Ceramide Phosphorylglycerol Phosphate A New Sphingolipid Found in Bacteria

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ABSTRACT

Ceramide phosphorylglycerol phosphate (CPGP) has been identified in the lipid extract of the anaerobic bacterium *Bacteroides melaninogenicus*. To our knowledge this is the first report of this lipid in biological material. The ceramide derivative contains two phosphates, an amide linked fatty acid and a dihydrosphingosine long chain base. Glycerol diphosphate (PGP) identified by paper and column chromatography can be isolated after mild acid hydrolysis of the ceramide derivative. Inorganic phosphate is liberated quantitatively on treatment of the PGP from the ceramide derivative with alkaline phosphatase. The proportions of the fatty acids found linked to the amide of the dihydrosphingosine (LCB) differ from those esterified to cardiolipin in this organism. The long chain base appears to consist of part of an homologous series of branched and normal LCB containing from 17 to 21 carbon atoms. Previous work has indicated that ceramide phosphorylethanolamine and ceramide phosphorylglycerol (CPG) are present in the lipid extracts of *B. melaninogenicus*. By analogy with phosphatidylglycerol synthesis, CPGP is postulated to be an intermediate in the synthesis of CPG.

INTRODUCTION

The anaerobic bacterium *Bacteroides melaninogenicus* has been shown to contain phosphate containing sphingolipids which account for half the extractible lipid phosphate (1). Sphingolipids are exceedingly rare in eubacteria (2). Ceramide phosphorylethanolamine (CPE), a rare sphingolipid previously reported in insects, protozoa and certain snails, and ceramide phosphorylglycerol (CPG), a lipid not previously reported, make up the major portion of the sphingolipid of *B. melaninogenicus* (1). A trace of a third phosphate containing sphingolipid was detected. In this study the trace ceramide has been identified as ceramide phosphorylglycerol phosphate (CPGP), a lipid not previously described in nature.

MATERIALS AND METHODS

Materials

The strain of *B. melaninogenicus*, the cultural conditions, harvesting procedures and methods for insuring cultural purity have been described in previous work (1,3). $H_3{}^{32}PO_4$ was supplied in plastic bottles by Tracerlabs, Waltham, Mass.

Column Chromatography

Fatty acid methyl esters were separated from ceramides or LCB on 1 g silicic acid columns (11 X 50 mm, Unisil, 100-200 mesh). The fatty acid methyl esters were eluted in 5 ml of chloroform. The dihydrosphingosine long chain bases (LCB) or ceramides were eluted with 5 ml of chloroform-methanol, (1:1) followed by 5 ml of methanol.

Glycerol phosphate esters derived from the lipids were eluted from 0.4 X 81 cm columns of Dowex-1 8X (200-400 mesh) in the formate form prepared as described (4,5). The esters were eluted with an ammonium formate-sodium borate gradient (4) or with 0.3 M ammonium formate pH 9.5 (Lester, unpublished method). The esters were desalted with Dowex-1 (100-200 mesh) as described in the text.

Paper Chromatography

Lipids were separated on silica gel loaded paper (Whatman SG-81) using solvents of chloroform-methanol-diisobutylketone-acetic acid-water (23:10:45:25:4 v/v), Solvent 1 in the first dimension and chloroform-methanol-diisobutylketone-pyridine-0.5 M ammonium acetate pH 10.4 (30:17.5:25:35:6 v/v), Solvent 2. Lipids were eluted from the silica gel loaded paper with a solvent of chloroform-methanol-19 mM ammonium hydroxide (20:20:1) by soaking the paper in 3 ml of solvent for 1 hr. The paper was then rinsed in three 1 ml portions of solvent. The recovery was quantitative.

Glycerol phosphate esters were separated on acid washed amino-cellulose paper (Whatman AE-81) (7). Solvents were 0.4% pyridine in 3 M formic acid and modified Wawszkiewicz solvent (5). This solvent contains 1.15 M ammonium acetate with 11.8 mM ethylenediaminetetra-

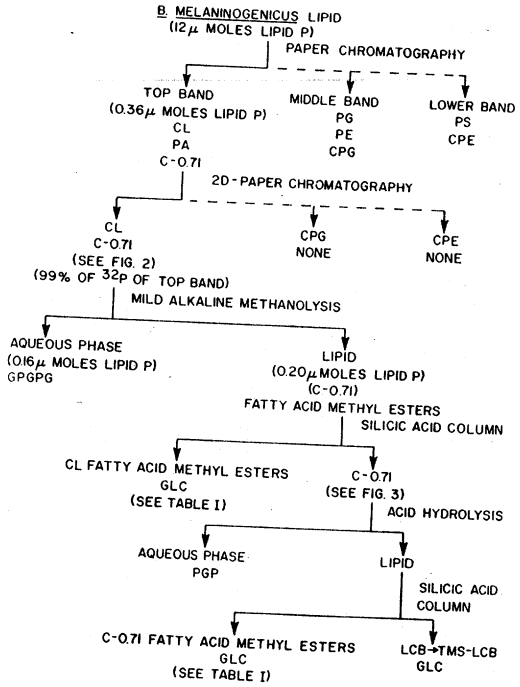

FIG. 1. Flow sheet for the purification of the unknown ceramide derivative.

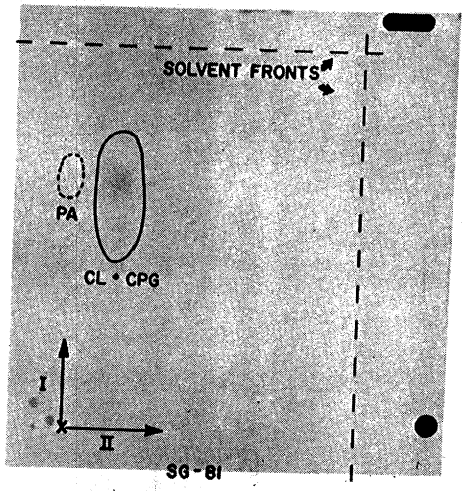

FIG. 2. Radioautogram of the chromatographic separation of the unknown ceramide derivative and CL from *B. melaninogenicus* grown with ^{32}P. See Materials and Methods and Reference 7.

acetic acid made to pH 5.0 with acetic acid and diluted 3 to 7 with 95% ethanolic 0.25 M ammonium hydroxide. Schleicher and Schuell 589 acid washed paper was used with ascending paper chromatography with the modified Wawszkiewicz solvent. The lipids were detected with the Hanes-Isherwood reagent for phosphate (4) or by periodate treatment followed by *o*-toluidine (4).

Gas Chromatography

Fatty acid methyl esters were prepared and separated on ethylene glycol succinate or SE-30 columns under the conditions described previously (8). Trimethylsilyl ether derivatives (TMS) of the LCB were prepared and analyzed as in an earlier study (1).

Measurement of Radioactivity

^{32}P was counted on paper disks in a scintillation spectrometer (7). Radioautograms were prepared with Kodak no-screen x-ray film (7). Illustrations of radioautograms were prepared by drawing the figures, copying the figure on a mylar sheet with the Xerox copier, then superimposing the developed film and the mylar sheet properly. The sheet and superimposed film were then photographed on a glow box.

Extraction and Analysis of the Lipid

Lipids were extracted from the bacteria by a modified Bligh and Dyer procedure (9). A 30 ml suspension of bacteria in 50 mM phosphate buffer pH 7.6 containing about 200 mg dry weight of cells was mixed with 75 ml of methanol and 37.5 ml of chloroform and shaken vigorously. The one phase system was allowed to stand overnight. Then 37.5 ml of chloroform and 37.5 ml of 1.0 M KCL solution containing glacial acetic acid (0.4% v/v) was added and the mixture shaken. After several hours the mixture separated into two phases. The lower layer containing the lipid was filtered through a 4 cm piece of Whatman No. 12 filter paper.

Purification of the Lipid

A flow chart of the purification of the unknown lipid is illustrated in Figure 1. A total of 12 μmoles of lipid phosphate isolated from cells grown in the presence of ^{32}P was spotted near the bottom edge of two silica gel impregnated papers. The lipids were separated into three bands by ascending chromatography in a solvent of chloroform-methanol-diisobutylketone-acetic acid-water (23:10:45:25:4 v/v). The bands were located by radioautography

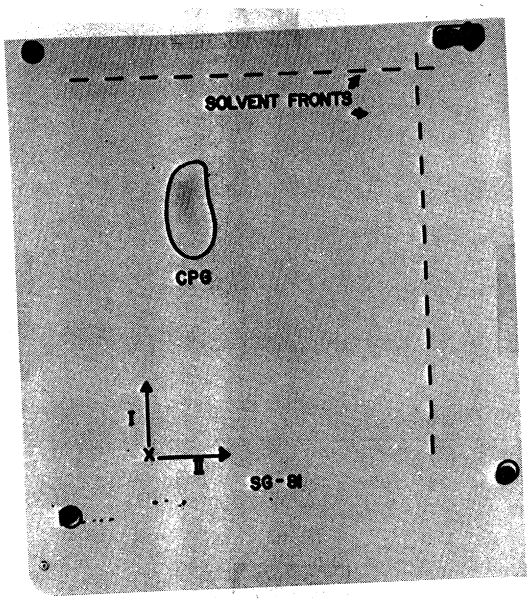

FIG. 3. Radioautogram of the unknown ceramide derivative after mild alkaline methanolysis which removes the CL. See Figure 2.

TABLE I

Distribution of Fatty Acids Between the Amide of Ceramide Phosphorylglycerol Phosphate and the Esters of Cardiolipin in *Bacteroides melaninogenicus*[a]

Fatty acid	Amide of CPGP	Ester of Cl
12:0	3.7	---
13:0, Br	2.8	0.9
14:0, Br	8.0	---
14:0	---	1.6
15:0, Br	25.8	64.6
15:0	10.5	---
16:0, Br	2.9	---
16:0	12.8	8.2
17:0, Br	---	8.6
18:0, Br	11.5	1.3
19:0, Br	0.8	5.4
19:0	0.3	7.3
20:0, Br	8.1	---
20:0	---	2.1
21:0	2.5	---

[a]Fatty acid methyl esters determined from the areas of response after GLC on ethylene glycol succinate columns. The data are given as the percentage of the total fatty acids recovered from the amide or the ester linkage.

and the lipids recovered. The top band contained lipids with the chromatographic mobility in other solvent systems (7) of cardiolipin (CL), phosphatidic acid (PA), and the trace ceramide derivative (R_f value 0.71). The middle band contained lipids with the chromatographic mobility in other systems of phosphatidylglycerol (PG), CPG and phosphatidyl ethanolamine (PE) (R_f value 0.52) and the lower band contained CPE and phosphatidylserine (PS) (R_f value 0.43). The lipids from the top band were recovered from the silica gel impregnated paper and spotted on sheets of this paper again. Two-dimensional chromatography separated the CL and ceramide derivative from the phosphatidic acid (Fig. 2). The CL plus ceramide derivative were recovered and accounted for 99% of the ^{32}P from the top band. Mild alkaline methanolysis was performed at 0 C for 2 hr and the KOH neutralized with Biorex 70 (a weakly acidic cation exchange resin (7). The water soluble glycerol phosphate ester derived from the diacyl lipid was then separated from the fatty acid methyl esters and the ceramide derivative using a sequence of three extractions with diethyl ether and a final extraction with chloroform to minimize emulsion formation (1). The mild alkaline methanolysis is complete in 2 hr (1).

Lipid phosphate was analyzed after digestion of the samples in perchloric acid (4); 0.16 μmoles of radioactive phosphate were recovered as a water soluble ester which co-chromatographed with unlabeled authentic diglycerol phosphorylglycerol (GPGPG) in two dimensions on aminocellulose paper (7). The remainder of the ^{32}P (0.20 μmoles lipid phosphate) was recovered in the organic solvent after the mild alkaline methanolysis.

The organic phase from the mild alkaline methanolysis contained fatty acid methyl esters derived from the CL. The fatty acid esters were separated from the ceramide derivative by silicic acid chromatography. The fatty acid esters were then analyzed by gas liquid chromatography (GLC). The ceramide derivative recovered from the silicic acid column was chromatographed in two dimensions on silica gel-loaded paper (Fig. 3). The ceramide derivative had identical chromatographic mobility before and after mild alkaline methanolysis (Fig. 2 and 3). This suggests the ceramide derivative contains no ester linked fatty acids. No fatty acyl esters were detected in ceramide phosphorylethanolamine (CPE) in this organism (1). The ceramide derivative did not react with periodate (4) before or after mild alkaline hydrolysis.

The ceramide derivative was hydrolyzed in methanolic 2 N HCl containing water (9.5% v/v) at 100 C for 2 hr (1,10). This procedure quantitatively liberates the amide linked fatty

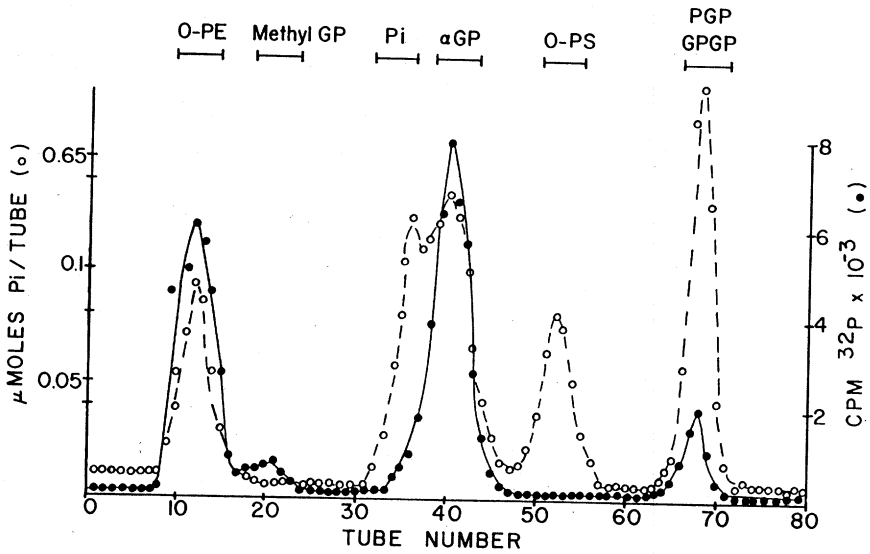

FIG. 4. Column chromatography of the water soluble products of the mild acid hydrolysis of the ceramides of *Bacteroides melaninogenicus* grown in the presence of ^{32}P. Fractions of 2.65 ml were collected, a portion removed to assay the ^{32}P and the remainder digested for the phosphate determination. The elution volumes of authentic compounds are illustrated at the top of the Figure. The recovery of ^{32}P was quantitative.

acid, the LCB and water soluble phosphate derivative (1). The fatty acids derived from the amide of the LCB were separated from the LCB by silicic acid chromatography of the organic phase of the hydrolysis mixture. These fatty acids were methylated (8) and separated by GLC. A comparison of the proportions of fatty acid components from the amide of the LCB and the fatty acids in ester linkage to CL is given in Table I. The fatty acids derived from the amide contain less 15:0, Br and more of the longer branched fatty acids than are found in the ester linkage. The LCB fraction was recovered from the silicic acid column and TMS derivatives prepared (1,11). TMS derivatives of the LCB had retention times corresponding to 16:0 (1.5%), 17:0,Br (39%), 18:0 (18%), 20:0, BR (4%), 20:0 (20%) and 21:0 (15%). The total detector response corresponded to 0.10 μmoles of TMS-dihydrosphingosine. This indicates that the molar ratio of LCB to phosphate in the ceramide was 1.00 to 2.05. The total response of the amide-linked fatty acid corresponded to 0.09 μmoles of methyl palmitate for an amide fatty acid to phosphate molar ratio of 0.92 to 2.00.

Identification of the Water Soluble Product of Acid Hydrolysis

To collect a large amount of the water soluble hydrolysis product of the unknown ceramide derivative for identification, the total lipid was deacylated by mild alkaline methanolysis and the fatty acids separated from the ceramide derivatives on a silicic acid column. The ceramide derivatives were hydrolyzed in 1 ml methanolic 2 N HCl containing 6.5 M water for 1 hr at 100 C. After cooling, 1 ml of water was added and the mixture extracted with two 2 ml portions of petroleum ether. The aqueous phase was then made to pH 10 with KOH and the petroleum ether extraction repeated twice. The aqueous phase was desalted by passing through a 5 X 300 mm column of Dowex 50-8X, 200-400 mesh, in the acid form and the ^{32}P recovered quantitatively. The HCl was removed in a stream of nitrogen. The ^{32}P labeled hydrolysis products were combined with authentic *o*-phosphorylethanolamine (*o*-pE), L-α-glycerol phosphate (αGP), inorganic phosphate (P_i), glycerol diphosphate (PGP) and *o*-phosphorylserine (*o*-pS) in 20 mM sodium borate pH 9.5 and loaded on a Dowex-1 column. The esters were then eluted from the column with an ammonium formate-sodium borate gradient (4). Esters containing ^{32}P were detected at the elution volumes of *o*-pE (43.3% of the ^{32}P), methyl-GP (4.5%), αGP (43.6%) and GPGP or PGP (8.5%). This is illustrated in Figure 4. The fractions corresponding to PGP or GPGP were combined and diluted to eight times their volume with distilled water. The sample was then pumped onto a 0.4 X 15 cm column of Dowex-1 -8X (100-200) mesh in the

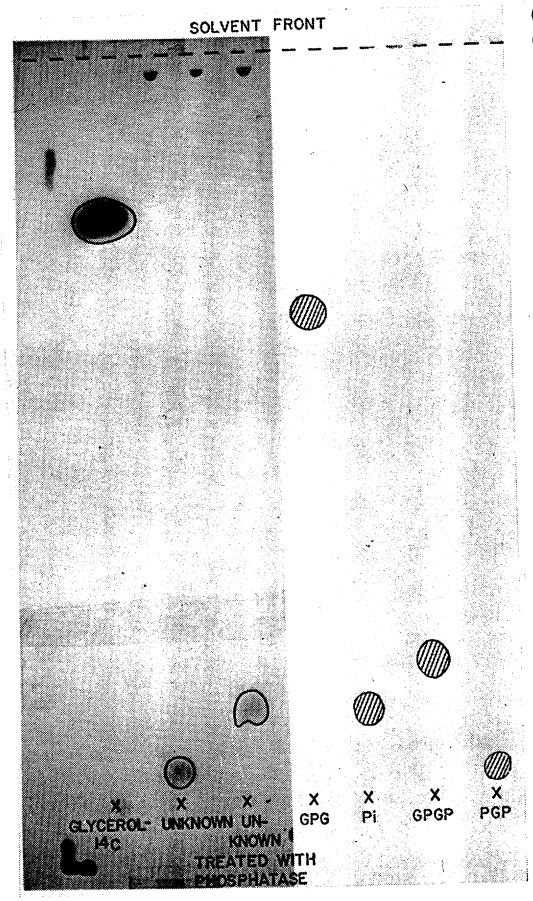

FIG. 5. Radioautogram of the separation of the water soluble glycerol phosphate esters derived from the trace ceramide of *Bacteroides melaninogenicus* grown in the presence of ^{32}P. The PGP fraction from the column illustrated in Figure 4 as desalted and chromatographed before and after treatment with alkaline phosphatase as described in Materials and Methods.

formate form. The column was washed with 3 column vol of 10 mM formic acid to remove the borate. The glycerol phosphate ester was then eluted with 0.3 M ammonium carbonate pH 8.0. The ^{32}P was quantitatively recovered. The ammonium carbonate was removed from the fraction containing the ^{32}P by boiling to dryness in a stream of nitrogen.

A portion of the ^{32}P containing ester was dissolved in 25 μl of 20 mM ammonium acetate pH 8.0. This was treated with 25 μl of alkaline phosphatase (1 mg/ml) from *Escherichia coli* (Worthington) for 3 hr at 25 C (5). The products of enzymatic hydrolysis and unhydrolyzed ester were applied to paper and subjected to ascending chromatography on acid washed paper with the modified Wawszkiewicz solvent (5). In this system the R_f values were glycerol, 0.80; glycerol phosphorylglycerol (GPG), 0.64; GPGP, 0.18; P_i, 0.15; and PGP, 0.05. A radioautogram of the ^{32}P-containing ester and its hydrolysis product together with a chromatogram of authentic standards is illustrated in Figure 5. The ^{32}P-containing ester had the chromatographic mobility of PGP. Only $^{32}P_i$ could be detected after alkaline phosphatase treatment. As a further identification the ester corresponding to the ^{32}P spot in Figure 5 was eluted from the paper with water, mixed with authentic P_i, GPGP and PGP and applied to a Dowex-1 8X (200-400 mesh) column. The esters were eluted with 0.3 M ammonium formate pH 9.5 as illustrated in Figure 6. The ^{32}P containing ester is clearly not GPGP.

RESULTS

Separation of the Unknown Ceramide Derivative

The lipid isolated by the procedure illustrated in Figure 1 was separated from CL. This is confirmed by the different proportions of fatty acids from the ceramide derivative and CL (Table I). Chromatography of the water soluble portion from the mild alkaline methanolysis performed on aminocellulose paper (7) indicated that there was no ceramide derivative in the GPGPG. The radioautograms illustrated in Figures 2 and 3 indicate that no CPE or CPG contaminate the unknown ceramide derivative. After mild alkaline methanolysis for 2 hr at 0 C the unknown ceramide derivative migrates as a single component in two dimensional paper chromatography (Fig. 3). Authentic CL is completely deacylated in 1.5 hr at 0 C and the phosphate can be quantitatively recovered in the aqueous phase (4,7).

Separation of the Ceramide Derivatives

A second purification procedure was used to confirm the results derived from the lipid isolated as in Figure 1. The total lipid extract from *B. melaninogenicus* grown with $H_3{}^{32}PO_4$ was subjected to mild alkaline methanolysis, the ceramide derivatives and fatty acid methyl esters from the diacyl lipids were recovered in the organic phase. The fatty acid methyl esters and ceramide derivatives were separated with a silicic acid column. The ceramide derivatives were then separated by chromatography on silica gel-loaded paper. The ceramide derivatives in the lipid sample accounted for 48% of the lipid phosphate. A radioautogram of the separated ceramide derivatives is illustrated in Figure 7. The two major ceramide derivatives correspond to CPE and CPG which have been identified previously (1). The distribution of

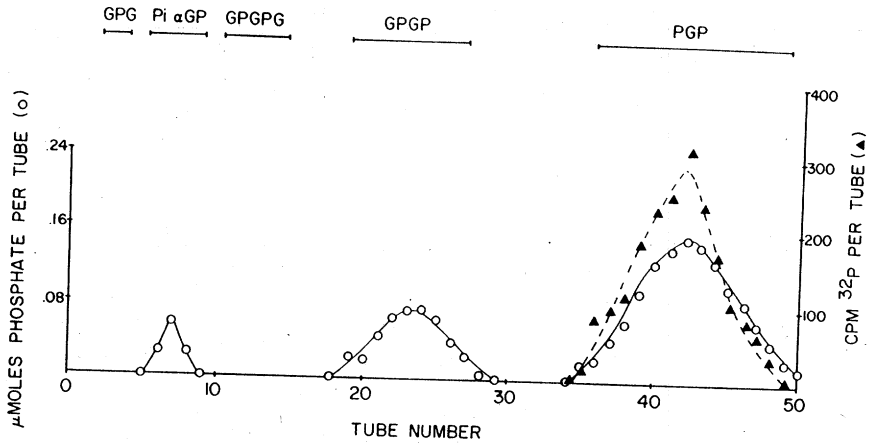

FIG. 6. Chromatography of the PGP isolated from the trace ceramide of *Bacteroides melaninogenicus* grown with ^{32}P. PGP was eluted from the paper chromatogram used for Figure 5 and chromatographed on Dowex-1 as described in Materials and Methods. See Figure 4. Each fraction contained 5.0 ml. The recovery of the ^{32}P was quantitative. The elution volumes of authentic glycerol phosphate esters are given at the top of the Figure.

^{32}P in the three ceramide derivatives was CPE, 45%, CPG, 51% and unknown 4%. The unknown ceramide derivative has the same chromatographic mobility when recovered and the chromatography repeated as that illustrated in Figure 3.

Water Soluble Product of Acid-Hydrolysis of the Unknown Ceramide Derivative

The ^{32}P-containing derivative obtained after hydrolysis of the unknown ceramide derivative cochromatographed with PGP in the paper chromatographic system illustrated in Figure 4 and the column chromatographic system illustrated in Figure 5. This was true with the unknown ceramide separated from other lipids chromatographically and then from CL by mild alkaline methanolysis as in Figure 1, from the unknown ceramide derivative separated from the other ceramide derivatives after mild alkaline methanolysis of the total lipid extract as in Figure 7, or from the hydrolysis product of the ceramide derivative mixture separated as in Figure 4.

The elution from a Dowex-1 column of the ^{32}P-glycerol ester recovered from the hydrolysis of the ceramide derivative is not exactly coincident with authentic PGP (Fig. 6). Perhaps there are two glycerol diphosphate esters in the lipid or one is an artifact of the hydrolysis. The column does indicate that the ester is not GPGP.

The absence of reactivity with periodate before or after mild alkaline methanolysis (Fig. 2, 3 and 7), the chromatographic mobility on paper (Fig. 5) and columns (Fig. 4,6) and the release of $^{32}P_i$ and only $^{32}P_i$ by alkaline phosphatase indicate that PGP is the ester liberated after acid hydrolysis of the minor component of the ceramide derivatives.

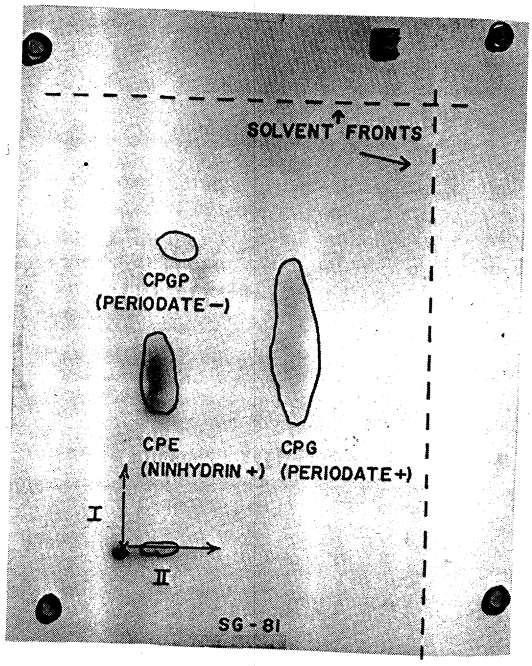

FIG. 7. Radioautogram of the chromatographic separation of the ceramides of *Bacteroides melaninogenicus* grown in the presence of ^{32}P as in Figure 2. The paper was then dipped in periodate solution followed by *o*-toluidine (4). A second paper was dipped in ninhydrin reagent (4).

DISCUSSION

The ceramide derivative described in this study contains two phosphates, an amide-linked fatty acid and one dihydrosphingosine homologue per molecule. The phosphate is probably linked to the 1 position of the LCB as in the other lipids in this organism (1). This ceramide derivative cannot be separated from cardiolipin by chromatography in two dimensions and its chromatographic mobility on silica gel-loaded paper is not affected by deacylation. No vicinyl hydroxyl groups can be detected before or after deacylation of the ceramide by mild alkaline methanolysis. After mild acid methanolysis PGP was recovered in the aqueous phase. The PGP was identified chromatographically. All the ^{32}P in $^{32}PG^{32}P$ was liberated as inorganic ^{32}P after treatment with alkaline phosphatase. It would appear that the structure of this ceramide derivative is ceramide-1-phosphoryl-1′-*sn*-glycerol-3′-phosphate. To our knowledge this lipid has not been described previously.

The ceramide derivative described in this study is homologous with phosphatidyl glycerol phosphate. A ceramide derivative homologous with phosphatidyl glycerol has also been detected in this organism (1). The presence of these two lipids suggests that the biosynthesis might also parallel the synthesis of phosphatidyl glycerol as described in *E. coli* (12). The CPG is present in about 10 times the concentration of the CPGP in *B. melaninogenicus*.

ACKNOWLEDGMENT

This research was supported by Grant GM-10285 of The Institute of General Medical Sciences, U.S. Public Health Service and Contract 12-14-100-9517(73) with the Agricultural Research Service, USDA administered by the E. Utiliz. Res. Dev. Div., Philadelphia, Penna. V. Rizza provided the organisms used in this study and Bernice Cooke assisted in the experiments.

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