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Disc gel electrophoresis of the vegatative cell-free extracts of strains of Clostridium botulinum types A, B, C, E, and F and the related nontoxic group showed limited value as a means for identifying these closely related microorganisms, since separation, though not consistent in all cases, could only be based on the number of protein fractions in the gel.

Enzyme staining of the protein-laden polyacrylamide gels of the strains showed single or multiple molecular forms for malic (NAD and NADP), isocitric (NAD), succinic (NAD), and lactic (NAD) dehydrogenases and alkaline phosphatase. Analyzing the enzyme patterns of the strains revealed that most of these enzyme systems are useful for distinguishing the types and the nontoxic strains.

A method which allowed two samples to be run in the same polyacrylamide gel showed that the differences between the total

protein patterns of two strains can be demonstrated clearly.

A type of iron bound protein (ferredoxin) was isolated from C. botulinum using a modification of the method recommended by L. E. Mortenson for isolating ferredoxin from Clostridium pasteurianum. The protein exhibited maximum absorption in the ultraviolet region near 260 mμ. Portions of the isolated iron bound protein were separated by disc electrophoresis, and following specific iron bound protein staining, showed a positive reaction in the same position in the gel column as first demonstrated using cell-free extract.

Evidence accumulated using cell-free extract of C. botulinum suggests that pyruvate is metabolized through a phosphoroclastic system as demonstrated in other clostridia. It is probable that the ferredoxin has the important role of electron mediator between pyruvic oxidase and hydrogenase for hydrogen evolution and acetyl phosphate formation.

A purposed system for the synthesis of aspartate and glutamate in C. botulinum incorporating the above enzymes including those of the phosphoroclastic system in a partial citric acid cycle and glyoxylate bypass was described.

A PHYSIOLOGICAL APPROACH TO A RAPID METHOD FOR IDENTIFYING *C. BOTULINUM*

by

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TABLE OF CONTENTS

LIST OF FIGURES

A PHYSIOLOGICAL APPROACH TO A RAPID METHOD FOR IDENTIFYING C. BOTULINUM

INTRODUCTION

A large volume of data has been published on the electrophoretic separation of total proteins and multiple molecular forms of enzymes. Much of this data is summarized in two symposia of the New York Academy of Sciences (Whipple, 1964; Wroblewski, 1961), in which most of the studies were carried out on enzyme systems and total protein of animal and plant tissues. The results suggested that the total protein and the enzyme patterns have taxonomic significance.

In bacteria, the electrophoretic separation of enzyme systems showed that multiple molecular enzyme forms can provide useful taxonomic data and expedite the identification of the Bacillus and Mycobacterium genera and some of the Gram negative asporogenous bacteria (Baillie and Norris, 1963; Cann and Willox, 1965; Norris and Burges, 1963). Such techniques were also employed to study mutationally altered proteins in Escherichia coli (Henning and Yanofsky, 1963). However, there is insufficient data available to show how the electrophoretic separation of protein and multiple molecular forms of enzymes may be generally useful in bacteria or at what taxonomic level the separation of groups of bacteria will occur. From the available data, this latter point will depend upon

which properties are selected for examination.

Identification and classification of Clostridium botulinum is now based on morphological, biochemical, and serological data. Comparative studies to determine the relative merits of the different methods are generally lacking. However, most present methods prove time consuming, expensive, or are frequently complicated by anomolous results (Slocum, 1964). Furthermore, investigators have encountered among the lipolytic clostridia many strains which are similar to one or other of the types of C. botulinum in every respect except for toxin production. Some investigators (Dolman, 1957; Hobbs, Roberts, and Walker, 1965) have suggested the existance of nontoxic C. botulinum mutants, which are indistinguishable from other nontoxic clostridia.

This study utilized disc gel electrophoresis to provide data for identifying rapidly the strains of C. botulinum types A, B, C, E, and F as well as the similar nontoxic strains. Malic, isocitric, succinic, and lactic dehydrogenases, and alkaline phosphatase showed multiple molecular forms as well as positional differences in the clostridial types. The differences were consistant for the strains, thus, offering a possible bases for identification.

Gels stained for the dehydrogenases showed clear areas suggesting oxidation of the reduced tetrazolium dye. Examination of these areas by a special staining technique showed a protein

containing iron. Previous investigations have shown the clostridia other than C. botulinum to contain ferredoxin (Valentine, 1964). This investigation was extended to isolate, identify, and compare the iron bound proteins (ferredoxins) from a proteolytic and a nonproteolytic type of C. botulinum as well as to examine their association, if any, with hydrogen evolution.

Finally, cell-free extracts of C. botulinum type E were analyzed for α-ketoglutaric dehydrogenase with the objective of clarifying questions concerning the existance of a classical citric acid cycle.

LITERATURE REVIEW

Botulism is a relatively rare neuroparalytic disease affecting both men and animals in many parts of the world. The disease generally results from the ingestion of a food containing toxic metabolites, which are produced by one of several known species of anaerobic clostridia, whose natural habitat is the soil (Dolman, 1964a).

The causative bacterium was isolated in 1897 by van Ermengem at Elleyelles, Belgium (as cited by Leighton, 1923) and named Bacillus botulinus. The term "botulinus" applied to a type of food poisoning caused by the ingestion of spoiled sausage (as cited by Jordon, 1917). Twenty years later, the Society of American Bacteriologist Committee on Classification of Bacterial Types recommended for this anaerobic bacillus the adoption of the generic term Clostridium, which included "pectridial as well as clostridial forms" (Winslow et al., 1917). So the name C. botulinum was acknowledged.

Frazier (1958) describes C. botulinum as a rod-shaped soil bacterium, saprophytic, spore-forming, gas-forming and strictly anaerobic. This species is subdivided into a number of types which differ from one another in that their toxins are immunologically different. Leuchs (as cited by Reddish, 1921), using horse antiserum, showed that two cultures of C. botulinum (one of them van

Ermengem's strain and the other one isolated by Landmann at Darmstadt) were morphologically and culturally similar, but the toxins produced were immunologically distinct (Reddish, 1921). Although these strains were designated as E (Ellezelles) and D (Darmstadt), they were subsequently reclassified (Gunnison and Meyer, 1929) and generally accepted as a nonproteolytic type B and an A strain, respectively (Tanner and Tanner, 1953).

Clostridium botulinum types A and B are frequent causes of human botulism and also responsible for limber-neck of chickens. Type A is found predominantly in the western part of the United States and is known to be more toxic than type B. Type B is widely distributed in most soils of the world.

During the 1920's there was a growing awareness of various forms of animal botulism as well as a growing recognition of new types of C. botulinum. Bengston (1922) isolated a type C strain from larvae of the greenfly (Lucila caesar), and Theiler in 1927 reported the isolation of a new type of clostridia, which was designated by Meyer and Gunnison (1928) as type D.

These two types were shown to affect wildfowl, cattle, horses, mink, and other animal species (Tanner, 1933; Dack, 1953). Their toxins have caused considerable animal loss in many parts of the world (Dolman and Murakami, 1961; Foster et al., 1965).

In 1934, Kurochkin and Emelyanchik reported three outbreaks

of human botulism near the Caspian Sea due to salted meat (as cited by Dolman and Murakami, 1961). Although the implicated organism resembled nonproteolytic C. botulinum, the toxin produced was unneutralized by any known type of bolulinum antitoxin. At the same time, a number of similar strains, isolated from sturgeon caught in the Sea of Azar (Dolman and Chang, 1953) were examined by Gunnison, Cummings, and Meyer (1935), who proposed the classification of these strains into a new type which they designated type E.

Type E differs from the other C. botulinum in habitat, isolated mainly from sea muds and fish intestines (Dolman et al., 1950; Nakamura et al., 1956); in the ability to grow and produce toxin at low temperatures (Dolman, 1957b; Johannsen, 1965); and in the thermolability of the toxin (Pedersen, 1955; Roberts and Ingram, 1965). The sources of over 50 known type E outbreaks reported in various countries since 1935, have invariably been raw or inadequately cooked products of fish and sea mammals (Dolman and Chang, 1953; Pederson, 1955; Nakamura, 1963; Kanzawa, 1963). Type E epidemiology and incidence are practically unalterable since the distribution of botulinum spores are in regional soil and off shore waters (Kanzawa, 1963; Foster et al., 1965), and are changed with difficulty over a period of many years by the dietetic and culinary habits of the local populations (Dolman and Iida, 1963; Kawabata and Sakaguchi, 1963; Nakamura et al., 1956).

A type F strain was isolated in 1960 by Moeller and Scherbel and confirmed later by Dolman and Murakami (1961). A second strain of type F was isolated from fish by Craig and Pilcher (1965). Type F spores are very sparsely distributed in nature, and because of this, they have been isolated only recently (Dolman, 1964b).

The types of C. botulinum are not biochemically or culturally distinguishable; however, as a group, they may be divided into two biochemical types, the one proteolytic and the other saccharolytic or fermentative in character, whose members do not hydrolyze coagulated native proteins.

Types A and B are regarded generally as being proteolytic; although a few strains of type B are known to be nonproteolytic (Frazier, 1958). Types C, D, and E are nonproteolytic and type F varies (Dolman, 1964b).

Identification

Although demonstration of the specific toxin enrichment culture filtrates constitutes valid proof of the presence of C. botulinum, isolation of pure cultures and identification thereof is the final and complete proof usually sought by investigators. Procedures for detection, isolation, and identification of these microorganisms, which ultimately incorporate morphological, biochemical, and serological data, have been described by various investigators; though

similar, these vary in detail. Comparative studies to determine the relative merits of the different methods are generally lacking. However, they do suffer from several inherent inadequacies. First, in many materials C. botulinum is present in relatively small numbers requiring the use of enrichment methods for detection. However, these methods generally enhance the growth of all anaerobic bacteria, many of which grow more rapidly than C. botulinum, and some of which may either prevent the outgrowth of the various types of botulinum or destroy the toxin (Slocum, 1964). Second, among the lipolytic clostridia, recent work has shown that there is a whole group of clostridia in addition to Clostridium sporogenes, which are similar to one or the other of the C. botulinum types in every respect except for their lack of toxin production. The taxonomy of these organisms is not clear at present. Moreover, these nontoxic clostridia appear to be much more widespread than the toxic C. botulinum.

Dolman (1957a) described a number of mutants of C. botulinum type E, which no longer produced toxin. Hobbs, Roberts, and Walker (1965) investigated strains of Dolman's OS variants and pointed out that these organisms differed in so many ways from C. botulinum that they were unlikely to be genetic mutants. They suggested, however, that if nontoxic mutants of C. botulinum were widespread, they were more likely to be the nontoxic clostridia which

have identical morphological and biochemical characteristics.

Attempts to discover a differential medium for the isolation and differentiation of clostridial organisms from each other as well as from other anaerobic organisms have mainly involved the use of egg yolk in various types of media (Slocum, 1964).

Willis and Hobbs (1958) described a modified egg yolk medium, which showed promise for the selective isolation of the clostridia. Using this medium, they were able to differentiate Clostridium welchii, Clostridium oedematiens, C. sporogenes, C. botulinum, Clostridium bifermentans, and Clostridium sordellii on the basis of their reaction. Willis and Hobbs (1959) found that with the addition of lactose and an acid indicator to the medium described by McClung and Toabe (1947), a presumptive diagnosis of pure cultures of all Nagler-positive clostridia was possible within 48 hours. A comparison of a number of media for the detection of saccharolytic and proteolytic properties of clostridia showed that the glucose-gelatin and lactose-egg yolk milk agar were the most useful for preliminary examination of pure cultures of clostridia, since together, they were able to provide a great deal of information rapidly and in an easy detectable form (Willis, 1959). Neomycin also was added for the selective isolation of pathogenic type clostridia. However, though the production of the pearly layer and opalescence on egg yolk type medium can be used for the isolation of C. botulinum types, the

specific types (A, B, and E) can not be conclusively differentiated unless the source of the organism is known and a toxicity test is performed (Willis, 1960).

Mouse protection tests, which are presently used extensively for identification purposes, are time consuming, expensive, and frequently complicated by anomalous results (Slocum, 1964).

Batty and Walker (1963) showed that the fluorescent antibody technique can be used to detect Clostridium septicum and Clostridium chauveoi in direct smears from pathological specimens. These closely related species are often found together in such specimens. Recently, fluorescent antisera prepared against heat-killed vegetative cells showed promise as a rapid method for the differentiation of strains of C. botulinum as demonstrated by Walker and Batty (1964a, b). They were able to differentiate between three important groups - the proteolytic A, B, and F, the nonproteolytic E, and the nonproteolytic C and D. However, in practice it was found that these antisera also stained some of the nontoxic clostridia.

Metabolism

Most of the early work included measurements and indentification of by-products resulting from fermentation activities and utilization of amino acids.

Dozier et al. (1924) working with Bacillus botulinus strain 38

observed that glucose increased the biological value of a nitrogen-rich, buffered medium by stimulating early reproduction, increasing the maximum number of viable organisms, and maintaining a higher level of growth throughout the experimental period. In addition, glucose increased the total amount of gas formed by approximately 33 percent.

Wagner et al. (1925) observed a considerable accumulation of ammonia, amino acids, and volatile acids in two percent peptone cultures of C. botulinum, the volatile acids being identified as a mixture of valeric, butyric, and acetic acids. Decreased ammonia production and the development of an acid reaction were observed in the glucose broth cultures.

Anderson (1924) reported that the gaseous products from a peptone broth culture included a mixture of carbon dioxide, hydrogen, and hydrogen sulfide. A CO_2/H_2 ratio of 18.3 was observed in two percent peptone cultures, while ratios of 5.7 and 3.8 were observed in broth cultures containing one percent formate or glucose.

Knight (1936) stated that the simplest medium which would support the growth of C. botulinum contained glucose and the amino acids, proline, glycine, leucine, alanine, lysine, and cystine together with traces of tryptophan and of the "sporogenes" growth factor. He suggested that C. botulinum may obtain its energy by means of the "Stickland reaction", a coupled oxidation-reduction

reaction between pairs of different amino acids. This was verified by Clifton (1939), who found that glycine and proline acted as hydrogen acceptors, while alanine and leucine acted as hydrogen donors in a manner similar to that reported by Stickland (1934) for C. sporogenes.

Clifton (1940a) working with types A and B noted that one percent glucose in a yeast extract, beef-brain broth culture was fermented directly, ethyl alcohol and carbon dioxide being the chief products in addition to small amounts of acetic acid, hydrogen, and lactic acid. About 25 percent of the glucose fermented by the resting-cell suspension was not accounted for, and no attempt was made to elucidate the metabolic pathway. He also noted that alanine was deaminatively oxidized to acetic acid, carbon dioxide, and ammonia in the presence of a suitable hydrogen acceptor such as glycine or proline. These results suggested that pyruvic acid may be an intermediate in alanine degradation as well as in the fermentation of glucose. He (Clifton, 1940b) observed that strains of types A and B, grown previously in the above glucose broth and, subsequently, suspended in phosphate buffer in a Warburg system flushed with hydrogen gas, utilized pyruvate with almost equimolar quantities of ethyl alcohol (tended to run a little high) and acetic acid being produced. This suggested that pyruvic acid was decarboxylated with the production of equimolar quantities of above by-products from the

dismutation of acetaldehyde. In general, the amounts of carbon dioxide recovered were a little less than the theoretical value, which suggested that it might have been utilized during fermentation. He suggested that if fermentation of glucose proceeded by the way of pyruvic acid, dismutation of acetaldehyde may account for a small amount of acetic acid produced, the bulk of acetaldehyde being reduced to alcohol by a hydrogen donor other than another molecule of acetaldehyde. Clifton (1940c) also showed that serine in washed suspensions of C. botulinum types A or B either in a hydrogen or a nitrogen atmosphere appeared to be deaminated with the formation of ammonia, carbon dioxide, ethyl alcohol, and acetic acid. He postulated that the mechanism was either the deamination of serine to pyruvic acid and ammonia, followed by fermentation of the former product or the simultaneous decarboxylation and deamination of serine to acetaldehyde, which was dismuted to ethanol and acetic acid. In the former mechanism, deamination might have been the controlling factor of the rate of utilization of serine by optimum pH shift. The results showed that the organism obtained a portion of its energy requirement by direct utilization of amino acids such as serine as well as through the Stickland reaction.

Simmons and Costilow (1962) demonstrated the presence of the Embden Meyerhof Pathway (EMP) in C. botulinum type A. They found that the enzymes of this pathway were induced sequentially,

gluco-kinase being the controlling enzyme. The absence of glucose-6-phosphate dehydrogenase in addition to the presence of the enzymes of the EMP system suggested that glucose was catabolized by this system.

A phosphoroclastic type reaction in which pyruvate was utilized with the production of one mole each of carbon dioxide, hydrogen, and acetyl phosphate per mole of pyruvate, was observed in glucose-adapted and non-adapted cells. The absence of nicotinamide adenine dinucleotide (NAD) or potassium phosphate resulted in almost complete inactivity.

Green (1966) working with type E vegetative cells and using a Warburg respirometric system under anaerobic conditions showed that the presence of a protein hydrolysate, preferable an enzymatic digest of casein, was required for vegetative cells to catabolize carbohydrates. A peptide factor and possible other factors from protein hydrolysates was suggested as the requirements responsible for this observation.

Isolation and Role of Ferredoxin in the Clostridia

Ferredoxin is a non-heme iron-containing protein, which functions as an electron carrier on the "hydrogen side" of the pyridine nucleotides (Arnon, 1965). Peck Jr. and Gest (1956) found that bacterial hydrogenase rapidly removed the single electron from

reduced methyl viologen yielding hydrogen. Subsequent reports from other laboratories showed the hydrogen evolving or utilizing reactions by bacteria to be strongly stimulated by methyl viologen (Valentine, 1964). These investigations focused attention on a natural low redox carrier and served as a guide for the development of assays for ferredoxin.

Methyl viologen is a member of the viologen dyes described by Michaelis and Hill (1933). This indicator is colorless in the oxidized state, while the reduced form exhibits a deep blue or violet color. Under physiological conditions (ph < 12) the dye is reduced by one electron. At 30° C, the normal redox potential of methyl viologen is -0.446 volts, and the potential of the system is independent of pH (Michaelis, 1933). Since the potential of the hydrogen electrode decreases with increasing pH, the potential of the dye system under alkaline conditions is more positive than that of the hydrogen electrode, whereas in acid solution it is more negative than that of the hydrogen electrode at the same pH.

In 1962, Mortenson et al. (1962) isolated ferredoxin from extracts of C. pasteurianum by specific absorption on diethylaminoethyl (DEAE) cellulose column. The ferredoxin, which adhered to the top of the column as a coffee-colored band, was eluted with phosphate buffer, dialyzed, and purified with amonium sulfate fractionation. Subsequent methods of isolation added sephadex column

chromotography (Buchanan, Lovenberg, and Rabinowitz, 1963; Lovenberg, Buchanan, and Rabinowitz, 1963). Later Mortenson (1964) improved the method by introducing 50 percent cold acetone extraction and the use of a 2-amino-2 (hydroxymethyl-1, 3-propanediol (tris)- HCl buffer as an eluent. This revised method showed a more purified form of ferredoxin in greater quantities. This protein was successfully substituted for methyl viologen in the stimulation of acetyl phosphate production and hydrogen evolution from ferredoxin-free extracts in the presence of pyruvate. This reaction was similar to the phosphoroclastic type described earlier by Koepsel and Johnson (1942), who observed the production of hydrogen upon the addition of pyruvate to cell-free extract of Clostridium butyricum in the presence of phosphate buffer and coenzyme A (CoA).

The phosphoroclastic system appears to be composed of pyruvic dehydrogenase, hydrogenase, and phosphotransacetylase, together with CoA and thiamine pyrophosphate. In this system ferredoxin has the important role of electron mediator between pyruvic dehydrogenase and hydrogenase for hydrogen evolution (Valentine, 1964).

Additional reactions in the clostridia also involve ferredoxin as an electron mediator. These reactions include dithionite (sodium hydrosulfite) oxidation coupled with hydrogen production, hypoxanthine oxidation coupled with the reduction of pyridine nucleotides,

formate oxidation coupled with the reduction of NAD, pyruvate oxidation coupled with urate reduction to xanthine, hydrogen oxidation coupled with nitrite and hydroxylamine reductions to ammonia, hydrogen oxidation coupled with nicotinamide adenine dinucleotide phosphate (NADP) reduction, pyruvate oxidation coupled with NADP reduction, pyruvate oxidation coupled with the reduction of nitrogen to ammonia, and hydrogen oxidation coupled with pyruvate production (Valentine, 1964). Ferredoxin-linked reactions in which the electron flows to and from ferredoxin show specificity. This reduced ferredoxin will react with substrates such as NAD, NADP, or urate only in the presence of specific reductases.

Tagawa and Arnon (1962) were the first to crystallize the ferredoxin of C. pasteurianum. These investigators found this protein to contain 10 atoms of iron and to have a molecular weight of 12,000 and a oxidation-reduction potential of -418 mV at pH 7.11. However, it was later found to have a molecular weight of 5,800 and to contain seven non-heme iron atoms and six or seven sulfide ions (Lovenberg, 1963). The sulfide called "labile sulfide" is loosely bound to the protein, as evident by its dissociation to hydrogen sulfide at acidic or basic pH (Lovenberg, 1963; Buchanan, 1963). This protein was found to have an isoelectric point of about 3.7 (Lovenberg, 1963). A larger number of acetic amino acids residues accounted for the overall acidic nature of this protein.

Ferredoxin was found in the following species of clostridia: C. pasteurianum, C. butyricum, Clostridium lactoacetophilum, Clostridium acidiurici, Clostridium tetanomorphum, Clostridium kluyveri, Clostridium thermosaccharolyticum, Clostridium cylindrosporum, C. sporogenes, and Clostridium nigrificans (Valentine, 1964). It was crystallized from the following species of clostridia: C. pasteurianum, C. tetanomorphum, C. cylindrosporum, C. aciduirici, and C. butyricum (Lovenberg, 1963). The crystalline preparations of ferredoxin exhibited distinct species-specific absorption in the ultraviolet regions of 280 to 300 mμ and a maximal light absorption at 390 mμ (Valentine, 1964). Lovenberg et al. (1963) showed that these clostridial ferredoxins have essentially the same catalytic and chemical properties, but differ slightly in amino acid composition.

MATERIALS AND METHODS

Microorganisms Used

The microorganisms were obtained from the following sources:

Quartermaster Food and Development Command, Natick, Massachusetts	C. botulinum type A strains 33A and 5A and type B strain 115B
National Canners Association, Washington, D. C.	C. botulinum type A strains 64-89, 73A, and 78A and type B strains 32B, 113B, and 213B
National Collection of Industrial Bacteria (NCIB), Torry Research Station, Aberdeen, Scotland	C. botulinum type C strain 4218 and type E strains VH, 4203, 4244, 4251, 4249, 4297, 4217, 4261, 4286, 4284, 4248, 4214, 4228, 4299, 4213, and 4239; the nontoxic strains 4285, 4277,

	4265, 4262, 4271, 4279, 4272, 4270, 4266, 4268, 4267, 4227, and 4263
Culture Collection, Oregon State University, Corvallis, Oregon	C. botulinum type F strain, C. pasteurianum, E. coli, and Micrococcus lactilyticus.

All strains were examined for total protein and enzymes using the disc electrophoretic method. C. botulinum strains 33A and NCIB 4261 and C. pasteurianum were employed in the ferredoxin and phosphoroclastic studies. C. botulinum strain NCIB 4261, M. lactilyticus, and E. coli were employed in the α-ketoglutaric dehydrogenase study, the last two microorganisms being used as positive controls.

Culture Media and Growth Conditions

Trypticase-proteose-glucose (TPG) medium consisting of 0.5 percent trypticase (Baltimore Biological Laboratory), 0.05 percent peptone (Difco), 1.0 percent glucose, and distilled water to 1000 ml adjusted to pH 7-7.3 was used to grow all the strains of C. botulinum included in the disc electrophoretic study. The medium (1 liter) was sterilized for 20 minutes at 15 pounds steam pressure. Immediately

after cooling, it was inoculated with a five percent inoculum from an 18 hour culture and incubated at 30° C anaerobically for 48 hours.

C. pasteurianum and strains of C. botulinum included in the ferredoxin, phosphoroclastic, and the α-ketoglutaric dehydrogenase studies were grown in 20 percent yeast extract (Difco), 20 percent peptone (Difco), and 1.0 percent glucose. The batches of medium adjusted to pH 7-7.3 in amounts of either two or 15 liters were sterilized for 20 and 60 minutes respectively, at 15 pounds steam pressure. Immediately upon cooling, each batch of medium was inoculated with a 10 percent inoculum from an 18 hour culture and incubated at 30° C anaerobically for 15-17 hours.

E. coli and M. lactilyticus were grown in nutrient broth (Difco) by inoculating with a 5 percent inoculum from an 18 hour culture and incubating aerobically at 37° C overnight.

Robertson's cooked meat medium, consisting of TPG medium and raw ground beef, was used to store all C. botulinum stock cultures. The medium was prepared by adding three grams of meat to 10 ml of TPG medium in a 30 ml screw cap tube and autoclaving for 10 minutes at 15 pounds pressure.

Each culture was checked for purity by Gram staining and by plating on blood agar. The blood agar was prepared by adding 10 percent defibrinated blood to blood agar base (Difco).

Each strain of C. botulinum was grown in nutrient gelatin

(Difco) by inoculating from a 18 hour culture and incubating anaerobically at 30° C for a minimum of five days. The proteolytic characteristic was determined by cooling the gelatin culture and observing for the presence of liquefication.

Identification

The identification of the strains of C. botulinum was checked biochemically by sugar fermentation tests and liver-veal-egg yolk agar (LVA), morphologically by blood agar, and immunologically by mouse toxicity tests.

The biochemical fermentations were determined on glucose, maltose, sucrose, fructose, sorbitol, glycerol, lactose, mannitol, salicin, and dulcitol. The sugars were dissolved in a base consisting of the TPG medium using phenol red as the indicator. The tubes were sterilized for 15 minutes at 15 pounds steam pressure.

The LVA medium was prepared by mixing egg yolk with an equal volume of sterile physiological saline. This mixture was added to sterile liver-veal agar base (Difco) (45° C) to a final concentration of 10 percent.

The mouse toxicity testing involved the use of the following antitoxins:

Type A botulinum antitoxin, equine serum, 10 units per ml

Type B botulinum antitoxin, rabbit serum, 28 units per ml

Type C botulinum antitoxin, equine serum, 10 units per ml

Type E botulinum antitoxin, equine serum, 10 units per ml. Five-tenths ml of the antitoxin prepared as directed was injected intraperitoneally into each of four 17-20 day old mice for each toxic specimen tested. After a minimum of 30 minutes the mice were injected with the toxin-containing medium.

The toxin-containing medium, the supernatant from a 3-4 day growing TPG culture, was diluted 1:5 and 1:20 with physiological saline, and five-tenths ml from each of the dilutions was injected intraperitoneally into two protected and two unprotected mice for each dilution. The mice were observed for 24 hours. Those showing progressive evidence of respiratory paralysis before death were assumed to have died from botulism.

Preparation of Cell Free Extract

Disc Electrophoretic Study

The cells were collected by centrifugation and washed 3-4 times with cold physiological saline. The washed cells were mixed into a thin paste with a buffer consisting of 5.7g of tris dissolved in 25.6 ml of 1 M H_3PO_4, diluted to 100 ml with distilled water and adjusted to pH 6.85-6.95. Then they were disrupted by sonification at 15,000 to 22,000 kc (Bronson Instruments, Inc., Model LS75,

Sonifier). In order to prevent too much damage to the enzymes, the cell mixture was held at 5° C and sonicated in 10 second-bursts with a 2 minute cooling period between each burst. Sonication was stopped when 80 percent or more ruptures were observed when examined by using a phase contrast microscope.

The sonicated material was centrifuged for 25 minutes at 11,000 x G at 5° C. The cell-free extracts were analyzed for enzymes within a period of 36 hours. Sonicates analyzed for protein were kept at 5° C for several days, centrifuged, and the cell-free extracts used within a period of 48 hours.

Ferredoxin, Phosphoroclastic, and α-Ketoglutaric Dehydrogenase Studies

The cells were collected by centrifugation and washed 3-4 times with cold 0.05 M potassium phosphate buffer adjusted to pH 7.0. They were then mixed with 0.05 M potassium phosphate buffer (pH 6.5) and placed into a glass homogenization flask together with three times their wet weight of glass beads (0.11 to 0.12 mm). They were then homogenized in a Bronwell carbon dioxide cooled mechanical homogenizer (MSK) at 4,000 cycles per minute. Disintegration was stopped when 80 percent or more ruptures were observed when examined by using a phase contrast microscope, usually after 2.5-3 minutes. The glass beads were removed by centrifugation at

3,000 x G for 10 minutes in a refrigerated centrifuge.

The homogenates of E. coli and M. lactilyticus were centrifuged at 11,000 x G for 20 minutes at 5° C to remove the cell debris. The homogenate of C. pasteurianum was diluted up to three times the original wet weight of the cells with hydrogen flushed 0.05 M potassium phosphate buffer and centrifuged at 38,000 x G for 15 minutes at 5° C (Lovenberg et al., 1963). Treatment of the homogenates of 33A and NCIB 4261 will be discussed later.

Electrophoresis

A modification of the disc gel electrophoretic method of Ornstein (1964) and Davis (1964) was used. Glass tubes (63 mm in length and 5 mm in diameter), in which the gels were made, were inserted into rubber caps so as to assume a vertical position. Five ml each of Small-Pore Solutions #1 and #2 were mixed and added to the glass tubes to within 1/2 inch of the tops by means of a 5 ml glass syringe. The ingredients constituting the small-pore solutions were as follows:

Small-Pore Solution #1

1 part A
2 part C
1 part distilled water
(pH 8.6 - 9.0)

(A) 1N HCl	48 ml
tris	26.6 g
N, N, N', N'-Tetra-methylenediamine	0.23 ml
distilled water to	100 ml
(pH 8.9)	

(C) Acrylamide	30 g
N, N'-Methylenebis-acrylamide	0.8 g
distilled water to	100 ml

Small-Pore Solution #2

Ammonium persulfate	0.14 g
distilled water to	100. ml

The small-pore mixture in each tube was overlaid with 1/4 inch column of distilled water. The small-pore gels were allowed to form for 40 minutes at room temperature. Upon gel formation, the unreacted monomer solution and water were removed by inverting the tubes and gently shaking and draining. A Large-Pore Solution (Canalco) was used to rinse and to form a 3/8 inch column on top of the small pore gel, which was overlaid by a 1/8 inch column of distilled water. A 15-watt daylight fluorescent lamp was set for 15 minutes about the same level as that of the tubes, parallel to the line of the tubes and about three inches away. Upon photopolymerization of the large pore gel, the water layer was removed, and a mixture containing 0.1 ml of Large-Pore Solution and varying concentrations of extract diluted with distilled water to a final volume of 0.1 ml was

added to each tube. The mixtures were photopolymerized for 20-30 minutes. Upon completion of photopolymerization, the tubes were removed from the rubber caps and inserted, sample gel uppermost into the rubber grommets of the upper buffer reservoir of the electrophoretic cell. Approximately 200 ml of a 1:10 diluted tris-glycine buffer containing 0.001 percent bromophenol blue solution were added to the upper reservoir. The buffer solution consisted of 6 g of tris, 28.8 g of glycine, and distilled water to one liter. The upper reservoir was placed upon the lower reservoir, which was filled with the same buffer minus the dye to within 1/2 inch of the top. The current was adjusted to approximately three mils per tube. Electrophoresis was continued until the dye front or pre-albumin had migrated about 25 mm into the small-pore gel (approximately 30 minutes). Upon completion, the protein-laden gels were removed from the tubes by rimming under distilled water with a 11 cm long needle. The power supply and the electrophoretic cell are shown in Figure 1.

The disc electrophoretic method was modified slightly in order to run two samples on the same disc column. The modification constituted placing the separator (a plastic coated paper) in the sample space at the top of the gel column, allowing the bottom of the separator to penetrate slightly into the large-pore gel, and filling each half with approximately 100 μl of each sample mixture.

Figure 1. Apparatus used in disc gel electrophoresis.

Polyacrylimide Gel Staining

Protein

The gels were stained for one hour in a one percent solution of naphthol blue black (Allied Chemical Company) in a seven percent acetic acid solution, after which, they were destained electrically in a seven percent acetic acid solution.

Enzyme

The method of enzyme analysis was based on the exposure of the protein-laden gels to various buffered enzymes systems at an incubation temperature of 37° C for varying amounts of time depending on the rate of formation of the colored bands and the desired intensity. The gels were assayed for malic, succinic, isocitric, and lactic dehydrogenases by the lactic dehydrogenase method proposed by J. M. Allen (Enzyme. . .) modified as follows: 7. 5 ml of 0. 05 M tris-HCl buffer; 3. 0 ml of 0. 5 M substrate; 3. 5 ml of nitro-blue tetrazolium (Sigma), 2 mg/ml; 5. 0 mg of either NAD (Sigma) or NADP (Sigma); and 0. 15 ml of phenazine methosulphate (Sigma), 2 mg/ml. The buffer and substrate were adjusted to the pH of the specific enzyme system, which in all cases except succinic dehydrogenase was pH 7. 5. The succinic dehydrogenase system was adjusted to pH 9. 0. In the case of the isocitric dehydrogenase system, 1. 0 ml

of 0.1 M magnesium chloride and 150 mg of sodium isocitrate (Sigma) were used. The alkaline phosphatase system as described by Burstone (1962) was modified as follows: 40 ml of 0.1 M tris-HCl buffer, pH 8.5; 10 mg sodium α-naphthyl acid phosphate (Sigma); 20 mg Fast Blue R R (Eastman Chemical Company); and 0.3 ml of 10 percent magnesium chloride solution.

Negative controls consisting of the enzyme system minus the substrate were run in parallel. Positive controls using E. coli extracts were also run in parallel.

A Densicord Recording Electrophoresis Densitometer was used to plot the protein and the enzyme patterns.

Iron Bound Protein

The iron bound protein stain was prepared and used as suggested by Canalco[1] in the following manner:

Stock Solution A

Sodium Acetate tri-hydrate		96 g
Acetic Acid (glacial)		42 g
Distilled water	to make	600 ml

Stock Solution B

2, 4 Dinitrose-1, 3 napthalenediol		150 g
absolute ethanol	to make	60 ml

[1] Canalco Industries, 4935 Cordell Avenue, Bethesda 14, Maryland.

Stock Solution C

Hydroquinone		6 g
absolute ethanol	to make	60 g

The following working solution was prepared just prior to use:

20 parts Stock Solution A
1 part Stock Solution B
1 part Stock Solution C

Sufficient working solution was added to each test tube to cover the gel completely. The gels were incubated at room temperature for a period sufficient to allow the iron protein-containing fractions to develop maximum color density (30-40 minutes).

Preparation of Ferredoxin

After each of the homogenates from NCIB 4261 and 33A was diluted to several volumes with cold distilled water, they were subjected to acetone extraction and DEAE-cellulose column separation as described by Mortenson (1964). The column used was two cm in cross section and three cm in length, and could handle the homogenates from 50 g wet weight of cells. The preparation was washed, and the ferredoxin eluted according to the method of Mortenson et al. (1962) with the exception that 1.0 M sodium citrate buffer adjusted to pH 5.0 was used for eluting the ferredoxin from NCIB 4261. The eluate from both types was dialyzed in distilled water at 5° C for 24 hours. The dialyzed ferredoxin was then precipitated by the addition of crystalline $(NH_4)_2SO_4$, 66 percent to 90 percent saturation at 0° C. Following centrifugation for complete recovery, the ferredoxin was

dissolved in small amounts of cold distilled water, and dialyzed for 24 hours against three liters of distilled water at 5° C with several changes. The dialyzed preparation was lyophilized and stored at -20° C under desiccation. Pure ferredoxin from C. pasteuranium was obtained through Sigma and was used as a control.

The ferredoxin in an amount of 10 μg was measured for an absorption spectrum in a Cary recording spectrophotometer (Model 11).

Preparation of Ferredoxin-Free Extract Fractions

The homogenates for NCIB 4261 and 33A were prepared using a similar method as that for ferredoxin. They were then diluted up to four times the original wet weight of the cells with hydrogen flushed 0.05 M potassium phosphate (pH 6.5). The beads used for homogenization were washed several times with the hydrogenated buffer, the washings decanted and pooled. After the homogenates and washings were flushed several minutes with hydrogen, they were ultracentrifuged at 40,000 x G for 45 minutes at 5° C. The clear supernatant was collected and passed through a cooled (5° C) DEAE-cellulose column in order to remove the ferredoxin. This fraction was lyophilized and stored anaerobically at -20° C. The particulate or insoluble fraction was then washed several times with the hydrogenated buffer, centrifuging as above to remove ferredoxin. This

fraction was lypholyzed and stored anaerobically at -20° C.

Ferredoxin-free extract of C. pasteurianum was prepared according to the method described by Mortenson et al. (1962).

Acetyl Phosphate Determination

The phosphoroclastic system proposed by Mortenson et al. (1962) was modified as follows: 100 μM potassium phosphate buffer, pH 6.5; 110 μM potassium pyruvate (Sigma); 0.5 mg CoA free acid form (Sigma); 15 mg of the soluble fraction; 30-40 mg of the soluble fraction, and distilled water to 1.0 ml. In the case of C. pasteurianum, 0.2 ml of the ferredoxin-free extract was used. The acetyl phosphate formed in a 1.0 ml aerobic incubated mixture was determined by the method of Lipmann and Tuttle (1945), and modified according to Lovenberg et al. (1963). In all cases, controls minus the ferredoxin or methyl viologen were run in parallel.

A standard curve for acetyl phosphate determination using succinic anhydride (Sigma) as an acetyl phosphate substitute was prepared according to the method described by Lipmann and Tuttle (1945). Varying concentrations of acetyl phosphate (Sigma) were used to check the accuracy of this curve.

Manometric Determination of Molecular Hydrogen Evolution

The soluble and insoluble ferredoxin-free extract fractions in

amounts of 15 mg and 30-40 mg, respectively, were assayed for hydrogenase activity by the manometric procedure described by Peck, Jr. and Gest (1956) with the exception that a Gilson Differential Respirometer Model G8, preflushed with helium, was used.

Hydrogen production resulting from activity of the phosphoroclastic system was analyzed manometrically as above utilizing the modified Mortenson system. The pyruvate and either the methyl viologen or the ferredoxin were placed in separate side arms and added to the reaction mixture in the main chamber of the vessel at zero time.

Hydrogen resulting from α-ketoglutaric dehydrogenase was measured manometrically utilizing the method of Valentine and Wolfe (1962) modified as follows: 100 μM of sodium α-ketoglutarate, 100 μM of potassium phosphate buffer (pH 6.5), 0.5 mg of CoA, 10 μM of methyl viologen, up to 0.5 ml of either NCIB 4261 or M. lactilyticus extracts, and distilled water to 2.0 ml.

In the first two analysis, controls minus ferredoxin or methyl viologen were run in parallel. In the latter analysis, controls minus α-ketoglutarate were run in parallel.

The identification of molecular hydrogen was based upon its absorption in the presence of a saturated methylene blue-palladium asbestos as described by Gest et al. (1950).

The spectrophotometric determination of NAD reduction by α-ketoglutaric dehydrogenase described by Kaufman et al. (1953) was used to determine this enzyme in NCIB 4261. E. coli was used as a positive control.

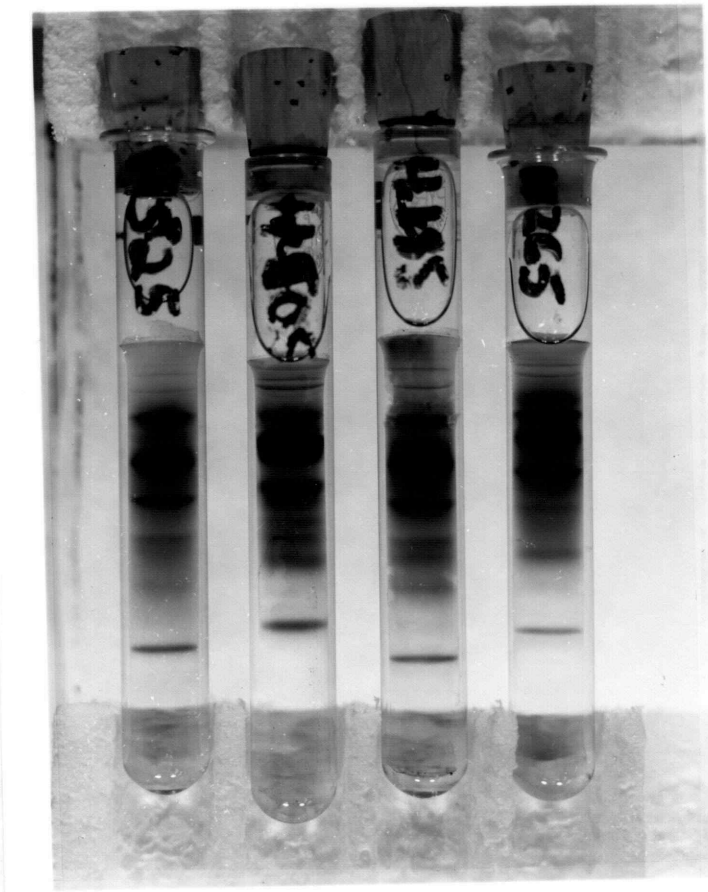
RESULTS

Gel Staining

Total Protein

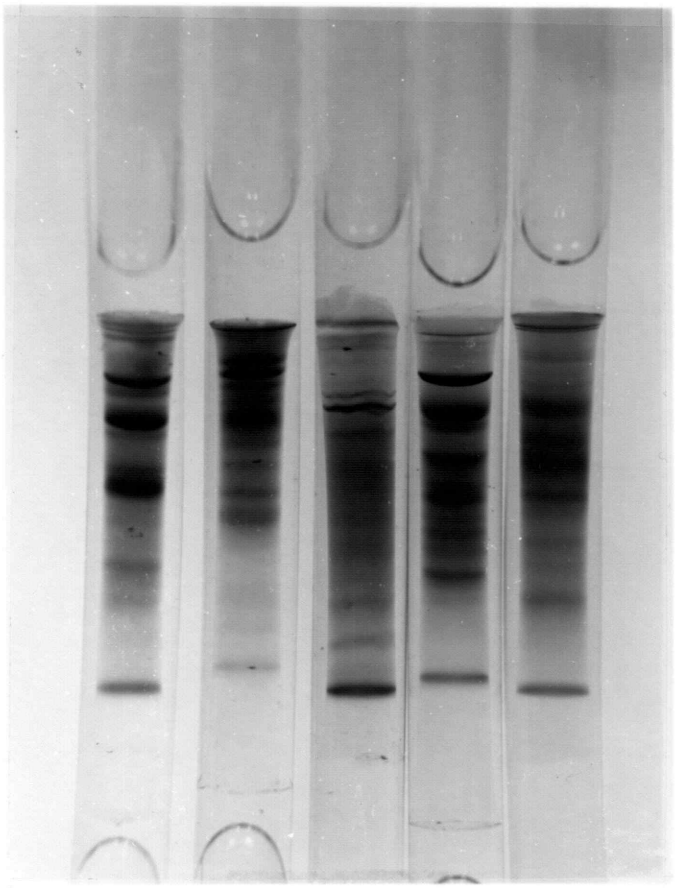
Reproducible total protein patterns were demonstrated for strains grown in the same medium on different occasions. This is shown for a strain (NCIB 4299) in Figure 2. The cell-free extract of a strain stored five to ten days at 4° C always gave essentially the same pattern (Figure 2). If the extract and debris were left mixed, the supernatant became more concentrated with age. However, the efficiency of extraction was found to vary considerably from one preparation to another, and the amounts of sample required to give a satisfactory protein stain varied from 10-100 μl.

The types of C. botulinum (A, B, C, E, and F) showed many protein bands in common. The minor differences when they existed, appeared only in the number of bands present. Two different methods of picturing the stained protein gels from the five types are shown in Figures 3 and 4. In Figure 4, the various protein bands as found in the gels are represented by numbers in each densiometric tracing. The higher the number the greater the distance of the protein fraction from the origin of protein migration. Although each type did show different numbers of bands the results were not



a b c d

Figure 2. Total protein stained gels of strain NCIB 4299 (type E) showing patterns obtained with extracts made from two separate cultures (a and b), and the same extract (b) stored at 4° C for 5 and 10 days (c and d, respectively).



115B	33A	NCIB 4218	VH	type F
(type B)	(type A)	(type C)	(type E)	

Figure 3. Total protein stained gels of C. botulinum types A, B, C, E, and F.

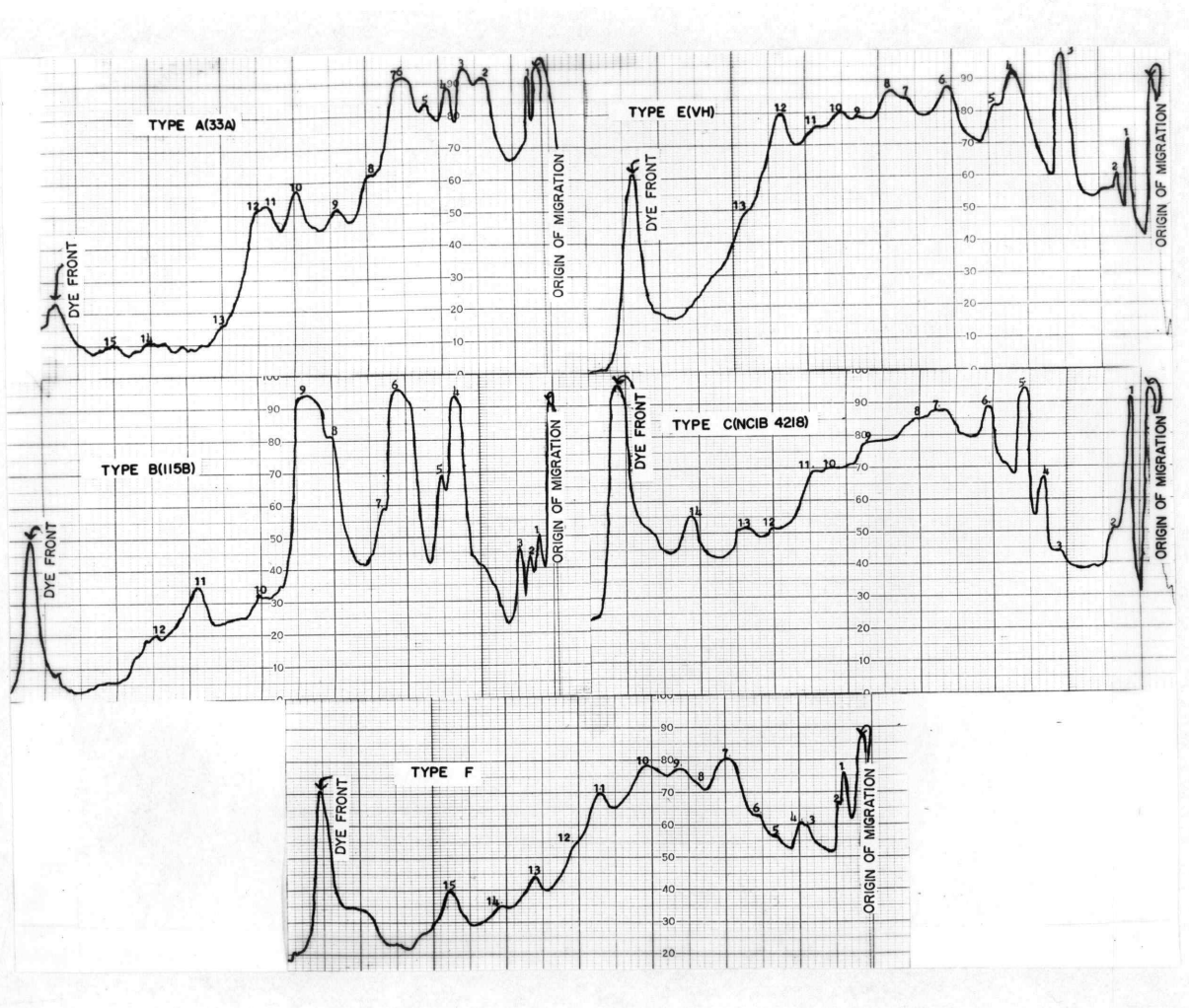


Figure 4. Densiometric tracings of the total protein stained gels of C. botulinum types A, B, C, E, and F.

always constant.

The nontoxic strains in general differed from the toxic types only in the number of protein bands present. The number of bands for some strains was identical to that shown by type E.

The strains of each type including the nontoxic group showed variation in the intensity of the stained bands (probably due to a variation in the concentration of each protein fraction) and, in some cases absence of common protein fractions. An example is shown in Figure 5 using different strains of type E.

A comparison of the protein patterns representing toxic and nontoxic strains in the same polyacrylamide gels is shown in Figures 6 and 7. When represented in this manner it is easier to note the differences in the protein patterns.

Enzymes

Malic dehydrogenase was demonstrated in all the strains of C. botulinum including the nontoxic group. The majority of strains showed multiple molecular forms of the enzyme. The staining intensity for each band and the number of bands varied among the strains of each type. An estimation of the R_f values for the bands in the gels showed that most strains have at least one band of activity in common. The malic dehydrogenase systems of the types and the nontoxic strains in the present of either NAD or NADP showed

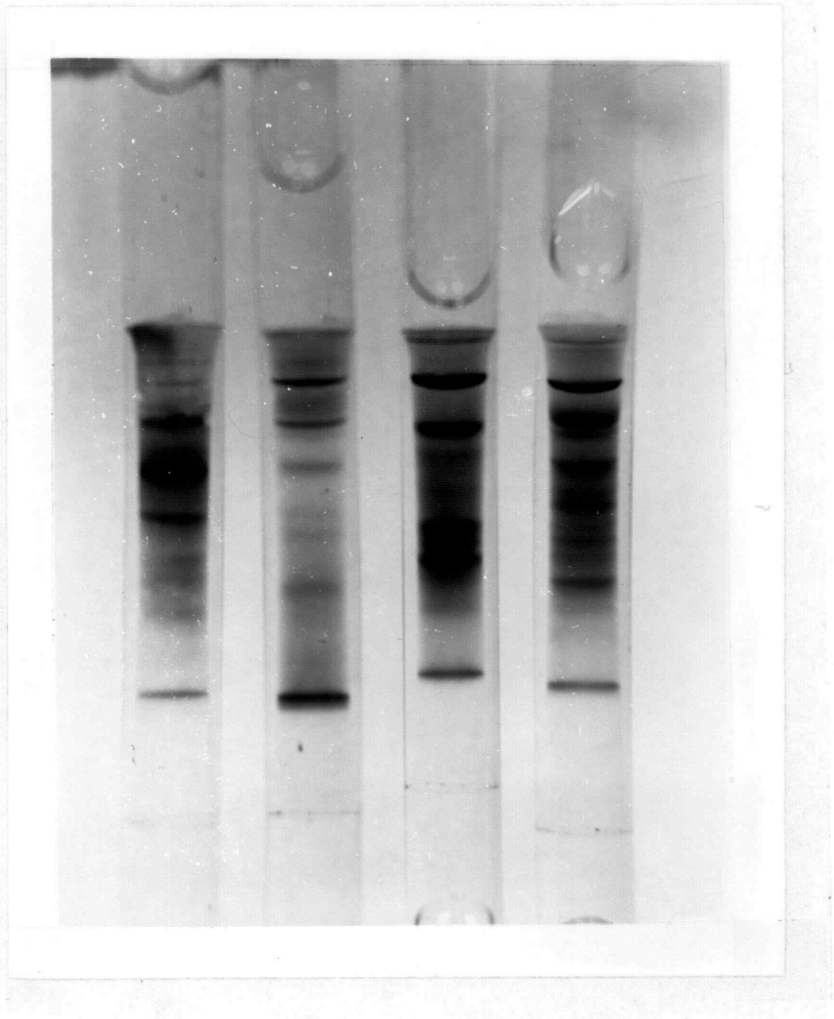


Figure 5. Total protein stained gels showing patterns of strains of C. botulinum type E.

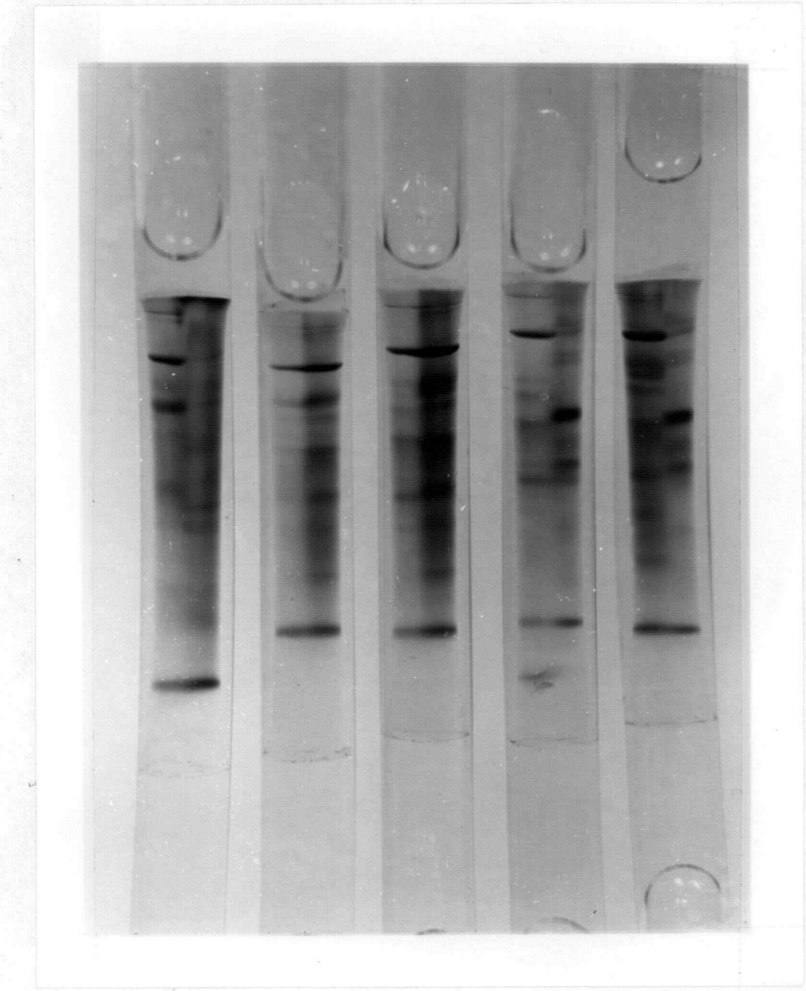


Figure 6. Total protein stained gels showing patterns of two strains of C. botulinum in each gel. Left to right, NCIB 4263, 33A (nontoxic, type A); NCIB 4263, 115B (nontoxic, type B); 33A, 115B (type A, type B); 33A, VH (type A, type E); and 115 B, NCIB 4203 (type B, type E).

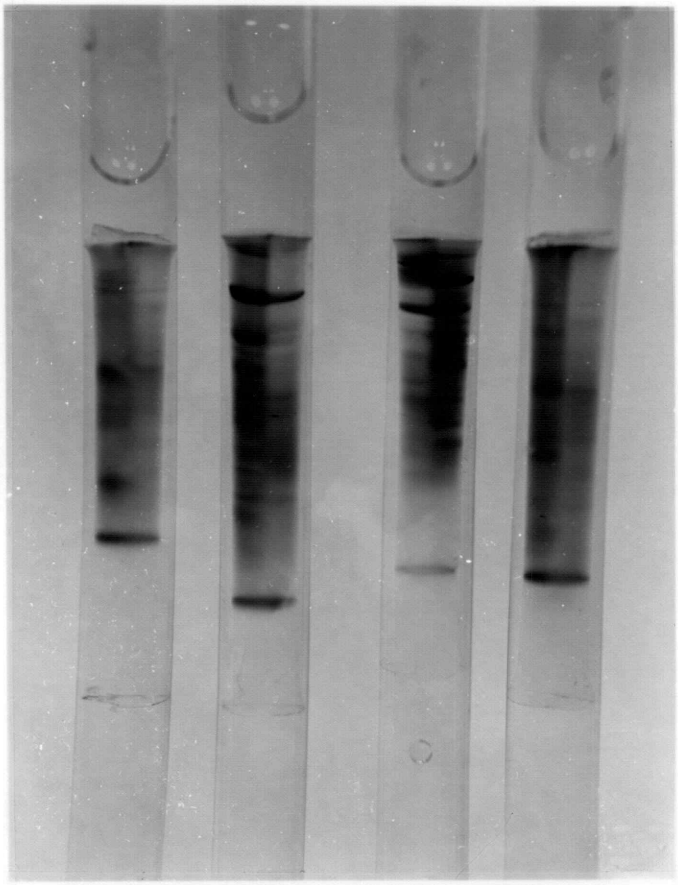


Figure 7. Total protein stained gels showing patterns of two strains of C. botulinum in each gel. Left to right, NCIB 4263, NCIB 4265 (nontoxic strains); NCIB 4268, NCIB 4266 (nontoxic strains); NCIB 4239, NCIB 4265 (type E, nontoxic); and NCIB 4249, NCIB 4268 (type E, nontoxic).

various patterns in common; these are shown in Figures 8 and 9, respective for the coenzyme.

In the staining of all the dehydrogenases, the band or bands indicating the position of the active enzyme appeared dark purple against a pale purple background, the lowermost band in each case being the dye front or pre-albumin.

All strains showed isocitric dehydrogenase activity in the presence of NAD. However, this enzyme was demonstrated in the strains of types A and B only during the pre-sporulation period. A few of the nontoxic strains showed two bands of activity, which differed only in staining intensity. In those variants showing the double bands, the band of greater intensity was positionally similar to that of the nontoxic strains. The position of the type E band was similar to that of types C and F but different from that of types A, B, and the nontoxic strains. The position of the bands of the types A and B was similar to each other, but different from the nontoxic group. The position of isocitric dehydrogenase on the gels demonstrated by each of the types and the nontoxic group is shown in Figure 10.

All strains showed single and double forms for succinic dehydrogenase. Both enzyme forms as shown in Figure 11 were similar among the strains of the types and the nontoxic group. In most strains the enzyme activity was slow. A slight increase in activity was produced by exposing the cells to higher buffer

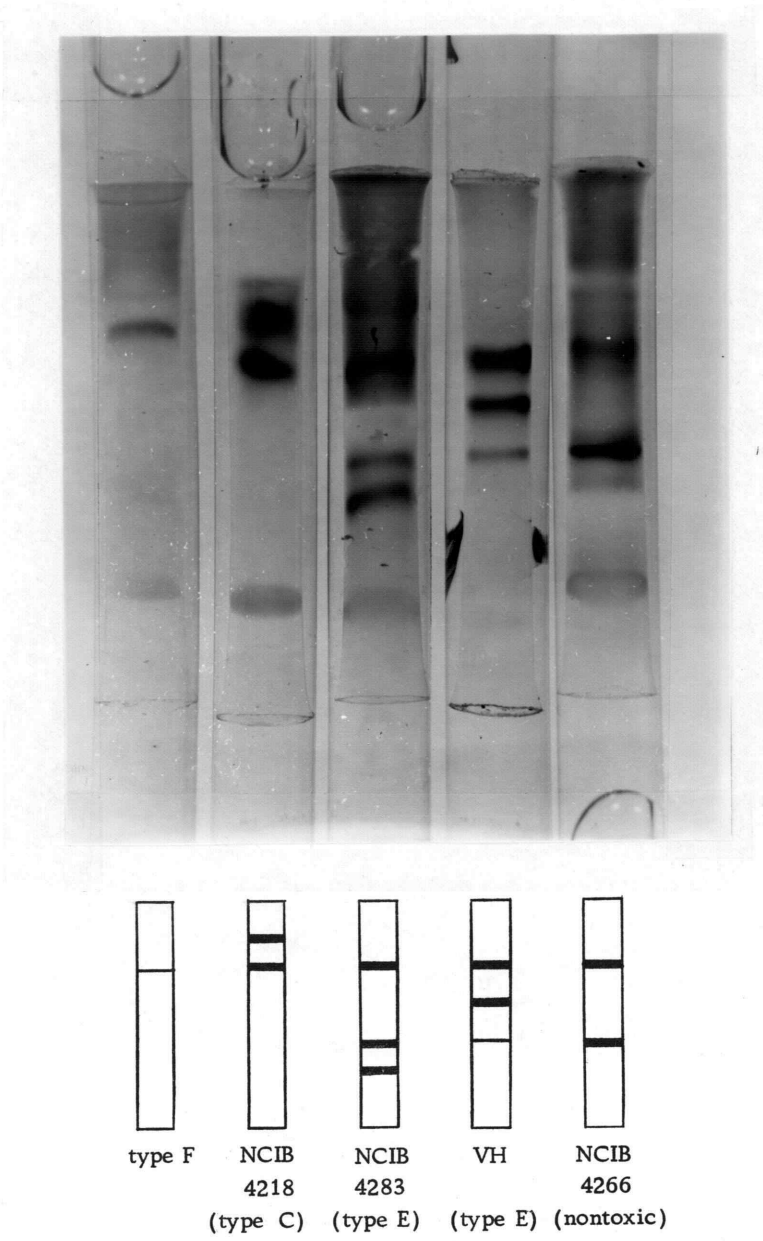


Figure 8. Enzyme stained gels showing patterns of the malic dehydrogenase (NAD) systems present in C. botulinum and the nontoxic group.

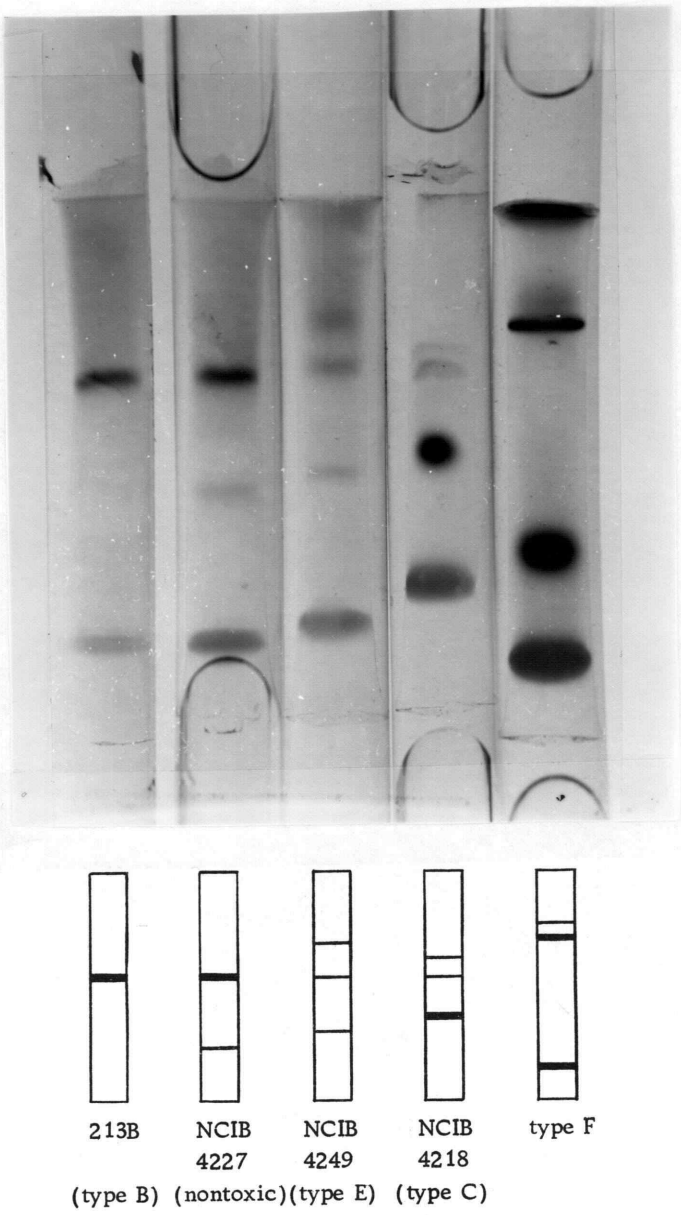


Figure 9. Enzyme stained gels showing patterns of the malic dehydrogenase (NADP) systems present in C. botulinum and the nontoxic group.

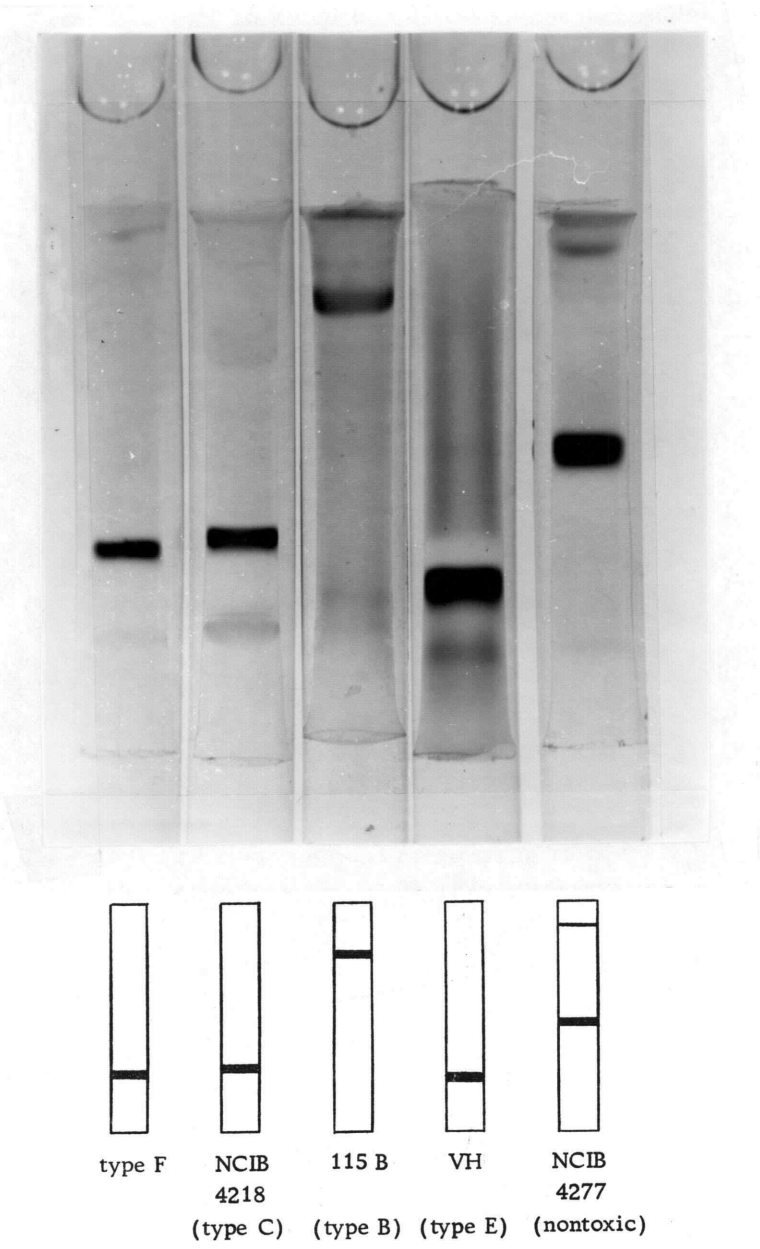


Figure 10. Enzyme stained gels showing positions of the isocitric dehydrogenases present in each of the C. botulinum types and the nontoxic group.

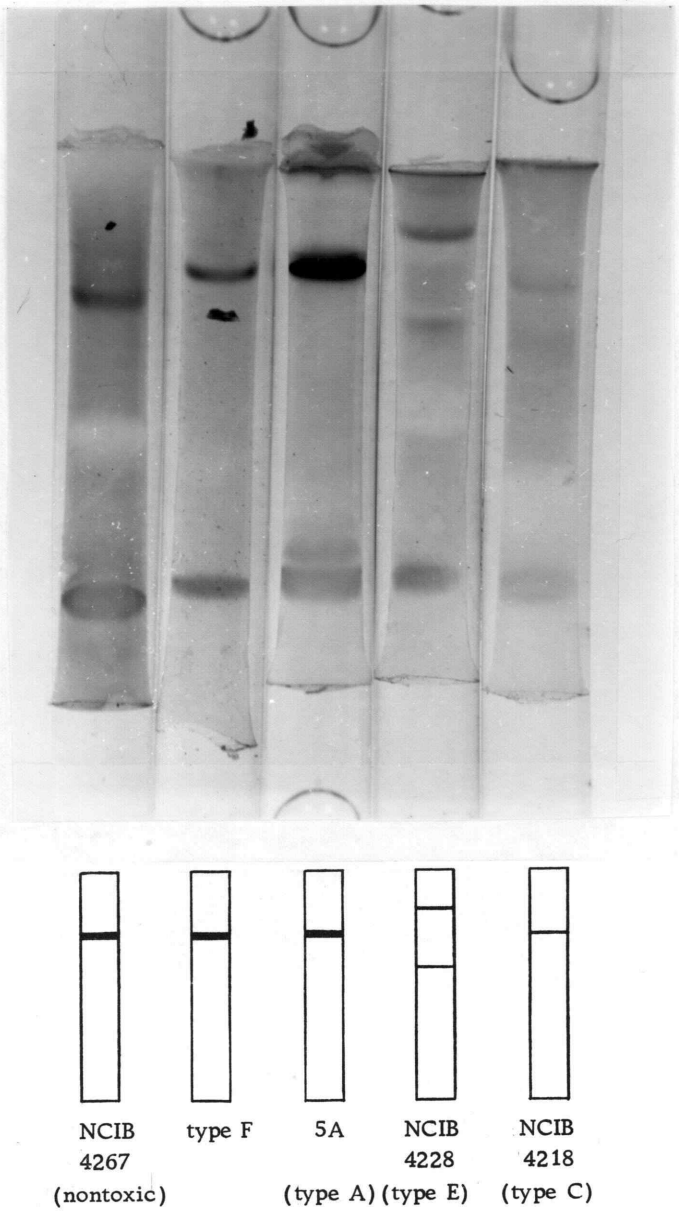


Figure 11. Enzyme stained gels showing the single and double forms of succinic dehydrogenase present in C. botulinum and the nontoxic group.

concentrations during sonification.

The presence of alkaline phosphatase was demonstrated in all the strains, the majority of which showed multiple molecular enzyme forms. Representative patterns are shown in Figures 12 and 13. The bands appeared dark brown against a light brown background. Types A, B, and some nontoxic strains showed similar patterns. The patterns observed for the strains of type E were different from those of types A, B, or the nontoxic strains. However some similarity was shown to types F and C.

All strains except those of types A (an exception was 5A) and B showed a single band of lactic dehydrogenase activity. The band shown by type F was positioned lower than that of type E and the nontoxic strains (Figure 13). Type E and the nontoxic strains showed similar positions. Strain 5A showed a band slightly above that shown by NCIB 4283 in Figure 13.

In many gels stained for dehydrogenase activity, one or more areas showed false positive reactions. Further investigation showed that this was caused by contamination in the NAD preparation. Since NAD cannot be isolated in pure form, it was necessary to decrease the amount used in the original staining methods. This limited the activity of the contaminants without influencing the true reaction.

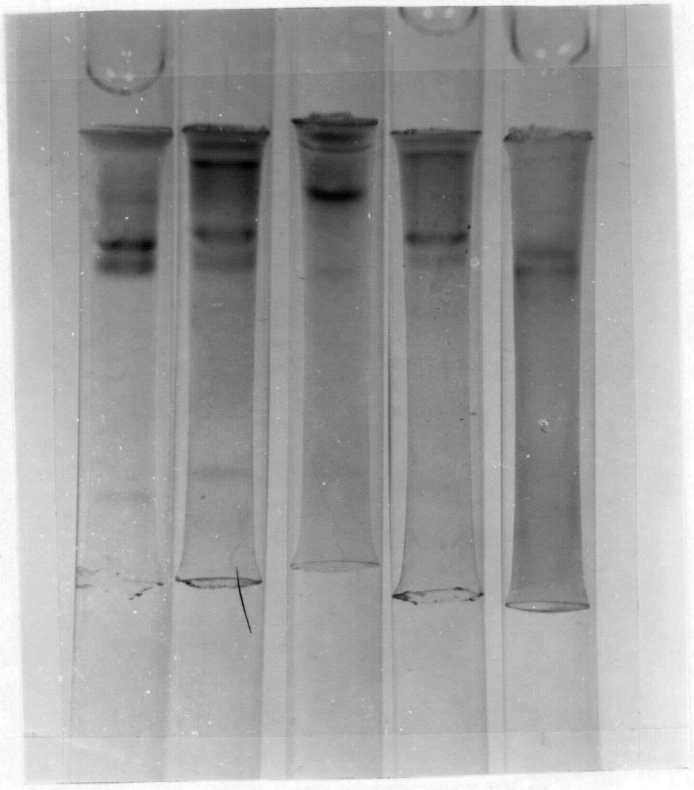


Figure 12. Enzyme stained gels showing patterns of the alkaline phosphatase systems present in C. botulinum and the nontoxic group.

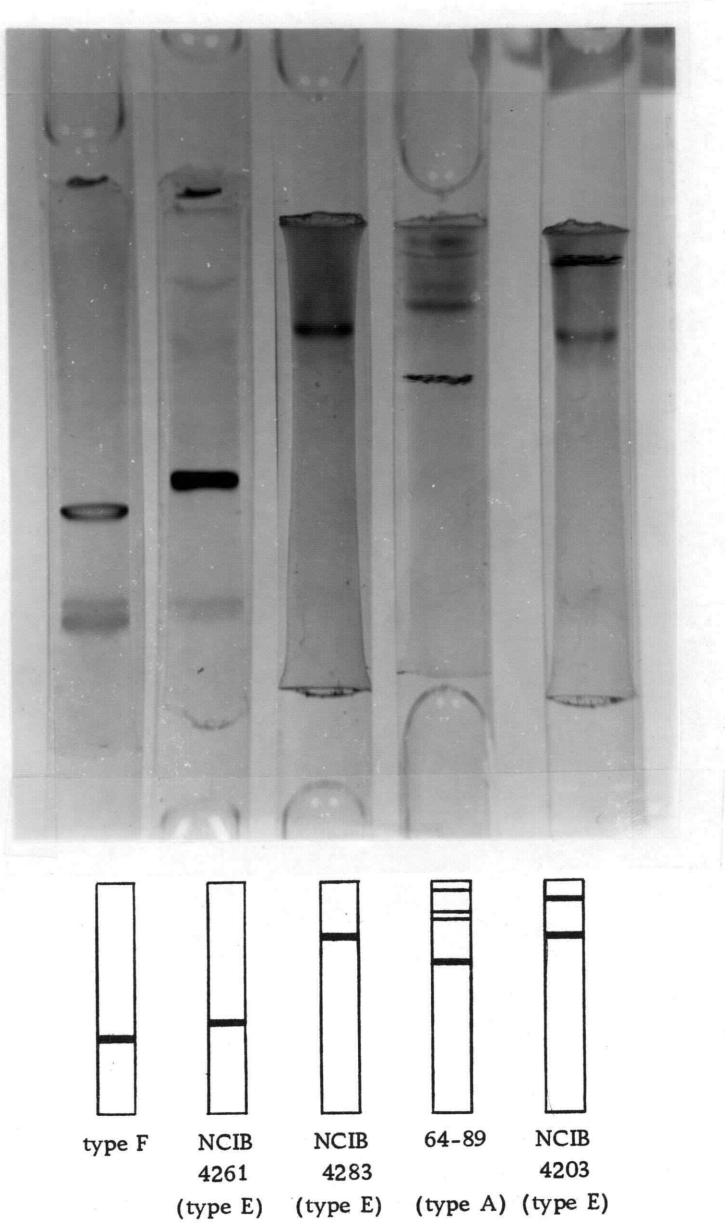


Figure 13. Enzyme stained gels showing patterns of the lactic dehydrogenase (first two on left) and the alkaline phosphatase systems present in C. botulinum and the nontoxic group.

Ferredoxin

The ferredoxin of C. botulinum types A (33A) and E (NCIB 4261) were separated by DEAE-cellulose column chromotography. Type A ferredoxin showed more of a reddish-brown color than did type E. The ferredoxins from both types as they occurred on the columns are shown in Figure 14.

Stained gels containing the purified ferredoxin from types A and E showed several additional protein bands, which may be impurities or possibly isoferredoxins. The type E ferredoxin band was lower on the gel column than that of the type A.

Ferredoxins were demonstrated by disc gel electrophoresis in the extracts from all the strains by iron protein staining and by comparing the positions of the iron bound proteins to that of a ferredoxin control. The area containing the ferredoxin was similar to that in which the reduced tetrazolium dye was oxidized (light area) upon exposure of the gel to the dehydrogenase staining systems. Figure 15 shows the positions on the gels of ferredoxins from purified preparations of C. botulinum types A and E and C. pasteurianum and from crude extracts of C. botulinum types A and E.

The maximum absorption spectra for types A and E were 262 mμ and 258 mμ, respectively (Figure 16). The maximum absorption peaks disappeared upon the reduction of the ferredoxins by the

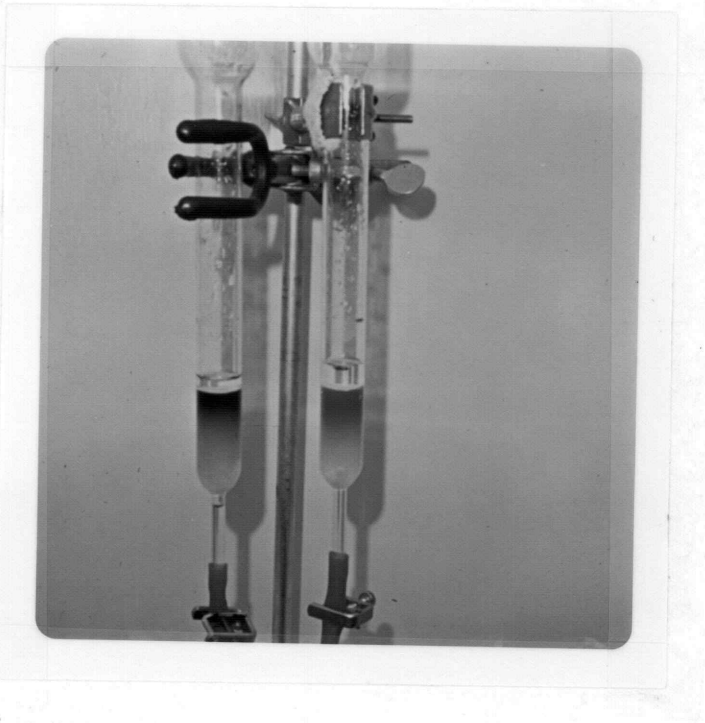


Figure 14. DEAE-cellulose columns containing the ferredoxins of NCIB 4261 (left) and 33A (right).

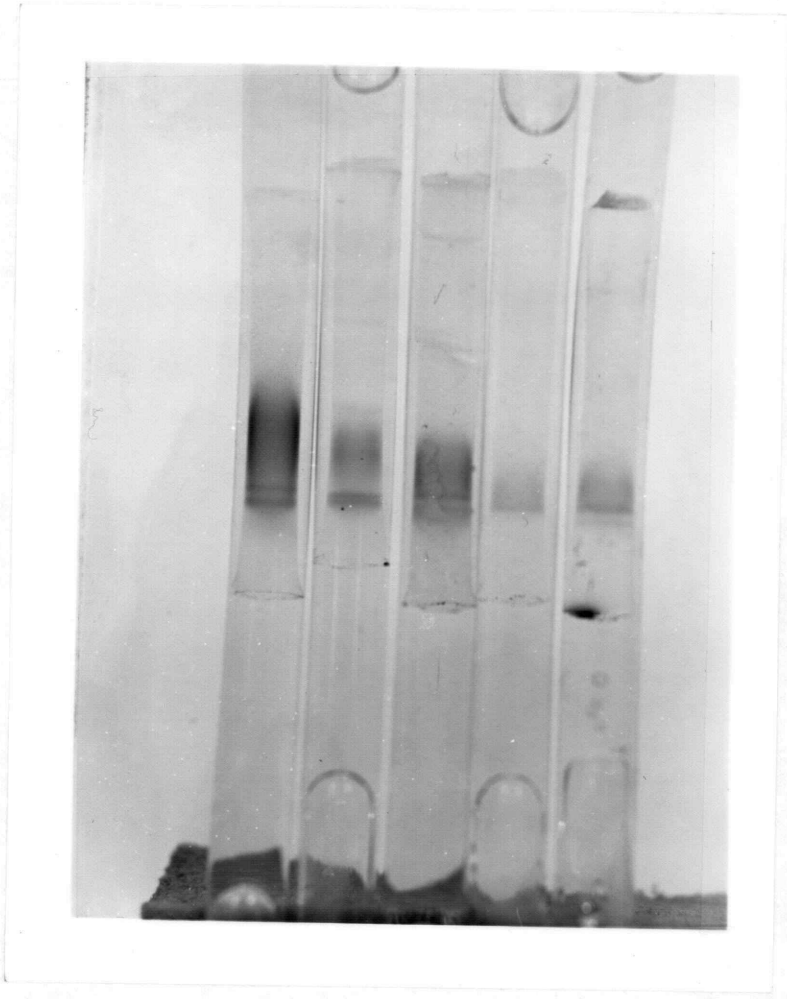


Figure 15. Gels stained for ferredoxin. Left to right, C. pasteurianum, C. botulinum type A, C. botulinum type E, C. botulinum type E crude extract, and C. botulinum type A crude extract.

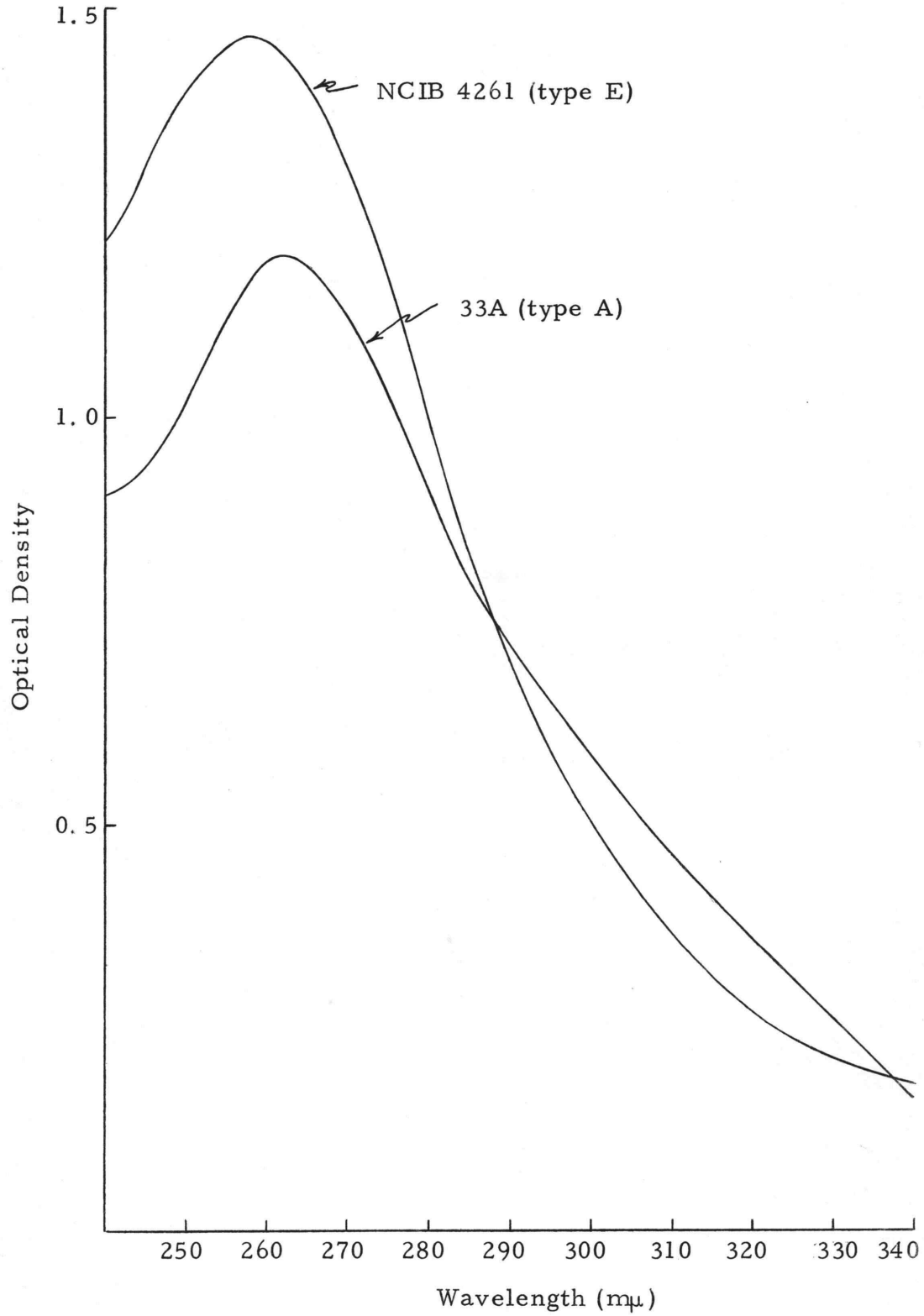


Figure 16. Absorption spectra of the ferredoxins of C. botulinum types E (NCIB 4261) and A (33A).

addition of sodium hydrosulfite.

Phosphoroclastic Reaction

Cell-free extracts prepared by centrifugation required 40,000 x G to obtain a clear preparation.

The particulate fractions of C. botulinum types A and E in the presence of methyl viologen and sodium hydrosulfite produced hydrogen, indicating hydrogenases (Figure 17). The soluble fractions under similar conditions showed no hydrogenase; on the other hand in the presence of pyruvate, CoA, and potassium phosphate, they reduced methyl viologen and produced small amounts of acetyl phosphate.

Combining the insoluble and soluble fractions of the strain with potassium phosphate, CoA, and pyruvate resulted in the production of hydrogen when either 16 μM of methyl viologen or 1.0 mg of strain specific ferredoxin was introduced into the system (Figure 18).

Using the above system for the type E strain and varying the concentration of methyl viologen or ferredoxin resulted in an increase in evolution of hydrogen per unit time (Figure 19). Hydrogen production in the presence of methyl viologen appeared to be linear to two μM and reaching a peak at four μM followed by a slight decrease. However with ferredoxin, the activity departed from a first

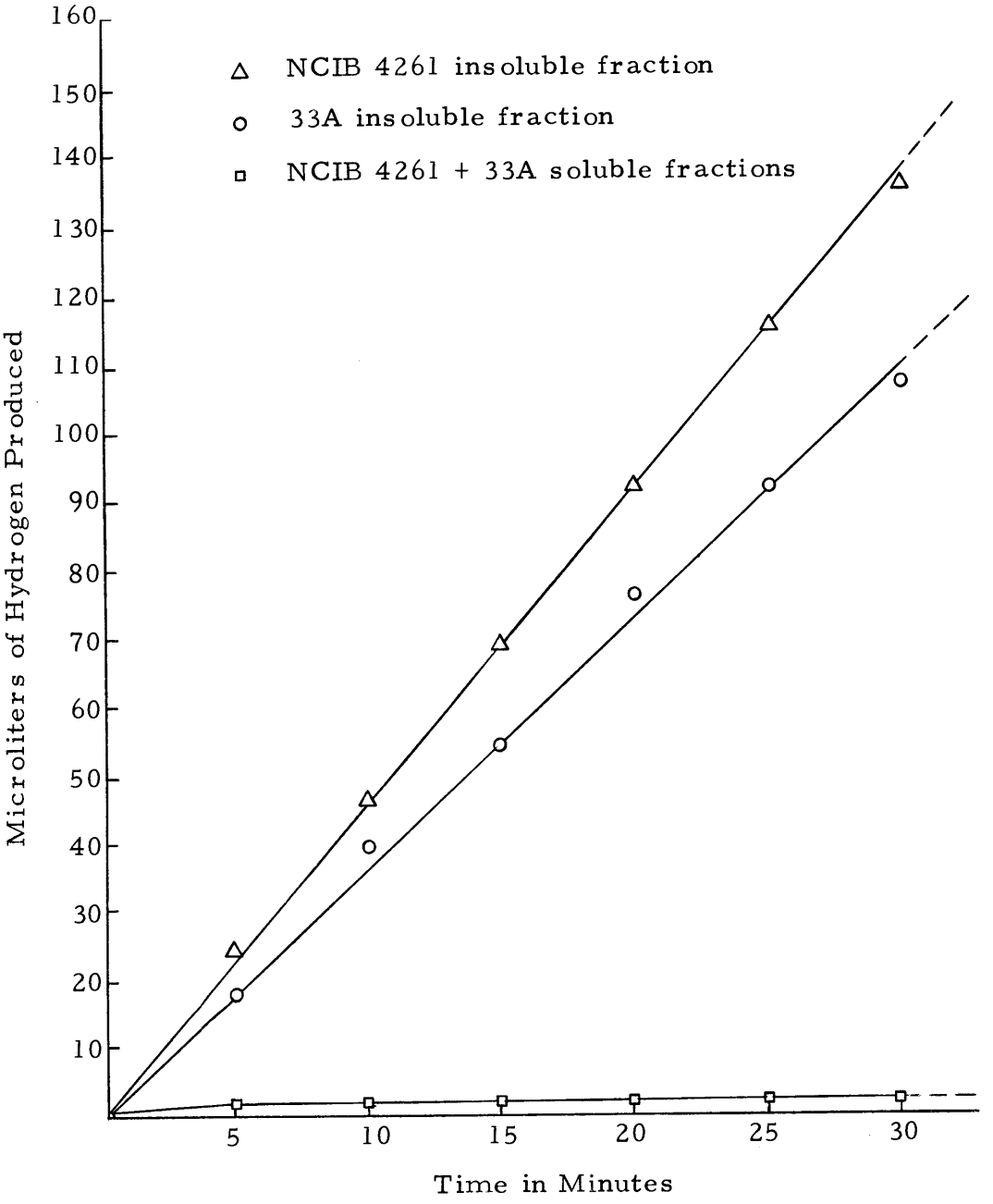


Figure 17. Hydrogen gas produced using hydrogenase containing insoluble fractions from types E and A and methyl viologen redox dye with sodium hydrosulfite electron donor.

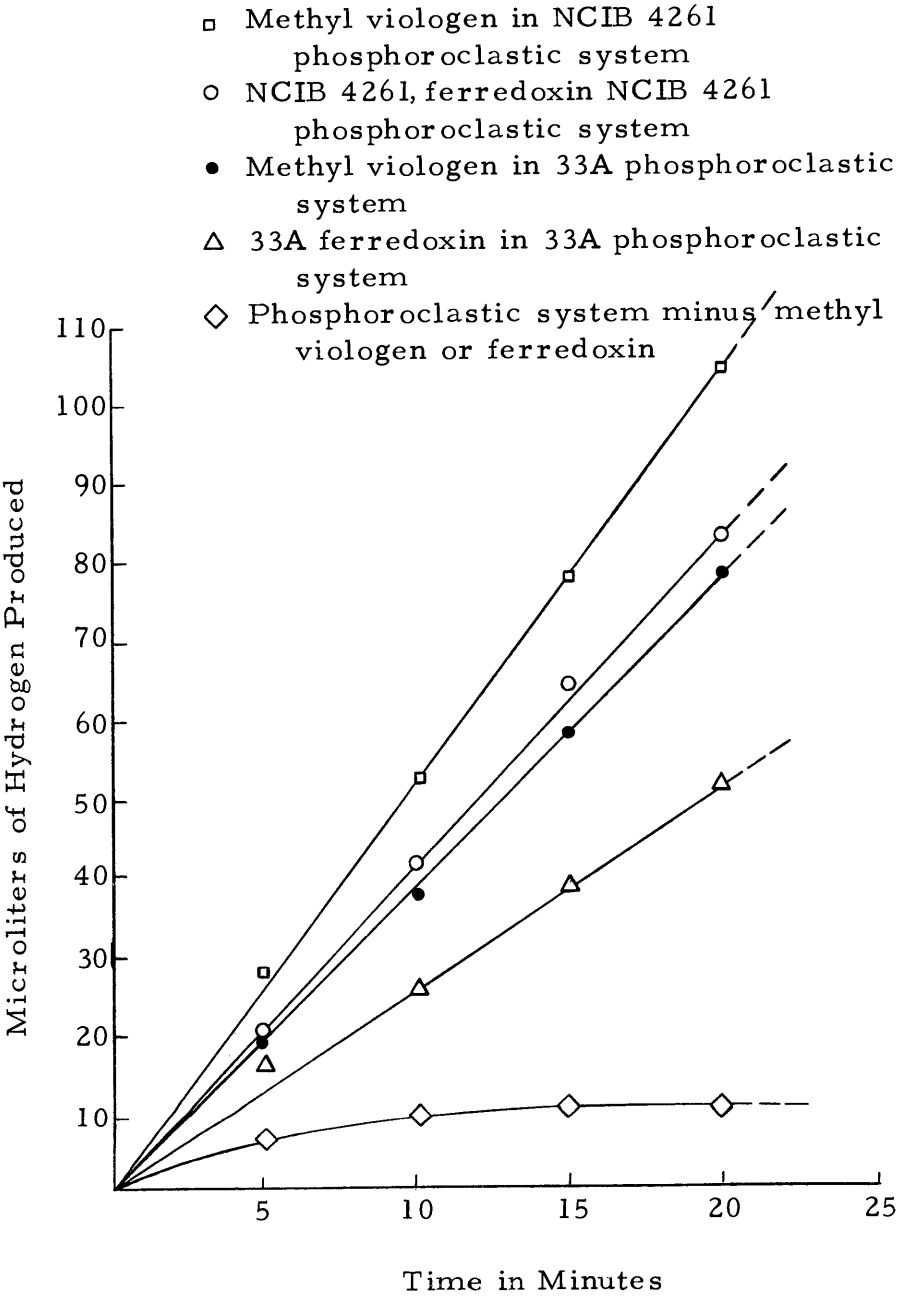


Figure 18. Hydrogen gas produced using methyl viologen or ferredoxin obtained from types A and B and a ferredoxin-free phosphoroclastic system obtained from each type.

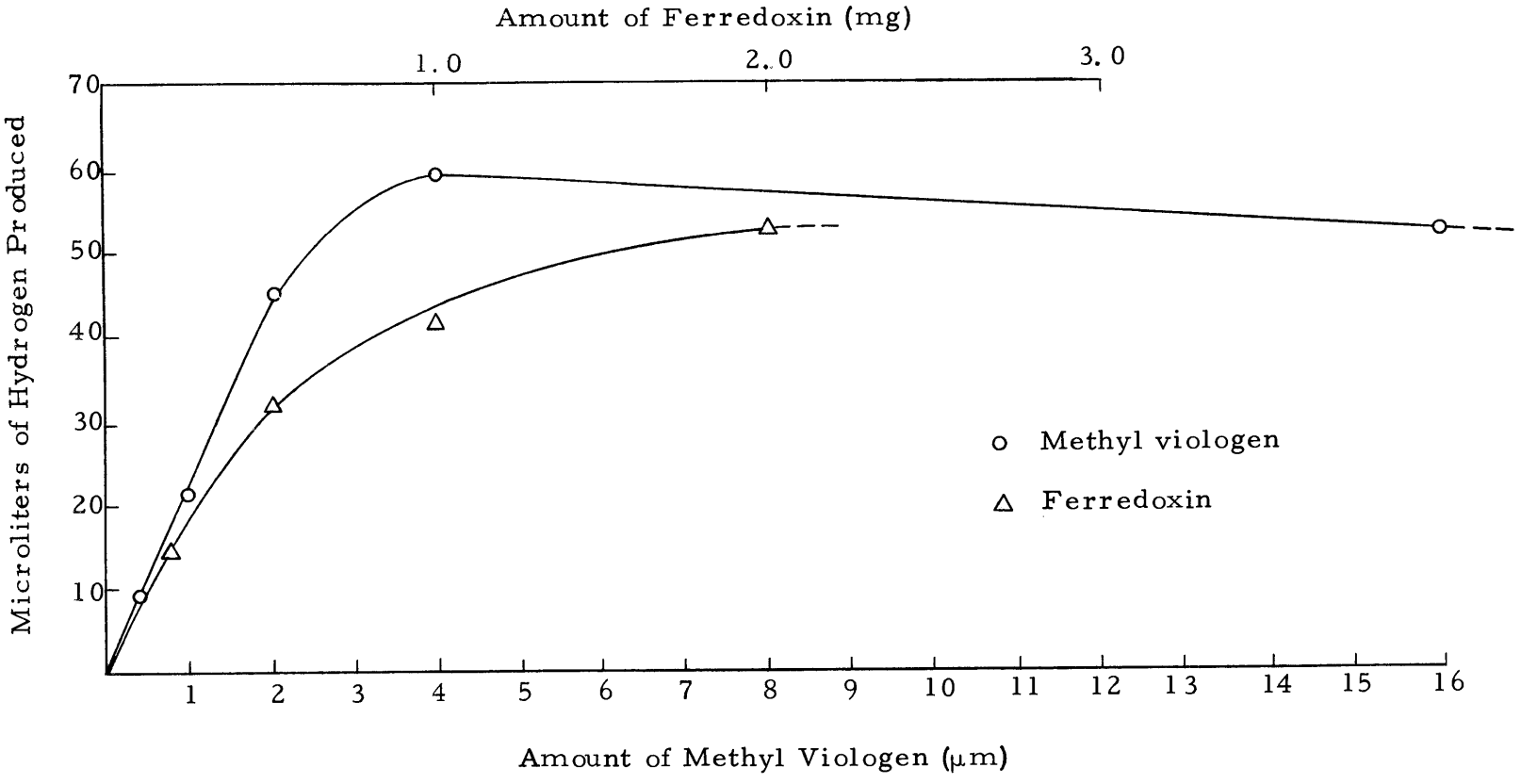


Figure 19. Hydrogen produced per 10 minute interval from methyl viologen or type E ferredoxin when either were introduced into a ferredoxin-free type E phosphoroclastic system.

order reaction at 0.5 mg and approached zero near two mg. Similar results for types A and E were observed in the measurement of acetyl phosphate using the above system (Figure 20).

A phosphoroclastic type reaction was observed in C. botulinum types A and E and C. pasteurianum irregardless of the source of ferredoxin (Figures 20 and 21).

α-Ketoglutaric Dehydrogenase

Cell-free extracts of NCIB 4261 analyzed both manometrically and spectrometrically were negative for α-ketoglutaric dehydrogenase.

Proteolytic Activity

The strains of C. botulinum types A and B liquefied the geletin within 36 hours, while strains of C. botulinum types E, C, and F and the nontoxic group were nonproteolytic.

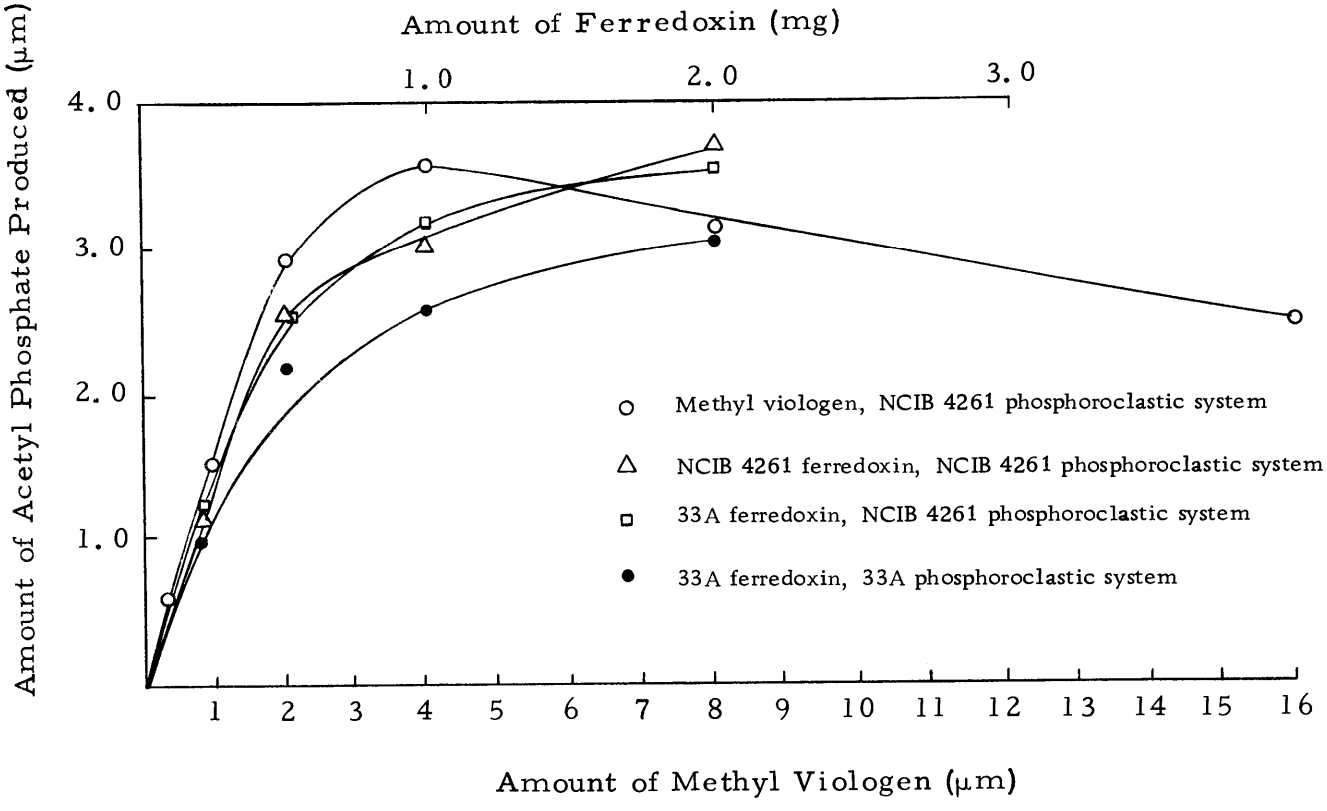


Figure 20. Acetyl phosphate produced per 10 minute interval from methyl viologen or type E ferredoxin when either were introduced into a ferredoxin-free type E phosphoroclastic system and from type A ferredoxin upon the introduction into types A and E ferredoxin-free phosphoroclastic systems.

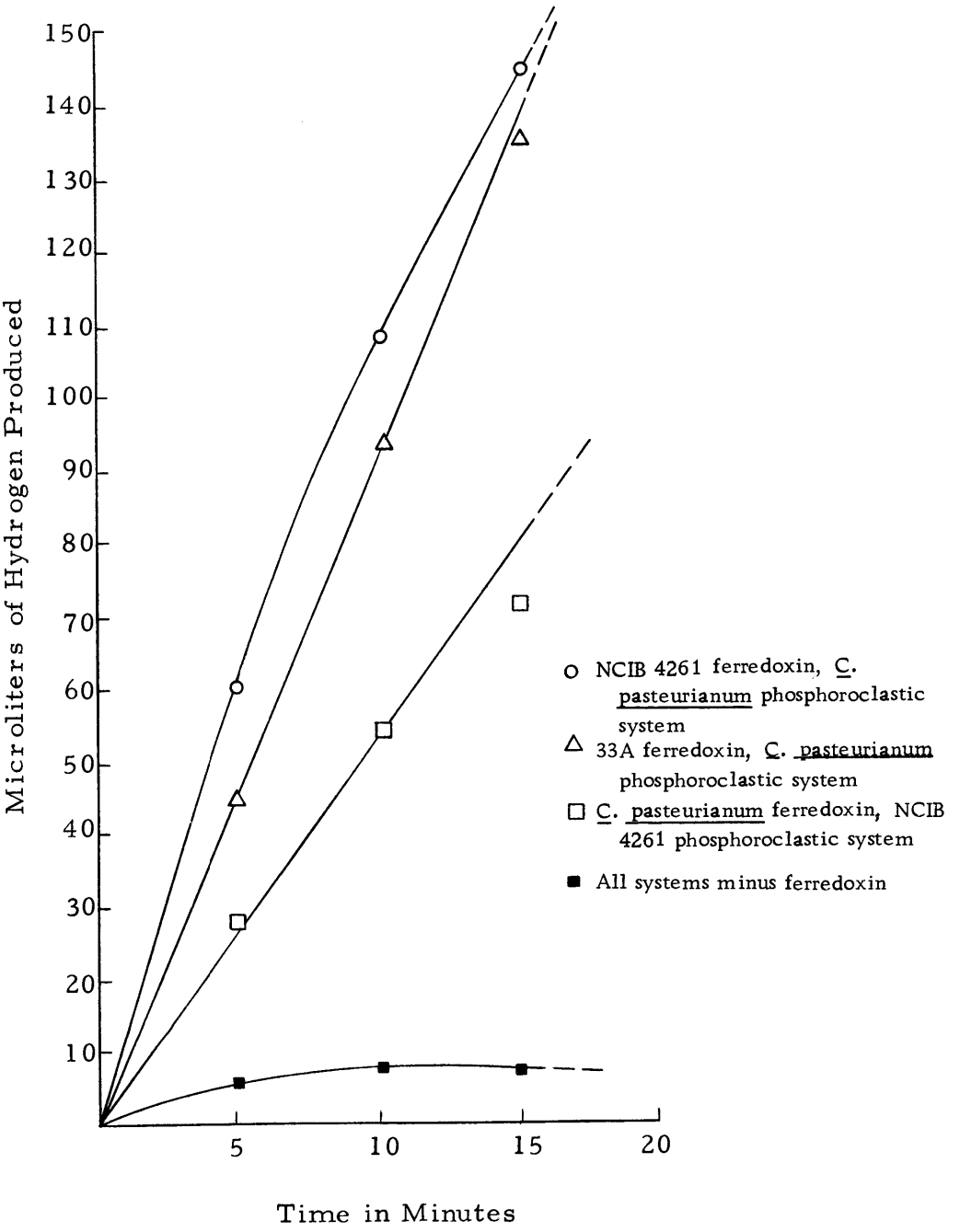


Figure 21. Hydrogen produced by the addition of C. bolulinum types A or E ferredoxins to C. pasteuranium ferredoxin-free phosphoroclastic system and the addition of C. pasteurianum ferredoxin to type E ferredoxin-free phosphoroclastic system.

DISCUSSION

Since the metabolic state of a microorganism may influence the number and types of proteins which can be electrophoretically separated, the disc electrophoretic study was restricted to investigation of C. botulinum strains grown under essentially the same cultural conditions (Wroblewski, 1961).

The results indicate that disc electrophoresis per se has only limited value for identifying the types as well as the nontoxic strains on the basis of total protein patterns. In view of the standardized procedure followed, a major cause of the discrepancies occurring between the protein patterns of strains of the same type may lie either in the culture extraction method, the electrophoretic manipulation, or the staining method. Aside from this, however, no explanation can be offered.

One of the chief difficulties encountered in this method was direct comparison of the gels. Theoretically, a given protein fraction should migrate at a rate which is relative to the marker dye and should always be the same irrespective of the distance run by the marker dye. This was usually not the case and, thereby, prevented the use of figures similar to the R_f values employed in chromatography. This difficulty was also encountered when the marker dyes of the various gels were run exactly the same distance.

The chief disadvantage of the disc method of electrophoresis is that different samples are run in separate gels, and precise identification of the bands in one gel with those in another is not always clearly defined, especially with the protein stained gels where 13-15 fractions were present in most of the samples. Thus even though the patterns of C. botulinum types and the nontoxic strains are demonstrably different from each other, it is not yet possible to indicate precisely which fractions are common to all. This disadvantage is not present in methods employing a large flat gel, in which samples are run side by side in the same gel. On the other hand, the disc method gives better separation of protein fractions and permits the use of larger volumes of a sample as well as achieving separation in a much shorter time. However, it is possible that increased resolution can also be obtained by using the disc method for the initial separation, after which each band can be further separated by running the disc gel in the opposite direction on a flat gel.

The application of the method which permitted two samples to be run in the same disc gel has circumvented some of this difficulty of direct comparisons. The results showed that the differences between two strains can be demonstrated quite clearly. This can also be used to show the precise comparison of enzyme patterns of the different samples. However, it does have the single disadvantage of

limiting the number of samples that can be compared directly. Then again it has all the advantages of disc gel electrophoresis.

The results showed that enzyme systems of C. botulinum when identified by gel electrophoresis may have definite potential in identifying the strains or types. However, more strains of the C. botulinum types and additional enzyme systems must be examined before any definite conclusion for future application can be made.

Of the enzyme systems investigated, malic, isocitric, and succinic dehydrogenases have not been demonstrated in strictly anaerobic bacteria. Alkaline phosphatase and lactic dehydrogenase have not been demonstrated in C. botulinum. The presence of lactic dehydrogenase in type A was found to be variable depending upon the strain. This appears to be evident in type B as suggested by the combined results of this study and that described by Clifton (1940) for strains of types A and B, in which the production of lactic acid was observed, implying the presence of lactic dehydrogenase (Mahler, 1966).

In most biological systems demonstrating succinic dehydrogenase, the enzyme was found to be associated with flavin adenine dinucleotide (FAD) and bound firmly to a peptide linkage (Conn and Stumpf, 1963). Since C. botulinum showed no succinic dehydrogenase activity in the presence of FAD and in the absence of NAD, the FAD-enzyme complex may not exist in this microorganism. If the

the complex were present in the gel upon analysis, its reduced form would have reacted with phenozine methosulfate making the negative controls positive (Mahler, 1966). If the protein were separated from FAD during extraction or electrophoretic treatments, it would be probable that the presence of FAD in the system would have produced activity, unless the site for FAD attachment was damage. At this stage, additional study is necessary in order to understand the full significance of this phenomenon.

The demonstration of multiple molecular enzyme forms or isozymes was not unexpected, since they have been demonstrated in other systems (Wroblewski, 1961). The occurrence of isozymes has been questioned or attributed to artifacts. However before a definite conclusion can be reached, a comparison of the purified multiple molecular forms on the basis of specific activities, kinetic constants, affinities of the reactants, physiological properties, and immunological characteristics must be made (Kaplan, 1963).

Differences between the ferredoxins of types A and E in maximum absorption spectrum, position on acrylamide gel, and elution characteristics from DEAE-cellulose column suggest structural heterogeneity. The ferredoxin of type E appears to be more electronegative as demonstrated by a greater mobility during electrophoresis and a greater affinity for DEAE-cellulose. Their absorption spectra differ from those of other clostridial ferredoxins, which

showed maximum absorption at 390 mμ and a distinct species-specific absorption in the ultraviolet regions at 280-300 mμ (Valentine, 1964).

The hydrogenases of types A and B were shown to be associated with the insoluble fractions of the cells. This is contrary to previous reports for the clostridia, which demonstrated soluble hydrogenases (Ackrell, 1966). The difference in activity of the hydrogenases between the types as shown in Figure 18 may be due in part to the method of preparation.

The formation of acetyl phosphate and the reduction of methyl viologen upon the introduction of the soluble fraction to a system containing methyl viologen, pyruvate, CoA, and potassium phosphate suggested that this fraction contains pyruvic oxidase and phosphotransacetylase.

The results shown in Figures 18, 19, and 20 strongly suggest the presence of a phosphoroclastic system in C. botulinum types A and E similar to that described for C. pasteurianum by Mortenson et al. (1963). This system contributes in part to the formation of hydrogen produced by the majority of C. botulinum strains through the electron transport action of ferredoxin and the use of hydrogen ions as terminal electron acceptors. It may lead to the formation of adenine triphosphate (ATP) through the ensuing reaction, ADP + acetyl phosphate $\rightleftarrows$ acetate + ATP, catalyzed by acetyl kinase. The

presence of this enzyme was described in C. botulinum type A by Simmons and Costilow (1962). This system (Figure 22) may also be a principle step in the synthesis of aspartate and glutamate (described later) from carbohydrate and acetate precursors.

The ferredoxins of the C. botulinum types A and E and C. pasteurianum were shown to be interchangeable without affecting the activity of the phosphoroclastic reaction. This is not surprising since similar results were demonstrated upon interchanging ferredoxins between bacterial species and plant and bacterial species (Valentine, 1964).

In addition to ferredoxin's role in the phosphoroclastic reaction, the results shown in Figure 18 strongly suggest that it may participate in the evolution of hydrogen from an aqueous non-biological system of sodium hydrosulfite as demonstrated in other clostridia (Valentine, 1964).

The dehydrogenases of isocitric, succinic, and malic in operation with the enzymes of the phosphoroclastic system may have roles in the formation of some essential amino acids and energy rich compounds necessary for cellular metabolism. The amino acids to be formed directly would be aspartate and glutamate. Glutamate would be derived from α-ketoglutarate by means of both or either transamination and/or the glutamic dehydrogenase reaction (Mahler, 1966). A reversible glutamic dehydrogenase reaction and

transamination involved in the formation of glutamate have been demonstrated in a few clostridia (Nisman, 1953). Aspartate would be derived from both or either fumarate and/or oxalacetate by means of a reaction catalyzed by aspartase and/or transamination, respectively (Mahler, 1966; Umbarger and Davis, 1962). Other essential amino acids could be formed from transamination involving glutamate and aspartate in individual reactions. Transamination involving glutamate and aspartate has been demonstrated in clostridia and other species of bacteria (Nisman, 1953; Mahler, 1966; Umbarger and Davis, 1962). These amino acids would then be available as substrates for use in the Stickland reaction, an important source of energy rich compounds in the clostridia (Nisman, 1953; Clifton, 1940).

A purposed system for the formation of aspartate and glutamate in C. botulinum is shown in Figure 22. This postulated system is based on the coordination of the results in this study with those of previous studies on the metabolism of C. botulinum and other clostridia in addition to generally accepted biochemical principles. The metabolic reactions, which have been demonstrated in C. botulinum are represented by unbroken arrows. Those assumed to be present, but have only been demonstrated in other microbial species are represented by the broken arrows. In this pathway isocitric is assumed to be formed in the same manner as in the citric acid cycle,

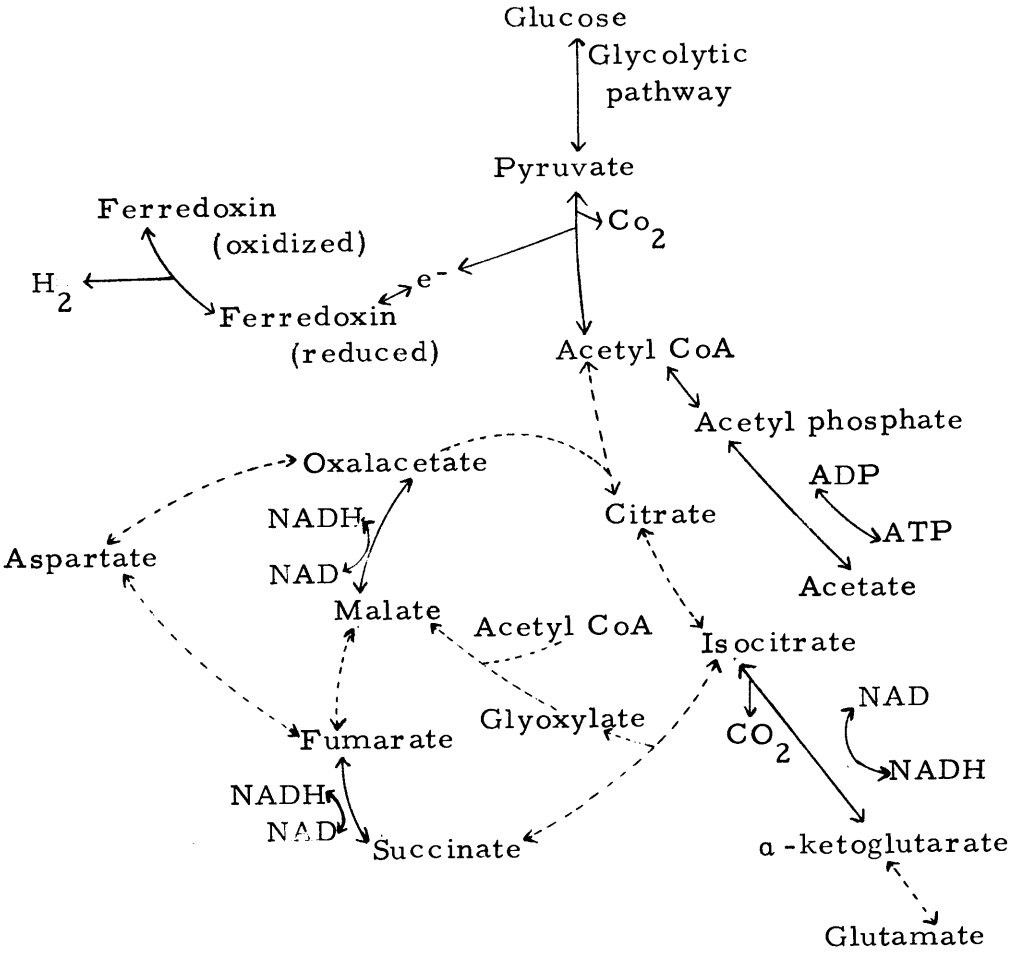


Figure 22. A proposed system in C. botulinum for the synthesis of aspartate and glutamate from carbohydrate and acetate sources.

originating from the condensation of acetyl CoA and oxalacetate (Mahler, 1966).

For the majority of C. botulinum strains, which are nonproteolytic, a system such as shown in Figure 22 would be feasible because of its capability of synthesizing some essential amino acids not provided in the cells' environment. On the other hand in the proteolytic C. botulinum strains, which are able to obtain essential amino acids exogenously, a more controlled type of amino acid synthesis must be considered.

Day and Costilow (1964a, b) observed that an increase in metabolic activity as well as acetate uptake occurred during the initiation stage of sporulation in C. botulinum. During the maturation of the spores, the metabolic activity decreased to the vegetative cell level of activity and the acetate level in the environment increased slowly. The results of this present study demonstrated isocitric dehydrogenase activity in the proteolytic C. botulinum only during the presporulation period.

These results suggest that during the vegetative state of the proteolytic C. botulinum cells, the enzymes contributing to the formation of citrate, isocitrate, and α-ketoglutarate are repressed because of the abundance of essential amino acids provided exogenously by the enzymatic degradation of proteins, polypeptides, or peptides. This repression ultimately prevents the formation of

aspartate and glutamate leaving only the phosphoroclastic system operational to form hydrogen, acetate, and ATP.

During the initial stages of sporulation when the energy demand is increased, the essential amino acid supply is limited by the cell's proteolytic ability. Thus the repressed enzymes are activated, and the microorganism obtains the essential amino acids exogenously and endogenously in an amount necessary to satisfy the increased metabolic demands of sporulation. During acetate utilization, this compound may be activated to form acetyl CoA via the acetyl kinase and the phosphotransacetylase reactions as a means of providing more carbon structures for the synthesis of aspartate and glutamate.

From the available evidence, it appears plausible that control of aspartate synthesis may be directly associated with the repressible enzymes (Figure 22). Such a concept would suggest the existance in C. botulinum of an enzyme similar to isocitritase, which catalyzes the formation of succinate from isocitrate and is common in microorganisms (Mahler, 1966; Conn, 1963). It is also possible that a type of malic synthetase may be present [the association of this enzyme with isocitritase is quite common in many microorganisms (Mahler, 1966)] to catalyzed the formation of malate from glyoxylate and acetyl CoA in providing available sources of carbon structures to be used in the formation of amino acids.

SUMMARY

Vegetative cell-free extracts of strains of C. botulinum types A, B, C, E, and F as well as the similar nontoxic strains were exposed to disc gel electrophoresis and examined for total protein and enzyme patterns. It was found that the types and the similar nontoxic group differed only in the number of protein fractions in the gels, and that this was not consistent.

Enzyme staining of the protein-laden polyacrylamide gels of the strains showed single or multiple molecular forms for malic (NAD and NADP), isocitric (NAD), succinic (NAD), and lactic (NAD) dehydrogenases and alkaline phosphatase. Analyzing the enzyme patterns of the strains revealed that most of these enzyme systems are useful for distinguishing the types and the nontoxic strains.

A method which allowed two samples to be run in the same polyacrylamide gel showed that the differences between the total protein patterns of two strains can be demonstrated clearly.

A type of iron bound protein (ferredoxin) was isolated from C. botulinum types A (33A) and B (NCIB 4261) using a modification of the method recommended by L. E. Mortenson for isolating ferredoxin from C. pasteurianum. This method involved acetone and diethylaminoethyl cellulose treatments followed by ammonium sulfate fractionation. Portions of the isolated ferredoxin were separated by

disc electrophoresis, and following specific iron bound protein staining, showed a positive reaction in the same position on the gel column as first demonstrated using cell-free extract. The purified ferredoxins of types A and E exhibited maximum absorption peaks at 262 mμ and 258 mμ, respectively.

Evidence accumulated from manometric and spectrometric analysis using cell-free extracts of C. botulinum suggests that pyruvate is metabolized through a phosphoroclastic system as demonstrated in other clostridia. It is probable that the ferredoxin has the important role of electron mediator between pyruvic oxidase and hydrogenase for hydrogen evolution and acetyl phosphate formation. The ferredoxins of the C. botulinum types A and E and C. pasteurianum were shown to be interchangeable without affecting the activity of the phosphoroclastic reaction.

A purposed system linking the dehydrogenases of malic, isocitric, and succinic and the enzymes of the phosphoroclastic system to the synthesis of aspartate and glutamate in C. botulinum was described. This postulated system incorporates a glyoxylate bypass associated with a partial citric acid cycle, some of the components of which serves as substrates in the reactions forming aspartate and glutamate. Such a system would be operational in the nonproteolytic strains during all stages of growth and only in the

proteolytic strains during intense metabolic activity as occurring in the pre-sporulation stage.

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