LONGITUDINAL OBSERVATIONS OF THE MICROFLORA ASSOCIATED WITH PROGRESSION OF INCIPIENT APPROXIMAL LESIONS IN CHILDREN

BY

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AD AUGUSTA PER ANGUSTA

V. Hugo

(Hernani, Act IV)

to Connie

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I give full credit to Dr. George Bowden, my advisor, for inspiration of the idea for this study, and I thank Dr. Bowden and Dr. Ian Hamilton for giving me the aspiration to pursue this endeavour. I wish, also, to thank Dr. Bowden for the consultation and consolation that he gave me during times of frustration and hesitation. Dr. Hamilton, as well, brought to me excitation and perspiration at moments of exasperation and desolation.

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A P P E N D I C E S

ABSTRACT

A longitudinal analysis has been made of the microflora associated with approximal incipient caries lesions and comparisons have been drawn between lesions which became larger, lesions which remained static and caries free control sites. Subjects were selected for the study on the basis of their dental status as revealed during regular six month recall examinations. Suitable subjects were those who showed one or more approximal incipient lesions and who also fitted other criteria such as good general health and availability for study. Parental consent was obtained in all cases. Twenty-two children, aged 4-9 years were selected and these children had a total of thirty-two lesions at the commencement of the study. Each test site (lesion) was paired in each child with a caries free control surface. Microbiological samples were taken at six or twelve week intervals over a period of one year. Radiographs of the sites were taken at the routine dental recall examination of the subjects at 6 month intervals. The subjects were instructed not to modify their dental hygiene practices throughout the study period. The routine radiographs were used to analyze the state of the lesions throughout the study. Incipient lesions which had progressed at 6 or 12 months were restored and, in some cases, the restored surface was sampled as a part of the study. The data from the radiographs was used to divide the lesions into two groups (a) progressive lesions and (b) static lesions. It was assumed that lesions which had progressed had been in an active caries state at some time during the study period.

The microbiological samples from each site were cultured on a range of media including selective media for some genera and species. The organisms identified included Streptococcus mutans, Streptococcus sanguis, Streptococcus mitior, Streptococcus milleri, Streptococcus salivarius, Actinomyces viscosus, Actinomyces naeslundii, Actinomyces odontolyticus, Neisseria species, and Veillonella. Lactobacillus strains were identified to the species level. The composition of the microflora at the sites was described as the percentage contribution of each of the organisms to the total cultivable flora. The data was analyzed to provide information on two parameters of the microflora at each site: (a) the mean percentage of the organism at a site over the total period of the survey (degree of colonization) and (b) the numbers of positive isolations of the organism at the site over the study period (persistence). These two parameters were used to define the degree of challenge that a given isolate gave to the enamel surface at a particular site. Colonization and persistence were compared for paired sites in a given subject using the student "t" test. Comparisons were drawn between progressive lesions, non-progressive lesions and control sites.

During the period of observation 14 of the 32 lesions progressed and they were restored. Simple observation of the data revealed that Lactobacillus was associated with 85% of the lesions which progressed and was never isolated from non-progressive lesions or control sites. Lactobacillus was always detected prior to the demonstration of progression by the routine radiograph. Statistical analysis of the microbiological data showed that some organisms showed a positive association with progression while others showed a negative association. Lactobacillus,

S. mutans and A. odontolyticus showed significant positive associations in both colonization and persistence. S. mitior and A. naeslundii showed significant negative associations with progression in both colonizations and persistence, however, A. viscosus showed a negative association only in colonization. Examination of the data for sites that had been restored showed that the amalgam was recolonized by all of the bacteria identified with the exception of A. odontolyticus and Lactobacillus. This suggests that these bacteria require a habitat which provides suitable niches for their survival, and that the restoration of the lesion destroys or alters this habitat.

The results support the evidence available implicating S. mutans and Lactobacillus in the etiology of caries. Another important aspect of the study is that it clearly associates Lactobacillus with lesion progression. Lactobacillus may be definitely implicated in the mechanism of progression or the presence of Lactobacillus may indicate a more extensive lesion than is shown by routine radiographs. In either case, a simple laboratory test based upon the isolation of Lactobacillus from approximal incipient lesions could be of value in helping the clinician to diagnose the state of the lesion. Such information is fundamental to the decision to restore the lesions or to attempt prevention with fluoride treatment.

CHAPTER I

INTRODUCTION

A) HISTORY OF CARIES

Through the ages, people of most races have suffered from the effects of tooth decay. As with other maladies, explanations for this disease were sought and many theories were put forward. The earliest theories were based on very little fact but were plausible explanations given the extent of general scientific knowledge at that time. These theories included from parasitic worms, imbalance of "vital humours" (Greek), destruction by an unidentified chemical agent and microbial parasites[1].

The birth of the modern theory of dental cavity formation is often attributed to W.D. Miller, in 1890[2]. He termed his hypothesis the "chemo-parasitic" theory because it stipulated that caries was the result of decalcification of dental tissue (enamel and dentine) by acids produced by microorganisms residing in the oral cavity. Miller's conclusions were based on a series of experiments which demonstrated that: (i) saliva mixed with different carbohydrate-containing foods and incubated at 37^{0} could decalcify the crown of a tooth, (ii) at least 30 species of oral bacteria could produce enough acid to cause dental caries, (iii) lactic acid was an end product of fermentation of carbohydrate-saliva mixtures, and (iv) a variety of microorganisms were

seen to invade carious dentin. The observations of plaque by Williams in 1897[3] added support to Miller's conclusions. Williams speculated that dental plaque provided the means of localizing the organic acids, produced by bacterial fermentation, against the tooth surface. It was proposed that plaque impeded the dilution and neutralization of the organic acids by saliva.

Stephan[4] was the first person to demonstrate these concepts *in vivo*. By use of minute pH electrodes placed in plaque on accessible tooth surfaces in the mouth, he was able to demonstrate the dramatic drop in pH that occurs after rinsing with glucose or sucrose. This was followed by a gradual return to "resting" pH after about 40 minutes. More recent studies[5,6] using indwelling telemetric sensors at interproximal surfaces, have shown that the period of pH depression probably lasts much longer than 40 minutes at these sites. It has also been observed that the pH response to a carbohydrate challenge is dependent on: (1) the type of carbohydrate, (2) concentrations of substrate, (3) the microbial composition of the plaque, (4) the amount of plaque and (5) the rate of salivary flow[5,6].

B) ETIOLOGY OF CARIES

From these and other observations it is apparent that dental caries is dependent on the relationship of 3 main environmental factors. These are: the host, the microflora

and the substrate. For the caries process to be active, these three environmental factors must be in a critical relationship. A fourth factor, time, must also be considered, as caries is a dynamic process. The host factor refers mainly to tooth and salivary characteristics. For instance, tooth morphology and tooth positioning[7] influence plaque accumulation and food retention and the fluoride content of enamel influences its solubility[8]. Salivary flow rate and salivary buffering capacity can influence pH responses in plaque[9]. The predominance of acidogenic and aciduric species of organisms in plaque promote the caries process[10]. Conversely, a high proportion of lactate-metabolizing organisms (e.g. Veillonella) [11] may exert a neutralizing effect on plaque pH. The type of substrate (carbohydrate), its concentration and the duration and frequency of its availability to plaque organisms will also influence the caries process[12,13,14].

C) CONTROL OF CARIES

Since all three principle factors must be present to produce caries, it should theoretically be possible to stop the disease by eliminating or modifying any one factor. For instance, it has been demonstrated that by eliminating fermentable carbohydrate from the oral cavity, the caries process is arrested[15]. Similarly, it has been shown, in animal experiments, that if the oral microflora is eliminated, caries will not occur[16]. However, it is impractical to con-

sider modifications of oral conditions of this type in human populations. Therefore, efforts to disrupt the caries process have been multifaceted in an attempt to minimize the caries potential of each factor. People have been encouraged to minimize frequency of carbohydrate intake[17] (particularly refined sugars); to eliminate dental plaque through brushing and flossing[18] and to fluoridate water supplies to decrease solubility of enamel by incorporation of fluoride[19]. These measures have resulted in a significant decrease in the rate of caries in industrialized countries[20].

D) SUSCEPTIBILITY OF HUMAN POPULATIONS TO DENTAL CARIES

Dental caries is generally regarded as a recent disease of mankind. Even so, evidence exists that caries was plaguing humans thousands of years ago. It spread from being a disease that affected a few teeth in a limited number of individuals to a situation where greater numbers of teeth were afflicted to a greater extent in a larger number of people[49].

The present world picture of dental caries is one of divergent trends[143]. In the developing countries there is a rapidly increasing incidence of dental caries[144] and a scarcity of dental health manpower. On the other hand, in the industrialized countries there is a marked decline in incidence of dental caries[145] and an adequate supply of dental

manpower. Apparently, the major influencing factor is a change to modern diet and dietary habits, particularly the consumption of greater amounts of commercial sugar-containing products[147]. A similar effect has been identified within industrialized societies, where higher consumption of refined carbohydrate is associated with lower socio-economic status[148]. Specific groups of people, such as native populations, within, or on the periphery of industrialized societies have exhibited patterns of disease that are related to dietary habits[150],[151].

The decline in caries incidence in industrialized countries became markedly noticeable in the early 1970's. The decline has been attributed to a combination of (1) the elimination of most of the accumulated caries, (2) widespread public health programs of fluoride brushing and/or rinsing, (3) availability of fluoride dentifrices and (4) water fluoridation[149].

In industrialized countries, the effect of fluoridation of water supplies on reduction of caries incidence in children is well documented[143],[152],[153] and statistics show an apparent decrease of about 50% in incidence of caries attributable to fluoridation of water supplies. However, there are also decreases in incidence in non-fluoridated areas. A number of authors[154],[155] have attributed much of this change to increased use of fluoridated dentifrices[155]. A reduction of caries incidence of 25% has been generally

attributed to fluoride toothpastes.

Along with an overall decreased incidence in caries, there has been a change in the susceptibility of specific sites on the teeth. Fissure caries now accounts for a larger portion of lesions, particularly in fluoridated areas[153]. However, approximal (smooth lesions) lesions are still observed[152,156]. Approximal lesions often appear to develop very slowly, remaining in an incipient stage of development for an indefinite period of time[157,158].

These changes in the incidence, in the susceptible dental sites and in the rate of development of dental caries have influenced everyday dental practice, especially in fluoridated areas, where the frequencies of recall examination and diagnostic bite-wing radiographs[159] are being reevaluated. The value of preventive intervention techniques, such as pit and fissure sealants is increasing[160]. Furthermore, the criteria for restoration of incipient approximal lesions are changing as evidence grows regarding their slow development[21].

E. DENTAL MORPHOLOGY AND CARIES LESIONS

The shape of teeth and the physical relationship of teeth to each other provide a diversity of environmental situations for microorganisms. Some areas, such as the facial and lingual surfaces of teeth, are relatively smooth and exposed to mechanical trauma during mastication, speech,

etc. These surfaces, therefore are less susceptible to significant bacterial colonization than are the more protected areas. By comparison, the surfaces of teeth adjacent to other teeth (approximal surfaces) are more isolated from mechanical trauma and the effects of saliva than the facial and lingual surfaces. Also, these protected areas of teeth tend to harbour food remnants more than the exposed areas. Therefore, metabolic substrate is more available in these isolated areas and bacteria have a better chance for survival. As a result, caries lesions are more prevalent in locations that provide a protective environment and availability of food than in more exposed locations[189].

The most caries susceptible areas of teeth are those surfaces with developmental defects such as fissures and pits[189]. These fissures are most common, and tend to be most pronounced, on the occlusal surfaces of teeth, particularly molar teeth. Pits and fissures offer isolated environments for bacterial incubation and readily trap food that is driven into their depths during mastication. The shape and depth of typical occlusal fissures even prevents the removal of plaque and food debris by tooth brushing. Other surfaces with pits and fissures, such as the buccal of mandibular molars and palatal of maxillary molars are quite susceptible to caries attack, also. This is particularly true when the pits and fissures are very pronounced. Approximal surfaces are the next most caries-susceptible surfaces, providing a

relatively isolated environment for microorganisms, but not as isolated as the depths of pits and fissures and not providing the inroads to the depths of enamel that are afforded by pits and fissures. As an example of the variation in susceptibility of surfaces, the likehood of decay of lower first permanent molars, in descending order, is: occlusal, buccal, mesial, distal and lingual. Not only is there variation and predictability in the caries susceptibility of different surfaces, but it has also been shown that the relative composition of the microflora varies from site to site [184,188].

It is important to note that inherent differences between surfaces and sites have resulted in a differential response to fluoride exposure. It has been demonstrated that in water fluoridated communities the over-all caries rate has decreased dramatically, but that the proportion of fissure caries (occlusal) has increased relative to smooth surface caries[152] Apparently, the benefits of fluoride deposition in surface enamel are much more significant on smooth surfaces. Furthermore, especially in fluoridated populations, approximal lesions have been shown to be much more slow-growing than occlusal lesions. pn fact, approximal lesions often appear to arrest progress and even remineralize or regress, whereas untreated occlusal lesions usually do not[158].

The progression of destruction in smooth surface lesions is quite different to that in fissure caries. Smooth sur-

face lesions, when sectioned longitudinally, are cone-shaped with the apex directed towards dentine. That is, there is a broad area of surface attack and a narrow advancing deep front. In contrast, the fissure lesion usually starts on the sides of the fissure wall and penetrates perpendicularly toward the dentino-enamel junction, guided by the direction of the enamel prisms, resulting in a broad advancing front. The cone-shaped lesion in fissure caries has its base towards dentine[190]. The clinical significance is that fissure caries tend to involve a broader area of dentine than might be expected from the clinical appearance and smooth surface caries involves a narrower area of dentine than expected[189].

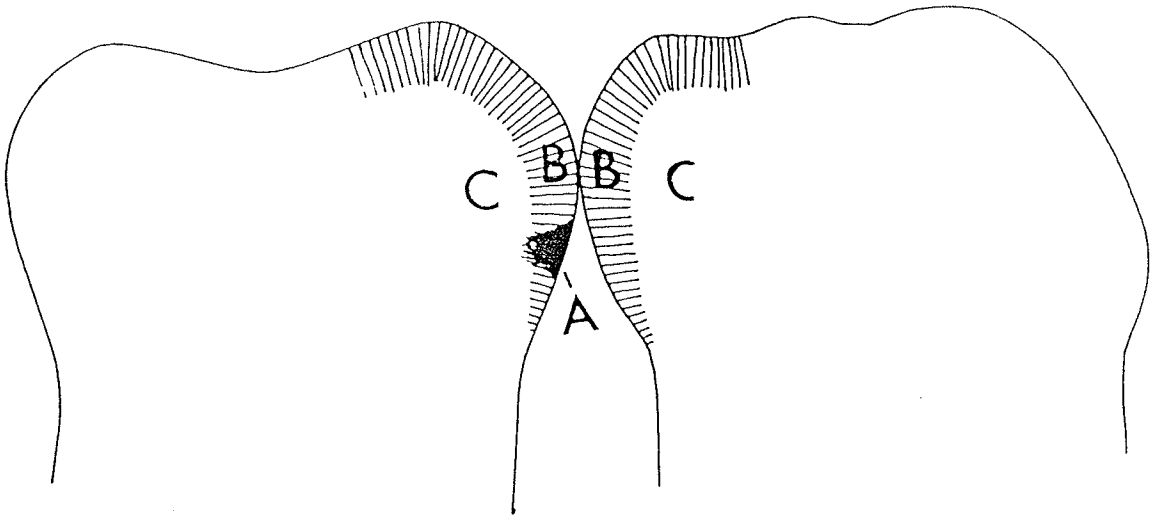
Therefore, because of differences in susceptibility to caries, microfloral composition, rate of caries progression and pattern of caries progression, fissure and smooth surface caries deserve separate consideration in terms of diagnosis, prognosis and treatment.

F) APPROXIMAL CARIES LESIONS

Approximal caries lesions occur on adjacent tooth surfaces which are in close contact. These lesions are described as smooth surface lesions as they do not originate in developmental pits or fissures. This is an important consideration when the initiation, progression, prevention or remission of these lesions is studied. Approximal

lesions are usually present immediately gingival to the contact area of the teeth (Figure 1). This location is relatively inaccessible and approximal lesions cannot be diagnosed clinically (by visual or tactile examination) until they have progressed to a very advanced stage. A lesion at this advanced stage poses a threat to the vitality of the dental pulp and restoration involves the removal of gross amounts of tooth structure. This difficulty of diagnosis by tactile or visual methods has meant that the dental bitewing radiograph has been used extensively by dentists on a regular (usually 6 monthly) basis for screening the approximal surfaces of posterior teeth. Radiographs are able to detect approximal lesions at a much earlier stage than the clinical examination and an experienced dentist can detect very minor radiolucencies in the approximal enamel. Such lesions are diagnosed as carious and subsequently restored with a dental filling. However, it has been shown that a precise diagnosis of the extent of approximal lesions cannot be made from radiographs[21]. On restoration the lesion may be more or less extensive than was expected from the radiograph. A recent study by Bille et al [21] which compared x-ray diagnosis to clinical findings at the time of restoration has emphasised the inaccuracy of radiographs. These authors [21] found that only 20% of the lesions which extented into the amelodentinal junction and 50% of lesions extending into dentine showed actual cavitation of the enamel surface. Furthermore, a similar study [22] of 827 operative treatments

Figure 1



Approximal caries lesion of deciduous molar tooth
(sagittal view)

A = lesion
B = enamel
C = dentine.

revealed that 462 or 56% of the restorations were placed in sites without actual cavitation. This study [22] also found that there was a greater tendency towards premature treatment in younger patients. It was determined that in patients under 16 years of age only 13% of approximal lesions restored showed actual cavitation at the time of restoration. This evidence places in question the value of bite-wing radiographs as the sole diagnostic tool for approximal caries. It also indicates that in many cases, restorations were placed into teeth where the surface enamel was intact and the lesions could remineralize. Accurate diagnosis can be seen to be an important consideration in these cases.

CHAPTER II

HISTOLOGY OF CARIES

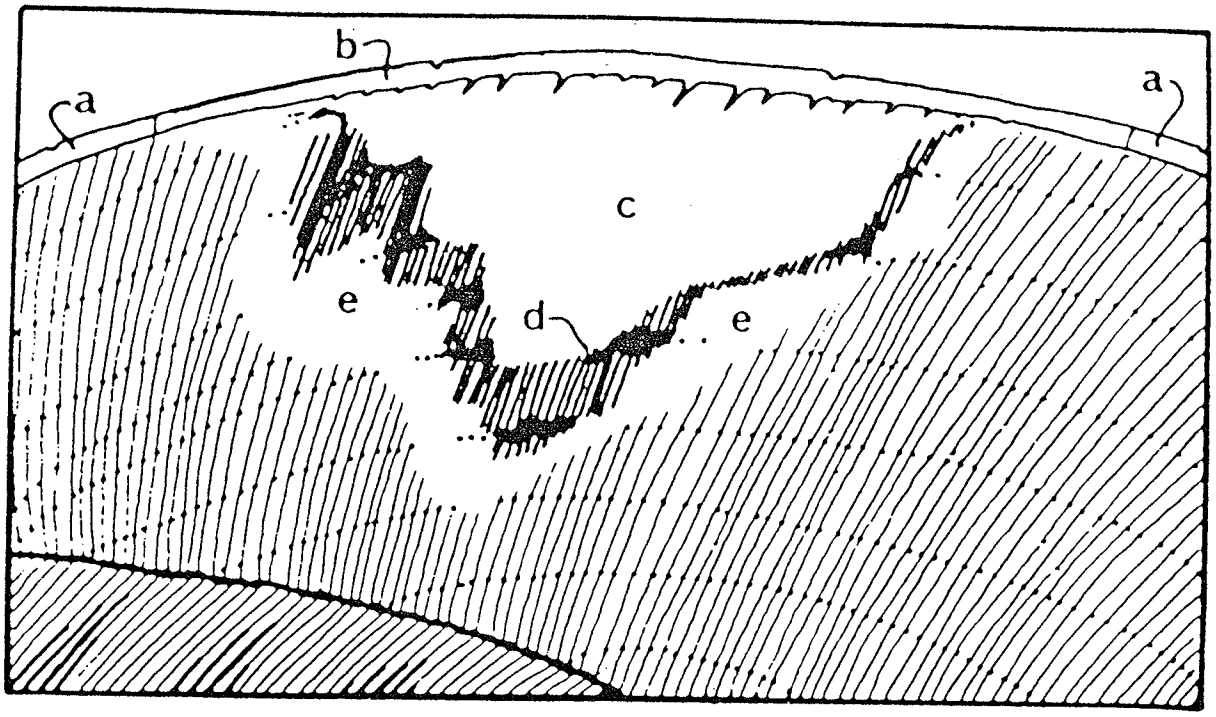
In order to understand and appreciate the nature of the caries process it is necessary to know the ultrastructural nature of the caries lesion. This is particularly true of the surface structure, which is of significance relative to remineralization.

1. FOUR ZONES

Histological examination of developing carious lesions of dental enamel has revealed some interesting phenomena. Normal human enamel is highly mineralized, being composed of about 97% mineral in the form of hydroxyapatite and 3% organic material and water. The caries process involves dissolution of the mineral component, resulting in an increase in the total pore volume of the enamel.

When viewed through a light microscope, longitudinal ground sections of early carious lesions are seen to be composed of several layers or zones. There has been controversy over the years as to the number of zones that are actually present. It seems as though much of the confusion is due to the fact that the zones are not all clearly demarcated. There are transitional areas between zones, and in cross section the zones overlap. In 1956, Darling [24] described three zones, while a year later Gustafson [25]

Figure 2



Schematic representation of the zones of the early smooth surface enamel caries lesion.

a - normal enamel surface

b - surface zone

c - body of the lesion

d - dark zone

e - translucent zone.

described seven zones. It has been generally agreed [26] that four zones can be delineated within the lesions. These are: (1) a translucent zone at the deepest part of the lesion : (2) a dark zone just above the translucent zone (3) the so-called body of the lesion above the dark zone and (4) a narrow, intact zone at the outer tooth surface. The third zone (body) occupies the bulk of the early lesion and shows the greatest degree of demineralization.

When examined with a polarizing microscope, normal enamel displays negative intrinsic birefringence. Birefringence refers to the property of a medium which causes it to show more than one index of refraction when transmitting plane polarized light. Intrinsic birefringence is characteristic of crystalline materials (such as tooth enamel) and is related to an asymmetrical alignment of chemical bonds, ions or molecules. A sign of birefringence is given to the structure based on the path taken by the slower and faster rays. Enamel has a negative intrinsic birefringence, relative to prism direction. Enamel, particularly carious enamel, contains pore spaces as well as prism structures. A mixed body of rodlets and pore spaces which contain material of a different refractive index from the rodlets produces a positive birefringence relative to the orientation of the rods. This is called "form birefringence". As dissolution of the mineral content takes place and pore volume increases, a positive form birefringence is produced by the

intercrystallite spaces. The observed birefringence is the visible result of the combination of positive and negative birefringent effects. Therefore, as demineralization and pore spaces increase, there is a transition from negative to positive birefringence. This produces an observed decrease in negative birefringence. When the pore volume is sufficient, positive birefringence will dominate and the observed effect will be a positive birefringence [23]. The birefringent effect is also dependent upon the medium occupying the pore spaces. The greater the difference between the refractive index of enamel and the substance (imbibition medium) occupying the pore spaces of enamel, the greater is the positive birefringence. The extent of the demineralization of the enamel can be quantitated by employing imbibition media of different refractive indices[27]. By varying the imbibition medium filling the pore spaces, a quantitative measurement of the pore volume can be made and the degree of demineralization calculated [27].

Two groups of imbibition media have been employed in this process. The first group consists of a series of aqueous solutions of potassium mercuric iodide or Thoulet's solution which provides a range of refractive indices. These media have been employed in studies by Darling [24,27], Silverstone [25] and others. The second group of imbibition media is comprised of the aliphatic alcohols and quinoline [23,28]. This group of media has the advantage that both the molecular

size and the refractive index increase in order from methanol through octanol. The observed birefringence is measured by instrumentation which calculates the total path difference at specific locations[27,28,29] within the enamel.

The development of these techniques has allowed researchers to examine the mineralization of the four zones of the early enamel lesions.

B. SURFACE ZONE

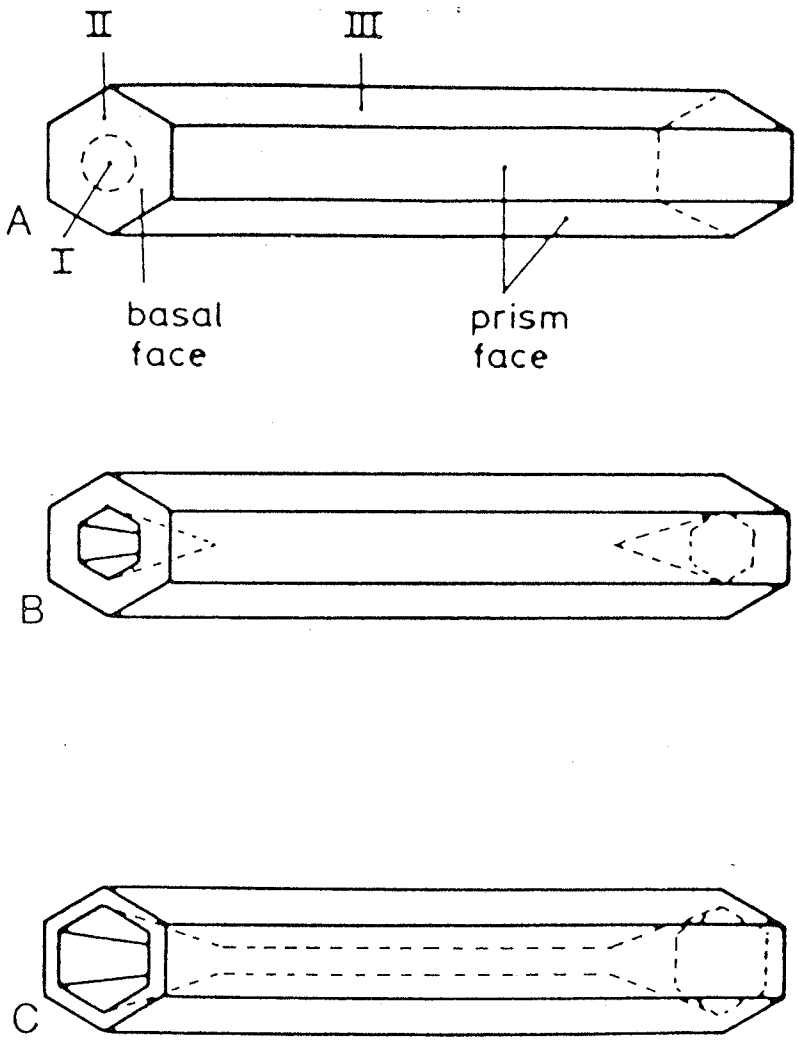
Early enamel lesions, with an intact enamel surface show, on micro radiographic and polarizing microscopic examination, a well mineralized surface zone about 30 um thick. The enamel immediately below this surface zone is, by contrast, highly demineralized. This phenomenon was first demonstrated on microradiographs in 1932 by Applebaum[34]. The intact surface zone was a surprise and puzzled early workers who attributed it to many causes, ranging from photographic artifacts[49] to a protective influence of an organic coating[36]. A number of investigators suggested that the surface zone was manifested due to special properties of the superficial enamel. These properties included a higher degree of mineralization[34], a high fluoride and low carbonate content[50] and the attraction of acid anions to the enamel surface which provided a protective coating[51]. Several studies have supported the idea that the surface zone is a result of greater resistance of the surface enamel to dissolution. Thewlis[52] (1940) and Brudevold (1948)[53] maintained that the

greater mineralization of the surface enamel was the critical factor. Isaac et al[54] cited higher a fluoride and lead content in surface enamel as contributing factors. Darling suggested that a greater amount of insoluble protein in surface enamel imparted a resistance to dissolution[27]. However, a number of studies[23,54] have shown that a fairly well-mineralized surface zone of enamel in caries can be created on enamel surfaces which have been abraded or where the surface enamel has been otherwise removed. It is suggested[26] that the surface zone remains well-mineralized by a process of reprecipitation of calcium and phosphate ions dissolved Plaque on the enamel surface, which is saturated with these ions may also be a source for ions which precipitate into the surface zone. Silverstone[26] suggests that the localized high fluoride ion concentration resulting from dissolution of the surface enamel would favour such precipitation. It is also significant that saliva is supersaturated with respect to calcium and phosphate ions and this would favour precipitation[211]. Therefore, it is likely that the surface zone is a product of both the special properties of surface enamel and the tendency for reprecipitation to occur at the surface of enamel associated with a lesion. The mineral loss within this surface zone is reported to be about 5% compared to sound, normal enamel[47]. Magnesium levels show no consistent difference between surface zone enamel and adjacent sound enamel. Both areas contain about 40% by weight less magnesium than sound interior enamel[41].

Ultimately in the progression of the carious lesion, the surface zone is demineralized. This event is considered to represent a late event in the progression of the lesion. Studying the progression of caries *in vitro*, Silverstone[23] described this stage as small areas of demineralization spreading from the outer enamel surface towards the body of the lesion. In the same study, Silverstone noted that the rate of progression of lesions increased significantly when the surface zone was disrupted.

Electron microscopy has shed some new light on the mechanisms involved in the production of early caries lesions. However, much of the information has been contradictory and many features that are visible at the light microscopic level have been difficult or impossible to identify at the ultrastructural level. In 1971, Mortimer and Trantner[55] utilized the scanning electron microscope to look at small enamel lesions. They reported that the Striae of Retzius appeared less demineralized than the surrounding enamel and they noted loss of material from prisms and from prism centres. These workers proposed from their findings that the carious attack involved the prism core and necks of prisms (Figure 3). However, much of the difficulty and controversy in ultrastructural investigation of both normal and carious teeth comes from the problem of preparing specimens of the hard tissue. This is coupled to the problem of determining which of the visible features are artifact. It is on this

Figure 3



Schematic representation of the initial dissolution of a hexagonal hydroxyapatite crystallite.

A - intact crystallite
- active sites of demineralization are indicated: I II III

B - initial etchpit formation

C - later stage - centre of crystal removed parallel to long axis.

basis that Poole and Silverstone[56] differed in their opinion of the ultrastructural features of carious enamel.

Using transmission electron microscopy, Johnson[57] reported an increase in intercrystallite spaces, suggesting a loss of mineral from the external surface of crystallites. Besides this slight etching of crystallites of the outer enamel, he reported defects in the enamel surface up to 4 um in width and 5 μm in depth. Johnson and other workers[57,58] also reported areas of demineralization at the centre of crystallites, giving them a hollow-tube type of appearance. It is important to note that no observable changes can be detected by electron microscopy in the first two zones of enamel caries. The changes noted here are all observed in the body of the lesion, where there is maximal demineralization. Therefore, no correlations have been made between the light microscopic and electron microscopic features of the translucent and dark zones.

Although conflicting evidence has been presented, the most outstanding ultrastructural investigations indicate that demineralization occurs from both inter and intraprismatic enamel, with a greater degree of susceptibility at the prism junction.

C. THE BODY OF THE LESION

The body of the lesion occupies the largest portion of small-enamel lesions. Longitudinal ground sections through

lesions imbibed with quinoline and viewed with transmitted light show the body of the lesion as a translucent zone with pronounced Striae of Retzius. When viewed through the polarizing microscope, sections in quinoline appear very similar to those viewed by transmitted light. When imbibed with water the body appears positively birefringent compared to the negatively birefringent normal enamel. Darling[27] reported a minimum pore volume of 5% at the periphery of the lesion, increasing to 25% or greater at the centre. In the same study, Darling reported alternate radioluscent and radiopaque lines about 30 μm apart running obliquely through the body of the lesion. He also noted, within the body of the lesion, a pattern of alternating radioluscent and radiopaque lines at right angles to the enamel surface and spaced 6-8 μm apart. In many sections the radioluscent lines were oriented at right angles to prism direction. Darling[27] suggested that these findings represented the result of acid invading the enamel through the Striae of Retzius with demineralization progressing along the interprismatic substance, through the cross striations of the prisms to the prismcores. Other workers, such as Crabb[45] disagreed with this theory. Crabb noted that the Striae of Retzius showed significant positive birefringence in quinoline and he explained this by proposing that the birefringence was associated with the slow progression of demineralization (as in the dark zone). He suggested that the striae actually presented obstacles (or areas of resistance) to progressive

demineralization. Further studies by Crabb[45,46], using quantitative microradiographic procedures, led to two theories suggesting, (1) that the alternate radiopaque and radiolucent bands in the body of the lesion were an expression of an inherent structural feature of enamel and not related to direction of spread of demineralization, however, their appearance was dependent on the extent of demineralization of the enamel, (2) that demineralization spread outwards from subsurface areas to the surface of the enamel. Mortimer[37], in two-dimensional microdensitometry studies, supported these ideas and suggested that as the alternate banding corresponded with the periodicity of the prisms of enamel, demineralization occurred along the prisms, with areas of greater susceptibility where they crossed the Striae of Retzius.

A number of studies have identified radiopaque, well-mineralized bands in the body of the lesion. Silverstone[48] found 15 lesions exhibiting these laminations in an examination of 100 carious lesions in deciduous molars. These radiopaque laminations follow a contour similar to the advancing front of the lesions. This suggests that the lamination defines the advancing front at various stages of lesion development and may be the result of a temporary period of arrest of the lesion.

Bergman and Lind[47], using quantitative microradiographic procedures, showed a wide variation in demineralization of

the body of the lesion. The degree of demineralization was independent of the depth of the lesion. The lowest mineral content observed was 29% by volume. This data is similar to that of Hallsworth et al, [41] who found an average reduction of 24% in mineral content of the body of the lesion compared to sound enamel. These authors also determined that there was an average reduction of 20.1% of magnesium by weight in the body of the lesion. These bands were often, but not always identified in early enamel lesions.

D. THE DARK ZONE

The "dark zone" of the enamel carious lesion can be demonstrated in 85-90% of lesions[28]. It is visible by transmitted light microscopy after imbibition with quinoline or Canada balsam and appears dark brown. On examination with polarized light after imbibition with quinoline, enamel lesions show the dark zone as positively birefringent. Studies wtih the polarizing microscope have yielded considerable information about this zone. Some confusion was created because this zone appeared positively birefringent and yet in contrast microradiographs showed that the dark zone was more mineralized than the body of the lesion, which appeared negatively birefringent[42]. Applebaum[32] in 1935 first proposed that the explanation for these observations might be a difference in permeability between the two zones. A convincing theory for the difference between the potential light and microradiographic data was not put forward until

1961. Darling et al (1961)[27] showed that if a variety of alcohols were used for imbibition media, the observed birefringence was more a product of the size and shape of the molecules of the imbibition media than their refractive indices. Darling et al [27] suggested that the dark zone contained micropores that show selectivity on the basis of size and partially exclude the larger molecules of imbibition media. The greater the molecular size of the media, the greater their exclusion from the micropores.

The origin of the micropores was explained by Silverstone et al[8],[28], who demonstrated that a dark zone could be created in a lesion without a dark zone if the lesion was remineralized. They ventured the theory that a combination of micropores and large pores develops in the dark zone by a process of remineralization of some of the large pores. This concept would support the school of thought that portrays the caries process as a system of constantly changing phases of demineralization and remineralization.

It is important to note that the presence of a dark zone appears to depend on a relatively slow rate of caries attack. This statement is based on information from several studies of the progression of caries lesions in vitro. Gray and Francis[60] used lactic acid in a hydroxyethyl cellulose base to produce enamel caries in vitro, within 96 hours, and showed that only a narrow dark zone was produced. Silverstone[61], using an in vitro system of gelatine gels of

varying gel concentration, demonstrated that when the demineralization process was slowed down so as to take several months, a dark zone was produced that was virtually identical to that of natural caries. He found that the concentration of the gel was the critical factor, demonstrating an inverse relationship between the gel concentration and rate of attack.

Histochemical data available for the dark zone is derived mainly from the micro-dissection study by Hallsworth et al[41]. These authors reported a 6% reduction in mineral content in the dark zone when compared to normal enamel. They felt that this figure might be a little high due to a possible bias in their method. Hallsworth et al also reported an average 12% reduction by weight in magnesium in the dark zone. Darling[27] demonstrated a 2 to 4% pore volume by examination of the dark zone by polarized light.

E. THE TRANSLUCENT ZONE

The translucent zone was first reported as a "light" zone in caries lesions by Williams[3] in 1897. In 1922, Mummery[32] described this zone as a 'translucent zone'. Considerable controversy then ensued over the nature of this zone, with some authors stating that it was an area of hypermineralization produced by a vital response[33,34]. Gustafson[25], in 1957, observed that the translucent zone appeared more negatively birefringent that normal enamel. He concluded that

the translucent zone was hypermineralized. However, later studies, using two-dimensional microdensitometry[37], established that the translucent zone was in fact demineralized enamel. The apparently greater negative birefringence of the translucent zone compared to the adjacent normal enamel has been explained[38] to be a result of the failure of the small pores of normal enamel to be filled with large molecules of quinoline inhibition media. This produces small amount of positive form birefringence. The pores of the demineralized translucent zone are large enough to accept the quinoline molecules and the positive form birefringent effect is not present. Thus the observed birefringence is slightly more negative than that of sound enamel.

Weatherall[39] and Hallsworth et al, [40,41] using microdissection and microchemical techniques, reported that the translucent zone had lost approximately 1.2% by volume of the mineral content. The same studies showed that the enamel of the translucent zone had a lower carbonate content and 12% by weight less magnesium. This data has established beyond doubt that the translucent zone is demineralized enamel.

CHAPTER III

INCIPIENT CARIES LESIONS

A) DEFINITION

The Concise Oxford Dictionary defines "incipient" as "beginning in an initial stage".

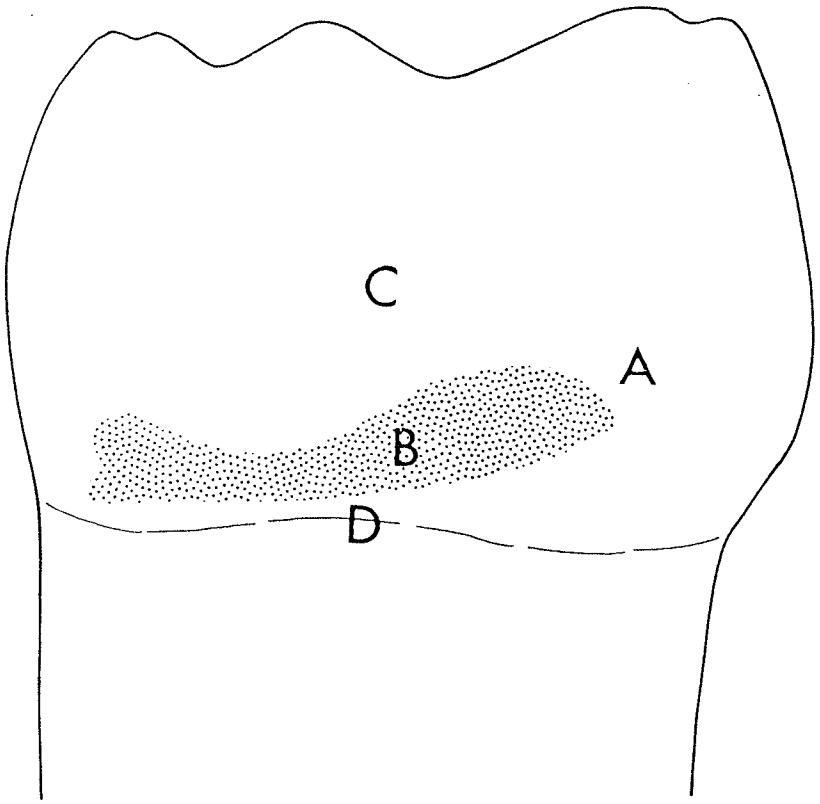
Webster's Third International Dictionary defines "incipient" as "beginning to be or become apparent" :commencing, initial".

B. CLINICAL APPEARANCE

Clinically, when a carious lesion occurs on a visible smooth surface or on an approximal surface of an extracted tooth, the earliest visible appearance of caries is an opaque white area, compared with the translucency of adjacent sound enamel. In fact, these incipient lesions are often termed "white-spot lesions".

When incipient lesions occur approximally they are found just cervical to the contact point of the tooth. Larger lesions follow the cervical curvature of the facet of the contact point to form a kidney-shaped contour (Figure 4). When examined with a dental explorer, the incipient lesion is found to have a surface indistinguishable from sound enamel, that is, the surface is hard and shiny[32]. Some incipient lesions are brown in colour, apparently due to absorption of exogenous staining material from the environ-

Figure 4



Diagramatic representation of an early (incipient) approximal caries lesion of a deciduous molar tooth.

A - intact enamel

B - incipient caries (white-spot lesion)

C - contact point

D - cervical margin.

ment. Usually it is assumed that brown, intact lesions have existed longer than white lesions and have absorbed these staining substances into their porous surfaces. These lesions are often called "brown-spot lesions". The extent of staining is probably related to specific environmental influences such as smoking.

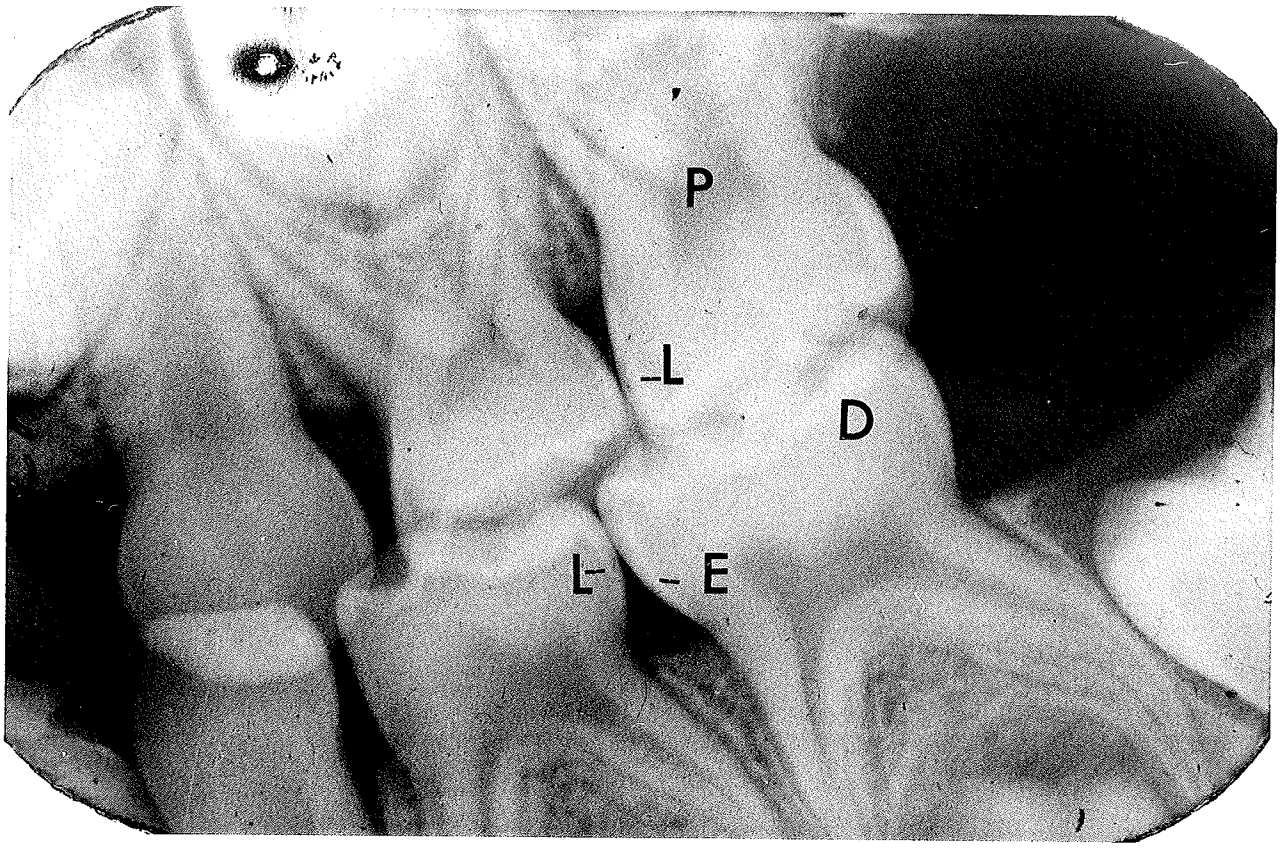
C) RADIOGRAPHIC APPEARANCE

Radiographically, incipient carious lesions appear as small radiolucent areas in the outer enamel (Figure 5). As previously described, radiographic diagnosis of the extent of carious penetration is very inaccurate. However, it is believed that at this stage the dentin is unaffected and the lesion does not necessarily require restoration[187].

D) CLINICAL SIGNIFICANCE

Once an incipient lesion is detected it must be monitored radiographically and clinically for evidence of progression. Lesions which do progress are restored. It is possible that incipient lesions may be arrested or reversed (remineralized) if the environmental conditions which initiated the lesion change or are changed (e.g. by improving local plaque control habits). This fact was demonstrated on a large scale, in vivo, in a study in Karlstad, Sweden[62]. Similarily, a study at the University of Alabama[63] illustrated that if strict oral hygiene criteria were met and maintained, apparent arrest of all incipient lesions, diag-

Figure 5



Radiograph illustrating two incipient approximal caries lesions.

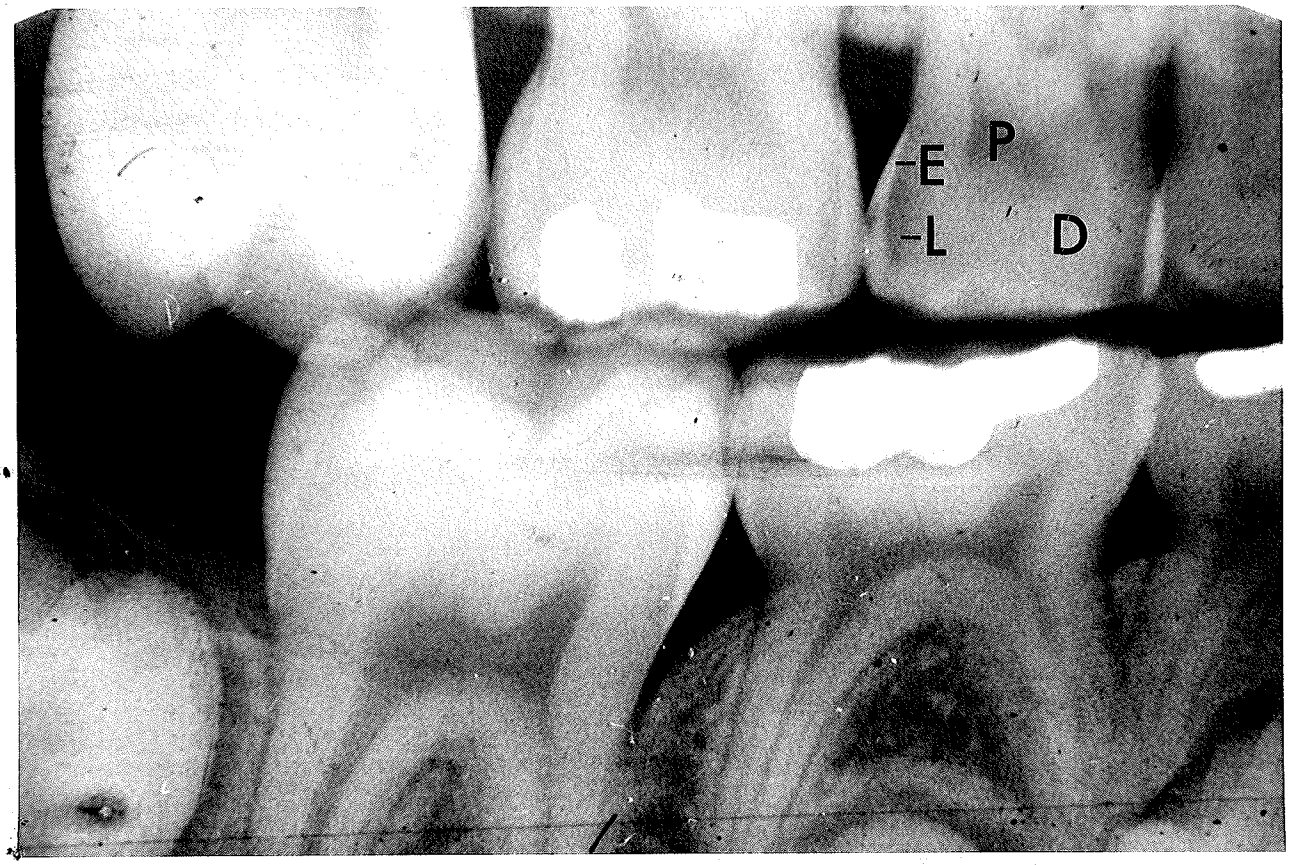
L - incipient approximal lesion

E - enamel

D - dentine

P - pulp.

Figure 6



Radiograph illustrating an advanced or "overt" approximal caries lesion.

L - caries lesion

E - enamel

D - dentine

P - pulp

nosed at the beginning of the study, could be accomplished.

Histologically, the incipient approximal lesion is similar to the early enamel lesion described in the preceding section, having an intact surface zone of enamel and subsurface zones called the body, dark zone and translucent zone. Generally, the incipient stage is said to be[64] that stage of development of the carious lesion beginning at the time that the lesion is first identifiable *in vivo* (clinically or radiographically). Detection by radiograph is possible when translucency is first lost in the surface enamel (white-spot formation) and approximal lesions *may* be detected as slight radiolucencies of the surface enamel. The incipient stage is past when surface breakdown occurs and actual *cavitation* of the lesion takes place (Figure 4). Therefore, the incipient carious lesion always has an intact surface zone of enamel. This description recognizes that there is a stage of development preceding the clinically identifiable lesion during which partial demineralization of the surface layer of enamel occurs, allowing passage of H^+ ions and subsequent subsurface demineralization[64].

E. REMINERALIZATION OF INCIPIENT CARIES LESIONS

A number of studies[62,63,65] have shown clinical evidence that the progression of incipient approximal lesions often ceases and the lesions may regress. Nygaard and Ostby[65] created experimental cavities on smooth surfaces of teeth *in*

vivo by mechanically creating an environment favourable for plaque retention. When incipient carious lesions were formed, the devices were removed from the teeth and the lesions appeared to remineralize. Some of the lesions disappeared completely, while others became discoloured but had hard surfaces. Silverstone[66] demonstrated, by quantitative assessment of enamel solubility, that acid etched enamel returned to normal solubility levels after 48 hours of exposure to oral fluids.

Silverstone and Poole[67, 68] exposed intact teeth with small enamel lesions to human saliva and synthetic calcifying fluids and subsequently examined the lesions histologically. Using adjacent, unexposed lesions as controls, they reported remineralization of lesions in saliva to a maximum depth of 100 μm, with most specimens limited to depths of 20-40 μm. Specimens exposed to calcifying fluids were modified throughout their entire depth. Silverstone[68] showed that the changes noted under the polarizing microscope were due to an increase in negative intrinsic birefringence, inferring that there was "deposition of oriented mineral crystallites". Silverstone speculated that the limited remineralization with saliva might be due to either limitation of penetration by the viscous nature of saliva or rapid surface mineralization by saliva, blocking ion movement to deeper areas of the lesion. The latter theory is supported by the fact that maximum remineralization with synthetic

calcifying fluids occurred when calcium concentrations were between 1 and 2 mM. When concentrations higher than 2 mM were used, the changes in mineralization were limited more to the surface of the lesion. It is believed[69] that at higher calcium concentrations, the more acidic phases of calcium phosphate are saturated and that they rapidly precipitate onto the enamel surface, blocking pores and limiting remineralization to the surface enamel.

Not only is remineralization of demineralized carious enamel possible *in vitro* and *in vivo*, but the remineralized enamel has been shown to be less soluble in acid than normal enamel. This is probably due to the formation of larger crystallites and the incorporation of fluoride into the remineralized enamel[70].

Table 1

Patterns of reactions used to identify species of Streptococcus isolates

Species	Gram	Catalase	H_2O_2 prod/n.	Mannitol	Sorbitol	Raffinose	Melibiose	Esculin	Arginine	Sucrose agar
S. mutans	+	-	-	+	±	±	±	±	±	H [a]
S. sanguis	+	-	+	-	-	±	±	+	+	H
S. mitior I	+	-	+	-	-	±	±	-	-	H
S. mitior II	+	-	+	-	-	±	±	-	-	S
S. salivarius	+	-	-	-	-	-	-	+	-	H/S
S. milleri	+	-	-	-	-	-	-	+	+	S [b]

a) H = Hard, adherent colonies on sucrose agar.

b) S = soft, non-adherent colonies.

Table 2

Patterns of reactions used to identify biotypes of S. mutans isolates

Biotype	Gram	Catalase	H_2O_2 Prod'n	Mannitol	Sorbitol	Raffinose	Melibiose	Esculin	Arginine	Sucrose agar	
I	+	-	-	+	+	+	+	+	+	-	H[1]
II	+	-	-	+	+	+	+	+	+	+	H
III	+	-	-	+	+	+	+	+	+	-	H
IV	+	-	-	+	±	-	-	-	-	-	H

1. H = hard, adherent colonies on sucrose agar.

CHAPTER IV

MICROBIOLOGY OF CARIES

A) INTRODUCTION

Microorganisms are essential to the development of dental carious lesions. This fact was clearly demonstrated by the studies of Orland et al [71,72], using gnotobiotic rats, who showed that germ-free rats do not develop caries even while on a highly cariogenic diet. Caries developed after the same rats were infected with an enterococcus and an enterococcus plus a pleomorphic bacterium and maintained on the same diet. However, there has been considerable disagreement over which organism or organisms are the prime etiological agents in the initiation and progression of human caries. Investigations have been complicated by the complexity of the oral flora and by the numbers of strains of organisms isolated from the lesions.

Gnotobiotic animals have been used to identify organisms capable of inducing caries. Monoinfections of animals with oral bacteria have pinpointed certain species with a high cariogenic potential. It has been found[99,103] in such studies, that Streptococcus mutans (several strains), a strain of S. salivarius [11], S. milleri [11], S. sanguis (several strains)[11], Peptostreptococcus intermedius, a strain of Lactobacillus acidophilus [136], L. casei [136] and Actinomyces [122] species can produce caries. Other oral organisms in experimental caries, particularly Veillonella species have

been identified as possible modifiers of the caries process because of their ability to degrade lactate[11]. In order to provide a basis for the discussion of the microflora associated with incipient lesions it is necessary to describe those bacteria which have been shown to be associated with caries in man.

B) BACTERIA ASSOCIATED WITH CARIES IN MAN

1) STREPTOCOCCUS MUTANS

In 1924 Clarke[93] isolated strains of Streptococcus from carious lesions in human teeth to which he gave the species name mutans. The species name derived from the varying morphology of the cells depending on the growth conditions. Thirty-six years later this organism was "rediscovered" by Sims who demonstrated the similarity of his isolate to the S. mutans described by Clarke, and deposited a culture (NTCC. 10449) with the National Type Culture Collection, England.

a) Characteristics

S. mutans is described as a group of non-motile, catalase negative Gram-positive streptococci[81] (Tables 1 and 2). S. mutans is usually alpha or non-hemolytic, however, some beta-hemolytic strains have been reported.

i) Cellular morphology

Cellular morphology is highly variable, depending on cultural conditions and varies from small coccoid organisms to short, wide rod shaped cells. The cells are usually arranged in short to medium length chains.

ii) Colonial Morphology

Colonial morphology varies with media used for cultivation. When grown on Mitis-salivarius agar, S. mutans forms highly convex to cushion-shaped, opaque colonies. Colonial morphology on sucrose containing medium is most commonly hard and rough, although smooth and mucoid variants have been reported. Smooth to rough reversion has been demonstrated after smooth strains have been cultured in experimental animals.

iii) Biochemical Differentiation

Shklair and Keene[79] originally separated S. mutans strains on the basis of biochemical tests and proposed five "biotypes" designated I to V. In general, these correlated with serotypes a to e. Later these workers incorporated serotypes f and g into their 5 designated biotypes. However, it has been demonstrated[80] that there are some discrepancies in this correlation between serotype and biotype (Table 1 and Table 2).

iv) Serological Identification of Strains

Bratthall[99] described five serotypes of S. mutans (a,b,c,d,e) and two other serotypes (f,g) were identified by Perch[78]. The specific antigens of each serotype have been purified and characterized chemically as cell-wall polysaccharides. Serotyping is the most convenient and relatively reliable means of subdividing strains within S. mutans.

v) Genotypic Identification of Strains

On the basis of analysis of DNA base composition and DNA homology, Coykendall[81] suggested that strains of S. mutans could be identified as one of four (I to IV) "genospecies". He found strong correlation between these four genetic groupings and serotypes c, b, a, and d.

vi) Metabolism

Acid Production

S. mutans has been reported[84] to be a homofermentative lactic acid producing organism. In the presence of excess glucose, the major fermentation product of S. mutans is lactate. However, when glucose availability is limited during chemostat growth, formate, acetate and ethanol are produced, together with lactate[84]. S. mutans utilizes sucrose at a faster rate than other oral bacteria, such as S. sanguis, S. mitis and Actinomyces viscosus[85].

Glucose Transport

Glucose transport in S. mutans utilizes a membrane associated phosphoenolpyruvate (PEP)-dependent transferase system; sucrose and lactose are transported by the same system[223]. Hamilton has shown[230] that at low pH the PEP system is repressed in S. mutans Ingbritt growing in continuous culture and another, as yet unidentified system is utilized for glucose transport. Further studies have indicated that

the alternate system is driven by proton motive force[228]. It was further shown that there was an increase in fluoride resistance at low pH[229]. The PEP-system is inhibited by fluoride by suppression of the enzyme enolase, which is involved in generation of PEP[226]. The aciduric and fluoride-adaptable behaviour of S. mutans are factors which may contribute to its cariogenic potential. It should be noted that fluoride resistance may be both phenotypic and genotypic and that Hamilton noted that freshly isolated strains were more inherently resistant to fluoride than a strain which had been transferred many times in the laboratory[111].

Intracellular Polysaccharide

Some strains of S. mutans are capable of producing large amounts of intracellular polysaccharide (IPS) in cytoplasmic granules when growing with excess sugar[230]. Under conditions of carbohydrate deprivation, the organisms degrade the IPS as an energy source and produce lactic acid, acetic acid and ethanol[227]. The ability to produce and store IPS has been suggested to be a factor in the cariogenic nature of S. mutans[86] however, it has been demonstrated in animal experiments[87] that strains that produce little IPS can be highly cariogenic. On the other hand, mutants of serotype c, which produce little IPS, have been shown to be less cariogenic than normal strains[88]. Therefore, it seems that IPS may be only one of the factors contributing to the cariogenicity of

S. mutans.

Extracellular Polysaccharides

S. mutans also synthesizes extracellular polysaccharides (glucans and fructans) from sucrose by way of the enzymes glucosyltransferase and fructosyltransferase. These polysaccharides, particularly glucans, are considered to be extremely important in dental plaque formation because they are water insoluble and promote retention of the bacteria to the tooth surface. Therefore, they provide a bulking effect to plaque and tend to insulate the deeper areas of plaque from the oral environment. Differences have been observed in glucan production between strains of S. mutans. Trautner et al[89] found that serological type d strains synthesized significantly greater amounts of glucan than type c strains and that the ratio of insoluble to soluble glucans was higher in type d strains. This finding may be relevant to the observation made by others[90] that serotype d is more often associated with subjects with high caries rates.

b) Ecology

i) Distribution

S. mutans is most frequently isolated from the human oral cavity, however, it has also been isolated from human[93] and rat feces[95]. In wild animals, it has been isolated from the Patas monkey[96], the Indian Fruit bat[96], wild rats living in sugar cane fields[97] and Rhesus monkeys[98]. It is, however,

relatively rare in animals other than man.

ii) Acidogenicity and Aciduricity

Many oral organisms are glycolytically very active. In fact, as illustrated by Hamilton and Bowden[223], there are strains of other organisms, such as S. mitior which, under controlled conditions, can be glycolytically much more active than S. mutans, however, S. mutans is able to grow at pH values significantly lower than S. mitior [223]. It is theorized that in the isolated environment of organized dental plaque the highly aciduric nature of S. mutans would give this organism a competitive advantage over most other oral organisms.

The combination of this acidogenic nature and the ability to synthesize intracellular polysaccharides in S. mutans may provide a potential to produce and maintain an acidic habibat within the plaque community.

iii) Mutacins

Strains of S. mutans can produce bacteriocins[91] and this may provide an ecological advantage in the microenvironments of dental plaque by inhibiting competing bacteria. Hamada and Ooshima[92] proposed that the bacteriocins of S. mutans be called "mutacins". These substances have been shown to be active against a wide variety of oral organisms[92]. Information on the mechanism of action of mutacins is limited, but there is evidence that they inhibit synthesis of

DNA, RNA and proteins in susceptible strains of bacteria.

c) Pathogenicity

i) Animal Studies

Animal models have been used extensively to study the cariogenicity of S. mutans, although some of the earlier studies[99 101] used strains of streptococci not specifically identified as S. mutans. In 1960, Keyes[100,101] demonstrated that experimental hamsters, fed a high-sucrose diet, would not develop caries. He termed these animals caries-inactive hamsters since they were apparently free of indigenous oral microorganisms that could induce dental caries. However when Keyes implanted "a cariogenic streptococcus" in the mouths of these animals, they developed caries. Moreover, the organisms were passed from parent to offspring, demonstrating the "infectious and transmissable nature of experimental dental caries". Many streptococcal strains isolated from the human mouth have been shown to be cariogenic in a variety of animal models, including monkeys[102], gerbils[103], mice[104], rats and hamsters[105]. The majority of these cariogenic streptococci are strains of S. mutans. However, it must be recognized that other organisms besides S. mutans can induce caries in animals[106].

ii) Human Studies

Studies of humans also strongly implicate S. mutans as a prime etiologic agent in caries.

In a study of children 13 to 14 years old, Littleton et al

[107] found that S. mutans was isolated in plaque from all carious lesions but in only 23% of samples from sound enamel surfaces. Loesche[108] examined the microflora of carious and non-carious occlusal fissures and found that 71% of caries fissures contained S. mutans, comprising more than 10% of the viable count. On the other hand, 70% of non-carious fissures contained no detectable S. mutans. More recently, Loesche and Straffon[109] and Huis in'tVeld et al [90], have demonstrated a close association between the presence of S. mutans and occurrence of caries.

iii) Summary

There seems little doubt, based on both human and animal studies, that Streptococcus mutans is a significant factor in the caries process. Certain characteristics of S. mutans have been identified which seem likely to be relevant to the cariogenicity of this organism. These properties are:

i) The highly aciduric nature of S. mutans
ii) Its capacity to colonize hard, smooth surfaces.
iii) Its high rate of metabolism of sucrose and lactic acid production
iv) Its ability to produce large amounts of insoluble extracellular polysaccharides.
v) Its ability to produce and store large amounts of intracellular polysaccharides.
vi) Its ability to produce broad-spectrum bacteriocins.

2) STREPTOCOCCUS MITIOR

a) CHARACTERISTICS

i) Cellular Morphology

The cells are Gram positive spherical or ellopsoidal 0.6 - 0.8 μm in diameter. Long chain formation is usual in broth cultures.

ii) Colonial Morphology

Colonial morphology includes rough and smooth variants with frequent reversion of rough to smooth upon subculturing in broth. Alpha-hemolysis of blood agar occurs when incubated aerobically. A soft, non-adherent colony or a hard adherent colony may be formed on sucrose agar[175].

iii) Biochemical Identification

Most strains do not hydrolyze arginine and esculin while fermentation of raffinose is variable (Table 1). S. mitior is peroxidogenic but does not ferment inulin, sorbitol or mannitol.

v) Metabolism

The final pH range in glucose broth is 4.2 - 5.8, averaging about 4.5. Acid is also produced from sucrose and maltose. Extracellular polysaccharides may be formed and many strains form intracellular polysaccharides[175]. Hydrogen peroxide is produced when the organism is grown aerobically.

iv) Serological Identification of Strains

No group antigen has been shown but many serological

types have been illustrated by the precipitin test. Serological reactions seem to be of little value in identification of this species[174].

b) Ecology

S. mitior is a common component of the resident flora of the human respiratory tract and a regular constituent of dental plaque. Because S. mitior actively ferments glucose and sucrose and achieves low final pH values in broth cultures, it is conceivable that it may contribute to a cariogenic situation when present in plaque.

c) Pathogenicity

No strains identified specifically as S. mitior have been reported to produce experimental caries in gnotobiotic animal studies. However, because of the difficulties in separating these organisms from other similar streptococci, such as S. sanguis, this may not be completely valid. The ability of S.mitior to achieve low pH values in broth cultures by fermentation of glucose and sucrose makes it a potential pathogen in the caries process. Carious dentin yields a high proportion of S. mitior [173].

3) STREPTOCOCCUS SANGUIS

a) Characteristics

i) Cellular Morphology

Spherical or ovoid, Gram-positive cocci 0.0 - 1.2 um in diameter. The species name S. sanguis is derived from the

fact that it is often isolated from the blood of patients with subacute bacterial endocarditis. In broth the cells occur in medium to long chains[175].

ii) Colonial Morphology

On Mitis-Salivarius agar S. sanguis produces small zoogleic colonies with a firm consistency and which are imbedded in the medium. Many S. sanguis strains produce spreading zones around the colonies indicative of twitching motility when cultured on blood agar. Hemolysis on blood agar is alpha-type[175,179]. A hard adherent colony is produced on sucrose agar.

iii) Biochemical Identification

S. sanguis strains hydrolyze arginine and esculin and produce acid from glucose, maltose, sucrose, lactose and often raffinose. Mannitol, sorbitol and xylose are not fermented. All strains recognized as definitely S. sanguis produce extracellular glucans (dextran) from sucrose (Table 1).

iv) Serological Identification of Strains

Five serological types have been identified, with some strains having more than one type-specific antigenic determinant. A close relationship has been recognized between S. sanguis and group H streptococci, but serological studies have failed to define specific groups and simplify the serology[178].

v) Metabolism

The final pH in glucose broth is 4.6 - 5.2. Hydrogen peroxide is produced when grown aerobically. S. sanguis strains produce predominantly soluble extracellular glucans (dextran) from sucrose.

b) Ecology

Carlsson demonstrated that the main habitat of S. sanguis in humans is the oral cavity and particularly in dental plaque[180]. Low levels of S. sanguis have also been reported in human feces[182]. S. sanguis is often implicated as a pathogen in bacterial endocarditis[181].

c) Pathogenicity

S. sanguis is often found to be the predominant streptococcus in dental plaque and Loesche[162] has noted that it tends to predominate in plaque from non-carious tooth surfaces. The capabilities of S. sanguis to ferment sucrose and glucose and produce extracellular glucans plus the low pH values reached in broth culture would seem to implicate it as a potential cariogenic organism. In fact, gnotobiotic experimental animals monoinfected with strains of S. sanguis have developed dental caries[87]. This organism may contribute to the caries process prime etiologic agent.

4. STREPTOCOCCUS SALIVARIUS

a) Characteristics

i) Cellular Morphology

S. salivarius is Gram positive with spherical or ovoid cells 0.8 - 1.0 μm in diameter. Chain length is extremely variable[175].

ii) Colonial Morphology

Colonial morphology may be rough or smooth with reversion from rough to smooth on subculturing in broth. This organism is non-hemolytic on blood agar and does not produce peroxide. When grown on sucrose agar most strains produce soluble levans (fructans) which contribute to the formation of typical large, mucoid, dome-shaped colonies. Some strains are said to produce insoluble dextrans[183].

iii) Serological Identification of Strains

Although several group antigens other than recognized Lancefield grouping antigens have been identified for S. salivarius, the majority of strains can be classified into two types (I and II). Type I reacts with Lancefield group K antiserum and accounts for 75% of strains isolated from the respiratory tract.

iv) Biochemical Identification

Acid is usually produced from fermentation of glucose, sucrose, maltose and raffinose. Mannitol and sorbitol are usually not fermented. Final pH in glucose broth is in the range 4.0 - 4.4. Esculin is usually hydrolyzed by this organism but arginine is not. Extracellular levan from sucrose formation is usual.

b) Ecology

S. salivarius is most frequently isolated from the surface of the oral soft tissues and in particular the tongue and it is usually found in saliva, possibly being dislodged from the tissue surfaces. It is frequently identified as a constituent of dental plaque, but some disagreement exists as to whether it is usually an integral part of the plaque ecology or whether it exists there transiently, being deposited from saliva[184].

c) Pathogenicity

Although a strain of S. salivarius reportedly has produced experimental caries when inoculated into the mouths of gnotobiotic animals, clinical findings do not associate the presence of, or the proportion of, S. salivarius in plaque with caries incidence. This organism is infrequently isolated as the pathogen in subacute bacterial endocarditis[185].

5. VEILLONELLA

a) Characteristics

i) Cellular Morphology

Representatives of this genus are anaerobic, non-motile, small, spherical, Gram-negative cocci. They are found in pairs, masses and short chains.

ii) Colonial Morphology

Lactate agar media containing 7.5µg/ml of vancomycin favor isolation. Colonies on poured plates are 1 to 3 mm. in diameter and are smooth and sometimes lens-shaped or diamond or heart-shaped and opaque grayish white[112].

iii) Genotypic Identification of Strains

Two species of Veillonella have been recognized, V. parvula and V. alcalescens. DNA analysis has recently shown that these two organisms are homologous [224].

iv) Metabolism

Veillonella are strictly anaerobic and do not ferment any carbohydrates or polyols. During growth, lactate is metabolized to propionic acid, acetic acid, CO_2 and H_2 while resting cells also metabolize pyruvic, oxaloacetic, malic, fumaric and succinic acids[112]. In contrast to Neisseria, Veillonella are generally cytochrome-oxidase negative. Veillonella are sensitive to the antibiotics penicillin, erythromycin, and bacitracin and resistant to vancomycin, streptomycin and neomycin[112].

b) Ecology

Veillonella occur in the natural cavities of humans and some animals. They are found in the human mouth, respiratory tract[113] and intestinal tract[114].

c) Pathogenicity

These organisms are normally present without any pathogenic effects, although they have been identified in the

mixed flora of suppurative infections of periodontitis, pulmonary gangrene, tonsilitis and appendicitis[115]. The role of Veillonella in these infections is not clear. However, they have been shown to contain lipopolysaccharides with endotoxic characteristics[116]. Therefore, they may play a significant role in the progression of these infections. Veillonella have been shown to contribute significantly to plaque formation once colonisation of tooth surfaces has been accomplished by other organisms. The role of Veillonella in the dental caries process has not been precisely established. It is speculated that the ability to metabolize lactate may give Veillonella a protective role in caries by reducing the size and duration of pH decreases in plaque. This concept has been supported by a study utilizing germ-free rats[11] although more recent studies in man may not support it[161]. No direct relationship has been defined between Veillonella and periodontal disease. Veillonella is present in subgingival plaque of normal, clinically healthy mouths.

6. ACTINOMYCES

i) Cellular Morphology

Actinomyces are Gram-positive, non-motile, non-sporing organisms which occur as branching rods and filaments of varying length[129].

ii) Colonial Morphology

Actinomyces grow well on blood agar or agar with serum. A. viscosus and A. naeslundii grow relatively rapidly producing colonies of 2-3 mm in 48 hours. The colonies are white or cream in colour and generally of a soft consistency, they are usually matt and sometimes have a granular surface. In contrast A. odontolyticus produces brownish colonies of 2-3 mm often with a metalic sheen. Older colonies (4-7 days) of A. odontolyticus grown on blood agar produce a brown/red pigmentation. A. israelii grows slowly, its colonies only becoming macroscopically visible after 4-7 days incubation. Two colony forms occur, one is not distinctive, being domed, white, with entire edge. The second form is rough, irregular white 1-2 mm in diameter and typical of of A. israelii. In general serotype 1 strains of A. israelii produce the rough and serotype II strains produce the soft colonies.

iii) Biochemical Characteristics

The Actinomyces are generally actively saccharolytic and have little or no activity against proteins. Biochemical tests such as fermentation or hydrolysis of different substrates are of little value in identification of species. Actinomyces are best identified by chemotaxonomic analysis of the cell wall components or serology[129,200].

iv) Serological Characteristics

Serology has proved most valuable in the characterization and identification of Actinomyces [129]. most of the species

have more than one serotype (Table 3).

In some cases gel diffusion of cell sonicate has been attempted as an identification method but it lacks the convenience of the FA technique. More recently a simple whole cell agglutination test has proved valuable in the initial separation of isolates (Fillery personal communication). This method has the advantage of being easily applicable to the type of study undertaken here.

b) Ecology

The are uniquely oral organisms and have only been isolated from the oral cavity or the tonsils of man and animals[200, 129]. This genus is present in large numbers on the surface of the teeth[161]. and can be easily isolated from carious dentine[128, 132]. Actinomyces colonise the mouth relatively early in life and there appears to be some selectivity with A. viscosus colonising younger children[209]. All of the species of Actinomyces with the exception of A. bovis can be isolated from man. A. bovis has only been isolated from animals[129].

c) Pathogenicity

Although almost all isolations of Actinomyces species are from the oral cavity of man and animals, actinomycotic infections can occur anywhere in the body[129]. Many of these infections may be traced to the oral cavity as the source of infection via bites or oral trauma[201, 202]. However, some of

Table 3

Characteristics used to identify Actinomyces isolates

Species		Gram	Catalase	O_2 Growth	Starch NYD	Mannitol	Colony Pigment	Agglutination A. viscosus	A. naeslundii	A. odontolyticus	A. odontolyticus
A. naeslundii	A	+	-	+	-	-	W[a)]	-	+	-	-
	B	+	-	+	-	-	W	+	+	-	-
A. viscosus II	A	+	+	+	-	-	W	+	-	-	-
	B	+	+	+	-	-	W	+	+	-	-
A. odontolyticus	I	+	-	+	-	-	R	-	-	+	-
	II	+	-	+	-	-	R	-	-	-	+
A. israelii		+	-	-	+	+	W	-	-	-	-[b)]

a) W = white

R = Red

b) As A. israelii usually autoagglutinates, identification is also based on gel diffusion of cell sonicates.

these infections cannot be linked directly to the oral cavity and are believed to be the result of transient bacteremias. Bacteremias have been demonstrated after oral surgical procedures and even toothbrushing[203] and it has been demonstrated that the frequency and extent of the bacteremias increases inversely with the level of oral hygiene[204]. Inhalation has been identified as a potential route for pulmonary actinomycosis[125].

Actinomyces species have been implicated in both dental caries and periodontal disease. A. naeslundii and A. viscosus both synthesize extracellular polymers which may facilitate adherence to tooth surfaces, enabling them to colonize. Actinomyces species have also been shown to aggregate in saliva[205]. In addition A. naeslundii will aggregate with streptococci[206] and A. viscosus has been shown to aggregate with Veillonellla[207]. These aggregation properties would certainly enhance plaque formation. A. viscosus and A. naeslundii have been shown to induce fissure[115] and root surface[120] caries in gnotobiotic rat studies. A. odontolyticus is a species that was recognized by Batty in 1958[128], when it was isolated from deep carious dentine. Some years later, Edwardsson[132] also made a definite association between this organism and deep carious dentine. Although little is known about the ecology of A. odontolyticus the indications are that it is involved with the later stages of development of caries lesions. A. viscosus and A. naeslun-

dii have been shown to initiate periodontitis in gnotobiotic rats and hamsters[119,208] and since they are usually present in gingival crevices of humans, it is possible that they may play a role in periodontal disease in man.

7. ORAL LACTOBACILLI

a) Characteristics

i) Cellular Morphology

Lactobacilli are described[191] as rod-shaped, varying from long, slender forms to short coccobacilli. Chaining of cells is common. Motility is unusual. These are Gram-positive organisms which tend to become gram-negative with age or increasing acidity of the media. They are non-sporing.

ii) Colonial Morphology

Colonial morphology varies with the species, however, colonies are characteristically unpigmented white to grey-white with the odd light yellow variant. Colonies are one to three millimeters in diameter and usually rough or dull textured.

iii) Metabolism

Lactobacilli are generally microaerophilic in nature, although some anaerobic forms are known[226]. Lactobacillus metabolism is fermentative and these organisms are characteristically saccharoclastic. At least half of the end-product carbon is lactate, other end-products of metabolism may be acetate, formate, succinate, carbon dioxide and etha-

nol[191]. Lactobacillus species are classified as either homofermentative or heterofermentative. Homofermentative species generally produce lactic acid as 85% or more of the the end-product of glucose metabolism[130]. Heterofermentative species produce about 50% lactic acid as end-product of glucose metabolism, plus CO_2, ethanol and acetic acid[130]. Fermentation of carbohydrates varies from species to species and provides a basis for species identification (Table 4).

b) Ecology

Lactobacillus species occur commonly in the mouths of humans, however, they are normally present in low numbers in dental plaque[192]. Studies[133] have shown that strains of lactobacilli have a low affinity for enamel surfaces relative to such bacteria as Streptococcus sanguis. The aciduric nature of Lactobacillus suggests that acid habitats[133] may favour their colonization. The oral lactobacilli have been studied in detail[131] and have been considered since the early days of oral microbiology[137] significant component of the oral flora.

Simple screening system for the identification of Lactobacillus isolates

	Gas production Glucose	Gas production Gluconate	Acid from ribose	Hyd Aesc.	Cello.	Acid from Mannit.	Melib.	Raff.	Melez.
THERMOBACTERIUM									
L. acidophilus	-	-	-	+	+	-			
L. salivarius	-	-	-	-	-	+			
STREPTOBACTERIUM									
L. casei	-	+	+				-	-	
L. plantarum	-	+	+				+	+	
BETABACTERIUM									
L. fermentum	+	+	+	-	-				-
L. cellobiose	+	+	+	+	+				-
L. buchneri	+	+	+	+	-				+
L. brevis	+	+	+	+	-				-

The most common oral species of Lactobacilli are: [131]

Homofermentative	Heterofermentative
L. casei	L. fermentum
L. acidophilus	L. brevis
L. plantarum	L. buchneri
L. salivarius	L. cellobiosus

L. casei and L. fermentum are the most common oral species[131]. Lactobacilli are frequently isolated from deep carious dentine[132] and salivary counts of Lactobacilli are seen to increase with greater caries activity.

c) Pathogenicity

Lactobacilli have been found to represent only a very small portion of the plaque flora[192]. This fact led Loesche and Syed[108] to speculate that Lactobacilli may be secondary contributors to the caries process rather than primary factors.

The low affinity of Lactobacilli for the enamel surface[133] and the coincidence of Lactobacilli appearance with development of carious lesions led Fitzgerald[134] to speculate that Lactobacilli favour the environmental conditions offered by active carious lesions. They therefore colonize as a consequence of the carious lesion rather, than being an etiologic factor in caries formation.

Lactobacillus counts in saliva have been used in the past to monitor caries susceptibility. Whatever the precise relationship is between Lactobacillus and caries formation, Sims[135] has shown that elimination of Lactobacillus from the oral cavity results in arrest of the caries process.

Lactobacillua have been tested for their ability to induce caries by inoculation into gnotobiotic animals. These experiments have shown that few strains of Lactobacillus can induce caries and the caries that were induced were confined to occlusal pits and fissures[136]. This evidence fits well with the finding that Lactobacillus has low affinity for the tooth surface[133].

The evidence seems to point to Lactobacilli being involved with the caries process with the potential of being an etiologic agent, particularly in later stages of development of caries lesions.

C. MICROBIOLOGY OF LONGITUDINAL STUDIES OF CARIES

Relative to the total volume of microbiological research of caries, there is a minimal amount of information derived from longitudinal microbiological examination of the caries process.

One of the earliest comprehensive studies was reported by Enright et al in 1932[137] who discovered, after an initial,

single bacteriological analysis of clinically diagnosed "Caries Active" and "Caries Immune" patients, that the caries process is not necessarily constant. Therefore, an accurate analysis can be made only through repeated, longitudinal analyses. The results of their repeated observations of the same patients led Enright et al [137] to conclude that the presence of lactobacilli was a "prognostic indicator" for caries activity.

Prior to the study of Enright et al, [137] Jay[138] had published the results of a less extensive longitudinal study which concluded that "Bacillus acidophilus" appeared in the oral cavity prior to the clinical onset of dental caries and that such changes in the oral microflora could be predictive of caries attack.

It should be noted that both of these studies relied heavily on the use of selective media that probably significantly influenced the final results Furthermore, some difficulties with taxonomy existed which resulted in some researchers identifying organisms as Lactobacilli and others identifying the same organisms as Streptococci. Microbiological methods of analysis have been refined over the years to give a more accurate picture of the bacterial composition of plaque. In 1939, Bibby[139] suggested that, based on all of the relevant bacteriological information available at that time, the dental caries process was probably due to an association of several different organisms.

Hemmens et al [140] reported a study in 1946 which observed longitudinally and in great detail the microflora of plaque sampled from approximal surfaces of erupting premolars. The microbiologic information gathered was then related to the development of or absence of dental caries. They found that the organisms most significantly related to development of caries were lactobacilli. They also noted a high incidence of what they called "aciduric streptococci" at active caries sites. These workers reported a decreased incidence of Leptotrichia, Actinomyces, alpha-hemolytic Streptococcus, S. mitior fusiform bacteria and Neisseria associated with the progression of lesions.

Ikeda et al [141], reported a study which identified the microflora of plaque from three surfaces of initially non-carious teeth. Samples were obtained and teeth inspected every three months for one year. Caries initiation was correlated with the presence of Streptococcus mutans in the plaque on the site before caries was detected. Caries was not observed when S. mutans was not isolated. However, the authors neglected to calculate such correlations for lactobacilli. Lactobacilli were found in only low numbers prior to caries onset and some sites became carious without detectable lactobacilli. They suggested that lactobacilli may be involved in progression of lesions, but that they make little or no contribution to caries initiation.

A study carried out in the Netherlands by Huis in't Veld et al [90] demonstrated that caries development occurs predominantly on tooth surfaces that harbour relatively high numbers of S. mutans (5%) in the plaque associated with the lesion site. Furthermore, this study pointed to serotype d S. mutans as the most common serotype in caries active subjects.

In the same year, Loesche and Straffon[109] published the results of a longitudinal study of the microflora of occlusal fissures. They concluded from their observations that sometimes Lactobacillus may also be a prime agent, with very low or even undectectable levels of S. mutans. Furthermore, they noted that levels and proportions of what they identified as S. sanquis tended to be higher in caries-free fissures.

A longitudinal study[142 | 146] has been made in which initial caries-free approximal surfaces of premolar teeth in children were observed with periodic sampling of plaque from the test sites. A detailed microbial analysis of the plaque samples was related to caries activity or inactivity. They concluded from their results that domination of the microflora by S. mutans at a site of caries was not essential for caries to develop. Furthermore, in a small number of sites (2 of 15) which became carious, S. mutans was never isolated. They did note a trend to increased numbers and isolation frequencies for both S. mutans and Lactobacillus at

sites after caries detection.

The evidence available from these longitudinal studies offers us the best understanding of the relationships between specific microorganisms and the caries process. Based on this information one must conclude that no single organism is exclusively associated with the initiation of caries. However, it appears that S. mutans is an organism that is most frequently a prime etiologic agent in the caries process. Other organisms, notably Lactobacillus seem to be closely associated with the caries process. Furthermore, longitudinal studies have demonstrated that the microflora of plaque associated with caries active sites is in a constant state of flux, probably because the environmental conditions change constantly as the lesion progresses. This may result in a transitional progression, whereby the prime etiologic agent in the caries process changes as the local disease state progresses and local environmental conditions change.

CHAPTER V

RATIONALE FOR THE PRESENT STUDY

In populations with low caries incidence, particularly in water fluoridated areas, early approximal caries lesions often appear to develop very slowly, sometimes to not progress at all, and sometimes even to regress or remineralize[157,158].

These early approximal lesions in deciduous teeth of children with low caries incidence pose a diagnostic and treatment planning problem for clinicians. That is, whether these early lesions should be left and monitored radiographically every six months to ensure that those lesions that do progress are intercepted, or whether all lesions should be restored, no matter how small.

It would be beneficial if a reliable, less invasive and safer diagnostic test could be used to aid in treatment planning for these inaccessible areas of the teeth. Medical practitioners routinely use microbiologic tests to determine the etiology of disease processes and to monitor the status of infective diseases and to decide on the interventive therapy to be used. There seems no good reason why the dental practitioner should not follow the same procedures given sufficient information on the caries process.

Dental caries has a microbial etiology that has been

studied extensively and the caries process has been examined longitudinally. However the longitudinal clinical study of caries presents special difficulties. In order to study the caries process from the initial stages, it is necessary to sample plaque from a large number of caries-free but caries-susceptible surfaces. This method requires an enormous amount of laboratory work in order to monitor enough surfaces over a sufficient period of time to include a significant number of developing lesions in the study. In spite of much effort, the few good longitudinal studies reported[140,141,161] have a small number of lesions included in the data.

Therefore, this present study was designed to avoid the problem of too few lesions by observing the microflora associated with early enamel lesions (incipient) in the approximal surfaces of deciduous molar teeth. This allowed the study of all but the very initial stages of the caries process while providing a ready pool of caries active sites. Some of these lesions were expected to progress during the observation period and some were not. Therefore this study allowed for a microbiologic comparison to be drawn between lesions which progressed and those which were static. If significant microbiological differences could be identified between the progressive and non-progressive lesions, specific microbiologic tests might be designed to aid in diagnosis or in prediction of the progression of these early lesions. Such tests could allow monitoring of these early

lesions with less frequent exposure of children to x-rays. Thus, the study was intended to detect significant microbiological differences between lesions which progressed and static lesions. The intention was to use this data to develop simple tests to aid the clinician in the accurate diagnosis of incipient lesions.

CHAPTER VI

MATERIALS AND METHODS

A. SUBJECT GROUP

Twenty-two children ages 4 to 9 years were selected for this study. Most of the subjects were patients of the Children's Hospital Dental Clinic, Winnipeg, Manitoba. All subjects were on a six-month regular dental recall examination and topical fluoride treatment program and were pre-selected upon diagnosis of at least one incipient approximal caries lesion from bite-wing radiographs during a regular recall examination.

The nature and purpose of the study were explained to the parent or parents of each child. Parents who were willing to allow their child to participate in the study were asked to complete a questionnaire (Appendix 1). The questionnaire was reviewed, along with the medical history of the child and any potential subjects who showed a poor history of appointment-keeping, had a history of frequent antibiotic therapy, were significantly medically debilitated, or who showed signs of potentially exfoliating the teeth to be examined during the time of the study were eliminated from the subject pool. Parents of subjects chosen according to these criteria were then asked to sign a consent form (Appendix 2) signifying that they would allow their child to participate in the study. No subjects were lost during the

time of the study. Continued participation was encouraged by presentation of a small gift to each child at each visit. Parents and participating children were instructed that no changes in routine diet or oral hygiene practices were to be made during the study and these aspects were not monitored during the study.

Thirty-two incipient approximal carious lesions were available for study in the 22 children. Whenever possible the contralateral site to each lesion site in the same child was chosen as the control site. Control sites were all judged (from radiographs) to be caries-free.

B. DIAGNOSTIC CRITERIA FOR CARIOUS LESIONS

Radiographic diagnosis of carious lesions was determined by agreement of three experienced clinicians who examined radiographs independently and made diagnoses without consultation with each other.

1. INCIPIENT APPROXIMAL LESIONS were defined, for the purpose of this study, as early approximal enamel caries lesions that did not require restorative treatment. To be included in the study, incipient lesions had to be diagnosed as such by the independent observations of all three observers (Figure 5).

OVERT APPROXIMAL LESIONS were defined as caries lesions on approximal surfaces judged, from radiographs, to require

restoration (Figure 6).

3. PROGRESSION OF LESIONS

Progression of lesions was determined by a change in status from incipient to overt lesions. This method of defining progression allowed for a clearer determination of progression from radiographs, rather than attempting to judge fine degrees of progression. It has been demonstrated previously that it is very difficult, if not impossible to make fine determinations of progression of approximal carious lesions from serial radiographs, even with standardization of technique. Bite-wing radiographs were available at time 0, 6 and 12 months during the study and were all produced by the same X-ray machine, at the same settings, and by the same operator. Subsequent radiographs were reviewed by the three clinicians independent of previous graphs and without knowledge of the identity of the subject or the point of time of the subject in the study.

C) SAMPLING METHODS

Plaque from the approximal test and control sites was sampled by the methods reported previously by Bowden et al, [161, 260]. Samples were collected on either a six-weekly or twelve-weekly schedule for a period of twelve months. Any lesions which were diagnosed from radiographs, at 6 months, to have progressed to a point requiring restoration were resampled, restored and usually resampled after restoration.

These sites were then eliminated from the study. Likewise, most lesions diagnosed as requiring restoration at 12 months were resampled and all were restored.

The device for plaque collection consisted of a strip of flexible, steel-backed abrasive tape soldered to a 6 cm. piece of 0.036 inch orthodontic wire that provided a handle. The abrasive strip was shaped so that the end tapered to a point, with the working tip being approximately 2 mm by 10 mm in size. The collection devices were pre-sterilized and stored in sterile packages with sterile gauze and sterile scissors (121 ^{0}C for 20 minutes).

Cotton rolls were placed buccal and lingual to the tooth to be sampled to isolate the area. Sterile gauze was used to wipe the buccal surfaces of the teeth adjacent to the sample site. The point of the abrasive sampling device was placed just gingival to the contact point of the site to be sampled with the abrasive side toward the tooth to be sampled. The device was pushed in and out of the approximal space six times, removed, and the smooth side of the device wiped clean on a piece of sterile gauze. The tip of the abrasive strip containing the sample of plaque was then cut off with sterile scissors and dropped into a disposable sterile test-tube containing 1 ml of sterile reduced transport fluid (RTF)[143]. Thus the sample was maintained in a moist environment and the viability of anaerobic organisms was protected.

At the time each sample was collected, brief notes were made regarding each test and control site. It was noted and recorded as to the extent of plaque accumulation. accumulation. Any obvious gingival inflammation was also recorded and whether the sampled site bled when probed.

D. MICROBIOLOGICAL ANALYSIS

1. Culturing of Samples

The samples in RTF were sonicated using a Kontes Sonifier at a power setting of 4 for 30 seconds. The samples were then serially diluted 1:10; 1:100; 1:1000; 1:5000 times in RTF. Dilutions were cultured on: blood agar (Oxoid CM 271 Blood Agar Base No. 2) supplemented with haemin, Vitamin K_1 and 5% sheep blood (Atlas Laboratories, Winnipeg); blood agar with 7.5 µg/ml. Vancomycin (Eli Lilly); TYC agar with and without Bacitracn 0.2 units/ml [262] and Rogosa agar (Difco Laboratories). These cultures were incubated at 37^{0}C both aerobically and anaerobically for 72 hours (aerobic) and 120 hours and 120 hours (anaerobic) (80% N_2, 10% H_2, 10% CO_2).

2. Counting of Colonies

Colonies were counted on plates where dilutions yielded 50 to 200 colonies per plate. The total cultivable flora was estimated from supplemented blood agar plates incubated anaerobically for 120 hours. The counts for each identified

organism isolated were then converted into a percentage of the total cultivable flora.

3. Identification of Isolates

a) Tests used for the initial separation of isolates

i) Gram stain

The Hucker-Conn[214] modification (Lillie 1953[215]) of Gram stain was used to differentiate Gram positive from Gram negative cells.

ii) Catalase

The production of catalase was tested by the method described by Cowan and Steele[213].

iii) Atmospheric Requirements

All strains were tested for growth on blood agar plates in the following atmosphere: -

1) Aerobic

2) Anaerobic 80% N_2,10%H_2, 10% CO_2.

Plates for anaerobic incubation were placed in an anaerobic jar (Oxoid Canada) or an anaerobic chamber (Coy Man.Co. Ann Arbor,Mich) for 4 days at 37^0 C.

Plates for aerobic incubation were placed into a standard incubator at 37^0 C for 4 days.

b) Identification of species

The description of tests below is limited to those genera which were a) most commonly isolated and b) of significance in the in the study. These were Streptococcus, Actinomyces, Lactobacillus, Veillonella and Neisseria (Table 5).

c) Streptococci

The characteristics used to classify the oral species of Streptococcus follow those described by Hardie and Bowden[216]. (Table 1). The tests used are outlined below.

i) Carbohydrate Fermentation

A peptone broth[216] was used as the basal medium and test carbohydrates were added to this medium at a concentration of 0.5% w/v. Acid production was indicated by a change in colour of phenol red from red (pH 7.6) to yellow (pH 5.5). The four carbohydrates used in the tests were all heat stable and the medium with added carbohydrate was autoclaved at 115^{0}C for 20 mins.

ii) Production of H_2O_2.

Isolates for testing were grown on blood agar plates under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) for 24hrs and then grown for 16 hrs under anerobic conditions before testing. The initial anaerobic incubation with added CO_2 ensured good growth of the isolates and aerobic incubation encouraged H_2 production. The test was made by scraping colonies from the plate and putting them onto a reagent

Table 5

Initial separation of isolates

Cell morphology	Gram	Catalase	Aerobic Growth	Anaerobic Growth	Genus
Coccal	+	-	+	+	Streptococcus
Coccal	+	+	+	+	Staphylococcus Micrococcus
Rod	+	+	+	+	Actinomyces Rothia Bacterionema
Rod	+	-	+	+	Actinomyces Lactobacillus
Rod	+	+	-	+	Propionibacteria
Rod	+	-	-	+	Actinomyces Arachnia Eubacterium
Coccal	-	+	+	+	Neisseria
Coccal	-	-/+	-	+	Veillonella
Rod	-	-	-	+	Bacteroides Fusobacterium Capnocytophaga

strip sensitive to peroxide (Merckoquant strips Merck, Darmstadt, Germany). Positive isolates produced a definite blue colour within 2 mins.

iii) Hydrolysis of Esculin

Isolates were tested in esculin broth[216] which includes ferric ammonium citrate and strains which hydrolyze esculin cause the medium to lose its blue fluorescence and turn black.

iv) Hydrolysis of Arginine

Strains were grown in arginine broth [216] and the production of ammonia was tested by the addition of Nessler's reagent (BDH Chemical). Positive cultures gave a bright orange colour which rapidly faded.

v) Colony form on sucrose agar

Some oral streptococci produce extracellular glucosyl transferase enzymes which will catalyze the conversion of sucrose to glucans and levans. This activity can result in distinctive colony features e.g. adherence, hardness, gummy consistency. All streptococcal isolates were cultured on TYC (de Stoppelaer[218]) for 48 hrs at 37^{0}C under an anaerobic condition (80% N_2, 10% H_2, 10% CO_2). The colonies were examined under a dissecting microscope at a magnification of 10 times.

d) Actinomyces

The characteristics used to classify these organisms are selected from those described by Slack and Gerencser,[129] and Bowden and Hardie[200] (Table 5). The tests used are outlined below.

i) Carbohydrate fermentation

The Streptococcus fermentation basal medium was used with concentration of 0.5% w/v of test carbohydrates.

ii) Starch hydrolysis

Isolates were tested by growing them for 96 hrs on agar medium (Oxoid Blood agar base No. 2 CM271) with 1% w/v soluble starch (BDH Chemicals). Starch hydrolysis was tested by flooding the plate with Lugol's iodine. Positive isolates were surrounded by a halo of uncoloured medium on a blue/black background.

iii) Whole cell agglutination

Antiserum to A. naeslundii (strain ATCC 12104), A. viscosus II (strain WVU672), A. odontolyticus I (NCTC 9335), A. odontolyticus II (WVU482), A. israelii I (NCTC 4860) nd A. israelii II (WVU307) were prepared in rabbits following the methods described by Bowden and Hardie[219]. A simple whole cell slide agglutination test was used[226] in which a suspension of cells in saline from a plate culture was prepared on a microscope slide and a single drop of antiserum (1 in 10

in saline) was added. The slide was rocked from side to side and agglutination confirmed by examination under a dissecting microscope.

e) Lactobacillus

i) Rogosa agar

All catalase negative Gram-positive rods were cultured anaerobically for forty-eight hours on solid media selective for Lactobacillus, as described by Rogosa[210]. Catalase negative rods which grew aerobically and anaerobically were identified as Lactobacillus species.

ii) BIOCHEMICAL IDENTIFICATION OF SPECIES

A short series of biochemical tests, as described by Bowden et al [211], were used to identify the various species of Lactobacillus isolated (Table 4).

Carbohydrate fermentation was tested in the basal MRS broth[212]. The following carbohydrates were added at concentrations (w/v) of 2%, glucose, ribose, cellobiose, mannitol, mellibiose, raffinose and melizitose. Bromocresol purple was added as a pH indicator. Ribose and esculin solutions were filter sterilized while all other carbohydrate solutions were autoclaved at 115oC for twenty minutes. Glucose and sodium gluconate broths were used to test for gas production. After anaerobic incubation at 37^{0}C for forty-eight hours, pH determinations were made for each carbohydrate and the esculin broth was tested for hydrolysis by the addition

of 1% w/v ferric chloride. Strains positive for esculin hydrolysis gave a black colour.

f) Veillonella

i) Acid production

Gram-negative anaerobic cocci were cultured in 1.0% glucose broth to test for acid production. Cultures were incubated anerobically for forty-eight hours at 37^{0}C and pH determinations were recorded. No pH change was indicative of Veillonella.

ii) End-product analysis

Gram-negative anaerobic cocci with negative cytochrome-oxidase tests were cultured in a peptone broth[219] containing sodium thioglycollate, sodium lactate (1% w/v) and L-cysteine (0.05% w/v). Cultures were incubated at 37^{0}C for at least seventy-two hours and later analyzed by gas-liquid chromatography for the presence of volatile acids[231].

g) Neisseria

i) Cytochrome-oxidase (oxidase) test

All aerobic Gram-negative cocci were tested for cytochrome-oxidase activity by means of the Kovac's method[220]. A piece of filter-paper in a petri dish was impregnated with a 1% solution of dimethyl-p-phenylenediamine (Sigma, St. Louis, Mo) and a platinum-wire loop with some of the cultured organism was wiped on the filter paper. A positive reaction, indicative of Neisseria

was indicated by the development of a dark purple colour within ten seconds[231].

ii) Maltose fermentation

Gram-negative facultatively anaerobic, oxidase-positive cocci were cultured in a heart infusion broth containing maltose at a concentration of 1.0% w/v. Bromocresol green was added as a pH indicator. Cultures were incubated aerobically at 37^{0}C. for seventy-two hours and final pH determination was made. A pH decrease from 7.6 to 6.0 or less was considered indicative of carbohydrate fermentation. Neisseria were grouped as saccharolytic or asaccharolytic by this test.

iii) Polysaccharide Production on Sucrose Media

Isolates identified as Neisseria were cultured on heart infusion agar with sucrose 4.0% w/v. Cultures were incubated aerobically for seventy-two hours at 37^{0}C. and the plates were then flooded with Lugol's iodine[213] and the inoculated areas examined for colour-change. A dark blue-brown appearance of a culture was indicative of extracellular polysaccharide production.

E. STUDY DESIGN

Children were selected rather than adults for the study group for the following reasons. Incipient approximal lesions occur in permanent teeth and in adult patients as

well as deciduous teeth of children. Incipient approximal lesions in deciduous molar teeth were selected for study for several reasons. It is clinically more acceptable to avoid restoration of approximal incipiencies and observe them instead, particularly in children with low caries experience. Very often a slowly progressing or non-progressing lesion in a deciduous tooth does not become clinically significant before exfoliation of the tooth occurs. Also, the enamel in deciduous teeth is only about one-half as thick as in adult teeth. Therefore, there was a greater chance of having incipient lesions progress to the dentino-enamel junction in deciduous teeth during the 12-month period of observation.

The selection of a suitable control site for each lesion or test site was made on the basis that the control site should be subject to as similar an environment as the test site. Therefore, the identical site on the contralateral side of the mouth to the test site was chosen, where possible. If the contralateral site was missing, carious or restored, the nearest similar surface was chosen.

Since the objective of this study was to monitor the microbiology of plaque associated with incipient approximal lesions in children under normal conditions, the major decisions to be made in study design were: (1) how to monitor lesion progression, (2) how to sample the plaque in a relatively inaccessible location and (3) how long and how often

to monitor both aspects.

In consideration of how long to study each lesion and its control, it was decided that a twelve month period of observation would: (1) provide a reasonable number of samples from each site, (2) based on information about progression of approximal lesions, allow sufficient time for significant progression to occur, (3) allow enough lesions to be studied to be statistically significant and (4) be a reasonable length of time to request subjects to participate (which would encourage enrollment and minimize subject loss).

As previously explained in section B of this chapter, incipient approximal lesions are observable only by way of radiographs and bite-wing radiographs provide the usual and best clinical information. It was felt that in order to be clinically ethical, the study could obtain radiographs no more frequently than usual clinical standards allow. Therefore, radiographs could be available at time 0, 6 months and 12 months in a one year study. The diagnostic criteria are explained in section B of this chapter. Consideration was given to employing devices custom-made to fit the mouth of each subject to position the radiographic films in identical locations in serial radiographs. This would help to standardize the radiographic results. The use of standardized radiographs from custom-made film-holding devices could not be justified since the initial radiographs were not standardized. Reradiographing detected lesions

with a standardized device for the benefit of the study alone would be unethical. Furthermore, because the subjects were all in a mixed dentition stage of development during the study, changes to the teeth and jaws would make it virtually impossible to construct a device for each subject that would be truly stable over a 12 month period.

The method of plaque sampling was chosen because of the success of the technique as previously employed by Bowden et al [161]. The consistency of the results obtained longitudinally from each sampling site in that study was considered to be indicative of the validity of the method. A criticism of the method is that it does not sample precisely the plaque associated with the lesion, but obviously must obtain plaque from across the approximal surface. Also, because of the method of sampling and the small amount of plaque collected, it was not possible to quantify the plaque samples. Therefore, the data had to be managed by relating numbers of specific organisms as proportions of the total cultivable flora from each sample.

Frequency of plaque sampling was chosen to be once every 6 or 12 weeks. It was felt that eight samples over a 12 month period of observation would give a good profile of the microflora at each site. However, in some cases, difficulties were encountered in gaining access to subjects due to a variety of personal reasons ranging from subject illness to subject holidays. This necessitated a revision of sample

scheduling for some subjects to a twelve-weekly basis. These are not unusual problems and must be recognized as difficulties to be dealt with in human longitudinal studies.

F. RECORDING OF DATA

Colonies from each sample were counted and recorded according to the methods previously described. An estimate of the total cultivable flora from each sample was made by counting all colonies on supplemented blood agar incubated anaerobically for 120 hours. Since each sample was dispersed in 1.0 ml of RTF, all counts were calculated to reflect the numbers of organisms that existed in the original sample. Since 20 µl of each dilution was inoculated onto each plate, the counts were multiplied by 50 and then by the dilution factor for the plate counted. For example, if 25 colonies of a particular isolate were counted on a plate where the dilution of the inoculum was 1:1000 of the original sample, the calculation is: $50 \times 25 \times 1000 = 1.25 \times 10^6$ organisms. This allowed for comparison of numbers of organisms in each sample. The proportion of the total cultivable flora which each organism represented in each sample was calculated by dividing the number of each organism in the original sample by the estimate of the total cultivable flora for that sample and converting to percentage. This method allowed the comparison of relative proportions of similar organisms between samples.

G) ANALYSIS

1. DEFINITIONS

The numerical significance of an organism to the microflora of plaque from a particular site or group is described herein as the "degree of challenge" by that organism.

The degree of challenge has two components:

a) Degree of colonization - which is reflected by the mean of the percentages of the cultivable flora represented by an organism in all the samples.

b) Persistence - proportion (percentage) of positive isolations of an organism from all the samples.

Thus, these two percentage figures reflect the degree of colonisation by an organism over the total period of time of observation. This concept has been described previously in a similar longitudinal study by Bowden et al [161].

2) STATISTICAL COMPARISONS

a) Test Versus Control Site in each Subject

Although paired data was available from each test site (incipient lesion) and its designated control site (non-carious contralateral site), the number of samples from each pair of sites was too few (varying from 3 to 8) to allow significant intra subject statistical comparison.

b) Grouped Data

Grouping the data from similar sites provided statistically significant numbers for comparison. The groups of sites that were compared in regard to both mean percent of cultivable flora and percent positive isolations for each organism (genus or species) were:

Progressive lesions

Non-progressive lesions

Control sites (all 32)

Control sites for progressive lesions.

Comparison of means of paired data for each organism was made by means of the student t test, yielding a level of significance of association of each organism.

CHAPTER VII

RESULTS

A. DATA COLLECTED

1) SUBJECTS

Twenty-two subjects aged from four to nine years were included in the study. No subjects were lost from the study during their twelve month period of observation.

2) TEST SITES AND CONTROL SITES

Thirty-two incipient approximal caries lesions (test sites) and thirty-two control sites were included in the study.

3) SAMPLES COLLECTED

A total of three hundred and fourteen plaque samples were collected and examined. Test sites and their control sites were sampled at the same time and equal numbers of times. Each site was sampled from three to eight times, depending on the frequency of sampling and on the progression or non-progression of lesions. Table 6 shows the distribution of samples for different sites.

4) MICROORGANISMS ISOLATED

A total of 10,217 microorganisms were isolated from all of the samples collected. Table 7 shows the numbers of identified organisms isolated. The organisms not identified

Table 6. The distribution of the sites with lesions based upon the numbers of samples taken from each site.

Number of samples taken[a)]	Number of sites	
	Progressive lesions[a)]	Non-progressive lesions[b)]
3	3[b)]	0
4	7	0
5	3	15
6	1	0
8	0	3

a) An equivalent number of samples were taken from control sites.

b) Samples taken post restoration were not included.

Table 7

Distribution of total organisms isolated and identified

Organisms	Number	% of total
S. mutans	718	7.6
mitior	654	6.9
sanguis	763	8.0
salivarius	918	9.7
milleri	157	1.7
fecalis	66	0.7
A. viscosus	4382	46.3
A. naeslundii	471	5.0
A. odontolyticus	67	0.7
Lactobacillus	26	0.3
Veillonella	1068	11.2
Neisseria	167	1.8
Total	9.457	100%

to species level were categorized on initial grouping as belonging to groups outside of the specific interest of this study.

Generally, these figures of isolates (Table 7) corresponded well with the results from similar studies. The relative numbers of A. viscosus and A. naeslundii do not agree with the findings of Ellen[209] who found A. naeslundii to outnumber A. viscosus in subjects of this age-group. This was attributed to Ellen's use of a selective media for Actinomyces which may favour A. naeslundii.

5) LESION PROGRESSION

During the twelve month period of observation of each lesion and control site, fourteen of the thirty-two (44%) incipient lesions progressed to a point at which restoration was required. Eighteen of the lesions did not progress. One of the initially non-carious control sites developed a lesion during the study and this lesion progressed to a point requiring restoration. All sites that developed extensive lesions requiring restoration were restored and ten of the fifteen restored sites were sampled after restoration.

6. COLONISATION OF LESIONS AND CONTROL SITES

The results of the microbiological analyses are shown in Table 8 and Table 9. Table 8 shows the percentage of the

Table 8

The median values and ranges for the bacteria in samples from the lesion and control sites expressed as a percentage of the total cultivable flora.

GANISMS	PROGRESSIVE N = 14	NON-PROGRESSIVE N = 18	CONTROL = 32
mutans	9.7[a)]	3.3	6.6
	(4.1 - 16.3[b)]	(0 - 8.7)	(0.29 - 12.4)
mitior	3.9	5.4	3.6
	(0 - 9.2)	(0 - 13.4)	(0 - 6.5)
sanguis	6.1	8.5	6.4
	(0.5 - 13.5)	(0 - 16.5)	(1.5 - 11.5)
salivarius	5.7	6.3	8.5
	(1.6 - 12.5)	(0 - 13.5)	(1.6 - 14.4)
viscosus	28.9	32.8	32.3
	(20.3 - 39.8)	(25.0- 49.3)	(24.4 - 42.2)
naeslundii	0.25	4.5	2.3
	(0 - 4.8)	(0 - 20.1)	(0 - 5.3)
dont.	0.75	0	0
	(0 - 6.6)	(0 - 0.6)	(0 - 0.02)
obacillus	0.66	0	0
	(0 - 5.1)	0	0
lonella	10.6	8.9	9.4
	(4.8 - 13.6)	(0 - 12.0)	(5.4 - 15.3)

a) Median value. b) Range.

Table 9. The median values and ranges for the percentage isolation frequency of bacteria in samples from the lesion and control sites

GANISMS	PROGRESSIVE N = 14	NON-PROGRESSIVE N = 18	CONTROL N = 32
mutans	100[a]	75	75
	(60 - 100)[b]	(0 - 100)	(0 - 100)
mitior	50	100	66.6
	(0 - 100)	(0 - 100)	(0 - 100)
sanguis	100	87	100
	(50 - 100)	(0 - 100)	(0 - 100)
salivarius	100	100	100
	100	(20 - 100)	(40 - 100)
viscosus	100	100	100
	100	100	100
naeslundii	50	80	80
	(0 - 100)	(0 - 100)	(0 - 100)
odont.	50	0	0
	(0 - 100)	(0 - 40)	(0 - 25)
tobacillus	75	0	0
	(0 - 100)		
llonella	100	100	100
	(66 - 100)	(0 - 100)	(83 - 100)

a) Median value. b) Range.

total cultivable flora for the significant organisms. This pooled data is expressed as median values (first line) and ranges of percentages, (in brackets) from lowest to highest for progressive lesions, non-progressive lesions and control sites. Table 9 shows the frequency of isolation of each organism (in percentage) from progressive lesions, non-progressive and control sites. This is expressed as median values and ranges from lowest to highest values.

B. ANALYSIS OF DATA

1. MICROBIOLOGY OF CONTROL SITES

Plaque sampled from control sites was microbiologically similar to data previously reported for approximal plaque in children[90 166] (Tables 8 and 9). Species of Streptococcus and Actinomyces were consistently isolated from all samples from all control sites. The following organisms were isolated at the following frequencies in the samples from the control sites: S. mutans 68%; S. sanquis 80%; S. mitior 80%; A. naeslundii 81% and S. salivarius 84%. Veillonella was isolated from all but three samples from control sites. A. odontolyticus was isolated from only two samples from control sites. Lactobacillus species were never isolated from control sites.

2. MICROBIOLOGY OF PROGRESSIVE LESIONS

The microbiological findings in samples from progressive lesions were similar to control sites with respect to A. viscosus and S. salivarius. The percentage of isolation was 89% for S. mutans, 85% for S. sanguis, 42% for S. mitior and 45% for A. naeslundii. Veillonella was isolated from all but one sample. (Table 9).

Very significant differences were noted between the microflora of the progressive lesion and that of the control sites with respect to the isolation of A. odontolyticus and Lactobacillus. A. odontolyticus was isolated from 45% of samples from progressing lesion sites. This organism was not isolated at any time from five of the fourteen progressive lesions. Lactobacillus was isolated from 49% of samples from progressing lesions but was absent from all of the samples from three of the fourteen sites.

3. MICROBIOLOGY OF NON-PROGRESSIVE LESIONS

The microbiological findings from the sites of non-progressive lesions more closely resembled the results from control sites than from progressive lesions. Isolation frequencies for the most prevalent organisms in non-progressive lesions were: S. mutans 71%; S. sanguis 85%; S. mitior 80%; S. salivarius 86% and A. naeslundii 74%. Veillonella was isolated from all but seven samples and five of those samples were from one subject who was consistently negative. A. odontolyticus was isolated six times, somewhat more often

than from control sites. Notably, Lactobacillus was not isolated from any non-progressive lesions (Table 9).

4. SIGNIFICANT DIFFERENCES BETWEEN THE MICROFLORA OF LESIONS AND CONTROL SITES

The most significant differences between the microbiology of different sites was with respect to the isolation frequencies for A. odontolyticus and Lactobacillus. A. odontolyticus was isolated significantly more frequently from progressive lesions ($p < 0.01$) than from non-progressive lesions or control sites ($p < 0.01$) (see Table 10). An even more striking difference is seen in the isolation frequencies of Lactobacillus which was detected only at progressive lesions ($p < 0.01$) and never at control sites or non-progressive lesions (Table 9).

A comparison (Table 10) of the isolation frequencies and mean percentage of the cultivable flora of progressive lesions and control sites by means of the student t test revealed a significant positive association between increased colonisation by S. mutans, Lactobacillus and A. odontolyticus and lesion progression. Progressive lesions were found to have higher numbers and more frequent isolations of these organisms than control sites.

Comparison of non-progressive lesions and their control sites by means of the student t test showed no significant differences in the microflora of either mean percentage of

Table 10

Comparison of the isolation frequencies and mean % of the cultivable flora from lesions and their control sites.

Organism	Isolation frequencies	Mean %
S. mutans	$p < 0.05$	$p < 0.05$
S. mitior	NS	NS
S. sanguis	NS	NS
S. salivarius	NS	NS
Lactobacillus	$p < 0.01$	$p < 0.05$
A. odontolyticus	$p < 0.01$	$p < 0.05$
A. naeslundii	NS	NS
A. viscosus	NS	NS
Veillonella	NS	NS

NS = does not reach $p < 0.05$

Table 11

The association of various genera and species with incipient lesions which progressed.

	Nature of Association			
	POSITIVE		NEGATIVE	
ORGANISM	IF[a]	M%[b]	IF	M%
S. mutans	$p < 0.05$	$p < 0.01$		
S. salivarius	$p < 0.05$	NS[c]		NS
Lactobacillus	$p < 0.01$	$p < 0.05$		
A. odontolyticus	$p < 0.01$	$p < 0.05$		
Veillonella	NS	$p < 0.05$	NS	NS
S. mitior			$p < 0.05$	$p < 0.05$
A. naeslundii			$p < 0.01$	$p < 0.1$
A. viscosus			NS	$p < 0.05$
S. sanguis	NS	NS	NS	NS

a) Isolation frequency

b) Mean percentage cultivable flora.

c) Did not reach $p < 0.1$.

the cultivable flora or isolation frequency of organisms. (Data not shown).

When the isolation frequencies and mean percentage of cultivable flora for the various organisms from progressive lesions were compared to non-progressive lesions utilizing the student t test, both positive and negative associations were revealed (Table 11). Positive associations are those instances where increased colonisation by an organism (genus or species) is associated with lesion progression.

Positive associations (Table 11 - left hand column) were made for S. mutans, S. salivarius, A. odontolyticus and Veillonella.

Negative associations are those instances where decreased colonisation by an organism is associated with lesion progression.

Negative associations were noted for S. mitior, A. naeslundii and A. viscosus.

A comparison between the results from control sites of progressive versus those of non-progressive lesions showed a significant difference only for S. mutans. This organism was isolated significantly more often ($p < 0.01$) and in significantly greater proportions ($p < 0.01$) from control sites of progressive lesions. Veillonella was also isolated more often from control sites of lesions however, the signifi-

Table 12

Mean values for % frequency of isolation for bacteria isolated from control sites of progressive and non-progressive lesions.

ORGANISMS	Control sites of progressive lesions n = 14	Control sites of non-progressive lesions N = 18	Significance
S. mutans	92	65	$p < 0.01$
S. mitior	61	70	NS
S. sanguis	91	94	NS
S. salivarius	95	100	NS
A. viscosus	100	100	NS
A. naeslundii	80	84	NS
A. odontolyticus	0	0	NS
Lactobacillus	0	0	NS
Veillonella	84	100	NS

NS indicates failure to reach a $p < 0.1$ level of significance.

Table 13

Mean values for % of total cultivable flora of bacteria from control sites of progressive and non-progressive lesions

Organisms	Control sites of progressive lesions n = 14	Control sites of non-progressive lesions N = 18	Significance
S. mutans	8.4	4.7	$p < 0.01$
S. mitior	3.9	4.1	NS
S. sanguis	6.8	7.2	NS
S. salivarius	7.5	8.7	NS
A. viscosus	32.4	32.9	NS
A. naeslundii	2.7	2.1	NS
A. odontolyticus	0	0.01	NS
Lactobacillus	0	0.0	NS
Veillonella	8.9	9.9	NS

NS indicates failure to reach a $p < 0.01$ level of significance.

cance did not reach the level of $p_{<}$ 0.1 (Table 12 and 13).

5. COMPARISON OF PROGRESSIVE LESIONS BEFORE AND AFTER RESTORATION

Ten progressive lesions were sampled after they were restored. The values determined for percentage contribution to total cultivable flora and frequency of isolation of significant organisms were recorded before and after restoration (Tables 14 and 15) and compared by the student t test (Tables 14 and 15). S. mutans, Lactobacillus Veillonella and A. odontolyticus were found to be present in greater proportions before restoration than after restoration. It was found that A. odontolyticus and Lactobacillus did not readily recolonize the restored sites of progressive lesions (Table 15). In fact, Lactobacillus did not recolonize any restored sites.

6. LESION PROGRESSION RELATED TO SUBJECT AGE

The twenty-two subjects ranged in all from four to nine years. There were fifteen incipient lesions studied in children four to six years of age at the beginning of the study and seventeen lesions observed in children seven to nine years old. Eight of the fifteen lesions in four to six year olds (53%) progressed and were restored (Table 16). Six of the seventeen lesions in seven to nine year old (35%) progressed and were restored. Because the numbers in each

Table 14

Mean % of total cultivable flora from sites of progressive lesions before and after restoration.

n = 10

Organisms	Pre-restoration	Post-restoration	Significance
S. mutans	11.3	6.9	$p < 0.05$
S. mitior	3.7	3.8	NS
S. sanguis	6.9	6.2	NS
S. salivarius	6.8	8.2	NS
A. viscosus	23.4	27.5	NS
A. naeslundii	2.4	2.4	NS
A. odontolyticus	0.9	0	$p < 0.05$
Lactobacillus	0.8	0	$p < 0.05$
Veillonella	11.2	8.1	$p < 0.05$

NS designates that significance did not reach $p < 0.1$.

Table 15

% Frequency of isolation from sites of progressive lesions before and after restoration.

Organisms	Pre-restoration	Post-restoration
S. mutans	100	90
S. mitior	70	80
S. sanguis	80	80
S. salivarius	100	100
A. viscosus	100	100
A. naeslundii	100	100
A. odontolyticus	60	20
Lactobacillus	80	0
Veillonella	100	100

group are small it would incorrect to formulate a firm conclusion on the basis of this data, however, it appears that younger children with incipient approximal lesions may be at greater risk of having the lesions progress than older children. This may simply reflect a difference in the speed of the caries process in different individuals. Perhaps in many of the older children with incipiencies, it simply took longer for the lesions to become radiographically evident, and the slower process resulted in no evidence of progression during the study period.

Table 16

Subject age at beginning of study and progression of lesions

Subject Age	No. of Lesions	No. progressed	% progressed	Grouped %
4	3	2	66%	
5	6	2	33%	53%
6	6	4	66%	
7	8	3	38%	
8	7	3	43%	35%
9	2	0	0 %	

CHAPTER VIII

DISCUSSION

A. MICROBIOLOGY OF PROGRESSION OF INCIPIENT APPROXIMAL LESIONS

1. RELATIONSHIP TO PREVIOUS STUDIES

This study employed methods similar to those used in other longitudinal studies of dental caries[147] [122]. A significant difference between this study and others that have been published is that this study selected, for examination, areas of enamel that had already been diagnosed as having an early or incipient enamel lesion. This pre-selection allowed a longitudinal microbiological comparison of incipient lesions which appeared to be active and those which appeared inactive.

The observations made in this study, as will be discussed, are similar to those made in other studies of caries and do tend to implicate similar organisms in the caries process.

2. LACTOBACILLUS AND PROGRESSION OF CARIES

The association of Lactobacillus with progression of incipient lesions was particularly remarkable. This study detected Lactobacillus only at the sites of incipient lesions which progressed and never at non- progressive lesions or control sites. Furthermore, Lactobacillus was

never isolated from the sites of restored lesions.

The nature of this association is open to speculation. It may be significant that Lactobacillus was usually found to represent only a very small percentage of the total cultivable flora ($< 1\%$). The obvious question that arises is whether an organism apparently present in such low numbers could be a significant active factor in the caries process. This is, perhaps, evidence that Lactobacillus is opportunistically associated with later stages of caries. The aciduric nature of this organism may predispose it to adopting a habitat where there is a well-established and isolated acidic environment. However, consideration should be given to the possibility that the Lactobacillus detected in this study represented only stray or peripheral organisms. That is, if Lactobacillus is associated with the cavitation phase of carious lesions, there may be a reservoir of Lactobacillus in the depths of the lesion where the environment is likely more isolated and more acidic[132]. The plaque sampling technique employed in this study would have picked up surface plaque from the lesion which contained only a relatively small number of the organisms present. Whether Lactobacillus is involved in later stages of caries progression or opportunistically present at later stages is not clear from the evidence of this study.

Furthermore, because of the inaccuracy of diagnosis of caries from radiographs, it is possible that Lactobacillus

was found at the site of lesions that were initially much larger than diagnosed from radiographs. That is, some of the progressive lesions sampled may have involved more than surface enamel when included in the study and may have already reached a stage beyond incipiency. Nevertheless, the evidence from this study clearly indicates that the presence of Lactobacillus at the site of an approximal lesion is indicative of progressive activity in that lesion.

It would be beneficial to undertake a further study which would obtain enamel samples from approximal lesions and relate surface histology to microbiologic findings in an effort to clarify the relationship between presence of Lactobacillus the stage of lesion progression and the state of the surface enamel.

3. S. MUTANS AND PROGRESSION OF CARIES

The results of this study reemphasize the close association of S. mutans with the caries process previously established by many other studies. This organism was isolated significantly more often and in significantly higher proportions from progressive or active lesions. This indicates a significant role for this organism in the progression of incipient approximal lesions.

These results also illustrate the ubiquitous nature of S. mutans in dental plaque. It was isolated regularly from inactive lesions and control sites as well as active

lesions. The presence of S. mutans in plaque is not, therefore, evidence of clinical caries activity at that site. Once we become able to detect very early carious changes in enamel, changes that are not yet clinically detectable, we may discover that S. mutans is associated with early stages of caries that occur much more universally on the surface of teeth than previously suspected. These early changes may be part of a cyclical process of demineralization and remineralization that in many cases may never reach the stage of being clinically detectable.

Serotypes (d and g) of S. mutans have been implicated as being more closely associated with active caries than other serotypes[90]. S. mutans isolated in this study were differentiated biochemically according to biotype as suggested by Shklair and Keen (Table 2). It was found that Biotype I predominated in both progressive and non-progressive lesions, with no significant differences in Biotype IV, corresponding to serotypes d and g. (Table 17). These results are contradictory to these of Huis In'tVeld et al who found serotypes d and g closely associated with caries activity.

It is possible that levels of S. mutans at a given site might be an indication of caries activity. However, the wide-spread presence of S. mutans in dental plaque, evidenced in this study, would reduce the reliability of such an evaluation. A similar type of study as this, with identification of serotypes of S. mutans, might provide more

Table 17

Median values for isolation frequency of biotypes of S. mutans from progressive and non-progressive lesions.

BIOTYPE	Serotype	n = 54 Progressive lesions		n = 90 Non-progressive lesions	
		No.	%	No	%
I	a	45	83	72	80
II	b	0	0	0	0
III	c/e/f	0	0	5	6
IV	d/g	4	7	5	6
Unidentified	5	9		8	9

N.B. - Samples from which no S. mutans were detected were subtracted from the total number of samples from those sites.

valuable indications.

4. ACTINOMYCES ODONTOLYTICUS AND PROGRESSION OF CARIES

The observations of this study indicate an association between A. odontolyticus and progressive caries lesions. The nature of the relationship is not clear. That is, it is not apparent whether this organism is a significant influence in the caries process or whether it simply finds a niche in the advanced lesion. The association of A. odontolyticus with progression has not been widely recognized in the past. However, it is noteworthy that this organism was first described by Batty upon isolation from carious dentin[108]. Edwardsson[115] also reported its frequent isolation from the advancing front of deep carious lesions. This study found that A. odontolyticus was usually isolated in very low numbers (means of percentage of total cultivable flora = 0.75%). Since many previous isolations were from deep carious dentin, consideration must be given to the possibility that the organisms isolated in the surface plaque samples may have been "strays" originating from deep in the lesion. This would suggest, similar to the speculation about Lactobacillus, that the presence of A. odontolyticus may be indicative of loss of the intact layer of surface enamel.

A combined histological and microbiological study to relate the microflora directly to the stage of lesion devel-

opment might clarify the relationshuip of both Lactobacillus and A. odontolyticus to the caries process.

5. VEILLONELLA AND PROGRESSION OF CARIES

Although Veillonella was almost universally present in the plaque samples taken for this study, it was isolated in significantly higher numbers from progressive lesions than from non-progressive lesions. This finding seems to contradict the evidence from animal studies[205] which suggested that this organism could inhibit the caries process. The theory hinged on the lactate metabolizing characteristic of Veillonella. In the present study, the higher levels of Veillonella at progressive sites seem to indicate that high levels of Veillonella do not inhibit caries activity by moderating pH through metabolism of lactate. Based on the animal studies mentioned and the nature of this organism, the expectation was to find higher levels of Veillonella at non-progressive sites.

A possible explanation for the higher levels of Veillonella at progressive sites is that the organism thrives better where there are higher or more constant levels of lactic acid, as would be expected at caries-active sites.

6. BACTERIA WITH NEGATIVE ASSOCIATIONS WITH CARIES PROGRESSION

Several organisms were discovered, in this study, to be isolated less frequently and/or in lower proportions at progressive lesions. This relationship has been termed a negative association.

S. mitior was found significantly less frequently and in lower percentages at progressive lesions. A similar finding was made by Loesche and Straffon [157] for S. sanguis who noted a relationship between S. mutans and S. sanguis which they described as a ratio of proportions of the two organisms. A higher S. mutans to S. sanguis ratio was indicative of caries activity or potential and a lower ratio was indicative of caries inactive plaque. It is possible that both this study and that of Loesche and Straffon are describing the same phenomenon. The means of identifying S. sanguis in the previous study was by colonial morphology, which was much less specific than the methods used in the present study. Because of the colonial similarities between S. mitior and S. sanguis it is quite possible that both studies are describing the same organism. The higher S. mutans and lower S. mitior levels at caries active sites may be a reflection of competition between the two organisms. At sites where S. mutans dominates its greater cariogenic potential results in caries progression.

A. naeslundii was found less frequently and in lower numbers and A. viscosus was found in significantly lower numbers at progressive sites. These negative associations have

not been commonly reported in previous studies. However, in a comprehensive longitudinal study, reported in 1946, Hemmens et al [121] described a similar observation for Actinomyces. They also reported decreases for Leptotrichia, alpha hemolytic streptococci, fusiformbacteria and Neisseria.

It is understandable that if increased proportions of one or more organisms with cariogenic potential, such as S. mutans, results in caries activity this activity will be associated with decreased proportions of other organisms. From an ecological viewpoint, this shift could be due to differences in the environment which favour some organisms more than others, rather than a direct antagonism between organisms. This is referred to as "autogenic succession"[222]. A progressive site, therefore, is one at which the ecological factors favour the cariogenic organisms. This statement is supported by the observation in this study that the microbiologic findings at non-progressive sites were indistinguishable from control sites. In both situations, during the period of observation, the plaque was non-cariogenic because ecological conditions did not sufficiently favour cariogenic organisms.

7. RECOLONIZATION AFTER RESTORATION

Tables 14 and 15 show the findings at the sites of progressive lesions just before restoration and then one month

or more after restoration. The most notable feature is the failure of Lactobacillus to recolonize at all at these sites. Also significant is the decreased colonization by A. odontolyticus after restoration. Means of the percentages of total cultivable flora (Table 14) show decreases in relative numbers of S. mutans, A. odontolyticus, Lactobacillus and Veillonella at a level of significance. of p 0.05. Although the sample size is small (n = 10), this data suggests that the local environment is changed significantly when lesions are restored. The decreased levels of these organisms suggest that the environment is less suitable for aciduric organisms. This is an example of "allogenic succession", wherein a community is altered because the habitat is altered by non-microbial factors[222]. In the case of restored caries lesions, this change may be due to the altered surface composition (enamel replaced by amalgam) or, probably more likely, the elimination of the cavity. Cavitation provides a more protected, isolated environment that is more suitable for maintenance of highly acidic conditions[232]. This would favour the aciduric organisms. Elimination of the cavity alters this situation and continuous low pH conditions are no longer present. This would no longer favour the aciduric organisms and would explain the decrease or elimination of their numbers after restoration found in this study.

8. FLUORIDE RESISTANCE OF LACTOBACILLUS

The significant association of the presence of Lactobacillus and caries progression revealed by this study renewed our interest in this organism. In the course of conducting an extensive series of tests for species identification of some of the Lactobacilli isolated during the study, it was observed that these organisms demonstrated a strong resistance to fluoride inhibition.

A freshly isolated strain from the longitudinal study was used by Dr. I. Hamilton[171] to investigate its fluoride resistance in continuous culture. The strain of L. casei examined was observed to survive in a complex medium with glucose limitation with little change in cell numbers at pH ranging from 7.0 to 3.8. Below pH 3.8, the organism was inhibited and cell numbers in the chemostat dropped. It was found that when the pH in the chemostat was dropped from 7.0 to 5.0, fluoride resistance (pH 5.5 and 10.5mM NaF) developed within twenty-four hours. Reestablishment of pH 7.0 in the chemostat resulted in a return to the initial F sensitivity (pH 4.5 mM and 5.3 mM NaF). Exposure to 20 mM NaF fluoride in the continuous culture media caused no drop in cell numbers until the pH fell below 6.0. The addition of 20 mM NaF to the continuous culture produced F^- resistance of 16 mM at pH 5.5. Glycolytic activity was monitored during the manipulation of pH and fluoride concentration in continuous culture and was found to be fairly constant. It was notable that 10.5 mM NaF fluoride added to continous

culture stimulated glycolysis except when the organism was adapting to a pH decrease in the media. The conclusions were that Lactobacillus possesses an inducible resistance to fluoride and this resistance appears to be associated with a unique characteristic of the organism in relation to intracellular pH.

This knowledge of the fluoride and acid resistance of Lactobacillus may be important to the role of Lactobacillus in the caries process, particularly in a water-fluoridated area or where children receive regular fluoride mouthrinses. The apparence of Lactobacillus in later stages of the development of the caries lesion may be a reflection of the favourability of highly isolated, constantly acidic conditions which we would expect to find in a cavity. In such an environment, particularly in the presence of fluoride, Lactobacillus would probably have an ecological advantage over other oral, acidogneic organisms, including S. mutans.

9. SIGNIFICANCE OF THE FINDINGS

The association of Lactobacillus with progressing incipient lesions could be a significant etiologic factor or simply an opportunistic phenomenon occurring later in the caries process. In either case their presence could be a significant diagnostic indicator of the state of the lesion. A simple test could be employed, using a highly selective

medium, to check for the presence of Lactobacillus in plaque from an incipient approximal lesion. Such a test would allow dentists to monitor incipient approximal lesions and make more accurate diagnoses of the state of the lesions. This would involve fewer radiographs and therefore less x-irradiation of patients.

Before this method could be used on a widespread basis, it would be necessary to test the validity of this procedure by examining a much larger group of subjects than was used for this study. Furthermore, it would be desirable to be able to clinically and histologically assess the state of incipient lesions with and without the presence of Lactobacillus. The results of such a study would allow a far better correlation to be made between the presence of Lactobacillus and lesion progression than can be made by radiographic interpretation. The comparison could be made by performing comprehensive bacterial analysis on plaque from incipient lesions and then restoring them. In the restoration procedure, a clinical analysis could be made of the state of the lesion and the approximal enamel containing the lesions could be removed intact and retained for histological analysis.

The information gathered in the present study also suggests the possibility of employing localized chemotherapy to interfere in the progression of incipient approximal lesions. The demonstrated resistance of Lactobacillus to

fluoride suggests that a local chemotherapeutic treatment would have to include an agent in place of or in addition to fluoride. In fact, early results in an effort to find a suitable agent have found most strains of Lactobacillus tested to be resistant to chlorhexidine, particularly at low pH. (Cleghorn B, and Bowden, G. personal communication). Erythromycin has proven to be effective against these organisms. Theoretically, if a chemotherapeutic agent could be used to eradicate the cariogenic organisms at the site of an incipient approximal lesion, remineralization could be promoted by a simultaneous application of fluoride. The bacterial community may be altered by this treatment and undergo allogenic succession similar to that shown to be related to restoration in the present study.

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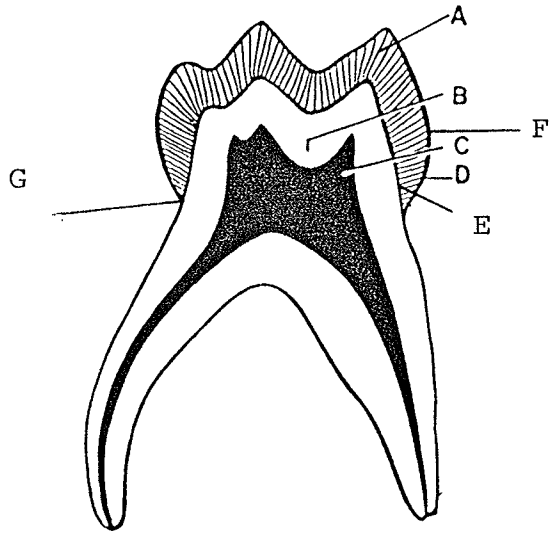
G L O S S A R Y

Approximal (adjective)	between the proximal surfaces of adjoining teeth.
Arch, Dental	the ensemble of teeth in either jaw in the form of an arch.
Buccal (adjective)	Pertaining to the cheek; towards the cheek or next to the cheek.
Cervical (adjective)	pertaining to the neck or to any cervix, i.e., that portion of a tooth near the junction of crown and root.
Contact area	the area of contact of one tooth with another in the same arch.
Crown (noun)	a name applied to that part of a tooth which is covered with enamel.
Dentin, dentine (noun)	the calcific tissue forming the body of a tooth, underlying the cementum and enamel.
Deciduous (adjective)	that which will be shed; specifically, the first dentition of man or animal.
Distal (adjective)	distant or farthest from a central point; away from the median line or the face, following the curve of the dental arch.
Enamel (noun)	the hard calcified tissue which covers the dentin of the crown portion of teeth.
Fissure (noun)	a deep ditch or cleft; a developmental linear fault found usually on the occlusal or the buccal surfaces of a tooth; commonly the result of the imperfect fusion of the enamel during tooth formation.
Gingiva (noun)	that part of the gum tissue and mucous membrane that immediately surrounds a tooth.

Lingual (adjective)	pertaining to or affecting the tongue. Next to, or toward the tongue.
Mesial (adjective)	toward or situated in the middle, as toward the median line of the dental arch.
Occlusal (adjective)	the surface of a molar or premolar tooth that meets the opposing teeth in the closure of the jaws.
Pit (noun)	a small, pointed depression in dental enamel, usually at the junction of two or more developmental grooves.
Proximal (adjective)	nearest, next, immediately preceding or following.
Pulp, dental	the highly vascular and innervated connective tissue contained by the pulp cavity of the tooth.
Radiograph (noun)	the photographic representation of opaque objects produced by the action of roentgen rays upon a sensitized plate or film.

Figure 7. Dental Anatomy Terminology



A. Enamel

B. Dentine

C. Pulp

D. Cervical Area

E. Dentino-enamel junction

F. Contact point

G. Cemento-enamel junction.

APPENDIX 1.

QUESTIONNAIRE

Re: Study by Dr. R. Boyar and Dr. G. Bowden, University of Manitoba, Faculty of Dentistry.

CHILD'S NAME:

BIRTHDATE:

	YES	NO
1. Has your child had any serious illness? If so, please specify: _____	___	___
2. Does your child take any pills or medications? If so, what ? _____	___	___
3. Has your child taken any anti-biotics during the last year?	___	___
4. Do you live in Winnipeg?	___	___
5. Does your child receive regular dental care?	___	___
6. Has this child's mother and/or father had many problems with dental decay?	___	___
7. Do you restrict your child's access to candy, soda pop and other snack foods?	___	___
8. If you have other children, have they had many problems with dental decay?	___	___

9. What do you think is the most important thing you can do to protect your child's teeth?

_____

_____

_____

Appendix 2.

CONSENT FORM

I do hereby consent to allow my child _____ to participate, as a subject, in the experimental study conducted by Dr. R. Boyar and Dr. G. Bowden.

I understand that dental plaque samples will be taken from my child's teeth at intervals of 6 or 12 weeks over a period of one year for purposes of bacterial analysis.

The nature and purpose of this study have been explained to me to my satisfaction.

I understand that I have the right to revoke this consent and withdraw my child from the study at any time.

Signed :

Date :

Relationship to child :

Witness :

Child's Name :

Birthdate :

Address :