STREPTOCOCCUS MUTANS 6715 WILD TYPE AND NON-PLAQUE FORMING MUTANTS: LIPID COMPOSITION AND EFFECTS OF EXOGENOUS FATTY ACIDS AND TRIGLYCERIDES

by

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S. MUTANS 6715 WILD TYPE AND NON-PLAQUE FORMING MUTANTS: LIPID COMPOSITION AND EFFECTS OF EXOGENOUS FATTY ACIDS AND TRIGLYCERIDES

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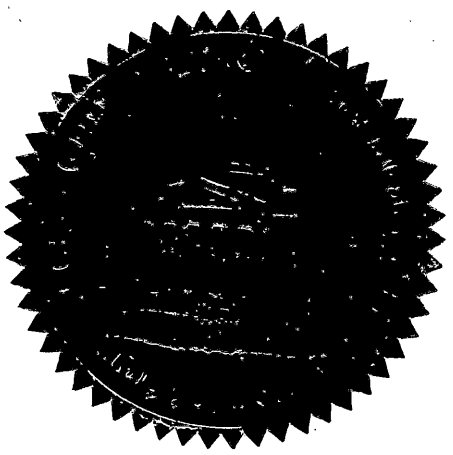

To Diana

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LIST OF ABBREVIATIONS

Abbreviations frequently used in the text include the following:

DGDG	Diglucosyl Diglyceride
DPG	Diphosphatidyl Glycerol
GM	Growth Medium
GTF	Glucosyltransferase
HDA	Heptane, Diisobutyl Ketone, Acetic Acid
LPC	Lysophophatidyl Choline
MBC	Minimal Bactericidal Concentration
MIC	Minimal Bacteriostatic Concentration
MGDG	Monoglucosyl Diglyceride
PA	Phosphatidic Acid
PC	Phosphatidyl Choline
PG	Phosphatidyl Glycerol
PIC	Plaque Inhibitory Concentration
SEB	Staphylococcus Enterotoxin B
T	Trace
TMS	Trimethylsilate
Tween 20	Polyoxyethylene Sorbitan Monolaurate
Tween 80	Polyoxyethylene Sorbitan Monooleate
WT	Wild Type

FATTY ACID NOMENCLATURE

Name	Carbon Number:Number of Double Bonds
Lauric Acid	12
Myristic Acid	14
Pentadecanoic Acid	15
Palmitic Acid	16
Hexadecenoic Acid	16:1
Heptadecanoic Acid	17
Stearic Acid	18
Octadecenoic Acid	18:1
Oleic Acid	18:1 cis-9
Vaccenic Acid	18:1 cis-11
Nonadecanoic Acid	19
Eicosanoic Acid	20
Eicosenoic Acid	20:1
Eicosadienoic Acid	20:2
Docosanoic Acid	22

GLOSSARY OF TERMS

CARIES - A localized, progressively destructive disease of the teeth that starts at the external surface with the apparent dissolution of the enamel by organic acids.

DENTAL CALCULUS - Dental plaque that has undergone mineralization of the matrix and microorganisms.

DENTAL PLAQUE - Microorganisms embedded in an extracellular matrix consisting of products of bacterial metabolism and substances from serum, saliva and diet.

DENTINE - The substance forming the mass of the tooth. About 20% is organic matrix, the inorganic fraction is mainly hydroxyapatite.

ENAMEL - The hard substance covering the exposed portion of the tooth. It is composed chiefly of hydroxyapatite, with an organic matrix comprising 0.5%.

MATERIA ALBA - An accumulation of microorganisms, dead exfoliated epithelial cells and food debris loosely adhereing to teeth.

PELLICLE - A film or scum on the surface of teeth.

SMOOTH SURFACE CARIES - Occurs on the non-biting surfaces of the teeth.

I. INTRODUCTION

A. Statement of the Problem

Dental caries is a multifactorial disease that can be found in human skulls dating back to 12,000 B.C. It is currently believed that dental plaque, a substance consisting of microorganisms and their byproducts, salivary debris and dietary components, is a precursor to dental caries and that caries is an infectious disease which requires a susceptible tooth, a diet conducive to caries formation, a cariogenic microbiota which excretes lactic acid, and time for these interactions to occur (White, 1975). It has been stated by Page (1974) that dental plaque is intimately related to the induction and progression of dental caries, and inflammatory gingival and periodontal disease. Previous studies have demonstrated that dietary lipids decrease caries activity in animals, and that some free fatty acids can reduce plaque formation *in vitro* without concomitant inhibition of microbial growth. The present study examines the effects of several free fatty acids and triglycerides on a cariogenic bacterium designated *Streptococcus mutans* 6715 and on plaque produced in the presence of this organism. It seeks to determine if there is a relationship between bacterial fatty acid content and plaque formation. By providing information on the effects of fatty acids on the chemical composition of the organism and on the formation of the bacterial component of dental

plaque, it is hoped that insight will be provided into the mechanisms of formation of this bacterial byproduct.

B. Review of the Literature

1. Theories of Caries Production

Dental caries is often considered to be a disease of modern society; however, Mummery in 1869; Von Lenhossek in 1919; and Leigh in 1935 demonstrated the presence of caries in human skulls from ancient times and the middle ages, as well as modern times (White, 1975). From these observations they concluded that dental caries was present in the human population of Europe between 12,000 and 3,000 B.C. (White, 1976). Mummery was further able to show that the amount of caries in the ancient population of Britain increased with the development of civilization beginning with the Neolithic Age (12,000 - 3,000 B.C.) and extending to the Iron Age (1,000 - 500 B.C.). The study by Leigh indicated that caries occurred in Egyptian populations during this period and that the disease was more prevalent among those eating refined diets.

Aristotle in 350 B.C. was possibly the first to notice the effect of host diet on caries (Guerini, 1969). He recognized that figs caused damage to the teeth and suspected that the effect was caused by their ability to adhere to the tooth, thus allowing "putrification" to take place on the tooth surface and cause decay. The effect of viscous, sweet foods on dental decay was again noted in the 14th century by Guy de Chaulic, the leading surgeon of his time, and by Pieter von Forest

in the 16th century (Guerini, 1969). At present there is a vast amount of information, both scientific and non-scientific which attests to the harmful effects of sugar containing foods on teeth. The scientific information on this subject is more fully reviewed by Gustafsson, et al. (1954); Grenby (1967); Newbrun (1967, 1974a, b); Makinen (1972); Nizel (1972); White (1975); and Bibby (1976). The offshoot of this scientific information can be observed in the increased interest and advertising for chewing gums and candies containing sucrose substitutes and the increased awareness by the consumer of the hidden sugar content of products.

Many theories have been put forth to explain the cause and progression of dental decay, ranging from the seventh century B.C. Assyrian legend that decay was caused by worms, a theory that was still supported in the 14th century A.D. by Guy de Chaulic to the chemo-parasitic theory of Miller in 1883 (White, 1975). Miller's theory has been modified only slightly over the years and is still considered to best explain the cause of tooth decay.

In 1683, van Leeuwenhoek provided the first clue that microorganisms might be involved in decay when he reported the presence of "animalicules" in scrapings from teeth (White, 1975). In 1819, Parmly revolted against the prevailing theories and proposed that caries began on the tooth surfaces where food putrified and eventually gained sufficient chemical power to dissolve the tooth (Newbrun, 1974). Support for Parmly's theory was supplied by Robertson in 1835 and by Regnart in 1838 (Newbrun, 1974). They experimented with dilute inorganic acids and were able to show that nitric and sulfuric acid "corrode" the

tooth's enamel and dentine. In 1847, Ficinus observed microorganisms in carious teeth which he termed "denticolae" (White, 1975). He was able to show that they grew in the enamel and destroyed the connection between the enamel prisms, and thus could penetrate to the dentin, where, he said, they caused decay. In the early 1860's Pasteur was able to show that microorganisms convert sugars to lactic acid by the process of fermentation. Emil Magitot in 1867 expanded this work by showing that acids produced during fermentation can cause the dissolution of tooth material (Newbrun, 1974). In 1867 Leber and Rottenstein regarded initial tooth decalcification as a chemical process (White, 1975). Once decalcification had occurred, they were able to show that the microorganism "Leptothrix buccalis" penetrated the dentinal tubule. Thus, by 1880 most of the elements required for production of dental caries had been described in the literature. In 1881 Milles and Underwood provided the final link by showing that an acid-producing micrococcus was present in carious dentin, a finding which led them to propose that decay in hard tooth structure was due to the action of acids and microorganisms (White, 1975). Miller in 1883 was able to extend the finding of Milles and Underwood and show: 1) that different foods mixed with saliva and incubated at 37^{o}C could decalcify the crown of a tooth; 2) several types of oral bacteria could produce enough acid to be of significance in dental caries; 3) lactic acid was a product of carbohydrate-saliva incubation mixtures and 4) several microorganisms of different types invaded carious dentine (White, 1975). From his work and that of the other investigators, Miller proposed his chemo-parasitic theory of dental decay. This stated that "dental decay

is a chemo-parasitic process consisting of two stages, decalcification or softening of the tissues and dissolution of the softened residue" (Newbrun, 1974). Miller also proposed that no single species of microorganism caused caries but rather that the process was mediated by oral microorganisms capable of acid production and protein digestion. Miller's theory was debated for the next 70 years until Orland showed in 1954 that germfree rats fed a highly cariogenic sucrose containing diet do not develop caries but that gnotobiotic rats on the same diet infected with combinations of enterococci and a proteolytic bacillus or an enterococcus and a pleomorphic bacterium do develop caries, thus demonstrating that caries is caused by bacteria and is an infectious process. Orland's work has since been confirmed by several independent studies, and the transmissability of cariogenic organisms has been shown by others (Fitzgerald and Keyes, 1960; Keyes, 1960; and Orland et al., 1954, 1955). Thus dental caries today is thought of as an infectious disease which demands a susceptible tooth, a diet conducive to caries formation, a cariogenic microbiota which excretes lactic acid and time for these factors to interact (White, 1975).

2. Dental Plaque

Soft deposits that tightly adhere to teeth have been recognized since 1683 when van Leeuwenhoek first used his microscope to examine tooth scrapings (White, 1975). These microbial deposits have been called by different terms by several authors: Buhlmann's fibers (Buhlmann, 1840); slime coating of denticolae (Ficinus, 1847); coating of leptothrix (Leber and Rottenstein, 1867); feltlike moss

(Williams, 1897); gelatinous plaque (Black, 1898) and several others (Newbrun, 1974; White, 1975). Today they are commonly referred to as dental plaque. In 1883 when Miller proposed his chemo-parasitic theory of dental caries it was thought that caries initiated in naturally retentive areas of teeth. This mechanism did not adequately explain smooth surface caries and thus Williams in 1897 proposed that smooth dental caries was caused by microorganisms enclosed in a gelatin-like substance on smooth enamel (White, 1975). Black in 1898 expanded this view by proposing that the gelatinous microbial plaque was formed by the action of a fungus acting on sucrose to produce an adhesive substance that held the organisms in place (White, 1975).

It is now known that plaque is a specific but variable structure resulting from colonization and growth of microorganisms on the surface of teeth, soft tissues and oral appliances (Page, 1974). It can be distinguished from the other dental deposits; acquired pellicle, materia alba, dental calculus and food debris by its structure and morphology. Dental plaque is an organized community of several species and strains of microorganisms of which Gram positive cocci are most common (Socransky and Manganeiello, 1971; Loesche, 1972). These microorganisms are embedded in an extracellular matrix of products of bacterial metabolism and substances from serum, saliva and diet. The extracellular matrix has been shown to alter the diffusion of substances in and out of the matrix, to serve as a storage site for fermentable carbohydrate, and to contain numerous inflammation-inducing and toxic substances (Page, 1974).

The characteristics of acquired pellicle, materia alba, dental calculus, food debris and other common dental deposits follow. Acquired pellicle is a structureless glycoprotein film, usually less than 1 μm thick and free of bacteria. This film is thought to be of salivary origin and it coats the tooth surface within minutes after a thorough mechanical cleaning. Materia alba is composed of microorganisms, dead exfoliated epithelial cells and food debris. It has little organization and loosely adheres to the teeth. Materia alba is an accumulation of the substance rather than bacterial growth. Dental calculus is plaque that has undergone mineralization of the matrix and microorganisms; however, its exposed surface is usually covered with viable organisms. Food debris are the residual food particles that remain in the mouth after eating.

The supragingival formation of plaque is thought to occur in two major steps: 1) the bacterial colonization of the tooth surface and 2) bacterial growth and maturation within the plaque matrix. The first step involves three general interactions. First the acquired pellicle is formed by a process of selective absorption of salivary constituents to the tooth surface. There are three components to this acquired pellicle based on their location. The first is a subsurface pellicle which extends 1-3 microns into defects in the enamel, next is surface pellicle and this covers the tooth and is approximately 0.2 microns thick. The final component is stained pellicle. This layer is 1 to 10 microns thick (Newbrun, 1975). The exact role of the acquired pellicle in colonization is not clear. In most cases the

pellicle enhances the aggregation of plaque forming bacteria and provides some components as substrates for these bacteria; however, there are instances in which colonization does not occur after the pellicle is deposited (Newbrun, 1975) and still other cases where colonization occurs without pellicle being deposited (Fitzgerald, 1968).

The second step in plaque formation is the absorption of oral bacteria to the acquired pellicle. This process is very rapid and highly selective as shown by the different efficiencies with which strains of bacteria adhere to the pellicle. Once this has occurred, the bacteria that have adhered to the pellicle can form distinct colonies. The outgrowth of these colonies, accumulation of salivary constituents, and extracellular bacterial polymers leads to the formation of the plaque matrix and thus to the third stage of colonization, the interbacterial interaction which serves to bind one organism to another to form a cohesive mass on the tooth surface (White, 1975).

This interaction can occur directly between organisms or it can occur by organisms being held in close proximity to each other by the matrix. The progression of this third stage leads to the second major area of plaque formation, bacterial growth and maturation within the plaque matrix. The plaque matrix contains substances from the saliva, products released by the bacteria, food debris and the microorganisms. It serves as a framework for the microorganisms, as a storage site for fermentable carbohydrate, it alters diffusion in and out of the structure and it contains inflammatory and toxic substances (Newbrun, 1975). The maturation of plaque brings about a change in its microbiology, thus early plaque contains mostly Gram positive cocci, short rods,

Neisseria and Nocardia. By the fourth day after initiation of plaque formation on a clean tooth, the number of cocci have decreased and organisms of the genera Leptothrix and Fusobacterium have increased. By day 10, vibrios and spirochetes can be detected and there is a general rise in the Gram negative population. The increase in the Gram negative population should not be taken to mean that the streptococci have lost their importance, since they still comprise well over 1/3 of the organisms present and thus form the largest single group present: S. mutans is a major constituent of this group.

Thus, dental caries is best thought of as a disease that can be associated with but not directly caused by plaque. The plaque provides an environment in which teeth and microorganism are held in close contact. The normal metabolism of the bacteria, especially S. mutans and species of Lactobacillus, produce organic acids as end products of carbohydrate metabolism. These organic acids, especially lactic acid, are strong enough to dissolve the tooth enamel if contact is maintained for a sufficient amount of time. The plaque matrix with its altered diffusion properties favors the retention of the acid. The breakdown of the enamel is a slow process and begins with only a small lesion in it. This lesion is then gradually enlarged until it eventually reaches the tooth dentine. Once the breakdown of enamel begins, the tooth is considered to have a carious lesion. With the exposure of the dentine, bacteria can invade more rapidly, eventually destroying the dentine. When the cariogenic process reaches this stage, the tooth is subjected to attack from both inside and out. These cariogenic lesions, if allowed to continue, will eventually destroy the tooth.

In summary, it is currently believed that dental plaque is a precursor to dental caries and that caries is an infectious disease which requires a susceptible tooth, a diet conducive to caries formation, a cariogenic microbiota and time for these interactions to occur.

3. Role of Streptococci in Caries

Streptococci are the most abundant oral bacteria (Drucker, 1971) and in 1924 Clarke was able to isolate a streptococcus from carious dentine. Clarke named this organism Streptococcus mutans because of its varying morphology. A description of this S. mutans appears in the 6th edition of Bergey's Manual (Breed et al., 1948) but was dropped from the 7th (Breed et al., 1957) and 8th editions (Buchanan and Gibbons, 1974). There has always been an interest in the role of streptococci in dental caries and the investigation into their role has greatly increased since Orland et al. (1955) demonstrated the role of streptococci in dental caries. The classification of oral streptococci has always been somewhat unsatisfactory because they do not easily fit into the four classical divisions of streptococci; pyogenic, viridans, enterococcal and the lactic groups. Because of this, they usually have been placed with the viridans group for lack of a better place. In 1960, Bisset and Davis emphasized the need for a method of classifying oral streptococci when they stated, "the oral flora contains many unclassifiable strains of ... streptococci". This need was also emphasized by Guggenheim in 1968. In an effort to better classify the oral streptococci, Carlsson (1968) proposed grouping cariogenic strains solely on bacteriological characteristics, and Drucker and Melville (1969, 1971)

proposed an Adamsonian classification (numerical analysis of phenotypic characteristics) of the oral streptococci using 27 characters. Drucker and Melville (1969, 1971) examined 252 strains of streptococci of oral origin. This group contained both cariogenic and non-cariogenic organisms. Their findings indicated that there were three well defined species which corresponded to organisms previously classified as S. mitis, S. salivarius, and S. sanguis.

A fourth species termed S. mutans by Edwardsson (1968) and Guggenheim (1968) was shown to be distinct from the other three. S. mutans are those streptococci expressing the following phenotypic characteristics: 1) the ability to ferment mannitol and sorbitol; 2) synthesis of an insoluble polysaccharide from sucrose; and 3) homofermentive lactic acid production (Newbrun, 1974). In 1970 Bratthall described five serotypes (a,b,c,d,e) of S. mutans. These serotypes did not correspond to any members of the Lancefield's serotypic groups. In 1974 Perch et al. confirmed Bratthall's findings and proposed two additional serotypes (f and g).

The S. mutans that comprise these seven serotypes are quite diverse at the molecular level. Thus members of any one serotype may contain differences in any of several enzymes (aldolases, invertases, glucosyltransferases), cell wall carbohydrates, a few biochemical reactions, fatty acid content and even morphology (Coykendall, 1976). Lambert and Moss (1976) determined the fatty acid content of 18 different strains of S. mutans that represent serotypes a,b,c,d,e and two that were not typed. They also determined the fatty acid content of five cultures of S. salivarius, five cultures of S. sanguis, three cultures of S. uberis,

two cultures each of S. mitis and unspecified Streptococcus species. Their data indicate that the fatty acid content of the bacteria divided the organisms studied into two distinct groups. The first group included S. mutans and S. salivarius; and the other group, S. sanguis, S. mitis, S. uberis and a Lancefield group C streptococcus. Lancefield group E streptococci did not fit either group. Thus, while they were able to show that the fatty acid content of S. mutans strains were similar to each other, they could not show that they were distinct from all other oral streptococci.

The guanine plus cytosine (G+C) ratio of the organisms classified as S. mutans is quite diverse ranging from 36 to 46%. When the S. mutans group is separated by G+C ratio, five distinct subspecies are present. However, only three of these subspecies contain organisms from a single serotype (Coykendall, 1976).

The phenotypic evidence derived since Clarke first reported the existence of S. mutans in 1924 seems to warrant the classification of S. mutans as a separate group in the genus Streptococcus. The classification of the individual strains of S. mutans into subspecies is more of a problem because of the great diversity within this group of organisms. Lambert and Moss (1976) sum up this dilemma by suggesting that the work of Bratthall (1970) (serotypes) and Coykendall (1976) (base composition) indicate that there are four major subspecies of S. mutans and that this separation is helpful to those interested in the genetic, metabolic, serological and epidemiology of S. mutans, while those interested in taxonomy or clinical aspects of S. mutans cannot justify this subdivision and favor the numerical classification of Carlsson (1968)

and Drucker and Melville (1969, 1971) which indicate S. mutans is a very homogeneous group.

As previously stated, Clarke isolated a streptococcus from carious dentine in 1924 which he termed Streptococcus mutans. This organism was not adequately described and interest in its role in dental caries dropped. In 1955, Orland et al. were able to demonstrate that caries in gnotobiotic rats could be initiated by an enterococcus combined with either a proteolytic bacterium or a pleomorphic bacterium. Orland and co-workers (1959) were later able to produce caries in similar rats using only an enterococcus. In 1960, Fitzgerald et al. were able to produce carious lesions in gnotobiotic rats using a strain of oral streptococcus. The organism they used was not an enterococcus or any currently (1960) recognized species of streptococcus (S. mutans had been dropped from the 1957 edition of Bergey's Manual). This streptococcus was also antigenically distinct from all of Lancefield's groups. The organism they used produced carious lesions in the rats' teeth that were very similar to those lesions observed in normal laboratory rats fed a cariogenic diet. Biochemical studies of this cariogenic organism indicated that it was acidogenic and homofermentive and thus was similar, if not identical, to the organism isolated by Clarke in 1924. Because of these similarities, this organism was called Streptococcus mutans. Since these initial observations others (Fitzgerald and Keyes, 1960; Fitzgerald and Fitzgerald, 1965; and Bowen, 1966) have been able to produce carious lesions in animals using S. mutans. Epidemiological studies on humans have indicated S. mutans occurs more frequently and in higher relative numbers in mouths with high caries

levels of carious sites within the mouth (Hardie and Bowden, 1976). These studies provide evidence for the strong association between S. mutans and dental caries. Other oral streptococci; S. faecalis, S. sanguis, S. salivarius and streptocci not identified as to species, have also been shown to produce caries in rats and hamsters maintained on cariogenic diets (Burnett et al., 1976). These organisms produce caries less frequently and less extensively in animals than do S. mutans.

The greater pathogenic potential of S. mutans has been attributed to its ability to produce high molecular weight extracellular dextrans that allow it to adhere tightly to the enamel on tooth surfaces (van Houte et al., 1971) and to its ability to produce large amounts of lactic acid which can demineralize the tooth and thus lead to decay (Charlton et al., 1971a, b). S. sanguis and S. salivarius are also acidogenic producing terminal pH's similar to S. mutans (pH 4.1). These organisms can also produce extracellular glucans (dextran or levan) but these are much less adherent than that produced by S. mutans and is a major reason for their decreased cariogenic potential (Burnett et al., 1976).

The importance of adherence to the cariogenic potential of S. mutans has been demonstrated by Michalek et al., (1975). These investigators were able to show that a mutant of S. mutans 6715 that exhibited approximately 87% less glucosyltransferase activity and 90% less adherence in vitro than the parental strain, produced significantly fewer smooth surface lesions in mono infected gnotobiotic rats than wild type 6715. Results analogous to these were obtained using mutants of S. mutans PS14

with similar characteristics. They were further able to demonstrate that other mutants of wild type 6715 that had greater glucosyltransferase activity and increased *in vitro* adherence caused more smooth surface carious lesions in mono infected gnotobiotic rats than the parental 6715. All of the mutants used in these experiments were identical to the parental strain with respect to streptomycin resistance, fermentation of mannitol and sorbitol and serotype. These findings demonstrate that a correlation exists between *S. mutans* pathogenesis in gnotobiotic rats, adherence and insoluble glucan synthesis.

The adherence of *S. mutans* to a smooth surface and the concurrent production of extracellular glucans serves as a base for formation and subsequent enlargement of the plaque matrix. The plaque matrix consists of saliva components, products released by bacteria, food debris and microorganisms. The plaque matrix provides an environment in which the microorganisms and teeth are held in close proximity to each other. This situation allows the organic acids produced by organisms such as *S. mutans* to remain in contact for sufficient time to dissolve the tooth enamel, expose the dentine thus providing the bacteria an opportunity to attack the dentine and destroy it.

In summary, *S. mutans* possess several characteristics that give it enhanced cariogenic potential. This cariogenic potential is manifested as *S. mutans'* ability to adhere to smooth surfaces better than other oral streptococci, to produce large amounts of lactic acid and to produce extracellular glucans which contribute to plaque formation.

4. Relation of Dietary Lipids to Caries

In 1935 Rosebury and Karshan reported that the prevalence of fissure caries in rats being fed a ground rice diet was significantly reduced when corn oil was added to their diet. They expanded the original work in 1939 and were able to show that paraffin oil, olive oil, Wesson oil, Crisco and lard are as effective in reducing caries in the rat as corn oil. From their results, they postulated that the effects of the corn oil and the other oils were due to their fat content rather than any specific ingredient or characteristic and that the effect of these substances on caries is exerted by free fat. Rosebury and Karshan (1939) proposed that the oils reduced caries by interfering with the impaction of food or by coating the tooth and food particle with an oily film that protected the tooth enamel against the acid products of fermentation or by preventing oral or bacterial enzymes from attracting the particles. Box (1940) was able to add credibility to part of the theory proposed by Rosebury and Karshan by showing that teeth suspended in dilute acid solutions and buffered at pH 5 were protected against decalcification by the addition of a small amount of sodium oleate to the acid solution. He was also able to demonstrate that teeth coated with a fine film of oleic acid were protected from decalcification when placed in dilute acid solutions.

By 1959 several reports had appeared in the literature showing the reduction of caries in animals by fats and oils: these included studies in various types of rats (Schweigert et al., 1946; Shaw, 1950; Wynn et al., 1953; Constant et al., 1954; McClure et al., 1956; Bavetta, 1959) and hamsters (Granados et al., 1949; Gustafson et al., 1953,

1955). The data obtained in several of these studies (Schweigert et al., 1946; Granados et al., 1949; Shaw, 1950; Bavetta, 1959) was unintentionally biased by the fact that the added fat or oil was substituted for an equal amount of carbohydrate, usually sucrose, which is now known to be highly cariogenic. These studies did show; however, that certain fats and oils can reduce caries, although probably not to the degree indicated, and that the amount or lack of unsaturation does not seem to be related to caries-inhibition (Gustafson et al., 1953; McClure et al., 1956). These authors also agreed that free fats or oils worked better than those found in food and that these fats and oils exerted their effect by preventing or lessening the adherence of food to the tooth and/or by protecting the tooth enamel from acid attack caused by fermentation of food in the mouth. It should be noted that none of the authors proposed that the fats and oils might reduce caries by affecting the bacterial flora of the mouth.

5. Bacteria and Lipids

Research examining the relationships between bacteria and fatty acids can be divided into four major areas: 1) the effect of fatty acids on the growth of bacteria and bacterial products; 2) the use of fatty acid content to aid in identifying bacteria; 3) the effect of environmental changes on fatty acid content and the manipulation of fatty acid content for membrane studies and 4) fatty acid synthesis and degradation.

a. Effects of Fatty Acids on Bacterial Growth and Products: The effect of fatty acids on bacteria was reported by Strong and Carpenter

(1942) who observed that low levels of linoleic acid stimulated the growth of Lactobacillus helveticus but that high concentrations inhibited growth. Kodicek and Worden (1945) expanded the findings of Strong and Carpenter to show that oleic, linoleic, and linolenic acids, or their sodium salts, inhibited the growth of L. helveticus and that stearic and palmitic acids stimulated growth. They were also able to show that linoleic acid exerted an inhibitory effect on the following Gram positive organisms: Streptococcus agalactiae, Staphylococcus albus, Bacillis anthracis, Listeria monocytogenes and Erysipelothrix rhusiopathiae. No inhibitory effect could be demonstrated on the Gram negative bacteria Escherichia coli or Proteus vulgaris. In 1959, Stephan reported the inhibition of 25 strains of Lactobacillus, and strains of Streptococcus and Staphylococcus, all of oral origin, and of mixed oral flora from plaque and saliva by alkaline salts of oleic and linoleic acids. These bacteria were also inhibited by straight chain saturated fatty acids between six and twelve carbons in length.

In 1954, Neiman reported the inhibition by long chain fatty acids of several Gram positive organisms; among them pneumococci, streptococci, staphylococci, lactobacilli, corynebacteria, listeria, bacilli, clostridia and mycobacteria and the Gram negative organisms, E. coli (some strains) and Haemophilus. He concluded from this that generally only Gram positive bacteria are susceptible to the action of fatty acids in small amounts although influences on Gram negatives have been observed.

Kodicek (1956, 1958) summarized the work on the effects of fatty acids on bacteria and listed these general conclusions: The greater the

inhibition, the more unsaturated the fatty acid; cis unsaturated fatty acids are more effective than their trans isomers; esterification of unsaturated fatty acids destroys their inhibitory ability; low concentrations are bacteriostatic while high concentrations cause lysis.

In a more recent work, Kabara et al. (1972) have investigated the effect of 15 fatty acids ranging from C-6 to C-20:4 on 12 Gram positive organisms and 8 Gram negative organisms. Their data indicated that lauric acid was the most inhibitory saturated fatty acid against a variety of organisms, while linoleic was the most inhibitory unsaturated fatty acid. Oleic acid was shown to be inhibitory toward group A streptococci. None of the fatty acids tested were effective against the Gram negative organisms (Proteus species, E. coli, Seratia marcescens, Klebsiella, Pseudomonas aeruginosa and Salmonella typhimurium).

Kabara et al. (1977) were further able to demonstrate the dodecanoyl monoglyceride was more active against a similar spectrum of Gram positive organisms than was lauric acid. Gram negative bacteria were not affected. Butcher et al. (1976) using Staphylococcus aureus reconfirmed Kodicek's analysis and McChesney et al. (1977) have obtained results similar to Kabara et al. (1972) with Streptococcus mutans 6715. Miller et al. (1977) have reported that saturated fatty acids up to palmitic and unsaturated fatty acids between C-16 and C-20 inhibit the growth of Neisseria gonorrhoeae.

In summary, fatty acids have been shown to inhibit a large variety of Gram positive organisms and a few Gram negative types (Neisseria, Haemophilus, some strains of E. coli and Pasteurella pestis). The

effectiveness of inhibition by a fatty acid appears to depend upon its chain length; degree of unsaturation; the isomeric form of the double bond; and whether the fatty acid is free or esterified (Kodicek, 1956, 1958), the former being more inhibitory.

In 1945 Kodicek and Worden postulated that unsaturated fatty acids might exert their effect on bacteria in either of two ways. The first involved a direct chemical action upon the metabolism of the bacteria or upon the availability of some metabolite present in the medium. They had no evidence for this mechanism. The second explanation, based on the finding of Adam (1941) that the monolayer formed by unsaturated fatty acids is different from that formed by saturated fatty acids, postulated a physicochemical mechanism in which the unsaturated fatty acids formed a monolayer around the bacteria and changed the permeability of the adjacent surfaces, exerted some chemical influence or altered the surface tension, thus interfering with bacterial division.

In 1972 Singer and Nicolson postulated that unsaturated fatty acids may affect bacteria by becoming incorporated into the membrane during its synthesis and thus altering its 'fluidity'. Kabara and co-workers (1972) who had just completed surveying the effects of 15 fatty acids on a variety of Gram positive and Gram negative bacteria stated that the mode of action of the fatty acids cannot be wholly explained in physicochemical terms, i.e. surface-tension activity, and that the explanation probably resided in a more complex mechanism. They suggested that interrelationships between fatty acid and carbohydrate metabolism might offer an explanation. Sheu et al. (1972, 1973) and Freese et al. (1973) were able to demonstrate that the inhibition of E. coli and B.

subtilis by fatty acids was caused by the uncoupling of substrate transport and oxidative phosphorylation from the electron transport system. Miller et al. (1977) have concluded that the uncoupling of oxidative phosphorylation and the blocking of electron transport or both are major mechanisms by which fatty acids inhibit N. gonorrhoeae. They were not able to rule out the possible effect of the fatty acids on membrane fluidity.

Butcher et al. (1976) proposed that the growth inhibition of Staph. aureus by externally applied unsaturated fatty acids might occur because these fatty acids were being incorporated into the membrane, upsetting those systems that are sensitive to membrane fluidity. They further postulated that the unsaturated fatty acids may act in a manner similar to certain membrane fusing agents such as lysolecithin (Lucy, 1970).

Altenbern (1977) also working with Staph. aureus, was able to show that the production of Staphylococcus enterotoxin B (SEB) was increased when the organism was grown in the presence of saturated fatty acids at pH 8 and decreased when grown in the presence of unsaturated fatty acids. To explain these findings, Altenbern postulated that if an optimal ratio of saturated to unsaturated fatty acids of membrane lipids promoted maximum SEB formation, then it might be expected that higher than optimal ratios would show increased suppression of the enterotoxin production. In an attempt to validate this hypothesis, the Staph. aureus was grown in the presence of 2-adamantanone, a compound known to increase the fluidity of Pseudomonas phaseolicola membranes. The study showed that S. aureus grown in the presence of this compound imitates the effect of unsaturated fatty acids on both growth and SEB formation. The concept

of altered control of SEB secretion by fatty acid composition and membrane fluidity is being investigated (Altenbern, 1977).

Initial studies by McChesney et al. (1977) on the effect of lauric, oleic and linoleic acid on S. mutans 6715 have indicated that oleic and linoleic acid cause an increase in the total unsaturated fatty acid content of this bacterium and that all three of the fatty acids reduce the amount of dental plaque formed in vitro. These studies have also indicated that the amount of total extracellular glucosyltransferase is increased when S. mutans 6715 is grown in either the presence of lauric or linoleic acid. Oleic acid was not tested. They have also been able to show that non-plaque forming mutants of 6715 have an increased percentage of unsaturated fatty acids and in this respect are similar to S. mutans 6715 grown in the presence of either oleic or linoleic acid. They postulated that the decrease in dental plaque formation observed when S. mutans 6715 is grown in the presence of fatty acids may result from an alteration in the membrane due to the incorporation of the fatty acids thus changing the ratio of saturated to unsaturated fatty acids, resulting in an alteration in the membrane's ability to control or regulate the glucosyltransferases that are released or associated with the cell surface. A decrease in cell bound glucosyltransferase has been shown by Spinell and Gibbons (1974) to decrease the amount of glucan, a major component of dental plaque, to which the S. mutans cells can bind.

An increase in the amount of extracellular glucosyltransferase (GTF) activity present has also been shown to occur when S. mutans OMZ 176 or S. salivarius is grown in the presence of Tween 80 (poly-

ethylene glycol sorbitan monooleate) (Umesaki et al., 1977 and Wittenberger et al., 1978). Both groups proposed that the increase in extracellular GTF activity was due to either a direct or indirect effect on enzyme synthesis or indirectly by promoting secretion of the enzyme. The extracellular GTF activity of S. mutans 6715 has also been shown to be stimulated by lysophosphatidyl choline (LPC) but not by lauric acid (Harlander and Schachtele, 1978). Their last finding differs from that of McChesney et al. (1977). This difference may be related to the medium used. Preliminary studies by McChesney and Bulkacz (unpublished data) have indicated that medium composition can alter the response of GTF to fatty acid stimulation. Harlander and Schachtele (1978) propose that the increased extracellular GTF activity results from a stabilization of the GTF enzyme by LPC, and that LPC could associate with the enzyme either during or after exretion from the S. mutans cell.

In summary, bacterial growth and some bacterial properties are known to be altered by fatty acids but how they exert their effect is largely unknown.

b. Use of Fatty Acids in Bacterial Taxonomy: The use of gas-liquid chromatography as an aid in the chemotaxonomy was proposed by Abel et al. in 1963 from their studies of enterobacteria. In 1964, Kates was able to show a correlation between bacterial family and lipid composition for Enterobacterioceae, Actobacteriaceae, Rhizobiaceae, Pseudomonadaceae, Lactobacillaceae, Bacillaceae, Micrococcaceae, Corynebacteriaceae, and Mycobacteriaceae. Since these initial studies, several investigators have examined the fatty acid content of a variety of organisms including streptococci (Drucker, 1972); Bacillus (Kaneda, 1967);

Clostridium (Moss and Lewis, 1967); Vibrio (Brian and Gardner, 1968); Neisseriae and Moroxella (Jantzen et al., 1974); Corynebacterium (Moss and Cherry, 1968); Listeria (Rainer et al., 1968); Pseudomonas (Moss and Dees, 1976) and Peptococcus and Peptostreptococcus (Moss et al., 1977).

Four groups, Drucker et al. (1973, 1974); Sharma and Newbrun (1975); Lambert and Moss (1976): and Szabo et al. (1978) have published data pertinent to this dissertation on the fatty acid composition of various strains of S. mutans including 6715. The data of Szabo et al. (1978) and Lambert and Moss (1976) agrees closely with each other and differ from the others in that no polyunsaturated or branched chain fatty acids were present. Preliminary data on the fatty acid content of WT. 6715 presented by McChesney et al. (1977) agrees with that of Szabo et al. (1978) and Lambert and Moss (1976). The major fatty acids present in all cases were palmitic, octadecenoic and eicosenoic. The relationship between S. mutans fatty acid content and the division of S. mutans into four subspecies has been discussed in more detail elsewhere. The relationship between lipid composition and the classification of bacteria is perhaps best summed up by Shaw's (1974) statement that, "assuming no previous knowledge of bacterial classification, except perhpas the Gram stain, the classification of bacteria solely on lipid composition would ... produce a scheme ... very similar to the one presently accepted."

c. Effects of Environment on Bacterial Fatty Acids: The third area examines the effect of environmental changes on the fatty acid content of bacteria and its manipulation for membrane studies. The work in

this area can be divided into sections: in the first section, the effects of different environmental conditions on the organism have been studied. Before looking more closely at some of these changes, it should be stated that virtually all of the lipids in Gram positive bacteria are associated with the cytoplasmic membrane or its appendages. Gram negative bacteria follow the same pattern as Gram positive bacteria; however, they also contain lipids outside the cell membrane in the cell wall structure. A lipid moiety is also associated with cell wall protein and polysaccharide. Neither class of bacteria store lipids in the form of triglycerides (fats) to any great extent and those bacteria that do store lipid, store it as poly-β-hydroxybutyate.

The most widely studied environmental condition has been the effects of temperature on fatty acid content (Farrell and Rose, 1967). However, medium composition (Farshtchi and McClung, 1970), age of the culture growth phase (Kates et al., 1964), and pH (Drucker et al., 1975) have also been studied. All of these parameters have been shown to alter the fatty acid content of organisms (Davies et al., 1970). The changes that these environmental alterations cause are consistent for all organisms studied, although, the particular changes are different from organism to organism. Thus, when the normal growth temperature of an organism is lowered, the amount of unsaturated fatty acids present is increased. At higher than normal temperatures, the amount of unsaturated fatty acids is decreased and the saturated fatty acids increased (Farrell and Rose, 1967). This difference in fatty acid content is also seen when organisms that normally grow at elevated temperatures, and thus have more saturated fatty acids are compared to organisms that

normally grow at low temperatures, and thus have increased amounts of unsaturated fatty acids.

The effect of medium composition on fatty acid content has been studied by several investigators, among them are Marr and Ingraham (1962) (*E. coli*); Farshtchi and McClung (1970) (*N. asteroides*); Drucker et al (1974) (*S. mutans*); and Dharwal et al. (1977) (*Mycobacterium phlei*). They were all able to show that changes in medium composition caused a change in the fatty acid profile; however, changes that occurred in the four organisms were not similar. The changes in fatty acid composition caused by a change in medium composition seen to depend on two things. First, the organism involved and second, the actual nutritional change (i.e., limiting N_2 will have a different effect than limiting the carbon source) (Marr and Ingraham, 1962).

The effect of pH on bacterial fatty acid composition has been studied by Drucker et al. (1975). He was able to show that if the environmental pH was held constant and the organism (*S. mutans* 10832) allowed to grow, then a definite difference in fatty acid content could be observed for the various pH's. They concluded from this study that for *S. mutans*, variation in pH is not an important factor as long as it is grown on a fermentable carbohydrate because the organism lowers its environmental pH with its normal production of lactic acid. This study indicates that while pH does effect fatty acid content of a bacterium, the extent and type of effect is likely to depend largely upon the bacterium and its environment.

The age of bacterial cultures (growth phase) has been shown to effect the fatty acid content of bacteria by Marr and Ingraham (1962);

Law et al. (1963) and Szabo et al. (1978). The change that occurred in all cases was a decrease in the mono-unsaturated fatty acids and an increase in the appearance of cyclopropane fatty acids. Davis et al. (1970) postulated that this change may "toughen" the membrane thus increasing the chance of cell survival.

The manipulation of the fatty acid content of Acheoleplasma laidlawii B, and E. coli either by growing them in the presence of an exogenous fatty acid or by using a fatty acid auxotrophic mutant has enabled several investigators to study the composition and functioning of bacterial membranes and has added insight into the nature of eucaryotic membranes. Reviews of this area of work have been provided by Salton (1971); Razin (1975); Cronan and Gelmann (1975) and the works of Silvert et al. (1968); Rodwell (1971); Chapman and Urbina (1971); Esfahani (1971); Rosen and Hackette (1972); Bayer et al. (1977) and Saito and McElhany (1977) are representative of the current research in the field, and all have extensive bioliographies that provide an excellent starting point for further investigation of this area. Work conducted in this area by Rodwell (1971) and Saito and McElhany (1977) enabled us to postulate that S. mutans 6715 grown in the presence of exogenous fatty acids could incorporate them into their lipids.

d. Fatty Acid Synthesis and Degradation: The final area in which the association of fatty acids and bacteria have been examined is the determination of the enzymes and pathways involved in the synthesis and degradation of fatty acids. The work in this area is not directly related to the main thrust of this dissertation and thus will not be

covered in this review.

The information provided here reviews the literature pertinent to understanding the interrelationships between dental caries, dental plaque, oral microorganisms and lipids. It is my hope that the information provided will allow those unfamiliar with this area to better understand the problem presented by dental caries and the importance of determining the effects of substances that decrease the formation of an important precursor of dental caries; dental plaque.

In summary, it has been shown that dental caries, oral microorganisms and their products are related. Furthermore, dental plaque, a substance consisting of microorganisms, their byproducts, salivary debris and dietary components is a precursor of dental caries and in particular S. mutans and its products appear to be most intimately related to caries production. Studies have demonstrated that dietary lipids decrease caries activity in animals and that some free fatty acids reduce plaque formation in vitro without concomitant inhibition of microbial growth. The data that will be presented in this dissertation seeks to determine if there is a relationship between the fatty acid content of S. mutans and its ability to form plaque.

II. MATERIALS AND METHODS

A. Propagation of Streptococcus Mutans 6715 and Mutants of 6715

Streptococcus mutans was obtained from Dr. Rachel Larson at the National Institute for Dental Research, Bethesda, Maryland. Non-plaque forming mutants of this strain, designated 19, 23 and 24A, were obtained from Dr. J. Bulkacz, Department of Oral Biology, School of Dentistry, Medical College of Georgia.

The cultures were grown in a growth medium (GM) which contained trypticase peptone, (BBL, Cockeysville, MD.) 5 grams/liter; yeast extract (Difco, Detroit, MI.) 5 grams/liter; K_2HPO_4 0.029M; $MgSO_4$ 0.2mM; $FeCl_3 \cdot 6H_2O$ 0.004mM; $MnCl_2 \cdot 4H_2O$ 0.003mM and 2% w/v glucose (Jordan et al. 1960). The pH was adjusted to 7.1 with 6 normal HCl. When plaque production was required, 5% w/v sucrose was substituted for glucose. Cultures were incubated at 36°C in an atmosphere of 95% N_2 - 5% CO_2 for 22 hours and then harvested by centrifugation at 10,000 x g at 4°C. Cells not used immediately were lyophilized and stored at -20°C. When required by the experimental protocol, S. mutans 6715 was grown in the same medium supplemented with the indicated concentrations of lauric acid, linoleic acid, oleic acid, palmitic acid, eicosadienoic acid, trilaurin or trilinolein (Supelco, Inc., Bellefonte, PA.). Stock solutions of these compounds containing 5,000 µg/ml were prepared in 100% ethanol and added to the medium to yield the appropriate final concentration.

B. Determination of Minimal Inhibitory Concentration and Minimal Bactericidal Concentration

The minimal inhibitory (bacteriostatic) concentration (MIC) and the minimal bactericidal concentration (MBC) was determined for the following fatty acids and triglycerides; lauric acid, linoleic acid, eicosadienoic acid, trilaurin and trilinolein. The 5,000 µg/ml stock solutions, prepared in ethanol, were diluted with medium to an initial concentration of 500 µg/ml: serial two-fold dilutions of this were made with the medium. The first assay tube contained 500 µg/ml of the compound being tested.

Two tenths of a milliliter of growth medium containing 2% w/v glucose was delivered to all assay tubes. To each of these tubes was added 0.1 ml of a S. mutans 6715 culture containing 10^5 organisms per milliliter, as determined by optical density, and 0.2 ml of the appropriate drug dilution. The positive control contained 0.4 ml medium and 0.1 ml of organisms. The negative control was 0.5 ml of media and no inoculum. The cultures were incubated at 36°C in an atmosphere of 95% N_2 - 5% CO_2 for 48 hours, at which time the MIC was determined. The MIC of each compound for S. mutans 6715 is defined as the lowest concentration of compound at which there is no macroscopic evidence of growth after 48 hours of incubation. At the end of this incubation period, the MBC for each compound was determined by taking samples from the MIC tube, the tube one concentration lower and the tubes two concentrations higher and plating these samples on petri dishes containing Todd Hewitt agar. The plates were incubated for 48 hours under the previously stated conditions and examined for growth. The MBC is defined as the

lowest concentration of compound at which no growth is detected.

C. In Vitro Plaque Production and the Effect of Free Fatty Acids and Triglycerides

In order to determine the effects of lauric acid, linoleic acid, oleic acid, eicosadienoic acid, trilaurin and trilinolein on *in vitro* plaque production, plaque was produced by a modification of the procedure of McCabe et al. (1967). Culture tubes (18x150 mm) containing 10 ml of growth medium that was 5% w/v in sucrose and the appropriate dilution of test agent were inoculated with 10^8 *S. mutans* 6715 per milliliter as determined by optical density. An identical tube without test agent was used as a positive control Sterile 20 gauge nichrome steel wires 15 cm in length were mounted in rubber stoppers covered with aluminum foil and suspended in the medium in the culture tubes. The cultures were incubated at 36°C in an atmosphere of 95% N_2 - 5% CO_2 for 48 hours. The wires were transferred to fresh medium and inoculum every other day for 14 days. At the end of this time, the wires were transferred to tubes containing sterile 0.85% NaCl for scoring. The amount of plaque produced was compared to the positive control and scored on a scale of 0 to 4+. Zero represented no plaque production and 4+ very heavy plaque production.

To validate the visual scoring, dry weights of plaque produced in the presence of differing concentrations of lauric acid were determined and correlated with the visual scoring. The plaque covered wires were removed from the tubes containing culture medium, organisms and the fatty acid and rinsed once in distilled water, once in 10% formalin and

again in distilled water. The plaque coated wires were placed in 18x150 mm test tubes and dried 18 hours at 70°C. They were then weighed and dried for another 8 hours and reweighed. The plaque wires and test tubes reached a constant weight after the initial 18 hours of drying. Upon completion of the second weighing, the plaque wires were suspended in distilled water for one hour to soften the plaque. The plaque was then scraped and washed from the wires. The wires were returned to their original test tubes and the drying schedule repeated until a constant weight was achieved. The difference in the weight of the plaque wire and test tube, with and without plaque, was considered to be the weight of the plaque.

D. Lipid Extraction

In order to ensure that any excess of the exogenously added fatty acids or triglycerides were not loosely adhering to S. mutans 6715, it was necessary to determine the adequacy of the washing procedure used during the harvesting of the cells. It was also necessary to determine the completeness of the lipid extraction procedure to ensure the accurate determination of the lipids present, to enable the quantitation of the individual lipids of the glycolipid and phospholipid classes and to allow accurate determination of the weight percent contribution the neutral, glycolipid and phospholipid fractions made to the total. The above were determined by two series of experiments.

The first set of experiments was designed to determine the optimum amount of washing between harvesting and extraction and also whether 0.85% saline or 0.15M phosphate buffered saline (pH 8.2) made from 0.15M

monopotassium phosphate and 0.15M disodium phosphate dissolved in 0.85% saline was better for this procedure.

Two 50 ml cultures each of WT. S. mutans 6715, WT. 6715 + 2.5 µg/ml linoleic acid and WT. 6715 + 10 µg/ml of lauric acid were grown in the medium under the conditions previously described. The cells were harvested by centrifugation at 10,000 x g for 10 minutes and the supernatant removed. One culture of each was then washed with 10 ml of 0.85% saline, the other with 10 ml of the phosphate buffered saline. The cells were harvested by centrifugation as described and each wash was collected separately, lyophilized and stored for analysis of fatty acid content by gas chromatography. This procedure was used to collect each of the five washes.

The first, second, third and fifth washes from each sample were analyzed for fatty acid content by gas chromatography. The first three washes with saline and phosphate buffered saline appeared to be equally effective. The first wash for each sample with either solution was the only wash of the first three to contain measurable fatty acid peaks. The second and third washes did not contain such peaks. By the fifth wash with either plain saline or buffered saline, the control cultures (WT. 6715) showed a slight increase in detectable fatty acids suggesting possible disruption of the cells. This increase was larger with the phosphate buffered saline. An increase of a similar magnitude was observed with the fatty acid supplemented cultures. It should be pointed out that the total area occupied by the fatty acid peaks in each sample of the wash was never greater than 100 units, while the total peak area for a bacterial fatty acid profile is normally between 7,000 and 10,000

units. It is unlikely that even the largest peak (20 units) observed in these experiments would be observed in a lipid extract analyzed at normal attenuations.

The second set of experiments determined the adequacy of the lipid extraction from WT. S. mutans 6715 by the procedure of Vorbeck and Marinetti (1965). A 50 ml culture of WT. 6715 was incubated under the previously stated conditions in growth medium containing 2% glucose and 0.5 μCi/ml of $1^{-14}C$ acetate. The cells were harvested by centrifugation (10,000 x g at 4°C for 10 minutes), washed twice with saline and then extracted by the procedure of Vorbeck and Marinetti (1965). The extraction mixture was filtered and made 20% in 0.85% saline. The cellular residue was re-extracted with 15% EDTA in NaOH at pH 7.5. The supernatant from this extraction was filtered, evaporated to dryness and resuspended in $CHCl_3$;MEOH (2:1) and made 20% in 0.85% saline. The cellular residue was re-extracted with $CHCl_3$;MEOH; conc. HCl (20:10:0.1). The supernatant was filtered and made 20% in 0.85% saline.

The aqueous and organic phases from the above were allowed to separate and the aqueous layer removed. The interface of each was washed twice with 0.85% saline and the aqueous layer removed after each washing. Sufficient methanol was added to make the phases homogeneous. A portion of each organic layer was counted to determine the amount of radioactivity present. The procedure of Vorbeck and Marinetti (1965) removes 91.6% of the radioactive lipids while the EDTA extract removes less than 0.1% of the lipids. However, the acid extraction yields an additional 8.4% of labeled lipids and significantly increases the total yield of radioactive lipids over the procedure of Vorbeck and Marinetti

(1965). Subsequent analysis of the organic extracts determined that the lipids extracted by the Vorbeck and Marinetti procedure yield 8.75 µg of phospholipid phosphorus and the acid extract, 2.55 µg. The EDTA extract did not contain detectable levels of phospholipid phosphorus. To ensure that the acid extraction had not caused the breakdown or hydrolysis of the additional lipids extracted, a sample from the Vorbeck and Marinetti extract and the acid extract were subjected to paper chromatography and the radioactive lipids detected by autoradiography. Both extracts were similar and no evidence of breakdown or hydrolysis of the lipids could be detected on the neutral or polar lipid chromatograms of the extracted sample.

The two preceding series of experiments resulted in development of the following procedure which was used for the extraction of lipid from S. mutans 6715 in the present studies. Cells were harvested by centrifugation at 10,000 x g at 4°C and washed twice with 0.85% NaCl. Lipids were extracted by a modification of the procedure of Vorbeck and Marinetti (1965). If lyophilized bacteria were extracted, they were first ground into a fine powder with a mortar and pestle. Extraction was done by adding absolute methanol to the cells and then heating at 65°C for 15 minutes. After cooling to room temperature, sufficient chloroform was added to give a final chloroform:methanol ratio of 2:1. The ratio of solvent to the wet weight of bacteria was 1 ml/10 mg. The resulting mixture was stirred for one hour. Cellular residue was removed by filtering through Whatman #41 filter paper. The residue was then re-extracted by stirring with fresh chloroform:methanol:concentrated HCl in a ratio of 20:10:0.1 (Wells and Dittmer, 1965) for 40

minutes and refiltering. The bacterial residue on the filter paper was washed with several volumes of chloroform:methanol 2:1. Sufficient 0.85% NaCl was then added to the combined extracts to make the final volume 20% saline (Folch, Lees and Stanley, 1957).

The organic and aqueous phases from the above procedure were allowed to separate and the aqueous layer removed. The interface was washed twice with 0.85% NaCl and the aqueous layer removed after each washing. Sufficient methanol was added to make the two phases homogeneous. The extract was evaporated under vacuum at 37°C and dryness assured by two successive evaporations in the presence of benzene; absolute ethanol 4:1. The resulting residue was dissolved in chloroform and filtered through a sintered glass funnel. If the extracted lipids were to be stored, the chloroform was evaporated under nitrogen and replaced with hexane and stored at -20°C.

All the solvents used were reagent grade and were obtained from Fisher Scientific Products, Atlanta, GA. The chloroform, methanol and hexane were redistilled in an all glass apparatus before use.

E. Paper Chromatography and Quantitation of Individual Glycolipid and Phospholipid Classes

To separate and quantitate individual glycolipid and phospholipid classes, Whatman SG81 silica-gel impregnated paper was spotted with a lipid solution containing not more than 15 µg of phospholipid phosphorus (Marinetti, 1962). The solvent system for neutral lipids was N-heptane; diisobutyl ketone; glacial acetic acid 85:15:1 (HDA); for polar lipids chloroform; methanol; 5N ammonium hydroxide 64:34:4 for the first dimension; and chloroform; diisobutyl ketone; pyridine; methanol; acetic

acid; formic acid; water 33:10:25:20:8:2:2 for the second dimension (Wuthier, 1976).

Lipids were visualized by dipping the dried chromatograms in 0.015% Rhodamine 6-G and then examining them under ultraviolet light. Those lipids containing free amino groups were detected by spraying the dried chromatograms with 0.5% Ninhydrin and those containing choline by immersing dried unstained chromatograms in 2% phosphomolybdic acid and 2% aqueous stannous chloride in 2% HCl. Phosphate containing lipids were visualized with Phosphay (Supelco, Inc., Bellefonte, PA.). Lysophospholipids were detected by immersing dried unstained chromatograms in 0.05% aqueous malachite green. The procedure for all of the above tests were those described by Ansel (1964). Vicinal glycols and glycolipids were detected using Schiff's reagent according to the procedure of Sastry and Kates (1964).

Individual lipid classes were identified by comparing them to known standards run in the same solvent systems, to published Rf values and by their reactions with the above color tests.

The individual phospholipid classes in the phospholipid fraction derived from column chromatography (see below) were separated using paper chromatography and the solvent systems previously described. The individual phospholipid classes were visualized with 0.015% Rhodamine 6-G, as previously described, the spots circled, cut out and placed in 18x150 mm test tubes. The phospholipids in each spot were extracted according to the method of Marinetti et al. (1959). The recovery of known amounts of phospholipid standards by this method was approximately 94%. The amount of phosphorus in each spot was determined by the method

of Marinetti (1962). Spots of the same size but not containing phospholipids were subjected to the same procedure in order to determine the phosphorus content of the paper. The results were expressed as a percentage of the total phospholipid phosphorus which the individual phospholipid class contained.

The individual glycolipid classes in the glycolipid fraction derived from column chromatography were separated, visualized and the spots removed from the chromatogram as previously described. The individual paper spots were placed in separate polyallomer tubes which had several small holes punched in the bottom. These tubes were suspended in 15 ml conical centrifuge tubes. To remove the glycolipids, the paper spots were saturated with chloroform; methanol; water 64:32:4 and centrifuged for 5 minutes in an IEC table top centrifuge at the number 6 setting. This procedure was repeated until each spot had been washed with 4 ml of solvent. The solvent was evaporated to dryness under N_2 and the anthrone positive material in the residue determined according to the method of Radin, Lavin and Brown (1955). Spots of paper the same size but not containing glycolipids were subjected to the same procedure in order to determine content of the anthrone positive material in the paper. The results were expressed as a percentage of the total anthrone positive glucose material the individual glycolipid class contained.

F. Column Chromatography

Lipids were separated by column chromatographic methods for quantitation of the individual glycolipid and phospholipid classes and for

determining the fatty acid profiles of these classes as well as that of the neutral lipid class. A slurry of 5 grams of Unisil silicic acid 100-200 mesh (Clarkson Chemical Co., Williamsport, PA.) in 30 ml of chloroform was poured into a glass column 1.25 cm i.d. The final height of the column was 12 cm. The column was washed with 100 ml of $CHCl_3$, 50 ml $CHCl_3$ that was 20% in methanol, 50 ml of chloroform containing 2 ml of 2,2 dimethoxy propane and 50 ml of chloroform. A sample containing 500 µg of phospholipid phosphorus was applied to the column and washed in with two 3 ml aliquots of chloroform. The flow of the column was 3 ml/minute. Neutral lipids were eluted first with 180 ml of chloroform. Glycolipids were eluted next with 700 ml of acetone. Phosphate containing lipids were eluted last with 180 ml of methanol. Ten milliliter fractions were collected with an automatic fraction collector (ISCO, Lincoln, NE.). The elution of the column follows the procedure used by Rouser, Kritchevsky and Yamamoto (1976).

Aliquots of each column fraction were assayed for carbohydrate using the anthrone reaction and the procedure of Radin, Lavin and Brown (1955). Phosphorus was determined by the procedure of Harris and Popat (1954) as modified by Marinetti (1962).

The individual lipid classes in each fraction were determined using paper chromatography. The methods used in this determination are described elsewhere.

The weight percentage of each fraction (neutral lipid, glycolipid and phospholipid) contributed was determined for all samples. The weight percentage was determined by collecting the solvent with which each fraction was eluted, evaporating the solvent under vacuum at 37°C and

weighing the collection vessel containing the lipid. The lipid was removed from the vessel with chloroform; the vessel dried and reweighed. The difference in the two weights was considered the weight of the lipid for that fraction, which was then expressed as a percentage of the total lipid applied to the column.

G. Determination of Hexoses Present in the Glycolipid Fraction of S. mutans 6715 Lipids

To determine which sugar or sugars were present in the glycolipid fraction of S. mutans 6715, thin layer plates coated with silica gel H without binder (Applied Science Labs, State College, PA.) were spotted with 250 µg of lipid from the glycolipid fraction of S. mutans 6715. The solvent system was chloroform; methanol; water 65:24:4 (Renkonen and Luukkonen, 1976). Lipids were detected with iodine vapor and outlined. Individual lipid spots from the thin layer plates were scraped into separate glass columns containing a 5mm plug of glass wool and the lipids eluted with 100 ml of chloroform; methanol 2:1. The chloroform; methanol was evaporated to dryness under N_2 and the residue resuspended in hexane for storage. A 20 to 30 µg sample of the lipid was analyzed for the presence of hexoses. Trimethylsilate (TMS) derivatives of the hexoses were made according to the procedure of Dawson (1972). The methanolic-HCl and the Sit-Prep® kit for making the TMS derivatives of the hexoses were obtained from Applied Science Laboratories. The TMS derivatives were separated on a glass column 182 cm in length and 2 mm i.d. The column was packed with Chromosorb W and the liquid phase was 3% S.E. 30 (Applied Science Laboratory, State College, PA.). The carrier gas

was purified N_2 at a flow rate of 30 ml/minute, the flow rate of H_2 was 25 ml/minute and that of air was 250 ml/minute. The column temperature was 175°C and the temperature of the detector was 230°C. The hexoses were identified by comparing their retention times to known standards.

H. Radioactive Labeling of Bacterial Lipids

Bacterial lipids were labeled with 1-^{14}C sodium acetate to aid in the detection and identification of the lipids present in S. mutans 6715 and to determine the efficiency of the lipid extraction procedure for this organism. Fifty ml of growth medium containing 2% glucose was inoculated with 0.6 ml of an 18 hour culture of S. mutans 6715. Radioactive 1-^{14}C sodium acetate (55.7 mCi/mmol) was then added to the medium (0.5 μCi/ml) and the organisms grown anaerobically in an atmosphere of 95% N_2 - 5% CO_2 at 36°C for 22 hours. At the end of the 22 hours, the cells were harvested by centrifugation at 10,000 x g at 4°C for 10 minutes and washed twice with saline. The saline was removed and 7 ml of absolute methanol was added to the pelleted organisms and the mixture heated at 65°C in a water bath for 15 minutes, then allowed to cool. Fourteen ml of chloroform was added to the tubes and the suspension stirred for one hour. The supernatant was filtered through Whatman #41 filter paper to remove any cellular residue (Vorbeck and Marinetti, 1965). The cellular residue remaining in the tube was re-extracted with chloroform; methanol; conc. HCl 20:10:0.1 (Wells and Dawson, 1965). The suspension was stirred for 40 minutes and then filtered to remove the cellular residue. The extraction of lipid was

continued according to the procedure outlined in the Lipid Extraction section of Materials and Methods.

Approximately 8 µg of phospholipid phosphorus was spotted on Whatman SG81 silica-gel impregnated chromatography paper. Neutral lipids were developed with N-heptane; diisobutyl ketone; glacial acetic acid 85:15:1 and polar lipids with chloroform; methanol; 5N ammonium hydroxide 64:34:4 for the first dimension and chloroform; diisobutyl ketone; pyridine; methanol; acetic acid; formic acid; water 33:10:25:20:8:2:2 for the second dimension (Wuthier, 1976).

Radioactive lipids were visualized by autoradiography using Kodak X-omat RP film XRP-1 (Kodak, Rochester, N.Y.). The amount of radioactivity in each visualized lipid was determined by cutting the spot from the original chromatogram and counting the radioactivity in a Beckman LS 330 liquid scintillation spectrometer (Beckman Instruments, Fullerton, CA.) using Aquasol (New England Nuclear, Boston, MA.) as the cocktail.

I. Preparation of Fatty Acid Methyl Esters for Gas Chromarography

Two procedures were used to form methyl esters for gas chromatography. In procedure No. 1, lyophilized bacteria, or non-lyophilized bacteria were used without prior extraction of the lipid. In procedure No. 2, the lipids were extracted first and then methylated. Method No. 1 was preferred because it was faster, required less sample and the results were comparable with those of procedure No. 2. Procedure No. 1 was used for the total fatty acid profiles of S. mutans 6715 grown in the presence or absence of fatty acids or triglycerides and for the non-plaque forming

mutants, SM19, SM23, and SM24A. Procedure No. 2 was used for the fatty acid profiles of the neutral, glycolipid and phospholipid classes of the above organism and additives.

In procedure No. 1, 10 to 40 mg of bacteria were mixed with 5 ml of 5% NaOH in 50% methanol in screw cap test tubes with teflon lined caps and heated for 15 minutes at 100°C in a water bath. The tubes were removed, allowed to cool and the pH adjusted to 2 with 6N HCl. Four milliliters of BF_3-MeOH (Supelco, Inc., Bellefonte, PA.) were added, the tubes and their contents flushed with N_2, resealed and heated for 10 minutes at 100°C. They were then allowed to cool before adding 10 ml of saturated NaCl and mixing. Ten milliliters of chloroform; hexane 1:4 were than added to the tubes, the phases allowed to separate and the organic layer removed and saved. The aqueous layer was re-extracted with 10 ml of chloroform; hexane 1:4 and the two organic layers combined and evaporated to dryness under N_2. The residue was resuspended in hexane for storage and analysis by gas chromatography. This procedure is method B of Moss, Lambert and Mervin (1974) with an increase in the amount of time from 5 minutes to 10 minutes used in the methylation step.

In procedure 2, the lipids were first extracted from the bacteria according to the methods previously outlined. The lipid extract was evaporated to dryness and mixed with 2 ml of BF_3-MeOH (Supelco, Inc.. Bellefonte, PA.) in screw cap test tubes with teflon lined caps. The tubes were flushed with N_2 and heated for 45 minutes at 100°C in a water bath. The tubes were cooled and 2 ml of water and 4 ml of hexane were added and mixed. The phases were allowed to separate and the

organic layer removed and evaporated to dryness. The residue was resuspended in hexane for storage or analysis by gas chromatography. Procedure 2 is the method used by Morrison and Smith (1964).

J. Gas Chromatography of Fatty Acid Methyl Esters

A Packard 420 gas chromatograph (Packard, Downers Grove, IL.) equipped with a flame ionization detector was used for analysis of the samples. Samples were analyzed on a 182 cm metal column with a 2 mm i.d. that was packed with Chromosorb W (Supelco, Inc., Bellefonte, PA.). The liquid phase was Hi Eff. 2BP (ethylene glycol succinate) that was 15% by weight (Applied Science Laboratory, State College, PA.). The carrier gas was purified N_2 at a flow rate of 30 ml/minute, the flow rate of H_2 was 25 ml/minute and that of air 250 ml/minute. The column temperature was 183°C and the temperature of the detector 230°C. The solvent was hexane. A speedomax recorder (Leeds and Northrup, Milano, Italy) was used at a chart speed of 2.5 cm/minute to trace the shape, height and relative retention time of each peak. Assignments for each peak were obtained from a semilogarithmic plot of relative retention time versus chain length and degree of unsaturation of standard mixtures of fatty acid methyl esters. The relative retention times of standard mixtures of the methyl esters of iso and anteiso branched chain fatty acids, of cyclic fatty acids containing 17, 19 or 21 carbons and of hydroxy fatty acids of 14, 16 or 18 carbons were also determined and plotted against chain length. Peak areas were obtained by multiplying the height by the width at half height. The relative percent of each fatty acid methyl ester was determined by dividing the area under each peak by the

total peak areas. The accuracy of the record response was insured by comparing the measured values obtained for fatty acid standard mixtures with their stated percent composition.

III. RESULTS

A. Determination of Minimal Inhibitory Concentration and Minimal Bactericidal Concentration

The minimal inhibitory (bacteriostatic) concentration (MIC) and the minimal bactericidal concentration (MBC) against S. mutans 6715 was determined for the following compounds: lauric acid, linoleic acid, oleic acid, eicosadienoic acid, trilaurin and trilinolein. Their inhibitory ability was examined over the range of 500 µg/ml to 1.95 µg/ml by use of serial two-fold dilutions in growth medium.

The MIC of each compound for S. mutans 6715 was defined as the lowest concentration of compound at which no macroscopic evidence of growth was observed after 48 hours of incubation. At the end of this incubation period, the MBC for each compound was determined by centrifuging the organisms out of the assay medium and streaking them on Todd Hewitt agar plates. The MBC was defined as the lowest concentration of compound at which no growth was detected on the plates after 48 hour incubation.

The results of these experiments are presented in Table 1. For all the compounds tested, there was never more than one dilution difference between the bactericidal and bacteriostatic concentrations.

Table 1.

BACTERIOSTATIC AND BACTERICIDAL CONCENTRATIONS FOR S. MUTANS 6715.

Stock solutions of the compounds listed were prepared in ethanol at a concentration of 5000 μg/ml for addition to the medium. The MIC was determined using two-fold dilutions in test tubes. The MBC was determined by plating samples from the MIC tube, the tube one concentration lower and the tubes two concentrations higher on Todd-Hewitt agar plates, and determining at which concentration no growth occurred. Cultures were incubated at 36°C in 95% N_2 - 5% CO_2 for 48 hours. All experiments were performed in triplicate and each experiment contained duplicate tubes for each concentration.

a. Minimal Inhibitory Concentration

b. Minimal Bactericidal Concentration

Table 1

BACTERIOSTATIC AND BACTERICIDAL CONCENTRATIONS FOR S. MUTANS 6715

Lipid	MIC[a]	MBC[b]
	µg/ml	
Lauric Acid	62.5	125
Linoleic Acid	31.25	31.25
Oleic Acid	62.5	62.5
Eicosadienoic Acid	31.25	62.5
Trilaurin	250	250
Trilinolein	250	250

They demonstrate that free fatty acids and triglycerides can be both bacteriostatic and bactericidal and that for the compounds tested, the free fatty acids are bactericidal at concentrations at least 50% below those of the triglycerides and that linoleic acid, which contains two cis double bonds, is the most potent bacteriostatic and bactericidal agent. The difference in the MICs and MBCs observed between free fatty acids and the triglycerides can most likely be attributed to the greater ease with which the free acids can enter the bacteria and be utilized by them.

B. In Vitro Plaque Production and the Effect of Free Fatty Acids and Triglycerides

To determine the effects of lauric acid, linoleic acid, oleic acid, eicosadienoic acid, trilaurin and trilinolein on in vitro plaque production, plaque was produced and scored by a modification of the procedure of McCabe et al. (1967). These procedures are described in detail in Materials and Methods.

The amount of plaque produced was compared to the positive control and scored on a scale of 0 to 4+, zero representing no plaque production and 4+ very heavy plaque production. A test agent was considered to be effective when it reduced the amount of plaque formed, as compared to the control, to the 1+ level or less at a concentration of test agent at least 50% more dilute than the MIC concentration. The concentration of test agent that produced a 1+ level or less of plaque was considered the plaque inhibitory concentration (PIC).

Figure 1 shows the relationship between visual score and plaque dry weight when S. mutans was grown over a range of 0 to 62.5 µg/ml of lauric acid. The difference between the plaque dry weight of 1+ plaque and 3+ or 4+ plaque is significant at a level $P < .001$. Figures 2 and 3 are examples of plaque scored visually as 1+ and 4+ respectively. Figure 4 is an example of plaque produced in either the absence of lauric acid (4+) or in the presence of various concentrations of lauric acid, scored as 0 through 3+.

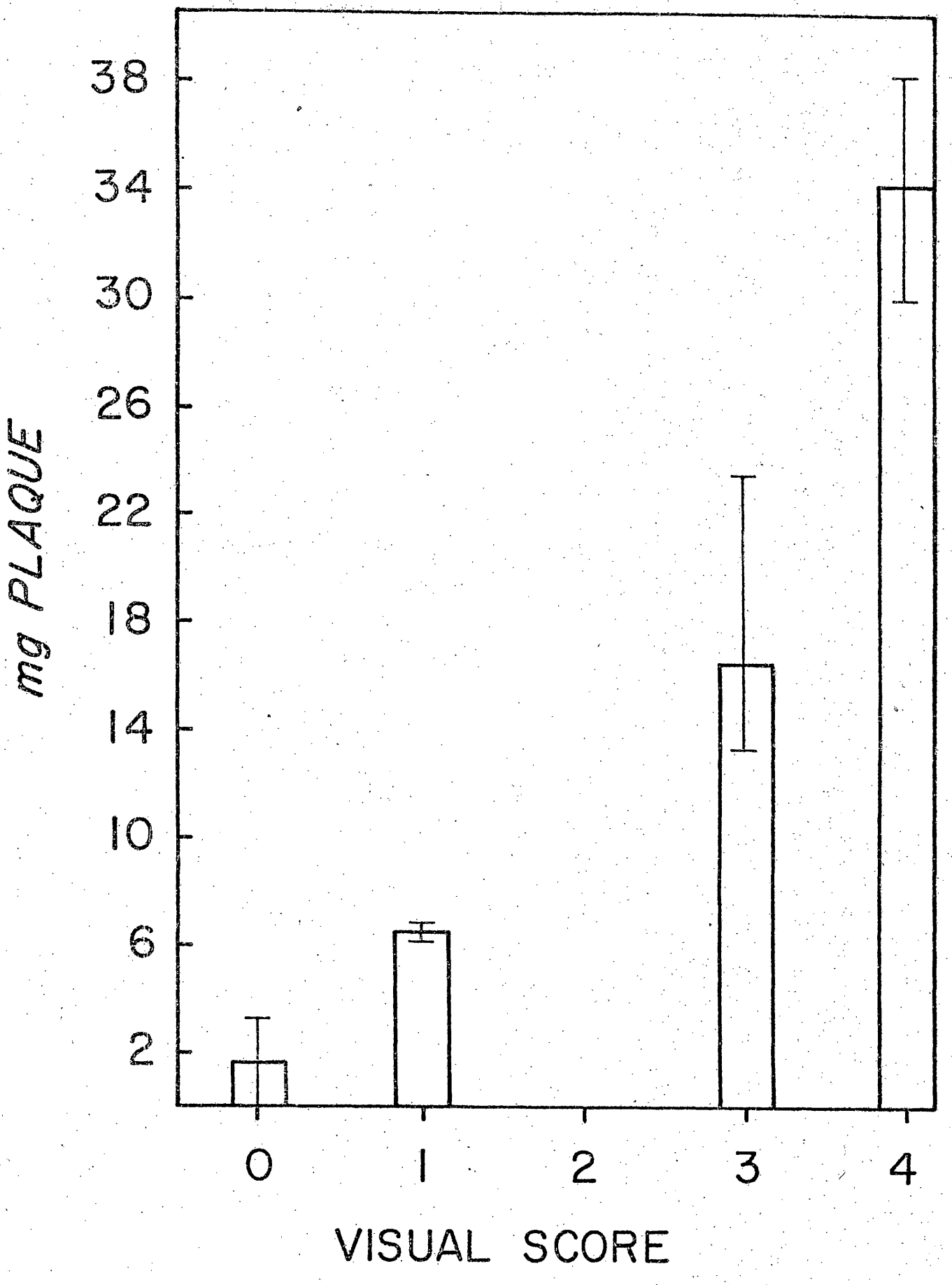
Table 2 presents the results of the plaque inhibition studies. It shows that the free fatty acids are more efficient in reducing plaque than are the triglycerides and that linoleic acid is the most efficient of those free fatty acids tested.

Table 3 is a composite of Tables 1 and 2 and more clearly shows the relationship between the MIC, the MBC and the PIC for the fatty acids and triglycerides. The data in the table show that only free fatty acids reduced plaque to the desired level at concentrations substantially below the MIC and MBC.

Figure 1.

VISUAL PLAQUE SCORE VERSUS PLAQUE DRY WEIGHT IN THE PRESENCE OF VARYING CONCENTRATIONS OF LAURIC ACID.

In vitro plaque was produced by a modification of the method of McCabe et al. (1967). At the end of two weeks, the wires containing plaque were rinsed in distilled water, scored visually, fixed in formalin, rinsed again in distilled water and dried to a constant weight. The plaque wires were then suspended in distilled water the the plaque removed from the wires. The wires were dried to a constant weight. The difference in the two weights was considered to be the weight of the plaque. The difference between plaque dry weight of 1+ plaque and 3+ or 4+ plaque is significant at a level $P < .001$. The amount of lauric acid used ranged from 0 µg/ml for the 4+ plaque to 62.5 µg/ml for the 0 plaque score.


38
34
30
26
22
18
14
10
6
2
mg PLAQUE
0
1
2
3
4
VISUAL SCORE

Figures 2 and 3.

VISUAL PLAQUE SCORE.

In vitro plaque was produced by a modification of the method of McCabe et al. (1967). At the end of two weeks, the wires containing plaque were rinsed in distilled water and scored visually. The plaque in Figure 2 scored 1+ and was produced in the presence of 35 µg/ml of lauric acid. The plaque in Figure 3 scored 4+ and was produced in the absence of lauric acid.

Figure 2.

Visual Plaque

Scored 1+

Figure 3.

Visual Plaque

Scored +4

Figure 4.

VISUAL PLAQUE SCORED 0, 1+, 2+, 3+ and 4+.

In vitro plaque was produced by a modification of the method of McCabe et al. (1967). At the end of two weeks, the wires containing plaque were rinsed in distilled water and scored visually. From left to right, the plaque was scored; 0, 1+, 2+, 3+ and 4+. The lauric acid concentration in the respective tubes was; 62.5 µg/ml, 35 µg/ml, 20 µg/ml, 15 µg/ml and 0 µg/ml.

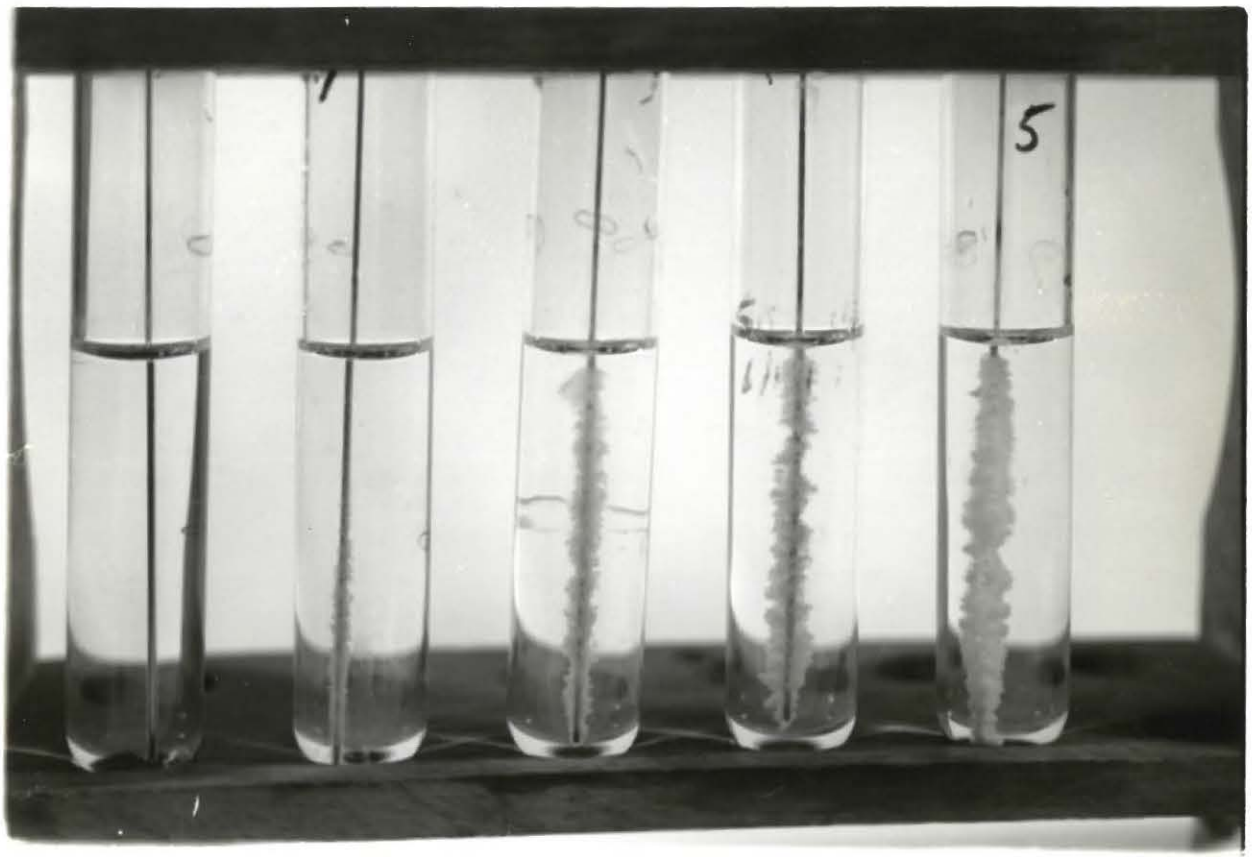


Figure 4.

Visual Plaque Score 1+, 2+, 3+, 4+

Table 2.

PLAQUE INHIBITORY CONCENTRATION OF VARIOUS FATTY ACIDS AND TRIGLYCERIDES.

In vitro plaque was produced by a modification of the procedure of McCabe et al. (1967). Culture tubes containing 10 ml of growth medium that was 5% w/v in sucrose and the appropriate dilution of test agent were inoculated with 10^8 *S. mutans* 6715. Identical tubes without a test agent were used as positive controls. Sterile nichrome steel wires were suspended in the medium and incubated at 36°C in 95% N_2- 5% CO_2 for 48 hours. The wires were transferred to fresh medium and inoculum every other day for two weeks. At the end of this time, the wires were transferred to tubes containing sterile 0.85% saline for scoring. Each experiment was performed in triplicate and each experiment had duplicate tubes for each concentration.

a. Concentration that reduced the amount of plaque formed, compared to the control, to the 1+ level or less.

Table 2

PLAQUE INHIBITORY CONCENTRATIONS (PIC) OF VARIOUS FATTY ACIDS AND TRIGLYCERIDES

	PIC[a] µg/ml
Lauric Acid	31.25
Linoleic Acid	7.8
Oleic Acid	31.25
Eicosadienoic Acid	31.25
Trilaurin	> 200
Trilinolein	> 200

Table 3.

BACTERIOSTATIC, BACTERICIDAL AND PLAQUE INHIBITORY CONCENTRATION OF FATTY ACIDS AND TRIGLYCERIDES FOR S. MUTANS 6715.

Stock solutions of the compounds were prepared in ethanol at a conentration of 5000 µg/ml for addition to the medium. The MIC was determined using the two-fold tube dilution method, the MBC was determined by plating samples from tubes used for the MIC determination and determining at which concentration no growth occurred. Plaque was produced by a modification of the method of McCabe et al. (1967). The plaque wires were transferred to fresh medium containing 5% w/v sucrose and inoculum every other day for two weeks. At the end of this time, the wires were transferred to tubes containing sterile 0.85% saline for scoring. Each experiment was performed in triplicate and each had duplicate tubes for each concentration.

a. Minimal Inhibitory Concentration

b. Minimal Bactericidal Concentration

c. Plaque Inhibitory Concentration

Table 3

BACTERIOSTATIC, BACTERICIDAL AND PLAQUE INHIBITORY CONCENTRATIONS OF FATTY ACIDS AND TRIGLYCERIDES FOR S. MUTANS 6715

Lipid	MIC[a]	MBC[b]	PIC[c]
		µg/ml	
Lauric Acid	62.5	125	31.25
Linoleic Acid	31.25	31.25	7.8
Oleic Acid	62.5	62.5	31.25
Eicosadienoic Acid	31.25	62.5	31.25
Trilaurin	250	250	> 200
Trilinolein	250	250	> 200

C. Quantitation of the Neutral Lipid, Glycolipid and Phospholipid Fractions from Column Chromatography

The extracted lipids from six liter cultures of S. mutans 6715 grown in the presence or absence of exogenous fatty acids or triglycerides or from cultures of the non-plaque forming mutants SM19, SM 23 and SM24A were separated into neutral, glycolipid and phospholipid fractions by silicic acid column chromatography. Ten ml fractions of the neutral lipid, glycolipid and phospholipid fractions were initially assayed for phosphorus and glucose in order to insure the purity of the fraction. Figure 5 shows graphically the results obtained from the fractions of the parental 6715 and is representative of results obtained when 6715 plus free fatty acids or triglycerides or the non-plaque forming mutants were fractionated. In all cases, the glycolipids were eluted with the first 500 ml of acetone. No phospholipid phosphorus was detectable in the glycolipid fraction. The phospholipid fraction was eluted with 180 ml of methanol and contained all of the recoverable phospholipid phosphorus. Recovery of the phosphorus was greater than 98%. This fraction contained less than 8% of the total anthrone positive material, due presumably to the non-specific reaction of the anthrone reagent with the glycerol moieties of phosphatidyl glycerol and diphosphatidyl glycerol. (Diphosphatidyl glycerol (500 µg) gives an absorbance reading equivalent to .58 µg of anthrone positive material and 250 µg of free glycerol gives a similar value.) The weight percentage each fraction contributed was determined by weighing the lipid contained in each fraction and then expressing this as a percentage of the total lipid applied to the column. The percentages for each preparation are shown in Table 4.

Figure 5.

COLUMN CHROMATOGRAPHIC SEPARATION OF GLYCOLIPIDS AND PHOSPHOLIPIDS.

500 µg of phospholipid phosphorus was added to a column containing 100-200 mesh silicic acid. Neutral lipids (not shown) were eluted with 180 ml $CHCl_3$, glycolipids with 700 ml of acetone and phospholipids with 180 ml of MEOH. Ten ml fractions of the neutral, glycolipid and phospholipid fractions were assayed for phosphorus and glucose. The graph is representative of results obtained when S. mutans 6715, S. mutans 6715 + free fatty acids or triglycerides or the non-plaque forming mutants were fractionated.

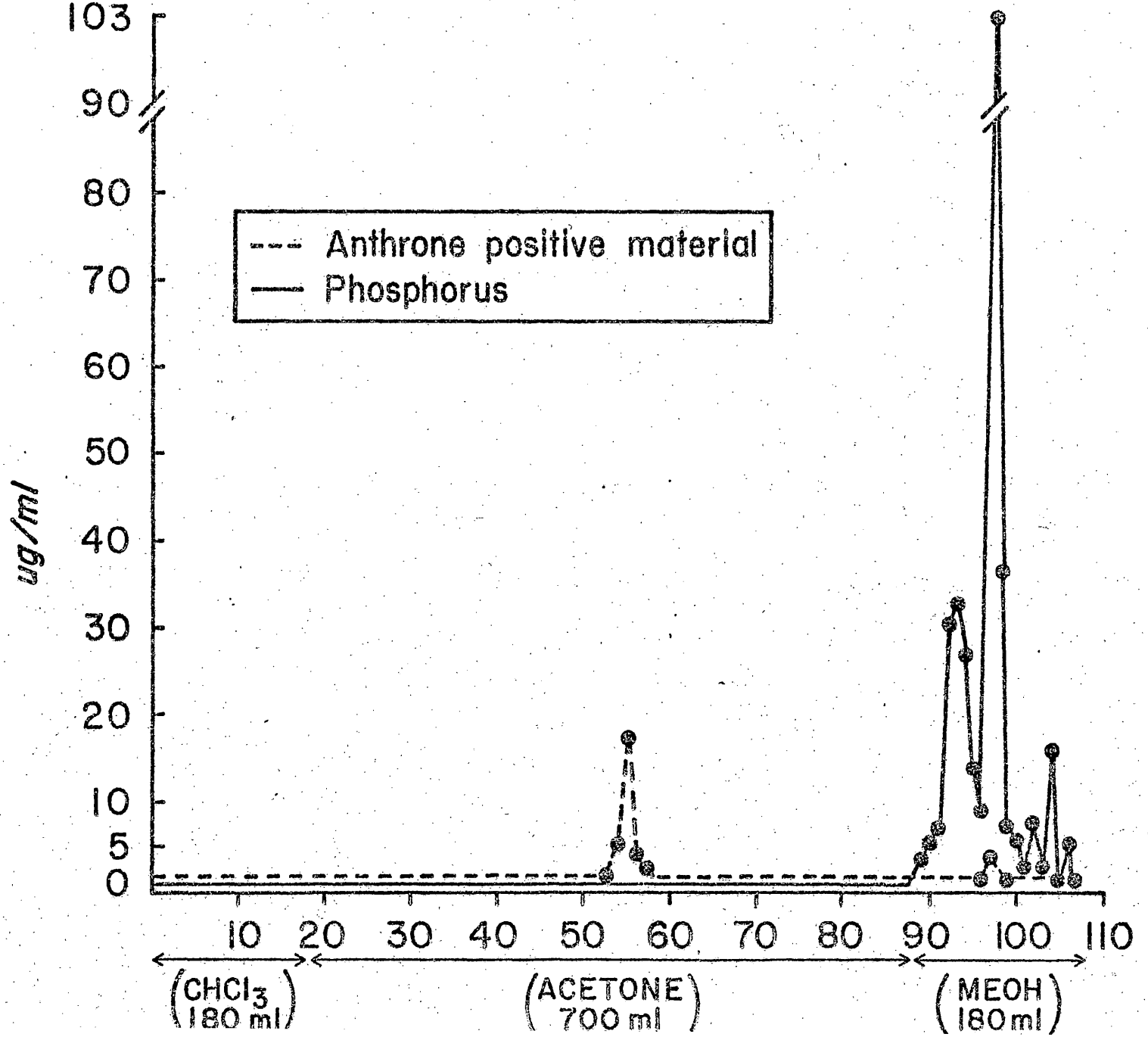

103
90
80
70
60
50
40
30
20
10
5
0
ug/ml
Anthrone positive material
Phosphorus
10
20
30
40
50
60
70
80
90
100
110
(CHCl3 180 ml)
(ACETONE 700 ml)
(MEOH 180 ml)

Table 4.

PERCENT OF TOTAL LIPID COMPRISING THE NEUTRAL LIPID, GLYCOLIPID AND PHOSPHOLIPID FRACTIONS.

The weight percentage each fraction contributed to the total was determined by weighing the lipid in each major fraction from the column and then expressing this as a percentage of the total lipid applied to the column. The results are the averages from three separate determinations and are expressed ± standard deviation.

Table 4

PERCENT OF TOTAL LIPID COMPRISING THE NEUTRAL LIPID, GLYCOLIPID AND PHOSPHOLIPID FRACTIONS

Organism + Lipid	Percent of Total Lipid by Weight		
	Neutral Lipid	Glycolipid	Phospholipid
6715	3.7 ± .15	67.2 ± 2.84	29.1 ± 2.37
6715 + 10 µg/ml Lauric Acid	13.1 ± .92	64.3 ± 2.42	22.5 ± 1.42
6715 + 2.5 µg/ml Linoleic Acid	10 ± .53	70.4 ± 2.81	19.6 ± 1.47
6715 + 10 µg/ml Oleic Acid	10.5 ± .85	61.2 ± 2.46	28.2 ± 1.47
6715 + 10 µg/ml Trilaurin	2.8 ± .26	66.4 ± 2.46	30.8 ± 1.56
6715 + 10 µg/ml Trilinolein	4.1 ± .26	49.2 ± 1.99	46.7 ± 2.31
SM 19	2.3 ± .17	56.9 ± 1.87	40.8 ± 1.97
SM 23	1.6 ± .1	60.9 ± 3.37	37.5 ± 2.55
SM 24A	3.6 ± .25	67.7 ± 1.71	28.6 ± 2.2

Table 5.

PERCENT OF TOTAL GLUCOSE IN INDIVIDUAL LIPIDS OF THE GLYCOLIPID FRACTION.

The individual glycolipid classes in the glycolipid fraction derived from column chromatography were separated by paper chromatography. They were visualized with Rhodamine G, cut from the paper and removed from the paper with $CHCl_3$; MEOH; H_2O (64:32:4). The glucose present was determined by the anthrone reaction, and is expressed as a percentage of the total glucose present. The values presented are the averages from three separate determinations and are expressed ± standard deviation.

a. monoglucosyl diglyceride

b. diglucosyl diglyceride

Table 5

PERCENT OF TOTAL GLUCOSE IN INDIVIDUAL LIPIDS OF THE GLYCOLIPID FRACTION

Organism + Lipid	Percent of Total Glucose	
	MGDG[a]	DGDG[b]
6715	28.4 ± 2.4	71.6 ± 2.82
6715 + 10 µg/ml Lauric Acid	36.7 ± 1.25	63.3 ± 2.05
6715 + 2.5 µg/ml Linoleic Acid	40 ± 2.31	60 ± 2.57
6715 + 10 µg/ml Oleic Acid	26.6 ± 1.74	73.4 ± 1.74
6715 + 10 µg/ml Trilaurin	21.4 ± 1.80	78.4 ± 2.33
6715 + 10 µg/ml Trilinolein	36 ± 1	64 ± 1.5
SM 19	42.2 ± 2.26	57.2 ± 1.90
SM 23	30.8 ± 1.76	69.2 ± 1.62
SM 24A	25.2 ± 1.56	74.8 ± 1.73

are expressed as a percentage of the total glucose which the individual glycolipid classes contain.

Lauric and linoleic acid, the two most effective antiplaque agents of those tested, reduce the amount of the diglucosyl-diglyceride (DGDG) present and increase the monoglucosyl-diglyceride (MGDG) present in 6715. This trend is also observed with trilinolein which does not reduce plaque to the 1+ level at a concentration 50% more dilute than the MIC concentrations and also for the non-plaque forming mutant SM19. Oleic acid, a moderately effective antiplaque agent, has no effect on the DGDG or MGDG concentration of 6715 and the non-plaque forming mutants SM23 and SM24A have DGDG and MGDG amounts that do not differ substantially from the parental 6715. S. mutans 6715 grown in the presence of trilaurin, which is not an effective antiplaque agent, differs from the others in that a marked increase in the amount of DGDG present and a decrease in the MGDG is observed.

Table 6 reports the results of the quantitation of individual phospholipid classes in the organisms. The results are expressed as a percentage of the total phospholipid phosphorus that the individual phospholipid classes contain. The data in this table indicate that those fatty acids which were shown to reduce plaque accumulation also reduced the amount of diphosphatidyl glycerol present and increased the amount of lysophosphatidyl choline present. Changes in the phosphatidyl choline and phosphatidyl glycerol classes were also observed when these fatty acids were present but the changes were not consistant. This suggests that each fatty acid may effect these classes differently. In contrast to this, trilaurin had virtually no effect on the percentage of each

Table 6.

PERCENT OF TOTAL PHOSPHORUS IN INDIVIDUAL LIPIDS OF THE PHOSPHOLIPID FRACTION.

The individual phospholipids classes in the phospholipid fraction derived from column chromatography were separated by paper chromatography. They were visualized with Rhodamine G, cut from the paper and extracted from the paper by the method of Marinetti et al. (1959). The phospholipid phosphorus present in each spot was determined by the method of Marinetti (1962). The recovery of known amounts of phospholipid standards by this method was approximately 94%. The values presented are the averages from three separate determinations and are expressed ± standard deviation.

a. lysophosphatidyl choline

b. phosphatidic acid

c. phosphatidyl choline

d. phosphatidyl glycerol

e. diphosphatidyl glycerol

T = < 1%

Table 6

PERCENT OF TOTAL PHOSPHORUS IN INDIVIDUAL LIPIDS OF THE PHOSPHOLIPID FRACTION

	Percent of Total Phospholipid Phosphorus				
Organism + Lipid	LPC[a]	PA[b]	PC[c]	PG[d]	DPG[e]
6715	6 ± .12	-	22.2 ± 1.82	3.8 ± .2	68 ± 2.44
6715 + 10 µg/ml Lauric Acid	14.4 ± .62	T	7.4 ± .53	26 ± 2.35	51.9 ± 3.49
6715 + 2.5 µg/ml Linoleic Acid	14 ± .51	-	16.6 ± .46	16.7 ± 1.22	52.7 ± 2.75
6715 + 10 µg/ml Oleic Acid	13.5 ± .35	1.2 ± .1	25.2 ± .95	5.1 ± .31	55 ± 1.85
6715 + 10 µg/ml Trilaurin	5.9 ± .15	-	20.8 ± .55	4.1 ± .36	69.2 ± 1.31
6715 + 10 µg/ml Trilinolein	9 ± .47	-	17.1 ± 1.35	9 ± .46	64.9 ± 2.67
SM 19	8.5 ± .55	-	23.8 ± 1.41	6.2 ± .35	61.5 ± 2.36
SM 23	4.6 ± .15	T	6.7 ± .44	8.9 ± .61	79.1 ± 1.80
SM 24A	3.8 ± .20	2.1 ± .06	11.2 ± .62	12.5 ± .80	70.4 ± 2.08

class present. Trilinolein altered the percentages of each class in a manner similar to that of the free fatty acids, but the magnitude of the change was greatly reduced. The percentages of the phospholipid classes composing the phospholipid fraction of the non-plaque forming mutants were different from those of the parental type but were not consistant among the mutants. The phospholipid class percentages of SM23 and SM24A do not resemble those of 6715 grown in the presence of free fatty acids. SM19 shows a decrease in the diphosphatidyl glycerol and an increase in lysophosphatidyl choline. These changes in SM19 are in the same direction as those observed when WT. 6715 is grown in the presence of lauric, linoleic or oleic acid, but are much smaller.

E. Determination of Fatty Acid Profiles for S. mutans 6715, 6715 in the Presence of Free Fatty Acids or Triglycerides and for Non-Plaque Forming Mutants of 6715

To determine if there was a relationship between the organism's fatty acid content and its ability to form plaque, a series of experiments were performed in which the fatty acid profile for S. mutans 6715 grown in the presence of absence of free fatty acids and triglycerides was determined. The fatty acid profiles of non-plaque forming mutants of 6715 (SM19, SM23, SM24A) grown in the presence or absence of free fatty acids was also determined.

The fatty acid profiles for S. mutans 6715, and the non-plaque forming mutants, SM19, SM23 and SM24A, are presented in Table 7. The data in the table show that all of the non-plaque forming mutants have

Table 7

FATTY ACID COMPOSITION OF S. MUTANS 6715 AND NON-PLAQUE FORMING MUTANTS.

The total cellular fatty acid profile for S. mutans 6715 and non-plaque forming mutants of 6715 was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the averages of values obtained from six separate cultures. T = < 1%.

a. vaccenic and oleic acid

Table 7

FATTY ACID COMPOSITION OF S. MUTANS 6715 AND NON-PLAQUE FORMING MUTANTS

Bacterial Strain	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
6715	-	13.7	3.2	41.1	4.9	-	5.6	22.6	-	-	-	8.5	-	-	63.6	36
SM 19	-	4.3	4.2	32	5.3	-	6.3	31.5	-	-	1.3	14.2	-	-	48.1	51
SM 23	-	4.1	-	35	3.8	-	7.8	31.8	-	-	-	16	-	-	46.9	51.6
SM 24A	-	5.4	4.2	31.9	3.3	-	8.6	31.1	-	-	-	14.8	-	-	50.1	49.2

a greater percent of fatty acids in the unsaturated form than does the plaque forming wild type. The mutants also have an increase in the percent of octadecenoic and eicosenoic acid present and a decrease in the percents of myristic and palmitic acid present. The percentage of pentadecanoic acid is increased in SM19 and SM24A. This fatty acid was not detected in the total fatty acid profile of SM23. These data suggest that the ability of S. mutans 6715 to form plaque and the inability of the mutants to form plaque may be related to the total percentage of unsaturated fatty acids present or to the amount or ratio of specific fatty acids.

Before determining if the decrease in the plaque forming ability of the organism could be directly related to its fatty acid composition, it was necessary to determine what effect the various concentrations of fatty acids had on the total fatty acid content of S. mutans 6715. The experiments that generated the data in the next two tables were intended to answer this question.

The data in Table 8 is from an experiment designed to determine if the degree of change observed in an organism's fatty acid content was strictly concentration dependent or if after a certain concentration of exogenously added fatty acid was achieved, the rate at which the changes occurred decreased. Eicosadienoic acid was chosen for this study because it is a long chain polyunsaturated fatty acid that is not a normal constituent of S. mutans 6715 and has a retention time as determined by gas chromatography that permitted its peak to be easily detected and measured. Because of this property, if an increase in the percentage of incorporation occurred by increasing the concentration of

Table 8.

FATTY ACID COMPOSITION OF S. MUTANS 6715 GROWN IN INCREASING CONCENTRATIONS OF EICOSADIENOIC ACID.

The total cellular fatty acid profile for S. mutans 6715 grown in increasing concentrations of eicosadienoic acid was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the averages of values obtained from three separate cultures for each concentration tested. T = < 1%.

a. vaccenic and oleic acid

Table 8

FATTY ACID COMPOSITION OF S. MUTANS 6715 GROWN IN INCREASING CONCENTRATIONS OF EICOSADIENOIC ACID

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacteria + Lipid																
6715 + 2.5 µg/ml Eicosadienoic Acid	-	5.2	3.6	38.6	T	-	4.9	24.7	-	-	T	11.6	10.8	-	52.3	47.1
6715 + 10 µg/ml Eicosadienoic Acid	-	4.2	T	35.4	T	-	6.2	20.3	-	-	-	10.8	21.9	-	45.8	53
6715 + 15 µg/ml Eicosadienoic Acid	-	3.6	2.8	34.4	T	-	4.3	19.4	-	-	T	8.6	25.8	-	45.1	53.8
6715 + 20 µg/ml Eicosadienoic Acid	-	4.8	1.9	33.8	T	-	5.1	18.6	-	-	T	8.6	26.2	-	45.6	53.4
6715 + 31.25 µg/ml Eicosadienoic Acid	-	4.3	T	32.6	T	-	4	16.3	-	-	-	6.7	34.7	-	40.9	57.7

eicosadeinoic acid, this increase could be easily followed. Eicosadienoic acid also had a MBC and PIC that were larger than linoleic acid and thus allowed a greater range of concentrations to be studied. The data in the table indicate that once an exogenous concentration of 10 μg/ml of eicosadienoic acid is reached there is no change in the saturated to unsaturated fatty acid ratio until the bacteriostatic and plaque inhibitory concentrations of 31.25 μg/ml are reached, and that a large majority of this change is brought about by an increase in the exogenously added eicosadienoic acid. The data indicated that the changes occurring in the fatty acid content slowed considerably when the exogenous fatty acid concentration was 30% or more of the plaque inhibitory concentration.

To determine if this observation was valid for fatty acids that inhibited plaque at concentrations below the bacteriostatic concentration, experiments were conducted in which the fatty acid content of 6715 was determined at an exogenous fatty acid concentration at or above the plaque inhibitory concentration and at a concentration of exogenous fatty acid at least 30% below this. The data from these experiments are presented in Table 9 and show that there is only a slight difference in the percentage of saturated and unsaturated fatty acids present for the high and low concentration of both lauric and oleic acid. The change in the percentage for the high and low concentration of eicosadienoic acid is less than 5% and can be accounted for largely by the increase in the percentage of eicosadienoic acid present. These findings indicated that we could conduct the remainder of the experiments in this area at concentrations of exogenous fatty acids approximately 30%

Table 9.

FATTY ACID COMPOSITION OF S. MUTANS 6715 AT A HIGH AND LOW CONCENTRATION OF SPECIFIC FATTY ACIDS.

The total cellular fatty acid profile for S. mutans 6715 grown in the presence of a high and low concentration of the indicated fatty acids was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the averages of values obtained from four separate cultures for each concentration tested. T = < 1%.

a. vaccenic and oleic acid

Table 9

FATTY ACID COMPOSITION OF S. MUTANS 6715 AT A HIGH AND LOW CONCENTRATION OF SPECIFIC FATTY ACIDS

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacteria + Lipid																
6715 + 10 µg/ml Lauric Acid	12.1	8.9	-	44.4	1.2	-	8.1	16.2	-	-	-	8.4	-	-	73.5	25.8
6715 + 40 µg/ml Lauric Acid	9.4	11	-	42.9	1.5	-	9	16.8	-	-	T	8.4	-	-	72.3	26.7
6715 + 10 µg/ml Oleic Acid	-	2.9	-	21.4	2.9	-	3.1	55.9	-	-	-	13.5	-	-	27.4	72.3
6715 + 35 µg/ml Oleic Acid	-	3.1	-	17.7	2.5	-	3.4	66.4	-	-	-	6.6	-	-	24.2	75.5
6715 + 10 µg/ml Eicosadienoic Acid	-	4.2	T	35.4	T	-	6.2	20.3	-	-	-	10.8	21.9	-	45.8	53
6715 + 31.25 µg/ml Eicosadienoic Acid	-	4.3	T	32.6	T	-	4	16.3	-	-	-	6.7	34.7	-	40.9	57.7

below the plaque inhibitory concentration. For the fatty acids and triglycerides that did not inhibit plaque, a concentration of 10 μg/ml of test agent was used. These concentrations of free fatty acids and triglycerides permitted all the cultures of 6715, regardless of the fatty acid or the added triglyceride, to reach a similar optical density reading after 22 hours of growth. The non-plaque forming mutants, SM19, SM23 and SM24A, were grown in the same concentrations of the fatty acid as the wild type.

In order to determine if the lack of plaque forming ability observed in SM19, SM23 and SM24A could be directly related to fatty acid content, experiments were performed in which separate cultures of S. mutans 6715 were grown in the presence of lauric, linoleic or oleic acid. The purpose of these experiments was to determine if the incorporation of any of these fatty acids into WT. 6715 would cause this organism's fatty acid profile to resemble that of the mutants. These three fatty acids had been previously shown to be effective plaque reducing agents (see Table 2). S. mutans 6715 was also grown in the presence of either eicosadienoic or palmitic acid, both of which had been shown not to be effective as plaque reducing agents. These fatty acids were used to determine if their effects on the fatty acid content of WT. 6715 would be similar to those that were effective plaque reducers and because preliminary data had suggested that appearance of eicosadienoic acid might be responsible for the antiplaque activity of linoleic acid. However, Table 2 shows that the concentration of eicosadienoic acid required to inhibit plaque production is more than 4 times greater than the concentration of linoleic acid required. The triglycerides,

trilaurin and trilinolein were used in order to determine if their effects on fatty acid content were similar to that of lauric and linoleic acid. The non-plaque forming mutants, SM19, SM23 and SM24A, were grown in the presence of palmitic and linoleic acid to insure that they retained the ability to incorporate exogenous fatty acids and to determine if the level of incorporation was similar to that of the wild type.

The data in Table 10 shows the percentages of the individual fatty acids that are present in 6715 and 6715 grown in the presence of the various free fatty acids and triglycerides. The data demonstrates that wild type 6715 can incorporate all of the exogenous fatty acids tested into its lipids as well as the fatty acids from the triglycerides. The data also shows that wild type 6715 is capable of elongating linoleic acid (a fatty acid that is not normally present in the bacterium) by two carbons. The incorporation of a fatty acid is reflected either by its appearance in the profile or by an increase in the percentage present. The exogenous fatty acid also causes an alteration in the percentages of the other fatty acids present and also in total percentages of saturated and unsaturated fatty acids present. This last manifestation of change could be the most significant because a change in the ratio of saturated to unsaturated could lead to an alteration in bacterial properties that might be expressed phenotypically. One of the possible phenotypic expressions observed for 6715 grown in the presence of lauric, linoleic or oleic acid could be decreased plaque production. The data in Table 10 does not permit a statement to be made about the relationship between bacterial fatty acid content and plaque production,

Table 10.

FATTY ACID COMPOSITION OF S. MUTANS 6715 AND S. MUTANS 6715 GROWN IN THE PRESENCE OF FATTY ACIDS OR TRIGLYCERIDES.

The total cellular fatty acid profile for S. mutans 6715 grown in the presence of fatty acids or triglycerides was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the averages of values obtained from six separate cultures for each fatty acid. T = < 1%.

a. vaccenic and oleic acid

Table 10

FATTY ACID COMPOSITION OF *S. MUTANS* 6715 AND *S. MUTANS* 6715 GROWN IN THE PRESENCE OF FATTY ACIDS OR TRIGLYCERIDES

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacteria + Lipid																
6715	-	13.7	3.2	41.1	4.9	-	5.6	22.6	-	-	-	8.5	-	-	63.6	36
6715 + 10 µg/ml Lauric Acid	12.1	8.9	-	44.4	1.2	-	8.1	16.2	-	-	-	8.4	-	-	73.5	25.8
6715 + 2.5 µg/ml Linoleic Acid	-	8.1	3	39.9	2.7	-	6.8	19.7	8.2	-	-	9	2.3	-	57.8	41.9
6715 + 10 µg/ml Oleic Acid	-	2.9	-	21.4	2.9	-	3.1	55.9	-	-	-	13.5	-	-	27.4	72.3
6715 + 10 µg/ml Eicosadienoic Acid	-	4.2	T	35.4	T	-	6.2	20.3	-	-	-	10.8	21.9	-	45.8	53
6715 + 10 µg/ml Palmitic Acid	-	8.8	1.2	66.1	9.6	-	1.8	10.1	-	-	-	2.3	-	-	77.9	22
6715 + 10 µg/ml Trilaurin	3.2	5.4	1.6	36.4	4.5	-	5.5	31.1	-	-	-	12.2	-	-	52.1	47.8
6715 + 10 µg/ml Trilinolein	-	6.3	1.1	36.7	4.8	-	4	29.8	6.2	-	-	10.7	-	-	48.1	51.5

but one cannot help but observe that the mutants that do not form plaque have a fatty acid content that is quite different from the plaque forming parental type. (Table 7).

Table 11 presents data from a series of experiments designed to determine if the fatty acids from trilaurin and trilinolein are incorporated into 6715 to the same degree as free forms of the fatty acid. The data indicate that the incorporation of free lauric acid into 6715 is approximately 4 times greater than the fatty acids from trilaurin, even though the fatty acid concentrations are similar (49.9 μM for lauric acid and 46.8 μM for the fatty acids in trilaurin). Free linoleic acid is incorporated into 6715 only 1.3 times greater than the fatty acids from trilinolein; however, the fatty acid content of linoleic acid (8.9 μM) is approximately 4 times less than the fatty acid content of trilinolein (33.9μM). This data indicates that the free fatty acids are much more easily incorporated into the bacterial fatty acids. This difference in ease of incorporation may partially explain why the MIC and MBC and the plaque inhibitory concentration for the free fatty acid is a minimum of 50% less than the values for the triglycerides. The lack of eicosadienoic acid in trilinolein grown culture's suggests that there may have to be a certain amount of linoleic acid present in the bacterial cell before it can be elongated to eicosadienoic acid. If this is the case, then elongation of linoleic acid may serve as a way of decreasing the amount of linoleic acid present and thus reducing its effects on the organism.

Tables 12 and 13 contain the results from experiments conducted to determine if the non-plaque forming mutants of S. mutans 6715, SM19, SM23 and SM24A retained the ability to incorporate exogenous fatty acids

Table 11.

COMPARISON OF THE FATTY ACID COMPOSITION OF S. MUTANS 6715 GROWN IN THE PRESENCE OF TRIGLYCERIDES OR THEIR COMPONENT FATTY ACID.

The fatty acid profile of S. mutans 6715 grown in the presence of a triglyceride or their component fatty acid was determined by gas chromatography. Fatty acid methyl esters were formed as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the averages of values obtained from six separate cultures for each concentration.

a. vaccenic and oleic acid

Table 11

COMPARISON OF THE FATTY ACID COMPOSITION OF S. MUTANS 6715 GROWN IN THE PRESENCE OF TRIGLYCERIDES OR THEIR COMPONENT FATTY ACID

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacteria + Lipid																
6715	-	13.7	3.2	41.1	4.9	-	5.6	22.6	-	-	-	8.5	-	-	63.6	36
6715 + 2.5 µg/ml Linoleic Acid	-	8.1	3	39.9	2.7	-	6.8	19.7	8.2	-	-	9	2.3	-	57.8	41.9
6715 + 10 µg/ml Triiinolein	-	6.3	1.1	36.7	4.8	-	4	29.8	6.2	-	-	10.7	-	-	48.1	51.5
6715 + 10 µg/ml Lauric Acid	12.1	8.9	-	44.4	1.2	-	8.1	16.2	-	-	-	8.4	-	-	73.5	25.8
6715 + 10 µg/ml Trilaurin	3.2	5.4	1.6	36.4	4.5	-	5.5	31.1	-	-	-	12.2	-	-	52.1	47.8

and if the level of incorporation was similar to that of the wild type. Table 12 presents the data obtained when 6715 or the mutants were grown in the presence of 10 μg/ml of palmitic acid. The increase in the percentage of palmitic acid present in the organisms ranged from 25 to 27% and in all cases resulted in the lowering of the percentages of octadecenoic and eicosenoic acids. The percentage of pentadecanoic acid was also lowered when WT. 6715, SM19 or SM24A was grown in the presence of palmitic acid.

This result was similar to that observed when WT. 6715 was grown in the presence of lauric acid, oleic acid, trilaurin or trilinolein (Table 10). Pentadecanoic was not detected in SM23. These data show that the non-plaque forming mutants retain their ability to incorporate palmitic acid, a representative long chain saturated fatty acid and that the incorporation results in a greater percentage of the total fatty acids being in the saturated form.

Table 13 reports the results from a series of similar experiments in which linoleic acid was substituted for palmitic acid. All of the mutants are able to incorporate linoleic acid. SM19 and SM24A incorporate the fatty acid to a degree similar to that of the wild type, while SM23 incorporates more than 3 times as much. The increased incorporation of linoleic acid by SM23 markedly lowers the palmitic acid content of the organism. Two of the mutants, SM23 and SM24A, have retained the ability of the wild type to elongate linoleic acid to eicosadienoic acid. The third mutant, SM19, shows no evidence of being able to elongate linoleic acid. The percentage of pentadecanoic acid present was lowered when WT. 6715, SM19 or SM24A was grown in the presence of linoleic

Table 12.

FATTY ACID COMPOSITION OF S. MUTANS 6715 AND NON-PLAQUE FORMING MUTANTS OF 6715 GROWN IN THE PRESENCE OF PALMITIC ACID.

The fatty acid composition of 6715 and of the non-plaque forming mutants of 6715 grown in the presence of palmitic acid was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the averages of values obtained from three separate cultures for each organism. T = < 1%.

a. vaccenic and oleic acid

Table 12

FATTY ACID COMPOSITION OF S. MUTANS 6715 AND NON-PLAQUE FORMING MUTANTS OF 6715 GROWN IN THE PRESENCE OF PALMITIC ACID

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacteria + Lipid																
6715	-	13.7	3.2	41.1	4.9	-	5.6	22.6	-	-	-	8.5	-	-	63.6	36
6715 + 10 µg/ml Palmitic Acid	-	8.8	1.2	66.1	9.6	-	1.8	10.1	-	-	-	2.3	-	-	77.9	22
SM 19	-	4.3	4.2	32	5.3	-	6.3	31.5	-	-	1.3	14.2	-	-	48.1	51
SM 19 + 10 µg/ml Palmitic Acid	-	1.8	T	58.8	5.2	-	3.6	19.8	-	-	-	10	-	-	64.2	35
SM 23	-	4.1	-	35	3.8	-	7.8	31.8	-	-	-	16	-	-	46.9	51.6
SM 23 + 10 µg/ml Palmitic Acid	-	4.8	-	58	6.6	-	3.6	19.6	-	-	-	7.4	-	-	66.4	33.6
SM 24A	-	5.4	4.2	31.9	3.3	-	8.6	31.1	-	-	-	14.8	-	-	50.1	49.2
SM 24A + 10 µg/ml Palmitic Acid	-	7.5	3.2	57.6	6.4	-	5.2	14.2	-	-	-	5.8	-	-	73.5	26.4

Table 13.

FATTY ACID COMPOSITION OF S. MUTANS 6715 AND NON-PLAQUE FORMING MUTANTS OF 6715 GROWN IN THE PRESENCE OF LINOLEIC ACID.

The fatty acid composition of 6715 and of the non-plaque forming mutants of 6715 grown in the presence of linoleic acid was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the averages of values obtained from three separate cultures for each organism. T = < 1%.

a. vaccenic and oleic acid

Table 13

FATTY ACID COMPOSITION OF S. MUTANS 6715 AND NON-PLAQUE FORMING MUTANTS OF 6715 GROWN IN THE PRESENCE OF LINOLEIC ACID

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacteria + Lipid																
6715	-	13.7	3.2	41.1	4.9	-	5.6	22.6	-	-	-	8.5	-	-	63.6	36
6715 + 2.5 µg/ml Linoleic Acid	-	8.1	3	39.9	2.7	-	6.8	19.7	8.2	-	-	9	2.3	-	57.8	41.9
SM 19	-	4.3	4.2	32	5.3	-	6.3	31.5	-	-	1.3	14.2	-	-	48.1	51
SM 19 + 2.5 µg/ml Linoleic Acid	-	2.1	T	30.2	6.9	-	7.8	24.8	7.8	-	T	19.4	-	-	40.1	58.9
SM 23	-	4.1	-	35	3.8	-	7.8	31.8	-	-	-	16	-	-	46.9	51.6
SM 23 + 2.5 µg/ml Linoleic Acid	-	1.2	-	24.5	5.4	-	3.9	23.5	25.7	-	-	11.6	4.1	-	29.6	70.3
SM 24A	-	5.4	4.2	31.9	3.3	-	8.6	31.1	-	-	-	14.8	-	-	50.1	49.2
SM 24A + 2.5 µg/ml Linoleic Acid	-	2.6	3.3	34.1	5.4	-	6.4	23.2	6.1	-	-	14.3	4.5	-	46.4	53.5

acid. The decrease in the percentage was not as great as when these organisms were grown in the presence of palmitic acid. The data in this table show that the non-plaque forming mutants have retained their ability to incorporate linoleic acid, a long chain unsaturated fatty acid and that two of the mutants have retained the ability to elongate this fatty acid. The percentage of fatty acid appearing in the unsaturated form was increased in the 4 organisms tested.

The total fatty acid profiles of WT. 6715, 6715 grown in the presence of free fatty acids or triglycerides and of the non-plaque forming mutants, showed that certain free fatty acids (linoleic and oleic acid) can alter the fatty acid profiles of 6715 so that they are similar in some respects (increased percentage of total unsaturation, increased percentage of eicosenoic acid and a decreased percentage of myristic and palmitic acid) to the profiles of the non-plaque forming mutants. These same fatty acids have been shown to reduce plaque accumulation in vitro, however, the total fatty acid profile data are not sufficient to adequately support the hypothesis that the fatty acid profiles and decreased plaque accumulation are directly related.

In order to further investigate if a relationship exists between fatty acid profiles and plaque accumulation, the profiles of the neutral, glycolipid and phospholipid fractions were determined individually. The profiles of the individual fractions were analyzed to determine if subtle changes in a particular lipid fraction were responsible for the reduction in plaque accumulation. The results of these determinations are reported in the following six tables.

Table 14.

FATTY ACID CONTENT OF THE NEUTRAL LIPID FRACTION OF S. MUTANS 6715 GROWN IN THE PRESENCE OR ABSENCE OF FATTY ACIDS OR TRIGLYCERIDES.

The fatty acid content of the neutral lipid fraction from column chromatography of S. mutans 6715 grown in the presence or absence of fatty acids or triglycerides was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the average values obtained from three separate preparations. Each preparation was analyzed in duplicate. T = < 1%.

a. vaccenic and oleic acid

Table 14

FATTY ACID CONTENT OF THE NEUTRAL LIPID FRACTION OF S. MUTANS 6715 GROWN IN THE PRESENCE OR ABSENCE OF FATTY ACIDS OR TRIGLYCERIDES

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacteria + Lipid																
6715	-	10.8	3.5	47.7	2.7	2	5.9	22	-	1.3	2	1.9	-	T	73.2	26.6
6715 + 10 µg/ml Lauric Acid	48.5	11.4	4.8	19.8	T	-	10.9	2.1	-	-	T	-	-	-	95.4	2.1
6715 + 2.5 µg/ml Linoleic Acid	-	7.2	9	32	5.8	-	5.2	21.6	15.7	-	-	2.6	-	-	53.4	45.7
6715 + 10 µg/ml Oleic Acid	-	8.9	2	12.7	1.1	-	6.3	64.7	-	-	T	3.2	-	-	29.9	69.4
6715 + 10 µg/ml Trilaurin	1.5	6.8	6.2	36.8	2	12.6	3.7	19.8	-	3.9	1.4	3.4	-	1.4	74.3	25.2
6715 + 10 µg/ml Trilinolein	-	6.4	5.5	41.4	1.4	1.8	4.5	19.2	8.6	T	1.6	3	-	4	66	32.2

in the presence of trilaurin or trilinolein. The change in the percentage of total saturated and unsaturated fatty acids present is partially a reflection of the change in the percentage of the added fatty acid or fatty acid from the triglyceride and partially to adjustments in the other fatty acid percentages to accommodate the incorporation or increases in a specific fatty acid.

The large increases detected by gas chromatography in the lauric, linoleic and the octadecenoic fatty acids is also reflected as a substantial increase in the total weight percent of the neutral fraction when 6715 is grown in their presence (see Table 4).

Table 15 shows the fatty acid profiles for the neutral lipid fraction of W.T. 6715 and for the non-plaque forming mutants, SM19, SM23 and SM24A . Two of the mutants, SM19 and SM23, have percentages of total saturated and unsaturated fatty acids that are quite different from the parental 6715 but that are very similar to each other. SM19 achieves these percentages by using a greater spectrum of fatty acids (C-17 and C-22) than does SM23. The higher levels of total unsaturated fatty acids observed in these two mutants is also seen when WT. 6715 is grown in the presence of linoleic or oleic acid (Table 4). SM24A has a total saturated and unsaturated fatty acid percentage nearly identical to 6715 but has done so by using different percentage of myristic, pentadecanoic, palmitic, heptadecanoic, nonadecanoic, eicosanoic and eicosenoic acid. Nonadecanoic is present in the neutral fraction of WT. 6715, 6715 grown in the presence of trilaurin or trilinolein and in all three non-plaque forming mutants. This fatty acid does not appear in their glycolipid or phospholipid fraction nor is it present in the neutral, glycolipid or phospho-

Table 15.

FATTY ACID CONTENT OF THE NEUTRAL LIPID FRACTION OF *S. MUTANS* 6715 AND OF ITS NON-PLAQUE FORMING MUTANTS.

The fatty acid content of the neutral lipid fraction from column chromatography of *S. mutans* 6715 and its non-plaque forming mutants was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the average values obtained from three separate preparations. Each preparation was analyzed in duplicate. T = < 1%.

a. vaccenic and oleic acid

Table 15

FATTY ACID CONTENT OF THE NEUTRAL LIPID FRACTION OF S. MUTANS 6715 AND OF ITS NON-PLAQUE FORMING MUTANTS

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacterial Strain																
6715	-	10.8	3.5	47.7	2.7	2	5.9	22	-	1.3	2	1.9	-	-	73.2	26.6
SM 19	-	17.3	13.6	13.9	6.8	3.2	6.5	14.6	-	1.8	5.2	15.1	-	T	61.5	36.5
SM 23	-	8.6	1.6	41.6	2.6	-	5.1	27.6	-	2.6	4.9	5.2	-	-	64.4	35.4
SM 24A	-	3	12.6	38.4	1	4.2	6.2	21.5	-	2.1	5	4.8	-	-	71.5	27.3

lipid fractions of 6715 grown in the presence of free fatty acids.

The fatty acid compositions of the glycolipid fractions of WT. 6715, and 6715 grown in the presence of lauric, linoleic, oleic acid, trilaurin or trilinolein are presented in Table 16. The most striking difference between these fractions and the neutral lipid fractions are the large decreases in the percentages of the particular exogenous fatty acids that are incorporated into the glycolipids. This decrease is most marked when 6715 is grown in the presence of lauric acid. The percentage of lauric acid in the neutral fraction was 48.5% while the glycolipid fraction was only 2.9%. Similar decreases, although not as great, are seen with both linoleic and oleic acid. Lauric acid and linoleic acid which were detectable in the neutral fraction when 6715 was grown in the presence of trilaurin and trilinolein, are not detected in the glycolipid fraction when 6715 is grown in their presence. The remainder of the changes that occur when 6715 is grown in the presence of lauric, linoleic or oleic acid are quite diverse and do not appear to follow any obvious pattern. The only apparent link that these changes have is that the shifts in the percentages of the fatty acids occur to accommodate the incorporation of the fatty acid in whose presence WT. 6715 is grown. The changes in the individual fatty acid percentages for 6715 grown in the persence of trilaurin or trilinolein varied less than 2% in most cases from the wild type. These changes are not considered to be significant.

The data presented in Table 17 is the fatty acid composition of the glycolipid fraction of the three non-plaque forming mutants of S. mutans 6715. These mutants all differ from the wild type by having a lower

Table 16.

FATTY ACID CONTENT OF THE GLYCOLIPID FRACTION OF S. MUTANS 6715 GROWN IN THE PRESENCE AND ABSENCE OF FATTY ACIDS OR TRIGLYCERIDES.

The fatty acid content of the glycolipid fraction from column chromatography of S. mutans 6715 grown in the presence or absence of fatty acids or triglycerides was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the average values obtained from three separate preparations. Each preparation was analyzed in duplicate. T = < 1%.

a. vaccenic and oleic acid

Table 16

FATTY ACID CONTENT OF THE GLYCOLIPID FRACTION OF S. MUTANS 6715 GROWN IN THE PRESENCE AND ABSENCE OF FATTY ACIDS OR TRIGLYCERIDES

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacteria + Lipid 6715	-	16.6	1.2	40.5	1.2	-	4.6	24.2	-	-	T	10.2	-	-	62.9	35.6
6715 + 10 µg/ml Lauric Acid	2.9	28.9	T	39.8	3.9	-	3.3	14.1	-	-	-	6.5	-	-	74.5	24.5
6715 + 2.5 µg/ml Linoleic Acid	-	17.2	1.4	38.3	3.7	-	4	18	7.4	-	-	7.8	2.1	-	60.9	39.0
6715 + 10 µg/ml Oleic Acid	-	7.9	T	31.7	2.8	-	4.5	34.9	-	-	T	16.9	-	-	44.1	54.6
6715 + 10 µg/ml Trilaurin	-	15.4	1.6	38.6	T	-	3.8	28.2	-	-	-	12	-	-	59.4	40.2
6715 + 10 µg/ml Trilinolein	-	15.6	T	40	T	-	4.4	26	-	-	T	11	-	-	60	37

Table 17.

FATTY ACID CONTENT OF THE GLYCOLIPID FRACTION OF *S. MUTANS* 6715 AND ITS NON-PLAQUE FORMING MUTANTS.

The fatty acid content of the glycolipid fraction from column chromatography of *S. mutans* 6715 and its non-plaque forming mutants was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the average values obtained from three separate preparations. Each preparation was analyzed in duplicate. T = < 1%.

a. vaccenic and oleic acid

Table 17

FATTY ACID CONTENT OF THE GLYCOLIPID FRACTION OF S. MUTANS 6715 AND ITS NON-PLAQUE FORMING MUTANTS

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacterial Strain																
6715	-	16.6	1.2	40.5	1.2	-	4.6	24.2	-	-	T	10.2	-	-	62.9	35.6
SM 19	-	5.8	2.2	32.4	1.7	-	5.4	35	-	-	-	17.8	-	-	45.8	53.9
SM 23	-	10.5	-	41.2	T	-	4.2	30	-	-	T	13.4	-	-	55.9	43.4
SM 24A	-	8.6	3.3	35.4	T	T	7	26.1	-	-	T	18	-	-	54.3	44.1

percentage of myristic acid, and higher percentage of octadecenoic and eicosenoic acid. The mutants also have a higher percentage of total unsaturated fatty acids than the parental strain. When WT. 6715 is grown in either the presence of linoleic or oleic acid, the glycolipid fraction has a larger percent of unsaturated fatty acids than does the wild type (Table 16). However, only when 6715 is grown in the presence of oleic acid does its glycolipid fraction follow the same trend observed with the non-plaque forming mutants; decreased myristic acid and increased octadecenoic and eicosenoic acid.

Table 18 contains the data for fatty acid composition of the phospholipid fraction for parental 6715, and 6715 grown in the presence of the free fatty acids or triglycerides. The percentage of the exogenously supplied lauric, linoleic or oleic in the neutral lipid fraction is greater than that observed in the glycolipid or phospholipid fraction. The percentages of the exogenously supplied fatty acids are very similar in these two fractions. The changes observed in the phospholipid fraction when 6715 is grown in the presence of lauric, linoleic or oleic acid are quite varied, with the greatest amount of change occurring when lauric acid is used. The changes observed in individual fatty acid percentages when 6715 is grown in the presence of trilaurin or trilinolein are quite small and differ only marginally from the wild type.

Two changes of interest are seen to occur in the glycolipid and phospholipid fractions, one involving parental 6715 grown in lauric acid and the other when it is grown in the presence of linoleic acid. When cultures of 6715 are supplemented with lauric acid only low levels

Table 18.

FATTY ACID COMPOSITION OF THE PHOSPHOLIPID FRACTION OF S. MUTANS 6715 GROWN IN THE PRESENCE OR ABSENCE OF FATTY ACIDS OR TRIGLYCERIDES.

The fatty acid content of the phospholipid fraction from column chromatography of S. mutans 6715 grown in the presence or absence of fatty acids or triglycerides was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the average values obtained from three separate preparations. Each preparation was analyzed in duplicate. T = < 1%.

a. vaccenic and oleic acid

Table 18

FATTY ACID COMPOSITION OF THE PHOSPHOLIPID FRACTION OF S. MUTANS 6715 GROWN IN THE PRESENCE OR ABSENCE OF FATTY ACIDS OR TRIGLYCERIDES

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacteria + Lipid																
6715	-	15.5	2.4	36.8	1	-	4	28.6	-	-	T	11	-	-	58.7	40.6
6715 + 10 µg/ml Lauric Acid	2.4	24.5	T	42.2	4.3	-	3.3	17.5	-	-	-	5	-	-	72.4	26.8
6715 + 2.5 µg.ml Linoleic Acid	-	16	1.1	38	4.3	-	3.8	20	8.1	-	-	6.6	1.8	-	58.9	40.8
6715 + 10 µg/ml Oleic Acid	-	7.4	T	27.8	3.5	-	4.8	42.5	-	-	T	13	-	-	40	59
6715 + 10 µg/ml Trilaurin	T	16.1	2.7	38.2	T	T	3.8	27.8	-	-	-	9.8	-	-	60.8	37.6
6715 + 10 µg/ml Trilinolein	-	13.1	1.4	42	T	-	4.2	28.6	T	-	T	9.6	-	-	60.7	38.2

(< 3%) of incorporation are observed in the glycolipid and phospholipid fractions. These lower levels of lauric acid are accompanied by approximately a 10% increase in the myristic acid present. This finding suggests that the exogenously added lauric acid is being elongated before its incorporation into the individual lipids in these fractions.

The other change of interest occurs when 6715 is grown in the presence of linoleic acid. This change involves the appearance of eicosadienoic acid in the phospholipid and glycolipid fractions. This fatty acid is also detected in the total fatty acid profile of WT. 6715 grown in the presence of linoleic acid. It does not appear in the non-plaque forming mutants SM19, SM23 and SM24A of S. mutans 6715. As indicated previously, it was initially thought that eicosadienoic acid might be related to the antiplaque activity of linoleic acid, but this does not appear to be the case.

The date in Table 19 show the fatty acid composition of the phospholipid fraction of the mutants, SM19, SM23 and SM24A. A pattern analogous to that observed in the mutant's glycolipid fraction occurs in the phospholipid fraction; a decrease in the percentage of myristic acid present and an increase in the percentage of octadecenoic and eicosenoic acid present. As in the glycolipid fraction, the mutants' phospholipid fraction has a greater percentage of unsaturated fatty acids. The phospholipid fraction from 6715 grown in the presence of oleic acid follows a similar pattern with respect to the aforementioned fatty acids.

The data in Tables 20, 21 and 22 are summaries of the data from the previous six tables. These tables (20, 21 and 22) combine the neutral, glycolipid and phospholipid fraction fatty acid profiles so that these

Table 19.

FATTY ACID COMPOSITION OF THE PHOSPHOLIPID FRACTION OF *S. MUTANS* 6715 AND ITS NON-PLAQUE FORMING MUTANTS.

The fatty acid content of the phospholipid fraction from column chromatography of *S. mutans* 6715 and its non-plaque forming mutants was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the average values obtained from three separate preparations. Each preparation was analyzed in duplicate. T = < 1%.

a. vaccenic and oleic acid

Table 19

FATTY ACID COMPOSITION OF THE PHOSPHOLIPID FRACTION OF S. MUTANS 6715 AND ITS NON-PLAQUE FORMING MUTANTS

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacterial Strain																
6715	-	15.5	2.4	36.8	1	-	4	28.6	-	-	T	11	-	-	58.7	40.6
SM 19	-	6	1	36.4	2	-	4.8	35.7	-	-	-	13.7	-	-	48.2	51.4
SM 23	-	8.9	-	40.2	T	-	3.9	35.2	-	-	T	10.8	-	-	53	46
SM 24A	-	6.6	2.1	36.2	T	T	8	29.8	-	-	T	15.7	-	-	52.9	45.5

Table 20.

FATTY ACID CONTENT OF THE NEUTRAL LIPID, GLYCOLIPID AND PHOSPHOLIPID FRACTIONS OF S. MUTANS 6715 GROWN IN THE PRESENCE OR ABSENCE OF TRIGLYCERIDES.

The fatty acid composition of the neutral lipid, glycolipid and phospholipid fractions from column chromatography of S. mutans 6715 grown in the presence or absence of triglycerides was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the average values obtained from three separate preparations. Each preparation was analyzed in duplicate. T = < 1%.

a. vaccenic and oleic acid

Table 20

FATTY ACID CONTENT OF THE NEUTRAL LIPID, GLYCOLIPID AND PHOSPHOLIPID FRACTIONS OF S. MUTANS 6715 GROWN IN THE PRESENCE OR ABSENCE OF TRIGLYCERIDES

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacteria + Lipid																
6715 Neutral	-	10.8	3.5	47.7	2.7	2	5.9	22	-	1.3	2	1.9	-	T	73.2	26.6
6715 Glycolipid	-	16.6	1.2	40.5	1.2	-	4.6	24.2	-	-	T	10.2	-	-	62.9	35.6
6715 Phospholipid	-	15.5	2.4	36.8	1	-	4	28.6	-	-	T	11	-	-	58.7	40.6
6715 + 10 µg/ml Trilaurin Neutral	1.5	6.8	6.2	36.8	2	12.6	3.7	19.8	-	3.9	1.4	3.4	-	1.4	74.3	25.2
Glycolipid	-	15.4	1.6	38.6	T	-	3.8	28.2	-	-	-	12	-	-	59.4	40.2
Phospholipid	T	16.1	2.7	38.2	T	T	3.8	27.8	-	-	-	9.8	-	-	60.8	37.6
6715 + 10 µg/ml Trilinolein Neutral	-	6.4	5.5	41.4	1.4	1.8	4.5	19.2	8.6	T	1.6	3	-	4	66	32.2
Glycolipid	-	15.6	T	40	T	-	4.4	26	-	-	T	11	-	-	60	37
Phospholipid	-	13.1	1.4	42	T	-	4.2	28.6	T	-	T	9.6	-	-	60.7	38.2

Table 21.

FATTY ACID CONTENT OF THE NEUTRAL LIPID, GLYCOLIPID AND PHOSPHOLIPID FRACTIONS OF S. MUTANS 6715 GROWN IN THE PRESENCE OF FATTY ACIDS.

The fatty acid content of the neutral lipid, glycolipid and phospholipid fractions from column chromatography of S. mutans 6715 grown in the presence of fatty acids was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the average values obtained from three separate preparations. Each preparation was analyzed in duplicate. T = < 1%.

a. vaccenic and oleic acid

Table 21

FATTY ACID CONTENT OF THE NEUTRAL LIPID, GLYCOLIPID AND PHOSPHOLIPID FRACTIONS OF S. MUTANS 6715 GROWN IN THE PRESENCE OF FATTY ACIDS

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacteria + Lipid																
6715 + 10 µg/ml Lauric Acid Neutral	48.5	11.4	4.8	19.8	T	-	10.9	2.1	-	-	T	-	-	-	95.4	2.1
Glycolipid	2.9	28.9	T	39.8	3.9	-	3.3	14.1	-	-	-	6.5	-	-	74.5	24.5
Phospholipid	2.4	24.5	T	42.2	4.3	-	3.3	17.5	-	-	-	5	-	-	72.4	26.8
6715 + 2.5 µg/ml Linoleic Acid Neutral	-	7.2	9	32	5.8	-	5.2	21.6	15.7	-	-	2.6	-	-	53.4	45.7
Glycolipid	-	17.2	1.4	38.3	3.7	-	4	18	7.4	-	-	7.8	2.1	-	60.9	39.0
Phospholipid	-	16	1.1	38	4.3	-	3.8	20	8.1	-	-	6.6	1.8	-	58.9	40.8
6715 + 10 µg/ml Oleic Acid Neutral	-	8.9	2	12.7	1.1	-	6.3	64.7	-	-	T	3.2	-	-	29.9	69.4
Glycolipid	-	7.9	T	31.7	2.8	-	4.5	34.9	-	-	T	16.9	-	-	44.1	54.6
Phospholipid	-	7.4	T	27.8	3.5	-	4.8	42.5	-	-	T	13	-	-	40	59

Table 22.

FATTY ACID CONTENT OF THE NEUTRAL LIPID, GLYCOLIPID AND PHOSPHOLIPID FRACTIONS OF THE NON-PLAQUE FORMING MUTANTS OF S. MUTANS 6715.

The fatty acid content of the neutral lipid, glycolipid and phospholipid fractions from column chromatography of the non-plaque forming mutants was determined by gas chromatography. Fatty acid methyl esters were prepared as described in Materials and Methods. The values for each fatty acid are expressed as a percentage of the total fatty acid content and are the average values obtained from three separate preparations. Each preparation was analyzed in duplicate. T = < 1%.

a. vaccenic and oleic acid

Table 22

FATTY ACID CONTENT OF THE NEUTRAL LIPID, GLYCOLIPID AND PHOSPHOLIPID FRACTIONS OF THE NON-PLAQUE FORMING MUTANTS OF S. MUTANS 6715

	Percent of Total Fatty Acid Content															
	12	14	15	16	16:1	17	18	18:1[a]	18:2	19	20	20:1	20:2	22	Sat.	Unsat.
Bacteria + Lipid																
SM 19 Neutral	-	17.3	13.6	13.9	6.8	3.2	6.5	14.6	-	1.8	5.2	15.1	-	T	61.5	36.5
Glycolipid	-	5.8	2.2	32.4	1.7	-	5.4	35	-	-	-	17.8	-	-	45.8	53.9
Phospholipid	-	6	1	36.4	2	-	4.8	35.7	-	-	-	13.7	-	-	48.2	51.4
SM 23 Neutral	-	8.6	1.6	41.6	2.6	-	5.1	27.6	-	2.6	4.9	5.2	-	-	64.4	35.4
Glycolipid	-	10.5	-	41.2	T	-	4.2	30	-	-	T	13.4	-	-	55.9	43.4
Phospholipid	-	8.9	-	40.2	T	-	3.9	35.2	-	-	T	10.8	-	-	53	46
SM 24A Neutral	-	3	12.6	38.4	1	4.2	6.2	21.5	-	2.1	5	4.8	-	-	71.5	27.3
Glycolipid	-	8.6	3.3	35.4	T	T	7	26.1	-	-	T	18	-	-	54.3	44.1
Phospholipid	-	6.6	2.1	36.2	T	T	8	29.8	-	-	T	15.7	-	-	52.9	45.5

fractions may be compared more readily to each other for the WT. 6715, 6715 grown in the presence of free fatty acids or triglycerides or for SM19, SM23 and SM24A. In all cases in which 6715 was grown in the presence of either the free fatty acids of the triglycerides, the percentage of the particular fatty acid or the fatty acid from the triglyceride was greatest in the neutral lipid fraction and between 2 and 20 times less in the glycolipid and phospholipid fractions, if present at all. The percentages in these two fractions were similar. In those organisms (6715, SM19, SM23 and SM24A) not grown in the presence of free fatty acids or triglycerides, the neutral lipid fraction contained the highest percentage of saturated fatty acids. The phospholipid fraction contains a greater percentage of unsaturated fatty acids than did the glycolipid fraction except for SM19 and for 6715 grown in the presence of trilaurin. The three fractions of SM19, SM23 and SM24A all contain a higher percentage of unsaturated fatty acids than do the corresponding fractions of the parental 6715. This suggests that the changes occurring in these mutants that have resulted in the loss of their ability to form plaque also have affected their fatty acid composition.

While these changes in the fatty acid composition of the fractions cannot be directly linked to plaque reduction, it is not unreasonable to expect that the changes in the fatty acid composition of these fractions may alter or effect bacterial structures or functions in which the individual lipids of the fractions are involved and this may indirectly effect plaque production.

IV. DISCUSSION

The ability of free fatty acids to inhibit the growth of various bacteria has been known for several years (Strong and Carpenter, 1942; Kodicek and Worden, 1945; Stephan, 1950; Neiman, 1954; Kabara et al., 1972; Sheu et al., 1972, 1973; Freese et al., 1973; Butcher et al., 1976; and Miller et al., 1977). Kodicek and Worden (1945) proposed that unsaturated fatty acids might inhibit bacteria in either of two ways. The first proposed a direct chemical action upon the metabolism of the bacteria or upon the availability of some metabolite present in the medium. Their second explanation postulated a physicochemical mechanism in which unsaturated fatty acids formed a monolayer around the bacteria and changed the permeability of the adjacent surfaces, exerted some chemical influence or altered the surface tension, thus interfering with bacterial division. Since Kodicek and Worden's initial proposal, several studies have attempted to determine the mechanism of fatty acid inhibition of bacteria.

The works of Sheu et al. (1972, 1973) and Freese et al. (1972) have demonstrated that the inhibition of E. coli and B. subtilis by fatty acids is caused by the uncoupling of substrate transport and oxidative phosphorylation from the electron transport system. Miller et al. (1977) have concluded that the uncoupling of oxidative phosphorylation and the blocking of electron transport or both, are major mechanisms by which

fatty acids inhibit N. gonorrhoeae. Other investigations (Kabara et al., 1972 and Butcher et al., 1976) have not yielded such definitive answers. The lack of a clear cut mechanism for fatty acid inhibition in the 30 bacteria (including several streptococci) studied by Kabara et al. (1972) led them to state that the mode of action of the fatty acids cannot be wholly explained in physicochemical terms, i.e. surface tension activity, and that the explanation probably resides in a more complex mechanism. Butcher et al. (1976) have proposed that the inhibition of Staph. aureus by externally applied unsaturated fatty acids might occur because the incorporation of these fatty acids into the membrane might upset those systems that are sensitive to membrane fluidity. The mechanism of fatty acid inhibition is perhaps best summarized by Kabara's statement; "More quantitative data are needed before any hypothesis concerning the mechanism of fatty acid action against microorganisms can be put forward."

The data presented here show that lauric, linoleic, oleic, and eicosadienoic acid and trilaurin and trilinolein can be both bactericidal and bacteriostatic for S. mutans 6715 and that the free fatty acids are more inhibitory than the triglycerides. These findings are in agreement with reports that show free fatty acids are more inhibitory than their esterified derivatives (Kabara et al., 1972). Lauric, linoleic and oleic acid were chosen for this study because unpublished studies by Schuster, Lankford, Kearse and McChesney indicated that these fatty acids reduced dental plaque formation in vitro as well as in vivo while palmitic and myristic acid did not. Palmitic and myristic acid are normal constituents of S. mutans 6715 lipids while lauric and linoleic acid are not (Lambert and Moss, 1976). Oleic acid has been

shown to comprise less than 19% of the octadecenoic acid present in S. mutans 6715 (Szabo et al., 1978). Eicosadienoic acid was chosen because initial studies showed the presence of a fatty acid containing twenty carbons and two double bonds when WT. 6715 was grown in the presence of linoleic acid. It was postulated that this fatty acid might arise from the elongation of linoleic acid and might be related to the antiplaque activity of linoleic acid. The data presented in Table 2 does not confirm this role for eicosadienoic acid. Trilaurin and trilinolein were used to determine if their effects on fatty acid content were similar to that of free lauric and linoleic acid.

Anticaries acitivity in animals by fats and oils has been reported by several authors (Schweigert et al., 1946; Granados et al., 1949; Shaw, 1950; Wynn et al., 1953; Gustafson et al., 1953, 1955; Constant et al., 1954; McClure et al., 1956; Bavetta, 1959). The acitivity of these fats and oils was thought to occur by coating either the bacteria or the surface to which they adhere (Kodicek and Worden, 1945). The data reported here demonstrates that free lauric, linoleic or oleic acid can reduce the amount of plaque formed in vitro by S. mutans 6715 at concentrations that are well below their respective bacteriostatic concentrations and suggests that reduction of plaque formation in vivo might account for some of the anticaries activity seen.

In order to determine if a direct relationship exists between bacterial fatty acid content and plaque production, a series of experiments was performed which showed that the fatty acid profile of wild type S. mutans 6715 and non-plaque forming mutants of this parental strain are quite different from each other and that both the wild type and mutants

retain the ability to incorporate exogenously added fatty acids into their bacterial lipids (Tables 7, 12 and 13).

The total fatty acid profiles presented here for WT. 6715 are like those reported by Lambert and Moss (1976) and are very similar to those of Szabo et al. (1978). However, the results of Szabo et al. (1978) differ from ours and Lambert and Moss' in that Szabo and co-workers reported the presence of trace amounts (less than 2%, no values given) of cyclic fatty acids and lauric acid in WT. 6715. The small amounts of lauric acid present are probably from either breakdown products of longer chain fatty acids or from the uptake of preformed fatty acids from the medium.

Cyclic fatty acids in bacteria have been reported by Law et al. (1963) to accumulate in the stationary phase of growth, thus the presence of cyclic fatty acids in S. mutans 6715, as reported by Szabo et al. (1978) has been attributed by these investigators to the use of a longer growth period. The length of time the organisms were allowed to grow was not stated, only that cells were harvested from the stationary phase of growth. Our studies did not reveal the presence of cyclic fatty acids in the total or fraction fatty acid profiles of S. mutans 6715.

Neither Lambert and Moss (1976) , Szabo et al. (1978), nor our own data confirm the presence of polyunsaturated fatty acids as reported by Sharma and Newbrun (1975) or of significant amounts of branched chain fatty acids as reported by Drucker et al. (1973, 1974) and Drucker and Veazey (1977) in S. mutans. Polyunsaturated fatty acids are not common in bacteria (Lehninger, 1975). The presence of the polyunsaturated

fatty acids in S. mutans B-14, as reported by Sharma and Newbrun (1975), is most likely the result of the uptake and incorporation by the bacteria of these preformed fatty acids from the medium.

The presence of significant amounts of branch chain fatty acids in S. mutans reported by Drucker (1973, 1974) and Drucker and Veazey (1977) has not been confirmed by either Szabo et al. (1978) or the present study. Lambert and Moss (1976) reported the presence of trace amounts (less than 2%) of branched chain fatty acids in S. mutans, but no indication was given as to which strains of the 18 strains of S. mutans they analyzed contained them. Drucker et al. (1973, 1974) and Drucker and Veazey (1977) reported the presence of these branched chain fatty acids in only three strains of S. mutans (JC2, D282 and NCTC 10832). The presence of such fatty acids in S. mutans may be the result either of uptake and incorporation by the bacteria of these fatty acids from the medium or they may be present only in certain strains of S. mutans.

We also have been able to demonstrate that when parental 6715 is grown in the presence of any of the three plaque reducing fatty acids, the total fatty acid profile as well as the individual fatty acid profiles of the neutral, glycolipid and phospholipid fractions are changed and that some of these changes mimic the patterns of the non-plaque forming mutants, i.e. an increased total percentage of unsaturated fatty acids is present in the neutral, glycolipid and phospholipid fractions as well as the total profile when WT. 6715 is grown in the presence of linoleic or oleic acid. Increases in octadenenoic and eicosenoic acid in these fractions and in the total fatty acid profile are also observed

when WT. 6715 is grown in the presence of oleic acid. The incorporation of these free fatty acids into the bacterial lipids has been reported by Sinensky (1971), Goldfine et al. (1976) and Umesaki et al. (1977). The particular alterations that the incorporation of lauric, linoleic or oleic acid brought about were not totally expected and appeared to be partially a reflection of the change in the percentage of the exogenously added fatty acid and partially an adjustment by the bacteria in the other fatty acid percentages in order to accommodate the incorporation or increases in a specific fatty acid.

The most pronounced change occurred in the neutral lipid fraction when 6715 was grown in the presence of fatty acids or triglycerides and was expressed as a large increase in the percent of the particular exogenously added fatty acid.

The neutral lipid fraction of WT. S. mutans 6715 and WT. 6715 grown in the presence of trilaurin or trilinolein and the non-plaque forming mutants, SM19, SM23 and SM24A, all contain nonadecanoic acid. Nonadecanoic acid was absent from the neutral lipid fraction of WT. 6715 grown in the presence of lauric, linoleic or oleic acid. This fatty acid was not detected in the glycolipid or phospholipid fractions nor in the total fatty acid profile of WT. 6715, WT. 6715 grown in the presence of free fatty acids or triglycerides, or in the non-plaque forming mutants.

The absence of nonadecanoic acid from the total fatty acid profiles is most likely due to an inability to detect the small amounts of this fatty acid present. The neutral lipid fraction of all the organisms tested contained less than 4% of the total lipid present and of this 4%,

only approximately 2% was nonadecanoic acid (.08% of the total cellular lipid). The only exceptions to this occurred when WT. 6715 was grown in the presence of free fatty acids. The neutral lipid fractions of these cultures contained approximately 12% of the total cellular lipid, a large portion of which is most likely due to the high levels of the exogenously added fatty acids present (Table 14). The high concentrations of these exogenously added fatty acids may either mask the presence of nonadecanoic acid or those organisms grown in the presence of lauric, linoleic or oleic acid may not elongate pentadecanoic acid. The absence of heptadecanoic acid from the total fatty acid profile as well as from the fraction fatty acid profiles from WT. 6715 grown in the presence of lauric, linoleic or oleic acid add support to the second possibility.

The lack of nonadecanoic acid from all of the glycolipid and phospholipid fractions would seem to indicate that this fatty acid occurs mainly as the free fatty acid and not as an esterfied derivative. The failure to detect the incorporation of this saturated fatty acid into all the glycolipid and phospholipid fractions may be due to either its very low intracellular concentration and/or to the general trend of decreased incorporation of saturated fatty acids into the glycolipid and phospholipid fractions.

Neither the total fatty acid profile of S. mutans 6715 grown in the presence of plaque reducing fatty acids nor the fatty acid profiles of the individual neutral, glycolipid and phospholipid fractions showed patterns with characteristics consistent enough to demonstrate a direct relationship between the fatty acid content of 6715 and its ability to

form plaque. The data does, however, suggest that the fatty acid content might influence plaque production indirectly. The evidence for this is as follows: The non-plaque forming mutants have fatty acid profiles that are similar to each other but quite different from the parental 6715. (All of the non-plaque forming mutants have a greater percent of fatty acids in the unsaturated form than do the plaque forming wild type. The three non-plaque forming mutants also have an increased percentage of octadecenoic and eicosenoic acid present and a decrease in the percentage of myristic and palmitic acid present.) Lauric, linoleic and oleic acid all reduce the accumulation of plaque in vitro at concentrations that are well below their bacteriostatic concentrations and fatty acid changes in major lipid fractions (glycolipids and phospholipids) do occur when 6715 is grown in the presence of the plaque reducing fatty acids.

Preliminary evidence to support the indirect involvement of fatty acids in reducing plaque production was provided by McChesney et al., (1977). They were able to show an increase in the total extracellular glucosyltransferase activity, an enzyme required for plaque production, present in culture medium when cultures of 6715 were grown in either the presence of lauric or linoleic acid. Oleic acid was not tested. They postulated that the increase in total extracellular glucosyltransferase activity could be due to an alteration in the membrane's ability to control or regulate the release of glucosyltransferase, or that which becomes associated with the cell surface. They further state that a decrease in bound enzyme could reduce the organism's ability to form plaque.

Harlander and Schachtele (1978) have reported the lysophosphatidyl choline (LPC) stimulates the activity of the glucosyltransferase enzyme and increases glucan formation. They proposed that the high affinity of LPC for glucosyltransferase may be due to a physical property of phosphoglycerides (a polar hydrophilic head and a non-polar hydrophobic tail) and that this physical property may give rise to the multiple forms of the enzyme isolated during purification, variations in the forms of the enzyme produced under various growth conditions and the ability of partially purified enzyme to attach specifically to S. mutans cell surface. It was further proposed that the enzyme could associate with phospholipids during or after release from the cell. The increased levels of glucosyltransferase activity they report also were observed by McChesney et al. (1977) for 6715 grown in the presence of either lauric or linoleic acid and by Wittenberger et al. (1978) when WT. 6715 was grown in the presence of Tween 80.

The growth of S. mutans 6715 in the medium of Jordan et al. (1960) containing 2% w/v glucose, produces a final extracellular pH of between 5 and 5.5. The internal pH of WT. 6715 is postulated to be higher than the external pH (Kashket and Wong, 1969). At these pHs, a large portion of the exogenously added fatty acid has both a polar and a non-polar end, and in this respect is similar to one of the physical properties of phosphoglycerides. If Harlander and Schachtele's (1978) proposal that LPC can bind to glucosyltransferase because of a polar head and non-polar tail and thus activate the enzyme is correct, then it is possible that fatty acids with a polar and a non-polar end may be able to bind to these same sites on the enzyme. The binding of the fatty acids to

the enzyme is postulate to prevent or decrease the amount of binding of LPC to the glucosyltransferase. This postulated decrease in binding of LPC may relate to lauric, linoleic and oleic acids' ability to reduce plaque formation *in vitro*.

The association of fatty acid with enzyme may reduce the enzyme's ability to associate with the *S. mutans* 6715 cell. This could manifest itself as an increase in total extracellular glucosyltransferase when in fact it was decreased attachment. Spinell and Gibbons (1974) have shown that a decrease in cell bound glucosyltransferase decreases the amount of glucan, a major component of dental plaque, which the *S. mutans* cells can bind and thus indirectly reduces plaque. A reduction in the activity of glucosyltransferase also would reduce the amount of glucan formed and be manifest as decreased dental plaque production.

The explanation of the ability of lauric acid, linoleic acid and oleic acid to reduce plaque formation at concentrations 50% or more below their MBC while eicosadienoic acid and the triglycerides trilaurin and trilinolein do not, may be related to their configuration as well as to their being present within *S. mutans* 6715 in a large excess (see Table 14). The configuration of linoleic acid (the most effective plaque reducing fatty acid) with its two cis double bonds and lauric acid (the next most effective fatty acid) with its twelve carbon chain are completely different from any fatty acid normally present in WT. 6715. Oleic acid, the least effective antiplaque fatty acid of the three, differs from vaccenic acid (the major octadecenoic acid of *S. mutans* (Szabo et al., 1978)) in only the position of the double bond, cis 9 versus cis 11. Thus, it may be that the configuration of these

three fatty acids which are different from the fatty acids normally present in S. mutans 6715 allows them to more readily associate with the glucosyltransferase enzyme and that the presence of excess amounts of them in the free form favors this association.

It is further suggested that eicosadienoic acid, (which has a configuration around the double bonds similar to that of linoleic acid but is two carbons longer) is not as effective an antiplaque agent because the increased carbon chain length hinders its association with the glucosyltransferase enzyme. Trilaurin and trilinolein may not be as effective as lauric and linoleic acid because of their failure to obtain sufficiently high intracellular concentrations to favor their association with the glucosyltransferase enzyme.

This explanation of how fatty acids might reduce dental plaque is consistent with the data in Tables 3, 8 and 9 that indicate that once a fatty acid concentration of less than 30% below the PIC is reached, there is little change in the fatty acid profile until the PIC concentration or higher is reached, and any change that does occur is mainly in the exogenously added fatty acid.

Using the above theory to explain the data in these tables would suggest that the exogenously supplied fatty acids are readily incorporated into bacterial structures until an exogenous fatty acid concentration 30% below the PIC is reached. At this point the bacterial cell begins accumulating an excess of the exogenously added free fatty acid until a level is reached at which the excess is able to associate with nearly all the intracellular glucosyltransferase enzymes (the PIC concentration) and thus reduce their activity. These enzymes are then

released from the bacterial cell with the fatty acids still associated, or free enzyme may associate with extracellular fatty acid. Such an association with fatty acid may prevent activation of the enzyme by either intracellular or extracellular LPC (Harlander and Schachtele, 1978). The difference in a fatty acid's ability to become associated with the enzyme or to reach specific intracellular levels could account for the differences in PIC's.

The above assumes that fatty acids, or for that matter LPC, do not directly affect the synthesis of the glucosyltransferase enzyme but that they merely act on preformed enzyme. Wittenberger et al. (1978) and Umesaki et al. (1977) have suggested that Tween 80 (polyoxyethylene sorbitan monooleate) stimulates the synthesis of glucosyltransferase either directly or indirectly. To accept this hypothesis, one must also be willing to consider that the glucosyltransferase enzyme produced by oral streptococci is not a constitutive enzyme as suggested by Gibbons and Nygaard (1968); Guggenheim and Newbrun (1969) and Carlsson et al. (1969). There is evidence to support the contention that the glucosyltransferase enzyme is not a constitutive enzyme (Carlsson and Elander, 1973; Janda and Kuramitsu, 1976; Schachtele et al., 1976) and thus its synthesis might be regulated in some unrecognized manner by the fatty acids.

Wittenberger et al. (1978) have been able to show that Tween 80 which increased extracellular glucosyltransferase (Wittenberger et al., 1978; Umesaki et al., 1977) does not act directly on the enzyme to stimulate catalytic activity nor does it appear to act on S. salivarius exclusively by altering cell permeability. They also showed that Tween

80 enhanced the rate of glucosyltransferase synthesis to a greater extent than fructosyltransferase and that the stimulation by Tween 80 was not a general effect on all protein synthesis. Tween 20 (polyoxyethylene sorbitan monolaurate) also increased extracellular glucosyltransferase levels. Sodium oleate (10 μg/ml) was reported not to stimulate glucosyltransferase levels but no actual data were given. They concluded from these findings that Tween 80 either directly or indirectly affected the synthesis of glucosyltransferase in S. salivarius. However, they could not totally exclude a direct effect of Tween 80 on the membrane and thus rule out that Tween 80 might facilitate the release of intracellular glucosyltransferase, preventing the enzyme from regulating its own synthesis.

Like Tween 80, Tween 20 also increases extracellular levels of glucosyltransferase (Wittenberger et al., 1978). Since a major component of Tween 80 and Tween 20 is their fatty acid (oleic and lauric acid respectively) and preliminary findings by McChesney et al. (1977) indicated that linoleic and lauric acid both stimulate extracellular glucosyltransferase levels, it is reasonable to postulate that the fatty acid moieties may be responsible for the increase in glucosyltransferase levels observed with the Tweens. If this is the case, then it is equally likely that the free fatty acids could increase extracellular glucosyltransferase levels by a similar mechanism. Neither Wittenberger et al. (1978) nor Umesaki et al. (1977) have been able to link the increased levels of glucosyltransferase produced by S. mutans in the presence of Tween 80 to increased plaque production either in vitro or in vivo. Similarly, McChesney et al. (1977) showed that two of the most effective

antiplaque fatty acids also increase the level of extracellular glucosyltransferase.

The explanation of this apparent contradiction may be related to a particular fatty acid's ability to stimulate or prevent the regulation of the enzyme's synthesis, thereby allowing more enzyme to be produced, and/or facilitate its release from the cell. Effective antiplaque fatty acids (lauric, linoleic and oleic acid) may alter the enzyme whose production it stimulates and by so doing decrease the glucosyltransferase's ability to adhere to the bacterial cell. Those lipids which are not effective antiplaque agents (eicosadienoic acid, trilaurin and trilinolein) may not effectively alter glucosyltransferase levels because of configuration or chain length (eicosadienoic acid) or because they do not reach sufficiently high intracellular levels (trilaurin and trilinolein). It is unlikely that any fatty acid normally found in S. mutans would affect glucosyltransferase levels and plaque forming ability in this manner unless it was involved in the control of the synthesis of glucosyltransferase. A control mechanism of this type has not been found.

Another possible explanation of how fatty acids reduce plaque accumulation could be that those that reduce plaque formation have a greater ability than non-inhibitory ones to bind or associate with sites on the bacterial cell that are adjacent to the glucosyltransferase binding sites, thus impairing binding of the glucosyltransferase enzyme, possibly through steric hindrance. The result of this would be less bound enzyme which Spinnell and Gibbons (1974) have shown leads to less glucan formation and less plaque. The functioning of lauric, linoleic

and oleic acid in this manner would be consistent with the preliminary data of McChesney et al. (1977), and it is not in conflict with the data of Harlander and Schachtele (1978) which suggests that LPC binds to the enzyme and not the bacterial cell.

It appears that free fatty acids do not inhibit plaque formation by producing an altered lipid profile in WT. 6715, although it does appear that there is an increased level of unsaturated fatty acids present in WT. 6715 under plaque reducing conditions and in non-plaque forming mutants. Possible explanations for the mechanism by which fatty acids reduce plaque formation are: 1) specific free fatty acids could bind to the enzyme and reduce the enzyme's ability to associate with the S. mutans 6715 cell; 2) particular free fatty acids may stimulate or prevent the regulation of the synthesis of glucosyltransferase thereby allowing more enzyme to be produced, and/or facilitate its release from the cell; effective antiplaque fatty acids may alter the enzyme and by so doing, decrease its ability to adhere to the bacterial cell; 3) fatty acids that reduce plaque accumulation could have a greater ability than non-inhibitory ones to bind or associate with sites on the bacterial cell that are adjacent to the glucosyltransferase binding sites, thus impairing binding of the enzyme by steric hindrance. These possible explanations are not inconsistent with our data and the findings of others.

V. SUMMARY

1. Lauric, linoleic, oleic and eicosadienoic acid and the triglycerides, trilaurin and trilinolein can be both bactericidal and bacteriostatic for *S. mutans* 6715.

2. Free fatty acids are bactericidal and bacteriostatic at lower concentrations than their esterified derivatives.

3. Visual plaque score and plaque dry weight can be correlated, validating the visual scoring method.

4. Lauric, linoleic and oleic acid reduce the amount of plaque formed *in vitro* by *S. mutans* 6715 at concentrations that are below their respective bacteriostatic concentrations.

5. The fatty acid profile of *S. mutans* 6715 was determined and is in agreement with published data.

6. The fatty acid profiles of non-plaque forming mutants of WT. 6715 are quite different from the profile of WT. 6715.

7. Exogenously added lauric, linoleic, oleic, palmitic and eicosadienoic acid and the triglycerides, trilaurin and trilinolein all alter the fatty acid content of WT. 6715.

8. Exogenously added linoleic acid results in the production of a fatty acid containing twenty carbons and two double bonds. This fatty acid is not normally present in WT. 6715.

9. Exogenously added palmitic and linoleic acid both alter the fatty acid profile on the non-plaque forming mutants (SM19, SM23 and SM24A) of WT. 6715.

10. Incorporation of exogenous fatty acids into S. mutans 6715 rises rapidly until a concentration of fatty acid approximately 30% below the plaque inhibitory concentration is reached. At this point, the amount of incorporation slows and most of the subsequent changes are reflected as an increase in the percentage of the exogenously added fatty acid.

11. Free fatty acids are incorporated into S. mutans in greater amounts than are the fatty acids from the comparable triglycerides.

12. Quantitatively lipids of the glycolipid class account for the majority of the lipids present in WT. 6715, in the non-plaque forming mutants, SM19, SM23 and SM24A, and in WT. 6715 grown in the presence of either lauric acid, linoleic acid, oleic acid, trilaurin or trilinolein. Phospholipids comprise the next most abundant class followed by the neutral lipids.

13. Glucose is the only sugar present in lipids of the glycolipid class for the organisms and additives mentioned in #12.

14. The glycolipid fraction contains two lipids and quantitatively diglucosyl diglyceride is the most abundant lipid in this fraction for the organisms and additives mentioned in #12.

15. The phospholipid fraction contains five phospholipids. Quantitatively diphosphatidyl glycerol is the most abundant, comprising 52 to 79% of the phospholipid fraction for the organisms and additives mentioned in #12.

16. Lauric, linoleic or oleic acid and the fatty acids from trilaurin and trilinolein appear as a higher percentage in the neutral fraction than in the glycolipid or phospholipid fractions.

17. The neutral lipid fraction of the non-plaque forming mutants of WT. 6715 is different from that of the parental 6715.

18. Lauric, linoleic and oleic acid are incorporated into the glycolipid and phospholipid fractions of S. mutans 6715 when the organism is grown in their presence.

19. S. mutans 6715 incorporates less than 1% of the fatty acids from either trilaurin or trilinolein into either its glycolipid or phospholipid fractions.

20. The glycolipid and phospholipid fractions of the non-plaque forming mutants of WT. 6715 have a higher percentage of total unsaturated fatty acids than do the parental 6715.

21. Neither the total fatty acid profile of S. mutans 6715 grown in the presence of plaque reducing fatty acids nor the fatty acid profiles of the individual neutral, glycolipid and phospholipid fractions could demonstrate that an obvious direct relationship existed between the fatty acid content of S. mutans 6715 and its ability to form plaque.

22. The data does, however, suggest that the fatty acid content might influence plaque production by this organism indirectly.

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