JOURNAL OF BACTERIOLOGY, Mar., 1967, p. 1079-1088
Vol. 93, No. 3

Indentification and Localization of the Fatty Acids in *Haemophilus parainfluenzae*

DAVID C. WHITE AND RICHARD H. COX

Department of Biochemistry and Department of Chemistry, University of Kentucky Medical Center, Lexington, Kentucky

Received for publication 7 December 1966

Haemophilus parainfluenzae was capable of synthesizing 22 fatty acids. These fatty acids were equivalent to 4% of the bacterial dry weight. These fatty acids were localized in the membrane-wall complex, which contained the respiratory pigments, the quinone, and the phospholipids. The fatty acids which could be extracted with organic solvents comprised 86% of the total fatty acids of the cell. These fatty acids were distributed as 98% in the phospholipids and 1.9% in the neutral lipids, of which 0.5% were free fatty acids. Palmitic, palmitoleic, oleic, and vaccenic acids comprised 72% of the total fatty acids and were found almost exclusively in the phospholipids. The phospholipids also contained the cyclopropane fatty acids. The neutral lipids contained significant proportions of the odd-numbered branched and straight-chain fatty acids. The principal free fatty acids were *n*-dodecanoic and pentadecenoic acids. The nonextractable wall complex contained 14% of the total fatty acids. These wall fatty acids were rendered soluble only after saponification. The wall fraction contained all of the β-hydroxymyristic acid and most of the myristoleic and pentadecenoic acids. The significance of the distribution of fatty acids between nonesterified, neutral lipid, phospholipid, and nonextractible wall remains to be determined.

Haemophilus parainfluenzae has an obligatory requirement of a membrane-bound electron transport system for growth (22). The bacterium has the capacity to extensively modify the composition of the membrane in response to changes in the environment (17). The changes in the electron transport system have been shown to result in a new composition that is more effective in the new environment (18). These modifications can be used to delineate some of the control mechanisms involved in the formation of the membrane complex (20). The function of this electron transport system is dependent on the structural integrity of the membranes. Any disturbance of the lipids by solvents results in the loss of respiratory activity (21). However, under carefully controlled conditions, the quinone can be extracted and respiratory activity can be partially restored on addition of the quinone (21). The identity of these essential lipid components and the relation of changes in the composition of the lipids to changes in the proportions of the respiratory pigments need to be known before study of the role of lipid in the formation of the membrane can be made. The fatty acids of *Haemophilus* have been characterized, and their cellular localization has been determined. Evidence will be presented to show that the fatty acid pattern of *Haemophilus* is complex and that the localization of the fatty acids is apparently not random.

MATERIALS AND METHODS

Growth of bacteria. The strain of *H. parainfluenzae*, harvesting procedures, conditions for growth and preservation of the bacteria, and medium have been described (17).

Dry weight. The dry weight was determined after drying in vacuo to constant weight as described previously (22).

Isolation of the fatty acids. About 25 g (dry weight) of frozen bacterial pellets was suspended in 400 ml of 3 N KOH containing 50% (v/v) ethyl alcohol and was refluxed for 12 hr. The cooled saponification mixture was then extracted with 0.2 volume of petroleum ether, and the organic phase was discarded. The aqueous phase was then acidified to *p*H 2.0 with HCl and extracted with 0.2 volume of petroleum ether. This was repeated with fresh petroleum ether four times. The combined petroleum ether extracts were dried over anhydrous Na_2SO_4 + $NaHCO_3$ (4:1, w/w) and were evaporated to a small volume in vacuo.

For studies of the localization of the fatty acids, 340 mg (dry weight) of bacteria harvested in the stationary phase of growth was saponified for 2 hr at 100 C in tubes sealed with Teflon-lined screw caps.

Extractions with petroleum ether were mixed vigorously for 5 min on the Vortex mixer. The combined petroleum ether extracts from the acidified aqueous phase were taken to dryness carefully in a stream of nitrogen at 20 C. The fatty acids were extracted from the lipid extracts with two portions of 0.47 M Na_2CO_3. The acids were then recovered from the aqueous phase by extraction with three portions of petroleum ether after acidification.

Methylation of the fatty acids. The method of Metcalfe and Schmitz (9), which involves boron trifluoride, was compared with a modification of the method of Lorette and Brown (8). The methylation with boron trifluoride leads to the development of a brown color which did not appear with the dimethoxypropane-induced transmethylation. Consequently, the method involving dimethoxypropane was used routinely, although no difference could be detected between the preparations with the gas chromatograph. The modification of the method involving dimethyoxypropane was suggested by T. O. Henderson. Fatty acids were suspended in 0.1 ml of benzene and, to this, 2 ml of 1.39 M HCl in dry methanol and 0.2 ml of 2,2-dimethoxypropane were added. After 12 hr at room temperature, 2 volumes of water was added, and the mixture was extracted with three portions of petroleum ether. The combined extracts were dried over solid Na_2SO_4 plus $NaHCO_3$ (4:1) for at least 1 hr and the volume was reduced in a gentle stream of nitrogen. No extraneous peaks from polymerization of the dimethoxypropane could be detected when mixtures of known fatty acids were methylated.

Hydrogenation of the fatty acid methyl esters. The hydrogenation was performed essentially as described by Kaneshiro and Marr (7) to differentiate between olefinic and cyclopropane fatty acid esters. The olefin esters were completely hydrogenated in 1 hr at room temperature, in methanol, at 1 atm of hydrogen with a 5% platinum catalyst on charcoal. The cyclopropane fatty acids were hydrogenated in glacial acetic acid at 2 atm of hydrogen, with a PtO_2 catalyst at room temperature for 12 hr in a Parr hydrogenation apparatus.

Dehydration of hydroxy fatty acids. Dehydration of β-hydroxymyristic acid was accomplished by heating for 10 min at 100 C in H_2SO_4 as described by Slepecky and Law (14). Ice was then added, and the fatty acid was extracted with three portions of petroleum ether. The fatty acid was then methylated.

Localization of the double bonds in the fatty acids. The method of von Rudloff (13) as modified by Chang and Sweeley (3) was used to localize the double bonds in the fatty acid methyl esters. The dicarboxylic acid fragments were methylated and identified by their retention times on columns of silicone oil SE-30. The acid fragments from the omega end of the enoic fatty acids were identified from their retention times on Poropak Q.

Extraction of lipid. Pellets obtained by centrifugation of bacterial suspensions were extracted with 30 ml of chloroform-methanol (2:1) in stainless-steel centrifuge tubes. Complete suspension was obtained by exposure to sonic vibration. After 30 min of extraction, the mixture was centrifuged and the supernatant fraction was filtered through a small plug of glass wool. The pellet was re-extracted twice and the chloroform-methanol solution was partitioned against 0.7 M NaCl (4). The removal of nonlipid contaminants by chromatography on Sephadex was done as described by Wells and Dittmer (16). The other method of lipid isolation involved a modification of the method described by Bligh and Dyer (2). The bacteria were suspended in 30 ml of water, and 75 ml of methanol and 37.5 ml of chloroform were added. The suspension was mixed vigorously and allowed to stand for several hours. Then 37.5 ml of chloroform and 37.5 ml of water were added, and the suspension was shaken and allowed to separate. The bottom layer was filtered through Whatman no. 12 filter paper before lipid analysis.

Separation of neutral and phospholipids. Lipid preparations were dehydrated by evaporation to dryness and were resuspended in benzene-absolute alcohol (4:1, v/v) several times and then resuspended in chloroform. The lipids were then applied to a column (1 by 6 cm) containing 2 g of silicic acid (100 to 200 mesh Unisil). The neutral lipids were eluted with 40 ml of chloroform and the phospholipids with 20 ml of methanol. This method was developed by J. C. Dittmer.

Phosphate analysis. Lipids were digested in 23.3% (v/v) perchloric acid for 1 hr at 200 C and were analyzed for phosphate by the method of Bartlett (1) as adapted for the Technicon AutoAnalyzer by R. L. Lester.

Fatty acid determination. Fatty acids were determined colorimetrically (11) as modified by W. J. Lennarz. Fatty acids were isolated from saponification mixtures and resuspended in chloroform-heptane (1:1). Palmitic acid was used as the standard.

Fatty acids are designated as follows. The subscript of C indicates the number of carbon atoms in the chain, the superscript B (C^B) indicates a fatty acid containing two methyl groups, the superscript cyclo indicates a cyclopropane fatty acid, the superscript =,9 indicates the fatty acid is monoenoic with the ,9 indicating that the olefinic double bond is between carbons 9 and 10 counting from the carboxyl end, and the superscript OH indicates the fatty acid is hydroxylated.

Gas chromatography. A dual-column gas chromatograph (model 402, F & M Scientific Co., Avondale, Pa.) was used in this study. The hydrogen flame-detectors gave a response that was within ± 1% of linearity between detector currents of 2×10^{-11} and 2×10^{-9} amp with serial dilutions of methyl palmitate. Analytical columns were of U-shaped glass tubes that had an internal diameter of 3 mm and were 1.8 meters long. The glass preparative columns were 6 mm in internal diameter and 1.8 meters long.

Ethylene glycol succinate was used as the liquid phase to make polar columns. Gas Chrom P, 60/80 mesh, was coated with 15% (w/w) ethylene glycol succinate. The preparative column was made in a similar way by use of 80/100 mesh Chromosorb W. Analytical columns were run isothermally at temperatures of 130 and 150 C. The flow of carrier gas (helium) was 84 ml/min, with a head pressure of 60 psi. Hydrogen at a head pressure of 18 psi (flow rate

of 65 ml/min) and air at a head pressure of 30 psi (flow rate of 250 ml/min) were used. The flash heater and detectors were heated to 210 and 230 C. Under these conditions, the efficiency of the column for methyl palmitate was 1,908 theoretical plates and the resolution between methyl stearate and methyl oleate was 3.15. Five mμmoles of methyl palmitate produced a current density in the detector of 6.4×10^{-11} amp and a resulting area of 197 mm² on the recorder paper. Preparative columns of this liquid phase were run isothermally at a temperature of 180 C. The flow of helium was 100 ml/min with the use of an 8:1 split ratio for the stream splitter with the preparative columns. The individual esters were collected in glass melting-point tubes that were inserted in tubes of Teflon surrounded by copper as recommended in F & M Technical paper 32. This produces a temperature gradient and essentially quantative recovery.

Nonpolar analytical columns were made of 2.3% (w/w) General Electric silicone oil SE-30 on 80/100 mesh Diatoport S. In this column, the flow of helium was 84 ml/min, of air, 380 ml/min., and of hydrogen, 50 ml/min. The temperature was held at 150 C. Under these operating conditions, the efficiency of this column was 2,530 theoretical plates for methyl palmitate and the resolution of methyl stearate and methyl oleate was 5.18. A 5-mμmole amount of methyl palmitate produced a current density in the detector of 2.1×10^{-11} amp and an area of 609 mm² on the recorder. The analytical column was used at 110 C and the flow of helium of 34 ml/min, to separate the dimethyl esters of the carboxylic acid fragments of the monoenoic esters cleaved with periodate-permanganate.

Fatty acid fragments of monoenoic esters cleaved by periodate-permanganate were separated by analytical columns of 80/100 mesh Poropak Q. The temperature of the column was 235 C, the flash heater, 310 C, and the detector, 330 C. The flow rates of helium, air, and H_2 were 75, 340, and 35 ml/min, respectively.

All columns were packed between small plugs of silane-treated glass wool. The percentage composition of saturated and monoenoic standard mixtures of fatty acid methyl esters from C_8 to C_{20} determined with the analytical columns was within 1% of that predicted for both major and minor components. The area of each response was either calculated from the disc integrator tracing or by the product of the peak height and the width at half peak height. Both methods gave similar results.

Infrared spectra. The infrared spectrum of the hydroxy fatty acid was measured in a 0.05-ml cell with NaCl windows with a Perkin-Elmer 337 spectrophotometer. The solvent was carbon tetrachloride.

Materials. Fatty acids that were used as standards were purchased from the Applied Science Laboratories, State College, Pa. Gas chromatographic materials were purchased from F & M Scientific Division of Hewlett-Packard Co., Avondale, Pa., and the Applied Science Laboratories. The β-hydroxymyristic acid standard was a gift of W. J. Lennarz and the C_{17}^{cyclo} acid was a gift of J. H. Law.

RESULTS

Identification of the fatty acid methyl esters. Known mixtures of saturated, monoenoic, branched, hydroxylated, and cyclopropane fatty acid methyl esters were chromatographed on the polar and nonpolar columns. The logarithms of the retention times were then plotted against the number of carbon atoms in the ester, and the points were connected by straight lines as has been established by James (6). Then chromatographic analysis of the *Haemophilus* fatty acid methyl esters was performed with the same conditions. The bacterial fatty acid esters were then tentatively identified by their positions on these graphs. The positions of the *Haemophilus* fatty acid methyl esters are marked with open circles and the known fatty acid esters are marked with the crosses in Fig. 1 and 2. The mixtures of fatty acid methyl esters were then hydrogenated under conditions in which only the olefinic double bonds were hydrogenated, as well as under conditions where the cyclopropane rings were saturated. The changes resulting after hydrogenation and chromatographic analysis are given in Table 1 and the changes in retention time are illustrated in Fig. 1 and 2. The percentage composition of the mixture of fatty acid methyl esters was calculated from chromatograms derived from analyses on polar and nonpolar columns. These results are illustrated in Table 2. The agreement for the composition of each fatty acid between the two columns corroberates the identification by use of the retention times.

Saturated fatty acids. The series of fatty acids C_{10}, C_{13}, C_{14}, C_{15}, C_{16}, C_{17}, C_{18}, C_{19}, C_{20}, and C_{21} had the expected retention times and percentage composition when assayed with both the polar and nonpolar columns. The C_{10} was most easily distinguished when the columns were run at 130 C. The C_{21} could not be detected on the polar ethylene glycol succinate column except in the lipid extracts as it was obscured by the C_{14}^{OH}. The branched C_{16}^{B} and C_{18}^{B} had the expected retention times from measurements with the iso C_{15}^{B} and anti-iso C_{14}^{B} as standards. The proportions of each as determined with each column agree. The retention times indicate that the branch is either iso or anti-iso, as branching near the center of the molecule has been found to shorten the retention time significantly.

Olefinic fatty acids. The retention times of the $C_{13}^{=}$, $C_{14}^{=}$, $C_{15}^{=}$, $C_{16}^{=}$, and $C_{18}^{=}$ indicate that these fatty acids are monoenoic when compared with standards. On hydrogenation in neutral solvent, there was a quantitative conversion to the expected saturated fatty acid. This result is

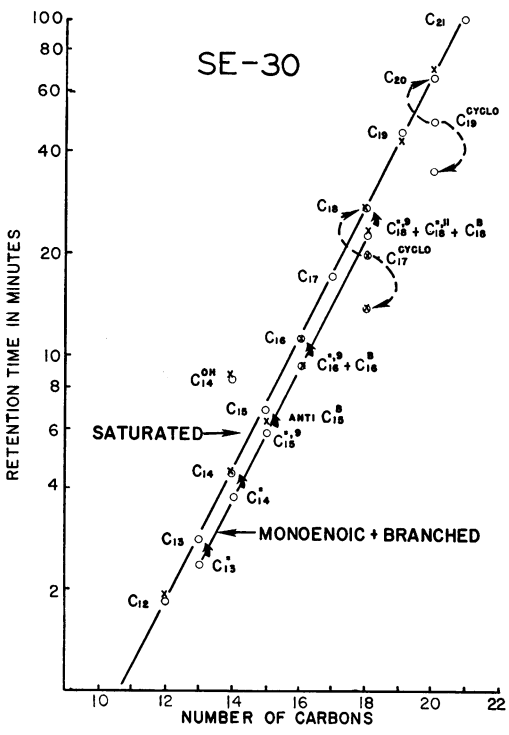

FIG. 1. *Logarithm of the retention time versus the number of carbon atoms in the gas chromatographic analysis of fatty acid methyl esters of Haemophilus parainfluenzae. The liquid phase of the column was silicone oil SE-30 and the temperature of the column was 150 C, with the other conditions as described in Materials and Methods. Symbols: ○ indicates Haemophilus fatty acid methyl esters, and ×, the known standards. Arrows with solid lines indicate changes in retention time which occur when hydrogenation is carried out in methanol. This results in saturation of the olefinic double bonds. Arrows with broken lines indicate the additional changes when hydrogenation is carried out in acetic acid, which results in saturation of the cyclopropane fatty acid methyl esters.*

illustrated in Table 3. The $C_{15}^{=}$, $C_{16}^{=}$, and $C_{18}^{=}$ acids were trapped individually as they emerged from preparative columns. The fatty acid methyl esters were then split at the olefinic double bond by the periodate-permanganate procedure, and the fragments were identified (Fig. 3). The $C_{15}^{=}$ gave rise to a C_9 dicarboxylic acid and a C_6 monocarboxylic acid, which indicates the acid is $C_{15}^{=,9}$. The $C_{16}^{=}$ acid gave rise to a C_9 dicarboxylic acid and a C_7 monocarboxylic acid, which indicates that the acid is palmitoleic acid. The $C_{18}^{=}$ fatty acid gave rise to C_9 and C_{11} dicarboxylic acids and C_9 and C_7 monocarboxylic acids. The areas of response indicated that the C_{11} dicarboxylic acid and the C_7 monocarboxylic acid fragment constitute 64 and 68% of the $C_{18}^{=}$ fragments, respectively. The C_9 dicarboxylic acid and the C_9 monocarboxylic acid represent 36 and 32% of the $C_{18}^{=}$ fragments, respectively. Consequently, the $C_{18}^{=}$ consists of 66% vaccenic acid and 34% oleic acid. Insufficient amounts of the $C_{13}^{=}$ or $C_{14}^{=}$ could be collected to allow determination of the position of unsaturation.

Cyclopropane fatty acids. The cyclopropane fatty acid methyl esters on hydrogenation in acetic acid disappeared and gave rise to about 15% of the saturated straight-chain fatty acid methyl ester and 85% of a branched-chain fatty acid ester with a shorter retention time than the iso or anti-iso branched-chain acid esters (Table 4 and Fig. 1). A sample of C_{17}^{cyclo} behaved identically to one of the acids in *Haemophilus* when subjected to these procedures. The other cyclopropane fatty acid in *Haemophilus* behaved as expected for C_{19}^{cyclo} acid.

Hydroxy fatty acids. The hydroxy fatty acid ester had the same characteristic late retention time on polyester columns as authentic β-

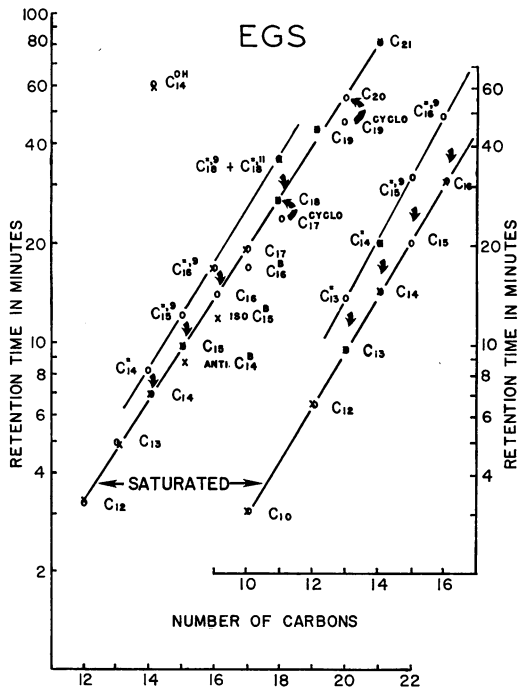

FIG. 2. *Logarithm of the retention time versus the number of carbon atoms in the gas chromatographic analysis of methyl esters of fatty acids of Haemophilus parainfluenzae. The liquid phase was ethylene glycol succinate (EGS). The analysis as shown in the left-hand graph represents experiments with the column temperature at 150 C and in the right hand graph at 130 C. Symbols are as in Fig. 1.*

TABLE 1. *Effect of hydrogenation on the fatty acid methyl esters of Haemophilus parainfluenzae*[a]

Acid	Ethylene glycol succinate column hydrogenation		Silicone oil SE-30 column hydrogenation		
	Neutral solvent	Acetic solvent	Acid	Neutral solvent	Acetic solvent
C_{10}*	U	U	C_{10}*	U	U
C_{13}	I	I	$C_{13}^{=}$	D	D
$C_{13}^{=}$*	D	D	C_{13}	I	I
C_{14}	I	I	$C_{14}^{=}$	D	D
$C_{14}^{=}$	D	D	C_{14}	I	I
C_{15}	I	I	$C_{15}^{=,9}$	D	D
$C_{15}^{=,9}$	D	D	C_{15}	I	I
C_{16}^{B}	U	U	C_{14}^{OH}	U	U
C_{16}	I	I	$C_{16}^{B} + C_{16}^{=,9}$	P	P
$C_{16}^{=,9}$	D	D	C_{16}	I	I
C_{17}	U	U	C_{17}	U	U
$C_{18}^{B} + C_{17}^{cyclo}$	U	P	C_{17}^{cyclo}	U	D
C_{18}	I	FI	$C_{18}^{B} + C_{18}^{=,9} + C_{18}^{=,11}$	D	D
$C_{18}^{=,9} + C_{18}^{=,11}$	D	D	C_{18}	I	FI
C_{19}	U	U	C_{19}	U	U
C_{19}^{cyclo}	U	D	C_{19}^{cyclo}	U	D
C_{20}	U	I	C_{20}	U	I
$C_{21} + C_{14}^{OH}$	U	U	C_{21}	U	U

[a] Fatty acids are listed in the order of their emergence from the columns. Asterisks indicate that the data are taken from columns run at 130 C; all other data were taken from columns run at 150 C. The changes after hydrogenation in neutral solvents, which saturate only olefinic double bonds, and in acetic acid, which hydrogenates both olefinic and cyclopropane fatty acids, are indicated as follows: P, the peak partially disappears; D, the peak completely disappears; I, the peak increases; FI, the peak increases more after hydrogenation in acetic acid than the increase in neutral solvent; and U, no change in the response.

hydroxy-myristic acid methyl ester (Fig. 2). Infrared spectroscopy of the methyl ester after isolation from the preparative column indicated carbonyl stretching at 5.75 μ, hydroxyl stretching at 3.10 μ, and carbon-hydrogen stretching at 3.3 μ. The isolated fatty acid was dehydrated in sulfuric acid, and the resulting α–β unsaturated fatty acid methylated. The retention time at 140 C of the β-hydroxymyristic acid methyl ester on the polyester column was 61.8 min. The retention time of the presumed α–β unsaturated ester was 23.05 min. After hydrogenation of the α–β unsaturated ester, the retention time was 7.31 min and that of authentic methyl myristate was 7.30 min. The long retention time of the α–β unsaturated fatty acid ester agrees with that from an experiment with authentic β-hydroxymyristic acid. This identification procedure was suggested by J. H. Law.

Total composition of the fatty acids of H. parainfluenzae. The concentration of fatty acids in *H. parainfluenzae* assayed in the stationary phase of growth was approximately 150 μmoles/g or about 4% of the dry weight. The content of fatty acid methyl esters as determined by gas chromatography with methyl palmitate as standard, the colorimetric analysis with palmitic acid as standard, and the total dry weight of the methyl esters varied from 3.8 to 4.5% of the dry weight.

If several gas chromatographic analyses are performed, the total fatty acid composition can be determined. The C_{10}, C_{12}, C_{13}, $C_{13}^{=}$, $C_{14}^{=}$, and C_{16}^{B} were best determined when the columns were held at 130 C. The C_{18}^{B} and C_{17}^{cyclo} were determined before and after hydrogenation in acid from the ethylene glycol succinate column. Hydrogenation in alcohol was used to differentiate C_{18}^{B} from $C_{18}^{=}$ with the SE-30 column. The proportions of $C_{18}^{=,9}$ and $C_{18}^{=,11}$ require the collection of the combined $C_{18}^{=}$ methyl esters from preparative columns and the determination of the proportions of the fragments obtained after periodate-permanganate treatment. The results of such an analysis are illustrated in Table 5 Three fatty acids, C_{14}, C_{16}, and $C_{16}^{=,9}$, made up 88.1% of the total fatty acids. These three plus C_{14}^{OH}, $C_{15}^{=,9}$, and C_{18} accounted for 96.8% of the fatty acids.

The Proteose Peptone (Difco) medium used to grow the bacteria contains 0.224 μmole of fatty

TABLE 2. *Per cent composition of the fatty acid methyl esters of Haemophilus parainfluenzae as determined by gas chromatography with columns of ethylene glycol succinate (EGS) and silicone oil (SE-30)*[a]

Acid	EGS	SE-30
C_{12}	0.8	0.8
C_{13}	<0.1	<0.1
C_{13}^{-}	0.1	0.2
C_{14}	27.9	25.4
C_{14}^{-}	0.8	0.8
C_{14}^{OH}	11.8	11.8
C_{15}	1.0	0.6
C_{15}^{-}	7.4	7.0
C_{16}^{B}	1.0	1.0
C_{16}^{-}	17.9	18.8
C_{16}	24.8	25.3
C_{17}	0.1	0.1
C_{17}^{cyclo}	0.4	0.6
C_{18}^{B}	2.3	2.6
C_{18}^{-}	2.5	2.3
C_{18}	2.2	2.6
C_{19}	0.2	0.1
C_{19}^{cyclo}	0.1	0.1
C_{20}	0.3	0.4

[a] Percentages calculated from the total of all peak areas (height × width at half peak height) from gas chromatographic analyses measured as in Fig. 1 and 2. The C_{16}^{B} and C_{16}^{-} as well as the C_{18}^{B} and the C_{18}^{-} proportions were determined with the SE-30 column before and after hydrogenation in methanol. The C_{17}^{cyclo} and C_{18}^{B} proportions were determined before and after hydrogenation in acetic acid when examined with the EGS column.

TABLE 3. *Conversion of the monoenoic fatty acid methyl esters from Haemophilus parainfluenzae by hydrogenation in neutral solvents to the corresponding saturated methyl esters*[a]

Acid	Before hydrogenation	After hydrogenation	Per cent recovered
C_{13}^{-}	5	0	
C_{13}	2	7	100
C_{14}	540	605	
C_{14}^{-}	34	0	105
C_{15}	16	118	
C_{15}^{-}	116	0	90
C_{16}	3,920	6,454	
C_{16}^{-}	2,400	120	103
C_{18}	280	437	
C_{18}^{-}	170	18	102

[a] The amount of each fatty acid is expressed in square millimeters determined as in Table 2. The C_{13} data were taken from analysis with an SE-30 column with the chromatograms normalized to equivalent areas for C_{12}. The C_{14} data were taken from an EGS column run at 130 C and normalized with C_{12}. The data for C_{15}, C_{16}, and C_{18} were taken from analysis with EGS columns before and after hydrogenation, and normalized to equivalent areas of C_{14}^{OH}. The per cent recovered represents the percentage of monoenoic acid that was found after hydrogenation in the saturated fatty acid, compared with that expected for the monoenoic acid present. Hydrogenation was performed in methanol as described in Materials and Methods.

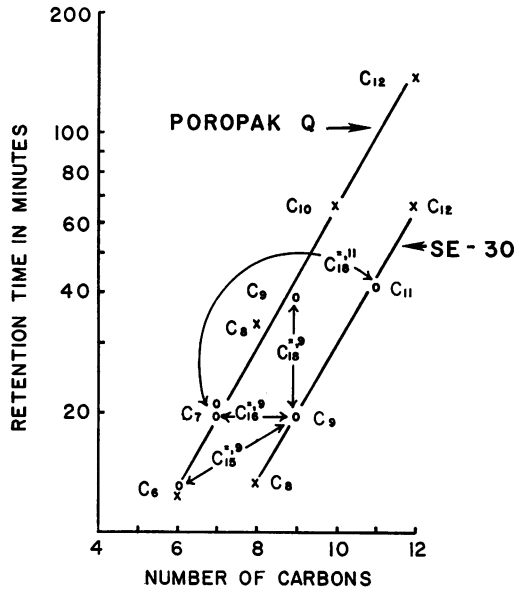

FIG. 3. *Logarithm of the retention time of fragments of monoenoic fatty acid methyl esters treated with periodate-permanganate and compared with the number of carbon atoms. On the left is the curve obtained by chromatography on Poropak Q of the free monocarboxylic acids from the omega end of the fatty acids. On the right is the curve obtained by chromatographic analysis on silicone oil SE-30 of the dimethyl esters of the dicarboxylic acid fragments from the carboxyl end of the fatty acids. Each monoenoic was isolated by preparative gas chromarography before treatment with periodate-permanganate. Arrows indicate the fragments from each monoenoic acid; symbols are as in Fig. 1. The retention times of the right-hand curve are plotted at 10 times actual value for convenience.*

acid per liter. The fatty acids consisted of 35% C_{14}, 60% C_{16}, 3% C_{16}^{-}, and 2% linoleic acid. During a growth cycle in this media, the bacteria can synthesize about 90.0 μmoles of fatty acid per 600 mg (dry weight). The fatty acids in the media could account for only 0.12% of the C_{14}, 0.10% of the C_{16}, or 0.03% of the C_{16}^{-} found in the bacteria grown in this medium. The linoleic acid did not appear in the bacteria. *Haemophilus*

TABLE 4. *Conversion of cyclopropane fatty acid esters to the branched-chain and saturated esters by hydrogenation in acetic acid*[a]

Acid	After hydrogenation in methanol	After hydrogenation in acetic acid	Per cent recovered
C_{17}^{B}	0	69	
C_{17}^{cyclo}	73	0	
C_{18}	464	482	112
C_{19}^{B}	0	21	
C_{19}^{cyclo}	18	0	
C_{20}	56	59	130

[a] The amounts of fatty acid esters were calculated as in Table 3 with analysis obtained from SE-30 columns after hydrogenation in a neutral solvent, and were compared with the results after hydrogenation in acetic acid. Methods of hydrogenation are given in Materials and Methods. Chromatograms were normalized with C_{19} to equal areas. The per cent recovery compares the amount of cyclopropane ester with the total amount of the new branched-chain ester plus the increase in saturated ester. The retention time of the branched ester that appears after hydrogenation in acetic acid indicates that the branching is near the middle of the chain (see Fig. 1).

appeared capable of synthesizing each of the 22 fatty acids it has been shown to contain.

Cellular localization of the fatty acids. If a suspension of *Haemophilus* in phosphate buffer was subjected to sonic vibration (21) and then centrifuged at 104,000 × *g* for 40 min, resuspended to volume, and recentrifuged, the enzymes of the Embden-Meyerhof-Parnas and hexose monophosphate shunt pathways are quantitively recovered in the supernatant fraction (22). The pellet contains the membrane-bound electron transport system consisting of the flavoprotein dehydrogenases (19); the respiratory quinone, 2-demethyl vitamin K_2 (21); the substrate-reducible cytochromes and cytochrome oxidases (23); and the ability to utilize oxygen in the presence of substrate. Electron microscopy of the pellet reveals typical membrane fragments (15). This membrane preparation contains all the phosphorous soluble in chloroform-methanol. This lipid-soluble phosphorus accounts for all the phospholipid in the bacteria. No fatty acids can be recovered from the supernatant portion after saponification. The total fatty acids can be recovered from the membrane fragments. Consequently, the fatty acids, the phospholipids, the respiratory quinone, and the respiratory pigments are all a part of the membrane system.

Extraction of the lipid from H. parainfluenzae. Fatty acids and lipid phosphorus could be extracted efficiently from *Haemophilus* with chloroform-methanol, 2:1 (Table 6). Two extractions with chloroform-methanol totally removed the phospholipids. Extraction with acidified chloroform-methanol did not remove any further phospholipid. The rapid and effective extraction procedure of Bligh and Dyer (2) produced 50.32 μmoles of P per g (dry weight), compared to 50.39 μmoles of P per g (dry weight) for the sum of two extractions with chloroform-methanol.

Partition of fatty acids between residue and lipid. The fatty acid composition of the lipids and the residue of both the chloroform-methanol and Bligh and Dyer (2) methods gave similar patterns (Table 7). The residue after lipid extraction had a peculiar composition, consisting of most of the C_{14}^{-} and $C_{15}^{-,9}$, and all of the C_{14}^{OH}, in the bacteria. Very little of the major fatty acid C_{16} and little of the $C_{16}^{-,9}$ or C_{18}^{-} was left in the wall after extraction. None of the C_{10}, C_{16}^{B}, C_{17}, C_{17}^{cyclo}, C_{19}, C_{19}^{cyclo}, C_{20}, or C_{21} was found in the residue.

Distribution of fatty acids between the neutral

TABLE 5. *Composition of fatty acids of Haemophilus parainfluenzae grown to late stationary phase*[a]

Acid	Per cent of total
C_{10}	0.006
C_{12}	0.1
C_{13}	0.07
C_{13}^{-}	0.006
C_{14}	19.33
C_{14}^{-}	0.21
C_{14}^{OH}	4.80
C_{15}	0.8
$C_{15}^{-,9}$	1.61
C_{16}^{B}	0.03
$C_{16}^{-,9}$	31.26
C_{16}	37.52
C_{17}	0.03
C_{17}^{cyclo}	0.08
C_{18}^{B}	0.01
$C_{18}^{-,9}$	0.37
$C_{18}^{-,11}$	0.56
C_{18}	2.31
C_{19}	0.27
C_{19}^{cyclo}	0.14
C_{20}	0.45
C_{21}	0.07

[a] Proportions of the fatty acid methyl esters were determined as the areas as in Table 2 from analyses by gas chromatography before and after hydrogenation or cleavage with periodate-permanganate, as described in the text. Table 5 gives the percentage composition of the fatty acid samples used for Tables 7, 8, and 9, and represents bacteria in the stationary phase of growth.

and phospholipids. If carefully dried lipid extracts are placed on a silicic acid column, the neutral lipids can be quantatively separated from the phospholipids. This method, described in Materials and Methods, represents a technique developed by J. C. Dittmer. The fatty acid composition of the neutral and phospholipids are tabulated in Table 8. The neutral lipids represented 1.6% of the total lipid fatty acids. Significant proportions of the $C_{13}^{=}$, C_{17}, C_{18}^{B}, and C_{19} were found in the neutral lipid.

Distribution between free and esterified lipid fatty acids. The lipid extract was partitioned with 0.47 M Na_2CO_3 as described in Materials and Methods to remove the nonesterified fatty acids. The results of this analysis are given in Table 9. Only 0.4% of the fatty acids was not esterified. Comparison with Table 8 indicates that the $C_{13}^{=}$, C_{17}, C_{18}^{B}, $C_{18}^{=}$, and C_{19} found in the neutral lipid did not exist as free fatty acids. These bound fatty acids represented 20% of the neutral lipid fatty acid and 0.32% of the total fatty acid composition.

TABLE 6. *Extraction of lipids from Haemophilus parainfluenzae*[a]

Fatty acid	Extractions		
	CM-1	CM-2	CMA
C_{14}	79	8	8
$C_{15}^{=}$	94	0	6
C_{16}	95	2	3
$C_{16}^{=}$	100	0	0
C_{18}	85	15	0
$C_{18}^{=}$	92	6	0
Total lipid	97	1	2
Lipid phosphate	98	2	0

[a] Results are expressed as the percentage of total lipid fatty acid. Fatty acids were determined from gas chromatograms after saponification and are expressed as percentage of total lipid fatty acid. CM-1 refers to a chloroform-methanol (2:1) extract (30 ml/400 mg, dry weight) performed as described in Materials and Methods. CM-2 refers to a second extraction of the residue of the CM-1. CMA refers to an extraction of the residue of CM-2 in which 0.8 ml of concentrated HCl was added to 3 ml of chloroform-methanol. Each lipid extraction was treated with Sephadex (16) to remove nonlipid contaminants. The yield of lipid fatty acids accounted for 86% of the total bacterial fatty acids determined colorimetrically with palmitic acid as standard.

TABLE 7. *Localization of fatty acids in the lipid and nonextractible residue of Haemophilus parainfluenzae*[a]

Acid	Extraction with chloroform-methanol		Extraction according to Bligh and Dyer	
	Lipid	Residue	Lipid	Residue
C_{10}	100	0	100	0
C_{12}	96	4	88	12
C_{13}	86	14	88	12
$C_{13}^{=}$	82	18	76	24
C_{14}	69	32	66	34
$C_{14}^{=}$	12	87	10	90
C_{15}	26	74	27	73
$C_{15}^{=}$	63	37	61	39
C_{16}^{B}	100	0	100	0
C_{16}	99	0.2	99	0.1
$C_{16}^{=}$	99	0.7	99	0.9
C_{17}	100	0	100	0
C_{17}^{cyclo}	100	0	100	0
C_{18}^{B}	88	12	80	20
C_{18}	94	6	89	11
$C_{18}^{=}$	99	1	99	0.3
C_{19}	100	0	100	0
C_{19}^{cyclo}	100	0	100	0
C_{20}	100	0	100	0
C_{21}	100	0	100	0
C_{14}^{OH}	0	100	0	100
Per cent of total fatty acid	86.8	13.2	84.9	15.1

[a] The proportions of each fatty acid are expressed as the percentage of the sum of the nonextractable residue fatty acid plus the lipid fatty acid. The recovery measured as the sum of lipid and residue fatty acid ranged between 95 and 110% of the total fatty acid in the bacteria before extraction.

DISCUSSION

H. parainfluenzae has a fatty acid composition that resembles gram-negative bacteria in some aspects. The localization of the β-hydroxymyristic acid in the nonextractable wall complex (10), the preponderance of the vaccenic over the oleic in the $C_{18}^{=}$ acids (7), and the lack of significant free fatty acids in the lipid (J. H. Law, Bacteriol. Proc., p. 129, 1961) are typical of *Escherichia coli.* The localization of the C_{10} fatty acid in the phospholipid in *Haemophilus* differs from its localization in the wall of *E. coli* (J. H. Law, Abstr. Meeting Am. Chem. Soc., p. 10C, 1962). In addition, the wall residue fraction of *Haemophilus* contains most of the $C_{14}^{=}$, C_{15}, and a part of the C_{13}, $C_{13}^{=}$, $C_{16}^{=}$, C_{18}^{B}, and C_{18} fatty acids. This specific distribution of these fatty acids is not common in other bacteria.

The principal fatty acid in *Haemophilus*, as in other bacteria, is palmitic acid (12), and in *Haemophilus* this acid is localized exclusively in the phospholipids. The phospholipids are readily extractable and contain 84% of the fatty acids of the bacteria. The localization of the cyclopropane fatty acids exclusively in the phospholipids would be expected from the study of the biosynthesis of the cyclopropane ring (5).

The principal neutral lipid consists of three isoprenologues of 2-demethyl vitamin K_2, with side chains of C_{25}, C_{30}, and C_{35}. In stationary-phase cultures of bacteria, this lipid is present in concentrations up to 4.5 μmoles/g, dry weight (0.29% of the dry weight; 21). The major fatty acids of the neutral lipids are $C_{13}^{=}$, C_{17}, C_{19}, and C_{18}^{B}. The neutral lipid contains some component to which most of the $C_{13}^{=}$, C_{17}, C_{18}^{B}, C_{18}, $C_{18}^{=}$, and C_{19} of the neutral lipid fatty acids are bound. The free fatty acids are mainly C_{12}, C_{15}, and $C_{15}^{=,9}$.

It is hoped that further studies of the changes in the proportions of phospholipids during modification of the electron transport system will allow correlation with changes in the specific distribution of the fatty acids. Perhaps some insight can be gained not only into the function of phospholipid in membrane formation but also into the relatively specific distribution of fatty acids in the lipid fractions of the bacteria.

TABLE 8. *Distribution of fatty acids between neutral and phospholipids in Haemophilus parainfluenzae*[a]

Acid	Neutral lipid (1.6%)[b]	Phospholipid (98.4%)[b]
C_{10}	—	100
C_{12}	8	92
C_{13}	5	95
$C_{13}^{=}$	28	72
C_{14}	2	99
$C_{14}^{=}$	<1	99
C_{15}	1	99
$C_{15}^{=}$	<1	99
C_{16}^{B}	—	100
C_{16}	<1	99
$C_{16}^{=}$	<1	99
C_{17}	17	83
C_{17}^{cyclo}	—	100
C_{18}^{B}	70	30
C_{18}	12	88
$C_{18}^{=}$	15	85
C_{19}	74	26
C_{19}^{cyclo}	—	100
C_{20}	—	100
C_{21}	—	100
Phosphate	0	100.1

[a] Neutral and phospholipids were separated on silicic acid as described in Materials and Methods. The sum of the neutral and phospholipid fatty acids represented between 93 and 113% of the total fatty acid of the lipid.

[b] Per cent of total.

TABLE 9. *Distribution of free and esterified fatty acid in the lipid of Haemophilus parainfluenzae*[a]

Acid	Esterified (99.6)[b]	Free (0.4)[b]
C_{10}	100	0
C_{12}	90	10
C_{13}	100	0
$C_{13}^{=}$	100	0
C_{14}	99	<1
$C_{14}^{=}$	100	0
C_{15}	92	8
$C_{15}^{=}$	98	2
C_{16}^{B}	100	0
C_{16}	99	<1
$C_{16}^{=}$	99	<1
C_{17}	100	0
C_{17}^{cyclo}	100	0
C_{18}^{B}	100	0
C_{18}	100	0
$C_{18}^{=}$	100	0
C_{19}	100	0
C_{19}^{cyclo}	100	0
C_{20}	100	0
C_{21}	100	0

[a] The nonesterified fatty acids were extracted from the lipid with Na_2CO_3 as described in Materials and Methods. The total of nonesterified and esterified fatty acid represented between 93 and 114% of that found for the total lipid.

[b] Per cent of total.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant GM 10285 from the National Institute of General Medical Sciences.

We are indebted to J. C. Dittmer, J. H. Law, and W. J. Lennarz for their valuable discussions and for the gifts of fatty acid standards.

LITERATURE CITED

1. BARTLETT, G. R. 1959. Phosphorus assay in column chromatography. J. Biol. Chem. **234:** 466–468.
2. BLIGH, E. G., AND W. J. DYER 1959. A rapid method of total lipid extraction and purification. Can. J. Biochem. Physiol. **37:**911–917.
3. CHANG, T. L., AND C. C. SWEELEY. 1962. Studies of canine adrenal polyenoic acids: locating double bonds by periodate-permanganate

oxidation and gas-liquid chromatography. J. Lipid Res. **3**:170–176.
4. FOLCH, J., M. LEES, AND G. H. SLOANE-STANLEY. 1957. A simple method for the isolation and purification of total lipids from animal tissues. J. Biol. Chem. **226**:497–509.
5. HILDEBRAND, J. G., AND J. H. LAW. 1964. Fatty acid distribution in bacterial phospholipids. The specificity of the cyclopropane synthetase reaction. Biochemistry **3**:1304–1308.
6. JAMES, A. T. 1960. Qualitative and quantitative determination of the fatty acids by gas-liquid chromatography. Methods Biochem. Analy. **8**:1–59.
7. KANESHIRO, T., AND A. G. MARR. 1961. Cis-9,10-methylene hexadecanoic acid from the phospholipids of *Escherichia coli*. J. Biol. Chem. **236**:2615–2619.
8. LORETTE, N. B., AND J. H. BROWN, JR. 1959. Use of acetone dimethyl acetal in preparation of methyl esters. J. Org. Chem. **24**:261–262.
9. METCALFE, L. D., AND A. A. SCHMITZ. 1961. The rapid preparation of fatty acid esters for gas chromatographic analysis. Anal. Chem. **33**: 363–364.
10. NESBITT, J. A., III, AND W. J. LENNARZ. 1965. Comparison of lipids and lipopolysaccharide from the bacillary and L forms of *Proteus* P18. J. Bacteriol. **89**:1020–1025.
11. NOVAK, M. 1965. Colorimetric ultramicro method for the determination of free fatty acids. J. Lipid Res. **6**:431–433.
12. O'LEARY, W. M. 1962. The fatty acids of bacteria. Bacteriol. Rev. **26**:421–447.
13. VON RUDLOFF, E. 1956. Periodate-permanganate oxidations. V. Oxidation of lipids in media containing organic solvents. Can. J. Chem. **34**: 1413–1418.
14. SLEPECKY, R. A., AND J. H. LAW. 1960. A rapid spectrophotometric assay of alpha, beta-unsaturated acids and beta-hydroxy acids. Anal. Chem. **32**:1697–1699.
15. SMITH, L. 1966. The respiratory chain system of bacteria. *In* T. P. Sanger [ed.], Biological oxidations. John Wiley & Sons, Inc., New York.
16. WELLS, M. A., AND J. C. DITTMER. 1963. The use of Sephadex for the removal of nonlipid contaminants from lipid extracts. Biochemistry **2**: 1259–1263.
17. WHITE, D. C. 1962. Cytochrome and catalase patterns during growth of *Haemophilus parainfluenzae*. J. Bacteriol. **83**:851–859.
18. WHITE, D. C. 1963. Factors affecting the affinity for oxygen of cytochrome oxidases in *Haemophilus parainfluenzae*. J. Biol. Chem. **238**:3757–3761.
19. WHITE, D. C. 1964. Differential synthesis of five primary electron transport dehydrogenases in *Haemophilus parainfluenzae*. J. Biol. Chem. **239**:2055–2060.
20. WHITE, D. C. 1965. Synthesis of 2-demethyl vitamin K_2 in the electron transport system in *Haemophilus parainfluenzae*. J. Bacteriol. **89**: 299–305.
21. WHITE, D. C. 1965. The function of 2-demethyl vitamin K_2 in the electron transport system in *Haemophilus parainfluenzae*. J. Biol. Chem. **240**:1387–1394.
22. WHITE, D. C. 1966. The obligatory involvement of the electron transport system in the catabolic metabolism of *Haemophilus parainfluenzae*. Antonie van Leeuwenhoek J. Microbiol. Serol. **32**:139–158.
23. WHITE, D. C., AND L. SMITH. 1964. Localization of the enzymes that catalyze hydrogen and electron transport in *Haemophilus parainfluenzae* and the nature of the respiratory chain system. J. Biol. Chem. **239**:3956–3963.