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Plasmenylethanolamine: Growth Factor for Cholesterol-Reducing *Eubacterium*

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A plasmalogen, plasmenylethanolamine, is required for in vitro growth of strains of *Eubacterium* which convert cholesterol to coprostanol. Plasmenylethanolamine was isolated from calf brain by selective saponification of lipid fractions separated by thin-layer or column chromatography. Cholesterol-containing thioglycolate broth plus purified plasmenylethanolamine or its 2-lyso derivative supported growth of *Eubacterium* ATCC 21408 and a cholesterol-reducing *Eubacterium* isolated from baboon feces. Plasmenylethanolamine obtained from commercial sources also supported growth of these organisms, but none of a number of other pure lipids would support growth. Metabolism of the alkenyl ether group of plasmenylethanolamine occurred during growth.

Cholesterol is converted to coprostanol in the intestine by microbial reduction of the 5,6-double bond. Early attempts to isolate a cholesterol-reducing bacterium were unsuccessful, although mixed anaerobic cultures readily transformed cholesterol to coprostanol in vitro (6, 17). Eyssen et al. (9) isolated an organism from rat cecum which reduced Δ^5-3β-hydroxy steroids to the 5β-saturated derivatives. This strictly anaerobic, gram-positive bacillus (ATCC 21408) has been tentatively placed in the genus *Eubacterium*. Similar organisms have been isolated from human feces (16). Although it is generally accepted that cholesterol-reducing *Eubacterium* strains are responsible for the in vivo production of coprostanol, Crowther et al. (5) reported that numerous isolates of common intestinal bacteria were capable of reducing cholesterol to coprostanol. However, this conclusion has not been subsequently confirmed (16) nor did earlier reports support it (6, 9, 15, 17). We also could not detect coprostanol formation by any strains of the genera *Bacteroides, Clostridium, Fusobacterium, Lactobacillus, Megasphaera, Peptococcus,* and *Peptostreptococcus* or by other *Eubacterium* strains isolated from baboon feces (A. W. Brinkley and G. E. Mott, unpublished observations).

The cholesterol-reducing organisms apparently require cholesterol or other Δ^5-3β-hydroxy steroids and do not grow in conventional media (9). Limited growth has been reported in media supplemented with cholesterol in lecithin (9), but successful maintenance of these organisms requires the addition of homogenized brain or brain lipid extracts (8, 9, 16). Further metabolic characterization of these bacteria has been hampered by the complexity of brain-containing media.

The objective of the present experiments was to characterize the components in brain necessary for growth of cholesterol-reducing *Eubacterium* in vitro.

MATERIALS AND METHODS

Media. Standard brain medium was similar to that described by Snog-Kjaer et al. (17) and contained the following components, per liter: thioglycolate broth without dextrose or indicator (Difco, Detroit, Mich.), 24 g; K_2HPO_4, 3.8 g; KH_2PO_4, 1.2 g; resazurin (Difco), 1.0 mg; and lyophilized bovine brain, 30 g. The dry ingredients were suspended with water and homogenized with a Polytron homogenizer (Brinkmann Instruments Inc., Westbury, N.Y.). After the pH was adjusted to 7.2, 2 to 5 ml of medium was dispensed into screw-capped tubes (16 by 100 mm), autoclaved, and passed into an anaerobic chamber for reduction. This medium was used for routine maintenance of cholesterol-reducing bacteria and as a positive control medium.

Lecithin-cholesterol medium (LCM), a brain-free medium which alone will not support growth of cholesterol-reducing bacteria, contained the following components, per liter: thioglycolate broth without dextrose or indicator, 24 g; K_2HPO_4, 3.8 g; KH_2PO_4, 1.2 g; lecithin (Sigma Chemical Co., St. Louis, Mo.), 0.1 g; resazurin, 1.0 mg; and cholesterol (Sigma Chemical Co.), 2.0 g. All ingredients except cholesterol were dissolved in water. The cholesterol was dissolved in 15 ml of hot ethanol and added dropwise to heated (80°C) medium while stirring vigorously. The final pH was adjusted to 7.2. The medium was lyophilized to remove residual ethanol, resuspended with water, dispensed into screw-capped tubes, autoclaved, and passed into an anaerobic chamber. Additions of lipid fractions in organic solvents were made before lyophilization.

Bacterial strains and inoculum size. We isolated

a cholesterol-reducing bacterium from baboon feces by techniques similar to those reported by other investigators (9, 16). This organism is a strictly anaerobic, gram-positive diplobacillus which has been tentatively identified as a *Eubacterium* using the criteria of the Virginia Polytechnic Institute Anaerobe Laboratory (13). Our isolate is designated *Eubacterium* 403 and closely resembles *Eubacterium* ATCC 21408 (9); both organisms reduce cholesterol to coprostanol in vitro and produce extracellular fibers which cause solidification of the medium.

Experimental media were inoculated with standard brain medium stock cultures diluted in LCM. Carryover of coprostanol and other metabolites in the inoculum was minimized by inoculation with a 0.1-ml aliquot of a 10^{-2} to 10^{-5} dilution of the stock culture per ml of test medium.

Culture conditions. The cultures were maintained and the experimental studies were performed in a modified stainless-steel Blickman chamber (S. Blickman Co., Weehawken, N.J.) under anaerobic conditions. The oxygen removal system was similar to that reported by Aranki and Freter (1). The gas mixture contained 10% hydrogen, 5% carbon dioxide, and 85% prepurified nitrogen (Union Carbide, Houston, Tex.). Resazurin was added to all media as an Eh indicator. The chamber humidity was maintained at 40 to 50% relative humidity by a mechanical condenser. The cultures were incubated at 35°C in the chamber.

Growth estimation. Growth of these strains of *Eubacterium* cannot be determined by conventional methods since these organisms do not form colonies on agar media, and turbidimetric methods cannot be used because cholesterol-containing media are opaque. We estimated by dilution to extinction that a solidified culture contained 10^8 organisms per ml, although this method is complicated by aggregation of the organisms and trapping of cells by the fibrous products. Since other investigators (9, 16) have reported that cholesterol reduction is required for growth of strains of cholesterol-reducing *Eubacterium*, we assumed that coprostanol formation was evidence of growth. Under conditions where no coprostanol was formed, we confirmed lack of growth by the absence of cells on a Gram-stained smear.

Extraction and isolation of brain lipids. The scheme for isolation of the lipid fractions of brain is summarized in Fig. 1. Six grams of lyophilized calf brain was homogenized in 320 ml of chloroform-methanol (2:1, vol/vol, redistilled) (10) with a Polytron homogenizer. An 80-ml amount of deionized water was mixed with the homogenate, and two phases were allowed to separate. The lower phase containing the lipids was removed and evaporated to near dryness on a rotary evaporator. The lipid residue was resuspended immediately in 20 ml of redistilled chloroform-methanol (2:1) and stored at −20°C.

The crude brain lipid extract from about 45 mg of lyophilized brain was fractionated by thin-layer chromatography (TLC) on silica gel 60 precoated plates (20 by 20 cm, 0.25-mm thickness; EM Laboratories, Elmsford, N.Y.). Lipid standards (1 mg/ml in chloroform-methanol [2:1]) also were spotted (0.1 ml) on the TLC plate to determine migration characteristics of the major lipids found in brain. The plate was developed in chloroform-methanol-35% aqueous ammonium hydroxide, 56:24:3 (vol/vol/vol). After development the solvent was allowed to evaporate from the plate; the standards and a small portion of the sample were sprayed with 0.02% dichlorofluorescein in methanol, and the bands were observed under UV light. Eight distinct bands, numbered 1 to 8 from the origin (Fig. 2), were scraped from the plate separately and eluted with 3× 20-ml portions of chloroform-methanol (2:1, vol/vol). Each fraction was evaporated to dryness, resuspended in a small volume of solvent, and added to LCM as described above. Growth of the *Eubacterium* in these modified media was monitored by coprostanol formation.

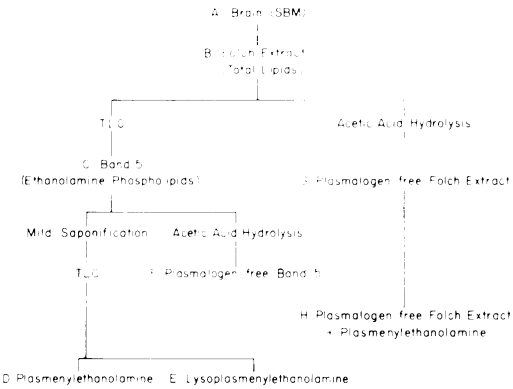

FIG. 1. *Summary of isolation procedure for lipid growth factors from lyophilized brain. Letters refer to specific lipid fractions tested (see Table 1). SBM, Standard brain medium.*

We prepared larger amounts of phospholipids from the Folch extract of brain by silicic acid column chromatography. Approximately 750 mg of crude brain lipids was fractionated into neutral lipid and phospholipid fractions on a 17.5- by 2.5-cm silicic acid column as described by Christie (4). The phospholipid fraction was then applied to a 9.8- by 1.8-cm silicic acid column, and the ethanolamine phospholipids were eluted with chloroform-methanol (80:20) after elution of the more polar lipids with 95:5 chloroform-methanol. Small amounts of phosphatidylserine were detected by TLC of the ethanolamine phospholipid fraction.

The TLC bands or column fractions with the same migration characteristics as ethanolamine phospholipids were further characterized. These fractions were evaporated to dryness, resuspended in CCl_4, and hydrolyzed by mild saponification in 0.027 N NaOH for 20 min at 37°C (7). This method will hydrolyze the diacyl lipids but leave intact the 1-alkenyl ether linkages characteristic of plasmalogens. The reaction mixture was extracted with 2:1 chloroform-methanol by the Folch procedure (10), and the lower phase was fractionated by TLC with chloroform-methanol-NH_4OH, 56:24:3, as solvent. Two bands were observed with the same migration as bands 3 and 5 previously detected.

The alkenyl ether linkages of lipids in various fractions were hydrolyzed with 90% acetic acid at 37°C for 18 h (12) and lyophilized before addition to media.

Evaluation of lipids. The brain lipid fractions and pure lipid standards were tested in LCM for their ability to support growth of the *Eubacterium*. The lipids were dissolved in chloroform-methanol (2:1) and added at a concentration of 1 mg/ml to LCM as described above. The cultures were incubated for 12 to 14 days at 35°C and then analyzed for coprostanol. The lipid standards tested were sphingomyelin, sphinganine, sphingosine, cerebrosides, sulfatides, phosphatidylethanolamine (PE), plasmenylethanolamine (PLE), lysophosphatidylethanolamine, phosphatidylcholine, lysophosphatidylcholine, phosphatidylserine, phosphatidylinositol, tripalmitin, linoleic acid, oleic acid, hexadecanol, 1,2-dipalmitoyl-3-hexadecyl ether, palmitaldehyde, palmityl palmitate, cholic acid, and gangliosides (Applied Science Laboratories, State College, Pa.).

Effect of PLE concentration on coprostanol formation. Twofold serial dilutions of PLE (formula weight, 744) were prepared in LCM with concentrations ranging from 0.01 to 2.69 mM. These preparations were then inoculated with *Eubacterium* 403 and incubated at 35°C. Each culture was sampled at 3, 5, and 7 days for coprostanol analysis.

Coprostanol analysis. A 0.1-ml aliquot of the cultures was saponified in 0.1 N ethanolic KOH at 55°C for 1 h and extracted with petroleum ether. The petroleum ether extract was evaporated to dryness under nitrogen gas, and the residue was dissolved in 0.1 ml of Tri-Sil/BSA (Pierce Chemical Co., Rockford, Ill.) and 0.1 ml of dimethyl formamide. The trimethylsilyl ether derivatives were synthesized by heating the mixture at 70°C for 40 min. The trimethylsilyl-ether derivatives were analyzed by gas-liquid chromatography at 250°C with flame ionization detection. Samples (5 μl) were injected on a column (3 feet [ca. 91 cm] by 4-mm ID) packed with 3% OV-17 (Applied Science Laboratories). Percent coprostanol formation by the cultures was calculated as a ratio of the coprostanol peak area divided by the sum of the peak areas of cholesterol and coprostanol and multiplied by 100.

The identities of cholesterol and coprostanol in the cultures were verified by co-chromatography of authentic standards and gas-liquid chromatography-mass spectrometry of the sterols.

Analysis of alkenyl ether linkages. The alkenyl ether linkages were determined in chloroform-methanol (2:1) extracts of the cultures by the I_2 uptake method of Gottfried and Rapport (11). The solvent extracts were evaporated to dryness, resuspended in methanol, and reacted with the I_2 reagent. The uptake of I_2 by the plasmenyl lipids was measured spectrophotometrically. Since plasmalogens are rather unstable, assays were performed by the I_2 uptake method on the same day as the extraction.

Phospholipid analysis. Phospholipid phosphorus of the purified PLE was determined by the method of Bartlett (2).

RESULTS

Isolation of PLE as a growth factor. The crude lipid extract (10) supported coprostanol formation by *Eubacterium* 403 comparable to that of whole brain; therefore, we fractionated the lipid extract into principal polar lipid classes by TLC (Fig. 2). TLC bands 3, 5, and 7 supported growth of the *Eubacterium* in LCM. Coprostanol formation in LCM plus band 5 (fraction C) was comparable to that observed in standard brain medium (Table 1). A similar fraction of ethanolamine phospholipids isolated by silicic acid column chromatography also supported growth (43% coprostanol) of the *Eubacterium*. Band 5 was a mixture of PLE and PE. Since pure PE did not support growth, we saponified

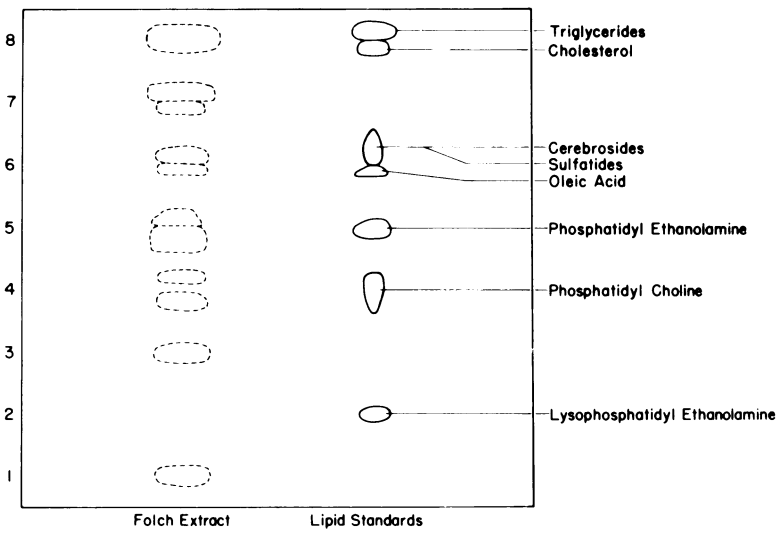

FIG. 2. *Thin-layer separation of brain lipids and pure lipid standards. Developing solvent was chloroform-methanol-35% NH_4OH, 56:24:3.*

TABLE 1. *Coprostanol formation by Eubacterium 403 in LCM containing various brain lipid fractions*

Lipid fractions	Coprostanol[a] (% of sterols)
A.[b] Brain (SBM)[c]	95
B. Folch extract of brain	84
C. Ethanolamine phospholipids, TLC separation (band 5)	87
D. PLE isolated from fraction C	48
E. Lyso-PLE isolated from fraction C	39
F. Plasmalogen-free ethanolamine phospholipids	0
G. Plasmalogen-free Folch extract	0
H. Plasmalogen-free Folch extract + PLE	97

[a] Mean of cultures from two to five experiments.
[b] Letters correspond to fractions from isolation scheme (Fig. 2).
[c] SBM, Standard brain medium.

the PE-plus-PLE mixture under mild conditions to selectively degrade PE. TLC of this saponified mixture separated pure PLE (Fig. 3) and a new band which had the same migration as 2-lyso-PLE (7). Both of these lipid bands supported growth of the *Eubacterium* in LCM (Table 1) with formation of fibers and solidification of the medium. These plasmalogens or plasmenyl lipids are a class of glycerophospholipids characterized by an alkenyl ether linkage in the 1 position (14). The purity of the PLE band was indicated by a phosphorus/alkenyl ether molar ratio of 1.02. No growth occurred in LCM supplemented with acid-hydrolyzed Folch extract, as indicated by lack of coprostanol formation and absence of cells by Gram stain. However, addition of acid-hydrolyzed Folch extract to LCM-plus-PLE enhanced coprostanol production, indicating that other brain lipids stimulate this activity. Acetic acid hydrolysis destroyed the growth-supporting activity of the PE-plus-PLE mixture (fraction F), as determined by sterol analysis and Gram stain (Table 1). PLE obtained from a commercial source also supported growth in LCM (60% coprostanol); however, no other lipid standard tested was active. Therefore, we concluded than an intact alkenyl ether linkage is a growth requirement of the cholesterol-reducing *Eubacterium* in vitro.

Catabolism of PLE. A culture of *Eubacterium* 403 in LCM plus 1 mg of PLE per ml was sampled daily for 10 days. The daily aliquots were extracted with chloroform-methanol (2:1) and assayed for alkenyl ether groups by the I_2 uptake method and for coprostanol by gas-liquid chromatography. Figure 4 shows that the *Eubacterium* catabolized the alkenyl ether linkage of PLE during coprostanol formation. Losses of PLE in an uninoculated control were less than 5% during the same period. Degradation of the PLE continued after coprostanol production had reached a maximum at 5 days.

Effect of PLE concentration on coprostanol formation. Figure 5 shows that coprostanol formation was directly related to PLE concentration during the first 3 days of growth; i.e., from 0 to 3 days increasing PLE concentrations resulted in greater coprostanol formation. However, the increases in coprostanol from days 3 to 5 and 5 to 7 appeared to be independent of the PLE concentration, as indicated by the constant difference between the three time points at each PLE concentration.

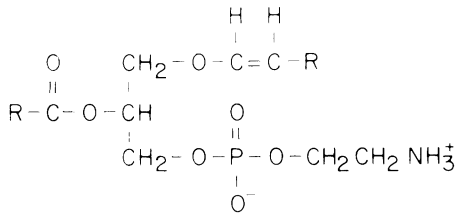

FIG. 3. *Pure plasmenylethanolamine; R = C_{11}–C_{18}.*

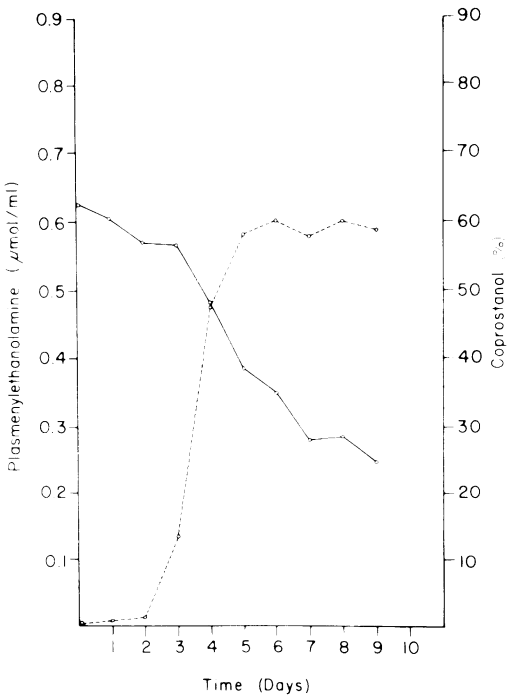

FIG. 4. *Degradation of PLE (solid line) and coprostanol (dashed line) formation by Eubacterium 403.*

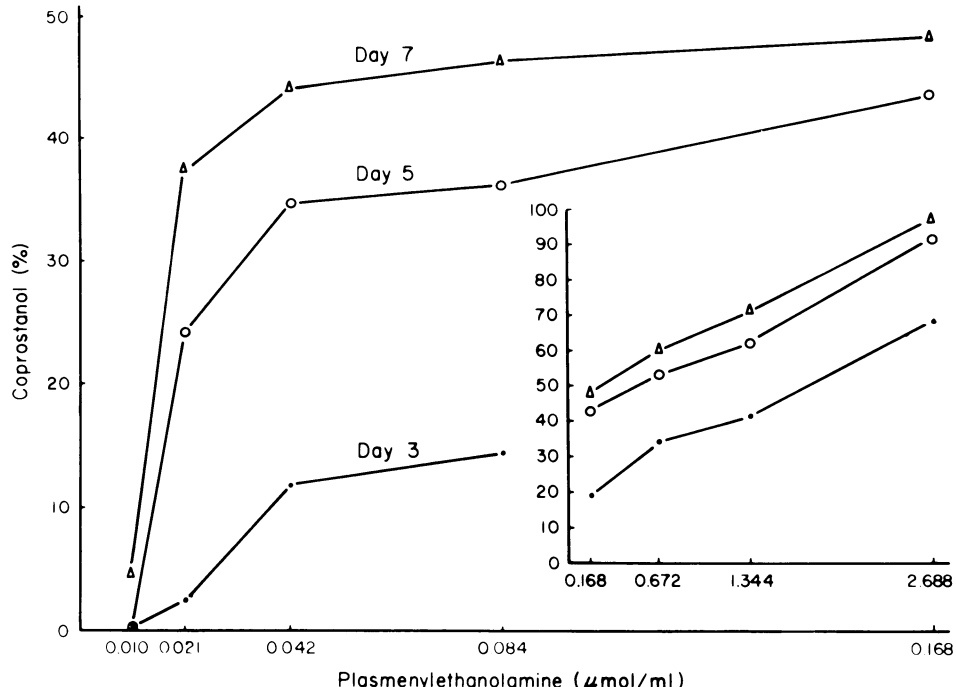

FIG. 5. *Effect of PLE concentration on coprostanol formation after 3 days* (●), *5 days* (○), *and 7 days* (△).

DISCUSSION

Supplementation of animal diets with brain enhances coprostanol formation in vivo (15, 16), and cholesterol-reducing organisms have been maintained in vitro only in media containing brain (9, 16). Although several investigators (9, 16) have shown that cholesterol is required for growth of these organisms, our results indicate that PLE also is required for growth in vitro. Brain has an unusually high plasmalogen content and a particularly high concentration of PLE compared to other tissues. This observation explains previous findings (9, 16) that cholesterol-containing media without brain did not allow growth of *Eubacterium* ATCC 21408. Hydrolysis of the plasmalogens by acetic acid destroys the growth-supporting activity of brain lipids, and addition of PLE to the acid-hydrolyzed lipids restores the activity. In the LCM-plus-PLE medium brain lipids hydrolyzed with acetic acid (i.e., plasmalogen-free) enhanced coprostanol formation, which suggests a role for other lipids also.

The sphingolipids may be the specific class of lipids which stimulate growth in LCM-plus-PLE since we have shown that this class of lipids is extensively metabolized by *Eubacterium* ATCC 21408 (Mott and Brinkley, Fed. Proc. **37**:1833, Abstr. no. 3092, 1978). This organism has sphingomyelinase (phospholipase C activity) and also cleaves sphingolipid bases to long-chain alcohols. However, none of the sphingolipids or their metabolic products would support growth in LCM without PLE. A previous report suggests that certain sphingolipids, e.g., cerebrosides, may enhance growth of cholesterol-reducing organisms in rats (15) since dietary fat plus the phrenosin fraction (mainly cerebrosides) from brain lipids increased coprostanol excretion. It is possible that this rather crude phrenosin fraction may have been contaminated with plasmalogens. However, it is unlikely that this fraction or even whole brain in the diet would stimulate coprostanol formation by supplying plasmalogens since the alkenyl ether linkage would be completely hydrolyzed by the acidic conditions in the stomach.

Wilson (19) demonstrated that type of dietary fat greatly affected coprostanol formation in rats. Linoleic acid, a polyunsaturated fatty acid, was a potent stimulator of coprostanol formation compared to saturated fatty acids. However, we have not observed a difference in coprostanol formation between groups of baboons fed diets high in either linoleic acid or saturated fat (Mott, unpublished observations).

Neither linoleic acid nor cerebrosides supported growth of the *Eubacterium* in vitro. How-

ever, in vivo these lipids may enhance growth of intestinal bacteria which produce plasmalogens required by the *Eubacterium*. Several intestinal organisms are known to produce plasmalogens (3, 18), and at least one, *Clostridium butyricum*, produces PLE. The possible symbiotic relationships these organisms may have with the *Eubacterium* and the plasmalogen concentrations in various parts of the baboon intestinal tract are under investigation.

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