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Influence of membrane lipid composition of Staphylococcus aureus on susceptibility to parabens.

Effimia Eriotou Bargiota

To the Graduate Council:

I am submitting herewith a thesis written by Effimia Eriotou Bargiota entitled "Influence of membrane lipid composition of Staphylococcus aureus on susceptibility to parabens.." I have examined the final electronic copy of this thesis for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Master of Science, with a major in Food Science and Technology.

P. Michael Davidson, Major Professor

We have read this thesis and recommend its acceptance:

H.O. Jaynes, F.A. Draughon

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Vice Provost and Dean of the Graduate School

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Date July 23 1985

INFLUENCE OF MEMBRANE LIPID COMPOSITION OF *STAPHYLOCOCCUS AUREUS* ON SUSCEPTIBILITY TO PARABENS

A Thesis

Presented for the

Master of Science

Degree

The University of Tennessee, Knoxville

Effimia Eriotou Bargiota

August 1985

DEDICATION

With love and appreciation, I dedicate this thesis to my parents. Through achievement of this goal, I can only hope that I have made them proud and that I have enable them to see through their dream for me.

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To my husband, Dimitrios Bargiotas, I extend my love and the acknowledgement that without his help this degree would have been all the more difficult to complete.

ABSTRACT

The relationship between staphylococcal lipid composition and resistance to parabens was investigated. Chloroform-methanol extractable lipids were determined gravimetrically. Phospholipid and fatty acid fractions were studied using thin layer chromatography, gas liquid chromatography and mass spectrometry. Consistent quantitative differences were found among the total lipid, phospholipid and fatty acid compositions of sensitive and resistant strains. Most notable was the fact that, in paraben resistant strains, the percentage of total lipid and the phosphatidyl glycerol fraction of phospholipids were greater than in paraben sensitive strains. Furthermore, paraben resistant strains showed decreases in cyclopropane-ring containing fatty acids as compared with sensitive strains. Since significant differences in total lipid, phospholipid and fatty acid fractions were noted, it was suggested that the resistance of Staphylococcus aureus to parabens was related to membrane lipid composition.

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CHAPTER I

INTRODUCTION

The mechanism by which phenolic compounds cause inhibition of microorganisms is not completely understood. Several researchers have proposed that these compounds act on the cytoplasmic membrane of the cell. The basis of these proposals is based upon the fact that these compounds cause leakage of intracellular compounds which indicates membrane permeability changes (Davidson and Branen, 1981). The antimicrobial activity of these compounds also increases as they become more soluble in lipids (Baranowski and Nagel, 1983). Since the cell membrane contains most of the lipid in bacterial cells, it follows that the antimicrobial activity of these compounds may be influenced by lipid composition. The actual mechanism by which these compounds act is most probably related to their direct effect on cellular enzyme systems located in the membrane or indirect effect of the same enzymes through interaction with membrane lipids.

In a recent study by Post (1982), it was found that four strains of Staphylococcus aureus had significantly different susceptibility to the phenolic antioxidant, butylated hydroxyanisole (BHA). Since the metabolic

processes of the cells had to be assumed to be similar, differences in susceptibility were most likely due to cell membrane composition.

The objectives of this study were twofold. First, the resistance of the four strains of S. aureus used by Post (1982) was determined with another group of phenolic antimicrobials, the alkyl esters of p-hydroxybenzoic acid (parabens). Secondly, the correlation of cytoplasmic membrane lipid composition (percentage lipid, phospholipids and fatty acids) to susceptibility was determined.

CHAPTER II

REVIEW OF THE LITERATURE

A. Parabens

Phenolic compounds have been used as antimicrobials since quite early in the history of medicine. According to Brock (1961), Lister in 1867 was first to use phenol in sanitizing equipment and in surgeries, and to observe that phenol prevented or at least reduced the chances of bacterial infections subsequent to wounds and surgery. Since that time, phenol has been replaced by many phenol derivatives, which are not as toxic and are more effective. Three such phenolic derivatives have been approved for foods in the United States. These are the methyl (21 CFR 184.1490) and the propyl (21 CFR 184.1670) esters of para-hydroxybenzoic acid which are generally regarded as safe (GRAS) when used at a maximum concentration of 0.1% each, and the n-heptyl paraben (21 CFR 172.145) which is an approved additive for selected foods at a concentration varying for each food product.

According to Aalto et al. (1953) all three parabens allowed for use in foods are very stable in air, cold and heat for pH ranges 3.0-8.0. Based on these advantages,

parabens are suitable antimicrobials for a variety of food products and they are preferred over benzoic acid, a compound which is restricted to use at pH 2.5-4.0 (Chichester and Tanner, 1972).

Another advantage of these compounds is that their toxicity is of a low order. Matthews et al. (1956) reported that the major toxic effect of methyl and propyl paraben in dogs was acute myocardial depression and hypotension and the effect was transient in nature and non-cumulative. They also determined that the oral LD_{50} values of the sodium salts of methyl and propyl parabens were 2000 and 3700 mg/kg respectively in chronic toxicity testing. Lueck (1980) reported a similar pattern of subclinical toxicity in that 500 mg/kg of methyl paraben caused no ill effects to rabbits over a 6-day period while 3000 mg/kg was toxic to the animals.

Parabens are metabolized in humans by first being absorbed from the gastrointestinal tract, then the ester linkage is hydrolyzed in the liver and kidneys and finally they are excreted, usually within 6 to 24 hours (Jones et al., 1956). Lueck (1980) stated that the p-hydroxy-benzoic acid formed after the hydrolysis of the ester linkage of the parabens was excreted in the urine unchanged, as p-hydroxy-hippuric acid or as esters of glucuronic acid.

Parabens show both antibacterial and antifungal activity, but they are most effective against molds. Lang and

Rye (1973) stated that in both cases inhibition increased as the alkyl chain length increased. Davidson (1983) added that, in general, increased activity with decreased polarity is more pronounced with Gram-positive than Gram-negative bacteria (Table 1 and 2). The differences in the resistance observed by different researchers within the same species are probably due to different strains used, differences in the incubation conditions (pH, time, temperature), media, assay techniques and analyses of the results (Davidson, 1983).

There are three possible targets for the antimicrobial action of phenolic compounds: (1) The cellular membrane, (2) genetic material, and (3) enzymes, (Eklund, 1980; Davidson and Branen, 1981). Most researchers have agreed that the mechanism by which parabens, and possibly all phenolic compounds, cause inhibition is a reaction with the cell membrane and/or the inactivation of essential enzymes. Beckett et al. (1959) observed that the phenolic compound hexylresorcinol caused the release of intracellular constituents from E. coli and changes in the light-scattering properties of the cells. Furthermore, they determined that the amount of membrane damage was proportional to the concentration of the compound used. Judis (1963) found that phenol and phenolic derivatives exert their lethal action by physical damage to the permeability barriers (cell membrane). Hamilton (1968) proposed that several antimicrobial

Table 1. Concentrations of esters of p-hydroxybenzoic acid necessary for total inhibition of growth of various bacteria

Microorganism	Concentration (ug/ul)				Reference
	Methyl	Ethyl	Propyl	Butyl	
Bacillus cereus	2000	1000	125	63	Aalto et al. (1953)
	-	-	400	12	Jurd et al. (1971)
	-	-	-	400	Kato and Shibasaki (1975)
Bacillus megaterium	1000	-	320	100	Lewis and Jurd (1972)
Sarcina lutea	4000	1000	500	125	Aalto et al. (1953)
Staphylococcus aureus	4000	1000	500	125	Aalto et al. (1953)
	-	-	400	-	Jurd et al. (1971)
	-	-	500	-	Pierson et al. (1980)
Streptococcus feacalis	-	-	400	-	Jurd et al. (1971)

Table 1. (Continued)

Microorganism	Concentration (ug/ml)				Reference
	Methyl	Ethyl	Propyl	Butyl	
Streptococcus faecalis	-	130	40	-	Lueck (1980)
Clostridium botulinum	1200	-	200	-	Robach and Pierson (1978)
Pseudomonas aeruginosa	4000	4000	8000	8000	Sokol (1952)
Pseudomonas fragi	-	-	4000	-	Moustafa and Collins (1969)
Escherichia coli	2000	1000	1000	4000	Aalto et al. (1953)
Salmonella typhosa	2000	1000	1000	1000	Aalto et al. (1953)
Serratia marcescens	800	490	400	190	Furr and Russel (1972)
Klebsiella pneumoniae	1000	500	250	125	Aalto et al. (1953)
Vibrio parahaemoliticus	-	-	50-100	-	Lee (1973)

Table 2. Concentrations of esters of p-hydroxybenzoic acid necessary for total inhibition of various fungi

Fungi	Concentration (ug/ml)				Reference
	Methyl	Ethyl	Propyl	Butyl	
Alternaria sp.	-	-	100	-	Jurd et al. (1971)
Aspergillus flavus	-	-	200	-	Jurd et al. (1971)
Aspergillus niger	1000	400	200	200	Aalto et al. (1953)
Penicillium digitatum	500	250	63	<32	Aalto et al. (1953)
Penicillium chrysogenum	500	250	125	63	Mallinckrodt, Inc. (n.d.)
Rhizopus nigricans	500	250	125	63	Aalto et al. (1953)
Byssochlamys fulva	-	-	200	-	Jurd et al. (1971)
Candida albicans	1000	1000	125	125	Aalto et al. (1953)
Saccharomyces cerevisiae	1000	500	125	63	Aalto et al. (1953)

compounds including phenolics share a common mechanism of action in which the adsorption of the compound on to the cell membrane is a critical step. Hugo and Franklin (1968) theorized that phenolic compounds were distributed between lipid and aqueous components of the cell, in accordance with the normal partition laws, and that when fattened cells (cells where the lipid content was increased due to the presence of glycerol in the growth medium) were used, phenols were partially distributed in the deposited lipid. Furr and Russel (1972a) showed that the parabens inhibited the growth of Serratia marcescens and that they induced leakage of intracellular constituents from non-growing cells, the greatest effects being with the higher esters used. Furthermore, they concluded (1972b) that the parabens initially bind to the cell wall of the bacterial cells, but their primary effect was to cause damage to the cytoplasmic membrane.

Much research has been done to find which part of the membrane was damaged by these compounds. Eletr (1974) and Singer et al. (1977) showed that hydrophobic spherical molecules lowered the transition temperatures for cell membranes, thus increasing the fluidity of lipid alkyl chains. Ingram (1976) observed differences in the membrane lipid composition and in the cell membrane turbidity of Escherichia coli in the presence of alcohols which are lipophilic compounds. She theorized that these changes were

due to the disorder of the bilayers caused when the alcohols were inserted into the hydrophobic interior of the membranes restricting the movement of the fatty acid chains. Ingram (1977) and Surak et al. (1980) found that cells adapted their membrane lipids to compensate for the presence of several phenolic compounds. They suggested that many of these changes could be attributed to attempts by the cells to maintain a homeoviscous membrane.

Sgaragli et al. (1977) theorized that the disruptive action of monocyclic compounds on biomembranes depended upon their interaction with hydrophobic and electrophilic regions of the membrane. They observed that monocyclic compounds with an aliphatic chain containing at least two carbon atoms, interacted with biomembranes producing solubilization of proteins. They also postulated that at least part of the action of the compounds on the membranes was due to a hydroxyl group found in some of the compounds tested. Davidson and Branen (1980), and Degre and Sylvestre (1983) determined that inhibition and lethality against bacteria were at least partially due to leakage of intracellular components following disruption of the cytoplasmic membrane when the phenolic antioxidant, butylated hydroxyanisole (BHA), was used.

Freeze et al. (1973) suggested that parabens somehow uncoupled both substrate transport and oxidative phosphorylation from the electron transport system and partially inhibited the electron transport system itself. Eklund (1980) postulated that growth inhibition caused by parabens appeared to be a consequence of transport inhibition.

Based on the above research, it is clear that the site of action of the phenolic compounds parabens is the cytoplasmic membrane. Most researchers agree that the possible targets of these antimicrobials are the lipids and the enzymes located in this osmotic barrier.

B. Bacterial Cell Membrane

It is now well documented that the major structural features (apart from shape and size) distinguishing prokaryotic bacterial cells from eukaryotic cells is related to the nature and organization of membrane systems, the nucleus and the "packing" of functions in membranous organelles (Salton, 1971). Anatomically, the majority of bacterial cells are relatively undifferentiated with respect to intracellular membrane-bound structures (Ghosh, 1974). Studies on the anatomy of the bacterial cell have established the presence of a plasma membrane and in many cases a well-developed internal membrane system referred to

as the "mesosome" (Salton, 1967). The bacterial cell has been found to lack a nuclear membrane and contain no separate mitochondrial organelles. Many metabolic functions which are localized in different organelles of the eukaryotic cell are found in the multifunctional plasma membrane of the bacterial cell (van Iterson, 1965). According to Salton (1971) the cell membrane is the major site of functional differentiation in the bacterial cell, giving rise to a variety of internal membranes or membrane systems, including mesosomes, photosynthetic vesicles, and membranes involved in spore formation. The structure, chemical composition, and physical properties of the bacterial plasma membrane have been found to be similar to other types of biological membranes.

According to Higgins and Shockman (1971), the bacterial cell is encased in an envelope structure in both Gram-positive and Gram-negative organisms. The details of the arrangement and interrelationships of the surface layers have been largely elucidated from the electron microscopy of thin sections. The organization of the envelope into a well defined, thick, and rigid outer cell wall and an underlying plasma and mesosome membrane system is characteristic of the great majority of Gram-positive bacteria. Strominger et al. (1959) stated that the walls of Gram-positive bacteria, which represent about 20% of the dry weight of the cells, have a relatively simple composition as compared to the more

complex walls of Gram-negative organisms. In the case of S. aureus, Ghuysen and Strominger (1963) found that the cell wall was composed of two polymers, a glycopeptide and a techoid acid.

In Gram-positive bacteria, the membrane structure underlying and running parallel to the wall usually has the appearance of a 75A unit membrane and it is referred to as the plasma membrane (Salton, 1967). Costerton et al. (1974) and Masayori (1979), studying bacterial envelopes, concluded that Gram-negative bacteria possess an additional external barrier which can exclude certain low molecular weight compounds from the cell and may have an important function in the retention of some enzymes. They stated that this additional external barrier contributed to the ability of Gram-negative microorganisms to live in an extraordinarily wide variety of environments, to exclude certain toxic molecules, and to prevent the access of antibodies to the Gram-negative cell.

The chemical structure of the cell membrane has been the subject of research since the end of nineteenth century. 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 (Sherbet, 1978). Today, it is generally accepted that the matrix of the

cellular membrane is a bimolecular leaflet of phospholipid molecules which are oriented with their polar heads on the outer surfaces of the bilayer, in contact with the aqueous environment. The interior of the membrane sandwich is composed of the hydrophobic lipid chains of the phospholipids. To this basic structure, protein, glycolipids, and other molecules are usually inserted in such a way as to confer on the bilayer the functional properties appropriate for the particular membrane (Wagner,1980). Sheppart (1972) found that this bilayer was asymmetrical with respect to the position of the membrane-associated proteins. Referring to the bacterial membrane chemical composition both Salton and Freer (1965) and Hoerl (1981) concluded that all bacterial membranes are generally composed of 60% protein and 40% lipid with smaller amounts of RNA (ranging from 0.8%-7.5%).

The biological membranes of all living organisms are dynamic chemical structures (Davies, 1973). In bacteria, the cytoplasmic membranes function as semipermeable barriers, sites of active transport, sites for respiratory enzymes, in waste removal, in cell wall biosynthesis and possibly as sites of DNA synthesis (Hughes, 1962; Kaback and Hong, 1973; Boyd and Hoerl, 1981).

The bacterial cell membrane must maintain a specific fluidity in order to accomplish its functions (Silvius et al., 1980). Miller (1980) suggested that fluidity is

controlled by homeostatic mechanisms within close tolerances. According to Fulco and Fujii (1980) and Kawagushi et al. (1980), this homeostasis can be accomplished by appropriate alterations of the relative rates of saturated and unsaturated fatty acid biosynthesis. Kates and Pugh (1980) suggested that phospholipid desaturases may play an important role in control of membrane fluidity, whereas Russell and Sandercock (1980) found that the regulation is achieved by modification of the phospholipid fatty acyl chain length. Nozawa (1980) found both fatty acid and phospholipid composition appreciably altered as a result of membrane fluidity alteration. It is clear that there are correlations between changes in phospholipid or fatty acid composition, enzymatic activities and membrane fluidity.

C. Bacterial Lipids

Fifty years ago, the pioneering work of Anderson on mycobacterial lipids gave the initial impetus to what was to be an explosive development of general interest in the study of bacterial lipids (O'Leary, 1967). Recently, according to Lechevalier (1977), chemotaxonomy has played an important role in systematics. The identification of lipids is used as a taxonomic aid, especially when morphological, physiological or immunological data fail to lead to an unequivocal

decision (Uchida and Mogi, 1973). It is of primary taxonomic character in defining the generic boundaries of the Nocardia-Mycobacterium-Corynebacterium group (Lechevalier, 1977). Etemadi and Convit (1974), usinglipid composition, were able to determine that lepromatous leprosy is caused by a Mycobacterium and not by a mixture of corynebacteria, mycobacteria and other bacteria as it was generally accepted.

Lipid analyses of subcellular fractions of Gram-positive and Gram-negative bacteria have revealed marked differences in both content and distribution. According to Vorbeck and Marinetti (1965), Gram-positive bacteria contain less lipid than Gram-negative bacteria. Little or no lipid has been found in the cell walls of Gram-positive organisms (Salton, 1964), and nearly all of the cellular lipid (94% or more) appears to be concentrated in the cell membrane (Vorbeck and Marinetti, 1965). In contrast, Gram-negative bacteria have been reported to contain 12-22% lipid in the cell wall (Salton, 1964).

According to Lechevalier (1977), the total lipid content of most bacteria is quite low, usually less than 5% by dry weight. Some exceptions to this rule include mycobacteria and certain allied forms such as corynebacteria of the diphtheria type and nocardiae, which commonly have as much as 20% lipid by dry weight.

There are several reports correlating the amount of lipid possessed by bacterial cells and their resistance to antibiotics and antibacterial agents. For example, Hill et al. (1963) found that naturally occuring cells of Streptococcus pyogenes with resistance to tetracycline possessed surface lipid. Similarly, Hugo and Stretton (1966), showed that the induced appearance of surface lipid on cells of Staphylococcus aureus was accompanied by an increase in the resistance of cells to penicillin-type antibiotics. Hugo (1967) and Brown and Watkins (1970) proposed that the amount and type of lipid in cells, and especially in cell envelopes, played a role in determining the drug sensitivity of many microorganisms. Hamilton however, (1968) showed that the extra lipid of the cell envelope of the fattened cells acted purely as a permeability barrier. Brown and Wood (1972) analyzed the lipid content of cells and cell envelopes of Pseudomonas aeruginosa and their resistance to polymyxin. They used their results to support the theory that, within the cell envelope, there was a permeability barrier to the antibiotic and that the lipid content of the cells was intimately involved in this barrier. Pechey et al. (1974) suggested that there was a redistribution of the total neutral lipid to the surface of the cell resulting in higher amount of surface neutral lipid when cells were grown in the presence of gentamicin.

Hugo and Franklin (1968) and Furr and Russell (1972b), working with Staphylococcus aureus and Bacillus subtilis, respectively, found that uptake of phenolic compounds was greater with fattened than unfattened cells and that the compounds induced less leakage from these cells than from the normal cells. They theorized that this was due to the fact that drug molecules were absorbed to the surface of cells high in lipid whereas with normal cells many drug molecules will pass into the interior of the cell without being held near the surface. Supporting this were the findings of Hugo and Davidson (1973) where lipid depleted cells of Staphylococcus aureus were less resistant to a series of antimicrobial drugs, including phenolic compounds, than normal cells.

Lechevalier (1977) defines lipids as fatty acids, glycerides, fatty alcohols, gluco-, sulfo-, and peptidolipids, waxes, and hydrocarbons, or compounds containing these substances which are soluble in organic solvents. This definition excludes the lower fatty acids and alcohols with less than six carbons and fatty acids containing substances such as lipopolysaccharides, lipoproteins, and lipoteichoic acids as they are water soluble.

D. Fatty Acids

The greatest amount of data available on bacterial lipids concerns their fatty acid composition. In bacteria, most of the fatty acids are linked to larger molecules such as phospholipids, glycolipids, lipoproteins, lipopolysaccharides, and lipoteichoic acids while free fatty acids and glycerides are in the minority (Lechevalier, 1977). Fatty acids which are unique to bacteria include the b-hydroxy, cyclopropane, and branched-chain fatty acids. Asselineau (1966) reported that bacteria do not contain polyunsaturated fatty acids or sterols which are so common in other living forms.

In many cases, certain types of fatty acids predominate in a given taxon and may be of importance in indentifying microorganisms (Moss et al., 1974). Hydroxylated fatty acids are most frequently reported in Gram-negative bacteria (Shaw, 1974). They are found as components of lipopolysaccharides, ornithine-containing lipids, or the polymer, poly-b-hydroxybutyrate. Of particular interest are the mycolic acids, which are a-branched,b-hydroxy fatty acids, produced by members of the Mycobacterium-Nocardia-Corynebacterium group (Asselineau, 1966; Goren, 1972; Lederer, 1971). Differences in these unique fatty acids permit one to easily classify a given genera of this group (Lechevalier et al., 1971 and 1973).

Much work has been done to elucidate the fatty acid composition of the Gram-positive bacterium, Staphylococcus aureus. Vaczi et al. (1967) found that the fatty acid composition of this species depended on the environmental conditions present during growth (pH, atmosphere, temperature, and growth medium). White and Frerman (1968) reported that the majority of fatty acids of S. aureus were associated with complex lipids. The dominant fatty acids of the microorganism have been reported to be the methyl branched fatty acids, 12-methyltetradecanoic (a-C15:0), 14-methylhexadecanoic (a-C17:0), and 16-methyloctadecanoic (a-C19:0) (Theodore and Panos, 1973; Jantzen et al., 1974). Post (1982) found as major fatty acids of S. aureus the myristic acid (C14:0), pentadecanoic acid (C15:0) which included the a-C15:0, palmitic acid (C16:0), stearic acid (C18:0) and arachidonic acid (C20:0).

E. Phospholipids

Phospholipids occur primarily in association with membranes and play important roles in the structure and function of the cytoplasmic membrane, in resistance of bacteria and in virulence (Brown and Wood, 1972). They are often referred to as "polar" lipids because, although they are soluble in organic solvents, they have hydrophilic groups which serve to make one end of each molecule polar.

The opposite end of the phospholipid molecule is substituted with fatty compounds such as fatty acids, fatty aldehydes, or alcohols and is thus hydrophobic (Cronan and Gelman, 1975). White et al. (1971) reported that almost all enzymes involved in the synthesis of phospholipids are localized in the inner cytoplasmic membrane. Raetz (1978) observed a tight coupling of phospholipid and fatty acid synthesis in bacteria.

According to Rozgonyi et al. (1973), Komura et al. (1975a), and Beining et al. (1975), the phospholipids most commonly found in Staphylococcus aureus are: diphosphatidyl glycerol (also called cardiolipin), phosphatidyl glycerol, phosphatidyl ethanolamine, aminoacyl phosphatidyl glycerol, and phosphatidic acid. Each type of phospholipid has a specific function. For example, diphosphatidyl glycerol has a structural role (Vaczi, 1973). Phosphatidyl glycerol is a precursor of the phospholipids diphosphatidyl glycerol and the aminoesters of phosphatidyl glycerol. Vaczi (1973) believed that changes in the concentration of this phospholipid could result in rapid structural alterations of the bacterial membrane depending on environmental conditions. Phosphatidyl ethanolamine usually comprises the largest portion of phospholipids in many bacteria. Its main function is most probably structural (Ames, 1968). Amino acyl phosphatidyl glycerol has been reported to help the regulation of membrane charge by increasing the positively

charged groups of the cell surface to give protection against the penetration of protons. Its esters may also have roles in synthesis of proteins, transport of amino acids, and the biosynthesis of the cell wall (Vaczi, 1973). According to Lechevalier (1977) phosphatidic acid is the most important intermediate product of the biosynthesis of the phospholipids.

F. Complex Lipids

Glycolipids are compounds that contain "carbohydrates in combination with long-chain aliphatic acids or alcohols and which are extracted ... into organic solvents without prior use of hydrolytic procedures" (Shaw, 1970). This definition excludes the lipopolysaccharides which are water soluble, the degradation products of larger molecules arising through hydrolysis, and sugar-containing phospholipids. Sastry (1974) stated that the most widespread glycolipids among bacteria are the glycosyl diglycerides. These are the only glycolipids found in Staphylococcus aureus (Beining et al., 1975).

In the amino acid-containing lipid category the most important lipids are the ones with an ornithine moiety. They are found in Gram-negative bacteria and actinomycetes (Lechevalier, 1977).

Peptidolipids are very rarely found in bacteria. Barber et al. (1965), Laneelle et al. (1965), and Guinand and Michel (1966) isolated peptidolipids from mycobacteria and nocardiae.

According to Goren (1972) all sulfolipids reported from bacteria are glycolipid sulfates. These compounds are relatively unstable, undergoing spontaneous desulfation under mild conditions. They have been reported only in the species Mycobacterium tuberculosis (Goren et al., 1971), Halobacterium cutirubrum (Kushwaha et al., 1975), Sulfolobus acidocaldarius (Langworthy et al., 1974) and Rhodopseudomonas sphaeroides (Oelze and Drews, 1972).

Waxes are compounds composed of fatty acids linked to long-chain fatty alcohols. They appear to be widespread within Acinetobacter and Mycobacterium (Makula et al., 1975). Waxes have also been found in Micrococcus (Russell, 1974), Clostridium (Hobbs et al., 1971) and possibly Brucella melitensis (Thiele and Schwinn, 1973).

Hydrocarbons have strong structural analogies with fatty acids, occuring in straight-chain, monounsaturated or branched forms. They have been found in a variety of genera including Micrococcus (Morrison et al., 1971), Sarcina (Tornabene and Markey, 1971), Clostridium (Hobbs et al., 1971), and Escherichia (Naccarato et al., 1974).

Other lipids found in very few bacteria species are the lipid-containing iron-binding compounds (Ratledge and Snow, 1974) and bound lipids (Moss et al., 1973).

G. *Staphylococus aureus*

Staphylococcus aureus belongs to the family *Micrococcaceae* (Bergey's, 1975). They are Gram-positive, facultatively anaerobic cocci. Generally they are small in size (less than 1 um in diameter) and form distinctive grape-like clusters due to their division which takes place in three dimensions. They are non spore-formers, non-motile, catalase positive, occasionally encapsulated, and their optimum growth temperature is 35-37^{o}C. They show abundant growth on agar containing media forming yellow- or golden-colored colonies. The colors are due to carotenoid pigments. To differentiate *S. aureus* from the other *Staphylococcus* species, the ability to produce b-hemolysis on blood agar, coagulase production and the fermentation of mannitol are used.

Staphylococci are closely associated with man. According to Morse (1982), 30-40% of the human population are asymptomatic carriers of pathogenic strains in their nasopharynx and intestines. The microorganism is able to cause a variety of diseases including skin abcesses, bone infections, urinary tract infections, cystitis, meningitis, Ritter's disease, infections of intestines, pneumonia, toxic shock syndrome and food poisoning.

According to Tartakow and Vorperian (1981) the most common of all food poisonings is that caused by Staphylococcus aureus. The disease has symptoms including nausea, vomiting, abdominal cramps and diarrhea. The incubation period in man ranges from 30 minutes to 8 hours with most illnesses occuring 2-4 hours after ingestion of the food (Banwart, 1983). The food poisoning is an intoxication caused by simple, water soluble proteins called enterotoxins which are produced by certain strains of coagulase positive S. aureus. There are six immunologically distinct enterotoxins which are represented by the letters A, B, C, D, E, and F. The enterotoxins are relatively heat stable and inactivation may be influenced by the pH and composition of the heating medium (Humber et al., 1975). Tatini et al., (1976) stated that normal thermal processing of foods (e.g. cooking or pasteurization) cannot completely inactivate these toxins. Further, he found that the heated toxins had greater biological activity than the unheated toxins when tested at the same dose level. Since enterotoxin cannot be inactivated by the usual heat treatment of food, foods containing 10^5 to 10^6 cells of staphylococci per gram are discarded as possible food hazards (Banwart, 1983).

CHAPTER III

MATERIALS AND METHODS

A. Determination of Differences in Resistance of *Staphylococcus aureus* Using p-Hydroxybenzoic Acid Esters

Test Organisms

Four strains of *S. aureus* were obtained from the Food Technology and Science Microbiological Laboratory. The strains, which were the same as those used by Post (1982), included ATCC 12600, A100, S-6, and LP. All strains were stored at 4°C on trypticase soy agar (TSA) (BBL, Cockeysville, MD) and subcultured every month on the same medium.

Test Compounds

Propyl paraben and a mixture of methyl and propyl parabens (2:1) (Fisher Scientific Co., Fair Lawn, NJ) were dissolved in 95% ethanol to final concentration 6% and 12% (w/v), respectively. These solutions were filter sterilized using a membrane filter (0.45 um) and used immediately. Dilutions were made in filter-sterilized 95% ethanol.

Test Media

The growth media were trypticase soy broth (TSB) (BBL) and TSA. A peptone solution, 0.1% (w/v), was used as a diluent for cultures grown in TSB and for enumeration. The media were dispensed appropriately and autoclaved at 121°C for 15 min. Antimicrobial solutions were added to the TSA which had been cooled in a water-bath to 50°C.

Determination of Growth Curve

Four flasks containing 100 ml of TSB, were inoculated with each of the four strains of S. aureus so that the initial concentration was 10^6-10^7 CFU/ml. The flasks were incubated at 37°C and sampled at 1 hr intervals up to 12 hr. Samples were plated using the Spiral Plater System (Spiral Systems, Inc., Cincinnati, OH). The plates were incubated at 37°C for 48 hr.

Determination of Minimum Inhibitory Concentration

Ranges for testing were based upon prior studies of the antimicrobial agents (Davidson and Branen, 1981). Intervals of 50 ppm were used to determine inhibition. One millimeter of the appropriate antimicrobial was added to a media bottle containing 99 ml of liquid TSA at 50°C and plates were poured. One S. aureus strain was diluted in peptone buffer and a 0.1 ml aliquot spread plated to obtain a final population of 10^5-10^6 colony forming units (CFU) per plate.

Controls were plates on which 0.1 ml sterile 95% ethanol was spread plated. Inoculated plates were incubated at 37°C and observed for growth at 48 hr. The concentration which inhibited growth was termed the "minimum inhibitory concentration" (MIC) (Barry, 1976).

Determination of Inactivation Over Time

Propyl paraben (Fisher Scientific Co.) was dissolved in 95% ethanol to a final concentration equal to 100 times the MIC. An aliquot (1 ml) of the antimicrobial was added to 98 ml sterile TSB followed by inoculation of 1 ml of S. aureus to obtain a final concentration of 10^6-10^7 CFU/ml. Samples were taken at 0.5 hr intervals and enumerated using a Spiral Plating System (Spiral Systems, Inc., Cincinnati, OH). The plates were incubated at 37°C for 48 hr.

B. Determination of Membrane Total Lipid, Fatty Acid and Phospholipid Composition

Lipid Extraction

Trypticase soy broth containing each strain at the late log-phase was centrifuged for 20 min at 14,000 x g. The precipitate was washed with peptone buffer and recentrifuged for 20 min at 14,000 x g.

Lipid extraction was a modification of the method described by Blich and Dyer (1959). The pellet containing the bacterial cells was weighed and suspended in 30 ml of water to which was added 75 ml of methanol and 37.5 ml of chloroform. The suspension was mixed vigorously and allowed to stand for four hours. Chloroform (37.7 ml) and water (37.5 ml) were then added, the suspension was shaken and layers were allowed to separate. The bottom, chloroform, layer was filtered through Whatman No.1 filter paper, transferred to a graduated cylinder and the volume recorded. The chloroform layer contained the purified lipid.

Total Lipid Determination

To analyze for total lipid, 10 ml of the chloroform layer was placed in each of three pre-weighed, dried beakers, evaporated to dryness under nitrogen and put in a vacuum oven (Thelco, Chicago, IL.) until constant weight was achieved. The beakers were weighed and the total lipid content per gram of cells was determined on wet basis.

Phospholipid Determination

Thin layer chromatography was used for the separation and identification of phospholipids. The method used was a modification of that described by Watkins (1982). Thirty milligrams of the lipid extract were dissolved in 1 ml chloroform and 10 ul portions spotted in 10 applications on

pre-activated silica gel thin layer plates (20 x 20 cm, 250 um thick; Whatman, Inc. Clifton, NJ). Reference compounds were lecithin, sphingomyelin, phosphatidylinositol, phosphatidylglycerol, phosphatidylethanolamine, phosphatidylserine, and diphosphatidylglycerol (cardiolipin). They were obtained from Sigma Co. (St. Louis, MO), dissolved in appropriate solvents and applied to the plates in the same manner as the samples.

Chromatograms were developed in tanks lined with No. 1 filter paper (Whatman) in which the mobile phase had equilibrated for 10 min before plates were inserted. The mobile phase consisted of chloroform:methanol:water, 120:52.2:7.5 (v/v). Plates were developed for 1 hour (or until solvent front reached the top).

Chromatograms were air dried for 10 min, and sprayed lightly with 50% sulfuric acid, followed by charring at 130^{o}C for 45 minutes. The chromatograms were scanned on a Schoeffel Spectrodensitometer 3000 equipped with a SDC 300 Density computer (Schoeffel Instrument Corp., Westwood, NJ). Phospholipid composition was determined as a relative percentage.

Determination of Fatty Acid Composition

Fatty acid composition was determined according to the method described by Moss, et al. (1974). Bacterial cells (10 mg) were transferred to a test tube (16 x 150 mm) containing 5 ml of 5% (w/v) NaOH in 50% (v/v) aqueous methanol. The tubes were sealed with Teflon-lined caps and the cells saponified for 30 minutes at 100°C. After the saponificate was cooled, the pH was reduced to 2.0 with 6 N HCl. Methyl esters of free fatty acids were formed by adding 5 ml of 12% boron trichloride-methanol reagent (w/v) (Supelco, Inc. Bellefonte, PA) and heating the mixture for 5 minutes at 80°C. The fatty acid methyl esters were then extracted twice from the cooled mixture with 10 ml of chloroform:hexane 1:4 (v/v). A few drops of saturated NaCl solution was added to enhance the separation. The solvent layers containing the fatty acid methyl esters were combined in a 50 ml beaker and evaporated to volume 0.2 ml under a gentle stream of nitrogen. A small amount of Na_2SO_4 (anhydrous sodium sulfate crystals) was added to remove moisture and the samples were stored at -20°C in screw-capped tubes until analyzed by gas liquid chromatography (GLC).

Methyl esters 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.). Major fatty acids were identified by

comparison of retention times with those of a pure bacterial fatty acid methyl ester standard (Supelco, Inc.).

The chromatographic conditions used consisted of the following:

Column: 3 m x 2 mm ID glass packed with 3% SP-2100 DOH on 100/120 Supelcoport.

Column Temperature: Temperature programmed from 150° to 225°C at 4°C/min.

Flow Rate: 20 ml per minute nitrogen at 50 psi column back pressure.

Sample Size: 3 ul of extract, 1 ul of standard.

Detector: Flame ionization.

The identification of the fatty acids was confirmed using mass spectrometry (Shimadzu GC-9A; Shimadzu, Ltd. Kyoto, Japan). Mass spectra were obtained in the EI mode at 20 and 70 ev. Tentative identifications of peaks were made by comparing sample spectra with those in the literature (Budzikiewicz et al., 1967), retention times, molecular weights or similarities to spectra of a like family of compounds analysed in the same manner as the samples. The sample size was 3ul of extract. The standard was a pure bacterial fatty acid methyl ester (Supelco, Inc.).

C. Statistical Analysis of Data

Analysis of variance was performed on the data to compare the resistance of the four strains and the differences in total lipid, fatty acid, and phospholipid composition of the four strains. Results presented are means of data obtained from two trials run in duplicate for each experiment. Where appropriate, significant difference among means was measured by Duncan's Multiple Range test (Steel and Torrie, 1960). Significant differences among means are reported at $P<0.05$.

CHAPTER IV

RESULTS AND DISCUSSION

A. Determination of Differences in Resistance of *Staphylococcus aureus* Using p-Hydroxybenzoic Acid Esters

Determination of Growth Curve

Four strains of *Staphylococcus aureus*, LP, A100, S-6, and ATCC 12600, were grown in trypticase soy broth for 12 hr and enumerated at 1 hr intervals (Fig. 1). The first objective of this experiment was to determine if there were differences in the growth of the microorganisms which could interfere with the inhibition studies. The second objective was to find the time needed to grow the microorganisms in order to harvest them at the late log phase required for some of the experiments which followed.

No difference in any of the growth curves was found among the four strains (Fig. 1). All showed a similar pattern of growth having a lag time of 3 hours, log phase 3-8 hours and a stationary phase starting at 8 hours of growth. To harvest microorganisms at the late log phase the microorganisms were grown in TSB for 7 hours.

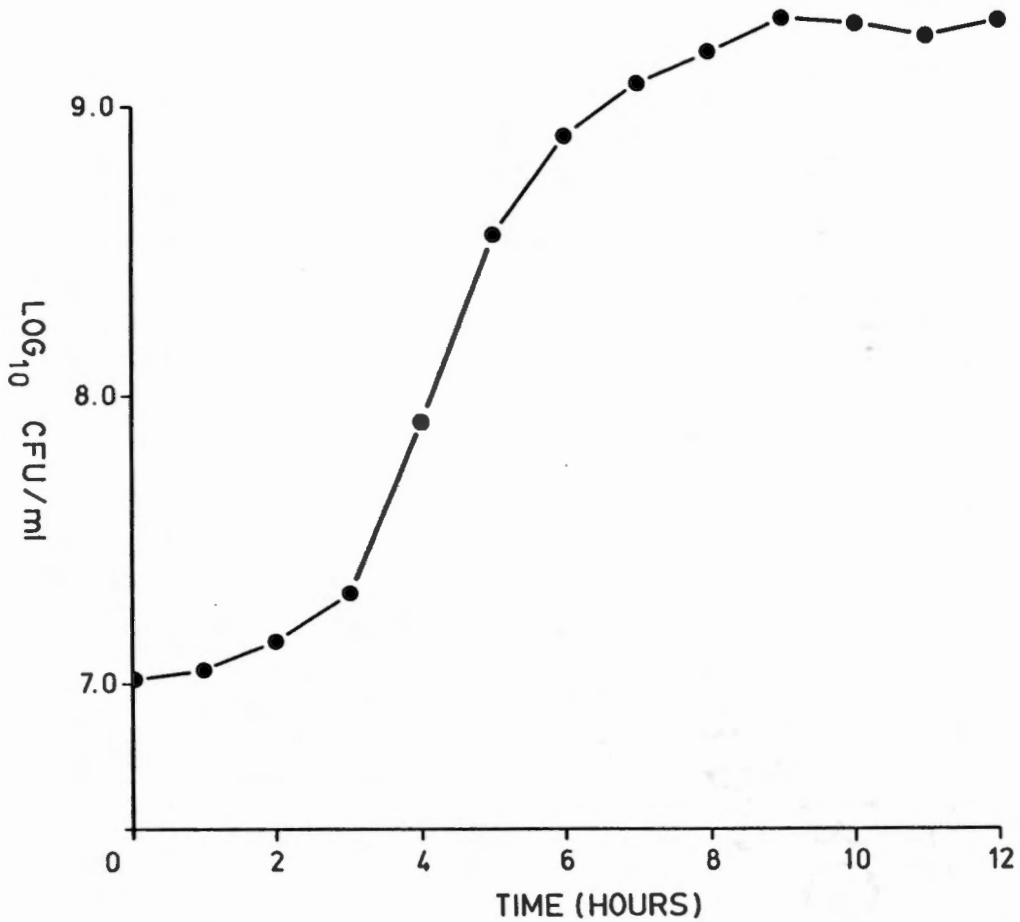

Fig. 1. The growth curve of four strains of Staphylococcus aureus (ATCC 12600, A100, S-6 and LP) grown in TSB at 37°C, where each point represents the mean of two replicates per strain.

Determination of Minimum Inhibitory Concentration

Four strains of Staphylococcus aureus were grown in the presence of propyl paraben or a mixture of methyl and propyl parabens (2:1) using intervals of 50 ug/ml. The objective of this experiment was to determine the minimum amount of antimicrobial necessary to cause inhibition of the growth of the microorganisms. This data would be used to determine if a correlation existed between resistance patterns of the microorganisms and their percentage total lipid, phospholipid or fatty acid composition.

The minimum inhibitory concentrations (MIC) of propyl paraben for the four strains were found to be similar (Table 3). The MIC for the LP and ATCC 12600 strains were significantly higher than that of the A100 and S-6 strains. Because the results were expressed as growth or no growth, no difference in resistance was found between the LP and ATCC 12600. The growth of ATCC 12600 on the trypticase soy agar (TSA) plates containing 350 ug/ml however was less dense than for the LP strain in both runs. These data indicated that the LP strain was more resistant than ATCC 12600 at the same antimicrobial concentration. The same pattern was observed for the A100 and S-6 strains. Although growth occured for both strains at 300 ug/ml propyl paraben, the amount of growth for the A100 strain was much more dense. Since the concentration intervals of the test compound were 50 ug/ml, it was possible that the LP and A100

Table 3. Minimum inhibitory concentrations[a] for propyl paraben against four Staphylococcus aureus strains on trypticase soy agar after incubation at 37°C for 48 hr.

Strain . . .	LP	ATCC 12600	A100	S-6
MIC	400[b]	400[b]	350[c]	350[c]

[a] means shown represent two replications

[b,c] means in rows with different superscripts are significantly different ($P<0.05$).

strains had MIC's near the upper limit of the range while the ATCC 12600 and the S-6 were near the lower limit.

The results obtained in this study were comparable to those of other studies reported by Davidson and Branen (1981). In this previous report, propyl paraben had an MIC 400-500 ug/ml for Staphylococcus aureus. The difference in the MIC values between the present study and those reported previously was most probably due to the difference in the strains used, experimental designs, or growth conditions.

The minimum inhibitory concentrations of the methyl and propyl paraben mixture (2:1) for the four strains ranged between 800-900 ug/ml (Table 4). The MIC of the LP strain was 900 ug/ml and was the highest of all strains, followed by that of the ATCC 12600 which was 850 ug/ml. Finally, the A100 and the S-6 strains showed no growth on the plates containing 800 ug/ml of the antimicrobial mixture. As was the case with the propyl paraben the amount of growth observed on the plates containing 750 ug/ml of the methyl and propyl paraben mixture was much greater for the A100 strain than the S-6 strain. This difference indicated that the MIC for the S-6 strain was probably slightly lower than that of the A100 strain.

The resistance showed by all four strains was an innate resistance since the microorganisms had not been exposed to the antimicrobials before the experiment nor were changes induced in any of their cell constituents. Therefore,

Table 4. Minimum inhibitory concentrations[a] for methyl and propyl paraben (2:1) against four Staphylococcus aureus strains on trypticase soy agar after incubation at 37°C for 48 hr.

Strain . . .	LP	ATCC 12600	A100	S-6
MIC	900[b]	850[c]	800[d]	800[d]

[a] means shown represent two replications.

[a,b,c] means in rows with different superscripts are significantly different ($P<0.05$).

differences in resistance were natural and not acquired as was the case in work reported by Hugo and Stretton (1966), Hugo and Franklin (1968) and Furr and Russell (1972b).

Determination of Inactivation Over Time

The four strains of Staphylococcus aureus were exposed to propyl paraben in TSB at a concentration equal to that of the MIC (Fig. 2). Samples were taken at 0.5 hr intervals and enumerated. The objective of this experiment was to determine the inactivation of the microorganisms over time and its relationship to the percentage lipid, phospholipid and fatty acid composition.

The LP strain was found to be more resistant than the other three strains (Fig.2). No difference in resistance was observed among the ATCC 12600, A100 and S-6 strains. It must be pointed out that the resistance observed in these tests was to an antimicrobial concentration equivalent to the MIC of propyl paraben for the LP strain. This explained the higher rate of death observed for the other three strains since their MIC for propyl paraben was lower than that of the LP strain. This was also most probably the reason why no difference was observed in the resistance among ATCC 12600, A100 and S-6.

This experiment also showed a definite difference between the LP and ATCC 12600 strains. Therefore, the assumption that the ATCC 12600 was less resistant than the

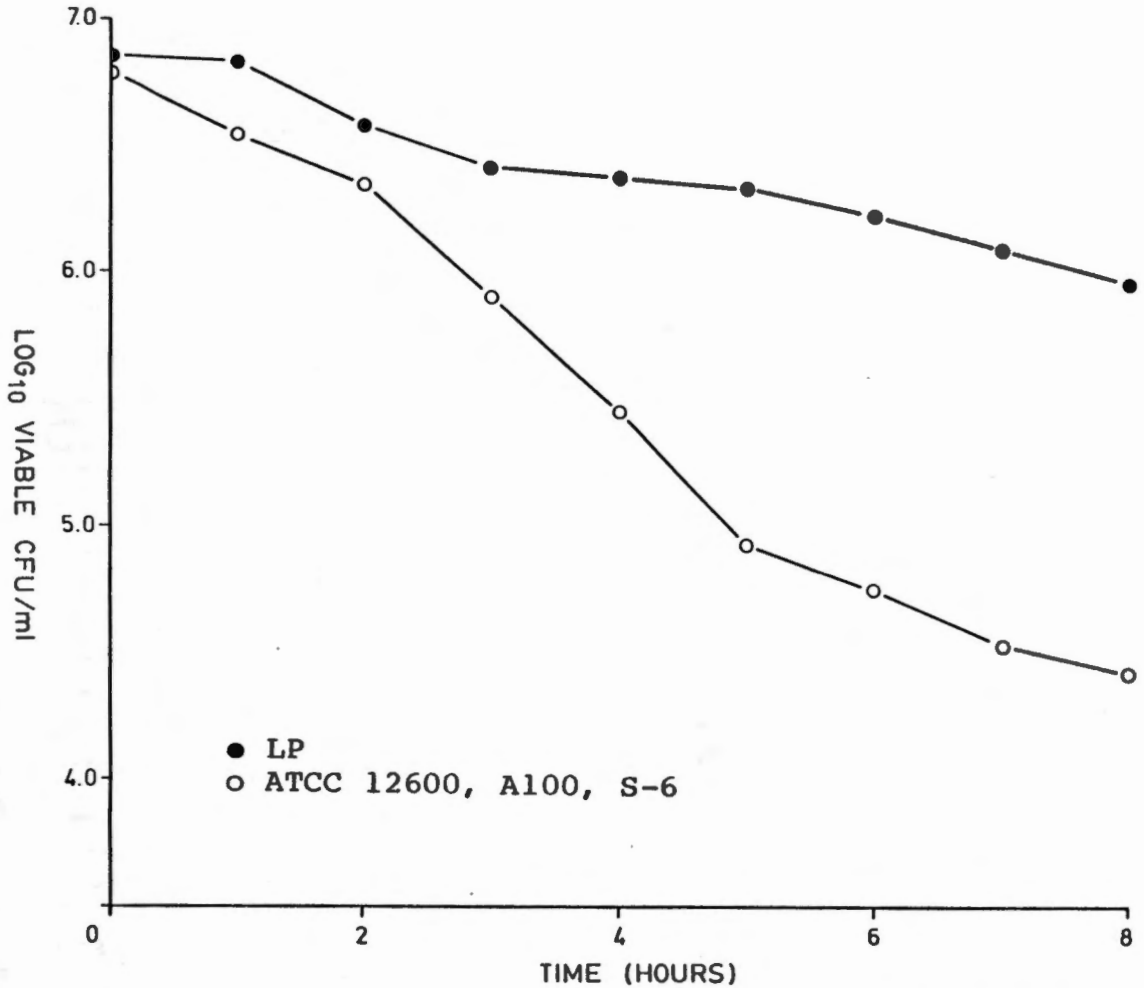

Fig. 2. The inactivation over time of four strains of S. aureus in the presence of 400 ug/ml propyl paraben in trypticase soy broth at 37°C, where each point represents the mean of two replicates.

LP strain based on the amount of growth shown on the MIC plates was probably correct. A similar design where the antimicrobial would have been used at a concentration equal to that of the MIC of the A100 and the S-6 strains would probably have distinguished the resistance of these two strains. As with the MIC, the inactivation over time studied the innate resistance of the microrganisms since they were not exposed to the antimicrobial substance prior to the experiment, nor were changes in any of their cell constituents induced.

The inactivation over time study and the MIC procedure were two different methods to measure the resistance of the four strains. The former was a study where the inactivation caused by a single concentration of the antimicrobial was studied over a period of time. In the MIC design, time was constant (48 hr) while the antimicrobial concentration was varied. Both procedures had their weak and strong points. In the case of the MIC, the results were read as growth or no growth and the amount of growth was not taken into consideration. The inactivation over time was a more detailed study where the behavior of the microorganism was observed over a period of time. Therefore, the reaction of the microorganism was known for any time within the limits of the study. Although it was more detailed, it was restricted to the specific concentration studied and had to follow the MIC procedure. It was necessary to run the

inactivation study when no conclusions could be drawn from the MIC experiment as to the resistance of LP and ATCC 12600 to propyl paraben.

B. Determination of Membrane Total Lipid, Fatty Acid, and Phospholipid Composition

Total Lipid Determination

The total lipid of each of the four strains was extracted and the lipid content per gram of cells was determined. The objective of this experiment was to find if a correlation existed between the total lipid content of the cells and the difference in resistance among the four strains of Staphylococcus aureus.

Statistical analysis of the results showed that there was significant difference among all the four strains used (Table 5). The LP strain contained the most lipid, followed by the ATCC 12600 strain. The A100 had less lipid than the ATCC 12600 and more than the S-6 strain. A significant correlation coefficient ($P<0.05$) was found between the amount of lipid in the cells and the resistance to propyl paraben ($r=0.77$) or the methyl/propyl mixture ($r=0.89$). Therefore, the amount of total lipid was probably related to the resistance of the microorganisms. The results also agreed with the literature, where cells with more lipid are more resistant to a variety of antimicrobial agents, than cells with less lipid.

Table 5. Percentage lipid composition[a] of four strains of Staphylococcus aureus (LP, ATCC12600, A100 and S-6) grown in trypticase soy broth at 37°C

	Strain			
	LP	ATCC 12600	A100	S-6
Lipid (%)	3.85[b]	3.37[c]	3.27[d]	2.82[e]
SE[f]	0.04	0.01	0.06	0.02

[a] means shown represent two replications .

[b-e] means with different superscripts are significantly different ($P<0.05$).

[f] standard error.

The fact that several antibiotics and antimicrobial substances have shown an increase in their antimicrobial activity when their lipid solubility increased have led to the suggestion that the cellular lipid may be a factor in determining the succeptibility of bacteria to these compounds. Several studies in the past (Hugo and Stretton, 1966; Hugo, 1967; Brown and Watkins, 1970; Brown and Wood, 1972; Furr and Russell, 1972b) concluded that increase in the lipid content of the cells resulted in a marked increase in the dose necessary to inhibit growth, and that lipid depletion caused increased sensitivity of bacterial cells.

The role of the lipid solubility of the parabens, as measured by their chloroform-buffer distributions, (Baranowski and Nagel, 1983) was suggested to be of equal importance to the resistance of cells as the amount of lipid. Support for the general hypothesis on the role of lipid to resistance comes from a consideration of the difference in sensitivity of Gram-positive and Gram-negative bacteria to the esters of p-hydroxybenzoic acid. The former are, in general, sensitive to the parabens, the latter are not, and yet the mode of action appears to be the same (Davidson and Branen, 1981). However, while the Gram-negative bacterial cell walls contain a large amount of lipid (up to 25%), Gram-positive bacterial cell walls normally contain little or no lipid (Salton, 1964).

All of the above studies were conducted on bacteria where their lipid content was artificially changed. It is interesting to note however that Hill et al. (1963) showed that freshly isolated pathogenic streptococci possessing more cell lipid than laboratory strains showed higher resistance to tetracycline. This indicated that an increase in cellular lipid could occur in vivo leading to a change in resistance to the antibiotics or antimicrobial substances.

Furr and Russell (1972b) showed that the uptake of parabens was the result of a general dissolution of the esters on the "cell surface." From the non-specific physicochemical nature of this adsorption they suggested that inhibition was caused by changes in the membrane structure, resulting from the breakage of hydrogen bonds, etc., rather than loss or inactivation of specific enzymes.

In considering these data and the findings of Furr and Russell (1972b) it was reasonable to suppose that the parabens would distribute themselves between the lipid and the aqueous components in the cell, in accordance with the normal distribution laws. Due to their hydrophobic and hydrophilic groups the parabens tend to orientate in an oil/water interface, if such an interface is available. In the case of cells containing more lipid, it is likely that phenol is immobilized with the alkyl side immersed in the cell lipid and the phenolic hydroxy-group projecting into the aqueous environment. Therefore, it could well be that

many antimicrobial molecules are thus trapped and sensitive areas in the cytoplasmic membrane are protected. This theory received support from the data obtained by Hugo and Franklin (1968). They found that the amount of material leaking from fattened cells (cells which were artificially induced to contain more lipid) was smaller than the amount leaking from normal cells.

On the basis of these findings, the following working hypothesis can be proposed. Parabens are adsorbed on to the cell membrane. The amount of the antimicrobial adsorbed and its rate of adsorption may be a relatively non-specific physicochemical phenomenon. Resistance results from a greatly decreased penetration of the antimicrobial substance to the membrane. In this case penetration was decreased due to the higher amount of total cell lipid.

Phospholipid Determination

The relative percentage phospholipid was determined from the total extractable lipid using thin layer chromatography. The objective of this experiment was to find if a correlation existed between the relative phospholipid percentages and the differences in resistance found among the four strains of *Staphylococcus aureus*.

The phospholipids found in the four stains examined were phosphatidyl ethanolamine, phosphatidyl glycerol, a phosphatidyl inositol-like compound, phosphatidyl serine, cardiolipin and an unknown with RF equal to 0.70 (Table 6). In comparing these results with the literature (Rozgonyi et al., 1973; Komura et al., 1975a; Beining et al., 1975) we found that phosphatidyl inositol and phosphatidyl serine were not reported previously in Staphylococcus aureus. In the case of phosphatidyl serine, it was found in small amounts in only two of the strains examined and was therefore probably not found in all strains of S. aureus. In contrast, the compound with the same RF as phosphatidyl inositol was found in all four strains in appreciable amounts. This may be due to the following two factors. Firstly, the phospholipid may have a very closely related RF under the same chromatographic conditions to another phospholipid or lyso-phospholipid whose standard was not examined in this study. This theory was supported by the fact that aminoacyl phosphatidyl glycerol and phosphatidic acid have been found in other studies with S. aureus (Rozgonyi et al., 1973; Komura et al., 1975a; Beining et al., 1975). Secondly, these differences may be attributed to differences in the strains examined, the growth conditions, and the point of maturity of the cells used. It was not unusual that phosphatidic acid was not detected in this experiment since it is the most important intermediate

Table 6. Relative percentage ± standard deviation of phospholipid fractions of four strains of Staphylococcus aureus (LP, ATCC 12600, A100 and S-6) grown in trypticase soy broth at 37°C

		Relative Percentage Phospholipid			
Phospholipid	RF	LP	ATCC 12600	A100	S-6
Phosphatidyl serine	0.24	$0.00^{c}\pm0.00$	$0.00^{c}\pm0.00$	$2.89^{b}\pm0.60$	$0.61^{c}\pm0.53$
Phosphatidyl inositol-like	0.27	$5.14^{cd}\pm1.72$	$7.40^{c}\pm0.27$	$14.46^{b}\pm0.19$	$3.38^{d}\pm0.83$
Cardiolipin	0.39	$0.00^{b}\pm0.00$	$1.07^{b}\pm2.01$	$0.00^{b}\pm0.00$	$1.23^{b}\pm1.48$
Phosphatidyl glycerol	0.41	$15.39^{b}\pm2.50$	$0.45^{c}\pm0.63$	$0.00^{c}\pm0.00$	$0.00^{c}\pm0.00$
Phosphatidyl ethanolamine	0.59	$72.59^{c}\pm3.36$	$78.08^{bc}\pm6.61$	$72.78^{c}\pm1.22$	$89.96^{b}\pm2.52$
Unknown	0.70	$6.89^{bc}\pm2.58$	$13.00^{b}\pm2.93$	$9.88^{bc}\pm0.43$	$4.82^{c}\pm0.32$

[a] means shown represent two replications

[b,c,d] means in rows with different superscripts are significantly different ($P<0.05$)

product of the biosynthesis of phospholipids. Its conversion therefore is rapid and consequently it is usually detected in bacteria in small amounts. Aminoacyl phosphatidyl glycerol is a derivative of phosphatidyl glycerol and helps the regulation of membrane charge. Its formation depends on enviromental conditions.

A statistical analysis of the results showed significant differences among all the phospholipids except for cardiolipin. Phosphatidyl ethanolamine was the major phospholipid which was in accordance with the results obtained from previous studies (Rozgonyi et al., 1973; Komura et al., 1975a; Beining et al., 1975). The most important difference was that found in the phosphatidyl glycerol fraction among the four strains. The LP strain, which was the most resistant to the antimicrobials, had the highest amount of phosphatidyl glycerol of all four strains. A significant correlation coefficient ($P<0.05$) was found between the amount of phosphatidyl glycerol in the cells and the resistance as measured by MIC to the methyl/propyl mixture ($r=0.86$). In contrast, no significant correlation was detected using propyl paraben ($r=0.59$).

Van Deenen (1972) has shown that the permeability of a membrane is partially determined by its lyso-phosphatidyl glycerol (a phosphatidyl glycerol derivative) content. Vaczi (1973) stated that two phospholipids can be derived from phosphatidyl glycerol. These are cardiolipin and the

amino acid esters of phosphatidyl glycerol. The possibility that phospholipids with two opposite charges could be synthesized from the same precursor could insure, depending on the environmental conditions, a rapid structural alteration of the bacterial membrane to adverse conditions.

Based on the above findings the following hypothesis could be proposed. The LP strain which contained the highest amount of phosphatidyl glycerol could alter its membrane charge rapidly, depending on the environmental conditions, as it could have been in the inactivation study, by forming the appropriate end-product phospholipid for each environmental situation. Therefore, it is able to control better its membrane permeability than the other three strains and resist higher amounts of antimicrobial substances than the ATCC 12600, A100 and S-6 strains.

Phosphatidyl serine, phosphatidyl ethanolamine and the phosphatidyl inositol-like compound also showed significant relative percentage differences among the four strains of S. aureus. These differences were not correlated to resistance and the biological role of these phospholipids in bacteria was either structural or unknown.

Determination of Fatty Acid Composition

The fatty acid composition of the four strains of Staphylococcus aureus was determined using gas liquid chromatography. The objective of this experiment was to determine if a relationship existed between the types or relative percentages of individual fatty acids and the difference in resistance found among the strains.

The major fatty acids found in the four strains were the C14:0, a-C15:0, 2-OH C14:0, C16:0, C17:0 , C17:0, C18:1, C18:0, C19:0 , and C20:0 (Table 7). The relative percentages of each fatty acid varied among the strains. Three unknowns with retention times higher than that of the C20:0 were present in all four strains. While the compounds have not been identified at the present time, their mass spectra indicated that they were not fatty acids but did belong to the same family of compounds. Their mass spectra showed the same base peak as benzoates (Budzikiewicz et al., 1967).

The major fatty acids found in the four strains agreed to some extent with the results of other researchers. Theodore and Panos (1973) and Jantzen et al. (1974) found that the dominant fatty acids of Staphylococcus aureus were the methyl branched chain fatty acids a-C15:0, a-C17:0 and a-C19:0. Post (1982) using the same four strains used in this study reported as major fatty acids the C14:0, C15:0, a-C15:0, C16:0, C18:0 and C20:0. Two of the methyl branched

Table 7. Mean relative percentages from two replications of fatty acid fractions from four strains of Staphylococcus aureus (LP, ATCC 12600, A100 and S-6) grown in trypticase soy broth at 37°C.

	Relative Percentage Fatty Acid			
Fatty Acid	LP	ATCC 12600	A100	S-6
3-OH C12:0	0.44^{a}	0.30^{c}	0.37^{b}	0.35^{b}
C14:0	8.64^{a}	5.49^{c}	5.81^{c}	7.94^{b}
a-C15:0	15.90^{a}	11.36^{c}	11.37^{b}	12.10^{b}
2-OH C14:0	34.11^{b}	38.51^{a}	40.53^{a}	28.76^{c}
Unknown	1.11^{a}	0.87^{b}	0.73^{c}	0.96^{b}
C16:1	1.00^{b}	1.45^{b}	1.53^{b}	2.66^{a}
C16:0	6.93^{d}	7.07^{c}	7.30^{b}	7.58^{a}
C17:0 Δ	0.83^{d}	9.22^{c}	11.31^{b}	12.13^{a}
C17:0	5.37^{a}	1.33^{bc}	0.00^{c}	1.65^{b}
C18:1	3.66^{ab}	2.47^{bc}	1.64^{c}	4.16^{a}
C18:0	14.82^{a}	11.89^{b}	12.83^{b}	12.83^{b}
C19:0 Δ	0.00^{c}	3.44^{a}	2.16^{b}	4.39^{a}
C19:0	1.36^{a}	1.03^{a}	1.35^{a}	1.96^{a}
C20:0	5.78^{a}	5.00^{ab}	2.65^{bc}	1.88^{c}
Others	0.05^{a}	0.57^{a}	0.42^{a}	0.65^{a}
Total	100.00	100.00	100.00	100.00
Unsaturated/	4.66/	3.92/	3.17/	6.82/
Saturated	95.34	96.08	96.83	93.18

^{a,b,c,d} means in rows with different superscripts are significantly different ($P<0.05$).

chain fatty acids, the a-C17:0 and a-C19:0, which have been reported as existing in S. aureus were not present in the microorganisms used in this study, most probably because of the difference in the strains, growth conditions or analytical system used.

Statistical analysis showed that all the fatty acids except the C19:0 were significantly different. No consistency was found with respect to the relative percentages of saturated or unsaturated fatty acids or to the ratio of unsaturated to saturated fatty acids among the more or less resistant strains. These results were similar to those reported by Post (1982) in that no relationship was found between the unsaturated to saturated total fatty acid ratio and the resistance of the microorganisms. Therefore, no definite conclusions could be drawn concerning the saturated and unsaturated fatty acids and cell resistance. The only fatty acids showing a relationship with resistance were those containing the cyclopropane ring. In both, C17:0Δ and C19:0Δ resistance was inversely proportional to the amount of these fatty acids. A significant correlation coefficient ($P<0.05$) was found between the sum of the relative percentages of the cyclopropane-ring containing fatty acids and the resistance to propyl paraben ($r=-0.72$) or the methyl/propyl mixture ($r=-0.92$).

Dunnick and O'Leary (1970) found that, in all the cases they studied, the antibiotic resistant organisms contained a

lower concentration of cyclopropane acids than was found in the corresponding sensitive strains. Introduction of a cyclopropane ring lowers the melting point below that of analogous saturated straight-chain acids (Dunnick and O'Leary, 1970). Therefore, the presence of cyclopropane in membranes and envelopes may, in part, be responsible for the nature and integrity of these structures, and thus, affect the ability of parabens to react with or pass through these structures.

CHAPTER V

CONCLUSIONS

The four strains of Staphylococcus aureus (LP, ATCC 12600, A100, and S-6) used in this study were shown to have different resistance to the esters of p-hydroxybenzoic acid tested. According to this research this difference is at least partly due to the difference in the total lipid content of the cells. It seems most logical to think that the lipids as a whole play a protective role to the bacterial cell. The hydrophobic part of the parabens is adsorbed by the lipids resulting in a decreased penetration of these molecules in the membrane. The phosphatidyl glycerol fraction of the phospholipids and the fatty acids containing the cyclopropane ring appeared to be also related to this resistance. The former by their ability to form phospholipids with opposite charges can control the permeability of the membrane. The latter by influencing the membrane fluidity may affect the ability of parabens to react with or pass through the membranes.

The differences found in the total lipids and the lipid constituents of these bacterial cells is most probably related to differences in enzymes or enzyme systems. Conformational diversities in the lipid bilayer among the

four strains would cause differences among the hydrophobic interactions of the membrane lipids and the enzymes located in the membrane. Therefore, the membrane activity would be different among the bacterial strains.

Whether the difference found in the resistance is attributed solely to the differences in the lipids or the enzymes, or both, is not known. In all cases, however, differences in the genetic make-up among the bacterial strains is most probably the cause for differences in resistance.

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