DETECTION AND DIFFERENTIATION OF CLOSTRIDIUM BOTULINUM BY PYROLYSIS - GAS - LIQUID CHROMATOGRAPHY

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ABSTRACT

DETECTION AND DIFFERENTIATION OF
CLOSTRIDIUM BOTULINUM BY
PYROLYSIS-GAS-LIQUID
CHROMATOGRAPHY

By

Ricky D. Cone

The objective of this study was to investigate the feasibility of using pyrolysis-gas-liquid chromatography (PGLC) for identification and detection of growth and toxin production by *Clostridium botulinum*. Ten strains representing types A, B, and E of *C. botulinum* were used in the study.

Vegetative cell cultures (10 hr incubation at 32 C) and spore cultures (36 hr incubation at 32 C) of all 10 strains were grown in Trypticase-peptone-sucrose-yeast extract medium (TPSY). Five type E strains were also grown in Multipeptone-sucrose-nutramino acids medium (MSN). Lyophilized samples of these cultures were subjected to PGLC analysis and the resulting pyrograms were examined for differences in elution patterns among (1) vegetative cells grown in each medium, (2) spores grown in each medium, (3) spores and vegetative cells grown in each medium, (4) vegetative cells grown in different media, and (5) spores grown in different media.

The possibility of using PGLC to detect growth and toxin production of all 10 strains of C. botulinum was investigated using a modification of the dialysis sac culture technique described by Vinet and Fredette (1951). The inoculum was added to the physiological saline (0.89%) which was outside the dialysis sac and nutrients were provided by dialysis of TPSY medium across the membrane from inside the sac.

The PGLC analysis was conducted for the cultures grown for 5 days at 32 C as well as the dialysate supernatant fluid (DSF) which remained after centrifugation of the cultures. Three control samples consisting of (1) uninoculated DSF, (2) uninoculated DSF plus purified toxin, and (3) a 1.0 mg sample of partially purified toxin were analyzed in the same manner as other DSF samples to assist in determining whether either growth or toxin production could be detected.

Differences in pyrograms suitable for identification purposes at the type level were obtained for spores and vegetative cells from both media, but only a few reliable differences were obtained at the strain level. It was possible to differentiate pyrograms of spores from those of vegetative cells grown in the same medium, and distinct differences among cultures of the same serotype harvested from different media were noted.

The dialysis sac technique was found to be useful in detecting growth of C. botulinum. Identification of the type of C. botulinum which had grown in the medium was possible. Toxin production, however, was not detectable by PGLC using this technique.

DETECTION AND DIFFERENTIATION OF *CLOSTRIDIUM BOTULINUM* BY PYROLYSIS-GAS-LIQUID CHROMATOGRAPHY

By

Ricky D. Cone

A THESIS

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INTRODUCTION

There has been a substantial increase during the last decade in studies related to the microorganism Clostridium botulinum. This interest was primarily brought about by the increase in outbreaks of type E botulism throughout the world (Iida et al., 1958; Dolman and Iida, 1963), and in the United States in 1963 (Anonymous, 1964).

Botulism is a rare, but highly fatal, disease for which no adequate clinical diagnostic tool is available. The study of botulism in foods usually involves (1) demonstration that the filtrate of a suspected food sample contains type specific toxin by use of animal neutralization tests and (2) cultural procedures to isolate and identify the causative microorganism. The use of animals in toxin assay procedures is limited by (1) the availability of animals to many laboratories, (2) the cost of the animals, (3) the lack of proper facilities in most laboratories for handling animals, (4) variability in response to toxin among species of animals, and (5) differences in injection methods among workers. Conventional methods of identification of C. botulinum are also time consuming and often fail to provide reliable results.

Since pyrolysis-gas-liquid chromatography has been used successfully for the rapid identification of other genera and species of bacteria (Oyama and Carle, 1967; Reiner, 1967; Reiner and Ewing, 1968), it seemed feasible that this technique might also be used for identification of cultures of C. botulinum. This method would not only provide adequate identification of cultures, it might also greatly decrease the minimum time of 5 to 7 days required for detection and identification of cultures by conventional methods.

The use of pyrolysis-gas-liquid-chromatography for the identification of these microorganisms as well as detection of their growth and toxin production was investigated. The effects of the growth medium and of per cent sporulation on the resulting elution patterns after pyrolysis were also studied.

LITERATURE REVIEW

Toxin Detection Methods and Culture Differentiation of Clostridium botulinum

Most of the methods used in the detection and identification of clostridia involve the use of the blackening reaction which occurs when sulfite and iron are present in the isolation medium. Wilson and Blair (1924) were the first to use this technique which has since undergone many modifications. The modification most commonly used today is the one described by Mossel (1959) in which 0.05% (w/v) $Na_2SO_3 \cdot 7H_2O$ and 10 ppm polymyxin B sulfate were added to the medium. Angelotti *et al.* (1962) reported that the incorporation of sulfadiazine and polymyxin in the Mossel based medium containing 0.05% sulfite increased the selectivity of the medium. More recently, Gibbs and Freame (1965) described the use of a differential reinforced medium for isolating clostridia. These workers also include a review of previous selective and differential media that have been used for clostridia.

The organisms of the group *Clostridium botulinum*, however, are of primary importance to food microbiologists, and the previously mentioned techniques fail to provide

the rapidity and ease which are necessary in isolating and differentiating them on a routine basis. Morphological and biochemical characteristics of the organisms of this group are traditionally used for identification purposes. These methods are thoroughly discussed in studies and reviews by Seddon (1922), Meyer and Gunnison (1928), Schoenhol (1928), Gunnison and Meyer (1929), Townsend (1929), Robinson (1930), Gunnison, Cummings, and Meyer (1936), Hazen (1937), Dolman and Chang (1952), Eales and Turner (1952), and Dolman and Murakami (1961).

Techniques for the isolation of C. botulinum from heavily contaminated materials have been reviewed by Kautter and Bartram (1967). McClung (1967) has provided a review of methods used for isolation and identification of C. botulinum up to 1966, and Kautter (1968) has compiled a more recent review of methods of isolation and identification as well as detection of toxin production by C. botulinum.

Several workers have reported the production of a precipitate and the formation of a layer having a "pearly" (iridescent) appearance adjacent to and covering the colonies of the different types of C. botulinum on egg yolk agar medium that may be useful in isolating and differentiating these bacteria (McClung and Toabe, 1947; Willis and Hobbs, 1958; Willis and Hobbs, 1959). The former workers used Trypticase (BBL)-peptone-agar as the

basal medium for this method while the latter used beef infusion agar. The use of liver-veal-agar as the basal medium for the egg yolk reaction was investigated by Hobbs, Stiebrs and Eklund (1967). They observed that this medium was less efficient for isolating C. botulinum type E while it was just as effective as the other two in isolating C. botulinum types A and B as well as C. sporogenes.

The fluorescent antibody technique has proven useful in detecting the presence of C. botulinum microorganisms in sample material as well as in confirming the type of various isolates detected. Russian workers including Kalatina (1960) and Bulatova and Kabanova (1960) were among the first to find this technique useful for identifying types of C. botulinum when they observed that certain types of luminescent sera would react only with certain types of C. botulinum. Batty and Walker (1963) and Walker and Batty (1964) were able to separate C. botulinum into three groups using fluorescent antibody staining: (a) proteolytic types A, B, and F; (b) nonproteolytic type E and D; and (c) nonproteolytic type E. Boothroyd and Georgala (1964) also found that fluorescent antibody staining showed considerable promise as a tool for isolation and/or identification of C. botulinum. These same workers (Georgala and Boothroyd, 1967) later indicated that more work was required on

the serological characteristics of cells, spores, and flagella of each type before the technique can be widely accepted. Walker and Batty (1967) further observed that the fluorescent antibody technique could be used in the identification of toxic as well as non-toxic strains of C. botulinum. Ward, Carol, and Garrett (1967), however, were unable to obtain consistent results with respect to toxin production and fluorescence when mouse-toxin tests were compared to fluorescent detection of toxin.

Hobbs and Anderson (1967) used polyacrylamide gel electrophoresis to study relationships between C. botulinum and non-toxic clostridia or non-toxic mutants. They observed that protein patterns of vegetative cell extracts of all strains of type E examined were essentially the same and that each of the types A, B and C gave different characteristic patterns.

Midura et al.(1967) examined the possibility of using immunofluorescence for detection of C. botulinum types A, B, and E. They reported that this technique could be quite useful as a presumptive indicator of the presence of C. botulinum cells. Midura et al. (1968) further combined the use of this technique with animal tests to detect growth and toxin production by C. botulinum type E in experimentally inoculated packages of turkey roll. They were able to obtain a time relationship between the presence of vegetative cells,

determined by immunofluorescence, and the presence of toxin as demonstrated by animal tests.

Immunological techniques have also been reported to be useful in detecting toxins of C. botulinum. Rycaj (1956), Yafayer and Chepeler (1961), and Sinitsyn (1961) indicated that passive hemagglutination of botulinal antitoxin-coated, tanned sheep red blood cells was as sensitive in detecting botulinal toxins as animal assays. Johnson et al. (1966) were able to detect one LD_{50} mouse unit of botulinal toxin using hemagglutination. Vermilyea, Walker and Ayres (1968) have employed the Ouchterlony immunodiffusion technique to detect toxins of C. botulinum. They indicated, however that the mouse assay was a more sensitive method for detecting toxin since the toxin had to be concentrated with Sephadex when the gel diffusion technique is used.

More recently, Duda and Slack (1969) have demonstrated toxin production by C. botulinum type A using electron microscopy. Upon electron microscopic examination of ultrathin sections of spheroplasts of the organism which had been exposed to ferritin-labeled type A antitoxin, they observed the presence of ferritin-labeled antibody in the outer spore coats in the vegetative cell cytoplasm. No ferritin-labeled antibody was observed in the spore cortex or spore core.

Detection, Classification, and Identification of Microorganisms by Gas-Liquid-Chromatography

The utilization of gas-liquid-chromatography (GLC) as an analytical tool became a reality when James and Martin (1952) made the first successful separation of volatile fatty acids. Since that time the use of GLC has probably developed more rapidly than any other analytical technique.

Abel, deSchmertzing, and Peterson (1963) were the first to investigate the feasibility of using GLC for the classification of microorganisms. They extracted the lipid components of the microorganisms, transesterified them to component carboxylic acid methyl esters, and resolved the esters by gas chromatography. The elution patterns which they obtained permitted them to distinguish between families of Enterobacteriaceae, Bacillaceae, Micrococcaceae, and Parvobacteriaceae. Differentiation of genera and species was however, rather difficult.

Cattaneo et al. (1964) using a similar method of examining methyl esters of bacterial fatty acids by GLC were unable to demonstrate significant differences among strains of the same type of mycobacteria from various sources. They did, however, show noticable variation in elution patterns from one type of Mycobacterium to another.

Brown and Cosenza (1964) observed differences in methyl esters of fatty acids among Gaffkya, Micrococcus, and Veillonella by GLC. No attempt was made by these workers to differentiate between closely related species. Yamakawa and Ueta (1964), however, were able to demonstrate differentiation at the species level in the genus Neisseria with this technique.

Garner and Gennaro (1965) were able to classify bacteria more readily than previous workers using GLC. On the basis of "fingerprints" obtained from GLC analysis of pyro-distillates of bacterial cultures using a flame ionization detector, they were able to differentiate between Aerobacter aerogenes, Aerobacter cloacea, Aerobacter lipolyticus, Escherichia coli, Escherichia aurescens, and Eschericia anaerogenes. Preparation of the material for GLC analysis, however, was rather complicated.

Bassette and Claydon (1965) found that GLC analysis of head-space vapors of volatile microbial metabolic products of growth in deodorized milk could be useful for characterizing bacteria. Pure cultures of microorganisms known to have widely different characteristics gave markedly different chromatographic patterns while related bacteria produced similar patterns. However, similar patterns were sufficiently different to enable differentiation of these bacteria.

Bawdon and Bassette (1966) used this technique to differentiate E. coli and A. aerogenes.

The preparation of methyl esters from fatty acids has continued to be an important technique in the differentiation of bacteria by GLC. Moss and Lewis (1967) examined methyl esters of fatty acids extracted from whole cells of 41 strains representing 13 species of Clostridium by GLC. Upon both visual and quantitative comparisons of chromatograms they were able to rapidly differentiate Clostridium perfringens, Clostridium sporogenes, and Clostridium bifermentans from each other and from 10 other species of clostridia. Steinhauer, Flentge, and Lechowich (1967) on the basis of their studies have also supported the suggestion that cellular lipid patterns could be a valuable means of identification of closely related bacteria.

Moss et al. (1967) examined the fatty acid methyl esters of 27 isolates of Corynebacterium acnes by GLC and observed that each strain possessed a similar profile which was characterized by a large percentage of C-15 branched-chain acids.

These workers felt that their findings, along with other similarities of these cultures to those of the genus Propionibacterium, strengthened previous suggestions that C. acnes should be classified in the genus Propionibacterium. Moss et al. (1969) used the same technique

and further proposed a similarity between anaerobic corynebacteria and a group of propionibacteria which contained C-15 saturated branched chain fatty acids in the iso-form. The corynebacteria were distinctly different from another group of propionibacteria which contained primarily anteiso fatty acids. Lewis, Weaver, and Hollis (1968) have also observed a difference in the fatty acid composition of various species of Neisseria by use of GLC.

Henis, Gould and Alexander (1966) investigated the possibility of detecting and differentiating microorganisms by GLC analysis of unique metabolic products which the organisms produce. Upon examination of ether extracts of several species of Bacillus as well as individual strains of E. coli, A. aerogenes and Pseudomonas aeruginosa using a gas chromatograph equipped with flame ionization and electron capture detectors, several products were observed. Both qualitative and quantitative differences in the production of such products as acetic, propionic, and butyric acids, ethyl alcohol, diacetyl, acetoin, and 2,3-butanediol made it possible to detect differences among genera, species and strains. Mitruka and Alexander (1967) further observed the sensitivity of the electron capture detector in detecting such microbial metabolites as acetoin and diacetyl in the picogram range.

Mitruka and Alexander (1968) found that GLC analysis of metabolic products enabled them to detect the presence of Proteus vulgarus, Streptococcus faecalis, Streptococcus liquefaciens, E. coli, Bacillus cereus and Bacillus popilliae in 2 to 4 hr old cultures inoculated with less than 1 x 10^4 cells per ml. A somewhat longer period of 7 to 12 hr was required for the detection of prcducts formed by A. aerogenes, E. coli K-12, Staphylococcus aureus, and Salmonella typhimurium.

More recently, Mitruka and Alexander (1969) have shown that bacterial growth may be even more easily detected by the use of cometabolism which involves the use of substrates which are presumed to yield products detectable at low concentrations. A halogenated compound which was incorporated in the growth medium was metabolized by the bacterium but was not a growth substrate, and a 7,000, 20,000, and 1,000 fold increase in GLC detectability was obtained in S. typhimurium, E. coli, and S. aureus respectively.

Differentiation of bacteria has also been accomplished by GLC analysis of ether extracts of the metabolic products produced by washed bacterial cells suspended in buffered glucose (O'Brien, 1967). The cells in this experiment were considered to be resting or non-growing since the organisms grow poorly or not at all in this chemically defined medium.

Cecchini and O'Brien (1968) reported a procedure to differentiate E. coli from other bacteria by direct GLC analysis of the culture medium. They were able to detect E. coli even in the presence of A. aerogenes and other microorganisms using special growth conditions since only the E. coli produced ethyl alcohol.

Moore, Cato and Holdeman (1966) have observed that fermentation patterns may be useful in characterizing and identifying microorganisms. Examination of fermentation patterns of 20 species of clostridia by GLC showed that the relative proportions and concentrations of the alcohols may be useful in this respect.

MacGee (1968) has observed distinct differences in elution patterns when alcohol extracts of widely different genera of microorganisms were subjected to GLC analysis. Only quantitative or no differences in chromatograms, however, were observed between three strains of E. coli.

Holdeman and Brook (1968) have found that GLC analysis of chloroform extracts of culture fluids in combination with GLC analysis of prepared derivatives of extracted compounds can be used in the identification of these compounds. They have tentatively identified 18 amines and 23 fatty acids from various strains of clostridia by this technique.

One of the most recent methods of characterizing bacteria by GLC involves the preparation of trimethylsilyl (TMS) derivatives of whole-cell hydrolysates (Farshtchi and Moss, 1969). These workers observed significant differences in the TMS profiles of Listeria, Neisseria, and Clostridium as well as Clostridium perfringens (two types) and Clostridium sporogenes. Differences in the two types of C. perfringens were believed to reflect either strain variations or differences among serological or toxigenic types within the same species.

Pyrolysis-Gas-Liquid Chromatography

The use of pyrolysis as an adjunct to gas chromotography has recently gained considerable importance. This technique is particularly useful in the polymer industry since it provides the rapidity necessary in the identification of polymers and polymer structures (Perry, 1967). Dal Nogare and Juvet (1966) have described thoroughly the major developments which have taken place in the use of pyrolysis-gas-liquid chromatography (PGLC) as an analytical tool and McKinney (1964) has compiled a comprehensive outline of the literature concerning the use of this technique between 1960 and 1963. Perry (1964) has also reviewed the techniques and potentialities of PGLC.

Advances in pyrolysis techniques have been achieved through improvements in apparatus design as well

greater control of experimental variables. The former has been aided by the work of Honaker and Horton (1965) who described a simple and inexpensive constant temperature pyrolyzer and Cox and Ellis (1964) who used a packed tubular furnace microreactor. The latter workers were able to identify polymeric materials and evaluate the effect of changes in reactor geometry and temperature on the number of pyrolysis products. Stanford (1965) described the use of glass capillary tubes open in one end to hold the material to be pyrolyzed in the pyrolyzer coil. Further advances in apparatus design were made by Barbour (1965) who described the use of a valved pyrolyzer in which the pyrolysis chamber could be isolated during pyrolysis. This allowed the pyrolysis products to be instantaneously injected into the gas chromatograph.

Information concerning the structure of organic compounds was obtained by Beroza and Sarmiento (1964) when they used a carbon skeleton technique to degrade the compounds. Burr et al. (1964) in another attempt to identify pyrolysis products found that methane and benzene were the only pyrolytic products of toluene when hydrogen was used as the carrier gas. Parsons (1964) was able to identify pyrolytic materials on the basis of their molecular weight by using a gas density balance technique in which the density of the carrier gas was varied.

The use of PGLC as an analytical tool encompasses many different areas. Those most commonly studied include analysis of proteins, peptides, and amino acids (Winter and Albro, 1964; Merrit and Robertson, 1967), and degradation of such products as porphyrins (Levy et al., 1964), polymeric materials (Feuerberg and Weigel, 1964) including cellulosic polymers (Groten, 1964), polyolefins (Cieplinski et al., 1964; Kolb et al., 1965; Deur-Siftar, 1967), polymers of polystyrene and acrylic resins (Esposito, 1964; Esposito and Swann, 1965; Spagnolo, 1968), aliphatic alcohols (Dhont, 1964), dialkyl phosphates and o,o-dialkyl dithiophosphates (Hanneman and Porter, 1964), and phthalate esters of tertiary alcohols (Rutherford and Funk, 1964).

Stack (1967) has investigated the possibility of using PGLC in dental work. Changes in the organic matrix of enamel of teeth as they decayed could be detected. Kirk (1967) has also examined the possibility of using this technique in crime laboratories for identification of unknown materials.

Brodasky (1967) observed that both low and high temperature PGLC could be used in the differentiation of selected antibiotics. He further observed that structural characterization of antibiotics could be accomplished using this technique.

Reiner (1965) was the first to investigate the feasibility of using PGLC for the identification of bacteria. Upon pyrolysis of cells of E. coli (18 different antigenic strains), Shigella sp., group A Streptococcus pyogenes (4 types), and mycobacteria (10 different pathogenic and nonpathogenic forms) each strain produced its own unique pyrogram and different cultures of the same strain yielded similar profiles. Reiner (1967) in a further study using additional microorganisms which were somewhat difficult to classify, reconfirmed his earlier report that PGLC could be used in the classification of microorganisms. Reiner and Ewing (1968) further observed that this technique could be used to differentiate two cultures of E. coli which differed only in their flagellar, H, antigens. These antigens were composed almost entirely of protein, thus, indicating that slight protein differences have a strong influence on pyrograms obtained.

Oyama and Carle (1967) have also reported the use of PGLC for the identification of bacteria. They converted the pyrograms which they obtained into a digital form and analyzed these with a computer. They obtained bar graphs from the computer data which enabled them to differentiate among 7 fungi grown in malt extract as well as 4 microorganisms grown in trypticase soy broth.

METHODS AND MATERIALS

Microorganisms

The microorganisms studied in this experiment include 2 strains of *Clostridium botulinum* type A, 2 strains of *C. botulinum* type B, and six strains of *C. botulinum* type E (5 toxic strains and 1 non-toxic variant). Type A strains used were 62 and 78 and type B strains used were 169 and 213. The type A and B strains were all obtained from Dr. C. F. Schmidt, Continental Can Co., Chicago, Illinois. The strains of type E used and their sources were:

Strain	Source
Vancouver Herring (VH)	Dr. C. F. Schmidt Continental Can Company Chicago, Illinois
Kalamazoo (KAL)	Mr. R. W. Johnston Food and Drug Administration Detroit, Michigan
Seattle Forks (SF)	Dr. J. T. Graikoski Bureau of Commercial Fisheries Ann Arbor, Michigan
517	Mr. Donald A. Kautter Food and Drug Administration Washington, D.C.
A6247	Mr. R. W. Johnston Food and Drug Administration Detroit, Michigan

Strain	Source
066B nontoxic (NT)	Mr. Haim M. Solomon Food and Drug Administration Washington, D. C.

Culture Media

Spores and cells of all 10 strains of C. botulinum investigated were grown in a medium consisting of 5.0% Trypticase (BBL), 0.5% peptone (Difco), 0.2% sucrose, 1.0% yeast extract (Difco), and 0.1% sodium thioglycolate adjusted to pH 7.2 (TPSY).

In addition, 5 type E strains were grown in a medium consisting of 2.5% Multipeptone (Fisher), 0.2% sucrose, 1% nutramino acids (Fisher) and 0.2% sodium thioglycolate (MSN). One liter Erlenmeyer flasks containing approximately 1 liter of the growth medium (TPSY or MSN) were inoculated with 15 ml of a 16 to 18 hr actively growing subculture prepared in the same medium of each of the C. botulinum strains and incubated at 32 C. Duplicate flask cultures were prepared for each strain.

Toxin production and growth were studied by growing each of the 10 strains of C. botulinum in 1 liter Erlenmeyer flasks filled with physiological saline solution (0.89%) in which a dialysis sac filled with 250 ml of TPSY medium (normal concentration) was suspended. The ratio of the medium to saline solution was approximately 1:4 (v/v). To prevent carry-over of materials from the

inoculation medium to the saline solution, the inoculum (14 ml of 16 to 18 hr actively growing culture) was centrifuged 10 min at 1000 x g using a Sorvall RC-2 refrigerated centrifuge. The culture was then resuspended using 5 ml of sterile distilled water and inoculated into the saline solution. The flasks were again incubated at 32 C and duplicate flask cultures were prepared from each strain.

Harvesting Cells and Spores

Cultures incubated at 32 C for 8 to 10 hr in TPSY and MSN media to obtain vegetative cells were then immediately cooled to 4 C to prevent sporulation, dispensed into 250 ml centrifuge bottles, and centrifuged for 15 min at 5000 x g using a Sorvall RC-2 centrifuge refrigerated to 4 C. The cells were then washed by centrifuging as above and resuspended in 200 ml of sterile distilled water until they appeared clean by microscopic examination (required 5 to 8 washings). After the final washing the cells were suspended in 100 ml of sterile distilled water and stored at 2 C.

Spore crops of C. botulinum type E were grown in both media and harvested after 24 to 30 hr incubation when spores were free from their sporangia. Longer incubation of 30 to 36 hr was necessary to obtain adequate sporulation of type A and B strains. It was then

necessary to treat these latter cultures with lysozyme (0.75 mg lysozyme per ml of 0.1N KCl) for 4 to 6 hr at 32 C to obtain free spores. All washings and storage procedures of spores and vegetative cells were the same.

No attempt was made to separate spores and cells using the dialysis sac technique. All cultures were incubated for 5 days and harvested in the manner previously described. Since the type A and type B cultures consisted of a considerable percentage of vegetative cells, lysozyme was not used. The dialysate supernatant fluid (DSF) was separated from the microorganisms by centrifugation and retained for subsequent analysis. The DSF was filtered through a Millipore membrane filter (0.80 μ pore size) to remove any organisms not removed by centrifugation and stored in glass bottles at 2 C.

Direct Microscopic Count

Direct microscopic counts were made on all culture samples using a Petroff Hausser counting chamber. One ml of each culture was added to either 3 ml or 7 ml of 30% glycerol solution depending on the concentration of the microorganisms. One loop of the suspension was placed under the cover slip on the counting chamber and the microorganisms in 80 small squares were counted. Total counts were then calculated using the following equation:

$$\text{total count} = \frac{(\text{Number cells counted})(\text{Dilution})(20)(10^6)}{\text{Number small squares counted}}$$

The direct counts gave an indication of the total count as well as the per cent sporulation. Refractile bodies were considered to be spores.

Toxicity Assays

Swiss Webster white mice weighing 15 to 20 g were injected intraperitoneally with 0.1, 0.2, and 0.5 ml of serial dilutions of each DSF sample. Prior to injection the samples were digested for 60 min at 37 C with 1% trypsin (Difco, 1:250) in an equal volume of 0.05 M sodium phosphate buffer, pH 6.0, and dilutions made in 0.05 M sodium phosphate buffer. The highest dilution at which those mice injected died was used to calculate the minimum lethal dose (MLD).

Controls

Control samples consisted of DSF treated in the same manner as other DSF samples with the exception that no inoculum was used. A second control consisted of DSF to which was added partially purified toxin of the VH strain of C. botulinum type E prepared by Mr. Alexander Emodi, Department of Food Science, Michigan State University. The partially purified toxin was added to the uninoculated DSF to give a final toxin titer of 1.0 x 10^6 MLD per ml. A lyophilized sample of the same

partially purified toxin was also retained for subsequent analysis.

Lyophilization of Samples

All cultures as well as the DSF and controls were lyophilized using a Virtis Freeze Mobile. Approximately 5 ml of each culture or sample was added to a 10 ml freeze drying ampoule. The contents were then shell-frozen using a dry ice and alcohol bath and lyophilized for 8 to 10 hr under a vacuum of 0.025 mm Hg.

Preparation of Column Material

Column material used for pyrolysis-gas-liquid chromatography (PGLC) analysis consisted of a 15% high temperature stabilized ethylene glycol adipate (EGA) liquid phase (Analabs, Hamden, Conn.) coated on 90 to 100 mesh Anakrom ABS (Analabs). Fifteen grams of EGA were carefully weighed into a 600 ml beaker and dissolved in several hundred ml of acetone. One hundred grams of the solid support were then added to the liquid phase solution and stirred for several minutes using a magnetic stirrer. The beaker and contents were placed on a steam bath to evaporate the solvent. Constant stirring with a glass rod was done throughout the evaporation to insure uniform coating, and a slow moving stream of nitrogen was blown over the material to help the solvent escape as it vaporized. After the material was as dry

as could be achieved using this technique, it was placed in a round bottom flask which was connected to a Rinco rotary vacuum apparatus. A vacuum was applied using a water aspirator, and the remaining solvent removed while applying a slight amount of steam as the flask turned. The material was then weighed to determine the percentage of liquid phase actually obtained.

Packing and Conditioning Columns

Prior to packing the columns, the packing material was screened through an 80 mesh wire sieve to remove any large clumps which might cause inefficient packing. The packing material was added to the top of the column through a funnel. The column was simultaneously vibrated with a vibrator tool and a vacuum drawn on the bottom of the column to uniformly pack the material in the column. Caution was taken, however, not to vibrate the material too severely since this could break some of the particles. Glass wool was used in each end of the column after packing to hold the material in place.

Columns were conditioned for 2 weeks at 240 C. They were further conditioned overnight during use to facilitate removal of pyrolytic materials retained by the column.

Pyrolysis Apparatus

A Hewlett-Packard Model 80 Pyrolysis Unit (F & M Scientific Division, Avondale, Pa.) was used to pyrolyze the samples. This instrument utilized an automatic pyrolysis cycle in which there was a 60 sec delay after activation of the switch, thus allowing the gas chromatograph to stabilize. After the delay period the jaw-style probe element which contained the sample was energized for 12 sec to bring about pyrolysis at 1200 C. The probe was fitted to the injection port of the gas chromatograph with an adaptor, thus allowing the volatile components to be swept directly into the column upon pyrolysis.

Gas Chromatograph

A Hewlett-Packard Model 5750 B research gas chromatograph (F & M Scientific Co., Avondale, Pa.) equipped with dual-flame ionization detectors and a Moseley 7128 A dual pen strip chart recorder were used for analysis of pyrolytic products. A special adapter was added to the injection port of the chromatograph to facilitate use of the pyrolysis unit.

Analysis of Samples

Lyophilized samples were analyzed by pyrolysis gas chromatography using the following operating parameters:

Column: 72 x 3/16" copper refrigeration tubing

Sample Size: 0.9 - 1.1 mg for spores and cells

1.3 - 1.7 mg for DSF and controls

Coating: 15% high temperature stabilized ethylene glycol adipate (Analabs)

Support: 90 to 100 mesh Anakrom ABS (Analabs)

Carrier Gas: Helium at pressure of 60 psi

Column A--50 ml per min flow rate

Column B--67 ml per min flow rate

Hydrogen: 12 psi

Air: 33 psi

Temperatures: Pyrolysis--1200 C for 12 sec

Detectors--250 C

Injection Port--230 C

Column--programmed at 10 C per min from 50C to 240 C.

Upper Limit Interval: variable

Chart Speed: 0.25 in per min

Sensitivity and Attenuation: 10^2 x 8 for spores and cells; variable for DSF and controls.

Interpretation of Results

Results obtained were interpreted by visual examination of the pyrograms. The presence or absence of peaks, the ratio of peak heights with relationship to each other,

and retention times were particularly useful. No quantitation or identification of peaks was attempted in these preliminary studies.

RESULTS AND DISCUSSION

Effects of Sample Size

Initial examination of several of the pyrograms appeared to reveal considerable differences in the quantity of material being eluted between strains and types as well as relative ratios of peak heights for each strain. These differences appeared to be caused by an inability to accurately control the amount of sample being pyrolyzed. The use of the jaw-style pyrolysis probe made it impossible to weigh the sample more accurately than to the nearest 0.1 mg.

The consistency with which the sample was packed into the probe also appeared to influence the elution pattern. If the sample was packed too firmly some of the products were not swept instantaneously into the column upon pyrolysis. However, if samples were too loosely packed they were often blown from the probe by the carrier gas when it was inserted into the injection port.

Some of the pyrolytic products were retained by the column support material during extended periods of use of the columns. The size of the sample as well as the physical and chemical characteristics of the

pyrolytic products might have contributed to this problem. In any event, these products contributed to baseline drift and often gave the false effect that one peak was proportionally higher than another. This problem occurred only occasionally in the pyrograms presented since it was eliminated by allowing the columns to condition at 240 C for a minimum of one hr twice a day when the columns were being used constantly. In addition the columsn were conditioned overnight at 240 C. The pyrograms in which sample size and baseline drift caused variations are discussed in their respective sections.

Levy (1967) has also indicated the importance of the effect of sample size and other operating parameters on the pyrolysis products. He observed that secondary reactions between radicals and fragments formed upon rupture of primary bonds during pyrolysis were affected by the quantity of material pyrolyzed. He also indicated that the rate of decomposition of the material pyrolyzed was dependent upon the film thickness of the sample in relationship to the geometry of the source of heat.

Janák (1960) observed that pyrolytic products of samples in the microgram range were often independent of the final temperatures of pyrolysis and gave better reproducibility from one laboratory to another. Ehrler and Frijouf (1968) also indicated that sample size as

well as final temperature, rate of temperature rise, and residence time in the reactor, greatly influenced pyrolysis elution patterns.

The final temperature and rate of temperature rise were readily controlled by the pyrolysis apparatus used in this study. Residence time, however, was probably influenced by sample packing while the sample size, as stated previously, was difficult to accurately control.

Analysis of Vegetative Cells

Pyrograms obtained upon pyrolysis-gas-liquid chromatography (PGLC) analysis of vegetative cells of various types and strains of Clostridium botulinum are shown in Figures 1 (types A and B), 2 (type E in Trypticase medium), and 3 (type E in Multipeptone medium). Examination of elution patterns for types A and B revealed distinct differences in peaks e, h, o, and r. Strain 62A was distinguishable from strain 78A and strains of type B by the large peak o. The type B strains were not readily differentiated from each other, however, the larger quantity of material eluted in peaks e, h and r allowed them to be differentiated from strains of type A. There also appeared to be some quantitative differences in peak d, but these failed to be consistent for any one strain or type. All other differences mentioned were present in duplicate as well as replicate analyses of the same sample.

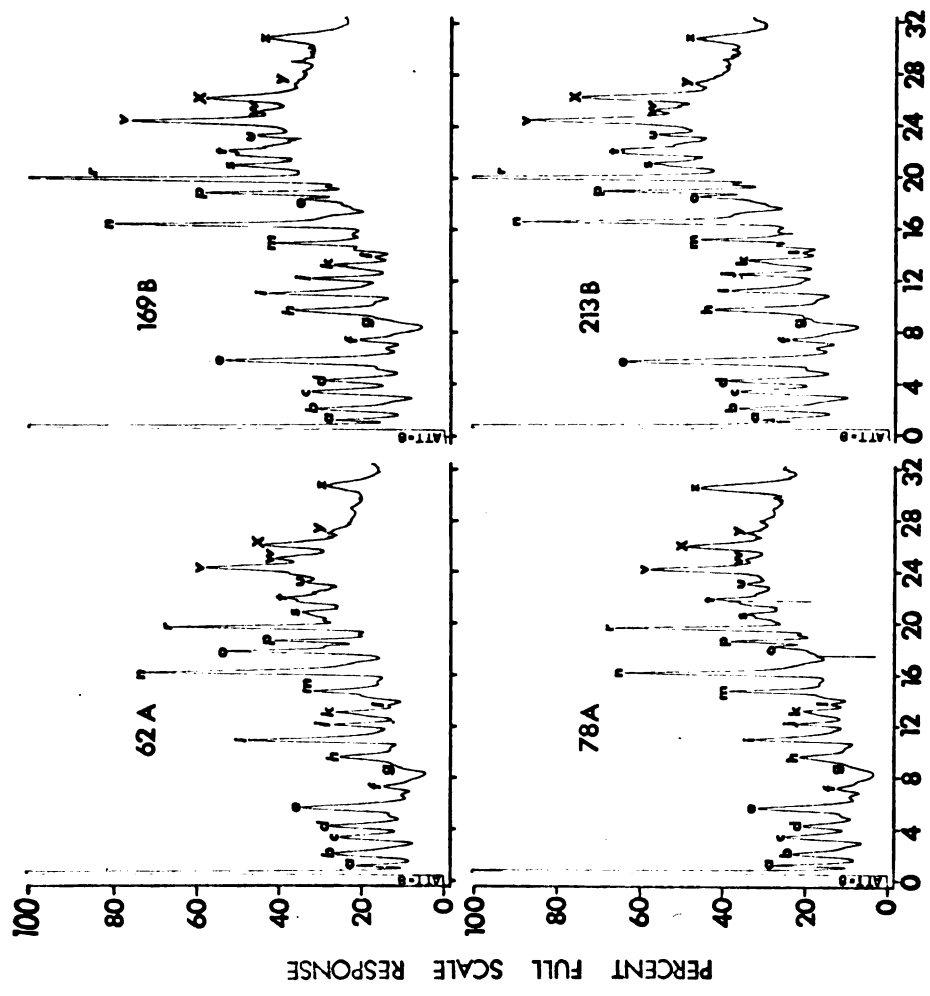

Figure 1. Pyrograms from vegetative cells of four strains of Clostridium botulinum types A and B grown in Trypticase-peptone-sucrose-yeast extract medium. The capital letters denote the type and Att is the attenuation.

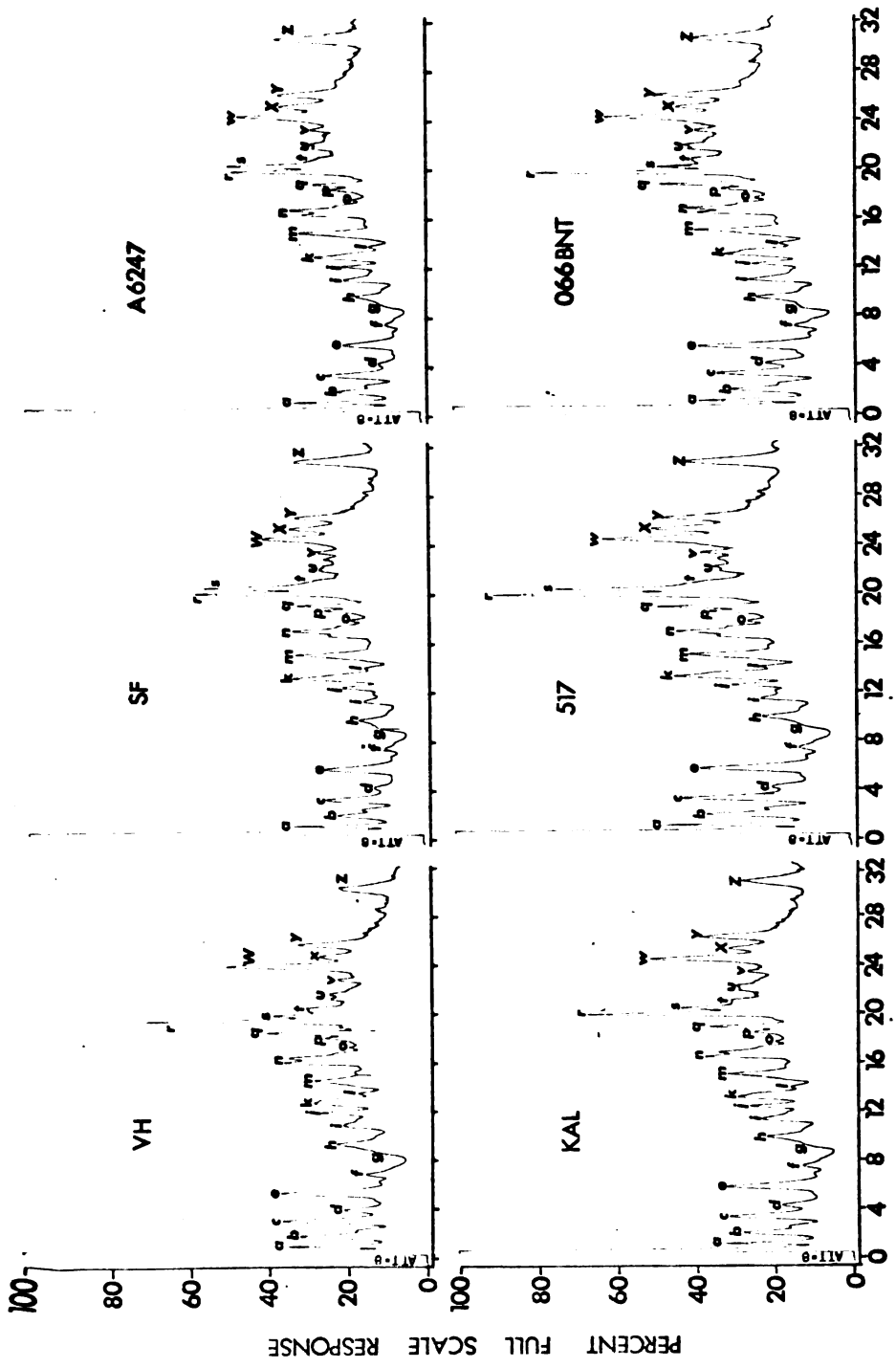

Figure 2. Pyrograms from vegetative cells of six strains of Clostridium botulinum type E grown in Trypticase-peptone-sucrose-yeast extract medium. Att is the attenuation.

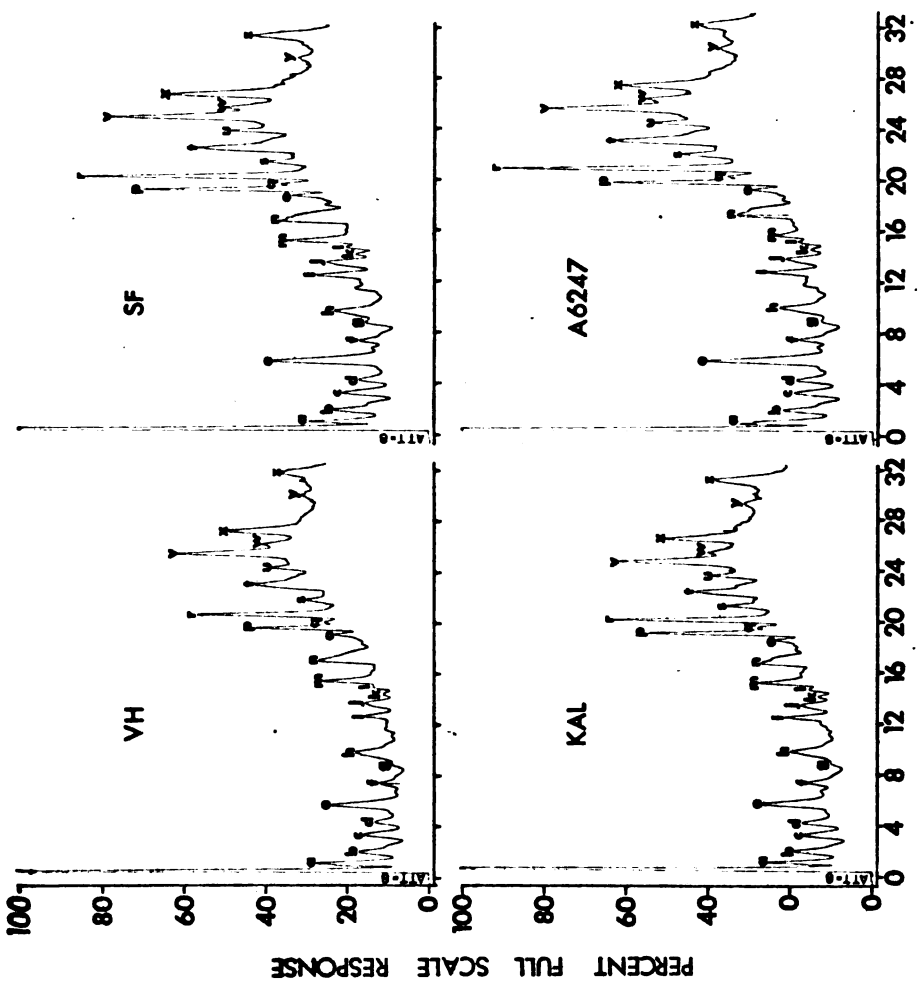

Figure 3. Pyrograms from vegetative cells of four strains of Clostridium botulinum type E grown in Multipeptone-sucrose-nutramino acids medium. Att is the attenuation.

Pyrograms of type E vegetative cells grown in Trypticase-peptone-sucrose-yeast extract medium (TPSY) showed no consistent differences among the 6 strains examined. There were quantitative differences in peaks k, n, r, and s, but identification on this basis was not possible. These quantitative differences might have been caused by variability in the growth rate of the cultures. This seemed quite possible upon examination of Table 1. Strains SF and 517 gave higher direct microscopic counts and higher concentrations of eluted material in peaks k and s. Peak n was also different for these two strains than for the others examined.

No differences were observed in pyrograms obtained from 5 strains of type E grown in Multipeptone sucrose-nutramino acids medium (MSN). Upon initial examination of the pyrograms in Figure 3 and part B of Figure 4, there appeared to be differences in the relative proportion of peaks p and r to peak v. This was apparently caused by increased drifting in the baseline between 16 and 22 min retention time. The pyrolysis of a possibly greater amount of sample with strains SF and A6247 might have also added to this effect. These phenomena were discussed earlier in the section concerning effects of sample size.

Vegetative cells of types A and B grown in TPSY medium gave definite differences in pyrograms from those

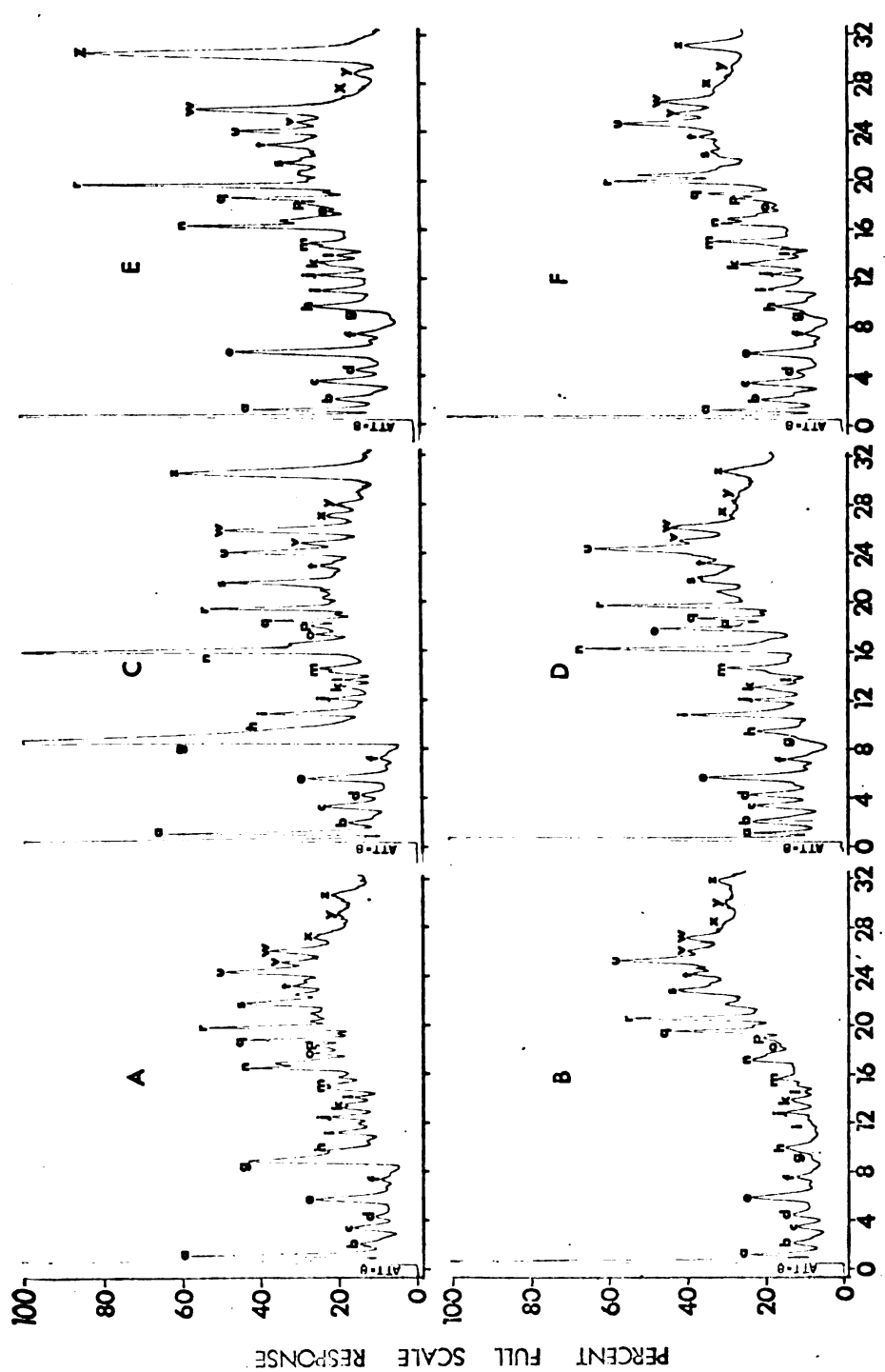

Figure 4. Comparison of pyrograms from Clostridium botulinum A) spores and B) vegetative cells of type E grown in Multipeptone-sucrose-nutramino acids medium; C) spores and D) vegetative cells of type A grown in Trypticase-peptone-sucrose-yeast extract medium; and E) spores and F) vegetative cells of type E grown in Trypticase-peptone-sucrose-yeast extract medium.

TABLE 1.--Direct microscopic counts of vegetative cells of Clostridium botulinum types A, B, and E grown in two different media.

Type	Strain	Total Count (organisms/ml)	
		MSN	TPSY
A	62	--	8.3×10^8
A	78	--	7.3×10^8
B	169	--	5.9×10^8
B	213	--	1.6×10^8
E	VH	2.4×10^8	5.0×10^8
E	KAL	1.2×10^8	7.5×10^8
E	SF	1.0×10^8	12.0×10^8
E	517	1.1×10^8	15.0×10^8
E	A6247	0.6×10^8	7.5×10^8
E	066BNT	--	5.5×10^8

of type E grown in the same medium. Parts D and F of Figure 4 illustrate the differences in representative strains of type A and type E. Since pyrograms of type A and type B were somewhat alike similar differences exist between types A and B compared to type E. Considerable variations were noted in peaks a through e while peaks i and n were much larger in type A than in type E. An additional peak which was not present in type A and type B appeared between peaks r and s in type E.

Reiner (1967) and Reiner and Ewing (1968) observed differences at the sub-species level in pyrograms of microorganisms including strains of Escherichia coli which differed only in their flagellar antigens. Their results, however, were based on years of research and only slight differences in one or two peaks.

The fact that few differences were found at the strain level in this study was difficult to explain. It appeared, however, that the use of the different column material from that used by the other workers might have been a possible answer. On the other hand, identification of C. botulinum at the strain level by PGLC may be more difficult than with other organisms previously tested. A difference in one peak however, was noted in strain 62A, and there were definite variations at the type level which made identification possible on this basis.

Analysis of Spores

Elution patterns obtained upon PGLC analysis of spores of C. botulinum types A and B grown in TPSY medium are shown in Figure 5. Strain 78A was readily distinguishable from other strains of type A and type B examined by the large quantity of material eluted in peaks g, i, and s. Once again, however, these differences appeared to be more quantitative than real since sporulation of strain 78A (Table 2) was much better than

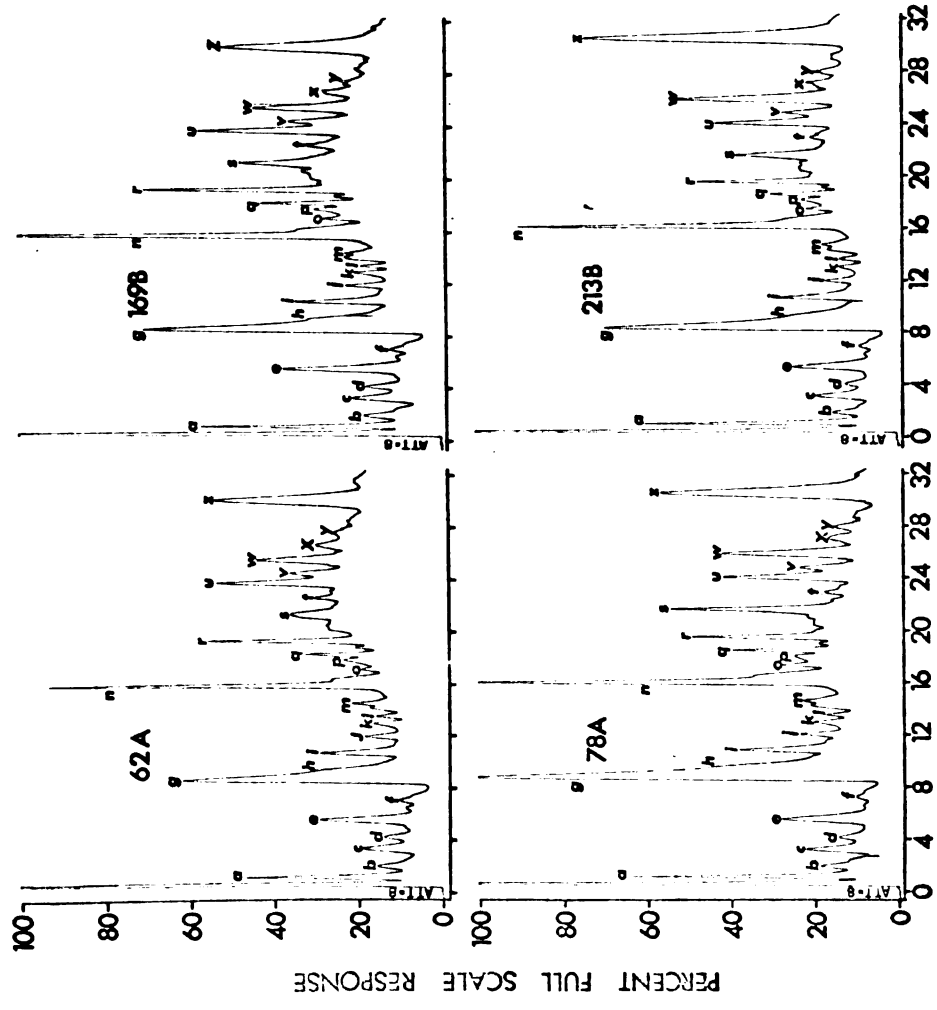

Figure 5. Pyrograms from spores of four strains of Clostridium botulinum types A and B grown in Trypticase-peptone-sucrose-yeast extract medium. The capital letters denote the type and Att is the attenuation.

TABLE 2.--Direct microscopic counts and per cent sporulation of cultures of *Clostridium botulinum* types A, B, and E grown in two different media.

Type	Strain	Culture Medium			
		MSN		TPSY	
		Total Count (organisms/ml)	Sporulation (%)	Total Count (organisms/ml)	Sporulation (%)
A	62	--	--	12.0×10^8	54.8
A	78	--	--	9.5×10^8	94.8
B	169	--	--	9.0×10^8	65.6
B	213	--	--	9.5×10^8	85.4
E	VH	3.8×10^8	76.3	5.2×10^8	93.5
E	KAL	2.2×10^8	83.2	5.8×10^8	91.3
E	SF	4.3×10^8	98.6	5.5×10^8	89.4
E	517	2.0×10^8	91.3	2.4×10^8	84.3
E	A6247	3.3×10^8	72.5	5.8×10^8	75.6
E	066BNT	--	--	4.9×10^8	78.4

sporulation for the other strains of type A and type B. Peaks w and z were often higher in strain 213B than in other strains, but this was not always consistent. In general, no differences were consistent and dependable enough to be used for identification purposes. Even strain 62A which had shown some differences at the vegetative cell stage did not produce a significantly different spore pyrogram.

Figure 6 illustrates the pyrograms for spores of type E grown in TPSY medium. With the exception of differences in the quantity of sample pyrolyzed, all of the elution patterns appeared to be the same. Strain 517 did show a smaller concentration of some components, but this was probably caused by poor growth since Table 2 indicates that the total count of this strain was considerably less than counts for the other strains. The differences in peak z also failed to be sufficiently consistent among duplicate and replicates of the same culture to be used for identification purposes.

Elution patterns of type E spores grown in MSN medium provided some rather interesting results (Figure 7). Strain VH was readily differentiated from all other type E strains examined by the tremendous size of peak n. In addition, peak e was slightly smaller in VH and an additional peak was present between peaks a' and b'. The additional peak appeared to have been brought about by

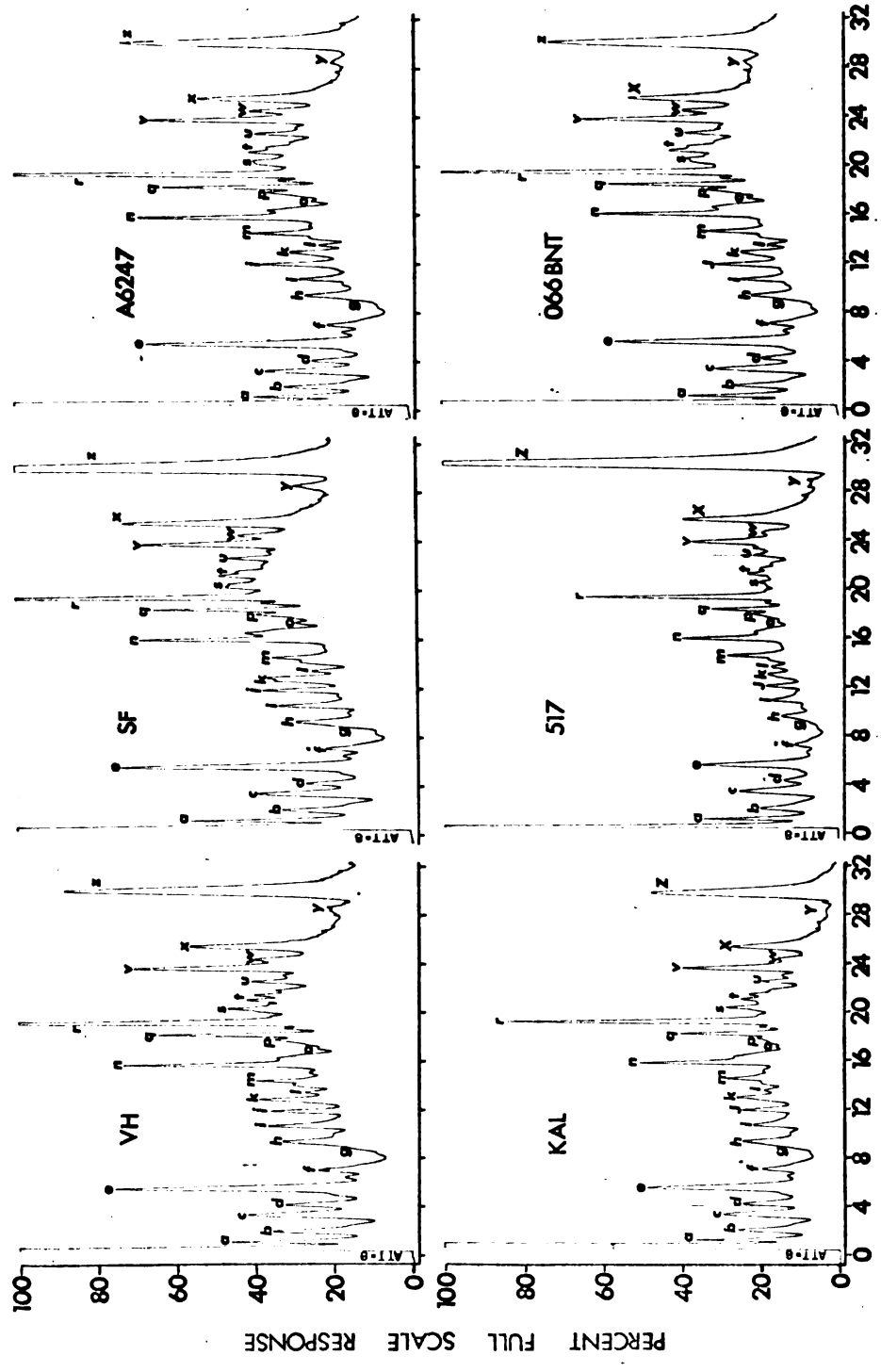

Figure 6. Pyrograms from spores of six strains of Clostridium botulinum type E grown in Trypticase-peptone-sucrose-yeast extract medium. Att is the attenuation.

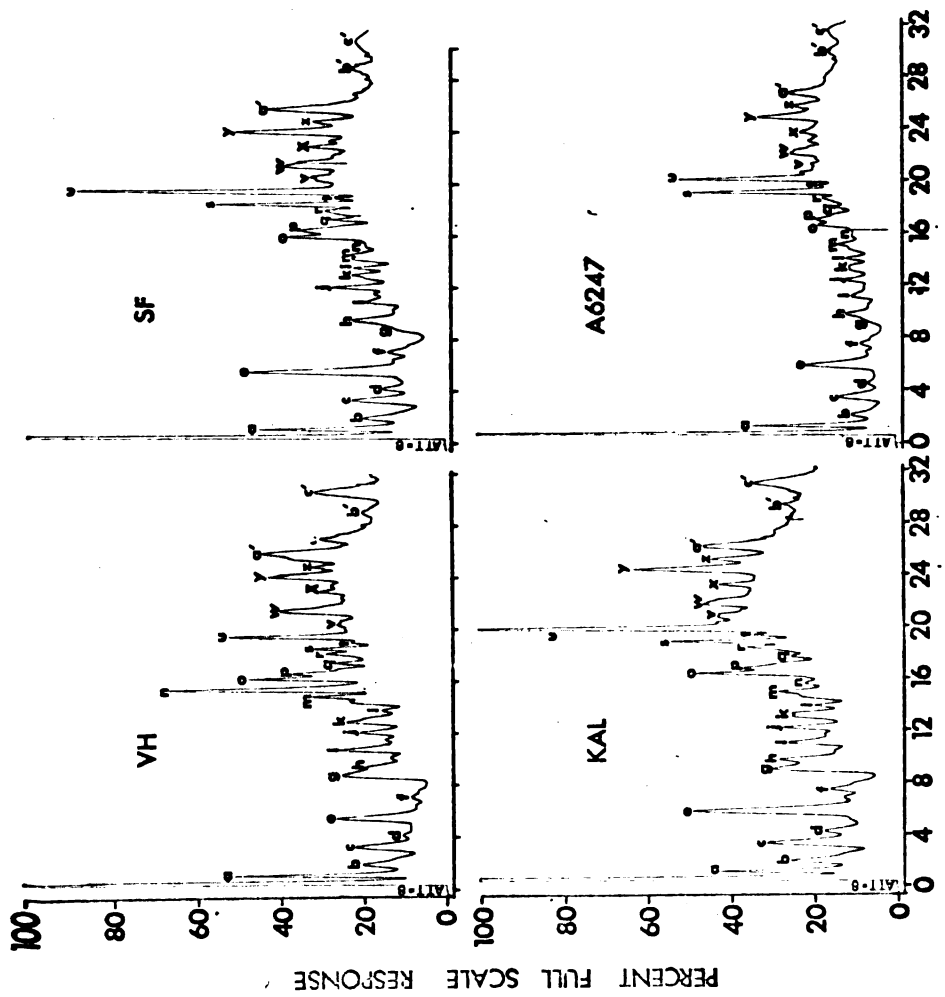

Figure 7. Pyrograms from spores of four strains of Clostridium botulinum type E grown in Multipeptone-sucrose-nutramino acids medium. Att is the attenuation.

differences in the retention time of peaks eluted after 24 min. Strain A6247 first appeared to be different from the other type E strains grown in MSN, but closer investigation revealed that this was caused by the pyrolysis of a smaller amount of sample for this strain. Variations in peaks g and h were initially thought to be caused by differences in growth and sporulation. Examination of the data in Table 2, however, failed to indicate any correlation between these peaks and growth or sporulation. None of the differences, with the exception of those in strain VH, were useful for identification purposes. No attempt was made to determine why strain VH gave a characteristic elution pattern.

A comparison of pyrograms for spores of representative cultures of type A and type E grown in TPSY medium is provided in parts C and E of Figure 4. Distinct and consistent differences occurred in peaks g, h, i, n, r, s, x, and z. These peaks permitted easy differentiation of type A spores and thus type B spores from spores of type E.

No studies have previously been undertaken to differentiate strains and types of cultures by pyrolysis of their spores. Differences in elution patterns, however, were expected since these spores were formed from the same vegetative cells in which differences were observed. Once again major differences were found at the type level while only a few differences were noted among strains of the same type.

Comparison of Spores and Vegetative Cells

Pyrograms of spores and vegetative cells of representative strains of C. botulinum types A and E are shown in Figure 4. Consistent differences were observed between spores and cells of each type (type B being similar to type A) of C. botulinum investigated. Differences which were common among all types included those in peaks g, n, and z. Types A and B showed additional differences in peaks a through d and peaks w, x, and y. Vegetative cells of all types also had a larger peak between peaks r and s.

Since spores have been shown to be physiologically different from vegetative cells (Murrell, 1967), differences in their pyrograms were expected. The presence of such materials as calcium and dipicolinic acid in spores as well as lysis of the sporangia and formation of new coat layers in spores, made them completely different bodies than the cells from which they were formed.

The presence of spores in cultures may strongly influence the pyrograms obtained and thus prove to be a limiting factor in the use of pyrolysis for the identification of those microorganisms which form spores. With the exception of strain 78A, however, the sporulation data in Table 2 failed to indicate any correlation between per cent sporulation and elution patterns. A

closer quantitative examination of those peaks related to sporulation might have revealed some correlation in this respect. Peaks g, n, and z might have been particularly useful in this respect.

Since lysis and other physiological changes besides sporulation occurred in the cultures as they grew older, apparent differences in spores and cells might also be partially attributed to the age of the culture. The data collected in this experiment failed to either support or contradict this theory since spores and cells of each strain were harvested at only one time.

Effect of Medium

The type of medium in which the cultures were grown was found to have a pronounced effect on the pyrograms obtained for both vegetative cells and spores of *C. botulinum* type E. Since types A and B would not grow in MSN medium, only type E strains were used for this study.

Differences in elution patterns of vegetative cells grown in MSN and TPSY media are shown in parts B and F of Figure 4. Distinct quantitative differences, always characteristic for their particular medium, were noted in peaks c, k, m, n, q, s, and z. Peak i was also absent in the MSN pyrograms while there was a larger additional peak between peaks r and s in the TPSY programs.

Parts A and E of Figure 4 illustrate the differences in elution patterns for spores grown in MSN and TPSY media respectively. Several characteristic differences were noted in the concentrations of peaks a, e, g, n, r, w, and z. Peak x was absent in spores grown in TPSY and peak z was much larger in spores from TPSY.

Oyama and Carle (1967) have also indicated that the growth medium had an influence on the pyrograms which they obtained when they grew Candida pulcherrima in malt extract and in trypticase soy broth. They further observed that organisms grown in similar media gave similar pyrograms.

The two media used in this experiment showed definite similarities as well as definite differences in pyrograms for the same culture. Since both media are similar in that they are rich in nitrogen, this might account for many of the similarities. The differences were probably caused by the presence of yeast extract as well as varying concentrations of other nitrogen-rich materials.

Detection of Growth and Toxin Production

An attempt was made to detect growth and toxin production of various types and strains of C. botulinum using a modification of the dialysis sac technique described by Vinet and Fredette (1951). Since the majority of the toxin should have been present in the supernatant fluid after growth and centrifugation of the

cultures, it was proposed that this toxin might be detectable by PGLC. The dialysis sac technique was used to limit and control the availability of nutrients to the cultures, thus preventing possible interference of these compounds with the pyrograms subsequently obtained. This was discussed in the previous section of the Results and Discussion concerning the effect of the medium on pyrograms.

No retention value with respect to molecular size has been provided for dialysis tubing, therefore, it was not possible to accurately predict what nutrients were available to the growing cultures except that they had to be less than approximately 30,000 M.W. Since the nutrients were limited, some of the cultures failed to grow and sporulate as readily as others (Table 3). Also, many of the vegetative cells lysed because of the prolonged period of incubation for 5 days. Microorganisms which were harvested were thus not defined as either spores of cells. In fact, the per cent sporulation data in Table 3 is questionable since many of the vegetative cells had lysed and thus the data actually represents apparent sporulation at the end of incubation.

The pyrograms obtained upon PGLC analysis of these organisms are shown in Figures 8 and 9. They illustrate the same type of differences as those found in regular TPSY and MSN media. Strains of type A and type B were

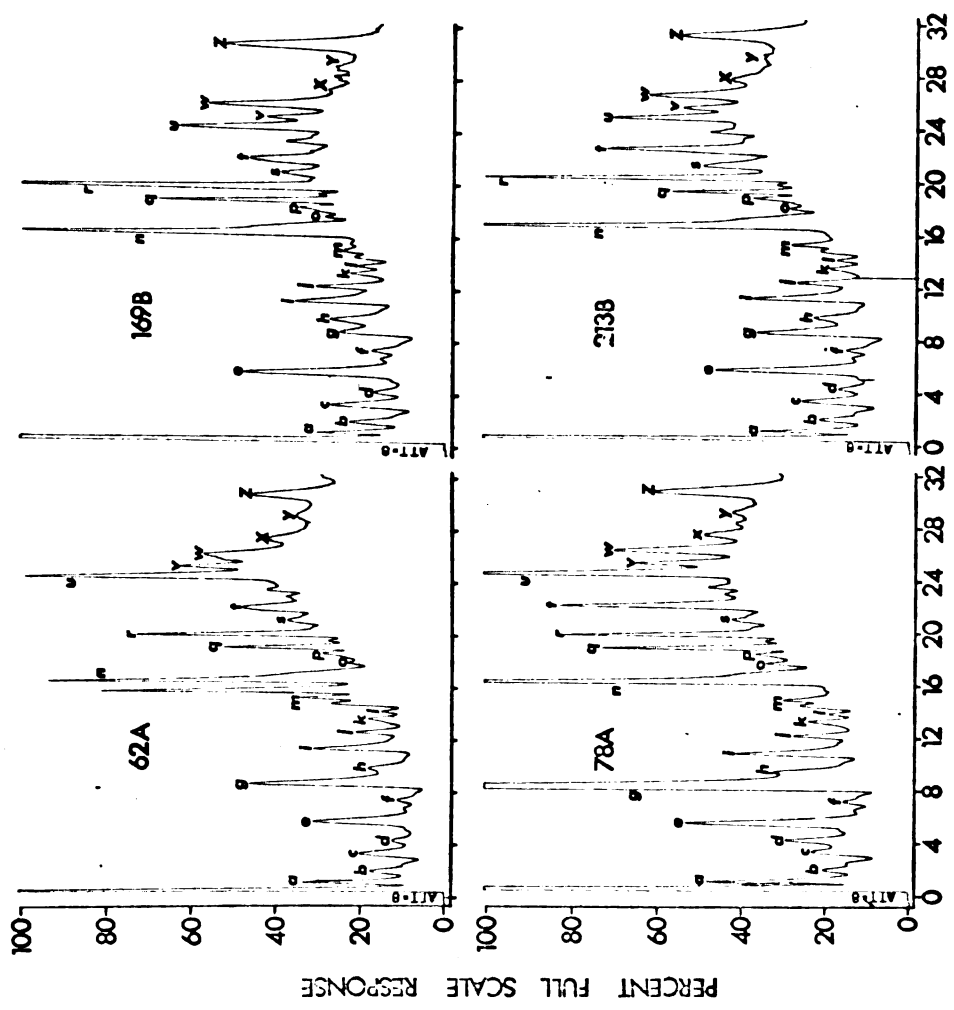

Figure 8. Pyrograms from cultures of four strains of Clostridium botulinum types A and B grown using the modified dialysis sac technique. The capital letters denote the type and Att is the attenuation.

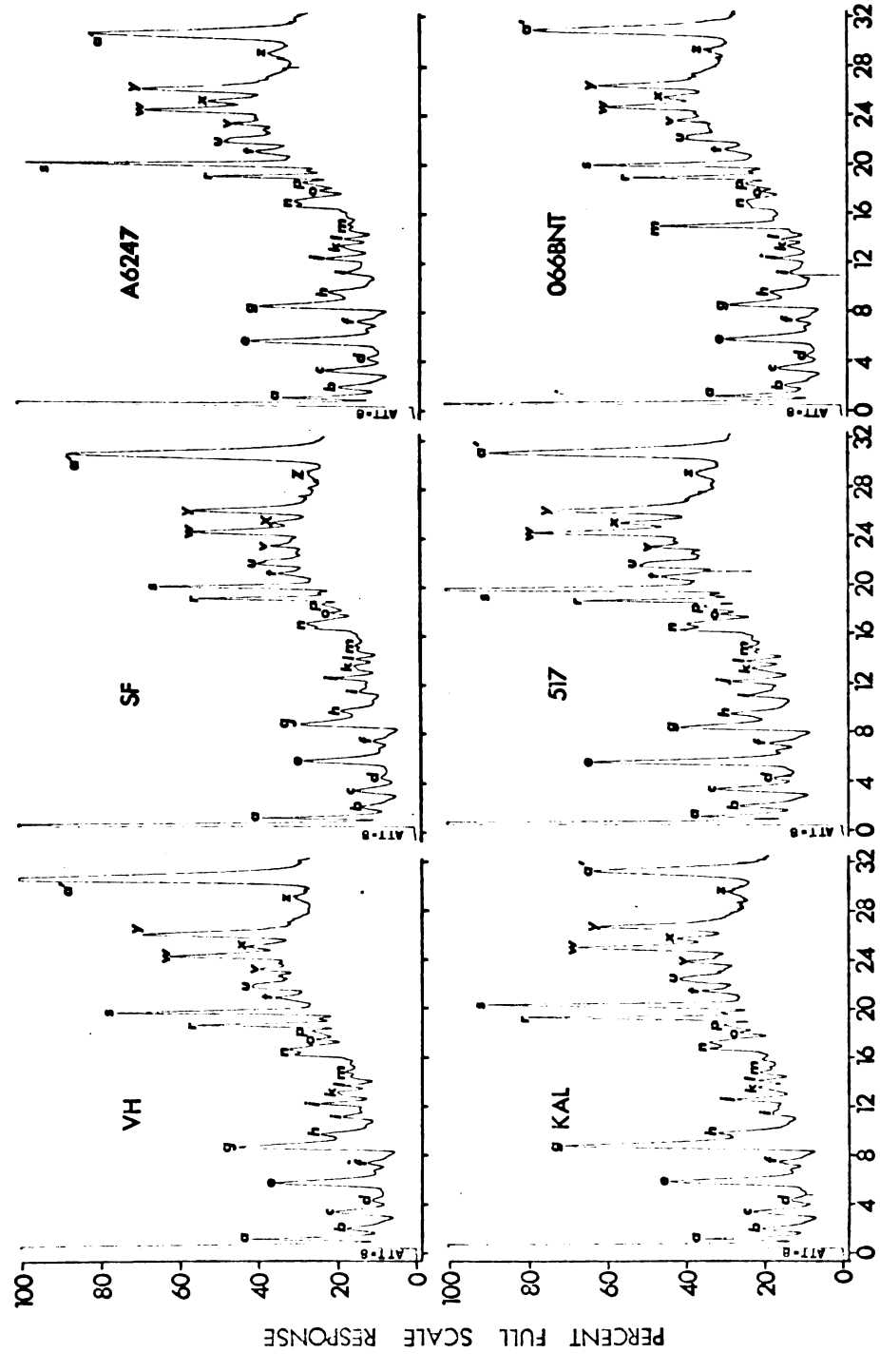

Figure 9. Pyrograms from cultures of six strains of Clostridium botulinum type E grown using the modified dialysis sac technique. Att is the attenuation.

TABLE 3.--Direct microscopic counts and per cent sporulation of strains of Clostridium botulinum types A, B, and E using the modified dialysis sac technique.

Type	Strain	Direct Count	% Sporulation
A	62	8.4×10^7	91.7
A	78	6.8×10^8	82.4
B	169	6.9×10^7	58.3
B	213	3.9×10^8	62.4
E	VH	1.5×10^8	84.8
E	KAL	4.6×10^8	80.8
E	SF	2.8×10^8	69.8
E	517	1.4×10^8	75.4
E	A6247	8.3×10^7	90.0
E	066BNT	1.4×10^8	92.4

slightly different in peaks g and u. Strain 62A produced an additional characteristic peak between peaks m and n. Elution patterns for strains of type E were identical with the exception of slight differences in peaks e and m. Because the cultures were grown under such "nonideal" conditions, these differences were not reliable enough to be used for identification purposes.

Elution patterns obtained by PGLC analysis of the dialysate supernatant fluid (DSF) of cultures of C. botulinum are illustrated in Figure 10. The DSF of strain

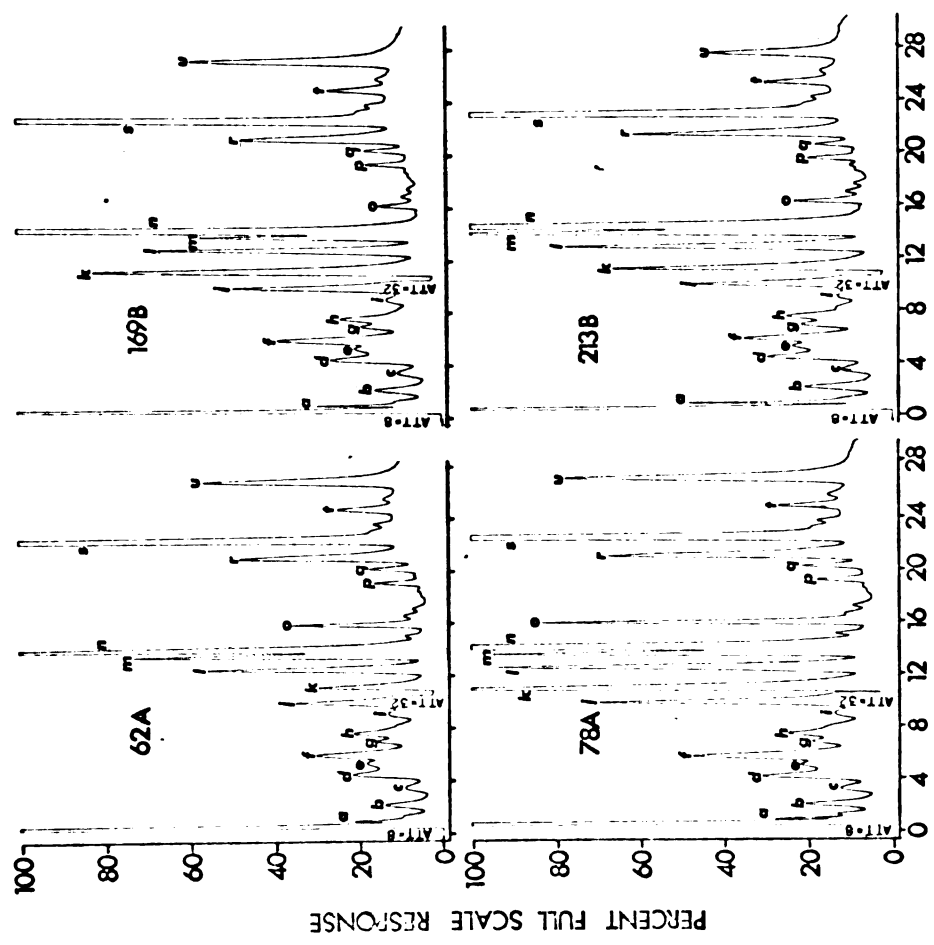

Figure 10. Pyrograms of the dialysate supernatant fluid remaining after centrifugation of four strains of Clostridium botulinum types A and B from the dialysate from the modified dialysis sac technique. Capital letters denote the type and Att is the attenuation.

78A was distinguished from that of other strains of type A and type B by the large peak k. Once again this difference might be quantitative since strain 78A was observed to grow better than the other strains (Table 3).

Differences in peak o of type A DSF and type B DSF were consistent and could not be correlated with growth rate. Type A could thus be distinguished from type B by this peak. Variation in peaks m and u, however, were not consistent enough for identification purposes.

Pyrograms from the DSF of type E strains are shown in Figure 11. They failed to indicate any differences in elution patterns which were consistent and significant enough for identification purposes of strains within the serotype E. The quantitative differences in peaks c, k, and m were probably caused by variability in the total amount of growth as well as lysis of many of the vegetative cells in some cultures.

The differences in pyrograms for the DSF of types A and B from those of type E DSF were quite distinct. Parts D, E and F of Figure 12 illustrate the differences in the DSF elution patterns of types E, A, and B respectively. These differences were probably caused by physiological differences between the three types. Since type A and type B were proteolytic, they produced and broke down different compounds than type E which was nonproteolytic and primarily saccharolytic with respect

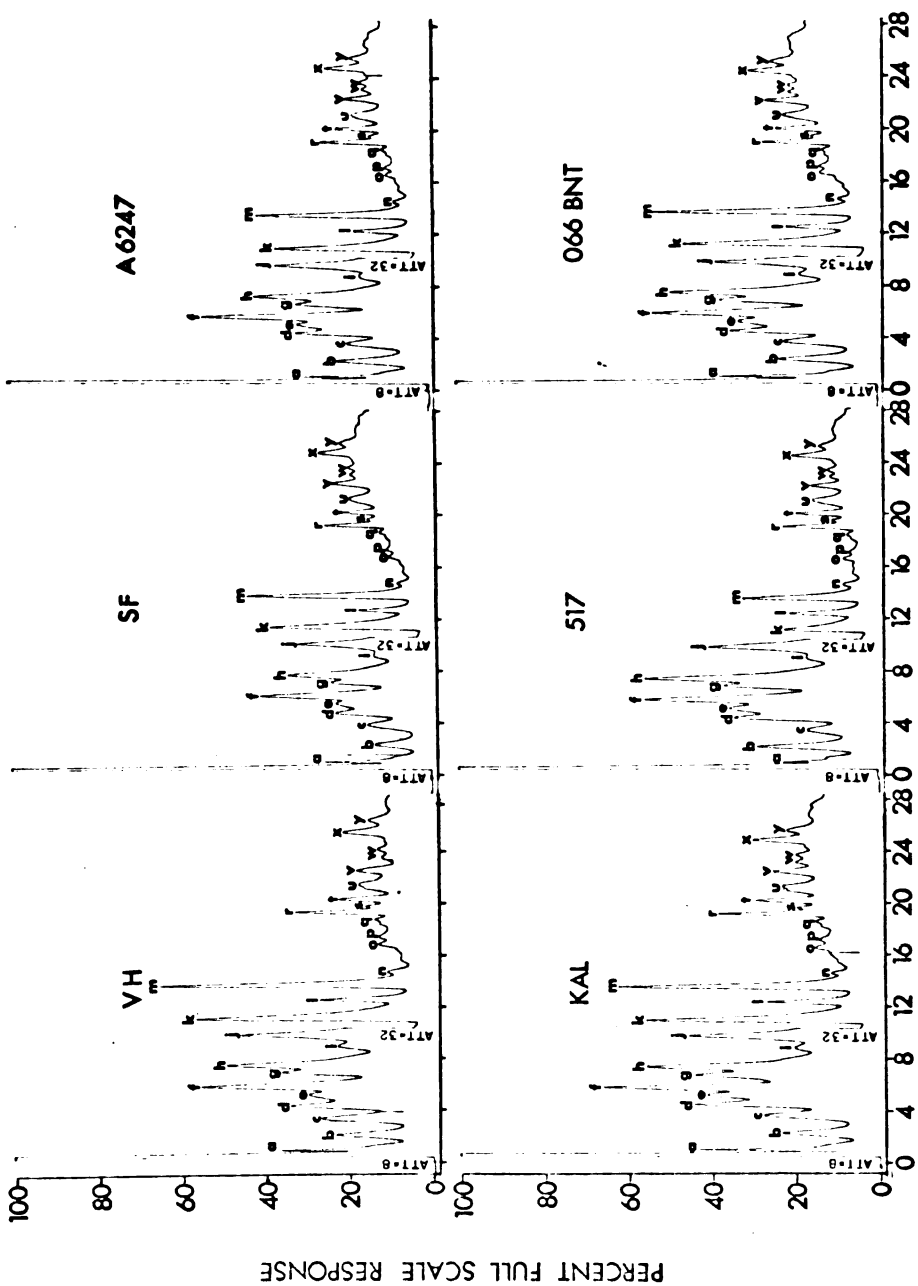

Figure 11. Pyrograms of the dialysate supernatant fluid remaining after centrifugation of six strains of Clostridium botulinum type E from the dialysate from the modified dialysis sac technique. Att is the attenuation.

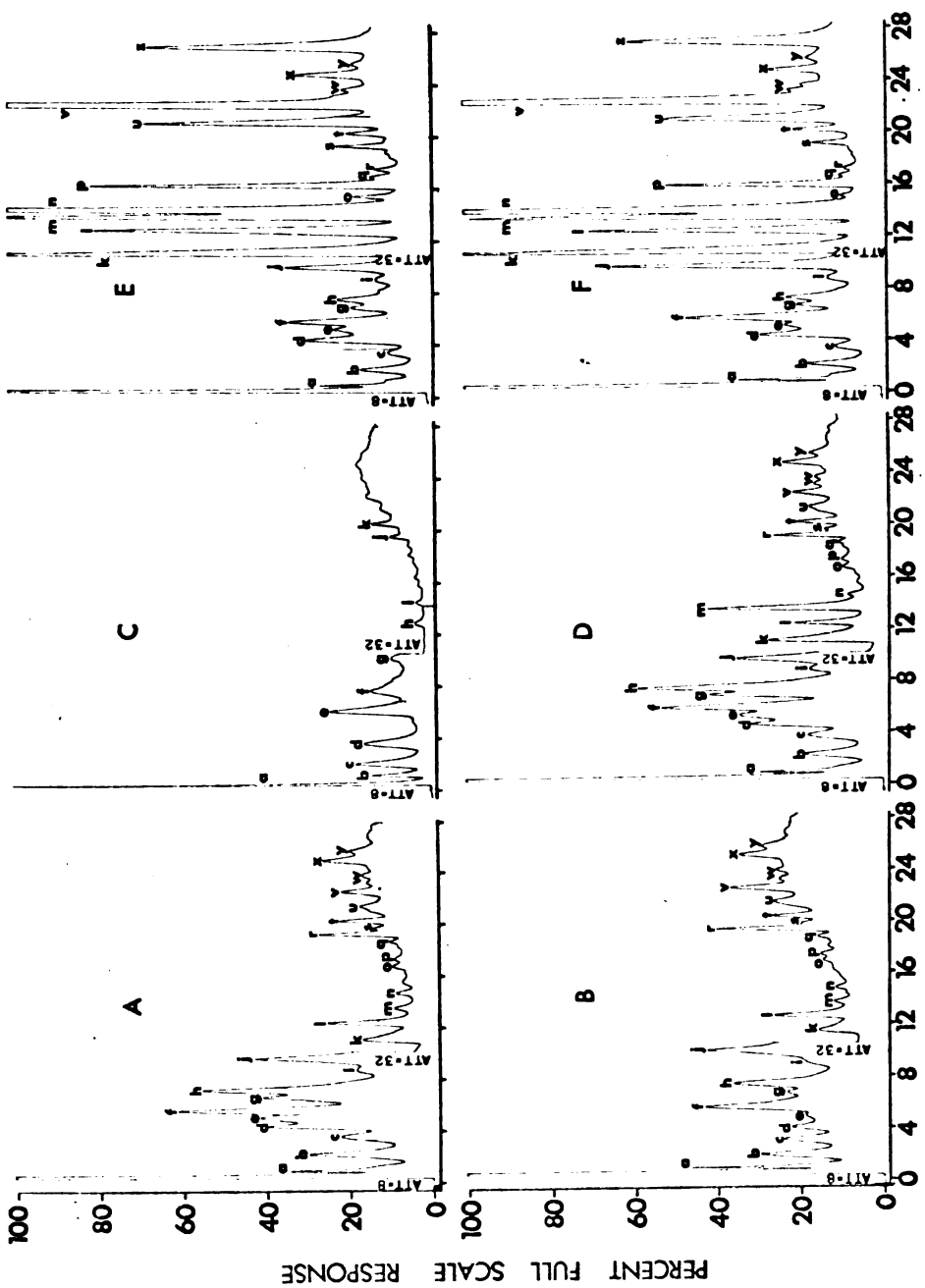

Figure 12. Comparison of pyrograms for samples consisting of A) uninoculated dialysate supernatant fluid (DSF); B) uninoculated DSF plus partially purified toxin; C) 1.0 mg of partially purified toxin; D) DSF of type E Clostridium botulinum; E) DSF of type A Clostridium botulinum; and F) DSF of type B Clostridium botulinum.

to physiology. Types A and B have also been shown to be markedly less saccharolytic than type E. These physiological differences were described by Dolman (1964).

Several types of control samples were analyzed to evaluate the feasibility of using PGLC to detect growth and toxin production. The elution pattern of the first control which consisted of an uninoculated sample of the DSF is shown in part A of Figure 12. The pyrogram was identical to the one for the DSF of type E (part D, Figure 12) with the exception that peaks k and m were considerably smaller in the control. The patterns of type A and type B were of course quite different. This indicated that growth of C. botulinum could be detected in a dialyzed medium.

The feasibility of toxin detection by PGLC was determined by the addition of a partially purified toxin of C. botulinum type E strain VH to uninoculated DSF giving a final toxin titer about equal to that of the actual cultures (Table 4). A 1.0 mg sample of the toxin was also subjected to PGLC analysis.. These results are shown in parts B and C of Figure 12.

Essentially all of the pyrolytic products of the toxin were eluted in small peaks between 0 and 15 min retention time. Since the amount of toxin actually present under normal conditions would only be a fraction of that used for this analysis, it appeared that detection

of the actual toxin in the growth medium was impossible. The toxin peaks were hidden by peaks from other pyrolytic products.

TABLE 4.--Toxin titers of various strains of *Clostridium botulinum* types A, B, and E grown in duplicate in trypticase-peptone-sucrose-yeast extract medium.

Type	Strain	Toxin Titer (MLD/ml)	
		1	2
A	62	2.0×10^5	2.0×10^5
A	78	4.0×10^4	4.0×10^4
B	169	1.0×10^2	4.0×10^2
B	213	4.0×10^2	4.0×10^2
E	VH	2.0×10^4	1.0×10^5
E	KAL	1.0×10^5	2.0×10^5
E	SF	2.0×10^3	2.0×10^3
E	517	1.0×10^3	1.0×10^3
E	A6247	1.0×10^5	4.0×10^4
E	066BNT	not toxic	not toxic

The fact that some of the peaks in the DSF plus toxin control were different than those in the uninoculated DSF control was probably caused by the nature as well as the amount of toxin which was added. Only those peaks in the first 11 min of retention time were changed,

further indicating that this was the area in which toxin was eluted but not readily detected under these experimental conditions.

SUMMARY AND CONCLUSIONS

Experiments were conducted to determine the feasibility of using pyrolysis-gas-liquid chromatography (PGLC) for the identification of various types and strains of Clostridium botulinum. The use of this technique for detecting both growth and toxin production by the same microorganisms was also investigated.

Vegetative cells (10 hr incubation at 32 C) and spores (36 hr incubation at 32 C) of 10 strains representing types A, B, and E C. botulinum were grown in Trypticase-peptone-sucrose-yeast extract medium (TPSY) and the resulting cultures analyzed by PGLC. Vegetative cells and spores of five strains of type E were also harvested from Multipeptone-sucrose-nutramino acids medium (MSN) and analyzed in the same manner as those grown in TPSY.

Examination of pyrograms for vegetative cells revealed differences in elution patterns which were of sufficient significance to permit differentiation at the type level. Pyrograms of types A and B indicated only slight but significant differences from each other. The pyrograms of type A and B, however, were both quite different from those of type E strains. The only consistent difference at the strain level occurred in strain 62 of

type A. It was readily differentiated from other strains by the size of one of its peaks. Several other slight differences occurred in elution patterns of pyrograms of type E vegetative cells grown in both media, but these differences appeared to be caused by changes in the amount of sample pyrolyzed or variations in growth and sporulation of the cultures. No differences suitable for identification purposes were observed in pyrograms of type E strains grown in either TPSY or MSN media.

Only differences at the type level were detected among pyrograms of the three types of spores grown in TPSY. Other variations were believed to be related to the size of the sample pyrolyzed or differences in growth and percentage of sporulation. In any event, these differences at the strain level were not sufficiently reliable for identification purposes.

Spores of type E grown in MSN did show consistent differences at the strain as well as the type level. Strain VH was easily differentiated from the other strains of type E.

Pyrograms of spores were different than those of vegetative cells for all strains studied. This may prove to be a limiting factor in the use of PGLC for the identification of microorganisms which form spores, since the pyrogram appeared to be dependent on the presence or absence of spores. The age of the culture might also be

related to these differences in spores and vegetative cells.

The effect of the growth medium on the elution patterns of pyrograms from 5 strains of type E was also observed. Significant differences between pyrograms from type E strains grown in MSN and TPSY media were easily detected in each of the strains examined. This indicated that the growth medium may be a limiting factor in the use of this technique for identification of C. botulinum as well as other microorganisms.

A modification of the dialysis sac technique described by Vinet and Fredette (1951) was used to determine the feasibility of detecting growth and toxin production by PGLC.

The inoculum was added outside the dialysis sac and nutrients were provided by dialysis across the membrane from inside the sac. Pyrograms of the cultures harvested from the growth medium after 5 days incubation at 32 C revealed only the same type of differences in elution patterns as those observed for the subcultures grown in normal flask cultures in MSN and TPSY media. The resulting pyrograms were difficult to evaluate since the unusual conditions under which the cultures were grown made it practically impossible to obtain a synchronous relationship between cultures with respect to growth and sporulation. Lysis of vegetative cells caused by

prolonged incubation of cultures might also have contributed to the variability. Reliable differences in pyrograms once again occurred at the type level but not at the strain level for the modified dialysis sac grown cultures.

Comparison of pyrograms obtained from samples of the dialysate supernatant fluid (DSF) with those obtained from three control samples that consisted of (1) uninoculated DSF, (2) uninoculated DSF plus partially purified toxin, and (3) a 1.0 mg sample of partially purified toxin revealed that PGLC could be used to detect growth of C. botulinum in this dialyzed medium. It was even possible to determine which type of C. botulinum had been grown in the medium. Detection of toxin production, however, was impossible since the toxin was present in such small quantities with respect to other compounds that its pyrolytic products were hidden among peaks from other pyrolytic products of the culture medium.

In general, PGLC appeared to show promise as an analytical tool for identification of C. botulinum at the type level. The investigation of other column materials and other operating parameters, however, is necessary to determine the usefulness of this technique at the strain level since this study revealed only a few reliable differences. Pyrolysis-gas-liquid chromatography may also be extremely valuable for detection of growth of

C. botulinum. Further studies must be conducted, however, to determine whether growth in actual food products can be detected.

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