Electron Capture Gas Chromatographic Detection of Acethylmethylcarbinol Produced by *Neisseria Gonorrhoeae*

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Acethylmethylcarbinol (acetoin) production by *Neisseria gonorrhoeae* and other *Neisseria* species was established by gas-liquid chromatography and by mass spectrometric data. Sixty-nine isolates of *Neisseria* were tested by incubating them in a chemically defined fluid medium. The medium was extracted with organic solvents and derivatized with heptafluorobutyric anhydride for gas chromatography and mass spectrometry. Cultures of 58 of the same strains were tested with the conventional Voges-Proskauer reagents, and results were compared with those of gas-liquid chromatography. When glucose was used as an energy source, *N. gonorrhoeae*, some *N. meningitidis*, and *N. lactamica* produced enough acetoin in 16 h to be detectable by either method, whereas other *Neisseria* species produce amounts detectable only by gas chromatography. The conventional acethylmethylcarbinol test with the chemically defined medium and maltose as an energy source might be used to develop methods that would differentiate certain members of the genus, including the pathogenic species.

The production of acethylmethylcarbinol (AMC) is recognized as a common criterion for the identification of several organisms in the family *Enterobacteriaceae*. The enzymes necessary for the production of this compound are commonly found among microorganisms (17). For a review of AMC production, readers are referred to an article by Eddy (11). AMC production by several of the *Neisseria* was established by Berger (1) and Vandekerkove et al. (15). We established its presence in cultures of *N. gonorrhoeae* and other *Neisseria* while attempting to identify metabolic products produced by these organisms. These products were detected by electron capture gas-liquid chromatography (EC-GLC).

Gas-liquid chromatography has been shown to be a valuable technique for identifying metabolic products of the *Neisseria* (6). During the course of our investigation of the *Neisseria*, we noticed a recurring peak in the gas chromatographic profiles that we later identified by gas chromatography-chemical ionization mass spectrometry (GC-CMS) as AMC. The purpose of this study was to investigate the use of EC-GLC to detect AMC production and to study its production within the genus *Neisseria*.

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Carolina in partial fulfillment of the requirements for the Ph.D. degree.)

MATERIALS AND METHODS

Organisms. The following organisms were obtained from the Center for Disease Control stock cultures: *N. gonorrhoeae* strains 12-51970-5, 38-51970-5, 35-51970, 33-51970, 30-51970, 27-51970, 26-51970, 22-51970, 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 type A, CDCK-1 (derived from ATCC 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 Y, B-4390 Slaterus 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-14, and N-15; *N. flavescens* strains Mississippi, N-159, and N-155; *N. catarrhalis* (8) strains KC-179, II PG-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. haemolysans* (14) strains CDCK-2 (derived from ATCC 10379) and KC-648; and *N. cinerea* strain KC-796.

Three strains of *N. elongata* (2), M-2, 7823/71, and 8554/71, were obtained from K. Bøvre and E. Holten, University of Oslo, Norway.

In addition, six auxotyped strains of *N. gonorrhoeae* (7), 27628, 27629, 27630, 27631, 27632, and 27633, were obtained from David Gibbs, Center for Disease Control postdoctoral fellow.

We verified all cultures by Gram stain, oxidase test, and carbohydrate tests as described (16). In addition, all meningococci were serotyped as described (10). Stock cultures were maintained by

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freezing at -50°C in Trypticase soy broth containing 21% glycerin. Although this maintenance procedure was generally satisfactory for at least a year's storage, some strains of *N. gonorrhoeae* were difficult to preserve in this manner.

Media. Working cultures were maintained on GC base medium (Difco) with a defined supplement as described by White and Kellogg (16). The growth medium was *Neisseria* defined fluid medium (NEDF) as described by Catlin (9), with the following exceptions: (i) all stock solutions that could not be autoclaved 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. Maltose (0.5%) was substituted for glucose in the NEDF base and used as a substrate for selected organisms.

Cultural conditions. One loopful (2-mm loop) of culture was inoculated into 10 ml of NEDF in a 50-ml Erlenmeyer flask fitted with a cotton plug. Flasks were incubated for 16 h at 35 to 36 $^{\circ}\text{C}$ in a candle extinction container that had been placed on a rotator (approximately 120 rpm).

Detection of AMC by conventional method (Voges-Proskauer). Alpha-naphthol and potassium hydroxide-creatine reagents were used (12). One milliliter of a 16-h culture was placed in a glass culture tube (12 by 75 mm), and 10 drops of α -naphthol reagent and 5 drops of KOH-creatine reagent were added with a disposable Pasteur pipette. The mixture was shaken well and allowed to stand for 1 h. An uninoculated medium control, a known positive AMC culture (*Enterobacter cloacae*), and a known negative AMC culture (*Escherichia coli*) were used. The control cultures were incubated in NEDF in the same manner as the *Neisseria*. A positive test was indicated by the appearance of a pink to red color beginning at the surface of the test medium. Color development usually occurred within 15 to 30 min.

Detection of AMC by gas chromatography method. Whole 16-h cultures (10 ml) were acidified to about pH 2 (pH paper) with 0.2 ml of 50% H_2SO_4 and extracted with 20 ml of diethyl ether (Baker-ethanol stabilized) to remove acids and ether soluble alcohols. The pH 2 extract (ether layer) was stored for future analysis, and the residual aqueous layer was then made basic (pH 10 to 11) with 1 ml of 8 N NaOH and reextracted with 20 ml of pesticide-quality chloroform (Matheson). This pH 10 extract was derivatized using heptafluorobutyric anhydride (HFBA) by the methods outlined by Brooks et al. (3). The sample was placed in 100 μl of ethyl ether, and 1.4 μl of this solution was routinely injected.

Gas chromatography. A Barber-Colman series 5000 gas chromatograph equipped with dual tritium 300-mCi EC detectors was used. The instrument was fitted with two glass U-shaped columns (0.6-cm inner diameter by 7.3-m length). Both columns were packed with 3% OV-1 coated on 80/100-mesh high-performance Chromosorb W (Analabs, Inc.). The column temperature was held at 90 $^{\circ}\text{C}$ for 8 min and then programmed for a linear increase of 5 $^{\circ}\text{C}/\text{min}$ to 220 $^{\circ}\text{C}$ and held at this temperature for 26 min. The temperature of both the injector and the detectors

was 220 $^{\circ}\text{C}$. Oxygen-free nitrogen (Matheson) was used as the carrier gas at a flow rate of 40 ml/min with a detector scavenge gas flow rate of 10 ml/min. Dual recorders were operated at 1 mV with a chart speed of 0.5 inch/min.

A Perkin-Elmer model 900 gas chromatograph equipped with a ^{63}Ni 10-mCi EC detector was also used. The detector was operated in the frequency pulse modulated mode (13) in the manner described by Brooks et al. (3). This instrument was equipped with dual coiled glass columns (0.3-cm inner diameter by 7.3-m length). One column was packed with 3% OV-1 (nonpolar column) and the other with TABSORB (polar column) (Regis Chemical Co.). This instrument was operated isothermally for 8 min at 90 $^{\circ}\text{C}$ and then programmed for a linear increase of 4 $^{\circ}\text{C}/\text{min}$ to 225 $^{\circ}\text{C}$. The injector temperature was 225 $^{\circ}\text{C}$; the manifold and detector temperatures were 250 $^{\circ}\text{C}$. A mixture of argon-methane (95:5) was used as the carrier gas at a flow rate of 50 ml/min with a detector scavenge gas flow rate of 18 ml/min. The recorder was operated at 1 mV with a 0.5-inch/min chart speed.

Mass spectrometry. A Dupont model 21-491B mass spectrometer interfaced with a Varian 2700 gas chromatograph was used. The resolution of the instrument was about 1,000, and the temperature of the ion source was 220 $^{\circ}\text{C}$. The instrument was operated in the chemical ionization mode with methane as reagent gas. Electron energy was 70 electron volts. The gas chromatograph was equipped with a coiled stainless-steel column (0.3-cm inner diameter by 7.3-m length) packed with TABSORB. The effluent from the column was split so that part of the effluent monitored by an EC detector with nitrogen as the scavenger gas. The gas chromatograph was operated for 8 min isothermally at 90 $^{\circ}\text{C}$ and then programmed for a linear increase of 4 $^{\circ}\text{C}/\text{min}$ to 225 $^{\circ}\text{C}$. Helium was used as the carrier gas with a flow of 36 ml/min.

RESULTS

Figure 1 (chromatograms B and C) shows comparative EC-GLC profiles of the pH 10 derivatized extracts of two *Neisseria* species cultured in NEDF. These are compared with a medium control (Fig. 1D) that was extracted in the same manner as the cultures. Also shown (Fig. 1A) is a chromatogram of an AMC-derivatized standard (3-hydroxy-3-butanone, 82% pure; Eastman Organic Chemicals). The peak for the AMC standard shown in the figure represents about 155 pmol.

Identification of AMC was made with GC-CMS and further confirmed by EC-GLC on three different types of gas chromatographic columns, 3% OV-1, TABSORB, and 3% Dexsil 410 (Analabs). The latter two column materials have polar or moderately polar characteristics. The GC-CMS base peak (fragment of highest intensity) was detected at $m/e = 71$ (Fig. 2). This fragment possibly represents the $[\text{CH}_3\text{COCHCH}_3]^+$ ion. The molecular ion + 1

FIG. 1. EC-GLC chromatograms of HFBA derivatives. (A) Acetoin standard; (B and C) pH 10 culture extracts; (D) pH 10 medium control extract. The analyses were made on a 3% OV-1 column. Peak 1, Acetylmethylcarbinol (acetoin); peak 2, 2, 3-butanediol.

($M + 1$), which shows the mass of the AMC-HFBA molecule

was detected at $m/e = 285$, and an $M - 15$ fragment was detected at $m/e = 270$. Fragments originating from the HFBA-derivatized portion of the molecule were present at $m/e = 69$ [CF_3] $^+$, $m/e = 169$ [$\text{CF}_2\text{CF}_2\text{CF}_2$] $^+$, and $m/e = 213$ [$\text{CF}_2\text{CF}_2\text{CF}_2\text{COO}$] $^+$. Mass spectra and retention times obtained from HFBA derivatives of the AMC standard and the culture extract were identical. In addition to the identification AMC, we tentatively identified 2,3-butanediol

FIG. 2. Chemical ionization mass spectra of the AMC-HFBA derivative from a pH 10 extract of *N. gonorrhoeae* cultured in NEDF medium.

by EC-GLC (polar and nonpolar columns) (Fig. 1 and 3, peak 2).

Tables 1 and 2 show the *Neisseria* cultures tested for AMC by the conventional and EC-GLC methods. All *N. gonorrhoeae* isolates cultured in NEDF with glucose (NEDFG) were AMC positive in 16 h by both the Voges-Proskauer and EC-GLC tests. When EC-GLC was used, each of eight gonococcal isolates was also positive for AMC when cultured in NEDF with maltose (NEDFM). There was approximately a fourfold difference in AMC production between the gonococci and the meningococci when they were grown in NEDFM (Table 1). Four isolates of *N. meningitidis* (CDC 15090 type B, M-158 type D, B-4390 Slaterus type Z, and 1170 type C) cultured in NEDFG gave negative Voges-Proskauer tests, but each was positive by the EC-GLC method. With either NEDFG or NEDFM, the three *N. lactamica* cultures produced large amounts of AMC as determined by either EC-GLC or the conventional detection method. AMC was a major product detected from the two cultures of *N. haemolysans* (*Gemella haemolysans*). The other *Neisseria* produced varying amounts of AMC or none at all as measured by EC-GLC (Table 1).

The age of the growth medium may effect AMC production because stronger reactions were observed on media that were freshly prepared than on media that were 6 to 8 weeks old. Heavy growth of the organisms may not be necessary for AMC production as judged by one

isolate of *N. gonorrhoeae* that grew poorly in the NEDF media but gave positive tests by both conventional and EC-GLC methods.

DISCUSSION

To our knowledge the production of AMC and 2,3-butanediol by *N. gonorrhoeae* has not been previously reported. Berger (1) and Vandekerckove et al. (15), using conventional tests, reported AMC production by some of the other species of *Neisseria*. The AMC produced by *N. gonorrhoeae* and *N. meningitidis* is probably derived from the metabolism of carbohydrates and the NEDF medium is conducive to its production. Removal of cells by centrifugation before extraction showed that the AMC was not a constituent of the cell wall. Changing the carbohydrate source from glucose to maltose definitely affected AMC production in both strains of *N. meningitidis* and *N. gonorrhoeae*. Whereas *N. meningitidis* strains that were poor producers of AMC in NEDFG medium became good AMC producers in NEDFM medium, *N. gonorrhoeae* strains produced decidedly less AMC in NEDFM medium (Fig. 3 and Tables 1 and 2). It is not known whether strains of *N. gonorrhoeae* are actually metabolizing maltose or utilizing other components of the medium. Since substitution of carbohydrates other than glucose or maltose also makes changes in the gas chromatographic profiles of the two organisms (data to be presented elsewhere), it is possible that maltose is utilized by

FIG. 3. EC-GLC chromatograms of HFBA derivatives of pH 10 extracts from spent culture media. The analyses were made on a 3% OV-1 column. Peak 1, Acetylmethylcarbinol; peak 2 = 2,3-butanediol.

strains of *N. gonorrhoeae*; however, further studies are needed to furnish proof of maltose utilization. Conflicting reports of AMC production between their data and ours can probably be attributed to the differences in the media, cultural conditions, and the sensitivities of the detection methods.

By the EC-GLC method, we were able to

detect about 23 and 93 nmol of underivatized acetoin diluted in ethyl ether on TABSORB and on 3% OV-1 columns, respectively. Derivatization of AMC gave much sharper peaks on both types of columns and also increased the sensitivity of detection about 600 times. We found that chloroform was a much better solvent than ethyl ether for extraction of AMC from spent

TABLE 1. Results of electron capture gas-liquid chromatography test for acetylmethylcarbinol produced by *Neisseria* species grown in NEDFG and NEDFM

Species	NADFG			NEDFM		
	No. tested	No. positive	nmol/10 ml of NEDF	No. tested	No. positive	nmol/10 ml of NEDF
<i>gonorrhoeae</i>	23	23	1,000 ^a	8	8	500 ^a
<i>meningitidis</i> (include all serotypes)	10	10	— ^b	10	10	2,000
<i>lactamica</i>	3	3	1,000	3	3	2,000
<i>catarrhalis</i>	5	4	— ^c	1	1	250
<i>sicca</i>	3	3	250	1	1	500
<i>flavescens</i>	3	1	100	1	0	
<i>flava</i>	3	3	250	1	1	500
<i>perflava</i>	3	1	250	2	1	250
<i>subflava</i>	5	2	250	2	1	400
<i>mucosa</i>	2	1	250	1	1	400
<i>caviae</i>	2	0		1	0	
<i>ovis</i>	1	1	250	1	0	
<i>cinerea</i>	1	1	100	1	1	100
<i>elongata</i>	3	0		1	1	100
<i>haemolysans</i>	2	2	1,000	0	0	

^a Concentrations are approximate.^b Six isolates 1,000, three isolates 500, one isolate 100.^c Two isolates 250, two isolates 100.TABLE 2. Voges-Proskauer test for acetylmethylcarbinol with *Neisseria* species grown in NEDFG and NEDFM

Species	NEDFG			NEDFM		
	No. tested	No. positive	Reaction strength ^a	No. tested	No. positive	Reaction strength
<i>gonorrhoeae</i>	16	16	+++	10	10	++
<i>meningitidis</i> (includes all serotypes)	10	6	+++	10	10 ^b	+++
<i>lactamica</i>	3	3	+++	3	3	+++
<i>catarrhalis</i>	5	2	++	2	2	++
<i>sicca</i>	3	2	+	1	1	++
<i>flavescens</i>	3	0		1	0	
<i>flava</i>	3	1 ^{c, d}	+	1	1 ^d	++
<i>perflava</i>	1	1	+	1	1	+
<i>subflava</i>	5	0		1	1	+
<i>mucosa</i>	0	0		1	1	+
<i>caviae</i>	2	0		1	0	
<i>ovis</i>	1	0		1	0	
<i>cinerea</i>	1	1	+	1	1	+
<i>elongata</i>	3	0		1	0	
<i>haemolysans</i>	2	2	++	0	0	

^a +, Weakly positive; ++, intermediate; +++, strongly positive.^b B-4390 Slaterus Z positive in 48 h.^c Variable.^d Same isolate.

culture media on the basis of the following observations: (i) the AMC peak appeared in large quantities in the chloroform fraction after prior extraction with ethyl ether; and (ii) when the initial extraction (pH 2) was made with chloroform instead of ethyl ether, a sizeable amount

of the AMC was recovered in that fraction (as indicated by relative EC-GLC peak size). Attention is directed to the profiles obtained from *N. gonorrhoeae* and *N. meningitidis* grown in the glucose and maltose NEDF media (Fig. 3). *N. gonorrhoeae* produced full-scale AMC and

2,3-butanediol peaks (Fig. 3A, peaks 1 and 2) in the glucose medium. However, when the maltose medium was used, a considerable decrease was noted in these products, especially 2,3-butanediol (Fig. 3B, peak 2). This was true of all gonococci tested. Isolates of *N. meningitidis* grown in the maltose medium gave full-scale peaks of AMC and 2,3-butanediol, but a considerable decrease was noted in these products when the glucose medium was used (compare Fig. 3D with Fig. 3C). This trend was true of all meningococcal serotypes tested; however, there were quantitative differences among different serotypes. This difference in metabolic activity with different energy sources may be useful as an additional means to differentiate the gonococci from the meningococci. Other differences in the EL-GLC profiles were noted when different energy sources were used (Fig. 3, all chromatograms, peaks 3–5).

Profile similarities and differences were often more apparent with the frequency pulse modulated mode ^{63}Ni EC detector (FPMM-ECD). The FPMM-ECD is more quantitatively reliable than the direct current EC detector, is less affected by overloading, and is much less affected by contamination from column bleed and from reagents. We recommend the FPMM-ECD method of operation.

EC-GLC is an excellent tool for detecting a variety of metabolic products (3–5). The HFBA derivatization and EC-GLC procedures recommended in this study are practical and selective for compounds containing hydroxyl and amine groups. This selectivity, along with the sensitivity, simplicity, rapidity, and reproducibility of the techniques, offers great potential for routine laboratory use in detecting AMC as well as many other types of compounds. The EC-GLC profiles thus obtained can be used to aid in identification of *Neisseria*. In addition, useful differential test methods for some *Neisseria*, including the two pathogenic species, might be developed that use the conventional AMC test with the defined medium. As shown in Tables 1 and 2, there was a significant difference among species in the amount of AMC produced by all strains of *N. gonorrhoeae* and *N. meningitidis* grown in NEDFM medium. This quantitative difference was demonstrated by both test procedures. Differences in AMC production as detected by conventional AMC test (Table 2) might provide the basis for a differential test that could distinguish between some members of the genus. It is also possible that the NEDF medium could be further modified so that the differences among members of the genus could be more easily recognized. For example, biotin

might be deleted from the medium to eliminate growth of *N. lactamica* (9), thus permitting differentiation between *N. lactamica* and *N. meningitidis*.

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