DEFENCE MECHANISMS OF THE MOUTH

With Particular Reference to Patients with Sjögren's Syndrome

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Thesis

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PREFACE

The work undertaken in this thesis was undertaken in the University of Glasgow during 1973-1975 while the author was a Lecturer in Oral Medicine and Pathology in Glasgow Dental Hospital and School.

Most of the work in this thesis is original and was mainly undertaken individually by the author, with some assistance by technical staff, directly supervised by the author.

Parts of the work of this thesis have been published or presented at scientific meetings:-

PUBLICATIONS

1. Local environmental factors in the host resistance to the commensal microflora of the mouth (with Mason, D.K.) in "Host Resistance to Commensal Bacteria" Ed. MacPhee, I.T., Churchill-Livingstone, Edinburgh, 1972.

2. Changes in the Oral Flora of Sjögren's Syndrome (with Mason, D.K.) Journal of Clinical Pathology, 27, 416, 1974.

3. Defence Mechanisms of the Mouth, in Applied Physiology of the Mouth, Ed. Levalle, C.B.L., Wright, Bristol, 1975.

4. The Physiological Responsiveness of Oral Mucosa: the role of Saliva (with Mason, D.K.) in "Oral Mucosa in Health and Disease" Ed. Dolby, A.E., Backwell Oxford 1975 (In Press).

PAPERS PRESENTED AT SCIENTIFIC MEETINGS

1. A New Medium for the Isolation of Oral Gram Negative Bacteria. International Association for Dental Research, Glasgow, 1971.

2. Local Environmental Factors in the Host Resistance to the Commensal Microflora of the Mouth. (with Mason, D.K.) Symposium on Host Resistance to Commensal Bacteria, Edinburgh, 1972.

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SUMMARY

The three ecological components of the mouth are, the host tissues, saliva and the commensal micro-flora. Normally these components interact to protect the oral tissues from invasion and colonization by potentially pathogenic micro-organisms. Patients suffering from Sjögren's Syndrome develop severe xerostomia due to a progressive destruction of salivary gland tissue, and although the changes in the host tissues, and saliva have received much study, little attention has been focused on the effect of these changes on the oral microbial flora.

Microbiological assessment of the oral flora of patients with Sjögren's Syndrome showed that there was an increase in the numbers of Candida albicans and Staphylococcus aureus and a reduction in the numbers of Streptococcus salivarius, Veillonella, Neisseria pharyngis and Staphylococcus salivarius, when compared with normal control subjects. The aetiological factors related to these changes in the oral flora were considered to be complex, with many factors involved, including atrophy of the oral mucosa and a reduction in the volume and pH of saliva. The details of a new medium which was used for the isolation and enumeration of Veillonella species in these studies, was reported.

Since intrinsic salivary antimicrobial substances, and microbial antagonistic mechanisms of the oral flora are thought to be the main factors which protect the mouth against infection by potentially pathogenic micro-organisms, it was decided to assess the antimicrobial activity of these two factors. The antimicrobial activity of mixed and centrifuged saliva collected from normal subjects was assessed using a new technique which possessed a number of advantages over the techniques used previously. The results of this study, suggested that the appearance of Staphylococcus aureus and Candida species in the mouth of patients with severe Sjögren's Syndrome was due to changes in the commensal microflora and the environment of the mouth, rather than to the loss of inhibitory factors derived from saliva.

The antagonistic activity of Type cultures of eleven oral commensal bacteria was assessed against Candida albicans, Staphylococcus aureus and Escherichia coli. All the Type cultures possessed some antimicrobial activity, but since alpha-haemolytic streptococci possessed most activity, it was concluded that these streptococci, were of prime importance in protecting the oral tissues against invasion by non-commensal micro-organisms. The chance finding of a difference in the inhibitory activity of two strains of Streptococcus sanguis suggested that the inhibitory activity of all strains of a bacterial species could not be assumed to be the same.

The next logical step was to compare the antimicrobial activity of alpha-haemolytic streptococci isolated from patients with Sjögren's Syndrome with streptococci isolated from healthy controls. The results of this study showed that the activity of streptococci isolated from control subjects was significantly greater than the activity of streptococci isolated from patients with Sjögren's Syndrome. It was concluded that the presence of Candida albicans, Staphylococcus aureus and Coliform bacilli in the oral flora of patients with Sjögren's Syndrome was related to the absence of certain alpha-haemolytic streptococci which possessed a wide spectrum of antimicrobial activity.

A retrospective study of the routine microbiology results from oral samples of Sjögren's patients, showed that, Candida albicans, Staphylococcus aureus and Colifrom bacilli, were present in the mouth before any therapy was started. Since moderate to large numbers of Staphylococcus aureus and Candida albicans were isolated from the mouth of a number of Sjögren's patients at relatively regular intervals over a period of years, it appeared that these micro-organisms could be regarded as a major component of the commensal flora of patients with Sjögren's Syndrome. A prospective study on the clinical response to antifungal therapy is necessary, and it was suggested that the use of artificial saliva, with added antifungal agents may be a satisfactory method of treating the oral symptoms of Sjögren's Syndrome. In addition the possibility was raised, of restoring the antimicrobial activity of the oral microflora by

re-colonizing the mouth of Sjögren's patients with a strain of alpha-haemolytic streptococcus with antifungal and antibacterial activity.

DEFENCE MECHANISMS OF THE HUMAN MOUTH

1.1 INTRODUCTION

The three main ecological components of the mouth are the host tissues, saliva and the oral microbial flora. The anatomy, physiology, biochemistry and pathology of the first two components have been studied in great detail, but relatively little similar detailed knowledge is available concerning the oral microbial flora. It appears that all three components possess factors which potentially could play a part in the defence of the oral and dental tissues. However, there is little direct evidence that any of the factors are active in vivo, and interactions among the factors rarely have been considered. In order to gain some fundamental information concerning the nature and role of some of the factors believed to be active in the protection of the mouth, the present investigation into oral defence mechanisms with special reference to patients with Sjögren's syndrome was initiated.

1.2 THE ORAL ENVIRONMENT

The human mouth is lined with a stratified squamous mucous membrane which consists of a superficial epithelial layer and a deeper connective tissue layer. Although the oral mucous membrane has this basic structure in all parts of the mouth, it is modified in certain regions, according to function. The oral mucosa is interrupted by teeth if they are present and is closely related to the tooth surface by means of the epithelial attachment. In addition, the mucosal surface is pierced not only by the ducts of the parotid, submandibular and sublingual glands, but also by the numerous small ducts of the accessory salivary glands scattered throughout the oral mucous membrane. A thin film of saliva therefore bathes the surface of the mucosa during waking hours and contained in the salivary layer are polymorphonuclear leukocytes, epithelial squames and the commensal oral microflora. The general environment of the outer layer of the oral mucosa could, therefore, be described as possessing a somewhat rough surface, interrupted by teeth and the orifices of ducts, coated with micro-organisms and moistened with saliva.

The main ecological components of the mouth are therefore the oral and dental tissues, saliva, and the oral microbial flora. Usually the complex interactions of these components result in a state which is recognised as normal and healthy, but when the interactions become deranged a state may result which is regarded as abnormal and recognised as disease. The mechanisms by which health is preserved in the face of potential disease, that is the defence mechanisms of the mouth, can be divided into non-specific immunity and specific immunity. Non-specific immunity can be regarded as the resistance displayed by an animal that has never experienced contact with a particular pathogenic micro-organism either as a pathogen or as a related non-pathogenic variant. Non-specific mechanisms are usually sufficient to prevent invasion of the host tissues by the vast majority of micro-organisms. In spite of their obvious importance to the health of man, little detailed knowledge is available concerning the non-specific immune mechanisms (Humphrey and White 1970). However these remarkable mechanisms are occasionally powerless to prevent infection by micro-organisms and the host after recovery from the initial infection is often able to resist a second onslaught from the same micro-organism for variable periods of time. This specific immunity does not exist except as a result of a previous exposure to the micro-organism. The investigations in this thesis are concerned with the protective role of non-specific immunity in the human mouth.

1.3 THE HOST TISSUES

1.3.1 Anatomical Factors

Anatomical factors which tend to protect the oral tissues from trauma during functional activity are well recognised. In ideal circumstances, the morphology of teeth and their spatial relationships one to another, prevents food being forced between them, and thereby protects the gingival tissues from injury. Due to malalignment of teeth, faulty fillings and mouth breathing to mention a few of the factors involved (Manson 1971), the ideal is rarely attained and trauma to the oral mucosa and gingivae along with food stagnation and dental plaque formation results. The most severe areas of stagnation in the mouth are the occlusal fissures and the site below the contact points

between teeth, and it is in these areas that the bulk of dental plaque is found. Microstagnation areas have been demonstrated on the surface of enamel, and these surface irregularities are probably favourable sites for the development and attachment of dental plaque and calculus (Boyde 1971).

The stratified squamous epithelium of the mouth is keratinized to varying degrees throughout the mouth. It has a well developed capacity to respond to irritation by hyperplasia and is rarely inflamed and ulcerated by primary bacterial infection. The crevicular epithelium which lines the gingival crevice on the other hand is thin, non-keratinized often inflamed and ulcerated due to the action of bacteria, and appears to be an inadequate barrier to prevent bacterial products entering the underlying connective tissues. The junction between oral epithelium and enamel is mainly adhesive in nature (Selvig 1971) and represents a region of mechanical weakness which under mechanical stress may allow the epithelium to break away from the enamel, permitting micro-organisms and their products access to the connective tissues. The damage which results may be repaired but the host tissues have been altered and further damage is likely. The close contact between the crevicular epithelium and the tooth surface does however minimise the penetration of micro-organisms and saliva into the sulcus region. In general, it would appear that the anatomical relationships and the physical properties of the teeth and periodontium may severely limit the ability of the tissues to resist the attack of the commensal oral flora, especially in the form of dental plaque.

1.3.2 Epithelial Cells and Microbial Adherence

Superficial epithelial cells from the oral mucosa are constantly being shed into saliva, and there is evidence that these desquamated cells are still bio-chemically active, for Friedman and Tonzetich (1968) reported that the glucolytic activity of whole saliva was related to the presence of epithelial cells. The desquamated epithelial cells carry variable numbers of adherent bacteria (Bradley 1948). Selective attachment of bacteria to certain specific epithelial cells or surfaces is relatively common among the indigenous

microbiota of mamalians. This selectivity is shown particularly in the colonization of the oral mucosa and teeth by streptococci. Streptococcus mutans is found only on the surfaces of teeth, while Streptococcus salivarius is rarely isolated from teeth, but is present on the surface of the tongue in large numbers (Gibbons 1972). It is generally thought that the adherence of bacteria to oral epithelial cells is due in part to the possession of an outer trypsin-sensitive capsule like substance which has been observed using the electron microscope in several species of streptococci (Ellen and Gibbons 1972; Gibbons et al 1972; Liljermark and Gibbons 1972). It seems likely that the bacterial "capsule" adheres to the plasma membrane of the epithelial cell either because of the stickiness of the "capsule" or because the desmosomal areas of the exfoliated cells act as binding sites (Collan and Sainio 1970). Since the epithelial cells and their attached bacteria are subsequently swallowed, this mechanism is believed to protect the integrity of the oral mucosa since any micro-organism which starts to colonize its surface is quickly removed. A similar mechanism involving the superficial cells of the crevicular epithelium probably operates in the clearance of micro-organisms from the normal gingival crevice.

One can theorise that in an environment such as the mouth where the mucosal surface is exposed to a constant flow of saliva, bacteria must either adhere to the surface of teeth, dentures, or epithelial cells, or else multiply at a rate which exceeds the dilution rate caused by saliva, if they are to colonize this environment. Microbial growth in the mouth appears to occur at a slow overall rate (Gibbons 1964), and it is likely this rate is slower than the dilution rate resulting from the flow of saliva. In these circumstances the ability of micro-organisms to adhere to an exposed surface, or become mechanically entrapped in a protected niche, would appear essential for successful colonization.

1.3.3 Leucocytes

There is a close relationship between the crevicular exudate and salivary polymorphonuclear leucocytes, since it is widely believed that the main source of salivary leucocytes is via the gingival crevice.

This view is supported by Wright (1961, 1962) who reported that the concentration of leucocytes was very low in the mouths of both young children and edentulous adults. Klinkhamer (1968) believes that oral polymorphonuclear leucocytes are present in two forms, the orogranulocyte and the salivary corpuscle. The orogranulocyte is actively phagocytic and is covered with a layer of isotonic mucus derived from the intrinsic mucous salivary glands. The salivary corpuscle is non-phagocytic and moribund due to exposure to the hypotonic mixed saliva. However the length of time the leucocytes remain active in vivo before becoming inactivated by the serous secretions is not known.

Wright and Jenkins (1953) found that the saliva of caries free individuals contained four times as many leucocytes as caries active individuals. However Friedman and Tonzetich (1968) in a similar study could find no such relationship. Finally Schiött and Löe (1970) have shown that the salivary leucocyte count varies not only from person to person but also varies in a diurnal fashion in any individual person. These findings cast considerable doubt on the significance of reports dealing with the relationship of salivary leucocyte counts and dental caries and periodontal disease.

Attström (1971) has reviewed the role of polymorphonuclear leucocytes in the pathogenesis of periodontal disease. Neutrophils are essential for the defence of the individual against micro-organisms and it is known that in neutropenic patients oral ulceration may develop (Gates 1969). In experimentally induced inflammatory processes intense neutrophil accumulations cause tissue destruction Weissmann (1967); but in leucopenic animals neutrophil accumulation is decreased and tissue destruction is less, when compared to non-neutropenic animals. The tissue damage has been attributed to the release of lysosomal enzymes from disintegrating neutrophils. In periodontitis neutrophils accumulate in the gingival crevice and it is thought that these cells may damage the periodontium through release of their lysosomal enzymes.

1.3.4 Temperature

The temperature of the mouth is about 37°C and probably does not vary to any extent although the temperature of the most anterior part of the mouth, i.e. the labial mucosa is likely to be 2-3°C below this figure. It is known that many pathogenic fungi of man in particular the fungi which commonly infect the skin, are inhibited when grown at 37°C. Therefore the fact that fungal infections other than those caused by Candida species are rare in the mouth may be related in part to the constant 37°C environment of the oral mucosa having an inhibitory effect on fungal growth.

1.4 SALIVA

1.4.1 Composition

Saliva is derived from the secretions of three pairs of large glands, the parotid, submandibular and sublingual glands and numerous smaller glands located in the labial, buccal retromolar, glossopalatine, palatine, tonsillar and lingual areas of the oral submucosa (Orban 1972). Saliva flows continuously during waking hours at a rate of 0.3 ml/min (Becks and Wainwright 1943). On leaving the salivary gland ducts, the secretions are sterile, but on entering the mouth they are modified by the addition of desquamated epithelial cells, small quantities of gingival exudate, polymorphonuclear leucocytes from the gingival tissues, and the oral microbial flora. This mixed secretion is termed mixed or whole saliva, to which the submandibular glands contribute from 37-62%, the parotid glands 21-62% (Kerr 1961) while the sublingual glands contribute 3-5% (Schneyer and Levin 1955 a and b) and the minor glands supply about 6-8% (Dawes and Wood 1973a).

Saliva consists of a complex mixture of inorganic and organic substances. The main electrolytes in human saliva are sodium, potassium, calcium, chloride, bicarbonate, inorganic phosphate and thiocyanate with small amounts of fluoride, iodide and magnesium. The composition of these electrolytes is different for each set of salivary glands and alters with variations in flow rate (Burgen and Emmelin 1961; Schneyer and Schneyer 1967; Dawes 1969).

The main organic constituents of saliva consist of salivary mucoids, amylase, albumen, lysozyme, gamma-globulins and urea with smaller amounts of blood group substances, vitamins, amino acids, ammonia, glucose, lactate, citrate, kallikrein, various enzymes and factors concerned in blood coagulation. In addition, mixed saliva contains variable amounts of organic and inorganic substances derived from gingival exudate, degenerating leukocytes and epithelial cells and the metabolic products of the very complex and varied oral microbial flora. These very variable additions to salivary secretions affect the detailed analysis of salivary components (Ellison 1967). Detailed tables of the constituents of saliva are given by Mason and Chisholm (1975) and Ferguson (1975).

1.4.2 Mucosubstances

Salivary mucosubstances are responsible for lubricating, physically protecting, waterproofing and mechanically cleansing the oral mucosa. Mucosubstances are complex molecules consisting of protein and polysaccharide components linked together by covalent chemical bonds. The protein component is always a single chain forming, as it were, a central backbone for the molecule. Polysaccharide side-chains are attached to the protein backbone and the number and nature of these determine to a great extent the properties of the molecules. Mucosubstances can be divided into glycoproteins and proteoglycans (mucopolysaccharides) on the basis of structural characteristics (Barrett 1971).

Salivary mucins are basically glycoprotein in nature and although the carbohydrate side-chains comprise quite a large part of the molecule, they show some properties normally associated with polysaccharides. It is the properties of this group of glycoproteins which give saliva its lubricating properties. Salivary mucus coats foodstuffs with a lubricant layer which assists chewing and swallowing. Similarly the surfaces of the tongue, oral mucosa and teeth have a lubricant coating in order that the complex interactions of these tissues involved in the production of speech can occur. The precise nature and composition of this continuous mucous coat or sheath as it is sometimes termed, is unknown. The mucus in direct contact with the

superficial epithelial cells of the oral mucosa is most likely derived from the secretions of the minor salivary glands, whose ducts open onto the mucosal surface. Until recently little was known about the precise biochemical composition of minor gland secretions, but Dawes and Wood (1973b) have shown that the main anion in the labial minor mucous gland secretions of humans is chloride, and that no amylase and very little bicarbonate or phosphate is present in these secretions. In addition the human lip mucous gland secretions have a very low buffering capacity compared to that of stimulated secretions from the major salivary glands. Hensten-Pettersen (1975) has demonstrated a high concentration of virus-haemagglutination inhibition activity and blood group substances in both palatine and labial minor gland secretions. Adams (1973) in an investigation of the relationship of saliva and the surface of the oral mucosa described the development and appearance of a "fuzzy" coat on the outermost surface of oral epithelial cells. This layer contained mainly acidic mucopolysaccharide, and appeared to consist of two components; the first and innermost component being derived from microgranules within the cell, and the outer component from salivary mucopolysaccharides and glycoproteins. It has also been shown that the survival rate of Hela cells which are subjected to changes in osmotic pressure is enhanced if the cells are protected by a "coat" of mixed saliva (Adams 1973).

1.4.3 Mechanical Washing Action

The salivary layer coating the oral mucosa is not stagnant but is constantly renewed by secretions from both minor and major salivary glands. Bloomfield (1921, 1922) has shown that salivary mucus takes a direct and relatively constant course along specific routes towards the oropharynx and is finally swallowed. Micro-organisms and foreign particles are "trapped" in the mucus and eventually destroyed by the gastric juice. This mechanical washing action is probably an important factor in limiting the microbial population of the mouth and preventing primary infection of the oral mucosa. In addition, it is probable that the layer of salivary mucus protects the underlying mucosa from the harmful effects of noxious chemical, microbial toxins and minor trauma.

1.4.4 Salivary Glycoprotein

It has been found for some time that a thin protein layer covers the surface of enamel and that with time the surface layer of enamel gradually becomes impregnated with organic material. This protein layer has been called the acquired enamel pellicle (Dawes et al 1963). There is evidence to support the theory that the pellicle is derived from specific salivary glycoproteins which are absorbed onto the enamel surface (Armstrong 1971; Mayhall 1970). Bibby (1971) has stated that the acquisition of organic material is believed to modify the form of early enamel caries and to contribute to the increased caries resistence and pigmentation of old teeth. Thus salivary glycoprotein would appear to have a protective function. On the other hand a high molecular weight glycoprotein has been isolated from mixed saliva which causes certain oral bacteria to selectively adhere to the tooth surface as well as causing mutual adhesion of bacteria in developing dental plaque (Hay et al 1971). In addition to having a protective function, salivary glycoprotein may therefore have a detrimental effect on the host tissues by promoting plaque formation thereby allowing large numbers of oral commensal bacteria to proliferate in close contact with the host tissues.

1.4.5 Crevicular Exudate

A number of views have been put forward regarding the occurrence and nature of the crevicular exudate. Löe and Holm-Pedersen (1965) reported that healthy gingivae did not exhibit a flow of fluid, but that exudation commenced with gingival inflammation and was proportional to the severity of the inflammation. These results were contrary to those of Bjorn et al (1965) who described small but definite amounts of fluid in gingival areas considered to be free of inflammation. These contradictory results can be explained by differences in the methods used for sampling and demonstrating the exudate and also differences as regards the clinical criteria used to designate healthy gingivae.

The composition of crevicular fluid is characteristic of an inflammatory exudate as judged by the sodium potassium ratio (Krasse and Egelberg 1962) the plasma protein concentration (Brandtzaeg 1965)

and lysozyme content (Brandtzaeg and Mann 1964). The other main constituents of crevicular exudate are, acid phosphatase (Sueda et al 1967), alkaline phosphatase (Ishikawa and Cimasoni 1970), beta glucuronidase (Bang et al 1970), a fibrinolytic enzyme system (Gustaffson and Nilsson 1961) and urea (Golub et al 1971).

Green and Kass (1970) have demonstrated antibacterial activity in the gingival sulci of mandibular rabbit incisors, using radio-labelled bacteria. Two mechanisms were described, one which killed the bacteria in situ and another which mechanically removed the bacteria due apparently to the rinsing action of the crevicular exudate. The various protective mechanisms which are involved in the gingival crevice area have been reviewed by Brandtzaeg (1966). Although the amounts and importance of this exudate are uncertain, the evidence suggests that it exerts a protective influence in the crevice and it is possible that it contributes to the antibacterial functions of saliva.

1.4.6 Hydrogen ion Concentration and Buffering Capacity

The pH of mixed saliva varies widely in any one individual; the normal range is 5.6 to 7.6 (Brawley 1935) with an average value of 6.7. Many factors affect the pH and buffering capacity of saliva the more important factors being salivary flow rate and the duration of stimulation (Dawes 1970). Although bicarbonate is the most important buffer in saliva, phosphate probably plays a small part (Lilienthal 1955). While the pH range for growth of many micro-organisms is relatively wide, the pH necessary for maximal growth is usually within narrow limits. It is likely that when a mixture of micro-organisms are competing for limited nutritional factors, the pH of the environment may have a selective action on the survival and growth of particular species. Generally a low pH tends to favour the survival and growth of aciduric bacteria and a high pH tends to favour the survival and growth of proteolytic bacteria. An example of the effect of salivary pH on the oral flora is the fact that the carrier rate of Candida albicans in healthy young adults with a salivary pH of 5.0-5.5 was 90% compared with a carrier rate of 56% in subjects with a pH of 6.5-7.0 (Young et al 1951).

1.4.7 Oxygen Tension

Depending on their oxygen requirements bacteria can be divided into a number of groups; those requiring free oxygen are obligatory aerobes; those that can live and multiply with or without free oxygen are called facultative anarobes; whilst those which grow only in the absence of free oxygen, i.e. under conditions of a high reducing intensity or low oxidation-reduction potential (O/R) are called obligatory anaerobes.

Mixed saliva possesses reducing properties as a result of the presence of reducing substances in salivary secretions and as a result of biochemical reactions of oral bacteria (Jenkins 1966 a). It is known that the oxygen tension (Eh) of various parts of the mouth is very low e.g. the maxillary buccal folds 0.4%, the mandibular buccal fold 0.3%, and the posterior part of the tongue 12.4%. It is also likely that even lower oxygen tensions are present deep in the crypts of the tongue papillae and in the tonsillar area (Eskow and Loesche 1971). A low O/R potential is associated with periodontal disease, (Kenney and Ash 1969) the mean O/R of periodontal pockets being -47.6 mV while healthy gingival sulci in the same individuals and also healthy controls gave a mean reading of +74 mV.

Since the general environment of the mouth tends to have a low oxygen tension and moderate reducing properties, strict aerobic bacteria and those micro-organisms which grow more profusely in the presence of oxygen than in its absence will be at a severe disadvantage as far as colonization of the mouth is concerned. This may explain why Pseudomonas aeruginosa and various Bacillus species which are strict aerobes and present widely in nature are rarely isolated from the mouth.

1.4.8 Lysozyme

Salivary lysozyme was first described by Fleming (1922) and acts by breaking the 1-4 link between N-acetyl muramic acid and N-acetyl glucosamine which are the two main mucopeptides of the bacterial cell wall (Chipman and Sharon 1969). Salivary lysozyme enters the mouth from a number of different sources; the major and the minor salivary

glands (Hoerman et al 1956; Hensten-Pettersen 1975), the tissue exudate which flows from the gingival crevice (Brandtzaeg and Mann 1964), and from the salivary leucocytes. Since lysozymes from different sources have qualitatively the same biological activity but different primary structures and specific activities (Jolles 1967), it is not surprising that a mucopolysaccharide present in the submandibular-sublingual secretions can selectively inhibit the action of parotid lysozyme (Hoerman et al 1956; Simmons 1952). This factor has no effect on lysozyme derived from the submandibular-sublingual secretions themselves.

There are conflicting reports as regards a relationship between dental disease and salivary lysozyme concentration. Some workers hold that there is no such relationship due to the broad overlap in values between the test and the normal control subjects. Other workers hold that there is an inverse relationship (Burnett and Scherp 1968a). To some extent these contradictory results can be explained by the fact that there are a variety of techniques available to assay lysozyme. These techniques have differing degrees of accuracy and specificity. Also many workers have failed to take into account the heterogeneous nature of salivary lysozyme. The effect of lysozyme on oral commensal bacteria was examined by Gibbons et al (1966). They found that a high concentration of lysozyme had no inhibitory effect on the growth of many oral bacteria. Coleman et al (1971) have described lysis of cariogenic and non-cariogenic streptococci with lysozyme but only if sodium lauryl sulphate was present.

It is interesting to note, however, that lysozyme combined with complement and colostral IgA, was able to lyse E. coli (Adinolfi et al 1966). There is doubt whether this system could function in the mouth due to the anti-complementary nature of saliva, but it is possible that similar interactions may have a protective function in the gingival crevice. Therefore on the evidence available the role of lysozyme in dental and oral disease is in considerable doubt.

1.4.9 Thiocyanate Dependent Factors

Dogon and Amdur (1970) have suggested that two thiocyanate-dependent antibacterial systems are present in human parotid saliva. The first system consists of thiocyanate, and an unidentified salivary protein component which functions under aerobic and anaerobic conditions (Dogon et al 1962; Dogon and Amdur 1965). The thiocyanate reacts with an acceptor within the bacterial cytoplasm under the influence of an enzyme in the salivary secretions. The antimicrobial factor does not cause immediate lysis of the bacteria and is effective only in actively growing organisms apparently killing them by inhibiting some essential growth factor (Zeldow 1961).

The second system consists of a peroxidative enzyme, probably lactoperoxidase, thiocyanate and hydrogen peroxide and functions only in aerobic conditions (Klebanoff and Luebke 1965). Lactoperoxidase is bound to the bacterium and in the presence of hydrogen peroxide, the thiocyanate is oxidised to dicyanoxide (Oram and Reiter 1968). However Hogg and Jago (1970) have discounted Oram's findings and suggested that the active end product was either cyanosulphurous acid or cyanosulphuric acid.

Factors which influence the assay of these antibacterials have been investigated by Bartles et al (1969). The effect of smoking on the antibacterial activity of mixed saliva has been reported by Courant (1967). He noted that the saliva of smokers contained a higher concentration of thiocyanate and possessed an increased antibacterial activity when compared with the saliva of non-smokers.

It is important to note that much of the experimental work into the nature of these factors has been carried out with pure parotid secretions and not with "mixed saliva". Furthermore, the activity of Klebanoff's factor is inhibited by catalase, whereas Dogon's factor is not. Since catalase is produced by certain oral micro-organisms and by the cellular activities of the host, it is possible that Klebanoff's system is not active in vivo. The antibacterial effect of these factors on a limited number of lactobacillus and Streptococcus species has been fully demonstrated, but the effect on other commensal oral

bacteria is unknown. There is evidence that Escherichia coli and perhaps other coliform bacilli are inhibited by thiocyanate dependent factors (Klebanoff et al 1966).

1.4.10 Green's Factor

Green described a bacteriolytic factor in the saliva of caries free individuals (Green 1958, 1959; Green and Wilson 1963). The saliva from caries active individuals either contained a very low concentration of the factor or was absent. The factor which Green believed was a gamma-globulin appeared to be absorbed onto the surfaces of susceptible bacteria which subsequently lysed (Green 1966). The factor was most effective against lactobacilli and a few streptococci. Since Geddes (1972) in a reassessment of Green's work was unable to demonstrate the factor in caries free parotid saliva, the importance of Green's factor is in considerable doubt.

1.4.11 Lactoferrin

Lactoferrin is an iron binding protein which has been described in tears, saliva, nasal, bronchial and gastrointestinal secretions, seminal fluid, cervical mucus, endometrium and urine (Masson et al 1966 a; Masson et al 1968 a, b). It is likely that lactoferrin is secreted locally, since it is present in the epithelial cells of human bronchial glands (Masson et al 1966b) and submaxillary glands (Masson and Heremans 1966). Lactoferrin has also been found in the cytoplasmic granules of human polymorphonuclear leucocytes (Baggiolini et al 1970; Leffel and Spitznagel 1972).

It has been suggested that the iron binding properties of lactoferrin are of importance in protecting mucosal surfaces against infection. The precise way in which lactoferrin accomplishes such a protective role is unknown. However bacteria have an absolute requirement for iron and many micro-organisms produce iron binding compounds in iron deficient media (Sussman 1974). Both host and parasite have evolved elaborate iron transport systems based on iron binding proteins and chelating agents, and Glynn (1972) has suggested that the ability of a micro-organism to acquire iron from the host and

bacteria is unknown. There is evidence that Escherichia coli and perhaps other coliform bacilli are inhibited by thiocyanate dependent factors (Klebanoff et al 1966).

1.4.10 Green's Factor

Green described a bacteriolytic factor in the saliva of caries free individuals (Green 1958, 1959; Green and Wilson 1963). The saliva from caries active individuals either contained a very low concentration of the factor or was absent. The factor which Green believed was a gamma-globulin appeared to be absorbed onto the surfaces of susceptible bacteria which subsequently lysed (Green 1966). The factor was most effective against lactobacilli and a few streptococci. Since Geddes (1972) in a reassessment of Green's work was unable to demonstrate the factor in caries free parotid saliva, the importance of Green's factor is in considerable doubt.

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the ability of the host to deny it to the microbe constitutes a battle of chelating agents. There is at the moment no experimental results dealing with the antibacterial activity of lactoferrin on the commensal oral flora. It is known however that lactoferrin either by itself or in conjunction with specific antibody can inhibit the growth of or destroy various micro-organisms; Staphylococcus albus and Pseudomonas aeruginosa (Masson et al 1966 b), Escherichia coli (Bullen et al 1972), and Bacillus subtilis (Oram and Reiter 1968). It is interesting that lactoferrin and lysozyme are usually found together in mucosal and glandular secretions since it is known that chelating agents potentiate the antibacterial activity of lysozyme (Repaske 1956).

1.4.12 Fluoride

The action of fluoride in reducing dental caries is beyond dispute but its mode of action is controversial (Jenkins 1967). It is well established that to obtain the full effect of fluoride on caries reduction, it must be present in drinking water during the calcification of enamel. It is believed that fluoride is incorporated into the apatite crystals of enamel during calcification and by converting a proportion of the crystals to fluorapatite lowers the solubility of the enamel in acid. There is, however, also evidence that some benefit can be obtained from post-eruptive exposure to fluoride alone. The fluoride content in saliva is 0.1 - 0.2 parts per million and this is mainly in the ionic form (Jenkins 1966b). It is still uncertain whether these low concentrations in saliva can influence the tooth directly. However Jenkins et al (1969) and Edgar et al (1970) studied dental plaque in a number of subjects before and after fluoridation of a public water supply. They found that after fluoridation the ionic fluoride concentration of dental plaque was increased. They also found that after incubating this same plaque in a sucrose solution for 15 minutes the fall in plaque pH was decreased when compared with the fall measured before fluoridation. These results support the view that the fluoride present in plaque is mainly bound to bacteria and that it is the action of fluoride on the plaque bacteria which reduced subsequent acid production (Van Houte et al 1969).

1.4.13 Immunoglobulins

The presence of antibody in saliva was noted in 1892 by Sanarelli, and the finding of antibodies in other secretions led to the concept of "local immunity". Thereafter several workers reported that local antibody-like activity existed in saliva and this activity could be protective (Kraus and Kono 1963). The finding of gamma-globulin in human parotid and whole saliva (Ellison et al 1960; Mandel and Ellison 1961; Kraus and Sirisinha 1962) suggested that this activity could indeed be antibody. Tomasi and his co-workers (1965) demonstrated that IgA found in human parotid saliva was an "11S" as opposed to the IgA in serum which was predominantly "7S". The main difference between the two types of IgA was shown to be due to the possession of an unique glycoprotein chain, the so called secretory component, by 11S S-IgA, (Tomasi and Calvanico 1968).

Whole saliva contains secretory IgA (S-IgA) as the major immunoglobulin with considerably less IgG and IgM. All the secretory IgA is derived from the salivary glands, while the IgG and IgM is derived mainly from the gingival exudate with a small component particularly IgM coming from the salivary secretions (Brandtzaeg 1972; Holmberg and Killander 1971). It appears that S-IgA is quantitatively the predominant immunoglobulin in parotid, submandibular and whole saliva, and on this basis alone, it is reasonable to assume that S-IgA is the most likely antibody type to affect the ecology of the oral cavity. It is of interest that S-IgA decreases with increase in flow rate and it has been suggested that the high concentration of S-IgA occurring in saliva secreted when there is minimal stimulation, e.g. during sleep, confers a protective antibacterial activity on the oral tissues (Mandel and Khurana 1969).

Several investigators have reported a correlation between the level of IgA in saliva and the dental caries state of the individual; Lehner et al (1967) reported high levels of IgA in whole saliva of caries free individuals versus caries active patients, while Shklair et al (1969) found no difference in parotid or whole salivary immunoglobulin concentration in either group of individuals, and Zengo et al (1971) found IgA elevated in submandibular but not the

parotid secretions of the caries free compared to the caries active patients. Studies have also been carried out on the effects of antibody on experimental dental disease in animals (Fitzgerald and Keyes 1962; Bowen 1969; Gaffer et al 1971; and Tanzer et al 1973). The results of these studies suggest that salivary antibody can effect both the development of experimental dental caries and produce inhibitory effects on the micro-organisms causing the disease. However, due to the relatively small number of animals involved and the fact that only one investigator used monkeys (Bowen 1969), the other workers using either rats or hamsters, further investigations are necessary before the role of the immune system in human dental disease can be accurately assessed.

A number of functions have been suggested for S-IgA in saliva. Antibacterial activity due to specific immunoglobulins have been demonstrated in saliva (Kraus and Kono 1963; Sirisinha 1970; Taubman and Smith 1973), and Brandtzaeg et al (1968) found that bacteria removed from the mouth had IgA absorbed to them. However the mechanisms by which S-IgA eliminates bacteria are not understood, although they seem to be different from the antibacterial activity of other immunoglobulin classes. IgA cannot fix complement (Heremans et al 1963; Ishizaka et al 1965), and therefore complement dependent bacteriolysis does not appear to be involved in the interaction of salivary antibody and micro-organisms. Furthermore, various components of the complement system are lacking in parotid and submandibular saliva (Brandtzaeg 1966; Genco and Taubman 1973).

The concept of "blocking antibody" was introduced by Good and Rodey (1970), who suggested that a basic function of S-IgA in the gastro-intestinal tract was to interfere with the absorption of ingested antigens through the gut mucosa. Since IgA coats the surface of the oral mucosa (Heremans et al 1966), secretory antibody could block the uptake of bacterial products and antigens in a similar manner thereby preventing the penetration of antigenic material into the oral mucosa. Another way in which this mechanism might assist in the protection of the mucous membrane is by inhibiting microbial adherence, either to the surface cells of the oral and dental tissues,

or to one another. Williams and Gibbons (1972) specifically inhibited the adherence of certain strains of Streptococcus mitis and Streptococcus salivarius to buccal epithelial cells with natural S-IgA antibody derived from saliva, while Taubman (1973) reduced the colonization of teeth by Streptococcus mutans in rats, by immunization procedures which stimulated salivary IgA antibody production. Viral replication on the mucosal surface can be prevented by IgA antibody (Ogra et al 1968). There is also evidence that specific secretory IgA may have a blocking effect on certain vital functions of microbial metabolism (Evans and Genco 1973).

The finding of naturally occurring specific S-IgA antibodies which inhibit epithelial adherence of members of the oral flora may have important implications in the pathogenesis of infection and the interaction of pathogens and commensal bacteria. It is generally assumed that once small numbers of a pathogen are introduced onto a mucosal surface, the pathogen somehow overgrows the indigenous flora and achieves numerical predominance. There is however little evidence to support this assumption. If we look at this situation with regard to adherence, a number of interesting possibilities become evident (Gibbons 1974). If a pathogen enters the mucosal environment of a non-immune individual, then unlike the commensal bacteria whose adherence is regulated by salivary antibodies, the pathogen could adhere and colonize unimpeded by secretory antibodies. This would enable small numbers of a pathogen to proliferate on the mucosal surface in a relatively short period of time. Once the pathogen has colonized the site in large numbers, it would stimulate an antibody response. The response is likely to be greater than that to most indigenous bacteria since it is probable that the pathogen will have caused tissue damage by means of toxins or lytic enzymes. Consequently the adherence of the pathogen would become inhibited to a greater extent than most of the indigenous flora and the pathogen would become selectively inhibited. An immune state would now exist whereby the individual would be protected against re-infection by the same serotype.

1.5 ORAL COMMENSAL MICROFLORA

1.5.1 Composition

It was established many years ago that the oral flora varies from one site to another, and there is little doubt that different areas of the mouth support relatively specific microbial populations (Socransky and Manganiello 1971). The main "ecological" niches of the human mouth are, the tongue, oral mucosa, tonsillar region, dental plaque and the gingival crevice area, while the organisms in saliva are regarded as a transient population derived mainly from the tongue (Krasse 1954; Gibbons et al 1964). Although a comprehensive survey of the microbiology of the oral cavity was carried out by Morris (1953 a, b, 1954 a, b, c, d) and recent reviews have been contributed by Burnett and Scherp (1968b), Socransky (1970), Socransky and Manganiello (1971), Davies (1972), Nolte (1973) and Hardie and Bowden (1973), there is virtually no information concerning the oral flora of edentulous individuals wearing complete upper and lower dentures. The majority of investigations of the oral flora have been concerned with saliva or dental plaque with relatively few studies investigating the flora of the soft tissues of the mouth.

1.5.2 Microbial Interactions

While the taxonomy and nutritional activity of the oral microbial flora have been actively studied in recent years, little detailed work has been carried out to elucidate the role of the oral flora in protecting the oral and dental tissues from disease. The concept of microbial antagonism and the use of the antibacterial properties of human commensal bacteria was conceived in the early days of microbiology. In 1877 Pasteur and Joubert (Florey 1946) noted that the growth of Bacillus anthracis in tissue was inhibited by "common" micro-organisms and they suggested that the latter might be used to treat anthrax. Later in 1885 Cantani (Florey 1946) tried to destroy Mycobacterium tuberculosis in human lungs by the inoculation of a mixture of non-pathogens; and Ennerich (Florey 1946) who in 1887 tried to prevent the spread of anthrax by deliberately infecting patients with Beta-haemolytic streptococci. With these studies in

mind Florey (1946) tried to harness microbial antagonisms for therapeutic use, without success.

Microbial antagonisms may be defined as the inhibition of growth and destruction of one microbial species by a member of the same or of an unrelated species. There are a number of possible mechanisms by which successful antagonists may produce their effect; they may compete successfully with pathogens for essential growth factors, or they may create an inhibitory environment by altering pH or redox potential, or they may elaborate a variety of antibiotic substances (Sanders 1969).

Experimental studies have shown that certain members of the oral microbial flora possess antagonistic activity directed against potential pathogens and against other members of the oral commensal flora. Indigenous bacteria of the mouth and upper respiratory tract have been shown to have antagonistic activity against the following pathogens: Staphylococcus aureus (Myers 1959; Boris 1968; Sanders 1969), Lancefield Group A Streptococci (Rosebury et al 1956; Bartles et al 1960; Sanders 1969), Neisseria meningitidis (Rosebury 1962), Coliforms (Sprunt and Redman 1968; Sprunt et al 1971), Corynebacterium diphtheriae (Thomson and Shibuya 1946; Myers 1959), and Streptococcus pneumoniae (Johanson et al 1970).

It has also been demonstrated that microbial antagonisms are active in controlling the commensal microbial flora of the human mouth by means of bacteriocines (Green and Dodd 1956; Kelstrup and Gibbons 1969, Kelstrup et al 1970; Donoghue 1972; Rogers 1972; Schlegel and Slade 1972). Microbial inhibition of a broader spectrum due to the production of other antibacterial substances have been reported by Parker (1970), and Holmberg and Hallander (1972). In subsequent investigations Holmberg and Hallander (1973) attributed the bactericidal activity of Streptococcus sanguis due to the production of hydrogen peroxide.

It appears therefore that certain oral commensal bacteria especially alpha-haemolytic streptococci, play an important role in

controlling the composition of the oral microflora, and preventing the colonization of the mouth and throat by potentially pathogenic bacteria.

1.6 AIM AND DESIGN OF THE PRESENT STUDY

From the review of the literature dealing with the environment and the defence mechanisms of the mouth, it is clear that the factors involved in preserving the oral and dental tissues in a state of health, are many in number and complex in action. It is also clear that few of the factors have been shown to have any direct effect in vivo in protecting the oral and dental tissues from infection.

The three main ecological components of the mouth are the oral tissues, saliva and the oral microbial flora, and it is reasonable to expect that if one of these components is greatly altered, for example the virtual cessation of salivary secretion, then the ecology of the mouth would be disturbed, the defence mechanisms would become ineffective, and a disease state supervene. A severe reduction in salivary secretion occurs in patients with Sjögren's syndrome, and although the changes which occur in the salivary glands and oral dental tissues have been widely described, little is known concerning the probable changes which occur in the commensal oral microflora. This thesis presents the results of a number of microbiological studies carried out on a group of patients with Sjögren's syndrome, to assess the effect of reduced salivary secretion on the composition of the oral microbial flora, and to investigate the effect of such changes on the oral flora, the host tissues and the defence mechanisms of the mouth.

The results from a routine microbiological investigation of a few patients with Sjögren's syndrome suggested that large numbers of micro-organisms not usually present in the oral microflora of normal individuals, were present in the microflora of Sjögren's patients. In order that the significance of these results could be assessed, a prospective microbiology study of Sjögren's patients and healthy controls was carried out (Chapter 2). The results showed that the Sjögren's group had significantly greater numbers of Candida albicans,

and Staphylococcus aureus in their mouths compared to the control patients.

A second microbiology study was carried out therefore to assess the composition of the commensal oral flora in patients with Sjögren's syndrome and in addition to note the salivary pH values and clinical changes in the oral mucosa (Chapter 4). Since the semi-selective culture medium used to isolate Veillonella species from oral samples in Chapter 4 was developed by myself and has not been previously described, details of the composition, method of preparation and use are given in Chapter 3.

Marked changes were found in the commensal flora, in the salivary pH and oral mucosa in patients with Sjögren's syndrome (Chapter 4). From the results of Chapters 2 and 4 it was clear that a breakdown had occurred in the factors protecting the mouth from invasion by non-commensal bacteria, and also in the factors controlling the composition of the oral microbial flora. Since the main factors concerned with protecting the mouth from invasion by non-commensal bacteria are the mechanical cleansing action of saliva, the antibacterial factors present in saliva and antagonistic substances produced by members of the commensal oral flora, it was decided to carry out a series of experiments to assess the importance of these factors with regard to the microbiological changes found in patients with Sjögren's syndrome.

In Chapter 5, the antimicrobial activity of mixed saliva was assessed, using Escherichia coli, Staphylococcus aureus and Candida albicans as the indicator organisms. Since previous methods had a number of disadvantages, and tended to give rather variable results, a new technique was developed which enabled the antimicrobial activity of saliva to be tested in conditions very close to the in vivo situation, with consistent results. The results of this investigation suggested that the micro-organisms present in mixed saliva are of more importance than the intrinsic salivary antimicrobial factors in protecting the mouth from invasion with exogenous micro-organisms.

The microbial antagonistic activity of a number of type strains of oral commensals was assessed using Escherichia coli, Staphylococcus aureus and Candida albicans as the indicator organisms (Chapter 6). The laboratory technique used to assess the microbial antagonistic activity was similar to the technique used to assess the anti-microbial activity of mixed saliva in Chapter 5. Although this technique has not been used before in assessing microbial antagonisms, it has many advantages over the previously used methods. The results of this study enabled some conclusions to be reached concerning the importance of the loss of certain species of oral commensal bacteria on the defence mechanism of the mouth.

Since one of the conclusions of Chapter 6 was that alpha-haemolytic streptococci were of importance in protecting the mouth from invasion and colonization by exogenous micro-organisms, a study was carried out to assess the antagonistic activity of alpha-haemolytic streptococci in the mouth of Sjögren's patients compared to that of healthy controls (Chapter 7). The technique used was that described in Chapter 6. The results of this study suggested that loss of certain strains of alpha-haemolytic streptococci were of importance in the appearance of large numbers of exogenous micro-organisms in the mouth of Sjögren's patients.

The effect of the experimental findings on the clinical management of the oral manifestations of Sjögren's Syndrome were discussed in Chapter 8.

THE ORAL FLORA IN SJÖGREN'S SYNDROME

2.1 INTRODUCTION

Sjögren's syndrome, first described in 1933 (Sjögren 1933) consists of chronic inflammation of the lacrimal and salivary glands leading to dryness of the eye (keratoconjunctivitis sicca) and dryness of the mouth (xerostomia), and in a proportion of cases lacrimal and or salivary gland enlargement may also be present. In 50-60% of patients the disease may be associated with a connective tissue disorder, usually rheumatoid arthritis but occasionally also with polymyositis, polyarteritis nodosa, progressive systemic sclerosis (scleroderma) and systemic lupus erythematosis. The term "sicca complex" is applied to these cases of Sjögren's syndrome not associated with rheumatoid arthritis or other connective tissue disease. Sjögren's syndrome occurs predominantly in middle aged females but has no direct relationship to the menopause.

Common oral symptoms are dryness of the lips and the oral mucosa with the secretion of thick ropy saliva. The signs and symptoms of Sjögren's syndrome vary in severity but may be sufficiently severe to interfere with mastication and produce angular cheilitis and atrophy of the oral mucosa. An increase in the incidence of dental caries following the development of xerostomia due to Sjögren's syndrome has been reported by Bloch et al (1965).

It is generally accepted that the composition of the commensal oral flora is controlled by complex interactions among the oral micro-organisms themselves (Bjornesjo 1950), the host tissues, and the mechanical washing action and antimicrobial activity of saliva (Bibby et al 1938; Van Kestern et al 1942; Kerr and Wedderburn 1958; MacFarlane and Mason 1972). If one of these factors is greatly altered, for example virtual cessation of salivary secretion, changes might be expected to occur in the oral flora. This study was carried out to discover whether a difference existed in the oral flora of patients with xerostomia due to Sjögren's syndrome compared with normal healthy controls.

2.2 MATERIALS AND METHODS

2.2.1 Patients

The study comprised 20 patients, 10 diagnosed cases of Sjögren's syndrome and 10 healthy controls. The test and control groups were matched for age, sex, denture and dental status; the results are shown in Table 2.1. The diagnosis of Sjögren's syndrome was based on the criteria described by Bloch et al (1965). Both patients and controls were questioned regarding a history of xerostomia and of the associated symptoms of Sjögren's syndrome. None of the control patients had symptomatic or clinical dry mouth and parotid salivary flow studies were carried out only on the 10 patients with Sjögren's syndrome. The flow studies were performed by the use of a modified Carlson-Crittenden cup, with an outer chamber of diameter 20 mm, and an inner chamber of diameter of 10 mm, (Carlson and Crittenden 1910; Mason et al 1966). Parotid saliva was collected from each patient after stimulation with 10% citric acid. The flow rate results were assessed as normal or abnormal after comparing with the normal range as defined by Chisholm (1970) and Whaley et al (1973). None of the patients or controls gave a recent history of taking antimicrobial agents. The criteria for a diagnosis of candidosis were, clinical evidence of oral inflammation, together with smears from the inflammed area showing yeasts in the "hyphal" form, and a swab yielding a moderate growth of a Candida species.

2.2.2 Microbiology

There are many technical difficulties related to sampling, culturing, identifying and enumerating the oral microbial flora (Burnett and Scherp 1968b). In addition to the technical problems, variations can occur in the oral flora between individuals, and also within the same individual from day to day, due to such factors as diet (Bowen 1970), antibiotics (Handelman and Hawes 1965), and various types of dental treatment (Shklair and Mazzarella 1961). The combination of technical difficulties and environmental variations generally leads to a situation where the best that can be expected is a qualitative assessment with little more than a rough estimate of the proportion in which different micro-organisms are present. Few

workers would claim that their differential counts gave more than an indication of the relative proportions of micro-organisms within a given sample.

In view of the apparently inherent difficulty in accurately enumerating the oral flora, and since there were no previous reports on the microbial flora of Sjögren's patients, I decided to use a relatively crude method of sampling, to cover as wide an area of the oral tissues as possible. The sampling technique which seemed most appropriate was swabbing various areas of the mouth with cotton wool swabs, which had been lightly moistened in sterile peptone water to facilitate the removal of organisms from the dry mucosa of the Sjögren's patients. It was accepted that this relatively crude sampling technique would demonstrate only very marked differences in the oral flora between the Sjögren's and control patients, for it was thought that only gross differences would prove to be statistically significant.

Four separate areas of the mouth were sampled for the micro-biological investigations; the fitting surface of the upper denture or palate, the dorsum of the tongue, the right tonsillar area, and the buccal mucosa in the region of the right parotid duct orifice. Each area was sampled by means of a firmly taken cotton wool swab. The swab was immediately immersed in 1 ml of sterile deionized water, thoroughly agitated and squeezed to transfer the sampled material to the fluid. All the swabs were taken by one operator, and before salivary flow studies were carried out. Within 15 minutes of the time of sampling 0.05 ml of each sample was inoculated onto the following culture plates; 1 blood agar, 1 Sabouraud's, 1 MacConkey's and 1 salt agar plate. The inoculum was spread evenly over the surface of each plate by means of a bent glass rod. All plates were examined and the numbers of yeasts (Sabouraud's agar), Staphylococci (salt agar) and Coliforms (MacConkey's agar) noted. Sample colonies from each of the plates were removed, and identified by the following means. Yeasts by means of germ tube production and sugar fermentation reactions, Staphylococcus aureus by coagulase production and Coliform organisms by the techniques used by Cowan and Steel (1966). The number of

colonies of each organism isolated from the four areas of the mouth were graded as shown in Table 2.2. By adding the grading values for each of the areas of the oral cavity a mouth score (0-12) for each organism was obtained.

2.3 RESULTS

2.3.1 Patients

Table 2.1 shows a comparison of the clinical data of the two groups. The ratio of females to males was 9-1 in both groups; the mean age of the control group was slightly higher than the Sjögren's group. Eight of the Sjögren's patients had dentures and two had natural teeth, where as only one of the control patients had natural teeth. To summarise, both groups were predominantly elderly females with complete dentures. In seven patients no measurable parotid secretion was obtained; three patients had reduced secretion and none of the patients had a flow rate which was within normal limits, Table 2.3.

2.3.2 Microbiology

The individual and total mouth scores for Candida albicans, Staphylococcus aureus and Coliform bacilli in the Sjögren's and control groups are shown in Tables 2.4, 2.5 and 2.6 respectively. All 10 Sjögren's patients had Candida albicans in their mouth; eight also had Staphylococcus aureus present and 3 patients had, in addition, various Coliform bacilli. The total mouth score for the 10 Sjögren's patients was Candida albicans 80, Staphylococcus aureus 74 and Coliform bacilli 21. Although patients with no measurable parotid secretion tended to have a higher mouth score than those with reduced flow rate there were exceptions, e.g. patient A8 with no measurable parotid flow but a mouth score of 3. Four of the control group subjects had yeasts present in their mouths, and one of these had, in addition, a few colonies of Staphylococcus aureus. Two patients had Staphylococcus aureus alone present. None of the control patients had any Coliform bacilli present. The total mouth scores for the 10 control patients was Candida albicans 10, Staphylococcus aureus 9 and Coliform bacilli 0.

Clinical Diagnosis	No. of Patients	Sex		Mean Age (Years)	No. with denture	No. Dentulous
		Male	Female			
Sjögren's syndrome	10	1	9	56	8	2
Control Patients	10	1	9	58	9	1

TABLE 2.1 AGE AND SEX DISTRIBUTION OF PATIENTS WITH SJÖGREN'S SYNDROME AND CONTROL SUBJECTS

Grade	
0	Absent
1	1 - 10
2	11 - 40
3	Over 40

TABLE 2.2 GRADING STANDARDS FOR NUMBER OF COLONIES ISOLATED

Sjögren's Patient	Salivary Flow
A 1	x
A 2	-
A 3	x
A 4	x
A 5	x
A 6	x
A 7	-
A 8	x
A 9	-
A 10	x

Salivary Flow

- Reduced secretion

x No measurable flow

TABLE 2.3 SALIVARY FLOW RATES FOR 10 PATIENTS WITH SJÖGREN'S SYNDROME

Candida Albicans			
Sjögren's		Control	
A 1	10	0	C 1
A 2	12	2	C 2
A 3	9	0	C 3
A 4	11	0	C 4
A 5	7	1	C 5
A 6	4	3	C 6
A 7	8	0	C 7
A 8	3	4	C 8
A 9	5	0	C 9
A 10	11	0	C 10
Total Score	80	10	

Candida Albicans

Sjögren's v Controls

$p = < 0.001$

TABLE 2.4 INDIVIDUAL AND MOUTH SCORES FOR 10 PATIENTS WITH SJÖGREN'S SYNDROME COMPARED WITH 10 CONTROL PATIENTS

Staphylococcus Aureus			
Sjögren's		Control	
A 1	8	0	C 1
A 2	11	0	C 2
A 3	10	0	C 3
A 4	11	0	C 4
A 5	3	1	C 5
A 6	9	0	C 6
A 7	10	5	C 7
A 8	0	0	C 8
A 9	0	3	C 9
A 10	12	0	C 10
Total Score	74	9	

Staphylococcus Aureus

Sjögren's v. Controls

$P = < 0.001$

TABLE 2.5 INDIVIDUAL AND MOUTH SCORES FOR 10 PATIENTS WITH SJÖGREN'S SYNDROME COMPARED WITH 10 CONTROL PATIENTS

Coliform Bacilli			
Sjögren's		Control	
A 1	0	0	C 1
A 2	11	0	C 2
A 3	4	0	C 3
A 4	0	0	C 4
A 5	0	0	C 5
A 6	0	0	C 6
A 7	6	0	C 7
A 8	0	0	C 8
A 9	0	0	C 9
A 10	0	0	C 10
Total Score	21	0	

Coliform Bacilli
Sjögren's v Controls
$P = < 0.5$

TABLE 2.6 INDIVIDUAL AND MOUTH SCORES FOR 10 PATIENTS WITH SJÖGREN'S SYNDROME COMPARED WITH 10 CONTROL PATIENTS

Citric Acid	25 g
Essence of Lemon	40 ml
Glycerol to 2 litres	
Yellow colouriser	

For Use

One teaspoonfull of concentrate to one cup of water.

TABLE 2.7 CONSTITUENTS OF GLYCEROL AND LEMON MOUTHWASH

Since evaluation of the numbers of micro-organisms produced a numerical score for each patient it was felt undesirable to assume that the scores were normally distributed. Therefore it was decided to analyse the data by non-parametric methods, the appropriate test being the Mann and Whitney U test (Siegel 1956). Using this test, the difference in the numbers of Candida albicans and Staphylococcus aureus between the Sjögren's and control groups yielded a value of $P = < 0.001$; the difference in the numbers of Coliform bacilli was not significant. The yeasts isolated from both the Sjögren's and the control groups were identified as Candida albicans. Two of the Coliform bacilli were identified as Escherichia coli and one as Enterobacter liquifaciens.

2.4 DISCUSSION

The results of this preliminary study show that with regard to yeasts and Staphylococcus aureus, there are distinct differences in the oral flora of patients with Sjögren's syndrome compared with normal healthy controls.

The precise reason for the increase in the incidence and number of these micro-organisms is not known. However, from the published work on the antimicrobial activity of saliva, it is known that at least some strains of Escherichia coli are inhibited by the thiocyanate peroxidase antilactobacillus system (Klebanoff et al 1966). Many strains of Staphylococcus aureus are inhibited by saliva (Bibby et al 1938), but the nature of the inhibitory factor is not known. In contrast to E.coli and Staphylococcus aureus, saliva from normal healthy adults appears to have no inhibitory effect on Candida albicans. Lehner (1965) has shown that patients with Candidosis secrete antibodies against the yeast in their saliva. The loss of these inhibitory factors together with the malfunction of the mechanical washing action of saliva, may partially explain our findings.

It is of interest to compare the results of the present study with those of Llory et al (1971) who reported changes in the salivary and dental plaque microbial flora of patients before, during and after their salivary glands had been irradiated in the treatment of

cervico-facial carcinoma. They reported an increase in the number of cariogenic lactic bacteria (Streptococci and Lactobacilli) and a fall in salivary pH to 5.38, some weeks after completion of irradiation. In addition they found small numbers of Staphylococcus aurues and various Coliform bacilli, both before and after irradiation. In a later paper Llory et al (1972) showed that there was an increase in the numbers of yeasts isolated from saliva and dental plaque after irradiation, and these results agree with the present study.

In relation to the number of yeasts present in the Sjögren's group, it is interesting to note that Young et al (1951) investigating the number of yeasts in the mouths of healthy young adults, found that the numbers of yeasts isolated, varied inversely with salivary pH. For example, the few subjects with a salivary pH of 5-5.5 had a 91% carrier rate for yeasts, compared with 52% carrier rate at salivary pH 6.5. It is known that patients with xerostomia due to irradiation for carcinoma have low salivary pH values (Frank et al 1965). However no salivary pH values for patients with Sjögren's syndrome are present in the literature.

Glycerol and lemon mouthwash was used four to five times per day by all the Sjögren's patients to help alleviate their symptoms of xerostomia, and it is interesting to speculate on the effect the mouthwash has on the oral environment, and commensal microflora. The constituents of the mouthwash and the instructions for use are shown in Table 2.7. The pH of the mouthwash diluted for use is very low, 3.8, and due to the glycerol content the mouthwash tends to cling to the surface of the mucosa for some time.

Few of the members of the commensal oral flora can utilize glycerol, but it is known that Staphylococcus aurues, Escherichia coli and Enterobacter liquefaciens ferment glycerol with the production of acid (Bergey 1948; Topley and Wilson 1964; Cowan and Steele 1974). Thus the presence of a relatively high concentration of glycerol in the mouth of the Sjögren's patients is perhaps another explanation for the high incidence of Staphylococcus aurues and the appearance of Coliform bacilli in the oral flora of Sjögren's patients.

There is no direct evidence concerning the utilization of glycerol by Candida albicans, but Rao et al (1960) have shown that one of the main pathways of glucose dissimilation was the Embden-Meyerhof pathway. Rao and his co-workers demonstrated a high activity of glycerol dehydrogenase in Candida albicans and these results suggest an interconversion of dehydroxyacetone phosphate and glycerol, with conversion of the former to glyceraldehyde 3-phosphate. By this means glycerol utilization is linked with the main Embden-Meyerhof glycolytic pathway. There is no precise information concerning the end products of the utilization of glycerol by Candida albicans. Pyruvic acid is the usual end product of the Embden-Meyerhof pathway, but the fate of pyruvate produced can vary tremendously depending on the species of micro-organism involved and on the environment (Dawes and Large 1973).

The majority of the oral streptococci are unable to ferment glycerol, but about 8% of strains of Streptococcus mutans (Colman and Williams 1972) which is regarded as cariogenic in man possesses this activity (Krasse et al 1968; Ikeda et al 1973). In a Sjögren's patient with natural teeth, the continual long term use of glycerol and lemon mouthwash could actively encourage the colonization of the teeth by Streptococcus mutans with resulting extensive carious destruction.

It would appear from these findings that the use of a glycerol and lemon mouthwash may play an active role in the microbial changes noted in the oral flora of Sjögren's patients.

A number of antagonistic mechanisms have been demonstrated by various oral commensal micro-organisms which tend to prevent potentially pathogenic bacteria colonizing the mouth and initiating infection (Chapter 1.5.2). It is reasonable to speculate that if oral commensal bacteria with antagonistic activity were lost from the oral environment then potential pathogens normally denied growth in the mouth, could colonize and subsequently infect the oral tissues. Since no attempt was made in this study to investigate changes in the commensal flora of Sjögren's patients, there is no direct proof either way.

None of the Sjögren's group had active yeast infections at the time of the microbiological investigation. However, an examination of the case histories of the Sjögren's group both before and after the microbiological tests, revealed that 3 patients had no history of infection, 5 had candidosis on one occasion and 2 patients had candidosis on more than one occasion, i.e. 70% of patients in the Sjögren's group had a history of candidosis.

Recently Higgs and Wells (1972) have demonstrated a high incidence of iron deficiency and Lehner (1972) has described cell mediated defects in patients with chronic mucocutaneous candidiosis. Iron deficiency anaemia and other nutritional deficiencies result in dystrophic changes in the oral mucosa, which in turn may predispose these tissues to candida infection. Subsequently due to proliferation of the yeast the antigen load gradually increases leading to the development of the state of immune tolerance. It is interesting to note that patients with rheumatoid arthritis have a high incidence of iron deficiency anaemia (Collins 1935), and that impaired delayed hypersensitivity reactions have been demonstrated by Leventhal et al (1967) in patients with Sjögren's syndrome. Since only 3 of the 10 Sjögren's patients had rheumatoid arthritis, cell mediated defects would appear to be unrelated to candidosis in this study.

2.5 CONCLUSIONS

The clinical implications of these findings is that we should not accept the inflamed dry mucosa seen in Sjögren's syndrome as inevitable due to decreased salivary flow rate; from these results it is just as likely that the inflammation is due to, or associated with, active Candida infection. Thus patients with xerostomia due to Sjögren's syndrome should be kept under regular clinical and microbiological surveillance for recurrence of candidiosis. It is clear that systemic factors influencing host resistance to microbial disease have to be considered as well as local changes in the oral environment when arranging treatment for these patients.

The appearance of numerous yeasts, Staphylococci and in some instances Coliform bacilli in the oral flora of patients with severe

Sjögren's syndrome, suggests that loss of saliva leads to a breakdown in the protective mechanisms which normally prevent these micro-organisms colonizing the mouth in large numbers. Although it is possible to speculate about the reasons for the appearance of these micro-organisms in Sjögren's patients, this study yielded no definitive evidence supporting any one factor. However this investigation suggested a number of pathways for further study.

A NEW SELECTIVE MEDIUM FOR THE ISOLATION OF VEILLONELLA FROM THE MOUTH

3.1 INTRODUCTION

One of the oral commensal species which was studied in Chapter 4 was Veillonella. Since the semi-selective culture medium used to isolate and enumerate Veillonella was developed by myself and has not been published previously in detail, it is appropriate to describe the composition, method of preparation and use of the culture medium in this Chapter.

Veillonella species are commonly present in the human mouth and are strictly anaerobic Gram negative cocci 0.3-0.5 μ in diameter usually arranged in pairs or masses. The generally accepted description of the oral species is that of Rogosa (1964 and 1965). Although Veillonella grow well on blood agar they are difficult to isolate from mixed specimens since they tend to be overgrown by facultative streptococci and other oral bacteria. A selective medium for the isolation and enumeration of Veillonella using basic fuchsin and streptomycin as the inhibitory agent was described by Rogosa (1956). In a later paper vancomycin was substituted for streptomycin (Rogosa et al 1958). The medium described below was developed as a result of an investigation to find a selective medium for the isolation of the Gram negative members of the oral flora.

3.2 MATERIALS AND METHODS

The composition of the medium was as follows:

Basal Medium

Neutralised bacteriological peptone (Oxoid)	24.4 g
Purified agar (Oxoid)	14.6 g
L-cysteine hydrochloride (B.D.H.)*	0.6 g
Yeast extract (Oxoid)	6.0 g
Potassium nitrate (B.D.H.)*	1.2 g
Deionized water	1000 ml

* B.D.H. Chemicals Ltd., Poole, Dorset.

The constituents were dissolved at 100°C and when molten the pH was adjusted to 7.4 and 25 ml of a 0.2% solution of bromothymol blue was added. The medium was dispensed in 100 ml amounts and autoclaved at 120°C for 15 minutes.

The inhibitory agent and various growth factors which were heat sensitive were prepared separately as stock solutions.

Glucose

A 10% solution of glucose (B.D.H. Analar grade) was prepared in deionized water and sterilized by Tyndallization.

Sodium Lactate

A 70% W/W solution of sodium lactate (B.D.H.) was sterilized by Tyndallization.

Vitamin K and Haemin Solution

(i) Menadione Solution A 100 mg were added to 20 ml of 95% ethyl alcohol, and sterilized by millipore filtration.

(ii) Haemin Solution B 50 mg of haemin (B.D.H.) were dissolved in 1 ml of N.NaOH then added to 100 ml of deionized water. This solution was autoclaved at 115°C and 15 lb/sq in for 15 min.

(iii) One ml of solution A was added to 100 ml of solution B.

Teepol 610 (Shell Chemical Company)

A 1% solution of Teepol 610 was prepared in deionized water and autoclaved at 115°C and 15 lb/sq in for 15 min.

To prepare the complete medium the following volumes of the stock solutions were added to 100 ml of the basal medium.

100 ml of the basal medium were melted then cooled to 55°C when the following volumes of the stock solutions were added:

Sodium Lactate	1.2 ml
Glucose	10 ml
Vitamin K/Haemin	1.2 ml
Teepol 610	1.2 ml

The final mixture was dispensed in 15 ml amounts into sterile plastic petri dishes, allowed to solidify, and dried at 50°C for 10 minutes before use.

Two experiments were carried out to find the concentration of teepol which would allow maximum growth of Veillonella and at the same time completely inhibit the Gram positive members of the oral flora. In the first experiment, plates were prepared with concentrations of teepol ranging from 0.005 to 0.5% and inoculated with a standard loop-ful of emulsified dental plaque. The plates were placed in an anaerobic jar and incubated for two days at 37°C. The Gas Pak system (B.B.L.)* was used to obtain anaerobic conditions, and anaerobiasis was monitored by the use of methylene blue strips (B.B.L.).* Gram films were made from the resultant growth and selected colonies were incubated aerobically and anaerobically on blood agar plates to assess their sensitivity to oxygen. Oxidase negative, strictly anaerobic Gram negative cocci were accepted as Veillonella.

In the second experiment the growth of Veillonella on a medium containing 0.01% teepol and on a medium containing 0.1% teepol was compared. An anaerobic coccus Hare's Group (V) N.C.T.C. 9805 (National Collection of Type Cultures London) was used to assess the inhibitory activity of the two concentrations of teepol. A smooth emulsion of an overnight blood agar culture of N.C.T.C. 9805 was made in one ml of sterile peptone water, and serial dilutions from 10^{-1} to 10^{-8} were prepared in the same medium. Using an Eppendorf pipette 20 μl of each dilution was placed in the appropriate sector of the 0.01% and 0.1% teepol plates. The plates were incubated anaerobically for two days. The number of resultant colonies were counted at the dilutions where this was possible. A third experiment was carried out to assess whether 0.01% teepol medium had any inhibitory effect on Veillonella species. The techniques used were as previously described in experiment two, except that growth on blood agar and 0.01% teepol medium were compared.

3.3 RESULTS

The results of the first experiment (Table 3.1) showed that the Gram positive flora of the mouth was totally inhibited at at concentration

* Baltimore Biological Laboratories; Division of Becton, Dickinson and Company, Maryland, U.S.A.

Final % Concentration of Teepol 610	Growth of Veillonella	Growth of Gram Negative Rods	Growth of Gram Positive Bacteria
0.005	+	+	+
0.01	+	+	-
0.05	+	+	-
0.1	+	+	-
0.15	+	-	-
0.5	-	-	-

+ = Growth - = No Growth

TABLE 3.1 GROWTH OF VEILLONELLA, ANAEROBIC GRAM NEGATIVE RODS AND GRAM POSITIVE ORGANISMS ON MEDIA CONTAINING VARYING CONCENTRATIONS OF TEEPOL 610

Dilution of Veillonella N.C.T.C. 9805	Number of Colonies on 0.01% Teepol Agar	Number of Colonies on 0.1% Teepol Agar
10^{-1}	+++	+++
10^{-2}	+++	++
10^{-3}	+++	2
10^{-4}	+++	0
10^{-5}	++	0
10^{-6}	20	0
10^{-7}	1	0
10^{-8}	0	0

TABLE 3.2 COMPARISON OF 0.01% TEEPOL AGAR AND 0.1% TEEPOL AGAR FOR ISOLATING VEILLONELLA (N.C.T.C. 9805)

of 0.01% teepol. On the other hand 0.15% teepol was necessary to inhibit the Gram negative oral flora with the exception of Veillonella, and a concentration of more than 0.5% teepol was required to completely inhibit the growth of Veillonella.

In the second experiment (Table 3.2) absence of growth occurred at a 10^{-4} dilution of Veillonella on the 0.1% teepol medium, compared with a dilution of 10^{-8} on the 0.01% teepol medium. It would appear that although 0.1% teepol supports the growth of Veillonella it is markedly inhibitory in nature when compared to 0.01% teepol medium.

In the third experiment (Table 3.3) no significant difference was found between the growth of various dilutions of Veillonella cultured on blood agar and 0.01% teepol agar. It can be concluded from these experiments that 0.01% teepol agar has no inhibitory effect on Veillonella, while effectively inhibiting the oral Gram positive flora.

On teepol medium, Veillonella produce green colonies 2-3 mm in diameter with raised centres and a rough or smooth edge after anaerobic incubation for 1-2 days. The colonial morphology of Veillonella was strikingly different as regards size and colour when compared to the other Gram negative bacteria which grow anaerobically on 0.01% teepol plates. These organisms which include Haemophili, Vibrio and Fusobacteria produced small colonies 1 mm or less in diameter, and were mainly clear or yellow in colour. In addition many of these organisms take 4-7 days to produce macroscopic colonies and if the plates are examined after 24-48 hours a "pure" culture of Veillonella is often present.

3.4 DISCUSSION

Teepol is a detergent used in many laboratories and pharmacies for cleaning glassware. The active agent of teepol is based on the sodium and potassium salts of alkyl sulphates and alkyl aryl sulphonates. Teepol is supplied in two forms, Teepol L and Teepol 610. Since Teepol 610 has a standard composition whereas the composition of Teepol L varies with different batches, the former reagent must be

Dilution of Veillonella N.C.T.C. 9805	Number of Colonies on 0.01% Teepol Agar	Number of Colonies on Blood Agar
10^{-1} to 10^{-4}	+++	+++
10^{-5}	+	+
10^{-6}	6	8
10^{-7}	2	4
10^{-8}	2	3

TABLE 3.3 COMPARISON OF BLOOD AGAR AND 0.01% TEEPOL AGAR FOR ISOLATING VEILLONELLA (N.C.T.C. 9805)

used for media preparation. Teepol 610 has been used in place of bile salts as a selective agent in relation to the bacteriological examination of water, (Jameson and Emberley 1956) but the inhibitory effect on the oral microflora has not previously been described.

The results of these experiments show that teepol medium (0.01%) is non-inhibitory to Veillonella when compared with blood agar but there is no equivalent information concerning Rogosa's media. The isolation of Veillonella from a sample does not mean that the culture medium used has no inhibitory properties, and this was clearly demonstrated when media containing 0.1% and 0.01% teepol were compared. A pour plate technique is usually used in Rogosa's medium while surface plating is used in the teepol medium. The teepol medium is primarily intended for routine isolation of Veillonella, and in this situation surface plating is more suitable than a pour plate technique.

No difficulty has been experienced in the isolation of Veillonella from saliva, tongue scrapings, throat swabs and dental plaque. The teepol medium described can be used as an alternative to Rogosa's medium for the isolation of Veillonella, and it has the added advantage of inhibiting the oral Gram positive flora while having little effect on the Gram negative flora.

3.5 CONCLUSIONS

Teepol medium (0.01%) is non-inhibitory to Veillonella when compared with blood agar, and is suitable for use in isolating and enumerating Veillonella from oral samples.

THE COMMENSAL MICROFLORA IN PATIENTS WITH SJÖGREN'S SYNDROME

4.1 INTRODUCTION

In view of the large numbers of Candida albicans and Staphylococcus aureus isolated from patients with severe Sjögren's syndrome (Chapter 2), it seemed reasonable to assume that some changes would be found among the members of the oral commensal microflora. Since there is no information in the literature about changes in the commensal flora of patients with Sjögren's syndrome, the present study was carried out. At the same time, changes in the other two main components of the oral environment, namely saliva and the oral mucosa, were investigated. The flow rate and pH of saliva was measured and gross morphological changes in the oral mucosa and at the angles of the mouth were assessed.

4.2 METHODS

4.2.1 Patients

The study comprised 20 patients, 10 diagnosed cases of Sjögren's syndrome and 10 healthy controls. The test and control groups were matched for age, sex, and denture status. The diagnosis of Sjögren's syndrome was based on the criteria described by Bloch et al (1965). Both patients and controls were questioned regarding a history of xerostomia and of the associated symptoms of Sjögren's syndrome. None of the control patients had symptomatic or clinical dry mouth.

4.2.2 Measurement of Parotid Flow Rate

Parotid salivary flow rate studies were carried out only on the 10 patients with Sjögren's syndrome. The flow studies were performed by the use of a modified Carlson-Crittenden cup, with an outer chamber of diameter 20 mm and an inner chamber of diameter 10 mm (Carlson and Crittenden 1910; Mason et al 1966). Parotid saliva was collected from each patient after stimulation with 10% citric acid for one minute. The flow rate results were assessed as normal or abnormal after

comparing with the normal range as defined by Chisholm (1970) and Whaley et al (1973).

4.2.3 Measurement of Salivary pH

Since there was virtually no saliva present in the mouths of the Sjögren's patients, it was technically impossible to use pH electrodes to measure salivary pH. Two ranges of Merk Spezialindikator pH strips were used, one range measuring pH values from 4.0-7.0 and the other range measuring from 6.5-10.0. Merk indicator strips had a number of advantages compared to conventional pH indicator papers: the pH paper was mounted on individual flexible plastic strips, and was colourfast, with a sensitivity of 0.2-0.3 of a pH unit.

Each patient was instructed to collect saliva in the floor of his mouth, and after one minute, the pH strips (one of each pH range), were soaked in the collected secretions in vivo. The pH value was immediately noted. After a few minutes the test was repeated, and the average of the two pH results was recorded.

4.2.4 Assessment of the Oral Mucosa and Angles of Mouth

The clinical changes in the tongues of the 10 Sjögren's patients and 10 control subjects were graded as described by Bertram (1967).

Grade 1 - Slight reddening, slight fissuring and minor atrophy of the filiform papillae on the tip of the tongue.

Grade 2 - Moderate reddening and fissuring, with sporadic papillary atrophy.

Grade 3 - Pronounced reddening, total papillary atrophy and severe lobulation or deep fissuring. Grade 3 also included a red atrophic completely smooth lacquered tongue.

Angular cheilitis was defined as an eroded and erythematous non-vesicular lesion radiating from the angle of the mouth.

4.2.5 Microbiology

Four separate areas of the mouth were sampled; the fitting surface of the upper denture, the palatal mucosa, the dorsum of the tongue and the right tonsillar area. Each area was sampled by means of a firmly taken cotton wool swab. The swab was immediately immersed in 1 ml of sterile peptone water (Oxoid) and thoroughly agitated and squeezed to transfer the sampled material to the fluid. All swabs were taken by one operator, and before salivary flow studies were carried out. Each sample was treated in a Whirlimixer (Fisons Scientific Apparatus, Leicestershire), for one minute to disperse any clumps of micro-organisms present in the sample. Within an hour of sampling, 0.02 ml of each sample was added to the following culture plates by means of an Eppendorf pipette: one blood agar, one teepol plate, and two Mitis Salivarius Agar plates. The blood agar plates were prepared by adding 5% sterile horse blood to Columbia agar base (Oxoid). The teepol plates were prepared as described in Chapter 3, and the Mitis Salivarius Agar plates (M.S.A.) were prepared according to the manufacturer's instructions (Oxoid). The sample added to each plate was carefully plated out for separate colonies, ensuring that as much of the plate surface as possible was utilized. The blood agar and one M.S.A. plate was incubated aerobically for two days at 37°C. The teepol plates were incubated anaerobically at 37°C for two days using a Baird and Tatlock anaerobic jar, and the Gas Pak system of Becton, Dickinson and Company, Maryland. Fresh methylene blue indicator strips (Becton, Dickinson and Company, Maryland) were used to check that strict anaerobiasis was achieved. One M.S.A. plate was incubated anaerobically for 24 hours at 37°C and then left at room temperature for a further 24 hours, before examination of colonial morphology (Jordan et al 1968). After incubation the plates were examined, and the numbers of Streptococci (M.S.A. and blood agar), Veillonella (teepol agar), Neisseria and Staphylococcus salivarius (blood agar) noted. Typical colonies were removed from each plate and identified using the following criteria:-

Veillonella: Green colonies 2-3 mm in diameter with raised centres and a rough or smooth edge, which on aerobic and anaerobic sub-culture onto blood agar, grow only under anaerobic conditions. On Gram film

the bacteria were Gram negative cocci in masses.

Neisseria pharyngis: Strictly aerobic Gram negative diplococci, which were oxidase and catalase positive; acid was produced from glucose, maltose and sucrose, but lactose was not fermented. Catalase activity was assessed as described by Cowan and Steele (1974), and Oxidase activity by Kovac's method (Kovac 1956). Sugar fermentation reactions were carried out on enriched nutrient agar slopes which contained 1% W/V of the sugar (Analar Grade, British Drug House). Details of the composition of the sugar fermentation reaction medium are given in Appendix 1.

Staphylococcus salivarius: Catalase variable Gram positive cocci; facultative anaerobes producing round entire convex colonies, grey to white in colour about 1 mm in diameter, mucoid in character and very adherent to the agar surface. The organism was coagulase negative, and reduced nitrate beyond nitrite. In doubtful cases, further biochemical tests were carried out as described by Gordon (1967). The coagulase and nitrate reduction tests were carried out using the methods described by Cowan and Steele (1974).

Streptococcus salivarius: Gram positive cocci which formed large pale blue and opaque zoogleic colonies $>$ 2 mm in diameter on aerobic M.S.A. plate (Carlsson 1967, 1968).

Streptococcus sanguis: Gram positive cocci which formed small zoogleic colonies (0.5-1 mm in diameter)with a firm consistency. The surrounding agar surface was deformed, and the colonies were attached so firmly to the medium that the zoogleic part of it could not be removed from the medium with a nichrome wire loop (Carlsson 1965). Both the aerobic and anaerobic M.S.A. plates were examined for the presence of colonies resembling Streptococcus sanquis.

Streptococcus mitis: Gram positive cocci with a flat or low convex elevation and a diameter rarely exceeding 1 mm in diameter. The colonies are soft in consistency and easily removed from the agar surface (Carlsson 1968). Both aerobic and anaerobic M.S.A. plates were examined for the presence of colonies resembling Streptococcus mitis.

Streptococcus mutans: Gram positive cocci which formed high convex, light blue zoogleic colonies (0.5-1 mm in diameter) reminiscent of frosted glass on anaerobically incubated Mitis Salivarius Agar (Krasse 1966).

4.2.6 Grading of Bacterial Numbers

Since Streptococcus salivarius produced distinctive mucoid colonies on aerobic M.S.A. and Veillonella produced distinctive green colonies on teepol agar, a direct count of the number of typical colonies recovered from the samples was made. Since Neisseria were isolated in mixed culture on blood agar, the number of colonies present was assessed by adding oxidase reagent (Kovac 1956) to the agar plate and then counting the number of oxidase positive colonies with a typical morphology and Gram staining reaction. Staphylococcus salivarius was isolated in mixed culture on blood agar. The colonies were relatively easily recognised and counted, due to their colonial appearance, their adherence to the surface of the agar plate, and to the fact that they were oxidase negative. The colonial numbers of Streptococcus salivarius, Veillonella, Neisseria pharyngis and Staphylococcus salivarius isolated from the four areas of the mouth were graded as shown in Table 4.1. By this means, individual scores for each of the four areas of the mouth were obtained, and by adding the scores for the tongue, palate, denture and throat, a total mouth score (0-12) was obtained. In the case of Streptococcus sanguis, Streptococcus mitis and Streptococcus mutans, no attempt was made to count the number of colonies isolated from the samples, only the presence or absence of each species was recorded.

4.3 RESULTS

4.3.1 Patients

Table 4.2 shows a comparison of the clinical data of the two groups. The age distribution was similar in both the Sjögren's and control groups, the female to male ratio was 9:1 and all the patients and controls had complete upper and lower dentures.

Grade	Number of Colonies
0	Absent
1	1 - 10
2	11 - 50
3	Over 50

TABLE 4.1 GRADING STANDARDS FOR THE NUMBER OF STREPTOCOCCUS SALIVARIUS, NEISSERIA PHARYNGIS, VEILLONELLA AND STAPHYLOCOCCUS SALIVARIUS ISOLATED FROM THE SJÖGREN'S PATIENTS AND CONTROL SUBJECTS

	Sjögren's	Controls
Number of Subjects	10	10
Sex Distribution:		
Male	1	1
Female	9	9
Age Distribution:		
31 - 40	1	0
41 - 50	0	1
51 - 60	4	3
61 - 70	2	3
71 - 80	3	3
Number with complete upper and lower dentures	10	10

TABLE 4.2 AGE, SEX AND DENTURE STATUS OF 10 SJÖGREN'S AND 10 CONTROL PATIENTS

4.3.2 Parotid Flow Rate, Angular Cheilitis and Atrophy of the Tongue

The parotid flow rate values, and the clinical assessment of the tongue and angles of the mouth in the Sjögren's patients are shown in Table 4.3. No measurable flow rate was recorded in 90% of the Sjögren's patients, and one patient had a reduced parotid secretion. Angular cheilitis was present in 5 (50%) of the patients, and 9 (90%) had clinical evidence of atrophy of the tongue (5 Grade 3, 3 Grade 2, and 1 Grade 1). None of the control group had angular cheilitis or atrophic changes in the tongue mucosa.

4.3.3 Salivary pH

It was assumed that the salivary pH values in the Sjögren's patients and control subjects were normally distributed and that the data was amenable to analysis by parametric methods. Comparison of the salivary pH values of the Sjögren's and control group of subjects was thus made using the Student's 't' test.

The salivary pH values for the two groups of patients are shown in Table 4.4. The mean pH value for the Sjögren's group was 5.5 and for the control group 6.4. When the results were analysed using the Student's 't' test, the difference between the two groups was found to be significant ($P = < 0.01$).

4.3.4 Microbiology

The individual and total mouth scores for Streptococcus salivarius, Neisseria pharyngis, Veillonella and Staphylococcus salivarius which were isolated from the tongue, palate, denture and throat of the Sjögren's patients and the control group, are shown in Tables 4.5, 4.6, 4.7 and 4.8 respectively.

Since evaluation of the numbers of micro-organisms produces a numerical score for each patient and control it was felt undesirable to assume that the scores were normally distributed. Therefore it was decided to analyse the data by non parametric methods, the appropriate test being the Mann and Whitney U test (Siegel 1956).

Sjögren's Patients	Parotid Flow Rates	Angular Cheilitis	Atrophic Tongue Grade
A 1	x	- ve	3
A 2	x	+	3
A 3	x	+	1
A 4	x	- ve	0
A 5	x	- ve	2
A 6	x	+	3
A 7	-	- ve	2
A 8	x	+	3
A 9	x	+	3
A 10	x	- ve	2

x No measurable flow + Positive

- Reduced secretion - ve Negative

TABLE 4.3 PAROTID FLOW RATE VALUES, AND THE CLINICAL ASSESSMENT OF THE TONGUE AND ANGLES OF THE MOUTH IN 10 SJÖGREN'S PATIENTS

Salivary pH Values			
Sjögren's		Controls	
A 1	5.0	6.2	C 1
A 2	5.3	6.6	C 2
A 3	6.0	6.2	C 3
A 4	5.3	6.2	C 4
A 5	5.3	7.0	C 5
A 6	5.3	5.8	C 6
A 7	7.2	6.2	C 7
A 8	5.3	7.2	C 8
A 9	5.0	6.0	C 9
A 10	5.5	6.8	C 10
Mean	5.52	6.42	
Standard Deviation	0.55	0.45	

Comparison of Sjögren's Group v Control Group

$t = 3.57$

$P = < 0.01$

TABLE 4.4 COMPARISON OF THE SALIVARY pH VALUES FOR 10 SJÖGREN'S PATIENTS AND 10 CONTROLS

The difference in the scores for Streptococcus salivarius between the two groups was significant in all sites sampled ($P = < 0.001$). The difference in the scores for Neisseria pharyngis was significant in the throat, palate and tongue samples ($P = < 0.01$), and also in the denture samples ($P = < 0.05$). The difference in scores for Veillonella was significant for the palate, denture and tongue samples ($P = < 0.001$) and also for the throat samples ($P = < 0.01$). The difference in scores for Staphylococcus salivarius was significant in the denture and palate samples ($P = < 0.001$) and also in the tongue and throat samples ($P = < 0.01$). The difference in the total mouth scores for Streptococcus salivarius, Veillonella and Staphylococcus salivarius was significant ($P = < 0.001$) as was the scores for Neisseria pharyngis ($P = < 0.01$).

The isolation rate of Streptococcus mitis, Streptococcus sanguis and Streptococcus mutans from the four sample sites is shown in Table 4.9. The number of times Streptococcus mitis was isolated from the four oral sites in the control patients was 37 (92%) and in the Sjögren's patients 35 (88%). Streptococcus sanguis was isolated from 19 (48%) of the control group samples but was never isolated from any of the Sjögren's patients. Streptococcus mutans was never isolated from either the control or Sjögren's groups.

4.4 DISCUSSION

The results of this study show that with regard to Streptococcus salivarius, Neisseria pharyngis, Veillonella species and Staphylococcus salivarius, there are statistically significant differences in the commensal oral flora of the Sjögren's group of patients compared with that of normal healthy control patients. The precise reason for the differences is not clear, but a number of possible explanations can be suggested.

4.4.1 The Effect of Atrophic Changes in the Oral Mucosa on the Commensal Microflora

In health the anterior part of the tongue is studded with numerous closely set projections or papillae. There are three kinds of papillae, filiform, fungiform and vallate. The filiform papillae

Streptococcus Salivarius											
	Sjögren's					Controls					
	Tongue	Palate	Denture	Throat	Total	Total	Throat	Denture	Palate	Tongue	
A 1	2	0	0	0	2	10	3	2	2	3	C 1
A 2	0	0	0	0	0	7	1	1	2	3	C 2
A 3	1	0	0	2	3	11	3	3	2	3	C 3
A 4	1	0	0	1	2	12	3	3	3	3	C 4
A 5	0	0	0	0	0	12	3	3	3	3	C 5
A 6	0	0	0	0	0	11	3	2	3	3	C 6
A 7	0	0	0	0	0	0	0	0	0	0	C 7
A 8	0	0	0	0	0	6	3	0	0	3	C 8
A 9	0	0	0	0	0	11	3	2	3	3	C 9
A 10	0	0	0	0	0	12	3	3	3	3	C 10
Total	4	0	0	3	7	92	25	19	21	27	

Streptococcus salivarius : Sjogren's v Control

Tongue $P = < 0.001$ Palate $P = < 0.001$ Denture $P = < 0.001$ Throat $P = < 0.001$ Total $P = < 0.001$

TABLE 4.5 SCORES FOR STREPTOCOCCUS SALIVARIUS ISOLATED FROM THE TONGUE, PALATE, DENTURE AND THROAT OF 10 SJÖGREN'S AND 10 CONTROL PATIENTS

Neisseria Pharyngis											
	Sjögren's					Controls					
	Tongue	Palate	Denture	Throat	Total	Total	Throat	Denture	Palate	Tongue	
A 1	0	0	0	0	0	9	3	0	3	3	C 1
A 2	0	0	0	0	0	12	3	3	3	3	C 2
A 3	0	0	0	0	0	12	3	3	3	3	C 3
A 4	0	0	0	0	0	0	0	0	0	0	C 4
A 5	0	0	0	0	0	10	3	2	2	3	C 5
A 6	1	0	0	0	1	2	0	0	2	0	C 6
A 7	3	3	3	3	12	3	0	0	0	3	C 7
A 8	0	0	0	0	0	10	3	2	2	3	C 8
A 9	0	0	0	0	0	11	3	2	3	3	C 9
A 10	0	0	0	0	0	11	3	2	3	3	C 10
Total	4	3	3	3	13	80	21	14	21	24	

Neisseria Pharyngis : Sjogren's v Control

Tongue P =<0.01 Palate P =<0.01 Denture P =<0.05 Throat P =<0.01 Total P =<0.01

TABLE 4.6 SCORES FOR NEISSERIA PHARYNGIS ISOLATED FROM THE TONGUE, PALATE, DENTURE AND THROAT OF 10 SJÖGREN'S AND 10 CONTROL PATIENTS

Veillonella											
	Sjögren's					Controls					
	Tongue	Palate	Denture	Throat	Total	Total	Throat	Denture	Palate	Tongue	
A 1	0	0	0	0	0	3	2	1	0	0	C 1
A 2	3	2	0	2	7	5	0	2	0	3	C 2
A 3	0	0	0	0	0	12	3	3	3	3	C 3
A 4	0	0	0	0	0	9	2	2	2	3	C 4
A 5	0	0	0	0	0	12	3	3	3	3	C 5
A 6	0	0	0	0	0	12	3	3	3	3	C 6
A 7	0	0	0	0	0	3	0	0	0	3	C 7
A 8	0	0	0	0	0	12	3	3	3	3	C 8
A 9	1	0	0	2	3	12	3	3	3	3	C 9
A 10	0	1	0	2	3	12	3	3	3	3	C 10
Total	4	3	0	6	13	92	22	23	20	27	

Veillonella : Sjogren's v Control

Tongue P =<0.001 Palate P =<0.001 Denture P =<0.001 Throat P =<0.01 Total P =<0.001

TABLE 4.7 SCORES FOR VEILLONELLA SPECIES ISOLATED FROM THE TONGUE, PALATE, DENTURE AND THROAT OF 10 SJÖGREN'S AND 10 CONTROL PATIENTS

Staphylococcus Salivarius											
	Sjögren's					Controls					
	Tongue	Palate	Denture	Throat	Total	Total	Throat	Denture	Palate	Tongue	
A 1	0	0	0	0	0	12	3	3	3	3	C 1
A 2	0	0	0	0	0	12	3	3	3	3	C 2
A 3	0	0	0	0	0	10	3	2	2	3	C 3
A 4	1	1	0	2	4	12	3	3	3	3	C 4
A 5	0	0	0	0	0	9	2	2	2	3	C 5
A 6	0	0	0	0	0	0	0	0	0	0	C 6
A 7	0	0	0	0	0	0	0	0	0	0	C 7
A 8	0	0	0	0	0	12	3	3	3	3	C 8
A 9	0	0	0	0	0	12	3	3	3	3	C 9
A 10	0	0	0	0	0	12	3	3	3	3	C 10
Total	1	1	0	2	4	91	23	22	22	24	

Staphylococcus salivarius : Sjogren's v Control

Tongue $P = < 0.01$ Palate $P = < 0.001$ Denture $P = < 0.001$ Throat $P = < 0.01$ Total $P = < 0.001$

TABLE 4.8 SCORES FOR STAPHYLOCOCCUS SALIVARIUS ISOLATED FROM THE TONGUE, PALATE, DENTURE AND THROAT OF 10 SJÖGREN'S AND 10 CONTROL PATIENTS

	Sjögren's Group					Control Group				
	Tongue	Palate	Denture	Throat	Total Isolates	Total Isolates	Throat	Denture	Palate	Tongue
Streptococcus Mitis	8	9	9	9	35	37	10	9	9	9
Streptococcus Sanguis	0	0	0	0	0	19	4	7	5	3
Streptococcus Mutans	0	0	0	0	0	0	0	0	0	0

TABLE 4.9 ISOLATION RATE OF STREPTOCOCCUS MITIS, STREPTOCOCCUS SANGUIS AND STREPTOCOCCUS MUTANS FROM ORAL SAMPLES REMOVED FROM 10 SJÖGREN'S AND 10 CONTROL PATIENTS

are by far the most numerous type. They are conical in shape and consist of a central connective tissue core, covered by stratified squamous epithelium which is keratinized especially towards the tip. The fungiform papillae are roundish projections and are non-keratinized, while the vallate papillae (six to eight in number), are much larger than the other types of papillae, and contain taste buds (Scott and Symons 1974). In health, the papillae and the clefts between papillae support the growth of a distinctive microbial flora (Gordon and Gibbons 1966). However, if the papillae are totally lost or reduced in number, as occurs in many patients with Sjögren's syndrome, the surface area of mucosa available for bacterial colonization would become markedly reduced, and the distinctive micro-environment of the tongue would cease to exist. On theoretical grounds therefore it is likely that severe atrophy of the mucous membrane of the tongue would lead to qualitative and quantitative changes in the flora normally found in that site.

The main oral ecological niche of Streptococcus salivarius, Neisseria, Veillonella and Staphylococcus salivarius is the tongue (Gordon and Gibbons 1966). Specific and preferential adherence of Streptococcus salivarius, Veillonella and Neisseria to the cells of the human tongue and buccal mucosa has been demonstrated by Gibbons (1972). The incidence and adherence activity of the bacteria mentioned above, is shown in Table 4.10. Streptococcus salivarius and Veillonella are present in large numbers and are strongly adherent to the tongue. Neisseria species are present in relatively small numbers on the tongue and have a low adherence capability. Staphylococcus salivarius is present in moderate numbers on the tongue, but no experimental work has been reported concerning the adherence of this organism to the superficial epithelial cells.

The absence of salivary secretions coating the oral mucosa may also affect the numbers of bacteria which are able to adhere to the superficial cells. Adams (1973) described a "fuzzy" coat on the outermost surface of oral epithelial cells, which consisted of an inner layer derived from the epithelial cells, and an outer layer derived from salivary glycoprotein. He demonstrated that the outer layer was absent in patients with Sjögren's syndrome. Since salivary

	Proportions Found Indigenously on Tongue	Experimentally Observed Adherence to Tongue Surface
Streptococcus Salivarius	High	High
Veillonella	High	High
Neisseria	Low	Low
Staphylococcus Salivarius	High	?
Streptococcus Sanguis	Moderate	Moderate

(After Gibbons 1972)

TABLE 4.10 ABILITY OF BACTERIA TO ADHERE TO THE TONGUE AS RELATED TO THEIR PROPORTIONS FOUND INDIGENOUSLY

glycoproteins have been shown to aggregate oral bacteria (Hay et al 1971), it is possible that the "salivary layer" is necessary for bacterial adherence to occur to surface epithelial cells. If this was the case, the numbers of commensal bacteria adhering to the surface of the mucous membrane of patients with severe Sjögren's syndrome would be greatly reduced.

Mandel and Khurana (1969) have shown that at low salivary flow rates, a high concentration of secretary IgA (S-IgA) was present in saliva. It is possible that at some stage in the disease process the saliva of patients with Sjögren's syndrome will contain relatively high concentrations of S-IgA. Williams and Gibbons (1972) have shown that the adherence of certain strains of Streptococcus salivarius to epithelial cells can be reduced by specific normally occurring salivary S-IgA. The antibodies appear to react with bacterial surface antigen and hinder attachment of the organism to epithelial cells. Gibbons (1974) believes that this immunologically controlled mechanism is important in controlling the composition of the oral microbial flora. It is possible that in Sjögren's patients the concentration of such S-IgA antibodies is greatly increased with the result that the adherence of Streptococcus salivarius to mucosal cells is completely inhibited instead of partially reduced, as occurs in the mouth under normal conditions. In these circumstances, Streptococcus salivarius would probably be dislodged from the mucosal surface, and due to the high levels of salivary antibodies, be unable to achieve re-attachment. Since Streptococcus salivarius would be unable to adhere to any part of the mouth, the organism would disappear from the oral flora after a period of time. It may be possible that similar mechanisms account for the loss of Staphylococcus salivarius, Veillonella and Neisseria pharyngis from the Sjögren's patients.

It would seem reasonable to expect that another effect of the loss of tongue papillae would be on the oxygen tension of the tongue especially in the inter-papillary crypt area. Eskow and Loesche (1971) have shown that the oxygen tension of the anterior part of the tongue is 16.4%. This value is for the surface of the tongue and it is likely that considerably lower oxygen tension values are present

within the papillary crypts. In addition mixed saliva is known to have reducing properties (Jenkins 1966a) and it is probable that the virtual absence of saliva would change the general environment of the mouth towards a high oxygen tension. The combined effect of loss of the filiform papillae and the reducing properties of saliva would tend to cause loss of anaerobic conditions in the mouth, particularly the tongue, with a consequent reduction in the numbers of strict anaerobes such as Veillonella species.

4.4.2 The Effect of pH on the Oral Microflora

There have been no previous studies on the salivary pH of patients with Sjögren's syndrome, with which the results of the present study can be compared. However, Frank et al (1965) have shown that the salivary pH of patients with xerostomia due to irradiation therapy for carcinoma of the salivary gland was low, 5.38 ($\pm$ 0.26) and these results are very similar to those recorded in the present study.

It is not clear whether the low salivary pH values recorded in the Sjögren's patients is caused by a reduction in salivary flow or by an increase in the numbers of aciduric micro-organisms due to the altered oral environment. At low salivary flow rates, the salivary pH is reduced (Jenkins 1966b) and it is likely that the buffering capacity of saliva is also low. It is not surprising therefore that the oral environment is markedly acid in patients with Sjögren's syndrome. As a result of the acid environment, aciduric micro-organisms such as yeasts will tend to proliferate and reduce the salivary pH further by fermenting dietry carbohydrate. Since the mechanical washing activity of saliva is lost in patients with severe Sjögren's syndrome, food debris tends to cling to the mucosa and is slowly cleared from the mouth. The amount of carbohydrate available for microbial metabolism in the mouth of patients with Sjögren's syndrome is likely to be greater, and to be present for a longer duration compared to patients with a normal salivary flow.

The effect of glycerol and lemon mouthwash on the oral environment and in the numbers of coliform bacilli, Staphylococcus aureus and Candida albicans, was discussed in Chapter 2. All but one

of the Sjögren patients in this study (A 7) used the mouthwash, and this must be regarded as a possible contributing factor to the low salivary pH values which were recorded.

The virtual absence of Veillonella in the flora of the Sjögren's patients may have an effect on the low salivary pH found in this study. Lactic acid and lactate are commonly produced by the fermentation of carbohydrate by oral bacteria with a resulting decrease in the pH of the surrounding environment. Since it has been shown that Veillonella can utilize lactate with the production of propionic acid, acetic acid, carbon dioxide and hydrogen with an overall increase in pH (Regosa 1964) large numbers of Veillonella would tend to prevent a decrease in pH. Mikx et al (1972) has provided some experimental evidence in support of this mechanism, by showing that in gnotobiotic animals, cariogenic streptococci when inoculated with Veillonella, produce less caries compared to animals inoculated with the streptococcal species alone.

4.4.3 The Normal Microflora of Patients with Complete Upper and Lower Dentures

Since little has been published about the normal oral flora of edentulous individuals who wear complete upper and lower dentures (Socransky and Manganiello 1971), there are no definitive studies with which to compare the microbiology results of the control group in the present investigation. Carlsson et al (1969) in one of the few detailed papers on this topic, reported on the prevalence of Streptococcus sanguis, Streptococcus mutans and Streptococcus salivarius in the saliva and on the upper denture of 20 normal denture wearers. Streptococcus salivarius was isolated from the denture and saliva of all 20 subjects (100%) while Streptococcus sanguis and Streptococcus mutans were isolated from both sites in 17 (85%) and 14 (70%) of subjects respectively. Generally, Streptococcus sanguis and Streptococcus mutans constituted a higher percentage of the streptococcal flora on the denture compared to saliva and vice versa for Streptococcus salivarius. In the present study, Streptococcus salivarius was isolated from the dentures of 8 (80%) of the ten control subjects, and Streptococcus sanguis from 7 (70%) of the

controls. Streptococcus mutans was not isolated from either site in any of the control subjects. Therefore the results of this study are in close agreement with Carlsson's study with the exception of the isolation rate of Streptococcus mutans. The reason why the present study failed to demonstrate the presence of S. mutans on dentures may be explained by the different laboratory methods used in the two studies. Carlsson sampled the teeth and base plate of the denture while only the fitting surface of the denture was sampled in this study. In addition a sample dilution technique was used by Carlsson and his co-workers, which was sensitive enough to demonstrate the relatively small numbers of S. mutans in their samples. It is unlikely that the rough screening method employed in the present study would be sufficiently sensitive, to demonstrate small numbers of S. mutans.

4.4.4 The Microflora of Patients with Xerostomia

There are no previous studies on the oral commensal flora of patients with Sjögren's syndrome with which to compare the results of this study. However, Llory et al (1971 and 1972) reported on the microbial composition of saliva and dental plaque of a group of patients who had received irradiation therapy for carcinoma of their salivary glands. By sampling the mouths of the patients before and after irradiation therapy Llory and his co-workers, were able to show that by the fifth week after the completion of irradiation therapy Streptococcus salivarius had virtually disappeared from the mouth. At about the same time, there was a marked reduction in the numbers of Neisseria, Veillonella and Corynebacteria isolated from the oral samples. From two weeks onwards the numbers of Streptococcus mitis, Streptococcus mutans and Lactobacilli increased reaching a maximum value which remained relatively constant for a number of years, by about the fifth to sixth week after completion of irradiation therapy. Llory and his co-workers noted that the marked rise in the numbers of Streptococcus mitis appeared to compensate for the loss of Streptococcus salivarius. These findings are in many respects similar to the results of the present study, although it must be noted that a direct comparison cannot be made since Llory gave no details of the age, sex, or denture status of his patients, nor any information concerning

salivary flow rate or atrophic changes in the oral tissues. Since dental plaque was sampled in Llory's studies, it is probable that all his patients possessed at least a few natural teeth. In both studies Streptococcus salivarius virtually disappeared from the oral flora, and the numbers of Neisseria and Veillonella were greatly reduced. Since the number of Lactobacilli and Corynebacteria were not assessed in the present study and the numbers of Staphylococcus salivarius were not assessed in Llory's study, no comparison can be made concerning these bacteria. The increase in the number of Streptococcus mutans in the saliva and dental plaque described by Llory was not found in this investigation. This discrepancy may be explained by the fact that the tooth surface is the main habitat of Streptococcus mutans (Carlsson 1967) and whereas Llory's patients were dentulous, the Sjögren's patients were edentulous. It is possible that an increase in the numbers of Streptococcus mitis compensated for the loss of Streptococcus salivarius from the Sjögren's patients, as described by Llory and his co-workers, since colonies resembling Streptococcus mitis were the only Streptococcal species isolated from the majority of the Sjögren's patients (Table 4.9).

4.4.5 The Effect of Microbial Antagonistic Mechanisms

The effect of the marked reduction in the numbers of Streptococcus salivarius, Veillonella, Neisseria pharyngis and Staphylococcus salivarius in Sjögren's patients on the antagonistic activity of the oral microbial flora is not clear. Since a series of experiments were carried out to investigate the role of microbial antagonistic mechanisms in preventing the colonisation of the mouth by foreign micro-organisms (Chapters 6 and 7), detailed consideration of this topic will be discussed in these Chapters.

4.5 CONCLUSIONS

This study has shown that severe xerostomia due to Sjögren's syndrome leads to changes in the commensal oral microflora; Streptococcus salivarius, Veillonella, Neisseria pharyngis and Staphylococcus salivarius all but disappear from the oral flora. The reasons for the loss are complex and include atrophy of the oral mucosa,

and a reduction in the volume and pH of saliva. The significance of the loss of these micro-organisms on the defence mechanisms of the mouth is investigated and discussed in Chapter 6.

A NEW METHOD OF ASSESSING THE ANTIMICROBIAL ACTIVITY OF SALIVA AGAINST ESCHERICHIA COLI, STAPHYLOCOCCUS AUREUS AND CANDIDA ALBICANS

5.1 INTRODUCTION

It is generally accepted that mixed saliva and pure parotid and submaxillary saliva possess antimicrobial activity against certain exogenous micro-organisms and also against members of the oral microflora (Bibby et al 1938; Kerr and Wedderburn 1958; Hamon and Klebanoff 1973). Two main antimicrobial systems have been described in mixed saliva and pure parotid and submaxillary saliva; lysozyme (Chauncey et al 1954; Hoerman et al 1956) and two thiocyanate dependent factors, one described by Dogon and Amdur (1962 and 1965) and the other by Klebanoff and Luebke (1965). One possible reason for the overgrowth of yeasts, staphylococci and coliforms in the mouth of patients with xerostomia due to Sjögren's syndrome (Chapter 2), is a reduced concentration of these intrinsic salivary antimicrobial factors. It can be argued that if salivary antimicrobial factors are important in protecting the oral tissues from infection, then saliva from healthy individuals would be inhibitory to various non-commensal micro-organisms. Therefore to test this hypothesis it was decided to assess the antimicrobial activity of mixed saliva from ten normal individuals against Candida albicans, Staphylococcus aureus and Escherichia coli.

In the past the antimicrobial activity of mixed and pure saliva has been measured by well plate techniques (Bibby et al 1938; Kerr and Wedderburn 1958) and by direct contact methods (Klebanoff and Luebke 1965; Dogon and Amdur 1965). Since none of the techniques used to date have reproduced the complex in vivo situation, the results obtained cannot be readily applied to the human mouth. In order to overcome these difficulties it was decided to develop a new technique to assess the antimicrobial activity of saliva, which closely resembled the oral in vivo situation.

5.2 METHODS

5.2.1 Patients and Saliva

The salivary samples investigated were collected from five males and five females aged between 19 and 32. All the patients were dentate and had no recent history of antibiotic therapy. Mixed non-stimulated saliva was collected from the subjects between 9.00 a.m. and 10.00 a.m. on the day of the experiment. Half of the saliva was stored at 4°C in a glass bijou bottle until required for use, while the other half was centrifuged at 15,000 r.p.m. for one hour at 4°C in a refrigerated ultracentrifuge (Measuring and Scientific Equipment Ltd., Crawley, Sussex). The supernatant was removed carefully and stored at 4°C until required. Each experiment was set up within 4 hours of the salivary specimens being collected. The antibacterial activity of half of the mixed and half of the centrifuged salivary samples was inactivated by boiling for 20 minutes. The sterility of the boiled salivary samples and the unheated supernatant of the centrifuged saliva was assessed by plating out 0.02 ml of the samples on blood agar plates. The cultures were incubated aerobically for 3 days and the number of colonies which resulted was noted.

5.2.2 Preparation of Pour Plates

The basal medium for the pour plates was 1.0% purified agar (Oxoid) in deionized water. The pH of the agar was adjusted to 6.5 using N/10 sodium hydroxide. The agar was dispensed in 7 ml amounts and autoclaved at 120°C for 15 minutes. Type cultures were used to assess the antimicrobial activity normally present in saliva against coliforms, yeasts and staphylococci. The type cultures were Escherichia coli N.C.T.C. 10418, Staphylococcus aureus N.C.T.C. 6571 and Candida albicans M.R.L. (Mycological Reference Laboratory, London) 3118C. The inoculum for the pour plates was prepared by incubating the test organisms on the following culture media at 37°C for 18 hours; Escherichia coli and Staphylococcus aureus on blood agar and Candida albicans on Sabourauds agar (Oxoid). The growth which resulted was harvested using a nichrome wire loop, added to 10 ml of peptone water, and thoroughly mixed using a Whirlimixer (Fisons Scientific Apparatus, Leicestershire). The cultures were then centrifuged at 2,000 r.p.m.

for 20 minutes. The supernatant was poured off, and the concentrated cultures were diluted in deionised water as shown in Table 5.1. The type cultures were subcultured each week onto fresh blood agar or Sabouraud's plates and after overnight incubation at 37°C were stored at 4°C. A heavy inoculum was used to subculture the micro-organisms in order to prevent the development of atypical mutants. Bottles of the basal medium were melted and then placed in a thermostatically controlled water bath to stabilised at 50°C. The volumes of the diluted cultures which were added to the 7 ml amounts of agar are shown in Table 5.1. The micro-organisms and agar were thoroughly mixed, then poured into plastic Petri dishes which were allowed to solidify at room temperature. The resulting pour plate was very thin (2 mm) and it was essential to pour the plates on a horizontal bench top to ensure an overall equal thickness of agar. The pour plates were converted into a number of small agar discs, by using a sterile metal cork borer with an external diameter of 4 mm (Figure 5.1). The plates were stored at 4°C until required for use.

5.2.3 Experimental Technique

The experiments to assess the antimicrobial activity of saliva were carried out in disposable plastic World Health Organisation (W.H.O.) serology plates. A few weeks prior to use, the plates were cut into short lengths (6 wells by 6 wells proved a suitable size) sealed in Mediplast sterilizing film and sterilized using ethylene oxide. When required for use the plastic wells were removed from their protective covering and placed in sterile square plastic dishes (Sterilin) as shown in Figure 5.2. The composition of the salivary samples which were added to the wells of the serology plate are shown in Table 5.2. Since there was a possibility that heated saliva would not be able to support the growth of all the micro-organisms, extra nutrients were added in the form of Eugon Broth (Becton, Dickinson and Company, Maryland). The constituents of Eugon Broth are shown in Appendix 2. All the samples were added to the wells using a graduated automatic pipette (R. B. Turner).

Three agar discs containing the test organisms were placed in each well by means of a fine dental syringe needle, bent at the tip to

Micro-organism	Dilution in Deionized Water	Amount Added to 7 ml of Basal Medium
Escherichia coli	1/500	0.015 ml
Staphylococcus aureus	1/500	0.015 ml
Candida albicans	1/10	0.001 ml

TABLE 5.1 DILUTION OF BACTERIA IN DEIONIZED WATER AND THE AMOUNT OF DILUTED CULTURE ADDED TO 7 ml OF BASAL MEDIUM TO PREPARE ONE POUR PLATE OF EACH TEST MICRO-ORGANISM

Well Number	Nature of Sample	Volume
1	Eugon Broth	0.1 ml
2	Mixed Saliva	0.1 ml
3	Heated Mixed Saliva	0.1 ml
4	Mixed Saliva + Eugon Broth	0.05 ml 0.05 ml
5	Heated Mixed Saliva + Eugon Broth	0.05 ml 0.05 ml
6	Centrifuged Saliva	0.1 ml
7	Heated Centrifuged Saliva	0.1 ml
8	Centrifuged Saliva + Eugon Broth	0.05 ml 0.05 ml
9	Heated Centrifuged Saliva + Eugon Broth	0.05 ml 0.05 ml

TABLE 5.2 NATURE OF THE SALIVARY SAMPLES USED IN THE EXPERIMENTS TO ASSESS THE ANTIMICROBIAL ACTIVITY OF SALIVA, AGAINST ESCHERICHIA COLI, STAPHYLOCOCCUS AUREUS, AND CANDIDA ALBICANS

FIGURE 5.1 A POUR PLATE WHICH HAS BEEN CONVERTED INTO NUMEROUS SMALL AGAR DISCS BY MEANS OF A STERILE CORK BORER.

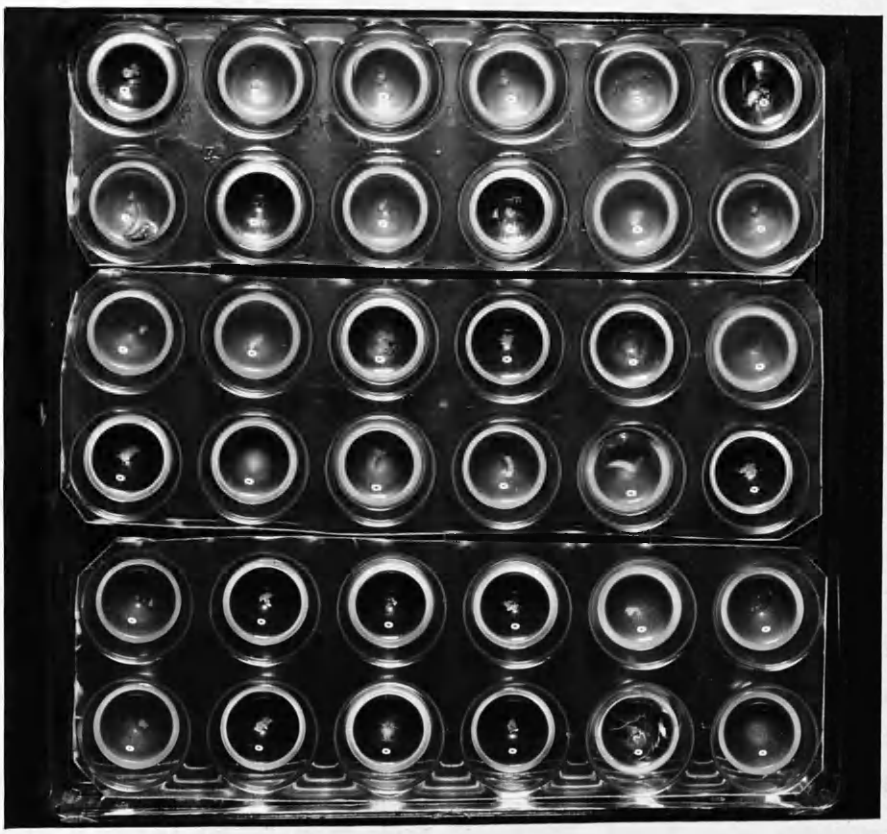

FIGURE 5.2 WELLS OF A DISPOSABLE PLASTIC WORLD HEALTH ORGANISATION SEROLOGY PLATE CONTAINING VARIOUS SALIVARY SAMPLES.

form a small hook (Figure 5.3). The square plastic plates containing the wells were then sealed in a moist chamber and incubated at 37°C for 18 hours. After incubation the agar discs were removed from each well by means of the hooked syringe needle and placed on the pre-marked lid or base of a plastic petri dish (Figure 5.4). The discs were examined for the presence or absence of growth using an inverted microscope (Leitz) (Figures 5.5 and 5.6). The degree of growth in each agar disc was compared with that found in the Eugon Broth control and was graded as shown in Table 5.3. By this means, the degree of inhibition caused by the salivary samples could be assessed.

The discs which showed no growth were transferred to the surface of a blood agar plate and reincubated overnight at 37°C. The discs were examined the next day by the techniques already described, and the presence or absence of growth recorded. By this means it was possible to gain some information about the bactericidal or bacteriostatic nature of the inhibitory factor in the test fluid under investigation. The change of pH of the salivary samples which occurred during the experimental period, was measured since it was possible that a very high or low pH could cause inhibition of the test micro-organisms. To measure the initial pH of the salivary samples, a duplicate set of salivary samples under test were prepared, and the pH measured by adding to each well two Merk Spezialindikator strips (4.0-7.0, 6.8-9) which had been cut into small lengths (Figure 5.7). At the end of the experiment when the agar discs had been removed from the wells the pH of each test was measured by the same technique.

5.2.4 Statistics

Since evaluation of the degree of inhibition of the test micro-organism by each salivary sample produced a numerical score, it was felt undesirable to assume that the scores were normally distributed. Therefore it was decided to analyse the data by non-parametric means, the appropriate test being the Mann and Whitney U Test (Siegel 1956).

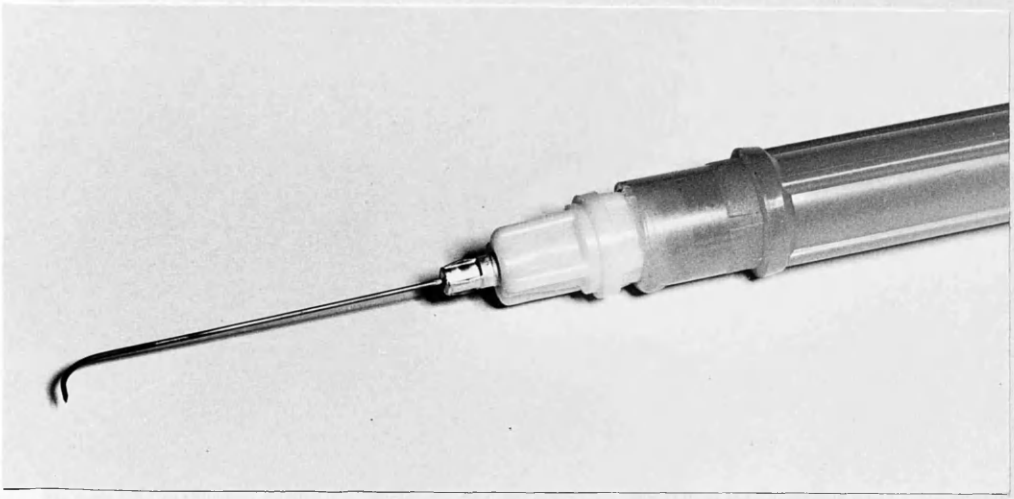

FIGURE 5.3 A DENTAL SYRINGE NEEDLE USED TO TRANSFER THE AGAR DISCS FROM THE POUR PLATE TO THE SALIVARY SAMPLES.

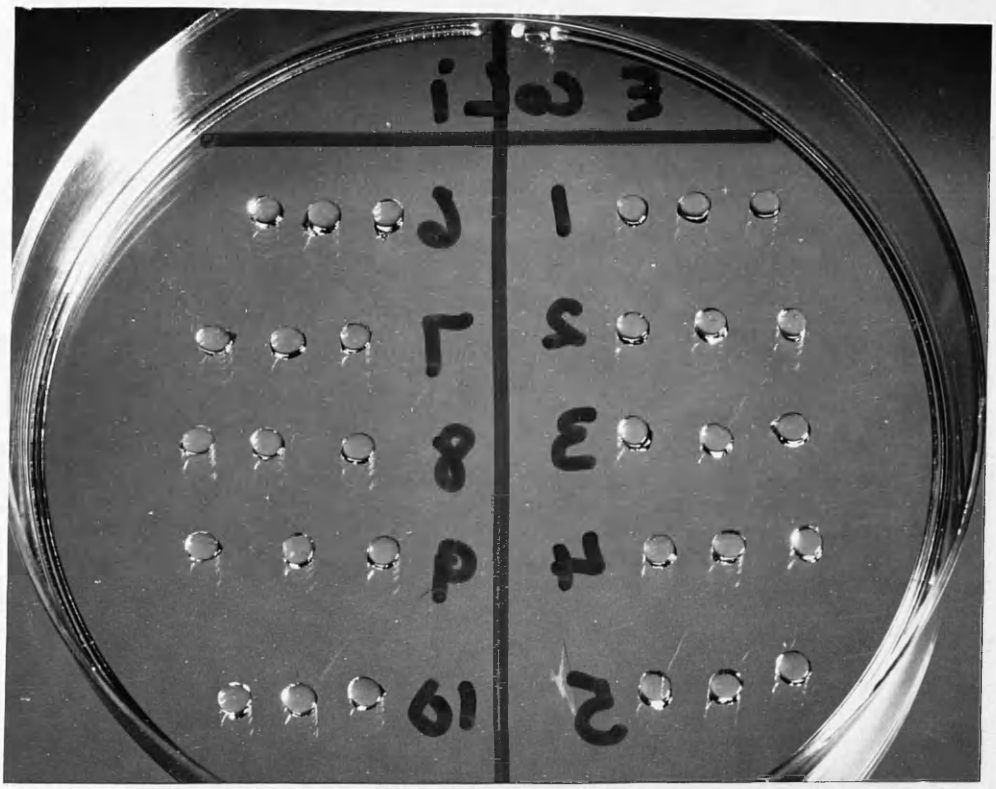

FIGURE 5.4 AGAR DISCS CONTAINING ESCHERICHIA COLI, PREPARED FOR MICROSCOPICAL EXAMINATION, AFTER OVERNIGHT INCUBATION AT 37°C IN VARIOUS SALIVARY SAMPLES.

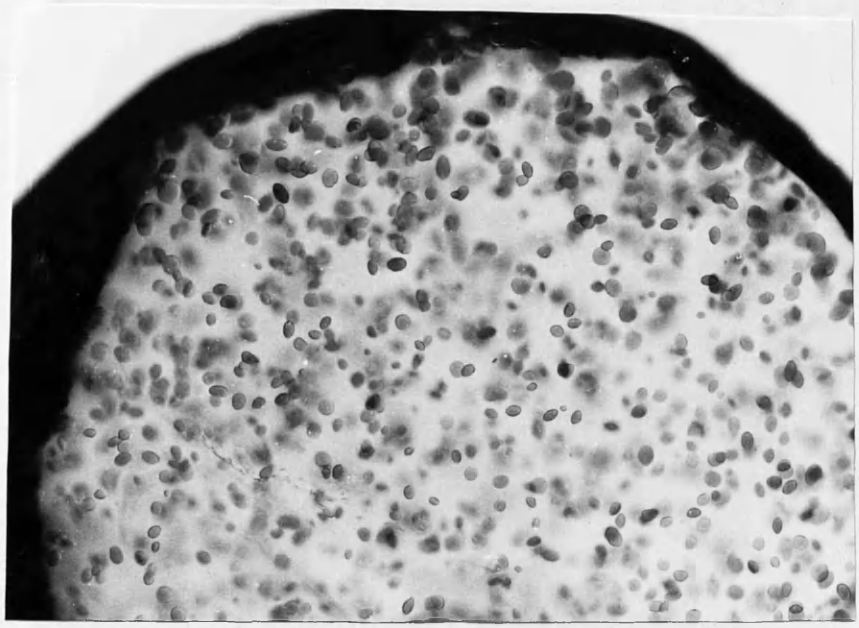

FIGURE 5.5 THE MICROSCOPICAL APPEARANCE OF AN AGAR DISC CONTAINING STAPHYLOCOCCUS AUREUS WHICH WAS INCUBATED IN BUGON BROTH FOR 18 HOURS AT 37°C. NUMEROUS COLONIES OF THE TEST MICRO-ORGANISMS CAN BE CLEARLY SEEN. (x5).

FIGURE 5.6 THE MICROSCOPICAL APPEARANCE OF AN AGAR DISC CONTAINING STAPHYLOCOCCUS AUREUS WHICH WAS INCUBATED IN MIXED SALIVA FOR 18 HOURS AT 37°C. TOTAL INHIBITION OF THE TEST MICRO-ORGANISMS HAS OCCURRED AND NO COLONIES ARE EVIDENT. (x5).

	Grade
Growth equal to or greater than Eugon Broth control disc	0
Growth greater than one quarter of the Eugon Broth control disc	1
Growth equal to or less than one quarter of the Eugon Broth control disc	2
No evidence of growth	3

TABLE 5.3 METHOD OF SCORING THE GROWTH OR INHIBITION OF GROWTH OF ESCHERICHIA COLI, STAPHYLOCOCCUS AUREUS AND CANDIDA ALBICANS

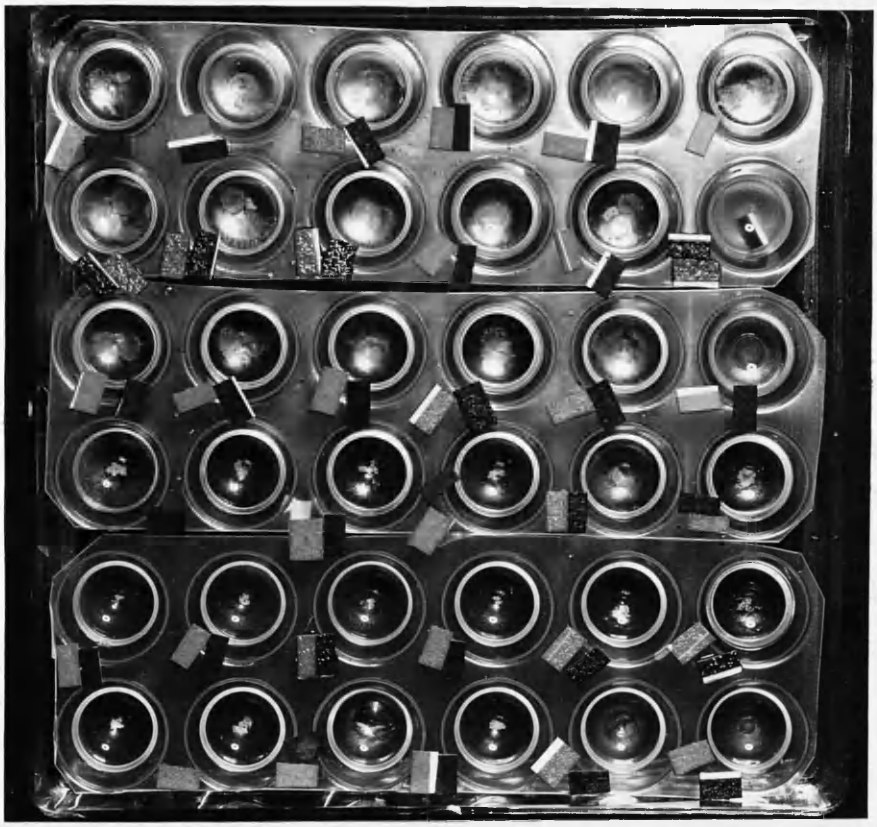

FIGURE 5.7 THE METHOD OF ASSESSING THE pH OF THE SALIVARY SAMPLES AFTER OVERNIGHT INCUBATION AT 37°C. THE LIGHT COLOURED STRIPS HAVE A pH RANGE OF 6.8-8.9, AND THE DARK COLOURED STRIPS A RANGE OF 4.2-6.8.

5.3 RESULTS

5.3.1. Sterility of Centrifuged Saliva and Heated Controls

All samples of heated mixed and centrifuged saliva were sterile on testing. Of the ten samples of non-heated centrifuged saliva, one sample was sterile, and the remaining nine samples yielded less than 5,000 colonies per ml of saliva. Since it has been estimated that mixed saliva contains on average 750 million micro-organisms per ml (Burnett and Scherp 1968), the nine centrifuged salivary samples although unsterile, contained about 150,000 times fewer organisms per ml compared with the original mixed salivary samples.

5.3.2 Escherichia coli

The results of the inhibitory action of saliva on Escherichia coli are shown in Table 5.4. Mixed saliva and mixed saliva plus Eugon Broth significantly inhibited Escherichia coli ($P \leq 0.001$) when compared to the Eugon Broth control, while the heated control mixed saliva samples possessed virtually no inhibitory activity. Centrifuged saliva, significantly inhibited Escherichia coli ($P \leq 0.001$) while centrifuged saliva plus Eugon Broth and the two heated centrifuged saliva control samples yielded no inhibitory activity. When the inhibitory activity of the mixed and centrifuged salivary samples were compared the mixed secretion possessed more inhibitory activity ($P \leq 0.01$). Eighteen (23%) of the 80 salivary samples tested completely inhibited Escherichia coli; of these 12 (66%) were bacteriostatic and 6 (33%) were bactericidal in nature. A comparison of the antibacterial activity of the mixed and centrifuged salivary specimens showed that 89% of the activity was present in the mixed salivary samples.

The pH values recorded at the start and after the completion of the experiments involving Escherichia coli are shown in Table 5.7. At the start of the experiments 74 (93%) of the salivary samples had a pH value within the range 7.6 to 6.5. At the end of the experiment 63 (79%) of the samples had a pH within the 7.6-6.5 range and 11 (13%) yielded values in excess of 7.6. The rise in pH values tended to be found in the mixed and centrifuged heated control samples to which

	Escherichia Coli				
	Salivary Sample	Total Inhibition Score 10 Subjects	Number of Samples Yielding No Growth	Number Bacteriostatic	Number Bactericidal
1	Eugon Broth Control	0	0	0	0
2	Mixed Saliva	28	8	7	1
3	Mixed Heated	4	0	0	0
4	Mixed + Eugon Broth	28	8	4	4
5	Mixed Heated + Eugon	0	0	0	0
6	Centrifuged Saliva	15	2	1	1
7	Cent. Heated	4	0	0	0
8	Cent. + Eugon Broth	2	0	0	0
9	Cent. Heated + Eugon	0	0	0	0

Salivary samples 2 v 1; 4 v 1; 6 v 1; $P = \leq 0.001$ Salivary samples 2 v 6; $P = \leq 0.01$

Salivary samples 3 v 1; 5 v 1; 7 v 1; 8 v 1; 9 v 1; Not significant

TABLE 5.4 THE TOTAL INHIBITORY SCORES OF SALIVARY SAMPLES FROM 10 SUBJECTS AGAINST E.COLI, AND DETAILS OF THE BACTERIOSTATIC OR BACTERICIDAL NATURE OF THE INHIBITION OBSERVED

pH Values	7.6	7.6-6.5	6.4-5.8	5.8
pH at start of Experiment	1	74	5	0
pH at completion of Experiment (E. coli)	11	63	0	6
pH at Completion of Experiment (S. aureus)	2	49	3	26
pH at Completion of Experiment (C. albicans)	12	56	4	8

TABLE 5.7 THE pH VALUES OF SALIVARY SAMPLES BEFORE AND AFTER THE EXPERIMENTS TO ASSESS THE ANTIMICROBIAL ACTIVITY OF SALIVA FROM 10 SUBJECTS AGAINST E.COLI, S. AUREUS AND C. ALBICANS

Eugon Broth had been added. Of the 18 salivary samples which completely inhibited the growth of Escherichia coli, 17 (94%) had a final pH of 7.6-6.5.

5.3.3 Staphylococcus aureus

The results of the inhibitory action of saliva on Staphylococcus aureus are shown in Table 5.5. Mixed saliva and mixed saliva plus Eugon Broth significantly inhibited Staphylococcus aureus ($P \leq 0.001$) when compared to the Eugon Broth control. The inhibitory activity of the heated mixed salivary samples was significant ($P \leq 0.01$) while the heated mixed salivary samples plus Eugon Broth possessed virtually no inhibitory activity. However, when the inhibitory activity of the mixed salivary samples was compared with that of the heated mixed salivary control samples, the difference was highly significant ($P \leq 0.001$). Centrifuged saliva and heated centrifuged saliva significantly inhibited the growth of Staphylococcus aureus ($P \leq 0.001$) when compared to the Eugon Broth controls, but when the inhibitory activity of the centrifuged samples was compared with that of the heated centrifuged controls the difference was significant ($P \leq 0.01$). Centrifuged saliva plus Eugon Broth and the heated control samples plus Eugon Broth possessed almost no inhibitory activity. When the inhibitory activity of mixed and centrifuged saliva was compared, no significant difference was found. However, when the inhibitory activity of mixed saliva plus Eugon Broth was compared to centrifuged saliva plus Eugon Broth, the mixed secretion possessed significantly more inhibitory activity ($P \leq 0.001$). Twenty seven (34%) of the 80 salivary samples tested completely inhibited Staphylococcus aureus; of these 18 (66%) were bacteriostatic and 9 (33%) bactericidal in nature. A comparison of the antimicrobial activity of the mixed and centrifuged salivary samples showed that 74% of the activity was present in the mixed salivary samples.

The pH values recorded at the start and after the completion of the experiments involving Staphylococcus aureus are shown in Table 5.7. At the start of the experiments 74 (93%) of the salivary samples had a pH value within the range 7.6-6.5. At the end of the experiments 49 (61%) of the samples had a pH within the 7.6-6.5 range and 26 (33%)

	Staphylococcus Aureus				
	Salivary Sample	Total Inhibition Score 10 Subjects	Number of Samples Yielding No Growth	Number Bacteriostatic	Number Bactericidal
1	Eugon Broth Control	0	0	0	0
2	Mixed Saliva	30	10	6	4
3	Mixed Heated	7	0	0	0
4	Mixed + Eugon Broth	30	10	5	5
5	Mixed Heated + Eugon	0	0	0	0
6	Centrifuged Saliva	23	6	6	0
7	Cent. Heated	11	1	1	0
8	Cent. + Eugon Broth	2	0	0	0
9	Cent. Heated + Eugon	0	0	0	0

Salivary samples 2 v 1; 4 v 1; 6 v 1; 7 v 1; 2 v 3; $P = < 0.001$
Salivary samples 3 v 1; 6 v 7; $P = < 0.01$
Salivary samples 5 v 1; 8 v 1; 9 v 1; Not significant

TABLE 5.5 THE TOTAL INHIBITORY SCORES OF SALIVARY SAMPLES FROM 10 SUBJECTS AGAINST S. AUREUS, AND DETAILS OF THE BACTERIOSTATIC OR BACTERICIDAL NATURE OF THE INHIBITION OBSERVED

yielded values less than 5.8. The fall in pH value tended to be found in the heated centrifuged samples, and in the mixed and centrifuged heated samples which contained Eugon Broth. Of the 27 salivary samples which completely inhibited Staphylococcus aureus, 22 (81%) had a final pH of 7.6-6.5.

5.3.4 Candida Albicans

The results of the inhibitory action of saliva on Candida albicans are shown in Table 5.6. Although none of the salivary samples completely inhibited the growth of the yeast, mixed saliva consistently markedly inhibited the growth of Candida albicans and this inhibitory activity was significant when compared with the Eugon Broth control, and all the other salivary samples ($P \leq 0.001$). The inhibitory activity of the centrifuged samples ($P \leq 0.01$), the heated centrifuged samples ($P \leq 0.025$), and the heated mixed salivary samples ($P \leq 0.05$), were significant when compared with the Eugon Broth controls. However when the inhibitory activity of these samples was compared with one another, no significant difference was demonstrated.

The agar discs from the mixed salivary specimens which showed marked inhibition of Candida albicans were examined to assess the type of inhibition, and this proved to be fungistatic, although as a general rule fewer colonies of yeasts were noted compared to the Eugon Broth controls.

The pH values recorded at the start and after the completion of the experiments involving Candida albicans are shown in Table 5.7. At the start of the experiment 74 (93%) of the salivary samples had a pH value within the range 7.6-6.5. At the end of the experiments 56 (70%) of the salivary samples had a pH value within the range 7.6-6.5; 12 (15%) had a pH greater than 7.8, and 8 (10%) had a pH of less than 5.8. The rise in pH was due mainly to the results obtained from one individual where a final pH of 8.1 was recorded for eight of the ten samples tested. Of the ten mixed salivary samples which markedly inhibited the growth of Candida albicans 8 (80%) had a final pH of 7.6-6.5.

	Candida Albicans		
	Salivary Sample	Total Inhibition Score 10 Subjects	Number of Samples Yielding No Growth
1	Eugon Broth Control	0	0
2	Mixed Saliva	19	0
3	Mixed Heated	7	0
4	Mixed + Eugon Broth	4	0
5	Mixed Heated + Eugon	0	0
6	Centrifuged Saliva	9	0
7	Cent. Heated	8	0
8	Cent. + Eugon Broth	0	0
9	Cent. Heated + Eugon	0	0

Salivary samples 2 v 1; 2 v 6; $P = < 0.001$

Salivary sample 6 v 1; $P = < 0.01$

Salivary sample 7 v 1; $P = < 0.025$

Salivary sample 3 v 1; $P = < 0.05$

TABLE 5.6 THE TOTAL INHIBITORY SCORES OF SALIVARY SAMPLES FROM 10 SUBJECTS AGAINST C.ALBICANS

5.4 DISCUSSION

5.4.1 Advantages of the New Technique Compared to Previous Methods of Assessing the Antimicrobial Activity of Saliva

The Well Plate Method

In the well plate method as described by Bibby et al (1938) and Kerr and Wedderburn (1958), the test organisms under test were added to a variety of complex solid culture media, in a concentration such that almost confluent growth resulted after incubation. Discs of agar were removed from the pour plate using a hollow sterile metal tube and saliva (usually about 0.1 ml) was added to fill the well or reservoir thus produced. Usually the plates were stored at 4°C for 24-48 hours after the salivary samples had been added to allow the antibacterial factors in saliva to diffuse into the agar medium and contact the test micro-organism. The plates were then incubated at 37°C for one to two days, and examined macroscopically for evidence of inhibition of growth around the saliva filled wells. In some instances the diameter of the zone of inhibition was measured in an attempt to quantitate the salivary antibacterial activity, but generally the results were so variable that only the presence or absence of growth was noted.

There are a number of technical factors which complicate the interpretation of the results obtained using the well plate method of assessing the antimicrobial activity of saliva. Since bacterial culture media are complex mixtures of protein, carbohydrate, lipid and inorganic salts, the possibility of an interaction occurring between the culture medium and saliva is always a possibility. In addition, the test micro-organisms may be stimulated to produce biologically active substances when growing in a culture medium which they do not produce in vivo. The interaction of saliva, culture medium and test micro-organism, may inhibit or falsely stimulate the production of various antibacterial factors. In the new technique the test micro-organisms are embedded in inert agar, and the agar discs are added directly to the salivary sample. Since no culture medium is employed the possibility of an interaction between the culture medium, test micro-organism and salivary sample is eliminated.

In the well plate method it is difficult to ensure that complete diffusion of the test solution into the culture medium occurs satisfactorily. If care is not taken when cutting and removing the agar discs to form the wells, the agar surrounding the well may be pulled away from the base of the Petri dish. When the test fluid is added to the well it may escape between the lower surface of the agar and the base of the Petri dish. The surface area of agar which allows diffusion of the test fluid is limited to the inner surface of the well and in a thin agar plate, this may be relatively small. The viscosity of saliva varies among individuals and if very viscous saliva is under test it may prove impossible to fill the well with the exact amount of saliva with the result that a marked under- or overfilling of the well occurs. In addition, it is likely that viscous saliva will diffuse more slowly into agar than "watery" saliva. In the well plate technique therefore the presence or absence of a zone of inhibition may be due not to differences in the antibacterial activity of saliva, but to differences in the viscosity of saliva. In an attempt to control differences in the diffusion rate of salivary specimens, a pre-incubation period of 24-48 hours at $4^{o}C$ has been used.

The new technique requires no pre-diffusion period; the salivary samples are set up and incubated at $37^{o}C$ within a few hours of their collection. The problems associated with diffusion of salivary samples are greatly reduced in the new technique since the agar discs containing the test micro-organism are thin (1 mm) and the test fluid is able to diffuse into the disc on all sides compared to the very limited area available for diffusion in the well plate method. The new technique mimics the in vivo situation relatively accurately since the test micro-organism is separated from the test fluid by only a thin layer of inert agar, and there is a large volume of saliva relative to the number of test micro-organisms. The well plate method, on the other hand, bears little similarity to the in vivo situation, since a small volume of saliva reacts with a large number of test bacteria which are embedded in a complex solid culture medium. Two other advantages of the new technique are that the effect of pH in the experimental situation, and the bacteriostatic or bactericidal nature of the inhibition noted, can be ascertained. The equipment used in

the new technique is standard in most laboratories and a large number of tests can be carried out in a relatively short period of time. A possible initial disadvantage of the new technique is the difficulty which may be encountered in working with the small discs of agar. This technique can prove somewhat frustrating and time consuming at first, but with a little practice and experience the technique becomes relatively simple.

Direct Contact Methods

There are two main methods of assessing the antimicrobial activity of saliva using direct contact methods. In the first method a standard inoculum of the test micro-organism is added to the salivary specimen and the mixture incubated at 37°C. Aliquots of the mixture are removed at the start and at various times during the period of incubation, and plated out on solid culture media. After overnight incubation the number of colonies of the test micro-organism which were isolated from the various samples removed during the experimental period are counted. If inhibition of the test micro-organism has taken place, the numbers of the test organism isolated from the test fluid during the experimental period decrease with time.

There are a number of potential inaccuracies in the direct contact method described above. There are inherent gross inaccuracies in enumerating bacteria by colony counting techniques and the results must be critically examined by statistical analysis if significant results are to be obtained. Inaccuracies in bacterial counting may occur in the direct technique especially when biological fluids are being tested, due to agglutination of the test micro-organism by the test fluid. In colony counting techniques it is usually assumed that one colony arises from the growth of one bacterium, but if agglutination has occurred during the experiment, then one colony may arise from a clump of ten or twenty micro-organisms. If the possibility of agglutination is not considered, the decrease in the numbers of the test organism may be attributed falsely to the antibacterial activity of the test fluid. The importance of this problem in assessing the anti-candidal activity of serum has been demonstrated by Chilgren et al (1968). The direct contact technique is close to the in vivo

situation, but there are difficulties in interpretating the results, and unless great care is taken, the conclusions reached may be of questionable accuracy. The new technique does not suffer from these difficulties. The growth or lack of growth of the test micro-organisms is assessed in the new technique by direct microscopical means, which is more accurate, reproduceable, and less time consuming compared to the colony counting techniques. The new method is unaffected by agglutination since the test micro-organism is firmly embedded within an agar gel.

In the second method of assessing the antimicrobial activity of saliva using a direct contact technique, a reduction in the numbers of the test micro-organism added to the salivary secretion is assessed by measuring the change in turbidity of the mixture during a period of hours using a spectrophotometer (Dogon and Amdur 1965; Klebanoff and Luebke 1965). This method can be used only with pure, or centrifuged and filtered salivary secretions, since the presence of micro-organisms or human cells would invalidate the results. Agglutination of the test organism by the test fluid during the experimental period could invalidate the results of this system. Since mixed saliva cannot be used when a turbidmetric method of assessing microbial inhibition is used, the results of experiments using this technique cannot be taken to represent the in vivo situation.

5.4.2 Inhibition of Escherichia coli by Saliva

Inhibition of Escherichia by saliva has been described by a number of workers; Van Kestern et al (1942), Bjornesjo (1950), Klebanoff et al (1966), and Hamon and Klebanoff (1973).

Using a well plate technique to investigate the antimicrobial factors of saliva, Van Kestern et al (1942) showed that mixed and centrifuged mixed saliva inhibited a number of micro-organisms including Escherichia coli. Centrifuged saliva tended to be less potent than uncentrifuged saliva, and heating the salivary specimen at 75°C for five minutes abolished the antibacterial activity. By treating the salivary samples in various ways before testing them for antibacterial activity, Van Kestern and her co-workers were able to

conclude that saliva contained at least two antibacterial factors, one which resembled lysozyme and had no inhibitory effect on Escherichia coli and a second factor distinct from lysozyme which could inhibit Escherichia coli.

Bjornesjo (1950) using both a well plate technique and a direct contact method, showed that the inhibitory activity of saliva against Escherichia coli was more likely to be due to competition between the commensal oral flora and Escherichia coli for available nutrients than to the action of intrinsic salivary antibacterial factors. If we accept Bjornesjo's conclusions about the inhibitory nature of saliva the results of the present study should have shown that the inhibitory activity of mixed saliva was greater than that of centrifuged saliva, due to the virtual absence of commensal micro-organisms in the centrifuged salivary samples. The addition of Eugon Broth to the mixed salivary samples should have abolished the inhibitory activity of these secretions since sufficient nutrients would have then been available to support the growth of E. coli and the commensal microflora. The results of the present study show that the difference in inhibition between the mixed and centrifuged samples of saliva was significant ($P = <0.01$), but the addition of Eugon Broth to the mixed salivary specimens did not abolish the inhibitory activity of these samples. It is possible, but unlikely, that the Eugon Broth added to the mixed saliva samples was so rapidly utilized by the commensal flora that no nutrients were available for E. coli. A more likely explanation is that the salivary flora produced antibacterial substances against E. coli both in mixed saliva itself and when Eugon Broth was added. It could be argued that since nine out of the ten centrifuged samples of saliva were not sterile, sufficient salivary bacteria would have been present to compete with E. coli for the available nutrients. The fact that the total inhibitory score for the centrifuged salivary samples was 15 out of a possible score of 30 could be taken to support that hypothesis. However, if there were sufficient micro-organisms present in the centrifuged samples to successfully compete with E. coli for available growth factors, then when Eugon Broth was added, marked inhibition of the test organism should have resulted as was the case in the mixed salivary samples with added Eugon Broth. In fact the samples

of centrifuged saliva plus Eugon Broth had no inhibitory activity and this suggests that the small numbers of salivary organisms present in these samples were insufficient to inhibit the growth of E. coli by competitive means. Another possible explanation for the inhibitory activity of the centrifuged salivary samples is that centrifugation removes many growth factors from mixed saliva. Centrifuged saliva would then be deficient in growth factors for E. coli and it would be reasonable to expect that heated centrifuged saliva would also be deficient. This was not the case, since no inhibition occurred in the heated centrifuged salivary samples. The probable explanation of this finding is that boiling saliva liberates growth factors normally unavailable for bacterial growth.

Klebanoff et al (1966) described a peroxidase-thiocyanate-hydrogen peroxide system in parotid saliva which inhibited the growth of Escherichia coli. Peroxidase and thiocyanate are normally present in pure parotid saliva but hydrogen peroxide is not normally present and must be added to activate the system. In vivo the source of hydrogen peroxide is probably certain members of the oral commensal flora, since Hamon and Klebanoff (1973) have shown that Streptococcus mitis can produce hydrogen peroxide in sufficient quantity to activate the peroxidase-thiocyanate system. Hamon and Klebanoff (1973) using a direct contact method and measuring inhibition by spectrophotometric means, showed that saliva which had been centrifuged and sterilized by filtration, failed to inhibit the growth of Escherichia coli, but when Streptococcus mitis was added to the salivary samples, the growth of Escherichia coli was markedly inhibited. These workers also showed that heated saliva plus S. mitis supported the growth of E. coli but when lactoperoxidase was added to this system inhibition of the test organism occurred. Hamon's explanation of these results was that the peroxidase-thiocyanate-hydrogen peroxide system was responsible for the inhibition of E. coli, the hydrogen peroxide being supplied by S. mitis. However there is reason to believe that this peroxidase system may not be active in vivo. It is known that catalase can inactivate the system by decomposing the hydrogen peroxide component. Catalase has been demonstrated in saliva (Deakins 1934 and 1941) and it is known that a number of oral commensal bacteria are catalase

producers; Neisseria and diphtheroids (Cowan and Steele 1974), Haemophilii (Sims 1970) and Staphylococcus salivarius (Gordon 1967). Since it has been estimated that one molecule of catalase can decompose 2,000,000 molecules of hydrogen peroxide in one minute, it is clear that very little catalase would be required to inactivate the peroxidase antibacterial system (West and Todd 1961). Klebanoff et al (1966) has shown that ascorbic acid and cysteine can inhibit the peroxidase system. Ascorbic acid is present in mixed saliva (Chauncy et al 1954) and cysteine is present in the Eugon Broth used in this study (see Appendix 2).

Assuming that Hamon and Klebanoff's peroxidase system can function in vivo and as mentioned earlier there is doubt about this assumption, the results of the present study should have shown marked inhibition by the mixed salivary samples, and reduced inhibition when Eugon Broth was added, due to inactivation of the peroxidase system by the cysteine which is present in Eugon Broth. The centrifuged salivary samples should have possessed no inhibitory activity since the oral flora which produces the hydrogen peroxide component of the peroxidase system had been removed by centrifugation. However the addition of Eugon Broth to mixed saliva did not abolish the antimicrobial activity of these samples, and partial inhibition of E. coli by the centrifuged salivary samples was noted. Therefore, the results of the present study do not agree with the results which might be expected if the peroxidase system was the main inhibitory factor in the inhibition of E. coli by saliva.

The present study has demonstrated that mixed saliva can inhibit the growth of E. coli and that the inhibitory activity is more bactericidal than bacteriostatic (Table 5.4). It is unlikely that inhibition of E. coli by saliva was due to adverse pH conditions since almost all the salivary samples which caused inhibition had a pH both before and after the experiment in the range 7.6-6.5, which is within the pH range for growth of E. coli. This study suggests that the production of antibacterial substances and competitive inhibition of E. coli for limited nutrients by the commensal salivary flora are responsible for the inhibitory action of saliva on E. coli. The

peroxidase system described by Hamon and Klebanoff (1973) appears to play little part in this inhibitory activity.

5.4.3 Inhibition of Staphylococcus aureus by Saliva

Inhibition of S. aureus by saliva has been described by a number of workers; Bibby et al (1938), Van Kestern et al (1947), Bjornesjo (1950), Kerr and Wedderburn (1958), and Hamon and Klebanoff (1973).

Bibby et al (1938) concluded from their investigations that oral micro-organisms had little importance in the antimicrobial activity of saliva, since mixed and centrifuged saliva were equally potent. However they also showed that the inhibitory activity of the centrifuged deposit of saliva which was rich in bacteria was only slightly less potent than that of mixed and of centrifuged saliva in inhibiting the growth of Staphylococci.

Van Kestern (1942) in a study on the properties of the antibacterial factors of saliva reported that the factor which inhibited the growth of S. aureus was inactivated by heat ($75^{o}C$ for 5 minutes) but was unaffected by filtration. They concluded that the factor was not lysozyme, but was similar to the factor which inhibited lactobacilli.

Bjornesjo (1950) assessed the antimicrobial activity of saliva against two Staphylococcal strains and concluded that inhibition was caused by competition between the commensal flora and the Staphylococci for the limited nutritional factors present in saliva. He showed that while Staphylococcus aureus proliferated slowly in heat or filter sterilized mixed saliva, the addition of 0.2% peptone water to the salivary samples resulted in a marked increase in the growth of S. aureus. Bjornesjo demonstrated that if the concentration of mixed saliva in a peptone/mixed saliva mixture was less than 25%, the inhibitory activity of the saliva was reduced.

If the inhibition of S. aureus was due to lack of essential nutrients in saliva, or competition for available nutrients between

S. aureus and the commensal microflora, or a combination of both as suggested by Bjornesjo (1950) then the results of the present study should have shown the following pattern of inhibition: mixed and centrifuged saliva should have inhibited the growth of S. aureus, where as mixed saliva plus Eugon Broth and centrifuged saliva plus Eugon Broth should have possessed no inhibitory activity. The results of the present study agree with this hypothesis, except that mixed salivary samples plus Eugon Broth markedly inhibited S. aureus. It is possible that since the composition of these samples consisted of 0.1 ml saliva and 0.1 ml Eugon Broth the oral commensal flora was still able to compete successfully for the additional nutrients and so inhibit the growth of S. aureus. The other interpretation of these results is that in addition to competitive inhibition, the salivary microflora can produce antimicrobial substances which are active against S. aureus in mixed saliva and that the addition of Eugon Broth stimulates rather than inhibits the production of these substances. The slight inhibitory activity of the heated mixed and heated centrifuged samples, is probably due to variations in the concentration of growth factors in the salivary samples tested from different individuals.

Kerr and Wedderburn (1958) using a well plate technique demonstrated antibacterial activity against S. aureus in 66% of pure parotid salivary samples, 75% of submaxillary secretions, and in 18% of mixed salivary samples. The marked difference in inhibitory activity between the pure salivary secretions and the mixed salivary samples suggests that the inhibitory factor is inactivated when it enters the oral environment. These results suggest that antimicrobial factors isolated from pure salivary secretions cannot be assumed to be fully active in vivo. Generally speaking, Kerr and Wedderburn's results show that pure salivary secretions possess antimicrobial activity which is independent of the oral microflora.

It is interesting to note that Williams and Powlen (1959) have shown that pure parotid saliva cannot support the growth of S. aureus, and at pH values above 7.8 is bactericidal. If the pH is less than 7.8, or the saliva is artificially buffered, the

parotid secretions exert a bacteriostatic effect on the Staphylococcus.

Hamlon and Klebanoff (1973) have shown that peroxidase, thiocyanate and hydrogen peroxide could inhibit the growth of S. aureus. They also demonstrated the inhibitory effect of catalase on the peroxidase system. Since Staphylococci are strongly catalase positive it is surprising that the peroxidase system is able to inhibit the growth of S. aureus. As mentioned earlier (5.4.2) it is unlikely that the peroxidase system is active in vivo.

If the peroxidase system was the main inhibitory factor involved in the present technique, one would have expected inhibition of S. aureus by the mixed salivary samples, no inhibition by the mixed plus Eugon Broth samples due to the inhibitory effect of cysteine on the peroxidase system, and no inhibition by the centrifuged samples due to a lack of hydrogen peroxide producing bacteria in these samples. Since all these samples inhibited the growth of S. aureus in the present study, it would appear unlikely that the peroxidase system was involved in inhibiting S. aureus.

The results of the present study have shown that mixed and centrifuged saliva can inhibit the growth of S. aureus and that the inhibitory activity is more bacteriostatic than bactericidal. It is unlikely that inhibition of S. aureus was due to adverse pH conditions since almost all the salivary samples which caused inhibition had a pH both before and after the experiment in the range 7.6-6.5, which is within the pH range for growth of S. aureus. The present study suggests that lack of essential nutrients in saliva, competitive inhibition and the production of antimicrobial substances by the salivary microflora are responsible for the inhibitory action of saliva on S. aureus. The peroxidase system described by Hamon and Klebanoff (1973) appears to play little part in this inhibitory activity.

5.4.4 Inhibition of Candida albicans by Saliva

There is relatively little information concerning the inhibition of Candida albicans by saliva.

Williams and Powlen (1959) have shown that Candida albicans remained viable in parotid saliva with no significant change in total numbers for up to 72 hours. Lehrer (1969) described an antifungal system consisting of human myeloperoxidase, hydrogen peroxide and either potassium iodide, sodium chloride or potassium bromide, which was rapidly lethal to C. albicans. Since sodium chloride, hydrogen peroxide and a peroxidase are present in saliva, it is possible that this system may regulate the number of yeasts in the mouth. However, Knight and Fletcher (1971) have shown that this peroxidase system is not active in mixed saliva against C. albicans. Knight and Fletcher (1971) presented experimental data which suggested that the inhibition of C. albicans by mixed saliva was due to depletion of the glucose content of saliva by oral micro-organisms. They showed that the addition of glucose to mixed saliva abolished the inhibitory effect and permitted C. albicans to proliferate.

Hamon and Klebanoff (1973) inhibited the growth of Candida tropicalis with a system which consisted of lactoperoxidase iodide and Streptococcus mitis. However, the concentration of iodide required to activate this system was in excess of that normally present in saliva. Since catalase inhibits this system, it is unlikely that it is active in vivo.

If depletion of glucose by salivary organisms is important in the inhibition of C. albicans by saliva, one would have expected the following results in the present study; inhibition by mixed saliva, no inhibition by mixed saliva plus Eugon Broth since glucose is present in that culture medium, and no inhibition by the centrifuged salivary samples since virtually all the salivary bacteria had been removed. The results of the present study agree with those postulated, except that the centrifuged salivary samples and the centrifuged salivary samples plus Eugon Broth were moderately inhibitory to C. albicans. A possible explanation of the inhibitory effect of the centrifuged samples is that centrifugation tends to reduce specific growth factors from the mixed secretion which are necessary for maximum growth of C. albicans.

The results of the present study show that only mixed saliva had the ability to inhibit the growth of C. albicans to any extent. Centrifuged saliva also possessed some inhibitory activity, but this was significantly less than that of mixed saliva ($P = <0.001$). It is unlikely that inhibition of C. albicans by saliva was due to adverse pH conditions since almost all the salivary samples which caused inhibition had a pH before and after the experiment in the range 7.6-6.5 which is within the pH range for growth of C. albicans. This study suggests that competition between the salivary microflora and C. albicans for growth factors, probably glucose, is responsible for the inhibitory action of saliva on C. albicans

5.4.6 The Antimicrobial Activity of Saliva and the Flora of Patients with Severe Sjögren's Syndrome

The present study has shown that mixed saliva does not support the growth of E. coli, S. aureus and C. albicans, due to the combined effect of a number of factors; insufficient nutrients in saliva, competitive inhibition of the test micro-organisms by the salivary microflora and the production of antimicrobial factors by salivary commensal bacteria. The antibacterial factors which are derived from the salivary secretions appeared to have little inhibitory effect on the micro-organisms studied. Thus the appearance of Staphylococci, Coliforms and yeasts in the oral flora of patients with severe Sjögren's syndrome would appear to be related to changes in the commensal microflora rather than to loss of the intrinsic anti-microbial factors which are present in saliva.

Normally the oral tissues are coated with a layer of saliva which in nutritional terms would appear to bearly support the growth of E. coli, S. aureus and C. albicans. This layer is constantly renewed and cleansed by the mechanical washing action of saliva. Any nutrients which are available are quickly utilized by the vast numbers of commensal oral bacteria which in addition may secrete antibacterial substances against non-commensal micro-organisms. In patients with severe Sjögren's syndrome a layer of saliva does not coat the surface of the oral tissues, and the mechanical washing action of saliva is severely impaired. The environment of the

surface of the oral mucosa consists of areas of stagnant saliva, desquamated epithelial cells, and food debris. As shown in Chapter 4, the commensal microflora coating the surface of the mucosa is drastically reduced qualitatively and probably quantitatively. The ability of the microflora which remains to compete with non-commensal micro-organisms for available growth factors and to produce anti-microbial substances is also probably reduced. The oral environment of patients with severe Sjögren's syndrome would appear therefore to be a suitable niche for the proliferation of certain non-commensal micro-organisms, and with virtually no protective mechanisms in operation, long term colonization may occur.

5.5 CONCLUSIONS

The new technique developed to assess the antimicrobial activity of saliva is rapid, simple to perform and gives clear cut results. The pH of the test solutions before and after incubation can be measured and an assessment of the bactericidal or bacteriostatic nature of the inhibition observed can be made.

Inhibition of E. coli and S. aureus by saliva appeared to be due to a combination of insufficient nutrients in saliva, competitive inhibition of the test micro-organisms by the salivary microflora and the production of antimicrobial factors by the salivary commensal bacteria.

Inhibition of C. albicans appeared to be due to competition between the commensal oral flora and C. albicans for available nutrients, probably glucose.

The results of this study suggest that the appearance of S. aureus, Candida species and Coliforms in the mouth of patients with severe Sjögren's syndrome is due to changes in the commensal microflora and the environment of the mouth, rather than the loss of inhibitory factors derived from saliva.

THE ANTIMICROBIAL ACTIVITY OF ELEVEN MEMBERS OF THE ORAL COMMENSAL MICROFLORA AGAINST ESCHERICHIA COLI, STAPHYLOCOCCUS AUREUS, AND CANDIDA ALBICANS

6.1 INTRODUCTION

The results of the investigations described in Chapters 2, 4 and 5, have tended to suggest that the appearance of yeasts, staphylococci and coliforms in the oral flora of patients with Sjogren's syndrome, was closely related to marked changes in the commensal oral flora, and in the host tissues. There have been a number of reports which have shown that alpha-haemolytic streptococci from the oro-pharynx of healthy individuals possess the ability to inhibit the growth of Staphylococcus aureus (Myers 1959; Sanders 1969) and various coliform bacilli (Sprunt and Redman 1968). Also it has been shown that if the number of alpha-haemolytic streptococci in the mouth was reduced by antibiotic therapy, Staphylococcus aureus, various coliforms and Candida species invaded and colonized the oro-pharynx (Myers 1959; Sprunt and Redman 1968; Seelig 1966). When the antibiotic therapy was discontinued the number of alpha-haemolytic streptococci returned to normal, and the staphylococci, coliforms and yeasts were eliminated from the oro-pharyngeal region. From these reports it would appear that microbial antagonistic mechanisms play an important part in protecting the mouth against the invasion of potential pathogens.

There is little detailed knowledge concerning the identity of the micro-organism involved in microbial antagonistic protective mechanisms. The experiments described in this Chapter were carried out in an attempt to identify the members of the oral flora which possessed the ability to inhibit the growth of Escherichia coli, Staphylococcus aureus and Candida albicans. It was decided to use the laboratory technique which had been developed to assess the antimicrobial activity of saliva (Chapter 5) in the present study; broth fluid cultures of the commensal oral bacteria being substituted for the salivary samples.

6.2 MATERIALS AND METHODS

6.2.1 Oral Commensal Bacteria

The following Type cultures (National Collection of Type Cultures, Colingdale, London) were used to assess the inhibitory activity of the oral microbial flora against Escherichia coli, Staphylococcus aureus and Candida albicans: Streptococcus mitis (N.C.T.C. 10712 F.W. 75), Streptococcus sanguis (N.C.T.C. 10904 - 804), Streptococcus sanguis (N.C.T.C. 7864), Streptococcus salivarius (N.C.T.C. 7366), Streptococcus milleri (N.C.T.C. 10708), Streptococcus mutans (N.C.T.C. 10449), Neisseria pharyngis (N.C.T.C. 4590), Neisseria catarrhalis (N.C.T.C. 4103), Corynebacterium flavidum (N.C.T.C. 764), Corynebacterium hofmanii (N.C.T.C. 231) and Staphylococcus salivarius (N.C.T.C. 189).

Pure cultures of the Type micro-organisms in Todd-Hewitt Broth, Eugon Broth and heated mixed saliva were used to assess their anti-microbial activity against Escherichia coli, Staphylococcus aureus and Candida albicans. Todd-Hewitt Broth was used since it supported the growth of all the type cultures under test and in addition contained a bicarbonate-phosphate buffer which ensured that the pH of the medium during microbial growth remained in the region of 7.0. It was thought that the presence of the buffer system would prevent inhibition of the test micro-organisms due to adverse pH conditions produced by the growth of the commensal bacteria. The constituents of Todd-Hewitt Broth (T.H.B.) are shown in Appendix 3. Eugon Broth also supported the growth of all the type micro-organisms used in the experiments, but contained dextrose and possessed no buffer system. In the case of Eugon Broth therefore, the fall in pH produced by the fermentation of dextrose by the commensal bacteria could be sufficient to cause inhibition of the test micro-organisms. The constituents of Eugon Broth are shown in Appendix 2.

Heated mixed saliva was used to simulate the environment of the mouth. Saliva was collected from one individual (a 32 year old dentate male) between 9.00 a.m. and 10.00 a.m. on the day of the experiment. Non-stimulated saliva was used, and after heating for

20 minutes at 100°C, the saliva was tested for sterility by adding 0.02 ml to a blood agar plate which was incubated aerobically at 37°C for 2 days. From the results of Chapter 5 it was known that heated mixed saliva supported the growth of Escherichia coli, Staphylococcus aureus and Candida albicans. However, it was not known whether heated mixed saliva would support the growth of the oral commensal Type bacteria used in this experiment. Since all the organisms are commensals of the mouth, and with the exception of Streptococcus mutans and Corynebacterium flavidum, commonly isolated from saliva, it was assumed that heated mixed saliva would either support the growth of the bacteria or prove to be bacteriostatic.

Each week the commensal type cultures were subcultured onto blood agar plates, incubated at 37°C for 18 hours and then stored at 4°C. A heavy inoculum was used for subculture, rather than a single colony, to prevent the emergence of atypical mutants. On the day before the experiment was to be performed, the type cultures were plated out on blood agar plates and incubated overnight at 37°C. The next morning using a standard Nichrome wire loop (Medical Wire and Equipment Company (Bath) Ltd., Wiltshire), 0.25 ml of the appropriate culture medium was added to an area of the blood agar culture where a confluent growth of the commensal organism was present. The bacterial colonies which were covered by the culture medium were emulsified in the fluid and a loopful of the mixture was transferred to 2 ml of the same culture medium in a glass bijou bottle. The cultures were thoroughly agitated using a Whirlimixer (Fisons Scientific Apparatus, Leicestershire) and then incubated at 37°C for four hours. At the end of the incubation period, 0.2 ml of each culture was added to the appropriate well of a plastic World Health Organization serology plate which had been sterilized and prepared as described in Chapter 5 5.2.

6.2.2 Pour Plates and the Test Micro-organisms

The preparation of pour plates and the small agar discs containing the test organisms, Escherichia coli (N.C.T.C. 10418), Staphylococcus aureus (N.C.T.C. 6571) and Candida albicans (M.R.L. 3188C) was carried out as described in Chapter 5 5.2. Three

discs containing either Escherichia coli, Staphylococcus aureus or Candida albicans were added to each well using a hooked dental syringe needle. The serology plates were placed in a moist chamber and incubated at 37°C for 18 hours. The discs were removed from the wells and examined for the presence or absence of growth as described in Chapter 5 5.2. The pH of each well was measured at the end of each experiment using small strips of pH indicator paper (Merk). No attempt was made to assess the bactericidal or bacteriostatic nature of the inhibitory activity observed.

Discs containing either Escherichia coli, Staphylococcus aureus or Candida albicans, were added to 0.2 ml amounts of each of the three culture media used in the experiments to act as controls. The pH of each control was measured at the end of the experimental period. The degree of growth which occurred in the test cultures was compared with the growth which occurred in the control cultures and graded as follows: (a) growth equivalent to that of the control disc; (b) partial inhibition, i.e. growth less than the control, and (c) complete inhibition of growth when compared with the control cultures.

6.3 RESULTS

6.3.1 The Growth of Escherichia coli, Staphylococcus aureus and Candida albicans in Todd-Hewitt Broth, Eugon Broth and Heated Mixed Saliva

In all instances, an almost confluent growth of the test organisms in the agar discs was observed in the three culture media. The final pH values of the test organisms in the different culture media after incubation at 37°C for 18 hours are shown in Table 6.1. The pH values of the Todd-Hewitt Broth and heated mixed saliva cultures tended to be alkaline whereas the Eugon Broth cultures were markedly acid.

6.3.2 The Inhibitory Activity of Type Cultures of Oral Commensal Bacteria in Todd-Hewitt Broth, Eugon Broth and Heated Mixed Saliva against Escherichia coli

The results of this series of experiments are shown in Table 6.2. The T.H.B. cultures of Streptococcus mitis and Streptococcus

	Escherichia Coli	Staphylococcus Aureus	Candida Albicans
Todd-Hewitt Broth	8.4	7.0	7.4
Eugon Broth	5.2	5.0	5.3
Heated Mixed Saliva	7.4	6.8	7.0

TABLE 6.1 FINAL pH VALUES OF 18 HOUR CULTURES OF ESCHERICHIA COLI, STAPHYLOCOCCUS AUREUS AND CANDIDA ALBICANS IN TODD-HEWITT BROTH, EUGON BROTH AND HEATED MIXED SALIVA

sanguis (N.C.T.C. 7864) totally inhibited the growth of Escherichia coli; the other Type cultures had no inhibitory activity. Eugon Broth cultures of Streptococcus mitis, Streptococcus sanguis (7864), Streptococcus salivarius and Streptococcus milleri totally inhibited, and Streptococcus sanguis (10904) partially inhibited the growth of Escherichia coli. The other Type cultures had no inhibitory activity. Heated mixed salivary cultures of Streptococcus mitis and Streptococcus sanguis (7864) totally inhibited and Streptococcus sanguis (10904) partially inhibited the growth of Escherichia coli. The other Type cultures had no inhibitory activity. The final pH values of the various cultures are shown in Table 6.2. It is unlikely that the activity of the Type cultures which produced inhibition of Escherichia coli was due to adverse pH conditions since there was little difference between the final pH of the inhibitory cultures, the control cultures (Table 6.1), or the Type cultures which failed to inhibit the growth of Escherichia coli.

6.3.3 The Inhibitory Activity of Type Cultures of Oral Commensal Bacteria in Todd-Hewitt Broth, Eugon Broth and Heated Mixed Saliva, against Staphylococcus aureus

The results of this experiment are shown in Table 6.3. The T.H.B. cultures of Streptococcus mitis and Streptococcus sanguis (7864) totally inhibited and Corynebacterium flavidum and Corynebacterium hofmanii partially inhibited the growth of Staphylococcus aureus. None of the other Type cultures possessed any inhibitory activity. Eugon Broth cultures of Streptococcus mitis, Streptococcus sanguis (7864), Streptococcus salivarius and Streptococcus milleri totally inhibited and Streptococcus sanguis (10904) and Neisseria pharyngis partially inhibited the growth of Staphylococcus aureus. None of the other Type cultures had any inhibitory activity. Heated mixed saliva cultures of Streptococcus mitis, Streptococcus sanguis (7864 and 10904), Neisseria pharyngis, Neisseria catarrhalis and Corynebacterium flavidum totally inhibited the growth of Staphylococcus aureus. None of the other cultures had any inhibitory activity. The final pH values of the various cultures are shown in Table 6.3. It is unlikely that the activity of the Type cultures which produced inhibition of Staphylococcus aureus was due to adverse pH conditions, since there was little difference between the final pH of the

	Todd-Hewitt Broth				Eugon Broth				Heated Mixed Saliva			
	No Growth	Partial Growth	Equal Growth	Final pH	No Growth	Partial Growth	Equal Growth	Final pH	No Growth	Partial Growth	Equal Growth	Final pH
Strep.mitis	+	-	-	8.1	+	-	-	7.0	+	-	-	6.8
Strep. sanguis *	-	-	+	8.4	-	+	-	5.8	-	+	-	7.0
Strep. sanguis +	+	-	-	8.1	+	-	-	5.9	+	-	-	6.8
Strep.salivarius	-	-	+	8.4	+	-	-	4.5	-	-	+	7.0
Strep. milleri	-	-	+	8.1	+	-	-	4.7	-	-	+	7.0
Strep. mutans	-	-	+	8.1	-	-	+	4.7	-	-	+	7.0
N. pharyngis	-	-	+	8.4	-	-	+	5.0	-	-	+	7.0
N. catarrhalis	-	-	+	8.4	-	-	+	5.0	-	-	+	7.0
C. flavidum	-	-	+	8.1	-	-	+	5.0	-	-	+	6.8
C. hofmanii	-	-	+	8.1	-	-	+	4.7	-	-	+	7.0
Staph.salivarius	-	-	+	8.1	-	-	+	4.7	-	-	+	7.0

TABLE 6.2 INHIBITION OF ESCHERICHIA COLI BY TYPE CULTURES OF ELEVEN ORAL COMMENSAL MICRO-ORGANISMS IN TODD-HEWITT BROTH, EUGON BROTH AND HEATED MIXED SALIVA, WITH THE FINAL pH VALUES FOR EACH TEST

* N.C.T.C. (10904 - 804)

\+ N.C.T.C. (7864)

	Todd-Hewitt Broth				Eugon Broth				Heated Mixed Saliva			
	No Growth	Partial Growth	Equal Growth	Final pH	No Growth	Partial Growth	Equal Growth	Final pH	No Growth	Partial Growth	Equal Growth	Final pH
Strep. mitis	+	-	-	6.2	+	-	-	5.0	+	-	-	5.0
Strep. sanguis*	-	-	+	7.0	-	+	-	5.0	+	-	-	6.6
Strep. sanguis+	+	-	-	7.0	+	-	-	5.3	+	-	-	6.8
Strep.salivarius	-	-	+	7.2	+	-	-	4.4	-	-	+	7.0
Strep. milleri	-	-	+	7.2	+	-	-	4.7	-	-	+	7.0
Strep. mutans	-	-	+	7.0	-	-	+	4.7	-	-	+	7.0
N. pharyngis	-	-	+	7.0	-	+	-	5.5	+	-	-	6.8
N. catarrhalis	-	-	+	7.0	-	-	+	5.0	+	-	-	6.8
C. flavidum	-	+	-	7.0	-	-	+	5.0	+	-	-	6.8
C. hofmanii	-	+	-	7.2	-	-	+	4.7	-	-	+	7.2
Staph.salivarius	-	-	+	7.2	-	-	+	5.5	-	-	+	7.0

TABLE 6.3 INHIBITION OF STAPHYLOCOCCUS AUREUS BY TYPE CULTURES OF ELEVEN ORAL COMMENSAL MICRO-ORGANISMS IN TODD-HEWITT BROTH, EUGON BROTH AND HEATED MIXED SALIVA, WITH THE FINAL pH VALUES FOR EACH TEST

* N.C.T.C. (10904 - 804)

\+ N.C.T.C. (7864)

inhibitory cultures, the control cultures (Table 6.1), or the Type cultures which failed to inhibit the growth of Staphylococcus aureus.

6.3.4 The Inhibitory Activity of Type Cultures of Oral Commensal Bacteria in Todd-Hewitt Broth, Eugon Broth and Heated Mixed Saliva, against Candida albicans

The results of this experiment are shown in Table 6.4. The T.H.B. cultures of Streptococcus mitis, Streptococcus sanguis (7864) and Neisseria pharyngis totally inhibited and cultures of Corynebacterium flavidum and Corynebacterium hofmanii partially inhibited the growth of Candida albicans. None of the other Type cultures possessed any inhibitory activity. None of the Eugon Broth cultures completely inhibited the growth of Candida albicans but cultures of Streptococcus mitis, Neisseria catarrhalis and Corynebacterium flavidum partially inhibited the growth of the test micro-organism. No inhibitory activity was shown by any other Type culture. None of the heated mixed saliva cultures inhibited the growth of Candida albicans, but cultures of Streptococcus mitis, Streptococcus sanguis (7864), Streptococcus mutans, Neisseria catarrhalis, Corynebacterium hofmanii and Staphylococcus salivarius, partially inhibited the growth of the test organism. No inhibitory activity was shown by any other Type culture. The final pH values of the various cultures are shown in Table 6.4. It is unlikely that the activity of the Type cultures which inhibited Candida albicans was due to adverse pH conditions, since there was little difference between the final pH of the inhibitory cultures, the control cultures (Table 6.1), or the Type cultures which failed to inhibit the growth of Candida albicans.

The results show that of the eleven micro-organisms tested cultures of Streptococcus mitis and Streptococcus sanguis (7864) in Todd-Hewitt Broth, Eugon Broth and heated mixed saliva, consistently inhibited the growth of Escherichia coli, Staphylococcus aureus and Candida albicans. All the commensal type cultures possessed some inhibitory activity, but the extent of the activity was minimal in many instances. For example, the only inhibitory activity of Streptococcus mutans and Staphylococcus salivarius was partial inhibition of Candida albicans in heated mixed saliva.

	Todd-Hewitt Broth				Eugon Broth				Heated Mixed Saliva			
	No Growth	Partial Growth	Equal Growth	Final pH	No Growth	Partial Growth	Equal Growth	Final pH	No Growth	Partial Growth	Equal Growth	Final pH
Strep. mitis	+	-	-	7.0	-	+	-	6.8	-	+	-	6.8
Strep. sanguis*	-	-	+	6.4	-	-	+	5.0	-	-	+	6.8
Strep. sanguis +	+	-	-	7.0	-	-	+	5.0	-	+	-	6.8
Strep.salivarius	-	-	+	7.0	-	-	+	4.4	-	-	+	7.0
Strep. milleri	-	-	+	7.0	-	-	+	4.7	-	-	+	7.0
Strep. mutans	-	-	+	6.8	-	-	+	4.7	-	+	-	7.0
N. pharyngis	+	-	-	7.0	-	-	+	5.5	-	-	+	7.0
N. catarrhalis	-	-	+	8.4	-	+	-	4.4	-	+	-	6.8
C. flavidum	-	+	-	6.8	-	+	-	5.9	-	-	+	7.0
C. hofmanii	-	+	-	7.4	-	-	+	6.8	-	+	-	7.0
Staph.salivarius	-	-	+	7.0	-	-	+	6.8	-	+	-	6.8

TABLE 6.4 INHIBITION OF CANDIDA ALBICANS BY TYPE CULTURES OF ELEVEN ORAL COMMENSAL MICRO-ORGANISMS IN TODD-HEWITT BROTH, EUGON BROTH AND HEATED MIXED SALIVA, WITH THE FINAL pH VALUES FOR EACH TEST

* N.C.T.C. (10904 - 804)

\+ N.C.T.C. (7864)

6.4 DISCUSSION

6.4.1 Inhibition of Escherichia coli, Staphylococcus aureus and Candida albicans by the Oral Commensal Flora

Since Type cultures have not been used in the past to assess the antimicrobial activity of oral commensal bacteria against Escherichia coli and Staphylococcus aureus, there are almost no previous investigations with which the results of the present experiment can be compared. Rosebury et al (1954) using bacteria isolated from the human mouth, showed that four strains of Streptococcus mitis were able to inhibit the growth of Staphylococcus aureus. Other workers have shown that many strains of oral alpha-haemolytic streptococci were able to inhibit Staphylococcus aureus (Myers 1959; Sanders 1969), and Escherichia coli (Sprunt and Redman 1968). These results agree with the findings of the present studies, since the oral commensal bacteria which showed most inhibitory activity against Escherichia coli and Staphylococcus aureus were Streptococcus mitis and Streptococcus sanguis (7864), which are both alpha-haemolytic on blood agar.

The technique used in the present study to assess the microbial antagonistic activity of commensal oral bacteria, has not been used by previous workers. However, since the results of this and past studies are in broad agreement, it would seem reasonable to conclude that the new method is an effective alternative to the methods used previously to assess the antimicrobial activity of micro-organisms.

There have been no previous studies carried out to investigate the role of specific species of oral commensal bacteria in inhibiting the growth of Candida albicans. The present study has shown that a number of oral commensal bacteria have the ability to totally or partially inhibit Candida albicans. Although Streptococcus mitis was the only Type culture which consistently inhibited Candida albicans, all the other Type cultures with the exception of Streptococcus sanguis (10904 - 804), Streptococcus salivarius and Streptococcus millerii, possessed some anti-candidal activity. It is interesting to note that Liljemark and Gibbons (1973) showed that Streptococcus miteor and Streptococcus salivarius but not Streptococcus mutans, were

able to prevent to colonization of the mouth and gastro-intestinal tract of gnotobiotic rats by Candida albicans. They showed that the inhibitory mechanism was probably due to competitive adherence between the yeast and streptococci for attachment to the oral epithelial cells. The reason for Streptococcus mutans failing to prevent colonization of the mouth by Candida albicans was probably related to the fact that Streptococcus mutans is unable to attach to the surface of oral epithelial cells (Gibbons and Van Houte 1971). The mechanism by which the endogenous oral flora interferes with the adherence of Candida albicans to the epithelial surface, is likely to be due to competition for or modification of epithelial receptor sites, or my enzymatically altering the surface of the yeast cells. It is possible that members of the oral commensal flora other than Streptococcus mitis and Streptococcus salivarius may be able to prevent colonization of the oral tissues by yeasts, and perhaps by other potential pathogens, by means of competitive adherence. If this mechanism can function in the human mouth, the combined action of competitive adherence, and the production of antagonistic substances, by certain commensal bacteria, could at least partially explain the virtual absence of Candida albicans, Escherichia coli and Staphylococcus aureus from the normal oral flora.

6.4.2 The Nature of the Inhibitory Activity of the Oral Commensal Flora

There are three main ways in which antagonism between bacterial species can occur. They are as follows: the elaboration of antibiotic substances or of bacteriocines, the depletion of essential substrates from the growth medium, and the creation of adverse physiological conditions, for example alteration of pH or oxygen tension. There is little information concerning the nature of the antimicrobial activity of alpha-haemolytic streptococci against Escherichia coli, Staphylococcus aureus and Candida albicans. Sprunt and Redman (1968) in their study on the inhibitory activity of alpha-haemolytic streptococci against various coliform bacilli, showed that the inhibition was caused by neither adverse pH conditions nor depletion of essential nutrients by the streptococci. Hydrogen peroxide produced by the streptococci appeared to be involved in the

inhibition of a few coliforms, but the majority were unaffected by hydrogen peroxide. Sprunt and Redman concluded that the inhibition of coliforms by alpha-haemolytic streptococci was caused by a number of different mechanisms, and that different strains of streptococci probably inhibited the test micro-organisms in different ways. Myers (1959) in a study on the inhibitory activity of viridans streptococci on Staphylococcus aureus concluded that the active substance produced by the streptococci, was an extra-cellular antibiotic agent which was inactivated by heating at 56°C for 1 hour.

Although of no direct importance in the present discussion, it is interesting to consider the nature of antimicrobial substances produced by alpha-haemolytic streptococci against organisms other than the three examined in this study, and also antimicrobial substances produced by other streptococcal species. Sanders (1969) demonstrated that the inhibitory activity of viridans streptococci against Lancefield Group A streptococcci was due to depletion of essential nutrients and induction of an acidic environment. Wolff and Duncan (1974) described inhibition of a number of bacteria including Escherichia coli and Staphylococcus aureus, by Lancefield Group A streptococci. They concluded that the inhibitory agent was not a bacteriocin, but a low molecular weight bactericidal factor, or possibly an antimetabolite. Finally Streptococcus salivarius has been shown to inhibit the growth of pneumococci (Johanson et al 1970), Corynebacterium diphtheria, Staphylococcus albus and Neisseria catarrhalis (Rosebury et al 1954). Therefore it would appear that oral commensal bacteria inhibit the growth of other micro-organisms by a number of different mechanisms, and that relatively little is known about the precise nature of these mechanisms.

6.4.3 The Significance of the Results of the Present Study on the Changes noted in the Oral Flora of Patients with Sjögren's Syndrome

The results of Chapter 4 showed that there was a highly significant reduction in the numbers of Streptococcus salivarius, Neisseria pharyngis and Staphylococcus salivarius in the oral flora of patients with Sjögren's syndrome compared to normal controls. In the present study, a type culture of Streptococcus salivarius in

Eugon Broth but in neither of the two other culture media, totally inhibited Escherichia coli and Staphylococcus aureus. A type culture of Neisseria pharyngis totally inhibited Candida albicans in Todd-Hewitt Broth, partially inhibited Staphylococcus aureus in Eugon Broth but had no inhibitory activity against Escherichia coli. A type of culture of Staphylococcus salivarius partially inhibited Candida albicans in heated mixed saliva, but had no inhibitory effect on Staphylococcus aureus or Escherichia coli. Since the anti-bacterial activity of these oral commensal type cultures against Escherichia coli, Staphylococcus aureus and Candida albicans was relatively small, it may be assumed that the loss of these commensal bacteria from the oral flora would be unlikely to cause the overgrowth of yeasts and staphylococci previously reported in patients with Sjögren's sysndrome. However only one strain of each commensal bacterium was investigated in this study, and it is possible that if a number of strains of Streptococcus salivarius, Neisseria pharyngis and Staphylococcus salivarius had been tested, an increase in the inhibitory activity may have resulted. Although within the limits of this study Streptococcus salivarius, Neisseria pharyngis and Staphylococcus salivarius, appeared to have little activity against the test micro-organisms, these and other commensal bacteria may well protect the mouth, by inhibiting potential pathogens not tested in the present study or by means of mechanisms not active in the present experimental system, for example adherence inhibition (Liljemark and Gibbons 1973).

Streptococcus mitis and Streptococcus sanguis (7864) were shown to possess marked inhibitory activity against Escherichia coli, Staphylococcus aureus and Candida albicans, and it is likely that the loss of these streptococci from the oral flora of Sjögren's patients would lead to the appearance of staphylococci and yeasts as described in Chapter 2. Streptococcus mitis was isolated from 9 out of 10 patients with Sjögren's syndrome and from all 10 control patients, but due to technical difficulties the precise number of colonies isolated were not counted. As a general rule, there appeared to be fewer alpha-haemolytic colonies isolated from the Sjögren's patients compared to the control subjects. Streptococcus sanguis was never

isolated from patients with Sjögren's syndrome. It is interesting to note the difference in antimicrobial activity between the two strains of Streptococcus sanguis studied. Streptococcus sanguis (7864) regularly inhibited the three test micro-organisms, whereas Streptococcus sanguis (10904 - 864) failed to inhibit Candida albicans and only partially inhibited Escherichia coli, and Staphylococcus aureus in certain culture media. This difference in activity suggests that the inhibitory action of all strains of a particular bacterial species cannot be assumed to be the same, and that the only accurate method of classifying a bacterium as inhibitory or non-inhibitory is by experimental means.

If the antimicrobial activity of the alpha-haemolytic flora is an important factor in preventing the colonization of the mouth by yeasts and staphylococci, then it might be expected that the activity of the alpha-haemolytic streptococcal flora in Sjögren's patients would be less than the activity in control subjects. It is unlikely that a simple comparison of the numbers of Streptococcus mitis and Streptococcus sanguis in the flora of the two groups of patients would give an accurate estimation of the inhibitory activity of the alpha-haemolytic streptococcal flora of the two groups due to the high probability of a variation in activity among strains of these streptococci. Therefore in order to demonstrate a difference in the antimicrobial activity between the two groups, a comparison of the inhibitory activity of alpha-haemolytic streptococci isolated from the Sjögren's patients and from the control subjects would be necessary.

6.5 CONCLUSIONS

All eleven members of the oral flora which were tested for antimicrobial activity against Escherichia coli, Staphylococcus aureus and Candida albicans were shown to possess some inhibitory activity.

The alpha-haemolytic streptococci, Streptococcus mitis (N.C.T.C. 10712 - F.W. 75) and Streptococcus sanguis (7864) possessed most inhibitory activity, and consistently inhibited the

three test micro-organisms.

It would appear that alpha-haemolytic streptococci are important in protecting the oral tissues from invasion and colonization by potentially pathogenic micro-organisms.

A difference in the inhibitory activity of two Type strains of Streptococcus sanguis was demonstrated. The implication of this finding, is that the inhibitory activity of all strains of a bacterial species cannot be assumed to be the same.

A COMPARISON OF THE ANTIMICROBIAL ACTIVITY OF ALPHA-HAEMOLYTIC STREPTOCOCCI ISOLATED FROM EIGHT PATIENTS WITH SJÖGREN'S SYNDROME AND FROM EIGHT CONTROL SUBJECTS

7.1 INTRODUCTION

The results of the experiments reported in Chapter 6 showed that the oral commensal bacteria which possessed most antimicrobial activity against Escherichia coli, Staphylococcus aureus and Candida albicans were alpha-haemolytic streptococci, Streptococccus mitis and Streptococcus sanguis (7864). In addition, the experiments tended to suggest that the antimicrobial activity of all strains of a particular bacterial species, could not be assumed to be the same. In view of these results it seemed reasonable to postulate that if alpha-haemolytic streptococci were important in preventing the colonization of the mouth by Escherichia coli, Staphylococcus aureus and Candida albicans, then the inhibitory activity of streptococci isolated from normal individuals should be statistically greater than the activity of streptococci isolated from patients with severe Sjögren's syndrome. In order, to test this theory, the present study was carried out.

7.2 MATERIALS AND METHODS

7.2.1 Sjögren's Patients and Control Subjects

All the patients with Sjögren's syndrome, and all the control subjects studied in this series of experiments, had been included in the studies carried out in Chapter 4. The diagnosis and measurement of the parotid flow rate of patients with Sjögren's syndrome were as described in Chapter 4.2.1 and 4.2.2.

7.2.2 Escherichia coli, Staphylococcus aureus and Candida albicans

The test organisms used in this study were Escherichia coli N.C.T.C. 10418, Staphylococcus aureus N.C.T.C. 6571 and Candida albicans M.R.L. 3118C. The preparation of pour plates and the

small agar discs containing these micro-organisms was as described in Chapter 5.2.2.

7.2.3 Alpha-haemolytic Streptococci

Four separate areas of the mouth were sampled: the fitting surface of the upper denture, the palatal mucosa, the dorsum of the tongue and the right tonsillar area. Each area was sampled by means of a firmly taken cotton wool swabs. The swab was immediately immersed in one ml. of sterile peptone water (Oxoid), and thoroughly agitated and squeezed to transfer the sampled material to the fluid. All swabs were taken by one operator and before the salivary flow studies were carried out. Each sample was treated in a Whirlimixer (Fisons Scientific Apparatus, Leicestershire) for one minute to disperse any clumps of micro-organisms present in the sample. Within one hour 0.02 ml. of each sample was added to a blood agar plate by means of an Eppendorf pipette. Each sample was plated out to ensure that single colonies would result after incubation. The blood agar plates were incubated at 37°C under aerobic conditions for 18 hours. Usually three or four alpha-haemoltyic colonies were removed from the blood agar cultures of the tongue, denture, palate and throat samples, and plated out for purity on fresh blood agar plates. After overnight incubation at 37°C, a smear was made from each sub-culture and stained by Gram's stain, to check that all the bacterial cultures consisted of Gram positive cocci. Those sub-cultures which were alpha-haemolytic on blood agar and consisted of Gram positive cocci, were stored at 4°C until required for use (usually a maximum of 48 hours). On the day before the experiment was due to be carried out, the purified cultures were plated out on fresh blood agar plates and incubated at 37°C for 18 hours.

Todd-Hewitt Broth was the culture medium used to assess the antimicrobial activity of the alpha-haemolytic streptococci. This medium was selected, since it supported the growth of all the alpha-haemolytic streptococci and also the test micro-organisms, Escherichia coli, Staphylococcus aureus and Candida albicans. The buffer system which is incorporated in Todd-Hewitt Broth ensured that

inhibition of the test organisms due to adverse pH conditions would be very unlikely. An additional reason for using this medium was that Type cultures of Streptococcus mitis and Streptococcus sanguis (7864) in Todd-Hewitt Broth, were shown to inhibit the growth of Escherichia coli, Staphylococcus aureus and Candida albicans (Chapter 6).

A standard nichrome wire loop (Medical Wire and Equipment Co. (Bath) Ltd., Wiltshire) which delivered 0.25 ml. was used to add a constant volume of Todd-Hewitt Broth to each of the cultures of alpha-haemolytic streptococci. The colonies covered by the Todd-Hewitt Broth were emulsified in the fluid, and then 0.25 ml. of the mixture added aseptically to two ml. volumes of Todd-Hewitt Broth in glass bijou bottles. The broth cultures were incubated at 37°C for four hours. Using the technique described in Chapter 5.2.3, 0.2 ml. of each culture was added to the wells of a pre-sterilized World Health Organisation plastic serology plate. Three agar discs containing the appropriate test micro-organisms were added to the broth cultures in the plastic serology plate, and the plates incubated at 37°C for 18 hours. The discs were then removed and examined microscopically for the presence or absence of growth. The pH of each culture was measured before and after the completion of the experiment, by the pH indicator method described in Chapter 5.2.3.

A Todd-Hewitt Broth cultures of each of the three test micro-organisms was set up in each batch of tests to act as a growth control. In addition, a Todd-Hewitt Broth culture of Streptococcus mitis (N.C.T.C. 10712 F.W. 75) was included in each experiment to act as an inhibition control. The degree of growth or inhibition of the test micro-organisms by the alpha-haemolytic streptococcal cultures was assessed by comparing the agar discs removed from the streptococcal cultures with the discs removed from the two control cultures. Growth equal to or greater than the Todd-Hewitt Broth control culture was recorded as equal growth; growth less than the control Todd-Hewitt Broth culture but greater than the control Streptococcus mitis culture was recorded as partial inhibition, and inhibition equal to that of the Streptococcus mitis culture was

scored as total inhibition.

7.2.4 Statistics

In order to investigate the significance of the inhibitory activity of the alpha-haemolytic streptococci isolated from the Sjogren's patients and the Control subjects, it was decided to use the X^2 Test for two Independent Samples (Siegel 1956). This test was selected, because, the two groups of patients were not related, and the method of scoring used was not truly numerical, and therefore, unable to meet the requirements of the 't' test.

7.3 RESULTS

7.3.1 Sjögren's Patients and Controls

Eight female patients with severe Sjögren's Syndrome and eight control subjects (seven female and one male) were studied. The age distribution of the Sjögren's patients and the control subjects is shown in Table 7.1. All the patients and controls had complete upper and lower dentures. None of the Sjögren's patients had a measurable parotid flow rate, and none of the control subjects had a symptomatic or clinical dry mouth.

7.3.2 Todd-Hewitt Broth Control Cultures

In each series of experiments, the Todd-Hewitt Broth Control cultures of Escherichia coli, Staphylococcus aureus and Candida albicans yielded almost confluent growth, and the Streptococcus mitis cultures completely inhibited the growth of all three test micro-organisms. The initial pH of the Todd-Hewitt Broth cultures of the test micro-organisms was 7.4 and the final pH values after 18 hour incubation at $37^{\circ}C$ were, Escherichia coli 8.4, Staphylococcus aureus 7.0 and Candida albicans 7.2. The initial pH of the Streptococcus mitis control cultures was 7.0-7.4, and the final pH values were Escherichia coli 7.2, Staphylococcus aureus 7.0 and Candida albicans 7.2.

	AGE IN YEARS			
	35-49	50-59	60-70	> 70
Sjögren's Patients	1	2	2	3
Control Subjects	1	2	3	2

TABLE 7.1 AGE RANGE OF EIGHT PATIENTS WITH SJÖGREN'S SYNDROME AND EIGHT CONTROL SUBJECTS

7.3.3 The Antimicrobial Activity of Alpha-haemolytic Streptococci Isolated from Patients with Sjögren's Syndrome and Control Subjects

One hundred and fifteen strains of alpha-haemolytic streptococci were isolated from the oral flora of eight patients with severe Sjögren's Syndrome, and the same number of strains were isolated from eight control subjects, giving a total of 230. The results of the experiments to assess the antimicrobial activity of the 230 strains of streptococci against Escherichia coli, Staphylococcus aureus and Candida albicans are shown in Table 7.2. For statistical purposes, the number of streptococcal strains which completely and partially inhibited the test organisms were added together, to give a single final score. Using the X^2 test the number of streptococcal strains isolated from the control subjects which inhibited the growth of Escherichia coli, Staphylococcus aureus and Candida albicans was highly significant ($P = < 0.001$), when compared with the number of streptococcal strains isolated from the Sjögren's patients. The pH values of the alpha-streptococcal cultures measured before and after the experimental period were compared with the pH values recorded for the Todd-Hewitt Broth Control cultures, and virtually no difference was noted. It can be assumed therefore that adverse pH conditions played little or no part in the inhibition of the test micro-organism.

7.4 DISCUSSION

There are no previous investigations with which to compare the results of the present study. There appears to be little doubt that within the limits of the experimental methods used in this study, that, the presence of yeasts, staphylococci and coliforms in the oral flora of patients with Sjögren's Syndrome is related to a marked reduction in the numbers of certain oral alpha-haemolytic streptococci which possess a wide spectrum of anti-microbial activity.

7.5 CONCLUSIONS

There is a highly significant difference between the anti-microbial activity of alpha-haemolytic streptococci isolated from control subjects and the activity of streptococci isolated from

	SJÖGREN'S PATIENTS			CONTROL SUBJECTS		
	No Inhibition	Partial Inhibition	Complete Inhibition	Complete Inhibition	Partial Inhibition	No Inhibition
Escherichia coli	111	1	3	40	9	66
Staphylococcus aureus	101	5	9	59	6	50
Candida albicans	101	10	4	9	33	73

Inhibition of Escherichia coli — Control v Sjögren's $X^2 = 49.7$ $P = < 0.001$

Inhibition of Staphylococcus aureus — Control v Sjögren's $X^2 = 50.1$ $P = < 0.001$

Inhibition of Candida albicans — Control v Sjögren's $X^2 = 18.5$ $P = < 0.001$

TABLE 7.2 A COMPARISON OF THE INHIBITORY ACTIVITY OF 115 STRAINS OF ALPHA-HAEMOLYTIC STREPTOCOCCI ISOLATED FROM EIGHT PATIENTS WITH SJÖGREN'S SYNDROME AND 115 STRAINS OF ALPHA-HAEMOLYTIC STREPTOCOCCI ISOLATED FROM EIGHT CONTROL PATIENTS, AGAINST ESCHERICHIA COLI, STAPHYLOCOCCUS AUREUS AND CANDIDA ALBICANS.

patients with Sjögren's Syndrome.

The presence of Candida albicans, Staphylococcus aureus and Escherichia coli in the oral flora of patients with Sjögren's Syndrome is likely to be related to the absence of strains of alpha-haemolytic streptococci which possess antimicrobial activity.

THE EFFECT OF THE EXPERIMENTAL FINDINGS ON THE CLINICAL MANAGEMENT OF THE ORAL MANIFESTATIONS OF SJÖGREN'S SYNDROME

8.1 INTRODUCTION

The oral problems of patients with Sjögren's Syndrome are associated with decreased salivary flow. Patients experience difficulty in swallowing and mastication, have an increased fluid intake, develop abnormalities in taste and sensation, and may suffer from oral mucosal soreness and ulceration (Bloch et al 1965 and Whaley et al 1973). In order to alleviate the discomfort of patients with severe Sjögren's Syndrome, it is important that the oral mucosa is kept as moist as possible, and to this end, the patients studied in this thesis were instructed to use a glycerol and lemon mouthwash, four to five times a day, or as required, (for details of the mouthwash see Table 2.7.

The possibility was raised in Chapter 4.4.2 that, due to its low pH, glycerol and lemon mouthwash may have contributed to the low salivary pH found in Sjögren's patients. It was suggested in Chapter 2.4 that since Staphylococcus aureus, and Candida albicans can utilize glycerol, while the oral commensal flora on the whole cannot utilize glycerol, that continual use of the mouthwash could be related to the appearance of these micro-organisms in patients with Sjögren's Syndrome. In order to clarify this situation, a retrospective study of the microbiological reports of patients with Sjögren's Syndrome was carried out to discover whether or not yeasts, staphylococci or coliforms were present in the patients oral flora before the glycerol and lemon mouthwash was first prescribed.

8.2 MATERIALS AND METHODS

The Sjögren's patients investigated in this Chapter had been studied previously in earlier Chapters (2 or 4). The affected areas of the mouth were sampled by means of cotton wool swabs and a smear was usually made from the lesion and stained by Gram's Stain. The swabs were cultured on blood agar (Oxoid) and Sabouraud's Agar (Oxoid),

and incubated overnight at 37°C. The Candida species, Staphylococci and the coliform bacilli which were isolated from the swabs were identified as described in Chapter 2.2.2.

8.3 RESULTS

The results of the retrospective microbiological study on seven patients with Sjögren's Syndrome are shown in Table 8.1. Five of the seven patients had microbiological investigations carried out before they started using the glycerol and lemon mouthwash, and the remaining two patients had used the mouthwash for 14 days before being assessed microbiologically. The microbiological results of the oral samples from the five patients who were investigated before the mouthwash was prescribed were as follows: moderate to large numbers of Candida albicans were present in all five patients, in four patients moderate numbers of Staphylococcus aureus were noted, and two patients had in addition a moderate growth of coliform bacilli. Samples from the two patients who started the mouthwash two weeks before the first microbiological assessment was carried out, yielded a heavy growth of Candida albicans and a moderate growth of Staphylococcus aureus. Therefore it would appear that the initial appearance of yeasts, Staphylococci and coliforms in the flora of Sjögren's patients was not related to the use of glycerol and lemon mouthwash.

Most of the Sjögren's patients investigated in Chapter 2 and 4 had attended Glasgow Dental Hospital for some years, and in that time, a number of routine microbiological investigations were carried out. The results of the microbiological status of 12 Sjögren patients over a period of a few years are shown in Table 8.2. In addition to the seven patients who had been microbiologically assessed before or soon after starting the mouthwash, a further five patients were studied who had been using the mouthwash for many months before any microbiological assessment was made. Until June 1975, the 12 Sjögren's patients were microbiologically assessed on a total of 65 occasions. Five of the patients were investigated on more than seven occasions, three patients were investigated on five occasions, and four patients

SJÖGREN'S PATIENTS	CANDIDA ALBICANS	STAPHYLOCOCCUS AUREUS	COLIFORM BACILLUS
A	+ + +	-	-
B	+ + +	+ + +	+ +
C	+ + +	+ + +	+
D	+ + +	+ + +	-
E	+	+ +	-
F	+ + +	+ + +	-
G	+ +	+ +	-

\+ = Light growth
\+ + = Moderate growth
\+ + + = Heavy growth
\- = No growth

TABLE 8.1 THE INCIDENCE OF CANDIDA ALBICANS, STAPHYLOCOCCUS AUREUS AND COLIFORM BACILLI IN ORAL SAMPLES REMOVED FROM SEVEN PATIENTS WITH SJÖGREN'S SYNDROME BEFORE (PATIENTS A TO E), OR WITHIN FOURTEEN DAYS (PATIENTS F AND G) OF FIRST USING GLYCEROL AND LEMON MOUTHWASH

SJÖGREN'S PATIENTS	NUMBER OF TIMES PATIENTS SAMPLED	NUMBER OF TIMES YEASTS ISOLATED	NUMBER OF TIMES STAPH. AUREUS ISOLATED	NUMBER OF TIMES COLIFORM BACILLI ISOLATED
A	2	1	0	0
B	8	6	5	0
C	5	5	3	0
D	2	1	1	1
E	8	6	4	0
F	5	1	4	0
G	7	7	5	0
H	7	6	4	1
I	3	1	3	1
J	11	8	3	0
K	2	2	2	0
L	5	2	0	0
TOTAL	65	46	34	3

TABLE 8.2 THE RESULTS OF A RETROSPECTIVE STUDY OF THE ROUTINE MICROBIOLOGICAL REPORTS OF TWELVE PATIENTS WITH SJÖGREN'S SYNDROME FROM THE TIME OF FIRST PRESENTATION TO JUNE 1975

on less than three occasions. Of the 65 microbiological assessments carried out, moderate numbers of Candida albicans were isolated from oral swabs on 46 occasions (71%), Staphylococcus aureus on 34 occasions (42%) and Coliform bacilli on 3 occasions (4%). In almost all cases antifungal agents (Nystatin or Amphotericin B) were prescribed for the Sjögren's patients in an attempt to eliminate the yeasts from the mouth.

8.4 DISCUSSION

The results of this study strongly suggest that the use of glycerol and lemon mouthwash does not lead to colonization of the mouth of patients with Sjögren's Syndrome by Candida albicans, Staphylococcus aureus and coliform bacilli. It is interesting to note that in spite of antifungal therapy, Candida albicans was isolated from the mouth of the 12 Sjögren's Syndrome patients on 46 occasions over a period of a few years. On microbiological grounds the antifungal agents at best appeared to reduce the number of yeasts but rarely eliminated the organisms from the mouth, since withdrawal of the antifungal agent usually lead to the appearance of moderate numbers of yeasts on the oral tissues by the time of the next appointment. It was not possible to assess critically the effect of the antifungal therapy on the oral lesions by retrospective study, since there was insufficient clinical data contained in the case notes. A prospective study would be necessary to obtain accurate information concerning the clinical response to antifungal therapy, and it is intended to carry out such a study in the near future. It may be that long term antifungal therapy will be necessary, and if this is the case, a mouthwash containing the antifungal agent may be a suitable method of drug administration. No attempt was made in most instances to remove the staphylococci from the mouth. It would appear therefore that in many patients with Sjögren's Syndrome, Staphylococcus aureus and Candida albicans are able to successfully colonize the oral tissues.

There are a number of factors which potentially could reduce the effectiveness of antifungal therapy in patients with severe Sjögren's Syndrome. The clinical records showed that Nystatin or Amphotericin B. lozenges or creams were commonly used to treat the lesions of oral candidosis. In patients with severe xerostomia lozenges are unsuitable,

since the patients do not produce sufficient saliva to dissolve the lozenge, and so release the antifungal agent. A suspension of the antifungal agent would appear to be a more satisfactory method of treatment in these cases. It is possible that glycerol and lemon mouthwash may reduce the efficiency of the antifungal agents, by producing an environment with a low pH and a high concentration of glycerol, which perhaps tends to stimulate rather than reduce the growth of Candida albicans. Another possible explanation for the failure of the antifungal therapy in patients with Sjögren's Syndrome is that host protective mechanisms which normally prevent yeasts from proliferating within the mouth are severely disorganised (Chapter 5, 6 and 7). It is probably that in normal circumstances, antifungal agents and host defence mechanisms combine to erradicate yeasts from the oral tissues, but in Sjögren's patients, virtual loss of protective factors means that the antifungal agents must act alone.

Two main alternatives to glycerol and lemon mouthwash have been described; synthetic saliva containing carboxymethylcellulose, sorbitol and optimal quantities of salts (Matzker and Schreiber 1972), and artifical saliva prepared from extract of bovine salivary glands (S-Gravenmade et al 1974). There appears to be a number of disadvantages in using synthetic saliva, namely the unpleasant taste, the large daily requirement (17 ml.) and the sticky irritant accumulations which develop at particular sites in the mouth. The bovine saliva, on the other hand appears to possess good moistening and lubricating properties, and small quantities (3-5 ml. per day) are required. It is possible that the use of bovine saliva with, added antifungal agents may be a more suitable mouthwash than the glycerol and lemon mouthwash used by the Sjögren's patients in this study. Unfortunately, the artificial saliva is still at an experimental stage, and cannot be obtained commercially.

In the past, local treatment of the oral symptoms of patients with Sjögren's Syndrome, has been concerned with restoring the mechanical function of the oral tissues by means of mouthwashes which possess similar properties, with regard to wetting and lubrication, as saliva. This thesis has shown that in addition to these

mechanical problems, microbiological problems should be considered in the management of patients with severe Sjögren's Syndrome. Since it appears that the presence and persistence of Candida albicans and Staphylococcus aureus is due mainly to loss of various oral commensal bacteria, especially alpha-haemolytic streptococci, it is tempting to speculate whether the restoration of certain strains of alpha-haemolytic streptococci to the flora of patients with Sjögren's Syndrome, might restore the host parasite relationships in favour of the host tissues, and lead to the permanent removal of yeasts and staphylococci. Since Streptococcus mitis (N.C.T.C. 10712 F.W. 75) has been shown to possess antimicrobial activity against, Escherichia coli, Staphylococcus aureus and Candida albicans (Chapter 6) and a similar strain has been shown to inhibit the colonization of the mouth of gnotobiotic rats by Candida albicans by means of adherence inhibition (Liljemark and Gibbons 1973), this streptococcus would appear to be an excellent choice to restore the antimicrobial activity of the Sjögren's patients. As mentioned in Chapter 1.5.2 the destruction of a pathogenic micro-organism in vivo by intentially infecting the host with a commensal micro-organism has long been the dream of bacteriologists since it was suggested by Pasteur and Joubert in 1891 (Florey 1946). Unfortunately this procedure, although attractive in theory has had little success in practice, with one exception. Shinefield et al (1966) and Boris (1968) have shown that epidemic infections in infants caused by Staphylococcus aureus (phage type 80/81) could be terminated by intentionally colonizing the infants with a strain of Staphylococcus aureus (502A) which had a very low pathogenicity. Therefore, if a successful method of colonizing the mouth of Sjögren's patients with Streptococcus mitis could be developed, it is possible that the apparent long term colonization of the mouth of these patients by Candida albicans and Staphylococcus aureus could be reversed, perhaps permanently.

8.5 CONCLUSIONS

Candida albicans, Staphylococcus aureus and Coliform bacilli, were present in the mouth of patients with Sjögren's Syndrome before a glycerol and lemon mouthwash was prescribed.

Candida albicans and Staphylococcus aureus, may be regarded as resident commensal micro-organisms in the oral flora of many of the patients studied with severe Sjögren's Syndrome, due to their long term carriage in moderate to large numbers on the oral tissues.

The clinical response to the use of antifungal agents in the treatment of oral candidosis in Sjögren's patients is uncertain, and a prospective study is necessary.

The use of artifical saliva with added antifungal agents may be a more satisfactory form of therapy for reducing the oral problems of Sjögren's patients.

It is possible that restoration of the antimicrobial activity of the oral microflora of Sjögren's patients may be achieved by re-colonizing the mouth with Streptococcus mitis (N.C.T.C. 10712 F.W. 75).

APPENDIX 1

Sugar Base

Tryptone (Oxoid)	10.0g
Neutralised Bact. Pept. (Oxoid)	5.0g
TryptoneT. (Oxoid)	5.0g
Cystine Hydrochloride (British Drug House)	0.5g
Sodium sulphate (British Drug House)	0.5g
Purified agar (Oxoid)	7.5g
Deionised water	1000 ml.

A. Dissolve in steamer.

B. Adjust pH of base to 7.2

C. Add 40 ml. of phenol red

D. Divide into 100 ml. amounts autoclave (15^{o}C for 15 mins).

Sugar Solution

Prepare a 10% solution of sugar in deionised water and sterilise by Tyndallisation.

A. Add 10 ml. of sugar solution to 100 ml. of basal medium which has been previously melted and cooled to 50^{o}C.

B. Pour aseptically into bijous, and form slopes.

APPENDIX 2

Eugon Broth

Becton Dickinson and Company, Cockeysville, Maryland, 21030, U.S.A.

Formula in Grams per litre of Deionized Water.

Trypticase Peptone.	15.0
Phytone	5.0
Sodium chloride	4.0
Sodium sulphate	0.2
L - Cystine	0.7
Dextrose	5.5

Final pH 7.0

APPENDIX 3

Todd-Hewitt Broth

Oxoid Limited, Wade Road, Basingstoke, Hampshire, RG24 OPW.

Formula in Grams per litre of Deionised Water.

Infusion from 450g fat-free minced beef	10.0
Tryptone	20.0
Dextrose	2.0
Sodium Bicarbonate	2.0
Sodium chloride	2.0
Disodium phosphate	0.4

pH 7.8 (approximately)

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