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DETERMINATION OF STREPTOCOCCAL-HYALURONIDASE IN HUMAN CARIOUS DENTIN

By

Saryubala Parikh

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A Thesis Submitted to the Faculty of the Graduate School of Loyola University in Partial Fulfilment of the Requirements for the Degree of Master of Science

June
1964

LIFE

Saryubala Parikh was born at Balasinor, India on December 30, 1928. Her elementary education was received at Vanita Vishram Girls' High School (Bombay), and her secondary education was received at the Modern High School (Bombay).

From June, 1943 to May, 1945, she attended Khalsa College (Bombay), and finished her Intermediate Science Course. From June 1945 to May 1947, attended to St. Xavier's College (Bombay) and finished Bachelor of Science (Honors) in Microbiology as principal, and Zoology as subsidiary subject. From June, 1950 to 1954, she attended Sir C. E. M. Dental College and Hospital (Bombay), and obtained the degrees of B. D. S. and L. D. S. C. P. S.

From 1954 to 1956, she practiced general dentistry in Bombay and also served part time at Suruodaya Hospital (Bombay). In the year 1956, she joined Sir C. E. M. Dental College and Hospital as a junior lecturer in the Department of Pathology and Bacteriology, and from 1958 until 1961, she also served in Department of Periodontia.

From September 1961 to May, 1962, she was a post graduate student in dentistry at the University of Illinois, where she also did research work under Dr. Massler and Dr. Bahn (Northwestern University), and published a paper on "Microorganisms in Active and Arrested Carious Lesions of Dentin."

She started the graduate studies at Loyola University, School of Dentistry in June, 1962.

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TABLE OF CONTENTS

Chapter		Page
I.	INTRODUCTION.	1
II.	REVIEW OF THE LITERATURE.	3
III.	MATERIALS AND METHODS.	24
IV.	RESULTS AND FINDINGS.	31
V.	DISCUSSION.	40
VI.	SUMMARY AND CONCLUSIONS	47
BIBLIOGRAPHY.		50
APPENDIX.		58

CHAPTER I

INTRODUCTION

According to recent literature, streptococci are considered to be a major causative group of microorganisms of deep dentinal caries, but their modes of action and approach of carious lesions are still the problems of continuing investigation.

At present, it is thought that streptococcal hyaluronidase (HASE) may be one agent causing dentin-caries. It may be reasoned that HASE, an enzyme, which can hydrolyse hyaluronic acid (HA), a mucopolysaccharide of dentine, can result in a physico chemical change comparable with dentin caries.

Among the hemolytic (beta) streptococci, Group A non-mucoid type (Pike, 1948), Group B, and C (Rogers, 1946, 1948; Pike, 1946, 1948; Meyer 1941; Werkman 1951; Burnett 1957), Group G (Crowley 1944), produce HASE.

Among non-hemolytic (gamma) Streptococci, one strain of strept. faecalis and one strain of strept. mitis have been found to produce HASE (Rosan, 1961).

All of the above mentioned strains of streptococci which produce HASE, have been obtained from sources other than dental caries; therefore, additional studies of different groups of streptococci obtained from dentinal caries are necessary to

determine the presence or absence of HASE.

It was, therefore, proposed to collect, the available streptococci from *actively carious teeth involving dentin, and by means of suitable cultural methods study their HASE activity by qualitative test.

All the HASE producing streptococci were further classified. Sera typing as well as fluorescent antibody typing (F.A.T.) technic have been utilized for the classification of hemolytic streptococci. The viridans type and non-hemolytic streptococci have been classified by growing them on Chapman's (salivarious-mitis) medium, 6.5 percent NaCl broth and inulin fermentation test.

It was found that not only hemolytic streptococci, strept. faecalis, and strept. mitis produce HASE, but few other non-hemolytic as well as viridans group (unidentified) also produce HASE.

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*Actively carious lesion (Miller 1959; Parikh 1963) color maybe pale yellow, lightly pigmented, or pale brown. The consistency maybe soft, friable, cheezy, necrotic mass in the superficial zone, while the dentin deeper to the lesion is soft and decalcified in nature, if the lesion is not very deep; i.e., in between necrotic zone and pulp.

Active caries is usually found in children, and young adults. The tooth is generally painful and if untreated, caries progresses rapidly, usually exposing pulp.

CHAPTER II

REVIEW OF THE LITERATURE

Composition of Teeth:

All teeth are composed of enamel, dentin, cementum, and pulp. The enamel, ectodermal in origin, is highly calcified and is the hardest structure of the tooth. It contains about 96 percent inorganic salts, and four percent of organic matter and water. The dentin, mesodermal in origin, contains about 60 percent of inorganic salts and 40 percent organic matter and water (Orban 1944-1962).

In contrast to dense enamel, dentin is tubular in nature and protoplasmic processes from the odontoblasts in the pulp pass through these tubules. Thus, the dentin is a vital tissue, while the enamel is not. The pulp is mesodermal in origin and consists mainly of cellular and fibrous elements that are present in loose connective tissue. It is in direct contact with the dentin via odontoblastic processes and any damage to dentin will have a reactive action from the vital pulp (e.g. formation of sclerosed dentin) (Orban 1944-1962).

Oral Bacterial Flora and Dental Caries:

1. Human Dental Caries:

The relation to microorganisms, found in the oral cavity,

to dental caries has been considered to be of great importance since the earliest days of bacteriological studies.

The teeth are constantly bathed in the saliva containing microbial life and debris. Such intimate contact with teeth, therefore, may play a major role in the production of dental caries.

Leber and Rotenstein (1867) described the presence of microorganisms in carious dentin.

Underwood and Milles (1881) reported that caries was completely dependent on the presence as well as proliferation of the bacteria. They found the presence of micrococci and some oval organisms in the sections of the carious dentin.

Miller (1883) demonstrated the presence of some filamentous forms, which he called Leptothrix, within the tubules of the carious dentin. In 1890, he isolated 22 different kinds of microorganisms from the human mouth. He reported that caries is essentially a process of decalcification resulting from the action of acid, produced by bacteria.

Goadby (1903) reported that Bacillus necrodentalis, an acid producer, was the causative agent of decalcification of enamel and dentin, but during his further studies (1910) he stated that certain streptococci were the active cause of caries.

Hartzell et al (1917) found viridans type of streptococcus in all cases, in deeper, advanced carious lesions and claimed

for it a leading role in the process, of dental caries.

McIntosh et al (1922) were able to isolate a bacillus, which they called Bacillus acidophilus-odontolyticus. This organism was aciduric as well as acidogenic and was able to produce acid which softened enamel.

Clarke (1924) obtained, from the carious teeth a cocco-bacillary form, slightly oval in shape and present in chain, which he called Streptococcus-multans.

Bunting et al (1925) reported that lactobacillus-acidophillus was the specific organism for the production of dental caries in human mouth, at least during the initial stage.

Hemmens et al (1946) studied carious lesions and dental plaques in children, and were able to isolate and classify 27 different microorganisms, including streptococci and lacto-bacilli. However, lactobacilli were found more likely to be causative of initiation of the enamel lesions, while strep-tococci were more persistently associated with advanced caries of the dentin. This finding was confirmed by Harrison (1948), and Thoma (1960).

Appleton (1944, 1950) stated that the dental caries is not a specific bacterial disease. It is a complex process. Not any single, but many known as well as unknown, some general and some local factors, may be involved in its production. Different combinations of the several factors may produce the same result

of disintegration of enamel and dentin. The pulp, because of its close connection with dentin, must be regarded as at least potentially infected in every tooth, in which dental caries has involved the dentin.

Robinson (1952) stated that dental caries is a disease which results from the metabolic activity of microorganisms. Any one or group of acidogenic organisms are no doubt of great importance in the initiation of dental caries.

Tunnicliff et al (1938) carefully excavated carious dentin and inoculated it in 0.2 percent dextrose brain broth. They isolated smooth and rough types of viridans streptococci, as the predominent organisms. In some cases this greening streptococcus was the only cultivable organism to grow in the culture medium.

Reymond Hayes (1942) did clinical and bacteriological investigations on 340 pulp therapy cases and found varieties of microorganisms in these pulps, but the majority of them were infected by non-hemolytic streptococci and viridans type. B hemolytic streptococcus was found in only one pulp of the patient suffering from scarlet fever.

Besic (1943) studied the fate of bacteria left beneath the filling in a tooth. Lactobacilli were found to die out in all ten tested cases, while streptococci survived at least for one year, thus they were found to be more prevalent and more

resistant.

Burnett et al (1951) were able to cultivate only cocci, lactobacilli and filamentous forms from 15 cases of the advanced carious lesions in the dentin in human beings. Of the three types, the cocci were the most abundant.

Shiere et al (1951) reported that strep. salivarious, though a normal inhabitant of the saliva in human mouth, may be considered as a potential etiological agent of dental caries. This organism is capable of converting some carbohydrates to lactic acid, thus lowering the pH to 5.0-4.0. This pH is sufficient for decalcification of enamel; moreover, etching of the intact enamel surface was demonstrated in vitro.

Yardani et al (1959) reported that microaerophilic streptococci were the organisms most frequently found in the floor of the cavities of initial caries in human teeth. They drilled the carious cavities in the upper molar teeth and allowed the dentin to fall on sterile mirrors. These carious scrappings were then inoculated in the Difco Brain Heart Infusion broth enriched with 5 percent tomato juice and bromcresol green as an indicator. The organisms cultivated and isolated were:

Pure Streptococci	-	43 cases
Streptococci and lactobacilli	-	2 cases
Streptococci and others	-	8 cases
Pure lactobacilli	-	1 case

Diptheroids - 1 case

Micrococci - 5 cases

None, i.e., Sterile - 5 cases

Cohen et al (1960) selected for bacteriologic study (in vivo) the deciduous molars from 30 children between ages 4-8 years. Culture media such as Brewer's thioglycolate medium, blood Agar, tomato juice agar, lactobacillus-selection agar were used for the isolation and identification of the organisms. Incubation was carried out at 37°C for 48 hours.

Besides, other varieties of organisms, seventy percent were strept. salivarious, while strept. mitis and hemolytic streptococci were ten percent each.

Strauss et al (1960) isolated Group D streptococci from 21.9 percent of children's saliva, who required dental caries treatment but from only five percent of caries free as well as periodontal infection free children. This suggests the association of Group D streptococci with caries in children.

2. Animal Dental Caries:

Many research workers have studied the relation of dental caries and streptococci, by using laboratory animals, mainly rats and hamsters, for various experiments.

Wakeman et al (1948) found that four times more of enterococci than lactobacilli were available from the carious teeth of cotton rats.

Shaw (1954) demonstrated that it was not possible to produce caries in rats in the absence of carbohydrates even if the animals were desalivated. He also found that lesions already developed became arrested in the absence of carbohydrates from the diet. This suggests that aciduric and acidogenic organisms are the causative organisms of dental caries, and streptococci are acidogenic. Furthermore, it also suggests that carious lesions cannot be initiated by proteolytic organisms.

Orland (1955) reported that caries did not occur in "germ free" animals even though they were fed on cariogenic diet.

"Germ Free" Method Used by Orland: Thirteen white rats from Lobund Colonies were kept throughout the complete experimental study in a sealed "germ free" rearing unit (investigated by Reyniers, 1946). These unitchambers were so devised that absolute aseptic conditions were strictly maintained. The animals used for the experiment were also called "germ free" because they were descended through several generations from ceasarean-born, hand fed "germ free" rats. All the animals were fed by mouth, on sterile but cariogenic diet (consisting mainly of carbohydrates), yet not a single, even microscopic, carious lesion was developed. However, when these animals were inoculated orally with Strept. faecalis (an enterococcus) in conjunction with other known proteolytic organisms, all animals developed caries during the 150 days' test period, but they

found that Strept. faecalis was the only organism capable of producing caries in these rats.

While in another experiment done by Kite, Shaw, and Sognnaes's (1950), the normal rats, not "germ free," were fed by stomach tubes. All the microorganisms present in the mouths of these animals were thus deprived of their food substrate (because of tube feeding, no food was available in oral cavity). This procedure thus resulted in complete absence of caries in the teeth of these animals.

Both of these experiments show that microorganisms as well as cariogenic food substrate are necessary to produce caries, absence of either of these failed to produce any caries.

Fitzgerald (1960) was able to isolate five strains of Streptococci from carious lesions in hamsters. All of these Streptococci were found to be actively acidogenic on a variety of carbohydrates. He was able to induce dental caries in a strain of albino "caries-inactive" hamsters by oral inoculation of single or pooled cultures of such Streptococci.

Decalcification of dentin due to metabolic activity of microorganisms, prior to their invasion:

Seltzer (1942) found that microorganisms could not be cultured from approximately 20 percent of human carious dentinal lesions. However, his study was directed toward the efficiency or sterilizing agents and no further discussion or explanation

was offered.

Van Amerongen (1953) using both bacteriologic and histologic methods could not find any organisms in the dentin under properly excavated cavities in humans. He stated that some of the tubules which contained grampositive particles resembling microorganisms were found to be dentinal fibrils cut tangentially, and were not bacteria.

Hanazawa (1923) reported that in the acute form of dental caries decalcification precedes bacterial invasion. There is a zone of softened dentin in which no bacteria can be found.

Black (1936) says, "The dentin is continuously softened in advance of the growing organisms, so that there is a little space softened around them that contains no microorganisms."

Thoma (1941) stated that in advanced acute caries a considerable layer of dentin becomes decalcified, bacteria invade the dentinal tubules and infect the odontoblastic processes.

Kronfeld (1949) in his histologic studies of carious dentin, indicated that very few bacteria were found in the "zone of active destruction," while many were present in the upper half, in the area prior to dentin decalcification.

Bernick et al (1954) in electron micrographic studies of carious dentin showed that the most superficial zone consists of soft, crumbly, necrotic mass, and a heavy concentration of microorganisms of the coccoid and bacillary type, while the

deeper zone even though decalcified in nature contains very few microorganisms, and the farthest zone away from the lesion may not contain any microorganisms. This report was supported by Miller (1959), who by means of histological studies of carious human teeth, described the layering of active dentinal caries by dye penetration technic.

Parfitt (1955) believed that decalcification extends beyond the limit of the bacteria, in spite of the presence of wide, extending dentinal tubules.

MacGregor et al (1956) studied 100 human carious teeth, bacteriologically as well as correlated with histological findings, and concluded that softening of dentin must precede the invasion of organisms in the majority, at least, of carious teeth.

Jolly and Sullivan (1960) showed that the deeper layers of the dentin between the pulp and the carious lesions were sterile in all instances, whereas, the superficial layer of carious lesion constantly showed the presence of cultivable microorganisms. They used both bacteriological and histological methods, and thus believed that decalcification precedes proteolysis.

Parikh (1963) in studies of "microorganisms in active and arrested carious lesions of dentin," found that the softening of dentin probably precedes the actual presence of Cultivable

microorganisms.

Enzyme Hyaluronidase (HASE) and Hyaluronic Acid (HA):

HASE is the name given to an enzyme, which is responsible for the hydrolysis of HA and an acid polysaccharide. The enzyme HASE can be obtained from mammalian testes, leeches, some of the bacteria and certain azo proteins (Meyer 1941).

Testicular HASE: Bull's testis is the usual source of testicular HASE. It is stable at pH 4.6-9 (Yaeger 1959).

Among bacteria, HASE produced by cl. welchi Type A is found to be the most potent (Chain and Duthie - 1940).

Some of the hemolytic Streptococci (Groups A, B, C, and G) are found to produce this enzyme (Meyer 1940, 1941; McClean 1941; Crowley 1944, 1951; Rogers 1945, 1946, 1948; Pike 1948). Production and activity of Streptococcal HASE are quite variable and differ from similar enzymes of some of the microorganisms in their pH sensitivity (Meyer 1941; Hale 1944; Humphrey 1946; Rogers 1946, 1948; Pike 1948; and Steinman 1960). It is stable at pH 7 (Hale 1944), except in few experiments of Rogers (1948), where optimum activity was obtained at pH 5.6. It also differs in its adaptive production (Rogers 1945, 1946), in the products of hydrolysis and also in its antigenic specificity (Hobby 1941; McClean 1941). It is not affected by oxygen and copper (Hale 1944). It can be obtained in pure powder form, though stable at alkaline pH-7-8, can readily be inactivated by acidic pH-4.6,

and heat, at 60°C for ten minutes (Steinman 1960).

There are many methods by which HASE determination can be done, i.e., either qualitative or quantitative. They are as follows:

1. Viscosimetric determination (Bergenstal et al 1948);
2. Turbidimetric determination (Pike 1948);
3. Mucin-clot prevention method (Crowley 1944);
4. Fluorescent antibody technic, first developed by Coons and Kaplan (1942).

<u>Enzyme Hyaluronidase (HASE) and the "Spreading Factor":</u>

It is well known that many infections caused by hemolytic Streptococci are generalized in nature rather than localized. This is true because some enzymes produced by these organisms have an ability to act as a "spreading factor" (S.F.). The Enzyme HASE, which is produced by some groups of Streptococci, has also the ability to act as a S.F.

Chain and Duthie (1940) reported that enzyme HASE hydrolyses HA with the liberation of N-Acetylglucosamine and glucuronic acid. According to them HASE was found in all sources of S.F., no spreading activity was encountered in the absence of HASE.

Meyer (1941) has proved that all HASEs can act as S.F., but all S.F.s are not HASEs. This was confirmed by Duran-Reynals (1942).

Duran-Reynals (1942) during the study on tissue permeability

and the S.F. in infection, reported that the part played by S.F. in Streptococcal infection is more complex because HASE which acts as S.F. is present in some strains of Streptococci, whereas, its substrate, Hyaluronic Acid (HA) is present in the capsules of others.

Seastone (1939) reported isolation of non-antigenic mucoid polysaccharide from Group C hemolytic Streptococci, and its quantitative estimation. The turbidity was determined in a Klett photoelectric colorimeter. For comparison, standards of various thickness glass plates were used.

McClean (1941) worked on some strains of Streptococcus pyogenes and found that all strains of Group B Streptococci so far examined produce HASE, but one strain produces capsules. The capsules were not affected by HASE and, therefore, he considered them as not composed of HA.

Hale (1944) reported that many previous workers (McClean 1941; Madinaveitia and Quibell 1940) have failed to prove the presence of HASE by viscosity reduction technic, because of the acid pH of the medium (4.6) they used. He also used this viscosimetric technic for the determination of HASE activity from Groups A and C Streptococci and found the maximum activity at pH 7.

Crowley (1944) used the mucin clot prevention test for the determination of HASE production and found that capsules of

Group A Streptococci were composed of HA, because they were lysed by an enzyme produced by some hemolytic Streptococci studied by Meyer et al (1941) and McClean (1941). This enzyme was reported to be identical to the diffusing factor (S.F. of Duran Reynals) present in tests and also possess the same type of activity produced by the clostridia group. All HASE producing strains were non-capsulated only 2/308 strains of Group A Streptococci (Types 4 and 22) showed HASE activity while 48/68 strains of Group C and Group G Streptococci tested showed HASE activity.

Rogers (1945) used protein and pepton free media, for the production of HASE by various groups of organisms. He found that all of the organisms require an adequately buffered medium. The HASE produced by Streptococci and cl. welchi was in proportion to the amount of hyaluronate (HA) added to the medium.

Strains of lancefield Group A Streptococci, Group C and Type 7 (Griffiths), Staphylococci and clostridia group were found to be HASE producers.

During later years (in 1948), Rogers reported that streptococci, lancefield Group C and C_7 grown inside dialysis sacs surrounded by a medium without any HA, could produce highly potent HASE, at neutral pH (about 7).

Robert Pike (1948) utilized both mucin clotprevention and turbidity reduction tests for the determination of the

HA, as well as HASE from different strains of Beta hemolytic Streptococci. He reported that Group A Streptococcus, nonmucoid type produces HASE, while Streptococcus mucoid type produces HA. He used Brain heart infusion broth with ten percent horse serum, 0.1 percent glucose and 0.001 percent phenol red as an indicator. This medium was quite suitable for the growth of Streptococci producing HA as well as HASE. Streptococci were then maintained in Brain heart infusion broth containing five percent rabbit blood.

Bergenstal et al (1948) discussed a viscosimetric determination of HASE in human semen. They found that absence of enzyme HASE in semen was always associated with absence of sperms. (HASE was present in all specimens where sperms were present).

Tolksdorf et al (1949) did a turbidimetric assay of HASE after incubating the mixture of enzyme (HASE) and substrate (HA) at 37.5°C in an acetate Nacl buffer at pH 6. Turbidities developed after diluting the mixture with acetate buffer at pH 4.2 and adding *acidified protein solution. These authors reported that hydrolysis of HA is markedly affected by the nature and concentration of the buffer thus interfering with the development of turbidity. According to Seastone et al (1939) the

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*(Horse serum plus human serum diluted 1:10 with acetate buffer at pH 4.2 and then acidified to pH 3.1.) The readings were taken by means of a turbidometer at wave length 600 mu.

age of acidified protein solution also influences the development of turbidity. Furthermore, the reaction of protein with HA increases the turbidity.

Lisanti (1950) used dialysed saliva for the determination of HASE activity. Viscocity was measured by means of Ostwald-Viscosometer at 25°C. He stated that the HASE activity of saliva may be a factor in the initiation or progress of dental caries, and may also help in spreading dentinal caries by causing dissolution of tubular contents.

Engel (1950) reported a very interesting phenomenon of "softening and solution of dentin." He stated that "the presence of HASE-like activity in carious dentin may be associated with Streptococcal invasion. Hyaluronidase does not depolymerise dentin and apparently does not attack carious dentin, however, it is possible that HA derivative may be one of the soluble terminal residues of the carbohydrate-protein complex of carious dentin. Such a substrate could then serve as a substrate for HASE." He also thought that pulpal invasion by microorganisms is enhanced by this HASE.

Another evidence of caries production by HASE is stated by Steinman (1960). Seventy rats, 18 days of age, were used and were sacrificed at 28 days of age, and teeth were examined histologically. He incorporated a synthetic anti-HASE (prepared

by the *method of Rogers and Spensley 1954), one percent by weight into the food of 40 rats, which were also fed on **cariogenic diet as the other group of 30 rats. The animals, which received the anti-HASE, showed a significantly reducing number of incipient caries than those that did not receive this anti-HASE. This study led him to conclude that bacterial HASE may play an important role in production of dental caries.

Warren et al (1951) studied the enzyme HASE produced by the strains of hemolytic-Streptococci, Group A. He used infusion broth (Baltimore Biological Laboratory) for the culture of the organisms, and found that good enzyme titres were present throughout the successive transfers of repeated subculturing of the organisms. By using a double concentration of the dehydrated medium, a marked increase in the HASE activity was noticed. This suggests that besides pH of the medium, the quality and quantity of the medium has great influence on the HASE production.

Wenner et al (1951) reported the specificities of HASE produced by 115 strains of Groups A, B, C, and G Streptococci. Each strain was grown in buffered Todd-Hewitt medium for about

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*Sulphonic acids of hydroquinone and catechol were used as a synthetic polyanionic inhibitors of HASE.

**Casein 24, Sucrose 65, salts USP XIV4, Corn Oil 5, Dehydrated Liver, Vitamin Mix I, and Choleine 0.2 parts.

15-18 hours. The mucin clot prevention test of McClean (1941) was utilized by him for the quantitative estimation of the enzyme HASE titre. A titre of 1:16 was considered as a satisfactory titre. (It was then stored in sealed glass ampules at -70°C.) The author found that HASEs produced by Groups A and B are serologically distinct and differ from the HASEs produced by Groups C and G, which are serologically indistinct.

Faber (1953) utilized the turbidimetric method for the determination of Anti-Streptococcal HASE (ASH) in sera of 500 patients suffering from rheumatic fever, glomerulonephritis, tonsilitis and rheumatoid arthritis.

Emmert et al (1955) studied in vitro the production of streptococcal HASE, its isolation, and its use as an antigen. Strept. C_7 (Rogers 1948) was cultivated at 37°C for three days in T.H. broth (Todd and Hewitt used this medium in 1932).

In 1957 the authors used Strept. C_7 (Rogers 1945) not only for the high production of the enzyme HASE but also to demonstrate the localization of sites of absorption of Streptococcal HASE by means of fluorescent antibody technic reported by Coons et al (1942, 1950) who established the Fluorescent-Antibody technic (F.A.T.) or Immuno-Histo-Chemical method for the determination of the pneumococcal antigen. Tissue section of kidney, lung, spleen, liver and skin near the eye of the mouse injected with Streptococcal HASE were used for specific fluorescent

antibody globulin technic.

In 1959 Emmert and Turner studied Streptococcal Group C HASE by means of F.A.T. as suggested by Coons and Kaplan. Intravenous injection of Strept. HASE into the mouse was found to be widely distributed into the body, however, selective absorption by specific cells was found on different tissues of the animals. Sections of spleen, lymphnodes, heart, muscles, bone and joint tissues, showed the presence of Streptococcal HASE by means of yellow-green fluorescence of antigen-antibody complex after the treatment with fluorescene isocyanate bound rabbit Anti-HASE serum. All the samples of rabbit Anti-HASE sera were tested (in vitro) for the detection of potent antibody titre by means of turbidity-reduction technic.

Rosan (1959) reported HASE production by Strept. mitis and Strept. faecalis (1961). In both of these experiments indirect fluorescent antibody typing technics were performed.

Yaeger and Truman (1959) established a technic of localization of testicular HASE by using F.A.T. Rabbit-anti-bovine-HASE serum and goat-anti-rabbit-globulin serum were prepared for the use of a double layer F.A.T. The antigens of a purified bull testicular HASE were localized in the sections of bull testis. They found fluorescence in the seminiferous epithelium in the region of spermatocytes and spermatogonia.

Nace et al (1961) devised a new technic for cellular-

localization of the antigen possessing enzymatic activity. This they called "Echo technic." Lactate dehydrogenase (LDH) found in the frog, rana pipiens, was one of the antigens used for the study. A planchet from an agar diffusion or immuno-electrophoretic pattern prepared with fluorescent antibody was stained to identify the lines produced by the antigen. The agar planchet containing each such line was dissected out and was placed on fixed frog tissue section. They could determine the antigen-fluorescent antibody complex dissociation at pH 10.5 or 3.5. The "free" fluorescent antibody was then allowed to diffuse into the tissue to react with LDH, the pH was adjusted to neutrality and sections were examined histochemically, after thorough washing.

Steinman (1962) used the turbidimetric method to study the production of HASE from streptococci obtained from rat dental caries. The organisms were cultivated in a standard broth containing yeast extract, peptone, and dextrose. Incubation was carried out at 37°C for 24 hours. The presence of HASE was detected by using:

1. A solution containing known HASE;
2. The Streptococcal culture;
3. Streptococcal solution heated at 60°C for 10 minutes

(to destroy the enzymatic action) was used as a control.

The amount of HASE absorbed by acidified protein and HA

(Tolkdorf 1949) was measured by a colorimeter. The turbidity decreases as the HASE is absorbed by the reagent. Thus, greater the decrease in turbidity, greater the transmittance due to the presence of HASE. He also studied the localization of rat oral streptococcus in incipient dental caries by means of fluorescent antibody technic.

Moody et al (1963) reported the use of F.A.T. and cultural-precipitin group procedures for identifying Group A Streptococci from paired throatswabs. They found that more positive (cultured-organisms) specimens were obtained by using both of these technics instead of any one of them.

CHAPTER III.

MATERIAL AND METHOD

Eighty freshly extracted, actively carious teeth involving the dentin were obtained from the Oral Surgery Clinic of Loyola University School of Dentistry. All teeth were collected regardless of race, age, or sex of the patient. The teeth were wrapped in a dry, sterile 2 x 2 piece of gauze, and stored in the refrigerator at 4°C.

Within two hours following extraction, each tooth was washed with cold running tap water to free it from blood, and food debris. It was then dried with a piece of sterile gauze and the outer surface was sterilized with a sterile piece of gauze soaked in 70 percent alcohol. The excess alcohol was squeezed off to prevent deeper penetration into the carious lesion. The tooth and the cutting end of the chisel were covered with sterile gauze during the entire chiseling process, done by the help of a sterile mallet. The tooth was split into two halves through the carious lesion.

The soft carious dentin was scooped out with a sterile excavator and transferred to approximately 15 ccs. of fluid *thioglycolate medium (DIFCO) contained in a tall screw-capped

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*This medium is widely used to obtain the growth of aerobic, microaerophilic as well as anaerobic group of organisms.

pyrex tube. Tubes with screw-caps were preferred to tubes with cotton plugs, to assure prevention of Air-contamination during storage.

These tubes were then incubated at 37°C for 24 hours. Growth was present at the end of 24 hours' incubation in almost all of the tubes. Those tubes which did not show any growth after 24 hours, were further incubated til seven days, and every day they were examined for the growth of the organisms. At the end of the seventh day, if there was no growth, the cultures were considered sterile.

Microscopic Examination of the Cultured Organisms:

A platinum loop having a diameter of 4mm. was used for the preparations of the smears. These smears were stained with Gram's Stain. The cultures, which revealed the presence of streptococci-like organisms, were further transferred to blood agar plates obtained from Baltimore Biological Laboratory. One loopful of the culture material was used for inoculation by streaking blood agar. These plates were also incubated at 37°C for 24 hours, and wherever necessary they were incubated for 48 hours to obtain a good growth of streptococci.

Three types of growth of streptococci were cultured; alpha, gamma and beta respectively. Smears made from several of these colonies were confirmed morphologically by Gram's Staining. An inoculum from these colonies was then transferred to tubes

containing Todd-Hewitt broth. This medium is favorable for the growth of streptococci (Todd and Hewitt 1932; Wenner et al 1951; Emmert et al 1955). Incubation was carried out as usual. These pure cultures were stored in the refrigerator for further use.

Alpha and gamma (non-hemolytic streptococci) streptococci were further differentiated by growing them on salivarious-mitis medium, in 6.5 percent Nacl. broth medium and Inulin fermentation test.

A stab culture of isolated alpha and gamma streptococci was made in Tripticase Soya Agar medium, and inulin paper-strip was incorporated in the medium, allowing half the portion of the strip to remain outside the medium. The tubes were incubated at 37°C for 24 hours.

Strept. salivarious ferments inulin, a positive test of which is the change in the color of the medium from pink to yellow, due to the production of lactic acid.

The typing of beta hemolytic streptococci was accomplished by means of a serologic technic and fluorescent antibody technic.

Typing of Beta Hemolytic Streptococci

Serologic Technic:

The autoclave method of Rantz and Randall (1956) for the preparation of a streptococcal-extract was used. The streptococci were grown in approximately 20 ccs. of T. H. broth for 24 hours. The material was then centrifuged for 20 minutes at

3000 r.p.m., to sediment the growth completely. The supernatant fluid was discarded and the organisms were resuspended in 0.5 cc. of 0.85 percent sodium chloride, by gently shaking the tube. This tube was then autoclaved for 15 minutes at 15 lbs. pressure (121°C.), and then centrifuged a second time, the supernatant solution being used for the precipitin test.

Bacto-streptococcus antisera types, A, B, C, D, E, F, G, K, N, O, and MG were available in dry powdered form. They were rehydrated by adding one cc. of distilled water in each vial. Equal amount of the required antiserum (2-3 cms.) and the prepared streptococcal extract was allowed to react for 10-15 minutes in a capillary tube (0.7 mm. inside diameter and 75-90 mm. long).

Normal serum and culture and culture controls were used for comparison. A positive reaction seen as a precipitate occurs in 10-15 minutes.

<u>Fluorescent-Antibody Technic:</u>

Seven smears were made from each of the pure culture of beta hemolytic streptococci. Each smears was flooded with group A, B, C, D, E, F, and G rabbit antiserum conjugated with fluorescein isocyanates and allowed to react for at least 30 minutes. From the beginning of the procedure until the end, the slide with the smears, were kept in covered petri dishes containing moist filter papers, in order to avoid rapid evaporation, and

to increase the time of reaction, at room temperature. The petri dish with the slide, was kept covered with the tin foil, to exclude light (Yaeger 1959). At the end of the reaction the slide was thoroughly rinsed with three changes of buffered saline solution, dried in air and examined under the fluor-oil immersion lens using glycerol. The ultra violet light was produced using an HBO-200 mercury vapor bulb light source. Filter combination for fluorescence microscopy were used as desired, for the color of observable fluorescence to apple green. Brightest fluorescence with dark field condenser was obtained by using ultra violet irradiation, 350-400 mμ. The primary filter used was c-5970 (3 mm.) and the secondary was w-2B.

Qualitative Test for Hyaluronidase (HASE)

Based on the methods of Seastone (1943) and Pike (1948), ten ccs of Beef Heart Infusion Broth (B.H.I.), containing ten percent of horse serum, one percent of glucose, and 0.001 percent phenol red, was inoculated with streptococcal culture from T. H. Broth, and incubated at 37°C for four hours.

The substrate was prepared as follows:

To 0.8 ccs of the serum glucose infusion broth, 0.2 mgms. of Potassium Hyaluronate were added and the pH was adjusted to 7.4 with 1N. NaOH. 1:10.000 dilution of merthiolate was added as a preservative.

After the required incubation, *(4, 24, or 48 hours, or seven days as necessary) the cultured broth was centrifuged for ten minutes at 3000 r.p.m. One cc of the supernatant from this culture was added to 0.8 ccs of the substrate in an ice water-bath. The pH was adjusted to 7.4 whenever necessary as seen by the phenol red indicator. This substrate-supernatent mixture was then incubated in a water bath at 37°C for 30 minutes and returned to ice water bath. 0.1 cc of horse serum, 0.1 cc of sterile serum glucose infusion broth and 2 mls. of acid serum reagent+ were added, and the mixture was allowed to react for 30 minutes at room temperature. Any turbidity which developed was read by means of Coleman's spectro photometer at 660 mμ.

Acid serum reagent (according to Seastone, 1943) clear, non-hemolysed horse serum was diluted 1:10 with 0.5 M acetate buffer at pH 4.2. The reaction was then brought to pH 3.1 with HCL. and merthiolate added as 1:100,000 final dilution. This reagent was prepared at least 24 hours before the test and stored in the refrigerator.

The control tube for the standardization of the reading was

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*After four hours' incubation, the cultures were tested for HASE activity. Those which did not show any activity (which was read by turbidometer of Coleman's photoelectrometer) were further incubated for 24 hours, or 48 hours, or seven days, as needed. After each incubation, they were tested for the production of HASE. Cultures which did not show any activity even after seven days' incubation were considered HASE-negative.

prepared by adding equal amounts of sterile broth and acid serum reagent.

Another tube containing 0.8 cc of substrate, 1.1 cc of sterile broth, 0.1 ml of horse-serum, and 2 ccs of acid serum reagent, was used for the comparative study of HA, and reaction of HASE. (If it is produced by the test organisms) with HA in test culture.

Another experiment was performed using: 0.2 ccs of glacial acetate acid, 0.13 gm. of Na. acetate and 2.87 ccs of ethyl alcohol, as serum reagent instead of 2 ml of acid serum reagent. HA acid present in the broth was precipitated out, and turbidity produced during these 30 minutes at room temperature, and was read by the same instrument at 660 mμ.

However, both these methods are used for the precipitation of HA, the first one is much easier to work with than the latter one, because of the ease of using previously prepared acid serum reagent, also the mixture, though turbid, is more homogenized in appearance.

CHAPTER IV.

RESULTS AND FINDINGS

Growth in Fluid Thioglycolate Medium:

After 24 hours incubation at 37°C, 73 tubes showed growth while seven tubes showed no sign of macroscopic growth.

After 48 hours incubation at the same temperature, two more tubes showed growth while the other five did not. These were further incubated for five more days, but none of them was positive either macroscopically or microscopically (Table I). Therefore, these five tubes were considered as sterile and then discarded.

TABLE I.

Hours of Incubation	* Number of Positive Cultures	** Number of Negative Cultures	*** No Growth (Sterile)	Total Number of Tubes Inoculated	Medium Used
24 (1 day)	73	7	--	80	Fluid Thioglycolate
48 (2 days)	75	5	--	80	
168 (7 days)	75	5	5	80	

*Positive = Macroscopic growth present.
**Negative = No Macroscopic growth present.
***Sterile = No Macroscopic or microscopic growth present.

Microscopic Examination:

Smears prepared from the positive cultures were examined under the oil immersion lens of the microscope.

Out of these 75 culture tubes, eight showed pure growth of bacilli, which morphologically resembled lactobicilli; two showed marked pleomorphism, although they were Gram+ bacilli were in pairs, in long chains, filamentous and single.

Five out of remaining 67 cultures were found to be purely of streptococci-like organisms. These five cultures were obtained from actively carious teeth, with deep dentinal decay not involving the pulp.

Sixty two culture tubes had mixed type of organisms, Gram+ cocci, either in clusters, in chain (short as well as long) or even in pairs, as well as Gram+ bacilli in many cases. In three instances Gram - cocci, diplococci and in three others Gram - bacilli were also present. No further isolation of the organisms other than streptococci was done.

Cultured Organisms on Blood Agar:

Out of the five pure cultures of streptococci-like organisms in fl. thioglycolate medium, one was beta hemolytic and four were viridans or alpha hemolytic streptococci.

From the 62 cultures containing mixed organisms, 45 more streptococcal cultures were obtained while the remaining 17 cultures were other organisms as shown in the Table II.

TABLE II.

Total Number of Specimens Cultivated	Total Number of Positive Cultures	Total Number of Negative Cultures	Organisms Cultivated in Thioglycolate and on Blood Agar Plate Medium	Total Number of Positive Cultures	Total Number of Streptococci Available
80	75	5	Alpha hemolytic strept.	33	50 (in pure form)
100%	93.75%	6.25%	Gamma hemolytic strept.	12	
			Beta hemolytic strept.	5	
			Strept. mixed with other organisms.	60	
			Gram positive bacilli in pure form-lactobacilli like organisms	8	
			Gram positive bacilli-like lactobacilli mixed with other organisms	30	
			Gram positive cocci-like Staph.	10	
			Gram negative diplococci cocci-like N. catarrahalis	3	
			Gram negative bacilli	3	
			Yeast and fungus	7	
			Sterile cultures-No growth of any kind	5	

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Colony Characteristics of Available Streptococci on Blood Agar Medium:

Alpha viridans-green or partial hemolytic streptococci (33 available in pure form):

The colonies were small, pin point, smooth and glossy as well as rough form. Some of them showed more hemolysis than others, characterized by more greenish zone.

Gamma hemolytic streptococci (12 were obtained in pure form):

These colonies were also small in size, showing no hemolytic zone. On Chapman's Medium: Characteristic, mucoid, big heaped up colony of strept. salivarious is very typical.

Beta hemolytic streptococci (five strains were obtained):

The colonies were (small) clear, dew drop-like. Smallest of all (compared to alpha and gamma), pin point, smooth, showing complete hemolysis.

Growth in T. H. Medium: (Figure 4)

In general, most of the colonies of strept. salivarious were big, fluffy and suspended throughout the medium. Strept. mitis were much smaller, more compact in nature than strept. salivarious, and also suspended in the medium. Strept. feacalis showed uniform turbidity with heavy deposit at the bottom of the medium.

Colonies of beta hemolytic streptococci were more of

granular type, scattered throughout the hazy medium with precipitation at the bottom of the tube. Though the majority of these streptococci showed the appearance as described above, it was not characteristic for all of them. For example, in many instances such characteristics were interchangeable and, furthermore, many unidentified streptococci which were also cultivated from the carious dentin showed the similar type of growth pattern.

Determination of HASE-Producers Streptococci Grown in S.G.B.H.I. Broth:

After four hours' incubation, six cultures of streptococci (out of 50) showed HASE activity, while after 24 hours, seven more showed HASE activity and only one more culture showed HASE activity after 48 hours. After seven days, none of the remaining cultures showed any HASE activity (Table IV).

Among the five beta hemolytic streptococci, three were found to be HASE producers, one slight in activity, was classified as Group A, while the strongest in activity was Group C. The third one was Group B. The other two beta types did not fit in any of the sera typing as well as fluorescent typing technic (Figure 12).

Among the six alpha hemolytic streptococci which showed enzymatic activity, two were found to be strept. mitis, while the other four could not be identified as any of the type

specific, but they were in the form of very long chains (Figure 1a).

Among the four non-hemolytic types, strept. faecalis, was a much stronger HASE producer than the other three. The other, which was a mild enzyme activity producer, was identified as strept. salivarious.

The two remaining HASE producers remained unidentified. They produced HASE after four hours. The organisms which did not show any HASE activity after 24 hours, 48 hours, and seven days were among these unidentified groups.

Some of the cultures among non-HASE producers showed even more turbidity than the standardized substrate tube. This suggests that some strains of streptococci produce HA instead of HASE (as reported by Seastone 1943; Pike 1948).

The streptococci secured from dentinal decay were found to be acidogenic. The serum glucose broth which was at pH 7.4 before the inoculation of the organisms was found to be at pH between 4.2-4.8 at the end of 24 hours, which was indicated by phenol red, present in the medium. Color of the medium turned to yellow from pinkish brown.

TABLE III.

GROWTH CHARACTERISTICS OF

Name of the Streptococci-Classified	Fluid Thyoglycolate Medium	Blood Agar	Chapman or Salivarious-Mitis Medium
Beta Hemolytic Streptococci. Groups A, B, and C.	Growth below the surface level; microaerophilic uniform turbidity	Clear hemolysis, pin point, dew drop-like colonies	--
Strept. Salivarious	Microaerophilic	No hemolysis	Big, mucoid bluish, raised up colonies, Approx. 0.7-1mm in diameter
Strept. Mitis	-do-	Viridans-type marked greenish zone showing partial hemolysis	Small, smooth, dark-blue, raised up colonies
Strept. Faecalis	-do-	No hemolysis	Growth appearance similar to mitis except colonies were slightly bigger but much smaller than salivarious
	N.B. In some culture tubes stallactite-type of growth present. Fig. 3		

CLASSIFIED HASE PRODUCERS

Todd-Hewitt Medium	S.G.B.H.I. Broth	6.5 Percent NaCl Medium	Inulin Fermentation
Uniform granular growth through-out the medium with some depo-sit at the bot-	Uniform turbi-dity with some deposit at the bottom. Final pH of the medium between 4.2-4.8	--	--
Big, fluffy, isolated colonies	same as that of beta hemolytic		+
small, compact isolated colonies.	-do-	- no growth	-
Uniform turbidity and heavy sediment	-do-	+ growth present	-

TABLE IV.

HYALURONIDASE PRODUCING STREPTOCOCCI

Types of Organisms Obtained	Total Number of Specimens	VARIATIONS IN SPECIMENS			Total HASE Positive
		Alpha	Gamma	Beta	
Streptococci	50	33	12	5	14
Group A	1	--	--	1	1
Group B	1	--	--	1	1
Group C	1	--	--	1	1
Streptococcus- Salivarious	6	--	6	--	1
Strept. Mitis	9	9	--	--	2
Strept. Faecalis	3	--	3	--	1
Unidentified	29	24	3	2	7

CHAPTER V.

DISCUSSION

It was found that growth was present in 75 out of 80 cultured specimens (approximately 94 percent), obtained from deep, soft carious dentin. This fact proves that microorganisms are closely related to the production of dental caries. It was also proved by Orland (1955) that caries could not occur in "germ free" rats, in absence of microorganisms even if the animals were fed on cariogenic diet. Kronfeld (1949), Barker (1935), and Zander (1940) also noted the presence of microorganisms histologically in the deeper layers of carious dentin, approaching the sound dentin.

While five out of the same 80 cultures (six percent) were found to be sterile even after seven days' incubation, this supports the data presented by Black (1924), Parfitt (1955), Jolly et al (1961), and Parikh (1963), that decalcification preceeds the actual invasion of microorganisms. Seltzer (1942) found that microorganisms could not be cultured from approximately 20 percent of carious dentinal lesions. Yardani (1959) also found five sterile cultures out of 65 cases of carious dentin.

From all those 75 (positive) cultures, streptococci were

isolated in 50 cases (approximately 67 percent), which proves the close association of these organisms to dentinal caries. This finding generally agrees with those of Harrison (1948), Shiere (1951), and Yardani (1959), who reported that streptococci are more persistantly associated with advanced caries of dentin in humans.

The serum glucose infusion broth in which these streptococci were grown for the determination of HASE was found to be quite acidic, pH between 4.2-4.8. This demonstrated that all the streptococci which were isolated from carious dentin were acidogenic. This result confirms the reports of Miller (1890), Goadby (1903), Hartzell et al (1917), Clarke (1924), Harrison (1948), and Steinman (1961) that active acid producing microorganisms may be a contributor to caries production. Furthermore, McIntosh (1922), Bunting (1925), Yardani (1961) have stated that not only acidogenic but also aciduric microorganisms may play an important role in caries production. Lactobacilli and streptococci possess both these properties. However, Besic (1943) suggested that streptococci, being more resistant than lactobacilli, are also microaerophilic in nature and survive better at greater depths of carious teeth. This present work confirms the microaerophilic nature of streptococci. All the isolated streptococci were slightly microaerophilic.

Hartzell et al (1917) and Harrison (1948) have reported

that lactobacilli are the cause of initial carious lesions, especially in the enamel caries, while streptococci probably have special importance in advanced dentinal decay.

Even though all these reports favor the etiologic role of streptococci in production of dentinal caries, the studies of other authors need some more evaluations. Turner et al (1951) was able to cultivate a virus from carious dentin, while Sims (1960), and Kondo et al (1961) have reported the presence of viellonella organisms in the saliva of patients possessing actively carious teeth.

The ground substance of dentin consists of mucopolysaccharide, mainly a carbohydrate-protein complex, and enzyme HASE hydrolyses HA which is also a mucopolysaccharide. The determination of streptococcal HASE in carious dentin was done on these bases to find out any correlation between the enzyme HASE, HA, and caries production.

It was found in the present study that 14 strains out of 50 cases, 28 percent of, isolated alpha, gamma and beta streptococci produce HASE, between 4 and 168 hours.

HASE activity was determined by reduction in the turbidity of the solution containing supernatent of streptococcal culture and potassium hyaluronate (HA). At the same time, turbidity was found to be increased in the remaining 36 specimens. This increase in turbidity may be interpreted as increase in HA,

which might have been produced by the streptococci. Pike (1948) has reported that non-mucoid type of Group A streptococci produce HASE while mucoid type produce HA. This was noted by an increase in the turbidity of the solution.

In this study it was found that maximum amount of HASE was produced in cultures incubated between 4-24 hours, and no enzymic activity was noted after seven days. Meyer et al (1961) obtained a more potent enzyme from one strain, in a 16 hour culture, than after a six or 24 hour period. Rogers found the maximum amount of HASE produced by Group C_7 streptococcus after 24 hours, during the active phase of growth. Pike (1948) found that seven out of 14 strains of Group A streptococcus destroyed a part of the HA present in the solution after 24 hours, while other seven strains completely destroyed it after seven days. Group C "human type," strain 517, and Group A, strain A21 showed maximum HASE concentration after four hours.

During the present work, only 14 out of 50 strains produce HASE and remaining 36 failed to show any HASE activity. This may be attributed partly due to the presence of horse serum in the medium. Pike (1948) again has reported that presence of normal horse serum in the culture medium reduced the HASE level of Group A, Strain A21 type of streptococci more than 90 percent.

Though HASE activity was not measured quantitatively in our experiment, turbidimetric reading suggested that some strains of

streptococci, especially Group C and one strain of salivarious, showed greater amount of HASE activity. This was seen by lesser turbidity in the solution. This result resembles that of Pike (1948) where he found that the presence of serum definitely increased the HASE activity of Strain A 192, while some of the strains were not definitely affected in increase or decrease of HASE activity.

The qualitative test for HASE detection used in this study was found to be easy to work and uncomplicated in manipulation. The acid serum reagent which was used could be prepared beforehand, thus, less time was consumed and more accurate results (though qualitatively) were obtained.

To secure optimum HASE activity, streptococci were grown in culture medium (S.G.B.H.I. broth) at pH 7.4. The pH concentration of the medium is very important in case of streptococcal HASE; for example, HASE activity is destroyed or inhibited at acidic pH. Madinaveitia and Quibell (1940), and McClean (1941) failed to demonstrate streptococcal HASE because of acidic pH 4.6 which they employed in the medium. This failure was due, as Hale (1944) showed, to the rapid inactivation of the enzyme at pH 4.6. Later McClean (1943) chose pH 7.0 for the standard viscosity reduction assay. Hale (1944) showed that streptococcal HASE could be estimated viscosimetrically at pH 7.0. Rogers (1948) also was in favor of pH 7 for streptococcal HASE

but in his study, unexpectedly he found that one strain of streptococcus C_7 showed maximum HASE activity at pH 5.6. This was also true for staphylococcal HASE (Staph. Humphrey). Pike (1948) utilized pH 7.4 and found maximum HASE production by Group A streptococci non-mucoid type.

Above mentioned findings suggest the ability of HASE to hydrolyse HA, and thus, may play a role in production of dentinal caries. Engel (1950) believed that presence of HASE-like activity in carious dentin may be associated partly with streptococcal invasion. He reported that, "HASE does not depolymerise dentin and apparently does not attack carious dentin. However, it is possible that a HA derivative may be one of the soluble terminal residues of the carbohydrate-protein complex of carious dentin. Thus, softening and solution of dentin in caries may be attributed to this HASE-HA reaction." Presence of HASE activity of some of the viridans (they were in majority among the isolated streptococci, 33 out of 50 cases) and non-hemolytic type of streptococci (next in series, 12 out of 50 cases), besides hemolytic one, suggest that enzyme HASE produced by these streptococci may have some important role in caries production.

When seen under fluorescent microscope, the apple-green color of fluorescing streptococci treated with specific fluorescent isocyanate antibody, was a more reliable test for their group specificity than in sero typing, the precipitation seen

in small capillary tubes, formed by antigen-antibody reaction is difficult to read. Furthermore, the preparation of the extract from the test organisms (streptococci) is time consuming and also needs control sera for comparison.

All the available HASE-producing streptococci could not have been typed out with the above methods of fluorescent specificity of the antisera and fluorescence antibody which were used in this study. Additional specific antisera and fluorescent antibody are needed to meet the wide range of streptococci present in dental caries.

CHAPTER VI.

SUMMARY AND CONCLUSIONS

Bacteriologic study was done on 80 freshly extracted carious teeth, to determine the presence of streptococci, and their ability to produce HASE.

Inoculation from carious dentin was first made in fluid thioglycolate medium. Isolation of the streptococci was then done by using blood agar, salivarious-mitis medium, 6.5 percent Nacl. broth and inulin fermentation test. All streptococcal cultures were grown and maintained in T.H. broth before transferring them to serum glucose beef heart infusion broth medium, for the determination of HASE activity. All cultures were incubated at 37 C for 24 hours, 48 hours, or even seven days when and where necessary.

After 24 hours' incubation, 73 out of 80 cultures showed the growth in thioglycolate medium, while two more (i.e., 75 cultures) showed growth after 48 hours and remaining five did not show any growth even after seven days; therefore, they were considered to be sterile.

Out of these 75 cultures, five were found purely to be streptococci-like organisms (microscopically) while eight were of Gram positive bacilli (like lactobacilli), remaining showed

mixed type of organisms such as staphylococci, Gram negative cocci, Gram negative bacilli, streptococci and lactobacilli-like organisms.

Streptococci were isolated from 50 out of 80 cultures (62.5 percent). Out of these 50 cultures, 33 (66 percent) were viridans type, 12 (24 percent) non-hemolytic, while five (10 percent) were of hemolytic type.

All these available streptococci were found to be high acid producers, final pH of the medium was between 4.2 and 4.8.

Typing of hemolytic streptococci was done by means of sera typing as well as fluorescence-antibody typing technic. Groups A, B, and C were thus typed out from five strains of hemolytic streptococci available in pure form out of the 80 cultured specimens.

Determination of streptococcal HASE was done by using qualitative test (based on Seastone 1943 and Pike 1948). The cultures for the test were inoculated in S.C.B.H.I. broth containing phenol red as an indicator, and incubated for four hours, 24 hours, 48 hours, or seven days, as necessary.

It was found that 14 out of 50 strains of streptococci produce HASE (28 percent). Among these were found Groups A, B, and C streptococci, Str. Salivarious (one strain), strept. mitis (two strains), strept-faecalis (one strain), and seven unidentified strains of viridans as well as non-hemolytic type.

From the results of the experiment the following may be concluded:

Streptococci are found to be in close association with deep dentinal decay. Whether they are the etiological agents for the production of dentinal caries, cannot be established by this study only, because of the presence of many other organisms.

Viridans type of streptococci are found in majority (62.5 percent), and some of them produce HASE, which may play a role in the production of dentinal caries. However, more detailed studies on the streptococcal HASE activity are necessary before establishing its etiologic role in the production of caries.

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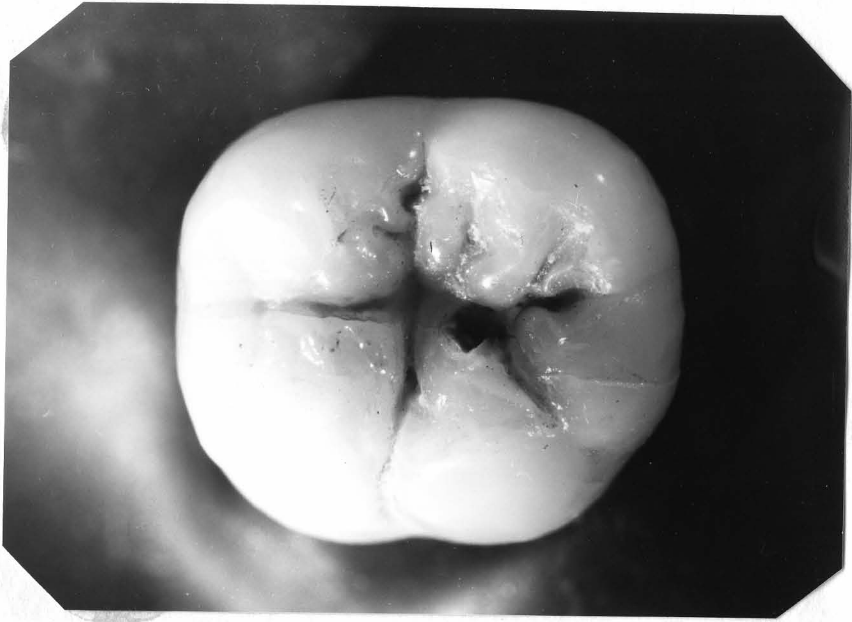
APPENDIX

FIGURE 1

Occlusal view of the crown of a molar tooth showing carious lesion.

FIGURE 2

Longitudinal section of the molar through carious lesion.



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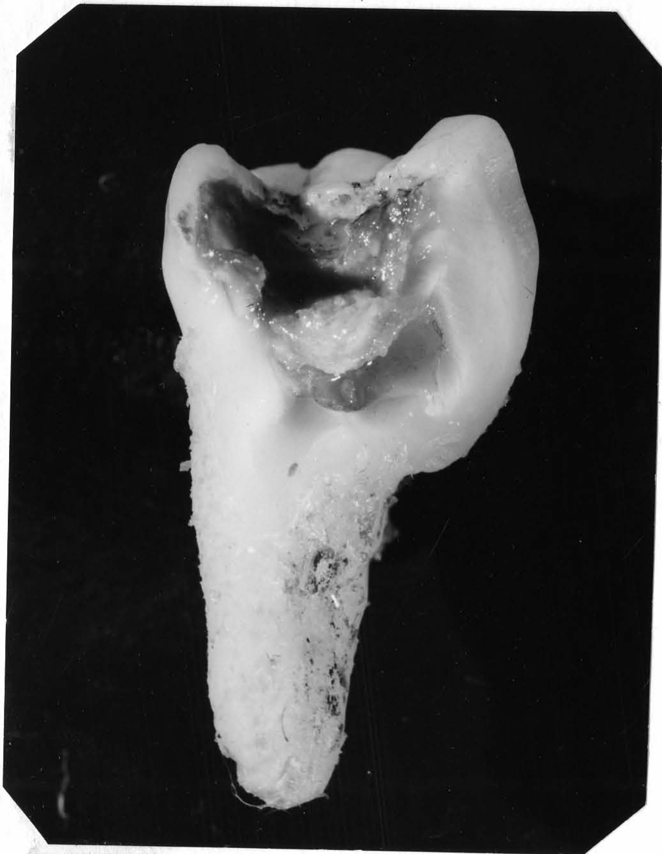

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FIGURE 3

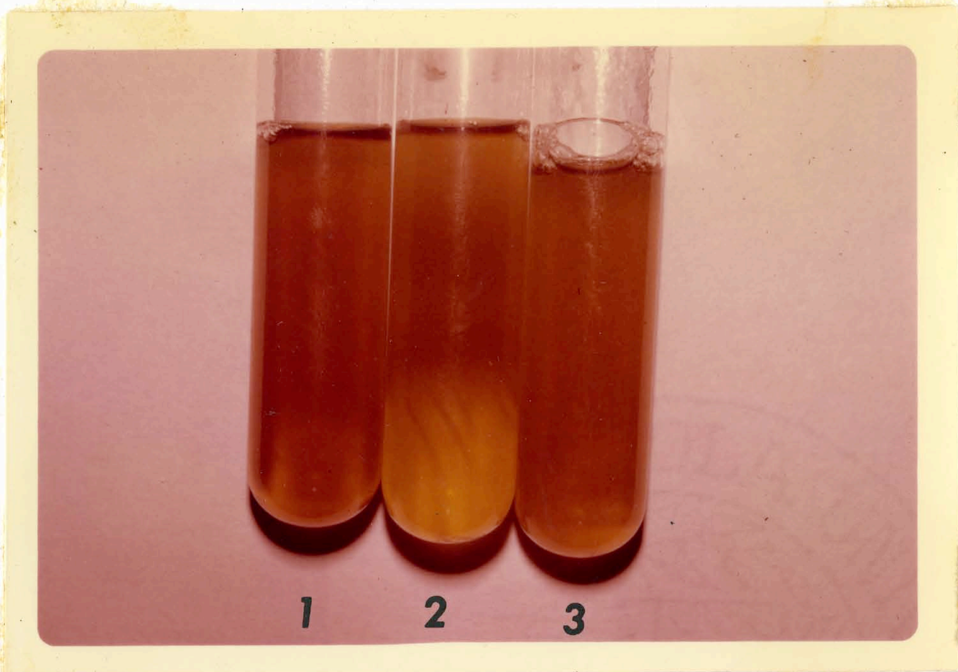
Growth of streptococci in fluid Thioglycolate Medium:

1.) Uniform turbidity throughout the Medium;
2.) Typical stalactite growth of streptococci;
3.) Uniform turbidity plus some deposit at the bottom of the tube.

FIGURE 4

Growth of streptococci in Todd-Hewitt Medium:

1.) Uniform turbidity;
2.) Big fluffy colonies of Strept. Salivarious, suspended throughout the Medium;
3.) Smaller and compact colonies of Strept. Mitis, suspended throughout the Medium;
4.) Very fine granular growth throughout the Medium (not seen very clearly);
5.) Same type of growth as in Figure 4, but old culture.



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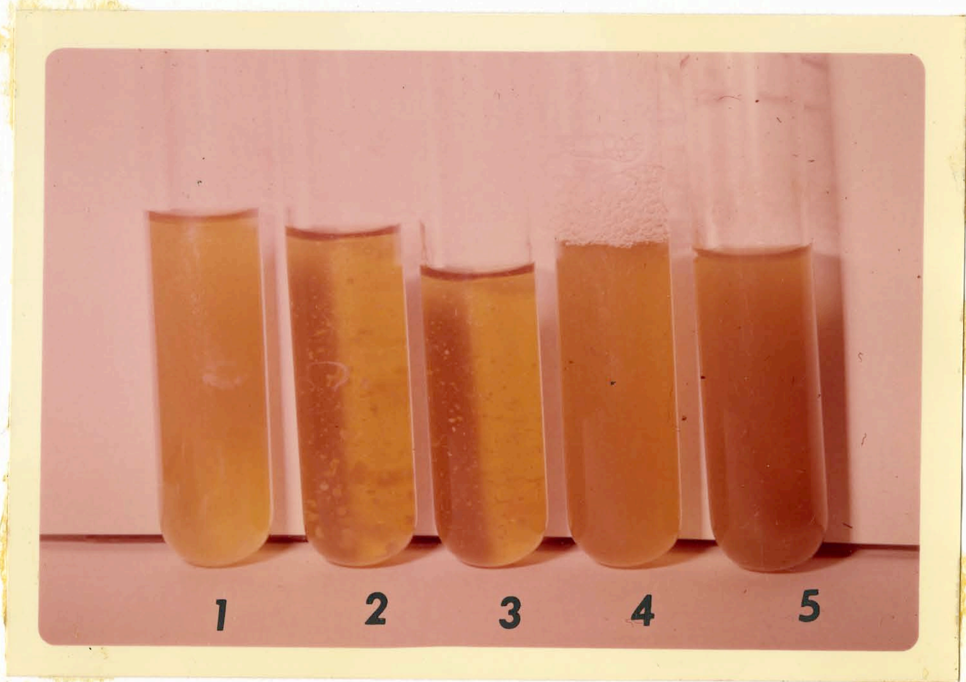

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FIGURE 5

1.) Growth of Strept. Faecalis in Todd-Hewitt Medium; uniform turbidity with granular deposit at the bottom;
2.) Streptococci grown on Blood Agar Medium;
 a.) After forty-eight hours' incubation;
 b.) After twenty-four hours' incubation;
3.) Inulin fermentation test:
 a.) Positive (Strept. Salivarious); P
 b.) Negative (Strept. Mitis). N

FIGURE 6

Qualitative test: HASE activity
a.) Control tube (broth + Serum Reagent). Note the clear solution;
b.) HASE. Positive but less than Number 5;
c.) Control tube (HA. + broth + Serum Reagent). Note the opaque solution;
d.) HASE. Negative; opaque solution;
e.) Mild HASE. Positive;
f.) Marked HASE. Positive; solution almost as clear as the tube in Number 1.

5

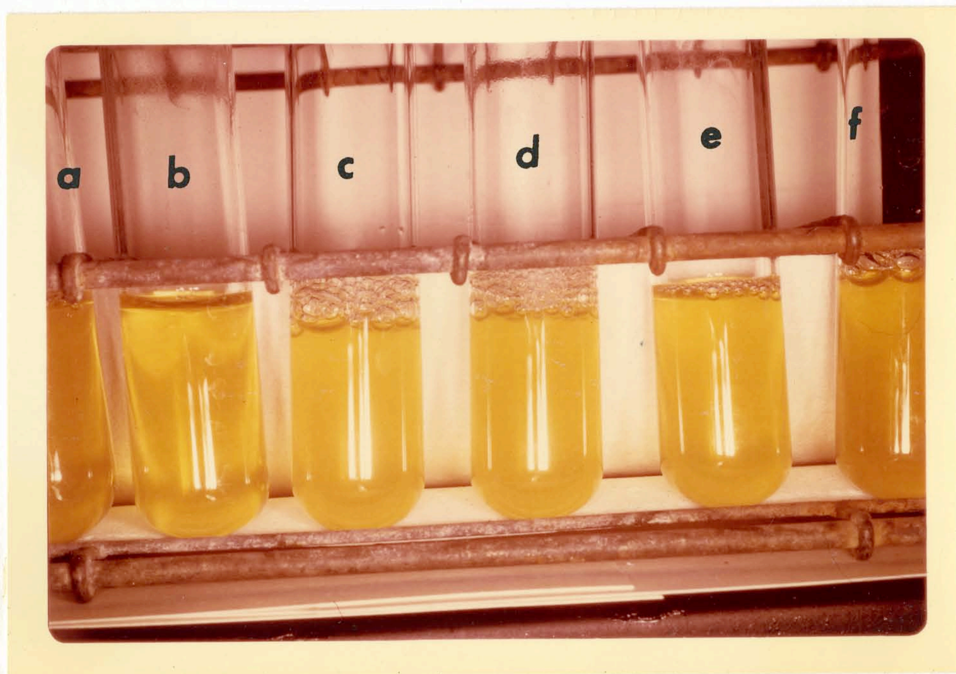

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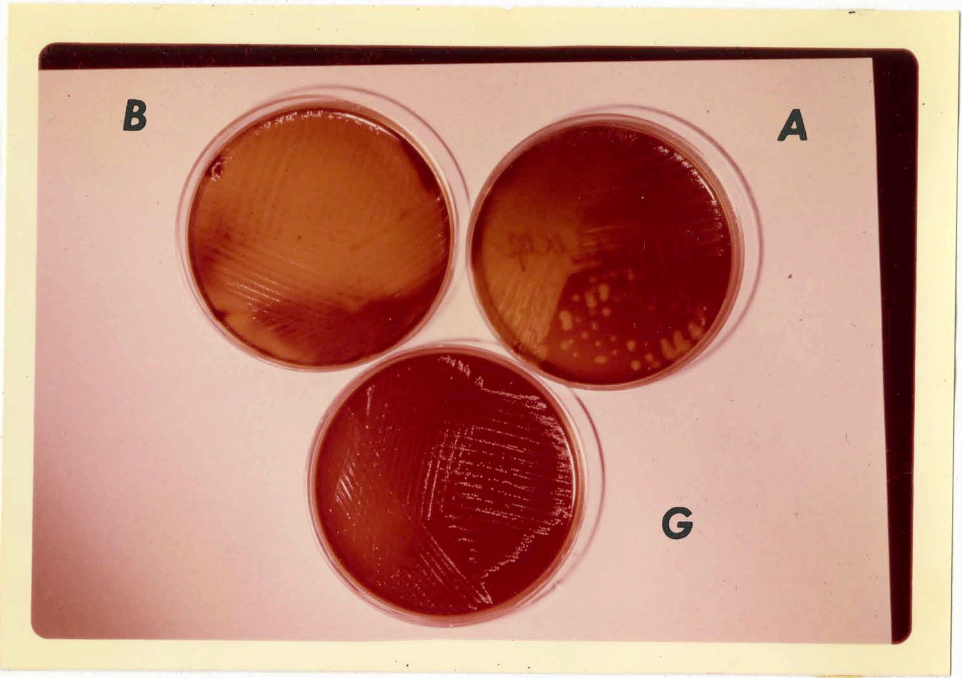
FIGURE 7

Different types of growths shown by Streptococci isolated from carious dentin:

1.) Beta hemolytic--showing complete hemolysis;
2.) Alpha hemolytic--showing partial hemolysis;
3.) Gamma hemolytic--showing no hemolysis.

FIGURE 8

Typical mucoid raised colonies of Strept. Salivarious on Chapman Medium. (Growth shown by arrows.)



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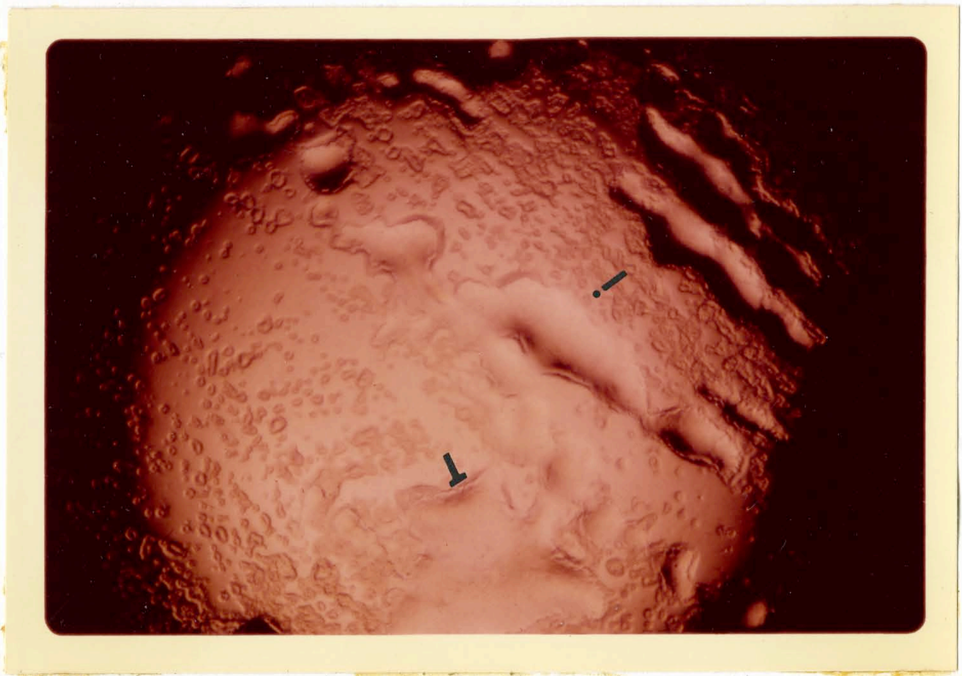

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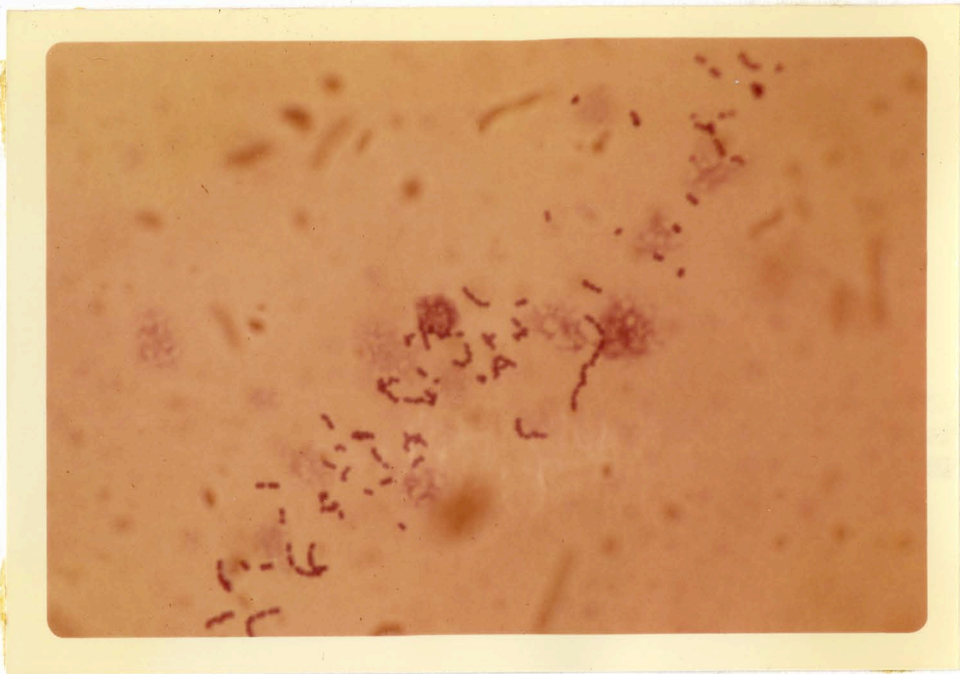
FIGURE 9

Streptococci--some in pairs, some in very short chains of even three to five organisms. Very few in medium size chains.

FIGURE 10

Very long chain of Streptococci.
(Smear prepared from culture grown in Todd-Hewitt Medium. Organism when grown on Blood Agar Medium showed partial hemolysis--Alpha type.)



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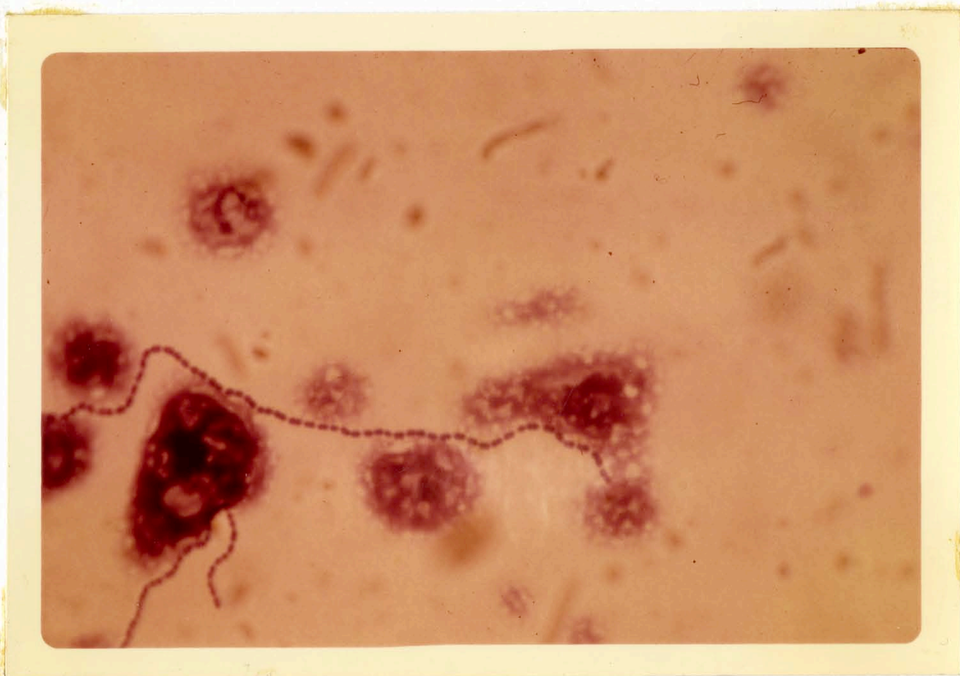

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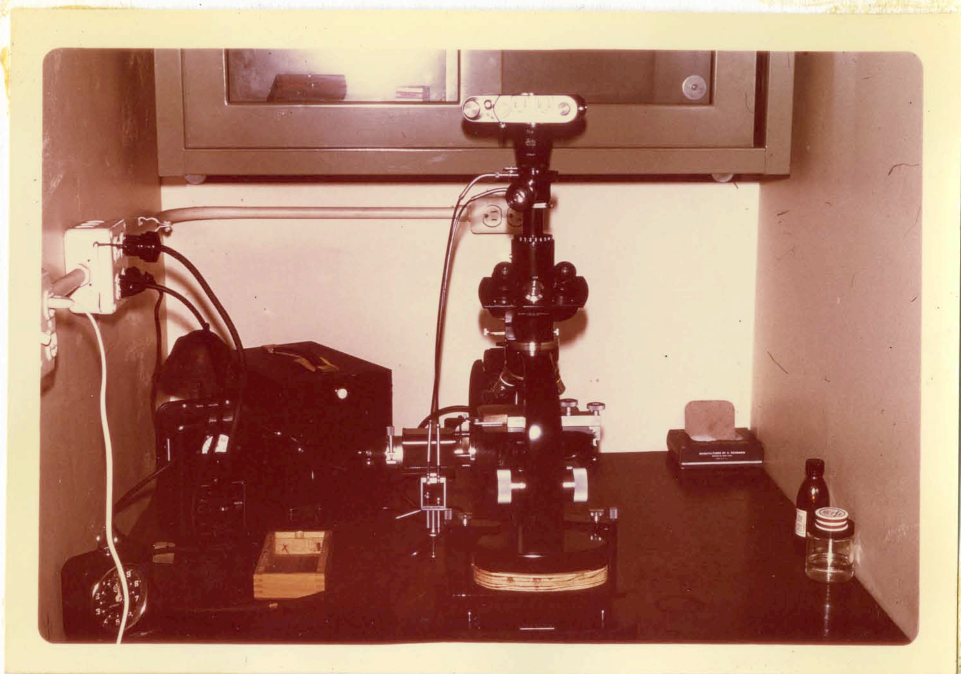
FIGURE 11

Complete equipment of fluorescent microscope with photographic camera at the top.

FIGURE 12

Hemolytic Streptococci Group B.
Note the various chains of fluorescing organisms against the dark as well as non specific fluorescing background in some area.
Fluorescence--bright apple-green color.



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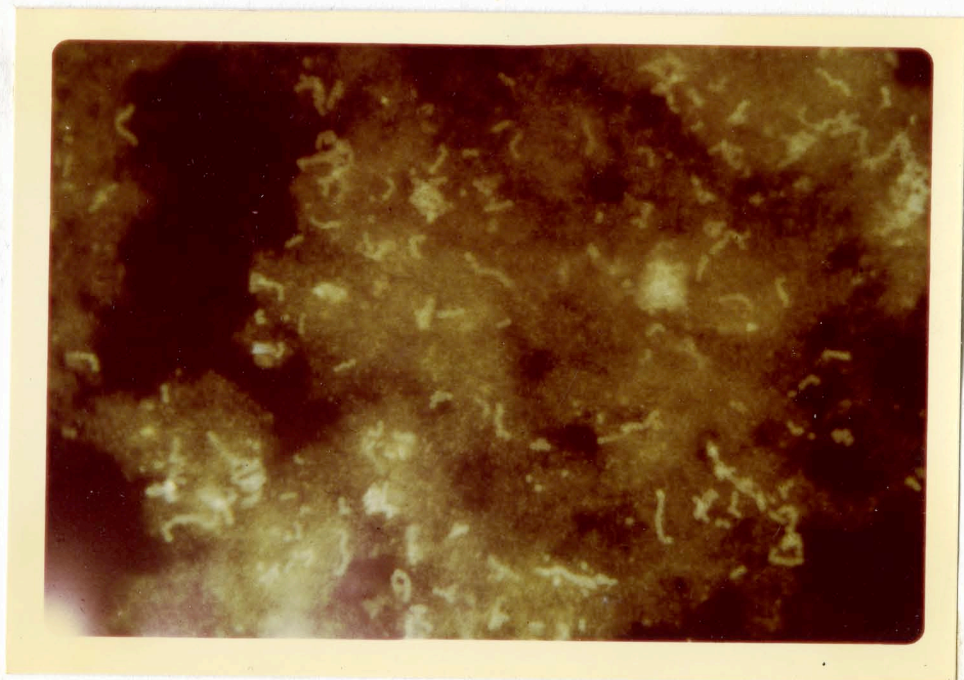

12

APPROVAL SHEET

The thesis submitted by Dr. Saryubala R. Parikh has been read and approved by three members of the faculty of the Graduate School.

The final copies have been examined by the director of the thesis and the signature which appears below varifies the fact that any necessary changes have been incorporated, and that the thesis is now given final approval with reference to content, form and mechanical accuracy.

The thesis is therefore accepted in partial fulfillment of the requirements for the Degree of Master of Science.

May 25, 1964
Date

Patrick D. Sato
Signature of Advisor