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Phospholipids and Fatty Acids of *Neisseria gonorrhoeae*

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The phospholipids and fatty acids of two strains of *Neisseria gonorrhoeae* of different penicillin susceptibilities were examined. The phospholipids, which comprise about 8% of the dry weight of the cells, consisted of phosphatidylethanolamine (70%) and phosphatidylglycerol (20%); small amounts of phosphatidylcholine and traces of cardiolipin were also present. Growing and stationary-phase cells were similar in content and composition of phospholipids except for phosphatidylcholine, which increased two- to fivefold in the stationary-phase cells. The fatty acids of the phospholipids were characterized by two major acids, palmitic and a C16:1, with myristic and a C18:1 acid present in smaller amounts. The fatty acids present in purified phospholipid fractions varied considerably in relative proportions from fraction to fraction. No significant difference in the composition of phospholipids from the two strains was evident. Large amounts of β-hydroxy lauric acid were detected only after saponification of the organisms. Differences in the lipid composition between the gonococcus and other gram-negative bacteria are discussed.

No detailed studies on the lipid composition of *Neisseria* species have been reported. One study from the 1940s (21) of the cellular constituents of *Neisseria gonorrhoeae* included limited qualitative data on the lipid composition; lecithin, a cephalin, and a sphingolmyelin were reported to be present. Some information on the fatty acids of *Neisseria* species is available (1, 16, 17). This report contains a description of the phospholipid and fatty acid composition of two strains of *N. gonorrhoeae*, one sensitive to penicillin and the other relatively resistant to the antibiotic.

MATERIALS AND METHODS

Microorganisms. The two isolates used in this study were specimens from the diagnostic bacteriology laboratory of the Beth Israel Hospital. They were identified by colonial morphology, Gram stain, oxidase test, and carbohydrate fermentations. The two strains were selected on the basis of sensitivity to penicillin G. One strain, B-21, was sensitive to less than 0.01 U/ml; the other strain, 48-744, was resistant to more than 1.0 U of the drug per ml, as determined by streaking a loopful of a suspension of approximately 10^6 cells/ml on chocolate agar plates containing varying concentrations of penicillin and observing growth after incubation for 48 h. Stock cultures of these organisms were maintained at −60 C after quick freezing of the cell suspensions in skim milk.

Culture media and cell growth. The following semisynthetic liquid medium (in grams per liter) was used: proteose peptone no. 3 (Difco), 10.5 g; soluble starch, 1.0 g; KH_2PO_4, 1.0 g; K_2HPO_4, 4.0 g; NaCl, 5.0 g. These ingredients were dissolved in hot distilled water and sterilized in the autoclave. After cooling, sterile solutions of glucose (5.0 g) and $NaHCO_3$ (0.42 g) were added. A 10-ml amount of supplement B (Difco) was finally added. Agar (1.5%) was added to the mixture before autoclaving for solid media.

An overnight growth of cells on solid medium was suspended in a small volume of the liquid medium. The cell suspension was added to the liquid medium to give a population of 10^7 to 2×10^7 cells/ml. Cultures (1.5 liters in 6-liter flasks) were shaken at 37 C. For exponential-phase cells, the cultures were allowed to attain a cell density of 2×10^8 to 4×10^8 cells/ml before harvesting; for stationary-phase cells, 24-h-old cultures were collected.

Extraction of lipid. The cells were centrifuged and washed with normal saline and distilled water. They were either used directly or lyophilized before lipid extraction with chloroform-methanol (2:1, vol/vol) overnight. The suspension was centrifuged briefly, and the supernatant was collected. The pellet was again extracted for 1 h and centrifuged, and the combined supernatants were then washed once with normal saline. The lower phase was evaporated under nitrogen, and the lipids were dried over NaOH pellets at reduced pressure.

To detect cellular fatty acids, the washed growing cells were saponified, extracted, and methylated as previously described (18). Lipid phosphorus was determined, after digestion of lipids with 70% perchloric acid, according to the method of Dawson (7).

Column chromatography. For fractionation, total lipids derived from 200 to 400 mg of lyophilized cells were used. Phospholipids were separated from the neutral lipids on an activated silicic acid (100 mesh; Clarkson Chemical Co., Williamsport, Pa.) column (1 by 24 cm). Neutral lipids were first eluted with 3 column volumes of chloroform. The column

was then eluted with 3 volumes of acetone, followed by 3 volumes of methanol-chloroform (1:2, vol/vol) to obtain the phospholipids. The fractions were first dried under a stream of nitrogen and then over NaOH pellets to a constant weight.

Thin-layer chromatography. The phospholipids were dissolved in chloroform and spotted as a thin band on precoated, semipreparative silica gel plates. The plates were developed with chloroform-methanol-water (65:25:2, vol/vol/vol). The plates were dried in air and covered with Saran Wrap, leaving a small part of the plate for visualization of phospholipids by iodine vapor. The iodine-stained areas were marked and compared with the spots from a standard mixture of phospholipids run on the same plate. The Saran Wrap was removed, and silica gel from unexposed areas corresponding to the various spots was carefully scraped off. The phospholipid fractions were purified by repeating the thin-layer chromatography until each fraction gave only one iodine-staining spot corresponding in R_f to the authentic standard. These fractions were used for fatty acid analysis.

Quantitation of the phospholipids. Cells were grown in 250 ml of medium containing 0.5 mCi of $H_3{}^{32}PO_4$. The amount of potassium phosphate in this medium was reduced to 25% of that in the normal growth medium, and the amount of ^{32}P added was sufficient to maintain a constant specific activity of the medium during the experiment. The lipids were extracted and subjected to thin-layer chromatography as mentioned above. The plate was scanned for radioactivity in a Packard radiochromatogram scanner. Silica gel from areas under the peaks was removed and tranferred to vials containing Bray's fluid (5) for counting in a Beckman scintillation counter. The amount of each component was determined as the percentage of total radioactivity recovered in the samples.

Hydrolytic procedures and paper chromatography. The phospholipids were hydrolyzed in dilute alkali by the method of Tarlov and Kennedy (23). The water-soluble decylated products were chromatographed on Whatman no. 1 filter paper, descending, in a solvent system of water-saturated phenol–ethanol–acetic acid (50:5:6, vol/vol/vol). After development, the paper was dried overnight in a stream of air.

Acid hydrolysis was done at 100 C for 4 h in 1 N HCl in sealed ampoules. The hydrolysates were extracted twice with petroleum ether, and the aqueous phase was evaporated to dryness. The residue was dissolved in water and subjected to descending chromatography with the solvent system phenol–*n*-butyl alcohol–formic acid (80%)–water (50:50:3:10, wt/vol/vol/vol) saturated with solid KCl on Whatman no. 1 paper which had been previously dipped in 1 N KCl and dried.

Ninhydrin (0.2%) in butanol was used to identify free amino groups and Dragendorff reagent (20) was used to identify choline. Phosphorus-containing spots were detected by the method of Hanes and Isherwood (12). The chromatograms of ^{32}P-labeled derivatives obtained after alkaline hydrolysis were scanned for radioactivity in the radiochromatogram scanner.

Fatty acid analysis. The total phospholipids and purified phospholipid fractions were saponified and methylated with boron trifluoride-methanol reagent (18). The esters were dissolved in small volumes of hexane and stored at −20 C. Gas-liquid chromatography was performed in a Packard gas-liquid chromatography apparatus using a 6-foot (ca. 183-cm) column of 10% diethylene-glycol succinate coated on 80/100-mesh Chromosorb W-AW (Chemical Research Services, Inc.), using an isothermal run at 165 C. The peaks of methyl esters were identified by comparison of retention times with the retention times of methyl ester standards, as well as by cochromatography with individual standards. In preliminary experiments, β-hydroxy palmitic acid and β-hydroxy myristic acid standards were used to determine the location of the β-hydroxy lauric acid peak by employing the method of Farquhar et al. (11). The identification was later confirmed by cochromatography with standard β-hydroxy lauric acid. Peak areas were calculated by triangulation, and the percentage of each acid was determined from the ratio of the area of its peak to the toal area of all peaks.

RESULTS

Growth. Both strains grew well in liquid medium with doubling times of 36 to 42 min. The cells grew exponentially to 8×10^8 to 10×10^8 cells/ml, as determined by both spectrophotometric readings and viable counts. Cell clumping was minimal during this period but became increasingly noticeable as the cells entered the stationary phase of growth. Macroscopic clumping was observed in 24-h-old cultures; the viable counts at this stage varied from 10^6 to 5×10^6 cells/ml. The colonial morphology of both strains on solid medium was that of type 3 by the criteria of Kellog et al. (15).

Lipid content. Cells of both strains contained 8.2 to 10.4% of the dry weight as lipid. The exponential- and stationary-phase cells were similar in total lipid content (Table 1). The lipid was predominantly phospholipid (greater than 80%), as determined by column chromatography and by phosphorus analysis of the total lipid; the remainder was neutral lipid since the acetone fractions from the silicic acid columns did not contain any material. The proportion of phospholipid to total lipid increased in stationary-phase cells as compared to exponential cells in both strains. From gravimetric data, stationary-phase cells of BI-21 and 48-744 contained 9.4 and 6.2% more phospholipid in total lipid, respectively, than the corresponding exponential cells; similar figures derived from phosphorus estimation data were 12% for BI-21 and 2.7% for strain 48-744.

Phospholipids. Thin-layer chromatography of the phospholipids revealed four iodine-staining spots corresponding in R_f to those of cardiolipin (CL), phosphatidylethanolamine (PE), phosphatidylglycerol (PG) and phosphatidylcholine (PC). PE and PC were further verified by positive ninhydrin and Dragendorff reactions, respectively (20). Glycerol phosphate esters, obtained by alkaline hydrolysis of phospholipids, were subjected to paper chromatography. Various components were identified by comparison with published R_f values (8) and with our own standards. Five phosphate-positive spots were obtained. The fastest moving spot (R_f 0.93) gave a positive Dragendorff reaction (20) and was identified as glycerylphosphorylcholine; the spot of glycerylphosphorylethanolamine (R_f 0.6) was ninhydrin positive. Of the remaining three phosphate-positive spots, the two with R_f values of 0.46 and 0.37 were identified as deacylated products of PG and CL, respectively. The slowest moving spot (R_f 0.18) was identified as inorganic phosphate.

The presence of ethanolamine and choline in phospholipids was further confirmed by paper chromatography of products of acid hydrolysis. Each hydrolysate gave one ninhydrin-positive spot (R_f 0.13) and one positive for Dragendorff reaction (R_f 0.8), which corresponded with R_f values of standard ethanolamine and choline. The quantitative composition of the phospholipid is shown in Table 2. In both phases of growth, the predominant component was PE, comprising 69 to 75% of the total phospholipid. PG was the next most abundant phospholipid, whereas CL was present in very small amounts. The amount of PC was small in the growing cells of both strains, but showed a two- to fivefold increase in the stationary-phase cells.

Fatty acid composition. The phospholipids obtained after column chromatography were examined for fatty acids (Table 3). Palmitic acid and a C16:1 acid were the major acids comprising 75 to 80% of the total fatty acids present. Myristic acid and a C18:1 acid were present in small amounts. In the penicillin-sensitive strain (BI-21), unsaturated acids were present in larger amounts than the saturated ones in growing cells; the situation was reversed in stationary-phase cells. In the resistant strain, saturated acids predominated in both phases of growth, although the predominance was striking in the growing cells.

TABLE 1. *Lipid composition of N. gonorrhoeae*

Strain	Growth stage	% Dry wt of cells	
		Total lipid[a]	Phospholipid[b]
BI-21	Exponential	10.4	8.4, 8.7
	Stationary	9.2	8.3, 8.8
48-744	Exponential	8.2	7.4, 7.6
	Stationary	8.6	8.3, 8.2

[a] By gravimetric estimation.

[b] First number: By gravimetric estimation of chloroform-methanol fraction from the silicic acid column. Second number: Calculated from phosphorus estimation of total lipid, taking 800 as an average molecular weight of phospholipid.

TABLE 2. *Phopholipids of N. gonorrhoeae*[a]

Phospholipid	Strain BI-21		Strain 48-744	
	Growing bacteria	Stationary-phase bacteria	Growing bacteria	Stationary-phase bacteria
CL	0.8	0.3	0.9	1.3
PE	74.6	69.7	70.3	69.4
PG	22.2	18.5	22.8	19.3
PC	2.3	11.4	4.5	9.9

[a] ^{32}P-labeled phospholipids were subjected to thin-layer chromatography. After scanning, silica gel from areas under radioactive peaks was scraped off and counted. Values are percentages representing the average of two experiments.

TABLE 3. *Fatty acids in total phospholipids of N. gonorrhoeae*

Strain	Growth stage	Fatty acid										
		12:0[a]	12:1	12:2	14:0	14:1	14:2	16:0	16:1	18:0	18:1	18:2
BI-21	Exponential	T[b]	ND	T	6	T	T	36	43	T	12	1
	Stationary	3	T	T	13	T	T	36	40	ND	7	ND
48-744	Exponential	T	ND	T	5	T	T	56	24	1	13	1
	Stationary	3	ND	T	6	T	T	42	33	T	15	ND

[a] Number to left of colon refers to number of carbon atoms; number to right of colon refers to number of double bonds.

[b] Number refers to percentage of total fatty acids; T, less than 1%; ND, not detected.

Each purified phospholipid fraction was also analyzed for fatty acids (Table 4). The majority of fractions from both strains had similar fatty acid profiles; only two fractions differed in containing C10:0-OH and one differed in containing an acid tentatively identified as C16:2. Fatty acids with 16 and 18 carbon atoms were the major fatty acids accounting for 63 to 96% of the total acids, although there were large variations in the proportions in various fractions. No consistent pattern emerged between the two strains or between the exponential- and stationary-phase cells of the same strain. In general, however, most fractions contained more unsaturated fatty acids than saturated ones. In the PC fractions of both strains, the C18:2 acid became the predominant component in the stationary-phase cells whereas it was not detected in the PC derived from the growing cells. The C10:0-OH acid was found only in PC (exponential) and PG (stationary) fractions of strain 48-744. Fatty acids with more than 18 carbon atoms were not detected, although the columns were run long enough to detect acids containing up to 24 carbon atoms.

β-Hydroxy lauric acid has been reported to be a major cellular fatty acid in several gonococcal strains (16, 17) and was not extractable by lipid solvents (17). Since only minor amounts of this acid were detected in various phospholipid fractions, we examined the whole-cell fatty acids after saponification of growing cells. Both strains contained the hydroxy acid (Table 5), although its proportion was smaller in the penicillin-resistant strain than in the penicillin-sensitive strain. Palmitic and a C16:1 acid were the other major acids present, as expected.

DISCUSSION

The data presented show that the phospholipids and fatty acids of *N. gonorrhoeae* resemble those of other gram-negative organisms (3, 14, 19, 22) with some exceptions. (i) The gonococcus is among the few gram-negative bacteria that

TABLE 4. *Fatty acids in individual phospholipids of N. gonorrhoeae*

Strain	Growth phase	Phospholipid	Fatty acid												
			12:0[a]	12:2	10:0 —OH	14:0	14:1	14:2	16:0	16:1	16:2	12:0 —OH	18:0	18:1	18:2
B-21	Exponential	CL	2[b]	ND	ND	3	T	1	32	23	ND	2	18	17	1
		PE	1	ND	ND	4	T	T	36	44	ND	T	2	11	1
		PG	1	1	ND	5	2	2	34	20	ND	3	4	21	2
		PC	4	ND	ND	4	5	4	22	23	ND	7	11	19	ND
	Stationary	CL	3	2	ND	2	3	2	24	19	ND	5	6	28	6
		PE	T	T	ND	6	T	T	40	36	ND	T	1	13	1
		PG	T	2	ND	9	T	T	41	31	ND	T	3	9	3
		PC	T	T	ND	1	T	T	15	4	ND	T	5	20	49
48-744	Exponential	CL	T	T	ND	6	T	T	21	23	ND	T	7	31	7
		PE	T	T	ND	3	T	T	42	36	ND	T	1	14	3
		PG	T	T	ND	7	T	1	50	24	ND	1	3	9	2
		PC	ND	ND	11	4	3	3	27	24	ND	8	8	12	ND
	Stationary	CL	1	4	ND	4	1	3	16	13	6	2	12	36	T
		PE	T	1	ND	5	T	T	33	24	ND	T	2	32	T
		PG	T	ND	25	5	1	T	15	10	ND	5	38	ND	ND
		PC	T	3	ND	T	T	T	21	12	ND	T	10	16	36

[a] Number to left of colon refers to number of carbon atoms; number to right of colon refers to number of double bonds; OH refers to β-hydroxy acid.

[b] Numbers refer to percentage of total fatty acids; T, less than 1%; ND, not detected.

TABLE 5. *Total fatty acids of N. gonorrhoeae*

Strain	Fatty acid										
	12:0[a]	14:0	14:1	14:2	16:0	16:1	12:0 —OH	18:0	18:1	18:2	14:0 —OH
BI-21	12[b]	8	T	T	20	36	19	ND	3	ND	ND
48-744	7	8	T	T	35	30	8	2	7	ND	2

[a] Number to left of colon refers to number of carbon atoms; number to right of colon refers to number of double bonds; OH refers to β-hydroxy acid.

[b] Numbers refer to percentage of total fatty acids; T, less than 1%; ND, not detected.

contain PC; the PC content of the stationary-phase cells is two- to fivefold higher than that of the growing bacteria. (ii) In many gram-negative bacteria the amounts of PG and CL vary markedly in growing and stationary-phase cells (19); however, there is little variation in the relative proportions of the major phospholipids of the gonococcal strains studied except for PC, as mentioned previously. (iii) Cyclopropane fatty acids and acids with more than 18 carbon atoms, generally found in gram-negative bacteria (3, 9, 14, 22), were not detected in the gonococci. (iv) β-Hydroxy lauric acid was detected in small amounts in all phospholipid fractions and in large amounts after saponification of the whole cells. (v) The proportion of unsaturated fatty acids increased markedly in PC in the stationary-phase bacteria; this was due largely to the emergence of a C18:2 acid as the predominant component.

The importance of these differences between gonococcal lipids and those of most other gram-negative bacteria is not clear. Possibly the lipid changes which occur on entry of bacteria into stationary phase have survival value; the paucity of such changes in the gonococci may be related to the rapid loss of viability of the organisms.

We found no consistent differences in lipid composition between the penicillin-resistant and -sensitive strains examined. In gram-positive cocci, increase in the content of cellular lipid was found to be related to increased resistance to penicillin (13). This has not been reported in gram-negative bacteria, although resistance to other antibiotics has been correlated with higher lipid content (2, 4, 6) or altered composition (9). Other surface components may also determine interaction of cells with antibiotics; ampicillin-resistant mutants of *Escherichia coli* had a different carbohydrate composition of the lipopolysaccharide as compared to the parent strain (10). Detailed carbohydrate analysis of gonococcal strains is in progress.

The importance of the presence of β-hydroxy lauric acid in large amounts in the cells (after saponification of the bacteria) remains unclear, although its potential use as a marker for detection of pathogenic *Neisseria* has been suggested (17), it may be possible to detect this acid in infected material in small amounts by gas-liquid chromatography.

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LITERATURE CITED

1. Adams, G. A. 1971. The chemical composition of cell wall lipopolysaccharide from *Neisseria sicca*. Can. J. Microbiol. 49:243–250.
2. Anderes, E. A., W. E. Sandine, and P. R. Elliker. 1971. Lipids of antibiotic-sensitive and resistant strains of *Pseudomonas aeruginosa*. Can. J. Microbiol. 17:1355–1365.
3. Asselineau, J. 1966. The bacterial lipids. Holden-Day, Inc., San Francisco.
4. Benbough, J. E., and G. A. Morrison. 1967. The molecular basis of an inhibition by tetracyclines. J. Gen. Microbiol. 49:469–485.
5. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1:279–285.
6. Chang, C., R. E. Molar, and J. C. Tsang. 1972. Lipid content of antibiotic-resistant and -sensitive strains of *Serratia marcescens*. Appl. Microbiol. 24:972–976.
7. Dawson, R. M. C. 1960. A hydrolytic procedure for the identification and estimation of individual phospholipids in biological samples. Biochem. J. 75:45–53.
8. Dawson, R. M. C. 1967. Analysis of phosphatides and glycolipids by chromatography of their partial hydrolysis or alcoholysis products, p. 163–189. *In* G. Marinetti (ed.), Chromatographic analysis of lipids, vol. 1. Marcel Dekker, Inc., New York.
9. Dunnick, J. K., and W. M. O'Leary. 1970. Correlation of bacterial lipid composition with antibiotic resistance. J. Bacteriol. 101:892–900.
10. Eriksson-Greenberg, K. G., K. Nordstrom, and P. Englund. 1971. Resistance of *Escherichia coli* to penicillin. J. Bacteriol. 108:1210–1223.
11. Farquhar, J. W., W. Insull, P. Rosen, W. Stoffel, and E. H. Ahrens. 1959. The analysis of fatty acid mixtures by gas-liquid chromatography. Nutr. Rev. 17(Suppl. August):1–30.
12. Hanes, C. S., and F. A. Isherwood. 1949. Separation of the phosphoric esters on filter paper chromatograms. Nature (London) 164:1107–1110.
13. Hugo, W. B., and R. J. Stretton. 1966. The role of cellular lipid in the resistance of gram-positive bacteria to penicillin. J. Gen. Microbiol. 42:133–138.
14. Kates, M. 1964. Bacterial lipids. Adv. Lipid Res. 2:17–90.
15. Kellog, D. S., Jr., I. R. Cohen, L. C. Norins, A. L. Schroeter, and G. Reising. 1968. *Neisseria gonorrhoeae*. II. Colonial variation and pathogenicity during 35 months in vitro. J. Bacteriol. 96:596–605.
16. Lambert, M. A., D. G. Hollis, C. W. Moss, R. E. Weaver, and M. L. Thomas. 1971. Cellular fatty acids of nonpathogenic *Neisseria*. Can. J. Microbiol. 17:1491–1502.
17. Moss, C. W., D. S. Kellog, Jr., D. C. Farshy, M. S. Lambert, and J. D. Thayer. 1970. Cellular fatty acids of pathogenic *Neisseria*. J. Bacteriol. 104:63–68.
18. Moss, C. W., and V. J. Lewis. 1967. Characterization of clostridia by gas chromatography. Appl. Microbiol. 15:390–397.
19. Randle, C. L., P. W. Albro, and J. C. Dittmer. 1969. The phosphoglyceride composition of gram-negative bacteria and the changes in composition during growth. Biochim. Biophys. Acta 187:214–220.
20. Skipsi, V. P., and M. Barclay. 1969. Thin layer chromatography of lipids, p. 541–548. *In* J. M. Lowenstein (ed.), Methods in enzymology, vol. 14. Academic Press Inc., New York.
21. Strominger, H. E., H. Ackerman, and C. M. Carpenter. 1944. Studies on gonococcus. 1. Constituents of the cell. J. Bacteriol. 47:129–139.
22. Sud, I. J., and D. S. Feingold. 1970. Mechanism of polymyxin B resistance in *Proteus mirabilis*. J. Bacteriol. 104:289–294.
23. Tarlov, A. R., and E. P. Kennedy. 1965. The beta-galactoside permease system and the metabolism of phospholipids in *Escherichia coli*. J. Biol. Chem. 240:49–53.