Identification of Some Basic Extractable Compounds Produced by *Neisseria gonorrhoeae* and *Neisseria meningitidis* in a Defined Medium

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Electron capture gas-liquid chromatography and mass spectrometry have been used to identify some of the basic extractable heptafluorobutyric anhydride reactive compounds found in a defined medium after 20 h of growth by *Neisseria gonorrhoeae* and *N. meningitidis*. Acetoin, 2,3-butanediol, pyrroline, and 1,3-diaminopropane were identified by both gas chromatography and mass spectrometry; 2-hydroxy-pyrrolidine and 3-aminomethyl-pyrrolidine were tentatively identified by mass spectrometry. A possible origin of the amines is through enzymatic oxidation of polyamines.

Morse et al. (3, 4) reported that they used electron capture gas-liquid chromatography (EC-GLC) to detect several basic extractable compounds produced by various species of *Neisseria* cultured in a defined medium. Since two species of this genus are important bacterial pathogens, we decided to attempt to identify some of these compounds so that their possible origin might be established and they would thus be more useful for identifying or classifying *Neisseria* species.

MATERIALS AND METHODS

Reagents. The chloroform used in the study was of nanograde quality (Mallinckrodt). The heptafluorobutyric anhydride (HFBA) was reagent grade (Pierce Chemicals); 2,3-butanediol and 1,3-diaminopropane were Aldrich reagent grade; 3-pyrroline was obtained from K and K Laboratories. Ethanol was undenatured absolute (99.5%), and the pyridine was spectrometric grade (Mallinckrodt).

Standards. Quantities of 189 μ g of 3-pyrroline, 9.8 μ g of 2,3-butanediol, and 8.9 μ g of 1,3-diaminopropane were prepared in 0.1 ml of chloroform and derivatized with HFBA as indicated below.

Cultures. *N. gonorrhoeae* F62, LB999, and 190 and *N. meningitidis* D3201 and M269 were each inoculated into 4 ml of a modified *Neisseria* defined fluid medium with glucose as described (3, 4) and incubated for 20 h. Identification of the organisms was confirmed by standard methods used at the Center for Disease Control (1). Derivatives were prepared for analysis by mass spectrometry and EC-GLC as described below.

Preparation of derivatives. Samples of 4 ml of the spent culture medium were acidified to about pH 2 with 0.1 ml of 50% (vol/vol) H_2SO_4 . The acidified samples were extracted by vigorous shaking with 20 ml of chloroform. The chloroform extracts from the acidified samples were stored for future use, and the residual aqueous phase was then made basic (about

pH 10 to 11) with 8 N NaOH and reextracted with 20 ml of chloroform. The basic extracts were concentrated under a stream of clean, dry air and derivatized with HFBA, pyridine, and ethanol as previously described (2). The derivatized samples in chloroform were evaporated to about 1 drop (but not to dryness), and 0.1 ml of a final solvent consisting of either ethyl ether (for mass spectrometry) or xylene-ethanol 50:50 (for EC-GLC) was added. Samples of 4 μ l were used for GLC-mass spectrometry and 1.4 μ l for EC-GLC analysis.

Apparatus. The derivatives were analyzed on Perkin-Elmer gas chromatographs, models 900 and 3920, which were equipped with frequency pulsed modulated ^{63}Ni electron capture detectors and dual glass columns of 0.3 cm (ID) by 7.6 m (length). The columns were packed with 3% OV-101 liquid-phase material coated on Chromosorb W 80/100 mesh. For analysis of the HFBA derivatives, the instrument was held isothermally at 90°C for 8 min, programmed for a linear increase of 4°C/min to 225°C, and held at 225°C for 24 min. A mixture of argon and methane (95:5) was used as the carrier gas at a flow rate of 50 ml/min. Flush gas was added to increase the flow at the detector to 67 ml/min. The detector temperature was set at 275°C. The standing current was set at 2, and the attenuation was 512 for programmed analysis and 64 for isothermal analysis. A Beckman recorder was used with an input signal of 1 mV and a chart speed of 76.2 cm/h (0.5 inch/min).

Gas chromatography-mass spectrometry. An LKB model 9000 mass spectrometer equipped with a System Industry system 150 computer package and a DuPont model 21-491B mass spectrometer interfaced with a Varian 2700 gas chromatograph were used to obtain mass spectra. The 21-491B was equipped with a chemical ionization source, and the interfacing gas chromatograph contained an electron capture detector. The resolution of both instruments was approximately 1,000. The effluent from the LKB gas chromatograph was monitored by a total ion current detector. The gas chromatographs were equipped with

0.3-cm (ID) by 3.6-m (length) glass columns packed with Chromosorb W 80/100 mesh (AW-DMCS H.P.) coated with 3% OV-1. Helium was used as the carrier gas, and methane was used as reagent gas for chemical ionization. The LKB gas chromatograph was programmed at 5°C/min from 90 to 225°C, and the Varian GC was programmed at 4°C/min from 90 to 225°C. Both instruments were then held isothermally at 225°C for 48 min.

RESULTS

The chromatogram shown in Fig. 1 is the profile obtained from *N. meningitidis* D3201. The strains of *N. meningitidis* and *N. gonorrhoeae* used in this study produced the same compounds in sufficient concentration to be detected by mass spectrometry. However, this does not mean that all strains of *N. meningitidis* and *N. gonorrhoeae* will produce the same patterns shown in Fig. 1 (see reference 1). Identification by mass spectrometry and gas chromatography of peak 1 (Fig. 1) was described previously (3). The mass spectrum of peak 2 is shown in Fig. 2. The compound had been determined both by

FIG. 1. EC-GLC chromatogram of HFBA derivatives prepared from a pH 10 chloroform extraction. Analyses were made on a 3% OV-101 column.

FIG. 2. Peak 2: Computerized mass spectra of di-heptafluorobutyl 2,3-butanediol analyzed at 30 eV. Background components have been removed by the computer.

mass spectral analysis of known and unknown and by EC-GLC retention time comparison of known and unknown to be the heptafluorobutyl derivative of 2,3-butanediol. The molecular ion at mass to charge ratio (m/e) 482 was missing. By lowering the electron voltage from 70 to 30 eV, the fragments shown in Fig. 1 were obtained. There was an M-15 peak at m/e 467. The fragment at m/e 240 was possibly obtained by a cleavage between the 2 and 3 carbons to yield

The fragments at m/e 69, 169, 197, and 213 are indicative of fragmentation of the heptafluorobutyl groups. The base peak at m/e 56 is probably the ion

Figure 3 shows the fragmentation pattern obtained from peak 3. Both the mass spectral analysis of known and unknown and the EC-GLC retention times of known and unknown showed the compound to be heptafluorobutyl pyrroline. There was a strong molecular ion at 265. The pyrroline ring is evident in the fragments at m/e 146 (Fig. 4A), 96 (4B), 78 (4C), and 68 (4D). The fragments at 69 and 169 are typical fragments of the heptafluorobutyl group.

Figure 5 shows the mass spectrum obtained from peak 4 (Fig. 1). On the basis of mass spectral evaluation, the compound was tentatively identified as heptafluorobutyl-2-hydroxy-pyrrolidine. There was a weak molecular ion at m/e 283. There was a loss of H_2O to form the fragment at m/e 265 (Fig. 4E), and a ring cleavage to form the fragment at m/e 240 (4F). The fragment at m/e 226 was formed by elimination

FIG. 3. Peak 3: Computerized mass spectra of heptafluorobutyl pyrroline with background components removed by the computer.

FIG. 4. Mass spectral fragments of basic extractable compounds of *Neisseria*.

of CH_2 from the fragment at m/e 240 (Fig. 4G). The fragment at 86 is the 2-hydroxy-pyrrolidine ring, which remains after the heptafluorobutyryl group has split off. The fragments present at m/e 69 and 169 indicate the presence of HFBA.

Figure 6 shows the mass spectrum obtained from peak 5. This peak has been identified, both by mass spectral analysis of known and unknown and by EC-GLC retention times of the known and unknown, as the HFBA derivative of 1,3-diaminopropane. The molecular ion is present at m/e 466. The elimination of one heptafluorobutyryl group produced the fragment at m/e 254. The fragment at m/e 240 is possibly formed by cleavage of a bond alpha to the amine group

to leave $\text{CF}_3\text{CF}_2\text{CF}_2\text{C}(=\text{O})\text{NH}-\text{CH}_2\text{CH}_2$. The fragments at m/e 226 and 237 are possibly $\text{CF}_3\text{CF}_2\text{CF}_2\text{C}(=\text{O})\text{NH}-\text{CH}_2^+$ and $\text{CF}_3\text{CF}_2\text{CF}_2\text{C}(=\text{O})\text{NH}-\text{CH}_3$, respectively. The fragment at m/e 84

appears to be $\text{CH}_2=\text{CH}-\text{CH}_2-\text{NH}-\text{C}(=\text{O})^+$. The fragment at m/e 72 was probably formed by the loss of both heptafluorobutyryl groups to leave the ion $\text{NH}^+-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}$. The fragments at 69, 138, and 169 were formed by fragmentation of the heptafluorobutyryl groups.

Figure 7 shows the mass spectrum of peak 6. The compound has been tentatively identified by its mass spectra as heptafluorobutyryl-3-aminomethyl pyrrolidine. The molecular ion is present at m/e 492. There was a loss of CH_3 to form the fragment at m/e 477 (Fig. 4H). The fragment at m/e 323 was formed by the loss of $\text{CF}_3\text{CF}_2\text{CF}_2$ to form (4I). There was further cleavage of the carboxyl group from this ion to

FIG. 5. Peak 4: Computerized mass spectra of heptafluorobutyryl-2-hydroxy-pyrrolidine. Background has been removed by the computer.

form the fragment at m/e 295 (4J). The fragments at m/e 279 and 266 are possibly represented by (4K) and (4L). The fragments at m/e 254, 252, and 226 appear to be formed by loss of HFBA (Fig. 4M, N, and O). The fragment at m/e 110 might have been formed by cleavage of HFBA followed by rearrangement to give the ion (4P). The fragments at m/e 82 and 84 were formed by cleavage of HFBA and nitrogen from the molecule, followed by rearrangement to give the ions (4Q) and (4R). The fragments at m/e 69 and 169 were formed by the fragmentation of the HFBA group.

DISCUSSION

Little has been published concerning amine production by *Neisseria* species. Morse et al. (3, 4) reported detection of basic extractable compounds produced by strains of *N. gonorrhoeae* and *N. meningitidis* that reacted with HFBA to form electron-capturing derivatives. Weaver and Herbst (5) reported an amine oxidase produced

FIG. 6. Computerized mass spectra of diheptafluorobutyl-1,3-diaminopropane with background removed by the computer.

FIG. 7. Peak 6: Computerized mass spectra of diheptafluorobutyl aminomethyl pyrrolidine with background removed by the computer.

by strains of *N. perflava* that oxidized the polyamines spermine and spermidine to 1,3-diaminopropane. They further speculated that α -aminobutyraldehyde was formed by oxidation of putrescine and that this compound could undergo spontaneous oxidation to form pyrroline. We have detected formation of putrescine, but it does not accumulate in most cultures, which could indicate that it is an intermediate. The investigators (5) further speculated that *N*-(3-aminopropyl)- α -aminobutyraldehyde could be formed and then could undergo cyclization to

form *N*-(3-aminopropyl)-pyrrolidine. Although they did not mention other *Neisseria* species which produce the diamine oxidase, the amines we are reporting may quite possibly be formed during growth in a manner similar to that postulated for *N. perflava*. More research is needed to either confirm or disprove the hypothesis. In either case, identification of these compounds should increase our understanding of the metabolic characteristics of this very important group of organisms.

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