Analysis of Clostridium botulinum Toxigenic Types A, B, and E for Fatty and Carbohydrate Content

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Lyophilized, 48-hr log-phase vegetative cells were extracted with chloroform-methanol (2:1, v/v) and ethanol-ether (3:1, v/v) and then saponified with methanolic KOH. Gas-liquid chromatography of the methyl esters of extractable fatty acids revealed distinctive "pattern profiles" of Clostridium botulinum toxigenic types "A," "B," and "E." C. perfringens type "A" and Escherichia coli strain "B" were also studied in a similar manner and were found to give pattern profiles which were distinct even from those obtained for the C. botulinum microorganisms. Amino sugar content of the five microorganisms was determined by using a Beckman amino acid analyzer. The molar ratio of glucosamine to that of galactosamine was found to be of further assistance in distinguishing the individual microorganisms.

There has been increased interest in the bacterial lipid and other chemical constituents of the genus Clostridium, both from a physiological standpoint and as a means of species differentiation (6, 17, 22, 24, 27).

Several approaches to this species differentiation have been explored. Henis et al. (10) used gas chromatographic analysis of bacterial metabolic products to provide a means for rapid detection and differentiation of several different microorganisms. Moore (20) also utilized gas chromatography for the study of the genus Clostridium and demonstrated that the patterns obtained allowed species differentiation.

Fugate and Hansen (Bacteriol. Proc., 1969, p. 46–47) reported preliminary results of an investigation in the use of lipid and carbohydrate patterns obtained by gas-liquid chromatography (GLC) of whole cell extracts to distinguish toxigenic C. botulinum strains. Kimble et al. (13) also reported a detailed study of the fatty acid composition in whole-cell extracts of C. botulinum and suggested that variances in composition served as a means of differentiating the chiefly proteolytic C. botulinum types A and B from the nonproteolytic types E and F.

It is well established that carbohydrates confer antigenic specificity to many bacterial cell components (2, 15). Thus, it would be of interest to determine whether C. botulinum vegetative cells contain carbohydrate moieties in the easily extractable lipid portion of their cell surface. Since members of this genus are frequently involved in food poisoning outbreaks, it would be advantageous to characterize the lipid and carbohydrate content of selected, single strains of C. botulinum as a basis for a rapid, preliminary means of identification. Moore (20) has pointed out that gas chromatography allows simple and rapid identification of species through characteristic compounds, although such analyses do not replace necessary morphological and biochemical studies.

The primary purpose of this investigation was to quantitatively characterize the lipid and carbohydrate content of whole cells of three strains of toxigenic C. botulinum. The three strains selected for extensive study (type E, Beluga; type B, 169B; type A, 62A) were found to be representative of several strains which were investigated during this study. Since this represents an investigation of single strains and since there are a large number of strains which were not included in this study, it may be necessary in the future to substitute other single strains for these clostridia so that the strains chosen are representative of the majority of the strains in each toxigenic type. It is good to remember that these strains constitute a diverse group of microorganisms (9, 21).

MATERIALS AND METHODS

Microorganisms. The cultures of C. perfringens type A and C. botulinum type E strains Beluga, 8E, and D-8; type B strains 169B and 115B; and type A

1 A preliminary report of this investigation was presented before the 69th Meeting of The American Society for Microbiology, Miami Beach, Fla., 4-9 May, 1969.
strains 62A, 73A, and 426A were obtained from the Bureau of Foods and Pesticides, Food and Drug Administration, Washington, D.C. Preliminary work with these strains indicated that type E strain Beluga, type B strain 169B, and type A strain 62A should be selected for further study as representative strains. Escherichia coli B was a gift from Sol Haberman (deceased) of Baylor University Medical Center, Dallas, Tex.

Cultural conditions. Clostridium tetani cultures were grown in 15-liter quantities in the Trypticase-peptone-glucose broth of Schmidt, Nank, and Lechowich (25). A 2.0% (of total volume) inoculum of a 12-hr culture was used when the cells were grown in large quantities. Vegetative cultures were incubated for 36- and 48-hr periods at 30 C. The cells were harvested by centrifugation and washed four times in 0.85% (w/v) NaCl and lyophilized in a VirTis lyophilizer before extraction procedures.

Cultures of E. coli B were grown and harvested as reported by Brian and Gardner (3) for the detection of cyclopropane fatty acids in bacterial lipids. Cultures were grown in 25 ml of Trypticase Soy Broth (TSB) and 3% agar at 35 C for 18 hr. These 18-hr cultures were then used as the inoculum for 6 liters of TSB with 3% agar. Cultures were incubated for 24 hr at 35 C. Cells were harvested by centrifugation and washed four times with 0.85% (w/v) NaCl. These cells were lyophilized.

GLC. All GLC analyses were performed with a Barber-Colman model 5000 equipped with flame ionization detection and having a flow rate of 40 ml of hydrogen per min and 150 ml of air per min.

Polar column number 1. An all-glass column (180 cm by 3 mm) with 8% LAC-728 on 1% phosphoric acid-precoated 100/120 mesh Anakrom AB was used. Nitrogen flow rate was 40 ml/min at 16 psi. The column was temperature-programmed during analysis from 100 to 250 C at 5 degrees per min. Injector temperature was 250 C, and detector temperature was set at 260 C.

Nonpolar column number 2. An all-glass column (180 cm by 3 mm) with 8% SE-33 on 100/110 mesh Gas-Chrom Q was used. Nitrogen flow and temperature conditions were the same as described for column number 1.

Hexose column number 3. An all-glass column (90 cm by 3 mm) with 4% XE-60 and 3% neopentyl-glycol-adipate on 100/110 mesh Gas-Chrom Q was used. Nitrogen flow rate was 50 ml/min at 18 psi. The column was temperature-programmed from 180 to 250 C at 10 degrees per min. Injector temperature was 250 C, and detector temperature was 260 C.

Analytical methods: lipids. One gram of lyophilized cells was extracted with chloroform-methanol (2:1), v/v and then with ethanol-ether (3:1, v/v) as shown in Fig. 1. The combined lipid extracts (number 1 and number 2) were concentrated under a stream of nitrogen. The combined extracts were washed by the procedure of Folch et al. (7) to begin lipid characterization. The upper phase lipids were made to a 10.0-ml volume. A 5.0-ml portion of each sample was saponified with 5.0 ml of 15% KOH in methanolic aqueous solution (1:1, v/v) for 1 hr at 80 C.

Fig. 1. Flow chart and outline of procedures used for analysis of microorganisms.

The remaining sample was chromatographed on diethylaminoethyl cellulose by the method of Creech (5) and Rouser et al. (23) to remove nonlipid contaminants. Thin-layer chromatography of the lipid mixture was accomplished by using the techniques of Mangold (18).

Nonsaponifiable material was extracted three times with 5-, 3-, and 2-ml volumes of mixed ethers (ligroin diethyl ether, 1:1, v/v) successively, and the combined extract was stored at 0 C for hexose analysis. The extract residue of cells was adjusted to pH 2.0 with 6 N HCl, and the saponifiable acids were extracted with the mixed ethers three times by using 5, 3, and 2 ml successively. The remaining aqueous portion was stored at 0 C for hexose analysis.

A sample of the extracted fatty acid was esterified with methanolic BCl as reported by Metcalf and Schmitz (19) and determined by GLC analysis on a polar column. The fatty acid methyl esters were then hydrogenated immediately for 20 min in 5 ml of chloroform-methanol (2:1, v/v) with 100 mg of 5% platinum on charcoal and chromatographed on the same column. The fatty acid esters were brominated as described by Brian and Gardner (3) and chromatographed on the same column. A second sample of the extracted fatty acids was treated similarly but chromatographed on a nonpolar column; as a result, six chromatograms were available for each microorganism studied. With both columns, quantitative results agreed within 5% of stated composi-
tion for the National Institutes of Health fatty acid standards (Applied Science Laboratories Inc., State College, Pa., reference 11). Fatty acids were identified by the method suggested by Kimble et al. (13).

Analytical methods: hexoses. Hexose content was determined by the method of Kim et al. (12). In most experiments, 10 mg of extract (scheme B-9 and 10, Fig. 1) was hydrolyzed in 7.5 ml of 0.25 N sulfuric acid plus 500 mg of Dowex 50-X12, 200 to 400 mesh (H⁺ form) ion-exchange resin in evacuated sealed ampoules at 100 C for 24 hr. The hexoses, in a portion of the hydrolysate, were converted to the alditol derivatives by the procedure of Kim et al. (12).

Thin-layer chromatography of a second portion of the hydrolysate was performed on glass plates coated with Adsorbosil-3 (Applied Science Laboratories, Inc.) in 0.1 N boric acid at a thickness of 0.5 mm. A mixture of butanol-acetone-water (40:50:10) was used as the developer. Silver nitrate spray followed by NaOH spray and heating at 100 C for 10 min was used for visualization. Ninhydrin in acetone was employed to locate the amino sugars.

Analytical methods: amino sugars. A 5-mg amount of dried lipid solvent extracts (scheme B-5, 9, Fig. 1) was hydrolyzed in 6 N HCl for 18 hr at 110 C. The hydrolysates were examined with an amino acid analyzer (model 120 C; Beckman Instruments, Inc., Palo Alto, Calif.) for amino sugars after being extracted with diethyl ether which removed non-covalently bound fatty acids. The analyzer was fitted with a 20-cm column packed with PA-35 spherical resin and maintained at 55 C. The buffer system was 0.35 N sodium citrate at pH 5.26, and a flow rate of 70 ml/hr was maintained.

A 10-mg amount of extracted whole cells (scheme A-1, Fig. 1) was hydrolyzed in 2 ml of 6 N HCl under reduced pressure at 110 C. After 18 hr, the sealed ampoules were broken and the filtered hydrolysates were examined for amino sugar content by using the same conditions.

The amino acid analyzer was calibrated with amino acid standard calibration mixture type 1 (Beckman Instruments, Inc.) and with glucosamine and galactosamine standards prepared to a concentration of 0.125 μmole per 0.1 ml.

Analytical methods: amino acids. Amino acid content of the whole cell was determined after the whole cell mass had been through the lipid extraction procedures. A 10-mg sample (scheme A-1) was hydrolyzed in 6 N HCl for 24 hr at 110 C. Hydrochloric acid was removed by repeated evaporation to dryness from water solution. These hydrolysates were then freed from noncovalently bound fatty acids by extraction with diethyl ether, redried, and dissolved in sodium citrate buffer (pH 2.2) for analysis. The amino acid analyzer operating conditions were the same as previously described.

RESULTS

The total lipid extracted accounted for 3.3, 3.0, 3.2, and 4.0% of the dry weight of C. botulimum vegetative cell types A, B, E and C. perfringens, respectively, with percentages based on 48-hr vegetative cells cultured as described above.

A comparison of the different fatty acid "pattern profiles" obtained from the microorganisms studied is shown in Fig. 2. E. coli data were included throughout the experiment, as suggested

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**Fig. 2.** Pattern profiles obtained when lipid extracts of microorganisms studied were chromatographed on LAC 728, column number 1.
by Brian and Gardner (3), to facilitate analysis of the fatty acid data. The lipid portions of *C. botulinum* types A, B, and E were found to contain essentially the same fatty acids but in different concentrations. Tetradecanoic (C:14) and hexadecanoic (C:16) acids were found to be the predominant fatty acids in the whole cell extracts of types A, B, and E. Together they make up over 50% of the total fatty acids. Table 1 lists the fatty acids identified in the five microorganisms studied. The results agreed favorably with those reported by Kimble et al. (13).

Figure 2 also shows that a distinctive pattern profile is obtained from *C. perfringens*, which is different in that relatively large percentages of dodecanoic (C:12) and tetradecanoic (C:14) were found. Smaller percentages of hexadecanoic (16.6%) and octadecanoic acids (1.3%) were also present, which agrees with results reported by Moss and Lewis (22).

The lipids of *C. botulinum* types A, B, and E are essentially the same in their fatty acid composition. The quantitative distribution of the various fatty acids were found to differ, but only with the additional information supplied by the amino acid analyzer may they be distinguished easily. However, the limited number of strains examined does not rule out the possibility that the differences observed between types are actually differences between individual strains. A comparison of our data with those of Kimble

### Table 1. Fatty acid composition of microorganisms studied relative to total fatty acid composition

| Fatty acid            | E. coli B | *C. botulinum* type E, Beluga | *C. botulinum* type A, 62A | *C. botulinum* type B, 169B | *C. perfringens* type A |
|-----------------------|-----------|--------------------------------|-----------------------------|-----------------------------|-------------------------|
| Octanoic              | Trace     | 0.6 Trace                       | Trace                       | 0.3                         | Trace                   |
| Decanoic              | 0.8       | 0.3 Trace                       | Trace                       | 0.2                         | 0.8                     |
| Dodecanoic            | 2.8       | 0.6 Trace                       | Trace                       | 3.3                         | 21.0                    |
| Tridecanoic           | —         | 0.4 Trace                       | Trace                       | 0.3                         | 0.4                     |
| Tetradecanoic         | 7.6       | 17.4 Trace                      | 12.5                        | 19.8                        | 11.0                    |
| 7,8-Tetradecenoic     | 1.0       | — Trace                         | 0.9                         | —                           | 1.2                     |
| Pentadecanoic         | 2.8       | 4.9 Trace                       | 1.4                         | 1.4                         | 4.3                     |
| Hexadecanoic          | 37.3      | 28.3 Trace                      | 40.7                        | 42.4                        | 16.6                    |
| 9,10-Hexadecanoic     | 10.0      | 12.5 Trace                      | 7.5                         | 2.9                         | 13.4                    |
| Heptadecanoic         | 2.5       | 2.0 Trace                       | 0.4                         | 0.4                         | 1.2                     |
| Δ-Methylene hexadecanoic | 12.0   | 1.0 Trace                       | 2.2                         | 0.6                         | —                       |
| Octadecanoic          | 2.0       | 1.4 Trace                       | 1.4                         | 1.2                         | 9.3                     |
| 11,12-Octadecanoic    | 14.1      | 3.4 Trace                       | 7.3                         | 3.4                         | 8.3                     |
| 5,6,11,12-Octadecadienoic | 0.3   | 0.3 Trace                       | 0.3                         | —                           | 0.8                     |
| Nonadecanoic          | 0.6       | 0.3 Trace                       | 0.3                         | 0.3                         | 1.0                     |
| Δ-Methylene octadecanoic | 2.8     | 0.5 Trace                       | 17.7                        | 4.2                         | Trace                   |
| Eicosanoic            | 0.3       | 0.1 Trace                       | 0.3                         | —                           | 1.6                     |
| Heneicosanoic         | 0.2       | 0.2 Trace                       | 0.9                         | 0.2                         | 1.0                     |
| Δ-Methylene docosanoic | Trace  | — Trace                         | 1.7                         | 0.2                         | —                       |
| Tetracosanoic         | 0.9       | Trace                           | 0.5                         | —                           | —                       |

* Results expressed as per cent of total.
  
* Not found.
  
* Did not occur to an extent greater than 0.1%.

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FIG. 3. Chromatograms obtained during qualitative analysis of lipids obtained from Clostridium botulinum type A.
et al. (13) does substantiate preliminary findings that a similarity does exist between strains of the same toxigenic type.

The chromatograms of *Clostridium* type A after methylation, hydrogenation, and bromination are shown in Fig. 3. Cyclopropane fatty acids containing 17, 19, and 23 carbon atoms were demonstrated to be present in each of the *Clostridium* types studied. *E. coli* was used as a standard of reference in detecting the cyclopropane fatty acids, since *E. coli* is a ready source of these acids, especially for GLC identification procedures (3). *E. coli* was found to have 37.3% hexadecanoic acid (C:16), with 12.0% cyclopropane methylene-hexadecanoic acid (C:17) and 2.8% cyclopropane methylene-octadecanoic (C:19) acid.

No hexose was detected by GLC of the alditol acetate derivatives from any of the lipid extracts of *Clostridium* vegetative cells.

*C. perfringens*, on the other hand, contains mannose, which distinguishes it quite readily from the *Clostridium* types studied. After hydrolysis, lipid solvent extracts from *E. coli* were found to contain glucose, fucose, galactose, and rhamnose. The results obtained in the separation of the amino sugars in the Beckman amino acid analyzer (model 120C) are shown in Table 2. The molar ratio of galactosamine to glucosamine appears to be quite useful in distinguishing the microorganisms studied in that it varied from 1:2 in *Clostridium* type A, 2:3 in type B, and 4:1 in type E. In contrast, the ratio for *C. perfringens* was 1:8 and 2:1 for *E. coli* B.

The amino acid residues detected after whole cell hydrolysis failed to reveal significant differences between the *Clostridium* species studied.

### DISCUSSION

The lack of carbohydrate in the lipid of the *Clostridium* species studied may be one of the major factors in explaining the findings of Lynt et al. (16). They reported that somatic antigens were common to all *C. botulinum* strains studied. This may well be due to the lipid composition and corresponding low antigenic specificity of this class of chemical constituents. Solomon et al. (26) expressed the belief that serological classification of the clostridia would require that the entire antigenic makeup, including the flagellar, somatic, and spore antigens of the organisms, be considered. An alternative approach to the identification of clostridia by purely serological techniques could be by GLC. Abel et al. (1) suggested that differences in chemical composition of microorganisms be detected by gas chromatography and used for their classification, since gas chromatography offers the necessary degree of sensitivity, rapidity, and selectivity required.

The need for more rapid bacterial species identification, especially with genera associated with food-borne illness, is quite apparent. Several novel methods, including immunological and enzymatic, have been proposed (14). The present investigation suggests that principles of immunochromatography and gas chromatography can also be combined and utilized in this respect.

There are several possible approaches to the problem of rapid identification of toxigenic *Clostridium* species. The present report demonstrates that analysis of the whole cell is preferable to analysis of sonically disrupted and enzyme-cleansed cell walls when rapidity is desired. The use of whole cells in preference to disrupted cells was investigated originally since whole cells gave promise of more rapid analysis. The unique pattern profiles obtained through the use of GLC analysis of fatty acids coupled with the amino sugar ratios for the microorganisms studied appears to offer a rapid means of differentiation.

This investigation is being continued through a study of additional strains to determine whether the strains reported are as representative for *Clostridium* as those investigated for *C. perfringens* by Moss and Lewis (22).

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### TABLE 2. Ratio of glucosamine to galactosamine in microorganisms studied

| Microorganism          | Amt present* | Molar ratio (Glu-NH₂:Gal-NH₂) |
|------------------------|--------------|-------------------------------|
|                        | Glu-NH₂ (amoles) | Gal-NH₂ (amoles) |
| *Clostridium botulinum*|              |                               |
| type A                 | 0.400        | 0.800                         |
| type B                 | 0.425        | 0.650                         |
| type E                 | 0.775        | 0.200                         |
| *C. perfringens* type A| 0.050        | 0.388                         |
| Enterobacter coli      | 0.100        | 0.063                         |

* A 10-mg amount of extracted whole cells was hydrolyzed in 6 N HCl under reduced pressure at 110°C for 18 hr, evaporated to dryness, and dissolved in 5 ml of buffer (pH 2.2). Glu, glucosamine; Gal, galactosamine.
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