THE EFFECT OF OXIDATION-REDUCTION POTENTIAL ON THE OUTGROWTH, TOXIN PRODUCTION, AND CHEMICAL INHIBITION OF *CLOSTRIDIUM BOTULINUM* TYPE A SPORES

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INTRODUCTION

Clostridium botulinum, the causative agent for botulism, is recognized as a potentially severe public health hazard by the food industry since very large numbers of commercial food products could become contaminated with *C. botulinum* and endanger the health of the consumers. Food production and preservation is a rapidly growing industry and the development and implementation of adequate processes and strict quality assurance procedures must be constantly emphasized. The production and distribution of adulterated food products implicated in outbreaks of botulism bring about many adverse consequences for the food processor such as financial and legal responsibilities.

The potential hazard of *Clostridium botulinum* is demonstrated in the development of the thermal processes for low acid canned foods. Such processes must reduce the probability of survival of *C. botulinum* spores which may be present in the food to an insignificant level. Another practice in the food industry used to inhibit or destroy *C. botulinum* spores is the use of nitrite in cured meat production. Similarly other chemicals have been utilized to retard or inhibit the presence and proliferation of toxic microorganisms. Among these are sodium chloride, sucrose, glycerol, and the benzoates and sorbates. With these food additives, technologists in the food industry have been able to develop many shelf stable products.

In investigations on the use of chemicals for inhibition of *C. botulinum*, it is necessary to carefully control the variables inherent

in the experimental design. Such variables include: the type and strain of the organism, the cell type (whether spore or vegetative cell), inoculum size, temperature of incubation, and the composition, pH, and oxidation-reduction potential (Eh) of the medium. If these variables are maintained at levels optimal for growth of the test organism, only the specific efforts of the inhibitory substances may be observed.

Foods are diverse in composition and display a wide range of Eh values. The control of *C. botulinum* growth and toxin formation by manipulation of the oxidation-reduction potential in foods may provide important technology for future product development and preservation. The following study was designed to determine the effect of the oxidation-reduction potential of the medium on the outgrowth, toxin production, and chemical inhibition of *Clostridium botulinum* type A spores.

REVIEW OF LITERATURE

A. Clostridium botulinum type A and Botulism

Human botulism, a foodborne intoxication, is caused by *Clostridium botulinum* types A, B, E, and F. The microorganism is an anaerobic, sporeforming, gram positive bacillus, motile by means of pertrichous flagella. The most outstanding characteristic of *C. botulinum* is the type-specific, heat labile, protein exotoxin which is produced during vegetative cell growth and released upon cell lysis. Ingestion or injection of this toxin may cause death in man by a neuro-paralytic mechanism (Boroff, *et al*. 1971). Identification of *C. botulinum* types can be made by *in vivo* toxin neutralization tests. Determination of cultural characteristics allows differentiation of the *C. botulinum* species into physiological groups with the proteolytic types A and B, and non-proteolytic type E being the ones most frequently involved in human botulism (L. DS. Smith, 1975). The natural habitat of *C. botulinum* is the soil and it has been isolated from soil throughout the world. Its distribution appears to be on a regional basis in the United States with type A predominately isolated in the western states and type B in the soils of the eastern states (Riemann, 1968). Types A, B, and F were the only strains of *C. botulinum* found off the coast of Southern California (Eklund and Poysky, 1968). Investigative studies in the Pacific have shown type A present in the soils of Hawaii and China (Meyer and Scheonlotz, 1922). The spores of type A along with types B, C, and D were found in soil samples of Australia

(Eales and Gillespie, 1947). Further north, type A has been demonstrated in Sweden (Fahraeus, 1949), and Great Britain (Haines, 1942). However, the occurrence of type E is more regional than that of the other types, it being confined to the bottom sediments of fresh and marine waterways (L. DS. Smith, 1975). Botulism in man is almost always caused by ingestion of poorly preserved food in which _C. botulinum_ has grown. The resistance of its spores to drying and heat allow them to become dustborne and contaminate a variety of foodstuffs such as preserved vegetables and meats (Lewis and Hall, 1968). During the 23 year period of 1950-1973, the majority of diagnosed human cases of botulism in which the type was determined was attributed to type A (USPHS, 1974a); thus considerable research interest has been devoted to this etiological agent.

B. Growth, Toxin Production and Chemical Inhibition of _C. botulinum_

For types A and B the optimum growth temperature is 35 to 37 C, while 25 to 30 C is the optimum temperature for toxin production as summarized in a review by Hobbs (1976). However, Bonventure and Kempe (1959) reported the rate of toxin synthesis and toxin yield for their strain of type A was maximum at 37 C. The non-proteolytic type E was found to grow best at 30 C (Riemann, 1968). The proteolytic strains of types A and B having a minimum toxin synthesis and growth temperature of 10 to 18 C are less tolerant of low temperatures than type E (Bonventure and Kempe, 1959). The minimum for type E being reported as 3.3 C (Schmidt, _et al_. 1961). The maximum growth temperature for proteolytic strains is between 40 and 50 C (Hobbs, 1976).

Studies by Townsend, et al. (1954) and Dozier (1924) on type A outgrowth and toxin production in a variety of food products adjusted to various levels of pH found that growth may or may not occur at pH values between 4.8 and 5.0 depending upon the substrate. The reported limiting pH values for growth of spore inocula of types A and B are 4.6 to 5.0 (Ingram and Robinson, 1951) (See Table 1). Growth was found to occur at pH 5.25 to 5.30 but not below pH 5.25 for an inoculum of vegetative cells and spores of type A in brain infusion media (Meyer and Kietzman, 1957). Growth and toxin production by C. botulinum in home canned pears at pH 5.8 in which lactobacillus was also isolated was reported by Meyer and Gunnison (1929). Maximum toxin levels for C. botulinum type A were demonstrated between pH 5.5 and 8.0; however, the toxin was reported to be much more stable at pH 6.5 and below (Bonventure and Kempe, 1959). From an analysis of published data, Riemann, et al. (1972) indicated that by changing the pH from 7.0 to 5.0 allowed decreasing concentrations of sodium chloride to inhibit the growth and toxin formation of C. botulinum types A and B. Toxin production without apparent growth in a nutrient medium containing 10.5% NaCl was reported by Tanner and Evans (1953). Greenberg, et al. (1959) reported that 9% NaCl inhibited toxin formation in perishable cured meats inoculated with C. botulinum types A and B spores. Complete inhibition of growth and toxin production of types A and B spores by 8.5 to 10.5 percent sodium chloride was reported by Lechowich (1968) in a summary of his experiments and others (Segner, et al., 1966; Baird-Parker and Freame, 1967;

Table 1. pH limits for outgrowth of Clostridium botulinum.*

Type of C. botulinum	Inoculum	Lowest pH permitting Germination	Lowest pH permitting Outgrowth
A and B	Spores		4.6 - 5.0
A	Spores	5.3	5.3
A	Vegetative cells		5.0
B	Spores (30 C)	5.0	5.0
B	Spores (20 C)	5.0	no growth
B	Vegetative cells		5.3
E	Spores	5.0	5.3
E	Vegetative cells		5.3
E	Spores in TPG (30 C)		5.03
E	Spores in TPG (8 C)		5.90
E	Spores in liver broth	5.01	5.22
E	Spores at 15.6 C		5.2
E	Spores at 10.0 C		5.4
E	Spores at 7.2 C		5.6
E	Spores at 5.0 C		6.0

*From Lechowich (1968)

Ohye and Christian, 1967; Emodi and Lechowich, 1968a, Emodi and Lechowich, 1968b) (See Table 2). In addition, Lechowich (1968) summarized the concentrations required for inhibition by sucrose (Table 2). The inhibitory level of sucrose (50 to 55%) for growth of types A and B vegetative cells, was demonstrated by Bever and Halvorson (1948).

Sodium nitrate and sodium nitrite have been utilized as meat curing agents for a long time. They have also been demonstrated as successful inhibitors of _C. botulinum_ in these meat products. Christiansen, _et al_. (1974; 1975) have clearly shown that toxin formation by type A and type B strains of _C. botulinum_ in various cured products is inhibited by nitrite. However, there exists some controversy about the nature of the inhibitive mechanisms. Tarr, (1962) reported the inhibitory action was due to hydroxylamine formation during nitrate reduction which inhibited catalase and allowed hydrogen peroxide accumulation in the medium. Jay (1970), on the other hand, believed the formation of nitric oxide was the major inhibitive mechanism with inhibition in cheese possibly due to the increased redox potential.

Bicarbonate requirements for the germination and outgrowth of _C. botulinum_ spores have long been known. Treadwell, _et al_. (1958) demonstrated necessity for bicarbonate or carbon dioxide in the medium to stimulate germination. The rate and extent of germination was shown to increase with increasing concentration of bicarbonate up to 8.3 mM by Rowley and Feehery (1970) for a strain of type A in a

Table 2. Inhibitory levels of NaCl and sucrose required to inhibit *Clostridium botulinum* under optimal conditions.*

Type of *C. Botulinum*	Inhibitory substance	Inhibitory concentration percent (w/v)	Inhibits
A and B	NaCl	8.5 - 10.5	Growth of vegetative and spore inocula.
A and B	NaCl	6.5 in aqueous phase of ham.	Proteolysis and putrefaction--not toxin production.
A and B	NaCl	9.0	Toxin production.
A and B	NaCl	8.2	Growth of vegetative inocula.
E	NaCl	5.0	Spore outgrowth.
E	NaCl	5.8	Growth of vegetative cells.
E	NaCl	6.5 (>4.8)	Growth of vegetative cells.
E	NaCl	4.9	Spore outgrowth.
A and B	Sucrose	50-55	Growth of vegetative cells.
B	Sucrose	30	Growth of vegetative cells.
B	Sucrose	31	Spore germination.
E	Sucrose	38.5	Spore outgrowth.

*From Lechowich (1968)

chemically defined medium. A distinct stimulation of germination for the spores of C. *bifermentans* and C. *sporogenes* due to the addition of carbon dioxide to a complex medium was reported by Holland, *et al*. (1970). In addition, Ando and Iida (1970) concluded that bicarbonate was essential for germination of C. *botulinum* in their chemically defined media. Reports suggesting the active stimulant is carbon dioxide and not bicarbonate in the germination of C. *sporogenes* were made by King and Gould (1971). This conclusion was made on the observance that bicarbonate stimulation was greater at a lower pH value at which carbon dioxide would be the active species.

C. Oxidation-Reduction Potential

1. Theory

The quantitative measure of the tendency of a metal ion, a nonmetal ion, or a compound to donate or accept electrons is termed an oxidation-reduction potential. The oxidation potential of the substance refers to its donating tendency and is utilized extensively in physical chemistry. The reduction potential on the other hand, is the measure of the acceptance of electrons. These oxidation and reduction potentials are associated to chemical equilibria by the equation:

$$Eh = Eo + \frac{RT}{nF} \ln \frac{[\text{Oxidized}]}{[\text{Reduced}]}$$

in which R = gas constant, T = absolute temperature, F = Faraday constant, n = number of electrons per gram equivalent transferred

in the reaction, Eh = reduction potential of the reaction with reference to the standard hydrogen electrode, Eo = standard oxidation-reduction potential of the reaction, [Oxidized] and [Reduced] = molar concentrations of the reactants in the system. Dependent on the temperature, Eo, and the ratio of oxidized and reduced reactants in the system, the oxidation-reduction potential, Eh, is also found to be a function of the hydrogen ion concentration of the system as demonstrated:

$$Eh = Eo + \frac{RT}{nF} \ln \frac{[Ox.]}{[Red.]} + \frac{RT}{nF} \ln \frac{Ka}{[H^+] + Ka}$$

where Ka is the equilibrium constant for the acid dissociation of the reductant, i.e.

$$\frac{[Red.]\ [H^+]}{[H\ Red.]} = Ka$$

To facilitate comparison of Eh measurements taken at varying pH values, (pH X) Leistner and Mirna (1959) used the equation:

$$Eh7 = E_{observed} + E_{reference} + 2.303\ (RT/F)\ (pH\ X\ -7.0)$$

to determine the oxidation-reduction potential adjusted to pH 7.0, (Eh7). The scale of oxidation-reduction potentials was determined using the hydrogen electrode as the common standard whose potential was arbitrarily set at 0.0 mv. Potentials of unknown systems may be conveniently determined by measuring their potential difference from that of the standard hydrogen electrode. Standard half-cells of known potential with reference to the hydrogen electrode (i.e. calomel, silver-silver chloride) may also be used to determine unknown potentials when coupled to a half cell consisting of an inert surface

such as gold or platinum. This surface must be in contact with the system in which the electron flow is to be measured. Such measurements are made by connecting a potentiometer to the system. Hewitt (1950) offers a comprehensive discussion of biochemical and organic chemical systems along with their characteristic oxidation-reduction potentials.

2. Germination, growth and toxin production

Morris and O'Brien (1971) reviewed several hypotheses on the effect of oxygen and oxidation-reduction potential on growth of anaerobic microorganisms including the following: (1) oxygen itself is the toxic agent and is invariably lethal to the organism; (2) an organism which lacks catalase (e.g. a species of Clostridium), is poisoned by the H_2O_2 which it forms when reducing some of the supplied oxygen; (3) anaerobes only flourish in media of low Eh (usually in the order of -150 to -400 mv at pH 7.0) and in the presence of free oxygen, this low redox potential could neither be attained nor maintained; and (4) oxygen is so much more avid an electron acceptor than the normal terminal oxidants of fermentation, that with oxygen present the anaerobe is unable to maintain the intracellular concentration of electron donors, such as NAD(P)H, that are required for biosynthesis and growth. Each of these hypotheses has had its proponents and detractors, but none has yet emerged as a unitary explanation of obligate anaerobiosis.

The toxicity of oxygen for certain microorganisms was first proposed by Pasteur (1861). This hypothesis was supported by Liefson

(1931) who found inhibition of C. botulinum sporulation and growth at high oxygen levels. Morris and O'Brien (1971) concluded that the termination of C. acetobutylicum growth under anaerobic conditions was due to dissolved oxygen in the medium and not the elevated Eh, when using artificial electron acceptors. Momentary exposure to air during anaerobic incubation resulted in considerable reduction of a C. botulinum 62A spore population (Wynne and Foster, 1948a). Holland, et al. (1969) reported that germination of PA 3679 S2 spores appeared to be inhibited by oxygen and not the Eh of the complex media.

However, there have been numerous studies reported in which the redox potential and not the free oxygen in the medium is believed to be the limiting growth factor. Favorable oxidation-reduction potentials were determined to be an essential requirement for growth of Clostridium welchii by Reed and Orr (1943). Aubel and Houget (1945) observed that C. saccharobutyricum cells when exposed to air were not seriously injured or killed. In experiments utilizing C. sporogenes and Bacteriodes vulgatus, the Eh level of the medium and not the oxygen tension was reported to be the factor limiting growth (Hanke and Katz, 1943). Dubos (1929a, 1929b) suggested that prior to growth of anaerobic bacteria, a favorable oxidation-reduction potential must be established.

The clostridia are incapable of aerobic growth at a high oxidation-reduction potential because they are deficient in enzymes capable of utilizing oxygen. The catalase theory of anaerobic growth was postulated by McLeod and Gordon (1923a, 1923b) as result of their

experimental findings that anaerobes are devoid of the enzyme catalase. In the presence of oxygen, hydrogen peroxide will be formed and accumulated by the reductive processes of bacteria and will kill organisms lacking this enzyme. Another enzyme that strict anaerobes do not synthesize is superoxide dismutase discovered by McCord and Fridovich (1969). This is the enzyme that will catalyze the reduction of the superoxide (O_2^-) radical to hydrogen peroxide. This compound is the result of the univalent reduction of oxygen and is much more toxic than hydrogen peroxide. Bacteria vary enormously in the enzyme systems they possess and hence in their reaction to oxygen supplies and in the redox potential developed in their cultures under different conditions (Hewitt, 1950).

Growth of anaerobic bacteria may be impossible when oxidation-reduction conditions are unsuitable (i.e. upper Eh limits for growth). Fildes (1929) found that the germination of *Bacillus tetani* (*Clostridium tetani*) spores was retarded as the Eh of the medium was raised toward a positive Eh limit above which germination was inhibited. Growth of *Bacteriodes* was reported inhibited in media poised at a potential higher than +150 mv at pH 6.6 (Vennesland and Hanke, 1940). Hanke and Bailey (1945) found that growth of *C. perfringens* was inhibited at a Eh of about +150 mv at pH 6.0, while Barnes and Ingram (1956) showed a limit of +250 mv at pH 6.0 for the same species. Pearson and Walker (1976) reported that *C. perfringens* could grow at an initial Eh7 (i.e. Eh adjusted to pH 7.0) of +320 mv but not at +350 mv. Kaufmann and Marshall (1965) demonstrated that an oxidation-reduction

potential greater than +144 mv inhibited the growth of *C. botulinum* in sterilized whole milk. The inhibitive redox potential for *C. sporogenes* at pH 7.0 was reported to be about +150 mv, while for *C. tetani* it is +110 mv and *C. histolyticum* +90 mv (cited by Smith and Holdeman, 1968).

Enhancement of anaerobic growth by the addition of certain compounds capable of lowering the Eh of the medium has been reported by several investigators. Growth of anaerobes was obtained in the presence of air when the potential of the medium was adequately lowered by addition of reducing agents (i.e. cysteine, glutathione, and thioglycolate) (Quastel and Stephenson, 1926). Klieger and Guggernheim (1938) obtained replication of *C. welchii* cells in the presence of air when ascorbic acid was added to the medium. They proposed that the ascorbic acid lowered the Eh of the medium to a favorable level for growth. Reed and Orr (1943) demonstrated that sodium thioglycolate, cysteine, ascorbic acid, and sodium formaldehyde sulfoxylate produced poised oxidation-reduction potentials in peptone broth near the optimum for pathogenic clostridia. Thioglycolate was found to enhance germination of spores for PA 3679 S2 held in suboptimal oxygen concentations (Holland *et al.*, 1969).

Potter (1911) was probably the first to report the development of reduced conditions due to bacterial growth. He proposed that the decomposition of the growth medium components by bacterial metabolism caused the lowering of the redox potential. Coulter and Isaacs (1929) believed that the removal of oxygen by bacterial growth

initially reduced the Eh with the reducing substances released upon lysis of the cell further affecting the O/R potential. Douglas et al., (1973) using foil electrodes to detect Eh changes, attributed the fall in the O/R potential to liberation of H_2 and metabolites into the medium during growth. Earlier Boyd and Reed (1931a, 1931b) found that hydrogen gas was capable of lowering the potential and proposed that actively metabolizing bacteria produced hydrogen gas which then reduced the Eh of the medium. The use of polished electrodes was found to be more sensitive for monitoring Eh changes in actively growing bacterial cultures as compared to unpolished ones (Jacob, 1970). Using a polished electrode Douglas and Rigby (1974) concluded from experiments with C. butyricum that the Eh was reduced by hydrogen gas liberated during emergence and not during germination or outgrowth.

3. Chemical inhibition of growth and toxin production

The oxidation-reduction potential of various media has been found in several studies to have a marked effect on the inhibition of growth by chemical agents. Hanke and Bailey (1945) demonstrated that the growth limiting Eh for C. welchii, C. sporogenes, and C. histolyticum was significantly affected by hydrogen ion concentration. The combined effect of sodium chloride concentration and the Eh of the medium was reported to have an inhibitory effect on vegetative cell growth by C. perfringens (Mead, 1969). Growth was prevented at Eh levels of +195 and +92 mv when the medium contained 5% sodium chloride. However, growth was initiated when the Eh was poised at +66 mv in the same medium. Oxygen was utilized in these experiments to adjust the O/R

potentials of the media. More recently Smith and Pierson (1975) reported that differences in Eh under highly reduced conditions have an effect on growth limiting concentrations of chemical inhibitors. They observed no difference in growth of C. botulinum type E spores in trypticase soy broth at Eh levels of -80 and -190 mv. However, such growth inhibitors as 4% NaCl, 35% sucrose, and hydrogen ion (pH 5.0) were more effective at -80 than at -190 mv.

METHODS AND MATERIALS

A. Maintenance and Identification of the Test Strain

A vegetative culture of Clostridium botulinum type A, 10755A (NCTC 2021) in chopped meat glucose medium was obtained from the VPI&SU Anaerobe Laboratory, Blacksburg, Virginia. This strain was used in all experiments performed. The anaerobic methods of Holdeman and Moore (1975) for media preparation, culture identification, and maintenance were utilized. The vegetative broth culture was transferred to chopped meat glucose medium and incubated at 37 C for 24 hours. Roll tubes of Brain Heart Infusion agar (BHIA) were streaked to obtain isolated colonies of the organism. The BHIA consisted of the following:

Brain Heart Infusion Broth (Fisher)	3.7 g
Bacto-yeast extract (Difco)	0.5 g
Agar	0.25 g
Resazurin	0.0001 g
Vitamin K-heme solution	1.0 ml
Cysteine hydrochloride	0.05 g
Distilled water	100.0 ml

An isolated colony was picked and inoculated into chopped meat glucose medium. Following 24 hours incubation at 37 C, the culture was transferred to chopped meat medium and maintained at room temperature as the stock culture. Each month during the study, the culture was transferred to fresh chopped meat medium. The test strain identity was confirmed by biochemical tests, gas chromatographic analysis of volatile fermentation products and toxicity tests as described by Holdeman and Moore (1975).

B. Preparation of Media

1. Growth vessel media

The basal assay medium used in all growth experiments was trypticase soy broth (TSB) (BBL). To maintain consistency of Eh, pH, and growth, each dehydrated medium used was from the same control lot. Growth, toxin production and Eh7 were monitored in the following types of media:

a. Prereduced trypticase soy broth (BBL) with 0.05% cysteine containing:

(1) no additional components
(2) 30%(w/v) sucrose
(3) 35%(w/v) sucrose
(4) 40%(w/v) sucrose
(5) 7.0%(w/v) sodium chloride
(6) 6.0%(w/v) sodium chloride
(7) 5.0%(w/v) sodium chloride
(8) hydrochloric acid adjusting pH to 5.10
(9) hydrochloric acid adjusting pH to 5.20
(10) hydrochloric acid adjusting pH to 5.30

b. Oxidized trypticase soy broth (BBL) with 0.05% cysteine containing:

(1) no additional components
(2) 30%(w/v) sucrose
(3) 6.0%(w/v) added sodium chloride
(4) 5.0%(w/v) added sodium chloride

(5) hydrochloric acid adjusting pH to 5.20

(6) hydrochloric acid adjusting pH to 5.30

The media used for growth curves and Eh7 measurements in the electrode vessel were prepared by the following procedure. A one liter round bottom distilling flask (Corning Glass Works, Corning, NY) was filled with 3N hydrochloric acid for 16 hours and rinsed with distilled water to remove any substances which might produce variations in the Eh7 readings. The dry ingredients for the basal medium were added to the flask and rehydrated in 750 ml of distilled water. In order to prevent foaming during sparging and growth, a silicone anti-foaming agent, "Siliclad" (Becton, Dickinson, and Co., Parsippany, NJ) was added to the medium to produce a final concentration of 300 ppm. For the experiments on inhibition by sucrose or sodium chloride, the compounds were added to the medium at this point in their specified amounts. To eliminate traces of oxygen and other dissolved gases, the medium was boiled and refluxed for 15 minutes in the flask fitted with a refluxing chimney (Holdeman and Moore, 1975). The anaerobic medium was cooled in ice water while being sparged with prepurified nitrogen which was passed through a copper-filled furnace (Sargent-Welch, Skokie, IL) to remove any oxygen in the gas. Cysteine hydrochloride was then added to the cooled medium to a concentration of 0.05% and the pH was adjusted to pH 7.0 ± 0.1 with 5N hydrochloric acid or 8N sodium hydroxide. The prereduced medium was stoppered, placed in a press clamp and autoclaved at 121 C for 15 minutes. The medium was cooled at room temperature or placed in a cold water bath.

The oxidized medium was prepared as above, but sparged with compressed air rather than oxygen-free nitrogen during cooling after refluxing. To induce further oxidation of the medium, the stoppered flask was agitated at 180 RPM in a cold water bath to cause mixing of the air in the headspace with the medium. Media utilized in experiments on inhibition by high hydrogen ion concentration were adjusted to a pH value of 5.7 with concentrated hydrochloric acid prior to sterilization. The desired pH values for the media were obtained by aseptically adding 5N hydrochloric acid to the medium after it had been sterilized and dispensed in the electrode vessel.

2. Media for enumeration

The medium used for determination of growth was peptone-yeast extract agar (PYA; 2.5% agar) which consisted of the following:

Peptone (Difco)	15.0 g
Bacto-yeast extract (Difco)	7.5 g
Cysteine hydrochloride	0.37 g
Resazurin	0.00075 g
Salts solution (Holdeman and Moore)	30 ml
Distilled water	750 ml
Agar	18.75 g

PYA was prepared and dispensed in size 18 x 142 mm rubber stoppered anaerobic culture tubes (Bellco Glass Inc., Vineland, NJ) according to the general methods of Holdeman and Moore (1975). Dilution blanks consisted of prereduced 0.1% peptone.

C. Electrode Vessel

1. General apparatus

The electrode vessel and apparatus used in all growth experiments is illustrated in Fig. 1 (M. V. Smith, Ph.D. Thesis, VPI&SU, Blacksburg,

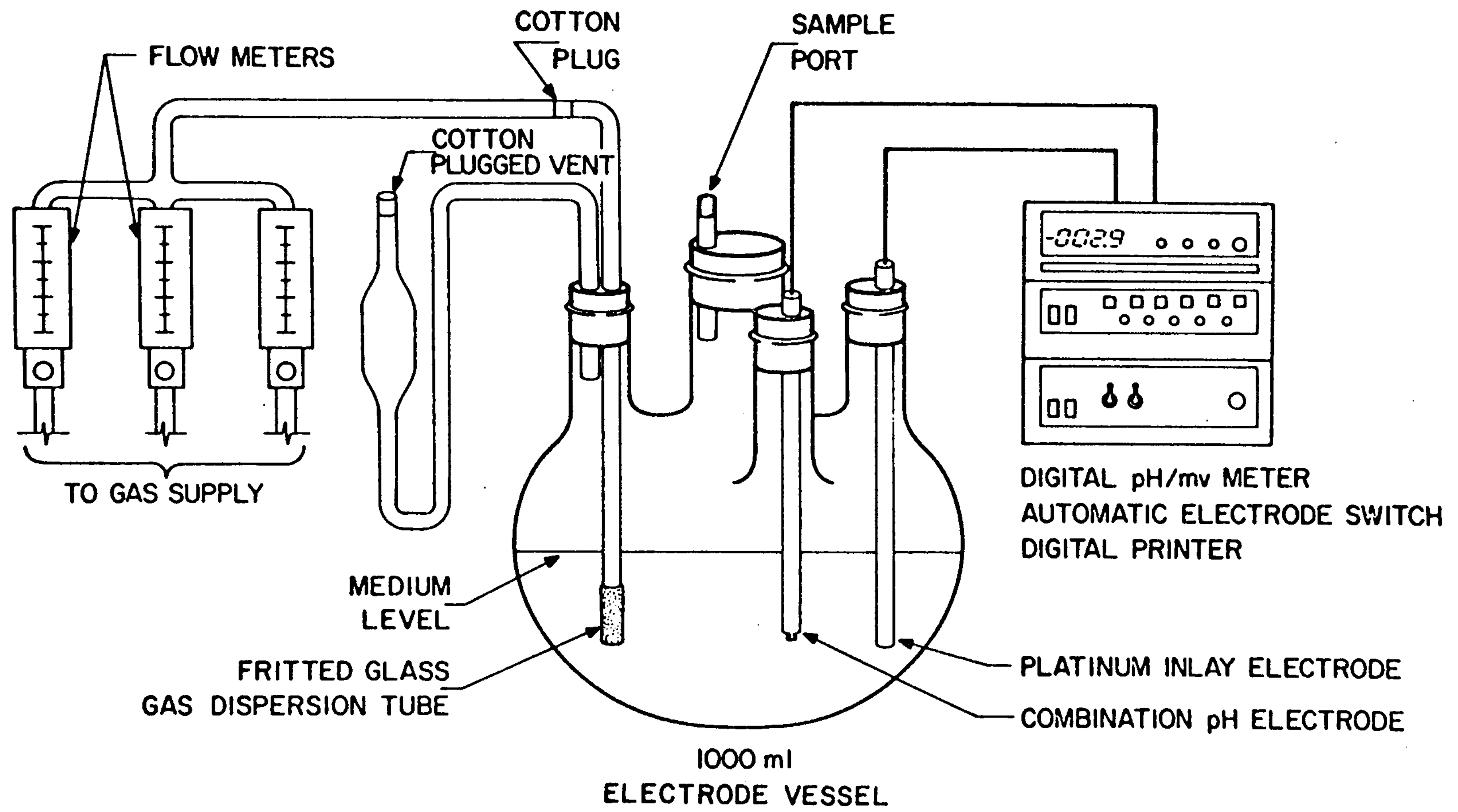


Fig. 1. Electrode-Growth Vessel

Virginia, 1975). This apparatus allowed the pH and Eh of the growth medium to be continuously monitored. Agitation of the medium was obtained by a magnetic stirring bar rotated by a submersible magnetic stirrer. The electrode vessel was composed of a one liter four necked distilling flask (Corning Glass Works, Corning, NY) fitted with a combination pH electrode (Corning No. 476051) and a platinum inlay electrode (Corning No. 476060) in two of the necks. The electrodes were placed in rubber stoppers to insure airtight connections to the electrode vessel. To allow various gases to enter and mix with the medium, the third neck was fitted with a rubber stoppered cotton-plugged glass dispersion tube and a glass tube connected to a cotton-plugged vent used for the gas exit. The fourth neck was arranged similarly with an air-tight screw cap tube which was utilized for dispensing media into the vessel and sample collecting. Glass and pure gum rubber tubing were used for all gas connections. Flow meters (Air Products and Chemicals, Inc., Allentown, PA) were arranged in a series in order to mix and control the gases. All gases used (i.e. prepurified nitrogen and anaerobic grade carbon dioxide) were obtained from Industrial Gas and Supply, Bluefield, WV. The nitrogen was passed through a copper-filled furnace as outlined previously to remove any oxygen before it was bubbled through the medium.

The platinum and pH electrodes were connected to a Model 801 digital pH/mv Meter (Orion Research Inc., Cambridge, MA), a Model 855 Automatic Electrode Switch (Orion), and a Model 851 Digital Printer (Orion), which allowed continuous monitoring and automatic recording

of each electrode potential. Temperature of the medium was maintained at 35 C by placing the electrode vessel in a water bath heated by a Haake Model E52 constant temperature circulator (Haake Instruments, Inc., Rochelle Park, NJ).

To obtain maximum sensitivity in measuring Eh, the platinum electrode was polished with a jeweler's rouge polishing cloth (Bulava Watch Co., Flushing, NY) prior to each experiment.

Preceeding each experiment, the entire electrode vessel was sterilized as outlined below. The four necked flask with stirring bar, attached sample port, and cotton-plugged glass tubing was sterilized by autoclaving for 15 minutes at 121 C. The vessel was placed within a hood under a germicidal ultraviolet lamp upon removal from the autoclave. Sterilization of the electrodes was carried out by immersion in 0.15% sodium hypochlorite for 20 minutes, followed by rinsing six times in sterile, distilled water and drying with sterile cheesecloth. They were then fitted in the electrode vessel. On completion of assembly, the apparatus was removed from the hood, placed in the water bath and connected to the gas flow lines.

2. Media dispensing and equilibration

The anaerobic medium was aseptically dispensed in the electrode vessel upon completion of preparation, sterilization, and cooling as described in the preceeding section. Prior to dispensing the medium, prepurified oxygen-free nitrogen (OFN) was used to flush the electrode vessel and apparatus for one hour at a rate of 100 ml/minute, to remove any air present which might oxidize the medium. The prereduced medium

at this point was immediately dispensed into the electrode vessel while being sparged with OFN (See Fig. 2).

Oxygen-free prepurified nitrogen was used to sparge the medium in the vessel during all equilibration periods and growth experiments. A period of two to four hours was necessary for the Eh of the medium to come to equilibrium. Carbon dioxide, used to promote germination of the spores, was then added continuously and mixed with the OFN so that a constant pH of 0.3 pH units less than the system containing no carbon dioxide was established in the flask before inoculation. This required a period of one to two hours. The carbon dioxide sparging was terminated upon germination of the spores as seen by phase contrast microscopy.

D. Eh and pH Calculations

Measurement of pH values were recorded in millivolts rather than pH units to facilitate continuous, non-interrupted data collection. Calibration of pH was achieved by use of a standard curve of pH versus millivolts prepared by using the following standard buffers (pH 4.02, 6.98, 9.94, obtained from Harleco, Co., Philadelphia, PA) at 35 C.

Standardization of the platinum electrode was performed in a potassium phthalate buffer, pH 4.0, saturated with quinhydrone at 35 C. At this temperature, this system has a known Eh of +447 mv. The equation, $+447=E_{obs}+E_{Ag - AgCl}$, was used to obtain the potential with reference to the Ag - AgCl half cell ($E_{Ag - AgCl}$). The platinum electrode was standardized prior to each set of experiments to insure accuracy and consistency of the Eh measuring system. The Eh of all

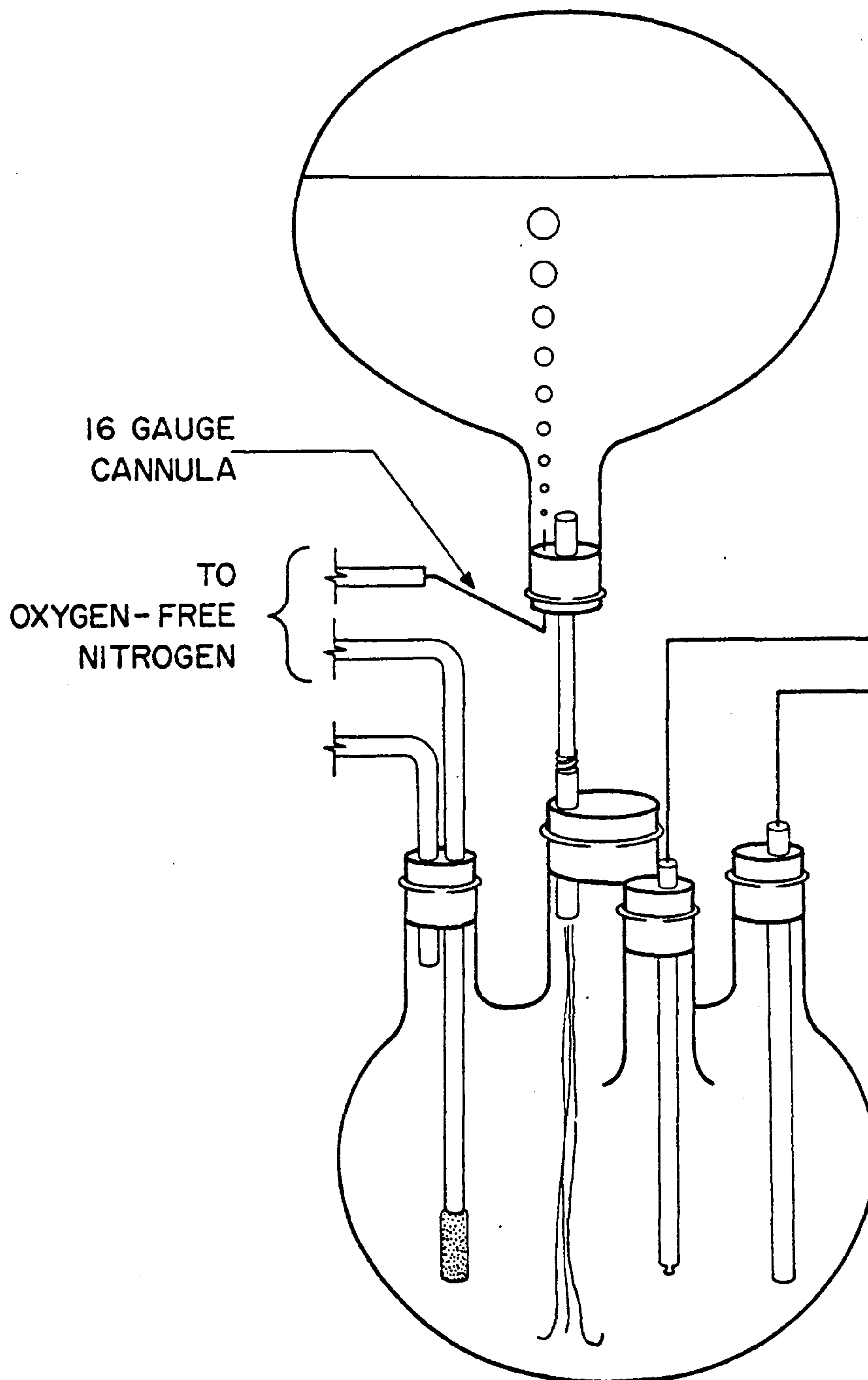


Fig. 2. Anaerobic dispensing of prereduced medium into the electrode vessel.

data points can be calculated from the E_{obs} by the equation: $Eh = E_{obs} + E_{Ag-AgCl}$. All Eh values recorded were adjusted to pH 7.0 to eliminate the effect of pH on the Eh of the system by use of the formula of Leistner and Mirna (1959): $Eh7 = E_{obs} + E_{ref} + 2.303\ (RT/F)\ (pH\ X - 7.0)$ where E_{obs} = the measured potential, E_{ref} = the reference electrode potential, 2.303 (RT/F) = 61.1 at 35 C and (pH X - 7.0) = the pH corrective term.

E. Growth and Toxin Studies

1. Preparation of inocula

Inocula for all growth studies consisted of spores of the 10755A strain of type A *Clostridium botulinum* prepared as outlined by Rowley and Feeherry (1970). The spore crops were harvested by aerobic centrifugation and washed eight times with sterile distilled water. The washed spores were suspended in sterile distilled water to a concentration of 5.5×10^8 viable spores per ml and stored at 4 C. The viable spore count was determined in PYA, incubated at 35 C for 48 hours by the roll tube methods of Pierson, *et al*., (1974), and Shoemaker and Pierson, (1976). Upon examination by phase contrast microscopy, sporulation of the preparation was estimated to be 95%.

2. Determination of growth and toxin production

The electrode vessel was inoculated anaerobically with spores prepared by dispensing 0.1 ml of the spore suspension into 9.9 ml of prereduced 0.05% cysteine 0.1% peptone (pH 7.0). This preparation was heat shocked for ten minutes at 80 C before 1.0 ml was aseptically

introduced into the vessel to produce a final concentration of approximately 10^3 spores/ml.

Spore outgrowth was quantitatively determined on periodic samples by duplicate roll tube counts in PYA medium incubated at 35 C for 48 hours as described in the preceeding section. Germination of the spores was estimated by observing loss of refractility as seen by phase contrast microscopy.

3. Toxin assay

At selected times during all growth studies except those with sodium chloride as the chemical inhibitor, 4.0 ml samples were aseptically removed from the growth vessel for toxicity testing. The samples were centrifuged at 20,000 x g for ten minutes at 4 C to remove all vegetative cells and debris. One milliliter of the sample supernatant fluid was diluted in 9.0 ml of a gelatin-phosphate buffer (0.1 percent gelatin in 0.05M sodium phosphate at pH 6.2). Quantitation of the toxin produced by the test strain was estimated by quantal mouse assay determining LD_{50} (Tint and Gillen, 1961). White male ICR mice weighing 18-20 grams were each injected intraperataneally with the 0.5 ml of the diluted sample. Six mice were used per group and deaths were recorded over 96 hours.

In the particular experiments where growth was expected to be inhibited, toxin screening was performed on the samples by injecting four mice each with 0.5 ml of sample preparation diluted to 1/10 of the original. The mice were observed for 24 hours and if death

occurred, the sample which had been stored at 4 C was quantitated as previously described.

For those growth studies in which sodium chloride was used as the growth inhibitor, 12.0 ml samples were taken for toxicity testing and centrifuged at 20,000 x g for ten minutes at 4 C. The samples were prepared by four different treatments in order to determine if sodium chloride affected toxin production or the preformed toxin. All samples were quantitated as outlined above. First, 6.0 ml of the supernatant was dialyzed (Dialyzer tubing, Fisher Scientific Products, Raleigh, North Carolina) in one liter of sterile phosphate buffer (0.05M sodium phosphate at pH 6.2) for 2 hours at 4 C to remove the NaCl. One milliliter of this treatment was serially diluted and injected. Another 4.5 ml of this treatment was mixed with 0.5 ml of a 1.0% solution of trypsin (Difco 1:250, Difco Laboratories, Detroit, Michigan) and incubated at 35 C for 30 minutes before serially diluting and injecting. Second, from the remaining 6.0 ml of non-dialyzed sample supernatant fluid, 1.0 ml was serially diluted and injected while another 4.5 ml was trypsin treated as described previously before dilution and injection.

All incidental dilutions of the dialyzed and non-dialyzed samples were accounted for in the quantitation. No toxin samples were stored at 4 C for longer than 24 hours except those from the growth experiments containing no chemical inhibitors. These samples were stored at 4 C for no longer than 48 hours.

F. Toxin Stability

For determination of toxin stability during refrigerated storage, a 48 hour culture of the test strain in thiotone-yeast extract glucose broth (TYGB) was centrifuged at 20,000 x g for ten minutes at 4 C to remove all vegetative cells and debris. The supernatant fluid was then divided into four 1.0 ml samples which were added to four different screw cap tubes containing 9 ml of the sterile gelatin-phosphate buffer (pH 6.2). One tube was quantitated immediately while the remaining three were stored at 4 C and tested after 24, 48, and 72 hours. All samples were quantitated as outlined in the preceeding section.

RESULTS AND DISCUSSION

A. Identification of the Test Strain

The biochemical tests which were performed to identify the test strain are presented in Table 3. The results are typical for *C. botulinum* and support the identification of the test strain as *Clostridium botulinum* type A (Holdeman and Moore, 1975). Upon gas chromatographic analysis of the volatile fatty acid end products of fermentation for strain 10755A, acetic and butryic acids were identified as the major products from the chopped meat carbohydrate medium as shown in Fig. 3. Smaller amounts of propionic, isobutryic, and isovaleric acids were also detected. These products are typical for a proteolytic strain of *C. botulinum* type A (Holdeman and Moore, 1975). The test strain was found to produce type A botulinum toxin by *in vivo* neutralization tests.

B. Determination of Reference Half-Cell Potential

The Ag-AgCl reference half-cell was found to have a value of +205 ± 5.0 mv at 35 C. This was based on the average of 15 measurements throughout the course of this study.

C. Growth, Toxin Production, and Eh7 Studies in Electrode Vessel

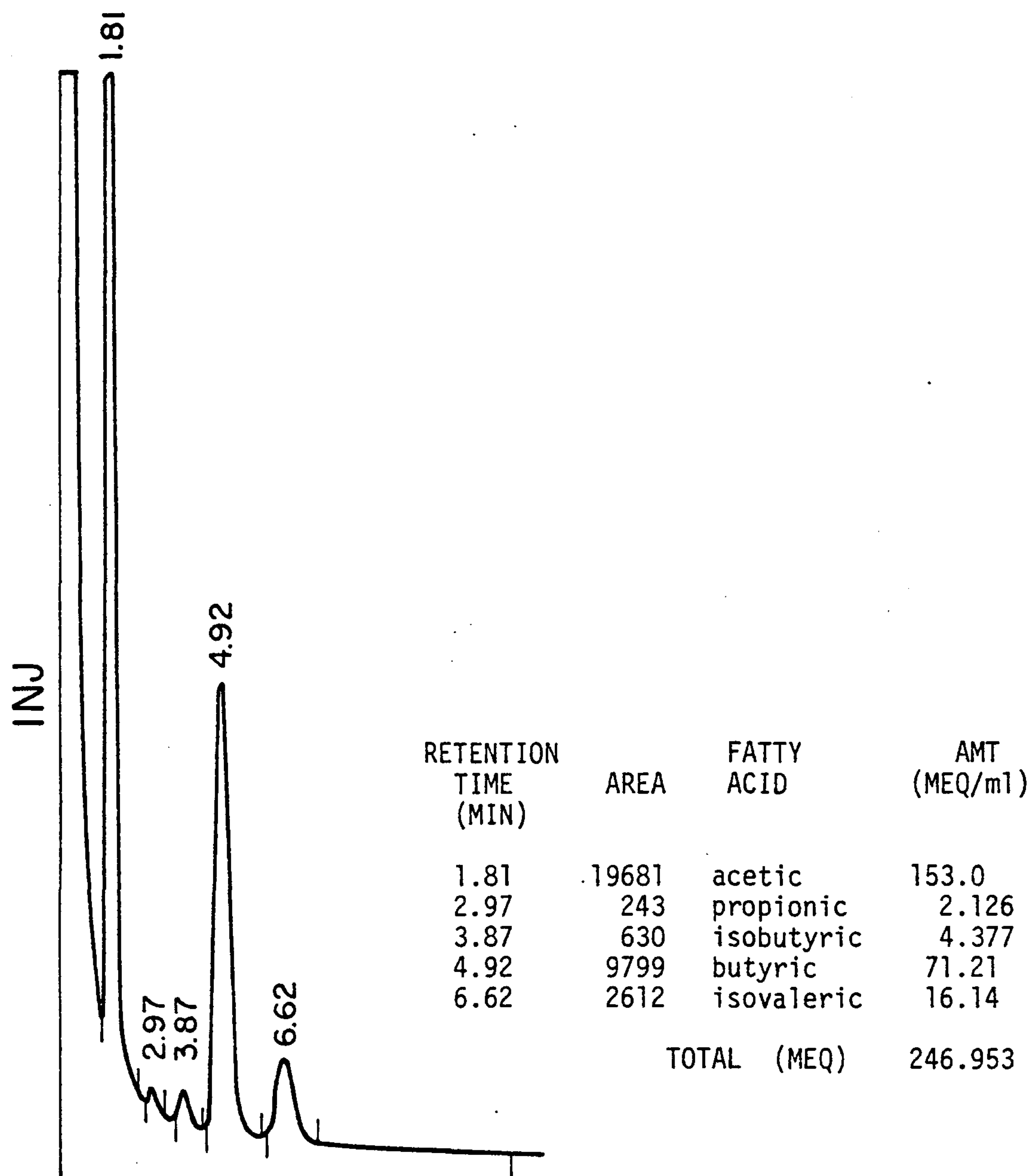
1. Uninhibited growth and toxin production in oxidized and reduced TSB.

Results of the uninhibited studies on outgrowth and toxin formation by the test strain are presented in Fig. 4.

Table 3. Growth and biochemical tests completed for identification of the test strain (*Clostridium botulinum* type A).

Test Performed	Results
Gram stain	Gram + rods
Spore stain	Subterminal spores
Starch hydrolysis	-
Starch pH	non-acid
Meat digestion	+
Indol production	-
Milk	digested
Gelatin	digested
Esculin hydrolysis	+
Esculin pH	non-acid
Lipase	+
Acid pH in PY medium with:[1]	
fructose	-
glucose	-
maltose	-
lactose	-
mannitol	-
mannose	-
melibiose	-
sucrose	-
xylose	-

[1]Test considered positive in peptone-yeast extract medium (PY) plus carbohydrate when the pH is lowered from 6.8 to less than 6.0



RETENTION TIME (MIN)	AREA	FATTY ACID	AMT (MEQ/ml)
1.81	19681	acetic	153.0
2.97	243	propionic	2.126
3.87	630	isobutyric	4.377
4.92	9799	butyric	71.21
6.62	2612	isovaleric	16.14
		TOTAL (MEQ)	246.953

Fig. 3. Results of gas chromatographic analysis of ether extracted volatile fatty acid products of Clostridium botulinum type A, strain 10755A, in chopped meat carbohydrate medium.

Growth of the culture was found to occur simultaneously with the Eh7 drop of the growth medium. Approximately the same incubation time was necessary in each type of medium for the drop in Eh7 to begin.

There was no significant inhibition of outgrowth, as measured by the roll tube counts, between the oxidized and reduced media. A maximum cell population of approximately 2.5×10^9 cells/ml was attained after 20 hours of incubation at 35 C in both oxidized and reduced TSB (Fig. 4). The lack of difference in growth of the test strain in these media, which differ only in oxidation-reduction potential, is consistent with previous studies showing that the Eh7 of the medium (ranging from -40 to -185 mv) does not have a significant effect on growth of _C. botulinum_ type E spores (M. V. Smith, 1975).

Toxin formation by the test strain was also found to be very similar in the two types of media. For both growth curves, toxin was not detected until after 24 hours of incubation (Fig. 4). The maximum toxin strength attained in the reduced TSB was 66,000 LD_{50}/ml while in the oxidized TSB 54,000 LD_{50}/ml was detected. These titers were reached after 72 hours of incubation at which time the culture was well into its stationary phase.

2. Inhibited growth and toxin production in oxidized and reduced TSB.

a. Sodium chloride

The comparison of growth and Eh7 changes for oxidized and reduced TSB containing 5% added NaCl is shown in Fig. 5. Growth and Eh7

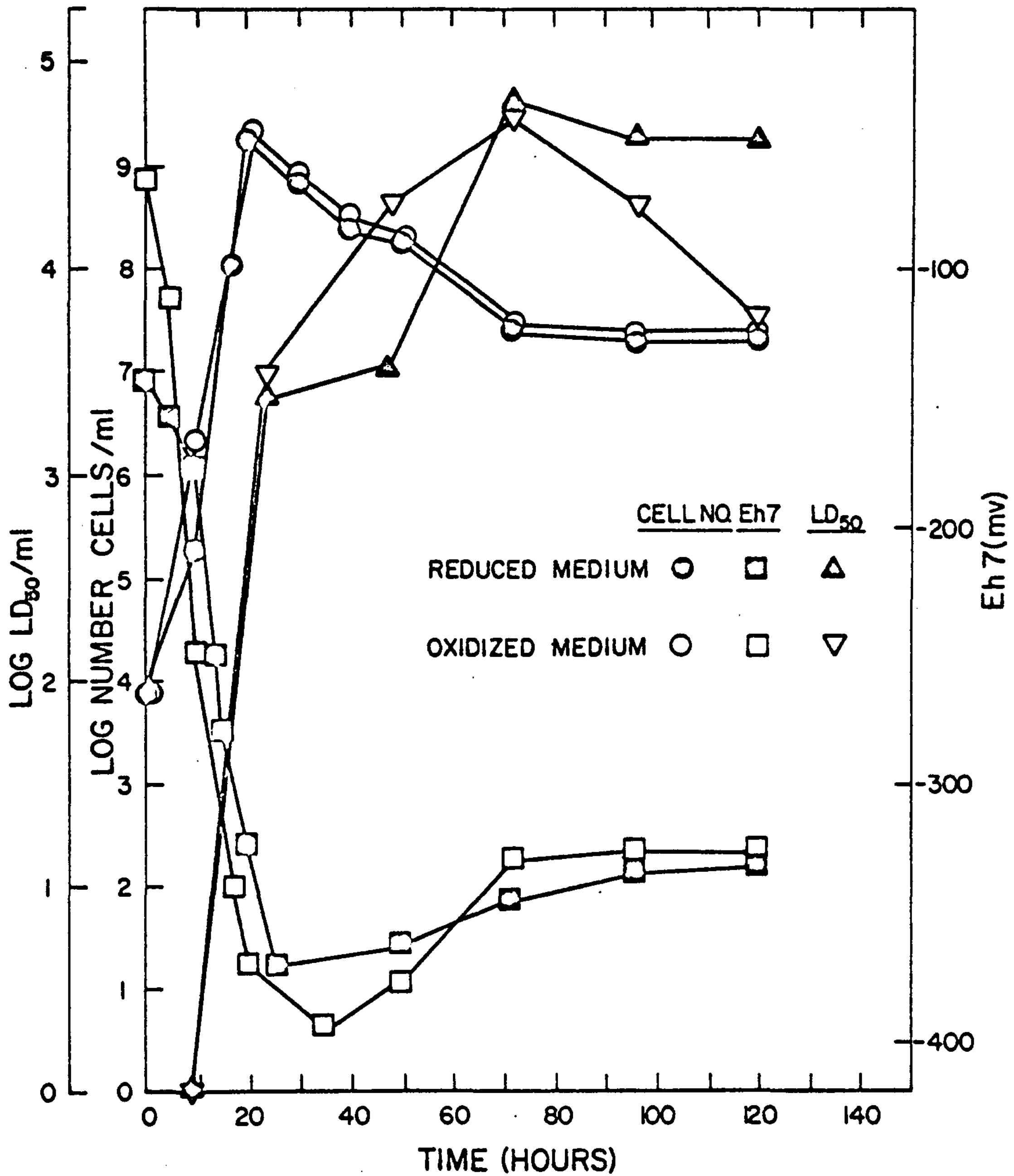


Fig. 4. Growth and toxin production of C. botulinum type A and Eh7 changes in oxidized and prereduced trypticase soy broth (pH 6.8) plus 0.05% cysteine.

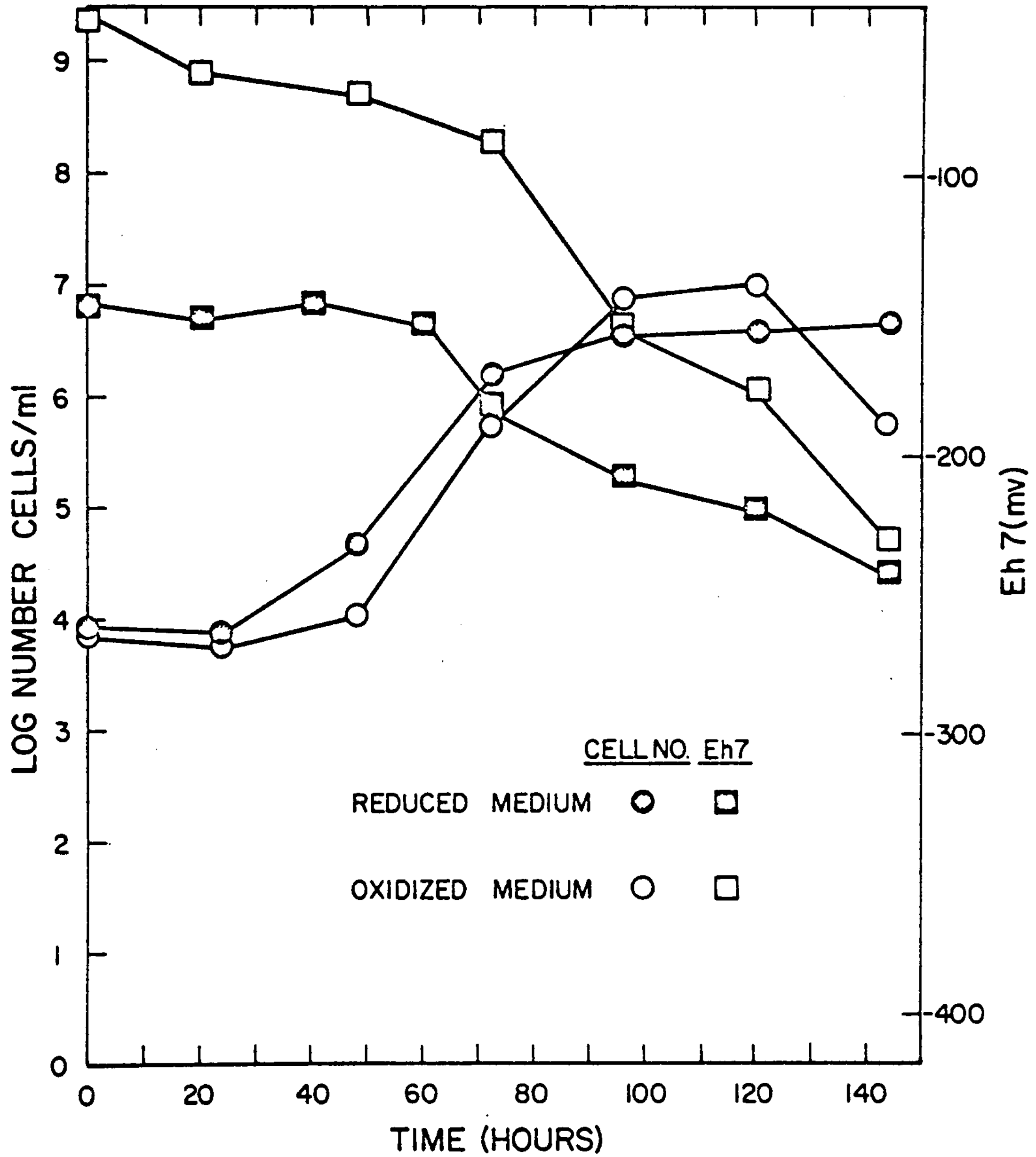


Fig. 5. Growth of C. botulinum type A and Eh7 changes in oxidized and prereduced trypticase soy broth (pH 6.8) plus 0.05% cysteine and 5% added sodium chloride.

reduction started roughly 30-40 hours after inoculation of the flask for the reduced medium. In the oxidized medium, the growth and Eh7 changes were initiated after approximately 48 hours of incubation. The oxidized TSB plus 5.0% NaCl induced a slightly increased lag phase in the growth of the test strain through 72 hours of incubation. However, a higher maximum cell number was eventually attained in the oxidized medium as compared to the reduced medium. This may occur once the Eh7 of the medium has been sufficiently reduced to a favorable level by the growth of the culture. This is in agreement with the results obtained by Boyd and Reed (1931a, 1931b).

For the analysis of toxin formation in these two media, the culture supernatant fluid samples were given four different treatments (no treatment, trypsinized, dialyzed, and dialyzed-trypsinized) in order to determine if 5% NaCl added had an effect on toxin formation or the preformed toxin. The results of these treatments on the quantitation of toxin in the reduced TSB plus 5% NaCl is presented in Fig. 6. Toxin formation was not detected until 72 hours after inoculation of the flask. Maximum toxin strength was found to be 6320 LD_{50}/ml at the end of 144 hours of growth for the dialyzed-trypsinized samples. There appears to be significant difference between the treatment except for the trypsinized sample at 144 hours. Variation in the toxin titer detected for this treatment may have resulted from trypsin inactivation of the toxin in the presence of the salt. Fig. 7 illustrates the effects of the four treatments on toxin quantitation in the oxidized TSB plus 5% NaCl. Probably due to the

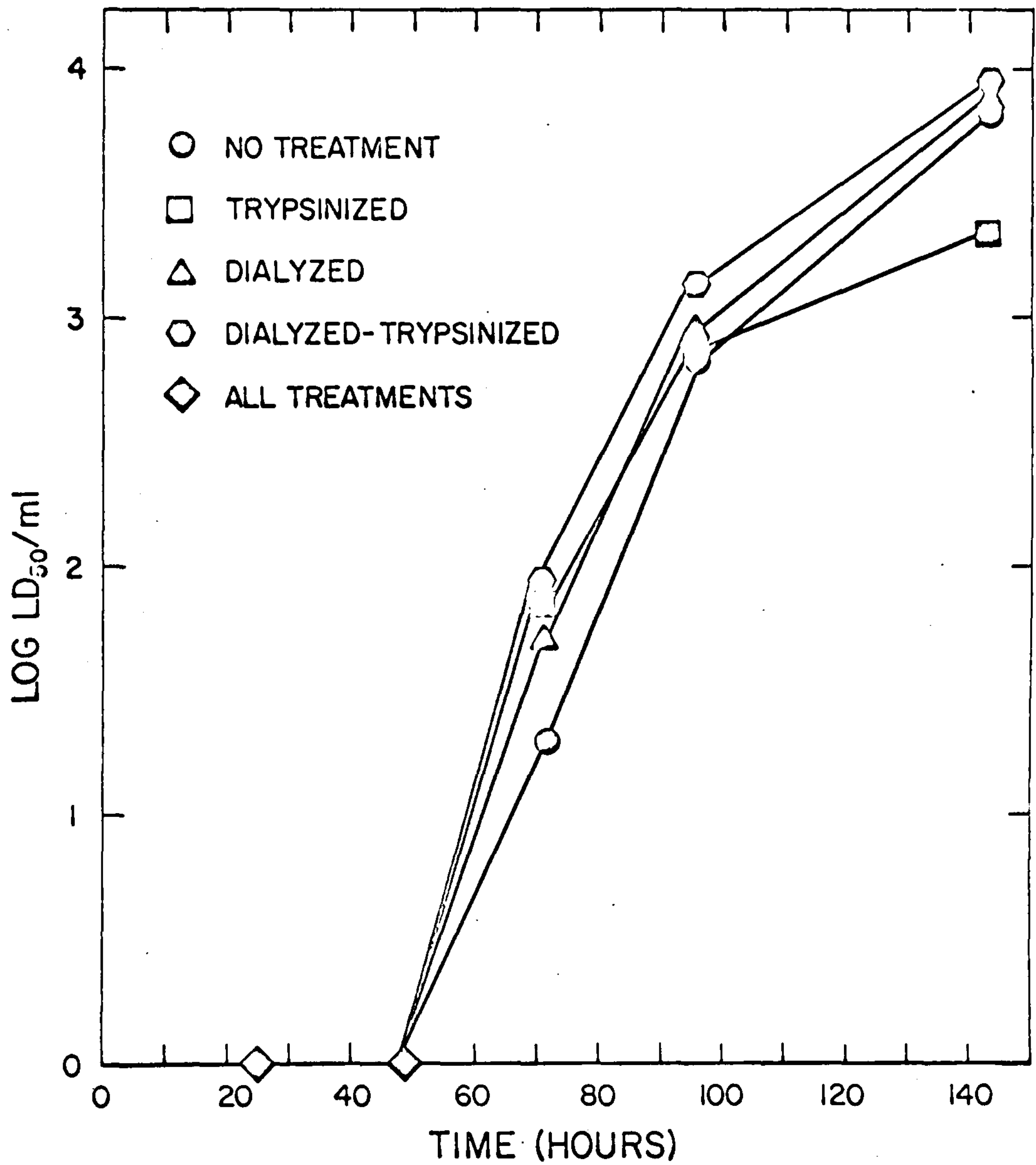


Fig. 6. Toxin production by C. botulinum type A in prereduced trypticase soy broth (pH 6.8) plus 0.05% cysteine and 5% added sodium chloride.

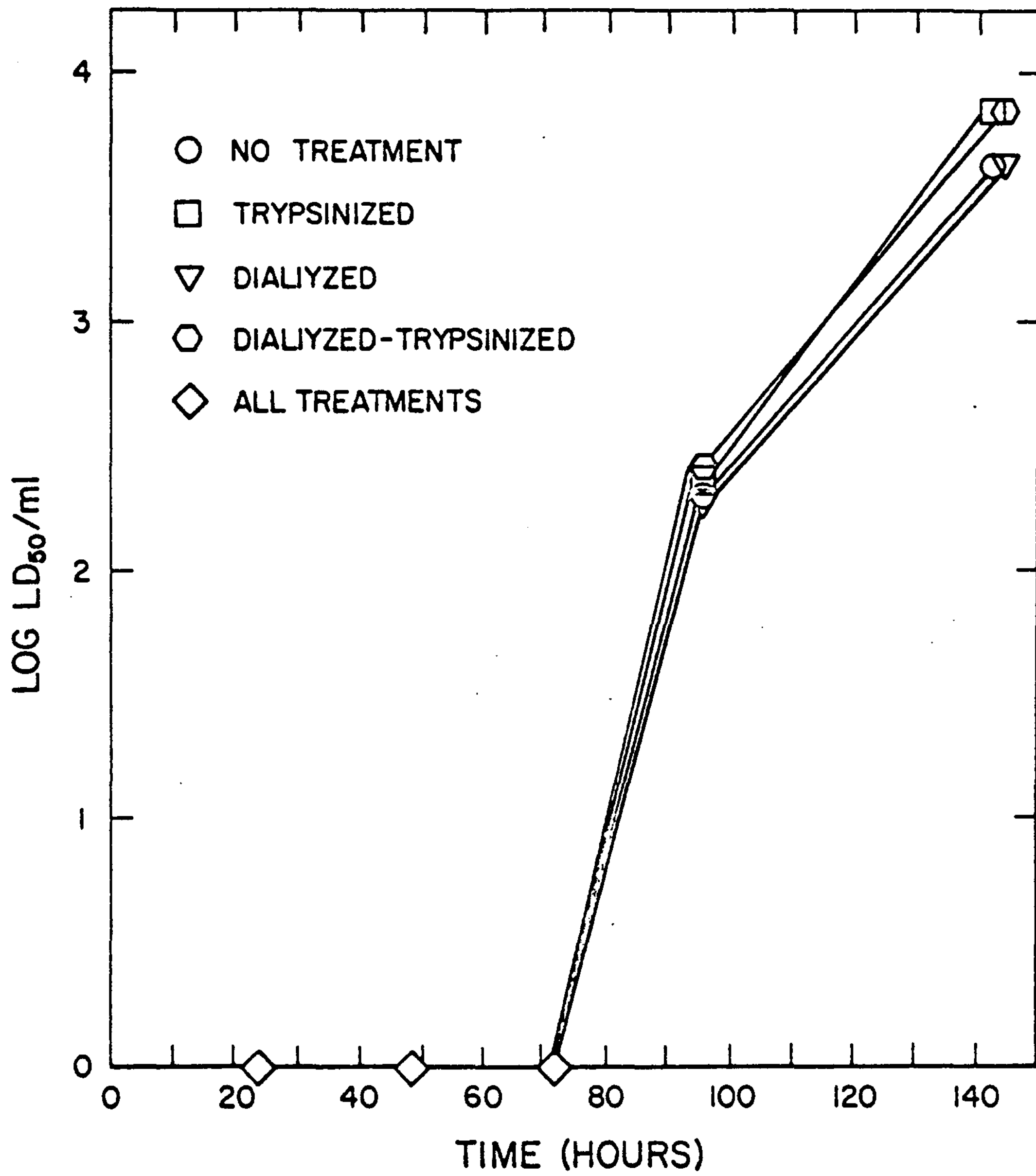


Fig. 7. Toxin production by C. botulinum type A in oxidized trypticase soy broth (pH 6.8) plus 0.05% cysteine and 5% added sodium chloride.

delay in growth in this medium, toxin was not detected until after 96 hours of incubation with the maximum toxin titer of 7000 LD_{50}/ml attained after 144 hours of incubation. Again no significant difference in toxin quantitation was obtained by the sample treatments indicating that sodium chloride at this concentration had no direct effect on toxin formation or preformed toxin, but rather an indirect affect through its growth inhibiting properties.

To investigate the levels of sodium chloride necessary to completely inhibit growth of the test strain, 6% NaCl was added to both oxidized and reduced TSB. The results are shown in Fig. 8. In the reduced medium, growth and Eh7 changes began at 100 hours with a maximum cell number of 5.0×10^5 cells/ml attained after 144 hours. Neither change in cell number or Eh7 reduction due to growth was detected in the oxidized medium through 144 hours of incubation. The spores remained phase bright and heat resistant. Toxin was not detected in all four treatments for both the oxidized and reduced media (Fig. 8).

Complete inhibition of the test strain in reduced TSB was achieved when 7.0% NaCl was added to the medium (Fig. 9). Neither germination, toxin production, or Eh7 reduction occurred through 144 hours of incubation. The initial spore inoculum remained phase bright and heat resistant.

Inhibition by sodium chloride can be considered one of moisture limitation where the sodium chloride lowers the water activity (Jay, 1970). These results indicate that in determining the concentration of sodium chloride needed for inhibition of the test strain, it must

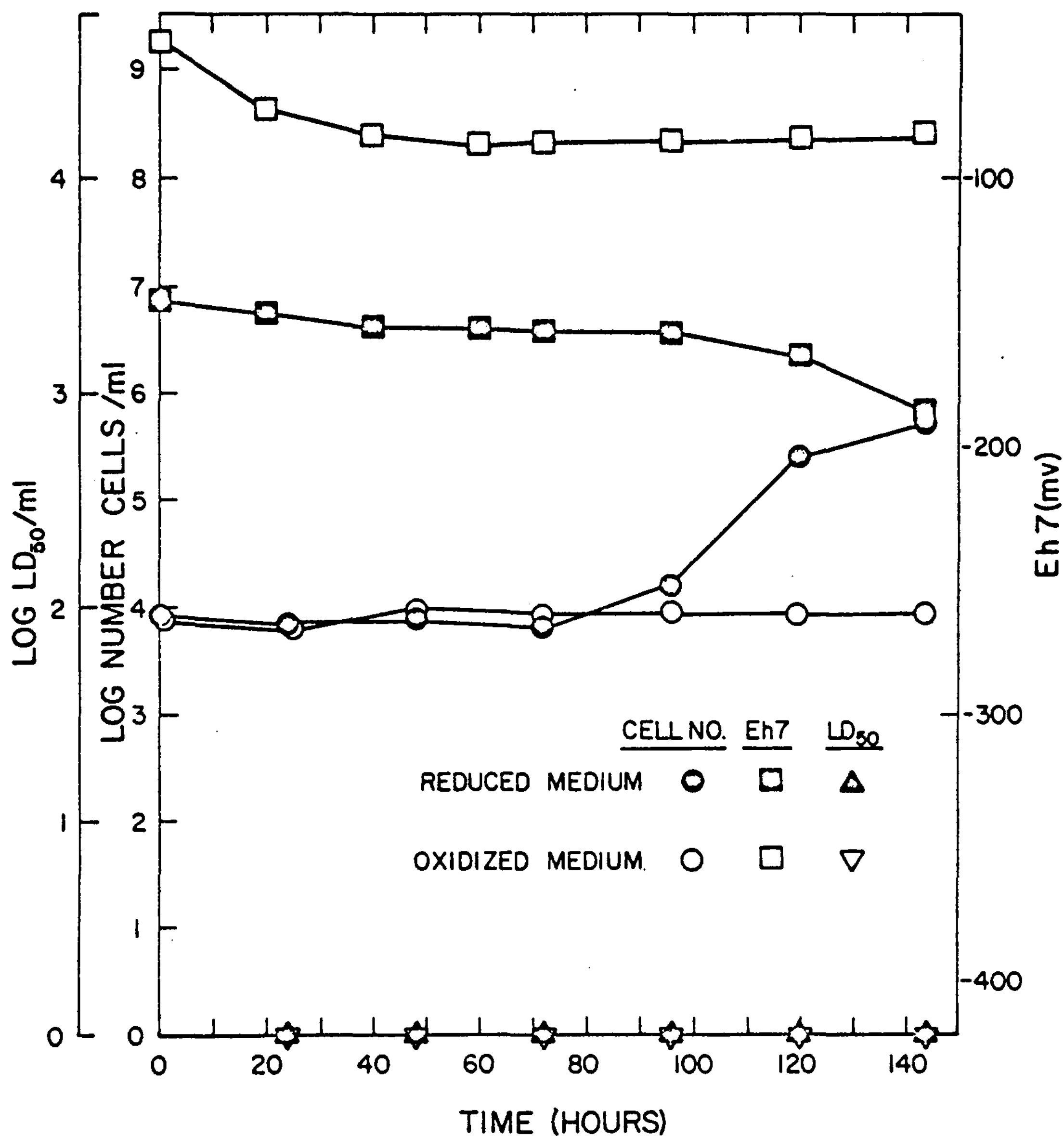


Fig. 8. Growth and toxin production of C. botulinum type A and Eh7 changes in oxidized and prereduced trypticase soy broth (pH 6.8) plus 0.05% cysteine and 6% added sodium chloride.

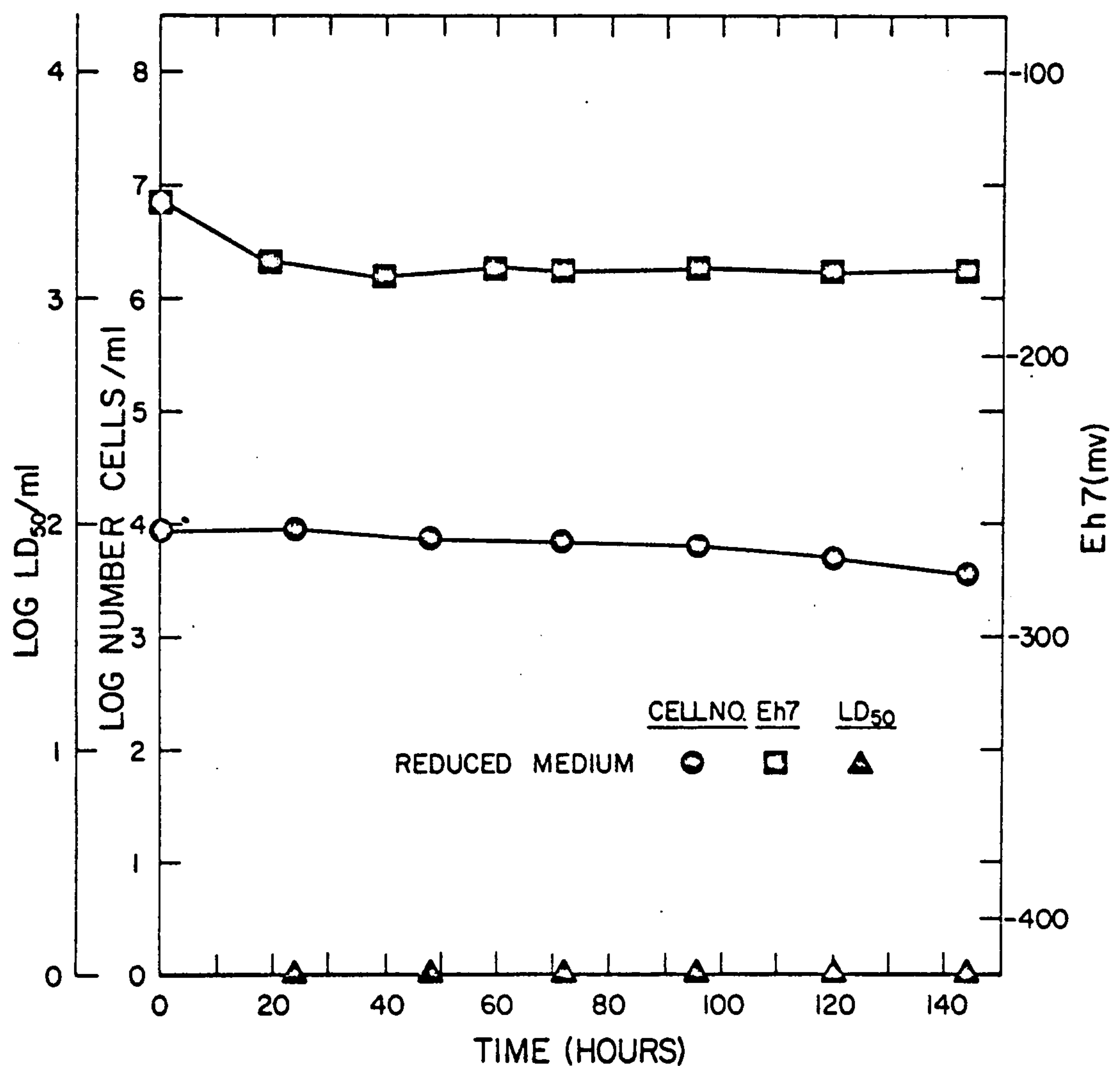


Fig. 9. Growth and toxin production of C. botulinum type A and Eh7 changes in prereduced trypticase soy broth (pH 6.8) plus 0.05% cysteine and 7% added sodium chloride.

be considered that the Eh7 has a definite influence on the inhibitive levels. For complete inhibition of C. botulinum in a reduced medium as compared to an oxidized medium, more sodium chloride may be necessary.

The requirement of 8.5 - 10.5% sodium chloride for complete inhibition of C. botulinum type A strains has been cited (Lechowich, 1968). Spencer (1967) reported growth limiting concentrations of sodium chloride in a cooked meat slurry to be 7.8% for type A spores and 6.9 - 7.8% for type B spores. Toxin formation by types A and B spores was inhibited in perishable cured meats containing 9% NaCl (Greenberg, et al., 1959). The inhibition levels required for this test strain in reduced TSB (7.0% added NaCl) indicated agreement with published data. Results obtained in the oxidized TSB found that germination was inhibited for up to 144 hours when 6.5% sodium chloride (6.0% added) was present. The spores were probably further inhibited by the oxidized state of the medium.

Previous work by Mead (1969) demonstrated similar results on the growth limiting salt concentrations for C. perfringens as affected by the Eh of the medium. In media containing 5% salt and poised by dissolved oxygen, he showed that at Eh levels of +195 and +92 mv growth was inhibited while at an Eh level of +66 mv growth occurred.

b. Sucrose

The effect of Eh7 and 30% sucrose on the inhibition of the test strain is presented in Fig. 10. The reduced medium showed a marked increase in the lag phase with the maximum cell population of 6.0×10^7 cells/ml being detected after 72 hours of incubation. In the medium

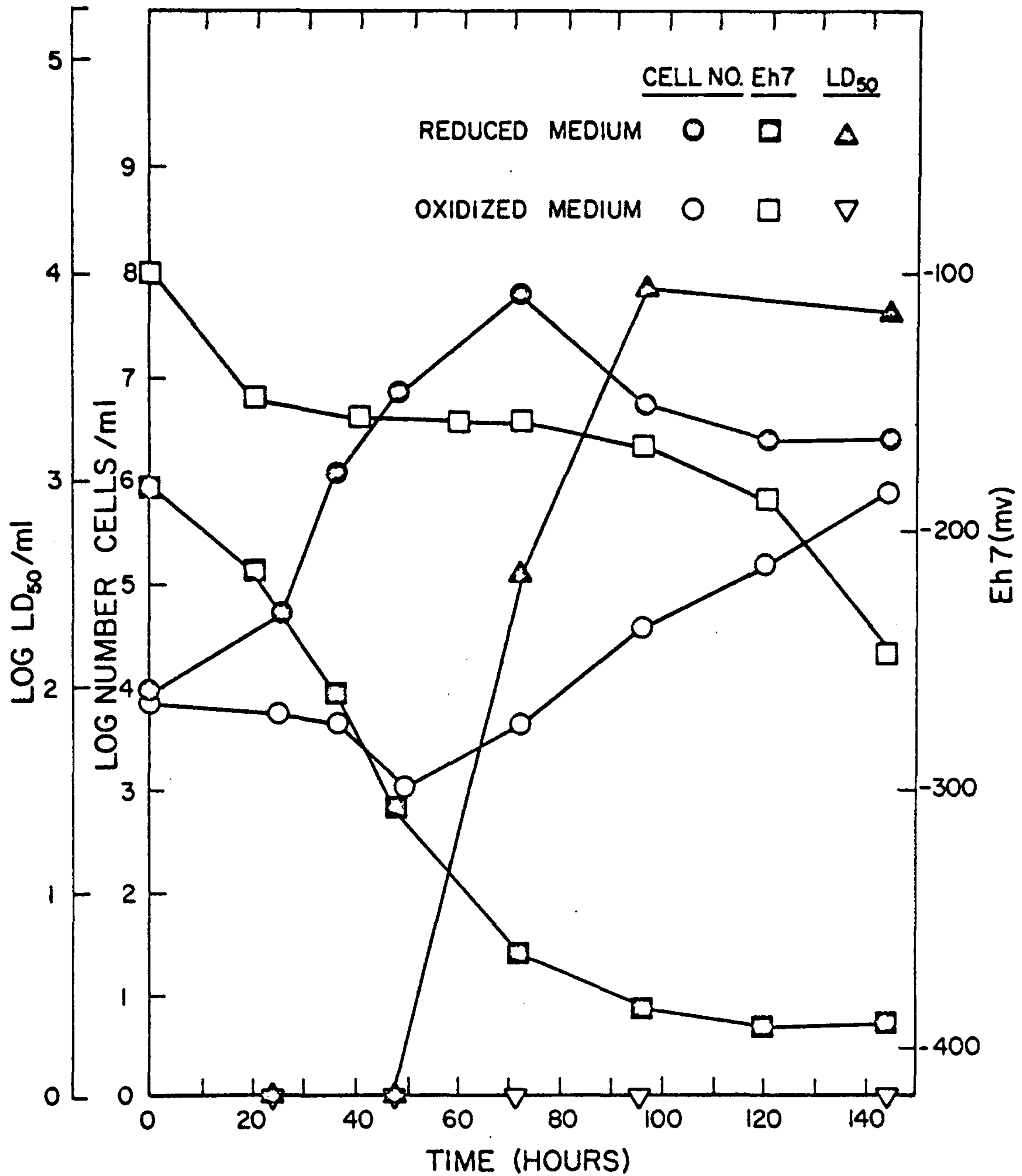


Fig. 10. Growth and toxin production of C. botulinum type A and Eh7 changes in oxidized and prereduced trypticase soy broth (pH 6.8) plus 0.05% cysteine and 30% sucrose.

poised at a higher redox potential a lag in growth was observed for up to 72 hours. The extent of growth was far less in the oxidized TSB with the culture reaching a maximum number of only 8.5×10^5 cells/ml after 144 hours incubation.

Toxin was not detected in the reduced medium until after 72 hours of incubation with the culture reaching its maximum toxicity at 96 hours with approximately 8300 LD_{50}ml (Fig. 10). Toxin formation in oxidized TSB was less than the detection level of 20 LD_{50}/ml.

The differences in growth and toxin production between the two media are attributed to their methods of preparation resulting in oxidized and reduced TSB since both contained the same amount of sucrose. In a previous investigation in similar media, it was reported that growth differences for *C. botulinum* type E was not the result of inhibitory substances formed during the oxidation of the medium components, but rather the redox potential of the medium (M. V. Smith, 1975).

Upon increase of the sucrose concentration to 35%, growth in reduced TSB was sufficiently inhibited that no toxin was detected for up to 144 hours (Fig. 11). The maximum cell number attained over this period was 4.8×10^4 cells/ml which was less than a one log cycle increase over the initial inoculum of 7.3×10^3 cells/ml. Growth experiments were not performed in oxidized TSB containing 35% sucrose; however, further inhibition of the test strain would be expected due to the increased Eh7.

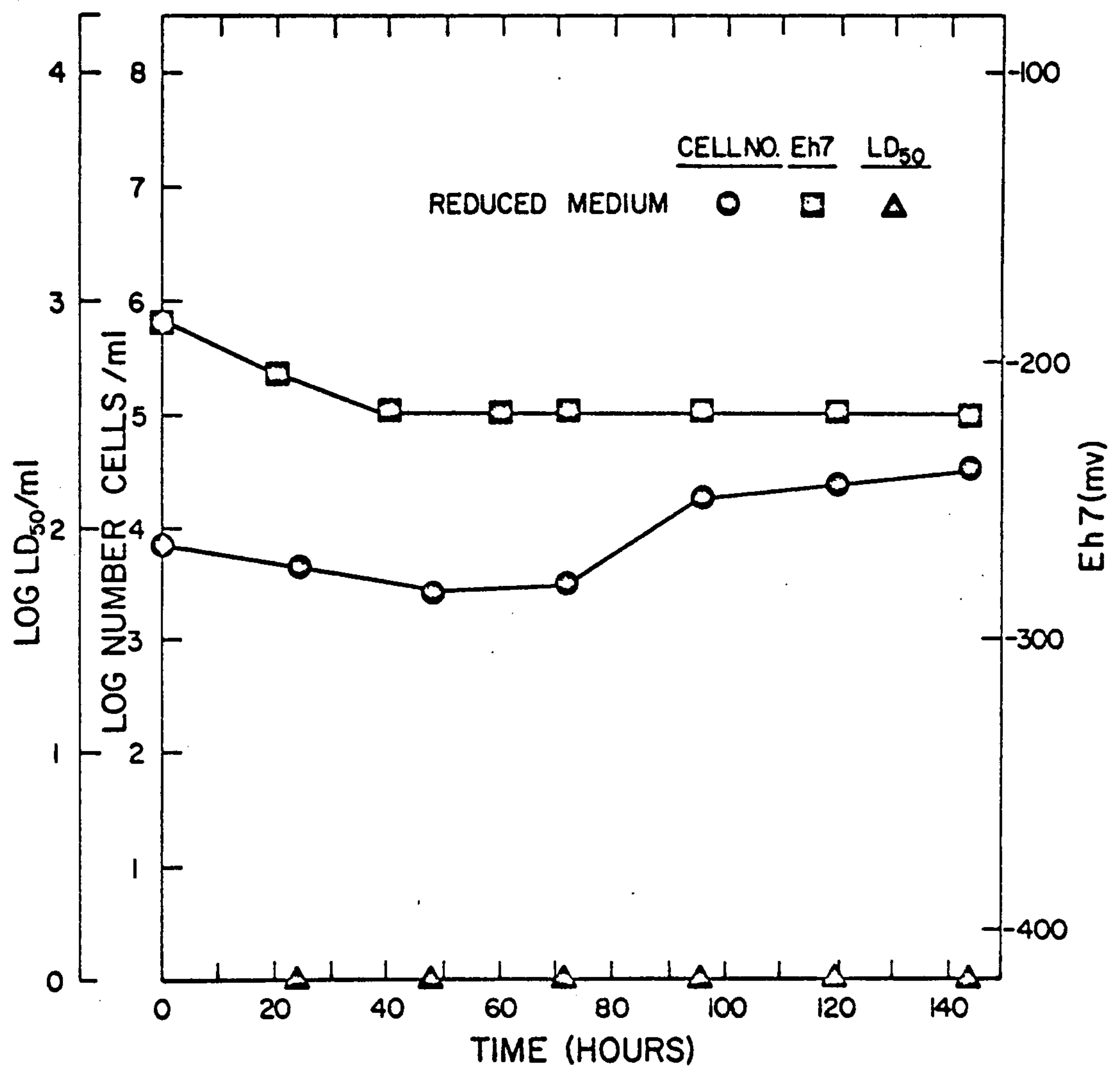


Fig. 11. Growth and toxin production of C. botulinum type A and Eh7 changes in prereduced trypticase soy broth (pH 6.8) plus 0.05% cysteine and 35% sucrose.

Very few data exist on sucrose inhibition of _C. botulinum_ type A spores. Bever and Halvorson (1948) reported that 50 to 55% sucrose was necessary for inhibition of types A and B vegetative cells. In another investigation, a sucrose concentration of greater than 31% was found necessary to inhibit germination of type B spores (Beers, 1957). In comparison of results from this study to previous investigations, the variations in strains used and conditions under which they were tested should be considered.

Inhibition of outgrowth for the test strain was achieved in reduced TSB containing 40% sucrose (Fig. 12). Neither growth, toxin formation or change in Eh7 due to growth was detected for up to 144 hours. Reduction of the viable count to less than that of the initial inoculum is attributed to germination as observed by phase contrast microscopy and eventual death of the spores in the 40% sucrose medium. This same phenomenon would also account for slight reduction of the viable count obtained in the two sucrose media (30% and 35%) previously discussed.

The initial Eh7 for both oxidized and reduced TSB in growth studies utilizing sucrose as the chemical inhibitor was substantially lower (40 mv) than the initial Eh7 of TSB not containing sucrose. This might be attributed to the partial breakdown of the sucrose (a non-reducing sugar) to glucose and fructose (reducing sugars), thus increasing the concentration of reducing compounds in the system. Breakdown of this compound could occur either in the refluxing of the medium during preparation or the sterilization step in which the medium is autoclaved at 121 C and 15 psi for 15 minutes.

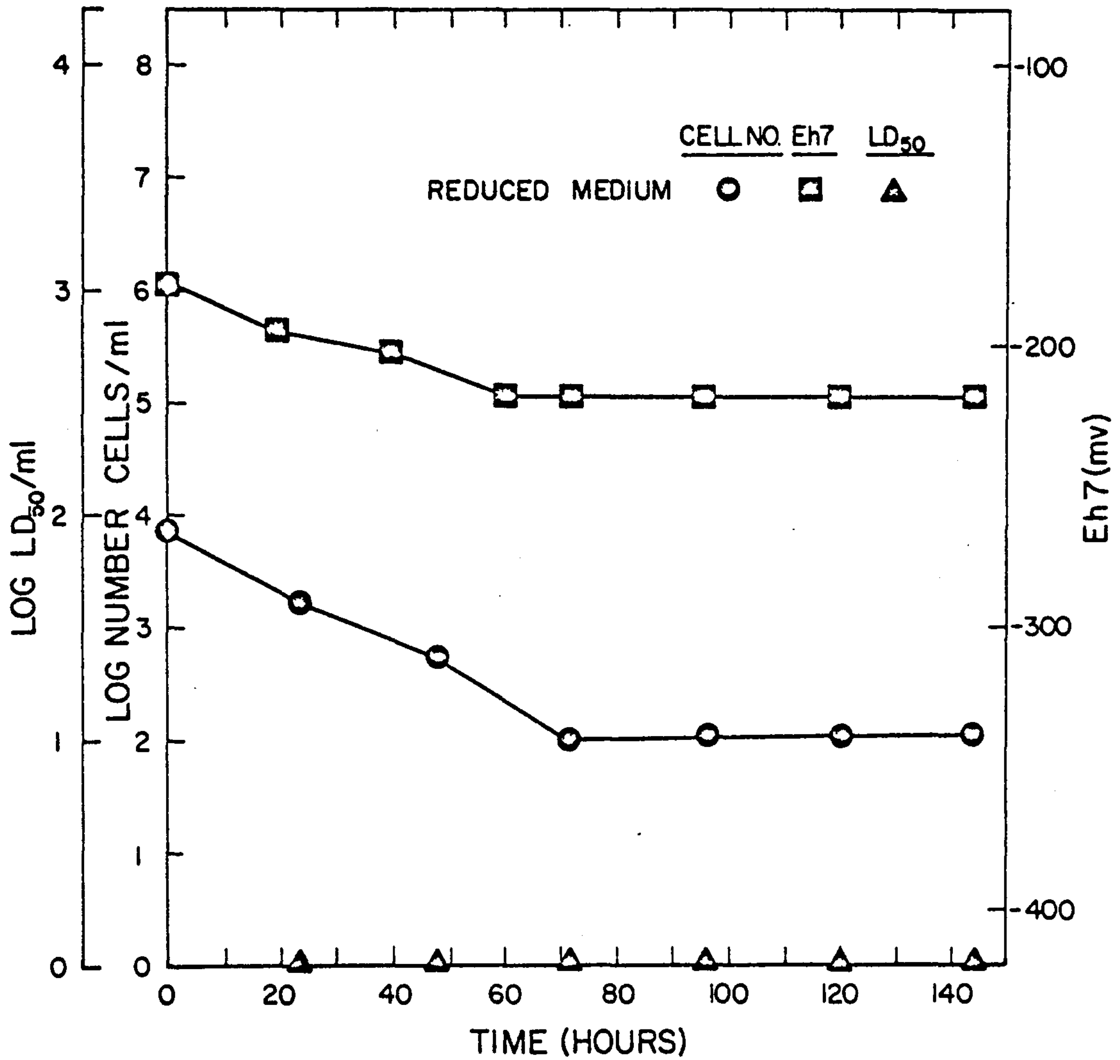


Fig. 12. Growth and toxin production and C. botulinum type A and Eh7 changes in prereduced trypticase soy broth (pH 6.8) plus 0.05% cysteine and 40% sucrose.

c. Hydrogen ion

Growth, toxin formation, and Eh7 changes by the test strain in oxidized and reduced TSB at pH 5.3 are presented in Fig. 13. The media reached a maximum cell population after 48 hours incubation with 4.3 x 10^8 cells/ml in the oxidized and 5.5 x 10^8 cells/ml in the reduced. However, there appeared to be a slightly longer lag phase for growth in the oxidized medium at this hydrogen ion concentration. Eh7 reduction began approximately 10 hours after inoculation of the flask for both media.

Toxin formation in the oxidized and reduced TSB at pH 5.3 was very similar (Fig. 13). In both media, toxin was not detected until the 48th hour of incubation. Maximum toxin formation in the oxidized medium occurred at 72 hours with 20,000 LD_{50}/ml while in the reduced medium the maximum titer of 20,000 LD_{50}/ml was not detected until after 96 hours. This may be explained by the more rapid death rate of the vegetative cells in the oxidized TSB as compared to the death rate in the reduced TSB. Thus, more toxin may be present due to the increased cell lysis in the oxidized TSB.

When the pH of the oxidized and reduced TSB was lowered to 5.2, further inhibition of growth and toxin production by the test strain was observed (Fig. 14). Growth and Eh7 changes in the reduced TSB began roughly 10-20 hours after inoculation of the flask with a maximum cell population of 3.8 x 10^8 cells/ml attained after 48 hours. Growth was not initiated in the oxidized TSB until after 24 hours of incubation and the maximum cell number of 3.7 x 10^6 cells/ml was not detected until 48 hours later.

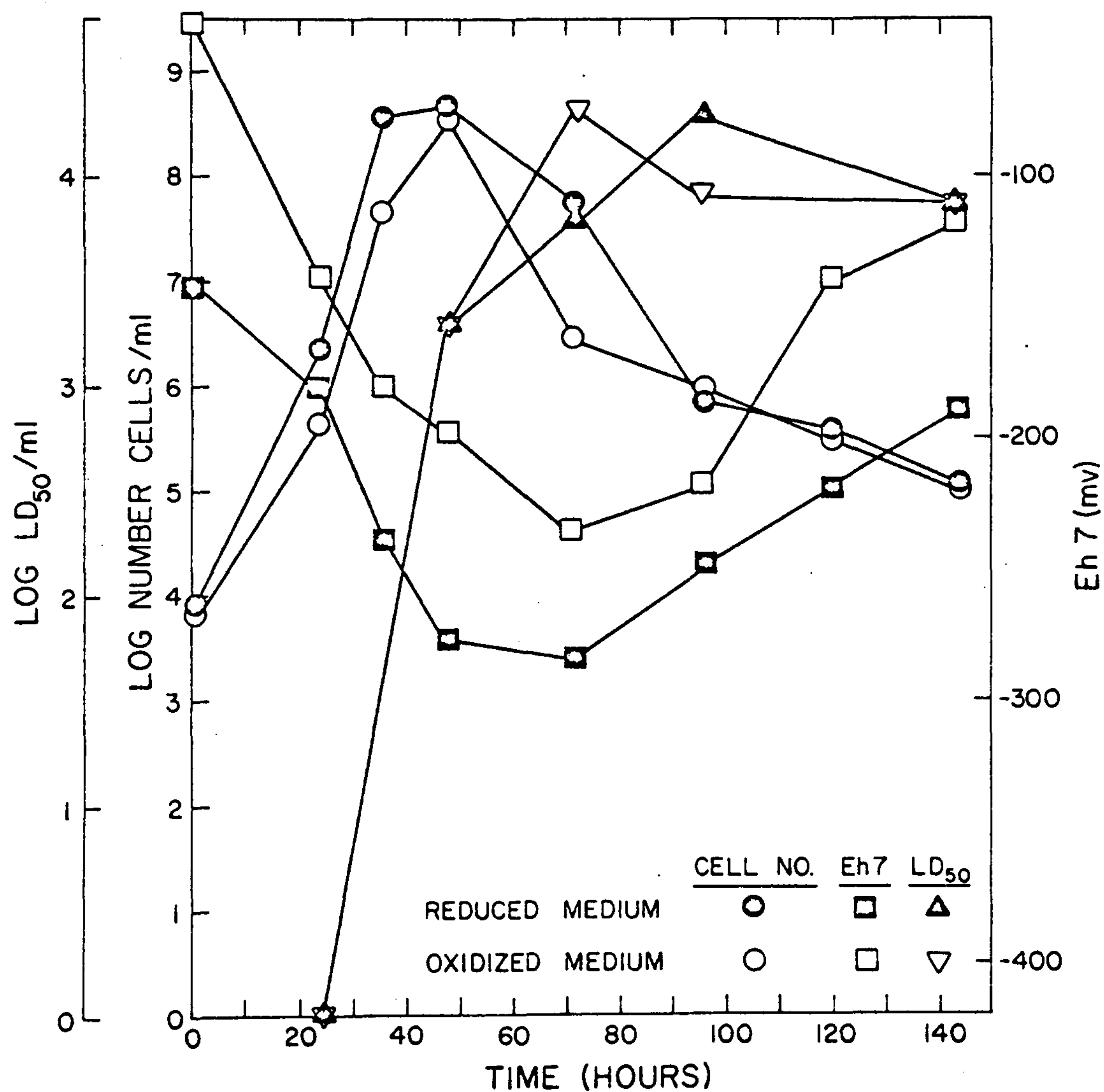


Fig. 13. Growth and toxin production of C. botulinum type A and Eh7 changes in oxidized and prereduced trypticase soy broth (pH 5.3) plus 0.05% cysteine.

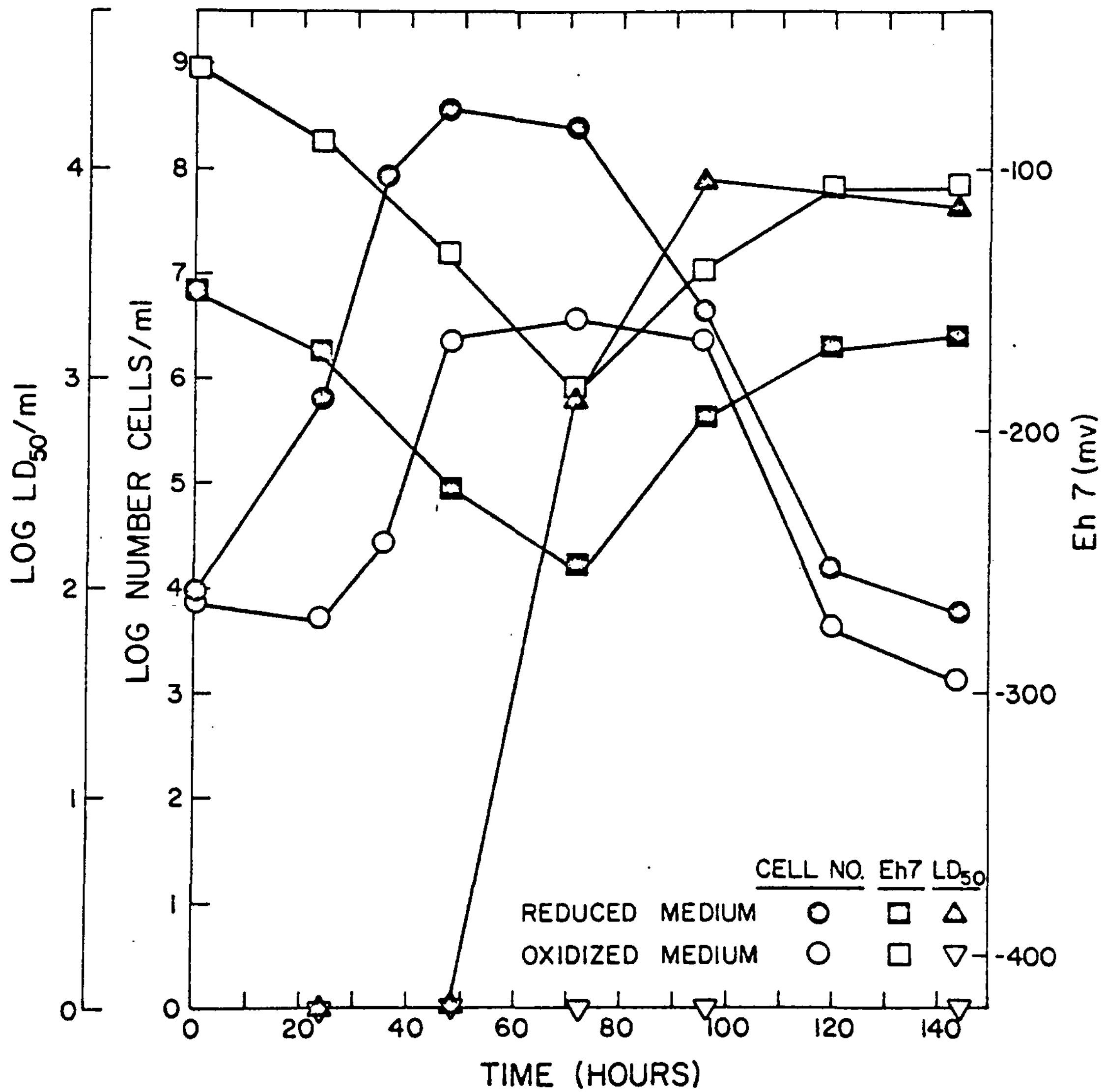


Fig. 14. Growth and toxin production of C. botulinum type A and Eh7 changes in oxidized and prereduced trypticase soy broth (pH 5.2) plus 0.05% cysteine.

Toxin formation in the reduced medium was first detected at 72 hours of incubation and the maximum toxin titer of 9,100 LD_{50}/ml was found at 96 hours of incubation (Fig. 14). Toxin was not detected in the oxidized medium through 144 hours of incubation.

Upon lowering the pH of the reduced TSB to 5.1, inhibition of germination for the test strain was observed (Fig. 15). Through 144 hours of incubation, growth, toxin production, or Eh7 changes were not detected. The initial spore inoculum remained phase bright and heat resistant.

Past workers have reported the relationship between Eh and pH (Liestner and Mirna, 1959). It was shown that the Eh of the system would increase as the pH of the same system is decreased. Hanke and Bailey (1945) have reported that the pH of a system has a marked effect on the growth limiting Eh for certain anerobes. As the lower pH limit is approached, both the hydrogen ion concentration and the resultant high Eh may be inhibitory factors determining whether or not growth occurs.

More recently, M. V. Smith (1975) reported that the Eh of the system has a marked effect on the limiting pH for growth of *C. botulinum* type E spores. Data shown in Fig. 13 and 14 support this work. In Fig. 14 both media contained the same hydrogen ion concentration (pH 5.2), however, the reduced medium allowed a higher maximum cell population to be reached as compared to the oxidized medium. Also growth was not sufficient in the oxidized medium for detectable levels of toxin to be present.

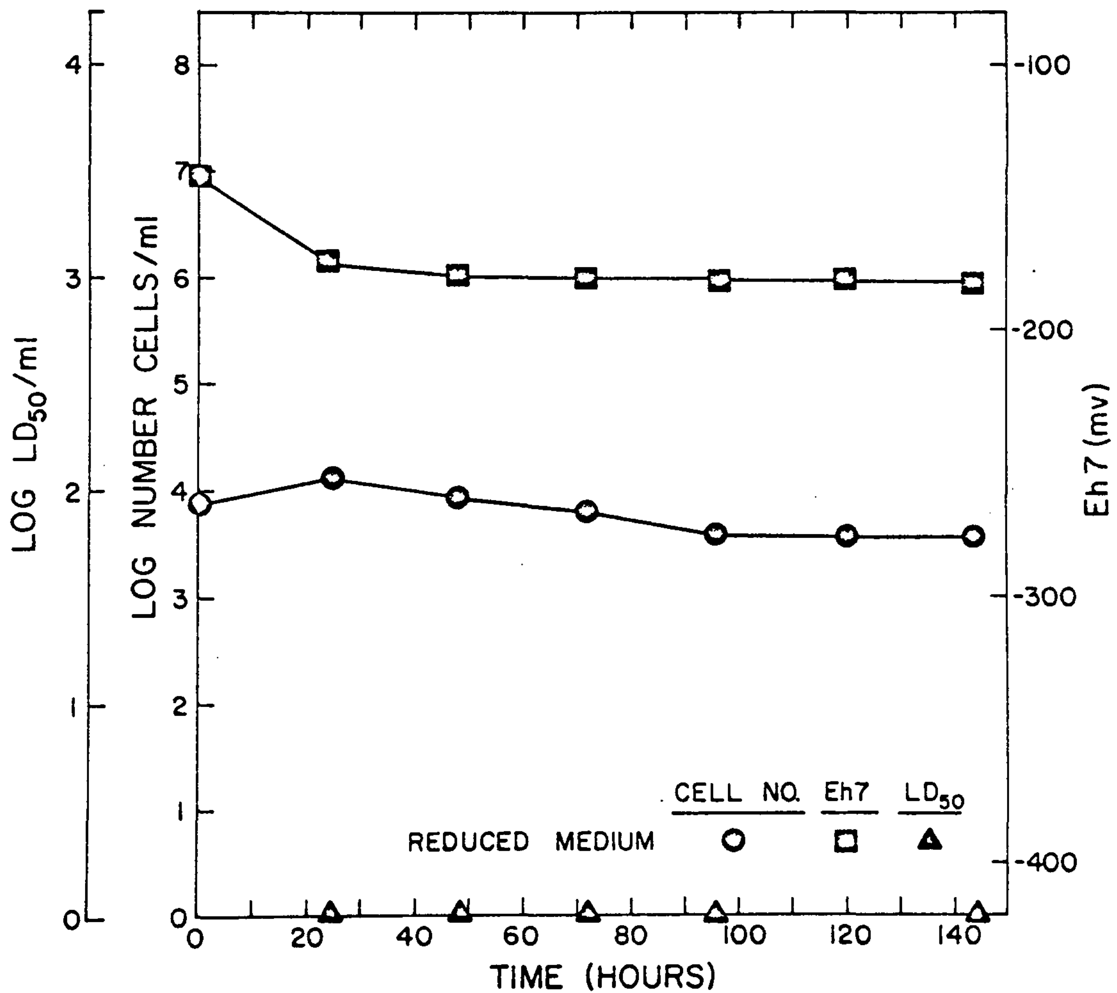


Fig. 15. Growth and toxin production of C. botulinum type A and Eh7 changes in prereduced trypticase soy broth (pH 5.1) plus 0.05% cysteine.

Other investigators have reported the outgrowth of 62A and 213B spores was inhibited at pH 4.8 to 5.0 depending on the substrate (Townsend, et al., 1954). Meyer and Kietzman (1957) found that a pH of 5.25 and below was inhibitory for a vegetative cell inoculum of type A. Therefore, the observed lag in growth for the test strain in the results presented in Fig. 14 may be due to the inability of the vegetative cells to grow, rather than inhibition of germination.

D. Toxin Stability

The toxin of C. botulinum type A strain 10755A decreased in toxicity from 6300 LD_{50}/ml at zero hours to 3724 LD_{50}/ml at 24 hours upon storage at 4 C. Refrigerated storage of the toxin sample for 24 hours did not significantly influence the results of the toxin quantitation studies. However, for toxin samples stored for 48 and 72 hours at this temperature, there appeared to be a greater loss in toxin strength. Toxin titers after 48 hours storage dropped to 2000 LD_{50}/ml while 72 hours of storage resulted in a decrease of titer to 600 LD_{50}/ml.

SUMMARY AND CONCLUSIONS

The effect of oxidation-reduction potential (Eh) on the growth, toxin formation, and chemical inhibition of C. botulinum type A spores was investigated. Strict anaerobic procedures and prereduced media were utilized so only the effect of the Eh would be observed.

Growth, toxin formation, and Eh7 experiments were performed in an airtight four necked flask containing electrodes for measuring Eh and pH and attached tubing for exchange and mixing of various gases.

In both oxidized (autoclaved under air) and reduced (autoclaved under nitrogen) media, there were no significant differences observed in the growth and toxin production of the test strain under unihibited growth conditions. A maximum cell population and toxin titer were attained in both media at the same time during incubation.

However, in the experiments with the growth limiting concentrations of various chemicals, the effect of Eh was quite pronounced. The addition of 6.0% sodium chloride (6.5% total) to reduced TSB (pH 6.8) allowed moderate growth to occur without toxin formation. However, when the same amount of sodium chloride was added to oxidized TSB (pH 6.8), growth and toxin formation were inhibited through 144 hours of incubation. On the incorporation of 30% sucrose into the growth medium, a significant lag phase was observed in the oxidized TSB (pH 6.8) as compared to the reduced TSB at the same pH. Also growth was not sufficient enough in the oxidized media to allow toxin to be detected. The same phenomenon occurred in TSB when adjusted to a pH

of 5.2. In the reduced TSB (pH 5.2), moderate growth and toxin formation occurred while in the oxidized TSB (pH 5.2) an increased lag phase and a decreased maximum cell population resulted with no toxin being detected through 144 hours.

From the results obtained in this growth study, it would appear that one should consider the Eh of the system when determining the growth limiting concentrations of various chemical inhibitors (i.e. sodium chloride, sucrose, hydrogen ion) for anaerobic bacteria. This preliminary data suggests that the manipulation of oxidation-reduction potential may prove useful in the control of toxic microorganisms in food systems. However, further investigation with other strains of *C. botulinum* is warranted. Also growth studies should be conducted in food systems to determine if they have the ability to poise the oxidation-reduction potential at inhibitory levels. Use of such a phenomenom in a food may very well depend on the chemical composition of that food since various reducing compounds may be present.

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THE EFFECT OF OXIDATION-REDUCTION
POTENTIAL ON THE OUTGROWTH, TOXIN PRODUCTION
AND CHEMICAL INHIBITION OF CLOSTRIDIUM
BOTULINUM TYPE A SPORES

by

Leslie A. Smoot

(ABSTRACT)

The effect of oxidation-reduction potential (Eh) on the outgrowth, toxin production, and chemical inhibition of *Clostridium botulinum* type A spores was investigated. Growth, toxin formation, and Eh7 changes were monitored in an airtight four necked flask containing electrodes for continuous measurement of Eh and pH and connections for the exchange and mixing of prepurified nitrogen and anaerobic carbon dioxide. Strict anaerobic procedures and prepurified media were utilized to facilitate controlled conditions.

In the electrode vessel the media were poised at the different Eh levels by sparging the prereduced media with oxygen free nitrogen or by sparging oxidized media (media autoclaved under air) with the nitrogen gas.

Outgrowth, toxin production, and Eh7 changes were measured under various growth conditions. In both oxidized (Eh7 = -60mv) and reduced (Eh7 = -145mv) media, there were no significant differences observed in the growth and toxin formation under uninhibited growth conditions.

However, growth and toxin production were further delayed and/or decreased in the oxidized media as compared to the reduced media during inhibition by sodium chloride (5.5% and 6.5%), hydrogen ions (pH 5.2 and 5.3), and sucrose (30%).