Sonderdruck aus
Hoppe-Seyler's Zeitschrift für Physiologische Chemie
Walter de Gruyter & Co, Berlin · New York
Hoppe-Seyler's Z. Physiol. Chem. Printed in Germany
Bd. 356, S. 715 - 725, Juni 1975

Sphingolipid Metabolism in *Bacteroideaceae*

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(Received 27 December 1974)

Dedicated to Professor Dr. Dr. G. Weitzel on the occasion of his 60th birthday

Summary: The lipid composition of the anaerobic *Bacteroides thetaiotaomikron* has been analyzed. Sphingomyelin, ceramide phosphinicoethanolamine, free even-numbered and branched chain sphingosine bases and ceramide represented about 50% of the total lipid extract. The main ester phospholipid was phosphatidylethanolamine.

The alkali-stable sphingophospholipids were predominantly *N*-acylated with 3-hydroxypalmitic acid, whereas the ester phospholipids are preferentially substituted with normal even and odd-numbered and branched-chain fatty acids.

When *Bacteroides* was grown in a medium supplemented with labelled palmitic acid, this fatty acid was utilized for acylation reactions and to a large extent for the de novo synthesis of sphinganine. This long-chain base was incorporated into the sphingolipids and was also present in free form.

The 3-hydroxypalmitic acid present in sphingolipids is not derived from palmitic acid, since labelled palmitate did not serve as a precursor. Free sphinganine added to the culture medium was also utilized efficiently for the biosynthesis of the sphingolipids by growing *Bacteroides* cultures.

The $^{3}H/^{14}C$ ratio in sphingomyelin and ceramide phosphinicoethanolamine is the same, when [1-^{14}C]palmitic acid and [3-^{3}H]sphinganine serve as precursors.

Sphingomyelin, which is usually only present in higher animals, is synthesized de novo in this *Bacteroides* strain.

Sphingolipid-Stoffwechsel in Bacteroideaceae

Zusammenfassung: Die Lipidzusammensetzung des anaeroben *Bacteroides-thetaiotaomikron*-Stammes (NTCC 10582) wurde analysiert. Sphingomyelin, Ceramidphosphinicoäthanolamin, freie geradzahlige verzweigte langkettige Sphingosinbasen und Ceramid bilden zusammen etwa 50% des Gesamtlipidextraktes. Das hauptsächlich vorkommende Esterphospholipid ist Phosphatidyläthanolamin.

Die alkalistabilen Sphingolipide sind vorwiegend mit 3-Hydroxypalmitinsäure *N*-acyliert. Gerad-

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Enzymes:
3-Dehydrosphinganine synthetase, acyl-CoA:serine *C*-2-acyltransferase (decarboxylating), EC 2.3.1.?, not yet listed.
3-Dehydrosphinganine reductase, D-sphinganine:NADPH oxidoreductase, EC 1.1.1.?, not yet listed.

und ungeradzahlige Fettsäuren liegen als Substituenten der Esterphospholipide vor. Markierte Palmitinsäure, die zum Kulturmedium von *Bacteroides* gegeben wurde, diente sowohl als Substrat der Acylierungsreaktion als auch der De-novo-Synthese von Sphinganin. Diese Base liegt in freier Form und eingebaut in die Sphingolipide der *Bacteroides*-Zelle vor.

Die 3-Hydroxypalmitinsäure der Sphingolipide leitet sich nicht von der Palmitinsäure ab, da radioaktiv markierte Palmitinsäure nicht als Vorstufe verwendet wird. Auch das zum Kulturmedium zugefügte freie Sphinganin wird in hoher Ausbeute für die Biosynthese der Sphingolipide verwendet.

Das identische $^{3}H/^{14}C$-Verhältnis in Sphingomyelin und Ceramidphosphinicoäthanolamin nach gleichzeitiger Zugabe von [1-^{14}C]Palmitinsäure und [3-^{3}H]D-*erythro*-Sphinganin als Vorstufen, weist auf die gemeinsame Vorstufe des Ceramids dieser Sphingolipide hin.

Das Vorkommen und die Biosynthese von Sphingomyelin in diesen Anaerobiern ist ungewöhnlich, da Sphingomyelin meist nur in höheren Tierarten synthetisiert wird.

Sphingolipids have been regarded as complex lipids confined to eucaryotic cells particularly of mammalian origin. However, Carter et al. recently described branched-chain sphingosine bases in flagellates[1]. White et al.[2-5] discovered that *Bacteroides melaninogenicus* contained ceramide phospholipids such as ceramide phosphinicoethanolamine and ceramide phosphinicoglycerol with iso-long-chain bases such as 17-methyloctadecasphinganine, 16-methylheptadeca-, 15-methylhexadeca- and normal octadecasphinganine. Lev et al.[6] and Morrison[7] detected a number of long chain bases in sphingolipids of rumen bacteria and rumen protozoa.

Lev et al.[8] described the growth-promoting activity of vitamin K as a stimulation of sphingolipid synthesis in particular of 3-dehydrosphinganine synthetase.

The enzymes of sphingolipid biosynthesis, 3-dehydrosphinganine synthetase, 3-dehydrosphinganine reductase, a NADPH dependent enzyme, of ceramide and ceramide phospholipid synthesis are ill defined largely because they are membrane bound. The only enzyme purified so far is 3-dehydrosphinganine reductase. Its lipoprotein nature and lipid requirement have been demonstrated[9].

Since sphingolipids represent the predominant lipid constituents the anaerobic *Bacteroideaceae* may prove a suitable source of the enzymes involved in the biosynthesis of the long-chain bases and the complex sphingolipids of this organism. Results of studies initiated on the sphingolipid synthesis in *Bacteroides thetaiotaomikron* are reported in this publication.

Experimental

Growth of Bacteroides thet.

Bacteroides thet. was preincubated in 10 m*l* Rosenow medium at pH 7.2. 1 *l* cultures were grown in a peptone-yeast-glucose extract medium, PYE medium (15 g peptone, 5 g NaCl, 2 g meat extract, 10 g yeast extract, 0.3 g cysteine hydrochloride, 10 g glucose, 0.5 g Bactoagar, distilled water ad 1000 m*l*). Controls were carried out by plating on blood agar. Bacterial growth was measured at 660 nm in 1 h intervals for 70 h. 0.8 - 1.4 g of bacteria/*l* medium were generally obtained within 50 h. The gas chromatographic analysis of unesterified short-chain fatty acids, which documented the presence of acetic, propionic, isobutyric and isovaleric acid further support the homogeneity of the *Bacteroides thet.* cell strain[10,11]. The fatty acids were extracted from 10 m*l* of acidified medium (pH 2) with 5 m*l* diethyl ether, the solution dried over $MgSO_4$, concentrated and immediately analyzed by gas-liquid chromatography. Bacteria were harvested by centrifugation at 12000 × *g* at 4 °C for 20 min. The washed bacteria were suspended in 50 m*l* distilled water, lyophilized and stored at −20 °C until used. Lipids were extracted in the following way: 1 g lyophilized bacteria were stirred at room temperature in 100 m*l* chloroform/methanol 2:1 for 6 h and the extraction repeated with the same solvent mixture and twice with chloroform/methanol 1:2. The combined extracts were washed with 1/5 of their volume 1% NaCl and the lower phase concentrated under nitrogen.

10 - 20 mg lipid samples in 10 m*l* chloroform/methanol were hydrolyzed with $HgCl_2$[12] (0.4 m*l* 1% $HgCl_2$ in 0.1M acetic acid) at 37 °C for 2 h. Then 2 m*l* 1% sodium EDTA was added, centrifuged and the lower phase concentrated under nitrogen. Mild alkaline hydrolysis was carried out according to Dawson[13]. 10 - 20 mg lipids were dissolved in 9 m*l* methanol/carbon tetrachloride

(5:1) and 1 ml 1N NaOH and incubated at 37 °C over night. 1 ml 1N HCl was added followed by 7 ml water, 3 ml methanol and 33 ml chloroform. The lower phase was washed twice with 1/5 of its volume of 1% NaCl and then concentrated to dryness.

Complex lipids were separated according to Gaver and Sweeley[14]. 20 g silicic acid (1.5 x 23 cm column) was used for 20 to 50 mg lipids. 500 ml chloroform, 40 ml acetone and 80 ml acetone/methanol 9:1 and 500 ml methanol were used for bulk separation. Protein was determined according to Lowry[15] and phosphorus according to Rouser et al.[16]. Long-chain bases were characterized by gas liquid chromatography as bis-(trimethylsilyl) ethers of their *N*-acetyl derivatives on a 3% SE 30 column at 230 °C and of their long-chain aldehydes released by periodate oxidation[17] on 2 m 2.5% EGS columns at 160 °C or 1 m 15% EGS columns at 155 °C. Hexoses of glycolipids were released by acid hydrolysis with 1N HCl reduced with sodium borohydride, acetylated and analyzed by gas liquid chromatography as described before[18].

Ceramide phosphinicoethanolamine was methylated with CH_3I as described for the corresponding dimethyl-derivative[19]. Radio thin-layer chromatography was carried out with a Packard radio thin-layer or a Berthold scanner. Radioactive precursors [1-^{14}C]- and [^{3}H]palmitic acid, [3-^{3}H]D(+)-*erythro*-sphinganine and [1-^{14}C]palmitoyl[3-^{3}H]sphinganine were synthesized in this laboratory[20]. Mass spectrometry was carried out with a Varian MAT mass spectrometer model CH5 combined with a gas chromatograph. Spectra were recorded at 70 eV.

Results

I. Analytical studies

Bacteroides thetaiotaomikron contains about 5.3 to 5.7% lipids, and up to 55% of these are phospholipids. The thin-layer analysis of the total lipid extract is represented in Fig. 1. When the total lipids are submitted to $HgCl_2$ and mild alkaline hydrolysis approximately 50% of the bacterial phospholipids are not hydrolyzable (Table 1).

Since the sphingolipids were of primary interest in this study the total lipid extract of the bacteria was first treated with $Hg^{2\oplus}$ ions, in order to hydrolyze plasmalogens, which are possibly present in this bacterial strain, followed by alkaline hydrolysis of the ester lipids. The remaining lipids were then separated by silicic acid column chromatography into a neutral lipid fraction consisting of the fatty acids, an acetone/methanol fraction which contained cerebroside derived from the medium and sphinganine, and a methanol fraction, which still contained long-chain bases but mostly sphingomyelin and ceramide phosphinicoethanolamine, Fig. 1. The individual lipids of each fraction were purified by preparative thin-layer chromatography. The purified lipids were then characterized by a number of techniques described below. These analyses revealed the following sphingolipids: free long chain bases, ceramide, ceramide phosphinicoethanolamine and sphingomyelin.

Free long chain base fraction: the free sphingosine bases isolated from the acetone/methanol (9:1) and methanol fractions by preparative thin-layer chromatography were characterized as follows:

a) the R_F-value in thin-layer chromatography of the ninhydrin-positive base was identical with that of sphinganine (chloroform/methanol/2N ammonia 40:10:1)[21].

b) Gas chromatography-mass spectroscopy of the *N*-acetyl-bis(trimethylsilyl) derivative (Fig. 2) and in addition periodate oxidation of the free base followed by gas chromatographic analysis of the long chain aldehyde (Fig. 3) proved that the main free long chain base is 15-methylhexadecasphinganine.

Table 1. Distribution of *Bacteroides thet.* lipids (% of weight) in fractions of the column separation before and after $HgCl_2$-alkaline hydrolysis

Fraction	% of total weight	
	Before	After alkaline hydrolysis
Chloroform	34.3	48.4
Acetone/methanol	21.9	28.0
Methanol	43.8	23.6

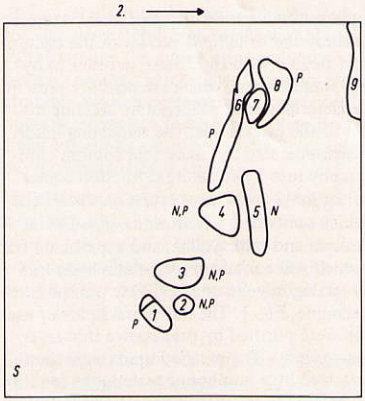
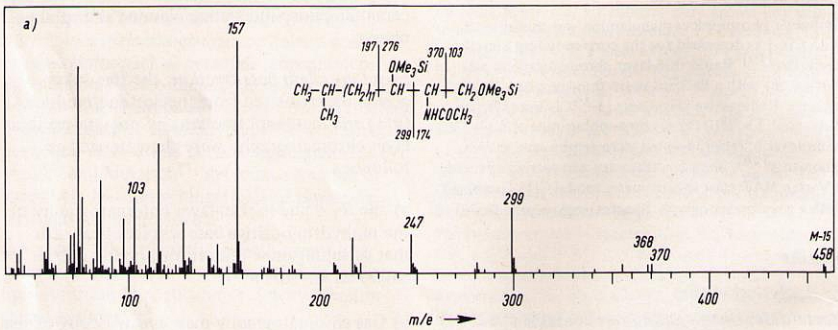


Fig. 1. Two-dimensional thin-layer chromatogram of total lipids of *Bacteroides thetaiotaomikron*.

1. Dimension: chloroform/methanol/water 65:25:4;
2. Dimension: chloroform/methanol/conc. ammonia 60:35:4

1 sphingomyelin, 2 phosphatidylserine, 3 ceramide phosphinicoethanolamine, 4 phosphatidylethanolamine, 5 sphinganine (15-methylhexadecasphinganine), 6 phosphatidylglycerol, 7 cerebroside, 8 cardiolipin, 9 ceramide, fatty acids, triglyceride, S = start; N = ninhydrin-positive; P = phosphate positive.



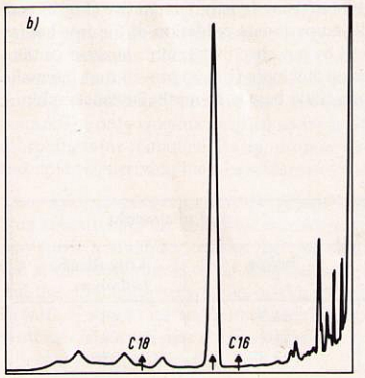


Fig. 2. Combined gas-liquid chromatography-mass spectrometry of silylated *N*-acetylated free long-chain base fraction isolated from the total lipid extract of *Bacteroides thet.* Column temp. 220 °C, 1% SE 30, column length: 2 m.

The fragments *m/e* 458 (M-15), 368 (M-15-90) and 103,174,197,280,299 and 377 are identical with those reported by White et al.[2] for *Bacteroides melaninogenicus*.

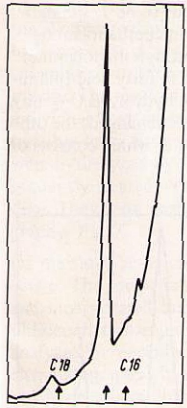


Fig. 3. Gas liquid chromatogram of long chain aldehyde liberated from the free base by periodate oxidation. Column temp. 155 °C, 2,5% EGS, column length: 2 m.

Ceramide was identified as follows: preparative thin-layer chromatography of the acetone/methanol fraction in solvent system chloroform/methanol 15:1 yielded a band with R_F identical to that of test ceramide (*N*-palmitoylsphinganine). This ceramide was acid hydrolyzed, and the fatty acids were separated from the long-chain bases. The fatty acids were methylated and treated with acetic anhydride in pyridine and the long-chain base *N*-acetylated and silylated. They were both analyzed by combined gas-liquid chromatography and mass spectroscopy. Again the only long-chain base present in ceramide was 15-methylhexadecasphinganine. The main acyl residue which was identified as 3-hydroxypalmitic acid by this combined technique was accompanied by traces of long-chain normal and iso fatty acids (Fig. 4).

Ceramide phosphinicoethanolamine, which is the predominant sphingolipid in *Bacteroides thet.* was purified for characterization by silicic acid chromatography. It proved to be pure in two solvent systems (chloroform/methanol/water 65:25:4 and chloroform/methanol/2N NH_4OH 60:35:8). Hydrolysis with *Bacillus cereus* phospholipase C yielded ceramide and phosphonoethanolamine, the former being identified by thin-layer chromatography (solvent system: chloroform/methanol 15:1), and by acid hydrolysis to yield the 15-methylhexadecasphinganine and 3-hydroxypalmitic acid. Phosphonoethanolamine was isolated and identified from the aqueous phase of the phospholipase C hydrolysis by thin-layer chromatography on CM-cellulose plates (solvent system: methanol/water/formic acid 70:13:7). Additional methylation of the ninhydrin-positive phosphosphingolipid with methyl iodide yielded sphingomyelin, Fig. 5. The double band is due to ceramide species with hydroxy- and non-hydroxy fatty acids.

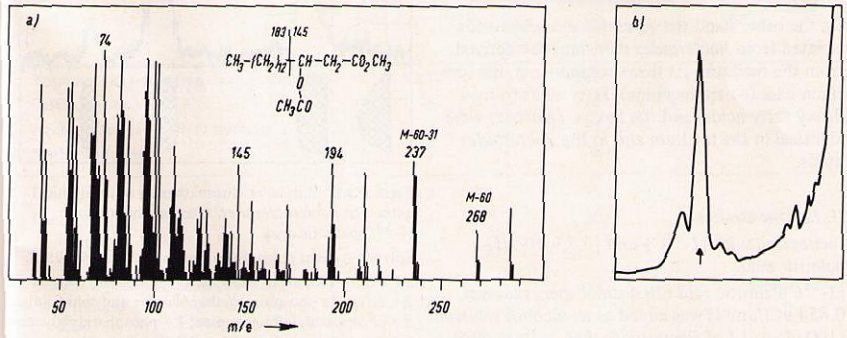


Fig. 4. Combined gas-liquid chromatography-mass spectrometry of *O*-acetylated hydroxy fatty acid methyl esters, isolated from ceramide of *Bacteroides thet.* lipids.
Column temp. 195 °C, 2.5% EGS, column length: 2 m.

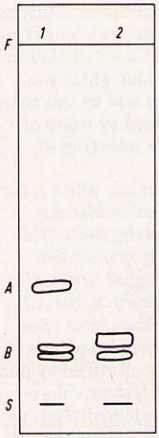


Fig. 5. Thin-layer analysis of 1) components of methanol fraction (A = ceramide phosphinicoethanolamine, B = sphingomyelin) and 2) reaction products of methanol fraction after *N*-methylation with methyl iodide.
S = start F = front. Solvent system: chloroform/methanol/water 65:25:4.

Sphingomyelin isolated from the methanol fraction was identified by its chromatographic behaviour, since it had a retention time identical with brain sphingomyelin and stained positively with Dragendorf's reagent and the phosphate reagent of Dittmer et al.[22]. Phospholipase C released ceramide, which consisted of the branched C_{17}-long-chain base and mainly 3-hydroxypalmitic acid. Phosphonocholine cochromatographed with an authentic sample under the conditions given for phosphonoethanolamine.

On the other hand the ceramide monohexoside isolated from *Bacteroides thet.* must be derived from the medium. Its three components, the long-chain base (4*t*-sphingenine), fatty acids (α-hydroxy fatty acids) and the hexose (glucose) were identical in the medium and in the *Bacteroides* lipids.

II. Isotope studies

Incubation with [1-^{14}C]- and [6,7,9,10-^{3}H]-palmitic acid

[1-^{14}C]Palmitic acid (20.4 μmol spec. radioact. 0.854 μCi/μmol) was added as an alcohol solution (100 μl) to 1 *l* of *Bacteroides thet.* culture. 86% of this radioactivity was recovered in the cell pellet. Five radioactive peaks showed up in the radio thin-layer chromatograms, Fig. 6a, which on further analysis turned out to be 1. sphingomyelin, 2. ceramide phosphinicoethanolamine and sphinganine, 3. phosphatidylethanolamine, 4. phosphatidylglycerol and 5. fatty acid (palmitic acid). After mild alkaline hydrolysis (Fig. 6b), only peak 1 and 2 remained unchanged, the other bands disappeared into band 3, which consists of

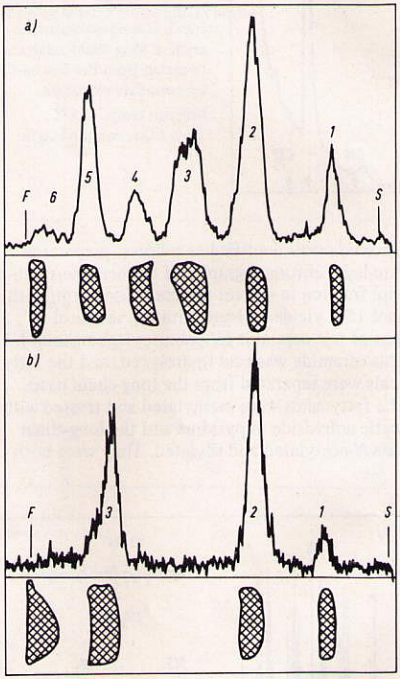


Fig. 6. Radio thin-layer chromatogram of total lipid extract of *Bacteroides thet.* after incubation with [1-^{14}C]palmitic acid.
Solvent system: chloroform/methanol/water 65:25:4.
a) Before alkaline hydrolysis: 1 = sphingomyelin; 2 = ceramide phosphinicoethanolamine and sphinganine; 3 = phosphatidylethanolamine; 4 = phosphatidylglycerol; 5 = free fatty acid (palmitic acid); 6 = ceramide.
b) After alkaline hydrolysis: 1 = sphingomyelin; 2 = ceramide phosphinicoethanolamine and sphinganine; 3 = fatty acids, ceramide.

free fatty acids. The chloroform-soluble lipids of the alkaline hydrolysate were separated according to Vance et al.[23] into the fatty acid fraction, ceramide phosphinicoethanolamine and sphingomyelin. The result is summarized in Table 2. The individual lipids were further purified by preparative thin-layer chromatography. The total lipids were hydrolyzed by acid methanolysis and subsequently treated with acetic anhydride in pyridine. They were analyzed by radio gas chromatography, Fig. 7.

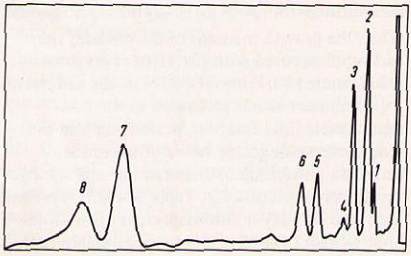


Fig. 7. Gas-liquid chromatogram of fatty acid methyl esters from total lipid extract of *Bacteroides thet.*.
The esters were acetylated. 1 = Methyl pentadecanoate; 2 = methyl isopentadecanoate; 3 = methyl palmitate; 4 = methyl palmitoleate; 5 = methyl stearate; 6 = methyl oleate; 7 = methyl 3-acetoxypalmitate; 8 = unknown substance. Column temp. 170 °C; 2.5% EGS; column length: 2 m.

Of the fatty acids only palmitic acid was radioactive. The long-chain bases in their free form and incorporated into ceramide, ceramide phosphinicoethanolamine and sphingomyelin were analyzed by periodate oxidation and radio gas chromatography. In every case only palmitaldehyde was radioactive, indicating that [1-^{14}C]palmitic acid had been utilized predominantly for the long-chain base biosynthesis. When the long-chain fatty acids of ceramide, ceramide phosphinicoethanolamine and sphingomyelin were analyzed by radio gas chromatography, the mass-peak of methyl 3-hydroxypalmitate occurred again, but no fatty acid was radioactive. Identical results were obtained with [6,7,9,10-^{3}H]-palmitic acid. The fatty acid methyl esters of the hydrolysate of the total lipid mixture were again separated by preparative radio thin-layer chromatography into hydroxy- and normal fatty acid methyl esters (solvent system: 1,2-dichloroethane). The hydroxy fatty acid fraction proved again to be radioinactive.

Table 2. Distribution of radioactivity in alkali stable lipids of *Bacteroides thet.* after incubation with [1-^{14}C]palmitate.

Fraction	Absolute radioact. × 10^{-6} [dpm]	Relat. radioact. [% of total]	$\frac{^{14}C_{fatty\ acid}}{^{14}C_{base}}$
Total lipid extract after alkali hydrolysis	8.37	100	
$CHCl_3$ fraction	3.68	44	
Acetone/methanol (9:1) fraction	1.47	17.6	
Methanol fraction	3.22	38.4	
Free base	1.12	25.5	
Ceramide	1.31	30	0.24
fatty acid	0.2		
base	0.83		
Ceramide phosphinicoethanolamine	1.73	39.5	0.24
fatty acid	0.23		
base	1.15		
Sphingomyelin	0.22	5	0.25
fatty acid	0.035		
base	0.138		

Incubation with [3-^{3}H]D(+)-erythro-sphinganine

When the growth medium of *Bacteroides thet.* was supplemented with [3-^{3}H]D(+)-*erythro*-sphinganine (10.4 μmol/*l*), 31% of the radioactivity administered was recovered in the non-saponifiable lipid fraction, present only in the long-chain sphingosine bases of ceramide, ceramide phosphinicoethanolamine and sphingomyelin as summarized in Table 3. Fig. 8 represents the radio thin-layer chromatogram of the total lipid extract (a) and of the unsaponifiable radioactive lipids (b) which amount to 82% of the lipid extract.

The analysis of the long-chain bases of ceramide, ceramide phosphinicoethanolamine and sphingomyelin proved that the long-chain base had been incorporated unchanged into the complex sphingolipids. Periodate oxidation of the long-chain bases which were released from the purified lipids by acid hydrolysis yielded exclusively radioactive palmitaldehyde in radio gas chromatography.

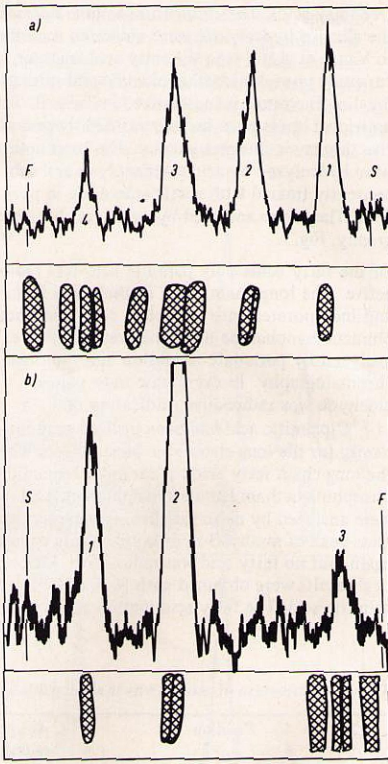


Fig. 8. Radio thin-layer chromatogram of total lipid extract of *Bacteroides thet.* after incubation with [3-^{3}H]D-*erythro*-sphinganine.

Solvent system: chloroform/methanol/water 65:25:4; S = start; F = front.

a) Before alkaline hydrolysis, 1 = sphingomyelin; 2 = ceramide phosphinicoethanolamine and free long-chain base; 3 = phosphatidylethanolamine and unidentified sphingolipid; 4 ≈ ceramide. *b) Total lipids after* mild alkaline hydrolysis, 1 = sphingomyelin; 2 = ceramide phosphinicoethanolamine and sphinganine; 3 = ceramide.

Table 3. *Distribution of radioactivity* in alkali-stable lipids of *Bacteroides thet.* after incubation with [3-^{3}H]D(+)-*erythro*-sphinganine (10.4 μmol; 1.42 × 10^7 dpm ^{3}H) in 1 *l* culture.

Fraction	Absolute *radioact.* × 10^{-6} [dpm]	Relat. radioact. [% of total]
Total lipid extract after mild alkaline hydrolysis	4.42	100
Chloroform fraction	0.18	4.8
Acetone/methanol fraction	1.33	35.4
Methanol fraction	2.25	59.8
Free base	1.07	31.8
Ceramide	0.79	20.8
Ceramide phosphinicoethanolamine	1.44	42.7
Sphingomyelin	0.16	4.7

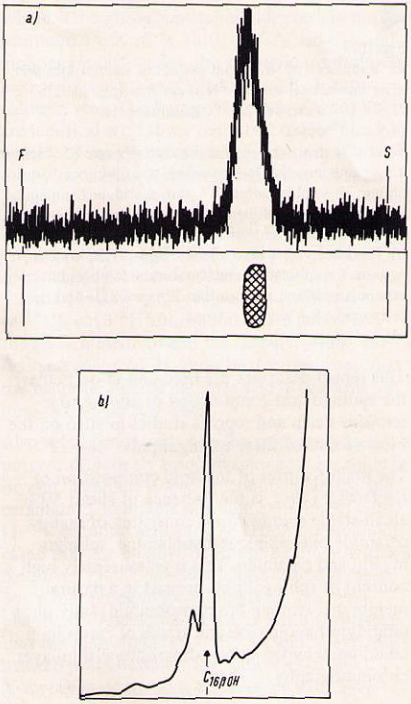


Fig. 9.
a) Radioscan of purified ceramide phosphinicoethanolamine after growth of the bacteria in a medium supplemented with [3-^{3}H]sphinganine. Solvent system: chloroform/methanol/water 65:25:4. S = start; F = front.
b) Gas chromatogram of *O*-acetylated fatty acid methyl esters obtained from ceramide phosphinicoethanolamine. The R_F-value is identical with methyl 3-acetoxypalmitate.

Again only 3-hydroxypalmitic acid was identified as the only acetylated methyl ester in gas-liquid chromatography. No radioactivity was found in the fatty acid fraction released from each sphingolipid purified by preparative thin-layer chromatography. Fig. 9a and 9b illustrate the purification of ceramide phosphinicoethanolamine and the gas-liquid chromatographic analysis of its fatty acyl substituents.

Incubation with [3-^{3}H]D(+)-erythro-sphinganine and [1-^{14}C]palmitic acid

The separation and analytical procedures of the total lipid extract of *Bacteroides thet.* which was grown in a medium supplemented with [3-^{3}H]-D(+)-*erythro*-sphinganine (11.5 μmol, spec. radioact. 0.632 μCi/μmol) and [1-^{14}C]palmitic acid (20.4 μmol, specif. radioact. 0.864 μCi/μmol) were those applied in the previously described experiments. 73% of the ^{3}H activity and 60% of the ^{14}C-activity were recovered and distributed as shown in Fig. 10a and 10b. The quantitative analysis is summarized in Table 4. Four sphingolipid fractions were obtained, each of which was purified for analysis by preparative thin-layer chromatography. It is remarkable, that the bacteria contain considerable amounts of the free long-chain base sphinganine, part of it as the unchanged substrate. But there is a large amount of newly synthesized sphinganine, as can be deduced from the ^{14}C radioactivity derived from [1-^{14}C]-palmitic acid. In fact periodate oxidation of the long-chain base fraction yielded considerable amounts of [^{14}C]palmitaldehyde, (Table 4). The long-chain base sphinganine incorporated as the only radioactive long-chain base of the individual sphingolipids (as shown by the oxidative degradation of the base component of ceramide, ceramide phosphinicoethanolamine and sphingomyelin), is more strongly labelled with ^{14}C than with ^{3}H as shown by the isotope ratio. We therefore conclude that the sphinganine synthesized de novo from palmitate is at least equally well or better utilized in the biosynthesis of the complex sphingolipids than the ^{3}H-labelled long-chain base.

The absence of any radioactivity in the fatty acids of the complex sphingolipids but the presence of only 3-hydroxy palmitic acid is in agreement with the results of the isotope experiments with labelled palmitic acid.

Discussion

Sphingolipids are rather uncommon lipids in bacteria. So far only *Mycoplasma gallisepticum*[24], *Bacteroides ruminicola*[25] and *Bacteroides melaninogenicus*[3] have been found to contain sphingolipids. Particularly the sphingolipids of the latter strain have been analyzed in more detail[4].

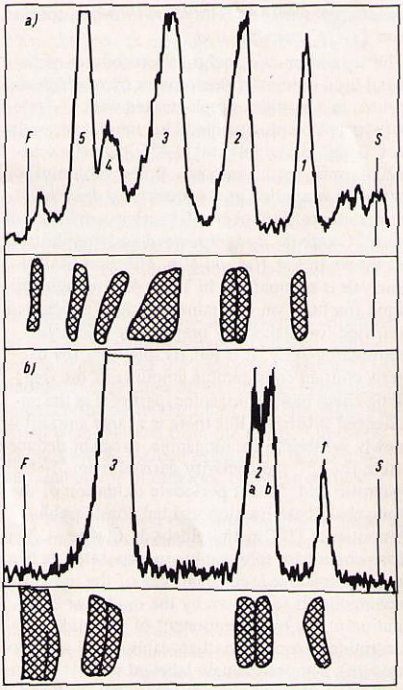


Fig. 10.
a) Radioscan of total lipid extract of *Bacteroides thet.* after incubation with [3-^{3}H]D-*erythro*-sphinganine (1.4×10^6 dpm) and [1-^{14}C]palmitic acid (1.9×10^6 dpm).
Solvent system: chloroform/methanol/water 65:25:4. 1 = sphingomyelin; 2a = ceramide phosphinicoethanolamine; 2b = sphinganine; 3 = phosphatidylethanolamine and unidentified sphingolipid; 4 = phosphatidylglycerol; 5 = palmitic acid and ceramide, S = start; F = front.
b) Radioscan after mild alkaline hydrolysis; same solvent system. 1 = sphingomyelin; 2 = ceramide phosphinicoethanolamine and sphinganine; 3 = ceramide and free fatty acids; S = start; F = front.

This report describes the lipid and in particular the sphingolipid composition of another *Bacteroides* strain and reports studies in vitro on the biosynthesis of these sphingolipids.

The main features of the lipid composition of *Bacteroides thet.* is the presence of about 50% alkali-stable sphingolipids consisting of mainly ceramide phosphinicoethanolamine, sphingomyelin and ceramide. This is an extremely high content of sphingolipids present in a natural membrane. Among the phospholipids only phosphatidylethanolamine and traces of cardiolipin could be detected in two-dimensional thin-layer chromatography.

Table 4. *Distribution of radioactivity in* alkali-stable lipids of *Bacteroides thet.* after incubation with [1-^{14}C]-palmitic acid (20.4 µmol = 3.95×10^7 dpm/l) and [3-^{3}H]D(+)-*erythro*-sphinganine (11.5 µmol = 1.6×10^7 dpm/l).

Fraction	Absolute radioact. $\times 10^{-6}$ [dpm]		Relat. radioact. [% of total]		$^3H/^{14}C$
	^3H	^{14}C	^3H	^{14}C	
Free base	4.92	3.52	52.3	41.2	1.40
Ceramide					
base	0.80	0.87	8.5	10.2	0.92
fatty acid	–	–	–	–	–
Ceramide phosphinicoethanolamine					
base	3.32	3.74	35.3	43.7	0.89
fatty acid	–	–	–	–	–
Sphingomyelin					
base	0.37	0.42	3.9	4.9	0.88
fatty acid	–	–	–	–	–

Whereas the glycerophospholipids contain normal saturated 14:0, 16:0, 18:0, iso-C_{17}, iso-C_{19} and monoenoic fatty acids, the sphingophospholipids are predominantly acylated with 3-hydroxypalmitic acid. The long chain bases of these lipids are identical with those described before[4], namely 15-methylhexadecasphinganine, 17-methyloctadecasphinganine and traces of sphinganine.

In order to find out whether all sphingophospholipids including sphingomyelin are biosynthesized by *Bacteroideaceae*, radioactive precursors, [1-^{14}C]- and [^{3}H]palmitic acid and [3-^{14}C]- or [3-^{3}H]sphinganine were added to the growth medium and the labelled lipids purified and analyzed. It could be demonstrated that the bacteria utilized palmitic acid efficiently for long-chain base synthesis.

Also sphinganine added to the medium is incorporated effectively into the sphingolipids sphingomyelin, ceramide phosphinicoethanolamine and ceramide. The double labelling experiment with [1-^{14}C]palmitic acid and [3-^{3}H]sphinganine indicated that the de novo synthesis of the long-chain base from [1-^{14}C]labelled palmitic acid is rapid. Since the sphingophospholipids are the main membrane lipid constituents, it is not surprising that the long-chain fatty acid present in excess is utilized preferentially. The almost exclusive *N*-acylation of the long-chain bases with 3-hydroxypalmitic acid, which is not derived from palmitate is a puzzling observation. Current experiments aim at determining the origin of this fatty acid and its function in sphingophospholipids.

Furthermore the results on the biosynthesis of these sphingolipids encourage further studies in vitro on the enzymes and the regulation of the enzymes involved in the biosynthesis of the long chain bases and the more complex sphingophospholipids derived therefrom.

The work reported in this paper has been supported by the *Deutsche Forschungsgemeinschaft*.
The purity of the *Bacteroides* strain used in these experiments has been checked by Professor *G. Pulverer*, Institut für Bakteriologie und Hygiene, Universität Köln. We gratefully acknowledge this cooperation.

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