Identification of *Neisseria* by Electron Capture Gas-Liquid Chromatography of Metabolites in a Chemically Defined Growth Medium

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A dual-purpose study was carried out in an attempt to develop a rapid, sensitive method to identify *Neisseria* species by gas chromatography and to learn more about the metabolism of these organisms. Sixty-nine isolates of *Neisseria* were grown in a chemically defined fluid medium; the spent medium was extracted sequentially at pH 2 with diethyl ether and at pH 10 with chloroform. The pH 10 extracts were derivatized with heptafluorobutyric anhydride and analyzed by electron capture gas-liquid chromatography. The resulting spent culture medium electron capture gas-liquid chromatography profiles showed several qualitative and significant quantitative differences among the *Neisseria* species potentially useful in separating and identifying these organisms. Putrescine and cadaverine, which were present in the spent culture medium of some *Neisseria*, including *N. gonorrhoeae*, were tentatively identified. Substituting carbohydrates for the chemically defined medium containing glucose in the base medium produced altered profiles with increased quantitative and qualitative differences.

Laboratory identification of *Neisseria* species is presently based on few criteria, routinely including: colony characteristics; Gram-staining reaction and cellular morphology; oxidase activity; and formation of acid from such carbohydrates as dextrose, maltose, sucrose, fructose, and sometimes lactose (Table 1). In addition, specific fluorescent conjugates and the ability to grow in the presence of vancomycin, colistin, and nystatin are used (24, 32). Not all *Neisseria* isolates can be identified by these methods for several reasons. Production of acid often varies from the expected patterns. Failure to form acid from one or more of the carbohydrates or to do so weakly is common within the genus. Furthermore, there is some evidence that sulfadiazine-resistant meningococci do not ferment maltose (21). Thus, additional criteria to identify the *Neisseria* are needed.

The effectiveness of gas-liquid chromatography (GLC) in analyzing cellular or metabolic products of *Neisseria* has been investigated. Analyses of cellular fatty acids of selected species have been performed, and a variety of long-chain fatty acids have been identified (9, 22, 23, 27). Brooks et al. (10) analyzed the hydroxy acids found after the growth of *Neisseria* on agar medium. Differences in GLC profiles among several species and the effects of medium com-

ponent variation on the metabolic profiles were demonstrated (10). These investigations emphasized the need to develop a chemically defined medium essentially free from compositional variation. Also, the analysis of other metabolic products such as amines might provide additional *Neisseria* identification criteria and also new data concerning their metabolism.

The production of amines by *Neisseria* has not been investigated. Practical methods introduced by Brooks et al. (6) for extracting spent culture medium, derivatizing with heptafluorobutyric anhydride, and analyzing by electron capture GLC (EC-GLC) offer selective and sensitive methods with which to study these compounds. The purpose of this investigation was to use EC-GLC to examine metabolites of *Neisseria* after they were grown in a defined medium. Additional objectives were to determine the reproducibility of EC-GLC profiles of *Neisseria* after they were grown in a defined medium and to evaluate the usefulness of the profiles for differentiating among various species.

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MATERIALS AND METHODS

Organisms. The following organisms were obtained from Center for Disease Control (CDC) stock

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TABLE 1. Conventional biochemical results of the *Neisseria* species studied, showing acid produced from carbohydrates and oxidase activity^a

Species	No. of isolates studied	Glucose	Maltose	Sucrose	Fructose	Lactose	Oxidase
<i>N. gonorrhoeae</i>	23	A	0	0	0	0	+
<i>N. meningitidis</i>	10 ^b	A	A	0	0	0	+
<i>N. lactamica</i>	3	A	A	0	0	A	+
<i>N. catarrhalis</i>	5	0	0	0	0	0	+
<i>N. sicca</i>	3	A	A	A	A	0	+
<i>N. flavescens</i>	3	0	0	0	0	0	+
<i>N. flava</i>	3	A	A	0	A	0	+
<i>N. perflava</i>	3	A	A	A	A	0	+
<i>N. subflava</i>	5	A	A	0	0	0	+
<i>N. caviae</i>	2	0	0	0	0	0	+
<i>N. mucosa</i>	2	A	A	A	A	0	+
<i>N. cinerea</i>	1	0	0	0	0	0	+
<i>N. ovis</i>	1	0	0	0	0	0	+
<i>N. elongata</i>	3	0	0	0	0	0	+
<i>N. haemolysans</i>	2	A	A	A	A	0	+

^a A, Acid produced from carbohydrate; 0, no acid produced from carbohydrate; +, oxidase positive.

^b Includes serotypes A, B, C, D, C + Y, Slaterus X, Y, and Z, and untypable.

cultures: *Neisseria gonorrhoeae* strains 12-51970-5, 38-51970-5, 35-15970, 33-51970, 30-15970, 27-15970, 26-15970, 22-15970, Grady 005, F-62 (colony type 1), 9 (colony type 1), 2686 (colony type 1), 268-4, 350-4, 354-4, and 356-6; *N. meningitidis* strains M-1036 serotype A, ATCC-CDC 15090 type B, M-269 type B, 1170 type C, B-4070 type C+Y, M-158 type D, KC-660 Slaterus type X, D-1796 Slaterus type Y, B-4390 Slaterus type Z, and 254-P untypable; *N. lactamica* strains A-2894, A-5906, and A-7515; *N. flava* strains N-13 and N-17; *N. perflava* strains B-491 and L-5; *N. subflava* strains B-886, B-2026, N-15, and N-14; *N. flavescens* strains MISS, N-159, and N-155; *N. catarrhalis* strains, genus *Branhamella* (28), KC-179, IIPG-70, 9176, 8176, and M-48; *N. sicca* strains N-5, L-5, and Chamblee; *N. caviae* strains KC-548 and KC-546; *N. ovis*; *N. mucosa* strains C-4497 and 7259; *N. cinerea* strain KC-796; and *N. haemolysans*, genus *Gemella* (28), strains ATCC-CDC-10379 and KC-648.

In addition, six auxotypes of *N. gonorrhoeae*, 27628, 27629, 27630, 27631, 27632, and 27633 (12), were obtained from David Gibbs, CDC. Also, three strains of *N. elongata* (4), M-2, 7823/71, and 8554/71, were obtained from K. Bøvre and E. Holten, University of Oslo, Oslo, Norway.

All cultures were identified by conventional bacteriological procedures. In addition, the meningococci were serotyped (14). The cultures were preserved by quick freezing (−50°C) in Trypticase soy broth (BBL) containing 21% glycerine. This method was generally satisfactory, and cultures remained viable for over a year.

Media. Working cultures were maintained on GC medium base (Difco) with a defined supplement as reported by White and Kellogg (32).

The medium used in EC-GLC studies was *Neisseria* defined fluid medium (NEDF) prepared as described (15), except as follows: (i) stock solutions 1, 3, 5, and 6 were autoclaved, whereas all others were sterilized by membrane filtration (0.22-μm pore size); (ii) 50 mg of ornithine was added per liter of medium; and (iii)

the final pH of the medium was adjusted to 7.3 ± 0.5 with sterile 1 N NaOH.

NEDF base medium was also prepared with maltose, sucrose, or lactose instead of glucose.

Cultural procedures and growth conditions. NEDF was dispensed in 10-ml amounts into 50-ml cotton-plugged Erlenmeyer flasks and allowed to reach room temperature before it was used. Sixteen- to 20-h cultures of *Neisseria* grown on GC medium base were used as inoculum. A 2-mm loop was used to inoculate one loopful of cells into each flask; flasks were placed in a candle extinction container and incubated at 35 to 36°C on a rotary shaker (approximately 120 rpm) for 16 h. An uninoculated flask served to check medium sterility and later was used as a medium control for the GLC determinations.

So that a large number of flasks could be incubated at one time, a thin-layer chromatography slide-holding box was used as a candle extinction container. Three of four candles were used in this essentially gas-tight container. The box had a shelf unit, accommodated 30 to 40 flasks, and fit conveniently on a rotary shaker (New Brunswick Scientific Co.). A grid of adhesive tape fixed to the smooth inner surfaces of the box prevented movement of the flasks during rotation.

Extraction and derivatization procedure. After a 16-h incubation, 0.2 ml of 50% (vol/vol) H₂SO₄ was added to each flask, reducing the pH to about 2 (pH paper). The acidified medium was then extracted with 20 ml of diethyl ether stabilized with ethanol (Baker Chemical Co.), and the ether layer was stored at 4°C for future analysis (10). After the pH 2 extraction, 1.0 ml of 8 N NaOH was added to the residual aqueous fraction to raise the pH to approximately 10 to 11 (pH paper). This fraction was then re-extracted with 20 ml of Nanograde-quality chloroform (Mallinckrodt) to obtain the basic extractable compounds. Acetyl-methylcarbinol (AMC) and 2,3-butanediol (BDO) were not soluble in ethyl ether and were extracted with chloroform from the basic extraction. Subsequent derivatization for EC-GLC analysis was performed as

described (6).

GLC analysis. Most analyses of the derivatized samples were made on a Barber-Coleman series 5000 gas chromatograph equipped with dual (300-mCi) tritium electron capture detectors and dual U-shaped columns (0.6-cm ID by 7.3-m length). The columns were packed with Chromosorb W 80/100 mesh (DMCS H.P.) and coated with 3% OV-1 (Analabs, Inc.). The instrument was operated isothermally for 8 min at 90°C, after which the temperature was linearly increased by 5°C/min to 200°C and held for 26 min. The detector and injector temperatures were 200°C. Oxygen-free nitrogen (Matheson) was used as carrier gas at a flow rate of 40 ml/min, with a detector scavenger gas flow of 10 ml/min. A recorder input signal of 1 mV was used with a chart speed of 0.5 inch (1.27 cm)/min.

Some analyses were also made on a Perkin-Elmer model 900 gas chromatograph fitted with a polar column (Tabsoorb, Regis Chemical Co.) and a 3% OV-1 nonpolar column. The columns were coiled glass (0.3-cm ID by 7.3-m length). The instrument was equipped with a ⁶³Ni (10 mCi) electron capture detector operated in the frequency pulse-modulated mode in the manner described by Brooks et al. (11). The instrument was held at 90°C for 8 min, after which the temperature was linearly increased by 4°C/min to 225°C. The manifold, detector, and injector temperatures were 250, 275, and 225°C, respectively. The carrier gas (95% argon-5% methane) was set at a flow rate of 50 ml/min, with a detector scavenger gas flow rate of 17 ml/min. The recorder was operated under the same conditions as given for the Barber-Coleman.

All analyses were made within 24 h of making the derivatives. Attempts were made to identify tentatively the compounds detected in the cultures by comparing retention times with heptafluorobutyric anhydride-derivatized standards chromatographed under the same conditions on polar and nonpolar columns (7). A standard injection of 1.4 μ l was used throughout the study. All isolates were cultured and analyzed at least twice during the study.

RESULTS

The *Neisseria*, including 22 of 23 isolates of *N. gonorrhoeae*, grew well in NEDF medium. *N. haemolysans* did not attain the turbidity that the other species did in 16 h, and one isolate each of *N. gonorrhoeae* and *N. meningitidis* required an extended incubation period of >40 h to attain the same turbidity as the other cultures. The degree of growth was established through repeatedly culturing these organisms in NEDF and comparing them against McFarland standards. EC-GLC analysis of the repeated cultures gave essentially identical chromatograms. Generally, incubation for >16 h did not drastically alter the EC-GLC profiles of the *Neisseria*, but some compounds appeared in greater concentration. Good growth of the organisms in the medium was dependent on rotation. Cultures of *N. gonorrhoeae* that were not rotated did not

become turbid within 48 h.

Figure 1C shows a medium control that was processed in the same manner as the cultures. It is apparent that the medium control is free of electron-absorbing compounds, which was true for the controls throughout the study.

All the organisms studied were cultured in batches of media prepared from the same basic lot of components. EC-GLC profiles of one isolate of *N. gonorrhoeae* grown in several different batches of NEDF remained essentially the same (Fig. 1A and B). This reproducibility held true for all isolates.

The chromatograms of all of the gonococci tested in NEDF with glucose were qualitatively similar, but there were major quantitative differences in the peaks labeled 1, 15, 17, 27, 28, and 29 (Fig. 2A).

Figure 2B shows the EC-GLC profile of a meningococcus. Serotypes B, C+Y, and Slaterus X, Y, and Z were all quite similar to the profile shown in Fig. 2B. Peaks 2 and 7, AMC and BDO (25), in the profiles of serotypes C and D were one-third to one-half scale. The C and D profiles were nearly identical. In the serotype A profile, peaks 10, 15, and 28 were almost absent, and peaks 26 and 29 were one-third less than is shown in Fig. 2B. The EC-GLC profile of the untypable strain (254-P) had the greatest similarity to the gonococcal profiles but had several quantitative differences, especially in peaks 19, 23, 24, and 25.

The metabolic profiles of *N. lactamica* grown in NEDF with glucose (Fig. 2C) were similar to those shown for meningococci (Fig. 2B); however, a major consistent difference was full-scale deflection of products represented by peaks 15 and 20 and 26. These profiles and those of *N. gonorrhoeae* and *N. meningitidis* are sufficiently similar to justify designating them as group 1.

Figure 3 shows additional metabolic groups of *Neisseria*. *N. flava*, *N. perflava*, and *N. subflava* profiles formed group 2a (Fig. 3A). *N. sicca*, *N. mucosa*, and *N. cinerea* profiles were very similar to those of group 2a and were designated group 2b (Fig. 3C). *N. (Branhamella) catarrhalis*, *N. caviae*, and *N. ovis* profiles formed group 3 (Fig. 3B). There were subgroups in group 3. Subgroup 3a gave nearly full-scale or full deflection of AMC and BDO, and peak 19 was missing. AMC and BDO were nearly absent in subgroup 3b.

Figure 4A shows the *N. flavescens* profile, which is designated group 4. This organism produced only trace amounts of AMC and BDO (peaks 2 and 7), a fact that easily differentiated it from group 1 and 2 organisms. The consistent presence of peak 19 differentiated it from group

FIG. 1. Gas chromatograms of chloroform extracts (pH 10) of duplicate cultures of *N. gonorrhoeae* in different batches of NEDF medium (A and B) and a medium control (C). Heptafluorobutyric anhydride derivatives of the extracts were analyzed by using an OV-1 column and an electron capture detector. R, Residual chloroform.

3 organisms. *N. elongata*, the only rod-shaped organism in the genus (4), differed greatly from the other species of *Neisseria* and is designated group 5 (cf. Fig. 4B with the other *Neisseria* profiles and note that almost all peaks are missing). The results of this study and the fact that *N. elongata* has a morphology different from that of other members of the genus present good evidence for removal of this organism from the genus *Neisseria*. *N. (Gemella) haemolysans* (Fig. 4C), which has already been removed from the genus *Neisseria* (28), produced an abundance of AMC, and little else, and was designated group 6.

The profiles shown in Fig. 5 are those from an isolate of *N. gonorrhoeae* grown in NEDF with glucose (Fig. 5A), NEDF with maltose substituted for glucose (Fig. 5B), and NEDF without carbohydrate (Fig. 5C). The purpose of this experiment was to determine whether a different

carbohydrate would cause profile changes valuable in differentiating *Neisseria* species. Compare peaks 2, 7, 10 (this peak may be more than one compound), 12 (greatly increased in the absence of carbohydrate), and 23 to 30 (Fig. 5A, B, and C). *N. gonorrhoeae* grew equally well in the presence of glucose or maltose, but different by-products were produced. For example, reduced amounts of AMC and BDO (peaks 2 and 7) but increased amounts of peaks 10, 17, 23, 24, 25, 26, and 27 occurred with maltose.

In a wider range of carbohydrate substitution with selected isolates, glucose, maltose, sucrose, or lactose was used in the NEDF base medium. Lactose was chosen because of the action of *N. lactamica* on it (18). Isolates of *N. gonorrhoeae*, *N. meningitidis*, and *N. lactamica* were cultured on all four of the carbohydrates. Differences in the profiles as the species and sugars were varied existed mostly in AMC and BDO peaks and

FIG. 2. Gas chromatograms of heptafluorobutyric anhydride-derivatized chloroform extracts (pH 10) of *N. gonorrhoeae* (A) and *N. meningitidis* (B) cultured in NEDF medium. R, Residual chloroform.

peaks 10 and 19. Measurement of the pH of the spent NEDF media was a useful indicator of expected activity. With an initial pH of 7.3, the spent medium pH was depressed to 5.0 to 5.5 when the carbohydrate was degraded by the strains of *N. gonorrhoeae* (glucose), *N. meningitidis* (glucose, maltose), and *N. lactamica* (glucose, maltose, lactose). When these strains grew but did not degrade the carbohydrate, the pH of the spent medium ranged from 6.8 to 7.0. Strains of *N. catarrhalis*, *N. cinerea*, and *N. flavescens* grown on NEDF with either glucose or maltose lowered the original pH of the spent medium from 7.3 to between 6.4 and 6.8.

N. meningitidis and *N. lactamica* appeared to make more products when they were grown in the presence of maltose and lactose, but as with *N. gonorrhoeae* the number of products varied with the different carbohydrates. These data are still being evaluated. One notable sim-

ilarity was found in the use of sucrose, in the presence of which all three species produced considerably less BDO (peak 7) and less AMC (peak 2).

Some analyses were performed on OV-1 (non-polar) and TABSORB (polar) columns with a Perkin-Elmer instrument equipped with a frequency pulse-modulated mode electron capture detector (11). With this instrument, tentative identification of putrescine and cadaverine (not shown) was made by comparing retention times of derivatized standards with the culture products. These products were detected in the pH 10 culture extracts of all *Neisseria* species except *N. elongata* and *N. (Gemella) haemolysans*.

DISCUSSION

Our first objective was to find a defined medium that supported good growth of the *Neis-*

FIG. 3. Gas chromatograms of heptafluorobutyric anhydride-derivatized chloroform extracts (pH 10) showing group profiles of several species of *Neisseria*. R, Residual chloroform.

seria. A number of chemically defined preparations were tried unsuccessfully, including those developed by Frantz (17), Kenny et al. (20), Hunter and McVeigh (19), and some modifications of our own based on tissue culture media. NEDF medium, when properly prepared, gave good growth with a clean EC-GLC background. Because it is chemically defined, NEDF can be prepared repeatedly to the same specifications. Medium reproducibility is a major concern if standardized EC-GLC profiles are to be obtained among laboratories. Media containing complex components such as peptones, autolysates, or serum can, and usually do, vary in composition among lots. This variation can affect the background, the metabolism, and sometimes the growth of the organisms (10). Although EC-GLC background peaks can be accounted for with controls, the response of the organisms to the differences in the medium may not be predictable. In addition to the need for reproducible media, the extraction, derivatization, and injection procedures must be reproducible. The last three require technical mastery through practice.

Quality control measures should be applied to the water used to make the medium (1, 19, 30). Certain *Pseudomonas* species can grow in distilled-water reservoirs (13, 16) or demineralizers and produce compounds detectable by EC-GLC.

FIG. 4. Gas chromatograms of heptafluorobutyric anhydride-derivatized chloroform extracts (pH 10) of NEDF cultures of *Neisseria* species. Observe that three additional groups are formed and that *N. elongata* and *N. haemolysans* are distinctly different from the other *Neisseria* species. R, Residual chloroform.

Statements have been made about the relative biochemical inactivity of the *Neisseria*, especially in the case of *N. gonorrhoeae* (3, 29, 33). These organisms may be fastidious in their growth requirements but that they are metabolically active is evidenced by the number and quantity of compounds detected by EC-GLC in 16-h cultures of *Neisseria* in a chemically defined medium. It is evident that EC-GLC can be an important analytical tool to aid in identifying *Neisseria* species.

A number of pathways are involved in the glucose metabolism of various *Neisseria* (2, 26, 31). Glycolytic formation of lactic acid with subsequent oxidation to pyruvate and then to acetate has been recognized in both *N. gonorrhoeae* (2, 26) and *N. meningitidis* (31). Vanderkerkove and co-workers (31) also established the production of AMC from glucose by *N. meningitidis* and certain other species of *Neisseria*. We have established the production of AMC by *N. gonorrhoeae* and other *Neisseria* species and have tentatively identified BDO (25).

The production of amines by the *Neisseria* has not been studied previously. Putrescine and cadaverine were tentatively identified on both polar and nonpolar columns from *Neisseria* cultures grown in the defined medium. Until now only a few of the peaks from the pH 10 extracts

FIG. 5. Gas chromatograms of heptafluorobutyric anhydride-derivatized chloroform extracts (pH 10) of a single isolate of *N. gonorrhoeae* cultured in NEDF (A), NEDF with maltose substituted for glucose (B), and NEDF without carbohydrate (C). R, Residual chloroform.

had been identified; however, recent mass-spectral studies completed in this laboratory (submitted for publication) show that most of these basic extractable compounds are amines. These and other metabolites identified *in vitro* may provide clues for future research on the *in vivo* activities of the *Neisseria*. The techniques used in this study have also been used to detect bacterial metabolites *in vivo* (8).

Modifying the growth medium can alter the metabolism of gonococci. We found that substituting maltose for glucose resulted in significant qualitative and quantitative differences in the metabolic end products. Although routine tests for the identification of *Neisseria* species depend on acid production from one or more carbohydrates, it is now evident that *N. gonorrhoeae* can utilize maltose but produce no acidic end

products by alternate pathways of 3-carbon fragment metabolism. Through such manipulation of media compounds, it is possible to alter the EC-GLC profiles of *Neisseria* and obtain EC-GLC profiles that show greater differences among species.

There is increasing support for the expanded use of GLC in clinical microbiology to aid in disease diagnosis (5). We feel that our study results add to this support. Efforts should be made to refine further these and similar techniques to provide useful new and more reliable identifying criteria for the clinical laboratory.

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