

THE INHIBITION OF GERMINATION AND GROWTH
OF CLOSTRIDIUM BOTULINUM 62A BY
BHA, BHT, TBHQ AND 8-HYDROXYQUINOLINE

by

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INTRODUCTION

Botulism has long been recognized as a worldwide public health hazard. The toxin produced by its causative agent, Clostridium botulinum, is one of the most potent lethal agents known. Botulism, if not treated properly in special health care facilities, often results in death. Due to widespread distribution of canned foods and other potentially susceptible foods there is potential for extensive and rapid outbreaks of botulism if food is improperly processed or handled. Botulinum poisoning has historically been a problem in home canning as evidenced by the frequent reporting of botulism from home-canned foods.

Preservation of foods for the prevention of botulinum poisoning is accomplished in many ways including pickling, curing, thermal processing, smoking, drying, salting and the addition of chemical preservatives. Often combinations of these methods are used. Therefore, the character and quality of our food is in part a result of techniques used to prevent growth and toxin production of C. botulinum. The addition of nitrite to foods, done in part for its antibotulinal activity, has come under question by consumer groups and regulatory agencies. Therefore, widespread research has been conducted to find a replacement for nitrite in cured meats. Since thermal processing is based on the destruction of C. botulinum spores, new methods of improved botulinal control may allow reduction in processing time and the concomitant increase in canned food quality.

The phenolic antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary-butyl hydroquinone (TBHQ)

have been found to possess activity against a wide variety of microorganisms. Several studies have reported BHA and BHT to be effective against C. botulinum. Recently studies have shown the inhibition of growth and toxin production of C botulinum by 8-hydroxyquinoline (8-OHQ). Much research is needed in order to determine the potential efficacy of these compounds in food preservation systems.

The effects of various food preservatives on growth from spores have been studied by many researchers. Most investigations, however, have not distinguished between the antimicrobial effects on vegetative cell growth and on spore germination. Since germination is a prerequisite for vegetative growth from C. botulinum spores, knowledge of each of these two processes is essential for a complete understanding of botulinal spoilage and control. The following study was designed to determine the extent and nature of the antibotulinal activity of BHA, BHT, TBHQ AND 8-OHQ by examining inhibition of both growth and spore germination of C. botulinum.

REVIEW OF LITERATURE

A. Clostridium botulinum and Botulism

Clostridium botulinum is characterized by its ability to produce heat-resistant spores and its production of an extremely poisonous neurotoxin. Types A, B, E and F have been implicated in human botulism while types C and D cause botulism in animals. Type G has been isolated from soil in Argentina but as yet has not been involved in foodborne botulism (Giminez and Ciccarelli, 1970).

Food-borne human botulism results from the ingestion of botulinum toxin. Type A is the most potent type known, 1.2 ng/kg being the minimum lethal dose for mice and 1 ng/kg being the estimated minimum lethal dose for humans (Gill, 1982).

Clostridium botulinum is a rod-shaped gram-positive obligate anaerobe, widely distributed throughout the world (Smith, 1977). Meyer (1956) reported the isolation of C. botulinum spores from North America, Australia, China, Sweden, England and Germany. Type B is most frequent in Europe and in the eastern U.S. Type A is the predominant type in the western U.S. Type E is the most common type found in aquatic environments (Smith, 1975).

Distribution of C. botulinum types is reflected in records of reported botulism. Type E accounts for 46% of reported outbreaks in Japan, Canada and Scandinavia in the twentieth century. Fish and marine mammals are the most common vehicles in these areas. Outbreaks in Europe have usually been caused by Type B, while Type

A botulism is the most predominant in the western U.S. (Dolman, 1964).

From 1899 to 1979 there were 2,028 reported cases from 785 outbreaks of human foodborne botulism in the United States. Nearly all cases were from improperly home-canned low acid foods, smoked fish and fermented seafoods (U.S. Dept. HEW, 1979; U.S. Dept. HHS, 1981a; U.S. Dept. HHS, 1981b). Most outbreaks have involved only a few cases but more extensive outbreaks have occurred, including 17 cases from smoked chub in 1963, 58 cases from home-canned peppers in 1977 and 34 cases from a three-bean and potato salad in 1978 (U.S. Dept. HEW, 1979; U.S. Dept. HHS, 1981a).

Botulism has the highest mortality rate of any food poisoning known (Murrell, 1976). From 1972 to 1979 botulism accounted for 35% of deaths reported to be caused by foodborne illness. Type A has been the most lethal type (U.S. Dept. HEW, 1979; U.S. Dept. HHS, 1981a; U.S. Dept. HHS, 1981b). Botulinum toxin is neuroparalytic in nature and functions by interfering with cholinergic synapses, thus paralyzing the nervous system (Boroff and DasGupta, 1971; Brooks, 1966). This may occur within a few hours to several days after ingestion, and usually between 12 and 36 hours (U.S. Dept. HHS, 1979). The disease is generally more severe when symptoms appear earlier (Brooks, 1966; Smith, 1975). Initial symptoms include nausea, vomiting, abdominal pain and diarrhea. Later headache, dizziness, double vision, dysphagia, constipation and gradual motor neuron dysfunction occur. Death usually results from respiratory paralysis (U.S. Dept. HHS, 1979).

The mortality rate for the period 1899 to 1949 was above

60%, but since then there has been a gradual decrease. The fatality/case ratio was 16% for the period from 1970-1977 and 5% for 1978 and 1979 (U.S. Dept. HEW, 1979; U.S. Dept. HHS, 1981a; U.S. Dept. HHS, 1981b). This trend is due to improved ventilation assistance for patients as well as more rapid diagnosis and treatment with antitoxin.

B. Growth and Toxin Production of *Clostridium botulinum*

It has been concluded that *C. botulinum* has the potential to grow and produce toxin in most types of food above pH 4.5 (Dack, 1964). Although it is true that growth and toxin production has been found to occur in many foods and in a wide variety of laboratory media, there are well-studied parameters which have been found to influence botulinal growth.

Minimum nutritional requirements have been studied in detail. Specific amino acids, mineral salts and growth factors such as thiamin, biotin and p-amino benzoic acid have been found to be required for growth and toxin production (Campbell and Frank, 1956; Dolman, 1964; Elberg and Meyer, 1939). Rich organic media such as cooked meat medium and Trypticase peptone broth satisfy all these requirements (Duff et al., 1957; Benedict, 1980). Proteinaceous and non-proteinaceous foods such as meats and vegetables also satisfy the nutritional growth requirements of *C. botulinum*. Bonventre and Kemp (1959a) found that addition of glucose gives the highest yields of cells and toxin. Holdeman (1967) found that glucose and yeast extract is required for maximum toxin production by *C. botulinum* Type F. For optimum growth,

media should be free of all dissolved oxygen and have an oxidation-reduction potential below -200 mv (Benedict, 1980). However, these requirements depend on the particular strain of C. botulinum tested and also are interactive with each other. Some investigators found C. botulinum capable of growth in media that had been exposed to oxygen, provided that the oxidation-reduction potential was low enough (-80 mv) (Smoot and Pierson, 1979).

Table 1 is a summary of some pertinent literature regarding the influence of temperature, pH and Aw (water activity) on C. botulinum growth and toxin production. There have been many different values reported for the limiting temperature and pH values for botulinal growth and toxin production. This is due to differences in substrate, inoculum size, strain and method of testing. Boventre and Kempe (1959b) found their strain of Type A to produce toxin best at 37°C. Type A toxin has been reported to be most stable at 30°C (Pederson, 1955). In general, Type E and nonproteolytic Type B and F strains have minimum growth temperatures about 10 degrees lower than Type A and proteolytic Type B strains (Walls, 1967). Maximum toxin production occurs at a slightly higher temperature for proteolytic strains than for non-proteolytic strains. It is well documented that C. botulinum Type E is psychrotrophic. This distinguishes Type E from Types A and B, since the latter types are not capable of growth at temperatures below 10°C (Ohye and Scott, 1953; Bonventre and Kempe, 1959b). Schmidt et al. (1961) found that the minimum temperature for growth and toxin production of Type E is 3.3°C, although as much as 30 to 60 days at this temperature

Table 1. Summary of limiting conditions reported for growth and toxin production of Clostridium botulinum.

				<u>References</u>
Type A	temperature	growth range	10-50°C	9,2,8,4
		growth optimum	35-37°C	
		toxin production optimum	25-37°C	
pH	4.4-8.0	growth, 5.3-8.3	toxin	7,6,13
Aw	>0.93 (<50-55% sucrose, <8.5-10.5% NaCl)			5,1
Type B	temperature	growth range	10-50°C	9,2,8
		growth optimum	37°C	
		pH	>4.4	
Aw	>0.93 (<50-55% sucrose, <8.5-10.5% NaCl)			5,1
Type E	temperature	growth range	3.3-45°C	10,3,5,12,11
		growth optimum	30-35°C	
		toxin production optimum	30°C	
pH	4.8-8.0			7,6
Aw	>0.95 (<38.5% sucrose, <5-6% NaCl)			7,1
Type F	temperature	growth range	>4°C	14,15
		growth optimum	30-37°C	

- | | |
|----------------------------------|----------------------------------|
| 1. Baird-Parker and Freame, 1967 | 9. Ohye and Scott, 1953 |
| 2. Bonventre and Kempe, 1959b | 10. Ohye and Scott, 1957 |
| 3. Grecz and Arvay, 1982 | 11. Pederson, 1955 |
| 4. Hobbs, 1976 | 12. Schmidt <i>et al.</i> , 1961 |
| 5. Kautter, 1964 | 13. Smelt <i>et al.</i> , 1982 |
| 6. Lechowich, 1968 | 14. Smith, 1977 |
| 7. Murrell, 1976 | 15. Walls, 1967 |
| 8. Ohye and Christian, 1967 | |

was required to produce detectable amounts of gas and toxin.

The minimum pH at which C. botulinum will grow or produce toxin is generally regarded to be 4.6 (Ingram and Robinson, 1951; Ohye and Christian, 1967). However, Smelt et al. (1982) reported growth below pH 4.6 in a medium containing soy protein or casein. Minimum pH values reported vary largely due to the substrate used for growth (Townsend et al., 1954; Dozier, 1924). Botulinum toxin is more stable at pH 6.5 and below (Bonventre and Kempe, 1959b).

Water activity (A_w) greatly influences botulinal activity. The minimum A_w for C. botulinum Type A and B is 0.93, while for Type E it is 0.95 (Baird-Parker and Freame, 1967). A_w can be reduced by increasing the solute concentration. Addition of sugar or salt therefore, can reduce the A_w so that botulinal growth is inhibited. Table 1 includes some concentration values of sucrose and sodium chloride which are effective in preventing growth and toxin production by C. botulinum. At least 55% sucrose or 10.5% NaCl is effective for types A and B while at least 38.5% sucrose or 6% NaCl is needed to prevent growth of type E (Murrell, 1976; Kautter, 1964; Baird-Parker and Freame, 1967).

The effects of the above-mentioned factors on botulinal growth are interrelated in that each may be influenced by the other factors. Baird-Parker and Freame (1967) found that with lower pH a higher A_w was required for growth. Segner et al., (1966) found that inhibition of growth from Type E spores by pH was affected by the incubation temperature and the level of inoculum. Riemann et al.,

(1972) reported that inhibition of C. botulinum Types A and B growth and toxin production by sodium chloride was dependent on the pH. They found that by decreasing the pH from 7.0 to 5.0 the concentration of sodium chloride needed for inhibition was lowered. Inoculum size has much to do with growth and toxin production. The larger the inoculum, the sooner the appearance of growth and toxin, and the stricter the conditions necessary for their inhibition (Reed and Orr, 1943). This is probably due to variation in sensitivity within the population; the larger populations having the greatest number of more resistant members.

C. Control of Growth and Toxin Production of Clostridium botulinum in Foods

Early methods for control of spoilage by Clostridium botulinum were salting and drying. Later, methods of fermenting and smoking were introduced. Current methods of preservation include thermal processing, curing, pickling, pasteurization, smoking, drying, salting and low temperature storage. Often combinations of these methods are used.

Thermal processing of low acid foods in hermetically sealed containers serves to achieve "commercial sterility" i.e. the destruction of all pathogens including Clostridium botulinum as well as the more thermoduric microorganisms which could cause spoilage at normal storage temperatures. Adequate heat processing of low-acid canned foods (pH >4.6 and A_w >0.83) is achieved with an F_0 of ≥ 2.78

minutes (a sterilizing value equal to or greater than 2.78 minutes at 250°F (Murrell, 1976). This treatment achieves a "botulinum cook" or a 12D process where D value is the time necessary for 90% destruction of the C. botulinum spores present. Thus, thermal processing of canned low-acid foods aims to destroy all but 10⁻¹⁰% of C. botulinum spores in the food. The particular food to be processed may require a greater sterilization value particularly if high concentration of fats or carbohydrates are present. Molin and Snygg (1967) showed that spores have higher apparent heat resistance when suspended in nonaqueous lipid as opposed to suspension in water. The pH of heating medium is also important. Zezones and Hutchings (1965) examined the heat resistance of C. botulinum Type A spores in spaghetti sauce of varying pH. They found the D₂₄₀ values to be 0.128, 2.6, 4.91 and 5.15 at pH 4, 5, 6 and 7, respectively. Heat resistance of C. botulinum spores is increased by lowered Aw (Murrell and Scott, 1966) and decreased by increased sodium chloride concentration (Duncan and Foster, 1968).

The condition under which spores are formed influences heat resistance. Heat resistance is known to be greater for spores produced at higher temperatures. Sugiyama (1951) found that spores of types A and B formed at 37°C were of higher heat resistance than those formed at 24° or 29°C. It has been suggested that spores grown at higher temperatures take up more Ca²⁺ (Murrell, 1967) and it has been shown that when Ca²⁺ in spores is replaced with other divalent cations the heat resistance decreases (Foerster and Foster, 1966). Thus, high

heat resistance may depend upon the presence of sufficient amounts of Ca^{2+} or other metal ions present in the sporulation medium. Increasing the chain length of a fatty acid added to the sporulation broth has also been reported to raise the level of spore heat resistance (Sugiyama, 1951). In general, proteolytic strains of C. botulinum are more heat resistant than nonproteolytic strains (Eklund et al., 1967).

Unfortunately, the thermal treatment used for sterilization of low acid canned foods adversely affects the organoleptic quality of many foods. Therefore, some products receive less than a botulinum cook, with F_0 values as low as 0.05-0.6 minutes. These foods include corned beef, luncheon meats, deviled ham, potted meat, hot dogs and bacon (Lechowich, et al., 1978). In order to adequately protect against C. botulinum poisoning these foods are preserved with a combination of salt, nitrite and refrigerated storage. Nitrite has been used for centuries as a preservative for ham and bacon. It does not enhance destruction of C. botulinum spores during heating nor inhibit germination of spores. Its function is to prevent growth and toxin formation of germinated spores which have survived the mild heat treatment (Pivnick, et al., 1970). Sodium nitrite, however, is not an ideal preservative. Amounts added to meats are rapidly depleted with storage and questions have been raised about the potential carcinogenicity of nitrosamines which are formed from the reaction of nitrite with secondary and tertiary amines (Crosby, 1976; Wasserman, 1978). This has prompted much research to find a suitable replacement for nitrite.

Botulinal control by means of acidification is also in use today. Pickling in vinegar solutions to reduce the pH below that permitting botulinal growth is used for such products as pickled herring, pigs' feet, eggs, certain vegetables and sausages. Fermentation methods also acidify and are used with many types of sausages as a means of preservation. Acidification methods for meats have several disadvantages, as pointed out by Sofos and Busta (1980). These include problems with reproducibility of pH, flavor impairment, viability of starter cultures and microenvironments of varying pH. Methods of food preservation are continually being researched in many laboratories.

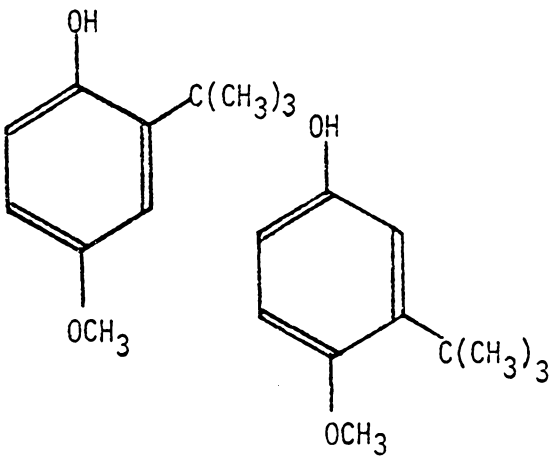
D. BHA, BHT, TBHQ and 8-Hydroxyquinoline

The search for new and improved methods for preventing pathogenic and spoilage microorganisms from growing in foods has led to the discovery of several promising antimicrobial compounds. The antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT) and tertiary-butyl hydroquinone (TBHQ) have been shown to have antimicrobial properties in addition to their antioxidant function. Antimicrobial activity of these antioxidants has been demonstrated with several microorganisms including bacteria, molds, yeasts and viruses. Foodborne pathogenic bacteria found to be inhibited include Staphylococcus aureus, Escherichia coli, Salmonella typhimurium, Vibrio parahaemolyticus, Clostridium

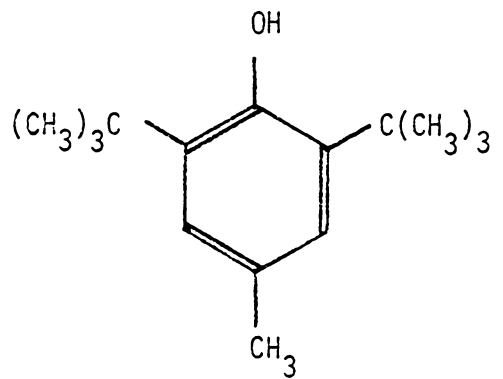
perfringens and Clostridium botulinum (Branen, et al., 1980). 8-Hydroxyquinoline (8-OHQ) has been found to exhibit activity against botulinal growth and toxin production (Reddy, et al., 1982; Pierson and Reddy, 1982; Reddy and Pierson, 1982). Other investigators have also found 8-OHQ to inhibit growth of Staphylococcus aureus and Escherichia coli (Rubbo, et al., 1950).

The structures of BHA, BHT, TBHQ and 8-OHQ are represented in Figure 1. BHA, BHT and TBHQ are the most frequently used antioxidants, along with propyl gallate, in the United States (Sims and Fioriti, 1980). They are widely used in the food industry to inhibit the autooxidation of fats and oils. In 1976, about 9 million pounds of BHT were produced for use in food (Roberts, 1981). These compounds are used in food packaging materials or as direct food additives. They are currently listed as "Generally Recognized as Safe" (GRAS) by the Food and Drug Administration for use in foods at a maximum concentration (alone or in combination) of 0.02% (200 µg/g) based on the lipid content of the food (Sims and Fioriti, 1980; Branen, 1975). Some products such as cereals, cake mixes and dried potato products have specific limiting allowed concentrations of antioxidant based upon the total weight of the product.

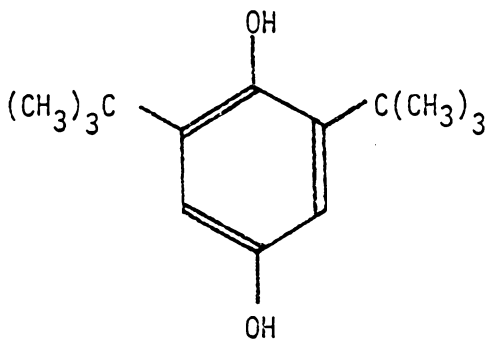
BHA and BHT have had the most extensive toxicity testing among the phenolic antioxidants. The testing has been carried out with several animal species and also in humans. Pathological testing of BHA has revealed low potential for harmful effects (Branen, 1975).



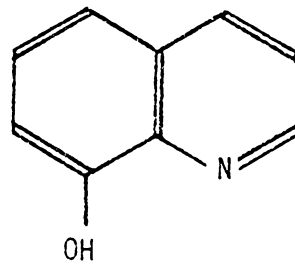
Butylated hydroxyanisole (BHA)
(a mixture of two isomers)



Butylated hydroxytoluene
(BHT)



Tertiary-butyl hydroquinone
(TBHQ)



8-Hydroxyquinoline
(8-OHQ)

Figure 1. Chemical Structures of BHA, BHT, TBHQ and 8-OHQ.

Dietary BHA is metabolized and excreted rapidly in animals and man (Astill, et al., 1962). Hodge et al., (1964) fed dogs 100 $\mu\text{g/g}$ BHA for one year without adverse effects. They found no BHA stored in the body tissue. However, there is still controversy regarding potential carcinogenicity of BHA (Anonymous, 1982).

BHT has had a history of conflicting and controversial toxicity testing. Dietary BHT has been found to cause marked hypertrophy of the liver, but studies designed to determine carcinogenicity have been contradictory (Stuckey, 1972). No adverse effects have been noted at normal levels of use (Branen, 1975). In 1977 FDA proposed interim restrictions on levels of BHT allowed in foods until additional toxicity studies could be performed. A committee was formed, but concluded that there was no conclusive evidence that BHT posed a public health hazard at current use levels. The committee recommended that additional toxicity studies be carried out but that the status of BHT should remain GRAS and allowed at existing levels. In response, the National Cancer Institute performed feeding studies with rats and mice. They fed rats and mice up to 6,000 $\mu\text{g/g}$ BHT in feed for over two years. After examining the animals carefully for tumors they concluded that "under the conditions of this bioassay, BHT was not carcinogenic" (U.S. Dept. HEW, 1979a).

This study has not put an end to controversy concerning the safety of BHT. Soon after the NCI study results were published the Center for Science in the Public Interest made a statement urging

a halt to the use of BHT in foods because of some uncertainties in the way tumors had been examined in the NCI study (CSPI, 1979). Since then, other toxicity studies have been reported. Nakagawa et al., (1981) reported several carcinogen-metabolizing enzymes which are stimulated by BHT. Their study and others (Wattenberg, 1975; Wattenberg, 1972; Weisburger, et al., 1977; Ulland, et al., 1973) have demonstrated that BHA and BHT may actually help the body to metabolize carcinogens, thus decreasing cancer incidence in animals fed diets containing cancer-causing agents. Branen (1975) reviewed toxicity testing of BHA and BHT and concluded that at levels of 500 times the estimated daily consumption, BHA and BHT appear to be free of injurious effects. Studies have shown that TBHQ has toxicity comparable to that of BHT (Lewis and Tatken, 1980).

A wide variety of phenolic compounds are used as active ingredients in commercial disinfectants due to their ability to prevent growth from spores. Among these compounds are 8-OHQ and its salts (Prindle and Wright, 1977). Toxicity testing has shown that high doses of 8-OHQ are quite toxic to rats and mice and have been reported to be carcinogenic in some cases. Few studies, however, have been done with oral administration of 8-OHQ. One such study reported an LD₅₀ higher (lower toxicity) than those reported for BHT or TBHQ (Lewis and Tatken, 1980). If 8-OHQ is to be petitioned to FDA for use in foods much oral toxicity testing must be done.

The antimicrobial effects of the phenolic antioxidants have been subject of many investigations. Initially, Ward and Ward (1967) reported only slight inhibition of Salmonella senftenberg

775W in the presence of 10,000 µg/g BHT. Since 1975 when Chang and Branen (1975) found 150 µg/ml BHA to prohibit growth of Staphylococcus aureus, numerous studies have shown the phenolic antioxidants to exhibit strong antimicrobial activity.

BHA and BHT have been shown to have inhibitory effects upon molds, (Fung, et al., 1977; Chang and Branen, 1975; Kim, et al., 1978) viruses (Snipes, et al., 1975; Wanda, et al., 1976; Kim et al., 1978), protozoa (Surak, et al., 1976) and bacteria. The inhibition of Staphylococcus aureus has been the most studied bacterial inhibition. In general, 150-200 µg/ml BHA has been found to prevent growth. Ayaz (1980) found 200 µg/ml BHA and 116 µg/ml BHT each to inhibit S. aureus in brain heart infusion broth. Chang and Branen (1975) and Davidson et al., (1979) reported 150 µg/ml BHA to inhibit S. aureus in Trypticase soy broth and in nutrient broth. Other investigators have reported inhibition of S. aureus growth at levels of 200 and 400 µg/ml (Shih and Harris, 1977; Van Tassell, et al., 1978). TBHQ has been found to inhibit S. aureus in an agar medium at a level of 30 µg/g (Erickson and Tompkin, 1977).

Salmonella typhimurium inhibition by BHA depends upon the strain and medium used for testing. Chang and Branen (1975) reported 400 µg/ml BHA to totally inhibit growth in nutrient broth while Van Tassell et al., (1978) found growth of S. typhimurium to be only slightly retarded at up to 400 µg/ml BHA in Trypticase soy broth. Another strain of S. typhimurium was inhibited by 150 µg/ml BHA in Trypticase soy broth (Davidson, et al., 1979).

Enteropathogenic Escherichia coli is inhibited by 400 µg/ml BHA in nutrient broth (Chang and Branen, 1975) while 400 µg/ml only slightly inhibits growth of another strain of E. coli (Shih and Harris, 1977). Robach et al., (1977) illustrated the importance of the medium used for testing when he found Vibrio parahaemolyticus to be inhibited by 50 µg/ml BHA in Trypticase soy broth, but that 400 µg/g BHA was required for the same inhibition of growth in a crab-meat homogenate.

Inhibition of Pseudomonas has been found to be quite variable, depending on the strain tested (Davidson and Branen, 1980a; Davidson and Branen, 1980b). P. fluorescens was inhibited by 150 µg/ml BHA while more than 400 µg/ml was necessary to prevent growth of P. fragi.

Several strains of Clostridium perfringens were prevented from growing by 150 µg/ml BHA in Fluid Thioglycollate Medium at 37°C (Klindworth, et al., 1979). At 45°C, however, 150 µg/ml BHA was not completely inhibitory for all strains tested.

A few investigators have studied inhibition of growth from Clostridium botulinum spores. Tompkin et al., (1978) reported that 1000 µg/g but not 200 µg/g TBHQ showed some inhibition of growth from spores in the presence of 50 ppm nitrite in a comminuted canned cured meat product. Other investigators studying inhibition of botulinal growth have used a prerduced Thiotone yeast extract glucose medium (Robach and Pierson, 1979; Reddy, et al., 1982; Reddy and Pierson, 1982). Robach and Pierson (1979) found growth and toxin production of C. botulinum to be inhibited by 50 µg/ml BHT and

200 µg/ml TBHQ. In another study using a buffered system (Reddy and Pierson, 1982) BHA, BHT and TBHQ exhibited similar inhibition of C. botulinum except that BHA appeared to be slightly less effective. The results of their study showed that the phenolic antioxidants are quite effective against C. botulinum at pH 6.0 and 7.0 when grown in liquid media. In another study, Pierson and Reddy (1982) found BHA, BHT and TBHQ to inhibit C. botulinum less effectively in a comminuted pork system. Concentrations of 1000 µg/g of the chemicals were required for a 3-4 day delay of the first detected toxic sample.

In contrast to the phenolic antioxidants, few inhibition of growth studies with 8-OHQ have been carried out. Rubbo et al., (1950) found growth of Staphylococcus aureus to be inhibited by 10 µg/ml 8-OHQ and Escherichia coli by 625 µg/ml in broth media. Fungi have been found to be inhibited at a concentration of 5 µg/ml (Uri and Szabo, 1950). Streptococcus has been found to be inhibited by 20 µg/ml 8-OHQ (Liese, 1927). The same investigator reported growth inhibition of E. coli and Salmonella typhosa by 100 µg/ml 8-OHQ while 200 µg/ml was required for inhibition of growth of S. schottmuelleri. Pierson and Reddy (1982) studied inhibition of Clostridium botulinum by 8-OHQ. They found 50 µg/ml 8-OHQ to be effective in the prevention of toxin production and cell growth. In a comminuted pork system they found enhanced inhibition of C. botulinum by 8-OHQ. Levels of 200 µg/g delayed package swelling and toxin formation at 27°C for at least 60 days while 40 µg/g inhibited for 7 days, although a few of their samples were found to be toxic after 4 days at a level of 80 µg/g.

E. Germination of *Clostridium botulinum* Spores

The transformation of a bacterial spore into a vegetative cell can be described as a process consisting of three stages: activation, germination and outgrowth. Although many previous workers have used the term "germination" to describe this entire process, it is more descriptive and useful to separate the three stages when referring to inhibition of growth from spores.

Activation is a result of treatments which do not in themselves initiate germination, but rather cause the spore to become more susceptible to the action of germinants. Dormant spores do not necessarily germinate when placed under favorable conditions; often activation is a prerequisite (Keynan and Evenchik, 1969). The most common method for activating *Clostridium botulinum* and other spores for germination is "heat shocking". Type A *C. botulinum* spores are maximally activated by a heat shocking treatment of 80°C for 10-20 minutes (Ando, 1973). Temperatures of about 60°C for 10 minutes has been reported as optimum for *C. botulinum* Type E activation (Ando and Iida, 1970; Ando, 1971). Other agents used to activate spores include exposure to low pH, reducing agents, calcium dipicolonic acid, ionizing radiation and several chemicals such as D-cycloserine and urea (Keynan and Evenchik, 1969). Requirements for activation have been found to be specific for particular species in many cases.

Once a spore has been activated it is capable of germination

if placed in the proper environment. Germination is a degradative process in which characteristic properties of the spore are lost. These include resistance to heat, radiation, pressure, desiccation and various chemical agents. The sequence of spore changes, in order, include loss of heat resistance, loss of chemical resistance, loss of calcium and dipicolinic acid, excretion of peptidoglycan, increase in stainability with basic dyes, loss of refractivity to light and decrease in optical density (Gould, 1970). Often decrease in optical density, loss of refractivity, increase in stainability and loss of heat resistance are used as criteria for estimation of extent of germination. Loss of refractivity is determined by counting spores which have turned from phase bright (refractile) to phase dark (non-refractile), using phase contrast microscopy. Germination, as opposed to outgrowth which transforms the germinated spore into the vegetative cell, involves no synthesis of new cell structures and involves no DNA or RNA synthesis.

The germination process is generally viewed as a "trigger reaction" because of the irreversible nature and sudden kinetics of detectable germination following a lag period (Keynan, 1978). No model to date for description of the trigger reaction has satisfied all scientists. Some view it as a metabolic reaction or series of reactions requiring energy and others view it as more of a physical process which is allosterically stimulated by germinants. Loci of action of germinants have not been conclusively determined (Smoot and Pierson, 1982).

For optimal germination of C. botulinum Type A, L-alanine

or cysteine, lactate and bicarbonate have been found to be required (Ando, 1973). Rowley and Feeherry reported rapid germination of C. botulinum 62A spores in medium containing L-cysteine, bicarbonate and thioglycollate (1970). Lactate has been found to apparently act as a cogerminant for clostridial spores (Ando, 1973; Ando and Iida, 1970). Some studies have suggested that carbon dioxide and not bicarbonate itself is the germinating influence (King and Gould, 1971; Treadwell, et al., 1958). Bicarbonate enhancement of germination has been demonstrated by several researchers for optimal germination of C. botulinum (Rowley and Feeherry, 1970; Ando, 1973; Ando and Iida, 1970). Sodium nitrite has been found to stimulate germination at high concentrations under certain conditions. Under other conditions sodium nitrite was found to inhibit germination (Ando, 1973). Glucose, lactic acid, L-alanine and bicarbonate were shown to be responsible for optimal germination of C. botulinum Type E spores (Ando and Iida, 1970). Overall, the requirements for other species has been found to vary considerably. Germinants described include various amino acids, ribosides, salts, carbohydrates and assorted chemical germinants (Gould, 1970; Gould and Dring, 1972; Smoot and Pierson, 1982).

Other environmental factors have been researched in some detail. The pH range for germination of C. botulinum Types A and B spores has been found to be pH 4.6 to 9.0. Optimal germination occurs between pH 6.5 and 7.0 (Rowley and Feeherry, 1970; Ando, 1973; Ando and Iida, 1970). Type E germination occurs optimally at pH 6.6 and is prevented at pH 5.3 (Ando and Iida, 1970). Ando and Iida (1970) found

that germination of C. botulinum Type E spores was not affected by initial Eh. In their experiments medium Eh decreased as germination proceeded. Treadwell et al., (1958), demonstrated that germination of C. botulinum is not affected by the level of oxygen in the medium. Other species of Clostridium have been shown to require anaerobic conditions for germination (Hitzman, et al., 1957). Spore germination has been found to occur at an Aw nonpermissive for vegetative growth. Baird-Parker and Freame (1967) found C. botulinum spores to germinate at Aw as low as 0.89. The temperature range for germination of Type A C. botulinum is 4-70°C with the optimum being 37°C (Ando, 1973; Roberts and Hobbs, 1968). The optimum temperature for germination of Type E spores was reported by Ando and Iida (1970) to be 37°C. They found only slight germination when they decreased the temperature to 10°C or raised it to 45°C. Grecz and Arvay (1982), however, found C. botulinum Type E strain VH to germinate optimally at 10°C and poorly at 37°C. This may demonstrate heterogeneity among the Type E strains.

Several chemicals have been shown to inhibit spore germination. Inhibition by nitrite has already been mentioned. Winarno et al., (1971) found increasing concentration of EDTA to inhibit germination of C. botulinum 62A spores. Treadwell et al., (1958) found C. botulinum germination to be inhibited by Thioglycollate. 15% sodium chloride prevented germination of several Bacillus species (Gould, 1964). Lower concentrations of sodium chloride were found to be inhibitory to cell growth but not germination. Other food preservatives studied by Gould included nisin, subtilin, sodium nitrite, sodium

benzoate and sodium sorbate. It was found that these compounds were ineffective at the levels tested for the prevention of germination of Bacillus, but that growth after germination was inhibited. Some researchers have found 0.2% sorbic acid to inhibit spore germination of C. botulinum (Sofos, et al., 1979). Smoot and Pierson (1981) reported potassium sorbate to inhibit germination of C. botulinum and B. cereus T spores at levels of 5,200 µg/ml and 3,900 µg/ml, respectively, at pH 5.7.

Several hydrophobic compounds capable of inhibiting germination have been identified. Phenols have been studied the most due to their use as sporostatic agents in disinfectant solutions. The disinfectants phenol and cresol have been shown to inhibit spore germination in Bacillus species (Parker and Bradley, 1968; Schmidt, 1955; Parker, 1969; Russell, et. al., 1979). Phenol at a concentration of 2000-2500 µg/ml has been found to prevent germination of spores as well as prevent cell growth (Russell, 1965; Yasuda, 1982). Chlorocresol is active in blocking germination at a concentration of 1000 µg/ml (Russell, 1974; Russell, et. al., 1979). This inhibition was shown to be reversible by washing of the spores (Parker, 1969). Alcohol has been studied by many researchers including Trujillo and Laible (1970). They found inhibition of germination of Bacillus spores to increase with increasing chain length. Reversibility of this inhibition was also demonstrated. Other hydrophobic compounds capable of inhibiting spore germination are linolenic, linoleic and oleic acids (Foster and Wynne, 1948). Parabens also are reported to inhibit spore germination

(Russell, et al, 1979; Watanabe and Takesue, 1976). The longer the carbon chain, the more effective the inhibition of germination (Watanabe and Takesue, 1976). Reversibility was demonstrated for this inhibition. Inhibition of germination demonstrated for the above hydrophobic compounds indicates that other compounds such as BHA, BHT, TBHQ and 8-OHQ may possess similar activity. However, more work must be done with Clostridium species.

The effect of spore environment on germination including temperature, pH, A_w , germinant concentrations and concentration of germination inhibitors is extremely complicated and interdependent. At lower pH values germination of C. botulinum Types A and E spores is inhibited at a higher A_w (Baird-Parker and Freame, 1967). Depending on the pH and other conditions nitrite may stimulate or inhibit C. botulinum spore germination (Ando, 1973). Concentration of divalent cations is also known to influence spore germination (Gould, 1970; Ando, 1981). C. perfringens spores germinate to a greater extent when in the presence of increased concentration of Ca^{2+} , Sr^{2+} , Ba^{2+} and Mn^{2+} while Mg^{2+} decreases the amount of germination (Ando, 1981). This effect is greater with non heat-activated spores. Food composition affects the limiting range of pH and temperature allowing germination and also influences the availability and activity of germinants and germination inhibitors.

Research involving the effects of food preservatives on spore germination has been lacking. Many researchers have studied inhibition of growth from spores and have not differentiated between

inhibition of spore germination and inhibition of vegetative growth (Riemann, 1972). Knowledge of each of these processes is essential to a complete understanding of the chemical inhibition of C. botulinum since spore germination must occur prior to growth and toxin production.

MATERIALS AND METHODS

A. Preparation of the Test Organism

Clostridium botulinum type A, strain 62A was chosen since it is one of the most studied strains of C. botulinum. Spores were produced by the biphasic culture method of Anellis et al. (1972). Spores were harvested by centrifugation (5,090 X G for 10 min) and washed in 4°C sterile distilled water six times or until debris was removed. The procedure of Rowley and Feeherry (1970), employing a lysozyme-trypsin treatment, was used in order to remove cell debris from spores and to eliminate any vegetative cells. Spores were then centrifuged and washed with 4°C sterile distilled water in order to remove enzyme-digested material and to obtain a clean spore suspension. The spores were adjusted to a concentration of about 1.5×10^{10} spores/ml with sterile distilled water and stored at 3°C until use. Spores were checked periodically by phase contrast microscopy and found to contain at most 4% germinated spores.

B. Preparation of Media and Chemicals

1. GO Medium

The complex liquid medium used for all vegetative growth experiments and some germination experiments consisted of 1% Thiotone (BBL, Cockeysville, MD.), 1% yeast extract (BBL) and 0.4% glucose in 0.05 M sodium phosphate buffer. This medium was termed GO medium since it supported both germination and outgrowth. GO medium was prepared

anaerobically by boiling the ingredients for ten minutes to remove dissolved oxygen and then cooling in ice under oxygen-free CO_2 . The pH of the medium was adjusted with sodium hydroxide and hydrochloric acid while sparging with oxygen-free nitrogen. The medium was dispensed into culture tubes (18 x 150 mm; Bellco Glass, Inc., Vineland, N.J.) and stoppered under oxygen-free nitrogen and then autoclaved 15 minutes at 121°C . When G0 medium was used for germination experiments, filter sterilized solutions of sodium bicarbonate and chloramphenicol were added to a concentration of 20mM and 10 $\mu\text{g}/\text{ml}$, respectively, in the medium after autoclaving. Sodium bicarbonate enhanced the germination rate while chloramphenicol prevented outgrowth of germinated spores. Chloramphenicol had no effect upon rate or extent of germination (data not shown).

2. G Medium

Germination inhibition experiments were also done with a defined liquid medium consisting of 40 mM L-alanine, 10 mM L-lactic acid and 20 mM sodium bicarbonate in 0.08 M sodium-potassium phosphate buffer. This medium was termed G medium since it supported only germination. G medium was prepared anaerobically by boiling the buffer for ten minutes and then cooling it in ice while sparging with oxygen-free CO_2 . After cooling, the other medium constituents were added as dry chemicals and the pH was adjusted in the same manner as for G0 medium. G medium was subsequently transferred to CO_2 -filled sterilized culture tubes and stoppered using the VPI Anaerobic Culture System (Bellco Glass, Inc., Vineland, N.J.).

3. Chemicals

BHA, BHT, TBHQ and 8-OHQ (Sigma Chemical Co., St. Louis, MO and Aldrich Chemical Co., Milwaukee, Wis.) were prepared as filter sterilized (Millipore Corp., Bedford, Mass., 0.22 μm) solutions in 95% ethanol since the compounds are relatively insoluble in water. Since initial experiments revealed that ethanol was somewhat inhibitory to germination, 1% ethanol was added to all media. Adding a consistent amount of ethanol to each tube allowed only the effect of the chemical inhibitors to be observed. Ethanol at this concentration had no detectable effect on growth rate. Solutions of sodium bicarbonate and chloramphenicol were prepared as aqueous solutions and filter sterilized (0.22 μm).

C. Growth Studies

1. Conditions for growth studies

Studies were done in order to determine the inoculation procedure yielding maximum growth rates for growth inhibition experiments. The procedure determined to be optimal was used throughout the study. Approximately 4.5×10^7 spores per ml were inoculated into 10 ml of G0 medium and incubated for 6 hours. One milliliter of this culture was then transferred to another tube of G0 medium and incubated for 4 hours. One tenth milliliter was then used as inoculum for growth inhibition studies. This inoculum level was about 1×10^6 cells/ml and corresponded to an initial optical density of 0.02. Inoculation of this magnitude allowed slight increases in cell numbers to be detected.

Extent of growth was determined by monitoring the optical density at 600 nm with a Bausch and Lomb Spectronic 20 Spectrophotometer. Percent inhibition of growth was determined relative to control tubes containing 1% ethanol and no chemical after 12 hours incubation (beginning of stationary phase).

Strict anaerobic conditions were maintained throughout the study in order to prevent oxidation of medium components. Addition of chemicals and inoculations were performed anaerobically using the VPI Anaerobic Culture System. pH values were accurate to ± 0.1 unit following addition of all medium components and before inoculation. No attempt was made to determine change in pH during growth or germination experiments. All experiments were performed in duplicate and at 37°C.

2. Reversibility of growth inhibition

Experiments on the reversibility of growth inhibition by the chemicals were done with G0 medium (pH 7.2). After incubating cells in the presence of an inhibitory amount of chemical, the cells were harvested by membrane filtration (0.22 μm) and resuspended both in the presence and the absence of each chemical.

D. Germination Studies

1. Conditions for germination

Preliminary studies showed maximum activation of C. botulinum 62A spores to be from 10 to 20 minutes for 80°C. For subsequent studies, spores in screw cap tubes were heat shocked at 80°C for 15 minutes prior to each experiment and kept on ice until used.

For germination experiments, spores were added to an initial optical density of between 0.30 and 0.35 which was equivalent to about 4.5×10^7 spores per ml. Germination was monitored by the decrease in optical density at 600 nm and calculated relative to the initial O.D.. Since BHT and 8-OHQ caused changes in optical density independent of spore germination when incubated in G0 medium, germination was estimated as the percent of phase dark spores (i.e. germinated spores) viewed under phase contrast microscopy. Experiments with BHT in G medium were also treated this way. For all other experiments, optical density change was used in order to estimate extent of germination. In selected experiments, spores were examined by phase contrast microscopy in order to confirm extent of germination. Percent inhibition was calculated relative to germination observed in control tubes containing 1% ethanol and no chemical.

2. Reversibility of germination inhibition

Experiments designed to determine whether the inhibition of germination with each chemical was reversible were carried out in G medium at pH 7.2. After 120 minutes incubation in the presence of an inhibitory concentration of chemical the medium was diluted one to eight with G medium containing no chemical. This procedure was used instead of the membrane filtration procedure since the latter procedure did not allow adequate resuspension of spores. Optical density or % phase dark spores were monitored throughout the experiment.

3. Loss of heat resistance

To determine whether the inhibition of germination by BHA,

BHT, TBHQ or 8-OHQ prevented loss of spore heat resistance, experiments were performed with G medium (pH 7.2). Spores were incubated in the presence of an inhibitory amount of each chemical and 1 ml samples were pipetted at intervals into a pretempered (80°C) 99 ml phosphate-buffered dilution blank. Spores which survived 80°C for 30 min were determined to be heat resistant and were enumerated using peptone yeast extract agar (PYA) (incubated at 37°C for 48 hours). The roll tube methods of Pierson et al. (1974) were used and PYA was prepared by the methods of Holdeman et al. (1977).

RESULTS AND DISCUSSION

A. Chemicals and Vegetative Cell Growth

1. Concentration and pH

Experiments were done to determine the effect of BHA, BHT, TBHQ and 8-OHQ on C. botulinum 62A vegetative cell growth. Figure 2 shows the growth response of C. botulinum cells in the presence of increasing concentrations of TBHQ in G0 medium at pH 6.2. As the concentration of TBHQ was increased, inhibition of growth increased. Complete inhibition was observed for up to 12 hours with 250 $\mu\text{g/ml}$ TBHQ. Only TBHQ at pH 6.2 is presented since this response was typical for all chemicals and pH values, although specific inhibitory concentrations were different for each chemical. Table 2 is summary data for experiments with each chemical at each pH value. Data is presented as percent inhibition relative to control tubes of the same pH so that only the effect of chemicals is considered, and the inhibitory effect of pH is eliminated. Results show that each of the four chemicals were generally more effective as the pH was lowered from 7.2 to 5.7.

As the pH decreased from 7.2 to 5.7, the concentration of chemicals required for at least 95% inhibition of growth decreased from 60 to 40 $\mu\text{g/ml}$, 20 to 10 $\mu\text{g/ml}$, 250 to 100 $\mu\text{g/ml}$, and 20 to 10 $\mu\text{g/ml}$ for BHA, BHT, TBHQ and 8-OHQ, respectively. The enhancement of inhibitory activity with decreasing pH is in agreement with results obtained in other investigations with different microorganisms. Stern et al, (1979) found that BHA was more effective for preventing growth of

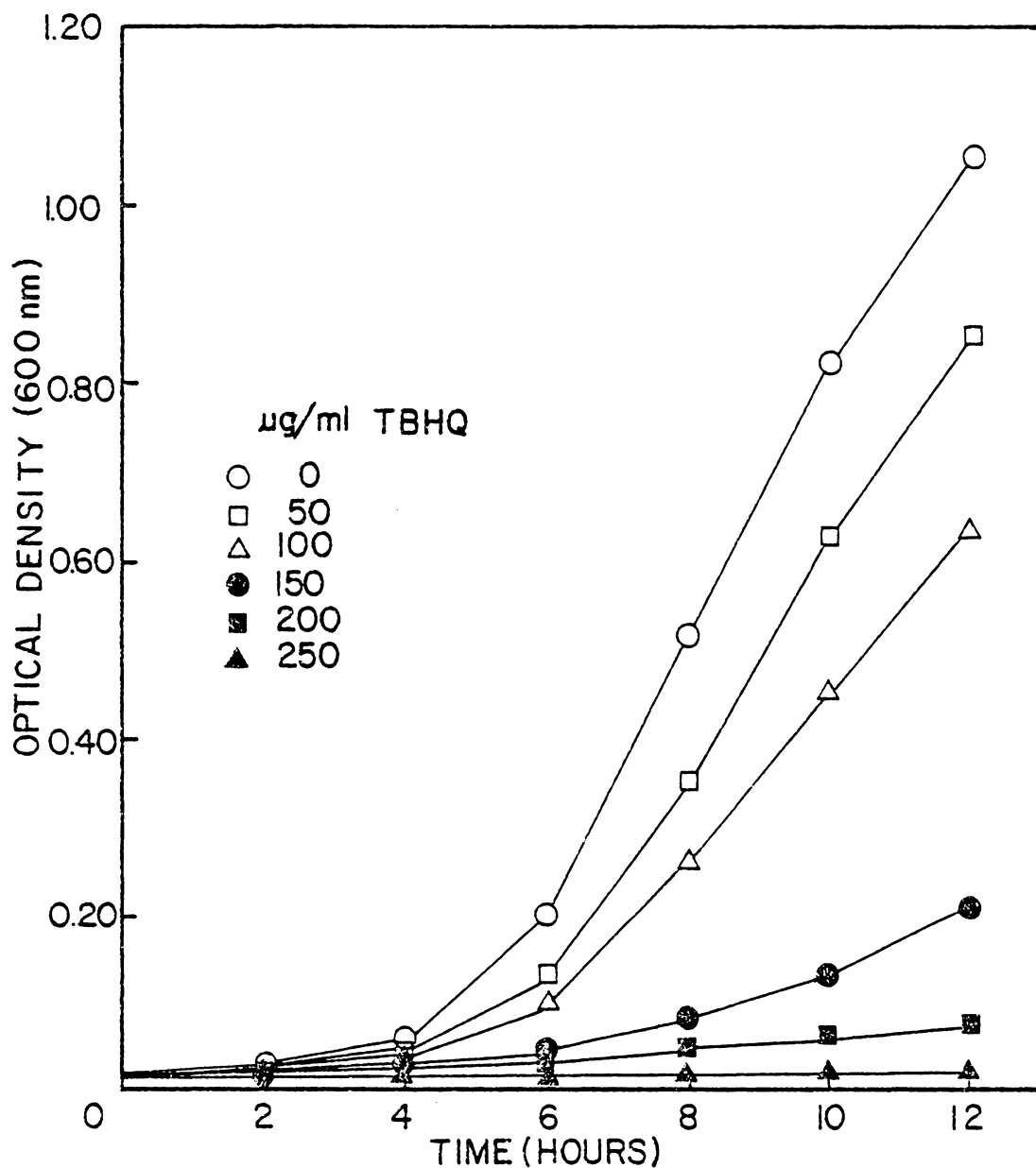


Figure 2. Growth of *C. botulinum* 62A in G0 medium (pH 6.2) at 37 C in the presence of various concentrations of TBHQ.

Table 2. Percent inhibition of growth of Clostridium botulinum 62A by various concentrations of BHA, BHT, TBHQ and 8-OHQ after 12 hours of incubation in GO medium at 37°C:^a

Chemical	Concentration (µg/ml)	pH 7.2	pH 6.7	pH 6.2	pH 5.7
BHA	20	13	9	10	23
	30	15	14	55	93
	40	30	33	99	98
	50	90	97	98	96
	60	97	97	98	98
BHT	5	5	6	74	51
	10	16	94	99	99
	15	94	98	100	97
	20	100	100	95	100
TBHQ	50	19	10	25	24
	100	40	39	77	98
	150	80	84	99	99
	200	93	97	98	98
	250	98	96	98	99
8-OHQ	10	84	93	90	96
	20	97	99	100	97

^aPercent inhibition =

$$\left(1 - \frac{\% \text{ fall in O.D.}^b \text{ in presence of chemical}}{\% \text{ fall in O.D. in absence of chemical at same pH}} \right) \times 100$$

^bMeasured at 600 nm, average of duplicate tubes.

Staphylococcus aureus as the pH was lowered. Klindworth et al., (1979) observed this with Clostridium perfringens. Other investigators found BHA and 8-OHQ to be more effective at pH 6.0 than 7.0 for preventing growth from C. botulinum spores, but that TBHQ and BHT were equally effective at these pH values after 7 days of incubation (Reddy and Pierson, 1982).

BHT and 8-OHQ were the most effective in preventing cell growth, followed by BHA and TBHQ. In general, three to four times more BHA was required than BHT for the same degree of inhibition. BHT and 8-OHQ were approximately equal in effectiveness. Ayaz et al., (1980) reported BHT to be a more effective growth inhibitor than BHA for several strains of Staphylococcus aureus. A study of growth from spores, however, showed that BHA is more effective than BHT for preventing C. botulinum out-growth and toxin production when incubated up to 4 days (Robach and Pierson, 1979). These results will be discussed in detail later (page 61).

2. Added cations

The effect of various divalent cations on the inhibition of growth by BHA, BHT, TBHQ and 8-OHQ was determined. Cations were added as chloride salts to GO medium at pH 7.2 prior to inoculation with cells. Addition of Zn^{2+} , Mg^{2+} , Sr^{2+} , Mn^{2+} and Ca^{2+} at concentrations of 100 $\mu\text{g/ml}$ had no observable effect on inhibition of growth by the chemical inhibitors. Hg^{2+} , Cu^{2+} and Co^{2+} at 100 $\mu\text{g/ml}$ were found to be inhibitory to growth and therefore their effect on growth inhibition could not be determined. Fe^{2+} , although showing no effect on the inhibition of

growth by BHA, BHT or TBHQ, had some influence on the activity of 8-OHQ. Ferrous ions appeared to decrease the effectiveness of 8-OHQ at pH 7.2. 10µg/ml 8-OHQ inhibited growth by 84% at 8 hours incubation while the combination of 100 µg/ml Fe²⁺ plus 10 µg/ml 8-OHQ resulted in only 31% inhibition. 20 µg/ml 8-OHQ inhibited growth by 97% at 8 hours when used alone, but growth inhibition decreased to 42% when cells were incubated in the presence of 20 µg/ml 8-OHQ plus 100 µg/ml Fe²⁺. The effect of iron on ability of nitrite to inhibit C. botulinum growth has also been investigated (Tompkin et al., 1978b, Tompkin et al., 1979). These investigators found that addition of iron lowered the efficiency of nitrite for C. botulinum growth inhibition. They also demonstrated increased antibotulinal activity by nitrite when EDTA was added to the growth medium. In these studies available iron was important for growth in the presence of nitrite. Addition of EDTA enhanced growth inhibition by nitrite, possibly due to removal of available iron. Results of the present study may support this theory since 8-OHQ is capable of sequestering iron (Albert et al., 1953). 8-OHQ may have chelated iron, making it unavailable for cell use. Added iron may have bound to 8-OHQ, sparing iron in the medium essential for cell growth, thus reducing inhibition of growth by 8-OHQ.

3. Reversibility of Growth Inhibition

In order to further characterize growth inhibition by each of the four chemicals, experiments were performed to determine whether the inhibition is reversible. After incubation for 12 hours in the

presence of an inhibitory amount of each chemical, cells were removed by filtration and resuspended in medium without chemical (Figures 3, 4, 5 and 6). Cells incubated with 60 $\mu\text{g}/\text{ml}$ BHA, 250 $\mu\text{g}/\text{ml}$ TBHQ or 20 $\mu\text{g}/\text{ml}$ 8-OHQ grew rapidly once the chemicals were removed, thus demonstrating reversibility of growth inhibition. Reversible growth inhibition by BHT was not demonstrated by the above procedure. In contrast to the other chemicals, cells incubated in the presence of BHT did not grow within the 12 hour incubation period once the chemical was removed. This may indicate stronger adsorption of BHT molecules to the cell membrane, penetration of BHT into the cell or irreversible damage to essential cell components by BHT.

B. Chemicals and Germination

1. Concentration and pH

Spores were incubated in GO medium with chloramphenicol in the presence of BHA, BHT, TBHQ and 8-OHQ in order to study germination inhibition. The germination response curves for TBHQ at pH 6.2 (Figure 7) represent data similar to other germination responses measured by optical density decrease. In these curves optical density values decreased more slowly for higher concentrations of each chemical. With TBHQ at pH 6.2 optical density decreased to about 60% in the absence of chemical while 200 $\mu\text{g}/\text{ml}$ TBHQ allowed only 2% decrease in optical density after the 6 hour incubation period. The results of germination inhibition experiments with varying concentrations of each chemical and pH in GO medium are shown in Tables 3, 4, 5 and 6. As the pH decreased

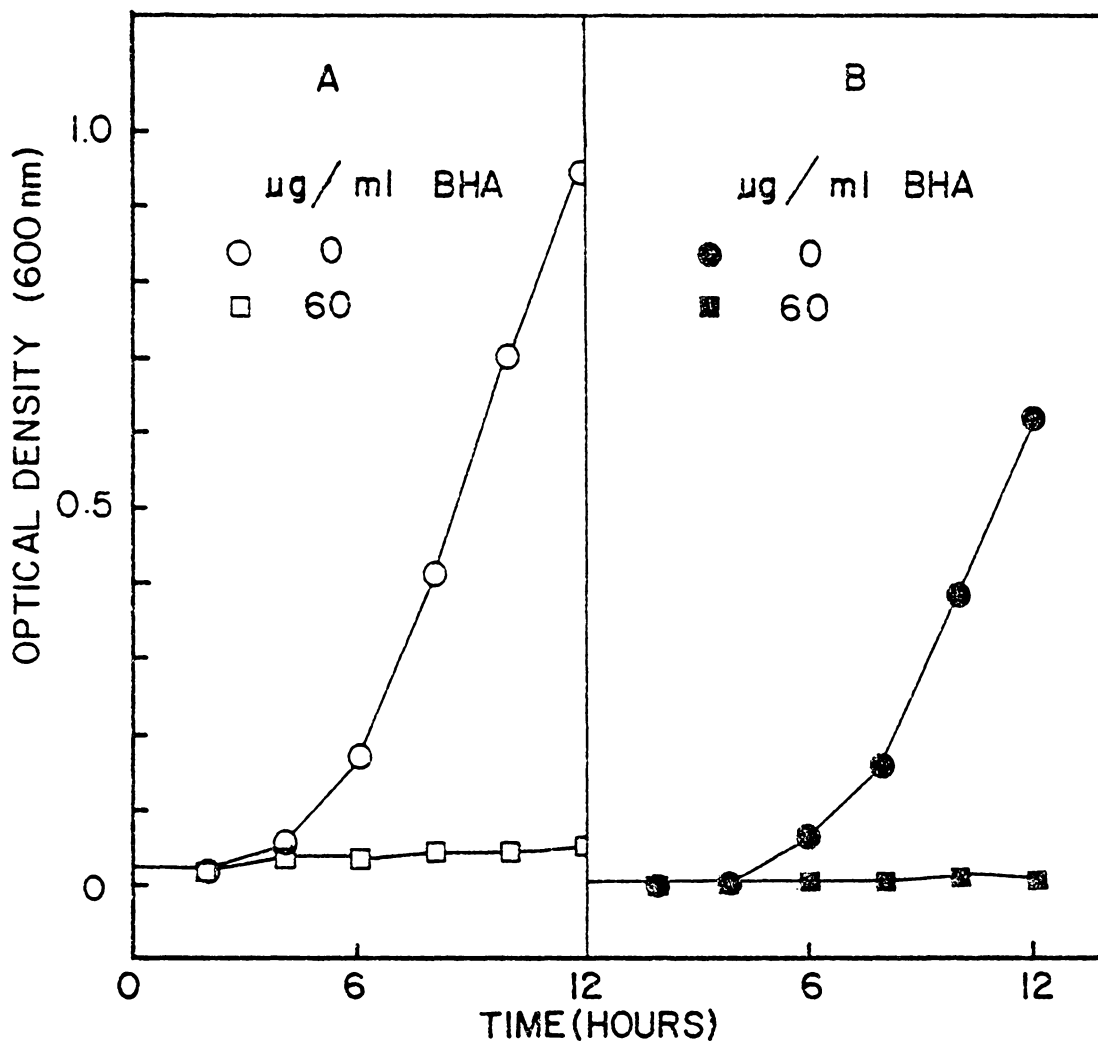


Figure 3. The reversibility of *C. botulinum* 62A growth inhibition by BHA. (A) Cells were incubated in G0 medium (pH 7.2) with or without BHA. (B) After 12 hours, the cells incubated in the presence of BHA were harvested by membrane filtration and resuspended in G0 medium (pH 7.2) with or without BHA.

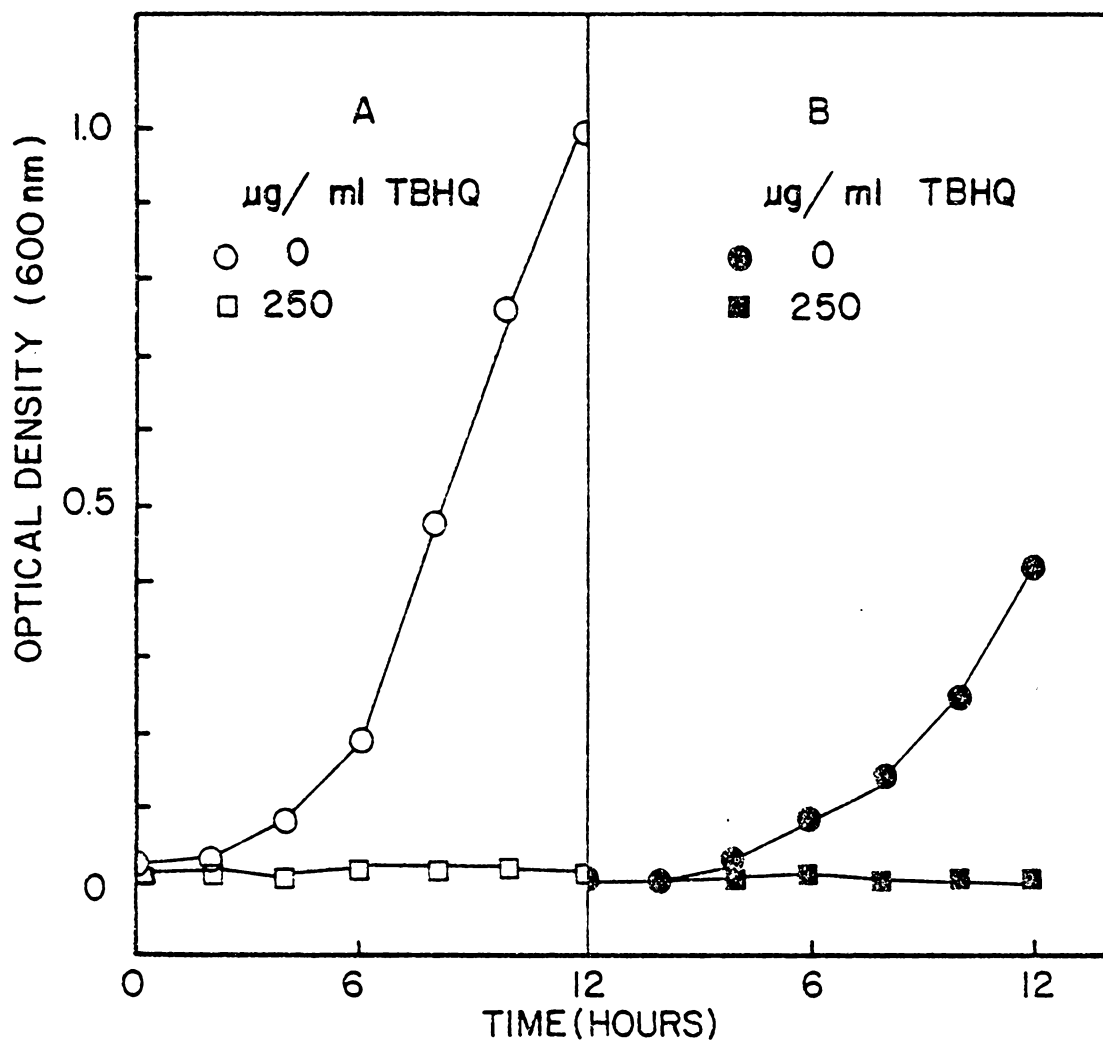


Figure 4. The reversibility of *C. botulinum* 62A growth inhibition by TBHQ. (A) Cells were incubated in G0 medium (pH 7.2) with or without TBHQ. (B) After 12 hours, the cells incubated in the presence of TBHQ were harvested by membrane filtration and resuspended in G0 medium (pH 7.2) with or without TBHQ.

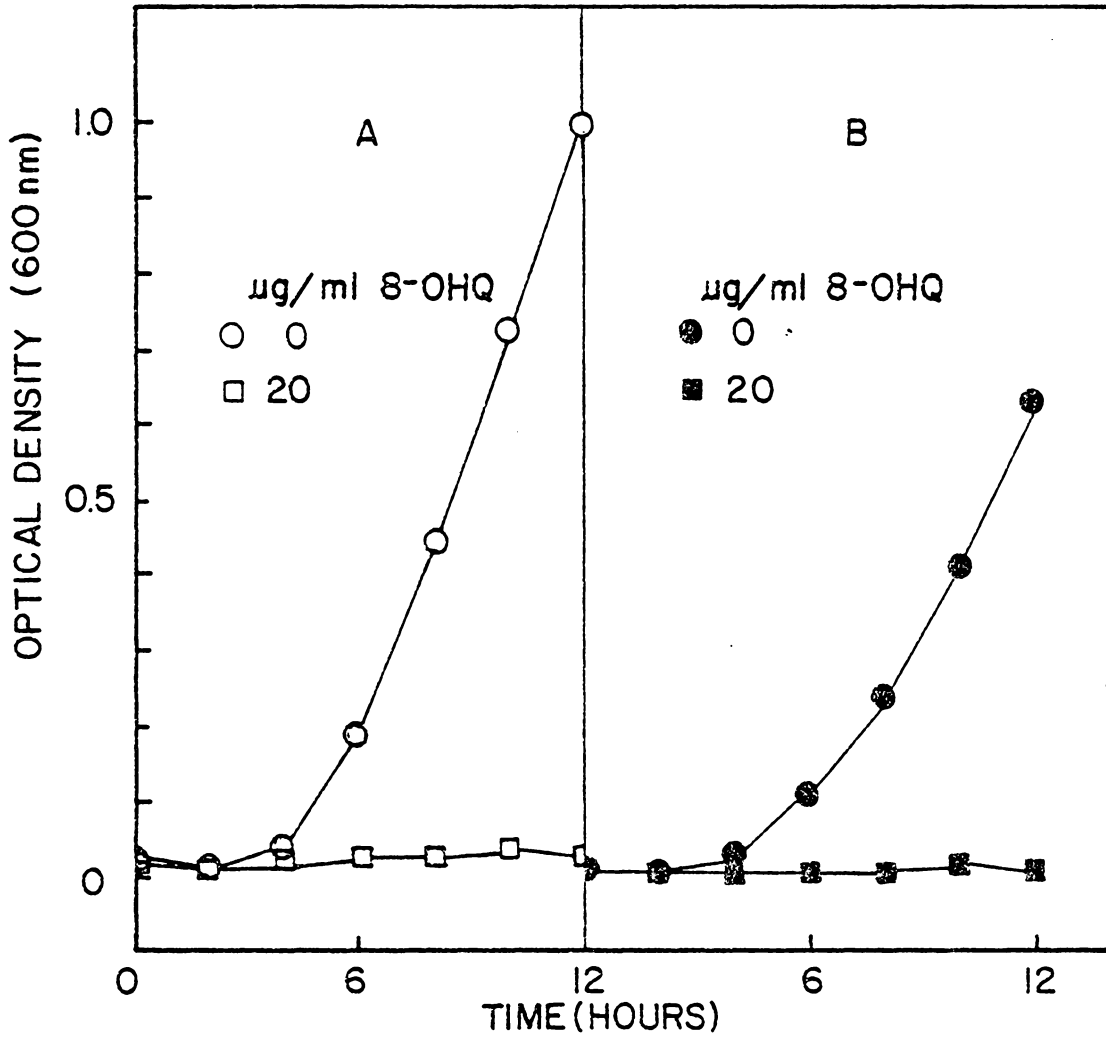


Figure 5. The reversibility of *C. botulinum* 62A growth inhibition by 8-OHQ. (A) Cells were incubated in G0 medium (pH 7.2) with or without 8-OHQ. (B) After 12 hours, the cells incubated in the presence of 8-OHQ were harvested by membrane filtration and resuspended in G0 medium (pH 7.2) with or without 8-OHQ.

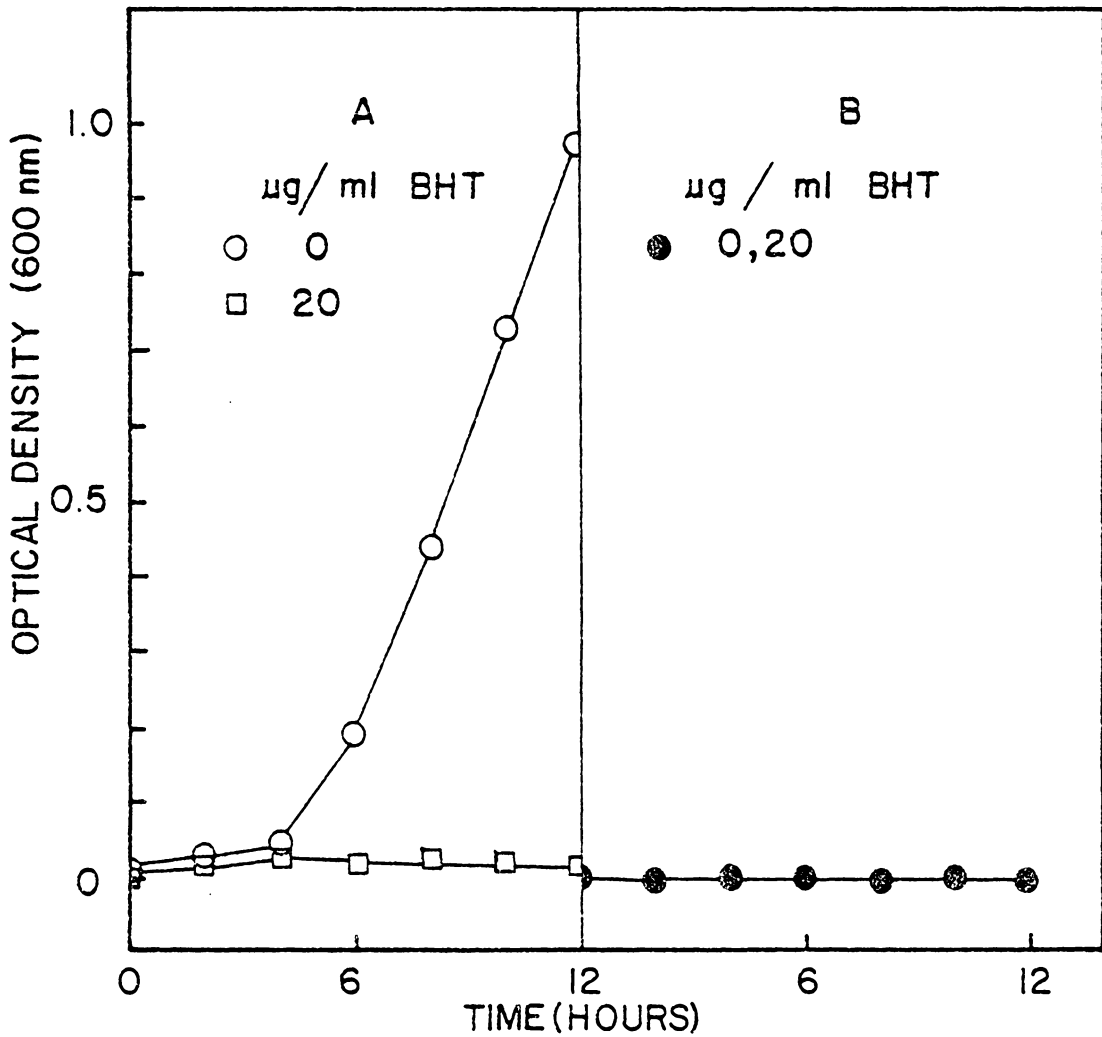


Figure 6. The reversibility of *C. botulinum* 62A growth inhibition by BHT. (A) Cells were incubated in GO medium (pH 7.2) with or without BHT. (B) After 12 hours, the cells incubated in the presence of BHT were harvested by membrane filtration and resuspended in GO medium (pH 7.2) with or without BHT.

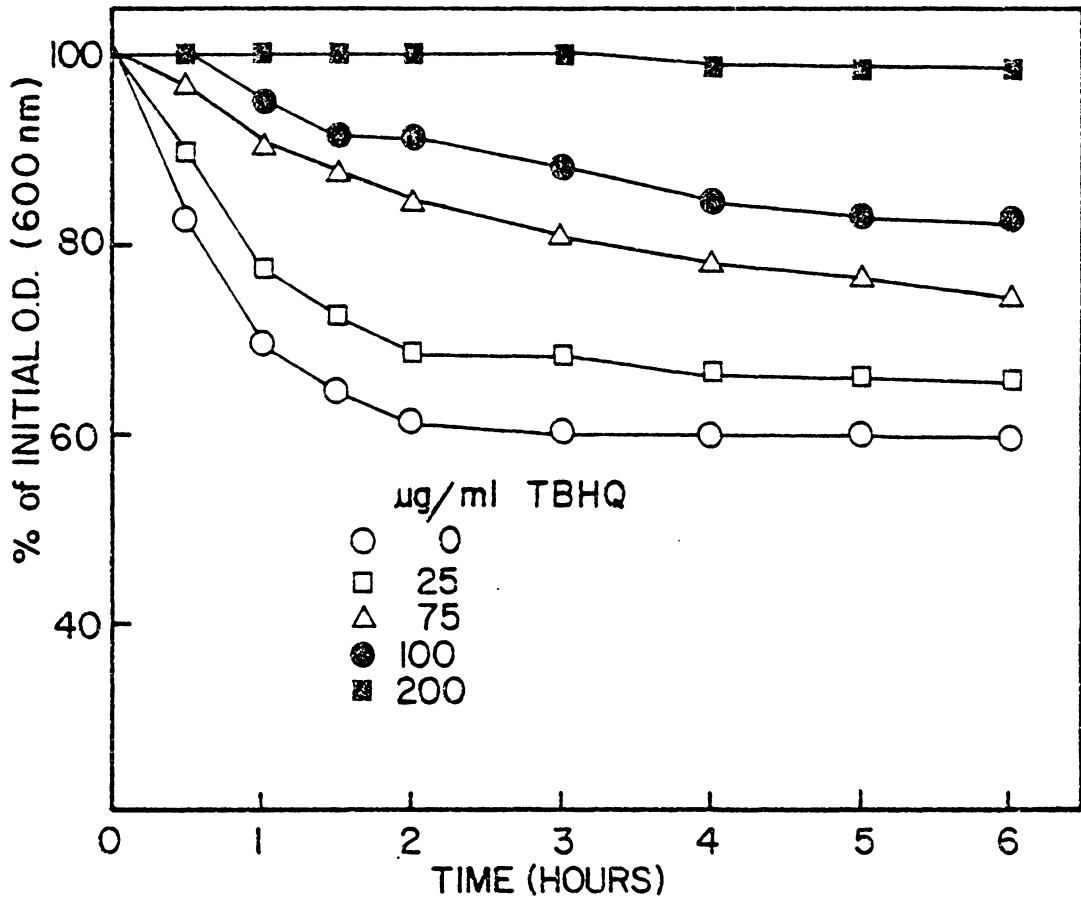


Figure 7. Germination of *C. botulinum* 62A in G0 medium (pH 6.2) in the presence of various concentrations of TBHQ.

Table 3. Effect of BHA concentration on the germination of Clostridium botulinum 62A spores in GO medium at 37°C.

pH	Concentration ($\mu\text{g/ml}$)	% Fall in O.D. ^a 2 hrs	6 hrs	% Germination ^b 6 hrs	% Inhibition ^c 2 hrs
7.2	0	41	41	99	—
	25	10	16	37	75
	50	2	4	10	95
6.7	0	38	41	64	—
	25	7	35	65	82
	50	0	2	5	100
6.2	0	39	42	97	—
	25	2	9	26	96
	50	2	3	18	96
5.7	0	27	30	95	—
	25	0	9	26	100
	50	0	2	4	100

^aMeasured at 600 nm, average of duplicate tubes.

^bPercentage of phase dark spores as determined by phase contrast microscopy.

^cCalculated as in Table 2.

Table 4. Effect of BHT concentration on the germination of Clostridium botulinum 62A spores in GO medium at 37°C.

pH	Concentration (µg/ml)	Percent Germination ^a		Percent Inhibition ^b 2 hrs
		2 hrs	6 hrs	
7.2	0	100	100	—
	25	59	93	41
	50	57	85	43
	100	49	89	51
	400	35	81	65

6.7	0	96	100	—
	25	35	85	64
	50	37	87	62
	100	36	69	62

6.2	0	79	95	—
	25	24	62	70
	50	31	74	61
	100	32	76	60
	400	24	65	70

5.7	0	64	91	—
	25	50	69	22
	50	22	35	66
	100	24	52	62
	400	21	40	67

^aPercentage of phase dark spores as determined by phase contrast microscopy.

^b% Inhibition =

$$\left(1 - \frac{\% \text{ germination in presence of chemical}}{\% \text{ germination in absence of chemical at same pH}} \right) \times 100$$

Table 5. Effect of TBHQ concentration on the germination of Clostridium botulinum 62A spores in G0 medium at 37°C.

pH	Concentration ($\mu\text{g/ml}$)	% Fall in O.D. ^a 2 hrs	6 hrs	% Germination ^b 6 hrs	% Inhibition ^c 2 hrs
7.2	0	45	45	100	—
	25	33	40	97	26
	75	28	37	91	38
	100	15	30	80	66
	200	3	18	36	93
	300	0	0	7	100
6.7	0	49	49	97	—
	25	33	42	64	32
	75	34	41	82	31
	100	19	30	52	62
	200	5	10	11	90
	300	3	5	4	94
6.2	0	38	38	97	—
	25	30	33	78	20
	75	15	25	48	60
	100	8	17	45	77
	200	0	2	5	100
	300	0	2	1	100
5.7	0	24	29	89	—
	75	8	13	42	66
	100	0	3	12	100
	200	2	2	4	94
	300	0	0	2	100

^a Measured at 600 nm, average of duplicate tubes.

^b Percentage of phase dark spores as determined by phase contrast microscopy.

^c Calculated as in Table 2.

Table 6. Effect of 8-OHQ concentration on the germination of Clostridium botulinum 62A spores in G0 medium at 37°C.

pH	Concentration ($\mu\text{g/ml}$)	Percent Germination ^a		Percent Inhibition ^b
		2 hrs.	6 hrs	2 hrs
7.2	0	97	99	—
	50	71	95	27
	100	54	68	44
	200	5	12	95

6.7	0	94	99	—
	50	53	93	44
	100	14	53	85
	200	2	30	98

6.2	0	82	96	—
	50	62	83	24
	100	4	0	95

5.7	0	60	88	—
	50	50	51	17
	100	4	9	93
	200	0	2	100

^aPercentage of phase dark spores as determined by phase contrast microscopy.

^bCalculated as in Table 4.

from 7.2 to 5.7 concentrations of BHA, TBHQ and 8-OHQ necessary for 90% or more inhibition of germination decreased from 50 to 25 $\mu\text{g/ml}$, 200 to 100 $\mu\text{g/ml}$ and 200 to 100 $\mu\text{g/ml}$, respectively. BHT was much less effective than the other chemicals. Concentrations of BHT up to 400 $\mu\text{g/ml}$ did not inhibit germination more than 70%, possibly due to the low solubility of BHT at these levels in aqueous media.

2. Inhibition in G medium

In order to study germination inhibition by the chemicals in a medium that would support only germination, germination experiments were also done with G medium. This medium included only factors essential for maximum germination rate. BHA and TBHQ (Tables 7 and 8) inhibited spore germination by more than 90% at concentrations above 50 and 200 $\mu\text{g/ml}$, respectively, at pH 7.2. These concentrations were similar to those for GO medium experiments. BHT, as in GO medium experiments, failed to prevent C. botulinum spores from germinating (data not presented). Up to 400 $\mu\text{g/ml}$ BHT allowed at least 30% germination at each pH tested. 8-OHQ (Table 9), however, was capable of stronger inhibition in G medium than in GO medium. At each pH tested, 75 $\mu\text{g/ml}$ 8-OHQ was capable of greater than 90% inhibition of germination in G medium, while 100 to 200 $\mu\text{g/ml}$ 8-OHQ was required for similar inhibition in GO medium (Table 6). This may be due to germination-promoting factors such as amino acids, cations or glucose which were absent from G medium, but present in GO medium.

3. Added cations

Concentration of divalent cations is known to influence spore

Table 7. Effect of BHA concentration on the germination of Clostridium botulinum 62A spores in G Medium at 37°C.

pH	Concentration ($\mu\text{g/ml}$)	Percent Fall in O.D. ^a 2 hrs	Percent Inhibition ^b 2 hrs.
7.2	0	41	—
	25	21	49
	50	2	96

6.7	0	42	—
	25	15	63
	50	0	100

6.2	0	3	—
	25	3	92
	50	0	100

5.7	0	26	—
	25	2	94
	50	1	95

^aMeasured at 600 nm, average of duplicate tubes.

^bCalculated as in Table 2.

Table 8. Effect of TBHQ concentration on the germination of Clostridium botulinum 62A spores in G medium at 37°C.

pH	Concentration ($\mu\text{g/ml}$)	Percent Fall in O.D. ^a 2 hrs	Percent Inhibition ^b 2 hrs
7.2	0	41	—
	25	34	16
	75	21	49
	100	9	78
	200	3	92

6.7	0	42	—
	25	34	18
	75	20	52
	100	10	76
	200	4	90

6.2	0	34	—
	25	21	38
	50	10	71
	75	4	87
	100	1	96

5.7	0	31	—
	25	14	55
	50	6	82
	75	6	80
	100	3	90

^a Measured at 600 nm, average of duplicate tubes.

^b Calculated as in Table 2.

Table 9. Effect of 8-OHQ concentration on the germination of Clostridium botulinum 62A spores in G medium at 37°C.

pH	Concentration ($\mu\text{g/ml}$)	Percent Fall in O.D. ^a 2 hrs	Percent Inhibition ^b 2 hrs
7.2	0	41	—
	25	38	8
	50	27	35
	75	0	100
	100	0	100
6.7	0	43	—
	25	39	9
	50	21	51
	75	3	94
6.2	0	34	—
	25	27	22
	50	5	85
	75	0	100
5.7	0	31	—
	25	20	35
	50	8	74
	75	3	91

^aMeasured at 600 nm, average of duplicate tubes.

^bCalculated as in Table 2.

germination (Gould, 1970). Chloride salts of various cations were therefore added to germination media in order to determine their effect on inhibition by the chemicals. Addition of 100 $\mu\text{g/ml}$ Zn^{2+} , Co^{2+} , Mg^{2+} , Cu^{2+} , Sr^{2+} , Mn^{2+} or Ca^{2+} did not affect the rate of germination in G medium at pH 7.2 in the presence of 100 $\mu\text{g/ml}$ 8-OHQ, 50 $\mu\text{g/ml}$ BHA, 25 $\mu\text{g/ml}$ BHT, 200 $\mu\text{g/ml}$ TBHQ or no chemical (data not shown). Although 100 $\mu\text{g/ml}$ Fe^{2+} did not affect inhibition of germination by BHA, BHT, or TBHQ there was an apparent reduction in the inhibitory effect of 8-OHQ. 8-OHQ at 100 $\mu\text{g/ml}$ inhibited germination by 100% while spores incubated in the presence of 100 $\mu\text{g/ml}$ 8-OHQ plus 100 $\mu\text{g/ml}$ Fe^{2+} (1:0.38 Fe^{2+} , 8-OHQ ratio) germinated by 22% as determined by phase contrast microscopy. The effect of iron on inhibition of germination by 8-OHQ may be due to the ability of 8-OHQ to chelate iron as discussed earlier.

4. Loss of heat resistance

Experiments were conducted to determine whether the inhibition of germination prevented loss of heat resistance. Loss of heat resistance is an early event in the germination process and is known to precede a decrease in optical density and loss of refractility (phase brightness) (Hsieh and Vary, 1975). Results are presented in Figure 8 for BHA, TBHQ and 8-OHQ. Spores incubated in the absence of inhibitor germinated and lost heat resistance while spores incubated in the presence of 50 $\mu\text{g/ml}$ BHA, 200 $\mu\text{g/ml}$ TBHQ or 200 $\mu\text{g/ml}$ 8-OHQ did not germinate and retained heat resistance. The prevention of loss of heat resistance with BHT was less clear (data not shown) due to the inability of BHT to inhibit germination as effectively as the other

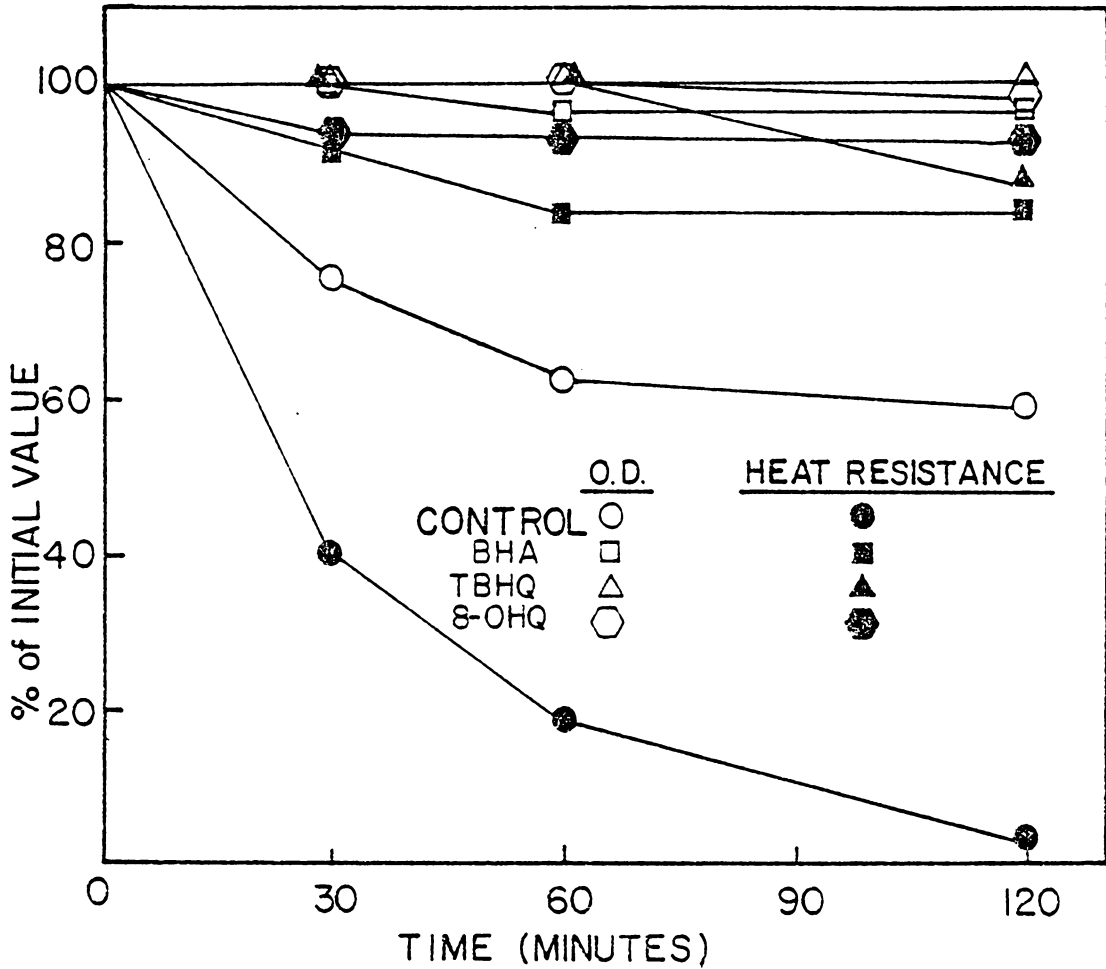


Figure 8. Effect of BHA, TBHQ and 8-OHQ on the germination of *C. botulinum* 62A spores in G medium at pH 7.2. Germination was measured as both the number of spores remaining heat resistant and fall in O.D. (600 nm). Concentrations were 50 $\mu\text{g}/\text{ml}$ BHA, 200 $\mu\text{g}/\text{ml}$ TBHQ and 200 $\mu\text{g}/\text{ml}$ 8-OHQ.

chemicals. Spores in the presence of 25 µg/ml BHT, however, showed a decreased loss of heat resistance relative to control tubes containing no chemical. Therefore, all four chemicals were found to prevent loss of heat resistance, indicating that they block germination at an early step.

5. Reversibility of germination inhibition

Reversibility of germination inhibition was studied with G medium at pH 7.2 for each of the chemicals. After spores were incubated in the presence of an inhibitory concentration of chemical the medium was diluted with G medium containing no chemical. For each chemical, dilution (reduction in concentration) allowed rapid and complete germination (Figures 9, 10, 11 and 12). Although BHT did not inhibit spore germination to the same degree as BHA, TBHQ or 8-OHQ, dilution with medium without BHT allowed rapid germination whereas dilution with medium containing BHT continued to prevent maximum germination. Thus, reversibility of germination inhibition was demonstrated for all four chemicals, indicating no permanent injury affecting spore germination. Other workers have demonstrated reversible inhibition of Bacillus spore germination by hydrophobic compounds such as alcohols and chlorocresol (Parker, 1969; Trujillo and Laible, 1970; Watanabe and Takesue, 1976).

C. Theories for Inhibition of Growth and Germination

1. Growth inhibition

Since vegetative cell growth is a very complicated process there are many theories possible for the mechanism of its inhibition by the

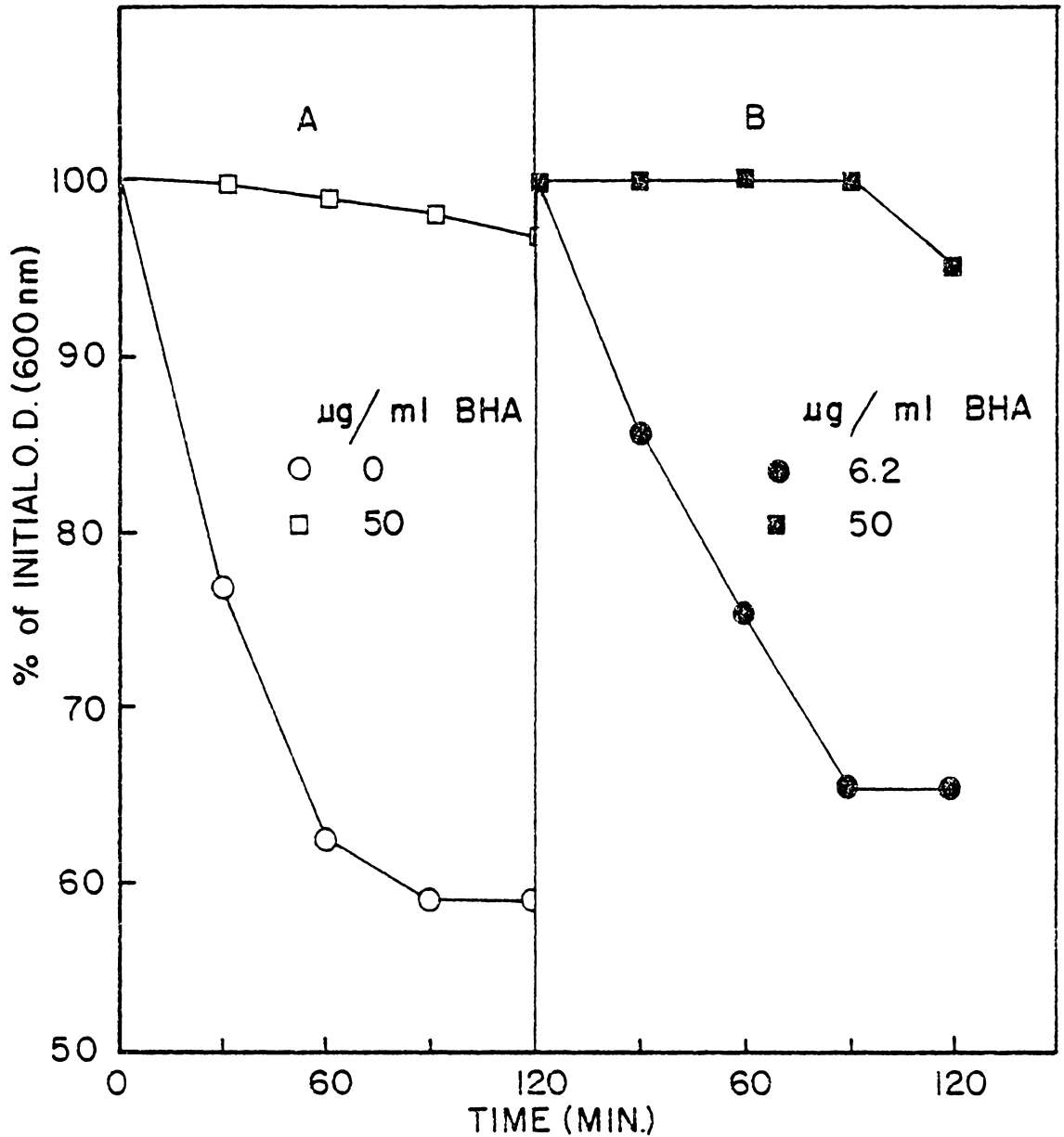


Figure 9. The reversibility of *C. botulinum* 62A spore germination inhibition by BHA. (A) Spores were incubated in G medium (pH 7.2) with or without BHA. (B) After 120 min, the spore suspension incubated with BHA was diluted one to eight with G medium (pH 7.2) with or without BHA.

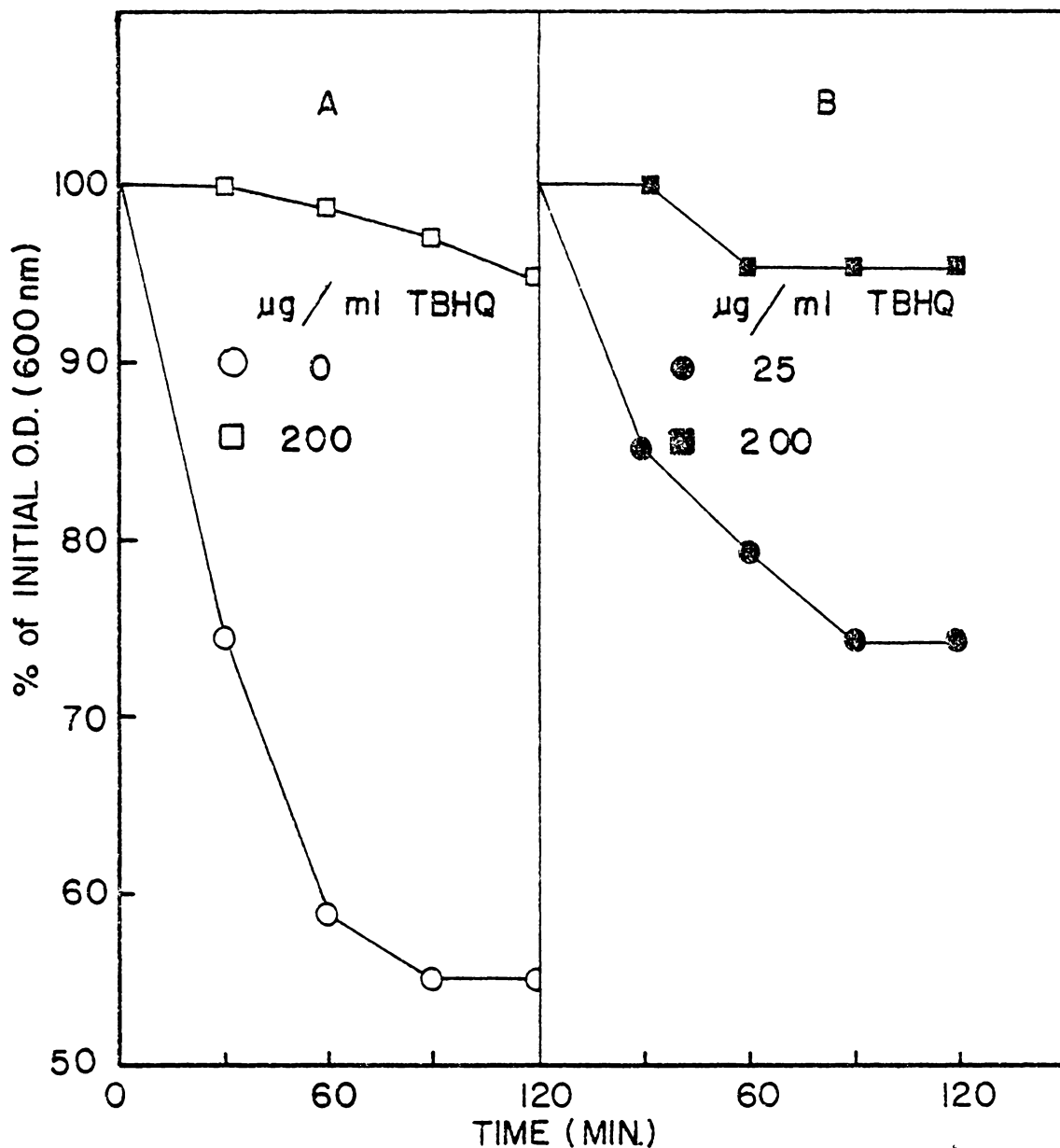


Figure 10. The reversibility of *C. botulinum* 62A spore germination inhibition by TBHQ. (A) Spores were incubated in G medium (pH 7.2) with or without TBHQ. (B) After 120 min, the spore suspension incubated with TBHQ was diluted one to eight with G medium (pH 7.2) with or without TBHQ.

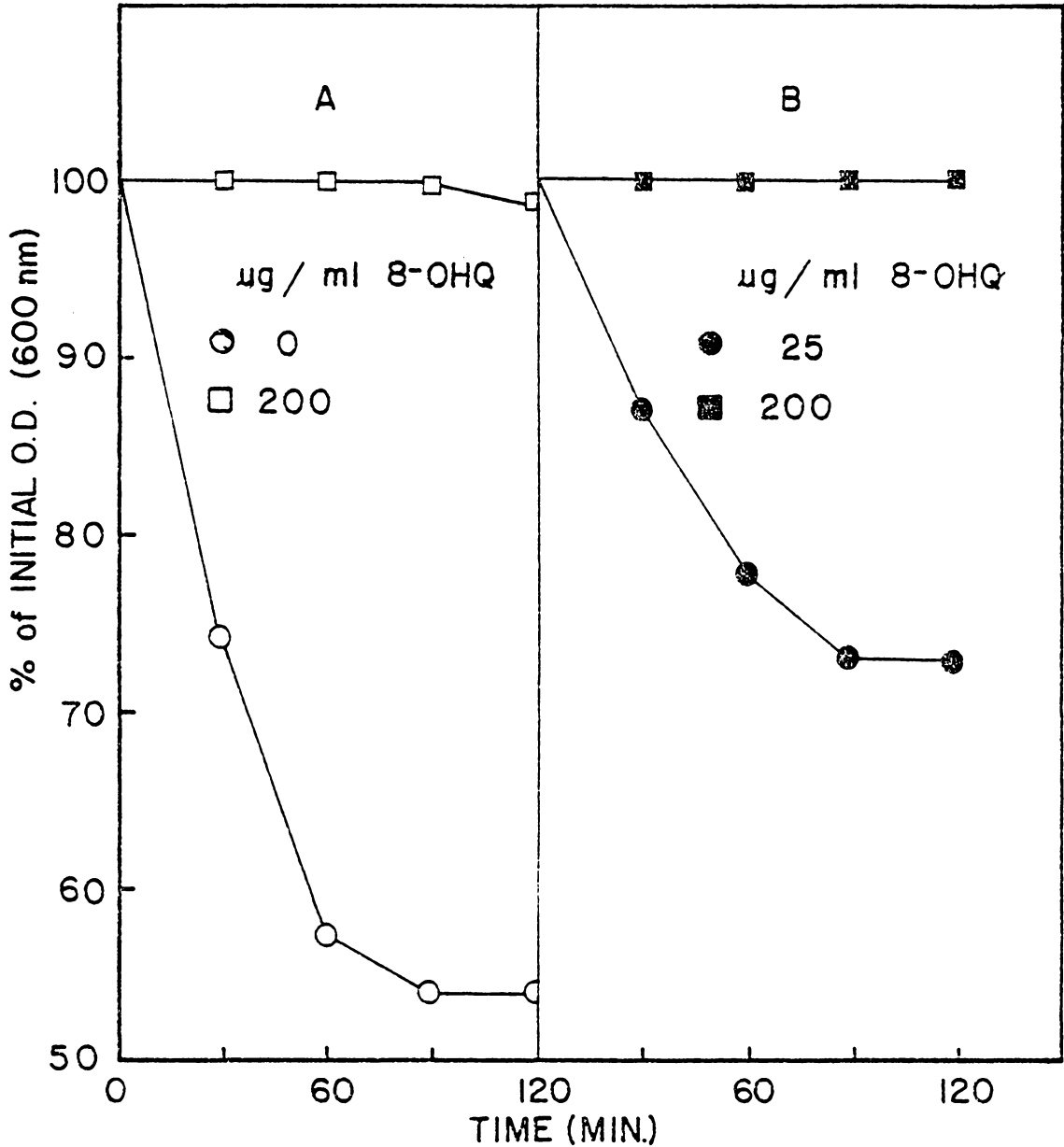


Figure 11. The reversibility of *C. botulinum* 62A spore germination inhibition by 8-OHQ. (A) Spores were incubated in G medium (pH 7.2) with or without 8-OHQ. (B) After 120 min, the spore suspension incubated with 8-OHQ was diluted one to eight with G medium (pH 7.2) with or without 8-OHQ.

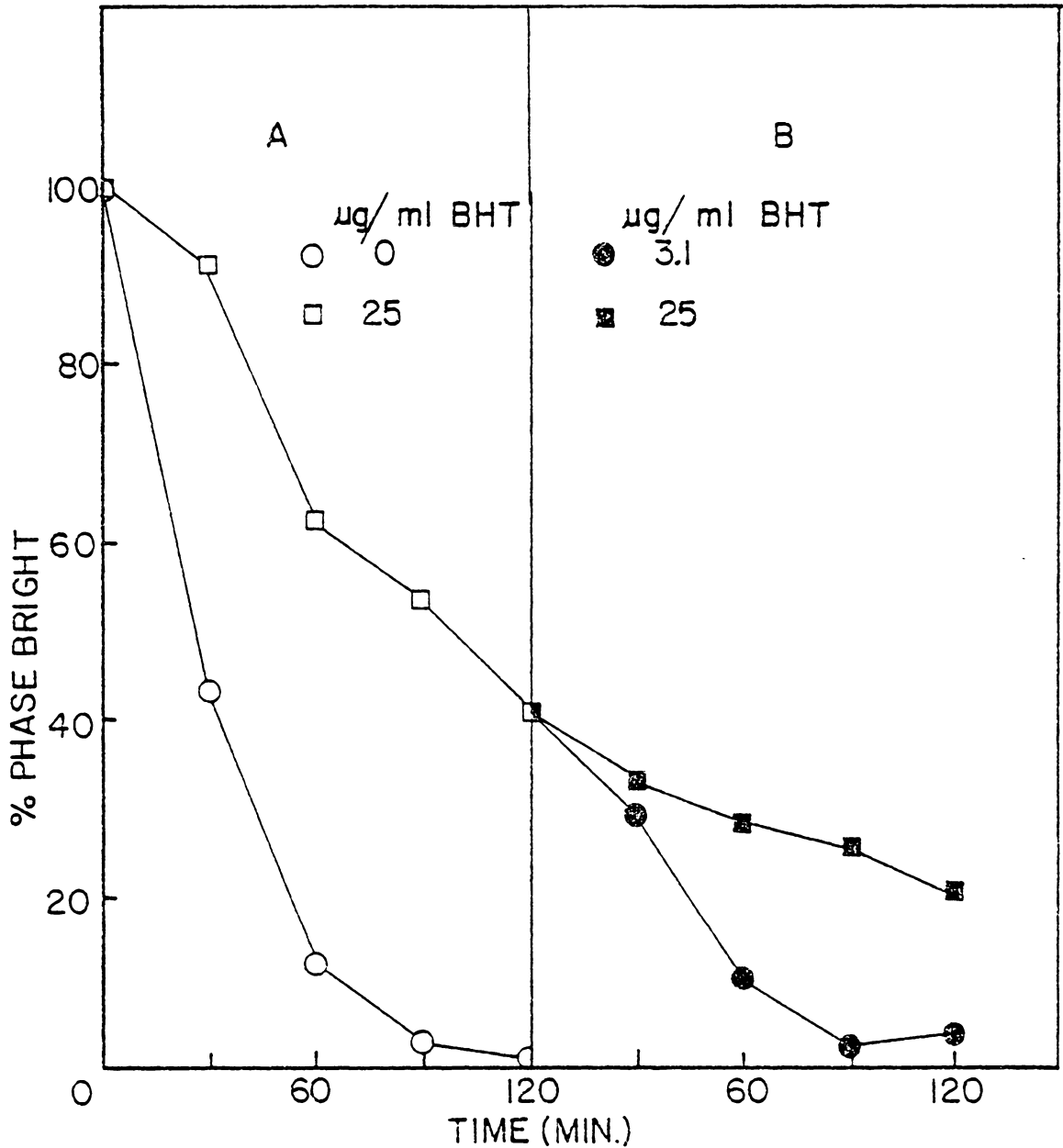


Figure 12. The reversibility of *C. botulinum* 62A spore germination inhibition by BHT. Germination was measured as % of phase bright (ungerminated) spores. (A) Spores were incubated in G medium (pH 7.2) with or without BHT. (B) After 120 min, the spore suspension incubated with BHT was diluted one to eight with G medium (pH 7.2) with or without BHT.

chemicals tested. The compounds studied in this report, BHA, BHT, TBHQ and 8-OHQ are similar in their hydrophobic character and reducing ability while 8-OHQ has the ability to chelate metals. These properties may be responsible for their antimicrobial activity.

Repression of growth may be due to interference with lipid arrangement within the cell membrane due to the hydrophobic nature of the four chemicals. This could result in leakage of vital cellular components. Some investigators have found this phenomenon to occur with BHA and BHT in Pseudomonas and Tetrahymena (Davidson and Branen, 1980a, 1980b; Surak et al., 1976).

The phenolic nature of BHA, BHT, TBHQ and 8-OHQ may allow intercalation with cell membrane lipids. Therefore, the compounds may be able to incorporate into the cell membrane and interfere with cellular transport mechanisms or enzyme systems essential for cell function.

Surak (1977) reported that BHA and TBHQ appear to inhibit DNA, RNA and protein synthesis in a species of protozoa. Thus, the phenolic compounds could penetrate the cell membrane and interfere with enzymes necessary for the expression of genetic information inside the cell.

BHA, BHT, TBHQ and 8-OHQ may react with hydrophobic active or allosteric sites on enzymes exposed on the outside of microbial cells in such a way that they are prevented from functioning. Since hydrophobic bonding is a weak interaction such inhibition would be expected to be reversible. This theory is supported by the fact that BHA, TBHQ and 8-OHQ inhibit C. botulinum reversibly.

Gardner (1977) suggested that since chelating agents are known

to inactivate thiol enzymes and coenzymes, the antibacterial activity of 8-OHQ may be related to chelating properties. Winarno et al., (1971) found another chelating agent, EDTA, is capable of inhibiting germination of C. botulinum spores. Of the eight hydroxyquinoline isomers only 8-OHQ has the ability to chelate and is also the only isomer to prevent bacterial growth (Gardner, 1977). 8-OHQ may prevent growth of C. botulinum by depriving it of available iron or by sequestering iron in reactive enzyme sites.

Albert et al., (1953) proposed that the lethal action of 8-OHQ is dependent upon a balanced ratio between amounts of 8-OHQ and iron present in the medium. They proposed that the 1:2 iron chelate of 8-OHQ could penetrate the cell membrane where it could then dissociate into a 1:1 chelate and free 8-OHQ. The 1:1 chelate could then inactivate enzymes by combining with metal-binding sites inside the cell, and the free 8-OHQ could inactivate enzymes by binding metallic prosthetic groups. It has been found that the antibacterial activity of 8-OHQ is decreased with insufficient iron (Rubbo et al., 1950). Pierson and Reddy (1982) found 8-OHQ to inhibit outgrowth of C. botulinum more effectively in comminuted pork than in a liquid laboratory medium. The enhanced activity of 8-OHQ in pork may have been due to additional iron present. Fe^{2+} and 8-OHQ in a ratio of 1:0.38 added to GO medium inhibited C. botulinum growth less than when 8-OHQ alone was added (this work). Since the addition of iron was shown to decrease antibacterial activity of 8-OHQ it appears that a balance of iron and 8-OHQ concentrations may be important for optimum inhibition of growth. Some iron may be

necessary for antibacterial activity of 8-OHQ, but too much may reduce activity. Added iron may decrease the activity of 8-OHQ by reducing the concentration of free 8-OHQ or the 1:1 iron chelate of 8-OHQ, and favoring the 1:2 chelate more.

2. Germination inhibition

The ability of BHA, BHT and TBHQ to inhibit germination of spores may be related to their antioxidant function. These antioxidants may donate electrons to surface receptors and in some way impair germinant receptor function. This theory is supported by the fact that BHA is more effective than BHT as an antioxidant and is also more effective in preventing germination, as demonstrated in this study.

It has been suggested that reductive cleavage of disulfide bonds in the spore coat proteins is involved in the process of spore germination (Vinter, 1961). BHA, BHT and TBHQ may, by virtue of their reducing power, break these disulfide bonds and thus gain entrance to hydrophobic regions inside the spore coat. This theory involves both the reducing character and the hydrophobic character of the chemicals.

As with vegetative cell inhibition, BHA, BHT, TBHQ and 8-OHQ may, because of their phenolic nature, react with hydrophobic active sites on the spore coat in order to inhibit spore germination. These sites could be enzymatic or allosteric in nature. The sites could also be active in the physical triggering of germination. Binding of the chemicals to these sites may prevent germinants such as L-alanine from reacting and thus prevent germination. This theory is supported by the fact that several hydrophobic compounds have been found to inhibit

spore germination (Parker and Bradley, 1968; Parker, 1969; Russell et al., 1979 Schmidt, 1955; Russell, 1965; Trujillo and Laible, 1970; Foster and Wynne, 1948; Watanabe and Takesue, 1976). Inhibition caused by hydrophobic attractions would be expected to be reversible. Supporting this theory is the finding in this study that inhibition by BHA, BHT, TBHQ and 8-OHQ is reversible in nature and that other hydrophobic compounds capable of germination inhibition have also been found to be reversible (Parker, 1969; Trujillo and Laible, 1970; Watanabe and Takesue, 1976).

It was mentioned earlier (page 35) that studies involving growth from spores have found BHA to be more inhibitory than BHT (Robach and Pierson, 1979) while others have found BHT to be more inhibitory than BHA toward growth of other organisms (Ayaz et al., 1980). Results presented here indicate that this is due to the ability of BHA to inhibit germination more effectively than BHT while BHT is more effective than BHA in vegetative growth inhibition. The above phenomenon indicates that the inhibition of growth of cells occurs by a different mechanism than the inhibition of germination. BHT and 8-OHQ were found to be more effective inhibitors of growth while BHA and TBHQ each inhibited germination and growth to the same extent. Although pH change affected the inhibition of growth and germination in a similar way, effectiveness of the chemicals differed from the two types of inhibition.

SUMMARY AND CONCLUSIONS

The inhibition of Clostridium botulinum 62A cell growth and spore germination by BHA, BHT, TBHQ, and 8-OHQ was studied. Growth inhibition experiments were done with a complex liquid medium (GO medium) that supported both germination and outgrowth. When GO medium was used for germination experiments sodium bicarbonate and chloramphenicol were added to concentrations of 20 mM and 10 µg/ml, respectively, in order to enhance germination rate and prevent outgrowth of germinated spores. Germination inhibition experiments were done using GO medium and a defined liquid medium composed of 40 mM L-alanine, 10 mM L-lactic acid and 20 mM sodium bicarbonate in 0.08 M sodium-potassium phosphate buffer.

Cell growth was monitored by increase in optical density (600 nm) and germination was monitored either by optical density decrease (600 nm) or by increase in percent phase dark spores viewed under phase contrast microscopy.

Results showed that as the concentration of each chemical was increased the rate of germination or growth decreased at pH values of 7.2 to 5.7. The effectiveness of BHA, BHT, TBHQ, and 8-OHQ in the inhibition of growth or germination increased as the pH was lowered from 7.2 to 5.7. This held true for each concentration of each chemical tested. Overall, the chemicals showed effective inhibition of C. botulinum germination and growth over a wide pH range, although BHT did not inhibit germination by more than 70% at any concentration or pH

tested. BHT and 8-OHQ were found to be more effective inhibitors of growth than of germination while BHA and TBHQ appeared to inhibit these two processes to the same extent. At pH 7.2 50 $\mu\text{g/ml}$ BHA, 200 $\mu\text{g/ml}$ TBHQ and 200 $\mu\text{g/ml}$ 8-OHQ were the minimum concentrations necessary for inhibition of germination by at least 90%. BHT at 400 $\mu\text{g/ml}$ failed to inhibit germination to this degree, possibly due to its low solubility in aqueous media at this concentration. Minimum concentrations at which growth at pH 7.2 was inhibited by at least 95% were 60 $\mu\text{g/ml}$ BHA, 20 $\mu\text{g/ml}$ BHT, 250 $\mu\text{g/ml}$ TBHQ and 20 $\mu\text{g/ml}$ 8-OHQ.

The inhibition of germination by 50 $\mu\text{g/ml}$ BHA, 400 $\mu\text{g/ml}$ BHT, 250 $\mu\text{g/ml}$ 8-OHQ and 250 $\mu\text{g/ml}$ TBHQ was found to block the loss of spore heat resistance, indicating inhibition of germination at an early point in the germination process. The inhibition of growth and germination was shown to be readily reversible for each chemical with the exception of the inhibition of growth by BHT. When 20 $\mu\text{g/ml}$ BHT was removed from the presence of cells after 12 hours incubation no growth was apparent for at least 12 hours after resuspension in the same medium without chemical. Iron was found to influence the ability of 8-OHQ to inhibit growth and germination. Added iron decreased the effectiveness of 8-OHQ, which demonstrates the importance of medium constituents to the inhibition of C. botulinum growth and germination.

BHA, BHT, TBHQ, and 8-OHQ show potential application in food systems as antimicrobials. The best methods for application must be determined in order to potentiate their activity. Spraying, dipping, soaking, pumping, and direct formulation are possibilities for some

food products. Pre-processing and post-processing application of these chemicals should also be studied. Ahmad (1979) found that by spraying BHA in a propylene glycol and water solution onto processed cheese there was a decrease from 400 to 150 $\mu\text{g/g}$ necessary in order to prevent mold growth for seven days. Russell and Loosemore (1964) found that heat resistance of Bacillus spores was decreased when phenol was applied prior to heating. Although there are reports that the presence of lipid decreases the effectiveness of BHA (Ahmad, 1979; Robach et al., 1977; Klindworth, et al., 1979), Pierson and Reddy (1982) found that 8-OHQ more effectively inhibited growth from C. botulinum spores in a meat system than in a liquid synthetic medium. They applied 8-OHQ to the meat as a formulation ingredient.

More work needs to be done in order to determine the effect of BHA, BHT, TBHQ, and 8-OHQ on botulinal inhibition in combination with such compounds as sodium chloride, sugar, acidulants and other potential botulinal inhibitors such as the parabens, nisin, potassium sorbate and hypophosphite.

The information reported here shows that BHA, BHT, TBHQ and 8-OHQ are effective inhibitors of growth from C. botulinum 62A spores. They are able to suppress both germination of spores and growth of cells over a wide range of pH in low concentrations. BHA, BHT and TBHQ have greater potential since they are already listed as GRAS. The results presented in this report are important to the development of antimicrobial applications for the preservation of foods as well as to the further study of potential inhibitors of Clostridium botulinum.

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THE INHIBITION OF GERMINATION AND GROWTH
OF CLOSTRIDIUM BOTULINUM 62A BY
BHA, BHT, TBHQ AND 8-HYDROXYQUINOLINE

by

Frederick K. Cook

(ABSTRACT)

The effect of butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary-butyl hydroquinone (TBHQ) and 8-hydroxyquinoline (8-OHQ) upon cell growth and spore germination of Clostridium botulinum type 62A was studied using a complex liquid medium. The inhibition of spore germination was further investigated using a defined liquid medium containing L-alanine, L-lactic acid and NaHCO_3 .

Cell growth was monitored by optical density (600 nm) increase and germination was monitored either by optical density (600 nm) decrease or by increase in percent phase dark spores viewed under phase contrast microscopy. Strict anaerobic conditions were maintained throughout the study.

As the concentration of each chemical was increased the rate of germination or growth decreased. This occurred for each pH tested. BHA, BHT, TBHQ and 8-OHQ were more effective inhibitors of growth and germination as the pH was lowered from 7.2 to 5.7. At pH 7.2 50 $\mu\text{g/ml}$ BHA, 200 $\mu\text{g/ml}$ TBHQ and 200 $\mu\text{g/ml}$ 8-OHQ were the minimum concentrations necessary for 90% inhibition of germination. BHT (400 $\mu\text{g/ml}$) inhibited

germination by 65% at pH 7.2. Minimum concentrations at which growth at pH 7.2 was inhibited by 95% were 60 $\mu\text{g/ml}$ BHA, 20 $\mu\text{g/ml}$ BHT, 250 $\mu\text{g/ml}$ TBHQ and 20 $\mu\text{g/ml}$ 8-OHQ. Inhibition of growth and germination by each chemical was found to be reversible with the exception of the inhibition of growth by BHT.