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Structure of diabolic acid-containing phospholipids isolated from *Butyrivibrio* sp.

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The structures of the diabolic acid-containing phospholipids of *Butyrivibrio* S2 grown in the presence of palmitic acid have been investigated. Generally they consist of two conventional bacterial phospholipid or galactolipid structures linked by esterification through a single diabolic acid residue. The main lipid consists of the butyroyl ester of *sn*-1-alkenylglycero-3-phospho-1'-*sn*-glycerol joined in this way to the butyroyl ester of *sn*-1-alkenyl-3-galactosylglycerol by esterification of the vacant 2-hydroxy groups of the alkenyl-substituted glycerol molecules. A lipid that possesses a palmitoyl group rather than one of the butyroyl groups of the latter structure has also been detected. The lipid occurring in the second highest concentration consists of two molecules of *sn*-1-alkenylglycero-3-phospho-*sn*-1'-glycerol butyroyl ester linked through diabolic acid in a similar manner to the main lipid. Other lipids with the latter structure either minus a butyroyl group or with a palmitoyl group, instead of one of the butyroyl groups, exist as minor components.

In the preceding paper (Hazlewood *et al.*, 1980) we describe the isolation of a number of new phospholipids containing esterified $(C_{16:0})_2$ diabolic acid (Klein *et al.*, 1979) as an intrinsic part of their structure after the growth of the fatty-acid auxotroph *Butyrivibrio* S2 in the presence of palmitic acid. In the present paper we report the structures of some of these unique large phospholipid molecules with mol.wts. exceeding 1500. In elucidating lipid structure, the method adopted has been to split off recognisable fragments of the molecule by chemical and enzymic means and thus to build up a picture of the structure as a whole. By esterification of its carboxylic acid groups, diabolic acid links either two molecules with an alkenylglycerophosphoglycerol-type structure, or one of these latter molecules with an alkenylglycerogalactose-type structure.

Experimental

Growth of Butyrivibrio S2 and isolation of its complex lipids

The methods used for growing the general fatty-acid auxotroph, extracting the lipids and isolating the main components in a state of purity have been described in the preceding paper (Hazlewood *et al.*, 1980). The numbers used in this latter paper for denoting individual lipids isolated by t.l.c. are continued in the present paper (numbers refer to the spot from which the lipid was isolated). The labelling of the hydrophobic moieties in each complex lipid achieved by always culturing the organism in the presence of [1-^{14}C]palmitic acid helped greatly in the analysis of some of the component molecular entities and was also invaluable for locating by a non-destructive method the degradation products of the complex lipids, so that these could be analysed or subjected to further degradation.

Degradation procedures

Alkaline alcoholysis. This was used to selectively split the complex lipids at carboxylic ester bonds (Dawson *et al.*, 1962; Brockerhoff, 1963). In general, the lipid (1.7 μmol/ml) was dissolved in a mixture containing carbon tetrachloride/methanol/1 M-NaOH (40:103:3, by vol.) and incubated at 43°C for various times (3–125 min). Normal fatty-acid acyl links tended to break more readily than those of the esterified carboxylic acid groups of diabolic acid. Consequently by carrying out the alkaline alcoholysis of a lipid such as lipid 14 for various times a whole series of partially saponified intermediates could be isolated. After saponification alkali was removed on a cation-exchange resin or by neutralization with ethyl formate (Dawson *et al.*, 1962) and after evaporation the lipid and hydrophilic products were distributed in chloroform/methanol/water (8:4:3, by vol.) or butanol/water (1:1, v/v).

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The esterified butyric acid proved to be much more easy to remove with alkali than esterified long-chain carboxylic acids, and methanolic NH_3 was usually effective. A sample of the lipid (1 μmol/ml) was incubated (5–15 min) with a mixture of equal volumes of methanol and aq. conc. NH_3. Room temperature was usually adequate for removing the butyroyl groups, although sometimes a higher temperature was needed and the mixture was heated at 70–100°C in a sealed tube. The hydrolysis medium was removed *in vacuo* and the products were distributed between chloroform and water. In general a butyroyl group esterified to galactose was more sensitive to ammoniacal hydrolysis than one esterified to glycerol.

Removal of aldehydogenic groups. Alkenyl groups of plasmalogens were removed as long-chain aldehydes by catalytic hydrolysis with a $HgCl_2$/HCl mixture. The lipid (2 μmol) was incubated at 43°C with 1 ml of a solution containing 5.35 mM-$HgCl_2$ and 0.98 mM-HCl in carbon tetrachloride/methanol/water (25:48:4, by vol.). Generally 20 min sufficed, but this was extended up to 4.5 h when the hydrolysis of the alkenyl group proved to be intractable. The hydrolysis mixture was taken to dryness *in vacuo* and the products were distributed in chloroform/methanol/water (8:4:3, by vol.).

Phospholipase A_2. In general, the phospholipid (0.7 μ-atom of P) was incubated in 1 ml of 0.1 M-Tris/HCl buffer, pH 7.1, containing 0.8 mM-$CaCl_2$. To the mixture was added 20 units of phospholipase A_2 from *Naja naja* venom (Koch–Light or Sigma) and 0.5 ml of methanol/diethyl ether (1:19, v/v) to assist the hydrolysis (Wells & Hanahan, 1969). The sealed tube containing the mixture was rotated in a water bath at 37°C for times up to 24 h after which the diethyl ether was blown off with a stream of air and to the residual incubation medium was added 4 ml of chloroform/methanol (2:1, v/v). The lipid digestion products were recovered from the lower chloroform-rich phase.

Phospholipase C. The phospholipid (4 μ-atoms of P) was dried down from solvent in a tube containing several glass beads. To the lipid residue was added 2 ml of 0.1 M-Tris/HCl buffer, pH 7.1, 0.2 ml (200 units) of phospholipase C (from *Bacillus cereus*, Boehringer) and 1 ml of diethyl ether. The tube was gassed with N_2, sealed and rotated at an oblique angle so that the beads tumbled for 24 h at 37°C. The lipid products in the digest were removed by extracting several times with diethyl ether until no radioactivity remained in the aqueous phase. To isolate any butyroylglycerophosphate (obtained for example in the digestion of lipids 14 and 22) the aqueous phase was treated with 36 vol. of chloroform/methanol (1:1, v/v) and the precipitate containing the ester and buffer salts etc. was collected by centrifuging. The precipitate was extracted with cold methanol (3 ml), which preferentially dissolved the butyroyl ester. The methanolic extract was then precipitated with an equal volume of chloroform and the process was repeated. The butyroyl ester was purified sufficiently for its presence to be verified by t.l.c. or paper ionophoresis by comparison with an authentic marker (Clarke *et al.*, 1976).

Separation of degradation products

Usually the products still contained hydrophobic groupings and could be conveniently separated and isolated by preparative t.l.c. on silica-gel plates (Merck F_{254}). Because of the presence of aldehydogenic groups in many of them, alkaline solvents were almost invariably used of the type described in the preceding paper (Hazlewood *et al.*, 1980). Location of lipids was by autoradiography after which the appropriate bands could be scraped off and the lipids eluted with chloroform/methanol (2:1, by vol.) and methanol.

Structure of glycerophosphate

1- or 3-Phosphoglycerols (α) were separated from 2-phosphoglycerol (β) by t.l.c. on silica-gel plates (Merck F_{254}) with methanol/propanol/conc. NH_3 (1:1:1, by vol.) as solvent. The plate was washed with diethyl ether and sprayed with the reagent of Vaskovsky *et al.* (1975) for detecting phosphorus.

The proportion of glycerophosphate in the *sn*-glycerol 3-phosphate form was determined spectrophotometrically on the isolated glycerophosphate fraction by using glycerol 3-phosphate dehydrogenase (EC 1.1.1.8) (Biochemica information, Boehringer, Mannheim, 1973 pp. 109–110).

Detection of 1-alkenylglycerol

Borate-impregnated thin-layer plates for separating α- and β-monoacylglycerol-type structures (Thomas *et al.*, 1965; Wood & Snyder, 1967) were prepared by spraying silica-gel plates (Merck F_{254}) with a 10% (w/v) solution of boric acid in methanol/water/conc. NH_3 (5:4:1, by vol.). After drying, the plates were activated by heating overnight at 100°C. They remained satisfactory for a period of about 2 weeks: if any crystalline deposit became visible on the surface they were rejected. Authentic 1-alkenylglycerol for use as a marker was prepared by incubating ox heart plasmalogen with phospholipase C (*Clostridium welchii*) followed by alkaline alcoholysis.

Action of glycosidases

The galactolipid (0.2 μmol) was incubated at 37°C with 0.5 ml of 0.1 M-Tris/HCl buffer, pH 7.1, containing 0.1% Triton X-100, 4 mM-$MgSO_4$ and 0.11 g of sweet-almond glycosidase (Koch–Light) or 10 mg of lactase (Sigma). The mixture was contained in a stoppered glass tube, gassed with N_2 and rotated continually with several glass beads for the

48h incubation. Only minimal hydrolysis of the galactolipids was obtained (10%).

Analysis of residues

The analysis of the various groups and molecular entities such as galactose, glycerol, alkenyl, fatty acyl, butyroyl and phosphorus in the lipid degradation products are described in the preceding paper (Hazlewood *et al.*, 1980).

Results and discussion

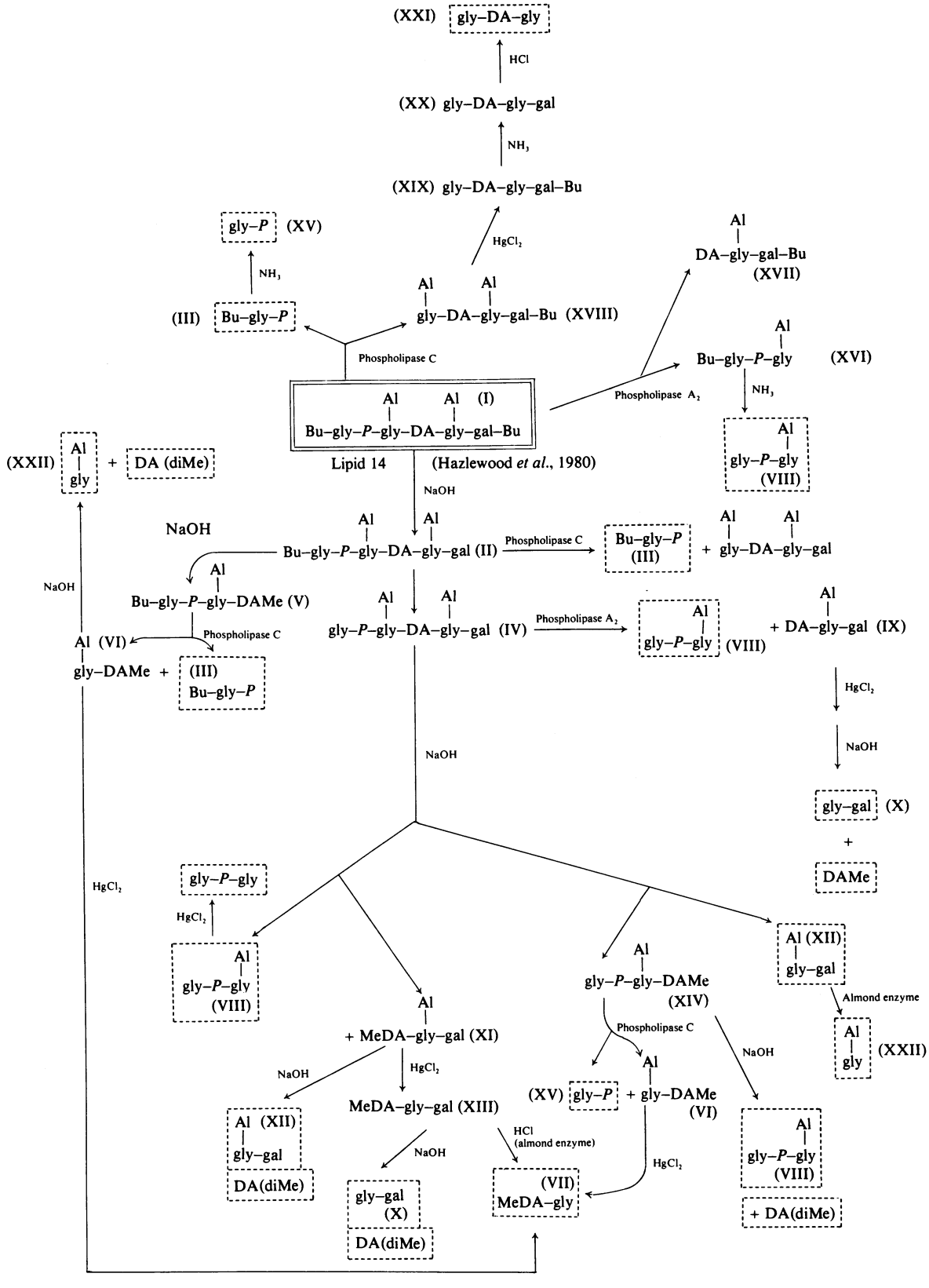
The predominant lipid (lipid 14, Hazlewood *et al.*, 1980) present in *Butyrivibrio* S2 grown in the presence of palmitic acid has the components P, galactose, glycerol, butyrate, diabolic acid and (alkenyl + acyl) groups in the molar ratio approx. 1:1:3:2:1:2 (Hazlewood *et al.*, 1980). Structural degradation was carried out chemically and enzymically as shown in Fig. 1, which also indicates the basic structure (I) of the lipid revealed by such studies.

Sequential analysis by t.l.c. of the products obtained when lipid 14 was treated with methanolic alkali indicated successive methanolysis of the acyl ester groups giving a range of intermediary products (Fig. 2). The immediate product formed represents the parent lipid minus the butyroyl group esterified to the galactose residue (II) (Figs. 1 and 2). This can be deduced by the treatment of this initial product containing one butyroyl residue with phospholipase C, which gives the butyroyl ester of glycerophosphate (III), an identifiable product (Clarke *et al.*, 1976), which is also formed when the complex lipid (I, Fig. 1) itself is treated with the same enzyme. Subsequently the main site of alkaline methanolysis is via the removal of the second butyroyl group giving the product (IV). However some methanolysis also occurs at the ester bond between the diabolic acid and glycerol residues giving the product (V), which still contains a single butyroyl residue and a methyl group introduced on to the exposed carboxylic acid grouping of diabolic acid. Treatment of this latter substance (V) with phospholipase C gives again the butyroyl ester of glycerophosphate (III) and the alkenylglycerol ester of diabolic acid (methyl ester) (VI). Removal of the aldehyde with $HgCl_2$ from structure (VI) gives the identifiable ester of diabolic acid and glycerol (VII) (still as a methyl ester). In contrast treatment of the main debutyroylated product (IV) with phospholipase A_2 gives the identifiable product alkenylglycerophosphoglycerol (VIII) (Clarke *et al.*, 1976) as well as the aldehydogenic substance (IX). Removal of the alkenyl group from structure (IX) with $HgCl_2$ and alkaline methanolysis gives the identifiable diabolic acid monomethyl ester and water-soluble glycerogalactose (X).

The debutyroylated lipid (IV) is decomposed by further alkali treatment to give initially four products depending on which carboxylic acid grouping of diabolic acid is released first. If methanolysis occurs at the phosphate end of structure (IV), then the identifiable alkenylglycerophosphoglycerol (VIII) is formed as well as the monomethyl ester of diabolic acid still esterified to alkenylglycerogalactose (XI). Further alkaline methanolysis of the latter produces diabolic acid dimethyl ester and the recognisable alkenylglycerogalactose (XII). Alternatively structure (XI) may be treated with $HgCl_2$ to give structure (XIII), which on saponification gives water-soluble glycerogalactose (X) and diabolic acid dimethyl ester. Alternatively acid hydrolysis [0.15M-HCl in methanol/water (3:1, v/v); 2–5min 100°C] or treatment with sweet-almond glycosidases gives the monomethyl ester of diabolic acid linked to glycerol (VII) in low yield. This indicates that the carboxylic acid grouping of diabolic acid is esterified directly to glycerol rather than galactose at the sugar end of the molecule, a conclusion confirmed later by the isolation of diglycerodiabolate. Since this glycerol molecule is linked therefore to diabolic acid, an alkenyl group and galactose, the butyroyl group on the sugar side of the molecule can only be esterified to the galactose residue.

Alternatively if the carboxylic ester linkage of the diabolic acid proximal to the sugar end of structure (IV) is cleaved first by the alkali treatment then the products are structure (XIV) and the identifiable alkenylglycerogalactose (XII). Further saponification of structure (XIV) gives the recognizable alkenylglycerophosphoglycerol (VIII) and diabolic acid dimethyl ester. Alternatively structure (XIV) may be treated with phospholipase C giving water-soluble glycerophosphate (XV) and alkenylglycerodiabolic acid monomethyl ester (VI), which on removal of the aldehydogenic group with $HgCl_2$ gives the monomethyl ester of diabolic acid esterified to glycerol (VII).

Further information regarding the structure of lipid 14 was obtained by direct treatment of the intact lipid (I, Fig. 1) with enzymes. Phospholipase A_2 hydrolysed structure (I), producing the butyroyl ester of alkenyl glycerophosphoglycerol (XVI) and compound (XVII), which indicated that the diabolic acid was linked to the middle hydroxy group of the glycerol on the phosphorus side of the lipid, since the enzyme acts specifically at this position. Treatment of compound (XVI) with NH_3 released butyric acid and the recognisable alkenylglycerophosphoglycerol (VIII). Phospholipase C hydrolysis of the intact lipid (I, Fig. 1) gave as well as butyroylglycerophosphate (III) the residual molecule (XVIII). This latter lipid proved useful for confirming that the diabolic acid was esterified to two glycerol residues. The aldehydogenic groups were


(XXI) gly–DA–gly
HCl
(XX) gly–DA–gly–gal
NH3
(XIX) gly–DA–gly–gal–Bu
gly–P (XV)
HgCl2
NH3
(III) Bu–gly–P
Al Al
gly–DA–gly–gal–Bu (XVIII)
Phospholipase C
Al
DA–gly–gal–Bu
(XVII)
Al
Bu–gly–P–gly (XVI)
NH3
Phospholipase A2
Al (I)
Bu–gly–P–gly–DA–gly–gal–Bu
Lipid 14 (Hazlewood et al., 1980)
Al
gly–P–gly
(VIII)
(XXII) Al gly + DA (diMe)
NaOH
Al Al
Bu–gly–P–gly–DA–gly–gal (II)
Phospholipase C
Bu–gly–P
(III)
+ gly–DA–gly–gal
NaOH
Al
Bu–gly–P–gly–DAMe (V)
Al Al
gly–P–gly–DA–gly–gal (IV)
Phospholipase A2
Al
gly–P–gly
(VIII)
+ DA–gly–gal (IX)
Al (VI)
gly–DAMe +
Phospholipase C
(III)
Bu–gly–P
HgCl2
NaOH
gly–gal (X)
+
DAMe
HgCl2
gly–P–gly
HgCl2
Al
gly–P–gly
(VIII)
Al
+ MeDA–gly–gal (XI)
Al
gly–P–gly–DAMe
(XIV)
Al (XII)
gly–gal
Almond enzyme
Phospholipase C
NaOH
Al
gly
(XXII)
NaOH
HgCl2
(XV) gly–P + gly–DAMe
(VI)
MeDA–gly–gal (XIII)
Al (XII)
gly–gal
DA(diMe)
HCl
(almond enzyme)
NaOH
HgCl2
Al
gly–P–gly
(VIII)
+ DA(diMe)
gly–gal
(X)
DA(diMe)
(VII)
MeDA–gly

removed from compound (XVIII) with $HgCl_2$ (4.5 h, 45°C) giving the product (XIX) in 70% yield and also a minor substance that still gave a reaction for aldehydes and is probably a cyclic acetal. Structure (XIX) was treated with NH_3 to release butyric acid. Even at room temperature considerable secondary degradation also occurred, but by stopping the reaction at 12 min the optimal accumulation (25% yield) of the primary product (XX) was obtained. A very brief treatment (75 s) of compound (XX) with 2 M-HCl at 100°C removed the galactose and gave a substance that analysed as diglycerodiabolate (XXI) and was chromatographically identical with the product obtained by phospholipase C and $HgCl_2$ treatment of the non-galactose-containing phospholipid (22) (see below).

From the above results the derived formula of the predominant complex lipid is as given in Fig. 3. The galactose is denoted as being in the furanose form because of the paper-chromatographic properties of the separated galactoglycerol, which ran much faster in n-propanol/ethyl acetate/water (24:13:7, by vol.) than authentic galactopyranosylglycerol. The furanose designation of the galactose was also suggested by the acid lability of the glycosidic linkage (Reeves *et al.*, 1964). The *sn*-1-position of the aldehydogenic group on the phosphate side of the lipid was shown not only by the results of phospholipase A_2 attack, but also by taking compound (VI) and producing an alkenylglycerol (XXII) by alkaline methanolysis. The behaviour of this on a borate-impregnated thin-layer plate indicated that the glycerol was substituted in a terminal hydroxy position. The configuration of the terminal glycerophosphate residue was examined by taking the butyroylglycerophosphate (III) split off from the intact lipid I by phospholipase C action, and removing the butyroyl group by NH_3 treatment. T.l.c. of the glycerophosphate (XV) indicated that the phosphate was esterified to a terminal hydroxy group (α-glycerophosphate), but this glycerophosphate was not reduced by glycerophosphate dehydrogenase, so presumably the product is *sn*-glycerol 1-phosphate. In contrast the susceptibility to phospholipase A_2 indicates the other glycerophosphate bond has the *sn*-glycerol 3-phosphate configuration. The position of the glycerol–galactose link has not been ascertained, but in conformity with the almost universal pattern of bacterial glycolipids it has been expressed in Fig. 3 as the 3-galactosyl-*sn*-glycerol. The problem of the site of attachment of the butyroyl groups on the terminal glycerol and galactose residues has not been solved because of the extreme ease with which esterified short-chain fatty acids can migrate between adjacent hydroxy groups in a molecule such as glycerol or galactose (Brockerhoff & Jensen, 1974). With long-chain fatty acids in a structure such as bacterial 3-*sn*-phosphatidyl-1′-(3′-acyl)-*sn*-glycerol, the acyl group is esterified to the 3′-hydroxy group (Olsen & Balou, 1971) and by analogy the butyroyl is also probably on the 3-position of the terminal glycerol residue of lipid 14. Although acylated sugar residues are not uncommon among the bacterial glycolipids (Shaw, 1970), there is little evidence to predict the likely site of attachment of the butyroyl group to the galactose residue, although in *Bifidobacterium bifidum* long-chain acyl groups can be attached to the 2- or 3-positions (Veerkamp, 1972). The susceptibility of the glycerogalactose (X) residue to β-galactosidase and analogy with other bacterial galactolipids containing galactofuranosyl residues (Plackett, 1967; Veerkamp, 1972) would indicate that it is an *O*-β-D-galactofuranosylglycerol linkage.

The lipid occurring in the second highest concentration (lipid 22, Hazlewood *et al.*, 1980) contained no sugar residue detectable by direct analysis or by examination of its decomposition products. Analysis of its molecular residues (Hazlewood *et al.*, 1980) indicated the simplest molecular ratios for diabolic acid/P/glycerol/butyrate/(aldehydogenic + acyl) groups as 1:2:4:2:2.

Enzymic and chemical procedures used to degrade the lipid are illustrated in Fig. 4. Incubation of lipid 22 (XXIII, Fig. 4) with phospholipase C liberated all the phosphorus as a product that could be easily identified as butyroylglycerophosphate (III) and a lipoidal product (XXIV). When the butyroylglycerophosphate (III) was hydrolysed in methanolic NH_3 the butyroyl group was removed and α-glycerophosphate (XV) was identified by paper ionophoresis (Clarke & Dawson, 1972) and t.l.c. (see the Experimental section). The glycerophosphate could not be decomposed by glycerophosphate dehydrogenase, so presumably it has the *sn*-glycerol 1-phosphate configuration; calf intestinal alkaline phosphatase liberated unesterified

Fig. 1. *Chemical and enzymic methods used for degrading ^{14}C-labelled phospholipid 14 obtained from Butyrivibrio S2 cultured with [1-^{14}C]palmitic acid*

Intermediaries were isolated by t.l.c. and autoradiography. Abbreviated versions of the various molecular residues have been used for convenience as follows: Bu, butyroyl; Al, alkenyl; DA, diabolic acid; gly, glycerol; gal, galactose; *P*, phosphate; Me, methyl. Products contained in broken-line squares can be identified by comparison with standard compounds. The enzymic and chemical degradations carried out are described in the Experimental section.

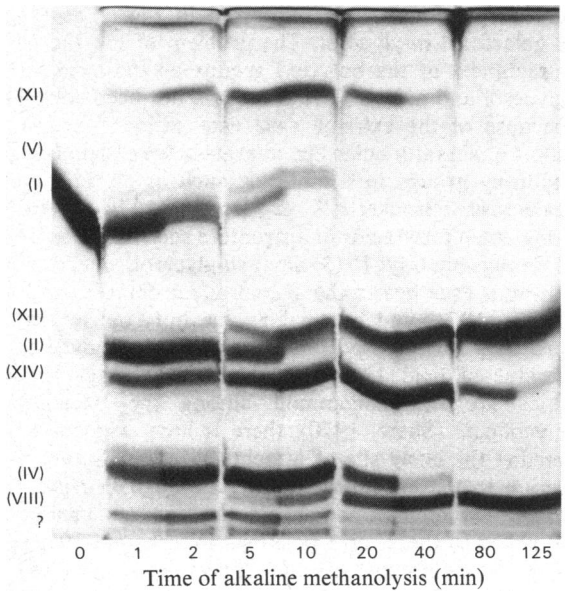


Fig. 2. *Degradation of the predominant lipid with methanolic NaOH*

The original lipid was incubated with methanolic alkali and samples were taken at various times for analysis of the lipoidal components by t.l.c. (chloroform/methanol/conc. NH_3, 100:30:3, by vol., with 30min overrun, followed by a second run with chloroform/methanol/conc. NH_3, 50:10:1, by vol., in the same direction) development by autoradiography. The Roman numerals refer to compounds assigned in Fig. 1. Lipid (I) (i.e. lipid 14 in Hazlewood *et al.*, 1980) is rapidly destroyed by loss of the butyroyl group attached to the galactose residue producing compound (II). The second butyroyl group can then be lost giving the product (IV). However, some methanolysis of the esterified diabolic acid before this can occur giving the fast-running product (V). Further decomposition of compounds (V) and (IV) then occurs, giving the terminal product alkenylglycerophosphoglycerol (VIII) with some transient intermediary accumulation of this compound still esterified to the monomethyl ester of diabolic acid (XIV). The other terminal product is alkenyl glycerogalactose (XII), again through the intermediary of this compound with the monomethyl ester of diabolic acid still attached to compound (XI). The identity of the two very-slow-running transient intermediates is unknown, although they contain both P and galactose: possibly they represent intermediaries formed through the presence of some fatty-acyl groups in the starting lipid (I) (Hazlewood *et al.*, 1980), presumably in place of the alkenyl grouping. The dimethyldiabolic acid formed as a final product is very fast running and is lost in the overrun of the t.l.c. plate.

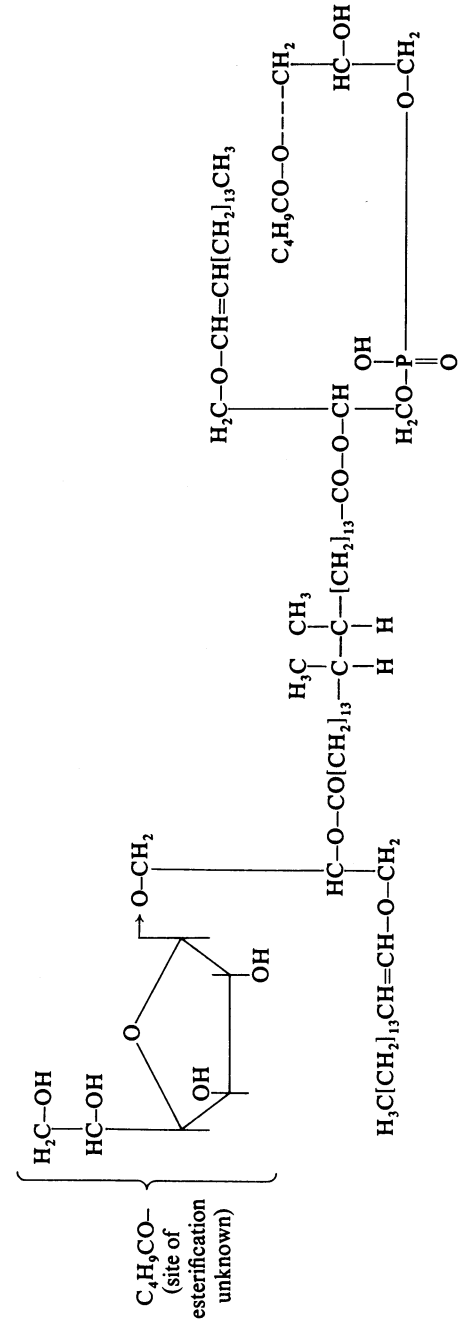


Fig. 3. *Structural formula of Lipid 14*

The butyroyl group on the glycerol residue can be replaced by a palmitoyl group (lipid 11).

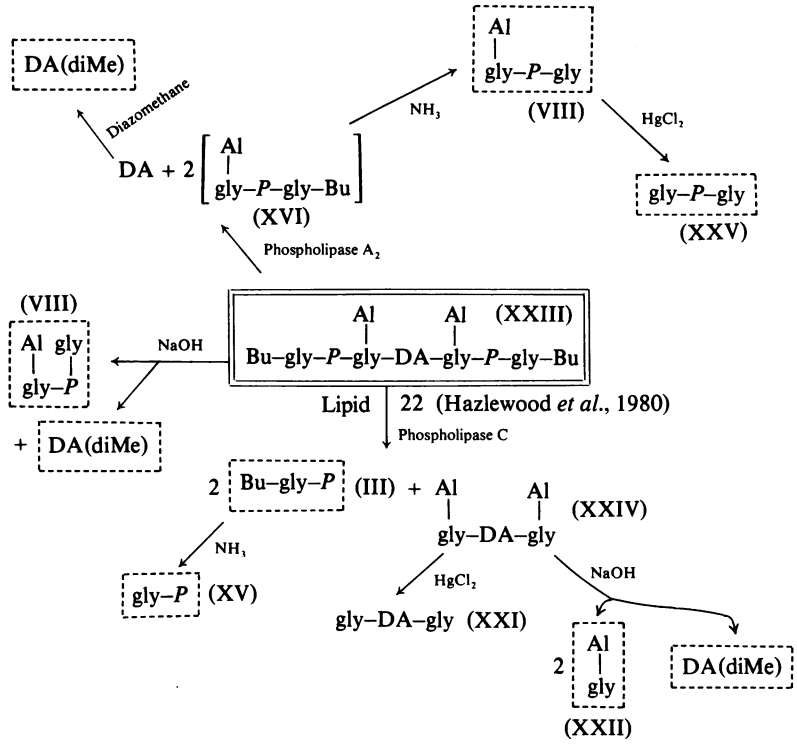


Fig. 4. *Chemical and enzymic methods used for degrading ^{14}C-labelled phospholipid 22*
Other details are as described in the legend to Fig. 1.

glycerol that could be detected by paper chromatography. The liberation of butyroylglycerophosphate, the residue analysis and analogy with the structure of lipid 14 suggested therefore that lipid 22 consisted of two molecules of alkenylglycerophosphoglycerol butyroyl ester linked by esterification with diabolic acid between the vacant hydroxy groups of the alkenyl-substituted glycerols. This surmise was strengthened by finding that on methanolic NaOH treatment, the end products could be identified as the dimethyl ester of diabolic acid and alkenylglycerophosphoglycerol (VIII).

The lipoidal product of phospholipase C hydrolysis (XXIV) contained aldehydogenic groups and diabolic acid in the molar ratio 2.04:1. It was treated with $HgCl_2$ to liberate the aldehydogenic groups: this gave a product that consisted of diabolic acid and glycerol in the molar ratio 1:2.06 and that is assumed to be two glycerol molecules esterified to the two carboxylic acid groups of diabolic acid (XXI). In addition product (XXIV) was treated with methanolic NaOH giving as the final products diabolic acid dimethyl ester and an alkenylglycerol (XXII). The behaviour of the latter product on t.l.c. plates impregnated with borate was equivalent to glycerol with the alkenyl group linked to a terminal hydroxy group.

The intact lipid (XXIII) was readily attacked by phospholipase A_2. Diabolic acid could be extracted from the incubation medium with diethyl ether, whereas the phosphate-containing portion remained water-soluble. The diabolic acid was converted into its dimethyl ester by passing diazomethane through its ethereal solution, and on t.l.c. this co-chromatographed with authentic $(C_{16:0})_2$ diabolic acid dimethyl ester (Klein *et al.*, 1979). The phosphate-containing product could be extracted from the diethyl ether-extracted incubation medium with chloroform and proved to be alkenylglycerophosphoglycerol butyroyl ester (XVI) in 87% yield. This compound had also been split from the main lipid (lipid 14) by phospholipase A_2 (Fig. 1, XVI). On treating compound (XVI) with NH_3/methanol to remove its butyroyl group (analysis showed that it contained one butyroyl group per P atom) the identifiable alkenylglycerophosphoglycerol (VIII) was produced. On further treatment of this with $HgCl_2$, palmitaldehyde and water-soluble glycerophosphoglycerol (XXV) were produced. The former was identified by g.l.c. (Clarke *et al.*, 1976) and the

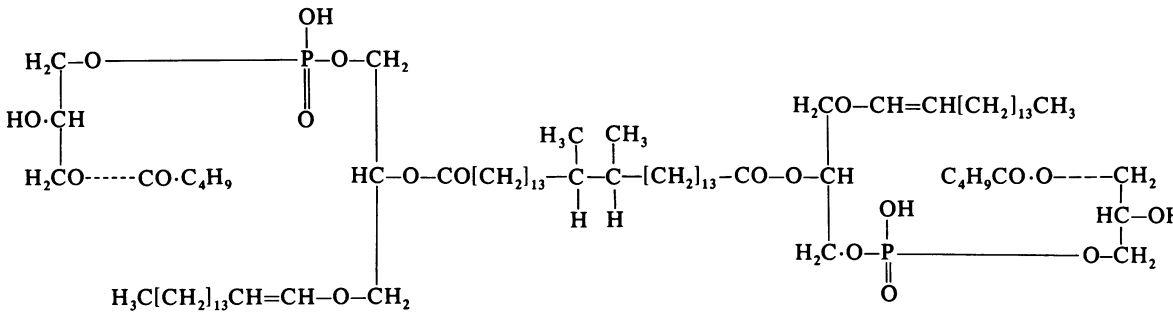


Fig. 5. *Structural formula of Lipid 22*

Similar structures exist in which one (lipid 25) or two butyroyl groups (lipid 26) are missing or one butyroyl group can be replaced by a palmitoyl group (lipid 20).

latter by paper ionophoresis (Dawson *et al.*, 1962). The phosphate ester ran identically with bis(glycero)phosphate prepared by the deacylation of phosphatidylglycerol ($M_{P_i} = 0.78$).

From the above evidence, it is suggested that the structure of lipid 22 is that denoted in Fig. 5. The esterification of the diabolic acid at the *sn*-2-position and the phosphate at the *sn*-3-position of the central glycerol molecules is defined by the specificity of phospholipase A_2; the terminal position of the alkenyl group in the isolated alkenylglycerol confirms this assignation. The end glycerols on the other hand have the *sn*-glycerol 1-phosphate configuration. The position of the esterified butyroyl group has again not been proven, although for reasons given previously one suspects that they may be terminal on the end-glycerol residues (Olsen & Balou, 1971).

Although lipids 14 and 22 were the only phospholipids whose structures were positively elucidated, we have evidence that some of the other diabolic acid-containing lipids are structurally related. Thus lipid 20, whose analysis indicates that the P/glycerol/diabolic acid/acyl/alkenyl/butyroyl ratio approximates to 2:4:1:1:2:1 (Hazlewood *et al.*, 1980) is probably lipid 22 (Fig. 5) in which one of the butyroyl groups has been replaced by a palmitoyl residue. This conclusion is confirmed by the production of the butyroyl ester of glycerophosphate and lysophosphatidic acid by the action of phospholipase C. Similarly lipid 11 when analysed differs from lipid 14 (Fig. 3) by having a palmitoyl group replacing a butyroyl group (Hazlewood *et al.*, 1980) and presumably the same inference can be drawn regarding its structure. When phospholipase C acts on lipid 11, lysophosphatidic acid can be identified as a product, indicating that the palmitoyl group replaces the butyroyl group on the P end of the molecule rather than the galactose end. From their chromatographic properties and analysis, lipids 25 and 26 probably have the same structure as lipid 22 (Fig. 5) minus one (lipid 25) or two (lipid 26) butyroyl groups. The ninhydrin-reacting lipids (lipids 28 and 29) contain ethanolamine linked to a phosphate residue. They also contain galactose, diabolic acid and butyroyl residues.

It is seen therefore that virtually all the phospholipids of *Butyrivibrio* S2 grown in the presence of palmitic acid consist of two molecules of more conventional alkenylglycerols linked by esterification through the $(C_{16:0})_2$ dicarboxylic acid, diabolic acid. There is a dearth of the usual phospholipids found in bacteria. Although the precise function of such unique lipids is unknown, two possibilities are apparent. First, the organism contains powerful phospholipases and glycolipases (Hazlewood & Dawson, 1979), which are present in its plasma membrane (G. P. Hazlewood and K. Y. Cho, unpublished work), and it is possible that such unusual structures would inhibit autodigestion of the plasma-membrane lipids. Secondly, the vicinal methyl groups in the centre of the diabolic acid would undoubtedly have a fluidizing effect on the plasma-membrane lipids (Hauser *et al.*, 1979), which would probably help the survival of the organism growing in the excess of saturated fatty acids as exists in the rumen.

We have little precise information on the biosynthetic route for such complex lipids. Presumably the diabolic acid structure is formed by dehydrogenation condensation at the penultimate carbon atoms of two fatty acid molecules (Klein *et al.*, 1979). However, theoretically this type of reaction could also occur if the fatty acid residues were esterified in a lipid structure. Thus if two molecules of the butyroyl ester of 2-acyl-1-alkenyl-*sn*-glycero-3-phospho-1′-*sn*-glycerol already shown to be present in the organism as a minor component (lipid

15; Hazlewood *et al.*, 1980) were dehydrogenated in this way, lipid 22 (Fig. 5) would be formed. Or if one molecule of lipid 15 interacted with a molecule of the 2-acyl-1-alkenyl-3-*sn*-galactosylglycerol, butyroyl ester (lipid 5) then the main lipid component (lipid 14, Fig. 3) would result. Experiments are required to test these possibilities.

References

Brockerhoff, H. (1963) *J. Lipid Res.* **4**, 96

Brockerhoff, H. & Jensen, R. G. (1974) *Lipolytic Enzymes*, p. 68, Academic Press, London and New York

Clarke, N. G. & Dawson, R. M. C. (1972) *Biochem. J.* **127**, 113–118

Clarke, N. G., Hazlewood, G. P. & Dawson, R. M. C. (1976) *Chem. Phys. Lipids* **17**, 222–232

Dawson, R. M. C., Hemington, N. & Davenport, J. B. (1962) *Biochem. J.* **84**, 497–501

Hauser, H., Hazlewood, G. P. & Dawson, R. M. C. (1979) *Nature (London)* **279**, 536–538

Hazlewood, G. P. & Dawson, R. M. C. (1979) *J. Gen. Microbiol.* **112**, 15–27

Hazlewood, G. P., Clarke, N. G. & Dawson, R. M. C. (1980) *Biochem. J.* **191**, 555–560

Klein, R. A., Hazlewood, G. P., Kemp, P. & Dawson, R. M. C. (1979) *Biochem. J.* **183**, 691–700

Olsen, R. W. & Balou, C. E. (1971) *J. Biol. Chem.* **246**, 3305–3313

Plackett, P. (1967) *Biochemistry* **6**, 2746–2754

Reeves, R. E., Latour, N. G. & Lousteau, R. J. (1964) *Biochemistry* **3**, 1248–1249

Shaw, N. (1970) *Bacteriol. Rev.* **34**, 365–377

Thomas, A. E., III, Scharoun, J. E. & Ralston, H. (1965) *J. Am. Oil Chem. Assoc.* **42**, 789–792

Vaskovsky, V. E., Kostetsky, E. Y. & Vasendin, I. M. (1975) *J. Chromatogr.* **114**, 129–141

Veerkamp, J. H. (1972) *Biochim. Biophys. Acta.* **273**, 359–367

Wells, M. A. & Hanahan, D. J. (1969) *Biochemistry* **8**, 414–424

Wood, R. & Snyder, F. (1967) *Lipids* **2**, 161–171