INFECTION AND IMMUNITY, Sept. 1994, p. 3753–3760
0019-9567/94/$04.00+0

Vol. 62, No. 9

Distribution of 3-Hydroxy $iC_{17:0}$ in Subgingival Plaque and Gingival Tissue Samples: Relationship to Adult Periodontitis

FRANK C. NICHOLS*

Department of Periodontology, University of Connecticut School of Dental Medicine, Farmington, Connecticut 06030

Received 17 February 1994/Returned for modification 25 March 1994/Accepted 6 June 1994

Gram-negative organisms incorporate hydroxy fatty acids into the lipid A moiety of lipopolysaccharide (LPS), and in the case of some members of the family *Enterobacteriaceae*, hydroxy fatty acids are incorporated exclusively into lipid A. However, a limited number of *Bacteroides* species have been shown to incorporate several classes of 3-hydroxy fatty acids, particularly 3-hydroxy $iC_{17:0}$, into constitutive lipids as well as LPS. The present study examined the distribution of hydroxy fatty acids in two periodontal pathogens, *Prevotella intermedia* and *Porphyromonas gingivalis*, by employing a phospholipid extraction procedure (E. G. Bligh and W. J. Dyer, Can. J. Biochem. Physiol. 37:911–917, 1959) which partitioned constitutive lipids into the organic solvent phase and LPS into the aqueous phase. The distribution of hydroxy fatty acids within organic solvent and aqueous extracts of these bacterial species was then compared with the distribution in subgingival plaque samples isolated from either gingivitis or severe periodontitis sites as well as the distribution in gingival tissue samples. The organic solvent and aqueous extracts were hydrolyzed under strong alkaline conditions, and the free fatty acids were treated to form pentafluorobenzyl-ester, trimethylsilyl-ether derivatives. Hydroxy fatty acid levels were quantified by using gas chromatography–negative-ion chemical ionization–mass spectrometry. By using this approach, the mean values of the 3-hydroxy $iC_{17:0}$ recovered within organic solvent extracts of *P. gingivalis* strains ranged from 56 to 63% of total 3-hydroxy $iC_{17:0}$. Substantially less 3-hydroxy $iC_{17:0}$ (<5%) was recovered in organic solvent extracts of *P. intermedia*. By comparison, 75% of the 3-hydroxy $iC_{17:0}$ in periodontitis subgingival plaque samples was recovered in organic solvent extracts, while only 43% of the 3-hydroxy $iC_{17:0}$ in gingivitis plaque samples from the same patients was recovered in organic solvent extracts. However, 3-hydroxy $iC_{17:0}$ was recovered essentially only in organic solvent extracts of both healthy or mildly inflamed and periodontitis gingival tissue samples. The preferential recovery of 3-hydroxy $iC_{17:0}$ in tissue lipids indicates that gingival tissues do not harbor significant levels of subgingival plaque organisms which contain 3-hydroxy $iC_{17:0}$. Furthermore, these results indicate that LPS from these organisms is not prevalent in gingival tissues. Finally, these results indicate either selective penetration of certain bacterial lipids into gingival tissues or that 3-hydroxy $iC_{17:0}$ is metabolically transferred from bacterial lipids into gingival tissue lipids.

Long-chain 3-hydroxy fatty acids are covalently linked to the lipid A component of lipopolysaccharide (LPS), a cell wall constituent of virtually all gram-negative bacteria. Nonoral members of the family *Enterobacteriaceae* generally contain 3-hydroxy myristic acid ($C_{14:0}$) as the predominant ester- and amide-linked hydroxy fatty acid within LPS (19) and essentially exclude 3-hydroxy $C_{14:0}$ from complex lipids (16). Except for *Bacteroides* species (11, 12), LPS isolates from common periodontal pathogens typically contain 3-hydroxy myristic acid as the predominant hydroxy fatty acid (2, 10). However, *Bacteroides* species contain substantial amounts of 3-hydroxy $iC_{17:0}$ [iso-branched D-(−)-3-hydroxy-15-methylhexadecanoate] (11), which is covalently linked to both LPS (8, 9, 14, 28) and complex lipids, particularly phospholipids (12). According to Mayberry (12), up to 50% of the 3-hydroxy $iC_{17:0}$ in selected strains of *Bacteroides fragilis* and *Bacteroides asaccharolyticus* is covalently linked within cellular lipids with all lipid-associated hydroxy fatty acid held in amide linkages. Ester-linked 3-hydroxy $iC_{17:0}$ was not demonstrated within lipid extracts in these *Bacteroides* species (12) nor was 3-hydroxy $C_{14:0}$ recovered in the lipid extracts. Little is known concerning the distribution of hydroxy fatty acids in lipids and LPS for those periodontal organisms which contain 3-hydroxy $iC_{17:0}$.

The application of phospholipid extraction procedures to bacterial samples would be expected to separate bacterial lipids, recovered in the organic solvent phase, from LPS, which remains in the aqueous phase. Assuming that bacterial hydroxy fatty acids are not also synthesized in human tissues, the recovery of covalently linked hydroxy fatty acids in aqueous and organic extracts of biological samples should provide an indirect assessment of LPS- and lipid-associated hydroxy fatty acid. Of the common bacterial hydroxy fatty acids, most can be synthesized in mammalian tissues during either beta or alpha oxidation processes, albeit in different epimer forms. However, no reports which demonstrate 3-hydroxy $iC_{17:0}$ synthesis in mammalian tissues exist. Therefore, the recovery of 3-hydroxy $iC_{17:0}$ in organic solvent and aqueous extracts of biological samples should reflect the recovery of bacterial lipids or LPS, respectively, from those organisms containing 3-hydroxy $iC_{17:0}$. The first goal of this study was to confirm the organic and aqueous solvent partitioning characteristics of LPS isolates from *Porphyromonas gingivalis* and *Prevotella intermedia*. Following this, the organic-aqueous distribution of hydroxy fatty

* Corresponding author. Mailing address: Department of Periodontology, University of Connecticut School of Dental Medicine, 263 Farmington Ave., Farmington, CT 06030. Phone: (203) 679-2363.

acids was evaluated in bacterial samples, subgingival plaque samples isolated from gingivitis and periodontitis sites, and gingival tissue samples excised from healthy or mildly inflamed sites or periodontitis sites. Comparison of the hydroxy fatty acid distribution between plaque and tissue samples indicates that 3-hydroxy $iC_{17:0}$ is selectively incorporated into gingival tissue lipids and is significantly elevated in gingival tissues from adult periodontitis sites.

MATERIALS AND METHODS

Lyophilized bacterial samples of *P. intermedia* (ATCC 33563) and *P. gingivalis* (ATCC 33277) (generously provided by Paulette Tempro) were grown in pure culture as described previously (15) and then centrifuged. The bacterial pellets were pooled and washed twice with phosphate-buffered saline prior to lyophilization. Frozen samples of oral strains of *P. gingivalis* obtained from gingivitis and periodontitis sites (generously provided by W. E. C. Moore) were also analyzed. Bacterial LPS isolates from *P. intermedia* and *P. gingivalis* were prepared as described previously (15). Bacterial samples and bacterial LPS isolates were analyzed for hydroxy fatty acid content as described below.

Subgingival plaque samples were collected from patients selected from the postgraduate or undergraduate periodontic clinic at the University of Connecticut School of Dental Medicine. Patients were selected if they were between 30 and 60 years of age and presented with generalized gingivitis and at least two sites demonstrating severe adult periodontitis (sulcus depths of ≥7 mm with attachment loss of ≥50%). Each patient received a complete periodontal examination at least 1 week prior to plaque sampling, and the selected sites were not probed immediately prior to plaque sampling. For each plaque sample, the site characteristics (probing depth, loss of attachment, and diagnosis) were recorded. Gingivitis sites were randomly selected from teeth which demonstrated gingival inflammation (gingival index of ≥1) but no loss of attachment. After informed consent was obtained, the site was isolated and supragingival plaque was removed with a curette. The site was then thoroughly irrigated with water and dried. After the site was reisolated with cotton rolls, subgingival plaque samples were obtained by inserting a coarse paper point to the base of the diseased sulcus and allowing 10 s to elapse before removing the point. The samples were maintained at −20°C until processing.

Gingival tissue samples, normally discarded during routine periodontal surgical procedures, were retained for lipid analyses. Minimally inflamed or healthy gingival tissue samples were obtained during crown-lengthening procedures from sites with no attachment loss. Periodontitis gingival tissue samples were obtained from sites with sulcus depths of ≥5 mm and attachment loss of ≥30%. All sites had received scaling and root planing at least 1 month prior to surgery, and tissue samples were not obtained from third molar or other extraction sites. The samples included interproximal gingival tissues as well as the collar of gingival tissue which immediately surrounded the tooth. Surgical procedures utilized only local anethesia, and gingival tissue samples were excised by sharp dissection. Tissue samples were stored at −20°C in sealed tubes until homogenization and lipid extraction (see below). Each tissue sample was thawed, weighed, and homogenized in extraction solvent as described below.

Lipid extraction and separation. Bacterial and plaque samples and gingival tissue homogenates were processed by the phospholipid extraction procedure of Bligh and Dyer (1) as modified by Garbus (4). Briefly, 0.5 ml of H_2O and 2 ml of $CHCl_3$-methanol (1:2, vol/vol) were added to each sample, and the samples were vortexed. After the samples were left standing for 2 h, 0.75 ml of $CHCl_3$ and 0.75 ml of 0.5 M K_2HPO_4–2 M KCl were added to each sample. The samples were vortexed, and the lower $CHCl_3$ phase (lipid phase) was removed and dried under N_2 gas. The aqueous phase, expected to contain LPS, was retained, and the hydroxy fatty acid content was analyzed as described below. Blank paper point samples were extracted in parallel with points containing subgingival plaque samples.

Fractionation of bacterial and plaque lipids by high-performance liquid chromatography (HPLC) was accomplished by using a μPorsil column (0.5 by 30 cm; silica gel; Waters Associates, Milford, Mass.) eluted with the following gradient: 14 min with 100% solvent A (hexane-isopropanol-water, 6:8:0.75, vol/vol/vol), 14 to 34 min with a linear gradient of 100% solvent A to 100% solvent B (hexane-isopropanol-water, 6:8:1.4, vol/vol/vol), and then 34 to 60 min with 100% solvent B (5, 7). Lipid samples were dissolved in solvent A (150 μl) and eluted at 0.4 ml/min with 2-min fractions. The fractions were read at 206 nm and dried under N_2. A defined quantity of each lipid fraction was then analyzed for either free or bound hydroxy fatty acid.

Lipid analysis by gas chromatography-mass spectrometry. All derivatizing agents were obtained from Pierce Chemical Corp. (Rockford, Ill.). Nonadecanoic acid ($C_{19:0}$, 30 ng, internal standard; Matreya, Inc., Pleasant Gap, Pa.) was added to each sample of bacterial extract, LPS isolate, or lipid fraction. When alkaline hydrolysis was employed, the samples were treated with 4 N KOH (0.5 ml, 100°C, 90 min), and the hydrolysate was acidified with concentrated HCl. The acidified samples were then extracted with $CHCl_3$ (1 ml, three times) and dried under a stream of N_2 gas. When acid hydrolysis was employed, bacterial or LPS samples were treated with 2 N HCl (1 ml, 100°C, 12 h), and the free fatty acids were extracted with $CHCl_3$ (1 ml, three times) and dried under a stream of N_2 gas. When intrinsic free fatty acid levels were determined, no hydrolysis step was employed.

The dried fatty acid samples were dissolved in acetonitrile (30 μl) and treated with 35% pentafluorobenzyl bromide in acetonitrile (10 μl) and diisopropylethylamine (10 μl). The solution was heated for 20 min at 40°C and evaporated to dryness under N_2 gas. The resultant pentafluorobenzyl esters were treated with *N-O*-bis(trimethylsilyl)-trifluoroacetamide (50 μl) and incubated overnight. The lipid derivatives were applied to a Hewlett-Packard (Avondale, Pa.) model 5890 gas chromatograph interfaced with a model 5988A mass spectrometer. Samples were applied to an HP-1 (Ultra-1, 12 m by 0.2 mm; Hewlett-Packard) column held at 100°C. Samples were injected by using the splitless mode and a temperature program of 2°C per min to 240°C. The mass spectrometer was used in the negative ion-chemical ionization mode with an ion source temperature of 100°C, an electron energy of 240 eV, and an emission current of 300 mA. Methane was used as the reagent gas and was maintained at 0.75 torr (ca. 100 Pa). Known amounts of 2-hydroxy $C_{14:0}$, $C_{16:0}$, and $C_{18:0}$ and 3-hydroxy $C_{14:0}$, $C_{16:0}$, $C_{17:0}$, and $C_{18:0}$ (Matreya) were analyzed for selected ion retention times and signal responses relative to the internal standard ($C_{19:0}$). Quantification of hydroxy fatty acids from bacterial samples was accomplished by using selected ion monitoring of the appropriate base peak ions, and extraction losses were corrected by using the $C_{19:0}$ internal standard. The retention time of the unbranched 3-OH $C_{17:0}$ standard was approximately 1.3 min longer than that of the predominant form of 3-hydroxy $C_{17:0}$ recovered from *P.*

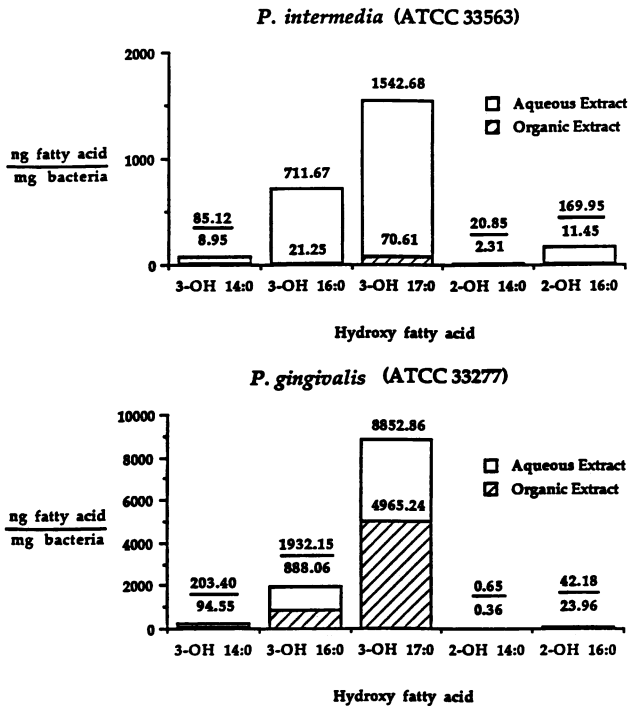

FIG. 1. Distribution of hydroxy fatty acids in organic solvent and aqueous extracts of *P. intermedia* (ATCC 33563) and *P. gingivalis* (ATCC 33277). Lyophilized bacterial samples were processed as described in Materials and Methods. Each stacked histogram bar shows the recovery of each hydroxy fatty acid in the respective organic solvent and the aqueous extracts. Each histogram bar is labeled to indicate the total hydroxy fatty acid recovered (top number) and the organic-extracted hydroxy fatty acid (bottom number).

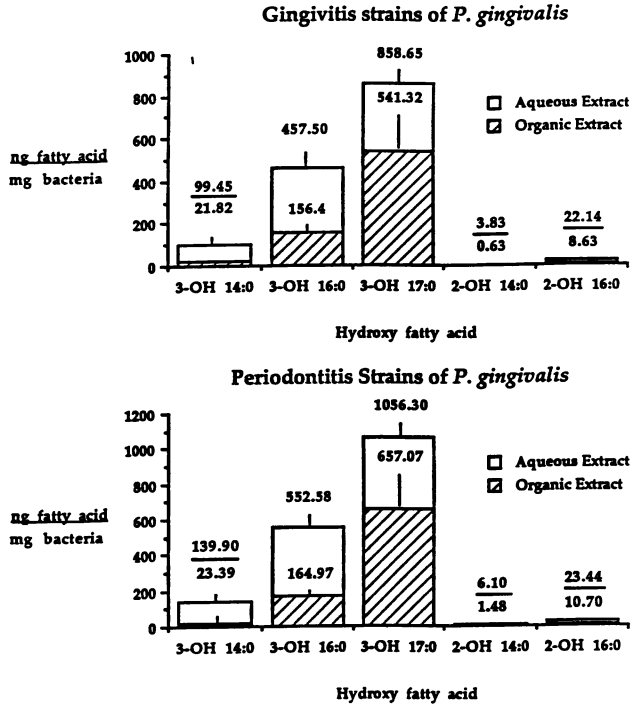

FIG. 2. Distribution of hydroxy fatty acids in organic solvent and aqueous extracts of *P. gingivalis* strains isolated from gingivitis and periodontitis sites in human subjects. Frozen bacterial samples were processed as described in Materials and Methods. Each stacked histogram bar shows recovery of each hydroxy fatty acid in the respective organic solvent and the aqueous extracts. Each histogram bar is labeled to indicate the total hydroxy fatty acid recovered (top number) and the organic-extracted hydroxy fatty acid (bottom number). The results are shown for three gingivitis and three periodontitis strains of *P. gingivalis*.

intermedia and *P. gingivalis*, previously described as an iso-branched form (11, 12).

Anthrone procedure. The carbohydrate content of LPS samples was determined by using the sulfuric acid-anthrone method of Roe (22).

Data analysis. Statistical testing included the Student *t* test, and numeric data ranges as well as error bars in figures are depicted as the standard errors of the means. Data were analyzed by site rather than subject for individual plaque and gingival tissue samples.

RESULTS

LPS isolates from *P. intermedia* and *P. gingivalis* prepared by the method of Westphal and Jann (26) were further extracted by using the method of Bligh and Dyer (1). The recovery of anthrone-reactive carbohydrate was determined in the organic extract of each LPS isolate and compared with that of the unextracted LPS samples. The organic solvent extract of *P. gingivalis* LPS contained 3% of the total anthrone-reactive carbohydrate, whereas less than 1% of the carbohydrate in *P. intermedia* LPS was recovered in the organic extract. Therefore, *P. intermedia* and *P. gingivalis* LPS isolates demonstrate limited organic solvent solubility, which is in agreement with solubility characteristics of LPS isolates extracted from *Enterobacteriaceae* (17).

Figures 1 and 2 show the relative distribution of hydroxy fatty acids between organic solvent and aqueous extracts of *P. intermedia* and *P. gingivalis* (based on the dry mass of bacteria). Figure 1 shows the hydroxy fatty acid distribution in ATCC strains of *P. intermedia* and *P. gingivalis* (samples generously provided by Paulette Tempro), and Fig. 2 shows the distribution in strains of *P. gingivalis* isolated from gingivitis and periodontitis sites in human subjects (samples generously provided by W. E. C. Moore). The higher content of hydroxy fatty acid on a mass basis for the ATCC strains is likely related to lyophilization of these samples, whereas the strains provided by W. E. C. Moore were frozen without lyophilization and likely hold considerable cell-associated water. A substantial fraction (greater than 50%) of 3-hydroxy $iC_{17:0}$ in *P. gingivalis* bacterial samples was recovered in constitutive lipids; slightly lower proportions of 3-hydroxy $C_{16:0}$ and 3-hydroxy $C_{14:0}$ were recovered in cellular lipids. In contrast, only a small fraction of the 3-hydroxy fatty acid was held within cellular lipid (less than 5%) in *P. intermedia* samples. Importantly, the organic-aqueous distribution of 3-hydroxy $iC_{17:0}$ was remarkably similar in ATCC, gingivitis, and periodontitis strains of *P. gingivalis*.

Organic solvent extracts from *P. gingivalis* (ATCC 33277) were fractionated by HPLC, as shown in Fig. 3. The distribution of 3-hydroxy $C_{16:0}$ and 3-hydroxy $iC_{17:0}$ varied considerably between bacterial lipid fractions. A similar distribution of 3-hydroxy fatty acids was observed within lipid extracts from *P. intermedia*, although the total hydroxy fatty acid recovery was considerably lower compared with that of *P. gingivalis* lipids (data not shown). Free hydroxy fatty acids were recovered at

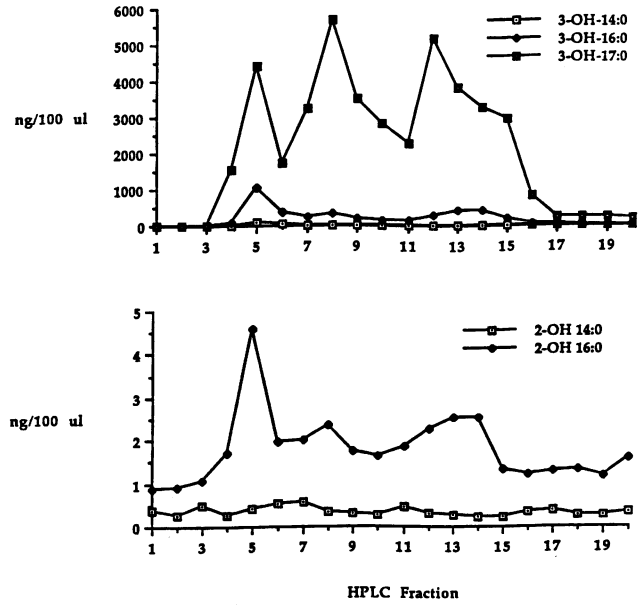

FIG. 3. Hydroxy fatty acid content of *P. gingivalis* constitutive lipids fractionated by HPLC. Lipids were extracted from 200 mg of *P. gingivalis* (lyophilized) by using the method of Bligh and Dyer. The lipid extract was dissolved in 1 ml of solvent A (hexane-isopropanol-water, 6:8:0.75, vol/vol/vol), 150 μl was applied to a μPorsil column (0.5 by 30 cm, silica gel; Waters Associates) and eluted with the following gradient: 14 min with 100% solvent A, 14 to 34 min with a linear gradient of 100% solvent A to 100% solvent B (hexane-isopropanol-water, 6:8:1.4, vol/vol/vol), and then 34 to 60 min with 100% solvent B. Lipids were eluted at 0.4 ml/min in 2-min fractions, and hydroxy fatty acid content was determined in 100-μl aliquots of each fraction. HPLC of authentic standards revealed that phosphatidylinositol elutes in fractions 3 to 5, phosphatidylethanolamine elutes in fractions 9 to 12, and phosphatidylcholine elutes in fractions 21 to 23. However, 3-hydroxy $iC_{17:0}$ was not recovered in fractions 20 to 30 of separated lipid extracts from *P. gingivalis*.

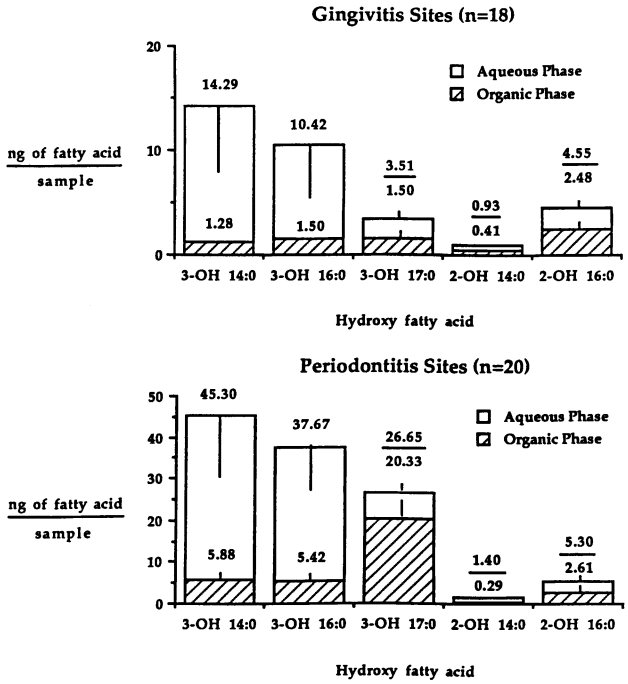

FIG. 4. Distribution of hydroxy fatty acids in subgingival plaque samples isolated from either gingivitis sites or severe adult periodontitis sites in human subjects. Two gingivitis and at least two periodontitis sites were sampled from nine patients (see Materials and Methods for disease site characteristics), and the samples were processed as described in Materials and Methods. Stacked histogram bars show the mean recovery of each hydroxy fatty acid in the respective organic solvent and aqueous extracts from either gingivitis or periodontitis sites. Each histogram bar is labeled to indicate the total hydroxy fatty acid recovered (top number) and the organic solvent-extracted hydroxy fatty acid (bottom number). Vertical lines indicate the standard errors of the means for all sites. Statistical testing evaluated differences by site (gingivitis versus periodontitis) rather than by subject. The mass ratio of 3-hydroxy $iC_{17:0}$ in organic solvent extracts relative to that in aqueous extracts was significantly different between gingivitis and periodontitis sites ($P < 0.05$, Student's t test).

negligible levels (≤0.1%) relative to the covalently linked fatty acids (data not shown).

The organic-aqueous distribution of hydroxy fatty acids was evaluated in subgingival plaque samples isolated from gingivitis and periodontitis sites. For each patient, subgingival plaque samples were collected from two gingivitis and at least two periodontitis sites, and the relative distribution of 3-hydroxy fatty acids in organic and aqueous extracts was determined for each plaque sample. Lipid and aqueous extracts were subjected to alkaline hydrolysis only. The summarized results for nine subjects are shown in Fig. 4. The mass ratio of 3-hydroxy $iC_{17:0}$ in the organic solvent phase relative to the aqueous phase was significantly different ($P < 0.05$, Student's t test) in the periodontitis plaque samples compared with the gingivitis plaque samples. The distribution of all other hydroxy fatty acids was not significantly altered between gingivitis and periodontitis sites. Additional experiments examined the distribution of hydroxy fatty acids in plaque samples taken from patients exhibiting only gingivitis (data not shown). Analysis of these samples revealed detectable levels of 3-hydroxy $iC_{17:0}$ levels only in the aqueous extracts.

Figure 5 shows the distribution of hydroxy fatty acids in HPLC-separated lipids extracted from pooled subgingival plaque samples isolated from severely involved periodontitis sites in four patients. For each patient, the pooled plaque samples were extracted and the isolated lipids were separated by using the same chromatographic system described in the legend to Fig. 3. The HPLC separation in Fig. 5 represents the average of the four HPLCs and shows the elevated recovery of 3-hydroxy $iC_{17:0}$ in specific fractions. Negligible levels of free 3-hydroxy $iC_{17:0}$ were recovered in the same fractions (data not shown). However, preparation of derivatives without prior alkaline hydrolysis demonstrated measurable recovery of free 3-hydroxy $C_{16:0}$ within fractions 4 to 6 but at very low levels relative to the covalently linked fatty acid. This indicates that free hydroxy fatty acids are not prevalent within lipid extracts of plaque from periodontitis sites.

Figure 6 shows the organic-aqueous distribution of hydroxy fatty acids in gingival tissue samples taken from healthy or mildly inflamed (Fig. 6a and c) and adult periodontitis (Fig. 6b and d) sites. In contrast to the subgingival plaque samples (Fig. 4), 3-hydroxy $iC_{17:0}$ was recovered primarily in the organic solvent extracts of both healthy or mildly inflamed and periodontitis tissue samples; there was negligible recovery in the aqueous extracts. In addition, the recovery of 3-hydroxy $iC_{17:0}$ was significantly elevated in the organic solvent extracts of periodontitis tissue samples compared with that of gingivitis samples ($P < 0.02$, Student's t test). All other hydroxy fatty

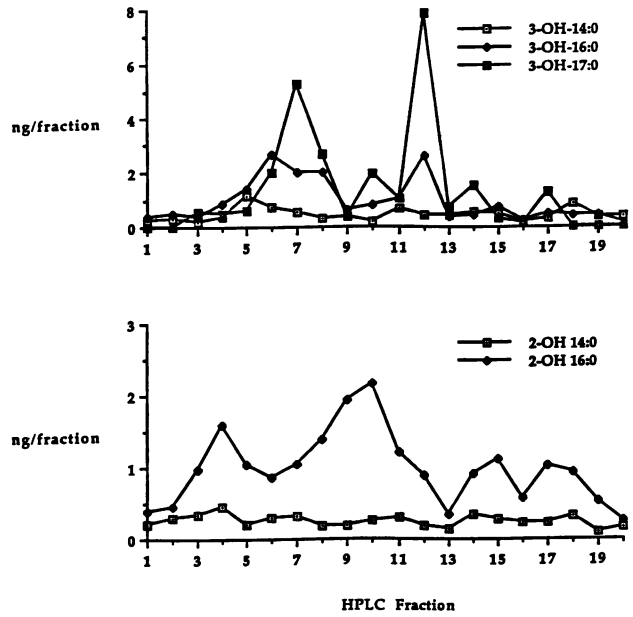

FIG. 5. Hydroxy fatty acid content of subgingival plaque lipids fractionated by HPLC. For each subject (n = 4), subgingival plaque samples from severe periodontitis sites were pooled and extracted by the method of Bligh and Dyer. The lipid extracts were dissolved in 150 μl of solvent A and applied to the column as described in the legend to Fig. 3. Fractions were dried under a stream of N_2 gas, and the hydroxy fatty acid content was determined for each fraction. The HPLC chromatogram represents the average of four chromatograms derived from four different pooled plaque samples. Each pooled plaque sample represents the combined subgingival plaque isolates taken from severe periodontitis sites in one subject.

acids were not significantly elevated in either the organic or aqueous extracts of periodontitis tissue samples. However, 2-hydroxy 16:0 was recovered in very impressive amounts within gingival tissue samples and was slightly elevated in the periodontitis tissue samples (Fig. 6b and d) compared with that of the healthy or mildly inflamed samples (Fig. 6a and c).

Figures 7 and 8 show the distribution of hydroxy fatty acids in HPLC-separated lipids extracted from either healthy or mildly inflamed tissue samples (Fig. 7) or periodontitis tissue samples (Fig. 8). Each tissue sample was homogenized, and the extracted lipids were separated by using the chromatographic system described in the legend to Fig. 3. The most notable difference between healthy or mildly inflamed samples and periodontitis tissue samples was the elevated recovery of 3-hydroxy $iC_{17:0}$ in periodontitis tissue samples within a limited number of fractions. The recovery of 3-hydroxy $iC_{17:0}$ was not elevated in the corresponding HPLC fractions from healthy or mildly inflamed samples. As with the lipid extracts from plaque samples, negligible levels of free hydroxy fatty acids were recovered in lipid extracts from gingival tissue samples (data not shown). Also notable was the impressive recovery of 2-hydroxy $C_{16:0}$ in gingival tissue samples from periodontitis sites. In contrast to the elevated recovery of 3-hydroxy $iC_{17:0}$ within a limited number of HPLC fractions, the elevated recovery of 2-hydroxy $C_{16:0}$ was observed in many HPLC fractions.

DISCUSSION

Bacteria, plaque, and gingival tissue samples were routinely treated with 4 N KOH (100°C for 90 min) to release covalently linked hydroxy fatty acids. Treatment with 4 N KOH did not cause measurable degradation of authentic hydroxy fatty acid standards (data not shown). Although it is reported that amide-linked fatty acids require strong acidic hydrolysis conditions to release fatty acids (3, 6), other reports indicate that amide-linked hydroxy fatty acids are released under strong alkaline conditions (20, 21, 27), similar to those used in the present study.

Additional experiments evaluated the release of hydroxy fatty acids from bacterial and plaque samples with acid hydrolysis (2 N HCl, 100°C, 12 h). In general, acid hydrolysis yielded equal or slightly less hydroxy fatty acid than alkaline hydrolysis and generated higher amounts of derivative products which caused premature degradation of gas chromatography column separations (data not shown). The reduced recovery of hydroxy fatty acids following acid hydrolysis is consistent with a previous report demonstrating acid-catalyzed condensation reactions resulting in 3-acyloxy fatty acid complexes (6). Formation of 3-acyloxy fatty acid complexes reduces the levels of free hydroxy fatty acids recovered after acid hydrolysis, which is consistent with the lower recovery of hydroxy fatty acids after treatment with acid than after treatment with alkali. Although the acyloxy complexes are cleaved with a subsequent alkaline hydrolysis (27), the residual acid degradation products would still be expected to cause premature degradation of gas chromatography column separations. Therefore, the release of covalently linked hydroxy fatty acids in organic solvent and aqueous extracts utilized only alkaline hydrolysis conditions.

The present investigation demonstrated that 56 to 63% of the 3-hydroxy $iC_{17:0}$ in *P. gingivalis* strains is recovered within complex lipids and considerably lower levels of 3-hydroxy $iC_{17:0}$ are recovered in lipids of *P. intermedia*. Exhaustive extraction of bacterial samples with repeated chloroform washes did not substantially increase the fraction of 3-hydroxy $iC_{17:0}$ recovered in bacterial lipids (data not shown). The recovery of 3-hydroxy $iC_{17:0}$ in *P. gingivalis* strains is consistent with the distribution of hydroxy fatty acids in certain *Bacteroides* species (12). Fractionation of lipids extracted from *P. gingivalis* by HPLC demonstrated that 3-hydroxy $iC_{17:0}$ is recovered predominantly in three major peaks and lesser amounts are recovered in other fractions. HPLC fractionation of lipids extracted from subgingival plaque samples revealed 3-hydroxy $iC_{17:0}$ predominantly in two peaks. However, HPLC fractionation of lipids from periodontitis tissue samples demonstrated 3-hydroxy $iC_{17:0}$ predominantly in one major peak. Although the plaque samples and tissue samples were not isolated from the same patients, the recovery of 3-hydroxy $iC_{17:0}$ in a limited fraction of gingival tissue lipids compared with bacterial or plaque lipids suggests that specific bacterial lipids containing 3-hydroxy $iC_{17:0}$ selectively penetrate gingival tissues in periodontitis sites.

Although the subgingival plaque samples from periodontitis sites contained greater quantities of hydroxy fatty acids than samples from gingivitis sites, the increased numbers of gram-negative organisms within periodontitis plaque samples does not explain the recovery of 3-hydroxy $iC_{17:0}$ primarily in lipid extracts in periodontitis sites. The distribution of 3-hydroxy $iC_{17:0}$ between organic solvent and aqueous extracts of gingivitis or periodontitis plaque samples could be related to differences in the particular species and/or strains of subgingival organisms which colonize either gingivitis or periodontitis sites. Typically, *P. gingivalis* is recovered in higher amounts within subgingival plaque from adult periodontitis sites (13), which is consistent with the observed elevated recovery of 3-hydroxy $iC_{17:0}$ in organic solvent extracts of periodontitis plaque samples. However, the percentage of 3-hydroxy $iC_{17:0}$

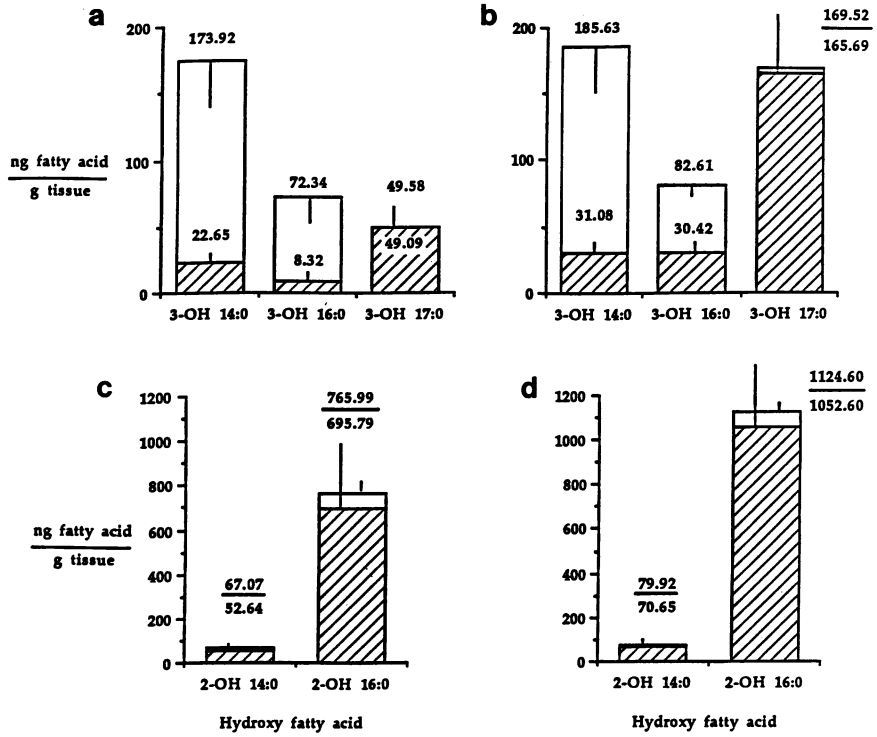

FIG. 6. Distribution of hydroxy fatty acids in gingival tissue samples taken from either healthy or mildly inflamed sites (n = 18) (a and c) or adult periodontitis sites (n = 19) (b and d). Tissue samples were excised during routine periodontal surgical procedures and frozen, and the samples were processed as described in Materials and Methods. Stacked histogram bars show the mean recovery of each hydroxy fatty acid in the respective organic solvent (cross-hatched area) and aqueous extracts (open area) averaged for all healthy or mildly inflamed (40.9 ± 4.2 mg per sample) and adult periodontitis (45.7 ± 3.9 mg per sample) samples. Each histogram bar is labeled to indicate the total hydroxy fatty acid recovered (top number) and the organic solvent-extracted hydroxy fatty acid recovered (bottom number). Vertical lines indicate the standard errors of the means. Statistical testing evaluated differences by site (gingivitis versus periodontitis) rather than by subject. The recovery of 3-hydroxy $iC_{17:0}$ in organic solvent extracts was significantly different between gingivitis (healthy or mildly inflamed) and periodontitis sites ($P < 0.02$, Student's t test).

recovered in organic solvent extracts from periodontitis plaque samples exceeds the percentage of 3-hydroxy $iC_{17:0}$ recovered in the organic extracts from any of the *P. gingivalis* strains evaluated, including those recovered from periodontitis sites. This evidence indicates that the distribution of 3-hydroxy $iC_{17:0}$ in organic solvent and aqueous extracts of laboratory-grown strains of *P. gingivalis* cannot explain the observed distribution of 3-hydroxy $iC_{17:0}$ in periodontitis plaque samples.

The distribution of 3-hydroxy $iC_{17:0}$ in organic and aqueous extracts of gingival tissue samples indicates that little 3-hydroxy $iC_{17:0}$ is recovered in the aqueous extracts. According to previous reports, the lipid extraction procedure of Bligh and Dyer will extract 94% of the sample phospholipid (1). This extraction efficiency would result in 6% of the 3-hydroxy $iC_{17:0}$ remaining in aqueous extracts of gingival tissue samples. In fact, 3% of the 3-hydroxy $iC_{17:0}$ was recovered in aqueous extracts of periodontitis samples. Given that negligible amounts of 3-hydroxy $iC_{17:0}$ are present in aqueous extracts of gingival tissues, LPS from *P. intermedia*, *P. gingivalis*, and *Bacteroides* species in periodontal pockets either minimally penetrates gingival tissues or is cleared rapidly from gingival tissue after penetration. Although previous reports have demonstrated penetration of gingival tissues by LPS isolates from *Leptotrichia buccalis* (18) and *Escherichia coli* (23), it is not established whether LPS from *P. intermedia* or *P. gingivalis* penetrates gingival tissues. If LPS from *P. intermedia* or *P. gingivalis* is rapidly cleared from gingival tissues, recovery of 3-hydroxy $iC_{17:0}$ would be expected in the systemic circulation. However, additional experiments did not detect 3-hydroxy $iC_{17:0}$ in plasma samples (700 μl per sample) isolated from eight patients with generalized severe adult periodontitis. It is possible that hydroxy fatty acids are released from LPS within gingival tissues and the free hydroxy fatty acids are incorporated locally into tissue lipids. Additional experiments will examine this possibility.

That 3-hydroxy $iC_{17:0}$ was recovered in gingival tissues predominantly within organic solvent extracts of both healthy or mildly inflamed and periodontitis tissue samples indicates a capacity for gingival tissues to accumulate 3-hydroxy $iC_{17:0}$ within lipids. The 2-hydroxy fatty acids were also recovered primarily in the gingival tissue organic solvent extracts, and the levels of 2-hydroxy $C_{16:0}$ were quantitatively impressive. In contrast, 3-hydroxy $C_{14:0}$ and 3-hydroxy $C_{16:0}$ were recovered primarily in aqueous extracts of tissue samples. Furthermore, the levels of 3-hydroxy $iC_{17:0}$ recovered in organic extracts were significantly elevated in periodontitis tissue samples compared with healthy or mildly inflamed tissue samples. Other hydroxy fatty acids were not significantly different between gingivitis and periodontitis tissue samples. More importantly, the recovery of 3-hydroxy $iC_{17:0}$ predominantly within tissue lipids could explain the elevated recovery of 3-hydroxy $iC_{17:0}$ in organic solvent extracts of periodontitis plaque samples; i.e., host cells present in the periodontal sulcus incorporate 3-hydroxy $iC_{17:0}$ preferentially into cellular lipids and shift the distribution of 3-hydroxy $iC_{17:0}$ between organic solvent and aqueous extracts in plaque samples from periodon-

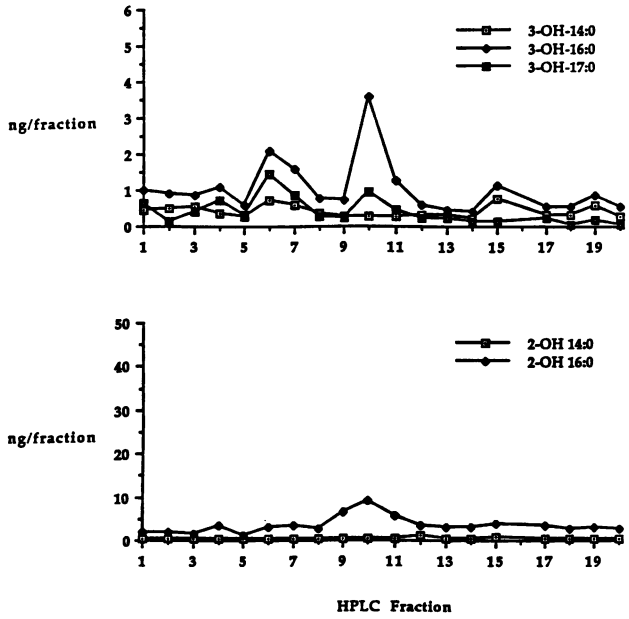

FIG. 7. Hydroxy fatty acid content of gingival tissue lipids fractionated by HPLC. Gingival tissue samples (62.75 ± 9.14 mg; n = 4) excised from healthy or mildly inflamed sites were homogenized and extracted by the method of Bligh and Dyer. The lipid extract from each tissue sample was dissolved in 150 μl of solvent A and fractionated by HPLC by using the method described in the legend to Fig. 3. Fractions were dried under a stream of N_2 gas, and the hydroxy fatty acid content was determined for each fraction. The HPLC chromatogram represents the average of four chromatograms derived from four different gingival tissue samples.

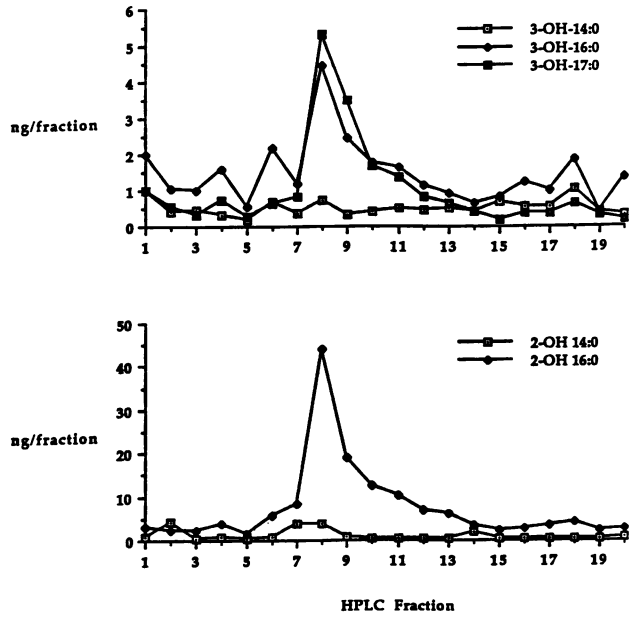

FIG. 8. Hydroxy fatty acids recovered in HPLC-separated lipids extracted from periodontitis gingival tissue samples. Gingival tissue samples (60.85 ± 8.96 mg; n = 4) excised from severe adult periodontitis sites were homogenized and extracted by the method of Bligh and Dyer. The lipid extract from each tissue sample was dissolved in 150 μl of solvent A and fractionated by HPLC by using the method described in the legend to Fig. 3. Fractions were dried under a stream of N_2 gas, and the hydroxy fatty acid content was determined for each fraction. The HPLC represents the average of four chromatograms derived from four different gingival tissue samples.

titis sites. However, additional work is required to clarify this possibility. The recovery of 3-hydroxy $iC_{17:0}$ predominantly in organic solvent extracts of healthy or mildly inflamed and periodontitis tissue samples indicates that 3-hydroxy $iC_{17:0}$ is deposited in gingival tissue lipids independent of the distribution of 3-hydroxy $iC_{17:0}$ in subgingival plaque. If gingival tissues harbored substantial levels of organisms which contain 3-hydroxy $iC_{17:0}$, the distribution of 3-hydroxy $iC_{17:0}$ between organic solvent and aqueous extracts of tissue samples would likely parallel the distribution found in subgingival plaque or laboratory-grown strains of *P. intermedia*, *P. gingivalis*, and *Bacteroides* organisms (12). The results presented here indicate that contamination of tissue by subgingival plaque organisms which contain 3-hydroxy $iC_{17:0}$ is not a prominent feature of either healthy or mildly inflamed or periodontitis tissue samples.

The primary recovery of 3-hydroxy $iC_{17:0}$ in the organic solvent extracts of gingival tissue samples together with the differential recovery of 3-hydroxy $iC_{17:0}$ in HPLC-separated lipids from plaque or gingival tissue samples indicates that gingival tissues allow selective penetration of specific bacterial lipids and perhaps incorporate 3-hydroxy $iC_{17:0}$ into intrinsic tissue lipids. The impressive levels of 2-hydroxy $C_{16:0}$ in gingival tissues (approximately 1 μg of 2-hydroxy 16:0 per g of tissue) is also striking. Altered alpha-oxidation resulting in elevated accumulation of 2-hydroxy phytanic acid in tissues has been described for Refsum's disease (24, 25). The observed accumulation of 2-hydroxy $C_{16:0}$ in gingival tissues occurs in otherwise healthy human subjects and may be elevated in periodontitis tissue samples. Because subgingival plaque samples contained negligible amounts of 2-hydroxy $C_{16:0}$, it appears that the accumulation of 2-hydroxy $C_{16:0}$ results from host metabolic processes within the gingival tissues. Whether the accumulation of 2-hydroxy $C_{16:0}$ in gingival tissues is related to bacterial lipid exposure remains to be established.

In summary, an average of 75% of the 3-hydroxy $iC_{17:0}$ in periodontitis subgingival plaque samples is recovered in organic solvent extracts while the remainder is recovered in aqueous extracts. However, in gingivitis plaque samples from the same subjects, less than 50% of the 3-hydroxy $iC_{17:0}$ is recovered in organic solvent extracts. In contrast, analysis of gingival tissue samples revealed that 3-hydroxy $iC_{17:0}$ is recovered essentially only in organic solvent extracts from healthy or mildly inflamed or periodontitis samples. The selective recovery of 3-hydroxy $iC_{17:0}$ in gingival tissue lipids indicates minimal invasion of tissues by subgingival organisms containing 3-hydroxy $iC_{17:0}$ and further suggests that LPS from these organisms is not prevalent in gingival tissues. That 3-hydroxy $iC_{17:0}$ was recovered in significantly elevated levels within gingival tissue lipids from periodontitis samples compared with that from healthy or mildly inflamed samples may indicate a role for 3-hydroxy $iC_{17:0}$-containing lipids, other than LPS, in the pathogenesis of periodontitis.

ACKNOWLEDGMENTS

I thank Paulette Tempro and W. E. C. Moore for providing the bacterial samples used in this investigation. I also thank the Postgraduate Trainees in Periodontology for their assistance in identifying patients and collecting samples for this study.

This work was supported by Public Health Service grant DE-07208.

REFERENCES

1. **Bligh, E. G., and W. J. Dyer.** 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37**:911–917.
2. **Brondz, I., and I. Olsen.** 1989. Chemical differences in lipopolysaccharides from *Actinobacillus* (*Haemophilus*) *actinomycetemcomitans* and *Haemophilus aphrophilus*: clues to differences in periodontopathogenic potential and taxonomic distinction. Infect. Immun. **57**:3106–3109.
3. **Christie, W. M.** 1982. Lipid analysis: isolation, separation, identification and structural analysis of lipids, p. 51–55. Pergamon Press, Oxford.
4. **Garbus, J., H. F. DeLuca, M. E. Loomans, and F. M. Strong.** 1963. The rapid incorporation of phosphate into mitochondrial lipids. J. Biol. Chem. **238**:59–63.
5. **Geurts Van Kessel, W. S. M., W. M. A. Hax, R. A. Demel, and J. De Gier.** 1977. High performance liquid chromatographic separation and direct ultraviolet detection of phospholipids. Biochim. Biophys. Acta **486**:524–530.
6. **Haeffner, N., R. Chaby, and L. Szabo.** 1977. Identification of 2-methyl-3-hydroxydecanoic and 2-methyl-3-hydroxytetradecanoic acids in the "Lipid X" fraction of the *Bordella pertussis* endotoxin. Eur. J. Biochem. **77**:535–544.
7. **Hax, W. M. A., and W. S. M. Geurts Van Kessel.** 1977. High-performance liquid chromatographic separation and photometric detection of phospholipids. J. Chromatogr. **142**:735–741.
8. **Johne, B., and K. Bryn.** 1986. Chemical composition and biological properties of a lipopolysaccharide from *Bacteroides intermedius*. Acta Pathol. Microbiol. Immunol. Scand. Sect. B **94**:265–271.
9. **Johne, B., I. Olsen, and K. Bryn.** 1988. Fatty acids and sugars in lipopolysaccharides from *Bacteroides intermedius*, *Bacteroides gingivalis*, and *Bacteroides loescheii*. Oral Microbiol. Immunol. **3**:22–27.
10. **Mashimo, J., M. Yoshida, K. Ikeuchi, S. Hata, S. Arata, N. Kasai, K. Okuda, and I. Takazoe.** 1985. Fatty acid composition and Shwartzman activity of lipopolysaccharides from oral bacteria. Microbiol. Immunol. **29**:395–403.
11. **Mayberry, W. R.** 1980. Hydroxy fatty acids in *Bacteroides* species: D-(−)-3-hydroxy-15-methylhexadecanoate and its homologs. J. Bacteriol. **143**:582–587.
12. **Mayberry, W. R.** 1980. Cellular distribution and linkage of D-(−)-3-hydroxy fatty acids in *Bacteroides* species. J. Bacteriol. **144**:200–204.
13. **Moore, W. E. C.** 1987. Microbiology of periodontal disease. J. Periodontal Res. **22**:335–341.
14. **Nair, B. C., W. R. Mayberry, R. Dziak, P. B. Chen, M. J. Levine, and E. Hausmann.** 1983. Biological effects of a purified lipopolysaccharide from *Bacteroides gingivalis*. J. Periodontal Res. **18**:40–49.
15. **Nichols, F. C., J. F. Peluso, P. J. Tempro, S. W. Garrison, and J. B. Payne.** 1991. Prostaglandin E release from monocytes treated with lipopolysaccharides isolated from *Bacteroides intermedius* and *Salmonella typhimurium*: potentiation by gamma interferon. Infect. Immun. **59**:398–406.
16. **Osborn, M. J.** 1979. Biosynthesis and assembly of the lipopolysaccharide of the outer membrane, p. 15–34. *In* M. Inouye (ed.), Bacterial outer membranes: biogenesis and functions. John Wiley & Sons, Inc., New York.
17. **Raetz, C. R. H.** 1987. Biosynthesis and pharmacologic properties of *Escherichia coli* lipid A, p. 229–245. *In* M. Inouye (ed.), Bacterial membranes as model systems. John Wiley & Sons, Inc., New York.
18. **Ranney, R. R., and E. H. Montgomery.** 1973. Vascular leakage resulting from topical application of endotoxin to the gingiva of the beagle dog. Arch. Oral Biol. **18**:963–970.
19. **Rietschel, E. T., C. Galanos, O. Luderitz, and O. Westphal.** 1982. The chemistry and biology of lipopolysaccharides and their lipid A component, p. 183–229. *In* D. Webb (ed.), Immunopharmocology and the regulation of leukocyte function. Marcel Dekker, New York.
20. **Rietschel, E. T., H. Gottert, O. Luderitz, and O. Westphal.** 1972. Nature and linkages of fatty acids present in the lipid-A component of *Salmonella* lipopolysaccharides. Eur. J. Biochem. **28**:166–173.
21. **Rietschel, E. T., O. Luderitz, and W. A. Volk.** 1975. Nature, type of linkage, absolute configuration of (hydroxy) fatty acids in lipopolysaccharides from *Xanthomonas sinensis* and related strains. J. Bacteriol. **122**:1180–1188.
22. **Roe, J. H.** 1955. The determination of sugar in blood and spinal fluid with anthrone reagent. J. Biol. Chem. **212**:335–343.
23. **Schwartz, J., F. Stinson, and R. Parker.** 1972. The passage of tritiated bacterial endotoxin across intact gingival crevicular epithelium. J. Periodontol. **43**:270–276.
24. **Steinberg, D., J. H. Herndon, B. W. Uhlendorf, C. E. Mize, J. Avigan, and G. W. A. Milne.** 1967. Refsum's disease: nature of the enzyme defect. Science **156**:1740–1742.
25. **ten Brink, H. J., D. S. M. Schor, R. M. Kok, B. T. Poll-The, R. J. A. Wanders, and C. Jakobs.** 1992. Phytanic acid α-oxidation: accumulation of 2-hydroxyphytanic acid and absence of 2-oxophytanic acid in plasma from patients with peroxismal disorders. J. Lipid Res. **33**:1449–1457.
26. **Westphal, O., and K. Jann.** 1965. Bacterial lipopolysaccharides: extraction with phenol-water and further applications of the procedure, p. 83–91. *In* R. L. Whistler (ed.), Methods in carbohydrate chemistry. Academic Press, Inc., New York.
27. **Wollenweber, H. W., and E. T. Rietschel.** 1990. Analysis of lipopolysaccharide (lipid A) fatty acids. J. Microbiol. Methods **11**:195–211.
28. **Wollenweber, H. W., E. T. Rietschel, T. Hofstad, A. Weintraub, and A. A. Lindberg.** 1980. Nature, type of linkage, quantity, and absolute configuration of (3-hydroxy) fatty acids in lipopolysaccharides from *Bacteroides fragilis* NCTC 9343 and related strains. Infect. Immun. **144**:898–903.