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THE PRODUCTION OF A BACTERICIDAL MONOGLYCERIDE IN MURINE ABSCESES THAT ARE GENERATED BY STAPHYLOCOCCUS AUREUS

The Ohio State University

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THE PRODUCTION OF A BACTERICIDAL MONOGLYCERIDE IN MURINE ABSCESSES THAT ARE GENERATED BY STAPHYLOCOCCUS AUREUS

DISSERTATION

Presented in Partial Fulfillment of the Requirements for the Degree Doctor of Philosophy in the Graduate School of The Ohio State University

By

Howard David Engler, B.A.

* * * * * *

The Ohio State University

1986

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To my parents and grandparents, whose love, encouragement and support enabled me to reach this point
I would like to extend my sincerest heartfelt appreciation to my adviser, Dr. Frank A. Kapral, for his guidance, wisdom, patience, and caring. My gratitude also extends to the members of my advisory committee, Dr. Norman L. Somerson, Dr. Abramo C. Ottolenghi, and Dr. Raymond W. Lang for their time and advice, and to Dr. Bernard U. Bowman for graciously consenting to substitute for Dr. Lang who had to resign from the committee because of other commitments. My thanks also go to Shelley Smith for her fine technical help and friendship, and to "the ladies in the kitchen" --- Beverly Dandrea and Sharon Bailes --- for all of their help and kindness. Finally, I would like to thank Cherri, whom I have known through most of my graduate school years, for having been a part of my life.
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Major Area of Study: Host-Parasite Interactions
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INTRODUCTION

It is more than 110 years since Von Recklinghausen in 1871 found "micrococci" in the kidneys of a patient who died of pyaemia, and Klebs in 1872 first suggested that the organism, which he called Microsporon septicum, was the cause of the diseased state observed (Elek, 1959). Hueter later called the cocci found in pus "monads", and Billroth in 1874 classified the organisms found in putrefying tissues and body discharges into Micrococcus, Diplococcus, Streptococcus and Gliacoccus. In 1880 Pasteur was the first to cultivate in liquid medium small spherical organisms from pus, and he considered these to be the cause of suppuration. The following year Sir Alexander Ogston, a Scottish surgeon, showed conclusively for the first time that a cluster-forming coccus produced inflammation and suppuration and was the cause of certain pyogenic abscesses in man (Baird-Parker, 1972). Ogston in 1882 named those micrococci that arranged as a bunch of grapes Staphylococcus. The first to isolate and grow staphylococci in pure culture was Rosenbach in 1884, and while studying their characteristics he described the two species Staphylococcus (pyogenes) aureus and Staphylococcus (pyogenes) albus.

Today, Staphylococcus aureus is one of the most important bacteria causing disease in humans. Although numerous antistaphylococcal antibiotics have been developed during the past 40 years, infections
with *S. aureus* continue to represent a major cause of morbidity and mortality, primarily due to the development of plasmid-mediated resistance by the organism, first to penicillin and more recently to methicillin (Willett, 1984). While localized staphylococcal skin infections are one of the most common of all infectious disease processes, *S. aureus* may produce disease in almost every organ and tissue of the body. *S. aureus* infections may spread by extension from the skin to contiguous tissues, or by way of the lymphatics and then the blood (Youmans, 1980), producing diseases which include furuncles, carbuncles, osteomyelitis, arthritis, pneumonia, meningitis, deep tissue abscesses, brain abscesses, endocarditis, and breast abscesses in the nursing mother. Infections with *S. aureus* are characterized by intense suppuration, necrosis of local tissues, and a tendency for the infected area to become walled off with the formation of a pus-filled localized abscess (Sherris, 1984), within which various toxins can be elaborated. These toxins, when disseminated, account for the symptoms associated with the different disease states.

While the most common type of staphylococcal lesion is the abscess, there was little knowledge, however, about the interactions that occur between the host and the organism which may be determinants for the survival of the organism within such lesions. This was principally attributed to the lack of an appropriate experimental model system for studying the events which occur during the development of the abscess. It has been shown that humans and other species
are resistant to a subcutaneous inoculation of up to $10^6$ staphylococci (Elek, 1956; Elek and Conen, 1957). Consequently, in order to establish a localized infection, experimental models have had to utilize either incisions (Moller and Rydberg, 1969; Winn et al., 1979), a foreign substance (James and MacLeod, 1961; Noble, 1965; Taubler and Kapral, 1966; Agarwal, 1967; Hays and Mandell, 1974; Isenberg et al., 1976), prior tissue damage (Anderson, 1972), or a large inoculum (Kapral, 1974). The conditions produced, however, in no way reproduce a naturally acquired infection that involves a small number of staphylococci which become established in the host, multiply, and give rise to disease.

Kapral and others, while examining the survival of *S. aureus* in vivo, discovered that inoculation of staphylococci into the peritoneal cavity of mice provides a method for generating abscesses in a quantitative manner. These abscesses are easily recoverable and are free of any extraneous host tissue. With this model, it was then possible to study the interactions that influence the survival of *S. aureus* within these lesions.

When greater than $2 \times 10^8$ non-encapsulated *S. aureus* strains possessing the clumping factor on the cell surface are inoculated into the peritoneal cavity of mice, the cocci are promptly clumped as a result of the interaction of the bound coagulase with the fibrinogen present in the peritoneal fluid (Kapral, 1966). Encapsulated strains or strains that lack the clumping factor remain dispersed when introduced into the peritoneal cavity; encapsulated
strains resist phagocytosis and subsequently multiply and release toxins, killing the host, while clumping factor-negative strains are subject to phagocytosis and are eliminated from the peritoneal cavity. Introduction of fewer than $2 \times 10^8$ cocci that possess the clumping factor does not lead to clumping, and neutrophils entering the region can phagocytize the organisms with a minimal release of toxin, resulting in the survival of the host.

When $2 \times 10^8$ or more cocci are inoculated, clumping occurs, and by the second hour post-infection neutrophils entering the region during the ensuing inflammatory response form a thick layer around the aggregated organisms. Phagocytosis is minimal because only those cocci at the periphery of the clumps are readily accessible to the leukocytes. After five to six hours, the clumps, together with associated leukocytes, consolidate into a few large clusters that contain almost the entire inoculum (Kapral, 1966). Such large doses of *S. aureus* usually release so much alpha toxin that the host is killed within six to twelve hours (Kapral, 1966; Kapral, 1974). However, by culturing toxigenic strains in the absence of carbon dioxide suitable inocula can be prepared so that alpha toxin elaboration in the peritoneal cavity is subsequently curbed (Kapral, unpublished data), and the host survives, permitting long-term observation of the host-parasite interaction.

Neutrophils near the clumped cocci are quickly destroyed, but neutrophils that newly arrive continue to adhere to the exterior of the lesion and this results in the enlargement of the structure.
After 24 hours, a layer of eosinophilic material, presumably fibrin, surrounds the neutrophil-covered clumps, and by 48 hours connective tissue deposition begins. By the fourth day the lesion is enclosed within a vascularized connective tissue capsule and has matured into a typical staphylococcal abscess. At this time, the abscess consists of (from center to periphery) a core of densely packed cocci, a broad zone of acellular debris, a region of disintegrating leukocytes, a zone of intact leukocytes, a layer of eosinophilic material, and a vascularized connective tissue capsule (Kapral et al., 1980). Although a large inoculum is required for the establishment of these abscesses and multiplication is not observed, this method of infection does provide a suitable model for studying the later processes involved in the survival of *S. aureus* within abscesses.

All strains of *S. aureus* examined are found to manifest one of three patterns of survival within peritoneal abscesses (Kapral et al., 1980; Dye and Kapral, 1981a). Some strains are found to be rapidly destroyed in the abscess, while other strains are found to persist for up to three months without multiplying but also without any notable loss in viability. The third pattern observed is one in which a strain's population first decreases, then increases to near initial levels by the tenth day, and thereafter slowly and progressively declines.

Since neutrophils make up a sizable portion of the abscess, phagocytosis would seem to be a reasonable explanation for the elimination of the staphylococci. However, after only three hours
post-inoculation, no staphylococci are detected in the proximity of viable neutrophils and viable intact neutrophils are not present near the clumped staphylococci located in the core of the abscess (Kapral et al., 1980). Phagocytosis, therefore, is not responsible for the different patterns of survival that are observed.

When the survival of staphylococci is examined in abscesses that are removed from the peritoneal cavity and homogenized in saline, a substance that is bactericidal for certain S. aureus strains is found to be present within the homogenates (Dye and Kapral, 1980; Dye and Kapral, 1981a; Kapral and Dye, 1981). This bactericidal activity is not detectable before twelve hours post-inoculation, but subsequently the amount of activity increases rapidly until a peak is reached at seven to ten days, after which levels gradually diminish (Shryock et al., 1986). By its resistance to heat, catalase, and pH extremes, the staphylocidal material is not attributable to leukocytic myeloperoxidase, and by its lack of solubility in weak acid, its resistance to trypsin, iron, and hematin, and its increased activity in 1 M NaCl, the staphylocidal material is not attributable to leukocytic cationic proteins (Dye and Kapral, 1980; Kapral and Dye, 1981). The bactericidal activity is found to reside only in the neutral lipid fraction extracted from abscess homogenates, and subsequent fractionation of the lipid extract reveals that the bactericidal material is an unsaturated fatty acid that comprises less than 0.5% of the free fatty acid pool (Dye and Kapral, 1981b).
Different *S. aureus* strains are found to differ considerably in sensitivity to this staphylocidal fatty acid (SFA) in *in vitro* assays, and their sensitivity correlates with the ease with which they are eliminated within abscesses (Dye and Kapral, 1981a). Those strains most sensitive to the SFA are destroyed rapidly *in vivo*, whereas those strains most resistant are able to persist for long periods of time within abscesses. Strains of intermediate sensitivity demonstrate a delayed pattern of elimination from abscesses. This differential activity manifest by the SFA is not demonstrated by any other unsaturated fatty acids known to be inhibitory to various organisms, such as palmitoleic, oleic, and linoleic acids, or by fats that could release bactericidal fatty acids, such as mesenteric and epididymal fats (Dye and Kapral, 1981b). The SFA appears to be a unique primary determinant for the survival of staphylococci within abscesses.

Although never isolated or chemically identified, a number of properties distinguish the SFA from other long chain unsaturated fatty acids with similar bactericidal activity (Table 1). First, the SFA has a specific activity against staphylococci at least 10-100 times greater than that of conventional long chain unsaturated fatty acids (Dye and Kapral, 1981b). Second, the SFA demonstrates differential activity; it is 10-400 times more active against certain *S. aureus* strains than against other strains (Dye and Kapral, 1981a). Third, whereas the antibacterial activity of unsaturated fatty acids has been shown to increase after oxidation or peroxidation (Gutteridge et al., 1974), the activity of the SFA is destroyed by oxidation.
Fourth, while most unsaturated fatty acids have a markedly reduced bactericidal activity after catalytic reduction and subsequent formation of a saturated compound, no such loss in activity occurs after reduction of the SFA. Fifth, whereas long chain fatty acids have a higher antibacterial activity in an acid environment rather than in an alkaline or neutral environment (Hart et al., 1962; Galbraith and Miller, 1973; Kondo and Kanai, 1972), there is no significant change found in the activity of the SFA over the pH range 5.5 to 9.0. Finally, the SFA cannot be found in normal tissue such as fat, spleen, liver, muscle, or kidney (Dye and Kapral, 1981b), while long chain unsaturated free fatty acids can be detected. The bactericidal activity of both the SFA and long chain unsaturated fatty acids is destroyed by esterification (Kondo and Kanai, 1977) and calcium (Galbraith et al., 1971; Galbraith and Miller, 1973), while the SFA is also found to be inhibited by phospholipids (Grandel, 1981) and staphylococcal delta toxin (Chamberlain and Kapral).

Histological studies of frozen abscess sections stained with the lipid stain Oil Red O show that lipids accumulate within these lesions during their development (Kapral et al., 1983). Prior to eight hours after staphylococcal infection virtually no lipid accumulation is detected. However, after twelve hours, cells that are apparently macrophages are present at the periphery of the neutrophil layer and are found to contain lipid droplets in their cytoplasm. By 24 hours, a continuous band of lipid-containing cells is seen at the periphery of the abscess. As the vascularized connective tissue capsule
develops, lipid accumulation increases and many extracellular lipid droplets are observed immediately beneath the capsule. Lipid appears as droplets of various sizes, and although the greatest concentration of droplets always occurs beneath the capsule, over time droplets are found to disperse from the periphery of the abscess throughout the leukocyte layer and the zone of acellular debris until smaller extracellular droplets are noted in the abscess core where the tightly packed viable staphylococci are located. After seven days, there is no change in the distribution of the droplets, although a slight increase in the average droplet size is observed for the four weeks that follow. These findings suggest that the macrophage may be the source of the staphylocidal fatty acid.

Studies have shown that macrophages, when exposed to inflammatory stimuli that perturb their surface membranes, generate and secrete large amounts of biologically active compounds that are metabolites of the fatty acid arachidonate (Humes et al., 1977; Doig and Ford-Hutchinson, 1980; Scott et al., 1980). More than 25% of the total fatty acid content of the macrophage membrane is composed of arachidonic acid (Scott et al., 1980). In response to certain stimuli, including phagocytizing particulates such as zymosan (Bonney et al., 1978) or immune complexes of IgG or IgE and antigen (Bonney et al., 1979; Rouzer et al., 1982b), or soluble agents such as the tumor-promoting agent phorbol myristate acetate (Humes et al., 1978), as well as a wide variety of hormones, which include epinephrine and bradykinin (Van den Bosch, 1980), calcium ionophores, ultra-violet
light, or simple mechanical agitation (Kuehl and Egan, 1980), macrophages can release up to 50% of their arachidonic acid content (Scott et al., 1983). The great majority of the arachidonic acid in mammalian cells is esterified in the fatty acyl chains of glycerophospholipids, almost exclusively in the 2-acyl position (Irvine, 1982). There is therefore a requirement for the activation of the calcium-dependent phospholipase A2 to release arachidonate from phospholipid reserves. Once free, the arachidonic acid is metabolized by the cyclooxygenase or lipoxygenase pathways to form the twenty-carbon unsaturated biologically active compounds known as eicosanoids (Wolfe, 1982).

If arachidonate reacts with the enzyme cyclooxygenase, the arachidonic acid is oxygenated to form the various prostaglandins and/or thromboxanes. Macrophages have been found to release large amounts of prostaglandin E2, prostaglandin I2, and thromboxane (Humes et al., 1977; Morley et al., 1979; Kuehl and Egan, 1980). If arachidonic acid reacts with the enzyme lipoxygenase, there is production of hydroperoxy- or hydroxy- eicosatetraenoic acids (HPETE's or HETE's), as well as the formation of the leukotrienes. A major metabolite of macrophages has been found to be leukotriene C, also known as the slow-reacting substance of anaphylaxis (SRS-A) (Bach et al., 1980; Rouzer et al., 1980a and b; Scott et al., 1983). Leukotriene C can be produced in increased amounts when macrophages are treated with the calcium ionophore A23187 (Conroy et al., 1976; Bach and Brashler, 1978), which functions in stimulating
phospholipase A₂. Glutathione, a major non-protein sulfhydryl compound in most cells that plays an important role in the protection of cellular constituents against oxidative damage, is a direct precursor of leukotriene C and is used as a cofactor in prostaglandin E₂ biosynthesis (Rouzer et al., 1982a). A requirement for an adequate intracellular pool of glutathione has been demonstrated for the synthesis of both of these metabolites by macrophages.

The aim of this study was to attempt to determine whether the SFA might be an intermediate in arachidonic acid metabolism leading to the synthesis of eicosanoids. In the process, an unidentified lipid was isolated and its characteristics were compared to those of the SFA. Finally, the involvement of calcium and the possible role of the second messenger inositol triphosphate in the production of the SFA was explored.
MATERIALS AND METHODS

Animals.

Sprague-Dawley Swiss White ICR female mice (Harlan Laboratories, Indianapolis, IN) were used as the experimental animal.

Staphylococcus aureus Strains.

*S. aureus* strain 18Z, which has been previously described (Kapral, 1966; Kapral and Miller, 1971; Kapral et al., 1980) was used to generate intraperitoneal abscesses. This strain produces both alpha and delta toxin.

*S. aureus* strains TG and 303, which have been previously described (Kapral and Miller, 1971; Dye and Kapral, 1980; Dye and Kapral, 1981a), were used as indicator strains in the bactericidal assay. Strain TG produces alpha, beta, and delta toxins, while strain 303 elaborates only beta toxin. These represent strains with low and high sensitivity to the staphylocidal fatty acid, respectively (Dye and Kapral, 1980; Dye and Kapral, 1981a). For routine assay of bactericidal activity, strain 303 was used as the indicator organism.

All of the strains used are able to produce the soluble coagulase and clumping factor.

Staphylococcal Suspensions to Generate Abscesses.

*S. aureus* strain 18Z was grown in Trypticase soy broth (TSB) (BBL Microbiological Systems, Cockeysville, MD) at 37°C for 18-24
hours with constant shaking in the absence of carbon dioxide in order to minimize the production of alpha toxin. The cells were recovered by centrifugation and were then washed three times in TSB. The organisms were resuspended in TSB to give a final concentration of approximately $10^{10}$ cocci per ml. Suspensions were sealed in glass ampoules and stored at -70°C. Plate counts were performed to confirm bacterial concentrations.

**Staphylococcal Suspensions for Bactericidal Assay.**

Strains TG and 303 were grown in TSB at 37°C for 18-24 hours. The cells were washed three times in a diluent consisting of saline with 8% TSB, and were resuspended in the same diluent to give a final concentration of approximately $10^6$ cells per ml. These suspensions were also sealed in ampoules and stored at -70°C. Plate counts were done to verify the concentration of the organisms.

**Preparation of Abscess Homogenates.**

Groups of five or ten mice weighing 25-35 grams were inoculated intraperitoneally with $10^9$ *S. aureus* strain 18Z in a volume of 0.25 ml. After seven days, the animals were sacrificed and the abscesses were harvested, being careful not to remove any extraneous host tissue. The abscesses were homogenized in five or ten milliliters of saline using a motor-driven teflon pestle (Inframo, Wayne, NJ) in a glass mortar, and the homogenates were pooled. Homogenates were sealed in ampoules in aliquots of 0.5 ml and stored at -70°C.
Preparation of Lipid Extracts.

Lipids were extracted from abscess homogenates (AH) by the method of Dole and Meinertz (Dole and Meinertz, 1960). Previous studies have shown that the Bligh and Dyer modification of the Folch extraction procedure (Bligh and Dyer, 1959) and the procedure of Dole and Meinertz were equally effective in extracting the staphylocidal fatty acid from AH (Dye and Kapral, 1981b).

One-half ml of AH was combined with 2.5 ml of a mixture of isopropanol-heptane-2 N sulfuric acid (40:10:1, v/v/v) and was thoroughly mixed. After ten minutes, 1.5 ml of heptane and 1.0 ml of distilled water were added, and the phases were allowed to separate at room temperature. The upper heptane layer was removed to a lipid-clean vial, dried under nitrogen, and the lipid residue was weighed. The lipids were then dissolved in ethanol and stored at -20°C until needed.

Bactericidal Assay.

*S. aureus* strains TG and 303 were used as the indicator organisms to assay bactericidal activity. Suspensions of the appropriate indicator organism were thawed and diluted in saline containing 8% TSB to give a final working suspension concentration of $10^3$ cocci per ml.

To assay bactericidal activity, a slightly modified procedure of one previously described (Dye and Kapral, 1980, 1981a and b) was used. Abscess homogenates were first heated at 80°C for 30 min to eliminate the staphylococci present in the abscesses when they were harvested and homogenized. An initial 1:10 (v/v) dilution of the abscess
homogenates or of the lipid extract dissolved in ethanol was made in 2 M NaCl containing 2 mM EDTA, and serial two-fold dilutions of this initial dilution were then made in the same diluent. When ethanolic solutions were assayed care was taken that the ethanol concentration in the first tube was less than 5% (the toxic concentration for staphylococci was 12% ethanol).

To 0.6 ml amounts of each dilution was added 0.6 ml of the indicator organism working suspension. Several tubes containing 0.6 ml of the indicator organism suspension and 0.6 ml of diluent only served as controls. After a one hour incubation period in a 37°C shaking water bath, the surviving staphylococci were enumerated by plating 0.5 ml samples from each dilution and from controls in Nutrient agar (Difco Laboratories, Detroit, MI). Each assay was performed in duplicate.

A bactericidal unit (50% Lethal Dose [LD50]) was defined as that amount of AH or lipid extract which destroyed 50% of the indicator staphylococci during the one hour incubation period. The concentration of bactericidal activity was expressed as LD50 per ml of AH or LD50 per mg of lipid.

The LD50 endpoints were determined by linear regression analysis using the method of probit transformation (Finney, 1971). Analysis of the endpoints from multiple assays on the same homogenate showed that endpoints have a standard error of ±15%. This percent error does not necessarily apply when comparing the endpoints of different lipid suspensions since endpoints could be influenced by how well the lipids were dispersed in the suspensions.
Thin Layer Chromatography.

Lipids extracted from AH were chromatographed on Silica Gel G-coated glass thin layer plates (20 by 20 cm; Redi-Plates; Fisher Scientific Co., Pittsburgh, PA). Before use, plates were washed in ether-methanol (10:1, v/v), dried, and activated at 100°C for 30 min. After spotting lipids on the plate with a micro-pipette, plates were developed in a single direction in the appropriate solvent system. After air drying, the lipids were visualized by exposing the plates to iodine vapor in a sealed tank or by spraying the plate with 50% aqueous sulfuric acid containing 0.6% potassium dichromate and then charring at 180°C for 60 min.

Recovery of Lipids After TLC.

Lipids that were visualized by iodine were recovered from plates by scraping the silica gel containing a particular spot into a lipid-clean tube and extracting three times by shaking with ethyl ether-methanol (10:1, v/v) for 10 min. The solvent was evaporated to a small volume on a rotary evaporator (Rotavapor-R; Buchi; Switzerland), transferred to a lipid-clean tube, and then totally removed under nitrogen. The lipid residue was weighed, dissolved in ethanol or hexane, and stored at -20°C until needed.

Hydrolysis of the "Unidentified Lipid".

The procedure for hydrolysis of acyl glycerols has been previously described (Chernick, 1969). Briefly, an aliquot containing 50 µg of the lipid sample in 50 µl of hexane was placed in a 12 x 75 mm test tube, and 0.1 ml of 0.05 N tetraethylammoniumhydroxide in ethanol
(10% tetraethylammoniumhydroxide diluted 1:14 v/v in ethanol) was added to the tube. A glass marble was placed on top of the tube to prevent evaporation, and the contents were heated at 60°C for 30 min in a water bath. While still in the water bath, 0.1 ml of 0.1 N HCl was added to the tube, after which the tube was removed from the bath, the contents were mixed, and 1.8 ml of water was added. The free fatty acids were removed by extracting three times with 1.5 ml of hexane and combining the upper hexane phases. Glycerol remained in the 2 ml aqueous phase.

**Glycerol Determination.**

The procedures for the determination of the presence of glycerol have been described previously (Lambert and Neish, 1950; Hanahan and Olley, 1958). This method is based on the determination of formaldehyde which is produced by the oxidation of glycerol with sodium periodate. The formaldehyde is subsequently reacted with chromotropic acid in a strong sulfuric acid solution, and the violet-colored product that results is determined spectrophotometrically.

Control tubes (12 x 75 mm) containing 2 ml of 0.10, 0.25, and 0.50 micromoles of glycerol, and a water (blank) tube, were prepared. To each control tube and the tube containing an unknown quantity of glycerol acquired by hydrolysis of the "unidentified lipid", 0.1 ml of 10 N sulfuric acid and 0.5 ml of 0.1 M sodium periodate were added. The contents of each tube were mixed and held at room temperature for 5 min. To each tube 0.5 ml of 10% sodium bisulfite was then added, and the contents of each tube were mixed and held at room temperature
for another 5 min. A 0.5 ml aliquot was removed from each tube to a
16 x 150 mm tube, and 5.0 ml of the chromotropic acid reagent (made
by dissolving 0.5 grams of chromotropic acid sodium salt in 50 ml of
water and adding 200 ml of 12.5 N sulfuric acid) was added. The tubes
were heated in a boiling water bath for 30 min, after which they were
cooled to room temperature. A standard curve and the unknown amount
of glycerol was determined by reading the absorbance of the contents
of each tube in a spectrophotometer (Gilford 250; Gilford Instruments;
Oberlin, OH) at 570 nm.

Effect of FAME on the "Unidentified Lipid".

A lyophilized partially-purified FAME (Fatty Acid Modifying
Enzyme) preparation was dissolved in 0.01 M sodium phosphate buffer
(pH 6.0) to give a final concentration of 2.0 mg of FAME per 0.45 ml
of buffer.

An aliquot containing 200 µg of the "unidentified lipid"
dissolved in hexane was dispensed into a lipid-clean vial and the
hexane was evaporated under nitrogen. To the vial was added 0.45 ml
of the FAME solution and 0.05 ml of ethanol (alcohol substrate).
This mixture was incubated in a shaking water bath at 37°C for 1 hr.

Following the incubation, the lipids were removed by extracting
the mixture twice with 2.0 ml of ethyl ether-methanol (6:1, v/v) and
once with 2.0 ml of ethyl ether. The upper ether phases were combined
and evaporated to dryness under nitrogen. The extracted lipids were
dissolved in 50 µl of hexane, and the entire amount was spotted on a
TLC plate and chromatographed in a solvent system of petroleum
ether-ethyl ether-acetic acid (40:60:1, v/v/v).

Biochemicals.

Calcium ionophore A23187, arachidonic acid, glutathione, indomethacin, NDGA (nordihydroguaiaretic acid), phenidone (1-phenyl-3-pyrazolidone), 4-bromophenacylbromide, lasalocid, nonactin, valinomycin, phorbol myristate acetate, verapamil hydrochloride, and phosphatidylinositol 4,5-biphosphate sodium salt were obtained from Sigma Chemical Co., St. Louis, MO. Myo-inositol, myo-inositol 1,4,5-triphosphate, and guanosine-3',5'-cyclic phosphate (cyclic GMP) were obtained from Calbiochem, La Jolla, CA. EDTA (disodium ethylenediaminetetraacetate) was obtained from Baker Chemical Co., Phillipsburg, NJ. Tetraethylammoniumhydroxide was obtained from Eastman Kodak Co., Rochester, NY. Chromotropic acid sodium salt was obtained from Fisher Scientific Co., Fairlawn, NJ.

All lipid standards were purchased from Sigma Chemical Co.

Staphylococcal delta toxin was in lyophilized form and was previously prepared and assayed in this laboratory.

A partially-purified lyophilized FAME preparation was obtained from Shelley Smith of this laboratory.

A 0.01 M stock solution of the calcium ionophore A23187 was prepared in dimethyl sulphoxide and stored at -70°C between uses. Solutions of arachidonic acid, indomethacin, NDGA, phenidone, bromophenacylbromide, and verapamil hydrochloride were prepared in ethanol. Solutions of glutathione, EDTA, myo-inositol, myo-inositol triphosphate, phosphatidylinositol 4,5-biphosphate, and cyclic GMP were prepared in
water. A solution of lasalocid was made in methanol, nonactin was dissolved in chloroform, and solutions of valinomycin and phorbol myristate acetate were made in acetone.

**Solvents.**

All solvents used were of reagent or HPLC grade, and those used for extraction of lipids were redistilled in glass prior to use.
RESULTS

Attempts to Generate Eicosanoids.

Since previous studies indicated that the macrophage may be the source of the staphylocidal fatty acid (SFA), the possibility that the SFA might be one of the intermediates of arachidonic acid metabolism leading to the production of eicosanoids such as prostaglandins, thromboxanes, hydroperoxy- and hydroxy- eicosatetraenoic acids, or leukotrienes was examined. Abscess homogenates (AH) were treated with compounds likely to be involved in stimulating eicosanoid production. Previous investigators have used 1 μM calcium ionophore A23187 (Doig and Ford-Hutchinson, 1980), 40-80 μM arachidonic acid (Parnham et al., 1983), and 10 mM glutathione (Bach and Brashler, 1978) as minimum concentrations to stimulate the production of eicosanoids. Accordingly, to different aliquots of AH was added 10 μM calcium ionophore A23187, 100 μM arachidonic acid, and 10 mM glutathione, either separately or in various combinations. After incubating for 20 min in a 37°C shaking water bath, the AH was extracted and the recovered lipid fraction was assayed for bactericidal activity. Lipid extracted from AH incubated in the absence of any of the above biochemicals served as a control. If any of the additions were able to stimulate SFA synthesis, then an increase in the cidal activity of the AH would be expected.
The addition of the calcium ionophore A23187, whether alone or in combination, increased the bactericidal activity of the AH 10-fold (Table 2). The addition of arachidonic acid and/or glutathione to AH had no effect. The increase in bactericidal activity generated by the calcium ionophore A23187 was accompanied by a shift in the slope of the dose-response curve (Figure 1), indicating the formation of a new compound.

**Time Course with the Calcium Ionophore A23187**

AH were incubated with 10 μM calcium ionophore A23187 for various time periods at 37°C prior to lipid extraction and assaying the lipid fraction for bactericidal activity. The increase in bactericidal activity was dependent on the length of incubation of AH with the calcium ionophore A23187 (Table 3). Although bactericidal activity was increased even after a short incubation of only 5 min, maximal effects were not noted until 160-320 min. Further incubation resulted in a marked decrease in the cidal activity that was generated.

**Attempts to Inhibit the Generated Bactericidal Activity.**

To determine if the increased cidal activity generated by the calcium ionophore A23187 involved eicosanoid synthesis, various compounds known to inhibit prostaglandin and/or leukotriene synthesis were incubated with aliquots of AH for 20 min at 37°C prior to an additional 5 or 50 min incubation with 10 μM calcium ionophore A23187.

The inhibitors used were indomethacin (100 μM) (Salari et al., 1984), which inhibits cyclooxygenase and thus prostaglandin synthesis, NDGA (100 μM) (Salari et al., 1984), which inhibits lipoxygenase and
thus leukotriene synthesis, phenidone (100 µM or 1 mM) (Parnham et al., 1983), which inhibits both cyclooxygenase and lipoxygenase, 4-bromophenacyl bromide (100 µM) (Vargaftig, 1977), an "active site" inhibitor of phospholipase A2 which thus interferes with the release of arachidonic acid from phospholipids, and EDTA (1 mM), which chelates the calcium that is required to stimulate the release of arachidonic acid from phospholipids.

None of these compounds was able to interfere with the ability of the calcium ionophore A23187 to augment the bactericidal activity of AH (Table 4).

**Thin Layer Chromatography of Lipid Extracts.**

Total lipids extracted from untreated AH or from AH treated with 10 µM calcium ionophore A23187 for 200 min were examined by TLC in a solvent system of petroleum ether-ethyl ether-acetic acid (30:70:1, v/v/v). A previously unrecognized very polar lipid was detected (Figure 2, arrow). This lipid is present in small amounts in untreated AH but was markedly increased in amount after incubation with the calcium ionophore A23187. The data indicate that the intensity of the spot appeared to be dependent upon the length of incubation of AH with the calcium ionophore A23187.

**Partial Identification of the Newly Recognized "Unidentified Lipid".**

Identification of the newly discovered polar lipid was attempted on TLC plates by employing various spray detection reagents, including those used to detect phosphate (Vaskovsky and Kostetsky, 1968), the carbohydrate of glycolipids (Svennerholm, 1956), free amino groups
(Skipski et al., 1962), choline (Christie, 1982), the sialic acid of gangliosides (Svennerholm, 1957), and the amide group of sphingolipids (Bischel and Austin, 1963). The "unidentified lipid" did not appear to react with any of these reagents as compared to control standards.

The presence of carbohydrates or sphingosines could not be detected by reaction with anthrone or trinitrobenzenesulfonic acid, respectively, using methods previously described (Yamamoto and Rouser, 1970).

Subsequent studies showed that the "unidentified lipid" migrated to the same location on TLC as do the monoacylglycerides. Numerous solvent systems were examined, including petroleum ether-ethyl ether-acetic acid (30:70:1, v/v/v), ethyl ether-acetone (8:2, v/v), chloroform-methanol-water (80:10:2 and 65:25:4, v/v/v), and 100% chloroform, and in each case the Rf's of both the "unidentified lipid" and monoooleylglycerol (or monopalmitoylglycerol) were equivalent.

As hydrolysis of a monoacylglyceride would yield a free fatty acid and free glycerol, confirmation that the "unidentified lipid" is a glyceride could be made by its hydrolysis and the subsequent quantitation of any glycerol that is present. Accordingly, the "unidentified lipid" was recovered from TLC plates and an aliquot of the "unidentified lipid" was hydrolysed. The determination of glycerol was performed on the aqueous phase of the hydrolysate.

An aliquot of 50 μg of the "unidentified lipid" was found to contain 0.185 μmoles (17 μg) of glycerol.
Characterization of the Monoglyceride.

Bactericidal activity of the monoglyceride. The monoglyceride, as well as the other spots as a pool, were each recovered from TLC plates and were assayed for bactericidal activity against the two staphylococcal indicator strains, TG and 303. While the monoglyceride possessed a high specific bactericidal activity, as evidenced against the SFA-sensitive 303 strain, the rest of the lipids recovered as a pool from the TLC plate demonstrated little activity (Table 5). The monoglyceride was also much more active against the SFA-sensitive 303 strain than against the SFA-resistant TG strain (Figure 3), thus indicating that the monoglyceride possessed the same kind of differential activity as the SFA.

The effect of oxygen on the monoglyceride. To evaluate the effect of oxidation on the monoglyceride, a solution of the isolated monoglyceride was made in ethanol. An aliquot of this solution was stored at room temperature in sealed ampoules under oxygen, and another aliquot was similarly stored under nitrogen. After 48 hours, both samples were assayed for residual bactericidal activity. The activity of the monoglyceride was reduced by 75% by oxidation (Figure 4). This is similar to the oxygen lability of the SFA.

The effect of staphylococcal delta toxin on the monoglyceride. As Chamberlain and Kapral have shown that staphylococcal delta toxin is present within abscesses and that the SFA is neutralized by delta toxin, the effect of this protein on the newly discovered monoglyceride was examined. The bactericidal assay was performed, with slight
modifications, as follows: Once the serial two-fold dilutions of the monoglyceride were completed (volume in each tube = 0.6 ml), 0.2 ml of saline containing 20 μg of delta toxin was added to each tube, including controls, and the tubes were incubated at room temperature for 30 min. To each tube was then added 0.4 ml of the indicator organism working suspension, and the assay proceeded to completion as usual. Delta toxin was able to neutralize about 95% of the activity of the monoglyceride (Table 6).

The effect of lecithin on the monoglyceride. To examine whether lecithin (phosphatidylcholine) neutralizes the monoglyceride in the same way that phospholipids are known to neutralize the SFA, 0.2 ml of ethanol containing 20 μg of lecithin was added to the tubes containing the serial two-fold dilutions of the monoglyceride, and to the control tubes containing only the diluent. After a 30 min incubation at room temperature, 0.4 ml of the indicator organism working suspension was added to each tube, and the assay proceeded to conclusion as usual. Lecithin neutralized about 95% of the activity of the monoglyceride (Table 7).

The effect of calcium on the monoglyceride. To evaluate the effect of calcium on the monoglyceride, the bactericidal assay was performed, with modifications, as follows: Serial two-fold dilutions of the monoglyceride were performed in saline (0.9% NaCl), so that the EDTA that is present in the normally used diluent would not have an effect on the added calcium. To each dilution tube, and to each control tube containing only saline, was added 0.2 ml of saline containing
200 µg of CaCl₂. Following a 30 min incubation at room temperature, 0.4 ml of the indicator organism working suspension was added to each tube, and the assay proceeded as usual to conclusion. This concentration of calcium was able to reduce the bactericidal activity of the monoglyceride by 50% (Table 7).

The effect of FAME on the monoglyceride. A fatty acid esterifying enzyme that is produced by some S. aureus strains has recently been described (Kapral and Mortensen, 1985a and b). This enzyme, which goes by the name FAME, has been found to esterify the SFA and other fatty acids, thereby eliminating their bactericidal activities. Incubation of FAME with the monoglyceride and the subsequent chromatography on a TLC plate of the extracted lipids showed that the FAME preparation was able to convert the entire quantity of the monoglyceride to free fatty acids and fatty acid esters (Figure 5).

Search for the monoglyceride in other tissues. To determine whether the monoglyceride is produced in tissues other than abscesses, the spleen, kidney, liver, heart, brain, lung, or muscle was removed from either normal mice or from staphylococcal infected mice which had been inoculated intraperitoneally seven days earlier. Each tissue that was removed was homogenized in saline. Lipid extracts from untreated tissue homogenates or from tissue homogenates treated with 10 µM calcium ionophore A23187 for 200 min at 37°C were then spotted on a TLC plate and developed in a solvent system of petroleum ether-ethyl ether-acetic acid (30:70:1, v/v/v). The monoglyceride was developed alongside the tissue homogenates extracts as a control. The
monoglyceride spot could not be found in the extracts from any of the tissues, indicating that the monoglyceride is unique to the abscess, just as is the SFA.

**Concentration Dependency of Bactericidal Activity Generated by the Calcium Ionophore A23187.**

To examine whether varying concentrations of the calcium ionophore A23187 have the same effect on the bactericidal activity of AH, concentrations of 1 μM, 10 μM, and 100 μM calcium ionophore A23187 were added to aliquots of AH. After incubating for 50 min in a 37°C shaking water bath, the whole AH was assayed for bactericidal activity. The calcium ionophore A23187 produced a concentration-dependent increase in the cidal activity of AH, with a minimum concentration of 10 μM required to generate a measurable response (Table 8).

**Effect of Other Ionophores and Phorbol Myristate Acetate on the Bactericidal Activity of AH.**

To assess whether only the calcium ionophore A23187 had the ability of generating bactericidal activity in AH or if other ionophores could do the same, three other different ionophores were incubated with aliquots of AH for 50 min, and the whole AH was assayed for bactericidal activity. Lasalocid (X 537A), another calcium ionophore (Tamarit-Rodriguez et al., 1977), was able to increase the cidal activity at a minimum concentration of 100 μM (Table 8). The extent of this increase was not as great as that of the calcium ionophore A23187. The addition of nonactin and valinomycin, both potassium ionophores, had no effect at concentrations of 10 μM.
The calcium ionophore A23187 has been shown to have the ability to stimulate the initiation of the respiratory burst in neutrophils (DeChatelet, 1982), similar to the action of the tumor-promoting agent phorbol myristate acetate. To determine if the action of the calcium ionophore A23187 in generating bactericidal activity was possibly associated with a stimulation of the respiratory burst, 10 µM phorbol myristate acetate was incubated with an aliquot of AH for 50 min, and the whole AH was assayed for bactericidal activity. Phorbol myristate acetate had no effect on the activity of AH (Table 8).

Inhibition of Generated Bactericidal Activity by a Calcium Channel Blocking Agent.

Since previous studies suggested that a calcium ion flux was needed for the production of the monoglyceride and concomitant increase in bactericidal activity, as evidenced by the effects of the calcium ionophores A23187 and lasalocid, the ability of an agent that blocks the calcium channel to inhibit the generation of bactericidal activity was examined. An aliquot of AH was incubated with 10 µM verapamil, a calcium channel blocking agent, for 15 min at 37°C before the addition of 10 µM calcium ionophore A23187 and an additional incubation period of 50 min at 37°C (Kazanjian and Pennington, 1985). The bactericidal activity of this treated aliquot of AH was then assayed, as was the activity of an aliquot of untreated AH and an aliquot of AH incubated for 50 min with 10 µM calcium ionophore A23187. Verapamil was able to block the ability of the calcium ionophore A23187 to stimulate the production of the monoglyceride and thus generate
bactericidal activity (Table 9).

**Effect of the Calcium Ionophore A23187 on Abscess Homogenate Fractions.**

To examine further how the calcium ionophore A23187 may be acting, abscess homogenates were centrifuged at 39,000 x g (18,000 rpm; SS-34 rotor; Sorvall RC2B centrifuge; DuPont Co, Biotechnology Systems, Wilmington, DE) for 30 min and divided into a cell-free supernatant fraction and a sediment fraction. The sediment fraction was washed three times in saline. Each fraction, either separately or combined, was assayed for cidal activity before and after the addition of 10 μM calcium ionophore A23187 (Table 10). All of the bactericidal activity was normally found to reside in the sediment. When the calcium ionophore A23187 was added to each individual fraction, no effect was observed. However, when both fractions were combined, the calcium ionophore A23187 was able to generate cidal activity by production of the monoglyceride.

Calcium ionophore A23187 (10 μM) added to previously boiled (100°C, 30 min) AH did not generate any bactericidal activity (Table 11).

The calcium ionophore A23187 had no effect on the bactericidal activity of the total lipid fraction or the monoglyceride fraction already extracted from AH (Table 12).

**Inositol triphosphate involvement in monoglyceride production.**

Calcium ionophores are known to mimic the action of the second messenger inositol triphosphate (Berridge, 1985), which functions in releasing calcium from internal stores. To test the idea that a
second messenger might be involved in the synthesis of the monoglyceride, 10 μM inositol triphosphate was incubated with an aliquot of AH for 50 min at 37°C, and the bactericidal activity of the whole AH was assayed. The addition of inositol triphosphate to AH increased the cidal activity of AH by presumably stimulating monoglyceride production, similar to the calcium ionophore A23187 (Table 13). A time course study demonstrated that when AH were treated with 10 μM inositol triphosphate, the increase in cidal activity was sustained for 22 hours (Figure 6).

Phosphatidylinositol 4,5-biphosphate, the source of inositol triphosphate in vivo, and inositol, when added to AH, had no effect on the bactericidal activity (Table 13). Cyclic GMP, another second messenger known to activate the enzyme G-kinase, also had no effect when incubated with AH.

To determine if high extracellular calcium concentrations were important in generating the monoglyceride, 10 mM calcium (as CaCl₂) was added to AH directly, and after an incubation at 37°C for 50 min, the bactericidal activity was determined. The addition of calcium to AH had no effect (Table 13), even when the calcium ionophore A23187 was added along with the calcium.

The monoglyceride could not be found in various tissues from normal or staphylococcal infected mice, when these tissues were homogenized and treated with 10 μM inositol triphosphate.
DISCUSSION

Many investigators have described the ability of long chain unsaturated fatty acids to kill or inhibit the growth of a large number of bacteria. Generally, gram-positive bacteria are more sensitive to the antibacterial effects of long chain fatty acids than are gram-negative organisms (Sheu and Freese, 1973; Kabara and Vrable, 1977; Kanai and Kondo, 1979). This can be attributed to the fact that the probable site of action by long chain fatty acids for killing is the cytoplasmic membrane, and gram-negative organisms are resistant due to the presence of hydrophilic lipopolysaccharide molecules projecting from the outer membrane which act as a penetration barrier against hydrophobic substances (Sheu and Freese, 1973), while the cell wall of gram-positive organisms may adsorb and transport fatty acids onto the inner membrane. It has been demonstrated that the determinants which influence the bactericidal activity of fatty acids include chain length, degree of unsaturation, position of the double bonds, presence of a polar end group, and stereochemical configuration (Kanai and Kondo, 1979).

Oleic acid was found to kill group A streptococci by altering the integrity of the cell membrane (Speert et al., 1979). The in vitro growth of Neisseria gonorrhoea was found to be inhibited by unsaturated fatty acids with chain lengths of C16 to C20 (Miller et al., 1977).
Other studies have shown that the "bacteriocin" of Neisseria gonorrhea results from the degradation of phospholipids to inhibitory free fatty acids (Walstad et al., 1974). *Escherichia coli* (Fay and Farias, 1975), *Bordetella pertussis* (Pollock, 1949), *Pasteurella pestis* (Eisler and von Metz, 1968), and *Bacillus subtilis* (Sheu and Freese, 1972) have all been shown to be inhibited by fatty acids.

An antimycobacterial factor attributed to non-esterified fatty acids which arise during infection has been described (Kochan et al., 1972; Kochan and Golden, 1974; Kochan and Berendt, 1974). It was reported that antibacterial activity was noted in cell-free extracts of tissues from infected animals and in extracts of immune-activated macrophages. The investigators suspected that the antibacterial effect resulted from membrane-bound tissue lipases hydrolysing lipoproteins or phospholipids in the macrophage cell membrane, thus releasing bactericidal free fatty acids. The secretion of antimycobacterial fatty acids by normal and activated macrophages was later demonstrated (Hemsworth and Kochan, 1978), and it was suggested that macrophages can exert their antimycobacterial effect without phagocytosis.

The alveolar lining material of rats has been found to contain an antibacterial factor that has recently been attributed to free fatty acids (Coonrod and Yoneda, 1983; Coonrod et al., 1984). While inhaled bacteria are normally killed by alveolar macrophages, studies showed that *Staphylococcus aureus* was not killed readily by alveolar macrophages unless the organisms were first exposed to alveolar lining material (LaForce et al., 1973). Coonrod and others have shown that
the surfactant-containing fraction of alveolar lining material contains powerful antibacterial long chain unsaturated free fatty acids which are lethal for staphylococci, pneumococci, many streptococcal species, and Bacillus species. These free fatty acids have a possible role in early pulmonary host defenses.

The sensitivity of Staphylococcus aureus to certain long chain unsaturated fatty acids has been reported (Wynne and Foster, 1950; Butcher et al., 1976; Heczko et al., 1979; Lacey and Lord, 1981). Dye, Kapral, and others have described a model system that involves the destruction of S. aureus in intraperitoneal abscesses by a unique bactericidal free fatty acid. The average seven-day old staphylococcal abscess contains 3.75 mg of free fatty acids (Kapral and Mortensen, 1985b). Of this amount, 3.0 mg consists of a pool of unsaturated free fatty acids consisting mainly of linoleic and oleic acids, along with lesser quantities of palmitoleic, linoleic, and arachidonic acids. Unique to the staphylocidal fatty acid (SFA) not only is its high specific bactericidal activity, however, but also its ability to exhibit differential activity towards indicator S. aureus strains against which the common unsaturated long chain free fatty acids demonstrate the same activity. Although the presence in the abscess of such large quantities of common unsaturated free fatty acids may indicate their possible participation in the elimination of the organisms, it is believed that the SFA is a major element in the host-parasite interactions governing the survival of staphylococci within abscesses. As such, it is important to determine the mechanisms
involved in its production and to isolate and characterize this novel lipid or its precursor.

 Ionophores are distinguished by their ability to create specific pathways of ion permeability in cell membranes (Szabo, 1981). The calcium ionophore A23187 is a carrier of calcium ions and is also an initiator of phospholipase A₂ activity (Pickett et al., 1977). In the presence of calcium, calcium ionophore A23187 can stimulate arachidonic acid metabolism leading to the production of eicosanoid derivatives.

 Calcium ionophore A23187 produced a time- and concentration-dependent increase in the bactericidal activity of abscess homogenates (AH). Maximum generated bactericidal activity was seen after an incubation of 160 min with a minimum concentration of 10 μM calcium ionophore A23187. Why an incubation of 1280 min resulted in a decrease in the cidal activity produced is not known, but it is possible that the calcium ionophore A23187 cannot maintain its effectiveness in air for that long a period, or that any lipid that may have been synthesized was subsequently oxidized and its activity retarded.

 While it was originally thought that the increase in bactericidal activity might be a result of the synthesis of the SFA as an eicosanoid derivative, it was subsequently discovered that the increase was not prevented by various compounds known to inhibit prostaglandin and/or leukotriene production. The generated bactericidal activity therefore could not be due to the action of the calcium ionophore A23187 stimulating arachidonic acid metabolism.
The total lipid fraction from AH treated with calcium ionophore A23187 was found to contain a heretofore unrecognized polar lipid. While this lipid was subsequently noted to be present in small amounts in the lipids extracted from untreated staphylococcal AH, after treatment with calcium ionophore A23187 the amount of this polar lipid was greatly increased. The longer the time of incubation of AH with the calcium ionophore A23187, the greater the amount of the "unidentified lipid" that was produced. This increase in the amount of the "unidentified lipid" correlated with the increase in the bactericidal activity of the AH that was seen, suggesting that the calcium ionophore A23187 was bringing about the production of an unknown lipid that possessed bactericidal ability. Further studies suggested that this "unidentified lipid" was a monoglyceride.

The monoglyceride possessed many of the same properties as the SFA, and as such may be the precursor of the SFA. The monoglyceride demonstrated high specific bactericidal activity against the SFA-sensitive *S. aureus* strain, and it exhibited the differential activity that is the hallmark for distinguishing the SFA from other conventional fatty acids with similar bactericidal activities. The antimicrobial activity of monoglycerides has been investigated (Kabara and Vrable, 1977), and it was shown that esterification of a fatty acid to glycerol to form a monoacyl derivative generally results in more active derivatives. Preliminary studies have shown that 1-monoolein has bactericidal activity, but does not demonstrate differential activity (303: 2800 ED50/mg, TG: 1900 ED50/mg), suggesting that differential
activity is not a common occurrence among the monoglycerides.

Similar to the SFA, the bactericidal activity of the monoglyceride was reduced by 75% after exposure to oxygen for 48 hours. This is unlike conventional fatty acids whose activity is enhanced by oxidation. Staphylococcal delta toxin, lecithin, and calcium all have the ability to neutralize the activity of both the monoglyceride and the SFA. The findings of the action of delta toxin are of importance since Chamberlain and Kapral have demonstrated the presence of delta toxin within abscesses as early as nine hours post-infection, with peak amounts occurring after seven to ten days. The amounts of delta toxin produced in the abscess are of sufficient quantity to be of benefit to the organism's survival in the lesion.

FAME, an enzyme that is produced by some S. aureus strains, has been found to esterify the SFA and other fatty acids to certain alcohol substrates, thereby destroying their bactericidal activities (Kapral and Mortensen, 1985b). It is a heat labile and protease K-sensitive enzyme with maximal activity at pH 5.5-6.0 and at 37-40°C. Neither monovalent or divalent cations are required for activity. Glycerides have been found to be inhibitory to FAME, with triglycerides containing unsaturated fatty acyl chains demonstrating the greatest inhibition and monoglycerides being the least inhibitory. Those S. aureus strains lacking FAME, or strains able to produce only small amounts of the enzyme, are rapidly destroyed within abscesses. In the presence of ethanol, the FAME preparation was found to convert the monoglyceride into a mixture of free fatty acids, fatty acid esters,
and presumably glycerol. The fact that free fatty acids were found indicates that a staphylococcal lipase was also present in the partially purified FAME preparation. The presence of free fatty acids together with esterified fatty acids in the mixture suggests that the lipase was more effective in hydrolysing the monoglyceride than was FAME in esterifying the fatty acids released by the lipase. This finding could be due to the fact that ethanol was the sole alcohol available for esterification and ethanol is about 800-fold less effective for esterification than is cholesterol, the preferred substrate for FAME and presumably the substrate utilized within abscesses. It may be that lipase and FAME work hand-in-hand, first to release free fatty acids from complex bactericidal lipids and then to inactivate the resultant bactericidal fatty acids by esterifying them.

The bactericidal monoglyceride could not be found in homogenates of spleen, kidney, liver, heart, brain, lung, or muscle from either normal or staphylococcal infected mice, either before or after treatment with the calcium ionophore A23187 or inositol trisphosphate. The uniqueness of the monoglyceride and the SFA to the abscess suggests that the abscess environment provides special conditions which are conducive to the production of these staphylocidal lipids by the host but which are not available in normal tissues. It is not known what these conditions might be, but changes brought about by the inflammatory response may be a possibility.
In addition to the calcium ionophore A23187, the production of the monoglyceride in AH can also be stimulated by lasalocid, another ionophore with an affinity for calcium ions. Valinomycin and nonactin, both potassium (monovalent cation) ionophores, did not initiate synthesis of the monoglyceride. These results, together with the ability of verapamil, a calcium channel blocker, to block the production of the monoglyceride by the calcium ionophore A23187, suggest that a Ca\(^{++}\) flux was needed for the production of the monoglyceride.

Addition of the calcium ionophore A23187 to either the cell-free supernatant fraction of AH or to the washed sediment fraction did not result in the production of the monoglyceride and an increase in bactericidal activity. Production of the monoglyceride, however, was seen when the calcium ionophore A23187 was added to the recombined fractions. This suggests that some substance(s) present in the AH supernatant must act in conjunction with membranous components to synthesize the monoglyceride. Whether this substance is an enzyme, the calcium-binding regulator protein calmodulin, or some other substance is not yet known.

Berridge and others have described the second messenger, inositol triphosphate (Streb et al., 1983; Berridge, 1984; Berridge and Irvine, 1984; Biden et al., 1984; Joseph et al., 1984; Hokin, 1985), which is involved in the inositol phosphate cycle. The phosphatidylinositides represent a minor component of the total membrane phospholipids (Rasmussen, 1986). They exist in three forms: phosphatidylinositol, phosphatidylinositol 4-phosphate, and phosphatidylinositol...
4,5-biphosphate, in a ratio of approximately 90:5:5. These forms are rapidly interconvertible by the appropriate kinases and phosphatases.

Normally, what occurs in vivo is that some external signal, the first messenger, stimulates a receptor in the cell membrane which in turn stimulates a GTP-dependent regulatory G protein to activate a phosphodiesterase in the inner leaflet of the membrane. This enzyme is a phospholipase C which hydrolyzes phosphatidylinositol 4,5-biphosphate by cleaving the inositol phosphates from the glycerol backbone, giving rise to the second messengers inositol triphosphate and diacylglycerol. Inositol triphosphate is water soluble and diffuses into the cytoplasm, where it releases Ca\(^{++}\) from the intracellular stores in the endoplasmic reticulum. Just how inositol triphosphate releases internal calcium has not been fully established, but it has been postulated that the inositol triphosphate may either act on an internal receptor to stimulate release or it may inhibit the uptake mechanisms responsible for sequestering intracellular calcium (Berridge, 1984). Whatever the case, the released calcium can then activate a reaction alone or it can bind to calmodulin to form a complex which can activate enzymes to bring about a certain response. The diacylglycerol that is released from phosphatidylinositol 4,5-biphosphate remains within the cell membrane, where it can activate the enzyme C-kinase, a calcium- and phospholipid-dependent enzyme which also has a role in mediating cellular responses. Calcium ionophores are known to mimic the actions of inositol triphosphate (Berridge, 1985) by also releasing Ca\(^{++}\) from internal stores.
The addition of inositol triphosphate to abscess homogenates stimulated monoglyceride production and produced a time-dependent increase in the bactericidal activity, suggesting that a second messenger might be involved in the synthesis of the monoglyceride. The fact that neither arachidonic acid or phorbol myristate acetate, both of which are C-kinase activators (McPhail et al., 1984a and b; Nishizuka, 1984), could stimulate monoglyceride synthesis would suggest that C-kinase was not involved in monoglyceride production. The G-kinase, another enzyme that functions to bring about cellular responses (Berridge, 1985), also appears not to be involved in monoglyceride production, since cyclic GMP, a second messenger known to activate G-kinase, did not stimulate monoglyceride synthesis in AH. Merely increasing the extracellular calcium concentration by adding calcium directly to AH also could not stimulate the synthesis of the monoglyceride. This suggests that endogenous Ca++ released from intracellular stores is needed to activate the production of the bactericidal monoglyceride.

Why intracellular calcium suffices whereas extracellular calcium does not, is not understood, nor is it known how the release of intracellular calcium stores leads to monoglyceride synthesis in the abscesses. Since an intracellular calcium flux is known to activate phospholipase A2 to release arachidonic acid (Van den Bosch, 1980), it is possible that in the case of intracellular calcium stimulation of monoglyceride production lipases are activated which act on complex lipids or glycerides to release the monoglyceride. There is
even the possibility that it is the diacylglycerol released from phosphatidylinositol 4,5-biphosphate that is acted upon by a lipase. If the monoglyceride is the precursor of the SFA, the SFA is released spontaneously or by the action of other lipases.

It has still not been elucidated which cell type or types is/are involved in the synthesis of the SFA and the monoglyceride. The involvement of the macrophage has not been ruled out. Dvorak and others have described the presence of lipid bodies which occur with increased frequency in macrophages at sites of inflammatory, immunologic, or neoplastic processes (Dvorak et al., 1983). These macrophage cytoplasmic lipid bodies appear to represent a major site of intracellular storage and metabolism of products of arachidonic acid and perhaps other fatty acids that are taken up from the external environment. Studies by Schlager and Meltzer show that the activation of tumoricidal activity in the macrophage following lymphokine treatment is associated with a marked increase in the percentage of triglycerides and unsaturated fatty acids in the cells (Schlager and Meltzer, 1981). Whether the macrophage synthesizes the staphylocidal lipids or accumulates lipids from the extracellular environment which are then modified internally, and whether neutrophils, lymphocytes, or lymphokines are involved in the synthesis of the staphylocidal lipids, remains to be determined.
Figure 1. Bactericidal activity of lipids extracted from untreated abscess homogenates (AH) or from abscess homogenates treated with 10 μM calcium ionophore A23187 (iono.) for 20 min. Indicator strain is S. aureus 303.
Figure 2. Thin layer chromatography of lipids extracted from untreated or calcium ionophore A23187-treated abscess homogenates. Plate was developed in petroleum ether-ethyl ether-acetic acid (30:70:1, v/v/v) and charred. Arrow shows location of the "unidentified lipid".
Figure 3. Dose-response curves showing differential bactericidal activity of the monoglyceride. *S. aureus* strain 303 is SFA-sensitive (ED₅₀ = 0.6 µg) and *S. aureus* strain TG is SFA-resistant (ED₅₀ = 4.0 µg).
Figure 4. Bactericidal activity of the monoglyceride, which was maintained either under nitrogen or under oxygen for 48 hours. Indicator strain is *S. aureus* 303.
Figure 5. Thin layer chromatography of the monoglyceride before and after incubation with FAME. Plate was developed in petroleum ether-ethyl ether-acetic acid (40:60:1, v/v/v) and charred. Approximately 120 μg was spotted in each lane. (1) the monoglyceride, (2) the monoglyceride + FAME, (3) oleic acid, (4) ethyl oleate.
Figure 6. The increase in bactericidal activity of abscess homogenates following treatment with 10 μM inositol triphosphate for various periods at 37°C. Bactericidal activity is expressed as ED50/ml homogenate. Indicator strain is S. aureus 303.
<table>
<thead>
<tr>
<th></th>
<th>SFA</th>
<th>LCFA</th>
</tr>
</thead>
<tbody>
<tr>
<td>SPEC. ACTIV. (ED$_{50}$/mg)</td>
<td>(&gt;10^3)</td>
<td>(&lt;500)</td>
</tr>
<tr>
<td>DIFFERENTIAL ACTIV.</td>
<td>YES</td>
<td>NO</td>
</tr>
<tr>
<td>OXIDATION</td>
<td>DESTROYS ACTIV.</td>
<td>INCREASES ACTIV.</td>
</tr>
<tr>
<td>REDUCTION</td>
<td>NO EFFECT</td>
<td>REDUCES ACTIV.</td>
</tr>
<tr>
<td>pH (5.5-9.0)</td>
<td>UNIFORM ACTIV.</td>
<td>VARIABLE ACTIV.</td>
</tr>
<tr>
<td>ESTERIFICATION</td>
<td>DESTROYS ACTIV.</td>
<td>DESTROYS ACTIV.</td>
</tr>
<tr>
<td>Ca$^{++}$</td>
<td>INHIBITS</td>
<td>INHIBITS</td>
</tr>
<tr>
<td>PHOSPHOLIPIDS</td>
<td>INHIBITS</td>
<td>-</td>
</tr>
<tr>
<td>DELTA TOXIN</td>
<td>INHIBITS</td>
<td>-</td>
</tr>
<tr>
<td>IN NORMAL TISSUE</td>
<td>NO</td>
<td>YES</td>
</tr>
</tbody>
</table>


<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>LD50/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH</td>
<td>130</td>
</tr>
<tr>
<td>AH + 10 μM A23187</td>
<td>1260</td>
</tr>
<tr>
<td>AH + 1 mM Glutathione</td>
<td>140</td>
</tr>
<tr>
<td>AH + 100 μM Arachidonic Acid</td>
<td>140</td>
</tr>
<tr>
<td>AH + 10 μM A23187 + 1 mM Glutathione</td>
<td>1400</td>
</tr>
<tr>
<td>AH + 10 μM A23187 + 1 mM Glutathione + 100 μM Arachidonic Acid</td>
<td>1260</td>
</tr>
<tr>
<td>10 μM A23187 (control)</td>
<td>0</td>
</tr>
</tbody>
</table>

*B20 min incubation.*

TABLE 3
BACTERICIDAL ACTIVITY OF ABSCESS HOMOGENATES AFTER INCUBATION WITH CALCIUM IONOPHORE A23187

<table>
<thead>
<tr>
<th>TIME (min.)</th>
<th>LD50/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>30*</td>
</tr>
<tr>
<td>5</td>
<td>370</td>
</tr>
<tr>
<td>20</td>
<td>340</td>
</tr>
<tr>
<td>40</td>
<td>370</td>
</tr>
<tr>
<td>80</td>
<td>410</td>
</tr>
<tr>
<td>160</td>
<td>1010</td>
</tr>
<tr>
<td>320</td>
<td>1110</td>
</tr>
<tr>
<td>1280</td>
<td>220</td>
</tr>
</tbody>
</table>

*Prior to adding ionophore.
**TABLE 4**

**EFFECT OF VARIOUS INHIBITORS ON CIDAL ACTIVITY GENERATED BY CALCIUM IONOPHORE A23187**

<table>
<thead>
<tr>
<th>COMPOUND</th>
<th>% INHIBITION</th>
</tr>
</thead>
<tbody>
<tr>
<td>Indomethacin (100 μM)</td>
<td>0</td>
</tr>
<tr>
<td>Nordihydroguaiaretic Acid (100 μM)</td>
<td>0</td>
</tr>
<tr>
<td>Phenidone (100 μM, 1 mM)</td>
<td>0</td>
</tr>
<tr>
<td>Bromophenacyl Bromide (100 μM)</td>
<td>0</td>
</tr>
<tr>
<td>EDTA (1 mM)</td>
<td>0</td>
</tr>
</tbody>
</table>

*20 min incubation with inhibitor prior to addition of the ionophore.*
TABLE 5

BACTERICIDAL ACTIVITY OF LIPIDS EXTRACTED FROM SILICA GEL OF DEVELOPED* TLC PLATE

<table>
<thead>
<tr>
<th>LIPID</th>
<th>RF</th>
<th>STRAIN TG</th>
<th>STRAIN 303</th>
</tr>
</thead>
<tbody>
<tr>
<td>&quot;Unidentified Lipid&quot;</td>
<td>.15</td>
<td>270</td>
<td>1560</td>
</tr>
<tr>
<td>Pool of Other Spots (#2 - 7):</td>
<td></td>
<td>40</td>
<td>20</td>
</tr>
<tr>
<td>#2 (cholesterol)</td>
<td>.55</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#3 (diglycerides)</td>
<td>.63</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#4 (unidentified)</td>
<td>.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#5 (free fatty acids)</td>
<td>.80</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#6 (esters)</td>
<td>.87</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#7 (triglycerides)</td>
<td>.91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blank Area on TLC Plate</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Control)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Developed in petroleum ether-ethyl ether-acetic acid (30:70:1).
TABLE 6

EFFECT OF DELTA TOXIN ON THE BACTERICIDAL ACTIVITY OF THE MONOGLYCERIDE

<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>LD₅₀/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoglyceride</td>
<td>1850</td>
</tr>
<tr>
<td>Monoglyceride + Delta Toxin*</td>
<td>100</td>
</tr>
</tbody>
</table>

*Addition of 20 µg delta toxin per dilution of the monoglyceride. Incubation at 25°C for 30 min.

Hemolytic activity of toxin is 130 HD₅₀/mg.
## TABLE 7

**EFFECT OF LECITHIN (PHOSPHATIDYLCHOLINE) OR CALCIUM ON THE BACTERICIDAL ACTIVITY OF THE MONOLYCEERIDE**

<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>LD₅₀/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Monoglyceride</td>
<td>1850</td>
</tr>
<tr>
<td>Monoglyceride + Lecithin</td>
<td>110</td>
</tr>
<tr>
<td>*Monoglyceride</td>
<td>1400</td>
</tr>
<tr>
<td>*Monoglyceride + Ca⁺⁺</td>
<td>700</td>
</tr>
</tbody>
</table>

Addition of 20 µg of lecithin or 200 µg of Ca⁺⁺ per dilution of the monoglyceride. Incubation at 25°C for 30 min.

*Assayed in saline.
<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>LD₅₀/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH</td>
<td>1600</td>
</tr>
<tr>
<td>AH + 1 μM A23187</td>
<td>1900</td>
</tr>
<tr>
<td>AH + 10 μM A23187</td>
<td>3600</td>
</tr>
<tr>
<td>AH + 100 μM A23187</td>
<td>3100</td>
</tr>
<tr>
<td>10 μM A23187 (control)</td>
<td>0</td>
</tr>
<tr>
<td>100 μM A23187 (control)</td>
<td>0</td>
</tr>
<tr>
<td>AH + 10 μM Lasalocid</td>
<td>1600</td>
</tr>
<tr>
<td>AH + 100 μM Lasalocid</td>
<td>2500</td>
</tr>
<tr>
<td>10 μM Lasalocid (control)</td>
<td>0</td>
</tr>
<tr>
<td>100 μM Lasalocid (control)</td>
<td>0</td>
</tr>
<tr>
<td>AH + 10 μM Nonactin</td>
<td>1800</td>
</tr>
<tr>
<td>10 μM Nonactin (control)</td>
<td>0</td>
</tr>
<tr>
<td>AH + 10 μM Valinomycin</td>
<td>1900</td>
</tr>
<tr>
<td>10 μM Valinomycin (control)</td>
<td>0</td>
</tr>
<tr>
<td>AH + 10 μM Phorbol Myristate Acetate</td>
<td>1500</td>
</tr>
<tr>
<td>Phorbol Myristate Acetate (control)</td>
<td>0</td>
</tr>
</tbody>
</table>

*50 min incubation.
TABLE 9

EFFECT OF VERAPAMIL ON THE BACTERICIDAL ACTIVITY
GENERATED BY CALCIUM IONOPHORE A23187

<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>LD₅₀/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH</td>
<td>1600</td>
</tr>
<tr>
<td>AH + 10 µM A23187</td>
<td>3600</td>
</tr>
<tr>
<td>AH + 10 µM Verapamil + 10 µM A23187*</td>
<td>1900</td>
</tr>
<tr>
<td>10 µM Verapamil (control)</td>
<td>0</td>
</tr>
</tbody>
</table>

*15 min incubation with verapamil prior to additional 50 min incubation with A23187.
TABLE 10

EFFECT OF CALCIUM IONOPHORE A23187 ON ABSCESS HOMOGENATE FRACTIONS

<table>
<thead>
<tr>
<th>FRACTION</th>
<th>LD50/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>Supernatant</td>
<td>0</td>
</tr>
<tr>
<td>Sediment*</td>
<td>3100</td>
</tr>
<tr>
<td>Supernatant + Sediment*</td>
<td>3900</td>
</tr>
<tr>
<td>Supernatant + 10 μM A23187</td>
<td>0</td>
</tr>
<tr>
<td>Sediment* + 10 μM A23187</td>
<td>2600</td>
</tr>
<tr>
<td>Supernatant + Sediment* + 10 μM A23187</td>
<td>6800</td>
</tr>
</tbody>
</table>

*Washed three times with saline.

Incubation with A23187 was for 50 min.
<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>LD&lt;sub&gt;50&lt;/sub&gt;/mL</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH</td>
<td>60</td>
</tr>
<tr>
<td>AH + 10 µM A23187</td>
<td>540</td>
</tr>
<tr>
<td>Heated AH</td>
<td>&lt;10</td>
</tr>
<tr>
<td>Heated AH + 10 µM A23187</td>
<td>80</td>
</tr>
</tbody>
</table>

*100°C/30 min.

Incubation with A23187 was for 50 min.
TABLE 12
EFFECT OF CALCIUM IONOPHORE A23187 ON THE BACTERICIDAL ACTIVITY OF THE TOTAL LIPID FRACTION OR THE MONOGLYCERIDE EXTRACTED FROM ABSCESS HOMOGENATES (AH)

<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>LD$_{50}$/mg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total lipids</td>
<td>380</td>
</tr>
<tr>
<td>Total lipids + 10 µM A23187</td>
<td>370</td>
</tr>
<tr>
<td>Monoglyceride</td>
<td>1820</td>
</tr>
<tr>
<td>Monoglyceride + 10 µM A23187</td>
<td>2250</td>
</tr>
</tbody>
</table>

Incubation with A23187 was for 50 min.
**TABLE 13**

**BACTERICIDAL ACTIVITY OF ABSCESS HOMOGENATES (AH) INCUBATED* WITH PHOSPHOINOSITIDES, CYCLIC GMP, CALCIUM, OR CALCIUM IONOPHORE A23187**

<table>
<thead>
<tr>
<th>PREPARATION</th>
<th>LD50/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>AH</td>
<td>320</td>
</tr>
<tr>
<td>AH + 10 μM IP$_3^a$</td>
<td>1270</td>
</tr>
<tr>
<td>10 μM IP$_3$ (control)</td>
<td>0</td>
</tr>
<tr>
<td>AH + 10 μM PIP$_2^b$</td>
<td>280</td>
</tr>
<tr>
<td>10 μM PIP$_2$ (control)</td>
<td>0</td>
</tr>
<tr>
<td>AH + 10 μM Inositol</td>
<td>300</td>
</tr>
<tr>
<td>10 μM Inositol (control)</td>
<td>0</td>
</tr>
<tr>
<td>AH + 10 μM cGMP</td>
<td>310</td>
</tr>
<tr>
<td>10 μM cGMP (control)</td>
<td>0</td>
</tr>
<tr>
<td>AH + 10 mM Ca$^{++}$</td>
<td>340</td>
</tr>
<tr>
<td>10 mM Ca$^{++}$ (control)</td>
<td>0</td>
</tr>
<tr>
<td>AH + 100 μM A23187</td>
<td>1050</td>
</tr>
<tr>
<td>AH + 10 mM Ca$^{++}$ + 100 μM A23187</td>
<td>880</td>
</tr>
<tr>
<td>100 μM A23187 (control)</td>
<td>0</td>
</tr>
</tbody>
</table>

*50 min incubation.

$^a$Inositol 1,4,5-triphosphate

$^b$Phosphatidylinositol 4,5-biphosphate
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