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THE INTERRELATIONSHIP OF BACTERIAL LIPID COMPOSITION AND THE ANTIMICROBIAL ACTIVITY OF THE ANTIOXIDANT BUTYLATED HYDROXYANISOLE

A Dissertation

Presented for the

Doctor of Philosophy

Degree

The University of Tennessee, Knoxville

Laurie Sue Post

December 1982

3065056

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DEDICATION

With love and appreciation, I dedicate this dissertation to my mother, Gertrude Helen Post. Her memory embodies a great deal of understanding, wisdom, and humor--qualities which will see me through a lifetime.

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My deepest appreciation is extended to Dr. P. M. Davidson who saw me through the course of this study. His advice, patience, and encouragement were instrumental to the fulfillment of my academic goals. Appreciation is also extended to Dr. H. O. Jaynes, Dr. C. C. Melton, and Dr. J. Becker for their guidance and assistance as committee members as well as to the Department of Food Technology and Science for the assistance provided during the course of my graduate studies.

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Sincere appreciation is offered to my aunt, Doris Sanchez, for her understanding and loving concern.

To my father, Dr. Harold Post, I extend my love and the acknowledgment that he made this degree possible. Through achievement of this goal, I can only hope that I have made him proud and that I have enabled him to see through his dream for me.

ABSTRACT

Two species of Pseudomonas were grown in the presence of sublethal concentrations of the phenolic antimicrobial antioxidant butylated hydroxyanisole (BHA). The effect that BHA had in the growth media on the bacterial fatty acid and phospholipid composition and the relationship of any modifications of the composition to the susceptibility of the microorganism was determined. Microbial lipids were extracted and analyzed by gas chromatography. Phospholipids were assayed using thin layer chromatography.

Pseudomonas fluorescens was almost totally inhibited by the presence of 150 ppm BHA for 15 minutes. The relative percentage of unsaturated fatty acids in the total lipid fraction was 58.14%. Growth in the presence of 50-125 ppm BHA caused a decrease in the relative ratio of unsaturated to saturated fatty acids for both total and polar lipid fractions while resistance to the antimicrobial effects of BHA increased. Decreased susceptibility also paralleled a decrease in phosphatidyl ethanolamine content and an increase in phosphatidyl glycerol and phosphatidyl inositol contents. Modified lipid content and decreased susceptibility were maintained through one transfer in broth without BHA. Control cells of Pseudomonas fragi were relatively resistant to the effect of BHA. The relative percentage of unsaturated fatty acids in the total lipid fraction was 37.57% for the control population and treated cells maintained approximately 38% unsaturated fatty acids as well. Changes in the polar fatty acid and phospholipid composition of P. fragi paralleled

alterations in the bacterial susceptibility to BHA. When the lethal effects of BHA against a variety of both gram-positive and gram-negative organisms were determined in relation to the total and polar fatty acid composition of the cytoplasmic membrane, a definitive relationship between total and polar fatty acid composition and susceptibility of a bacterial species to BHA could not be elucidated. Increased levels of saturated fatty acids did not confer protection and cell gram reaction did not appear to be a strict indicator of bacterial susceptibility to BHA. The antimicrobial activity of BHA, while associated with changes in lipid composition, may be more directly mediated through alteration of the activity of membrane bound enzyme systems.

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CHAPTER I

INTRODUCTION

The introduction of a new food preservative compound into the marketplace is a costly and time-consuming process in view of the legal regulations concerning food safety. If a dual functionality could be elaborated for a currently approved food additive, then the compound could be more efficiently utilized (Kabara, 1981). Among the antioxidants currently approved for use is butylated hydroxyanisole (BHA). While BHA is added primarily to prevent oxidation in lipid-containing foods, the antimicrobial activity of this hydrophobic antioxidant has been well-documented (Branen et al., 1980).

Most evidence suggests that the inhibitory powers of BHA are in part based on disruption of the cytoplasmic membrane of the cell (Sgaragli et al., 1977; Branen et al., 1980). Growth of Pseudomonas fluorescens and Pseudomonas fragi in the presence of BHA has been found to result in changes in the fatty acid and phospholipid content of the cytoplasmic membranes of bacteria (Davidson and Branen, 1980b). Davidson and Branen (1980b) also found that the treatment of cells with BHA resulted in the loss of intracellular constituents through destruction of the cell permeability barrier.

The cytoplasmic membrane of the cell is composed of lipids (mostly in the form of phospholipids), proteins and polysaccharides (Lehninger, 1975). The lipids form a continuous nonpolar phase lending structural support to the membrane as well as serving a physiological

role in the functioning of membrane-associated enzymes (Cronan and Vagelos, 1972). As a nonpolar phenolic compound, the antimicrobial activity of BHA could be related to phenol and its derivative compounds. Phenols are known to disrupt the outer layer of cells, the site of most cellular lipid material (Prindle and Wright, 1977). Additional information on the mechanism of action of BHA will allow a better evaluation of its potential usefulness as an antimicrobial in a particular food and could serve to elaborate a lipid-mediated mode of action for an antimicrobial compound.

The objectives of these experiments were twofold. First, the effect BHA in the growth media had on the fatty acid and phospholipid compositions of P. fluorescens and P. fragi was determined and the relationship of any modifications of the composition to the antimicrobial activity of BHA was studied. Secondly, the lethal effect of BHA on several bacterial species representing both gram-positive and gram-negative organisms important in food spoilage or as pathogenic agents was determined. This study was undertaken to determine if a relationship existed between the total and polar fatty acid composition of the cytoplasmic membrane and the susceptibility of the organism to the antimicrobial effect of BHA and to determine if the effect was related to cell wall type.

CHAPTER II

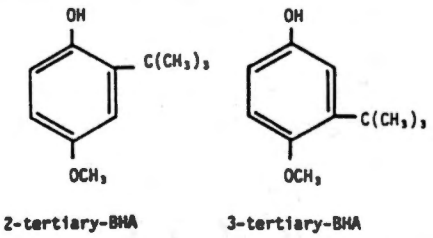
REVIEW OF THE LITERATURE

A. Butylated Hydroxyanisole as an Antioxidant And an Antimicrobial Agent

Butylated hydroxyanisole (BHA) is a phenolic antioxidant used to preserve food by retarding deterioration, rancidity, or discoloration due to oxidation of lipid ingredients. Unsaturated fatty acids are subject to oxidation at their double bonds to form free radicals and intermediate hydroperoxide cleavage products (Labuza, 1971). The free radicals will combine with oxygen to form a peroxy free radical which in turn can remove hydrogen from another unsaturated molecule to perpetuate a chain reaction. Hydroxyperoxides are further oxidized to aldehydes, semialdehydes, and acids which impart objectionable flavors to lipid containing foods. Antioxidants react to terminate the chain reaction through donation of a hydrogen atom to a free radical and in the process become free radicals themselves which are deactivated by oxidation to a quinone (deMan, 1976; Pokorny, 1971). Other synthetic antioxidants commonly used today include the phenolic compounds monotertiary butyl hydroquinone (TBHQ), propyl gallate, and butylated hydroxytoluene (BHT).

Butylated hydroxyanisole is found in an isomeric mixture of 15% 2-tertiary-butyl-4-hydroxyanisole and 85% 3-tertiary-butyl-4-hydroxyanisole (Johnson, 1971). The physical properties of BHA which are of commercial interest are listed in Table 1. BHA is considered generally recognized as safe for addition to food products. Regulations

Table 1. Physical properties of BHA which are of commercial importance in application of food systems

Structural formula (mixture of 2 and 3 isomers)	 2-tertiary-BHA 3-tertiary-BHA
Molecular weight	180
Physical appearance	White waxy tablets
Boiling range, 733 mm., °C	264-270
Melting range, °C	48-55
Odor	Slight

SOLUBILITY IN COMMON SOLVENTS

Solvent	Temperature, °C	Solubility, % (Approx.)
Water	0-50	Insoluble
Glycerol	25	1
Ethyl alcohol	25	50+
Diisobutyl adipate	25	60
Propylene glycol	25	70
Glyceryl monooleate	25	50
Coconut oil	25	40
Cottonseed oil	25	30
Corn oil	25	40
Peanut oil	25	40
Soya oil	25	50
Lard	50	50
Paraffin	60	60+

Adapted from Eastman Kodak Co., 1982.

issued by the Food and Drug Administration permit the use of BHA singly or in combination with other approved antioxidants in food products at a maximum concentration of 0.02% based on the weight of the fat or oil (including essential oil) content of the food product (CFR, 1981a). The Food, Safety, and Inspection Service of the U.S. Department of Agriculture allows the use of antioxidants in meat and poultry at an individual level of 0.01% by weight, or 0.02% in combination, based on the fat content of the food. Antioxidants are permitted in dry sausage at a level of 0.003% singly, or 0.006% in combination, based on the total weight of the food. Antioxidants are permitted in dried meats at a level of 0.01%, singly or in combination, based on the total weight of the food (CFR, 1981b, 1981c).

The wide distribution and the large consumption of food products containing antioxidants allows for extensive human exposure to these agents. The average daily intake of BHA has been estimated to be 0.1 mg/kg body weight per day (Branen, 1975). At consumption levels of 50 mg/kg/day (well above the estimated amount consumed by man), BHA exhibits a low toxicity in mammals. At levels of 500 mg/kg/day, BHA ingestion resulted in hypertrophy of the liver and proliferation of the smooth endoplasmic reticulum in the livers of rats and monkeys (Allen and Engblom, 1972; Branen et al., 1973; Gaunt et al., 1965). Sgaragli et al. (1977) found that monocyclic compounds, such as BHA, interact with biomembranes producing solubilization of proteins. Rat liver mitochondrial and lysomal membranes were disrupted by BHA, thereby interrupting mitochondrial respiratory function. Liver injury had also been reported earlier in a study by Wilder et al. (1960) in which dogs

had been fed BHA at a level of 250 mg/kg/day for a 15 month feeding period. While Allen and Engblom (1972) have reported that BHA caused nucleolar fragmentation and the presence of large intranucleolar fibrils in heptatocytes, no significant decrease in BHA, RNA or protein synthesis was reported in the livers of monkeys. Such pathological changes in the libers of laboratory animals were considered nonhepatotoxic since they were reversible when BHA was removed from the ration (Johnson, 1971). Other toxic effects attributed to BHA include inhibition of bradykinin function (Posati and Pallansch, 1970; Donaldson, 1973) and allergic reactions (Fisherman and Cohen, 1973).

Sgaragli et al. (1980) have investigated the possibility that an enzymatic system is present in the intestinal wall of the rat which is responsible for the metabolic inactivation of BHA after determining that the intraperitoneal LD_{50} of BHA in rats was one-half of that reported administered per os. Incubation of BHA with partially purified rat intestine peroxidase and hydrogen peroxide resulted in an oxidation product of BHA. Peroxidative oxidation at the intestinal wall may prove to inactivate some phenol derivatives potentially toxic to mammals.

The antimicrobial properties of such phenolic antioxidants as BHA have been the subject of many recent investigations. While BHA is added primarily to prevent oxidative rancidity in lipid containing foods, it has been shown to inhibit a wide range of microorganisms. Growth inhibition has been reported for the gram-negative bacteria Salmonella typhimurium, enteropathogenic Escherichia coli, Pseudomonas fragi, Pseudomonas fluorescens and Vibrio parahaemolyticus (Pierson et al., 1980; Davidson and Branen, 1981; Chang and Branen, 1975; Shih

and Harris, 1977; Davidson and Branen, 1980a; Robach et al., 1977). Inhibitory concentrations ranged from 50 to 400 ppm (Table 2).

Antimicrobial activity against gram-positive bacteria includes work with Staphylococcus aureus (Raccach, 1981; Lahellec et al., 1981). Chang and Branen (1975) found S. aureus to be quite sensitive and a 10^6 inoculum was totally inactivated by 150-200 ppm BHA. Ayaz et al. (1980) showed enterotoxin production to be inhibited at this concentration as well. Klindworth et al. (1979) showed that 150 ppm of BHA totally inhibited three strains of C. perfringens. Reddy et al. (1982) have found BHA to be effective in retarding C. botulinum growth and toxin production, although not to the extent found by Robach and Pierson (1979). Robach and Pierson found inhibitory levels of BHA to be significantly lower when used against single strains of C. botulinum (50 ppm) than did Reddy and coworkers who used a mixed inoculum of spores from Clostridium types A and B. The antibacterial effect of BHA in laboratory media against five strains of Bacillus was evaluated by Shelef and Liang (1982). Growth was inhibited by 75-100 ppm BHA.

BHA has been shown to exhibit antifungal properties against mycelial growth and aflatoxin production of Aspergillus flavus and A. parasiticus (Fung et al., 1977; Chang and Branen, 1975; Beggs et al. 1978). The antiviral activity of BHA was evaluated by Wanda et al. (1976). BHA was found to totally inactivate the bacteriophage Ø6, a lipid containing virus, at a concentration of 100 M. In work with the protozooan Tetrahymena pyriformis, Surak and coworkers (1976) showed DNA, RNA, and protein synthesis to be inhibited by 200 ppm BHA within 10 minutes after treatment.

Table 2. Concentrations of BHA necessary to totally inhibit various bacteria in laboratory media

Microorganism	Concentration (ppm)	References
Salmonella typhimurium	400	Pierson et al., 1980
	150	Davidson & Branen, 1981
Escherichia coli	400	Chang & Branen, 1975
		Shih & Harris, 1977
Vibrio parahaemolyticus	50	Robach et al., 1977
Pseudomonas fragi	400	Davidson & Branen, 1980a
Pseudomonas fluorescens	100	Davidson & Branen, 1980a
Staphylococcus aureus	150	Chang & Branen, 1975
	200	Ayaz et al., 1980
		Pierson et al., 1980
Clostridium perfringens	150	Klindworth et al., 1979
Clostridium botulinum	50	Robach & Pierson, 1979
Bacillus cereus	75-100	Shelef & Liang, 1982
Bacillus subtilis	75-100	Shelef & Liang, 1982
Bacillus megaterium	75-100	Shelef & Liang, 1982

As a nonpolar phenolic compound, the antimicrobial activity of BHA could be related to phenol and its derivative compounds (DeSmet et al., 1978; Kaye and Proudfoot, 1971). The antiseptic properties of phenol were recognized as early as 1860 by Lister in his work to develop aseptic surgical techniques (Pelczar et al., 1977). In high concentrations, phenol disrupts the outer layer of the cell and precipitates cell proteins. In low concentrations, death of the cell is due to inactivation of essential enzyme systems (Prindle and Wright, 1977). The effectiveness of phenolics is determined by such structural characteristics as the substitution of alkyl chains on the compound (Suter, 1941). An increase in the nonpolar character of the compound was found to increase antimicrobial activity.

Effects shown by BHA against Pseudomonas fluorescens and Pseudomonas fragi suggest disruption of the cytoplasmic membrane is at least partially responsible for inhibition. In recent work by Davidson and Branen (1980b), BHA was found to cause the leakage of intracellular constituents. Sgaragli et al. (1977) have also shown that BHA causes structural modifications in biomembranes.

Since small phenolic compounds readily penetrate membranes (Singleton, 1981), BHA may function as a membrane perturber in that it could insert into the lipid layer of the bacterial cell and disrupt the ordered state of the alkyl chains (Surak, 1980; Surak and Singh, 1980). Eletr et al. (1974) was able to show that adamantane, a quasi-spherical molecule used as a lipophilic perturber, disrupted the ordered state of the membrane lipids of Saccharomyces cerevisiae and thereby lowered the associated transition temperature of the membrane. At

lowered transition temperatures, a decrease in lipid saturation occurs. In an attempt to maintain membrane fluidity, a microorganism will alter the fatty acid composition of the cytoplasmic membrane in terms of increased synthesis of fatty acids (Marr and Ingraham, 1962; Sinensky, 1971). Such changes may affect the activity of membrane bound enzymes. In the same study by Eletr and coworkers, it was found that the activities of tightly bound membrane enzymes associated with oxygen uptake in cells of Saccharomyces cerevisiae reflected the physical state of the membrane lipids. More specifically, Exterkate (1977, 1979) found the activity of pyrrolidone carboxylylpeptidase in Streptococcus cremoris to be affected by n-butanol through alteration of the physiochemical state and organization of the membrane lipids. The activity of lactate dehydrogenase in E. coli was found to depend on the nature of unsaturated sites of membrane phospholipids (Esfahani and Wakil, 1972). Unsaturated sites are the determining factors in the fluidity of lipid molecules. Modification of lipid fluidity altered protein-lipid interactions within the membrane and therefore the activity of membrane bound enzymes. Eletr et al. (1973) determined whether the fluidity of the lipids in hepatic microsomal membranes was related to the activities of the membrane bound enzymes UDP-glucuronyl transferase and glucose-6-phosphatase. Enzymatic activity was found to be dependent on the physical state of the membrane lipids.

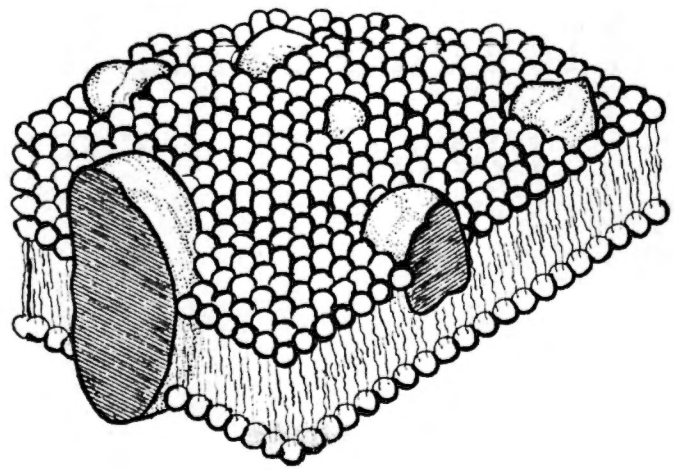
B. Bacterial Membrane Structure

Overton (1895) first proposed that membranes were composed of lipids due to the ease with which lipid soluble substances penetrated

the plasma membrane of the cell. Danielli and Davson (1935) proposed that lipids existed as a bilayer which was coated on both sides by proteins. Nonpolar fatty acyl chains were oriented inwards perpendicular to the membrane surface and polar groups of phospholipids occurred at the surface coated by proteins and polysaccharides. This concept of membrane structure was termed the "sandwich" or "unit" model (Sherbet, 1978).

The lipid bilayer concept did not account for the dynamic properties of lipid molecules, however. Lipid molecules were known to possess a rapid internal motion and a rotational motion along the axis of the molecule. Lateral movements of the molecules and exchange of lipids from one side of the bilayer to the other were reported (Singer and Nicolson, 1972). Recognitions of these motional properties, conferring fluidity to the membrane, led to the development of the fluid mosaic model by Singer and Nicolson (1972). This model proposes that phospholipids are arranged in a bilayer to form a fluid, liquid-crystalline matrix or core. Globular protein molecules penetrate into either side or extend entirely through the membrane (Figure 1). Thus the lipid bilayer is structurally asymmetrical. Proteins are free to diffuse laterally in two dimensions. Individual lipid molecules move laterally as well, leading to the characteristic properties of fluidity, flexibility, high electrical resistance, and relative impermeability to highly polar molecules (Singer, 1974, 1975).

Cellular membranes are essential to the life of the cell. The cytoplasmic membrane of the cell serves as an osmotic barrier, houses the active transport allowing for selective permeability (permeases) and is the site of many key enzymatic reactions involved



Adapted from Lehninger, 1975

Figure 1. The fluid mosaic model of membrane structure consists of a matrix of fluid lipid bilayer throughout which globular integral proteins are dispersed.

in metabolism and conversion of nutrient energy to ATP (Lehninger, 1975). Mitchell (1963) examined the enzymatic activities of various fractions of Micrococcus lysodeikticus and Staphylococcus aureus (Table 3). Certain enzymes were found to a small extent in the cytoplasm and to a much greater extent in the membrane fraction. Enzymes responsible for the biosynthesis of polymers for use in the cell wall are located in the membrane as well (Rogers and Perkins, 1968; Cronan and Gelman, 1975).

Environmental factors such as incubation temperature or composition of growth media can markedly alter the lipid composition of the bacterial membrane (Yang and Haug, 1979). Variations in growth temperature are well known to alter the proportions of individual fatty acids in bacteria and thereby shift the ratio of unsaturated to saturated fatty acids (Marr and Ingraham, 1962; DeSiervo, 1969; Joyce et al., 1960). Marr and Ingraham found the fraction of unsaturated fatty acids in the envelope phospholipids of E. coli to increase markedly as growth temperature decreased. Fukushima et al. (1976) found marked differences in the phospholipid polar head groups contained in the membrane lipid layer of Tetrahymena pyriformis when cells were grown at temperature extremes. Phosphatidylethanolamine decreased in concentration at lower temperatures. Khuller and Goldfine (1974) also found temperature adaptation to involve alterations in relative proportions of membrane phospholipids in Clostridium butyricum. At 25 C, 35% of the total phospholipid phosphorus was in the form of ethanolamine and N-methylethanolamine phosphoglycerides while at 37 C the proportion increased to 42% at the expense of the glycerol phosphoglycerides.

Table 3. Enzymes and activities at 25 C found in dialyzed fractions of Micrococcus lysodeikticus and Staphyloccus aureus

Enzyme	mu mole substrate/g/sec			
	M. lysodeikticus		S. aureus	
	Membrane	Cytoplasm	Membrane	Cytoplasm
Cytochrome oxidase	117	14.8		
Catalase	1.94×10^5	2.42×10^6		
Alcohol dehydrogenase	10	143		
NADH oxidase	500	49		
Glucose 6-phosphatase	0	0	4.3	39.8
α-Glycerophosphatedehydrogenase	0	0	10.3	9.8
Lactic dehydrogenase	44.0	<0.3	64.2	3.2
Acid phosphatase	0	0	25.5	2.0
β-Galactosidase	0	0		
Succinic dehydrogenase	44.1	2.1	4.7	<0.3
Formic dehydrogenase	0	0	16.6	<0.3
α-Oxoglutarate dehydrogenase	5.6	1.64		
Isocitric dehydrogenase	9.9	84.2	1.64	45.2
Malic dehydrogenase			19.8	<0.5
Malic enzyme			0	26.6
Glucose dehydrogenase			1.6	0.3

Adapted from Mitchell, 1963.

In a study using E. coli, Sinensky (1971) noted that at increased temperatures, there was a tendency to incorporate longer and more saturated fatty acids into phospholipids. This process was termed "homeoviscuous adaption" or the variation of fatty acid composition of membrane phospholipids in order to produce a membrane with constant fluidity at the temperature of growth (Jackson and Cronan, 1978). At higher temperatures, it was necessary for phospholipids to exhibit higher solid to liquid crystalline phase transitions--in order to restore membrane fluidity to a functional level. Homeoviscuous adaption played a role in regulating membrane bound enzyme activity so that appropriate reaction rates could be maintained when cells were grown at different temperatures. Enzymatic activity was dependent on fluidity of the lipid cofactor as Sinensky (1971) had shown in an earlier study where the specificity of the enzymes catalyzing phosphatidic acid synthesis was altered by temperature change.

C. The Lipid Classes of Bacteria

The total lipid content of most species of bacteria ranges between 1 and 10% of the dry cell weight (O'Leary, 1962). Certain cellular structures are known to be relatively rich in lipid; however, little is known of the specific fatty acids present. A characteristic consistent with various species are lipid or cytoplasmic inclusions. The accumulation of these lipid globules within the cell have been reported in Corynebacterium diphtheriae and Bacillus sp. (Burdon, 1958).

In the gram-positive cocci, cells contain almost no lipid that is not in the membrane (Rogers and Perkins, 1968). Only small amounts (1-3%) of lipid have been associated with the isolated cell walls of gram-positive bacteria. In contrast, gram-negative cell walls contain 10 to 20% lipid composed mostly of lipoprotein and lipopolysaccharide complexes (Salton, 1956; Weidel and Primosigh, 1958). A cell envelope structure has been proposed for gram-negative organisms containing 3 layers of lipid material: the cytoplasmic membrane, a lipoprotein layer, and a lipopolysaccharide layer (Kellenberger and Ryter, 1958).

According to Ames (1968), 70% of the cellular lipid in E. coli is located in the membrane. The cell membrane contains about 45% lipid and 55% protein, the lipids forming a continuous nonpolar phase (Lehninger, 1975; Salton, 1970). Phospholipids comprise the major lipid component of most biological membranes. Their role in membrane structure and transport is the subject of an increasing number of bacterial studies. Two distinct roles have been elucidated: (1) structural--in which phospholipids impart permeability and provide a matrix in which membrane proteins are embedded; (2) physiological--phospholipids are required for the function of certain membrane associated enzymes (Cronan and Vagelos, 1972).

Bacterial phospholipids tend to be of the phosphatidyl glycerol type. Carbohydrates, inositol and glycerol, are common constituents of those bacterial phospholipids that have been studied. Nitrogen containing compounds such as ethanolamine, and to a lesser extent choline, have also been detected (O'Leary, 1962; Kanfer and Kennedy, The phospholipids most common to the bacterial cells are phosphatidyl-

ethanolamine, phosphatidylglycerol, diphosphatidylglycerol (cardiiolipin) and phosphatidylserine (Thompson, 1980). In a study by Kito et al. (1975), phosphatidylethanolamine was found to predominate in E. coli. The lipids of Pseudomonas aeruginosa were examined by Hancock and Meadow (1969). Of the extractable lipids, 90% were phospholipids and phosphatidylethanolamine comprised the major component.

Free fatty acids were commonly found in a number of bacterial species including Corynebacterium diptheriae, Lactobacillus acidophilus, and Bacillus megaterium. The lipid content of Salmonella typhimurium consists almost entirely of free fatty acids. Fatty acids are usually palmitic, stearic, and octadecanoic acids. The purpose and intracellular distribution of these unesterified fatty acids has yet to be determined. The proportion of free fatty acids found in bacteria is high, 20% in some species, in comparison to plant or animal cells (Knaysi, 1951).

Of the neutral lipids, mono, di, and triglycerides are found in low levels, if at all, in bacterial cells. Esters of long chain fatty acids and long chain alcohols (waxes) are also uncommon (O'Leary, 1962).

Glycolipids have been reported as components of the lipids of mycobacteria, Staphylococcus aureus, Streptococcus lactis, and Streptococcus faecalis. In S. aureus, the glycolipid moiety was identified as a glucosyl diglyceride (Macfarlane, 1962). In Pneumonococcus, glycolipids accounted for 30% of the total lipids (Rogers and Perkins, 1968). Glycolipids are characterized by the combination of such sugars as glucose, galactose, arabinose, mannose, rhamnose, and trehalose with

one or more molecules of fatty acids or alcohols. Glycolipids compose the antibiotic substances of Pseudomonas and the lipopolysaccharide endotoxins of gram-negative bacteria (O'Leary, 1962). Glycolipids may play a metabolic role in the organism as well.

Lipoproteins or lipoamino acids have also been observed in C. perfringens and S. aureus (Macfarlane, 1962) and in lactic acid bacteria (Ikawa, 1963). In S. aureus, the associated amino acid was found to be lysine forming an amino acyl ester of phosphatidyl glycerol. Although such components may play a role in protein synthesis, very little is known at present as to their true function (O'Leary, 1962).

Bound lipids constitute a large proportion of total cellular lipids. These compounds are lipids which are combined with carbohydrates and proteins and are not readily extracted with organic solvents until first released through a hydrolytic procedure (O'Leary, 1962).

D. Fatty Acids of Bacteria

Bacteria are characterized by the presence of unusual fatty acids not seen in other organisms. The composition of bacterial lipids has long been known to depend on the strain studied, culture medium, growth temperature, and growth phase (Cronan, 1968; Daron, 1973). In work by Marr and Ingraham (1962), the lipids of young cultures of E. coli were found to contain large proportions of 16 and 18 carbon acids and small amounts of 17 and 19 carbon cyclopropane acids. In late logarithmic phase, cyclopropane content was greatly increased at the expense of unsaturated fatty acids. Therefore, precise figures as to percentages of fatty acid composition are of limited value. The groups of fatty acids

known to occur in bacteria are: unsaturated fatty acids, saturated straight chain acids, hydroxy acids, branched chain acids, and cyclopropane acids (Fulco, 1974; Kaneda, 1963a, 1963b).

Unsaturated 18 and 16 carbon fatty acids are more commonly identified in bacteria (Bloch et al., 1961). The occurrence of an unidentified 14 carbon acid (tetradecanoic) had been reported in C. diptheriae and chain lengths greater than 18 carbons have been noted only in the corynebacteria (Asano and Takahashi, 1951). Hexadecanoic (C16) and octadecanoic (C18) acids are commonly misidentified. Monounsaturated C18 fatty acids are often labeled oleic acid while, in actuality, they are cis-vaccenic acid. Vaccenic acid is the major octadecanoic acid present in L. casei (Hofmann and Tausig, 1955a).

Of the saturated fatty acids, chains of less than 12 carbon units comprise only small amounts. Straight chain saturated fatty acids longer than 28 carbon atoms have not been reported. Chains of 20-28 carbon units have been found in a limited number of species. Lignoceric acid (C24) has been reported in Lactobacillus acidophilus. The most common saturated fatty acids are lauric (C12), myristic (C14), palmitic (C16), and stearic (C18). Palmitic acid usually comprises the largest amount of saturated fatty acid content (O'Leary, 1962; Romero and Brenner, 1966).

Fatty acids with odd numbers of carbon atoms have also been reported. In their study with P. aeruginosa, James and Martin (1956) found evidence of C15 and C17 straight chain saturated acids. Fatty acids with 15 carbon atoms predominated in all of the Bacillus sp. examined by Kaneda (1967, 1969; Shen et al., 1970; Daron, 1970). A 17 carbon acid was reported in Pseudomonas fluorescens which comprised over

20% of the bound and free lipid extracts (Scheuerbrandt and Block, 1962).

Hydroxy and branched chain acids have been reported, although complete identification is still necessary (Forsyth et al., 1958). Several complex hydroxy acids have been found peculiar to C. diptheriae and a number of complex branched chain acids have been isolated from the corynebacteria (O'Leary, 1962). Several branched chain fatty acids have been found by Kaneda (1967, 1969) to be characteristic of Bacillus.

Lactobacillic acid was one of the first cyclopropane acids of which detailed information was available (Crowder and Anderson, 1932). This 19 carbon fatty acid has been identified in several Lactobacillus species and E. coli has been shown to contain a 19 carbon acid similar to lactobacillic (Law, 1961; O'Leary, 1959; Hofmann and Tausig, 1955a). Cyclopropane acids of 13, 15 and 17 carbons have been tentatively identified in C. butyricum (Goldfine, 1961; Goldfine and Bloch, 1961). Hancock and Meadow (1967) have also detected the cyclopropane acids 17:0 cy and 19:0 cy in extractable lipids of P. aeruginosa. Table 4 depicts the fatty acid spectra of several bacterial species.

E. Microorganisms of Food Spoilage and Pathogenic Significance

Antioxidants retard oxidation in a wide variety of food products which are also prone to microbial contamination when subjected to prolonged storage or abusive treatment. The use of an antioxidant with a dual antimicrobial activity such as BHA would allow for a more efficient utilization of a currently available food additive.

trophs such as Pseudomonas spp. are of commercial importance as spoilage

Table 4. Fatty acid spectra of some representative species of bacteria

	Lactobacillus casei	*Streptococcus* sp.	*Clostridium butyricum*	*Escherichia coli*
Fatty Acids[a]				
Saturated				
C10	2.1	0.5	0.5	0.3
C12	2.8	5.2	2.4	0.3
C14	2.1	4.4	0.4	0.7
C16	24.3	26.6	49.0	
C16+17[c]				85.4
C18	7.0	18.0	6.2	
C18+C19[c]				0.5
Unsaturated				
C16			17.0	
C18			7.9	
C16+C18	37.6	38.0		11.6
Cyclopropane				
C13			0.4	
C15			1.5	
C17			9.0	+[b]
C19	12.6		5.2	+

[a] Percent of total fatty acids

[b] Present in undetermined amount.

[c] Cyclopropane acid.

Adapted from O'Leary, 1962.

organisms. The bacteria Bacillus cereus, Clostridium perfringens, Escherichia coli, Salmonella spp., and Staphylococcus aureus are commonly implicated in outbreaks of foodborne disease in the United States (Table 5).

Bacteria of the genus Pseudomonas play a central role in the spoilage of refrigerated foods including meat, poultry, and milk products. With a generation time about 30% shorter than that of other species, psychotrophic pseudomonads dominate the aerobic spoilage flora of proteinaceous foods stored between 2 and 15 C (Ingraham and Stokes, 1959). Pseudomonas spp. and Achromobacter spp. account for approximately 50% or more of the total population of spoiled poultry meat and are most commonly implicated in the spoilage of fish (Ayres et al., 1980). Psychrotrophs predominate in the spoilage flora of fluid milk products (Jones and Langlois, 1977) and the bacterial spoilage of eggs is often caused by Pseudomonas spp. (Florian and Trussell, 1957).

Two species of Pseudomonas which are often recovered from spoiled refrigerated products are P. fluorescens and P. fragi. both of these species produce surface slime, synthesize extracellular proteolytic and lipolytic enzymes and produce a variety of volatile compounds which play a significant role in the degradation of food (Porzio and Pearson, 1975; Jay, 1978; Tarrant et al., 1973; Peterson and Gunderson, 1960; Lawrence, 1967; Berry et al., 1973). Of the food grade chemical compounds found inhibitory to psychrotrophs, malonic acid, sodium benzoate, and potassium sorbate have demonstrated activity against P. fragi (Moustafa and Collins, 1969). Potassium sorbate was also found to extend the lag phase of two strains of P. fluorescens in laboratory media (Robach, 1978). Inhibitory substances produced by lactic starter cultures such as Lactobacillus bulgaricus and Pediococcus cerevisiae

Table 5. Confirmed bacterial disease outbreaks in the United States, 1975-1979

	1975	1976	1977	1978	1979
	----------%----------				
B. cereus	2.43	2.17	----	5.71	----
C. perfringens	13.01	6.52	5.94	8.57	16.81
E. coli	----	----	----	0.95	----
Salmonella	30.89	30.43	40.59	42.86	36.97
Staphylococcus aureus	36.59	28.26	24.75	21.90	28.57
Other bacterial	17.08	32.62	28.72	20.01	17.65

From Center for Disease Control, 1981.

have also been studied as a means of psychrotroph control (Gilliland and Speck, 1975).

Escherichia coli is a normal inhabitant of the intestines. While the majority of these are nonpathogenic, reports incriminating E. coli as an etiologic agent of food poisoning involve a variety of foods including meat, fish, poultry, and dairy products (Mehlman et al., 1976). Only enteropathogenic (EEC) strains are capable of producing the food poisoning syndrome. Strains affecting humans are divided on the basis of whether they produce an enterotoxin or not. Enterotoxigenic strains cause cholera-like or infantile diarrhea. Nontoxin producing or enteroinvasive E. coli is associated with a Shigella-like syndrome (Collins-Thompson, 1980). A well documented foodborne EEC outbreak occurred in the U.S. in 1971 due to imported cheeses (Marier et al., 1973). Levels of 10^6-10^7 cell/g were found in the cheese which was imported from France. The lactic acid bacteria, Lactobacillus bulgaricus, is known to produce an antienterotoxic factor against EEC, although isolation of the cell free substance has proven difficult (Mitchell and Kenworthy, 1976).

Salmonellosis is one of the major foodborne illnesses affecting man and results from the ingestion of viable cells numbering from several million to several billion/gram. Symptoms, which include nausea, vomiting, abdominal pain, headache and diarrhea, are caused by endotoxin liberated from cells through the action of low stomach pH and proteases (Jay, 1978). Of the 1700 or more serotypes, apparently all can cause disease in man. Among the chief sources of salmonellosis in man are pork, poultry, eggs, meat and milk products (Collins-Thompson,

1980). Turkey and chicken are most often implicated when poultry products are cited as etiological agents. Salmonellae usually do not affect the appearance, smell or taste of the food in which they grow.

The primary habitat of Salmonella spp. is the intestinal tract of animals such as birds, reptiles, farm animals, and man. The disease spreads easily in a continuous cycle from animal to man, man to man and man to animal. Of the various serotypes involved in foodborne outbreaks, S. typhimurium is the most frequently encountered (Jay, 1978). Various commercially prepared and packaged foods were examined for the incidence of Salmonella spp. by Adinarayanan et al. (1965). Contaminated food included cake mixes, cookie doughs, dinner rolls and corn bread slices. Common vehicles for the contamination of convenience foods are dried milk and dried and frozen egg ingredients. The most vulnerable foods have proven to be those subjected to excessive handling or processing. The bacteria may be introduced during any of the stages of production, processing, storage or preparation of food products.

Chemical compounds which have proven to be inhibitory to Salmonella include some common food additives and natural constituents of food products. Hydrogen peroxide, sorbates, ethylene oxide and formic, stearic, citric, lactic, and phosphoric acids have demonstrated antimicrobial effects (Litchfield, 1980; Jay, 1978). Naturally occurring inhibitory substances have also been reported in onion, garlic, spices and cocoa (Busta and Speck, 1968; Litchfield, 1980). Volatile compounds

in milk and culture filtrates of dairy product starter cultures have also shown activity against salmonellae (Kulshrestha and Marth, 1975; Sorrells and Speck, 1970).

Staphylococcus aureus intoxication is one of the more common foodborne illnesses affecting man. The actual number of yearly outbreaks is difficult to determine since a majority of the cases go unreported. Symptoms appear within 2 to 4 hours after consumption of contaminated foods and are characterized by diarrhea, vomiting, prostration, and abdominal cramps. Recovery is complete within 24-48 hours (Jay, 1978). Staphylococcal food poisoning is produced by certain strains of S. aureus that also produce coagulase. Several reports have been made in which coagulase negative strains were found to be etiological agents; however, implication of coagulase positive staphylococci is most prevalent (Bergdoll, 1980). Upon growth in a suitable food product, an enterotoxin is elaborated and, upon ingestion of the toxin-containing food, symptoms arise. Staphylococcal enterotoxins are unique among bacterial toxins in being heat resistant proteins. However, less than 50% of S. aureus strains are enterotoxigenic. Evidence from food poisoning outbreaks indicate as little as 0.1 ug or less of enterotoxin might be sufficient to cause disease in man. However, a large population of cells (10^6 organisms/gram) is necessary before the food is rendered hazardous (Bergdoll, 1980).

The main reservoir of S. aureus in man is the nose and throat. Domesticated animals harbor the organism as well (Jay, 1978). Common food vehicles are most often convenience type foods made by hand and

improperly refrigerated after being prepared. These include salads, creme filled baked goods and casseroles. Coagulase positive staphylococci have been found in market meats, poultry, vegetables, milk and milk products and cheese (Tatini et al., 1976). Illness is easily prevented if foods are kept either below 40 or above 140 F until consumed. The parabens, a group of antimicrobial food additives, have been shown to be effective in the inhibition of gram-positive organisms such as S. aureus. Sorbates have been reported to inhibit staphylococci, as well (Jay, 1978).

Clostridium perfringens food poisoning has been a problem often associated with the food service industry, since most outbreaks are meat or poultry dishes which have been cooked and held for some time before serving (Avens, 1980). These gram-positive rods grow anaerobically. Vegetative cells are destroyed by the heat of thorough cooking, but heat resistant spores persist. As the food cools, germination and outgrowth occurs. An exotoxin is produced by sporulating cells in the last stages of sporulation. The toxin produces diarrhea and vomiting in man (Jay, 1978). The spores of food poisoning strains differ widely in their resistance to heat. Heat resistance may be decreased by exposure of endospores to the natural acid conditions of some foods. These organisms have also been reported to be inhibited by 5% NaCl (Jay, 1978). Proper cooking and post-cooking storage temperatures remain the best means of control of C. perfringens.

Food poisoning strains of C. perfringens exist in soil, water, dust, spices and the intestinal tract of man and other animals. Large numbers of cells/g of food are associated with this syndrome, minimum

numbers being approximately 5 x 10^8 (Nakamura and Schultze, 1970). With an optimum growth temperature of 37-45 C, the slower cooling rate of large meat cuts such as roasts allows for the development of a large population. Poultry, such as stuffed turkey and prepared "deep" dishes are often implicated, as well. As more foods are eaten outside the home and prepackaged convenience foods become an increasingly large part of the diet, more cases of this foodborne illness may be expected.

The type of food poisoning associated with Bacillus cereus is similar to that produced by C. perfringens. B. cereus is a gram-positive rod capable of forming heat resistant spores. This organism may also grow anaerobically (Collins-Thompson, 1980). The pathogenic agent in B. cereus food poisoning appears to be an enterotoxin. Foods implicated in outbreaks include soups, meat, fried rice, cooked vegetables, ice cream, and milk. Spores contained in dried foods and spices may also be a source of contamination. B. cereus is ubiquitious in nature and is found in soil, water, and air (Jay, 1978). This organism has been associated with foodborne illnesses in Europe since 1906, but its incidence in the United States is presumed to be quite low. As with C. perfringens, correct cooking and holding temperatures provide an effective means of control in the absence of a chemical system for preservation.

CHAPTER III

MATERIALS AND METHODS

A. Modification of Bacterial Fatty Acid Composition and Relationship to Bacterial Susceptibility

Test Organisms

Cultures of Pseudomonas fluorescens ATCC 15456 and Pseudomonas fragi ATCC 4973 were obtained from the American Type Culture Collection, Rockville, Maryland. The cultures were received in freeze-dried form and revived in 5 ml Trypticase Soy broth (TSB) at 26 C. Stock cultures were maintained on Trypticase Soy agar (TSA) slants at 4 C and transferred periodically to maintain viability. Commercially prepared media used in this study were obtained from BBL, Cockeysville, Maryland.

Preparation of BHA

A fresh stock solution of BHA was prepared prior to each experimental application. Butylated hydroxyanisole tablets were obtained from Eastman Chemical Products, Inc., Kingsport, Tennessee. The tablets were ground and dissolved in 95% ethanol to a final concentration of 4% (w/v) and sterilized by filtration through a 0.45 um Nalgene membrane filter unit (Sybron Corp., Rochester, New York). Appropriate amounts of the 4% BHA stock solution were aseptically dispensed into sterile flasks of media.

Lethality of BHA

Pseudomonas fluorescens and P. fragi were incubated in the presence of various sublethal concentrations of BHA and then challenged

with several lethal concentrations of BHA. Survivors were enumerated through plate counts. Prior to an experiment, transfers were made from the appropriate stock cultures to two 125 ml flasks of TSB and incubated 24 hours at 26 C in an agitating water bath to serve as starter cultures. A 1% aliquot was taken from each of the two starter cultures and added to a duplicate series of flasks containing TSB and 0, 50, 100, and 125 ppm BHA for P. fluorescens and 0, 50, 100, 200, and 300 ppm BHA for P. fragi. The incubation period for each flask was adjusted in order to obtain approximately the same final population level for each concentration of BHA. This was done through calculation of generation times for the growth of each species in the presence of the varying amounts of BHA. For P. fluorescens, the culture containing 0 ppm BHA (control) was incubated 24 hours at 26 C. At the 50 and 100 ppm levels, a 36 hour incubation period was required. A 48 hour incubation period was used at 125 ppm. Cells grown in the presence of 125 ppm BHA were transferred into media containing no BHA and incubated 24 hours in order to determine if changes in susceptibility would be maintained or were a temporarily acquired phenomenon.

For P. fragi, 0 and 50 ppm concentration levels were incubated 24 hours at 26 C. Levels of 100 and 200 ppm were incubated 36 hours and 300 ppm was incubated 48 hours. Controls were inoculated containing TSB and 95% ethanol at concentrations equivalent to the amounts added as a carrier for the varying levels of BHA.

After appropriate incubation periods, a 1% aliquot of each culture was added to a series of flasks containing 0.05M phosphate-0.1% peptone buffer at pH 7.0 and BHA at concentrations of 0 to 450 ppm. Survivors were enumerated by plate counts on TSA as described

by Speck et al. (1976) and Harrigan and McCance (1976) after 0, 5, and 15 minutes incubation at 26 C in an agitating water bath. Plates were incubated at 26 C for 24 hours. Controls were run. The experiment was replicated twice and each replicate was performed in duplicate.

Lipid Extraction and Fractionation

Microbial lipids were extracted through a modification of the Folch extraction (Folch et al., 1951, 1957) and separated into neutral and polar lipid fractions by a modified silic acid slurry method described by Murty et al. (1960). Two 1000 ml volumes of each culture were grown on separate occasions in media containing 0, 50, 100, or 125 ppm BHA for P. fluorescens and 0, 50, 100, 200, or 300 ppm BHA for P. fragi. Incubation times were as indicated previously. Cells of P. fluorescens grown at 125 ppm were carried through one transfer in broth with no BHA.

The cells were harvested and washed with deionized water using centrifugation at 8000 x g for 10 minutes. Each replication was treated independently and identically. The washed cells from each replication were resuspended in 100 ml in deionized water and the cells precipitated using 5% (w/v) trichloroacetic acid. The cell suspension was divided and placed into 50 ml capacity polypropylene centrifuge tubes (Nalge/Sibron Corp., Rochester, New York) and centrifuged (6000 x g). The cell precipitates were weighed and combined, then homogenized 3 minutes at high speed with 17 ml/g chloroform/methanol (2:1) solution in a

laboratory blender. Calcium chloride (0.02%) solution (2 ml/10 ml extract) was added to the crude extract which was allowed to separate into two phases in a separatory funnel. The lower lipid containing phase was placed into a 50 ml screw-capped tube (teflon-lined cap) and washed with small amounts of Folch Upper Phase solution (3 parts chloroform + 48 parts methanol + 47 parts calcium chloride solution). The upper phase which formed was removed with a Pasteur pipette and discarded. The lipid containing phase was stored under nitrogen at -20 C until fractionation.

Fifty grams of silica gel (60-200 mesh) were activated by heating at 110 C for 20 hours and cooling in a desiccator for 1 hour prior to use. The silica gel, a portion of the stored total lipid extract, and 200 ml chloroform were combined to form a slurry which was stirred for 5 minutes on a magnetic stirrer prior to being transferred to a coarse sintered glass (Buchner type) filter (350 ml capacity, 40-60 um porosity fritted disc) funnel and being filtered by suction. Five 50 ml portions of chloroform were used to wash the silica gel. The filtrate, containing neutral lipids, was evaporated to 25 ml using a flash rotary evaporator. The silica gel was removed to a beaker and mixed with 200 ml methanol for 5 minutes on a magnetic stirrer. The slurry was filtered as before with four 50 ml portions of methanol. The filtrate, containing polar lipids, was evaporated to 25 ml. Both fractions were stored under nitrogen at -20 C.

Esterification and Analysis of Fatty Acid Composition

Methyl esters of total and polar lipid fractions were formed by the following procedure. The remaining total lipid extract for each replicate of each growth concentration and a portion of the corresponding polar fraction were evaporated to dryness on a flash rotary evaporator. One ml benzene, 2 ml methanol, and 1 ml boron trifluoride-methanol reagent (Supelco, Inc., Bellefonte, Pennsylvania) were added to each tube which was sealed and boiled 30 minutes in a water bath. The methyl esters were extracted from the cooled mixture with three 5 ml aliquots of petroleum ether. A few drops of saturated salt solution were added to enhance separation of the lipid containing phase from the aqueous phase. The upper phase, containing methyl esters, was removed with a Pasteur pipette to a tightly stoppered tube and stored under nitrogen at -20 C.

Methyl esters of total and polar lipid fractions were analyzed for fatty acid composition using a Shimadzu GC mini-2 Gas Chromatograph (Shimadzu, Ltd., Kyoto, Japan) on a GP 3% SP-2100 DOH Supelcoport column (Supelco, Inc., Bellefonte, Pennsylvania). Major fatty acids were identified by comparison of retention times with those of pure bacterial fatty acid methyl ester standards (Supelco, Inc., Bellefonte, Pennsylvania) and by mass spectroscopy using a Hewlitt Packard Model 5710A Gas Chromatograph interfaced with a Hewlitt Packard Model 5980 Mass Spectrometer at a 70 eV potential (Woodward and van Gent, 1960; James, 1960).

Phospholipid Analysis

Thin layer chromatography (TLC) was used to separate and identify the components of the polar lipid fraction (Nelson, 1975). The solid support was preactivated 250 um silica gel (80 Å) on 20 x 20 cm glass plates (Analytical TLC Precoated plates, LK5O, Whatman, Inc., Clifton, New Jersey). A one dimensional TLC was run using a chloroform:methanol:acetic acid: water (10:40:20:20:10) system. Identification of phospholipids was confirmed in a second system of chloroform:methanol:water:28% aqueous ammonia (130:70:8.0:0.5). Phospholipids were identified by comparison of sample R_f values with those obtained for known standards. The standards used were cardiolipin (disphosphotidylglycerol), phosphatidyl inositol, phosphatidyl ethanolamine (L-α-cephalin), L-α phosphatidyl-DL-glycerol, and L-α-phosphatidyl serine (Sigma Chemical Co., St. Louis, Missouri). Percentages of each phospholipid were determined by scanning densitometry using a Schoeffel Spectrodensitometer 3000 equipped with a SDC 300 Density Computer (Schoeffel Instrument Corp., Westwood, New Jersey).

Influence of Incubation Temperature

Cells of Pseudomonas fluorescens ATCC 15456 were incubated in TSB at 5 C for 10 days, 10 C for 4 days, 25 C for 24 hours, and 35 C for 48 hours. Cultures were grown in duplicate flasks and the experiment was replicated once. For each duplicate flask, a 1% aliquot of culture was added to a series of flasks containing 0.05M phosphate-0.1% peptone buffer at pH 7.0 and BHA at concentrations from 0 to 350 ppm. Survivors were enumerated by plate counts on TSA after 0,

5, and 15 minutes of incubation at 26 C in an agitating water bath. For each incubation temperature, two 1000 ml volumes of culture were grown and extracted for microbial lipids by the method previously described. Total and polar lipids were analyzed for fatty acid composition by gas-liquid chromatography as before.

B. Fatty Acid Composition as an Indicator of Bacterial Susceptibility to BHA

Test Organisms

The bacteria tested included the gram-negative species: Escherichia coli ATCC 11775, Pseudomonas fluorescens ATCC 15456, Pseudomonas fragi ATCC 4973, Salmonella anatum and the gram-positive species: Bacillus cereus ATCC 14579, Staphylococcus aureus ATCC 12600, Staplylococcus aureus NR, Staphylococcus aureus A-100, Staphylococcus aureus S-6, and Clostridium perfringens ATCC 12917. The two species of Pseudomonas were obtained and maintained as previously described. Cultures of E. coli, S. aureus ATCC 12600, B. cereus and C. perfringens were obtained from the American Type Culture Collection, Rockville, Maryland. The cultures were received in freeze-dried form and were revived in 5 ml TSB except for C. perfringens which was revived in 10 ml tubes of Cooked Meat medium (Oxoid USA, Inc., Columbia, Maryland) plugged with a vaspar seal and incubated 48 hours at 37 C. The cultures were maintained in the Cooked Meat medium at 4 C until required for use. Staphylococcus aureus was incubated at 35 C and E. coli and B. cereus at 32 C. Stock cultures were maintained on TSA slants at 4 C and transferred periodically to maintain viability.

Cultures of Salmonella anatum and Staphylococcus aureus NR were obtained from the Food Technology and Science Department culture collection and stock cultures were maintained on TSA slants at 4 C. S. aureus A-100 and S. aureus S-6 were obtained in freeze-dried form from Dr. M. S. Bergdoll, Food Research Institute, University of Wisconsin, Madison, Wisconsin. S. aureus A-100 is a potent enterotoxin producer. The cultures were revived in 5 ml TSB at 37 C and transferred to TSA slants for storage at 4 C. The cultures were transferred periodically to maintain viability. Commerically prepared media used in this study were obtained from BBL, Cockeysville, Maryland, unless otherwise indicated.

Lethality of BHA

The lethal effect of BHA against several bacterial species was determined as previously described. All organisms except for C. perfringens were treated in the following manner. Cells were grown in duplicate flasks of TSB at the optimal temperature for each organism in an agitating water bath to serve as starter cultures. A 1% aliquot of each culture was transferred to fresh duplicate flasks of broth and reincubated. A 1% inoculum of each culture was added to a series of flasks containing 0.05M phosphate-0.1% peptone buffer at pH 7.0 and BHA at concentrations from 0 to 400 ppm. Survivors were enumerated by plate counts on TSA after 0, 5, and 15 minutes of incubation at the optimum temperature for the particular organism.

Duplicate cultures of C. perfringens were grown in 125 ml Fluid Thioglycollate medium without indicator (Oxoid USA, Inc., Columbia, Maryland) in screw-top bottles at 37 C. One transfer was made into

fresh media to insure a viable culture. For each duplicate bottle, a 1% aliquot of culture was added to a series of flasks containing 20% Fluid Thioglycollate medium without indicator and 0 to 400 ppm BHA. Survivors were enumerated by plate counts on TSA containing additional reducing agents (0.25 g/L L(-) cystine and 0.5 g/L sodium thioglycollate) under anaerobic conditions as provided by an Oxoid anaerobe jar and system.

Lipid Extraction, Fractionation, and Analysis

Microbial lipids were extracted as before using a chloroform-methanol procedure and separated into neutral and polar lipid fractions by a modified silicic acid slurry method. Methyl esters of total and polar lipid fractions were prepared and analyzed for fatty acid composition using gas-liquid chromatography. Major fatty acids were identified by comparison of retention times with those of pure fatty acid methyl ester standards and by mass spectroscopy.

C. Statistical Analysis

All data were analyzed by analysis of variance using the Statistical Analysis System (SAS) of The University of Tennessee Computer Center. The experimental design was a factorial of a completely randomized block, either growth concentration BHA x treatment concentration BHA x time x replication or treatment concentration BHA x time x replication.

Where appropriate, significance among means was measured by Duncan's Multiple Range Test (Steel and Torrie, 1960). Regression

analyses were performed to determine the type of relationship between treatment, susceptibility, and/or fatty acid composition.

CHAPTER IV

RESULTS AND DISCUSSION

A. Modification of Bacterial Fatty Acid Composition and Relationship to Bacterial Susceptibility

Two species of Pseudomonas were grown in the presence of sublethal concentrations of the phenolic antioxidant/antimicrobial butylated hydroxyanisole (BHA) and subsequently challenged with varying concentrations of BHA while suspended in a non-nutritive buffer. The objective of this experiment was to determine what effect, if any, BHA in the growth media might have on the fatty acid composition of the microorganism and the possible relationship of any modifications of the fatty acid composition to the susceptibility of the microorganism.

Lethal Effect of BHA

A wide variance in susceptibility to the action of BHA was seen for P. fluorescens and P. fragi. P. fluorescens proved more susceptible than P. fragi to the antimicrobial activity of BHA. The dose response curves for P. fluorescens grown in the presence of 0-125 ppm BHA and subsequently challenged with concentrations of BHA to 450 ppm for 15 minutes are depicted in Figure 2. Appendix C contains regression equations for all plotted curves. The value for the LD_{90} (concentration of BHA required to reduce the viable population by 90%) were as follows: 120 ppm for the control; 166 ppm for cells grown in 50 ppm BHA; 130 ppm for cells grown in 100 BHA; $\geq$ 450 ppm for cells grown at 125 BHA.

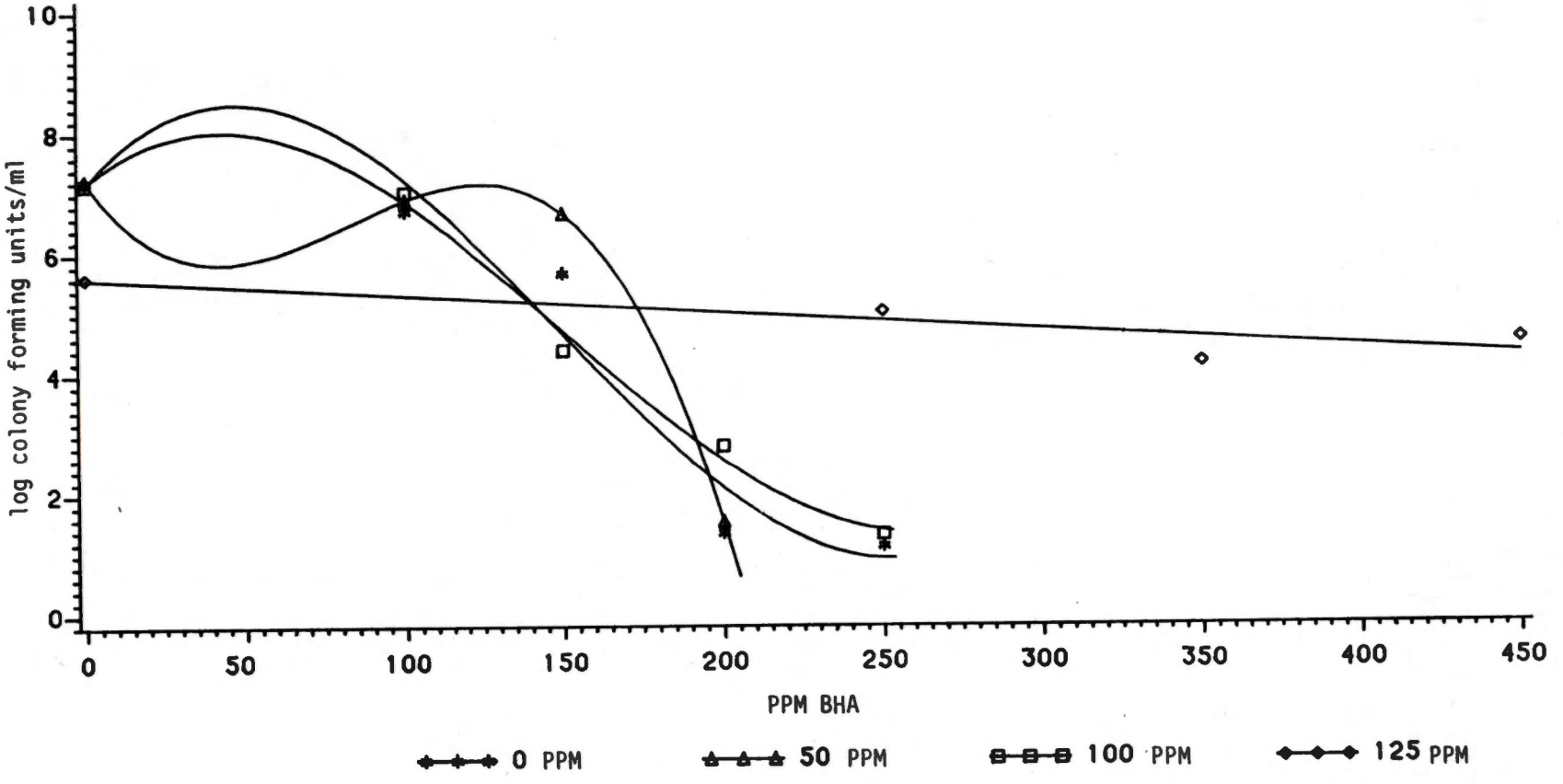


Figure 2. Dose response curves for Pseudomonas fluorescens grown in the presence of 0-125 ppm BHA and subsequently challenged with varying concentrations of BHA for 15 minutes.

For the control and 100 ppm cultures, little reduction in cell numbers occurred until a treatment concentration of 100 ppm after which an increase in treatment concentration elicited a corresponding reduction in cell number. Cell numbers were reduced to approximately log one at 250 ppm BHA. For the 50 ppm culture, cell numbers were not reduced until a treatment concentration of 150 ppm, after which a more rapid decline in population occurred than that evidenced in the control or 100 ppm cultures. Cell numbers were reduced to log one at 200 ppm BHA.

The growth of P. fluorescens was inhibited in media containing 125 ppm. An attempt has been made prior to the experimental procedure to synchronize cell numbers in each of the 4 growth cultures in order to minimize effect due to cell number. However, cell numbers were consistently suppressed in growth media with 125 ppm. After 48 hours of incubation, cell numbers were approximately 2 log cycles lower than a 24-hour control population. Cells which were grown in 125 ppm BHA, however, exhibited an extremely high tolerance to subsequent exposure to BHA. Cell numbers remained fairly constant in the presence of up to 450 ppm BHA for 15 minutes. The lethality rates of various challenge concentrations of BHA on P. fluorescens cultures grown in the presence of 0-125 ppm BHA are depicted in Figures A-1 through A-5.

While it can be said that the lethal effect of BHA was greatly influenced by prior exposure of P. fluorescens to subinhibitory levels of BHA in the growth media, no definitive pattern of influence emerges. Growth in 50 ppm BHA appears to cause an initial resistance to subsequent BHA treatment levels below 150 ppm after which a rapid decline in cell numbers occurs. Growth in 125 ppm causes the cells to be almost totally

resistant to the action of BHA. However, growth in 100 ppm BHA causes the cells to be only slightly more resistant than the control population and certainly less resistant than the 50 ppm culture as evidenced by their respective LD_{90} values of 130 ppm for the 100 ppm culture and 160 ppm for the 50 ppm culture. Generally, growth of P. fluorescens at nontoxic levels of BHA appeared to increase its subsequent resistance to normally lethal concentrations of BHA. Resistance may increase during growth in the presence of BHA through change in cellular structure, thereby decreasing permeability to the inhibitor. Phenolic compounds are known to denature proteins and increase cell permeability (Davis et al., 1973). The antimicrobial activity of BHA has been theorized to be in part membrane-mediated (Davidson and Branen, 1980b). A change in membrane structure, possibly through adjustment of lipid content, may prove to be a protective measure in maintaining the membrane barrier function or may act to preserve the necessary environment for enzyme-substrate interactions. The adaptive response may also be due to a decrease in the levels of activity of membrane-bound enzymes since it is also possible that the mechanism of inhibition by phenolic antioxidants may be related to inactivation of essential enzymes (Davidson and Branen, 1981).

The 125 ppm culture appeared to be almost totally resistant to the action of BHA. Drug-resistant mutants are known to appear spontaneously within a population of are directed by the presence of the drug (Davis et al., 1973). Microbial cultures evolve rapidly due to large populations, short generation times, and strong selective pressures which allow for extremely rapid selection. BHA resistant mutants have been selected for by the presence of BHA in the growth media at levels of 125 ppm.

Some kinds of drug resistance increase adaptively during growth in the presence of the inhibitor (Davis et al., 1973). In contrast to genotypic resistance, such induced phenotypic resistance is lost after several generations of growth without the drug. The loss in susceptibility of cells grown in the presence of 125 ppm BHA to the antimicrobial action of BHA was found to be maintained through one transfer into broth without BHA (Figure 3). This suggests a genotypic change which arose through mutation and selection rather than a phenotypic adaption by the organism; however, additional transfers would be necessary to assure a maintenance of resistance through several generations. Work by Davidson (1979) has also indicated that P. fluorescens may become tolerant to BHA. This point is an important factor in the application of BHA as an antimicrobial in food systems. BHA could prevent growth by competitive flora while allowing spoilage of the product by resistant strains of P. fluorescens.

Pseudomonas fragi cells were grown in the presence of 0 to 300 ppm BHA and challenged with 0-450 ppm BHA for 15 minutes (Figure 4). Control cells were relatively resistant to the action of BHA with an LD_{90} of $\geq$400 ppm. When grown in the presence of 50 ppm the initial response of the cells was similar to the control; however, above 250 ppm the lethal effect of BHA became evident. Population numbers were suppressed in cultures containing 100 ppm BHA. Afer 36 hours of incubation cell numbers were one log lower than a 24-hour control culture. With subsequent BHA treatment, some lethal effects were found at levels as low as 100 ppm. The LD_{90} values were 355 for the 50 ppm culture and 247 for the 100 ppm culture.

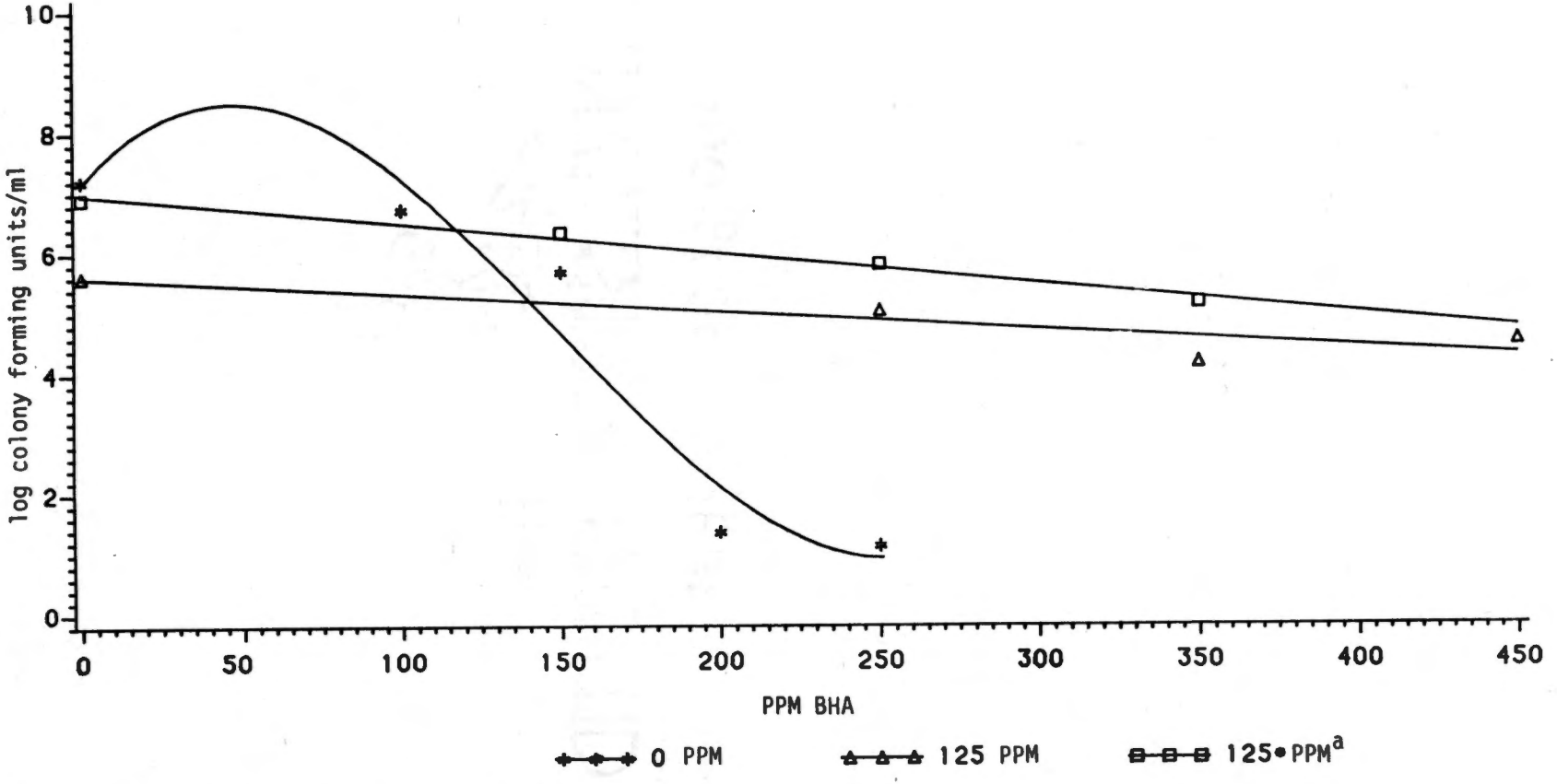


Figure 3. Dose response curves for Pseudomonas fluorescens grown in media containing 0 and 125 ppm BHA and for cells grown in the presence of 125 ppm BHA carried through one transfer in broth with no BHA. Cells were subsequently challenged with varying concentrations of BHA for 15 minutes.

[a]Cells grown at 125 ppm BHA carried through one transfer in broth with no BHA.

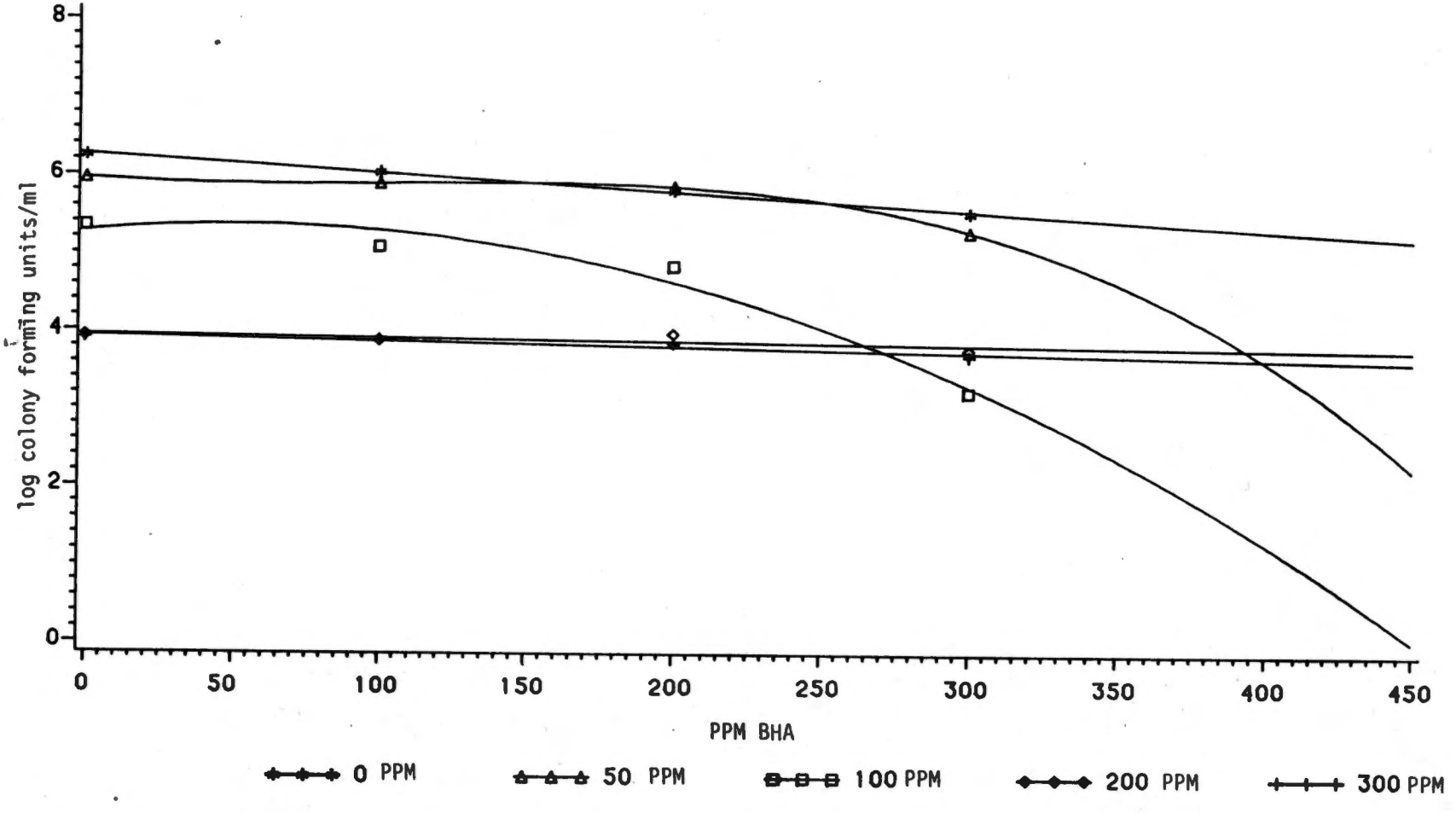


Figure 4. Dose response curves for Pseudomonas fragi grown in the presence of 0-300 ppm BHA and subsequently challenged with varying concentrations of BHA for 15 minutes.

Cultures grown in 200 and 300 ppm BHA had lower total cell numbers after 36 and 48 hours of incubation than the control after 24 hours of incubation. Cell numbers were nearly 2.5 log cycles lower. As with P. fluorescens, a selection process may have occurred leaving a population virtually unaffected by the action of BHA with LD_{90} values in excess of 450 ppm (Davis et al., 1973). Cultures grown at 50 and 100 ppm BHA may have represented a transition from a fairly resistant control through cultures made more susceptible to the action of BHA through sensitization by growth in the substance until a smaller, more highly resistant culture was selected. The lethality rates of various challenge concentrations of BHA on each P. fragi culture grown in the presence of 0-300 ppm BHA are depicted in Figure A-6 through A-10.

Modification of Fatty Acid and Phospholipid Content and Relationship to Lethality of BHA

The fatty acid composition of the total and polar lipid fractions from P. fluorescens grown in Trypticase Soy broth (TSB) in the presence of various concentrations of BHA are depicted in Tables 6 and 7. The fatty acids comprising the largest relative percentage for all growth medias were C16:0, C16:1, and C18:1. In the presence of 50-125 ppm BHA, the relative unsaturated to saturated fatty acid ratio for both total and polar lipid fractions decreased significantly ($P<0.05$).

In the total lipid fraction, a decrease in C16:1 was noted with increasing concentrations of BHA in the growth media. Relative percent unsaturation contributed by C16:1 and C18:1 declined from 58% to 47.8%. A steady increase in the relative percentages of 12 and 14 carbon fatty acids occurred as well ($P<0.05$). In the polar lipid fraction, the relative percent unsaturation contributed by C18:1 and C16:1 declined

Table 6. Fatty acid composition of the total lipid fraction from Pseudomonas fluorescens grown in Trypticase Soy broth at 26 C in the presence of various concentrations of butylated hydroxyanisole

Fatty Acid[a]	BHA in growth media (ppm)			
	Control	50	100	125
	Relative percent peak area			
12:0	1.00	2.25	7.97	8.44
14:0	0.60	0.85	0.94	0.96
16:1	35.52	27.40	27.26	27.00
16:0	33.16	32.18	32.97	31.27
17:0[b]	3.01	1.03	0.56	0.66
18:1	22.39	23.45	21.85	20.80
18:0	3.68	11.61	7.90	9.90
Others	0.64	1.23	0.55	0.97
Unsaturated/ Saturated	1.38	1.03	0.97	0.92

[a] First number denotes number of carbon atoms, second number is number of double bonds.

[b] Total concentration of α17:0, 17:0Δ , 17:0.

Table 7. Fatty acid composition of the polar lipid fraction from Pseudomonas fluorencens grown in Trypticase Soy broth at 26 C in the presence of various concentrations of butylated hydroxyanisole

Fatty Acid[a]	BHA in growth media (ppm)			
	Control	50	100	125
	Relative percent peak area			
11:0	---	---	---	0.39
12:0	0.45	0.25	0.29	0.44
13:0	---	---	---	1.81
14:0	0.58	0.53	1.27	10.19
15:0[b]	0.51	0.15	0.81	10.93
16:1	35.79	29.50	25.30	25.04
16:0	31.60	36.42	34.88	21.38
17:0[c]	1.11	5.51	1.39	6.76
18:1	25.38	22.67	26.37	22.76
18:0	3.60	4.68	8.85	---
Other	0.98	0.29	0.84	0.30
Unsaturated/ Saturated	1.58	1.09	1.07	0.92

[a]First number denotes number of carbon atoms, second number is number of double bonds.

[b]Total concentration of α15:0 and 15:0.

[c]Total concentration of α17:0, 17:0Δ, and 17:0.

significantly from 61% to 47.8% over growth concentrations from 0 to 125 ppm (p 0.05). The saturated fatty acid C18:0 was eliminated in the 125 ppm culture while relative percentages of 14 and 15 carbon fatty acids increased to major proportions over the growth concentration range. Eleven and 13 carbon fatty acids were present in the 125 ppm culture only.

The data presented are in general agreement with the recent findings of Davidson and Branen (1980b). While the relative percentages of the major fatty acids C16:0, C16:1, and C18:1 are in close agreement, in their study, C18:0 was not detected in the polar or total lipid fractions of P. fluorescens. They also reported two unidentified peaks with a suggestion that one was cyc 17:0. In this study, several species of 17 carbon fatty acids were detected. Scheuerbrandt and Block (1962) had also reported the presence of a 17 carbon acid that comprised over 20% of the lipid content in P. fluorescens. Davidson and Branen (1980b) found that the relative ratio of unsaturated to saturated fatty acids declined significantly in the total lipid fractions from control to 50 ppm extracts. However, they attributed the decline to a decrease in oleic acid (C18:1) while in the present study a loss in unsaturation between the control and 50 ppm extracts occurred due to a drop in C16:1 content. No overall change in the relative unsaturated fatty acid ratio of the polar fraction was found in the study by Davidson and Branen while a decrease in C16:1 and C18:1 contents caused a decline in the relative percent unsaturated fatty acids in the polar fraction of this experiment. Differences in quantitative data were probably due to differing experimental procedures including growth media and gas chromatographic techniques.

A modified lipid content was maintained in both total and polar lipid fractions through one transfer of cells grown in 125 ppm BHA to broth containing no BHA (Tables 8 and 9). Although the individual fatty acid contents differed from that of both the control and 125 ppm cultures, decreased levels of the unsaturated fatty acids C16:1 and C18:1 persisted in the transferred culture and in this respect resembled cells of the 125 ppm culture. Maintenance of decreased susceptibility to the action of BHA through one transfer had also been observed.

Overall, a steady decline in the relative ratio of unsaturated fatty acids occurred with increasing concentrations of BHA in the growth media (Figure 5) for both total and polar fractions of P. fluorescens. The decrease in relative unsaturation was accompanied by increased resistance to the antimicrobial effects of BHA (Table 10). An increase in the relative percentage of saturated fatty acids and decreased susceptibility were maintained through one transfer in broth with no BHA which tends to support a relationship between relative unsaturated fatty acid content and cellular susceptibility to the action of BHA.

The major fatty acids detected in polar and total lipid extracts of P. fragi were also C16:0, C16:1, and C18:1 (Tables 11 and 12). In a study by Miller et al. (1971), palmitic acid (C16:0) was found to be the major lipid component synthesized by P. fragi. They also found that palmitic, palmitoleic (C16:1) and an 18 carbon acid possessing one double bond comprised more than 93% of the free fatty acid content.

Table 8. Fatty acid composition of the total lipid fraction from Pseudomonas fluorescens grown in Trypticase Soy broth at 26 C in the presence of 125 ppm BHA and carried through one transfer in broth with no BHA

Fatty Acid[a]	BHA in growth media (ppm)		
	Control	125	125[b]
	Relative percent peak area		
12:0	1.00	8.44	5.05
14:0	0.60	0.96	1.18
16:1	35.52	27.00	24.16
16:0	33.16	31.27	35.45
17:0[c]	3.01	0.66	1.35
18:1	22.39	20.80	19.58
18:0	3.68	9.90	12.07
Others	0.64	0.97	1.16
Unsaturated/ Saturated	1.38	0.92	0.78

[a]First number denotes number of carbon atoms, second number is number of double bonds.

[b]Cells grown at 125 ppm BHA carried through one transfer in broth with no BHA.

[c]Total concentration of α 17:0, 17:0Δ, 17:0.

Table 9. Fatty acid composition of the polar lipid fraction from Pseudomonas fluorescens grown in Trypticase Soy broth at 26 C in the presence of 125 ppm BHA and carried through one transfer in broth with no BHA

Fatty Acid[a]	BHA in growth media (ppm)		
	Control	125	125[b]
	Relative percent peak area		
11:0	---	0.39	6.26
12:0	0.45	0.44	0.46
13:0	---	1.81	1.50
14:0	0.58	10.19	1.02
15:0[c]	0.51	10.93	6.06
16:1	35.79	25.04	27.52
16:0	31.60	21.38	29.19
17:0[d]	1.11	6.76	7.52
18:1	25.38	22.76	16.21
18:0	3.60	---	3.81
Others	0.98	0.30	0.45
Unsaturated/ Saturated	1.58	0.92	0.78

[a]First number denotes number of carbon atoms, second number is number of double bonds.

[b]Cells grown at 125 ppm BHA carried through one transfer in broth with no BHA.

[c]Total concentration of α15:0 and 15:0.

[d]Total concentration of α17:0, 17:0$_{\Delta}$, and 17:0.

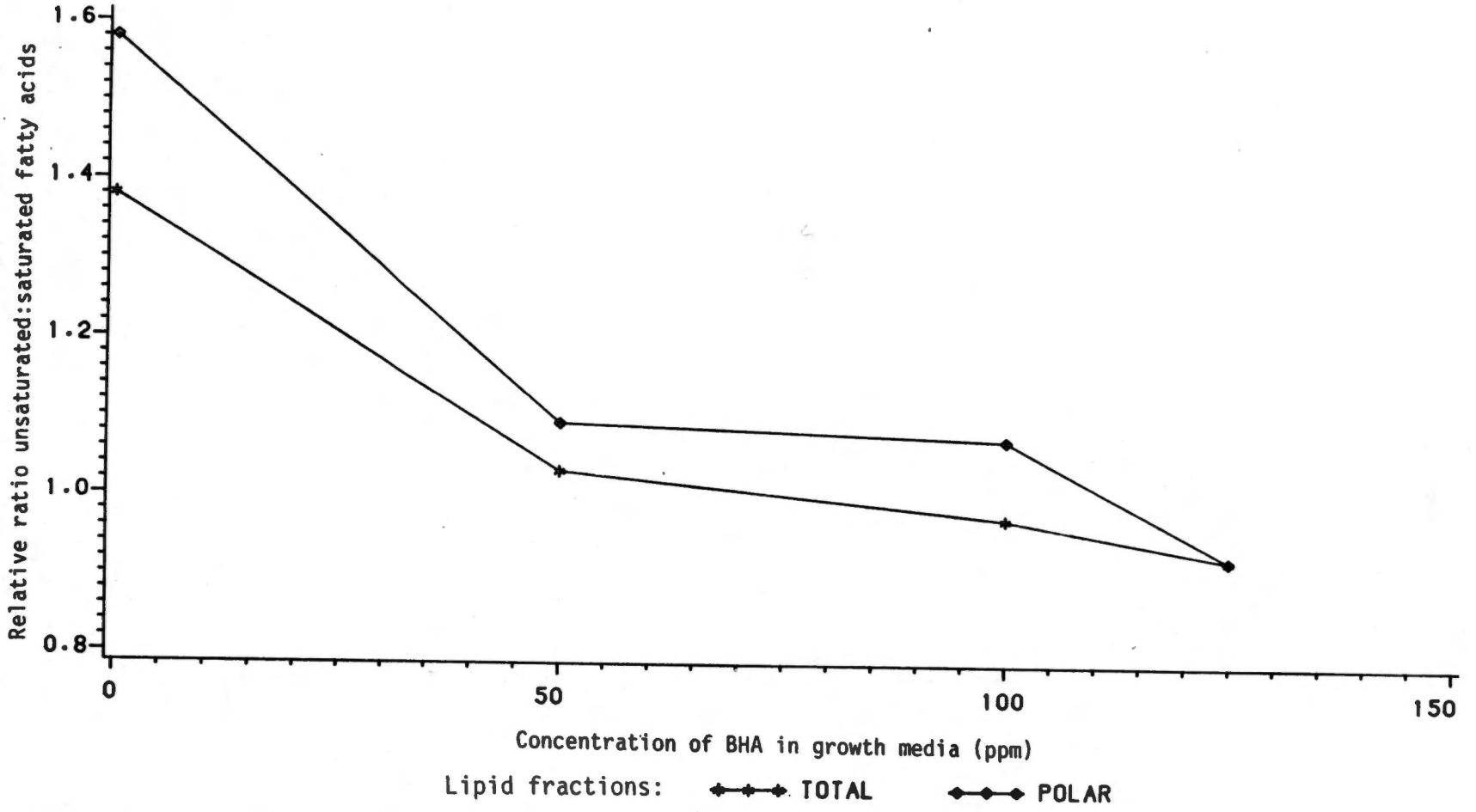


Figure 5. Relative unsaturated:saturated fatty acid ratios for polar and total lipid fractions of Pseudomonas fluorescens grown in the presence of 0-125 ppm BHA.

Table 10. LD_{90} values and relative percent unsaturated:saturated fatty acid ratios for total and polar lipid fractions of *Pseudomonas fluorescens* grown in the presence of 0-125 ppm BHA

Concentration BHA	LD_{90}	Ratio Relative Percent Unsaturated: Saturated Fatty Acids	
		Total Fraction	Polar Fraction
0	120	1.38	1.58
50	166	1.03	1.09
100	130	0.97	1.07
125	$\geq$450	0.92	0.92

Table 11. Fatty acid composition of the total lipid fraction from Pseudomonas fragi grown in Trypticase Soy broth at 26 C in the presence of various concentrations of butylated hydroxyanisole

Fatty Acid[a]	BHA in growth media (ppm)				
	Control	50	100	200	300
	Relative percent peak area				
12:0	1.42	2.81	5.00	4.15	3.59
14:0	1.57	3.96	1.09	1.32	1.02
15:0[b]	2.97	1.94	1.53	1.33	0.83
16:1	14.84	8.12	14.93	12.46	15.46
16:0	27.95	24.45	22.57	20.63	27.82
17:0[c]	11.07	8.44	8.47	7.53	3.06
18:1	22.73	30.00	23.03	25.88	24.87
18:0	14.64	14.29	13.68	12.20	11.67
19:0	2.35	5.40	4.48	12.62	8.64
Unknown	---	---	2.07	2.10	3.57
Others	0.46	0.59	5.22	1.88	3.04
Unsaturated/ Saturated	0.60	0.62	0.61	0.62	0.68

[a]First number denotes number of carbon atoms; second number is number of double bonds.

[b]Total concentration of α15:0 and 15:0.

[c]Total concentration of α17:0, 17:0Δ, and 17:0.

Table 12. Fatty acid composition of the polar lipid fraction from Pseudomonas fragi grown in Trypticase Soy broth in the presence of various concentrations of butylated hydroxyanisole

Fatty Acid[a]	BHA in growth media (ppm)				
	Control	50	100	200	300
	Relative percent peak area				
11:0	---	---	0.26	1.73	---
12:0	0.35	0.27	0.29	1.75	1.47
14:0	0.88	0.83	3.43	1.47	1.63
15:0[b]	1.11	1.99	4.09	1.08	2.56
16:1	18.66	22.58	27.21	13.23	36.17
16:0	36.66	33.75	27.46	27.00	31.45
17:0[c]	18.59	17.83	9.62	6.06	1.88
18:1	17.67	14.93	19.06	34.00	21.66
18:0	3.35	2.23	3.29	9.88	2.02
19:0	1.25	5.08	3.01	---	0.89
Others	1.53	2.84	2.11	3.80	0.27
Unsaturated/ Saturated	0.57	0.60	0.86	0.90	1.37

[a]First number denotes number of carbon atoms; second number is number of double bonds.

[b]Total concentration of α15:0 and 15:0.

[c]Total concentration of α17:0, 17:0Δ, and 17:0.

In the total lipid fraction, a steady decrease in the relative percentages of C15:0, C17:0, and C18:0 fatty acids occurred with treatments from 0 to 300 ppm. The relative percentages of other fatty acids differed according to treatment level with no established trends. Fatty acids such as C19:0 increased to a relative percentage of 12.62%, at 200 ppm BHA, which dropped to 8.64% with a treatment level of 300 ppm. However, both values were significantly higher than that of the control with 2.35% ($P<0.05$), indicating an actual change in the relative percentage of C19:0 with BHA treatment.

Davidson and Branen (1980b) had reported an increase in the relative unsaturated to saturated fatty acid ratio with BHA treatment. In this study such an increase was slight (Figure 6). For control cells the unsaturated fatty acids C16:1 and C18:1 comprised a total relative percentage of 37.6%. Cells grown in 50 to 200 ppm BHA contained 38% C16:1 and C18:1, and cells from the 300 ppm culture contained 40% C16:1 and C18:1. No significant increase in C16:1 occurred with treatment in this study as had been reported in the work by Davidson and Branen. They had attributed the rise in unsaturation to an increase in the relative percentage of C16:1. In this study, saturated 18 and 19 carbon fatty acids were identified which had not been detected in their work.

The fatty acid content of P. fragi differed both qualitatively and quantitatively from P. fluorescens. Unlike P. fluorescens, 15 and 19 carbon fatty acids were identified as components of the total lipid fraction of P. fragi and 17 carbon fatty acids comprised a larger proportion of the total lipid composition.

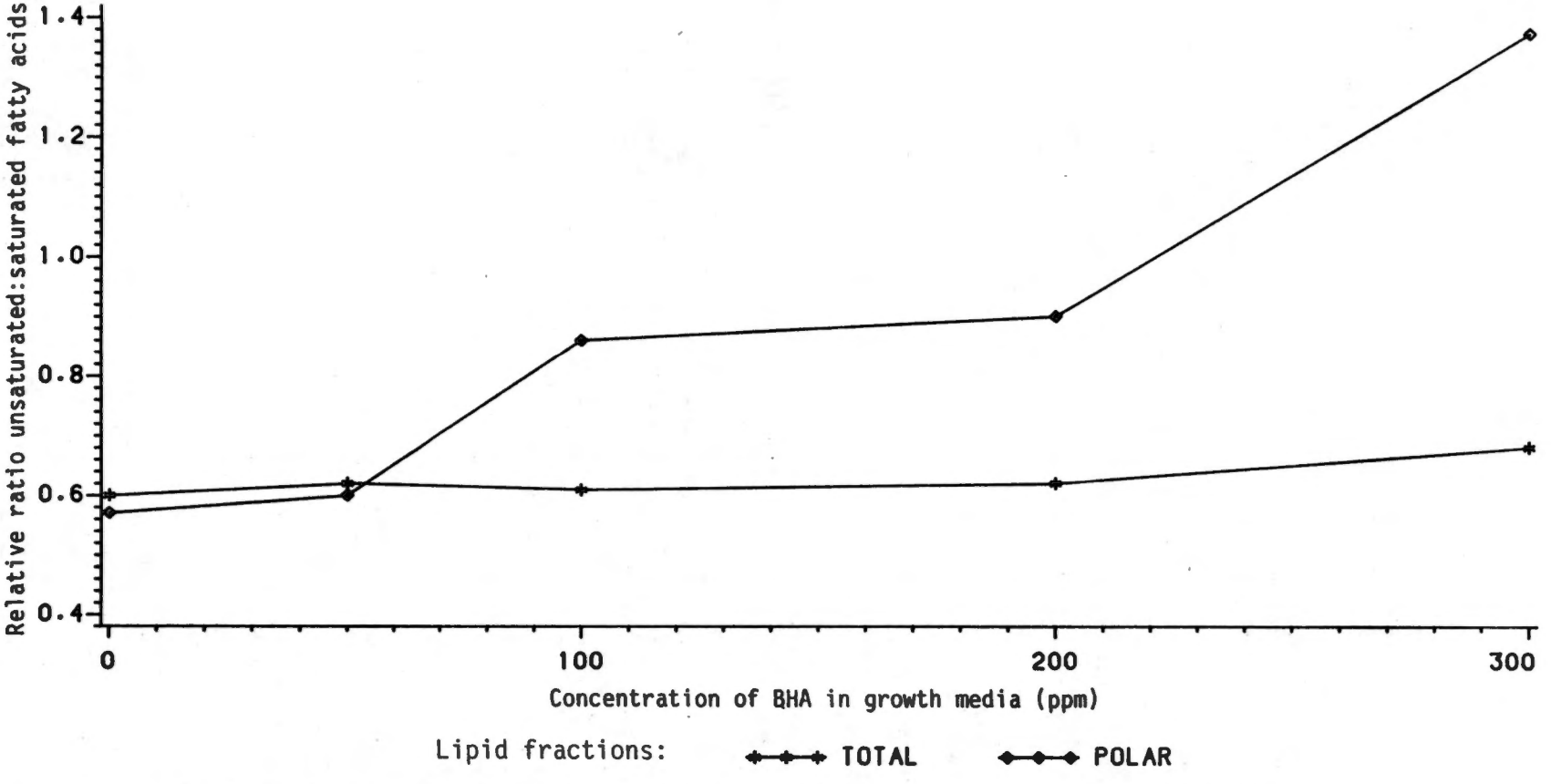


Figure 6. Relative unsaturated:saturated fatty acid ratios for polar and total lipid fractions of Pseudomonas fragi grown in the presence of 0-300 ppm BHA.

The polar lipid composition of P. fragi was characterized by seemingly random changes in fatty acid composition with BHA treatment from 0 to 300 ppm. Like the study by Davidson and Branen (1980b), the relative unsaturated to saturated fatty acid ratio increased with BHA concentration in the growth media ($p<0.05$) (Figure 6). The control population contained 36.3% C16:1 and C18:1 which rose to 47.8% in the 300 ppm culture. The major fatty acids detected were C16:0, C16:1, and C18:1.

An 11 carbon fatty acid was identified in the polar fraction for cultures treated with 100 and 200 ppm. A 19 carbon fatty acid was also identified which was detected in all polar fractions except that from the 200 ppm culture. One consistent trend was noted for the 17 carbon fatty acid group where significant decreases in relative percentages occurred with increasing BHA treatment. This had been reported for the total lipid fraction as well. The saturated 18 carbon fatty acid comprised a smaller relative proportion of the polar lipid fraction than those of the total.

No relationship between the proportion of unsaturated fatty acids in P. fragi and susceptibility could be identified. While an increase in the relative percentage unsaturated fatty acids occurred in the polar fraction with increasing BHA concentration, no corresponding increase in inhibition occurred as with P. fluorescens (Table 13).

The phospholipid fractions of P. fragi and P. fluorescens were separated and examined using thin layer chromatography. Phosphatidyl inositol (PI), phosphatidyl glycerol (PG), and phosphatidyl ethanolamine (PE) proved to be the major phospholipids present. For both organisms,

Table 13. LD_{90} values and relative percent unsaturated:saturated fatty acid ratios for total and polar lipid fractions of *Pseudomonas fragi* grown in the presence of 0-300 ppm BHA

Concentration BHA	LD_{90}	Ratio Relative Percent Unsaturated: Saturated Fatty Acids	
		Total Fraction	Polar Fraction
0	$\geq$400	0.60	0.57
50	355	0.62	0.60
100	247	0.61	0.86
200	$\geq$450	0.62	0.90
300	$\geq$450	0.68	1.37

PE was found in the largest proportions followed by PG. These results agree with the findings of Davidson and Branen (1980b) and Hancock and Meadows (1969) who reported that the majority of phospholipids in Pseudomonas spp. were phosphatidyl ethanolamine with lesser amounts of phosphatidyl glycerol. The relative percentages contained in each organism grown in the presence of various concentrations of BHA are listed in Table 14.

Davidson and Branen (1980b) reported no significant differences in the proportions of phospholipids of P. fluorescens between the control and a culture treated with 50 ppm BHA. In this study, P. fluorescens exhibited an increased proportion of PI and PG over that of the control with increasing growth concentration of BHA. A parallel decrease in the relative percentage of PE occurred as well (Figure 7). Cells grown at 125 ppm carried through one transfer in broth with no BHA exhibited phospholipid levels close to that of the control; PI-1.33%, PE-69.83%, and PG-28.84%.

For the control population of P. fragi relative percentages of phospholipids were 24.97% PI, 42.60% PE, and 32.43% PG. Figure 8 illustrates an increase in the proportion of PE over that of the control with treatment levels of BHA in excess of 100 ppm. A drop in the proportion of PE occurred at the 50 ppm treatment level. The relative proportion of PG varied with BHA treatment level; however, no established trend was elaborated. PI levels declined slightly after an initial rise in percentage at the 50 ppm level. As Davidson and Branen (1980b) had reported, cells grown at 100 ppm did have a higher relative percentage of phosphatidyl glycerol and lower proportion of phosphatidyl ethanolamine than the control ($p<0.05$).

Table 14. Relative percentages of the phospholipid fractions of Pseudomonas fragi and Pseudomonas fluorescens grown in the presence of various concentrations of butylated hydroxyanisole

Sample	Relative Percentage		
	Phosphatidyl Inositol	Phosphatidyl Ethanolamine	Phosphatidyl Glycerol
P. fluorescens			
Control	1.33	69.83	24.84
50 ppm BHA	2.39	53.87	43.74
100 ppm BHA	4.84	51.28	43.88
125 ppm BHA	6.00	51.22	42.78
125[a] ppm BHA	1.75	76.17	22.08
P. fragi			
Control	24.97	42.60	32.43
50 ppm BHA	29.92	36.29	33.79
100 ppm BHA	24.88	38.41	36.71
200 ppm BHA	22.95	50.44	26.61
300 ppm BHA	20.89	47.01	32.09

[a]Cells grown at 125 ppm BHA carried through one transfer in broth with no BHA.

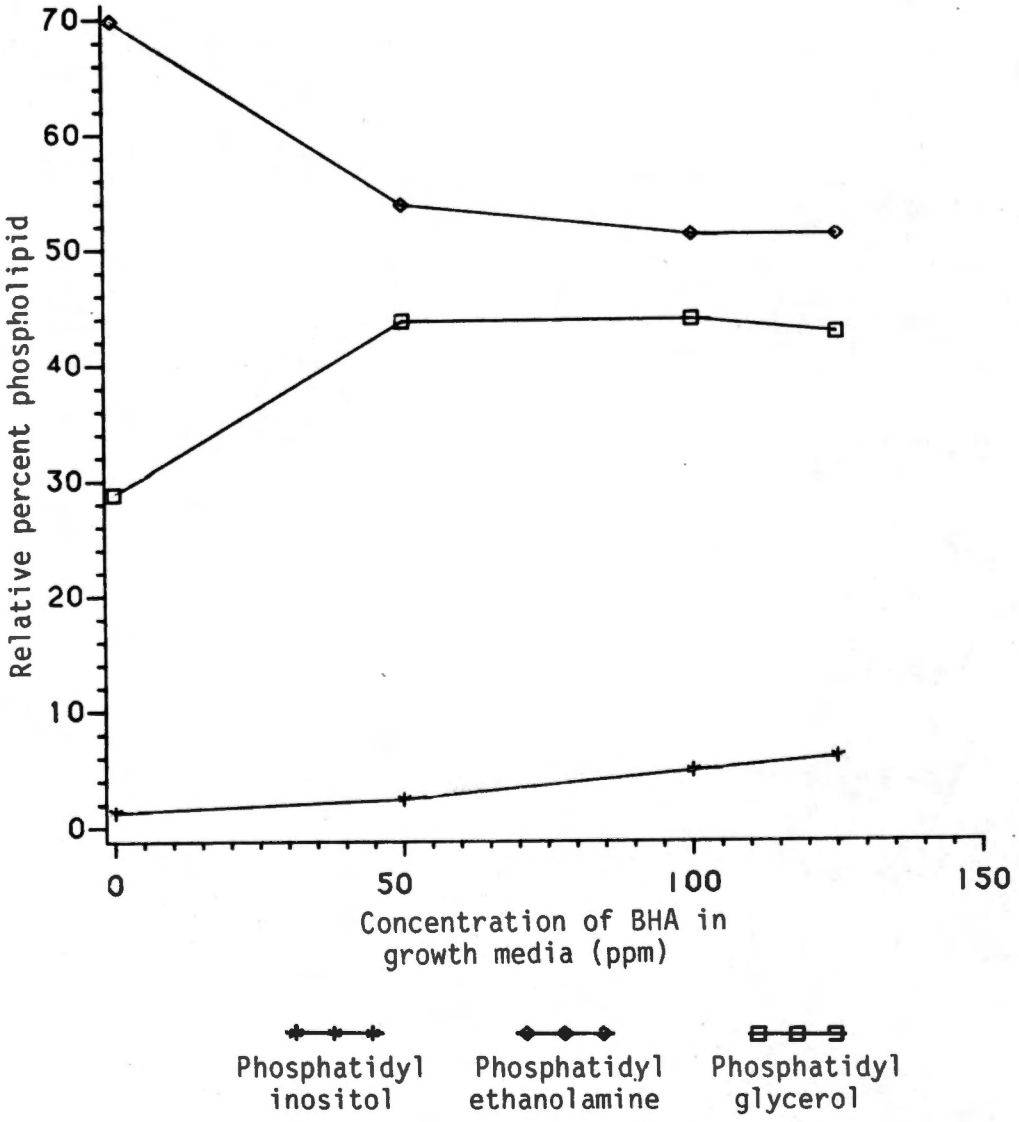


Figure 7. Relative percentages of the major phospholipids of Pseudomonas fluorescens grown in the presence of 0-125 ppm BHA.

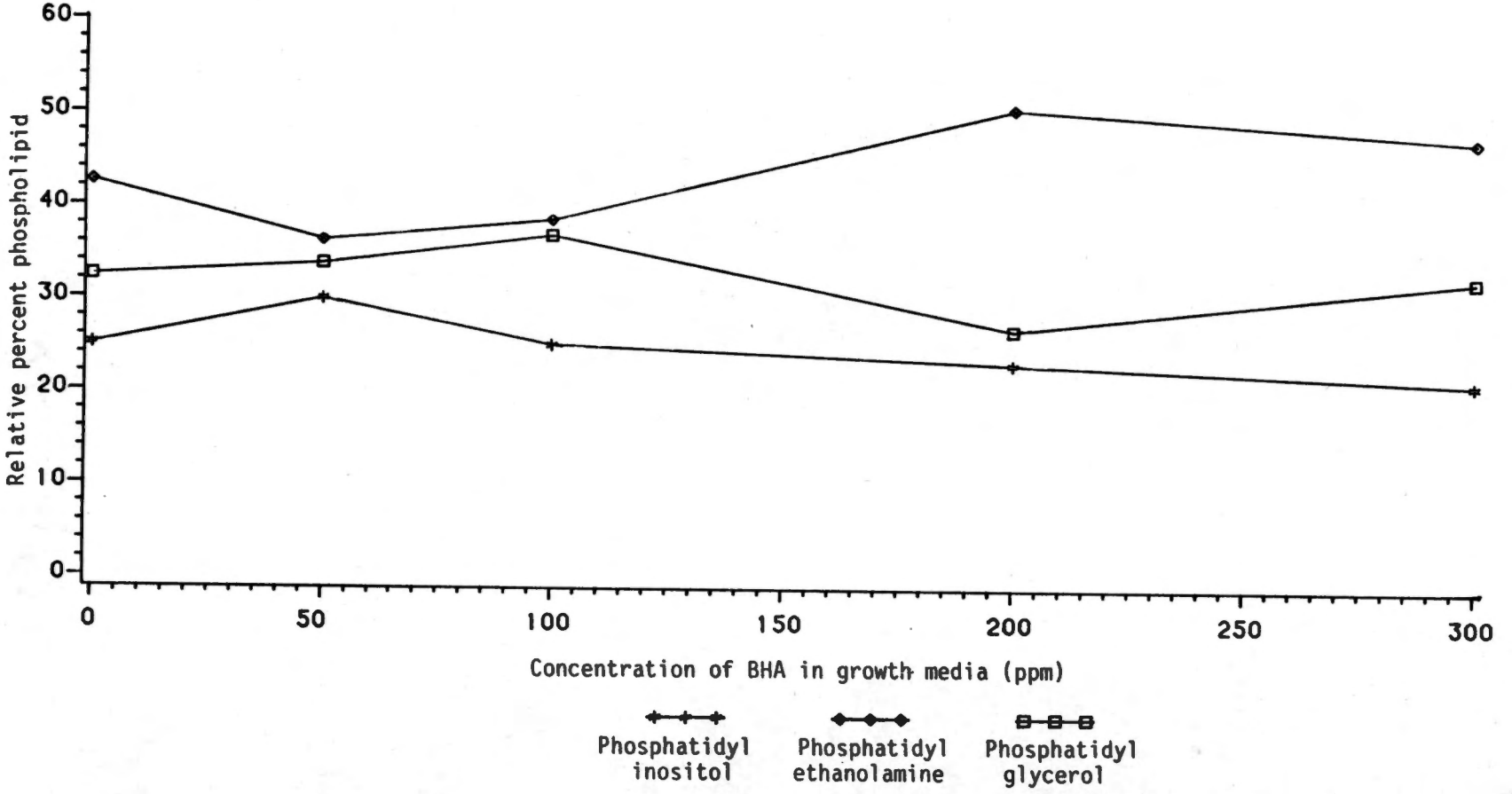


Figure 8. Relative percentages of the major phospholipids of Pseudomonas fragi Grown in the presence of 0-300 ppm BHA.

For both P. fluorescens and P. fragi, growth in the presence of BHA altered phospholipid composition as well as total and polar fatty acid contents. This may have resulted in membrane alterations. The physical state of membrane lipids, of which phospholipids are most significant, is of critical importance to the activities of membrane bound enzymes (Eletr et al., 1974). Exterkate (1977) had reported the activity of pyrrolidone carboxylyl peptidase in Streptococcus cremoris to be dependent on the physiochemical state and organization of the membrane components. In a study by Ingram (1977), cells of E. coli were found to contain an altered fatty acid and phospholipid composition when grown in the presence of a variety of organic solvents and food additives. She theorized that changes in individual phospholipids components could be expected to alter the charge distribution of the membrane. This effect as well as changes in fatty acid composition could affect protein-lipid interactions as well as organization.

Influence of Incubation Temperature

This experiment was undertaken in order to determine if the fatty acid composition of P. fluorescens could be altered by incubation temperature to effect the same susceptibility changes or lack thereof as observed when cells were incubated in the presence of BHA. As determined previously in this study, growth in the presence of increasing concentrations of BHA appeared to cause a decrease in the relative percentage of unsaturated fatty acids for both the total and polar lipid fractions of P. fluorescens. This increase in the relative percentage of saturated fatty acids was paralleled by increased resistance to the action of BHA. The membrane lipid composition of

E. coli was found to be regulated by incubation temperature at the time of synthesis (Cronan, 1975). As growth temperature increased, E. coli incorporated increasing proportions of saturated and long chain fatty acids into phospholipids (Sinensky, 1974). This experiment would, therefore, tend to support a lipid mediated mode of action for BHA if the same changes in susceptibility occurred when the fatty acid composition was altered as a function of growth temperature.

As the temperature of growth was lowered from 35 to 5 C, the relative proportion of unsaturated fatty acids increased in both polar and total lipid fractions of P. fluorescens. This was in agreement with the findings of such researchers as Sinensky (1974) and Cronan (1975). Figures 9 and 10 depict the relative percentages of the major fatty acids in total and polar lipid fractions of P. fluorescens as altered by temperature. The combined relative percentage of the unsaturated fatty acids C16:1 and C18:1 was 54.01% (total lipid fraction) and 55.12% (polar lipid fraction) at 35 C which increased to 82.70% (total lipid fraction) and 74.55% (polar lipid fraction) at 5 C. Gaughron (1947) had found that cells cannot grow at temperatures below the solidification point of their lipids. The degree of fluidity of the lipid bilayer must be maintained within narrow limits to allow proper membrane function. The cell adapts to changing temperature by altering lipid composition in such a way as to restore membrane fluidity to a functional level (Fukashima et al., 1976). An increase in the synthesis of unsaturated fatty acids at low temperatures is such an adaption.

The dose response curves for P. fluorescens grown at 5, 15, 25, and 35 C are depicted in Figure 11. Cells of P. fluorescens grown

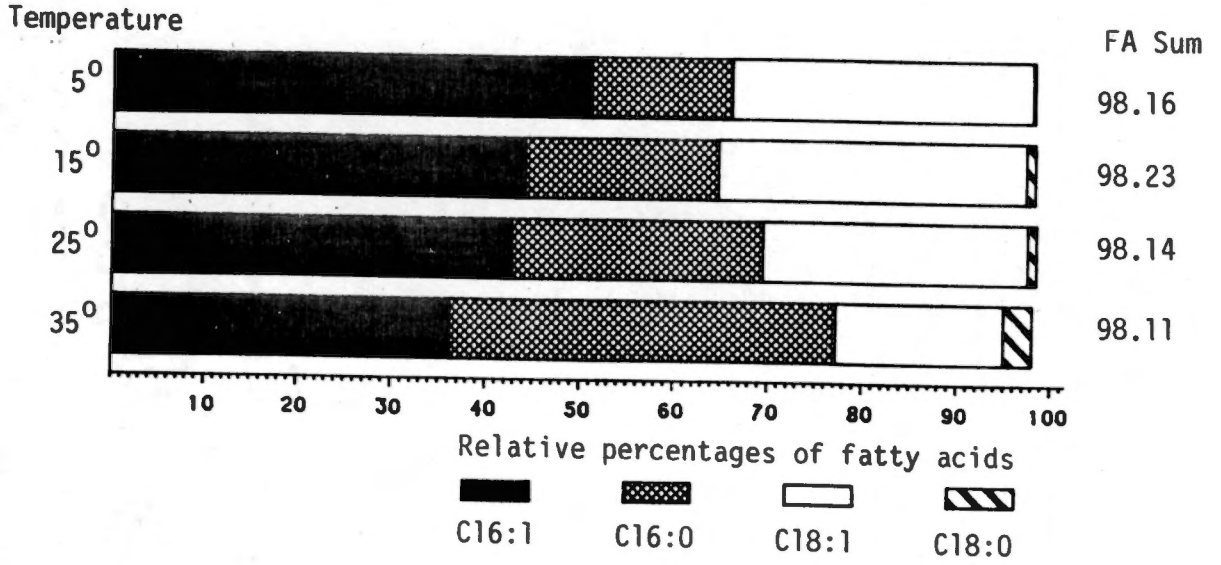


Figure 9. Relative percentages of major fatty acids for total lipid fractions of Pseudomonas fluorescens grown in Trypticase Soy broth at 5, 15, 25 and 35°C.

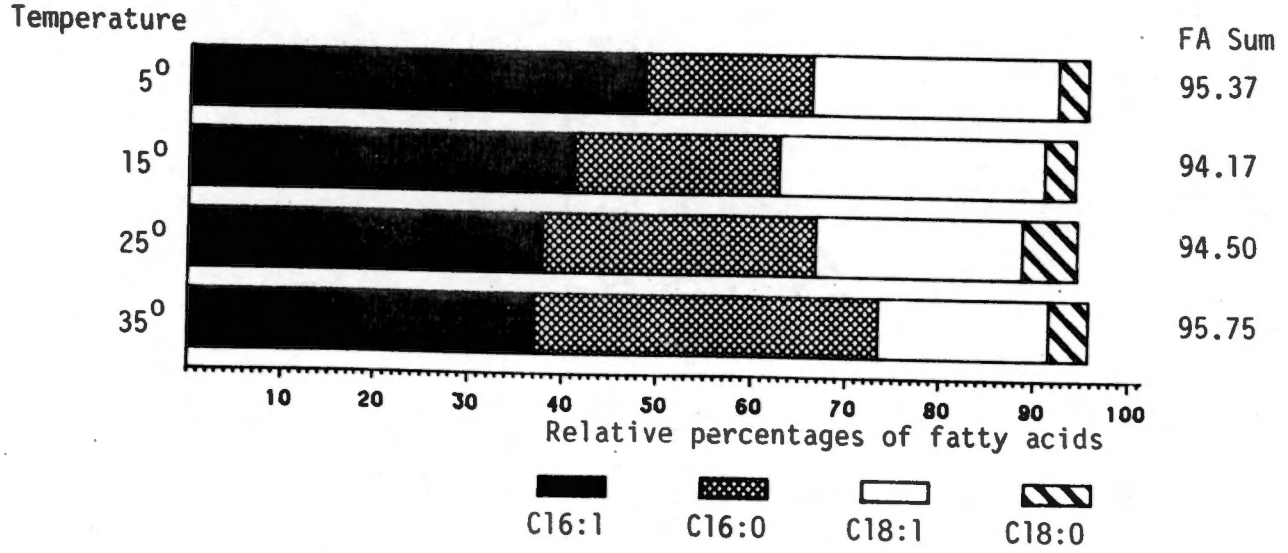


Figure 10. Relative percentages of major fatty acids for polar lipid fractions of Pseudomonas fluorescens grown in Trypticase Soy broth at 5, 15, 25 and 35°C.

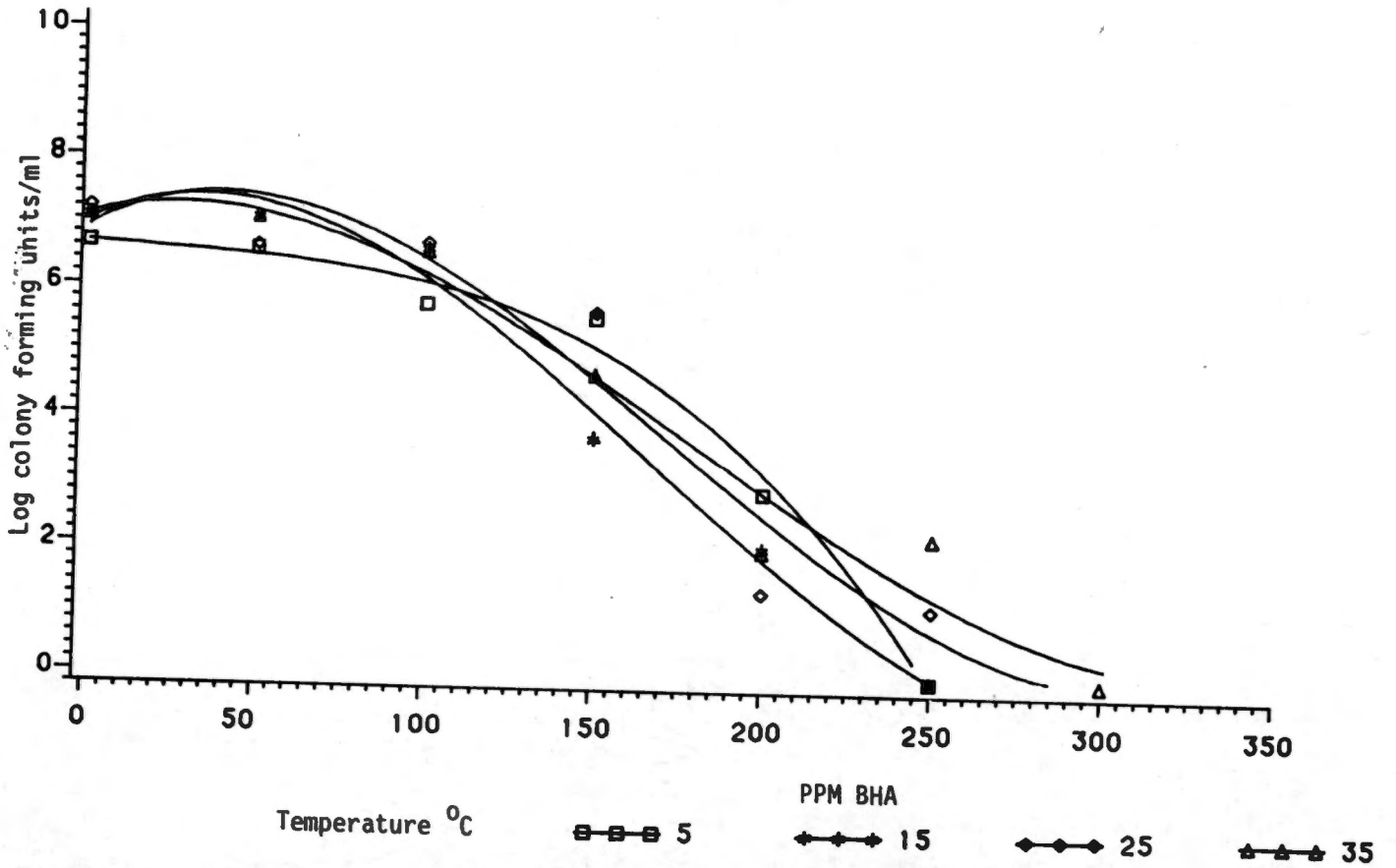


Figure 11. Dose response curves for Pseudomonas fluorescens incubated at 5, 15, 25 and 35°C and subsequently challenged with varying concentrations of BHA for 15 minutes.

at 5 C proved most resistant to the action of BHA with an LD_{90} value of 137 ppm. The LD_{90} values for cells grown at 15, 25, and 35 C were 108 ppm, 115 ppm, and 117 ppm respectively. An increase in susceptibility occurred as the cells were grown at higher temperatures which favored the increased synthesis of saturated fatty acids. The relationship of LD_{90} values to the relative percentage of unsaturated fatty acids at each growth temperature is depicted in Figure 12 (total lipid fraction) and Figure 13 (polar lipid fraction). These results are in direct contrast to findings reported earlier in this study. An increase in the relative percentage of saturated fatty acids as induced by growth of P. fluorescens in the presence of BHA conferred increased resistance on the part of the cells to subsequent treatment with BHA. An increase in the relative percentage of saturated fatty acids as induced by growth temperature decreased the resistance of P. fluorescens to the action of BHA. It may be noted that, in contrast to P. fluorescens, P. fragi showed no such relationship between the relative ratio of unsaturated to saturated fatty acids and susceptibility to the action of BHA but remained highly resistant despite alterations in fatty acid composition induced by growth in BHA. The inhibitory action of BHA may not be mediated through direct alteration of individual fatty acids but may occur as a result of alteration in the nonspecific interaction between membrane integral proteins and surrounding lipids. Eletr et al. (1973) had reported that an enzyme requirement for a given level of activity appeared to be satisfied by a bulk property of the lipid moiety rather than an absolute requirement for individual phospholipid molecules.

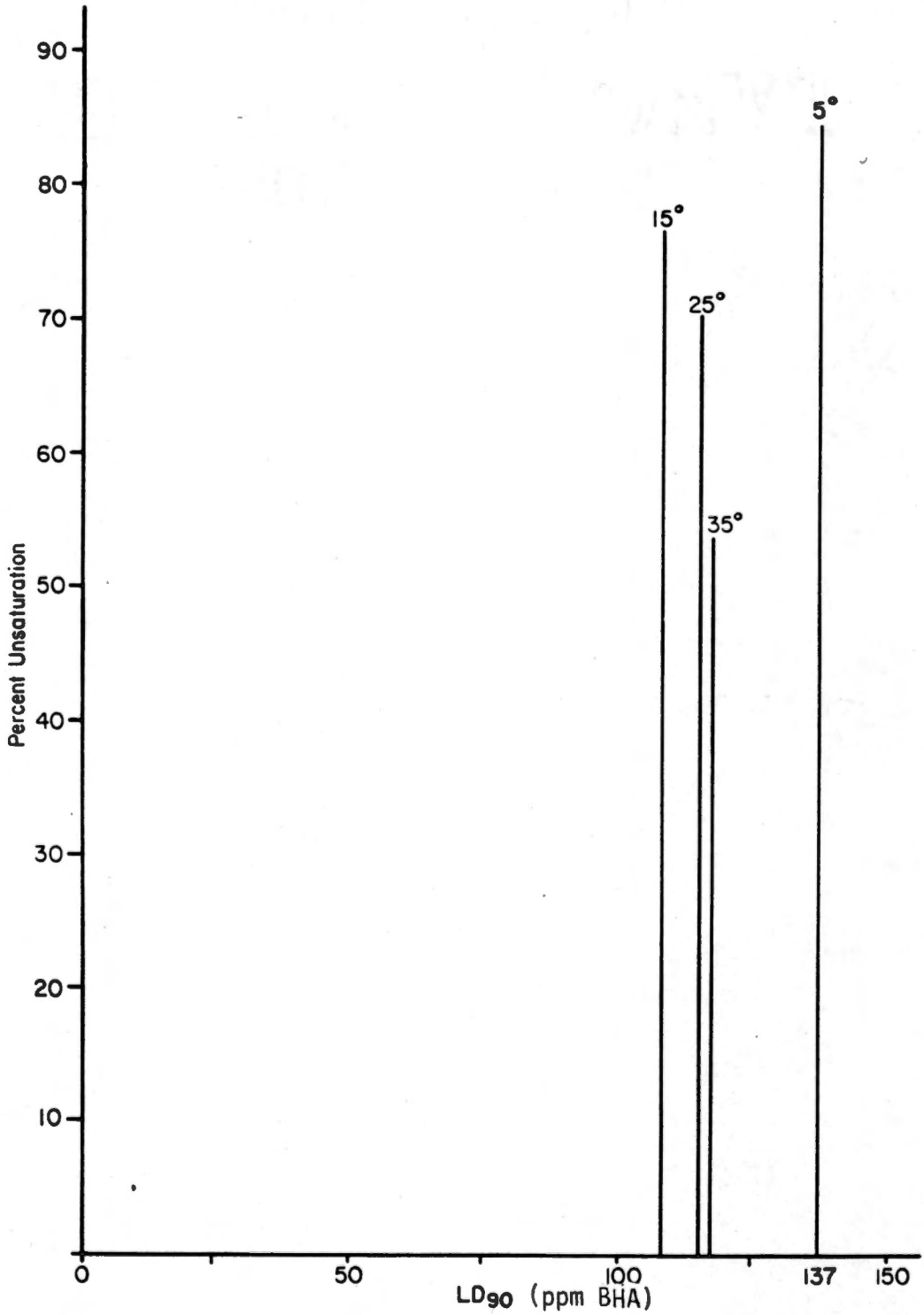


Figure 12. LD_{90} versus relative percent unsaturation for the total fatty acid composition of Pseudomonas fluorescens grown at 5, 15, 25 and 35°C.

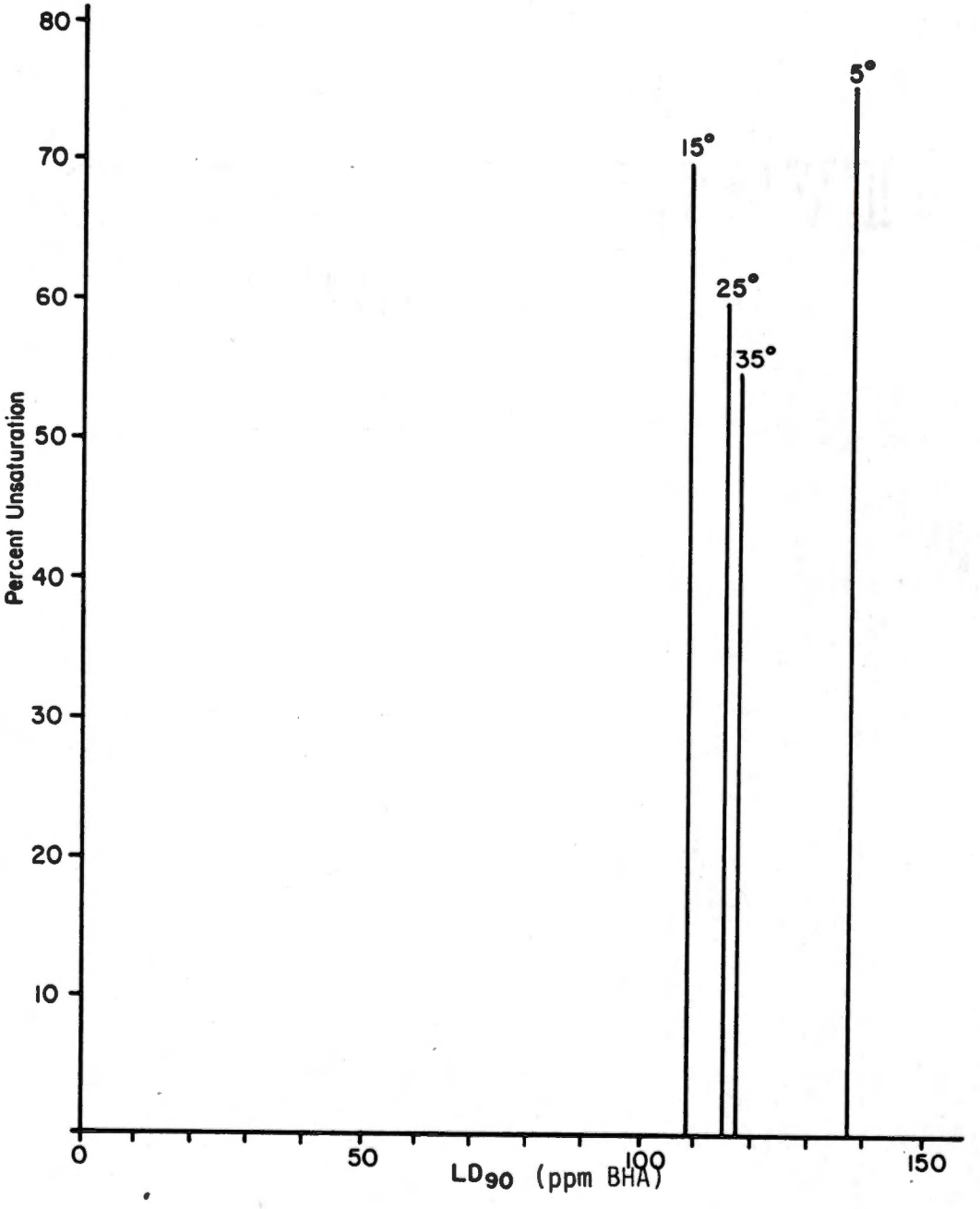


Figure 13. LD_{90} versus relative percent unsaturation for the polar fatty acid composition of Pseudomonas fluorescens grown at 5, 15, 25 and 35°C.

B. Fatty Acid Composition as an Indicator of Bacterial Susceptibility to BHA

The total and polar lipid composition of several bacterial species was examined in order to determine (1) if there was a relationship between total and/or polar fatty acid composition of the cytoplasmic membrane and susceptibility of the organism to BHA, and (2) to determine if the effect was related to cell wall type (gram-positive or gram-negative).

Lethal Effect of BHA

The dose reponse curves for all organisms tested are depicted in Figures 14-23. S. aureus 12600 (Figure 17) illustrates a highly resistant gram-positive organism. No significant reduction in cell number was found until treatment concentrations of over 350 ppm were used. Clostridium perfringens, in contrast, shows almost total inhibition with treatments over 150 ppm BHA for 15 minutes (Figure 21). Of the gram-negative species, E. coli proved one of the more resistant, with less than a 2 log cycle decrease at 400 ppm BHA (Figure 19). Salmonella anatum was reduced to a population of less than log one by 250 ppm (Figure 20).

The values of the LD_{90} or dose required to cause a 90% reduction in gram-negative and gram-positive bacterial survivors are listed in Table 15. A wide range of susceptibilities was noted within the same gram reaction category. An interesting feature was the varying effects of BHA within several strains of the same species as seen with S. aureus. Two strains (S. aureus 12600, S. aureus NR) had

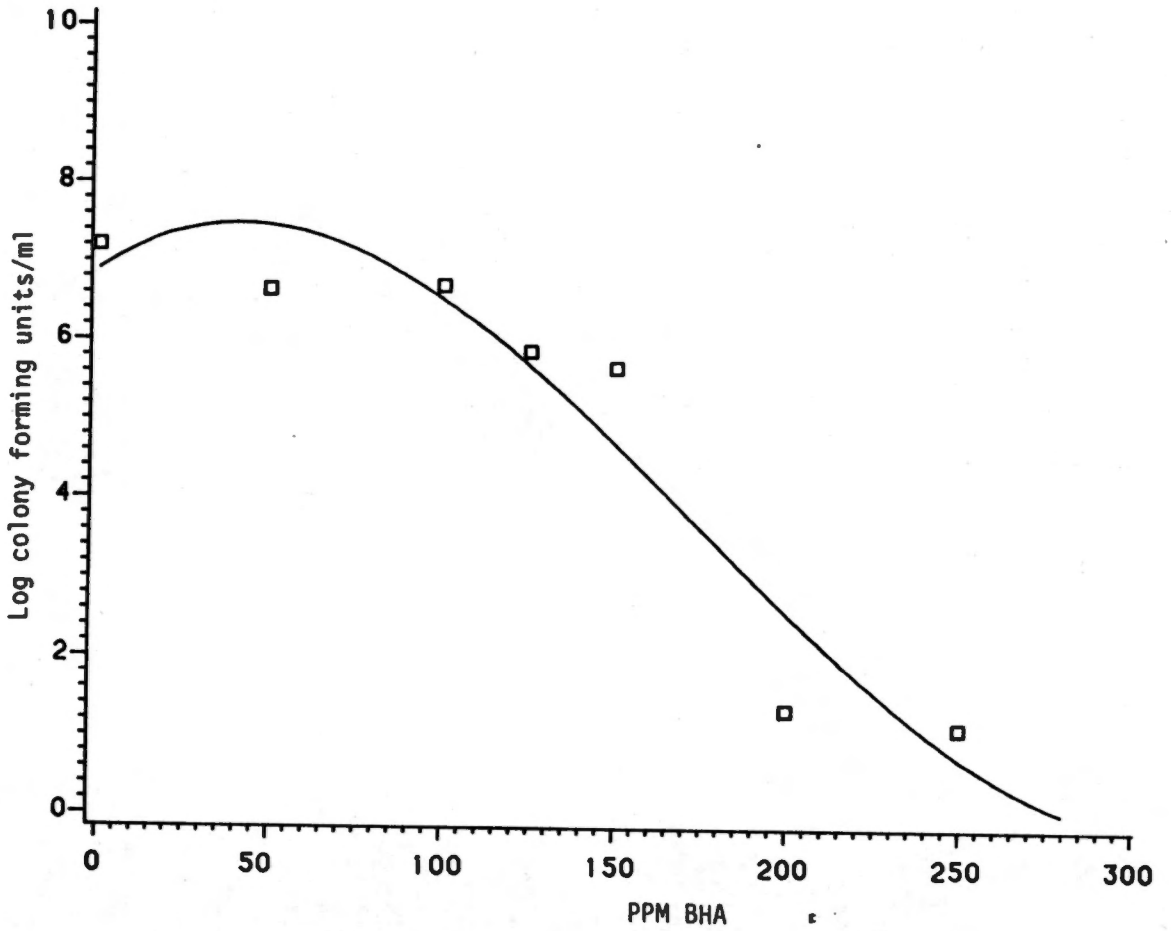


Figure 14. Dose response curve for Pseudomonas fluorescens in 0.05M phosphate-0.1% peptone buffer treated with varying concentrations of BHA for 15 minutes.

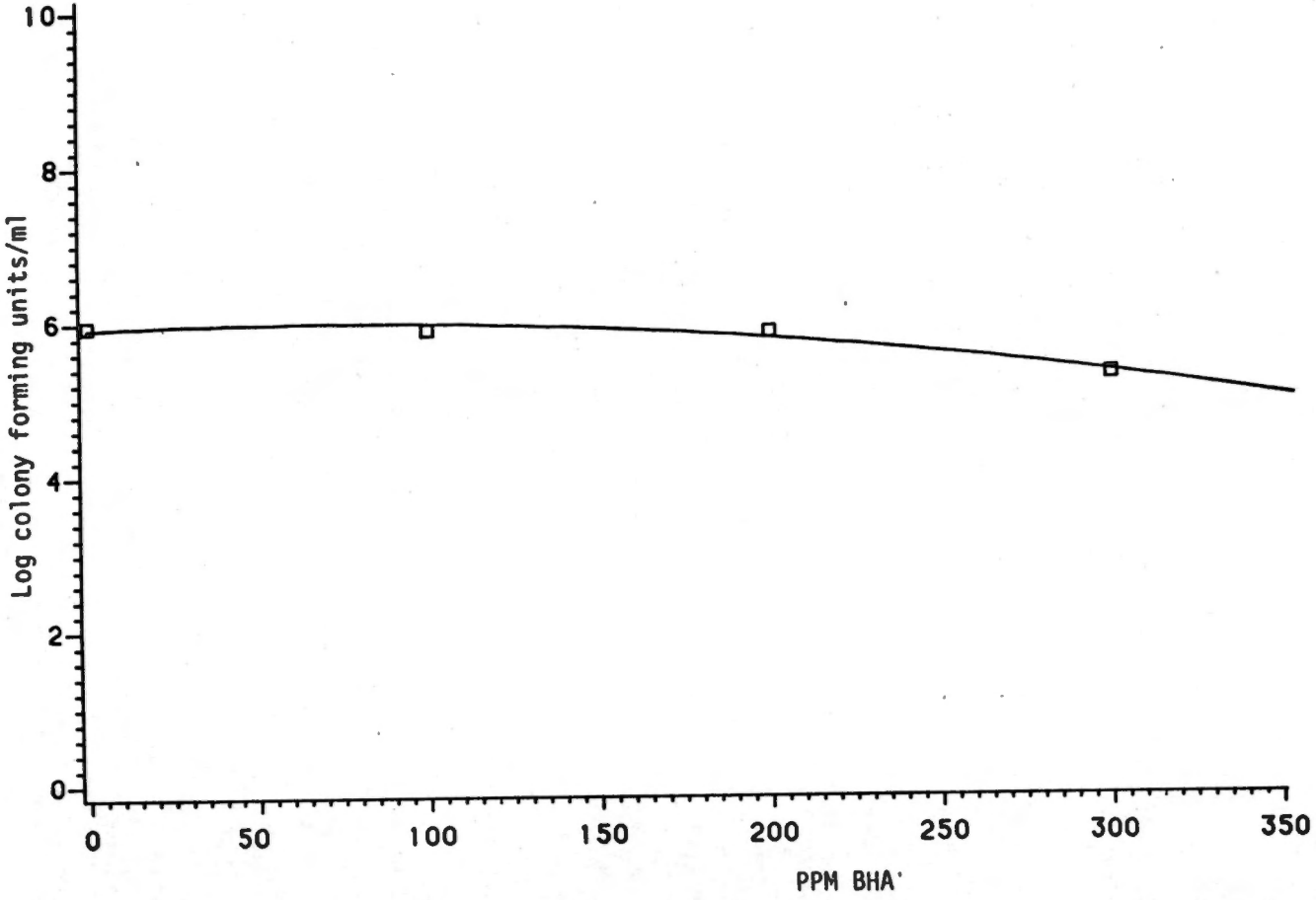


Figure 15. Dose response curve for Pseudomonas fragi in 0.05M phosphate-0.1% peptone buffer treated with varying concentrations of BHA for 15 minutes.

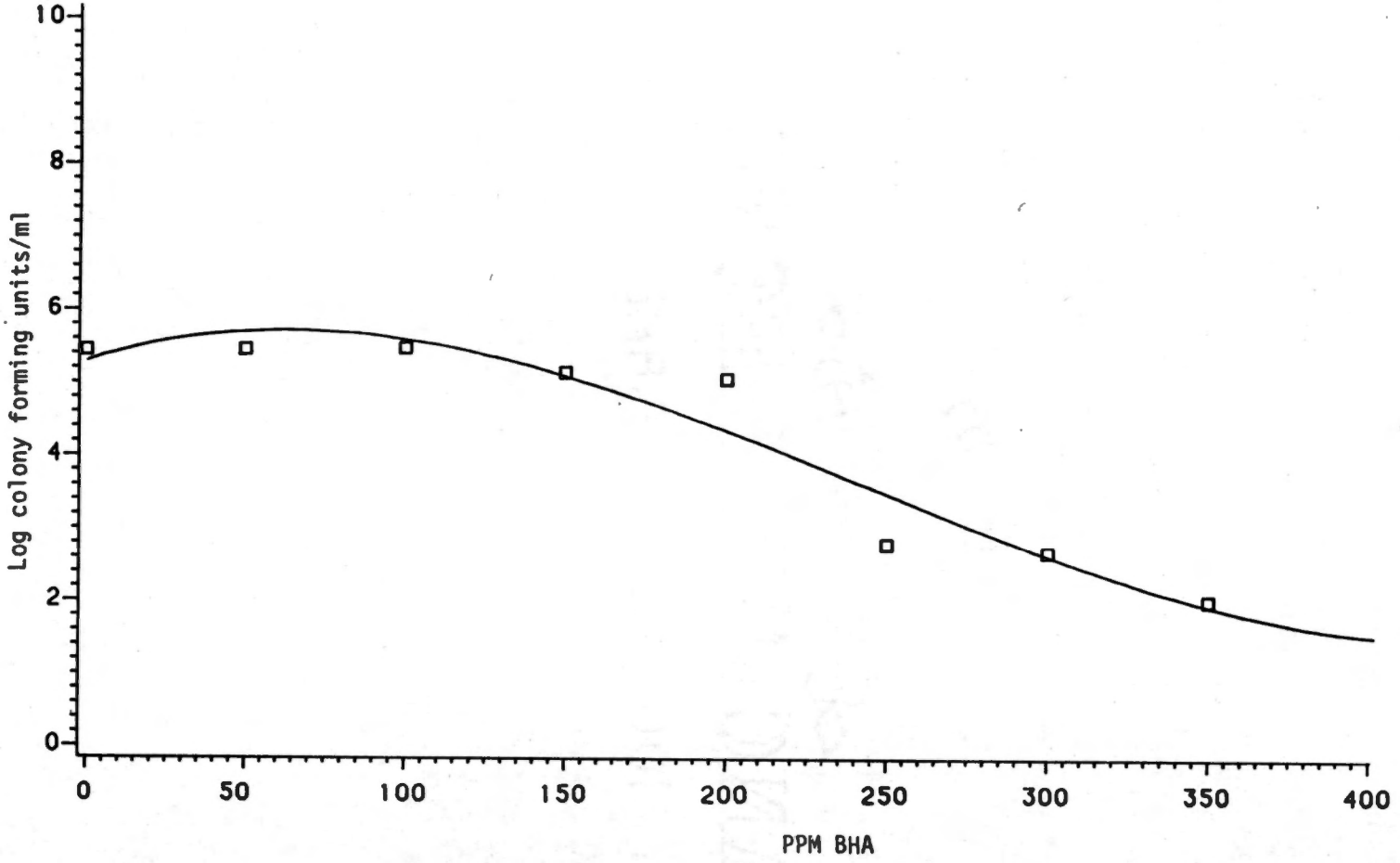


Figure 16. Dose response curve for Bacillus cereus in 0.05M phosphate-0.1% peptone buffer treated with varying concentrations of BHA for 15 minutes.

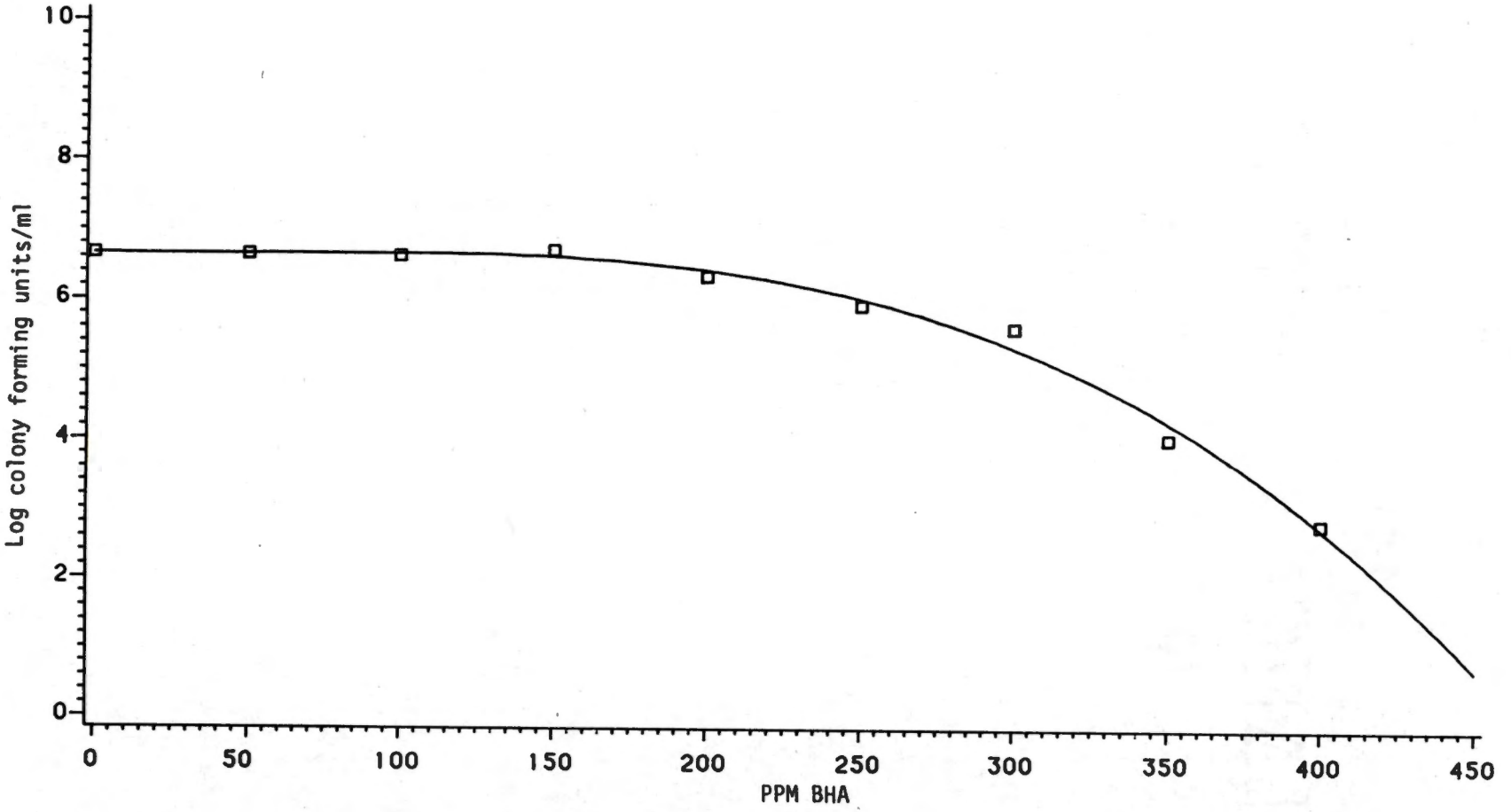


Figure 17. Dose response curve for Staphylococcus aureus 12600 in 0.05M phosphate-0.1% peptone buffer treated with varying concentrations of BHA for 15 minutes.

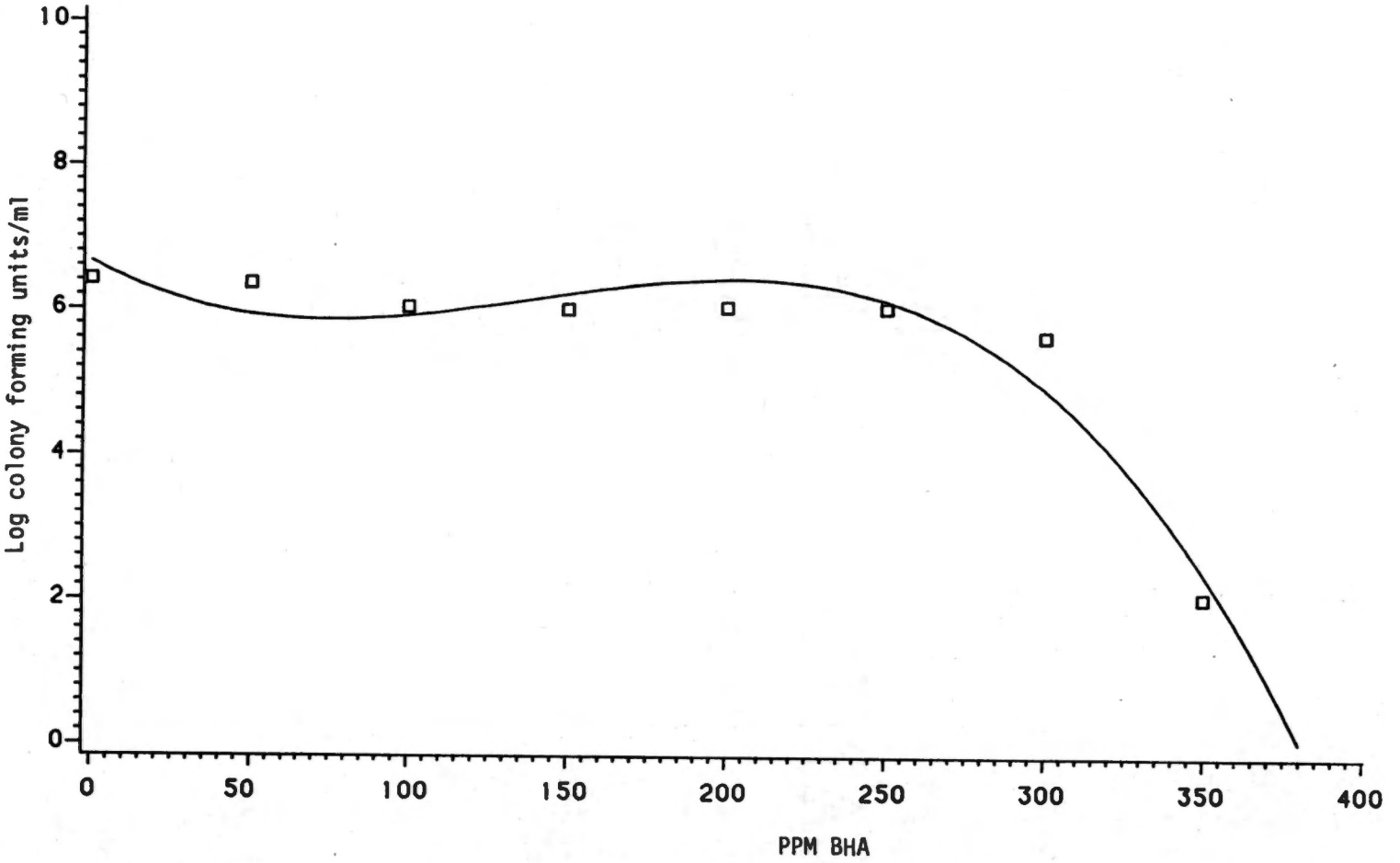


Figure 18. Dose response curve for Staphylococcus aureus NR in 0.05M phosphate-0.1% peptone buffer treated with varying concentrations of BHA for 15 minutes.

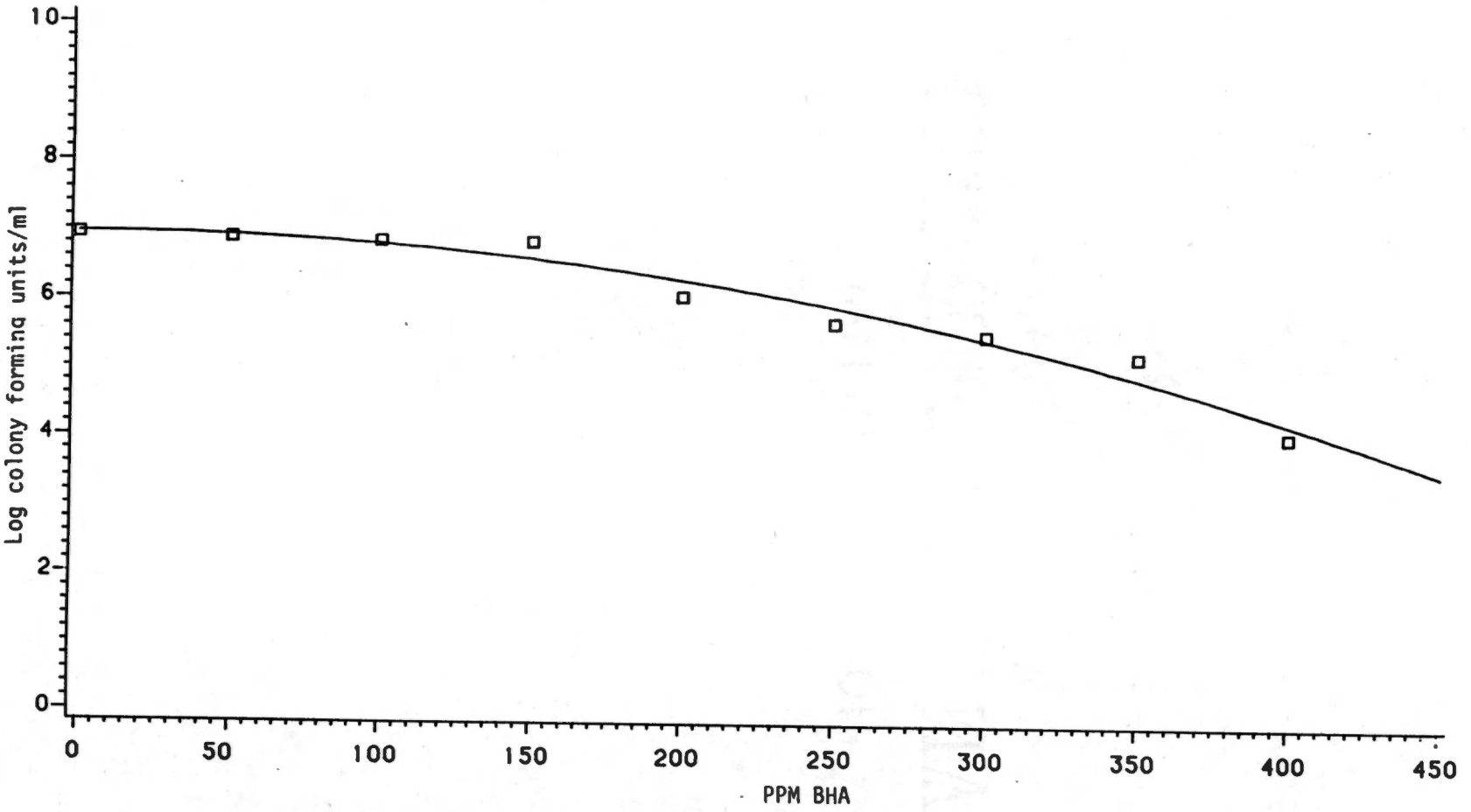


Figure 19. Dose response curve for Escherichia coli in 0.05M phosphate-0.1% peptone buffer treated with varying concentrations of BHA for 15 minutes.

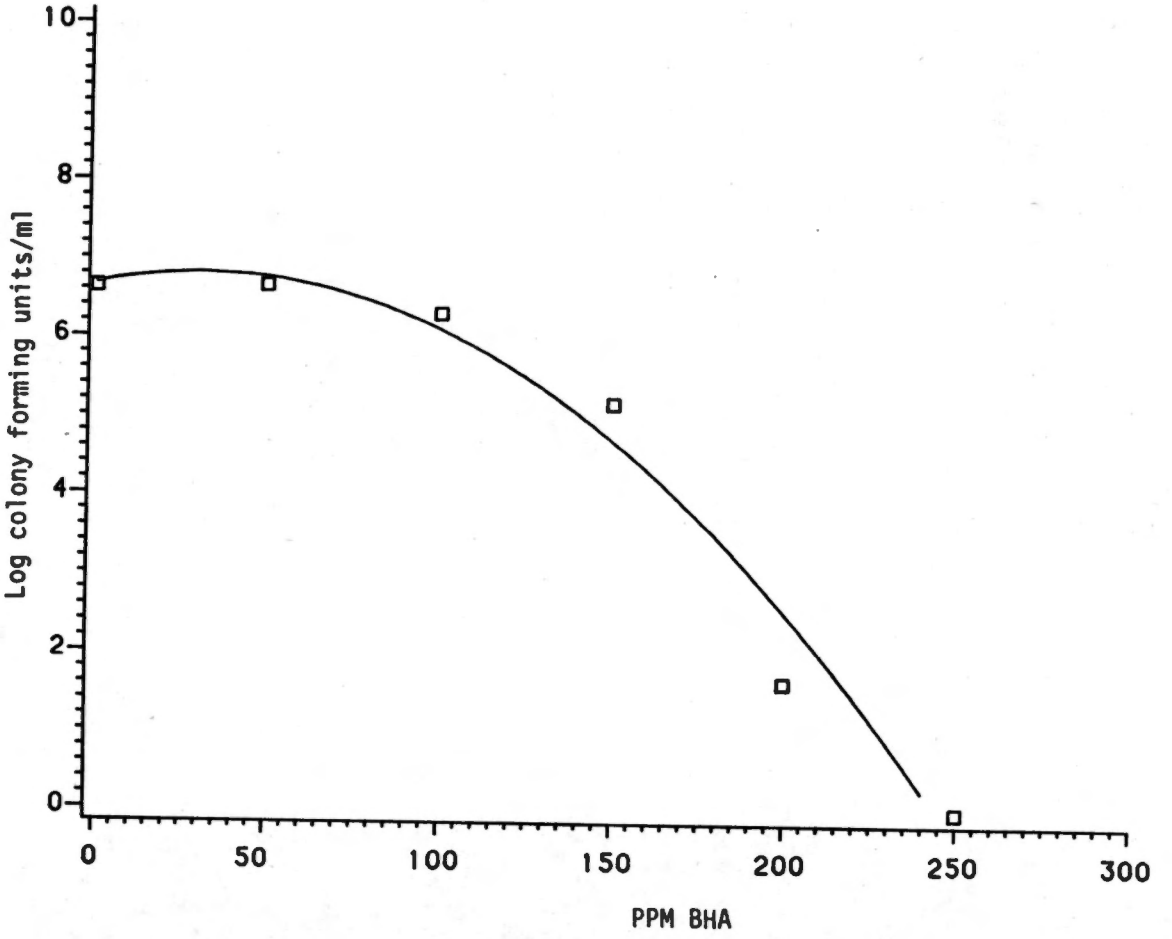


Figure 20. Dose response curve for Salmonella anatum in 0.05M phosphate-0.1% peptone buffer treated with varying concentrations of BHA for 15 minutes.

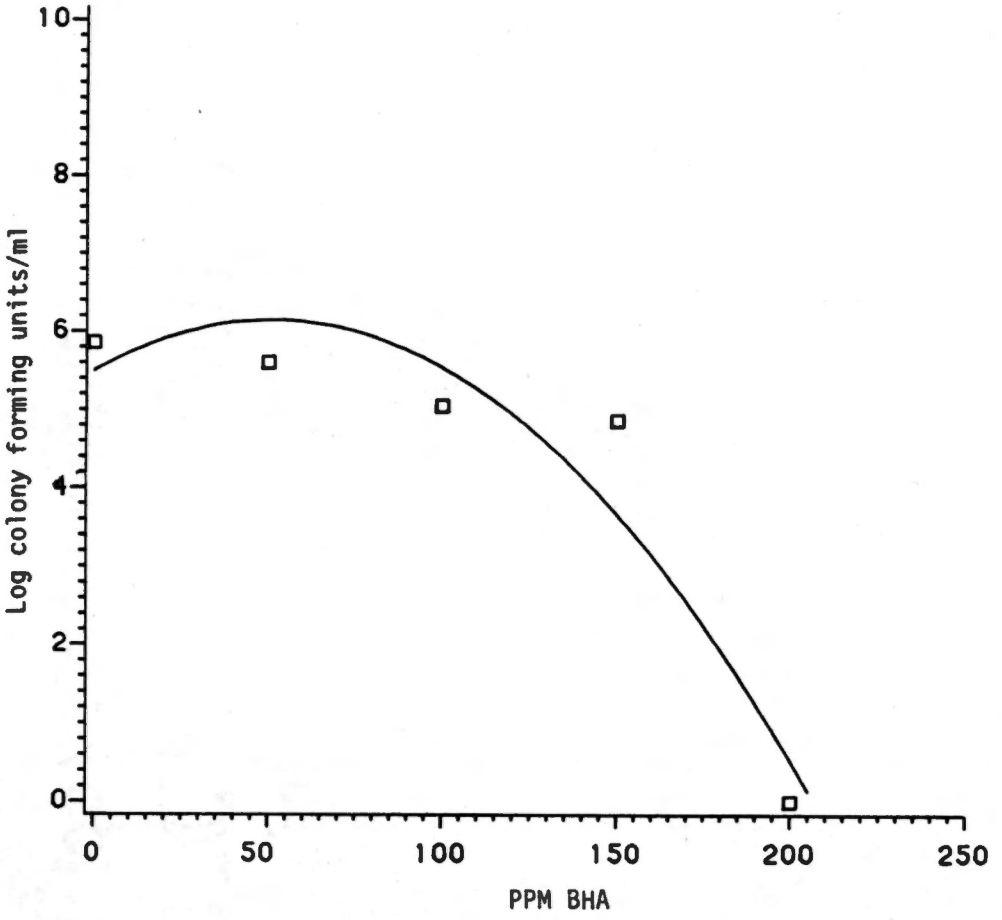


Figure 21. Dose response curve for Clostridium perfringens in 0.05M phosphate-0.1% peptone buffer treated with varying concentrations of BHA for 15 minutes.

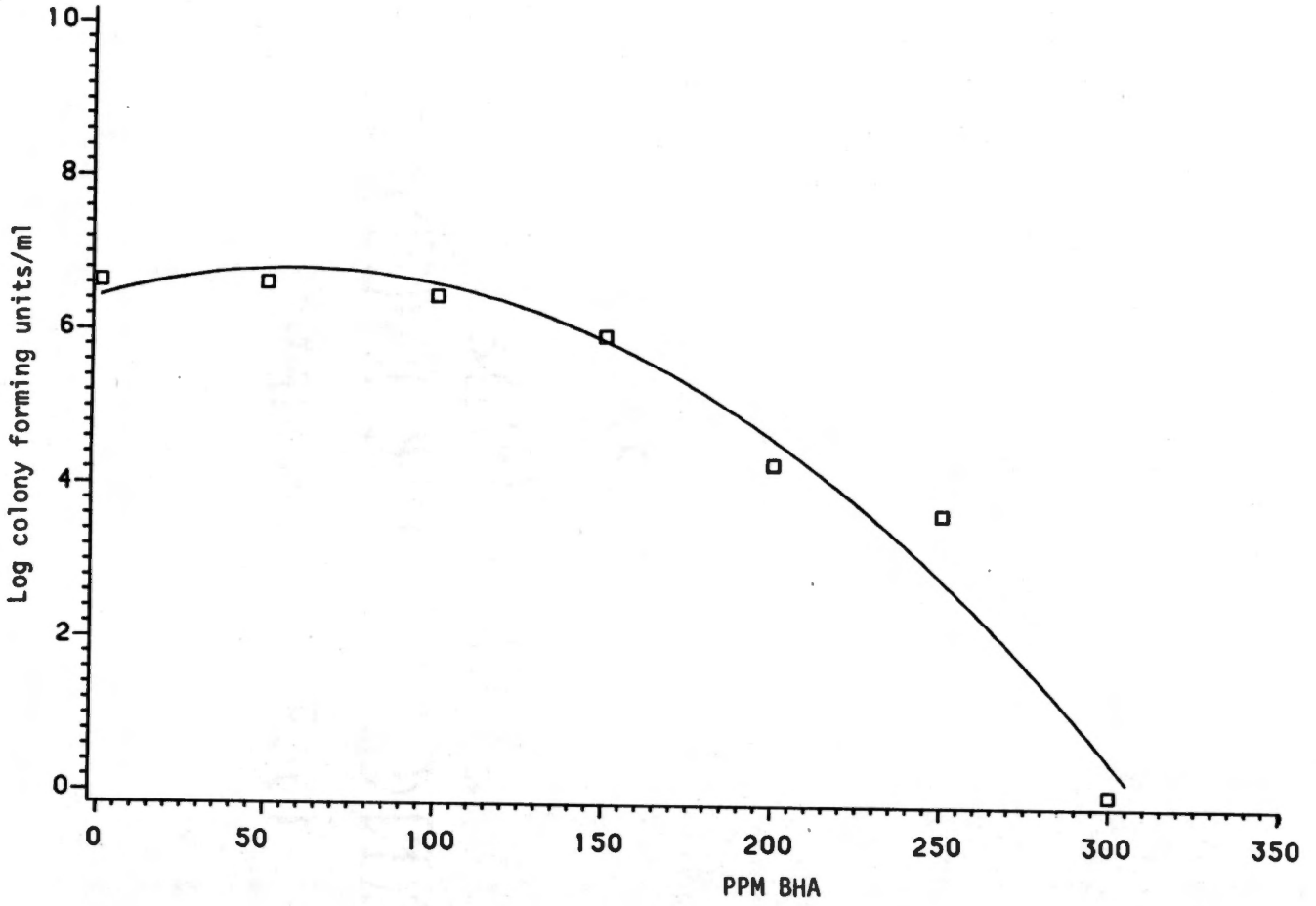


Figure 22. Dose response curve for Staphylococcus aureus S-6 in 0.05M phosphate-0.1% peptone buffer treated with varying concentrations of BHA for 15 minutes.

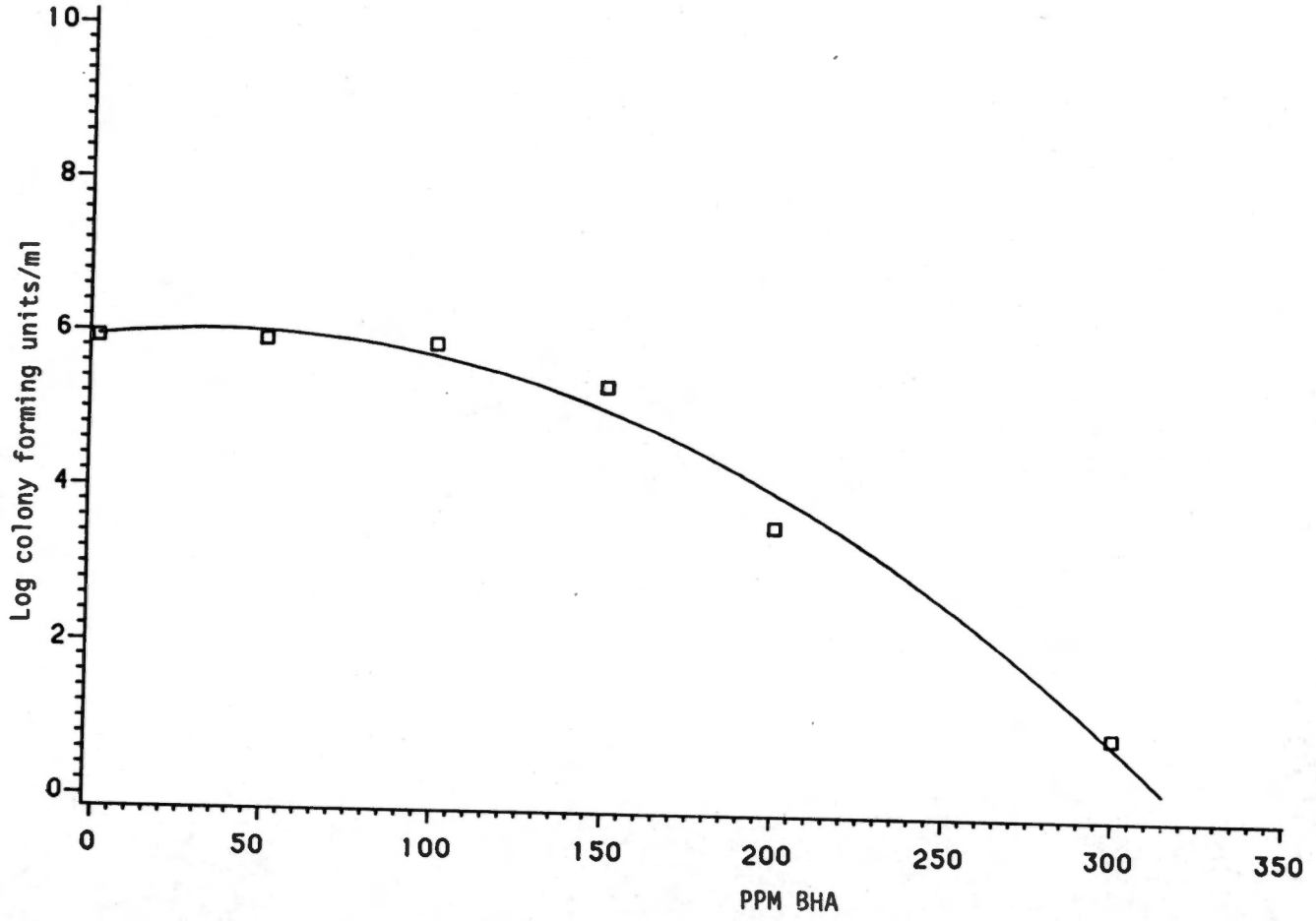


Figure 23. Dose response curve for Staphylococcus aureus A-100 in 0.05M phosphate-0.1% peptone buffer treated with varying concentrations of BHA for 15 minutes.

Table 15. Dose of BHA required to cause a 90% reduction in bacterial survivors (LD_{90}) at 15 minutes

Microorganism	LD_{90} (ppm BHA)
gram-negative	
Pseudomonas fluorescens	117
Pseudomonas fragi	347
Escherichia coli	243
Salmonella anatum	125
gram-positive	
Bacillus cereus	220
Clostridium perfringens	110
Staphylococcus aureus 12600	293
Staphylococcus aureus NR	304
Staphylococcus aureus S-6	166
Staphylococcus aureus A-100	155

an LD_{90} value of approximately 300 ppm, while two strains proved susceptible with values of 166 ppm (S. aureus S-6) and 155 ppm (S. aureus A-100).

These findings are in general agreement with the published susceptibilities of most of these organisms to BHA. The highly susceptible nature of P. fluorescens in contrast to P. fragi was also observed by Davidson and Branen (1980a). The relative resistance of E. coli had been observed by Chang and Branen (1975) and Shih and Harris (1977). Both research teams reported that concentrations of 400 ppm BHA were necessary to totally inhibit the bacteria in laboratory media. Klindworth et al. (1979) noted the relative sensitivity of C. perfringens to the action of BHA. They determined that 150 ppm were necessary to totally inhibit this organism.

In this study, B. cereus occupied an intermediate level in the ranking of susceptibility to the action of BHA while Shelef and Liang (1982) reported the organism to be highly susceptible with a reported requirement of only 75-100 ppm BHA necessary to totally inhibit the organism. While the LD_{90} values of two strains of S. aureus tested in this study, S. aureus S-6 and S. aureus A-100, are in close agreement with the 150-200 ppm values for total inhibition found by Chang and Branen (1975) and Pierson et al. (1980), two other stains were more highly resistant. The relative sensitivity of S. anatum reported in this work is in sharp contrast to the highly resistant nature of another Salmonella species, S. typhimurium, reported by Pierson et al. (1980).

Aside from the obvious strain or species influence, many other environmental factors affect the antimicrobial activity of BHA. Klindworth et al. (1979) found that very young or old cultures of C. perfringens exhibited increased susceptibility to the action of BHA. pH was also found to play a varied role (Klindworth et al., 1979; Ayaz et al., 1980). Temperature as well as the presence of lipid was shown to affect activity. In a study by Davidson and Branen (1980a), low temperatures were reported to reduce BHA solubility into the lipid portion of the organism, thereby reducing lethality. The antimicrobial potency of BHA was also found to be diminished in lipid containing media or foods due to the partitioning of the BHA into the lipid phase causing the compound to be unavailable to act on microbes (Klindworth et al., 1979; Shelef and Liang, 1982).

It appears possible that such factors as strain or species differences in membrane structure or environmental influences can govern the action of BHA possibly through the key mechanism of BHA solubility in the lipid portion of the bacteria. As Eletr et al. (1974) had found, the membrane perturbing agent adamantane solubilized into the hydrophobic regions of the membrane, causing disruption of the symmetry of packed alkyl chains on the phospholipid molecules. Likewise, most action sustained by monocyclic compounds such as BHA depends on interaction with hydrophobic and electrophilic regions of the membrane and the extent of resultant lipid solubility (Sgaragli et al., 1977). It would follow then that any one or a combination of factors which results in the decreased solubility of BHA in the

membrane lipid layer could lead to decreased effectiveness of the antioxidant as an antimicrobial compound.

Fatty Acid Content and Relationship to Bacterial Susceptibility

Figures 24 and 25 show the total and polar fatty acid composition for 8 of the 10 bacterial species examined. Staphylococcus aureus 12600 was taken to represent the resistant strains of S. aureus and S. aureus A-100 was taken to represent the susceptible strains of S. aureus. The full fatty acid spectra for both total and polar fractions of all four staphylococci strains are listed in Tables B-1 and B-2. Although all four compositions are similar, S. aureus 12600 appears more closely related to S. aureus NR and S. aureus A-100 more closely follows S. aureus S-6 in the total relative unsaturated to saturated fatty acid ratios.

The fatty acids which constituted a major proportion of the total lipid compositions were consistently found to be unsaturated and saturated 16 and 18 carbon fatty acids. This occurred for all species examined except C. perfringens where saturated 11, 13 and 17 carbon fatty acids composed a larger portion of the total lipid content. Other fatty acids constituting a large part of the fatty acid complements were 15 and 17 carbon fatty acids. S. aureus A-100 contained a relative percentage of approximately 40% C15:0. These findings are in agreement with those of O'Leary (1962). He had reported that unsaturated 16 and 18 carbon fatty acids were most commonly identified in bacteria and the most common saturated fatty acids were C12:0 through C18:0. In this study, 19 carbon fatty acids were found for E. coli, S. anatum, C. perfringens, and the four strains of S. aureus,

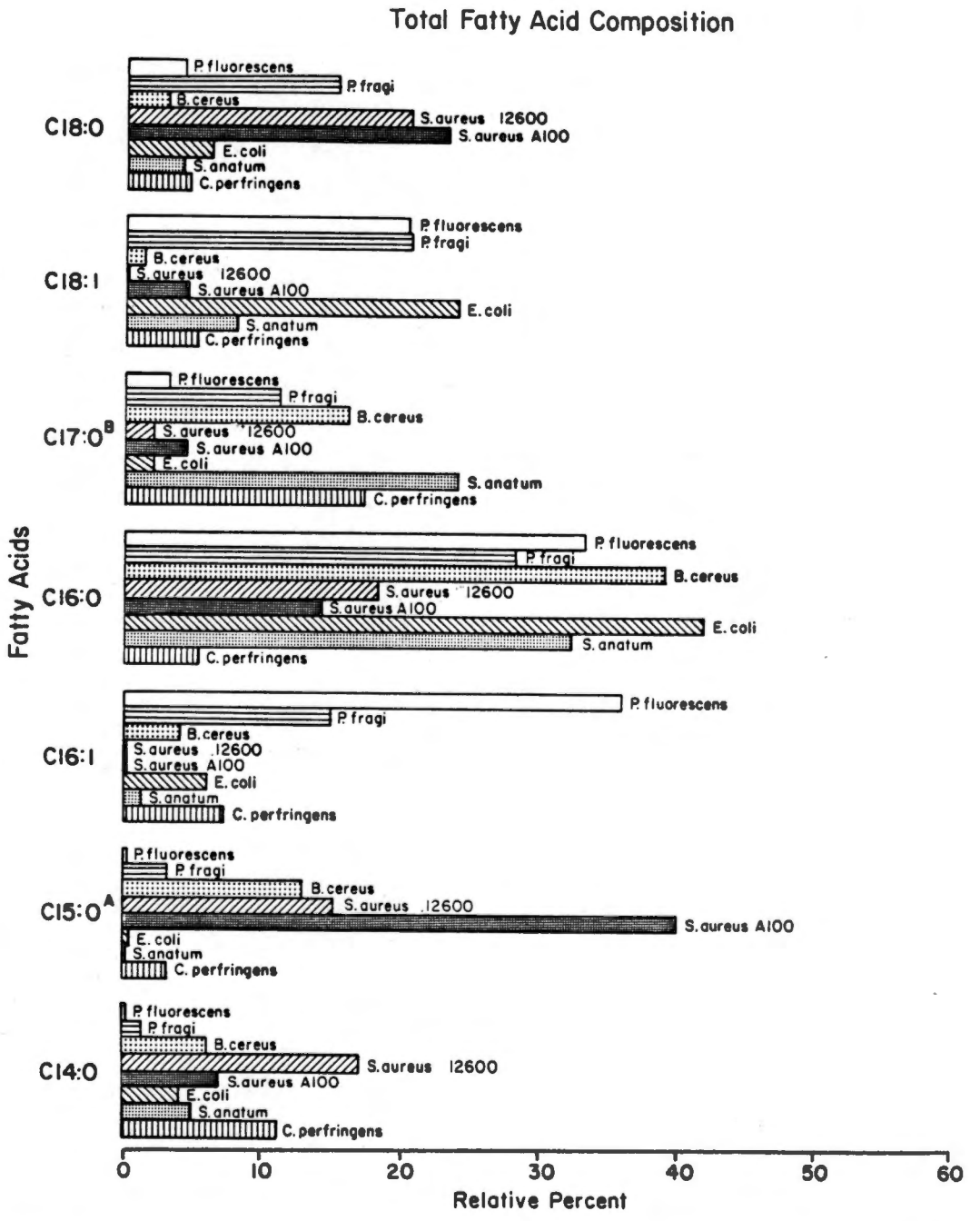


Figure 24. Spectra of total fatty acid compositions for several bacterial species (relative percentages of fatty acids).

[a]Total concentration of α15:0 and 15:0.

[b]Total concentration of α17:0, 17:0Δ, and 17:0.

[c]Total concentration of 19:00 and 19:0 Δ .

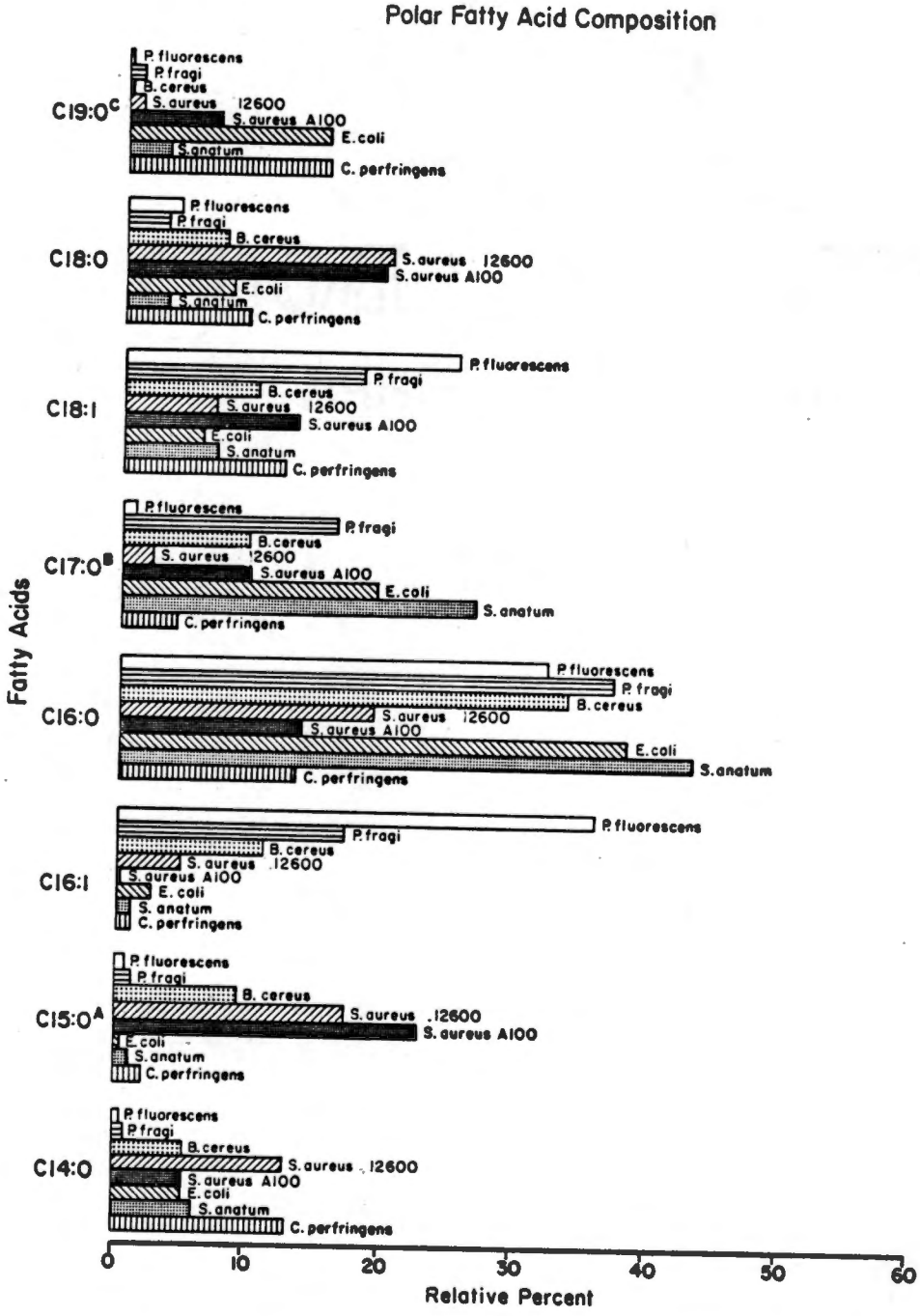


Figure 25. Spectra of polar fatty acid compositions for several bacterial species (relative percentages of polar fatty acids).

[a]Total concentration of α15:0 and 15:0.

[b]Total concentration of α17:0, 17:0Δ, and 17:0.

[c]Total concentration of 17:0 and 19:0Δ.

and a 20 carbon fatty acid was identified in total lipid fractions from E. coli, S. anatum, and S. aureus (12600). Law (1961) had also reported the presence of a 19 carbon acid similar to lactobacillic acid in E. coli.

The total lipid fractions for all organisms contained a higher relative ratio of saturated to unsaturated fatty acids except P. fluorescens where unsaturated fatty acids predominated. The major unsaturated fatty acids were C16:1 and C18:1. The relative percentages of unsaturated fatty acids for total and polar fractions as compared to the relative percentages of the saturated fatty acids C16:0 and C18:0 are depicted in Figures 26 and 27. For the total lipid fraction of P. fluorescens the relative percentage of unsaturated fatty acids was 58%, for P. fragi 38%, for B. cereus 2.0%, for S. aureus 12600 0.83%, for E. coli 29%, for S. anatum 9.5%, for C. perfringens 12% and for S. aureus A-100 4.6%. Tables B-3 and B-4 depict full fatty acid spectra for the total and polar lipid fractions of E. coli, S. anatum, C. perfringens, and B. cereus. Sample chromatograms for the total lipid fractions of each organism are depicted in Appendix D.

The polar lipid fractions were shown to have similar relative ratios of saturated to unsaturated fatty acids except for E. coli, which showed decreased levels of unsaturated 18 carbon fatty acids and B. cereus which showed increased levels of unsaturated 18 carbon fatty acids as compared to the total composition. As in the total lipid fractions, 16 and 18 carbon fatty acids predominated as well as C17:0. In this fraction, 19 carbon fatty acids constituted a relative percentage of about 15% of the fatty acid complements for E. coli and C. perfringens (Figures 26 and 27).

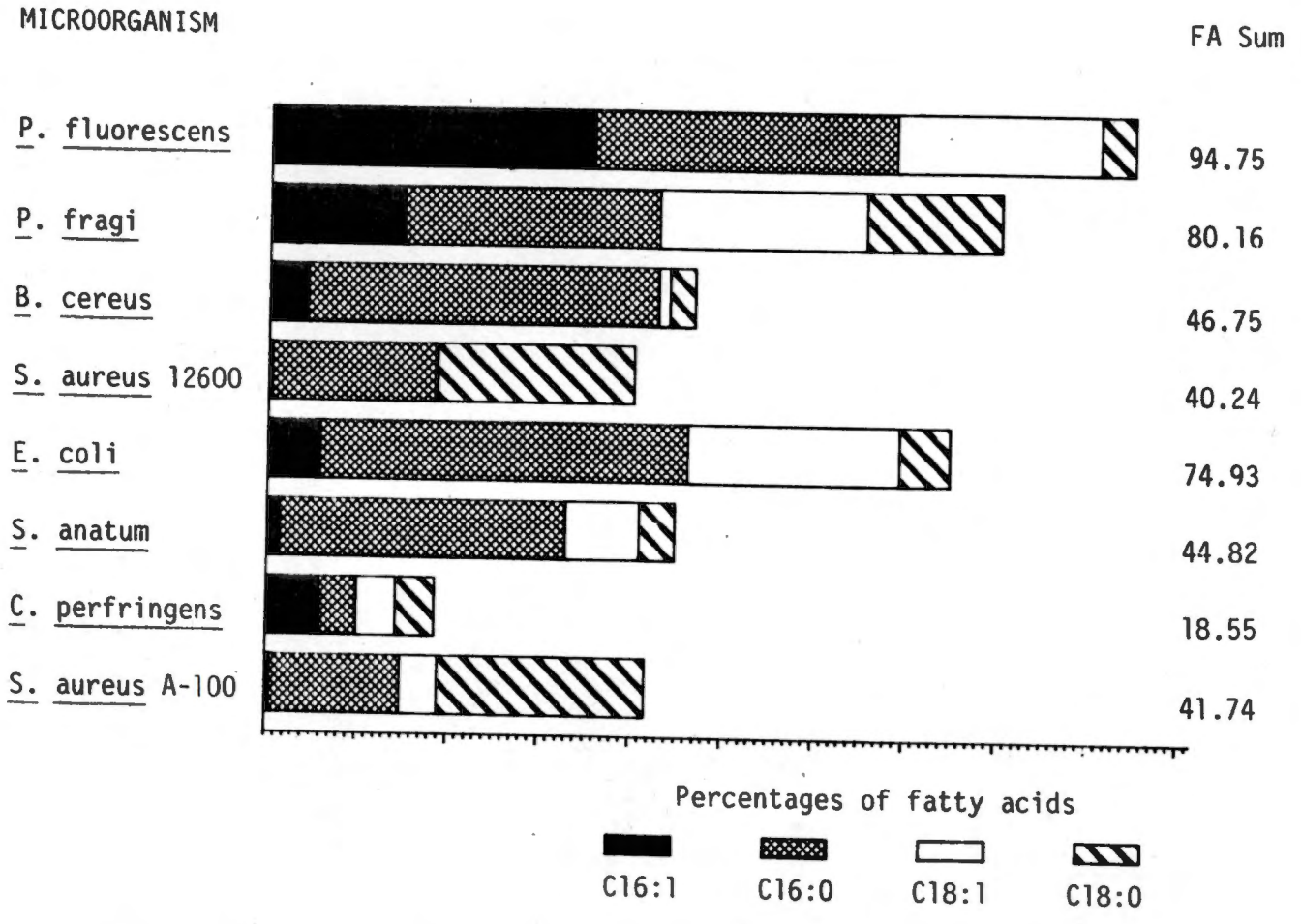


Figure 26. Relative percentages of major fatty acids for total lipid fractions.

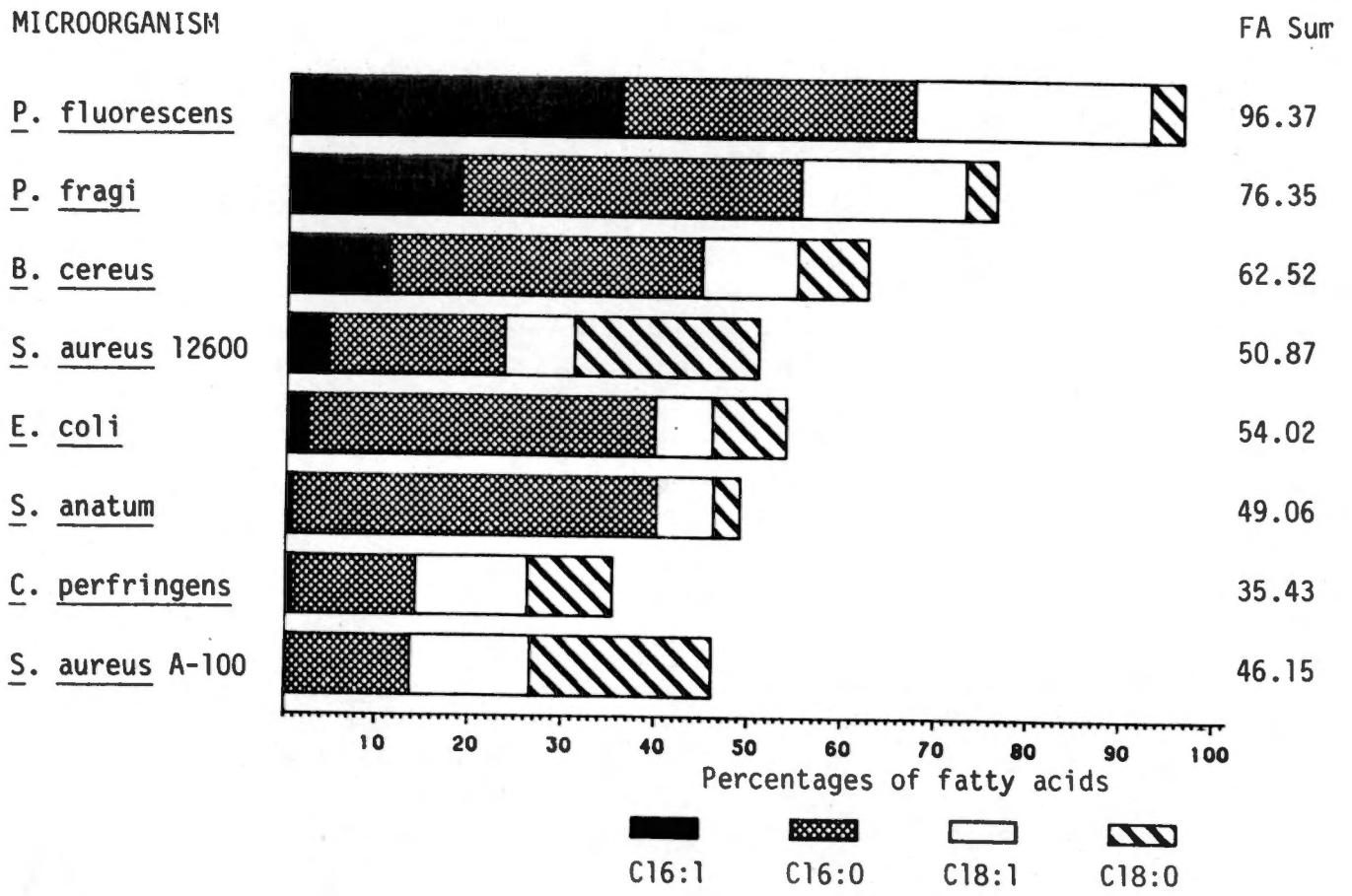


Figure 27. Relative percentages of major fatty acids for polar lipid fractions.

The relative percentage of C15:0 for S. aureus was 23%.

The relationship of LD_{90} to the relative percentage of unsaturated fatty acids was also studied (Figures 28 and 29). In the case of P. fluorescens, increased susceptibility to BHA was accompanied by a higher ratio of unsaturated to saturated fatty acids, whereas this relationship was lacking in the other susceptible species such as S. anatum and C. perfringens. Also, although all four strains show low levels of unsaturated fatty acids, S. aureus is divided as to susceptibility. Despite wide dissimilarities in the lethal effect of BHA, S. aureus 12600 and S. aureus A-100 were shown to be quite similar in fatty acid make-up. The polar lipid fractions showed the same relative relationship of LD_{90} to percent unsaturation.

Cell gram reaction does not appear to be a strict indicator of bacterial susceptibility to BHA. The literature suggests that the antimicrobial activity of BHA appears greatest against gram-positive bacteria while gram-negative species are less sensitive due to the protective nature of the lipoprotein/lipopolysaccharide layer of the cell wall (Branen et al., 1980). In this study, both gram-positive (Staphylococcus aureus 12600, S. aureus NR) and gram-negative species (P. fragi, E. coli) showed increased resistance to the action of BHA. Increased sensitivity was demonstrated, as well, with the gram-negative organism, P. fluorescens, and the gram-positive organism, C. perfringens.

While a definitive relationship cannot be elucidated between total or polar fatty acid composition and susceptibility of a bacterial species to BHA, i.e., increased saturation does not confer protection,

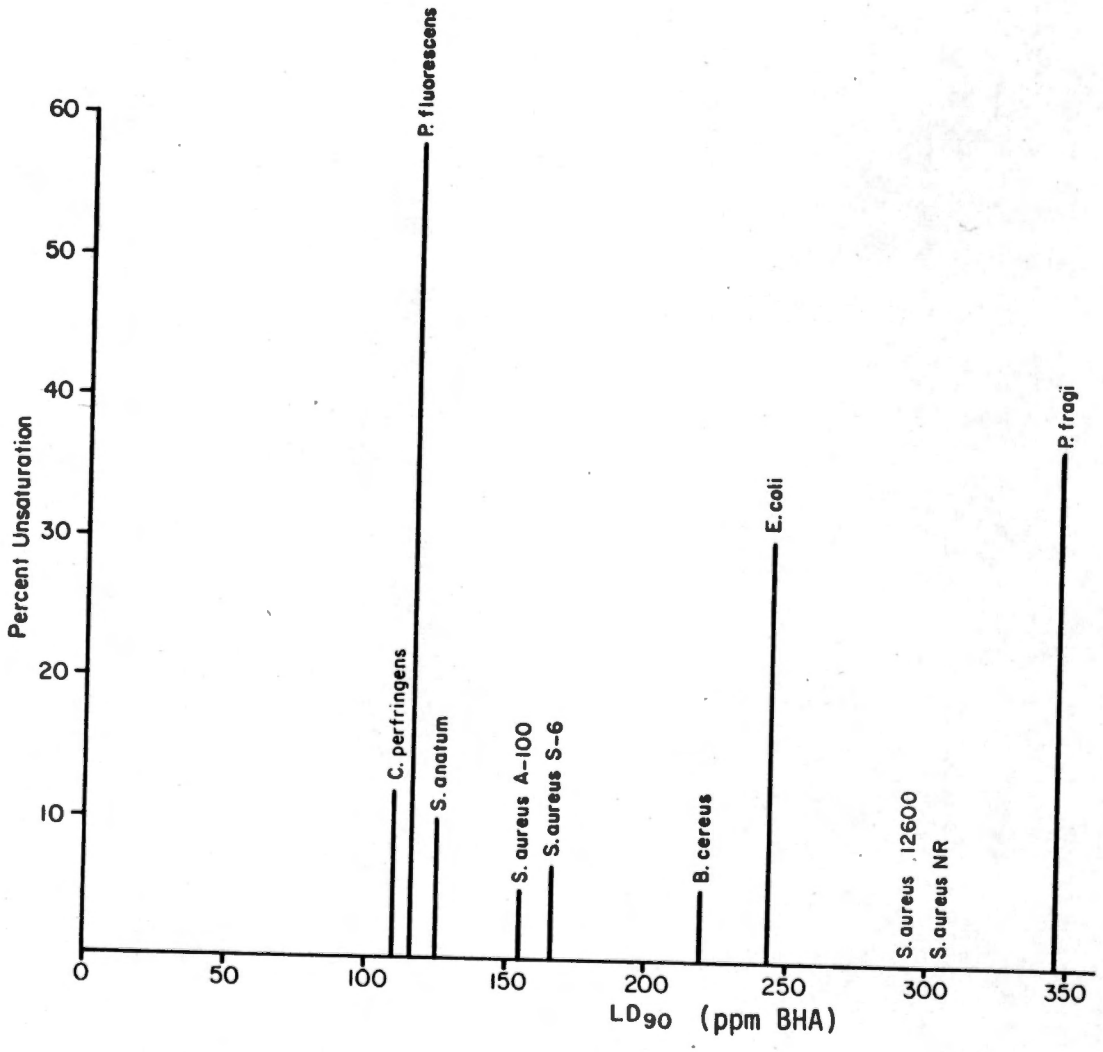


Figure 28. LD_{90} versus relative percent unsaturation for the total fatty acid compositions of several bacterial species.

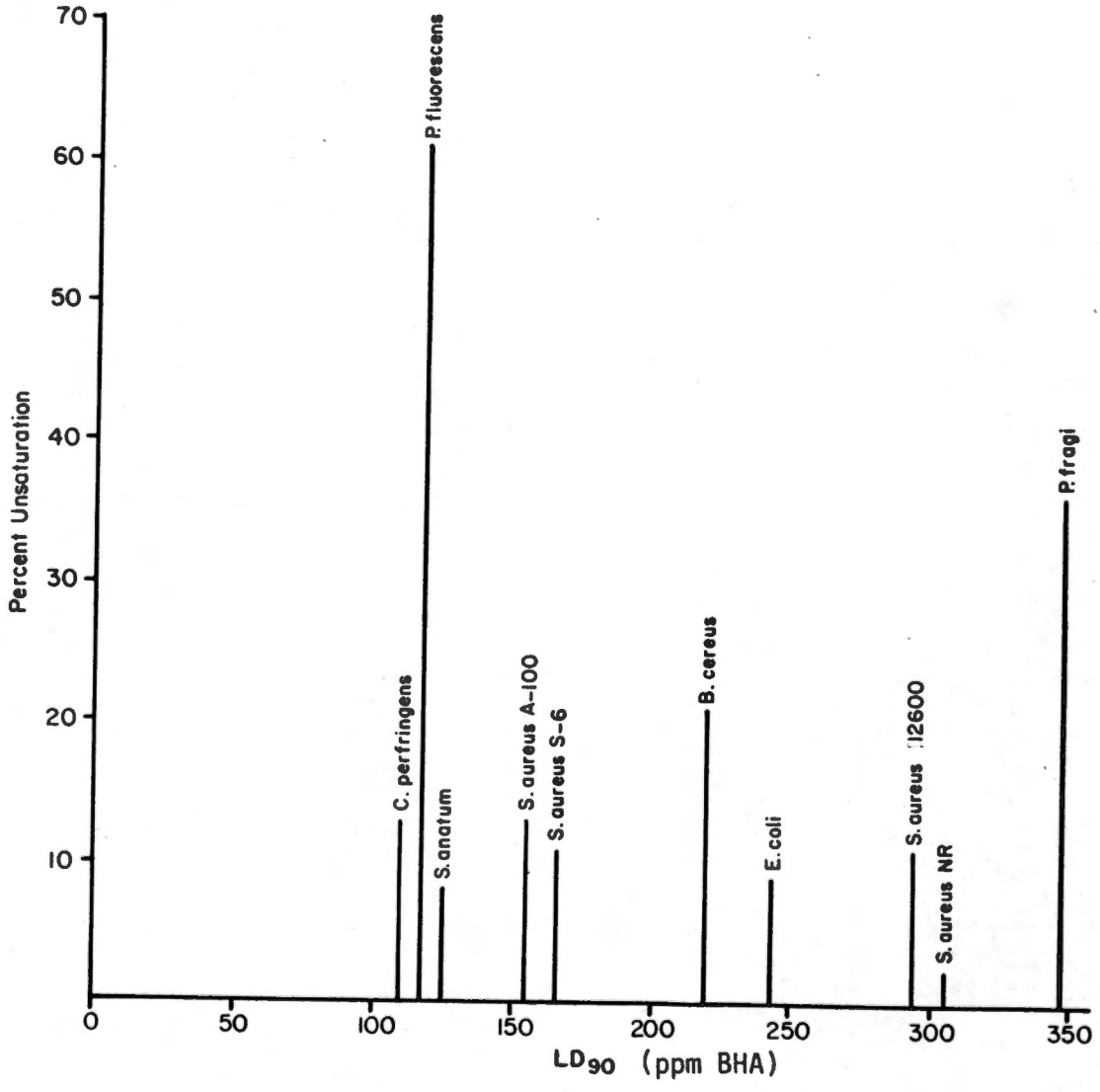


Figure 29. LD_{90} versus relative percent unsaturation for the polar fatty acid compositions of several bacterial species.

further investigation with other hydrophobic antimicrobials is required before a direct relationship theory may be discarded. The results of this study suggest that the action of BHA, although membrane mediated, may be directed toward the activity of membrane enzyme systems.

CHAPTER V

CONCLUSIONS

Growth of P. fluorescens and P. fragi in the presence of BHA caused some significant changes in the fatty acid composition and phospholipid content of the organisms which paralleled a modified susceptibility to the antimicrobial activity of BHA. Control cells of P. fragi were relatively resistant to the effect of BHA. The relative percentage of unsaturated fatty acids in the total lipid fraction was 37.57% for the control population and treated cells maintained approximately 38% unsaturated fatty acids as well. Changes in the polar fatty acid and phospholipid composition of P. fragi paralleled alterations in the bacterial susceptibility to BHA.

Pseudomonas fluorescens was almost totally inhibited by the presence of 150 ppm BHA for 15 minutes. The relative percentages of unsaturated fatty acids in the total lipid fraction was 58.14%. Growth in the presence of 50-125 ppm BHA caused a decrease in the relative ratio of unsaturated to saturated fatty acids for both total and polar lipid fractions while resistance to the antimicrobial effects of BHA increased. Decreased susceptibility also paralleled a decrease in phosphatidyl ethanolamine content and an increase in phosphatidyl glycerol and phosphatidyl inositol contents. Modified lipid content and decreased susceptibility were maintained through one transfer in broth without BH

It would appear that the content of unsaturated fatty acids affected the cellular susceptibility of Pseudomonas fluorescens to

the action of BHA. Growth in the presence of BHA resulted in an increase in the content of saturated fatty acids and an increase in resistance. It would then follow that P. fragi was conditioned to resist the action of BHA by virtue of its high level of saturated fatty acids. However, when P. fluorescens was grown at temperatures favoring an increase in the saturated fatty acid content, no corresponding increase in resistance was noted. While changes in lipid composition in response to growth in the presence of BHA may be due to protective measures initiated by the cell or a toxic effect of BHA, it does not appear that the lethal effect of BHA is directly related to these changes. The acquired resistance on the part of P. fluorescens may have been due to BHA induced alterations in metabolic pathways associated with the membrane rather than a direct relationship to fatty acid composition. The number, type, and/or arrangement of the membrane phospholipids may play a role in the proper functioning of associated enzyme systems and alterations of the lipid matrix may affect the activity of membrane-bound enzymes.

When the lethal effects of BHA against a variety of both gram-positive and gram-negative organisms were determined, a definitive relationship between total and polar fatty acid composition and susceptibility of a bacterial species to BHA could not be elucidated. Increased levels of saturated fatty acids did not confer protection and cell gram reaction did not appear to be a strict indicator of bacterial susceptibility to BHA.

The antimicrobial activity of BHA, while associated with changes in lipid composition, may be more directly mediated through alteration

of the nonspecific interaction between membrane integral proteins and surrounding lipids. Eletr et al. (1973) had proposed that an enzyme requirement for a given level of activity appeared to be satisfied by a bulk property of the lipid moiety rather than an absolute requirement for individual phospholipid molecules. Marcelja (1976) determined that a change in the order of lipid molecules surrounding a protein led to an indirect lipid mediated interaction between membrane integral proteins. The most important changes in lipid structure were restricted to the "first neighbor" phospholipids surrounding a protein. When two proteins within the membrane approached, the regions of modified lipid structure surrounding each molecule overlapped. Depending on the relative strength of the direct protein-protein interaction in comparison with the hydrophobic interaction of proteins with the lipid chains, proteins could remain separated by a layer of lipids or come into direct contact to cause protein aggregation. Growth in the presence of BHA caused changes in the order of lipid molecules and possibly in the actual amount of lipid material which could cause protein aggregation and disruption of enzyme systems.

The results of this study suggest that the action of BHA, although membrane-mediated, may be directed toward the activity of membrane-bound enzyme systems. Further investigation with other hydrophobic antimicrobials is required, however, before a more direct lipid-mediated mode of action may be disregarded as the basis for the antimicrobial activity of BHA.

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APPENDICES

APPENDIX A

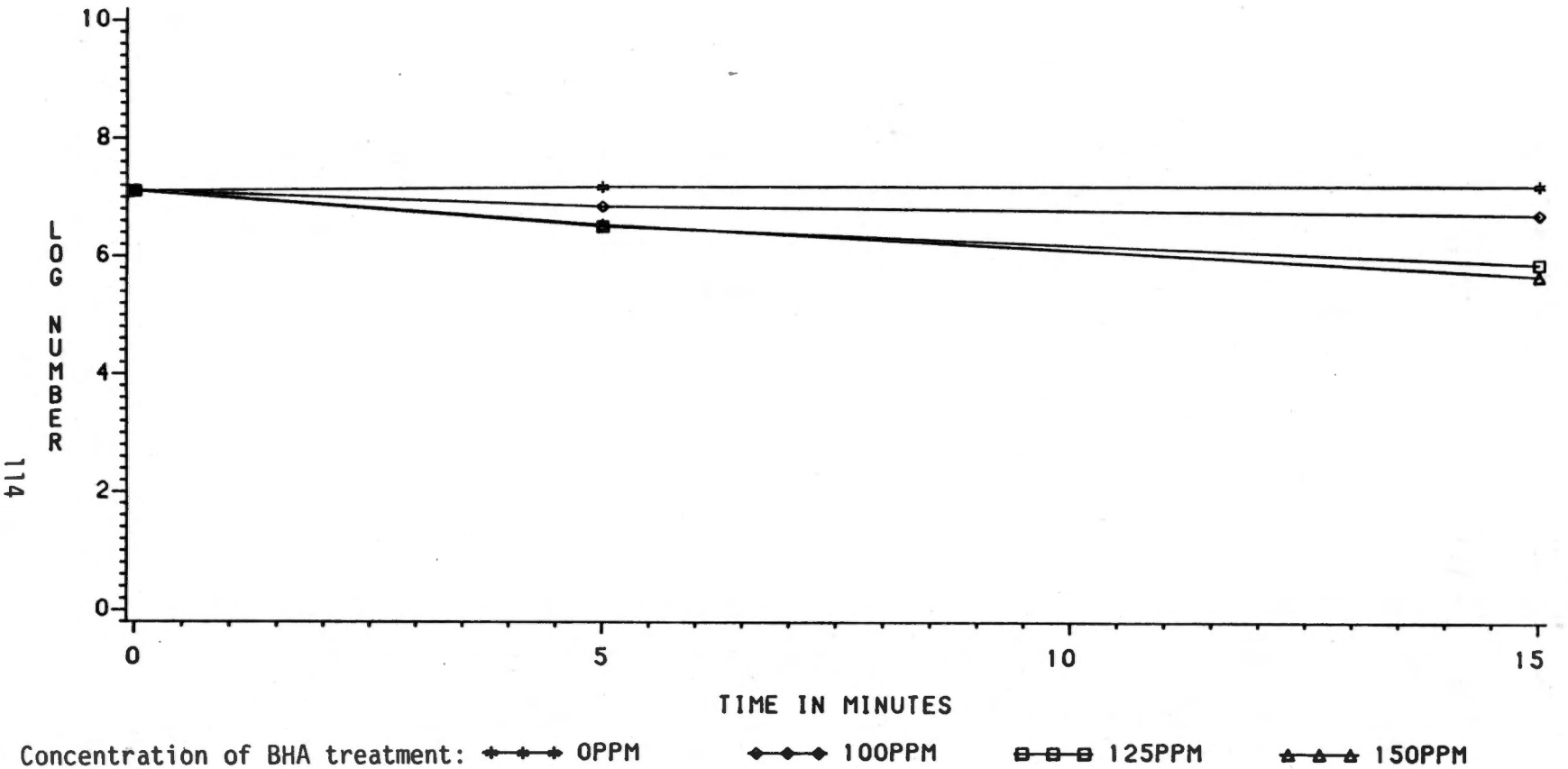


Figure A-1. Lethality rate of butylated hydroxyanisole on Pseudomonas fluorescens grown in the presence of 0 ppm BHA at 26 C.

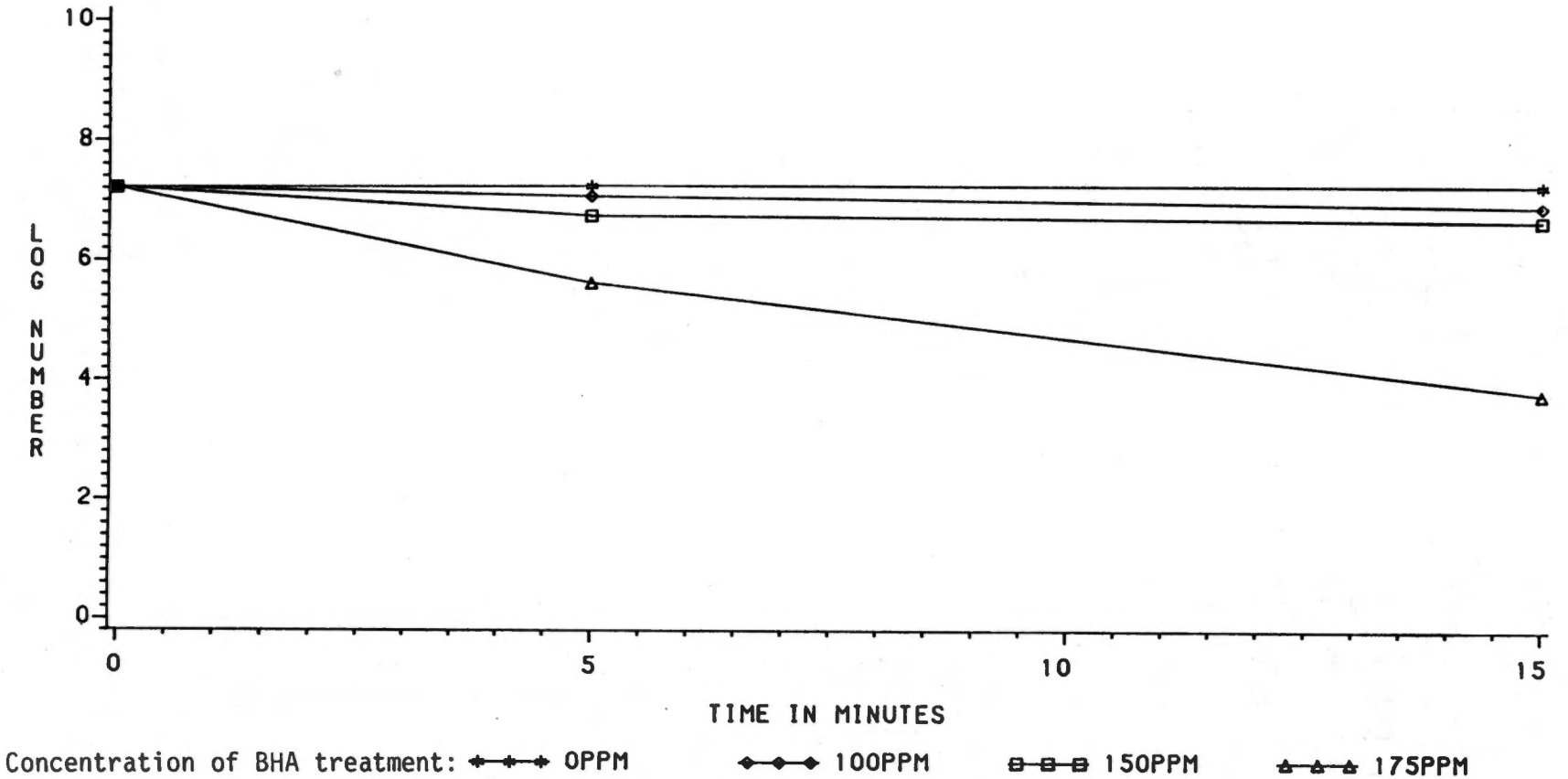


Figure A-2. Lethality rate of butylated hydroxyanisole on Pseudomonas fluorescens grown in the presence of 50 ppm BHA at 26 C.

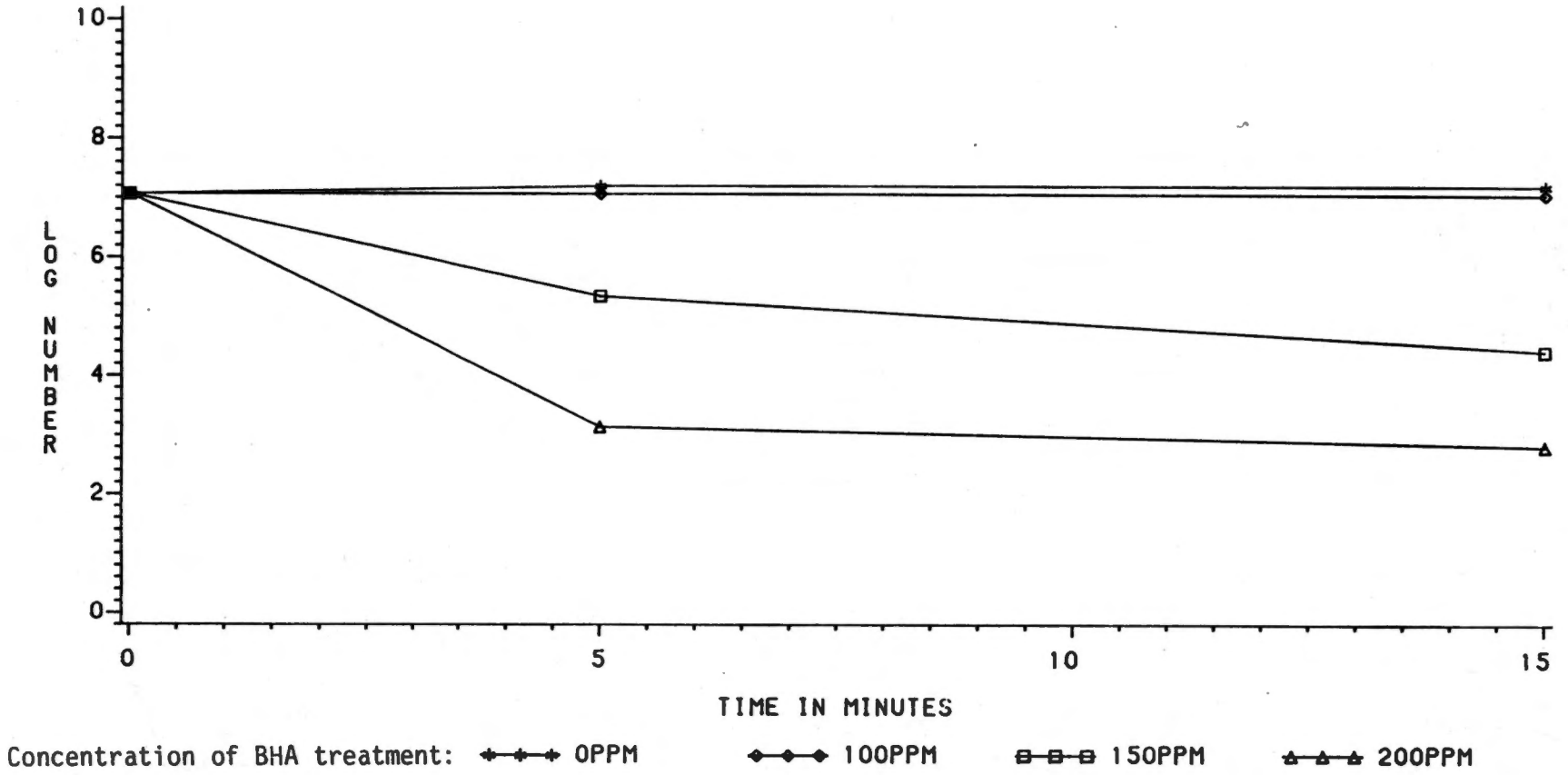


Figure A-3. Lethality rate of butylated hydroxyanisole on Pseudomonas fluorescens grown in the presence of 100 ppm BHA at 26 C.

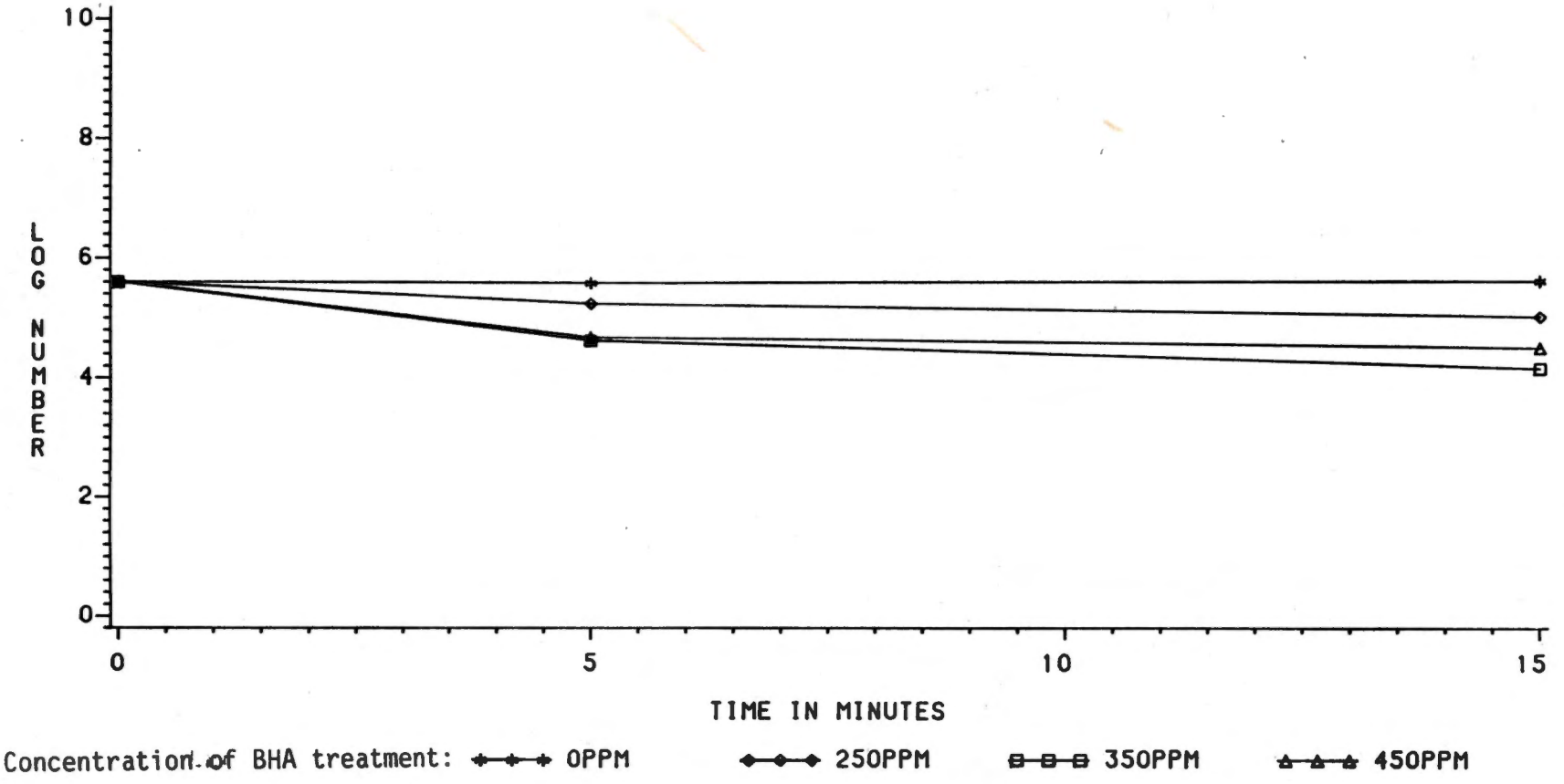


Figure A-4. Lethality rate of butylated hydroxyanisole on Pseudomonas fluorescens grown in the presence of 125 ppm BHA at 26 C.

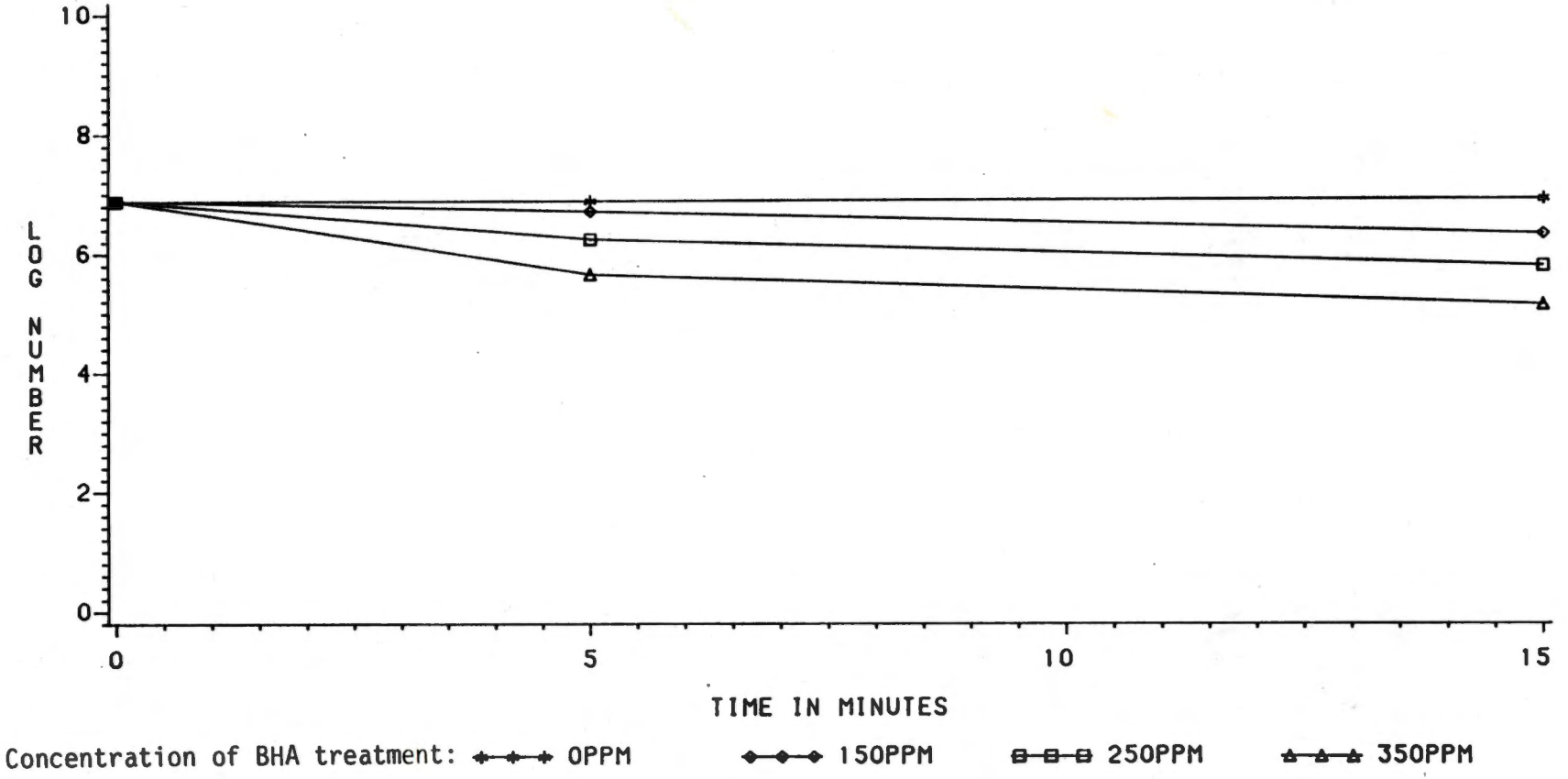


Figure A-5. Lethality rate of butylated hydroxyanisole on Pseudomonas fluorescens grown in the presence of 125 ppm BHA at 26 C and carried through one transfer into broth containing no BHA.

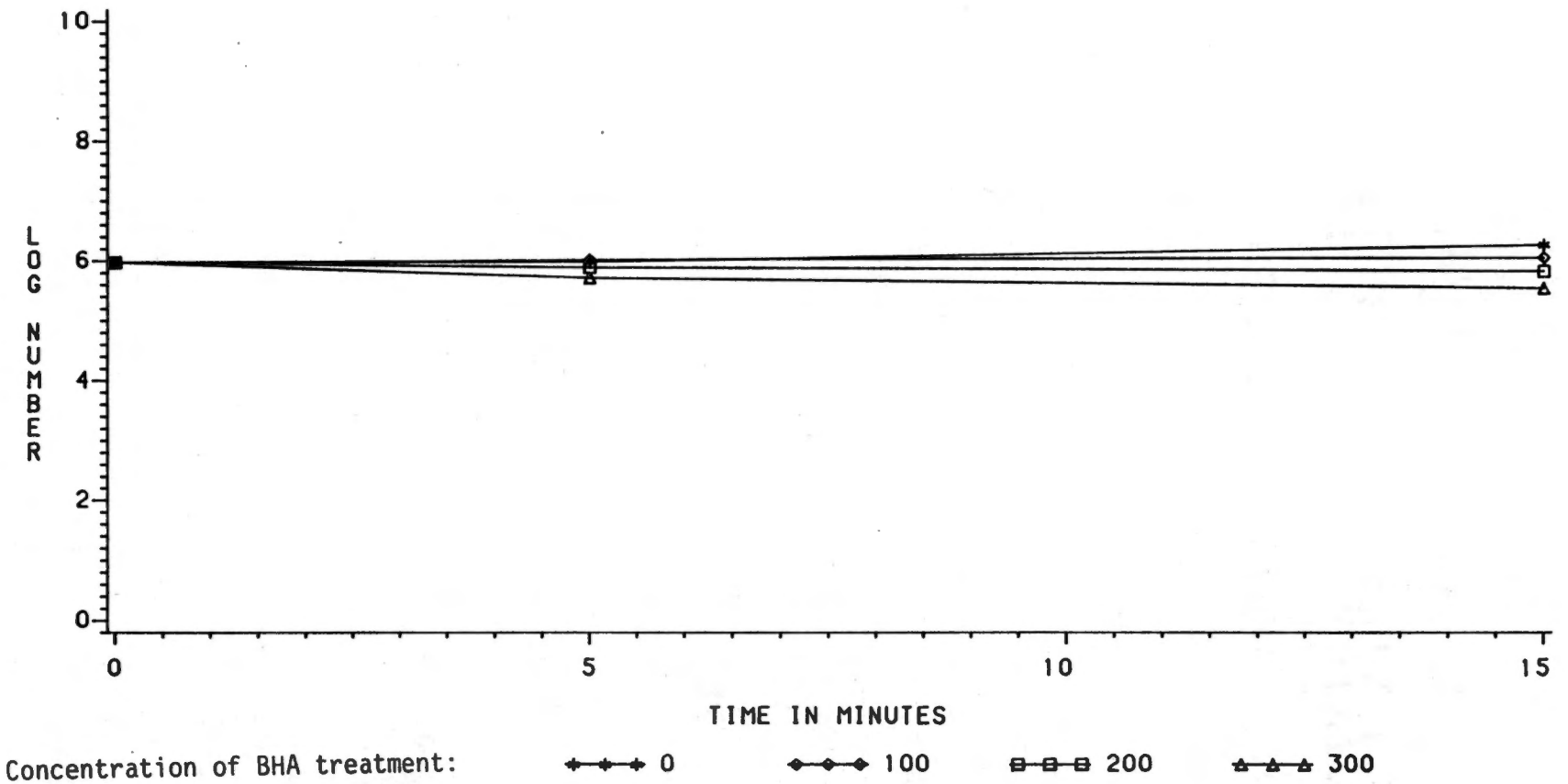


Figure A-6. Lethality rate of butylated hydroxyanisole on Pseudomonas fragi grown in the presence of 0 ppm BHA at 26 C.

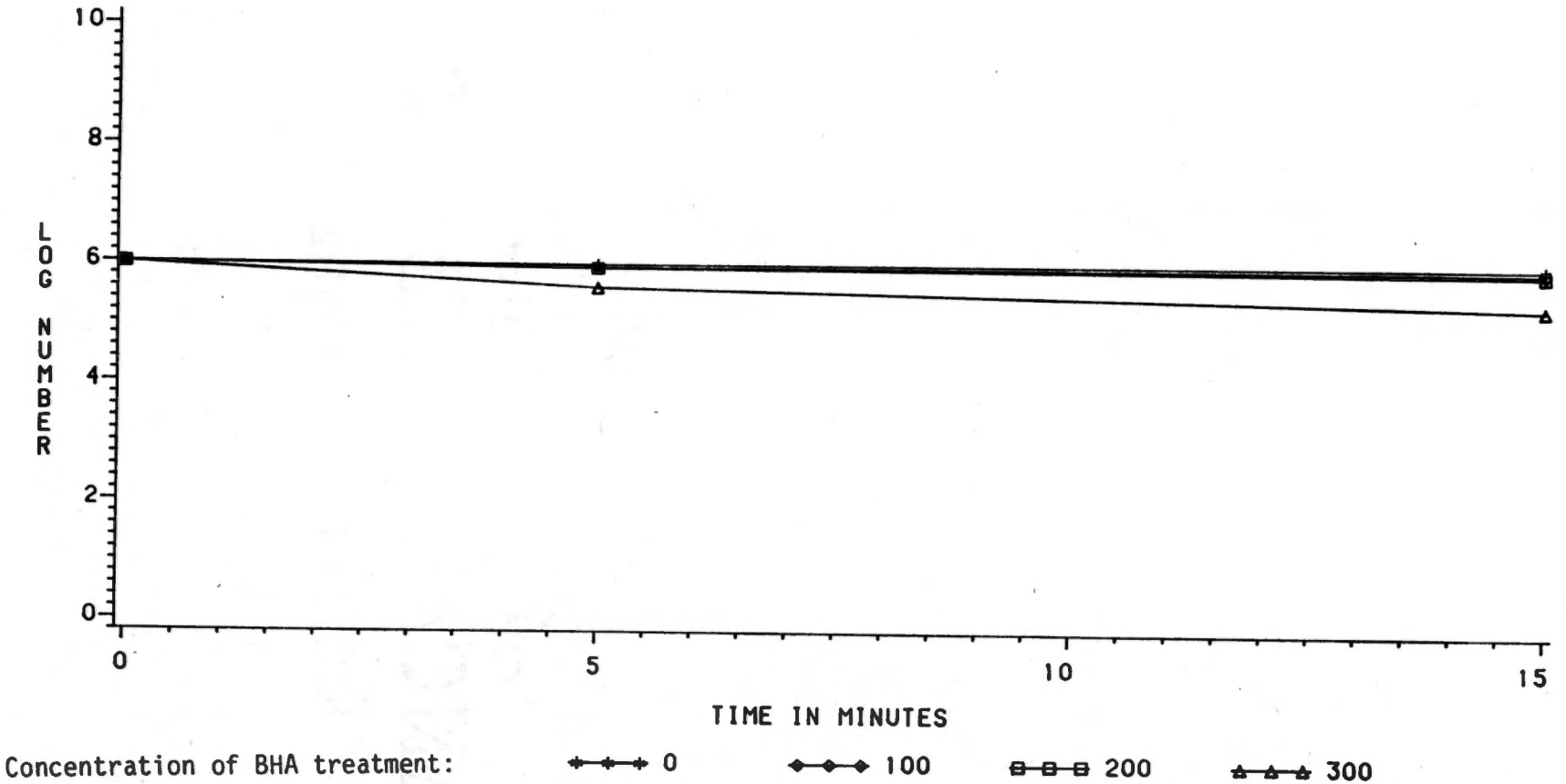


Figure A-7. Lethality rate of butylated hydroxyanisole on Pseudomonas fragi grown in the presence of 50 ppm BHA at 26 C.

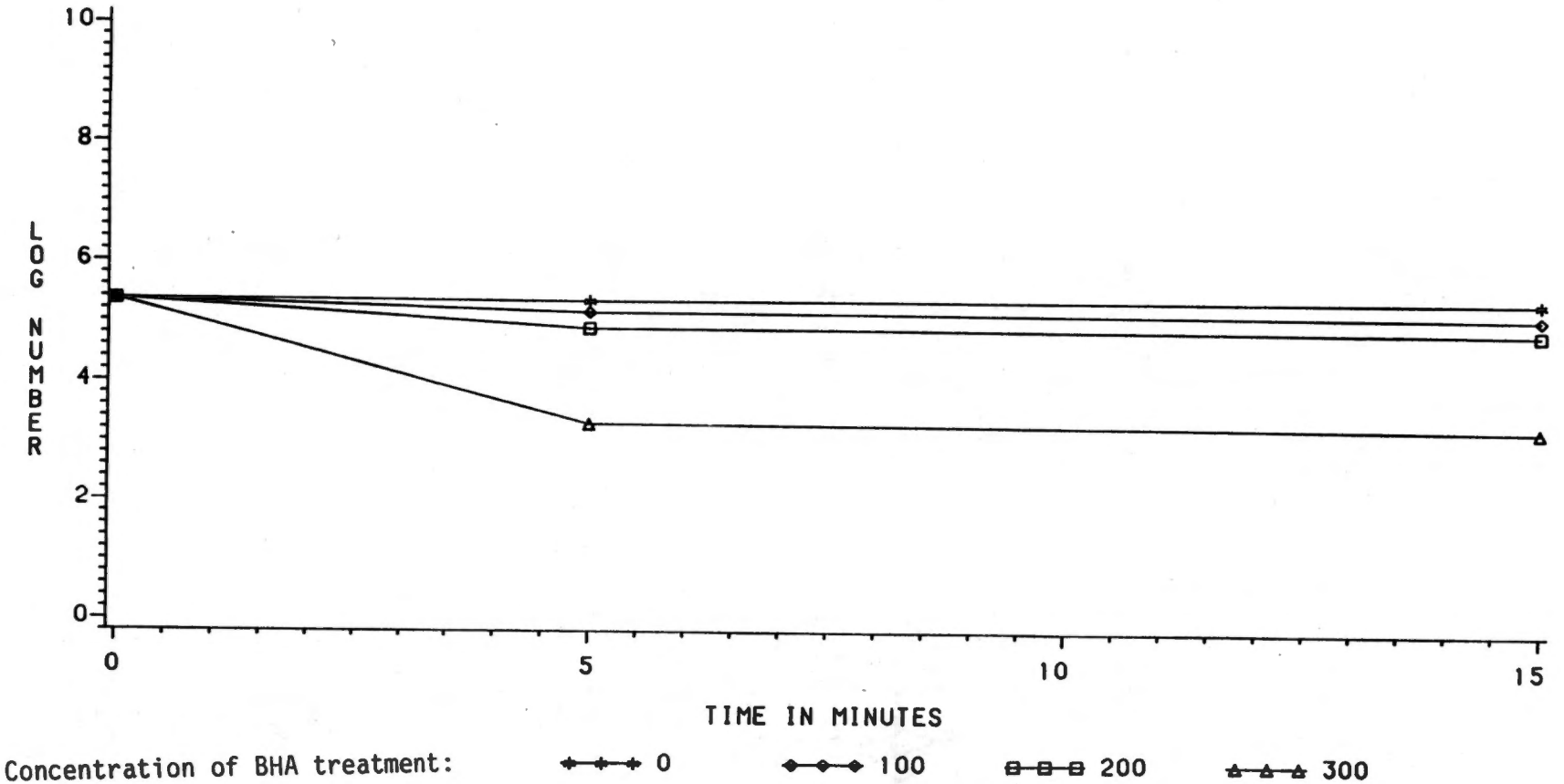


Figure A-8. Lethality rate of butylated hydroxyanisole on Pseudomonas fragi grown in the presence of 100 ppm BHA at 26 C.

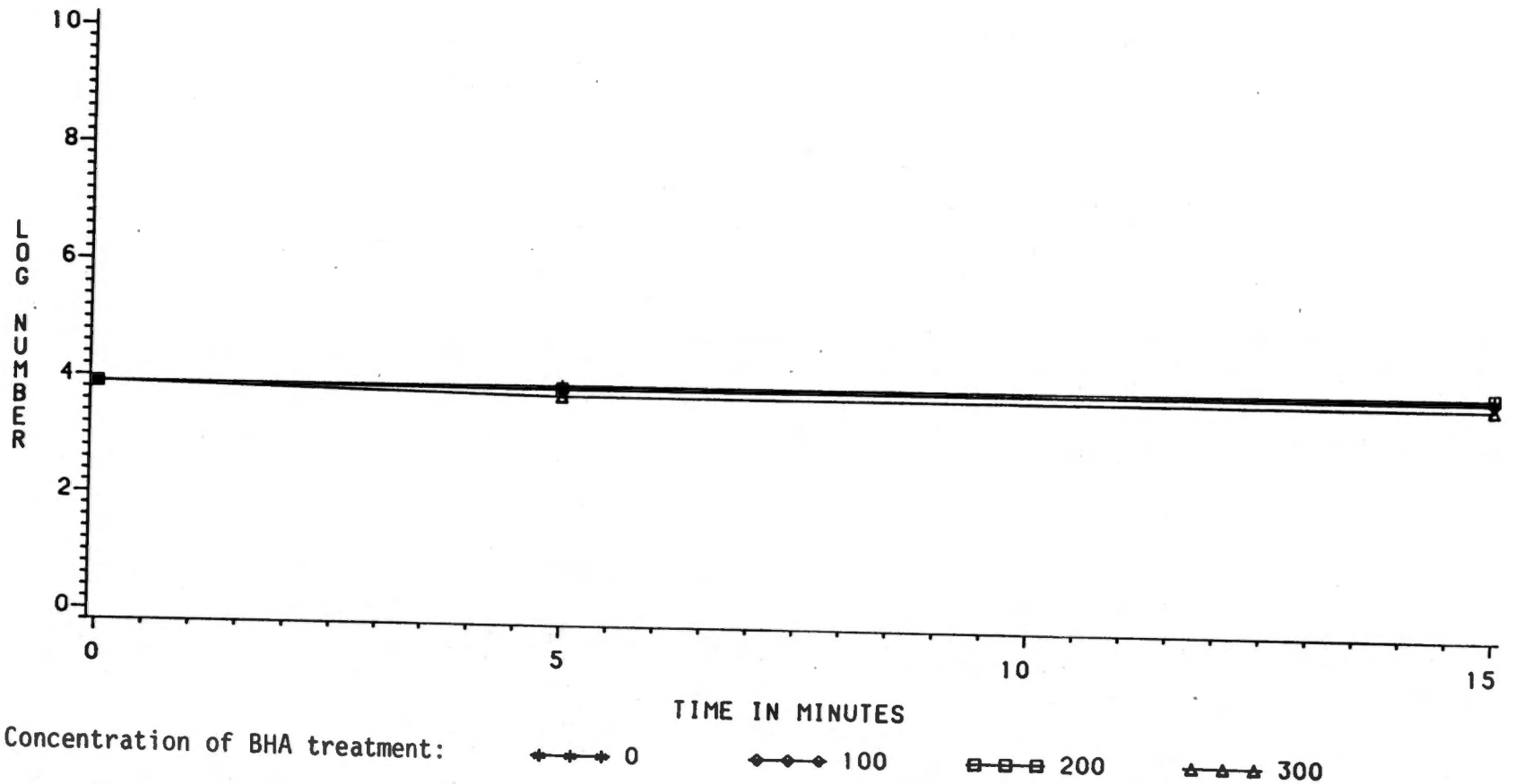


Figure A-9. Lethality rate of butylated hydroxyanisole on Pseudomonas fragi grown in the presence of 200 ppm BHA at 26 C.

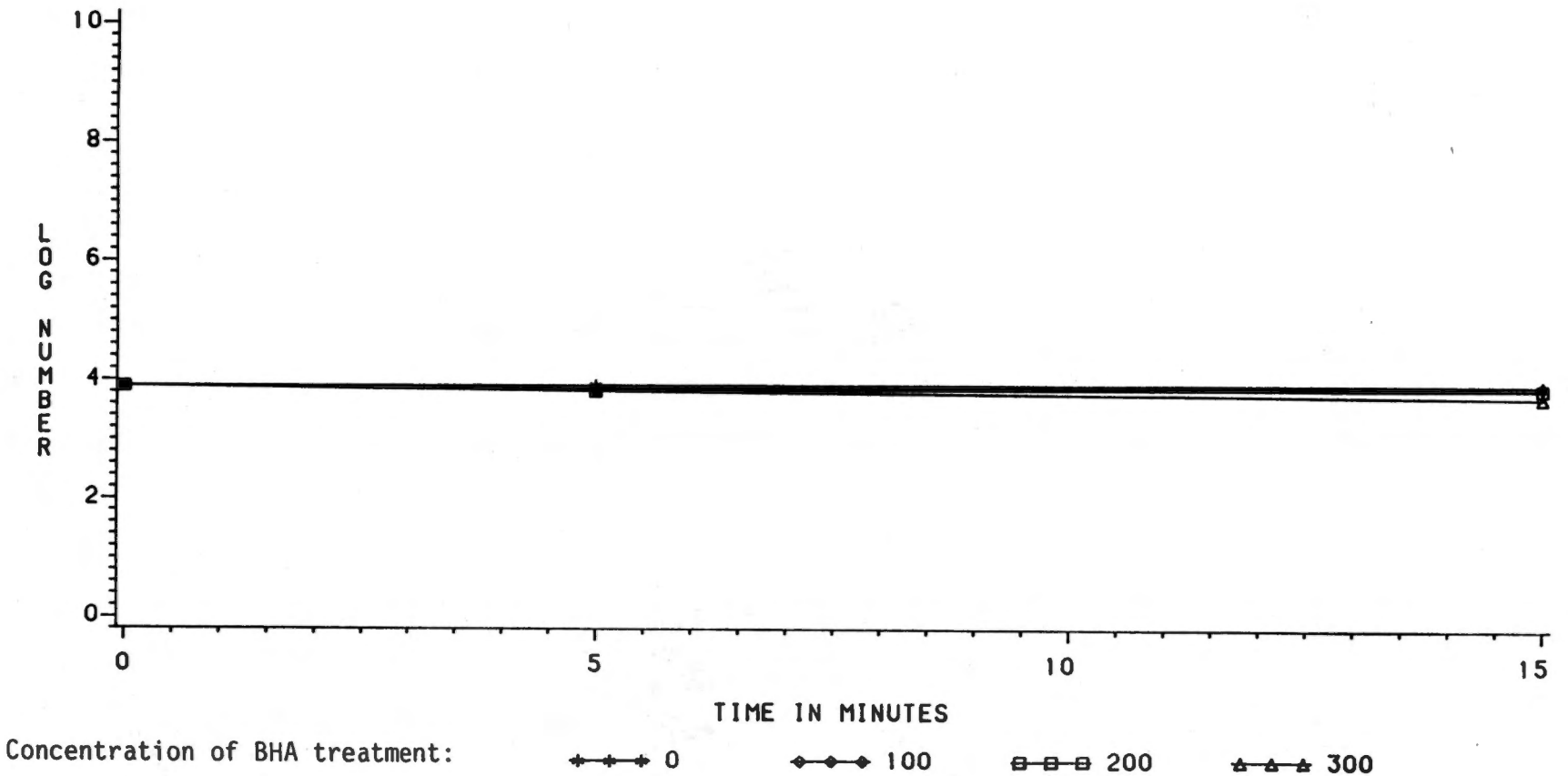


Figure A-10. Lethality rate of butylated hydroxyanisole on Pseudomonas fragi grown in the presence of 300 ppm BHA at 26 C.

APPENDIX B

Table B1. Fatty acid composition of the total lipid fractions from four strains of Staphylococcus aureus (relative percent peak areas)

Fatty Acid[a]	Staphylococcus aureus	Staphylococcus aureus NR	Staphylococcus aureus S-6	Staphylococcus aureus A-100
Unknown	1.66	2.98	2.69	1.81
13:0	0.18	---	0.11	---
3:OH 12:0	2.33	3.65	3.19	2.62
14:0[b]	17.24	23.46	12.96	6.77
15:0[b]	15.46	18.61	23.26	40.40
16:1	0.45	0.06	1.36	0.46
16:0[c]	17.88	19.37	19.53	14.47
17:0[c]	0.94	1.03	1.15	4.28
18:1	0.38	1.01	5.56	4.12
18:0[d]	21.53	27.47	29.07	22.72
19:0[d]	1.83	1.67	1.11	1.45
20:0	19.38	---	---	---
Others	0.74	0.69	0.07	1.20
Unsaturated/ Saturated	0.83/99.17	1.07/98.93	6.92/93.08	4.58/95.42

[a]First number denotes number of carbon atoms, second number is number of double bonds.

[b]Total concentration of α15:0 and 15:0.

[c]Total concentration of α17:0, 17:0Δ, and 17:0.

[d]Total concentration of 19:0 and 19:0Δ.

Table B2. Fatty acid composition of the polar lipid fractions from four strains of *Staphylococcus aureus* (relative percent peak areas)

Fatty Acid[a]	Relative Percent Peak Area			
	Staphylococcus aureus	*Staphylococcus aureus* NR	*Staphylococcus aureus* S-6	*Staphylococcus aureus* A-100
Unknown 1	1.83	---	1.55	1.15
13:0	0.41	1.62	1.00	---
3:OH 12:0	4.71	1.50	2.34	3.10
14:0[b]	12.83	8.66	7.45	4.91
15:0[b]	17.79	10.96	13.91	22.79
3:OH 14:0	1.04	---	---	---
16:1	4.47	0.70	3.28	0.24
16:0	19.16	10.17	19.28	13.49
Unknown 2	---	---	4.44	---
17:0[c]	2.17	0.94	1.30	9.66
18:1	7.54	1.01	8.00	12.87
18:0[d]	19.70	14.05	36.47	19.55
19:0[d]	0.55	36.42	0.21	6.74
Unknown 3	---	---	---	2.48
20:0	6.35	13.78	0.37	2.37
Others	1.45	0.19	0.34	0.65
Unsaturated/ Saturated	12.01/87.99	1.71/98.29	11.28/88.72	13.11/86.89

[a]First number denotes number of carbon atoms, second number is number of double bonds.

[b]Total concentration of α15:0 and 15:0.

[c]Total concentration of α17:0, 17:0Δ, and 17:0.

[d]Total concentration of 19:0 and 19:0Δ.

Table B3. Fatty acid composition of the total lipid fractions from several bacterial species (relative percent peak areas)

Fatty Acid[a]	Escherchia coli	Salmonella anatum	Clostridium perfringens	Bacillus cereus
11:0	0.22	---	14.58	---
Unknown 1	---	---	---	1.79
12:0	0.29	0.46	1.41	3.06
Unknown 2	---	---	6.57	6.77
13:0	---	---	13.94	2.87
3OH 12:0	---	2.55	5.60	---
14:0[b]	3.61	5.64	10.90	5.86
15:0[b]	0.63	---	2.78	12.46
3OH 14:0	---	---	6.40	2.61
16:1	6.02	1.36	7.08	4.05
16:0[c]	41.59	31.47	5.12	38.71
17:0[c]	2.12	24.28	14.69	16.34
18:1	23.91	8.13	4.66	1.23
18:0[d]	5.73	3.87	4.76	2.72
19:0[d]	6.47	5.27	1.48	---
20:0	4.58	17.07	---	---
Others	4.74	---	---	1.53
Unsaturated/ Saturated	29.04/70.96	9.49/90.51	11.7488/26	5.28/94.72

[a]First number denotes number of carbon atoms, second number is number of double bonds.

[b]Total concentration of α15:0 and 15:0.

[c]Total concentration of α17:0, 17:0Δ, and 17:0.

[d]Total concentration of 19:0 and 19:0Δ.

Table B4. Fatty acid composition of the polar lipid fractions from several bacterial species (relative percent peak areas)

Fatty Acid[a]	Escherichia coli	Salmonella anatum	Clostridium perfringens	Bacillus cereus
2OH 10:0	---	0.09	---	0.98
Unknown 1	---	0.31	---	0.96
12:0	---	0.70	16.12	1.52
Unknown 2	---	---	---	5.13
13:0	0.72	---	0.78	0.57
3OH 12:0	2.34	0.68	1.23	3.29
14:0[b]	5.55	5.86	15.34	4.62
15:0[b]	0.58	0.84	1.83	8.99
3OH 14:0	1.91	0.83	---	1.80
16:1	2.38	0.75	0.75	11.12
16:0	37.55	39.33	13.56	33.67
17:0[c]	19.34	23.88	3.82	9.63
18:1	6.08	6.18	11.97	10.21
18:0	8.01	2.80	9.15	7.52
Unknown 3	---	---	6.04	---
19:0[d]	15.49	2.81	14.84	---
20:0	---	14.94	4.55	---
Unsaturated/ Saturated	8.46/91.54	6.93/93.07	12.72/87.28	21.33/78.67

[a]First number denotes number of carbon atoms, second number is number of double bonds.

[b]Total concentration of α15:0 and 15:0.

[c]Total concentration of α17:0, 17:0Δ, and 17:0.

[d]Total concentration of 19:0 and 19:0Δ.

APPENDIX C

REGRESSION EQUATIONS FOR PLOTTED CURVES

Figure 2. Dose response curves for Pseudomonas fluorescens grown in the presence of 0-125 ppm BHA and subsequently challenged with varying concentrations of BHA for 15 minutes.

culture growth in the presence of 0 ppm BHA

$$Y = 7.14667116 + 0.06168585X - 0.00078605X^2 + 1.7584906E\text{-}06X^3$$

culture growth in the presence of 50 ppm BHA

$$Y = 7.24000000 - 0.07531667X + 0.00120175X^2 - 4.8383333E\text{-}06X^3$$

culture growth in the presence of 100 ppm BHA

$$Y = 7.17831873 + 0.04278766X - 0.00059210X^2 + 1.3132390E\text{-}06X^3$$

culture growth in the presence of 125 ppm BHA

$$Y = 5.62000000 + 0.01717464X - 0.00012170X^2 + 1.7328571E\text{-}07X^3$$

Figure 3. Dose response curves for Pseudomonas fluorescens grown in media containing 0 and 125 ppm BHA and for cells grown in the presence of 125 ppm BHA carried through one transfer in broth with no BHA. Cells were subsequently challenged with varying concentrations of BHA for 15 minutes.

culture grown in the presence of 0 ppm BHA

$$Y = 7.14667116 + 0.06168585X - 0.00078605X^2 + 1.7584906E\text{-}06X^3$$

culture grown in the presence of 125 ppm BHA

$$Y = 5.62000000 + 0.01717464X - 0.00012170X^2 + 1.7328571E\text{-}07X^3$$

culture grown in the presence of 125 ppm BHA transferred once into broth with no BHA (125*)

$$Y = 6.90250000 - 0.00279250X$$

Figure 4. Dose response curves for Pseudomonas fragi grown in the presence of 0-300 ppm BHA and subsequently challenged with varying concentrations of BHA for 15 minutes.

culture growth in the presence of 0 ppm BHA

$$Y = 6.49625000 - 0.23925000X$$

culture growth in the presence of 50 ppm BHA

$$Y = 6.60500000 - 1.12625000X + 0.56875000X^2 - 0.09250000X^3$$

culture growth in the presence of 100 ppm BHA

$$Y = 4.60437500 + 1.00862500X - 0.33437500X^2$$

culture growth in the presence of 200 ppm BHA

$$Y = 4.49500000 - 0.97708333X + 0.47000000X^2 - 0.06791667X^3$$

culture growth in the presence of 300 ppm BHA

$$Y = 4.00750000 - 0.07075000X$$

Figure 11. Dose response curves for Pseudomonas fluorescens incubated at 5, 15, 25 and 35 C and subsequently challenged with varying concentrations of BHA for 15 minutes.

culture growth at 5 C

$$Y = 6.66311508 - 0.00393261X + 3.1972222E\text{-}07X^3$$

culture growth at 15 C

$$Y = 6.99785714 + 0.02594405X - 0.00042943X^2 + 8.5666667E\text{-}07X^3$$

culture growth at 25 C

$$Y = 7.21473214 + 0.00131732X - 0.00011345X^2$$

culture growth at 35 C

$$Y = 7.09994048 + 0.01396806X - 0.00028017X^2 + 5.2611111E\text{-}07X^3$$

Figures 14-23. Dose response curves for organisms challenged with varying concentrations of BHA for 15 minutes in 0.05M phosphate-0.1% peptone buffer.

Pseudomonas fluorescens

$$Y = 6.89483777 + 0.03032027X - 0.00042081X^2 + 8.0407407E\text{-}07X^3$$

Pseudomonas fragi

$$Y = 5.95500000 - 0.00266250X + 2.9125000E - 05X^2 - 9.250000E\text{-}08X^3$$

Bacillus cereus

$$Y = 5.29401515 + 0.01435060X - 0.00013176X^2 + 1.8090909E\text{-}07X^3$$

Staplylococcus aureus 12600

$$Y = 6.65409091 - 0.00077238X + 1.8435065E\text{-}05X^2 - 1.024745E\text{-}07X^3$$

Staphylococcus aureus NR

$$Y = 6.6553030 - 0.02456627X + 0.00022575X^2 - 5.4429293E\text{-}07X^3$$

Escherichia coli

$$Y = 6.95712121 + 0.00010292X - 1.7194805E\text{-}07X^3$$

Salmonella anatum

$$Y = 6.675625 + 0.00922911X - 0.00014977X^2$$

Clostridium perfringens

$$Y = 5.91357143 + 0.00202214X - 0.00011379X^2$$

Staphylococcus aureus S-6

$$Y = 6.43851190 + 0.01244107X - 0.00010815X^2$$

Staphylococcus aureus A-100

$$Y = 5.94447024 + 0.00506000X^2 - 7.4059524E\text{-}05X^3$$

APPENDIX D

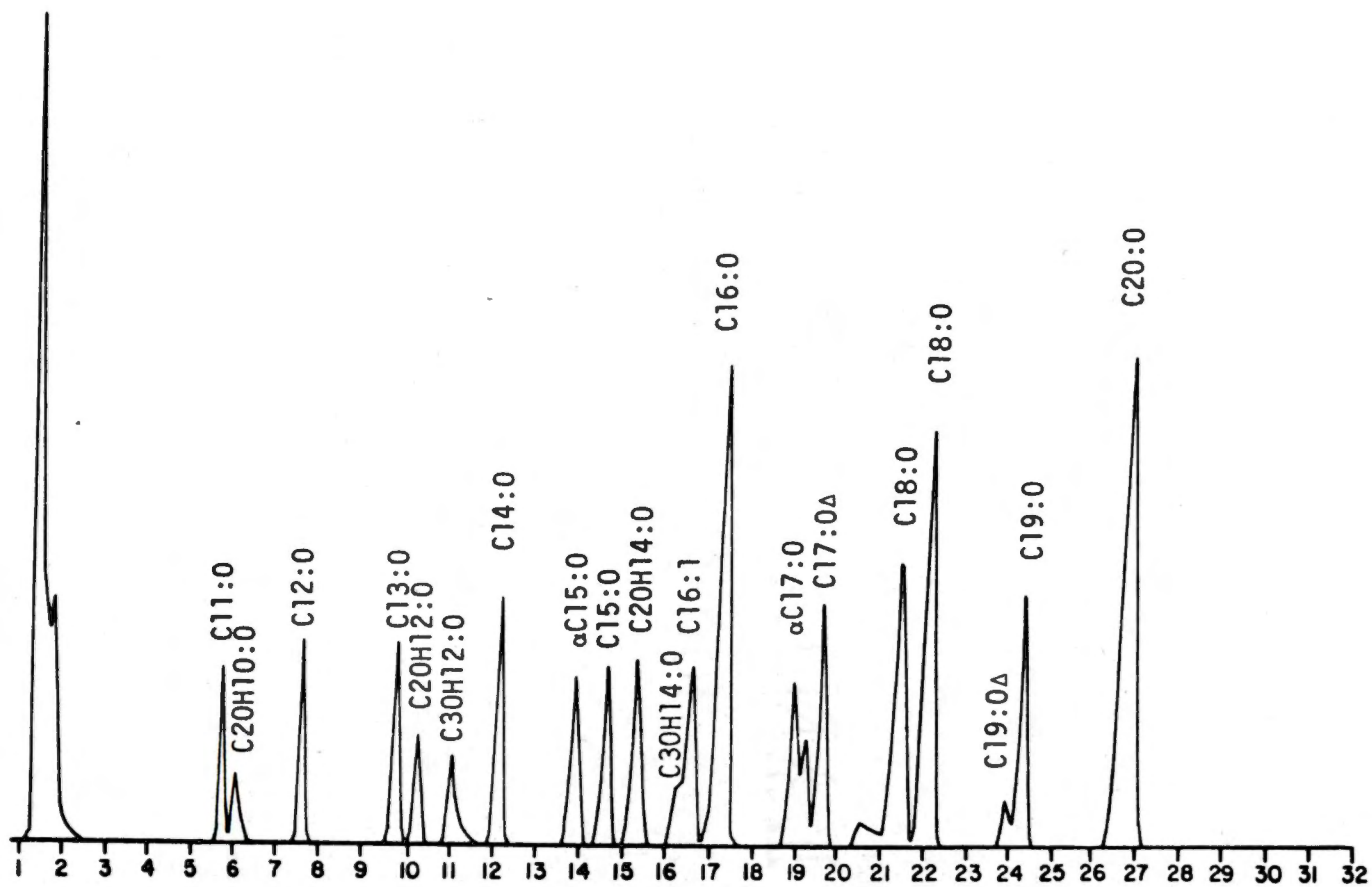


Figure D-1. Representative chromatogram of bacterial methyl ester standard (oven temperature 150-250 C/4^{0} per minute, injection temperature 250 C).

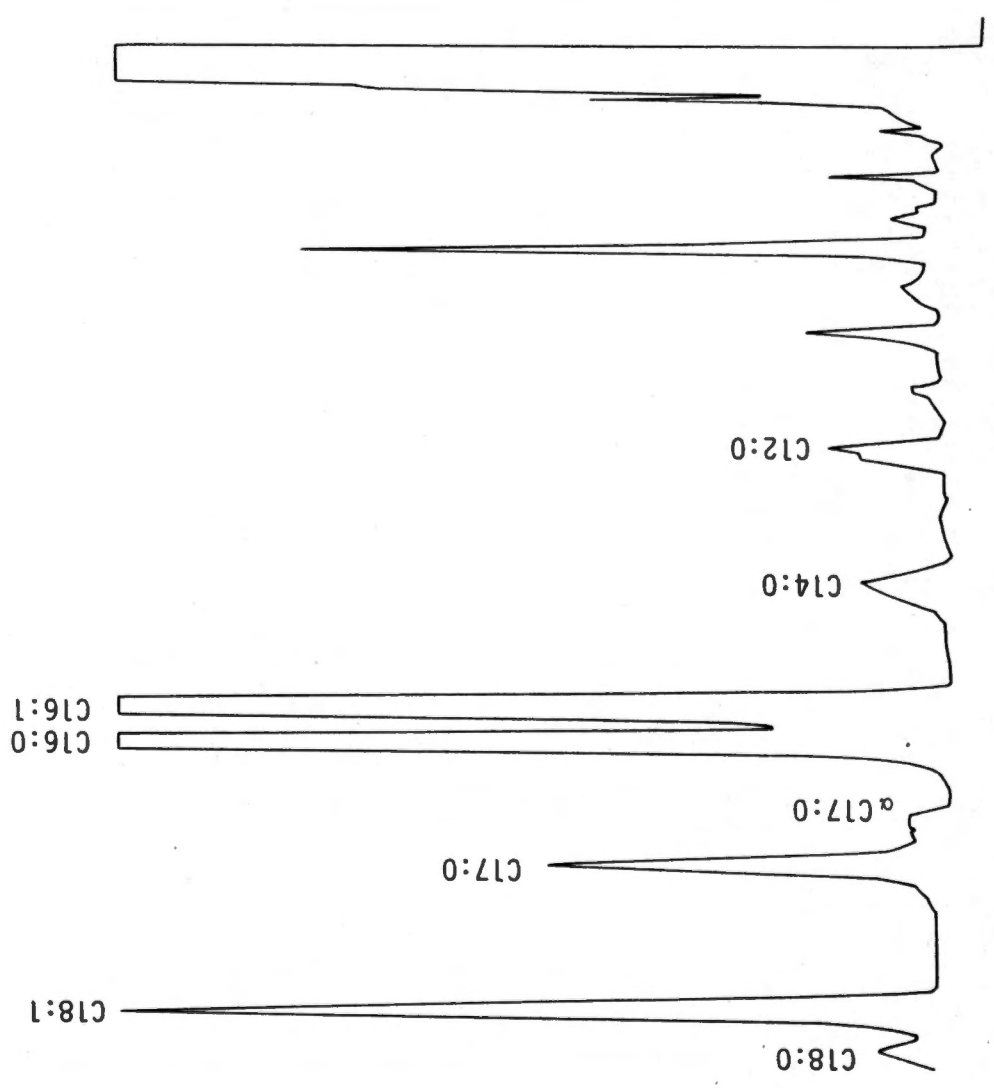


Figure D-2. Representative chromatogram of fatty acid methyl esters from the total lipid fraction of Pseudomonas fluorescens (oven temperature 150-225 C/4° per minute, injection temperature 250 C).

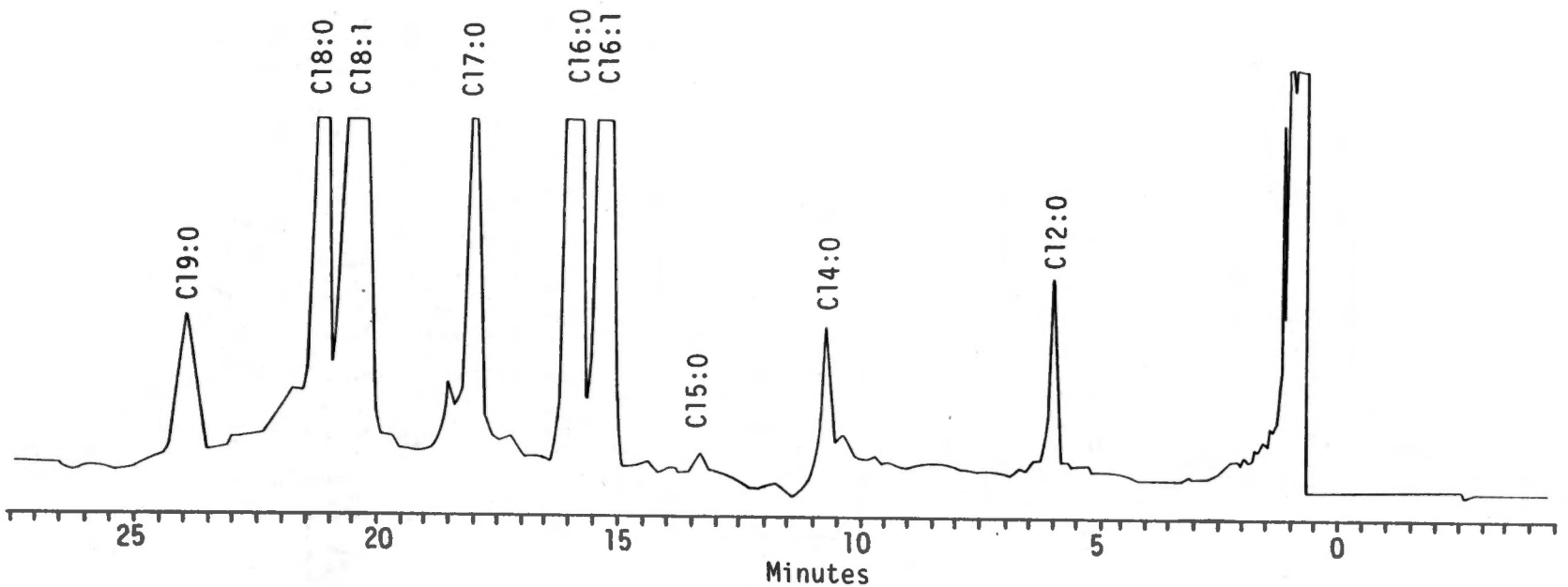


Figure D-3. Representative chromatogram of fatty acid methyl esters from the total lipid fraction of Pseudomonas fragi (oven temperature 150-225 C/4° per minute, injection temperature 250 C).

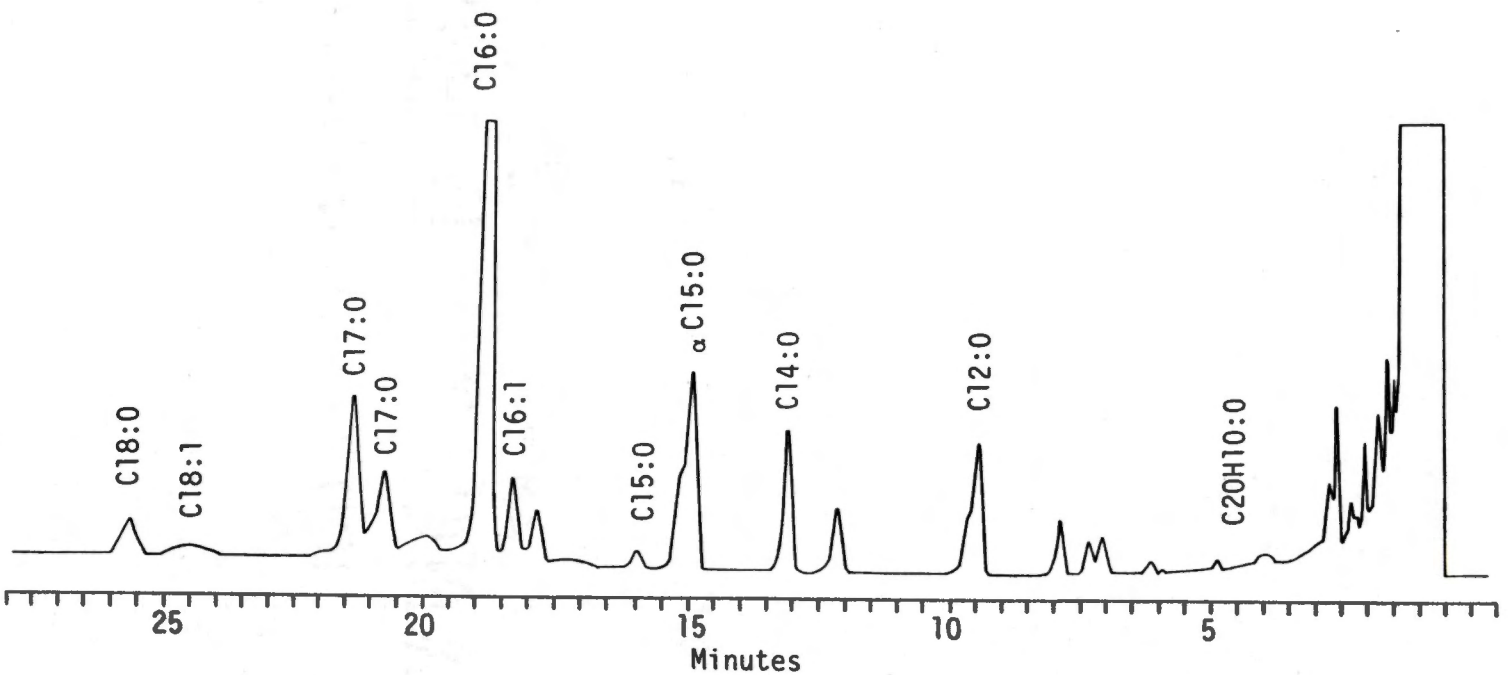


Figure D-4. Representative chromatogram of fatty acid methyl esters from the total lipid fraction Bacillus cereus (oven temperature 150-225 C/4° per minute, injection temperature 250 C).

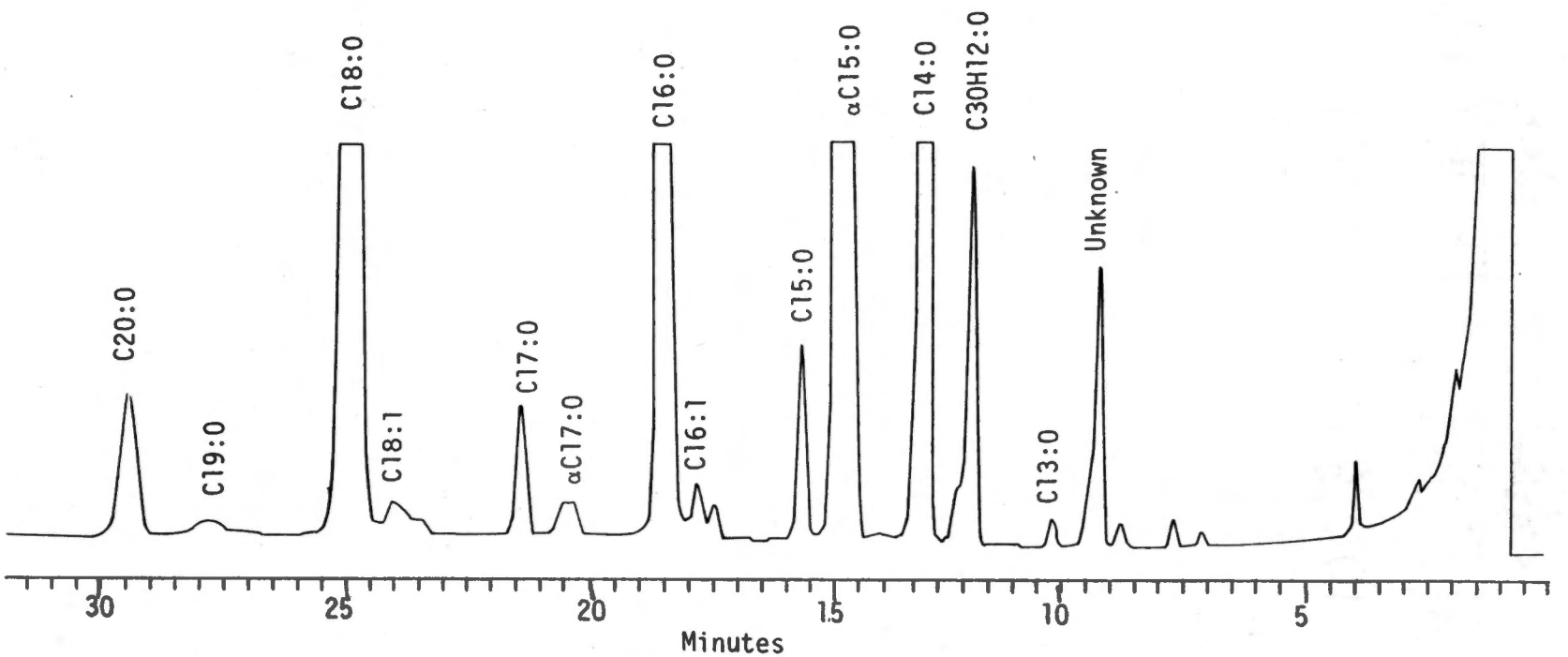


Figure D-5. Representative chromatogram of fatty acid methyl esters from the total lipid fraction of Staphylococcus aureus 12600 (oven temperature 150-225 C/4° per minute, injection temperature 250 C).

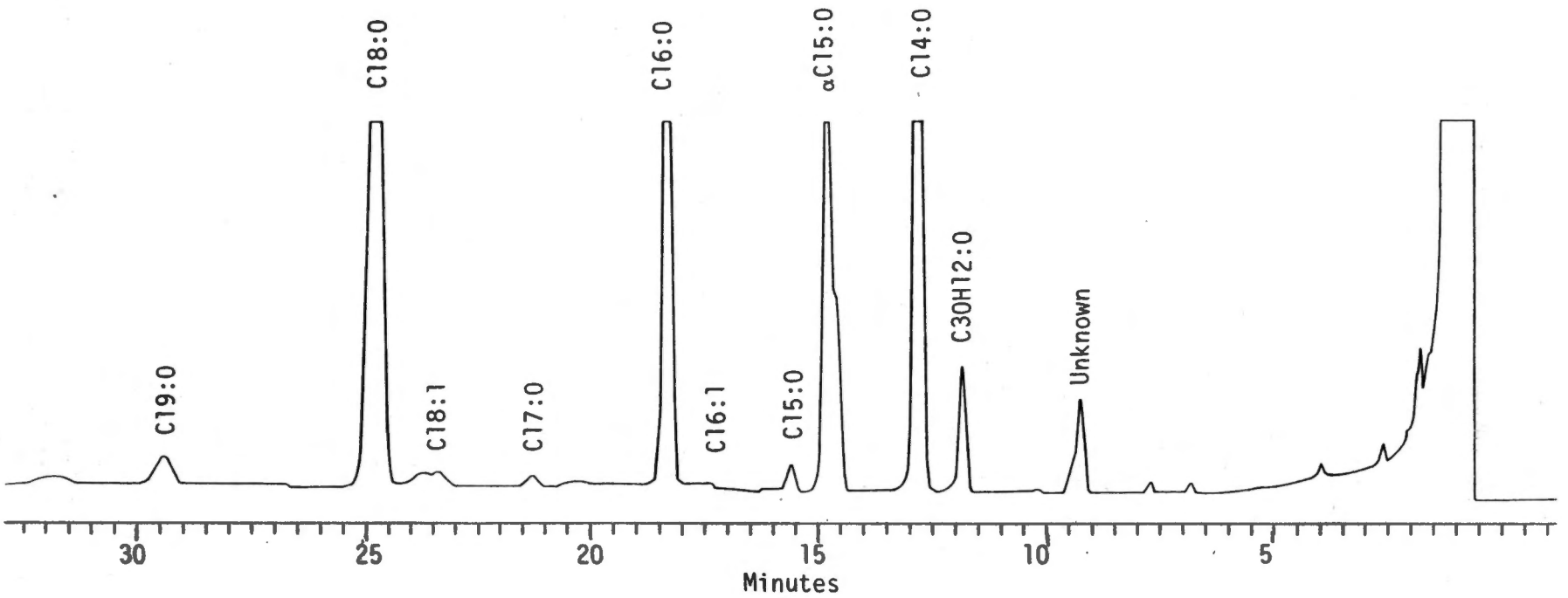


Figure D-6. Representative chromatogram of fatty acid methyl esters from the total lipid fraction of Staphylococcus aureus NR (oven temperature 150-225 C/4° per minute, injection temperature 250 C).

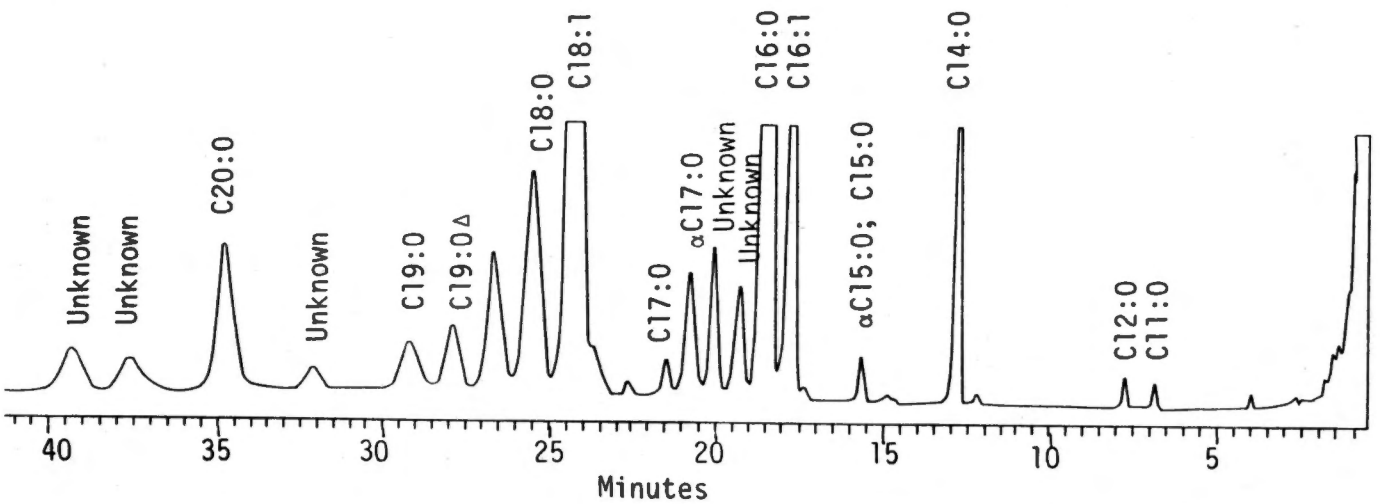


Figure D-7. Representative chromatogram of fatty acid methyl esters from the total lipid fraction of Escherichia coli (oven temperature 150-225 C/4° per minute, injection temperature 250 C).

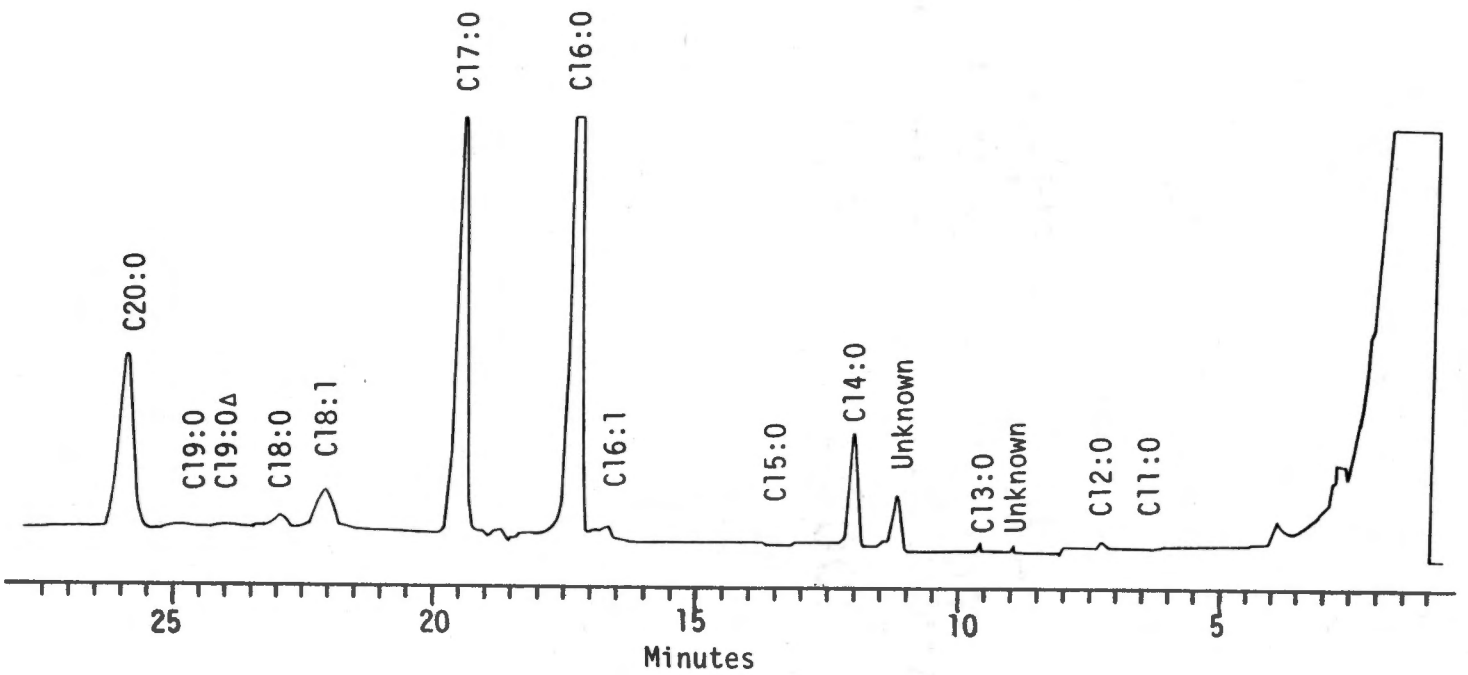


Figure D-8. Representative chromatogram of fatty acid methyl esters from the total lipid fraction of Salmonella anatum NR (oven temperature 150-225 C/4° per minute, injection temperature 250 C).

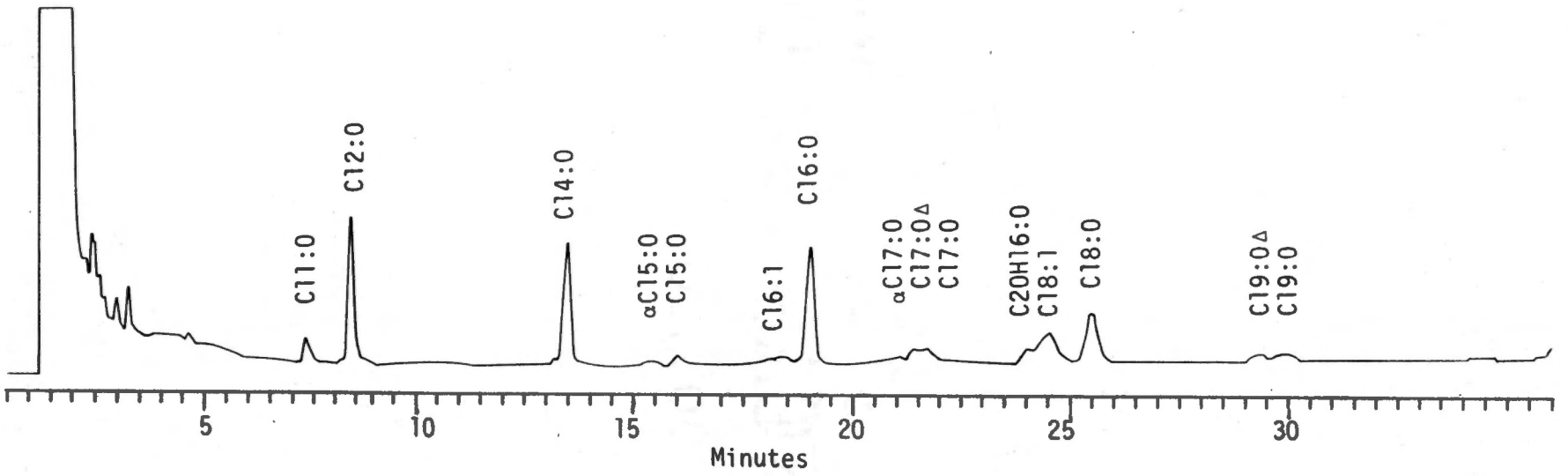


Figure D-9. Representative chromatogram of fatty acid methyl esters from the total lipid fraction of Clostridium perfringens (oven temperature 150-225 C/4° per minute, injection temperature 250 C).

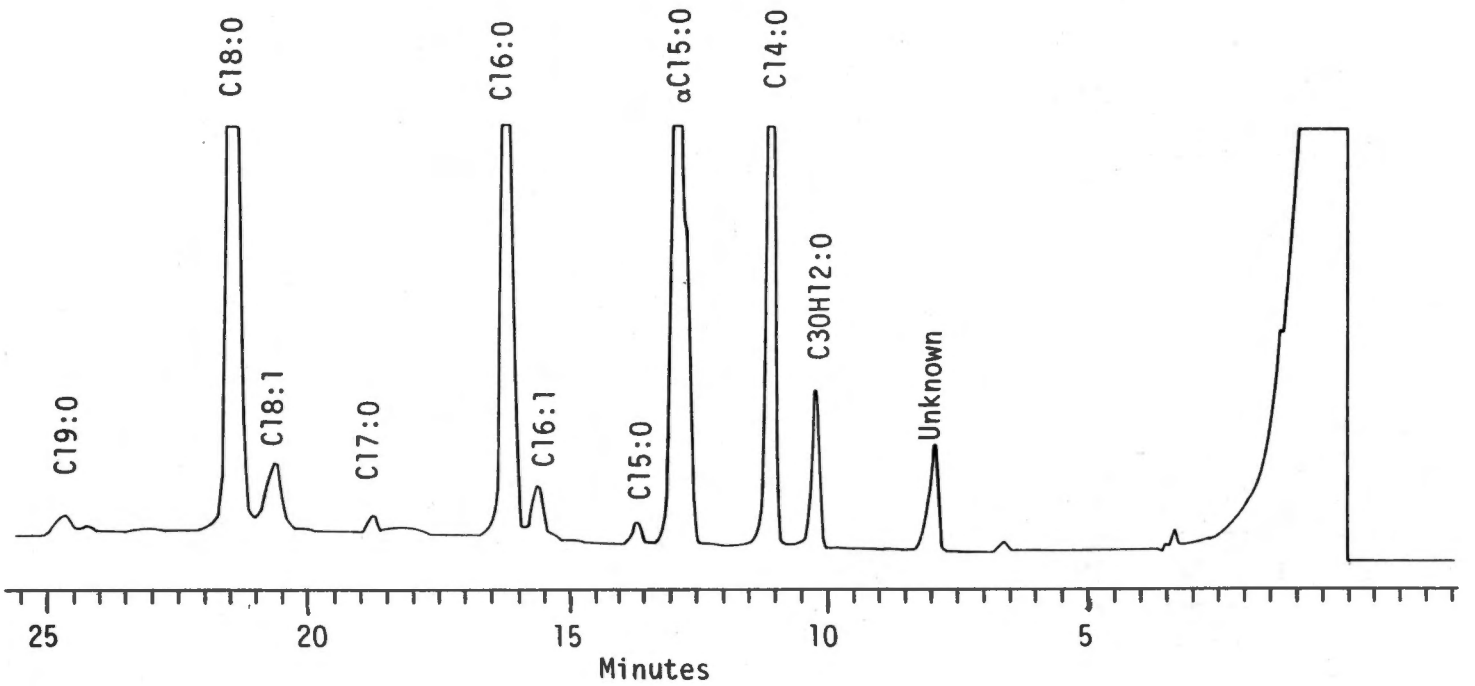


Figure D-10 Representative chromatogram of fatty acid methyl esters from the total lipid fraction of Staphylococcus aureus S-6 (oven temperature 150-225 C/4° per minute, injection temperature 250 C).

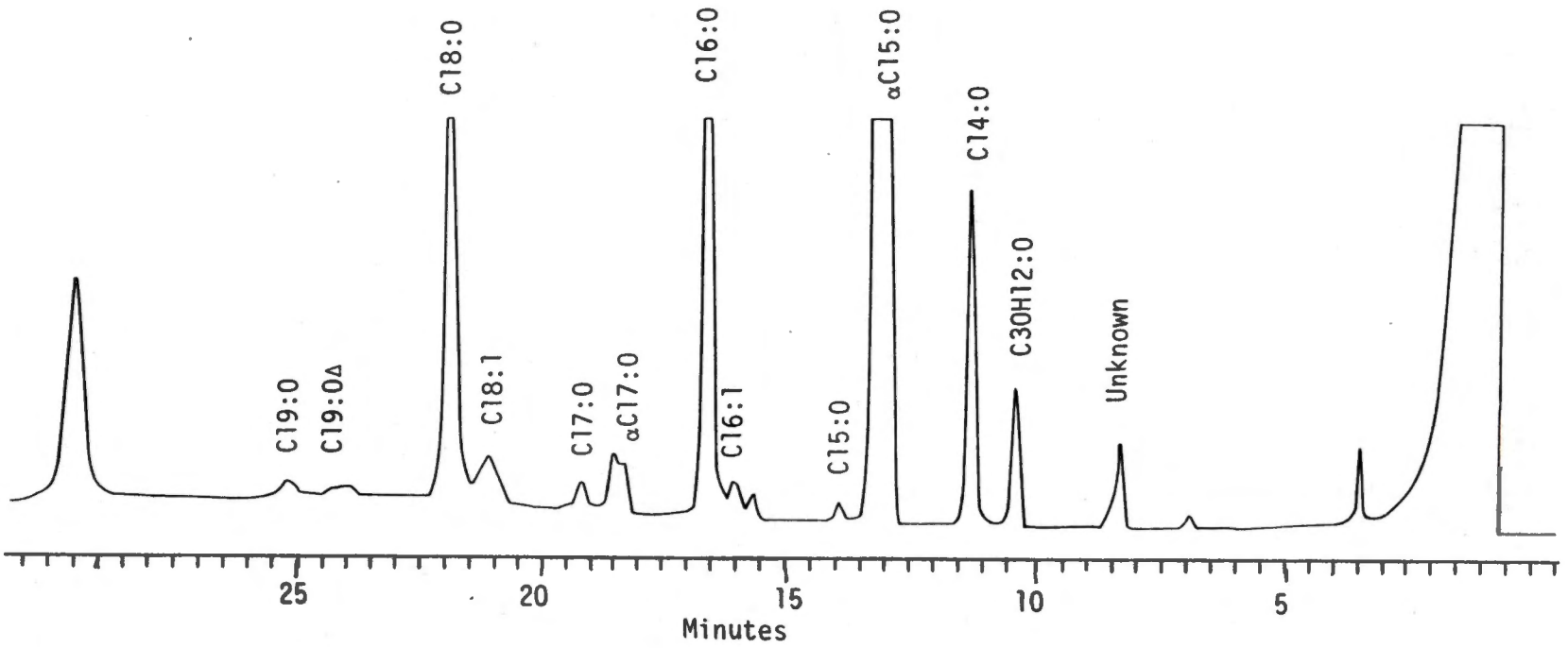


Figure D-11. Representative chromatogram of fatty acid methyl esters from the total lipid fraction of Staphylococcus aureus A-100 (oven temperature 150-225 C/4° per minute, injection temperature 250 C).

VITA

Laurie Sue Post was born on April 12, 1955, in New York City, New York, to Dr. Harold and Gertrude Helen Post. Upon graduating from John F. Kennedy High School, Silver Spring, Maryland, in June 1973, she entered the University of Maryland, College Park, and in May 1977 received a Bachelor of Science degree in Microbiology. She enrolled in the graduate school at the University of Maryland, College Park, and received a Master of Science degree in Food Science in December 1978. In January 1980, she entered The University of Tennessee, Knoxville, and began work towards a Ph.D. in Food Technology and Science.

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