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OCCURRENCE OF PLASMALOGENS IN ANAEROBIC BACTERIA

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Plasmalogens, aldehyde-containing phospholipids, were found to occur widely in all four fractions of ruminal microbes obtained after differential centrifugation of sheep rumen contents. Plasmalogens were also detected in ruminal and soil anaerobic bacteria which were obtained by enrichment cultures with various energy sources. They occurred widely in all strictly anaerobic bacteria and Propionibacterium tested. The strains included Desul fovibrio sp., Selenomonas ruminantium, Bacteroides ruminicola, Veillonel-la gazogenes, Peptostreptococcus elsdenii, Propionibacterium freudenreichii, Pro-pionibacterium shermanii, Clostridium saccharoperbutylacetonicum, Clostridium acetobutylicum, Clostridium perfrigens, Clostridium kaneboi, and Clostridium kainantoi. Although the ubiquity of plasmalogens in these anaerobic bac-teria was confirmed, the amount of plasmalogens per unit weight of cells varied considerably depending on bacterial species. Amount of plasmalogens
in Selenomonas and Propionibacterium also varied by cultural conditions.
However, plasmalogens were not detected in aerobic bacteria and in
facultative anaerobic bacteria even when they were cultured under strictly
anaerobic conditions.

It has been reported from this laboratory (1) that Selenomonas ruminan-tium var. lactilytica, a gram-negative anaerobic bacterium isolated from sheep rumen contents, contains plasmalogens as components of phospholipids. The reported occurrence of plasmalogens in other bacteria has been confined to some special anaerobic bacteria including Ruminococcus albus (2), Ruminococ-cus flavefaciens (2), Bacteroides succinogenes (3), and Borrelia sp. (4), those occur-ring in the rumen (5), and a soil anaerobe, Clostridium butylcum (6). KATZ and KEENEY have reported earlier that the whole bacterial fraction (7) and holotrich protozoa (8) harvested from rumen contents of a cow were rich sources of aldehyde-containing lipids. These results led the authors to ex-amine the occurrence of plasmalogens in various bacteria. The present data demonstrate that these aldehyde-containing phospholipids are detected widely among anaerobic bacteria but not in aerobic and facultative anaerobic bac-teria.

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MATERIALS AND METHODS

Fractionation of rumen microorganisms by differential centrifugation
Rumen contents were obtained through a rumen fistula from a sheep which was fed once a day on hay and concentrates. The content (1.5 liters) before the feeding was filtered through a sheet of cheese cloth. The filtrate was centrifuged at 27 x g for 5 min. The precipitate was collected and the supernatant was recentrifuged at 27 x g for 5 min. The precipitate was again collected and the centrifugation was repeated once more. The final super-natant was subjected to further fractionation as will be described later. The combined precipitate was suspended in a carbonate buffer (pH 6.8) and the suspension was centrifuged at 27 x g for 5 min. The precipitate was washed twice with water by centrifugation at 27 x g for 5 min. The washed precipi-tate was designated as Fraction 1. The supernatant from the spinning at 27 x g was centrifuged at 100 x g for 5 min. The precipitate was collected and the supernatant was recentrifuged at 100 x g for 5 min. The combined precipitate was suspended in a carbonate buffer (pH 6.8) and the suspension was centrifuged at 100 x g for 5 min. The precipitate was washed twice with water by centrifugation at 100 x g for 5 min. The water-washed precipitate was designated as Fraction 2. The supernatant from the centrifugation at 100 x g was fractionated by similar procedures and the water-washed Fractions 3 and 4 were obtained as the precipitates at 440 x g for 10 min and 10,000 x g for 30 min, respectively.

Media for enrichment culture of rumen or soil bacteria

As a basal medium for enrichment cultures of ruminal or soil bacteria,
a medium containing rumen fluid or soil extract was employed, respectively. The former contained 20% (by vol.) of rumen fluid, which was prepared ac-cording to the method of Allison et al. (9), 0.5% each of Trypticase (Balti-more Biological Laboratory, Baltimore, U.S.A.) and yeast extract (Daigo Eiyo Kagaku, Tokyo). An inorganic salt mixture, according to the composition of Bryant and Robinson (10), was included in the medium. The latter contained the same salt mixture, 0.5% each of Trypticase and yeast extract, and 70% (by vol.) of soil extract that was prepared according to the method of LOCH-HEAD and BURTON (11). To both media, either 1.25% of sodium DL-lactate, 1.9% of glycerol, 1 % of carboxymethylcellulose, 1 % of soluble starch or 1 % of casein supplemented with 0.05% each of glucose and cellobiose was added as energy sources. As reducing agents, 0.025% each of cysteine-HC1 and Na2S • 9H20 was added.
Media were freed from oxygen according to the method of HUNGATE
(12) with a slight modification. The media for ruminal or soil bacteria were flushed with 100% C02 or 5% C02-95% N2i respectively. When 100% C02 was used as the gas phase, 0.8% of Na2C03 was added to the media.

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Enrichment cultures of rumen and soil bacteria

One drop each of rumen contents collected from the sheep before the feeding or of a suspension of the experimental field soil of this Faculty (1 g in 10 ml sterilized saline) was inoculated into various media (10 ml). After culture for 24 hr, a drop of each culture was transferred into a fresh medium of the same composition. Incubation was continued again for 24 hr. After four such successive transfers, the culture (5 ml) was inoculated into a fresh medium (2,000 ml). Cells were harvested after 24-hr incubation, washed twice with water, and stored overnight in a deep freezer. The culture of ruminal or soil bacteria was carried out at 37° or 30°, respectively.

Pure bacterial strains and their growth conditions
Following bacterial strains were from the stock cultures of this labora-tory : Bacillus subtilis (TAM 1069), Pseudomonas fluorescens Migula (IFO 3461), Corynebacterium sepedonicum (IFO 3306), Streptomyces aureofaciens, Escherichia coli Migula, Rhodospirillum rubrum, Lactobacillus casei, Lactobacillus delbru-eekii, Leuconostoc mesenteroides, Streptococcus faecalis, Bacteroides ruminicola, Peptostreptococcus elsdenii, and Selenomonas ruminantium. Propionibacterium freudenreichii (IFO 12424) and Propionibacterium shermanii (IFO 12426) were from the Institute for Fermentation, Osaka. Clostridium acetobutylicum 179-121 and 314-48, Cl. kainantoi 182-72, Cl, kaneboi, Cl. perfrigens, and Cl. sac-charoperbutylacetonicum were obtained from the Department of Agricultural Chemistry, Kyushu University, Fukuoka. Clostridium species 3-97 and 4-52, and Desulfovibrio species were from the Institute for Agricultural Research, Tohoku University, Sendai. Veillonella gazogenes was obtained from the National Institute of Animal Health, Kodaira, Tokyo.
B. subtilis, Ps. fluorescens, and Coryn. sepedonicum were grown at 30° aerobically by shaking for 10, 10, and 12 hr, respectively, in a nutrient broth (pH 6.8) which contained 1 % of meat extract (Kyokuto Seiyaku, Tokyo) and polypeptone (Daigo Eiyo Kagaku, Tokyo), and 0.5% of NaCI. Streptomyces aureofaciens was grown at 30° for 24 hr under aerobic conditions in a medium containing 1 % of glucose, 0.1% of meat extract and yeast extract, and 0.2% of polypeptone (pH 7.2). E. coli was grown at 37° either aerobically by shak-ing for 8 hr or anaerobically for 24 hr in the nutrient broth. The anaerobic culture was conducted in 5% C02-95% N2.
R. rubrum was grown at 30° for 30 hr under light-anaerobic conditions (5% CO2-95% N2) in the G-5 medium described by BOSS (73). Lact. casei, Lact, delbrueckii, Leu. mesenteroides, and Streptococcus faecalis were grown at 30° for 20 hr anaerobically in a medium (pH 6.8) containing 1 % of yeast extract, polypeptone, and glucose, and 0.5% of sodium acetate in 5 % C02-95 % N2. Prop. freudenreichii and Prop. shermanii were grown at 30° for 20 hr either anaerobically in 100% N2 or standing for 20 hr in a medium (pH7.0) con-taining 1 % glucose, 0.5% of yeast extract, polypeptone, meat extract, and sodium acetate, and 0.25% of NaCI. Cl, acetobutylicum 179-121 and 314-48, Cl, kainantoi,

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Cl. kaneboi, Cl. perfrigens, and Cl. saccharoperbutylacetonicum were grown at 30° for 40 hr in the media described by Hongo et al. (14). Cl. sp. 3-97 and 4-52 were grown in a medium which contained 1% of polypeptone, 0.5% of meat extract and NaCI, and 0.2% of glucose. The strain 3-97 was cultured at 30° for 36 hr and the strain 4-52 at 37° for 20 hr. All clostridia were

grown in 5% C02-95% N2. Desulfovibrio sp. was grown at 30° for 48 hr under anaerobic conditions (5% C02-95% N2) in a medium containing 0.5% of yeast extract, 0.18% of sodium DL-lactate, 0.2% of MgSO4 •7H2O, 0.1% of NH4Cl and Na2SO4, 0.05% of K2HPO4i and 0.001% of FeSO4.7H2O. As reducing agents, 0.04% of sodium thioglycolate and 0.025% of cysteine-HC1 were added. Selenomonas ruminantium was grown either in the yeast extract-glucose medium for 8 hr or in the yeast extract-lactate medium with biotin for 18 hr. Details of the culture media were described previously (15). Bacteroides ruminicola was grown for 10 hr following the method of BRYANT and Ro-BINSON (10). Peptostreptococcus elsdenii was grown for 24 hr in a medium containing 0.2% of yeast extract, 1.25% of sodium DL-lactate, and the salt mixture of BRYANT and ROBINSON (10). V. gazogenes was grown for 24 hr in the medium described by OGIMOTO and SUTO (16). Selenomonas, Bac-teroides, Peptostreptococcus, and Veillonella were grown at 37° in 100% CO2 with supplementation of 0.8% of Na2CO3 to the culture media.
In every case, cells were harvested by centrifugation, washed twice with water, and stored overnight in a deep freezer prior to the extraction of lipids.

Extraction of phospholipids from microorganisms
Packed cells were extracted three times with ethanol-ether (3: 1, by vol.) and then three times with chloroform-methanol (1:3, by vol.). Each extrac-tion was performed at room temperature for 10 min. Extracts were combined, evaporated in N2 atmosphere to dryness and then re-extracted with ether. The ether extract was washed twice with water and used for the analysis of plasmalogens of bacterial fractions from rumen contents and enrichment cultures. Each lipid sample obtained as above from the pure culture was applied to a silicic acid column (1.5 x 3.0 cm). After neutral lipids were eluted with chloroform, phospholipid fraction was eluted with chloroform-methanol (1:1, by vol.). The phospholipid fraction was concentrated in N2 atmosphere and analyzed for plasmalogens.

Analytical procedures

Analysis of lipid materials in the extract was carried out by thin-layer chromatography with silica gel H (E. Merk, Darmstadt, Germany) as a layer of approximately 250 p thickness. Two solvent systems were employed. Sol-

vent A consisted of chloroform, methanol, and water (65: 25:4, by vol.) and
solvent B contained chloroform, acetone, methanol, acetic acid, and water (100:40: 20: 20:10, by vol.). Qualitative tests for phosphate, amino nitrogen, and aldehyde on thin-layer chromatograms were carried out as following.

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Phosphate was detected by spraying Zinzadze reagent (17). Amino nitrogen was developed by 1.5% ninhydrin in acetone. Fatty aldehyde was detected by spraying 0.5% methanol solution of 2, 4-dinitrophenylhydrazine containing 2 N HC1.

Quantitative determination of phosphate, amino nitrogen, and aldehyde
was performed by the following methods. For the determination of phosphate, samples were assayed by KING'S method (18) after perchloric acid digestion. Amount of phosphate was expressed as P. For the determination of amino nitrogen, samples were applied to thin-layer plates and, without chromato-graphy, were sprayed with 2 % ninhydrin in acetone and heat-treated at 50° for 30 min. The colored material was extracted with 70% ethanol and its

absorbancy was measured by a Hitachi FPW 4 colorimeter equipped with a 57 filter (570 mp). The amount of amino nitrogen was expressed as N with serine as a standard. For the determination of fatty aldehyde, samples were applied to thin-layer plates at the origin. To the origin, 2, 4-dinitrophenyl-hydrazine reagent was sprayed and plates were kept at room temperature for 2 min. After removal of methanol and HCl under reduced pressure, plates were developed with light petroleum (bp 30-60°) and ether (8: 2, by vol.) as a solvent system. Fatty aldehydes, as dinitrophenylhydrazones, were detected as a yellow spot which migrated to the area corresponding to Rf 0.5 to 0.6. Unreacted dinitrophenylhydrazine remained at the origin. The hydrazones were scraped from plates and suspended in chloroform. After centrifugation, the absorbancy of the extract was measured with the colori-meter at 370 mss. Aldehyde contents were expressed as myristic aldehyde equivalent.

Reagents
In order to avoid contamination of carbonyl compounds from reagents, following purification procedures were carried out. Methanol (1,000 ml) was refluxed for 2 hr with 2 g of 2, 4-dinitrophenylhydrazine and 3 ml of 98% phosphoric acid. After the reaction, methanol was collected by distillation. Ethanol was treated similarly. 2, 4-Dinitrophenylhydrazine was recrystallized from aldehyde-free methanol. Ether and chloroform were distilled before use.

RESULTS

Occurrence of plasmalogens in rumen microorganisms fractionated after dif-ferential centrifugation
In order to know the occurrence of plasmalogens in ruminal microorgan-isms, four microbial fractions were prepared by differential centrifugation. Fractions 1 and 2 mainly consisted of protozoa and large bacteria which were the mixture of so-called "Quin's organisms" (19) and selenomonads. Fraction 3 was predominated by various kind of rods and streptococci, and fraction 4 consisted of small cocci. Cell yields of the four fractions are

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shown in Table 1. Thin-layer chromatography of phospholipids from these bacterial fractions revealed that all of them exhibited two to three phosphate-containing spots. The main spot corresponding to Rf 0.46-0.47 in solvent A and to 0.42 in solvent B was positive for amino-nitrogen, fatty aldehyde, and phosphate, indicating that it contained plasmalogens. The minor spots cor-responding to Rf 0.12 (Fractions 3 and 4), 0.16 (Fraction 1), 0.22 (Fraction 2), and 0.34 (Fraction 4) in solvent A also contained plasmalogens. Fraction 3 gave a minor spot at Rf 0.76 in solvent A which contained fatty aldehyde and phosphorus but no detectable amino-nitrogen. Quantitative analysis for fatty aldehyde and phosphate in four lipid extracts is shown in Table 2. The data clearly demonstrate that the aldehyde-containing phospholipids are pre-sent in every fraction. The fractions 1 and 2, which contained protozoa,

" Quin's organisms ", and selenomonads, were most rich in these lipids.

Plasmalogen content of bacteria from rumen and soil enriched with various energy sources

The enrichment culture technique was employed in order to obtain re-presentative bacterial populations which grew at the expense of special energy sources included in the culture medium. As energy sources, sodium DL-lactate, glycerol, carboxymethylcellulose (CHIC), soluble starch, or casein with

and

Table 1. Yield of cells after differential centrifugation of rumen
Rumen contents (1.5 liters each) were fractionated by differential
cell yields of four fractions in dry weight basis were recorded.

contents.

centrifugation

Table 2. Analysis of lipid extracts of
for fatty aldehyde

cell
and

fractions from
phosphate.

rumen contents

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glucose and cellobiose was used. Since higher viable counts of ruminal bac-teria were obtained in 100% CO2 than in 5% C02-95% N2 and reverse was the case for soil bacteria (unpublished observation), 100% CO2 and 5% CO2-95%o N2 were used as the gas phase for the enrichment of ruminal and soil bacteria, respectively. Thin-layer chromatography in solvent A showed that all ruminal bacterial fractions enriched with various carbon sources contained

plasmalogens. The major spot was observed at Rf 0.45-0.47. Like the observation with the fractionated ruminal bacteria, a few minor spots con-taining plasmalogens were also detected. The analytical data for fatty alde-hyde, phosphate, and amino-nitrogen of lipid extracts from enriched ruminal and soil bacteria are recorded in Tables 3 and 4. The data clearly show that all enriched bacterial fractions contain plasmalogen phospholipids irre-spective of their origin.
Concerning the molar ratios of aldehyde to phosphate, higher values were obtained by rumen bacteria enriched with lactate, glycerol, or casein. The lowest value was recorded when enriched with starch. On the contrary, the molar ratios were higher with carboxymethylcellulose-, starch-, and casein-enriched soil bacteria. It might be concluded from these data that plasmalogens occur widely not only in rumen microorganisms but also in anaerobic soil bacteria.

Occurrence of plasmalogens in various bacteria in pure culture

The preceding experiments suggest that the occurrence of plasmalogens
is not restricted to certain special bacteria but is a general nature of anaero-bic bacteria. In order to substantiate this hypothesis, analysis was made

Table 3. Analysis of lipid
energy sources for

extracts of rumen microorganisms enriched with
fatty aldehyde, phosphate, and amino-nitrogen.

various

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with lipid extracts of various bacteria in pure culture. The results obtained as well as the reported occurrence of plasmalogens in various bacteria are summarized in Table 5. As shown, no plasmalogen was detected in the lipid extracts of aerobic bacteria including Ps. fluorescens and B, subtilis. Similarly, no plasmalogen was found in facultative anaerobes which include E. coli, R. rubrum, and various lactic acid bacteria, even when they were cultured under strictly anaerobic conditions. On the contrary, all strict anaerobes tested contained aldehyde-containing phospholipids. These strains include Cl. aceto-butylicum 179-121 and 314-48, Cl, kainantoi, Cl. kaneboi, Cl, perfrigens, Cl. saccharoperbutylacetonicum, Cl, sp. 3-97 and 4-52, Desulfovibrio sp., Seleno-monas ruminantium, Peptostreptococcus elsdenii, Bacteroides ruminicola, and Veillonella gazogenes. Plasmalogens were also detected in Prop. freudenreichii and Prop, shermanii. These strains were the only exception in that they contained plasmalogens even when cultured under semi-anaerobic conditions. No detectable amount of fatty aldehyde was found in the phospholipid frac-tions from Coryn, sepedonicum and Streptomyces aureofaciens, which are close-ly related to Bacteroidaceae as judged by morphological, physiological, and biochemical properties (20). These organisms failed to grow under the an-aerobic conditions employed.
Table 6 summarizes the analytical data for fatty aldehydes, phosphate,
and amino-nitrogen in the lipid extracts of representative anaerobes. The molar ratios of aldehydes to phosphate varied greatly among various bacteria. Significant difference was observed in the phospholipid compositions of Sele-nomonas and Propionibacterium depending on cultural conditions. Upon chromatography of lipid extracts on thin-layer plates with solvent A, samples from Desulfovibrio sp., Selenomonas ruminantium, Bacteroides ruminicola, and Clostridium sp. 3-97 and 4-52 gave the main spot at Rf 0.47-0.50 which showed positive reactions for fatty aldehyde, phosphate, and amino-nitrogen.

Table 4. Analysis of lipid extracts
various energy sources for

of soil microorganisms enriched
fatty aldehyde and phosphate.

with

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Table 5. Occurrence of plasmalogens in various bacteria in pure culture.
The occurrence of plasmalogens has been reported for bacteria indicated reference numbers in parentheses.

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with

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Table 5. Continued

Table 6. Analysis of lipid extracts of various bacteria for phosphate, and amino-nitrogen.

fatty aldehyde,

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Again, a few minor spots containing plasmalogens were detected in
sample except that from Bacteroides ruminicola.

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every

DISCUSSION

Biochemical studies on lipids have been focussed on complex lipids, especially phospholipids, since they are important constituents of cell struc-tures. Plasmalogens, aldehyde-containing phospholipids, have been identified in a wide variety of organisms especially belonging to an animal kingdom (21). Although much have been learned about bacterial lipids in the past decade, there have been a few reports which showed the presence of plasma-logens in bacterial cells. Thus, these lipids have been considered as the constituents of some restricted microorganisms.
Although the technique employed for the analysis of plasmalogens was still incomplete, namely, the lipid samples analyzed for plasmalogens were contaminated with other non-aldehyde phospholipids, the data presented here show that all fractions of microorganisms obtained by differential centrifug-ation of rumen contents and all bacterial fractions from rumen contents

which were enriched with various energy sources are rich in plasmalogens. This is in accord with the observations of KATZ and KEENEY (7, 8). More-over, the occurrence of plasmalogens is not confined to rumen microbes. Mixed populations of soil bacteria which were also enriched by various energy sources contain relatively large amounts of plasmalogens. The possibility that a similar type of bacteria was enriched from both sources, rumen con-tents and soil, may probably be excluded, since the enrichment culture of soil bacteria was done in 5% C02-95% N2. This condition has been found to be unsuitable for growth of most of rumen bacteria which require much higher partial pressure of C02 (unpublished observation).
As far as the present survey is concerned, all strict anaerobes in pure cultures contain appreciable amounts of plasmalogens. All aerobic or facul-tative anaerobic bacteria tested, on the contrary, do not contain detectable amount of plasmalogen even when cultured under strictly anaerobic conditions. Similarly, plasmalogen was not detected in all lactic acid bacteria tested which are known to be microaerophilic. However, plasmalogens were detected in Peptostreptococcus elsdenii, which is a strictly anaerobic lactic acid bacterium. It is of interest to note that Prop, freudenreichii and Prop. shermanii con-tained plasmalogens although they grew under semi-anaerobic conditions. It has been known that many strains of Propionibacterium are anaerobic to aerotolerant (22). Thus, it might be concluded that plasmalogens occur wide-ly in strictly anaerobic bacteria and Propionibacterium.
Fig. 1 illustrates the phylogenetic tree of bacteria after DE LEY (20) who reached this conclusion on the basis of morphological, physiological, and biochemical properties of each bacterial group. Bacteria in solid circles were found to contain plasmalogens and those in dotted circles contained no de-

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The phylogenetic tree shown above represents a part of the relationship described
by DE LEY (20). Since Aeromonas and Lachnospira positioned between Pseudomonas and Enterobacteriaceae were not analyzed for plasmalogen, these two genera were omitted from this figure. Similarly, Catenabacteria, Eubacteria, Bifidobacteria, and Ramino-bacteria placed between Clostridia and Propionibactericum, Micromonospora placed between Corynebacterium and Actinomycetes, and Butyribacterium placed between Clostridia and Heterolactics were omitted for the same reason.

tectable amount of plasmalogen. It appears that the occurrence
gens has an apparent relationship with the phylogenetic tree
DE LEY (20). Further analysis of plasmalogens is in progress.

of plasmalo-
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Fig. 1.
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Relationship between
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occurrence of plasmalogens and a phylogenetic tree

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