GAS CHROMATOGRAPHIC ANALYSIS OF AMINES AND OTHER COMPOUNDS PRODUCED BY SEVERAL SPECIES OF *CLOSTRIDIUM*

by

John B. Brooks

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APPROVED:

W. E. C. Moore, Ph. D.
Committee Chairman

Lillian V. Holdeman, Ph. D.

R. E. Benoit, Ph. D.

R. G. Saacke, Ph. D.

J. L. Hess, Ph. D.

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Blacksburg, Virginia 24061

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REVIEW OF THE LITERATURE

Biologically, amines are produced by plant enzymes, mammalian tissue cells, and microorganisms. They are formed by decarboxylation of amino acids (11, 13, 14, 32), degradation of polyamines, or by transamination.

Very little is known about the production of volatile amines by transamination, but one study (40) of cell-free extracts from the flowering plant *Mercurialis perennis* demonstrated enzymatic transamination of caproic aldehyde, isovaleraldehyde, isobutyraldehyde, propionaldehyde, acetaldehyde, and formaldehyde. Addition of ammonium salts had no effect, indicating synthesis by transamination. The catalytic amination by the transaminase occurred only if an appropriate amino acid was present to donate an amino group.

Amines are also produced in mammalian tissue. A study of the origin of tyramine and tryptamine in human urine indicated that these amines originated in unidentified tissue and were not bacterial in origin. Bachrach et al. (6) demonstrated the production of putrescine and spermidine by Ehrlich ascites tumor cells. Putrescine was utilized in the production of spermidine.

Many microorganisms produce amines (18, 41, 43). Bacterial cells usually contain small amounts of putrescine, indicating that it is synthesized by growing cells (48). Microorganisms isolated from the intestinal tracts of rats produce several amines (41). Bacteria isolated

from the cecum produced more amines than did the bacteria isolated from other areas of the tract. In another study (7), in which chromatographic techniques were used to detect phenolic and histamine derivatives in feces of infants and children, p-tyramine was the principal amine found. Histamine and N-acetylhistamine were found in infant stools, but were rare in stools of older children. Small amounts of substrates tentatively identified as N-methl-p-tyramine and m-tyramine were occasionally found in feces of older children.

Amines are generally considered to be toxic to higher animals and to some bacteria. Spermine administered parenterally has a potent nephrotoxic action and causes death of laboratory animals from renal tubular necrosis (35). Inhalation of vapors from putrescine or cadaverine is known to produce diarrhea and nausea in man. Culture filtrates containing no known toxic substance except amines have caused death in laboratory animals when injected intravenously (32). No reports are available concerning the toxic properties of many amines.

It has generally been assumed that in the quantities normally produced by microorganisms in the intestines of man, amines are detoxified by amino oxidases before they reach the blood stream (49). Some aliphatic amines inhibit monoamine oxidase activity in rats. This enzyme catalyses the oxidation of wide variety of physiological amines to the corresponding aldehydes and ammonia (45). Propylamine, amylamine, heptylamine, and ethylenediamine administered orally in lethal doses had almost no effect on monoamine oxidase activity. Secondary and tertiary saturated normal monoamines, diethylamine, dipropylamine,

dibutylamine, triethylamine, and isopropylamine in fatal doses, significantly inhibited monamine oxidase activity. Allylamine was the strongest inhibitor of monoamine oxidase in liver and brain tissue.

Little is known about the metabolism or possible function of amines. Of a series of 6 aliphatic and 2 cyclic amines, mycobacteria isolated from fish could metabolize only putrescine (15). Thermal denaturation profiles of ribosomal ribonucleic acid (RNA) from thermophilic and mesophilic organisms demonstrated that the presence of putrescine or spermidine increased resistance to denaturization of ribosomal RNA in the mesophilic organisms and spermidine was similarly active in the thermophilic organisms (17, 27).

Current studies of bacterial activity frequently emphasize carbohydrate metabolism. Only limited information is available concerning microbial metabolism of amines and amino acids. Study of amines produced by bacteria should prove valuable to the understanding of microbial toxicity, nitrogen metabolism, and may aid in identification and taxonomy of microorganisms.

The study of amine production by bacterial cultures has been hindered by the lack of analytical methods that are both sensitive and practical for routine use in diagnostic or taxonomic bacteriology. Previous analyses of amines in biological materials depended upon extraction of the amines from large volumes of culture medium. Cadaverine and putrescine have been distinguished from spermine and spermidine using a modification of Dragendorff's reagent with which only the latter

gave a red color (2). Later, several procedures were developed using ion-exchange column chromatography for the quantitative estimation of amines. These involved adsorption of the amine on acid adsorbents, such as Amberlite XE-64 or Dowex 50, followed by elution with salts or acids. The concentration of the amines was then determined by non-specific assay, such as color reaction with dinitrofluorobenzene (36). Each amine was tentatively identified by comparison of elution times with those of known compounds. Chromatographic position when used alone is not reliable. Tests to verify identification involved separation by paper chromatography, electrophoresis, color reaction with copper carbonate for the identification of polyamines, calculation of the ratio of the optical densities of the dinitrophenyl derivatives at 350 mμ and 390 mμ to distinguish between primary and secondary amines, and addition of isotopic markers (44). Hong and Connors (22) suggested a sensitive method for the determination of some primary and secondary amines based upon their conversion to N-substituted cinnamides by reaction with trans-cinnamic anhydride. After acetylation the cinnamide derivative was extracted with chloroform and measured spectrophotometrically. This test has only been used to detect known amines and only a limited number of amines were detected.

The successful application of gas chromatography for the analysis of many bacterial products, including alcohols, short chain fatty acids (20, 29), long chain fatty acids (1, 50) and gaseous products (37), suggests that this technique might be used for the analysis of amines.

Several methods for the detection of amines by gas chromatography have been developed since 1960. These procedures were developed for the analysis of pure amines or mixtures of pure amines. Amines present in drugs have been successfully separated by gas chromatography (8, 23). Fales and Pisano (16) separated phenolic and aromatic amines by gas chromatography. Arad-Talmi, et al. (5) separated allylamine, and butylamine from aqueous solutions using a copper column 2 1/2 m long, 5 mm I.D., packed with 20% carbowax 20M coated with 5% KOH on chromosorb W 30-60 mesh, a thermal conductivity detector, and temperature programming. Only five microliter samples of aqueous solutions were required. Vanden-Heuvel, et al. (46) prepared trifluoroacetic anhydride derivatives of several mono-and diamines, including putrescine and cadaverine, to increase volatility and give greater resolution by reducing column adsorption and tailing. Capella and Horning (9) separated some biologically important amines by preparation of hexamethyldisilazane derivatives. Hydroxyl groups were converted into trimethylsilyl-ether groups and primary amines were converted into eneamines or Schiff bases. Secondary amino groups remained unchanged. Known amines representing possible products of human metabolic pathways also were separated by gas chromatography as hexamethyldisilazane derivatives.

Morrissette and Link (30) reported differentiation of fatty nitrogen compounds as trifluoroacetyl derivatives, using a high temperature grease such as Apiezon L or silicone on an inert support previously treated with KOH. From mixtures of amines containing from

8 to 22 carbon atoms, linoleyl- and linolenyl amines were separated from stearyl- and oleylamine. Preparation of derivatives required only a few minutes.

The amino acids from which several of the amines are derived have small measurable vapor pressures, but the volatility is too low for direct gas chromatographic analysis (19). Several amino acids can be separated by gas chromatography as N-butyl-N-trifluoroacetyl derivatives, and N-trifluoroacetylamino acid methyl esters (24, 51 10). Trifluoroacetyl amino acid derivatives can be prepared and successfully chromatographed in two hours (10).

Amell, et al. (3) separated methylamine, dimethylamine, and ethylamine using o-toluidine as the liquid phase and Johns-Manville C-22 fire brick as the solid support. It was necessary to renew the packing periodically because the column gradually bled. Column bleeding was alleviated considerably by substituting 1-hexadecanol for the o-toluidine liquie phase.

Modification of gas chromatographic column substrates can reduce undesirable tailing and adsorption. Alkali pretreatment of column supports has played a major role in the development of a satisfactory packing for the separation of diamines (38). The exact role of the alkali is not understood, but the data suggest that the beneficial effect was not simply due to neutralization of "acidic sites." Coating of 20M Chromosorb with 10% Carbowax plus treatment with 5% KOH (w/w) prior to application of the liquid partition phase

gave chromatographic packing with superior properties for the separation of aliphatic diamines. Separation of primary, secondary, and tertiary amines of low molecular weight has been accomplished using Teflon coated with polyglycol 1500, and 4.4 Apiezon L. (26). Vessman and Schill (47) made a study of several column supports and liquid coating for the resolution of high-boiling amines (b.p. from 200° to 400° C). Acetyl derivatives of primary and secondary amines tested were separated without decomposition on a microglassbead support with a liquid phase consisting of 1% SE-30 (methyl substituted silicone elastomer), 0.8% QF-1 (fluorine substituted silicone fluid), and 0.8% NGSe (polyester of neopentylglycol substrate).

The use of gas chromatography for analysis of dilute solutions of amines such as those produced by bacterial cultures presents problems in addition to the necessity for selecting volatile derivatives and suitable column substrates. Gas chromatographic procedures for amine analysis depend upon satisfactory methods for extraction of the amines from aqueous solutions in detectable quantity, development of procedures for preparation of derivatives from the extraction, and selection of a column packing material and chromatographic conditions that will give good resolution and prevent adsorption and tailing that is characteristic of amines.

The presence of water increases retention time and decreases resolution (25, 38, 42). Attempts made to circumvent this difficulty include silvering the support (31) which proved to be tedious and of

little value. Powdered polyethylene has been used with some success to separate water-methanol mixtures. A mixed stationary phase of sorbitol and di (2-ethylhexyl) sebacate has been used to separate alcohols from water. Sorbitol was used to retain the water; however, sorbitol alone gave poor resolution of alcohols. Alcohols containing up to six carbon atoms were eluted prior to the water peak (33).

Drying the media under vacuum and resuspending the dehydrate in an organic solvent has been used with ion-exchange and paper chromatography in work with spermine, and spermidine (34). Another approach has been to employ an organic solvent that extracts the desired product from water in amounts sufficient for analysis (29). In the case of nitriles, mixtures of known composition were extracted from an aqueous HCl solution saturated with KCl using five volumes of o-dichlorobenzene (4). One disadvantage of using organic solvents is that they may obscure detection of compounds that have retention times close to that of the solvent. With suitable sample preparation, gas chromatography can provide the required sensitivity, accuracy, and simplicity for routine analysis of small culture samples.

From the available information, it appeared that a rapid and accurate test might be developed for analysis of amines produced in bacterial culture media. The present research was undertaken to develop such a procedure and determine its applicability for selected species of bacteria.

GAS CHROMATOGRAPHIC ANALYSIS OF AMINES AND OTHER COMPOUNDS PRODUCED BY SEVERAL SPECIES OF *CLOSTRIDIUM*[1]

Introduction

Microorganisms may produce amines by decarboxylation of amino acids, degradation of polyamines, condensation of amines, or possibly by transamination of aldehydes or other compounds (11, 14, 18, 21, 32, 40, 41, 43). Further study of amine production may prove important to our understanding of protein and amino acid metabolism by bacteria, may yield information concerning the toxic effects of bacteria, and may aid in identification and taxonomy of bacteria.

Comprehensive study of the production of amines by bacteria cultures has been hindered by the lack of analytical methods that are rapid and practical for the analysis of small samples of dilute aqueous solutions containing amines. Many amines are difficult to extract from aqueous solutions. They tend to adsorb on column packing materials, causing tailing and poor resolution. Conversion of amines to amine derivatives (46) and conditioning of various column packing materials (38) have been used to decrease tailing and increase resolution.

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Recent reports of analyses of mixtures of known amines using gas chromatography (5, 9, 30, 46) suggested that procedures suitable for routine analysis of bacterial cultures might be developed. The present study was undertaken to develop sensitive routine gas chromatographic tests for amines in whole cultures. Of secondary consideration was development of tests to detect additional bacterial products including higher alcohols, fatty acids, and unidentified neutral compounds that contain no free hydroxyl, carboxyl, or amino groups.

Several investigators have utilized gas chromatography for identification of fatty acids and have found them useful as an aid in differentiation of microorganisms (1, 29, 50). Previous procedures employed for isolation and identification of long chain fatty acids have not been practical for extensive use because they are complex and tedious.

Materials and Methods

I Cultures.

Three strains of each species of *Clostridium* were compared in most cases. The organisms tested and their V. P. I. strain nos. are listed below:

C. bifermentans: 2035, 2036, 2012, and 3131
C. botulinum type A: 1613, 1624, and 1550
C. botulinum type B proteolytic (P): 1612, 1541, and 1618
C. botulinum type B non proteolytic (NP): 1731
C. botulinum type C: 3803, 3804 and 1542
C. botulinum type D: 1543, 1615, and 3805
C. botulinum type E: 1623, 1545, and 1547
C. botulinum type F (NP): 1730 and 1549

C. botulinum type F (P): 1548
C. butyricum: 1717, 1622, and 1718
C. chauvoei: 2030, 2029, and 1527
C. histolyticum: 2032, 2031, and 1528
C. innocuum: 1614, 2019, and 2020
C. limosum: 0658, 1950, 1748B, and 1928
C. novyi type A: 1617, 1619, and 2021
C. novyi type B: 1602
C. perfringens type A: 1538, 0609, and 1537
C. perfringens type B: 1588
C. perfringens type C: 1539
C. perfringens type D: 1540
C. perfringens type E: 1589
C. septicum: 2865, 2874, and 2881
C. sordellii: 2013, 2025, and 2027
C. sporogenes: 1529, 2022, and 2026
C. tetani: 1608, 1609, and 2010

With the exception of *C. bifermentans* 3131, *C. botulinum* types C: 3803, 3804, and D 3805, *C. limosum* (previously listed as *C. bubalorum* but now recognized as *C. limosum*) 1950, 1748B, and 1928, *C. septicum* 2865, 2874, and 2881, the sources and cultural characteristics of the 62 strains representing 13 species of *Clostridium* have been described (29). These other strains have been characterized in this laboratory and conform to the description of each species (29).

II Sample Preparation.

1. Extraction of cultures. Six ml of pre-reduced chopped meat medium with 0.5% added glucose (CMG) at pH 7 were inoculated under oxygen-free CO_2 with approximately 0.1 ml of an actively growing culture.

C. botulinum cultures were incubated at 30°C, and cultures of all other species at 37°C.

After incubation for one week cultures were acidified with 50% (v/v) aqueous H_2SO_4 to approximately pH 2, as indicated by pH test paper. Acidified cultures were (Fig. 1, culture 1) extracted with 3 ml of spectrograde ethyl ether (Eastman Chemicals)* by shaking thoroughly for about one minute followed by centrifugation for approximately 2-3 minutes to break the ether-water emulsion. The ether layer was transferred to a 12 x 75 mm culture tube with a Pasteur pipette. Most of the organic acids, alcohols, ketones, adlehydes, and unidentified neutral compounds were removed in this extraction. A separate culture (Fig. 1, culture 2) was acidified and extracted with chloroform rather than ether when further characterization of these products was desirable.

To obtain amines, the acidified chloroform or ether-extracted culture residue was then made basic (pH 10-11) with 8N NaOH, as indicated by pH test paper and re-extracted with 3 ml of spectrograde chloroform (Fisher Scientific) by shaking thoroughly for about one minute followed by brief centrifugation. The aqueous supernatant fluid was decanted and discarded.

2. Preparation of fatty acid methyl esters. For satisfactory chromatographic elution, short and long chain fatty acids were converted

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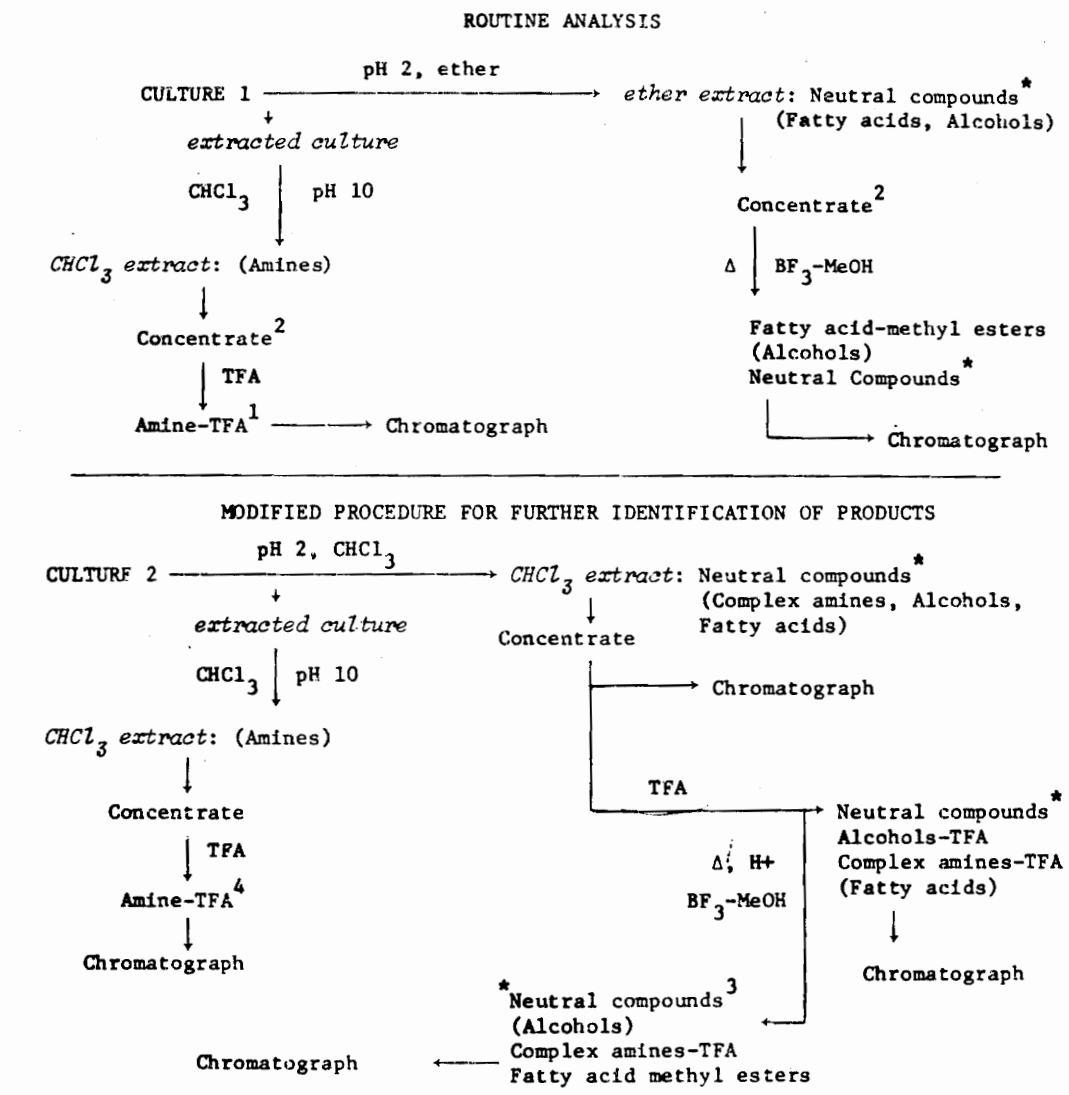
*Use of trade names does not constitute endorsement by the U. S. Public Health Service.



1. Some alcohols may carry through if present in large quantities.
2. May be further concentrated after the reaction is complete.
3. Sometimes missing, possibly due to dilution and evaporation.
4. Amine concentration decreased due to prior acid-chloroform extraction.

* Compounds that do not react with TFA or BF_3-methanol

( ) Not usually detected

Figure 1. Procedure for analysis of soluble products in broth cultures.

to methyl esters with boron trifluoride methanol (BF_3-MeOH) as follows:

$$R\text{-}CO_2H + MeOH \xrightarrow[H+]{BF_3} R\text{-}CO_2CH_3 + H_2O$$

The acid ether extract was concentrated to approximately 0.5 ml under a moderate stream of air led into a stoppered culture tube through a hypodermic needle and discharged through a similar needle into a hood. Concentration required approximately 4 minutes. Approximately 0.1 ml of BF_3-MeOH reagent (Applied Science Laboratories, Inc., State College, Pennsylvania) was added to the concentrated ether extract (all reagents were added dropwise, and volumes are approximate). The tube was heated in a boiling water bath for 4 minutes and cooled in a flowing stream of tap water for about 30 sec. Approximately 0.07 ml of a solution containing 14 parts chloroform to 1 part methanol were added and mixed by shaking to extract the fatty acid esters. Approximately 0.07 ml of 0.1N NaOH was then added to remove excess methanol and the mixture was briefly shaken. One tenth to one microliter of the light brown chloroform ball was chromatographed.

3. Preparation of trifluoroacetic anhydride derivatives of amines and alcohols. For satisfactory chromatographic elution, primary and secondary alcohols and amines were acylated with trifluoroacetic anhydride (TFA) as follows:

1. $R\text{-}CH_2OH\ (R_2\text{-}CHOH) + (CF_3CO)_2O \rightarrow R\text{-}CH_2OCOCF_3 (R_2\text{-}CHOCOCF_3) + CF_3COOH$
2. $R\text{-}NH_2\ (R_2\text{-}NH) + (CF_3CO)_2O \longrightarrow R\text{-}NHCOCF_3\ (R_2\text{-}NCOCF_3) + CF_3COOH$

The reaction is exothermic and no additional heat is required. Pyridine

(PY) catalyzes the reaction and improves acetylation. Tertiary alcohols and amines ordinarily are not acylated.

To test for amines, the chloroform extract of the alkalinized medium was concentrated for about 15 minutes under an air stream to approximately 0.09 ml; then 6 drops from a 22 gauge needle (ca. 8.4 µl) of a solution containing 14 parts TFA to 1 part pyridine were added and mixed by shaking. The mixture was stoppered and held for three minutes; ca. 0.07 ml of 4% HCl was added with shaking to remove excess pyridine and anhydride which bleed from the column and interfere with resolution. A cloudy chloroform ball then formed below the aqueous layer. One-tenth to one microliter of the chloroform was removed for chromatography. When free amines or a part of the aqueous layer are injected along with the chloroform layer, resolution of products is poor.

Moisture inactivates the TFA-Py mixture and causes formation of a precipitate in the reagent solution. To keep the reagent dry and to contain offensive fumes, the reagent was kept in a serum stoppered bottle.

Rarely, the TFA derivatives apparently revert to the free amines. If derivative formation is incomplete or the derivatives revert to free amines, the aqueous layer can be removed and the sample treated again.

4. Procedure for further identification of bacterial products. Neutral compounds which may include tertiary alcohols or complex tertiary amines are obtained in ethyl ether or chloroform extracts of

acidified cultures, and some of these compounds are detected in chromatograms of the untreated extracts. These compounds do not react with BF_3-MeOH or TFA. Primary and secondary alcohols and complex amines also were sometimes found in chloroform extracts of acidified cultures, but they are not detected until they are acylated. When the acylated mixture is subsequently methylated, the acylated alcohols are unstable (to heat in the presence of 4% HCl) and are no longer detected. The amines remain acylated and are still detected. The fatty acids are usually not detected until they are methylated.

For differentiation of neutral compounds, (compounds described here as "neutral compounds" do not contain free hydroxyl-, amine- or carboxyl groups) alcohols, complex amines and fatty acids; a chloroform extract of an acidified culture was concentrated to approximately 0.09 ml, and one-tenth to one microliter of the untreated concentrate was chromatographed. Only neutral compounds were detected in this sample.

The remaining concentrate was acylated as described (II-3). One-tenth to one microliter of the chloroform TFA treated extract was chromatographed. Neutral compounds, alcohol derivatives and acid extractable complex amine derivatives were detected in this sample.

After removal of the aqueous residue, the TFA treated solution was methylated with ca. 0.1 ml of BF_3-MeOH reagent as described above (II-2). The methylated solution was chromatographed. Complex amine derivatives and fatty acids were detected. Neutral compounds may or

may not appear in this sample depending on the amount of dilution or other undetermined factors.

The size of the above chromatographic samples ranged from 0.1 to 1.0 µl. The chloroform layer obtained in all three subsamples could be further concentrated for 10 to 30 seconds under an air stream to improve chromatographic peak resolution and facilitate identification. The above procedures are summarized in Figure 1.

III Chromatographic conditions.

Samples were analyzed on a Beckman GC-4 dual-column gas chromatograph with helium as the carrier gas at a flow rate of 48cc/min. (at 80°C). Dual hydrogen flame ionization detectors were used with a recorder speed of 1"/min. with an imput signal of 1 mv. Attenuation of 5×10^2 was used for amines and 2×10^2 for fatty acid esters, neutral compounds, and complex amine and alcohol derivatives. Chromosorb W; 80/100 mesh, acid washed, dimethyldichlorosilane treated, high performance (AW-DMCS, H.P.), coated with 3% SE 30 (Applied Science Laboratories, Inc.) was packed in dual stainless steel columns (1/8 inch O.D., 20 feet long). The column was temperature programmed for 1 minute at 80°C, a linear increase for 4 min. to 195°C, two minutes at 195°C, a linear increase for 2.25 minutes to 255°C, followed by sixteen minutes at 255°C. The temperature of the injector inlet was 220°C, of the detector line was 230°C, and of the detector was 270°C. The two columes were used alternately, except during identification of classes of compounds (II-4); then three samples (chloroform extract pH 2, acyl derivatives, and methyl esters) were analyzed in sequence on the same column. Tentative identification was made by

comparing retention times with those of derivatives of known compounds.

IV Standards.

A useful fatty acid standard contained 0.2 ml each of acetic and propionic, 0.1 ml each of isobutyric, butyric, isovaleric, valeric, isocaproic, caproic, heptanoic, caprylic, nonanoic, capric, and oleic acids, with 0.5 gm each of lauric, triedecanoic, myristic, pentadecanoic, palmitic, heptadecanoic, stearic, nonadecanoic, arachidic, docosanoic, and tetracosanoic acids, 0.1 ml of 50% H_2SO_4, and 3 ml of ethyl ether. Approximately 0.03 ml of this mixture was methylated and 3 to 4 µl of the resulting solution were used for chromatography (Fig. 2).

A suitable alcohol standard contained ca. 0.011 ml each of amyl-, butyl-, isoamyl-, phenyl-, hexyl-, and heptyl alcohols added to 1 ml of chloroform. Approximately 0.07 ml of the mixture was acetylated and 0.1 µl of the resulting solution was used for chromatography (Fig. 3).

The amine standard contained 0.56 ml methylamine (40% in water), 0.29 ml trimethylamine (25% in water), 0.35 ml ethylamine (70% in water), 0.15 ml n-propylamine, 0.08 ml isobutylamine, 0.09 ml 1-amino-2-propanol, 0.07 ml isoamylamine, 0.18 ml pyrrolidine, 0.08 ml n-hexylamine, 0.03 ml di-n-butylamine, 0.08 ml each of heptylamine and octylamine, 0.57 ml 1,3-diaminopropane, 0.088 ml β-phenylethylamine, 0.35 ml putrescine, 0.30 ml cadaverine, 6.0 ml each of 5% solutions of spermidine and spermine, made to a total volume of 65.2 ml by addition of 45.93 ml of chloroform. (Derivatives of the above compounds are eluted in the order listed.) To ca. 0.042 ml of the standard mixture

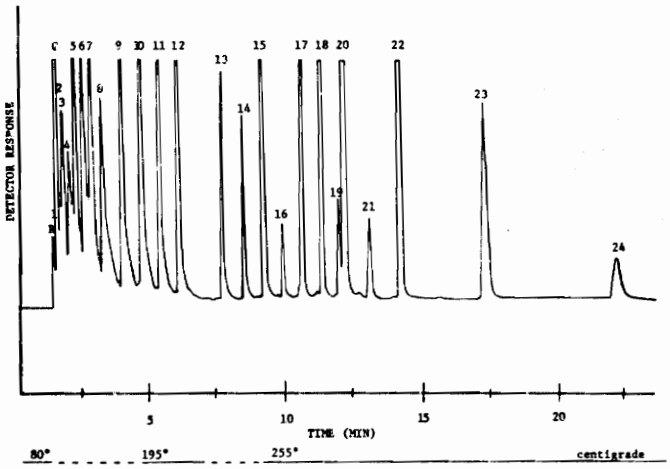


FIG. 2. Standard curve of fatty acid methyl esters.

R = reagent	6 = valeric	13 = lauric	20 = stearic
C = chloroform	7 = isocaproic	14 = tridecanoic	21 = nonadecanoic
1 = acetic	8 = caproic	15 = myristic	22 = arachidic
2 = propionic	9 = heptanoic	16 = pentadecanoic	23 = docosanoic
3 = isobutyric	10 = caprylic	17 = palmitic	24 = tetracosanoic
4 = butyric	11 = nonanoic	18 = heptadecanoic	
5 = isovaleric	12 = capric	19 = oleic	

— = isothermal

-- = linear temperature increase

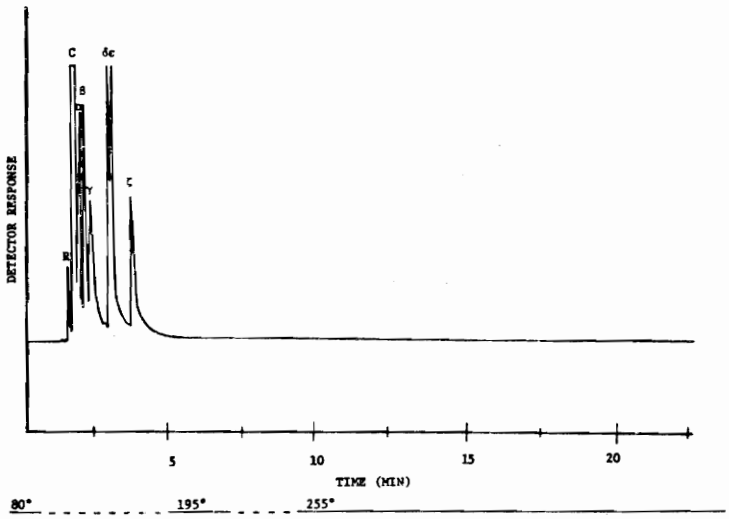


FIG. 3. Standard curve of alcohol trifluoroacetyl esters.

R = reagent
C = chloroform
α = butyl
β = isoamyl
γ = amyl
δ = phenol
ε = hexyl
ζ = heptyl

was added ca. 0.014 ml of an acylated tyramine tryptamine mixture prepared by adding 0.35 ml TFA plus 0.65 ml of chloroform to 0.062 gm of tyramine cloride, and 0.60 gm of tryptamine acylated as described above (II-3) and 0.6 to 0.8 µl of the resulting solution was chromatographed (Fig. 4).

Results and Discussion

Conditions suitable for amine production were determined with three strains of *C. tetani*. Amine production in peptone yeast extract with glucose, milk, gelatin (28, 29) chopped meat (39) and chopped meat with 0.6% glucose (CMG) media was compared. The maximum quantities of amines were produced in CMG at pH 6.5 to 6.7 and fewer kinds of amines were produced in the other media tested.

Maximum concentrations of amines were obtained after three weeks of incubation, but major amine products and recognizable chromatographic patterns were obtained with cultures of *C. tetani* after 24 hrs. of incubation. *C. botulinum* types A, B(P), F(P), *C. sporogenes*, and *C. novyi*, types A and B produce typical fatty acid and amine patterns in CMG after 48 hrs. of incubation.

Chromatograms of acylated and methylated extracts of uninoculated CMG medium are shown in (Fig. 5). Neutral compounds representing components of the uninoculated CMG medium were detected in the chloroform extract of the medium (Fig. 6).

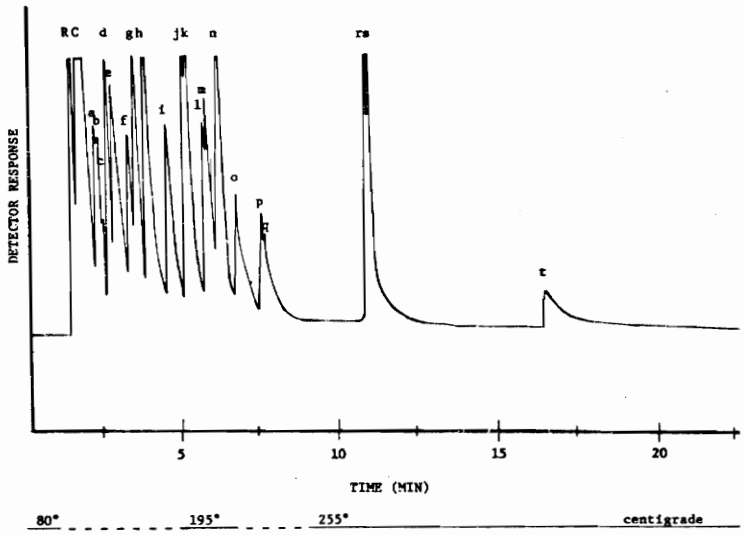


FIG. 4. Standard curve of amine trifluoroacetic anhydride derivatives.

R = reagent
C = chloroform
a = methylamine
b = trimethylamine
c = ethylamine
d = n-propylamine
e = isobutylamine
f = 1-amino-2-propanol
g = isoamylamine
h = pyrrolidine
i = hexylamine
j = di-n-butylamine
k = heptylamine
l = octylamine
m = 1,3-diaminopropane
n = β-phenylethylamine
o = putrescine
p = tyramine
q = cadaverine
r = tryptamine
s = spermidine
t = spermine

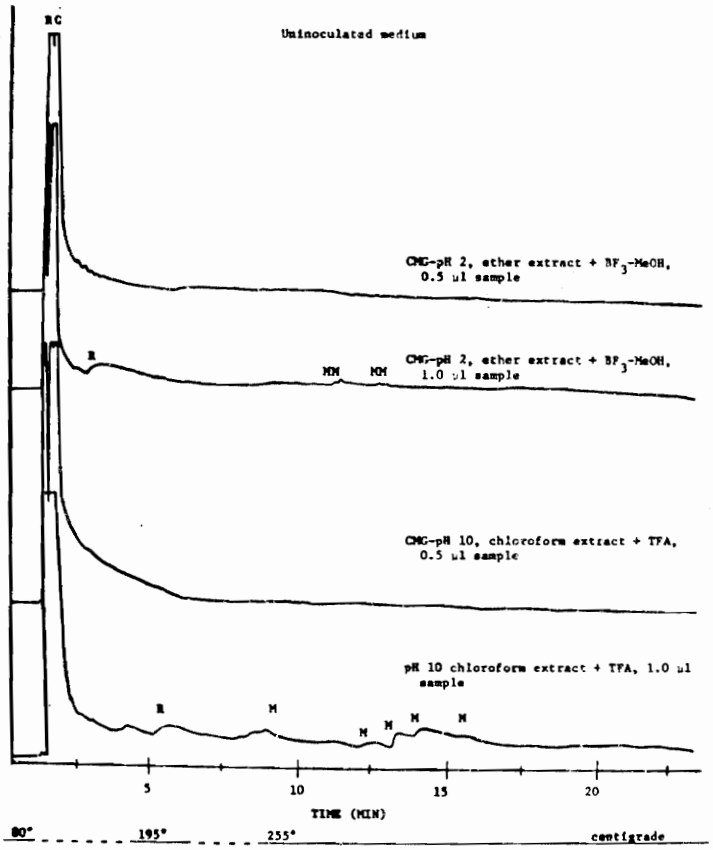


FIG. 5. Chromatograms of treated extracts of uninoculated medium.
R = reagent C = chloroform M = compounds in the medium

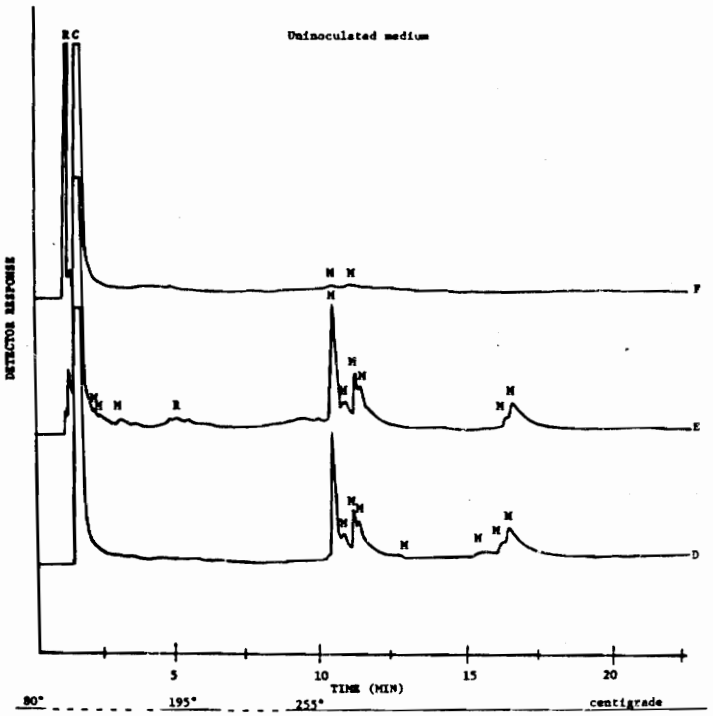


FIG. 6. Chromatograms of uninoculated CMG - pH 2, chloroform extract 1 µl.

R = reagent
C = chloroform
D = no reagent
E = plus TFA
F = plus TFA and BF_3 methanol
M = compounds in the medium

Fatty acid and neutral products.

As reported previously, short chain fatty acids are produced in large quantities by many of the cultures tested (29). The methylated ether or chloroform extracts of acidified whole cultures contained much smaller quantities of long chain fatty acids. Samples large enough to allow detection of long chain fatty acid esters (chain length longer than C10 and probably representing cellular fatty acids) generally contained such large amounts of neutral compounds and short chain fatty acid esters that these more volatile compounds were not resolved. With the present system two sample sizes were desirable. In the present study no attempt was made to improve resolution of the more volatile products by modification of the temperature program.

With proper sample sizes similar and characteristic chromatographic patterns were obtained for each of 3 strains of a species as illustrated in Figure 7, and different sets of patterns were produced by strains of other species (Figs. 8 and 9). The largest number of different neutral compounds and fatty acids were detected in culture extracts of *C. sporogenes* (Fig. 7). Some other species, such as proteolytic strains of *C. botulinum* type B and *C. botulinum* type A, produce similar patterns of these products as would be expected because their other metabolic properties have also been shown to be similar (21). Several of the species tested produced very few fatty acids as illustrated by chromatograms representing *C. septicum* (Fig. 8).

Neutral compounds that do not react with BF_3-MeOH or TFA were

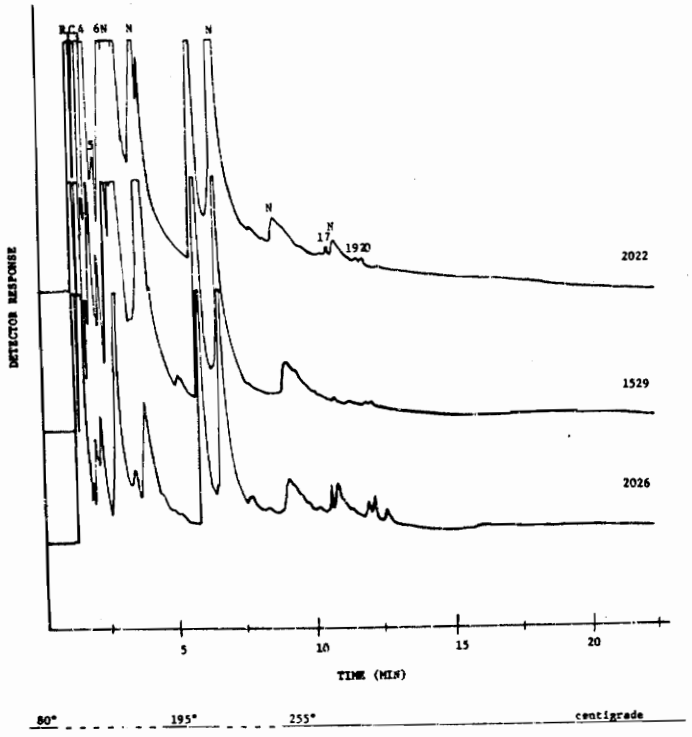


FIG. 7. *C. sporogenes*, fatty acid methyl esters and neutral products extracted with ether from acidified cultures at pH 2.

C = chloroform
N = neutral compounds
R = reagent
1 = acetic acid
3 = propionic acid
4 = butyric acid
5 = isovaleric acid
6 = valeric acid
17 = palmitic acid
19 = oleic acid
20 = stearic acid

unlabeled peaks = unidentified fatty acids.

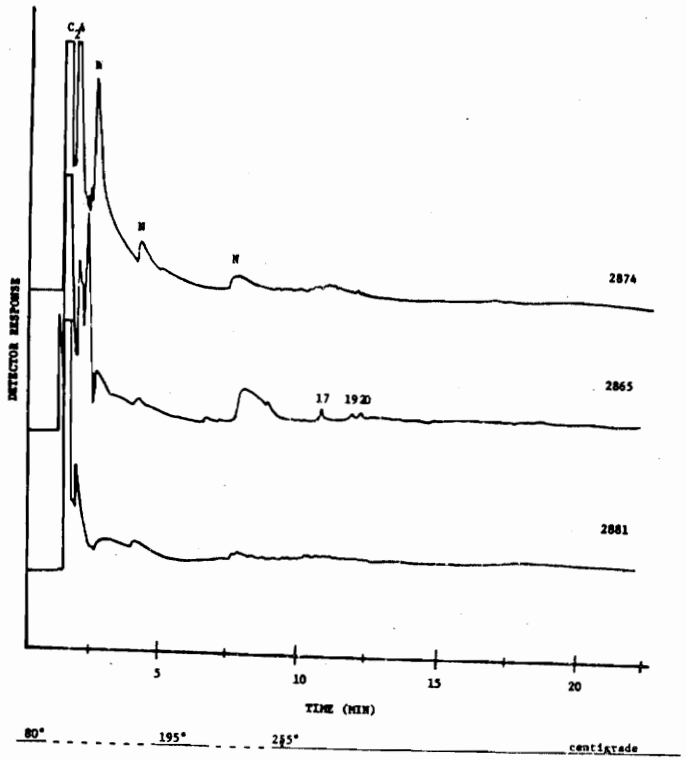


FIG. 8. *C. septicum*, fatty acid methyl esters and neutral products extracted with ether from acidified cultures at pH 2.

C = chloroform
N = neutral compounds
2 = propionic acid
4 = butyric acid
17 = palmitic acid
19 = oleic acid
20 = stearic acid
unlabeled peaks = unidentified fatty acids.

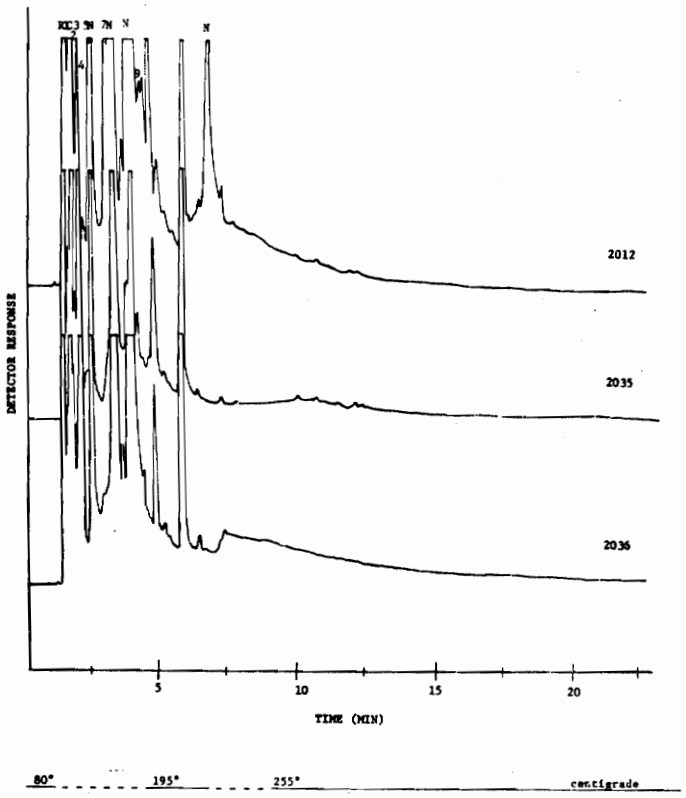


FIG. 9. *C. bifermentans*, fatty acid methyl esters and neutral products extracted with ether from acidified cultures at pH 2.

C = chloroform
N = neutral compounds
R = reagent
1 = acetic acid
2 = propionic acid
3 = isobutyric acid
4 = butyric acid
5 = isovaleric acid
7 = isocaproic acid
9 = heptanoic acid

unlabeled peaks = unidentified fatty acids.

frequently detected in the initial acid extract of cultures and these appeared in the methylated extract. Their elution profiles indicated that they have a different temperature-column affinity relationship than do the esters of fatty acids. The neutral compounds are more sensitive to changes in sample size than are fatty acid methyl esters. In Figure 9 the neutral compound eluted in 7 minutes was present in only one chromatogram (strain 2012); however, the product was detected in other preparations from strains 2035 and 2036. The reasons for this variation in detecting neutral compounds from similar cultures of the same strains have not been determined.

On the basis of fatty acid methyl esters and neutral products the 13 species of *Clostridium* tested were divided into seven groups in Table I (p. 44), and representative chromatograms are shown in Figures 7-9, Appendix I (AI) 2-10, and Appendix II (AII) 7.

Amine products.

A surprisingly large number of products was detected in the extracts of alkaline media that were previously extracted under acid conditions. As many as 50 or 60 different acylated compounds can be detected in extracts of individual cultures. Extraction of these compounds from alkalinized medium (pH 10) and failure to detect them without acylation, indicates that they most probably are amines. Retention times of 18 of the peaks are identical to retention times of TFA-derivatives of known amines.

Based on the size of chromatographic peaks obtained from

amines extracted from aqueous solutions treated in the same manner as cultures, it was estimated that as much as 1 μM of isoamylamine, 0.5 μM of β-phenylethylamine or approximately 1 μM of cadaverine per ml of culture were produced by some strains of clostridia. These concentrations of individual products indicate that amine concentrations exceeded 0.5% (weight) in the broth culture. Chromatographic peaks of the cadaverine derivatives were much smaller than those of β-phenylethylamine or isoamylamine. Cadaverine and other diamines are more difficult to extract from aqueous solutions and small chromatographic peaks represent disproportionately high aqueous concentrations.

Although there was noticeable variation in individual peaks that were detected among individual preparations, the general pattern, and often certain areas of the individual chromatograms, are distinctive for the different species tested. The major products detected in cultures of *C. sordellii* (Fig. 10) showed similarities to those obtained from cultures of *C. bifermentans* (Fig. 11); however the strains of *C. bifermentans* tested produced much larger quantities of β-phenylethylamine. This difference is evident even though the samples analyzed were larger in the case of *C. sordellii* (Fig. 10). The metabolism of the two organisms is similar and differientiation is usually made on the basis of urease production by *C. sordellii*.

As illustrated in Figure 12 cultures of *C. innocuum* characteristically contained a large number of low boiling (more volatile) amines. The many derivatives eluted within 5 minutes were obtained repeatedly in different preparations. In preparations from cultures of this species,

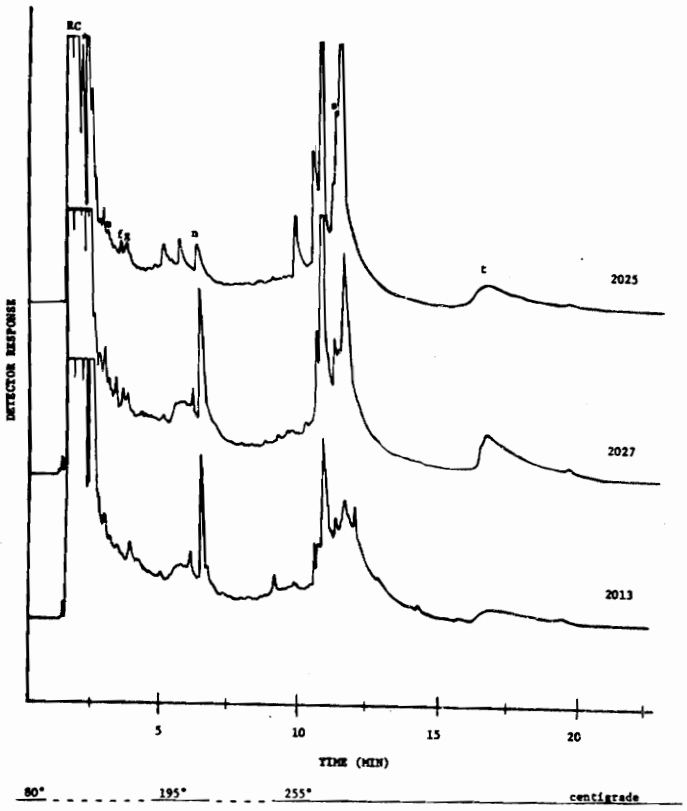


FIG. 10. *C. sordellii*, amine TFA derivatives extracted with chloroform from cultures at pH 10.

C = chloroform
R = reagent
* = alcohol or amine
e = isobutylamine
f = 1-amino-2-propynol
g = isoamylamine
n = β-phenylethylamine
s = spermidine
t = sperminde
unlabeled peaks = unidentified amines.

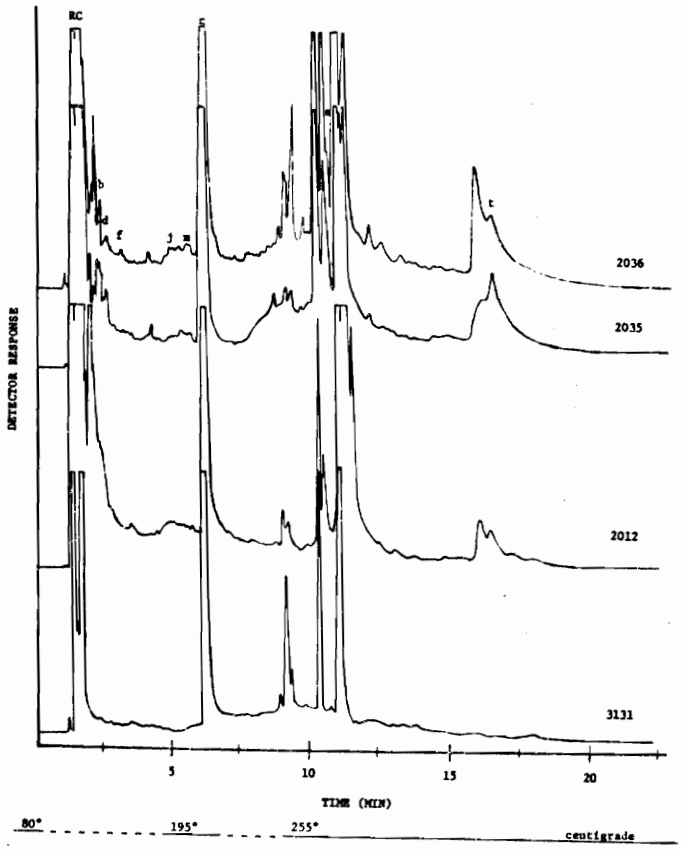


FIG. 11. *C. bifermentans*, amine TFA derivatives extracted with chloroform from cultures at pH 10.

C = chloroform
R = reagent
b = trimethylamine
d = n-propylamine
f = 1-amino-2-propanol
j = di-n-butylamine
m = 1,3-diaminopropane
n = β-phenylethylamine
s = spermidine
t = spermine

unlabeled peaks = unidentified amines.

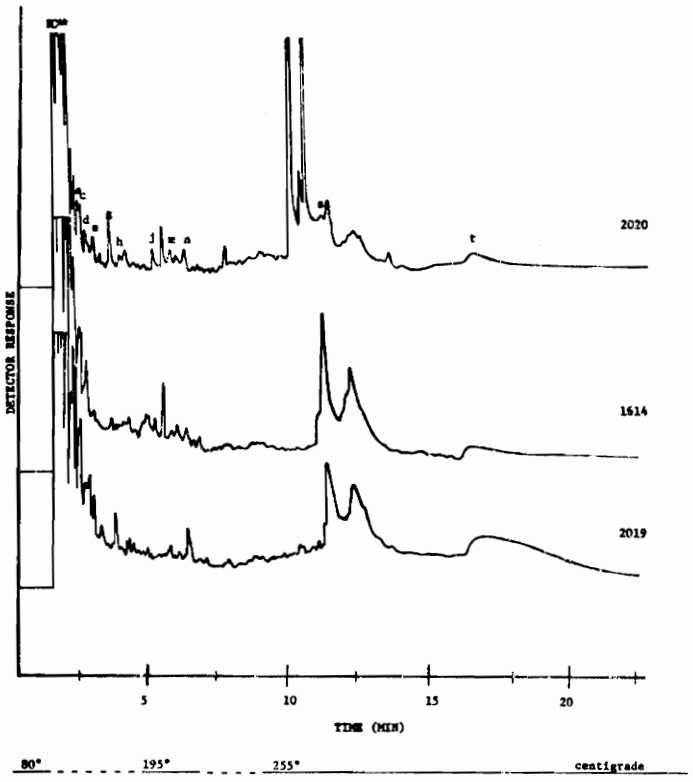


FIG. 12. *C. innocuum*, amine TFA derivatives extracted with chloroform from cultures at pH 10.

C = chloroform
R = reagent
* = alcohol or amine
a = methylamine
c = ethylamine
d = n-propylamine
e = isobutylamine
g = isoamylamine
h = pyrrolidine
j = di-n-butylamine
m = 1,3-diaminopropane
n = β-phenylethylamine
s = spermidine
t = spermine

unlabeled peaks = unidentified amines.

products eluted after 5 minutes were present in variable amounts between strains, but in all preparations only a relatively few higher boiling amines were detected.

Some of the variation observed between different strains demonstrates the sensitivity of the methods. Strain no. 1928 of *C. limosum* (Fig. 13) is a variant of this species. The chromatographic pattern of amine derivatives of this variant is atypical in that much smaller quantities of the major compounds are consistently produced. Dr. C. S. Cummins of this laboratory has found that cell walls of 1928 contain more galactose and less mannose than cell walls of the other strains and sera against cell wall antigens of strain 1928 do not agglutinate the other strains. Sera against cell wall antigens of the other strains do not agglutinate strain 1928. The other three strains are antigenically similar to each other and to most strains of *C. limosum*.

Another example of differences between strains of the same species is illustrated in chromatograms from cultures of *C. histolyticum* (Fig. 14). Repeated cultures of strain 2031 produced large quantities of putrescine and cadaverine while much less putrescine and cadaverine were detected in repeated cultures of strains 1528 and 2032. The cultural and biochemical characteristics were similar in all of these strains.

Cultures of *C. perfringens* type A also contained a large number of amines. Representative chromatograms for three strains tested are presented in Figure 15. A characteristic of these cultures is the presence of a large number of both high boiling and low boiling amine products.

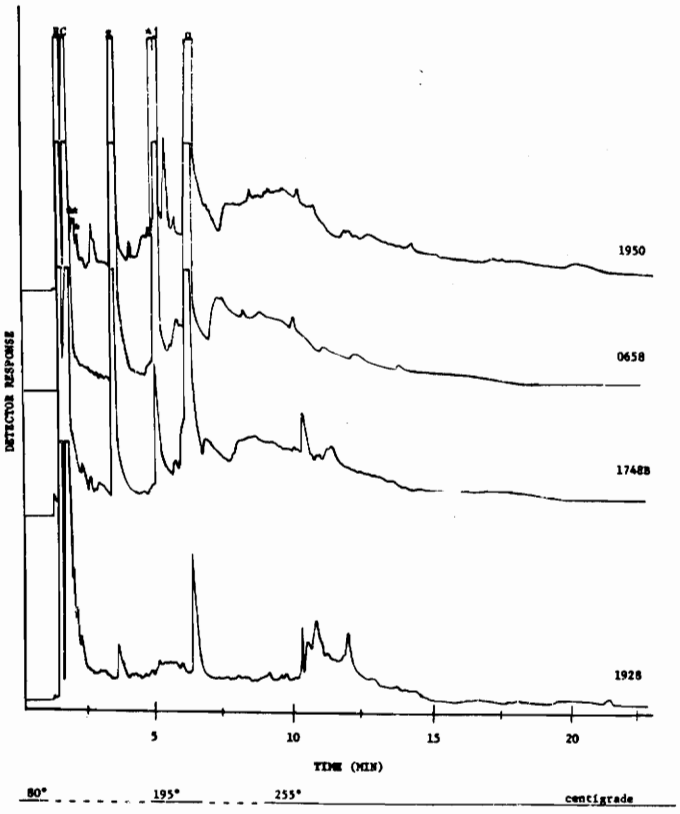


FIG. 13. *C. limosum*, amine TFA derivatives extracted with chloroform from cultures at pH 10.

C = chloroform
R = reagent
* = alcohol or amine
g = isoamylamine
j = di-n-butylamine
n = β-phenylethylamine

unlabeled peaks = unidentified amines.

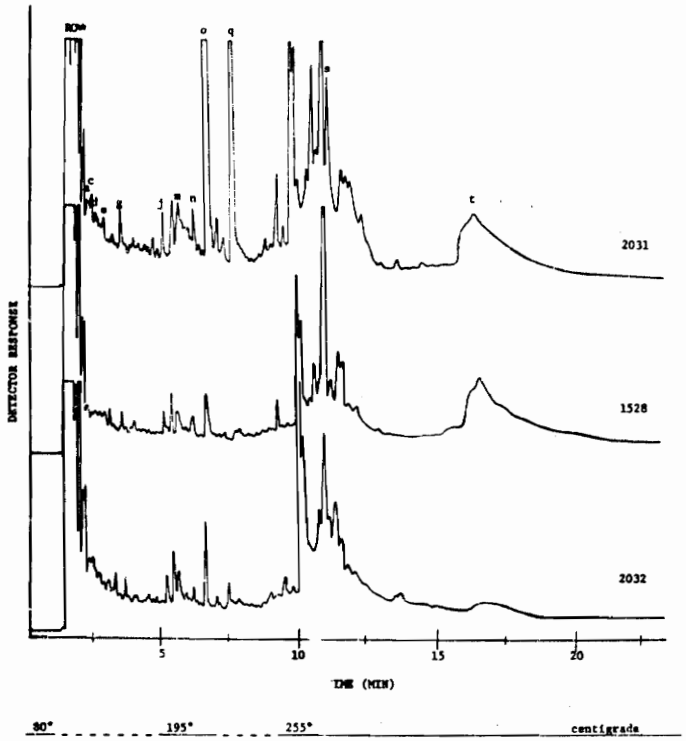


FIG. 14. *C. histolyticum*, amine TFA derivatives extracted with chloroform from cultures at pH 10.

C = chloroform
R = reagent
* = alcohol or amine
a = methylamine
c = ethylamine
d = n-propylamine
e = isobutylamine
g = isoamylamine
j = di-n-butylamine
m = 1, 3-diaminopropane
n = β-phenylethylamine
o = putrescine
q = cadaverine
s = spermidine
t = spermine
unlabeled peaks = unidentified amines.

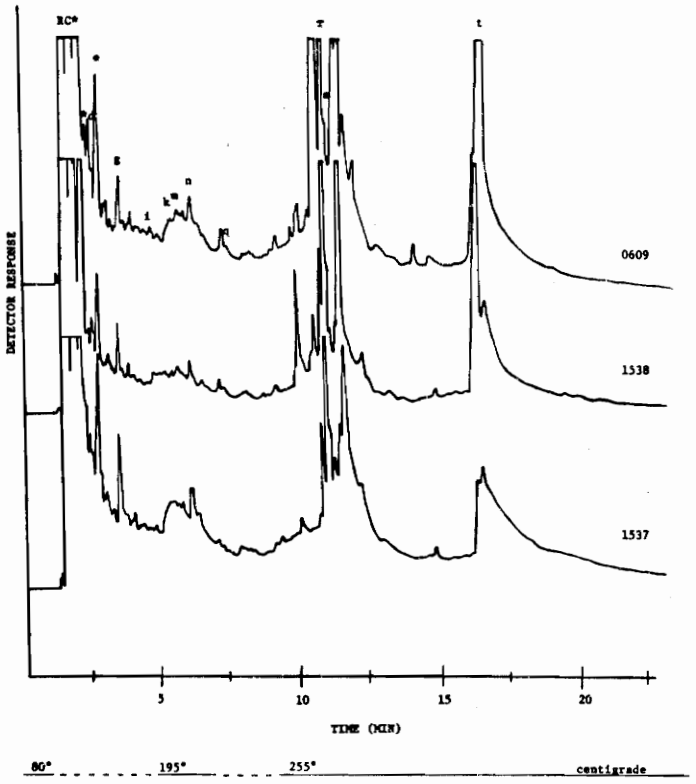


FIG. 15. *C. perfringens* type A, amine TFA derivatives extracted with chloroform from cultures at pH 10.

C = chloroform
R = reagent
* = alcohol or amine
d = n-propylamine
e = isobutylamine
g = isoamylamine
i = hexylamine
k = heptylamine
m = 1,3-diaminopropane
n = β-phenylethylamine
q = cadaverine
r = tryptamine
s = spermidine
t = spermine
unlabeled peaks = unidentified amines.

On the basis of their major amine products the 13 species of *Clostridium* were divided into 10 groups. The distinctive major amine products of each of these groups are summarized in Table II (p. 45), and representative chromatograms are shown in Figures 10-15, AI 11-13, and AII 3-6.

Alcohol, complex amine, and neutral compound products.

On the basis of alcohols, complex amines, and neutral compounds produced the 13 species of *Clostridium* tested were divided into 12 groups in Table II (p. 45), and representative chromatograms are shown in Figures 16-19 and AI 14-17.

Group 3 is the only group in which complex amines were detected in chloroform extracts of acidified medium (Fig. 16 peaks "u"), and on this basis the group was differentiated from group 4. The cultures of *C. botulinum* type E produced two neutral products that were found in the uninoculated medium. The two unlabeled peaks at 11.5 minutes were larger than they were in samples of uninoculated medium.

Some of the alcohol-TFA esters apparently have very little affinity for the column and elute in an unusual pattern. In Fig. 17, the alcohol, or mixture of alcohols, with a retention time of 4.5 minutes distinguishes cultures of *C. botulinum* type C from *C. botulinum* type D and *C. novyi* types A and B.

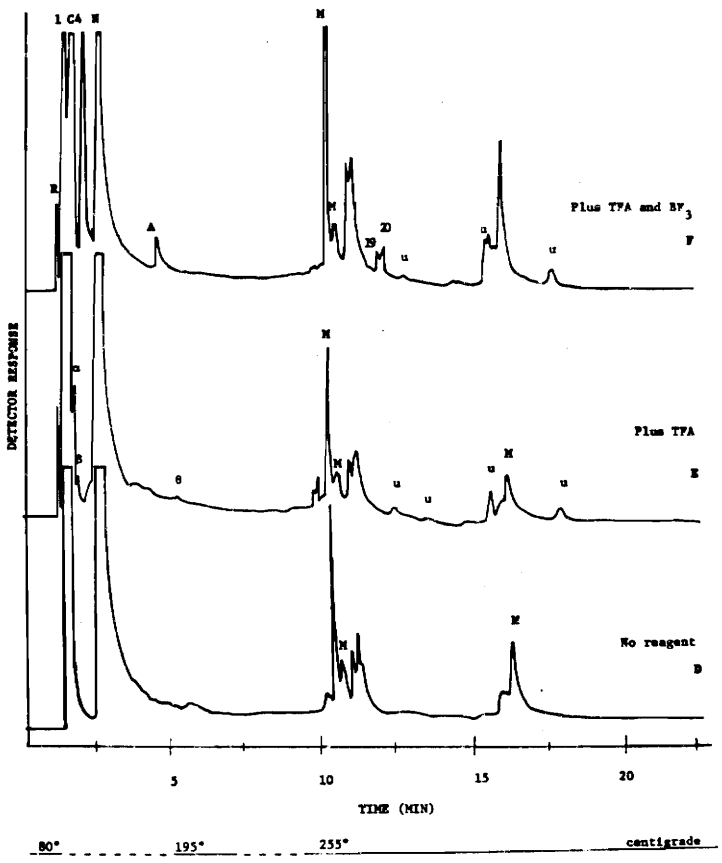


FIG. 16. Complex amines and other products extracted with chloroform from acidified cultures of *C. botulinum* type E.

A = unidentified fatty acid
C = chloroform
N = neutral compound
R = reagent
α = butyl-alcohol-TFA esters
β = isoamyl alcohol-TFA esters
θ = unidentified alcohol
M = compounds in medium
u = unidentified amines
1 = acetic acid
4 = butyric acid
19 = oleic acid
20 = stearic acid

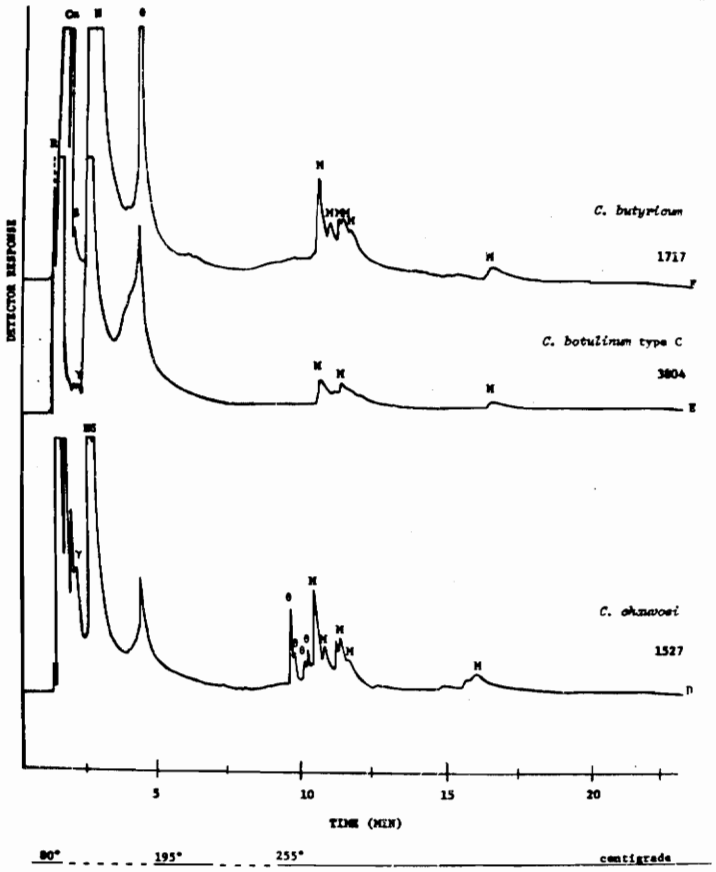


FIG. 17. Alcohol-TFA esters and neutral compounds extracted with chloroform from acidified cultures.

C = chloroform
N = neutral compounds
R = reagent
α = butyl alcohol
β = isoamyl alcohol
δ = phenol
Θ = unidentified alcohols
M = compound from the medium

Figure 18 illustrates the different classes of compounds extracted with chloroform from acidified cultures. Some neutral compounds (M and N) were detected without acylation or methylation (chromatogram D). When the extract was acylated, the TFA esters of alcohols were also detected (chromatogram E: α, δ, and Θ). When the acylated mixture was subsequently methylated, fatty acids were detected, but alcohol TFA esters were destroyed and no longer detected. Other neutral compounds were extremely sensitive to changes in sample size. The product (N) eluted after 2.9 minutes did not appear after methylation, probably as a result of dilution. That this product is not an alcohol was indicated by its appearance in the chromatogram of the untreated extract (chromatogram D). Its appearance in methylated ether extracts of another acidified culture of *C. tetani* (Fig. 19; strain 2010) further suggests it is a neutral compound and simply because of dilution, is no longer detectable after both acylation and methylation. On the other hand the product was not extracted from cultures of *C. tetani* at pH 7 (Fig 19, strain 1608); thus the true nature of this compound is not known.

The chromatograms of neutral compounds and esters of fatty acids extracted at pH 2, represented in Figure 19 (strain 2010), illustrate products detected in routine analysis of cultures. The absence of neutral compounds (N) in the $CHCl_3$ extract of strain 1609 at pH 10, indicates that these compounds are effectively removed by the initial extraction of the acidified medium.

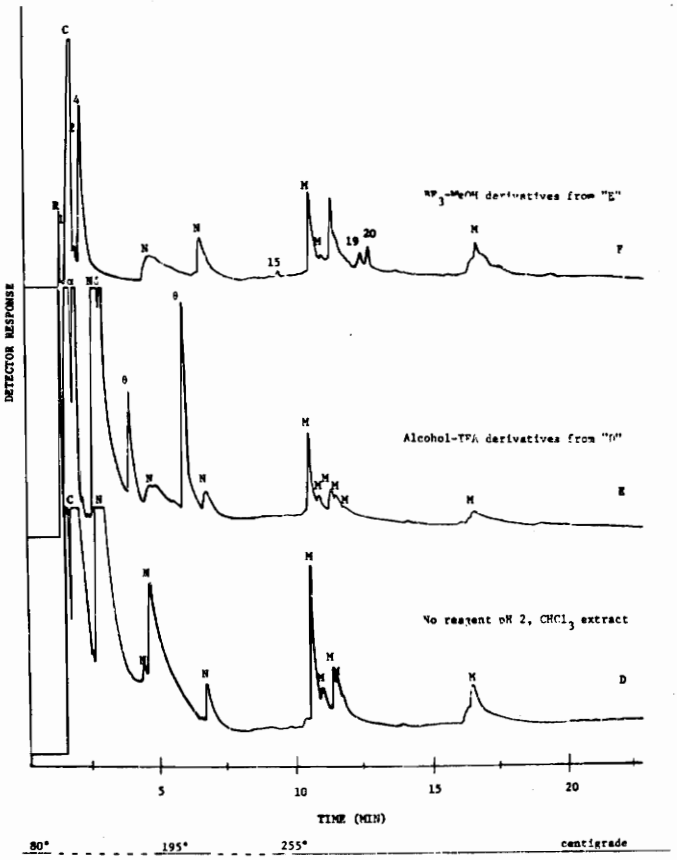


FIG. 18. Identification of classes of compounds extracted with chloroform from an acidified culture of *C. tetani* No. 1609.

C = chloroform
M = compound
N = neutral compounds
α = butyl alcohol
δ = phenol
Θ = unidentified alcohol

2 = propionic acid
4 = butyric acid
15 = myristic acid
19 = oleic acid
20 = stearic acid

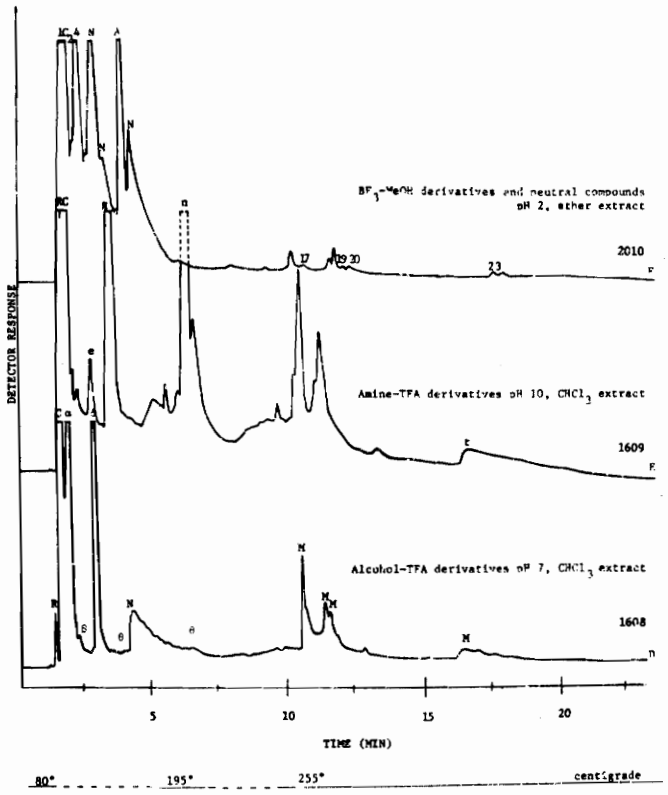


FIG. 19. Identification of classes of compounds extracted from cultures of *C. tetani*

A = unidentified fatty acid
C = chloroform
M = compounds from medium
R = reagent
1 = acetic acid
2 = propionic acid
4 = butyric acid
17 = palmitic acid
19 = oleic acid
20 = stearic acid
23 = docosanoic acid
e = isobutylamine
g = isoamylamine
n = β-phenylethylamine
t = spermine
α = butyl alcohol
β = isoamyl alcohol
δ = phenol
Θ = unidentified alcohol
in 2010 unlabeled peaks = unidentified fatty acids.
in 1609 unlabeled peaks = unidentified amines.

TABLE I

Characteristic fatty acid and neutral products of some species of *Clostridium*.

Organism	Group	Fatty Acid Products	Neutral Products
C. bifermentans	I	2, 3, 14, 4(S), 15, 16, 7, (6)	(2.6, 3.5, 4, 7)
C. sordellii		2, 3, 14, 4(S), 15, 16, 7, (6)	(2.6, 3.5, 4, 7)
C. botulinum A	II	2, 3(V), 14(S), 4, 15, 5, (6)	(3.5, 4, 7, 8.5, 11)
C. botulinum B (P)		2, 3(V), 14(S), 4, 15, 5, (6)	(3.5, 4, 7, 8.5, 11)
C. botulinum F (P)		2, 3(V), 14(S), 4, 15, 5, (6)	(3.5, 4, 7, 8.5, 11, 13.5)
C. sporogenes		2, 3(V), 14(S), 4, 15, 5, (6)	(3.5, 4, 7, 8.5, 11)
C. botulinum B(NP)	III	2, 4	(2.8)
C. botulinum E		2, 4, 3(S)	(2.8)
C. botulinum F(NP)		2, 4	(2.8)
C. botulinum D		2, 4	(2.8)
C. novyi A		2, 3(S), 4, 5(S)	[2.8, 7.6(V), 7.8(V)]
C. novyi B		2, 3(S), 4, 5(S)	(2.8)
C. botulinum C		2, 4	(2.8)
C. butyricum		2, 4	
C. chauvoei		2, 4	
C. perfringens A		2, 4	(2.8)(S)
C. perfringens C		2, 4	(2.8)(S)
C. perfringens B		2, 4	(2.8)(L)
C. perfringens D		2, 4	(2.8)(L)
C. perfringens E		2, 4	(2.8)(L)
C. limosum	IV	2	
C. histolyticum		2	
C. septicum	V	2, 4	(2.8, 3.2, 6.5)
C. innocuum	VI	2, 3, 4, (6.3)(V)	(3.2, 4, 4.4, 5.2, 7.1)
C. tetani	VII	2, 3, 4, (3.9)	(2.9, 3(S), 4.5)

TABLE II

Characteristic amines, alcohols, and neutral compounds produced by some species of *Clostridium*.

Organism	Group	Amines	Alcohols Neutral Compounds and Complex Amines	Group
C. bifermentans	A	βp(L), (9.3,9.4), HBA	(5.8,6.3), [N2.6,3.5,4,7(V)]	1
C. sordellii		βp, HBA	(5.8,6.3), [N2.6,3.5,4,7(V)]	
C. botulinum A	B	1, i4, 6, dB, HBA	4, i5, (5.9), [N3.5,4,7,8.5,11(V)]	2
C. botulinum B (P)		1, i4, 6, dB, HBA	4, i5, (5.9), [N3.5,4,7,8.5,11(V)]	
C. botulinum F (P)		1, i4, 6, dB, HBA	4, i5, (5.9), [N3.5,4,7,8.5,11(V)]	
C. sporogenes		1, i4, 6, dB, HBA	4, i5, (5.9), [N3.5,4,7,8.5,11(V)]	
C. botulinum B (NP)	C	d3, (9.9,10), HBA	4, i5, [5.9(S)], (N2.8), (A12.6, 13.5,15.8,18.2)	3
C. botulinum E		d3, (9.9,10), HBA	4, i5, [5.9 (S)], (N2.8), (A12.6, 13.5,15.8,18.2)	
C. botulinum F (NP)		d3, (9.9,10), HBA	4, i5, [5.9 (S)], (N2.8), (A12.6, 13.5,15.8,18.2)	
C. botulinum D	D	i5, βp,	4, i5, (N2.8)	4
C. novyi A		i5, βp,	4, i5, (N2.8)	
C. novyi B		i5, βp,	4, i5, (N2.8)	
C. botulinum C		i5, βp,	4, i5,5(S),(4.5),(N2.8)	5
C. butyricum		LBA, HBA, βp(V)	4, i5, (4.5), (N2.8)	
C. chauvoei		LBA, MHBA	4,i5,5,C6,(4.5,9.8,9.9,10.4, 10.5), (N2.8)	6
C. perfringens A	E	LBA, MHBA	4,OSCA, (N2.8)(S)	7
C. perfringens C		LBA, MHBA	4,OSCA, (N2.8)(S)	
C. perfringens B		LBA, MHBA	4,OSCA, (N2.8)(L)	
C. perfringens D		LBA, MHBA	4,OSCA, (N2.8)(L)	
C. perfringens E		LBA, MHBA	4,i4, OLCA, (N2.8)(L)	
C. limosum	F	i5, dB, βp (5)	4,OSCA, (N2.8,5.2,7.4)	8
C. histolyticum	G	d4, d5(V), MHBA	4,(2.8)	9
C. septicum	H	d4(S), d5	4, (4.7,4.9), (N2.8)	10
C. innocuum	J	i5, MLBA, HBA(S)	4, i5(S), (5.8),(N3.2,4,7.1)	11
C. tetani	K	i4, i5, βp	4(L), C6,(4.2,6.1),(N2.9,4.5, 6.8)	12

Legend for Tables I and II

A in parenthesis with numbers = retention times for acid extractable complex amines.

C = cyclic.

d before a number = diamine.

dB = di-n-butylamine.

HBA = high boiling amines (amines with retention time longer than 10.5 minutes).

i = iso.

(L) = large amount

LBA = low boiling amines (amines with retention time shorter than 3 minutes).

MHBA = many high boiling amines.

MLBA = many low boiling amines.

N = unidentified neutral compounds.

NP = non proteolytic.

Numbers in parenthesis or brackets = retention time of unidentified compounds in a particular group.

Numbers without parenthesis or brackets = the number of carbon atoms in acid, amine, or alcohol.

OLCA = other long chain alcohols present.

OSCA = other short chain alcohols present.

βp = β-phenylethylamine.

P = proteolytic.

(S) = small amount.

(V) = variable.

Some compounds listed in tables I and II are not labeled on representative chromatograms.

The above data indicate that a variety of amines are produced by the organisms tested, and that the quantity in some cases is quite high. The possibility exists that amines are produced in sufficient quantity to affect the pathogenecity of the organism. While there is some variation in the patterns produced by different strains of the same species this strain variation often agrees with information obtained by other methods as in the case of *C. limosum*. Neutral products were present in almost all organisms tested and were major products of some species. Further study and identification of these compounds is warranted. Alcohols, both short and long chain, were detected in several organisms and were valuable in grouping as indicated in Table III. For identification purposes simplification or refinement of the gas chromatographic patterns might be accomplished by alteration of group substrates, modification of chromatographic conditions, or exposing resting cell suspensions to individual amino acids or groups of amino acids.

TABLE III

Select species of *Clostridium* grouped on basis of compounds detected by gas chromatography

Organisms	Fatty Acids Neutrals	Amines	Alcohols Neutrals Complex amines	Summary
C. bifermentans	I	A	1	I A 1
C. sordellii				
C. botulinum A	II	B	2	II B 2
C. botulinum B (P)				
C. botulinum F (P)				
C. sporogenes				
C. botulinum B (NP)	III	C	3	III C 3
C. botulinum E				
C. botulinum F (NP)				
C. botulinum D		D	4	III D 4
C. novyi A				
C. novyi B				
C. botulinum C			5	III D 5
C. butyricum				III E 5
C. chauvoei			6	III E 6
C. perfringens A		E	7	III E 7
C. perfringens C				
C. perfringens B				
C. perfringens D				
C. perfringens E				
C. limosum	IV	F	8	IV F 8
C. histolyticum		G	9	IV G 9
C. septicum	V	H	10	V H 10
C. innocuum	VI	J	11	VI J 11
C. tetani	VII	K	12	VII K 12

NOTE: P = Proteolytic, NP = Non proteolytic

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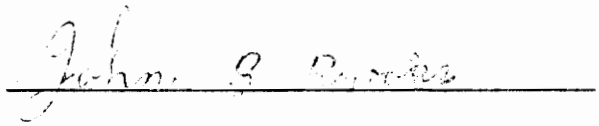
VITA

John B. Brooks was born on August 9, 1929, in Isabella, Tennessee, to Walter M. and Cosbie Brooks. He graduated from Murphy High School, Murphy, North Carolina, in 1949, and was employed by the Tennessee Copper Co.

In February, 1951 he was married to Mary Jane Hughes, Murphy, North Carolina. He was in the Army Signal Corps from 1954-1956 and served 18 months in Germany.

In August, 1962, he was graduated from the University of Western Carolina with a B. S. degree with majors in chemistry and biology. He has been employed by the U. S. National Communicable Disease Center in Atlanta, Georgia, as a biochemist since 1962, and has been engaged in graduate studies in biochemistry and microbiology at Virginia Polytechnic Institute since 1965.

He is a member of the Scientific Research Society of America and the American Society of Microbiology.



GAS CHROMATOGRAPHIC ANALYSIS OF AMINES AND OTHER COMPOUNDS PRODUCED BY SEVERAL SPECIES OF *CLOSTRIDIUM*

John B. Brooks

Abstract

Procedures are described for the routine analysis of amines and other compounds produced in bacterial cultures. Whole cultures were grown in six mls of chopped meat-glucose medium, acidified, and extracted with ethyl ether or chloroform to obtain organic acids, alcohols, ketones, aldehydes, and other neutral compounds. The residual broth was then made basic and extracted with chloroform to obtain the amines. For chromatographic analyses, alcohols and amines were converted to trifluoroacetyl derivatives. Organic acids were converted to their methyl esters. Analyses were made by a temperature-programmed gas chromatograph equipped with flame ionization detectors.

Sixty-two strains of clostridia, representing 13 species, were analyzed for soluble products in whole cultures. Many amines were found and total amine concentrations exceed 0.5% (weight) in cultures of several species examined. Neutral products were found to be major products of several species. Alcohols both short and long chain were detected and were valuable in differentiation of some groups of clostridia.

The 13 species were grouped as follows: on the basis of fatty acids and neutral products, 7 groups; on the basis of amine products, 10 groups; on the basis of alcohol and neutral products, 12 groups; and on the basis of amine, alcohol, and neutral products, 13 groups.

APPENDIX I

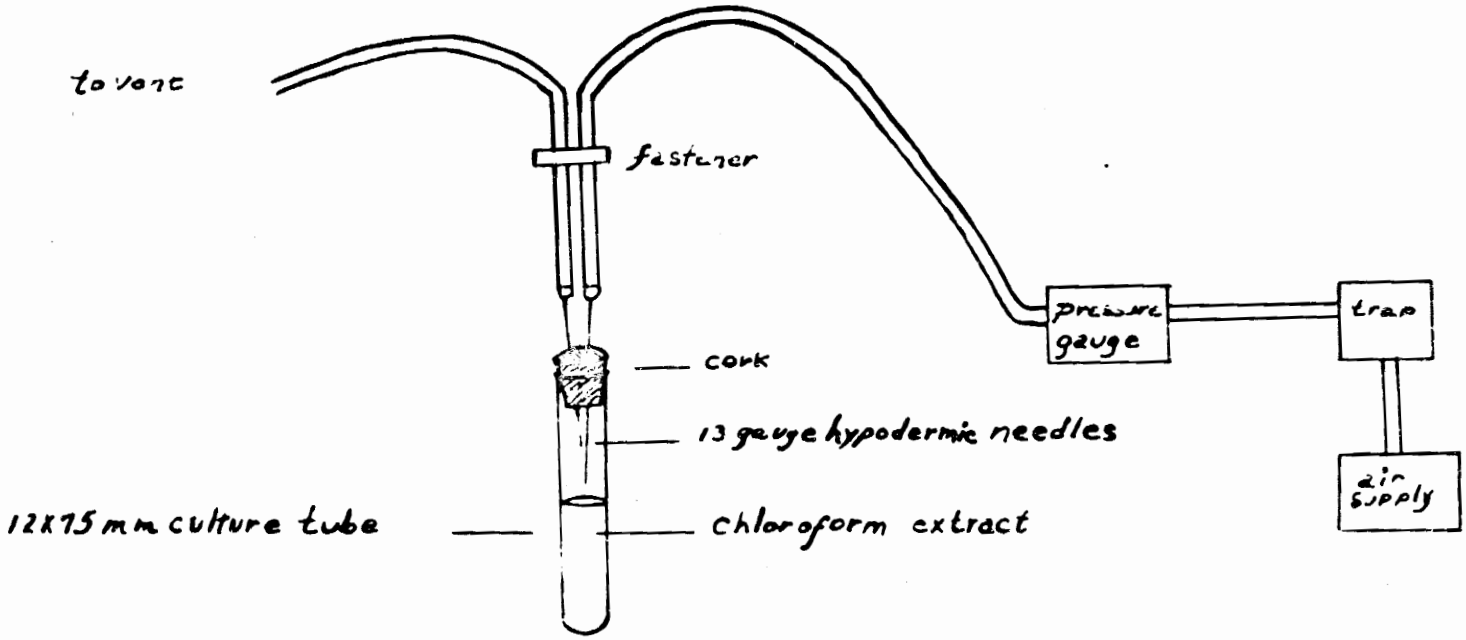


figure A I 1.

Concentrating Apparatus

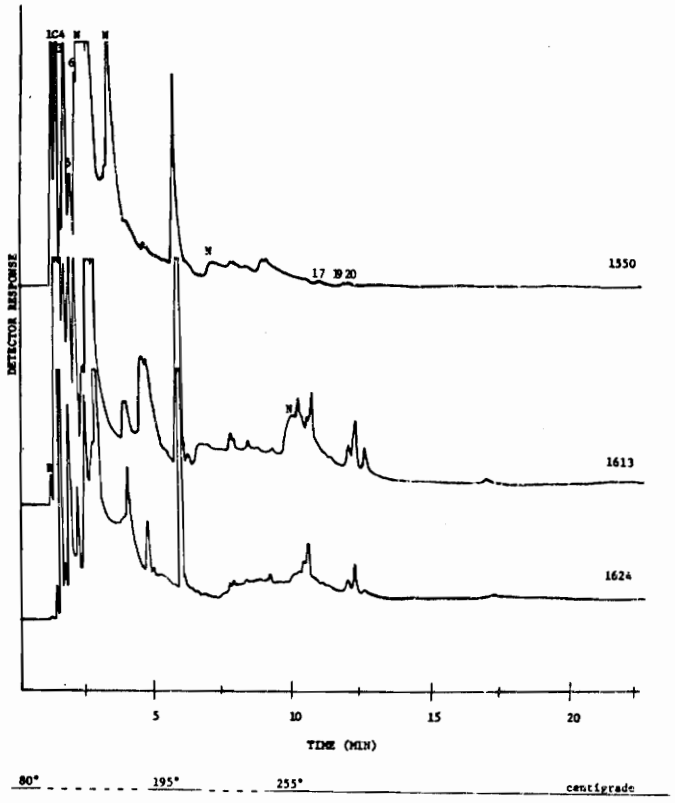


FIG. AI 2. *C. botulinum* type A, fatty acid methyl esters and neutral products extracted with ether from acidified cultures at pH 2.

C = chloroform
N = neutral compounds
R = reagent
1 = acetic acid
3 = isobutyric acid
4 = burtyric acid
5 = isovaleric acid
6 = valeric acid
17 = palmitic acid
19 = oleic acid
20 = stearic acid
unlabeled peaks = unidentified fatty acid.

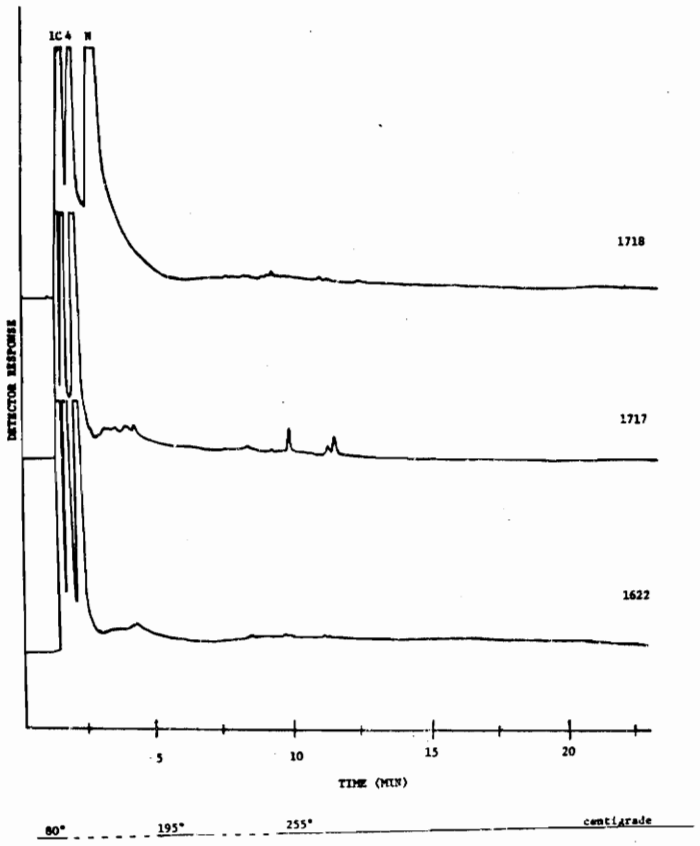


FIG. AI 3. *C. butyricum*, fatty acid methyl esters and neutral products extracted with ether from acidified cultures at pH 2.

C = chloroform
N = neutral compounds
1 = acetic acid
4 = butyric acid

unlabeled peaks = unidentified fatty acids.

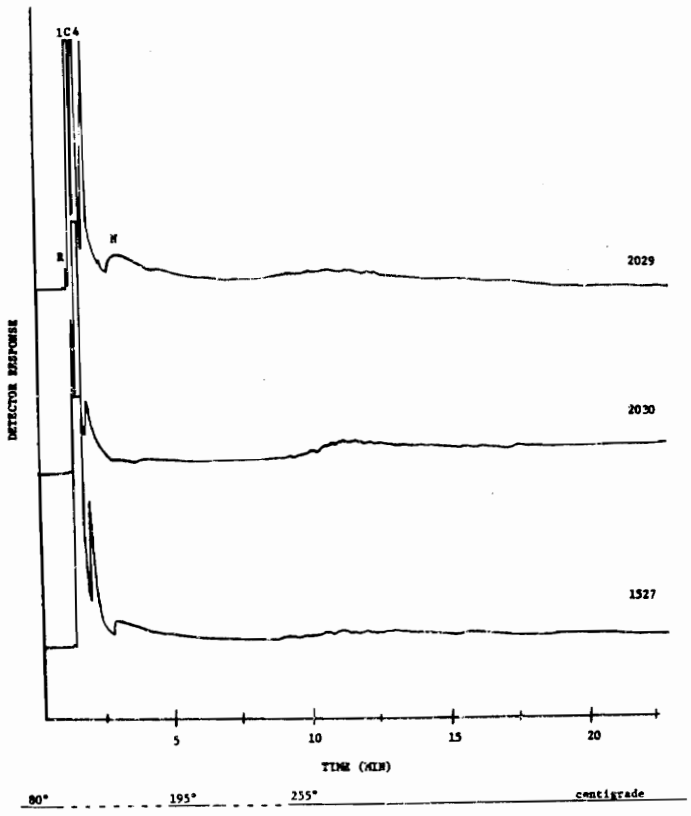


FIG. AI 4. *C. chauvoei*, fatty acid methyl esters and neutral products extracted with ether from acidified cultures at pH 2.

C = chloroform
N = neutral compounds

1 = acetic acid
4 = butyric acid

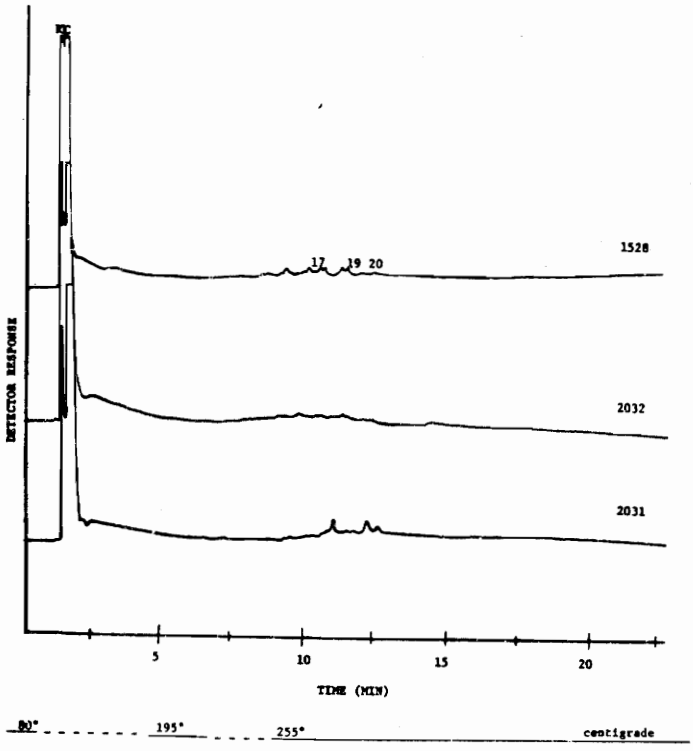


FIG. AI 5. *C. histolyticum*, fatty acid methyl esters and neutral products extracted with ether from acidified cultures at pH 2.

C = chloroform
R = reagent
1 = acetic acid

17 = palmitic acid
19 = oleic acid
20 = stearic acid

unlabeled peaks = unidentified fatty acids.

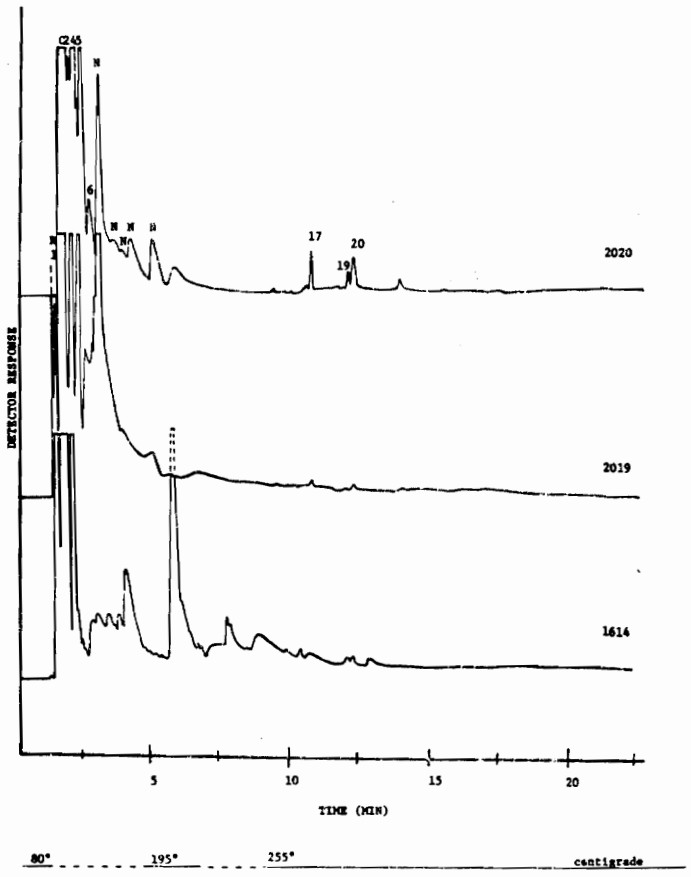


FIG. AI 6. *C. innocuum*, fatty acid methyl esters and neutral products extracted with ether from acidified cultures at pH 2.

C = chloroform
N = neutral compound
R = reagent
1 = acetic acid
2 = propionic acid
4 = butyric acid
5 = isovaleric acid
6 = valeric
17 = palmitic acid
19 = oleic acid
20 = stearic acid
unlabeled peaks = unidentified fatty acids.

Strain no. 1614 consistently produced larger amounts of the unidentified fatty acid with a retention time of 6.5. The amine products and results of other tests for this organism are typical of *C. innocuum*.

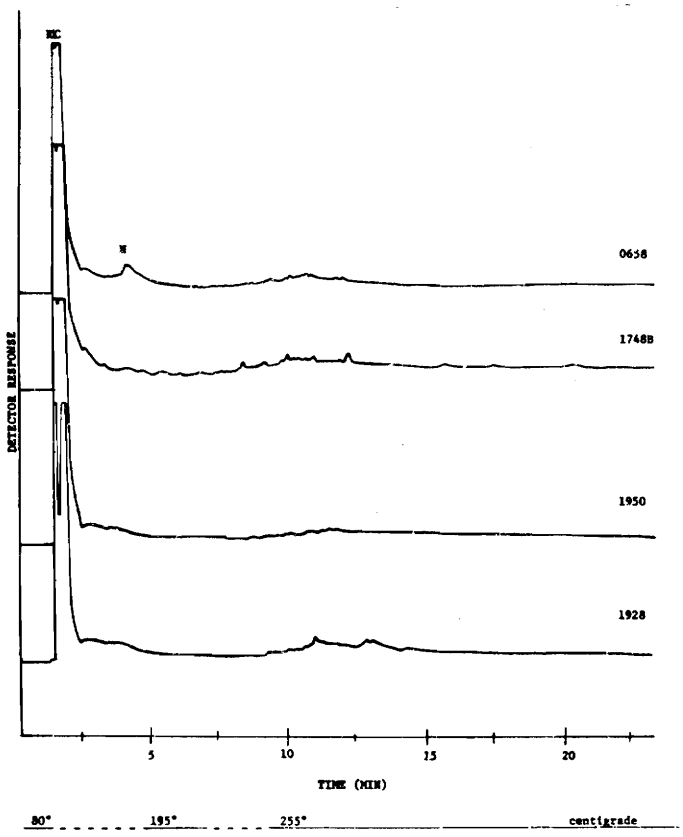


FIG. AI 7. *C. limosum*, fatty acid methyl esters and neutral products extracted with ether from acidified cultures at pH 2.

C = chloroform
R = reagent

1 = acetic acid
unlabeled peaks = unidentified fatty acids.

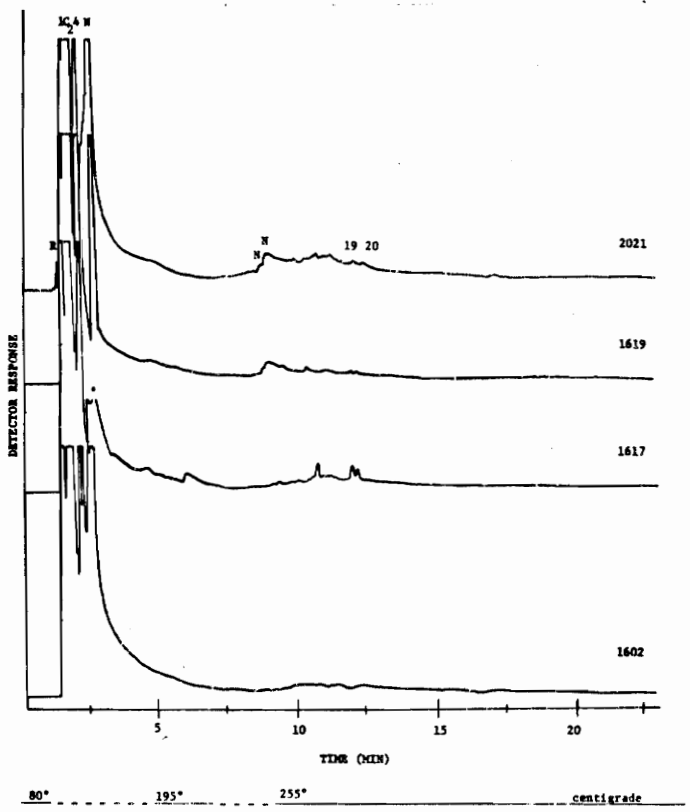


FIG. AI 8. *C. novyi* type A nos. 2021, 1619, and 1617, and type B no. 1602, fatty acid methyl esters and neutral products extracted with ether from acidified cultures at pH 2.

C = chloroform
N = neutral compound
R = reagent
1 = acetic acid
2 = propionic acid
4 = butyric acid
19 = oleic acid
20 = stearic acid
unlabeled peaks = unidentified fatty acids.

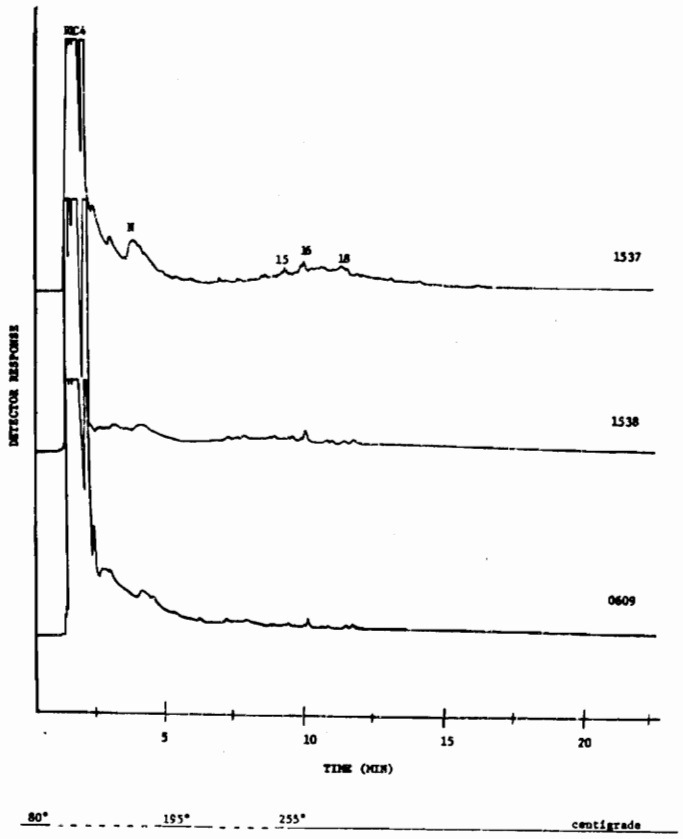


FIG. AI 9. *C. perfringens* type A, fatty acid methyl esters and neutral products extracted with ether from acidified cultures at pH 2.

C = chloroform
N = neutral compound
R = reagent
1 = acetic acid
4 = butyric acid
15 = myristic acid
16 = pentadecanoic acid
18 = heptadecanoic acid
unlabeled peaks = unidentified fatty acids.

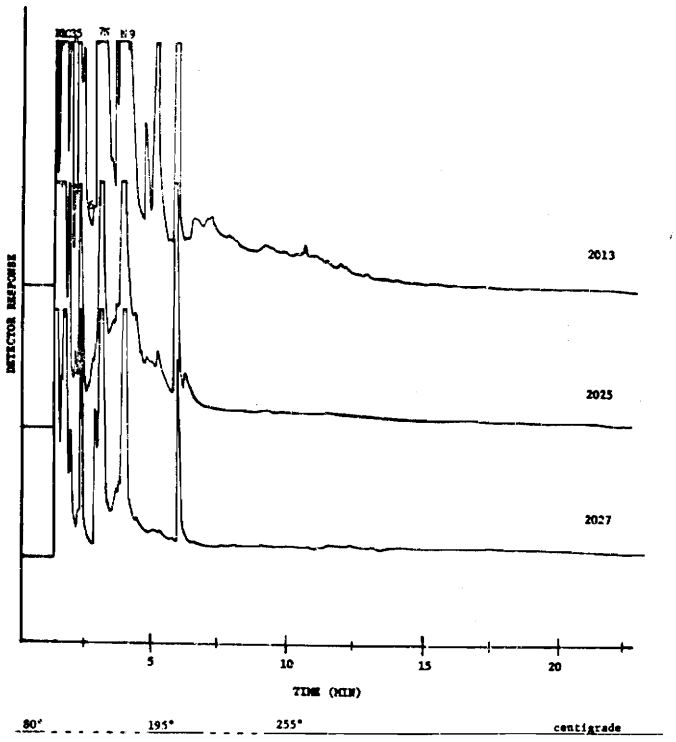


FIG. AI 10. *C. sordellii*, fatty acid methyl esters and neutral products extracted with ether from acidified cultures at pH 2.

C = chloroform
N = neutral compound
R = reagent
1 = acetic acid
3 = isobutyric acid
5 = isovaleric acid
7 = isocaproic acid
9 = heptanoic acid
unlabeled peaks = unidentified fatty acids.

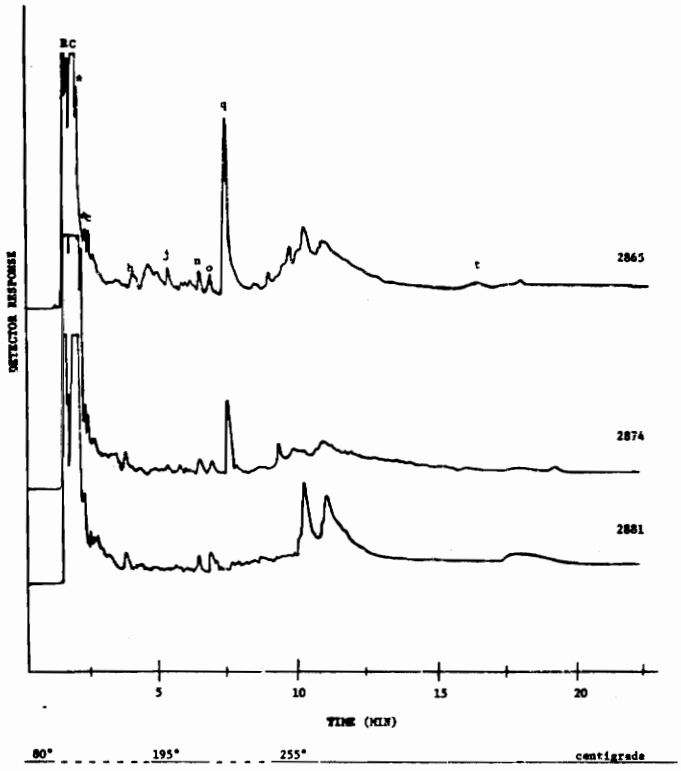


FIG. AI 11. *C. septicum*, amine TFA derivatives extracted with chloroform from cultures at pH 10.

C = chloroform
R = reagent
* = amine or alcohol
c = ethylamine
h = pyrrolidine
j = di-n-butylamine
n = 1,3-diaminopropane
o = putrescine
q = cadaverine
t = spermine
unlabeled peaks = unidentified amines.

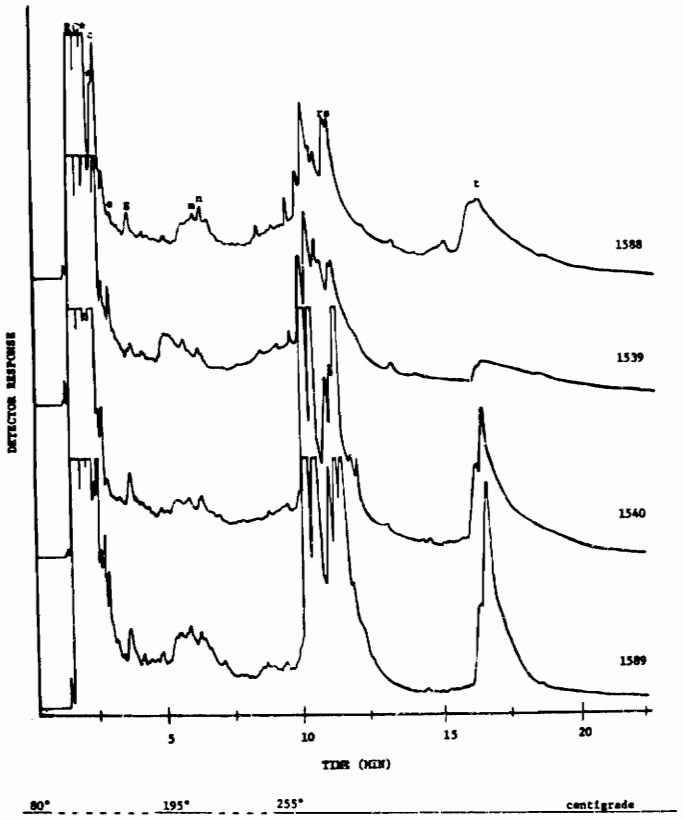


FIG. AI 12. *C. perfringens* type B no 1588, C no. 1539, D no 1540, E no. 1589, amine TFA derivatives extracted with chloroform from cultures at pH 10.

C = chloroform
R = reagent
* = amine or alcohol
c = ethylamine
e = isobutylamine
g = isoamylamine
m = 1,3-diaminopropane
n = β-phenylethylamine
r = tryptamine
s = spermidine
t = spermine
unlabeled peaks = unidentified amines.

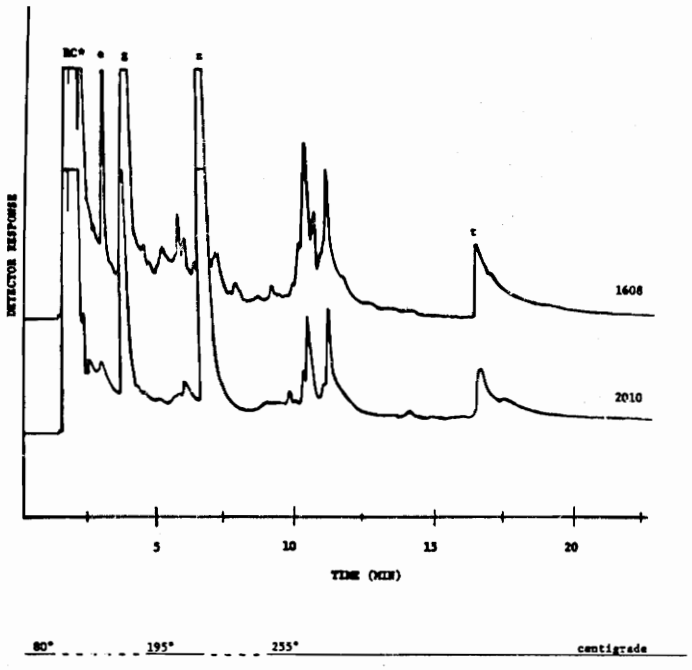


FIG. AI 13. *C. tetani*, amine TFA derivatives extracted with chloroform from cultures at pH 10.

C = chloroform
R = reagent
* = amine or alcohol
e = isobutylamine
g = isoamylamine
n = β-phenylethylamine
t = spermine
unlabeled peaks = unidentified amines.

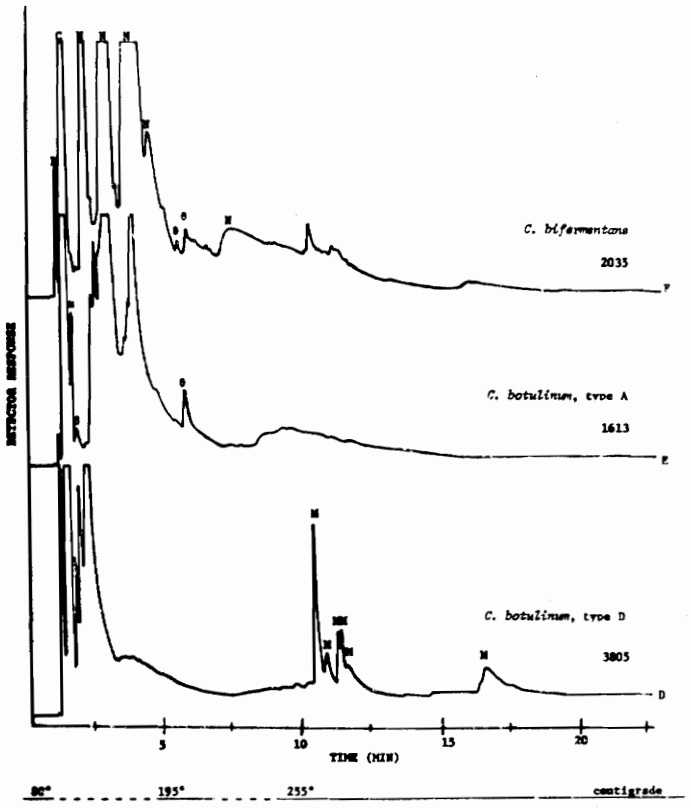


FIG. AI 14. Alcohol-TFA esters and neutral compounds extracted with chloroform from acidified cultures.

C = chloroform
M = compound from medium
N = neutral compound

α = butyl alcohol
β = isoamyl alcohol
θ = unidentified alcohol

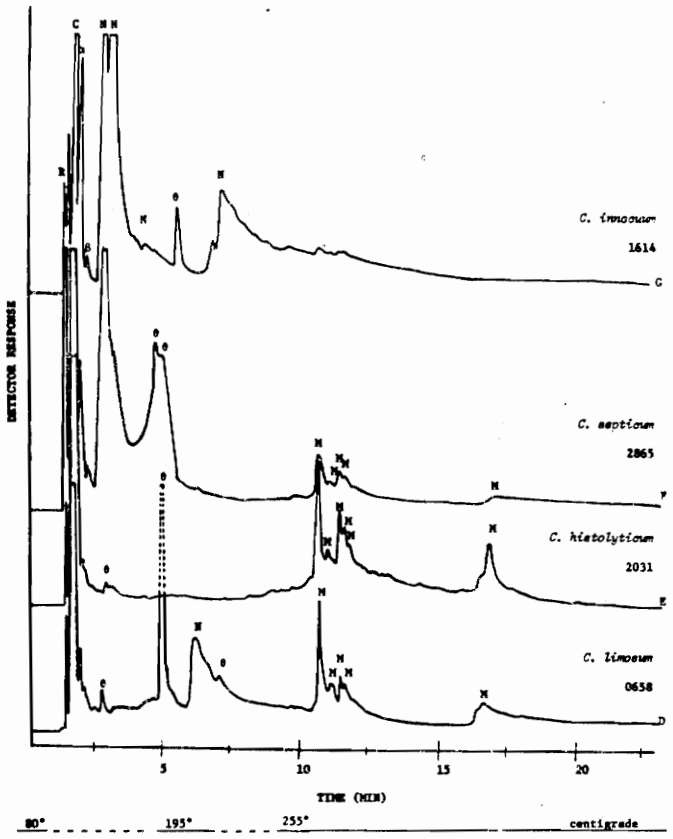


FIG. AI 15. Alcohol-TFA esters and neutral compounds extracted with chloroform from acidified cultures.

C = chloroform
M = compound from medium
N = neutral compound

α = butyl alcohol
β = isoamyl alcohol
θ = unidentified alcohol

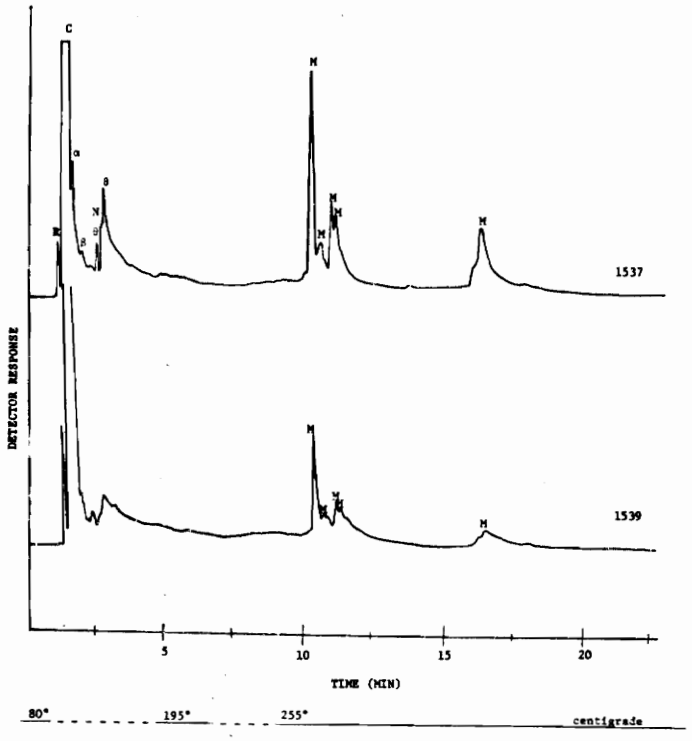


FIG. AI 16. Alcohol-TFA esters and neutral compounds extracted with chloroform from acidified cultures of *C. perfringens* type A no. 1537, and C no. 1539.

C = chloroform
M = compound from medium
N = neutral compound
α = butyl alcohol
β = isoamyl alcohol
θ = unidentified alcohol

Note the small amount of neutral compound with a retention time of 2.8 as compared with the large amount produced by strains of types B, D, and E fig. AI 17.

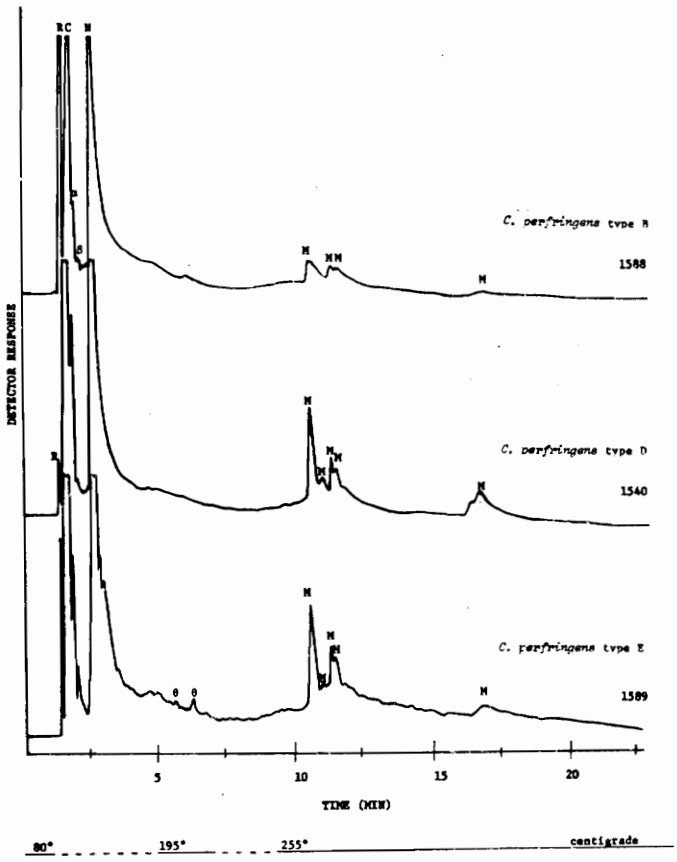


FIG. AI 17. Alcohol-TFA esters and neutral compounds extracted with chloroform from acidified cultures.

C = chloroform
M = compound from medium
N = neutral compound
α = butyl alcohol
β = isoamyl alcohol
θ = unidentified alcohol

Compare with Figure AI 16. The neutral compound with a retention time of 2.8 is produced in large amounts as compared to strains of type A and C, Fig. AI 16.

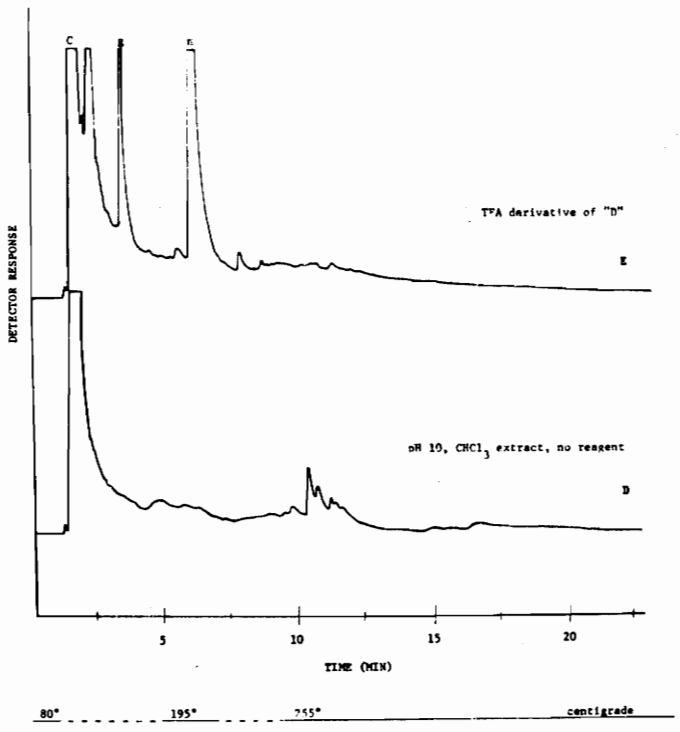


FIG. AI 18. Free amines and TFA derivatives of amines from a culture of *C. novyi* type A no. 1619.

C = chloroform
g = isoamylamine
n = β-phenylethylamine

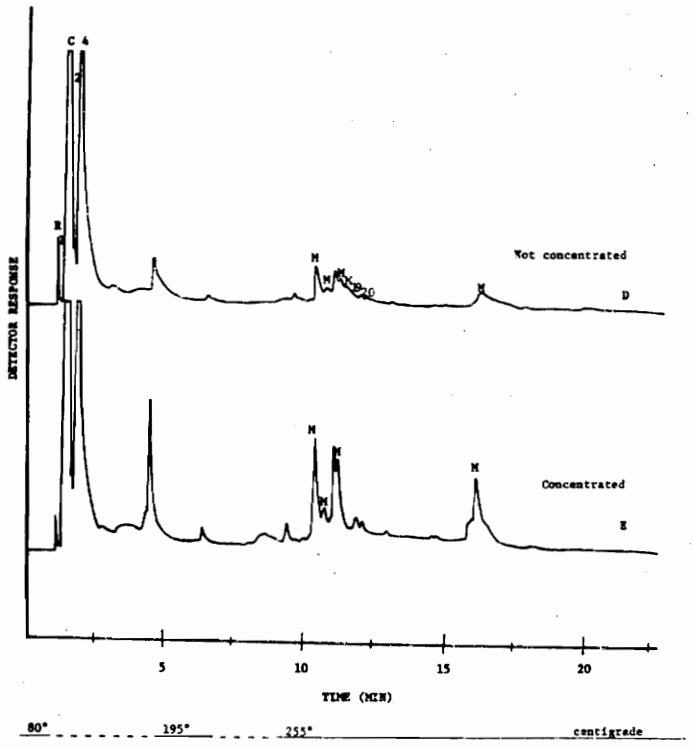


FIG. AI 19. Concentration effect. A chloroform extract of an acidified extract of a culture of *C. butyricum* treated with TFA and BF_3-MeOH.

C = chloroform
M = compound from medium
1 = acetic acid
2 = propionic acid
4 = butyric acid
19 = oleic acid
20 = stearic acid
unlabeled peaks = unidentified fatty acids.

Chromatogram E was concentrated 10 seconds by air after formation of methyl esters. Note the improved resolution and detection of compounds.

APPENDIX II

VARIATION AMONG STRAINS OF *CLOSTRIDIUM BOTULINUM* AND RELATED CLOSTRIDIA

Lillian V. Holdeman and John B. Brooks*

Anaerobe Laboratory, Department of Veterinary Science
Research Division, Virginia Polytechnic Institute
Blacksburg, Virginia 24061, U.S.A.

Summary. Three distinct physiological groups of *Clostridium botulinum* can be differentiated on the basis of reactions on 58 substrates, acid and alcohol products, and limited studies of protein metabolism. Some other species of clostridia also have similar characteristics.

Group I: *C. botulinum* type A, proteolytic strains of *C. botulinum* types B and F, and *C. sporogenes*.

Group II: *C. botulinum* types C and D and *C. novyi* type A.

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Group III: Non-proteolytic strains of *C. botulinum* types B and F, type E, and culturally similar organisms that are nontoxic.

Characteristics of the groups and variation within the groups are presented and indicate that the three cultural groups cannot be reliably subdivided on the basis of cultural reactions, morphological characteristics, or metabolic products other than toxins.

The most outstanding characteristic of a strain of *Clostridium botulinum* is the toxin that it produces. On the basis of the antigenic specificity of the toxins, the species has been divided into 6 types, A-F, with the type C having two antigenic sub-groups, C-alpha and C-beta. In most cases the cultural characteristics of the strains producing the toxin are only of secondary interest, and a strain producing a toxin antigenically and pharmacologically similar to another *C. botulinum* toxin is a strain of *C. botulinum*, no matter what other characteristics it exhibits, assuming of course that it is an anaerobic, sporeforming bacillus. Nevertheless, strains within types or groups share certain cultural and biochemical characteristics. The purpose of this paper is to report the cultural and biochemical variations and similarities that we have observed among different strains of the types and among strains of organisms

that are culturally similar to toxigenic strains.

CULTURES

Unless it is otherwise stated, the strains originally were identified in our laboratory. All labeled strains included in this study were also tested for toxicity and typed in our laboratory, using antitoxin obtained from the National Communicable Disease Center.

Labeled strains in Group I. *C. botulinum* type A, Prévot 146. *C. botulinum* type B (proteolytic): ATCC 7949 (NCA 213B), Prévot 24 NCASE, and Smith A-116. *C. botulinum* type F (proteolytic): Giménez 160 and Langeland strain. *C. sporogenes* (rhizoid colony) ATCC 7905 and Smith M-154. *C. sporogenes* (non-rhizoid colony), ATCC 7955 (NCA 3679) and ATCC 3584. *C. sporogenes* strain designated "ocean sediment," Smith 09 (Smith, 1968).

Strains in Group II. *C. botulinum* type C (toxic), Smith strains "C-alpha" and "C-beta." One of the two nontoxic strains had previously been toxic when we tested it. *C. botulinum* type D: the one toxic strain was obtained from Smith; of the two nontoxic strains, one had been toxic when we tested it previously and one was ATCC 9633 (South African strain). *C. novyi* type A: ATCC 3540, ATCC 17861, Prévot 669, and Smith "Prac B." Related clostridia: Three of these four nontoxic strains were received labeled as *C. botulinum* type C; one had been sent to us for identification.

Strains in Group III. *C. botulinum* type B (non-proteolytic):

Eklund 17B and Prévot 59. *C. botulinum* type F (non-proteolytic): Eklund 83F, Eklund 202F, and one identified by Smith. *C. botulinum* type E: ATCC 9564, "Tenno" strain from Hobbs, "Beluga" strain from Hobbs; Hobbs FT-18, Johannsen 1084, and Kautter 066B TOX. Related clostridia: strains labeled "typical" had cultural reactions similar to *C. botulinum* type E, but were nontoxic and include Hobbs strains 18E, 14G, 14D, and Kautter strains 066B NT (nontoxic), S-5, 28-2, 810; strains PM-15 and S-9 also were obtained from Kautter, but were more different, culturally, from the toxic strains in this group than are the strains labeled "typical."

METHODS

General methods. Cultures were grown by the anaerobic culture methods described by Moore (1966) and characterized using the tests described by Holdeman et al. (1967). Cultures were incubated in an atmosphere of 97-100% oxygen-free CO_2 for 5 days or 3 weeks. Cultural results were essentially the same at 5 days as at 3 weeks.

The pH in glucose broth cultures was determined with a pH meter after cultures had been incubated for 1, 3, and 5 or 7 days; for those cultures incubated for 3 weeks, also at 14 and 21 days. In comparing our results with those obtained by other investigators on the same strain, we have noted that we often detected complete liquefaction of gelatin when others do not and that we failed to detect fermentation of adonitol by strains which others found do

ferment adonitol.

The major products detected from growth in glucose medium, presented in Table 4, were determined by the methods described by Moore et al., 1966.

Detection of amines, long-chain fatty acids, and neutral compounds. Cultures were inoculated into 6 ml chopped meat-glucose medium and incubated for 7 days.

Culture extracts and derivatives were prepared as described in Fig. 1 and were chromatographed on a Beckman GC-4* gas chromatograph using dual flame detectors, dual stainless steel columns, with helium as the carrier gas at a flow rate of 48 cc per minute. Columns, twenty feet long and one-eighth inch in diameter, were packed with Chromosorb W, 80/100 mesh (AW-DMCS)HP**. Samples were run for 25 minutes; temperature was programmed from 80 to 255 C.

More details of the method and results with additional strains and species will be reported by one of us (JBB) in a doctoral dissertation.

RESULTS AND DISCUSSION

The strains of *C. botulinum* types A-F form three major groups

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*Use of trade names does not constitute endorsement.

**Chromosorb W, 80/100 mesh, acid washed, dimethyl-dichloro-silane; Applied Science Laboratories, P. O. Box 440, State College, Pennsylvania, U.S.A.

according to their cultural characteristics and metabolic products. Clostridia that have characteristics similar to those of *C. botulinum* also have been included in this grouping.

Group I. *C. botulinum* type A, proteolytic strains of *C. botulinum* types B and F and *C. sporogenes*. Characteristics of these strains are presented in Tables 1 and 4. No cultural or morphological characteristic that can be used to reliably separate the toxic from the nontoxic strains is known. One of the type B strains had rhizoid, "sporogenes-like" colonies when originally tested and again one year later when this culture was re-examined after lyophilization. One of the type A strains was non-hemolytic on agar containing horse blood and also produced no reaction on egg yolk agar. Although these cultures no doubt represent rare exceptions to the general rule that all strains of *C. botulinum* and *C. sporogenes* produce lipase on egg yolk agar and that rhizoid colonies are *C. sporogenes* rather than *C. botulinum*, these types of variation do occur occasionally.

At first we did not detect, with some of the organisms in Group I, the marked fermentation of glucose and maltose that usually is so characteristic of organisms in this group. However, when the concentration of peptone in the basal medium was lowered from 2 g/100 ml to 0.5 g/100 ml, weak acid production could be detected from both glucose and maltose.

In the medium containing 2% peptone, cultures produced as much total acid, quantitatively determined, in media without glucose as in media with glucose, indicating that little fermentation of glucose occurred. The total quantity of acids produced by the cultures in basal peptone-yeast medium was large (8 to 10 milliequivalents/100 ml).

In glucose cultures of the "ocean sediment" strain of *C. sporogenes*, the glucose was actively fermented and major amounts of lactic acid accumulated. This accumulation of lactic acid probably was responsible for the lower pH obtained with this strain in media with a fermentable carbohydrate (Table 1).

Group II. *C. botulinum* types C and D and *C. novyi* type A. Characteristics of strains in this group are given in Tables 2 and 4. Adequate study of this group of *C. botulinum* was not possible because there were so few toxic strains available for examination.

We became aware of the similarity between strains of *C. botulinum* type C and *C. novyi* type A when we were examining some nontoxic strains labeled *C. botulinum* type C (designated "related clostridia" in Table 2). When these cultures were grown in egg yolk agar, we noted an opaque zone (presumably due to lecithinase activity) in the agar around the colonies. This opaque zone extended far beyond the pearly zone that was over and immediately around the colonies and, except for intensity, was similar to the reaction on egg yolk agar exhibited by *C. novyi* type A strains. The opaque

zone produced by these "related strains" was much lighter than that produced by *C. novyi* type A strains, but it was nonetheless present. We have not determined whether these are nontoxic variants of *C. botulinum* type C (as labeled) or nontoxic strains of *C. novyi* type A that do not produce much lecithinase. There was no characteristic colonial or cellular morphology by which species of cultural group II might be differentiated.

Fermentation acids produced by the various strains in this group (Table 4) were similar. Acetic, propionic, and butyric acids were the major products from fermentation of glucose and lactate; acetic and butyric acids were produced from pyruvate. These acids were produced in different ratios from the different substrates.

Substrate	total acids*	ratio		
		acetic	propionic	butyric
Peptone-yeast (PY)	1.0	1	2	2
PY - glucose	4.7	1	4	5
PY - lactate	8.5	1	5	2.5
PY - pyruvate	4.8	1	0.5	1.8

*Total meq/100 ml acetic, propionic, and butyric acids or salts thereof.

Thus, a greater amount of acid and more propionic acid were produced from lactate than from glucose or pyruvate. We have found very few other species of anaerobic bacteria, either spore-

formers or non-sporeformers that, in our test system, produce appreciably larger amounts of propionate from lactate than from glucose.

Group III. *C. botulinum* type E, non-proteolytic strains of types B and F, and related organisms. Characteristics of strains in this group are given in Tables 3 and 4. A few of the toxic type E strains decreased the pH in basal peptone-yeast media so much that determination of acid production from carbohydrate was difficult. Results designated positive for acid production in Table 3 represent pH values at least 0.5 unit lower than that found in the basal medium.

In the group of nontoxic organisms that might be related to these non-proteolytic strains of *C. botulinum*, we included strains (designated "typical" in Table 3) that had cultural reactions similar to the toxic strains in group III and two strains (PM-15 and S-9) that were different culturally but had been found to be serologically related to toxic type E strains (Solomon et al., 1968).

In an effort to determine whether in the past we had examined other strains of clostridia that should be included in this group, we coded many of the characteristics that were uniformly positive and some that were uniformly negative for toxic type E strains. Then by computer we compared about 400 clostridia to select the strains that were identical in 21 of the 22 characteristics indicated.

To allow for a coding error or strain variation, absolute agreement was not requested. Positive characteristics coded were: gram-positive, motile, anaerobic, rods, forming gas from glucose, producing moderate or greater amounts of butyric and acetic acids, and fermenting glucose, fructose, maltose, mannose, sucrose. Negative characteristics coded were: no fermentation of lactose, no production of indol, no major amount of lactic acid from glucose, and no moderate or greater amounts of caprylic, heptanoic, caproic, valeric, isovaleric, isobutyric, or propionic acids from glucose. Production of toxin, hemolysin, lipase, or spores was not included in the comparison.

Because we required agreement of only 21 of the 22 characteristics listed, strains of *C. chauvoei* and of *C. butyricum* (both of which ferment lactose) were selected, as were two strains of non-spore-forming anaerobes and PM-15. With these exceptions, no other organism similar to the toxic organisms in group III was selected. Labeled *Clostridium* strains bearing more than 80 different species names and many unidentified clostridia were represented in the collection subjected to analysis. The S-9 strain was not selected because it ferments neither maltose, mannose, nor sucrose. Both S-9 and PM-15 appear to be more closely related to the toxic strains in group III than to any of the other clostridia that we can now recognize.

Amines long-chain fatty acids, and neutral compounds produced by strains of *C. botulinum* and related organisms. The following portion of this report concerning these soluble compounds present in culture fluids should be considered preliminary because we have studied only a few strains. Nevertheless, the general types of compounds that have been detected are of interest and are pertinent.

By using combinations of extraction methods and treatment with trifluoroacetic anhydride (TFA) or boron trifluoride methanol (BF3), we have been able to identify classes of compounds as well as a variety of individual compounds (Fig. 1). By comparing the retention times of peaks from treated culture extracts with those of known compounds similarly treated, we have tentatively identified 18 amines and 23 fatty acids. Identification was further substantiated by using several conditions of operation; e.g., different temperature programs, carrier gas flow rates, and column packings.

Unless present in very high concentrations, natural (neither methylated nor acylated) acids and most alcohols and amines do not elute from the Chromosorb W packing material; even then, resolution is poor and peaks are small and not well defined. To reduce their affinity for the packing material and permit elution of these compounds from the column, charged groups can be acylated (amino and hydroxyl groups) with TFA or methylated (carboxyl groups) with BF3. Natural aldehydes, ketones, and tertiary alcohols and amines will elute from the Chromosorb W.

An example of the differentiation of classes of compounds present in one culture fluid is presented in Fig. 2.

Bottom chromatograph, "medium": Compounds represented by peaks "h", "i", "j", "k", "l", "o", and "p" are present in $CHCl_3$ extracts of the acidified uninoculated medium and still are detected after acylation and methylation (chromatographs B and C).

Chromatograph A: Compounds extracted at pH 2 from the broth culture are represented by peaks "c" and "d". These probably are aldehydes or ketones. Such compounds may be partially or completely lost during acylation or methylation procedures, possibly from boiling, evaporation, or dilution (see peaks "c" and "d", chromatograph B and peak "d", chromatograph A).

Chromatograph B: After the $CHCl_3$ extract of acidified culture was treated with TFA, the TFA-alcohols (peaks "b" and "e") are chromatographed. The TFA derivatives represented by beaks "b" and "e" were not stable when acidified (1% HCl, final concentration) and heated; TFA-amine compounds are stable to such treatment, TFA-alcohol compounds are not and are lost during methylation procedures (see chromatograph C).

Chromatograph C: Fatty acids, extracted with either ether or chloroform, are not detected until they have been

methylated (peaks "a", "f", "g", "m", and "n").

TFA derivatives of amines subsequently extracted from this culture broth after the pH had been raised to 10 and the extract had been treated with TFA are shown in Fig. 3, bottom chromatograph. [Chromatographs of the uninoculated medium controls for the TFA-treated $CHCl_3$ extracts at pH 10 are shown in Fig. 8 (chromatographs B-1 and B-2). In all of the experiments reported here, one µl was the largest test sample injected.]

The chromatographs of TFA-amines from representative strains of cultural groups I, II, and III are shown in Fig. 3. Chromatographic profiles of amines produced by different strains in cultural groups I, II, and III are shown in Figs. 4, 5, and 6 respectively. The ordinates represent detector response and the abscissas represent 0 to 25 minutes.

The profiles for strains of type A, proteolytic type B, proteolytic type F, and *C. sporogenes* (cultural group I) are very similar (Fig. 4). We originally thought that the larger relative amount of n-dibutylamine-TFA consistently seen in chromatographs of the Langeland type F strain might be characteristic for all proteolytic strains of type F, but this is not true. The Giménez strain of type F, like other strains in cultural group I, has the lesser relative amount of n-dibutylamine.

In the chromatograph of the *C. sporogenes* cultural extract, the notch on peak "c" and the two notches on the preceding peak

appear to constitute a difference between *C. sporogenes* and the toxic strains. In other preparations from *C. sporogenes* cultures, these peaks are much smaller. Compounds eluted in 12 to 15 minutes have retention times similar to those of substances obtained from the uninoculated medium (Fig. 8, chromatograph B-2). In this area of the chromatographs, differences in peak heights may not be very reliable.

In interpreting these chromatographs, presence or absence of peaks and relative ratios of peak areas are more important than individual peak height. When the amount of sample is increased only a few hundreths of a microliter, some peaks may be completely obscured by the increased size of neighboring peaks; also, isolated smaller peaks may be greatly increased in height.

Strains of *C. botulinum* types C and D (toxic strains) and *C. novyi* type A (cultural group II) characteristically produce large quantites of *iso*-amylamine and phenethylamine (Figs. 3, 5).

The chromatographs of the amines produced by the non-proteolytic strains of types B and F and type E (cultural group III) are remarkably similar (Fig. 6). Note in particular the notched peak (Fig. 3, peak f; Fig. 6, preceding peak d), which has appeared in chromatographs of all of the strains in this group which we have examined, including some of the related clostridia (strains 810, S-9, and 28-2).

Except for the 3 nontoxic organisms mentioned, only toxic strains of *C. botulinum* have been examined for amines. We have studied at least 3 strains of each subgroup (or all of the strains available) and three strains each of *C. sporogenes* and *C. novyi* type A.

Amines produced by strains within each of the 3 cultural groups (as previously defined) were very similar and we have been unable to use this analysis to differentiate with certainty among strains within each group. Analysis of amines produced by these organisms extends and further confirms the previous observations that organisms within the groups are metabolically similar.

Chromatographic patterns of methylated ether extracts of whole cultures are shown in Fig. 7. [Chromatographs of the uninoculated medium controls for these methylated ether extracts at pH 2 are shown in Fig. 8, chromatographs A-1 and A-2.] Individual chromatographs in Fig. 7 represent the general types of fatty acids and neutral compounds produced by all members of each cultural group represented. Differences among the short-chained fatty acids, not so easily seen here, are similar to those previously described by Moore et al. (1966), who used direct isothermal chromatography of ether extracts of whole cultures on resoflex columns.

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TABLE 1. Reactions of some strains of *C. sporogenes* and proteolytic strains of *C. botulinum* types A, B, F (Cultural Group I)

Characteristic	*C. botulinum*			*C. sporogenes*		
	type A	type B	type F	rhizoid	not rhizoid	ocean sed.
	7*	5	2	17	2	1
Toxicity	+**	+	+	-	-	-
Digestion of:						
gelatin	+	+	+	+	+	+
milk	6/7	4/5	1/2	14/17	+	+
meat	+	+	+	+	1/2	-
Lowest pH (gluc.)	5.4-6.0	5.8-6.1	5.93	5.5-6.2	5.9-6.0	4.95
Acid in media with						
glucose	+	3/5	+	14/17	+	+
fructose	4/7	1/5	-	5/15	-	-
D(+)-maltose	1/7	1/5	-	-	-	+
sucrose	-	-	-	1/17	-	-
cellobiose	-	-	-	1/17	-	-
glycogen	1/7	-	-	1/17	-	-
D(+)-mannose	1/7	-	-	-	-	+
pectin	-	-	-	1/17	-	-
D-sorbitol	2/7	1/5	-	-	-	-
H_2S production	5/7	4/5	+	15/17	+	-
Hydrolysis of:						
esculin	+	+	+	+	+	+
starch	-	-	-	1/17	-	+
6.5% NaCl, gr.	3/7	4/5	1/2	12/17	1/2	+
Hemolysis[a]	6/7†	+	+	+ψ	+	+
Lipase[b]	6/7†	+	+	+ψ	+	+
Rhizoid colonies	-	1/5	-	+	-	+
Motile	5/7	+	+	15/17	+	+

All strains produce NH_3, are anaerobic, and produce gas in glucose agar deeps.

No strain produces acid from adonitol, amygdalin, L-arabinose, dextrin, dulcitol, DL-erythritol, esculin, D-galactose, glycerol, inositol, inulin, lactose, D-mannitol, melezitose,

TABLE 1. Continued

melibiose, raffinose, rhamnose, D(-)-ribose, salicin, L-sorbose, starch, trehalose, D-xylose.

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* number of strains

** + = positive for all strains, - = negative for all strains
#/# = strains positive/strains tested.

a = horse red blood cells

b = egg yolk agar

† = The same culture that does not lyse horse red blood cells also does not produce lipase.

Ψ = Both lipase positive and lipase negative variants consistently were observed in one culture. Subcultures from lipase positive colonies also contained variants that did not produce detectable lipase.

Note: Except where indicated, unusual reactions were not associated with any one strain.

TABLE 2. Reactions of some strains of *C. botulinum* types C and D, *C. novyi* type A, and related clostridia (Cultural Group II)

Characteristic	*C. botulinum* type D		*C. botulinum* type C		Related clost.	*C. novyi* type A
	2*	1	2	2	4	4
Toxicity	-**	+	-	+	-	+
Digestion of						
gelatin	+	+	+	+	+	+
milk	1/2	+	1/2	-	1/4	-
meat	-	-	-	-	1/4	-
Lowest pH (gluc.)	5.2-5.4	5.2	5.1-5.2	5.2-5.7	4.9-5.3	5.5-5.6
Acid from						
glucose	+	+	+	+	+	+
D-fructose	-	-	+	1/2	3/4	-
D-galactose	-	-	+	+	2/4	-
glycerol	+	-	+	-	2/4	2/3
inositol	+	+	+	+	+	1/4
D(+)-maltose	+	-	+	1/2	2/4	+
D(+)-mannose	+	-	+	+	+	2/4
melibiose	-	+	1/2	-	2/4	1/4
pectin	-	+	+	+	3/3	3/4
D(-)-ribose	+	-	+	-	+	1/4
adonitol	-	-	-	-	3/4	-
L-arabinose	-	-	+	-	2/4	-
cellobiose	-	-	1/2	-	1/4	-
dextrin	-	-	-	-	2/4	-
DL-erythritol	-	-	-	-	1/4	-
esculin	-	-	-	-	1/4	-
glycogen	-	-	1/2	-	1/4	-
inulin	-	-	-	-	2/4	-
raffinose	-	-	-	-	3/4	1/4
rhamnose	-	-	-	-	1/4	1/4
salicin	-	-	-	-	2/4	1/4
D-sorbitol	-	-	-	-	2/4	-
L-sorbose	-	-	-	-	2/4	-
starch	-	-	-	-	3/4	-
sucrose	-	-	1/2	-	2/4	1/4
trehalose	-	-	-	-	2/4	-
Indol prod.	-	-	-	1/2	-	-
6.5% NaCl, gr.	-	-	-	-	-	-

TABLE 2. Continued

Characteristic	*C. botulinum* type D		*C. botulinum* type C		Related clost.	*C. novyi* type A
	2*	1	2	2	4	4
Hemolysis[a]	+	+	2/3	+	+	+
Lecithinase[b]	-	-	-	-	weak	+
Lipase[b]	+	+	+	+	+	+
Rhizoid colony	-	-	-	-	-	-

All strains are anaerobic and produce gas in glucose agar deeps.

No strain produces H_2S or hydrolyzes esculin or starch.

No strain produces acid from amygdalin, dulcitol, lactose, mannitol, melezitose, xylose.

* number of strains tested

** + = positive for all strains, - = negative for all strains
#/# = strains positive/strains tested

a = horse blood cells

b = egg yolk agar

Note: Except where indicated, unusual reactions were not associated with any one strain.

TABLE 3. Reactions of strains of *C. botulinum* type E, non-proteolytic strains of types B and F, and of related organisms (Cultural Group III)

Characteristic	*C. botulinum* types			Related clostridia		
	B 2*	F 3	E 6	typical 8	PM-15	S-9
Toxicity	+**	+	+	-	-	-
Hemolysis[a]	+	+	+	+	+	+
Lipase[b]	+	+	+	+	+	+
Lecithinase[b]	-	-	-	-	-	-
Rhizoid colony	-	-	-	-	-	-
Digestion of						
gelatin	+	+	5/6	7/8	+	+
milk & meat	-	-	-	-	-	-
Lowest pH (gluc.)	5.3-5.6	5.0-5.4	5.0-5.5	5.0-5.5	5.2	5.45
Acid from:						
glucose	+	+	+	+	+	+
amygdalin	-	2/3	-	3/8	v	-
L-arabinose	-	-	-	1/8	-	-
cellobiose	-	-	-	-	+	-
dextrin	1/2	1/3	-	-	+	-
esculin	1/2	2/3	-	-	-	-
D-fructose	+	+	+	+	+	+
D-galactose	1/2	-	-	-	-	-
glycogen	+	-	-	-	-	-
inositol	-	+	-	1/8	-	-
D(+)-maltose	1/2	+	+	+	+	-
D-mannitol	-	-	-	-	+	-
D(+)-mannose	+	+	+	+	+	-
melezitose	-	-	+	4/8	-	-
pectin	1/2	1/3	5/6	7/8	+	+
D(-)-ribose	1/2	+	+	6/8	v	-
salicin	-	-	-	-	+	-
D-sorbitol	+	2/2	5/6	7/8	+	+
starch	1/2	+	4/6	6/8	+	+
sucrose	+	+	+	+	v	-
trehalose	+	+	5/6	7/8	+	-
D-xylose	-	-	-	1/8	-	-

TABLE 3. Continued

Characteristic	*C. botulinum* types			Related clostridia		
	B	F	E	typical	PM-15	S-9
	2*	3	6	8		
Hydrol. of:						
esculin	-	-	1/6	-	+	-
starch	-	+	4/6	6/8	-	+
6.5% NaCl, gr.	-	-	-	-	-	-
"Boticin" prod. †				3/4	-	-
Serol. related to E †			2/2	4/4	+	±

All strains are anaerobic, gram-positive, motile bacilli that form oval, sub-terminal spores. All strains produce gas in glucose agar deeps.

No strain produces H_2S, indol; nitrates not reduced.

No strain produces acid from adonitol (see discussion), dulcitol, erythritol, glycerol, inulin, lactose, melibiose, raffinose, rhamnose, sorbose.

* number of strains tested

** + = all strains positive; - = all strains negative, #/# = # positive/# tested.

v = variable results on replicate tests.

a = horse red blood cells

b = egg yolk agar

† = as reported by Kautter et al. (1966), Lynt et al. (1967), and Solomon et al. (1968).

Note: Except where indicated, unusual reactions were not associated with any one strain.

Table 4. MAJOR PRODUCTS OF *C. BOTULINUM* AND RELATED CLOSTRIDIAL GROUPS IN GLUCOSE MEDIA

Group I*		Group II		Group III	
ACETIC	3.0-4.0	ACETIC	0.0-1.0	ACETIC	1.0-1.5
propionic	0.2-0.3	PROPIONIC	1.5-2.5		
ISOBUTYRIC BUTYRIC † ISOVALERIC valeric isocaproic	3.0-4.0	BUTYRIC valeric	1.5-3.0	BUTYRIC	1.5-2.0
lactic Ψ	0.0-tr	lactic	tr-mod	lactic	tr-mod
succinic	0.0-tr	succinic	tr-mod	succinic	tr-mod
		formic	0.0-tr	formic	tr-mod
alcohols	mod				

Note: Upper case letters, underlined = products present in major amounts (>1.0 meq/100 ml)
Upper case letters = products present in moderate amounts (0.25-1.0 meq/100 ml)
Lower case letters = products present in minor amounts (<0.25 meq/100 ml)

Numerals = approximate concentrations in milliequivalents per 100 ml; tr = trace; mod = moderate

* Group I: *C. botulinum* type A, proteolytic strains of types B and F, and *C. sporogenes*

Group II: *C. botulinum* types C and D and *C. novyi* type A

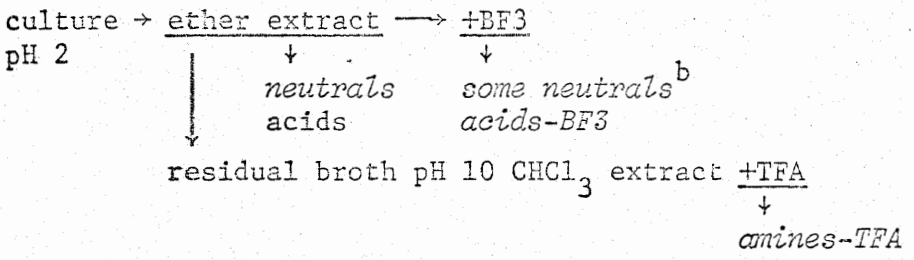
Group III: *C. botulinum* type E, non-proteolytic strains of types B and F, and related non-toxic clostridia

† One strain of *C. botulinum* type A is unusual in that very little butyric acid is produced. The other products of this strain are similar to those of the other organisms in this group.

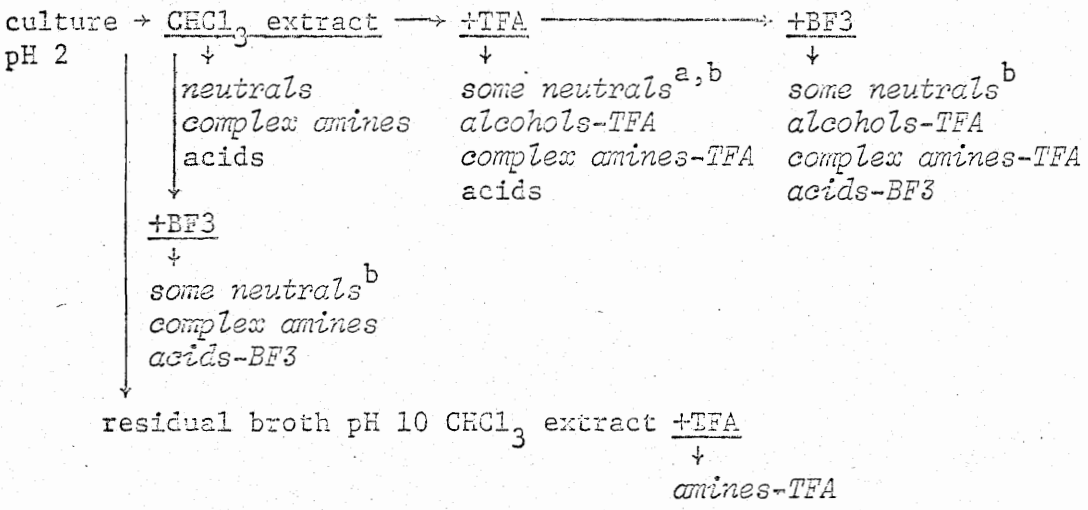
Ψ In cultures of 1 strain of *C. sporogenes* (labeled 'ocean sediment') major quantities of lactic acid accumulate. The other acids produced by this strain are similar to those produced by the other organisms in this group.

Figure 1. METHODS FOR ANALYSIS OF SOLUBLE COMPOUNDS IN CULTURE FLUIDS

A. Procedure for identification of fatty acids and amines



B. Procedure for identification of classes of compounds



Note; Compounds in italics will chromatograph.

BF3 = boron trifluoride methanol.

TFA = trifluoroacetic anhydride.

[a] Some neutral compounds form TFA derivatives; some do not.

[b] Some neutral compounds may be lost by dilution or evaporation during acylation or methylation procedures.

Figure 2. Differentiation of neutral compounds, fatty acids, and alcohols by formation of TFA derivatives followed by methylation. $CHCl_3$ extracts of acidified whole cultures, *C. botulinum* type C

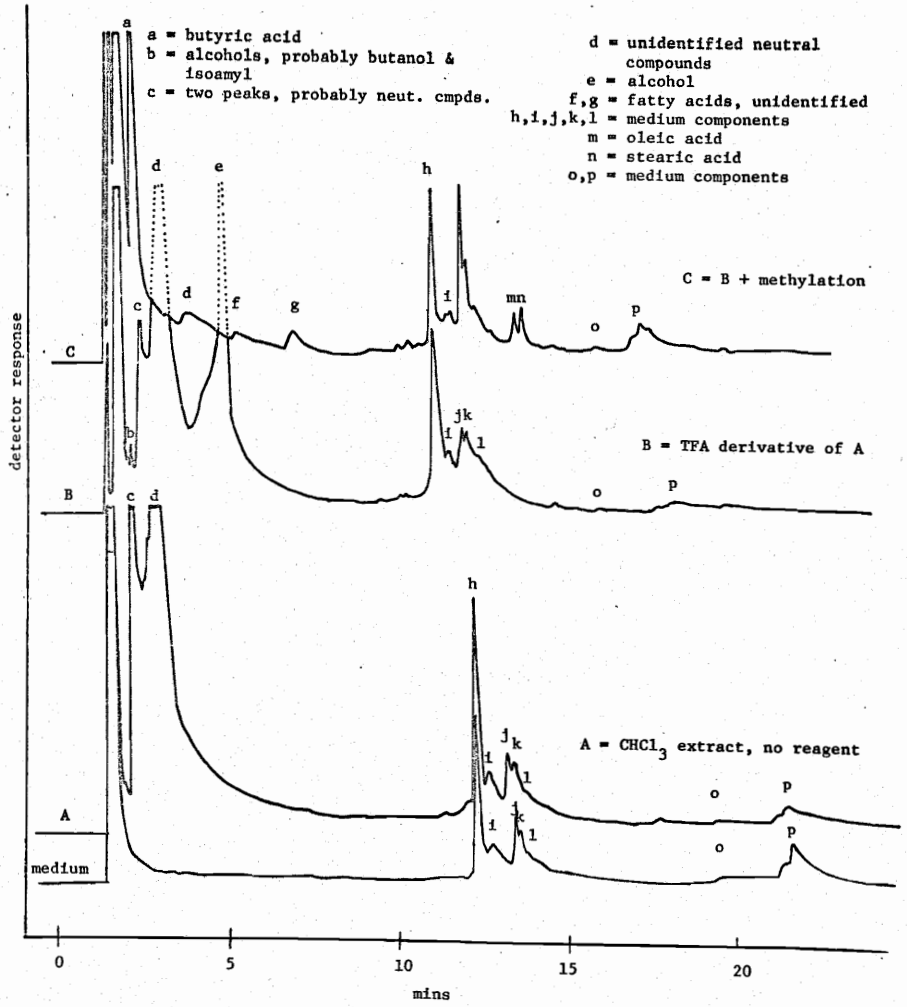


Figure 3. Chromatographs: TFA derivatives of $CHCl_3$ extracts of alkaline whole cultures of *C. botulinum*

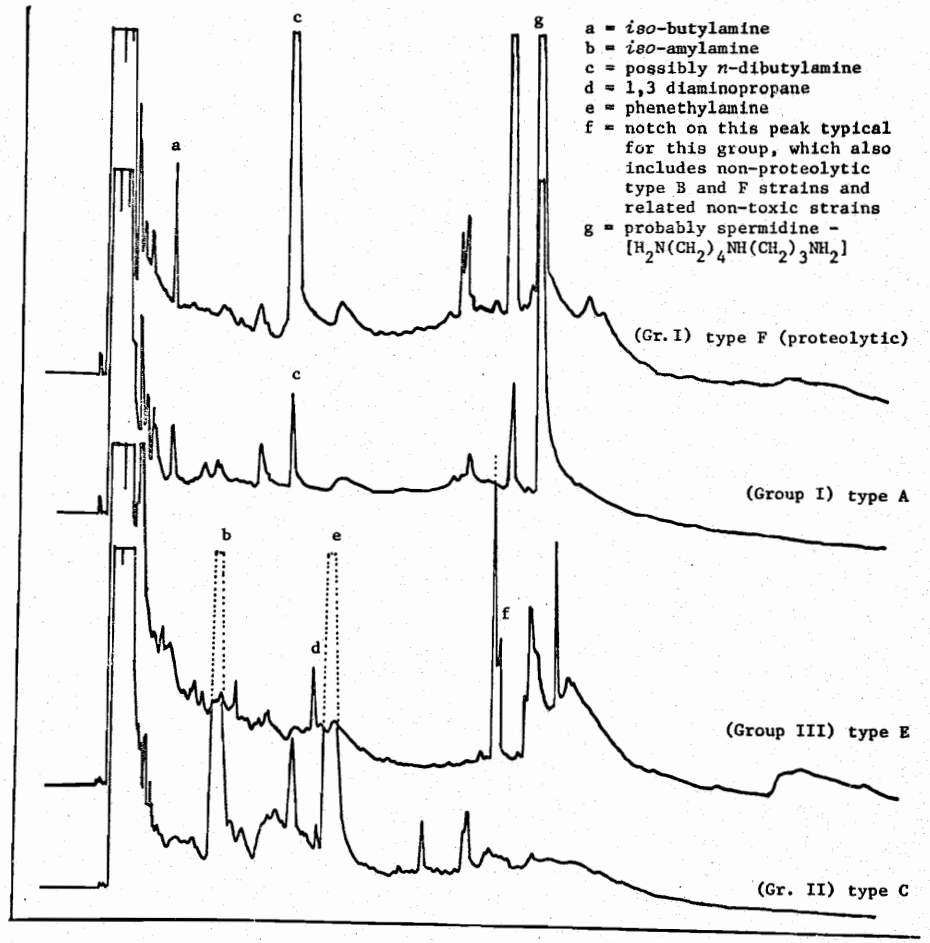


Figure 4. Chromatographs: TFA derivatives of $CHCl_3$ extracts of alkaline whole cultures of *C. sporogenes* and proteolytic *C. botulinum* strains, group I

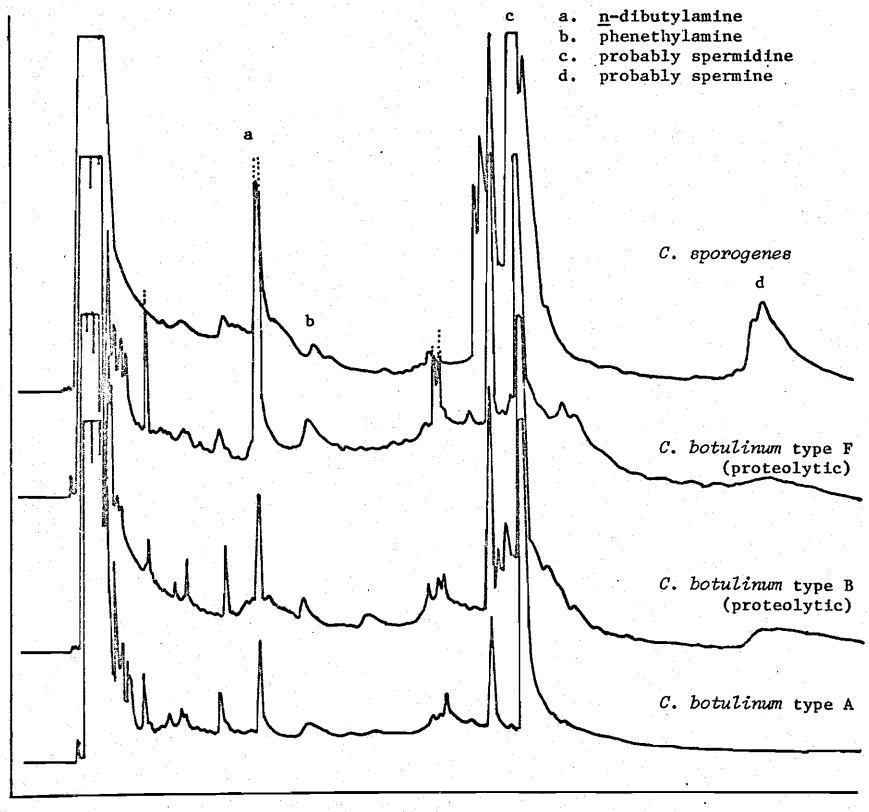


Figure 5. Chromatographs: TFA derivatives of $CHCl_3$ extracts of alkaline broth cultures of *C. botulinum* types C and D and *C. novyi* type A, group II

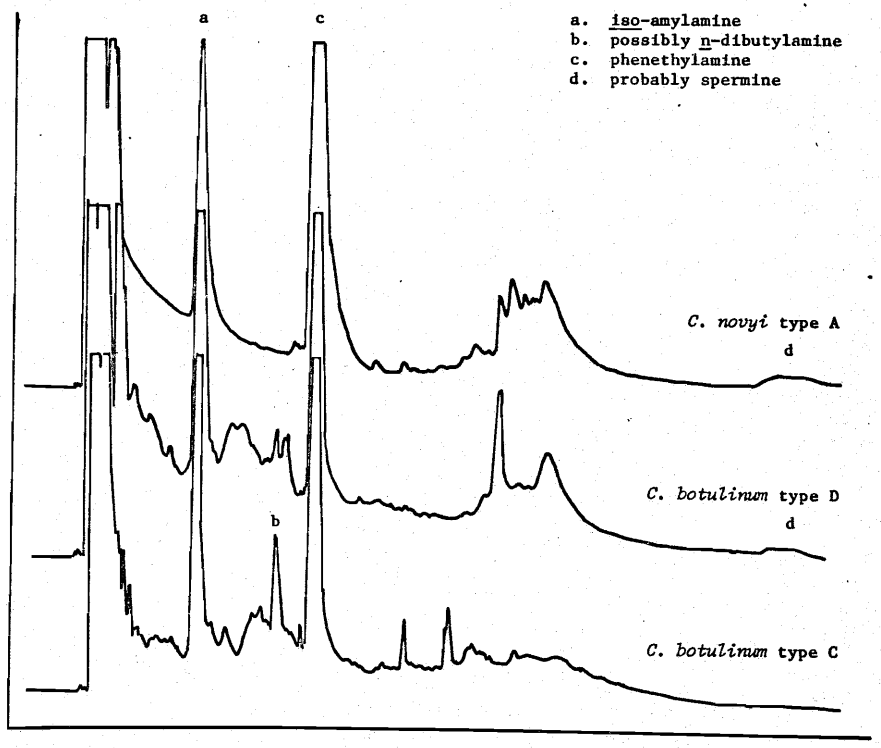


Figure 6. Chromatographs: TFA derivatives of $CHCl_3$ extracts of alkaline whole culture of non-proteolytic *C. botulinum* strains, group III

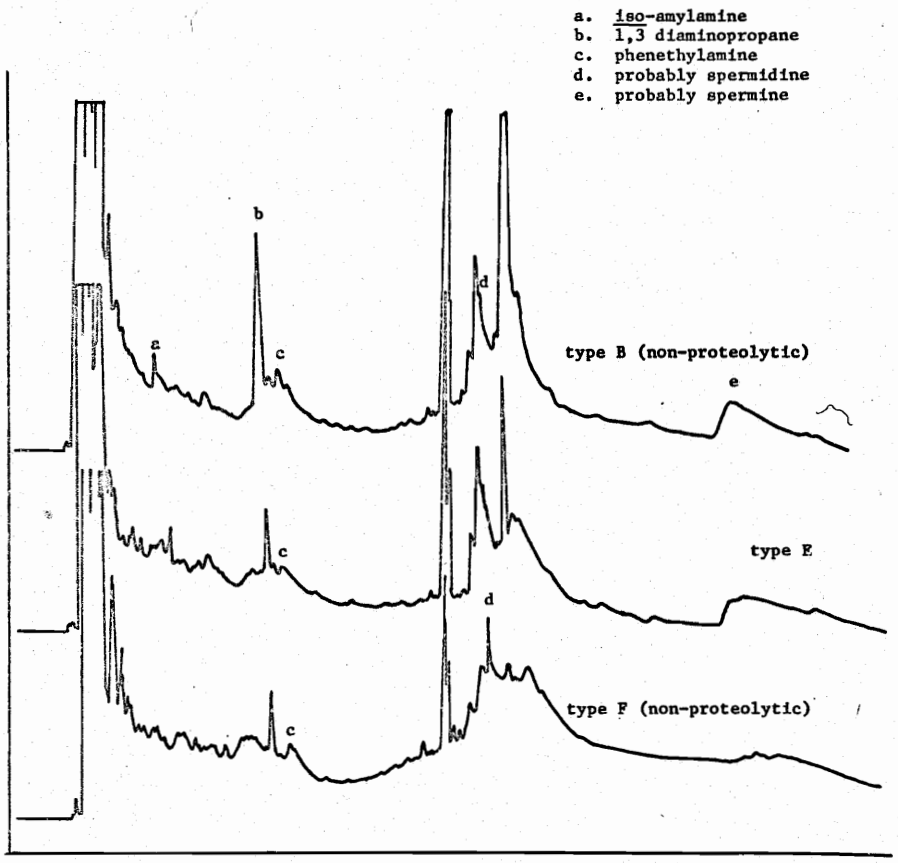


Figure 7. Chromatographs: methylated ether extracts of acidified whole cultures of *C. botulinum*

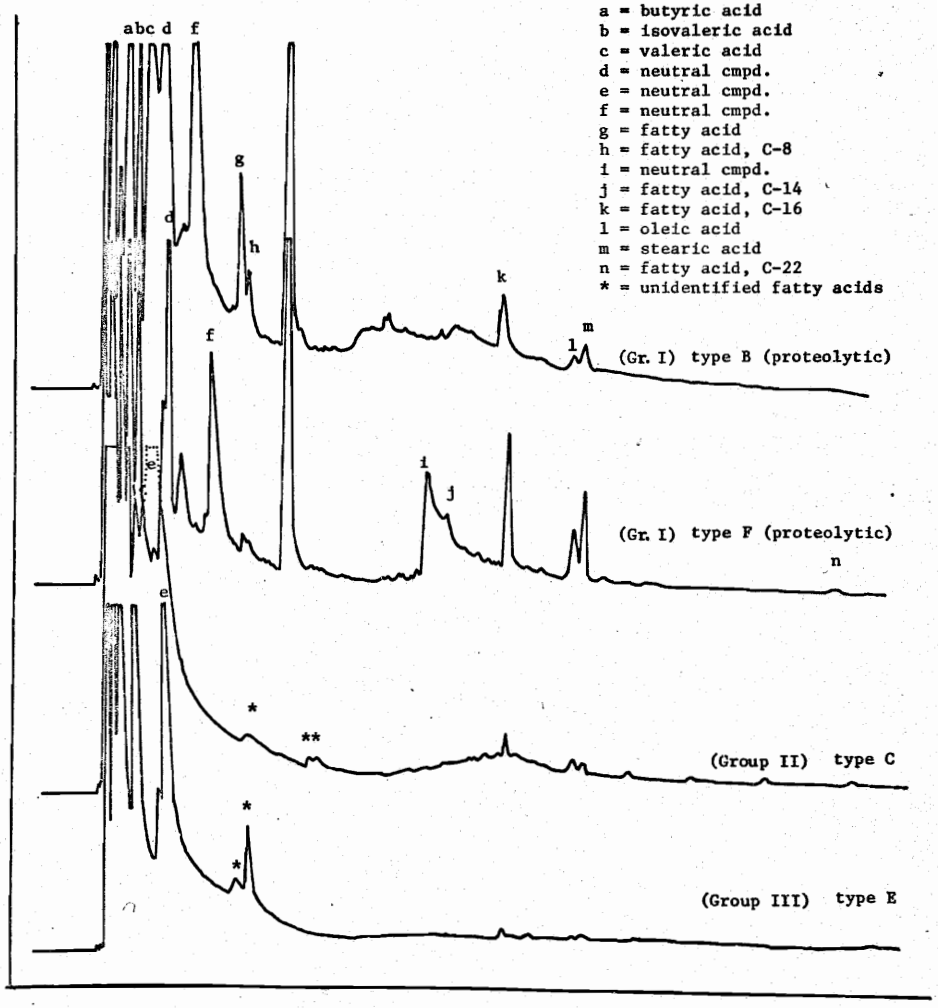


Figure 8. Chromatographs: uninoculated chopped meat medium