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Capnocytophaga spp. Contain Sulfonolipids That Are Novel In Procaryotes

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A group of unusual sulfonolipids was found in bacteria of the genus *Capnocytophaga*. One of these lipids, to which we have assigned the trivial name capnine, was isolated in 98% pure form and was identified, by infrared absorption spectrometry, high-resolution mass spectrometry, and other methods, as 2-amino-3-hydroxy-15-methylhexadecane-1-sulfonic acid. Another lipid appears to be an *N*-acylated version of capnine; after acid hydrolysis, its sulfur was recovered in a form chromatographically indistinguishable from that of capnine. The new lipids are related structurally to sphingosine and the ceramides, respectively, but differ markedly from those compounds in important respects, notably the presence of the sulfonate group. Some *Capnocytophaga* strains accumulated mostly capnine, whereas others accumulated mostly *N*-acylcapnine. All seven strains examined were found to contain the new lipids, in amounts ranging from 7 to 16 μmol/g of cells (wet weight). The lipids were found in isolated cell envelopes, where they were present in amounts ranging up to 400 mg/g of envelope protein; they are, accordingly, major cell components.

Bacteria of the genus *Capnocytophaga* (15) are small, gram-negative, fusiform cells that are chemoorganotrophic, require CO_2 for growth (i.e., are capnophilic), exhibit a strictly fermentative metabolism but are tolerant of oxygen, and exhibit gliding motility. These organisms were isolated from the tooth surface and the gingival crevice of healthy individuals as well as from periodontal lesions and are implicated in the etiology of some disease states (16, 19). The one species tested in gnotobiotic rats was able to effect bone resorption (11). Because of the putative ability of these organisms to cause inflammation, we undertook an investigation of their cell surface components with the intent of identifying those that have high endotoxic activity. In the course of fractionating the cell envelopes, we encountered a substance with unusual properties. This substance (Fig. 1) has been assigned the trivial name "capnine." Because capnine was present in large quantities, it was subjected to chemical studies. Experiments in which capnine was labeled with ^{35}S led to the detection of a related compound, *N*-acylcapnine (Fig. 1). Together termed the capnoids, these unusual lipids are major components of the cell envelope of *Capnocytophaga* spp. The capnoids bear an obvious structural relationship to the 1-deoxyceramide-1-sulfonic acid (Fig. 1) previously reported to be a minor lipid component of the diatom *Nitzschia alba* (1, 3), although the hydrocarbon chain of capnine differs from that of the corresponding moiety of the deoxyceramide sulfonic acid.

MATERIALS AND METHODS

Reagents. Solvent A was acetone-methanol-17 N NH_4OH, 5:5:1, (vol/vol).

Bacterial strains and cultivation of bacteria. The organisms used in this study were described previously (15). Cells were grown in Trypticase soy broth (BBL Microbiology Systems)-yeast extract-glucose-hemin or (in one case, strain 4) in Todd-Hewitt broth-glucose-hemin (details given in 15). Cultures were incubated without shaking at 37°C in GasPak (BBL) jars (small cultures) or in filled, sealed flasks (large cultures) and were harvested by centrifugation (10,000 × *g* for 15 min) at late log or early stationary phase.

Purification of capnine. Wet, packed cells of *C. gingivalis* strain 30N51 (100 g, from approximately 25 liters of culture) were stirred with 400 ml of acetone plus 400 ml of methanol until dispersed. Then 55 ml of 17 N NH_4OH was added and vigorous stirring was continued for 30 min. The mixture was filtered through paper, and the filter cake was washed with 300 ml of solvent A. To the combined filtrates were added 1 liter of water and 170 ml of glacial acetic acid (to give a pH of 5); a precipitate formed. The preparation was stored at 4°C overnight, and the precipitate was collected by centrifugation (10,000 × *g* for 15 min at 4°C) and was washed, by centrifugation, successively with 200-ml portions of ethanol, ethanol containing 0.1 N HCl, and again with ethanol. At each step, the mixture was stirred at room temperature until the solids were well dispersed and then was chilled at −20°C for at least 2 h before centrifugation. The precipitate was then washed twice with water (100-ml portions) at 4°C, dried in vacuo, and stored over a desiccant at −20°C. The yield was 150 mg (53% of the estimated cellular content). The method was scaled down for purification of [^{35}S]capnine from cells grown in the presence of [^{35}S]sulfate; in that procedure the yield was over 50%

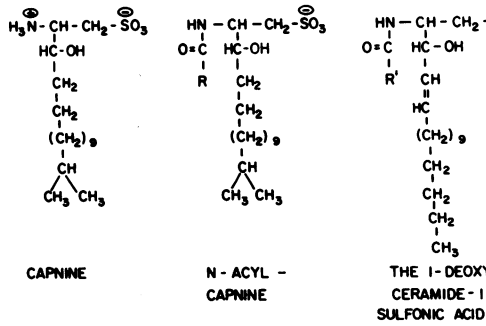

FIG. 1. *Structure of the capnoids and of the deoxyceramide sulfonic acid of N. alba (1, 3). R' represents a group of fatty acids, the major species being trans-3-n-hexadecenoic acid. The R group of N-acylcapnine has not been identified.*

of the radioactivity in capnoids (estimated as described below).

Labeling of capnoids with ^{35}S. Cells were grown in media containing $Na_2{}^{35}SO_4$ (specific activities given for individual experiments) for at least four generations (24 to 36 h). The cells were harvested and then washed three times (by centrifugation at 8,000 × *g* for 10 min) with 7% trichloroacetic acid and once with water (20 to 40 ml of each washing solution per g of wet cells). The washed cells were mixed with solvent A (at least 10 ml/g of wet cells), allowed to stand with occasional stirring for 30 min at room temperature, and centrifuged. The pellet was reextracted with solvent A and the two extracts were combined. Samples were taken for determination of radioactivity and for chromatography or purification of capnoids. Radioactivity in liquid samples was measured in a scintillation counter after evaporation of organic solvents under a stream of N_2 and mixing of the residue with 1 ml of 0.3% aqueous sodium dodecyl sulfate and 10 ml of Aquasol. Portions of the extracts to be chromatographed (in solvent system I) were (where necessary) taken to dryness under a stream of N_2 and redissolved in a small volume of solvent A.

The specific activities of inorganic sulfate in the culture media were calculated from direct determinations of the concentrations of radioactivity and determinations of the sulfate concentrations by a barium-precipitation method (17a). Values obtained for sulfate ion concentrations were 1.6 mM for Trypticase soy broth medium and 0.73 mM for the Todd-Hewitt medium.

Partial purification of *N*-acylcapnine. The solvent A extract of ^{35}S-labeled cells of *Capnocytophaga* sp. strain P7 (which contains a high proportion of *N*-acylcapnine) was evaporated to dryness under a stream of N_2 and dissolved in chloroform. The lipids were fractionated on a column of DEAE-cellulose (bed volume, 10 ml/g of wet cells) as described by Kates (12, Table 5.2, sequence 1) except that solvent A was substituted as the final, alkaline solvent that elutes sulfo- (and sulfono-) lipids. Eighty percent of the radioactivity applied to the column was recovered in this fraction, which was free from capnine but contained some nonradioactive impurities. The eluate was evaporated to dryness under N_2, dissolved in chloroform, washed three times with an equal volume of water (centrifugation was necessary to break the emulsion), again dried, and stored in chloroform solution at −20°C.

Degradative procedures. Acid hydrolysis was carried out in 6 N HCl at 100°C for 16 h, in sealed tubes under N_2. The hydrolysates were taken to dryness under a stream of N_2 and dissolved in solvent A (for thin-layer chromatography). In some cases dried hydrolysates were extracted with water and then centrifuged; the supernatants were tested for inorganic sulfate by addition of $BaCl_2$.

In attempts at saponification, capnine was mixed with 10% ethanolic KOH and heated at 100°C until dry. The residue was mixed with water, adjusted to pH 3 with HCl, and centrifuged; the supernatant was tested for inorganic sulfate, and the precipitate was dissolved in solvent A for chromatography.

Periodate oxidation of capnine was carried out on a 40-mg sample dissolved in 15 ml of 0.2 N aqueous NaOH; the mixture was incubated in the dark at 25°C, with stirring, for three successive 2-h periods, each of which was preceded by the addition of 2.5 ml of 0.5 M aqueous sodium metaperiodate. The mixture was then extracted with three 20-ml portions of diethyl ether, and the combined ether phases (aldehyde fraction) were filtered and then evaporated to dryness under a stream of N_2.

Periodate-permanganate oxidation of capnine (20 mg) was carried out as described by Kates (12, section 7.1.2.3). The resulting fatty acid fraction was taken to dryness under a stream of N_2, dissolved in 4 ml of 10% BCl_3 in methanol, allowed to boil for 4 min, and concentrated to about 1 ml under N_2. Water (3 ml) was added, and the fatty acid methyl ester was extracted with two 4-ml portions of petroleum ether, dried under N_2, and dissolved in acetone for gas chromatography-mass spectrometry.

Thin-layer chromatography. System I was silica gel G, developed with chloroform–methanol–7 N NH_4OH (70:30:5, vol/vol). The plates were equilibrated with the vapor phase of the solvent system for 15 to 30 min before addition of the developing solvent. System II was silica gel G, developed with *n*-propanol–17 N NH_4OH, (4:1, vol/vol). System III was silica gel H, developed with petroleum ether (boiling point, 35 to 60°C)–diethyl ether–glacial acetic acid (90:10:1, vol/vol).

The ninhydrin spray reagent was 0.5% ninhydrin in butanol. Rhodamine 6G was used as a spray reagent as described by Kates (12). For the periodate-Schiff test, plates were sprayed with 0.1 M sodium metaperiodate in 0.1 N NaOH (freshly prepared) until damp, allowed to stand for 20 min at room temperature, and dried. The plates were then sprayed with 10% sodium metabisulfite (in water, freshly prepared); after partial drying, spraying was repeated until iodine color was no longer produced. While still damp, the plates were sprayed with the Schiff reagent, prepared as described by Kates (12).

For autoradiography, plates (containing 8,000 dpm of ^{35}S in each lane) were dried to remove chromatographic solvents, sprayed with a 10% (wt/vol) solution of scintillation fluors in toluene, again dried, and placed in contact with X-ray film (Kodak XRP-5) for 3 days. The film was developed as recommended by the manufacturer.

Gas chromatography and mass spectrometry. Combined gas chromatography-mass spectrometry was performed by use of a Packard model 5985 gas chromatograph equipped with a 20-m capillary column (model 814012A) and an electron impact ionization device. The column temperature was programmed to remain at 125°C for 5 min and then to rise to 250°C at 8°/min. Mass spectra of capnine were obtained by use of an Associated Electrical Industries MS-9 (low resolution) and a DuPont MS-110B (high resolution) mass spectrometer.

Isolation of cell envelopes. *Capnocytophaga* sp. cells grown in the presence of ^{35}S were suspended in 50 mM KCl–10 mM Tris-hydrochloride buffer (pH 7.4 at 4°C) and (where indicated) sonicated (Branson Sonifier, 5 to 10 A, 30-s pulses at 0°C) until the cells were fragmented into particles a small fraction of their normal length. Sucrose (1.1-g/ml suspension) was added and dissolved; the preparations were overlayered with sucrose solutions containing the Tris buffer and were centrifuged at 100,000 × *g* (4°C) for 12 to 16 h. Fractions of 1-ml volume were collected; samples were taken for determination of protein (5). Additional samples were taken for determination of trichloroacetic acid-precipitable radioactivity; these contained 150 μg of protein, adjusted where necessary by addition of nonradioactive cells as carrier. Assay mixtures (1 ml) contained 7% (wt/vol) trichloroacetic acid, 10 mM nonradioactive Na_2SO_4, and 40 mM 2-mercaptoethanol; they were heated for 10 min at 70 to 80°C, cooled, and diluted with approximately 4 volumes of 5% trichloroacetic acid. The precipitates were collected quantitatively on filters (type HAWP-025, Millipore Corp.) that had been soaked in 5% trichloroacetic acid, washed four times with 10-ml portions of that solution, dried, and counted in a toluene-based scintillation fluid.

Materials. [^{35}S]sodium sulfate (>50 Ci/mol of S) and Aquasol were obtained from New England Nuclear; silica gel G thin-layer plates (250 μm thick), from Analabs, Inc.; DEAE-cellulose (DE-23), from Whatman, Inc.; scintillation fluors (blend 2a-70), from Research Products International; reagents for the protein determination, from Bio-Rad Laboratories; and fatty acid methyl esters and BCl_3 in methanol, from Applied Science Laboratories, Inc. Sucrose was the ultrapure enzyme grade from Schwarz/Mann. All other chemicals were of reagent grade.

RESULTS

Properties and structure of capnine. Elemental analysis of purified capnine gave the composition: C, 57%; H, 10.2%; N, 4.2%; O, 18%; and S, 9.1% (total, 98.5%). The structure shown in Fig. 1, which has the formula $C_{17}H_{37}NO_4S$, gave the theoretical values: C, 58%; H, 10.6%; N, 4.0%; O, 18.2%; and S, 9.1%. It would appear that capnine was isolated in the dipolar ionic form (Fig. 1) or in the free-acid, free-base form, and not as a salt. The elemental analysis accounts for 98.5% of the mass as C, H, N, O, and S; given the evidence for purity (described below) and the molecular weight of 351 found by mass spectrometry, these elements account for all but 5 atomic mass units per molecule. The amounts of N, O, and S, in turn, are precisely accounted for by functional groups that were shown, by means independent of the elemental analysis, to be present. It is unlikely that counterions were present.

On thin-layer chromatography in either solvent system I or II, purified capnine gave a zone that was ninhydrin reactive, though the color intensity was not so great as might have been expected from the behavior of capnine in the quantitative ninhydrin reaction; apparently, the zone is not well penetrated by the spray reagent. The zone was also visible as an opaque spot after the plates had been sprayed with butanol or water. In preparations purified from cells grown in the presence of [^{35}S]sulfate, the zone detected by these methods coincided precisely with the radioactivity that was detected autoradiographically. No impurities were revealed by the methods that detected capnine, nor were any apparent after spraying with aqueous rhodamine 6G, exposure to I_2 vapor, or charring after spraying with H_2SO_4. These last three methods do not detect capnine with high sensitivity, though a charred zone that was visible only under long-wavelength UV light, and a faint I_2 spot, were observed. The aqueous rhodamine solution apparently does not penetrate the capnine zone, which was visible as an opaque spot under room lighting but did not appear as a dark spot under UV light.

In neutral or acidic solvents, regardless of their polarity, the solubility of capnine was very limited. This may, perhaps, be understood in terms of the structure of the compound (Fig. 1); aggregates may be formed in which there are two ionic interactions between neighboring molecules and a hydrophobic interaction between the hydrocarbon side chains as well. In contrast, capnine was soluble in moderately polar solvents containing NH_4OH (in which the amino group is deprotonated). Capnine dissolved in 0.1 N aqueous NaOH, but, upon standing, the solutions became cloudy and flow birefringent.

Capnine could be crystallized by allowing saturated solutions in solvent A to evaporate slowly at room temperature. Although the crystallized form was not used for other experiments described in this report, the resulting white, fascicular crystals could be washed with cold methanol without great loss of material, were homogeneous and identical to capnine on thin-layer chromatography, and decomposed without melting between 325 and 335°C.

In the quantitative ninhydrin reaction (9), capnine gave a color yield (per mole of N) 85% as great as that given by alanine. This indicates that the N is present either as a primary amino group or as an ammonium salt. The latter pos-

sibility was ruled out by the infrared spectrum and by the observation that, when capnine was dissolved in excess 0.1 N NaOH and incubated for 16 h at 25°C over a trap containing H_2SO_4, no more than 1% of the ninhydrin reactivity was lost from the capnine solution, nor was any ninhydrin-reactive material found in the trap.

The chromatographic behavior of capnine (in systems I and II) was not altered by acid hydrolysis or saponification, an observation that supports the view that esters (including sulfate esters) and amide groups are absent. Capnine exposed to these degradative procedures did not release detectable inorganic sulfate, whereas comparable quantities of sodium dodecyl sulfate yielded easily detectable sulfate ion; again, this indicates the absence of sulfate esters.

The infrared spectrum of capnine (Fig. 2) reveals absorption bands at 3,360 and 3,160 cm^{-1} (N-H stretch), 1,590 cm^{-1} (-NH_2 deformation), and 1,525 cm^{-1} (-NH_3^+ deformation), and a complex of bands between 1,260 and 1,025 cm^{-1} (sulfonate group). These features are virtually superimposable upon the corresponding features of the spectrum of taurine (2-aminoethane sulfonic acid; 17). The observation of bands at 1,365 and 1,390 cm^{-1} is consistent with the presence of an isopropyl group. In contrast to the spectrum of the 1-deoxysphingenine-1-sulfonic acid derived from the deoxyceramide sulfonic acid of a diatom (1), capnine exhibited no absorption band at 960 cm^{-1} (*trans* double bond). The band exhibited by capnine at 660 cm^{-1} was judged to be too sharp, and at too low a wavenumber, to result from a *cis* double bond, especially since it was not accompanied by the expected band at about 1,650 cm^{-1}. The band at 660 cm^{-1} may result from absorption (S-C stretch) by the sulfonate group.

Mass spectrometry of capnine in the MS-9 mass spectrometer with the probe at −40°C revealed a molecular ion of mass (m) = 351 atomic mass units (at an intensity of 6% of that of the line, at m = 124, which was the most intense line above m = 100). The molecular ion observed is consistent with the structure shown in Fig. 1 ($C_{17}H_{37}NO_4S$). This molecular ion was not observed at higher temperatures, nor was it observed in the high-resolution mass spectrum obtained with the MS-110 mass spectrometer (Table 1), in which the largest ion observed had a mass of 333. The high-resolution feature of this method, plus the constraints on the composition of capnine imposed by the data from elemental analysis (one S, one N, and no more than four O, the remainder being C and H) permitted the assignment of a unique composition to this ion, $C_{17}H_{35}NO_3S$. In view of the observation of the ion of m = 351 at low temperature, and the evidence (cited below) that capnine has a hydroxyl group as well as a sulfonate group, the ion of mass 333 must represent M^+ minus H_2O. Capnine must dehydrate very readily in the mass spectrometer, for with one exception (the ion of mass 254) all species detected in the high-resolution spectrum were dehydrated. With the exception of the molecular ion, the same effect was observed in the low-temperature spectrum, which showed more of the dehydrated species than of ions that could have been the corresponding hydrated forms. All the major lines observed in the high-resolution spectrum were also major features of the low-temperature spectrum.

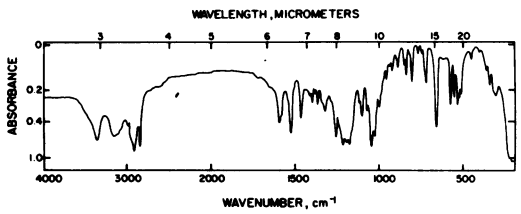

FIG. 2. *Infrared absorption spectrum of capnine (KBr pellet). The wave-number scale changes at 2,000 cm^{-1}.*

The line at m = 124 (Table 1), to which was assigned the unique composition $C_2H_6NO_3S$ and the structure of taurine minus H, locates the amino group of capnine on carbon 1 or carbon 2 (carbon 1 being that which bears the sulfonate group). The fragment of m = 238 (M^+ minus H_2O, minus CH_2SO_3H) places both the amino group and the remainder of the hydrocarbon chain on carbon 2. Strong lines at m = 318 (M^+ minus H_2O, minus CH_3) and at m = 290 (M^+ minus H_2O, minus C_3H_7) coupled with the absence of a line at m = 304 (M^+ minus H_2O, minus C_2H_5) permit the assignment of the gem-dimethyl groups at the terminus of the chain (iso structure).

After chromatography in system II, the capnine zone gave a strong positive reaction with the periodate-Schiff reagents (applied at alkaline pH, as described in Materials and Methods). Oxidation of capnine (40 mg) with periodate at alkaline pH yielded an aldehyde fraction (24 mg) that on chromatography in system III gave a major zone of R_f 0.55 (as detected with I_2 vapor) and two minor zones (R_f values, 0 and 0.2). The major zone stained a deep lavender when sprayed with the Schiff reagent alone. This component was distinct from capnine, which does not move from the origin in system III and which does not react with the Schiff reagent unless previously oxidized with periodate. The R_f (0.55) and staining behavior of the major product of periodate oxidation are characteristic of long-chain aldehydes (12). These results are consist-

TABLE 1. *High-resolution mass spectrum of capnine*[a]

Log relative intensity (approx)[b]	Determined mass (amu)	Composition assigned[c]	Structure (inferred)
3.7	124.0059	$C_2H_6NO_3S$	$H_2\overset{+}{N}=CH-CH_2-SO_3H$
0.6	136.0053	$C_3H_6NO_3S$	M^+ minus H_2O, minus $C_{14}H_{29}$
1.4	137.0148	$C_3H_7NO_3S$	M^+ minus H_2O, minus $C_{14}H_{28}$
2.6	150.0222	$C_4H_8NO_3S$	M^+ minus H_2O, minus $C_{13}H_{27}$
1.6	164.0358	$C_5H_{10}NO_3S$	M^+ minus H_2O, minus $C_{12}H_{25}$
0.3	178.0495	$C_6H_{12}NO_3S$	M^+ minus H_2O, minus $C_{11}H_{23}$
0.2	192.0677	$C_7H_{14}NO_3S$	M^+ minus H_2O, minus $C_{10}H_{21}$
0.04	206.0816	$C_8H_{16}NO_3S$	M^+ minus H_2O, minus C_9H_{19}
0.04	234.1176	$C_{10}H_{20}NO_3S$	M^+ minus H_2O, minus C_7H_{15}
3.9	234.2364	$C_{17}H_{30}$	M^+ minus H_2O, minus NH_3, HSO_3H
5.1	236.2538	$C_{17}H_{32}$	M^+ minus H_2O, minus NH_3, SO_3
3.3	238.2550	$C_{16}H_{34}N$	M^+ minus H_2O, minus CH_2SO_3H
0.2	248.1298	$C_{11}H_{22}NO_3S$	M^+ minus H_2O, minus C_6H_{13}
3.6	252.2680	$C_{17}H_{34}N$	M^+ minus H_2O, minus SO_3H
2.0	253.2754	$C_{17}H_{35}N$	M^+ minus H_2O, minus SO_3
2.4	254.2581	$C_{17}H_{34}O$	M^+ minus NH_3, SO_3
0.3	262.1476	$C_{12}H_{24}NO_3S$	M^+ minus H_2O, minus C_5H_{11}
0.3	276.1623	$C_{13}H_{26}NO_3S$	M^+ minus H_2O, minus C_4H_9
2.2	290.1775	$C_{14}H_{28}NO_3S$	M^+ minus H_2O, minus C_3H_7
1.3	316.2105	$C_{17}H_{32}O_3S$	M^+ minus H_2O, minus NH_3
2.9	318.2150	$C_{16}H_{32}NO_3S$	M^+ minus H_2O, minus CH_3
0.7	332.2286	$C_{17}H_{34}NO_3S$	M^+ minus H_2O, minus H
1.4	333.2326	$C_{17}H_{35}NO_3S$	M^+ minus H_2O

[a] All lines resulting from fragments of mass between 120 and 500 atomic mass units (amu) are reported, except for the following omissions: (i) some of the lines representing ions containing only C and H; (ii) a series of lines representing ions of the compositions $C_nH_{2n}H$ and $C_nH_{2n+2}N$; (iii) lines that were identified as resulting from fragments having a composition the same as one reported in the table, but containing ^{13}C or ^{34}S; and (iv) some of the lines of log relative intensity less than 0.3 on the scale used.

[b] Lines were recorded on photographic film, which was then scanned for absorbance. The data are areas under the absorbance peaks, normalized (on the basis of values for fragments containing rare isotopes) to give values that are approximately proportional to $\log_{10}$ beam intensity.

[c] Data were analyzed by computer subject to the constraints that the maximum numbers of hetero atoms were one N, four O, and one S. In all cases, the indicated composition was the only one having a theoretical mass that fell within the tolerance limits, which ranged from M ± 0.0021 amu (m = 120) to M ± 0.0026 amu (m = 333); in a few cases, expanded tolerance (not exceeding 0.0045 amu) had to be employed to assign a composition.

ent with the presence in capnine of a hydroxyl group vicinal to the amino group, that is, on carbon 3; such linkages are oxidized by periodate to yield aldehydes. No aldehyde products could be detected after attempts to oxidize capnine with periodic acid at acid pH, either with a spray system applied to thin-layer plates (13) or in suspensions of capnine in methanol (12). Presumably, reaction was prevented by the insolubility of capnine under these conditions. Oxidation of capnine (20 mg) with periodate and permanganate yielded a fatty acid fraction (13 mg) which, when converted to methyl esters, was homogeneous on gas chromatography (retention time, 14.0 min). After mixing with authentic standards, this substance could be separated from methyl isotetradecanoate (12.3 min), methyl *n*-tetradecanoate (12.9 min), methyl anteisopentadecanoate (14.2 min), and methyl *n*-pentadecanoate (14.6 min). The mass spectrum of the methyl ester derived from capnine showed a molecular ion at m = 256 (intensity, 26%); the most intense lines were at m = 74 (100%), m = 87 (90%), m = 143 (39%), and m = 213 (29%). The three most intense lines are characteristic of saturated, nonhydroxylated fatty acid methyl esters. The molecular ion indicates that the compound was a methyl pentadecanoate. In a number of determinations, the line at m = 213 (M^+ minus C_3H_7) was much more intense than that at m = 199 (M^+ minus C_4H_9), whereas the converse was true for authentic methyl anteisopentadecanoate (isopentadecanoate standards are not readily available). The results, then, are consistent with an isopentadecanoic acid structure for the fatty acid derived from capnine, as would be predicted from the high-resolution mass spectrum. The finding of a 15-carbon fatty acid after periodate-permanganate oxidation of capnine confirms the position of the hydroxyl group on carbon 3 (Fig. 1).

There remain two uncertainties in the structure of capnine. First, the configurations about the asymmetric carbons are unknown. Second,

there is a question of the possible presence of an internal branch in the capnine chain. Fragments resulting from cleavage of 6- to 10-carbon units from the isopropyl end of the chain were observed on mass spectrometry, with the exception of M^+ minus H_2O, minus C_8H_{17} (m = 220), which was not detected (Table 1). However, the lines representing removal of 7- and 9-carbon fragments were so weak that a chain branch at carbon 9 could neither be assigned nor ruled out. Capnine is probably synthesized from a fatty acid (discussed below), and such internal branches have not been found in fatty acids; for this reason, capnine is represented in Fig. 1 without an internal branch.

Detection of capnoids by labeling with ^{35}S. Chromatography of crude solvent A extracts from cells grown in the presence of [^{35}S]sulfate revealed the presence of two (and only two) labeled components (Fig. 3e, f, and g). The component that migrated more slowly is capnine. The authenticity of the [^{35}S]capnine standard used in this experiment (Fig. 3a) is established by the facts (i) that it was isolated in high radiochemical yield by a procedure identical to that used to prepare the capnine that was characterized chemically, (ii) that it was chemically and radiochemically homogeneous on thin-layer chromatography, and (iii) that the radioactive zone on the thin-layer plates coincided precisely with the zone detected with ninhydrin. The other lipids in crude extracts affect the chromatographic behavior of the capnoids; thus, the capnine standard, when chromatographed in pure form, did not behave in exactly the same way as the slower labeled component from crude extracts (Fig. 3). However, when this standard was mixed with a nonradioactive crude extract from *Capnocytophaga* sp. cells (Fig. 3b), it migrated the same distance as, and gave a zone of shape similar to, the slower, labeled component from the crude extracts (Fig. 3).

Occurrence and properties of *N*-acylcapnine. The faster, labeled component from crude extracts (Fig. 3) is apparently an N-acylated form of capnine. This component, in labeled form, has been partially purified in high-radiochemical yield, as described in Materials and Methods. The purified material was free from labeled capnine (Fig. 4a). Thin-layer chromatography followed by spraying with rhodamine revealed not only the *N*-acylcapnine (which, in contrast to capnine, is detected with great sensitivity by that method), but also a few nonradioactive impurities. *N*-acylcapnine gave only a faint spot when exposed to I_2 vapor.

Acid hydrolysis of the labeled *N*-acylcapnine yielded the ^{35}S in a form that exhibited the chromatographic behavior of capnine (Fig. 4b).

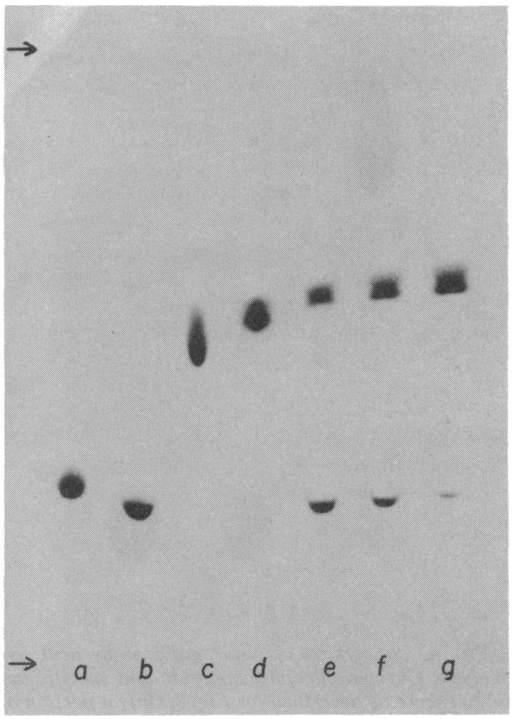

FIG. 3. *Identification of capnoids by thin-layer chromatography. The figure shows an autoradiogram of the developed thin-layer plate (system I). The same amounts of radioactivity were applied in each lane. (a) Purified [^{35}S]capnine (approximately 1 Ci/mol); (b) the [^{35}S]capnine plus a nonradioactive crude solvent A extract from Capnocytophaga sp. cells; (c) partially purified N-acyl[^{35}S]capnine (approximately 1 Ci/mol); (d) N-acyl[^{35}S]capnine plus the nonradioactive crude extract; (e, f, and g) crude solvent A extracts of washed cells that were grown in the presence of [^{35}S]sulfate (approximately 2 Ci/mol); (e) C. gingivalis 30N51; (f) C. ochracea 61-92; (g) unclassified Capnocytophaga species, strain 62. The arrows indicate the origin (lower) and solvent front (upper).*

The ^{35}S-labeled *N*-acylcapnine zone did not give a ninhydrin reaction, whereas the ^{35}S-labeled hydrolysis product gave a ninhydrin reaction that coincided with the radioactive zone. The results are consistent with the structure shown in Fig. 1: *N*-acylcapnine should not give a ninhydrin reaction, but should yield capnine on acid hydrolysis. Similarly, *N*-acylcapnine did not give a reaction with the periodate-Schiff spray reagents, whereas its sulfur-containing hydrolysis product did; again, this behavior is consistent with the proposed structure. Further study will be necessary to determine whether the hydrolysis product is, in fact, identical to capnine.

Capnoid content of *Capnocytophaga* spp. The radiochemical method has been used to

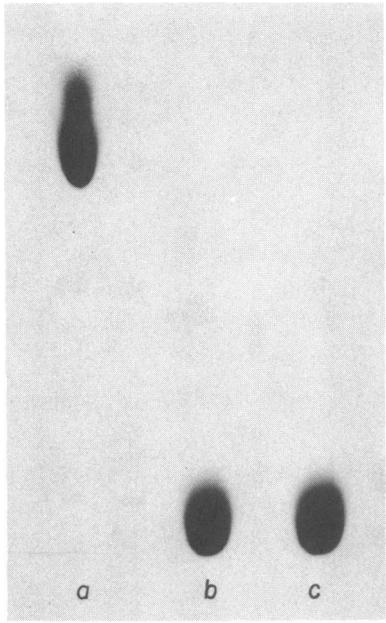

FIG. 4. *Conversion of N-acylcapnine by acid hydrolysis. Chromatographic methods and specific activities were as described for Fig. 3. Only a portion of the plate is shown; the remainder contained no detectable radioactivity. (a) Partially purified N-acyl[^{35}S]capnine; (b) N-acyl[^{35}S]capnine after acid hydrolysis; (c) [^{35}S]capnine isolated as such from cells.*

estimate cellular content of capnoids because (in contrast to bulk-isolation procedures) it engenders little loss of capnoids (and is therefore accurate) and because it permits the use of small cultures that are conveniently handled. The approach is to measure the amount of radioactivity (per unit mass of cells) that was incorporated from [^{35}S]sulfate into capnoids during extensive growth and then to isolate a pure sample of capnine from these cells and determine its specific radioactivity; the capnoid content is the ratio of these two quantities. Absolute capnoid contents of *Capnocytophaga* spp. cannot be estimated solely from cellular contents of capnoid radioactivity and the specific radioactivity of sulfate in the growth medium, for organosulfur compounds are present in the medium as well. This latter approach could be employed if the organisms were grown on media in which all the sulfur sources were labeled to a uniform specific activity; since *Capnocytophaga* spp. require complex media for growth, the method would be both difficult and expensive.

Radioactivity extracted by solvent A from cells that were grown in the presence of [^{35}S]-sulfate and then washed with trichloroacetic acid and water (to remove inorganic sulfate), as described in Materials and Methods, was taken as a measure of radioactivity in cellular capnoids. As shown in Fig. 3, all of the extracted radioactivity was found in capnoids. For the measurement to be accurate, it is also necessary that the extract contain essentially all of the cellular capnoids. Control experiments have shown that the procedure used to wash the cells is conservative of capnoids. Replicate samples of labeled, washed, and unwashed cells were extracted with solvent A. Measured samples of the extracts were chromatographed (inorganic sulfate remains at the origin), and the capnoid zones were located by autoradiography and scraped from the plate. Capnoids were eluted quantitatively from the silica gel with solvent A, and their radioactivity was determined. Washed cells yielded no less than 95% as much radioactivity as did unwashed cells; hence, the washing method (which is far more convenient than quantitative chromatography) resulted in little loss of capnoids. This has been demonstrated both for a strain (30N51) that contains mostly capnine and for one (P7) that contains mostly *N*-acylcapnine. It is also true that solvent A extracts virtually all the capnoids from the cells. When the residual insoluble matter (protein, nucleic acid, etc.) was dissolved by heating in sodium dodecyl sulfate solution and its radioactivity was determined, it was found that 99% of the radioactivity in the washed cells had been extracted by solvent A; this was true for every strain mentioned in this report. (Sulfur from inorganic sulfate is apparently incorporated into capnoids, but not into protein, by these organisms; protein sulfur is presumably derived from the peptides that are abundant in the growth medium.)

Cells of *C. gingivalis* (strain 30N51) were grown in cultures (250 ml, sufficiently large to yield 1 to 2 mg of purified capnine) in the presence of [^{35}S]sulfate (approximately 0.5 Ci/mol). The cells were harvested and weighed, and radioactivity in capnoids was determined as described above. From the solvent A extracts, pure samples of capnine were isolated as described in Materials and Methods (in a scaled-down procedure); that a single ninhydrin-reactive component was present was established by chromatography. Specific activities were determined from counting rates and quantitative ninhydrin assays, with weighed samples of pure, nonradioactive capnine used as standard. The value obtained for capnoid content (radioactivity in capnoids per cell mass, divided by capnine specific activity) was 10.0 ± 0.1 μmol of capnoids per g of wet cell weight (mean ± standard derivation of four independent determinations); this is equivalent to a capnine content of 0.35% of the

wet weight or 1.7% of the dry weight, or an even greater content if one takes into account that some of the capnoids are present as *N*-acylcapnine. The total lipid fraction of *C. gingivalis* 30N51 extracted by a modification of the procedure of Bligh and Dyer (12, section 3.4) amounted to 3.2% of the cell wet weight. The capnoids account for a minimum of 10% of the cellular lipids; if the acyl group of *N*-acylcapnine is a long-chain fatty acid, this value might be as high as 20% for organisms containing mostly the acylated form (depending upon the size of the *N*-acyl group).

It should be noted that this assay method may be applied to organisms containing mostly *N*-acylcapnine, if the extracted lipids are hydrolyzed in acid prior to the isolation of capnine. An incidental observation in the above experiment was that the capnine specific activity averaged 44% of the specific activity of sulfate in the growth medium, suggesting that about one-half of the capnoid sulfur was derived from inorganic sulfate.

To screen *Capnocytophaga* strains for capnoid content, we adopted a simpler procedure (Table 2). Comparability of the data depends upon the assumptions that the organisms all utilized inorganic sulfate and organosulfur compounds to the same relative extents and that the ratio of absorbance to cell mass was constant. Despite these limitations, the data serve to illustrate the fact that variation of capnoid content among different strains was unremarkable. In contrast, there was marked variation in the ratio of capnine to *N*-acylcapnine among different strains. This is evident from the autoradiogram presented in Fig. 3. Actual distributions (determined by counting capnoids eluted from the silica gel) ranged from 85% capnine (strain 30N51) to 96% *N*-acylcapnine (strain 62), with many intermediate cases noted. For this reason, it would seem that capnine, although probably a precursor of *N*-acylcapnine, is not simply a transient intermediate in biosynthesis.

TABLE 2. *Relative capnoid content of various strains of Capnocytophaga spp.*

Species	Strain	Relative capnoid content[a]
C. sputigena	4	72
Uncategorized	T3441	83
C. gingivalis	30N51	100
C. ochracea	21-54	123
Uncategorized	62	131
Uncategorized	P7	154
C. ochracea	61-92	156

[a] Relative capnoid content was calculated as radioactivity in capnoids, divided by the product of absorbance at 650 nm and sulfate specific activity. Values were normalized to that for strain 30N51 as 100. Each value is the mean of two independent determinations that agreed to within 5%. The cells were grown for at least four generations in medium (25 ml) containing [^{35}S]sulfate (approximately 2 Ci/mol). The absorbance (650 nm) of each culture was determined. Samples were taken for determination of radioactivity in capnoids. This was the radioactivity that was extracted from washed cells by solvent A; samples of the extracts taken for determination of radioactivity contained at least 10,000 dpm. For each organism, it was shown by chromatography that the extracted radioactivity was found only in capnoids. Samples of the culture media were counted for determination of sulfate specific activity.

Localization of capnoids in the cell envelope. When intact cells of *C. gingivalis* 30N51 were grown in the presence of [^{35}S]sulfate and centrifuged to buoyant equilibrium on discontinuous sucrose gradients (as described in Materials and Methods and Fig. 5), they banded at the 70%/55% sucrose interface (Fig. 5a). The fraction that accumulated there contained virtually all of the cellular protein as well as all of the trichloroacetic acid-insoluble radioactivity (which is a measure of relative capnoid content, for the reasons discussed above). In contrast, when the cells were sonicated, most of the capnoids, together with a smaller proportion of the cellular protein, were found at the 55%/42% sucrose interface, whereas most of the protein remained in the 70% sucrose solution (Fig. 5b). The fraction found at the 55%/42% sucrose interface represents cell envelopes; this is evident from two observations. The fact that this fraction had a lower buoyant density, and contained a lesser proportion of the total cellular protein, than intact cells indicates that it was enriched in lipid-containing structures. Electron micrographs of this fraction (thin sections of a pellet obtained by centrifugation, stained with uranyl acetate and lead citrate; not shown) revealed that it was composed of a nearly homogeneous collection of vesicles approximately 500 nm in diameter. These vesicles were bounded by the two concentric membranes with an intervening layer of amorphous material (peptidoglycan?) that are characteristic of the envelopes of intact cells; however, the vesicles were devoid of the densely staining internal contents (mostly protein) that are characteristic of intact cells.

The results indicate that a fraction that was enriched in envelopes was also enriched in capnoids; the same result was obtained for a strain (P7) that (unlike strain 30N51) contains a high proportion of *N*-acylcapnine. The specific radioactivity of the envelope fraction (Fig. 5b) is equivalent to a capnine content of 160 μg/mg of envelope protein; for strains with a higher capnoid content and a greater proportion of *N*-acyl-

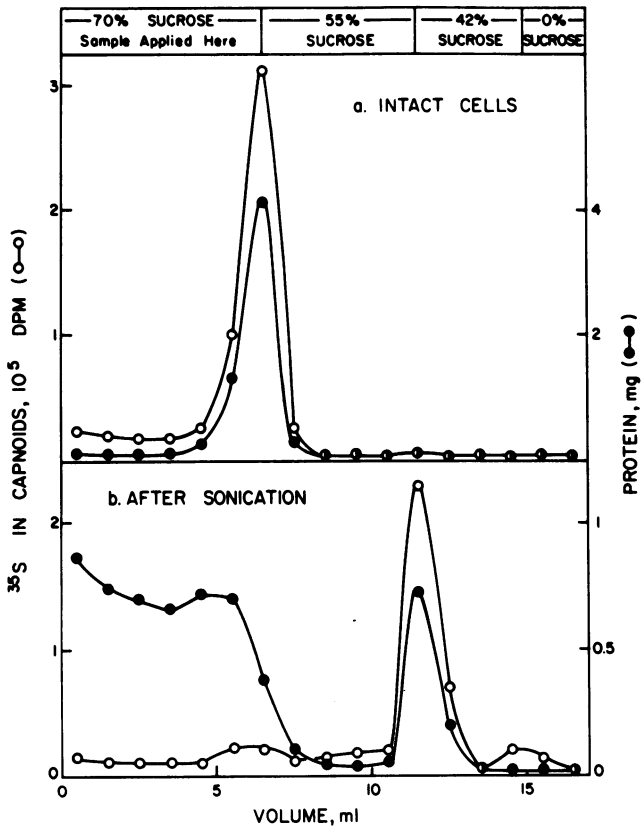

FIG. 5. *Localization of the capnoids in the cell envelope. Isopycnic sucrose gradient centrifugation (a) of C. gingivalis cells that were grown in the presence of [^{35}S]sulfate (0.73 Ci/mol) and (b) of the same material after sonication. Details are given in Materials and Methods.* (●) *Protein;* (○) *radioactivity in capnoids (trichloroacetic acid-insoluble material).*

capnine, this value might range up to 400 μg of capnoids per mg of protein, depending upon the molecular weight of *N*-acylcapnine.

DISCUSSION

The sulfonolipids we describe here are hitherto unrecognized components of procaryotic cells where, at least in *Capnocytophaga* spp., they are quantitatively significant components of the cell envelope. The most unusual feature of the capnoids is the sulfonate group. Most of the naturally occurring sulfonates are related structurally and metabolically to cysteic acid (alanine-3-sulfonic acid). The capnoids probably fall into this class as well, but are unusual in that they are lipids both in the structural sense and by virtue of their location in the cell envelope.

Only one lipid closely related to the capnoids is known to us, and that is the 1-deoxyceramide-1-sulfonic acid (Fig. 1) found in the diatom *N. alba* (1, 3). The 1-deoxysphingenine-1-sulfonic acid moiety of this lipid has in common with capnine the sulfonic acid, amino, and hydroxyl groups found on carbons 1, 2, and 3, respectively, but differs from capnine in having a 4-5 unsaturated, normal 18-carbon chain, whereas capnine has a saturated, iso 17-carbon chain. The deoxysphingenine sulfonic acid is a minor component of *N. alba*, in which it constitutes 0.13% of the cell dry weight or 0.6% of the cellular lipids (3); in contrast, the capnoids constitute at least 1.7% of the dry weight, and 10% of the lipids, of *Capnocytophaga* spp.

In addition to tbe sulfonolipids that contain a taurine moiety, we know of only one other lipid containing a sulfonic acid group, the sulfonquinovosyl diglyceride of plants (4), certain pseudomonads (20), and *Bacillus acidocaldarius* (14). This is a glycolipid bearing the sulfonic acid group on one of the sugar carbons and is unlikely to be related metabolically to the capnoids. A number of lipids that contain esters of sulfuric acid are known (reviewed in 8), though these are not common in bacteria.

Despite the presence of the sulfonate group and the structure of their side chains, capnine and *N*-acylcapnine are undoubtedly analogs of sphingosine and the ceramides, respectively. The initial step in sphingosine biosynthesis is the condensation of a palmitoyl group (activated as a thioester of coenzyme A) with serine, with concomitant loss of the serine carboxyl group, to yield 1-hydroxy-2-amino-3-ketooctadecane (6). It seems possible that capnine might be synthesized by a similar reaction. There are three possibilities for the origin of the taurine moiety; the fatty acyl group might condense with cysteic acid, or it might condense with cysteine (with subsequent oxidation of the sulfur to the sulfonate level), or the initial condensation might involve serine, with the latter addition of the sulfonate group, from phosphoadenosyl phosphosulfate, following dehydration of the condensation product at the 1-2 positions. These last two reactions are analogous to the formation of cysteic acid from phosphoadenosyl phosphosulfate and α-aminoacrylic acid (18); the α-aminoacrylic acid is formed by dehydration of serine. One of these reactions in which a sulfonate is formed from an eneamine and phosphoadenosyl phosphosulfate may well account for the incorporation of radioactivity from inorganic [^{35}S]sulfate into capnoids. It has been shown (2) that exogenous cysteine can supply the sulfur-containing moiety of the deoxyceramide sulfonic acid of the diatom *N. alba*. The observation that *Capnocytophaga* spp. incorporated radioactivity from [^{35}S]sulfate into capnoids, but not into protein, indicates that cysteine is not an obligatory intermediate in capnine biosynthesis; however, the observation (albeit indirect) that some of the capnoid sulfur was derived from exogenous organosulfur sources indicates that cysteine may be able to serve as a precursor. The simplest explanation would be that cysteic acid is the condensing moiety and that it may be derived either from oxidation of the cysteine supplied in the growth medium or from the condensation of α-aminoacrylic acid and phosphoadenosyl phosphosulfate. Multiple-pathway models for the condensation reaction cannot, at present, be ruled out.

In any of these modes of capnine biosynthesis, the first sulfonic acid product would presumably be 2-amino-3-keto-isoheptadecane-1-sulfonic acid; the hydroxyl group of capnine could be introduced by reduction of the keto group. It is interesting to note that, if capnine were formed as described, the fatty acyl group involved would be the isopentadecanoyl group. Saturated, 15-carbon, branched-chain fatty acids constitute more than half of the cellular fatty acids of *Capnocytophaga* spp. (10), though whether the branch structure is iso (13-methyl) or anteiso (12-methyl) is not yet clear. In a number of other gliding bacteria, the predominant structure is iso (7).

Interest in the capnoids stems not only from the fact that they are unusual biological compounds, but from two other sources as well. Sulfonates are frequently toxic to animal cells, and it is conceivable that the capnoids might play a role in the inflammatory process in periodontitis. The capnoids, which are major components of the cell envelope, might also confer some unusual properties upon the cell surface. For example, a high content of *N*-acylcapnine might result in a membrane with a high intrinsic surface negative charge. *N*-acylcapnine might also contribute to membrane fluidity, both via the iso structure of the capnine side chain and via mutual repulsion of the sulfonate groups. It would be of great interest if the capnoids were involved in some aspect of the gliding motility of *Capnocytophaga* spp. and other gliding bacteria in which we have detected their presence (unpublished data).

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