BIOCHEMICAL AND IMMUNOLOGICAL STUDIES OF PERIODONTAL DISEASE IN HUMANS WITH EMPHASIS ON THE ANALYSES OF BREAKDOWN PRODUCTS EMANATING FROM THE GINGIVAL CREVICE

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Abstract

Active periodontal disease is characterized by marked destruction of collagen in periodontally-involved tissues. Biochemical characterization of the disease is complicated as it is an episodic disorder that is believed to cycle through periods of high activity and quiescence. The first part of the study focused on the measurements and analysis of volatile sulphur compounds (VSC) collected at gingival sulci. Their concentrations in mouth air correlate with the severity of the disease. The second part was devoted to the evaluation of breakdown products of collagen metabolism found in the gingival crevicular fluid (GCF) in relation to periodontal disease.

A novel method and device was developed for collection and analysis of volatile compounds from specific gingival crevice sites. It demonstrated that volatile sulphur compound profiles of crevicular air differ from that of mouth air. It showed that total sulphur content of either inflamed or deep (PD ≥4 mm) periodontal sites was significantly higher than the noninflamed or shallow (PD<4 mm) sites ($p<.05$). The ratio of CH_3SH to H_2S was significantly higher in inflamed than noninflamed sites ($p<.05$) and in deep versus shallow sites ($p<.1$). This is the first known study to quantitate VSC directly from individual crevicular sites.

Furthermore, in the course of the study, methodology was developed for high performance liquid chromatography (HPLC) analysis of hydroxyproline (Hyp) from GCF using precolumn derivatization with phenylisothiocynate. The method was applied to a study of crevicular fluid collected from 30 periodontally-involved subjects that participated in a clinical study which evaluated spiramycin as an adjunct in treatment to scaling and root planing. The study compared Hyp values to several recorded clinical periodontal parameters. Analysis of hydrolyzed and unhydrolyzed samples indicated that the dominant source of Hyp was present in a peptide or bound form. Hyp did not correlate with pocket depth or crevicular fluid volume. Hyp levels measured at inflamed and noninflamed sites fluctuated at given examination points (0, 2, 8, 12, 24 weeks) and between these time points during the study. The most complete data were obtained for time points 0 and 12 weeks which were used to make the best longitudinal comparison between these two time

points. Accordingly, the Hyp levels in both treated groups at week twelve in periodontal sites that were inflamed at week zero and remained inflamed at week 12 were higher than in sites that remained noninflamed. This relationship was statistically significant in the spiramycin treated group ($p<.05$). In both treatment groups Hyp levels at week twelve were higher in healing sites that experienced a ≥ 2 mm gain of attachment between weeks 0 and 12, than sites that remained unresolved. Again in the spiramycin treated group this difference was found to be statistically significant ($p<.05$). Hence the study indicated an increase in Hyp levels in GCF during episodes of increased collagen metabolism. This metabolism occurred during both healing and active phases of periodontal disease.

A cross sectional study of GCF from inflamed and noninflamed periodontal sites was performed to confirm that Hyp content was higher in inflamed sites and to investigate the Hyp contribution from type I collagen and C1q sources. Again Hyp content was found to be higher in inflamed than noninflamed sites ($p<.001$). Using ELISA, type I collagen was found to contribute approximately 6-fold more Hyp than C1q to total Hyp content of fluid from inflamed and noninflamed sites. SDS/PAGE gels of GCF from inflamed sites exhibited more intense protein banding pattern than GCF from noninflamed sites. Western blot analyses showed markedly more intense staining for type I collagen peptides than for C1q reactive peptides. This finding, together with the ELISA assay results which indicated that C1q contributed less than 10% of total Hyp to both inflamed or noninflamed GCF, strongly implies that collagen is the dominant source of the total Hyp content in the processsed GCF samples.

The presence of higher VSC content and CH_3SH to H_2S ratio in crevicular air and higher Hyp content in GCF, can distinguish inflamed from noninflamed periodontal sites. These indicators can distinguish the presence of disease at specific sites at the time of examination. Since Hyp levels were found to be increased at periodontal sites that exhibited healing during a 3 month period, Hyp can also ascertain the effectiveness of periodontal therapy.

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1. INTRODUCTION

1.1 ORAL CAVITY

1.1.1 Structure

The oral cavity is a unique mixture of specialized hard and soft tissues. Teeth are comprised of enamel, dentin and cementum, and house a specialized connective tissue, the dental pulp. Salivary glands, either major or minor, are found in various parts of the mouth. The oral cavity has a mucosal covering which is clinically and biologically classified as masticatory, lining and specialized types. They vary in structure depending on the function of the region concerned.

Mucosal covery of the oral cavity has two basic components: epithelium and the connective tissue lamina propria. Separating the two components is the basal lamina, an epithelial product formed by the basal epithelial cells. Papillae of connective tissue project toward the epithelium, which in turn has ridges that protrude into the lamina propria. The basal lamina forms an interface between the connective tissue papillae and epithelial ridges (Bhaskar 1980).

Depending upon the region, the stratified squamous epithelial lining of the oral cavity can be keratinized, non-keratinized or parakeratinized (Cleaton-Jones and Fleisch 1973). The surfaces of more functionally stressed areas of the mouth such as the hard palate and gingiva are protected with a keratinized epithelium. However, in many individuals the gingival epithelium is parakeratinized (Bhaskar 1980). Gingival crevicular epithelium as well as delicate locations like alveolar, buccal and vestibular mucosae are composed of non-keratinized epithelium.

1.1.2 Environment

The environment of the oral cavity is complex having numerous anaerobic and aerobic sites. The emergence of teeth into the oral cavity results in the formation of a gingival crevice and an even more complex microenvironment. The gingival crevice is bounded by soft tissue on one side and

mineralized tissue on the other. From physical, metabolic and nourishment perspectives, the gingival crevice offers an unique environment where a wide variety of microorganisms flourishes. The superior aspect of the gingival crevice offers an aerobic environment, while deeper portions allow for anaerobic conditions . Microorganisms can attach to either the surface of the sulcular epithelium or to the enamel or cemental surfaces of the teeth. In addition, the gingival sulcus is bathed in a fluid that emanates from the underlying connective tissue adjacent to the junctional epithelium.

1.2 GINGIVAL CREVICE

1.2.1 Structure and function

The gingival epithelium is divided into several subclasses based upon anatomical location and histological characteristics. Included in a typical cross section of marginal gingiva are crevicular, junctional (attachment), as well as lining epithelia of free and attached gingivae and of alveolar mucosa (Brill and Krasse 1958). In particular, the crevicular epithelium extends from the gingival margin to the junctional epithelium, and forms a physical and biological boundary of the gingival crevice surrounding each tooth.

As the tooth erupts into the oral cavity, the reduced enamel epithelium of the emerging crown fuses with the oral epithelium. The reduced enamel epithelium (REE) that remains attached to the enamel of the tooth which has not yet erupted is termed the primary attachment epithelium. As the tooth erupts the REE becomes shorter thus causing a development of a shallow groove, the gingival crevice, between the gingiva and the surface of the tooth (Figure 1.1). This gingival sulcus extends from the apical attachment epithelium to the coronal gingival margin (Bhaskar 1980).

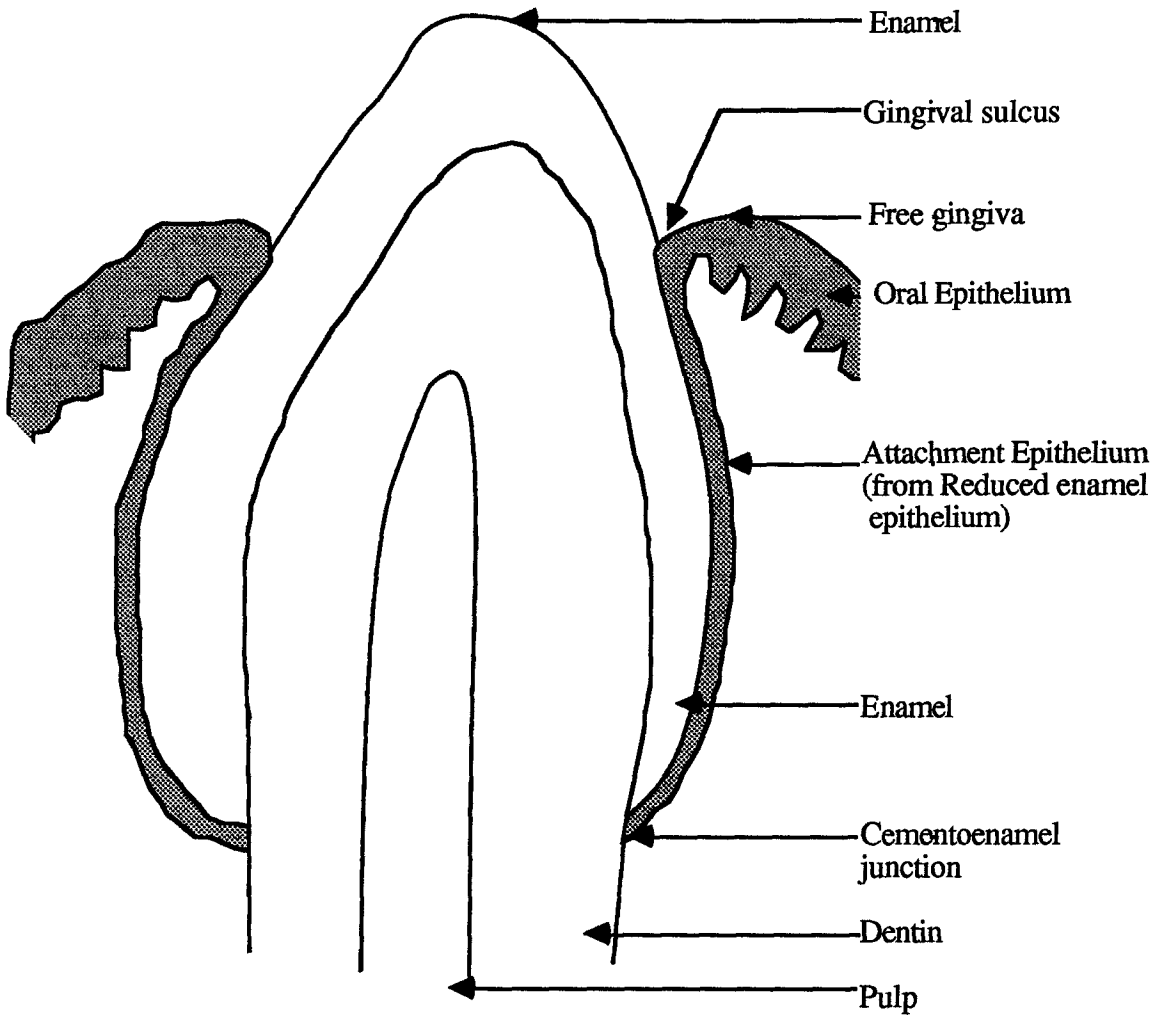

Figure 1.1: Attached epithelial cuff and gingival sulcus at an early stage of tooth eruption (Bhaskar 1980).

The non-keratinizing crevicular epithelium consists of three layers of cells, namely the stratum basale, stratum intermedium and stratum superficiale (basal, intermediate and superficial). Basal cells of both keratinizing and non-keratinizing epithelia are the same morphologically; however, the intermediate cells of non-keratinized epithelium are larger than those of the stratum spinosum found in keratinized epithelium, and contain no granular or cornified cells (Bhaskar 1980).

A number of authors have microscopically studied the cytostructure of the crevicular epithelium and have found it to resemble non-keratinizing oral mucosa (Listgarten 1964; Listgarten 1966; Schroeder and Theilade 1966; Weinstein et al. 1967; Freedman et al. 1968; Gavin 1968; Geisenheimer and Han 1971; Kaplan et al. 1977; Muller-Glauser and Schroeder 1982). The basal cells have a central ovoid nuclei bounded by a porous nuclear membrane, numerous basally placed mitochondria, Golgi apparartus in close proximity to the nucleus, and abundant amounts of polyribosomes. These features are suggestive of active protein synthesis. Intermediate cell layers show a decrease in the size of the nucleus and number of mitochondria, and also lack keratohyalin granules. These flattened cells display an increase in the number of tonofilaments, but they have no tendency to form prominent bundles as in keratinizing gingival epithelium. In the superficial layer of cells, cytoplasmic organelles are scarce and appear in a degenerative state. The cells lack a well defined nucleus, contain electron dense bodies, varible sized vesicles and intact desmosomes.

Crevicular epithelium is a specialized lining epithelium which is formed at the time of active eruption of the dentition and is lost when the teeth are exfoliated. It forms the soft tissue wall of the gingival crevice together with the junctional epithelium, and opposes the hard structure of the tooth. While this specialized structure has an important function of maintaining a barrier against masticatory forces and bacterial plaque toxins, it lacks a protective keratin layer.

1.2.2 Gingival vasculature and permeability

The lamina propria contains the vascular supply, innervation and lymphatics of the gingival tissues. Ultrastructurally, individual capillary loops are present in each of the connective tissue papillae. The gingival blood supply is derived from the supraperiosteal arterioles along the buccal and lingual surfaces of the alveolar bone, the arterioles that emerge from the crest of the interdental alveolar bone and to some extent from the arterioles within the periodontal ligament (Cohen 1954).

The permeability of crevicular epithelium to tissue fluid has been widely studied (Brill and Krasse 1958; Brill and Bjorn 1959; Brill and Krasse 1959; Thilander 1964). Using a fluorescein tracer, Brill and Björn found that tissue fluid permeates healthy gingival pocket tissues in dogs.

With the same tracer compound, Brill and Krasse observed that except for crevicular epithelium, fluorescein did not penetrate other regions of healthy oral mucosa.

In all three layers of crevicular epithelium, the intercellular spaces are wide and generally occupied by leukocytes, with neutrophils being the most common type. Some of these cells rupture as they migrate towards the surface of the crevicular epithelium. Thilander reported a general widening of the intercellular spaces after application to the pocket epithelium of leukocyte homogenate, containing proteases, amylases, peroxidases and lipases (Thilander 1964). This was ascribed to an effect on the intercellular substance and the mutual adhesion mechanism of the cells. Studies of the intercellular matrix of the oral mucosa have suggested that it is comprised of mainly polysaccharides and proteins (Wislocki et al. 1951; Thonard and Scherp 1959; Cancellaro et al. 1961; Klingsberg et al. 1961). It is believed that an enzymatic degradation of such constituents results in an increase in the uptake of water to maintain constant tonicity. This weakening of the adhesion effect may be responsible for widening of intercellular spaces (Thilander 1964).

In general, the intercellular compartment tends to be narrowest in the intermediate layer, slightly wider in the basal layer and widest in the most superficial layer (Geisenheimer and Han 1971). These continuous intercellular spaces provide an explanation for the passage of tissue fluid through the crevicular epithelium and not through other regions of healthy mucosa.

1.3 GINGIVAL CREVICULAR FLUID

1.3.1 Production

There are differences in the ratios of components between crevicular fluid and serum from which it is derived (Weinstein et al. 1967). This indicates that the fluid is not a transudate, but rather an inflammatory exudate (Oliver et al. 1969; Orban and Stallard 1969). This apparent selectivity can be related to two morphological features of crevicular epithelium (Gavin 1968). First, the basal lamina appears to form the only continuous barrier between the connective tissue

and the gingival pocket. Secondly, the presence of prominent microvilli suggests a modification in constituents of the intercellular spaces.

In 1971, Geisenheimer and Han, using electron microscopy, (E.M.), observed the presence of typical desmosomes in all cell layers of the crevicular epithelium (Geisenheimer and Han 1971). These intercellular bridges were most numerous in the intermediate layer, followed by the superficial layer, and the least in the stratum basale. They also found hemidesmosomes irregularly spaced along the basal surface of the basal cells, at the interface between crevicular epithelium and lamina propria.

An electron microscopic study by the same investigators showed that in marginal gingiva of clinically healthy tissue the manifestations of inflammation, such as dense cellular infiltrate, dilated vessels, ruptured cells and large amounts of edematous exudate are not observed (Geisenheimer and Han 1971). Their observations also indicated that clinically normal marginal gingiva will appear free of manifestations of inflammation when rigid standards of oral hygiene and tissue selection are applied.

A stratified squamous epithelium is considered to be an effective barrier to certain substances. It is most permeable to small lipid soluble non-polar materials (Thilander 1964). However, pocket epithelium does not display the same properties, as large polar serum proteins can pass through the crevicular epithelium (Brill and Krasse 1958; Brill and Bjorn 1959). Furthermore, pocket epithelium is thinner than the gingival epithelium and lacks a protective keratin layer.

Holmberg and Killander (Holmberg and Killander 1971) and others (Mann 1963; Egelberg 1964; Björn et al. 1965; Löe and Holm-Pedersen 1965; Hara and Löe 1969; Oliver et al. 1969), found gingival fluid flow higher in subjects with severe periodontitis. Extracellular passage of serum protein through crevicular epithelium is the most important route of flow in an inflamed gingiva, which exhibits widening of the intercellular spaces (Thilander 1963; Thilander 1968). Since similar concentrations of immunoglobulins were found in gingival fluid from inflamed gingiva as in serum, these investigators concluded that serum was the origin of various crevicular proteins such as IgA, IgG and IgM.

Crevicular epithelium has been described in relation to the periodontal pocket and underlying connective tissue. Its unique ability to allow passage of tissue fluid through its layers has been extensively studied. It is of immense interest how such a permeable mucosa can simultaneously act as an effective barrier to exogenous substances.

1.3.2 Composition

Whether the gingiva is inflamed or healthy is of crucial importance when evaluating the sulcular region. A potential problem in studying the gingival sulcus is an inadequate definition of the health status of the epithelium. The degree of inflammation has an effect on the turnover of the epithelial cells (Marwah et al. 1960). Conflicting results amongst researchers may in part be due to the studies being performed on gingival tissue specimens which have been classified normal and chronically inflamed .

Egelberg reported that the number of inflammatory cells was increased in inflamed gingival pockets (Egelberg 1963). However, the proportion of inflammatory cells appeared to be independent of the degree of inflammation. Differential counts of inflammatory cells showed 95-97% neutrophils, 1-2% lymphocytes, and 2-3% monocytes (Attström 1970). Although the proportion of leukocytes was found to be independent of the severity of inflammation, a positive relation was observed between the number of cells and the presence of inflammation.

Chemical analysis and observed ratios of various components of crevicular fluid differ from those of the serum samples. The Na+/K+ ratio of 2/1, which is much lower than in serum, support the earlier results of Krasse and Egelberg (Krasse and Egelberg 1962). The ratios of protein/ chloride and of protein/inorganic phosphorous were also reported much lower in serum. This indicated a decreased presence of higher anionic protein than in serum. Furthermore, since the proteins in the gingival fluid did not react with an anti-saliva sera, this implied that they were not derived from saliva.

Hara and Löe (Hara and Löe 1969), reported on the glucose, hexosamine and hexuronic acid content in crevicular fluid. They found glucose concentration in crevicular fluid three to six times

greater than in serum. The presence of hexosamine and hexuronic acid may represent breakdown products of bacterial action on host tissues. Similar substances are liberated during the inflammatory process by enzymatic degradation of the gingival connective tissue.

Components in gingival fluid were found to react with specific antisera to alpha G-, alpha A- and alpha M-globulins, albumin and fibrinogen (Brandtzaeg 1965). This suggested that plasma was the source of these proteins. Also, these components migrated upon electrophoresis, just as plasma proteins did, and were present in concentrations characteristic of plasma. Other reports have also indicated that the concentrations of IgG, IgA and IgM in crevicular fluid were very similar to serum (Holmberg and Killander 1971).

A significant amount of research has been devoted to enzyme characterization of crevicular fluid. Acid phosphatase, beta-glucuronidase, lysozyme, cathepsin D, proteases, alkaline phosphatase, and lactic dehydrogenase have all been found in gingival crevicular fluid (Brandtzaeg and Mann 1964; Sueda and Cimasoni 1968; Ishikawa and Cimasoni 1970). These enzymes, however, could have originated from either the host tissue and/or the plaque microflora.

1.3.3 Methods of collection

Sampling of gingival fluid has been performed by a variety of methods. These have included collection of subgingival washings, and collection using either capillary tubes or filter paper strips. The gingival washing technique proved to be quite cumbersome and time-consuming. This collection procedure was improved by using microcapillary tubes, which, in turn, more recently has been superseded by the use of filter paper strips. These advances simplified the collection and handling of the samples.

Sampling with capillary tubes provided the potential for quantitative analysis of a known volume of crevicular fluid. Initially the use of such micropipettes was merely to collect the fluid without evaluating the volume or the exact procedure for sampling (Krasse and Egelberg 1962; Mann 1963; Brandtzaeg and Mann 1964). Egelberg (Egelberg 1963), described his capillary tube sampling procedure as simply bringing the tube into the pocket and moving it slightly in mesial and

distal directions, two to four times. Kaslick and workers (Kaslick et al. 1968), described a technique for exact measurement of gingival fluid involving collection of fluid with capillary tubes of known inner diameter, followed by centrifugation and removal of sediment from the sample. This technique for collection and measurement of fluid volume has been subsequently adopted by other investigators (Sueda et al. 1969).

More recently, two filter paper strip methods have been employed to sample crevicular fluid. The first, referred to as the intracrevicular technique, involves the gentle insertion of the filter paper into the gingival sulcus. In the second, known as extracrevicular sampling, the strips are positioned on the vestibular surfaces of the gingivae and teeth, without penetrating the sulcus. The latter method is preferred for repetitive sampling from the same site as it does not disturb the sulcular environment.

The method of intracrevicular sampling was first described by Brill and co-workers (Brill and Krasse 1958; Brill 1959; Brill and Bjorn 1959). Filter paper strips used to absorb the fluid were inserted into the gingival crevice until minimal resistance was felt (Figure 1.2). This method was commonly used among researchers during the 1960's and early 1970's (Björn et al. 1965; Lindhe and Attström 1967; Weinstein et al. 1967; Hara and Löe 1969; Golub et al. 1971; Sandalli and Wade 1971; Golub et al. 1976; Ciancio et al. 1980). The strips were inserted in the crevices for a suitable sampling time before being removed and analyzed.

A slight variation of this method was introduced by Mann (Mann 1963). By inserting three filter paper strips into each crevice he not only limited the sampling to a specific area but also eliminated the chance of contamination by saliva. These three strips were used to dry the crevice and remained side by side in the crevice for three minutes. The central drying strip was removed and immediately replaced with a collecting strip. After five minutes the strip was removed for evaluation. This variation insured that any fluid collected after the drying sequence must originate from within the gingival sulcus.

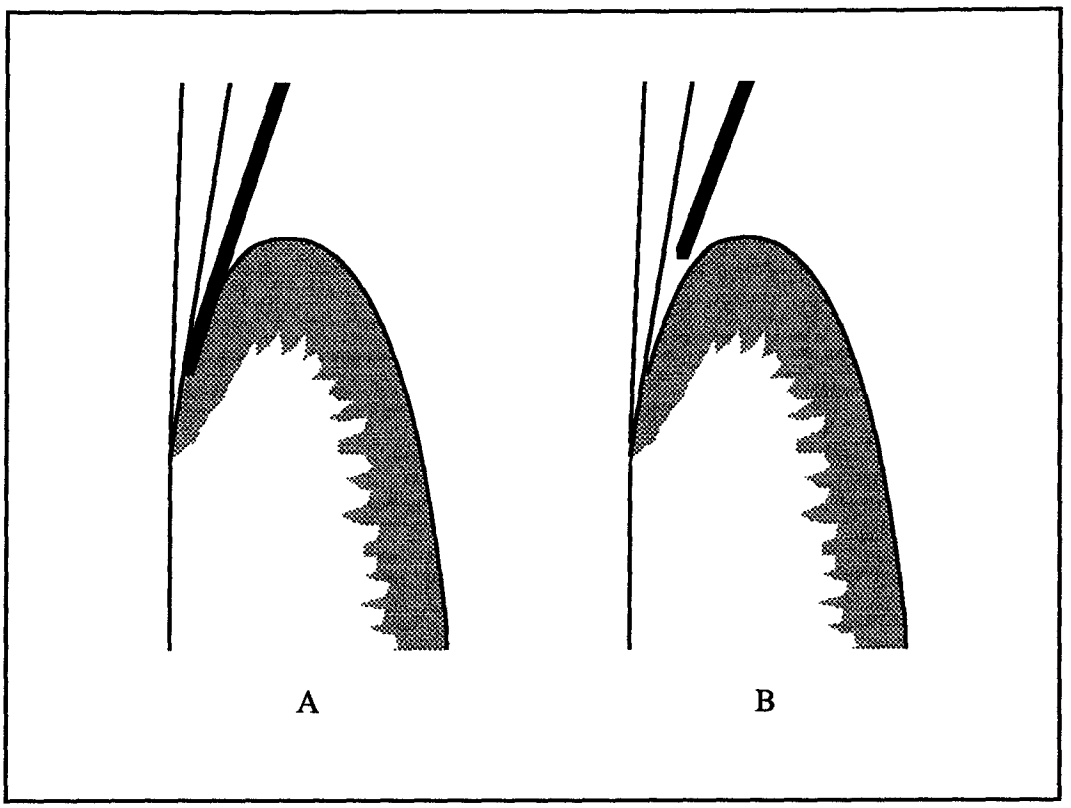

Figure 1.2: Intracrevicular sampling. 'A': For the Brill technique, the strip is passed into the crevice until resistance is felt. 'B': In the Löe and Holm-Pedersen technique, the end of the strip is placed at the entrance to the crevice. (Cimasoni 1983)

Yet another modification of the intracrevicular technique was to sample only at the sulcular orifice. In this technique, filter paper strips are placed at the entrance of the crevice, avoiding physical irritation of the crevicular epithelium (Löe and Holm-Pedersen 1965). Egelberg and Attström (Egelberg and Attström 1973), used this technique in preference to the initial intracrevicular method (Figure 1.2). They found that while the intracrevicular samples yielded greater volumes of fluid than orifice samples, this was not an advantage, since the orifice method showed less variation between the samples. This technique has since been adopted by other investigators (Egelberg 1966a-e; Waite 1976; Jameson 1979).

In addition to intracrevicular sampling, Brill and Krasse (Brill and Krasse 1958) described the extracrevicular method. Accordingly, strips of filter paper were placed against the vestibular surfaces of the teeth and gingivae. This method has been used by other researchers (Löe and Holm-Pedersen 1965), and is illustrated in Figure 1.3.

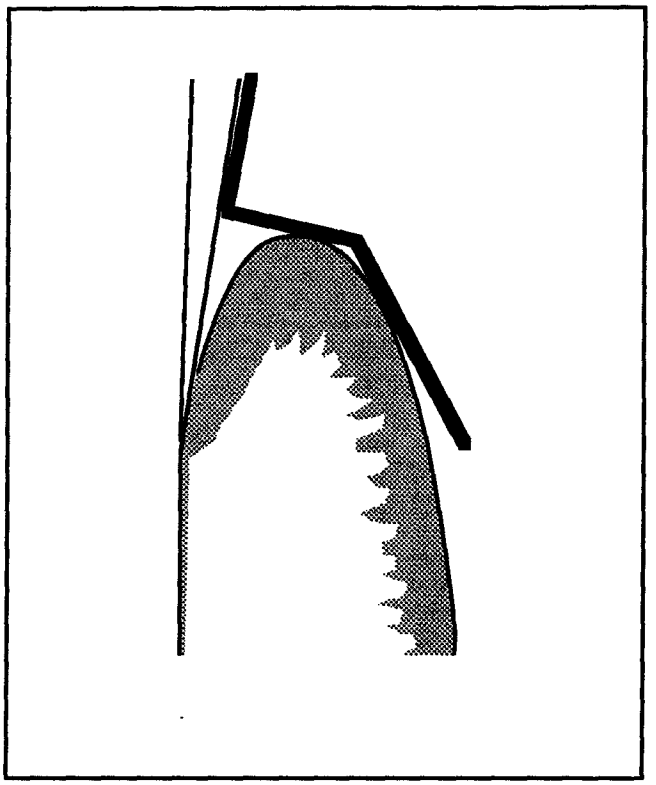

Figure 1.3: Extracrevicular sampling. The strip fits closely to the tooth surface, gingival margin and attached gingiva, thus bridging the entrance of the gingival sulcus. (Cimasoni 1983)

1.3.4 Volume

The amount of crevicular fluid collected using the available techniques is quite small and on occasion difficult to detect. In order to distinguish the presence of crevicular fluid on filter paper strips, Brill and Björn (Brill and Bjorn 1959) and others (Mann 1963; Weinstein et al. 1967), have

utilized fluorescein which passes from the systemic circulation to the oral cavity via the gingival sulcus, and is not found in the saliva. In these studies, subjects were administered sodium fluorescein prior to crevicular fluid collection. After fluid collection, fluorescence of the filter paper strips was viewed under ultraviolet light. This technique can be employed to detect GCF when small amounts of fluid are present.

Another method of evaluating crevicular fluid volume was effected by staining the filter paper strips. This technique, used to accentuate small volumes of gingival crevicular fluid (GCF) on filter paper strips, utilized ninhydrin staining (Orban and Stallard 1969). The resulting blue or purple colour, indicative of reaction with alpha-amino groups of amino acids, can be quantitated microscopically using a suitable grid pattern.

More recently, fluid soaked filter paper strips are analyzed electronically. In Jameson's studies (Jameson 1979), a gingival crevice fluid meter was employed. This instrument discriminates minute fluid volumes by the reduction in capacitance between two sensors when in contact with a standardized filter strip containing fluid under investigation. Use of this instrument had the potential of yielding a quick and suitable method for repeated fluid volume determinations. Furthermore, it obviated a need for injection of subjects with fluorescein.

The most widely used instrument today for analyzing crevicular fluid volume on filter paper strips is the Periotron 6000™. This device measures conductance of a fluid absorbed filter paper strip when placed between two electrodes. A digital readout between 0 to 200 is expressed, which is then related to a standard curve of known volume and corresponding Periotron values. In the past decade, the majority of crevicular fluid studies have performed crevicular fluid collection using filter paper strips, and volume measurements using a Periotron instrument.

1.3.5 Clinical significance

It was determined that crevicular fluid flow followed a circadian periodicity with the highest flow rate occuring in the late evening (Bissada et al. 1967). Using the Brill technique (Brill and Krasse 1958), Bissada and co-workers observed that after oral administration of sodium

fluorescein the maximum flow followed the evening crest in body temperature by about four hours. Moreover, a considerable variation was found in rate of flow between different individuals and also between different crevices of the same individual.

The gingival fluid flow during the menstrual cycle was studied by Lindhe and Attström (Lindhe and Attström 1967). They observed a small gradual increase of gingival exudation during the proliferative phase and a peak on the day of ovulation. Following ovulation the secretory phase showed a gradual decrease in gingival fluid flow. These findings suggested that perhaps an increase in female sex hormones results in a relatively higher gingival exudation, whereas a lower gingival fluid flow occurs during menstruation when the availability of female sex hormones is at the lowest level.

Numerous investigators examined the influence of diabetes upon the state of health of the periodontium. In comparing diabetic patients to healthy controls it was suggested that the severity and duration of diabetes appeared to have little effect upon periodontal diseases (Hove and Stallard 1970). No significant differences in pocket depth or gingival inflammation were found in a group of diabetic and control patients with similar plaque indices. While no difference was found in the severity of the inflammatory infiltrate in the two groups of patients, histological sections displayed a significantly higher incidence of vascular modifications in gingiva of diabetic patients (Cimasoni 1983). In contrast, Ringelberg and co-workers (Ringelberg et al. 1977) reported a higher flow rate of GCF in a group of diabetic children than in a control group of children.

There are conflicting reports as to the possible variation attributable to diabetes in the concentration of glucose in the GCF. In an early study which compared serum and GCF levels of glucose, it was determined that GCF glucose was up to six-fold higher than that found in serum (Hara and Löe 1969). Subsequently, Ficara and co-workers showed similar concentrations of glucose in GCF and serum of healthy and diabetic persons, especially in subjects exhibiting high blood glucose levels (Ficara et al. 1975). However, Weinberg and co-workers reported that glucose levels in GCF from clinically inflamed sites were only approximately 10% of that of serum (Weinberg et al. 1986).

A positive correlation has been reported between the degree of gingival inflammation and the amount of gingival fluid flow (Brill 1960; Mann 1963; Björn et al. 1965; Löe and Holm-Pedersen 1965; Orban and Stallard 1969; Rüdin et al. 1970; Wilson and McHugh 1971). This correlation has been confirmed by the majority of histological findings.

Perhaps of even greater significance is the fact that changes in gingival fluid flow rate may be a sign of subclinical inflammation. A longitudinal study of experimental gingivitis demonstrated a gradual increase in GCF flow even before clinical signs of gingivitis were evident (Löe and Holm-Pedersen 1965). This pre-gingivitis increase in the GCF flow prior to clinical evidence of disease during experimental development of inflammation has also been observed by several other investigators (Löe et al. 1967; Son et al. 1971; Egelberg and Attström 1973; Greenstein 1984). It is believed that these increases in GCF flow are a result of early changes in microvascular permeability in vessels adjacent to the junctional epithelium (Söderholm and Attström 1977).

Both cross-sectional and longitudinal studies have compared GCF volume with measured periodontal parameters. Both approaches have found strong correlations between the amount of gingival fluid collected and the Gingival Index (GI) scores (Björn et al. 1965; Löe and Holm-Pedersen 1965; Oliver et al. 1969; Wilson and McHugh 1971; Holm-Pedersen et al. 1975; Hancock et al. 1979; Kowashi et al. 1980; Solis-Gaffar et al. 1980). Moreover, gingival fluid flow was found to be dependent upon the degree of gingival inflammation but independent of pocket depth (Mann 1963; Suppipat et al. 1977).

The recognition that bacteria are a factor in the etiology of periodontal diseases created an interest in the use of antimicrobial drugs in periodontal therapy. Early work by Bader and Goldhaber (Bader and Goldhaber 1966), demonstrated that systemically administered tetracycline in dogs quickly emerged in the gingival fluid. The finding led to the use of antibiotic drugs in periodontal therapy. A variety of antibiotic drugs in conjunction with surgical and non-surgical periodontal therapy were evaluated (Mills et al. 1979; Ciancio et al. 1980; Walker et al. 1981). More recently, the finding that tetracycline and its derivatives inhibit collagenase activity has influenced their use in periodontal treatment (Golub et al. 1984; Golub et al. 1987).

1.4 CONNECTIVE TISSUE IN THE PERIODONTIUM

1.4.1 Collagen structure

The extracellular matrix is composed of two main classes of macromolecules; the collagens and the polysaccharide glycosaminoglycans (GAG's). Collagen is the major structural protein of the extracellular matrix and is the most abundant protein found in mammals. GAG's are usually bound to a core protein to form proteoglycans. Although collagens are the major proteins found in tissue they should not be considered as the only molecules of importance, since the function of the extracellular matrix is dependent on the structure of all its various components.

Collagen has a characteristic triple stranded helical structure. This rod-like conformation confers onto collagen its strength and rigidity, especially when individual collagen molecules are arranged in fibers and bundles. This helical structure, which accounts for 95% of the total collagen molecule, is composed of three separate alpha chains, each of which contains approximately 1000 amino acid residues, twisted together to form a left handed triple helix. Each collagen molecule is stabilized by interchain hydrogen bonds.

Formation of each component alpha chain is effected intracellulary. Each alpha chain follows a repeating amino acid pattern. Every third amino acid residue is a glycine residue (~333 residues / chain). The repeating sequence of amino acids is Gly-X-Y, where X and Y can be any amino acid. Generally the X position is occupied by a proline residue, whereas the Y position is frequently occupied by hydroxyproline. Except for proline (Pro) and hydroxyproline (Hyp), no other amino acid was found to exist in collagen in any recognizable pattern (Piez 1963; Hulmes et al. 1973). These amino acids each occur at about 100-120 sites per collagen molecule. Proline comprises approximately 12% of the amino acid residues in collagen, whereas Hyp is present in slightly lesser amounts, comprising 10 - 12% of the residues. These cyclic imino acids give ridigity to the whole collagen molecule and influence the left-handed helical structure of the individual alpha chains. Hydroxyproline residues not only stabilize the triple helical structure of collagen, but via

hydrogen bonds, cross link adjacent collagen molecules and contribute to stabilization of the structure (Ramachandran et al. 1975). Thus, this repeating amino acid configuration is required for stability and proper helical conformation.

In addition to the helical portion of the collagen molecule, there are short non-triple-helical regions found as extensions at the NH_2- and COOH- terminal ends of each alpha chain (Traub and Piez 1971). These so-called "extension" domains have functional roles. They are the areas where hydroxylysine is found which contributes to intra- and intermolecular cross-links in native fibrils. Such cross links stabilize the molecular arrangement of collagen and greatly decrease the solubility of these molecules.

1.4.2 Collagen types

There are at least fifteen genetically distinct types of collagen which have been thus far identified in the human body. The most useful way to classify collagen type has been to use a system based upon the function of the collagen form. It is practical to group the collagens into two main groups. The two classifications of collagens are those that form periodically banded fibers and those that do not (Burgeson and Morris 1987). A list of the known genetically distinct collagens is given in Table 1.1.

A. The interstitial collagens - Collagens that form broad-banded fibers		
Type I	$\{\alpha1(I)\}_2\alpha2(I)$	Found in most tissues except cartilage; major component of bone, tendon, skin, and dentin
Type I 'trimer'	$\{\alpha1(I)\}_3$	Some fetal tissues; product of certain malignant and normal cell lines
Type IIM	$\{\alpha1(IIM)\}_3$	Cartilaginous tissues
Type IIm	$\{\alpha1(IIm)\}_3$	Cartilaginous tissues
Type III	$\{\alpha1(III)\}_3$	Found together with type I; in relatively high concentrations in extensible tissues such as blood vessels, skin and gut
B. The minor collagens - Less abundant; do not form broad-banded fibers		
Type IV (Basement membrane collagen)	$\{\alpha1(IV)\}_2\alpha2(IV)$	Major component of all basal laminae
Type V	$\{\alpha1(V)\}_2\alpha2(V)$	Minor components of most tissues except cartilage; fiber forms unknown
	$\{\alpha1(V)\}_3$	
	$\{\alpha3(V)\}_3$	
	$\alpha1(V)\alpha2(V)\alpha3(V)$	
Type VI	$\{\alpha1(VI)\}_2\alpha2(VI)$	First identified in aortic intima, but now thought to have a broad, but yet unidentified, tissue distribution
Type VII (long chain collagen)	$\{\alpha1(VII)\}_3$	Identified in amniotic membrane and skin; believed to be associated with all stratified epithelia; may be anchoring fibril protein
Type VIII (endothelial cell collagen)	$\{\alpha1(VIII)\}_3$	Identified as product of a variety of cell types
Type IX (HMW-LMW)	$\alpha1(IX)\alpha2(IX)\alpha3(IX)$	Cartilaginous tissues
Type X (G collagen)		Product of cartilage hypertrophic cells
C. Minor cartilage collagens with unknown subunit compositions		
1α		Compositionally similar to type V; structures, fiber forms, and functions unknown
2α		
3α		Compositionally similar to type II.

Table 1.1: Genetically distinct collagens (Burgeson and Morris 1987)

The types of collagens that form large fiber groups are 300 nm long having periodic cross striations of 67 nm which are evident when studied by electron microscopy. Newly secreted collagen molecules aggregate to form collagen fibrils, which will combine to form a larger collagen fiber. Collagen fibers are the most common fiber found in connective tissue and are responsible for the rigidity and strength of the connective tissue. The major types of these banded fibers are collagen types I, II, and III, and these collagen types are refered to as the interstitial collagens.

Type I collagen is the most abundant and widely distributed form of collagen found throughout the connective tissues of the body. It is located in bone, tendon, skin, tooth structures, periodontal ligament and gingiva (Bornstein and Sage 1980). Early work on collagen was performed on neutral-salt soluble and acid-soluble collagen derived from tissues composed entirely of type I collagen (rat tail or tendon), or tissues in which this form is predominate. There is also an atypical form of type I collagen referred to as type I trimer. Its designation is $\{\alpha 1(I)\}_3$. Type I trimer was observed in a polyoma virus-induced mouse tumor (Moro and Smith 1977), cirrhotic liver (Rojkind et al. 1979), human skin (Uitto 1979) and diseased human gingiva (Narayanan et al. 1980; Narayanan et al. 1985). Whether this represents a separate gene product or is the recombination of three $\alpha 1(I)$ chains is still unknown.

Type II collagen is a homotrimer that has been identified to exist in two forms. Types IIM and IIm are used to designate major and minor forms that have been separated based upon amino acid sequencing (Butler et al. 1977). The chain composition is designated $\{\alpha 1(II)\}_3$. Type II collagen is the main constituent of cartilage, vitreous humour and intervertebral discs.

Type III collagen is the second most abundant collagen type after type I. Except for tendon and bone, it is found in the same connective tissues as type I . It, like type II, is a homotrimer designated as $\{\alpha 1(III)\}_3$. While the function of type III collagen has not been demonstrated, there appears to be a correlation between the proportion of type III to type I collagen and the extensibility of the connective tissue (Burgeson and Morris 1987). Relatively high concentrations of type III

have been isolated from skin, blood vessels and placental tissues (Miller 1976), as well as gingiva and periodontal ligament.

Collagens that do not form broad banded fiber systems are types IV through X. Although their main distinguishing feature is their inability to form broad-banded collagen fibers, they like all collagens contain both helical and non-helical structural domains.

Type IV collagen is referred to as the basement membrane collagen. It contains two genetically distinct α chains, and is designated as $\{\alpha 1(IV)\}_2\alpha 2(IV)$. This form of collagen is highly glycosylated and lacks the characteristic 67nm fibrillar structure of interstitial collagens (Kefalides et al. 1979). Studies using monospecific antibodies revealed that type IV collagen molecules were localized to the lamina densa of the basal laminae. It is believed that this collagen in the basal lamina is involved in membrane ultrafiltration, and in the anchoring of neighbouring cells during the development and maintenance of tissues (Martinez-Hernandez and Amenta 1983).

Although type IV collagen contains both helical and non-helical domains, the size and orientation differ from that observed in the interstitial collagens. The main triple-helical domain in type IV collagen is longer than that found in the interstitial collagens (Kühn et al. 1981). In addition to containing cysteine residues which form disulfide bonds within its triple helix, type IV collagen contains several regions with a disrupted Gly-X-Y sequence, creating susceptible regions to enzyme attack (Bornstein and Sage 1980). The amino and carboxy terminal domains of type IV collagen are also different. The majority of the amino terminal end is triple helical, and this minor triple helical region is separated from the major central triple helix by a small globular domain called NC-2 (Timpl et al. 1979a). The designation NC indicates that this globular portion is not susceptible to bacterial collagenase attack. In addition, this minor triple helical portion is the site where type IV collagen molecules combine and later become part of the basement membrane complex (Timpl et al. 1981a). The carboxy terminal globular domain is similar in size to that found for the interstitial collagens.

Studies pertaining to the synthesis of basement membrane reveal that type IV collagen is incorporated directly into the matrix as a procollagen entity (Heathcote et al. 1978). Structural

studies of polymeric type IV collagen using electron microscopy suggest that four molecules form a tetramer joined by disulfide and other covalent linkages (Kühn et al. 1981).

Type V collagen is composed of a group of molecules having genetically distinct chains and similar structure (Sage and Bornstein 1979). Presently three α chains with different chain organization have been identified. Studies employing monoclonal antibodies suggest that type V collagen is present throughout connective tissue stroma (Linsenmayer et al. 1983), although its function is not well understood. Other studies indicate that type V collagen may be a basement membrane component (Stenn et al. 1979), and since it has been detected on cell surfaces it has also been considered as a cytoskeleton collagen (Gay et al. 1981).

Collagens are found throughout the hard and soft tissue components of the periodontium and contribute significantly to its function. For example, in the gingiva, collagens comprise the majority of the protein, with more than 60% of the total tissue protein being collagen. For various components of the periodontium, the distribution of the collagen types are shown in Table 1.2

STRUCTURE	COLLAGEN TYPES	PERCENT OF TOTAL
Alveolar bone	I	100
Periodontal ligament	I, III, V*	84, 16-18, 1
Cementum	I, III	95, <5
Healthy gingiva	I, III, IV, V	91, 9, <1, <1
Edentulous ridge mucosa	I, III, IV	85, 14, <1

Table 1.2: Distribution of collagen types in periodontal structures (Schroeder and Page 1990). '*' denotes the the proportion is not known.

1.4.3 Intracellular and extracellular metabolism

Collagen is synthesized by fibroblasts which secrete a precursor form, procollagen, into the extracellular space. Once procollagen is in the extracellular compartment, it is converted into collagen following cleavage of the N- and C-terminal peptide domains. Each collagen molecule is approximately 300,000 daltons, comprised of 3 unbranched polypeptide chains of approximately 1000 amino acids in length. Each polypeptide α chain is coiled into a left handed helix, and all three α chains are assembled in the collagen molecule to form a right handed helix.

Collagen biosynthesis can be separated into several stages. First the DNA is transcribed into corresponding mRNA's, which are translated into various alpha chains. Intracellular processing via post-translational modifications are necessary for the formation of triple helical procollagen molecules. Extracellularly, secreted procollagens are processed into molecules which, for example, are crosslinked into fibers in types I and III collagen, or form molecular networks as in type IV collagen. The sequence of collagen synthesis is outlined in Figure 1.4.

Transcription of DNA into mRNA for the pro-alpha chains is effected in the usual manner as for any other protein. First, DNA is transcribed into pre-mRNA which contains exons and introns. These are further modified by excision and splicing to form cytoplasmic mRNA. These steps give rise to several types of mRNA's, which code for different forms of collagen. Furthermore, different sizes of mRNA have been identified for the pro-α2(I) chain (Myers et al. 1981), and the pro-α1(I) chains (Chu et al. 1982), for human type I procollagen.

Post-translational modifications of collagen can be separated into intracellular and extracellular events. Intracellularly, modifications such as hydroxylation and glycosylation of the polypeptide chains result in the formation of procollagen molecules. Once procollagen is secreted to the extracellular space, further modifications include cleavage of extension peptides and cross linking of collagen molecules.

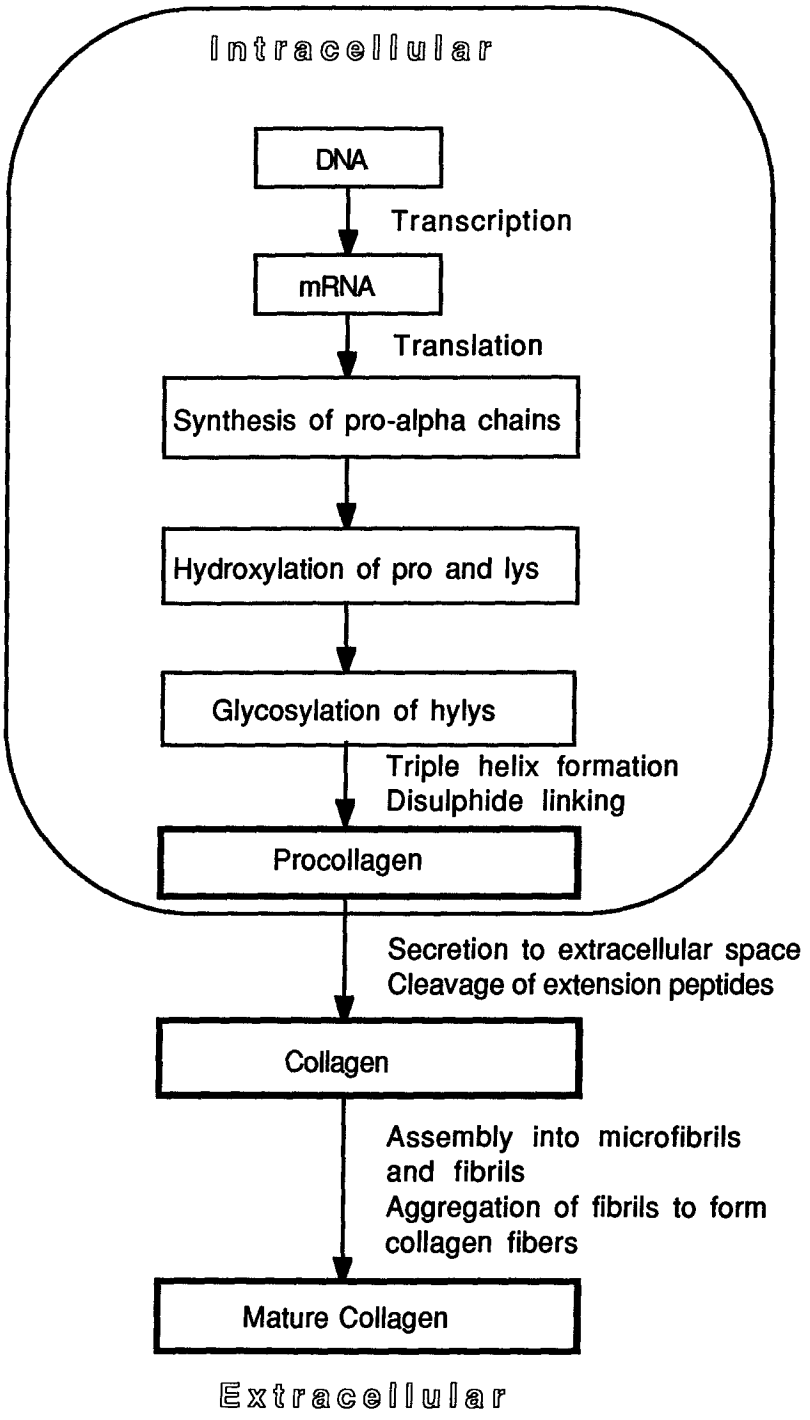

Figure 1.4: Schematic diagram showing the major steps in collagen biosynthesis. 'pro'= proline; 'lys'= lysine; 'hylys'= hydroxylysine.

The synthesis of the polypeptide chains occurs on membrane-bound ribosomes, and they pass through the membrane to the cisternae of the rough endoplasmic reticulum (Prockop et al. 1976). The pro-α chains are synthesized in a precursor form having an additional hydrophobic N-terminal pre-peptide sequence similar to sequences found for other secretory proteins. However, the pre-protein sequences of procollagen may be significantly longer than for other proteins. The pre-peptide in the newly synthesized pre-pro-α1(I) chain contains more than 100 amino acid residues (Palmiter et al. 1979; Sandell and Veis 1980). It is believed that the function of the pre-peptide is to bind the rough endoplasmic reticulum during polypeptide synthesis and perhaps play a role transporting the protein across the membrane (Davis and Tai 1980).

The pre-peptides of collagen are cleaved within the rough endoplasmic reticulum membrane during translation. One enzyme has been isolated and characterized from dog pancreas membranes (Strauss et al. 1979). This enzyme is inhibited by high concentrations of chymostatin and by some serine proteases. The specificity of this enzyme is uncertain as the amino acid sequence at the protein cleavage site can vary. However, incorporation of leucine and threonine analogues specifically inhibits the cleavage of the pre-protein segment (Hortin and Boime 1981). This would indicate that there is some level of control of the protease involved in the propeptide processing.

The next major intracellular modification is the hydroxylation of both proline and lysine residues. The hydroxylation of prolyl and lysyl residues are catalyzed by three distinct enzymes: prolyl 4-hydroxylase, prolyl 3-hydroxylase and lysyl hydroxylase. All three enzymes specifically require that the amino acid residue must be present in a peptide linkage (Kivirikko and Myllylä 1980), and that Fe^{2+}, 2-ketoglutarate, O_2 and ascorbate be present as co-factors. The resulting 4-Hyp, 3-Hyp and hydroxylysine (hylys) are found almost entirely in collagen with smaller amounts found in a few other proteins with collagen-like amino acid sequences (Adams and Frank 1980).

Further studies on the requirement for peptide substrates for enzymatic hydroxylation demonstrates a spatial requirement for hydroxylation of Pro and lysine residues. Studies on a number of peptides that the minimum sequence requirement for interaction with both prolyl 4-hydroxylase was attained with a X-Pro-Gly triplet, and for interaction, lysyl hydroxylase a X-Lys

-Gly triplet (Kivirikko and Myllylä 1980). In agreement with these sequence requirements both 4-Hyp and Hylys have been identified in collagen and collagen amino acid sequences. The only exceptions are for the sequence of X-Hyp-Ala in the first subcomponent of the first complement protein (C1q), and one sequence of X-Hyl-Ser or X-Hyl-Ala in some short non-helical end portions of collagen α chains (Gallop and Paz 1975).

The 3-Hyp has been identified in collagen only in the sequence Gly-3Hyp-4Hyp-Gly (Gryder et al. 1975). The reaction with peptidyl proline 3-hydroxylase appears to require a Pro-4Hyp-Gly sequence rather that Pro-Pro-Gly sequence which is probably not hydroxylated (Tryggvason et al. 1977). The prolyl 4-hydroxylase and lysyl hydroxylase is enhanced by the amino acid in the X position of the X-Y-Gly triplet being hydroxylated. Additionally, interaction with all three enzymes is influenced by amino acids in other parts of the peptide, peptide conformation and chain length (Prockop et al. 1976; Risteli and Kivirikko 1976; Risteli et al. 1976; Kivirikko and Myllylä 1980). The maximal velocity of the hydroxylation reaction is influenced by the structure of the amino acid in the 'X' position of the same triplet. The effect of increasing chain length is to decrease the reaction constant, K_m, of the peptide (Prockop et al. 1976; Kivirikko and Myllylä 1980). And finally, triple helical peptides do not allow for hydroxylation reactions to occur, which indicates that hydroxylation occurs before triple helical formation of pro α chains. However, within the non triple-helical domains of collagen pro α chains, the lysine before glycine order does not hold. Furthermore, Hylys that form in these regions are important since they are later converted to aldehydes which subsequently participate in cross linking collagen extracellularly (Olsen 1981).

Another intracellular event in the biosynthesis of collagen is the glycosylation of Hylys and asparagine residues in the propeptides. Two specific enzymes, galactosylhydroxylysyl transferase and glycosylgalactosylhydroxylysyl transferase, catalyze the O-glycosylation of hylys in the collagen domain of procollagen (Kivirikko and Myllylä 1979). The first enzyme, peptidyl hydroxylysine galactosyltransferase, couples a galactose sugar to certain Hylys's, while the

second, peptidyl galactosylhydroxylysine glucosyltransferase, transfers glucose to certain galactosylhydroxylysines.

Glycosylation takes place mainly while the pro α chains are still being assembled on the ribosomes, as there is no significant time lag between the hydroxylation of lysine and the glycosylation of Hylys (Kivirikko and Myllylä 1984). As in hydroxylation, glycosylation continues until the formation of procollagen triple helix prevents further reaction. Thus, the degree of glycosylation will depend upon the rate of triple helix formation. The extent of glycosylation is also different amongst the genetically distinct collagen types. This can be explained by regulation of the amounts of the single types of transferase enzymes.

Glycosylation is also influenced by several factors including peptide amino acid sequence, peptide chain length, and peptide conformation (Kivirikko and Myllylä 1979). The number of -X-Hyl-Gly- triplets in a polypeptide chain is important in the overall glycosylation, whereas the amino acid sequence in the region around Hyl residues may be unimportant (Anttinen and Hulkko 1980). The effect of peptide length is that the longer the chain the better it acts as a substrate. Triple helix formation prevents glycosylation, most likely due to steric hindrance with both transferases.

The carbohydrate donor for both peptidyl hydroxylysine glycosyltransferase enzymes is the corresponding uridine diphosphate (UDP) glycoside. Either UDP-galactose or UDP-glucose supplies the sugar moiety for the peptidyl Hylys residue. The reaction requires a bivalent cation, best served by manganese (Kivirikko and Myllylä 1979), although in vitro Fe^{2+} and Ca^{2+} have served as the cation cofactors (Myllylä et al. 1979).

The function of O-glycosylations in collagen is unknown. There have been suggestions that they play a role in the packing of collagen into fibrils. Intermolecular cross links are formed from glycosylated Hylys residues, and these units may have a role in the attachment of some cells to type IV collagen (Berman et al. 1980). However, glycosylation was found not to be a requirement for secretion of procollagen from cells, as secretion was not affected in patients with type VI

Ehlers-Danlos syndrome characterized by a deficiency of glycosylated and non-glycosylated Hylys residues (Quinn and Krane 1976).

Glycosylation of asparagine residues in collagen is affected by transfer of a mannose-rich oligosaccharide side chain from a dolichol phosphate intermediate. The acceptor site for the oligosaccharide side chain contains the triplet Asn-X-Thr-, in agreement with structural requirements for N-glycosyltransferases (Pesciotta et al. 1981). In type I procollagen most or all of the carbohydrate moieties reside in the C-terminal propeptide, whereas for type II procollagen, these carbohydrates are present in both the N-terminal and C-terminal propeptides. It has been suggested by some investigators that these aspargine-linked oligosaccharides are necessary to maintain a normal rate of procollagen secretion from the cell (Housley et al. 1980), but this view is not universally accepted (Kivirikko and Myllylä 1982).

The formation of intramolecular and intermolecular propeptide disulfide bonds contributes to the rate of formation and stability of triple helical procollagen. For the N-terminal peptides, in types I and II procollagens, only intrachain disulfide bonds are formed (Prockop et al. 1979). However, interchain disulfide bonds are found in the C-terminal propeptides for both these types of collagen. Type III procollagen has both N-terminal and C-terminal interchain disulfide bonds, and 2 additional interchain disulfide bonds at the C-terminal end of the triple helical domain. Both types IV and V procollagens consist of two or more polypeptide chains (Fessler et al. 1981a,b; Kumamoto 1981), which contain interchain disulfide bonds. The exception is for three pro α1(V) chains which do not form disulfide linkages (Fessler et al. 1981a,b; Kumamoto 1981).

As interchain disulfide bonds are found in the C-terminal region for types I, II and III procollagens, the C-terminal propeptides are considered to contain the information for interchain recognition and initial chain association (Rosenbloom et al. 1976). Supporting this concept is that interchain bonds between C-terminal pro-α chains are found when triple helix formation is prevented, whereas bonds between N-terminal pro-α chains in type III procollagen are not formed under the same situation. This indicates that disulfide bonds are formed earlier between C-terminal

propeptides than for N-terminal propeptides. Thus, triple helix formation from the three pro-α chains is likely to begin at the C-terminal end of these propeptides (Fessler et al. 1981c).

The hydroxyl groups of 4-Hyp are critical in that they stabilize the triple helix under physiological conditions (Prockop et al. 1976). With no Hyp residues present, the triple helix of collagen melts at approximately 25 °C, whereas with about 100 Hyp residues per α chain, the melting point rises to 40°C (Berg and Prockop 1973; Rosenbloom et al. 1973). Thus the formation of stable triple helices of collagen requires that approximately 50% of the proline residues incorporated into each pro-α chain become hydroxylated. Furthermore, it is known that non-helical pro-collagen is secreted by cells at one tenth the rate that normal pro-collagen is secreted (Kao et al. 1977; Kao et al. 1979).

Extracellular processing of collagens involves cleavage of extension peptides and formation of intermolecular cross links. The removal of the N- and C-terminal extension propeptides occurs via specific N- and C-terminal proteases. These are neutral endopeptidases and they require the presence of a divalent cation such as Ca^{2+}. In type I collagen for example, removal of extension peptides occurs via specific proteases after the secretion of the entire propeptide (Bornstein and Sage 1980). For type I procollagen, the cleavage site for the N-terminal protease in the pro-α1(I) chain is through a proline-glutamine bond (Hörlein et al. 1979). Furthermore, it is known that without removal of the propeptides, collagen fibril formation is inhibited (Prockop et al. 1979).

Fibril formation occurs spontaneously for interstitial collagen molecules once propeptides have been removed. Although the rate to which this can occur *in vivo* is difficult to assess, macromolecules like fibronectin and proteoglycans are thought to affect the rate of fibril formation (Kleinman et al. 1981).

Major collagen cross linking occurs via aldol condensation following oxidation of lysine and hylys residues by lysyl oxidase (Kuboki et al. 1981). The oxidative deamination of ε-amino groups in certain lysine and hylys residues yields reactive aldehyde groups termed allysine and hydroxyallysine respectively. These aldehydes can then condense with other lysine or hylys residues or with allysines to form covalent intermolecular cross linkages.

There are two main mechanisms for intracellular and extracellular degradation of collagen. Intracellular degradation of fragments of collagen fibrils is believed to occur via lysosomal metabolism. Extracellular degradation is also affected by enzymes, but is probably modulated by the tissue, cell type, and the disease state.

Newly synthesized collagen and collagen fibrils initially present extracellularly both can undergo intracellular collagen degradation. Degradation of newly synthesized collagen appears to serve both as a basal turnover rate phenomenon, as well as a mechanism for removing defective collagen. Intracellular degradation of extracellular collagen is believed to occur after disruption of the collagen fiber, with subsequent phagocytosis and digestion by the lysosomal enzymes.

It has been shown that 10 to 20% of newly synthesized collagen is degraded intracellularly, while the remainder is secreted to the extracellular environment. This degradation based on determinations for radiolabelled Hyp has been demonstrated for cells synthesizing types I, III, and IV collagen (Bienkowski et al. 1978a,b; Palotie 1983). The observation that this intracellular degradation is relatively consistent for a variety of collagen types, suggests that it may be a universal phenomenon.

In addition to the basal level of intracellular collagen degradation, the conformationally abnormal newly synthesized collagen is degraded at an enhanced rate. The role of degradation appears to eliminate defective pro-α chains that are unable to form a stable helix. Diploid human fibroblasts, which form pro-α chains that are deficient in 4-Hyp and do not form triple helical molecules, degrade approximately one third of their newly synthesized collagen (Berg et al. 1980). The same investigators also found that cis-4-Hyp, a proline analogue, prevents triple helix formation and significantly enhances the degradation of newly synthesized pro-α chains. Other studies demonstrated that the use of amino acid analogs not only rendered collagen more susceptible to proteolysis (Uitto and Prockop 1974), but that the proteolysis occurred within a short time after synthesis (Berg et al. 1984).

Enhanced intracellular degradation was also found to occur in collagen that was thermally denatured or altered by incorporation of amino acid analogues. Studies of incubated human skin

fibroblasts at 30°C and 42°C showed an increase in newly synthesized collagen degradation from 12% to 49% due to increased temperature (Steinmann et al. 1981). Increased intracellular collagen degradation was also observed in systems which block the enzyme prolyl hydroxylase (Kao et al. 1979), thus interfering with the conversion of Pro to Hyp. These studies support the concept that collagen conformation is important in the molecules' stability.

In addition to proteolysis of non-helical or defective collagen, another control mechanism has been demonstrated. Under-hydroxylated collagen is nonhelical at physiological temperature and pH, and it has been shown to be secreted at one tenth the rate of fully hydroxylated collagen (Kao et al. 1979).

Extracellular collagen degradation occurs via attack by enzymes in the matrix and to some extent by the actions of by-products of metabolism. The major enzyme responsible for collagen degradation is mammalian collagenase. Other enzymes, such as elastases, gelatinases, proteases, and bacterial collagenases also breakdown collagen. By-products of protein metabolism such as volatile thiol compounds have also been shown to disrupt interstitial collagens (Johnson et al. 1985, 1992).

Collagen fibrils are stable at physiologic temperature and pH, and are resistant to cleavage by proteases within their helical domains. If denatured by heat which disrupts the triple helix for example, the α chains can be cleaved by proteolytic enzymes. However, mammalian collagenases, which are metalloproteinases dependent upon Ca^{2+}, are able to cleave collagen at physiological temperatures into 3/4 and 1/4 fragments. Once these fragments are formed they spontaneously denature and can then be further degraded by proteases. In contrast to mammalian collagenase 3/4 and 1/4 cleavage products, the bacterial collagenase digests collagen to numerous small peptides that are readily dialyzable.

Mammalian collagenase cleaves interstitial collagen in a specific helical region. In type I collagen this cleavage occurs through the Gly-Ile bond in the α1(I) chain (residues 775-776), and through the Gly-Leu bond in the α2(I) chain. In the α chains of type III collagen, the cleavage

also occurs through the Gly-Leu bond. This forms the N-terminal 3/4 and C-terminal 1/4 fragments which spontaneously denature at 37°C.

Type III collagen differs from type I in that it can be degraded in its native form by enzymes other than mammalian collagenase. Type III collagen is susceptible to attack by other proteases such as elastase, trypsin, and thermolysin (Miller et al. 1976; Mainardi et al. 1980b), in the same region where collagenase acts. In addition, elastase is thought to further contribute to collagen degradation by cleaving the telopeptide region thus solubilizing the entire molecule (Starkey et al. 1977).

Mammalian collagenase is secreted in a latent form. Agents that convert the latent to an active form include proteases such as trypsin and plasmin, and organomercurial compounds. Metal chelating agents such as EDTA produce total inhibition, while thiol agents like cysteine and dithiothreitol produce partial inhibition of collagenase activity. Two additional collagenase inhibitors found in plasma are α2-macroglobulin and β_1-anticollagenase (Woolley 1984).

Type IV collagen is an integral part of basement membranes and its degradation may have a profound effect on the integrity of this matrix. In studies of collagen degradation it was found that type IV collagen was resistant to mammalian collagenase (Welgus et al. 1981). Several investigators have identified and characterized a type IV specific degrading enzyme. It is also a metalloproteinase that is activated by trypsin or mercuric chloride (Salo et al. 1983). The cleavage site was determined to be near the N-terminal portion of the molecule (Fessler et al. 1984).

Other proteinases are also capable of degrading type IV collagen. Pepsin extracted type IV collagen has been shown to be susceptible to a mast cell proteinase (Sage et al. 1979) and to a human neutrophil metallogelatinase (Murphy et al. 1980). Human leukocyte proteases were also found to degrade native type IV collagen (Mainardi et al. 1980a; Uitto et al. 1980). These results suggest that this lattice form of collagen is more labile than the fibrillar collagens of the interstitial group.

Metabolism of collagen in the periodontium varies with each collagen type and the rate differs from other tissues of the body. Studies based on conversion of radiolabelled Pro into labelled Hyp

demonstrated a faster rate of synthesis in oral tissues than in skin. It was also determined that the label was lost at a faster rate in gingival tissues than other connective tissues of skin, tendon and bone, indicating a more rapid turnover in the gingiva.

Additional studies on collagen metabolism in the periodontium have been performed using animal models (Sodek 1976; Sodek 1977; Sodek et al. 1977; Sodek 1978; Sodek and Limeback 1979). Sodek has demonstrated that although collagen turnover is higher in gingiva than in bone or skin, it is lower than in periodontal ligament (Sodek 1977; Sodek 1978). These different turnover rates are reflected in different half lives for collagen. For example, the half life for mid-root peridontal fibers is 7.5 days while for dentogingival fibers is 25 days. These studies have shown that collagen metabolism in the oral cavity is complex.

Destruction of periodontal connective tissue results from increased activity of tissue proteolytic enzymes, including matrix metalloprotease and plasminogen/plasmin cascades (Birkedal-Hansen 1988). Since mammalian collagenases are the only tissue proteases capable of degrading native collagen, they can initiate degradation of collagen.

Studies of collagenase activity in relation to severity of gingival inflammation have demonstrated an increased enzyme activity in inflamed human gingiva and crevicular fluid as compared to healthy tissue and fluid (Uitto and Raeste 1978; Overall et al. 1987). In addition, moderately inflamed tissue was found to release more latent collagenase than did severely inflamed gingiva. This latent form of the enzyme can be activated by trypsin or bacterial plaque (Uitto and Raeste 1978).

The sources of the collagenases and mechanisms of their activation were subsequently elucidated. The role of gingival, GCF and salivary interstitial collagenases in human periodontal diseases was investigated by Sorsa and co-workers (Sorsa et al. 1990). It was found that all three collagenases had M_r of 70K and existed predominantly in a latent form that could be activated by aminophenylmercuric acetate, gold thioglucose and hypochlorous acid. While several serine proteases also activated gingival and salivary collagenases, plasmin and plasma kallikrein were ineffective. The collagenases from all three sources degraded types I and II collagens at

approximately equal rates which were considerably faster than degradation of type III collagen. These findings indicated that the collagenases found in inflamed gingiva, GCF and saliva resemble the enzyme derived from neutrophils.

In contrast, an investigation of the collagenase activity in crevicular fluid of patients with juvenile periodontitis (JP) indicated that these samples contain an enzyme from a different source (Suomalainen et al. 1990). Gingival crevicular fluid from untreated JP pockets contained mammalian collagenase which cleaved type II at a markedly slower rate than types I and III collagens. This substrate specificity is characteristic of collagenases produced by fibroblasts, epithelial cells and macrophages. Furthermore, they found that *A. actinomycetemcomitans* isolated from subgingival plaque samples of these patients was able to release collagenase from PMN's *in vitro*. They postulated that due to a lack of normally functioning PMN's in the periodontium of JP patients, the bacteria activate resident periodontal cells to produce increased amounts of collagenase.

Regardless of the source of mammalian collagenases, they are important initiators of collagen degradation. These are the only known enzymes that degrade the native interstitial collagens via cleavage within their helical domain. Once collagen is cleaved into 3/4 tissue collagenae A (TC^A) and 1/4 tissue collagenase B (TC^B) fragments, it is subject to denaturation at body temperature and further degradation by gelatinases and proteases. Proteinases such as telopeptidase, elastase and cathepsin G can cleave the cross link regions and eliminate TC^A triple helical complexes, thus exposing the chains to further proteolytic degradation. Since bacterial collagenase can completely degrade collagen to small peptides, the demonstration of TC^A collagen cleavage fragments in moderate to severely inflamed gingival tissues (Overall et al. 1987), suggests a dominant role for mammalian collagenases in collagen destruction in inflamed gingiva.

1.4.4 Non-collagenous components

In addition to the major connective tissue protein collagen, other macromolecules, namely glycoproteins, glycosaminoglycans, proteoglycans and elastin account for most of the remaining

extracellular matrix (ECM) content in periodontal connective tissue. While extensive research has focused on collagen of gingiva and periodontal ligament, comparatively much less attention has been devoted to these other proteins.

Most extracellular proteins are glycosylated, and therefore, by definition are glycoproteins. Four types of carbohydrate linkages to protein have been distinguished. They are the proteoglycans which contain GAG's linked O-glycosidically via xylose to either serine or threonine, the collagens that have glucose and galactose O-glycosidically linked to Hylys, the mucins which contain oligosaccharide O-glycosidically linked to serine or threonine and a further group of glycoproteins that have oligosaccharide with a mannose core N-glycosidically linked to asparagine. Possible functions of these carbohydrate linkages include modification of physical properties, alterations to the susceptability of enzymatic degradation, regulation of metabolic behavior of the protein and organization of proteins within cells, in membranes and in the extracellular compartment (Hakomori et al. 1984).

Fibronectin (FN) is a major glycoprotein which is found in the extracellular spaces, cell surface, basement membranes and body fluids. It is synthesized by fibroblasts, endothelial cells and monocytes. It is a dimer consisting of polypeptide chains of approximately 2000 amino acid residues, which are linked together via disulfide bonds to form a molecule of approximately 450,000 daltons. FN has been implicated in a number of functions which have been observed *in vitro*. Various observed functions have been related to plasma, extracellular matrix organization, homeostasis and wound healing.

Fibronectin promotes cell attachment and spreading which are mediated by interaction of FN with cell surface receptors. A specific region of FN, of about 150,000 daltons, has been identified as the specific domain required for these activities (Sekiguchi and Hakomori 1980).

Fibronectin is also involved in organization of extracellular matrix and basement membranes. Its role in matrix organization is not well understood but is conjectured to be via binding of FN to collagen (Kleinman et al. 1976; Pearlstein 1976) and to proteoglycans (Yamada et al. 1980; Oldberg and Ruoslahti 1982). Covalent cross linkage of FN to collagen, fibrin and other

macromolecules occurs via the enzyme transglutaminase, which is thought to react with a glutamine residue on FN's N-terminal region (Mosher et al. 1980).

Fibronectin also plays a role in homeostasis and wound healing by participating in several different processes. These include homeostasis at the site of injury, clearance of cell debris and blood clots, formation of a temporary scaffolding and recruitment of granulation tissue.

FN is involved in homeostasis via interactions with several components. In early studies the mechanism was thought to occur via the interaction of FN with fibrin (Ruoslahti and Vaheri 1975), and crosslinkage of FN by transglutaminase. Further studies showed that FN is covalently cross linked to fibrin via factor VIIIa (Mosher 1975), which in turn mediates cell adhesion to fibrin (Grinnell et al. 1980). Activated platelets have been shown to express surface FN, whereas nonactivated platelets do not (Ginsberg et al. 1980). In addition, FN enhances the spreading of platelets on collagen surfaces (Grinnell et al. 1979). Furthermore, due to the above interactions, it has been suggested that FN plays a role in platelet adhesion to the subendothelium of injured blood vessels (Bensusan et al. 1978).

Clearance of cellular debris and blood clots from wound areas by macrophages is believed to be mediated by FN acting as a nonspecific opsonin (Zinkevich et al. 1982). Both monocytes and polymorphonuclear leukocytes have FN receptors on their cells surface (Bevilacqua et al. 1981; Pommier et al. 1984). Thus, via interaction with these receptors, FN may play a role in debris clearance by mononuclear phagocytosis.

The ability of FN to interact with a component of the first complement protein, C1q (Pearlstein et al. 1982), may be important in promoting wound healing and homeostasis. Since C1q structurally possesses a helical domain, aggregation of this molecule with FN may function as a scaffolding matrix. It has been recently shown that aggregated forms of C1q bind to the fibroblast C1q receptor (Bordin et al. 1990), and thus may play a role in fibroblast cell adhesion. Potentially these interactions of FN, C1q and fibroblasts may be important in directing the wound healing matrix. In addition, because biologically C1q has immunological activity, aggregation of this complement protein can participate in immune-mediated opsonization.

Studies on experimental granulation tissue have implicated FN for additional roles in wound healing. Not only was the concentration of FN increased after wounding, but also the factor VIIIa-mediated cross linked FN to fibrin served as a better substrate for fibroblast attachment and spreading than did fibrin alone (Grinnell et al. 1980). In addition, FN was found to act as a chemoattractant for both endothelial cells (Bowersox and Sorgente 1982) and fibroblasts (Postlethwaite et al. 1981). Furthermore, FN in granulation tissue was found to be primarily associated with fine fibers of type III collagen, while later on in wound healing when there is a predominance of thicker, mature type I collagen fibers, FN is no longer present (Kurkinen et al. 1980). This correlated with the observation that FN has the strongest association with type III collagen, when FN is experimentally exposed to all collagen types in their native state (Engvall et al. 1978).

Laminin is a glycoprotein which consists of three subunits of 200,000 - 220,000 daltons and one large subunit of 400,000 - 440,000 daltons, and is supported by interchain disulfide bonds (Timpl et al. 1979b). The small and large subunits are referred to as a and b subunits, and they are biologically and immunologically distinct (Rao et al. 1982). This large glycoprotein is an important component found within all basement membranes, where it serves as a major attachment glycoprotein for epithelial cells (Terranova et al. 1980). In addition to having domains for epithelial cells, laminin is able to bind both type I collagen and proteoglycans on the ends of its a and b subunits (Yamada 1983). Furthermore, laminin was found to have the capacity for adhesion to fibroblasts, with a similar affinity to that of FN (Couchman et al. 1983). Through the interactions with epithelial cells, proteoglycans, fibroblasts and collagen, laminin acts as an extracellular glue important for maintaining the integrity of basement membranes.

Glycosaminoglycans (GAG's), which are long unbranched polysaccharide chains composed of repeating disaccharide units, are another important class of macromolecules found in the periodontium. Formerly called mucopolysaccharides, the GAG's characteristically have one of two sugars in the repeating disaccharide always an amino sugar, either N-acetyl glucosamine or N-

galactosamine. Due to the presence of sulfate or carboxy groups on both or many of the sugar residues, GAG's are very negatively charged.

Glycosaminoglycan chains are synthesized by sequential addition of sugars from sugar nucleotide precursors. For example, UDP-glucuronic acid and UDP-N-acetylgalactosamine are precursors for chondroitin sulfate (Silbert 1964). For GAG's acting as side chains in proteoglycans, the protein core is synthesized first and then sites for monosaccharide transferases involved in GAG synthesis are presented (Roden 1980).

Proteoglycans comprise a large group of macromolecules that are ubiquitous in extracellular connective tissue (Lindahl and Höök 1978). Each is composed of a central protein core to which multiple GAG chains are attached via oligosaccharide linkages. The oligosaccharide linkage for most GAG's to this protein core is a trisaccharide consisting of galactosyl-galactosyl-xylose (Lindahl and Roden 1972). The xylose is glycosidically linked to the hydroxyl moiety of serine in the protein chain. Each proteoglycan molecule is composed of 2 or more different types of GAG's. The total number of GAG's can vary from one or two to more than a hundred chains per proteoglycan molecule (Heinegärd and Paulson 1984). This forms the basic proteoglycan unit. These units can then be covalently bound via a link protein to a central core of hyaluronic acid (Hardingham and Muir 1972), a linear polymer of alternating glucuronic acid and N-acetyl glycosamine units.

The function of proteoglycans and GAG's has mainly been derived from studies on cartilage (Comper and Laurent 1978). Proteoglycans are able to retain water and thus can produce an increase in tissue pressure. In cartilage, an increase tissue pressure is manifested in compressive stiffness, which has been shown experimentally to be related to the presence of the GAG components of proteoglycans (Kempson 1980). Studies by Comper and Laurent have indicated that proteoglycans limit diffusion of macromolecules through connective tissue (Comper and Laurent 1978). It is thought that due to their anionic and hydrated form, proteoglycans are responsible for maintaining tissue and cell functions via interaction with matrix and cell surface components.

The breakdown of matrix structure of GAG's and proteoglycans appear to be initiated by glycosidases and proteases (Fluharty 1982). It is believed that after enzymatic cleavage and disaggregation of matrix components, cells take up the fragments via pinocytosis and further degradation occurs via lysosomes .

Proteoglycans present in the periodontium are primarily secreted by the resident fibroblasts. The major proteoglycans located in the gingiva are chondroitin sulfate and dermatan sulfate (Shibutani et al. 1989). Dermatan sulfate, the most prominent GAG in gingival connective tissue is found uniformly distributed in the periodontium. Although chondroitin 4-sulfate also has a wide distribution, it is present in relatively much smaller amounts. Furthermore, chondroitin 6-sulfate is found only perivascularly.

Chondroitin sulfate is the predominant GAG in alveolar bone. Chondroitin 4-sulfate accounts for more than 90% of the total GAG's extracted, while smaller amounts of hyaluronic acid, dermatan sulfate, and heparin sulfate were also isolated (Waddington et al. 1989). In addition, proteoglycans in bone were found to be smaller in size than those present in the gingiva, but similar in size to those present in hard tissues of cementum and dentin (Bartold 1990).

Elastin, another major fibrous extracellular protein besides collagen, is a prominent component in connective tissues where resiliency is important. High concentrations are found in the aorta, large arteries, lung and skin. Elastin is composed of two distinct components: an amorphous elastin component which comprises 90% of the mature fiber, and a microfibrillar component of small fibrils 10-12 nm in diameter.

Tropoelastin, the precursor of elastin, is secreted by the cell as a 72,000 dalton polypeptide. These molecules are cross linked via the aldehyde groups of lysine formed by enzyme peptidyl lysine oxidase (Siegel 1980), which is the same enzyme involved in collagen cross linking. The resulting insoluble polymer which is highly cross linked serves as the elastomer. Although the same enzyme is involved, cross linking in elastin differs from that in collagen. Since there is no hylys and little histidine in elastin, they are not involved in the process. The final result of the

condensation reactions of four lysyl residues, is the formation of desmosine and isodesmosine, two cross linkage components unique to elastin.

1.5 VOLATILE SULPHUR COMPOUNDS

1.5.1 Substrates

Volatile suphur compounds (VSC) are a group of substances that are associated with and contribute significantly to halitosis. These compounds are emitted in breath and originate from oral and non-oral sources, such as nasopharyngeal, respiratory and gastro-intestinal systems (Sulser et al. 1939).

Most investigations on the mechanism of production of oral malodour were performed on putrescent saliva systems. Whole saliva contains a variety of substances including bacteria, desquamated epithelial cells, lysed leukocytes, food debris and blood. From this complex mixture an objectionable odour is generated within one hour of incubation (Massler et al. 1951). The reduction in intensity of putrefactive odour following thorough brushing of teeth and associated oral tissues demonstrates that the oral cavity is the principle source of breath malodour emitted via the mouth (Sulser et al. 1939).

Oral malodour is intensified in individuals whose saliva exhibits increased putrefactive activity (Tonzetich and Richter 1964). Investigations by Tonzetich and co-workers on head space analysis of putrescent systems, provided the initial insights on VSC substrates (Tonzetich and Kestenbaum 1960; Tonzetich and Richter 1964). Their studies showed that cellular elements are the main sources of substrate for the production of VSC. Chemical and organoleptic evaluations attributed the putrescence of putrefied saliva principally to acidic compounds. The VSC are produced via putrefaction by microorganisms of proteinaceous substrates, such as desquamated epithelial cells, blood cells and food debris (McNamara et al. 1972). In the process, the cells are disintegrated, the disulphide substrate is liberated and reduced to thiols; these serve as immediate precursors of VSC (Tonzetich and Johnson 1977). It is important to note that the addition of sulphur-containing

amino acids intensified the putrescence of incubated saliva. While substrates such as cysteine that possess free thiol groups, were the most immediate sources of malodour, disulphide substrates like cystine produced odour of similiar intensity but at a slower rate.

Oral malodour has also been correlated with the severity of periodontitis, and was shown to decrease following periodontal therapy (Tonzetich and Spouge 1979). The initial elevated malodour in periodontitis subjects is attributed to several factors. In periodontitis, increased numbers of desquamated epithelial cells and adherent bacteria are found in saliva (Dreizen et al. 1956). Furthermore, haemorrhage and increased crevicular fluid flow from affected periodontal sites provide increased sources of putrescible substrates (Cimasoni 1983).

1.5.2 Bacteria

Sulphur containing volatile compounds are formed through degradation of sulphur-containing substrates by oral microflora (Tonzetich and Kestenbaum 1960; Tonzetich and Carpenter 1971). At least five genre of anaerobic gram-negative oral microorganisms commonly found in the deepest region of periodontal pockets are capable of forming H_2S. Spirochaetes, fusiforms, vibrios, veillonellae and bacteroides species produce H_2S (Schwabacke and Lucas 1947; Breed et al. 1957; Omata and Braunberg 1960; Sawyer et al. 1962; Rogosa and Bishop 1964). *Porphyromonas asaccharolyticus* which produces profuse amounts of VSC (Tonzetich and McBride 1981), especially CH_3SH and $(CH_3S)_2$, is found in high numbers in the gingival crevice (Syed et al. 1981). This organism has been implicated as a primary pathogen in the etiology of periodontal disease.

1.5.3 Analysis of volatile compounds produced in the oral cavity

Individual malodorous components of breath and saliva have been identified using gas chromatographic (GC) and/or mass spectrometric analyses (Tonzetich and Richter 1964; Larsson 1965; Kostelc et al. 1980; Kostelc et al. 1981). Tonzetich introduced the GC method employing a highly sensitive and selective flame photometric detector (FPD) coupled with a GC unit (Brody and

Chaney 1966) for direct measurement of subnanogram quantities of VSC present in mouth air samples (Tonzetich 1971).

The direct GC analysis identified three volatile sulphur compounds in mouth air of all individuals (Tonzetich 1971). Hydrogen sulphide (H_2S) and methyl mercaptan (CH_3SH) were established as the primary volatile components which accounted for approximately 90 per cent of the sulphur content of mouth air. The remaining minor component was identified as dimethyl sulphide, $(CH_3)_2S$. Concentrated air samples also demonstrated the presence of dimethyl disulphide, $(CH_3S)_2$, with increased prevalence and concentration in periodontal cases (MacKay and Hussein 1978). Kaizu and co-workers provide convincing evidence that except for objectionable breath emitted by periodontally diseased individuals, the malodorous concentration of CH_3SH emanating from the oral cavity can be controlled by tongue scraping (Kaizu et al. 1978). Other investigators have also shown that VSC produced on the tongue surface in periodontal patients, contributes sixty percent of the total sulphide content of mouth air (Yaegaki and Sanada 1991). Reduction of objectionable breath from these patients was further accomplished by periodontal treatment which removed a significant portion of the VSC content from their mouth air.

Rizzo reported a positive correlation between the amount of H_2S produced in gingival crevice and the depth of corresponding periodontal pockets (Rizzo 1967). Similarly, mouth air studies in our laboratory have indicated that levels of VSC are considerably increased in the mouths of persons with periodontal disease. In a study of twenty periodontal subjects, the concentration of H_2S and CH_3SH correlated with the incidence and depth of periodontal pockets greater than 3 mm. Furthermore, the levels of both compounds were markedly reduced following curettage and corrective periodontal surgery (Tonzetich and Spouge 1979). Similar studies of salivary volatiles indicate that other volatile products of salivary putrefaction such as pyridine and its 2-, 3-, and 4-methyl analogues are present in elevated amounts in saliva collected from individuals with moderate to severe periodontitis (Kostelc et al. 1980).

1.5.4 Implications in periodontal disease

It has been previously reported that volatile thiol compounds, H_2S and CH_3SH produced through putrefactive activity in the gingival sulcus, have the capacity to increase the permeability of non-keratinized oral mucosa. Using a two-chamber diffusion apparatus, both ions, (^{35}S)-SO_4, and small molecules, (^{3}H)-PGE_2, were found to penetrate more readily across mercaptan exposed than untreated mucosa. This penetration followed a gradual diffusion kinetics until a steady state was reached (Ng and Tonzetich 1984). Using the same diffusion apparatus, it was found that the diffusion of ^{14}C- labelled LPS from E. coli followed a different pattern. Initially a component of the (^{14}C)-LPS was found to diffuse, which decreased with time, through both control and CH_3SH treated specimens. A marked increase in diffusion of (^{14}C)-LPS was obtained with tissues with prior exposure to CH_3SH (Tonzetich et al. 1987). These results indicated that CH_3SH increases the permeability of mucosa to ions, small molecules, and LPS. Thus one mechanism whereby thiol agents may contribute to the etiology of periodontal disease is by disrupting the integrity of the mucosal barrier.

Once volatile sulphur compounds penetrate the epithelial barrier, they can then react with the underlying connective tissue and accompanying cells. Human gingival fibroblasts were shown to be adversely affected when exposed to H_2S and CH_3SH. Exposure of cells to 10ng H_2S or CH_3SH/ml air/CO_2 during 26 hours of activation and 4 hrs of pulsing resulted in a 44-47% reduction in total DNA synthesis (Johnson et al. 1992).

Direct effect of VSC on collagen has been demonstrated in our laboratory. Exposure of type I rat-tail tendon collagen to H_2S and CH_3SH resulted in the conversion of some mature fibrillar (Tonzetich and Lo 1978) and acid-soluble forms (Johnson and Tonzetich 1985; Johnson et al. 1985) to a neutral salt-soluble product. CM cellulose and SDS/PAGE/fluorography analysis of both neutral salt-soluble and acid-soluble products generated from acid-soluble collagen reacted with radiolabelled H_2S, yielded radioactivity associated with $\alpha 1$, $\beta_{1,1}$, $\beta_{1,2}$, and $\alpha 2$ chains characteristic of type I collagen. Furthermore, it was determined that thiol groups (-SH) reacted with 2 or more active sites on the molecule (Tonzetich and Johnson 1986).

The demonstration of decreased DNA synthesis suggested that VSC inhibits a certain population of cells during their cell cycle or perhaps that there is a reduced availability of substrates. By investigating Pro transport of fibroblast cells, a 35% and 36% reduction in Pro transport was reported in the presence of 10ng of H_2S or CH_3SH/ml air/CO_2, respectively (Tonzetich et al. 1985). Subsequent staining of connective tissues with fluorescein diacetate and counter staining with ethidium bromide demonstrated damage to cells exposed to CH_3SH. Since the uptake of amino acids is a membrane-associated phenomenon, disruption of the integrity of cell membranes by H_2S and CH_3SH would affect protein synthesis.

Investigations of the effect of H_2S- and CH_3SH-treated human gingival fibroblast cultures on protein and collagen metabolism have also been reported (Johnson 1983). Exposure to H_2S decreased total protein synthesis by 18 to 20%, but did not alter the ratio of type I to type III procollagen, 8.5/1. CH_3SH-exposed fibroblast cultures showed a 35% decrease in total protein content. There was a reduction in type III collagen and almost a complete absence of type III procollagen. Furthermore, type I collagen content was reduced, whereas type I procollagen and/or type I protrimer increased approximately two-fold.

Investigations on the effect of CH_3SH on collagenolytic proteases and cAMP production were performed using human gingival fibroblast cultures. Following exposure to CH_3SH, cathepsin B activity increased by 20% , while cAMP content of CH_3S-exposed cultures was increased by 34% and by 58% in the same systems supplemented with IL-1 (Tonzetich et al. 1990).

The effect of CH_3SH on IL-1 secretion by leukocytes was studied using human tonsillar mononuclear cells. Exposure of these cells to 10 ng/ml of CH_3SH was as effective as $5x10^{-5}$ M 2-mercaptoethanol in augmenting a LPS augmentation of IL-1 production (Waterfield and Tonzetich 1989). This implies that CH_3SH may play a role in activation of the immune system.

Recent investigations by Ratkay and Tonzetich, have shown that exposure of T lymphocytes to CH_3SH resulted in a 30% increase in IL-1 production (Ratkay and Tonzetich 1992). In addition, exposure of fibroblasts to CH_3SH increased collagenase production. Thus, it is possible that

CH_3SH potentiates collagenase production either by direct interaction with fibroblasts, and/or via an IL-1 mediated pathway.

Recently Ouyang (Ouyang 1991) demonstrated that Zn ion can reverse the adverse metabolic effects of CH_3SH. It was found that 10^{-4} to $0.5x10^{-5}$ M Zn completely nullified the CH_3SH adverse effects on protein content and proline transport of human fibroblast cell cultures.

The described results suggest that VSC affects periodontal tissue directly as well as indirectly by disrupting the tissue and normal fibroblast cell function. Thus, once H_2S or CH_3SH gains entry into the tissue it can be involved in a number of vital biochemical reactions contributing to potentiation of periodontal disease.

1.6 PERIODONTAL DISEASE

1.6.1 Health versus disease state

The periodontium consists of four tissues surrounding each tooth. The cementum, alveolar bone, periodontal ligament and gingival tissues function in several ways to maintain the integrity of the dentition. They secure the teeth within bone, resist forces of mastication, maintain a barrier between the external and internal environments of the body, provide a source of cells to support growth and repair, and defend against noxious agents.

Periodontal disease is a process which destroys the tooth's supporting structures, the periodontium. Although periodontal disease is clinically complex and heterogeneous, it is categorized as an inflammatory process which, depending upon an individual's host response, results in loss of alveolar bone and loss of ligamentous attachment (Page and Schroeder 1981).

The different classifications of inflammatory periodontal disease are broadly divided into two categories. Gingivitis and periodontitis have been characterized as separate entities based upon histopathologic, bacteriologic and immunologic evidence, which have provided the basis for understanding the disease process. Initially a reversible inflammation that is induced by dental plaque which causes irritation in the gingival crevice, is considered gingivitis. Depending on

whether this source of irritation is removed, gingivitis can be present in either an acute or chronic form. Persistance of the inflammation can lead to deeper penetration into the underlying connective tissues. Extension of the inflammatory process causes proliferation and apical migration of the junctional epithelium, resulting in periodontal pocket formation. Loss of subjacent connective tissue and underlying alveolar bone are indicative of the state of periodontitis.

A number of different periodontitis lesions have been categorized. Children and adolescents can be affected by generalized or localized juvenile periodontitis. The localized form is characterized by rapid loss of attachment and distinct bone loss around the mandibular incisors and around the first molars. The generalized form also involves other teeth with the same distinct rapid bone loss. Adults can be affected by rapidly progressive or by adult periodontitis forms. Rapidly progressive periodontitis is seen most commonly in young adults in their twenties and early thirties. As its name implies, it is a rapid form which is characterized by markedly inflamed gingiva and significant bone loss (Page 1983). The disease may have periods of remission in which the gingiva appears clinically normal, however the patients are now known to exhibit defects in either neutrophil or monocyte chemotaxis. Adult periodontitis is the most common form of periodontitis. The age of onset is usually after 30 to 35 years of age. Unlike the other forms of periodontitis, there are no localized patterns of bone loss, no evidence of rapid progression and no systemic abnormalities.

Microorganisms have been implicated as the primary causative agents in the pathogenesis of periodontal disease. Both direct and indirect mechanisms of tissue breakdown have been described. Direct tissue destruction is a result of endotoxins, exotoxins, proteolytic enzymes and cytotoxic substances, which have been shown to disrupt tissue and cellular integrity (Ng and Tonzetich 1984; Kryshtalskyj and Sodek 1987; Overall et al. 1987; Uitto 1987). Indirect mechanisms involve activation of host cells in response to bacterial products such as antigens or mitogens. This host-mediated tissue injury is a result of inflammation, mediated via complement activation and liberated lymphokines from sensitized lymphocytes (Genco and Slots 1984).

Pathogenesis of inflammatory periodontal disease is characterized into four distinct stages (Page and Schroeder 1976). These are described by Page and Schroeder as the initial, early, established and advanced stages. Ultrastructural and histopathological features have been previously used to examine the stages of experimental gingivitis (Listgarten and Ellegaard 1973; Payne et al. 1975). The same techniques were applied to defining the nature of periodontal inflammatory lesions, with insight as to the progression of the disease. The following represents a summary of these stages as first described by Page and Schroeder (Page and Schroeder 1976).

One of the major difficulties in understanding the pathogenesis of periodontal disease is the inability to discriminate between normal and pathological tissue. In experimental animals, in which periodontal tissues are maintained plaque-free, leukocytes have been observed within the junctional epithelia (JE). It has generally been accepted that these features reflect enhanced levels of host defense mechanisms.

The response of the gingival tissues within 2 to 4 days to an accumulation of microbial plaque is localized to the region of the gingival sulcus. The initial lesion is characterized by acute exudative vasculitis, exudation of GCF, and increased migration of leukocytes into the J.E. and gingival sulcus. Other features include the presence of serum proteins, alteration of coronal J.E., and loss of perivascular collagen. The gingival tissues begin to show clinical signs and symptoms of gingivitis.

The early lesion develops within 4 to 7 days after the beginning of plaque accumulation. It is characterized by a dense infiltrate of lymphocytes, pathologic alterations in local fibroblasts and continuing loss of the collagen fibers supporting the marginal gingiva. Additional features include proliferation of the basal cells of the JE and an increase in the volume of GCF.

Two to three weeks after the early lesion the development of the established lesion occurs. It is distinguished by a predominance of plasma cells within the attached connective tissue, prior to any significant bone loss. Although the lesion is confined to the bottom of the sulcus, as in earlier stages, the plasma cells are not confined to the reaction site and are present along blood vessels deep within connective tissue between collagen bundles. Other features of the established lesion

include proliferation, apical migration and lateral extension of the JE, with or without pocket formation, and continued loss of connective tissue.

The established lesion may remain stable for years or it may develop into a progressive, destructive lesion. Host response to this stage of periodontal disease is vascular proliferation at the site of infiltration, while fibrosis and scarring may occur at sites distant from zones of continuing collagen loss. Factors causing the conversion to an advanced lesion are not totally understood.

The advanced lesion is characterized by extension of the lesion into alveolar bone and periodontal ligament with accompanying bone loss. Collagen loss continues subjacent to the pocket epithelium with fibrosis at more peripheral sites. This lesion continues to be dominated by the presence of plasma cells as in the established lesion. The result of these inflammatory changes is the formation of periodontal pockets. In addition, at this stage of disease, periods of acute exacerbation and periods of quiescence will occur.

The first three lesions, initial, early and established, are equivalent to the stages seen clinically in gingivitis. They account for the major portion of inflammatory gingivitis and periodontal disease. The advanced lesion is manifest as periodontitis and this is seen as alveolar bone loss, increased tooth mobility, pocket formation and periodontal abscess formation (Page and Schroeder 1976).

Evidence supporting the bacteriological etiology of periodontal disease has led to the evaluation of microbial species associated with different types of the disease. At clinically healthy sites, there are mainly gram-positive facultative anaerobic cocci and rods. Streptococci species predominate in normal flora as facultative anaerobic cocci. In addition, Actinomyces organisms, particularly *A. viscosus* and *A. naeslundi* are present as gram-negative rods (Slots 1977; Slots 1979).

In gingivitis, there is a shift in microflora in subgingival plaque toward a higher content of gram-negative forms. Established gingivitis is characterized by a shift from a Streptococcus to an Actinomyces dominated plaque. The organisms found in established gingivitis include *A. viscosus*, *A. israelii*, *Fusobacterium nucleatum*, *Campylobacter sputorum*, black-pigmented Bacteroides species and Veillonella species (Loesche and Syed 1978). Moore and co-workers, in

extensive analyses of microorganisms associated with human experimental gingivitis, found that the subgingival plaque in gingivitis also contains *A. naeslundi*, *A. odontolyticus*, *Streptococcus anginosus*, *Veillonella parvula* and Treponema species (Moore et al. 1982).

The composition of the subgingival flora associated with early onset forms of periodontal disease, especially juvenile periodontitis (JP), has been extensively studied. The bacteria reported in localized JP include *Actinobacillus actinomycetemcomitans* (Aa), Capnocytophaga, *Eikenella corrodens* and Bacteroides species, excluding *Porphyromonas gingivalis* (*P. gingivalis*) (Slots 1976; Slots 1979; Zambon et al. 1981; Mandel 1984).

The flora of adult forms of periodontitis is predominantly composed of gram-negative, anaerobic and motile organisms (Moore 1987). The most consistent bacteria recovered from periodontally-involved sites includes both *P. gingivalis* and Aa (Tanner et al. 1984; Dzink et al. 1988). With a recent emphasis on periodontally active sites, other micro-organisms found include *Wolinella recta*, other Bacteroides species and *Treponema denticola* (Moore 1987).

Certain bacteria appear to have important roles in the initiation and maintenance of the major forms of periodontal disease. For example, Aa and *P. gingivalis* are the significant organisms implicated in the pathogenesis of juvenile periodontitis (Zambon 1985) and adult periodontitis (Genco and Slots 1984), respectively. Although the various mechanisms by which these microorganisms participate in periodontal disease are well documented, there is a lack of evidence to establish a correlation between their presence and disease activity (Listgarten 1987). This is partly due to the fact that many periodontal pathogens are studied *in vitro*, which does not take into account the influence of *in vivo* conditions and that of the host response. It is also due to the lack of an acceptable standard to measure discrete periodontal disease activity.

The concept that periodontal disease is a gradual continuous process has been modified to an episodic disorder that cycles through active and quiescent phases of infection. Monitoring of individual periodontally diseased sites indicates that active destruction occurs in short bursts of time, followed by prolonged periods of tissue stability (Goodson et al. 1981; Goodson et al. 1982; Haffajee et al. 1983; Socransky et al. 1984). Paired measurements of attachment level at two

month intervals for one year revealed a significant increase in probable attachment loss in 2.8 percent of 3414 sites in 22 subjects. Attachment level changes that were deemed significant ranged between 2 and 5 mm. This observation and fluctuations found when monitoring rates of destruction during two month intervals are not consistent with a continuous disease process. Other authors have also found that attachment level loss in periodontal disease can occur in bursts of activity within a two month period (Kennedy and Polson 1973; Schroeder and Lindhe 1975; Lindhe and Ericsson 1979; Schroeder and Lindhe 1980). If periodontal tissue destruction occurs over short bursts of time at individual sites, then it would be beneficial to be able to detect such periods of high activity and possibly identify the active sites. This would aid in prevention and treatment monitoring of the affected sites.

1.6.2 Disease progression

The progression from gingivitis to periodontal disease is not totally understood. It has been assumed by clinicians that destructive periodontal disease is a result of progressive gingivitis. Although some investigators have found no firm evidence that this progression occurs (Schectman et al. 1972; Page et al. 1975), other studies indicate that gingivitis always precedes periodontitis (Saxe et al. 1967; Lindhe et al. 1973). It has been shown in the dog model that with plaque accumulation, gingivitis does progress to periodontitis (Lindhe et al. 1973).

The next consideration is whether periodontitis can occur without passing through a phase of gingivitis. The idea that periodontal inflammation and destruction are discontinuous is an observed characteristic in the advanced lesion (Page and Schroeder 1976). Evidence that gingival inflammation is not related to attachment loss comes from clinical studies in humans. Haffajee and co-workers found sites of progressive attachment loss through episodic bursts of activity, generally in the absence of clinical signs of gingivitis (Haffajee et al. 1983). They monitored 3414 individual periodontal sites and found no significant correlations of gingival redness, plaque, bleeding on probing or suppuration with periodontal destruction determined by probing attachment loss. In addition, of all sites that showed no significant attachment loss, the majority showed

positive signs for gingival redness, plaque, bleeding on probing and suppuration. These results indicated that some periodontal lesions occur with gingivitis as a precursor. However, in some cases gingivitis may not presage destruction of periodontal attachment.

The concept on the pattern of progression of the periodontal lesion has changed during the past two decades. Three models representing the progression of chronic destructive periodontal disease have been reviewed by Socransky and co-workers (Socransky et al. 1984). It was traditionally thought that periodontal disease was continuous in nature, following a linear model of progressive destruction. Once periodontal disease was initiated at a particular site, there was progressive attachment loss with time. This led to the belief that unless an affected tooth was treated the periodontal condition would worsen.

There are several reasons why the model for continuous or linear disease progression is incorrect. Firstly, studies have shown that attachment loss can occur very rapidly which is inconsistent with this model. Studies by Goodson and co-workers on progression patterns of advanced destructive periodontal disease have demonstrated significant increases in probeable attachment loss, between 2-5mm over two month intervals (Goodson et al. 1982; Haffajee et al. 1983). Secondly, the fact that there are a few sites that exhibit no changes in periodontal activity is inconsistent with a slow progressive destructive disease model. Two longitudinal studies by the same authors, each monitoring periodontal attachment for one year, have shown that the number of sites exhibiting significant attachment loss were 2.8% and 5.7%. And finally, data from animal studies indicate that periodontal disease does not progress in all lesions (Lindhe et al. 1975).

The second concept of chronic periodontal disease progression is the random burst hypothesis. In this model the cumulative extent of the destruction varies depending upon the site. Some sites show several bursts of activity, while other sites show no activity and may be free of periodontal disease throughout an individual's life. Sites that show activity may never demonstrate activity again or could be subject to further activity in the future. The term random is related to time and previous loss of attachment. There is good evidence to support the hypothesis that periodontal disease is not random with regard to site of involvement (Löe et al. 1978).

The third concept of disease progression was the asynchronous multiple burst model. According to this model, the majority of periodontal activity occurs within a few years of an individual's life. Sites can show repeated bursts of activity followed by prolonged periods of inactivity. The major difference from the random burst model is that multiple sites can break down within a relatively short period of time, which is then followed by prolonged periods of quiescence.

The complement system plays an important role in the pathogenesis of and protection in periodontal disease. Activation of complement by bacteria intiates a number of events including opsonization, chemotaxis and release of inflammatory mediators, and bacteriolysis via the membrane attack complex. Complement cleavage products have been isolated in GCF and observed in diseased gingival tissues. In a recent article, Schenkein (Schenkein 1991) reviewed the role of complement in periodontal diseases. Although a number of bacteria from individuals with periodontal diseases can activate complement, some of these organisms evade opsonization due to their proteolytic activity. Schenkein concluded that there is insufficient concrete evidence to indicate that complement activation occurs in human periodontal disease and that it is important in its pathogenesis or in protection against bacteria.

However, other investigators concluded that the presence of complement proteins in the crevicular fluid provides evidence for their activation in active disease. In comparing healthy and periodontally involved patients, the presence of C5 and decreased levels of C3 and C4 were found in diseased sites (Attström et al. 1975). More recent studies have also shown that complement cleavage in crevicular fluid can be used to distinguish pre- from post-treatment sites, and adult from juvenile periodontitis (Niekrash and Patters 1985; Niekrash and Patters 1986). Such observations may be useful in elucidating pathogenic mechanisms of the host.

Increased degradation of collagen is a characteristic feature of the onset and progression of periodontal diseases. Mammalian collagenases have a specific activity, whereby cleavage of the collagen molecule generates 3/4 (TC_A) and 1/4 (TC_B) fragments. The demonstration of tissue collagenase activity *in vivo*, elaborating TC_A and TC_B fragments, has been shown to correlate with

inflammation severity in human gingiva (Overall et al. 1987). The generation of 3/4 and 1/4 fragments is a critical step in collagen degradation since the fragments are unstable at physiological temperatures and are subject to further degradation by specific and non-specific gelatinases (Weiss 1976).

Since collagen loss occurs in both gingivitis and periodontitis, it is not surprising that collagenolytic activity in GCF has been correlated with the severity of periodontal involvement (Golub et al. 1976; Uitto and Raeste 1978; Kowashi et al. 1979; Kryshtalskyj and Sodek 1987; Villela et al. 1987). The studies by Kryshtalskyj and Sodek and by Villela and co-workers describe an active collagenase which degraded collagen into 3/4-a and 1/4-a fragments. This cleavage pattern is characteristic of mammalian, and not microbial, collagenase.

1.6.3 Clinical evaluations

Classical clinical parameters used to measure periodontal conditions include plaque index (Pl.I), gingival index (GI), pocket depth (PD), and bleeding on probing (BoP). It has been widely accepted that bacterial plaque is not just associated with inflammatory disease, but is the cause of this destructive process. While Pl.I is used as an indirect measure of potential inflammation of the gingival tissues, both GI and BoP provide direct evidence that inflammation is present at the time of examination. Measurements of increasing PD however, provide information that prior periodontal destruction has occurred. While all these indices indicate the presence of periodontal disease, they provide no information on disease activity at the time of examination.

The periodontal probe has been and continues to be one of the most useful and fundamental tools used in periodontal diagnosis. Assessment of periodontal defects such as pocket depths, width of keratinized tissue and gingival recession are measured with this instrument. However, statements as to the presence of active disease cannot be made when measuring such periodontal sites.

Retrospective disease activity can be ascertained by comparing changes in probing measurements obtained at different time points. Goodson and co-workers (Goodson et al. 1982)

have demonstrated that changes in probing periodontal attachment levels can be discriminated with a high degree of accuracy.

There is controversy as to the probing depth measurement and how it relates to JE attachment. Although the intent is to probe to the level of the cementoenamel junction (CEJ) (for a healthy tooth), there are reports that show that this does not always occur (Listgarten 1980). The degree of probing force and the diameter of the probe both contribute to the extent of the penetration of the instrument. In addition, the degree of inflammation in the periodontal tissues is also an important factor in how deep the probe penetrates. The greater the inflammation present, the deeper the probe penetrates the JE beyond its coronal extent, towards the connective tissue attachment (Hancock and Wirthln 1981).

The accuracy of probing measurements is further compromised in at least two ways. The markings on the probe can vary from 1 mm to 3 mm gradations, and therefore, accurate at best to 1mm. Another source of error is the probing force used by the operator. To improve upon these limitations, an electronic probe was developed which operates at a constant probing force and is accurate to 0.1 mm.

One of the first electronic probes that became available was the Florida probe (Florida Probe Corp., Gainesville, FL.). Due to its features of a constant probing force and a 0.1 mm resolution, this instrument compared to the conventional probe reduces the inter-operator variability in measuring pocket depth (Walsh and Saxby 1989).

Radiographs are frequently used to detect and assess periodontal disease. They offer information on the level of alveolar bone present and alterations in bony contour. However, single radiographs only yield information as to the present state of the periodontium. They only represent a one-frame look at the condition, and thus, only by comparing radiographs taken at different times can one retrospectively demonstrate disease activity.

There are a number of limitations in the use of radiographic interpretations. One of the main limitations of a radiograph is that it detects changes in bone density only after 30-50% of the mineral content of the bone has been lost (Bender and Seltzer 1961a,b). In addition, the

reproducibility of angulation and quality of film can obscure the subtle bone changes on the radiograph.

A method for comparing radiographs from different time points is possible through digitial subtraction radiography. This is an imaging modality commonly used in medical radiology, whereby each image is scanned by a computer and structures that have not changed density between examination points are subtracted to produce the image. The result is a generation of an image where mineralized changes have occurred. The benefit of this technology is that it not only eliminates operator subjectivity in examination, but that it can detect as little as a 5% change in the mineral content (Ortman et al. 1982).

A study of the relationship between attachment level loss and alveolar bone loss demonstrated that attachment loss precedes radiographic evidence of crestal bone loss during periods of disease activity (Goodson et al. 1984). In view of this result, comparison by subtraction radiography of films taken at different times can demonstrate changes in periodontal mineral content, but again would demonstrate disease activity retrospectively.

Parameters such as the gingival index and bleeding on probing are sensitive indicators of gingival inflammation. Several studies have shown correlations between the clinical signs of inflammation and histological evidence of inflammatory gingival lesions (Applegren et al. 1979; Greenstein et al. 1981; Abrams et al. 1984). However, these clinical signs pertain to the status of the gingiva alone and are only useful in diagnosing gingivitis and are of little value in identifying periodontitis, or being able to predict the onset of periodontitis.

Recognition of active destructive periodontal disease is difficult. Studies have shown that periodontal disease is cyclical and site specific, and that disease progresses for a relatively short time, soon replaced by longer or shorter periods of quiescence (Goodson et al. 1982).

1.6.4 Host interactions

Macrophages play an important role in the host response to periodontal disease. Direct stimulation of these cells by bacterial products, such as lipopolysaccharide (LPS), or by indirect stimulation by lymphokines leads to the production of collagenase and other proteolytic enzymes (Wahl and Mergenhagen 1988). Macrophage interaction and processing of cell surface bound antigens, gives rise to the production of monokines which amplify the immune response. One of the better characterized monokines is interleukin-1 (IL-1), a potent stimulator of T cells. IL-1 affects a number of target cells including thymocytes, T and B lymphocytes, fibroblasts and hepatocytes. Richards and Rutherford have recently studied IL-1 effects on the collagenolytic activity and prostaglandin-E (PGE) secretion by human periodontal ligament fibroblasts (PLFBl) and gingival fibroblasts (GFBl) (Richards and Rutherford 1988). They reported that while PLFBl and GFBl both responded to IL-1 by secreting PGE, only GFBl produced increased levels of collagenolytic activity.

Human GCF has been found to contain thymocyte activating factor (Mergenhagen 1984). In addition to macrophages, epithelial cells are capable of secreting an Epidermal Cell Thymocyte Activating Factor (ETAF), a protein which is similar if not identical to IL-1 (Luger et al. 1982). Since IL-1 and ETAF were found to be physiochemically and biologically similar, it is not possible to differentiate whether the source of activity is from epithelial cells or macrophages.

Analysis of gingival fluids collected from inflamed and noninflamed sites indicated that thymocyte growth promoting activity was significantly increased in the inflamed sites (Charon et al. 1982). They collected specimens of fluid by a gingival washing technique (Skapski and Lehner 1976), which is a crude method for collecting gingival samples. It is now preferred to collect GCF using either filter paper strips or calibrated microcapillary tubes, which allow for site specific sampling and quantitation of fluid volume.

For a considerable time, immunological mechanisms have been implicated in the pathogenesis of chronic periodontal disease. Cell mediated (Ivanyi et al. 1972) and humoral responses (Brandtzaeg 1966) have both been linked to progression of the disease. Initial opinions of

lymphocyte function within inflamed tissues were primarily based upon studies performed on peripheral blood lymphocytes (PBL) from patients with periodontal involvement (Campana 1981). As periodontal disease is localized to the supporting tissues of the tooth, activity of PBL may not reflect the activity of lymphocytes present within the lesion. In order to better understand the pathogenesis of the disease it is necessary to characterize cells present in the tissue. The study of functional characteristics of lymphocytes in chronically inflamed tissue will lead to a better understanding of the role of the immune system in periodontal disease.

As stated previously the progression from gingivitis to periodontitis proceeds from the initial lesion, through early and then established lesions, and finally to the advanced lesion. Histochemical and immunological studies of lymphoid cell subpopulations in experimental gingivitis in humans have characterized gingivitis as T-cell dominated (Seymour et al. 1983). The distinction between gingivitis and periodontitis is that in the established lesion of gingivitis there is no bone destruction, while the advanced lesion of periodontitis involves progressive bone loss. The switch from a stable lesion to a progressive lesion has been shown to be associated with a switch in lymphocyte populations, from T cells to B cells (Mackler et al. 1977). Phenotypic characterization of lymphocyte populations in established human periodontal disease suggests that it be considered as a B cell lesion (Seymour and Greenspan 1979).

The T lymphocyte role in the regulation of the immune system can range from enhancement to suppression. The involvement of T helper (T_H) and T suppressor (T_S) cells in periodontal lesions can be either protective or destructive, depending upon the type of antigen stimulation. If an antigen is T cell dependent, T_H cells would act protectively by stimulating B cells to become IgG, IgA or IgE secreting plasma cells. If however, an antibody response is T cell independent, as for LPS antigen, a polyclonal B cell activator, T_H cells would give rise to overstimulation of B cells. This would lead to an overproduction of antibodies and Type III hypersensitivity reactions. Alternatively, T_S cells in this instance would function in a protective role.

Cell-mediated immunity (CMI), has been shown to relate to the periodontal condition in studies of the lymphoproliferative response during human experimental gingivitis (Patters et al. 1979).

The sometimes protective, sometimes destructive consequences of CMI leaves one to question whether the host response is beneficial or harmful in cases of periodontal disease. Evidence is present to support both these contradictory roles of the immune response.

In studies performed on animals, variations in T cell populations resulted in differing effects on periodontal bone loss. Thymectomized (Tx) hamsters showed a greater bone loss then did sham-Tx controls (Barefoot and Silverman 1977). In contrast, studies on rats with an imbalance of T cell regulation, either congenitally athymic or neonatally Tx, demonstrated elevated bone loss relative to controls. Experiments involving T cell suppression offered similar contradictory results. T lymphocyte reduction from cyclosporin A treatment resulted in decreased bone loss (Guggenheim et al. 1981), while cyclophosphamide suppression resulted in dramatically increased bone loss after ligature placement (Sallay et al. 1982). Therefore, the question whether the host response is harmful or beneficial remains unresolved.

Studies of host response in experimental periodontal disease in rats indicate that changes in T cells, depending on the degree of sensitization and subset involved, result in differing effects on periodontal bone loss (Taubman et al. 1984b). When sensitized T lymphocytes are adoptively transferred, bone loss is increased, presumably through the mechanism of host synthesis of osteoclast activating factor (OAF) and other lymphokines. However, when athymic rats, having an imbalance of T_H and T_S cells, are reconstituted with a balanced population of thymus cells, bone loss is decreased (Yoshie et al. 1983). Although these results suggest that T cells play a role in the regulation of periodontal bone loss, further studies are necessary to determine the T cell subsets present and their influences at various stages of periodontal disease.

The application of immunocytochemical methods to histological sections has led to the quantitation of lymphocyte phenotypic variations in experimental gingivitis and in periodontal diseased tissues. Characterization of lymphocyte subpopulations in established periodontal disease was determined using indirect immunofluorescence to detect human thymocyte antigen, human myeloid antigen and IgG and IgM-bearing cells *in situ* in the lesion (Seymour and Greenspan 1979). The results indicated that the majority of lymphoid cells were IgM-positive/thymus antigen-

negative B cells, while relatively few thymus antigen-positive T cells were found. In a similar study of cryostat sections from patients with chronic marginal periodontitis, a three layer immunofluorescent technique was used to differentiate T helper cells (T_H) from T suppressor/cytotoxic cells (T_{SC}) (Johannessen et al. 1986). The ratio of T_H to T_{SC} was 1.13. These results are in agreement with Taubman and co-workers (Taubman et al. 1984a), who found a T_H/T_{SC} ratio of 1.1, by measuring immunofluoresence of extracted cells separated by Ficoll-Hypaque gradient centrifugation.

In studies of experimental gingivitis, Seymour and co-workers (Seymour et al. 1983), used two and three layer immunofluorescent techniques to characterize lymphocyte differentiation antigens, and conjugated fluorescein isothiocyanate (FITC) rabbit anti human-Ig for demonstration of plasma cells. Although these authors found a predominance of T cells throughout their 21 day study, further work was necessary to characterize T-cell subsets. These results became available in a subsequent immunohistological analysis of gingivitis in 1988, as Seymour and co-workers were able to identify T-cell subsets and the pattern of Class II major histocompatibility complex (MHC) antigens. Using a panel of monoclonal antibodies in an avidin biotin immunoperoxidase technique, the ratio of T_H cells (CD4+) to T_{SC}cells (CD8+) varied only slightly from 2.18 to 2.48 over the course of the lesion. A double staining immunofluorescence technique using FITC-conjugated OKT8 antibody and phycoerythrin-conjugated anti-Leu-15, was used to differentiate T suppressor from T cytotoxic subpopulations. Examination with an epifluorescence microscope revealed that all T8+ cells co-expressed Leu-15, indicating a suppressor rather than a cytotoxic phenotype, suggesting a regulatory role for these cells. In addition, the majority of T cells expressed the Class II MHC antigens HLA-DR and HLA-DQ; this implied that they were activated.

Although these are the first studies to classify T-cell subpopulations and determine their activation, the clinical data are insufficient to differentiate the periodontal status with respect to disease activity. The chronology, stage of development and current destructive activity in individual periodontal lesions, in the studies of experimental gingivitis and chronic marginal periodontitis, were not recorded. The limit of controlled experimental periodontal disease in

humans is to the gingivitis stage, as it would be unethical in humans to allow gingivitis to progress to a course of untreated experimental periodontitis. For this reason an indicator of active disease, such as hydroxyproline (Coil et al. 1987), is essential in ascertaining the activities of individual sites and predicting clinical changes in the periodontium. Markers for active disease would not only allow clinicians to monitor patient periodontal status but also enable scientists to correlate with clinical parameters with biological phenomena.

1.6.5 Treatment

The goal of periodontal therapy is to restore the periodontal tissues to a healthly state and function. The treatment is directed at reducing or eliminating bacterial plaque and resolving periodontal inflammation. This is accomplished by removal of local factors by conventional non-surgical methods of scaling and root planing. Following treatment, prevention of further disease depends mainly on the proper management of the bacterial plaque by mechanical and/or chemical means.

Scaling and root planing in the initial phase of treatment has been supplemented with antimicrobial drugs. The use of these antimicrobial agents can be divided into those for use in plaque control and those used in the elimination of subgingival microflora. A common therapeutic agent used for plaque control is chlorhexidine mouthrinse. When used on a daily basis it has been shown to reduce supragingival plaque. The use of this agent in subgingival irrigation may also prove to be effective in reducing the subgingival microflora.

Systemic antibiotics have also been used alone and as adjuncts with scaling and root planing in treatment of advanced forms of periodontal disease. A variety of antibiotics have been used including tetracycline (Listgarten et al. 1978; Helldén et al. 1979; Slots et al. 1979), penicillin (Helovuo and Paunio 1989), metronidazole (Jenkins et al. 1989; Soder et al. 1990; Loesche et al 1991, 1992), and spiramycin (Mills et al. 1979; Al-Joburi et al. 1989). Tetracycline when given systemically in conjunction with conventional therapy, showed only minor differences in microbial and clinical parameters when compared to scaling and root planing alone. Overall, the studies have

shown conflicting results regarding the benefit of systemically administered antibiotics in periodontal therapy.

There has been an increased interest on the methods and effects of local delivery of antibiotics to specific periodontal sites (Goodson et al. 1979; Goodson et al. 1983; Goodson et al. 1985; Goodson et al. 1991; Heijl et al. 1991). Local delivery has advantages of directing site specific treatment and maintaining high concentrations of antibiotic locally. Matrix systems for controlled drug release are employed to maximize and sustain the local concentration of therapeutic agent. Investigations in this area continue to evaluate matrix support systems and different antimicrobial agents.

Recent advances in the use of tetracycline in periodontal therapy have demonstrated a non-antimicrobial property of therapeutic significance. Golub and co-workers have shown that tetracycline directly inhibits the extracellular activity of a metalloproteinase collagenase (Golub et al. 1983; Golub et al. 1984; Golub et al. 1985). This agent may suppress connective tissue breakdown during active periods of periodontal disease. This important finding has expanded experiments into localizing this benefical property of tetracycline, and has led to the development of chemically modified tetracyclines (CMT) (McNamara et al. 1986; Golub et al. 1987). The latter agents have lost their antimicrobial properties but retain their anticollagenase activity (Golub et al. 1991). Although CMT's have not been approved for human use, they possess great potential in future treatment of periodontal disease.

After intial therapy, surgery may be required in more severe forms of the disease to further eliminate inflammation and/or improve the architecture of the periodontal tissues. Although surgical therapy is directed at the treatment of remaining periodontal pockets, creation of a more accessible contour which is conducive to more effective home care maintenance is also a goal. The main approaches to surgery involve resective, inductive and reattachment procedures. These surgical methods should be regarded as an extension of the basic principle of mechanical debridement performed during initial therapy.

1.7 INDICATORS OF PERIODONTAL DISEASE

An ideal indicator of periodontal disease should be able to do the following: 1) diagnose the presence of disease at the time of examination, 2) evaluate disease activity and identify active sites at the time of examination, 3) predict and determine the degree of future periodontal attachment loss thereby serving as a predictor of subsequent breakdown of periodontal tissues that would occur in absence of treatment, and 4) quantitatively monitor the response to therapy.

1.7.1 Clinical indicators

An accurate assessment of a patient's periodontal health is a key to the diagnosis and determination when periodontal therapy is required. As previously indicated, once a patient has a history of periodontal disease, the active phases of the disease are characterized by short bursts of activity, generally followed by longer periods of quiescence. For this reason it is difficult to assess precisely the progression of the disease.

At present the only methods available to clinically measure periodontal disease activity are to monitor retrospective changes in alveolar bone levels and changes in attachment level over time. Clinical parameters such as gingival index, bleeding on probing, crevicular fluid flow and plaque index, provide information on the state of gingival inflammation at a given examination point. A recent 2 year study on the relationship of gingival bleeding and supragingival plaque to attachment loss $\geq$ 2mm, confirmed that neither of these signs were prognosticators of attachment loss during this period (Kaldahl et al. 1990). Pocket depth, attachment and alveolar bone level, are indicators of patient history of periodontal involvement. Measurements of recent reductions in alveolar bone height and/or attachment level loss indicate that active disease has occurred. However, monitoring of these clinical parameters yields only a retrospective diagnosis of active disease.

The gingival crevice is an important milieu for the periodontal disease process. This is a logical location to seek for diagnostic aids for evaluating the destructive disease process. Crevicular fluid emanating from the crevice contains a multitude of substances. Although its composition is similar

to that of saliva, it differs from it with respect to bacteria, enzymes, immunological and inflammatory mediators, and by-products of metabolism. One means of investigating the complex nature of periodontal disease is by the analyses of this unique transudate.

1.7.2 Bacterial indicators

Studies of microorganisms in periodontal pockets of periodontitis subjects demonstrate increased numbers of gram-negative anaerobic rods and spirochetes as compared to unaffected gingival sulci. Evidence that bacteria contribute to the etiology of periodontal disease has prompted investigators to determine which microorganisms are associated with different forms of the disease. Although an overall increase in mircoorganisms is associated with periodontal conditions, their presence does not necessarily connote disease activity (Haffajee et al. 1983).

The progressive destruction of the periodontium is the result of an active state of the disease. At a given time point, a previously diseased periodontium can clinically be in a healing, stable, or a continuing active disease state. Although it is not presently known what triggers the transformation from a stable to an active disease state, it has been suggested that a significant number of pathogenic bacteria may cause or initiate the destructive process. Furthermore, the presence or increased numbers of certain bacteria may be indicative of the active disease state.

A number of microbiological assays have been used to monitor periodontal diseases. Culturing methods were first performed to detect and characterize bacteria associated with various forms of the disease. In localized juvenile periodontitis (LJP), Slots reported that Capnocytophaga, Aa, and *Wolinella recta* were the dominant isolated types (Slots 1976). In adult periodontitis, White and Mayrand found that in comparison to less inflamed sites the inflamed sites had a greater portion of gram-negative anaerobic rods, 32% of which were *P. gingivalis* (White and Mayrand 1981).

The culture results were often compared to clinical parameters to seek information as to the severity of the disease or the level of disease activity. By development of bacterial profiles it was intended to develop a diagnostic test for various forms of periodontal disease. Slots and co-workers determined the presence of Aa, *P. gingivalis* and *B. intermedius* in destructive

periodontitis in adults (Slots et al. 1986). Their definition of progressive disease was based on radiographic evidence of alveolar bone loss over a minimum period of two years. They found that at least one of these organisms was present in 99% of the investigated progressive lesions. However, their definition of progressive disease was actually a retrospective evaluation that periodontal disease had occurred.

Using immunological techniques, investigations of antibodies to specific oral microorganisms has enabled better understanding of certain periodontal conditions. It has been demonstrated that elevated serum antibody titers to Aa are present in individuals with localized juvenile periodonditis (LJP) (Ebersole et al. 1982; Ranney et al. 1982). Similarly serum IgG titers to *P. gingivalis* have been shown to be elevated in adult and rapidly progressive periodontal diseases (Mouton et al. 1981; Tolo and Schenck 1985). A recent study compared antibodies from both serum and GCF sources to *P. gingivalis* in patients with treated and untreated periodontal disease (Murray et al. 1989). The results showed that untreated periodontitis patients had significantly higher serum and GCF levels of IgG antibody to *P. gingivalis* than did either patients with treated periodontitis or gingivitis controls.

In a longitudinal study of serum antibodies to *P. gingivalis* in periodontitis patients, Mouton and co-workers (Mouton et al. 1987) observed an unexpected consistent serological dichotomy in chronic periodontitis patients. Throughout the one year study, half of the periodontitis group did not exhibit measureable levels of IgA, while the other half remained seropostive. Correspondingly, in comparison to healthy subjects the chronic periodontitis patients either exibited normal antibody titers or significantly increased levels of antibody reactive to *P. gingivalis*. Although it could be conjectured that low levels of antibody reflect low colonization, this may not be true since it has been reported that large numbers of specific microorganisms can be found in the absence of their corresponding specific antibody (Williams et al. 1985).

Microbial measurements can be useful in selecting and evaluating treatment, and for establishing recall schedules. Robertson and co-workers assessed subgingival microflora and clinical parameters at baseline and after 7 months in a group of severe peridontitis patients that

received scaling and root planing (Robertson et al. 1987). They found that after 7 months, the percentage of sites with *P. gingivalis* was significantly lower in patients with resolved than in patients with unresolved periodontal sites. However, they concluded that none of the clinical and mircobial measures used in the study could predict the response to repeated scaling and root planing.

A recent 30 month investigation by Listgarten and co-workers, on treated patients who were on a 3 month recall, compared disease recurrence with analyses of specific culturable bacteria (Listgarten et al. 1991). They found that the presence or absence of Aa, *Prevotella intermedia*, or *P. gingivalis*, was not a reliable predictor of future episodes of recurrent disease.

An indirect method for monitoring bacteria is by assessing microbial enzyme activity. One method is based on hydrolysis of a specific peptide, benzoyl-arginine naphthylamide (BANA), by a trypsin-like enzyme found in a limited number of microorganisms. Periodontal microflora capable of this cleavage are *Treponema denticola*, *P. gingivalis*, and Capnocytophaga (Schmidt et al. 1988). Clinical studies have shown that BANA cleavage is strongly associated with the presence of *T. denticola* and *P. gingivalis* (Bretz et al. 1990). The presence of these specific bacteria may be an important prognostic marker. A recent study that examined subgingival plaque for these two organisms before and after treatment indicated that *T. denticola* levels decreased at healing sites and either increased or remained the same at nonresponding sites (Simonson et al. 1992).

New detection methods for bacteria have enabled a more specific and expedient evaluation of species present at periodontal sites. The use of DNA probes offers improved sensitivity in microbial detection. A recent study compared culture methods and DNA probe analyses for the detection of Aa, *P. gingivalis*, and *B. intermedius*, in subgingival plaque samples (Savitt et al. 1988). The results indicated that probe assays frequently identified these pathogens in samples that were culture negative. DNA probes also revealed a better correlation on an individual patient basis between the presence of a pathogen and clinical evidence of disease.

There are a number of limitations to the currently used assays. The premise for their use is that specific microorganisms were present in healthy states, whereas others are associated with specific periodontal diseases. However, according to Listgarten (Listgarten 1988), an association between certain bacteria and clinical conditions does not make them causative agents, nor does it imply their presence is a useful diagnostic tool. It is possible that bacteria may be present in disease conditions because the environment is conducive to their growth and proliferation. In this case the presence of bacteria would be the result rather than the cause of the disease.

Significant variations in bacterial composition were found at healthy and diseased periodontal sites (Listgarten and Helldén 1978; Listgarten 1984). Furthermore it has been shown that most of the variability is due to differences among subjects rather than differences amongst diseased sites within subjects (Evian et al. 1982). This has obvious implications on pooling of bacterial samples and comparisons of healthy and diseased sites in the same individual.

Also of concern is the reliability of diagnostic tests, as the host response plays a role in determining whether the disease remains stable or progresses. The interpretations of microbiological tests are limited as they do not measure host-microbiologic interactions. Hence they cannot detect active states or predict the future progression of disease.

Diagnostic tests for bacterial species such as Aa and *P. gingivalis* may serve to identify sites and/or patients at risk for periodontitis. As there are no known longitudinal studies that compare and monitor specific subgingival microflora and disease progression, the ability of such a risk indicator is unknown. However, as discussed above, since the host response is important in determining the onset and progression of disease, the presence or number of pathogenic bacteria may be superfluous. The presence of the same pathogenic bacteria in one host may result in disease progression, whereas the same proportions of bacteria in another host may result in a stable lesion.

The use of microbial indicators to detect the presence of microorganisms is most useful in monitoring the effectiveness of therapy. They may become an important diagnostic aid to ensure that potentially pathogenic bacteria have been successfully eliminated. Although culture techniques

are able to identify a multitude of species and subspecies of microorganisms, they are not practical for routine use in clinical practice. DNA probes, immunofluoresence microscopy and enzyme markers would provide faster, more sensitive results, provided specialized laboratories or chairside tests are available to perform such analyses.

In light of these limitations, a more practical use of microbiological assays appears to be for monitoring treatment. Quantitation of specific bacteria known to be present in higher amounts at diseased sites can enable specific monitoring of treatment and targeting of those sites that require further elimination of bacteria.

1.7.3 Enzyme indicators

A multitude of enzymes are involved in the metabolism and pathogenesis of periodontal tissues. They can be derived from microbial pathogens and host sources. The identification and characterization of such enzymes may provide rationale for their use as potential markers of the disease process.

As previously described, the process of collagen degradation by host enzymes involves mammalian collagenases, which are produced by PMN's, fibroblasts, epithelial cells and macrophages. As this enzyme is secreted as a proenzyme, activation requires cleavage by other enzymes. This can be effected by such enzymes as neutrophil elastase, mast cell tryptase, and Cathepsin G.

Gingival crevicular fluid from periodontitis sites has been shown to possess active collagenolytic activity, whereas control and gingivitis sites exhibit latent collagenase and collagenase inhibitor complex (Kryshtalskyj et al. 1986). Subsequently it has been shown that the increased collagenolytic activity associated with periodontitis sites is accompanied by other enyzmes which further degrade collagen beyond the 3/4 and 1/4 fragments (Kryshtalskyj and Sodek 1987).

Other investigators have demonstrated higher collagenase activity in inflamed gingiva and crevicular fluid than in healthy tissue and fluid (Uitto and Raeste 1978; Overall et al. 1987).

Furthermore, it was found that relatively less inflamed tissue released more latent collagenase than the more severely inflamed gingiva. In a study of collagenase in different forms of periodontal disease, it was determined that amongst patients collagenase activity increased with the severity of the disease in the order of healthy < gingivitis < periodontitis (Villela et al. 1987). Amongst sites, a significant correlation was found between GCF collagenase activity and pocket depth in both chronic adult periodontitis and LJP. Furthermore, it has been observed that interstitial collagenase activity decreases after periodontal treatment (Hakkarainen et al. 1988).

The preferred methods of quantifying mammalian collagenase are quite elaborate and involve the use of radiolabelled collagen substrates for SDS/PAGE analysis of resultant breakdown products. This methodology is not adaptable for a simple, quick, or practical clinical test. Immunological methods would be useful in detecting collagenase except that they also detect the latent form of the enzyme. Thus, it appears that collagenase assays offer no additional diagnostic benefit over the conventional clinical methods in identifying the disease state.

A variety of other proteolytic enzymes are believed to play a role in the pathogenesis of periodontitis. It is likely that the destructive process is associated with increased levels of these enzymes because they are capable of degrading tissue components. Using synthetic substrates coupled to fluorogenic agents, serine proteinase activity was found in crevicular fluid. A tryptase-like activity was found in GCF collected from gingivitis and periodontitis patients (Cox and Eley 1989b). An expanded investigation employing synthetic peptides linked to fluorogenic agents was performed to detect cathepsin B and L, elastase, typtase and trypsin-like enzyme activities consistent with a host cell origin. Also, dipeptidyl peptidase-like activity (DPP IV), which can result from a mixture of host and bacterial sources, was also investigated. Cox and Eley (Cox and Eley 1989a) found that all 5 classes of enzyme activities were detected, and that it is possible to analyze all 5 activities using only 0.1 µl of GCF. Althougth enzyme concentrations varied widely at individual patient sites, elastase-like activity was generally the highest.

The same fluorogenic methods were also used to determine cathepsins B,H and L activities in GCF from chronic adult periodontitis and experimental gingivitis patients. Kunimatsu and co-

workers found higher levels of these cathepsins at sites with more severe signs of the disease (Kunimatsu et al. 1990). The total activity of each enzyme (per unit time) was positively correlated with the GCF volume, in contrast to a negative correlation found between specific activity of each enzyme in GCF (activity units per mg of protein) and GCF volume. From this the investigators concluded that these proteases are selectively released into the crevice. However, in the employed GCF collection procedure the first filter paper used to collect fluid during the first 30 seconds was discarded. This has critical consequences as it has been recently demonstrated that the concentration of host or bacterial substances is likely to be highest in the first collected sample of GCF (Lamster et al. 1989).

In an attempt to evaluate GCF proteases for their diagnostic potential, Eley and Cox employed the same 5 classes of fluorogenic substrates to compare protease activity in GCF with clinical parameters which included probing depth (PD), clinical attachment loss (CAL), gingival index (GI), bleeding index (BI) and plaque index (Pl.I) (Eley and Cox 1992). In this cross-sectional study they found that total enzyme activities had good diagnostic specificity and sensitivity as predictors of clinical parameters. They obtained the following order of correlations with different enzymes and parameters; cathepsin B/L- > elastase- > DPP IV- > trypsin- > tryptase-like activity and PD > CAL > GI > BI > Pl.I. A relatively high level of correlation with pocket depth infers that the total enzyme activities are, in part, dependent upon the size of the GCF reservoir in the pocket.

Lactate dehydrogenase (LDH), β-glucuronidase (BG) and arylsulfatase (AS) activities have also been evaluated in GCF (Lamster et al. 1985a-c; Lamster et al. 1988). LDH is a cytoplasmic enzyme and its occurence in extracellular fluid indicates cellular necrosis. Both BG and AS are ground substance degrading enzymes involved in degradation of GAG's, and are constituents of lysosomal granules.

Lamster and co-workers investigated these enzymes in a six month study to determine if their activities parallel clinical attachment loss seen in patients with chronic adult periodontitis (Lamster et al. 1988). In a subgroup of patients that was identified as displaying a localized form of disease

activity, clinical attachment loss could be substantiated by individual GCF samples that demonstrated BG activity at least 4 times the population mean baseline value. In contrast, LDH and AS did not provide a statistically significant measure of localized attachment loss in this group of patients. Both the sensitivity (89%) and specificity (89%) for the relationship of BG activity in GCF to detection and prediction of clinical attachment loss, indicated that the analysis had diagnostic value. However, more studies are required to further examine this relationship, since this subgroup of patients displaying localized disease activity consisted of only 4 patients.

Elastase relationship to periodontal health has been studied in both GCF and salivary systems. Zafiropoulos and co-workers (Zafiropoulos et al. 1991) performed a detailed cross sectional study that compared GCF elastase inhibitor complex (GCF ELPα_1-PI) with clinical indices and subgingival flora. They found that correlations between GCF ELPα_1-PI and *P. gingivalis* (r=0.642) or *B. intermedius* (r=0.774) were the highest for alveolar bone loss $\leq$ 20% and pocket depth $\leq$ 3mm, respectively. These results indicate the measurement of GCF ELPα_1-PI concentrations may be useful for evaluating sites with little or no tissue destruction.

In a recent investigation Palcanis and co-workers used a prototype elastase diagnostic kit to assess the potential use of elastase as a marker of periodontal disease activity {Dentsply, York PA}(Palcanis et al. 1992). Total elastase was significantly higher in sites demonstrating progressive attachment loss than in inactive sites and sites demonstrating bone loss. When the joint presence of bone loss and attachment loss were considered together, the sensitivity and specificity of the assay was 82% and 66% respectively. Since GCF elastase levels are significantly higher in sites demonstrating progressive attachment and bone loss assessed 6 months later, elastase may serve as a predictor for these changes.

The relationship of salivary elastase and collagenase to periodontal health has been assessed by Uitto and co-workers who studied the possibility of using salivary elastase detection from an oral rinse for the development of a simple periodontal disease screening test (Uitto et al. 1992). They found that elastase activity was markedly elevated in periodontitis and the activity decreased close to the normal levels following periodontal therapy. As a screening method for untreated patients, a

strong correlation was found between periodontitis and positive elastase test results. Similarly, salivary collagenase was significantly higher in periodontitis patients before than after treatment (Uitto et al. 1990).

It is anticipated that the enzymes present in the cytoplasm of dying cells would serve as markers of tissue destruction. Aspartate aminotransferase (AST) is such an intracellular enzyme that is released from impaired or dying cells. It is seen in increased amounts in the serum following myocardial infarction and during active hepatitis. Studies on AST levels in GCF were first performed in beagle dogs (Chambers et al. 1984). Recently this area of research has received considerable attention (Page et al. 1975; Persson et al. 1990a,b; Persson and Page 1990; Chambers et al. 1991; Cohen et al. 1991; Persson and Page 1992).

AST levels have been evaluated in gingivitis and periodontitis models. In human experimental gingivitis a statistically significant association was demonstrated between AST levels and gingival index scores for both developing lesions and resolving gingivitis (Persson et al. 1990b). In a 26 week study of AST in a ligature-induced periodontitis in beagle dogs, a correlation was found between elevated concentration of the enzyme and microscopic evidence of disease activity (Cohen et al. 1991).

Persson and co-workers assessed the relationship between GCF AST levels and active tissue destruction in treated chronic periodontitis patients (Persson et al. 1990a). They examined 25 patients at 3 month intervals over a 2 year period, and determined that the maximum enzyme level was significantly elevated at sites with confirmed disease activity and attachment loss. AST levels had median values approximately 600-800 IU at disease-active sites, compared to 400-500 IU at disease-inactive sites. These results supported the claim that an objective diagnostic test based upon AST levels could distinguish between disease-active and disease-inactive sites.

In a recent longitudinal study by Chambers and co-workers, AST levels were monitored in 31 patients with untreated adult periodontitis (Chambers et al. 1991). They found that only 2.6% (40 of the 1536 periodontal sites) lost 2 mm or more of attachment over the two year study. Although

strong correlations were found between AST levels and degenerated sites, associations were also observed with sites that maintained the same attachment level and sites that exhibited gingivitis.

Further evaluations were performed by Persson and co-workers to ascertain the diagnostic characteristics of crevicular fluid AST levels associated with periodontal disease activity (Persson and Page 1992). The ability of crevicular fluid AST activities at 600, 800, 1000, and 1200 μIU levels to recognize active disease was investigated. Eight of the 25 subjects investigated demonstrated 1 or 2 sites that lost ≥ 2mm of attachment during a 2 year period. The AST 800 μIU demonstrated a sensitivity of 0.93 and specificity 0.68 for attachment loss ≥ 2mm. AST 800 μIU was the most suitable cutoff point to distinguish sites at risk for future attachment loss from those that were unlikely to progress further. These results appear to be the most significant finding in crevicular fluid research in the past few years.

1.7.4 Immunological and inflammatory indicators

Immunological products and inflammatory mediators are produced by affected periodontal tissues. Since these substances play important roles affecting the pathogenesis of the disease, this led investigators to identify and relate them to the progression of periodontal disease.

Interleukin-1α and β are significant mediators of the destructive process of periodontal disease. They are produced by a number of cell types such as mononuclear phagocytes, PMN leukocytes and endothelial cells. Their effects include enhanced bone resorption and the production of prostaglandin E_2 and collagenase.

Investigations of IL-1β in gingival tissue in relation to periodontal disease have recently been performed. In one study comparing chronic adult periodontitis patients to healthy control subjects, IL-1β was consistently recovered from GCF of disease affected subjects whereas no IL-1β could be found in normal gingival tissue (Hönig et al. 1989). Although measureable attachment loss was associated with the presence of IL-1β, it was not statistically significant. In another study using immunofluorescent staining techniques, there were almost 3-fold more IL-1β staining cells in periodontally diseased tissue than in normal tissue (Jandinski et al. 1991).

IL-1 levels have also been measured in crevicular fluid. In a cross sectional study of GCF from adults with previous destructive periodontitis, measureable amounts of IL-1β were found at 58% of the examined sites which displayed a wide range of values (Wilton et al. 1992). Since no statistically significant correlations were found with measured clinical parameters, IL-1β could not be related to previous evidence of destructive periodontal disease. This lack of correlation with measurements like pocket depth is not surprising since pocket depth reflects the cummulative history of periodontal involvement which does not necessarily reflect the current disease activity.

Another study which examined IL-1α and IL-1β in disease active sites of untreated periodontitis patients found that 90% of GCF samples from tested sites contained measureable levels of IL-1, with more frequent occurrence of IL-1β (Masada et al. 1990). In addition, marked reductions in total IL-1 levels were observed after treatment. Furthermore, in a subset of these patients, IL-1 messenger RNA was detected in all sampled gingival tissue, demonstrating that IL-1 is produced and released locally. Although GCF IL-1 was recovered in a greater number of crevicular sites than in previous study, its presence was related only to a cross-sectional judgement of sites manifesting characteristic features of disease presence. A longitudinal study is required to compare levels of IL-1 in GCF with accurate measures of disease activity such as changes in attachment level or reductions in alveolar bone height. Only then would it be possible to assess the utility of IL-1 to serve as an indicator of active disease.

Immunoglobulins have been evaluated in GCF for class and subclass distributions and assayed as biologically active proteins against specific substrates. A recent investigaion by Reinhardt and co-workers examined IgG subclasses in GCF from active versus stable periodontal sites (Reinhardt et al. 1989). Significantly higher mean IgG1 and IgG4 concentrations were found in GCF at sites that exhibited ≥ 2mm of attachment loss than sites that were stable. These relationships remained significant when IgG subclass concentrations were adjusted per mg albumin. In addition, mean adjusted IgG subclass concentrations in GCF were generally higher than in serum, especially for IgG4 which displayed an active site GCF : serum ratio of 24.2 : 1.

Thus IgG4 concentrations may be useful as a disease indicator, as shifts towards higher IgG4 levels indicate immunopathological changes related to periodontitis.

Autoimmunity to collagen has been evaluated in patients with periodontal disease. An early study by Mammo and co-workers demonstrated an enhanced cellular immune response to native and denatured homologous type I collagen in patients with periodontal disease (Mammo et al. 1982). Subsequently this same laboratory demonstrated that serum levels of antibody to type I collagen was significantly higher in periodontally affected subjects than in the controls (Ftis et al. 1986). This was followed by a study on 20 patients of antibody to type I collagen in GCF from inflamed sites before and 6 weeks after treatment (Refaie et al. 1990). It was found that IgG antibody levels to collagen in GCF were significantly higher than in control sera, but that these levels were not significantly different from those in autologous sera. Furthermore, the levels of IgG antibody in GCF and autologous sera did not change significantly after periodontal treatment. This result is not surprising due to the 10 to 15 minute GCF sampling time employed in this study. Prolonged GCF sampling time greater than 10 minutes has been shown to dilute the GCF with serum proteins (Curtis et al. 1988). Thus in the above study, GCF and autologous sera initially meant to comprise two distinct sources, would be expected to possess similiar compositions.

In a recent study of autoimmunity to collagen in adult periodontal disease, immunoglobulin classes in sera and gingival tissue extracts were examined in patients with chronic adult periodontitis (Anusaksathien et al. 1992). While IgG and IgA antibodies to type I collagen were present in higher concentration in tissue extracts than in autologous serum, no significant differences were found for IgM antibodies. These results support the concept that the periodontium is a major site for production of collagen antibodies. In addition, their findings suggest a class switch of IgM to IgG in inflamed tissue, which may be the result of prolonged antigenic stimulation.

Although antibodies to collagen were found in periodontally affected individuals, they were only associated with the presence of periodontal disease. In regards to their use as an indicator of

periodontal disease, analysis for antibodies to collagen is another example of elaborate testing to determine what is already known by prior clinical evaluation.

In addition to IL-1β, another cytokine, tumour necrosis factor alpha (TNF-α), which is produced by activated monocytes and leukocytes and found in GCF, was examined as a possible indicator of periodontal disease (Rossomando et al. 1990). Although TNF-α levels were found consistently well above serum levels, the amounts recovered from individual sites varied widely and when compared to clinical recordings of periodontal disease status, no significant differences were found. In this cross-sectional study TNF-α could not predict periodontal health or disease. However, its presence in some pockets that were ≤ 3mm and absence in other pockets that were ≥ 4mm requires further investigation. It is possible that in a longitudinal study employing clinical attachment loss measurements to identify disease activity, TNF-α may be an indicator for early stages of disease activity.

Prostaglandin E_2 (PGE_2), a potent mediator of inflammation produced via the cyclooxygenase pathway from arachidonic acid, has been detected in GCF of experimentally induced periodontitis in the dog (Tubb et al. 1990). Studies have been made on the relationship of PGE_2 and $6KPGF_{1\alpha}$ to cAMP, IgG, IgM and α-2macroglobulin in GCF in adult periodontitis subjects (Sengupta et al. 1990). Twenty one days after scaling and root planing, the levels of all factors significantly decreased except for $6KPGF_{1\alpha}$ and cAMP which were essentially unchanged. There was a significant correlation between PGE_2, $6KPGF_{1\alpha}$, and cAMP before but not after treatment. The authors concluded that this correlation pattern indicates the involvement of PGE_2, $6KPGF_{1\alpha}$, and cAMP in inflammation of the periodontium.

The most significant studies on PGE_2 in GCF were performed by Offenbacher and co-workers (Offenbacher et al. 1986; Offenbacher et al. 1989). In cases of untreated periodontitis patients, GCF PGE_2 was found to substantially increase during active phases of periodontal destruction. A subsequent investigation in experimental ligature induced periodontitis in monkeys demonstrated that PGE_2 level increases at three months correlated with attachment loss and bone loss. In this model, PG levels peaked at 6 months and returned to baseline by 12 months. This work confirms

the earlier findings in the initial investigation that PGE_2 has value in diagnosing the disease active state.

1.7.5 By-products of metabolism

Byproducts of metabolism of the periodontium include cellular and extracellular tissue elements. One of the main features of active periodontitis is the destruction of the extracellular matrix. It is plausible to propose that crevicular fluid would contain byproducts of tissue destruction, and hence evidence of breakdown.

By monitoring crevicular fluid for breakdown products of connective tissue, it is possible to follow destruction of supporting tissue during active periodontal disease. In particular, Hyp, a unique amino acid which constitutes 10-12% of the collagen molecule and is not found in appreciable amounts in other tissue fluids, has been isolated in human crevicular fluid (Miller et al. 1982). It is anticipated that during episodes of active periodontal disease, it should be possible to follow collagen breakdown by measuring Hyp levels in GCF.

In our laboratory a highly sensitive and specific method has been developed for determinations of Pro and Hyp in biological systems using high performance liquid chromotography (Yaegaki et al. 1986). In particular, the method has been modified for evaluation of Hyp in crevicular fluid. In our earlier clinical study on 30 patients, we observed that Hyp levels in GCF were increased in periodontally involved sites, and that Hyp existed mainly in a peptide form (Coil and Tonzetich, 1986). Subsequently, it was determined that Hyp levels did not correlate with pocket depth or crevicular fluid flow. Emerging results at the time indicated that Hyp levels were associated with the level of epithelial attachment (Coil et al. 1987). It appeared that changes in Hyp levels preceded changes in attachment level by 2 to 4 weeks. Thus, Hyp could be promising as an indicator of active disease state.

Most of the Hyp in GCF may be derived either from fragments of tissue collagen or serum C1q. C1q, a subcomponent of the first complement protein, has a molecular weight of approximately 410,000 and contains 4.3% Hyp by weight. Svanberg used 0.02 M sodium acetate

to remove C1q from GCF samples (Svanberg 1987a,b). We have similarly removed C1q successfully by precipitating it with 0.5M acetate solutions, followed by centrifugation, which effectively removed 5 times the serum concentration of C1q added to crevicular fluid samples (Coil and Tonzetich 1988). Thus, it appears from these results that the main source of Hyp following this procedure is degraded collagen. However, it is still possible that C1q could be degraded by microorganisms to fragments that are not eliminated by the employed precipitation procedure.

Analysis of Hyp was performed in GCF and serum in three beagle dogs during a 5 week course in experimental gingivitis (Svanberg 1987a). Hyp concentration from individual sites showed an irregular pattern of high and low values during the 5 week study indicating that collagen metabolism was not a linearly continuous process. In a further study Svanberg examined Hyp content of GCF using a 9 day ligature-induced periodontitis beagle dog model (Svanberg 1987b). Collagen derived Hyp (total GCF minus serum value) was maximal 4 days after removal of ligature. No indication was given as to the extent of tissue destruction at this examination point. Although it appeared that Hyp was temporally related to inflammation, further investigations are required to determine the accuracy of this amino acid as an indicator of disease.

A number of GAG's have been detected from periodontal sites exhibiting previous destruction (Embery et al. 1982). Last and co-workers further investigated GAG's in GCF (Last et al. 1985). At sites of chronic gingivitis, hyaluronic acid was the only major component detected. Chondroitin-4-sulfate was also detected at periodontal sites that had $\geq$ 7mm pocket depth and radiographic evidence of bone loss, moderate pockets with some detectable alveolar bone crest loss, and at sites of LJP involvement. Since chondroitin-4-sulfate is a prominent component of bone, these results indicate that its detection in GCF may be a sensitive indicator of bone destruction.

Other potential markers of bone destruction have also been examined. In a preliminary investigation, Bowers and co-workers (Bowers et al. 1989) used a dot blot assay to examine GCF for the presence of osteonectin, bone phosphoprotein, and bone sialoproteins I and II. While neither of the bone sialoproteins were detected, osteonectin and bone phosphoprotein were found

in quantities that roughly correlated with the degree of periodontal involvement. This is the first known published work to examine GCF for this group of connective tissue-associated proteins.

Evaluations of protein composition of GCF may also prove useful in identifying a marker or markers of periodontitis. Baseline data of a longitudinal study on the protein composition of GCF from subjects without periodontitis indicated that four non-plasma derived proteins are routinely detected in the fluid (Curtis et al. 1990). It is believed that these represent products of normal turnover in the periodontium. It is anticipated the major metabolic changes that accompany destruction of periodontal tissues will be detectable in the GCF protein profile.

Elastin is a fibrous protein component of connective tissues, especially abundant in lungs, ligaments and arterial walls. Its degradation has been associated with degenerative disorders such as emphysema and arteriosclerosis. Desmosine (DES) and isodesmosine (IDE) are specific cross-linking amino acids found in elastin, and their presence in the tissue is indicative of elastin catabolism.

Although prominent elastin fibers are present in the periodontal ligaments of rabbit, dog, sheep and swine, they are found in the gingiva but not the periodontal ligament of man (Berkovitz et al. 1982). Oxytalan fibers are found in the periodontal ligament of man, having a similar distribution to elastin fibers in the above named animals. Although it is impossible to distinguish between them histochemically, these two fibers types are thought to be phylogenetically related.

HPLC methods for the detection of the specific cross linking amino acids, DES and IDE, have been performed on cell cultures (Muramoto et al. 1984) and tissue hydrosylates (Yamaguchi et al. 1987). Application of HPLC methods for analysis of crevicular fluid, will make it possible to detect DES and IDE at the nanogram level if they are present. This has not been attempted to date.

1.8 PROPOSED STUDY

1.8.1 Crevicular volatile sulphur production

In view of the apparent importance of VSC production in periodontal pockets (Coil and Tonzetich 1984; Coil and Tonzetich 1985), one of the purposes of this study was to develop a device for collection of volatiles from the gingival crevice, and to analyze and compare the composition of VSC in gingival sulci with those of mouth air. The study is intended to quantify the level of VSC production in periodontal crevice sites and relate it to clinical measurements of periodontal involvement. It is plausible that measurement of volatiles emanating from periodontal sites may reflect the level of disease activity occurring at the time of sampling.

1.8.2 Crevicular fluid hydroxyproline content as an indicator of disease activity

The only known study to quantitate hydroxyproline levels in GCF was work done by Miller and co-workers (Miller et al. 1982). Using pre-column derivatization followed by HPLC separation, Hyp was identified in GCF samples of three subjects. Since it has been demonstrated that active stages of periodontitis are accompanied by significant loss of collagen (Page and Schroeder 1976), it is conjectured that Hyp levels in GCF may reflect the level of collagen metabolism in the periodontium. By monitoring gingival fluid levels of Hyp, it may be possible to monitor the level of collagen breakdown occurring at specific periodontal sites. By comparing Hyp levels to clinically accepted periodontal assessments at specific periodontal sites, it would be possible to evaluate Hyp as an indicator for presence of periodontal disease. Attachment level measurements in a longitudinal study could also assess the ability of Hyp analyses to indicate disease activity.

1.9 SIGNIFICANCE

It is no longer appropriate to attempt to identify active periodontal disease using traditional clinical measurements because they only give retrospective evidence of periodontal disease. Pathology recorded by gingival indexes, bleeding on probing, suppuration and pocket depth, are inadequate measurements for predicting future changes in the level of periodontal attachment (Haffajee et al. 1983; Kaldahl et al. 1990). There is a strong need for a non-invasive, sensitive and specific test for periodontal disease activity.

In considering such a test, one must first accept the concept that periodontitis in man is an episodic disorder, occurring in short bursts followed by long periods of quiescence. Without this perception of the disease activity, the traditional clinical measures would be sufficient to designate the presence of disease if one accepts a linear model for progression of disease. Secondly, in recent years it is generally acknowledged that periodontitis, specifically periodontal disease activity, may be a site specific phenomena (Goodson et al. 1982; Haffajee et al. 1983).

The assessment of periodontal diseases remains controversial because of a lack of reliable diagnostic procedures which can differentiate previous periodontal disease from currently progressing or active periodontal destruction. Evaluations of disease progression have focused upon loss of epithelial attachment and loss of alveolar bone, which act only as retrospective analyses of disease activity. It is anticipated that correlation of Hyp levels in crevicular fluid with attachment level change will establish Hyp as a reliable predictor of disease activity.

2. MATERIALS AND METHODS

2.1 MATERIALS

Unless stated otherwise all reagents used in this study were of analytical grade and purchased from either Fisher Scientific Limited (Fairlawn, NJ, USA) or Sigma Chemical Company (St. Louis, MO, USA). All materials used for the High Performance Liquid Chromatography (HPLC) were purchased from Waters (Millipore Corp., Milford MA, USA). Acetonitrile, ethanol and methanol were HPLC chemical grade and obtained from BDH (BDH Chemicals, Vancouver, B.C.,Canada).

Materials and chemicals used for the electrophoresis and western blotting were purchased from Bio-Rad (Bio-Rad Laboratories, Richmond, CA, USA) and ELISA plates were from Falcon (Becton Dickinson and Co., Lincoln Park, N.J.,USA)

Antibodies were obtained from a number of sources. Anti-type I collagen was bought from Chemicon (Temecula, CA, USA) and Southern Biotechnology Associates Inc. (Birmingham, AL, USA), and donated by Dr. Sampath Narayanan (Seattle, WA, USA). Hybridoma cell lines producing monclonal antibodies to C1q were graciously donated by Dr. Linda Curtiss (Scripps Clinic, La Jolla, CA, USA). Polyclonal antibody to C1q was purchased from Calbiochem (Calbiochem Corp. La Jolla, CA, USA). Secondary antibodies, which included alkaline phosphatase conjugated goat anti-rabbit IgG and alkaline phosphatase conjugated goat anti-mouse IgG, were purchased from Calbiochem and BRL (Bethesda Research Laboratories, Gaithersburg, MD, USA), respectively. All other antibody products were purchased from Sigma.

2.2 VOLATILE SULPHUR COMPOUND STUDY

2.2.1 Development of a collection device for sampling crevicular air

The concepts surrounding the development of a suitable gingival crevice air collection device focused upon several ideal parameters. An ideal collection device should be easily fabricated, custom fit for each patient's crevicular site, provide a tight seal, be reusable, and be non-invasive to the tissues.

The initial approach was to fabricate a collection device using a common silicone based dental impression material. This was accomplished by the following procedures. First, using alginate impression material (Jeltrate^{TM}, L.D. Caulk Co., Miford, DE, USA), an impression was obtained of the patient's dental arch using a standard metal tray. Subsequently a dental cast, a replica of the patients' teeth, was made by pouring this impression in a die stone, (Densite^{TM}, Georgia-Pacific, Portlan, OR, USA). From this cast a segmental custom tray from acrylic was made that overlapped the crowns and gingival margins on several teeth adjacent to and including the test tooth (FASTRAY^{TM}, Harry J. Bosworth Co., Chicago, IL, USA). A section of micro polyethylene tubing (INTRAMEDIC^{TM}, Becton, Dickinson and Company, Parsippany, N.J., U.S.A.), measuring 0.76 mm I.D. x 1.22 mm O.D., was held against the buccal surface of the tooth at the level of the free gingival margin using dental floss interproximally. A medium body silcone based impression material (Reprosil^{TM}, L.D. Caulk Co, Miford, DE, USA) was mixed and placed in the segmental tray, which in turn was placed in the patient's mouth and pressed over the tooth to be sampled. To ensure that the tubing was completely surrounded by the impression material, it was positioned and held so that it contacted the edge of the segmental tray while the impression material was setting. The setting took five minutes during which time this newly forming sampling device was held stable in the patient's mouth.

After the impression had set, the sampling device was removed from the patient's mouth and the dental floss was removed from around the tubing. The portion of the tubing which protruded

from the inside of the impression material and rested against the free gingival margin was cut and discarded. This created a space above the free gingival margin from which crevicular air could be sampled, leaving two pieces of tubing protruding from the impression which served as inlet and outlet sampling ports. A peristaltic pump was attached to the micro polyvinyl tubing exiting on the same proximal surface of the gingival crevice to be sampled. The pump set at a flow rate of 0.6 ml/min. directed a flow of air across the gingival site and out through the micro tube at the opposite proximal surface.

Because it is impossible to obtain sufficiently large crevicular air samples for direct gas chromatographic analyses, it was necessary to pre-trap and concentrate the volatiles emanating from the gingival sites. The same methodology for collection and concentration of volatile organic compounds from human mouth air was adapted for use in collecting volatiles from crevicular air (Tonzetich et al. 1991). A Tenax-GC trap, which was used to collect volatile compounds, was placed between a vacuum pump and the subject's oral cavity. Tenax traps were constructed from 7 mm OD x 75 mm lengths of aluminum tubing, coated on the inside with teflon, packed with 0.7 g of Tenax-GC, and stoppered with glass wool end plugs. A total of six traps were used in the study.

Each Tenax trap was tested individually for absorption and desorptive capabilities. The retention times of Tenax-trapped sulphide components from mouth air were matched with those obtained by direct mouth air sampling. Room air was also analyzed in a similar manner to ensure that there was no sulphur contribution from the ambient air. Prior to use, a Tenax trap that was stored at -20°C in a stoppered test tube was removed and immediately desorbed on a modified Bendix Flasher at 120°C. It remained at room temperature for no longer than one hour before it was cooled and maintained in dry ice at -55°C while being utilized as a collection device.

Pilot runs designed to test the recovery of volatiles from crevicular air were performed to ascertain the validity of the sampling technique. After collecting the equivalent of 10 ml of crevicular air from several subjects, each Tenax trap was desorbed on the modified Bendix Flasher unit coupled to the gas chromatographic/ flame photometric detector (GC/FPD). As each Tenax

trap had been tested for its ability to retain and desorb volatile sulphur compounds prior to use and as none of the spectrums in these pilot runs revealed the presence of volatile sulphur compounds, this implied that there was a defect in construction or operation of the sampling device. In addition, since each patient exhibited VSC in their mouth air at the same appointment as the pilot runs for crevicular air sampling were performed, it was anticipated that there was a good possibility of detecting VSC in crevicular air samples.

After contemplating the possible causes for failure using this technique, it was decided to change the position of the peristaltic pump. Instead of pumping air across the gingival crevice and forcing it through tubing at the opposite proximal site, it was decided to aspire air from above the crevice. When the Tenax trap was placed between the gingival site and the peristaltic pump, measureable amounts of VSC were collected as evidenced by the recordings on the GC chromatograms.

The sampling device was further modified to allow for easier fabrication. Instead of producing a dental cast to construct a special custom segmental tray to take an impression, a soft acrylic denture reline material, Coe-soft^{TM} (Coe Laboratories Inc., Chicago, IL, 60658) was used to take an impression without using a tray by placing it directly over the tested crevicular site. This modification yielded the same volatile compound collection capabilities and allowed for easier chairside fabrication.

2.2.2 Experimental design

Twenty gingival crevicular sites, thirteen test and seven control, were selected from seventeen patients (12 male and 5 female, aged 24-67 yrs). Patients were selected on the basis of not having received previous periodontal treatment or antibiotic medication within the past six months and having no known systemic disease. Pocket depths were measured using a Marquis probe and recorded to the nearest millimeter by one competent examiner. Periodontal sites were separated into control and test groups based upon pocket depth measurements of $\leq$3 mm and $\geq$4 mm, respectively. Three patients that exhibited both shallow and deep crevices, were further classified

on the basis of bleeding on probing as having an inflamed or noninflamed site. Five patients contributed three and two sites to the inflamed and noninflamed groups, respectively. Clinical signs of inflammation based upon bleeding on probing evaluations were performed by the same examiner.

Patients were tested at least one week after periodontal probing and their periodontal status was confirmed immediately after VSC collection. The GC/FPD method developed by Tonzetich was employed for sampling mouth air (Tonzetich 1971). Patients were instructed to close their lips and breathe through their nose. After one minute, a 6 cm length of a 15 cm long piece of teflon tubing (0.3 mm ID.) connected to the GC sampling line assembly was inserted between the lips into the oral cavity. The subject then voluntarily stopped breathing while 15 ml of mouth air was aspired by means of a 25 ml Hamilton syringe, thus filling the 10 ml sample loop of the GC. The carrier gas was then directed through the sample loop and the 10 ml sample transferred onto the GC column for separation.

2.2.3 Collection of crevicular air

Collection of volatiles from gingival sulci was effected by sampling air from individual isolated periodontal sites. A small section of micro polyethylene tubing was held, as previously described, against the buccal surface of the tooth at the level of the gingival margin using dental floss interproximally. Coe-Soft was mixed and molded into a cube before it was fitted over the tooth and its adjacent tubing. After the material hardened, the impression was removed from the mouth and epoxy resin was used to secure the tubing as it exited the impression to the external portion of the acrylic. This created a closed system. Finally, the portion of tubing at the gingival margin was removed thereby creating inlet and outlet ports for sampling. Each custom sampling device had a 60 cm outlet port of tubing extend from the crevicular site to a Tenax trap, while a 15 cm inlet port of tubing extended from the collection device to ambient air.

These special devices for collection of volatiles were constructed for each site of each individual. They were prepared after the subjects provided mouth air samples for GC analysis.

The collection device was placed back in the mouth and the exit port tubing was connected via tygon tubing to a Tenax trap cooled to -55°C in dry ice. The other end of the trap was connected to a peristaltic pump and crevicular air was removed at a rate of 0.60 ml/min. for sixteen minutes. Only one device was required per site which allowed for repetitive sampling. Immediately after the collection of volatiles, the tenax trap was removed from the dry ice chamber and quickly detached from the tubing and prepared for GC analysis.

2.2.4 Gas chromatographic analysis of crevicular air

Immediately after collection, the compounds concentrated on Tenax traps were desorbed at 120°C using a modified Bendix Flasher coupled to the GC unit and analyzed by GC. The separations of volatile sulphur compounds were performed on a Tracor 550 Gas Chromatograph using a 7.315 x 3.2 mm fluorinated ethylene propylene (FEP) Teflon column packed with 5 per cent polyphenyl ether and 0.05 per cent phosphoric acid on 30-40 mesh Teflon (Micro Tek Instruments Corp., Austin, Texas). The temperature of the column and detector were 70°C and 150°C respectively. The pressures and flow rates of the employed high purity gases (Union Carbide Canada Ltd.) were : air carrier gas (55 psig)- 10 cc/min; H_2 (40 psig) - 80 cc/min; air (55 psig) - 60 cc/min. Sulphide profiles were recorded using a Shimadzu, C-R3A Chromatopac apparatus (Kyoto, Japan). Permeation tube standards were used to internally program calculations of sulphide content from recorded peak areas.

2.2.5 Identification and quantitation of volatile sulphides

The identification of sulphide components was based upon their retention times. The chromatographic pattern exhibited sulphide peaks and retention times (Rt) as follows: hydrogen sulphide, H_2S (Rt:115-130 sec.), methyl mercaptan, CH_3SH (Rt: 190-205 sec.), dimethyl sulphide, $(CH_3)_2S$ (Rt: 275-310 sec.), dimethyl disulfide, $(CH_3S)_2$ (Rt: 390-425 sec.). Standard plots of integrated peak area versus concentration for H_2S and CH_3SH are illustrated in figures 2.1 and 2.2 respectively. For sulphide peaks that were below the threshold quantitation level

setting of the recorder (100 mV/sec), extrapolation calculations were performed using peak weights of known peak areas and corresponding ng concentrations. For H_2S the relationship of ng to peak area was (y= .1266 + 1.556 exp-04 x - 1.214 exp-08 x^2 + 5.167 exp-13x^3 ; r^2=1.000), while for CH_3SH the ng to peak area was (y= .2223 + 1.189 exp-04 x - 3.634 exp-09 x^2 + 5.43 exp-14 x^3 ; r^2=1.000). The peak weight to ng relationships for $(CH_3)_2S$ and $(CH_3S)_2$ were based upon a similiar response factor and the % weight of sulphur of each compound compared to CH_3SH (52/67 and 68/67 respectively). Depending upon the amount of sampling time for each crevicular site, the total volume of trapped crevicular air was calculated and the levels of recorded sulphides were reported as ng/10 ml of crevicular air.

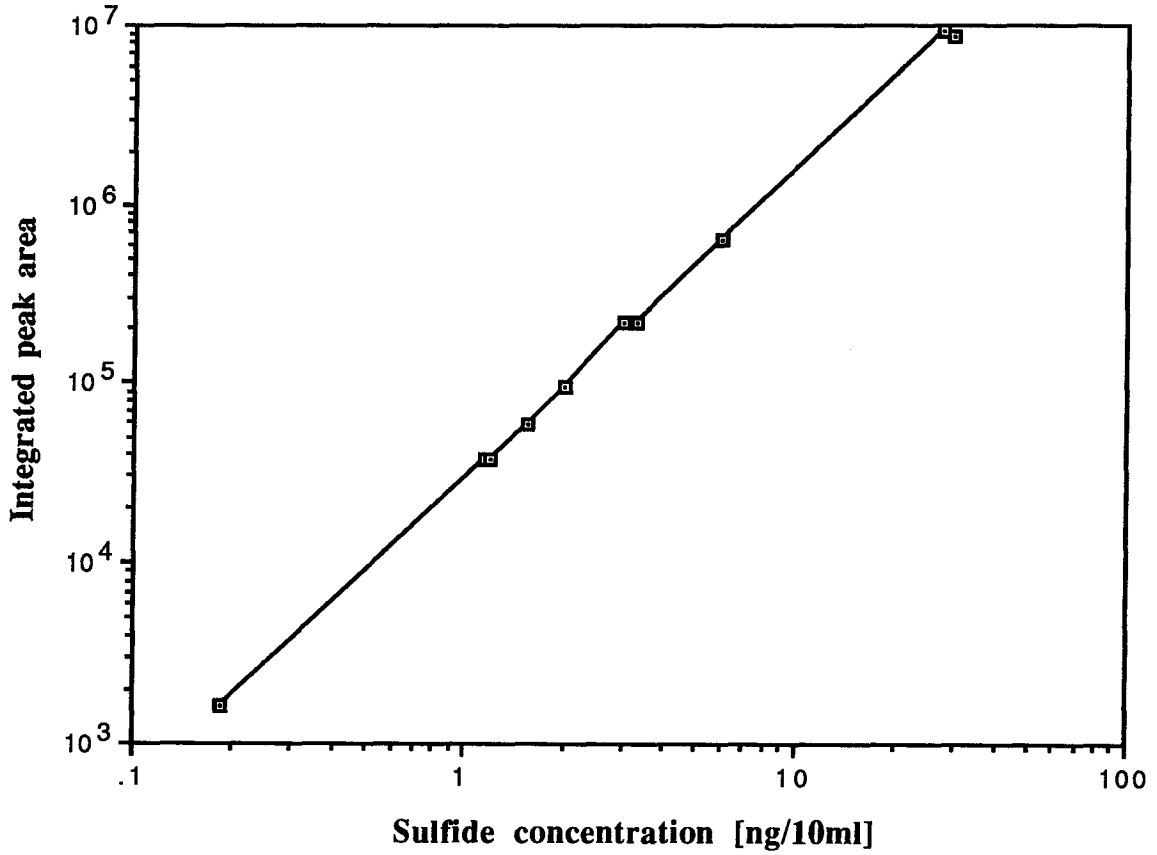

Figure 2.1: Standard plot for H_2S of integrated peak area versus concentration

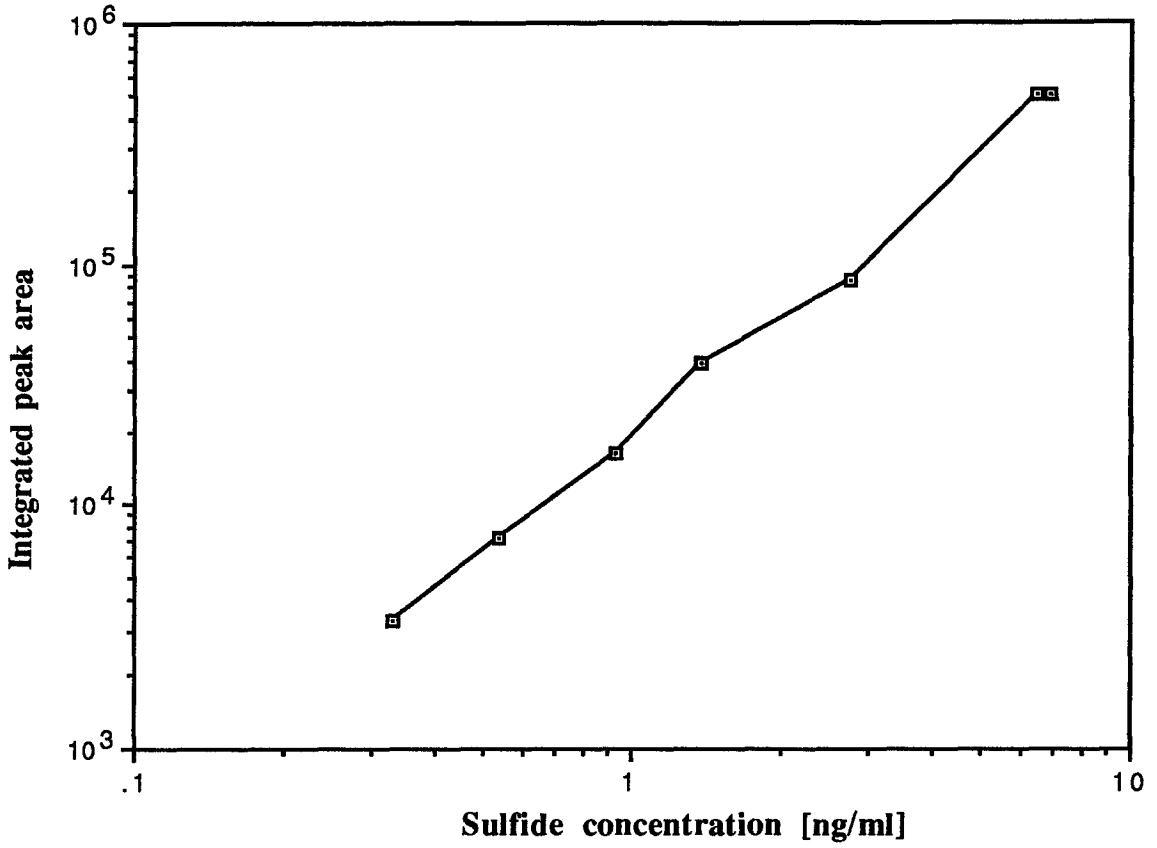

Figure 2.2: Standard plot for CH_3SH of integrated peak area versus concentration

2.2.6 Data analysis

The levels of crevicular VSC were converted to ng levels of sulphide per 10 ml of air, based upon flow rate and time of sample collection. This enabled comparisons of crevicular and mouth air sulphides to be performed on equivalent volumes of air. The various sulphide levels, the ratios of CH_3SH to H_2S and all methyl sulphides to H_2S, and total sulphur content in crevicular air were categorized according to pocket depth (shallow and deep) and inflammation (noninflamed and

inflamed). Comparisons based on student's *t*-test were made between mean values of sulphide ratios and total sulphur levels in crevicular air.

2.3 DEVELOPMENT OF HPLC HYDROXYPROLINE ANALYSIS

The objective was to develop and apply a sensitive, specific, and reproducible chromatographic procedure that would detect Hyp in crevicular fluid. In previous investigations Hyp content of gingival exudate was determined by colorimetric methods (Hara and Takahashi 1975). Recently the analysis of Hyp in GCF by an HPLC method was described by Miller and co-workers (Miller et al. 1982). A logical place to begin the current study was to evaluate the applicability of the latter HPLC method.

Miller and co-workers analyzed Hyp using precolumn derivatization with dansyl chloride (5-dimethylaminoaphthahalene-1-sulfonyl chloride) (Miller et al. 1982). Briefly, the procedure involved acid hydrolysis followed by incubation of the sample with dansyl chloride reagent for one hour at 55 °C. After drying, reconstitution in methanol, and filtration, the samples were injected onto a μ-bondpack C18 column. The components were eluted at a flow rate of 1.5 ml/min. using 0.1 M sodium acetate/acetonitrile buffer system, programmed with acetate/acetonitrile gradients as 95/5 constant for 10 minutes, increased to 80/20 during 15 minutes, followed by another gradient of 06/40 during 75 minutes. Fluorescence detection was effected using an excitation wavelength of 405 nm with a 485 nm emission cut off filter.

Our HPLC unit (Waters Assoc., Millipore Corp., Mississauga, ONT, Canada) consisted of a Model 721 programmable system controller, two Model 510 HPLC pumps, a Model 710 WISP, A Model 730 data module and a Model 481 LC UV variable-wavelength spectrophotometer. The system was coupled to a 10 cm x 8 mm (5 μm) reversed-phase Nova-Pak C18 Radial-Pak column (Waters Assoc.) used with a Z-module radial compression separation system (Waters Assoc.) and when required a Gilson Model 201 fraction collector (Gilson, France).

In using the dansyl chloride derivatization method, it was found that the standard Hyp peak was small, gave low responses, and variably appeared at retention times beyond 30 minutes of elution. In addition, location of the Hyp peak was at times uncertain, especially in test systems of crevicular fluid. Due to the above limitations, spiking of the sample with relatively large amounts of Hyp was required to identify the Hyp peak. Other investigators have also demonstrated that using this method Hyp is difficult to detect, as it has a low responsiveness and is very close to a glutamine peak (Wiedmeier et al. 1982).

Concurrent with the investigation for an appropriate Hyp analytic procedure, an efficient technique for analysis of Hyp and Proline (Pro) was developed in our laboratory by Yaegaki and co-workers (Yaegaki et al. 1986). A number of techniques were evaluated for determining Hyp and Pro in biological systems including derivatization with dabsyl and dansyl chlorides, and NBD-Cl (4-chloro-7-nitrobenzolfurazan). These methods were found complicated and limited in applicability due to poor separation, low recovery, low sensitivity, and complexity of analyses. For example, using radiolabelled Hyp and Pro, it was determined that derivatizing with dansyl chloride resulted in less than 50% recovery of the amino acid. As Hyp is found in extremely low concentrations in biological materials, its chromatographic peak in complex samples was overshadowed in previous Hyp derivatization analyses by large numbers of other constituents.

The method developed by Yaegaki and co-workers was based on the removal first of primary amino acids from the sample followed by analysis for secondary amino acids Hyp and Pro. Primary amino acids are removed by pre-column derivatization with *o*-phthalaldehyde (OPA) and separation by reversed phase HPLC. Subsequent pre-column derivatization of the void volume with phenylisothiocyanante (PITC) and reversed phase HPLC separates derivatized Hyp and Pro which are quantified using a UV detection system. Using this new method Pro and Hyp were readily analyzed without interference by other constituents.

This method was successfully used in evaluating collagen metabolism by human gingival fibroblasts cultured under different experimental conditions. The prior removal of primary amino

acids from the sample allows a clean chromatographic separation of Hyp and Pro, and hence, more accurate results. The procedure yields 93% recovery for both Hyp and Pro.

In crevicular fluid systems the volume of collected fluid is in the submicroliter range. Due to this volume limitation, it was necessary to reduce the manipulation of the sample steps to the minimum. For this reason the utilization of a single derivatizating step employing PITC, which reacts with both primary and secondary amino acids, was evaluated.

Using the above described improved modified HPLC method, a Pierce amino acid standard (Sigma), supplemented with 5 x 10^{-3} M 4-Hyp, was processed for the PITC derivatization according to the method outlined by Yaegaki and co-workers (Yaegaki et al. 1986). From this standard amino acid mixture the Hyp peak was cleanly separated from neighbouring Glu and Ser peaks. This was confirmed by spiking the mixture with radiolabelled Hyp (^{3}H-Hyp) with specific activity of 5.9 Ci/mmol (DuPont, Boston, MA, USA), and determination of radioactivity in collected peak fractions. Figure 2.3 displays the HPLC profile of the derivatized amino acid standard exhibiting a peak corresponding to Hyp with a retention time of 3 min 20 seconds. In pilot tests of crevicular fluid samples, the Hyp consistently remained separated from the Glu and Ser peaks. This is demonstrated by a crevicular fluid profile shown in figure 2.4. Hence two derivatization methods have been developed in our laboratory for the analysis of hydroxyproline (Hyp) and proline (Pro) in biological materials (Yaegaki et al. 1986). One of these, the single phenylisothiocyanate (PITC) derivatization method was most suitable as it yielded clean separations of Hyp and other amino acids in crevicular fluid, without the need for initial derivatization with *o*- phthalaldehyde to remove primary amino acids. However, if one requires accurate analysis of both Pro and Hyp then Yaegaki's two derivatization technique is a method of choice.

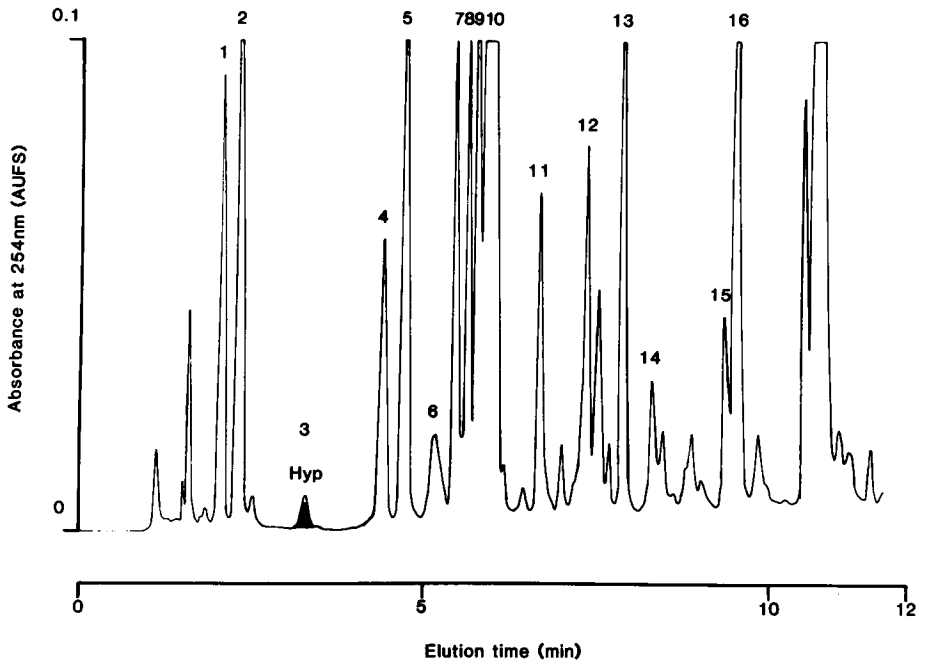

Figure 2.3: HPLC profile of PITC derivatized amino acid mixture. The black outline within the peak corresponding to Hyp indicates the location of the radioactivity. '1'= Asp; '2'= Glu; '3'= Hyp; '4'=Ser; '5'=Gly; '6'=His; '7'= Arg; '8'=Thr; '9'= Ala; '10'=Pro; '11'=Tyr; '12'=Val; '13'=Met; '14'= Cys; '15'=Ile; '16'=Leu.

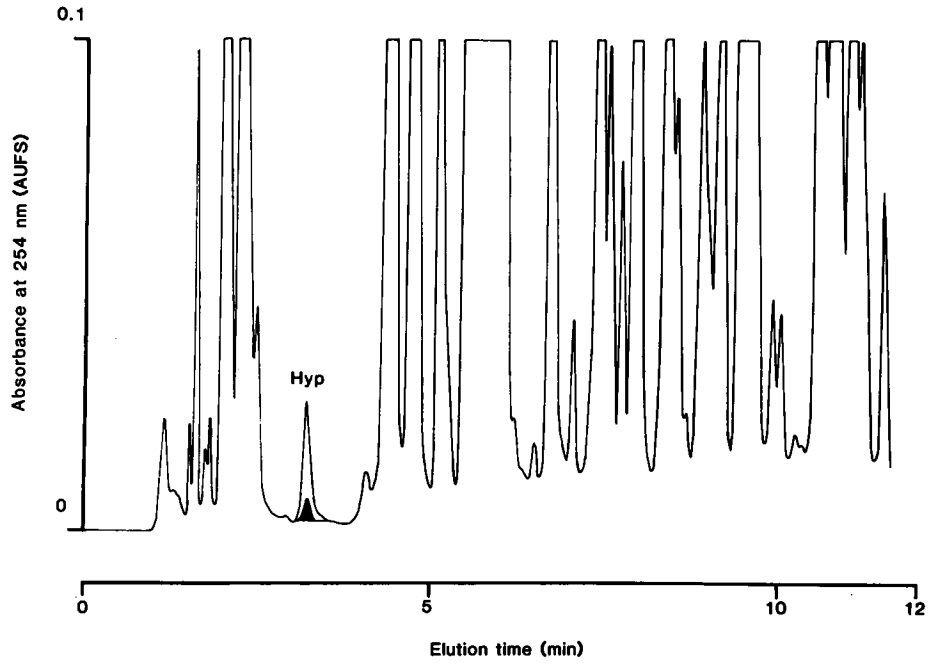

Figure 2.4: HPLC profile of PITC derivatized crevicular fluid. The black outline within the peak corresponding to Hyp indicates the position of the radioactivity.

2.4 SPIRAMYCIN STUDY

2.4.1 Experiment design

This study was performed on thirty patients selected from the Graduate Periodontal Clinic at the University of British Columbia. The objective of the study was to test the effect of antimicrobial treatment with spiramycin as an adjunct to scaling and root planing. Exclusion criteria for patient selection included the following: 1) the presence of juvenile periodontitis, 2) a history of periodontal therapy within the past six months, 3) the use of antibiotics in the last six months, 4) a history of diabetes, and 5) pregnancy or lactation. Each patient had a minimum of two teeth that were periodontally involved, each having at least one interproximal site of ≥7 mm pocket depth and interproximal contact with a natural tooth. If several teeth were found suitable, then the most posterior tooth in each arch was chosen, as they are the most difficult to maintain. For each tooth, four sites (distal buccal, mesial buccal, distal lingual and mesial lingual) were clinically evaluated using the following parameters: 1) plaque index, 2) crevicular fluid flow, 3) probing depth, 4) attachment level and 5) bleeding on probing.

For those patients that met the above criteria at the initial examination, alginate impressions were taken of patients' dental arches and poured in dental stone, and an acrylic stent was constructed over the resulting plaster casts. The acrylic was extended to within 3 mm of the gingival margins interproximally and where possible it extended two teeth beyond the selected test sites. The acrylic opposing each examinable interproximal site was marked with a vertical line, so that interproximal positioning of the periodontal probe would be reproducible at later examination periods.

The patients began the study within one week after the initial examination, which involved measurement and recording of the above clinical parameters at times 0, 2, 8, 12 and 24 weeks after the onset of the study. All clinical examinations were performed by the same experienced examiner. After plaque index was recorded for each site, supragingival plaque was removed with

a sterile curette. Following collection of crevicular fluid on filter paper strips and measurement of volume using a Periotron 6000 (Harco Electronics, Irvine, CA, USA), each paper strip was subsequently assayed for Hyp (Coil and Tonzetich 1986). This was followed by pocket depth and attachment levels measurements using a Marquis probe. It was imperative to adhere to this sequence of measurements so as not to interfere with the crevicular site prior to fluid collection.

2.4.2 Sample collection and measurement

Each site was isolated using cotton rolls and gently dried with air. A standardized filter paper strip (Periopaper^{TM}, Harco, Tustin, CA, U.S.A.), was positioned at each interproximal site and placed at the entrance of the crevice to collect fluid for five seconds. Crevicular fluid volume was immediately measured using a Periotron 6000 instrument and recorded. Each filter paper strip was placed in a 13 mm pyrex test tube which was sealed using parafilm wax. The relationship between the Periotron 6000 reading and fluid volume was determined using 0.0 - 1.2 µl of fetal calf serum and is depicted in figure 2.5.

2.4.3 HPLC analysis for hydroxyproline

Crevicular fluid was eluted from filter paper strips by soaking them for 24 hours in 100 µl of 5 mM Na_2HPO_4. The strips were then washed with 50 µl of 0.5 N acetic acid and left to soak for an additional one hour. After the two eluants were combined, they were placed in 1.5 ml Eppendorf tubes and centrifuged at 15,600 g for 30 minutes at 4° C. The supernatants were collected in 6 x 50 mm pyrex micro culture tubes (Corning glass works, Corning, NY). These sample tubes were then placed in a reaction vial, which could accomodate up to twelve samples and one Hyp standard. Further processing of the samples in the reaction vial was carrried out using the Pico-Tag Workstation (Millipore, Milford, MA).

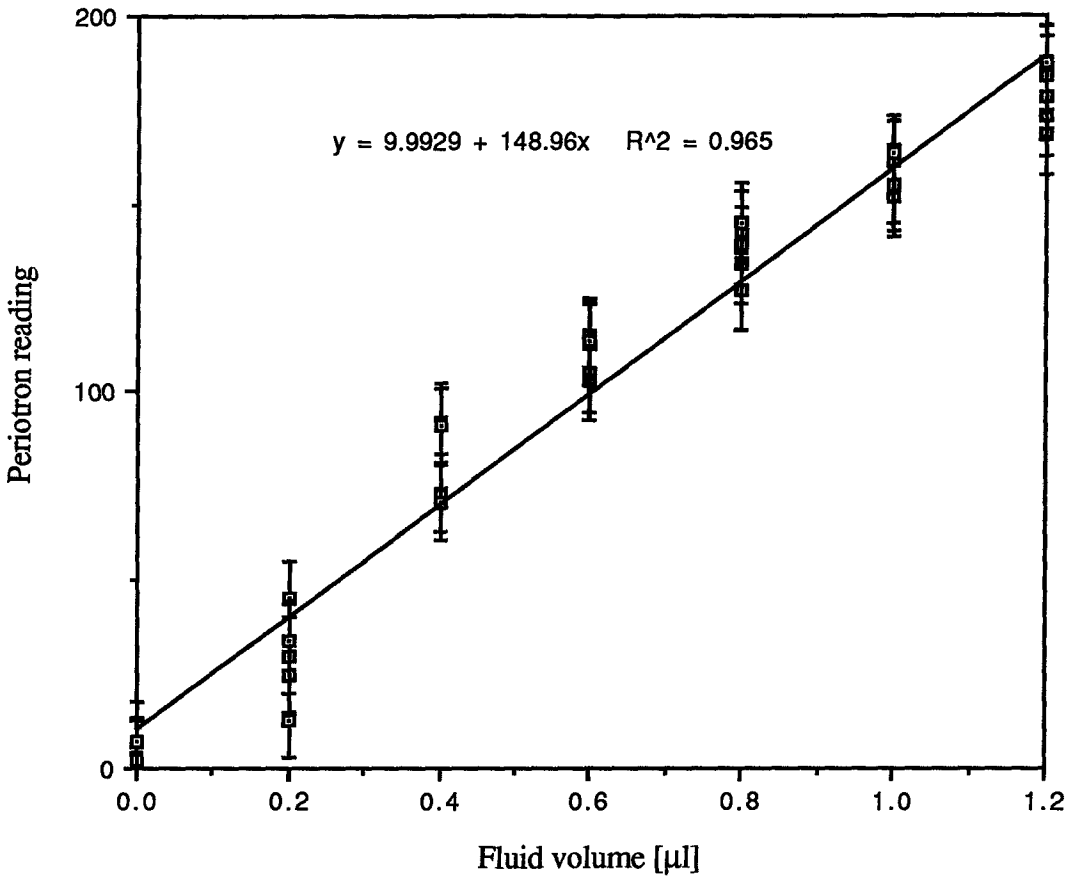

Figure 2.5: A standard plot of Periotron readings versus fluid volume using fetal calf serum-soaked Periotron filter paper strips

Samples were vacuum dried to 65 mtorr before being hydrolyzed in 350 µl of 6 N HCl containing 0.1% phenol, which was added to the bottom of each reaction vial. Oxygen was removed from reaction vials by three successive vacuum evacuations interposed with nitrogen flushing. After the third evacuation the reaction vial was sealed under vacuum and placed in the workstation oven, where the samples underwent hydrolysis at 150 °C for one hour. After cooling to room temperature the reaction vial was cleaned and dried, and each sample tube was wiped dry before being placed back into the reaction vial and dried to 65 mtorr.

Twenty microliters of freshly prepared redrying agent (ethanol: water: triethylamine 2: 2: 1) was added to each sample and again the reaction mixture was brought to dryness. Then 50 µl of freshly made PITC derivatizing reagent (ethanol: water: phenylisothiocyanate: triethylamine 7: 1: 1: 1), was added to each sample vial, vortexed for 15 seconds, placed back and sealed into the reaction vial, and left at room temperature for 20 minutes. Excess reagent was purged under vacuum at 65 mtorr, to remove all traces of PITC, which otherwise would interfere with subsequent amino acid separation.

Sample tubes were then removed from the reaction vial and processed for HPLC analysis. Samples were reconstituted in 200 µl of sample diluent (5 mM Na_2HPO_4 solution adjusted to pH 7.4 with 10% orthophosphoric acid combined with acetonitrile, 95:5, v/v) and vortexed for 30 seconds. The resulting mixtures were filtered through 0.45 µm HV SJHV004NS filters (Millipore, Milford, MA) using 1 cc tuberculin syringes, into individual limited volume inserts suspended within HPLC sample vials.

All analyses were performed on the same HPLC unit as described in section 2.4.3. In addition to the Nova-Pak C18 column, two Pico-Tag C18 columns (Waters Assoc.), quality controlled specifically for amino acid analyses, were also used during the study. Also, a temperature control monitored column heater (Waters Assoc.) replaced a water bath for maintaining a constant temperature of 38°C.

HPLC solvent buffers A and B were comprised of the following solutions. Non-organic water was prepared by passage of 18 MΩ pure water through a Norganic cartridge (Waters Assoc., Mississauga, Ont.,Canada). Buffer A of the mobile phase was composed of 60 ml of acetonitrile and 940 ml of 138 mM sodium acetate buffer containing 0.05% triethylamine (TEA), adjusted to pH 6.4 with glacial acetic acid Buffer B consisted of 60% acetonitrile in 40% non-organic water by volume. The flow program for the solvent gradient of buffers A and B is given below in Table 2.1. A 100 µl volume of sample was automatically injected onto the C_{18} stainless steel column maintained at 38 °C, for separation and determination of the phenylthiocarbamyl derivative of Hyp.

Each sample was separated over a period of 12 minutes followed by a gradient washing and re-equilibration for 13 minutes.

Time (min)	Flow (ml/min)	% A	% B	Curve
0.0	1.0	100	5	05
10	1.0	54	46	05
10.5	1.0	0.0	100	05
11.5	1.5	0.0	100	06
12.0	1.5	0.0	100	05
12.5	1.5	100	0.0	05
20	1.5	100	0.0	05
20.5	1.0	100	0.0	05
50	1.0	100	0.0	05

Table 2.1: Gradient program for separation of PITC-derivatized amino acid residues.

As described earlier, the retention time for Hyp was established using a radioactively labelled standard ^{3}H-Hyp. A standard amino acid mixture (Pierce) containing Hyp was included and processed with each set of 12 samples. This acted as an internal standard for each set of analyses, and monitored any fluctuations in detector response between sets of sample runs and/or separation differences amongst different columns employed. Standard plots for high and low levels of Hyp are displayed in figures 2.6 and 2.7, respectively.

2.4.4 Data analysis

Comparisons were made between Hyp levels and clinical measurements amongst the various data collection time points. Due to a variety of circumstances, such as the crevicular fluid samples not being available for processing, too low a collected fluid volume, chromatographic fluctuations that were uninterpretable, and significant HPLC instrument malfunctions over the course of the

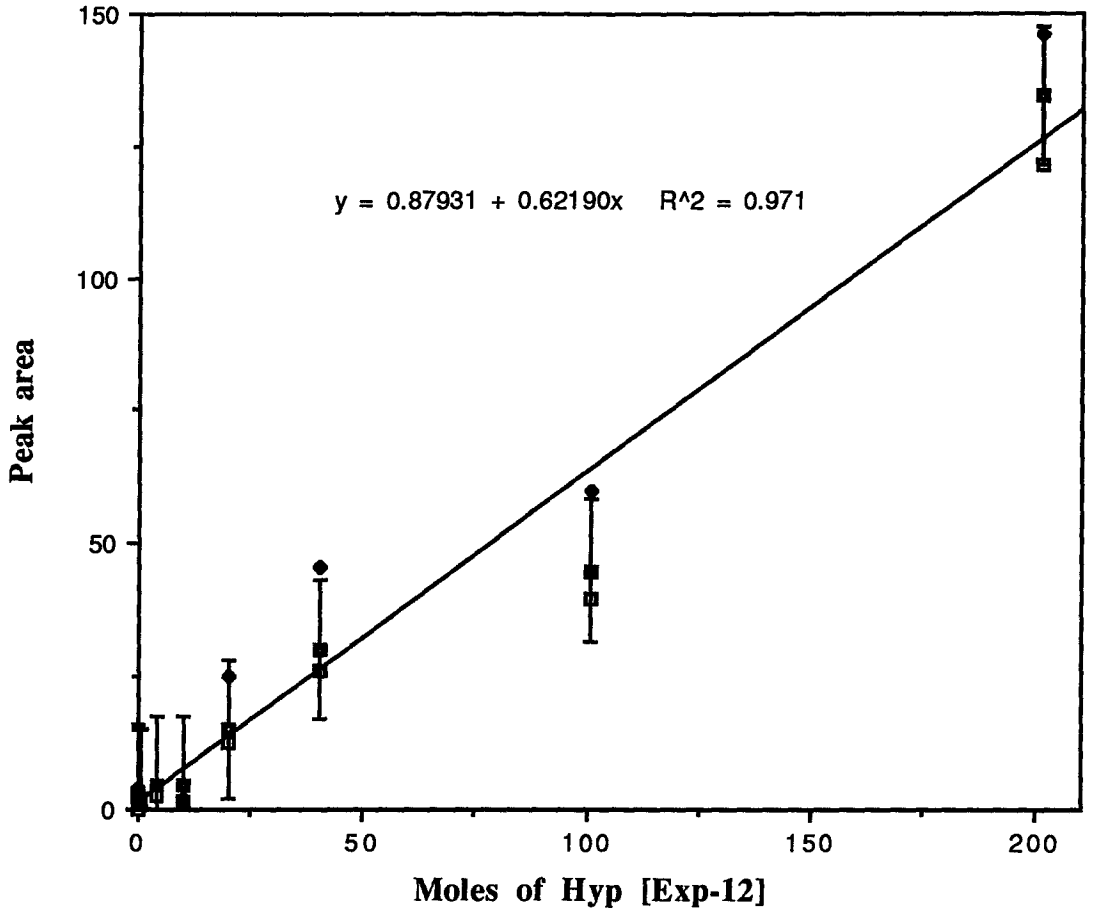

Figure 2.6: Standard plot for high concentrations of hydroxyproline.

study, Hyp data points for all examination periods were incomplete. After an in depth analysis of existing data points that were common to the same patient, it was determined that the most complete data was available for time points 0 and 12 weeks. Thus, Hyp analyses were compared to clinical measurements between weeks 0 and 12 of the study. This offered a comparison of parameters before and after periodontal treatment, a range of time that has commonly been used by other investigators (Lamster et al. 1988; Sengupta et al. 1988; Reinhardt et al. 1989; Kaldahl et al. 1990; Persson et al. 1990a; Chambers et al. 1991; Deas et al. 1991; Persson and Page 1992).

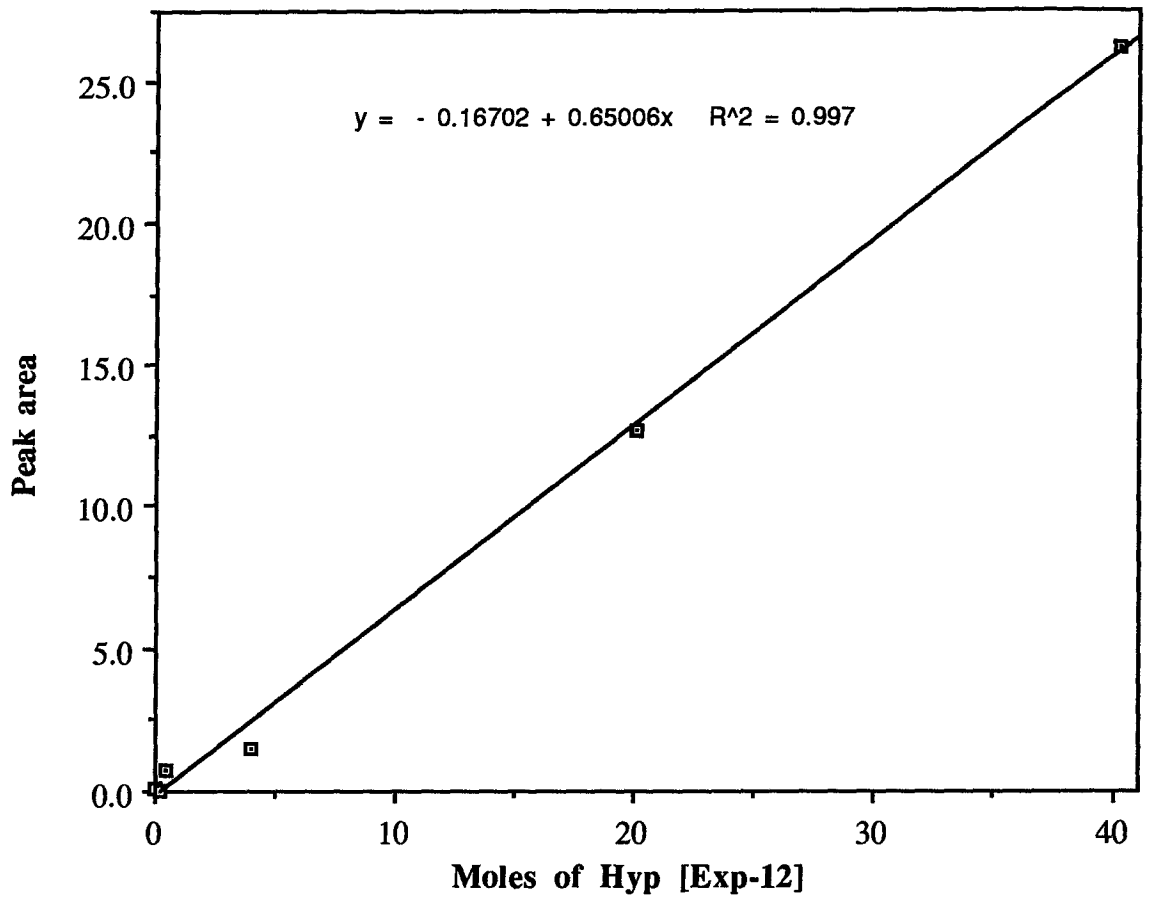

Figure 2.7: Standard plot for low concentrations of hydroxyproline.

2.5 INFLAMED AND NONINFLAMED PERIODONTAL SITES STUDY

2.5.1 Experimental design

The intent of this study was to confirm that more Hyp is found in inflamed periodontal sites, a result of the spiramycin study, and to determine the source of Hyp. During the spiramycin study a question arose regarding the contribution of Hyp from C1q, a subunit of the first complement component, to the total HPLC measured Hyp content of crevicular fluid. It is known that approximately 50% of the C1q molecule has a helical collagen-like structure containing 4.3% by

weight Hyp, and that C1q is normally present in the serum. Therefore, due to the leakage of plasma proteins through the junctional epithelium, it is likely that this protein can gain entry to the gingival crevicular fluid.

The processing of the crevicular fluid in the spiramycin study included acetate precipitation followed by subsequent centrifugation to reduce the contribution derived from C1q. Acetate precipitation has been reported to remove intact C1q molecules from crevicular fluid (Svanberg 1987a). Thus, a study was performed to ascertain the efficiency of C1q removal from crevicular fluid by precipitating the C1q with acetate buffer. A further study was also planned to investigate the efficiency of C1q removal by acetate in a crevicular fluid model containing type I collagen and C1q, using ELISA with antibodies to collagen and C1q. Therefore, it was necessary to employ a crevicular fluid model specifically to determine the effects on type I collagen and C1q.

Potential subjects were chosen for the study on the basis of criteria cited under section 2.4.1. In addition, subjects taking systemic medications that would influence gingival health such as anti-inflammatory drugs, including aspirin, within 4 weeks of the study were excluded.

After clinically examining each patient without probing, gingival crevicular fluid was collected with Periopaper from several periodontal sites that clinically appeared inflamed or noninflamed. Each site was isolated with cotton rolls, gently air dried and supragingival plaque was removed. Periopaper was placed at the orifice of the gingival crevice for 30 seconds. GCF volume was assessed using a Periotron 6000, and GCF samples were placed in 1.5 ml Eppendorf tubes containing 50 µl of 0.05 M sodium acetate and immediately placed in an ice bath, before being transferred to storage at -70°C until analysis. Then pocket depth and bleeding on probing measurements of all sites were performed using a Marquis periodontal probe.

As required the GCF samples that were stored at -70°C were thawed at room temperature before being centrifuged at 15,600 g for 30 min. to remove the precipitated C1q (Svanberg 1987a). The resulting supernatants were allocated for analyses as follows. A 10 µl aliquot was used for HPLC analysis for total Hyp present in each sample; while 25 µl of the original sample was utilized for ELISA experiments to determine the levels of type I collagen and C1q in the GCF. The

remaining GCF sample was divided equally and employed for SDS/PAGE gels and western blot analyses to ascertain collagen and C1q content at inflamed and noninflamed sites.

2.5.2 C1q antibodies

Analyses for C1q were performed using antibodies to it. Two hybridoma cell lines that produce monoclonal antibodies directed against C1q were graciously donated by Dr. Linda Curtiss (Research Instiute, Scripps Clinic, La Jolla, CA). These two hybridoma cell lines, 1H11 and 2A10, were shown to have the greatest binding specificity to the head and stalk regions of C1q respectively (Kilchher et al. 1985). The head and the stalk regions represented the globular and collagen-like helical tail regions of the C1q molecule.

Both the 1H11 and 2A10 hybridoma vials supplied 5 x 10^{-6} cells which were processed in the following manner. The frozen cells were quickly thawed by immersing the vials in warm water. The cells were transferred to a 10 ml sterile polyethylene tube containing 5 ml of DMEM, and centrifuged for 4 min. at 1500 g at room temperature to remove the medium. Each pellet was then reconstituted in 5 ml of HT medium and transferred to a 25 cm^2 culture flask and incubated at 37°C. The whole procedure was completed within 12 minutes.

The HT medium was made from the following ingredients: two ml. of HT stock solution which when reconstituted in 10 ml yielded 5 x 10^{-3} M hypoxanthine and 8 x 10^{-4} M thymidine, ten ml. of fetal calf serum, 0.5 ml. of Hepes stock solution, one ml. of 100 mM cis oxaloacetate stock solution, five ml. of mixed supplement (containing 1 ml Pen/Strep; 1 ml Na pyruvate (0.1M); 1 ml NEAA solution (0.01M); 0.1 ml vitamin C (5 mg/ml) and 2.0 ml L-glutamine (0.2M)), and 81.5 ml of the DMEM made up the total volume to 100 ml.

The cells were observed after two days and each day thereafter for changes in colour of the media, cell number and vitality. After one week the 1H11 cells reached a count of 1 x 10^6 cells/ml.. These cells were split equally into two culture flasks and supplied with an equal amount of HT medium. As the 2A10 cells showed no evidence of proliferation, they were reincubated at

37°C. After two additional days the 1H11 cells were diluted to a ratio of 1:3 and 1:2 of cells and HT medium, respectively. However, there was still no evidence of proliferation of the 2A10 cells.

Over the next few weeks the 1H11 cell line continued to grow in culture and the cells were expanded and stored in liquid nitrogen. The 2A10 cells did not proliferate and according to trypan blue vital staining (0.2% w/v) examination under the light microscope they were non-viable.

Two additional vials (Q58 and 2A) of 2A10 hybridoma cells were donated by Dr. Curtiss's laboratory and again processed in the same manner as described above. One day after incubation at 37 °C, the cells from each culture flask were examined. The Q58 cells appeared to be slightly more numerous and healthier than the 2A cells. However, after 5 days, the viable cell counts for the Q58 and 2A cells were 6×10^3 and zero, respectively. Continued careful monitoring and cell counting every second day indicated that the Q58 cell cultures also became non-viable after an additional two weeks of incubation.

Since the 1H11 cells were predictably proliferating and several vials had been stored in liquid nitrogen, it was decided not to further pursue antibody production from the 2A10 cell line. 1H11 antibody was known to be directed against the globular head of the C1q molecule. It was expected that this antibody would have less cross reactivity than the 2A10 antibody to collagen, because the latter binds to the collagen-like helical domain of C1q.

In order to increase the amount of C1q antibody (MAB-C1q) produced from 1H11 cells, mice were used to produce ascites fluid containing MAB-C1q. Five 9 week old Balb/c mice were subjected for 5 seconds to a CO_2 environment to make them drowsy before being primed with 0.5 ml of pristane i.p. One week later the mice received, in the same manner, a booster of an additional 0.5 ml of pristane. Three days later each mouse was injected with 0.5 ml i.p. of (5×10^6) 1H11 cells in HT medium. After 11 days two mice developed ascites tumors. Ascites fluid was harvested by puncturing the abdomen of each mouse with a 20 gauge needle and the fluid was collected drop-wise in a 10 ml polyethylene tube. Table 2.2 depicts the sequence of events.

Vari-able	Prstne Inj - 1	Prstne Inj-2	1H11 cell inj	Ascts Col.	Ascts Col.	Ascts Col.	Ascts Col.	Ascts Col.	Ascts Col.	Ascts Col.
Day	-11	-3	0	11	12	13	14	16	18	21
Vol-1	0.5	0.5	0.5	.75	5.0	2.5	2.5	7.0	3.0	2.5*
Vol-2	0.5	0.5	0.5	2.75	0.5**					

Table 2.2: Sequence of events for developing ascites fluid in Balb/c mice. 'Prstne' = pristane; 'Ascts' = ascites fluid; 'Inj' = injection; '*' = mouse was sacrificed; '**' = mouse expired

The specificity of monoclonal antibody to C1q (MAB-C1q) was evaluated using common tissue macromolecules and molecules of similar structure. These included C1q, collagen types I, III, and IV, fibronectin (FN), laminin, and albumin. This MAB-C1q was found highly sensitive and specific for C1q, and exhibited virtually no cross reactivity to the above mentioned components. This was evaluated using ELISA by matching optical densities of serially diluted C1q with other serially diluted substrates, and then determining their relative concentrations for a given level of C1q.

A polyclonal antibody, rabbit anti-human C1q, was obtained from Calbiochem. Specificity testing of this product was performed in the same manner and to the same components as the MAB-C1q. The results are displayed in Table 2.3. Even though both of these antibodies exhibited excellent specificity and high responsiveness, the monoclonal antibody was chosen for further analyses since it provided the best specificity to C1q.

Specificity	Monoclonal AB mouse anti-human C1q	Polyclonal AB rabbit anti-human C1q
C1q	100	100
human collagen type I	< 0.5	< 0.5
human collagen type III	< 0.5	< 0.5
human collagen type IV	< 0.5	< 0.5
human fibronectin	< 0.5	< 1
mouse laminin	< 0.5	< 0.5
human albumin	< 0.5	< 0.5

Table 2.3: Percent specificities of monoclonal and polyclonal C1q antibodies. Evaluated by matching optical densities of C1q with other substrates, and then determining their relative concentrations for a given level of C1q

2.5.3 Type I collagen antibodies

In order to perform type I collagen analyses, it was necessary to obtain appropriate antibodies to it. A considerable effort was spent in locating, obtaining, and testing suitable antibodies to type I collagen. The few commercial sources that offered the product had backorders, which actually translated into a reality that they were having problems with their hybridoma cells lines. Limited quantities were obtained from Seattle (Nayaranan, S., Seattle, WA), and preparations of unacceptable purity were available from another laboratory (Sodek 1990).

Finally a source of polyclonal rabbit anti-human type I antibody was obtained from Chemicon International. This antibody held great promise as suggested by the provided specificity sheet and the list of recent publications that had used this product. This antibody was tested using ELISA for specificity and cross reactivity with common tissue macromolecules and molecules of similar structure; included were types I, III, and IV collagen, FN, laminin and C1q. Comparisons of Chemicon's quoted specificities and specificities determined by our testing are displayed in Table 2.4.

Results of using this antibody to human type I collagen were not encouraging. Its sensitivity was poor since the alkaline phosphatase reaction took 24 hours to develop. In addition, the specificities to type I collagen were not acceptable as indicated by the obtained extensive and intense cross reactions. The reactivities demonstrated that type III collagen, type IV collagen, and C1q had much better reactivity to this antibody than the intended type I collagen, indicating a heteroclitic specificity. These shockingly unfavourable specificities were confirmed by repeated analyses. The results obtained in these inital experiments were subsequently confirmed by in-house testing of Chemicon's rabbit anti-human type I collagen antibody.

Substrate	Chemicon's % specificity	ELISA measured % specificity
human collagen type I	100	100
human collagen type II	0.4	NT
human collagen type III	0.8	120
human collagen type IV	0.4	195
human collagen type V	0.5	< 5
human fibronection	0.4	< 5
mouse laminin	0.4	NT
bovine collagen type I	0.7	NT
rat collagen type I	0.4	NT
chicken collagen type I	0.4	NT
C1q	NT	400

Table 2.4: Percent specificities to Chemicon's polyclonal rabbit anti-human type I collagen. Evaluated by matching optical densities of type I collagen with other substrates, and then determining their relative concentrations for a given level of type I collagen. 'NT'= not tested.

Selective adsorption experiments using mixed human sera were performed to try and reduce the cross reactivities. After sera adsorption of the type I collagen antibody ELISA evaluations were performed using C1q and types I and IV collagens. Unfortunately, it resulted in a marked decrease in the overall sensitivity of the antibody with relatively no change in relative specificity to these substrates.

Subsequently, another antibody to human type I collagen became available from Chemicon. This preparation was classified as a monoclonal antibody to human type I collagen raised in rabbits. Our ELISA evaluations demonstrated that this product also had significant cross reactivities, particularly to C1q, and types III and IV collagens.

Two additonal sources of antibody to type I collagen were evaluated. The first was a limited amount of rat anti-human type I collagen antibody graciously provided by Dr. S. Narayanan (University of Washington, Seattle, WA). The second was an affinity purified goat anti-human type I collagen antibody obtained from Southern Biotechnologies Associates Inc. (Birmingham, AL). Specificity testing of both of these antibodies demonstrated the appropriate specificity to collagen with no measurable cross reactivity to C1q. Finally, for reasons of specificity and availability, the affinity purified goat anti-human type I collagen antibody from Southern Biotechnologies was chosen for use in ELISA and western blotting experiments. Table 2.5 compares the specificities of all of the above mentioned type I collagen antibodies that were evaluated.

Substrate	Chemicon polyclonal	Chemicon monoclonal	Narayanan polyclonal	Southern Biotech. polyclonal
Collagen type I	100	100	100	100
Collagen type III	120	180	28	38
Collagen type IV	195	65	13	15
Fibronectin	< 5	NT	NT	NMR
Laminin	< 5	NT	NT	NMR
C1q	400	190	NMR	NMR

Table 2.5: Sources of antibody to type I collagen showing percentage specificity with related proteins. Evaluated by matching optical densities of type I collagen with other substrates, and then determining their relative concentrations for a given level of type I collagen. 'NMR'= no measurable response; 'NT'= not tested.

2.5.4 Crevicular fluid model

Because it was desirable to ascertain the relative contributions of type I collagen and C1q in GCF, crevicular fluid models were used to evaluate how these components are affected by our analyses and how they can be affected in the periodontal environment. To test the effectiveness of C1q precipitation, two experiments were performed. The first evaluated C1q influence on total Hyp content in GCF, after processing it in different buffer systems and analysis by HPLC. The second evaluated an acetate mixture of type I collagen and C1q after centrifugation, by ELISA using antibodies to these components. Also, the effect of proteolytic enzymes from pathogenic bacteria on the recovery of type I collagen was evaluated using ELISA.

The crevicular fluid collected on filter paper strips in the Spiramycin study was eluted in 5 mM Na_2HPO_4 and 0.5 N acetic acid which solubilizes neutral salt-soluble and acid-soluble compartments of collagen. The design of the experiment was to ascertain the efficiency of removal of C1q from the system by precipitating the C1q with acetate buffer.

Sample preparation and procedures for testing C1q removal in sample buffers are outlined in Figure 2.8. Crevicular fluid samples were collected on filter paper strips and the volume was determined using a Periotron 6000. Fluid was extracted twice from the filter paper strips using two 100 µl aliquots of 0.005 M phosphate buffer. The two eluents were combined and divided into phosphate buffer and acetate buffer groups. In each of these groups the eluents were further sub-divided: to one half human C1q was added at either one or five times the serum concentration (70 or 350 µg/ml); and to the other half no C1q was added. To each portion 100 µl of the appropriate buffer (either 0.005 M phosphate buffer or 0.5 N acetic acid) was added. The acetate buffer systems were centrifuged at 15,600 g for 30 minutes at 4°C to remove precipitated C1q. Then the eluents from both the phosphate and acetate systems were processed for HPLC Hyp analyses (Coil and Tonzetich 1988).

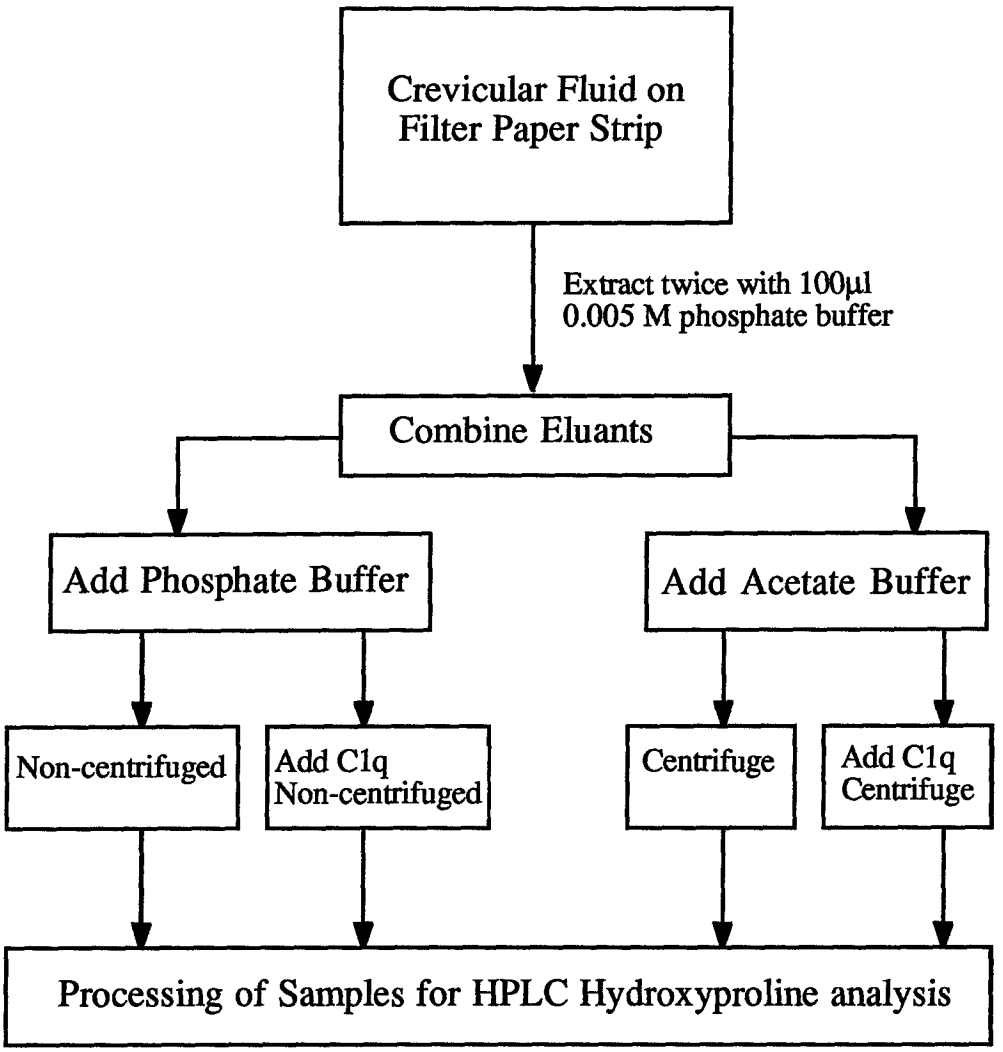

Figure 2.8: Flow diagram of C1q precipitation experiment

C1q removal in a sodium acetate buffer system from a crevicular fluid model was verified by ELISA. In the ELISA system, elimination of C1q from crevicular fluid was assayed using monoclonal antibodies to C1q (MAB-Cq). For this test one µl of 200 µg/ml human C1q was added to 49 µl of 0.05 M sodium acetate in a 1 ml Eppendorf tube. After centrifugation at 15,600g for 30 min at 4°C, 25 µl of the supernatent was transferred to an ELISA plate well. The pellet was resuspended in 25 µl of PBS and dispensed into an ELISA plate well. A 25 µl volume of

uncentrifuged sample was taken from a solution consisting of 1 µl of 200 µg C1q/ml in 49 µl PBS and added to an ELISA plate well. Sample volumes in each well were brought with PBS to a final volume of 50 µl. Incorporation of human C1q standards and ELISA analyses are described later in section 2.5.7.

Identical evaluations for type I collagen in sodium acetate buffer were also performed on 1 µl volumes of 1 mg/ml of human type I collagen standard.

To simulate an enzymatic activity in a periodontal environment 2 proteolytic enzyme sources were each incubated with type I collagen, and percent recovery of type I collagen was determined using ELISA. Bacterial collagenase (BC) and *P. gingivalis* bleb preparations (blebs) were individually combined with type I collagen . For this experiment two µl of BC, 2 µl of 1mg/ml type I collagen, and 2 µl PBS containing 50 mM calcium were added to an ELISA plate well. Similary 5 µl of blebs, 2 µl of type I collagen, and 3 µl of PBS containg 50 mM calcium were added to an ELISA plate well. After 1 and 2 hours of incubation at 37°C analyses for type I collagen were performed according to the ELISA analyses described in section 2.5.7.

2.5.5 Sample collection and measurement

Patients who satisfied the inclusion criteria were examined as potential patients for the study. Each patient received a thorough clinical examination of soft tissues, with particular attention focused on the gingiva. Periodontal sites that visually appeared to be inflamed, as indicated by lack of gingival stippling, papillary edema, erythematous papillary or marginal gingival were catalogued along with several periodontal sites that visually appeared clinically healthy.

All recorded sites were isolated and dried prior to sampling of crevicular fluid. Then a periotron filter paper strip was placed at the entrance to the crevice and fluid was collected for 30 seconds. After GCF volume was determined using a Periotron 6000, samples were placed in individual 1.5 ml Eppendorf tubes containing 50 µl of 0.05 M sodium acetate and maintained in an ice bath while being transferred to storage at -70°C.

All recorded sites were then probed using a Marquis periodontal probe to determine the pocket depth and evaluate bleeding on probing response. Pocket depths were measured to the nearest millimeter in the usual accepted manner. A positive bleeding on probing response was recorded for those sites that bled within 10 seconds of probing. Sites which bled immediately upon probing were also noted. A negative bleeding on probing response was assigned to those sites that did not show evidence of bleeding within 30 seconds after probing.

Patients were selected for the study if they exhibited at least one inflamed and one noninflamed periodontal site. In addition, sites were chosen that were not on the same tooth or that shared a common interproximal space. A total of fifty five subjects screened from a pool of patients who met all the above criteria were chosen for the study.

2.5.6 HPLC analysis for Hyp

HPLC was used to determine total Hyp content of inflamed and noninflamed GCF samples. Individual crevicular fluid samples were removed from -70°C storage, thawed and centrifuged at 15,600 g for 30 minutes. A 10 µl aliquot of the supernatant was pipetted from each Eppendorf tube and transferred to an individual 6 x 50 mm pyrex culture tube. Processing of samples for HPLC analyses was carried out using the Pico-tag Workstation in the same manner previously described in section 2.4.3.

Important points of emphasis for the handling samples include the following. The samples were hydrolyzed at 150 °C for one hour. After derivatization the samples were dried thoroughly to 65 millitorr, to remove all traces of PITC, which usually took 4 to 5 hours. This is an important step as incomplete drying would adversely affect the recovery of derivatized products.

All analyses were performed on the same HPLC assembly described in section 2.4.3, with the following changes in apparatus; an Automated Gradient Controller and a Model 740 data module (Waters Assoc.) were incorporated into the assembly unit and separation of the derivatized amino acids was effected using a Pico-tag C_{18} column.

Crevicular fluid samples from individual inflamed and noninflamed sites in 54 patients were analyzed. These samples were grouped together so that the inflamed and noninflamed samples collected from the same patient were included together in the same reaction vial. Amino acid standards included in each HPLC reaction vial yielded the same profiles, indicating excellent between-run precision for the analyses.

2.5.7 ELISA analyses

ELISA was used to test for the presence of type I collagen and C1q in GCF and crevicular fluid model. The primary antibodies employed in the study were affinity purified goat anti-human type I collagen antibody from Southern Biotechnology, and mouse monoclonal human C1q antibody from Dr. Curtiss. It was also necessary to use rabbit anti-human C1q (Chemicon). Secondary antibodies employed were alkaline phosphatase conjugated goat anti-mouse IgG (BRL) and alkaline phosphatase conjugated rabbit anti-goat IgG (Sigma).

For the type I collagen analyses, 55 paired samples of inflamed and noninflamed GCF were analyzed in the following manner. GCF samples stored at -70°C were thawed and centrifuged at 15,600 g for 30 min. Duplicate four µl aliquots of each sample were taken and individually added to 46 µl of phosphate-buffered-saline (PBS) in an ELISA plate well. Inflamed and noninflamed samples from the same patient were included on the same plate. Triplicate standards of human type I collagen were serially diluted from an initial concentration of 0.1 µg/50 µl. A total of four ELISA plates (Falcon) were used for these analyses.

Ninety-six-well ELISA plates were coated overnight at 4°C with individual 4 µl aliquots of the GCF samples in 46 µl of PBS and with 50 µl of serially diluted type I collagen as a standard, leaving lane 1 as a blank. After washing twice with PBS the nonsaturated binding sites of the plates were blocked with 2% casein/PBS during 2 hours of incubation at 37°C followed by two sequential washes with PBS. Fifty microliters of 1% casein/0.05% Tween 20/PBS buffer containing goat anti-human type I collagen antibody (1:5000 dilution) was added to all the wells and incubated at 37°C for one hour. After two washes with PBS/0.1% Tween 20, the plates were

incubated with 50 µl of alkaline phosphatase-conjugated rabbit anti-goat-IgG antibody (Calbiochem) in 1% casein/0.05% Tween/PBS (1:1000 dilution) for one hour at 37°C. The plates were then washed four times with PBS/0.1% Tween 20 and developed with 50µl/well (0.5 mg/ml) of p-nitrophenyl phosphate (Sigma) in diethanolamine buffer. The optical densities were measured after 6 hours at room temperature, at 405 nm using a Titertek ELISA recorder.

Analyses for C1q were performed in a similar manner on 55 paired samples of inflamed and noninflamed GCF. Three µl aliquots of each sample were taken in triplicate and individually added to 47 µl of PBS in an ELISA plate well. Inflamed and noninflamed samples from the same patient were included on the same plate. Triplicate standards of human C1q were serially diluted from an initial concentration of 0.1µg/50 µl. A total of six ELISA plates were used for these analyses.

Ninety-six-well ELISA plates were coated for 1.5 hours at 37°C with individual 3 µl aliquots of the GCF samples in 47 µl of PBS and with 50 µl of serially diluted human C1q as a standard, leaving lane 1 as a blank. The procedure was the same as described above for type I collagen analysis, except the secondary antibody used was alkaline phosphatase conjugated rabbit anti-goat IgG antibody (Sigma) in 50 µl of 1% casein/0.05% Tween/PBS (1:1000 dilution). The optical densities were measured after standing for 1 hour at room temperature.

In the same manner, 24 paired samples of inflamed and noninflamed GCF, already analyzed for type I collagen content, were again analyzed for C1q content using the polyclonal antibody, rabbit anti-human C1q. Duplicate three µl aliquots of each GCF sample were subjected to ELISA analysis as outlined above.

2.5.8 SDS/PAGE gel electrophoresis

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS/PAGE) was performed under non-reducing conditions to separate proteins of each crevicular fluid sample. For analysis, a 6 µl volume of each diluted GCF sample was combined with 4 µl of SDS/PAGE sample buffer containing distilled water (4 ml), 0.5 M Tris-HCl, pH 6.8 (1.0 ml), glycerol (0.8 ml), 10% (w/v)

SDS (1.6 ml), and 0.05% (w/v) bromophenol blue (0.2 ml). Samples were denatrued by placement in boiling water for 5 minutes.

Polyacrylamide gel electrophoresis was performed using mini-gels with stacking gel and running gel acrylamide concentrations of 4% and 7.5%, respectively. The gel dimensions were 8 cm (L) x 4 cm (H) x 0.75 mm (D) with 15 sample wells each of 3 mm width. The GCF samples were grouped together according to patient, having an inflamed site adjacent to a noninflamed site. A solution of prestained high range protein molecular weight standards (BRL, Burlington, Ontario, Canada), was added to a separate lane. Ten µl volumes of samples and standards were loaded into the wells using a micro syringe and electrophoresed at a constant voltage of 120V. Once the bromophenol blue marker reached the bottom of the gel, the electrophoresis was terminated and the gel was removed and placed in 40% methanol solution prior to silver staining.

Silver staining was performed using modified procedures developed by Oakley, Switzer and co-workers (Switzer et al. 1979; Oakley et al. 1980). Each gel was placed in 50 ml of the first fixative (57.5 g TCA; 17.25 g 5-sulfosalicylic acid, dihydrate; in 500 ml dH_2O) and gently shaken for 30 minutes. Following a brief transfer to dH_2O, the gel was exposed to the second fixative (50 ml of 2.5% glutaraldehyde) and shaken for 45 minutes. Following three 10 min. rinses with deionized water (dH2O), the gel was left overnight in 10% ethanol. A solution of diammine was prepared by titrating 2 ml of 19.4% $AgNO_3$ with base solution (10 ml 0.35% NaOH + 0.67 ml conc. NH_3OH) until the red precipitate disappeared. To this diammine solution 1.5 ml of additional base solution, 5 ml ethanol and 32 ml dH_2O were added. The gel was placed in a dish containing this solution and orbitally shaken for 15 minutes. The gel was then thoroughly rinsed with three aliquots of fresh dH_2O shaken for 20 min each, then left in 200 ml of dH_2O for two hours. Reducer, consisting of 0.5 ml 1% citric acid, 0.05 ml formaldehyde, and 10 ml ethanol brought to a final volume of 100 ml with dH_2O, was used to develop the stained gels. Once the desired stain intensity was reached, which usually took between 5 and 10 minutes, the gel was soaked in dH_2O for at least another day before drying. Over-stained gels or background was lightened by soaking the gel for 15 minutes in freshly prepared 5% sodium thiosulfate (w/v) in dH_2O.

2.5.9 Western blotting

Following SDS/PAGE, the gel was equilibrated in 50 ml of the blotting transfer buffer (25 mM Tris, 192 mM glycine, 20% v/v methanol). After equilibration was achieved by gently shaking the gel for 10 minutes in the transfer buffer, the solution was discarded and replaced with a fresh buffer. This process removed electrophoresis salts and detergents which would otherwise increase the conductivity of the transfer buffer and generate heat during the electrophoretic transfer. In addition, equilibration also allowed the gel to adjust to its final size, as the gel shrinks in the presence of methanol.

Powderless gloves were worn during gel equilibration and preparation of mini trans-blot materials in order to avoid contamination of the membrane. Four pieces of filter paper were cut to the size of the gel during electrophoresis. The same size of an Immobulin-P transfer membrane (Millipore Corp., Bedford, MA, U.S.A.), was cut and labelled in the top and bottom corners to assist with future orientation of the membrane. The membrane was wetted in an organic solvent by slowly sliding it at a 45° angle into methanol and allowing it to soak for 30 seconds. The membrane was then transferred to a dish containing transfer buffer and allowed to soak for at least 15 minutes prior to blotting. The filter papers along with the fiber pads were slid into transfer buffer at a 45° angle to avoid entrapment of air bubbles, and left to soak for a minimun of five minutes before the assembly of the mini trans-blot apparatus.

Prior to assembly of the transfer sandwich, the Bio-Ice cooling unit and 500 ml of transfer buffer precooled at 4° C, were placed in the buffer chamber containing a magnetic stirrer. The gel holder cassette was assembled sequentially to form a sandwich consisting of a fiber pad, two filter papers, gel, membrane, two filter papers and the remaining fiber pad. The assembly was continually immersed in transfer buffer during the assembly of all components. At the stage when the membrane was placed over the gel, a small glass test tube was rolled over the membrane to ensure an intimate contact between the gel and the membrane and also to remove any air bubbles trapped between the gel and the membrane. Following this step the remaining saturated two filter papers and fiber pad were placed over the membrane and the gel holder cassette was closed.

The assembly was placed in the buffer chamber so that that gel faced the cathode electrode and additional transfer buffer was added to within 1 cm of the upper edge of the chamber. Transfers were effected in 6 hours using an initial power setting of 35 V and 80 mA, which reached a final current level of 120-140 mA.

Immediately after the transfer, the membrane was ready for probing analysis for either C1q or type I collagen. Unless stated otherwise, all procedures were performed at ambient temperature. Using gloves and forceps, the membrane was placed in 50 µl of 2% casein/PBS as a blocking reagent, and shaken for a minimum of 2 hours or left overnight at 4° C. The membrane was transferred to another dish containing the first antibody solution. Either rabbit anti-human C1q (Calbiochem) or goat anti-human type I collagen (Southern Biotechnologies) were used in 50 milliliters of 1% casein/0.05 % Tween 20/PBS (1:1000 dilution), and gently shaken for 3 to 4 hours. The membrane received a brief rinse and two 50 ml. washes of 15 minutes each using PBS/0.1% Tween 20, employing an orbital shaker.

The membrane was then immersed in the second antibody solution which contained the corresponding species specific anti IgG - alkaline phosphatase conjugate. Anti rabbit IgG - AP conjugate or anti goat IgG - AP conjugate were used separately in 1:1000 or 1:2000 dilutions respectively, in fifty milliliters of 1%casein/0.05% Tween 20/PBS, which was agitated for 3 or 4 hours using an orbital shaker. Each membrane received a brief rinse and three 100 ml washes for 10 minutes each using PBS/0.1% Tween 20 under gentle shaking conditions.

The blot was developed using the bromochloroindolyl phosphate/ nitro blue tetrazolium (BCIP/NBT) substrate from BRL (Bethesda Research Laboraties, MD, U.S.A.), which generates an intense dark purple precipitate at the site of enzyme binding. During the final rinses of the membrane a fresh BCIP/NBT developing solution was prepared. One hundred microliters of NBT stock (0.1 g NBT in 2 ml of 70% dimethylformamide) was mixed in 25 ml of alkaline phosphatase buffer (100 mM NaCl, 5 mM $MgCl_2$,100 mM Tris, pH 9.5). Fifty microliters of BCIP stock (0.1 g BCIP in 2 ml of 100% dimethylformamide) were added to complete the developing reagent. The washed immunoblot was placed in a 14 cm petri dish containing the BCIP/NBT reagent. The

blot was gently shaken until the bands appeared. Some bands appeared within a few minutes, while the weaker bands required more than 15 minutes to appear. After the desired intensity of the bands were reached and before the background colour increased, the membrane was immersed in 20 mM EDTA which chelated Mg^{2+} ions and thereby stopped the precipitation reaction.

2.5.10 Data analysis

Comparisons of total Hyp levels generated by HPLC analysis were performed on inflamed and noninflamed sites in the same patient. They were evaluated using paired *t*-tests. ELISA determinations of type I collagen and C1q content were made using corresponding levels of standards plotted against the measured optical densities. Standard curves were generated for each ELISA plate analyzed. ELISA results for levels of C1q and type I collagen found in GCF aliquots, were normalized to the fluid volume of GCF initially collected. The units are reported in moles/µl GCF. Then based upon the amount of Hyp known to be present in C1q and type I collagen, these values were converted into moles Hyp/µl GCF, for each of these components. These in turn were related to the total moles of Hyp/µl GCF, which was determined by HPLC analyses. The values were expressed in % (C1q or type I collagen) Hyp contribution of total Hyp. These values were compared using the student's *t*-test. All statistical tests were performed at the 95% and 99% level of significance.

SDS/PAGE gels and western blots were qualitatively analyzed for banding pattern and presence and position of antibody reactions. Qualitative assessment of any differences between inflamed and noninflamed sites was performed where appropriate.

3. RESULTS

3.1 VOLATILE SULPHUR COMPOUNDS IN HUMAN GINGIVAL CREVICE

3.1.1 Volatile sulphur composition

Qualitative and quantitative differences were found in VSC compositions of mouth and crevicular air samples. Figure 3.1 compares profiles for Tenax-trapped mouth air and Tenax trapped crevicular air from the same patient. These are reproducible profiles exhibiting peaks corresponding to H_2S (Rt: 115-130 sec.), CH_3SH (Rt: 190-205 sec.), $(CH_3)_2S$, (Rt: 270-310 sec.), and $(CH_3S)_2$, (Rt: 390-425 sec.). Whereas both chromatograms exhibit profiles for H_2S, CH_3SH and $(CH_3)_2S$, the crevicular air sample shows significantly more CH_3SH and $(CH_3)_2S$. These profiles demonstrate that the composition of crevicular air can differ from that of mouth air.

The results further indicate that it is possible to collect sufficient amounts of VSC from specific gingival crevice sites for gas chromatographic analyses. Figure 3.2 compares profiles of Tenax-trapped crevicular air collected from two subjects. Whereas both chromatograms exhibit profiles of H_2S, $(CH_3)_2S$ and $(CH_3S)_2$, one chromatogram shows a prominent peak for CH_3SH, its concentration exceeding that of H_2S.

Of the 17 patients studied, three contributed to both test (≥4mm) and control (≤3mm) crevice groups, while the remaining subjects were divided into either group. Results of the gas chromatographic identification of VSC from control and test groups indicate that there are comparative differences in composition of mouth and crevicular air. Tables 3.1a and 3.1b depict the levels of H_2S, CH_3SH, $(CH_3)_2S$, and $(CH_3S)_2$, and the ratios for CH_3SH to H_2S and for all methyl sulphides (CH_3-S's) to H_2S, found in mouth and crevicular air samples respectively. Total sulphur content is also shown for crevicular air.

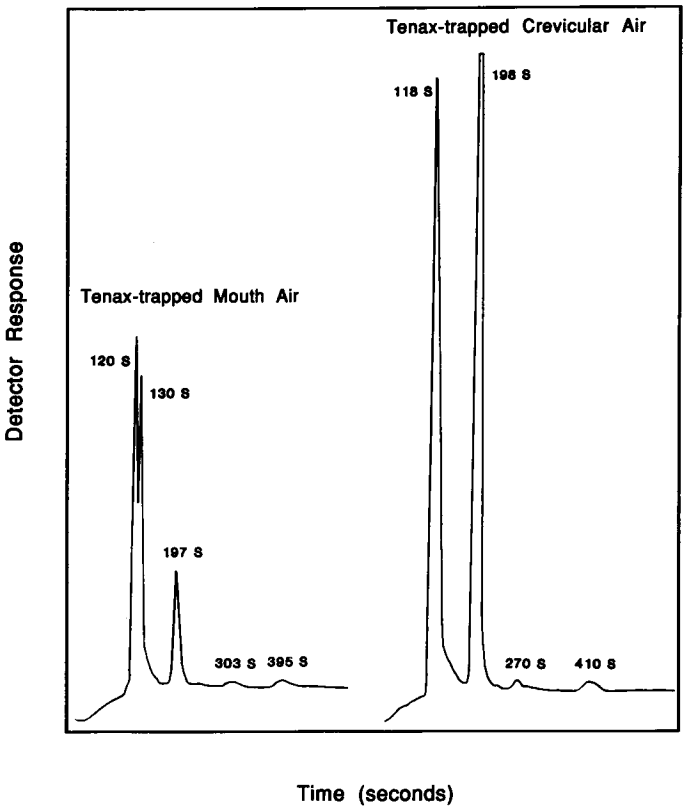

Figure 3.1: Comparative profiles of 10 ml of tenax-trapped mouth and crevicular air from the same subject.

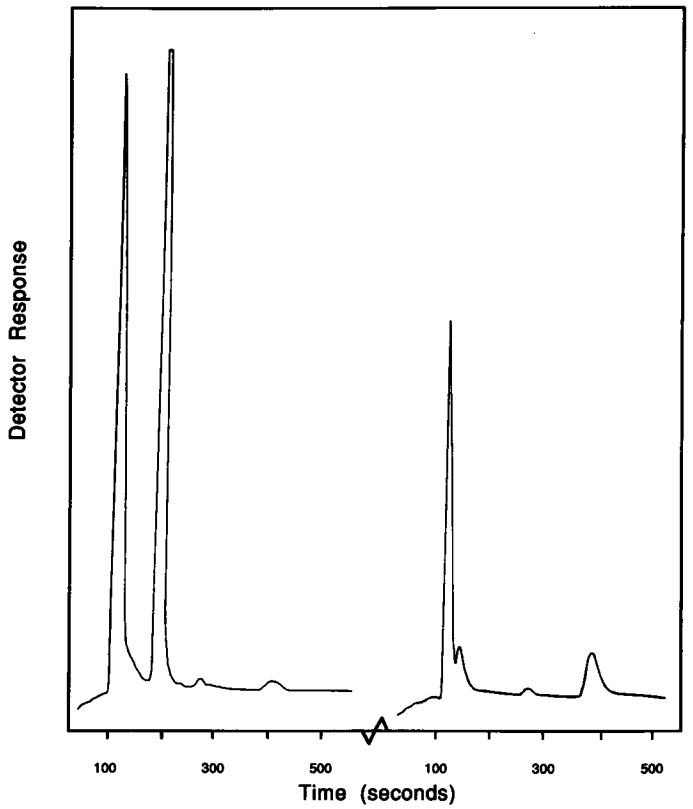

Figure 3.2: Comparative profiles of tenax-trapped crevicular air from two different subjects.

Mouth Air

Pnt	H_2S (ng)	CH_3SH (ng)	$(CH_3)_2S$ (ng)	$(CH_3S)_2$ (ng)	Ratio CH_3SH to H_2S	Ratio CH_3S's to H_2S
1	.99	1.07	.54	.00	1.08	1.63
2	.60	.55	.30	.00	.90	1.40
3	.78	.51	.19	.00	.66	.90
4	.75	.00	.00	.00	.00	.00
5	.48	.12	.09	.00	.24	.44
6	.68	.31	.11	.00	.45	.61
7	1.11	1.28	.58	.00	1.16	1.68
8	2.69	1.03	.61	.00	.38	.61
10	.60	.28	.00	.00	.47	.47
11	.52	.41	.00	.18	.78	1.12
4	.75	.00	.00	.00	.00	.00
12	.29	.00	.12	.00	.00	.40
13	.64	.00	.00	.00	.00	.00
5	.48	.12	.09	.00	.24	.44
3	.77	.51	.19	.00	.66	.90
14	.33	.35	.43	.00	1.05	2.32
15	3.19	.48	.66	.00	.15	.36
16	.48	.26	.00	.00	.55	.55
17	.60	.40	.67	.00	.66	1.79
18	.31	.27	.00	.00	.86	.86

Table 3.1a: Levels of sulphides found in 10 ml of mouth air. Ratios for CH_3SH to H_2S and for all methyl sulphides to H_2S are presented.

Crevicular Air

Pnt	PD* mm	Site	H_2S (ng)	CH_3SH (ng)	$(CH_3)_2S$ (ng)	$(CH_3S)_2$ (ng)	Total Sulphur	Ratio CH_3SH to H2S	Ratio CH_3S's to H2S
1	3	46MB	.33	.25	.41	.00	.99	.78	2.05
2	3	24MB	.42	.27	.35	.00	1.03	.65	1.48
3	3	46MB	.49	.05	.05	.08	.68	.11	.38
4	3	46MB	.44	.07	.00	.00	.51	.17	.17
5	3	36MB	.56	.07	.14	.14	.90	.12	.61
6	6	16MB	.64	.71	.10	.19	1.63	1.11	1.57
7	12	26MB	.74	.27	.64	.00	1.65	.37	1.24
8	4	46DB	.68	.46	.00	.00	1.14	.68	.68
10	5	15MB	.33	.65	.25	.00	1.22	1.97	2.71
11	6	26MB	.50	.15	.00	.24	.89	.31	.79
4	4	36DB	.63	.28	.27	.00	1.17	.44	.87
12	5	36DB	.52	.28	.11	.50	1.41	.55	1.74
13	8	26DB	.62	.23	.25	.27	1.37	.36	1.20
5	5	26MB	.51	.19	.20	.37	1.27	.37	1.49
3	4	36DB	.48	.47	.17	.86	1.98	.98	3.10
14	5	26MB	.00	.00	.00	.00	.00	.00	.00
15	4	12DB	.34	.57	.00	.00	.91	1.67	1.67
16	4	13MB	.38	.53	.00	.00	.91	1.41	1.41
17	4	25MB	.74	.59	.00	.00	1.33	.80	.80
18	4	12DB	.63	.00	.00	.00	.63	.00	.00

Table 3.1b: Levels of sulphides found in 10 ml of crevicular air. Ratios for CH_3SH to H_2S and for all methyl sulphides to H_2S, as well as the total sulphur content are presented. 'MB'= mesiobuccal; 'DB'= distobuccal.

H_2S was the dominant VSC found in both the mouth and crevicular air samples. CH_3SH was present in mouth air of all 5 control subjects and absent in 3 of the 10 test subjects, while in crevicular air it was detected in variable amounts in all test and control subjects. In controls, $(CH_3)_2S$ was detected in 4 out of 5 samples of both mouth and crevicular air. In test subjects, $(CH_3)_2S$ was detected in 6 of 10 mouth air and in 8 of 10 crevicular air samples. It is noteworthy that 3 of the 8 test subjects exhibiting $(CH_3)_2S$ in crevicular air did not exhibit detectable levels in mouth air. The fact that in these subjects the VSC composition of mouth air differed from crevicular air is additional support that the customized collection device created a closed system and that crevicular air is distinct from mouth air in the same patient.

3.1.2 Deep versus shallow and inflamed versus noninflamed sites

The frequency of appearance of VSC components in mouth and crevicular air of both control and test subjects is displayed in Table 3.2. The subjects in control and test groups were further subdivided into depth and inflammation categories. Of the 17 patients examined, three contributed to all four categories, having one periodontal site that was both shallow and noninflamed, while another site was both deep and inflamed. Five and ten subjects contributed VSC values for the control and test depth groups respectively (C: shallow and T: deep). Three of these patients contributed separate sites, while five additional subjects contributed two and three sites, for the control and test inflammation groups respectively (C: noninflamed and T: inflamed). The frequencies of mouth air VSC in the control and test inflammation groups, therefore, contain three contributions of the same value. The frequency of VSC in these four groups is tabulated as a number and percentage of times that each of the four examined sulphides were present.

In both mouth and crevicular air H_2S was the most frequently observed VSC in all four site categories. The occurrence of both CH_3SH and $(CH_3)_2S$ was similar, or slightly greater in crevicular air over mouth air. There appears also to be no great differences in frequency between control and test groups for both of these sulphides in mouth and crevicular air. However, $(CH_3S)_2$ does appear in greater frequency in crevicular air than mouth air. As was the case with

the other methyl sulphides, $(CH_3S)_2$ did not appear to predominate in either the control or test groups.

Table 3.3 portrays the levels of VSC per 10 ml of mouth or crevicular air from control and test sites. In both instances, H_2S was the VSC collected in higher amounts from all subjects regardless of site category. Furthermore, there was no evidence of dominance of H_2S in either of the control or test groups. Although CH_3SH was found to the same proportion in control and test groups for each of the mouth air samples, the levels of this sulphide were significantly different in control and test groups of crevicular air. In both the depth and inflammation categories, crevicular air levels of CH_3SH were higher in the test over the control groups at the 95% level of significance. The same trend held true for $(CH_3S)_2$, although in the crevicular air samples, the differences in the sulphide levels were not statistically significant. The levels of $(CH_3)_2S$ were similar in mouth air for both subcategories in control and test groups. However, in crevicular air, the levels of this sulphide were statistically greater at the 90% level of significance in the inflamed over the noninflamed group. No significant difference was found in the level of this sulphide between deep and shallow gingival crevices.

	Mouth Air				Crevicular Air			
Crevice Sites	H_2S No: {%}	CH_3SH No: {%}	$(CH_3)_2S$ No: {%}	$(CH_3S)_2$ No: {%}	H_2S No: {%}	CH_3SH No: {%}	$(CH_3)_2S$ No: {%}	$(CH_3S)_2$ No: {%}
C: shallow	5 {100}	4 {80}	4 {80}	0 {0}	5 {100}	5 {100}	4 {80}	2 {40}
C: noninfl	5 {100}	4 {80}	3 {60}	0 {0}	4 {80}	4 {80}	2 {40}	2 {40}
T: deep	10{100}	7 {70}	6 {60}	1 {10}	10{100}	10{100}	8 {80}	6 {60}
T: inflamed	6 {100}	5 {83}	4 {67}	0 {0}	6 {100}	6 {100}	3 {50}	2 {33}

Table 3.2: Frequency of VSC in mouth and crevicular air from Control (C) and Test (T) sites.

	Mouth Air				Crevicular Air			
Crevice Sites	H_2S ng {SD}	CH_3SH ng {SD}	$(CH_3)_2S$ ng {SD}	$(CH_3S)_2$ ng {SD}	H_2S ng {SD}	CH_3SH ng {SD}	$(CH_3)_2S$ ng {SD}	$(CH_3S)_2$ ng {SD}
C: shallow	.72{.19}	.45{.42}	.22{.21}	0{0}	.45{.09}	.14{.11}	.19{.18}	.04{.06}
C: noninfl	.53{.22}	.25{.20}	.14{.18}	0{0}	.42{.25}	.04{.04}	.04{.06}	.04{.06}
T: deep	.85{.68}	.39{.44}	.17{.23}	.02{.06}	.56{.12}	.37{.19}	.20{.18}	.24{.28}
T: inflamed	1.1{1.2}	.27{.22}	.19{.28}	0{ 0}	.47{.11}	.41{.17}	.13{.12}	.25{.38}

Table 3.3: Levels of VSC per 10 ml of mouth or crevicular air at Control (C) and Test (T) sites.

Figures 3.3 and 3.4 illustrate sulphide ratios found in crevicular air. Figure 3.3 demonstrates the sulphide ratios of CH_3SH/H_2S and total CH_3-S's/H_2S in control and test groups. A similar trend was found for both the ratios of CH_3SH to H_2S and all methyl sulphides to H_2S. Deeper crevicular sites exhibited higher sulphide to H_2S levels than the shallow sites. In particular, the CH_3SH to H_2S ratio of the test group was significantly greater than the control ($p<.10$). Figure 3.4 shows the sulphide ratio of CH_3SH to H_2S for the control versus test groups and compares the depth and inflammation categories. Comparisons in each category revealed that the ratio of CH_3SH to H_2S was greater in the test than the control groups. In both the deeper and inflamed sites the ratios were significantly larger ($p<0.1$ and $p<.05$) than the shallow and noninflamed sites, respectively.

Quantitation of the total VSC content in crevicular air of control and test subjects is displayed in Figure 3.5. It shows the same trend for the CH_3SH to H_2S ratio; the deeper and inflamed sites show higher total sulphur content than the corresponding shallow and non-inflamed sites. The total sulphur content in deeper sites was significantly different from shallow sites ($p<.05$). Similarly, the total sulphur content at inflamed sites was significantly different than for noninflamed sites ($p<.05$).

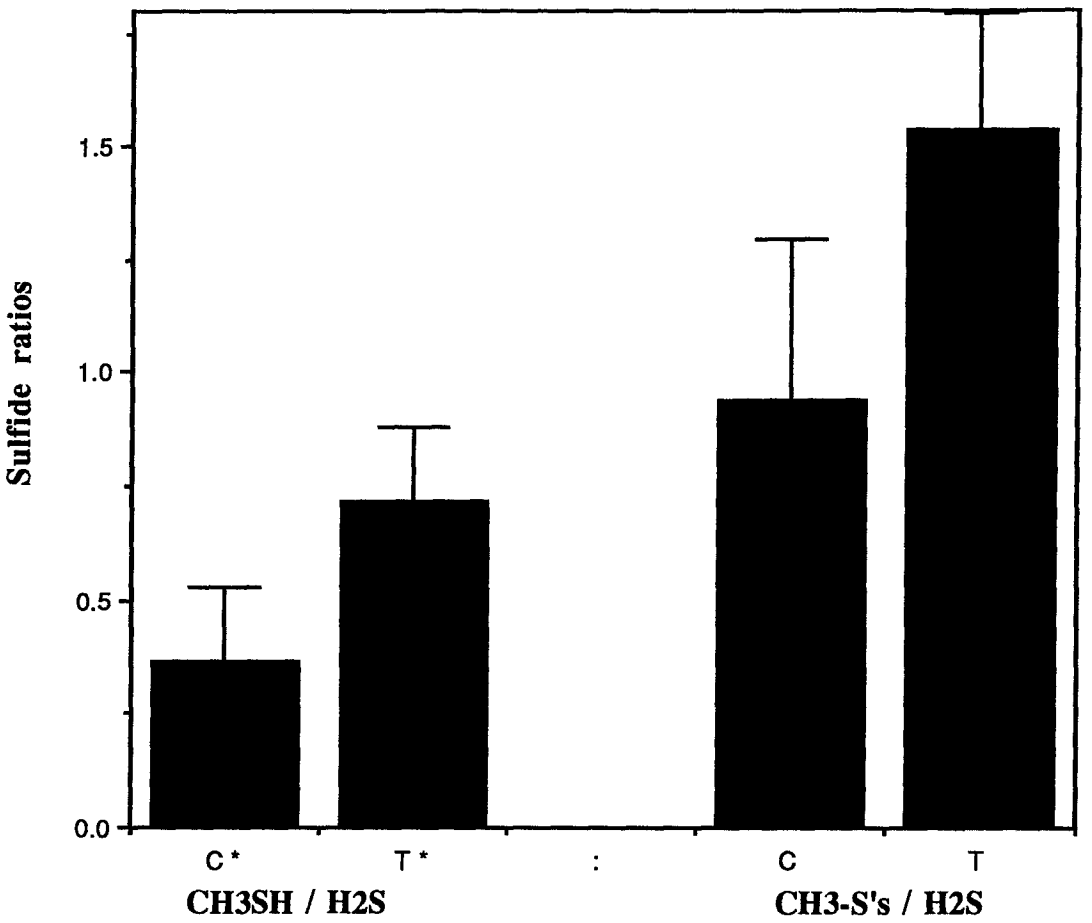

Figure 3.3: Ratios of CH_3SH to H_2S and total methyl sulphides (CH_3-S's) to H_2S from control (C) and test (T) sites of crevicular air. T* is not significantly greater than C* ($p>.05$). Error bars represent the standard error of the mean.

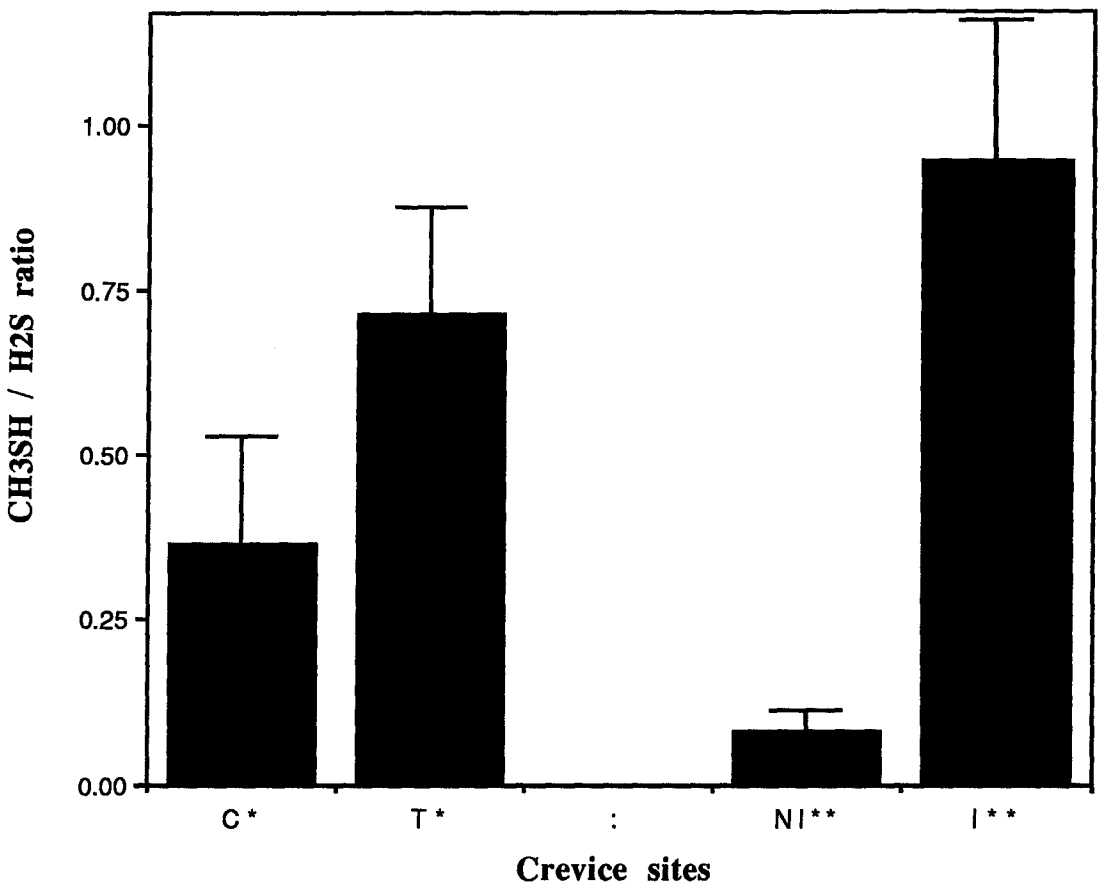

Figure 3.4: Ratios of CH_3SH to H_2S of control (C) and test (T) and noninflamed (NI) versus inflamed (I) crevicular sites. T* is significantly larger than C* ($p<.10$). I** is significantly larger than that NI** ($p<.05$). Error bars represent the standard error of the mean.

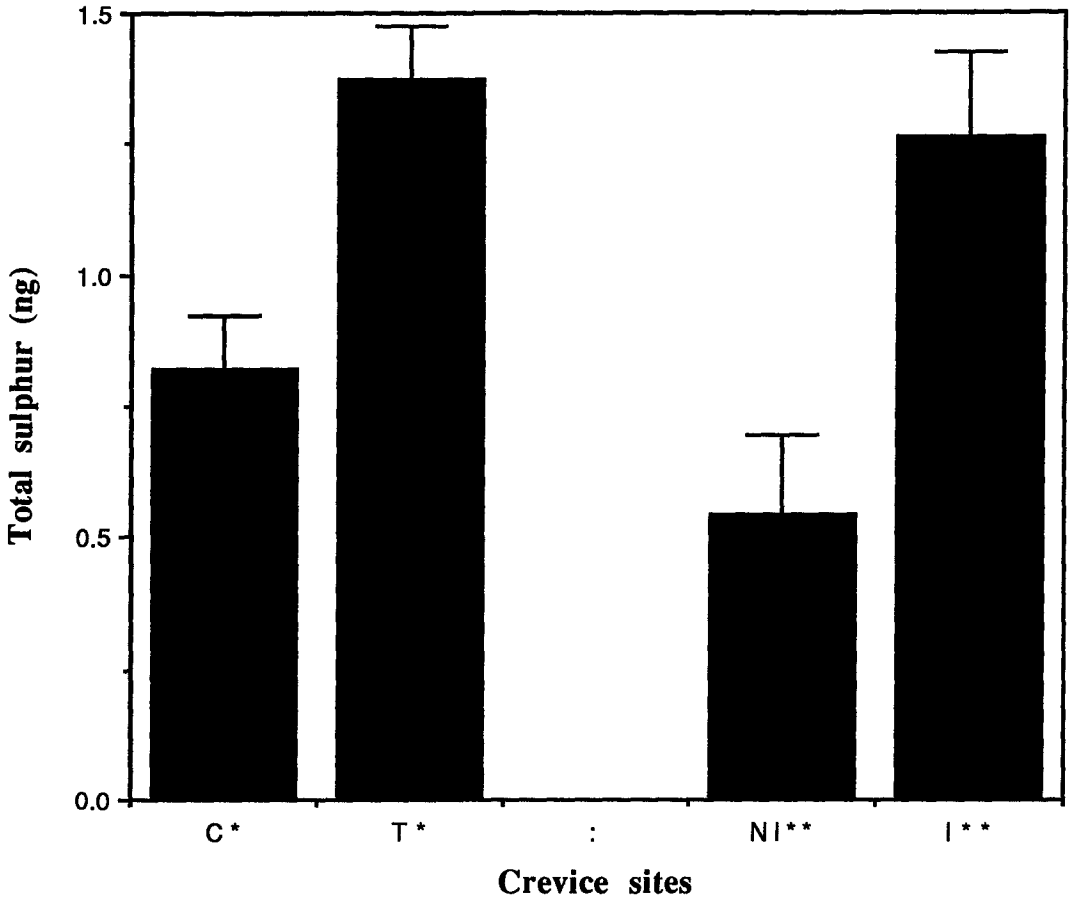

Figure 3.5: Total sulphur content of H_2S, CH_3SH, $(CH_3)_2S$, $(CH_3S)_2$ in crevicular air at control (C) versus test (T); and noninflamed (NI) versus inflamed (I) sites. T* is significantly different than C* ($p<.05$). I** is significantly different than NI** ($p<.05$). Error bars represent the standard error of the mean.

3.2 HYDROXYPROLINE LEVELS IN GINGIVAL CREVICULAR FLUID AND SPIRAMYCIN EFFECT ON PERIODONTAL SITES

3.2.1 Hydroxyproline content in hydrolyzed and unhydrolyzed samples

The results of HPLC analyses indicate that it is possible to demonstrate and quantitate Hyp levels using small volumes of crevicular fluid in the range of 0.2 to 1.0 µl collected on strips of

filter paper. HPLC analyses using PITC precolumn derivatization yielded distinctly different results for hydrolyzed and unhydrolyzed crevicular fluid. The analyses showed that acid hydrolysis yielded significantly more Hyp and other amino acid residues then did the unhydrolyzed samples. Most of the unhydrolyzed crevicular fluid samples exhibited no peak for Hyp and barely detectable presence of other amino acids. The corresponding hydrolyzed samples exhibited Hyp peaks and strong responses for remaining amino acids. Representative HPLC chromatograms of PITC-derivatized unhydrolyzed and hydrolyzed crevicular fluid are shown in Figures 3.6 and 3.7, respectively.

These results indicate that the preponderance of amino acid content of crevicular fluid is present in a peptide form, and a disproportionately lesser amount in a free unbound form. Consequently, the Hyp analyses of crevicular fluid collected during the spiramycin study were performed on the hydrolyzed samples.

3.2.2 Hydroxyproline levels in inflamed and noninflamed sites

All crevicular fluid samples were analyzed by reversed phase HPLC. The amount of Hyp was determined and presented as either moles of Hyp for the entire collected GCF sample or as moles of Hyp per µl of GCF. Hydroxyproline levels in the total GCF sample from inflamed and non-inflamed sites of both the spiramycin and non-drug treatment groups, for all examination periods of the study, are shown in Table 3.4. Parallel values for Hyp levels in moles of Hyp per µl of GCF are shown in Table 3.5. These Hyp levels represent mean values in inflamed or noninflamed categories during each examination point and for each drug treatment group.

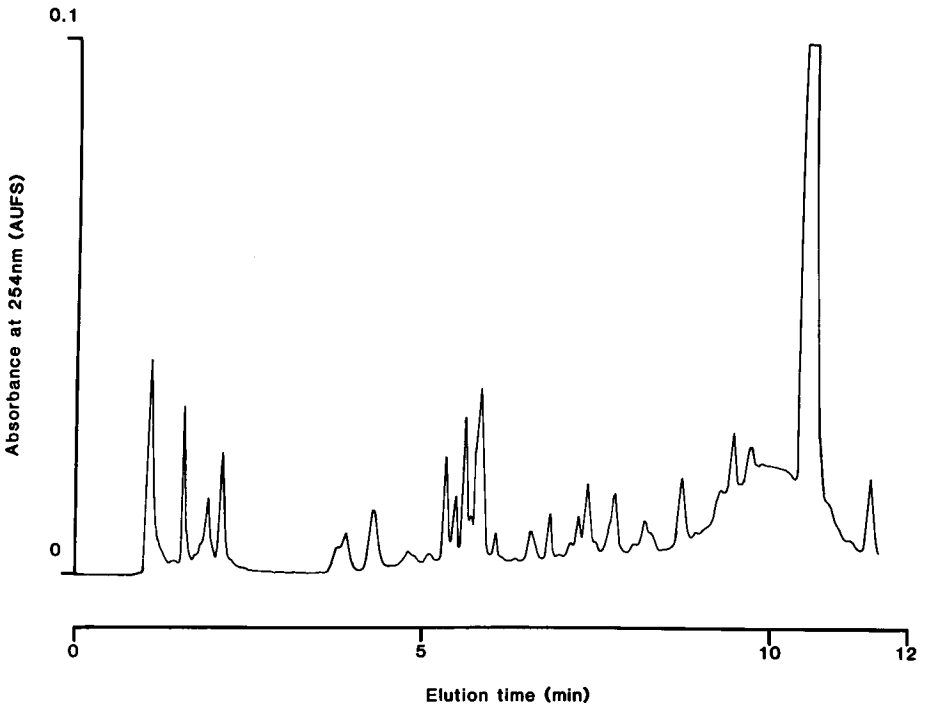

Figure 3.6: HPLC profile of unhydrolyzed PITC-derivatized crevicular fluid sample.

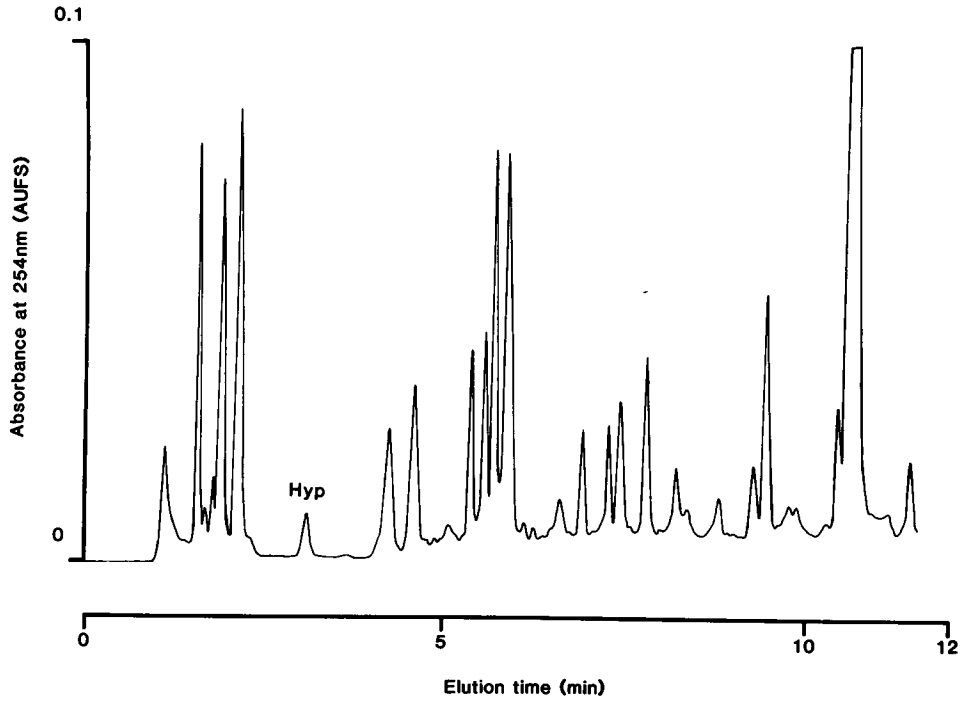

Figure 3.7: HPLC profile of hydrolyzed PITC-derivatized crevicular fluid sample.

Tables 3.4 and 3.5 are graphically presented in Figures 3.8 through 3.11. In Figures 3.8 and 3.9, in which the data is plotted as the total moles of Hyp per sample, the levels of Hyp are higher in the inflamed than in the noninflamed periodontal sites. This trend holds true for all examination points, regardless of the drug treatment group, with one exception. In Figure 3.8 at week two in the spiramycin treatment group, the Hyp levels for the noninflamed group are greater than those for the inflamed group, however this difference is not statistically significant at the 95% level of significance. But, in Figure 3.9 at week 8 in the non-drug group where the Hyp level is much higher for the inflamed group compared to the noninflamed group, this difference is statistically significant at the 95% level of confidence. All other comparisons of Hyp content in inflamed versus non-inflamed sites were not statistically significant ($p>.05$).

Figures 3.10 and 3.11 display Hyp levels per μl of GCF during all examination points for both inflamed or noninflamed sites, for either the spiramycin or non-drug treatment groups, respectively. Unlike the previous two figures, Hyp levels in Figures 3.10 and 3.11 follow no distinct trend at various examination points. In Figure 3.10, Hyp levels in inflamed sites are lower than found in noninflamed sites from weeks 0 to 12, but this trend is reversed at week 24. For the non-drug treatment group in Figure 3.11, the Hyp level in inflamed sites is less during the inital and final exam points, but slightly greater during the remaining exam points, when compared to Hyp levels in noninflamed sites. All comparisons of Hyp content in inflamed versus non-inflamed sites were not statistically significant ($p>.05$).

The average Hyp levels at zero time point for combined drug and non-drug groups are displayed in Table 3.6 and graphically represented in Figure 3.12. Although the inflamed periodontal sites show more total Hyp in the collected GCF sample (MHyp) than in noninflamed sites, an opposite relationship is seen for the Hyp levels calculated on the basis of Hyp per μl of GCF. For these weighted Hyp (WHyp) values, the Hyp level is slightly higher for the noninflamed versus the inflamed site. The difference in Hyp levels between inflamed and noninflamed sites, for either total Hyp or weighted Hyp, is not statistically significant ($p>.05$).

Hydroxyproline values for all examination points for both drug groups are listed in Table 3.7. Mean values of total Hyp for each time point are displayed for drug and non-drug groups in Figure 3.13. The drug group shows a trend of decreasing Hyp values through to week 12, which at week 24 reverses back to slightly above the week zero baseline level. The non-drug group exhibits a slight drop in Hyp value at week two, which is followed by a rise in week 8 and a further rise slightly above baseline in week 12, and ends with a decrease at week 24. In comparing spiramycin-treated versus non-drug treated groups the only significant differences in the total moles of Hyp in a given examination period are found in weeks 12 and 24. At week 12 the total moles of Hyp are significantly higher in the non-drug treated group than in the drug treated group ($p<.05$). Conversely, at week 24 the total moles of Hyp are significantly larger in the spiramycin treated group compared to the non-drug treated group ($p<.05$). In addition, there is a significant difference at the 95% level between total Hyp levels at week zero versus week 12 in the drug treatment group.

Mean values of weighted Hyp for each time point are displayed for drug and non-drug treatment groups in Figure 3.14. Although there is a difference in the levels of Hyp in spiramycin versus non-drug treatment groups at week zero, this difference is not statistically different ($p>.05$). For the spiramycin treated group, there is a trend for an increase in Hyp content throughout the examination periods. This is first observed as a significant rise in Hyp at week two compared to week zero ($p<.05$). Subsequent examination points exhibit rising Hyp levels above that of week zero. No other significant differences were found in the spiramycin treated group.

The general trend for the non-drug treatment group is for a decrease in Hyp levels after week zero. At week 12 there is a slight increase in Hyp, but the level is similiar to that found at week 2, and still below the level found at week zero. Although there is a tendency for Hyp levels to be below those found at week zero, no statistically significant differences in Hyp levels were found amongst the examination periods ($p>.05$).

Drug	Stat	Week 0		Week 2		Week 8		Week 12		Week 24	
		BP=1	BP=0	BP=1	BP=0	BP=1	BP=0	BP=1	BP=0	BP=1	BP=0
+	X	2.19	1.57	1.76	2.20	1.94	1.59	1.59	1.27	2.32	2.11
+	SD	1.91	1.77	1.37	2.28	1.99	1.30	3.21	1.21	1.73	1.52
+	n	61	15	40	59	26	31	25	38	7	23
+	SE	.24	.46	.22	.30	.39	.23	.64	.20	.65	.32
-	X	2.52	1.67	2.22	1.48	2.86*	1.51*	2.59	1.97	1.46	1.30
-	SD	4.93	1.08	3.40	0.84	2.63	1.32	2.66	1.79	1.92	1.19
-	n	50	27	44	40	27	39	30	27	17	45
-	SE	.70	.21	.51	.13	.51	.21	.49	.34	.47	.18

Table 3.4: Total Hyp levels in both the spiramycin (Drug= +) and non-drug (Drug= -) treatment groups. Units for Hyp are in moles {Exp -11}. 'BP=1'= bleeding on probing positive; 'BP=0' = bleeding on probing negative; X= mean; SD= standard deviation; n= number of periodontal sites; SE= standard error of the mean. '*' indicates a statistically significant difference at the 95% level of significance.

Drug	Stat	Week 0		Week 2		Week 8		Week 12		Week 24	
		BP=1	BP=0	BP=1	BP=0	BP=1	BP=0	BP=1	BP=0	BP=1	BP=0
+	X	4.15	4.38	4.61	8.17	5.31	5.78	4.70	6.87	10.52	6.00
+	SD	5.73	5.33	4.05	12.13	6.64	8.09	4.93	12.97	14.60	8.79
+	n	16	15	40	59	26	31	25	38	7	23
+	SE	0.73	1.38	0.64	1.58	1.30	1.45	0.99	2.10	5.52	1.83
-	X	6.20	6.46	5.42	5.01	4.50	3.99	5.32	4.89	2.90	4.63
-	SD	8.65	8.72	8.44	8.56	5.90	3.70	6.48	5.31	3.47	7.97
-	n	50	27	44	40	27	39	30	27	17	45
-	SE	1.22	1.68	1.27	1.35	1.14	0.59	1.18	1.02	0.84	1.19

Table 3.5: Weighted Hyp levels in both the spiramycin (Drug= +) and non-drug (Drug= -) treatment groups. Units for Hyp are in moles {Exp -11} per µl of GCF. 'BP=1'= bleeding on probing positive; 'BP=0' = bleeding on probing negative; X= mean; SD= standard deviation; n= number of periodontal sites; SE= standard error of the mean.

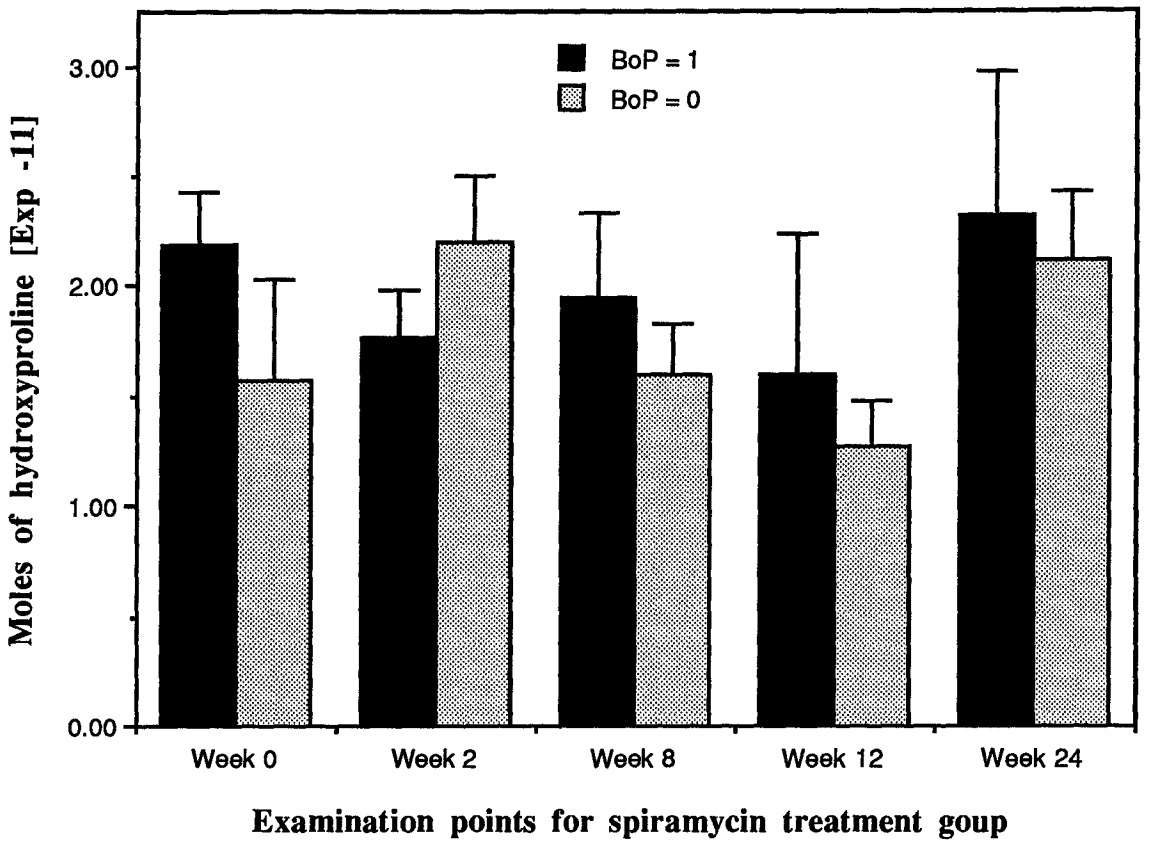

Figure 3.8: Total Hyp levels at periodontal sites from the spiramycin treatment group at all examination points of the study. Hydroxyproline units are in moles Hyp {Exp-11}. Error bars represent the standard error of the mean.

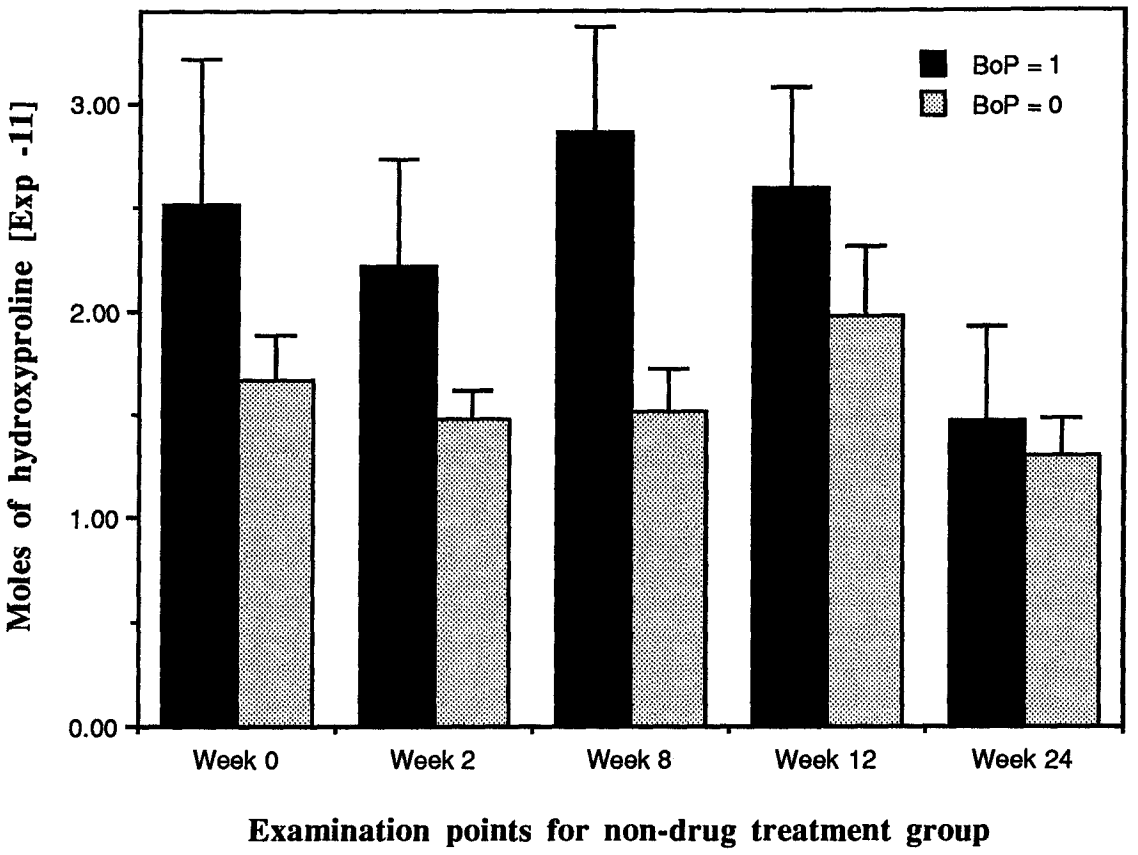

Figure 3.9: Total Hyp levels at periodontal sites from the non-drug treatment group at all examination points of the study. Hydroxyproline units are in moles Hyp {Exp-11}. Error bars represent the standard error of the mean.

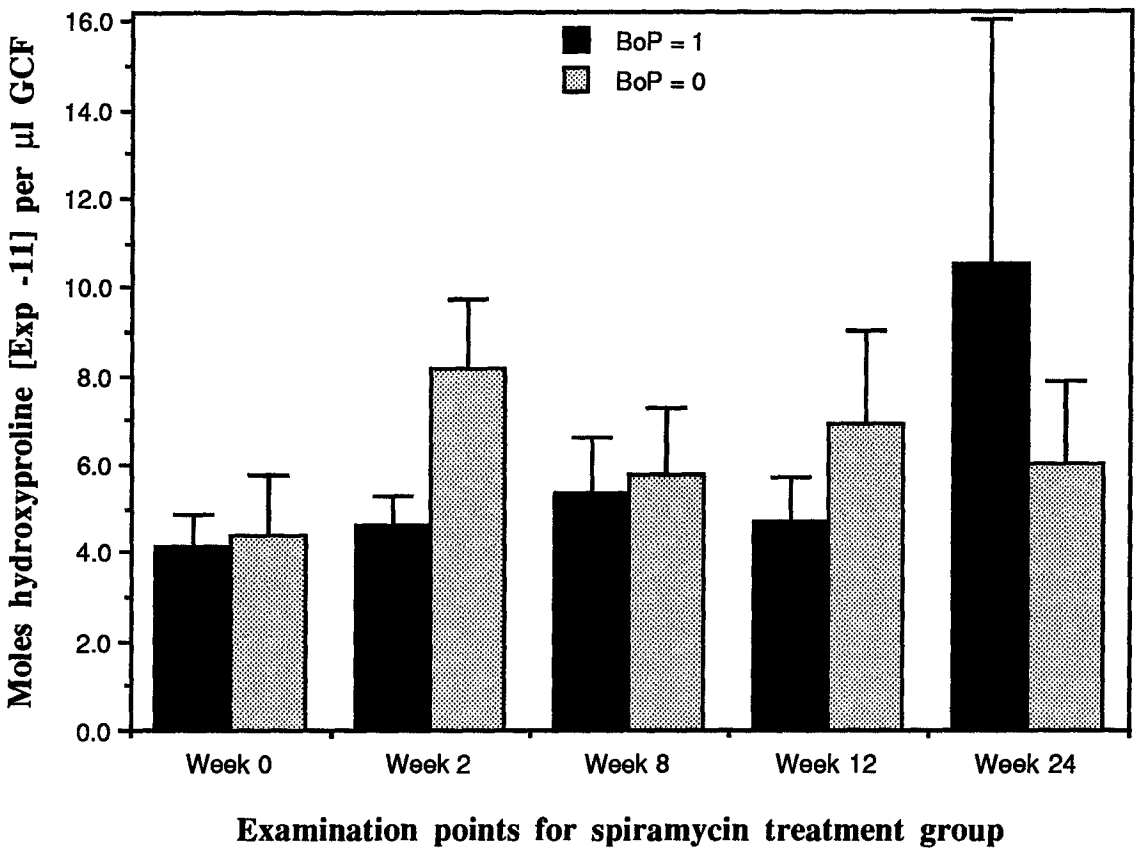

Figure 3.10: Weighted Hyp levels at periodontal sites from the spiramycin treatment group at all examination points of the study. Hydroxyproline units are in moles Hyp {Exp-11} per µl GCF. Error bars represent the standard error of the mean.

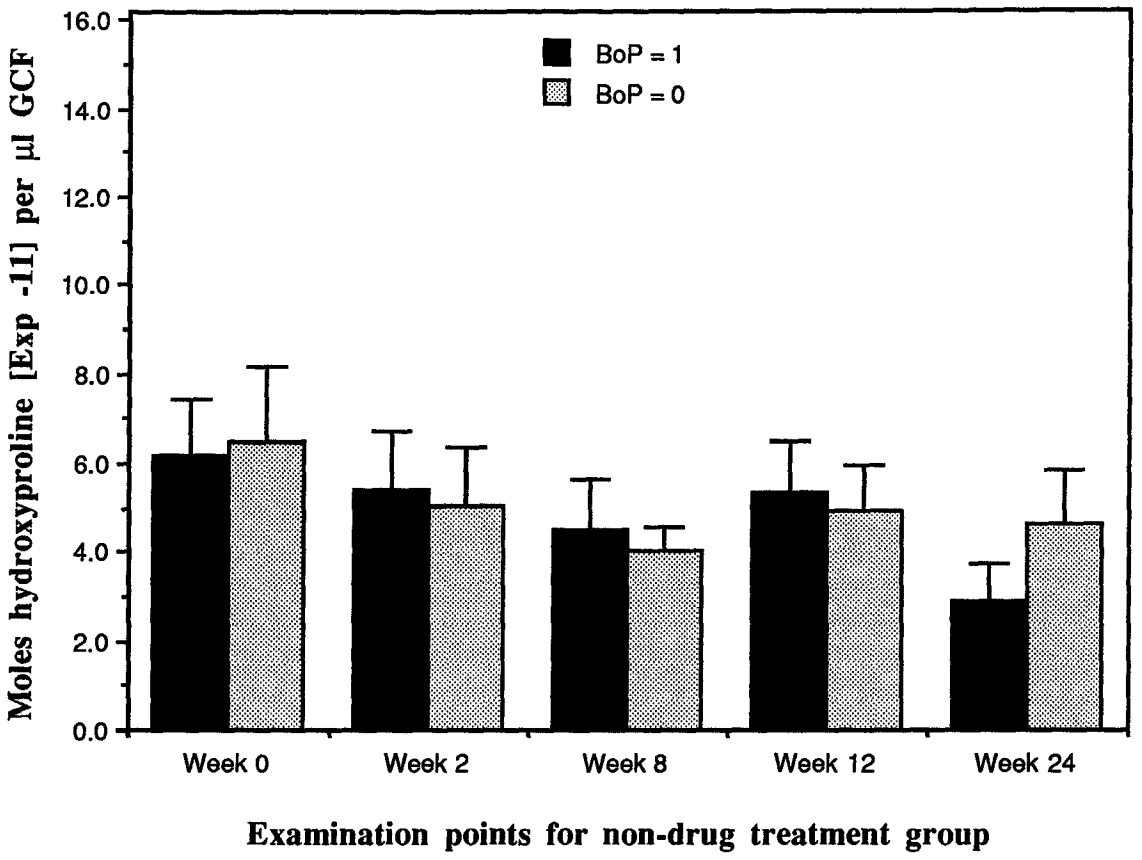

Figure 3.11: Weighted Hyp levels at periodontal sites from the non-drug treatment group at all examination points of the study. Hydroxyproline units are in moles Hyp {Exp-11} per µl GCF. Error bars represent the standard error of the mean.

Statistic	BoP = 1 WHyp	BoP = 1 MHyp	BoP = 0 WHyp	BoP = 0 MHyp
X	5.07	2.34	5.72	1.64
SD	7.231	3.58	7.68	1.34
n	111	111	24	42
SE	0.69	0.34	1.18	0.21

Table 3.6: Hydroxyproline values at week zero time point for inflamed and noninflamed sites for both spiramycin and non-drug treatment groups combined. WHyp= Moles Hyp {Exp -11} / µl GCF; MHyp= Moles Hyp {Exp -11}; X= mean; SD= Standard deviation; n= Number of periodontal sites; SE= Standard error of the mean.

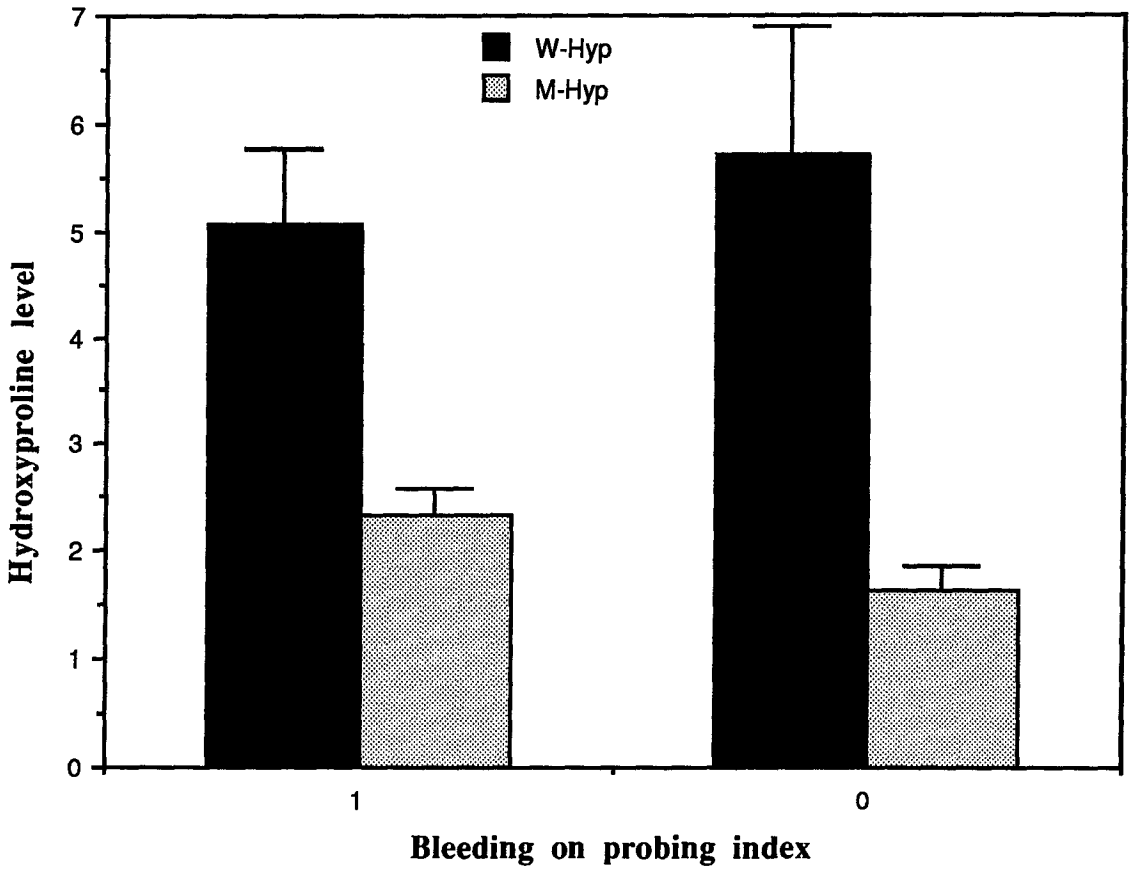

Figure 3.12: Hydroxyproline levels in inflamed and noninflamed periodontal sites at week zero time point. 'Mhyp'= Moles Hyp {Exp-11} for total GCF sample; 'Whyp'= Moles Hyp {Exp-11} per µl GCF. Error bars represent the standard error of the mean.

Drug	Stat	Week 0		Week 2		Week 8		Week 12		Week 24	
		WHyp	MHyp	WHyp	MHyp	WHyp	MHyp	WHyp	MHyp	WHyp	MHyp
+	X	4.12	2.06	6.73	2.02	5.57	1.75	6.01	1.40	7.05	2.16
+	SD	5.62	1.89	9.83	1.96	7.41	1.64	10.31	1.20	10.32	1.55
+	n	76	76	99	99	57	57	63	63	30	30
+	SE	.64	.22	.99	.20	.98	.22	1.30	.15	1.88	.28
-	X	6.29	2.22	5.23	1.87	4.12	2.06	5.12	2.30	4.16	1.34
-	SD	8.62	4.03	8.45	2.54	4.69	2.05	5.91	2.29	7.04	1.41
-	n	77	77	84	84	66	66	57	57	62	62
-	SE	.98	.46	.92	.28	.58	.25	.78	.30	.89	.18

Table 3.7: Hydroxyproline values at various exam points for both spiramycin (Drug= +) and non-drug (Drug= -) treatment groups. Stat= Statistic; WHyp= Moles Hyp {Exp-11} / µl GCF; MHyp= Moles Hyp {Exp-11}; X= Mean; SD= Standard deviation; n= Number of periodontal sites; SE= Standard error of the mean.

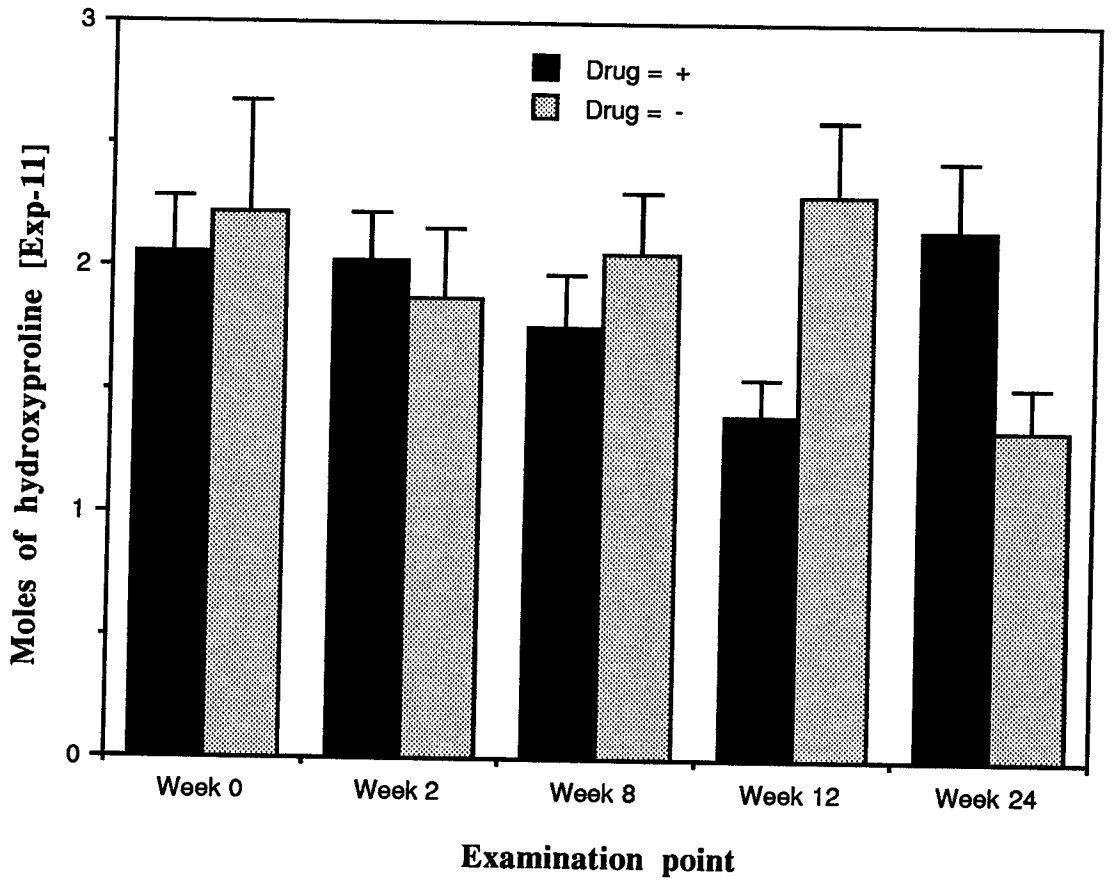

Figure 3.13: Total Hyp levels at all examination points for both the spiramycin and non-drug treatment groups. 'Drug = +' = spiramycin treated group; 'Drug = -' = non-drug group. Error bars represent the standard error of the mean.

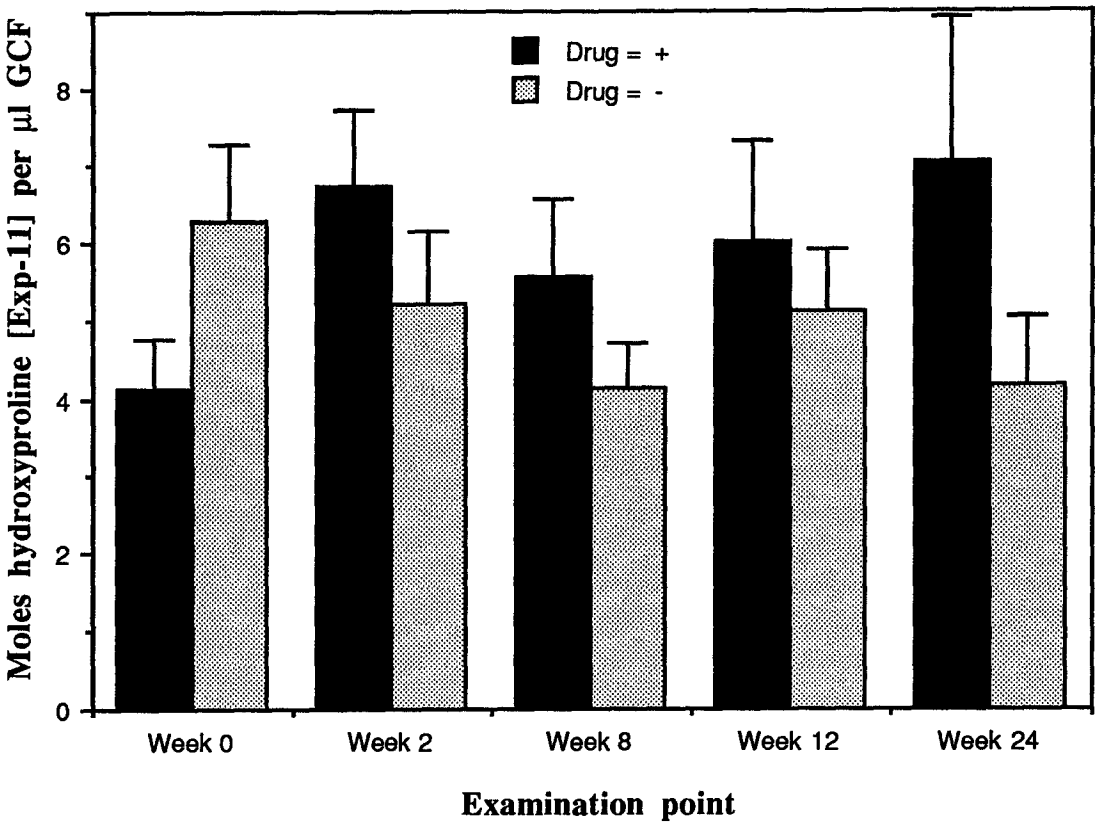

Figure 3.14: Weighted Hyp levels at all examination points for both the spiramycin and non-drug treatment groups. 'Drug = +' = spiramycin treated group; 'Drug = -' = non-drug group. Error bars represent the standard error of the mean.

Hydroxyproline values at week zero time point for shallow and deep periodontal sites that are either inflamed or noninflamed are given in Table 3.8. The mean, standard deviation, number of subjects and standard error are listed for all drug treatment groups. These data are graphically represented in Figures 3.15 through 3.17. For the combined drug treatment groups, the Hyp levels for shallow pockets show a different trend than for deeper sites. In Figure 3.15, the total Hyp in the collected sample is greater in inflamed versus noninflamed sites. This same trend is true for the weighted Hyp levels in shallow sites, showing that inflamed sites exhibit more Hyp than do the noninflamed sites. In pocket depths greater than or equal to 4 mm, Hyp levels for inflamed and noninflamed crevicular fluid are approximately the same. No statistically significant differences were found amongst these baseline Hyp measurements ($p>.05$).

Hydroxyproline levels were compared to bleeding on probing measurements in 127 periodontal sites of 17 patients that had complete clinical and Hyp analysis data for weeks zero and twelve of the spiramycin study. Bleeding on probing scores were compared amongst periodontal sites between these time points. The results indicated that inflamed periodontal sites at week zero which remained inflamed at week twelve had higher Hyp levels at week twelve than sites that remained noninflamed. In the drug group a significant difference was found between Hyp levels at week twelve in inflamed and noninflamed sites ($p<.05$). Tables 3.9 and 3.10 display mean Hyp values and bleeding on probing recordings at weeks 0 and 12, for spiramycin and non-drug treatment groups respectively.

3.2.3 Hydroxyproline levels versus attachment levels

In comparing Hyp levels to changes in attachment levels in 127 periodontal sites in the above spiramycin study, changes in probable attachment level were deemed significant if changes in measurements were greater than or equal to 2 mm. In both treatment groups Hyp levels were higher in sites that experienced a gain of attachment of $\geq$2 mm between weeks 0 and 12, than sites that remained unresolved. In the drug group at week twelve a significant difference was found in

Hyp levels between healing and nonresolved sites ($p<.05$). Changes in attachment level between weeks 0 and 12 for both treatment groups are displayed in Figure 3.18.

Drug	Statistic	Pocket Depth < 4mm				Pocket Depth ≥4mm			
		BoP=1 WHyp	BoP=1 MHyp	BoP=0 WHyp	BoP=0 MHyp	BoP=1 WHyp	BoP=1 MHyp	BoP=0 WHyp	BoP=0 MHyp
+ and -	X	8.55	3.52	6.72	1.58	4.26	2.06	4.39	1.71
+ and -	SD	12.49	7.39	9.06	1.44	5.10	1.78	5.29	1.24
+ and -	n	21	21	24	24	90	90	18	18
+ and -	SE	2.73	1.61	1.85	0.29	0.54	0.19	1.25	0.29
+	X	2.86	1.74	5.76	2.02	4.44	2.28	2.32	0.89
+	SD	1.70	0.89	6.46	2.19	6.25	2.06	2.03	0.35
+	n	11	11	9	9	50	50	6	6
+	SE	0.51	0.27	2.15	0.73	0.88	0.29	0.83	0.14
-	X	14.81	5.48	7.30	1.31	4.04	1.78	5.42	2.13
-	SD	16.14	10.59	10.49	0.68	3.19	1.34	6.15	1.32
-	n	10	10	15	15	40	40	12	12
-	SE	5.10	3.35	2.71	0.17	0.50	0.21	1.78	0.38

Table 3.8: Hydroxyproline values at week zero time point for both shallow and deep pockets subdivided into inflamed or noninflamed, for spiramycin (Drug= +), non-drug (Drug= -), and all (Drug= + and -) treatment groups. 'BoP=1'= Bleeding on probing positive; 'BoP=0'= Bleeding on probing negative; WHyp= Moles Hyp {Exp-11} / µl GCF; MHyp= Moles Hyp {Exp-11}; X= mean; SD= Standard deviation; n= Number of periodontal sites; SE= Standard error of the mean.

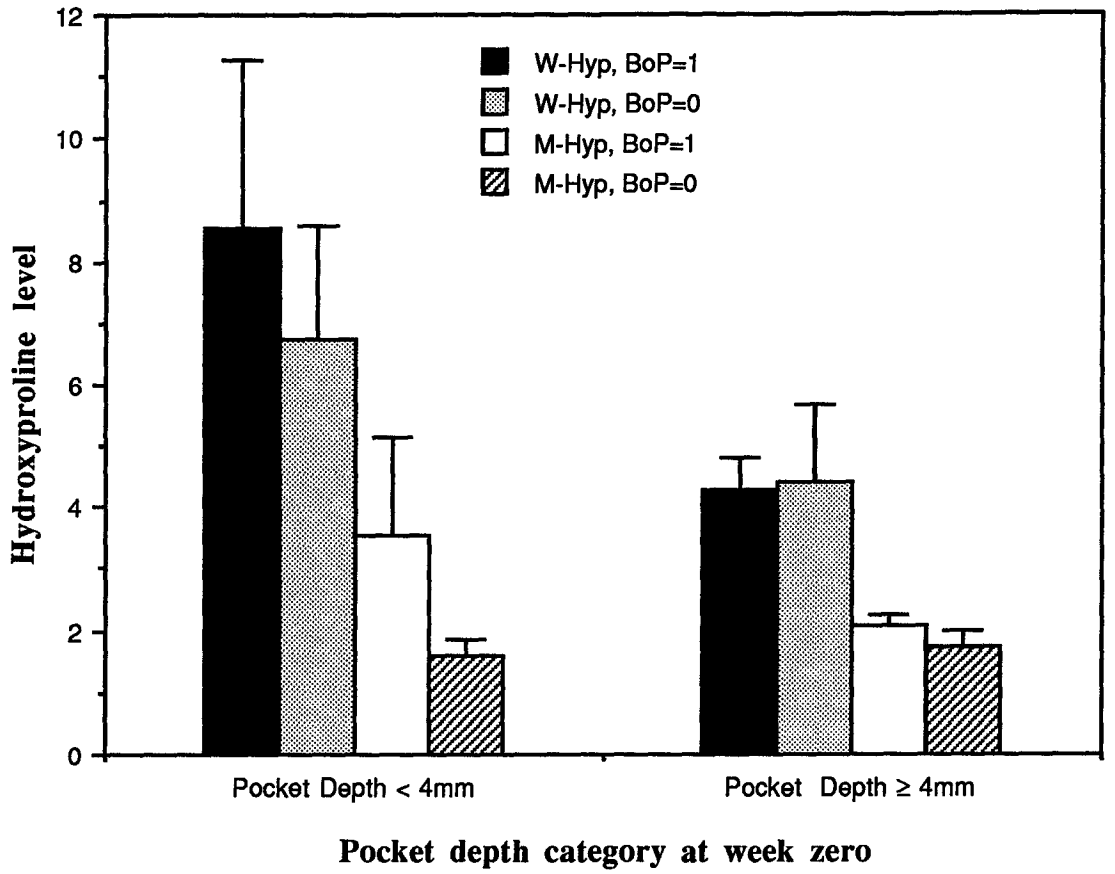

Figure 3.15: Hydroxyproline levels for combined drug treatment groups at week zero for shallow and deep pockets. W-Hyp= moles Hyp {Exp-11} / µl GCF; M-Hyp= moles Hyp {Exp-11}; 'BoP=1'= Bleeding on probing positive; 'BoP=0'= Bleeding on probing negative. Error bars represent the standard error of the mean.

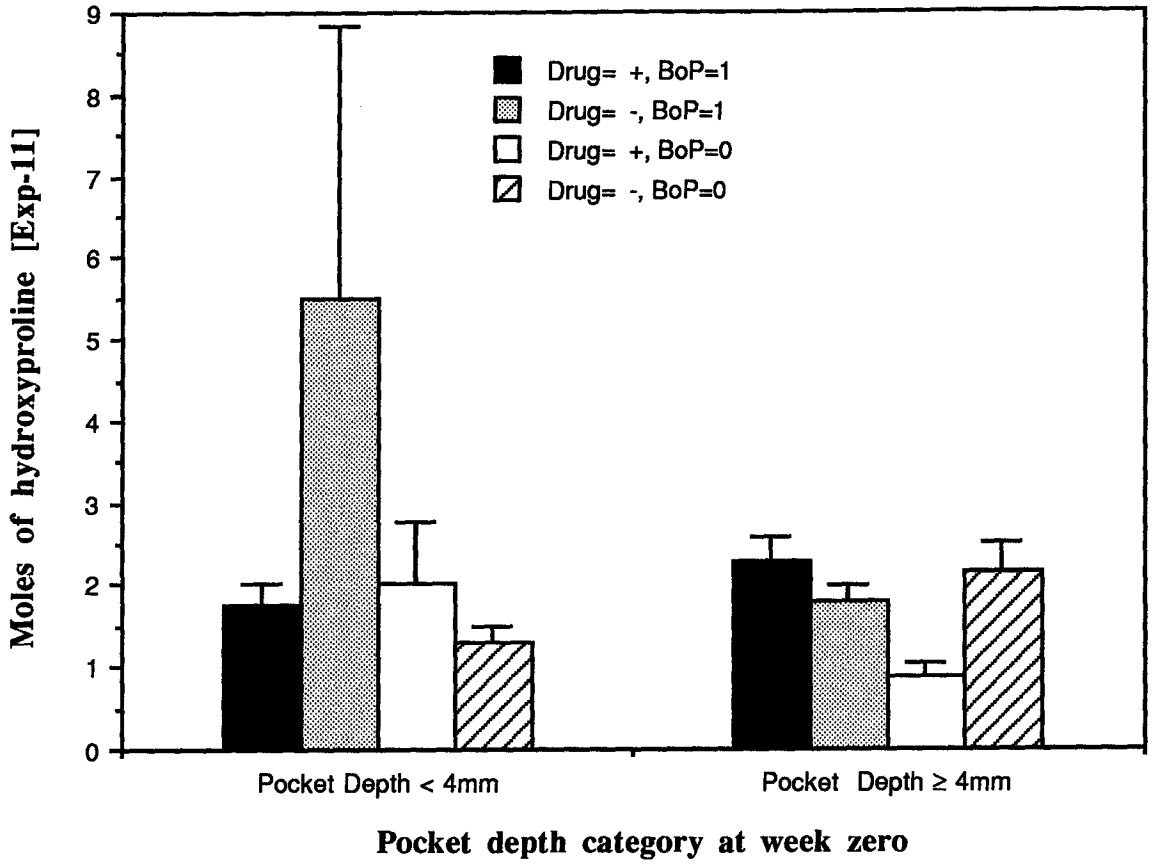

Figure 3.16: Total Hyp levels for both drug treatment groups at week zero for both bleeding on probing categories and shallow and deep pocket depths. 'Drug= +'= Spiramycin treatment group; 'Drug= -'= Non-drug treatment group; 'BoP= +'= Bleeding on probing positive; 'BoP= -'= Bleeding on probing negative Error bars represent the standard error of the mean.

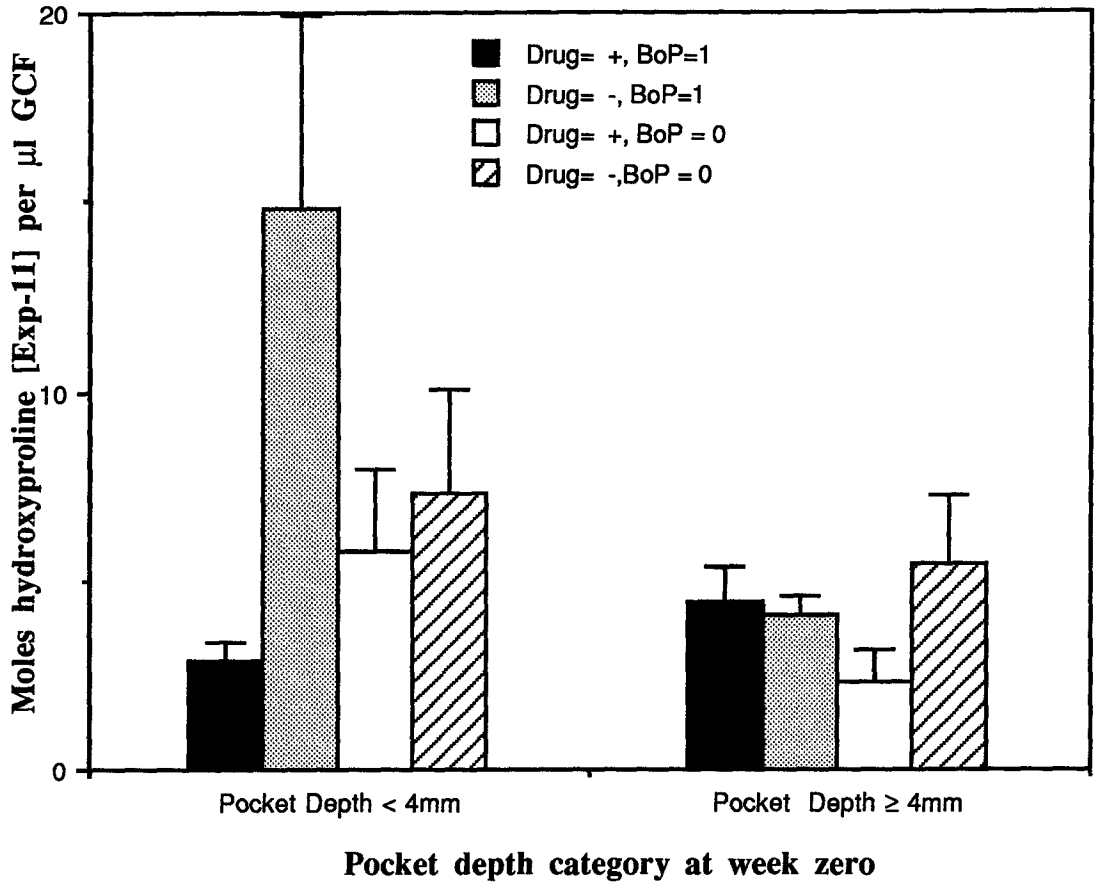

Figure 3.17: Weighted Hyp levels for both drug treatment groups at week zero for both bleeding on probing categories and shallow and deep pocket depths. 'Drug= +'= Spiramycin treatment group; 'Drug= -'= Non drug treatment group; 'BoP= +'= Bleeding on probing positive; 'BoP= -'= Bleeding on probing negative. Error bars represent the standard error of the mean.

N= 15 Week (0,12) BoP (+/+)				N= 26 Week (0,12) BoP (+/-)			
Week 0, BoP +		Week 12, BoP +		Week 0, BoP +		Week 12, BoP -	
MHyp {SE}	WHyp {SE}	MHyp {SE}	WHyp {SE}	MHyp {SE}	WHyp {SE}	MHyp {SE}	WHyp {SE}
1.43 {0.40}	2.81 {1.01}	0.72 {0.25}	1.58* {0.59}	1.67 {0.52}	2.74 {0.97}	0.23 {0.12}	0.45 {0.13}
N=2 Week (0,12) BoP (-/+)				N=11 Week (0,12) BoP (-/-)			
Week 0, BoP -		Week 12, BoP +		Week 0, BoP -		Week 12, BoP -	
MHyp {SE}	WHyp {SE}	MHyp {SE}	WHyp {SE}	MHyp {SE}	WHyp {SE}	MHyp {SE}	WHyp {SE}
4.02 {3.22}	3.97 {2.81}	0.55 {0.25}	1.22 {0.22}	0.41 {0.15}	0.80 {0.30}	0.41 {0.35}	0.23* {0.12}

Table 3.9: Hydroxyproline values and bleeding on probing measurements at weeks 0 and 12 in spiramycin treatment group. 'N'= number of subjects, 'Mhyp'=Moles Hyp {Exp-11}, 'Whyp'=Moles Hyp {Exp-11}/µl GCF, 'SE'=Standard error. '*'= Hyp level is significantly lower ($p<.05$).

N= 18 Week (0,12) BoP (+/+)				N= 28 Week (0,12) BoP (+/-)			
Week 0, BoP +		Week 12, BoP +		Week 0, BoP +		Week 12, BoP -	
MHyp {SE}	WHyp {SE}	MHyp {SE}	WHyp {SE}	MHyp {SE}	WHyp {SE}	MHyp {SE}	WHyp {SE}
2.80 {1.91}	3.83 {2.52}	0.71 {0.26}	1.47 {0.58}	1.23 {0.27}	2.75 {0.62}	0.36 {0.10}	0.75 {0.28}
N=9 Week (0,12) BoP (-/+)				N=18 Week (0,12) BoP (-/-)			
Week 0, BoP -		Week 12, BoP +		Week 0, BoP -		Week 12, BoP -	
MHyp {SE}	WHyp {SE}	MHyp {SE}	WHyp {SE}	MHyp {SE}	WHyp {SE}	MHyp {SE}	WHyp {SE}
1.11 {0.34}	2.29 {1.02}	0.05 {0.05}	0.18 {0.17}	0.85 {0.28}	2.59 {1.00}	0.53 {0.28}	0.81 {0.33}

Table 3.10: Hydroxyproline values and bleeding on probing measurements at weeks 0 and 12 in non-drug treatment group. 'N'= number of subjects, 'Mhyp'=Moles Hyp {Exp-11}, 'Whyp'=Moles Hyp {Exp-11}/µl GCF, 'SE'=Standard error.

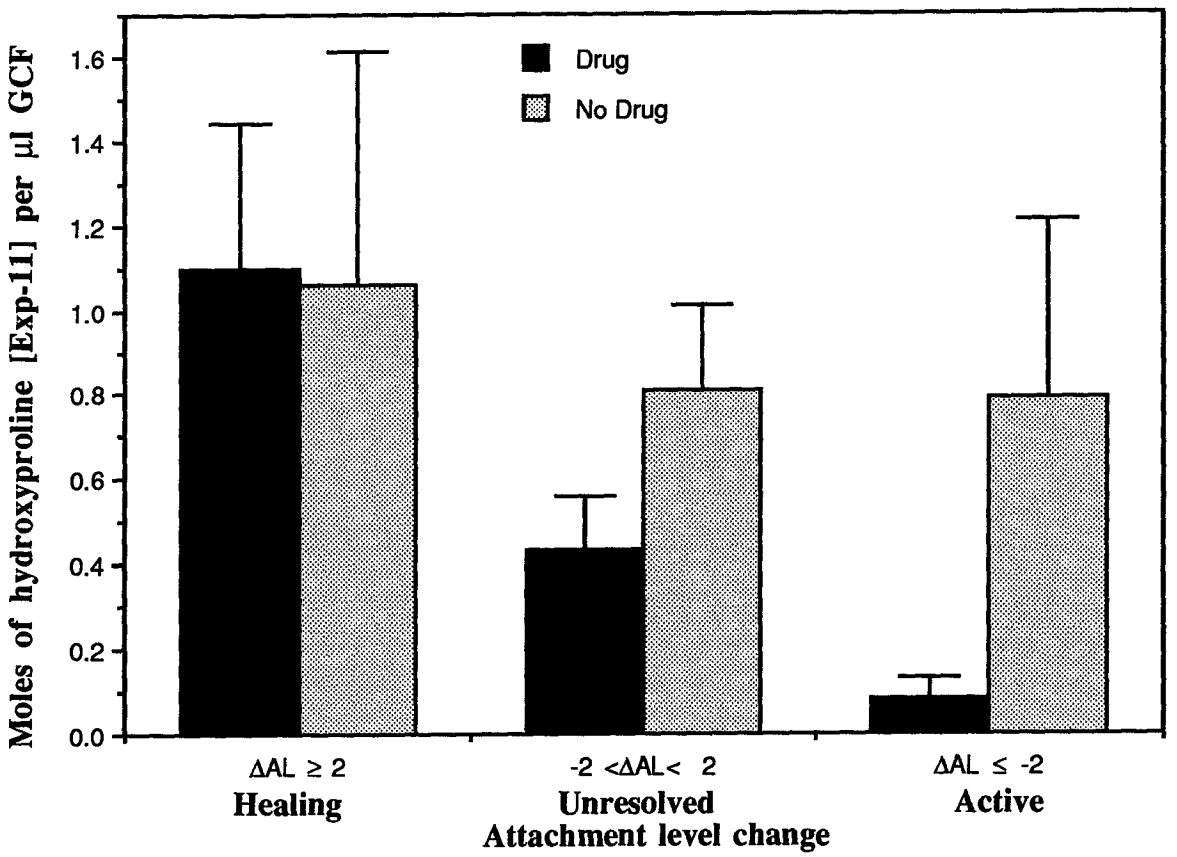

Figure 3.18: Hydroxyproline levels versus changes in attachment level between weeks zero and twelve for drug and non-drug groups. 'ΔAL ≥ 2'= healing sites; '-2 ≤ΔAL≤ 2'= unresolved sites; 'ΔAL< -2'= active sites. Error bars represent the standard error of the mean.

3.2.4 Effect of spiramycin on periodontal measurements

The effect of spiramycin versus placebo on bleeding on probing, pocket depth and attachment level measurements was also compared in 127 periodontal sites of 17 patients of the spiramycin study. The results indicated that the spiramycin treatment group had improvements in bleeding on probing, pocket depth and clinical attachment level between weeks 0 and 12. These changes exhibited in Table 3.11, were all greater in the spiramycin group than the corresponding changes in the placebo group. The changes in both treatment groups were statistically significant ($p<.05$).

Drug	Exam	N	GCF µl (SE)	% BoP	PD(Avg) mm (SE)	MAL(Avg) mm (SE)	Hyp(Avg) moles(E-11)/µl (SE)
YES	Wk 0	54	0.67 (.06)	76	4.7 (.31)	7.9 (.38)	2.41 (.56)
	Wk 12	54	0.45 (.09)	31	2.0 (.17)	6.0 (.31)	0.75 (.19)
NO	Wk 0	73	0.56 (.04)	63	4.8 (.25)	8.2 (.28)	2.92 (.71)
	Wk 12	73	0.49 (.04)	37	3.6 (.20)	7.4 (.23)	0.87 (.20)

Table 3.11: Periodontal measurements at weeks 0 and 12 for both drug and non-drug treatment groups. Drug = Spiramycin; Exam = either week 0 or week 12; N= number of periodontal sites examined; GCF= gingival crevicular fluid volume; SE= standard error or the mean; %BoP= percentage of sites with bleeding on probing; PD(Avg)= mean pocket depth; MAL(Avg)= mean measured attachment level; Hyp(Avg)= mean Hyp level (moles{Exp-11} / µl crevicular fluid).

3.3 BIOCHEMICAL ANALYSES OF CREVICULAR FLUID FROM INFLAMED AND NONINFLAMED PERIODONTAL SITES

3.3.1 Hydroxyproline levels in inflamed and noninflamed sites

The results of Hyp analysis from 54 patients who contributed crevicular fluid from both an inflamed and noninflamed periodontal site indicated that there was a distinct relationship between the levels of Hyp in inflamed versus noninflamed periodontal sites. The mean level of Hyp at inflamed sites was 684 (+/-SE 63) picomoles/µl GCF. At noninflamed sites the mean level of Hyp was 460 (+/-SE 53) picomoles/µl of GCF. Using the paired *t*-test, inflamed sites showed significantly higher Hyp levels than patient matched noninflamed sites ($p<0.001$). These results are displayed in Figure 3.19.

3.3.2 C1q content in crevicular fluid model

The effect of extraction buffers on the C1q content in crevicular fluid model systems was evaluated using HPLC and ELISA analyses. HPLC analysis was used to assess the effectiveness of C1q precipitation using both 0.005 M phosphate and 0.5 N acetic acid buffers. The results indicated that the addition of 1 µl of 350 µg/ml C1q to crevicular fluid collected from disease sites to the acetate buffer system followed by centrifugation precipitated most of the added C1q. According to HPLC Hyp analysis 65% reduction in added C1q was removed as a precipitate. Results also indicated that 1 µl of 350 µg/ml barely increased to total Hyp content found in crevicular fluid of diseased sites.

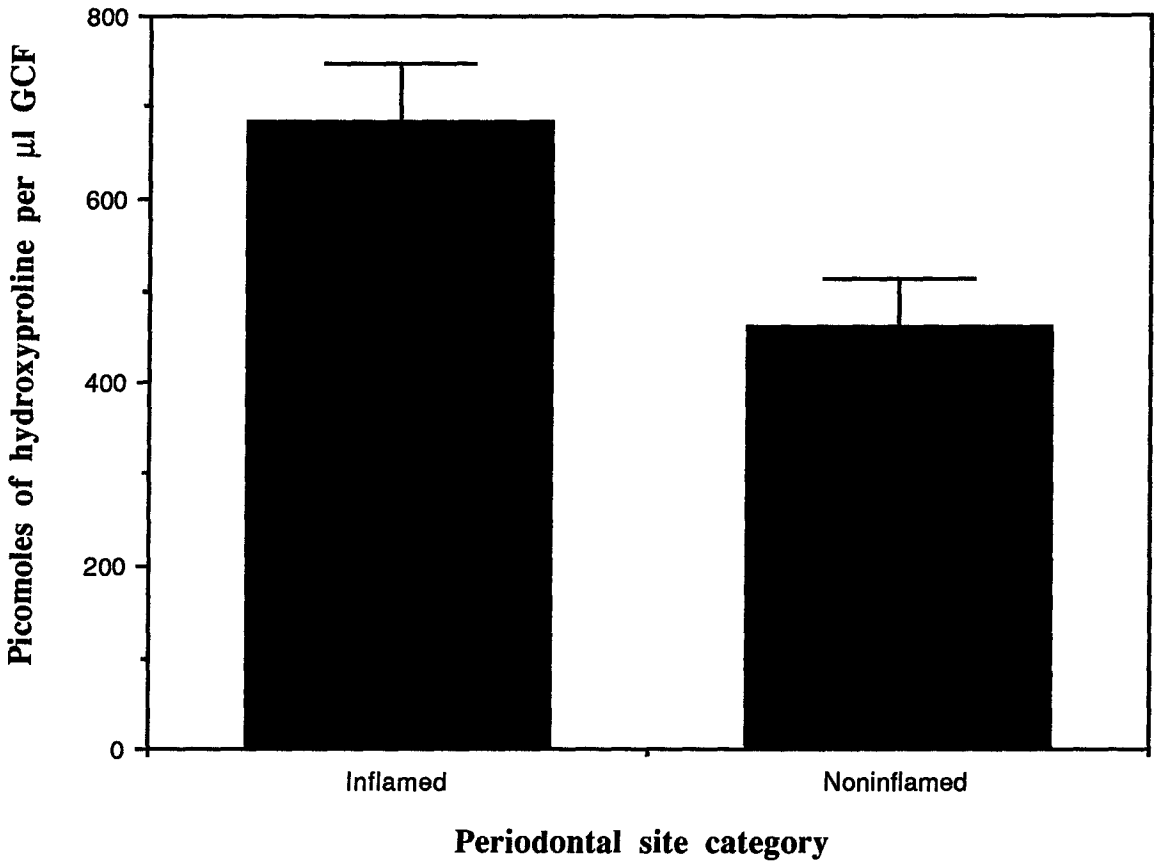

Figure 3.19: Weighted Hyp levels in inflamed and noninflamed periodontal sites as measured by HPLC. Error bars represent the standard error of the mean.

The removal of C1q in a sodium acetate buffer system from a crevicular fluid model was verified by ELISA. A 1 µl volume of 200 µg/ml C1q added to 49 µl of 0.05 sodium acetate buffer followed by centrifugation at 15,600 g for 30 min. at 4°C resulted in C1q removal from the supernatant. Employing monoclonal antibodies to C1q for ELISA, examination of the supernatant indicated that only 8.7% of the initial C1q remained in solution. Figure 3.20, which shows the percent recovery of C1q fractions following centrifugation, indicates that under the employed conditions 91.3 % of C1q was precipitated by acetate buffer.

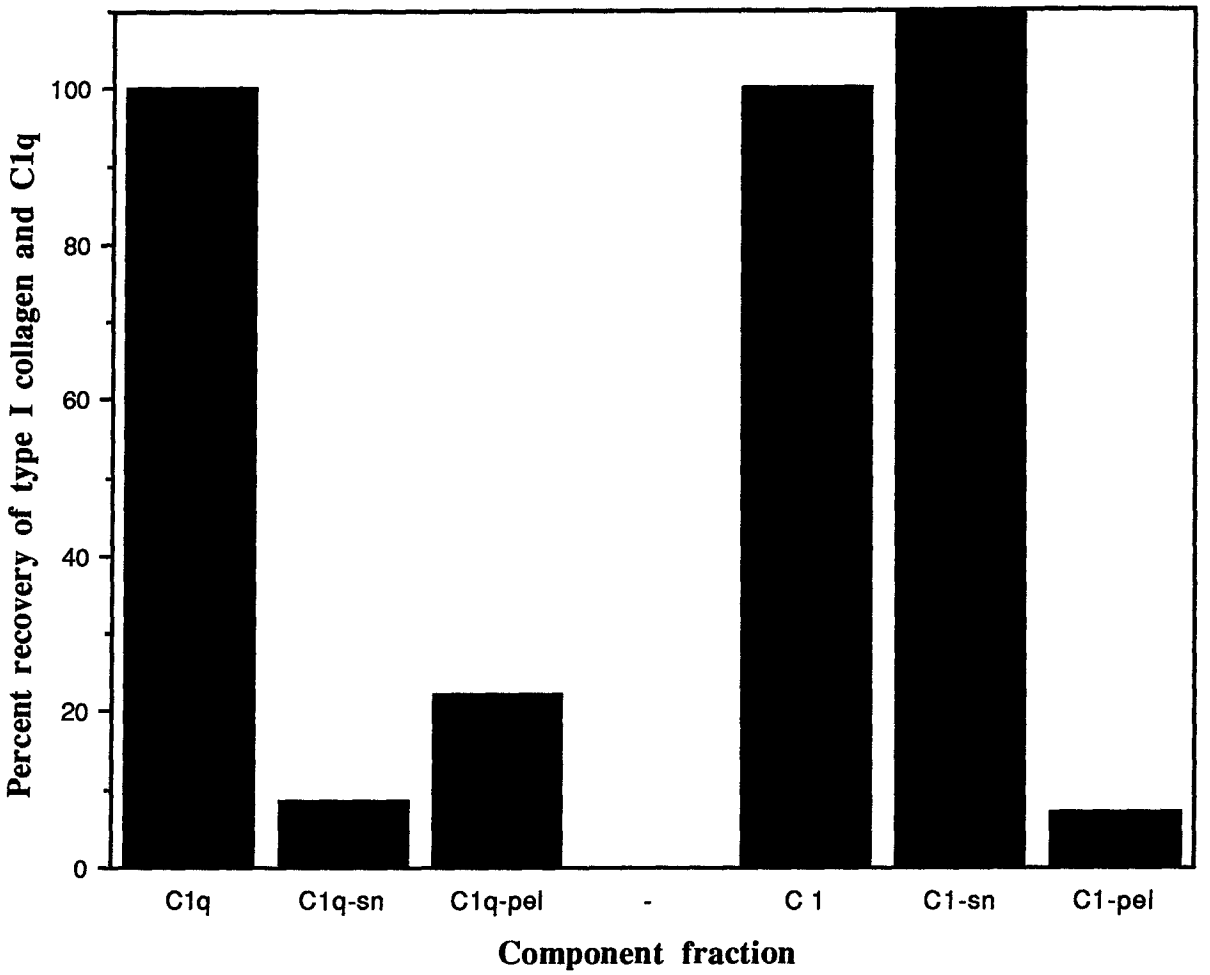

Figure 3.20: Type I collagen and C1q in a crevicular fluid model. ELISA measurements for the relative % weight of either type I collagen (C1) or C1q in supernatant (sn) and pellet (pel) after centrifugation at 15,600 g for 10 minutes compared to parallel non-centrifuged systems.

3.3.3 C1q content in crevicular fluid

The ELISA analyses for the content of C1q from inflamed and noninflamed crevicular fluid samples were performed using two different primary antibodies; the first was a monoclonal and the second a polyclonal antibody to C1q.

For this phase of the study fifty four subjects provided crevicular fluid samples from both an inflamed and noninflamed periodontal site. The ELISA results on inflamed and noninflamed crevicular fluid using the monoclonal antibody to C1q demonstrated widespread differences in the content of C1q between inflamed and noninflamed and amongst different crevicular sites. From the C1q content values of each crevicular site, a corresponding amount of Hyp contribution from C1q was determined on the basis that Hyp constitutes 4.3% of the C1q molecule. This Hyp level was compared to the overall total level of Hyp determined by HPLC. It was found that not only did the amount of C1q vary widely amongst crevicular sites, but that the amount of calculated Hyp contribution in number of cases far exceeded the maximum of 100%. These results indicated that at inflamed sites C1q contributed 125 %(+/-SE 125), while at noninflamed sites C1q contributed 221% (+/-SE 230) to the total Hyp content of GCF. This inferred that the variability of the C1q values, particularly those indicating a Hyp contribution greater than 100%, was due to an undetermined cross-reactivity to some other components in the sample.

Hence the ELISA experiments were repeated using a polyclonal antibody to C1q which was tested and found to have negligible cross-reactivity to collagen and closely related structures. GCF samples from matched inflamed and noninflamed periodontal sites of each patient were evaluated in 24 of the initial 54 subjects. Using this antibody it was demonstrated that Hyp contribition attributable to C1q to the overall levels of Hyp was small. It indicated that C1q accounted for only 6.91% (+/-SE 1.15) of total Hyp content of the GCF sample in inflamed sites and 9.85% (+/-SE 2.49) of the total Hyp content in fluid from noninflamed sites. These values are not statistically different ($p>.05$). A comparison of the C1q derived Hyp contribution from inflamed and noninflamed sites to total Hyp levels is presented in Figure 3.21. Furthermore, the ELISA assay using this polyclonal antibody resulted in a narrow range of C1q levels in GCF. The range of the data obtained with this antibody preparation was deemed a more accurate representation of the C1q content in GCF since none of the C1q values contributed more than 100% to the total Hyp levels determined by HPLC.

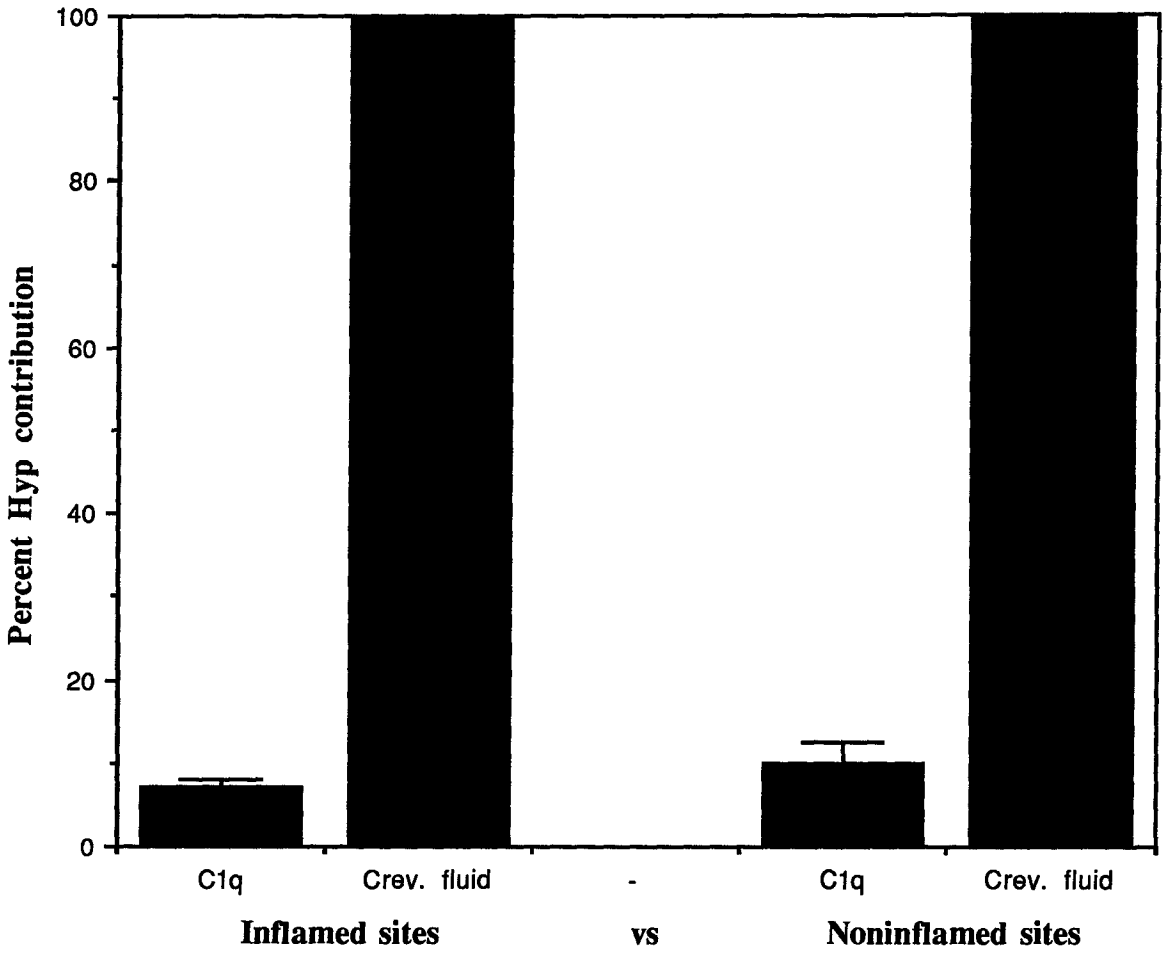

Figure 3.21: ELISA results using a polyclonal antibody to C1q. C1q derived Hyp contributions to crevicular fluid from inflamed and noninflamed sites compared to total Hyp content of crevicular fluid. Error bars represent the standard error of the mean.

3.3.4 Type I collagen in crevicular fluid model

The effects of sodium acetate buffer and proteolytic enzymes on the recovery of type I collagen were assessed in crevicular fluid models. After centrifugation of a 1 µl volume of 1 mg/ml type I collagen added to 49 µl of 0.05 sodium acetate buffer, collagen content was determined using polyclonal antibodies in an ELISA assay. Analysis of the supernatant indicated in Figure 3.20 that essentially all of the collagen was recovered following centrifugation. Thus, whereas whole

collagen is soluble in acetate and remains in the supernatant, the intact C1q is precipitated and removed by this procedure.

The reliability of an ELISA assay to quantitate degraded collagen was assayed in systems in which collagen was incubated with proteolytic enzymes. In this study type I collagen was incubated with either bacterial collagenase (BC) or bleb preparation from *P. gingivalis* (blebs), for one and two hour periods at 37°C. After one hour of incubation, 82.1% and 90.3% of the added collagen was detected in the BC and blebs' enzyme systems, respectively. After 2 hours of incubation the collagen content was further reduced to 78.8% and 77.7%. The results depicted in Figure 3.22 show that the employed enzymatic preparations caused a reduction of anti-collagen I determinants due to degradation of collagen which increased with incubation time.

3.3.5 Type I collagen in crevicular fluid

The content of type I collagen in crevicular fluid was determined from both inflamed and noninflamed sites in each of the 54 original subjects. Employing a polyclonal antibody to type I collagen in an ELISA assay, collagen content of the crevicular sites was determined on duplicate samples.

Each ELISA reading was converted to a molar value of collagen, which was then normalized to an amount of collagen per µl of crevicular fluid. Finally, a percentage of Hyp contributed by a collagen was calculated and compared to the total Hyp level as determined by HPLC analyses. The results indicated that in inflamed sites type I collagen contributed 27.68% (+/-SE 6.12) of the total Hyp content of GCF. In noninflamed sites type I collagen contributed 51.65% (+/-SE 9.56) to the total Hyp content. Analysis of ELISA results using the student's *t*-test indicated that noninflamed sites showed significantly greater amounts of collagen-derived Hyp than the inflamed sites ($p<.05$). Figure 3.23 exhibits the percent Hyp content derived from collagen measured from inflamed and noninflamed sites and total Hyp content of crevicular fluid.

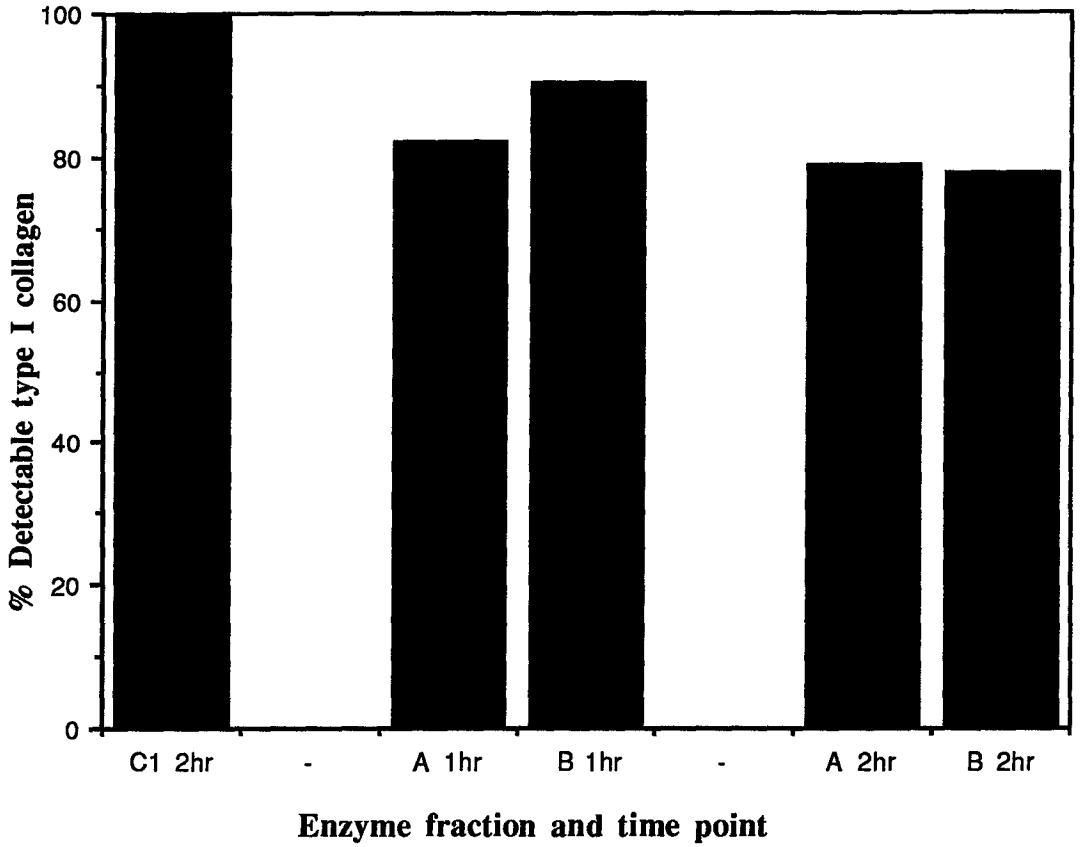

Figure 3.22: Detection of degraded collagen using ELISA, after one and two hour incubations with bacterial collagenase (A) and *P. gingivalis* blebs (B).

Comparisons of the amount of type I collagen and C1q in inflamed and noninflamed crevicular fluid were made on the 24 subjects whose GCF C1q levels were analyzed using the polyclonal C1q antibody. Figure 3.24 displays the mean ELISA values of moles of type I collagen and C1q per µl of GCF, from inflamed and noninflamed periodontal sites. In inflamed sites the mean type I collagen content was 0.65 (+/-SE .18) picomoles/µl GCF, while the content of C1q was 0.25 (+/-SE .04) picomoles/µl GCF. In noninflamed sites the mean type I collagen content was 0.82 (+/-SE .19) picomoles/µl GCF, while the content of C1q was 0.19 (+/-SE .04) picomoles/µl GCF. The results indicated that the moles of type I collagen were significantly higher then moles of C1q in GCF, for both inflamed and noninflamed groups ($p<.025$ and $p<.0025$ respectively).

Figure 3.25 depicts the mean ELISA values of moles of Hyp contributed by type I collagen and C1q per µl of GCF, from inflamed and noninflamed periodontal sites. In inflamed sites the mean Hyp contribution from type I collagen was 160 (+/-SE 43.9) picomoles/µl GCF, while the Hyp contribution from C1q was 33.85 (+/-SE 5.06) picomoles/µl GCF. In noninflamed sites the mean Hyp contribution from type I collagen was 202 (+/-SE 45.9) picomoles/µl GCF, while the Hyp contribution from C1q was 26.05 (+/-SE 5.02) picomoles/µl GCF. The results indicated the moles of Hyp contributed from type I collagen were significantly larger than moles of Hyp contributed by C1q per µl of GCF, for both inflamed and noninflamed groups ($p<.01$ and $p<.001$ respectively).

Figure 3.26 displays the combined type I collagen and C1q Hyp percent contributions to total Hyp content for inflamed and noninflamed periodontal sites from 24 subjects. The percent contributed from type I collagen derived Hyp is significantly greater than by C1q-derived Hyp, in both inflamed and noninflamed groups ($p<.01$ and $p<.0025$ respectively). Both the type I collagen and C1q-derived Hyp levels are lower in the inflamed sites than the corresponding Hyp levels in the noninflamed group.

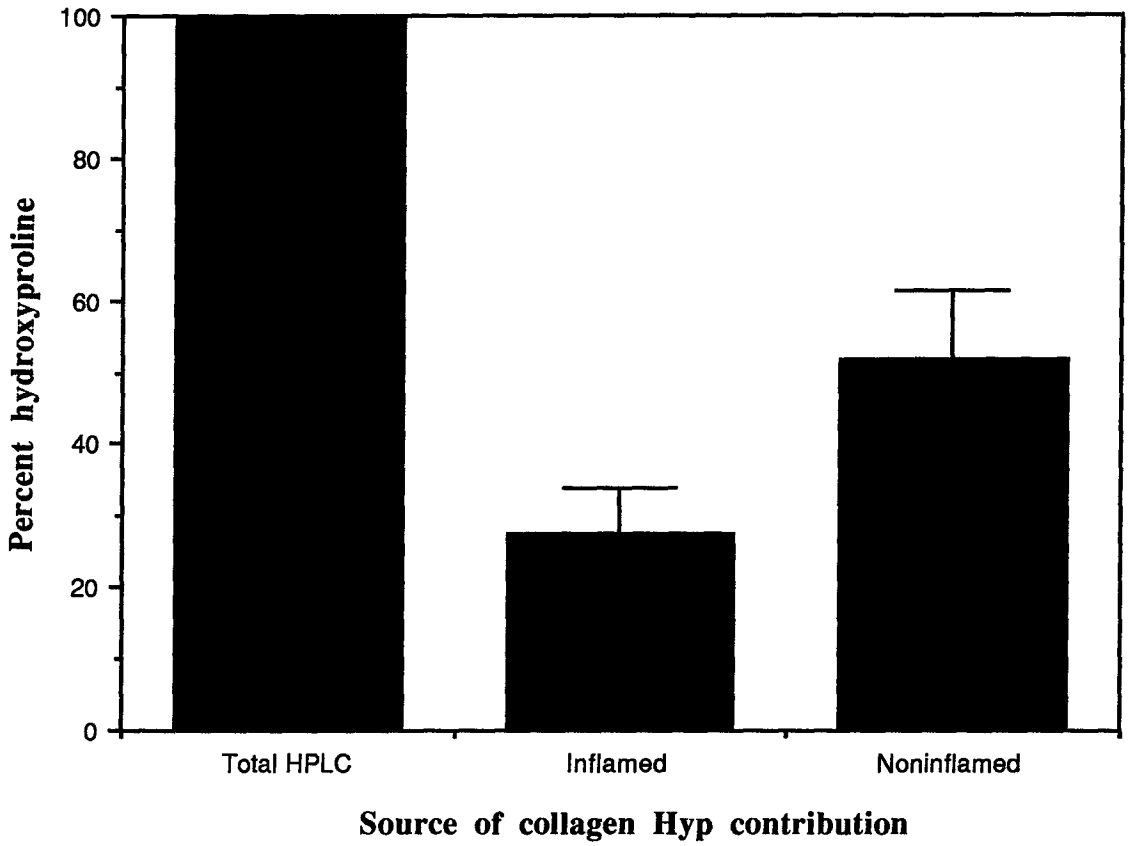

Figure 3.23: ELISA results using a polyclonal antibody to type I collagen. Type I collagen derived Hyp contributions to crevicular fluid from inflamed and noninflamed sites, compared to total Hyp content of crevicular fluid. Error bars represent the standard error of the mean.

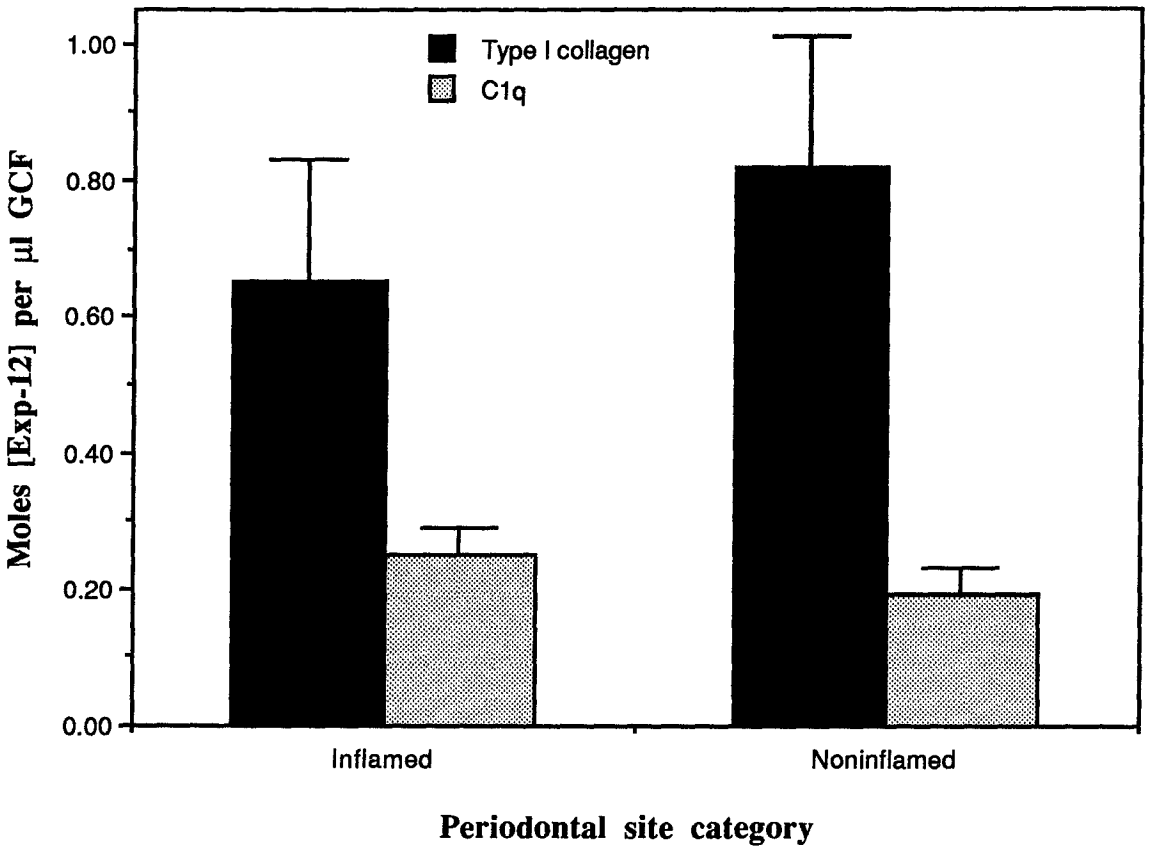

Figure 3.24: Moles {Exp -12} of type I collagen and C1q, per µl of crevicular fluid, in both inflamed and noninflamed periodontal sites. Error bars represent the standard error of the mean.

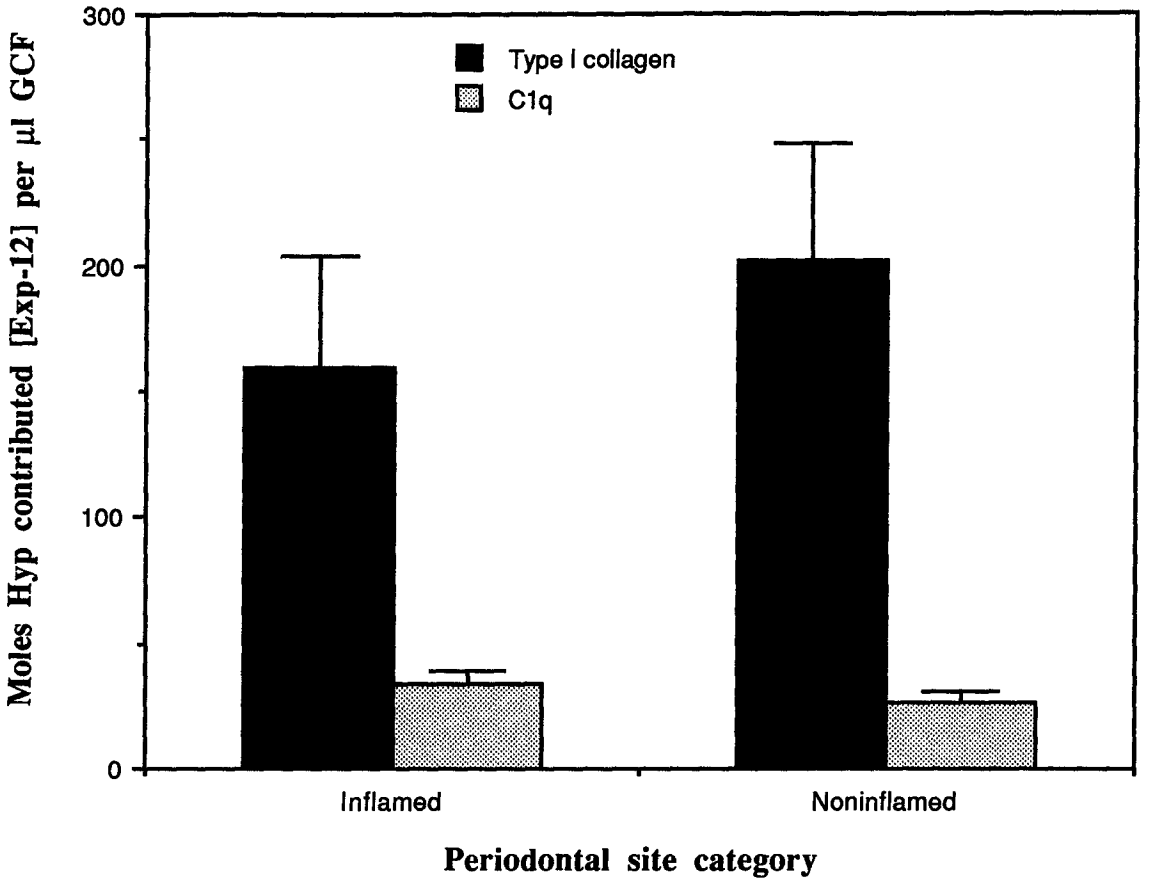

Figure 3.25: Moles of Hyp {Exp -12} contributed by type I collagen and C1q, per µl of crevicular fluid, from inflamed and noninflamed periodontal sites. Error bars represent the standard error of the mean.

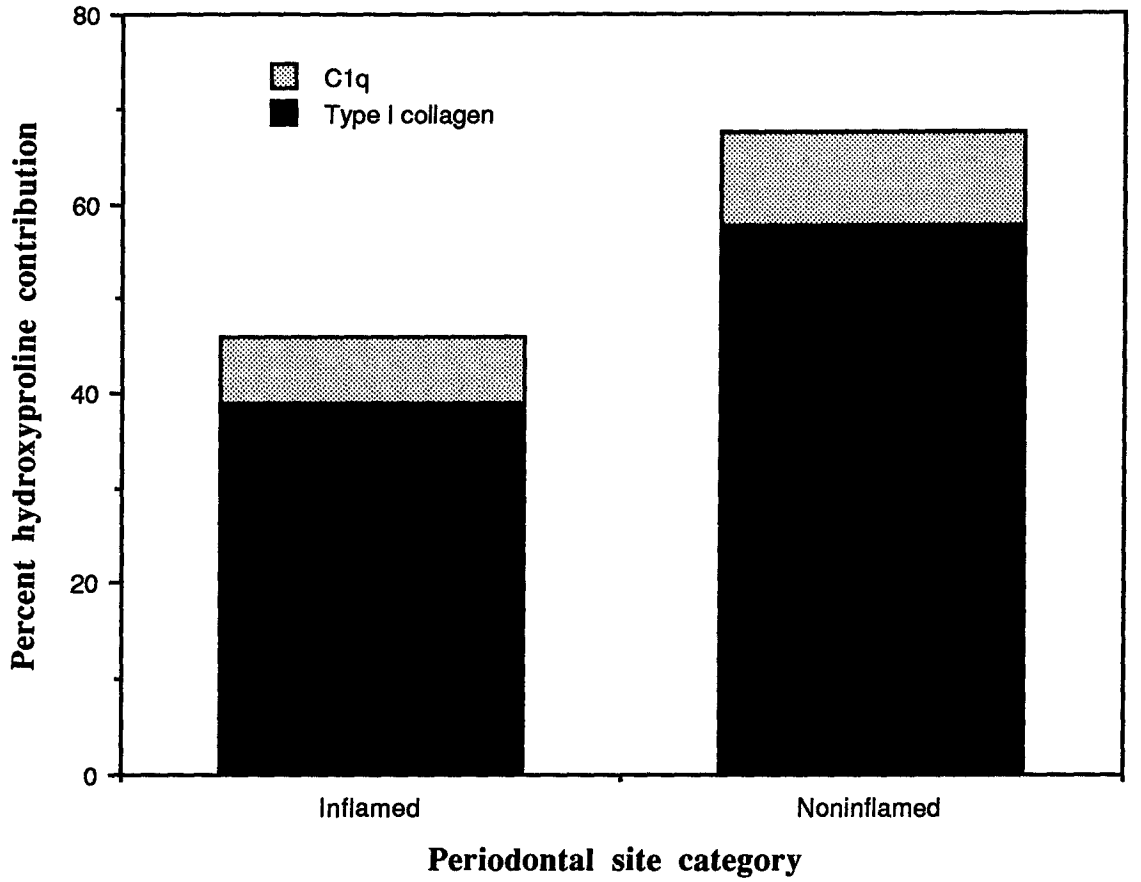

Figure 3.26: Crevicular fluid Hyp contributions from type I collagen and C1q from inflamed and noninflamed periodontal sites as measured by ELISA. The 100 percent level corresponds to the level of Hyp determined by HPLC.

3.4 IMMUMOLOGICAL FINDINGS IN CREVICULAR FLUID FROM INFLAMED AND NONINFLAMED PERIODONTAL SITES

3.4.1 SDS/PAGE gels

Native and denatured type I collagen and C1q preparations were separated by SDS/PAGE gels to determine banding profiles for these components. Figure 3.27 shows silver stained gel profiles of native and denatured forms of these components. Alternating denatured and native samples are shown for 0.5 μg of rat tail type I collagen (lanes 2, 3, 9, 10), 0.5 μg human placental type I

collagen (lanes 4, 5, 11, 12), and 0.2 μg human C1q (lanes 6, 7, 13, 14). Typical profiles for native type I collagen exhibiting β1,1, β1,2, α1 and α2 bands are seen in lanes 3, 5, 10, and 12. Denatured samples demonstrate a significant loss in these bands, particularly β1,1 and β1,2, and an emergence of numerous bands with molecular weight below 100K. A typical strong band for native AB chain of C1q at 66K is present in lanes 7 and 14, while a prominent band typical for denatured AB chain of C1q at 39K is present in lanes 6 and 13.

A SDS/PAGE profile of native and denatured pooled GCF from inflamed and noninflamed sites is shown is Figure 3.28. Pooled GCF from two groups of patient matched inflamed and noninflamed sites are represented. From a group F designated patient pool (lanes 7-10), native and denatured GCF samples exhibited more intense and diverse banding for inflamed than noniflamed sites. This same trend was also found in a group E designated patient pool (Lanes 11-14).

3.4.2 Western Blotting

A western blot of anti-human type I collagen and anti-human C1q reacted to duplicate type I collagen and C1q standards are shown in Figure 3.29. When lanes 1 through 7 were developed using anti-human type I collagen antibody, distinct reactions to type I collagen (lanes 4-7) but none to C1q (lanes 2, 3) were seen for both native and denatured preparations. Similarly, when lanes 9 through 15 were developed using anti-human C1q antibody, distinct and intense reactions were obtained to C1q (lanes 9, 10) with barely detectable reaction to native type I collagen (lanes 11, 13).

Western blot analyses were performed on denatured pooled GCF from four groups of patient matched inflamed and noninflamed sites. These substrates were developed using either anti-human type I collagen (Figure 3.30) or anti-human C1q (Figure 3.31). In anti-collagen developed blots, all four patient matched groups exhibited intense reactions for type I collagen with more intense reaction for small peptides in inflamed than noninflamed sites. In anti-C1q developed blots, all four patient matched groups exhibited extremely weak reactions for C1q in comparison to the C1q standard (lanes 7, 14).

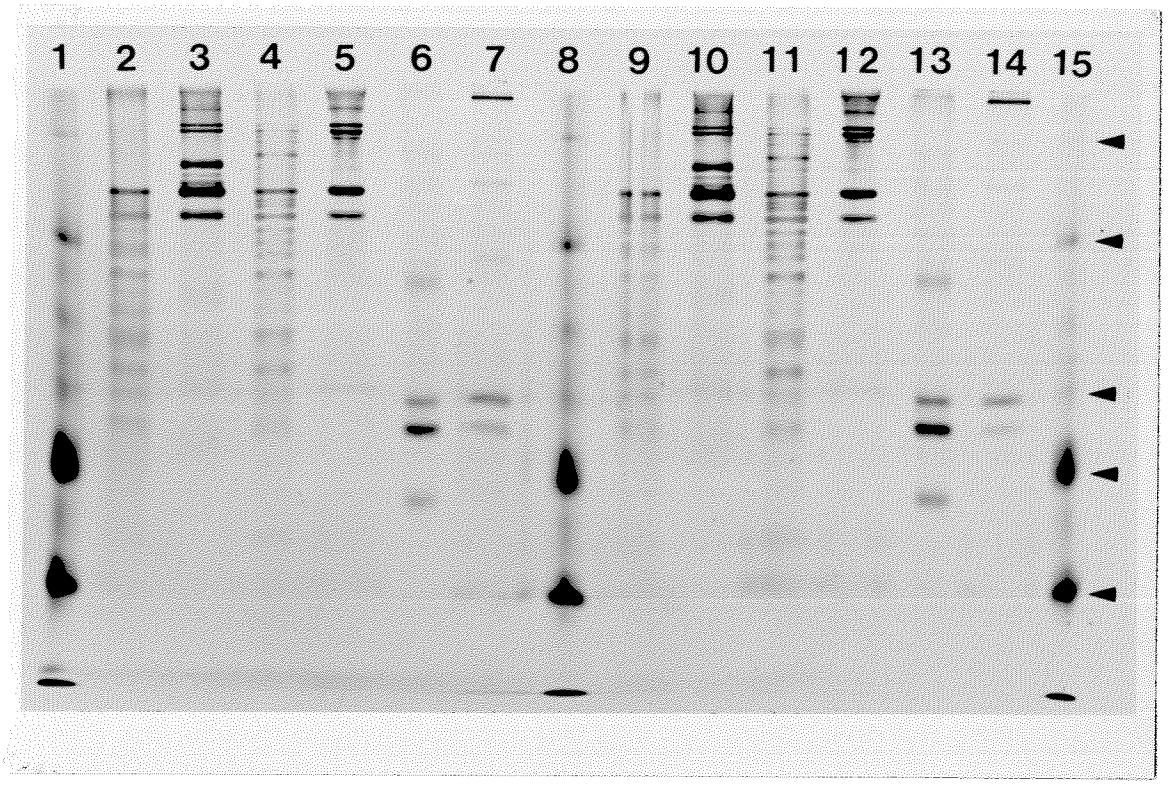

Figure 3.27 SDS/PAGE gel of type I collagen and C1q standards. Arrows indicate molecular weight markers, from top to bottom, respectively: 200,000 D (myosin H chain), 97,400 D (phosphorylase b), 68,000 D (bovine serum albumin), 43,000 D (ovalbumin), 29,000 D (carbonic anhydrase).

Lane 1: High molecular weight standard
Lane 2/9: Denatured (D) rat tail type I collagen
Lane 3/10: Native (N) rat tail type I collagen
Lane 4/11: D - human placental type I collagen
Lane 5/12: N - human placental type I collagen
Lane 6/13: D - human C1q
Lane 7:/14 N - human C1q
Lane 8:/15 High molecular weight standard

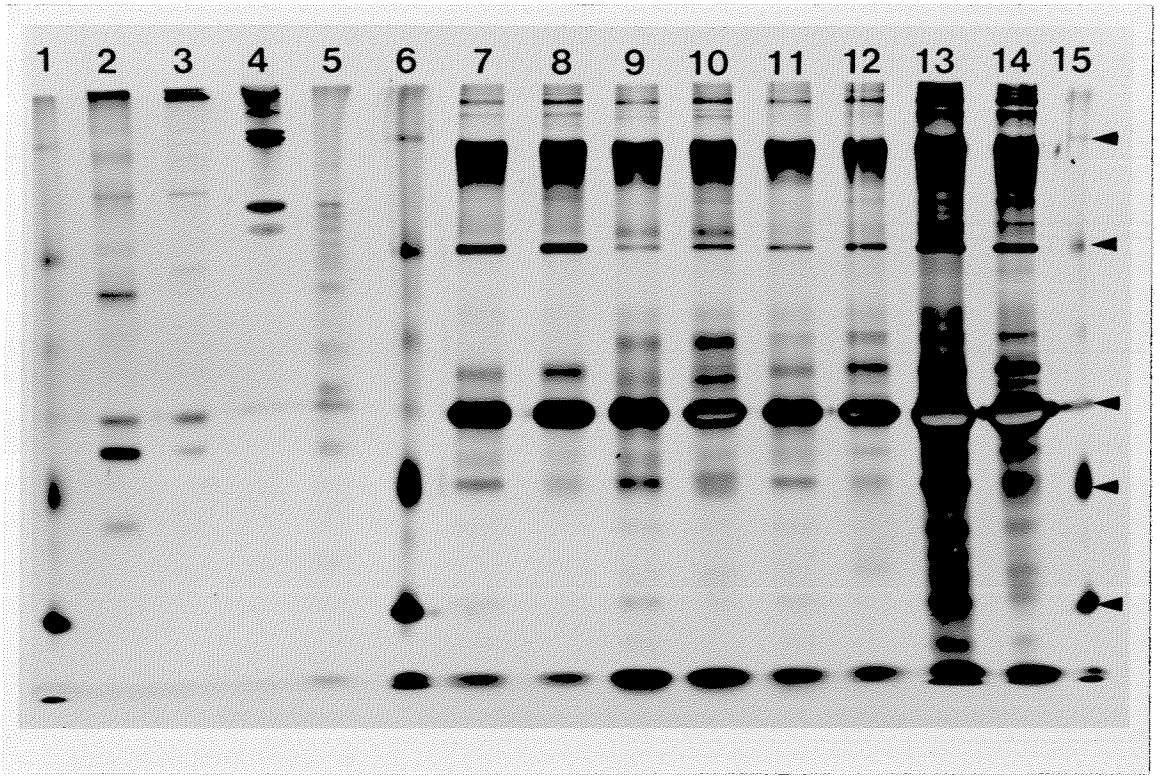

Figure 3.28 SDS/PAGE gel of native and denatured patient matched pooled GCF from inflamed and noninflamed periodontal sites. Arrows indicate molecular weight markers, from top to bottom, respectively: 200,000 D (myosin H chain), 97,400 D (phosphorylase b), 68,000 D (bovine serum albumin), 43,000 D (ovalbumin), 29,000 D (carbonic anhydrase).

Lane 1: High molecular weight standard
Lane 2: Denatured (D) human C1q
Lane 3: Native (N) human C1q
Lane 4: N - human placental type I collagen
Lane 5: D - human placental type I collagen
Lane 6 High molecular weight standard
Lane 7 Patient group F: D - noninflamed GCF
Lane 8: Patient group F: N - noninflamed GCF
Lane 9: Patient group F: D - inflamed GCF
Lane 10: Patient group F: N - inflamed GCF
Lane 11: Patient group E: D - noninflamed GCF
Lane 12: Patient group E: N - noninflamed GCF
Lane 13: Patient group E: D - inflamed GCF
Lane 14: Patient group E: N - inflamed GCF
Lane 15: High molecular weight standard

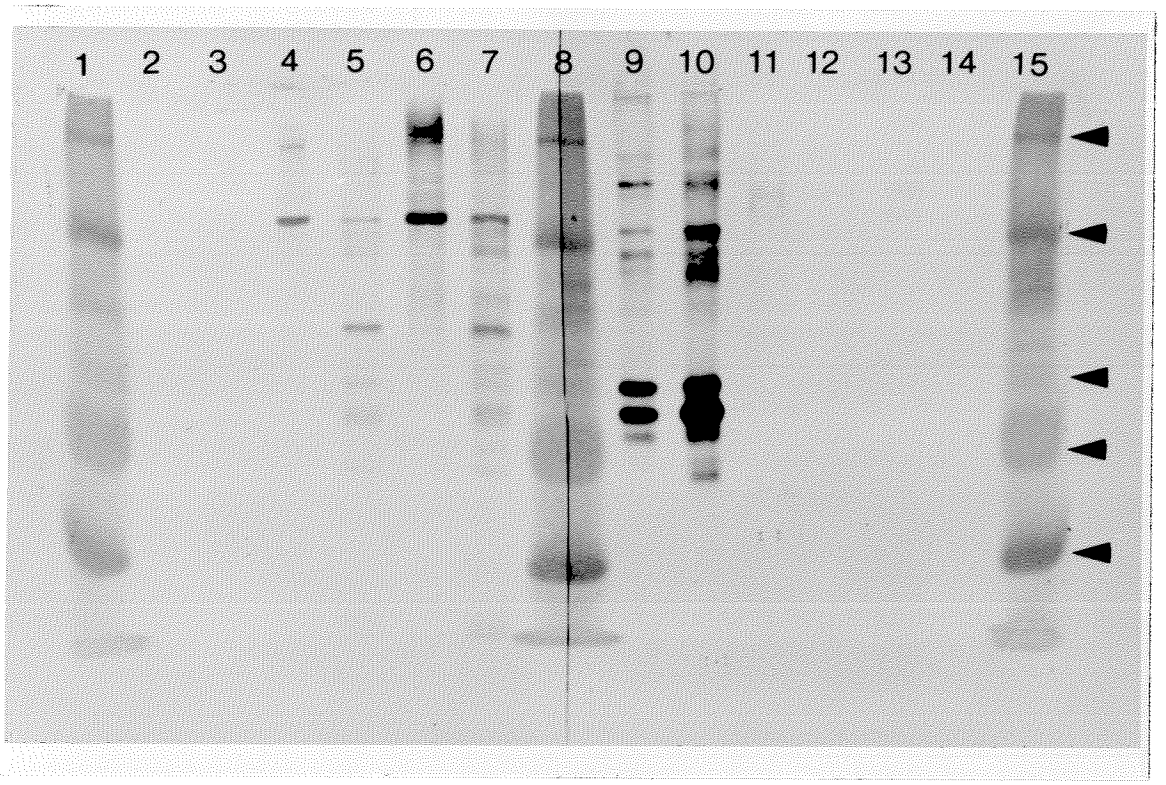

Figure 3.29 Western blot of type I collagen and C1q standards. Arrows indicate molecular weight markers, from top to bottom, respectively: 200,000 D (myosin H chain), 97,400 D (phosphorylase b), 68,000 D (bovine serum albumin), 43,000 D (ovalbumin), 29,000 D (carbonic anhydrase).

Lane 1: High molecular weight standard
Lane2: anti-human type I collagen reacted with Native (N) human C1q
Lane 3: anti-human type I collagen reacted with Denatured (D) human C1q
Lane 4: anti-human type I collagen reacted with N - human placental type I collagen
Lane 5: anti-human type I collagen reacted with D - human placental type I collagen
Lane 6: anti-human type I collagen reacted with N - type I rat tail collagen
Lane 7: anti-human type I collagen reacted with D - type I rat tail collagen
Lane 8: High molecular weight standard
Lane 9: anti-human C1q reacted with N - human C1q
Lane 10: anti-human C1q reacted with D - human C1q
Lane 11: anti-human C1q reacted with N - human placental type I collagen
Lane 12: anti-human C1q reacted with D - human placental type I collagen
Lane 13: anti-human C1q reacted with N- type I rat tail collagen
Lane 14: anti-human C1q reacted with D - type I rat tail collagen
Lane 15: High molecular weight standard

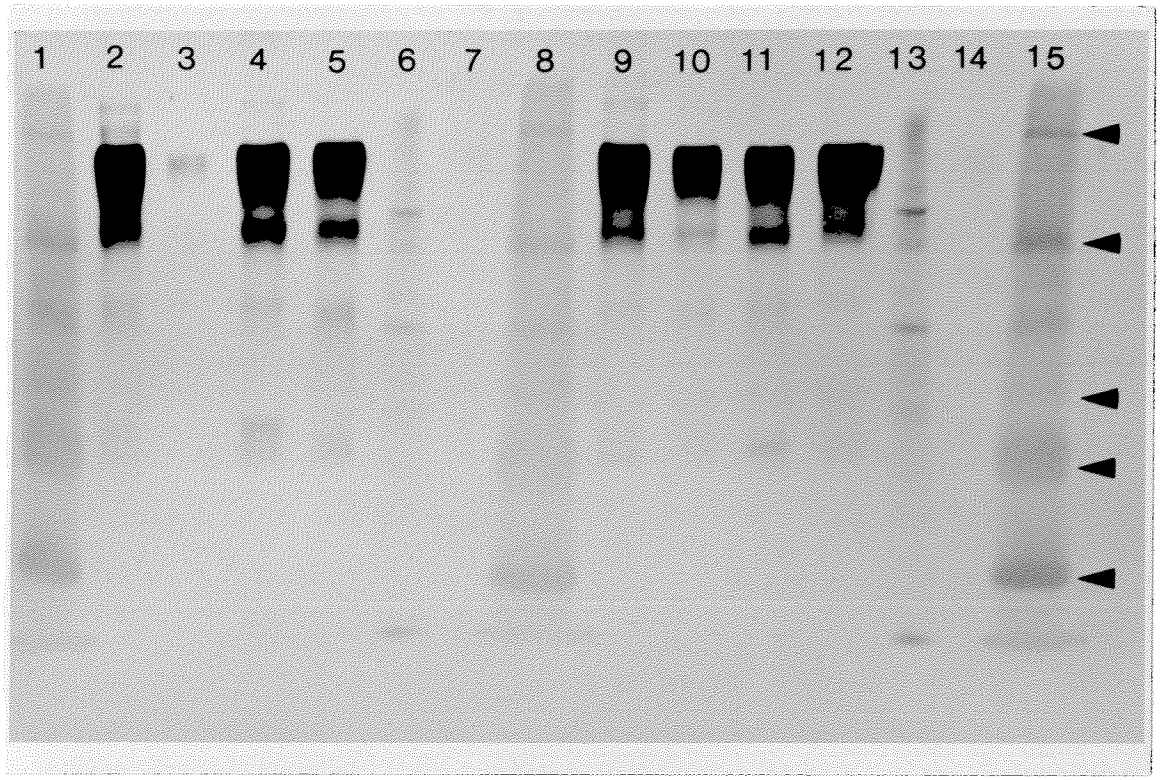

Figure 3.30 Western blot of anti-human type I collagen reacted with denatured patient matched pooled GCF from inflamed and noninflamed sites. Arrows indicate molecular weight markers, from top to bottom, respectively: 200,000 D (myosin H chain), 97,400 D (phosphorylase b), 68,000 D (bovine serum albumin), 43,000 D (ovalbumin), 29,000 D (carbonic anhydrase).

Lane 1: High molecular weight standard
Lane 2: Patient group A: Inflamed GCF
Lane 3: Patient group A: Noninflamed GCF
Lane 4: Patient group B: Inflamed GCF
Lane 5: Patient group B: Noninflamed GCF
Lane 6: Human placental type I collagen
Lane 7: Human C1q
Lane 8: High molecular weight standard
Lane 9: Patient group E: Inflamed GCF
Lane 10: Patient group E: Noninflamed GCF
Lane 11: Patient group F: Inflamed GCF
Lane 12: Patient group F: Noninflamed GCF
Lane 13: Human placental type I collagen
Lane 14: Human C1q
Lane 15: High molecular weight standard

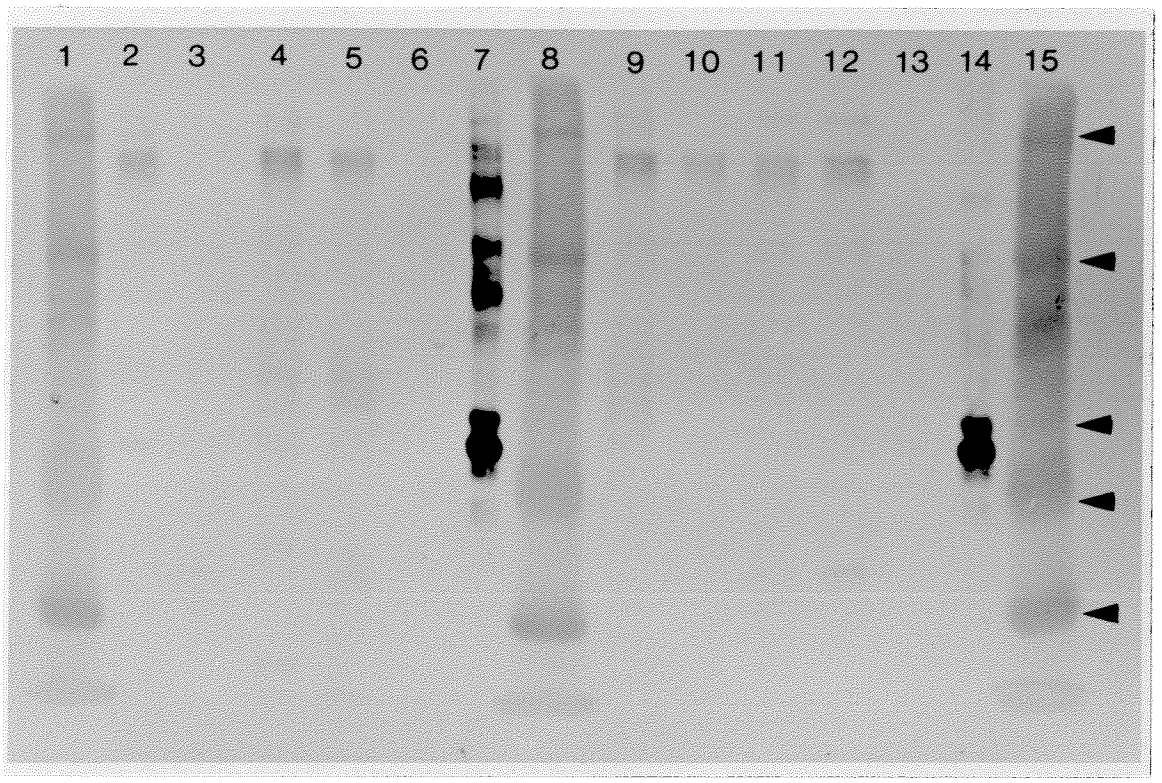

Figure 3.31 Western blot of anti-human C1q reacted with denatured patient matched pooled GCF from inflamed and noninflamed sites. Arrows indicate molecular weight markers, from top to bottom, respectively: 200,000 D (myosin H chain), 97,400 D (phosphorylase b), 68,000 D (bovine serum albumin), 43,000 D (ovalbumin), 29,000 D (carbonic anhydrase).

Lane 1: High molecular weight standard
Lane 2: Patient group A: Inflamed GCF
Lane 3: Patient group A: Noninflamed GCF
Lane 4: Patient group B: Inflamed GCF
Lane 5: Patient group B: Noninflamed GCF
Lane 6: Human placental type I collagen
Lane 7: Human C1q
Lane 8: High molecular weight standard
Lane 9: Patient group E: Inflamed GCF
Lane 10: Patient group E: Noninflamed GCF
Lane 11: Patient group F: Inflamed GCF
Lane 12: Patient group F: Noninflamed GCF
Lane 13: Human placental type I collagen
Lane 14: Human C1q
Lane 15: High molecular weight standard

4. DISCUSSION

4.1 VOLATILE COMPOUNDS IN THE ORAL CAVITY

This study demonstrates that it is possible to collect and analyze volatile sulphur compounds from specific gingival crevicular sites, and to compare their content to that of mouth air. This was accomplished through the development of an apparatus for collection and total retention of volatile compounds found in crevicular air. The study provides clear evidence that both the composition and content of sulphides from these two sources are distinct.

In contrast to sulphide levels of mouth air that were determined by other investigators under restricted conditions, the differences in crevicular air sulphides for the various parameters in this study were achieved on relatively low levels of VSC that were collected without any restrictions. Hence, their levels of VSC of mouth air are significantly greater than the concentrations found in the patients used in the present study. Our results indicate that it is possible to obtain statistically significant results regardless of the levels of VSC found in any patient. The fact that the comparisons were performed on the ratio of low amounts of sulphides found in the gingival crevice, underscores the sensitivity and significance of this comparison.

Furthermore, the studies of mouth air in periodontal patients also utilized an optimum sampling time for patient breath. Yaegaki (Yaegaki 1990; Yaegaki 1991), in his mouth air studies of periodontal subjects, assayed mouth air in the early morning under restricted conditions, which is known to yield the highest levels of sulphides (Tonzetich 1978). In the present study, the patients were sampled throughout the day, and were not restricted in eating, drinking or exercising oral hygiene procedures prior to testing, as is generally the case in mouth air studies. Hence, the levels of mouth air volatiles in this study are considerably lower than seen in controlled early morning mouth air studies. It is interesting that the ratio for CH_3SH to H_2S in crevicular air of control subjects (.37 +/- .18SE) is similiar to the CH_3SH to H_2S ratio in mouth air of the control group (.5) observed in another investigation (Yaegaki 1990). However, this sulphide ratio in test groups for the mouth air study of periodontally involved subjects (4.6) is quite different from that found in

crevicular air of either inflammed (.95 +/- .21SE) or deep (.71 +/- .16SE) gingival sites. The fact that the tongue is the principal source of mouth air sulphides in periodontal patients and that mouth air analyses were performed on early morning samples provides a feasible explanation for the disparity of the test group ratio. Yaegaki and Sanada found that the VSC production on tongues of patients with periodontal disease was significantly higher than those of controls, and that the CH_3SH / H_2S ratio was significantly reduced by removal of the tongue coatings (Yaegaki and Sanada 1991). From this they concluded that salivary putrefaction did not substantially contribute to the elevated ratio of mercaptan in mouth air and that in addition to periodontal involvement, tongue coatings play an important role in VSC production leading to an elevated concentration of methyl mercaptan in periodontally involved individuals.

It is noteworthy that the VSC content of an individual periodontal site is dependent upon the degree of gingival inflammation. Other investigators have shown that H_2S and CH_3SH content in mouth air correlated with the incidence and depth of periodontal pockets in excess of 3 mm. In addition, implementation of periodontal therapy consisting of gingival curettage and periodontal surgery was found to reduce levels of both H_2S and CH_3SH of mouth air (Tonzetich and Spouge 1979). Furthermore, Kaizu and coworkers, found that in a group of periodontal subjects with halitosis, that the CH_3SH content in mouth air was observed to have a higher correlation with gingival inflammation but showed no correlation with pocket depth or bone loss (Kaizu et al. 1978). The results of the current study are the first known to date that compare gingival inflammation with the volatile sulphur content of a specific periodontal site.

With regard to sulphide ratios, it is noteworthy that CH_3SH to H_2S was the most significant ratio investigated. A recent study by Yaegaki on the composition of H_2S and CH_3SH in mouth air of periodontally involved subjects, found that not only were the levels of CH_3SH greater than those of H_2S, but also that the ratio of CH_3SH to H_2S was significantly higher ($p < .05$) than for a control group (Yaegaki and Sanada 1991). Compared to the total methyl sulphide to H_2S ratio, the present study found that the CH_3SH to H_2S ratio was the only sulphide ratio in crevicular air that gave a statistically significant result ($p < .01$). Contributing factors to this result were frequencies

and amounts of dimethyl sulphide and dimethyldisulfide whose levels were below that of the methyl mercaptan. In addition, the levels of $(CH_3S)_2$ in deep and inflamed sites showed more variation in frequency and amount than did the levels of CH_3SH. The resulting large standard deviations of the means gave rise to levels of $(CH_3S)_2$ in control and test systems that were not significantly different ($p>.05$). The fluctuation and weak appearance of this sulphide are further evidence that methyl mercaptan is a preferred component to use for sulphide comparisons. Although CH_3SH occurred in all but one of the examined crevicular sites, the levels in control subjects were well below that for the corresponding test group. As the levels of both H_2S and CH_3SH are dependent upon a number of factors such as microbial activity and sampling time, it seems appropriate to compare the ratios of these sulphides as an indicator of the composition of VSC in the gingival crevice.

A related investigation which measured the levels of H_2S production in GCF concluded that the levels of H_2S correlated with both the gingival index and the crevicular fluid volume (Solis-Gaffar et al. 1980). This conclusion is in agreement with the present study, which shows that the levels of total sulphides is greater in inflamed over noninflamed sites. These investigators measured H_2S after each filter paper strip containing crevicular fluid was incubated with L-cysteine for 3 days. They reported that the generation of H_2S in the GCF could be due to either the presence or gram-negative organisms or cysteine desulfhydrase activity. In the present study, the levels of various sulphides were measured directly on individual crevices, as they existed *in vivo*.

The generation of higher levels of sulphides in deeper and inflamed gingival crevices over control systems suggests that such an environment is conducive for establishment of gram-negative anaerobic pathogens. Because H_2S and CH_3SH are known to be both directly and indirectly detrimental to mucosal tissues (Ng and Tonzetich 1984), the measurement and characterization of these volatiles achieved in this investigation could be a useful method for monitoring the initiation and activity of periodontal disease.

Not only can VSC serve as indicators for distinguishing inflamed from noninflamed sites, but they have been shown to have significant effects on periodontal tissue. There are several

mechanisms whereby the VSC can act on periodonal connective tissues. They can increase the permeability of the mucosa, change cell metabolism, and directly and indirectly alter the structure of the tissue components.

Ng and Tonzetich have shown that the permeability from epithelial surface to underlying connective tissue is potentiated by exposure of the epithelial surface to thiols (Ng and Tonzetich 1984). They observed that after exposure to mercaptan, both small ions and molecules such as PGE_2 penetrated more readily across the mucosa, thus providing indirect evidence for disruption of basement membrane components. Subsequently it has been shown that thiols degrade FN, laminin, and type IV collagen, all which are known to possess disulfide linkages, as evidened by analyses using SDS/PAGE gels (MacKay et al. 1989). Specifically it was demonstrated that mercaptan directly disrupted these known basement membrane components.

Once volatile sulphur compounds penetrate the epithelial barrier, they can then react with the underlying connective tissue and accompanying cells. Direct effects of VSC on collagen have been demonstrated. Exposure to either H_2S or CH_3SH of type I collagen resulted in the conversion of some of the mature fibrillar (Tonzetich and Lo 1978) and acid-soluble preparations (Johnson and Tonzetich 1985; Johnson et al. 1985) to a neutral salt-soluble product. Analyses of these products revealed that radiolabelled H_2S was associated with $\alpha 1$, $\beta_{1,1}$, $\beta_{1,2}$, and $\alpha 2$ chains characteristic of type I collagen. Furthermore, it was determined that thiol groups (-SH) reacted with 2 or more active sites on the molecule (Tonzetich and Johnson 1986). This provided evidence that thiols directly disrupt extracellular collagen, which may make it more susceptible to enzymatic degradation and contribute to increased collagen destruction observed in periodontal disease.

Methyl mercaptan was also found to have a more adverse effect on fibroblast cell metabolism. In the presence of 10 ng of H_2S or CH_3SH/ml air/CO_2, a 35% and 36% reduction in proline transport, respectively, was found in fibroblast cell cultures (Tonzetich et al. 1985). Further analyses using vital cell staining indicated damage to cells exposed to CH_3SH. Since the uptake of amino acids is a membrane-associated phenomenon, disruption of cell membranes would affect protein synthesis.

Other detrimental cellular effects of VSC have recently been described (Johnson et al. 1992). Exposure of fibroblast cell cultures to CH_3SH resulted in a 25% decrease in total protein content, and a 44% supression of DNA synthesis. There was also a reduction in type III collagen and almost a complete absence of type III procollagen. In addition, type I collagen content was reduced, whereas type I procollagen and/or type I protrimer was increased two-fold. These results demonstrated the adverse effects of CH_3SH on fibroblast cells.

Methyl mercaptan has also been shown to potentiate collagenolytic proteases, IL-1 and cAMP production by human gingival fibroblast cultures. Cathepsin B activity is increased by 20% while cAMP content of CH_3SH exposed cultures is increased by 34% (Tonzetich et al. 1990).

CH_3SH was also shown to activate the immune system. Recent investigations by Ratkay and Tonzetich, have shown that exposure of T lymphocytes to CH_3SH results in a 30% increase in IL-1 production (Ratkay and Tonzetich 1992). In addition, exposure of fibroblasts to CH_3SH increased collagenase production. Thus, it is possible that CH_3SH potentiates collagenase production either by interacting directly with the fibroblast cell, and/or via an IL-1 mediated pathway. It has been shown that IL-1 produces more collagenase and plasminogen activator, which under the influence of CH_3SH creates plasmin which converts procollagenase to collagenase. Richards and Rutherford have shown that recombinant IL-1 added to fibroblasts from the periodontal ligament and gingival connective tissue will induce the production of prostaglandin E_2 (PGE_2) and collagenase (Richards and Rutherford 1988). Since IL-1, PGE_2 and collagenase each play a role in tissue degradation, CH_3SH is seen here to potentiate the pathogenesis of periodontal disease.

Although thiols are considered inhibitory to collagenase we observed here that they also cause an increase in collagenase production. It is known that collagenase can be activated by organomercurials which may change the molecular conformation of the enzyme, indicating that the thiol-reacted collagenase can be reactivated. Whereas thiol groups activate bacterial collagenase, they inhibit mammalian collagenase. For example, cysteine normally is used to activate collagenase in bacterial systems.

The same situation is analogous to production of α2 macroglobulin (α2-MG) in periodontal pockets. Although one would expect increased inactivation of collagenase by increased levels of α2-MG in periodontal pockets, in reality, higher levels of active collagenase in periodontal pockets was observed (Uitto and Raeste 1978). It was postulated that there are other mechanisms, such as action of proteolytic enzymes, which cleave the α2-MG-collagenase complex to free collagenase (Uitto and Raeste 1978)

The present study is the first known successful attempt to collect and analzye VSC from individual periodontal sites using a non-invasive sampling technique. Thiols have been shown to disrupt basement membrane components and interstitial collagen molecules and stimulate the immune system. Exposure of fibroblast cell cultures to thiol compounds increases both IL-1 and cAMP production. These in turn increase the production of proteases, such as collagenase, elastase and plasminogen activator. These enzymes would lead to the destruction of collagen. The presence of VSC in periodontal pockets parallels the pattern of establishment of gram-negative micororganisms (Tonzetich and McBride 1981). These collagenase-producing organisms are strong producers of VSC which probably play a role in activation of their proteolytic enzymes.

4.2 HYDROXYPROLINE AS A MEASURE OF PERIODONTAL TISSUE ACTIVITY

The initial question was: can collagen breakdown products be detected at periodontal sites that are undergoing disease alteration? Since collagen makes up most of the protein in the periodontium, it was reasonable to study its metabolism. Collagen is known to have a unique amino acid composition, specifically the presence of Hyp and Hylys. A further question arises: can Hyp be quantified in periodontal sites?; furthermore, can Hyp levels be correlated with active periodontal breakdown? The hypothesis of this study was that increased levels of Hyp occur at periodontal sites undergoing active breakdown. It was conjectured that increased Hyp levels in GCF would reflect the amount of collagen metabolism in periodontal connective tissue.

Analyses of Hyp in body fluids and tissue have been used as measures of collagen metabolism in pathological states. Urinary Hyp excretion was found to be increased in a group of 11 subjects during phases of mild vitamin C deficiency (Hevia et al. 1990). In a study of 33 patients with chronic liver disease, both the urinary excretion of Hyp and the hepatic content of Hyp were increased in relation to the severity of the liver disease (Yamada et al. 1989).

At the time of our investigation the most recent study of Hyp in GCF was reported by Miller and co-workers (Miller et al. 1982). They used HPLC employing pre-column derivatization with dansyl chloride to measure Hyp content in gingival exudate. They found that the Hyp content ranged from 300 to 1480 ng/mg exudate weight.

In testing this dansyl chloride pre-column derivatization method, we observed that the standard Hyp peak small and that it gave low responsiveness and variably appeared at retention times greater than 30 minutes. The same limitations using dansyl chloride were also found by other investigators who demonstrated that the Hyp peak was difficult to detect, had a low responsiveness and was very close to the glutamine peak (Wiedmeier et al. 1982).

A recently developed HPLC method in our laboratory utilized derivatizations with PITC alone or in combination with OPA to detect both Hyp and Pro in biological materials (Yaegaki et al. 1986). In the crevicular fluid analysis, it was found that a single derivatization step using PITC was sufficient and reliable to separate Hyp from other amino acids. This one step technique yielded Hyp as a cleanly separate peak. However, the proline peak in the chromatogram remained unresolved and could only be separated from alanine using a combined OPA/PITC derivatization technique.

The results of Hyp analysis of GCF showed that there was more Hyp in hydrolyzed than in unhydrolyzed crevicular fluid. There are also more amino acids in general as evidenced by comparing profiles of hydrolyzed versus unhydrolyzed GCF. This observation implied that in GCF there was less Hyp in a free amino acid form than in bound or peptide form. This finding is in agreement with Hyp analyses of urine using HPLC. Hughes and co-workers demonstrated that free urinary Hyp levels are substantially lower (2-29 μmoles/24 hrs) than total Hyp urinary levels

(122-374 μmoles/24 hrs) in healthly individuals (Hughes et al. 1986). Therefore, all subsequent Hyp analyses were performed on hydrolyzed samples of GCF.

In the spiramycin data, Hyp levels were presented as either total moles of Hyp per GCF sample or in moles Hyp/μl of GCF. These units were used to compare the values of total Hyp in a given GCF sample versus Hyp level per unit volume. Comparisons with periodontal measurements were performed using both types of Hyp values.

The total Hyp content was generally higher for inflamed than for noninflamed sites at each time point in the study. This trend was seen in spiramycin and non-drug treatment groups, but was not observed for weighted Hyp values. At week zero, total moles of Hyp in each sample were found to be greater in inflamed (23.4 +/- 3.4 picomoles) then in noninflamed (16.4 +/-2.1 picomoles) sites. However, when weighted, Hyp values at inflamed sites (50.7 +/-6.9 picomoles) were slightly less than those at noninflamed (57.2 +/-11.8 picomoles) sites. None of these differences were large enough to be statistically significant ($p>.05$).

When Hyp values were examined in both treatment groups throughout the study regardless of clinical parameters, a distinct trend was evident. For weighted Hyp values, in comparison to the baseline, Hyp content in the spiramycin treated group was consistently higher than the values in the non-drug group. This was an important finding as it was later found that overall increases in weighted Hyp levels were present at week 12 for healing periodontal sites. However, total Hyp levels for all examination points for both drug and non-drug treatment groups showed no particular trend.

At week zero both weighted and total Hyp values were higher in inflamed than in noninflamed sites of pockets < 4 mm, whereas these Hyp measures were both similar in pockets ≥ 4 mm. When only pocket depths were considered both weighted and total moles were larger for pockets depths < 4 mm. However, none of these differences were statistically significant ($p>.05$).

Hyp content at inflamed and noninflamed periodontal sites of patients in the 24 week spiramycin study exhibited fluctuations at given time points (0, 2, 8, 12 and 24 weeks) and between time points throughout the study. These fluctuations were seen with both types of Hyp

measurements. Similar fluctuations were also seen in weekly measurements of Hyp by Svanberg in a 5 week experimental gingivitis study using a beagle dog model (Svanberg 1987a). This indicated to him that collagen metabolism was not a linear process. In a further beagle dog study using a 9 day ligature-induced periodontitis model, Svanberg found that collagen derived Hyp content was maximal four days after removal of the ligature. In our 24 week spiramycin study the variations of Hyp levels did not follow any distinct pattern. It was conjectured that since the Hyp values were derived from different sites at different time points, they were not being compared on an equal basis. Since there were no significant trends in Hyp values seen in either the spiramycin or non-drug treated group, this suggested that absolute values of Hyp content were not significant and useful for determining either the present disease state or the disease active state. Therefore, one may suggest that the data which would represent matched sites for different time points in the study may give better comparisions for Hyp levels.

Analyses of the data showed that the most complete matched data was available for 17 patients at examination points 0 and 12 weeks. Analyses of the 127 periodontal sites studied indicated that Hyp values correlated to bleeding on probing and attachment level changes. Hyp levels at week twelve in periodontal sites that were inflamed at week zero and remained inflamed at week 12 were higher than in sites that remained noninflamed. This relationship was stronger for weighted Hyp values than for total Hyp content of the GCF sample. In the spiramycin treated group a significant difference was found between Hyp levels at week 12 in these inflamed and noninflamed sites ($p<.05$). These results revealed it was necessary to examine the same site at different time points in order to detect and relate changes in Hyp content. Previous analysis of the entire data, not matched for site and patient, demonstrated non-significant fluctuations in Hyp content.

In this same group of 17 patients it was also determined that Hyp content was higher in sites that exhibited healing than sites that remained unresolved. This relationship was found to be true for weighted Hyp measures and not for total Hyp content found in the GCF sample. Healing was defined by a gain in attachment by ≥ 2 mm. Again, in the spiramycin treated group, this result was significant ($p<0.5$).

This result was in contrast to our preliminary observations of the data comparing Hyp content with attachment level changes. Preliminary analysis of data in the spiramycin study revealed that a gain in attachment between zero and subsequent examination intervals was accompanied by a decrease in Hyp content (Coil et al. 1987). These changes in Hyp levels were considered of great importance as it was anticipated that they precede changes in attachment level. However, this was only preliminary data and mainly observed changes in attachment level between weeks zero and week two of the study. This fall in Hyp content and improvement in attachment level was considered healing.

In Svanberg's ligature-induced periodontitis model in beagle dogs, it was also seen that 2 weeks following removal of ligature the Hyp values were significantly less than when the ligature was in place. This decrease in Hyp content was observed to persist for the remaining 4 weeks of the study. The Hyp values in this post-ligature phase were considered to represent collagen-derived Hyp, since total GCF Hyp was subtracted from serum Hyp. Thus, the maximal Hyp level at the time of ligature removal represents maximal collagen breakdown, whereas subsequent weeks indicate that collagen degradation is not as prominent.

Although no clinical measurements of the periodontium were presented in Svanberg's investigation, it is plausible that the four week period after ligature removal represents a stabilizing periodontal phase. Hyp levels have decreased as there is expected to be a 70% loss in collagen in the early destructive phase of periodontal disease (Page and Schroeder 1976). It is conjectured that after the stabilizing phase the periodontium would shift to a healing phase. This would be seen as improved attachment level, increased collagen production and increased collagen turnover. The latter would be reflected in a rise in Hyp content at healing sites. In the fourth and last week of the Svanberg study, the Hyp content was found to be the same or higher than the value at week 3 at 50% of the sites. This possibly represents a shift from a stabilizing phase to a healing phase.

It has been demonstrated that as much as 2 mm differences in probing attachment can occur due to inflammation of the junctional epithelium (Listgarten 1980). If early changes in attachment level occur, it is expected that they are due to improvements in the junctional epithelium, and do not

represent changes in functional attachment levels. Thus it is possible to observe early changes in attachment level measurements after therapy, which, in fact, only reflect changes in the inflammation of the JE. This phenomenon along with the decreased levels of Hyp found by Svanberg up to 4 weeks after ligature removal would explain why prelimary results during early examination points of the spiramycin study detected improvements in attachment levels with corresponding decreased in Hyp content.

As mentioned previously, our results showed that Hyp was increased in periodontal sites that exhibited healing as evidenced by a gain in probing attachment levels of ≥ 2mm. This finding was unexpected, and differed from our preliminary observations, where it was hypothesized that higher Hyp values would be found in clinically active sites. It was conjectured that sites that were undergoing periodontal breakdown would exhibit higher Hyp levels than other sites due to loss of connective tissue collagen. In fact, these sites which were losing probing attachment of ≥ 2mm over the twelve week period exhibited Hyp levels that were similar or less than sites that demonstrated no clinical change in attachment level. The finding that Hyp levels were higher in periodontal sites that were healing than sites that were unresolved was statistically significant in patients receiving spiramycin therapy.

In the spiramycin study it was of interest that weighted Hyp levels were highest in healing sites, as compared to those sites that either remained the same or even lost periodontal attachment over the twelve week time period. It was conjectured that the relative increase in Hyp levels was reflected by a higher turnover of collagen in healing sites. At all periodontally affected sites it was expected that 70% of the collagen was already lost during inflammatory periodonal disease (Narayanan and Page 1983). Once this collagen was lost, there would be relatively less collagen available for breakdown, due to a low amount present. This may provide an explanation as to why Hyp levels were lower in non-healing sites since they may represent a turnover of less collagen than that occurring in a healing site.

This result is in agreement with a recent investigation examining collagen metabolites in patients undergoing periodontal therapy. Talonpoika and co-workers examined procollagen III

aminoterminal propeptide (PIIINP) levels in GCF from periodontal patients before and after treatment (Talonpoika and Hämäläinen 1992). Using a radioimmune assay they found that there were significantly elevated PIIINP levels after periodontal therapy, which peaked at approximately 20 days post treatment and then gradually decreased back towards baseline levels by about day 40. These results indicated that the elevated PIIINP levels in GCF after periodontal treatment reflected an increased type III collagen synthesis in the gingiva. This is not only a significant finding from the standpoint of showing an increased presence of a collagen fragment in healing sites, but that the metabolism of collagen was over and above that found in the preexisting inflamed periodontal tissue. This was the only other known investigation to demonstrate that a specific collagen fragment, as a result of extracellular processing, was present in the GCF in greater amounts in healing periodontal sites than in pretreatment values.

One important distinction in the spiramycin study was that the Hyp determinations and clinical measurements were performed on a group of periodontitis-treated patients. The Hyp measurements beyond week 0 are taken from periodontal sites that have been identified to have a previous disease and have undergone treatment. Hyp values can be compared with clinical parameters only in relation to the effect of treatment. This is in contrast to studies that evaluate indicators in groups of untreated periodontitis subjects. In such patients, when a loss of attachment is observed, the disease process is unaltered by treatment therapies and is considered to occur spontaneously. Here the true effect of the disease process can be compared to a particular indicator being evaluated.

Some sites in the spiramycin study were observed to have lost ≥2 mm of attachment within 3 months of receiving therapy. They can be viewed differently than sites that had undergone clinical signs of attachment loss in untreated subjects, investigated in other studies. In treated sites the elements of the periodontium have been altered. Subsequent to treatment the host and microflora adapted to these new changes. By removing etiologic factors in the periodontium, the host could respond. It has been shown that an inflamed periodontal site that undergoes therapy can exhibit improved clinical probings values due to healing of the junctional epithelium (Listgarten 1980).

The next study involved the analyses of GCF from untreated patients who exhibited an inflamed and noninflamed site. The results indicated Hyp content was higher in inflamed periodontal sites than in noninflamed sites compared within the same patient. The difference was statistically significant ($p<0.001$) and in agreement with the spiramycin study of weighted Hyp measures at inflamed and noninflamed sites at weeks 0 and 12. Hence, both studies showed that Hyp can be used as an indicator of periodontal inflammation provided it is compared to healthy site measurements within the same patient.

Connective tissue degradation occurs as part of the active disease process. Monitoring for products of degradation would seem a logical means of following the course of the disease. Factors such as bacterial enzymes, and immunological factors are dependent upon the host response. In order to shift the balance away from a stable host response to a disease state, a change is required. The effect on connective tissue would represent shifts in host-parasite balance. Changes in connective tissue metabolism would reflect the status of the disease state.

A large number of indicators have been claimed to identify an active state of disease. Actually it is more correct to state that they represent a change that has occurred in the periodontium since the last examination point. It may be that the site has already undergone its destructive phase, and that the marker is merely an indication that the destruction has taken place. If, however, further clinical destruction is seen at the next time interval then this indicator should be considered to have represented a disease active state at the time of the previous examination. Furthermore, such an indicator would be considered a predictor, since it preceded clinical changes, such as future attachment level changes.

When evaluating a potential indicator of periodontal disease, one must consider that its usefulness will depend on whether it can determine or predict a particular state in a given patient. A good marker should differentiate a single positive test result for a condition in one individual from a test result in a control patient without the condition. For example, the range of values for a proposed indicator in both active and inactive disease states may overlap, and hence, for a given measured value for an individual, it would not be possible to predict with certainty his/her clinical

outcome. A good diagnostic marker should be quantitative and with reasonable confidence discriminate between one clincial outcome versus another.

There has been some controversy as to the detection of active disease sites using cutoff points in the measurements of changes in attachment level. Cut off points of ≥2 mm for periodontal disease activity considerations are discriminations that only recognize that changes in attachment level beyond that cutoff point are considered significant. This is thought to be an underestimation of actual sites undergoing disease activity. By using electronic probes with features of constant probing force and 0.1 mm resolution, it is possible to demonstrate earlier and more subtle changes in attachment level. It is expected that longitudinal monitoring of attachment level would detect more deteriorating sites that are actually undergoing changes in attachment level. It would be beneficial to monitor and detect more subtle changes in attachment level, which are below the threshold level. These sites may actually be undergoing destructive changes but go undetected and are not recognized until a considerable amount of attachment loss has occurred, which may be irreversible.

Several notable advances have been made toward the development of a diagnostic test for active periodontal disease. Analysis of crevicular fluid for aspartate amino transferase (AST) as an indicator of cell death, has been found to be significantly increased in GCF of patients that subsequently undergo periodontal degeneration as indicated by loss of periodontal attachment. Two well controlled studies by Perrson and co-workers have shown that increases in GCF AST correlates with loss of periodontal attachment and that GCF AST rises during experimental gingivitis and returns to normal when gingival health is restored (Persson et al. 1990a,b; Persson and Page 1992). These studies pose a question: Which cell death is being measured? Epithelial cells? Fibroblasts cells? Or is it simply host PMN's which are found in increased number in inflamed sites? Regardless of its source high levels of AST are a consequence of periodontal inflammation and destruction.

The reason why AST appears to be a good indicator is that is monitors a by-product of metabolism. Although AST is an enzyme, it is an intracellular enzyme whose presence

extracellulary implies cellular disruption. Such a product of metabolism would be seen in increased amounts during periods of high cell turnover. AST has been shown to act as a predictor as its levels were found to be increased before changes in attachment level were observed clinically. As it is considered a by-product of metabolism, concentrations of AST reflect the host-pathogen interaction balance, and indicate when a shift towards a pathogenic state is occurring.

Recently Uitto and co-workers have correlated salivary elastase levels with periodontal involvement in patients screened at the UBC dental clinic. Their results indicate that elastase activity in salivary rinses of periodontally involved subjects is significantly higher than in rinses from less severely involved periodontal patients and healthy controls. The sensitivity of this enzyme test identified true positive periodontitis patients 87%, and a true negative periodontitis patients 82 % of the time. The specificity of the test produced a low false positive rate of 18% and false negatives of 13%. This appears to be a good screening device to detect the existence of periodontal disease in the oral cavity.

In our studies, high levels of Hyp were found at specific periodontal sites during the disease state and at healing sites of treated patients. In terms of monitoring effectiveness of therapy, this information would be useful in identifying those sites which do not respond to treatment. In order to determine how Hyp levels respond in untreated patients, a further study is required that would monitor Hyp levels versus changes in attachment level.

4.3 CONTRIBUTION BY C1q AND COLLAGEN TO THE HYDROXYPROLINE CONTENT OF GINGIVAL CREVICULAR FLUID

During the spiramycin study a question arose regarding the relative contributions by collagen and serum derived C1q, to the total HPLC measured Hyp content of GCF. C1q, a subcomponent of the first complement component, has a molecular weight of approximately 409,600 and contains 4.3% Hyp by weight. It is found locally at the periodontal sites and binds strongly to fibrin, FN and laminin (Pearlstein et al. 1982; Entwhistle and Furcht 1988). C1q may play a role as part of a

scaffold during wound healing. C1q has been implicated to serve as an intercellular glue that acts as a matrix which is laid down at the periphery of tissue undergoing inflammation and perhaps pathological processes. It has been proposed that such a C1q matrix could act like a fibrin clot, and that the formation of C1q matrix precedes the fibrin clot.

In addition to forming a matrix, C1q has also been shown to bind to cells. *In vitro* experiments using periodontally-derived fibroblasts have shown that C1q acts as a cementing substance through a cell surface receptor (Bordin et al. 1990). Studies are in progress to determine the structure of this receptor. Thus, C1q not only acts as a matrix, but also binds cells of the connective tissue to form a diffusion barrier.

In order to determine the collagen-derived Hyp , it was necessary to remove C1q from GCF. To remove C1q from crevicular samples Svanberg (Svanberg 1987a,b) precipitated it with 0.02 M sodium acetate then removed it by centrifugation. We have also removed C1q by precipitating it with 0.5 M acetate solutions, followed by centrifugation, which was effective in removing from crevicular fluid samples 5 times greater concentrations of C1q than in serum (Coil and Tonzetich 1988). From these results it appears that the main source of Hyp following precipitation and centrifugation would be from collagen and possibly degraded C1q.

An additional experiment was performed to further investigate the contribution of Hyp from both C1q and collagen,and to confirm that higher levels of Hyp are present at inflamed periodontal sites. This study analyzed GCF collected from inflamed and noninflamed sites of 54 patients. The HPLC results confirmed that Hyp content was significantly higher ($p<.001$) in inflamed periodontal sites (684 +/- 63 picomoles/µl GCF) than in noninflamed sites (460 +/- 53 picomoles/µl GCF).

Aliquots of all GCF samples were examined for the presence of type I collagen using ELISA employing polycolonal antibodies to it. The results indicated that type I collagen was present in higher amounts in noninflamed sites than inflamed sites. A percentage of Hyp contributed by a collagen source was calculated and compared to the total Hyp level as determined from prior HPLC analyses. It was determined that type I collagen contributed 27.7 +/-6.1% of the total Hyp content

in GCF from inflamed sites. However, noninflamed sites contributed 51.7 +/-9.6% of the total Hyp content. This difference was statistically significant ($p<.05$).

Analyses of the same GCF for C1q was performed using ELISA employing monoclonal antibodies to C1q. These antibodies were harvested from hybridoma cells cultures, amplified in ascites fluid collected from mice, and were shown to be specific for C1q. The results obtained with this antibody preparation were unexpected. It was found that levels of C1q varied widely amongst the crevicular sites. When the C1q levels were determined and Hyp contribution was calculated and compared to total levels of Hyp, it was found that in a number of cases Hyp contribution far exceeded the maximum 100% level. This was unanticipated since the monocloncal antibody was tested for specificity and cross reactivity to related molecules. This antibody preparation was directed to the globular head region of C1q molecule and was expected to react specifically with C1q and not type I collagen (Kilchher et al. 1985). Therefore, these highly diverse C1q values in GCF are believed to be due an undetermined cross reactivity. Since this antibody was directed against the globular portion of C1q, it is possible that a cross reactivity may have occurred with another globular component.

When the ELISA was repeated using a polyclonal antibody to C1q the results demonstrated that the Hyp contribution by C1q to the overall levels of Hyp was minimal. C1q accounted for 6.9 +/- 1.1% of the total Hyp content in inflamed sites and 9.9 +/- 2.5% of the total Hyp content in noninflamed sites. The same trend as observed in collagen-derived Hyp also applied to C1q; a higher contribution of Hyp in noninflamed than in inflamed sites.

These ELISA results indicate that collagen is the major source of hydroxyproline in crevicular fluid with only very low amounts ascribed to C1q reactive peptides.

These results pose two perplexing questions. If Hyp content was higher in inflamed sites, why were the levels determined by ELISA of type I collagen and C1q higher in noninflamed sites? And secondly, what was the nature of the C1q found in the ELISA experiment? To solve these questions, crevicular fluid models were used to evaluate how type I collagen and C1q were affected by the analyses and how they could be affected in the periodontal environment.

Crevicular fluid models were used to evaluate the various forms of type I collagen and C1q that could be represented in a crevicular fluid sample. Due to enzymatic activity in the periodontium these parent molecules were expected to be present in various stages of degradation. It was conjectured that based upon the degree of cleavage, various sizes of fragments are present in crevicular fluid. These fragments could be separated in the following ways: ones that could or could not be precipitated in acetate (FrPPT+ or FrPPT-), and ones that could or could not react to antibodies directed against the parent molecule (FrAB+ or FrAB-). Conceptualization of which components would appear in crevicular fluid and acetate-precipitated GCF are shown in Figure 4.1.

The effectiveness of acetate precipitation was evaluated for both removal of C1q and the retention of type I collagen. It was found that 91.3% of C1q was removed in a 0.05 M sodium acetate buffer followed by centrifugation at 15,600g for 30 minutes at 4°C, while the level of type I collagen virtually was the same. This confirmed earlier precipitation reports which also removed C1q using acetate buffer (Svanberg 1987a,b). Thus the amount of C1q detected by ELISA would represent only that portion that would avoid precipitation and remain reactive to antibodies. In the case of collagen, the same type of fragments would also be present. However in the collagen system, it was expected that the fragments would not be precipitated.

The question remained: How could more collagen exist in noninflamed sites yet there be more Hyp present at inflamed sites? It was hypothesized that in inflamed sites molecules such as collagen and C1q were degraded further to smaller peptides due to increased proteolytic activity than in noninflamed sites, which resulted in reduced number of antigenic determinant sites. This possibility was investigated using type I collagen incubated with bacterial collagenase and *P. gingivalis* bleb preparation. The results indicated that less of the initial collagen was detected using ELISA with increasing incubation time. This demonstrated that there was a loss of antigenic determinants due to incubation of collagen with these proteolytic enzyme preparations.

Whole crevicular fluid	Acetate precipitation removes these components:	Supernatent after acetate precipitation and centrifugation
Collagen (100%)		Collagen
Collagen-Fr-AB(+)		Collagen-Fr-AB(+)
Collagen-Fr-AB(-)		Collagen-Fr-AB(-)
C1q (100%)	C1q (92%)	C1q (8%)
C1q-FrPPT(+)AB(+)	C1q-FrPPT(+)AB(+)	
C1q-FrPPT(+)AB(-)	C1q-FrPPT(+)AB(-)	
C1q-FrPPT(-)AB(+)		C1q-FrPPT(-)AB(+)
C1q-FrPPT(-)AB(-)		C1q-FrPPT(-)AB(-)

Figure 4.1: Type I collagen and C1q components in whole, precipitate and supernatant fractions conceptualized to occur in acetate treated crevicular fluid. 'Fr'= fragment; 'PPT'= precipitation; '(AB)'= antibody; '(+)'= sensitive; '(-)'= insensitive.

SDS/PAGE gels and western blot analyses provided further evidence on the composition of inflamed and noninflamed GCF. In pooled GCF samples there were more smaller fragment proteins present in inflamed GCF than noninflamed GCF. Western blots using antibodies to type I collagen demonstrated that there were more smaller fragments reactive to the antibody in inflamed sites than in noninflamed sites. The same trend applied to C1q. Markedly more intense reactions were obtained for type I collagen than for C1q western blots. These results provide a feasible explanation for ELISA results which indicated higher collagen and C1q content in noninflamed sites.

As the results from the ELISA experiments indicate, more type I collagen than C1q exists in the samples, hence more of the Hyp is attributed to a collagen source. The fractions of type I collagen and C1q have been considered with the aid of a crevicular fluid model. In this model it appears that the C1q component that was detected in GCF samples had to avoid precipitation and also be

antibody reactive. Earlier HPLC results showed that C1q at serum concentrations would give rise to barely detectable recordings for total HPLC analysis. This finding, together with the result that C1q contributed less than 10% to total Hyp in either inflamed or noninflamed GCF, indicated that C1q was not a prominent contributor to the total Hyp content of GCF.

5. CONCLUSIONS

The experiments of this thesis were designed to evaluate volatile sulphur production from gingival crevice, crevicular fluid for Hyp as an indicator of disease activity, and type I collagen and C1q for their contributions to Hyp levels in GCF. A device was developed for the collection of volatiles from the gingival crevice, while GCF was collected from the gingival sulcus using filter paper strips. Specific conclusions based on the analyses of these components are summarized in the following statements:

1. Volatile suphur compounds can be collected and analyzed from individual periodontal sites.
2. The total suphur content in crevicular air is higher at inflamed and deep, than corresponding noninflamed and shallow periodontal sites.
3. The ratio of CH_3SH to H_2S in crevicular air is significantly higher at inflamed and deep, than corresponding noninflamed and shallow periodontal sites.
4. The preponderance of Hyp content of crevicular fluid is present in a peptide form, and a disproportionately lesser amount in a free unbound form.
5. Since more type I collagen than C1q exists in GCF samples, the predominant source of GCF Hyp is attributed to a collagen source.
6. Hyp levels in GCF are reflective of the amount of collagen turnover at specific periodontal sites.
7. Higher Hyp levels were found at inflamed than noninflamed periodontal sites.

8. Higher Hyp levels were present at healing periodontal sites, than those that remained unresolved after spiramycin and scaling and root planing treatment.

6. RECOMMENDATIONS FOR FUTURE STUDY

In order to determine the response of Hyp GCF levels in untreated patients, a longitudinal study is required to monitor its levels versus changes in periodontal attachment. Since collagen metabolism is increased, as evidenced by elevated Hyp levels in periodontal sites that experienced healing after therapy, it is of interest to determine collagen metabolism at untreated periodontal sites. Measurements with high resolution, pressure sensitive periodontal probes can determine subtle changes in attachment level. This will allow monitoring of attachment level changes that might otherwise be below a cutoff threshold level and go undetected. This will permit closer comparison of Hyp levels to attachment level measurements.

A study requiring further investigation is the production and testing of CH_3SH reacted collagen antibodies as potential markers for periodontal disease activity. In a pilot study it was observed that polyclonal serum antibodies raised in mice by injection of CH_3SH treated collagen, reacted with GCF gave a clear differentiation between diseased and control sites (Ratkay et al. 1990). Intense ELISA reaction and western blot staining were observed for collagen in GCF from diseased sites and only a trace or absence of antigen response to that in GCF samples from control sites.

This result may be similar to that obtained with N-ethylmaleimide (NEM) by other investigators (Dawson et al. 1987). NEM-containing small protein fragments were shown to have strong antigenic properties. This may also be true for CH_3SH bound collagen fragments. It is of significance that antibodies formed from the above substances are different from the commercially available products. Antibodies from commercial sources generated by exposure of whole collagen

resulted in antibodies that are directed against the entire molecule. Hence, disruption and fragmentation of collagen would lead to loss of antigenic sites. This would explain why ELISA values for healthy sites showed more collagen than inflamed sites using commercially available antibodies. As Hyp values in GCF were significantly higher in sites that showed higher levels of metabolic activity (inflamed and healing sites), the development of this CH_3SH treated collagen antisera merits strong consideration.

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8. APPENDIX

Abbreviations used in this thesis

Aa	Actinobacillus actinomycetemcomitans
Ala	alanine
AS	arylsulfatase
AST	aspartate aminotransferase
BANA	benzoyl-arginine naphthylamide
BC	bacterial collagenase
BG	β-glucuronidase
belbs	*P. gingivalis* bleb preparations
BI	bleeding index
BoP	bleeding on probing
BRL	Bethesda Research Laboratories
CAL	clinical attachment loss
CEJ	cementoenamel junction

CM	carboxy methyl
CMI	cell mediated immunity
CMT	chemically modified tetracylines
DB	distobuccal
DES	desmosine
dH_2O	deionized water
DL	distolingual
DPP	dipeptidyl peptidase
EDTA	ethylenediaminetetraacetic acid
ELISA	enzyme linked immunosorbent assay
EM	electron microscope
ETAF	epidermal cell thymocyte activating factor
FN	fibronectin
g	gravity
GAG	glycosaminoglycans
GC	gas chromatography
GCF	gingival crevicular fluid
GI	gingival index
Gly	glycine
HPLC	high performance liquid chromatography
hylys	hydroxylysine
Hyp	hydroxyproline
IDE	isodesmosine
IL-1	interleukin- 1
Ile	isoleucine
JE	junctional epithelium
JP/LJP	juvenile periodontitis / localized juvenile periodontitis

LDH	lactate dehydrogenase
LPS	lipopolysaccharide
lys	lysine
MAB	monoclonal antibody
MB	mesiobuccal
MHC	major histocompatability complex
MHyp	moles of hydroxyproline
ML	mesiolingual
M_r	molecular weight
OAF	osteoclast activating factor
OPA	*o* - phthaldehyde
PBS	phosphate buffered saline
PD	pocket depth
PGE	prostaglandin E
PITC	phenylisothiocyanate
PlI	plaque index
PMN	polymorphonuclear leukocytes
Pro	proline
REE	reduced enamel epithelium
Rt	retention time
SDS/PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
Ser	serine
TCA	trichloroacetic acid
TC_A/TC_B	tissue collagenase A/B
TEA	triethylamine
T_H (CD4+)	T helper cells
TNF-α	tumor necrosis factor α

T_S	T suppressor cells
T_{SC} (CD8+)	T suppressor/cytotoxic cells
Tx	thymectomized
UV	ultraviolet
VSC	volatile sulphur compounds
WHyp	weighted hydroxyproline