GERMINATION AND GERMINATION-LIKE CHANGES IN SPORES OF *CLOSTRIDIUM BOTULINUM* TYPE E

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ABSTRACT

GERMINATION AND GERMINATION-LIKE CHANGES IN SPORES OF *CLOSTRIDIUM BOTULINUM* TYPE E

By

Kenneth Fox

Botulism food poisoning is usually caused by ingestion of a food product containing the toxic metabolites of a microorganism known as *Clostridium botulinum*. The toxin is liberated during outgrowth of the organism, which is most commonly found in food products as a spore. Before vegetative outgrowth can occur, the spore must germinate. Methods of preventing toxin production in foods may concentrate on the outgrowth period of growth cycle or the germination period. Research on germination of bacterial endospores has in the past been done mostly with the genus *Bacillus*, and most of the studies have dealt with germination as a distinct and separate phase of the life cycle of a sporulating organism. These studies attempt to evaluate germination of an anaerobe, *Clostridium botulinum* type E, in relation to subsequent outgrowth of the germinated spores. Whenever germination is considered as a single

phase, the problem of differentiation between true germination and germination-like changes occurs.

Spores of type E Clostridium botulinum were germinated in three test systems. The first system was a medium composed of multipeptone, sucrose, and nutramino acids (MSN) containing all the necessary nutrients for germination, outgrowth, and sporulation. The second medium was a single component system (3.5% sodium nitrite) which caused "germination-like" changes in the spores. The third system was a three component system which caused true germination of the spores, but could not support outgrowth since it lacked the necessary nutrients for outgrowth and sporulation. This system contained alanine, sodium bicarbonate, and glucose (ABG).

The three germination systems produced germination curves of different shapes. The MSN system yielded steadily increasing germination, the ABG system yielded steadily increasing germination but at a more rapid rate than in MSN. The sodium nitrite system gave a different pattern. The germination rate was very rapid initially, and then leveled off at approximately 100% germination. This system caused "germination-like" changes in the spores as seen under dark phase contrast microscopy. However, these spores did not undergo outgrowth when transferred to a complete growth medium. The conclusion is that the sodium nitrite

caused a response that appears to mimic that of true physiological germination; but since this chemical does not prepare the spore for or may even inhibit outgrowth, the process which it causes should not be considered germination. In this nitrite system neither a temperature optimum nor a pH optimum existed, whereas true temperature and pH optima were shown in the MSN and ABG systems. The shapes of the germination curves in the ABG and MSN systems were steadily increasing, but the nitrite system produced a curve rising rapidly to approximately 100% germination. Observation by phase contrast microscopy showed the spores in the nitrite system to "germinate" in a synchronous manner, whereas microscopic observation of spores in MSN and in ABG showed a sequential pattern of germination.

Spores which had been germinated in ABG underwent outgrowth when they were transferred to a complete growth medium (MSN).

It was concluded that the ABG system therefore represents true physiological germination, whereas the nitrite system represents a situation in which "germination-like" changes occur, but the spores are not prepared for outgrowth.

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IN SPORES OF
CLOSTRIDIUM *BOTULINUM* TYPE E

By

Ian
Kenneth Fox

A THESIS

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To my parents

for their patience
and encouragement
over the years

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CHAPTER I

INTRODUCTION

Food poisoning is a problem which concerns everyone who handles food. The most common type of food poisoning in recent years has been caused by *Clostridium perfringens* with *Staphylococcus* also playing a major role. Most of these cases of food poisoning are relatively mild, and the mortality is rather low. Botulism cases occur more infrequently but possess a rather high mortality rate of approximately 30 to 40%. Botulism as a disease is caused in a susceptible host by consumption of the toxic metabolites of an organism known as *Clostridium botulinum*. Microbiologically this organism is classified as a gram positive anaerobic spore forming rod. Serologically there are six types: A, B, C, D, E, and F. Those types which are pathogenic to human beings are A, B, and E, and recently F. These strains produce serologically different toxins, and the organisms are distinctly different physiologically. There are three distinct physiological groups of *Clostridium botulinum* differentiated on the basis of reactions on different substrates.

Group I consists of *C. botulinum* type A, proteolytic strains of *C. botulinum* types B and F, and *C. sporogenes*. Group II consists of *C. botulinum* types C and D, and *C. novyi* type A. Group III consists of nonproteolytic strains of *C. botulinum* types B and F, type E, and culturally similar organisms that are non-toxic. This classification is taken from Holdeman (1970).

When a proteolytic A, B, or F strain invades or is present in a food product, such as a can of corn, which meets the growth requirements (anaerobiosis, neutral pH, protein), the organism which most probably gained entry as a spore, germinates, grows, and produces a powerful neurotoxin. Other metabolic by-products besides toxin are produced such as gases, and protein decomposition products, primarily amines. A person planning to consume this food product would be given some warning due to the odor detected when the can was opened and in most cases would not consume the product.

When a food has been invaded by type E organisms, another problem generally develops. Type E organisms, being nonproteolytic and saccharolytic in nature, do not produce these amine compounds which give rise to off odors. Thus a potential consumer might not be warned of the inherent danger of the food product. In the past 10 years type E organisms have been found widely distributed among marine products. The Great Lakes region of the United States has been host to several outbreaks of type E botulism. Viable spores of these organisms have been isolated from the mud and waters of the Great Lakes as well as from many of the fish that live in these waters.

Toxin production by _C_. _botulinum_ occurs during outgrowth. Being a spore former, the organism spends much of its life cycle as a dormant spore. It is in this phase that the organism is generally found in nature. However, when the environmental conditions become suitable, the spore may germinate and grow into a mature vegetative cell and begin producing its toxin. If this environment is a food product which is subsequently consumed, then clinical botulism may result.

The prevention of botulism has been attempted in the past with only types A and B principally in mind. These types produce very heat stable and heat resistant spores. It is for this reason that the thermal processes for canned foods were developed back in the 1920's. A retort cook of approximately 45 min at 250 F will sterilize most cans of food weighing approximately one pound.

More recently type E _C_. _botulinum_ has been found in foods which normally do not receive a pressure process. This situation presents a new problem. How do we prevent the growth of an organism in a food which is not to be sterilized, such as a vacuum packaged fish product?

There are two general approaches to this problem. The first approach involves changing the environmental conditions so that the organism cannot undergo outgrowth, thereby preventing the release of the powerful toxin. The second approach involves preventing the germination of the spore. If the spore does not germinate, then outgrowth obviously cannot occur and no toxin is produced. These are two distinct problems, since the environmental conditions for germination are not necessarily the same as those for outgrowth of the organism.

This thesis will deal with aspects of the second approach, such as examination of germination and subsequent outgrowth. The literature does not report much work on germination requirements of *C. botulinum* type E. A necessary step in ultimately finding a preventative to germination consists of understanding and defining germination in chemically defined systems.

Much work has been done on germination of other spores primarily of the genus *Bacillus*. Most of the work to date has, in this author's opinion, not considered the consequences of outgrowth in characterizing and defining the process of germination. This thesis will attempt to clarify the definition of germination and discuss and evaluate some of the characteristics which differentiate true physiological germination from germination-like changes.

It is hoped that this foundation will enable further researchers to pursue the more applied aspects of prevention of germination in foodstuffs.

CHAPTER II

LITERATURE REVIEW

Characteristics of Spores

The bacterial endospore is a body formed by gram positive spore-forming rods of the genera Bacillus and Clostridium.

Robinow (1960), Murrell and Warth (1965), Murrell (1967), Walker et al. (1961), Warth et al. (1963b), and many others have reviewed the composition of bacterial endospores. The outermost envelope surrounding the spore is called the exosporium. This is a thin, delicate covering, which lies outside of the spore coat. Beneath the exosporium lies a layer or layers, the spore coat or coats, which give rise to the characteristic structure of the spore. Bradley and Williams (1957) demonstrated that the surfaces of spores have different patterns of ridges or grooves. More recent work by Hoeniger and Headley (1969) and Samsonoff et al. (1970) have confirmed these observations. The spore coat may be composed of more than one layer. The inner layer of the spore coat is separated from the spore cortex by a thin membrane.

The region below the inner spore coat is characterized by low electron density and was named by Robinow (1953) as the cortex. This region which occupies approximately half of the spore volume within the outer coat of Bacillus megaterium (Mayall and Robinow, 1957) is evident in the electron micrographs of thin sections of spores of both aerobic and anaerobic bacteria. Numerous authors have suggested that the cortex is the location of dipicolinic acid.

The dormant cytoplasm is separated from the cortex by a thin membrane called the spore wall (Hashimoto and Naylor, 1958). The spore wall is also of low electron density and is often not visible unless sections of acid-treated spores are examined. Cytological studies on the process of sporulation in bacteria have led to the conclusion that the spore envelope (exosporium and spore wall) is composed of distinct layers and that these layers are not derived from vegetative cell membranes.

The question of the permeability of bacterial spores is a question still not entirely resolved. Many attempts have been made to measure the water content of spores, often with conflicting results. Based on the high optical density of the spore, many workers have concluded that the water content of spores is very low. A number of older papers have been concerned with the water content of spores (Friedman and Henry, 1938; Waldham and Halvorson, 1954). This theory of water content has more recently been considered in light of the results obtained by Gerhardt and Black (1961). These workers found permeability of spores to be quite great, and that glucose permeated 40% of the weight or 51% of the volume of spores of B. cereus apparently by free diffusion since its uptake was relatively independent of environmental variables.

Another interesting question is how an anhydrous region of the spore can be created in an aqueous environment. The theory of a contractile cortex system in the bacterial spore would suggest a mechanism to dehydrate the protoplast and a mechanism for maintaining this state. The contractile cortex theory is compatible with the current data available on water permeability of bacterial spores (Lewis et al., 1960). Studies on water permeability and spore moisture content have been primarily associated with research on heat resistance (Fox and Pflug, 1968; and Fox and Eder, 1969).

The germination of spores of Clostridium botulinum has not been widely studied. Such parameters as temperature optima, pH optima, optimal substrate, germination and growth requirements and sporulation requirements are still not clearly understood. An understanding of these parameters is necessary if we are to solve the problems concerned with botulism food poisoning.

There are several factors accounting for the lack of information in the area of germination of C. botulinum type E spores. First of all, the organism being an anaerobe makes research more complicated than similar studies on aerobes such as the Bacillus sp. Secondly, the toxic nature of the organism limits the amount of investigation it receives. Thirdly, sporulation is occasionally erratic and the sporulation percentage is reduced, rendering the production of spore crops much more difficult.

Several physiological changes occur during germination of spores, and these changes can be used to monitor the germination process. Some of the most commonly used parameters are the following:

1. Loss of refractility and darkening of the spore as seen under phase contrast microscopy.
2. Reduction in the optical density of spores suspended in water when monitored at 600-610 nm.
3. Increased stainability with basic dyes.
4. The release into the suspending medium of dipicolinic acid and calcium.
5. Decrease in dry weight of the spores.
6. Swelling of the spore.
7. Loss of resistance to high temperature, dessication, radiation, and chemicals.

Campbell (1957) defined spore germination in bacteria as "the change from a heat resistant spore to a heat labile entity which may not necessarily be a true vegetative cell." The choice of which one of these parameters to use in a study of spore germination to most easily reflect the loss in heat resistance will depend upon the organism and the specific goals of the research project. The most general definition of germination is as follows: (Sussman and Halvorson, 1966) "Germination occurs when the first irreversible stage that is recognizably different from the dormant organism is reached, as judged by physiological and morphological criteria." However, in the present studies the subsequent ability of the germinated spore to produce outgrowth was also evaluated because of the relevance to the food poisoning phenomenon.

Description of the Germination Process

When a spore is placed in a surrounding environment suitable for germination, a number of events occur. The spore changes from a bright round to oblong body to a dark body with a slight increase in size when observed with the microscope. This process can occur in as short a time as two to three minutes. However, a microlag may occur which might be as long as several hours. Hashimoto et al. (1969) studied single cell germination by phase contrast microscopy. They divided the total germination time into two events: micro-lag and micro-germination. They further divided the second phase, microgermination time, into two phases. The first phase of microgermination involves water influx into the spore, loss of dipicolinic acid (DPA) and loss of thermostability. This results in swelling of the spore which initially is relatively low in water. When bacterial spores germinate, a considerable amount of organic matter leaches out of the cell. Powell and Strange (1953) demonstrated that a 30% decrease in the dry weight of spores of *Bacillus subtilis* and *Bacillus megaterium* occurs during germination. Approximately 50% of the germination exudate was identified as calcium-dipicolinic acid complex. Lee and Ordal (1963) demonstrated that 14 amino acids were present in the spore exudate, glutamic acid and lysine being the major constituents. Riemann (1963) has analyzed the excretions of amino acids and peptides from spores of a putrefactive anaerobe, 3679h, germinated by calcium-DPA and EDTA. The first stages of germination do not involve protein synthesis. Chloramphenicol, an effective inhibitor of protein synthesis, has no effect on the loss of heat resistance when spores are incubated in L-alanine and adenosine. The second phase of microgermination involves a further degradation of the cortex and a further loss of spore materials.

During the microlag, a degradation or modification of the surface of the spores occurs by enzymatic reactions triggered by specific germinants (Vary and Halvorson, 1968). The rapidity of the initial degradative reactions, which proceed endogenously, is represented by the microlag time. The termination of these reactions results in the initial rapid drop in refractility during which release of DPA and loss of heat resistance occur. After the release of DPA and partial hydration of the spore, further degradative reactions involving the dissolution of the cortex are initiated. These reactions, dependent on environmental factors, terminate with the release into the medium of components of the cortical peptidoglycan (Warth et al., 1963). At the end of this series of reactions, the spores are completely hydrated, fully stainable with basic dyes, and non-refractile when examined under dark phase contrast microscopy.

Uehara and Frank (1967) studied the sequence of events during germination of putrefactive anaerobe 3679 spores. Their results showed that germination of PA 3679 consists of a sequence of events in two separate regions of the spore. Early events that occur in the peripheral region (cortex) of the spore are followed by additional events in the central region (core) leading to complete germination. The initial germination step could be measured by DPA release, absorbancy decrease, gradual phase darkening of the periphery, and in some cases the loss of resistance to heat and toxic agents. The second barrier controls the final germination step, probably by controlling permeability of the core membrane.

Quite possibly hydration occurs during the final germination step since swelling of the core is observed during germination in most species (Robinow, 1953).

Occasionally a population of spores contains what have been labeled super-dormant spores (Gould et al., 1968). In any population, there are a certain number of spores which will never germinate, and a few of these may be super-dormant spores. The latter are generally difficult to detect. What causes this state of superdormancy is not known. Woese et al. (1958) have suggested that one important factor could be the distribution within the spores of a critical germination enzyme. If molecules of the germination enzyme are Poisonally distributed in a spore population, then the number of critical molecules could be so low in some spores as to result in a super-dormant state.

Initiation of Germination

Spores require some sort of "trigger" to cause them to begin the germination process. These triggers provide the activation necessary to break the dormant spore state. Various physical and chemical factors which serve as activators include temperature, light, moisture, and the composition of the medium.

Many spores require a "heat shock" or heat activation before they will germinate. Evans and Curran (1943) first systematically demonstrated that spores which did not germinate overcame this dormancy when heat activated. Later workers found that spores also were subject to other environmental stresses during heat activation. Keynan et al. (1964) have shown that heat activation can be prevented by such environmental stresses as low pH. Certain cations may interfere with activation when present during heating. Splittstoesser and Farkas (1966) have demonstrated an inhibition of heat activation of Bacillus popilliae spores in the presence of potassium ions. Heavy metals such as iron and copper inhibited the activation of spores of B. cereus T and of B. megaterium (Levinson and Hyatt, 1955; Murty and Halvorson, 1957). Heat activation has been shown to enhance germination caused by many chemical systems. In B. megaterium heat activation enhanced the rate of both L-alanine and glucose induced germination (Levinson and Hyatt, 1955). Keynan et al. (1961) found that the rate of germination of spores of B. cereus in the presence of calcium-DPA was not influenced by prior heat activation. Black (1964) observed that heat activation did not influence the rate of nitrate-induced germination. Hyatt and Levinson (1968) found that dormant spores of B. megaterium were activated for germination on glucose by heating them in an aqueous solution (but not if heated dry), by treating them with aqueous ethyl alcohol or by exposing them to water vapor. This activation, a reversible process, results in a spore which retains its typical heat resistance, refractility, non-stainability, and DPA content, but which is no longer dormant--that is, it is conditioned for germination. Heat activation of spores of B. cereus T (Keynan et al., 1964) could be accelerated by using reducing agents such as mercaptoethanol or thioglycollic acid or high concentrations of H^+ ions (pH below 5).

Curran and Evans (1945) recognized the phenomenon that the conversion from a dormant to an activated state is a reversible process since spores which had been activated could revert upon storage, but could be reactivated with a second heat treatment.

Chemical Trigger Agents

Chemicals may cause the germination of a spore by either of two basic mechanisms: 1) The chemical may affect the metabolism of the spore, or 2) the chemical may effect some physical action on the surface of the spore. The first category generally includes metabolites, while the second category commonly includes surface active agents. The chemical activators that are metabolites suggest that germination has an enzymatic basis for the following reasons (Sussman and Halvorson, 1966):

1. Germination agents are usually normal metabolites and in a number of cases disappear during germination.

2. Stereospecific binding sites can be recognized for germinating agents which are subject to competitive inhibition.
3. The temperature dependence of germination is that expected of an enzymatic reaction.
4. Germination can be blocked by a number of metabolic poisons.

A chemical triggering agent is one which will induce germination but whose continued presence is not necessary for germination. It has been known for a long time that L-alanine is able to induce germination in spores of B. subtilis (Hills, 1950; Powell and Hunter, 1955; Church et al., 1954). Keynan and Halman (1961) have investigated some of the properties of the L-alanine trigger mechanism in the germination of spores of B. licheniformis. Often delays in the germination of spores after the addition of the germinating stimulants have been observed in a number of cases, including L-alanine mechanism and the research discussed in this thesis (Woese et al., 1958).

Prior to germination, a spore may be first heat activated and then placed in a germination system, or the spore may not require activation. Much of the evidence available indicates that the prime event responsible for activation is not metabolic. Most workers in this field are of the opinion that the change from the dormant stage to an active one is accompanied by a change in the tertiary structure of some macromolecules of the spores (Keynan and Evenchik, 1969).

It has been suggested that activation changes the tertiary structure of a protein responsible for the dormant state of spores, and that activation could be considered as a reversible denaturation of the protein (Keynan et al., 1964). Activation at pH extremes occurs in the range at which proteins are denatured. Disulfide bond disrupting agents and reducing agents which induce activation also are known to cause denaturation of proteins. Ionizing radiation also can cause changes in the tertiary structure of macromolecules, either by direct action or by secondary effects of reactive free radicals from water, causing rupture of hydrogen bonds, peptide bonds, or other covalent linkages (Bacq and Alexander, 1961). Spores contain five times more sulfur than is found in vegetative cells (Vinter, 1961). This additional sulfur is concentrated in the spore coats as cystine. It is therefore possible that the macromolecules responsible for maintaining the dormant state are coat proteins rich in cystine, stabilized in a specific configuration by S-S linkages.

Very polar solvents rupture hydrogen bonds, causing changes in the tertiary structure of proteins. Experiments with polar solvents show that substances which cause denaturation of proteins primarily by H-bond breakage are very effective in producing rapid activation of bacterial spores even at low temperatures (Widdowson, 1967). While present kinetic evidence suggests that heat activation is more likely the result of breakage of a few strong bonds rather than many weak ones in the structure thought to be responsible for spore dormancy (Busta and Ordal, 1964), it is not known to what extent the breakage of H-bonds is involved in heat activation.

Keynan et al. (1964) have suggested that activation by heat, reducing agents, and low pH may involve a reversible reduction of disulfide bonds resulting either in an unblocking of an enzyme system, or in a change in the permeability of a structure controlling the dormant state of the spore. Freese and Cashel (1964) have advanced the following hypothesis for the activation induced by calcium and dipicolinic acid: The solution of Ca-DPA used for activation is essentially saturated, so that tiny crystals or complexes of this salt are formed. Such small Ca-DPA crystals or complexes may pull individual calcium or DPA ions away from spores. This process may either make sites more accessible that can subsequently react more efficiently with germinating agents such as L-alanine, or the permeability of spores may be increased by the Ca-DPA treatment. Riemann and Ordal (1961) also observed that equimolar amounts of calcium and dipicolinic acid are effective germination agents for bacterial spores of *B. subtilis*. These spores would germinate without heat activation, merely by the addition of the Ca-DPA mixture.

Most of the research on spore activation has shown that this activation process must be carried out in the presence of water. Murrell (1967) concluded that it is still not known if a small anhydrous region surrounded by an impermeable membrane barrier exists within the spore. Heat or ethyl alcohol may affect the structure of liquid water, permitting it to reach and hydrate the critical site in the spore. The identity of the site of spore activation is still unknown, but it may be an enzyme or enzyme system, dehydrated and inactive in the dormant spore, whose activity is required for germination (Hyatt and Levinson, 1968).

After the spore has been activated, the actual germination process begins. Ward and Carroll (1966) studied single amino acid germination of *C. botulinum* type E. These workers found limited germination with some amino acids. Glycine and L-cystine elicited the shortest germination times and the greatest amount of germination; phenylalanine or leucine, and alanine showed delays. Fujioka and Frank (1966) studied the nutritional requirements for germination of PA 3679 in a chemically defined medium. They concluded that in their chemically defined medium L-alanine was the primary germinant, and arginine and mineral salts (magnesium sulfate, iron sulfate, manganous sulfate, and sodium chloride) were secondary germinants.

When spores are germinated in a single amino acid solution, the germination is often incomplete. This incomplete germination may occur because of heterogeniety in populations of spores so that following incubation with a germinant for some time, the remaining ungerminated spores have germination requirements quantitatively and qualitatively different to those of the initial population (Foerster and Foster, 1966). It is possible that individual spores may differ in size, coat thickness, and permeability, metal, DPA content, content of free amino acids, or germination inhibitors, etc., so that different spores are in different states of readiness for rapid germination. Changes may occur in the medium during germination, causing incomplete or fractional germination.

A number of non-nitrogenous compounds have been reported to initiate bacterial spore germination. These include simple sugars, carbohydrates, and various normal metabolic intermediates in glucose metabolism (Stedman, 1956; Schmidt, 1955; Lawrence, 1955; Heiligman et al., 1956). Wynne and Foster (1948) observed that the requirement for carbon dioxide in spores of Clostridium sp. was absolute in chemically defined media and that oxaloacetic acid would replace carbon dioxide. The acids, L-malic, fumaric, and succinic, accelerated germination in C. botulinum and in B. megaterium but not in four other aerobes and four other anaerobes. Lactate, pyruvate, acetate, formate, succinate, fumarate, and malate have been reported as stimulants for germination of most species of Bacillus (Halvorson and Church, 1957). Hachisuka et al. (1955) and Lawrence (1955) found that although lactate was inactive and pyruvate was a poor germinating stimulant for spores of B. subtilis, both compounds were stimulants if the spores were preheated with these substrates. Levinson and Hyatt (1962) found that in unheated spores, the only nitrogenous germination stimulants were L-alanine, glucoseamine, and N-acetylglucoseamine, whereas heated spores germinated in response to nitrate, nitrite, and seven other amino acids.

A number of disaccharides have been reported to stimulate germination of spores. Most of the literature has been concerned with germination of the aerobic Bacillus genus, however. Maltose (Powell, 1951), lactose and sucrose (Hachisuka et al., 1955) have been shown to aid in germination of anaerobes. Products of glucose metabolism such as glycerol, acetate, and pyruvate have also been reported to be effective.

Glucose has been shown to initiate germination of several species of Clostridium (Wynne et al., 1954). Initiation of Clostridium bifermentans and Clostridium septicum spore germination by lactate plus amino acids was described by Gibbs (1964). Holland et al. (1969) used a medium consisting of L-alanine, sodium lactate, sodium bicarbonate and L-cysteine to germinate spores of Clostridia.

Studies on the metabolism of germinating spores of anaerobic bacteria have received little attention. Costilow (1962) observed a number of amino acid fermentations leading to CO_2 and H_2 in germination of spores of C. botulinum 62-A. During germination, the rate of amino acid fermentation increased; this rise was inhibited by chloramphenicol, an inhibitor of protein synthesis.

The ribosides have been reported to initiate germination in many spores mostly of the genus Bacillus. Powell and Hunter (1955) found that inosine was generally more active in initiating germination than was adenosine. It has been shown that inosine and adenosine were phosphorylated during or shortly following germination by incorporation of labeled phosphate into nucleoside phosphates (Srinivasan and Halvorson, 1961). Generally the most rapid germination is achieved by combinations of more than one germinant. The combination of L-alanine plus inosine or adenosine has been widely studied. In addition to organic germinants, spores generally require a certain concentration of ions such as sodium or calcium in the medium for maximal germination.

A number of chemicals which fall into the category of surfactants can be used to effect germination of spores. Rode and Foster (1960, 1962) have used the term chemical germination to refer to germination-like changes in spores produced by surface active agents and to distinguish this from physiological germination and mechanical germination. Foerster and Foster (1966) found that chemical germination with dodecylamine of different metal-type spores produced by ion-exchange took place at the same relative rates as physiological germination. Rowley and Levinson (1967) treated spores of B. megaterium with sodium thioglycollate for 30 min at 50 C. The spores remained refractile, but they became stainable, lysozyme sensitive and non-viable, and they lost DPA. Their percentage germination in glucose over 2 hr was markedly reduced. Rode and Foster (1966) found that when spores were exposed to an acid environment, there was an exchange of ions between the spore and its surrounding environment affecting dormancy, heat resistance and germinability.

Duncan and Foster (1968) showed that sodium nitrite alone was able to stimulate germination of PA 3679h spores, while Bester et al. (1968) showed a similar effect with C. butyricum. The process was accelerated by using increased concentrations of sodium nitrite, a low pH, and a high temperature of incubation. At low concentrations of nitrite (0.01 to 0.2%), a delay of 36 to 48 hr occurred before germination commenced at 37 C. Conversely, with 3.45% nitrite at 45 C and pH 6.0, most of the spores germinated within one hour. However, sodium nitrite inhibited germination by L-alanine, the degree of inhibition being influenced by nitrite concentration and pH (Labbe and Duncan, 1970). Hyatt and Levinson (1961) were able to induce germination of _B_. _megaterium_ spores with either KNO_3 or KNO_2, but only if the spores were first subjected to a sub-lethal heat treatment of 10 min at 60 C. Black (1964) also observed germination of _B_. _cereus_ strain _terminalis_ spores in 0.5 to 2.0 M $NaNO_2$ or KNO_2. The relatively low pH necessary for nitrite induced germination indicated possibly that undissociated nitrous acid is the effective compound.

Black (1964) had suggested that germination of _B_. _cereus_ spores by nitrite was effected by reduction, with nitrite serving as an electron donor, and possibly mediated by the activity of spore catalase. However, the fact that spores of PA 3679 and _C_. _botulinum_ type E (this thesis) have been shown to "germinate" in nitrite would indicate a non-specific action of nitrite, since presumably these spores are devoid of catalase.

Duncan and Foster (1968) suggested that if nitrite does act by reduction, it could conceivably result in the alteration of the tertiary structure of a spore protein, which in turn may be involved in the Ca-DPA complex. The increase in germination rate with increased temperature and increasing nitrite concentration may be a reflection of such structural alteration. The bacteriostatic action of sodium nitrite has been discussed by Castellani and Niven (1955).

Riemann and Ordal (1961) found that Ca-DPA could germinate a number of species of Bacillus and Clostridium. Germination was most rapid when equimolar calcium and DPA were used. The relationship of Ca-DPA germination to ionic germination is not clear. Rode and Foster (1962) found that Ca-DPA germination of spores of B. megaterium depended upon the presence of other ions, and that Ca-DPA could substitute for either germinant in the L-alanine + inosine germinating system. The exact mechanism of Ca-DPA induced germination is still not understood.

Spores have also been germinated with various enzymes. Lysozyme may cause changes in sensitized spores which are characteristic of those changes normally associated with germination. In addition to losing DPA, becoming phase dark, and heat sensitive, suspensions of lysozyme germinated spores become optically less dense. Continued incubation of spores in the presence of lysozyme causes loss of viability and eventually leakage of the spore contents into the medium. Sierra (1967) found that intact spores of B. subtilis are susceptible to subtilopeptidase attack, and this enzymatic reaction induces changes in the spore similar to those that take place during physiological germination. Germination with subtilopeptidase was almost completely inhibited by an excess of di-isopropyl fluorophosphate (DIPFP). L-alanine induced germination was not affected by DIPFP. Sierra concluded that subtilopeptidases initiate spore germination by releasing germination agents from the spores.

Effect of Physical Agents

Physical agents also have been reported to initiate spore germination. Rode and Foster (1960b) discussed the fact that the immediate result of abrading or cracking the surface layers of spores was to induce the germination-like changes usually associated with physiological spore germination. The fact that spores can be mechanically germinated suggests that the structural integrity of the coats and the cortex play an important role in maintaining the dormancy of the spores.

Clouston and Wills (1969) studied the effect of hydrostatic pressure as high as 500 atm on the initiation of germination of spores of *B. pumilus*. Initiation of germination occurred at pressures exceeding 500 atm and was the prerequisite for inactivation by compression.

General Process of Germination

The trigger reaction which causes the initiation of spore germination is not completely understood. The overall process of germination involves rapid depolymerization of murein, probably mostly from the outer cortex, and leakage from the spore of some of the resulting muropeptides along with calcium and dipicolinic acid. Changes in spore shape, volume, cytology, and optical properties presumably result more or less directly from the initial depolymerization reaction. If a major cause of spore dormancy and resistance is pressure exerted on the core by a contractile cortex, then it is easy to understand how activation of a cortex-lytic system could release the pressure and initiate germination (Alderton et al., 1964). The exact relation of Ca-DPA to this process is not entirely clear. There is still some question as to what part of the spore the Ca-DPA complex resides. However, the correlation of Ca-DPA to heat resistance and its loss during germination are well known.

Kinetics of Spore Germination

Any of the specific methods used to study spore germination may be used to follow the kinetics of germination. The method of using direct microscopic examination presents perhaps the best opportunity to discover the true kinetics of the organism. However, kinetic studies by direct microscopic examination are laborious, require specialized equipment, and often have to be repeated many times. The other most popular method of following kinetics of germinating spores is by use of turbidimetric measurements of populations of spores.

The early time course of germination of a spore population is sigmoidal, whether measured by optical changes or by the onset of heat sensitivity or by individual spore observation. Sometimes the first portion of the curve is not present, and this merely indicates a lack of a lag period. Vary and McCormick (1965) have done considerable work on characterizing the systems and kinetics of germinating spores. Germination at any point can be expressed as a fraction Y so that where OD_i is the initial optical density, OD_f is the final optical density, and OD_t is the optical density of the spore population after time t:

$$Y = \frac{OD_i - OD_t}{OD_i - OD_f}$$

A plot of 1/Y versus log t is linear:

$$\log 1/Y = - C \log t + \log \log (1/Y_o)$$

where Y_o is the value of Y where t = 1, and the slope is -C.

Heat activation affects the kinetics of bacterial spore germination. Pretreatment of bacterial spores at elevated temperatures for various periods of time affects the kinetics of germination. O' Connor and Halvorson (1961) have shown the effect of heat activation on lag time and rate of L-alanine induced germination. Heat activation generally caused the microlag to be decreased, and the rate of germination was increased. In addition, the response of spores to different concentrations of germinating agent is also influenced by prior heat treatment. The biochemical alterations underlying these changes are not well understood. Heat activation could release endogenous stimulants, alter the properties of the initial binding site, modify the rate of linked reactions, alter the permeability, or remove an inhibitor of germination. When germination is induced by Ca-DPA, the rate of change in absorbancy is relatively temperature independent, and not greatly affected by prior heat activation (Keynan and Halvorson, 1962). But the lengths of the lag period showed a temperature dependence characteristic of an enzymatic reaction. The ΔH for the shortening of the lag was similar to that for the rate of germination induced by L-alanine. These findings suggest that some metabolic reaction is taking place during the lag period of Ca-DPA induced spores.

Methods of Representing Germination Data

There are many methods of representing germination rate data for bacterial spores. Campbell et al. (1965) have used the plot of absorbancy versus time. This type of plot is shown in Figure 1. Other workers have plotted absorbancy versus time (Duncan and Foster, 1968). Others have plotted such things as percent transmittance versus time, and percent germination versus time. It is this author's opinion that a plot of percent transmittance versus time gives a more true picture of the way in which germination proceeds. The plot of absorbancy versus time tends to eliminate the S shape from the germination curve, and the S shape tells quite a lot about what is occurring. Figure 2 shows the same data as represented in Figure 1 but plotted as a function of percent transmittance versus time coordinate system. In this thesis, germination curves were chosen to be presented as percent transmittance versus time.

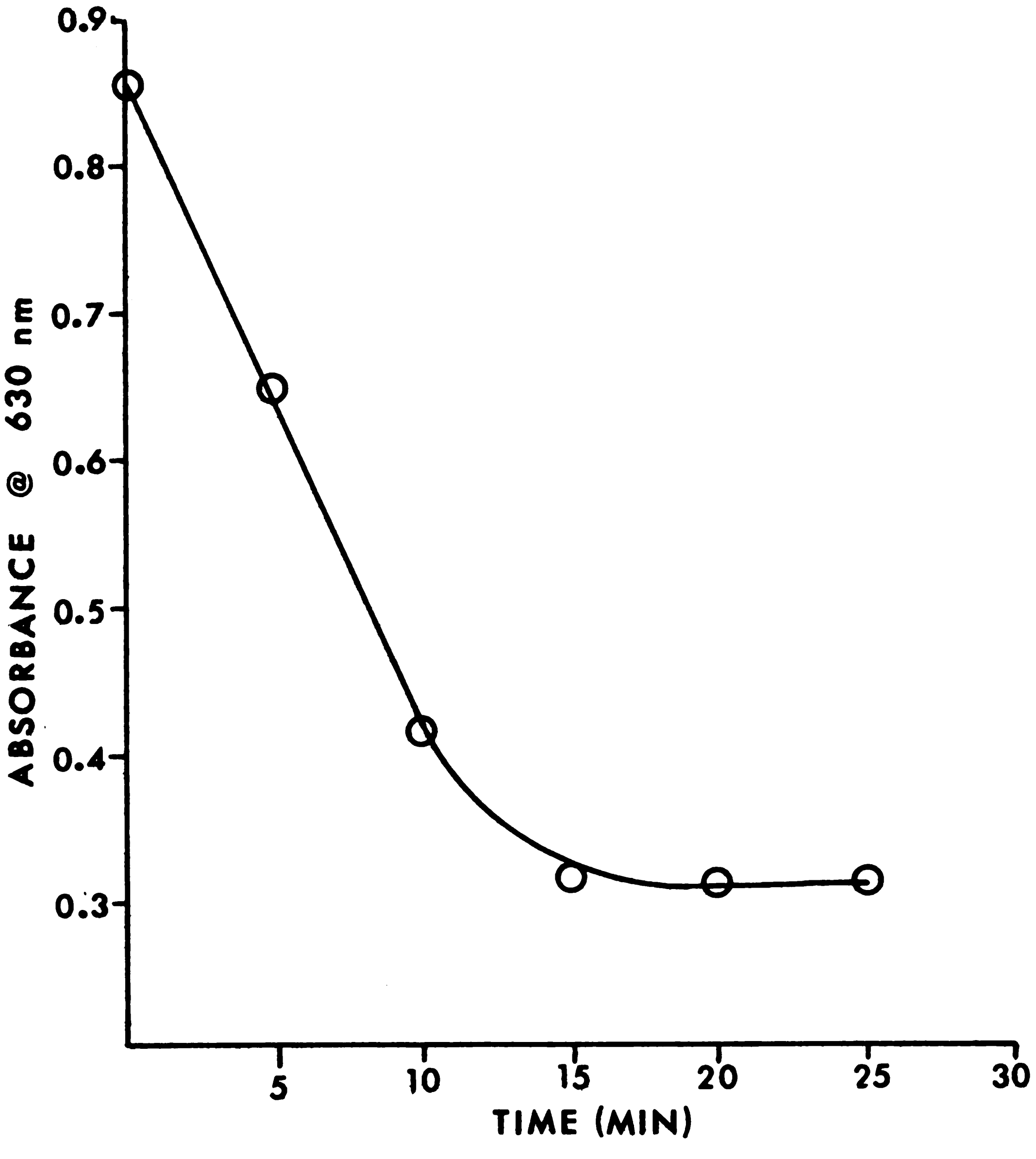

Figure 1. --Germination curve of Bacillus stearothermophilus plotted on absorbance versus time coordinates. (Campbell et al., 1965)

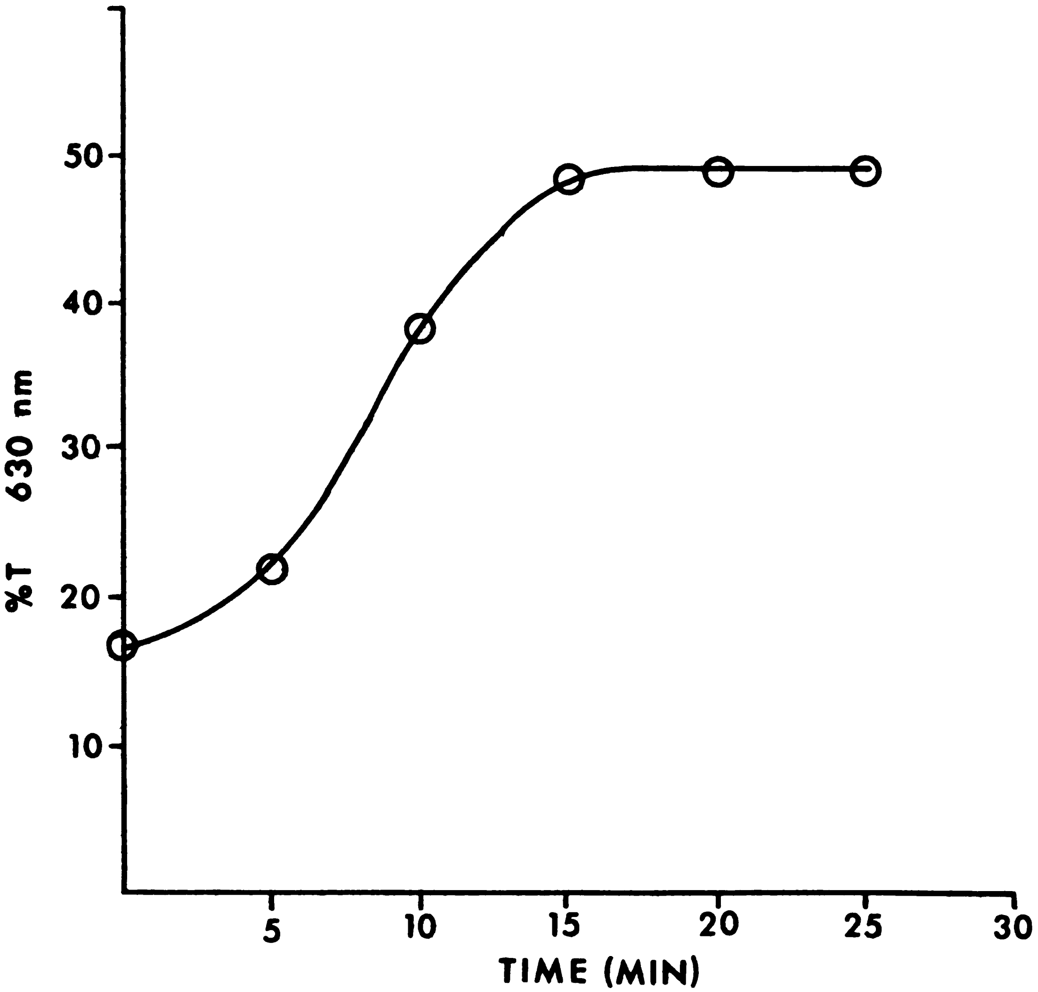

Figure 2. --Germination curve of Bacillus stearothermophilus plotted on percent transmittance versus time coordinates.

CHAPTER III

MATERIALS AND METHODS

Preparation of Spores

The organism used in this study was *Clostridium botulinum* type E Kalamazoo strain. This strain was obtained from Mr. R. W. Johnston, Food and Drug Administration, Detroit, Michigan. The organism was isolated by FDA in an outbreak in Kalamazoo, Michigan, in 1963, when smoked whitefish was identified as the food product.

Spores for this research were produced in a new medium containing the following ingredients:

Multipeptone (Fisher Scientific Co., Pitt., Penn.)	2.5%
sucrose	0.2
Nutramino acids (Fisher Scientific Co.)	1.0
sodium thioglycollate	0.2

This medium, termed MSN, when prepared in deionized distilled water has a pH of 6.9-7.0. Spore production was accomplished by inoculating one liter of the medium contained in a one liter screw-cap Erlenmeyer flask with one ml of a stock spore suspension. The flask was then incubated at 32 C for approximately 42 hr or until the maximum number of free spores was present when examined under dark phase contrast microscopy. If the incubation period was too short, fewer free spores were produced; and if the incubation period was too long, some of the free spores began to germinate, making the time of harvesting fairly critical. The spores were harvested by centrifugation at 5,000 rpm (4000 X g) for 15 min at 0 C. The pellet was collected and the spores were further washed three times in sterile distilled water. The spores were stored as a stock suspension in sterile water at a concentration of approximately 1×10^9 spores/ml. The stock suspension was kept at 4 C throughout the research. During the course of the research spores were produced from the spore stock as needed to obtain sufficient spores and to avoid the necessity of using aged spores for any particular experiment or experiments.

Procedure for Turbidimetric Germination Experiments

The turbidimetric population germination experiments were performed in the following manner. Five milliliters of the chemical germinant were dispensed into 13 X 100 mm screw-cap test tubes and one ml of the stock spore suspension containing approximately 1×10^9 spores/ml was added. The caps were tightened and the tube shaken slowly to avoid aeration, and the initial turbidimetric reading taken. The turbidimetric measurements were made on a Bausch and Lomb Spectrophotometer model 20 set at 600 nm wavelength. After the initial reading, the tubes were placed in a water bath at the appropriate incubation temperature (usually 32 C). The tubes were removed from the water bath at various time intervals and turbidimetric measurements were made. The readings were taken in percent transmittance for two reasons. First, the percent transmittance scale is more easily read than the optical density scale, thereby causing fewer errors in reading; and second, the percent transmittance could be read more rapidly with greater accuracy. Since it was desired to plot the germination curves in percent T, this method of reading the spectrophotometer proved reliable. Rapidity in reading the scale was important when 20 or 30 tubes were being handled at one time. Before each tube was inserted into the spectrophotometer the tube was shaken slowly to distribute the spores throughout the suspension, since the spores tend to settle during the incubation period.

Microscopic Observation Methods

Experiments were conducted in which individual spores were observed during germination and outgrowth. A system was designed to accomplish this task. Any system which permits observation of spores or bacteria undergoing germination or growth must meet the following requirements:

1. The system must allow the spores or bacterial cells to be in intimate contact with a liquid medium during the observation period.
2. The system must not allow the spores or bacterial cells to move around during the observation since their location must remain constant to allow observation over a long period of time, in many cases up to 5 hr.
3. The system must provide for anaerobic conditions in the case of _C_. _botulinum_.
4. The system must not allow the medium to dry out during the observation period. The water activity of the medium must remain relatively constant throughout the test period.
5. Once the spores have been added to the medium, the system must be rapidly set up for observation before the spores begin to germinate.

The system which will be described was designed to meet all of the above requirements.

Figure 3 shows a schematic diagram of the system used. A common 1 × 3 in glass microscope slide was used to support an agar mixture of the medium. Two milliliters of a 2X concentration

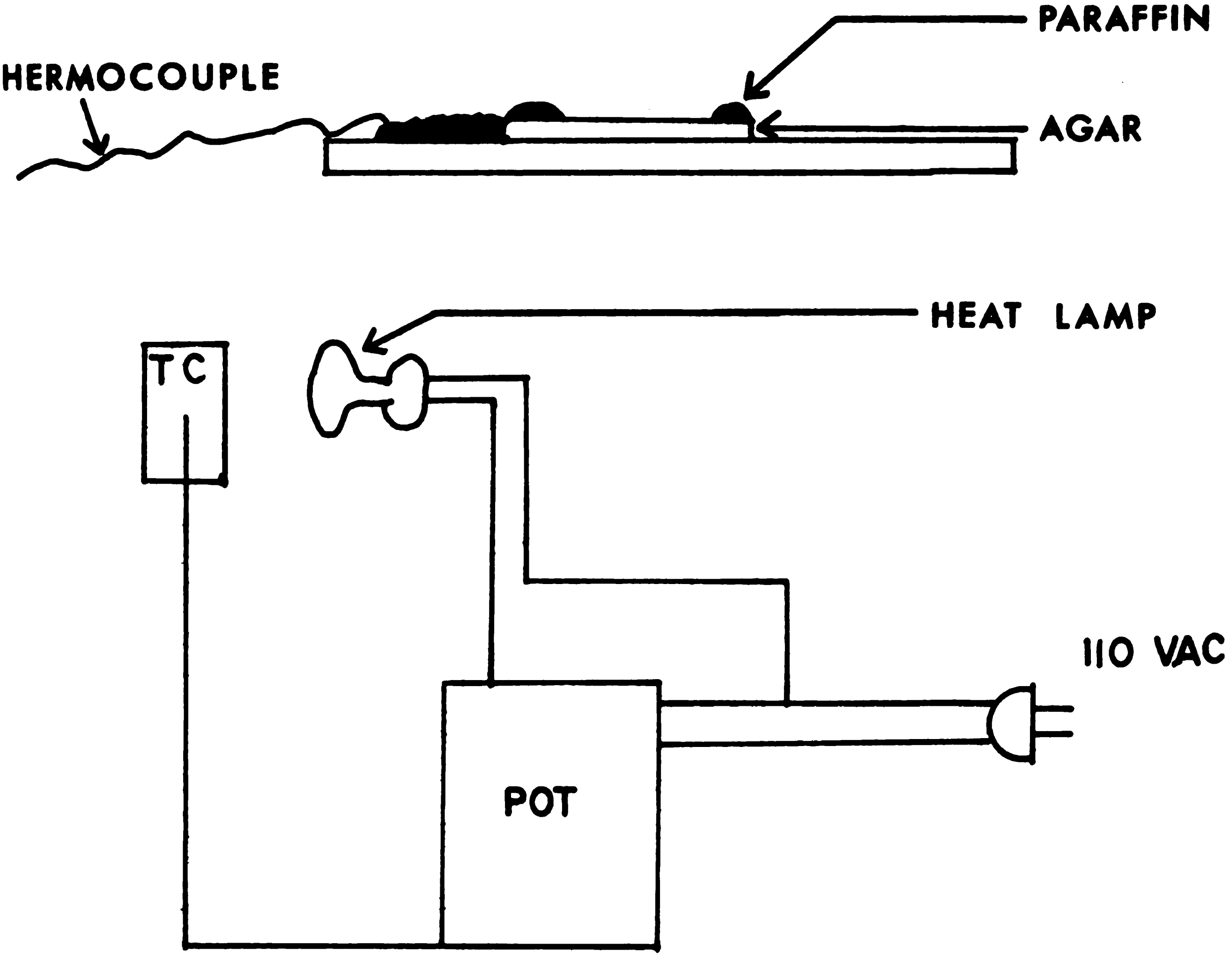

Figure 3. --Schematic diagram of microscope slide temperature control system.

of the germinant was mixed with 2 ml of a 4% noble agar solution. This 4 ml mixture was then at the desired concentration of germinants and 2% in noble agar. One milliliter of the agar solution was layered on the center of the slide. After the agar solidified it was trimmed to a square about 22 mm per side. An inoculum of 0.2 ml of a heavy spore suspension (approximately 1×10^{10} spores/ml) was deposited on the agar in the center of the square. Two-tenths milliliter of a 2X concentration of chemical germinant was added to the drop of spore suspension. The cover slip was then placed over the agar square. The spores at this point were resting in a liquid-solid interface, and both the solid and the liquid phases contained the same concentration of germinants. The edges of the cover slip resting on the agar surface were sealed with paraffin. A thermocouple (copper-constantan) was inserted into the paraffin and the lead wire further secured to the slide by additional paraffin. The thermocouple was connected to a single pen recording potentiometer (Wheelco, model 8000-2600). A normally closed SPST microswitch was taped onto the temperature scale on the potentiometer in such a manner that as the recording pen moved up scale it hit and tripped the microswitch. An infrared heat lamp was connected through a rheostat (to control intensity) to the microswitch and to the line voltage. When the recording pen hit the microswitch the circuit opened (normally closed switch), thus turning the heat lamp off. The heat lamp was mounted so it faced directly onto the microscope stage about 6 inches from the glass slide. While the heat lamp was off the microscope slide began to cool, and as the recording pen began to move down scale, the microswitch closed and the heat lamp came on again. This continual recycling of the heat lamp maintained the microscope slide at the desired temperature depending on the location of the microswitch on the potentiometer scale. To adjust the temperature, the microswitch was simply untaped, moved and retaped. The rheostat enabled the lamp intensity to be adjusted so the cycle interval was long, thus promoting longer life of the heat lamp.

Using this system it was possible to maintain the microscope slide at exactly 32 C or any other temperature above ambient within a tolerance of ±0.5 C.

At various time intervals throughout the germination period photographs were taken through the microscope using a Polaroid film back and a Nikon AFM Microflex automatic exposure shutter. These photographs were then studied to yield kinetics of spore germination and outgrowth.

Calcium and Dipicolinic Acid Analyses

To determine the loss of calcium and dipicolinic acid (DPA) contained in the type E spore, a large quantity of spores was needed.

These spores were obtained by using 30 one-liter flasks, each containing one liter of MSN medium. The spores were prepared as described previously. The three test germinating systems, MSN; 1% alanine, 2% sodium bicarbonate, and 3% glucose (ABG); and 3.5% sodium nitrite in 0.1 M citrate, 0.2 M phosphate buffer at pH 6.0 (NPB), were prepared and 150 ml of each were dispensed into each of 6 centrifuge bottles (a total of 18 bottles). The concentrations of the germinants were adjusted so that after 50 ml of a heavy spore suspension were added, the total 200 ml of liquid would contain the correct concentration of the components in the system. To start the experiment, 50 ml of a spore suspension containing approximately 1×10^{11} spores/ml were added to each centrifuge bottle. The eighteen centrifuge bottles were then placed in a water bath at 32 C. The bottles containing germinant solutions had also been previously tempered in the water bath, together with the stock suspension of spores. At various time intervals (0, 10, 15, 20, 30, and 90 min) three centrifuge bottles were removed from the water bath: one containing spores in MSN, one containing spores in NPB, and the third containing spores in ABG. Immediately after removal from the water bath the containers were plunged into an alcohol-dry ice solution to cool them rapidly, thus stopping the germination events. The spores were spun down in a refrigerated centrifuge maintained at 0 C. One hundred fifty milliliters of sterile distilled deionized water was added to each centrifuge bottle. The spores were mixed with the magnetic stirring bar contained in each bottle, and the spores were centrifuged again. This washing procedure was repeated two more times, for a total of three washings, to achieve removal of any remaining germinant or medium or any calcium or dipicolinic acid in the medium. After the final washing the spores in each centrifuge bottle were resuspended in 50 ml of sterile distilled deionized water, the original volume of the inoculum.

Twenty-five milliliters of this spore suspension of each of the 18 samples were placed into tared dried crucibles and placed in a drying oven at 150 C. The spores were dried for 24 hr. The remaining 25 ml were used for the dipicolinic acid assay. When the spore aliquots dried in the crucibles reached constant weight, they were weighed to obtain the dry weight of the spores. The crucibles were then placed in a muffle furnace and ashed at 500 C for 24 hr. These ashed samples were used for calcium determination by atomic absorption spectrophotometry.

The 25 ml aliquots to be used for the dipicolinic acid assay were handled in the following manner. The entire aliquot for each sample was added to a 50 ml centrifuge tube. The contents of each tube was acidified with 1.0 N HCl to a pH approximately 2.0. The samples were mixed and autoclaved for one hr at 121 C, each tube being covered with aluminum foil to prevent evaporation. After autoclaving the samples were cooled and 3.0 ml of 10% trichloroacetic acid were added to each tube. The tubes were allowed to stand 4 hr and were centrifuged at 5000 rpm (4000 × g) for 15 min. Four milliliters of the 28 ml supernatant from each sample were carefully removed and added to a spectrophotometer tube. One ml of a color reagent was added, the tubes mixed, and the optical density read at 440 nm on a Spectronic 20 Spectrophotometer.

The color reagent was made up as follows: A 0.5 M solution of sodium acetate was prepared and adjusted to a pH of 5.5. To that solvent was added 1% $Fe\,(NH_4)_2(SO_4)_2 \cdot 6H_2O$ and 1% ascorbic acid. This color reagent was prepared just a few minutes before being used, to prevent oxidation and deterioration.

The concentrations of dipicolinic acid in each sample were calculated by comparison of the optical density readings with those of the standard curve and the percent dry weight figure obtained by multiplying the value of DPA per ml × 28 ml × 100 and dividing by the dry weight of the spores used in that sample.

Calcium Determination

The ashed samples were removed from the crucibles by washing with 6 N HCl, using a total of 4 ml to remove all of the ashed material. In order to facilitate removal, initially one ml of 6 N HCl was added to each crucible and the crucibles heated on a hot plate just to the point of steam rising out of the crucible. The material from each crucible was added to a 5 ml volumetric flask. After the ashed spore samples had been transferred to the flask in 4 one-milliliter aliquots of HCl, one ml of lanthanide chloride was used to prevent interference by other ions.

A Beckman model 1301 atomic absorption spectrophotometer was used. The wavelength used was 422.6 nm. A standard curve was first run using known concentrations of calcium. The concentrations of calcium in the unknown samples were then calculated from the peaks generated by the standards.

Preparation of Toxin for Mouse Assay and Type Identification

Spores of *C. botulinum* type E Kalamazoo strain were grown in 10 ml of MSN and 10 ml of a 5% trypticase (Baltimore Biological Co.), 0.5% peptone, 0.5% sucrose and 1.0% yeast extract (TPSY) medium. The media were inoculated with 0.1 ml of the stock spore suspension. The media were incubated at 32 C for 24 hr, after which time the tubes were shaken and diluted 1:2 with buffered trypsin solution prepared according to the method of Iida (1970). A solution of 0.5 % trypsin (1:250, Difco) was prepared in gelatin-phosphate buffer (0.2% gelatin. 0.4% dibasic sodium phosphate).

The pH of the system was 6.2. A 1:2 dilution of the toxic media was made with this trypsin solution, and the mixture was digested for 1 hr at 37 C. After digestion, the mixture was cooled and working dilutions were prepared with gelatin phosphate buffer for injection into mice.

For the typing experiments, the mice were injected with the toxic material and also with specific type E antitoxin (obtained from the National Center for Disease Control, Atlanta, Georgia) in order to establish that the organism used was indeed type E C. botulinum. For both the typing and toxicity studies the results were expressed as the number of mice that survived. These results are discussed in the results and discussion section.

CHAPTER IV

RESULTS AND DISCUSSION

Toxin Production Results by Mouse Assay

Toxin production of Clostridium botulinum type E Kalamazoo strain was evaluated in two test media: MSN and TPSY (Emodi and Lechowich, 1969). Table 1 shows the results of the mouse assay.

Table 1. --Results of mouse assay for C. botulinum type E toxin. Toxin was digested with trypsin as described in text.

Dilution of toxin	Medium	Volume injected into mouse (ml)	Effective dilution	D = death S = survival
1:2000	TPSY	0.5 ml	1:4000	D
1:2000	TPSY	0.25	1:8000	D
1:2000	TPSY	0.10	1:20000	D
1:20000	TPSY	0.50	1:40000	S
1:200	MSN	0.10	1:2000	D
1:2000	MSN	0.50	1:4000	D
1:2000	MSN	0.25	1:8000	D
1:2000	MSN	0.10	1:20000	D
1:20000	MSN	0.50	1:40000	S

The data in this table indicate that toxin production was equally good in both media and that the end point for toxin production was between 1:20,000 and 1:40,000 mouse lethal doses per ml for both the MSN and TPSY media.

Table 2 shows the results of the type identification experiments used to confirm that the organism used was a good toxin-producing strain of *C. botulinum* type E.

Table 2.--Results of type identification studies of type E *Clostridium botulinum* by mouse assay.

Dilution of toxin	Medium	Volume injected into mouse (ml)	Volume of type E antitoxin injected	D = death S = survival
1:200	TPSY	0.10 ml	----	D
1:200	TPSY	0.10	0.20	S
1:200	MSN	0.10	----	D
1:200	MSN	0.10	0.20	S

Experiments with Sodium Nitrite As a Germinant

The literature review has mentioned that sodium nitrite has been observed to cause germination-like changes in some bacteria. These changes have not been evaluated in reference to outgrowth in the past.

Sodium nitrite was thought to be a possible germinant for spores of Clostridium botulinum type E. To determine this, sodium nitrite was prepared at different concentrations ranging from 0.1% to 10% in aqueous solution. When type E spores were incubated at 32 C in nitrite solutions, no germination was observed by turbidimetric measurements or stainability experiments. A buffer system was thought to be necessary to stabilize the system. The following experiment was performed. Nitrite solutions were prepared in citrate-phosphate buffer, pH 6.0 (0.1 M, 0.2 M) at the following concentrations: 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 5.5%. Five milliliters of each of these solutions were dispensed into screw cap spectrophotometer tubes, and one ml of a 1×10^9 spores/ml inoculum was added to each tube. The tubes were incubated at 32 C and percent transmittance readings were taken at periodic intervals. Figure 4 shows the results of this experiment. The optimum concentration for total germination response of sodium nitrite was 3.5%. The requirement for citrate-phosphate buffer may be one of two possibilities. The buffer may be necessary to stabilize the pH or the buffer may provide the necessary ionic strength to enable "germination" to proceed. A number of spore germinating systems have been reported to require some ions in solution with the test germinant. Perhaps this is such a system. Nitrite

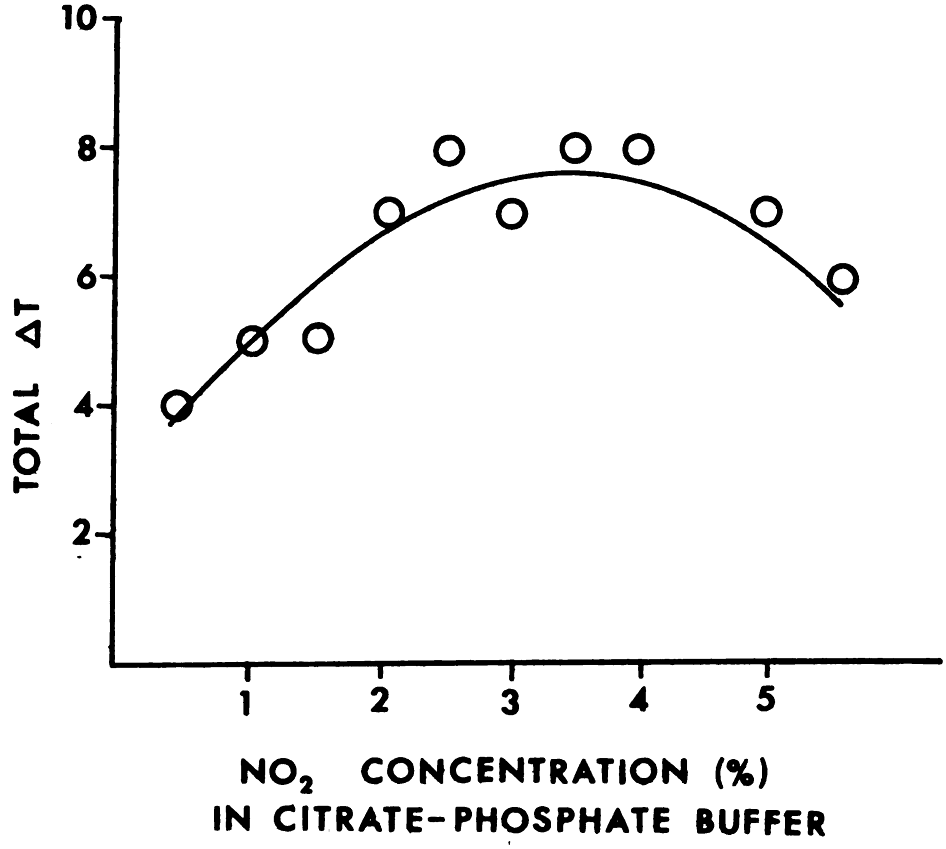

Figure 4. --Spore germination response of type E Clostridium botulinum in varying percents of sodium nitrite in citrate-phosphate buffer.

germination in the presence of plain phosphate buffer proved to be equally satisfactory. Citrate-phosphate buffer, however, was usable over a wider pH range than was phosphate buffer; therefore for the remainder of the experiments, citrate-phosphate buffer was used in all of the sodium nitrite germination solutions.

The concentration of citrate-phosphate buffer used in the nitrite germinating system was found to be important. Sodium nitrite solutions at a concentration of 3.5% were prepared in citrate-phosphate buffer, and in various dilutions of the 0.1 M citrate-0.2 M phosphate buffer. Type E spores were germinated at 32 C in these solutions, and the results of this experiment are shown in Figure 5. This figure shows that as the concentration of citrate-phosphate buffer decreased, so did the total amount of "germination." In all of the experiments reported here a total percent transmittance change of only 2 or 3 units was considered negligible. Therefore, when the buffer was infinitely dilute (that is, pure water used as the solvent), no germination was observed.

Effect of Nitrous Acid on the Germination of Type E Spores of C. botulinum

The possibility was examined that the positive effect of sodium nitrite on germination was caused by undissociated nitrous acid. Nitrous acid was prepared in the following manner. Sodium

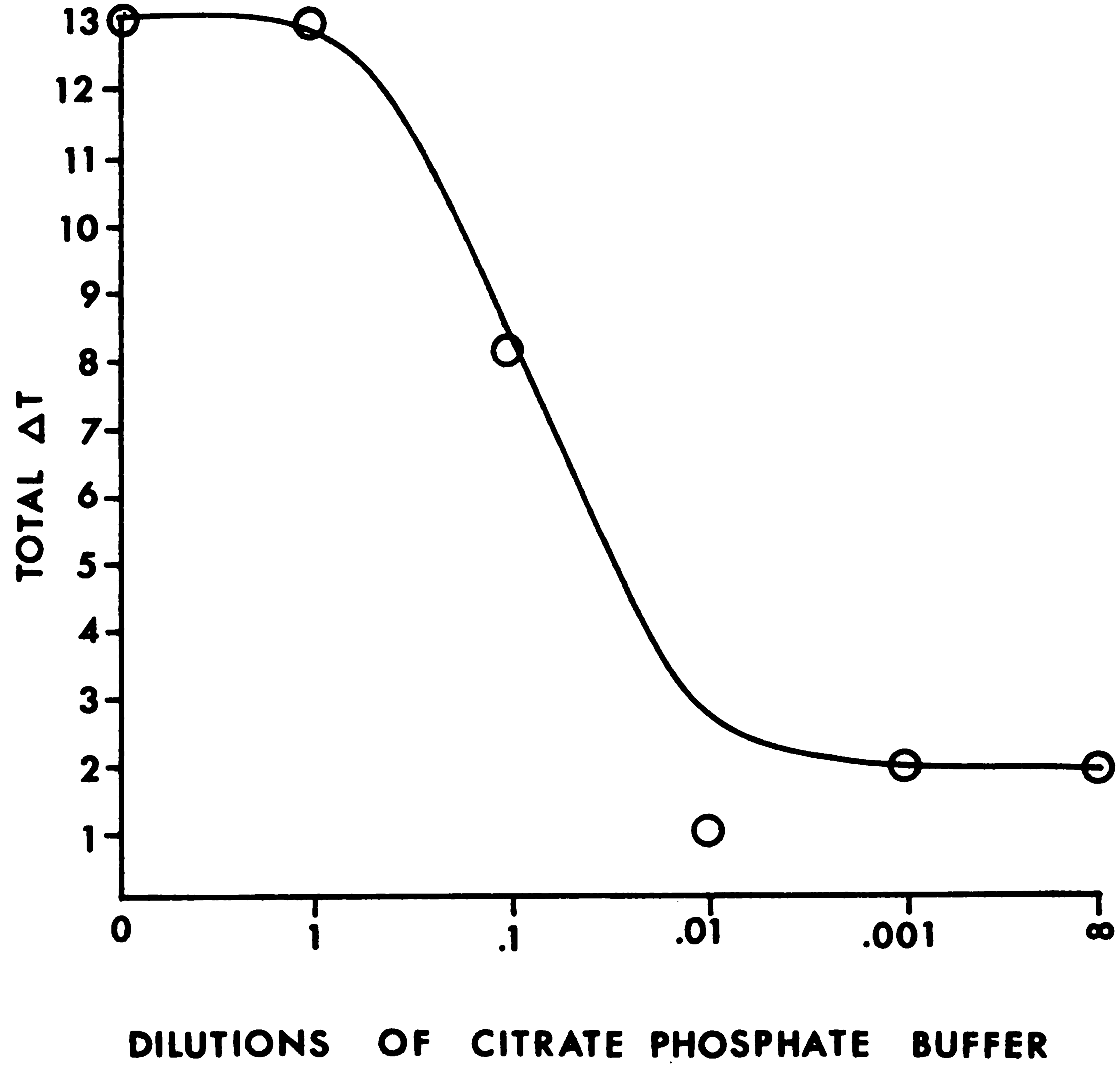

Figure 5. --Effect of buffer concentration on total germination response of type E spores of Clostridium botulinum in 3.5% sodium nitrite.

nitrite was reacted with hydrochloric acid to produce dinitrogen trioxide, a gas at room temperature, but a blue liquid at -20 C.

$$2\ NaNO_2 + 2\ HCl = 2\ NaCl + N_2O_3 + H_2O$$

$$N_2O_3 + H_2O = 2\ HONO$$

Dinitrogen trioxide was collected through a condenser maintained at approximately -40 C by pumping the alcohol portion of a dry-ice ethanol mixture through the condenser. As the dinitrogen trioxide condensed it dropped into a small beaker kept on dry ice. Three grams of N_2O_3 were produced and collected in the dry ice cooled beaker. This compound was rapidly dissolved in 100 g water at 0 C. The water was then quickly heated to 32 C and the spores were added, and the tube was closed to prevent the loss of gases, thus slowing down the rate of decomposition of the nitrous acid. This mixture produced an approximate 3% initial concentration of nitrous acid (HONO). The system was very crude, and the rate of decomposition of nitrous acid was not known. Even the initial concentration of nitrous acid was not accurately known. However, in this aqueous system the type E spores germinated faster than they did in a sodium nitrite plus citrate-phosphate buffer, giving some evidence that the nitrous acid may be the active principle, and that when a higher concentration of nitrous acid was used, the effect of the citrate-phosphate buffer was mitigated.

If this response observed with type E spores in the presence of sodium nitrite is to be considered true germination, then outgrowth must be examined. One hundred milliliters of sodium nitrite (3.5%) in citrate-phosphate buffer at pH 6.0 (NPB) were placed in a 200 ml centrifuge bottle. To this was added 10 ml of a 5×10^9 spores/ml spore suspension. The system was then incubated at 32 C for one hr, at which time approximately 100% of the spores were "germinated" when observed by dark phase contrast microscopy. The centrifuge bottle containing the spores in the NPB was centrifuged at 5000 rpm to remove the spores. The NPB was decanted and 100 ml of sterile water were added. The spores were mixed and then centrifuged again. This washing procedure was repeated two more times to assure removal of any nitrite adhering to the spore surfaces. After the final washing, 10 ml of sterile water were added to the spores followed by 100 ml of MSN medium. The centrifuge bottle was then incubated at 32 C for 48 hr to observe outgrowth. No outgrowth was observed. The spores were again centrifuged and removed from the MSN. The MSN was analyzed for nitrite by the AOAC method 23.013. This analysis indicated that 9 ppm (μg/g) nitrite was carried over from the spore germination medium (NPB). To show that 9 μg/g of nitrite was not inhibitory to outgrowth, MSN medium was prepared with 9 µg/g sodium nitrite, and inoculated with type E spores. The spores were incubated for 48 hr at 32 C. Good germination, growth, and sporulation resulted.

A nitrite inhibition experiment was conducted to determine how much nitrite is needed to inhibit growth of type E spores in MSN medium. The results of Table 3 indicate that growth still occurred at a nitrite concentration of 300 µg/g but not at 500 µg/g nitrite. Emodi and Lechowich (1969) found that 100-200 µg/g nitrite was inhibitory to type E growth only at low temperatures.

Table 3. --Nitrite inhibition of type E spores of *Clostridium botulinum* in MSN growth medium.

µg/g $NaNO_2$ in MSN	Relative turbidity of tube after 48 hr
0	++
50	++
100	++
150	+
200	++
250	++
300	+++
500	-
1000	-

The conclusion from these experiments was that the lack of outgrowth of the spores which had been "germinated" in NPB and transferred to MSN for outgrowth was due to some effect of the "germination" process and not the residual carry-over of nitrite into the growth medium.

Since sodium nitrite could "germinate" the spores, but not allow outgrowth, a search continued for a system which would be simple, and could elicit true physiological germination (germination with provision for subsequent outgrowth).

Experiments with Alanine, Sodium Bicarbonate and Carbohydrate Systems

Holland et al. (1969) studied the effect of various germinating systems on Clostridium sporogenes. These workers used a variety of amino acid and carbohydrate systems. One of the systems they used consisted of 100 mM L-alanine, 200 mM sodium bicarbonate, 20 mM sodium lactate, and 0.05% L-cysteine. This system was tested for C. botulinum and was found to yield good germination with type E spores. Figure 6 shows a typical germination curve of the spores in the Holland et al. germination system. It was subsequently found that the L-cysteine could be deleted with no change in rate or total amount of germination.

Substitution experiments for the carbohydrate to replace sodium lactate were conducted. A number of carbohydrates were

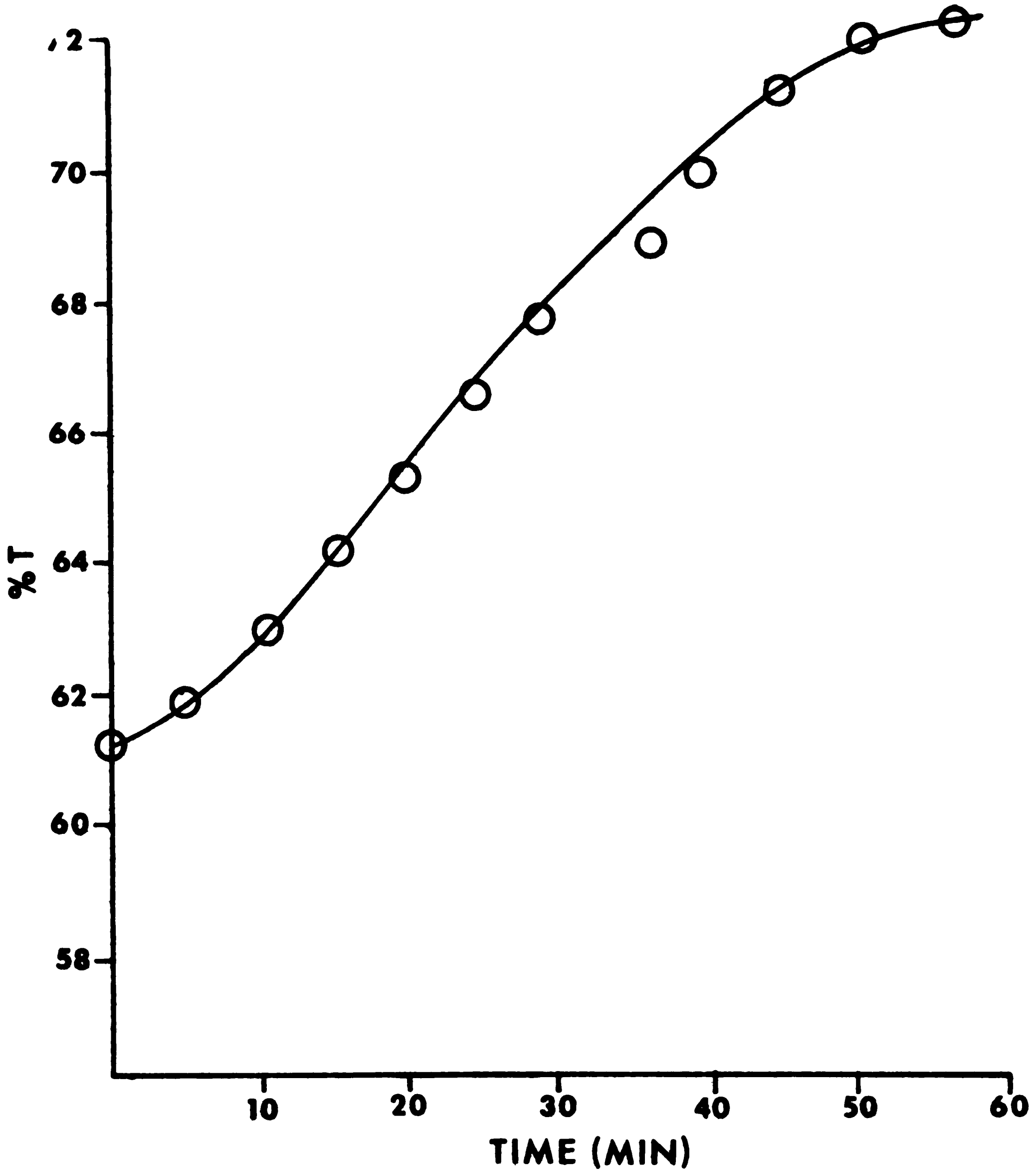

Figure 6. --Typical germination curve of type E spores of Clostridium botulinum in alanine, sodium lactate, sodium bicarbonate, and cysteine.

prepared at 3% and tested in a system containing 1% L-alanine, and 2% sodium bicarbonate. Table 4 shows the individual carbohydrates used and their total germination responses. This table shows that of 35 carbohydrates tested, only 8 gave total responses less than 7 transmittance units. Of these 8, four of them were alcohols whose activity decreased as the carbon chain length increased. It appeared that almost any simple carbohydrate (also starch, glycogen and dextrin) would suffice in combination with alanine, and sodium bicarbonate as a germinant for type E spores. Based upon this experiment, the remainder of the experiments using this system were performed with glucose as the carbohydrate source for both economic reasons and convenience. Deletion of the carbohydrate, alanine, or bicarbonate resulted in complete lack of germination.

This alanine, bicarbonate, glucose (ABG) system was tested for germination in relation to outgrowth in a similar manner as had been done with the nitrite system. When the spores were germinated in ABG and then centrifuged, washed and reinoculated into MSN growth medium, the spores grew normally when incubated at 32 C. Figure 7 illustrates photomicrographs taken of germinated spores undergoing outgrowth after being first germinated in ABG.

Substitution experiments for alanine using 1% aspartic, arginine, glycine, valine, leucine, isoleucine, tyrosine, tryptophan, serine and cysteine showed no germination.

Table 4. --Total germination response of type E spores of Clostridium botulinum in a system consisting of 1% L-alanine, 2% sodium bicarbonate, and 3% of the following carbohydrates in aqueous solution.

Carbohydrate	Total change in percent transmittance
glucose	13
maltose	14
mannitol	7
sorbose	13
galactose	14
ribose	4
arabinose	13
mannose	14
inositol	14
rhamnose	14
inulin	9
raffinose	11
trehalose	13
melezitiose	14
adonitol	13
glycerol	13
sorbitol	14
salicin	7
cellibiose	13
melibiose	7
fructose	12
xylose	11
lactose	11
sucrose	10
starch	13
dextrin	13
dulcitol	3
ethylene glycol	3
glycogen	6
lyxose	7
2-deoxyglucose	12
methanol	6
ethanol	5
propanol	3
butanol	1
water	0

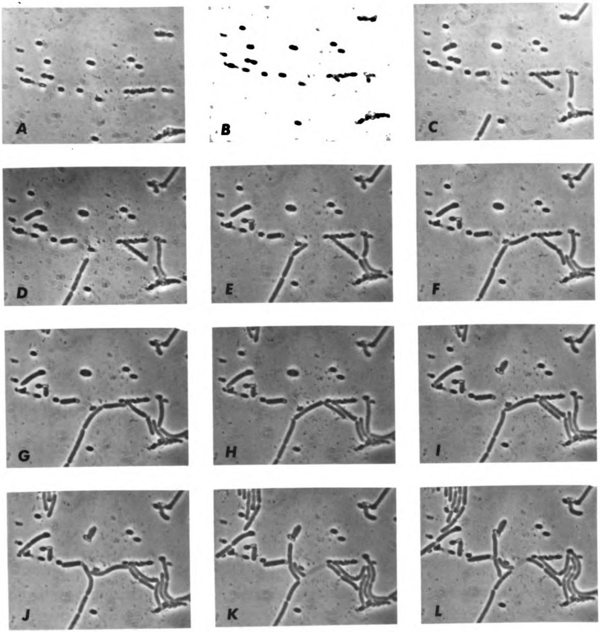

Figure 7. --Photomicrographs of spores of type E Clostridium botulinum undergoing outgrowth in MSN medium after being first germinated in ABG.
Time sequence: (min) 0, 120, 180, 210, 220, 230, 240, 245, 250, 260, 275, 285.

Characteristics of Three Test Germinating Systems

Observation of spores germinating in NPB, ABG, and the MSN medium using phase contrast microscopy showed differences in kinetics. Figures 8 and 9 show photomicrographs of spores germinating in NPB and in ABG. The photographs indicate that the spores in the NPB germinated in synchrony as opposed to the progressive germination as seen in the ABG. Figure 10 shows the germination curves obtained when the percent germination was ascertained from the photomicrographs by observing the number of spores which have germinated at any given time interval. This figure indicates that the germination curve for NPB was different from those of ABG and MSN. The NPB curve indicates rapid germination after a short lag to 100% germination at about 30 min. The ABG curve rose rapidly with no lag and then leveled off. The MSN curve rose slowly but steadily throughout the 100 min observation period.

The synchronous (all cells germinating together) nature of the germination in NPB is suggestive of a chemical rather than a physiological mechanism. Spores germinating physiologically would be expected to exhibit some probability function governing germination. There would be some spores which would germinate only after a long time period.

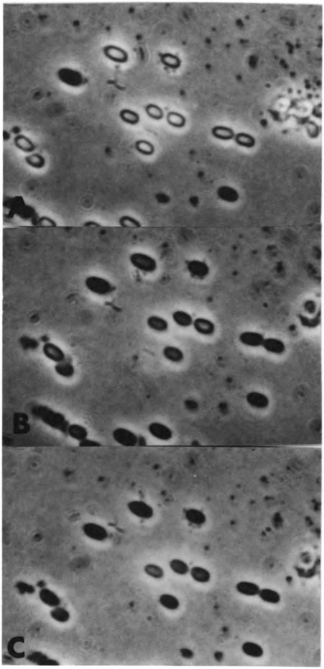

Figure 8. --Photomicrographs of type E spores of *Clostridium botulinum* "germinating" in nitrite plus buffer (NPB) system.
Time sequence: A = 0 time, B = 30 min, C = 130 min.

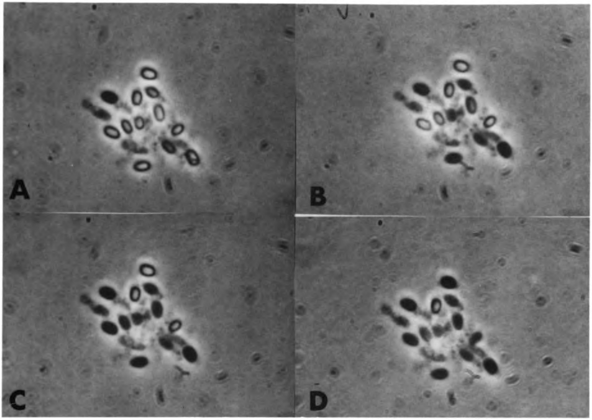

Figure 9. --Photomicrographs of type E spores of Clostridium botulinum germinating in alanine, sodium bicarbonate and glucose (ABG) system.
Time sequence: A = 0 time, B = 20 min, C = 39 min, D = 280 min.

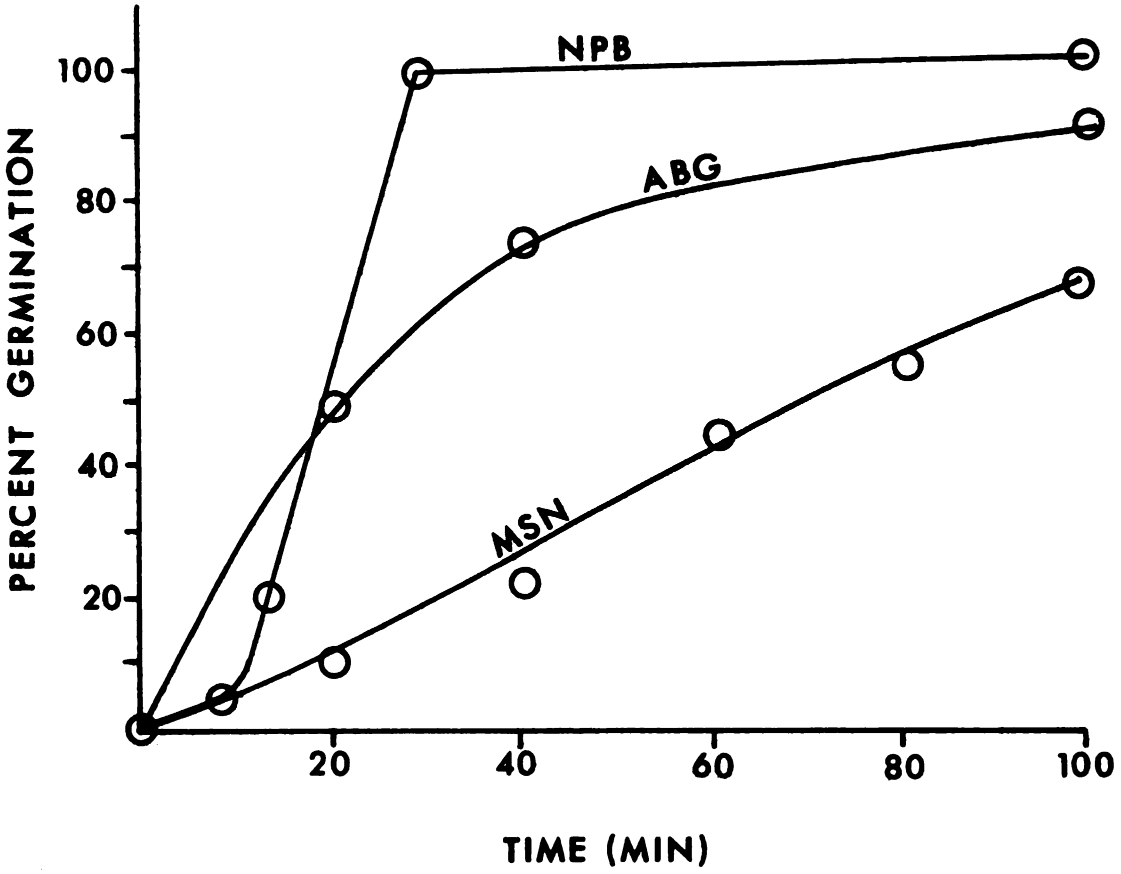

Figure 10. --Germination curves of spores of type E Clostridium botulinum. The data were obtained from photomicrographs of germinating spores.

When the kinetics of these three test germination systems were observed by means of population turbidimetric measurements, the results shown in Figure 11 were obtained. NPB produced the greatest amount of germination, while ABG and MSN produced less total germination response, respectively. However, the difference in shape of the curves is not apparent from this figure. These data were not obtained by the observation of individual spores germinating, but the effects were measured on an entire population of germinating spores. Subtle differences in the kinetics of individual spores during the germination process may be masked by other spores when the entire population is observed. Let us suppose that in the NPB 99% of the spores germinate in synchrony as has been previously discussed. This leaves 1% of the spores not germinating or germinating in an other than synchronous fashion. If the concentration of spores in the medium was 1×10^7 spores/ml, then 1% of these spores would represent a total of no less than 1×10^5 spores/ml. These 10^5 spores behaving differently than the other 99% are enough to mask the true kinetics, since 10^5 spores produce some turbidity by themselves. For this reason, it was felt that more characteristic kinetic observations are made when individual spores or bacteria are studied in environmental situations than when the entire population is observed. A possible argument against this method is the fact that when studying

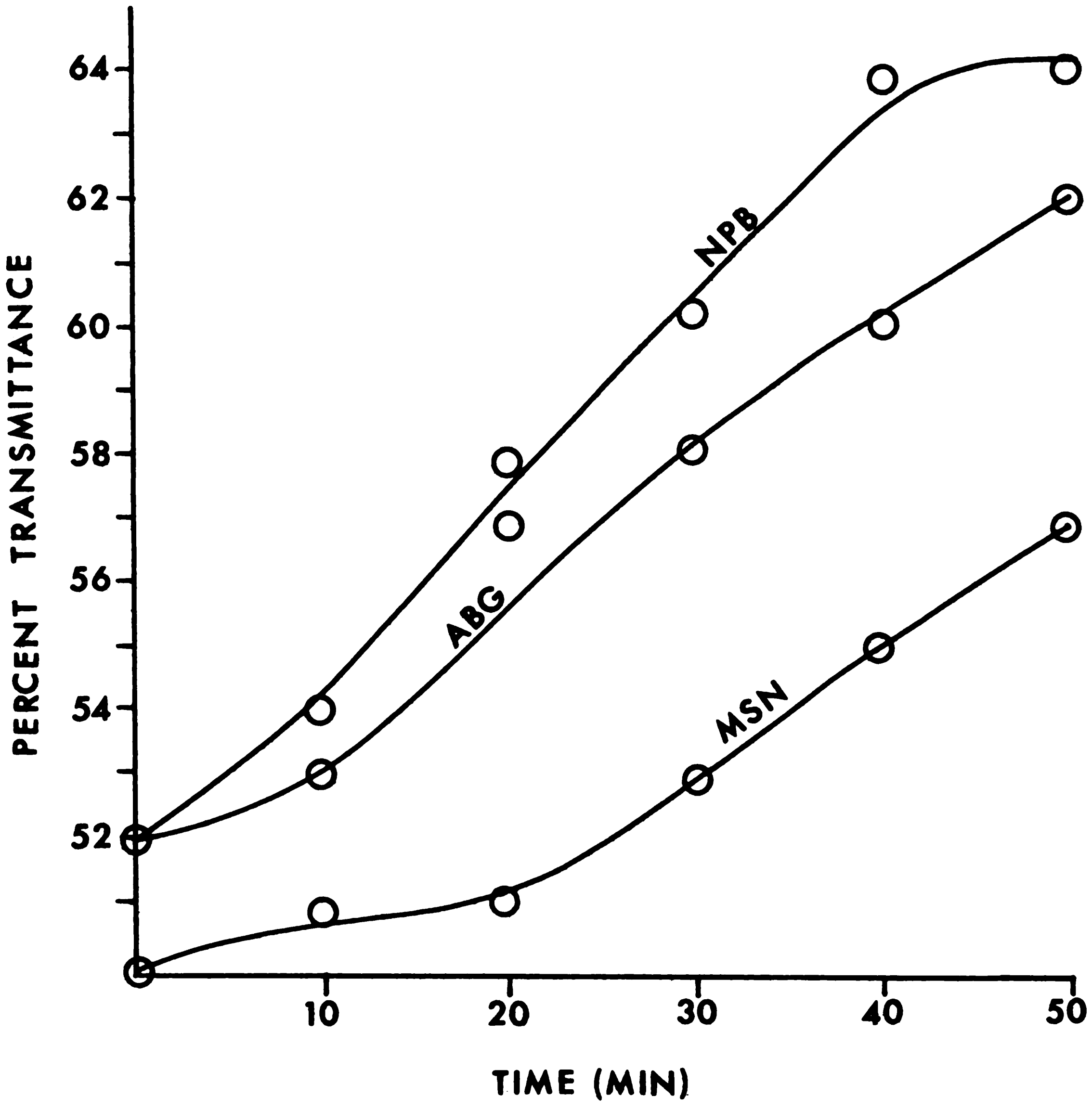

Figure 11. --Germination curves of spores of type E Clostridium botulinum in three test systems. Data were obtained by turbidimetric measurements.

individual spores the observer is selecting only about 50 individuals from a population of 1×10^9 cells and basing his conclusions on the behavior of these 50 cells. It must be assumed that the suspension is homogeneous and that these 50 individuals are characteristic of the entire population.

When the data for individual cell germination shown in Figure 10 were plotted on probability coordinates versus the logarithm of time, the result is shown in Figure 12.

This figure contains three straight lines representing germination curves. The nitrite curve is much steeper than those of ABG and MSN. This was a result of the synchronous rapid germination rate produced by sodium nitrite (NPB). The justification for the use of the probability scale on the ordinate is based upon the fact that a biological function seems to be dependent upon probability with respect to the homogeneity of the population. The reason for using the logarithmic scale for time on the abcissa was not understood. However, these coordinates yielded a straight line which is suitable for comparison purposes and also for quantitative relationships, since the straight lines can be described by a point, for example the 50% point, and a slope.

The germination data for spore populations presented in Figure 11 were re-plotted on probability versus logarithmic time

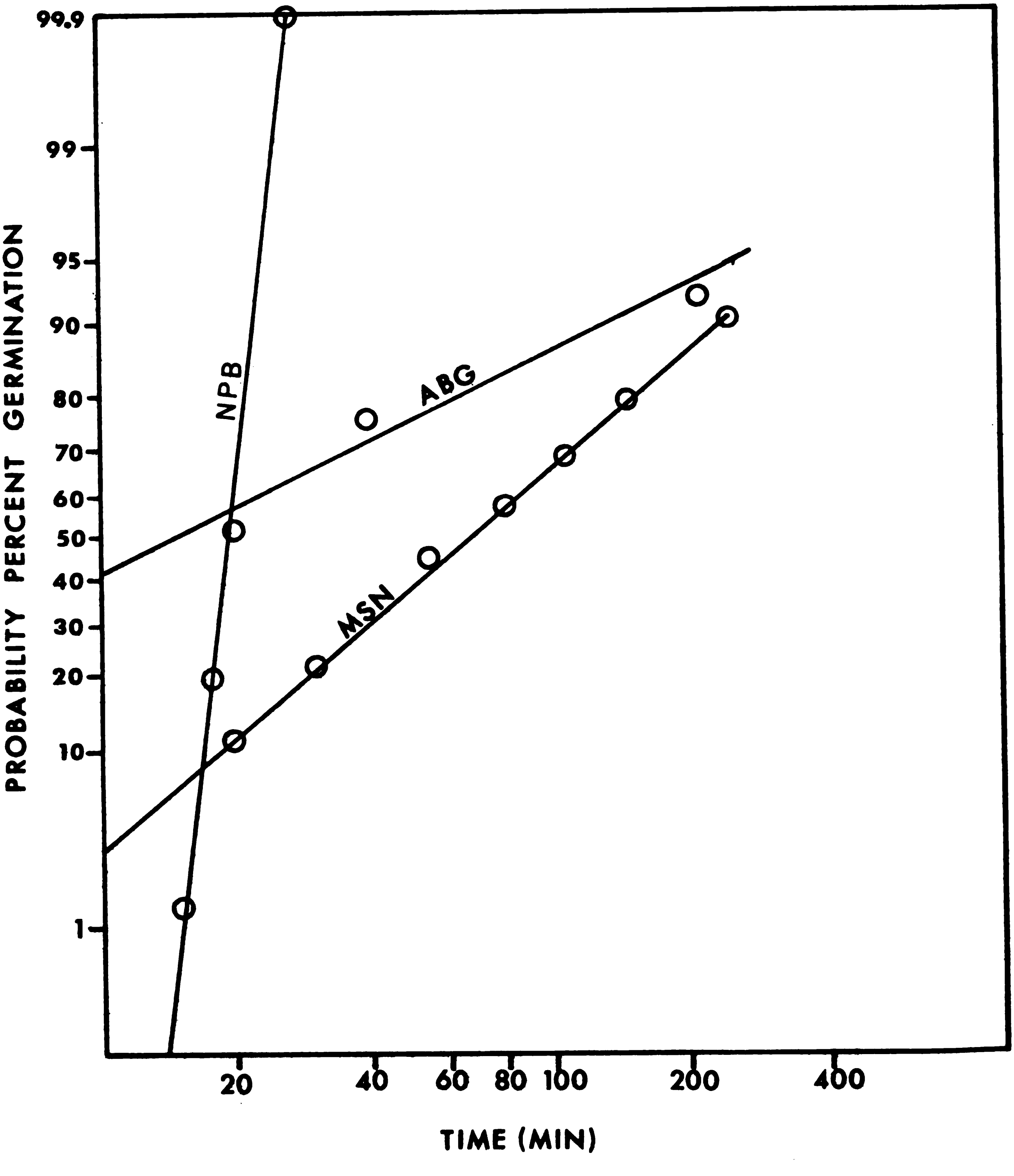

Figure 12.--Probability germination curves of spores of type E Clostridium botulinum in three test systems. The data were obtained from photomicrographs of single spore germination.

coordinates and are represented in Figure 13. The curves in this figure were irregular, and showed no consistent pattern. If the true germination kinetics are shown on a probability plot of single spore observation, then a probability plot of population turbidimetric kinetics will mask the true kinetics. The shapes of the curves on this figure are thought to result from a masking of the true phenomenon.

Figure 14 shows the results of the temperature optimum experiments for the three test germination systems. It can be seen that both the MSN and ABG germination curves exhibited true temperature optima. The NPB germination curve did not show such a true temperature optimum. The most obvious explanation for this type of phenomenon is that a chemical reaction rather than a physiological or biological reaction was controlling the total amount of germination. We would thus expect an increase in temperature to cause a greater amount of germination if the germination was caused by a chemical reaction. Both of the ABG and MSN germination curves indicated that little germination occurred at 50 C, a temperature at which many enzymes are inactive. Nitrite induced "germination" was still proceeding well at this temperature.

These particular data conflict with a theory previously discussed which mentioned that nitrous acid may be the active component in causing nitrite induced germination. As the temperature is raised,

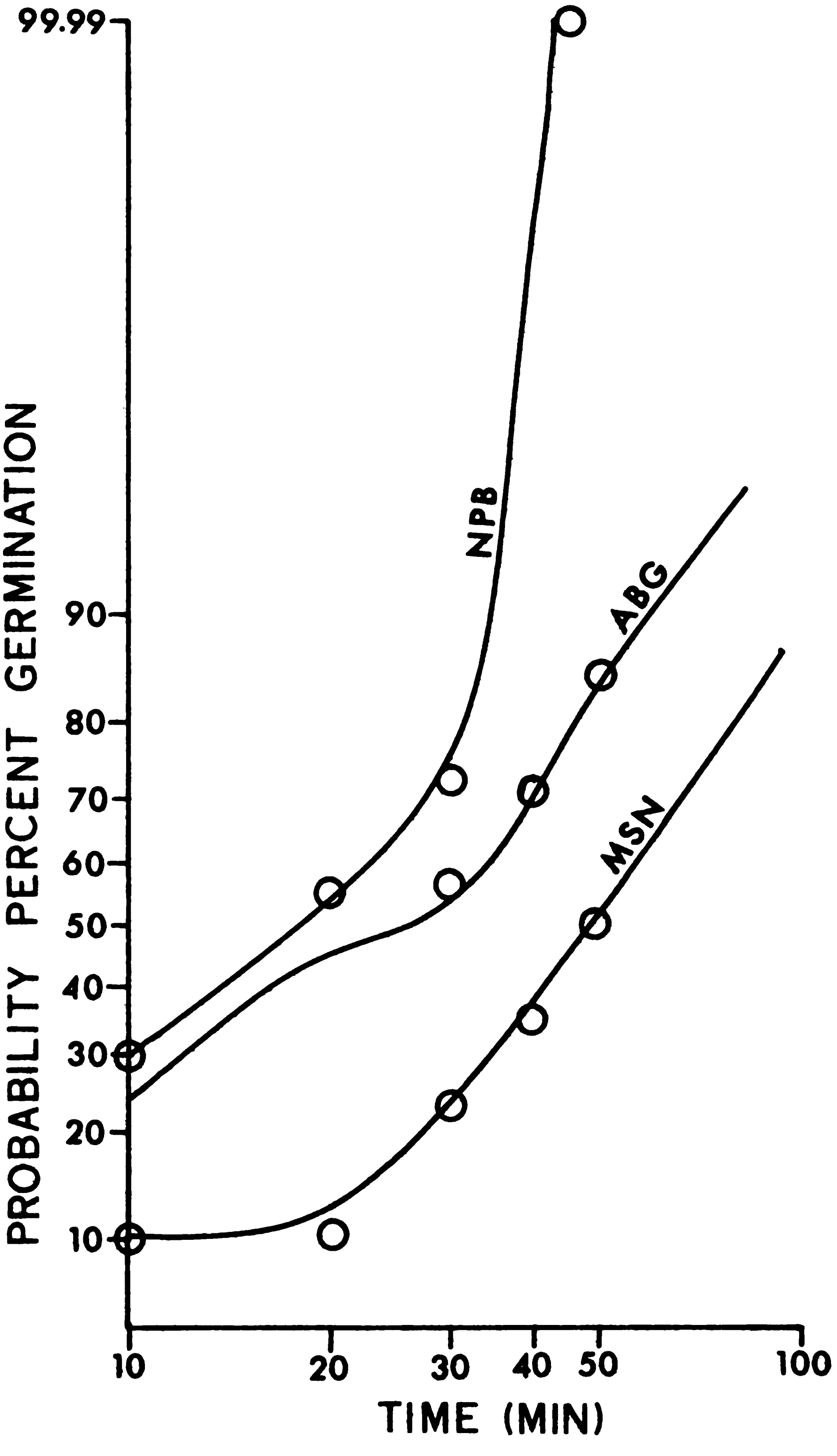

Figure 13. --Probability germination curves of spores of type E Clostridium botulinum in three test systems. The data are replotted from Figure 11.

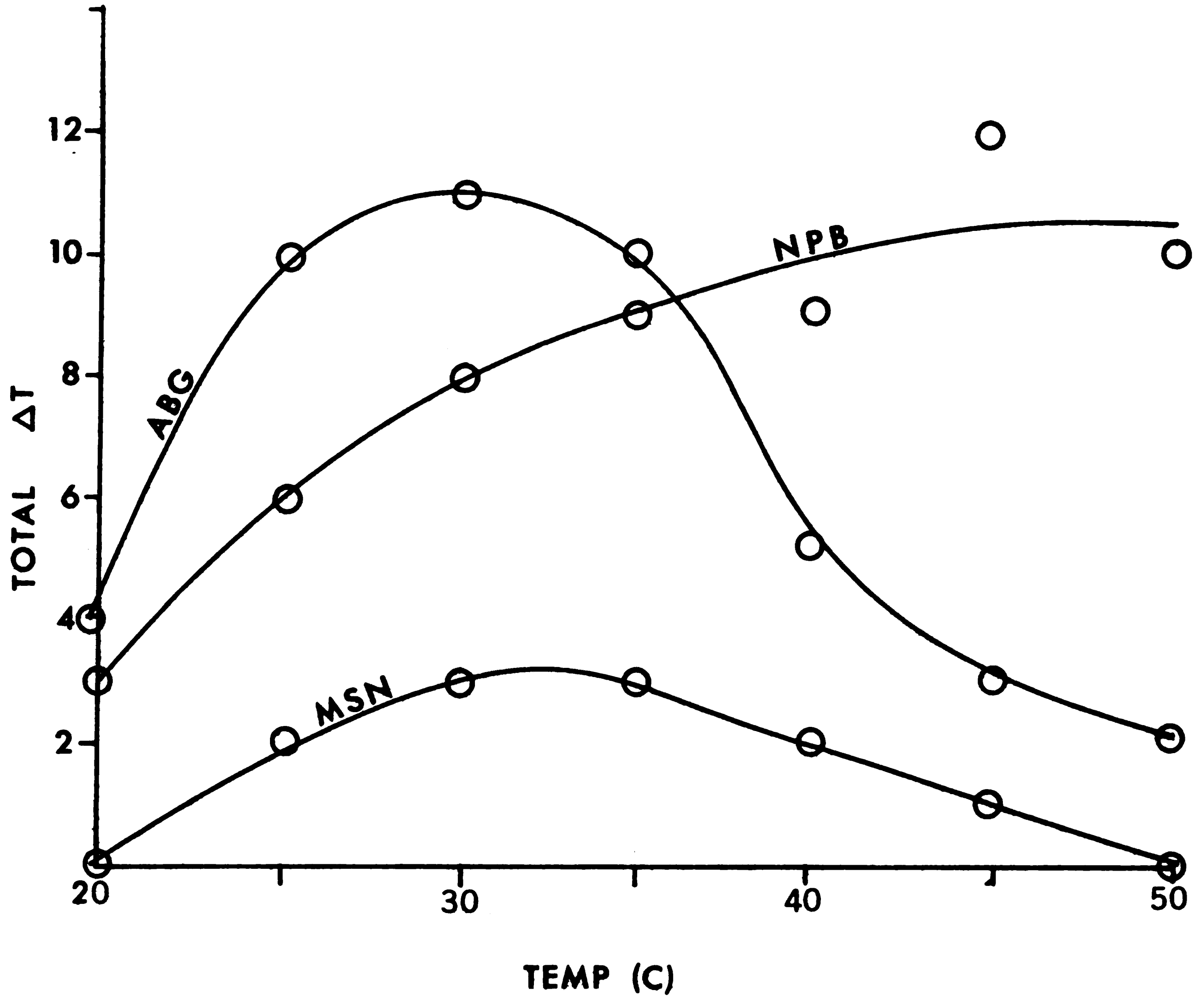

Figure 14. -- Total germination response of spores of type E <u>Clostridium</u> <u>botulinum</u> in three test systems at different germination temperatures.

we would expect the amount of nitrous acid still available in the undissociated state to decrease, and thus might expect the amount of germination also to decrease. However, as the temperature increased, so did the germination response. No explanation for this phenomenon is offered at this time.

Figure 15 presents the results of the pH optimum experiments for the three test germination systems. This figure shows that both the MSN and the ABG pH optimum curves exhibit true pH optima. These optima occur at around pH 7.0, and they begin to fall above pH 7.0. The curve for the NPB system begins to fall above pH 6.0, and does not exhibit an optimum. There are several possible explanations for this behavior. When the pH of a solution of sodium nitrite is raised above pH 6.0, various chemical reactions occur causing decomposition of the sodium nitrite. As the pH of a sodium nitrite solution is lowered, a greater percentage of the nitrite is present as undissociated nitrous acid. When the pH reaches 2 or lower, the nitrous acid decomposes and the nitrite is released as various gases such as nitrogen dioxide and nitrous oxide. It would be expected that the germination response would decrease below pH 2.0. However, in the normal pH range of 4 to 7, no optimum could be shown.

One of the commonly measured parameters of spore germination is stainability with basic dyes such as methylene blue.

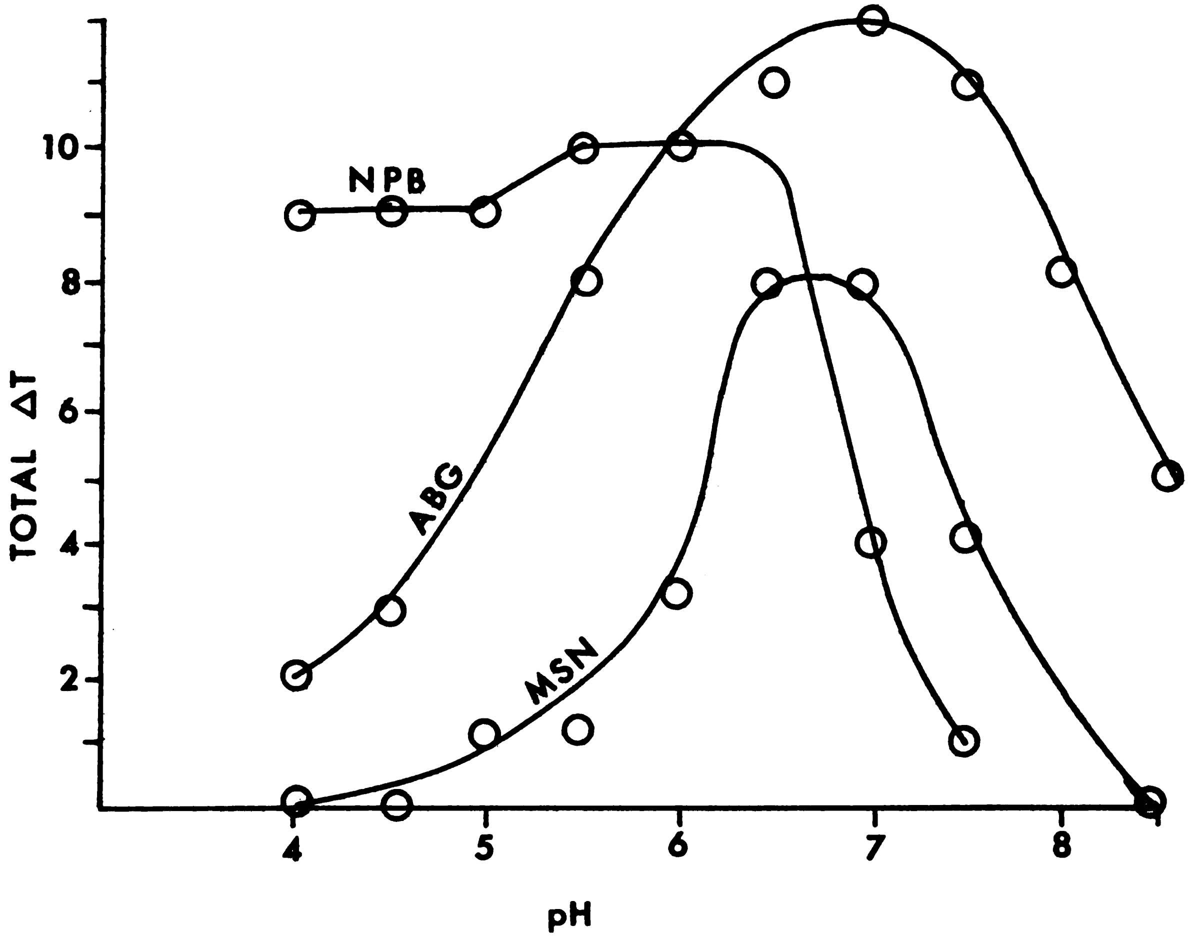

Figure 15. -- Total germination response of spores of type E Clostridium botulinum in three test systems to different germination pH.

Ungerminated spores do not take up the dye, whereas fully germinated spores are more permeable and take up the methylene blue. If the percent stained spores are followed with time, a germination curve results. Figure 16 presents germination curves for type E spores in the three test germination systems. Since this technique more closely followed single spore observation, it would be expected that the kinetics derived from this type of experiment would more nearly follow those obtained from single cell observation. The germination curve for the spores in the NPB system indicated a rapid initial rise to approximately 100%, with no further germination with time. The ABG curve showed a fairly rapid rate of germination, but not a single initial rise. The MSN curve showed a slight lag and then a steady rate of germination. Again, the germination response produced by nitrite (NPB) was not typical of the other two systems.

The effect of chemical inhibitors was studied in relation to germination in the three test media. Table 5 shows the effect of two chemical inhibitors, $HgCl_2$ and NaOCl, on the germination of type E spores in the three test germination systems. It can be seen from these data that the system most severely affected by the inhibitor was the MSN. The NaOCl prevented all germination in MSN. In NPB the addition of NaOCl to the nitrite solution still allowed approximately 50% germination. The NaOCl in the ABG system also allowed 50% germination.

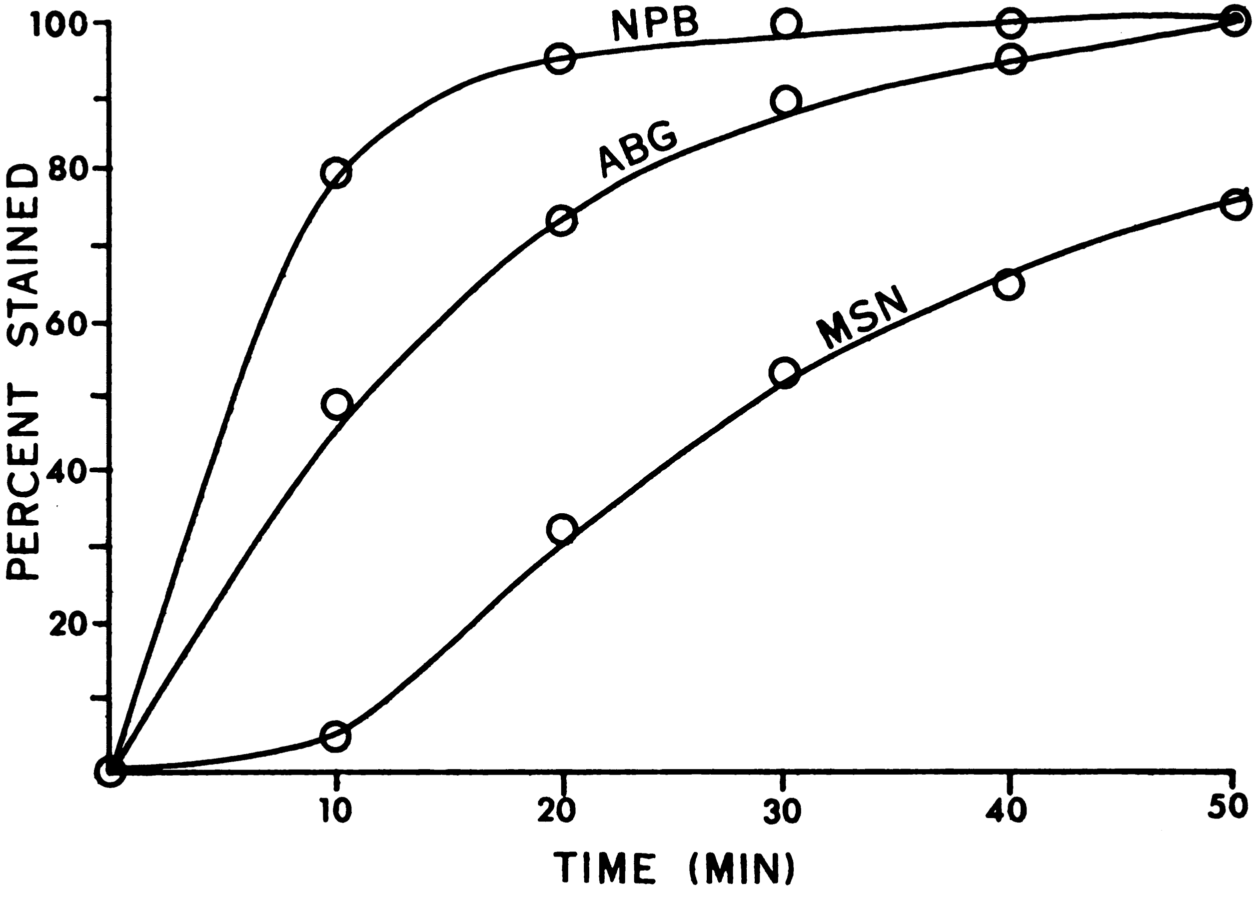

Figure 16. --Germination curves of spores of type E *Clostridium botulinum* obtained through stainability experiments with methylene blue.

Table 5. --Effect of two chemical inhibitors (0.002% $HgCl_2$ and 0.76% NaOCl) on the germination of spores of type E Clostridium botulinum in three test systems: NPB, ABG, and MSN.

Time (min)	Percent transmittance @ 600 nm									
	Tube Number									
	1	2	3	4	5	6	7	8	9	10
0	45	49	45	49	47	52	44	56	54	41
15	50	49	44	52	51	54	46	58	55	42
30	51	50	45	57	55	55	48	58	55	41
45	54	53	45	59	58	56	50	59	56	42
60	55	53	45	60	58	57	51	60	58	42
75	55	53	44	60	60	59	52	61	57	42
Total change	10	4	0	11	13	7	6	5	3	1

Legend:

Tube Number	Composition
1	MSN
2	MSN + 0.002% $HgCl_2$
3	MSN + 0.76% NaOCl
4	NPB
5	NPB + 0.002% $HgCl_2$
6	NPB + 0.76% NaOCl
7	ABG
8	ABG + 0.002% $HgCl_2$
9	ABG + 0.76% NaOCl
10	water

When these same chemical inhibitors were added 15 min after the start of the experiment, the results were different. Figure 17 shows the results of this experiment when NaOCl or

$HgCl_2$ was added to the spores in MSN after 15 min. Figure 18 shows the results for NPB, and Figure 19 shows the results for the ABG system. In the MSN system the inhibitors both appeared to possibly delay the rate and amount of germination. However, the ABG system showed sensitivity to $HgCl_2$ and little, if any, sensitivity to NaOCl. These results tend to indicate that once the germination process has begun, regardless of the type of mechanism, it proceeds with little, if any, hesitation when certain inhibitors are added. However, if these inhibitors were present initially in the germination medium, the process is slowed to some extent. Other workers have studied inhibition of bacterial spore germination. Prasad and Srinivasan (1969) found that methylanthranilate inhibited the germination of spores of aerobic bacilli without affecting growth and sporulation. The inhibition could not be reversed by removal of methylanthranilate. Other workers (Parker and Bradley, 1968; Sierra, 1968; and Slepecky, 1963) have reported a variety of metabolic inhibitors which can inhibit one or more steps in the process of spore germination. These inhibitors include ions, and heavy metals which indicate a possible enzymatic effect.

Surface active agents have been shown to initiate germination-like changes in many bacterial species (Hyatt and Levinson, 1968). Dodecylamine was tried as a surface active germinant for type E

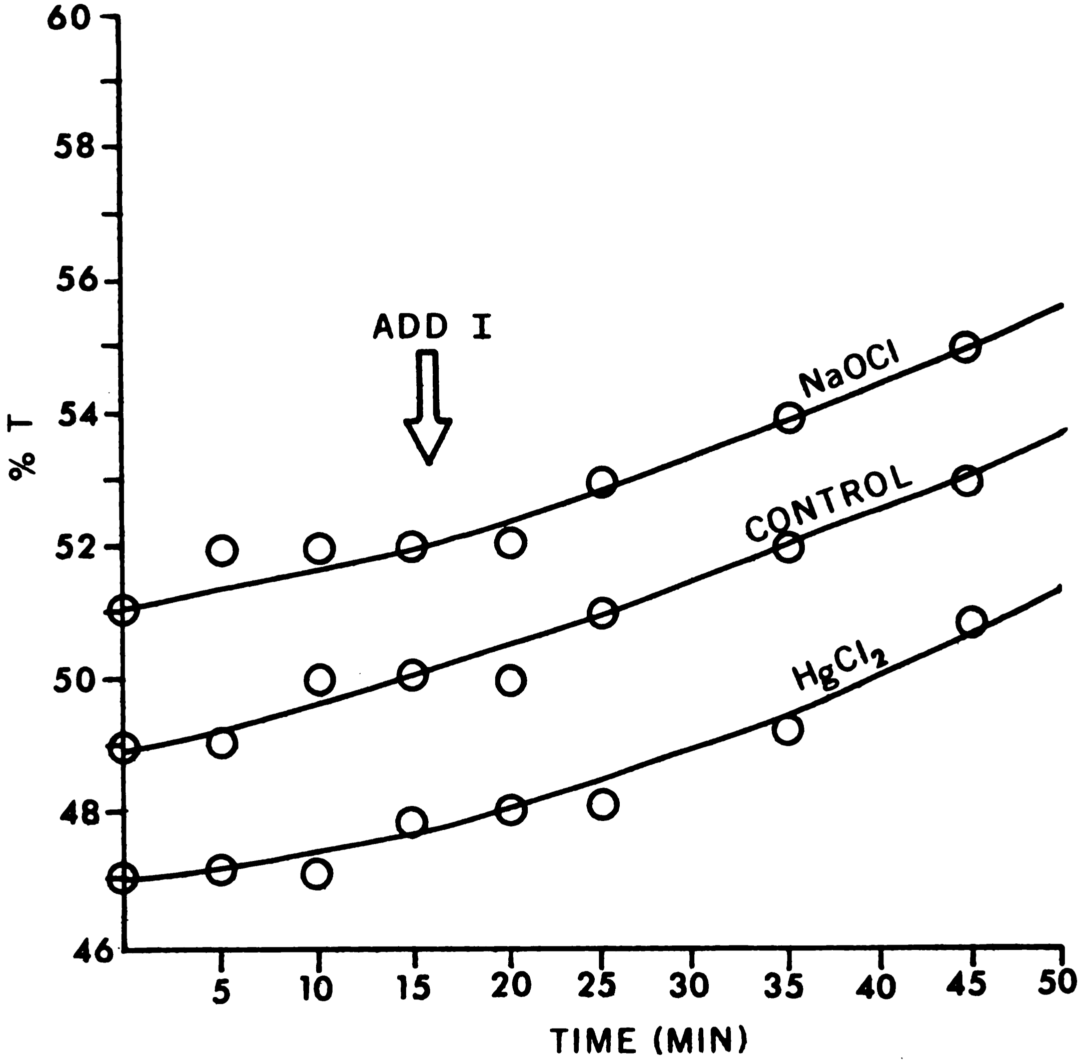

Figure 17. --Germination curves for Clostridium botulinum in MSN showing effect of two inhibitors, NaOCl and $HgCl_2$ on germination of type E spores.

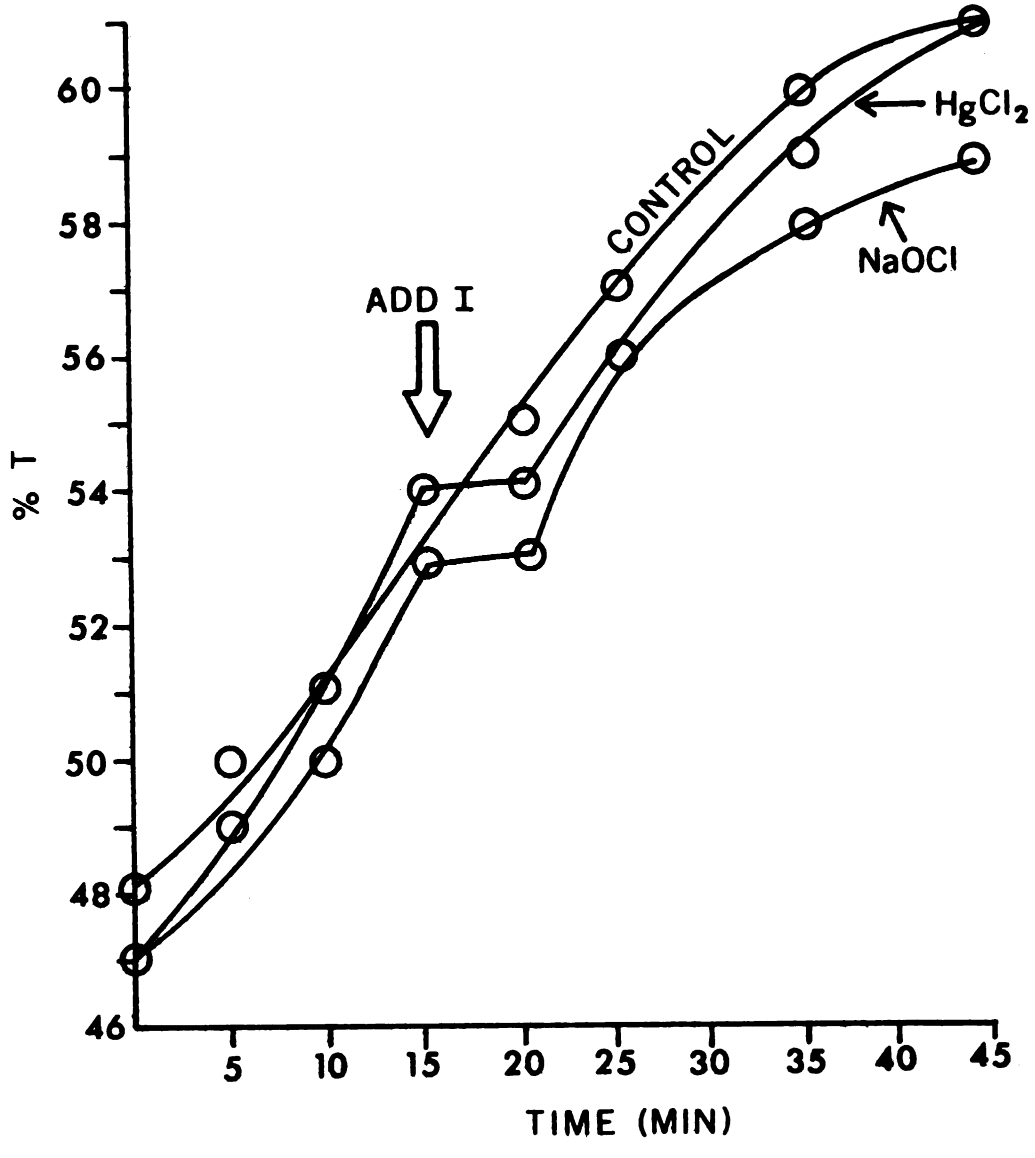

Figure 18. --Germination curves for Clostridium botulinum in NPB showing effect of two inhibitors, NaOCl and $HgCl_2$ on germination of type E spores.

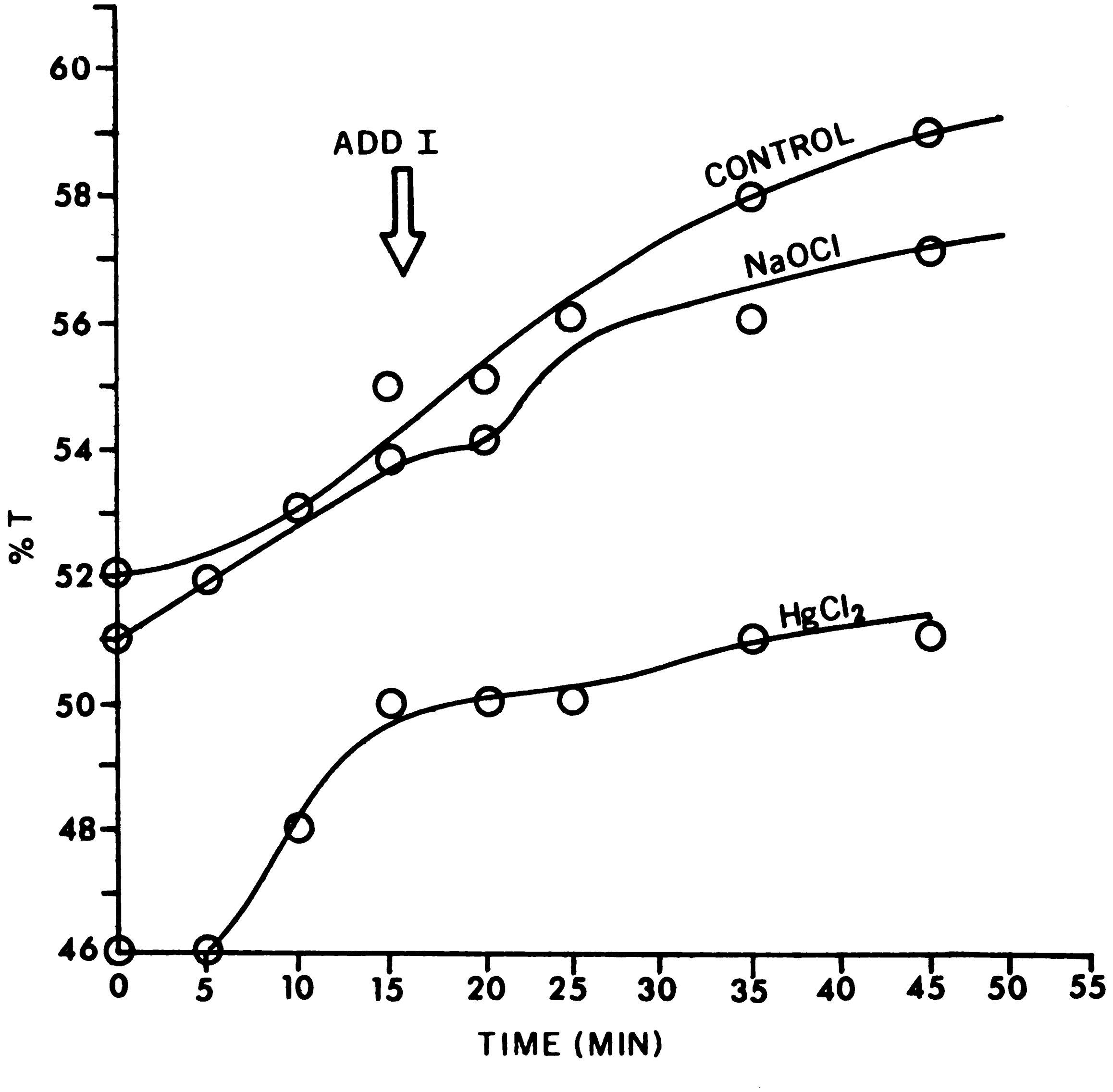

Figure 19. --Germination curves for Clostridium botulinum in ABG showing effect of two inhibitors, NaOCl and $HgCl_2$ on germination of type E spores.

spores of C. botulinum. Spores were incubated in an aqueous solution of dodecylamine (100 µg/ml) at 32 C. None of the spores germinated as measured by the turbidimetric technique. Another experiment was performed to determine if the dodecylamine in any way altered the permeability of the spore to cause a difference in germination kinetics when the spores were placed in a true germination system. Spores were treated in aqueous dodecylamine solution (100 µg/ml) at 32 C for 10, 20, 30, and 40 min. The spores were centrifuged, the dodecylamine decanted, and the spores resuspended in sterile distilled deionized water after these time periods. These spores were then inoculated into MSN broth after a single washing and incubated at 32 C. Germination was followed by turbidimetric measurements at various intervals. The results indicated that only the 40 min dodecylamine treated spores germinated to a greater extent than the control spores which received no dodecylamine treatment. The kinetics of this type of germination were similar to normal MSN germination kinetics. Figure 20 shows the results of this experiment. The germination curves are similar in shape, but the 40 min dodecylamine treated spores gave a greater total change in percent transmittance.

Experiments were conducted to determine the loss of calcium and dipicolinic acid from germinating spores of type E C. botulinum in three test systems: MSN, ABG, and NPB.

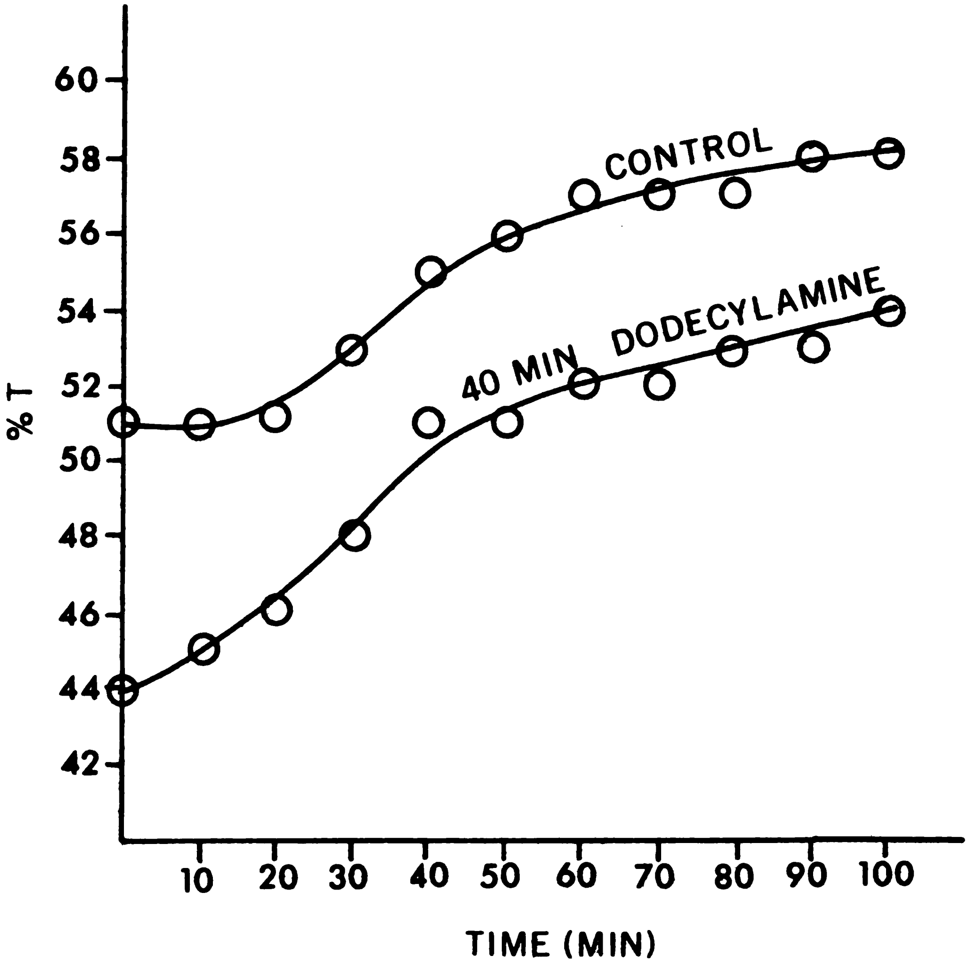

Figure 20. --Effect of pretreatment in 100 µg/ml dodecylamine of type E spores of *Clostridium botulinum* germinated in MSN.

Dipicolinic acid in dormant type E spores was determined by the method of Janssen et al. (1958), and a DPA level of only 0.5% was detected on a spore dry weight basis. This represents a very low DPA level when compared to the levels commonly found in other spores. Murrell and Warth (1965) listed 20 species of *Bacillus* spores having DPA contents (dry weight basis) from 5.06% to 13.55%. Similar studies with clostridia have revealed dipicolinic acid contents in the same range.

Calcium in type E spores of *Clostridium botulinum* Kalamazoo strain was shown by atomic absorption spectrophotometry also to be present only in the amount of 0.5%. This results in a calcium to dipicolinic acid molar ratio of 4:1. Murrell and Warth (1965), Lechowich and Ordal (1962) and others have discussed the relationship between Ca-DPA molar ratio and heat resistance. These workers found that most spores contained Ca-DPA ratios of 1:1. Perhaps the fact that the type E *C. botulinum* spores have a Ca-DPA ratio other than 1 may partially explain their low heat resistance (Dolman and Chang, 1953; Angelotti, 1970). Ito et al. (1970) have discussed the relation of the heat resistance of type E spores to those of types A and B *C. botulinum*.

Since the method of Janssen et al. (1958) did not permit reasonable accuracy below 20 µg/g, it was not possible to determine the loss of DPA to any degree with the type E spores, even with spore dry weights of approximately 0.05 g.

The results of the calcium determinations are shown in Table 6.

Table 6. --Percent calcium by dry weight in germinating spores of type E *C. botulinum* in three test germination systems.

Elapsed time during germination	Germination system		
	NPB	ABG	MSN
0 min	0.2752	0.5867	0.6522
5	0.3734	0.5022	0.5404
10	0.3493	0.4822	0.5221
15	0.2902	0.6010	0.5242
30	0.3185	0.5215	0.5928
90	0.2235	0.5882	0.5325

The above table illustrates that the percent calcium in the spores may have dropped during germination in the three test systems, but that these results could not be corroborated by statistical analysis. It was hoped that the loss of calcium could have been correlated with changes in refractility, stainability, and turbidity. Spore dry weights of 0.0421 to 0.0563 g were used in the calcium determination, and it was hoped that this level would give the desired results. This procedure also has several places where errors are undoubtedly committed, such as washing the spores to remove materials lost to the exudate, and washing the spores from the crucibles after ashing as was described in materials and methods. In order to accurately determine the loss of calcium in germinating spores of type E C. botulinum, one would probably have to use gram quantities of spores. When a large number of samples is to be run, it is possible that an entire experiment might consume 5 or more grams (dry weight) of type E spores. The present experiment required 1.8 g (dry weight) of spores. The first time that this experiment was conducted, the spores were grown in a 40 liter fermenter. However, this procedure yielded few free spores, and for this reason the spores were later grown in 30 one-liter Erlenmeyer flasks. Perhaps the best method for Ca determination would involve the use of Ca^{45} labeled spores.

The information available on the germination requirements of spores of the anaerobic bacteria such as Clostridium botulinum still lags far behind the data available on the aerobic bacilli. This research has tried to supply some needed data, but additional data are obviously required. It is hoped that the research reported here will stimulate more research concerning the spore forming anaerobes, which classically have been the major impetus for developing the technology of the canning industry. As was mentioned in the introduction, Clostridium botulinum is probably the one organism which is most important to the food industry and perhaps the one organism about which the least is known.

CHAPTER V

CONCLUSIONS

1. Not all systems which cause germination-like responses should be called true germination since if subsequent outgrowth of the germinated spores cannot be eventually obtained, then one may assume that the spores are sterile. We must keep this definition precise by amending it to say that until a medium is found which will permit outgrowth of the germinated spores, the spores will be considered dead.

2. Nitrite-induced germination appears to be a physio-chemical type of response rather than a metabolic or physiological one. The temperature responses, pH responses, shapes of germination curves, and rates of "germination" support this theory.

3. Microkinetics may ultimately prove to show the true responses of bacteriological phenomenon. The probability germination curves partially support this. Figure 12 presents regular straight lines obtained from microkinetics, whereas Figure 13

presents probability germination curves also, but these data were taken from turbidimetric population observations and the results are irregularly shaped lines. These irregularities may be caused by a masking of the true simple order of germination.

4. If nitrite can cause germination-like responses of _Clostridium botulinum_ type E, but not allow outgrowth as a result of the type of "germination," then food products with a high level of nitrite might be safe from growth of type E. However, the effect of low concentrations of nitrite in food substrates was not studied. It is not known whether low nitrite concentrations could trigger germination, and still allow outgrowth. This point needs to be studied.

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