To my Mother -

and the memory of A. J. B.

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SUMMARY

Part One of the thesis reports studies on the chemical structure of two membrane polar lipid components of H. cutirubrum.

For the first component, a monomeric phosphatidyl glycerophosphate (PGP) structure which was recently in dispute has been definitively established by NMR examination and osmometric molecular weight determination of methylated PGP derivatives, as well as by elemental analysis of a series of PGP salt forms.

The second component, previously never isolated, was found to have an unusual structure, viz. 1-sn-phosphatidyl-3'-sn-glycero-1'-sulfate. The close relationship of the lipid to PG was shown by their spectral similarity, the facile solvolysis of the lipid to PG and the partial chemical synthesis of the lipid by treatment of natural PG with a sulfating reagent. The presence of the sulfate moiety was established by spectral analysis procedures, in particular by ^{31}P-^{1}H heteronuclear spin-decoupling, as well as by elemental analytical data. Furthermore, solvolysis of the lipid in THF-HCl was shown to release sulfate ion quantitatively.

The sulfate moiety was shown to be esterified at the C-1 position of glycerol by comparison of the NMR chemical shifts of the S-OC$\underline{H}_3$ groups of the bacterial lipid, with that of the S-OC$\underline{H}_3$ groups of PG-2-S, synthesised by an unequivocal route.

Part Two of the thesis includes two series of synthetic studies. The first series describes the chemical synthesis and characterisation of the RRR-diastereoisomeric form of two extended-chain homologues (C_{21} and C_{22}) of the isoprenoid phytanic acid (C_{20}). The C_{21} acid was obtained by chain elongation via carboxylation of RRR-phytanyl magnesium iodide and the C_{22} acid by a malonate chain elongation of RRR-phytanyl iodide. The optical rotations of the C_{21} and C_{22} homologous methyl esters were of special interest in connection with earlier studies of the optical properties of branched-chain fatty acids, while their GLC behaviour was of interest with regard to studies in other laboratories on GLC separation and identification of diastereoisomeric isoprenoid acids formed by oxidation and chain elongation of meso-pristane and phytol.

The second series describes the synthesis and properties of monophytanyl ether analogues of phosphatidic acid, phosphatidyl glycerol and phosphatidyl glycerophosphate from synthetic 3-O-phytanyl-sn-glycerol. The lysophosphatide products were characterised by elemental and spectral analysis and their chromatographic and degradative behaviour was established. The possibility that lysophosphatides may play a role as intermediates in the anabolic and catabolic metabolism of the extreme halophiles enhances the usefulness of the data obtained.

RESUME

La structure chimique de deux des composés polaires de la membrane de H. cutirubrum fait l'objet de la première partie de la thèse.

Pour le premier composé, une structure monomère phosphatidyl glycérol (PGP), recemment contestée, a été établie d'une façon certaine par RMN et par la détermination osmométrique du poids moléculaire des dérivés méthyliques du PGP ainsi que par l'analyse élémentaire d'une série de sels du PGP.

Le deuxième composé, qui n'avait jamais été isolé auparavant, s'avéra d'une structure inhabituelle, le sn-glycéro-3'-sulfate-1' de sn-phosphatidyle-1. La similarité spectrale entre le lipide et le glycérol de phosphatidyle (PG), la solvolyse facile du lipide en PG, ainsi que la synthèse partielle du lipide en soumettant le PG naturel à l'action d'un agent de sulfatation ont démontre un rapport étroit entre le lipide et le PG. La présence d'un groupement sulfate a été établie par analyse centésimale, par des mesures spectrales, parmi lesquelles la RMN du proton avec découplage de spin hétéronucléaire ^{31}P-^{1}H. Il a été en outre montré que le sulfate était libéré quantitativement au cours de la solvolyse du lipide dans un milieu THF-HCl. Le groupement sulfate a été localisé en position C-1 du glycérol par comparaison en RMN du déplacement chimique du groupe S-OCH_3 du lipide bactérien avec celui d'un échantillon authentique de PG-2-S synthétisé d'une façon non équivoque.

La deuxième partie de la thèse comprend deux études synthétiques. La première décrit la synthèse et l'identification de la forme diastéréoisomérique $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{R}}$ de deux homologues (C_{21} et C_{22}) de l'acide phytanique (C_{20}). L'acide en C_{21} a été obtenu par l'extension de la chaîne en C_{20} en passant par la carboxylation de l'iodure de $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{R}}$-phytanyle magnésium, et l'acide en C_{22}, par l'extension de la chaîne de l'iodure de phytanyle à l'aide d'un malonate. Le pouvoir rotatoire des esters méthyliques homologues en C_{21} et C_{22} est spécialement intéressant si on les compare avec des études antérieures décrivant les propriétés optiques des acides gras à chaînes ramifiées. De plus le comportement en CPV des deux esters méthyliques est intéressant si on le compare à des travaux dans d'autres laboratoires se rapportant à la séparation par CPV et l'identification d'acides isoprénoïdes diastéréoisomères formés par oxydation et par extension de la chaîne du meso-pristane et du phytol.

La deuxième étude décrit la synthèse et les propriétés des analogues monophytanylés de l'acide phosphatidique, de PG et PGP, obtenus à partir du O-monophytanyle-3-sn-glycérol synthétique. Les produits lysophosphatidiques furent caractérisés par analyse centésimale et par spectrométrie ainsi que par chromatographie et par des processus de dégradation. La possibilité que les produits lysophosphatidiques puissent jouer le rôle d'intermédiaires dans le métabolisme anabolique et catabolique des halophiles extrêmes accroit l'utilité des résultats obtenus.

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LIST OF ABBREVIATIONS

AP-L	Apiezon-L
BDS	Butanediol succinate polyester
Bzl	Benzyl
CDP	Cytidine diphosphate
CTP	Cytidine triphosphate
DEGS	Diethyleneglycol succinate polyester
DHAP	Dihydroxyacetone phosphate
ECL	Equivalent chain length
GLC	Gas-liquid chromatography
NMR	Nuclear Magnetic Resonance
PA*	Phosphatidic acid
PAPS	3'-Phosphoadenosine-5'-phosphosulfate
PG*	Phosphatidyl glycerol
PGP*	Phosphatidyl glycerophosphate
PG-1-S*	1-sn-Phosphatidyl-3'-sn-glycero-1'-sulfate
PG-2-S*	1-sn-Phosphatidyl-1'-sn-glycero-2'-sulfate
SL*	Glycolipid sulfate
Ts	p-toluenesulfonyl
TLC	Thin layer chromatography

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

GENERAL INTRODUCTION

It is ironic that the salt traditionally used as a preservative against the microbial deterioration of foodstuffs and other proteinaceous substances should turn out to be the very compound specifically required for survival and growth by a specialised class of bacteria. These "halophilic" bacteria are to be found among the red-coloured algae and fungi which thrive in natural salt concentrates. During the last forty years, bacteriologists have described the taxonomy of the halophiles and their absolute requirement for salt [see reviews by Flannery (1956) and Ingram (1957)], but not until the pioneering work of Gibbons' group at NRC, Ottawa, Canada during the fifties were the biochemical aspects of halophilism studied.

One feature that emerged was the unique structure of the cell membrane of halophilic bacteria. Most cell membrane lipids contain long chain fatty acid esters of glycerol. The halophilic bacteria are unique in that the long chains in their membrane lipids are linked to glycerol by ether bonds. Claims have been made for the presence of dialkyl (ether) phosphatides in animal tissue (Popovic, 1965; Marinetti, 1959; Horrocks and Ansell, 1967) but only in halophilic bacteria has their presence so far been rigorously established. The unusual characteristics of the halophiles, especially those relevant to the chemistry of the cell membranes, are discussed in detail in the following sections.

I. Review of Halophilic Bacteria

1. Classification

The subject of halophilism in general has been reviewed by Larsen (1962) and the extreme halophiles in particular by Larsen (1967) and Kushner (1968).

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

(i) moderate halophiles which grow in concentrations of sodium chloride from 0.5 - 3.5 M;

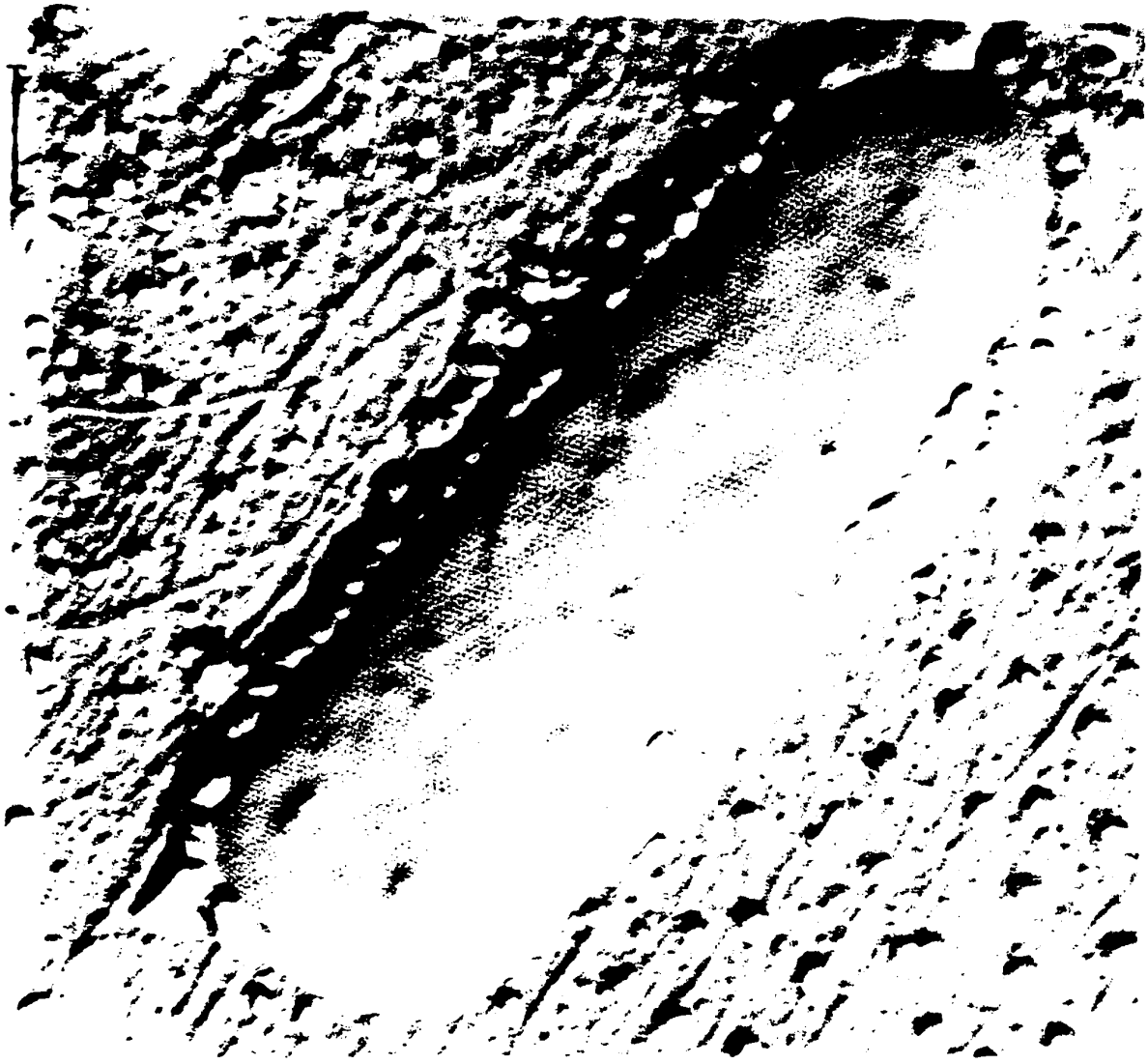
(ii) extreme halophiles which will not grow in less than 2.5 M sodium chloride and require saturated sodium chloride (ca. 5 M) for optimal growth.

Bergey's Manual (Seventh Edition, 1957) lists five species of extreme halophiles in the genus Halobacterium, family Pseudomonadaceae: H. cutirubrum, H. halobium, H. salinarium, H. marismortui and H. trapanicum. This genus consists of rod-shaped, Gram-negative, non-spore-forming, obligate aerobes which when motile are lophotrichously flagellate (Figure 1). Optimal growth occurs at a salt concentration of about 25% sodium chloride (4.3 M) and the minimum sodium chloride concentration required for growth is about 15% (2.5 M).

In addition to the Halobacteria there exist coccoid halophiles which require at least 12% sodium chloride (2 N) for growth, with optimum growth at a concentration of about 20% sodium chloride (3.2 M). Three such species have been assigned to the general Micrococcus and Sarcina, family Micrococcaceae: M. morrhuae, S. littoralis and S. morrhuae. They are non-spore-forming obligate aerobes, but are non-motile.

FIGURE 1

Surface of the extreme halophile, Halobacterium cutirubrum. The replica was prepared by J. Y. D'Aoust, who kindly supplied the photograph. x 51, 000



Extreme halophiles grow slowly even under optimum conditions. Larsen (1967) reports a generation time for Halobacteria of about 7 hr, and for halococci about 15 hr. The complex medium of Sehgal and Gibbons (1960) supports good growth and Onishi et al.(1965) have also developed a chemically-defined medium.

2. Salt Requirements

The most striking effect of progressive dilution of the saline environment of extreme halophiles is the change of their rod-like form to a spherical one, which finally lyses completely and irreversibly in 1 - 1.5 M salt (Abram and Gibbons, 1961). The requirements of the rod-form for Na^+ appear to be specific. If Na^+ is replaced by K^+ or NH_4^+ the integrity of the rod is not preserved (Kushner, 1964). However, Na^+, K^+ and Li^+ are equally effective in maintaining the integrity of mechanically-prepared envelopes, whose dissolution begins at 3 M sodium chloride and is almost complete at 1 M salt. Thus the specific requirement of the intact cell for Na^+ suggests that the role of Na^+ is not solely osmotic. Soo-Hoo and Brown (1967) showed that the protective effect of monovalent cations on the morphological integrity of H. halobium decreased in the same order as their hydrated volumes ($Li^+ > Na^+ > K^+ > NH_4^+$) and concluded that the salt concentration effect is a combination of electrostatic and osmotic factors. In accord with these observations, Kushner (1964) and Soo-Hoo and Brown (1967) found 0.5 M Mg^{++} to be more effective than 1 M Na^+ in preventing lysis of H. cutirubrum cells.

3. The Cell Envelope Structure

The reports of Brown and Shorey (1963) suggested that cells of H. halobium were bounded only by a triple layered plasma membrane, but later work (Cho et al., 1967; Stoeckenius and Rowen, 1967) showed that the cell envelope consisted of a wall and a plasma membrane. However, the cell envelope of the extremely halophilic bacteria differs distinctly from that of other gram-negative bacteria, in not possessing muramic acid and diaminopimelic acid. Analysis showed that the halophile envelope was largely lipoprotein containing $> 93\%$ of the lipids of the whole cell (Kushner et al., 1964, see Fig. 2).

The cell envelope requires a high concentration of monovalent ions (or a lower concentration of divalent ions) to prevent electrostatic disaggregation (Brown, 1963; Kushner et al., 1963). The charges responsible for this disaggregation include those of the carboxyl groups of aspartic and glutamic acids which are present in unusually high proportions in the membrane proteins, as well as the phosphate and sulfate groups of the acidic lipid components. Stoeckenius and co-workers (Stoeckenius and Rowen, 1966; Stoeckenius and Kunau, 1968) have fractionated the cell envelope of H. halobium, after dialysis against water, by differential centrifugation and sucrose density gradient centrifugation into the following four fractions;

[a] a soluble colourless, lipid-free fraction containing protein and hexosamines, which was considered to be the cell wall;

[b] a red fraction containing 40% lipid and 60% protein, presumably formed from cell membrane disaggregation;

[c] a purple fraction consisting of large membrane sheets of similar composition to [b];

[d] "intracytoplasmic membrane fraction" (collapsed gas-vacuoles), largely protein in nature.

These workers found that fractions [a] and [b] contain a high proportion of dicarboxylic amino acids in agreement with the view that disruption of the cell envelope upon removal of salt is due to high charge density at the envelope surface.

The colour of fraction [c] has been shown to be due to a complex of retinal and an opsin-like protein (Oesterhelt and Stoeckenius, 1971; Blaurock and Stoeckenius, 1971); the similarity of this complex to the visual pigment-containing membranes of the vertebrate eye suggested a possible photo-receptor function. The protein retinal complex was appropriately named bacteriorhodopsin.

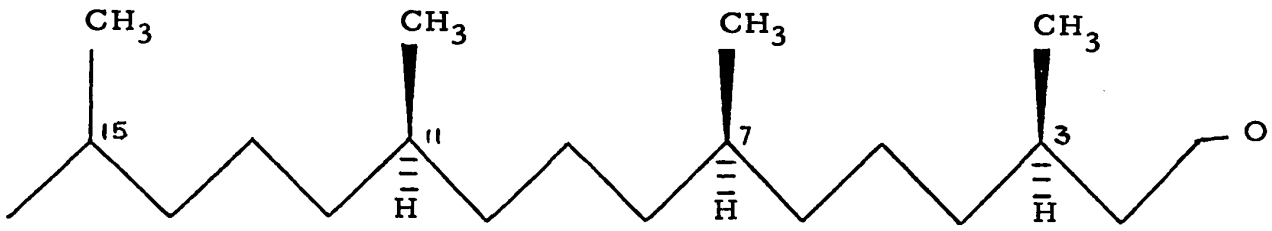
II. Chemistry of the Membrane Lipids

1. Total Cellular Lipids of H. cutirubrum

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

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

The polar (acetone-insoluble) lipids were recognised as atypical of Gram-negative bacteria in three respects. Firstly, the low nitrogen content (0.18%, N/P atomic ratio 0.09; Sehgal et al., 1962) indicated the presence of only traces of nitrogenous lipids. Secondly, analysis for phosphorus (P=4.3-4.6%) indicated an unusually high P lipid content. Thirdly, neither mild alkaline hydrolysis (Dawson, 1954) nor drastic hydrolysis of the lipids, released fatty acids, showing that the lipids were not derivatives of diacyl glycerols typically found in normal bacteria. Instead, hydrolysis gave 65-75% of a non-saponifiable material which was shown to be an asymmetrically substituted di-O-alkyl glycerol (Kates et al., 1965; Joo et al., 1968; Faure et al., 1963). Both alkyl groups were found to be 3,7,11,15-tetramethylhexadecyl- (or phytanyl) groups (Kates et al., 1965). Each of the three asymmetric centres of the phytanyl groups (C-3, C-7 and C-11) was shown to have the R-absolute configuration (Kates et al., 1967). Thus, the bacterial phytanyl group has the 3R, 7R, 11R, 15-tetramethylhexadecyl structure:



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

2. Molecular Structure of the Polar Lipids of H. cutirubrum

Chromatography of the polar (acetone-insoluble) lipids revealed the presence of two major components and four or five minor components (Fig. 2); (Sehgal et al., 1962; Kates et al., 1966). All of the components characterised to date are derivatives of 2, 3-di-O-phytanyl-sn-glycerol.

The major component (Spot 6 + 7, Fig. 2) was an acidic phosphatide which accounted for about 85% of the lipid P and about 60% of the weight of the polar lipids. Elemental analyses and degradative hydrolysis studies on the lipid indicated a phosphatidyl glycerophosphate structure (Structure 1, Fig. 3) in which ester-linked alkyl groups were replaced by ether-linked branched chain (phytanyl) groups. However, the data were not entirely unambiguous; the sodium and potassium salts isolated had a cation: P atomic ratio of 1:1, and only one ionisable acid group per atom of P (i.e. two acid groups per molecule of PGP) could be detected by phenolphthalein indicator, although three acid groups per molecule of PGP were required. It was for this reason that Faure et al, (1963), who had also arrived essentially at a PGP structure for this lipid, concluded that the phosphatide was a dimer in which two molecules of PGP were condensed to give a pyrophosphate structure (Structure 2, Fig. 3). The chemical synthesis of the monomeric structure 1 yielded a lipid identical with the natural substance (Joo and Kates, 1969). Furthermore, its stereochemical structure was shown by this synthesis to be 1-sn-phosphatidyl-3'-sn-glycero-1'-phosphate (Fig. 4, Structure B), in which the configuration of both glycerol moieties is opposite to that of diacyl-PGP found in other bacteria (Slotboom and Bonsen, 1970).

FIGURE 2

Tracing of chromatogram of total lipids of H. cutirubrum cells and envelopes. The chromatogram was stained with Rhodamine 6G and viewed under ultra-violet light. The fluorescent colours are indicated by the following abbreviations:

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

Broken lines indicate minor components. Identification of components:

Spots 1, 3	unidentified;
Spot 2	glycolipid sulfate (SL);
Spot 4	desulfated SL;
Spot 5	phosphatidyl glycerosulfate*;
Spots 6, 7	phosphatidyl glycerophosphate;
Spot 8	phosphatidyl glycerol;
Spot 9	pigments.

Solvent system:

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

* See Part One, this thesis

[Data from Kushner et al (1964].

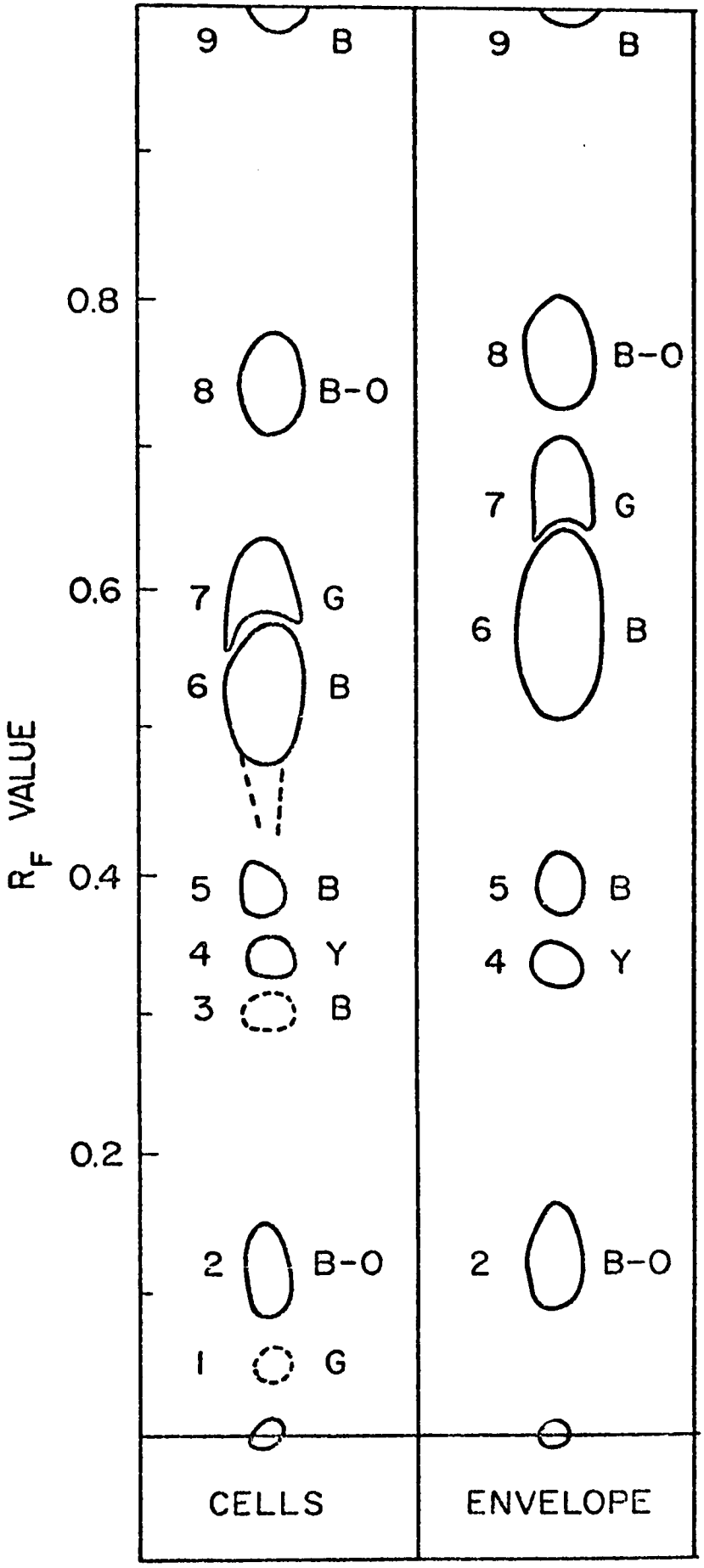
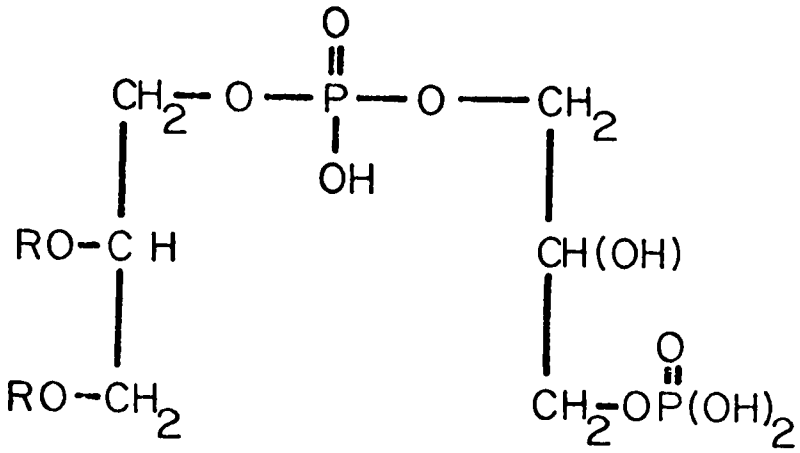

9
B
8
B-O
7
G
6
B
5
B
4
Y
3
B
2
B-O
1
G
0.8
0.6
0.4
0.2
R_F VALUE
CELLS
ENVELOPE

FIGURE 3

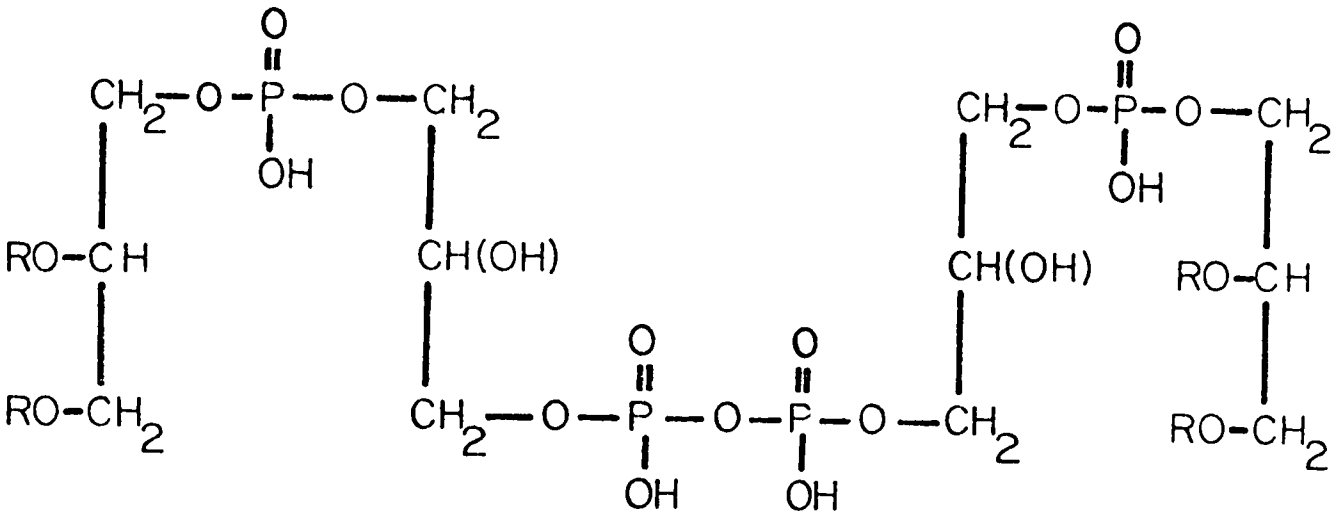
Chemical structures for the phosphatidyl glycerophosphate component in H. cutirubrum:

Structure 1 Monomeric structure proposed by Kates et al.(1963)

Structure 2 Dimeric pyrophosphate structure proposed by Faure et al. (1963)



Structure 1



Structure 2

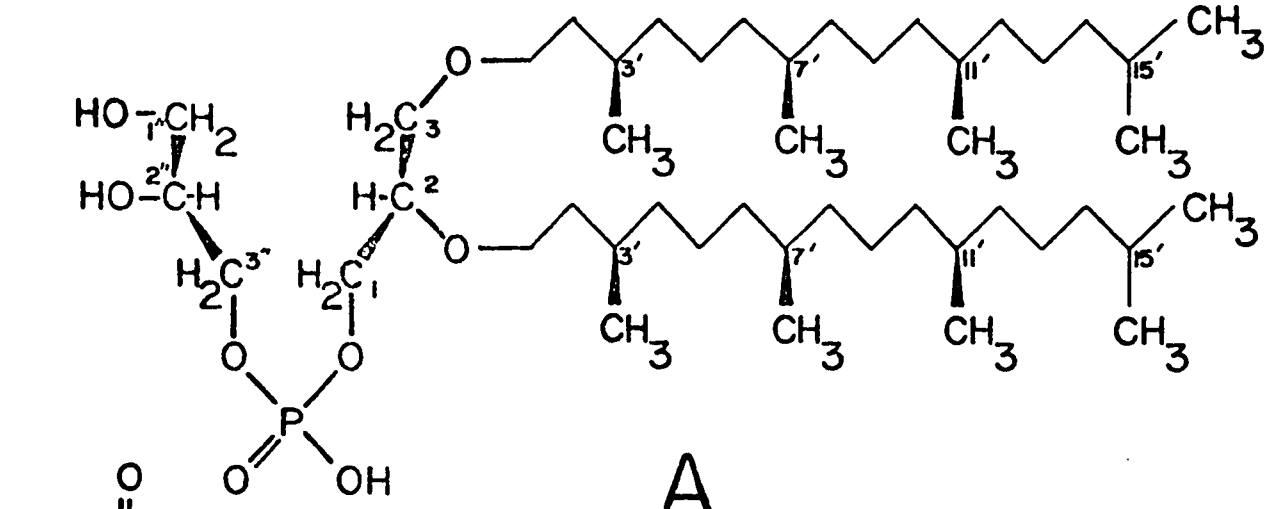
FIGURE 4

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

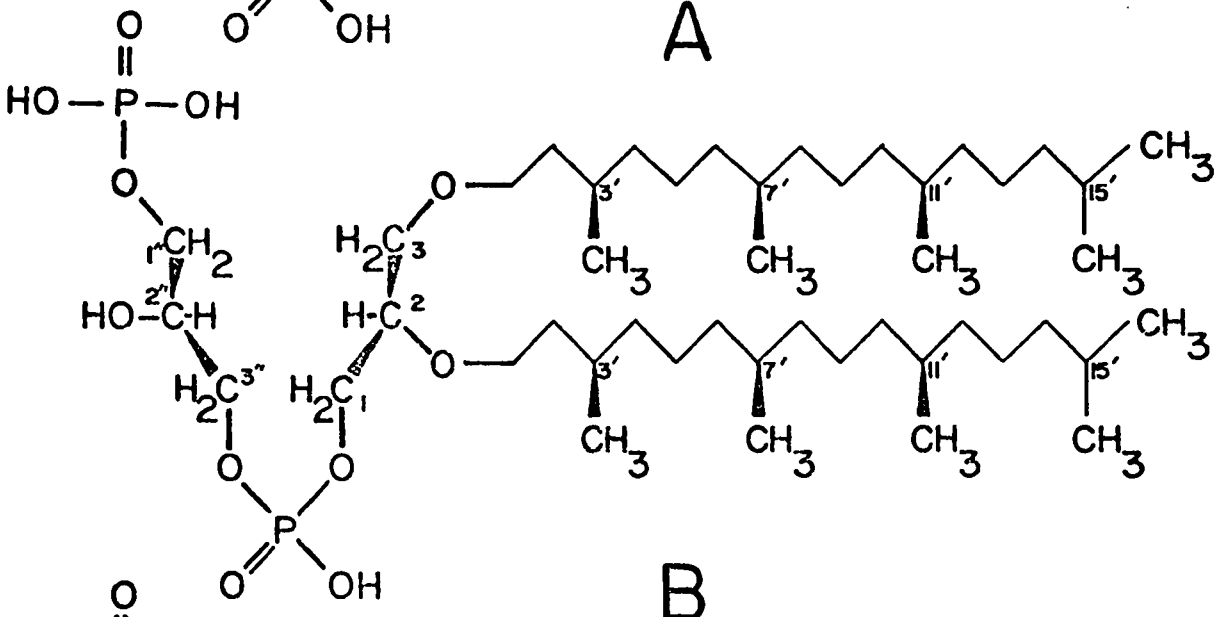
A 2, 3-Di-O-(3'R, 7'R, 11'R, 15'-tetramethylhexadecyl)-sn-glycero-1-phosphoryl-3''-sn-glycerol [1-sn-phosphatidyl-sn-3'-glycerol; a-PG]

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

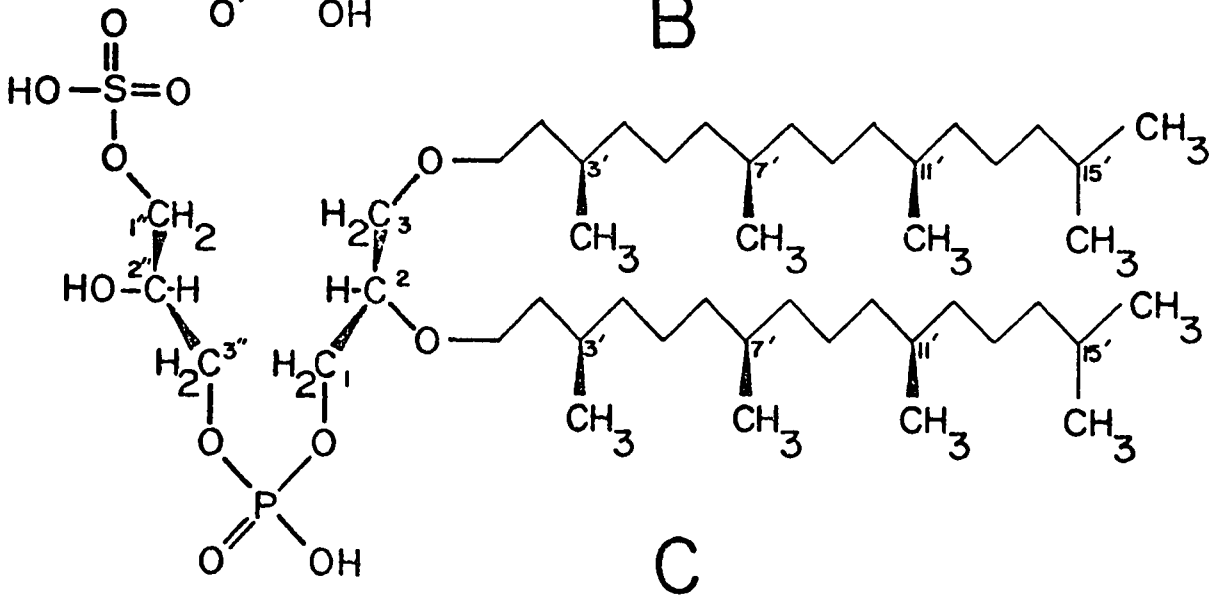
B 2, 3-Di-O-(3'R, 7'R, 11'R, 15'-tetramethylhexadecyl)-sn-glycero-1-phosphoryl-3''-sn-glycero-1''-sulfuric acid [1-sn-phosphatidyl-sn-3'-glycero-1'-sulfuric acid: PG-1-S]



A



B



C

In the study described in this thesis, the remaining question of the number of ionisable groups present per molecule was investigated by NMR studies of permethylated PGP and PGP-trimethyl ester and by an examination of a series of salt forms of PGP.

The other major lipid component (spot 2; Fig. 2) was a phosphorus-free glycolipid which accounted for 25-30% by weight of the total polar lipids (Kates et al., 1967). Analytical data showed it to be a sulfate ester of a glycolipid containing three moles of sugar and one mole of diphytanyl glycerol ether. The sugars were identified as glucose, mannose and galactose and their linkages were established as galactose-(1→6)-mannose-(1→2)-glucose-(1→1)-diphytanyl glycerol. The sulfate group was shown to be esterified at C-3 of galactose (Kates et al., 1967; Kates and Deroo, 1972). Optical rotation data suggest that glucose and mannose are α-linked but the terminal galactose is β-linked. The final structure proposed is 3-SO_3^--galp-β-(1→6)-manp-α-(1→2)-glup-α-(1→1)-sn-2,3-diphytanylglycerol (Kates and Deroo, 1972).

The minor components include three phospholipids (spots 3, 5, 8; Fig. 2) and two glycolipids (spots 1,4; Fig. 2). One of the phospholipids (spot 8; Fig. 2) accounting for 5% of the lipid P, has been isolated and identified as the diphytanyl ether analogue of phosphatidyl glycerol (Faure et al., 1964; Kates et al., 1966). Joo and Kates (1968, 1969) confirmed this structure and established the stereochemical structure as 1-sn-phosphatidyl-3'-sn-glycerol (Fig. 4; structure A) by chemical synthesis. Thus, as in PGP, the two glycerol moieties in this phospholipid have configurations opposite to those of the glycerol moieties in the diacyl-PG in plants and other bacteria (Slotboom and Bonsen, 1970).

A second minor phosphatide (spot 5, Fig. 2), accounting for 6% of the lipid phosphorus, had not previously been characterised, but preliminary studies suggested the presence of both phosphorus and sulfur in this lipid (Kates et al., 1968). The isolation and structure identification of this novel phosphosulfolipid was therefore undertaken and the study leading to its complete stereochemical structure is presented in detail in this thesis.

The third minor phospholipid (spot 3; Fig. 2), which accounted for $< 1\%$ of the lipid phosphorus, has not yet been isolated in sufficient quantity for structural studies.

Of the remaining glycolipids, one of them (spot 4; Fig. 2) proved to be the desulfated glycolipid sulfate; the second one (spot 1; Fig. 2) contains both sugar and sulfate and awaits further study.

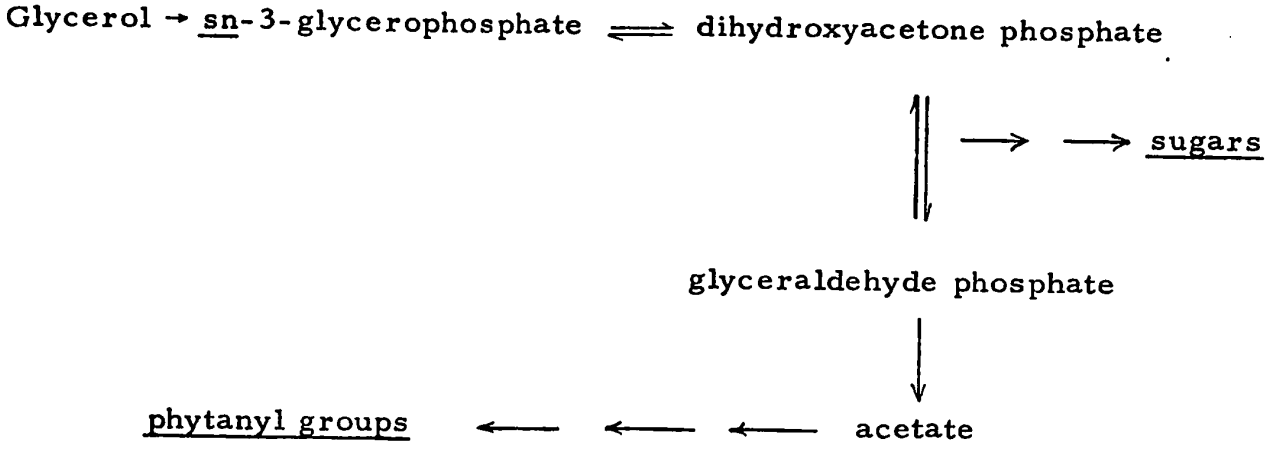
The major diphytanyl ether components have been found in all extremely halophilic bacteria studied, but not in the moderate, or non-halophilic bacteria (Kates et al., 1966; Marshall and Brown, 1968).

III. Biosynthesis of Membrane Ether Lipids in H. cutirubrum

1. Incorporation of Labelled Precursors into Polar Lipids by Whole Cells

Of the long-chain hydrocarbon group precursors, mevalonate-^{14}C showed the highest degree of incorporation (ca. 8%), followed by glycerol-1,3-^{14}C (2.0%) and acetate-1-^{14}C (1.90%) (Kates et al., 1968). Degradative studies of the labelled lipids showed that the ^{14}C label derived from acetate and mevalonate was almost entirely associated with the phytanyl groups, but that from glycerol was associated with water-soluble groups as well as phytanyl groups. These findings suggested that the predominant biosynthetic route for the hydrocarbon chains was the mevalonate pathway, although the individual steps in the pathway have yet to be elucidated with cell-free or purified enzyme systems. In particular, the reduction steps by which the fully saturated phytanyl chain is formed from the unsaturated dimethylallyl pyrophosphate intermediate remain to be established. Certainly, the reduction mechanism must be stereospecific in order to generate the D configuration (R absolute) at the C-3, C-7 and C-11 asymmetric centres of the phytanyl chain (Kates et al., 1967).

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



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

Information concerning the origin of the glycerol moiety in the diphytanyl ether was also obtained in these experiments. The glycerol moiety in the synthesised lipid retained 100% of ^{3}H from 1(3)-^{3}H-glycerol but complete loss of ^{3}H occurred with 2-^{3}H-glycerol. Thus, dehydrogenation at C-2 but not at C-1 (or C-3) must occur in the glycerol moiety. This eliminates involvement of aldo-keto isomerisations such as that between dihydroxyacetone phosphate (DHAP) and glyceraldehyde-3-phosphate. Unless DHAP exists in a physically separate pool where it cannot equilibrate with glyceraldehyde-3-phosphate, it does not appear to act as precursor of the glycerol moiety of the glycerol ether, as it does for the

monoalkyl glycerol ethers in other organisms (Snyder et al., 1970). A novel biosynthetic pathway must therefore exist for the glycerol diphytanyl ether; this pathway must also explain the fact that the configuration at C-2 in the glycerol moiety is opposite to that in natural diglycerides.

With regard to the phosphate and sulfate moieties of the polar lipids, studies with ^{32}P-orthophosphate and ^{35}S-sulfate have shown that a ready incorporation of the label occurs provided the concentration of unlabelled phosphate and sulfate in the medium is reduced (Kates et al., 1968). For cells harvested in the early stationary phase, the incorporation of ^{32}P largely paralleled the distribution of phosphorus among the lipid components, i.e. PGP accounted for 77% of the total ^{32}P incorporated, PG 8% and PGS 11%. When ^{35}S-sulfate was used as precursor, 80% of the isotope incorporated into the lipids appeared in glycolipid sulfate component (spot 2; Fig. 2) and about 15% was associated with the unidentified glycolipid sulfate component (spot 1; Fig. 2). The remaining 5% of radioisotope was associated with the unidentified phosphatide (spot 5; Fig. 2) which is the subject of study in Part One of this thesis.

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

Although ^{14}C-acetate is incorporated almost entirely into the phytanyl groups, low incorporation into fatty acids ($< 0.3\%$ of the total incorporation) was also observed (Kates et al., 1968). The presence of a fatty acid synthetase system could be demonstrated in a cell-free preparation from H. cutirubrum cells (Pugh et al., 1971) but its activity was considerably inhibited at the high salt concentration (4 M) of the normal growth medium. The role of the fatty acids produced in such low amounts has yet to be determined.

IV. Review of Derivatives of Phosphatidyl Glycerol and of Sulfolipids

All the phospholipids identified to date in extreme halophiles can be considered as derivatives of the diether analogue of phosphatidyl glycerol. Furthermore, two of the lipids are sulfate esters, viz. PGS and SL. It may be of interest, then,to provide a brief review of the presently known derivatives of PG (diacyl form) and of the known naturally occurring sulfolipids.

1. Phosphorylated Derivatives of Phosphatidyl Glycerol (Diacyl Form)

(a) Phosphatidyl glycerophosphate

PGP was first observed as an intermediate in the biosynthesis of PG in both bacterial and rat-liver particulate preparations by Kennedy and co-workers (Kiyasu et al., 1963; Kanfer and Kennedy, 1964). Its structure was shown to be sn-3-phosphatidyl-1'-(sn-glycero-3'-phosphate). Stanacev et al.(1969) and Marshall and Kates (1972) have also shown the intermediacy of PGP in PG biosynthesis by rat-heart mitrochondria and spinach leaf particulate fractions, respectively. PGP can also arise from phospholipase C degradation of both natural and synthetic cardiolipin (diphosphatidyl glycerol) as shown by De Haas et al.(1966). Coulon-Morelec et al. (1962) employed their acetolysis procedure to show that PGP was the major product of beef-heart cardiolipin degradation.

Definitive accounts showing an accumulation of PGP in whole cells are few. PGP is reported to be present in substantial amounts in Propionibacterium shermanii lipids after five days of growth (Prottey and Ballou, 1968) but the identification is based solely on chromatographic mobilities and the liberation of glycerol during acid hydrolysis. High proportions of the diphytanyl ether analogue of PGP have been established, however, in Halobacteria (Faure et al., 1963; Kates et al., 1963, 1965, 1971) as was described in detail earlier in this thesis.

(b) Diphosphatidyl glycerol (cardiolipin)

The structure of this lipid, discovered in beef heart by Pangborn (1947), provoked considerable controversy. Several polyglycero-polyphosphate structures with varying degrees of acylation were proposed, but a 1, 3-diphosphatidyl glycerol (DPG) structure is now generally accepted on the basis of analytical and enzymatic degradation data by several research groups (see review by Slotboom and Bonsen, 1970). Recent detailed studies by Nielsen (1971) also support this structure. The lipid is found in diverse tissues including those from animals and higher plants, as well as bacterial and yeast cells.

(c) Other polyglycerolphosphates

The cardiolipin structure in (b) does not, of course, exclude the possible occurrence of other related structures. Reports of complex polyglycerol phosphates have appeared in the literature. Ibbott and Abrams (1964) reported the isolation of a polyglycerolphospholipid in Streptococcus faecalis which had a fatty acid/P ratio of 5:2 and which may be acyl diphosphatidyl glycerol.

The phospholipids of Listeria monocytogenes contain diphosphatidylglycerol (45% of polar lipids) and bis-phosphatidylglyceryl phosphate (10%) (Kosaric and Carroll, 1971), in addition to phosphatidyl glycerol (45%).

Benns and Proulx (1971) have reported the enzymatic formation in E. coli of an hitherto uncharacterised phospholipid from PG. The biosynthesis of the lipid, tentatively assigned as a bis-phosphatidic acid, suggests a new pathway for the turnover of PG in E. coli.

2. Aminoacyl Esters of Phosphatidyl Glycerol ("Lipoamino Acids")

This type of PG derivative was discovered by Macfarlane (1962) in Clostridium welchii, the major amino acids present being alanine, and lysine. The alanyl-PG was shown by chemical synthesis to have the structure 3-sn-phosphatidyl-1'-(3'-O-L-alanyl)-sn-glycerol (Molotkovsky and Bergelson, 1966, 1967a, 1967b). Houtsmuller and van Deenen (1965) found that lysyl-PG in Staphylococcus aureus has the configuration 3-sn-phosphatidyl-1'-(2' or 3'-O-L-lysyl)-sn-glycerol. The 3-O-lysyl isomeric structure was later confirmed by chemical synthesis (Bonsen et al., 1967). Mycoplasma laidlawii is reported to synthesise the D-alanine ester of PG as well as the L-isomer (Koostra and Smith, 1969).

3. Acyl Derivatives of Phosphatidyl Glycerol

Acyl phosphatidyl glycerol has been tentatively identified in rabbit lung lipids (Body and Gray, 1967) and Mycoplasma (Plackett et al., 1970). Olsen and Ballou (1971) also isolated the lipid from Salmonella typhimurium, and established its structure as sn-3-phosphatidyl-1'-(3'-acyl)-sn-glycerol. Studies of NMR spectra and comparative rates of triphenylmethylation of the lipid were used to locate the third acyl group at the α-position of the monoacylglycerol.

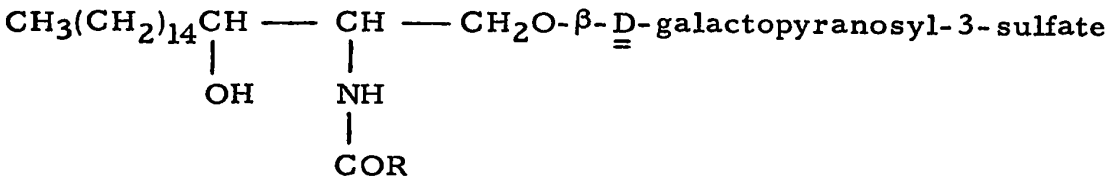
4. Glucosamine Derivatives of Phosphatidyl Glycerol

The first evidence for the existence of amino sugar derivatives of PG in bacteria was obtained by Op den Kamp et al.(1965). A variety of chemical and enzymatic degradations showed the compound, isolated from Bacillus megaterium, to be 3-sn-phosphatidyl-1'-[2'-(2-amino-2-deoxy-β-D-glucopyranosyl)]-sn-glycerol (Op den Kamp et al., 1965, 1966, 1967). A similar compound was reported present in Pseudomonas ovalis (Phizackerly et al., 1966), but the amino sugar was later shown to be α-glycosidically linked to the secondary hydroxyl group of PG (MacDougall and Phizackerly, 1969). Two positional isomers (2'- or 3'-O-amino sugar derivatives) containing a β-glycosidically linked amino sugar have also been detected in Bacillus megaterium (MacDougall and Phizackerly, 1969; Verheij et al., 1970). Bertsch et al.(1969) have also reported the presence of the 3'-glucosaminyl isomer in Bacillus megaterium.

5. Occurrence of Sulfolipids

(a) Cerebroside sulfate

This sulfolipid has been known since the work of Thudichum (1874) on the chemical composition of brain tissue a century ago. Its structure was finally established as a ceramide galactosyl-3-sulfate (Yamakama et al., 1962; Stoffyn and Stoffyn, 1963).



cerebroside sulfate

Not until 1958, however, was the existence of a sulfolipid demonstrated in tissue other than brain, namely plant tissue (Benson, 1963). Since then, sulfolipids have been identified in mammalian tissues, insects, fungi, protozoa and bacteria (see reviews by Baulieu, 1965; Stoffyn, 1966 and Haines, 1970).

(b) Glycolipid sulfates

A sulfolipid isolated from Mycobacterium tuberculosis appears to be responsible for the fixation of neutral red, a characteristic which distinguishes virulent from certain avirulent strains of the organism (Goren et al., 1970; see also review by Goren, 1972). This lipid was shown to be an acylated derivative of trehalose-2-sulfate*, namely 2,3,6,6'-tetraacyl-trehalose-2-sulfate. Highly branched-chain fatty acids are present in the ester, a situation which is reminiscent of the occurrence of the tetramethylhexadecyl alkyl chains in the glycolipid sulfate of extremely halophilic bacteria.

(c) Alkyl sulfates

Another interesting class of sulfatides, the long-chain alcohol sulfates, are present in algae (Haines and Block, 1962). Mayers and Haines (1967) characterised l-(S)-14-docosanediol-1,14-disulfate in the phytoflagellate Ochromonas danica. In addition, polyhaloalkyl disulfates containing up to six chlorine atoms per mole were detected. Haines (1970) points out that the unique feature in these lipids, which account for 3% of the dry weight of the alga, is the presence of ionic groups at both ends of the molecule.

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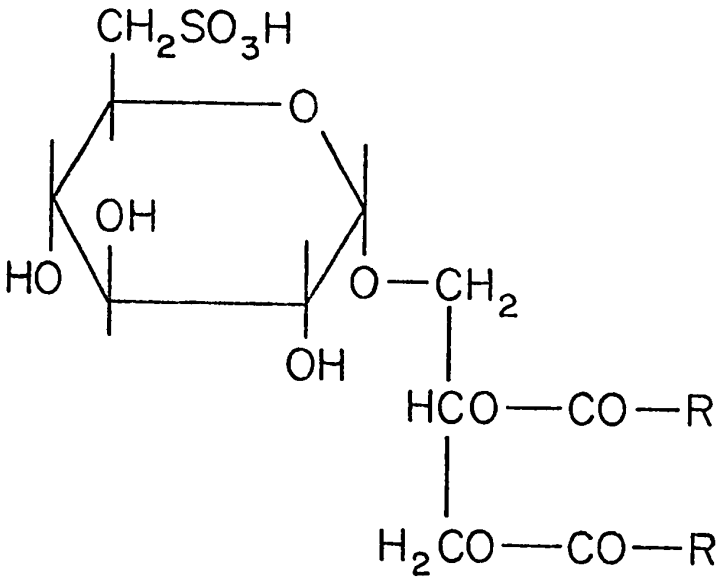
* Trehalose is a non-reducing α or β (1→1) disaccharide of D-glucose.

(d) Sulfonolipid of plants

The first discovery of a lipid containing a C-S linkage is credited to Benson et al.(1959) who reported the presence of a sulfolipid in algae which resisted liberation of sulfate ion by acid hydrolysis. The sulfolipid was finally shown by chemical synthesis to be a derivative of 6-deoxy-glucose-6-sulfonate with the structure (Benson, 1963):



6-sulfo-α-D-quinovopyranosyl-(1→3')-1', 2'-diacyl-sn-glycerol

V. Aims of the Research

The biochemist is intrigued by the existence of organisms which proliferate in an unusually demanding environment. The detailed studies of Kates and co-workers and the work of Faure and co-workers in the last decade provided considerable knowledge of the chemistry of diphytanyl ether lipids of the extreme halophiles. Some questions concerning the structure and identity of these lipids still remained open. This thesis, which is written in two parts, deals with some of these questions.

The first part of the thesis describes studies on the chemical structure of two phospholipid membrane lipid components. For one of them, the major phosphatide phosphatidyl glycerophosphate, the aim was to resolve some controversy about its structure. Kates and co-workers proposed a monomeric structure (1, Fig. 3) on the basis of degradative, analytical and synthetic work, while Faure and co-workers preferred a dimeric pyrophosphate structure (2, Fig. 3) for the compound. The controversy was resolved by the estimation of the total number of ionisable groups in PGP in three ways:

(i) NMR studies of methylated derivatives of PGP;

(ii) Molecular weight determination of the methylated derivatives and the free acid form of PGP;

(iii) Cation/P ratios of various salts of PGP.

The other structural study presented in this thesis is the complete chemical characterisation of a previously unknown polar component (spot 5; Fig. 2), including its chemical synthesis as confirmatory structure proof. To aid in its identification, labelling experiments were done using 1(3)-^{14}C-glycerol, 1-^{14}C-acetate and ^{32}P-orthophosphate and the isotopically-labelled component was isolated for degradative study.

The second part of the thesis describes the chemical synthesis of phytanyl group-derived lipids. The aim was to synthesise:

(i) Lyso-analogues (monophytanyl glycerophosphatides) of PA, PG and PGP. These compounds are not presently available from enzymatic cleavage of the existing diether lipids, although this method of preparation is commonly applied to their diester analogues. The compounds may be implicated in the biosynthesis of the diether phosphatides, and were in fact earlier considered to be present as minor components of the total polar lipids.

(ii) Extended branched-chain homologues (C_{21} and C_{22}) of $\underline{R}\underline{R}\underline{R}$-phytanic acid ($C_{20}$). These compounds were of interest since their physical properties were required for further correlation with existing data already used to establish the $\underline{R}\underline{R}\underline{R}$ configuration of the bacterial phytanyl group. These data are discussed in Part Two of this thesis.

MATERIALS AND METHODS

I. Solvents and Reagents

All solvents used were distilled over glass, a 10% forerun being discarded.

Anhydrous solvents were prepared as follows:

Tetrahydrofuran, diethyl ether and pyridine - the solvent was heated to reflux with lithium aluminum hydride for 1 hr, distilled and stored over nitrogen.

Chloroform - washed with an equal volume of water to remove ethanol, allowed to stand over anhydrous calcium chloride and distilled from phosphorus pentoxide. The anhydrous chloroform was stored over nitrogen in the dark.

Methanol - dried by the magnesium ethoxide procedure described by Vogel (A Textbook of Practical Organic Chemistry, Longmans, Green and Co. (London), 3rd Edition, p. 169).

Benzene - solvent heated to reflux over sodium metal for 1 hr and distilled.

Solvents for spectroscopic measurement were:

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

Carbon tetrachloride - "Baker Instra-analysed" GC Spectrophotometric Quality solvent.

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

The following specialised reagents were supplied by Aldrich Chemical Co. Inc. :-

Diphenyl chlorophosphate

Sulfur trioxide-pyridine complex (technical)

2,4,6-Triisopropylbenzenesulfonyl chloride

1,8-Dihydroxynaphthalene-3,6-disulfonic acid, disodium salt (Chromotropic acid)

Barium chloranilate.

II. Culture and Growth of Organism

1. Culture of Organism

The obligate aerobe, red-pigmented, extremely halophilic bacterium, Halobacterium cutirubrum, was the organism used throughout the present study. The organism was isolated, screened and purified originally by Dr. A.G. Lochhead at the Animal Research Institute, Research Branch, Canadian Department of Agriculture, Ottawa, Ontario. Stock cultures were maintained on algar slants by adding 2.0% agar to the growth medium.

2. Growth of Organism

Cells of Halobacterium cutirubrum were grown at 37°C on a rotary shaker in the complex standard liquid medium described by Sehgal and Gibbons (1960). The medium was composed of (g/l): casamino acids (Difco), 7.5; yeast extract (Difco), 10.0; trisodium citrate, 3.0; potassium chloride, 2.0; magnesium sulfate heptahydrate, 20.0; sodium chloride, 250,and ferrous sulfate heptahydrate, 0.05. The ingredients

were dissolved in 800 ml of distilled water, the pH was adjusted to 7.5 - 7.8 with 1 N sodium hydroxide and the medium was autoclaved for 15 minutes at 120°C. It was then filtered to remove any precipitate, the pH was adjusted to 6.5 - 7.0 with 1 N HCl and the medium made up to one liter. Some 20% of the magnesium and some phosphate are removed in the precipitate (Sehgal and Gibbons, 1960). However, sufficient of both ions remains in the medium for optimal growth. For routine cultures, the medium ingredients were dissolved in 1 liter distilled water without sterilisation and the pH was adjusted to 6.5 - 7.0 with 1 N HCl.

Cells used for inoculation were grown in the standard complex liquid medium (Sehgal and Gibbons, 1960) for 48 hrs. at 37°C; they were harvested by centrifugation at 6000 x g for 10 min., washed twice by resuspension in 4 M NaCl, 0.03 M KCl and 0.1 M $MgSO_4$ (hereafter referred to as the 4 M salt solution), centrifuged and finally suspended in the 4 M salt solution to give an optical density of 0.22 at 660 nm. Fifty ml of the medium in 250 ml Erlenmeyer flasks provided with a side-tube (12 cm length, 1.5 cm int. diameter) for turbidity measurements, were inoculated with 1 ml of this cell suspension. Incubation was carried out at 37°C on a rotary shaker at 150 r.p.m. Growth was estimated by turbidity measurements (optical density) at 660 nm on a Coleman Junior spectrophotometer using the uninoculated medium in a matched Erlenmeyer flask as a blank.

Cells were harvested in their stationary phase (130 hours; optical density in side tube, 0.611 $\approx$ 1.0 g dry weight of cells per litre of culture) by centrifugation at 8000 x g, washed twice in the 4 M salt solution and suspended in the same solution.

III. Lipid Extraction Procedure

1. Extraction of Total Cellular Lipids

Lipids were extracted by the method of Bligh and Dyer (1959) as modified by Kates et al.(1965, 1966). The washed cells were suspended in salt 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 in the dark 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. 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 under reduced pressure at 30 - 35°.

2. Acetone Precipitation of Polar Lipids

The red viscous total lipid residue was dissolved in a small volume of chloroform, diluted with ten volumes of acetone and cooled overnight (0°). The tan-coloured precipitate of phospholipids and sulfolipids obtained was washed several times with small portions of cold acetone, dried in vacuo, weighed and finally dissolved in chloroform to a known concentration. The combined acetone supernatants containing the neutral lipid fraction (hydrocarbons, pigments, etc.) was evaporated in vacuo to a small volume and dissolved in acetone to a known volume. In general, the acetone-insoluble lipids amounted to 91-95% of the total lipids.

IV. Chromatography of Lipids

1. Silicic Acid Impregnated Paper Chromatography

Polar lipids of Halobacterium cutirubrum and synthetic phosphatides 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).

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 sicilic 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 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).

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

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

Autoradiography: Lipids labelled with ^{32}P or ^{14}C were detected by autoradiography (Section VII.6).

2. Thin Layer Chromatography (TLC)

(a) Analytical TLC

For purity determination and for monitoring of reactions, thin layer chromatograms were run on 7.5 cm x 2.5 cm microscope slides or 20 cm x 20 cm plates coated with silica gel H (0.25 mm thickness). Spots were visualised by: (i) spraying with 40% sulfuric acid in ethanol followed by charring, or (ii) spraying with phosphate-detecting reagent of Dittmer and Lester (1964), or (iii) spraying with α-naphthol reagent for sugars. The Rf values quoted are the mean values of at least three independent determinations on microslides, with an error of ± 0.05.

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

(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° for 12 hr. Solvent systems used for the separation of neutral and polar lipids are given in the appropriate experimental sections.

For separation of total polar lipids of H. cutirubrum, the plates were streaked with a solution of the lipids (ca. 40 mg/plate) in chloroform-methanol (95:5, v/v) using a "Pelick Streaker" (Applied Science Laboratories, State College, Pa.). The plates were developed twice in pre-equilibrated rectangular jars lined with Whatman 3 MM paper, using chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v) as solvent. The lipid bands were visualised by spraying the air-dried plates with Rhodamine 6G solution (0.01%) and immediately viewing them under ultraviolet light (366 nm). The elution of the components is described in Experimental Procedures (Section I).

3. Column Chromatography

Synthetic neutral intermediate compounds were purified on columns 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 Ottawa sand was carefully applied to the silica surface. A solution of the substance in the minimum volume of the column solvent was introduced at the sand layer and the elution rate was adjusted according to the chromatographic similarity of the components to be separated. A rate of 5 ml per min was usually satisfactory.

Silicic acid used for the separation of acid-labile substances (trityl derivatives and acyl glycerols) was first stirred with 10% methanolic-sodium acetate solution for 1 hr, thoroughly washed with a series of solvents of diminishing polarity and then activated as usual. The solvent sequence was: methanol, methanol-chloroform (1:1, v/v), chloroform, chloroform-benzene (1:1, v/v), benzene.

V. Chromatography of Water-Soluble Hydrolysis

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

1. Phosphate Esters

Aliquots containing 10-15 μg P or 500-1000 cpm (^{14}C or ^{32}P) were evaporated to dryness under a stream of nitrogen, dissolved in about 20 μl of water and applied to a strip of Whatman No. 1 paper (19 x 57 cm). The esters were chromatographed for 12-16 hr in the following solvent systems:

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

(b) saturated phenol-water (107 g phenol in 38 ml of water, ascending technique);

(c) ethanol-ammonium acetate (1 M) (70:30, v/v, ascending technique).

The developed chromatograms of unlabelled components were stained with sulfosalicylic acid-ferric chloride reagent by a modification (Vorbeck and Marinetti, 1967) of the Wade and Morgan (1953) procedure. The dry chromatograms were immersed in an acetone solution of ferric chloride (1.5 g $FeCl_3.6H_2O$ in 30 ml of 0.3 N HCl diluted to 1 litre with acetone), dried again for 10 minutes and then immersed in a 1.25% solution of sulfosalicylic acid in acetone. The spots appeared white on a violet background.

Labelled components were first detected by autoradiography (see Materials and Methods Section, VII.6), and then the chromatogram was stained as described above to detect unlabelled components.

2. Reducing Sugars and Glycerol

Aliquots containing 100 - 200 µg of sugar or glycerol (or ca. 2000 cpm of labelled material) were concentrated under a nitrogen stream to near dryness. The residues were dissolved in a drop of water and chomatographed on a strip of Whatman No. 1 paper (19 x 57 cm) in solvent systems:

(a) pyridine-ethyl acetate water (2:5:5, v/v) upper phase, descending technique;

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

Sugars and polyols were detected by the following stains:

(a) silver nitrate-sodium hydroxide dip reagent*;

(b) periodate-tolidine stain**.

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* $AgNO_3$-NaOH reagent: solution A, 0.7 g silver nitrate in 3 ml water was diluted to 200 ml with acetone. Solution B, 0.8 g sodium hydroxide in 10 ml water was diluted to 200 ml with 95% ethanol.

** $NaIO_4$- o-tolidine reagent: solution A, 5 ml of 0.25 M sodium metaperiodate were diluted to 100 ml with acetone. Solution B, 2.12 g o-tolidine were dissolved in 6 ml of acetic acid and diluted with 49 ml of water and 950 ml of acetone.

VI. Physical Methods

1. Nuclear Magnetic Resonance Spectra

100 MHz NMR spectra were recorded in [^{2}H]-chloroform using a Varian HA-100 NMR spectrometer equipped with a ^{31}P-^{1}H spin-decoupler or a Varian T-60 instrument.

A 5% sample solution was routinely used in a standard thin glass NMR tube. However, the ^{31}P-^{1}H decoupled spectra obtained for methylated phosphatides and sulfophosphatides required more concentrated solutions in order to achieve an acceptable signal-to-noise ratio. Since the quantity of materials available for spectrometric study was often rather limited, a 50 µl micro-cell was used for these methylated substances which allowed concentrations of 100 - 200 mg/ml (10 - 20% by weight) to be used.

Tetramethylsilane (TMS) was used throughout as an internal reference; chemical shifts are reported as ppm (δ) relative to TMS taken as 0 ppm.

2. Infrared Spectra

Infrared spectra were obtained for thin liquid films between NaCl plates, or for ca. 1% solutions in carbon tetrachloride using a Beckman IR-20 double-beam spectrophotometer.

3. Optical Rotations

Optical rotations were measured for chloroform solutions at ambient temperature using a Perkin-Elmer polarimeter, Model 141, with digital readout. Because of the weak optical activity of most of the substances

investigated, readings were taken on sample solutions of at least 2% concentration. Care was taken to ensure that traces of silica arising from prior chromatography were completely removed by centrifugation. The optical rotations reported are the mean values of at least four independent readings of the polarimeter. Low optical rotations reported for the branched-chain methyl ester series (see Part Two) are mean values recorded on two separate Perkin-Elmer machines.

VII. Radioisotopic Procedures

1. ^{14}C and ^{32}P-Labelling of Cellular Lipids of *H. cutirubrum*

Cells of *H. cutirubrum* were grown in 100 ml of standard culture medium for extreme halophiles (Sehgal and Gibbons, 1960) containing 50 μC of either 1-^{14}C-acetate (57 mC/mM) or 1(3)-^{14}C-glycerol (15.3 mC/mM), or 1.0 mC of ^{32}P-sodium phosphate, essentially as described elsewhere (Kates *et al.*, 1968). The incubations were carried out at 37° with shaking at 120 rpm (rotary shaker) in 500 ml Erlenmeyer flasks equipped with a side-arm. Each culture was harvested in the early stationary phase [O.D. 0.92 after 65 hr (baffled flask) and O.D. 0.65 after 65 hr (unbaffled flask)] by centrifugation at 8000 x g. After removal of the supernatant medium, the cells were suspended in 3.2 ml of salt solution (4 M NaCl + 2 M KCl) and extracted by a modification of the Bligh and Dyer (1959) procedure.

2. Extraction of Labelled Lipids

To 3.2 ml of each of the cell suspensions in a 50 ml stoppered centrifuge tube were successively added 8 ml of methanol and 4 ml of chloroform and the mixture was shaken and left at room temperature for 1 hour. The monophasic systems were diluted with 4 ml each of chloroform and water and the phases were clarified by brief centrifugation. The chloroform phases were withdrawn by Pasteur pipette, washed with an equal volume of methanol-water (10:9, v/v) and evaporated to dryness with benzene in a stream of nitrogen. The residues were dissolved in 5 ml of chloroform, an aliquot was removed from each solution for counting on aluminum planchets, and the lipid components were then separated by chromatography on silicic acid-impregnated paper or on TLC plates as described below.

3. Isolation of ^{14}C-Labelled Components

The chloroform solution of ^{14}C-labelled total lipids was concentrated to ca. 200 µl and applied as a series of spots to silicic acid-impregnated Whatman 3MM paper by means of a glass capillary tube (ca. 10^5 cpm per paper, corresponding to about 2 mg lipid per paper). After development in diisobutylketone-acetic acid-water (40:25:5, v/v), the chromatogram was dried in the fume-hood and the radioactive areas were located by autoradiography (Fig. 5). The paper was cut horizontally into strips (approx. 1.0 - 1.5 cm in width) corresponding to each labelled component. Each strip was separately eluted by descending chromatography using 3.8 ml of chloroform-methanol-water (1:2:0.8, v/v). The eluates were diluted with 1.0 ml each of chloroform and water and centrifuged briefly. The chloroform phases were withdrawn, neutralised with 0.2 N methanolic ammonium hydroxide and evaporated to dryness with benzene in a stream of nitrogen. The residues were dissolved in 5 ml of chloroform, counted and examined for purity on TLC (see Fig. 5).

FIGURE 5

Autoradiographs of:

Left: silicic acid-impregnated paper chromatogram of ^{14}C-labelled polar lipids of H. cutirubrum

Right: thin-layer chromatogram of purified components:

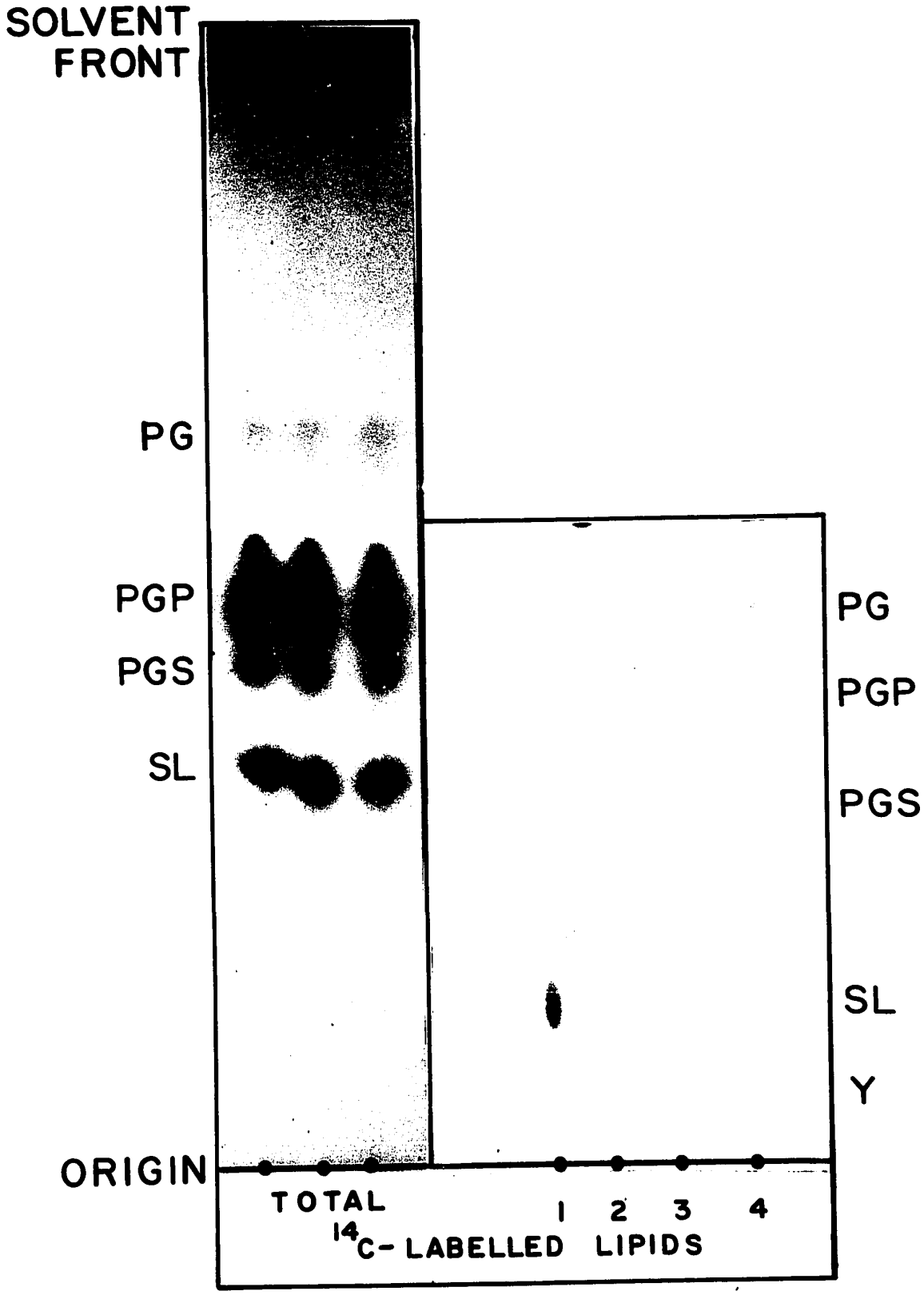
1. total polar lipids
2. phosphatidyl glycerosulfate
3. phosphatidyl glycerophosphate
4. phosphatidyl glycerol.

Component Y is unidentified.

Solvent systems:

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

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



4. Isolation of ^{32}P-Labelled Components

The ^{32}P-labelled lipids were chromatographed on TLC in chloroform-methanol-90% acetic acid (30:4:20, v/v); the plate was dried for 1 h in the fume-hood and exposed to an atmosphere of ammonia. The radioactive bands were located by autoradiography (Fig. 6) and each radioactive band was eluted with chloroform-methanol-diethyl ether (1:1:1, v/v). The eluates were concentrated to dryness under reduced pressure and the residues were freed from ammonium acetate by Bligh-Dyer partitioning (Materials and Methods, III.1) between chloroform and methanol-water. Each chloroform phase was evaporated to dryness in the presence of benzene and dissolved in 10 ml of chloroform again for counting. The purity of each labelled component (PGP, PG, PGS and one unidentified component) was examined by TLC (see Fig. 6).

5. Radioisotopic Counting

Aliquots of the chloroform solutions of the labelled lipids (containing 500 - 1000 cpm) were plated on aluminum planchets and counted with an end-window GM counter. The counting efficiency was 5% for ^{14}C and 28% for ^{32}P (counts for ^{32}P were corrected for decay).

6. Autoradiography

^{14}C and ^{32}P-labelled components on silicic acid-impregnated paper chromatograms were detected by stapling the dried chromatogram onto a sheet of Kodak no-screen X-ray film and exposing it in a light-proof folder for suitable periods, dependent on the activity of the spot. Appropriate exposure times were 24 hours per 1000 cpm per spot. After autoradiography unlabelled components were detected by Rhodamine 6G stain.

Labelled components on TLC plates were located by placing a pre-cut (20 cm x 20 cm) sheet of X-ray film directly on to the silica surface and exposing it in a light-proof folder as described for paper chromatographic separations.

FIGURE 6

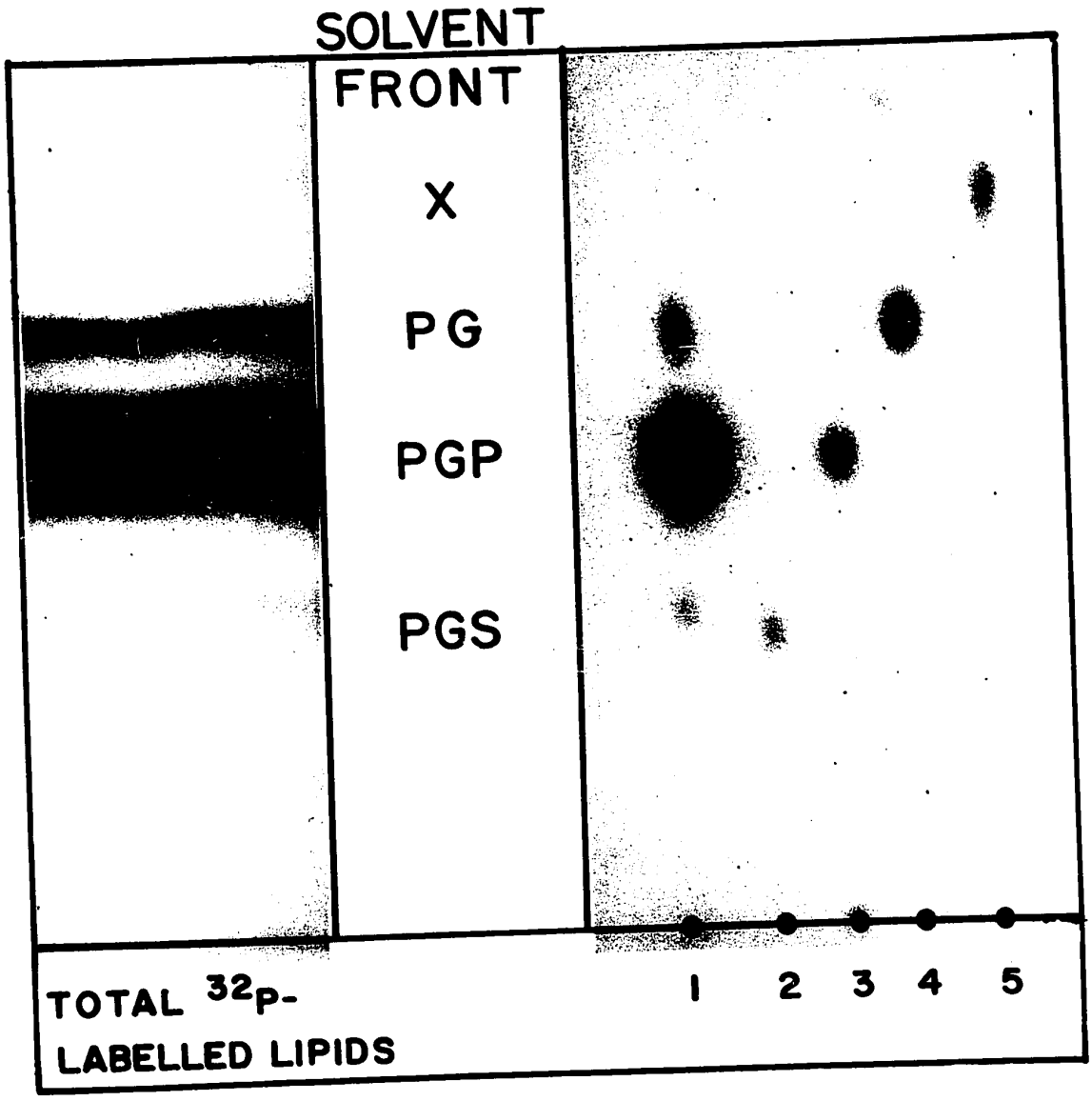
Thin-layer chromatograms and autoradiographs of ^{32}P-labelled polar lipids of H. cutirubrum and ^{32}P-labelled TLC purified polar lipid components.

Left: preparative TLC of ^{32}P-labelled polar lipids

Right:
1. Total polar lipids
2. Phosphatidyl glycerosulfate
3. Phosphatidyl glycerophosphate
4. phosphatidyl glycerol
5. Unidentified (tentative assignment based on Rf value, phosphatidic acid).

Solvent system:

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



VIII. Analytical Procedures

1. Phosphorus Determination

Phosphorus in natural and synthetic materials 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) and 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 sample 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.

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

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

*** Standard 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).

(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 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 (2 and 4 µg P) and a reagent blank. Beer's law was obeyed in the range 1 - 10 µg P (Fig. 7).

2. Glycerol Determination

Glycerol was determined after drastic hydrolysis of PGS by the method of Renkonen (1962).

The hydrolysate was partitioned between chloroform and methanol-water, according to a modification of the Bligh and Dyer procedure (1964) as described in Materials and Methods, Section III.1.

Aliquots of the methanol-water phase containing ca. 0.2 µmole glycerol was transferred to 15 ml glass-stoppered centrifuge tubes, the solvent was removed under a nitrogen stream and 0.1 ml of 10 N H_2SO_4 followed by 0.5 ml of 0.1 M aqueous sodium periodate solution* were added. The tube contents were mixed and allowed to stand at room temperature for 5 min; 0.5 ml of 10% sodium bisulfite solution** were added

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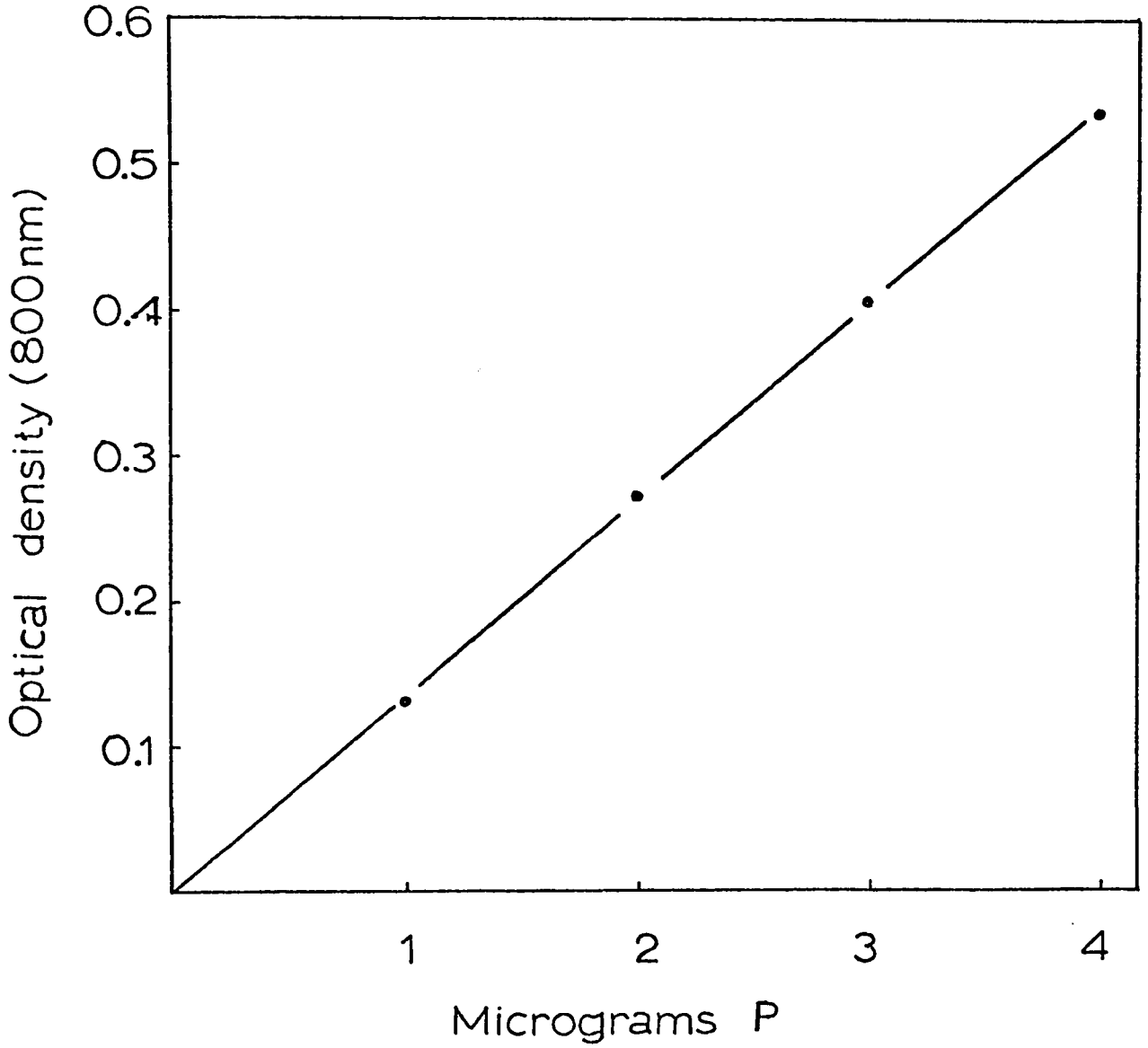
* 0.1 M Sodium Periodate: 2.14 g $NaIO_4$ dissolved in 100 ml distilled water.

** 10% Sodium Bisulfite: 10 g $Na_2S_2O_5$ dissolved in 100 ml distilled water.

FIGURE 7

Calibration curve for determination of P by the method of Bartlett (1959).



with thorough mixing and a 0.5 ml aliquot was transferred to a glass-stoppered centrifuge tube containing 5 ml of chromotropic acid reagent*. The tube was stoppered and heated in a boiling water bath for 135 min and cooled to room temperature during 30 min. The absorbance at 570 nm was read in a Coleman Junior II Spectrophotometer (12 mm cuvettes) against a reagent blank. The procedure was calibrated with known amounts of glycerol** (0.1 to 0.5 μmoles) carried through the hydrolysis as well as the colorimetric step (Fig. 8).

3. Cation Analysis

Ratios of cation/phosphorus were determined for intact phosphatide and sulfophosphatide salts by phosphorus analysis (see Section I) and cation analysis by flame photometry (Li, Na, K) or atomic absorption spectroscopy (Mg) using Pye Unicam and Jarrell-Ash atomic absorption spectrophotometers respectively. A chloroform solution of the lipid (ca. 1 mM) was partitioned between chloroform and methanol-water (10:9, v/v) by a modification of the method of Bligh and Dyer (1964); 1 ml of the chloroform solution was diluted with 2 ml of methanol and 0.8 ml of 0.5 N aqueous HCl and 1 ml each of chloroform and water were immediately added to give a biphasic system ($CHCl_3$-CH_3OH-H_2O, 10:10:9, v/v). The mixture was briefly centrifuged, the upper methanol-water phase was removed by Pasteur pipette, the chloroform phase was washed with ca. 3 ml of methanol-water (10:9, v/v) and the mixture was again centrifuged.

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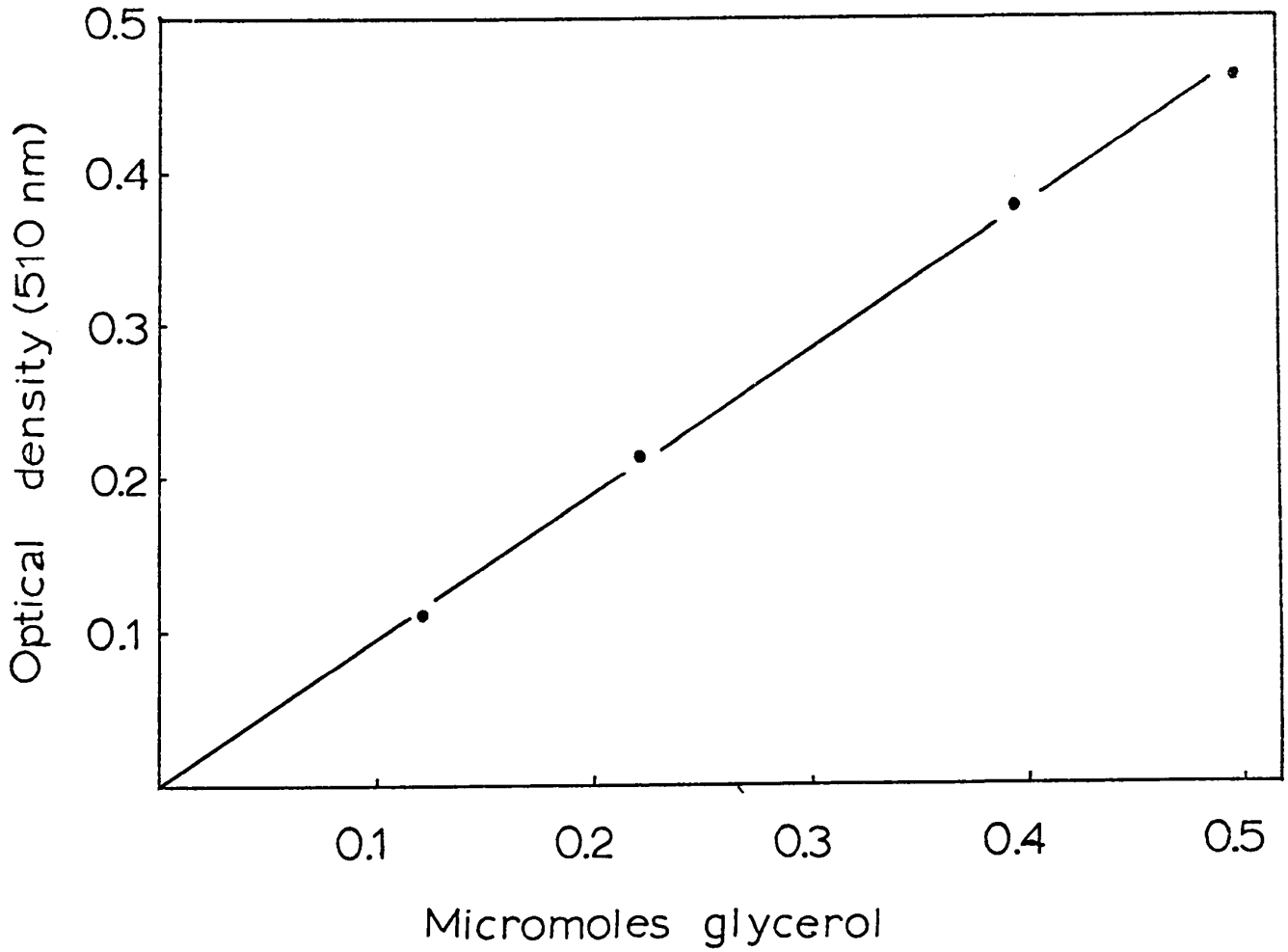
* Chromotropic Acid Reagent: 100 mg 1,8-dihydroxynaphthalene-3,6-disulfonic acid (disodium salt) dissolved in 10 ml distilled water and then diluted with 45 ml 24 N H_2SO_4.

** Glycerol Standard: 0.25 mmolar; 230 mg analytical grade anhydrous glycerol dissolved to 100 ml in distilled water; 1.0 ml of this solution diluted to 100 ml with distilled water. (1 ml contains 23 μg or 0.25 μmole glycerol).

FIGURE 8

Calibration curve for the determination of glycerol by the method of Renkonen (1962)



The washing was repeated; the methanol-water phases were combined and made up to 10 ml with methanol. Aliquots of this solution (2 ml; ca. 0.1 to 0.2 mM in cation) were evaporated to dryness under a nitrogen stream at 60° and the residues were dissolved in 2 ml of methanol-water (10:9, v/v). This treatment removed traces of chloroform present in the mixture, which strongly suppressed spectral emission of the cations (the suppression is illustrated graphically in Fig. 9). The solutions were analysed for cation, using solutions of LiCl, NaCl, KCl and $MgCl_2$ in methanol-water (10:9, v/v) as reference standards (Calibration curves, Figs. 10 - 13).

4. Ester Sulfate Analysis (Spencer, 1960)

In this procedure, the sulfate ester (PGS, K salt, ca. 16 mg, 16 μmole) was cleaved by mild acid-catalysed solvolysis in tetrahydrofuran (Mayers et al., 1969; Goren, 1971) (see Experimental Procedures, Section III. 5). The liberated inorganic sulfate was determined by a barium chloranilate assay as follows. The methanol-water phase of the solvolysis reaction mixture was made up to 10 ml, giving a solution containing ca. 1.6 μmole per ml. Aliquots (1.0 ml, containing ca. 50 μg S) were diluted in a centrifuge tube with 4.0 ml of ethanol and 0.5 ml of a 0.5 M acetate buffer (pH 4.0) containing 72.5 μg K_2SO_4 per ml. After agitation by vortex mixer, approximately 10 mg of solid barium chloranilate was added to each tube and the suspension was vigorously vortexed at 1 minute intervals during 10 minutes. The tubes were centrifuged at 3000 x g for 5 minutes, each supernatant was transferred to a 12 mm round cuvette with a Pasteur pipette and the absorption was read at 530 mμ. A reagent blank, and standard K_2SO_4 solutions (25 - 100 μg S) were also carried through the colorimetric procedure (Calibration curve, Fig. 14).

FIGURE 9

Suppression of spectral emission of potassium ion by chloroform

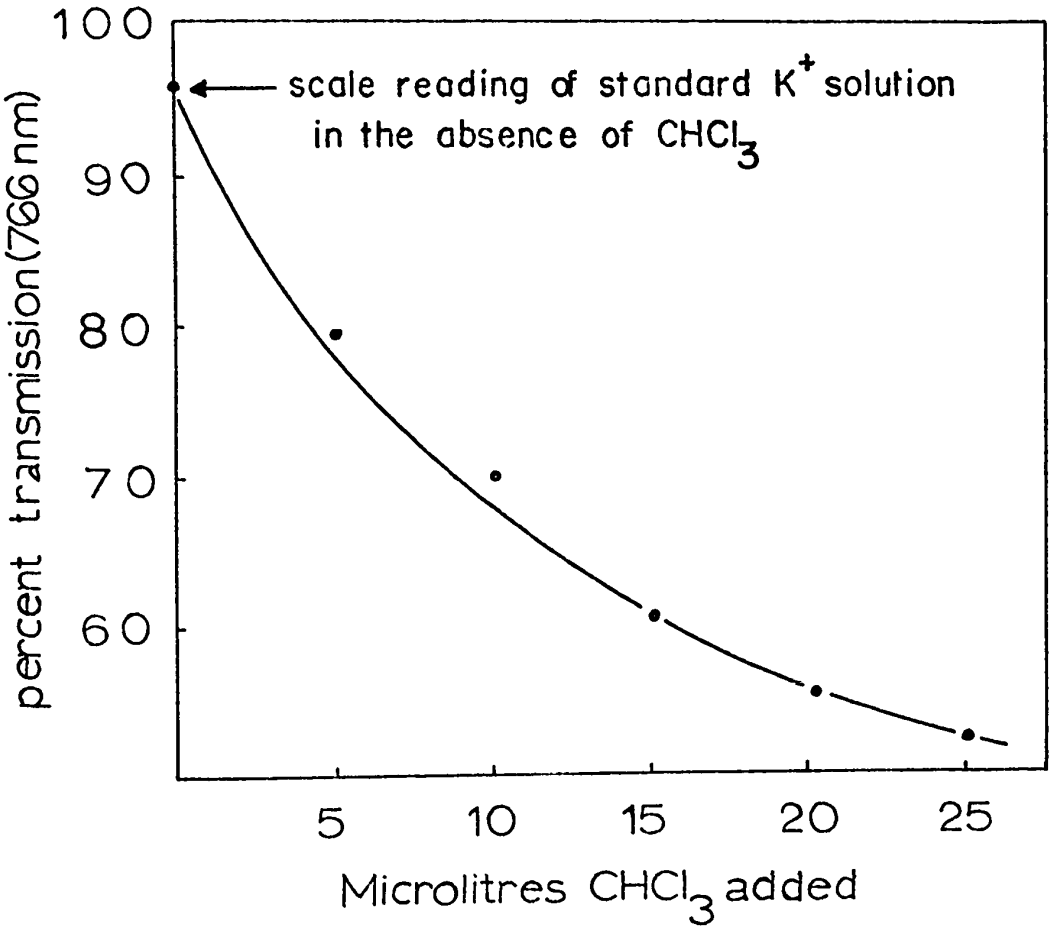


FIGURE 10

Calibration curve for the determination of lithium by flame photometry.

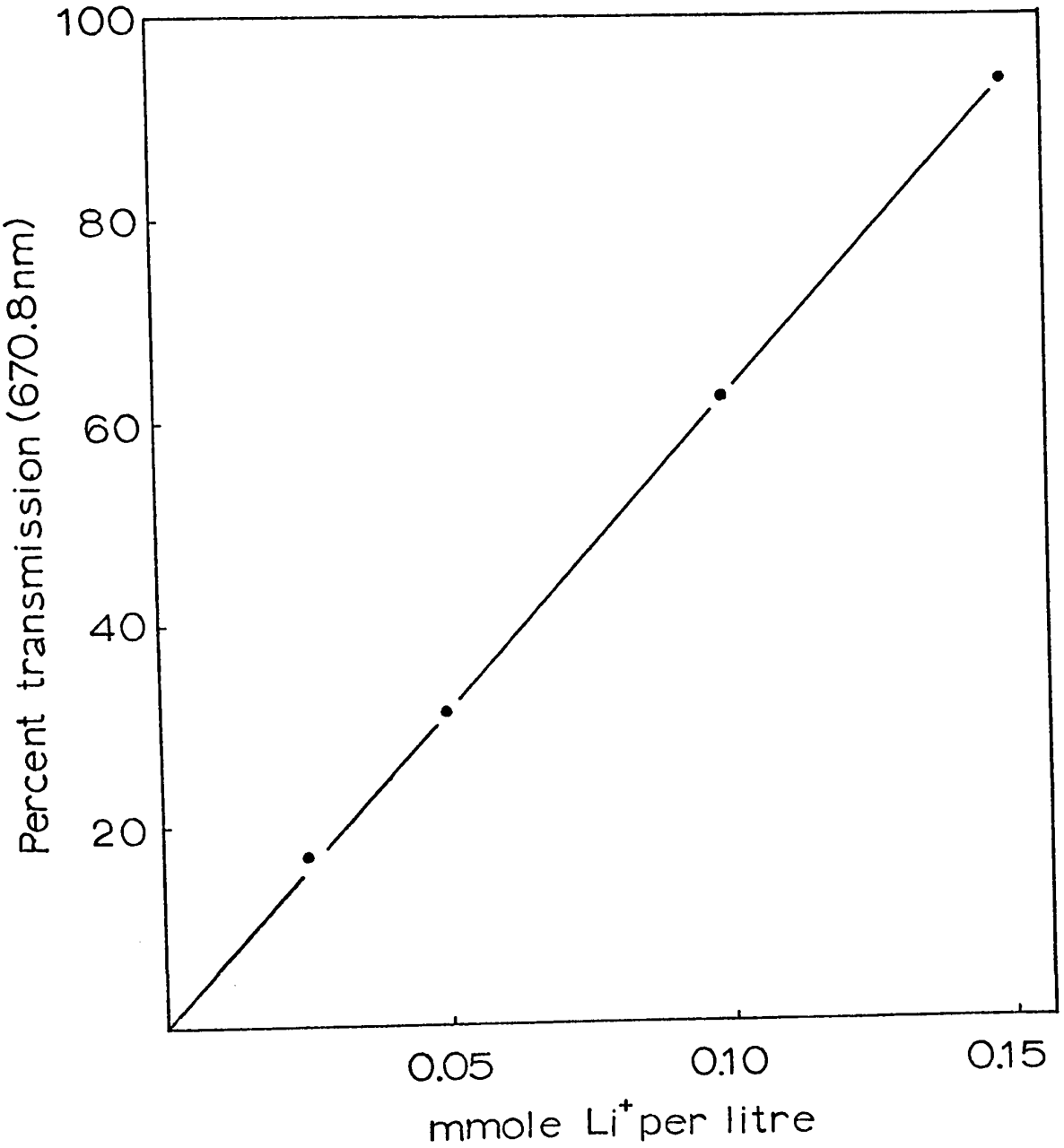

100
80
60
40
20
Percent transmission (670.8nm)
0.05
0.10
0.15
mmole Li+ per litre

FIGURE 11

Calibration curve for the determination of sodium by flame photometry.

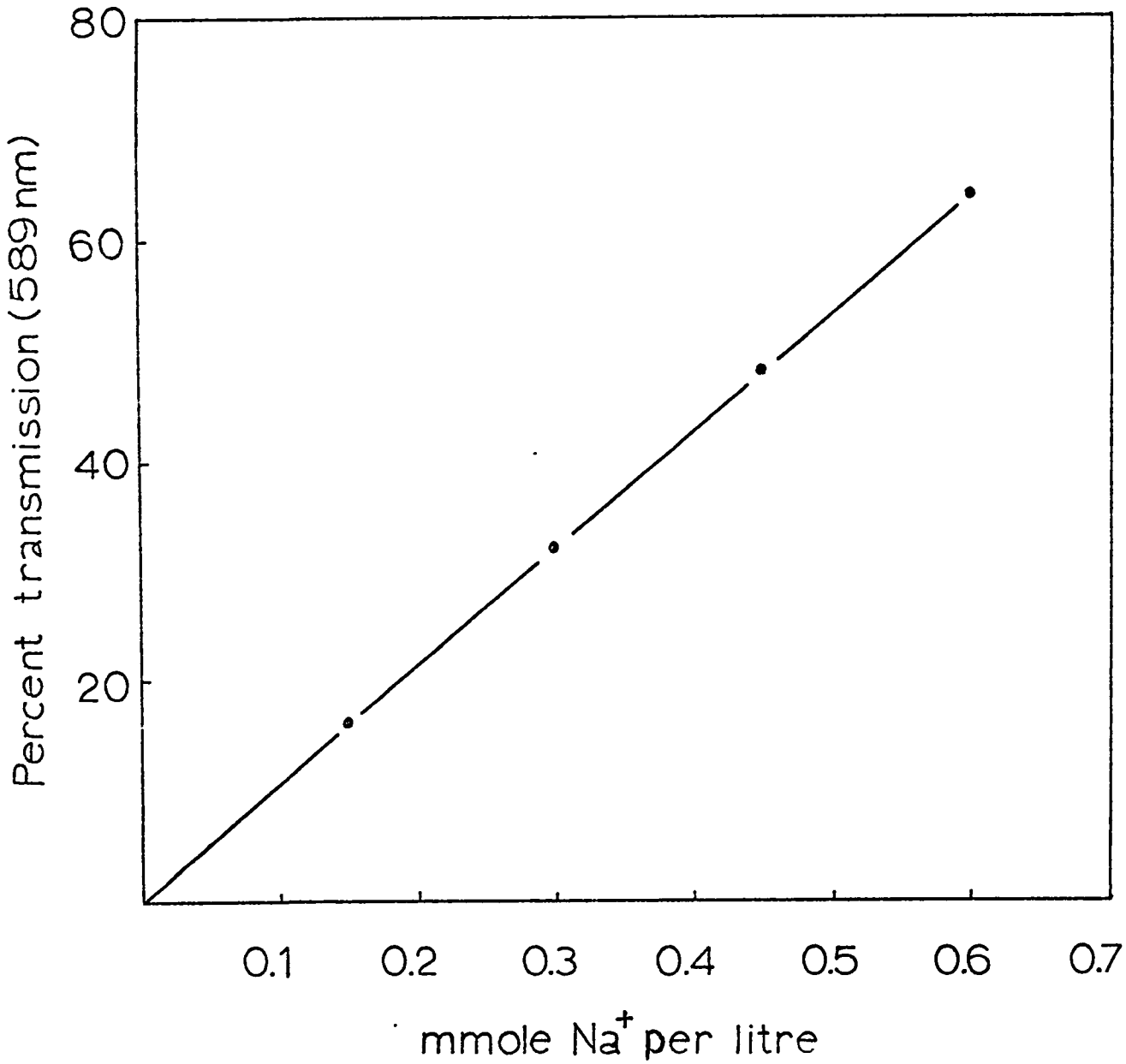

80
60
40
20
Percent transmission (589nm)
0.1
0.2
0.3
0.4
0.5
0.6
0.7
mmole Na+ per litre

FIGURE 12

Calibration curve for the determination of potassium by flame photometry.

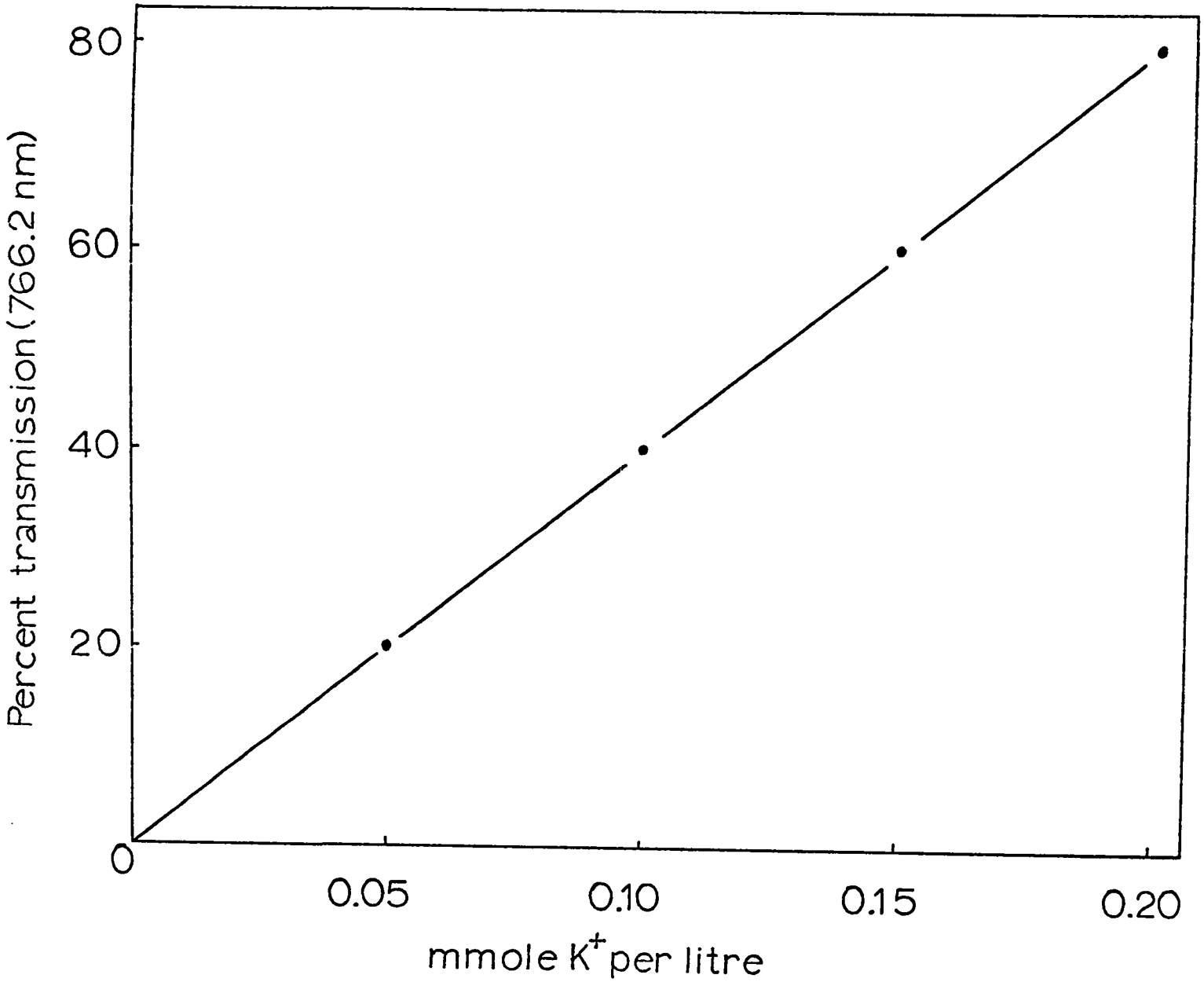


FIGURE 13

Calibration curve for the determination of magnesium by absorption photometry.

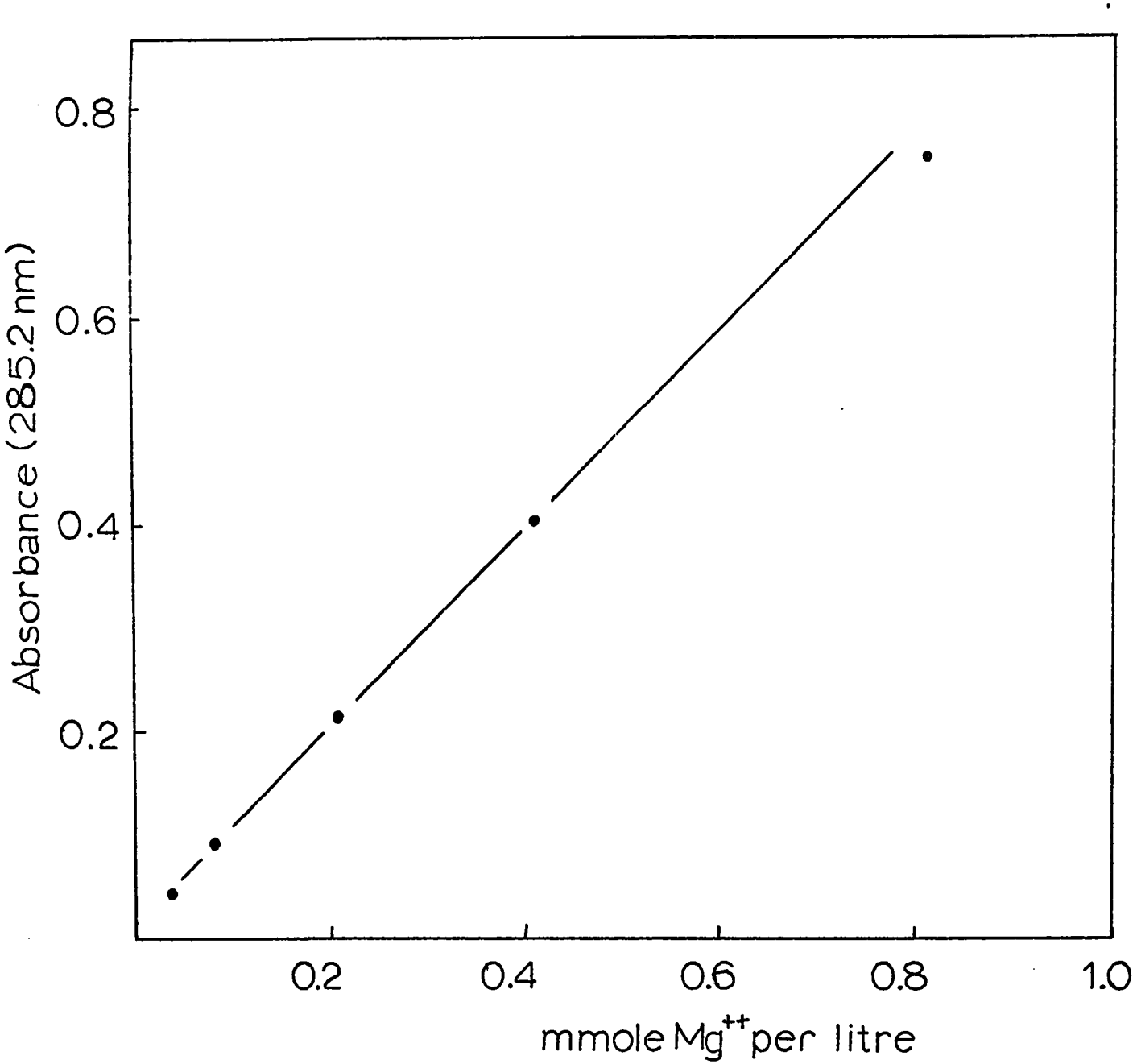
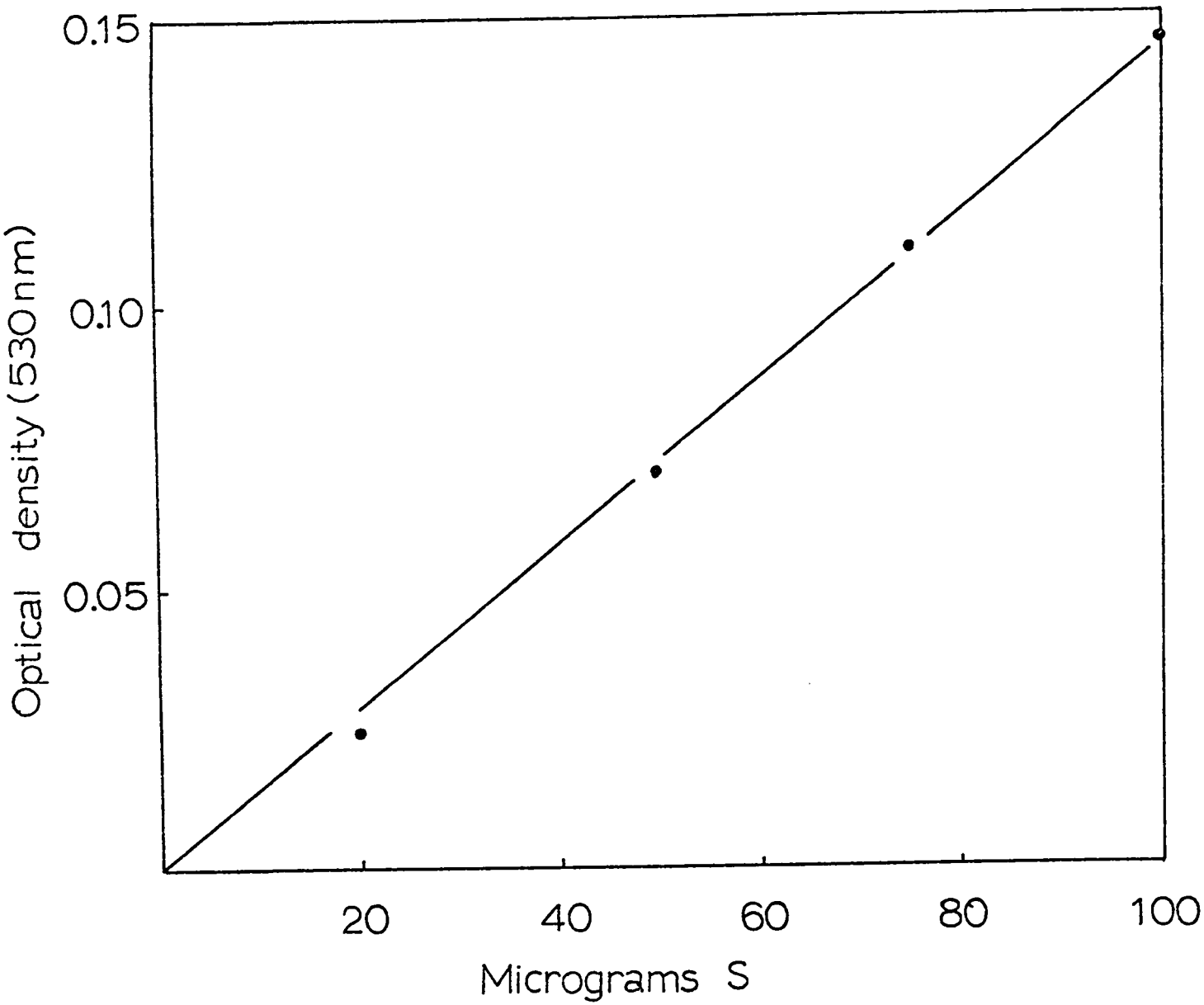

0.8
0.6
0.4
0.2
Absorbance (285.2 nm)
0.2
0.4
0.6
0.8
1.0
mmole Mg++ per litre

FIGURE 14

Calibration curve for the determination of sulfate by the method of Spencer (1960)


0.15
0.10
0.05
Optical density (530 nm)
20
40
60
80
100
Micrograms S

5. Gas-Liquid Chromatography

GLC data were obtained using a Carlo Erba Fractovap unit equipped with a flame-ionisation detector system. The coiled 3 ft. glass columns contained 10% butanediol succinate polyester on Gas-Chrom A.

The operating conditions were optimised as follows: column oven temperature 172°C, injector part temperature 225°C, detector block temperature 235°C, nitrogen carrier gas pressure 0.9 kg/cm^2.

PART ONE

STRUCTURAL STUDIES ON PHOSPHATIDYL GLYCEROPHOSPHATE, PHOSPHATIDYL GLYCEROSULFATE, AND PHOSPHATIDYL GLYCEROL

EXPERIMENTAL PROCEDURES

I. Fractionation of Diether Phosphatides

1. TLC

Total polar lipids of H. cutirubrum were fractionated by preparative thin-layer chromatography essentially as described by Joo and Kates (1969), but modified as follows: 480 mg of polar lipid were fractionated on twelve preparative TLC plates by double development in chloroform-methanol-30% ammonium hydroxide, as described in the chromatography section. The bands corresponding to PGP, PGS, PG and SL (Table 1) were combined and each mixture was eluted in a glass column with about 300 ml of chloroform-methanol-diethyl ether (1:1:1, v/v) [see Footnote (c), Table 1]. Each extract was diluted with benzene and brought to dryness under reduced pressure. 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 dried in vacuo. The four major components recovered from the plates accounted for 90 ± 2% by weight of the total lipids applied (Table 1). The purification of PGP, PGS and PG is described in the following sections.

TABLE 1

Preparative TLC Fractionation of Total Polar Lipids of H. Cutirubrum

Component[a]	Rf[b]	Mean Recovery, % wt.[c]	Mean Recovery, %P[c]
Glycolipid sulfate (SL)	0.24	22.9 ± 1.7	-
Phosphatidyl glycero-phosphate(PGP)	0.38	63.2 ± 1.1	81.0 ± 0.1
Phosphatidyl glycero-sulfate (PGS)	0.52	2.9 ± 0.5	5.2 ± 0.7
Phosphatidyl glycerol (PG)	0.78	3.8 ± 0.3	4.8 ± 0.3
Total recovery[d]	-	92.7 ± 2.0	90.9 ± 0.3

a All components are derivatives of sn-2, 3-di-O-phytanyl glycerol

b Preparative chromatography of total acetone-insoluble (polar) lipids (40 mg/plate) on silica-gel H in chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v) by double development.

c Material was recovered by elution of the silica with chloroform methanol-diethyl ether (1:1:1, v/v) in a glass column; recoveries are expressed as percent of lipid weight applied to the plates or as percent of P applied to the plates and are the mean of 4 chromatographic separations. The value for SL was obtained by further extraction of the silica with acid Bligh-Dyer solvent.

d Total recovery of P by elution with neutral Bligh-Dyer solvent (one phase) 78%, and by acid Bligh-Dyer solvent (one phase, 0.25 N HCl) 92%.

2. Column Chromatography

Total polar lipids of H. cutirubrum were also separated on "Unisil" silica. The column was prepared in chloroform (see Materials and Methods Section), and the lipid components were eluted with chloroform-methanol mixtures, the proportion of methanol being changed stepwise as given in Table 2. An LKB automatic fractinn collector was used for collection of fractions, which were monitored by TLC and pooled to give separate PGP, PG, PGS and SL components (Fig. 15). The pooled fractions were analysed for phosphorus and cation content, as described in Analytical Procedures.

The analytical data show (Table 2) that the lipids are eluted in a recurrent pattern which is dependent not only on the gross lipid structure, but also on the nature of the cation bound to the lipid. The first polar components (PGS, PGP and PG) are eluted as their magnesium salts. When the proportion of methanol is increased to 20%, sodium salts of PGS and SL are eluted; this fraction contains almost all the SL originally present in the lipid mixture. Proportions of methanol of 30% and higher elute PGP again, but this time as its sodium salt. The level of potassium ion in each fraction was negligible, although the potassium content of the "natural" lipid mixture applied to the column was ca. 12 equiv. -% (Table 14).

TABLE 2

Silicic Acid Column Chromatography of Total Polar Lipids (Natural Salt Mixture) of H. cutirubrum[a]

Fraction	Solvent % CH_3OH in $CHCl_3$	Volume Solvent (ml)	Lipid Eluted Wt. %	Identity of Lipid: Principal component	Identity of Lipid: Trace component	% P in Eluted fraction	Atomic Ratio: Na/P	Atomic Ratio: K/P	Atomic Ratio: Mg/P
1	6	100	1.30	PGS	-	3.30	-	-	0.93
2	6	150	6.60	PGP	PG	5.91	-	-	0.49
3	6	350	9.49	PGP	PG	6.16	-	-	0.51
4	8	500	3.49	PG	PGP	3.53	0.41	-	-
5	20	150	21.0	SL	PGS	0.26	1.1[b]	-	-
6	20	100	2.61	PGS	SL	2.23	1.8	-	-
7	30	250	40.7	PGP	-	6.32	1.0	0.13	-
8	40	200	11.9	PGP	SL	6.20	1.1	0.10	-
9	100	200	2.91	PGP	PGS	3.70	1.3	-	-

a Chromatography on 20 g "Unisil" silica (column dimensions 25 x 1.5 cm). Total weight applied to column 335 mg: recovery 318.3 mg (95%).

b Na/S atomic ratio. S was calculated from weight of lipid.

FIGURE 15

Thin-layer chromatogram showing the lipid composition of pooled fractions obtained by column chromatography of H. cutirubrum polar lipids

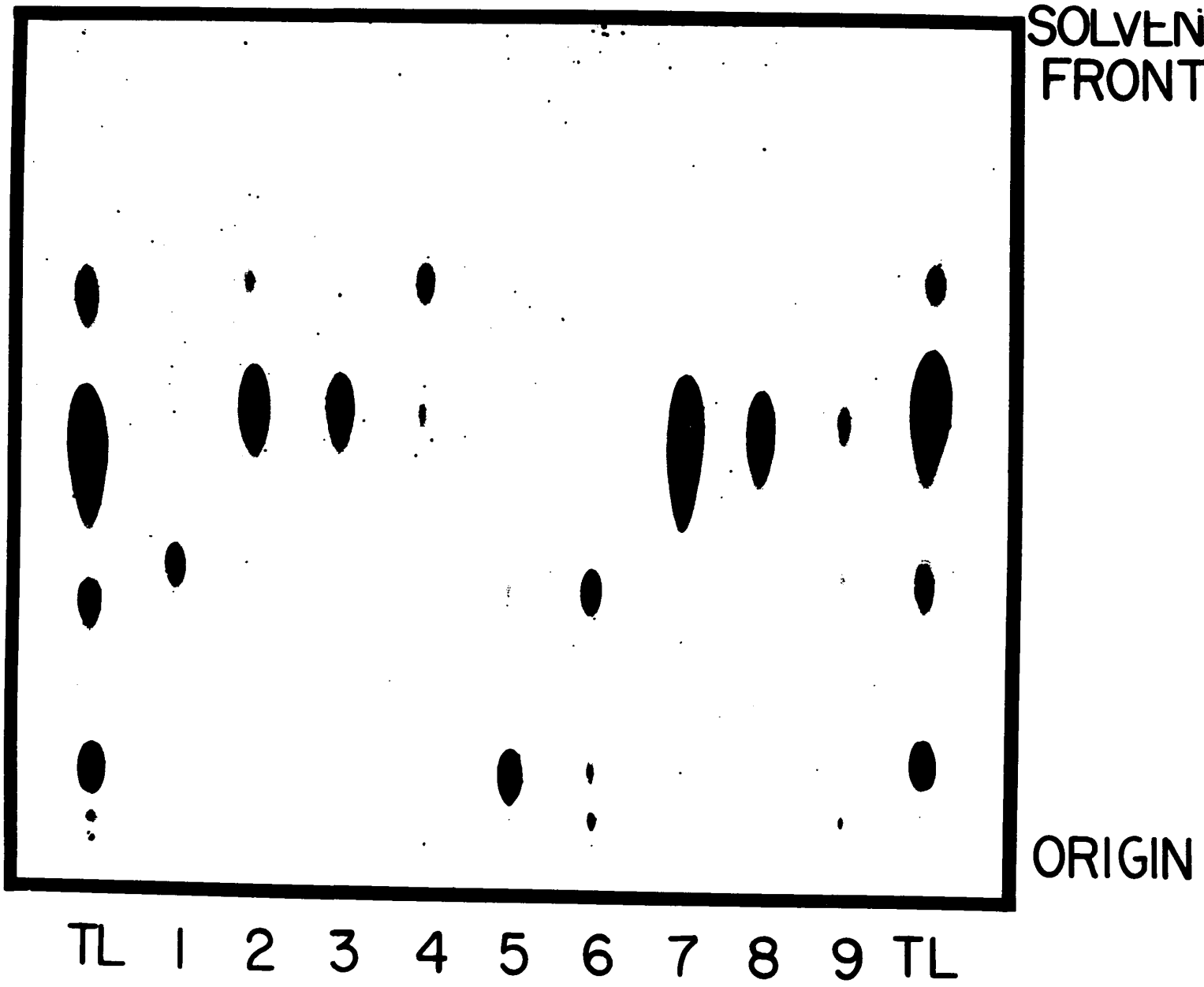
Column eluting solvents:

Fractions 1-3, chloroform-methanol (94:6)
Fraction 4, chloroform-methanol (92:8)
Fractions 5-6, chloroform-methanol (80:20)
Fraction 7, chloroform-methanol (70:30)
Fraction 8, chloroform-methanol (60:40)
Fraction 9, methanol (100)

TLC solvent:

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

TL = total (polar) lipids.



II. Phosphatidyl Glycerophosphate (PGP)

1. Isolation as "Natural" Salt Form

The PGP recovered from the TLC plates was freed from traces of the highly dextrorotatory glycolipid sulfate (SL) by preparative chromatography in chloroform-methanol-water (80:20:2, v/v) as described by Joo and Kates (1969). The chromatographically pure lipid (Fig. 16, Table 3) was obtained as a granular white solid consisting of Na, K, Mg, NH_4-PGP salts after repeated acetone precipitation (yield 41%, based on total polar lipids). $[\alpha]_D^{22}$ +0.84° (c, 4.34 in chloroform).

Anal. Found: P, 6.60; Na, 0.95; K, 0.22; Mg, 1.82; N, 0.90; Na + K + Mg + NH_4/P (equivalent ratio) 1.23.

2. Preparation of Free Acid Form

A solution of the TLC purified "natural" salt form of PGP (124 mg, 0.13 mmole) in 20 ml of chloroform-methanol (1:1, v/v) was acidified with 9 ml of 1.0 N aqueous HCl, and the biphasic mixture was centrifuged briefly. The chloroform phase, containing the acid form of the lipid, was washed with an equal volume of methanol-water (10:9, v/v), diluted with benzene, and evaporated to dryness under reduced pressure to give 109 mg (94%) of colourless oil. $[\alpha]_D^{22}$ +3.92° (c, 4.34 in chloroform).

Anal. Calc. for $C_{46}H_{96}O_{11}P_2 \cdot H_2O$ (905.20): C, 61.03; H, 10.91; P, 6.84.
Found: C, 61.44; H, 10.10; P, 6.89; mol. wt. (by osmometry), 1011.
The infrared spectrum (CCl_4) of the acid form is shown in Fig. 24A.

FIGURE 16.

Thin-layer chromatograms of total polar (acetone-insoluble) lipids and purified lipid components from H. cutirubrum developed in alkaline, acid and neutral solvent systems.

TL, total lipids: 1, glycolipid sulfate; 2, phosphatidyl glycerosulfate; 3, phosphatidyl glycerophosphate; 4, phosphatidyl glycerol.

Solvent systems:

A, chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v);
B, chloroform-methanol-90% acetic acid (30:4:20, v/v);
C, chloroform-methanol-water (65:25:4, v/v).

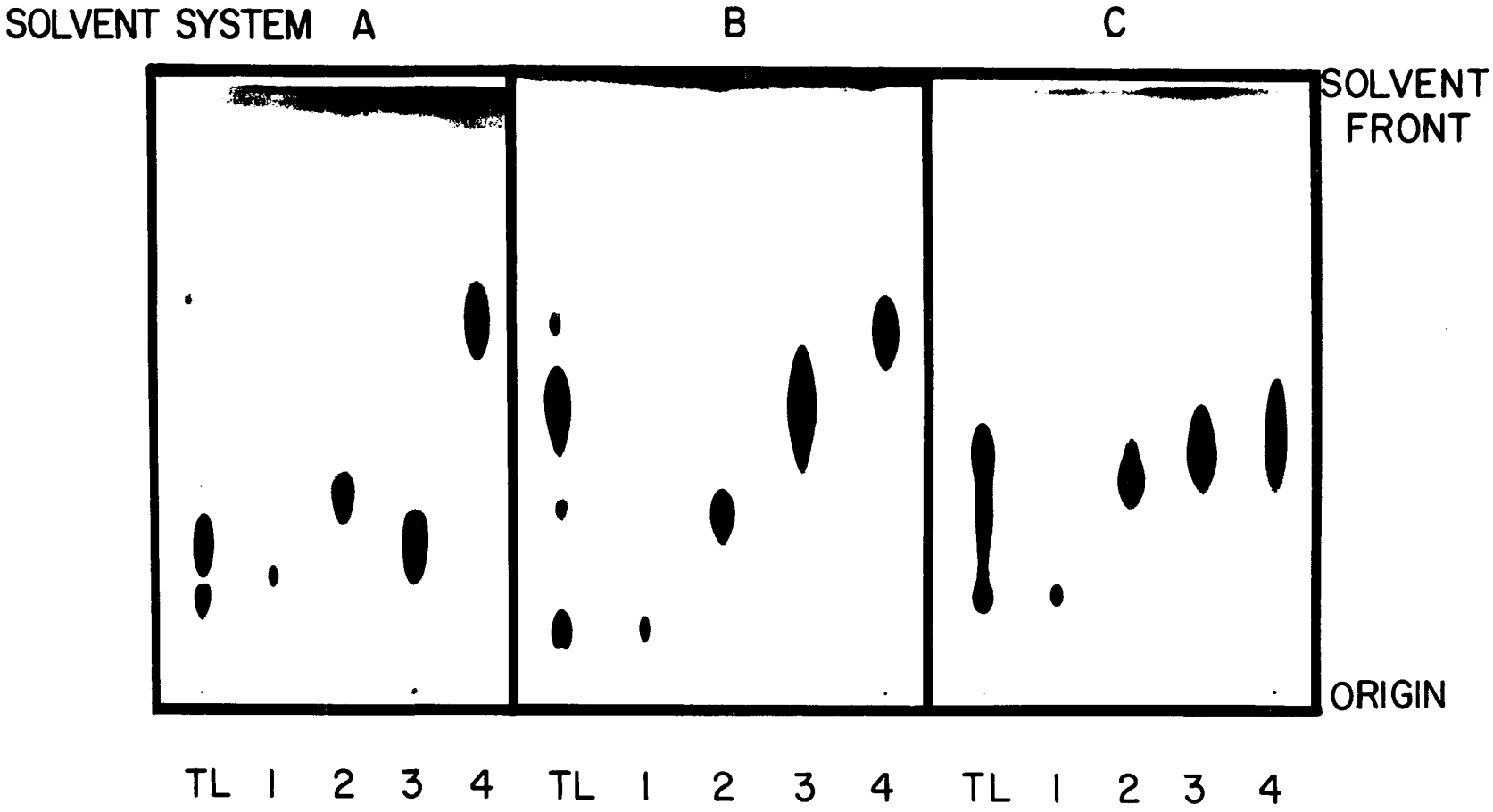


TABLE 3

Chromatographic Mobility of Polar Lipids of H. Cutirubrum

Lipid Component	Rf Value in Solvent System*			
	1	2	3	4
Glycolipid sulfate (SL)	0.46	0.15	0.13	0.22
Phosphatidyl glycero-phosphate (PGP)	0.70	0.58	0.23	0.55
Phosphatidyl glycero-sulfate (PGS)	0.62	0.38	0.37	0.43
Phosphatidyl glycerol (PG)	0.67	0.68	0.66	0.68

* TLC on silica gel H layers (20 x 20 x 0.025 cm) in solvent system 1, chloroform-methanol-water (65:35:5, v/v); 2, chloroform-methanol-90% acetic acid (30:4:20, v/v); 3, chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v). Chromatography on silicic acid-impregnated paper in solvent system 4, diisobutylketone-acetic acid-water (40:25:5, v/v).

3. Preparation of Monovalent Cation Salts

(a) Li_2-PGP, Na_2-PGP, K_2-PGP, $(NH_4)_2$-PGP

The free acid form of PGP (ca. 25 mg) in 2 ml of chloroform-methanol (2:1, v/v) was titrated to the phenol-phthalein end-point (external indicator) with 0.2 N methanolic LiOH, NaOH, KOH, or NH_4OH respectively. Each neutralised solution was concentrated to near dryness in a nitrogen stream, diluted with ten volumes of acetone and cooled to 0°. The precipitated salt was freed from traces of alkali by reprecipitation of its solution in 0.5 ml of chloroform-methanol (2:1, v/v) with cold acetone (5 ml), and then dried in vacuo at room temperature. The yields of salt form were 95-98%; elemental analysis figures for the salts are reported in Table 4.

(b) K-PGP, K_2-PGP, K_3-PGP

To a solution of PGP ("natural" salt form) in 30 ml of chloroform-methanol (1:1, v/v) (52 mg, containing 111 μmole lipid P) was added 13.5 ml of 0.5 N HCl; the biphasic system was centrifuged and the chloroform phase was washed twice with methanol-water (10:9, v/v). The chloroform solution of the free acid form of PGP was divided into three equal portions which were treated, respectively, with 1 equivalent, 2 equivalents and 3 equivalents of 0.051 N methanolic KOH per 2 equivalents of lipid phosphorus. The resulting potassium salt forms were isolated as solids by concentrating their solutions in the presence of benzene and precipitating with acetone in the usual way. The acetone precipitation of K_2-PGP and K_3-PGP was almost quantitative, but the mono-potassium salt was appreciably acetone-soluble, only about 60% of the lipid salt being precipitated. Elemental analyses for the three salts are given in Table 4.

TABLE 4

Analytical Data for Salt Forms of Phosphatidyl Glycerophosphate (Diphytanyl Ether Analogue)

PGP Salt Form	C, %		H, %		P, %		Cation, %	
	Found	Calc	Found	Calc	Found	Calc	Found	Calc
$(NH_4)_2[C_{46}H_{94}O_{11}P_2].H_2O$	58.80	58.88	10.90	11.07	6.65	6.59	2.60[a]	2.98
$Li_2[C_{46}H_{94}O_{11}P_2].H_2O$	60.20	60.29	10.83	10.55	6.70	6.76	1.55	1.51
$Na_2[C_{46}H_{94}O_{11}P_2].H_2O$	58.28	58.21	9.66	10.20	6.55	6.53	5.20[b]	4.84
$K[C_{46}H_{95}O_{11}P_2].H_2O$	58.58	58.57	10.56	10.37	6.61	6.57	4.89	4.16
$K_2[C_{46}H_{94}O_{11}P_2].H_2O$	56.21	56.30	9.80	9.86	6.40	6.31	8.68	7.97
$K_3[C_{46}H_{93}O_{11}P_2].H_2O$	54.35	54.19	9.43	9.39	5.90	6.08	11.04	11.51
$Mg[C_{46}H_{94}O_{11}P_2]$	60.49	60.75	10.15	10.42	6.81	6.75	2.87	2.67
$Ca[C_{46}H_{94}O_{11}P_2]$	59.44	59.71	10.21	10.24	6.64	6.70	4.41	4.33
$Ba[C_{46}H_{94}O_{11}P_2]$ [c]	54.16	54.03	9.32	9.27	6.06	6.04	13.18	13.43
$Ba[C_{46}H_{94}O_{11}P_2]$ [d]	54.05	54.03	9.20	9.27	6.02	6.04	12.89	13.43

a From Dumas-N analysis

b Corrected for glassware Na-blank

c Prepared using barium acetate

d Prepared using barium chloride according to Joo and Kates (1969).

4. Preparation of Divalent Cation Salts (Ca-PGP, Ba-PGP, Mg-PGP)

The calcium and barium salts were prepared by the addition of a slight excess of 20% aqueous calcium or barium acetate solution, respectively, to a methanolic solution of the ammonium salt of PGP (ca. 20mg/ml). The precipitated PGP divalent metal salt was centrifuged down, washed with methanol and reprecipitated from its chloroform solution by cold acetone. Another sample of the barium salt was prepared by treatment of the sodium salt of PGP with an excess of 20% aqueous barium chloride solution (Joo and Kates, 1969).

The magnesium salt was prepared by the addition of a slight excess of 20% methanolic magnesium acetate solution to a methanolic solution of PGP free acid. The turbid solution was diluted with chloroform and water to give a biphasic system (ratio of chloroform-methanol-water, 1:1:0.9, v/v); the chloroform phase was washed with methanol-water (10:9, v/v), and concentrated in the presence of benzene to a small volume. The magnesium salt of PGP was precipitated by the addition of 10 volumes of cold acetone, centrifuged down, washed with cold acetone and dried in vacuo. The yields of these salts were ca. 95%; their analytical data are given in Table 4.

5. Synthesis of Methylated Derivatives

(a) Phosphatidyl glycerophosphate, trimethyl ester (Me_3-PGP)

To a solution of 55 mg phosphatidyl glycerophosphate (free acid form) in 5 ml of diethyl ether was added an ethereal solution of diazomethane until a permanent yellow colour persisted in the mixture. After 15 min., the solution was concentrated to 2 ml under a nitrogen stream. TLC examination (Fig. 17) showed a major spot (Me_3-PGP) with Rf 0.58 and a minor one (Me_4-PGP) with Rf 0.71 in chloroform-methanol water (90:10:1, v/v).

FIGURE 17

Thin-layer chromatogram of methylated phosphatidyl glycerophosphate derivatives.

1 Phosphatidyl glycerophosphate (free acid form)

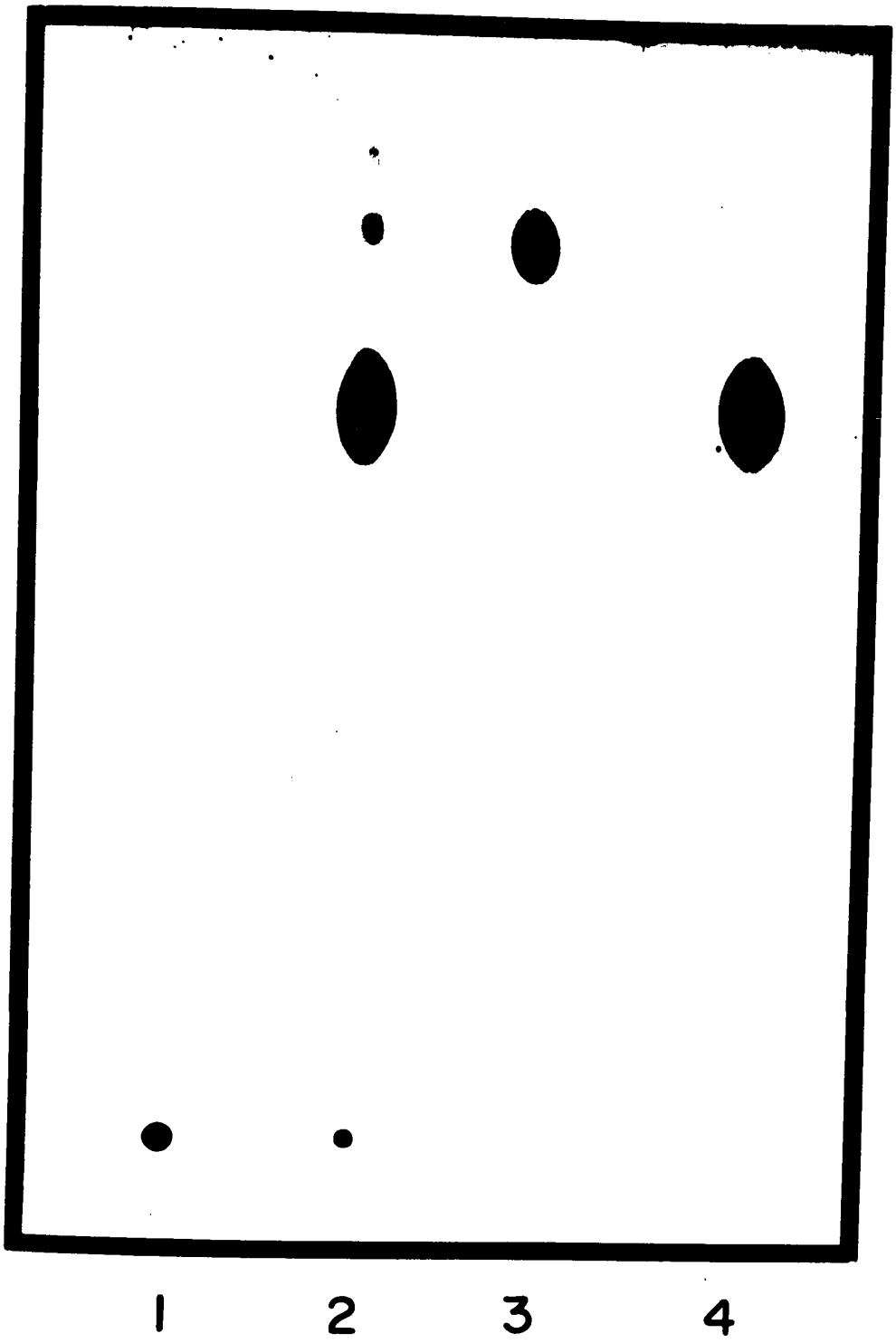
2 Reaction mixture after diazomethane treatment of phosphatidyl glycerophosphate (acid form) for 15 min.

3 Thin-layer chromatographically-purified trimethyl ester of phosphatidyl glycerophosphate (Me_3-PGP)

4 Thin-layer chromatographically-purified permethylated phosphatidyl glycerophosphate (Me_4-PGP)

Solvent system:

chloroform-methanol-water (90:10:1, v/v)



The methylated products were separated by preparative TLC in chloroform-methanol-water (90:10:1, v/v), yielding 49 mg (86%) of Me_3-PGP, and 7.8 mg (14%) of Me_4-PGP. $[\alpha]_D^{22}$ of Me_3-PGP, +4.3° (c, 4.9 in chloroform).

Anal. Calc. for $C_{49}H_{102}O_{11}P_2$ (929.2): C, 63.33; H, 11.06; P, 6.66; $5x(OCH_3)^*$, 16.70.

Found: C, 63.12; H, 10.93; P, 6.62; OCH_3, 16.00, 15.57; mol.wt. (by osmometry), 845, 883.

The infrared and NMR spectra of Me_3-PGP (Figs. 24C, 25A respectively) are discussed in the Results and Discussion Section.

(b) 2,3-Di-O-phytanyl-sn-glycero-1-phosphoryl-3'-sn-glycero-2'-O-methyl-1'-phosphate, trimethyl ester. (Permethylated phosphatidyl glycerophosphate, Me_4-PGP)

A solution of 106 mg (0.12 mmole) of the chromatographically pure free acid form of phosphatidyl glycerophosphate in 15 ml of methyl iodide was heated under reflux with 300 mg of freshly-prepared silver oxide (Hirst and Percival, 1963) for 1 hr. The reaction mixture was diluted with diethyl ether, silver salts were removed by centrifugation and the residue was washed twice with diethyl ether-chloroform (1:1, v/v). The combined supernatants were concentrated to an oil (110 mg) which showed one major product (Me_4-PGP on TLC (Fig. 17) with Rf 0.71 in chloroform-methanol-water (90:10:1, v/v/v). It was freed from traces of a fast-moving contaminant (1-O-methyl-2,3-di-O-phytanyl-sn-glycerol) by preparative TLC on silica gel using chloroform-diethyl ether (1:2, v/v); yield of chromatographically pure permethylated PGP (Fig. 17) 94 mg (85% yield); $[\alpha]_D^{22}$ +7.2° (c, 1.29 in chloroform).

* These values (and those for Me_4-PGP) have been corrected to include the propyl iodide formed from the glycerol moieties of the PGP derivative during the analysis (assuming 2 equivalents propyl iodide per PGP molecule) (Viebock and Schwappach, 1963). A blank methoxyl analysis on PGP (disodium salt) gave a value of 8.17% (theory based on 2 equivalents propyl iodide, 6.53%).

Anal. Calc. for $C_{50}H_{104}O_{11}P_2$ (943.3): C, 63.66; H, 11.11; P, 6.56; 6x$(OCH_3)^*$, 19.73.

Found: C, 63.38; H, 10.87; P, 6.49; OCH_3, 18.84, 18.63*; mol.wt. (by osmometry), 981, 970.

Physical data for Me_3-PGP, Me_4-PGP and PGP free acid are given in Table 5. Both methylated derivatives have much higher chromatographic mobilities and higher positive optical rotations than the free acid form of PGP (Fig. 17 and Table 5). The infrared and NMR spectra of Me_4-PGP (Figs. 24D, 25B respectively) are discussed in the Results and Discussion Section.

III. Phosphatidyl Glycerosulfate (PGS)

1. Isolation as "Natural" Salt Form

The PGS obtained from the TLC fractionation was usually chromatographically pure and was freed from Rhodamine by repeated precipitation by acetone as described for PGP. However, on some occasions the resolution between the PGS and PGP components was less clear and cross-contamination was inevitable. The pure components were then obtained by rechromatography in the same solvent system (Fig. 16; for Rf values see Table 3).

The purified "natural" salt form of PGS (a mixture of Mg, Na, and NH_4 salts) accounted for about 3% of the total polar lipids. The "natural" PGS had the following specific rotations in chloroform (c, 1.42): 589 nm, -1.41°; 578 nm, -0.35°; 546 nm, +0.14°; 436 nm, +0.70°; 365 nm, +0.99°.

Analytical data for the "natural" salt form are reported in Table 6.

TABLE 5

Physical Measurements on Methylated Derivatives of Phosphatidyl Glycerophosphate (Diether Analogue)

Compound	TLC Rf Values* 1	2	$[\alpha]_D^{**}$	M_D
PGP-Free Acid	0.0	0.0	+3.92°	+34.8°
PGP-Trimethyl Ester (Me_3-PGP)	0.28	0.58	+4.27°	+39.7°
Permethylated PGP (Me_4-PGP)	0.45	0.71	+7.21°	+68.0°

* Solvent systems: 1) $CHCl_3$-diethyl ether (1:2, v/v).

2) $CHCl_3$-CH_3OH-H_2O (90:10:1, v/v)

** In chloroform at 22°

TABLE 6

Analytical Data for Phosphatidyl Glycero-1-Sulfate

DATA	Mixed Salt (natural)		K Salt (natural)		K Salt (natural)		K Salt (synthetic)	
	Found	Calc.	Found	Calc.*	Found	Calc.**	Found	Calc.**
C, %	57.54	-	57.72	57.34	56.24	56.29	56.58	56.29
H, %	10.20	-	10.08	9.73	9.51	9.76	9.96	9.76
P, %	3.40	-	3.35	3.22	3.06	3.16	3.30	3.16
S, %	3.70	-	3.45	3.33	-	-	3.42	3.27
K, %	-	-	8.84	8.12	-	-	8.71	7.96
S/P, atomic ratio	1.05	1.00	1.00	1.00	-	-	1.00	1.00
K/P+S, atomic ratio	-	-	1.05	1.00	-	-	1.05	1.00
D:P:G≠ mole ratio	-	-			-	-	-	-
Equiv. weight	-	-	457 ± 10	443.6	-	-	-	-

* Calc. for $C_{46}H_{93}O_{11}PSK_2$ (963.45): analytical sample dried in vacuo at 65°.

** Calc. for $C_{46}H_{93}O_{11}PSK_2 \cdot H_2O$ (981.47): analytical samples dried in vacuo at room temperature.

≠ Phytanyl diether:phosphorus:glycerol mole ratio. Found 1.0:1.0:0.97; Calc. 1.0:1.0:1.0.

2. Preparation of Salts of PGS

(a) Potassium salt

A solution of 45 mg of the purified "natural" PGS in 10 ml of chloroform-methanol (1:1, v/v) was diluted with 4.5 ml of 0.5 N aqueous HCl. The biphasic system was briefly centrifuged, and the chloroform phase was washed with methanol-water (10:9, v/v), and evaporated to dryness in a stream of nitrogen. The residual PGS free acid was dried in vacuo (40.0 mg) and titrated in chloroform solution with 0.1 N methanolic KOH to the phenolphthalein end-point (0.0876 mequiv. consumed). The solution was diluted with benzene, concentrated to a small volume in a nitrogen stream, cleared by centrifugation, and diluted with ten volumes of acetone. After several hours at 0°, the precipitate was centrifuged down, dissolved in the minimum volume of chloroform, reprecipitated with cold acetone, and finally dried in vacuo; yield of potassium salt (white hygroscopic powder) 38 mg. It had the following specific rotations (c, 2.38 in chloroform): 589nm, +2.02°; 578nm, +2.14°; 546 nm, +2.29° ; 436 nm, +4.14°; 364 nm, +6.29°. Analytical data for the potassium salt of PGS are given in Table 6. The infrared spectrum (Fig. 27) is discussed in the Results and Discussion Section.

(b) Ammonium and sodium salts

These salts were prepared from the "natural" salt mixture by the method described for the potassium salt, the acid form of PGS (10 - 15 mg) being neutralised with 0.2 N methanolic ammonium or sodium hydroxide, respectively. Elemental analyses of these salts were not performed because of their limited supply. However, on account of their sensitivity to acid, the purity of the salts was checked by TLC after neutralisation, and again after isolation of the anhydrous salt forms.

(c) Magnesium salt

This salt was prepared by treating the acid form of PGS (ca. 15 mg) in 2 ml of chloroform with 2 ml of 10% methanolic magnesium acetate solution, and 1.8 ml of water. The biphasic system was briefly centrifuged; the chloroform phase was washed with methanol-water (10:9, v/v), and evaporated to near dryness in the presence of benzene. The magnesium salt of PGS was precipitated by the addition of 10 volumes of acetone, centrifuged down after cooling to 0°, and washed with cold acetone.

The optical rotation of the various salt forms are given in Table 7.

3. Synthesis of Dimethyl Ester (Me_2-PG-1-S)

Bacterial PG-1-S (K salt, 8 mg) was converted to the acid form as described previously, and the chloroform solution was treated with ethereal diazomethane until a permanent yellow colour was obtained. The solution was evaporated in a nitrogen stream in the presence of benzene, and the oily residue was dried in vacuo. The NMR and infrared spectra were recorded with minimum delay, because the ester, like the methyl esters of PG and PGP, decomposed fairly quickly to give diphytanyl glycerol ether. The infrared and NMR spectra (Figs. 27, 28 respectively) are discussed in the Results and Discussion Section.

TABLE 7

Optical Rotation Values[a] for Salt Forms of Phosphatidyl Glycerosulfate

Salt Form	$[\alpha]_D^{22}$	M_D
PGS-dipotassium salt	+2.02	+19.9
PGS-diammonium salt	+1.55	+14.6
PGS-magnesium salt	+0.58	+5.39
PGS-disodium salt	-1.75	-16.7

a Measured in chloroform solution

4. Degradative Procedures for PGS (Potassium Salt)

(a) Drastic hydrolysis with aqueous HCl

PGS (10 - 20 mg, 325 - 650 μgP) was hydrolysed in a sealed tube with 0.9 ml of 2 N aqueous HCl for 24 hr at 120° (Scheme 1). The hydrolysate was transferred to a 15 ml centrifuge tube with 1.0 ml of methanol and 1.0 ml of chloroform, the biphasic system was briefly centrifuged, and the chloroform phase was carefully withdrawn by Pasteur pipette. The chloroform-soluble hydrolysis product (2,3-di-O-phytanyl-sn-glycerol) was identified as described in Section 4(d), and the methanol-water phase was analysed for glycerol, and both inorganic and total P (see Analytical Procedures).

(b) Alkaline hydrolysis with methanolic-NaOH

Samples of PGS were hydrolysed in 4.5 ml of 0.8 N NaOH in 90% methanol at reflux temperature for two hours. The hydrolysis products were partitioned according to the modified Bligh-Dyer procedure (see Materials and Methods, Section III, 1) by adding 4 ml of chloroform and 3.1 ml of water to the cooled hydrolysate. The chloroform-soluble products were examined by TLC (see below).

(c) Hydrolysis with methanolic-HCl

PGS (unlabelled, 10-20 mg, or labelled, 1.5-2.0 x 10^4 cpm ^{32}P or ^{14}C) was hydrolysed in a 50 ml side-arm flask (Kates, 1964) with 4.5 ml of 2.5% methanolic-HCl (prepared by passing HCl gas (2.5 g) into 100 g of anhydrous methanol) at reflux temperature for 3 hr (Scheme 1). The hydrolysate was transferred to a 15 ml centrifuge tube with 5 ml of chloroform, 0.5 ml of methanol, and 4.5 ml of water as washing solvents, and the two phases were separated as described for the drastic hydrolysis procedure. The chloroform-soluble and water-soluble hydrolysis products were investigated as described below.

SCHEME 1.

Degradative procedure for 1-sn-phosphatidyl-sn-3'-glycero-1'-sulfate (Natural PG-1-S)

The enzymatic hydrolysis was carried out by incubation of the aqueous hydrolysate at 36° for 8 hr with a calf-intestine alkaline phosphatase preparation (Worthington) in the following system (total volume 1.0 ml): 0.4 ml of ^{14}C-labelled substrate solution (2000-3000 cpm), 0.3 ml of 1.0 M glycine-NaOH buffer (pH 9.5), 0.1 ml of 0.12 M magnesium acetate solution, 0.2 ml of enzyme solution (5 mg/ml in 0.2 M potassium bicarbonate solution).

The reactions were stopped by the addition of 2 ml of ethanol and the mixtures were centrifuged; each supernatant was concentrated to 1 ml in a stream of nitrogen and then passed through a column of Amberlite MB-3 ion exchange resin. The ion-free filtrates were concentrated to a small volume in a nitrogen stream and chromatographed, together with authentic ^{14}C-glycerol, on Whatman No. 1 paper in n-butanol-acetic acid-water (5:3:1, v/v) (Materials and Methods, V.2). As a control, the hydrolysate of PGP, containing 1,3-glycerol diphosphate (Kates et al., 1965) was also enzymatically degraded and the products chromatographed on paper.

5. Desulfation of Phosphatidyl Glycerosulfate

(a) Time-course study

PGS ("natural" salt form, 2μmole) was solvolysed following the general procedure of Mayers et al.(1969) or Goren (1971) for sterol or sugar sulfates, in 1.5 ml of anhydrous tetrahydrofuran-HCl (0.005 M)* at room temperature (Scheme 2). The reaction was monitored by TLC in chloroform-methanol-90% acetic acid (30:4:20, v/v) during a four-hour period.

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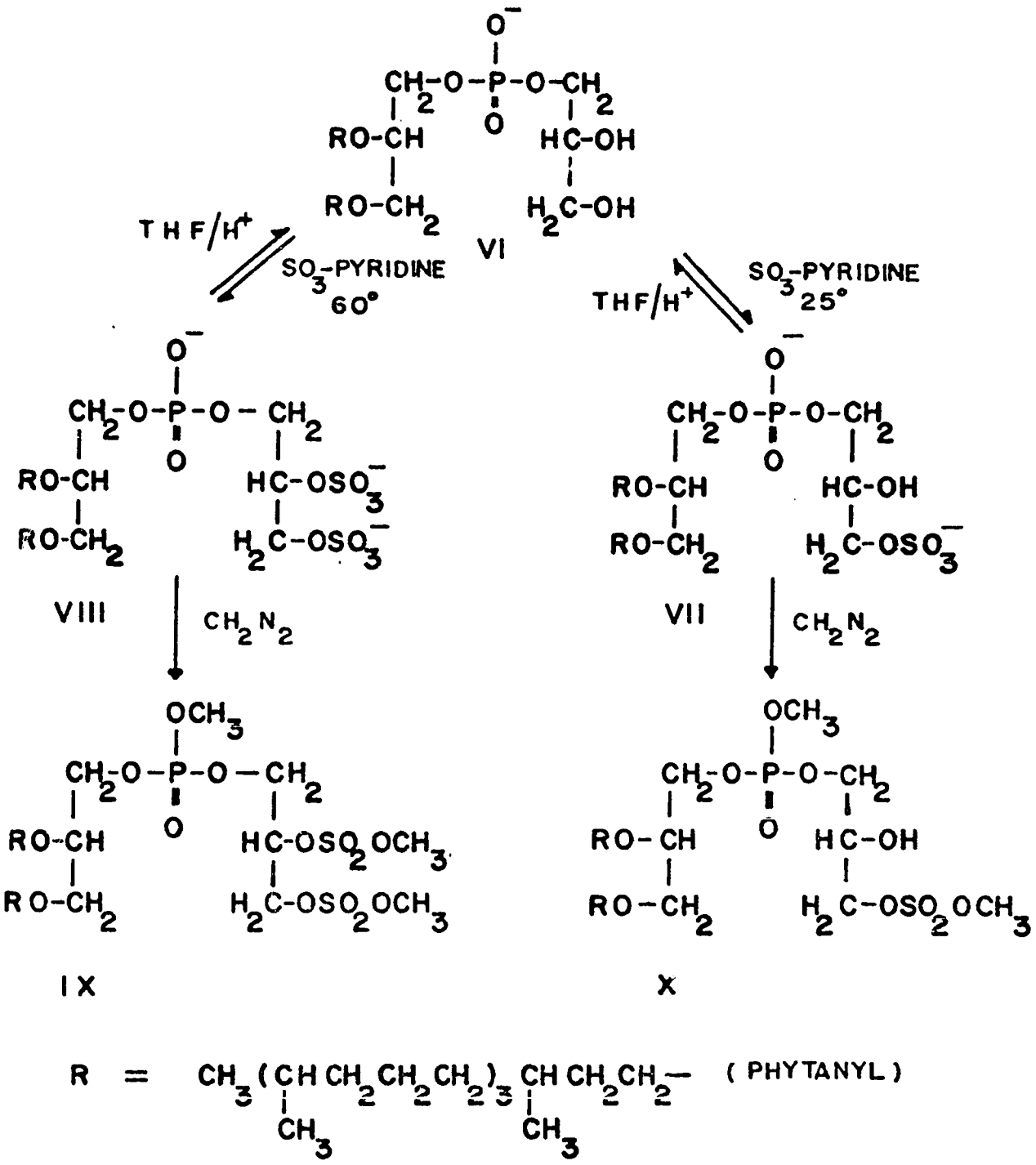
* The tetrahydrofuran, distilled from lithium aluminum hydride, was saturated with HCl gas and the resulting stock solution (ca. 3 M THF-HCl) was diluted with anhydrous THF to give a 0.005 M working solution. The acid solutions were stored no longer than 2 days, as they generated a substance on standing which interfered with TLC analysis of the solvolysate.

SCHEME 2

Synthesis (and desulfation) of 1-sn-phosphatidyl-sn-3'-glycero-1'-sulfate and 1-sn-phosphatidyl-sn-3'-glycero-1', 2'-disulfate.



Scheme II

The solvolysis was also performed with the pure potassium salts of PGS, PG-2-S, and PG-1,2-di-S, but the reactions were too rapid for time-course study.

(b) Identification of phosphatidyl glycerol from desulfation of PGS

Purified PGS (potassium salt, 16.5 mg, 16.8 μmole) was solvolysed in 5 ml of anhydrous tetrahydrofuran-HCl (0.005 N) and the solution was neutralised with 0.2 N methanolic ammonium hydroxide. The solvents were removed in a nitrogen stream and the residue was dissolved in 10 ml of chloroform-methanol (1:1, v/v).

The solution was diluted with 4.5 ml of 0.1 N aqueous HCl and briefly centrifuged. The two phases were separated and the chloroform phase was washed with ca. 3 ml of methanol-water (10:9, v/v) and then neutralised with 0.2 N methanolic NaOH. The sodium salt of PG (14.0 mg, 16.5 μmole, 98%) was precipitated by acetone from its concentrated solution in chloroform and characterised by its chromatographic mobility, its positive reaction with periodate-Schiff reagent and its optical rotation. Found: $[\alpha]_D^{22}$ +3.15° (c, 1.40 in chloroform); reported (Joo and Kates, 1969) for the sodium salt of bacterial PG (sn-1-phosphatidyl-sn-3'-glycerol) - $[\alpha]_D^{22}$ +3.43° (c, 2.36 in chloroform).

Anal. Calc. for $C_{46}H_{94}O_8PNa.H_2O$ (847.6): C, 65.30; H, 11.71; P, 3.66. Found: C, 65.31; H, 11.52; P, 3.48.

The methanol-water phase plus washings from the Bligh-Dyer partition was analysed for sulfate ion as described under Analytical Procedures.

Anal. Calc. release of sulfate ion 16.8 μmole; Found: 16.0 μmole (95.2% recovery of sulfate).

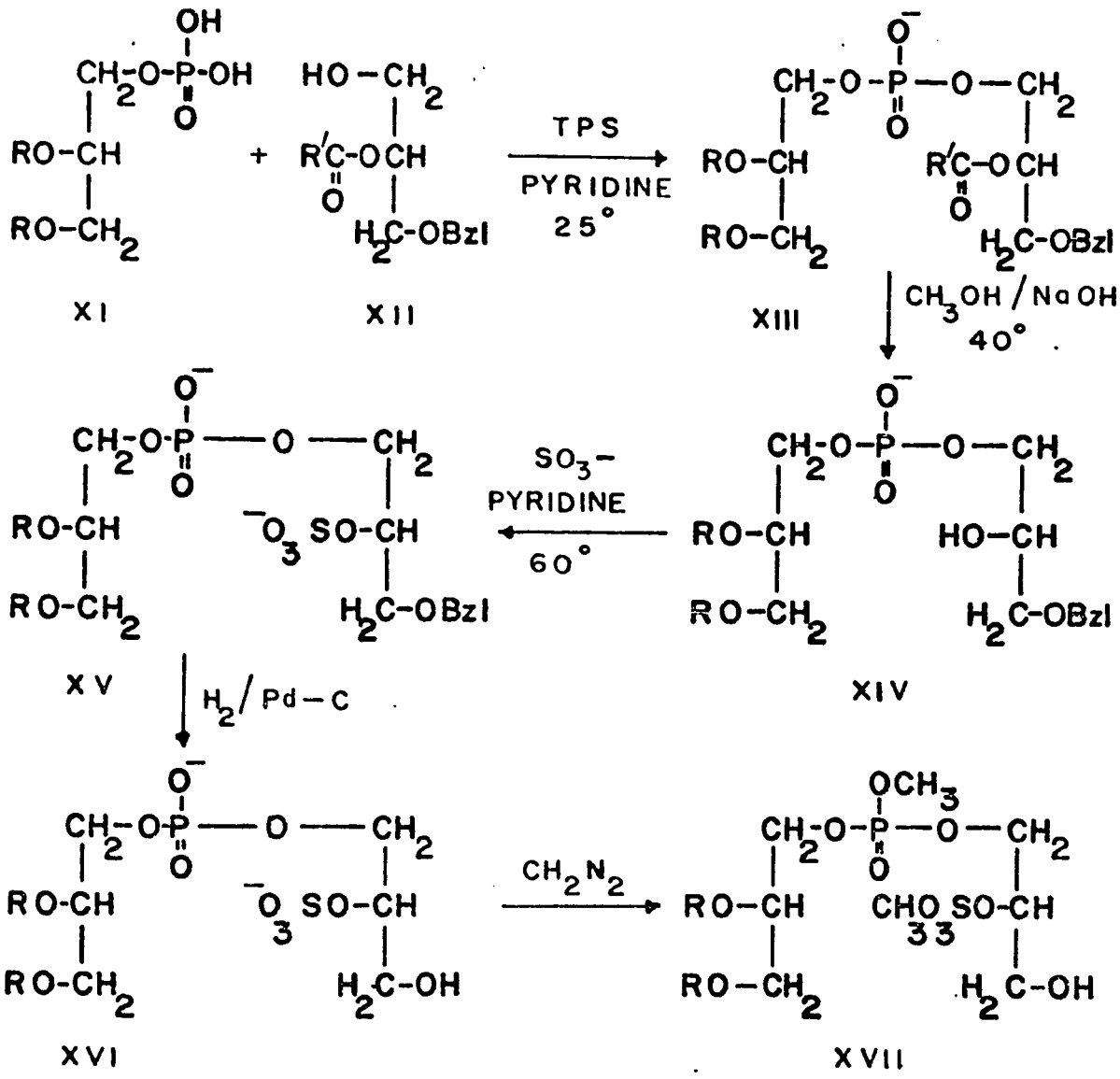
6. Synthesis of Phosphatidyl Glycerosulfate Isomers

Two approaches were used. The first involved direct sulfation of the potassium salt of the pure bacterial phosphatidyl glycerol (VI) (diphytanyl ether analogue), to give mono- and disulfated derivatives (Scheme 2). The physical and chemical properties of the potassium salt and the methyl ester of the monosulfate were compared to those of the natural phosphosulfolipid salt and ester, respectively, which were isolated and purified in the same manner.

The second approach was the unambiguous synthesis of the β-isomer, phosphatidyl glycero-2'-sulfate (XVI., Scheme 3) from 2,3-di-O-phytanyl-sn-glycerol obtained directly by methanolysis of the total polar lipids of Halobacterium cutirubrum. The configuration of the phytanyl diether had previously been established by synthesis (Kates et al., 1965, 1967). This diether was converted as described by Kates et al.(1971) to the free acid form of diphytanyl ether phosphatidic acid, which was readily condensed in the presence of triisopropylbenzenesulfonyl chloride in dry pyridine (Aneja et al., 1970) with 2-O-myristoyl-3-O-benzyl-sn-glycerol to give the partially blocked phosphatidyl glycerol (XIII., Scheme 3). The blocked PG was deacylated in warm methanolic NaOH and the benzylated product (XIV) was sulfated with SO_3-pyridine complex in dry benzene at 60°. Debenzylation was readily achieved by hydrogenolysis over palladium charcoal and the synthetic phosphosulfolipid (XVI) was isolated as its potassium salt, or converted to its methyl ester (XVII). The spectroscopic properties of these compounds were compared to those corresponding to the bacterial PGS.

SCHEME 3.

Synthesis of 1-sn-phosphatidyl-sn-1'-glycero-2'-sulfate



R = $CH_3(CHCH_2CH_2CH_2)_3CHCH_2CH_2-$ (phytanyl)
CH_3 CH_3

R′ = $CH_3(CH_2)_{12}-$

Scheme III

(a) Sulfation of phosphatidyl glycerol

(i) 2,3-Di-O-phytanyl-sn-glycero-1-phosphoryl-(3'-sn-glycero-1'-sulfate) [PG-1-S]. - A solution of "natural" salt of PG (42 mg, 52 μmole) in 5 ml of anhydrous benzene was stirred with sulfur trioxide-pyridine complex reagent (9.5 mg, 60 μmole) at room temperature for 6 hr. TLC examination showed a major spot (Rf 0.38 in chloroform-methanol-90% acetic acid, 30:4:20, v/v) corresponding to the monosulfated product, and traces of disulfated product (Rf 0.12). The mixture was cleared by centrifugation, concentrated in a nitrogen stream and chromatographed on silica plates in chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v). The monosulfated product was eluted with 250 ml of chloroform-methanol-diethyl ether (1:1:1, v/v) and the eluate was evaporated to dryness in the presence of benzene; the residue was dissolved in 10 ml of chloroform-methanol (1:1, v/v), cleared by centrifugation and diluted with 4.5 ml of 0.5 N aqueous HCl. After brief centrifugation of the biphasic system, the chloroform phase was neutralised with 0.2 N methanolic KOH, concentrated to small volume with benzene and diluted with ten volumes of acetone. After several hours at 0°, the precipitated potassium salt was centrifuged down, washed with cold acetone and dried in vacuo to give 38 mg (39 μmole, 75% yield) of chromatographically pure white powder. The synthetic product was indistinguishable on TLC from the natural (bacterial) PGS (Fig. 18). It had the following specific rotations (c, 2.26 in chloroform); 589 nm, $+2.88^{\circ}$; 578 nm, $+2.97^{\circ}$; 546 nm, $+3.32^{\circ}$; 436 nm, $+5.53^{\circ}$; 365 nm, $+8.50^{\circ}$.

Analytical data for the synthetic PGS are given in Table 6.

FIGURE 18.

Thin-layer chromatograms of natural (bacterial) phosphatidyl glycerosulfate and synthetic phosphatidyl glycerosulfate developed in alkaline and acid solvent systems.

1 Bacterial 1-sn-phosphatidyl-3'-sn-glycero-1'-sulfate (potassium salt)

2 Synthetic 1-sn-phosphatidyl-3'-sn-glycero-1'-sulfate (potassium salt)

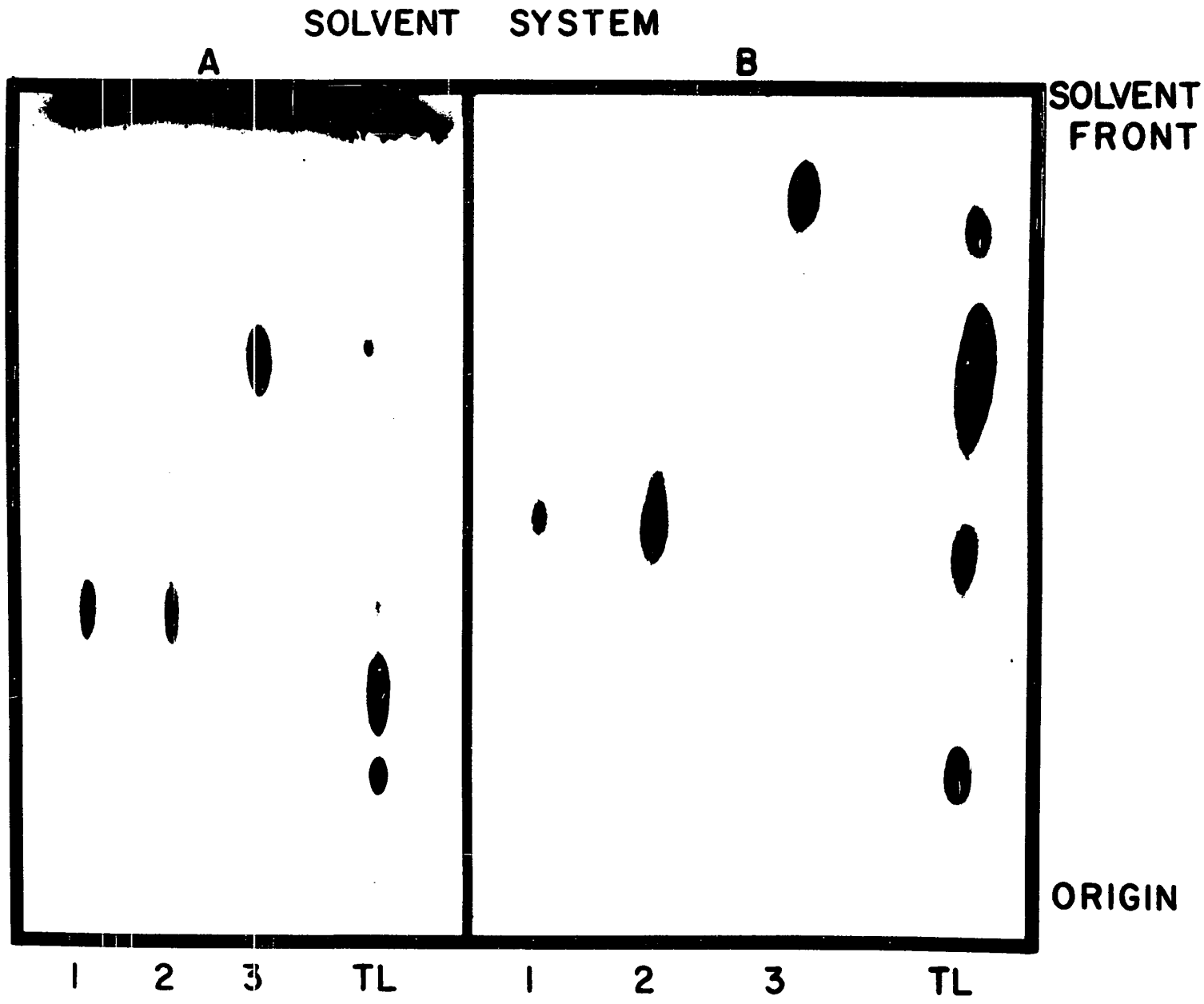
3 Bacterial 1-sn-phosphatidyl-3'-sn-glycerol

4 Total polar lipids of H. cutirubrum

Solvent systems:

A Chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v)

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



Dimethyl ester of synthetic PG-1-S

Synthetic PG-1-S (8 mg) was converted to its dimethyl ester as described for the bacterial PG-1-S (Rf methyl ester 0.68 in chloroform-methanol-water, 90:10:1, v/v). The infrared and NMR spectra (Figs. 35, 28B, respectively) are discussed in the Results and Discussion Section.

(ii) 2,3-Di-O-phytanyl-sn-glycero-1-phosphoryl-(3'-sn-glycero-1',2'-disulfate) [PG-1,2-di-S]

A solution of "natural" salt of PG (25 mg, 30 μmole) in 5 ml of anhydrous benzene was stirred with sulfur trioxide-pyridine reagent (16 mg, 0.10 mmole) at 60° for 1 hr. The mixture was centrifuged, the supernatant was concentrated in a nitrogen stream and the product was chromatographed in chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v) to remove a trace of monosulfated product (Rf PG-1,2-di-S, 0.12 and PG-1-S, 0.39). The disulfate was eluted from the silica and converted to its tripotassium salt as described for the monosulfated derivative; the precipitate was washed with cold acetone and dried in vacuo to give 18 mg (17 μmole, 57% yield) of chromatographically pure white powder.

It had the following specific rotations (c, 1.6 in chloroform): 589 nm, +4.06°; 578 nm, +4.13°; 546 nm, +4.31°; 436 nm, +6.96°; 365 nm, +11.08°.

The analytical data and the infrared spectrum of the tripotassium salt of PG-1,2-di-S are shown in Table 8 and Fig. 19, respectively.

TABLE 8

Analytical Data for Phosphatidyl Glycero-2-sulfate and Phosphatidyl Glycero-1, 2-disulfate

Data	PG-2-S, K salt		PG-1, 2-di-S, K salt	
	Found	Calc*	Found	Calc**
C, %	56.13	56.29	49.96	50.24
H, %	9.21	9.76	8.03	8.62
P, %	3.28	3.16	2.86	2.82
S, %	3.52	3.27	5.61	5.83
K, %	-	-	11.0	10.7
S/P atomic ratio	1.04	1.00	1.90	2.00
K/S+P atomic ratio	-	-	1.05	1.00

* Calc. for $C_{46}H_{93}O_{11}PSK_2 \cdot H_2O$ (981.47)

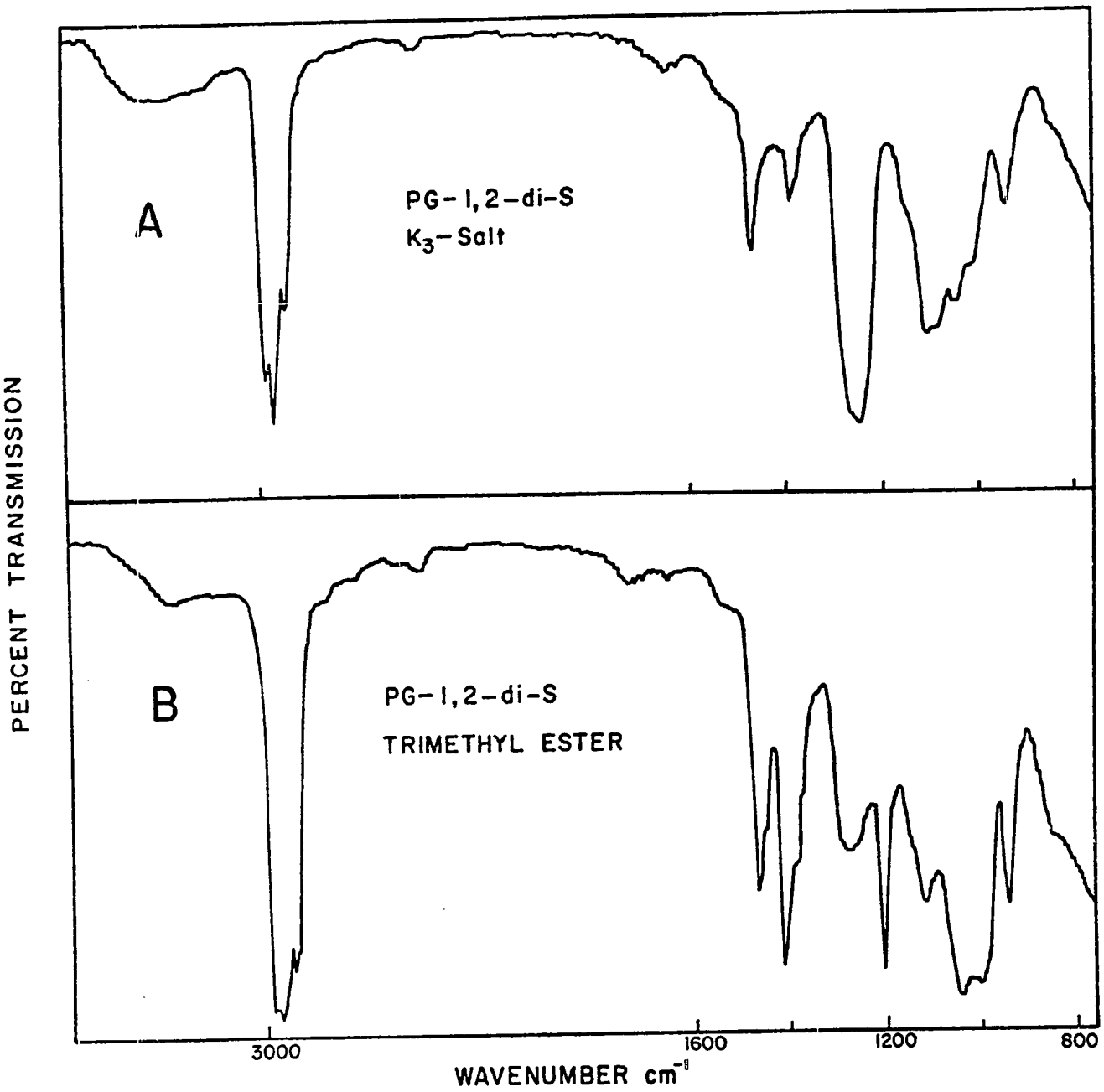
** Calc. for $C_{46}H_{92}O_{14}PS_2K_3 \cdot H_2O$ (1099.6)

FIGURE 19.

Infrared Absorption Spectra in CCl_4 of:

A 1-sn-phosphatidyl-3'-sn-glycero-1', 2'-disulfate, potassium salt

B 1-sn-phosphatidyl-3'-sn-glycero-1', 2'-disulfate, trimethyl ester


A
PG-1,2-di-S
K3-Salt
B
PG-1,2-di-S
TRIMETHYL ESTER
PERCENT TRANSMISSION
3000
1600
1200
800
WAVENUMBER cm-1

Trimethyl ester of PG-1, 2-di-S

PG-1, 2-di-S (6 mg) was converted to its methyl ester with diazomethane as described for PG-1-S.

The infrared spectrum of the trimethyl ester is shown in Fig. 19, and the NMR spectrum (Fig. 36) is discussed in the Results and Discussion Section.

(b) Synthesis of phosphatidyl glycero-2-sulfate, PG-2-S (XVI; Scheme 3)

2, 3-Di-O-phytanyl-sn-glycerol (natural diether)

This starting material was prepared from total (polar) acetone-insoluble lipids of Halobacterium cutirubrum after methanolic-HCl hydrolysis as described elsewhere (Joo et al., 1968). The diether was purified by silicic acid column chromatography; it had $[\alpha]_D^{22}$ +8.7° (c, 4.2 in chloroform); reported $[\alpha]_D^{22}$ +8.5° (Joo et al., 1968).

2, 3-Di-O-phytanyl-sn-glycerophosphoric acid (phosphatidic acid, diphytanyl ether analogue) (XI)

This compound was prepared by phosphorylation of the natural diether with diphenylphosphoryl chloride as described by Kates et al. (1971). The potassium salt had $[\alpha]_D^{22}$ +1.75° (c, 3.1 in chloroform); reported $[\alpha]_D^{22}$ +1.88°, +1.78° (Kates et al., 1971).

Anal. Calc. for $C_{43}H_{87}O_6PK_2 \cdot H_2O$: P, 3.74.

Found: P, 3.80.

The potassium salt was converted to the free acid form as described for PGS and PGP.

1, 2-Isopropylidene-sn-glycerol

This substance was synthesised from D-mannitol by the method of Baer and Fisher (1939), as modified by Wickberg (1958) and LeCocq (1964); it had $[\alpha]_D^{22}$ +14.9° pure substance; reported $[\alpha]_D^{22}$ +14.38° (Joo and Kates, 1969).

3-O-Benzyl-sn-glycerol

This compound was prepared from 1, 2-isopropylidene-sn-glycerol by a modification of the methods of Howe and Malkin (1951) and Sowden and Fischer (1941) as described by Kates et al.(1963). The fraction distilling in the range 152 - 154°/1 mm crystallised on standing (m. p. approximately 21°) and had α_D^{22} +5.90° (pure substance), reported (Joo and Kates, 1969) α_D^{22} +5.96° (pure substance).

2-O-Myristoyl-3-O-benzyl-sn-glycerol (XII)

This substance was prepared as described for the stearoyl compound by De Haas and van Deenen (1965), and was separated from the 3-O-myristoyl isomer by preparative TLC using sodium acetate-impregnated plates (see Materials and Methods, Section IV. 2b). Examination of the product by NMR showed that the 2-O-myristoyl compound (5.0 ppm) contained only a trace of the 3-O-myristoyl isomer (4.1 ppm).

2,3-Di-O-phytanyl-sn-glycero-1-phosphoryl-1'-(2'-O-myristoyl-3'-O-benzyl)-sn-glycerol (XIII)

The dried free acid form of 2,3-di-O-phytanyl-sn-glycero-phosphate (XI), prepared from the potassium salt (120 mg, 0.16 mmole) as described for PGP, was mixed with a solution of 2-O-myristoyl-3-O-benzyl-sn-glycerol (XII, 125 mg, 0.32 mmole) in 10 ml of anhydrous pyridine and brought to dryness under reduced pressure. The residue was dried in vacuo over phosphorus pentoxide for 12 hr, and then stirred with 20 ml of anhydrous pyridine and dry triisopropylbenzenesulphonyl chloride (194 mg, 0.64 mmole) at room temperature for 8 hr. The mixture was cooled on ice, stirred with 1 ml of water for 30 min and evaporated to dryness under reduced pressure. Trituration of the residual oil with cold diethyl ether precipitated triisopropylbenzenesulfonic acid which was removed by centrifugation and twice washed with diethyl ether. The combined ethereal supernatants were washed with water, 0.5 N hydrochloric acid and again with water, and brought to dryness under reduced pressure after addition of benzene.

Examination of the residual oil by thin-layer chromatography in chloroform-methanol-30% ammonium hydroxide (80:20:2, v/v) showed a major phosphate-positive spot with Rf 0.70. The crude product was purified by preparative thin-layer chromatography using double development first in chloroform-diethyl ether (3:1, v/v; Rf 0.00), then in chloroform-methanol-30% ammonium hydroxide (90:10:1, v/v; Rf 0.30). The product was eluted with chloroform-methanol-diethyl ether (1:1:1, v/v), converted to the potassium salt and precipitated by acetone, as described for PGS. The oily precipitate was washed with cold acetone and dried in vacuo; yield 137 mg (0.12 mmole, 75%) of chromatographically pure product XIII having $[\alpha]_D^{22}$ +2.90° (c, 4.1 in chloroform).

Anal. Calc. for $C_{67}H_{126}O_9PK$ (1145.8): P, 2.70.
Found: P, 2.66.

The infrared spectrum (liquid film) of compound XIII (Fig. 20B) showed absorption for carbonyl (1740 cm^{-1}), aromatic modes (3025, 3065, 3095, 1495, 690 cm^{-1}), phytanyl groups (2850, 2930, 2960, 1455, 1375-1365 [doublet] cm^{-1}), myristoyl $(CH_2)_n$ (730 cm^{-1}), bonded P = 0 (1240 cm^{-1}), C-O-C, P-O^- (1100 cm^{-1}) and P-O-C (1055 cm^{-1}). Hydroxyl absorption was absent.

2,3-Di-O-phytanyl-sn-glycero-1-phosphoryl-1'-(3'-O-benzyl)-sn-glycerol [3-O-benzyl-PG] XIV

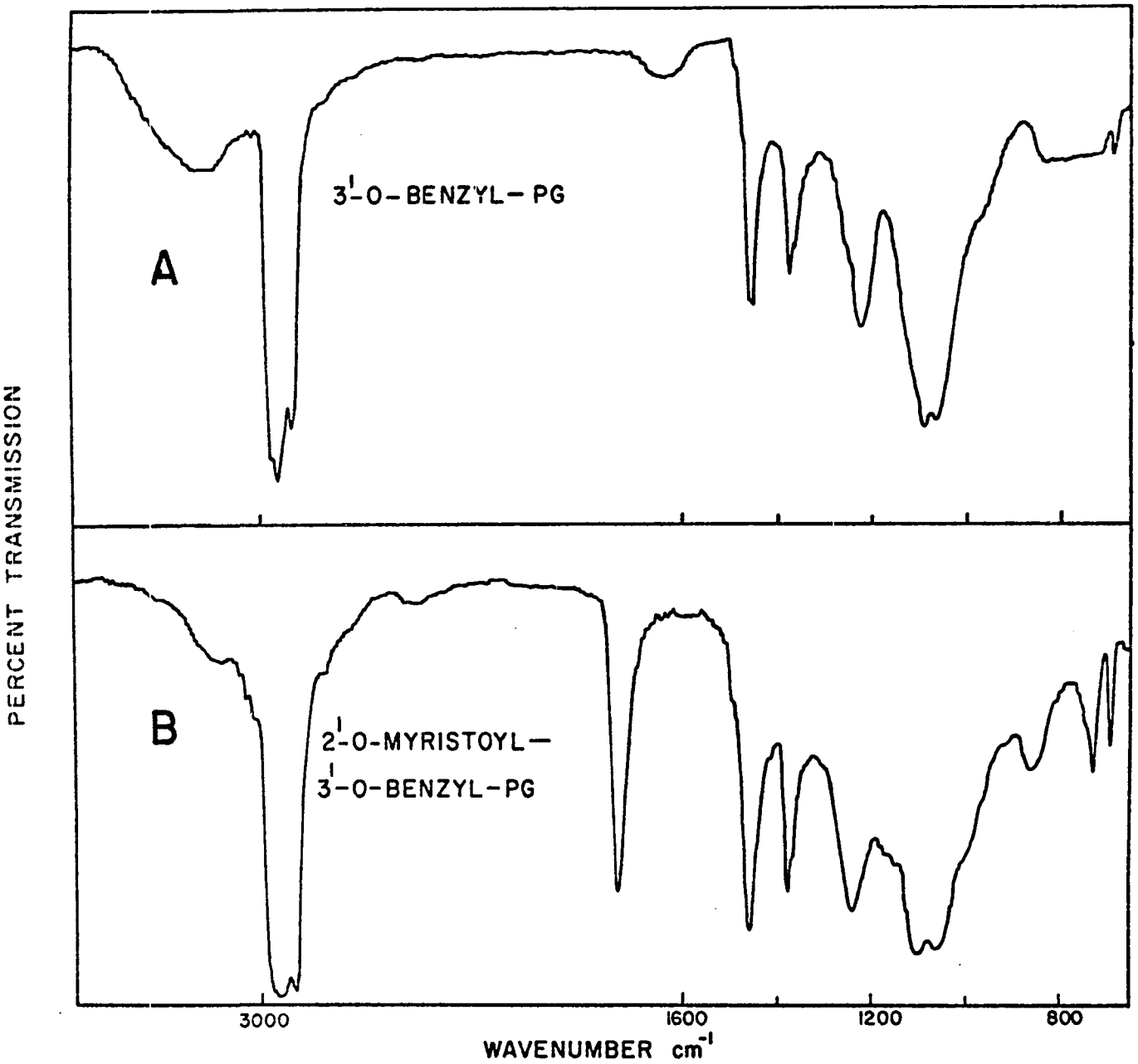
The 2-O-myristoyl derivative (XIII) was deacylated essentially by the procedure of Brockerhoff (1963) as modified recently (Marshall and Kates, 1972) as follows: a solution of XIII (potassium salt) (137 mg, 0.12 mmole) in 0.8 ml of chloroform and 1.2 ml of methanol was treated with 2.0 ml of 0.2 N methanolic NaOH at 40°. Deacylation was complete after 40 min (Rf of XIII and deacylated product XIV, 0.70 and 0.48 respectively, in chloroform-methanol-30% ammonium hydroxide, 80:20:2, v/v) whereupon 3.2 ml of chloroform, 0.8 ml of methanol and 3.6 ml of 0.5 N aqueous HCl were successively added to the cooled reaction mixture. The biphasic system was briefly centrifuged and the chloroform phase was neutralised with 0.2 N methanolic KOH, concentrated to small volume and diluted with acetone to separate the phospholipid product from methyl myristate. The oily precipitate was centrifuged down, washed with cold acetone and dried in vacuo to give 89 mg of product XIV (0.095 mmole, 79%). An analytical sample was obtained by preparative TLC in chloroform-methanol-30% ammonium hydroxide (80:20:2, v/v), followed by reconversion to the potassium salt as described above. It had $[\alpha]_D^{22}$ +1.03° (c, 2.43 in chloroform.

FIGURE 20

Infrared Absorption Spectra [A, in CCl_4: B, liquid film] of:

A 1-sn-phosphatidyl-1'-(3'-O-benzyl)-sn-glycerol, potassium salt. (3-O-benzyl-PG)

B 1-sn-phosphatidyl-1'-(2'-O-myristoyl-3'-O-benzyl)-sn-glycerol, ammonium salt



Anal. Calc. for $C_{53}H_{100}O_8PK$ (935.41): C, 68.05; H, 10.78; P, 3.31.
Found: C, 67.85; H, 10.21; P, 3.43.

The infrared spectrum (Fig. 20A) closely resembled that of the myristoyl derivative (Fig. 20B) except for the absence of carbonyl and $(CH_2)_n$ absorption (1740 and 730 cm^{-1} respectively), and the presence of hydroxyl absorption (3280 cm^{-1}, broad).

2,3-Di-O-phytanyl-sn-glycero-1-phosphoryl-1'-sn-glycero-3'-O-benzyl-2'-sulfate (XV)

A solution of compound (XIV) (potassium salt; 85 mg, 0.091 mmole) in 15 ml of anhydrous benzene was stirred at 60° for 3 hr with sulfur trioxide-pyridine complex reagent (30 mg, 0.19 mmole). The cooled mixture was cleared by centrifugation, the residue was washed with benzene and the combined supernatants were concentrated to a small volume under reduced pressure. The crude product was purified by preparative thin-layer chromatography in chloroform methanol-30% ammonium hydroxide (80:20:2, v/v; Rf, 0.18) and the desired product was eluted from the silica and converted to the potassium salt form as described for XIV; the yield of chromatographically pure compound XV (white powder) was 75 mg (0.071 mmole; 75%), having $[\alpha]_D^{22}$ -2.38° (c, 2.10 in chloroform).

Anal. Calc. for $C_{53}H_{99}O_{10}PSK_2H_2O$ (1055.6): C, 60.30; H, 9.65; P, 2.93; S, 3.04; S/P atomic ratio, 1.00.
Found: C, 60.91; H, 9.24; P, 2.95; S, 3.23; S/P atomic ratio, 1.06.

The infrared spectrum resembled that of 3-O-benzyl-PG (XIV) except for the relatively more intense absorption band centred at 1240 cm^{-1} (P = O, and asymmetric stretch S = O) and the sulfate bands at 1045 cm^{-1} (shoulder, symmetric stretch S = O), 940 cm^{-1} (C-O-S) and 840 cm^{-1} (C-O-S, weak) (Haines, 1971); hydroxyl absorption was absent.

Dimethyl ester of XV

The dimethyl ester was prepared as described for PG-1-S. The infrared spectrum showed sulfate absorption at 1405 and 1195 cm^{-1} and at 935 cm^{-1} (intense, C-O-S, secondary sulfate). Hydroxyl absorption was absent.

NMR assignments: phytanyl (78 H) 0.75 - 1.78 δ ; P-OCH_3, doublet 3.72, 3.83 δ (which collapsed to singlet 3.78 δ on ^{31}P-^{1}H spin-decoupling); S-OCH_3 , singlet 3.94 δ ; -$OCH_2C_6H_5$, 4.57 δ ; -$OCH_2C_6H_5$, 7.30 δ .

2,3-Di-O-phytanyl-sn-glycero-1-phosphoryl-1'-sn-glycero-2'-sulfate [PG-2-S] (XVI)

3-O-Benzyl phosphatidyl glycerosulfate (XV) (potassium salt, 40 mg, 0.038 mmole) was hydrogenolysed with palladium-charcoal catalyst (Hessel, 1954), in 6 ml of chloroform-methanol (1:1, v/v) at room temperature and pressure. After 30 min, the catalyst was removed by centrifugation and washed with 4 ml of chloroform-methanol (1:1, v/v); the combined supernatants were immediately diluted with 4.5 ml of 0.1 N aqueous HCl and the biphasic system was briefly centrifuged. The chloroform phase was neutralised with 0.2 N methanolic KOH, and the potassium salt of the PG-2-S (XVI) was isolated as described for compound XV. Yield, 35 mg (94%). $[\alpha]_D^{22}$ -6.08° (c, 1.65 in chloroform).

Analytical data for PG-2-S potassium salt are given in Table 8. The PG-2-S migrated as a single spot in three solvent systems (see Table 9); in each system the PG-2-S had slightly higher mobilities than the bacterial PG-1-S (Fig. 21).

The infrared spectrum of PG-2-S potassium salt (Fig. 38A) resembled the corresponding spectrum of PG-1-S, but differed in having more pronounced absorption bands at 935 cm^{-1} (C-O-S stretch, secondary sulfate), and 1040 cm^{-1} (S=O symmetric stretch, secondary sulfate).

TABLE 9

Chromatographic Mobilities of Some Sulfated Derivatives of Phosphatidyl Glycerol

Compound	Rf in Solvent System 1[a]	2[a]	3[b]
Phosphatidyl glycero-1-sulfate (natural)	0.30	0.33	0.41
Phosphatidyl glycero-1-sulfate (synthetic)	0.30	0.33	0.41
Phosphatidyl glycero-2-sulfate	0.33	0.39	0.44
Phosphatidyl 3-O-benzyl-sn-glycero-2-sulfate	0.48	0.54	-
Phosphatidyl glycero-1,2-disulfate	0.10	0.12	0.15
Phosphatidyl glycerol	0.67	0.66	0.68

a TLC on silica-gel H layers (20 x 20 x 0.025 cm) in solvent systems 1, chloroform-methanol-90% acetic acid (30:4:20, v/v), 2, chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v).

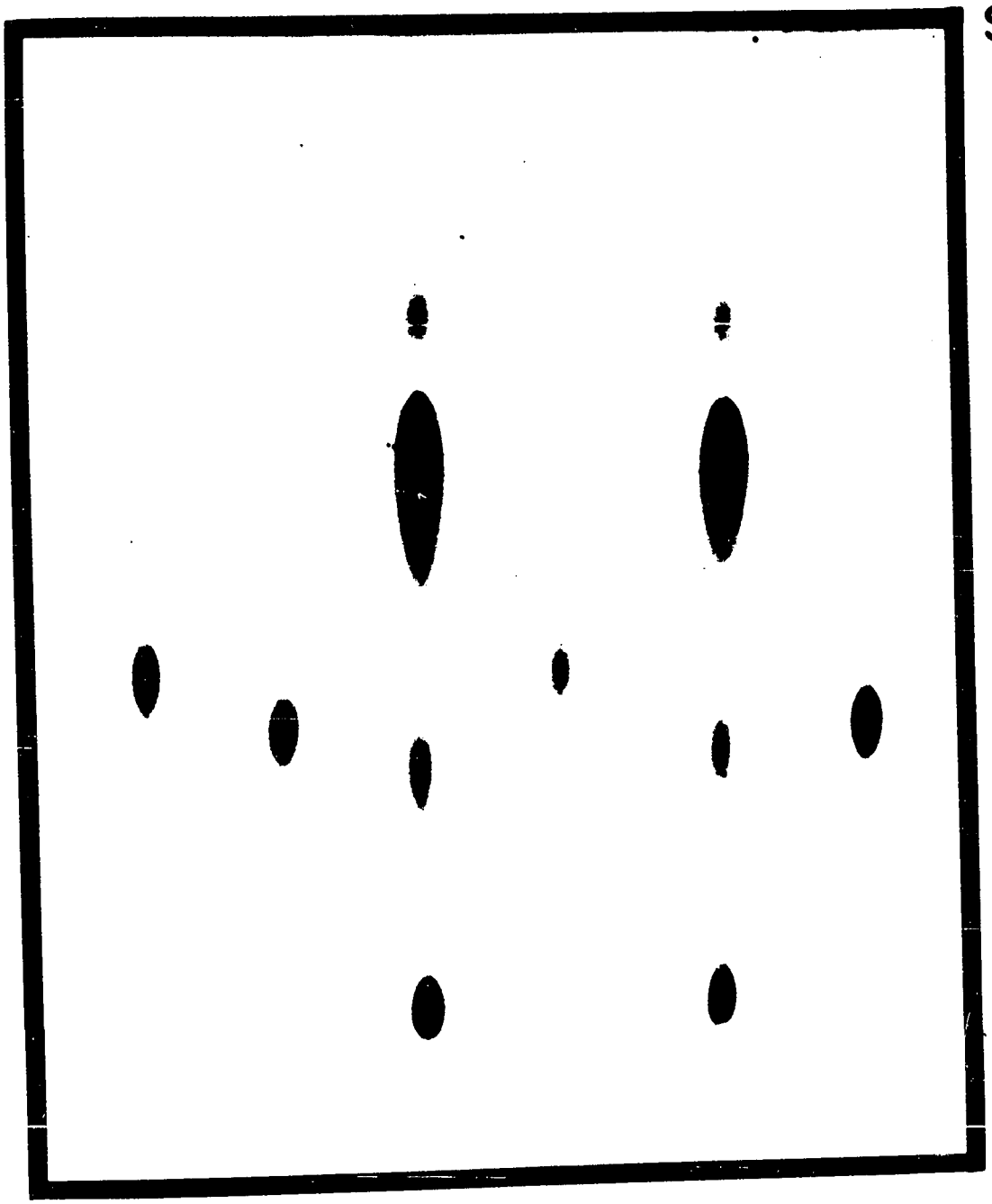
b Chromatography on silicic acid-impregnated paper in diisobutylketone-acetic acid-water (40:25:5, v/v).

FIGURE 21

Thin-layer chromatographic comparison of natural (bacterial) phosphatidyl glycero-1-sulfate (PG-1-S) and synthetic phosphatidyl glycero-2-sulfate (PG-2-S).

Solvent system:

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



Dimethyl ester of PG-2-S (XVII)

The ester was prepared by treating the acid form of the lipid with ethereal diazomethane as described for PG-1-S. The infrared and NMR spectra of the ester (Figs. 38B, 37B, respectively) are discussed in the Results and Discussion Section.

7. Preparation of 1, 3-di-O-phytanyl-sn-glycerol ("1, 3-diether")

2-O-benzyl-1, 3-di-O-phytanyl-sn-glycerol (Scheme 4)

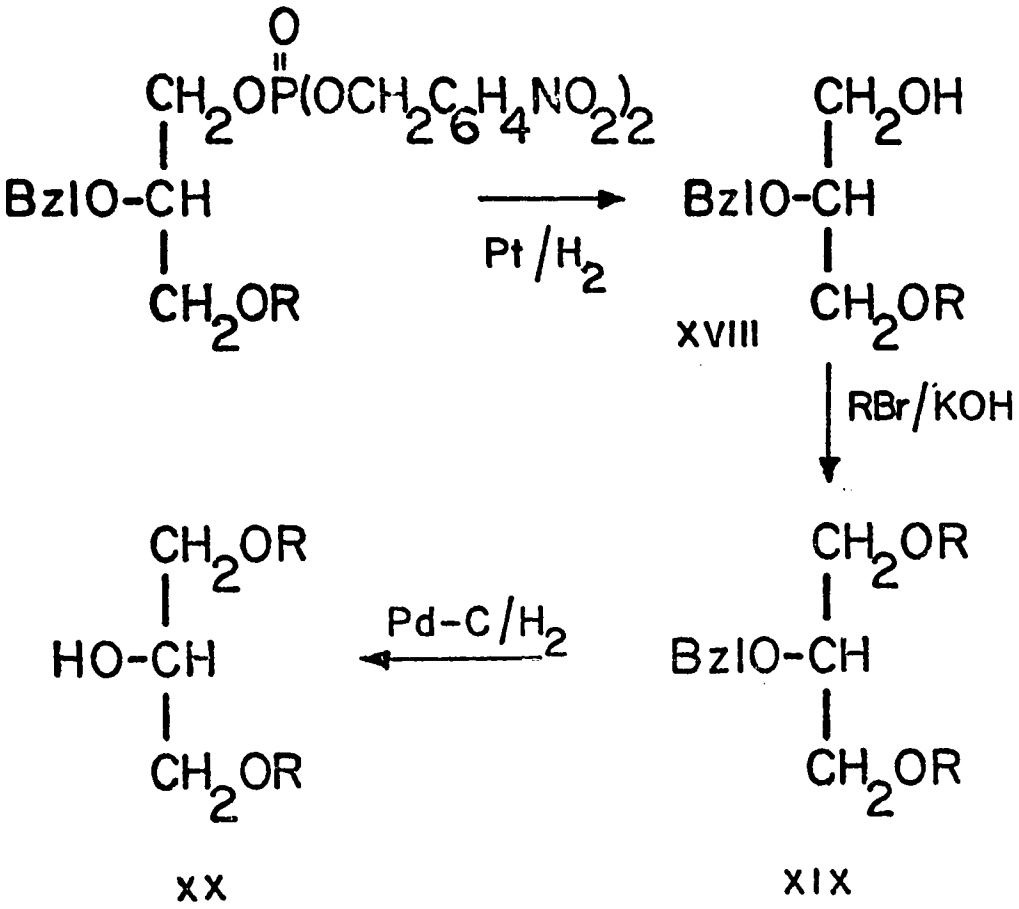
A stirred mixture of 2-O-benzyl-3-O-phytanyl-sn-glycerol (XVIII) (22 mg, 5 μmole) in 10 ml of anhydrous benzene was refluxed for 16 hr with DDD-phytanyl bromide (25 mg, 7 μmole) in the presence of 2.0 g of powdered KOH. The water formed was removed in a Soxhlet apparatus containing calcium carbide. The cooled reaction mixture was diluted with 20 ml of ice-water, and then neutralised with 10% H_2SO_4. The benzene phase was removed and the aqueous phase was extracted with three 10 ml portions of benzene. The combined benzene extracts were evaporated under reduced pressure to a colourless oil, TLC examination of which showed a major spot (Rf 0.45 in benzene) corresponding to 2-O-benzyl diether (XIX). The oil was hydrogenolysed without prior purification.

1, 3-di-O-phytanyl-sn-glycerol [1, 3-diether] (XX)

The benzylated diether XIX was hydrogenolysed in 5 ml of chloroform-methanol (1:1, v/v) using 50 mg of palladium-charcoal catalyst (Hessel, 1954) at room temperature and pressure. After 30 min the catalyst was centrifuged down, thoroughly washed with chloroform-methanol (1:1, v/v) and recentrifuged. Evaporation of the combined supernatants

SCHEME 4

Synthesis of 1, 3-di-O-phytanyl-sn-glycerol ("1, 3-diether")



R = phytanyl

gave an oil (36 mg) which showed a major spot on TLC (Rf 0.70 in chloroform-diethyl ether, 9:1, v/v) corresponding to the desired 1,3-diether. The product was freed from less-polar contaminants by preparative chromatography in the above TLC solvent, giving 18 mg (52%) of chromatographically pure material. The isomeric diethers (1,3- and 2,3-phytanyldiether) were only marginally separated on TLC in chloroform-diethyl ether mixtures, but better chromatographic resolution was obtained using ethyl acetate-carbon tetrachloride (1:5, v/v); Rf 0.78 and 0.62 for the 1,3- and 2,3-diether respectively (Fig. 22).

$[\alpha]_D^{22}$ +1.1°, M_D +7.2° (c, 1.2 in chloroform).

Anal. Calc. for $C_{43}H_{88}O_3$ (653.13): C, 79.07; H, 13.58.

Found: C, 79.37; H, 13.47.

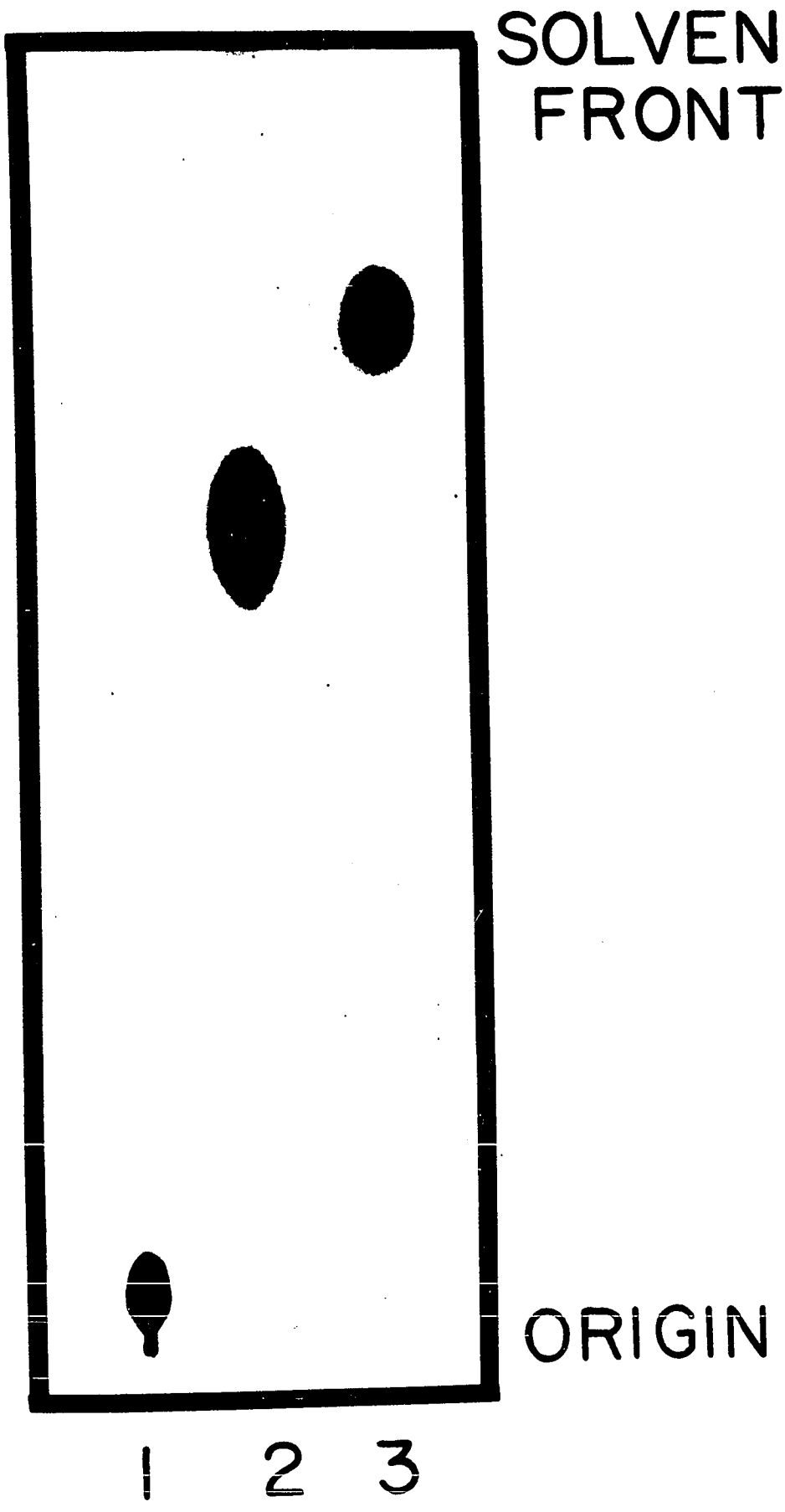
FIGURE 22

Thin-layer chromatogram of isomeric diphytanyl glycerol ethers.

1 Synthetic monophytanyl glycerol ether standard;
2 2, 3-Di-O-phytanyl-sn-glycerol obtained by acid hydrolysis of PGS;
3 Synthetic 1, 3-di-O-phytanyl-sn-glycerol.

Solvent system:

ethyl acetate-carbon tetrachloride (1:5, v/v)



IV. Phosphatidyl Glycerol (PG)

1. Isolation as "Natural" Salt Form

This substance was clearly resolved in the TLC fractionation and was obtained in the pure state after a single chromatographic separation (Fig. 16). The "natural" salt form of the lipid was precipitated as an oily solid by acetone.

2. Sodium Salt Form

The sodium salt of PG was prepared via the free acid form as described for the monovalent cation salts of PGP and was isolated as a solid by acetone precipitation. $[\alpha]_D^{22}$ +3.33° (c, 8.36 in chloroform); reported $[\alpha]_D^{22}$ +3.43° (Joo and Kates, 1969).

Anal. Calc. for $C_{46}H_{94}O_8PNa.H_2O$ (847.6): P, 3.66.

Found: P, 3.68.

3. Monomethyl Ester of Phosphatidyl Glycerol (Me-PG)

The ester was prepared by direct methylation of the free acid form of PG (10 mg) as described for Me_3-PGP. The product slowly decomposed to give phytanyl diether on standing at 5° both in chloroform solution and in the anhydrous solvent-free state. Accordingly, infrared and NMR spectra of the ester were obtained with minimum delay after removal of solvents *in vacuo*. $[\alpha]_D^{22}$ +4.12° (c, 1.02 in chloroform).

Anal. Calc. for $C_{46}H_{97}O_8P$ (809.2): P, 3.83.

Found: P, 3.89.

The NMR spectrum of the methyl ester (Fig. 29) is discussed in the Results and Discussion Section.

4. Synthesis of 2, 3-Di-O-phytanyl-sn-glycero-1-phosphoryl-2'-sn-glycerol [β-PG], Scheme 5.

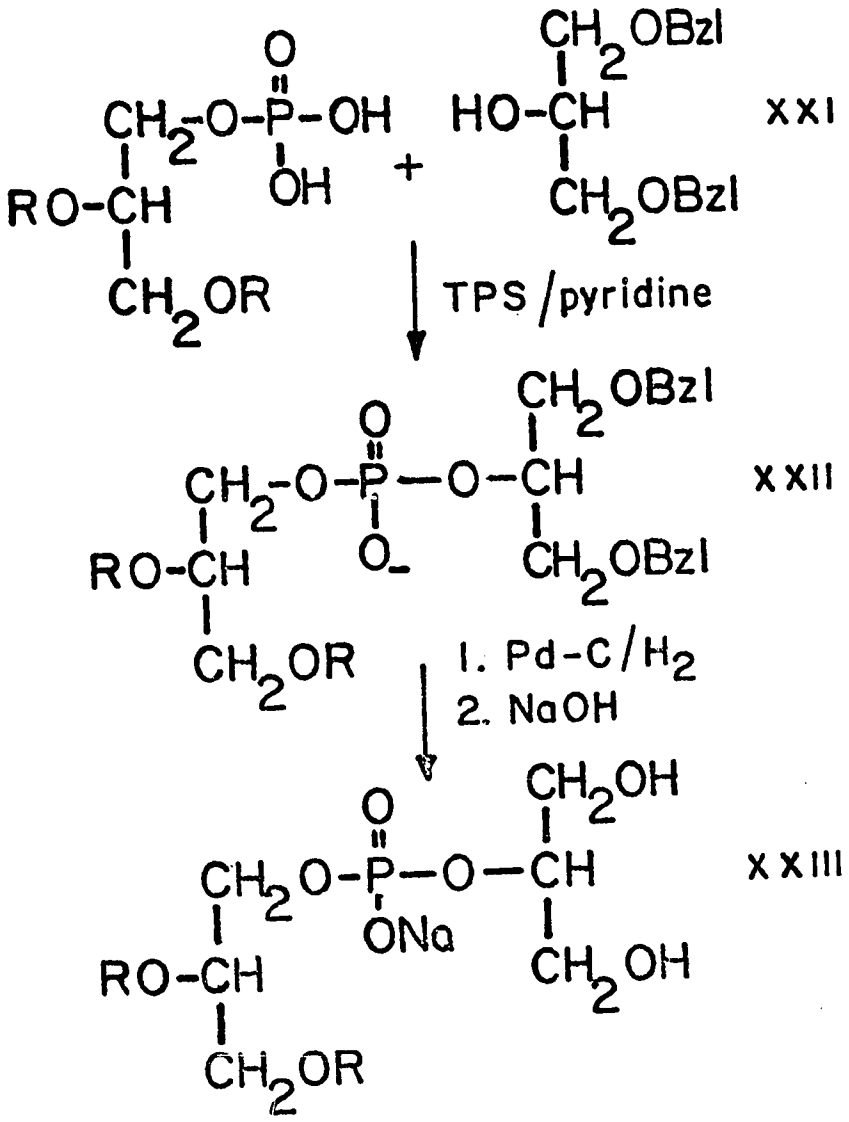
The starting materials were 2, 3-di-O-phytanyl-sn-glycerophosphoric acid and 1, 3-di-O-benzyl-sn-glycerol (XXI). The former was prepared by phosphorylation of 2, 3-di-O-phytanyl-sn-glycerol as described in Section III. 6b. The latter was synthesised from 1, 3-dichloro-2-propanol by the procedure of Fairbourne et al.(1931) and purified by vacuum distillation (b. p. 155°/0. 8 mm).

2, 3-Di-O-phytanyl-sn-glycerophosphoric acid (18 mg, 25 μmole) and 1, 3-di-O-benzyl-sn-glycerol (20 mg, 73 μmole) in 5 ml of anhydrous pyridine were stirred at room temperature for 24 hr in the presence of triisopropylbenzenesulfonyl chloride (35 mg, 116 μmole). The reaction mixture was processed as described for 1-phosphatidyl-sn-1'-(2'-O-myristoyl-3'-O-benzyl)-sn-glycerol (Section III. 6b). TLC examination of the residual oil revealed two phosphate-positive spots (Rf 0. 26 and 0. 92 in chloroform-methanol-water, 90:10:1, v/v). No phosphatidic acid (Rf 0. 0 in the above solvent) was detected.

The two phosphorus-containing products were isolated by preparative TLC in chloroform-methanol-water (90:10:1, v/v); the separated products were eluted with chloroform-methanol-diethyl ether (1:1:1, v/v) and neutralised with methanolic ammonium hydroxide as described for other acidic lipids. The more polar product, dibenzyl-β-PG (XXII) (18 mg, as ammonium salt) was hydrogenolysed in 5 ml of ethanol-chloroform (9:1, v/v) at room temperature and pressure over palladium-charcoal catalyst for 1 hr. Chromatographic examination of the product in three solvent systems showed a single spot with significantly higher mobilities than those of bacterial PG (Table 10 and Fig. 23). The debenzylated product (10 mg) was

SCHEME 5

Synthesis of 1-sn-phosphatidyl-2'-sn-glycerol (β-PG)



R = phytanyl

TABLE 10

Chromatographic Mobilities of α- and β-Phosphatidyl Glycerols

Compound	Solvent system 1	2	3
α-PG*	0.51	0.63	0.69
β-PG**	0.58	0.67	0.72
isomerised β-PG	-	-	0.69 + 0.72

* 1-phosphatidyl-3'-sn-glycerol (bacterial)

** 1-phosphatidyl-2'-sn-glycerol (synthetic)

Solvent systems: 1, chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v); 2, chloroform-methanol-90% acetic acid (30:4:20, v/v); 3, diisobutyl ketone-acetic acid-water (40:25:5, v/v), chromatography on silicic acid-impregnated paper according to Marinetti (1957).

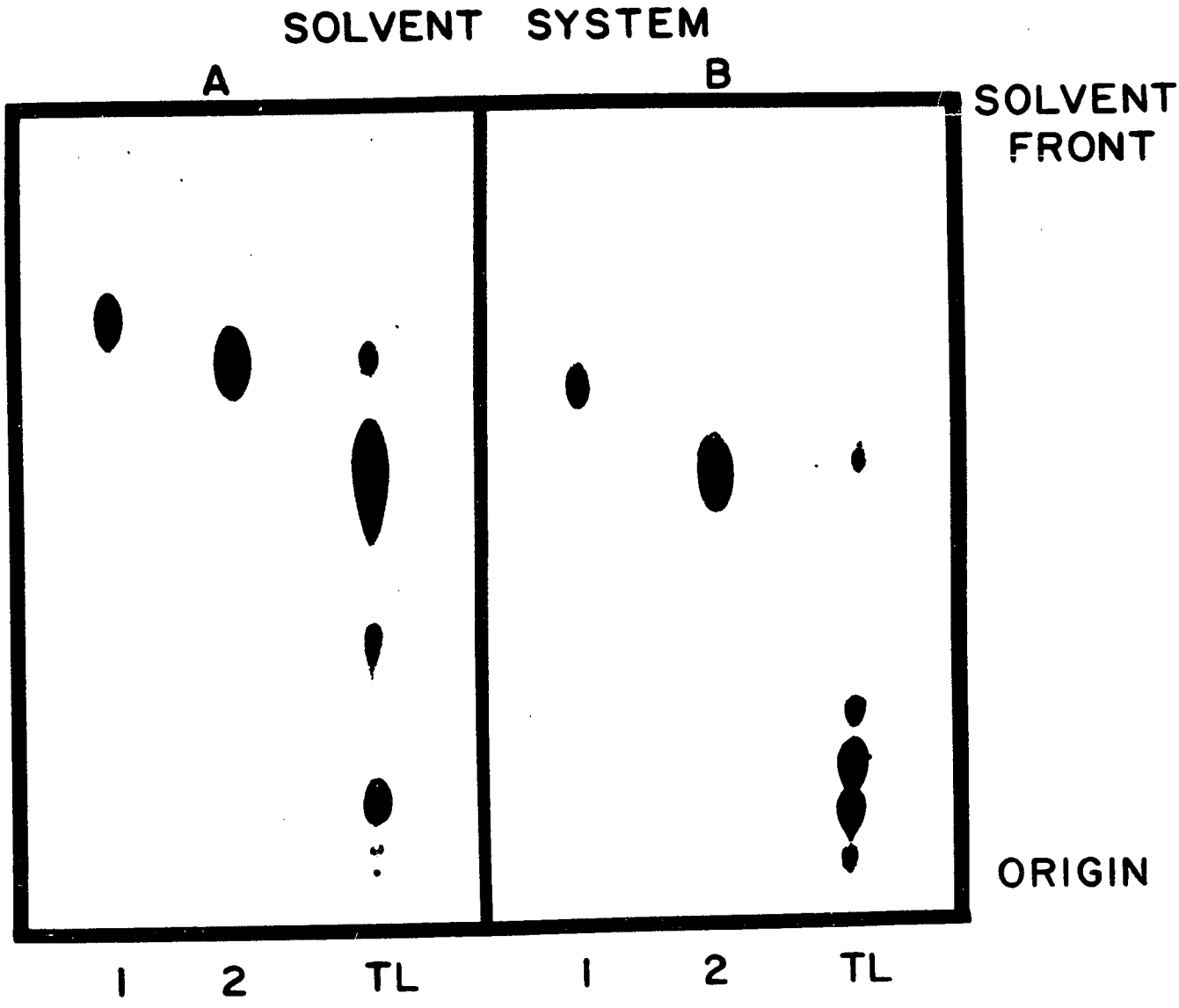
FIGURE 23

Thin-layer chromatographic comparison of natural (bacterial) phosphatidyl glycerol (α-PG) and synthetic 1-sn-phosphatidyl-sn-2-glycerol (β-PG)

1 Synthetic β-PG
2 Natural α-PG
TL Total polar lipids of H. cutirubrum

Solvent systems:

A chloroform-methanol-90% acetic acid (30:4:20, v/v)
B chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v)



converted to the sodium salt form (XXIII) and precipitated with acetone as described for the potassium salt of PGS; yield, 8 mg (38% based on phosphatidic acid). $[\alpha]_D^{22}$ +1.37° (c, 0.8 in chloroform).

Anal. Calc. for $C_{46}H_{94}O_8PNa.H_2O$ (847.6): P, 3.66.

Found: P, 3.60.

Pyrophosphate dimer of phosphatidic acid

The less polar phosphorus-containing product (10 mg, as ammonium salt) was converted to the potassium salt as described above.

Anal. Calc. for $C_{86}H_{174}O_{11}P_2(NH_4)_2.H_2O$ (1542.4): P, 4.01.

Found: P, 3.91.

Calc. for corresponding monohydrated dipotassium salt: P, 4.12. Found: P, 4.15.

5. Isomerisation of β-PG under Mild Acid Conditions

The β-isomer of PG (1 mg) was incubated at 37° with 2 ml of 0.025 N HCl in chloroform-methanol (1:1, v/v) for 15 min. The solution was diluted with 0.9 ml of water, the biphasic mixture was centrifuged and the chloroform phase was neutralised with 0.2 N methanolic ammonium hydroxide. Chromatography of the product on silicic acid-impregnated paper revealed a periodate-Schiff positive spot with Rf slightly lower than β-PG but identical to that of bacterial PG (Table 10); the original β-isomer of PG gave no reaction after periodate-Schiff staining, but it was detected with Rhodamine reagent.

RESULTS AND DISCUSSION

I. Structural Studies on Phosphatidyl Glycerophosphate (PGP)

The aim of the investigation was to determine whether the monomeric structure 1 or the dimeric structure 2 (Fig. 3) best represented the natural phospholipid. The solution to the problem was the demonstration of the presence of three ionisable acid groups per molecule of PGP. Evidence for the presence of these three groups was obtained from three sources which are separately discussed below.

1. Spectral Study of the Methylated Derivatives Me_3-PGP and Me_4-PGP

Treatment of the free acid form of PGP with ethereal diazomethane resulted in rapid esterification of the P-OH groups to form the trimethyl ester (Me_3-PGP) in high yield (86%). Concomitant formation of small amounts (14%) of the permethylated PGP derivative (Me_4-PGP) was observed (Fig. 17).

Permethylation of PGP (free acid form) was best achieved by methylation with methyl iodide and silver oxide under reflux conditions, an 85% yield of pure Me_4-PGP being obtained. Me_4-PGP could also be prepared by treatment of PGP (acid form) with excess ethereal diazomethane at room temperature in the presence of a catalytic amount of boron trifluoride ethereate. However, low yields (63%) of product were obtained as a result of Lewis acid-catalysed cleavage of the glycerol diether-phosphate linkage. Attempts to permethylate PGP (acid form) at room temperature

with methyl iodide-sodium hydride were not successful, because of rapid base-catalysed cleavage of the phosphodiester bonds. The reaction mixture after 4 hr was shown by TLC examination to contain both 1-O-methyl-2,3-di-O-phytanyl-sn-glycerol and phosphatidic acid dimethyl ester. Similarly, permethylation of Me_3-PGP with methyl iodide-silver oxide was not satisfactory because of extensive breakdown of the phosphodiester linkage.

Me_3-PGP was found to be much less stable than Me_4-PGP, the former showing decomposition when kept in chloroform solution at 5° for a week, while the latter remained unchanged for long periods under the same conditions. This behaviour is in keeping with the observation that the methyl esters of PG-1-S, PG-2-S and PG are unstable both in chloroform solution and in the pure dry state.

The infrared spectra of both Me_3-PGP and Me_4-PGP (Figs. 24C, 24D respectively) are consistent with those expected for methylated derivatives of PGP structure 1. Both spectra showed strong absorption bands for phytanyl groups (2950, 2920, 2860, 1455, 1375-1365 cm^{-1}), an unbonded triester P=O band (1275 cm^{-1}), cf. the hydrogen-bonded P=O band at 1210 cm^{-1} in the spectrum of K_2-PGP (Fig. 24B), and a P-O-C band (1040 cm^{-1}). Absorption bands for ionised P-O^- (1095 cm^{-1}) and bound water (1650 cm^{-1}) were absent in both spectra. No pyrophosphate absorption band was apparent. The spectrum of Me_3-PGP showed hydroxyl absorption at 3380 cm^{-1}. A pronounced band was present at 840 cm^{-1} in the spectra of both esters, but it was absent in the free acid and potassium salt spectra (Figs. 24A, 24B respectively).

FIGURE 24

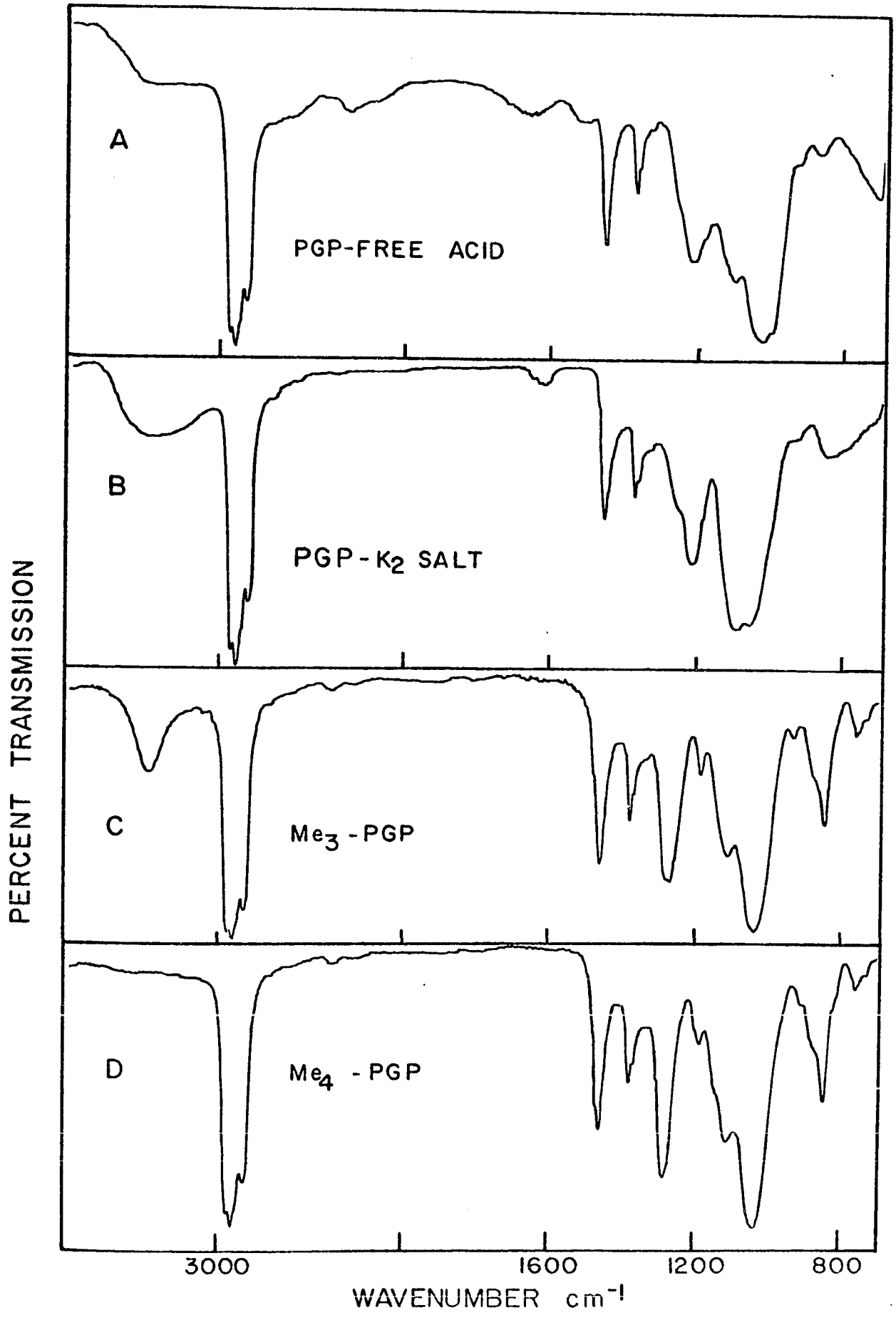
Infrared absorption spectra in CCl_4 of:

A 1-sn-phosphatidyl-3'-sn-glycero-1'-phosphoric acid

B 1-sn-phosphatidyl-3'-sn-glycero-1'-phosphate, dipotassium salt

C 1-sn-phosphatidyl-3'-sn-glycero-1'-phosphate, trimethyl ester (Me_3-PGP)

D 1-sn-phosphatidyl-3'-sn-glycero-2'-O-methyl-1'-phosphate, trimethyl ester (Me_4-PGP)



The 100 MHz NMR spectra of the methylated derivatives showed the expected signals for phytanyl group protons and glycerol derived methylene and methine protons (Fig. 25; Table 11): sharp methoxy signals at 3.72, 3.83 and 3.47 δ (p.p.m.) were present in the ^{31}P-^{1}H coupled spectrum of Me_4-PGP (Fig. 25B), but only the first two (3.73 and 3.84 δ) were present in that of Me_3-PGP (Fig. 25A). The spectra were simplified by ^{31}P spin decoupling: in each spectrum, a pair of methoxy signals collapsed to a single peak (3.80 δ for Me_3-PGP and 3.78 δ for Me_4-PGP), allowing their assignment to P-O$\underline{CH}_3$ groups. The broad multiplet at 3.99 - 4.32 δ gave rise to a narrowed multiplet at 4.06 - 4.21 δ on ^{31}P spin decoupling and is assigned to P-O$\underline{CH}_2$ groups. The signal at 3.47 δ in the spectrum of Me_4-PGP, however, was unchanged by ^{31}P spin decoupling and could thus be assigned to C-O$\underline{CH}_3$. Integration of the overall spectra and of the expanded downfield regions clearly showed the presence of three P-OCH_3 groups per molecule of Me_3-PGP and three P-OCH_3 groups plus one C-OCH_3 group per molecule of Me_4-PGP (Table 11). Since no P-O^- groups are present (infrared evidence) this indicates the presence of three ionisable hydroxyl groups attached to phosphorus per molecule of PGP.

This number of groups is compatible with the monomeric structure 1, but not with dimeric structure 2 (Fig. 3) which would require four such groups. However, the possibility existed that the bacterial PGP had the pyrophosphate structure 2 which was cleaved and converted to the monomeric Me_4-PGP during methylation with methyl iodide and silver oxide. This possibility is most unlikely, since the pyrophosphate linkage is known to be stable under mild alkaline conditions (Cornforth and Popjak, 1969), which would prevail in the permethylation procedure used. The

FIGURE 25

Nuclear magnetic resonance spectra (expanded downfield region; sweep width 250 Hz) in [^{2}H]-chloroform of:

A trimethyl ester of PGP (Me_3-PGP),

(a) ^{31}P decoupled spectrum

(b) ^{31}P-^{1}H normal spectrum

B permethylated PGP (Me_4-PGP)

(a) ^{31}P decoupled spectrum

(b) ^{31}P-^{1}H normal spectrum

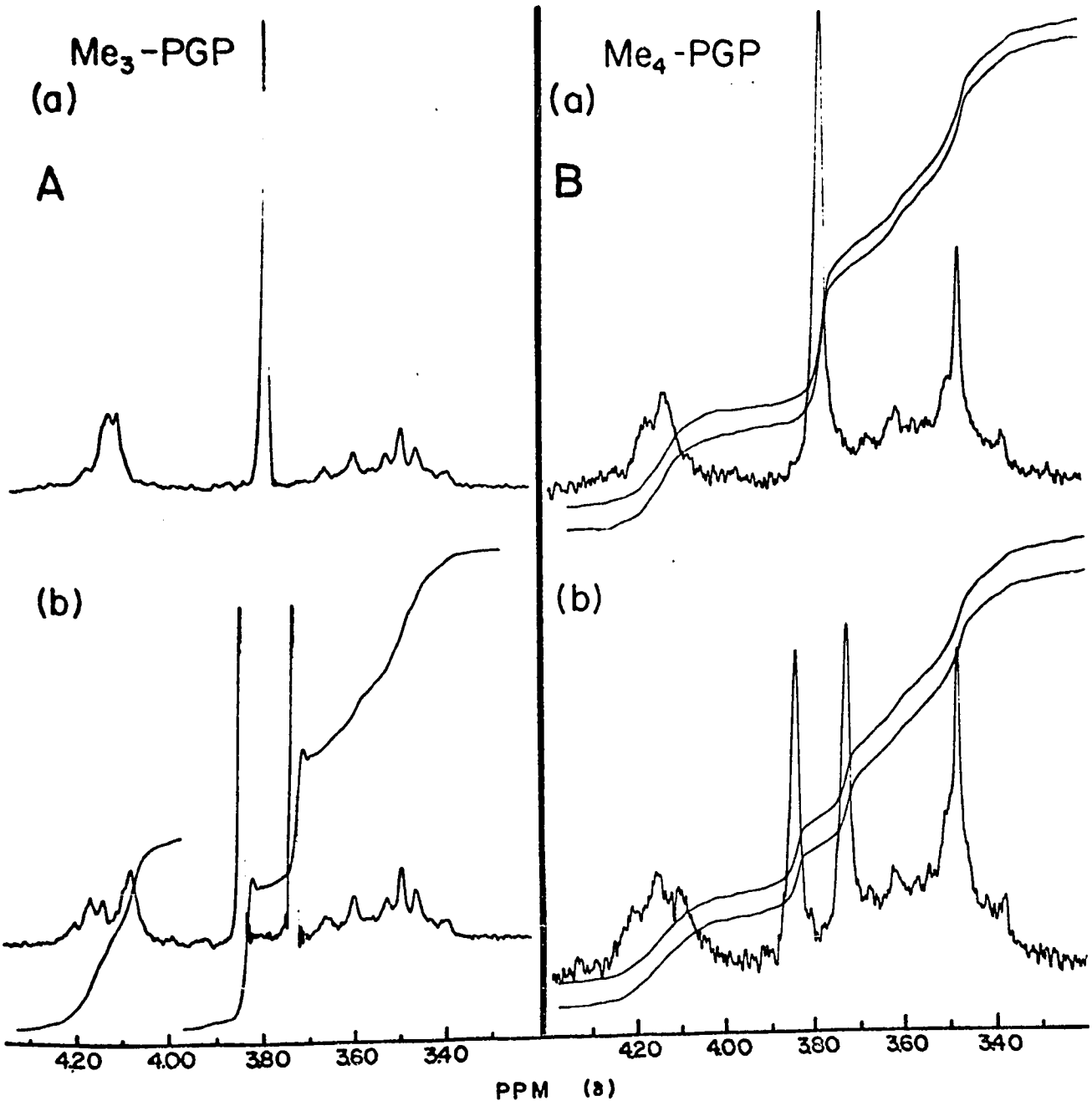

Me3-PGP
(a)
A
(b)
Me4-PGP
(a)
B
(b)
4.20
4.00
3.80
3.60
3.40
420
400
380
360
340
PPM (δ)

TABLE II

Chemical Shifts and Assignments of Proton Resonance Signals for Methylated Phosphatidyl Glycerophosphates

Group		Chemical Shift (δ) p.p.m.	Coupling Constant J_{P-H}(Hz)	No. of Protons	
				Calc.	Found
Me_3-PGP					
P-O$\underline{CH}_3$	coupled	3.73, 3.84	11.3	9	9.3
	decoupled	3.80			
P-O$\underline{CH}_2$	coupled	3.98-4.28			
	decoupled	4.07-4.22		6	6.4
$C_{20}H_{41}$-O$\underline{CH}_2$ $C_{20}H_{41}$-O$\underline{CH}$ HO-$\underline{CH}$ $C_{19}H_{39}\underline{CH}_2$		3.37-3.71		8	7.3
2x($C_{19}\underline{H}_{39}CH_2$)		0.72-1.80		78	78*
Me_4-PGP					
P-O$\underline{CH}_3$	coupled	3.72, 3.83	11.3	9	8.5
	decoupled	3.78			
P-O$\underline{CH}_2$	coupled	3.99-4.32		6	5.3
	decoupled	4.06-4.21			
C-O$\underline{CH}_3$	coupled	3.47		3	**
	decoupled	3.47			
$C_{20}H_{41}$-O$\underline{CH}_2$ $C_{20}H_{41}$-O$\underline{CH}$ $\underline{CH}_3$-O$\underline{CH}$ $C_{19}H_{39}\underline{CH}_2$		3.36-3.71		11	12.2
2x($C_{19}\underline{H}_{39}CH_2$)		0.67-1.70		78	78*

* 78 phytanyl protons per molecule taken as integral calibration standard

** C-O$\underline{CH}_3$ included in δ 3.36-3.71 integral

possibility of cleavage of a pyrophosphate bond by diazometholysis (Baer and Maurukas, 1955; Crone, 1964; Renkonen, 1968) in the diazomethane methylation procedure should also be considered. However, this possibility can be ruled out since exposure to diazomethane was very brief, relative to the long periods required for diazometholysis of glycerophosphatides. Furthermore, the dipotassium salt of the bacterial PGP could be recovered unchanged even after overnight treatment with diazomethane in chloroform-diethyl ether (1:1, v/v). Thus, if the bacterial PGP existed as the pyrophosphate structure 2, it would not have been cleaved during the diazomethane treatment and analysis of the methyl ester product would have indicated a ratio of P-OCH_3/P of 1:1, rather than the observed value of 3:2.

The quantitative estimation of methoxyl groups both by NMR and by analytical means gives strong support to the monomeric phosphatidyl glycerophosphate structure 1.

2. Osmometric Molecular Weight Values of Methylated PGP Derivatives (Me_3-PGP and Me_4-PGP)

The apparent molecular weight values determined by vapour pressure osmometry for the Me_3-PGP and Me_4-PGP derivatives and for the free acid form of PGP (Table 12) also gave support to monomeric structure 1. The duplicate values were obtained (using different instruments) for the samples whose elemental analyses are given in Experimental Procedures. The values vary by a maximum of about 6%.

TABLE 12

Apparent Molecular Weight determined by Vapour Pressure Osmometry for the Free Acid Form, Potassium Salts and Methylated Derivatives of Phosphatidyl Glycerophosphates[a]

PGP Derivative	Calculated Molecular Weight	Osmometric Molecular Weight[b]
PGP free acid[c]	905.2	1011
Me_3-PGP	929.2	845, 883
Me_4-PGP	943.3	981, 970
K-PGP[c]	943.3	2861
K_2-PGP[c]	981.4	8099
K_3-PGP[c]	1020	> 20000

a Diphytanyl ether analogue

b In chloroform solution

c Monohydrate

The maximum variation in two independent measurements was ±5%. These determinations thus appear to be a reliable assessment of molecular weight for such fully-esterified phosphatides. In contrast, however, osmometric molecular weight measurements for ionised (salt) forms of PGP invariably gave high values, which were markedly dependent on the mole ratio of cation/P in the lipid sample (Table 12). It follows that osmometric molecular weight determinations of ionised salt forms cannot be used directly in structure determination of acidic lipids.

3. Cation/P Ratios of PGP Salts

Hypothetically, the pyrophosphate structure 2 (Fig. 3) is capable of forming a series of monovalent cation salts having cation/P ratios of 1.0. The monovalent structure 1, however, should be capable of binding more cation and attaining a cation/P ratio of 1.5.

The cation/P ratios determined for a series of PGP salts are recorded in Table 13. The salts of monovalent cations which were obtained by base-titration of the acid form of PGP to the phenolphthalein end-point gave a ratio of unity. The divalent cation salts which were prepared by displacement reactions gave a mole ratio of 0.5. The barium salt prepared by two methods (see Experimental Procedures, II.4), gave the same ratio, although the analyses reported previously for the barium salt (Joo and Kates, 1969) gave a ratio of 0.75. Treatment of the free acid form of PGP with 1.5 equivalents of KOH per equivalent of P gave a tripotassium salt (K/P ratio = 1.5). Evidently, salts containing three equivalents of cation per mole are present in the natural total polar lipid mixture and in pure components isolated from these lipids, because their total cation/P ratio is invariably 1.2-1.3 (Table 14 and Experimental Procedures, II.1).

TABLE 13

Cation/P Atomic Ratios for Salts of Phosphatidyl Glycerophosphate

PGP Salt Form	Cation/P Atomic Ratio	
	Found[a]	Calc.
$(NH_4)_2[C_{46}H_{94}O_{11}P_2].H_2O$	0.88[b]	1.00
$Li_2[C_{46}H_{94}O_{11}P_2].H_2O$	1.03	1.00
$Na_2[C_{46}H_{94}O_{11}P_2].H_2O$	1.07[c]	1.00
$K[C_{46}H_{95}O_{11}P_2].H_2O$	0.59	0.50
$K_2[C_{46}H_{94}O_{11}P_2].H_2O$	1.07	1.00
$K_3[C_{46}H_{93}O_{11}P_2].H_2O$	1.48	1.50
$Mg[C_{46}H_{94}O_{11}P_2]$	0.54	0.50
$Ca[C_{46}H_{94}O_{11}P_2]$	0.51	0.50
$Ba[C_{46}H_{94}O_{11}P_2]$[d]	0.49	0.50
$Ba[C_{46}H_{94}O_{11}P_2]$[e]	0.48	0.50

a For elemental analyses see Table 4

b From Dumas-N analysis

c Corrected for glassware Na-blank

d Prepared using barium acetate method

e Prepared using barium chloride method

TABLE 14

Exchange of Cations bound to Phosphatidyl Glycerophosphate after Column Chromatography or TLC on Silicic Acid[a]

Compound	Equivalent cation/P ratio found				
	K/P	Na/P	Mg/P	NH_4/P	Total cation/P
PGP-K_2					
Before column chromatography	1.04	-	-	-	1.04
After column chromatography	0.16	0.33	0.32	-	0.81
PGP-"natural salt"					
Before TLC[b]	0.15	0.75	0.32	-	1.22
After TLC	0.03	0.19	0.70	0.30	1.22

a "Unisil" silicic acid (1.0 g) washed with chloroform, 20 mg of K_2-PGP applied, and elution done with chloroform-methanol (1:1, v/v) followed by pure methanol.
TLC was done on silica gel H in solvent chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v)

b Data from analysis of total lipids which is assumed to be representational of the major component PGP

These analytical results (Table 13) agree with those found earlier by Kates and co-workers (1965) in showing that the monovalent cation salts obtained by titration to the phenolphthalein end-point have a cation/P ratio of 1.0. The results thus appear to support dimeric structure 2 as suggested by Faure et al.(1963). A similar argument holds for the divalent salt series. Structure 2 should give a maximum divalent-cation/P atomic ratio of 0.5, while structure 1 should in theory give a ratio of 1.5. The observed ratio for these salts (0.5) again appears to support dimeric structure 2, but does not rule out structure 1. However, the definitive result is the isolation of a tripotassium salt having a cation/P ratio of 1.5; in fact, the three possible potassium salts of monomeric PGP were isolated as analytically pure solids, namely K-PGP, K_2-PGP and K_3-PGP. Their infrared spectra (Fig. 26) show clearly the stepwise increase in $P-O^-$ absorption band intensity at 1095 cm^{-1} expected for mono-, di- and tri-ionised phosphate species.

These findings therefore are compatible with the results obtained with the methylated derivatives of PGP and confirm the choice of structure 1 for bacterial PGP. The isolation of only the part-neutralised salts, e.g. K_2-PGP, after titration to the phenolphthalein end-point, may now be explained by the fact that the pK of the third ionisable phosphate group is above the end-point pH range of this indicator (Kates et al., 1965).

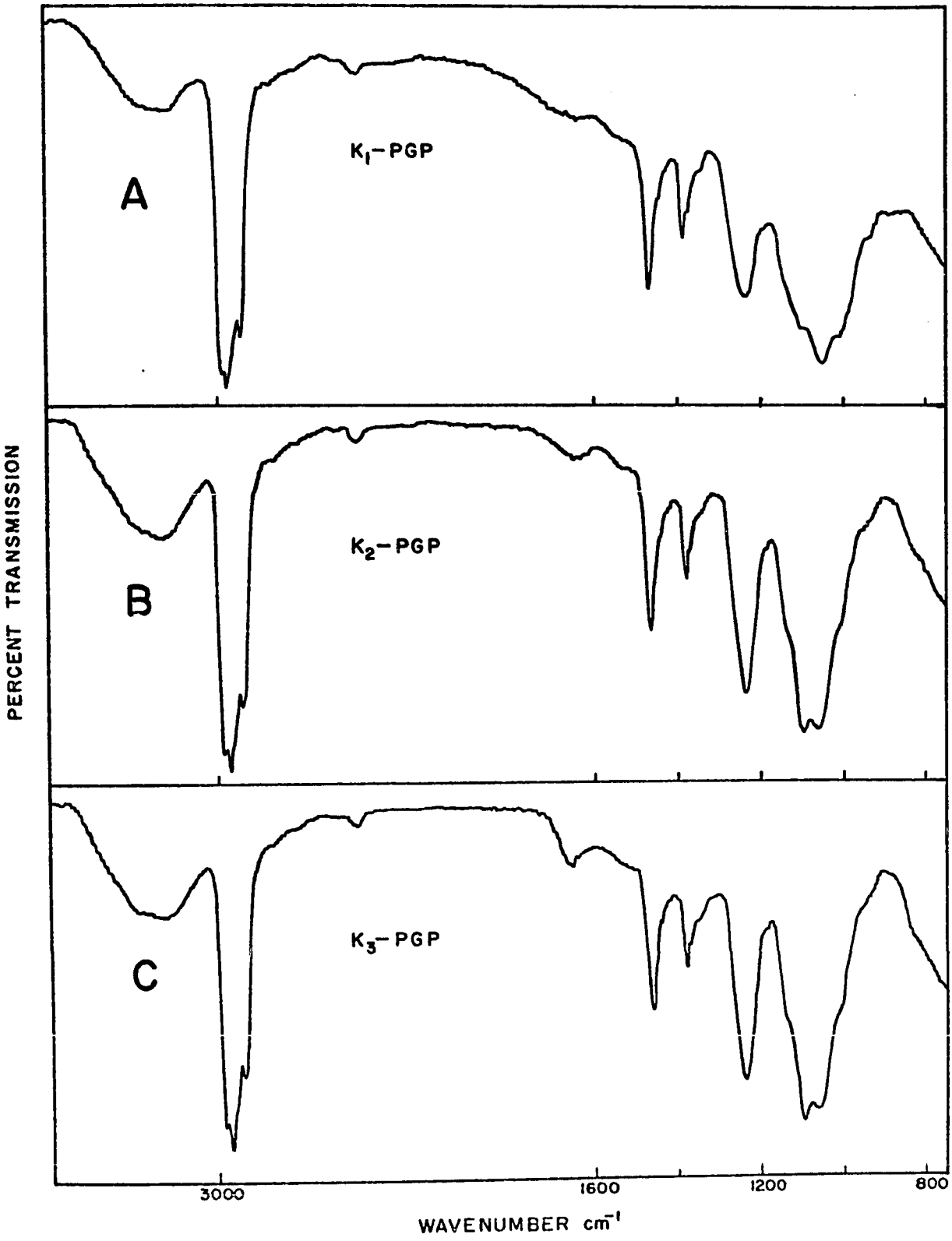
FIGURE 26

Infrared absorption spectra in CCl_4 of :

A phosphatidyl glycerophosphate, monopotassium salt

B phosphatidyl glycerophosphate, dipotassium salt

C phosphatidyl glycerophosphate, tripotassium salt



II. Physical Properties of PGP Salt Forms

The dependence of physical properties on the nature of the cation associated with PGP became increasingly apparent as the series of salts discussed in the preceding section was prepared. Variation in osmometric molecular weight has already been mentioned (Results and Discussion, I. 2); two further effects are discussed below.

2. Chromatographic Mobility

The magnesium and sodium salts of PGP were eluted separately on silicic acid columns using chloroform-methanol mixtures as eluting solvents (for details of the chromatography see Table 2 and Fig. 15). However, no separation could be achieved on TLC (silica gel H) using neutral, acid or alkaline aqueous solvents.

Earlier reports of the separation of lipids into different cation forms have come from Marinetti et al.(1958) and Rathbone (1962) who showed that phosphatidyl serine eluted as two distinct ionic forms, the sodium salt being retained longest. Renkonen (1968) showed that phosphatidic acid gave a similar elution pattern on alumina, silica and DEAE-cellulose, the calcium salt eluting before the sodium salt.

The tendency of the magnesium salt of PGP to elute before the sodium salt may reflect different degrees of inter-molecular association in the two salts, a characteristic which may be significant in membrane processes in the living cell.

Although this variation in chromatographic mobility complicates the fractionation of the acidic lipids, it should be possible to take advantage of it and find the distribution of cations among the various lipid components after lysis of the cell. Of course this would not necessarily reflect the nature of the binding sites in the living cell, because cation

exchange may occur during the cell lysis. However, the cation distribution found in the lipid components may be an indication of ion specificity by a given lipid. This approach has been used by Rayman et al. (1967) who isolated PGP exclusively as the magnesium salt after column chromatography of H. cutirubrum lipids and suggested that the lipid acted as a specific binding site for magnesium ion in the cell. However, this procedure may not be reliable; we find that chromatography of PGP samples on silica causes marked changes in their cation composition. Table 14 shows the cation exchange occurring in K_2-PGP after passage through a column of silicic acid. About 85% of the original potassium ion was exchanged for approximately equal proportions of sodium and magnesium ion. More evidence of exchange is afforded by analysis of the cation composition of PGP salt mixture after TLC in an ammoniacal solvent (Table 14). PGP showed a marked tendency to acquire magnesium ion during the TLC fractionation.

Nielsen (1971) also has reported cation exchange phenomena occurring during the chromatography of rat-liver phospholipids. He showed that the amounts of cations that could be extracted from the silica with dilute acid were compatible with the observed changes in the proportions of lipid-bound metal ions. It is clear from the results of these column exchange experiments that only silicic acid which has been rigorously freed of cations can give meaningful information about the original cation composition of lipid material. One possibility is the use of cation-free silica (e.g. acid-washed Florisil; Carroll, 1963) which should permit separation of the lipids into their salt forms that were liberated at the moment the cell was lysed in the Bligh-Dyer extraction. This may give information about the cation-binding abilities of the individual lipid components. Another modification that may be useful for preparative purposes, is the conversion of the total polar lipids to a single salt form and their subsequent chromatography.

2. Optical Rotation Measurements

The sign and magnitude of the molecular rotations of the PGP salts were dependent on the nature of the bound cation, as well as the cation/P ratio for a given cation (Table 15). No marked concentration dependence was observed in the concentration range 1.5 - 4.5 g lipid per 100 ml chloroform.

The absolute optical rotation values of the salt forms of PGP do not appear to correlate with any property of the cation involved; although it is evident that the divalent cation salts were all levorotatory and the monovalent cation salts possessed rotation values which diminished in the positive sense as the radius of the unhydrated cation decreased. However, these trends were not paralleled in a series of salt forms of PGS, where the magnesium salt was dextrorotatory and the sodium salt was levorotatory, and there seems little predictability in this phenomenon.

One consequence of this phenomenon is clear. A lipid should be converted to a single salt form before optical rotations are measured. The acid Bligh-Dyer procedure described in Materials and Methods is well suited to the interconversion of salt forms, and it also allows isolation of the free acid form of the lipid if desired. The free acid form of the lipid, in which cation effects are absent, is a suitable choice for optical rotation measurement, provided it has sufficient stability. It would not be a good choice for the phosphosulfolipid PGS whose free acid form is particularly labile. The free acid form of PGP and its methyl ester have a relatively high optical activity compared to the salts and so are useful as a further means of characterisation. In the characterisation of a new phosphatide it is thus desirable to measure the optical rotations of at least one salt, the free acid form and its methyl ester, especially for purposes of comparison with a synthetic product.

TABLE 15

Optical Rotation Values for the Free Acid Form and Various Salt Forms of Phosphatidyl Glycerophosphate[a]

Compound	$[\alpha]_D^{22}$ [b]	M_D^{22}
PGP free acid	+3.92	+34.8
K-PGP	+3.96	+36.2
K_2-PGP	+2.63	+25.3
K_3-PGP	+1.77	+17.7
Na_2-PGP	+0.70	+6.64
Li_2-PGP	0.00	0.00
$(NH_4)_2$-PGP	-0.51	-4.70
Ca-PGP	-0.68	-6.29
Mg-PGP	-1.65	-15.0
Ba-PGP	-1.65	-16.9

a Diphytanyl ether analogue

b In chloroform solution

II. Studies on Phosphatidyl Glycerosulfate

1. Isolation of PGS

Three factors made the isolation of the lipid difficult. Firstly, the component accounted for a relatively small proportion of the polar lipids (1 - 3%). Secondly, the chromatographic mobility of PGS is similar to that of PGP, the major phosphatide component (Table 3). Thirdly, PGS is extremely acid-labile when completely anhydrous, 0.0002 N HCl in tetrahydrofuran, for example, causing rapid decomposition. Even without solvent-assistance, quite low concentrations of acid were capable of promoting desulfation (the desulfation of PGS is separately discussed in a later section).

Resolution of the PGS component could be achieved by silicic acid column chromatography, the magnesium and sodium salt forms being separately eluted in a fairly pure state (see Column Chromatography of Polar Lipids, Experimental Procedures, I.2 and Fig. 15), but TLC fractionation allowed more efficient isolation of PGS.

Although the Rf values of PGS and PGP (Table 3) are quite different in an acetic acid-based solvent system, such as that of Kapoulos (1969), tailing of the PGP component inevitably resulted in contamination of PGS. In addition, the high concentration of acetic acid in the TLC solvent system was undesirable because the accumulation of acid with the product after extraction of the silica tended to promote desulfation of the lipid. This effect is particularly evident under anhydrous conditions. Prior exposure of the developed plates to ammonia gas reduced the acidity, but the ammonium acetate formed interfered with the visualisation of the lipid bands by Rhodamine 6G. The acetic acid solvent was, however, useful in analytical TLC.

The most satisfactory TLC-isolation of PGS was achieved by double development of the polar lipids in the ammoniacal solvent system (Table 1). Although the separation of the lipid components is marginal, the presence of ammonia retards the PGP component to a greater degree than the PGS component, and no tailing contamination resulted. Several elution methods were tried (Table 1); the most satisfactory recovery of lipid that involved least degradation of PGS was achieved with chloroform-methanol-diethyl ether (1:1:1, v/v) elution as described by Joo and Kates (1969).

The PGS was isolated after repeated acetone precipitation as a white deliquescent solid which resembled PGP in solubility characteristics. Both compounds are freely soluble in chloroform, benzene and tetrahydrofuran and almost insoluble in methanol and acetone. The chromatographic behaviour of PGS and PGP differed, however. PGS migrated as a single spot with similar Rf values in both acid and alkaline solvent systems on TLC, whereas PGP had markedly different mobility in these systems (Table 3).

The salt forms of PGS were stable indefinitely in the dry state at room temperature, and showed only slight degradation in chloroform solution at room temperature. However, degradation was rapid in anhydrous oxonium-ion forming solvents with traces of acid (see Desulfation of PGS, Experimental Procedures, Section III. 5). The free acid form of PGS was also very susceptible to desulfation under anhydrous conditions. The isolation of anhydrous PGS (acid form) for equivalent weight determination was therefore performed in glassware washed in 2 N NaOH solution prior to use.

2. Analytical Data

Chromatography of the PGS on silicic acid-impregnated paper revealed a single spot which fluoresced blue with Rhodamine 6G under ultraviolet light and which gave a positive phosphate reaction but gave negative tests for vicinal diol groups, unsubstituted and substituted amino and amido groups, and reducing sugars.

Elemental analysis of the natural salt mixture confirmed the presence of P and S in the molecule, their atomic ratio being 1:1 (Table 6). The levorotatory nature of this salt mixture (Table 7) suggested that a high level of sodium ion was present. The free acid form of the lipid consumed two equivalents of KOH which suggested the presence of two acidic groups per molecule and elemental analysis of the pure potassium salt obtained were consistent with those calculated for the monohydrated dipotassium salt of a sulfate ester of phosphatidyl glycerol (diphytanyl ether analogue; Structure C, Fig. 4).

3. Spectral Analysis

The infrared spectrum of the phosphosulfolipid potassium salt (Fig. 27A) resembled that of PG (diphytanyl ether analogue) and did not clearly distinguish sulfate absorption bands from phosphate bands. The sulfate band at 1250 cm^{-1} (asymmetric S=O stretch) was evident only as a shoulder on the strong P=O band (1220 cm^{-1}), but a weak S-O-C band at 840 cm^{-1} was present. The absence of carboxylate ion (and ester linkages) was shown by the absence of carbonyl absorption (1740 cm^{-1}).

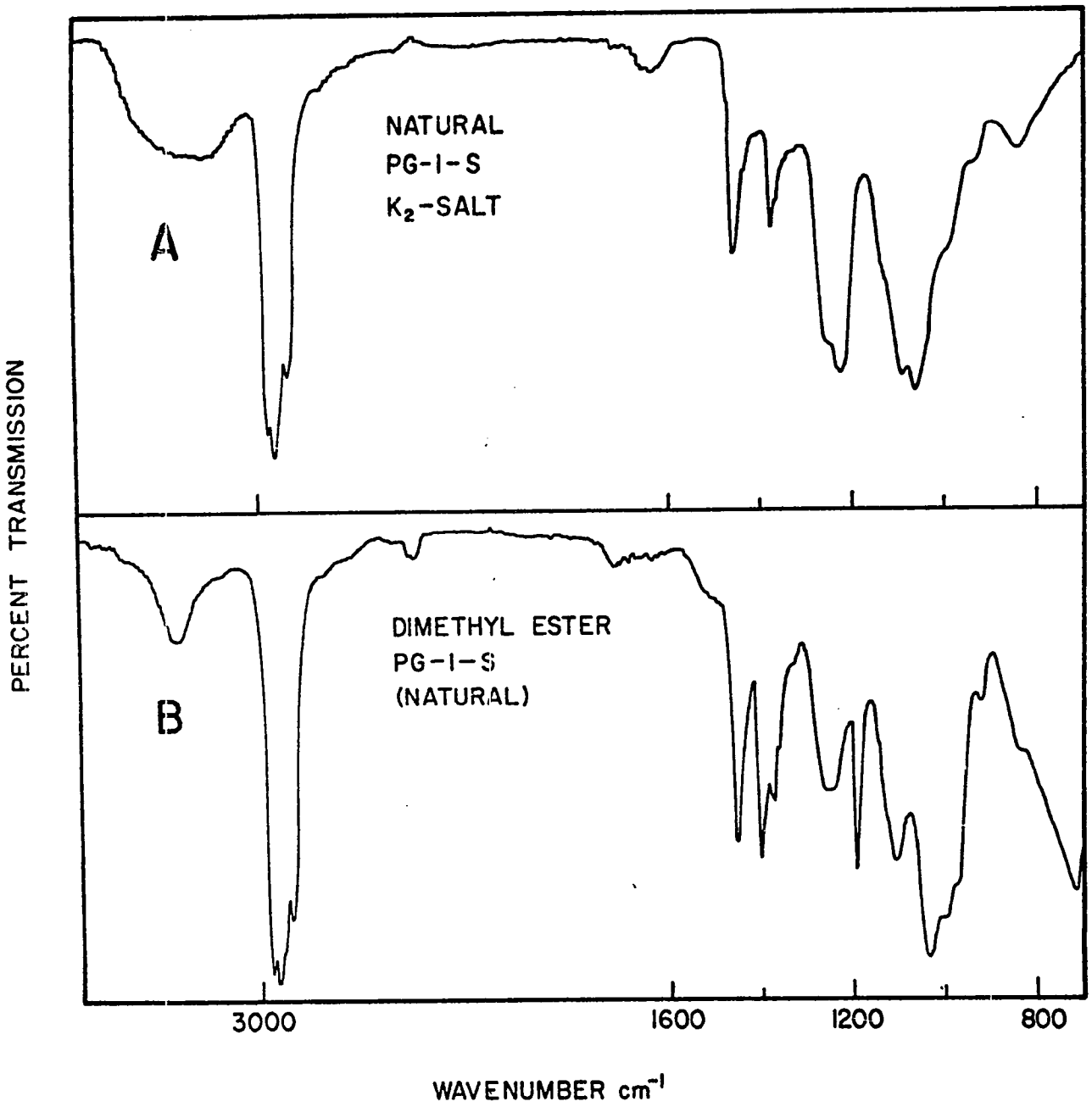
The presence of a sulfate group was revealed more clearly by the infrared spectrum of the dimethyl ester of PGS (Fig. 27B). Discrete sulfate ester absorption bands at 1198 and 1408 cm^{-1} (sharp, $-O-SO_2-O-$), and a weak absorption band at 845 cm^{-1} were evident, but little or no secondary sulfate absorption at 935 cm^{-1} (C-O-S) was apparent.

FIGURE 27

Infrared absorption spectra in CCl_4 of:

A 1-sn-phosphatidyl-3'-sn-glycero-1'-sulfate, dipotassium salt (bacterial PG-1-S)

B 1-sn-phosphatidyl-3'-sn-glycero-1'-sulfate, dimethyl ester (bacterial Me_2-PG-1-S)


NATURAL
PG-I-S
K_2-SALT
A
DIMETHYL ESTER
PG-I-S
(NATURAL)
B
PERCENT TRANSMISSION
3000
1600
1200
800
WAVENUMBER cm^{-1}

The 100 MHz NMR spectrum (Fig. 28A) of the dimethyl ester showed three methoxy ($-OCH_3$) signals (3.74, 3.86, 3.98 δ; Table 16), two of which (3.74, 3.86 δ, J_{P-H} 11.8 Hz) collapsed to a single peak (3.80 δ) when $^{31}P-^{1}H$ spin-decoupled, which allowed their assignment to $P-OCH_3$ groups (Kates and Hancock, 1971). The third signal (3.98 δ) remained unchanged during spin-decoupling and was assigned to $S-OCH_3$ groups; integration of the $P-OCH_3$ and $S-OCH_3$ signals indicated the presence of one $P-OCH_3$ and one $S-OCH_3$ group per molecule of phosphosulfolipid. The NMR spectrum of the monomethyl ester of PG (Fig. 29) also showed $P-OCH_3$ signals (3.73, 3.84 δ; Table 16) which collapsed on spin-decoupling to a single peak (3.79 δ); no signal at 3.98 δ was apparent.

4. Hydrolytic Degradation of PGS

Drastic hydrolysis of PGS (2 N aqueous HCl at 120°) liberated free glycerol, inorganic P_i, and a chloroform-soluble product (66% of the lipid weight) which was devoid of P and which had chromatographic mobility (Fig. 30), optical rotation and degradative behaviour (BCl_3 cleavage) identical with that of authentic 2,3-di-O-phytanyl-sn-glycerol. This product was also liberated by the alkaline hydrolysis procedure (Fig. 30). For the purpose of comparison, the isomeric diether 1,3-di-O-phytanyl-sn-glycerol was synthesised via 2-O-benzyl-3-O-phytanyl-sn-glycerol; it was shown to have different chromatographic mobility (Fig. 22) and optical rotation ($[\alpha]_D^{22}$ +1.1°) from the bacterial diether ($[\alpha]_D^{22}$ +8.5°).

The molar ratio of phytanyl diether:P:glycerol liberated by acid hydrolysis (0.97:1.00:1.01) was in accord with a phosphatidyl glycerol type of structure (Fig. 4A). Release of P_i under these hydrolysis conditions precluded the existence in this phosphosulfolipid of a C-P linkage which is present, for example, in the ether phospholipids of Tetrahymena pyriformis (Liang and Rosenberg, 1966).

FIGURE 28

Nuclear magnetic resonance spectra (expanded downfield region; sweep width 250 Hz) in [^{2}H]-chloroform of:

A dimethyl ester of 1-*sn*-phosphatidyl-3'-*sn*-glycero-1'-sulfate from *H. cutirubrum* (natural Me_2-PG-1-S)

a) ^{31}P decoupled spectrum

b) ^{31}P-^{1}H normal spectrum

B dimethyl ester of synthetic 1-*sn*-phosphatidyl-3'-*sn*-glycero-1'-sulfate (synthetic Me_2-PG-1-S)

a) ^{31}P decoupled spectrum

b) ^{31}P-^{1}H normal spectrum

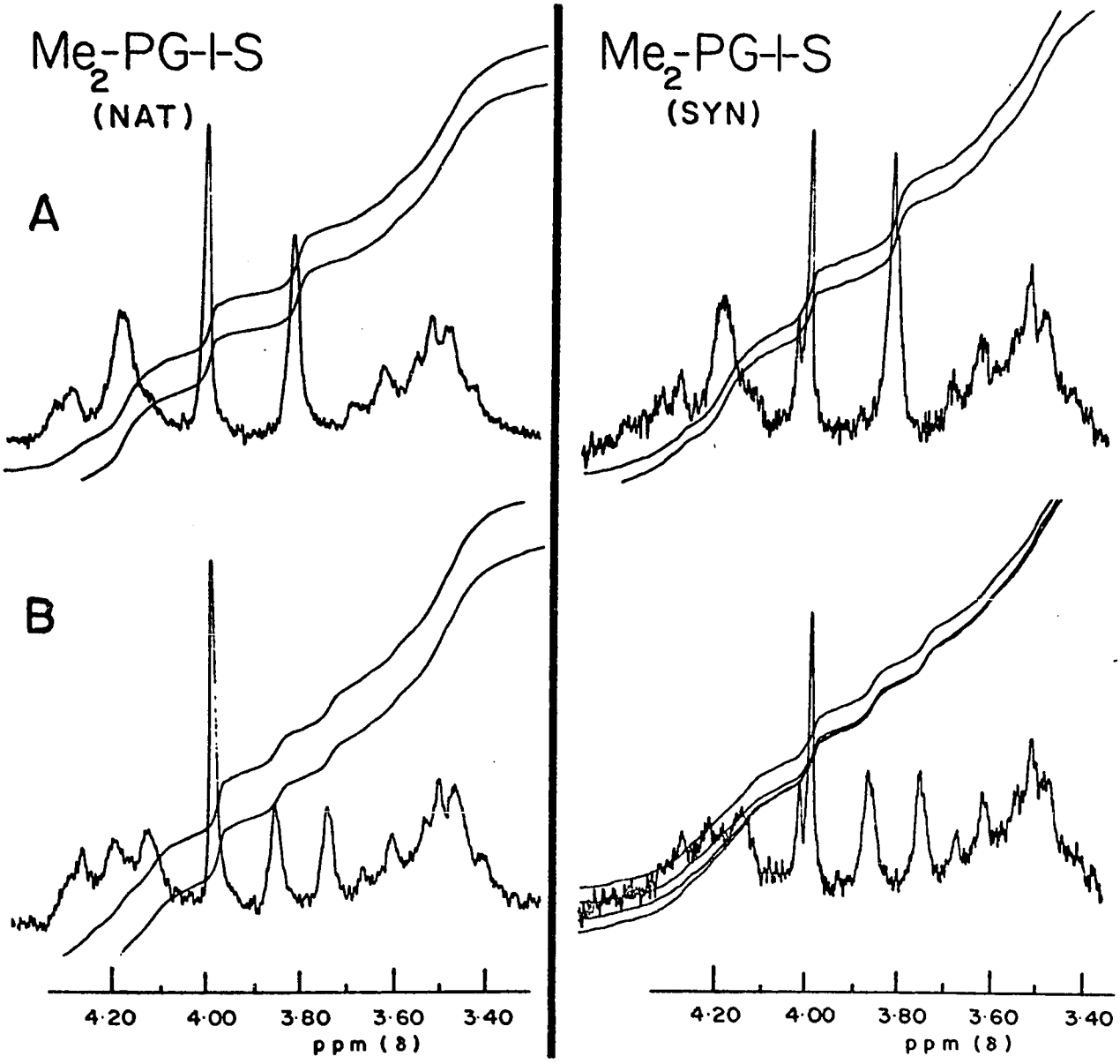

Me2-PG-I-S
(NAT)
A
B
4·20
4·00
3·80
3·60
3·40
ppm (δ)
Me2-PG-I-S
(SYN)
4·20
4·00
3·80
3·60
3·40
ppm (δ)

TABLE 16

PMR Chemical Shifts of P-OCH_3 and S-OCH_3 Groups in Methyl Esters of Phosphatidyl Glycerol and Phosphatidyl Glycerol Sulfates

	Chemical Shift (δ ppm)			
	P-OCH_3		S-OCH_3	
	$^{31}P-^{1}H$		$^{31}P-^{1}H$	
Compound*	Coupled	Uncoupled	Coupled	Uncoupled
Phosphatidyl glycerol	3.73, 3.84	3.79	-	-
Phosphatidyl glycero-1-sulfate (natural)	3.74, 3.86	3.80	3.98	3.98
Phosphatidyl glycero-1-sulfate (synthetic)	3.75, 3.86	3.80	3.98	3.98
Phosphatidyl glycero-2-sulfate	3.75, 3.86	3.81	4.01	4.02
Phosphatidyl glycero-1, 2-disulfate	3.76, 3.87	3.81	4.01, 4.04	4.01, 4.04

* All compounds are derivatives of sn-2, 3-di-O-phytanyl-sn-glycerol

FIGURE 29

Nuclear magnetic resonance spectra (expanded downfield region; sweep width 250 Hz) in [^{2}H]-chloroform of:

Dimethyl ester of 1-sn-phosphatidyl-3'-sn-glycerol

a) ^{31}P decoupled spectrum

b) ^{31}P-^{1}H normal spectrum

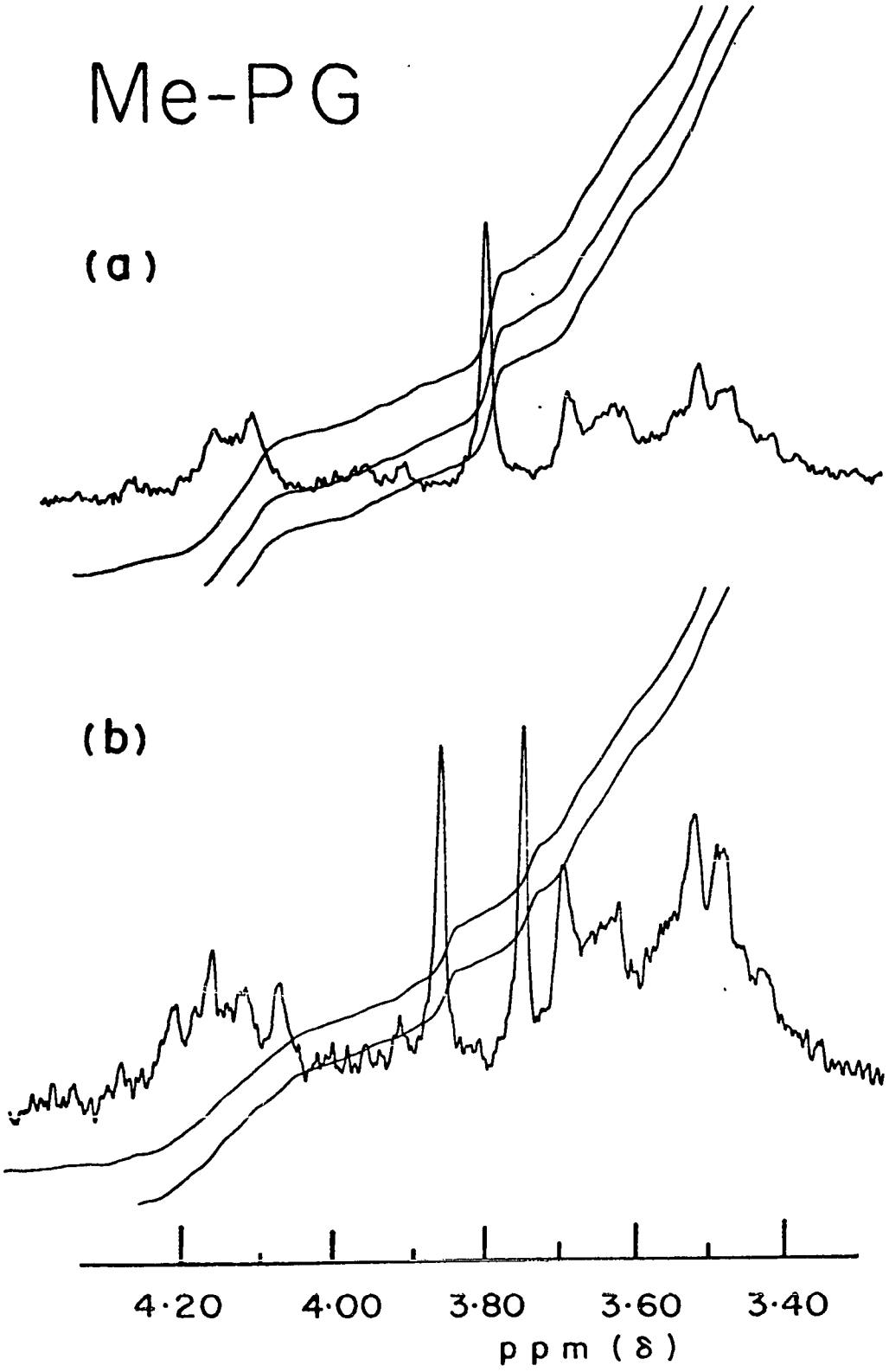

Me-PG
(a)
(b)
4·20
4·00
3·80
3·60
3·40
p p m (δ)

FIGURE 30

Thin-layer chromatogram and autoradiograph of ^{14}C-phytanyl diether obtained by acid and alkaline degradation of ^{14}C-phosphatidyl glycerosulfate.

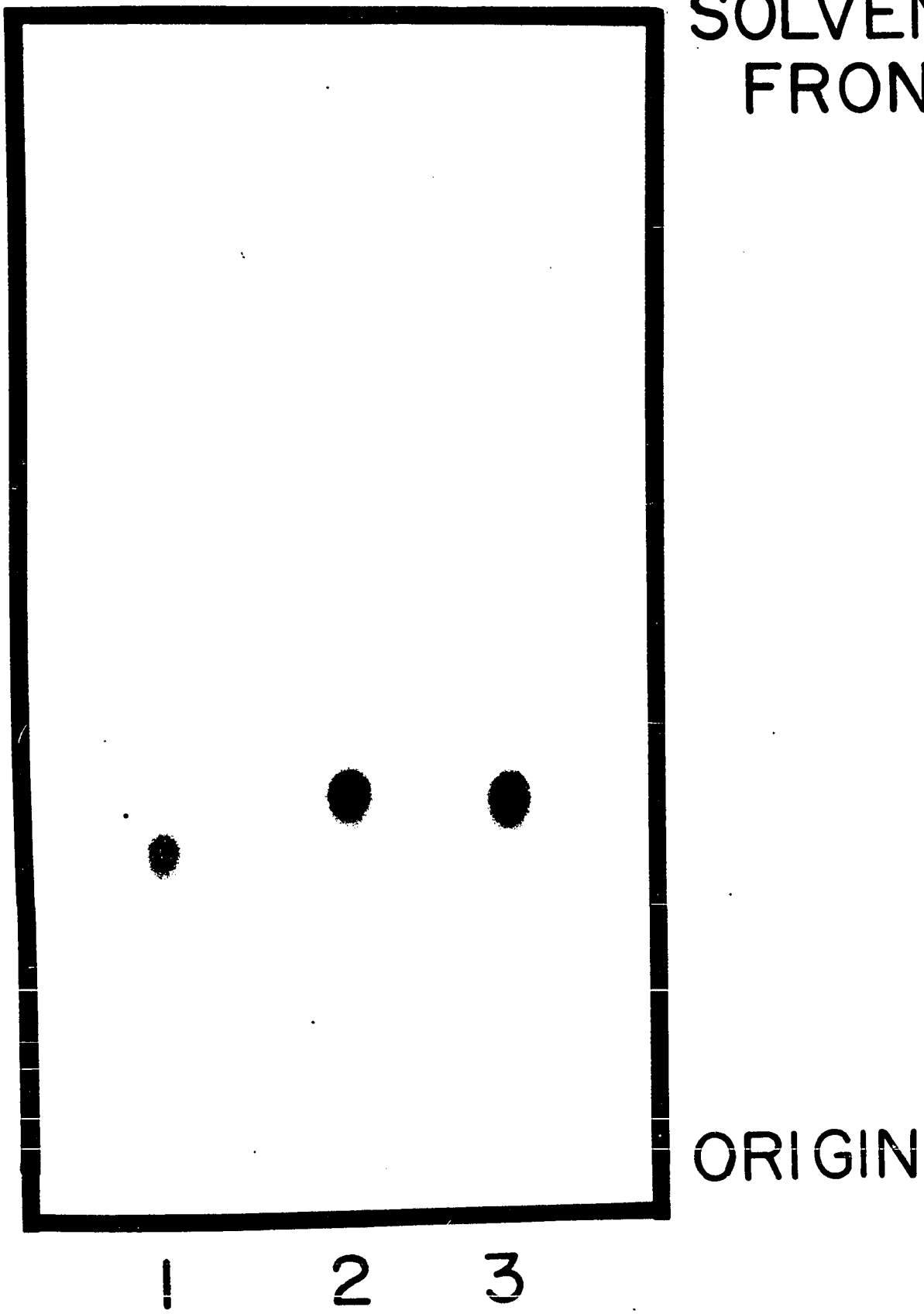
1 phytanyl diether standard

2 phytanyl diether obtained after 2.5% methanolic-HCl hydrolysis of phosphatidyl glycerosulfate

3 phytanyl diether obtained after 1.0 N methanolic KOH hydrolysis of phosphatidyl glycerosulfate

Solvent system:

chloroform-diethyl ether (20:1, v/v)


SOLVENT
FRONT
ORIGIN
1
2
3

The chloroform-soluble hydrolysis product (phytanyl diether) was accompanied by traces of material which chromatographed with mnno-phytanyl glycerol. The formation of this product has been observed during hydrolysis of total polar lipids of H. cutirubrum (Joo et al., 1968) and Kates et al.(1971) showed that it arose from breakdown of phytanyl ether linkages during hydrolysis. It now seems unlikely that a monophytanyl phosphatide analogous to a lysophosphatide is present in the lipids of H. cutirubrum.

Further evidence for a PG-type structure was obtained by methanolysis of the intact lipid (2.5% methanolic-HCl, 65°, 3 hr; Scheme 1) which liberated phytanyl diether and a water-soluble product which contained all of the lipid P in an organically bound form. This phosphate ester, after hydrolysis in 1.0 N HCl at 100° for 1 hr to cleave methyl esters, had identical chromatographic mobility to that of glycerophosphate in three solvent systems (Fig. 31) and it released free glycerol as the sole non-ionic product on treatment with alkaline phosphatase (Fig. 32).

5. Desulfation of PGS

Direct chemical evidence for the presence of a sulfate ester was obtained by subjecting the potassium salt of the phosphosulfolipid to mild acid-catalysed solvolysis in anhydrous tetrahydrofuran-HCl (0.005M) at room temperature. In accord with the reported acid-lability of sulfate esters in the presence of oxonium ion-forming anhydrous solvents (Goren, 1971; Haines, 1971), the phosphosulfolipid (K salt) was quantitatively desulfated within a few minutes. The rate of desulfation was dependent on the nature of the cation form of PGS; the "natural" salt mixture (Mg, Na, NH_4 salts) required longer times (up to 2 hr; Fig. 33) than did the potassium salt for complete desulfation. The acid-catalysed solvolysis was fully inhibited in the presence of water (ca. 10%) but not in the presence of

FIGURE 31

Paper chromatography and autoradiograph of the water-soluble acid degradation products of ^{14}C, ^{32}P-phosphatidyl glycerosulfate, ^{32}P-phosphatidyl glycerophosphate and ^{32}P-phosphatidyl glycerol.

1 ^{32}P-α-glycerophosphate standard

2, 5 and 6 methanol-water phases after Bligh-Dyer partition of acid hydrolysates from phosphatidyl glycerosulfate, phosphatidyl glycerol and phosphatidyl glycerophosphate respectively

3 ^{14}C-glycerol standard

4 ^{32}P-orthophosphate standard

7 1, 3-glycerol diphosphate standard

8 α-glycerophosphate standard

Solvent system:

n-butanol-acetic acid-water (5:3:1, v/v)

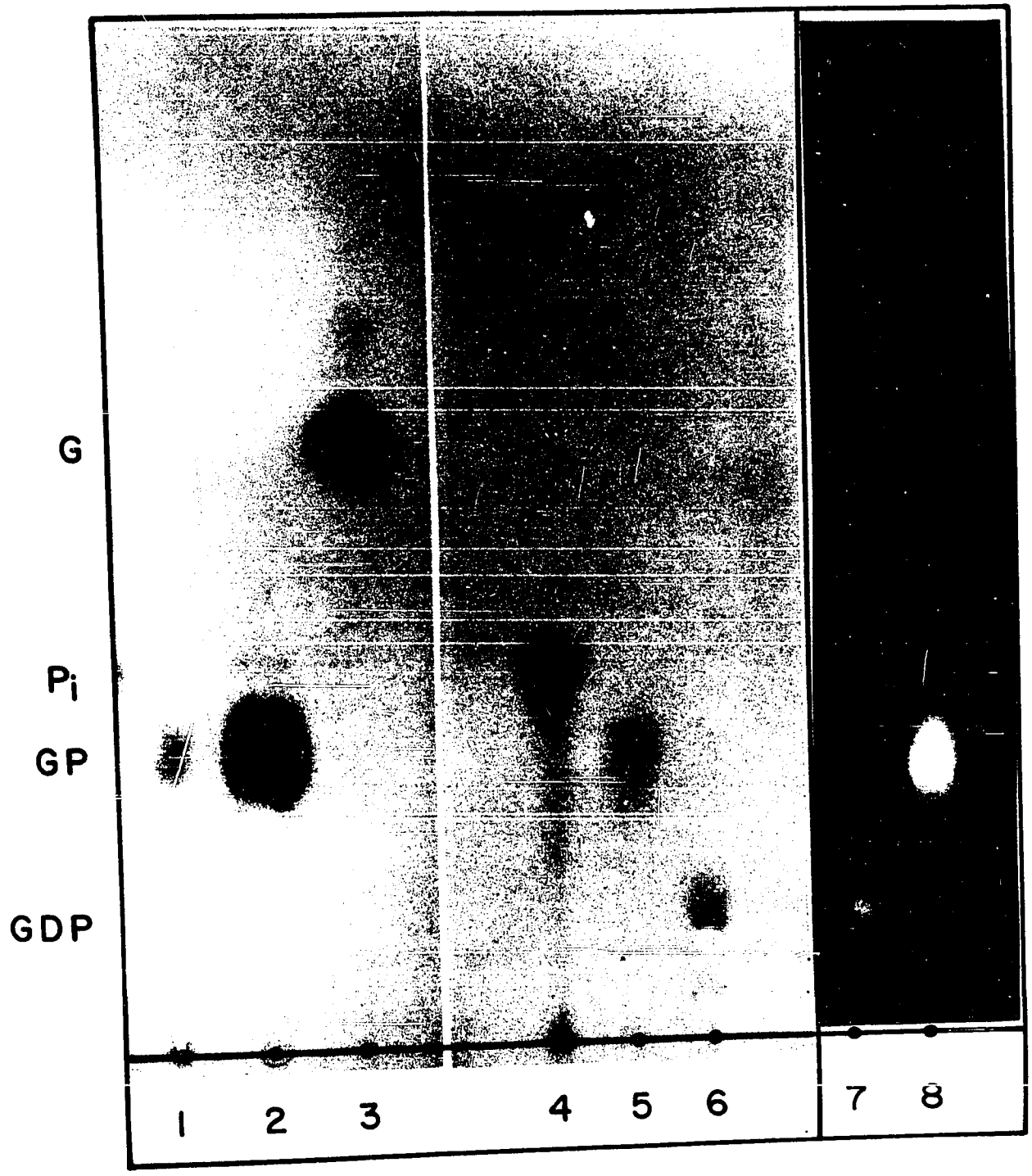

G
Pi
GP
GDP
1
2
3
4
5
6
7
8

FIGURE 32

Paper chromatogram and autoradiograph of ^{14}C-glycerol derived from both glycerol moieties of ^{14}C-phosphatidyl glycerosulfate

1 labelled product of alkaline phosphatase cleavage of ^{14}C-glycerophosphate derived from ^{14}C-phosphatidyl glycerosulfate

2 same as (1), using ^{14}C-phosphatidyl glycerophosphate as the source of glycerol

3 water-soluble product of BCl_3 cleavage of ^{14}C-phytanyl diether derived from ^{14}C-phosphatidyl glycerosulfate

4 ^{14}C-glycerol standard

Solvent system:

n-butanol-acetic acid-water (5:3:1, v/v)

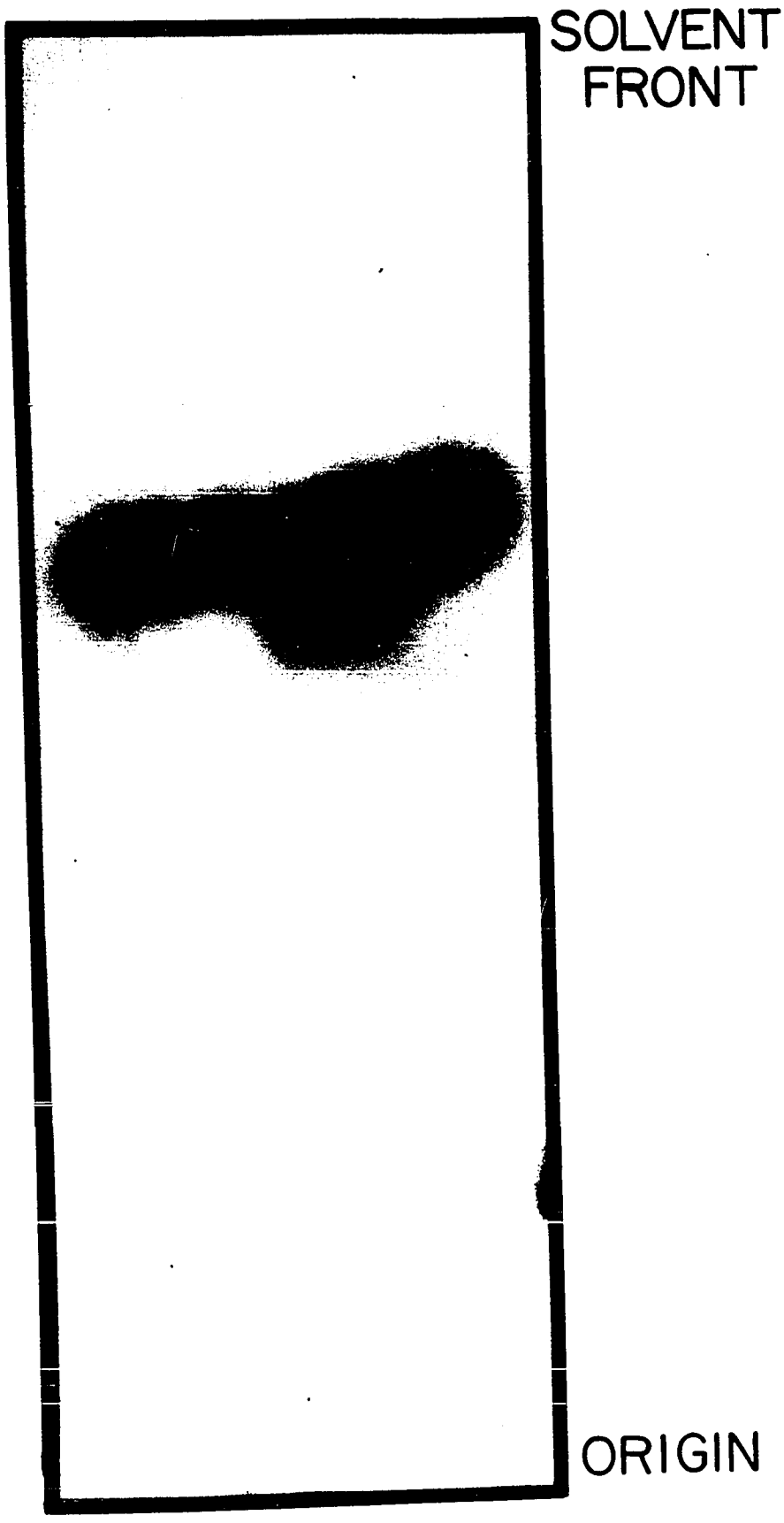

SOLVENT
FRONT
ORIGIN
1 2 3 4

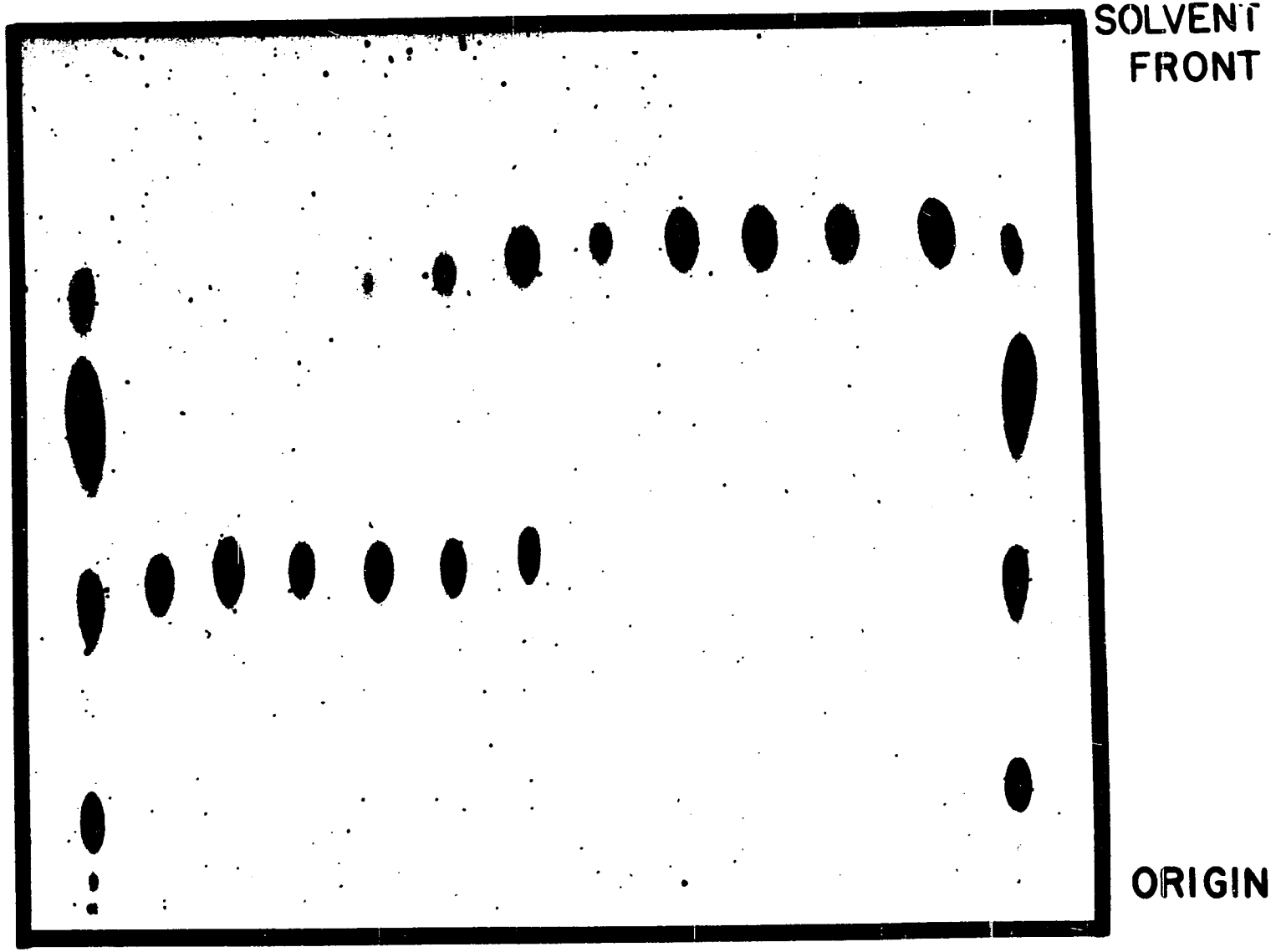
FIGURE 33

Thin-layer chromatogram showing a time-course study of the acid-catalysed solvolysis of phosphatidyl glycerosulfate in tetrahydrofuran-HCl

Solvent system:

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

The "natural" salt form of phosphatidyl glycerosulfate (a mixture of sodium, magnesium and ammonium salts was used and the acid concentration was 0.005 M).


SOLVENT FRONT
ORIGIN
TL PGS
PG TL
1 2 4 8 16 32 64 128 256
MINUTES

methanol (ca. 10%) (Fig. 34). The lipid product of solvolysis was identical with the bacterial PG (2,3-di-O-phytanyl-sn-glycero-1-phosphoryl-3'-sn-glycerol) with respect to chromatographic mobility (Fig. 33), positive staining with periodate-Schiff reagent, analytical data and specific rotation. Since the solvolysis mechanism involves retention of configuration (Haines, 1971) these results show that the phosphosulfolipid is a sulfate ester of 1-sn-phosphatidyl-3'-sn-glycerol, but have no bearing on the position of sulfation in the lipid.

6. Synthetic Studies

Final confirmation of a PG sulfate ester structure was obtained by direct monosulfation of the bacterial diether PG with one equivalent of SO_3-pyridine complex at room temperature (Scheme 2); the product obtained (K salt) was identical with bacterial PGS with respect to chromatographic mobility (Fig. 18), analytical data (Table 6), optical rotation, the infrared spectra of the potassium salt and methyl ester (Fig. 35) and the NMR spectrum of methyl ester (Fig. 28; Table 16).

Although sulfation at the 1-position most likely predominated, the possibility remained that sulfation of natural PG gave a mixture of 1- and 2-monosulfates. Indeed, the NMR spectrum of the methyl ester of the synthetic monosulfated PG obtained (Fig. 28; Table 16) shows, in addition to the major S-OCH_3 signal at 3.98 δ, a minor signal at 4.01 δ which is attributable to the -OCH_3 group of a secondary sulfate. Evidence for this discrimination between primary and secondary sulfate -OCH_3 signals was afforded by the NMR spectrum (Fig. 36; Table 16) of the trimethyl ester of PG-1,2-disulfate which was synthesised by sulfation of

FIGURE 34

Thin-layer chromatogram showing the effect on the acid-catalysed solvolysis of phosphatidyl glycerosulfate in tetrahydrofuran of (a) change in acid concentration and (b) the presence of hydroxylic solvents

TL total polar lipids of H. cutirubrum

1, 8 phosphatidyl glycerosulfate and phosphatidyl glycerol reference standards, respectively

2-7 incubation of 300 μg samples of phosphatidyl glycerosulfate for 12 hr in tetrahydrofuran-HCl under the following conditions:

2, 5×10^{-4} M THF-HCl

3, 1×10^{-3} M THF-HCl

4, 2×10^{-4} M THF-HCl

5, 4×10^{-5} M THF-HCl

6, 5×10^{-3} M THF-HCl + 10% methanol

7, 5×10^{-3} M THF-HCl + 10% water

Solvent system:

chloroform-methanol-9.0% acetic acid (30:4:20 , v/v)

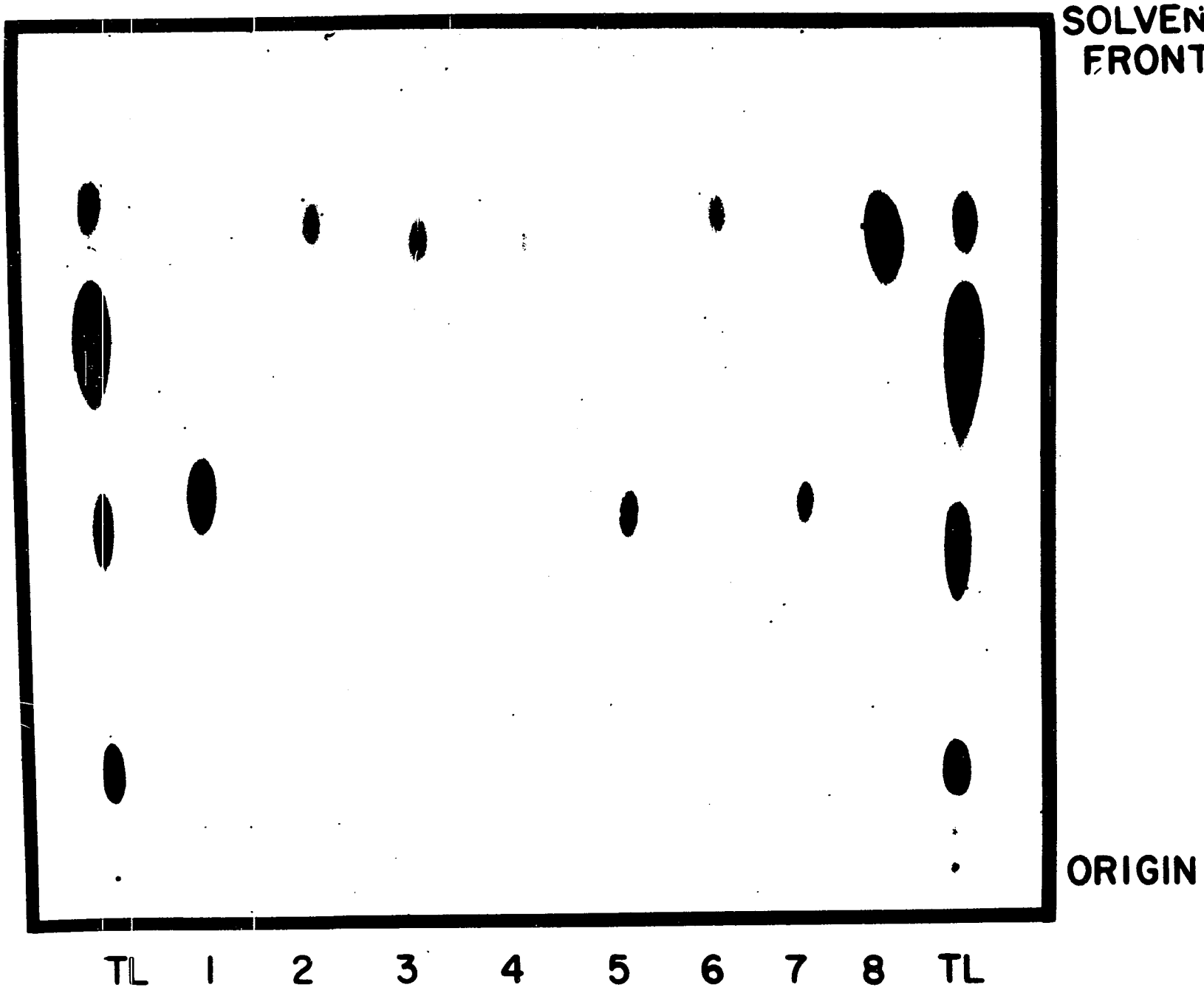

SOLVENT FRONT
ORIGIN
TL
1
2
3
4
5
6
7
8
TL

FIGURE 35

Infrared Absorption Spectra in CCl_4 of :

A 1-sn-phosphatidyl-3'-sn-glycero-1'-sulfate, dimethyl ester (bacterial Me_2-PG-1-S)

B 1-sn-phosphatidyl-3'-sn-glycero-1'-sulfate, dimethyl ester (synthetic Me_2-PG-1-S)

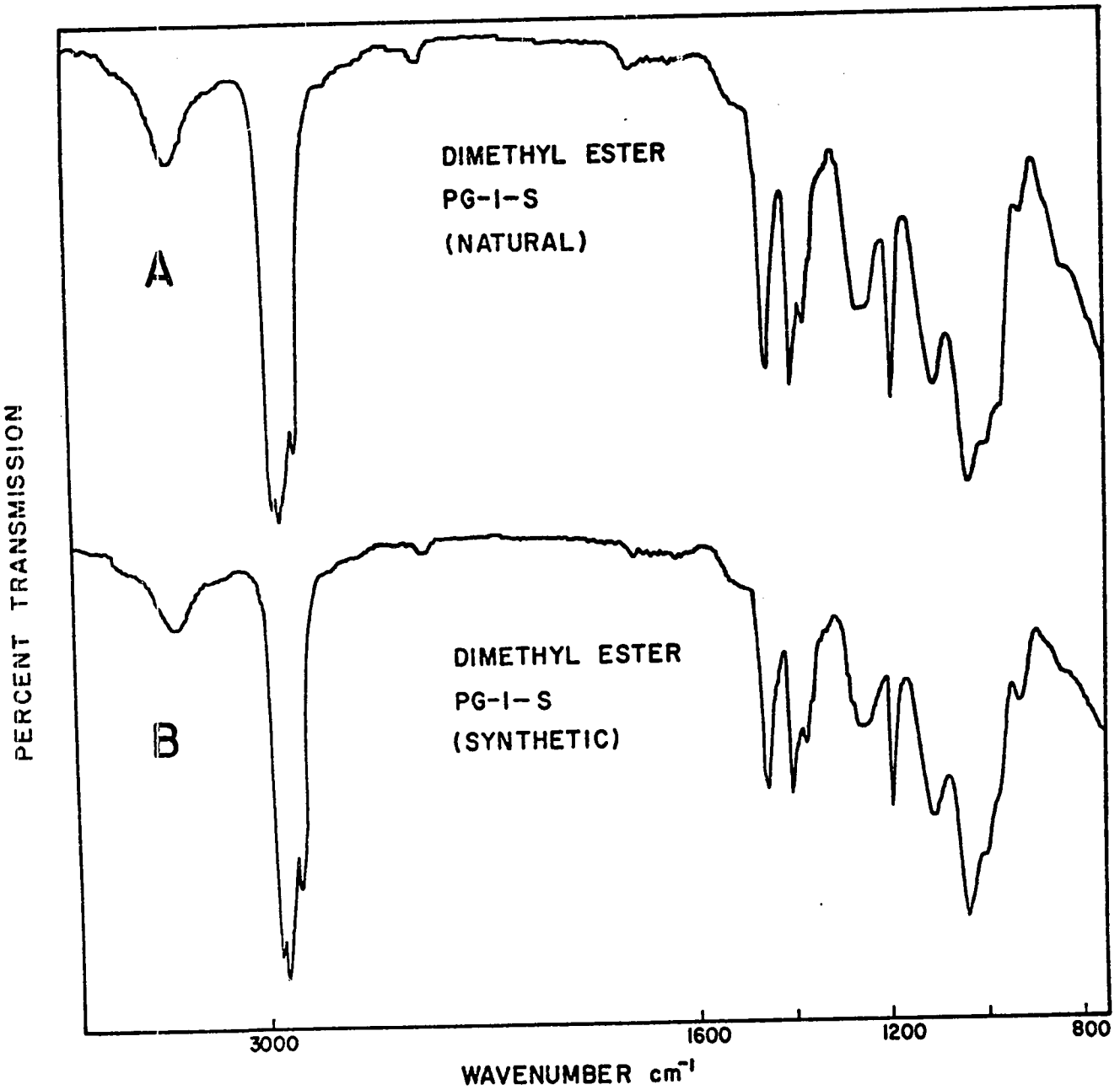

DIMETHYL ESTER
PG-I-S
(NATURAL)
A
DIMETHYL ESTER
PG-I-S
(SYNTHETIC)
B
PERCENT TRANSMISSION
3000
1600
1200
800
WAVENUMBER cm⁻¹

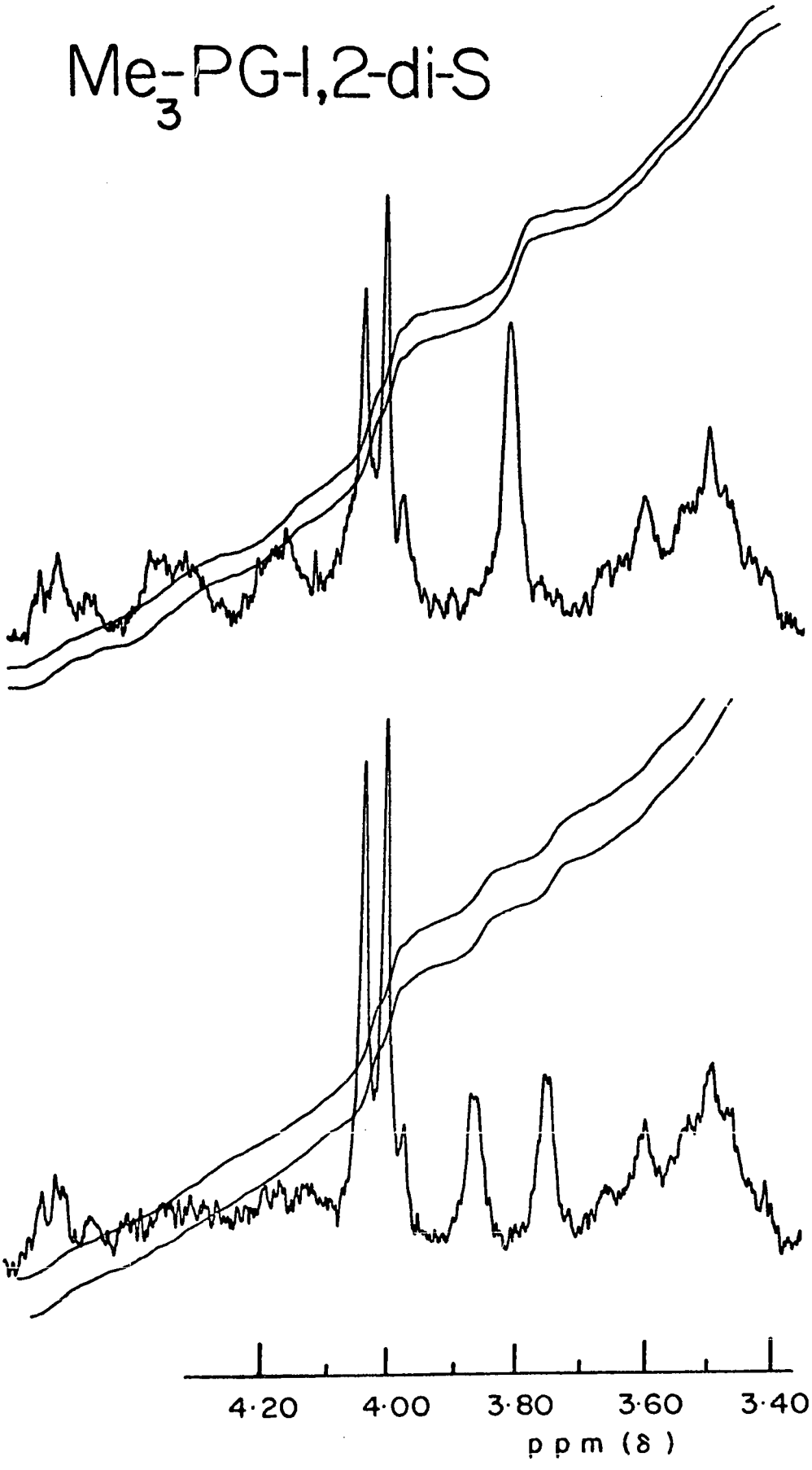
FIGURE 36

Nuclear magnetic resonance spectra (expanded downfield region; sweep width 250 Hz) in [^{2}H]-chloroform of:

Trimethyl ester of 1-sn-phosphatidyl-sn-3-glycero-1, 2-disulfate (Me_3-PG-1, 2-di-S)

a) ^{31}P decoupled spectrum

b) ^{31}P-^1H normal spectrum


Me3-PG-1,2-di-S
4·20
4·00
3·80
3·60
3·40
ppm (δ)

natural PG or PGS with excess SO_3-pyridine complex at 60° (Scheme 2). The NMR spectrum showed two sharp S-OCH_3 signals (4.01, 4.04 σ) which were not ^{31}P coupled, showing that signals from glycerol primary and secondary sulfate -OCH_3 groups differ in chemical shift by 3 Hz.

To establish the assignment for a primary sulfate -OCH_3 signal unambiguously, the NMR spectrum of the methyl ester of the authentic β-sulfate of PG (PG-2-S), synthesised as shown in Scheme 3, was examined. The S-OCH_3 signal at 4.01 σ observed (Fig. 37B and Table 16) could now be attributed to the secondary sulfate -OCH_3 group. This assignment established the S-OCH_3 signal at 3.98 σ in the NMR spectrum of the methyl esters of bacterial PGS and the synthetic monosulfated PG, as that of a primary sulfate group. Interestingly, it has recently been shown in this laboratory that the S-OCH_3 group signal in permethylated glycolipid sulfate (SL) is also at 4.01 σ (Kates and Deroo, 1972). Earlier studies have already shown that this sulfate moiety is secondary, being at the C-3 position of galactose.

In addition, the infrared spectrum of the methyl ester of PG-2-S (Fig. 38B) differed from that of the methyl ester derivative of natural PGS (Fig. 27B) in having a strong absorption band at 935 cm^{-1}. This lends support to the contention that the natural substance, in whose methyl ester spectrum absorption at 935 cm^{-1} is very weak, has a primary sulfate structure. Haines (1971) has observed that of the sulfate infrared absorption modes, the C-O-S stretching vibration mode is the most sensitive to the difference between primary and secondary sulfates, the former generally being above 990 cm^{-1} and the latter characteristically at 935 cm^{-1}.

These results thus unambiguously establish the structure of the bacterial phosphosulfolipid as 2,3-di-O-(3'7'11'15'-tetramethylhexadecyl)-sn-glycero-1-phosphoryl-3''-sn-glycero-1''-sulfate (Fig. 4C).

FIGURE 37

Nuclear magnetic resonance spectra (expanded downfield region; sweep width 250 Hz) in [^{2}H]-chloroform of:

A Dimethyl ester of 1-sn-phosphatidyl-3'-sn-glycero-1'-sulfate from H. cutirubrum (natural Me_2-PG-1-S)

a) ^{31}P decoupled spectrum

b) ^{31}P-^{1}H normal spectrum

B Dimethyl ester of synthetic 1-sn-phosphatidyl-1'-sn-glycero-2'-sulfate (synthetic Me_2-PG-2-S)

a) ^{31}P decoupled spectrum

b) ^{31}P-^{1}H normal spectrum

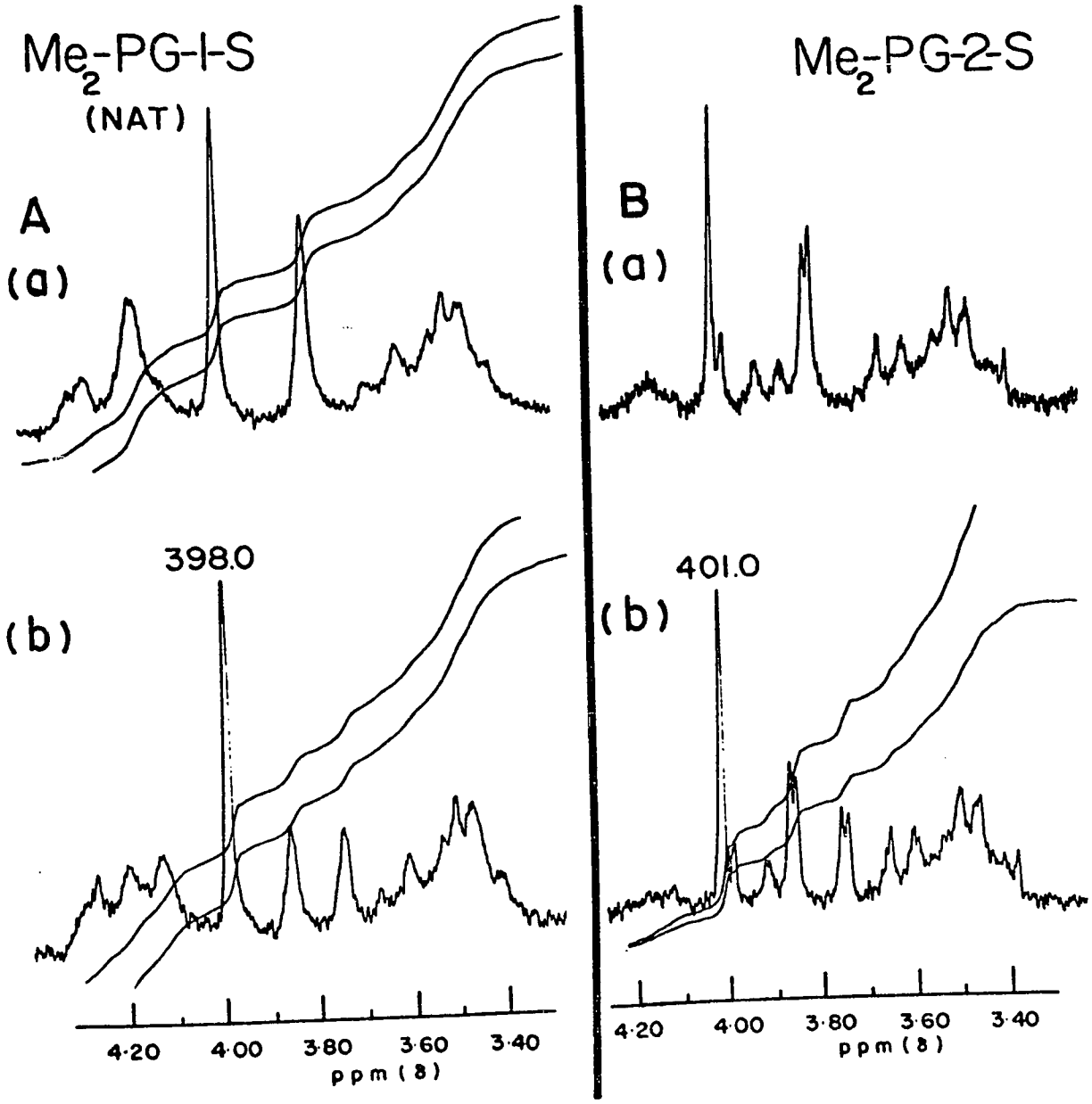
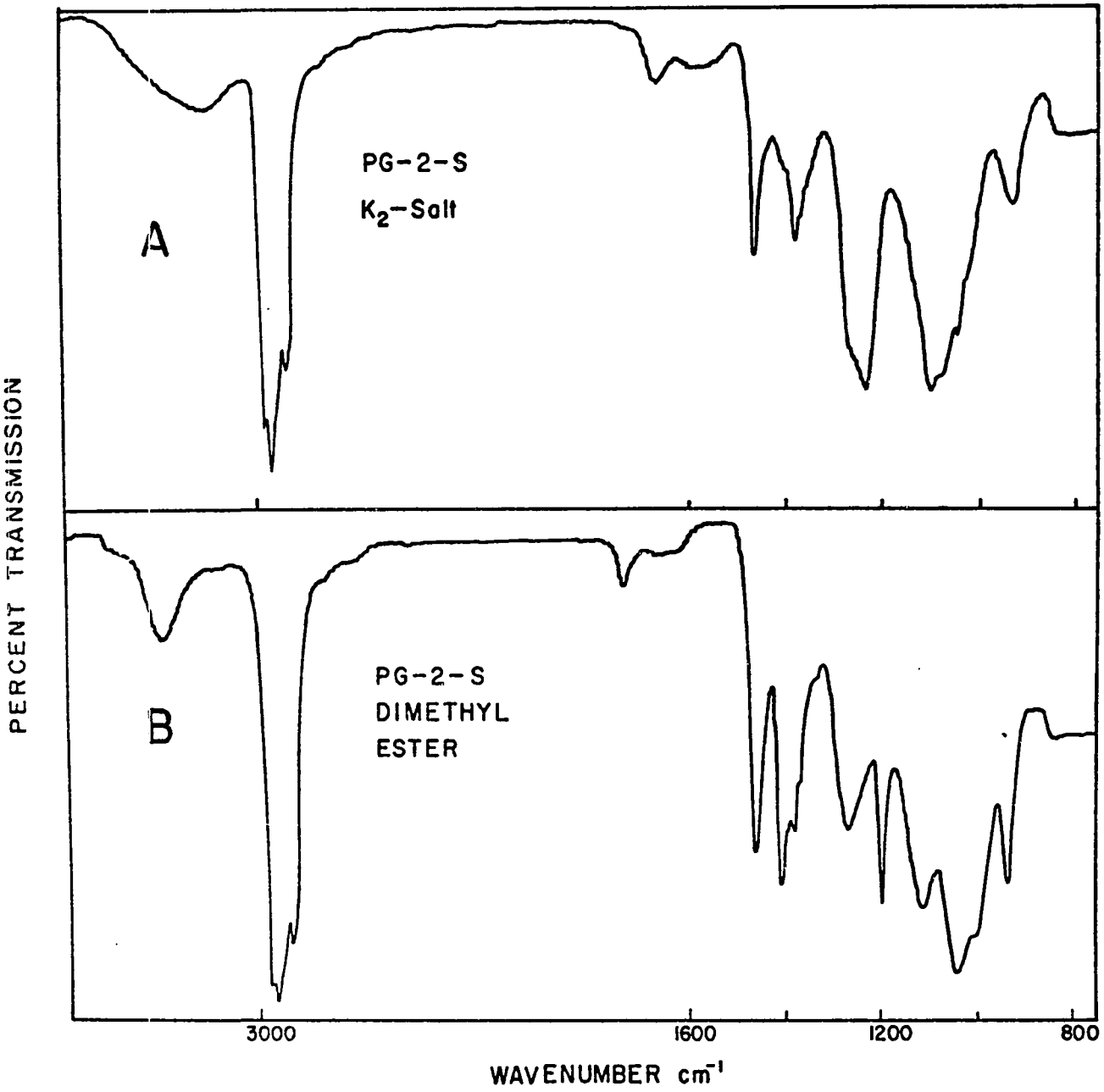

Me2-PG-I-S
(NAT)
A
(a)
(b)
398.0
4·20
4·00
3·80
3·60
3·40
ppm (δ)
Me2-PG-2-S
B
(a)
(b)
401.0
4·20
4·00
3·80
3·60
3·40
ppm (δ)

FIGURE 38

Infrared absorption spectra in CCl_4 of :

A 1-sn-phosphatidyl-1'-sn-glycero-2'-sulfate, dipotassium salt

B 1-sn-phosphatidyl-1'-sn-glycero-2'-sulfate, dimethyl ester


PG-2-S
K_2-Salt
A
PG-2-S
DIMETHYL
ESTER
B
PERCENT TRANSMISSION
3000
1600
1200
800
WAVENUMBER cm^{-1}

7. Synthesis of β-Linked PG for Comparison with PGS

Before elemental analyses and methylation studies were performed on PGS, the presence of sulfur in the molecule had not been recognised. Furthermore, acid hydrolysis data showed a ratio of P_i, glycerol and diphytanyl glycerol ether that was consistent with a PG-type structure. However, two preliminary observations were not in accord with such a structure.

Firstly, the chromatographic mobility of the intact lipid in acid or alkaline solvent systems was much lower than PG (Table 3). This difference in mobility could not simply be attributed to the presence of different salt forms of the same lipid. Kates et al.(1965) showed that the potassium salt and the free acid form of PGP were not resolved in an acidic solvent on silicic acid-impregnated paper. In addition, no TLC resolution of different salt forms was observed in alkaline or neutral solvent systems during PGP studies (described elsewhere in this thesis) even though the magnesium and sodium salts of PGP could be efficiently resolved by silicic acid column chromatography.

Secondly, the lipid gave a negative reaction to periodate-Schiff reagent, which precluded the presence of a vicinal diol grouping required for PG (Fig. 4A). Since no other water-soluble moiety had at that time been detected in the hydrolysate, the possibility was considered that the lipid possessed a β-linkage analogous to that proposed by Debuch and Rotsch (1966) for a phosphatide isolated from spinach leaves.

Therefore, the β-isomer of PG (1-phosphatidyl-sn-2'-glycerol) was synthesised by means of the mixed acid anhydride method (Aneja, 1970), using diphytanyl ether phosphatidic acid and 1, 3-dibenzyl glycerol (Scheme 5).

After removal of the benzyl blocking groups by catalytic hydrogenolysis, the synthetic β-isomer was converted to its sodium salt and compared chromatographically to the unknown lipid and to bacterial α-PG. It was found to have a higher mobility than either of these compounds (Table 10; Fig. 23).

This finding clearly showed that the isomeric β-PG structure was untenable for the unknown lipid and strengthened the alternative proposal that the compound was a substituted PG. It was of interest that the synthetic β-PG readily isomerised to the α-form after 15 min exposure to dilute acid. The lability of this type of phosphatide may account for the continued lack of definitive evidence for the existence of β-linked phosphatides in natural lipid mixtures, in spite of the attention focussed on them in former years (Baer et al., 1943; De Haas and van Deenen, 1965).

The co-product obtained in the condensation reaction for β-PG was also of some interest. It appeared to be a pyrophosphate dimer of phosphatidic acid. This conclusion was based on three observations:

(a) The infrared spectrum of the compound (potassium salt) in CCl_4 revealed a sharp P-O-P absorption band at 935 cm^{-1};

(b) The results of phosphorus analyses performed on the potassium and ammonium salts of the compound were in close agreement with the theoretical values for the proposed pyrophosphate structure;

(c) The product was cleaved by 2.5% methanolic HCl at room temperature in less than 5 min to give a product which chromatographed on TLC with phosphatidic acid in both acid and alkaline solvent systems.

The low yield in this synthesis of β-PG (38%) is therefore attributed to competition by pyrophosphate formation with the desired esterification of the 1, 3-di-O-benzyl glycerol. Condensations performed with a primary alcohol (for example, the formation of 2-O-myristoyl-3-O-benzyl-sn-glycerol) (see Scheme 3) did not generate enough pyrophosphate to be discernible on TLC. This is in agreement with the observations of Aneja (1970) who reports finding triisopropylbenzenesulfonylchloride a superior condensing agent to dicyclohexylcarbodiimide, the latter being quite prone to cause self-condensation of the phosphatidic acid.

IV. Possible Role of PGS in Membrane Structure and Function

1. Membrane Stability

The isolation of this new highly acidic phosphosulfolipid (PGS) from H. cutirubrum is in keeping with the established trend of finding acidic components in halophile membranes (see General Introduction). All of the polar lipid components characterised to date in H. cutirubrum contain negatively charged groups [PO-O$^-$, PO(O$^-$)$_2$ and SO$_2$O$^-$] ; no basic or zwitterionic lipid species have been detected. The lipid composition of the extreme halophile is thus in marked contrast to that of a typical gram-negative non-halophilic bacterium. For example, each member of a series of eight gram-negative bacteria studied by Randle et al.(1969) contained the basic or neutral phosphatidylethanolamine or phosphatidylcholine as major lipid component at all stages of growth. It is likely that the charges on the lipid and protein components interact electrostatically with the environmental cations and help to stabilise the membrane. Magnesium ion, in particular, is important in maintaining the stability of the cell envelope (Kushner and Onishi, 1966). Binding of acidic phosphatides with protein in the membrane may arise through chelation between basic amino acid residues (e.g. lysine, arginine) and lipid phosphate and sulfate groups, as well as by binding of lipids to hydrophobic residues in the proteins (McClare, 1967; Kates, 1972).

2. Selective Ion Transport

Polar lipids having charged head groups have been implicated in the control of ion permeability of the membranes in which they reside. Evidence is accumulating that it is the acidic lipids in particular that are involved in a cation selectivity which may in turn be related to a selective transport ability of the membrane. Quantitative experiments, for example,

using model bilayer membranes, show that phosphatidyl glycerol and diphosphatidyl glycerol are highly cation selective (Hopfer et al., 1970), while membranes containing neutral or zwitterionic lipids (phosphatidylethanolamine and diglucosyl diglyceride) are only slightly cation selective. Other workers have recently provided evidence for cation specificity involving acidic phospholipids. Papahadjopoulos (1971) showed that phospholipid vesicles composed of phosphatidyl glycerol or phosphatidylserine could discriminate between monovalent cations. Vesicles composed of neutral ionic lipids such as phosphatidylethanolamine and phosphatidylcholine possessed no demonstrable ability to discriminate between different cations under the same experimental conditions. Haest et al.(1972, in press) report that liposomes containing phosphatidyl glycerol (net negative charge) are cation and H^+ selective, while those containing lysyl-phosphatidyl glycerol (net positive charge) are anion selective. It is conceivable that this type of binding behaviour may be related to ion transfer processes across the membrane.

The possibility that the sulfate group in the newly characterised lipid of H. cutirubrum is specifically involved in the physiological mechanism of trans-membrane ion movement is also raised by in vitro studies performed on sulfated polysaccharides found in mammalian tissue. The affinities of heparin and chondroitin sulfate for alkali metal ions were found to differ. Heparin, a highly O- and N-sulfated mucopolysaccharide containing an excess of sulfate groups over carboxyl groups, bound potassium ion in preference to sodium ion (Salminen and Luomanmäki, 1963). On the other hand, chondroitin sulfates, which also bind cations, have not been shown to exhibit specificity in binding potassium and sodium ion (Dunstone, 1962; Farber et al., 1957). This observation may implicate the sulfate group in the specificity shown by heparin because in chondroitin sulfate the binding is shared between an equal number of weakly acidic carboxyl groups and strongly acidic sulfate groups.

The contention that the sulfate group may be of particular advantage to the organism in concentrating potassium ion against the ion gradient is supported by the hypothesis of Teunissen and Bungenberg de Jong (1938). These workers determined the concentration of alkali metal salt at which the charge of a polyelectrolyte is reversed. They found that the ability of a cation to change the charge of a polyelectrolyte depends on both the unhydrated and the hydrated size of the cation, as well as the polarizability of the anionic groups relative to water. "For anionic groups more polarizable than water (e.g., phosphate and carboxyl), the electrostatic immobilization of a cation is determined by its unhydrated size and thus small unhydrated sodium ions are bound in preference to potassium ions. However, for an anionic group of less polarizability than water (sulfate), the potassium ion, having a smaller hydrated diameter than sodium ion, is bound preferentially". Thus a highly acidic lipid such as PGS might be expected to bind potassium ion in preference to sodium ion. It would therefore be interesting to study ion transport in model membranes composed of this new lipid, for example in a "liposome" system prepared according to Bangham (1965).

3. Biosynthetic Involvement of PGS in PG and PGP Synthesis

It is already clear that a novel biosynthetic pathway must exist for PG and PGP, since both glycerol moieties in each of these lipids have the opposite configuration to those in the corresponding diester analogues found in non-halophiles (Kates, 1972). The participation of sn-glycerol-1-phosphate instead of the usual sn-3-isomer is ruled out by the demonstration that H. cutirubrum synthesises only the latter isomer (Wassef et al., 1970). Therefore, sn-3-GP cannot participate directly in this condensation. However, it may be utilised for synthesis of sn-1-glycerosulfate which could then be condensed with CDP-diether to form PGS, in an analogous reaction to that for the biosynthesis of PGP diester (Chang and Kennedy, 1967) :

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

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

Desulfation of PGS to give PG, followed by phosphorylation of PG with ATP should yield PGP with the correct stereochemistry:

(c) 1-sn-phosphatidyl-sn-3'-glycero-1'-sulfate →
1-sn-phosphatidyl-sn-3'-glycerol (PG)

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

Another possible pathway utilising sn-3-GP indirectly could involve synthesis of PG via CDP-sn-3-glycerol, as follows:

(e) sn-3-GP + CTP → CDP-sn-3-glycerol + PP

(f) CDP-sn-3-glycerol + 2, 3-diphytanyl-sn-glycerol →
sn-1-phosphatidyl-3'-sn-glycerol (PG)

PGP would then be formed by phosphorylation of PG with ATP as shown above [reaction (d)] and PGS by sulfation with PAPS:

(g) 1-sn-phosphatidyl-sn-3'-glycerol $\xrightarrow{PAPS}$ 1-sn-phosphatidyl-
sn-3'-glycero-1'-sulfate (PGS)

It must be emphasised that these pathways involving PGS are purely hypothetical and no direct evidence supporting them is available. However, the observation that ^{14}C-labelled diphytanyl glycerol ether is found in the ^{14}C-labelled total lipid extract from H. cutirubrum (unpublished observation made during the course of this work) may be taken as indirect evidence in favour of reaction step (f).

PART TWO

SYNTHESIS OF PHYTANYL GROUP DERIVED LIPIDS

I. Synthesis of Extended-chain Homologues of Phytanic Acid

1. Introduction

(a) Determination of the stereochemistry of the phytanyl group

The justification for the assignment of R-absolute configuration to each of the asymmetric centres of the phytanyl chain (C-3, C-7, C-11) is briefly discussed below.

The configurations at C-7 and C-11 of the bacterial group were shown by Kates et al.(1967) to be the same as in the phytyl chain of chlorophyll (i.e. 7R, 11R) by converting the bacterial phytanyl group to the C_{18} ketone (6,10,14-trimethylpentadecanone-2) which had the same rotation as the ketone obtained by oxidative cleavage of phytol*; both ketones were identical to an authentic sample of 6R, 10R, 14-trimethylpentadecanone-2.

The basis for the determination of the C-3 configuration was the observation by Abrahamsson et al.(1963) that the molecular rotations of monomethyl-branched carboxylic acids of the same absolute configuration alternate in sign and decrease in magnitude, as the methyl branch is moved to successive positions along the chain from the 2-position. Bacterial phytanyl groups were used to synthesise C_{19} (pristanic) and C_{20} (phytanic) carboxylic acids (C-2 and C-3 methyl-branched acids, respectively); the signs of their molecular rotations indicated a D-configuration at C-3 of the parent phytanyl group (Kates et al., 1967).

---

* A C_{20}-Δ^2-mono-unsaturated isoprenoid alcohol obtained from chlorophyll.

(b) Phytanic, pristanic and related isoprenoid acids

The natural occurrence of 3, 7, 11, 15-tetramethylhexadecanoic acid (phytanic acid) was observed first by Hansen and Shorland (1951) in butterfat and later its homologue 2, 6, 10, 14-tetramethylpentadecanoic acid (pristanic acid) was also isolated from the same source (Hansen and Morrison, 1964). Since then, there have appeared numerous reports of these isoprenoid acids in animal lipids and in marine oils (see Review by Smith, 1970). Refsum's syndrome, for example, is a hereditary metabolic disease (Klenk and Kahlke, 1963) characterised by an abnormal accumulation of phytanic acid in the body.

Samples of phytanic and pristanic acids isolated from natural sources do not necessarily have the same stereochemistry. Ackman and Hansen (1967) used open-tubular GLC columns to identify both $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{R}}$- and $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{S}}$-isomers in natural samples. The two diastereoisomers were revealed as partly-resolved peaks, the $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{R}}$-isomer eluting first. It is worthy of note in this context, that the elution patterns for phytol-derived samples of pristanic and phytanic acids clearly showed equal proportions of $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{R}}$- $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{S}}$-isomers, while those for the corresponding acids derived from Halobacterium lipids showed exclusively the $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{R}}$-isomer peak. This finding was in accord with the conclusions drawn by Kates and co-workers about the stereochemistry of the bacterial phytanyl chain.

The assignment of $\underline{\underline{R}}$-absolute configuration to C-3 of the phytanyl chain (Kates et al., 1967) depended on the characteristic variation of molecular rotation in pristanic and phytanic acids. It was of interest to see if further correlation of the phenomenon could be obtained with related isoprenoid acids. Only the $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{R}}$- $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{S}}$ stereoisomeric mixture of the C_{21} homologue had previously been synthesised (Fieser and Chamberlin, 1948;

Sen Gupta and Peters, 1966). The synthesis of the RRR-C_{21} and RRR-C_{22} homologues was therefore undertaken and the products were characterised by their spectral, chromatographic and optical properties. The results of this work have been published (Kates et al., 1972) and are presented in the following section of this thesis. The C_{21} and C_{22} compounds were also of interest in connection with studies in Ackman's laboratory on GLC separation and identification of stereoisomeric isoprenoid acids formed by oxidation and chain elongation of meso-pristane and phytol (Cox et al., 1970).

2. Experimental Procedures

(a) Gas-liquid chromatography

GLC was carried out on a glass column (2 m x 4 mm ID) of 10% butanediol succinate polyester on Gas-Chrom A at 172° and 0.9 kg/cm^2 inlet N_2 pressure (flame detector) in a Carlo Erba gas chromatograph.

Open tubular (capillary) column GLC was carried out on columns of stainless steel tubing (50 m x 0.25 mm ID) coated respectively with Apiezon-L (AP-L), or with diethylene glycol succinate (DEGS) or butanediol succinate (BDS) polyesters. These were operated in a Perkin-Elmer model 226 (DEGS) or model 900 (AP-L, BDS) under conditions shown in Table 18. The BDS capillary column was operated under conditions known to give partial resolution of the RRR-RRS diastereoisomeric pairs of the homologous C_{19}, C_{20}, C_{21} and C_{22} methyl esters (Ackman, personal communication). The capillary column data were obtained by Ackman and co-workers, Fisheries Research Board of Canada, Halifax Laboratory.

(b) Reference compounds

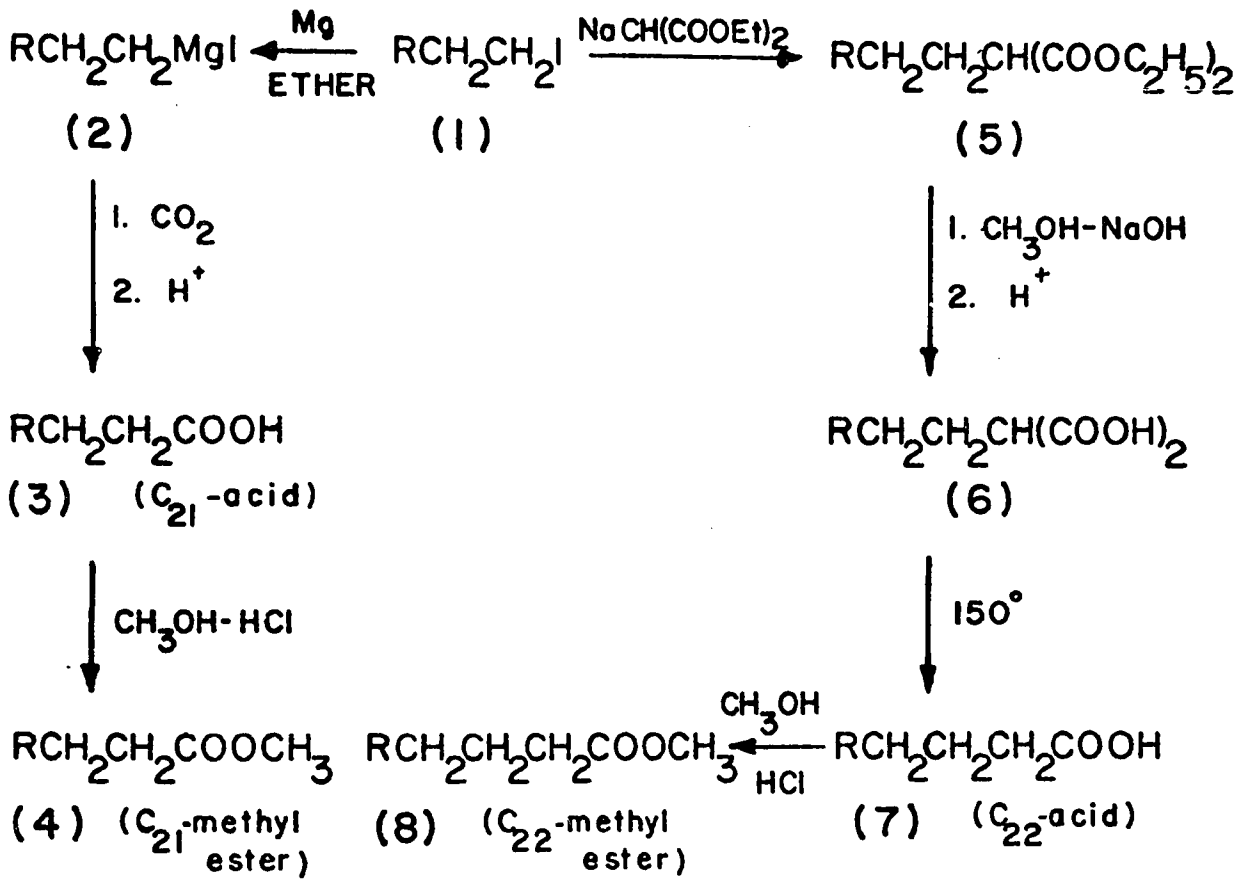
The 2R, 6R, 10R, 14-tetramethylpentadecanoic (C_{19}) and 3R, 7R, 11R, 15-tetramethylhexadecanoic (C_{20}) acids and their respective methyl esters were prepared as described previously (Kates et al., 1967).

(c) Methyl 4R, 8R, 12R, 16-tetramethylheptadecanoate [C_{21}-methyl ester], Scheme 6

RRR-phytanyl iodide (1) (1-iodo-3R, 7R, 11R, 15-tetramethylhexadecane) was prepared by hydriodic acid cleavage of the phytanyl glyceryl diether obtained from H. cutirubrum, as described elsewhere (Kates et al., 1967). To a solution of the iodide (60 mg, 0.147 mmoles) in 10 ml diethyl ether (freshly distilled from $LiAlH_4$) in a dry two-necked 50 ml round-bottom flask, equipped with an efficient condenser, a micro stirring bar and a $CaCl_2$ drying tube, was added an excess of magnesium turnings (25 mg; Grignard reaction quality). The mixture was brought to boiling, the reaction was initiated by addition of a crystal of iodine and refluxing was continued for 30 min. The ethereal solution of (2) was cooled on ice and injected by hypodermic syringe through a rubber septum on to 10 g of solid CO_2 in a two-necked 100 ml round bottom flask equipped with a $CaCl_2$-tube. The mixture was allowed to reach room temperature during 2 hours, then diluted with 5 ml of 6 N HCl and extracted with three 20-ml portions of ethyl ether. The combined extracts were concentrated to dryness in vacuo and the residual crude acid product (3) was freed from non-polar material by extraction of its solution in a mixture of 4.5 ml of methanol and 0.5 ml of 7 N aqueous NaOH with four 5-ml portions of petroleum ether (b.p. 30-60°). The methanol phase containing the sodium

SCHEME 6

Synthesis of methyl 4$\underline{R}$, 8$\underline{R}$, 12$\underline{R}$, 16-tetramethylheptadecanoate (C_{21}-methyl ester) and methyl 5$\underline{R}$, 9$\underline{R}$, 13$\underline{R}$, 17-tetramethyloctadecanoate (C_{22}-methyl ester)



$$R = CH_3\underset{\underset{CH_3}{|}}{CH}(CH_2CH_2CH_2\underset{\underset{CH_3}{|}}{CH}-)_3$$

salt of the acid was strongly acidified with 6 N HCl and the liberated free acid (3) was extracted with four 5-ml portions of petroleum ether (b.p. 30-60°). The acid obtained on evaporation of the solvent in a stream of nitrogen was converted to the methyl ester by heating under reflux in 4.5 ml of 2.5% methanolic HCl for 1 hr. After addition of 0.5 ml of water, the methyl ester (4) was extracted with several portions of petroleum ether and recovered by evaporation of the solvent; yield 31 mg (62% overall) of almost colourless oil. The C_{21}-methyl ester showed a single spot on TLC (Fig. 39; Table 17) in three solvent systems and a single peak on GLC on butanediol succinate polyester, both on packed (Fig. 40) and capillary columns (Table 18). It had the following specific rotations in chloroform (c, 1.88): 589 nm, -0.69° ; 578 nm, -0.43° ; 546 nm, 0.00°; 436 nm, -0.80°.

Anal. Calc. for $C_{22}H_{44}O_2$ (340.57): C, 77.58; H, 13.02.
Found: C, 77.27; H, 13.16.

FIGURE 39

Thin-layer chromatogram of $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{R}}$-$C_{19}$, C_{20}, C_{21} and C_{22} isoprenoid methyl esters.

S methyl stearate reference standard

1 methyl 2, 6, 10, 14-tetramethylpentadecanoate (C_{19})

2 methyl 3, 7, 11, 15-tetramethylhexadecanoate (C_{20})

3 methyl 4, 8, 12, 16-tetramethylheptadecanoate (C_{21})

4 methyl 5, 9, 13, 17-tetramethyloctadecanoate (C_{22})

Solvent system:

hexane-diethyl ether (97:3, v/v); double development

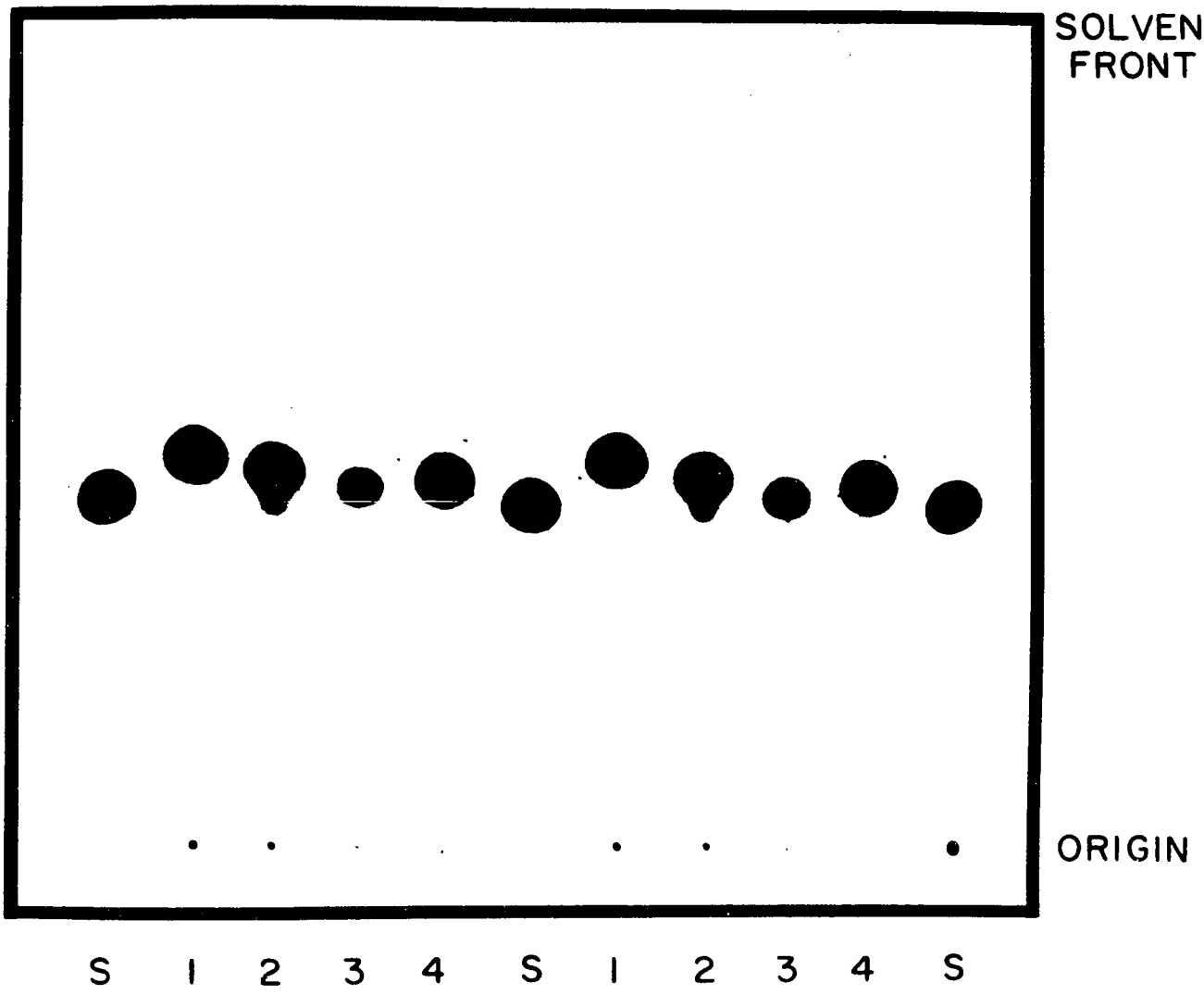

SOLVENT
FRONT
ORIGIN
S 1 2 3 4 S 1 2 3 4 S

TABLE 17

Physical Data for Homologous Saturated Isoprenoid Fatty Acid Methyl Esters

RRR-methyl Ester	TLC Rf[a] 1	2	3	$[\alpha]_D^b$	M_D
C_{19}[c]	0.70	0.55	0.58	-11.8°	-37°
C_{20}[c]	0.70	0.55	0.56	+3.5°	+11°
C_{21}	0.70	0.55	0.53	-0.69	-2.4°
C_{22}	0.70	0.55	0.54	-0.19	-0.67°

a On silica gel H in (1) petroleum ether (b.p. 30-60°) - diethyl ether-acetic acid (90:10:1, v/v); (2) benzene; and (3) hexane-diethyl ether (95:5, v/v).

b In chloroform at 22°

c Data from Kates et al.(1967)

FIGURE 40

GLC chromatogram of homologous $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{R}}$-$C_{19}$, C_{20}, C_{21} and C_{22} branched chain methyl esters.

Column conditions:

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

Temperature: 172°

Carrier gas: Nitrogen; pressure 14 lb/in^2 (0.9 Kg/cm^2)

S is solvent peak.

Broken line peak is methyl stearate reference standard.

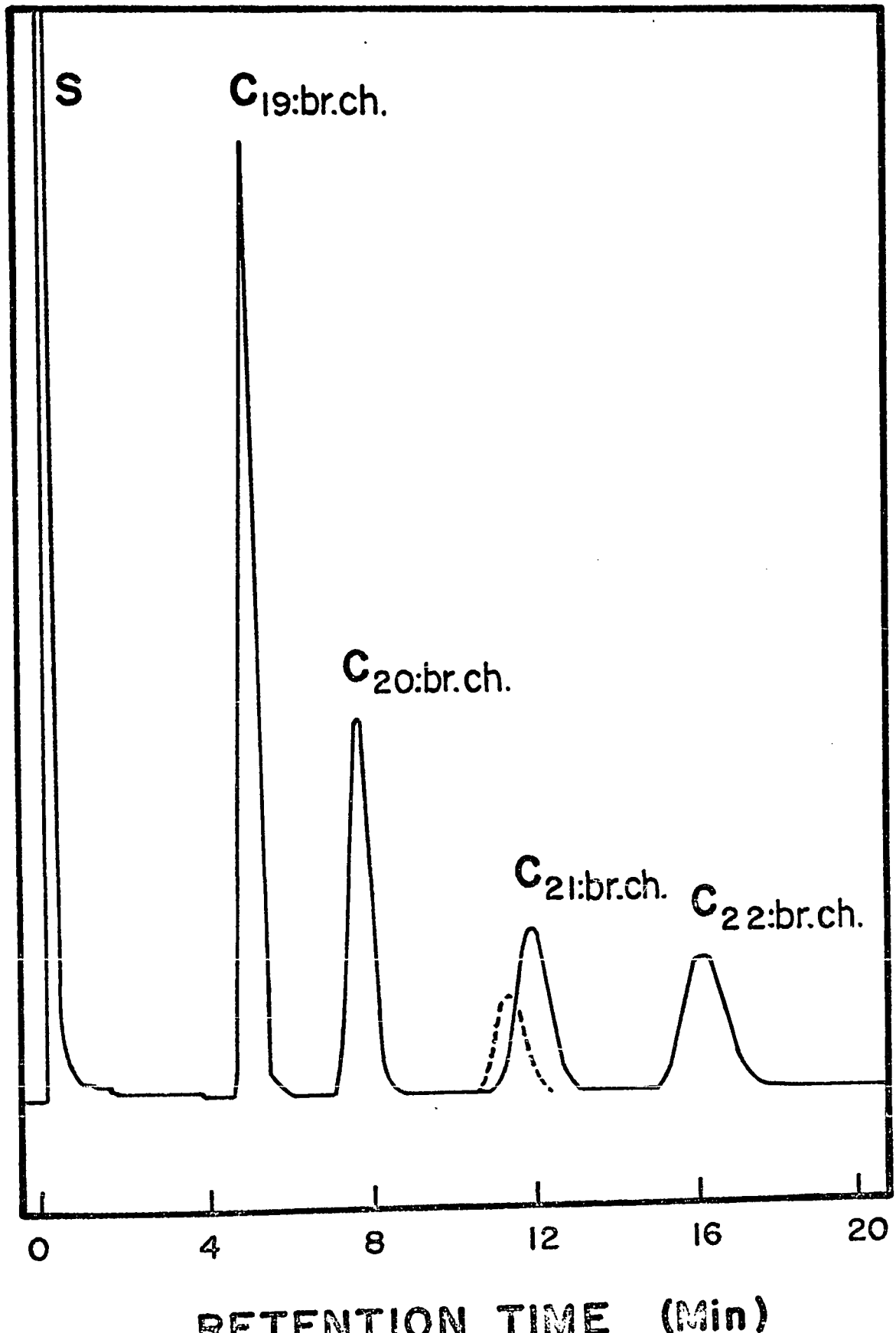

S
C19:br.ch.
C20:br.ch.
C21:br.ch.
C22:br.ch.
0
4
8
12
16
20
RETENTION TIME (Min)

TABLE 18

Gas-Liquid Chromatographic Data for Homologous Saturated Isoprenoid Fatty Acid Methyl Esters

RRR-methyl ester	AP-L Capillary column		DEGS Capillary column		BDS Capillary column		BDS Packed column	
	$R_{18:0}$[a]	ECL[b]	$R_{18:0}$[c]	ECL[b]	$R_{18:0}$[d]	ECL[b]	$R_{18:0}$[e]	ECL[b]
C_{19}	0.468	16.40	0.475	15.68	0.412	15.85	0.454	15.70
C_{20}	0.736	17.47	0.725	17.01	0.714	17.11	0.702	16.97
C_{21}	1.306	18.57	0.987	18.34	1.142	18.35	1.085	18.24
C_{22}	1.98	19.48	1.52	19.30	1.66	19.24	1.46	19.11

a Retention relative to methyl stearate on Apiezon-L (AP-L) at 180° and 60 psi (4 kg/cm^2) He.

b Equivalent chain length

c Retention relative to methyl stearate on diethylene glycol succinate polyester (DEGS) at 170° and 40 psi (2.7 kg/cm^2) of He.

d Retention relative to methyl stearate on butanediol succinate polyester (BDS) at 150° and 60 psi (4 kg/cm^2) of He.

e Retention relative to methyl stearate on butanediol succinate polyester at 172° and 14 psi (0.9 kg/cm^2) of N_2.

(d) Methyl 5R, 9R, 13R, 17-tetramethyloctadecanoate [C_{22}-methyl ester] (Scheme 6)

To 0.5 ml of a 0.65 M solution of sodium ethoxide in ethanol (prepared by dissolving 375 mg sodium metal in 25 ml of absolute ethanol) was added a solution of 40 mg (0.25 mmoles) of diethyl malonate (freshly distilled; b.p. 74-75°/5 mm) in 1 ml of absolute ethanol. The mixture was stirred at room temperature for 10 min, cooled to 10° and a solution of RRR-phytanyl iodide [(1); 102 mg, 0.25 mmole] in 1 ml of absolute ethanol-benzene (1:1, v/v) was added dropwise with stirring over a period of 10 min. After further stirring for 3 hours at 10-15°, followed by heating under reflux for 30 min, the reaction mixture was cooled, neutralised by the addition of one drop of glacial acetic acid and freed from solvents in vacuo on a rotary evaporator. The residual colourless oil (5) was heated under reflux in 12 ml of 0.5 N NaOH in methanol-water (5:1, v/v) for 1 hour. The cooled mixture was extracted with petroleum ether (30-60°) to remove non-saponifiable material and the free C_{23}-dicarboxylic acid (6) was liberated by strong acidification with 3 ml of 6 N HCl and extracted with petroleum ether: yield, 79 mg (82%) of oily product; Rf 0.07 on TLC in petroleum ether-diethyl ether-acetic acid (90:10:1, v/v; Rf of palmitic acid, 0.18). A liquid film of the diacid product (6) was decarboxylated by heating in an oil bath at 150° for 80 min. The C_{22}-monocarboxylic acid (7) product obtained had Rf 0.2 on TLC, similar to that of palmitic acid, in petroleum ether-diethyl ether-acetic acid (90:10:1, v/v). The free acid (7) was methylated with 2.5% methanolic-HCl, as described above for the C_{21} acid and the C_{22}-methyl ester (8) was purified by preparative TLC on silica gel H in petroleum ether-diethyl ether-acetic acid (90:10:1, v/v; Rf 0.72): yield, 43 mg (48% overall) of

product giving a single spot on TLC (Fig. 39); GLC showed it was 99.2% pure, containing 0.8% of C_{21}-methyl ester. It had the following specific rotations (c, 3.3 in $CHCl_3$): 589 nm, -0.2° ; 578 nm, -0.2° ; 546 nm, +0.1° ; 436 nm, +0.1°.

Anal. Calc. for $C_{23}H_{46}O_2$ (354.60): C, 77.90; H, 13.08.
Found: C, 77.27; H, 13.45.

3. Results and Discussion

The C_{21}-acid was synthesised (Scheme 6) by carboxylation ("dry-ice") of the alkyl Grignard reagent prepared from the RRR-phytanyl iodide (1), followed by treatment with mineral acid; the free acid (3) obtained was converted to the methyl ester (4) by methylation in boiling methanolic-HCl. The C_{22}-acid was prepared by reaction of the RRR-phytanyl iodide (1) with sodium diethylmalonate, followed by saponification of the diethyl dicarboxylate ester (5) and decarboxylation of the resulting dicarboxylic acid (6) by heating at 150°; the C_{22}-acid (7) was converted to the methyl ester (8) by treatment with methanolic-HCl.

The methyl esters of both acids were obtained in substantially pure form as oils. GLC analysis on capillary columns showed that the C_{21} ester was $>$ 99.9% pure with respect to isoprenoid homologues and contained $<$ 1% of its 4S, 8R, 12R-stereoisomer. The C_{22} ester was obtained in 99.2% purity; it contained 0.8% of the C_{21}-4R, 8R, 12R-isomer and was also $>$ 99% stereochemically pure.

The mass spectra of the C_{21} and C_{22} esters (Fig. 41) showed peaks corresponding to the expected parent ions and fragmentation patterns (Table 19) consistent with their saturated isoprenoid structures (see Sen Gupta and Peters, 1966). It is noteworthy that the base peak in the spectrum of the C_{22} ester is the McLafferty-derived ion in contrast to the β-cleavage derived base peak for the C_{19} - C_{21} homologues.

The infrared spectra of the C_{21} and C_{22} methyl esters (Fig. 42) also showed bands characteristic of saturated isoprenoid chains (see Sen Gupta and Peters, 1966):

(a) strong C-CH_3 absorption at 1460 cm^{-1} (CH-deformation mode):

(b) strong gem-dimethyl absorption at 1375-1365 cm^{-1} (doublet ; CH-deformations) and at 1170 cm^{-1} (skeletal vibration):

(c) methylene absorption at 730 cm^{-1} for $(CH_2)_3$ without any shoulder at 720 cm^{-1}: and

(d) a doublet at 988 and 1010 cm^{-1} for both the C_{21} and C_{22} ester characteristic of the C-4 or C-5 CH_3-branch.

The NMR spectra of both esters (Fig. 43) showed the expected signals for $COOCH_3$ protons (δ 3.74 ppm), CH_2 protons adjacent to the $COOCH_3$ group (multiplet centred at δ 2.30 ppm), other CH_2 protons as well as CH protons (δ 1.25 ppm; CH-protons included) and C-CH_3 protons (δ 0.92 - 0.83 ppm).

On TLC, the C_{21} and C_{22} esters had essentially the same mobilities in non-polar solvents as the C_{19} and C_{20} saturated isoprenoid methyl esters (Table 17); however, the latter esters consistently gave slightly higher Rf values inhexane-diethyl ether (95:5, v/v) than the C_{21} or C_{22} esters (Fig. 39).

FIGURE 41

Mass spectra of:

A methyl 4$\underline{\underline{R}}$, 8$\underline{\underline{R}}$, 12$\underline{\underline{R}}$, 16-tetramethylheptadecanoate (C_{21}-methyl ester)

B methyl 5$\underline{\underline{R}}$, 9$\underline{\underline{R}}$, 13$\underline{\underline{R}}$, 17-tetramethyloctadecanoate (C_{22}-methyl ester)

Ionisation voltage 70 eV; samples were introduced by a direct inlet system set at 150°.

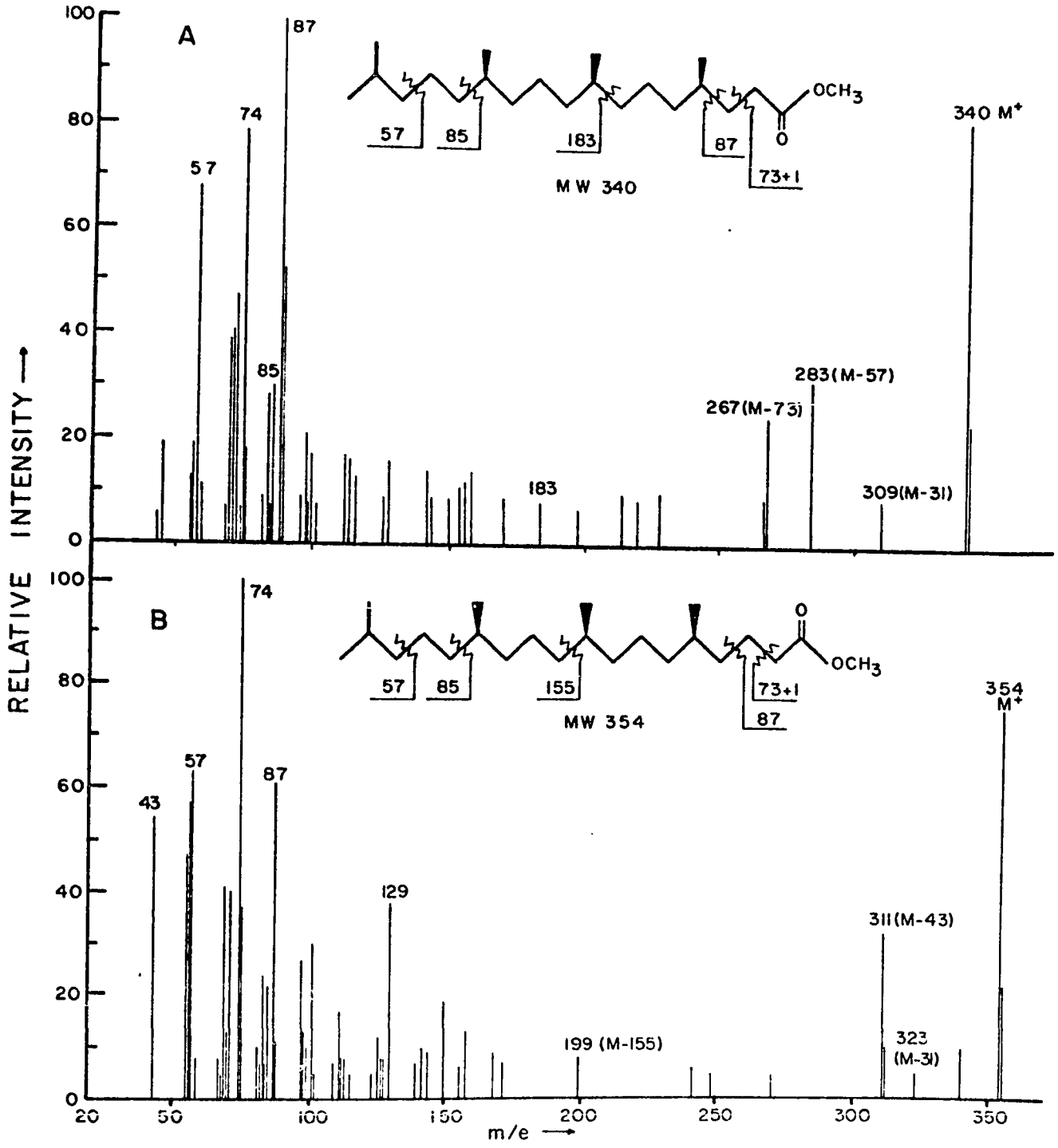

A
B
RELATIVE INTENSITY
m/e
MW 340
MW 354
340 M+
354 M+
283(M-57)
267(M-73)
309(M-31)
311(M-43)
323 (M-31)
199 (M-155)
OCH3

TABLE 19

Comparison of the Ions derived from Homologous RRR-Saturated Isoprenoid Fatty Acid Methyl Esters via McLafferty Rearrangement or β-Cleavage

Methyl Ester (RRR-isomer)	"McLafferty Derived" Ion		"β-Cleavage Derived" Ion	
	m/e	Relative intensity [a], %	m/e	Relative intensity [a], %
C_{19}	88	86	101	100
C_{20}	74	65	101	100
C_{21}	74	79	87	100
C_{22}	74	100	87	61

[a] Expressed as % of base peak (=100); see Figure 41

FIGURE 42

Infrared absorption spectra (liquid film) of:

A methyl 4$\underline{\underline{R}}$, 8$\underline{\underline{R}}$, 12$\underline{\underline{R}}$, 16-tetramethylheptadecanoate (C_{21}-methyl ester)

B methyl 5$\underline{\underline{R}}$, 9$\underline{\underline{R}}$, 13$\underline{\underline{R}}$, 17-tetramethyloctadecanoate (C_{22}-methyl ester)

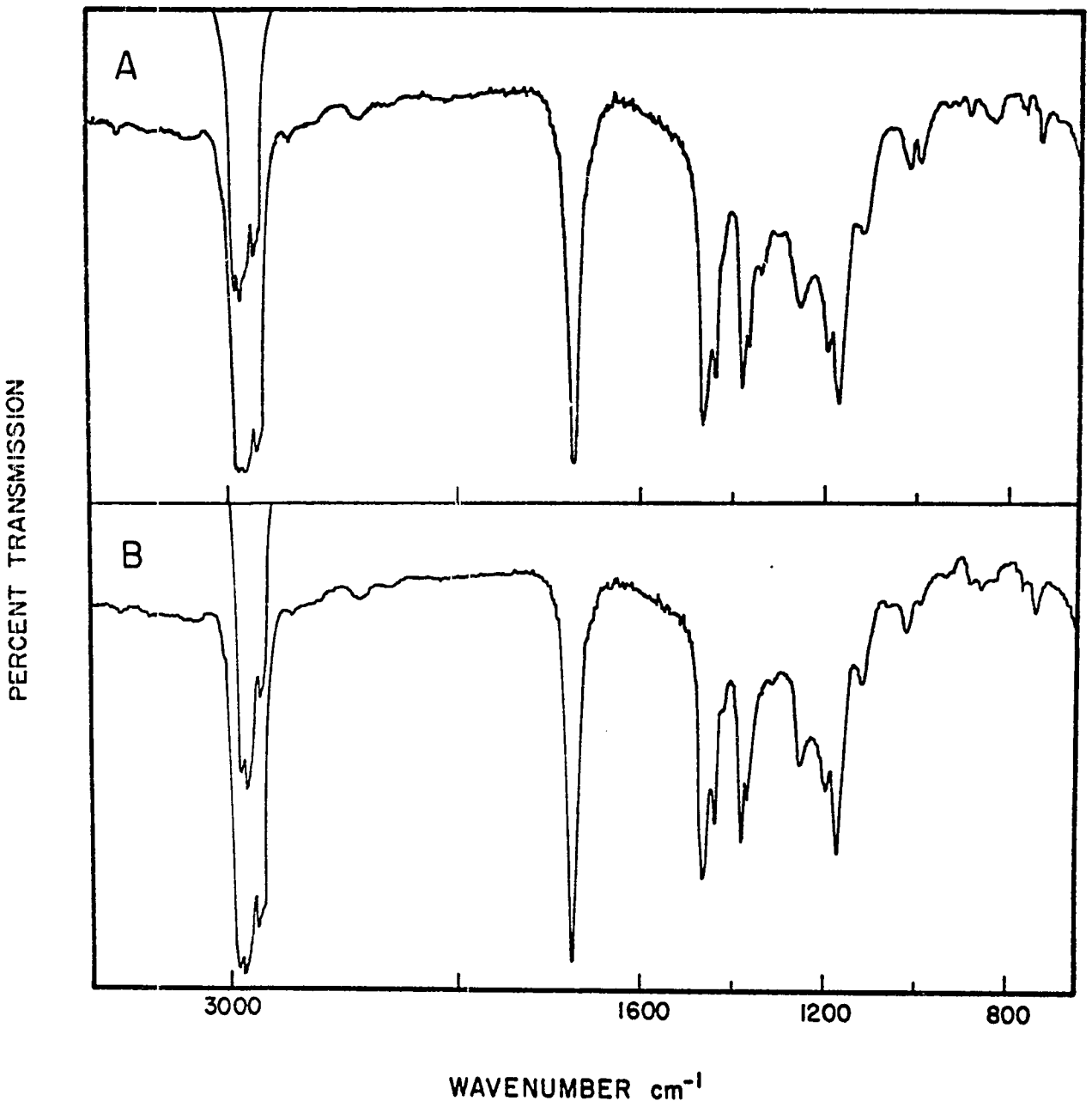
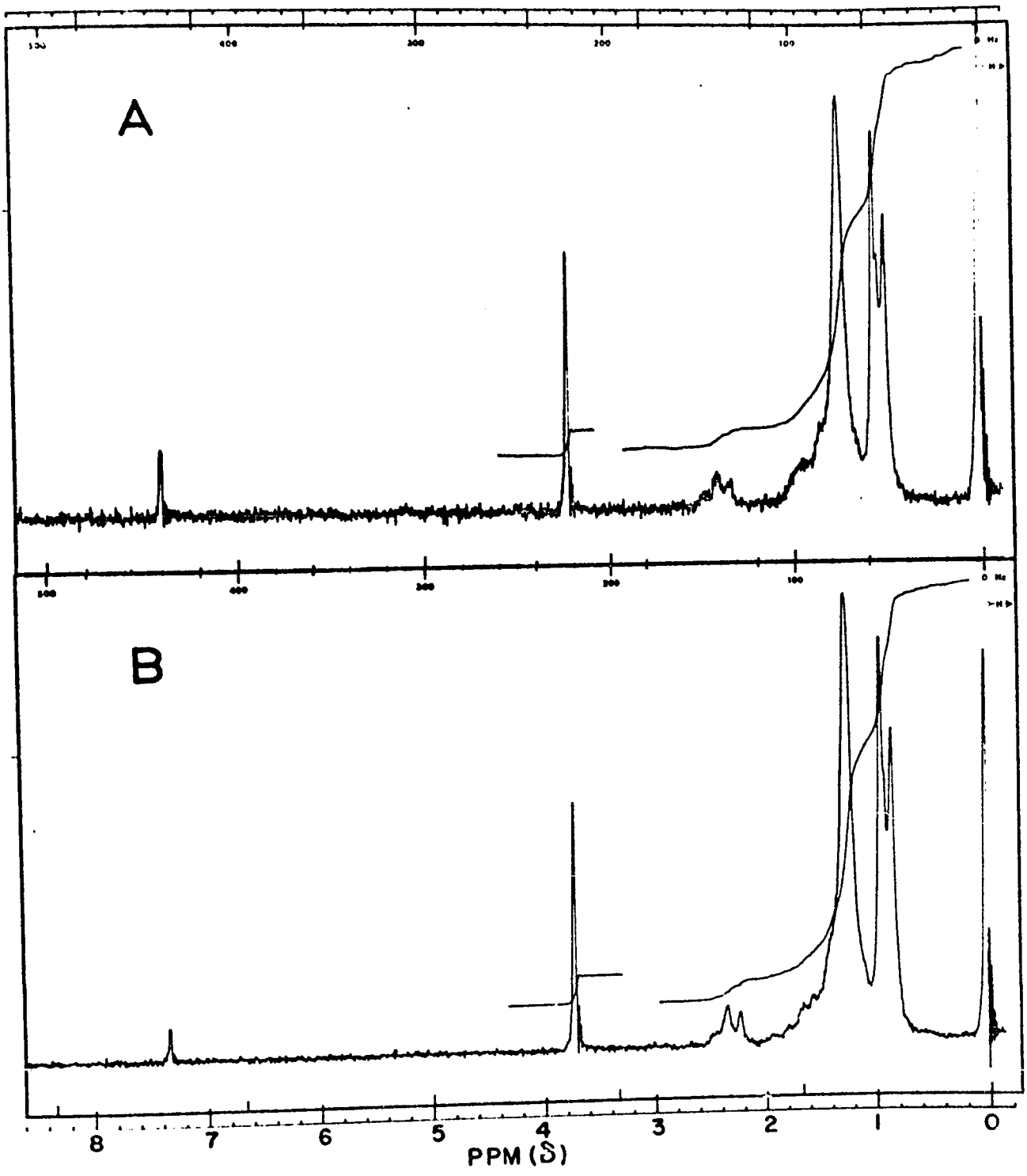

A
B
PERCENT TRANSMISSION
3000
1600
1200
800
WAVENUMBER cm⁻¹

FIGURE 43

Nuclear magnetic resonance spectra ([^{2}H]-chloroform solution) of:

A methyl 4$\underline{\underline{R}}$, 8$\underline{\underline{R}}$, 12$\underline{\underline{R}}$, 16-tetramethylheptadecanoate (C_{21}-methyl ester)

B methyl 5$\underline{\underline{R}}$, 9$\underline{\underline{R}}$, 13$\underline{\underline{R}}$, 17-tetramethyloctadecanoate (C_{22}-methyl ester)


A
B
8
7
6
5
4
3
2
1
0
PPM (δ)

The methyl esters of C_{19}, C_{20}, C_{21} and C_{22} isoprenoid acids were well resolved by GLC on BDS columns (Fig. 40). Comparative GLC retention data and equivalent chain length (ECL) values obtained under isothermal conditions on three commonly used liquid phases are recorded in Table 18 for the four homologous esters. The data reveal a relationship between the position of the first branch - methyl group and the ECL value, which has a bearing on the interaction of the methyl branches with the GLC liquid phase. Such interaction should be minimal for the non-polar phase, Apiezon-L and maximal for the polar phases, BDS and DEGS (Jamieson, 1970). Thus, the differences between ECL values on the polar phases and on Apiezon-L for each homologue should reflect the relative extent of interaction of the methyl groups with the polar liquid phase. For DEGS, BDS (capillary) and BDS (packed column) these differences are, respectively: C_{19}, 0.72, 0.55, 0.70; C_{20}, 0.46, 0.36, 0.50; C_{21}, 0.23, 0.22, 0.33; and C_{22}, 0.18, 0.24, 0.37. These findings indicate that a CH_3-branch at C-4 or C-5 interferes relatively less with substrate-liquid phase interaction and results in relatively higher retentions and ECL values, than a CH_3- branch at C-2 or C-3. These interaction effects clearly parallel the changes in the absolute values of the molecular rotations of the methyl substituents in the 2-, 3-, 4- and 5-methyl positions along the chain. The rest of the methyl substituents have relatively little effect and it can be predicted that the ECL values of C_{23} or C_{24} homologues (first methyl branch at C-6 and C-7, respectively) would be approximately those of the C_{22} methyl ester increased by 1.0 or 2.0, respectively (Ackman, 1972).

The molecular rotations of the homologous $\underline{R}\underline{R}\underline{R}$-$C_{19}$, C_{20}, C_{21} and C_{22} methyl esters (Table 17) show decreases in absolute values as the methyl-branching group is moved down the chain from C-2, as expected

from the studies of Abrahamsson et al.(1963). There is a slight discrepancy, however, in that the M_D values of the C_{21} and C_{22} esters are negative instead of positive, as expected for the D-4 and D-5-methyl acids (Abrahamsson, 1963). However, this may be explained by the fact that the other two asymmetric centres exert a negative contribution to the rotation (Kates, 1967), which overrides the positive contribution of the C-4 or C-5 asymmetric centres.

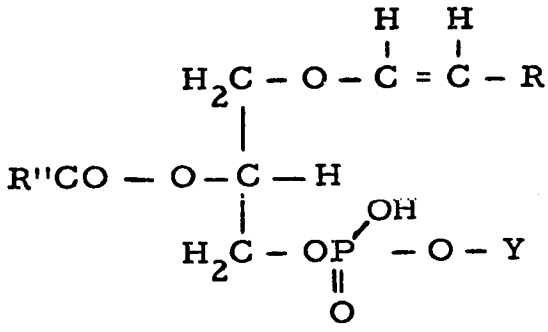
The physical data obtained are thus in general agreement with those obtained by Abrahamsson (1963) upon which was based the configuration assignment at C-3 of the bacterial phytanyl chain (Kates et al., 1967).

II. Synthesis of Monophytanyl Glycerol Ether Phosphatides (Lysophosphatides)

1. Introduction

The biochemistry of lipids that contain ether bonds has been reviewed by Snyder (1969). The following section presents a brief account of the occurrence of ether phosphatides and the chemical methods recently employed in their synthesis.

Diacyl derivatives of glyceryl monoethers, $ROCH_2CHOHCH_2OH$, occur in nature in the non-saponifiable liver lipids of fish. Glyceryl monoethers are also found as phospholipids in most animal tissues where they usually occur as monoether-monoester analogues of phosphatidylethanolamine and phosphatidylcholine. These analogues include a specialised class of monoether lipids known as "plasmalogens". The term plasmalogen denotes those lipids which contain a vinyl ether-linked alkyl group at C-1 of glycerol, i.e. they are derivatives of 1-alk-cis-1'-enyl-2-acyl-sn-glycerol. The phosphorylated derivatives have the general formula:



Y = choline, ethanolamine, etc.

The occurrence and properties of the plasmalogens have been reviewed by Rapport and Norton (1962); Klenk and Debuch (1963); Thiele (1964).

Monoether phosphatides containing a C-2 unsubstituted glycerol moiety have been isolated, but the investigators indicated that the native O-alkyl lipid was probably fully substituted (Carter et al., 1958). Monoether phosphatides that are unsubstituted at C-2 are usually referred to as "lysophosphatides". These monosubstituted derivatives are analogous to lyso-(monoester) derivatives which are formed by the action of phospholipase A on natural diester phosphatides and which are metabolically important as intermediates in the degradation and resynthesis of diester phosphatides (van Deenen and De Haas, 1966; Goldfine, 1968).

The chemical synthesis of glyceryl ether derivatives has been an active area of research since the pioneering work of Baer's group (Baer et al., 1944; Baer and Fischer, 1947). Phosphorylated 1-O-monoether derivatives have also been synthesised. Malkin and co-workers have prepared a series of phosphatidylethanolamine analogues from chimyl and batyl alcohols* (Baylis et al., 1958; Bevan and Malkin, 1960). Chacko and Hanahan (1968) have synthesised phosphatidylethanolamine and phosphatidylcholine derivatives of 1-O-selachyl-stearoyl alcohol*. Malkin's general procedure was to condense an acylated chimyl (or batyl) iodide with the silver salt of a suitably blocked phosphorylethanolamine. The monoether glycerols were prepared by alkylation of 1, 2-isopropylidene glycerol. Chacko and Hanahan devised a synthetic route to the 1-O-glyceryl ether in order to prepare monoether phosphatides with the natural D configuration. The 3-O-glyceryl ether, prepared from 1, 2-isopropylidene glycerol, was converted to the 1-O-glyceryl ether via the ditosylate in the

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* Chimyl alcohol: 1-hexadecylglyceryl ether
Batyl alcohol: 1-octadecylglyceryl ether
Selachyl alcohol: 1-cis-9'-octadecenylglyceryl ether

manner of Lands and Zschocke (1965). The monoether-monoacyl phosphatidylethanolamine was prepared by phosphorylation of the selachyl alcohol with phosphorus oxychloride and subsequent reaction with N-phthaloyl-ethanolamine.

The chemical synthesis of plasmalogens is difficult because of the presence of a reactive acid-labile cis vinylether bond, as well as an alkali-labile fatty acid ester bond. However, plasmalogen phosphatides have been synthesised. Slotboom et al.(1967) used pancreatic lipase to prepare the starting material, rac-1-alk-1'-enyl-2-acylglycerol from synthetic rac-1-alk-1'-enyl-2,3-diacylglycerol, for the synthesis of a plasmalogen lecithin. The lecithin plasmalogen, rac-1-hexadec-1'-enyl-2-oleyl-3-phosphorylycholine was then available by conventional methods.

A synthetic method using conventional techniques was reported by Serebrennikova et al.(1969). A phosphotriester of 1-alk-enyl-acyl glycerol was synthesised via the bromohydrin, anionically debenzylated and converted to the silver salt. The salt was coupled with N,N-dimethyl-ethanolamine chloride to give the N,N-dimethyl derivative of the plasmalogen phosphatide.

A partial synthesis of a lecithin plasmalogen was reported by Baumann et al.(1968). 1-Alk-cis-1'-enyl-sn-glycerol, obtained by lithium aluminum hydride reduction of beef heart lipids, was acylated and then converted into the phosphatide as described by Slotboom et al.(1967).

These synthetic procedures require as starting materials a 1-alk-cis-1'-enyl-sn-glycerol. Probably the most reliable method to date for its chemical synthesis is that of Gigg and Gigg (1968). In this method, sn-glycerol-2,3-carbonate is converted to a symmetrical alkanal di-(sn-glycerol-2,3-carbonate)acetal by reaction with an appropriate alkanal dimethylacetal. This derivative is then converted into cis-1-alkenylglycerol in three steps.

In the present study the chemical synthesis of the monophytanyl glycerol ether analogues of phosphatidic acid, phosphatidyl glycerol and phosphatidyl glycerophosphate was undertaken. The work was motivated by two factors:

(a) the presence of small amounts of monophytanyl glycerol derivatives in H. cutirubrum lipids was inferred from the observation that monophytanyl glycerol ether was always detected in trace amounts in the methanolysate of the lipids;

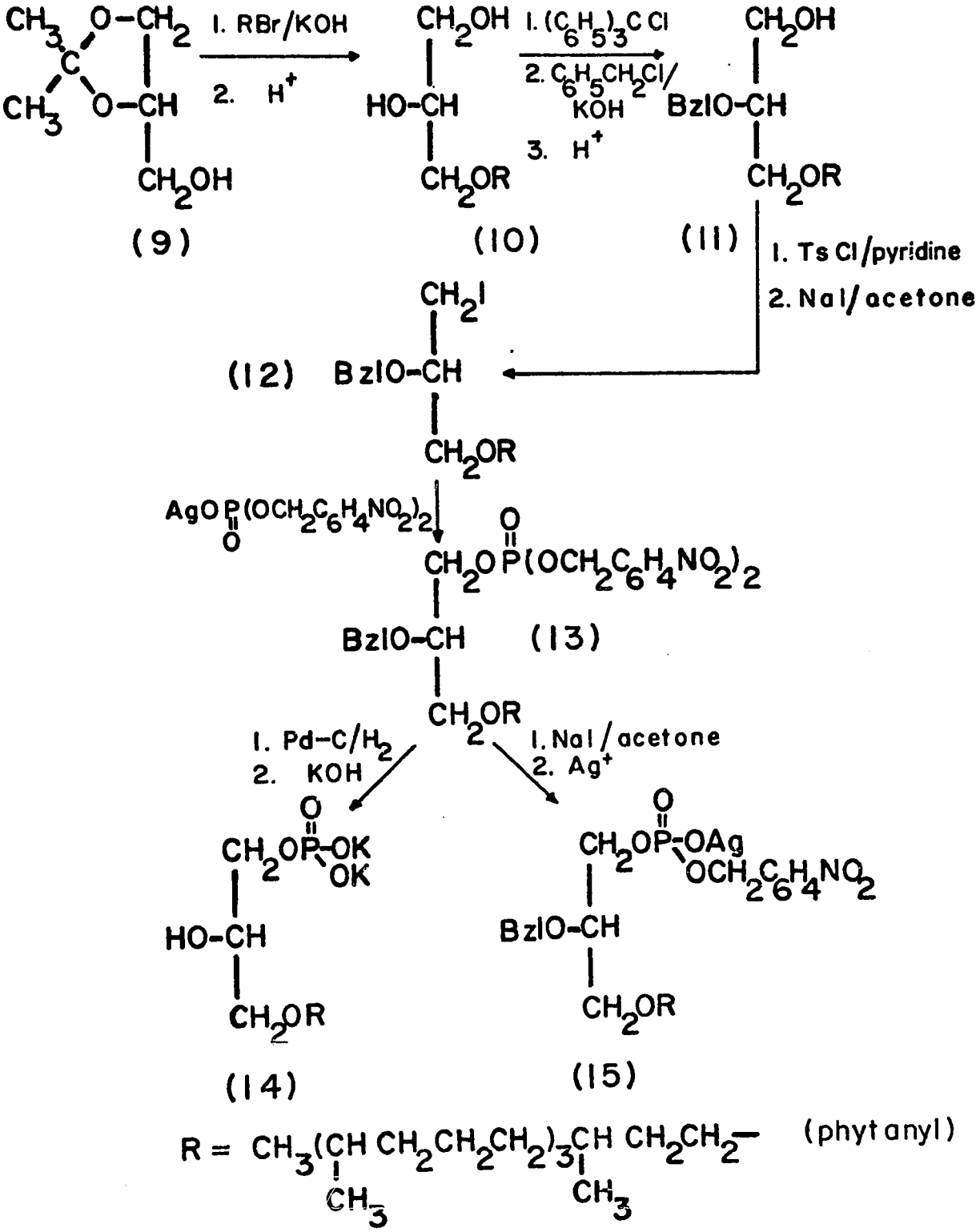
(b) the lysophosphatides were of significance as model compounds in biosynthetic studies carried out in this laboratory with H. cutirubrum. The intermediacy of lyso-derivatives in anabolic metabolism of the organism is a plausible possibility.

2. Synthetic Approach

The general approach to the synthesis of the monophytanyl glycerol ether phosphatides involved the condensation of a suitably blocked monosilver salt of the monophytanyl ether phosphatidic acid with a protected glycerol iodohydrin derivative, followed by removal of the blocking groups by mild procedures (see Schemes 7, 8 and 9). This procedure is analogous to that used previously by van Deenen and co-workers for the synthesis of O-aminoacylphosphatidyl glycerols (Bonsen et al., 1965; De Haas et al., 1966; Bonsen and van Deenen, 1967) and Joo and Kates (1969) for the synthesis of diether analogues of phosphatidyl glycerol and phosphatidyl glycerophosphate.

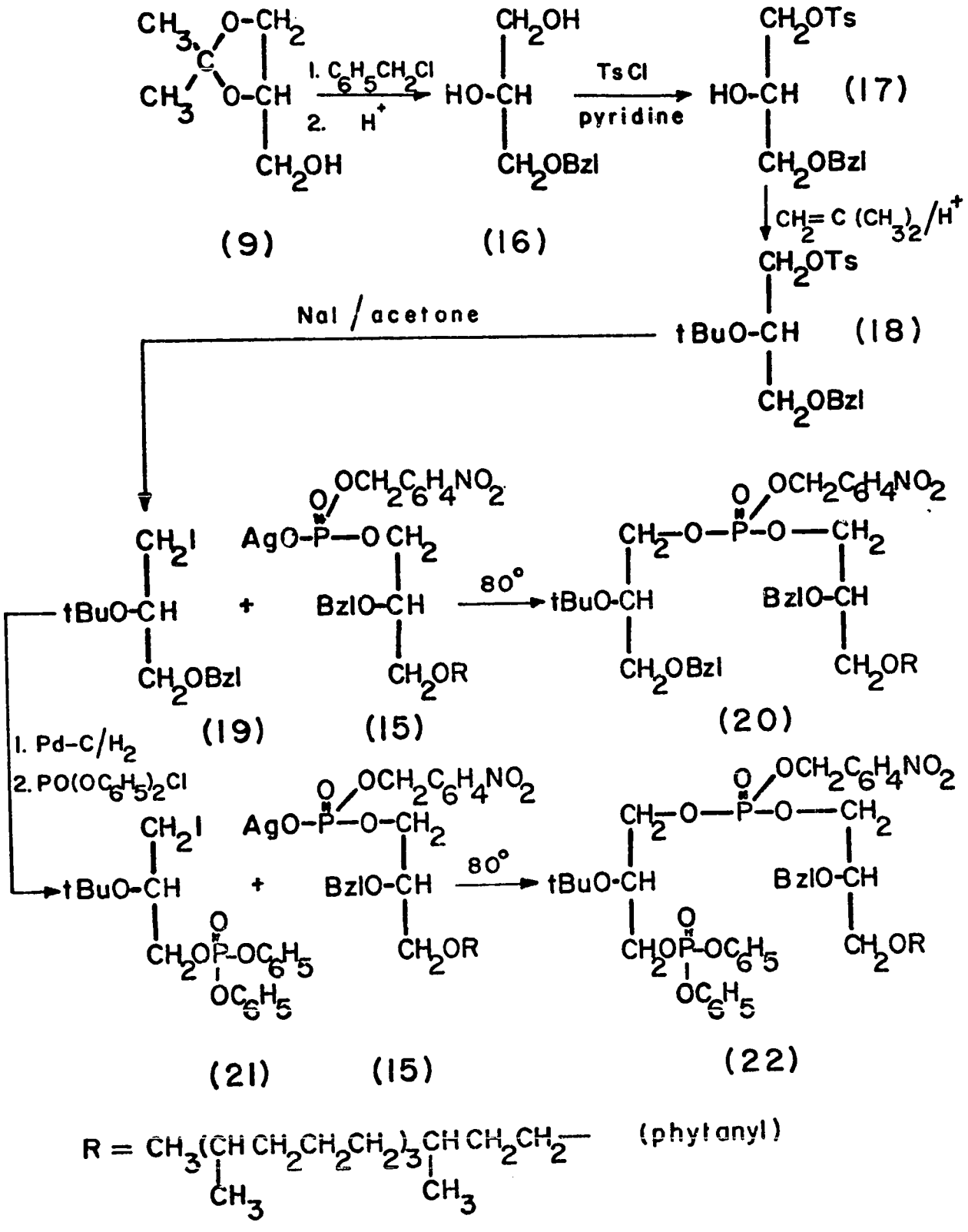
SCHEME 7

Synthesis of dipotassium monophytanyl ether phosphatidate (lysophosphatidic acid, dipotassium salt) and silver 1-(p-nitrobenzylphosphoryl)-2-O-benzyl-3-O-phytanyl-sn-glycerol


1. RBr/KOH
2. H+
1. (C6H5)3C Cl
2. C6H5CH2Cl/KOH
3. H+
1. Ts Cl/pyridine
2. NaI/acetone
1. Pd-C/H2
2. KOH
1. NaI/acetone
2. Ag+
(9)
(10)
(11)
(12)
(13)
(14)
(15)
R = CH3(CH CH2CH2CH2)3CH CH2CH2— (phytanyl)
CH3
CH3

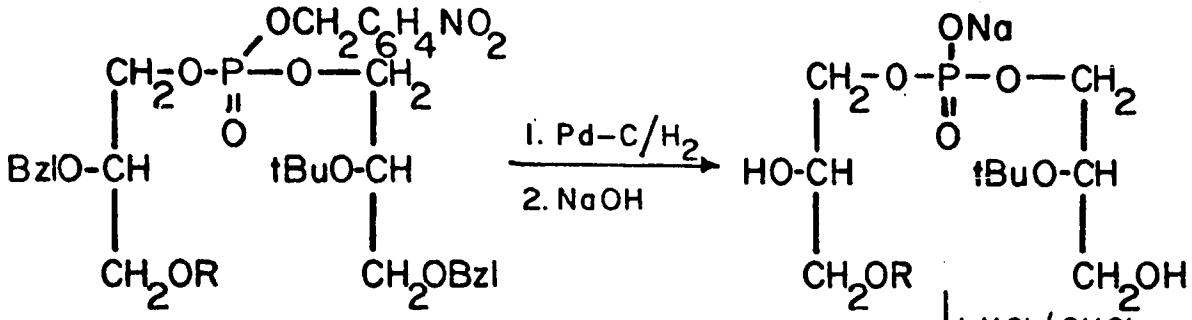
SCHEME 8

Synthesis of 3-O-phytanyl-2-O-benzyl-sn-glycero-1-p-nitrobenzyl-phosphoryl-1'-(2'-O-tert.-butyl-3'-O-benzyl)-sn-glycerol (fully blocked lysophosphatidyl glycerol)
and
3-O-phytanyl-2-O-benzyl-sn-glycero-1-p-nitrobenzylphosphoryl-1'-glycero-(2'-O-tert.-butyl-3'-diphenylphosphate) (fully blocked lysophosphatidyl glycerophosphate)


1. C6H5CH2Cl
2. H+
TsCl
pyridine
(9)
(16)
(17)
CH2=C(CH3)2/H+
(18)
NaI / acetone
80°
(19)
(15)
(20)
1. Pd-C/H2
2. PO(OC6H5)2Cl
(21)
(15)
(22)
R = CH3(CHCH2CH2CH2)3CHCH2CH2— (phytanyl)
CH3
CH3

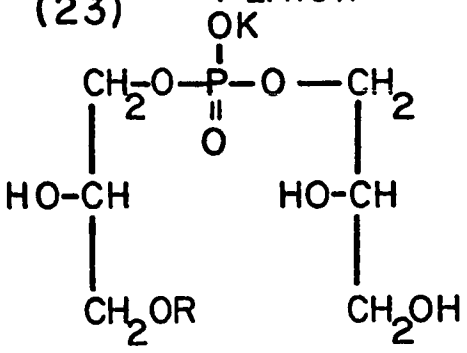
SCHEME 9

Synthesis of 3-O-phytanyl-sn-glycero-1-phosphoryl-1'-sn-glycerol (lysophosphatidyl glycerol) and 3-O-phytanyl-sn-glycero-1-phosphoryl-(1'-sn-glycero-3'-phosphate) (lysophosphatidyl glycerophosphate)

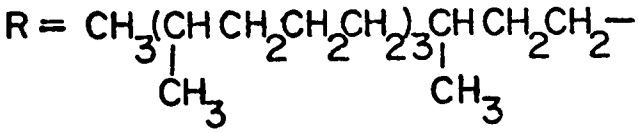


(20)

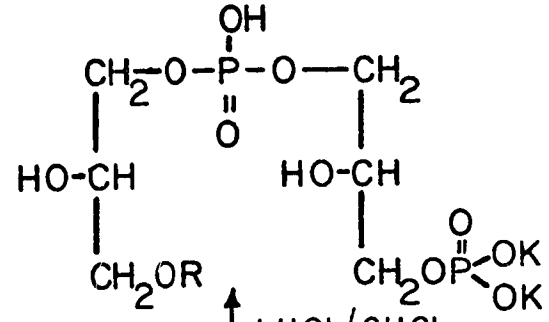
(23)



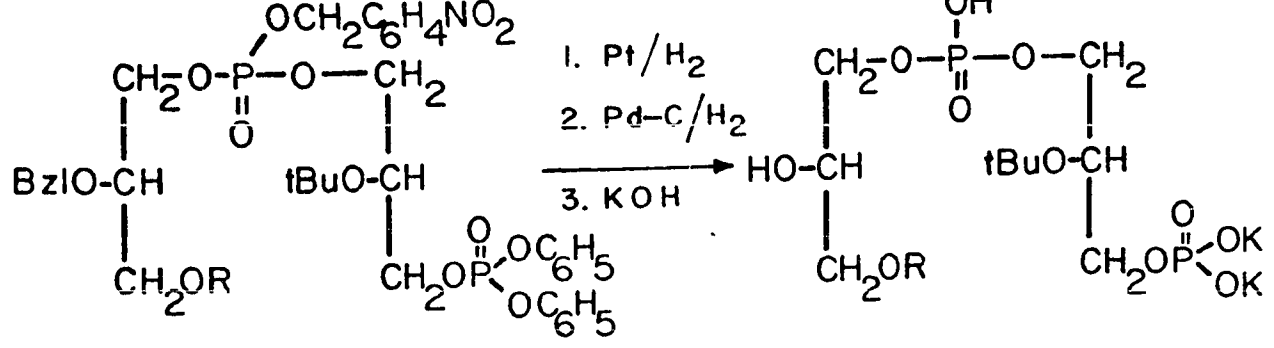
(24)



(phytanyl)



(26)



(22)

(25)

The starting material used was 3-O-phytanyl-sn-glycerol (10) which was synthesised by alkylation of 1, 2-isopropylidene-sn-glycerol (9) followed by removal of the blocking group, essentially as described by Joo et al.(1968). The 3-O-phytanyl-sn-glycerol (10) was converted to 2-O-benzyl-3-O-phytanyl-sn-glycerol (11) via the 1-O-trityl intermediate. The alcohol was tosylated and the tosylate was converted to the iodo derivative (12) by displacement reaction with iodide ion. Condensation of the iodo compound with silver di-p-nitrobenzylphosphate gave the phosphotriester (13), which was converted by catalytic hydrogenation directly to monophytanyl ether phosphatidic acid (14).

For the synthesis of lyso-PG and lyso-PGP (Scheme 9) partial anionic debenzylation of the triester (13) with sodium iodide, followed by treatment with silver ion, was used to prepare the required mono-p-nitrobenzyl silver salt intermediate (15). The substituted iodoglycerol used in the synthesis of lyso-PG was 1-iodo-2-O-tert.-butyl-3-O-benzyl-sn-glycerol (19) and that used in the synthesis of lyso-PGP was 1-iodo-2-O-tert.-butyl-3-diphenylphosphoryl-sn-glycerol (21). Both iodo compounds were synthesised according to Joo et al.(1969), as shown in Scheme 8. Condensation of the silver salt of the blocked monoether mono-p-nitrobenzyl phosphate (15) with iodo compound (19) or (21) gave the fully protected triesters (20) or (22), respectively. Removal of nitrobenzyl and benzyl groups from triester (20) and nitrobenzyl and phenyl groups from triester (22) was effected by catalytic hydrogenolysis; tert.-butyl groups were removed by treatment with anhydrous HCl in chloroform. The free acid forms of the resulting phosphatides were neutralised with ammonium hydroxide, purified by preparative TLC and converted to their respective potassium salt forms (24 and 26).

3. Experimental Procedures

(a) Synthesis of 3-O-phytanyl-1-O-phosphoryl-sn-glycerol (lysophosphatidic acid)

1, 2-Isopropylidene-sn-glycerol and 3-O-benzyl-sn-glycerol were synthesised as described in Part One, Experimental Procedures, III. 6.

RRR-RRS-phytanyl bromide was prepared from dihydrophytol [obtained from the catalytic hydrogenation of commercial phytol (Mann Laboratories)] as described by Kates et al. (1965) and had $[\alpha]_D^{22}$ -1.8° (c, 3.5 in chloroform), reported for RRR-RRS phytanyl iodide (Kates et al., 1965) $[\alpha]_D$ -0.75°.

RRR-Phytanyl bromide was prepared by cleavage of bacterial di-O-phytanyl glycerol ether with hydriodic acid, followed by conversion of the resultant phytanyl iodide to phytanyl bromide via the acetate and alcohol* intermediates (Joo et al., 1968). Alternatively, the phytanyl bromide could be obtained directly and in higher yield by cleavage of the diether at 0° with boron tribromide in carbon tetrachloride, essentially as described for cleavage with BCl_3 (Kates et al., 1965). The phytanyl bromide obtained by either procedure had $[\alpha]_D^{22}$ -3.47° (M_D -12.5°) (c, 12.6 in chloroform); reported for phytanyl iodide, M_D -25° (Kates et al., 1967).

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* RRR-phytanol $[\alpha]_D^{22}$ +2.58° (c, 6.1 in chloroform); reported (Kates et al., (1967) $[\alpha]_D^{22}$ +2.4°.

3-O-Phytanyl-sn-glycerol (monophytanyl glycerol ether) (10) was prepared from 1,2-isopropylidene-sn-glycerol using both RRR-RRS and RRR- phytanyl bromide, according to the method of Joo et al.(1968). The monoethers were purified by column chromatography on silicic acid and were eluted with chloroform. RRR-RRS-isomer $[\alpha]_D^{22}$ -2.10° (c, 3.4 in chloroform); reported (Kates et al., 1965) $[\alpha]_D^{22}$ -2.0° (c, 3.5 in chloroform. RRR-isomer $[\alpha]_D^{22}$ -0.71° (c, 3.9 in chloroform) and $[\alpha]_D^{22}$ -0.84° (c, 5.2 in chloroform); reported (Joo et al., 1968) $[\alpha]_D^{22}$ -0.94° (c, 2.0 in chloroform.

Anal. Calc. for $C_{23}H_{48}O_3$ (372.61); C, 74.13; H, 12.99.
Found: C, 74.02; H, 12.85.

Tri-p-nitrobenzyl phosphate, prepared from silver phosphate (Lipmann and Tuttle, 1944) was converted to silver di-p-nitrobenzyl phosphate as described by Zervas and Dilaris (1955, 1956). Both compounds analysed correctly for P. The silver salt was stored in the dry state at 5° in the absence of light.

1-O-Triphenylmethyl-3-O-phytanyl-sn-glycerol (1-O-trityl monoether)

A solution of 3-O-phytanyl-sn-glycerol [(10); 1.02 g, 2.75 mmole[in 20 ml of anhydrous pyridine was reacted with triphenyl chloromethane (2.00 g, 7.20 mmole) for 1 hr at 10° and then at room temperature for a further 24 hr. The reaction mixture was diluted with 25 ml of ice-water and the product isolated as described by Palameta and Kates (1966) for trityl mono-oleyl glycerol. The material obtained contained only traces of triphenylmethanol and glycerol monoether (Rf 0.77 and 0.35, respectively, on TLC in chloroform-diethyl ether, 3:1, v/v) in addition to the major spot corresponding to the 1-O-trityl-3-O-phytanyl-sn-glycerol (Rf 0.80 in chloroform-diethyl ether, 3:1, v/v). The impurities were

removed by chromatography on a column of silicic acid (25 g) (pre-washed with 100 ml of 90% methanol containing 5 g of sodium acetate) made up in petroleum ether (30-60°). Unwashed silicic acid columns in petroleum ether were found to be sufficiently acidic to detritylate the compound. Similar behaviour has previously been reported for tritylglycerylbenzyl ether (Buchnea and Baer, 1960). Elution with petroleum ether-benzene (2:1, v/v) gave triphenylmethanol. The lipid product was then eluted with benzene (500 ml) as a colourless oil which could not be induced to crystallise (1.52 g, 90%). $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{R}}$-$\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{S}}$ isomer; $[\alpha]_D^{22}$ -3.10° (c, 2.35 in chloroform).

Anal. Calc. for $C_{42}H_{62}O_3$ (614.92); C, 82.03; H, 10.16.
Found: $\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{R}}$-$\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{S}}$-isomer: C, 81.87; H, 9.81.

$\underline{\underline{R}}\underline{\underline{R}}\underline{\underline{R}}$-isomer: C, 82.54; H, 10.04.

The infrared spectrum (thin film) of the trityl monoether showed absorption for trityl (3100, 3070, 3040, 1600, 1490 cm^{-1}), hydroxyl (3600 sharp, 3450 cm^{-1} broad), phytanyl (2960, 2920, 2860, 1455, 1375-1365 cm^{-1}), and C-O-C ether groups (1110 cm^{-1}).

NMR assignments: phytanyl (39 H) 0.75-1.70 σ; glycerol methylene (6 H) 3.13-3.55 σ; glycerol methine (1 H) 3.90 σ; aromatic (15 H) 7.25-7.52 σ; hydroxyl (1 H) 2.40 σ.

1-O-Triphenylmethyl-2-O-benzyl-3-O-phytanyl-sn-glycerol

A mixture of 1-O-triphenylmethyl-3-O-phytanyl-sn-glycerol (510 mg, 0.83 mmole) in 50 ml of anhydrous benzene, 3.0 g of powdered KOH and 0.5 ml of freshly distilled benzyl chloride was refluxed for 18 hr. The water produced was trapped by means of a phase separating head. TLC examination of the solution showed a major spot (Rf 0.75 in benzene) corresponding to the benzylated product and a minor spot (Rf 0.65 in benzene) with the same mobility as dibenzyl ether.

The mixture was diluted with 100 ml of ice-water, the benzene layer was removed and the aqueous phase was extracted with four 20-ml portions of diethyl ether. The combined benzene and ethereal extracts were washed with water until the washings were neutral and the organic layer was dried by repeated evaporation with benzene under reduced pressure. The oil was freed of benzyl chloride by high-vacuum distillation and the residue was hydrolysed without further purification.

For analytical purposes, a portion (30 mg) was purified on a silica column (5 g) prepared in petroleum ether (30-60°); elution with a mixture of petroleum ether-benzene (1:1, v/v) gave 14 mg of colourless oil, whose infrared spectrum showed typical aromatic and phytanyl group absorption bands and no hydroxyl absorption bands.
RRR-RRS-isomer; $[\alpha]_D^{22}$ -2.78° (c, 6.0 in chloroform).

Anal. Calc. for $C_{49}H_{68}O_3$ (705.03): C, 83.49; H, 9.72.
Found: RRR-RRS-isomer: C, 83.18; H, 9.64.

2-O-Benzyl-3-O-phytanyl-sn-glycerol (11)

1-O-Triphenylmethyl-2-O-benzyl-3-O-phytanyl-sn-glycerol (500 mg, 0.71 mmole) was hydrolysed at reflux temperature in 60 ml of aqueous-methanolic HCl (methanol-water-conc. HCl, 18:1:1, v/v) for 6 hr. TLC examination showed a new slow-moving spot (Rf 0.27 in benzene) and two other spots corresponding to triphenylmethanol and dibenzyl ether.

The cooled hydrolysate was extracted with four 50-ml portions of petroleum ether (30-60°) and the combined extracts were washed with water until the washings were neutral. The solution was diluted with benzene and evaporated under reduced pressure. The residual yellow oil (408 mg) was purified on a column of silicic acid (20 g), prepared in petroleum ether (30-60°)-benzene (1:1, v/v); triphenylmethanol and then dibenzyl ether were eluted by this solvent (500 ml); elution with chloroform (600 ml) yielded 238 mg (72%) of the benzylated monoether (11) as a colourless oil, with Rf 0.56 and 0.27 in chloroform-diethyl ether (3:1, v/v) and benzene, respectively. An analytical sample was obtained by preparative TLC in chloroform-diethyl ether (3:1, v/v).

RRR-isomer: $[\alpha]_D^{22}$ +12.1° (c, 2.9 in chloroform)

Anal. Calc. for $C_{30}H_{54}O_3$ (462.73): C, 77.86; H, 11.76.

Found: RRR-RRS-isomer: C, 77.69; H, 11.56.

RRR-isomer: C, 77.52; H, 11.24.

The infrared spectrum (thin film) showed hydroxyl absorption (3460 cm^{-1}, broad) in addition to phytanyl and aromatic absorption.

NMR assignments: phytanyl (39 H) 0.75-1.65 δ; glycerol methylene and methine (7 H) 3.30-3.80 δ; benzylic methylene (2 H) 4.70 δ; aromatic (5 H) 7.35 δ.

1-O-p-Toluenesulphonyl-2-O-benzyl-3-O-phytanyl-sn-glycerol

To a solution of 2-O-benzyl-3-O-phytanyl-sn-glycerol [(11); 770 mg, 1.65 mmole] in 10 ml anhydrous pyridine at 0° was added freshly recrystallised p-toluenesulphonyl chloride (620 mg, 4.0 mmole) and the mixture was then stirred at 10° for 16 hours. Although no crystalline precipitate was evident, TLC examination indicated that tosylation was complete. The reaction mixture was poured into 100 ml of ice-water and stirred vigorously (10 min). The mixture was extracted with four 30-ml portions of petroleum ether (30-60°). The combined extracts were washed with water (100 ml), ice-cold 2 N aqueous HCl (100 ml), and water (100 ml) and dried by evaporation with benzene under reduced pressure to give the tosyl derivative as a colourless oil (809 mg, 82% yield); Rf 0.58 in chloroform. An analytical sample was obtained by preparative TLC in chloroform. RRR-isomer: $[\alpha]_D^{22}$ +1.43° (c, 6.8 in chloroform).

Anal. Calc. for $C_{37}H_{60}O_5S$ (616.91): C, 72.03; H, 9.80; S, 5.20.
Found: RRR-RRS-isomer: C, 72.06; H, 9.69; S, 5.71.
RRR-isomer: C, 71.93; H, 9.81; S, 5.35.

The infrared spectrum (thin film) showed tosyl group absorption (1182, 1195 cm^{-1} doublet) and aromatic absorption; no hydroxyl group absorption was apparent.

NMR assignment: phytanyl (39 H) 0.78-1.85 δ; CH_3-C_6H_4- (sharp singlet) 2.42 δ; methylene and methine (5 H) 3.30-4.20 δ; -O-CH_2-C_6H_5 (sharp singlet) 4.57 δ; C_6H_4-CH_3 (multiplet, centred 7.23 δ); -CH_2OSO_2- (doublet) 7.76 δ.

1-Iodo-2-O-benzyl-3-O-phytanyl-sn-glycerol (12)

Dry sodium iodide (900 mg, 6.00 mmole) was added to a solution of 1-O-p-toluenesulfonyl-2-O-benzyl-3-O-phytanyl-sn-glycerol (697 mg, 1.15 mmole) in 15 ml of anhydrous acetone; the solution was stirred at room temperature for three hours and then refluxed for 20 hr. The crystalline precipitate of sodium tosylate was removed by centrifugation and washed with two 5-ml portions of cold acetone. The combined supernatants were evaporated under reduced pressure to give an oily solid which was extracted with four 25-ml portions of diethyl ether. The ethereal solution was centrifuged and then washed with two 25-ml portions of 5% aqueous sodium thiosulfate solution and then water (50 ml). The organic phase was diluted with benzene and evaporated under reduced pressure to give the iodo derivative (12) as a colourless oil (640 mg, 97% yield). The product was used at once for the next step of the synthesis. RRR-isomer: $[\alpha]_D^{22}$ +2.09° (c, 2.2 in chloroform).

Anal. Calc. for $C_{30}H_{53}O_2I$ (572.63); C, 62.92; H, 9.33; I, 22.16.

Found: RRR-isomer: C, 63.07; H, 9.20; I, 21.08.

The infrared spectrum of the iodo derivative (thin film) showed no hydroxyl or tosyl group absorption.

NMR assignments: phytanyl (39 H) 0.78-1.85 δ; methylene and methine (7 H) 3.40-3.80 δ; O-C$\underline{H}_2$-C_6H_5 (sharp singlet) 4.73 δ; aromatic (5 H) 7.42 δ.

3-O-Phytanyl-2-O-benzyl-1-O-(di-p-nitrobenzylphosphoryl)-sn-glycerol (13)

A solution of 1-iodo-2-O-benzyl-3-O-phytanyl-sn-glycerol [(12), 620 mg, 1.08 mmole] in 15 ml of anhydrous toluene was heated to reflux with silver di-p-nitrobenzylphosphate (1.9 g, 4.0 mmole) in the absence of light for 20 hr. Silver salts were removed by centrifugation and washed twice with 5 ml portions of benzene; the combined supernatants were evaporated under reduced pressure. The residual yellow oil (777 mg) was purified by chromatography on a column of silicic acid (25 g). After elution of non-polar material (30 mg) with petroleum ether-benzene (1:1, v/v) the phosphorylated product [585 mg, 72% yield, (13)] was eluted with benzene.

An analytical sample was obtained by preparative TLC in chloroform-diethyl ether (3:1, v/v) as a faintly yellow oil, Rf 0.52.

RRR-RRS-isomer: $[\alpha]_D^{22}$ +2.5° (c, 1.20 in chloroform)

RRR-isomer: $[\alpha]_D^{22}$ +4.4° (c, 2.78 in chloroform).

Anal. Calc. for $C_{44}H_{65}O_{10}N_2P$ (812.95); C, 65.00; H, 8.06; N, 3.45; P, 3.82.

Found: RRR-isomer: C, 64.65; H, 7.82; N, 3.36; P, 3.92.

The infrared spectrum (thin film) showed strong absorption bands for nitro (1350, 1525 cm^{-1}), triester P=O (1285 cm^{-1}) and P-O-C (1035 cm^{-1}) groups, in addition to phytanyl and aromatic group absorption. Hydroxyl group absorption was absent (Fig. 44.1).

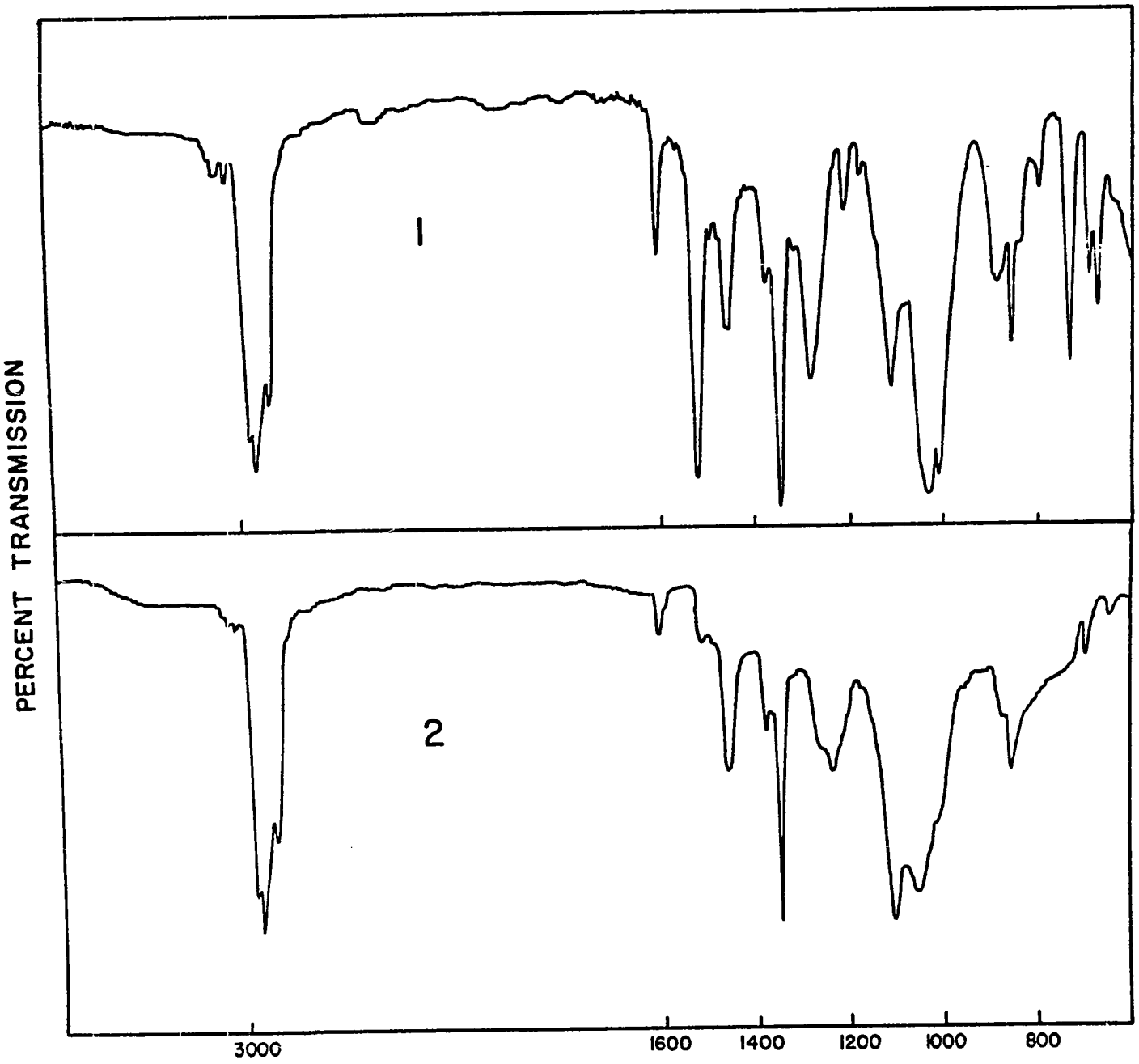
NMR assignments: phytanyl (39 H) 0.75-1.90 δ; methylene and methine (7 H) 3.35-4.42 δ; $-C\underline{H}_2OC_6H_5$ 4.70 δ; $-OC\underline{H}_2-C_6H_4NO_2$ 7.35 δ; $-O-CH_2-C_6\underline{H}_4NO_2$ (8 H) quartet, centred 7.84 δ.

FIGURE 44

Infrared absorption spectra (1, liquid film; 2, CCl_4) of:

1 3-O-Phytanyl-2-O-benzyl-1-(di-p-nitrobenzylphosphoryl)-sn-glycerol (fully blocked monoether phosphatidic acid)

2 3-O-Phytanyl-2-O-benzyl-1-(p-nitrobenzylphosphoryl)-sn-glycerol, sodium salt


PERCENT TRANSMISSION
1
2
3000
1600
1400
1200
1000
800

3-O-Phytanyl-1-O-phosphoryl-sn-glycerol, potassium salt.
[Lysophosphatidic acid (14)]

1-O-Di-p-nitrobenzylphosphoryl-2-O-benzyl-3-O-phytanyl-sn-glycerol [(13); 155 mg, 0.19 mmole] was hydrogenolysed in 10 ml of anhydrous ethanol at room temperature and atmospheric pressure with freshly prepared palladium-charcoal catalyst (from 200 mg palladium chloride). The reaction was complete in 1 hr, when the catalyst was removed by centrifugation and washed with two 5-ml portions of chloroform-ethanol (1:1, v/v). The supernatant and washings were combined and concentrated to dryness under reduced pressure. To a solution of the oil in 5 ml chloroform was added 5 ml methanol and 4.5 ml 1.0 N HCl, and the biphasic system was briefly centrifuged. The chloroform phase was neutralised to the phenolphthalein end point (external indicator) with 0.2 N methanolic KOH, evaporated in the presence of benzene to 0.5 ml, diluted with 5 ml of acetone and cooled on ice. The gelatinous precipitate which slowly formed was centrifuged, washed with 0.5 ml of cold acetone and reprecipitated from its chloroform solution by ten volumes of diethyl ether. The lysophosphatidic acid (14) was dried in vacuo to give 44 mg (45% yield) of chromatographically pure white solid (for Rf values see Table 20 and Fig. 47.1.

RRR-isomer: $[\alpha]_D^{22}$ +7.69° (c, 2.0 in chloroform).

Anal. Calc. for $C_{23}H_{47}O_6PK_2$ (528.87); C, 52.23; H, 8.98; P, 5.85.
Found: RRR-isomer: C, 51.98; H, 8.86; P, 5.83.

The infrared spectrum of lysophosphatidic acid (potassium salt) is shown in Fig. 47.1

(b) Synthesis of lysophosphatidyl glycerol

Sodium-1-O-(mono-p-nitrobenzylphosphoryl)-2-O-benzyl-3-O-phytanyl-sn-glycerol

A solution of 1-O-(di-p-nitrobenzylphosphoryl)-2-O-benzyl-3-O-phytanyl-sn-glycerol [(13); 270 mg, 0.33 mmole] in 20 ml of anhydrous acetone and sodium iodide (500 mg, 3.3 mmole) was refluxed with stirring for 6 hr. The solvent was evaporated under reduced pressure to give an oily solid, which was washed thoroughly with chloroform. The extract was cooled to 0° and centrifuged; the supernatant was evaporated to give a dark oil (448 mg), which was purified on a silicic acid column (20 g) prepared in hexane. Chloroform eluted a dark oil, whose infrared spectrum was consistent with that expected for p-nitrobenzyl alcohol. Chloroform-methanol (1:1, v/v) eluted the product as a brown oil (169 mg) with Rf 0.33 and 0.76 in chloroform-methanol (9:1, v/v) and chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v), respectively.

An analytical sample was obtained as a yellow gum by preparative TLC in chloroform-methanol-water (80:20:2, v/v), (Rf 0.50). The product was eluted from the silica with chloroform-methanol-diethyl ether (1:1:1, v/v). RRR-isomer $[\alpha]_D^{22}$ +4.29° (c, 4.2 in chloroform).

Anal. Calc. for $C_{37}H_{59}O_8NPNa$ (699.81): C, 63.60; H, 8.50; N, 2.00; P, 4.43.
Found: RRR-RRS-isomer: C, 63.61; H, 8.57; N, 2.09; P, 4.40.

The infrared spectrum (CCl_4) showed absorption bands for nitro (1350, 1520 cm^{-1}), diester P=O (1220 cm^{-1}), P-O-C (1050 cm^{-1}), and ionised P-O^- (1095 cm^{-1}) groups. Hydroxyl group absorption bands were absent.

Silver 1-(mono-p-nitrobenzylphosphoryl)-2-O-benzyl-3-O-phytanyl-sn-glycerol (15)

A solution of silver nitrate (170 mg, 1.0 mmole) in 7.5 ml acetone-water (2:1, v/v) was added to sodium 1-O-mono-p-nitrobenzylphosphoryl-2-O-benzyl-3-O-phytanyl-sn-glycerol (560 mg, 0.805 mmole) in 5 ml of acetone at 35°, with the exclusion of light. The oily precipitate of silver salt (15) was centrifuged down, washed with two 0.5 ml portions of cold acetone and dried by repeated evaporation in the presence of benzene under reduced pressure to give 600 mg of 15 (95% yield). An analytical sample was obtained by preparative chromatography as described for the corresponding sodium salt and the remaining product was used at once for the next step of the synthesis.

RRR-isomer $[\alpha]_D^{22}$ +6.13° (c, 1.70 in chloroform).

Anal. Calc. for $C_{37}H_{59}O_8NPAg$ (784.69): C, 56.63; H, 7.58; N, 1.79. P, 3.95.

Found: RRR-isomer: C, 57.21; H, 7.47; N, 1.57; P. 3.90

3-O-Phytanyl-2-O-benzyl-sn-glycero-1-p-nitrobenzylphosphoryl-1'-(2'-O-tert.-butyl-3'-O-benzyl)-sn-glycerol. [Fully blocked lysophosphatidyl glycerol, monophytanyl ether analogue (20)]

The dry silver salt of the blocked phytanyl monoether [(15); 310 mg, 0.39 mmole] was refluxed with stirring in anhydrous benzene with 1-iodo-2-O-tert.-butyl-3-O-benzyl-sn-glycerol [(19); 140 mg, 0.40 mmole] in the absence of light for 12 hr. The precipitated silver iodide was centrifuged down, washed with two 5 ml portions of benzene and the combined benzene solutions were concentrated to dryness under reduced pressure to give 350 mg of faintly yellow oil having Rf 0.60 in chloroform-diethyl ether (3:1, v/v). Traces of iodo starting material were removed by chromatography on silicic acid (20 g) using benzene (50 ml) as eluting solvent. The product (20) was eluted with benzene-chloroform (1:2, v/v). Solvents were removed under reduced pressure and the fully blocked lysophosphatidyl glycerol was dried in vacuo to give 247 mg of chromatographically pure product (71% yield). RRR-isomer: $[\alpha]_D^{22}$ +5.75° (in chloroform).

Anal. Calc. for $C_{51}H_{80}O_{10}NP$ (898.13); C, 68.21; H, 8.98; N, 1.56; P, 3.45. Found: RRR-isomer: C, 68.22; H, 9.16; N, 1.65; P, 3.48.

The infrared spectrum (Fig. 45.1) of a thin film showed absorption bands for phytanyl (2960, 2915, 2860, 1455, 1375-1365 cm^{-1}), nitro (1520, 1345 cm^{-1}), triester P=O (1280 cm^{-1}), P-O-C (1030 cm^{-1}) and aromatic (720, 790, 1500, 1600 cm^{-1}) groups. Hydroxyl absorption bands were absent.

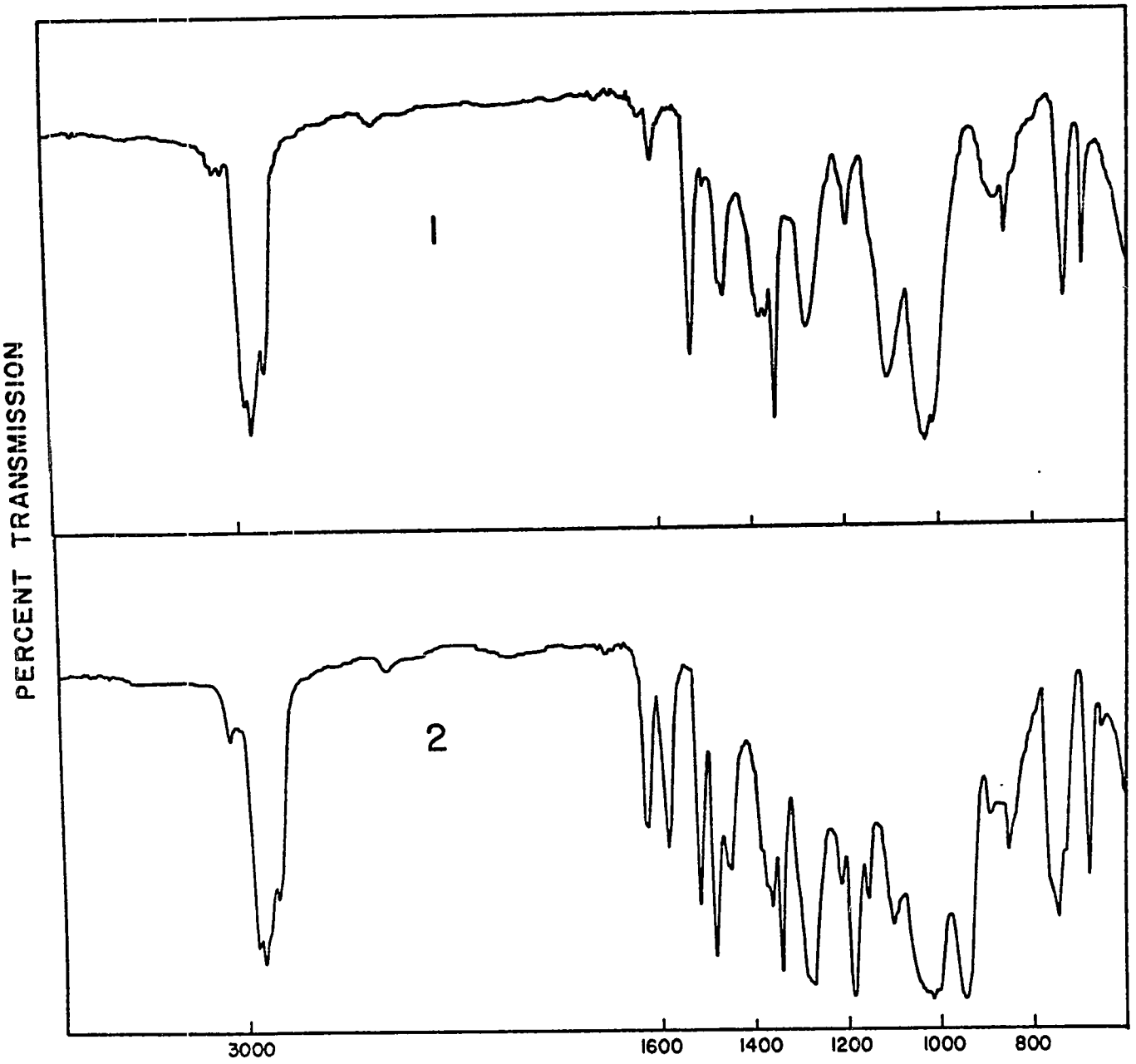
NMR assignments: phytanyl (39 H) and tert. butyl (9 H) 0.75 - 1.65 δ; glycerol methylene and methine (10 H) 3.30 - 4.40 δ; C_6H_5-CH_2 (4 H) 4.65 δ and 4.54 δ; $OCH_2C_6H_4NO_2$ (2 H) doublet, centred 5.20 δ; aromatic (14 H) 7.25 - 8.28 δ.

FIGURE 45

Infrared absorption spectra (liquid film) of:

1 3-O-Phytanyl-2-O-benzyl-sn-glycero-1-p-nitrobenzylphosphoryl-1'-(2'-O-tert.-butyl-3'-O-benzyl)-sn-glycerol (fully blocked lysophosphatidyl glycerol, monophytanyl ether analogue)

2 3-O-Phytanyl-2-O-benzyl-sn-glycero-1-p-nitrobenzylphosphoryl-1'-sn-glycero-(2'-O-tert.-butyl-3'-diphenylphosphate) (fully blocked lysophosphatidyl glycerophosphate, monophytanyl ether analogue)


PERCENT TRANSMISSION
1
2
3000
1600
1400
1200
1000
800

3-O-Phytanyl-sn-glycero-1-phosphoryl-1'-(2'-O-tert.-butyl)-sn-glycerol, sodium salt (23)

The fully blocked lysophosphatidyl glycerol [(20): 226 mg, 0.252 mmole] was hydrogenolysed in 15 ml anhydrous methanol with palladium-charcoal catalyst (freshly prepared from 250 mg palladium chloride) at room temperature and pressure during 2 hr. The catalyst was removed by centrifugation, washed with two 5 ml portions of chloroform-methanol (2:1, v/v) and the combined supernatant and washings were concentrated to near dryness. A solution of the oily product in 5 ml of chloroform was diluted with 10 ml of methanol and 4.5 ml of 1.0 N aqueous HCl and the mixture was gently shaken and briefly centrifuged. The chloroform phase was removed and neutralised at once with 0.2 N methanolic NaOH, to the phenolphthalein end point (external indicator); the solution was concentrated in the presence of benzene under a stream of nitrogen to a small volume and diluted with 10 volumes of acetone. After cooling on ice, the oily precipitate was centrifuged, washed with 0.2 ml of cold acetone and dried in vacuo. The yield of chromatographically pure 2'-O-tert.-butyl-lysophosphatidyl glycerol (23) was 119 mg, 78%; Rf 0.51 and 0.64 in chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v) and chloroform-methanol-water (65:35:5, v/v) respectively.

RRR-isomer $[\alpha]_D^{22}$ +8.8° (c, 2.4 in chloroform).

Anal. Calc. for $C_{30}H_{62}O_8PNa$ (604.77); P, 5.12.

Found: P, 4.97.

3-O-Phytanyl-sn-glycero-1-phosphoryl-1'-sn-glycerol, monopotassium salt [lysophosphatidyl glycerol (24)]

To a solution of the sodium salt of the tert.-butyl compound [(23); 100 mg, 0.17 mmole) in 3 ml of anhydrous chloroform at 0° was added 12 ml of an anhydrous solution of hydrogen chloride in chloroform (ca. 0.5 N). The mixture was kept in a stoppered centrifuge tube at 0° for 90 min, by which time formation of monophytanyl glycerol ether by cleavage of the phosphodiester linkage had begun. The reaction was stopped by the addition of 15 ml of methanol and 13.5 ml of water. The mixture was shaken, centrifuged briefly and the chloroform phase was immediately neutralised with 0.2 N methanolic ammonium hydroxide. The solution was diluted with benzene and evaporated to dryness under reduced pressure. The lyso-derivative (24) was freed from accompanying neutral impurities by precipitation of its solution in chloroform with acetone after conversion to the potassium salt as described previously. Yield: 41 mg (41%).

The desired product gave a phosphorus-positive spot on TLC with Rf 0.23 and 0.48 in chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v), and chloroform-methanol-90% acetic acid (30:4:20, v/v), respectively. An analytical sample was obtained by preparative TLC in chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v); the eluting solvent was chloroform-methanol-diethyl ether (1:1:1, v/v).

$[\alpha]_D^{22}$ +4.1° (c, 2.9 in chloroform).

Anal. Calc. for $C_{26}H_{54}O_8PK.H_2O$ (582.78); C, 53.58; H, 9.69; P, 5.32. Found: RRR-isomer: C, 53.58; H, 9.12; P, 5.12.

The infrared spectrum of lysophosphatidyl glycerol (CCl_4) is shown in Fig. 47.2.

(c) Synthesis of lysophosphatidyl glycerophosphate

3-O-Phytanyl-2-O-benzyl-sn-glycero-1-p-nitrobenzylphosphoryl-1'-sn-glycero-(2'-O-tert.-butyl-3'-diphenylphosphate). [Fully blocked lysophosphatidyl glycerophosphate, monophytanyl ether analogue (22)]

A suspension of the dry silver salt of the blocked monoether [(15); 310 mg, 0.40 mmole] in 25 ml of dry benzene was refluxed in the absence of light with stirring for 12 hr with 1-iodo-2-O-tert.-butyl-3-O-diphenylphosphoryl-sn-glycerol [(21); 207 mg, 0.42 mmole]. The precipitated silver iodide was centrifuged down and washed with two 5 ml portions of benzene.

The combined supernatant and washings were concentrated to dryness under reduced pressure, yielding 400 mg of faintly yellow oil which on TLC in chloroform-diethyl ether (3:1, v/v) gave essentially one spot (Rf 0.40). The product (22) was freed from traces of iodo starting material by column chromatography on 20 g silicic acid, which was eluted with benzene (50 ml); benzene-chloroform (1:1) 100 ml and chloroform (400 ml). The chromatographically pure product appeared in the chloroform eluate: yield 338 mg (82%). RRR-isomer $[\alpha]_D^{22}$ +5.38° (c, 2.1 in chloroform.

Anal. Calc. for $C_{56}H_{83}O_{13}NP_2$ (1040.2); C, 64.66; H, 8.04; N, 1.35; P, 5.96.

Found: RRR-isomer: C, 64.56; H, 7.90; N, 1.54; P, 5.91.

The infrared spectrum (Fig. 45.2) (thin film) resembled that of the blocked phosphatidyl glycerol analogue, but differed in having more intense triester P=O (1280 cm^{-1}) and aromatic absorption bands (1605, 1495 cm^{-1}). Hydroxyl absorption bands were absent.

The NMR spectrum also resembled that of the blocked phosphatidyl glycerol derivative, except for the absence of the benzylic methylene ($-CH_2C_6H_5$) signal at 4.54 δ.

3-O-Phytanyl-sn-glycero-1-phosphoryl-1'-(2'-O-tert.-butyl)-sn-glycero-3'-phosphate, [25]

Removal of the phenyl blocking groups from the pure fully-protected derivative (22) by hydrogenolysis over platinum proceeded smoothly. However, the removal of benzyl and p-nitrobenzyl blocking groups over palladium-charcoal caused extensive decomposition, the main product identified by TLC being monophytanyl glycerol ether. Three phosphate-containing products were also detected; one of them (Rf 0.15 in chloroform-methanol-30% ammonium hydroxide, 65:35:5, v/v) was likely to be the tert.-butyl derivative of lyso-PGP (25). This compound was isolated by preparative TLC in the solvent system above and converted to the ammonium salt (yield 8 mg).

Anal. Calc. for $C_{30}H_{62}O_{11}P_2(NH_4)_2$ (696.83): P, 8.88.

Found: P, 8.29.

The compound gave monophytanylglycerol ether as the sole chloroform-soluble product on 2.5% methanolic-HCl hydrolysis, and on the basis of this evidence and the P-analysis value, the 2-O-tert.-butyl-lyso-PGP structure can be tentatively assigned.

3-O-Phytanyl-sn-glycero-1-phosphoryl-1'-sn-glycero-3'-phosphate [26]

The tert.-butyllyso-PGP (25) was subjected to 0.5 M HCl in anhydrous chloroform to remove the tert.-butyl protecting group as described for the corresponding lyso-PG derivative (23).

The product was purified by TLC (Rf 0.52) in chloroform-methanol-30% ammonium hydroxide (12:10:2, v/v) [Rf diphytanyl ether analogue of PGP, 0.78], giving ca. 1 mg of chromatographically pure material. The Rf value of this material in chloroform-methanol-90% acetic acid was 0.40 and its methanolysis in 2.5% methanolic-HCl gave monophytanylglycerol ether.

It is considered likely that this material is the monophytanyl analogue of PGP (lyso-PGP) on the following basis:

1) chromatographic mobility relative to the diphytanyl ether analogue of PGP ;
2) TLC characterisation of its chloroform-soluble methanolysis product;
3) the authenticity of compound (22), the fully protected derivative of lyso-PGP.

However, complete analytical and spectral data are not available and this structure assignment must be taken as tentative.

(d) Hydrolysis of lysophosphatidic acid

Samples of lysophosphatidic acid (potassium salt) containing ca. 65 μg of P were hydrolysed under three different conditions as follows:

(i) the sample was heated in 4 ml of 2.5% methanolic hydrogen chloride under reflux for 1, 3 and 5 hr, respectively; to the cooled hydrolysate were added 4 ml of chloroform and 3.6 ml of water and the biphasic system was centrifuged. Each phase was analysed for total P.

(ii) The sample was treated with 1 ml of 2 N methanolic-HCl (prepared by diluting 10 ml of conc. HCl with 50 ml of methanol) in a sealed tube at 130° for 72 hr. The cooled tube was opened and the contents were quantitatively transferred to a centrifuge tube using successively 1.6 ml of water, 1.2 ml of methanol and 2 ml of chloroform. The two phases were analysed for P.

(iii) Samples were treated with 1 ml of 2 N aqueous HCl in a sealed tube at 130° for 12, 24 and 72 hr respectively. Each tube after hydrolysis was washed out with 0.8 ml of water, 2.0 ml of methanol and 2.0 ml of chloroform. The two phases from each tube were analysed for P.

4. Results and Discussion

(a) Monophytanyl phosphatidic acid

The synthesis of monophytanyl phosphatidic acid was carried out by a procedure analogous to that used by Kates et al (1971) for the synthesis of diphytanyl phosphatidic acid. The phosphorylation step in both procedures involved a glycerol iodohydrin condensation with silver di-p-nitrobenzyl phosphate, followed by catalytic removal of the aromatic protecting groups. However, the monophytanyl glycerol ether starting material for the lyso-PA synthesis was only available by direct chemical synthesis (see Scheme 7). Prior to the phosphorylation step the C-2 position of the glycerol moiety was protected by benzylation and the benzyl group was later removed by hydrogenolysis , a procedure mild enough to preclude phosphate group migration and consequent racemisation.

The lyso-PA dipotassium salt was obtained as a chromatographically and analytically pure solid. Unlike the diphytanyl ether PA (Kates et al., 1971), the salt's elemental analysis did not indicate the presence of water of hydration. The salt was freely soluble in chloroform and benzene, sparingly soluble in methanol and carbon tetrachloride and insoluble in acetone and diethyl ether. The molecular rotation of the dextrorotatory potassium salt of lyso-PA was higher than that of the diphytanyl PA potassium salt.

The chromatographic behaviour of the lyso-PA was typical of a primary phosphate, resembling that of diphytanyl ether PA (Kates et al., 1971). The chromatographic mobility was strongly dependent on the ionic form in which the lipid was chromatographed (Table 20 and Fig. 46). Thus, in acidic solvents, in which the migrating species would be largely the unionised free acid form, the initially applied potassium salt had the same

TABLE 20

Chromatographic Mobilities of Synthetic Lysophosphatidic Acid and Lysophosphatidyl Glycerol

Compound	Rf in Solvent System A	B	C
lyso-PA	0.00	0.54	0.62
lyso-PG	0.24	0.48	0.44

Solvent systems:

A Chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v)

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

C Diisobutylketone-acetic acid-water (40:25:5, v/v) on silicic acid-impregnated paper

FIGURE 46

Thin-layer chromatograms of lysophosphatidic acid in alkaline, acid and neutral solvent systems:

TL Total polar lipids H. cutirubrum

1 lysophosphatidic acid (monophytanyl analogue)

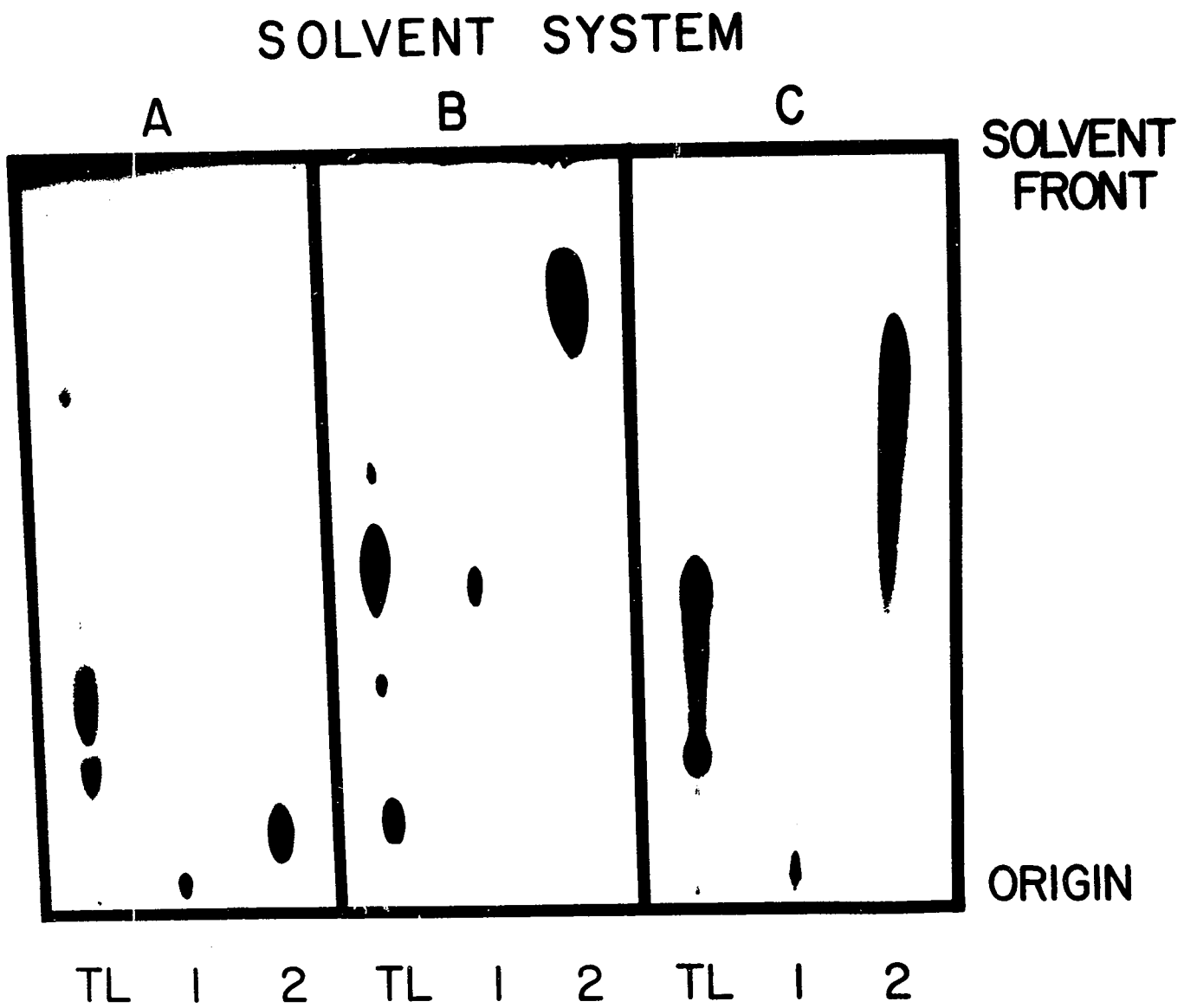
2 phosphatidic acid (diphytanyl analogue)

Solvent systems:

A chloroform-methanol-30% ammonium hydroxide (65:35:5, v/v)

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

C chloroform-methanol-water (65:35:5, v/v)


SOLVENT SYSTEM
A
B
C
SOLVENT FRONT
ORIGIN
TL 1 2 TL 1 2 TL 1 2

high mobility as the acid form (Fig. 46B). In basic solvents, the initially applied potassium salt and the free acid form had very low mobility (Fig. 46A). Neutral, or weakly acidic solvents were found unsuitable for chromatography of the lyso-PA as they caused severe streaking , presumably due to the formation of a mixture of molecular species having different degrees of ionisation (Fig. 46C). As expected, the lyso-PA possessed lower mobility than diphytanyl ether PA in all solvent systems used.

The infrared spectrum of the potassium salt (Fig. 47.1) resembled that of diphytanyl ether PA (Kates et al., 1971), showing in addition to phytanyl group absorption bands, P-O$^-$ (1090 cm^{-1}) and hydroxyl (3200 cm^{-1}) absorption bands. The solvent obscured the expected absorption at 1220 cm^{-1} for P=O, but two sharp bands (980 and 960 cm^{-1}) were evident, which were not present in the spectrum of the free acid form. These bands are unassigned although Kates et al.(1971) have reported a band at 980 cm^{-1} to be consistently present in the spectra of salt forms of long chain monoalkyl phosphates and of diacylphosphatidic acids.

The most striking chemical characteristic of the lyso-PA was its resistance to acid hydrolysis (see Table 21). Kates et al.(1971) have recently reported a comparable acid stability in diphytanyl ether PA. The lyso derivative could be recovered unchanged after 5 hr reflux with 2.5% methanolic-HCl, a treatment which, however, quantitatively cleaves the phosphodiester bond in the diether phosphatides of H. cutirubrum. Complete hydrolysis of the phosphate-glycerol linkage in lyso-PA was achieved after 72 hr reflux treatment with aqueous HCl at 130°. This resistance to hydrolysis was not merely a question of low lipid solubility in the aqueous acid, because the replacement of water by methanol, in which the free acid form of lyso-PA is freely soluble, gave rise to less hydrolysis under the same conditions [Hydrolysis (2), Table 21].

FIGURE 47

Infrared absorption spectra (1, $CHCl_3$; 2, CCl_4) of

1 monophytanylphosphatidic acid (potassium salt), lyso-PA

2 monophytanylphosphatidyl glycerol (potassium salt), lyso-PG

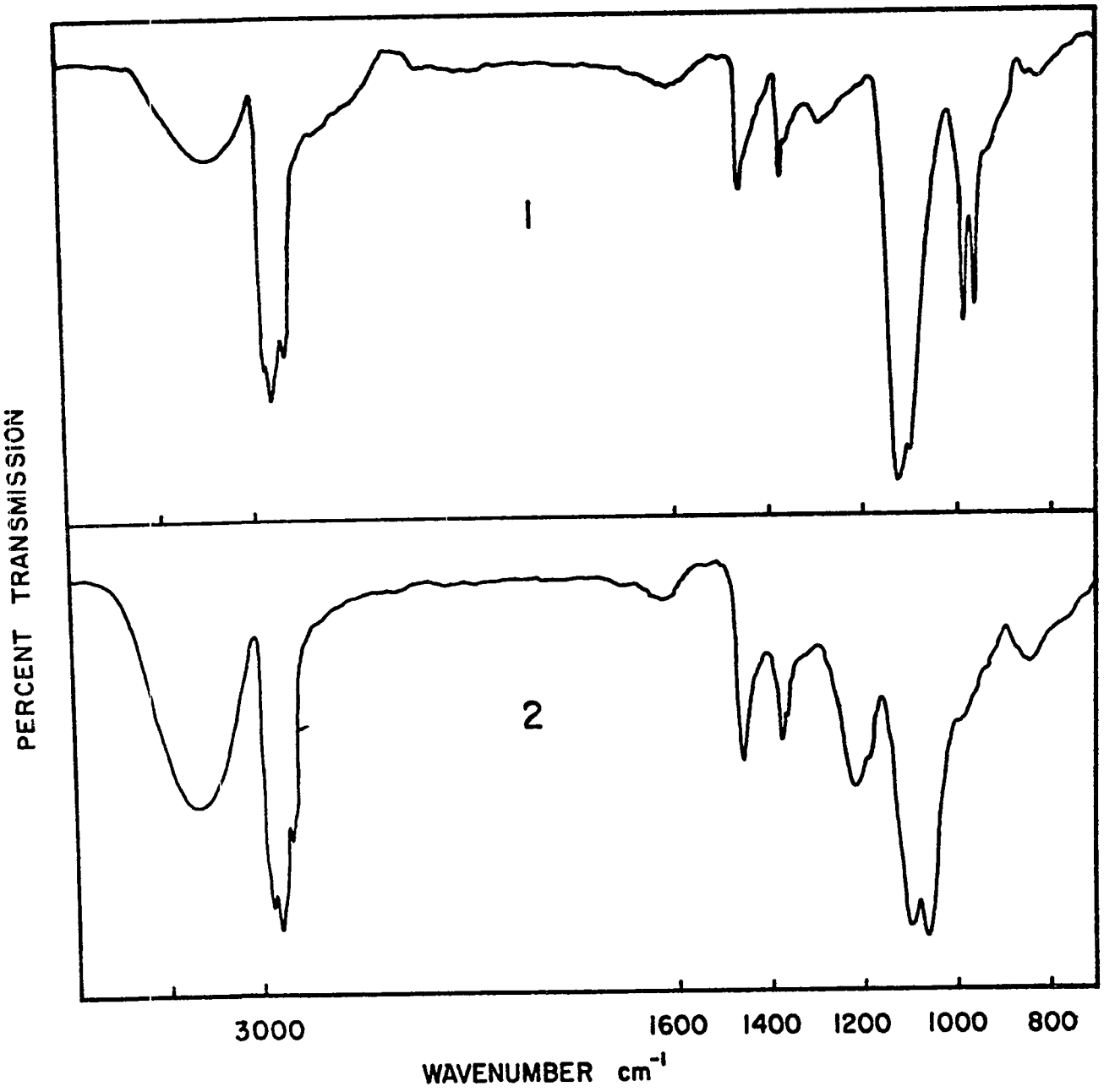

1
2
PERCENT TRANSMISSION
3000
1600
1400
1200
1000
800
WAVENUMBER cm-1

TABLE 21

Acid Hydrolysis of Monophytanyl Ether Phosphatidic Acid (Lysophosphatidic Acid)

Hydrolytic Agent	Time, Hour	Distribution of P, %	
		Methanol-water phase	Chloroform phase
(1) 2.5% methanolic-HCl (anhydrous)	5	0	100
(2) 2 N methanolic-HCl[a,b]	72	65	35
(3) 2 N aqueous-HCl[b]	12	86	14
(4) 2 N aqueous-HCl[b]	24	91	9
(5) 2 N aqueous-HCl[b]	72	100	0

a solution made by diluting 10 ml. conc. HCl to 60 ml. with methanol

b sealed tube hydrolysis at 120°

(b) Monophytanyl-phosphatidyl glycerol and monophytanyl-phosphatidyl glycerophosphate

The fully protected lysophosphatides (20) and (22) were obtained in good yield by the silver salt/iodide condensations (Scheme 8). Removal of the aromatic protecting groups from the lysophosphatidyl glycerol derivative proceeded smoothly, but the removal of the 2'-O-tert.-butyl blocking group with anhydrous $CHCl_3$-HCl was accompanied by some cleavage of the phosphodiester bond. The lypo-PG was isolated by acetone precipitation as a chromatographically pure solid after removal of contaminating monophytanylglycerol by preparative TLC. Elemental analysis data for the monopotassium salt were consistent with those expected for monohydrated lyso-PG and mild acid degradation gave monophytanylglycerol as the main lipid product, although traces of lysophosphatidic acid were detected in the hydrolysate. The formation of these two lipid hydrolysis products showed that unlike the hydrolysis of the known diphytanyl ether phosphatides, the lyso-derivative hydrolysis proceeds by cleavage on both sides of the phosphorus bridge, presumably because cyclisation can occur with the neighbouring hydroxyl groups of both glycerol moieties.

The potassium salt of lyso-PG was freely soluble in chloroform and benzene, sparingly soluble in carbon tetrachloride and methanol and insoluble in acetone. The molecular rotation of the potassium salt was slightly less than that of the diphytanyl ether analogue of PG.

The infrared spectrum (Fig. 47.2) of the potassium salt of lyso-PG showed phytanyl group absorption and strong hydroxyl (3320 cm^{-1}), P=O (1220 cm^{-1}), P-O^- (1095 cm^{-1}) and P-O-C (1055 cm^{-1}) absorption bands. The presence of bound water was revealed by the absorption at 1610 cm^{-1}.

Typical lyso-phosphatide chromatographic behaviour was shown by lyso-PG. The mobility in acid, alkaline and neutral solvent systems was less than that of the diphytanyl ether phosphatide (see Tables 3 and 20 for Rf values of diether and monoether phosphatides, respectively).

The removal of the blocking groups from the fully protected lysophosphatidyl glycerophosphate derivative led to extensive decomposition at the phosphodiester bond on both occasions, so that attempts were made to isolate the free lysophosphatide. Sufficient product was obtained (7 % yield) to permit the acquisition of chromatographic and hydrolytic data, but elemental and spectral analysis data were not obtained.

The lyso-PGP derivative appears to be too labile for synthesis in satisfactory yield by this route. The success of the triisopropylbenzene-sulfonyl chloride catalysed condensations which were used in Part One of this thesis suggest that a more likely route to the lyso-PGP may involve condensation between 2-O-benzylmonophytanyl phosphatidic acid and a 2-O-benzyl-3-(dibenzylphosphoryl)-glycerol. Catalytic hydrogenolysis of the benzyl groups under neutral conditions should give lyso-PGP in reasonable yield.

The possibility that lysophosphatides may play a role as intermediates in the anabolic and catabolic metabolism of the extreme halophiles enhances the usefulness of these syntheses and of the data obtained from the synthetic products.

CLAIMS TO ORIGINAL RESEARCH

1. A method has been developed for the estimation of the number of ionisable P-OH groups in phosphatidyl glycerophosphate (diphytanyl ether analogue), by heteronuclear spin decoupling experiments on two methylated derivatives of PGP. The method is also applicable to structure study of lipids containing a variety of acidic groups [e.g. PO(OH), SO_2(OH), COOH].

2. The controversy concerning the structure of the PGP component in H. cutirubrum lipids has been resolved by method (1) and also by molecular weight and analysis studies on a series of PGP salts.

3. A novel phosphosulfolipid has been isolated from H. cutirubrum; its chemical structure has been established as 2,3-di-O-phytanyl-1-phosphoryl-3-sn-glycero-1-sulfate using, in part, the NMR method (1).

4. Synthetic routes for the stereospecific syntheses of 3-O-phytanyl-sn-glycero-1-phosphoric acid (lysophosphatidic acid, monophytanyl ether analogue) and 3-O-phytanyl-sn-glycero-1-phosphoryl-sn-1-glycerol have been developed and the chromatographic and hydrolytic properties of the products have been studied.

5. The RRR-diastereoisomeric forms of two extended chain homologues (C_{21} and C_{22}) of the isoprenoid phytanic acid (C_{20}) have been synthesised by chain elongation reactions. The GLC behaviour of the methyl esters has been investigated.

6. The syntheses of 1,3-di-O-phytanyl-sn-glycerol and 2,3-di-O-phytanyl-1-phosphoryl-sn-glycero-2-sn-glycerol (β-PG) have been carried out.

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