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Biosynthetic studies of the polar lipids of *Halobacterium cutirubrum*. Formation of isoprenyl ether intermediates

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Biosynthetic studies were carried out on the complex polar lipids of the extreme halophile, *Halobacterium cutirubrum*. Cells were pulse-labeled with [^{32}P]phosphate, [^{14}C]mevalonate and [^{14}C]glycerol for short terms (1, 15, 30, 60 min); incorporation of radiolabels into total lipids and their distribution among individual lipid components were determined as a function of incubation time. Glycerol was incorporated 9-fold more rapidly into total lipids than mevalonate, but at the same rate as phosphate. Several intermediate components were detected by short-term (1–30 min) pulse labeling, some of which were shown to be allyl ether-bound isoprenyl intermediates of the major saturated diphytanylglycerol ether lipids, phosphatidylglycerophosphate (PGP), phosphatidylglycerol (PG), phosphatidylglycerosulfate (PGS) and sulfated triglycosyldiphytanylglycerol (S-TGD). These intermediate components were detected by their products of acid methanolysis, alkaline hydrolysis and Vitride reduction, before and after catalytic hydrogenation. Another intermediate detected was the isoprenyl ether analogue of phosphatidic acid, which showed a product-precursor relationship with PGP and PG for all three radioisotope precursors. Other intermediates remain unidentified, but their product-precursor relationship was established by their labeling patterns with the three radioisotope precursors. These studies suggest that the biosynthetic pathways for the major diphytanylglycerol ether phospho- and glycolipids involve formation of the respective isoprenyl intermediates that are reduced in a stepwise fashion to the fully saturated diphytanylglycerol ether phospho- and glycolipids. Chase studies, following a 24 h labeling period, showed that the major phospholipid (PGP) has a very low turnover rate for phosphate groups, but a 3-fold higher turnover of the phytanyl groups; the minor phospholipids PG and PGS have much higher phosphate turnover rates.

Introduction

Obligate extremely halophilic bacteria, which belong to the family Archaebacteria, characteristically synthesize isoprenoid and isopranoid hydrocarbon chains and only traces of straight-chain compounds, such as fatty acids [1,2]. All phospholipids and glycolipids are derived from 2,3-di-*O*-phytanyl-*sn*-glycerol (C_{20}-diether) rather than

Abbreviations: PGP, phosphatidylglycerophosphate; PG, phosphatidylglycerol; PGS, phosphatidylglycerosulfate; S-TGD, sulfated triglycosyldiphytanylglycerol; S-TeGD, sulfated tetraglycosyldiphytanylglycerol; TLC, thin-layer chromatography; PA, phosphatidic acid; MPG, monophytanylglycerol; Me, methyl; DPG, diphytanylglycerol; CI-MS, chemical ionization-mass spectrometry; GP, glycerophosphate.

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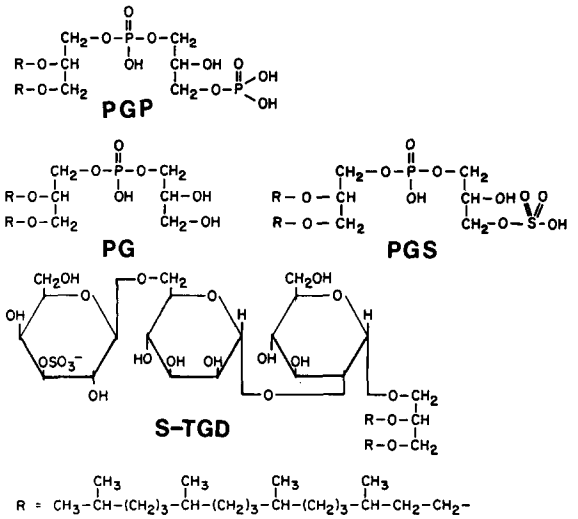


Fig. 1. Structures of major lipids of *H. cutirubrum*. PGP, PG, PGS, S-TGD and S-TeGD. All compounds are derivatives of 2,3-diphytanyl-*sn*-glycerol.

1,2-diacyl-*sn*-glycerol, and the major lipid components are phosphatidylglycerophosphate (PGP, diphytanyl ether analogue) and a sulfated triglycosyl-C_{20}-diether (S-TGD) [1,2] (Fig. 1). Minor components are the diphytanyl ether analogues of phosphatidylglycerol (PG) and phosphatidylglycerosulfate (PGS) (Fig. 1) and a sulfated tetraglycosyl-C_{20}-diether (S-TeGD) [3].

Relatively few studies have been made on the biosynthesis of these unusual C_{20}-diether-derived phospholipids and glycolipids [2,4,5]. However, it has been shown in whole cell labeling experiments with *Halobacterium cutirubrum* [6] that [^{32}P]phosphate labels all the phospholipids, [^{35}S]sulfate labels PGS, S-TGD and the minor glycolipid, S-TeGD, [^{14}C]acetate and [^{14}C]mevalonate label the phytanyl chains of all polar lipids, and [^{14}C]glycerol labels the phytanyl groups, the glycerol moiety of the C_{20}-diether and also the polar head-groups (glycerol phosphates, glycerol sulfate and sugars) of all polar lipids. These results show that the predominant pathway for biosynthesis of the hydrocarbon chains is the mevalonate pathway (starting from acetate) and that glycerol enters both the mevalonate pathway for phytanyl groups via acetate (recently, it was shown [7] that lysine labels the methyl and methine carbons of phytanyl groups, the remaining carbons being derived from acetate) and the glycolysis cycle for synthesis of glycerol phosphates and sugars [2,4,5]. Participation of glycerol in glycolysis was demonstrated by (i) its rapid conversion to *sn*-glycero-3-phosphate by the action of glycerol kinase and its oxidation to dihydroxyacetone phosphate by *sn*-3-glycerophosphate dehydrogenase in a cell-free system of *H. cutirubrum* [8,9] and (ii) the loss of 50% of ^{3}H from [1(3)-^{3}H]glycerol and complete loss of ^{3}H from [2-^{3}H]glycerol in sugar and phytanyl groups of the polar lipids [10]. Curiously, the glycerol in the diphytanyl glycerol moiety retained 100% of ^{3}H from [1(3)-^{3}H]glycerol, but none from [2-^{3}H]glycerol [10].

The results obtained so far indicated the presence of unusual and unique pathways of lipid biosynthesis in extreme halophiles. To extend our knowledge of these pathways, we have carried out short-term pulse-labeling studies of polar lipid biosynthesis in *H. cutirubrum*, and a long-term chase study, using the precursors [^{32}P]orthophosphate, [^{14}C]mevalonate and [^{14}C]glycerol.

Our findings suggest that the biosynthetic pathways for the major diphytanylglycerol ether phospholipids involve their respective allyl ether-linked isoprenyl intermediates, which undergo stepwise reduction to form the final saturated phytanyl ether phospholipids (see Fig. 1).

Materials and Methods

Reference standards

Authentic samples of individual diphytanylglycerol analogues of phospholipids (PG, PGP, PGS) and glycolipid sulfate (S-TGD) were isolated from *H. cutirubrum* cells and purified as described elsewhere [1]. Samples of mixed squalenes (squalene, dihydrosqualene and tetrahydrosqualene) [11], 2,3-diphytanyl-*sn*-glycerol and bacterioruberins [12] were isolated from the neutral lipids of *H. cutirubrum*. The diphytanylglycerol analogue of phosphatidic acid (PA) was synthesized by phosphorylation of diphytanylglycerol [13]. CDP-diether was synthesized from PA as described elsewhere [13]. 1- and 2-monophytanylglycerol (MPG) were synthesized as described previously [14]; monophosphate and pyrophosphate esters of phytanol, phytol, farnesol and

geranylgeraniol were synthesized as described previously [15]. Monophytanyldihydroxyacetonedimethylketal was synthesized by the procedure of Piantadosi et al. [16].

Culture of organism

Cells of *H. cutirubrum* were grown in complex medium [1] containing per liter: peptone (Oxoid), 11 g; trisodium citrate, 3 g; KCl, 2 g; $MgSO_4$, 9.8 g; NaCl, 250 g and $FeCl_2 \cdot 7\ H_2O$, 0.05 g (pH adjusted to 6.8–7.0), with shaking (120 rpm) for 72 h at 37°C. Cells from 100 ml culture were harvested by centrifugation, washed twice and suspended in 2 ml of basal salt solution (KCl, 2 g; $MgSO_4$, 9.8 g; NaCl, 250 g per l).

Growth of organism in present of labeled precursors

Pulse studies. The washed cell suspension (2 ml) from 100 ml culture was added to 96 ml of 'low phosphate medium' (complex medium containing only 5.5 g peptone per l; total inorganic phosphate 2 mg/100 ml) in a 500 ml side-arm flask, to give an absorbance of 0.6 at 660 nm. The cell suspensions (98 ml) were preincubated at 37°C until a definite increase in absorbance was observed (5 h, taken as zero time). A solution of one of the following radioactive precursors in 2 ml of low phosphate medium was added to the 98 ml cell suspension: (1) [^{32}P]orthophosphate (Amersham, 5 mCi, 'carrier-free', neutralized to pH 7); (2) DL-[2-^{14}C]mevalonic acid *N,N'*-dibenzylethylenediamine salt (50 μCi, 1.1 μmol; spec. act. 45 Ci/mol); (3) [U-^{14}C]glycerol (100 μCi, 0.58 μmol; 170 Ci/mol); or (4) [^{32}P]orthophosphate (4 mCi, 'carrier-free') plus [^{14}C]mevalonate (20 μCi, 0.44 μmol; spec. act. 45 Ci/mol). All incubations were done at 37°C, and suitable aliquots were taken as follows: for [^{32}P]orthophosphate, [^{14}C]mevalonate and [^{14}C]glycerol, 5–25 ml aliquots at 1, 15, 30 and 60 min; for the [^{32}P]phosphate-[^{14}C]mevalonate double-label experiment, 2 ml at 1, 5, 15, 30 min and 1, 2, 4 h, and 5 ml at 24 h. The absorbance (at 660 nm) of the culture was measured immediately prior to removal of each aliquot.

Chase studies. After removal of the 24 h sample in the [^{32}P]phosphate-[^{14}C]mevalonate double-label experiment, the remaining cells (81 ml) were harvested at 8000 × *g* at 10°C, washed twice with the complete complex medium, suspended in 100 ml of complete complex medium in a 500 ml side-arm flask, and incubated at 37°C with shaking. The absorbance of the culture was read at 660 nm, and a suitable aliquot (5 ml) of this culture was removed at 1, 5, 15 and 30 min and at 2 and 24 h, and the remainder at 48 h.

Extraction of lipids

Each aliquot of culture was immediately centrifuged at 10000 × *g* at 3°C and the cell pellet was extracted by the procedure of Bligh and Dyer [17] as modified by Kates [18] with 9.5 ml of $CHCl_3$/MeOH/H_2O (1 : 2 : 0.8, v/v) followed, after mixing, by addition of 2.5 ml each of chloroform and water to give two phases. The lower chloroform phase was removed and washed once with 2 ml of upper phase (MeOH/H_2O, 10 : 9, v/v), and a suitable aliquot of the chloroform phase was counted in Aquasol 2 for ^{14}C and ^{32}P.

Chromatography of total lipids and degradation products

The chloroform extracts were diluted with benzene and brought to dryness in a stream of nitrogen. The residual lipids were immediately redissolved in a small amount of $CHCl_3$ (0.1–0.2 ml) and subjected to thin-layer chromatography (TLC) on analytical precoated commercial plates (Baker or Brinkmann silica gel G, 0.25 mm thick), previously washed twice with $CHCl_3$/MeOH (1 : 1, v/v) and once with acetone, and activated at 110°C before use. The solvent systems were: A, $CHCl_3$/MeOH/CH_3COOH/H_2O (85 : 22.5 : 10 : 4, v/v), double development, or B, $CHCl_3$/MeOH/NH_4OH (65 : 35 : 5, v/v), for polar lipids; C, petroleum ether/ethyl ether/acetic acid (70 : 30 : 1, v/v), for nonpolar lipids. The radioactive spots were detected by autoradiography (Fuji X-ray film) [18] and were scraped into scintillation vials and counted for ^{14}C and/or ^{32}P. The results are expressed as μCi ^{14}C or ^{32}P incorporated per 100 ml of culture into total lipids, and as percentages of the total radioactivity incorporated into each lipid component. The TLC plates were then charred with H_2SO_4 [18] for detection of all lipids, including standard marker lipids. Tentative identification of labeled spots was based on TLC mobilities relative to authentic standards as well as by their degradation products (see Results).

Water-soluble products of methanolysis were chromatographed on TLC cellulose plates (Merck, DC-cellulose, 0.1 mm thick) in solvent systems: D, 1-butanol/acetic acid/water (50 : 30 : 10, v/v) or in solvent E, pyridine/ethyl acetate/water (2 : 5 : 5, upper phase); the latter solvent was also used for paper chromatography (Whatman). After detection of radioactive spots by autoradiography, the chromatograms were stained with the periodate-*o*-tolidine or silver nitrate-sodium hydroxide stain for detection of sugars and polyhydroxy compounds and with salicylsulfonic acid/ferric chloride for phosphate esters and inorganic phosphate [18].

Enzymatic hydrolysis

[^{32}P]- or [^{14}C]phospholipids (TLC eluted spots) or standard isoprenyl pyrophosphates were hydrolyzed in 0.1 M borate buffer (pH 8.2) with bacterial alkaline phosphatase (Sigma) at room temperature with shaking for 18 h [19]. After neutralization with 1.5 ml of MeOH/0.2 M HCl (2 : 1, v/v), the mixture was extracted with chloroform. Both chloroform and methanol/water phases were used directly for further analysis.

Catalytic hydrogenation

Total labeled lipids and individual labeled spots were hydrogenated in methanol solution with PtO_2 (Adams) catalyst at room temperature, as described elsewhere [18].

Methanolysis

To determine the distribution of radioactivity between the hydrophobic and water-soluble moieties of the lipids, TLC-scraped spots were methanolyzed in screw-capped tubes with 4.5 ml of 0.83 M or 2 M methanolic HCl at 70 °C for 5 h [18]; after addition of 0.5 ml water, the lipid moieties were extracted with petroleum ether; the petroleum ether and methanol/water phases were saved for further analysis (see below).

Reductive hydrogenolysis (Vitride reduction)

TLC-scraped spots were treated in screw-capped tubes with 2.5 ml dry ethyl ether/benzene (4 : 1, v/v) with 0.5 ml of Vitride reagent (Eastman Kodak, Rochester) at 37 °C with shaking for 1 h in a water-bath [18]. The mixture was cautiously acidified with 2 ml of methanol/2 M aqueous HCl (1 : 1, v/v), concentrated under a nitrogen stream, then diluted with 1.5 ml MeOH and 1.25 ml $CHCl_3$, mixed and centrifuged. The supernatant was removed and the insoluble material was washed twice with 3 ml of methanol/1 M HCl/chloroform (2 : 0.8 : 1). The combined supernatants and extracts (one phase) were diluted with chloroform and water to form a two-phase system. Both chloroform and methanol/water phases were saved for further analysis (see below).

Alkaline hydrolysis [18]

Lipids or TLC-scraped spots were hydrolyzed in screw-capped tubes with 1 ml of 1 M NaOH in $MeOH/H_2O$ (9 : 1, v/v) at 100 °C for 2 h; after addition of 1 ml of $CHCl_3$ and 0.8 ml of water and mixing, the chloroform and methanol/water phases were separated and treated as described below.

Analysis of hydrolysis products

Suitable aliquots of petroleum ether or $CHCl_3$ phases of the various hydrolysates, after counting for ^{14}C and ^{32}P, were concentrated to a small volume and subjected to TLC in solvent C. After autoradiography, ^{14}C-labeled spots were eluted by the Bligh and Dyer extraction procedure [18]; the $CHCl_3$ phases were counted for ^{14}C, brought to dryness under nitrogen, dried in vacuo and analyzed by HPLC or mass spectrometry before and after methoxylation or acetylation [18].

The aqueous methanolic phases obtained after alkaline hydrolysis were counted for ^{14}C and ^{32}P, then neutralized with Dowex-50, H^+, and concentrated to dryness. The residues were hydrolyzed in aqueous 1 M HCl at 100 °C for 1 h, concentrated to dryness under N_2, dried in vacuo over KOH, neutralized with methanolic NH_4OH (0.5 M) and chromatographed on TLC cellulose plates in solvent D or E.

The aqueous methanolic phases from the acid methanolysis were counted, brought to dryness, and treated with aqueous 1 M HCl, as described above, prior to chromatography on cellulose plates or on paper.

High-pressure liquid chromatography

High-pressure liquid chromatography was per-

formed on a reverse-phase column (Bondapak C_{18}, 39 mm × 30 cm) at room temperature with ultraviolet detection at 210 nm (LKB 2151 variable wavelength monitor) using isocratic elution with acetonitrile as solvent at a flow rate of 1 ml/min for mono- and diphytanylglycerols, phytol, isophytol, farnesol, geranylgeraniol and their methoxy derivatives; samples were injected in the respective eluting solvent. The isoprenol components were identified by comparison of their retention times relative to methyl palmitate with those of authentic isoprenol samples.

Mass spectrometry

The purified samples were analyzed by fast atom bombardment (negative FAB) and by chemical ionization-mass spectrometry (CI-MS) on a VG-7070E mass spectrometer with a digital RL02 data system and printonic printer.

Results

In vivo short-term labeling of total lipids from H. cutirubrum with [^{32}P]orthophosphate, [^{14}C]-mevalonate or [^{14}C]glycerol

When *H. cutirubrum* cells were incubated in a low phosphate medium with [^{32}P]orthophosphate, traces of ^{32}P were detected in the total lipids after the first minute of incubation; after a lag period of 15 min, incorporation of the tracer rapidly increased, reaching a value of 0.2% of the tracer supplied after 60 min (Table I). Under the same growth conditions but with [^{14}C]mevalonate as precursor, incorporation of ^{14}C into total lipids was detected after the first minute and showed a shorter lag and a lower rate than that of ^{32}P, but reaching a percent incorporation after 60 min of 0.6% (Table I).

In contrast, with [^{14}C]glycerol as precursor, incorporation of ^{14}C into total lipids occurred without a lag phase, and increased linearly with time to a maximum value of about 4.4% after 30 min (Table I). Calculations based on nmol radioisotope incorporated showed that at 30 min, [^{14}C]glycerol was incorporated 9-fold more rapidly into total lipids than [^{14}C]mevalonate, but at the same rate as [^{32}P]orthophosphate (Table I).

Distribution of radioisotopes among individual lipid components

The distribution of ^{32}P and ^{14}C among the lipid components was determined by TLC of the total

TABLE I

PULSE-LABELING STUDIES ON INCORPORATION OF [^{32}P]PHOSPHATE, [^{14}C]MEVALONATE AND [^{14}C]GLYCEROL INTO TOTAL LIPIDS OF *H. CUTIRUBRUM*

H. cutirubrum cells were grown in 100 ml of low phosphate medium, in presence of [^{32}P]phosphate (5 mCi), [^{14}C]mevalonate (50 μCi), or [^{14}C]glycerol (100 μCi). Total lipids were extracted and counted, as given in Materials and Methods.

Radioisotope precursor	Time (min)	Cell growth (absorbance)	Incorporation		
			μCi/100 ml	nmol [a]/100 ml	% of label supplied
[^{32}P]Orthophosphate	1	0.68	0.006	0.07	0.0001
	15	0.69	0.35	4.5	0.01
	30	0.71	1.96	25.3	0.04
	60	0.74	10.34	133.4	0.20
[^{14}C]Mevalonate	1	0.69	0.025	0.55	0.05
	15	0.70	0.079	1.73	0.16
	30	0.71	0.134	2.95	0.27
	60	0.73	0.287	6.31	0.60
[^{14}C]Glycerol	1	0.70	0.098	0.58	0.10
	15	0.71	2.14	12.6	2.14
	30	0.72	4.41	25.9	4.41
	60	0.75	4.44	26.9	4.44

[a] Calculated using the following specific activities: [^{32}P]orthophosphate, 77.5 Ci/mol P_i; [^{14}C]mevalonate, 45.5 Ci/mol and [^{14}C]glycerol, 170 Ci/mol.

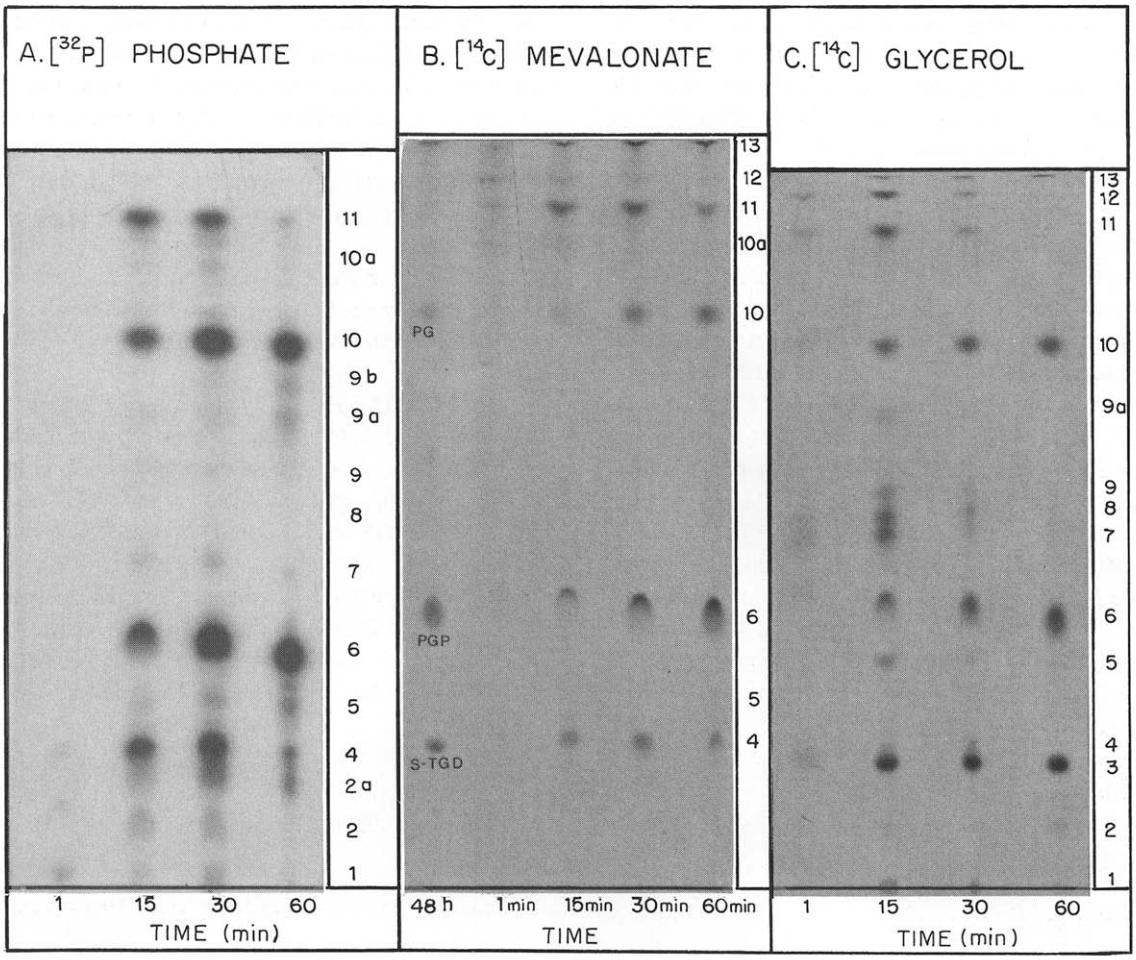


Fig. 2. Autoradiograms of TLC plates showing distribution of radiolabel among lipid components as a function of time: A, [^{32}P]phosphate; B, [^{14}C]mevalonate; C, [^{14}C]glycerol. TLC was carried out on Baker silica gel G plates (0.25 mm-thick layers) in solvent system A. Identification of spots: 1 and 2 (R_F 0.01 and 0.10, respectively), isoprenyl pyrophosphate; 3 (R_F 0.18), precursor of S-TGD; 4 (R_F 0.21) unidentified phospholipid precursor; 5 (R_F 0.33), precursor of PGS; 6 (R_F 0.38), precursor of PGP; 7, 8, 9 (R_F 0.50, 0.53, 0.56, respectively), unidentified glycolipid precursors; 10 (R_F 0.77) precursor of PG; 11 (R_F 0.91) precursor of PA; 12, 13 (R_F 0.97, 0.99, respectively), nonphosphorylated intermediates; 2a (R_F 0.16), 9a (R_F 0.65), 9b (R_F 0.69), unidentified phospholipids; 48 h, ^{14}C-labeled total lipids from cells double-labeled with [^{14}C]mevalonate and [^{32}P]phosphate for 48 h, and ^{32}P allowed to decay. R_F values of standard unlabeled phosphatides: phytyl pyrophosphate or geranylgeranyl pyrophosphate, 0.01, 0.10; S-TGD, 0.18; CDP-diether, 0.22; PGS, 0.33; PGP 0.38; PG, 0.78; PA, 0.92.

labeled lipids in solvent A, followed by autoradiography (Fig. 2) and counting of each labeled component. A total of fourteen ^{32}P-labeled components were detected (Fig. 2A), eight [^{14}C]mevalonate-labeled (Fig. 2B) and fourteen [^{14}C]glycerol-labeled (Fig. 2C) spots. Changes in percent distribution of radioactive label among the components during incubation with the three precursors (Fig. 3) were as follows:

(1) [^{32}P]Orthophosphate. At 1 min incubation, only four components were labeled, spots 1, 2, 4 and 11. The percentage of ^{32}P incorporated into spots 1 and 2 (which have R_F values similar to those of prenyl pyrophosphate [15]) accounted for 69% of the total, and decreased rapidly almost to zero at 60 min; the percent of ^{32}P in spot 11 (having an R_F value similar to that of PA), and spot 4 remained essentially constant during the

first 15 min, then decreased to low values at 60 min (Figs. 2A, 3A). The percentage of ^{32}P in spot 6 (with an R_F value close to that of PGP) increased from zero at 1 min to more than 50% of the total ^{32}P incorporation after 60 min. A parallel but lower percentage incorporation of ^{32}P occurred into spot 10 (having an R_F value similar to that of PG). Relatively low incorporations of ^{32}P occurred in spots 2a, 5, 7, 8, 9 (traces), 9a, 9b and 10a, but no ^{32}P was detected in spots 3, 12 or 13 at any time during the 60 min incubation. The complete absence of ^{32}P in spots 3, 12 and 13 was confirmed by the [^{32}P]phosphate-[^{14}C]mevalonate double-labeling experiment (data not shown).

These results (Fig. 3A) suggest a product-precursor relationship for the major ^{32}P-labeled components, as follows: 1, 2 → 11 → 4 → 6 → 10--→5.

(2) [^{14}C]Mevalonate. At 1 min incubation, most of the ^{14}C (46%) was present in fast moving spots 12 and 13; lower percentages of ^{14}C were present in spots 11 and 4 (Figs. 2B, 3B). The percentage of ^{14}C in spot 12 decreased rapidly during the first 30 min, while that in spot 13 remained constant; ^{14}C in spots 11 and 4 increased to a maximum at 15 min and decreased thereafter. Percentage incorporation of ^{14}C from [^{14}C]mevalonate into spot 6 almost paralleled that of ^{32}P into this component. Percentage incorporation of ^{14}C into spot 10 also paralleled the ^{32}P incorporation, but was at a lower level.

For ^{14}C incorporation from [^{14}C]mevalonate, the following product-precursor relationship is suggested by the data in Fig. 3B: 12 → 11 → 4 → 6 → 10--→5.

(3) [^{14}C]Glycerol. One-third of the ^{14}C incorporated at 1 min was associated with components 7, 8 and 9, the remainder being equally divided among spots 11, 12, 13 and 4 (Figs. 2C, 3C). Note that spots 7 and 8 were more highly labeled with ^{14}C than spot 9 (see Fig. 2C) during the labeling period 15–60 min. The percentage of ^{14}C in all of these spots decreased with time, while that in spots 3, 6 and 10 increased. Spot 3 (with an R_F value similar to that of S-TGD) accounted for

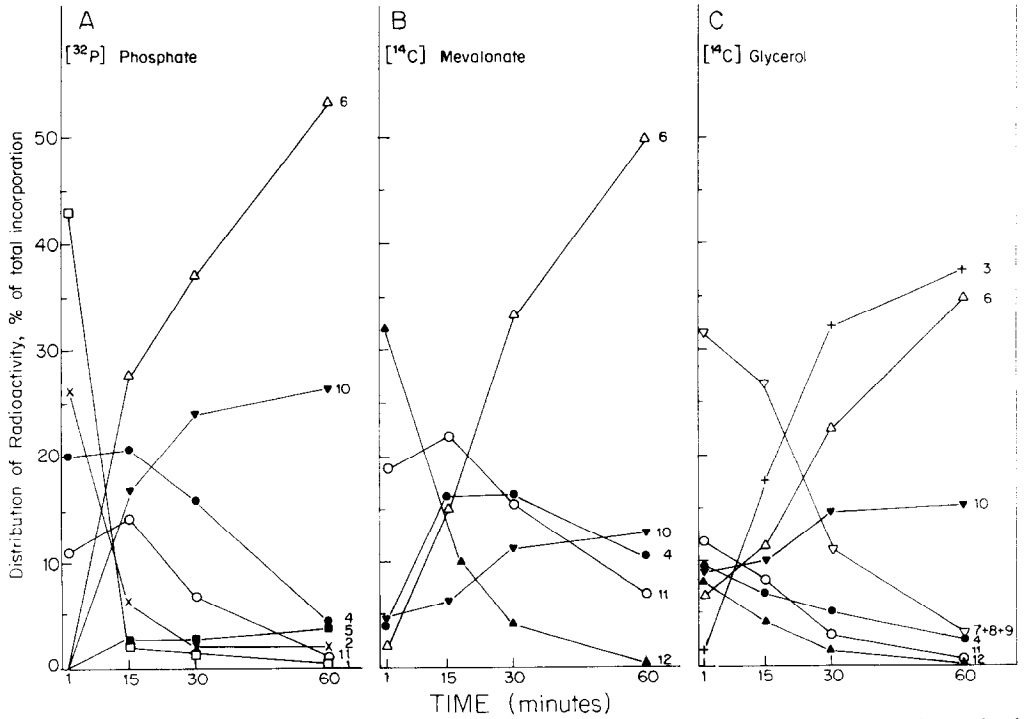


Fig. 3. Percentage distribution of radiolabel among individual lipid components as a function of time during pulse labeling: A, [^{32}P]orthophosphate; B, [^{14}C]mevalonate; and C, [^{14}C]glycerol. Labeling conditions were as in Table I. Identity of lipid components 1–13 are given in legend to Fig. 2.

a higher proportion of the ^{14}C than spot 6 (PGP) at 15, 30 and 60 min.

For ^{14}C incorporation from [^{14}C]glycerol, the product-precursor relationship appears to be (see Fig. 3C):

$$\begin{array}{l} \nearrow 7(8) \rightarrow 3 \\ 12(13) \rightarrow 11 \rightarrow 4 \rightarrow 6 \rightarrow 10 \dashrightarrow 5 \end{array}$$

Note that spot 3, being a sulfoglycolipid (see below), is not put on the main pathway for phospholipids (spots 5, 6 and 10).

Characterization and identification of labeled lipid components

In the short-term labeling (1–30 min) studies, it was observed that the TLC-separated labeled lipids (e.g., spots 6, 10 and 11) were very unstable towards acidic solvents. These observations suggested that the rapidly labeled lipid components may be unsaturated forms of the main phospholipid components, perhaps containing ether-linked phytyl groups instead of phytanyl groups; these unsaturated precursors appear to be progressively saturated during the first 60 min of incubation, but complete saturation does not occur until at least 24 h incubation (see below).

To obtain evidence for this suggestion, the total lipids labeled with [^{14}C]glycerol for 15–30 min as well as the individual components eluted from the TLC plates (Fig. 2) were hydrogenated (with Pt as catalyst) and examined by TLC in solvent A. Little or no change in mobility of the spots was observed after hydrogenation, except for spot 6 (R_F 0.38), which now showed two components, with R_F values of 0.38 (6R), 0.35 (6R′) and spot 4 (R_F, 0.21), which showed two components, with R_F values of 0.23 (4R) and 0.09 (4R′). Each separated component was then subjected to degradation by acid methanolysis, alkaline hydrolysis or Vitride reductive hydrogenolysis (Table II), and the products were examined by TLC in various solvents (Table III, Fig. 4) and also by CI-MS analysis, in comparison with those formed by the

TABLE II

DISTRIBUTION OF RADIOACTIVITY BETWEEN THE HYDROPHOBIC AND WATER-SOLUBLE MOIETIES OF LIPID COMPONENTS LABELED WITH [^{14}C]GLYCEROL

Labeled lipid components (scraped spots containing (5–50)·10^3 dpm per spot) were subjected to acid methanolysis, reductive hydrogenolysis (Vitride) or alkaline hydrolysis, as described in the text.

Spot No.	Recovery of ^{14}C (% of total)					
	acid methanolysis		alkaline hydrolysis		reductive hydrogenolysis (Vitride)	
	PE phase	$MeOH/H_2O$ phase	$CHCl_3$ phase	$MeOH/H_2$ phase	$CHCl_3$ phase	$MeOH/H_2O$ phase
3	13	86	97	3	58	18
3R	11	89	–	–	81	1
4 [a]	11	89	93	4	36	12
4R	38	61	–	–	72	8
4R′	45	55	95	5	45	22
6	30	70	67	33	55	27
6R, 6R′	38	62	94	4	50	35
7+8+9	traces	100	–	–	–	–
(7+8+9)R	traces	100	–	–	–	–
10	18	84	–	–	61	30
10R [a]	26	74	–	–	44	12
11 [a]	25	75	–	–	25	5
11R [a]	93	4	–	–	34	5
12+13	55	43	88	10	–	–
(12+13)R	63	35	–	–	–	–

[a] Low recoveries after Vitride hydrogenolysis are most likely due to trapping of water-soluble ^{14}C-labeled products in the aluminum salt precipitates.

unhydrogenated components. The degradation products expected for the major saturated diphytanylglycerol lipids (PG, PGP, PGS, S-TGD-1) are given in Table IV. It was thus possible to establish the identity, tentatively, of several of the lipid intermediates (spots 3, 5, 6, 10, 11), but others (spots 1, 2, 4, 7–9, 12, 13) remain unidentified because of insufficient material. The results are summarized as follows:

Spots 1 and 2. These components were labeled by [^{32}P]phosphate and [^{14}C]glycerol, but only very faintly with [^{14}C]mevalonate (Fig. 2). The mobilities of these components corresponded to those of phytanyl pyrophosphate or geranylgeranyl pyrophosphate (Fig. 2). Note that isoprenyl pyrophosphates give two spots with R_F values of 0.10 and 0.02 in solvent A, suggesting that spots 1 and 2 are most likely isoprenyl (phytyl- or geranylgeranyl) pyrophosphates [15]. However, the faint labeling of spots 1 and 2 with [^{14}C]mevalonate relative to the labeling with [^{14}C]glycerol is rather puzzling.

Spot 3. This component was labeled only with [^{14}C]glycerol and had TLC mobilities corresponding to the glycolipid sulfate (S-TGD) (Fig. 2).

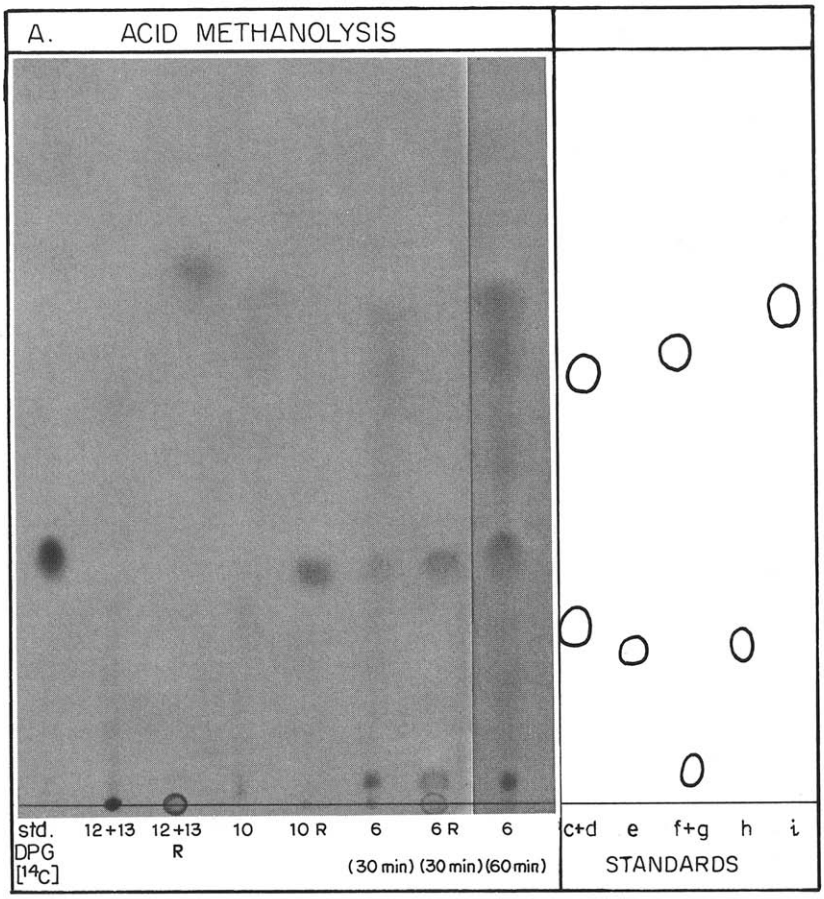


Fig. 4. Autoradiogram of TLC plates showing distribution of radiolabel among hydrophobic degradative products of short-term (15–60 min) pulse-labeled lipid components after: A, acid methanolysis; B, alkaline hydrolysis; C, reductive hydrogenolysis (Vitride). TLC was carried out on Brinkmann silica gel plates in solvent C (see Materials and Methods). Radioactive components were spotted as indicated on the TLC plates. Standard lipids spotted: a, hydrophobic products of PG; b, hydrophobic products of PGP; c, phytol; d, methoxyphytol; e, geranylgeraniol; f, MPG; g, methoxy-DPG; h, monophytanyldihydroxyacetonedimethylketal; i, squalene. [^{14}C]DPG standard was isolated from the hydrophobic methanolysis products of PGP after 48 h labeling with [^{14}C]mevalonate and [^{32}P]phosphate, and purified by TLC in solvent C. Identities of products are as shown in this figure, or as in Table III.

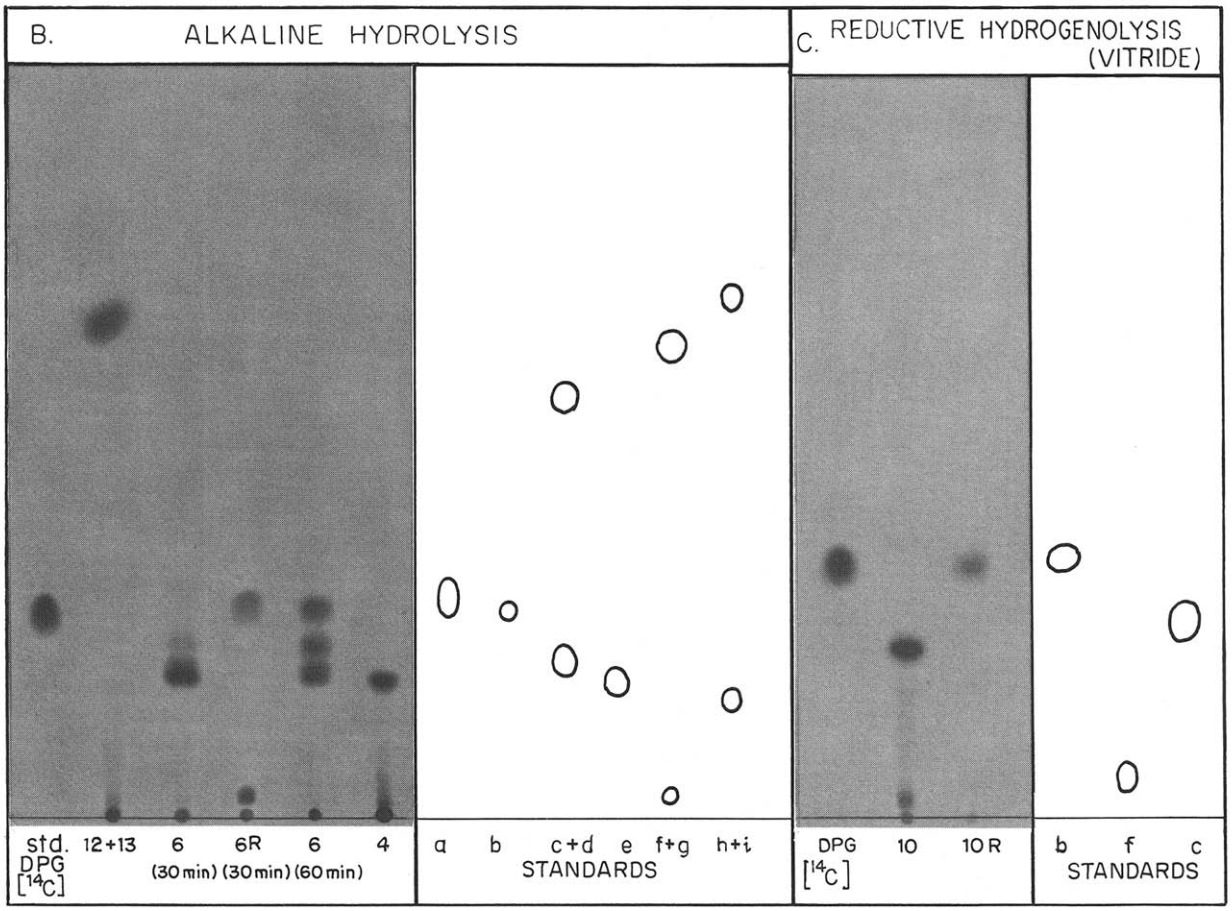


Fig. 4 (continued).

Spot 3 or hydrogenated spot 3 (3R) yielded, after acid methanolysis, mostly water-soluble radioactive products (90%, Table II) corresponding to glucose, galactose, mannose and polyols (Table III); [^{14}C]diphytanylglycerol was detected only in 3R (Table III). However, spot 3 or 3R was recovered (97%) intact after Vitride or alkaline treatment (Table III). These findings are consistent with spot 3's being a precursor of the glycolipid sulfate (S-TGD) (Table IV).

Spot 4. This component was labeled with all three precursors, and had TLC mobilities somewhat higher than those of spot 3 (S-TGD) but corresponding to that of CDP diether (Fig. 2, Table III). After methanolysis, spot 4 gave hydrophobic products (10% of ^{14}C, Table II) corresponding to isoprenyl methanolysis products (Table III), and water-soluble products (90% of ^{14}C, Table II) corresponding mainly to glycerol (Table III). Spot 4, after Vitride or alkaline treatment, gave an unidentified hydrophobic component (X_1) (Table III, Fig. 4B) and little or no radioactive material in the water-soluble products (Table II). After catalytic hydrogenation, spot 4R yielded only diphytanylglycerol (DPG) after either methanolysis or Vitride reduction (Table III). Identity of DPG was confirmed by CI-MS (see spot 6 below).

As mentioned above, spot 4R′ (R_F, 0.09 in solvent A) was obtained after catalytic hydrogenation of total lipids. After methanolysis or Vitride hydrogenolysis of spot 4R′, a major labeled hydrophobic product (45%) (Table II) was obtained, with TLC mobilities corresponding to monophytanylglycerol (Table III).

Spot 5. This component was weakly labeled in

TABLE III

CHROMATOGRAPHIC CHARACTERIZATION OF THE RADIOACTIVE DEGRADATION PRODUCTS OF SHORT-TERM PULSE-LABELED LIPID COMPONENTS OF *H. CUTIRUBRUM*

Individual lipids from cultures labeled with [U-^{14}C]glycerol for 15 and 30 min were subjected to acid methanolysis, alkaline hydrolysis or reductive hydrogenolysis (see Materials and Methods) and the products were chromatographed as described in footnotes a and b.

Lipid component			R_F [a] of hydrophobic products and tentative identity ($CHCl_3$ or pet. ether-soluble)			R_F [b] of water-soluble products			Identity
						methanolysis solvents			
Spot No. [c]	R_F in solvent A	tentative identity	methanolysis	alkaline hydrolysis	reductive hydrogenolysis (Vitride)	D	E (TLC)	E (paper)	
3	0.18	pre-S-TGD	0.00 (?)	0.00 (spot 3)	0.00 (spot 3)	0.11	0.11	0.22	Polyols
						0.15	0.15	0.34	Gal
						0.19	0.19	0.40	Glc
						0.23	0.24	0.50	Man
3R	0.21	S-TGD	0.00 (?)	0.00 (spot 3R)	0.00 (sport 3R)	–	0.15	–	Gal
							0.19		Glc
							0.24		Man
							0.53		Gly
4	0.21	unidentified	0.60 isoprenyl 0.70 products	0.03 MPG 0.20 *cpd* X_1	0.04 MPG 0.20 *cpd* X_1	0.54	–	–	Gly
4R	0.23	unidentified	0.31 DPG	0.31 DPG	0.31 DPG	0.44	–	–	cpd X_3
4R′	0.09	unidentified	0.04 MPG	–	0.04 MPG	–	–	–	
5 [d]	0.33	pre-PGS	0.31 DPG 0.04 MPG	–	–	–	–	–	
6	0.38	pre-PGP	0.03 MPG	0.20 *cpd* X_1	0.20 *cpd* X_1	0.53	0.58	–	Gly (major)
			0.31 DPG	0.24 cpd X_2	0.24 cpd X_2	0.07	0.01		GDP (minor)
			0.60 isoprenyl 0.67 products	0.31 DPG	0.31 DPG	0.24	0.04	–	unidentified
6R 6R′	0.38 0.35	PGP	0.31 DPG 0.03 MPG	0.00 (PGP) 0.03 MPG 0.28 DPG	0.00 (PGP) 0.04 MPG 0.31 DPG	0.07	–	–	GDP

7+8+9	0.50 0.53 0.56	unidentified	no labeled hydrophobic products		0.00 (spots 7, 8, 9,)	0.09 0.15 0.19	0.10 0.15 0.19	0.20 0.32 0.37	polyols Gal Glc
(7+8+9)R	0.50 0.53 0.56	unidentified	no labeled hydrophobic products		0.00 (spots 7, 8, 9)	–	–	0.20 0.32 0.37	polyols Gal Glc
10	0.77	pre-PG	0.60 isoprenyl 0.62 products 0.70	–	0.00 Pre-PG 0.03 MPG 0.21 cpd X_1	0.20 0.53	0.01 0.58	–	GP Gly
10R	0.77	PG	0.31 DPG	–	0.00 (PG) 0.34 DPG	0.21	–	–	GP
11	0.92	pre-PA	0.59 isoprenyl 0.62 products 0.67	–	0.00 Pre-PA 0.21 cpd X_1	0.28	–	–	unidentified
11R	0.92	PA	0.00 PA	–	0.00 PA 0.31 DPG	–	–	–	
12+13	0.97 0.99	unidentified	0.00 (sp. 12+13) 0.56 isoprenyl 0.61 products 0.66	0.00 (sp. 12+13) 0.67 uniden- tified	–	0.46	–	–	unidentified
(12+13)R	0.97 0.99	unidentified	0.00 (sp. 12+13R) 0.70 unidentified	–	–	0.45	–	–	unidentified

[a] TLC R_F values of ^{14}C-labeled hydrophobic products and lipid standards on silica gel G plates in solvent C: MPG, 0.03–0.04; monophytanyldihydroxyacetonedimethylketal, 0.15–0.17; geranylgeraniol (GG), 0.18–0.20; phytol, (Phy) or phytanol (Pha) 0.22–0.24; DPG, 0.31–0.34; dimethoxy-MPG, 0.40; methoxyphytol, 0.57–0.60; methoxy-DPG, 0.60; squalene 0.65–0.70; squalane, 0.70. Authentic samples of PG, PGP or PGS yielded DPG (R_F, 0.30–0.34) after acid methanolysis, alkaline hydrolysis or reductive hydrogenolysis; S-TGD gave DPG only after acid methanolysis; PA gave DPG only after reductive hydrogenolysis; phytanyl pyrophosphate or geranylgeranyl pyrophosphate gave, on acid methanolysis, three spots: methoxyphytol (R_F 0.58–60) or methoxy geranylgeraniol (R_F 0.56–0.58), methoxyisophytol or methoxyisogeranylgeraniol (R_F 0.62–0.64) and isoprene hydrocarbons (R_F 0.68–0.70).

[b] R_F values of ^{14}C-labeled water-soluble products and standards in solvents D and E (cellulose plates) and in solvent E (paper, mobilities relative to glycerol), respectively: glucose (Glc), 0.20, 0.19, 0.40; galactose (Gal), 0.15, 0.15, 0.34; mannose (Man), 0.24, 0.24, 0.50; glycerophosphate (GP), 0.20, 0.01–0.03, – ; glycerol diphosphate (GDP), 0.07–0.10, 0.00–0.01, – ; dihydroxyacetone phosphate (DHAP), 0.18, 0.01, – ; DHAP-dimethylketal, 0.36–0.39, 0.05, – ; P_i, 0.27, 0.01, – ; glycerol (Gly), 0.50–0.53, 0.58, 1.0; dihydroxyacetone (DHA), 0.50–0.53, 0.83, 1.40. On methanolysis of authentic standards, PG gave GP; PGP gave GDP, and S-TGD gave Glu, Gal, Man (mobilities of these products were as given for the respective water-soluble standards).

[c] R, reduced with PtO_2 catalyst.

[d] Spot 5 was labeled only weakly in short-term pulses; data shown are for 24 h pulse.

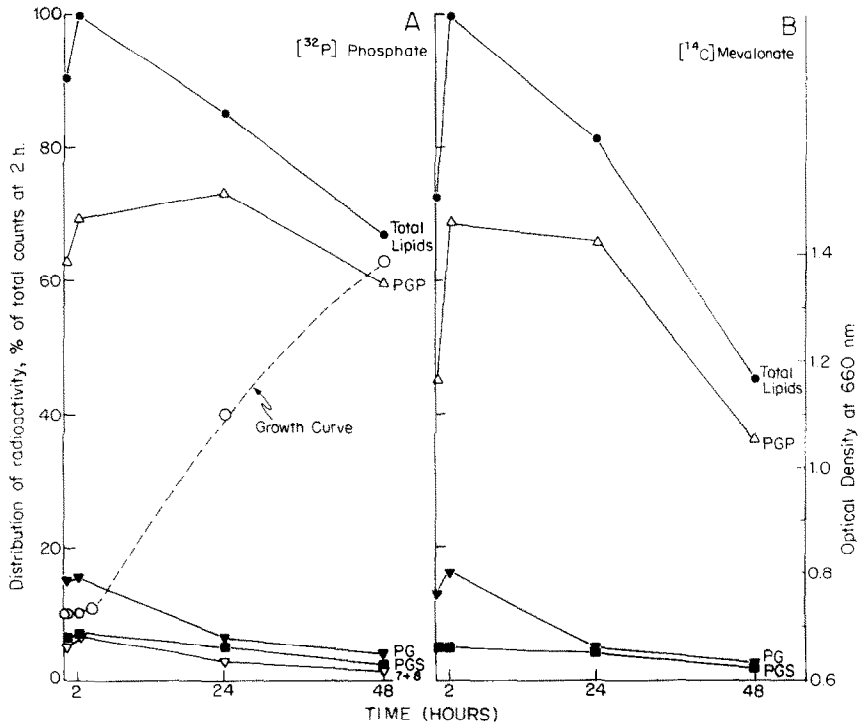


Fig. 5. Percentage distribution of radiolabel among individual lipid components as a function of time during a 48 h 'chase'. Labeling and chase conditions are as in the text. Identity of lipid components are as shown in this figure, or as in Fig. 2.

short-term pulses (up to 1 h) by all precursors used, and had mobilities corresponding to PGS. After long-term pulses (longer than 24 h), sufficient radioactive material was available for methanolysis, which yielded mostly diphytanylglycerol with small amounts of monophytanylglycerol (Table III, Fig. 6).

Spot 6. This component was labeled with all three precursors, and had R_F values in solvents A and B similar to those of the phytanyl ether analogue of PGP (Fig. 2). Methanolysis of spot 6 from 30 or 60 min pulses yielded radioactive hydrophobic products, accounting for 30% of total ^{14}C (Table II), corresponding to phytyl methanolysis products, diphytanylglycerol and monophytanylglycerol (Table III, Fig. 4A), along with water-soluble radioactive products (70% of ^{14}C, Table II) corresponding to glycerol, glycerol diphosphate and an unidentified component (Table III). Acid methanolysis of the catalytically hydrogenated spot 6R yielded mostly the [^{14}C]diphytanylglycerol and small amount of [^{14}C]monophytanylglycerol (Fig. 4A). Vitride hydrogenolysis or alkaline hydrolysis of spot 6 (30 min pulse) gave three radioactive hydrophobic products: the major one corresponding to the unidentified component, with an R_F value of 0.20 (X_1) in solvent C (identical with that from spot 4), and minor ones corresponding to another unidentified component, with an R_F value of 0.23–0.24 (X_2) in solvent C, and to diphytanylglycerol (DPG) (Table III, Fig. 4B). When spot 6 from the 60 min pulse was subjected to Vitride hydrogenolysis (results not shown) or alkaline degradation (Fig. 4B), the same products were formed, but the proportion of radioactivity was greatly increased in the unidentified spot (X_2) and in diphytanylglycerol. After catalytic hydrogenation of spot 6, however, all degradation procedures gave mainly [^{14}C]diphytanylglycerol with small amounts of [^{14}C]-monophytanylglycerol. Identity of DPG was confirmed by CI-MS, which showed a molecular ion with m/z 653 $(M + H)^+$ (calculated mol.wt., 652).

Spots 7, 8 and 9. These three components,

TABLE IV

EXPECTED PRODUCTS OF DEGRADATION OF SATURATED AND UNSATURATED DIETHER POLAR LIPID ANALOGUES [a]

Phospholipid or glycolipid [b,c]	Acid methanolysis	Alkaline hydrolysis	Vitride hydrogenolysis
PA (R = phytanyl) (X = H_2PO_3)	no reaction	no reaction	DPG (R = phytanyl) (X = H)
pre-PA (R = phytyl) (X = H_2PO_3) (spot 11)	MeO-phytol, MeO-iso-phytol, phytenes, + GP	no reaction	DPG′(R = phytyl) (X = H) + P_i
PG (R = phytanyl) (X = GP)	DPG (R = phytanyl) (X = H) + GP	DPG (R = phytanyl (X = H) + GP	DPG (R-phytanyl) (X = H) + P_i + glycerol
pre-PG (R = phytyl) (X = GP) (spot 10)	MeO-phytol, MeO-iso-phytol, phytenes + GP + glycerol	DPG′(R = phytol) (X = H) + GP	DPG′(R = phytyl) (X = H) + P_i + glycerol
PGP(R = phytanyl) (X = GDP)	DPG (R = phytanyl) (X = H) + GDP	DPG (R = phytanyl) (X = H) + GDP	DPG (R = phytanyl) (X = H) + P_i + glycerol
pre-PGP (R = phytyl) (X = GDP) (spot 6)	MeO-phytol, MeO-iso-phytol, phytenes + GDP + glycerol	DPG′(R = phytyl) (X = H) + GDP	DPG′(R = phytyl) (X = H) + P_i + glycerol
S-TGD (R = phytanyl) (X = sugars)	DPG (R = phytanyl) (X = H) + sugars + sulfate	no reaction	no reaction
pre-S-TGD (R = phytyl) (X = sugars) (spot 3)	MeO-phytol, MeO-iso-phytol, phytenes + sugars + sulfate	no reaction	no reaction

[a] See refs. 1 and 15.

[b] The structures of these lipids can be represented as (see also Scheme 1):

OR
OR
OX, where R and X are defined as indicated above.

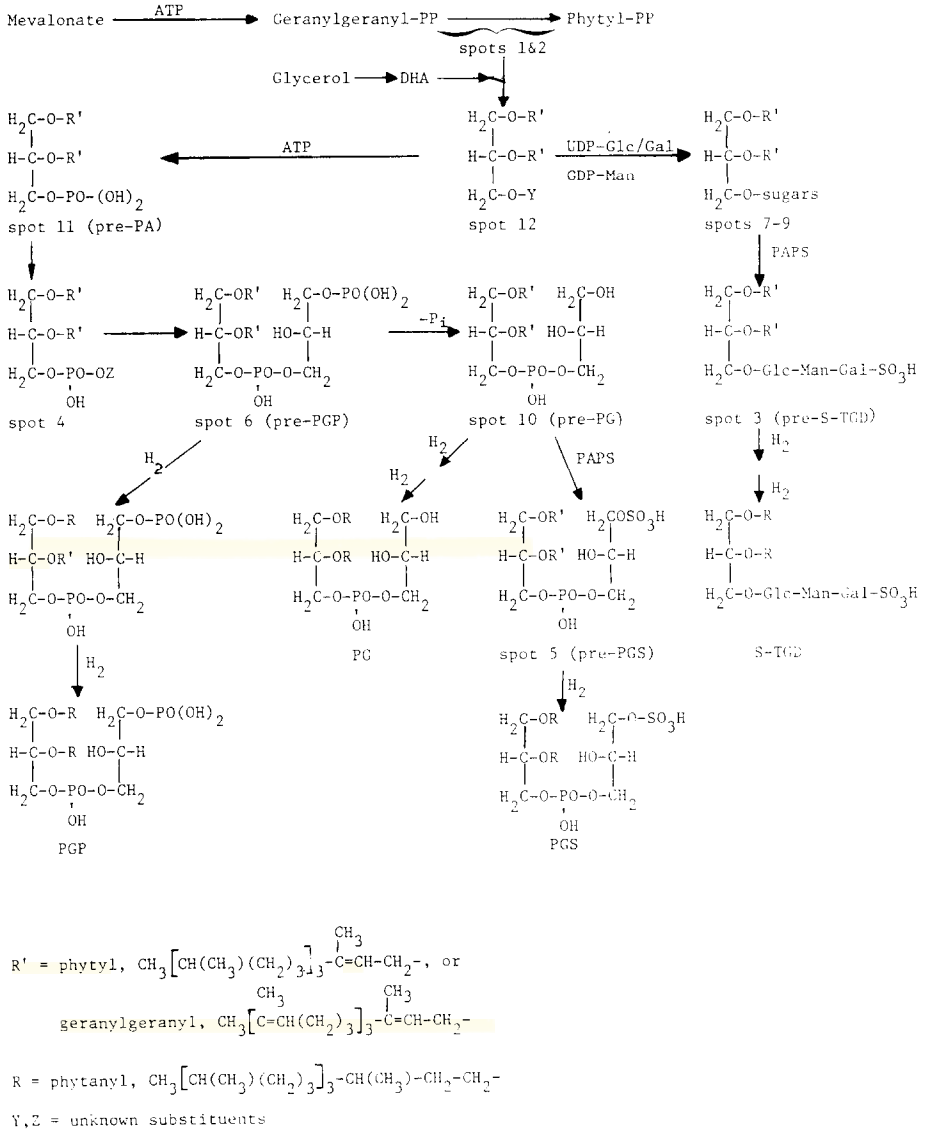
[c] Abbreviations are as given in Table III.

labeled mostly with [^{14}C]glycerol, slightly with ^{32}P but not with [^{14}C]mevalonate (Fig. 2), had R_F 0.50, 0.53, 0.56, respectively, in solvent A before and after hydrogenation (Table III). After methanolysis of combined spots 7, 8 and 9, virtually all of the ^{14}C activity appeared in the methanol/water phase (Table II) in three components. Two of the products had mobilities corresponding to glucose and galactose; the third had mobilities lower than those of hexoses, and appeared to be a polyol (Table III). Spots 7, 8 and 9 thus appear to be intermediates in the formation of glycolipids.

Spot 10. This component was labeled with all three precursors, and had TLC R_F values of 0.77 in solvent A and 0.46 in solvent B, before and after hydrogenation, similar to those of the phytanyl ether analogue of PG. After acid methanolysis, spot 10 gave radioactive hydrophobic products (accounting for 19% of total ^{14}C) corresponding to isoprenyl methanolysis products (Fig. 4A, Table III) together with radioactive

water-soluble products (accounting for 84% of total ^{14}C, Table III), including a major component with mobilities corresponding to glycerol, and a minor component with mobilities corresponding to GP (Table III). Vitride hydrogenolysis or alkaline hydrolysis of spot 10 gave hydrophobic products consisting mainly of the unidentified component (X_1) and some monophytanylglycerol (Table III, Fig. 4C). After catalytic hydrogenation, spot 10R yielded, on acid methanolysis, alkaline hydrolysis and on reductive (Vitride) hydrogenolysis, a single radioactive component with mobility in solvent C corresponding to diphytanylglycerol (Fig. 4C, Table III). Identity of this compound was confirmed by CI-MS (see spot 6).

Spot 11. This component was labeled with all three precursors (Fig. 2) and had TLC R_F values of 0.92 and 0.06 in solvents A and B, respectively, before and after hydrogenation, similar to those of authentic PA. On acid methanolysis, spot 11 gave radioactive hydrophobic products corresponding to isoprenyl methanolysis products, and an un-



R' = phytyl, $CH_3[CH(CH_3)(CH_2)_3]_3-C(CH_3)=CH-CH_2-$, or

geranylgeranyl, $CH_3[C(CH_3)=CH(CH_2)_3]_3-C(CH_3)=CH-CH_2-$

R = phytanyl, $CH_3[CH(CH_3)(CH_2)_3]_3-CH(CH_3)-CH_2-CH_2-$

Y,Z = unknown substituents

Scheme I. Proposed pathway for biosynthesis of polar lipids in *Halobacterium cutirubrum*. PP, pyrophosphate.

identified water-soluble radioactive component (Table III). Vitride hydrogenolysis, however, gave the unidentified hydrophobic product (X_1) (some unreacted spot 11 remained at the origin). After catalytic hydrogenation, spot 11R yielded, on methanolysis, the unchanged spot 11R, but on Vitride hydrogenolysis, now yielded diphytanylglycerol (Table III). Furthermore, negative fast atom bombardment-mass spectrometry of unhydrogenated spot 11 showed an ion peak with m/z 729 ($M^+ - 1$), compared to that of the synthetic saturated diphytanyl analogue of PA of m/z 731 ($M^+ - 1$). These results suggest that spot 11 is a phosphatidic acid ether analogue in which the ether-bound chains are C_{20}-isoprenyl instead of the C_{20}-isopranyl chains formed after 24 h or more pulse labeling.

Spots 12 and 13. These phosphate-free components had TLC R_F values of 0.99 and 0.98–0.96, respectively, in the acidic solvent A (Fig. 2B, 2C), and 0.87 and 0.71, respectively, in the basic solvent B, before and after catalytic hydrogenation. Both spots 12 and 13 comigrated with red carotenoid pigments (bacterioruberins [12,20]) in solvents A and B; spot 13 also comigrated with squalene in solvent A.

After acid methanolysis of spots 12 and 13, TLC of the hydrophobic products (containing 55% of total ^{14}C) in solvent C showed a major radioactive spot at the origin, and three faint spots corresponding to isoprenyl methanolysis products (Fig. 4A, Table III). The spot at the origin, when run in polar solvent A, showed a spot with mobility (R_F 0.98) similar to that of the intact spot 12 or 13. On alkaline methanolysis of spots 12 and 13, a major radioactive spot with TLC mobilities in solvent C corresponding to isoprene hydrocarbons e.g., squalene (Fig. 4B, Table III), and similar to that for the unhydrolyzed spots 12 and 13 was observed (data not shown). After acid methanolysis of the hydrogenated spots (12 and 13R), R a major radioactive spot with mobility similar to that obtained by alkaline hydrolysis of spots 12 and 13, was detected (Table III, Fig. 4A). These results suggest that spot 12 may be a substituted isoprenylglycerol diether (see Scheme I).

Chase studies

Cells were labeled for 24 h with a mixture of [^{32}P]phosphate and [^{14}C]mevalonate and then subjected to a 'chase' period in cold medium for up to 48 h.

The first 2 h of the chase represented an equilibration period during which the labeling of the total lipids increased by 10% in ^{32}P and 38% in ^{14}C (Fig. 5). Thereafter, total lipid labeling decreased by 33% for ^{32}P and 54% for ^{14}C over a 48 h period. However, it is interesting that the major phospholipid spot 6 (PGP), which accounted for about 70% of the total ^{32}P and ^{14}C, showed no decrease in either ^{32}P or ^{14}C over the 22 h after the equilibration period, but then showed losses in ^{32}P and ^{14}C of about 15 and 50%, respectively, after 48 h (Fig. 5). In contrast, PG, which

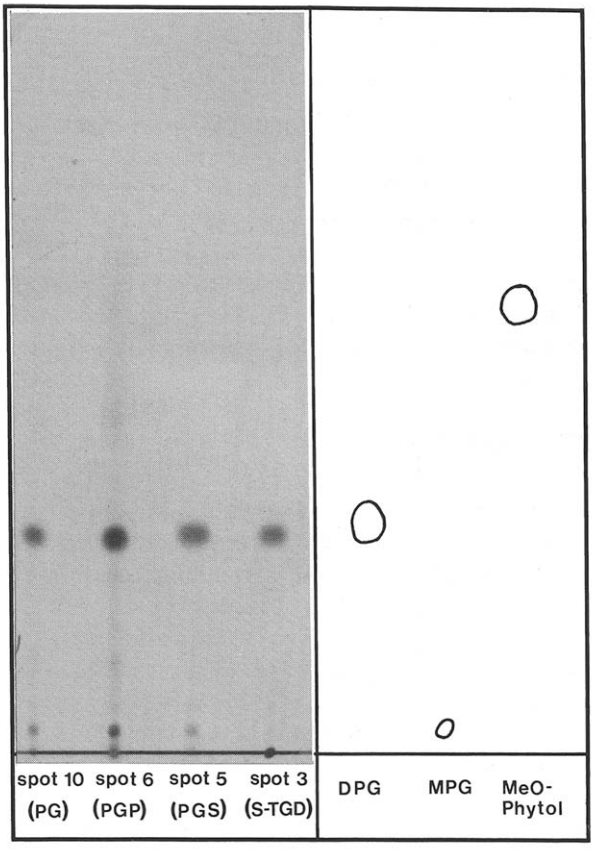


Fig. 6. Autoradiogram of TLC plates showing distribution of radiolabel among hydrophobic methanolysis degradative products of labeled lipid components from cells grown for 24 h with [^{14}C]mevalonate and [^{32}P]phosphate, followed by a 2 h chase in unlabeled medium. Material applied: methanolysis products of spot 10 (PG), spot 6 (PGP) spot 5 (PGS) and spot 3 (S-TGD); standards: DGP; MPG; MeO-phytol, methoxyphytol.

accounted for about 15% of total ^{32}P and ^{14}C, lost about 58 and 69% of its ^{32}P and ^{14}C, respectively, during the first 24 h of chase, and 72 and 84% of its ^{32}P and ^{14}C, respectively, after 48 h (Fig. 5). PGS, which accounted for only and 4% of the total ^{32}P and ^{14}C, respectively, showed losses of ^{32}P and ^{14}C of about 70 and 50%, respectively, over the 48 h chase.

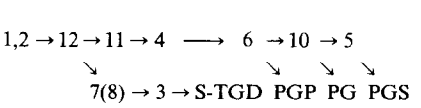
These results indicate that the major phospholipid PGP has a very low turnover rate with respect to phosphate groups, and a 3-fold higher turnover rate for the phytanyl groups. The minor phospholipid, PG, however, has an almost 5-fold greater turnover rate for phosphate groups than PGP and a 2-fold greater turnover rate for phytanyl groups. The other minor phospholipid, PGS, has about the same phosphate turnover rate as PG, but the turnover rate for phytanyl groups was much lower, being comparable to that for PGP. The glycolipid sulfate (S-TGD) (data not shown) was found to have a very low turnover rate with respect to phytanyl groups (30% in 48 h), a much lower rate than that of PGP.

Methanolysis of the major labeled lipids (PGP, PG, PGS, S-TGD), obtained after 24 h labeling followed by 2 h 'chase', yielded [^{14}C]diphytanylglycerol as major labeled hydrophobic product (Fig. 6).

Discussion

The results of the short-term (1–60 min) pulse-labeling studies show that transient intermediates of the phospholipids and glycolipids are formed with each of the three precursors used. With [^{14}C]glycerol, intermediates of the sulfated triglycosyldiphytanylglycerol (S-TGD) were also observed.

These intermediates are of two types: 'early' (1–15 min) and 'later' (15–60 min) (Fig. 3). The 'early' intermediates (spots 1, 2, 4, 7, 8, 9, 11, 12 and 13) represent short-term phospholipid (and glycolipid) precursors that appear within the first few minutes of the biosynthesis and are converted to the 'later' (longer term) intermediates (spots 3, 5, 6 and 10). The latter are then converted to the final saturated S-TGD, PGS, PGP and PG, respectively. The metabolic relationships between these intermediates and the final products, based on the pulse-labeling data (Fig. 3), appear to be as follows:



Both the 'early' and 'later' intermediates appear to be unsaturated compounds containing allyl ether-linked isoprenyl groups (phytyl or geranylgeranyl) on the basis of the following evidence: (i) acid methanolysis of 15–30-min-labeled 'later' intermediates (spots 3, 6 and 10) and also of the 'early' intermediates that are labeled within 1 min (spot 4, 11, 12 and 13) yielded isoprenyl methanolysis degradation products with TLC mobilities very similar to those of the methanolysis products of phytyl or geranylgeranyl derivatives (e.g., for phytyl derivatives: methoxyphytol, methoxyisophytol and phytenes; see footnote, Table III, and Ref. 15.); (ii) acid methanolysis of intermediate spots 3, 4, 5, 6, 10 and 11, after catalytic hydrogenation, yielded mostly the saturated diphytanylglycerol ether (DPG), and in some cases (spots 5 and 6), also the monophytanylglycerol ether (MPG) (Table III); (iii) alkaline hydrolysis or Vitride hydrogenolysis of spots 4, 5, 10 and 11 gave an unidentified spot (X_1), while spot 6 gave spot X_1 together with another unidentified spot (X_2) as well as some DPG (Table III, Fig. 4B). Spots X_1 and X_2 were converted to the saturated DPG by catalytic (Pt) hydrogenation (data not shown); (iv) the lower TLC mobilities in solvent C of spots X_1 and X_2 relative to the saturated DPG (see Fig. 4) are consistent with spot X_1 containing two isoprenyl groups and X_2 one isoprenyl and one isopranyl group. Thus, with spot 6, it is possible to discern the stepwise hydrogenation of the intermediate isoprenyl forms by comparison of the methanolysis products of spot 6 after 30–60 min pulses (Fig. 4). Note that the hydrogenation of spot 6 is still not complete after a 24 h pulse followed by a 2 h chase, while that of spots 10, 5 and 3 is completed during the same period of time (Fig. 6); (v) alkaline hydrolysis or Vitride hydrogenolysis of catalytically hydrogenated spots 4, 5, 6, 10 and 11 gave mostly DPG (but also some MPG in the case of spot 6) (Fig. 4, Table III).

Interpretation of these findings is based on the

products of acid methanolysis, alkaline hydrolysis and Vitride hydrogenolysis expected for the saturated diphytanylglycerol and unsaturated diphytylglycerol analogues of PA, PG, PGP, PGS and S-TGD (see Refs. 1 and 15), as summarized in Table IV. It may be concluded from the above evidence that the 'later' intermediates, spots 3, 5, 6 and 10, have the structures of S-TGD, PGS, PGP and PG, respectively, but contain allyl ether-linked isoprenyl groups instead of phytanyl groups. They may thus be designated as 'pre-S-TGD', 'pre-PGS', 'pre-PGP' and 'pre-PG', respectively (see Table IV). Similarly, of the 'early' intermediates, spot 11 may be designated as 'pre-PA' (see Table IV); spot 4 may be an isoprenylglycerol ether biosynthetic intermediate between 'pre-PA' and 'pre-PGP' (perhaps similar to CDP-diether); and spots 7–9 may be isoprenylglycerol glycoside intermediates of the 'pre-S-TGD' (Scheme I). With regard to the identity of spots 12 and 13, the latter may be an intermediate in the biosynthesis of the hydroxy-C_{50}-carotenoids, bacterioruberins [20], and spot 12 may be a nonphosphorylated isoprenylglycerol diether in which the hydroxyl group is blocked by a nonpolar acid- and alkali-stable group (to account for its high TLC mobility and acid and alkaline stability). Spot 12, from labeling kinetics (Fig. 3) appears to be an intermediate for both the phospholipids and the glycolipids (Scheme I).

The detection of allyl ether-linked isoprenyl intermediates of the phytanylglycerol analogues of phospholipids and glycolipids supports the hypothesis [21,22] of the involvement of an isoprenyl pyrophosphate to form the glycerol ether-linked isoprenyl intermediates that are subsequently hydrogenated to the fully saturated diphytanylglycerol analogues (see also Ref. 2). This hypothesis would also be in accord with the observed inhibition of polar lipid biosynthesis in *H. cutirubrum* by bacitracin [23], which binds isoprenyl pyrophosphates [24]. The present results do not allow a final conclusion concerning the identity of the isoprenyl pyrophosphate involved, although phytyl pyrophosphate and geranylgeranyl pyrophosphate would be likely candidates. However, both the TLC mobilities (Table III, Fig. 4) and HPLC retention times (data not shown) of the isoprenyl methanolysis products of spots 4, 5 and 10 are closer to those of the phytyl methanolysis products.

On the basis of the structures of the intermediates given in Table IV and their metabolic interrelationships (Fig. 3), the following tentative pathways for biosynthesis of the major polar lipids in *H. cutirubrum* is presented for discussion (Scheme I). Alkylation of a suitable glycerol derivative (most likely dihydroxyacetone, but not glycerol itself (see Ref. 2)) with an isoprenyl pyrophosphate (phytyl or geranylgeranyl pyrophosphate) to give the isoprenyl diether of the substituted glycerol derivative (spot 12) which is then either phosphorylated with ATP to give 'pre-PA' (spot 11) or glycosylated to give the intermediate glycolipids (spots 7–9). The latter would then be further glycosylated and sulfated to pre-S-TGD (spot 3) and finally reduced to the saturated S-TGD. The pre-PA (spot 11) appears to be converted to an intermediate substituted pre-PA (spot 4) which is transformed into pre-PGP (spot 6), stepwise reduction of which yields the saturated PGP. Dephosphorylation of pre-PGP would form pre-PG (spot 10) which, on hydrogenation, would give the saturated PG. Sulfation of pre-PG followed by hydrogenation would give the saturated PGS.

This complex pathway will require further study to identify, in particular, the isoprenyl pyrophosphate, the derived glycerol acceptor for the isoprenyl groups, the resulting isoprenyl diether product (spot 12) and the phospholipid intermediate, spot 4. Subsequent studies will employ a cell-free particulate system from *H. cutirubrum*, which has previously been used for study of carotenoid biosynthesis [25].

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