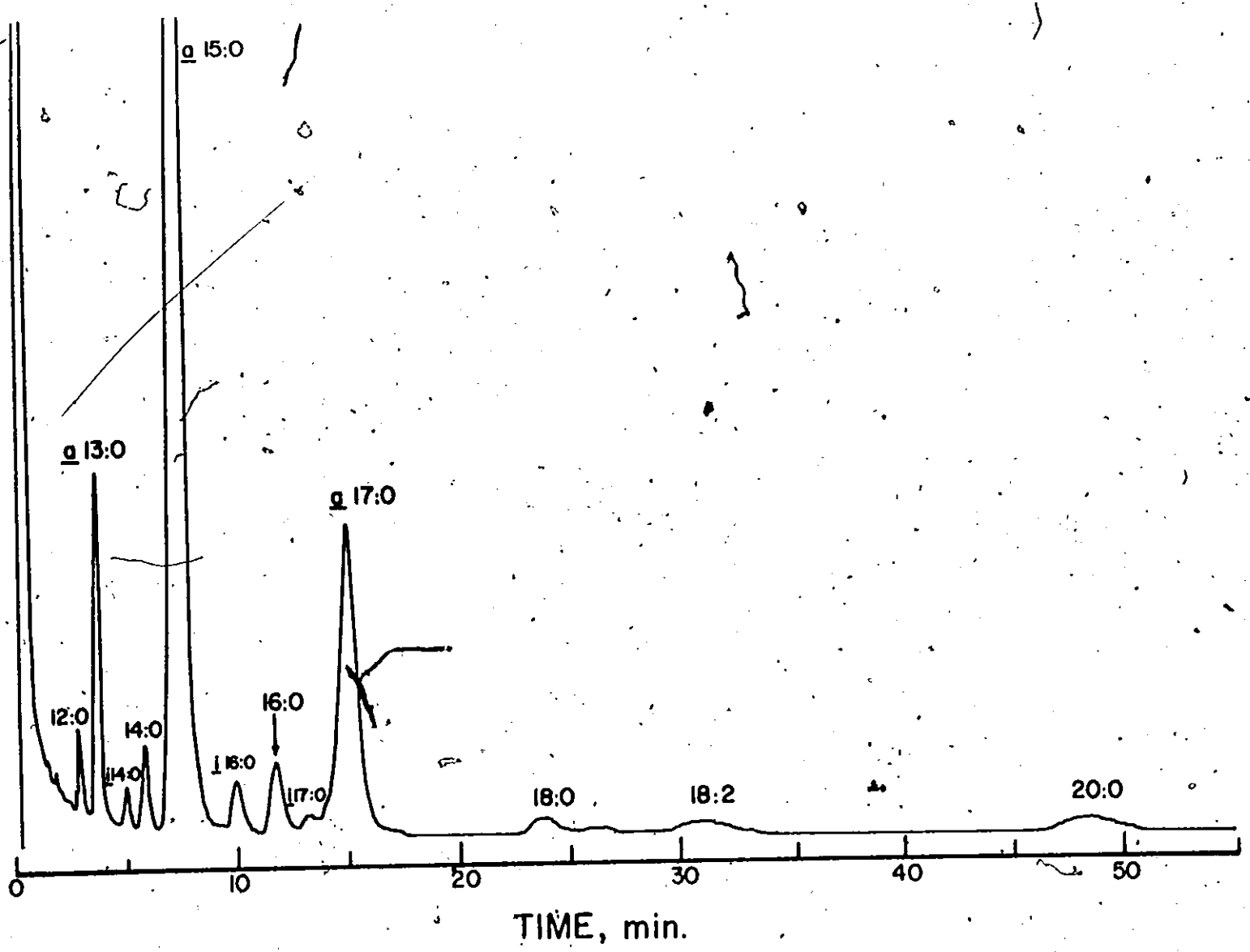

A
i 15:0
i 16:0
a 15:0
i 17:0
a 17:0
14:0
i 14:0
16:0
B
a 15:0
i 17:0
a 17:0
16:0
i 14:0
i 16:0
18:0
C
16:0
18:2
18:1
14:0
15:0
i 17:0
17:0
18:0
0
10
20
TIME, min.

FIGURE 12

GLC chromatogram of total fatty acids (analyzed as their methyl esters) of original-mixed culture.

Column conditions and abbreviations are given in Fig. 11.


a 15:0
a 13:0
a 17:0
12:0
14:0
14:0
16:0
16:0
17:0
18:0
18:2
20:0
0
10
20
30
40
50
TIME, min.

IV. Isolation of Individual Lipid Components of S. epidermidis

Chromatography of the total lipids on silicic acid column yielded the following main fractions (Table 13): fraction 1 (chloroform eluate), containing neutral lipids; fraction 2 (chloroform-acetone, 1:1 eluate), containing a small amount of monoglucosyl diglyceride (MGD) and traces of neutral lipids and phospholipid (PG); fraction 3 (acetone eluate), containing mostly glycolipid (DGD); fraction 4 (chloroform-methanol, 1:1 eluate), the main fraction (65% by wt.), containing the major phospholipid (PG), cardiolipin, glucophospholipid (GPDGD), X_2 and traces of X_1 and an unidentified phospholipid (spot 7, Fig. 9); and fraction 5 (methanol eluate), containing X_1, PA and traces of GPDGD and PG.

Fractions 2, 3 and 4 were subjected to repeated preparative TLC for final purification of MGD, DGD and GPDGD and PG respectively (Table 14). Preparative TLC of fractions 2 and 3 in chloroform-methanol-water (65:25:4) gave MGD and DGD in yields of 75% and 90% from each fraction respectively, accounting for 3.3 and 15.5% respectively, of the total lipids. TLC of Fr. 4 in the same solvent as above separated PG, cardiolipin and GPDGD. PG was repurified by TLC in solvent chloroform-methanol-90% acetic acid (30:4:20) and was recovered in 79% yield from fraction 4; it accounted for 60% of total lipids by weight and 77.5% of total lipid P. GPDGD was repurified by TLC in chloroform-methanol-water (65:35:8)

TABLE 13

Chromatography of Total Lipids from *S. epidermidis* on Silicic Acid Column*

Fraction	Solvent	Eluate (ml)	Recovery, % of total			Components†
			wt.	P	sugar	
1	$CHCl_3$	300	6.2	0.0	0.0	Neutral lipids
2	$CHCl_3$:Acetone (1:1)	400	2.4	1.1	2.6	MGD, PG (tr)
3	Acetone	400	16.0	0.0	41.6	DGD
4	$CHCl_3$:MeOH (1:1)	500	64.8	94.8	46.3	PG, GPDGD, CL, X_1, X_2
5	MeOH	600	6.6	5.8	3.8	PG (tr), X_1, PA

*Total lipid applied to column of Unisil silicic acid (2.8 x 38 cm; 50 g): 704 mg containing P, 2.6% and sugar 7.4%.

†Abbreviations: MGD, monoglucosyl diglyceride; DGD, diglucosyl diglyceride; CL, cardiolipin; X, unidentified phospholipid; GPDGD, glycerol phosphoryl diglucosyl diglyceride; PG, phosphatidyl glycerol.

TABLE 14

Preparative TLC Fractionation of Polar Lipids of S. epidermidis obtained from column Chromatography

Column fraction[a]	Component	Recovery[b]	
		% by wt.[c]	% P[d]
2	MGD	75 (3.3)	
3	DGD	90 (15.5)	
	GPDGD	13 (9.5)	12.7 (9.7)
4	CL	5.4 (3.5)	2.9 (2.7)
	PG	79 (60)	82 (77.5)

a. See Table 13.

b. Material was recovered by elution of the corresponding bands of silica with $CHCl_3$:MeOH:diethyl ether (1:1:1, v/v)(see Materials and Methods, II.C.2.b.). For details of solvent systems, see text. Recoveries are expressed as percent of lipid weight and lipid P of each fraction applied to the plate.

c. Values in parentheses are percentages of total lipid.

d. Values in parentheses are percentages of total lipid P.

and was recovered from Fr. 4 in 13% yield, it accounted for 9.5% of total lipids by weight and 9.7% of total lipid P (Table 14).

All lipid components before being subjected to further structural studies gave a single spot on TLC in neutral, acidic or basic solvent systems (Fig. 13). The R_f values of these lipids in various solvent systems are given in Table 15.

FIGURE 13

Thin-layer chromatograms of pure lipid components of S. epidermidis:

1. Glycerophosphoryl diglucosyl diglyceride
2. Diglucosyl diglyceride
3. Phosphatidylglycerol
4. Monoglucosyldiglyceride

Solvent systems:

A. chloroform-methanol-28% ammonia (65:35:5, v/v)

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

C. chloroform-methanol-water (65:35:8, v/v)


A
B
C
FRONT
1 2 3 4
1 2 3 4
1 2 3 4
ORIGIN

TABLE 15

Chromatographic Mobility of Individual Isolated Polar Lipids of S. epidermidis on TLC

Lipid Component	R_f Value in Solvent System*				
	1	2	3	4	5
Cardiolipin	0.50	0.69	0.85	0.45	0.70
Diglucosyl diglyceride (DGD)	0.49	0.50	0.46	0.60	0.58
Monoglucosyl diglyceride (MGD)	0.80	0.60	0.75	0.80	0.75
Phosphatidyl glycerol (PG)	0.50	0.46	0.42	0.52	0.54
Phosphoglycolipid (GPDGD)	0.08	0.10	0.06	0.04	0.28
Standards					
Cardiolipin (beef heart)	0.40	0.60	0.79	0.40	0.64
Digalactosyl diglyceride (runner bean)	0.40	0.52	0.50	0.65	0.62
Monogalactosyl diglyceride (runner bean)	0.75	0.68	0.79	0.85	0.71
Phosphatidyl glycerol (synthetic dioleoyl)	0.42	0.50	0.40	0.58	0.63

*Solvent systems, (v/v): 1, chloroform:methanol:28% ammonia (65:35:5)(Davidson and Stanacev, 1971); 2, chloroform:methanol:water (65:25:4)(Lepage, 1964); 3, chloroform:methanol:90% acetic acid (30:4:20)(Hancock and Kates, 1973); 4, chloroform:acetone:methanol:acetic acid:water (6:8:2:2:1)(Rouser et al., 1970); 5, chloroform:methanol:water (65:35:6)(Fischer, 1970).

V. Structural Studies of Glucophospholipid (GPDGD)

A. Characterization of GPDGD

The TLC pure component had staining properties (spot 2, Table 10) and R_f values (Table 15) given above. It gave analytical data for phosphorus, hexose, glycerol and fatty acid esters corresponding to those expected for the molecular composition of a glucophospholipid: Found, molar ratios for P: hexose:glycerol:acyl esters, 1:1.94:2.03:1.99; Calcd., 1:2:2:2 (Table 16). The compound had a specific rotation, $[\alpha]_D^{25} = -16.3^\circ$ and molecular rotation, $[M]_D = -170.7$ when measured in chloroform.

The infrared spectrum of GPDGD (Fig. 14) showed broad OH absorption at 3250 cm^{-1}, strong CH_2 and CH_3 absorption at 2940, 2850 cm^{-1} and 1465 cm^{-1}; isopropyl absorption at 1380 cm^{-1}. An ester C=O band is seen at 1740 cm^{-1}, a C-O-C stretch at 1165 cm^{-1}, a phosphate P=O at 1300 cm^{-1}, along with $P-O^-$ absorption between 1090-1100 and P-O-C at 1025 cm^{-1}. A C-OH (primary) absorption was overlapped with the P-O-C peak but the C-OH (secondary) absorption may be discerned at ca. 1110 cm^{-1} as a shoulder on the broad $P-O^-$ band.

B. Hydrolytic Degradation of GPDGD

The reactions used in the structural determination of GPDGD, consisting of both non-enzymatic and enzymatic hydrolyses are summarized in Scheme 3.

TABLE 16

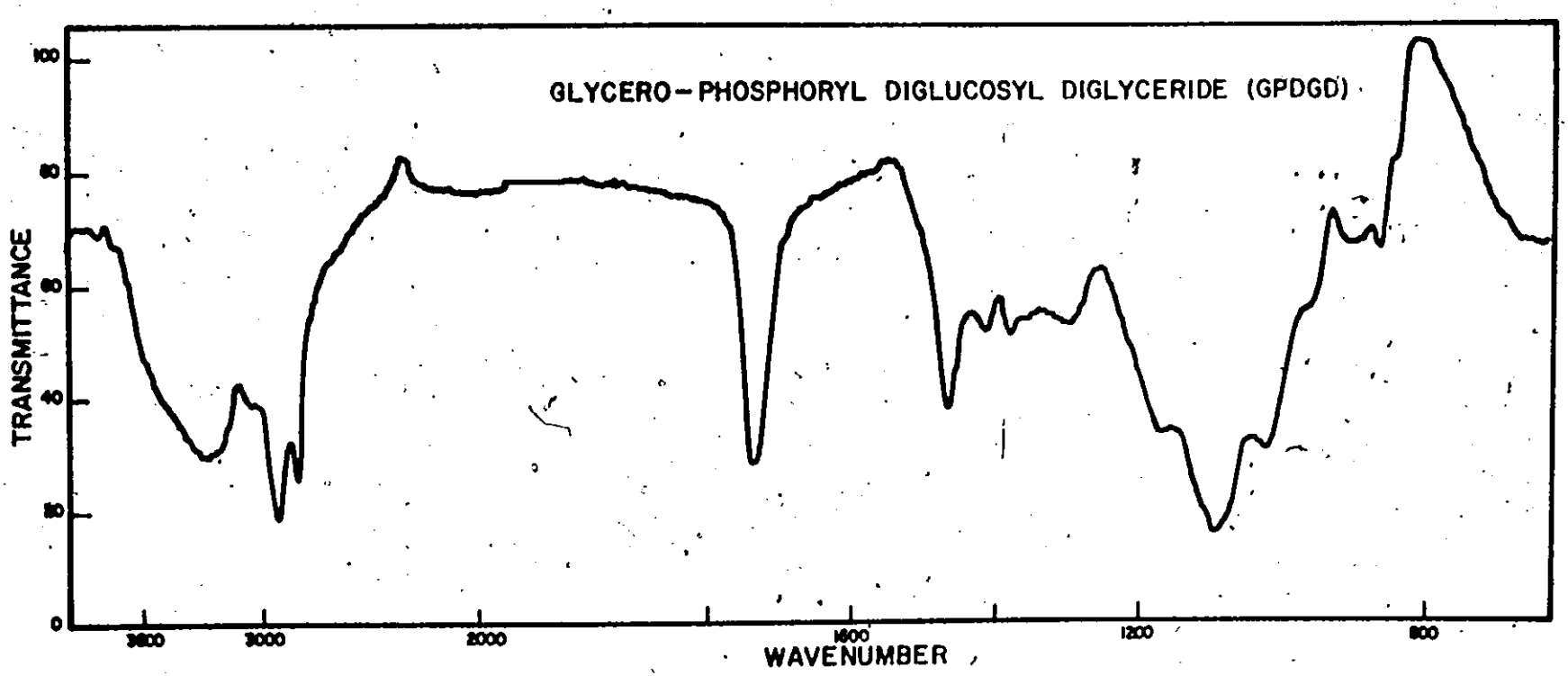
Analysis of Purified GPDGD and its Deacylation Product (GPDGG)

Composition	GPDGD		GPDGG	
	Found	Calc[a]	Found	Calc[b]
P, %	2.91	2.95	5.30	5.28
Hexose, %	32.8	34.2	62.1	61.3
Glycerol, %	17.5	17.5	30.8	31.4
Ester, %	46.7	47.6	-	-
Mole ratio's				
P:Hexose:Glycerol:Fatty ester	1:1.94:2.03:1.99	1:2:2:2	1:2.02:1.96	1:2:2
Optical rotation				
$[\alpha]_D^{25}$	-16.3[c]		-29.0[d]	
$[M]_D^{25}$	-170.7		-170.4	-147.9[e]

a. For $C_{49}H_{90}O_{20}P.NH_4$ (M.W = 1049.8)
b. For $C_{18}H_{34}O_{18}P.NH_4$ (M.W = 587)
c. In water
d. In $CHCl_3$
e. Calculated for all β-linkages (more detailed in Table 19)

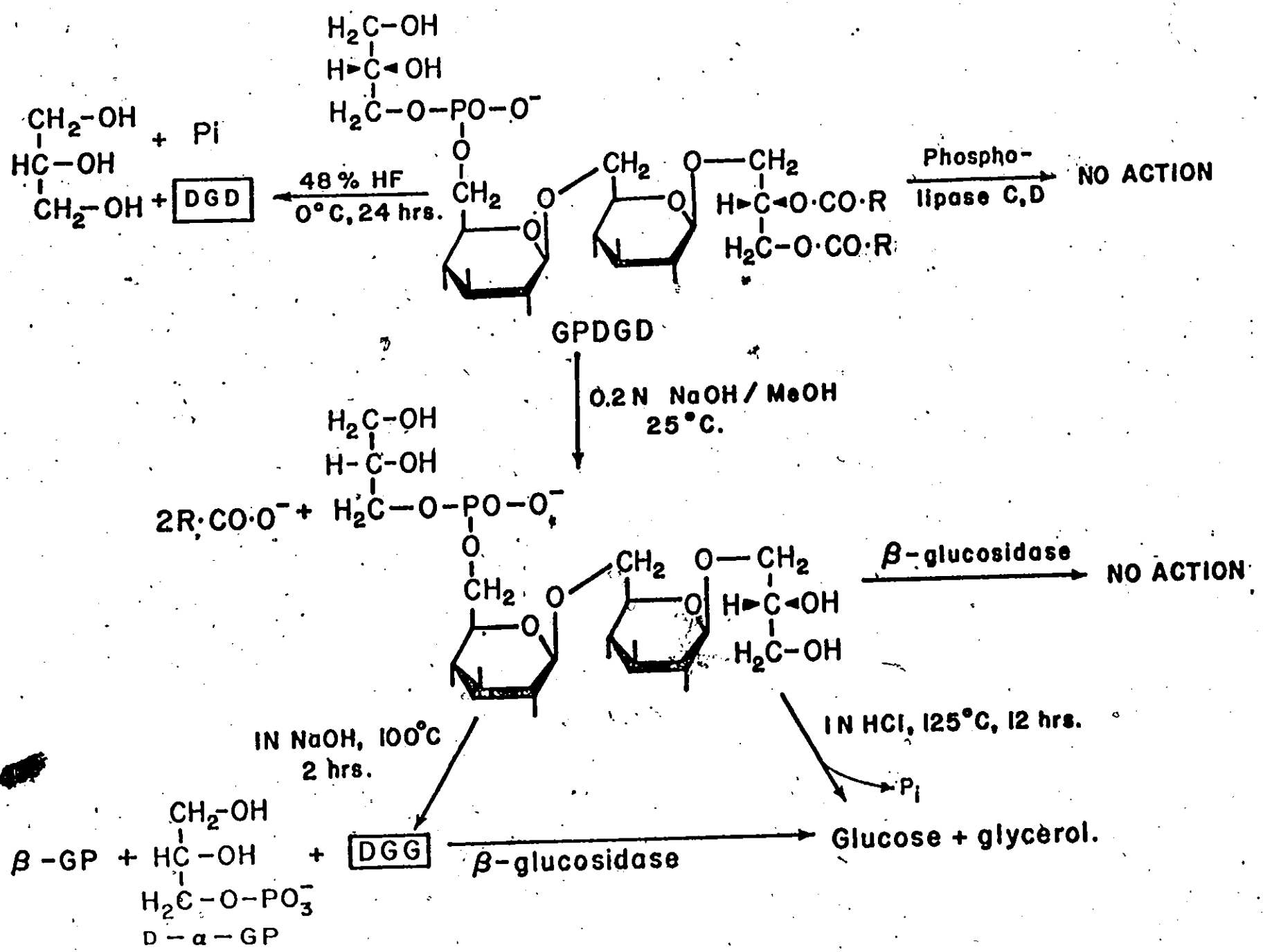
FIGURE 14

Infrared Spectrum of Glycerophosphoryl Diglucosyl Diglyceride, (ammonium salt form) in chloroform.


GLYCERO-PHOSPHORYL DIGLUCOSYL DIGLYCERIDE (GPDGD)
TRANSMITTANCE
100
80
60
40
20
0
3800
3000
2000
1600
1200
800
WAVENUMBER

SCHEME 3

Degradation sequences establishing the structure of glycerophosphoryl diglucosyl diglyceride.


Pi
DGD
48 % HF
0° C, 24 hrs.
Phospho-
lipase C, D
NO ACTION
GPDGD
0.2 N NaOH / MeOH
25° C.
2R·CO·O⁻ +
β-glucosidase
NO ACTION
1N NaOH, 100°C
2 hrs.
1N HCl, 125°C, 12 hrs.
Pi
β-GP +
DGG
β-glucosidase
Glucose + glycerol.
D-α-GP

1. Treatment with Hydrofluoric acid (48%, w/v)

As mentioned before, HF is known to cleave phosphodiesters and phosphomonoesters without attacking fatty ester linkages or glycosidic bonds (Archibald et al., 1968). As shown in Fig. 15 no diglyceride was detected after HF treatment of GPDGD (Fig. 15A) while 1,2- and 1,3-diglycerides were liberated from PG (Fig. 15B), 1,3-diglyceride resulting from isomerization of the 1,2-isomer (Shaw and Stead, 1972). The main chloroform soluble product obtained from the reaction of GPDGD with HF was a carbohydrate containing compound which co-chromatographed with the main glycolipid, DGD (spot 2, Fig. 16) found in the same organism, minor component, spot 2a and 2b (Fig. 16) were unreacted GPDGD and a lyso-compound of DGD (tentatively identified), respectively. The water soluble fraction of HF treated GPDGD contained glycerol and P_i as analyzed on paper chromatography in solvents 1 and 2 of Table 17.

These results indicate that in the structure of GPDGD, the glycerol and DGD moieties are linked by phosphodiester linkage (Scheme 3), since no diglyceride was liberated by HF hydrolysis. Therefore the two moles of fatty acid which were present per mole of GPDGD (Table 16) must be esterified at the vicinal OH of glycerol and not at those of glycerophosphate. Lack of hydrolysis of GPDGD with phospholipases C and D (see next section) also confirmed this conclusion.

FIGURE 15

Thin-layer chromatograms of the chloroform-soluble, breakdown products of:

A. GPDGD treated with 48% HF

B. PG treated with 48% HF

1. Standard DGD (isolated from S. epidermidis) and 1,2-dipalmitin
2. Untreated GPDGD
3. HF-treated GPDGD
4. HF-treated PG
5. Untreated PG
6. Standard 1,2-dipalmitin

a, DGD, GPDGD and PG respectively; b, 1,2-diglyceride; c, 1,3-diglyceride; d, e, f, free fatty acids.

Solvent system:

Petroleum ether-ethyl ether-acetic acid (80:20:1, v/v)

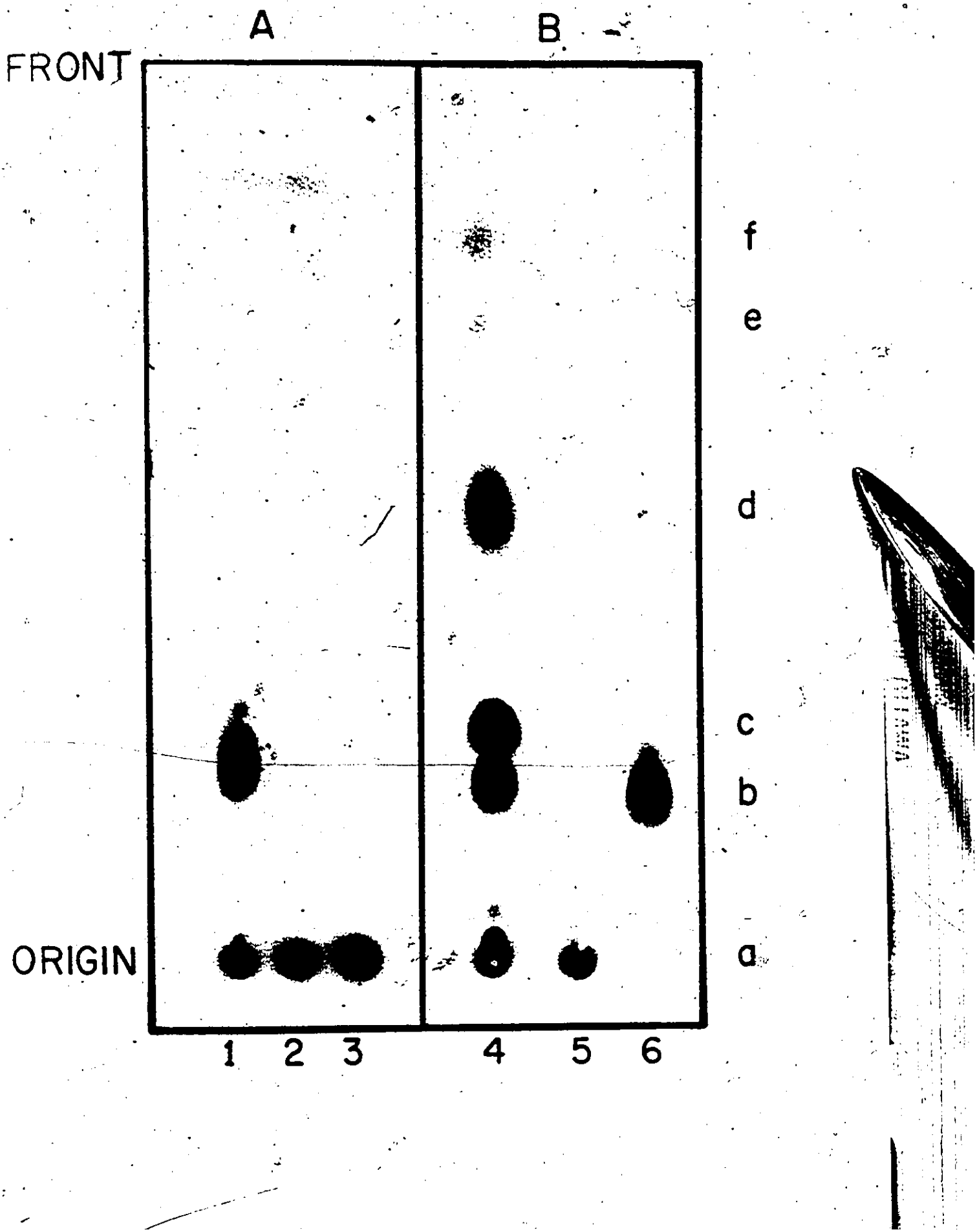

A
B
FRONT
f
e
d
c
b
a
ORIGIN
1
2
3
4
5
6

FIGURE 16

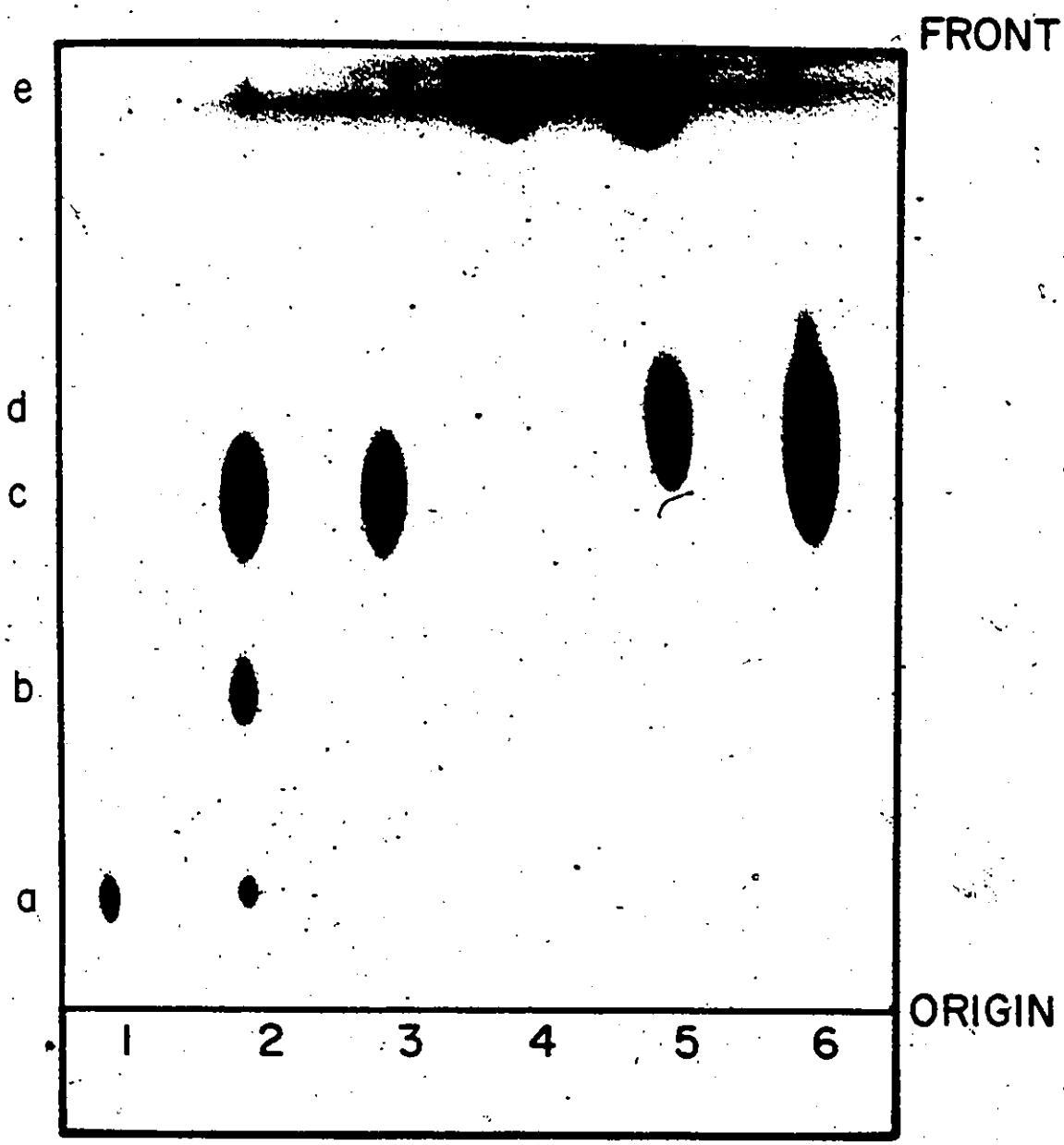
Thin-layer chromatogram of the chloroform-soluble, breakdown products of GPDGD and PG treated with 48% HF.

1. untreated GPDGD
2. HF-treated GPDGD
3. diglucosyl diglyceride isolated from *S. epidermidis*
4. standard 1,2-dipalmitin
5. HF-treated PG
6. untreated PG

a, GPDGD; b, lyso DGD (tentatively identified); c, DGD; d, PG; e, DG

Solvent system:

chloroform-methanol-28% ammonia (65:35:5, v/v)


FRONT
e
d
c
b
a
ORIGIN
1
2
3
4
5
6

2. Treatment with Phospholipases C and D

The mechanism of action of phospholipases C and D towards phosphoglycerides is known to be as shown in Scheme 4. If the glucophospholipid had a glycerophosphatide structure (Scheme 4, X = DGG), the products would be with phospholipase C, diglyceride plus phosphodiglucosyl glycerol and with phospholipase D, phosphatidic acid plus diglucosyl glycerol (DGG).

When GPDGD was treated with phospholipases C or D (see Experimental Procedures II.G), no hydrolytic breakdown of the substrate was detected. In contrast, PC (from egg lecithin) gave the expected products (Scheme 4) when treated with phospholipases C and D.

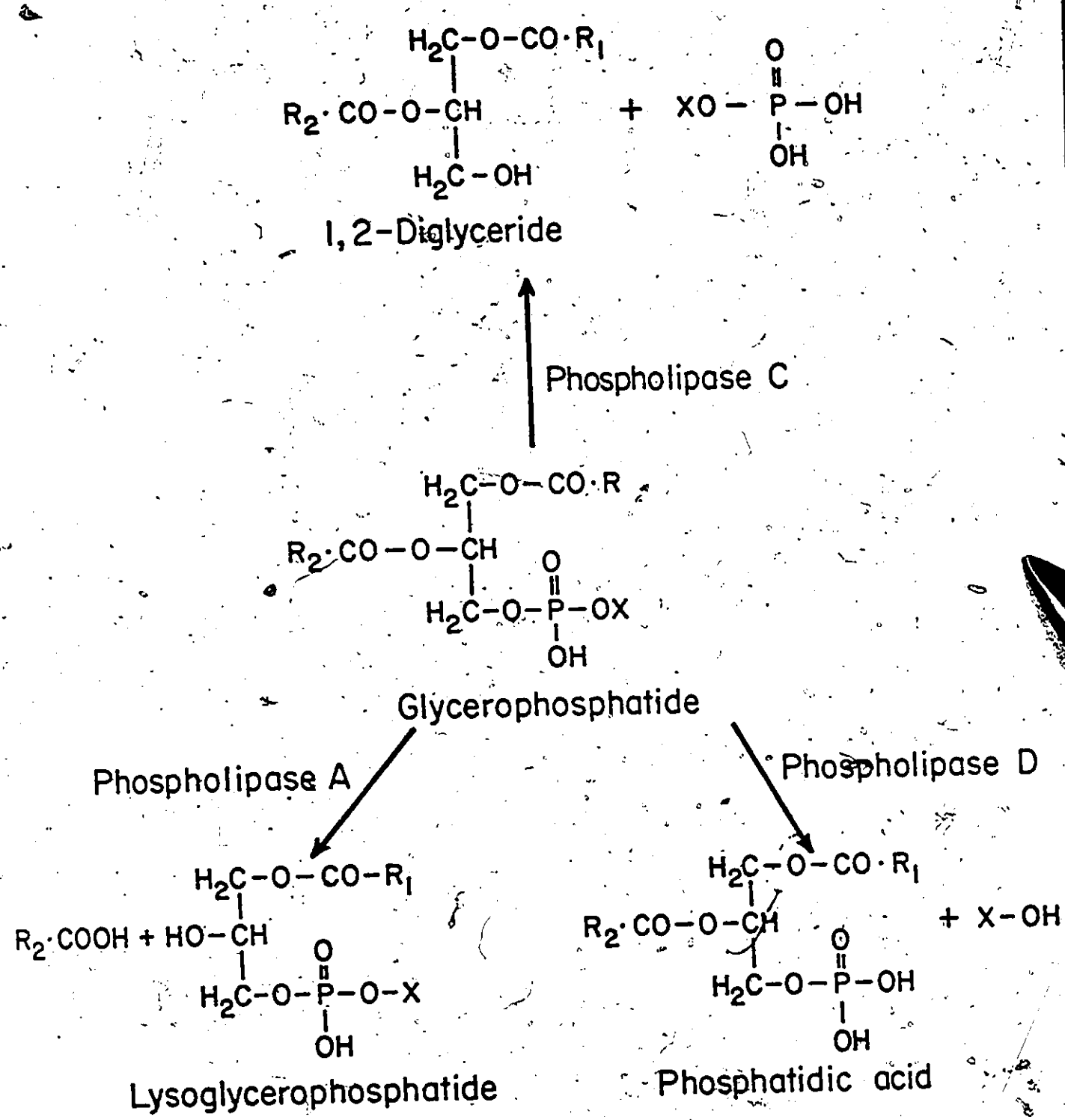
The negative result obtained with GPDGD confirmed that this compound was not a glycerophosphatide, i.e., the two fatty acyl groups were not esterified with the glycerophosphate moiety but rather with the glycerol moiety.

3. Deacylation (mild alkaline hydrolysis)

After mild alkaline hydrolysis (see Experimental Procedures II.A), a single water-soluble compound was obtained. The compound could be detected on paper chromatograms with periodate-Schiff reagent with which it rapidly gave a pink to purple colour. This is characteristic of formaldehyde which would be produced from a 1-substituted glycerol (Roberts et al., 1963). The deacylated product of

SCHEME 4

Site of action of phospholipases A, C and D on a glycero-phosphatide and products obtained from these enzymatic hydrolyses. (Modified from Van Deenen and De Haas, 1966).



Phosphatide	X
PC	$-CH_2-CH_2-\overset{+}{N}(CH_3)_3$
PG	$-CH_2-\underset{OH}{CH}-CH_2-OH$

GPDGD moved slightly slower than the deacylated glucophospholipid from Acholeplasma laidlawii (gift from Dr. N. Shaw) on paper chromatogram (R_f 0.39 and 0.42, respectively in solvent 3, Table 17).

The water-soluble component obtained was found to be non-reducing to Fehling's solution and had a specific rotation, $[\alpha]_D^{25} = -29^\circ$ and the molecular rotation, $[M]_D = -170.4$ (Table 16). The compound contained all the lipid phosphorus, hexose and glycerol in the molar ratio of 1:2.02:1.96 (Table16), similar to the ratios found for the intact lipid, indicating that the lipid contains only fatty acyl ester linkages and no alkyl ether bonds.

4. Acid Hydrolysis

Drastic acid hydrolysis of GPDGD (2 N HCl, 125°C in a seal tube) followed by chromatography of the water-soluble products yielded only one sugar component which co-chromatographed with authentic standard glucose (solvent 1, Table 17). The other two components were identified chromatographically as glycerol and inorganic phosphate (solvents 2 and 3, Table 17). The chloroform-soluble fraction contained only fatty acids with the same composition as that of the intact GPDGD (see Table 26).

Therefore from the information obtained from acid hydrolysis, the two hexose residues present in GPDGD were revealed as glucose. The stereo-configuration of the linkage

TABLE 17

R_f[a] Values of Various Water-Soluble Compounds (standards) which might be obtained from hydrolysis of lipid components

Component[b]	R_{Glu}[c]	R_f	
	Solvent 1	Solvent 2[d]	Solvent 3[e]
Pi	-	0.14	0.20
α-GP	-	0.30	0.31
G-PE	-	0.61	-
G-PG	-	0.36	0.34
G-PGPG	-	0.04	-
G-Gal	0.61	0.51	-
G-Gal-Gal	0.16	0.39	-
Galactose	0.85	-	-
Glucose	1.00	0.40	0.50
Mannose	1.20	-	-
Inositol	0.36	-	-
Erythritol	1.70	-	
Glycerol	2.3	0.70	0.79

a. Whatman No. 1, paper chromatography

b. Pi, inorganic phosphate; GP, glycerophosphate; GPE, GPG and GPGPG were deacylated products obtained from PE, PG and cardiolipin respectively; G-Gal and G-Gal-Gal were deacylated products obtained from monogalactosyl diglyceride and digalactosyl diglyceride (runner bean) respectively.

c. Relative to glucose in pyridine:ethyl acetate:water (2:5:5, v/v; upper phase), descending chromatography.

d. Phenol:water (5:2, w/w), ascending chromatography.

e. Propanol:10 N NH_4OH (3:2, v/v), ascending chromatography.

and the position between these two glucose were established as discussed below.

5. Strong Alkaline Hydrolysis

Drastic aqueous alkaline hydrolysis of GPDGD (1 N NaOH, 100°C) or its deacylated product yielded two water-soluble components, one of which was identified chromatographically as glycerophosphate (solvents 2 and 3, Table 17) and the other faster moving component gave a periodate-Schiff positive spot with chromatographic mobilities identical to that of the deacylation product obtained from DGD, the main glycolipid found in this organism (R_f 0.39 in solvent 2, Table 17). Glycerophosphate was the only phosphorus containing product and no sugar phosphate was detected.

The glycerophosphate was analyzed for α- and β-isomers by the method of Burmaster (1946) and found to contain 42% α- and 58% β-isomers. These two isomers accounted for 99.5% of the total water-soluble P (Table 18).

The chloroform-soluble fraction obtained from the hydrolysis was composed entirely of free fatty acids, having identical composition to that of the intact GPDGD (Table 26).

c. Configuration of Glycerophosphate

Since the glycerophosphate moiety is present originally as the α-isomer, in view of the periodate-oxidation results (see Results V.F.1), the presence of the β-isomer

TABLE 18

Analysis of the Glycerophosphates Liberated on Strong Alkaline Hydrolysis (1N NaOH, 2 hrs, 100°C)

Glycerophosphate Isomers	% of Total Water Soluble-P	
	Deacylated GPDGD	Deacylated PG
Total glycerophosphate[a]	99.5	99.0
α-glycerophosphate[a]	41.7	41.0
β-glycerophosphate[b]	58.3	59.0
sn-3-α-glycerophosphate[c]	0(0.0)*	19.7 (48.2)*
sn-1-α-glycerophosphate[d]	41.7(100)*	21.3 (51.8)*

*Values in parenthesis are given as % of α-GP

a) Method of Burmaster (1946)

b) Difference between total GP and α-GP

c) Glycero-3-phosphate dehydrogenase assay

d) Difference between α-GP and sn-3-GP

indicated that isomerization must have occurred during the alkaline hydrolysis. Such isomerization occurs in phosphodiesters via a cyclic phosphate intermediate (see Scheme 5) as shown previously (Baer and Kates, 1948), resulting in retention of configuration of the α-isomer.

The configuration of α-glycerophosphate was determined by the treatment with the stereospecific enzyme glycerophosphate dehydrogenase (see Materials and Methods II.E.8) which is specific for sn-glycero-3-phosphate and not for sn-glycero-1-phosphate. The α-glycerophosphate (α-GP) obtained from strong alkaline hydrolysis of deacylated GPDGD was not dehydrogenated at all by the enzyme, in contrast to the α-GP from the deacylated PG which was dehydrogenated to the extent of 50% (Table 18).

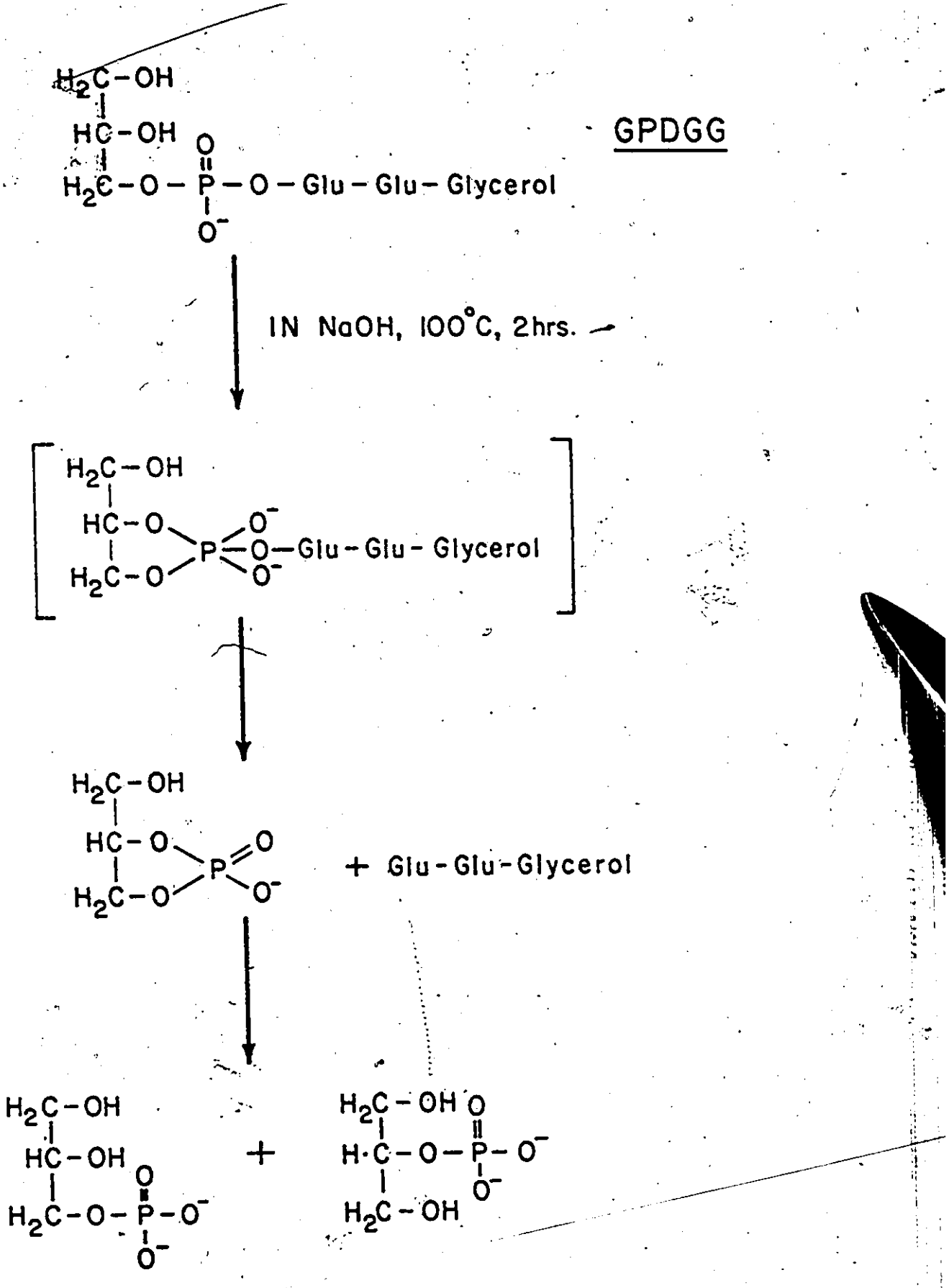
These results show that the configuration of α-GP in GPDGD is the unusual sn-glycero-1-phosphate while that from PG was an equimolecular mixture of sn-1 and sn-3 isomers, as expected for PG with the structure 1,2-diacyl-sn-glycero-3-phosphoryl-1'-sn-glycerol.

D. Location of Glycerophosphate

Since there are two glucose residues present in the structure of GPDGD (Table [illegible]), it remained to be determined which glucosyl portion is attached to the glycerophosphate (GP). The deacylated GPDGD (i.e., GPDGG) was treated with β-glucosidase along with the deacylated DGD

SCHEME 5

Mechanism for the strong alkaline hydrolysis of glycerophosphoryl diglucosyl glycerol. (Modifed from Baer and Kates, 1948).


GPDGG
1N NaOH, 100°C, 2hrs.
Glu-Glu-Glycerol
+ Glu-Glu-Glycerol
α-GP
β-GP

(i.e., DGG) of the same organism (see Experimental Procedures II.G.4). Even after prolonged incubation (30 hr) only traces of glucose (0.08 µmole) were released from GPDGG (1 µmole) while in the case of DGG, the reaction was complete within 24 hr (Fig. 17).

The results obtained, indicated that GP might be located on the terminal glucosyl residue where it is considered to block the attack by the enzyme. Another explanation, namely that GPDGD contains, besides GP linked to the internal glucose, an unknown substituent in position 6 of the terminal glucose, and this substituent was removed by strong alkaline hydrolysis to give diglucosyl glycerol (DGG) (see Results V.B.5). The other possibility is that GP may be linked to the internal glucose but in this position may inhibit the enzymatic hydrolysis of the terminal glycosidic linkage by steric hindrance even though the terminal unsubstituted glucose is available for cleavage. The position of glycerophosphate was established unambiguously by the methylation studies (see Results V.F.3).

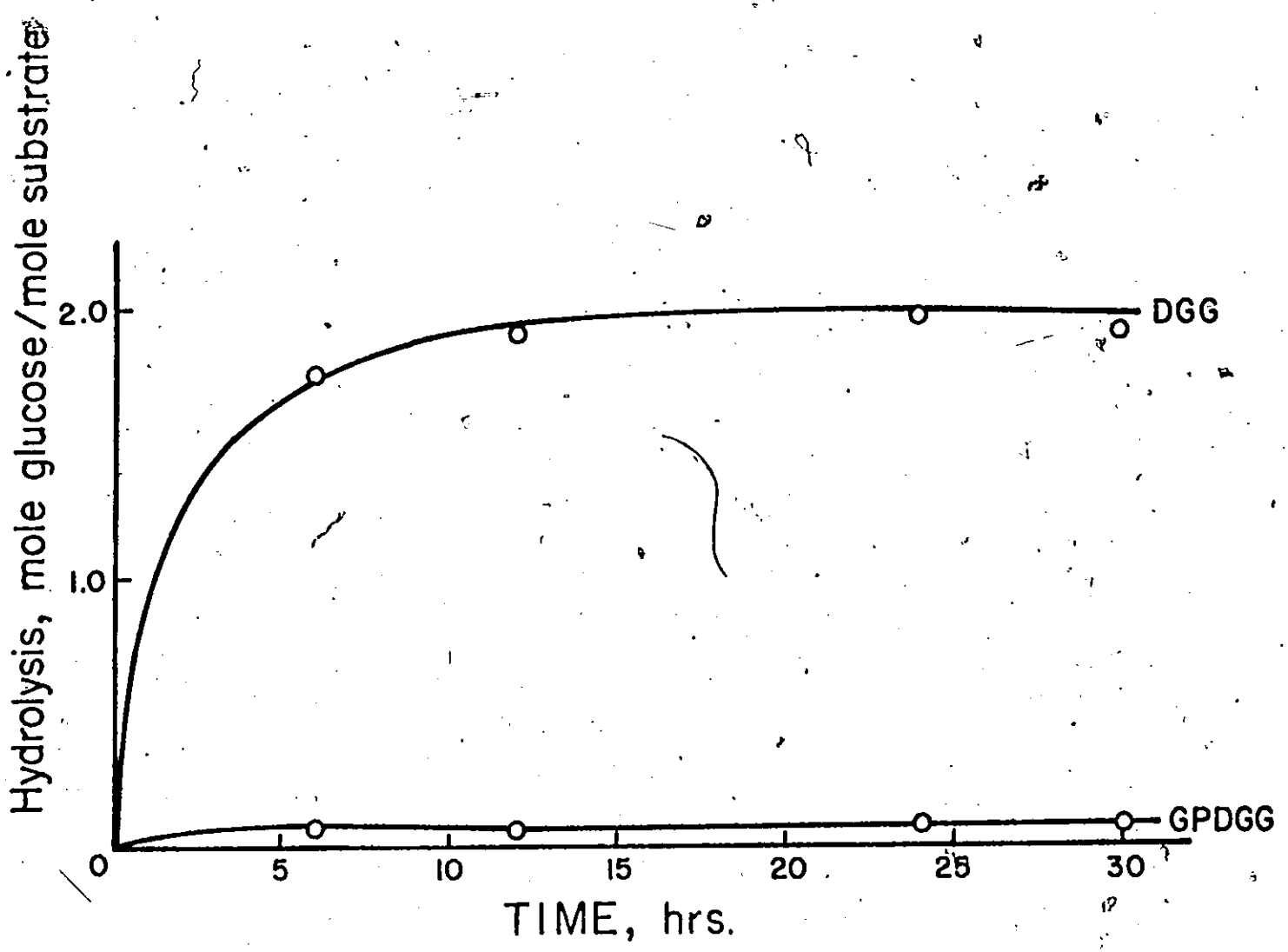
E. Determination of the Configuration of Glycosidic Linkages Present in GPDGD

Two glycosidic linkages are present in the structure of GPDGD, one between the two glucose residues, the other between the second glucose and glycerol. Several experiments were carried out to determine the configuration of these linkages.

FIGURE 17

Enzymatic hydrolysis of glycerophosphoryl diglucosyl glycer (GPDGG) and diglucosyl glycerol (DGG) with β-glucosidase (emulsin).

Assay system (vol., 3.2 ml): GPDGG (2.5 µmole P) or DGG (4.6 µmole glucose) in 1.6 ml water, 0.1 M phosphate buffer pH 6. (1.1 ml) and β-glucosidase (0.5 ml containing 0.3 mg protein The reaction mixture was incubated at 30°C and aliquots (0.2 were withdrawn at indicated times and analyzed for glucose release by the glucostat reagent.


Hydrolysis, mole glucose/mole substrate
2.0
1.0
0
5
10
15
20
25
30
TIME, hrs.
DGG
GPDGG

1. Treatment with the specific enzymes α-glucosidase and β-glucosidase

Pure diglucosyl glycerol (DGG) obtained from strong alkaline hydrolysis of GPDGG (see Experimental Procedures II.B) was treated with α-glucosidase (from yeast; free of β-activity) and β-glucosidase (emulsin, free of α-activity). Controls using maltose and cellobiose as substrate were performed at the same time. It was found that DGG was not attacked by α-glucosidase but was attacked by β-glucosidase and both glucose and glycerol were released as revealed by paper chromatography in solvent 1 (see Table 17). α-Glucosidase and β-glucosidase were shown to be active and specific by hydrolyzing only maltose and cellobiose, respectively. However, the β-glucosidase contained tightly bound glucose which was undialyzable and showed up when the enzyme alone was subjected to paper chromatography. The α-glucosidase used was reasonably pure and easily freed of glucose by dialysis.

The result obtained from enzymatic hydrolysis indicated that both glycosidic linkages were in β-configuration since only glucose and glycerol were detected. If the two linkages were in α- and β-configuration, monoglucosyl glycerol or maltose should be detected but none of these was observed. In order to establish conclusively the configuration of the glycosidic linkages, the optical rotation and the NMR spectrum of GPDGG were determined.

2. Optical Rotation Measurement of GPDGG

The "theoretical" values of molecular rotations of GPDGG were calculated according to Hudson's rule of optical rotation (Hudson, 1909), namely: "the molecular rotation of an optical active compound containing more than one asymmetric center can be calculated from the arithmetic sum of the molecular rotations of their optically active constituents." On this basis, the molecular rotations of GPDGG were calculated as follows:

a. Assuming all α-linkages, (sn-1-α-GP-glu-α-glu-α-glycerol)

$$[M]_{GPDGG} = [M]_{sn\text{-}1\text{-}GP} + 2[M]_{\alpha\text{-methylglucoside}} + [M]_{sn\text{-}3\text{-acylglycerol}}$$

$$= 2.99 + 2(308.6) - 18.1$$

$$= +602.1$$

b. Assuming all β-linkages, (sn-1-α-GP-glu-β-glu-β-glycerol)

$$[M]_{GPDGG} = [M]_{sn\text{-}1\text{-}GP} + 2[M]_{\beta\text{-methylglucoside}} + [M]_{sn\text{-}3\text{-acylglycerol}}$$

$$= 2.99 - 2(66.4) - 18.1$$

$$= -147.9$$

c. Assuming one α- and one β-glycosidic linkage,

$$[M]_{GPDGG} = [M]_{sn\text{-}1\text{-}GP} + [M]_{\alpha\text{-methylglucoside}} + [M]_{\beta\text{-methylglucoside}} + [M]_{sn\text{-}3\text{-acyl glycerol}}$$

$$= 2.99 + 308.6 - 66.4 - 18.1$$

$$= +227.1$$

Values for the $[\alpha]_D$ of the model compounds in the same solvent as GPDGG were taken from the literature. The calculated $[M]_D$ values of these model compounds and literature references are given in Table 19.

The specific rotation of the deacylated GPDGD, $[\alpha]_D^{25}$ was found to be -29.02°, which corresponded to $[M]_D$ - 170.4 (Tables 16 and 19). Comparison of the observed molecular rotation with the theoretical molecular rotations calculated for all α-, all β- or one α- and one β-linkages showed closest agreement with that for the all β-configuration (Table 19).

This finding was in accord with the results obtained from the enzymatic hydrolyses that both glycosidic linkages present were in the β-configuration.

3. Nuclear Magnetic Resonance Studies (NMR)

NMR studies were carried out to confirm the preceding indications that the glycosidic linkages of the glucosyl-glucosyl-glycerol in GPDGG were in the β-configuration.

The spectra of the α- and β-methyl-D-glucopyranoside show the chemical shift of the anomeric proton in β and α configurations respectively. The doublet at 4.68δ is due to H^β while the doublet at 4.20δ is due to H^α (H^β and H^α are the anomeric protons in α- and β-D-glucoside respectively). The area under each doublet, obtained by integration, represents 1H on the C-1 carbon atom of the sugar (Table 20; Fig.18)

TABLE 19

The Specific Rotation of the Model Compounds and the Calculated Molecular Rotation of GPDGG and DGG for various possible linkage configurations

Compounds	$[\alpha]_D^{20}$ in water	$[M]^+$	Reference
Model Compounds			
Methyl-O-α-D-glucopyranoside	+158.0	+308.6	Handbook of Chem. and Physics (CRC, 49 edit.)
Methyl-O-β-D-glycopyranoside	- 34.2	- 66.4	" " " "
sn-3-monopropionyl glycerol	- 12.2	- 18.1	Baer and Fischer, 1945
sn-1-glycerophosphate	+ 1.45*	+ 2.99	Baer and Kates, 1948
Calc. For GPDGG**			
a. all α-linkages		+602.1	
b. all β-linkages		-147.9	
c. one α, one β		+227.1	
Found For GPDGG	- 29.02	-170.4	
Calc. For DGG‡			
a. all α-linkages		+599.1	
b. all β-linkages		-150.9	
c. one α, one β		+224.1	
Found For DGG		-159.0	

+ [M], molecular rotation = $\frac{M[\alpha]}{100}$, where M = Molecular weight.

*Derived from value for barium salt of sn-3-GP in 2N HCl ($[\alpha]_D = -1.45^\circ$)

**See Results Section V.E.2

‡See Results Section VI.C.2

FIGURE 18

100 Mc NMR Spectra of Model Compounds

A. Methyl-O-α-D-glucopyranoside

B. Methyl-O-β-D-glucopyranoside

Solvent	D_2O
Temperature	Ambient
Sweep width	500 Hz
Sweep time	500 sec
Lock Signal	Acetone

See Table 20 for assignment of peaks

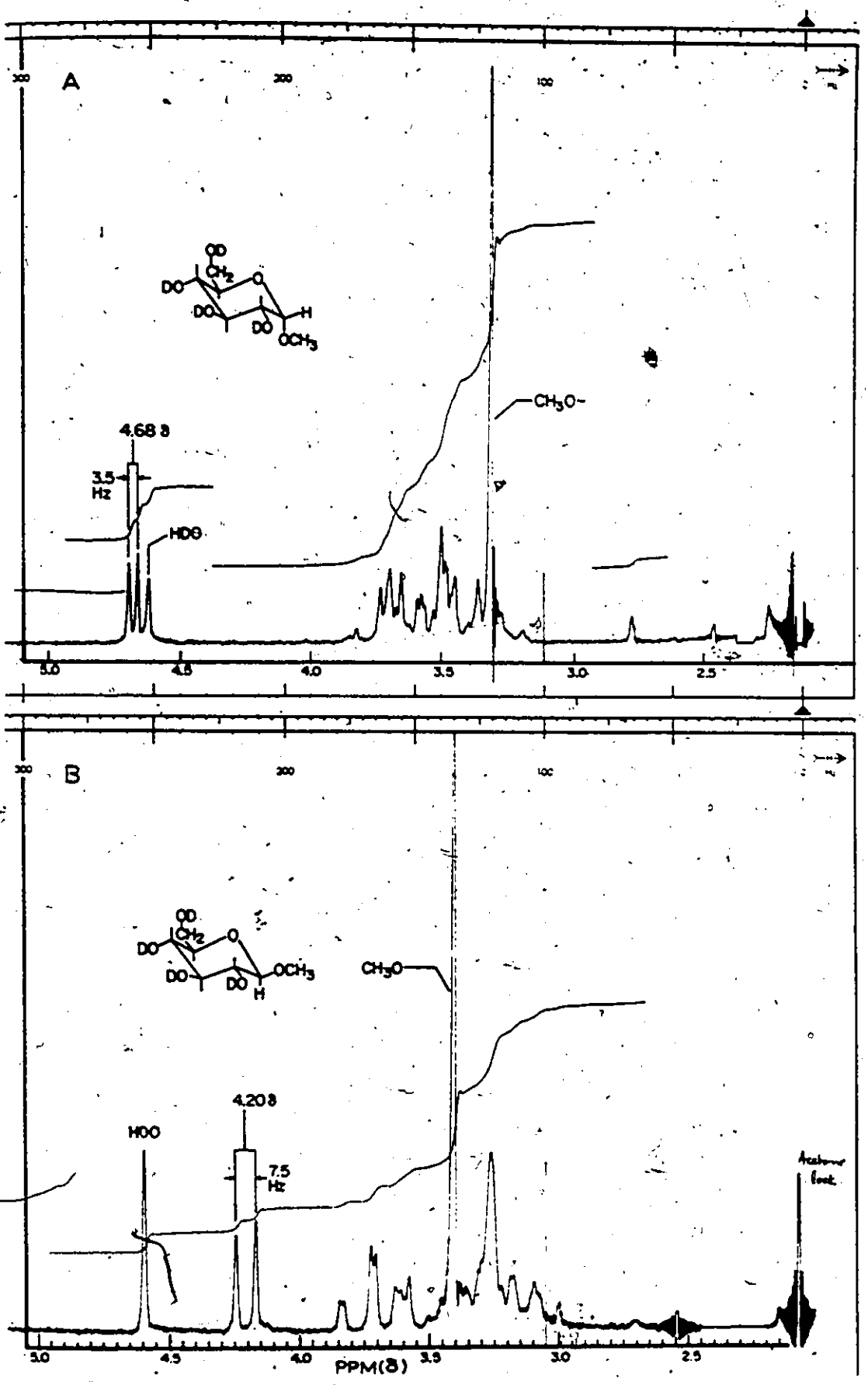

A
4.68 δ
3.5 Hz
HDO
CH3O-
5.0
4.5
4.0
3.5
3.0
2.5
B
CH3O
4.20 δ
HOO
7.5 Hz
Acetone
PPM(δ)

TABLE 20

Chemical Shifts (δ) and Coupling Constant (J) of NMR Signals for GPDGG[a], DGG[b] and the Standard Compounds[c]

Compound	Group	Chemical Shift (δ) ppm	Coupling Constant (J) Hz	Number of Protons	
				Calc	Found
GPDGG	anomeric protons	4.48, 4.52 (two doublets)	7.5,7.5	2.0	1.9
DGG	anomeric protons	4.25,4.31 (two doublets)	7.0,7.0	2.0	1.9
Standards					
α-methyl glucoside	β-anomeric proton (H^{β})	4.68 (doublet)	3.5	1.0	0.9
β-methyl glucoside	α-anomeric proton (H^{α})	4.20 (doublet)	7.5	1.0	1.0

a. See Fig. 19
b. See Fig. 22
c. See Fig. 18

The assignment of these signals is in accord with the fact that an axially oriented hydrogen (H^{α}) is more shielded than an equatorially oriented hydrogen (H^{β}) and should resonate at higher applied magnetic field. Furthermore, the spin-spin coupling constant ($J_{H_1-H_2}$) between the protons on C_1 and C_2 is 3.5 Hz in the case of α-methyl-D-glucoside (typical of axial-equatorial couplings) and 7.5 Hz for β-methyl-D-glucoside (typical of axial-axial coupling), Table 20.

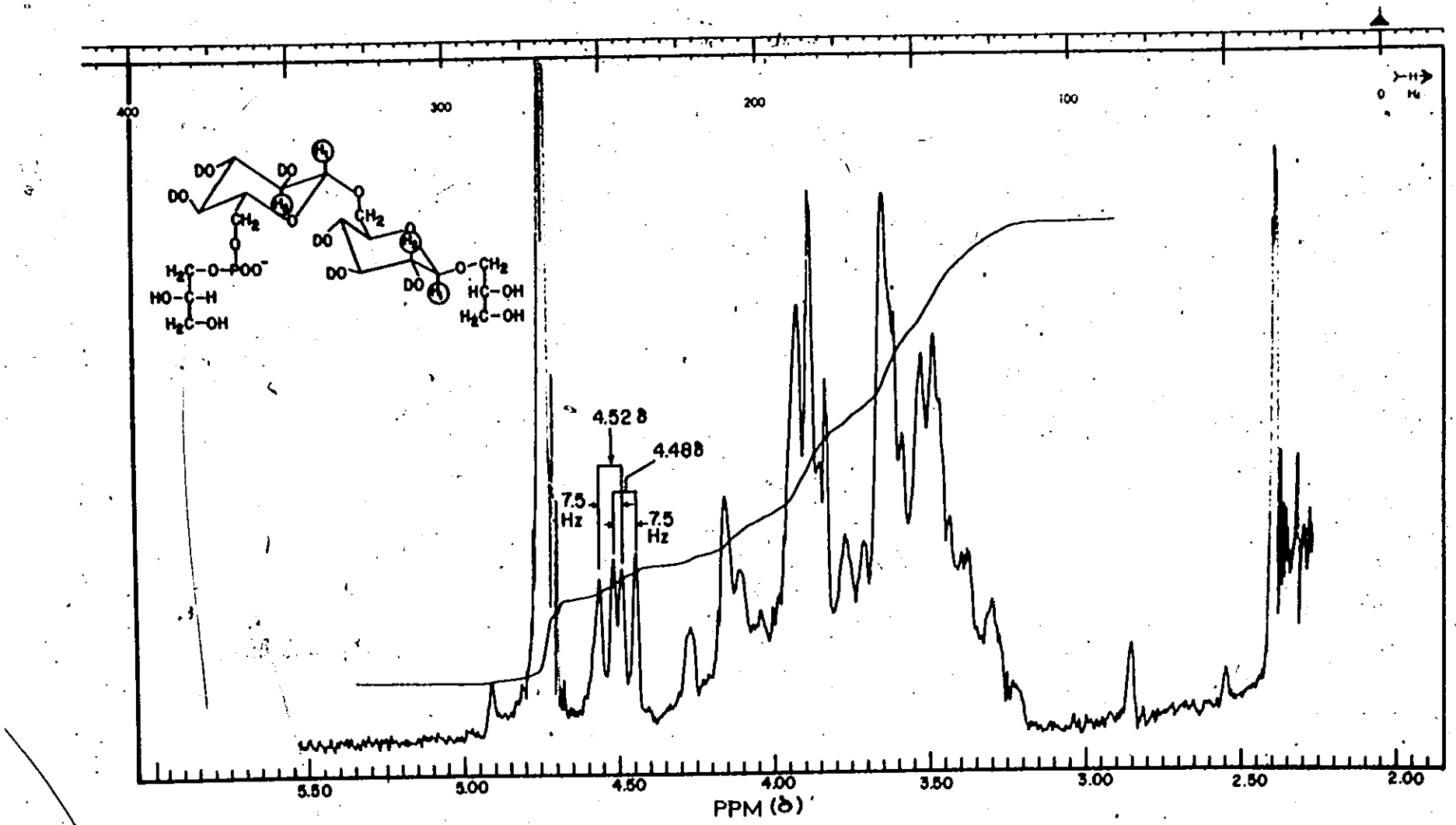
The 100 Mc NMR spectrum of GPDGG (Fig. 19) showed a series of four peaks centered at approximately 4.56δ. The center of this groups of peaks is approximately 24 Hz upfield from the HDO peak. This group of peaks is probably a pair of overlapping doublets, each doublet arising from one of the anomeric hydrogens. It is assumed that the upfield peak of the doublet of one C-1 hydrogen overlaps the downfield peak of the doublet of the C-1 hydrogen of the second sugar (4.48 and 4.52δ). Both doublets have the same coupling constant, i.e., 7.5 Hz, the same coupling constant as observed in β-methyl-D-glucoside (Table 20). It is possible, although not very probable that the two inner (overlapped) peaks are due to one of the C-1 hydrogens while the two outer peaks are due to the C-1 hydrogen of the other sugar. This is not probable since the coupling constant ($J_{H_1-H_2}$) of the inner peaks would be about 2 Hz which is rather smaller than that ever observed for any carbohydrate. Another possibility is

FIGURE 19

100 Mc NMR Spectrum of Glycerophosphoryl Diglucosyl Glycerol (GPDGG)

Solvent	D_2O
Temperature	Ambient
Sweep width	500 Hz
Sweep time	250 sec
Lock signal	Acetone

See Table 20 for assignment of peaks


400
300
200
100
0
Hz
4.528
4.488
7.5 Hz
7.5 Hz
5.50
5.00
4.50
4.00
3.50
3.00
2.50
2.00
PPM (δ)

that the first and second peaks of this group formed a doublet due to one of the C-1 hydrogens and that the third and fourth peaks comprise a doublet due to the other anomeric hydrogen. However, the coupling constant observed in this case would be 5.5 Hz which is in between the value for axial-axial coupling (≈7 Hz) or axial-equatorial coupling (≈3 Hz) mentioned before. Therefore, the assignment in which the two doublets give the coupling constant 7.5 Hz is the one that should represent the anomeric protons of the two sugar moieties. There was no doublet peak present in the region where one would expect to find anomeric protons of α-linked glucosides (i.e., downfield from the HDO peak). The peaks in the region 3.4δ-4.3δ are due to the protons of the sugars other than the anomeric protons and to the protons of the glycerol moiety. However, this group does not include -OH protons since these have been exchanged with D_2O. Any of these rapidly dissociated protons remaining would appear in the HDO peak.

This analysis of the NMR spectrum confirmed the linkage configuration obtained from enzymatic hydrolyses and optical rotation measurements, since the line positions and the coupling constant (7.5 Hz) of the peaks, of the anomeric protons were characteristic of β-D-glucosides, thus ruling out the presence of α-linkages.

F. Determination of the Positions of the Glycosidic Linkages

The deacylated product of GPDGD was nonreducible to Fehling's solution, indicating that the OH of anomeric carbons of both sugars were linked with -OH groups of the other sugar and glycerol. Three different methods were carried out to determine the position of the glycosidic linkage.

1. Quantitative Analysis of Periodate Consumption

On periodate oxidation, GPDGG consumed about 6 moles of $NaIO_4$ per mole of the compound with the liberation of about 2 moles of formaldehyde (see Scheme 6 and Table 21). Periodate oxidation of the deacylated product from the main glycolipid (DGG) was carried out at the same time and was found to be about 5 moles/mole with the liberation of 1 mole of formaldehyde per mole of DGG (Table 21). These values for periodate consumption agreed closely with those calculated for a 1 → 6 linkage between the sugars both in GPDGG and for DGG (see Table 21). The mole of formaldehyde released from DGG confirms the presence of terminal vicinal-OH groups expected from a 1,2-diglyceride structure in the original DGD. The formation of 2 moles of formaldehyde from GPDGG indicates the presence of two pairs of terminal vicinal OH's, one from the glycerol of the DGG moiety and the other from the glycerophosphate moiety. The latter moiety must therefore be the α-isomer and not the β-isomer.

SCHEME 6

Reactions represent "Smith Degradation" process of glycerophosphoryl diglucosyl glycerol.

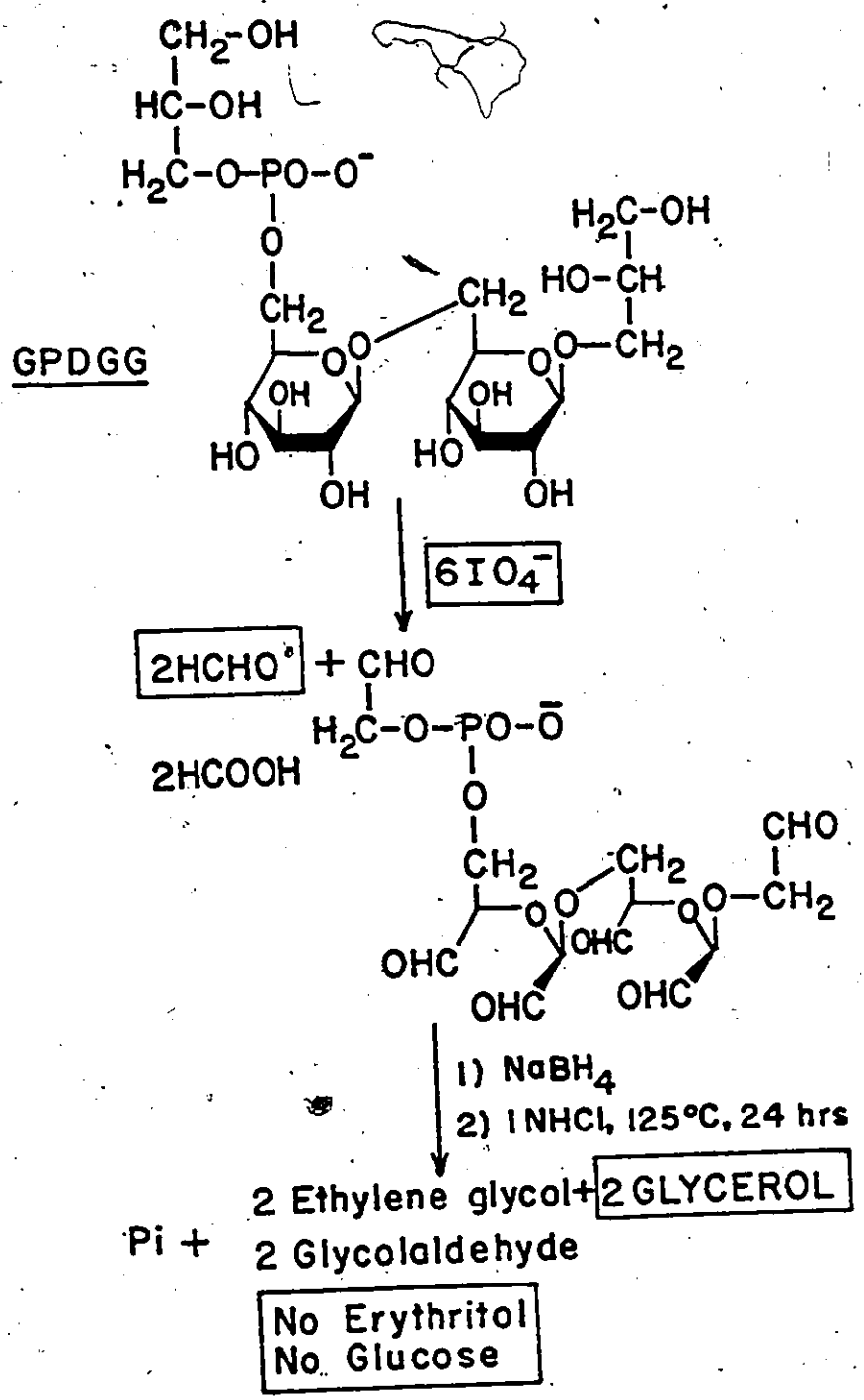

GPDGG
$6IO_4^-$
2HCHO
2HCOOH
1) $NaBH_4$
2) 1NHCl, 125°C, 24 hrs
2 Ethylene glycol + 2 GLYCEROL
Pi + 2 Glycolaldehyde
No Erythritol
No Glucose

TABLE 21

Periodate Consumption and Formaldehyde Formation of GPDGG and DGG

Calculated for Linkage	GPDGG*		DGG**	
	IO_4^- uptake moles/moles	HCHO released moles/moles	IO_4^- uptake moles/moles	HCHO released moles/moles
1 → 2	5	2	4	1
1 → 3	4	2	3	1
1 → 4	5	2	4	1
1 → 6	6	2	5	1
Found	6.11	2.10	4.85	1.15

*See structure in Scheme 6

**See structure in Scheme 9

2. Smith Degradation

Additional proof for the 1 → 6 glycosidic linkage in DGD and GPDGD was obtained by means of the Smith degradation procedure (see Experimental Procedures II.E.2). The reaction scheme and the final products expected from a GPDGG having a 1 → 6 glycosidic linkage between the glucose units are shown in Scheme 6. Table 22 summarizes the products expected from the other isomers.

After Smith degradation of the bacterial GPDGG and DGG, only glycerol was detected on paper chromatograms of the products (solvent 1, Table 17) or by GLC of the acetylated products (Table 23). No erythritol or glucose was detected; small molecules such as glycolaldehyde, glyceraldehyde and ethylene glycol could not be detected due to their loss by volatilization during the work-up procedure. Cellobiose was used as a control and gave glycerol and erythritol as expected (Tables 22 and 23). Glucose would be formed only when the linkage is 1 → 3 while erythritol would be formed only when the linkage is 1 → 4 (Table 22). Since no glucose or erythritol was detected among the degradation products, the presence of these two possible linkages (1 → 3 or 1 → 4) in GPDGD and DGD is thus excluded. Glycerol would be obtained either from a 1 → 2 or 1 → 6 linkage, but the 1 → 2 linkage is eliminated by the finding that the periodate uptake was about 6 moles/mole (1 → 6 linkage) rather than 5 moles/mole expected for a 1 → 2 linkage (Table 21).

TABLE 22

The Expected Hydroxy Products Obtained from Smith Degradation* of Various Glycosidic Linkages

Glycosides	Degradation products (mole/mole glycoside)			
	Glycosidic linkage between two glucose residues			
	1 → 2	1 → 3	1 → 4	1 → 6
DGG	2 glycerol 1 glycolaldehyde 1 glyceraldehyde 1 ethylene glycol	1 glycerol 1 glycolaldehyde 1 glucose 1 ethylene glycol	1 glycerol 2 glycolaldehyde 1 erythritol 1 ethylene glycol	2 glycerol 2 glycolaldehyde 1 ethylene glycol
GPDGG	2 glycerol 1 glycolaldehyde 1 glyceraldehyde 2 ethylene glycol	1 glycerol 1 glycolaldehyde 1 glucose 2 ethylene glycol	1 glycerol 2 glycolaldehyde 1 erythritol 2 ethylene glycol	2 glycerol 2 glycolaldehyde 2 ethylene glycol
Cellobiose			1 glycerol 1 glycolaldehyde 1 erythritol	

* See Scheme 6 and 9 for degradation reactions

TABLE 23

Smith Degradation Products Obtained from GPDGG and DGG*

Compound	Relative Retention**	Identification
Standards		
ethylene glycol	0.14	
glycerol	1.00	
erythritol	4.17	
Degradation products from		
GPDGG	1.00	glycerol
DGG	1.01	glycerol
Cellobiose peak #1	1.01	glycerol
#2	4.15	erythritol

*Analyzed by GLC as the acetylated derivative on 10% BDS (Gas-Chrom W) at 180°C.

**Relative to triacetin (1,2,3-triacetyl glycerol); retention time 5 min.

The results of the Smith degradation thus are consistent with the presence of a 1 → 6 glycosidic linkage between the two glucose moieties in GPDGD.

The lack of reducing ability of GPDGG (negative reaction with Fehling's solution) and a rapid staining with periodate-Schiff reagent indicated that the other glycosidic linkage in GPDGG is between C_1 of glucose to C-1 or C-3 of glycerol.

Therefore these studies showed that the glycosidic linkages position in GPDGG were glucose (1 → 6) glucose (1 → 1 or 3) glycerol.

3. Methylation of GPDGD

The location of the α-GP and the position of the glycosidic linkage in GPDGD was established unambiguously by the results obtained on methylation of GPDGD.

The permethylated GPDGD finally obtained showed no free -OH absorption in the infrared. The NMR spectrum of the permethylated GPDGD (Fig. 20A, Table 24) showed the following: an envelope of overlapping sharp signals (CH_3 branches and terminals of the fatty acyl chains) at 0.81δ - 0.95δ; a sharp methylene signal ($-CH_2-$ of the fatty acyl chains) at 1.23δ; broad signals attributable to the protons α to the carbonyl of the fatty acyl groups ($-CH_2-\overset{O}{\overset{\|}{C}}-O-$) at 2.20δ - 2.40δ; and a group of signals between 3.35δ and 3.60δ is assigned to the $C-O-CH_3$ groups of the sugar rings and the

FIGURE 20

100 Mc NMR Spectra of Permethylated Glycerophosphoryl Diglucosyl Diglyceride

A. ^{31}P-^{1}H normal spectrum

B. ^{31}P decoupled spectrum

Solvent	$CDCl_3$
Temperature	Ambient
Sweep width	250 Hz
Sweep time	250 sec
Lock signal	Tetramethylsilane

See Table 24 for assignment of peaks

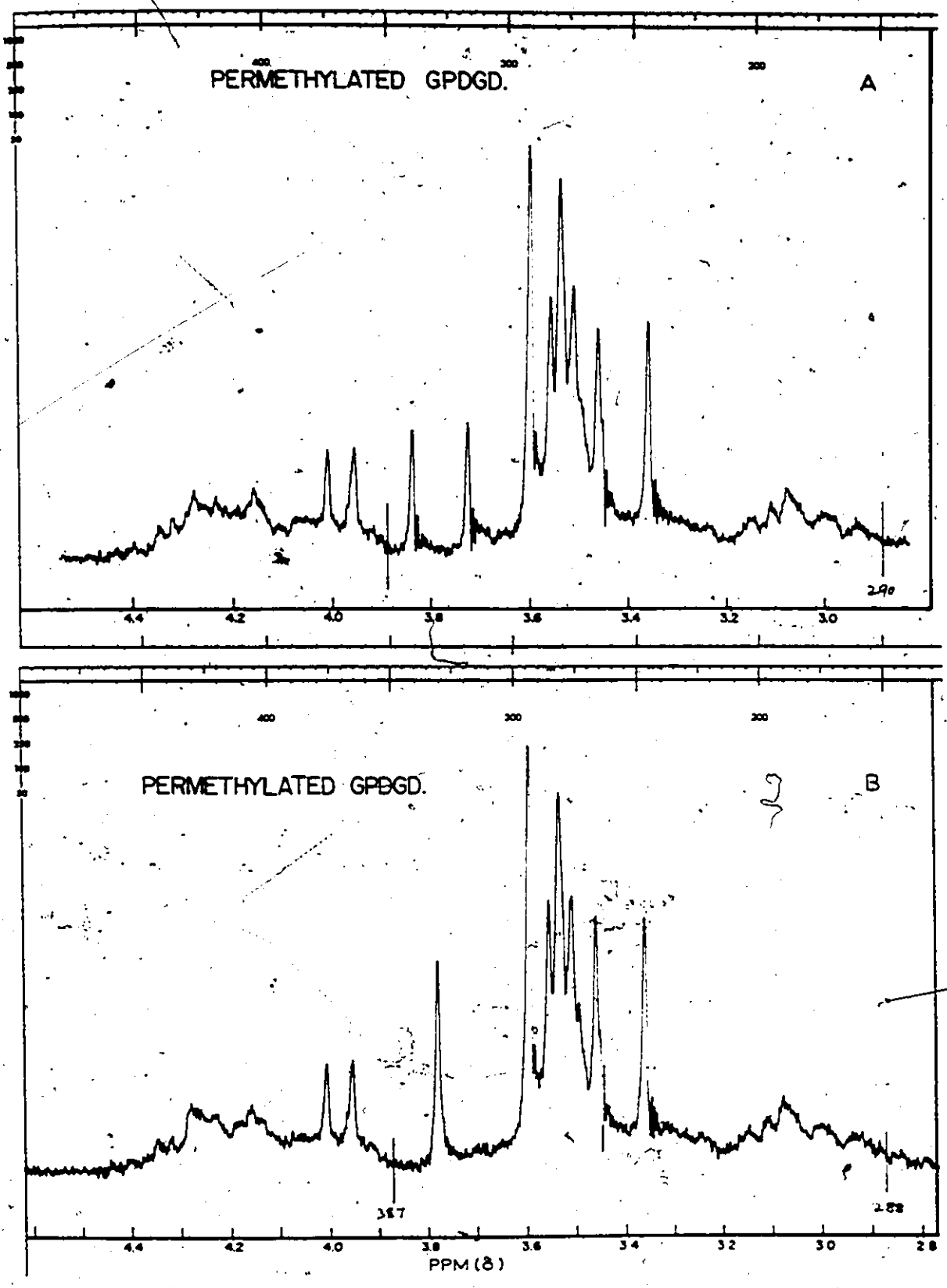

PERMETHYLATED GPDGD.
A
2.90
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
PERMETHYLATED GPDGD.
B
3.67
2.88
4.4
4.2
4.0
3.8
3.6
3.4
3.2
3.0
2.8
PPM (δ)

TABLE 24

Chemical Shifts and Assignments of NMR Signals

for Permethylated GPDGD*

Group	Chemical Shift (δ) ppm	Coupling Constant (J) Hz	No. of Protons** Calc.	Found
CH_3–, branches and terminals of fatty acid chains	0.81–0.95		12+	16
$-CH_2-$, fatty acid chain	1.23		48+	51
$-CH_2-\overset{O}{\overset{\|}{C}}-O$, fatty acyl esters	2.20–2.40		4	4.4
C-O-CH_3, sugar ring and glycerol	3.35–3.60		24	24
P-O-CH_3 coupled	3.73,3.84	11.5	3	2.6
decoupled	3.78			
Anomeric protons	3.95,3.99	6.5	2	1.8

*See Scheme 7 for the structure.

**24 methoxy protons per molecule taken as integral calibration standard.

+Calculated from fatty acid analysis data (Table 26).

glycerol moiety. Two sharp signals at 3.73δ and 3.84δ (equivalent to 3 protons) with a coupling constant (J) of 11.5 Hz were collapsed to a single peak (3.78δ) when the spectrum was simplified by ^{31}P-spin decoupling (Fig. 20B). This corresponds to the assignment for the P-O-CH_3 group (Kates and Hancock, 1971). The last two sharp signals at 3.95δ and 3.99δ (2.5 protons) with a coupling constant (J) of 6 Hz which were not changed when the spectrum was decoupled are assigned to the anomeric protons of the two sugar rings even though the coupling constant was slightly lower than that previously reported for anomeric protons of β-glycosidic linkage (7-7.5 Hz, Table 20).

After drastic acid hydrolysis of the permethylated GPDGD (1N HCl, 125°C, 48 hr in a sealed tube), followed by $NaBH_4$ reduction and acetylation of the reduction products obtained from the hydrolysis (Scheme 7), only one methylated sugar corresponding to the alditol acetate of 2,3,4-O-trimethyl glucose was detected by GLC. The other products obtained were the acetylated derivatives of 1,2-dimethyl glycerol and glycerol (Table 25).

The fact that only one partially methylated glucose, the 2,3,4-O-trimethyl derivative was obtained indicated that the two glucose moieties in GPDGD must be linked by a 1 → 6 glycosidic linkage and the C_6-position of the terminal glucose moiety must be attached to the α-glycerophosphate moiety. No other arrangement could yield only the 2,3,4-O-trimethyl glucose.

SCHEME 7

Methylation analysis of permethylated glycerophosphoryl diglucosyl diglyceride.

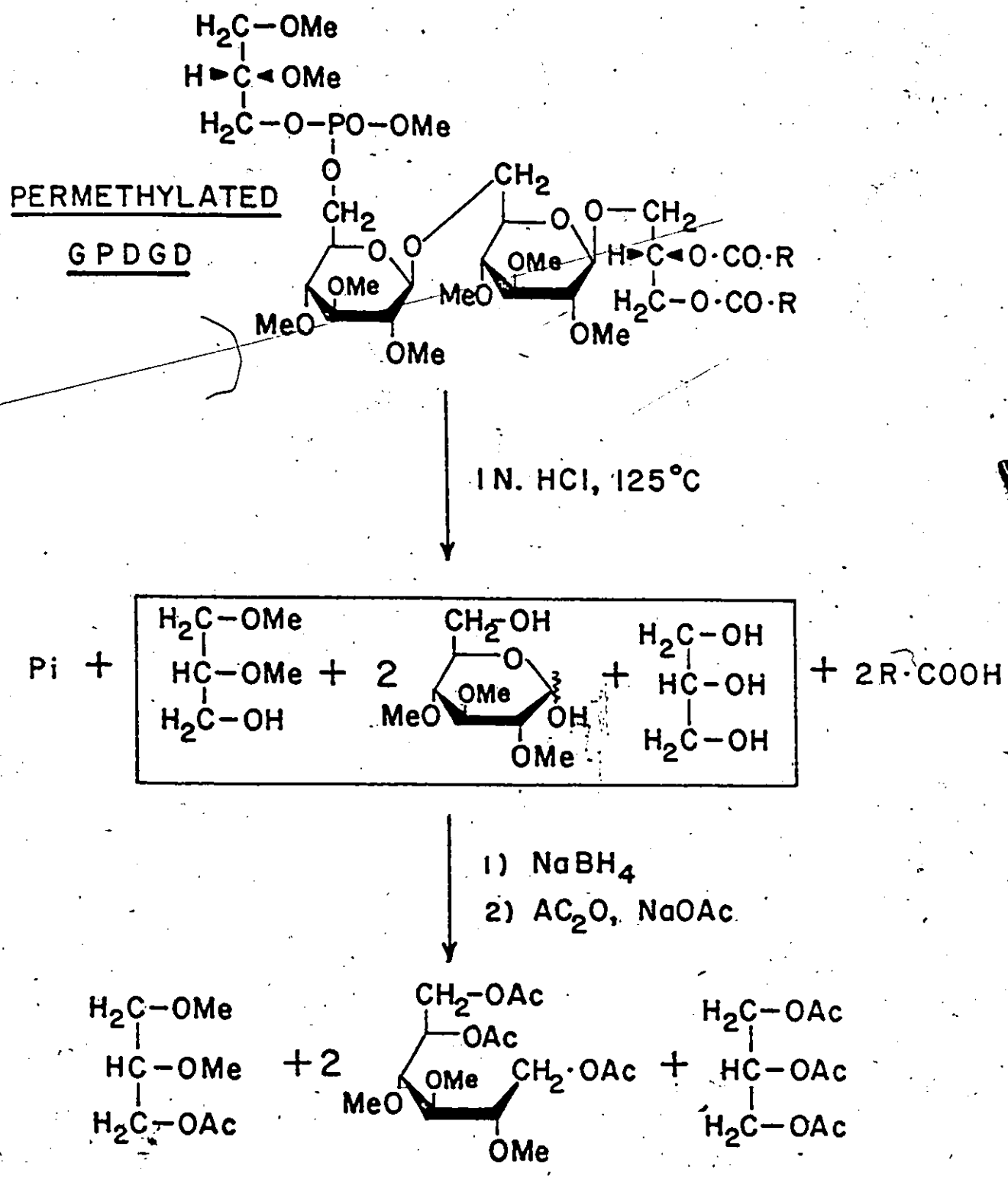

PERMETHYLATED
GPDGD
1 N. HCl, 125°C
Pi +
+ 2R·COOH
1) NaBH4
2) Ac2O, NaOAc
+2

TABLE 25

Hydrolysis Products of Permethylated GPDGD and DGG

Components[a]	Relative Retention[b]		
	Standard	GPDGD	DGG
1,2-dimethyl glycerol	0.05	0.06	0.05
glycerol	0.29	0.29	-
2,3,4,6-tetramethyl glucose	1.0	-	1.02
2,4,6-trimethyl glucose	1.82	-	-
2,3,6-trimethyl glucose	2.05	-	-
2,3,4-trimethyl glucose	2.17	2.17	2.19
3,4,6-trimethyl glucose	2.74	-	-

[a]Analyzed by GLC as the partially methylated alditol acetates on 10% BDS (Gas-Chrom W) at 200°C.

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

These studies, therefore, establish conclusively that in GPDGD the glucose units are joined by a 1 → 6 glycosidic linkage and that the α-GP is esterified on the -OH group of C_6 of the terminal glucose moiety, thus confirming the results obtained from quantitative periodate oxidation and Smith degradation studies.

G. Fatty Acid Composition of GPDGD

Gas liquid chromatographic analysis of the fatty acids of GPDGD showed that the major fatty acids were a 15:0 (60%), a 17:0 (23%), i 17:0 (10%) and 18:0 (4%) (Table 26). The amount of a 15:0 was essentially the same as in MGD but lower than that of PG and DGD (Table 26).

TABLE 26

Fatty Acid Composition of Purified Lipid Components from S. epidermidis

Expressed in % of total area

Fatty acid*	Retention relative** to methyl myristate		Lipid Component‡			
	BDS	SE-30	GPDGD	PG	DGD	MGD
i 14:0	0.85	0.91	0.2	-	0.2	0.2
14:0	1.0	1.0	-	-	-	0.1
i 15:0	1.20	1.24				
a 15:0	1.25	1.28	60.0+	74.6+	70.5+	57.8+
i 16:0	1.66	1.68	0.8	-	0.6	1.0
16:0	1.98	2.13	1.7	-	1.2	1.6
i 17:0	2.28	2.75	9.5	7.8	7.3	10.3
a 17:0	2.44		23.4	17.6	17.8	24.0
18:0	3.81	4.56	4.3	-	2.3	5.0

*See Table 12 for abbreviations.

**Conditions were the same as in Table 12.

‡See list of abbreviations.

+i-15:0 , was the minor component and was not detectable in presence of large proportion of a-15:0. See Table 31.

VI. Structural Studies of the Main Glycolipid (DGD)

A. Characterization of DGD

The TLC pure component gave analytical data (Table 27) for hexose, glycerol, fatty acyl esters, corresponding to those expected for a diglycosyl diglyceride (mole ratios; glycerol:hexose:acyl esters; found, 1:1.83:1.80, calcd., 1:2:2; Table 27). The compound had a specific rotation $[\alpha]_D^{25} = -18.2^\circ$ and molecular rotation, $[M]_D = -160.1$ (Table 27) when measured in chloroform.

The infrared spectrum of DGD (Fig. 21) showed strong OH absorption at 3400 cm^{-1}, strong CH_2 and CH_3 absorption at 2940, 2850 and 1470 cm^{-1}; $-CH_3$ branches at 1385 cm^{-1}; ester -C=O at 1740 cm^{-1} and C-O-C at 1180 cm^{-1}; C-O (secondary alcohol at ca. 1100 cm^{-1} and C-O (primary alcohol) as a shoulder at ca. 1020 cm^{-1} on the strong C-O (secondary alcohol).

B. Hydrolytic Degradation of DGD

The hydrolytic reactions used for structural determination of DGD are shown in Scheme 8.

1. Deacylation

Mild alkaline hydrolysis (Experimental Procedures II.A) of DGD gave a single water-soluble compound with R_{Glu} (solvent 1, Table 17) 0.20, R_f values in solvents 2 and 3 (Table 17) 0.43 and 0.45 respectively similar to that of

TABLE 27

Analysis of Purified Diglucosyl Diglyceride (DGD) and its Deacylated Product (DGG)

Composition	DGD		DGG	
	Found	Calc[a]	Found	Calc[b]
glycerol, %	11.7	10.5	21.4	22.1
hexose, %	41.0	41.0	86.4	86.5
ester, %	54.3	56.8	-	-
Mole ratio's				
glycerol:hexose:ester	1:1.83:1.80	1:2:2	1:2.08	1:2
$[\alpha]_D^{25}$	-18.2°[c]		-38.23°[d]	
$[M]_c$	-160.1		-159.0	-150.9[e]

a. For $C_{46}H_{86}O_{15}$ (M.W = 878)

b. For $C_{15}H_{28}O_{13}$ (M.W = 416)

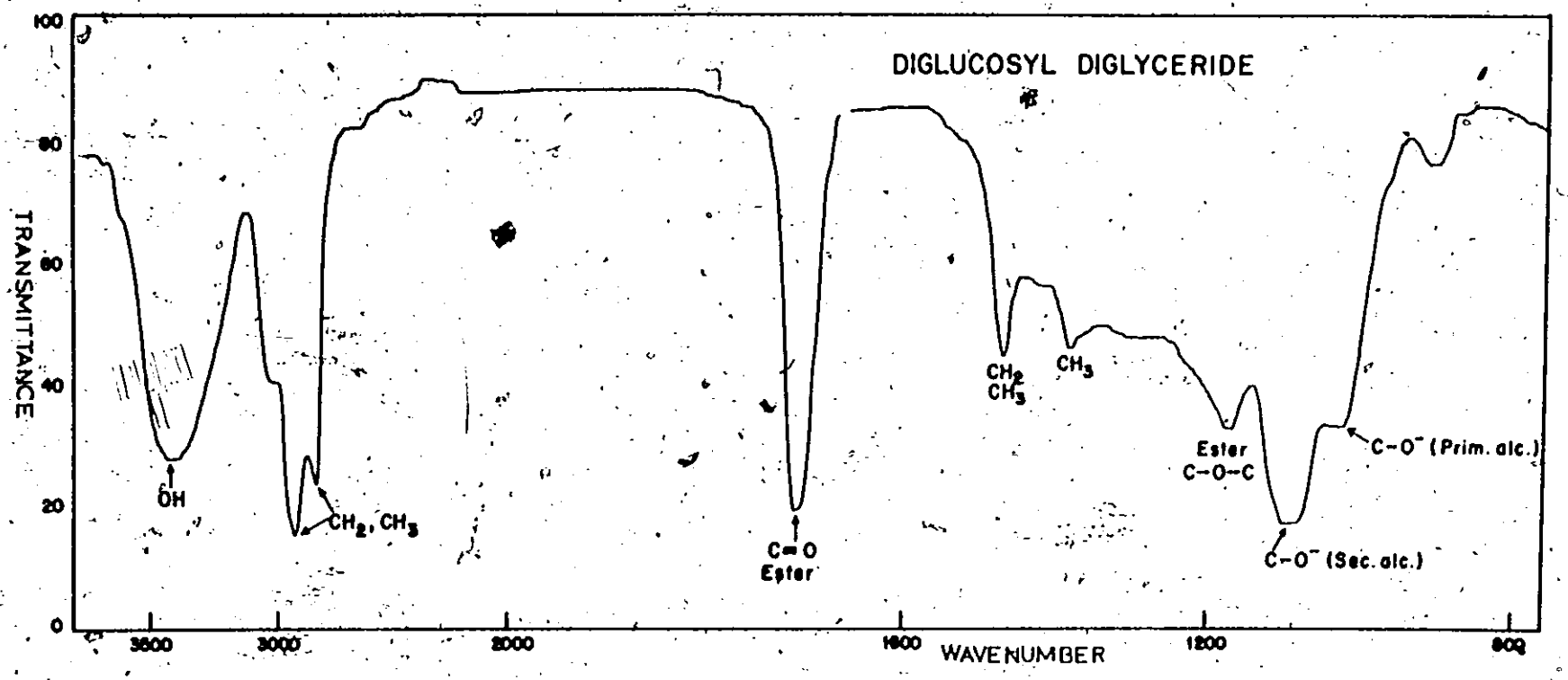
c. In $CHCl_3$

d. In H_2O

e. Calc. for all β-glycosidic linkages (see Table 19).

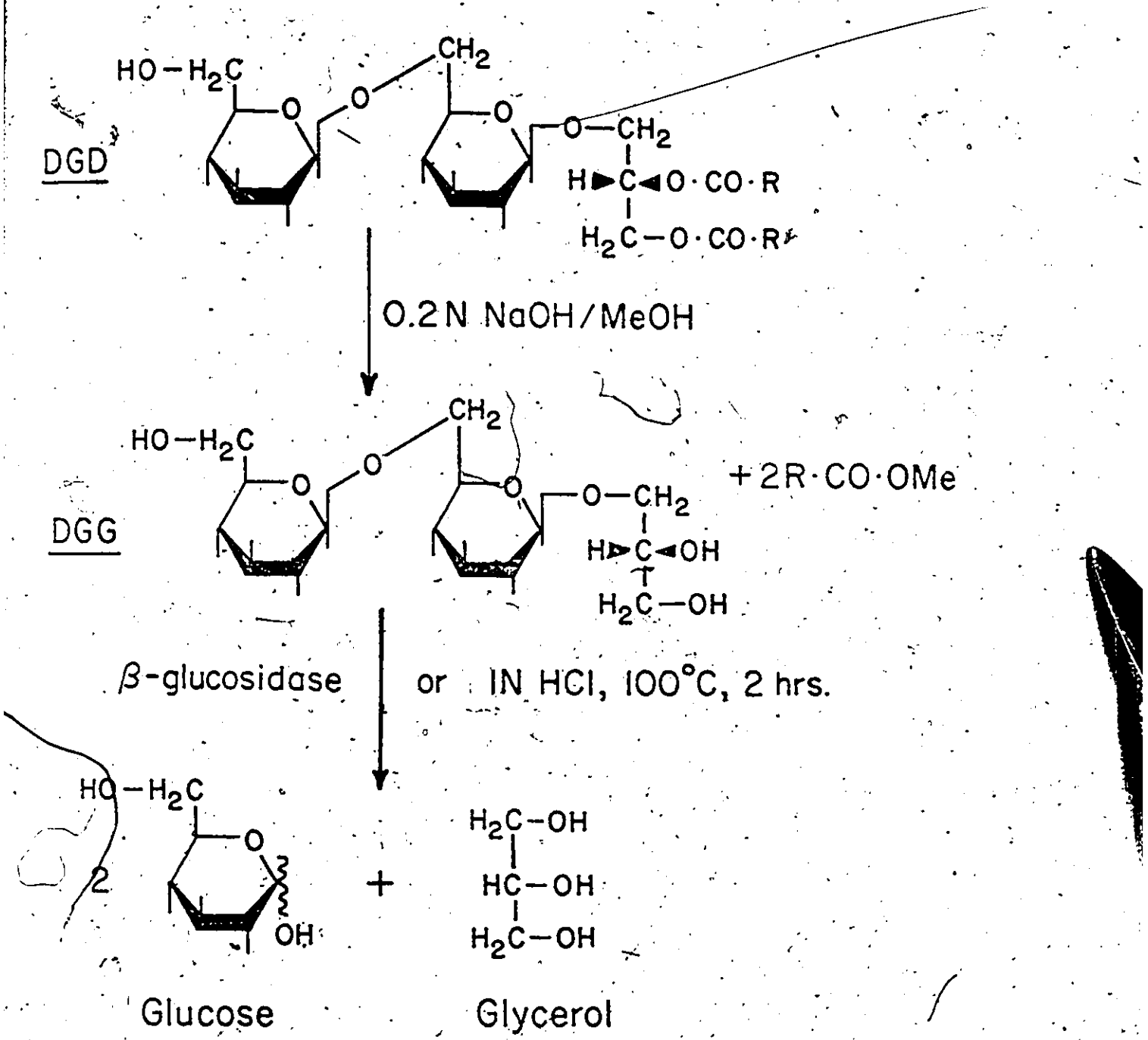
FIGURE 21

Infrared Spectrum of Diglucosyl Diglyceride in Chloroform


DIGLUCOSYL DIGLYCERIDE
TRANSMITTANCE
100
80
60
40
20
0
3600
3000
2000
1600
1200
800
WAVENUMBER
OH
CH2, CH3
C=O
Ester
CH2
CH3
CH3
Ester
C-O-C
C-O⁻ (Prim. alc.)
C-O⁻ (Sec. alc.)

SCHEME 8

Degradative sequences establishing the structure of diglucosyl diglyceride.


DGD
0.2N NaOH/MeOH
DGG
+2R·CO·OMe
β-glucosidase
or 1N HCl, 100°C, 2 hrs.
2
Glucose
Glycerol

G-Gal-Gal (Table 17). The deacylated product from DGD gave positive stain (yellow colour) with periodate-tolidine reagent and periodate-Schiff reagent (pink colour) and had the same chromatographic properties as those of the sugar-containing product obtained from strong alkaline hydrolysis of GPDGD (see Results V.B.5). The compound was found to be non-reducing to Fehling's reagent and had a specific rotation, $[\alpha]_D^{25} = -38.23^\circ$ in water and $[M]_D = -159.0$ (Table 27). It gave analytical data for hexose and glycerol in the molar ratio by 1:2.08 (Table 24) expected for a diglycosyl glycerol.

2. Acid Hydrolysis

Hydrolysis of DGD with 1N HCl (100°C) yielded two water-soluble products which were identified chromatographically as glucose and glycerol (solvent 1, Table 17). Therefore, it was revealed that the hexose moiety of DGD was glucose, the same as in GPDGD (see Results V.B.4). The chloroform soluble fraction was composed entirely of free fatty acids which had the same composition as those from the intact DGD (Table 26).

C. Determination of the Configuration of Glycosidic Linkages

Two glycosidic linkages are present in the structure of DGD, one between the two glucose units and the other between the disaccharide and the glycerol; as occurs also in

GPDGD. Both glycosidic linkages were found to have the β-configuration the same as in GPDGD by the following methods:

1. Treatment with α- or β-glucosidase

The deacylated product of DGD, i.e., DGG was found to be hydrolyzed by β-glucosidase and not by α-glucosidase. The products obtained were identified by paper chromatography as glucose and glycerol; approximately 2 moles glucose per mole DGG were released from DGG and the reaction was complete in 24 hr (Fig. 17).

Therefore, both glycosidic linkages in DGD are the β-configuration, as found also for DGG obtained from DPDGD by strong alkaline hydrolysis.

2. Optical Rotation

The specific rotation, $[\alpha]_D^{25}$ of the deacylated DGD (DGG) was -38.2° and the molecular rotation $[M]_D$ was -159.0 (Table 27). This value of $[M]_D$ was compared with the theoretical values calculated for various configuration of glycosidic linkages (see Results V.E.2 and Table 19). Since the observed $[M]_D$ value (-159.0) was closer to the value calculated for the β-linkages (-150.9) rather than those calculated for two α-linkages (+599.1) or one α- and one β-linkage (+224.1)(Table 19). This finding indicated that both glycosidic linkages in DGD have the β-configuration.

3. Nuclear Magnetic Resonance Studies

The NMR spectrum of DGG showed two doublets centered at 4.25 and 4.31 with the coupling constant 7.0 Hz (Fig. 22 and Table 20). As mentioned in the NMR studies of GPDGG (see Results V.E.3), these two doublets were due to the two anomeric protons of the two sugar moieties present in DGG. The coupling constant (J) and the relative location of these two doublets (i.e., upfield from HDO peak) were similar to those of the corresponding signals in the spectrum of the model compound, β-methyl glucoside but were different from those for the α-methyl glucoside (Table 20).

These results together with those from specific glucosidase studies and optical rotation measurements, therefore show that the configuration of both glycosidic linkages in DGD have the β-configuration, the same as in GPDGD.

D. Determination of the Position of the Glycosidic Linkage

The linkage position between the two glucose moieties of DGD was found to be the same as those in GPDGD by the following methods:

1. Periodate Consumption and Smith Degradation

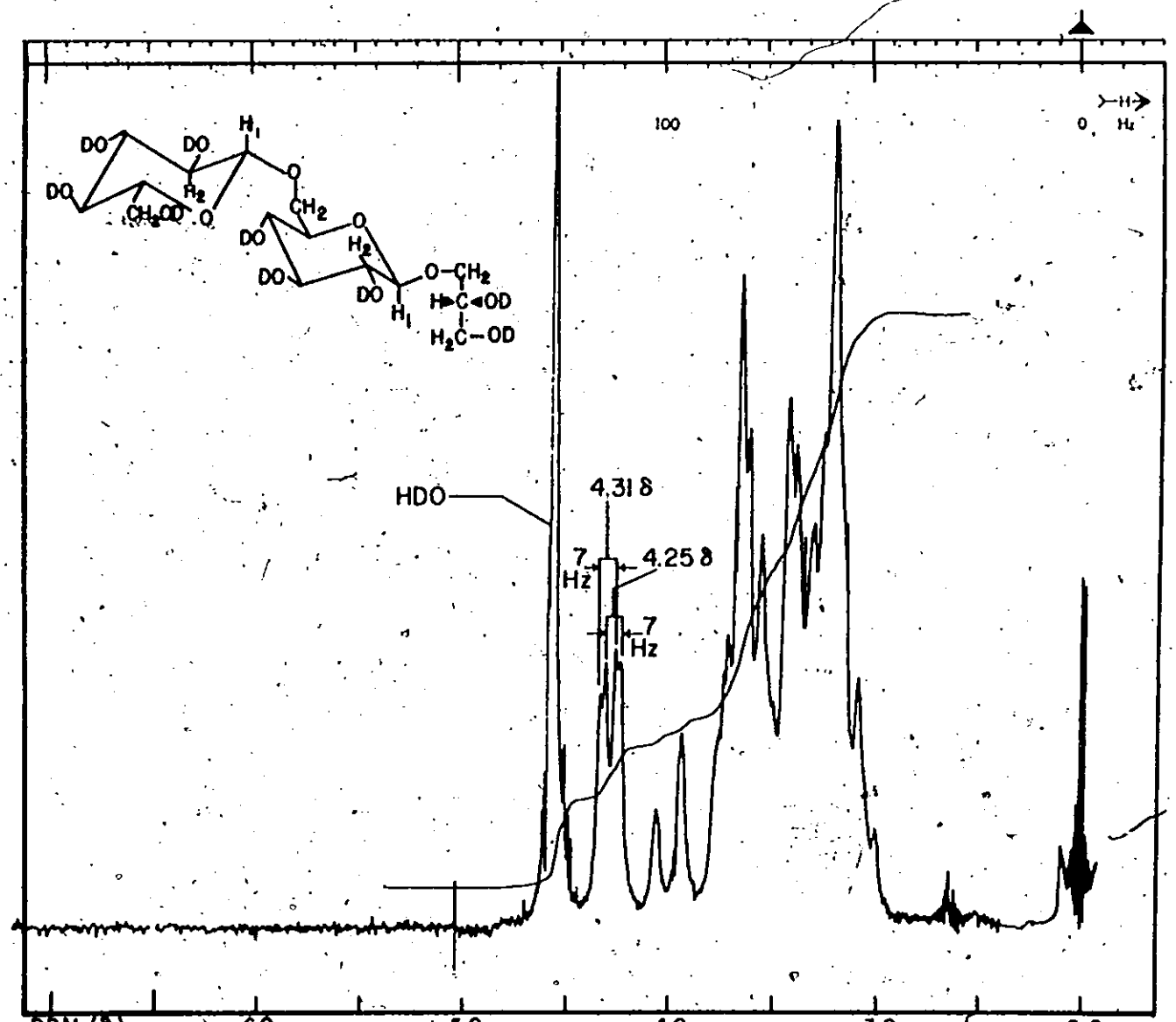
DGG consumed 4.85 moles of $NaIO_4$ per mole of the compound with the liberation of 1.15 moles of formaldehyde (Table 21). When the oxidation products were reduced with

FIGURE 22

100 Mc NMR Spectrum of Diglucosyl Glycerol (DGG)

Solvent	D_2O
Temperature	Ambient
Sweep width	1000 Hz
Sweep time	500 sec
Lock signal	Acetone

See Table 20 for assignment of peaks


100
0 Hz
HDO
4.31 δ
7 Hz
4.25 δ
7 Hz
PPM (δ)
6.0
5.0
4.0
3.0
2.0

$NaBH_4$, followed by acid hydrolysis (Smith degradation, Scheme 9), only glycerol was detected and no glucose or erythritol were found when examined by paper chromatography or GLC of their acetylated derivatives (Table 23). As discussed before (see Results V.F.2), the absence of erythritol and glucose excluded the possibility of a 1 → 3 and 1 → 4 linkage (Table 22) and the consumption of about 5 moles of $NaIO_4$ per mole of DGG indicated a 1 → 6 linkage and not a 1 → 2 linkage (Table 21).

The lack of reducing ability of DGG (negative Fehling's test) and a rapid staining with periodate-Schiff reagent indicated that the other glycosidic linkage in DGG is between C_1 of glucose and C_1 or C_3 of glycerol.

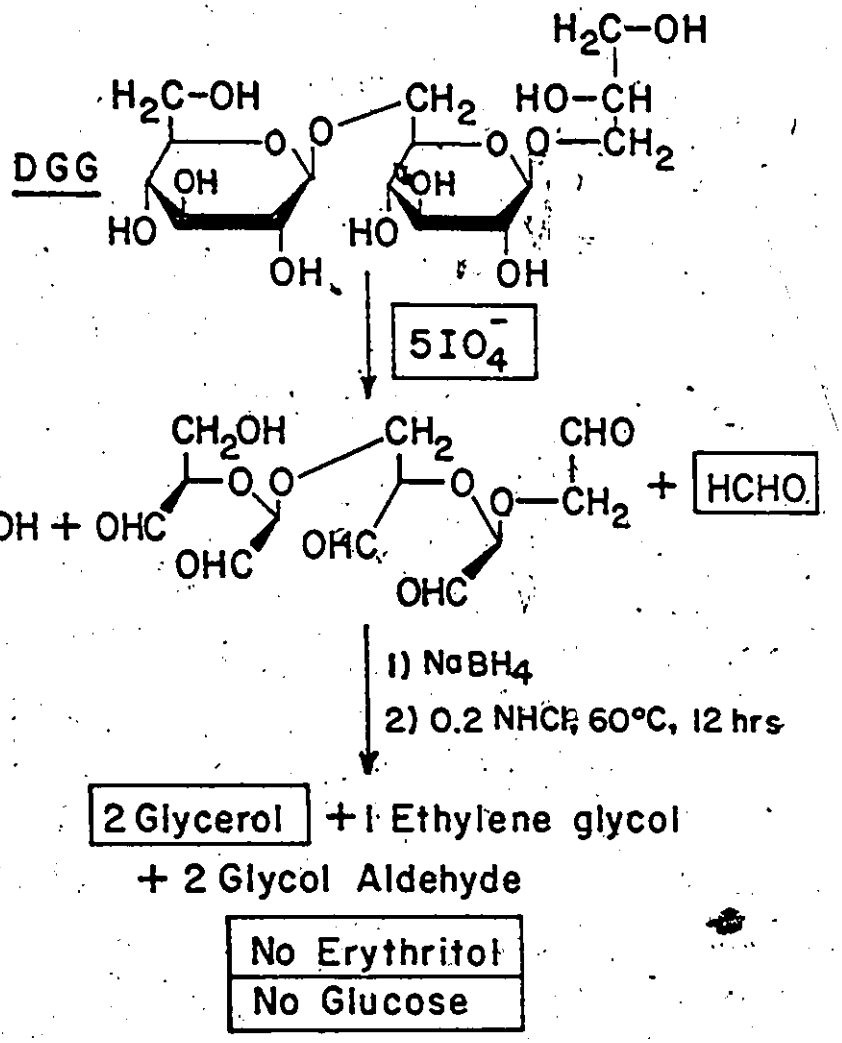
Therefore, these studies showed that the glycosidic linkages position in DGG were also glucose (1 → 6) glucose (1 → 1 or 3) glycerol, the same as those in GPDGD.

2. Methylation Studies

The product obtained by exhaustive methylation of DGG (Experimental Procedures II.F.1), showed no free OH absorption in the infrared. Acid hydrolysis of the permethylated DGG, yield partially methylated sugars corresponding to 2,3,4,6-O-tetramethyl glucose and 2,3,4-O-trimethyl glucose (Scheme 8) which were detected by GLC of their methyl glycosides (Table 28) or of alditol acetate derivatives (Table 25). In addition, 1,2-di-O-methyl glycerol was detected as the acetylated derivative. The formation of

SCHEME 9

Reactions represent "Smith Degradation" process of Diglucosyl glycerol.


DGG
$5IO_4^-$
2HCOOH + OHC
HCHO
1) $NaBH_4$
2) 0.2 NHCl, 60°C, 12 hrs
2 Glycerol + 1 Ethylene glycol
+ 2 Glycol Aldehyde
No Erythritol
No Glucose

SCHEME-10

Methylation analysis of permethylated diglucosyl glycerol.

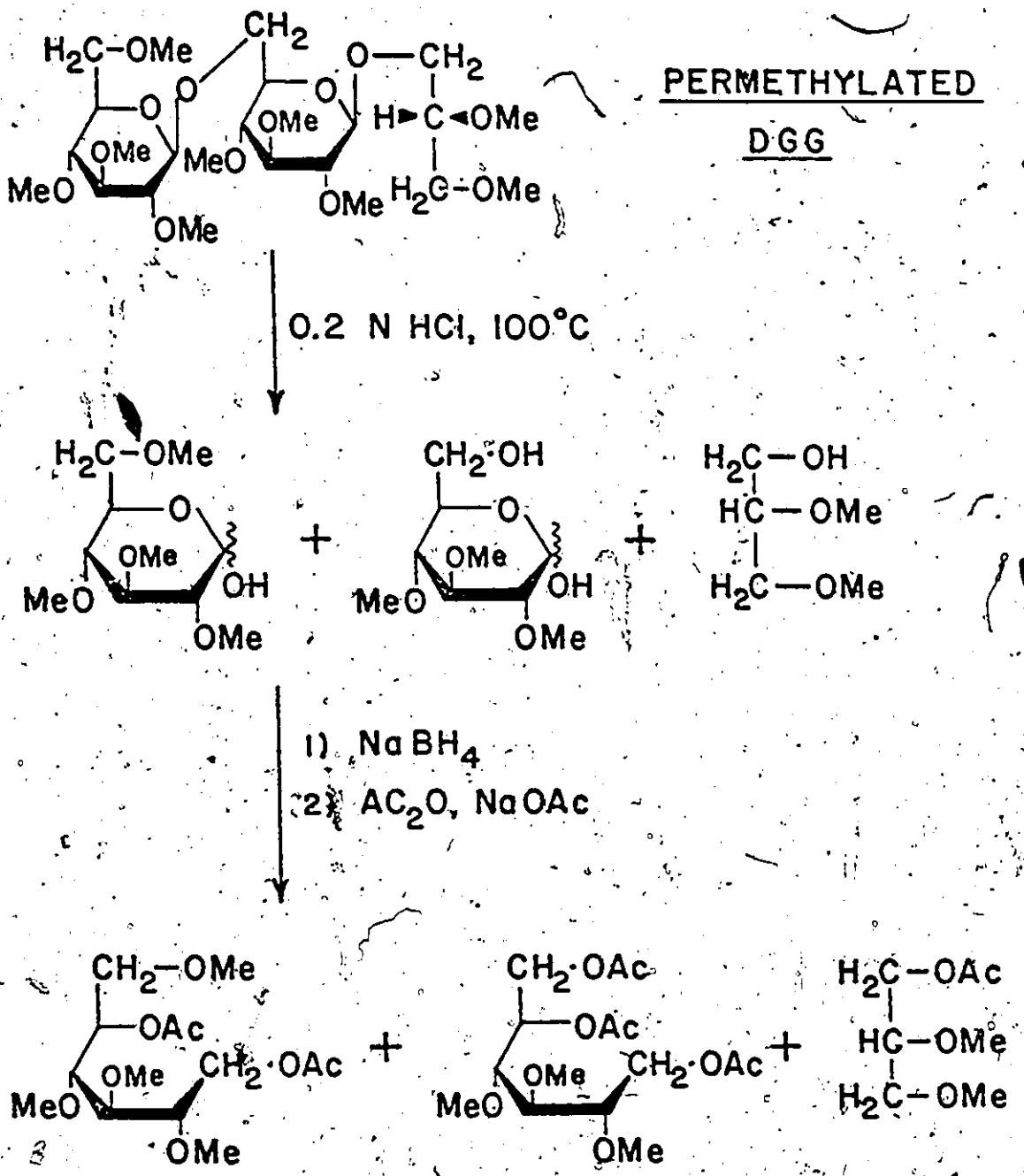

PERMETHYLATED
DGG
0.2 N HCl, 100°C
1) NaBH4
2) Ac2O, NaOAc

TABLE 28

Relative Retention Times on GLC* of Methyl Glycosides of Partially Methylated Sugars Obtained from Acid Hydrolysis of Permethylated DGG

Methyl glycoside	Relative Retention Times**	
	Standards	From DGG
2,3,4,6-tetra-O-methyl-D-glucose	1.00 1.36	1.06 1.40
2,3,4-tri-O-methyl-D-glucose	1.98 2.63	2.00 2.63
3,4,6-tri-O-methyl-D-glucose	2.36 2.55	
2,3,6-tri-O-methyl-D-glucose	2.54 3.18	

*Column 3% ECNSS-M on Gas Chrom. A at 175°C.

**Values are relative to 2,3,4,6-tetra-O-methyl-β-D-glucopyranoside with a retention time of 10 min. Each methyl glycoside gives two peaks (α- and β-anomers).

these partially methylated products indicate that both glucose moieties must be joined by a 1 → 6 linkage and internal glucose is joined to the C_3-OH (or C_1-OH) group of the glycerol moiety of DGG.

The three methods, quantitative uptake of sodium periodate, Smith degradation and the methylation of DGG thus establish the presence of a 1 → 6 linkage between the two sugar moieties in DGD. The DGG moiety of DGD must therefore be the same as the DGD moiety in the structure of GPDGD.

E. Fatty Acid Composition of DGD

Gas liquid chromatographic analysis of the fatty acids of DGD showed that the major acids were a 15:0 (71%), a 17:0 (18%), i 17:0 (7%) and 18:0 (2%)(Table 26). The fatty acid composition of DGD was similar to that of PG but rather different in the percentage of main fatty acids (a 15:0 and a 17:0) from those of GPDGD and MGD.

VII. Structural Studies of the Minor Glycolipid (MGD)

A. Characterization of MGD

The TLC pure component gave analytical data (Table 29) for hexose, glycerol, fatty acyl esters, corresponding to those expected for a monoglycosyl diglyceride (mole ratios; glycerol:hexose:acyl esters, found, 1:1.06:2.16; calcd., 1:1:2; Table 29).

The infrared spectrum of MGD (Fig. 23) showed free OH absorption at 3400 cm^{-1}, strong CH_2 and CH_3 absorption at 2940, 2850 and 1470 cm^{-1}; $-CH_3$ branches at 1385 cm^{-1}; ester C=O at 1740 and C-O at 1180 cm^{-1}; C-OH (secondary alcohol) may be discerned at ca. 1110 as a shoulder on the strong C-O (primary alcohol) band at 1075 cm^{-1}.

B. Hydrolytic Degradation of MGD

The hydrolytic reactions used for structural determination of MGD are shown in Scheme 11.

1. Deacylation

Mild alkaline hydrolysis of MGD gave a single water-soluble product with R_{Glu} 0.70 in solvent 1 and R_f 0.58 in solvent 2 (see Table 17). It rapidly developed a pink colour with the periodate-Schiff reagents, indicative of formaldehyde produced from a 1-substituted glycerol (Roberts et al., 1963) and was found to be non-reducing to Fehling's solution. Since there was not enough material, optical rotation measure-

TABLE 29

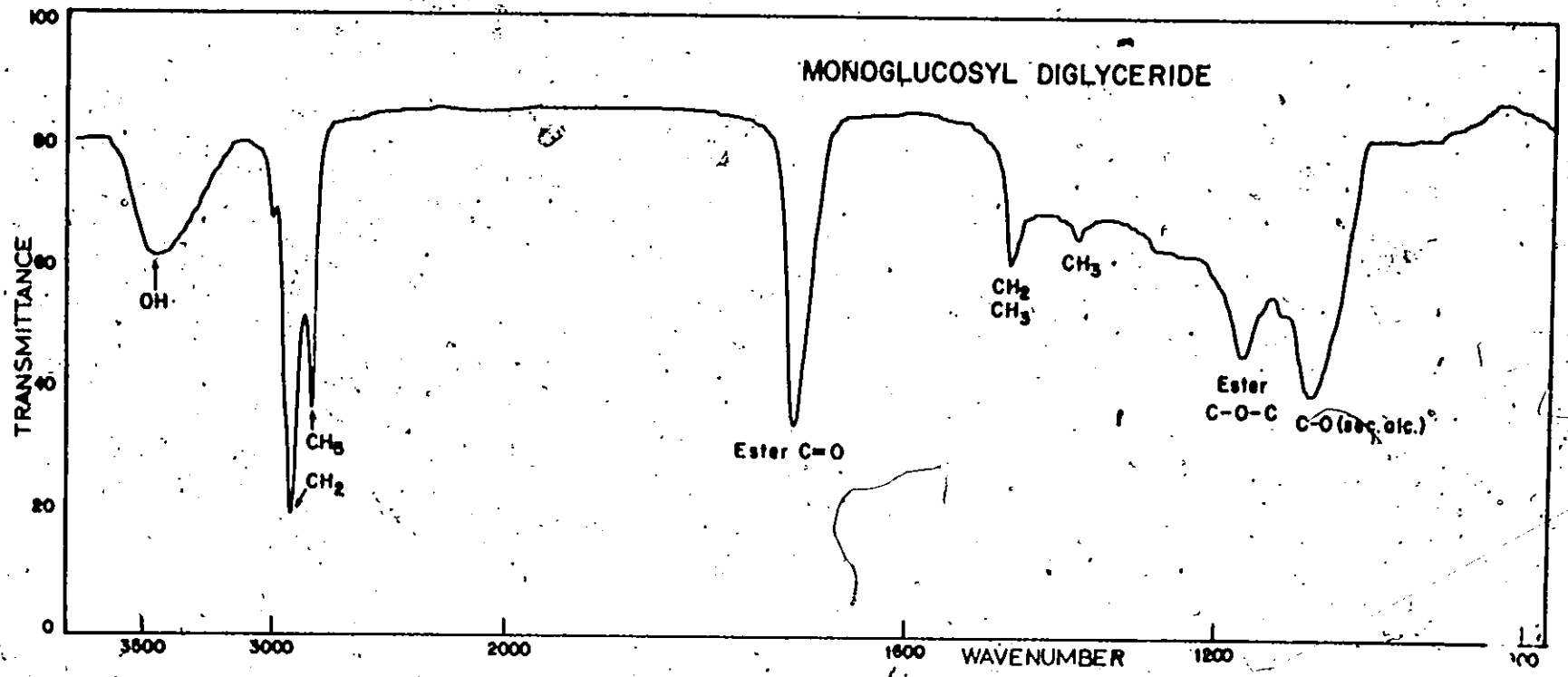
Analysis of Purified Monoglucosyl Diglyceride (MGD)

Composition	MGD	
	Found	Calc*
glycerol, %	12.7	13.7
hexose, %	23.4	24.8
ester, %	66.8	70.0
Mole ratio's		
glycerol:hexose:ester	1:1.06:2.16	1:1:2

*For $C_{41}H_{78}O_{10}$ (M.W. = 730.0)

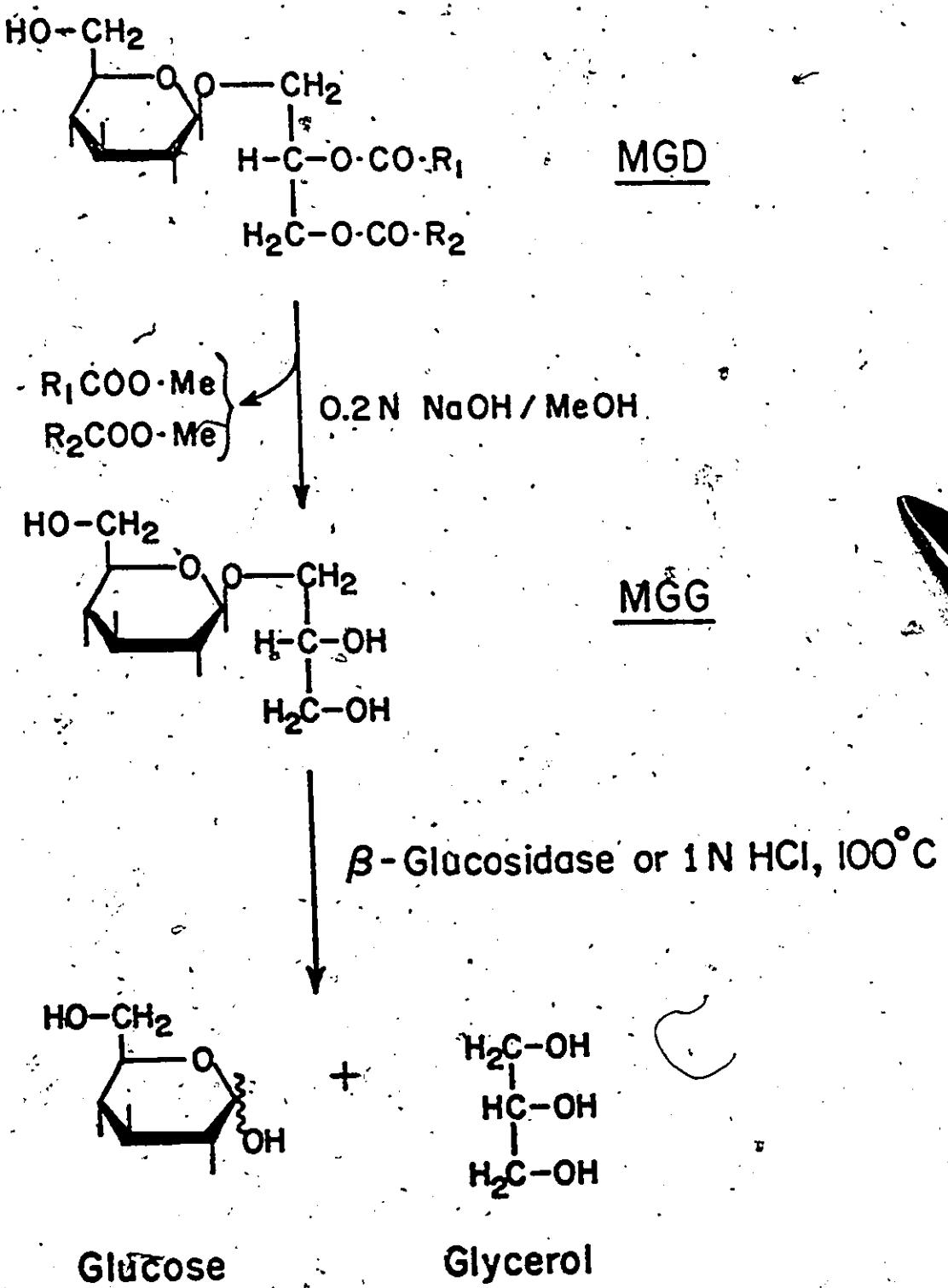
FIGURE 23

Infrared Spectrum of Monoglucosyl Diglyceride in Chloroform


MONOGLUCOSYL DIGLYCERIDE
TRANSMITTANCE
100
80
60
40
20
0
OH
CH3
CH2
Ester C=O
CH2
CH3
CH3
Ester
C-O-C
C-O (sec. alc.)
3600
3000
2000
1600
1200
WAVENUMBER

SCHEME 11

Degradative sequences establishing the structure of monoglucosyl diglyceride.


MGD
$R_1COO\cdot Me$
$R_2COO\cdot Me$
0.2 N NaOH / MeOH
MGG
β-Glucosidase or 1 N HCl, 100°C
Glucose
Glycerol

ment and constituent analysis (hexose, glycerol) could not be done.

2. Acid Hydrolysis

Hydrolysis of MGD with 1N HCl (100°C) yielded two water-soluble products which were identified chromatographically as glucose and glycerol. The chloroform-soluble fraction was composed entirely of free fatty acids which had the same composition as that from the intact MGD (Table 26).

C. Configuration and the Position of Glycosidic Linkage

Only one glycosidic linkage is present in the structure of MGD, i.e., between glucose and glycerol moieties. Since there was not enough material, only treatment with specific α- and β-glucosidase was used to determine the configuration. The deacylated product of MGD was found to be hydrolyzed by β-glucosidase and not by α-glucosidase. The products obtained were identified by paper chromatography as glucose and glycerol.

Since the deacylated compound was found to be non-reducible by Fehling's solution and rapidly gave pink when stained with periodate-Schiff reagent, therefore it was assumed that the glycosidic linkage is between C_1 of glucose and C_3 (or C_1) of glycerol, i.e., glucose (1 → 1 or 3)glycerol.

D. Fatty Acid Composition of MGD

Gas liquid chromatographic analysis of the fatty acids of MGD showed that the major acids were a 15:0 (58%), i 17:0 (10%), a 17:0 (24%) and 18:0 (5%)(Table 26), similar to those observed in GPDGD.

VIII. Structural Studies of the Main Phospholipids (PG)

A. Characterization of PG

The TLC pure component gave analytical data for phosphorus, glycerol, fatty acyl esters, corresponding to those expected for phosphatidylglycerol (mole ratios; phosphorus:glycerol:acyl esters, found 1:1.93:1.90; calcd., 1:2:2; Table 30). The compound had a specific rotation, $[\alpha]_D^{25} = +7.97^\circ$ in chloroform and a calculated molecular rotation, $[M]_D = 57.9$ (Table 30). The fatty acids in this bacterial lipid were isolated quantitatively and consisted mainly of C_{15} and C_{17} branched acids, predominantly the anteiso-acid (Table 26); the mixed acids were optically active ($[\alpha]_D^{25}$ of methyl esters in chloroform $+10.2^\circ$).

The infrared spectrum of PG (Fig. 24) showed OH absorption at 3250 cm^{-1}, strong CH_2 and CH_3 absorption at 2940, 2850 and 1470 cm^{-1}, CH_3 branch absorption at 1385 cm^{-1}. A broad band between 1230-1180 cm^{-1} were ester C-O-C and phosphate P=O, a weak phosphate $P-O^-$ absorption may be discerned at 1090 cm^{-1} as a shoulder on the strong P-O-C band at 1065 cm^{-1}.

B. Hydrolytic Degradation of PG

Structural determination of PG was based on both non-enzymatic and enzymatic hydrolysis studies as shown in Scheme 12.

TABLE 30

Analysis of purified Phosphatidyl Glycerol (PG) and its deacylated product (GPG)

Composition	PG		GPG	
	Found	Calc*	Found	Calc**
P, %	4.26	4.24	11.9	11.8
Glycerol, %	24.3	25.2	67.8	70.0
Ester, %	65.7	68.9	-	-
Mole ratio's				
P:glycerol:ester	1:1.93:1.90	1:2:2	1:1.94	1:2
[α]	+7.97+	-	0.0†	0.0
[M]	+58.2		0.0	0.0

*for $C_{37}H_{73}O_{10}P.NH_4$ (M.W = 730.6)

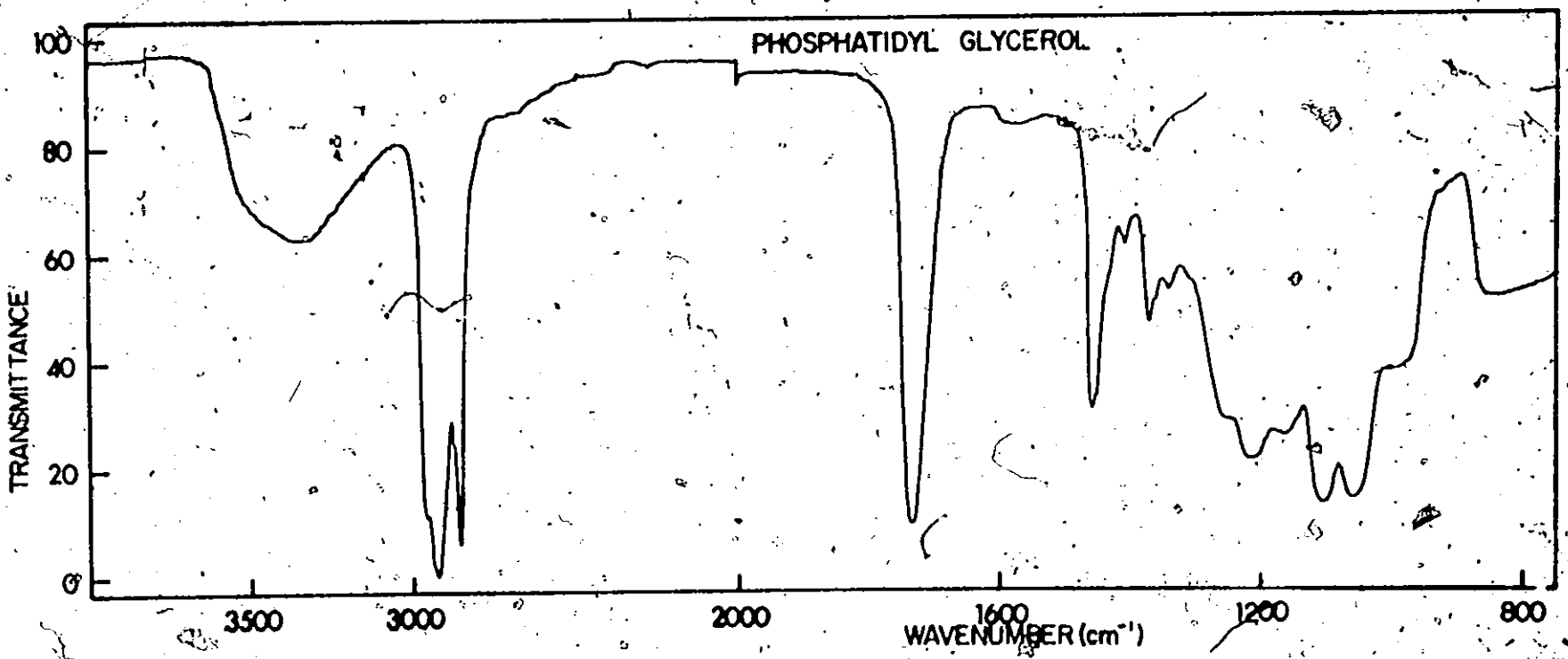
**for $C_6H_{14}O_8P.NH_4$ (M.W = 263)

+in $CHCl_3$

†in water

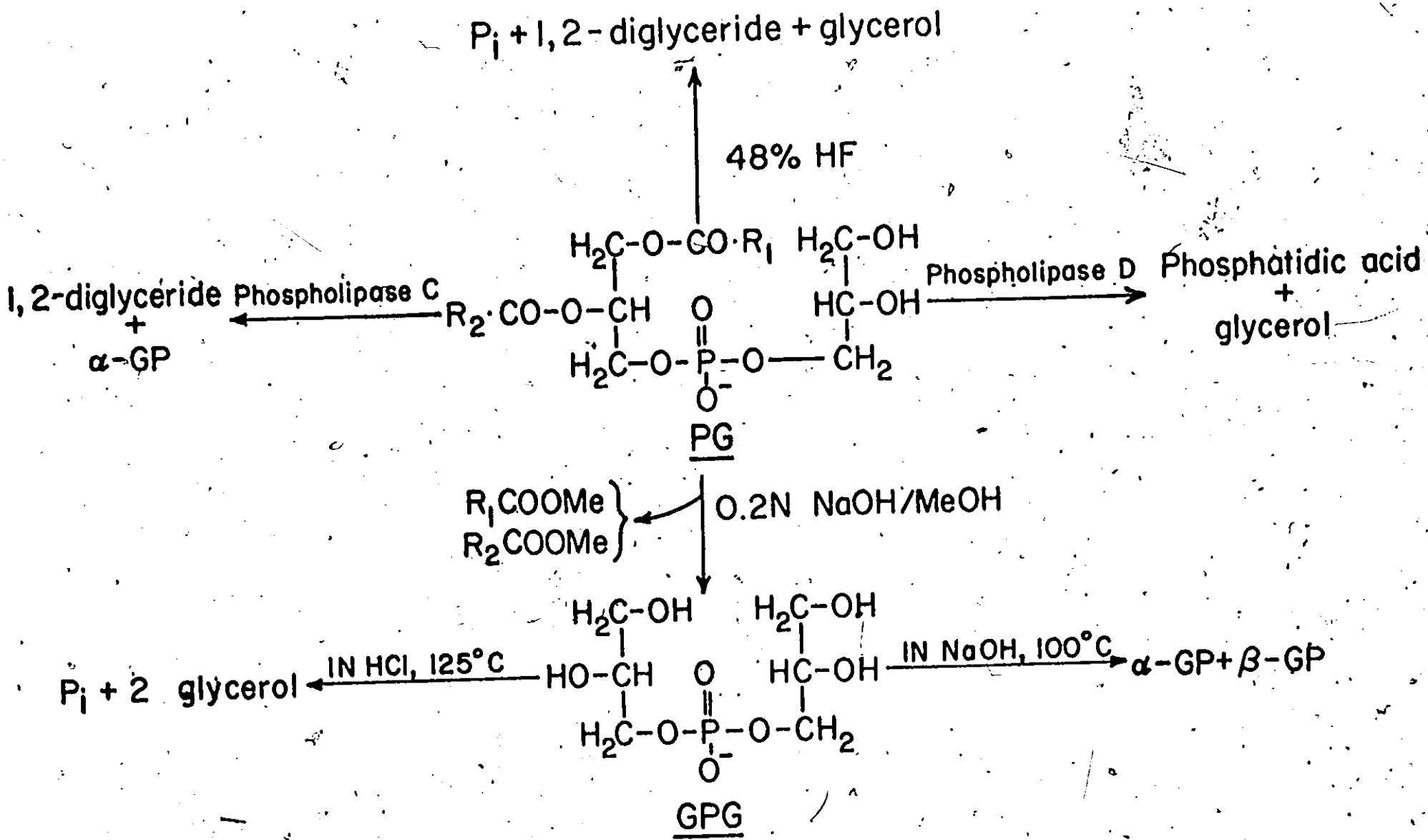
FIGURE 24

Infrared Spectrum of Phosphatidylglycerol (sodium salt form) in carbontetrachloride


PHOSPHATIDYL GLYCEROL
TRANSMITTANCE
100
80
60
40
20
0
3500
3000
2000
1600
1200
800
WAVENUMBER (cm⁻¹)

SCHEME 12

Degradative sequences establishing the structure of phosphatidylglycerol.


Pi + 1,2-diglyceride + glycerol
48% HF
H2C-O-CO·R1
H2C-OH
1,2-diglyceride
+
α-GP
Phospholipase C
R2·CO-O-CH
O
HC-OH
Phospholipase D
Phosphatidic acid
+
glycerol
H2C-O-P-O-CH2
O-
PG
R1COOMe
R2COOMe
0.2N NaOH/MeOH
H2C-OH
H2C-OH
Pi + 2 glycerol
1N HCl, 125°C
HO-CH
HC-OH
1N NaOH, 100°C
α-GP+β-GP
H2C-O-P-O-CH2
O-
GPG

1. Deacylation

Mild alkaline hydrolysis (0.2 N NaOH/MeOH) of PG gave a single water-soluble compound, having R_f value in solvent 2, Table 17, identical to that of the deacylated compound of PG isolated from E. coli (R_f 0.36). The compound gave a pink stain with the periodate-Schiff reagent which developed more rapidly than with the intact lipid (PG) indicating that the deacylated compound contained more terminal vicinal OH groups than the parent lipid. These results are consistent with the deacylated product being GPG.

The deacylated compound contained inorganic phosphate and glycerol in the molar ratio of 1:1.94 (Table 30) and was optically inactive, indicating that two α-substituted glycerols must have opposite configurations.

2. Treatment with 48% HF

As mentioned for GPDGD (see Results V.B.1), after treatment of PG with 48% HF, 1,2- and 1,3-diglycerides were detected in the chloroform phase (Figs. 15 and 16) and glycerol and P_i were detected in the aqueous phase. The liberation of diglyceride indicated that the two fatty acyl groups present in the lipid (Table 30) were both esterified on one of the two glycerol moieties as expected for a PG and not for a lyso-bis-phosphatidic acid.

3. Enzymatic Hydrolysis of PG

PG from S. epidermidis was found to be completely hydrolyzed by phospholipases A, C and D. The reactions are indicated in Scheme 4.

Treatment of PG with phospholipase A yielded two chloroform-soluble products which were identified chromatographically as lyso PG and free fatty acid (Fig. 25A). Reaction of PG with phospholipase C produced a water-soluble product, which was paper chromatographically identical to standard α-glycerophosphate (solvent 2, Table 17), and a chloroform-soluble product identified by TLC as 1,2-diglyceride (Fig. 25B). With phospholipase D, phosphatidic acid was detected as the chloroform-soluble product (Fig. 25C) and free glycerol was detected as the water-soluble product (solvent 2, Table 17).

The results of the enzymatic hydrolyses together with those from the HF hydrolysis and the periodate-Schiff staining behaviour confirm the presence in the PG component of a 1,2-diacyl-sn-glycero-phosphoryl group linked to an α-hydroxy group of glycerol.

4. Strong Alkaline Hydrolysis

Drastic aqueous alkaline hydrolysis of PG (1 N NaOH, 100°C) or its deacylated product yielded only one water-soluble component identified by paper chromatography as glycerophosphate (solvent 2, Table 17).

FIGURE 25

Thin-layer chromatograms of the chloroform-soluble products obtained from enzymatic hydrolyses of PG with phospholipases A, C and D.

A. Treatment with phospholipase A

1. untreated-PG
2. chloroform-soluble products of PG hydrolysis : lyso PG (lower spot) and free fatty acid (at solvent front)

Solvent system: chloroform-methanol-90% acetic acid (30:4:20, v/v)

B. Treatment with phospholipase C

1. untreated PG
2. 1,2-diglyceride obtained from treated-PG
3. standard 1,2-dipalmitin

Solvent system: n-hexane-ethyl ether-glacial acetic acid (70:30:1, v/v)

C. Treatment with phospholipase D

1. phosphatidic acid obtained from treated-PG
2. untreated-PG
3. standard phosphatidic acid

Solvent system: chloroform-methanol-28% ammonia (65:35:5, v/v)

FIGURE 25

Thin-layer chromatograms of the chloroform-soluble products obtained from enzymatic hydrolyses of PG with phospholipases A, C and D.

A. Treatment with phospholipase A

1. untreated-PG
2. chloroform-soluble products of PG hydrolysis: lyso PG (lower spot) and free fatty acid (at solvent front)

Solvent system: chloroform-methanol-90% acetic acid (30:4:20, v/v)

B. Treatment with phospholipase C

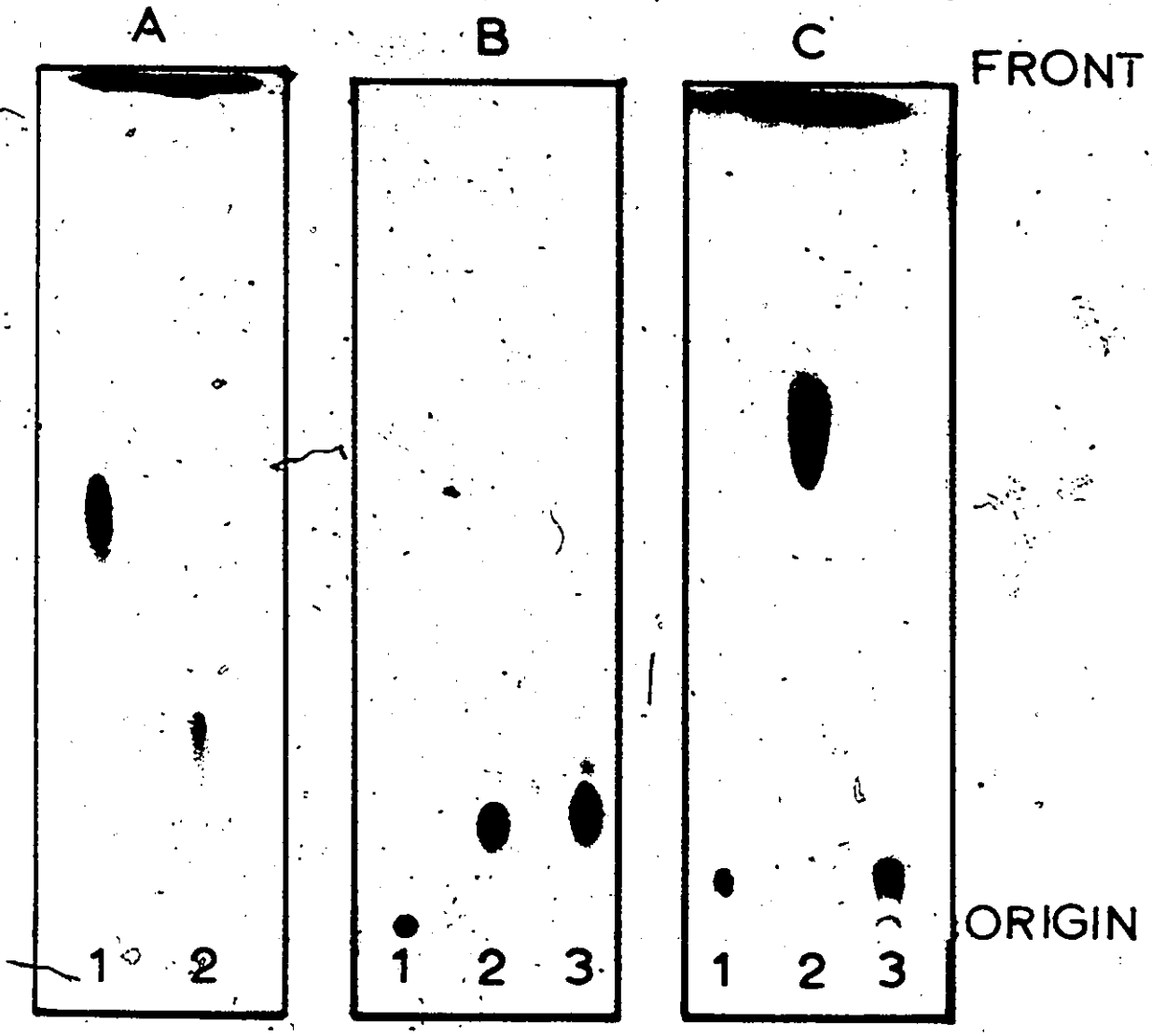
1. untreated PG
2. 1,2-diglyceride obtained from treated-PG
3. standard 1,2-dipalmitin

Solvent system: n-hexane-ethyl ether-glacial acetic acid (70:30:1, v/v)

C. Treatment with phospholipase D

1. phosphatidic acid obtained from treated-PG
2. untreated-PG
3. standard phosphatidic acid

Solvent system: chloroform-methanol-28% ammonia (65:35:5, v/v)


A
B
C
FRONT
ORIGIN
1 2
1 2 3
1 2 3

The glycerophosphate obtained consisted of two isomers: α-isomer (41%) and β-isomer (59%) (Table 18). These two isomers accounted for 99% of the total water-soluble P (Table 18). The glycerophosphate and glycerol moieties of PG were present originally as the α-isomer, in view of the rapidly staining pink with periodate-Schiff-reagent of its deacylated compound; the β-isomer arose from isomerization during the alkaline hydrolysis, (see Results V.B.5 and Scheme 5).

C. The Configuration of PG

In regard to the configuration of the phosphatidyl moiety of PG, the experiment with snake venom phospholipase A indicated the 3-sn-phosphatidyl configuration, since the enzyme only acts on 1,2-diacyl derivatives of sn-glycero-3-phosphate but not on those of sn-glycero-1-phosphate (Van Deenen and De Haas, 1963). The optical inactivity of the deacylated compound from PG (Table 30) indicated that the second glycerol moiety must have the configuration opposite to the first one, i.e., sn-glycero-1-phosphate. This was confirmed by the finding that the α-glycerophosphate obtained after strong alkaline hydrolysis of PG was a mixture of sn-glycero-3-phosphate (48%) and sn-glycero-1-phosphate (52%) (Table 18).

The experiment described above unambiguously establish the structure of PG from S. epidermidis as

1,2-diacyl-sn-glycero-3-phosphoryl-1'-glycerol, the same as reported before by Benson and Miyano (1961) for PG from photosynthetic tissue and confirmed by Haverkate and Van Deenen (1965).

D. Fatty Acid Composition of PG

GLC analysis of the fatty acids of PG showed that the major acids were a 15:0 (74.6%), i 17:0 (7.8%) and a 17:0 (18%) (Table 26), similar to those observed in other lipid components of S. epidermidis, except 18:0 was not detected.

The positional distribution of the fatty acids was investigated after hydrolysis of PG by phospholipase A from snake venom which liberates specifically the fatty acids from the 2-position (Van Deenen and De Haas, 1963). After separation of the hydrolysis products (lyso PG and fatty acids) on TLC (Fig. 25A), GLC analyses showed that the 2-position contained exclusively the a 15:0 fatty acids (Table 31, Fig. 26). Position 1, however, contained both i 15:0 (15%) and a 15:0 (21%) as well as both i 17:0 (18%) and a 17:0 (40%). In total fatty acids of PG, i 15:0 was present in very small amount (11%) compared to a 15:0 (89%) (Table 26), and the column used was not sufficiently efficient to separate them. However, when a large part of a 15:0 was removed by phospholipase A treatment, i 15:0 could be detected among the fatty acids, in the 1-position (Fig. 26). The single peak obtained for the fatty acids in the 2-position (Fig. 26) was clearly due to only the a 15:0 acid because it was sharper than the corresponding peak observed in total fatty acids of PG.

FIGURE 26

GLC chromatograms of fatty acid methyl esters of PG:

A. Total fatty acids of PG

B. Fatty acid at β(2)-position of PG, obtained from PG treated with phospholipase A (snake venom)

C. Fatty acid at α(1)-position of PG, obtained from lyso PG (after treated-PG with phospholipase A).

Column conditions and abbreviations were given in Fig. 11.

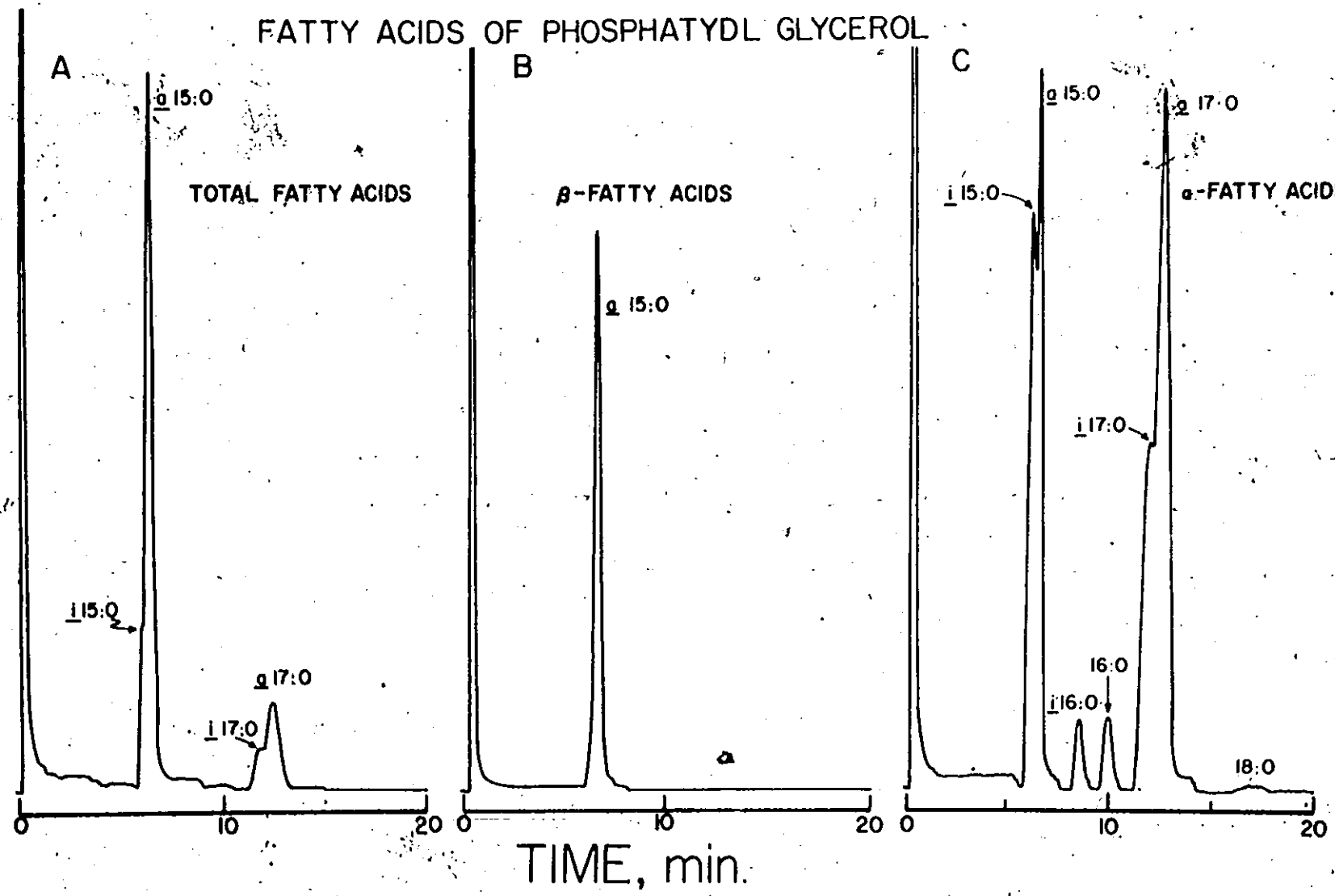

FATTY ACIDS OF PHOSPHATYDL GLYCEROL
A
a 15:0
TOTAL FATTY ACIDS
i 15:0
a 17:0
i 17:0
B
β-FATTY ACIDS
a 15:0
C
a 15:0
a 17:0
i 15:0
α-FATTY ACIDS
i 17:0
16:0
i 16:0
18:0
0
10
20
TIME, min.

TABLE 31

Positional Distribution of Fatty Acids in "PG".

Expressed in % of total area under fatty acids peaks*

Fatty Acids**	Area, %		
	Total	1-position+	2-position‡
i 15:0	74.6§	15.1	-
a 15:0		20.9	100.0
i 16:0	-	2.6	-
16:0	-	3.3	-
i 17:0	7.8	18.3	-
a 17:0	17.6	39.5	-

*By GLC on 10% BDS at 180°C with N_2 as carrier gas (0.8 kg/cm^2), See Fig. 26.

**For abbreviations, see Table 12.

+From isolated lyso PG.

‡Released by phospholipase A treatment.

§Contained mostly a 15:0 and only small amount of i 15:0 which was not clearly detectable on the tracing chromatogram (Fig. 26).

IX. Effect of Salt on Lipid Composition of S. epidermidis

A. Total Lipid Content and Lipid Composition

Bacteria grown in Sehgal and Gibbons medium containing increasing sodium chloride concentrations showed only slight increase in total lipid content (Table 7) and no qualitative differences in lipid composition, the pattern of components being essentially the same as shown in Fig. 10 for S. epidermidis grown in 10% NaCl containing medium. However, quantitative changes in composition were observed, especially with the phosphorus containing lipids, PG, GPDGD and cardiolipin (Fig. 27, Table 32). The amount of PG decreased from 67% to 50% while the amounts of cardiolipin and GPDGD increased from 0.5 to 11% and 5 to 15% respectively, as the sodium chloride level was increased from 0% to 25%. On the other hand the amounts of glycolipids (DGD and MGD) were only slightly changed with increasing of salt in the media.

B. Fatty Acid Composition

The lipids of cells grown at each of the salt concentrations contained qualitatively the same fatty acid components (Table 33). However, quantitative change in the proportion of the major fatty acids (a 15:0 and a 17:0) were observed when salt concentration was increased from 15% to 25% (Table 33). The percentages of a 15:0 were practically the same (≈38% in cells grown in the range 0-15% NaCl but

appeared to be higher (75%) when cells were grown in 25% NaCl. The percentages of a 17:0 were relatively the same throughout the range 0-25% NaCl, while the percentages of i 17:0 and i 19:0 decreased when the percentages of NaCl in the medium increased. The percentage of minor fatty acid components did not change signfficantly in the range 0-15% but were generally lower in cells grown in 25% NaCl.

TABLE 32

Lipid Composition of S. epidermidis grown in Sehgal and Gibbons media Containing Various NaCl Concentrations

Lipid Component*	Lipid Composition, moles %				
	NaCl conc., %				
	0	5	10	15	25
Cardiolipin (CL)	0.5	0.7	0.9	1.6	10.8
Diglucosyl diglyceride (DGD)	20.5	17.7	18.9	22.8	19.8
Glycerophosphoryl diglucosyl diglyceride (GPDGD)	5.3	6.0	7.2	9.7	14.5
Monoglucosyl diglyceride (MGD)	6.6	5.9	4.0	3.5	5.2
Phosphatidyl glycerol (PG)	67.1	69.7	69.0	62.4	49.6

*See Fig. 2 7.

FIGURE 27

The effect of sodium chloride concentration in the growth medium (Sehgal and Gibbons, 1960) on the polar lipid composition of S. epidermidis. Values of each lipid component are given as moles % (obtained from sugar or P-analysis) of total lipids. Each lipid component was separated from Total lipids of cells grown in various salt concentrations media by two dimensional TLC in the solvent systems shown in Fig. 10.

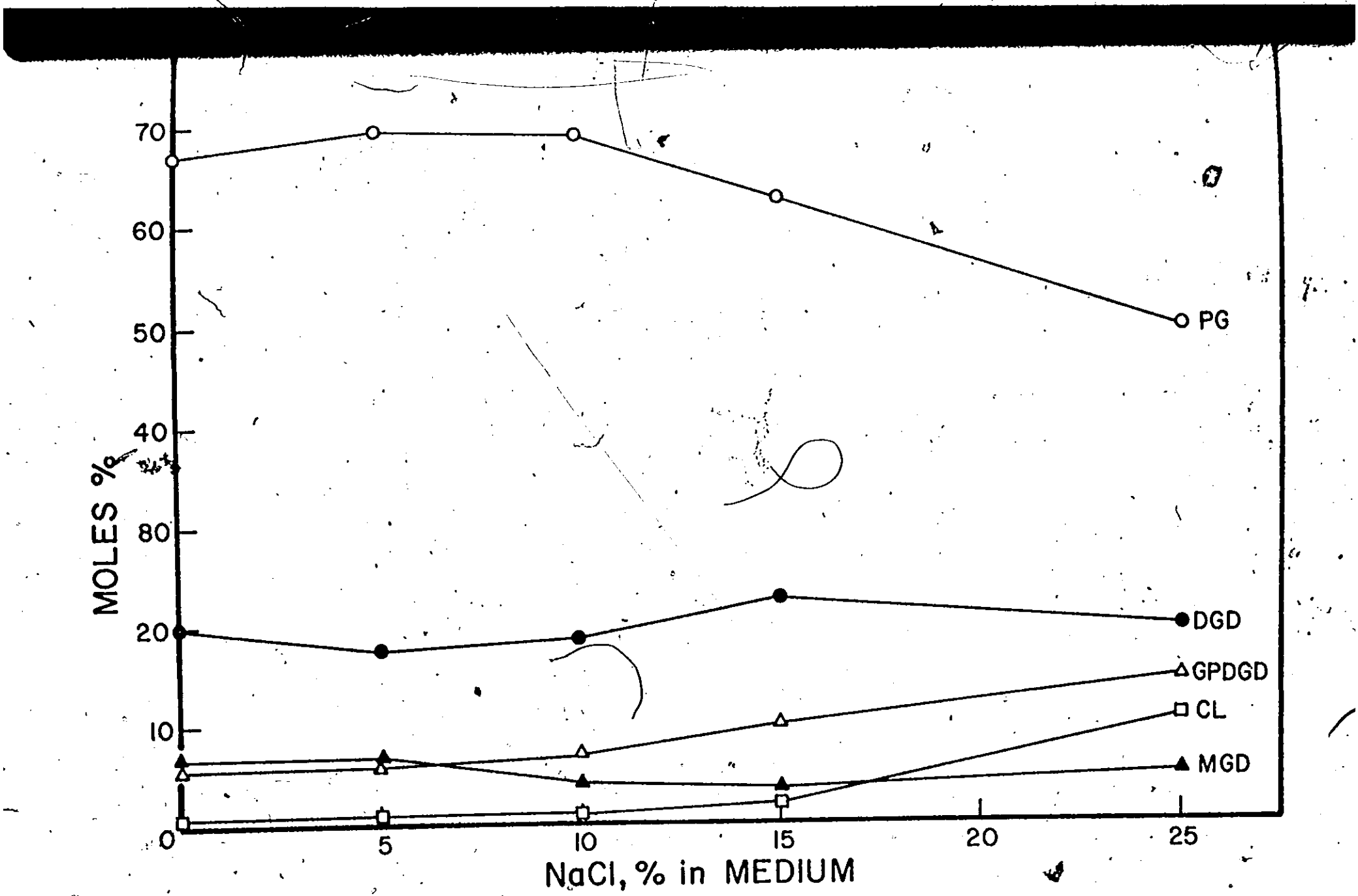

70
60
50
40
80
20
10
0
MOLES %
5
10
15
20
25
NaCl, % in MEDIUM
PG
DGD
GPDGD
CL
MGD

TABLE 33

Fatty Acid Composition of Total Lipids of S. epidermidis Grown in Sehgal and Gibbons Media Containing Various NaCl Concentration

Expressed in % of total area under fatty acid peaks

Fatty Acids*	Retention Relative to 14:0 Me**	NaCl conc., %				
		0	5	10	15	25
a 13:0	0.66	0.2	0.2	0.1	0.4	0.4
i 14:0	0.87	0.4	0.3	0.3	0.3	0.4
14:0	1.00	0.3	0.3	0.22	0.4	0.4
a 15:0	1.28	58.0	58.1	57.4	57.6	74.6
i 16:0	1.70	1.7	1.1	0.9	0.9	1.6
16:0	2.00	2.0	2.0	1.9	2.4	1.3
i 17:0	2.38	8.6	8.0	6.6	4.9	-
a 17:0	2.49	17.0	16.8	16.4	16.8	15.8
i 18:0	3.32	0.41	0.3	2.4	0.2	0.4
18:0	3.87	3.40	3.6	3.8	4.7	1.0
18:1	4.30	0.7	0.4	0.6	1.1	0.4
i 19:0	4.64	3.1	2.9	2.3	1.7	0.6
a 19:0	4.86	1.7	2.4	2.4	2.7	1.4
20:0	7.55	2.1	3.0	3.5	4.5	2.1

*For abbreviations, see Table 12.

**GLC on 10% BDS column at 180°C with N_2 as carrier gas (0.8 kg/cm^2).

X. Metabolism of Polar Lipids of S. epidermidis

A. Incorporation of ^{14}C and ^{32}P into Total Lipids

S. epidermidis grown in "low phosphate" Sehgal and Gibbons medium (10% NaCl) in the presence of [^{32}P] orthophosphate and [1-^{14}C]glycerol (Fig. 28) had a generation time 130 min, which was longer than that in the "phosphate rich" medium (60 min) at the same concentration of NaCl (see Results I.C). This is most likely due to the higher amount of cells used (O.D. =0.5) and the lower amount of nutrients (casamino acids and yeast extract) in the "low phosphate" medium.

^{14}C from [1-^{14}C]glycerol was incorporated into total lipids much faster than ^{32}P from [^{32}P]orthophosphate (Fig. 28). Maximum incorporation of [^{14}C]glycerol (4% of total ^{14}C) was achieved after 10 min but decreased slightly during exponential phase and remained fairly constant during stationary phase. In contrast incorporation of ^{32}P into phospholipids was slower than that of [^{14}C]glycerol and increased continuously during exponential and stationary phase; at 180 min, 0.9% of the [^{32}P]orthophosphate in the medium was incorporated into phospholipids.

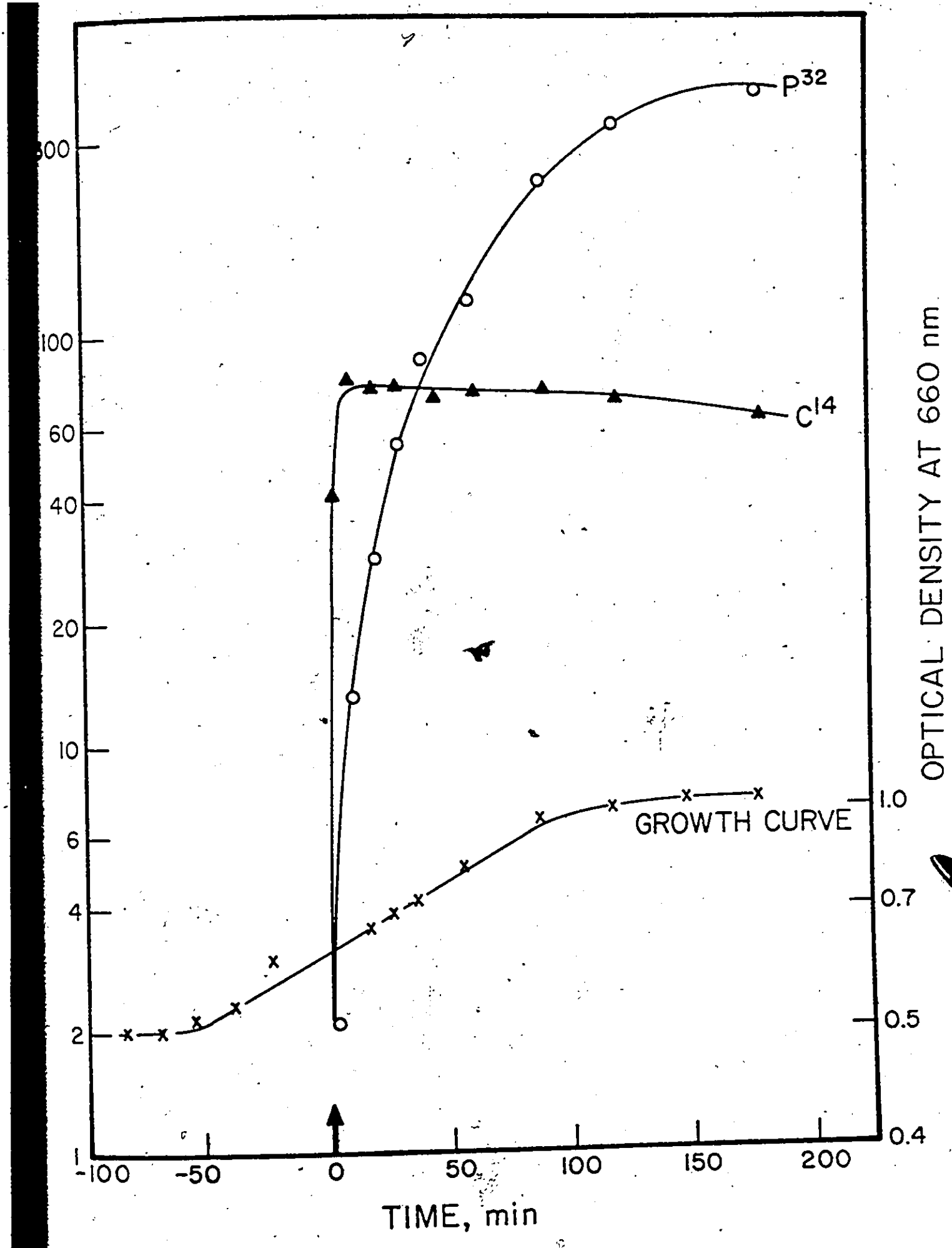
B. Distribution of ^{14}C and ^{32}P among Lipid Components During Growth Cycle

Typical autoradiograms of two dimensional TLC plates showing separation of ^{32}P and ^{14}C labelled lipids

FIGURE 28

Incorporation of [^{32}P] orthophosphate and [1-^{14}C] glycerol into total lipids of S. epidermidis.

Cells were grown in "low phosphate" Sehgal and Gibbons (1960) medium containing 10% sodium chloride to early exponential growth phase, the above radioactive precursors were added at the time indicated by arrow and cells were allowed to grow as shown in the growth curve. Aliquots (5 ml) were taken at various times after the addition of radioactive precursors and lipids were extracted and counted.


P32
C14
GROWTH CURVE
OPTICAL DENSITY AT 660 nm
TIME, min
300
100
60
40
20
10
6
4
2
1
1.0
0.7
0.5
0.4
-100
-50
0
50
100
150
200

after short term (3 min) and long term (180 min) labelling are presented in Figs. 29 and 30 respectively. In the solvent systems used (Fig. 30), the PG and DGD components were not well resolved. TLC was then repeated in a suitable solvent for separation of glycolipid from phospholipid (Fig. 31). In this way radioactivity in DGD can be measured separately from that in PG. The labelled components were scraped off from the TLC plate and counted. The values for ^{32}P and ^{14}C activity of each lipid component during the growth cycle are given in Table 34 and the results are also presented as percent distribution of ^{32}P and ^{14}C among the different lipid components as a function of incubation time after addition of the radioactive precursors (Fig. 32). It may be seen that the percent of ^{14}C- and ^{32}P in PA was high (ca. 38%) during the first 3 min of incubation, thereafter declining to low values at 20 min. At the same time, the percent ^{32}P and ^{14}C in PG showed a corresponding increase, indicating a precursor-product relationship between PA and PG (Fig. 32).

Other precursor-product relationships which may be discerned are between PG and cardiolipin and between PG and GPDGD. The decrease in percent of [^{32}P]PG was accompanied by a corresponding increase in percent of [^{32}P]cardiolipin and [^{32}P]GPDGD between 20 and 60 min of incorporation period; no further changes percentagewise occurred after 60 min. The percentage decrease in [^{14}C]PG also corresponded with

FIGURE 29

Autoradiogram of two dimensional thin-layer plate of [^{32}P] and [^{14}C] total lipids of S. epidermidis obtained from cells grown for 3 min in the medium described in Fig. 28.

Solvent systems: First direction, chloroform-methanol-28% ammonia (65:35:5, v/v); Second direction, chloroform-methanol-90% acetic acid (30:4:20, v/v).

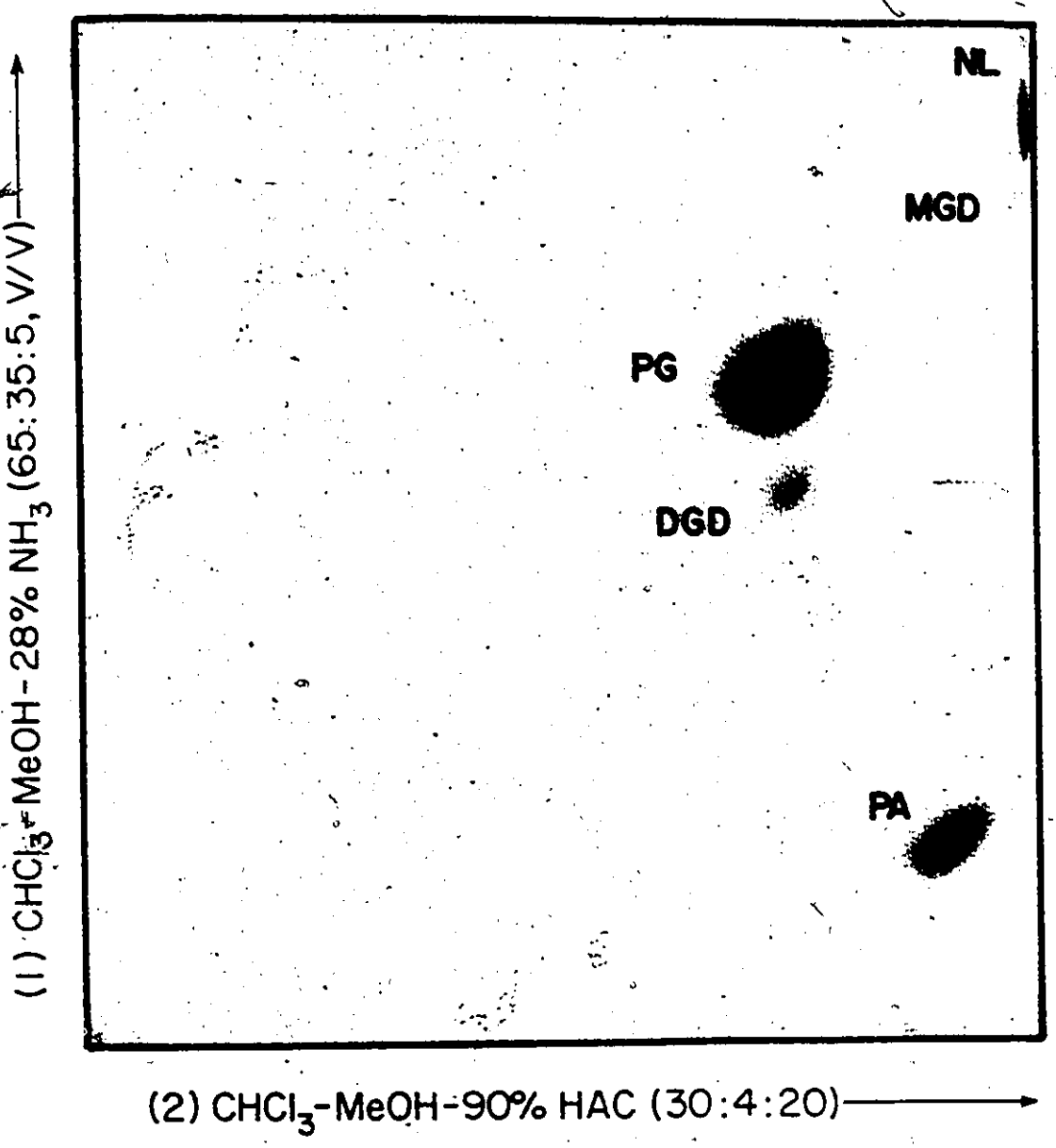

NL
MGD
PG
DGD
PA
(1) $CHCl_3$-MeOH-28% NH_3 (65:35:5, V/V)
(2) $CHCl_3$-MeOH-90% HAC (30:4:20)

FIGURE 30

Autoradiogram of two dimensional thin-layer plate of [^{32}P] and [^{14}C] total lipids of S. epidermidis obtained from cells grown for 180 min. in the medium described in Fig. 28.

Solvent systems: the same as in Fig. 29.

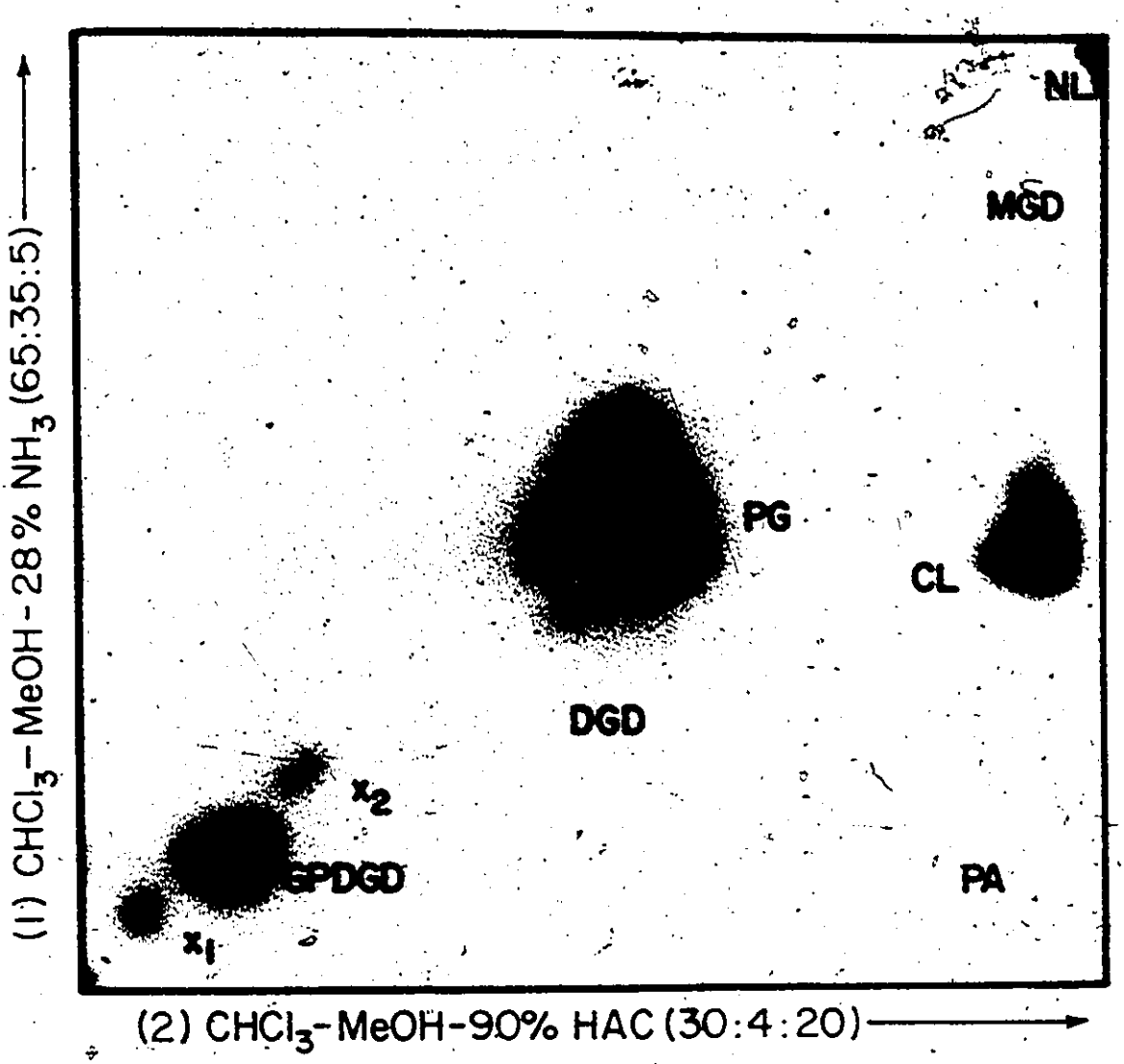

NL
MGD
PG
CL
DGD
x_2
SPDGD
PA
x_1
(1) $CHCl_3$–MeOH–28% NH_3 (65:35:5)
(2) $CHCl_3$–MeOH–90% HAC (30:4:20)

FIGURE 31

Autoradiogram of two dimensional thin-layer plate of [^{32}P]and [^{14}C]total lipids of S. epidermidis for separation of glycolipid from phospholipid components. Total lipids analyzed on this plate are the same as in Fig. 30.

Solvent systems:

First direction, chloroform-methanol-water (65:25:4, v/v)

Second direction, acetone-acetic acid-water (100:2:1, v/v).

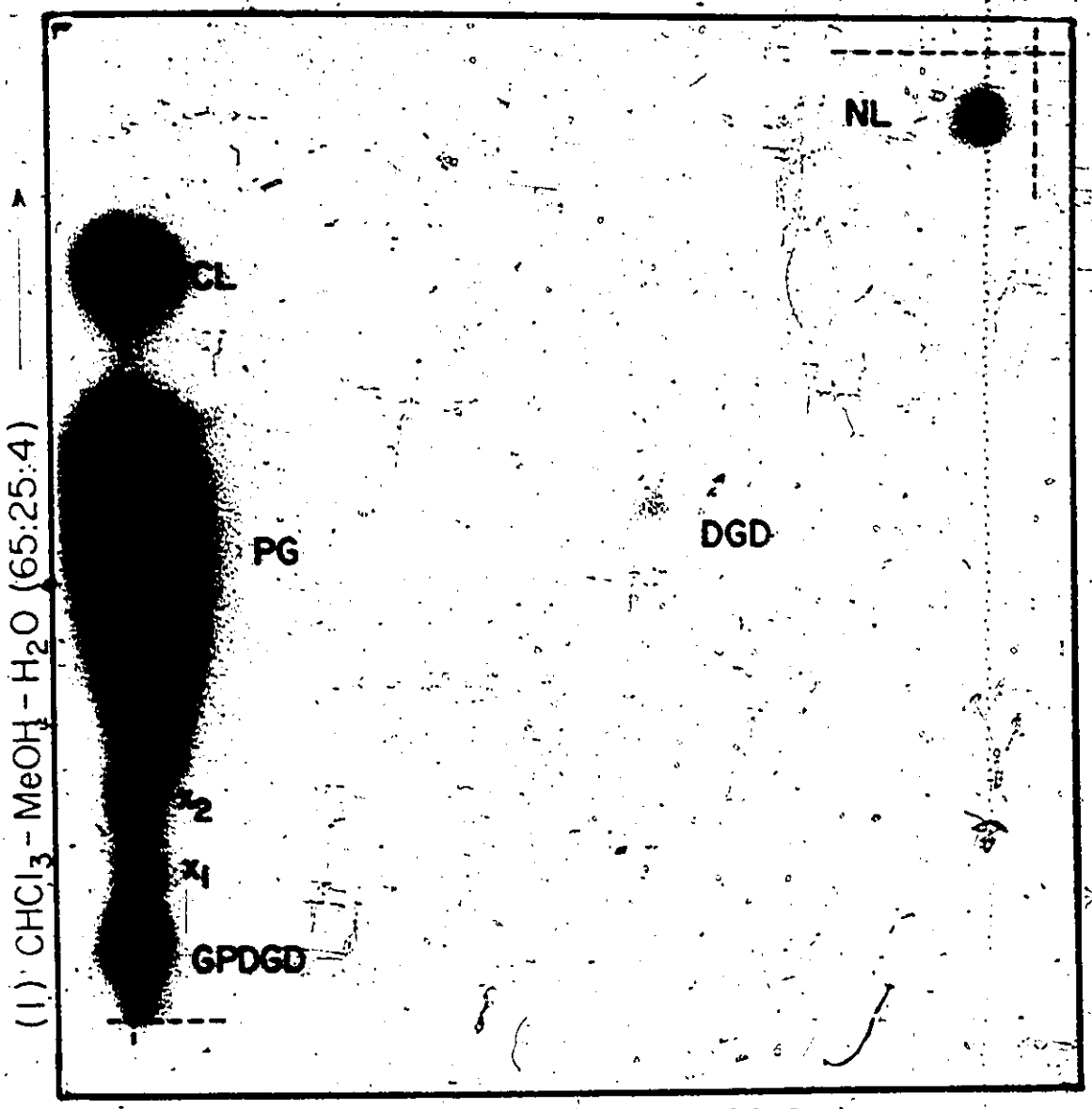

NL
CL
PG
DGD
X2
X1
GPDGD
(1) $CHCl_3$ - $MeOH$ - H_2O (65:25:4)
(2) Acetone - Acetic acid - water (100:2:1)

TABLE 34

Distribution of ^{32}P and ^{14}C activity among Lipid Components* of S. epidermidis grown in the presence of [^{32}P]orthophosphate and [1-^{14}C]glycerol+

Time (min)	Activity, CPMx10^{-3}/ml Culture										
	PA		PG		GPDGD		CL		MGD	DGD	NL
	^{32}P	^{14}C	^{32}P	^{14}C	^{32}P	^{14}C	^{32}P	^{14}C	^{14}C	^{14}C	^{14}C
3	0.81	5.60	1.12	27.9	0.1	0.33	0.07	0.7	2.27	3.13	1.24
10	2.96	0.95	9.17	60.0	0.97	1.26	0.21	1.50	0.40	11.5	3.56
20	1.35	0.73	24.5	59.0	2.55	0.73	1.06	1.73	1.53	6.48	3.86
30	1.37	0.60	36.9	51.1	8.26	4.48	8.26	2.40	1.72	6.56	7.54
40	2.48	0.42	63.0	42.2	18.6	2.85	14.2	2.22	0.83	6.19	7.65
60	1.36	0.84	74.2	39.5	25.6	4.77	25.9	4.12	1.03	4.89	9.21
90	0.48	0.80	140.8	38.3	51.5	7.94	50.8	5.82	0.58	6.04	13.40
120	← ND† →										
180	1.19	0.13	225.4	20.8	89.1	13.2	80.4	11.6	0.70	5.48	11.85

*See Figs. 29, 30 and 32.

+in "low phosphate" Sehgal and Gibbons (1960) medium containing 10% NaCl

†not determined

FIGURE 32

Percent distribution of ^{14}C and ^{32}P activities among individual lipid components of S. epidermidis cells grown for short periods of time with [1-^{14}C] glycerol and [^{32}P] orthophosphate in the medium described in Fig. 28. Lipid components fractionated on two dimensional TLC (Figs. 29, 30 and/or 31) were scraped into vials and counted. Values are given as % of total cpm recovered from each plate. Bacterial density at the time of lipid extraction is indicated on the growth curve.

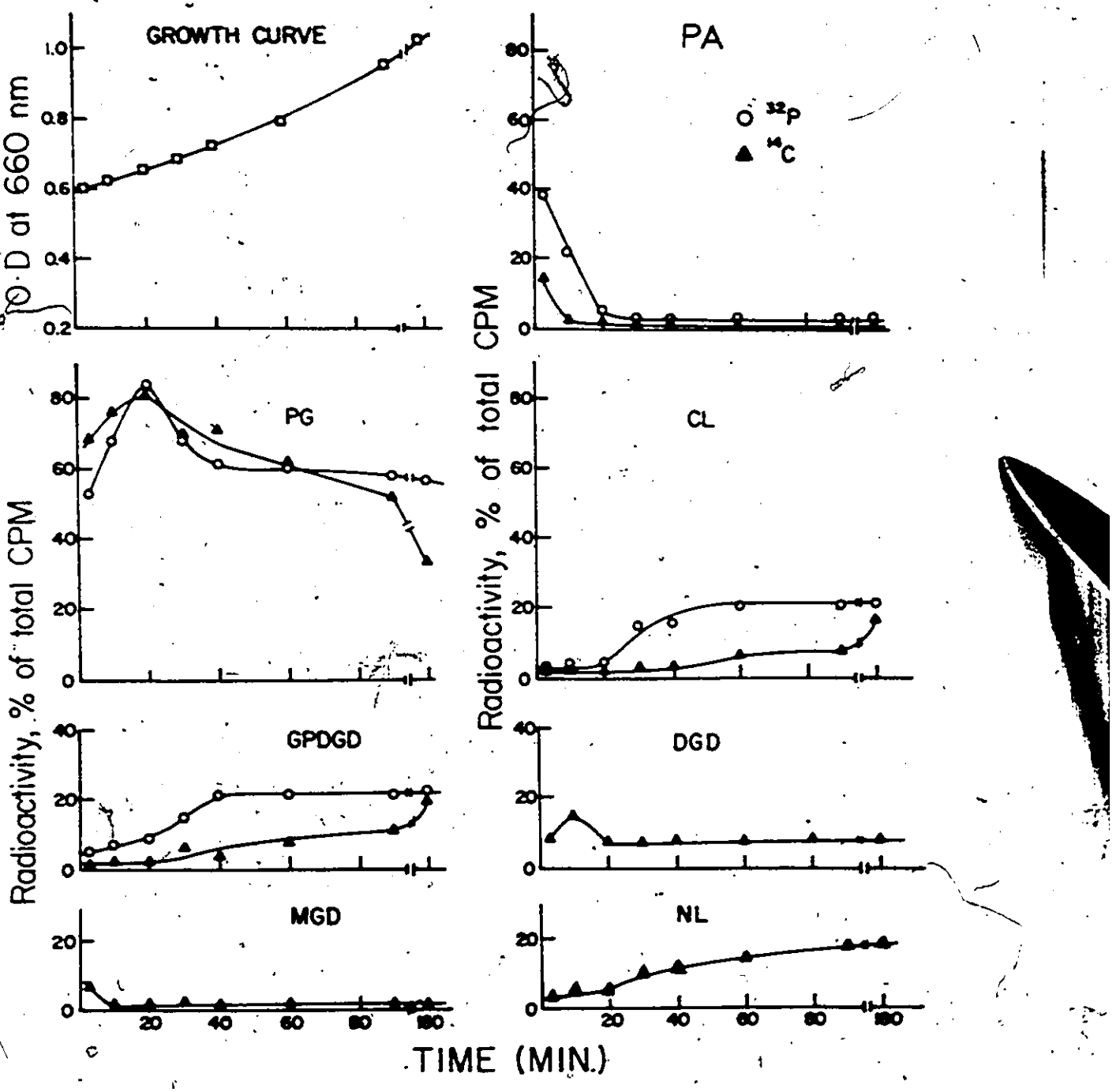

GROWTH CURVE
O.D at 660 nm
PA
^{32}P
^{14}C
PG
CL
GPDGD
DGD
MGD
NL
Radioactivity, % of total CPM
TIME (MIN.)

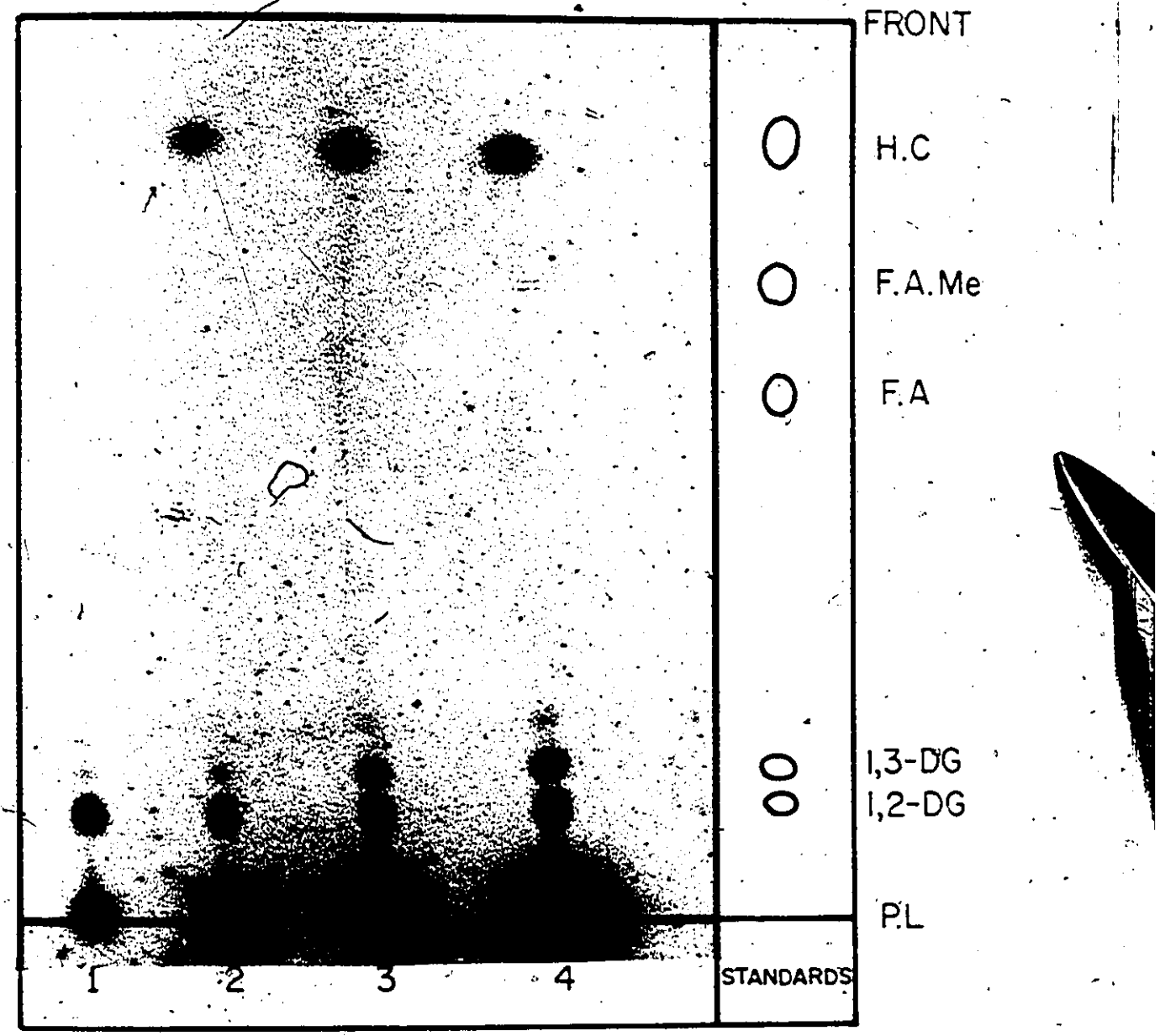
FIGURE 33

Autoradiogram of thin-layer plate of [^{32}P] and [^{14}C] total lipids of S. epidermidis in non-polar solvent systems showing the composition of neutral lipid components. The total lipids were obtained from cells grown in the medium as described in Fig. 28 at various periods of time as follows:

1. 3 min
2. 10 min
3. 60 min
4. 180 min

Abbreviations: PL, polar lipids; DG, diglyceride; FA, free fatty acid; F.A.Me, fatty acid methyl ester; HC, hydrocarbon.

Solvent system: petroleum ether-ethyl ether-acetic acid (80:20:1, v/v)


FRONT
H.C
F.A.Me
F.A
1,3-DG
1,2-DG
P.L
1
2
3
4
STANDARDS

percentage increases in [^{14}C]cardiolipin and [^{14}C]GPDGD but the decrease in ^{14}C activity of PG and the increases in [^{14}C] cardiolipin and [^{14}C] GPDGD were continuous during the period of incorporation 20 min to 180 min. Since labelling of GPDGD and cardiolipin occurred almost simultaneously, it is most probable that cardiolipin is not the precursor of GPDGD or vice versa (Fig. 32).

The percent of [^{14}C]MGD decreased in the first 20 min of incubation, thereafter remaining constant at level of 1% during the remainder of the growth cycle. Concomittantly, the [^{14}C] DGD increased to a maximum at 10 min, thereafter decreasing to a constant value at 20 min. Thus, there is most likely precursor-product relationship between MGD and DGD (Fig. 32).

The percent activity of [^{14}C] neutral lipid (mainly, 1,2- and 1,3-diglyceride and free fatty acids (Fig. 33) increased steadily between 20 and 90 min incubation.

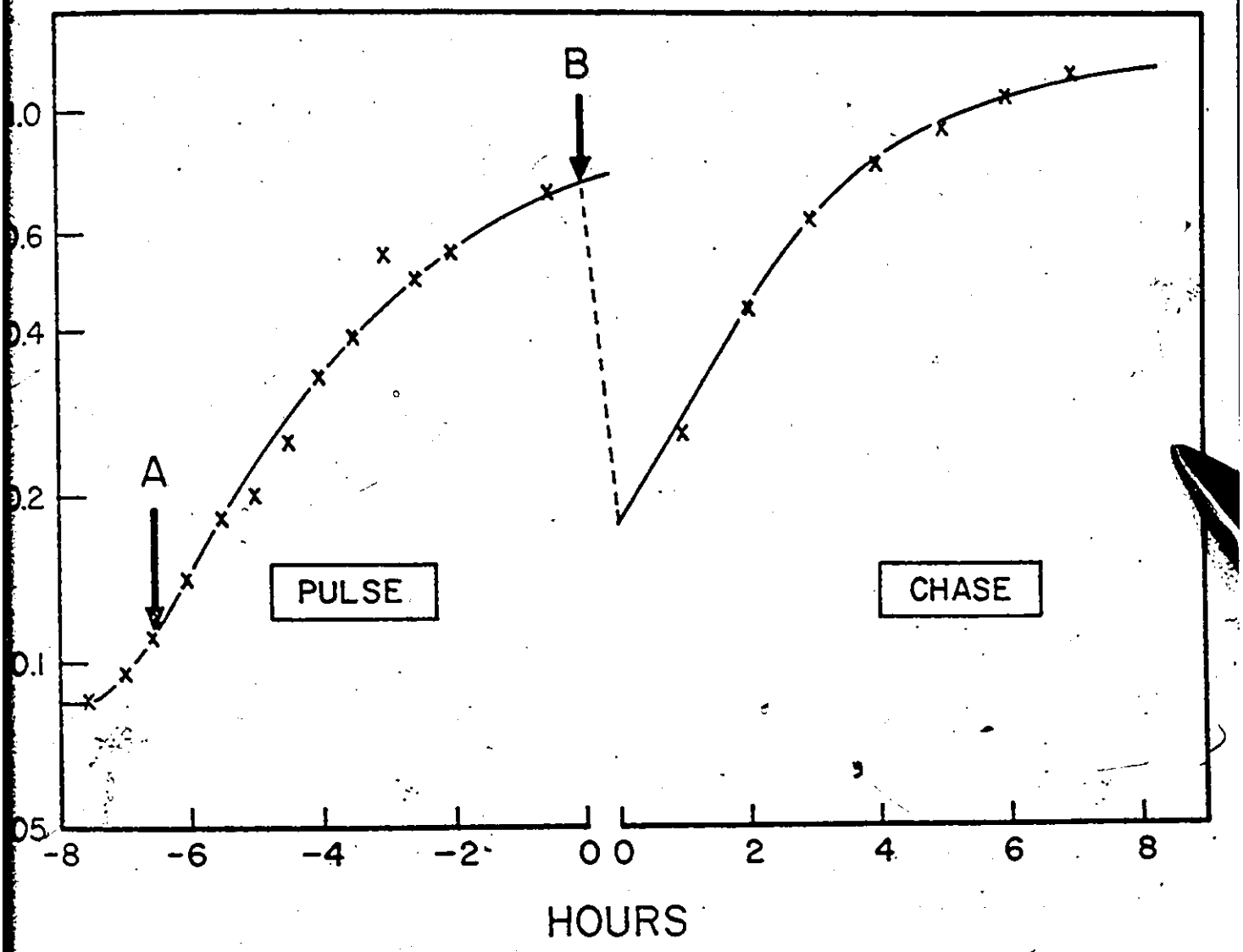
C. Turnover of Lipid Components (Pulse-Chase Studies)

Growth curves of S. epidermidis cells during the "pulse" and "chase" periods are given in Fig. 34.

The turnover of total lipids labelled with ^{32}P and ^{14}C during growth of the cells in non-radioactive medium is shown in Fig. 35A. It may be seen that the loss of ^{32}P was more rapid than the loss of ^{14}C during exponential growth and the loss of both was relatively slow during the early stationary growth.

FIGURE 34

Growth of S. epidermidis (measured as O.D. at 660 nm) in the presence of [^{32}P] orthophosphate and [1-^{14}C] glycerol in a pulse-chase experiment. Cells were grown in 100 ml of "low phosphate" medium (Sehgal and Gibbons, 1960, containing 10% NaCl); [^{32}P] orthophosphate (1 mC_i) and [1-^{14}C] glycerol (50 μC_i) were added at point A; labelled cells were harvested at point B, and resuspended in 450 ml non-radioactive, "phosphate-rich" medium (Sehgal and Gibbons, 1960) containing 10% NaCl). Generation times: "pulse" curve, 82 min, "chase" curve, 79 min.


B
A
PULSE
CHASE
1.0
0.6
0.4
0.2
0.1
0.5
-8
-6
-4
-2
0
0
2
4
6
8
HOURS

Autoradiograms of TLC plates of total lipids throughout the chase experiment were similar to that found at the end of the pulse experiment (180 min, see Fig. 30), except that MGD was not detected during the chase period and spots X_1 and X_2 were observed. Again labelling of DGD was determined from TLC plates run in the solvent systems shown in Fig. 31.

Distribution of ^{32}P and ^{14}C activities of each lipid component during the chase are given in Table 35 as percent of total cpm; the absolute counts as cpm per ml culture during the chase period are plotted in Fig. 35. It is apparent that the loss of ^{32}P and ^{14}C observed in total lipids are mostly due to the loss of ^{32}P and ^{14}C in PG, the latter having the highest rate of loss of ^{32}P during the first 1.5 bacterial doublings (1 generation = 79 min, Fig. 34). In four hours (≈3 bacterial doublings), 90% of ^{32}P and 40% of ^{14}C were lost from PG whereas the remaining ^{32}P and ^{14}C both appear to be fairly stable during stationary phase of growth (Fig. 35B). The ^{32}P and ^{14}C activities of PA decreased similarily during the chase period (Fig. 35C). In contrast, both ^{32}P and ^{14}C of cardiolipin showed initial increases to maximum values during exponential growth, then decreasing to fairly constant values in stationary phase (Fig. 35C). GPDGD also showed a rapid increase in ^{32}P and ^{14}C during exponential growth (Fig. 35D) but thereafter showed a continuous decrease to low values in stationary

phase. For both cardiolipin and GPDGD, changes in ^{32}P and ^{14}C were parallel throughout the chase period. DGD lost ^{14}C activity during exponential growth and remained constant after this period, whereas ^{14}C of neutral lipids showed an initial increase and then also remained constant after two hours (Fig. 35D). The ^{32}P and ^{14}C activities of X_1 and X_2 during the chase period are not plotted but the values are given in Table 35.

TABLE 35

Turn over of ^{32}P and ^{14}C activities of [^{32}P] and [^{14}C] lipid components* during growth in non-radioactive medium+. Expressed in % of total CPM

Time (hr)	Activity, % of total CPM													
	PA		PG		GPDGD		CL		DGD	NL	X_1		X_2	
	32_P	14_C	32_P	14_C	32_P	14_C	32_P	14_C	14_C	14_C	32_P	14_C	32_P	14_C
0	1.0	1.3	92.0	73.6	3.1	3.0	2.1	2.7	9.4	8.7	2.0	1.4	-	-
1	0.9	1.4	82.0	66.8	10.9	8.6	4.4	3.7	5.2	10.8	1.8	3.2	-	-
2	1.1	1.6	61.9	58.7	13.1	5.8	20.9	8.2	5.9	13.0	1.8	2.4	0.4	0.5
3	1.4	1.4	51.3	57.8	18.1	9.2	25.2	11.1	6.0	11.2	2.9	2.8	1.1	0.6
4	0.8	1.0	63.4	60.6	17.9	5.5	14.0	4.7	7.2	17.4	2.8	3.1	1.1	0.5
5	1.4	1.0	61.1	63.5	17.9	4.0	15.9	8.3	7.0	14.2	2.1	1.3	1.6	0.6
6	0.8	0.7	70.1	62.9	5.2	1.8	18.2	4.7	7.0	21.3	0.9	1.1	0.9	0.4
7	←						ND†							→
8	0.7	0.9	75.0	62.3	1.7	0.4	21.5	5.9	9.0	21.7	0.5	0.5	0.5	0.2

*See Figs. 29, 30 and 35.

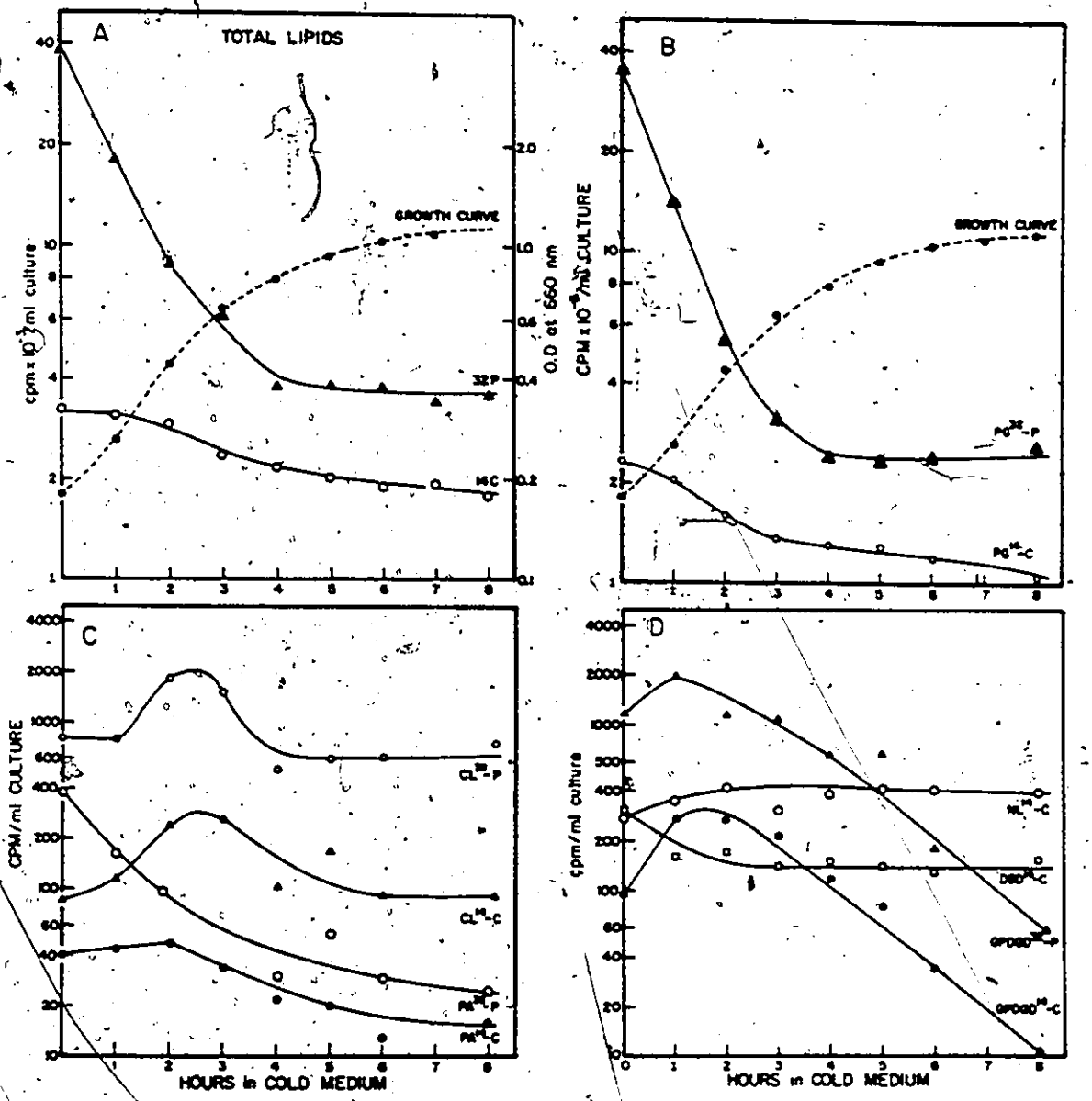
+in "phosphate rich" Sehgal and Gibbons (1960) medium containing 10% NaCl

†not determined

FIGURE 35

Turnover of ^{32}P and ^{14}C in total lipids and in the individual lipid component of S. epidermidis. Cells were obtained from the "chase" experiment. After a "pulse" labelling with [1-^{14}C] glycerol and [^{32}P] orthophosphate for 340 min (Fig. 34); 50 ml aliquots were withdrawn at the times indicated and optical density (at 660 nm) measurement and lipid extraction were performed on each aliquot. The lipid components were separated on two dimensional TLC (Figs. 30,31) and counted for ^{14}C and ^{32}P activities. Values are given as cpm per ml culture.

A, total lipids; B, phosphatidylglycerol; C, cardiolipin and phosphatidic acid; D, neutral lipids, diglucosyl diglyceride and glycerophosphoryl diglucosyl diglyceride.


A
TOTAL LIPIDS
GROWTH CURVE
cpm x 10³/ml culture
O.D at 660 nm
32P
14C
B
GROWTH CURVE
CPM x 10⁻³/ml CULTURE
C
CPM/ml CULTURE
HOURS in COLD MEDIUM
D
cpm/ml culture
HOURS in COLD MEDIUM

DISCUSSION

I. Lipids of Nocardia Species

The major phospholipids found in the species of Nocardia studied here were PC, lyso PG and PG (Tables 9,11). PC has been found as a major phospholipid component of Nocardia coeliaca (Yano et al., 1969) together with PE, PI and cardiolipin. In the order Actinomycetales, the presence of PC so far has been reported only in the Nocardia coeliaca but not in other species of Nocardia nor in other members of this order (e.g., Streptomyces or Mycobacterium). Hagen et al. (1966) suggested a correlation between bacterial PC and intracytoplasmic membrane structure, and demonstrated PC to be present in Hyphomicrobium and Nitrocystis oceanus, organisms which have such structures. Ikawa (1967), in his reviews of bacterial phospholipids, observed that PC is present in bacteria requiring highly efficient electron transport, and he speculated that PC-containing bacteria might be the more advanced evolutionary form.

The two unidentified phospholipids (spot 2 and 8, Fig. 8) were not PE since both are ninhydrin negative (Table 11); however, one of them, spot 2, might be PI, since it had relatively low mobilities in several solvent systems (see Materials and Methods II.C.2.c) close to those reported for PI (Kates, 1972a). Free PI was reported to be present in N. coeliaca and in Micromonospora (Tabaud et al., 1971) but

not in N. polychromogenes or in Streptomyces griseus (Kataoka and Nojima, 1967). The latter two organisms contained monomannoside derivative of PI which was not found in the species of Nocardia studied here. The phospholipid pattern of this Nocardia species resembles that of N. coeliaca more than any other species of Nocardia.

Glycolipids found in a few strains of N. polychromogenes and N. coeliaca have been identified as acylated glucose (Brennan et al., 1970) and diglucosyl diglyceride (Khuller and Brennan, 1972). Two unidentified glycolipids were found in the Nocardia species studied (spots 3 and 6, Table 11 and Fig. 8). On the basis of the R_f value on TLC of the fast moving glycolipid (spot 6), it may be a diglycosyl diglyceride. The slower moving one (spot 3) could not be acylated sugar due to its low mobility, and may perhaps be a triglycosyl diglyceride.

The fatty acid composition of the Nocardia species studied here (Table 12) is similar to those of N. coeliaca (Yano et al., 1969), N. polychromogenes (Kataoka and Nojima, 1967) and N. leishmanii (Yano et al., 1970). The major components were highly branched chain fatty acids (iso 15:0, iso 16:0, iso 17:0 and anteiso 17:0). However, palmitic acid which was present in fair amounts (13-20%) in these Nocardia was found only in small amounts (3%) in the Nocardia species studied here. Moreover, tuberculostearic acid (10-methyl stearic acid) was found in small to trace amounts in

N. leishmanii and N. coeliaca but was not found in N. polychromogenes nor in our species of Nocardia. Thus, it is possible that, branched chain fatty acids are typical of the genus Nocardia, and differences in the presence of minor fatty acid components such as palmitic acid or tuberculostearic acid may be due to species differences.

In general, it can be seen that the lipid composition of the Nocardia species studied here is similar to that of N. coeliaca more than other species of Nocardia. Further studies should be done to identify or to confirm the types of phospholipids and glycolipids present in this species. Such detailed studies on the lipids might help to identify the species of the Nocardia studied.

II. Lipids of Wallemia sebi

Phospholipids of W. sebi were tentatively identified as PC, PS, PE and a "glucophospholipid" (probably a PI-mannoside). The occurrence of PC is more common in fungi than in bacteria (Mangnall and Getz, 1973). The phospholipid composition of W. sebi is similar to that found in other species of fungi such as in yeasts, Saccharomyces cerevisiae (Suomalainen and Nurminen, 1970), Candida lipolytica (Kates and Paradis, 1973) or in Humicola grisea (Mumma et al., 1971) in which PC, PE, PI and PS were the major phospholipids (in decreasing proportions) and PG is rarely found. Although PI-mannosides are uncommon in fungi, the presence of complex inositides containing mannose in Baker's yeast has been reported (Lester and Steiner, 1968; Trevelyan, 1968). Thus, the "glucophospholipid" found in W. sebi may very well be a PI-mannoside derivative on the basis of its similar R_f value to that reported for PI-mannoside (Yano et al., 1969). The absence of PI in W. sebi may be due to its being entirely in the form of mannoside derivatives.

The three fast moving glycolipids observed in W. sebi may be monoglycosyl diglyceride, acylated monoglycosyl and acylated diglycosyl diglyceride because of their relative high mobilities on TLC. The possibility that these glycolipids might be glycosphingolipids or cerebrosides known to be present in Baker's yeast (Trevelyan, 1968) could be

eliminated since glycosphingolipids have much lower mobilities (Kates, 1972a).

The fatty acid composition of W. sebi is typical of fungi in which unsaturated fatty acids are present as major components. The unsaturated fatty acids found in W. sebi are linoleic and oleic acids (60% and 13%, respectively) together with palmitic acid (23%). In examining the available literature dealing with the fatty acids of fungi in the class Fungi Imperfecti in which W. sebi is placed, the general pattern of major fatty acids in this class is found to consist of oleic, linoleic and palmitic acid (see Shaw, 1966; Erwin, 1973). The differences among the genera are: (i) the occurrence of fair amounts of linoleic acid in a few species such as in Candida scotti (28%) (Kates and Baxter, 1962), Humicola grisea (19%), and H. nigrescens (12%) (Mumma et al., 1970); (ii) the presence or absence of palmitoleic acid and (iii) the relative proportions of oleic acid and linoleic acids. The fatty acid composition of W. sebi is qualitatively the same as that in Microsporum gipseum (Wirth et al., 1964), Trichophyton rubrum (Wirth and Anand, 1964), Hirsutella giganta (Shaw, 1965) and Sporotrichium exile (Mumma et al., 1970). In these species, linolenic and palmitoleic acids are absent and the proportion of linoleic, palmitic and oleic acids are present in decreasing order of abundance, as was found in W. sebi. The similarities in fatty acid pattern of W. sebi with those of the other members of the Fungi Imperfecti is consistent with its classification as a fungus in this class.

III. Lipids of Staphylococcus epidermidis

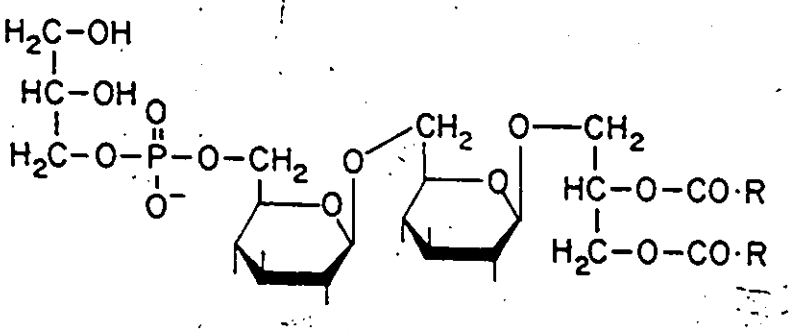
Values for lipid content of S. epidermidis obtained by the two methods of extraction namely, Bligh and Dyer (1959) and hot-isopropanol extraction (Kates and Eberhardt, 1957), were very similar (3.1% and 4.4%, respectively). Hot-isopropanol is known to stop the action of hydrolytic enzymes. Since the lipids obtained by both procedures had the same pattern, it can be concluded that the lipid components of S. epidermidis studied in this work are "free" membrane lipids and were not derived from "bound" forms covalently linked to other membrane components such as proteins or teichoic acids. Although the isopropanol extraction gives a slightly higher lipid yield than the Bligh and Dyer procedure, it is more time consuming and less convenient. Thus, all the lipid extractions were performed by Bligh and Dyer procedure.

The polar lipids of S. epidermidis contained five phospholipids (phosphatidyl glycerol, cardiolipin, phosphatidic acid, X_1 and X_2), two glycolipids (diglucosyl - and monoglucosyl diglyceride) and a novel glucophospholipid (Fig. 8). Of these, the four components, phosphatidylglycerol, diglucosyl- and monoglucosyl diglyceride and the glucophospholipid were isolated and their structures established to be as shown in Fig. 36.

FIGURE 36

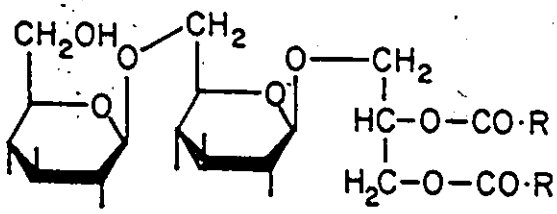
Final structures established for the polar lipid components of S. epidermidis. Values in brackets are given as percentages by weight of total lipids.

(a)



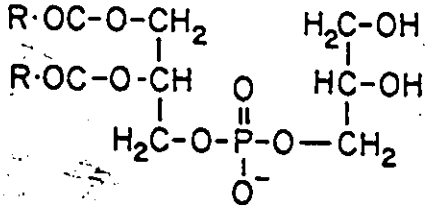
GPDGD (9.5%)

(b)



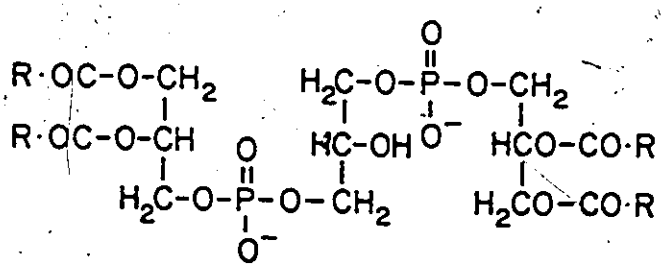
DGD (15.5%)

(c)



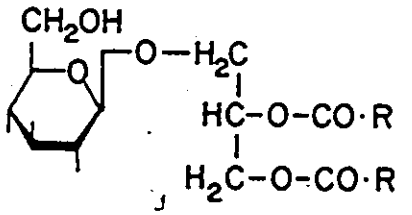
PG (60.0%)

(d)



CL (3.5%)

(e)



MGD (3.3%)

A. Glucophospholipid (GPDGD)

On the basis of the results reported above, the structure of this lipid component is most likely to be 3-O-[sn-glycero-1-phosphoryl-6'-O-(β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranosyl)]-1,2-diacyl-sn-glycerol (Fig. 36a). The elucidation of this structure was primarily based on chemical analysis of the lipid constituents (P, glycerol, fatty acid and sugar) of both intact lipid and its deacylated product (Table 16) as well as the IR spectrum (Fig. 14). The configurations of the glycerophosphate moiety and the glycosidic linkages were determined by the use of specific purified enzymes, and also in the case of the glycosidic linkages by optical rotation and NMR. The (1→6) position of the glycosidic linkage between the two glucose moieties was determined by quantitative periodate consumption of the deacylated product (Table 21), by Smith degradation (Table 22) and by permethylation analysis (Table 25). The periodate method, although simple and relatively quantitative is subject to considerable error and may lead to erroneous interpretation of the linkage position (see Table 21). The results obtained from periodate consumption were therefore confirmed by examination of the oxidation products ("Smith degradation") and finally by methylation analysis of the methylated GPDGD. Since all three methods gave the same results, the structure shown in Fig. 37 is most likely correct.

This structure is similar to that established for the glucophospholipid in Streptococcus faecalis, S. hemolyticus (Fischer et al., 1973) and Acholeplasma laidlawii (Shaw et al., 1972) (Fig. 3a) except that the disaccharide moiety in the glucophospholipid of the latter three organisms is kojibiose (2-O-α-D-glucopyranosyl-D-glucopyranose) whereas in S. epidermidis, it is gentiobiose (6-O-β-D-glucopyranosyl-D-glucopyranose). However, the glucophospholipid of S. epidermidis differs further from that of A. laidlawii (Shaw et al., 1972) in the configuration of glycerophosphate moiety. In the latter organism, the configuration of glycerophosphate moiety is sn-glycero-3-phosphate whereas in S. epidermidis, it is sn-glycero-1-phosphate.

The structure of the glucophospholipid of S. epidermidis is different from that reported for Staphylococcus aureus by Short and White (1970). In S. aureus, the structure of the glucophospholipid proposed by these authors was 3-sn-phosphatidylglucose (Fig. 3e). Characterization of this structure was based on chemical analysis showing that the lipid contained two moles of fatty acids, one mole of glucose and one mole of glycerol per mole of phosphate. Partial acid hydrolysis yielded glucose and glycerophosphate, and mild alkaline hydrolysis yielded a non-reducing glucoside. Treatment with phospholipase C gave diglyceride and low yields of glucose-1-phosphate and treatment with phospholipase D gave traces of phosphatidic acid.

Shaw and Stead (1972), in their study on the structure of glucophospholipid in A. laidlawii examined the glucophospholipid of S. aureus and found that the deacylation product of the glucophospholipid from S. aureus did not correspond chromatographically to synthetic glucose-6-phosphoryl glycerol or α,β-glucose-1-phosphoryl glycerol, but rather to the glycerylphosphoryl diglucosyl glycerol from the corresponding glucophospholipid in A. laidlawii. They suggested that the structure of the glucophospholipid of S. aureus may be similar to that in A. laidlawii (Fig. 3a). On this basis the glucophospholipid in S. epidermidis is probably different from that in S. aureus. However, further detailed studies are needed to establish the structure of the S. aureus glucophospholipid with certainty.

It is interesting to note that the glycolipid portion of the glucophospholipid of S. epidermidis is the same as the main glycolipid found in this bacterium, namely 3-O-[β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranosyl]-1,2-diacyl-sn-glycerol (Fig. 36b). A structural relationship between the glucophospholipid and the diglucosyl diglyceride components has also been observed in A. laidlawii (Shaw et al., 1972) and in Streptococci (Amborn and Pieringer, 1971; Fischer et al., 1973a), indicating a biosynthetic relationship between these two lipids.

The function of phosphoglucolipid in the cell membrane of these bacteria is not known. However, the recent

isolation of lipoteichoic acids from Lactobacilli (Wicken and Knox, 1970) and S. faecalis (Toon et al., 1972) which contain covalently bound lipid, has suggested a possible function for glucophospholipid as a component of a lipoteichoic acid. Treatment of lipoteichoic acid from S. faecalis with hydrogen fluoride yielded diglucosyl diglyceride together with diglyceride and monoglyceride (Toon et al., 1972). The presence of the neutral lipids suggests that the lipid component may be a phosphoglycolipid rather than the glycolipid itself (Toon et al., 1972). The lipid portion of lipoteichoic molecule may interact hydrophobically with other membrane lipids and in this way the polymer of the teichoic acid chain can be attached to the membrane surface (Knox and Wicken, 1973). The unbound glucophospholipid is usually present in small quantities in most bacteria with the exception of A. laidlawii, where it is a major component.

B. Diglucosyl Diglyceride (DGD)

This lipid component is the main glycolipid, accounting for 15.5% of total lipids. On the basis of the results given in Section Results VI, the structure of the deacylated product was established as: 3(1)-O-[β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranosyl]-sn-glycerol.

The configuration of the diglyceride portion of the diglucosyl diglyceride was not determined in this study, but it is reasonable to assume that the diglyceride is a

1,2-diacyl-sn-glycerol, the same as that shown to be present in the PG of this bacterium (see Results VIII.C). This assumption is based on the fact that diglycerides are precursors of bacterial glycolipids (Lennarz and Talamo, 1966; Pieringer, 1968), and that the glycosyltransferase catalyzing the biosynthesis of glycolipids is specific only for the 1,2-diacyl-sn-glycerol isomer (Pieringer, 1968).

Thus, the structure of the intact diglucosyl diglyceride is most likely to be: 3-O-[β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranosyl]-1,2-diacyl-sn-glycerol (Fig. 36b).

This structure is identical with that of the major diglucosyl diglyceride in S. aureus (Polonovski, 1965; Brundish et al., 1966). However, Polonovski (1965) also reported the presence of another isomer, namely O-[β-D-glucopyranosyl-(1→3)-O-β-D-glucopyranosyl]-diglyceride. In contrast, the results obtained from Smith degradation and methylation studies of the diglucosyl glycerol from S. epidermidis exclude the presence of this or any other isomer. The diglucosyl diglyceride found in S. epidermidis is also has identical structure to that in Bacillus subtilis (Brundish et al., 1966) and in B. cereus (Saito and Mukoyama, 1971).

C. Monoglucosyl Diglyceride (MGD)

On the basis of the results given in Results VII.I, the structure of this minor glycolipid (3.3% of total lipids)

is 3-O-[β-D-glucopyranosyl]-1,2-diacyl-sn-glycerol (Fig. 36e). The configuration of diglyceride moiety was not determined and was assumed to be a 1,2-diacyl-sn-glycerol (see Discussion for the structure of diglucosyl diglyceride in this section).

In most bacteria, the monoglycosyl diglyceride component usually has a structure corresponding to that of the diglycosyl diglyceride component, since the monoglycosyl compound is the biosynthetic precursor of the diglycosyl compound (see Introduction VI.A.2). The monoglycosyl diglyceride therefore does not usually accumulate in significant amounts but a few organisms, do contain monoglycosyl diglycerides as major components, e.g. Pseudomonas diminuta (Wilkinson, 1969), species of Arthrobacter (Shaw and Stead, 1971) and Mycoplasma laidlawii (Shaw et al., 1968).

D. Phosphatidylglycerol

This is the main phospholipid of S. epidermidis and accounts for 60% by weight of total lipids. From the results obtained (see Results VIII), the structure of the main phospholipid was established as 1,2-diacyl-sn-glycero-3-phosphoryl-1-sn-glycerol (Fig. 36c). Phosphatidylglycerol with this structure and configuration has been isolated previously from spinach leaves (Haverkate and Van Deenen, 1965), and this structure has been confirmed by chemical synthesis (Saunders and Schwarz, 1966).

Macfarlane (1961) isolated PG in large amounts from whole cells of Micrococcus lysodeikticus; its specific rotation was $[\alpha]_D$ + 5° in ethanol, similar to that of the PG isolated from S. epidermidis ($[\alpha]_D$ + 7.97° in chloroform). The difference in rotation is probably due to differing amounts of optically active branched chain (anteiso) fatty acids present in both bacteria (cf. Tables 4 and 26). The fatty acids of PG isolated from M. lysodeikticus had $[\alpha]_D$ +3° in chloroform (Macfarlane, 1961) whereas that of S. epidermidis is +10.2° in the same solvent. On this basis, PG in the latter organism has a higher content of anteiso acids.

Other minor polar lipids present in S. epidermidis are cardiolipin, phosphatidic acid and two unidentified phospholipids (X_1 and X_2, Fig. 9). The two unidentified phospholipids may be amino acid derivatives of PG. However, it was observed that free amino acids if present in the lipid extracts give spots with the same chromatographic properties as X_1 and X_2. Further studies such as mild alkaline deacylation must be done on the isolated X_1 and X_2 lipids to establish whether they are amino acyl derivatives of PG, such as lysyl-PG.

The phospholipid and glycolipid composition of S. epidermidis is very similar to that of S. aureus. In both species, PG, diglucosyl diglyceride, glucophospholipid, cardiolipin and monoglucosyl diglyceride in decreasing amounts were found. The differences are in PE and lysyl-PG which were

reported to be present in S. aureus (Short and White, 1970; Haest et al., 1972) but were not detected in S. epidermidis. The level of lysyl-PG in S. aureus was found to be greatly dependent on pH of the growth medium (Houtsmuller and Van Deenen, 1965). Cells of S. aureus grown at pH 4.7, contained high percentages of positively charged lysyl-PG (about 80%) whereas cells grown at pH 7.0 contain low level of lysyl-PG but high percentage of the negatively charged, free PG (about 55%) (Houtsmuller and Van Deenen, 1965). Based on evidence obtained from a considerably reduced permeability for Rb^+ of lysyl-PG liposomes compared to that of PG-liposomes, it was suggested that the high level of lysyl-PG formed when cells are grown in low pH medium was involved in control of membrane ion permeability (Haest et al., 1972). Moreover, Hopfer et al. (1970) also found that bilayer membranes prepared from PG are far more permeable to protons than that of lysyl-PG.

Lysyl-PG appears to be absent, or present only in small amounts, if X_1 or X_2 prove to be lysyl-PG. S. epidermidis is grown in the complex medium of Sehgal and Gibbons (1960) at a neutral pH (6.5 - 6.8), under which conditions the cells would not require the positively charged lysyl-PG to control its H^+ permeability. White and Frerman (1967) suggested that lysyl-PG is rather susceptible to enzymatic hydrolysis during lipid extraction and to prevent such hydrolysis, they

acidified the culture medium to pH 2.0 prior to harvesting S. aureus cells. Since such a high pH might destroy cellular polar lipid components, it was considered inadvisable to include this acidification step here before harvesting of the cells. Thus, the low level or absence of lysyl-PG in S. epidermidis may be due to enzymatic hydrolysis during extraction. On the other hand, it is possible that lysyl-PG might not be synthesized at all in S. epidermidis.

The absence of PE and the presence of diglucosyl diglyceride is a rather common occurrence in Gram-positive bacteria and S. epidermidis fits into this general pattern. Phosphatidylethanolamine is more typical of Gram-negative bacteria (Kates, 1964; Ikawa, 1967) and glycosyl diglycerides are more characteristic of Gram-positive bacteria (Shaw, 1970) and most bacteria contain one or the other of these two lipids. However, in certain Gram-positive organisms they occur together, e.g. S. aureus (Short and White, 1970), Bacillus cereus T, and B. subtilis W23 (Minikin et al., 1971).

During the growth cycle of B. cereus, there is an interrelationship between these two neutral polar lipids. The level of PE decreases concomitantly with increasing level of diglucosyl diglyceride to a maximum at the stationary phase (Minikin et al., 1971). These observations suggest that these two lipid types may have a similar and perhaps interchangeable role in bacterial membranes (Minnikin et al., 1971). Studies on model bilayer membranes prepared from bacterial

phospholipids and glycolipids showed that those prepared from PE and diglucosyl diglycerides have similar slight selectivity to cations, whereas those involving PG and cardiolipin are highly cation selective (Hopfer et al., 1970).

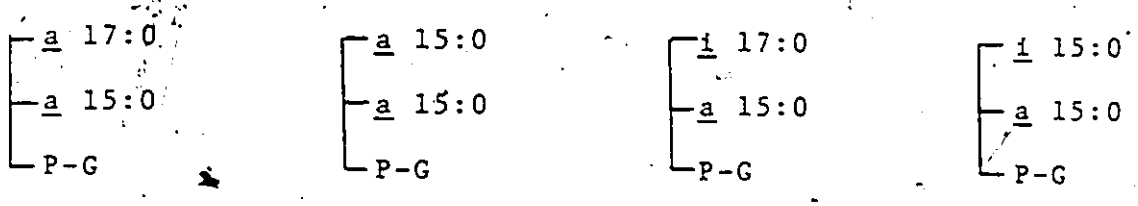
Generally, it can be seen that most cell membranes contain both net charged-phospholipids (e.g. PG, lysyl-PG or cardiolipin) and neutral polar lipids (e.g. PC, PE or glycolipids). Polar lipids having charged groups may be expected to play a role in ion selectivity of the membranes in which they reside whereas the neutral polar lipids might be involved in non-electrolyte permeability (Hopfer et al., 1970). It is, therefore, possible that biological membranes in general have a requirement for one or more acidic polar lipids and in addition, one or more neutral polar lipids and that within each class the lipids may be to some extent interchangeable (Minnikin et al., 1971).

E. Fatty Acid Composition and Positional Fatty Acid Distribution

Fatty acid patterns of each lipid component of S. epidermidis studied are similar (Table 26), suggesting that the diglyceride moiety of each lipid may be derived from the same phosphatidic acid precursor (see Introduction VI). The total fatty acid composition of S. epidermidis reported here is qualitatively similar to that reported

before in this species (Bergh et al., 1964) and to that of S. aureus and other bacteria in the family Micrococcaceae (Table 4).

From the results of the positional distribution of fatty acids in phosphatidylglycerol (Table 31), the following four molecular species of phosphatidylglycerol in S. epidermidis may be present:



Abbrev.: P = phosphate group, G = glycerol

The positional distribution of fatty acids in PG studied here is the same as found in phospholipids of Listeria monocytogenes in which C_{15} branched chain fatty acids were preferentially esterified to the 2-position whereas the branched chain C_{17} and palmitic acid were at the 1-position (Kosaric and Carrol, 1971). The presence of mainly C_{15} branched chain fatty acids at the 2-position and C_{17} branched chain fatty acids at the 1-position were also found in PE of Bacillus cereus (Saito and Sato, 1968) and in Nocardia leishmanii (Yano et al., 1970).

In animal and plant tissues, the 2-position of phospholipid is largely occupied by unsaturated fatty acids and among the saturated fatty acids, the shorter chain fatty

acids such as C_{14}-C_{16} show a greater tendency to occupy the 2-position than longer chains (Lands, 1965; Haverkate and Van Deenen, 1965; Couch and Saloma, 1973). In bacteria, the positional distribution of fatty acids is similar to that in animal and plant tissues (McElhaney and Tourlette, 1970a) except for Clostridium butyricum in which C_{16} was located mainly at the 2-position while 16:1 and cyclopropane acids (17:0 and 19:0) were at the 1-position (Hildebrand and Law, 1964). It was suggested that this may reflect a methylation specificity superimposed by the cyclopropane fatty acid synthetase (Hildebrand and Law, 1964).

The positional distribution of fatty acids in GPDGD, DGD and MGD was not analyzed since suitable specific lipases were not available. Saito and Mukoyama (1971) and Fischer et al. (1973a) used lipase from Rhizopus delemar to study the positional distribution of fatty acids in glycolipid and glucophospholipid from B. cereus and Streptococci respectively. The R. delemar lipase is known to attack the terminal ester linkage of the "synthetic" triglyceride (Hanahan et al., 1960), however, the reaction with glycolipid is slower than with the usual triglycerides (Saito and Mukoyama, 1970). The positional distribution of fatty acids found in glycolipid and/or glucophospholipid was the same as that for the phospholipids present in the respective bacteria (Saito and Mukoyama, 1971; Fischer et al., 1973a). Therefore, it is reasonable to assume that in S. epidermidis, the

positional distribution of fatty acids in GPDGD, DGD and MGD may be the same as found in PG. This would further support the contention that the diglyceride moiety of PG, GPDGD, DGD and MGD may be derived from the same phosphatidic acid precursor.

IV. Effect of Salt on Growth and Lipid Composition of S. epidermidis

Results on the effect of salts on growth of S. epidermidis (Figs. 4, 5) indicated that S. epidermidis is a halotolerant bacterium which grows best in the absence of added salt but can tolerate salt in the medium up to near saturation. Clearly, these cells must be adapted to growth and survival in a high salt medium and such adaptation may involve changes in membrane components, such as lipids and proteins, or in cytoplasmic enzymes.

The lipid content of cells grown in media of increasing salt concentration remained fairly constant (Table 7). However, the proportion of PG decreased while that of cardiolipin and GPDGD, increased with increasing salt concentration; the proportions of the glycolipid components (DGD and MGD) remained unchanged (Fig. 27). A similar effect was reported in S. aureus, in that the percentage of cardiolipin was markedly increased while that of PG diminished with the rise in the sodium chloride concentration (0-10%) (Yoshioka et al., 1972).

Generally, bacteria growing in media containing high sodium chloride concentrations (extreme halophiles, moderate halophiles, or halotolerant organisms) usually contain high contents of acidic lipids. For instance, in H. cutirubrum, all of the membrane polar lipids are acidic

(see Kates, 1972); in a halotolerant-moderate halophile (Pelleg and Tietz, 1971,1973), an acidic glycolipid (glucuronosyl diglyceride) and acidic glucophospholipid were found together with PG and cardiolipin; in S. aureus and S. epidermidis, high contents of PG were present along with cardiolipin and glucophospholipid. Thus, it is likely that these acidic lipids might play an important role in controlling ion permeability of cells grown in salt media. Quantitative experiments on model bilayer membranes prepared from PG or cardiolipin indicated that they were highly cation selective (Hopfer et al., 1970). Moreover, it has been shown that acidic phospholipids such as PG and PS are capable in model membranes of discriminating between univalent cations whereas neutral ionic phospholipids such as PE and PC are not (Papahadjopoulos, 1971). The author suggested that discriminating between monovalent cations in biological membranes could depend upon carboxyl as well as a phosphate group. The acidic glucophospholipid such as GPDGD present in S. epidermidis might perhaps be capable of carrying out an ion selectivity function similar to that demonstrated for PG and PS.

The observed reciprocal increase in percentages of cardiolipin and GPDGD and decrease in percentage of PG with increasing salt concentration of the medium (Fig. 27) suggest that the highly acidic cardiolipin and GPDGD may be more effective than PG in controlling ion permeability when

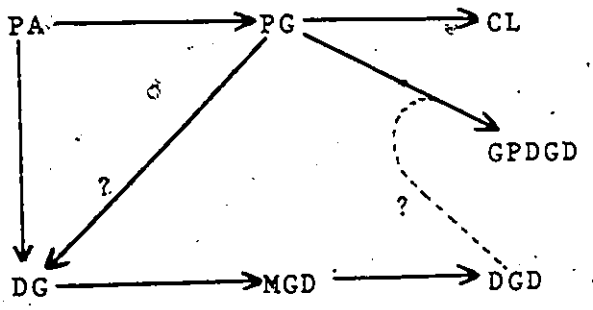
cells are grown in a high salt medium. The decreased proportion of PG might be explained by increased turnover of PG to cardiolipin and GPDGD stimulated by high salt. The unchanged level of neutral glycolipids (DGD and MGD) with increasing concentration of sodium chloride in the growth medium is consistent with the view that neutral polar lipids are not involved in membrane ion permeability (Hopfer et al., 1970).

The total fatty acid composition of S. epidermidis grown in medium of salt concentration in the range of 0-15% was practically constant, but when the salt concentration was increased to 25%, the proportion of anteiso 15:0 fatty acid increased greatly from 58 to 75% (Table 32). Uchida and Moji (1972) reported that Pediococcus sovae, a halotolerant organism, showed little change in fatty acid composition with increasing salt concentration in the medium (0 to 18%). The increase in percentage of anteiso 15:0 fatty acid at the high salt concentration (25%) observed here might be due to partial inhibition of chain elongation of branched 15:0 to branched 17:0 fatty acids by high salt concentration.

In summary, it may be concluded that polar groups such as phosphates or hydroxyl groups of acidic glucophospholipid, probably play a more important role than the nonpolar acyl chains in controlling ion permeability of the membrane.

V. Metabolism of Membrane Lipids of S. epidermidis

Time course of incorporation of [^{32}P]orthophosphate and [1-^{14}C]glycerol into individual lipid components during growth of S. epidermidis cells revealed a sequence of labelling of the membrane lipids (see Fig. 32 and Results X) which suggests the following precursor-product relationship among the lipid components:



The biosynthesis of phosphatidylglycerol (PG) from phosphatidic acid (PA) and cardiolipin (CL) from PG have been demonstrated clearly in E. coli and in other bacteria (see Introduction VI). The presence of these pathways in S. epidermidis is supported by the sequential labelling of PA, PG and cardiolipin (Fig. 32), suggesting a product-precursor relationship between PA and PG and between PG and cardiolipin. Diglyceride (DG) may be formed by the action of phosphatidic acid phosphatase on PA as found in E. coli (Van Den Bosch and Vagelos, 1970), and also by action of phospholipase C on PG. The increase in percentage of ^{14}C in neutral lipid (mainly diglyceride, Fig. 33) at the

expense of ^{14}C in PG during the growth period suggests that breakdown of PG to DG was occurring. However, no definite conclusions concerning the existence of these pathways for DG in S. epidermidis can be made from the present results, since diglyceride was not specifically determined. In most strains of E. coli, phospholipase C was not detected (Bright-Gaertner and Proulx, 1972) but it has been found in many other genera of bacteria such as Clostridium welchii (Ikezawa et al., 1964), Bacillus cereus (Haverkate and Van Deenen, 1964) and in S. aureus (Doery et al., 1965). The presence of phosphatidic acid phosphatase and phospholipase C has not previously been reported in S. epidermidis, to the best of my knowledge.

Diglyceride may serve as the precursor of monoglucosyl diglyceride (MGD) which in turn is the precursor of diglucosyl diglyceride (DGD). This biosynthesis pathway has been demonstrated in Streptococcus faecalis (Pieringer, 1968) and in other bacteria (see Introduction VI). In S. epidermidis, the labelling sequence of MGD and DGD (Fig. 32) suggests a product-precursor relationship between these two glycolipids, consistent with the operation of this pathway.

Biosynthesis of glucophospholipid in S. faecalis has been shown to involve both PG and diglucosyl diglyceride as precursors (Pieringer, 1972). In S. epidermidis, the labelling pattern (Fig. 32) suggests that PG may be the donor of the sn-glycero-1-phosphate moiety of GPDGD, since both

the ^{14}C and ^{32}P percentage activity of PG decreased while these activities increased in GPDGD. However, the percentage ^{14}C activity of DGD did not change with increasing ^{14}C-activity in GPDGD. One explanation of this observation is that the rate of conversion of DGD to GPDGD may be equal to the rate of biosynthesis of DGD from MGD. Alternatively, some other precursor (as yet unidentified) of the glucosyl diglyceride moiety may be involved. The presence of PA and MGD accounting for considerable proportions of radioactivity in the early growth period (up to 10 min) is consistent with the explanation that they are intermediates in lipid biosynthesis.

In the pulse-chase experiment, ^{32}P is "chased" out of PG faster than [^{14}C]PG, during exponential growth (Fig. 35) but thereafter up to the stationary phase both ^{32}P and ^{14}C decrease in parallel. This pattern of turnover of ^{32}P- and ^{14}C-activities of PG is the same as that observed in the total lipids (Fig. 35). During the early chase period (2 hr), the increase in both ^{32}P and ^{14}C in GPDGD and cardiolipin concomitant with the decrease in ^{32}P and ^{14}C radioactivity of PG, supports the conclusion from the pulse experiment (Fig. 32) that both cardiolipin and GPDGD are synthesized from PG. In the early stationary phase, radioactivities in cardiolipin, PG and DGD reached fairly constant levels whereas those of GPDGD continued to decrease to very low values (Fig. 35). Thus, turnover of GPDGD is much more

rapid than that of DGD, PG or cardiolipin in S. epidermidis. In contrast, the glucophospholipid in S. aureus was metabolically stable while DGD turned over (Short and White, 1970), just the reverse of that in S. epidermidis.

These observations suggest that in S. epidermidis, the main catabolic enzymes involved in lipid turnover would perhaps be phospholipase C, and phospholipase A together with lysophospholipase. The latter two enzymes acting in sequence were thought to be concerned with the degradation of phospholipids in E. coli (Bright-Gaertner and Proulx, 1972; Albright et al., 1973). In S. aureus, phospholipase A, lysophospholipase and phospholipase C activities were found in the culture fluids of the organisms (Doery et al., 1965). These enzymes have not been reported in S. epidermidis. The radioactivity lost from PG may be converted to other lipid components such as cardiolipin, GPDGD, diglyceride, as well as to non-lipid, water-soluble compounds as suggested by Kanfer and Kennedy (1963). However, definitive conclusions concerning the operation of the above pathways for metabolism of lipids in S. epidermidis can only be based on further studies with cell-free system

APPENDIX I

CHARACTERISTICS AND IDENTIFICATION OF THE MICROORGANISMS EXAMINED IN THIS THESIS

A. Staphylococcus epidermidis

A pure culture of this bacterium was isolated by Dr. J. Harwig, Food and Drug Directorate, Department of National Health and Welfare, Ottawa, Canada. The organism was found to be a colourless, Gram-positive coccus and was identified as Staphylococcus epidermidis by Dr. C. E. Par (above address) on the basis of the following tests (see Introduction I.A.):

Mannitol Fermentation	Positive
Catalase Production	Positive
Indole Production	Negative
Coagulase Production	Negative
Acetoin Production	Negative
Glucose Fermentation (anaerobic)	Positive
Gas from Anaerobic Glucose Fermentation	Negative
Glucose Fermentation (aerobic)	Positive
Gas from Aerobic Glucose Fermentation	Negative

B. Wallemia sebi

A pure culture was isolated by Dr. J. Harwig and sent to Dr. A. C. Stolk, Centralbureau Voor Schimmelcultures, Oosterstratt, Netherland for identification.

His description and identification of this organism was as follows (Stolk, 1971):

"Wallemia sebi (Fr.) v.Arx (Sporendonema sebi Fr., Hemispora stellata Vuill.) is characterized by arthrospores (spores which develop by disarticulation of hyphae). In this species the disarticulating hyphal structures are produced by large flask-shaped phialides. Young spores are because of their development as arthrospores somewhat cubical, but mostly they become very soon globose. They show yellowish or reddish colours, their walls are slightly roughened and they measure 2.5-3.5μ.

The species is strongly osmophilic, growing best on media as malt +20% sucrose, or malt +25% NaCl. The cultures are somewhat restricted and show brown colours. Identification of this species is entirely based on its morphological characteristics, which are rather unique. Cytological or biochemical tests were not necessary for its identification.

C. A Nocardia sp.

A pure culture of this bacterium was isolated and identified as a Nocardia sp. by Dr. M. B. Gochnauer, Biology Department, University of Ottawa, Ottawa, Canada, who carried out the tests for characterization of the organism as follows:

The organism is an aerobic, Gram-positive, obligate halophile which grows in media containing at least 10 percent

NaCl with the optimum at 15-20 percent NaCl. On a solid medium, this organism produces branched aerial and substrate mycelia. The latter occasionally fragments. Long chains of conidia are produced on both aerial and substrate mycelia. The spores are oval and have smooth surfaces as shown by scanning and transmission electron microscopy. The aerial mycelium is white at all salt concentrations. The substrate mycelium is pigmented; the colour produced is dependent on the salt concentration. In media containing 25% NaCl or more it is yellow. As the concentration of salt is decreased, the substrate mycelium becomes darker. In the presence of 10 and 12.5% NaCl, it is dark brown and the pigment diffuses out into the medium. The pigments developed in about seven days of growth.

The morphology of the two genera Nocardia and Streptomyces are very similar but can be distinguished by determination of the chemical composition of the cell wall. The following results of cell wall analyses of this organism were obtained: (1) the presence of meso-diaminopimelic acid as determined by the method of Becker et al. (1964); (2) the presence of large amounts of arabinose and galactose using the methods of Becker et al. (1965). These findings establish the identity of the organism as a Nocardia sp.

CLAIMS TO ORIGINAL RESEARCH

1. Lipid components of a Nocardia species and of Waksmania sebi have been identified chromatographically, and total fatty acid composition determined. The lipid composition of these two species have not previously been reported.

2. Complete structural determinations of the polar lipid components of Staphylococcus epidermidis have been carried out.

3. A novel glucophospholipid has been isolated from S. epidermidis and its chemical structure has been established as 3-O-[sn-glycero-1-phosphoryl-6'-O-(β-D-glucopyranosyl-(1→6)-O-β-D-glucopyranosyl)]-1,2-diacyl-sn-glycerol. The complete structure of glucophospholipids in other Staphylococcus species has not previously been determined.

4. Studies on the effect of sodium chloride concentration (0 to 25%) in the growth medium on the lipid composition of S. epidermidis reveal considerable changes in the proportions of charged polar lipids and relatively small changes in percentages of anteiso-C_{15} fatty acid. These changes may be involved in control of ion permeability of the cell membrane.

5. Metabolism of membrane lipids has been studied in intact cells of S. epidermidis, by means of "pulse-chase" experiments using [^{32}P]orthophosphate and [1-^{14}C]glycerol.

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