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Detection of hyaluronan and hyaluronidase in human whole saliva and parotid saliva



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# DETECTION OF HYALURONAN AND HYALURONIDASE IN HUMAN WHOLE SALIVA AND PAROTID SALIVA

by

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**THESIS**

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**San Francisco**

In honor of

Lawrence K. Low

and

in memory of

Mary Y. Low

*- for all their love, encouragement, and support*

## PREFACE

The biology of hyaluronan has been subject to investigation for several decades because of its unique physiological characteristics. Traditionally, this high molecular weight glycosaminoglycan has been recognized for its contribution to the structure and organization of the extracellular matrix. However, the elevated levels of hyaluronan during fetal wound healing, a prime example of rapid tissue proliferation and regeneration, has garnered renewed interest in recent years. Wound healing is a complex series of biological events which have been shown to be fundamentally different in the fetus and adult. Clinical observations that intraoral wounds heal in a similar fashion as the scarless fetal wound is intriguing. In a pursuit to unravel the mechanisms of intraoral tissue repair, this investigation provides new evidence for the healing properties of saliva.

I gratefully acknowledge the enduring support of my graduate thesis committee including Dr. Robert Stern, Dr. M. Anthony Pogrel, and Dr. Dorothy Rowe. Their expertise knowledge, wisdom, and encouragement have greatly contributed to a remarkable educational experience. I especially appreciate Dr. Stern for providing me the opportunity to perform this research in his laboratory as well as for promoting an enriched daily learning environment. In addition, the

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# CHAPTER 1: INTRODUCTION

The biology of hyaluronan (HA) and its catabolic enzyme, hyaluronidase (HA'ase), has rapidly become a field of intense interest in recent years due to its important contributions to the extracellular matrix (ECM). Due to their ubiquitous nature, HA and HA'ase in biological systems are involved in complex processes. HA metabolism is vitally important to several fundamental physiological and cell biological functions such as ECM homeostasis, cell proliferation, and locomotion (Laurent and Fraser, 1992). Furthermore, the clinical uses of HA as a medicinal agent in the treatment of joint disease (Rydell et al., 1970; Strachan et al., 1990), as an ophthalmic surgical device (Miller and Stegmann, 1983), as a diagnostic marker for disease (Engstrom-Laurent, 1989; Engstrom-Laurent and Laurent, 1989), or as a therapeutic agent to accelerate wound healing (Hellstrom and Laurent, 1987; Casadel et al., 1989; King et al., 1991) underline the need to understand more thoroughly the metabolism of HA.

In the oral environment, the presence and possible roles of HA and HA'ase have been subject to little investigation. The remarkable observation that intraoral wounds heal rapidly with little or no scarring even without suturing (Peakock, 1984) elicits questions regarding similarity to fetal wound healing. In comparison to adult wound repair, the fetal environment

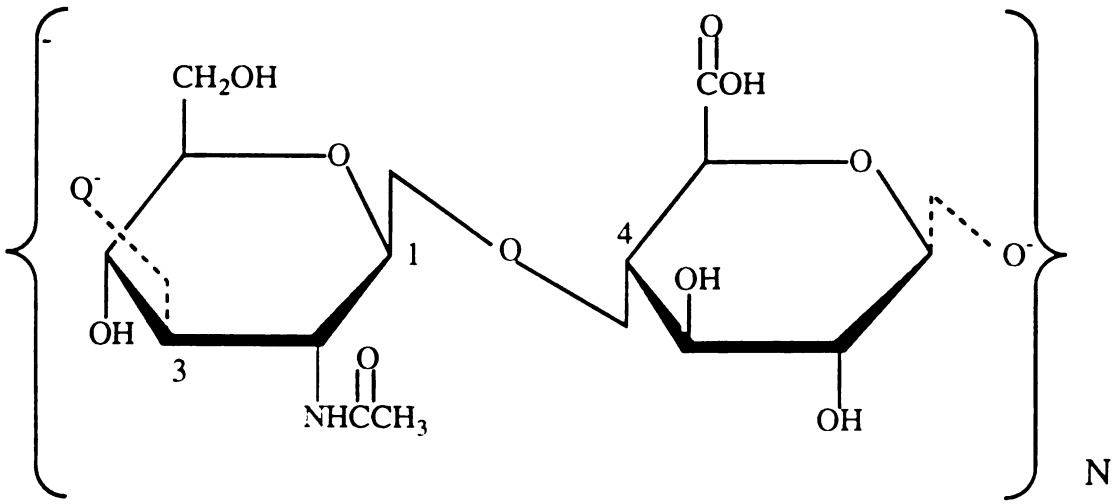
exhibits numerous differences such as an enriched milieu of growth factors and an abundance of ECM components, particularly HA (Longaker and Adzick, 1991). In addition, the oral mucosa does not appear to age like the skin as demonstrated by the observation that the oral mucosa of a healthy 20-year-old person resembles that of an 85-year-old (Wolff et al., 1991). Moreover, comparison of healthy, nonmedicated older persons with others of the same age who were medically compromised reveals few substantiative differences in the oral mucosa as well as in the overall oral health and function (Ship and Baum, 1993). The interaction of saliva with the oral mucosa may be responsible possibly for these intriguing properties (Hutson et al., 1979). To better understand the role of HA and HA'ase in the oral cavity as well as in other biological systems, an overview of these vital ECM components is presented.

## I. Hyaluronan

Since its first isolation from the vitreous body of the eye by Meyer and Palmer (1934), HA has been shown to be ubiquitously distributed among the organs and tissues of the mammalian body. Although soft connective tissue contains the highest concentration, common sources for isolation of HA include synovial fluid, umbilical cord, rooster comb, and some Streptococcal strains of bacteria. Established by enzymatic and chemical studies, the chemical and physical structure of

HA was first described by Meyer as a linear polymer of a repeating disaccharide unit composed of ß-1,4-D-glucuronic acid-ß1,3-N-acetyl-D-glucosamine (Meyer, 1958) (Figure 1-1). Originally the HA polymer was termed hyaluronic acid since it was isolated from the vitreous body (hyaloid = vitreous = glassy) and because it contained uronic acid. Later, however, it was realized that under physiological conditions sodium replaces all carboxyl groups on uronic acid and thus, sodium hyaluronate was the more appropriate name. Balazs et al. (1986) proposed that the term, hyaluronan, be adopted to alleviate confusion as well as to conform with standard polysaccharide nomenclature.

Because of its chemical structure, HA is a member of the heterogeneous glycosaminoglycan (GAG) family in which repeating disaccharide units, composed of hexosamine glycosidically linked to either uronic acid (glucuronic or iduronic) or galactose, form negatively charged polysaccharide chains. The family of GAGs identified presently also include chondroitin-4-sulfate, chondroitin-6-sulfate, dermatan sulfate, keratan sulfate, heparan sulfate, and heparin. However, HA is uniquely distinguished from other GAG family members by its lack of a covalently linked peptide. Referred to as proteoglycan (PG) 'monomers', the other GAGs are covalently attached to a core protein and thus, do not normally exist as free polymers *in vivo*. Rather, in the major




N-Acetyl-D-Glucosamine D-Glucuronic Acid

Figure 1-1: Structure of hyaluronan repeating dissacharide unit

PG, aggrecan, an aggregate of PG monomers forms as the core proteins noncovalently associate with link proteins along the length of the HA chain in a "bottle brush" configuration (Hardingham and Muir, 1973). Another characteristic feature of HA is that it does not possess any sulfated groups typically found in all other GAGs.

## II. Physiological Roles of Hyaluronan

The physical properties of HA confer to it the several physiological roles which have been assigned. Consisting of 200 - 10,000 disaccharide units (Toole, 1990), the molecular mass of HA in normal tissues typically exceeds $10^6$ (Hook, 1984), but may vary from $1 \times 10^5$ to $5 \times 10^6$ (Toole, 1990). The extensive secondary structure, stabilized by hydrogen bonds and water bridges, is known to stiffen HA which accounts for the unusual viscosity of solutions (Scott, 1992). In solution, the long unbranched HA chains form an overall expanded coil structure which has the capacity to hold 1000-fold more water than the polymer (Fessler and Fessler, 1966). Consequently, HA maintains a large volume:mass ratio when incorporated with PGs and in turn, provides turgidity and compressive strength on tissues (Sweeney and Weiss, 1992). This highly hydrated sphere of HA also can act like an ion-exchange resin permitting electrostatic interactions which form the basis for some of the various properties of HA such as its influence on osmotic pressure and ion transport and

distribution (Sebag, 1992). Although the exact mechanism has not been elucidated, an additional function of the expanded macromolecular HA structure includes keeping migrating cells apart during morphogenesis (Toole et al., 1984). Also, it has been suggested that as water becomes trapped by HA, an enormous swelling of the connective tissue stroma ensues to create channels for cell movement by separation of the collagen layers.

Historically, HA was described as a predominant component of the "ground substance" or "cementing substance" of subcutaneous tissues. This amorphous, colorless, and transparent material appeared like "ground glass" and was thought to act only as a space filler between cells and fibers. Today the roles of HA in modulating various cell functions contributing to the overall integrity and dynamics of the ECM are becoming increasingly significant. HA is recognized for its major role in providing structural and functional support. HA participates in several macromolecular physiological roles including provision of structural framework in cartilage (Heinegard and Oldberg, 1989), homeostasis of the ECM (Laurent, 1970; Comper and Laurent, 1978), steric interactions to exclude other macromolecules (Laurent, 1970; Comper and Laurent, 1978), and lubrication of joints and tissues (Ogston and Stanier, 1953; Gibbs et al., 1968).

More recently, an increased appreciation of HA and its degradation products has occurred because of their role in growth, development, and repair of tissues. For example, levels of HA are elevated during embryological development (Toole and Gross, 1971; Toole 1981; Kujawa and Tepperman, 1983; Kujawa et al., 1986; Kulyk and Kosher, 1987), wound healing (Longaker et al., 1991), and tumorigenesis (Knudson et al., 1989). As a major component of the ECM, HA binds numerous PGs which function in cell adhesion and migration. These dynamic processes are mediated though interactions between the PG polysaccharide chains and other major ECM components. Hence, cell adhesion proteins such as fibronectin and laminin, structural elements consisting of collagen and elastin, HA, and the PGs comprise an interactive network, responding to one another or cell surfaces through specific protein or oligosaccharide recognition sites (Jackson et al., 1991). For example, the tripeptide sequence, Arg-Gly-Asp (or R-G-D) (Ruoslahti and Pierschbacher, 1987), recognizes the integrin receptor superfamily on cell surfaces which in turn, transmits information between the ECM and the cytoskeleton of the cell membrane (Hynes, 1987). Recently, sequence analysis of pig liver HA'ase has revealed a R-G-D site (Stern, unpublished data).

Considerable attention has been focused toward the critical balance of HA and HA'ase in basic biological processes

including cell proliferation, locomotion, and differentiation (Orkin and Toole, 1980; Toole et al., 1989; Balazs et al., 1951; Turley, 1984). During embryogenesis, for example, predictable changes in HA synthesis and breakdown occur. In the early stage of morphogenesis, cell growth, proliferation, and migration predominate and HA levels characteristically rise (Toole, 1976; Laurent and Fraser, 1992). In early chick embryogenesis, the mesodermal cells of the limb bud (day 3 - 3.5) synthesize high concentrations of HA. Moreover, a maximal rate of HA synthesis is observed prior to the mitotic process and thus, HA is implicated in promoting division by permitting cell detachment from the supporting matrix (Brecht et al., 1986).

Involvement of HA in cell locomotion is most obvious during morphogenesis. HA synthesis predominates when embryonic cell migration is initiated in a variety of cell types such as neural crest (Derby and Pintar, 1978), chondrocytes (Toole et al., 1989), smooth muscle cells (Boudreau and Rabinovitch, 1991), corneal fibroblasts (Toole, 1982), and heart cushion cells (Markwald et al., 1978). The ability of HA to occupy a large volume is believed to provide the open space for cell migration. For example, the developing murine salivary gland demonstrates increased quantities of HA present at its migrating edge (Bernfield et al., 1984). Additionally, the role of HA in promoting cell locomotion is especially

important in tumor invasion. Increased levels of HA are detected in tumors and may correlate with tumor aggressiveness in some cases (Knudson et al., 1989). Moreover, a novel HA receptor, termed Receptor for HA Mediated Motility (RHAMM), has been identified to regulate directional locomotion of tumor cells when HA is bound (Turley, 1992).

Although HA stimulates proliferation and migration, an opposite effect is observed in differentiation. The presence of HA inhibits differentiation and therefore, its removal by its degradative enzyme, HA'ase, is necessary for this stage to proceed (Toole et al., 1972; Kujawa et al., 1986). Concurrently, the proliferative phase and HA synthesis cease.

The biosynthesis of HA is directed via hyaluronate synthase, a 50-kD eukaryotic protein identified by Prehm (1989). Normally GAGs are synthesized by the addition of sugars to the nonreducing end of the growing oligosaccharide chain in the Golgi apparatus (Jackson et al., 1991). However, the mechanism of HA synthesis differs significantly in that its sugars are added to the reducing end of the polymer (Prehm, 1989). Furthermore, elongation occurs inside the plasma membrane rather than in the Golgi, and subsequently, HA is translocated into the extracellular space with the nonreducing end in the lead.

## III. Catabolism of Hyaluronan

To fully understand the importance of HA during development, differentiation, and other cell biological functions, the catabolism of this unique polyanionic polysaccharide chain requires considerable attention. Identified as early as 1928 (Duran-Reynals, 1928), HA'ase originally was described as a "spreading factor" because of its ability to facilitate the diffusion of toxins, vaccines, and dyes into tissue (Meyer and Rapport, 1952). Later, this "spreading factor's" enzymatic capacity was acknowledged when the enzyme was observed to rapidly depolymerize the HA-rich environment of the vitreous humor which subsequently, decreased in viscosity (Chain and Duthrie, 1940). As a result, much interest has been focused to elucidate HA'ase participation in altering HA and PGs in numerous cell functions including proliferation (Balazs and Holmgren, 1950; Fiszer-Szafars and Nadal, 1977), motility (Hakanson et al., 1980), differentiation (Fiszer-Szafars, 1984), adhesion (Barnhart et al., 1979), and phagocytosis (Goggins et al., 1968b; Forrester and Balazs, 1980).

Depolymerization of HA in mammals is achieved through the concerted action of three enzymes, hyaluronidase, ß-glucuronidase, and ß-N-acetylglucosaminidase (Roden et al., 1989). Hyaluronidase (EC 3.2.1.35) is characterized by its mechanism of endoglycosidic cleavage in which the internal ß(1-4) sugar linkages are cleaved. Following exhaustive

digestion with this endo-ß-N-acetylhexoaminidase, the major degradation product is the tetrasaccharide, GlcUA→GlcNAc→GlcUA→GlcNAc (Roden, 1980). Smaller amounts of disaccharide, hexasaccharide, and higher oligosaccharide also are found. Additionally, catabolism of other GAGs including chondroitin-4-sulfate and chondroitin-6-sulfate is believed to be catalyzed by hyaluronidase and have a central role in the function of lysosomal HA'ases in particular (Fenger, 1982). In comparison, ß-glucuronidase (EC 3.2.1.31) and ß-N-acetylhexosaminidase (EC 3.2.1.30) are exoglycosidases which cleave terminal monosaccharides from the non-reducing terminus in a stepwise manner (Arbogast et al., 1975).

Despite expectations for a relatively stable molecule, the turnover of HA is exceedingly rapid for a connective tissue structural component. Fraser and Laurent (1989) estimate 10 - 100 mg of HA are turned over daily in adult humans. Measured by intravenous injection of $^{3}$H-labeled hyaluronan into humans, circulating HA, originating from the peripheral tissues via the lymph (Laurent and Laurent, 1981), was determined to have a half-life of 2.5 - 5.5 minutes (Laurent and Fraser, 1986). Furthermore, the liver was established as the principal site of catabolism of HA present in the circulation, although the kidneys and spleen were reported also to play an additional role (Laurent and Fraser, 1986; Laurent and Fraser, 1991). Further investigations into HA degradation in the liver have

revealed its uptake by hepatic endothelial cells (Smedsrod et al., 1990), the presence of specific receptors for HA endocytosis on these cells (Forsberg and Gustafson, 1991), and following endocytosis, transport to lysosomes for degradation by HA'ase, ß-glucuronidase, and ß-N-acetylglucosaminidase (Roden et al., 1989). However, HA is present only at low concentrations in the circulation (Laurent and Laurent, 1981). Before reaching the circulation, the majority of the body's HA is taken up and degraded in the lymph nodes which exhibit a high capacity for HA catabolism (Fraser et al., 1988).

Recent evidence demonstrates local degradation of HA in tissues such as the eye, skin, intestine, and joints (Laurent et al., 1988; Reed et al., 1990; Brown et al., 1991; Laurent and Reed, 1991). Rapid turnover is observed in each of these tissues which may facilitate the participation of HA in physiological regulatory mechanisms of various processes such as embryological development, wound healing, and tumorigenesis (Laurent and Fraser, 1992).

## IV. Wound Healing Properties of Saliva

Beyond doubt, saliva is important for the preservation and maintenance of oral health (Mandel, 1987; 1989). The complexity of saliva bestows a multitude of functions including lubrication, protection to aid oral defenses, modulation of bacterial populations, and maintenance of

calcified dental tissue integrity (Fox et al., 1985). However, many salivary components have been poorly investigated (Herrera et al., 1988).

The role of saliva in wound healing has been implicated by many investigators (Hutson et al., 1979; Li et al., 1980; Niall et al., 1982; Bodner, 1991). Self and communal licking and grooming among mammals are instinctive behaviors which become more pronounced following injury (Hart and Powell, 1990). Antibacterial substances, growth factors, and immunoglobulins in saliva are delivered to the wound by licking, which are postulated to promote wound healing (Bodner, 1991). For example, the antibacterial properties of salivary lysozyme were assumed to contribute to the wound healing capacity of saliva (Tenovou et al., 1987). More recently, interest has focused on the effect of epidermal growth factor (EGF) in wound healing since it is detected in most human saliva samples (McGurk et al., 1990). Evidence suggests EGF accelerates the rate of wound healing in animals (Niall et al., 1982; Brown et al., 1989). Additional findings to support the contribution of saliva to wound healing show decreased healing rates of cutaneous, gingival, and extraction wounds following sialadenectomy of all major salivary glands in animal models (Hutson et al., 1979; Bodner and Dayan, 1990; Shen et al., 1979; Bodner et al., 1991).

## V. Fetal and Adult Wound Repair

Demonstration of the HA-rich fetal wound matrix by DePalma et al. (1989) has pioneered a new approach to understanding the scarless fetal wound. During rapid tissue proliferation, regeneration, and repair, HA is known to be a key structural and functional component of the ECM. Hence, the increased levels of HA and HA'ase inhibitors and low HA'ase levels may modulate fetal wound healing by orchestrating healing by regeneration rather than by scarring (Longaker et al., 1991; Longaker and Adzick, 1991). In a parallel analogy, intraoral wound healing may function in a similar manner since these substances are integral components of intraoral wound repair (Bertolami and Ellis, 1986).

Adult mammalian wound repair is the sum of a complex series of biochemical events which include hemostasis, inflammation, proliferation, and remodeling. In a surgically created wound, the acute inflammatory reaction is initiated almost immediately with leukocytes, erythrocytes, and platelets adhering to the endothelium (Peacock, 1984). Vasodilation, increased vascular permeability, and diapedesis of primarily acute inflammatory cells occur. In this early phase of wound healing, HA levels are dramatically elevated to aid in cell proliferation (Bertolami and Donoff, 1978) and to facilitate cell migration in order to restore cellular continuity (Abetangelo et al., 1983). By approximately three days after

wounding, the cellular phase of repair commences with fibroblasts proliferating and migrating into the wound (Peacock, 1984). On the fourth or fifth day post-wounding, HA levels decline due to increased HA'ase levels which remain elevated for several days before diminishing (Bertolami and Donoff, 1982). As a result, HA is degraded and levels continue to gradually decrease during the repair process (Alexander and Donoff, 1980; Weigel et al., 1986). In turn, smaller molecular weight HA fragments are produced which are known to be relatively potent angiogenic agents (West et al., 1985). In addition, sulphated GAG levels are elevated (Weigel et al., 1986). More importantly, the removal of HA in healing wounds signals a change from cellular proliferation to cellular differentiation (Toole et al., 1972). Hence, differentiation, neovascularization, collagen synthesis, and tissue organization follow (Toole and Trelstad, 1971; Toole, 1972). Wound maturation ensues as the amount of PGs and fibroblasts decrease (Peacock, 1984). As the remodeling phase continues the end result is the formation of a scar composed of cross-linked collagen. Therefore, considering this pattern of specific cellular behaviors, the rate of wound healing may be measured as a function of the timing of the appearance and subsequent disappearance of HA'ase (Pogrel et al., 1989).

## VI. Hyaluronidase Activity in the Oral Cavity

The presence and possible roles of HA and HA'ase in saliva

have been subjected to little investigation, especially in recent years. Mahler and Lisanti (1952) identified HA'ase activity of presumably bacterial origin in human whole saliva, but failed to detect enzyme activity in parotid saliva. In another study of human whole saliva, Rolvstead et al. (1958) attempted to establish baseline data on HA'ase activity, but the detection method lacked sensitivity and specificity. Over 20 years ago, Hopps and Prout (1972) distinguished between tissue and bacterial HA'ases in human whole saliva on the basis of distinct pH profiles. However, the complexity of the microflora in whole saliva precluded interpretation to distinguish whether these enzymes were indeed of tissue origin or bacterial origin. On the other hand, Chauncey (1961) reported that HA'ase, among other human salivary enzymes such as arylsulfatase and catalase, was of purely microbial origin.

HA'ase activity has been demonstrated in human gingival crevicular exudate (Schultz-Haudt et al., 1954; Dewar, 1958; Courant et al., 1965) as well as in the gingival homogenates of humans (Goggins et al., 1968a; D'Allaird et al., 1969). This activity may be related to periodontal tissue destruction or it may represent progression to the cellular differentiation stage in healing. Moreover, this activity may represent enzyme of host tissue or bacterial origin, but more conclusive data have not been reported until recently. The bacterial HA'ase activity in human gingival crevicular fluid

has been shown to be higher in inflamed sites than in normal areas (van Palenstein-Helderman and Hoogeveen, 1976). Both HA and HA'ase activity of lysosomal and bacterial origin have been detected in gingival exudate from sites of chronic gingivitis in adults (Last and Embery, 1987). In addition, animal studies in dogs have demonstrated HA'ase activity in both saliva and gingival crevicular fluid. However, these studies are also limited and outdated (Tynelius-Bratthall and Attstrom, 1972; Tynelius-Bratthall, 1972; Tan and Bowness, 1968).

Therefore, salivary HA and HA'ase appear to be timely and important subjects for investigation. The specific objective of this study is to determine the levels of HA and HA'ase in unstimulated and stimulated parotid saliva and whole saliva in normal human adults. Toward this end, two rapid, reliable, and sensitive assays based on a cartilage-derived biotinylated HA-binding protein (HABP), will be employed to measure HA and HA'ase concentrations (Fosang et al., 1990; Stern and Stern, 1992). In addition, saliva samples will be evaluated for endogenous HA'ase inhibitors. It is postulated that parotid saliva is a potential source for the tissue HA'ase enzyme previously identified in whole saliva (Hopps and Prout, 1972). Furthermore, the overall goal of this study is to assess in saliva the levels of the major elements involved in HA metabolism under normal, uninjured conditions as a preliminary

investigation to define the mechanisms of intraoral wound healing. It is proposed that intraoral wounds heal in a similar manner to scarless fetal wounds in part by the precise regulation of endogenous HA and HA'ase in salivary secretions.

# CHAPTER 2: MATERIALS AND METHODS

## I. Description of the Study Population

Individuals participating in this study included 8 male and 2 female normal healthy volunteers between 19 and 34 years of age. Subjects were required to be neither taking medication nor receiving regular medical care for the treatment of systemic disease, to exclude individuals with possible salivary dysfunction or salivary flow interference.

## II. Collection of Saliva Samples

Unstimulated and stimulated pure parotid saliva and mixed whole saliva samples were collected under standardized conditions similar to collection methods utilized in salivary flow rate studies (Baum, 1981; Heft and Baum, 1984; Tylenda et al., 1988). Participants were seen for 2 hours at 1 p.m. or 3 p.m. by one clinician (M.L.L.) at the University of California at San Francisco Oral and Maxillofacial Surgery Clinic. All subjects were not allowed to have anything by mouth for a minimum of 2 hours prior to saliva collection. This included refraining from eating, drinking, gum chewing, smoking, and oral hygiene procedures.

After the orifice of the left Stenson's duct and surrounding oral mucosa were carefully wiped with a dilute iodine solution (Betadine), parotid saliva was collected first by duct

cannulation using a flexible polypropylene sialography catheter (MeadoxSurgimed, Oakland, N.J.). The saliva secreted during the initial 2 minutes of outflow was discarded since during this period salivary components can vary significantly (Dawes, 1969). Approximately 2 ml of parotid saliva was then collected and stored in a separate 15 ml polystyrene conical tube until all samples were obtained on ice to prevent degradation of salivary proteins by digestive enzymes, primarily from microorganisms and degenerating host cells (Soderling, 1989). After collection of parotid saliva, mixed saliva was aspirated from the floor of the mouth utilizing a 10 ml Monojet syringe. All unstimulated secretions were procured first and followed by collection of stimulated samples using 2% citric acid as the gustatory stimulus for salivation. The citrate was applied uniformly over the dorsal surface of the tongue with a cotton swab for 5 seconds in 30 second intervals until approximately 2 to 5 ml of saliva were obtained. The time required to obtain each sample was monitored and the final volume measured.

### III. Pretreatment of Saliva Samples

Immediately following collection of all samples, the saliva was prepared for analysis and storage. Each saliva sample was treated with 0.1% ethylenediaminetetraacetic acid (EDTA) to prevent the formation of calcium proteinates which may affect the saliva protein composition (Cowman et al., 1983;

Soderling, 1989). Generally, sugar moieties released from glycoproteins in whole saliva form a calcium-rich protein precipitate with a concomitant increase in saliva viscosity (Soderling, 1989). Furthermore, pure salivary gland secretions also may precipitate after storage in the freezer in the absence of chelating agents. The saliva samples were clarified by centrifugation at 12,400 x *g* for 20 minutes at 4°C to remove any insoluble material. The supernatents were stored at -20°C until use; after thawing at 4°C, all samples were kept on ice while being prepared for analysis.

**IV. Protein Assay**

Protein content of each saliva sample was determined by the BioRad Protein Microassay (BioRad Laboratories, Richmond, CA). One hundred sixty microliters (μl) of a 30-fold dilution of saliva in calcium- and magnesium-free phosphate buffered saline (PBS) was added to 40 μl of concentrated dye reagent (Coomassie Brilliant Blue G-250, phosphoric acid, methanol) in a 96-well microtiter plate (Costar, Data Packaging, Cambridge, MA) and mixed thoroughly. After 5 - 60 minutes, the absorbance of samples was measured at 595 nanometers (nm) in a Titertek Multiscan Plus MK II automated plate reader (Labsystems, Helsinki, Finland). PBS served as the reagent blank and bovine serum albumin (BSA) (Sigma, St. Louis, MO) was used to generate a standard curve. Reported values represent the mean protein content of saliva samples assayed

in triplicate which were obtained from the standard curve.

**V. ELISA-like Assay for Hyaluronan**

Quantitation of the HA content in saliva was determined using an ELISA-like assay as described by Fosang et al. (1990). This procedure is modeled after the basic concept of a competitive ELISA. In brief, a fixed amount of HA is immobilized on a solid support, and a fixed quantity of a HA-specific indicator molecule is allowed to competitively bind to HA attached to the support or in the test solution. Prepared from human umbilical cords, HA (ICN Biochemicals, Costa Mesa, CA) was applied first to a microtiter plate. One hundred $\mu$l aliquots of 0.1 mg/ml HA in 0.42 mM N-hydroxysulfosuccinimide (Pierce) and 3.2 mM 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (Sigma, St. Louis, MO) were adsorbed to the 96-well microtiter plate (Covalink-NH, Nunc) for 17 hours at 4°C. After the coupling of HA to the wells, the plates were rinsed three times with a solution of 2 M sodium chloride (NaCl) and 41 mM magnesium sulfate in PBS (Buffer A). All subsequent incubations were followed by triplicate washes using Buffer B which consisted of Buffer A with the addition of 0.05% Tween 20 (Fisher Scientific, Fair Lawn, NJ).

A biotinylated HABP served as the indicator molecule, similar to a labeled antibody, to "competitively" bind HA in saliva

samples and subsequently decrease the HABP available to bind to HA-coated wells.

Using a modified technique of Tengblad (1979), HABP was prepared from bovine nasal cartilage (Pel Freeze, Rogers, AZ) and biotinylated according to Stern (1993). This cartilage-derived peptide from proteoglycan core protein specifically and irreversibly binds to HA (Goldberg, 1988). Hence, when coupled to an immunoperoxidase-linked avidin-biotin reaction, this complex is easily detected. An equal volume of undiluted saliva sample and diluted biotinylated HABP were incubated at 37°C for 1 hour. HABP was diluted 100-fold in 25 mM sodium phosphate, 1.5 NaCl, 0.3 M guanidine-HCl, 0.08% BSA, and 0.02% sodium azide, pH 7.0. A standard curve for each ELISA plate was established using serial dilutions of Healon$^{R}$, a highly purified, high molecular weight fraction of sodium hyaluronate (Pharmacia AB, Uppsala, Sweden) prepared in PBS. A negative control to serve as reagent blank and positive control to determine the maximal binding of HABP to HA fixed onto the plate were included also.

Following blocking of nonspecific binding with 300 µl/well of 0.5% nonfat dry milk in PBS at 37°C for 30 minutes, 100 µl of the samples preincubated with HABP were added to the plate in triplicate. The remaining biotinylated HABP in the sample was allowed to bind to the HA-coated wells for 1 hour at 37°C and

then to be detected utilizing the avidin-biotin-peroxidase technique. The avidin-biotin complex (ABC) reagent (Vector, Burlingame, CA) was prepared in PBS containing 0.1% Tween 20 as directed and 100 µl/well of the reagent was incubated for 30 minutes at room temperature. Finally, after five thorough washes with Buffer B, the substrate, 0.5 mg/ml o-phenylenediamine (Calbiochem, San Diego, CA) in 33 mM citrate, 67 mM dibasic sodium phosphate, pH 5.3, and 0.015% hydrogen peroxide, was added to complete the enzymatic reaction. Utilizing a Titertek Multiscan Plus MK II automated plate reader, absorbance values were obtained at 492 nm. Reported mean saliva HA concentrations represent the remaining percentage of biotinylated HABP bound to the plate as related to the standard curve for known concentrations of Healon$^{R}$ HA.

**VI. ELISA-like Assay for Hyaluronidase Activity**

Developed in our laboratory by Stern and Stern (1992), the ELISA-like assay for hyaluronidase activity has been shown to be 10,000 times more sensitive than the Reissig technique (Reissig et al., 1955) which has been the most widely employed procedure in years past. Based upon the hydrolysis of HA adsorbed to microtiter plates by HA'ase, the first step of the assay involved the addition of equal portions of 0.4 mg/ml HA (ICN Biochemicals, Costa Mesa, CA) dissolved in water and 0.2 M carbonate buffer, pH 9.2. One hundred µl aliquots of this coating solution were adsorbed to 96-well microtiter plates

(Costar, Data packaging, Cambridge, MA) for 17 hours at 4°C. Following three rinses with PBS, triplicate 100 µl aliquots of diluted saliva samples and *Streptomyces* hyaluronidase (Calbiochem, San Diego, CA) were incubated at 37°C for 17 hours to allow HA hydrolysis to proceed. Each assay plate included serial dilutions of *Streptomyces* hyaluronidase in 0.5 M sodium acetate, 0.05 M NaCl, and 0.02% BSA at pH 5.0 (Stern, 1993) to establish a standard curve. Negative and positive controls were included for each assay plate similar to those used in the ELISA-like assay for HA. The rates of HA hydrolysis by enzyme preparations were both dose- and time-dependent, demonstrating that a valid unit of enzyme activity was actually being observed.

To characterize the optimal conditions for salivary HA'ase activity, a 10-fold dilution of saliva was prepared in various buffers. Characterization included comparison of different enzymatic buffers, range of pH, and salt and albumin concentrations. Prepared according to Gomori (1955) with minor modifications, 0.5 M sodium acetate, 0.05 M citrate-0.1 M sodium phosphate, 0.1 M sodium phosphate, and 0.1 M sodium formate enzymatic buffers were evaluated first at various pH values ranging from pH 3.7 to 6.8. Each buffer also contained 0.05 M NaCl and 0.02% BSA. Because collected saliva quantities were limited, characterization assays were performed primarily with samples collected from one

individual. Upon confirmation of the optimal enzymatic buffer for both the parotid and whole saliva, the effects of various salt concentrations ranging from 0 to 0.5 M were examined in the presence of 0.02% BSA. Next, to verify the pH optima for salivary HA'ase, the range of pH values was extended from pH 3.3 to 7.3. Further characterization of the HA'ase activity included investigating the contribution of albumin ranging from 0 to 0.2% BSA by assaying saliva under the optimal conditions determined in the previous experiments. Upon determining the buffers for optimal enzymatic activity, each solution was examined using a 1:10 dilution of saliva to confirm that the buffering capacity was sufficient throughout the enzymatic reaction.

The newly defined conditions for optimal salivary HA'ase activity were utilized to assay the remaining saliva samples. Unstimulated and stimulated parotid saliva samples were assayed in a buffer consisting of 0.05 M citrate, 0.1 M sodium phosphate, 0.5 M NaCl, and 0.02% BSA at pH 3.7. Since two peaks of HA'ase activity at different pH values were detected in mixed saliva, all unstimulated and stimulated samples were assayed under both conditions as follows: 0.05 M citrate, 0.1 M sodium phosphate, 0.05 M NaCl, 0.02% BSA at pH 4.0 and 0.1 M sodium phosphate, 0.05 M NaCl at pH 6.8. To exclude the contribution of any exoglycosidase activity, 1 mM saccharic acid 1,4-lactone (Sigma) was added to each of the optimally

defined buffers.

Following HA hydrolysis incubation, the plate was rinsed three times with PBS and non-specific binding was blocked as described for the ELISA-like assay for HA. Detection of the remaining HA in the plate following HA'ase digestion was performed using 100 µl/well of biotinylated HABP diluted 40-fold at 37°C for 1 hour.

Because of the keratan sulfate chains present on the HABP (Figure 2-1), the biotinylated signal was amplified using an anti-keratan sulfate (α-KS) monoclonal antibody and a secondary biotinylated antibody. Prior to each of the remaining incubations, the plate was rinsed three times with PBS containing 0.05% Tween 20. One hundred µl/well of α-KS (Seikagaku Corporation, Tokyo, Japan) diluted 1:1000 in PBS with 0.1% Tween 20 were added and incubated at room temperature for 30 minutes. The secondary antibody, biotinylated anti-mouse IgG (Vector, Burlingame, CA) diluted 1:200 in PBS with 0.01% Tween 20, was added in 100 µl aliquots and incubated at room temperature for 30 minutes. Detection of the biotinylated complex was accomplished in the identical manner as described previously for the ELISA-like assay for HA.

The relative hyaluronidase activity represents the difference

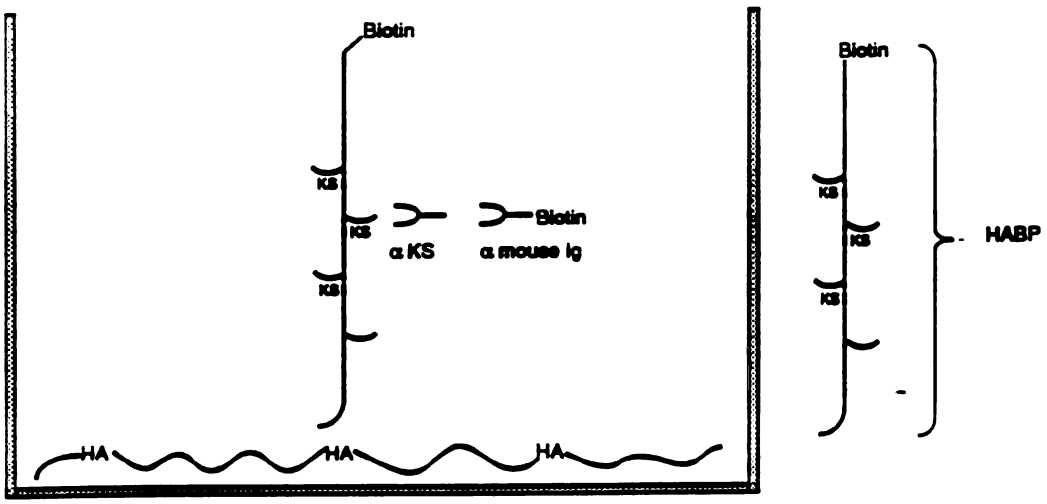



Figure 2-1: ELISA-like assay for HA'ase activity (Stern and Stern, 1992). Schematic diagram represents detection of HA remaining following enzymatic degradation by HA'ase. A cartilage-derived biotinylated HABP specifically and irreversibly binds to HA on microtiter plate. The biotinylated signal is amplified by detection of keratan sulfate (KS) chains located on the HABP using an anti-keratan sulfate (α-KS) monoclonal primary antibody and biotinylated secondary antibody (α-mouse Ig).

in absorbance between control wells with no HA'ase exposure and sample wells containing the enzyme. The units of salivary HA'ase activity are expressed in National Formulary Units (NFU) which is defined as the amount of enzymatic activity required to reduce turbidity by 50% in 30 minutes, representing 50% hydrolysis of the substrate.

Since the salivary hyaluronidase activity was detected at low levels, saliva was lyophilized, concentrated 10-fold by resuspension in 0.85% NaCl (Cowman et al., 1983), and assayed under optimal enzymatic conditions. Due to limited quantities of pure parotid saliva, only mixed saliva was examined.

To establish the integrity of the salivary HA'ase, different conditions were investigated. To determine the effect of storage conditions (- 20°C) on salivary HA'ase activity, mixed saliva was evaluated following multiple rounds of freezing and thawing. In addition, the prevention of protease degradation in mixed saliva was investigated in the presence of a 1:50 dilution of protease inhibitor cocktail (PIC) consisting of 0.8 M EDTA, 2 M Tris, 1 mM p-hydroxy-mercuribenzoate (Sigma), 1 mM benzamidine hydrochloride (Kodak), and 5 mM N-ethylmaleimide (Sigma). Furthermore, following a 2.5 hour incubation at 37°C, mixed saliva was assayed for the HA'ase activity remaining.

HA'ase inhibitors may be present in saliva and thus, $2.5 \times 10^{-4}$ NFU/ml *Streptomyces* HA'ase was assayed for activity with unstimulated parotid or whole saliva serially diluted in the bacterial enzyme buffer. The percent inhibition was calculated as follows: $\%I = 1 - [(A_{max} - A_{sample}) / (A_{max} - A_{min})]$ where $A_{max}$ represents the absorbance of wells without exposure to HA'ase, $A_{min}$ is the absorbance of wells with exposure to HA'ase and no inhibitor, and $A_{sample}$ is the absorbance of wells exposed to both HA'ase and sample containing inhibitor (Stern and Stern, 1992).

**VII. Dot Blot Analysis**

Immunorecognition of salivary hyaluronidase was performed by dot blot analysis using polyclonal antibodies against commercially purified bovine testicular HA'ase prepared by colleagues in the laboratory (Gakunga, unpublished data). Two $\mu$l of undiluted parotid and mixed saliva were spotted onto nitrocellulose membranes (BioRad Laboratories, Richmond, CA). Serial dilutions of bovine testicular HA'ase (Wydase) in PBS were included as positive controls while 3% BSA (Sigma) served as the negative control.

To block non-specific binding, the dry membrane was incubated in PBS containing 0.01% Tween 20 for 30 minutes. All incubations were performed on an orbital shaker (American Rotator) at room temperature and followed by two rinses with

0.1% Tween 20 in PBS for 5 minutes each. Since the commercial bovine testicular HA'ase was found to contain albumin, the HA'ase antibody preparation was purified first using an Affigel 10 column to remove any anti-albumin antibodies that may have been present. Additionally, a 100-fold dilution of anti-HA'ase antibody was incubated in a 1:1 mixture of PBS + 0.1% Tween 20 and 3% BSA for 30 minutes prior to exposure to the membrane. The membrane was then incubated with this primary polyclonal antibody, anti-bovine testicular HA'ase, solution for 30 minutes. A biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA), diluted 1:200 in equal volumes of PBS + 0.1% Tween 20 and 3% BSA, served as the secondary antibody to detect the bound anti-HA'ase antibody in a 30 minute incubation. Colorized detection of the biotinylated secondary antibody was achieved using the ABC reagents (Vector, Burlingame, CA) prepared in 0.1% Tween 20 in PBS for 30 minutes and followed by the addition of 3,3' - diaminobenzidine (DAB) (Vector, Burlingame, CA) as substrate which was utilized according to the manufacturer's instructions.

# CHAPTER 3: RESULTS

## I. Pretreatment and Storage of Saliva for Analysis of Enzyme Activity

Initial experiments focused on the preservation of saliva samples to ensure that the HA'ase enzymatic activity could be maintained. Because some proteins can become trapped (Soderling, 1989), the removal of salivary sediment by centrifugation was investigated to determine its effect on HA'ase activity. In addition, since calcium-rich protein precipitates often form in whole saliva, as well as in pure salivary secretions following storage at - 20°C (Soderling, 1989), treatment with 0.1% EDTA (Cowman et al., 1983) was evaluated. Figure 3-1 demonstrates the enhanced HA'ase activity in whole saliva following treatment with 0.1% EDTA and clarification by centrifugation. In addition, storage of the saliva at - 20°C prior to pretreatment had little effect on HA'ase activity. Furthermore, the addition of 0.1% EDTA had no effect on the activity of *Streptomyces* HA'ase standards.

As shown in Figure 3-2, consecutive rounds of freezing and thawing had no deleterious effect on HA'ase activity in mixed saliva. However, the addition of a protease inhibitor cocktail slightly decreased the HA'ase activity and thus, was not included in saliva samples (Figure 3-3). The ability of

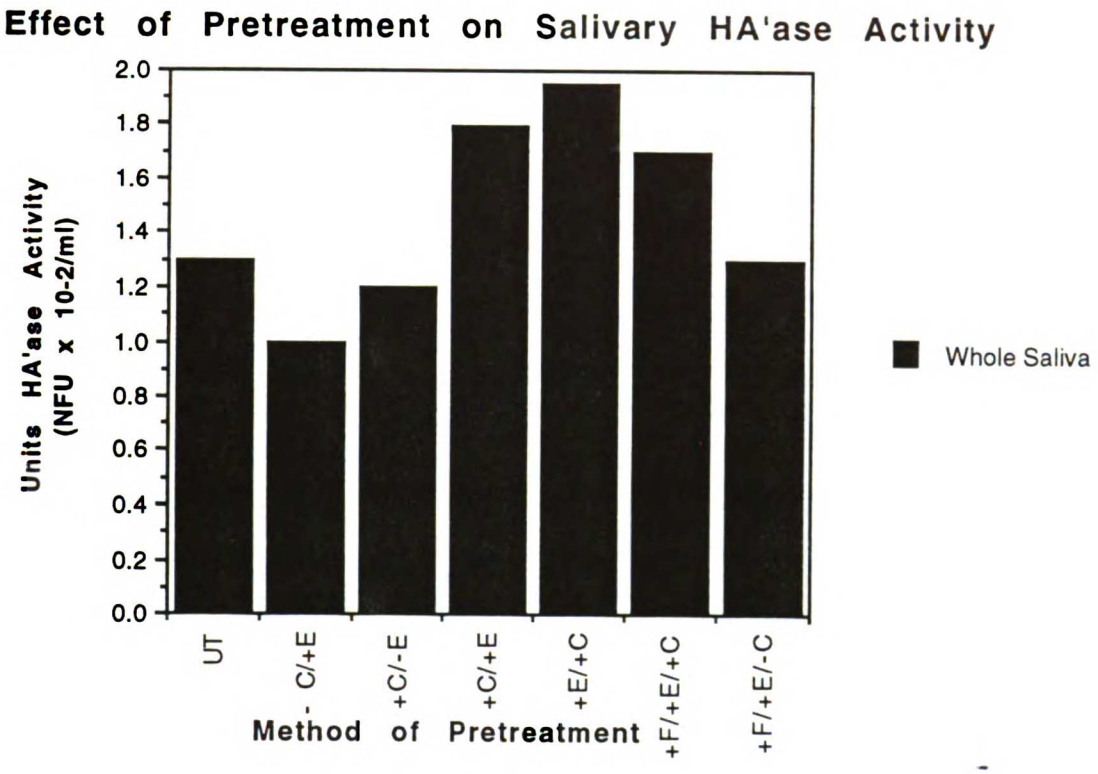



**Figure 3-1: Comparison of HA'ase activity in pretreated saliva samples. Unstimulated whole saliva samples were assayed for HA'ase activity in citrate-phosphate buffer, 0.5 M NaCl, pH 3.7 following different pretreatment procedures. The various conditions examined included (listed in the order of treatment): untreated (UT); no centrifugation (-C) with 0.1% EDTA (+E); centrifugation (+C) with no EDTA (-E); centrifugation (+C) with 0.1% EDTA (+E); 0.1% EDTA (+E) with centrifugation (+C). Samples were also assayed for HA'ase activity with storage at - 20°C (+F) prior to pretreatment procedures. Optimal activity was obtained in the presence of 0.1% EDTA followed by centrifugation.**

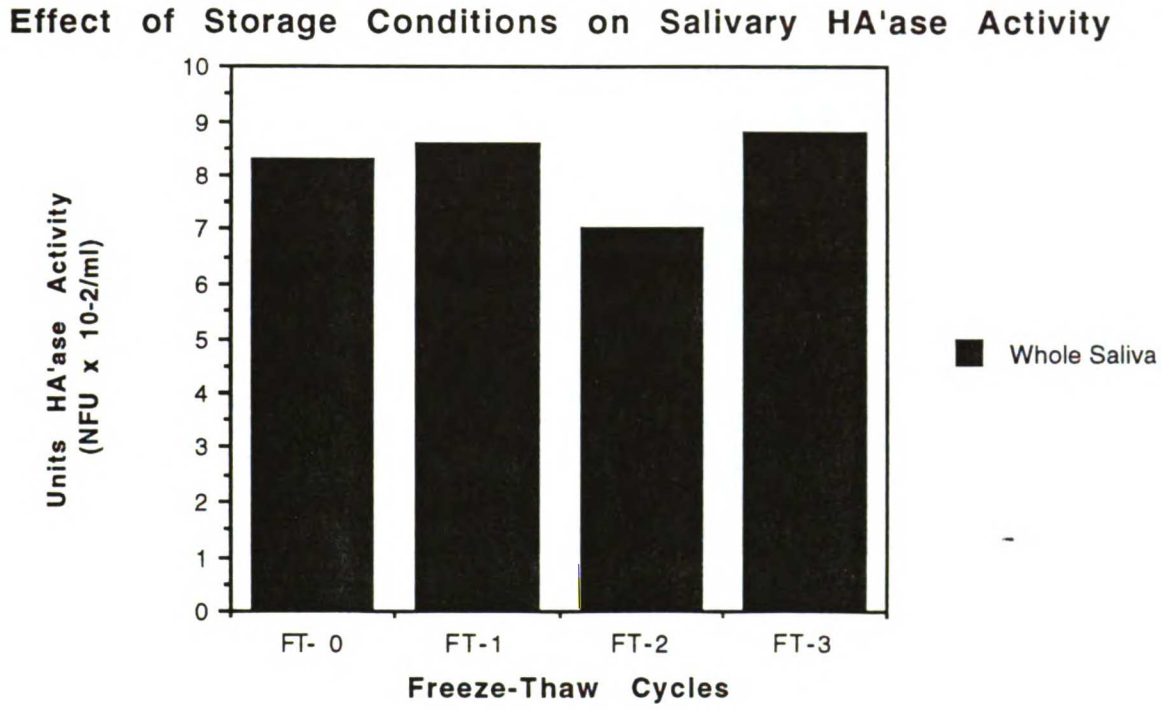



Figure 3-2: Salivary HA'ase activity following multiple freeze-thaw cycles. After 3 consecutive rounds of freezing in an 1:1 ethanol and propanol/dry ice mixture and thawing at 4°C, aliquots of each round were compared with untreated whole saliva for HA'ase activity in citrate-phosphate buffer, 0.5 M NaCl, pH 3.7. Multiple freeze-thaw cycles had little effect on apparent activity.

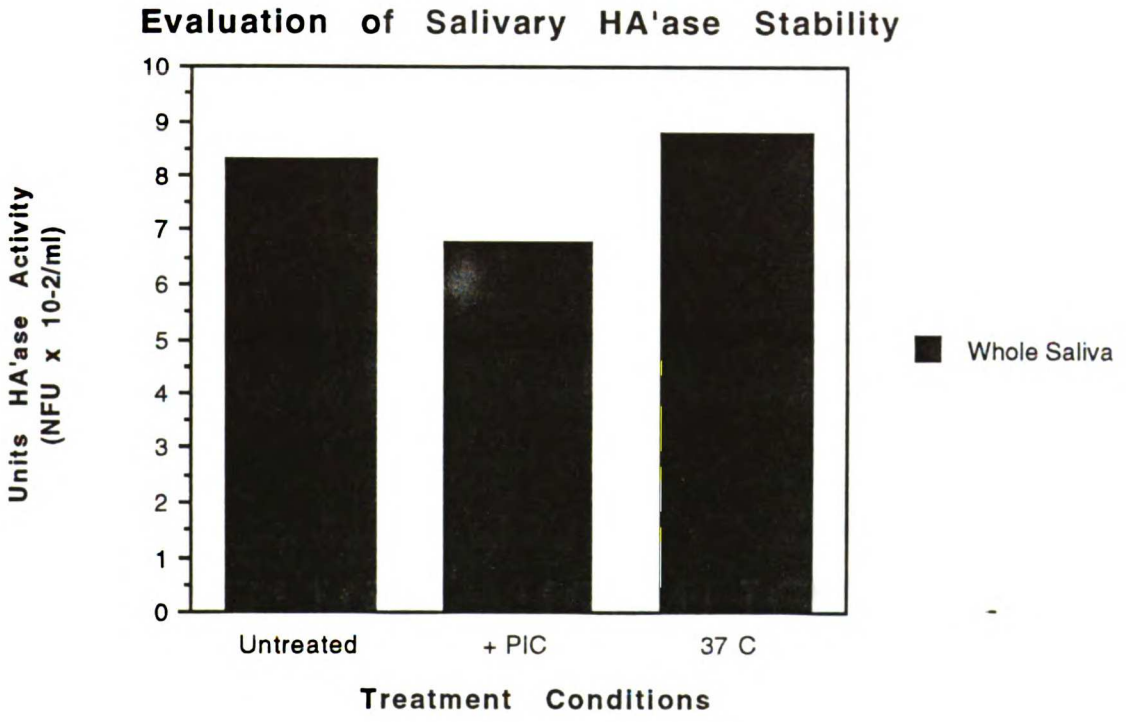



**Figure 3-3: Assessment of HA'ase Activity with PIC or a prolonged 37°C incubation.** Samples of unstimulated whole saliva were evaluated for maintenance of HA'ase activity in citrate-phosphate buffer, 0.5 M NaCl, pH 3.7 with the addition of a 1:50 dilution of PIC or a 2.5 hour incubation at 37°C without PIC. An approximate 10% reduction in activity was apparent with PIC whereas little effect on activity was observed with a prolonged 37°C incubation.

salivary HA'ase to endure a prolonged incubation period (2.5 hours) at 37°C was confirmed also.

**II. Protein Concentration**

Unstimulated parotid saliva exhibited the highest protein concentration at 1.15 ± 0.58 mg/ml (mean ± standard deviation) (range 0.43 - 2.22 mg/ml) as determined by the BioRad Protein Microassay (Figure 3-4). The protein content in stimulated parotid saliva showed a tendency to decrease with a mean concentration of 0.95 ± 0.61 mg/ml (range 0.03 - 1.83 mg/ml). However, this trend was not apparent in unstimulated and stimulated whole saliva samples which contained 0.98 ± 0.28 mg/ml (range 0.64 - 1.64 mg/ml) and 1.26 ± 0.49 mg/ml (range 0.59 - 2.12 mg/ml) protein, respectively.

**III. Determination of Hyaluronan Concentration in Parotid Saliva and Mixed Saliva**

The ELISA-like assay for HA was utilized to determine the HA levels in saliva samples as shown in Figure 3-5. The greatest HA levels were detected in unstimulated whole saliva with a mean concentration of 459.2 ± 338.1 ng/ml (range 148 - 1270 ng/ml) (Figure 3-5A). Mean HA concentrations decreased to 175.7 ± 105.9 ng/ml (range 73 - 375 ng/ml) in stimulated whole saliva secretions. However, slightly elevated levels of HA were detected in 2 individuals. The mean HA content for unstimulated and stimulated parotid saliva was 98.3 ± 85.7

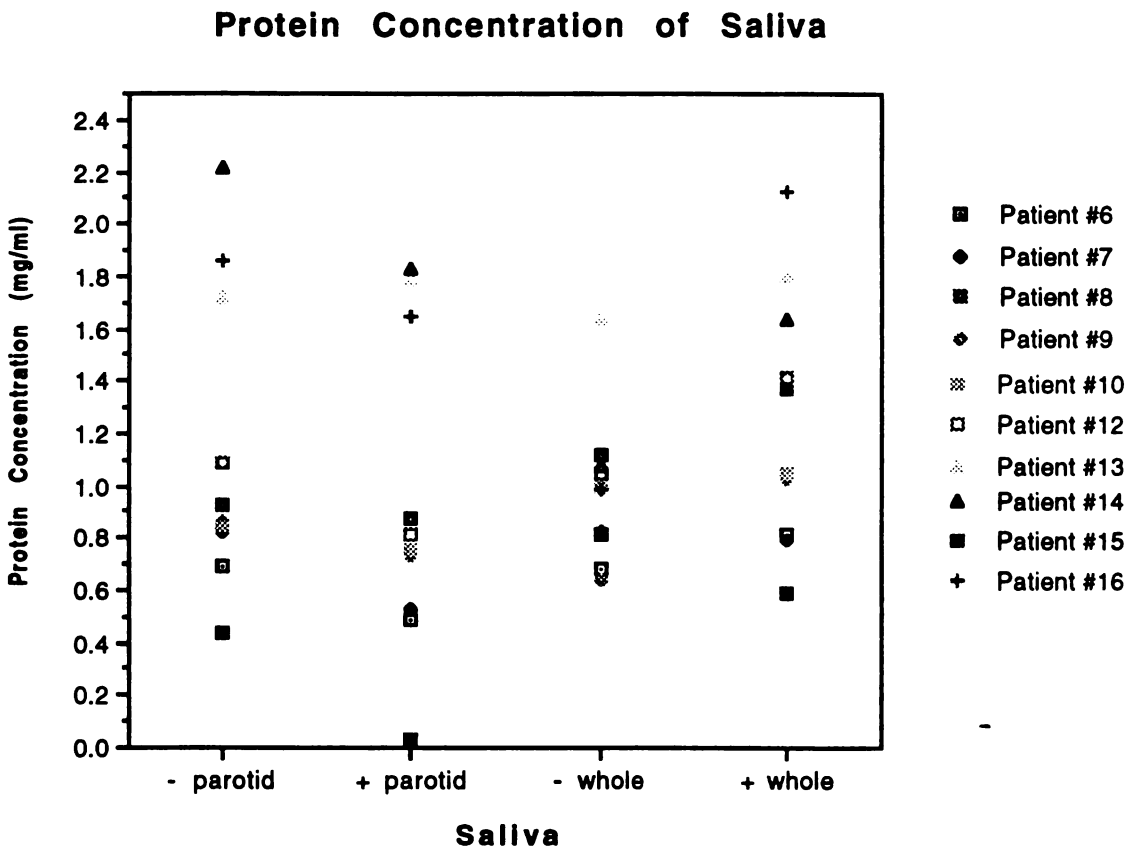



Figure 3-4: Distribution of protein content in unstimulated and stimulated parotid saliva and whole saliva. Each sample was diluted 1:30 and assayed using the BioRad Protein Microassay kit according to the manufacturers instructions. Salivary protein concentrations (mg/ml) were determined relative to absorbance values ($OD_{595}$) obtained for the BSA standard curve. A slight decrease in protein content was apparent in stimulated parotid saliva as compared to the unstimulated parotid samples. This trend was not observed in whole saliva samples.

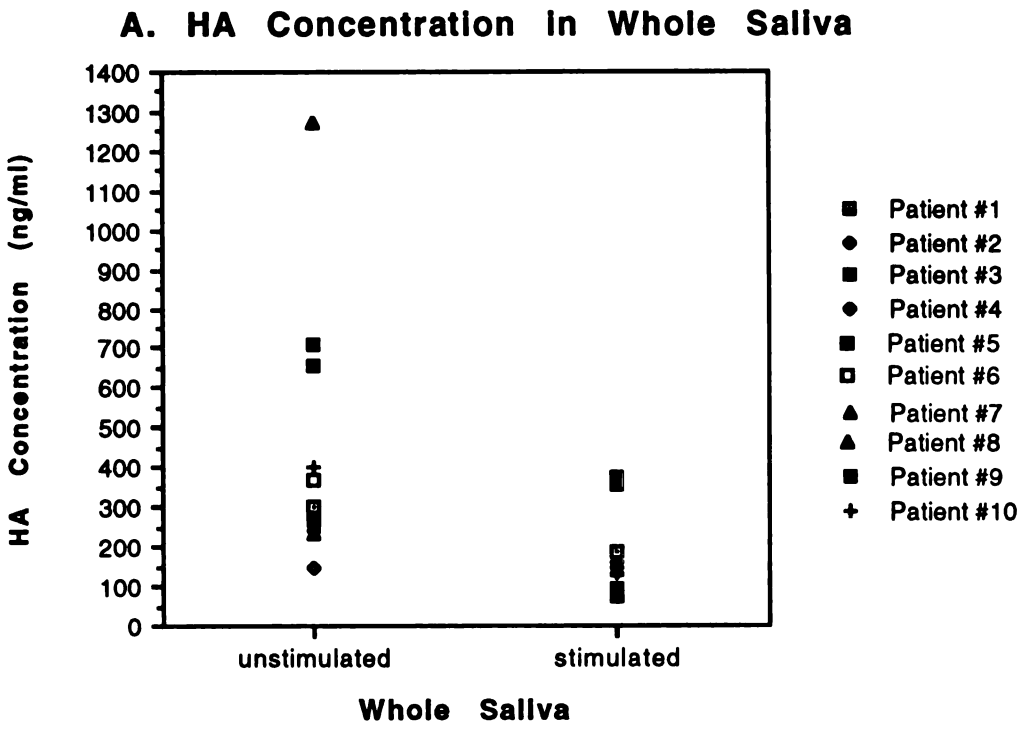
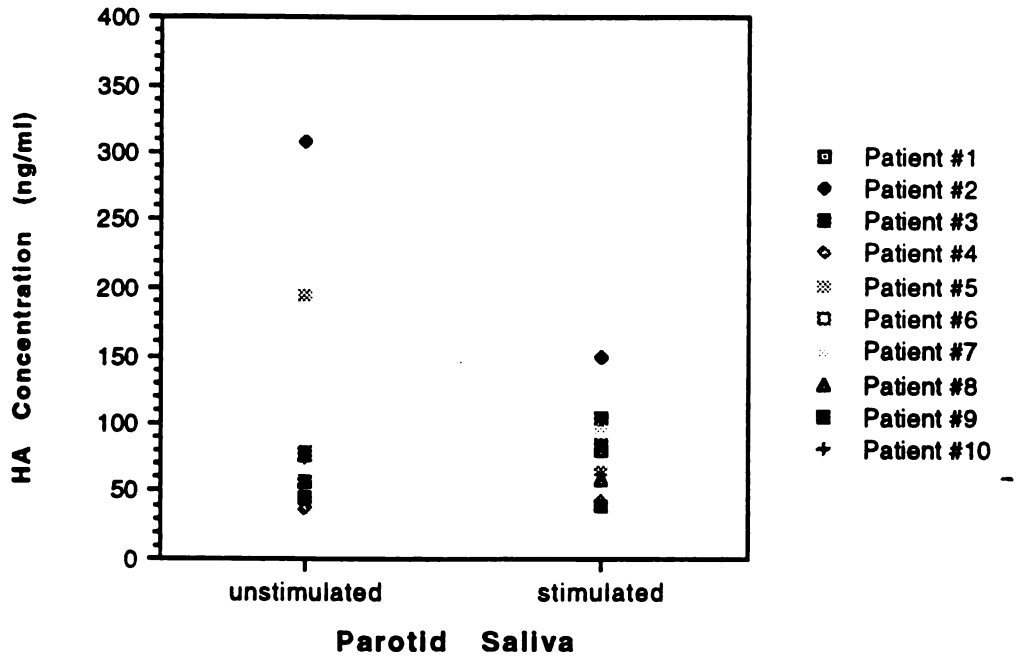



Figure 3-5: Distribution of hyaluronan content in unstimulated and stimulated (A) whole saliva and (B) parotid saliva. The mean HA content of 10 subjects was determined by the ELISA-like assay for HA using a biotinylated HABP as the indicator molecule. Absorbance values ($OD_{492}$) were compared to a Healon$^{R}$ HA standard curve to determine the HA content (ng/ml) in saliva samples. Unstimulated saliva has higher levels of HA in both whole and parotid saliva.

**B. HA Concentration in Parotid Saliva**



ng/ml (range 37 - 307 ng/ml) and 82.7 ± 33.4 ng/ml (range 40 - 150 ng/ml), respectively (Figure 3-5B). Although the mean HA concentration appeared to decrease in stimulated parotid saliva, 8 subjects displayed approximately equivalent or increased HA content in the stimulated secretions.

**IV. Characterization of Hyaluronidase Activity in Parotid Saliva and Mixed Saliva**

In the initial screen of whole saliva for the presence of HA'ase activity in citrate-phosphate buffer, 0.5 M NaCl, pH 3.7 or phosphate buffer, 0.5 M NaCl, pH 6.2, HA'ase activity was detected only in the citrate-phosphate buffer as measured by the ELISA-like HA'ase assay (data not shown). Hence, this buffer was used to demonstrate the proportional dose-dependence of HA'ase in whole saliva as seen in Figure 3-6. Linearity began at a 1:10 dilution of saliva and thus, all samples were prepared at this concentration to measure HA'ase activity throughout the remainder of the investigation. This was in accordance with Afify et al. (1993) in that any biological extract must be diluted first to demonstrate this proportionality to HA'ase enzyme activity.

Several enzymatic solutions were compared for optimal salivary HA'ase activity since each varies with the pH range of buffering capacity. The preliminary pH profile of unstimulated whole saliva revealed two peaks of activity in

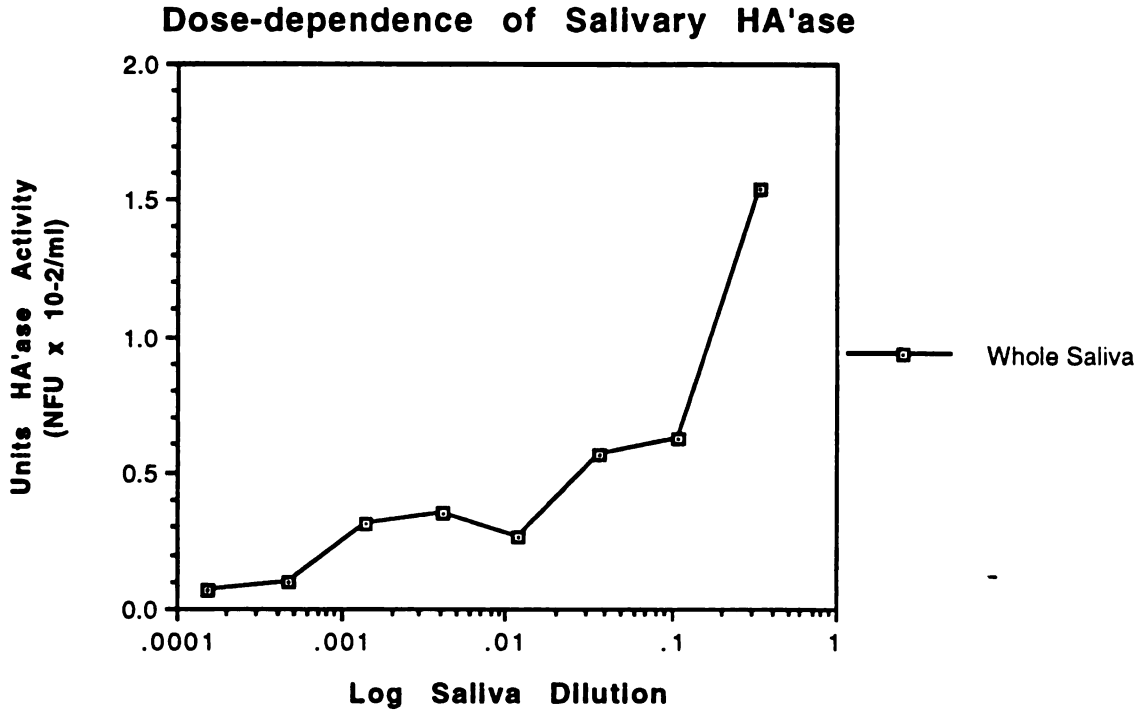



Figure 3-6: Dose-dependence of HA'ase activity in whole saliva. Serial 1:3 dilutions of unstimulated whole saliva in citrate-phosphate buffer, 0.5 M NaCl, pH 6.2 were examined for activity using the ELISA-like assay for HA'ase. Linearity began approximately at a 1:10 dilution of saliva which was subsequently used for all remaining HA'ase assays.

citrate-phosphate buffer, pH 3.7 and phosphate buffer, pH 6.8 (Figure 3-7). Each buffer contained 0.05 M NaCl and 0.02% BSA. However, under both conditions, no HA'ase activity was detected in parotid saliva at the low salt concentration. Including a comparison with formate buffer, a similar pattern of results for whole saliva samples was obtained using a 0.5 M NaCl concentration in the enzymatic buffer (data not shown).

Investigation of the effects of salt concentration on enzymatic activity revealed interesting differences between parotid and mixed saliva. Unstimulated whole saliva demonstrated elevated HA'ase activity levels in 0.05 M NaCl at the acidic peak of activity while the neutral peak showed little effect of differing salt concentrations (Figure 3-8A). In comparison, unstimulated parotid saliva displayed activity only in citrate-phosphate buffer containing 0.5 M NaCl at pH 3.7 (Figure 3-8B). Because of the importance of high salt concentrations for HA'ase activity in parotid saliva, a comparison of different enzyme buffers at pH 3.7 confirmed that citrate-phosphate buffer was optimal for this enzyme (Figure 3-9).

A more detailed pH profile ranging from pH 3.3 to 7.3 of unstimulated parotid and mixed saliva is shown in Figure 3-10. Whole saliva demonstrated a slight shift in the acidic activity peak to pH 4.0 while the neutral activity peak

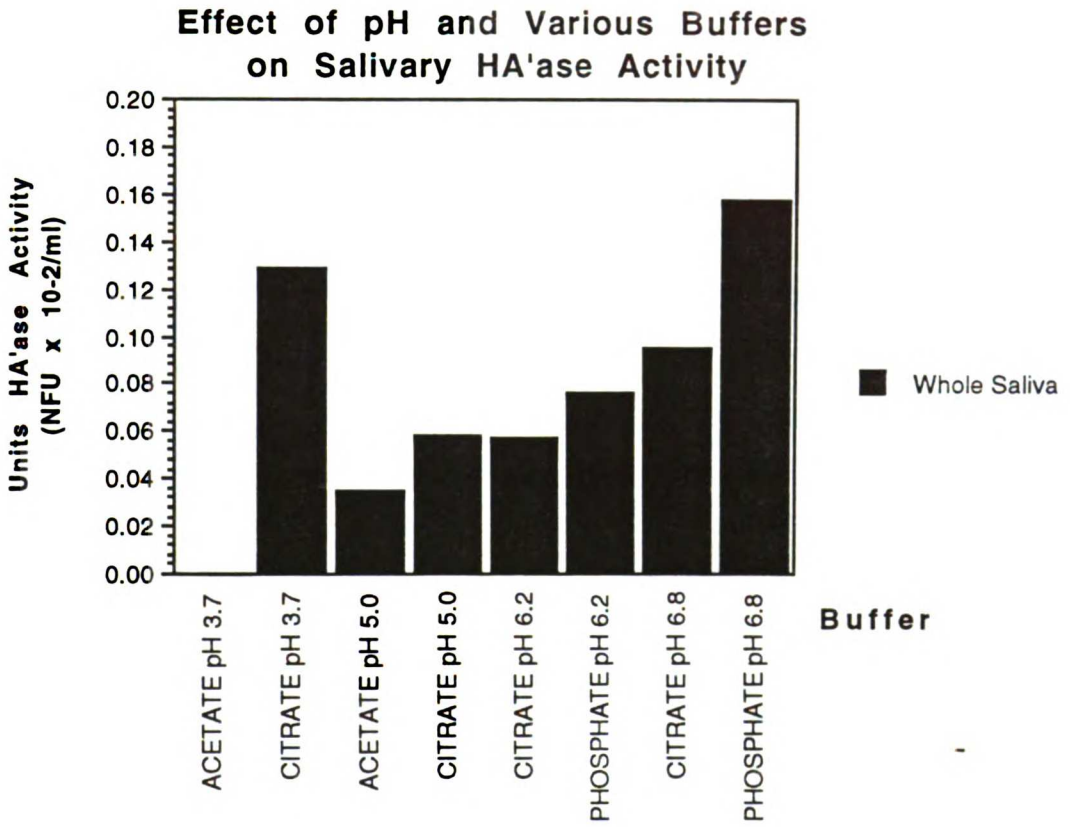



**Figure 3-7: Comparison of pH and enzymatic buffers for optimal salivary HA'ase activity.** Various enzymatic buffers were evaluated within their maximal range of buffering capacity for optimal HA'ase activity in unstimulated whole saliva and parotid saliva. All buffers contained 0.05 M NaCl and 0.02% BSA. A bimodal distribution was apparent in whole saliva. No activity was detected in parotid saliva.

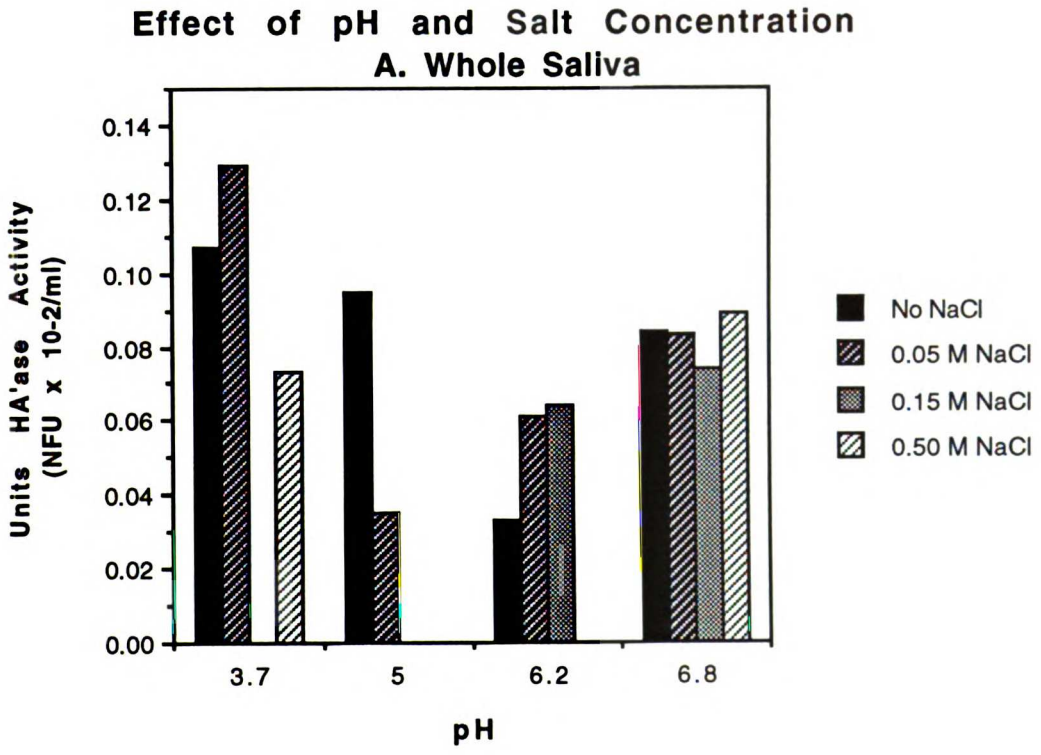
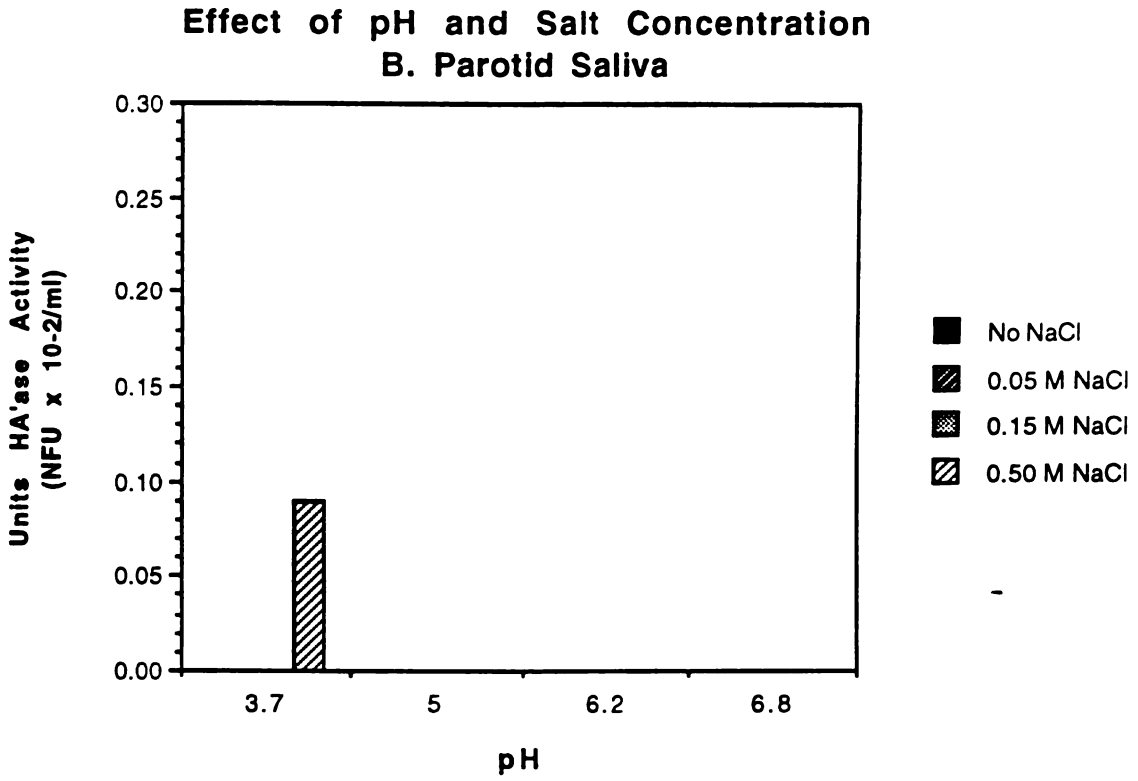



Figure 3-8: pH and salt curve of whole saliva and parotid saliva. A comparison of citrate-phosphate buffer, pH 3.7 and pH 5.0, to phosphate buffer, pH 6.2 and pH 6.8, at various salt concentrations examined the HA'ase activity in unstimulated (A) whole saliva and (B) parotid saliva. All buffers contained 0.02% BSA. Two major peaks activity were observed at pH 3.7 and 6.8 in whole saliva whereas parotid saliva displayed activity only at pH 3.7.


Effect of pH and Salt Concentration
B. Parotid Saliva
Units HA'ase Activity
(NFU x 10-2/ml)
0.30
0.25
0.20
0.15
0.10
0.05
0.00
3.7
5
6.2
6.8
pH
No NaCl
0.05 M NaCl
0.15 M NaCl
0.50 M NaCl

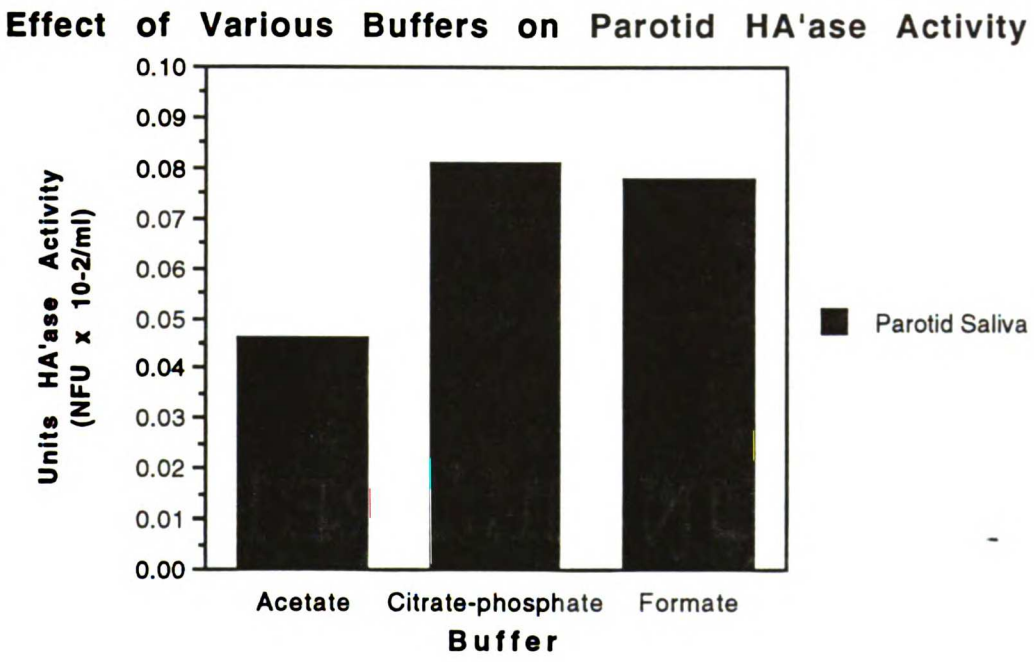



**Figure 3-9: Comparison of enzymatic buffers at pH 3.7 for optimal salivary HA'ase activity in unstimulated parotid saliva. Acetate, citrate-phosphate, and formate buffers containing 0.5 M NaCl and 0.02% BSA were assessed to determine the optimal conditions for parotid HA'ase activity. Citrate-phosphate buffer appeared to be the optimal buffer for the acid-active HA'ase in parotid saliva.**

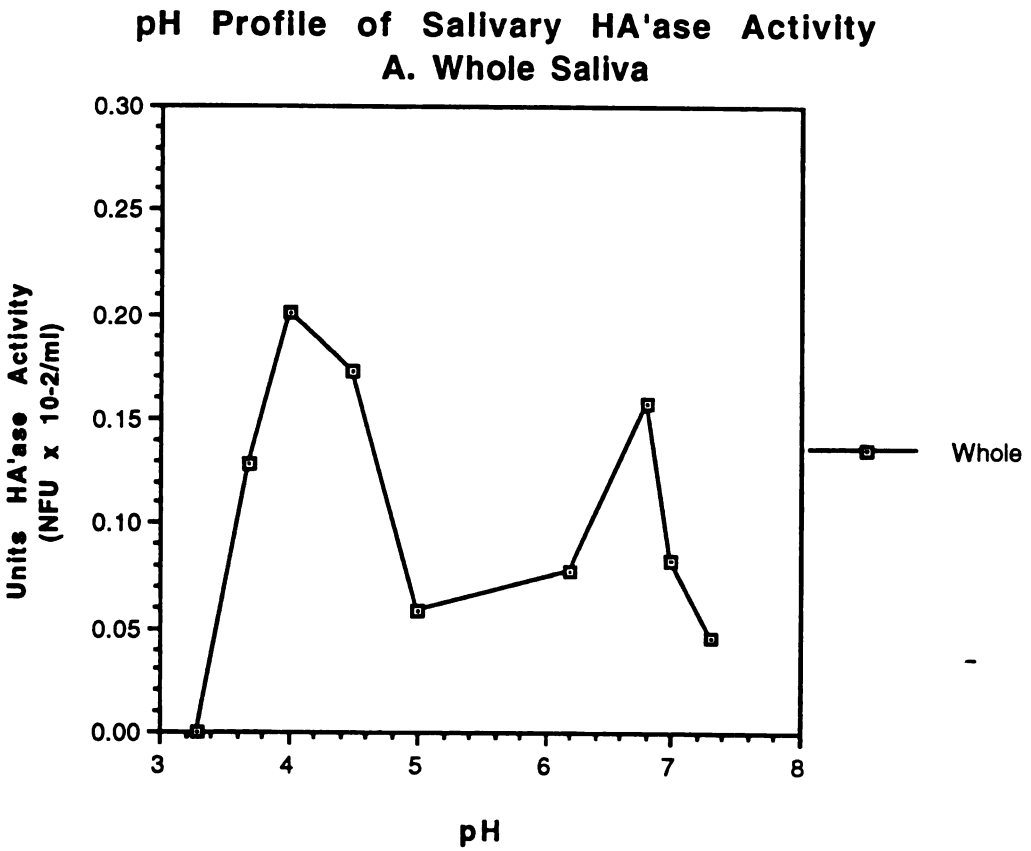
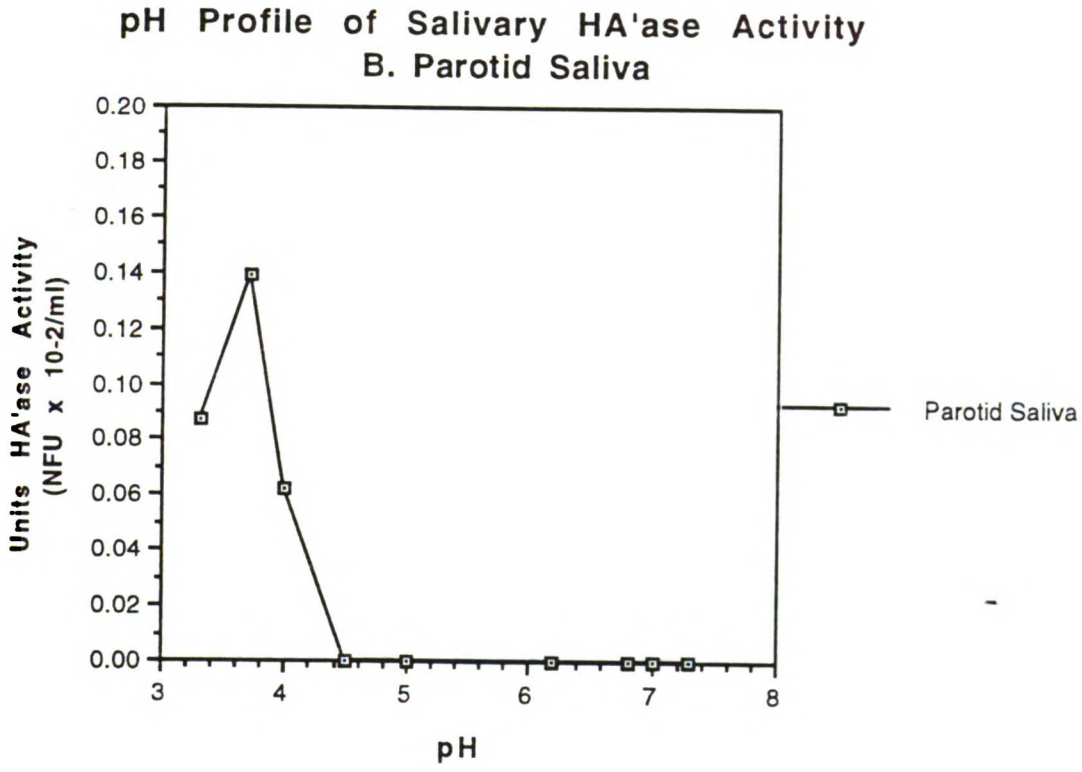



Figure 3-10: Extended pH curve for whole saliva and parotid saliva. (A) Unstimulated whole saliva demonstrated two major peaks of HA'ase activity, pH 4.0 and pH 6.8, when assayed in enzymatic buffers containing 0.05 M NaCl and 0.02% BSA. Again, a bimodal pH curve of optimal enzyme activity was apparent. (B) Unstimulated parotid saliva exhibited one activity peak at pH 3.7 in the presence of 0.5 M NaCl and 0.02% BSA. A unique pH profile for parotid saliva HA'ase was evident by its optimal activity only at low pH.


pH Profile of Salivary HA'ase Activity
B. Parotid Saliva
Units HA'ase Activity
(NFU x 10-2/ml)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
3
4
5
6
7
8
pH
Parotid Saliva

remained at pH 6.8. This shift in the acidic peak of activity was confirmed in whole saliva from two other individuals (data not shown). Parotid saliva reproducibly exhibited peak HA'ase activity at pH 3.7 with no indication of the presence of any neutral enzyme.

The contribution of albumin to HA'ase activity was examined. Parotid and whole saliva samples were diluted in the optimally defined buffer, thus far, and assayed with increasing concentrations of BSA. A unique phenomenon was apparent in comparing the different HA'ase activities of parotid and whole saliva (Figure 3-11 and 3-12). Although the acidic HA'ase activity in both parotid and whole saliva appeared to increase with the addition of 0.02% BSA, the neutral activity in whole saliva tended to decrease by a mean 42% as compared to the complete exclusion of BSA in the dilution buffer.

The effect of concentration of whole saliva by lyophilization was studied with hope of increasing the detectable levels of activity in the ELISA-like assay for HA'ase. Following lyophilization and resuspension at a 10-fold concentration, neutral HA'ase activity was less than doubled as indicated in Figure 3-13.

**V. Hyaluronidase Activity in Parotid Saliva and Mixed Saliva**

Following definition of the optimal conditions for HA'ase

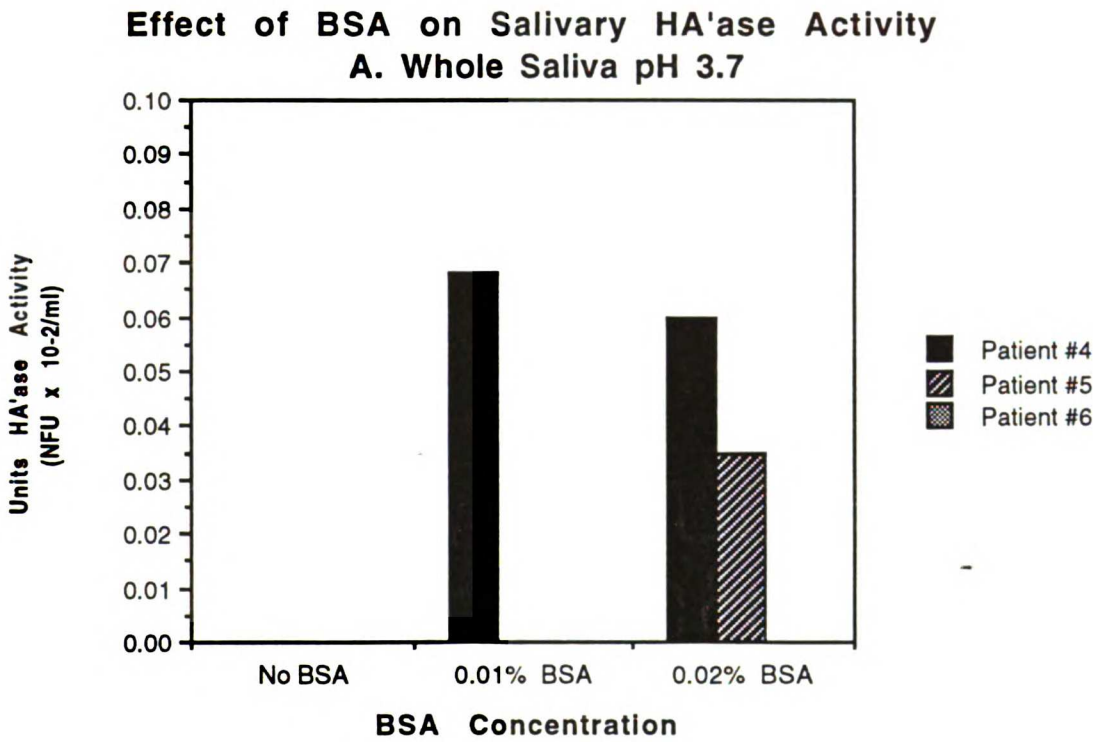
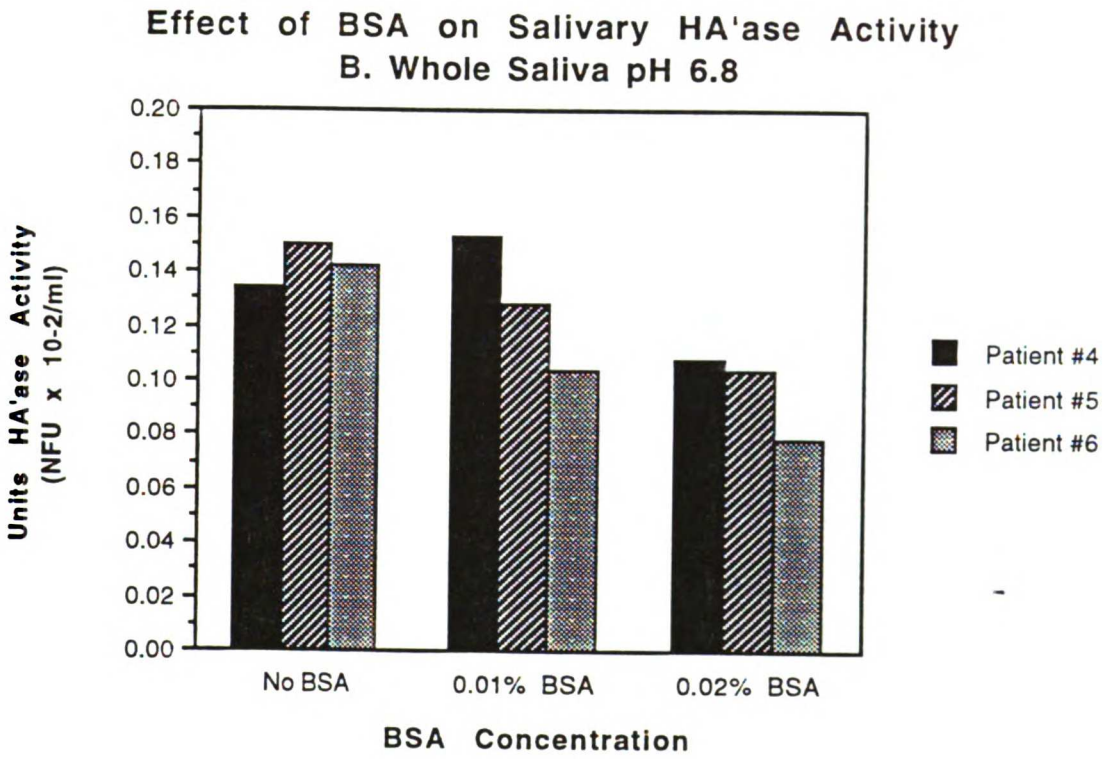



**Figure 3-11: Whole saliva HA'ase activity in the presence of various concentrations of BSA. Unstimulated whole saliva was evaluated for optimal activity in (A) citrate-phosphate buffer, 0.05 M NaCl, pH 3.7 and (B) phosphate buffer, 0.05 M NaCl, pH 6.8. Samples from 3 subjects are shown. The acid-active enzyme appeared to exhibit elevated HA'ase activity with 0.02% BSA whereas the neutral-active enzyme tended to be optimally active in the absence of BSA.**


Effect of BSA on Salivary HA'ase Activity
B. Whole Saliva pH 6.8
Units HA'ase Activity
(NFU x 10-2/ml)
0.20
0.18
0.16
0.14
0.12
0.10
0.08
0.06
0.04
0.02
0.00
No BSA
0.01% BSA
0.02% BSA
BSA Concentration
Patient #4
Patient #5
Patient #6

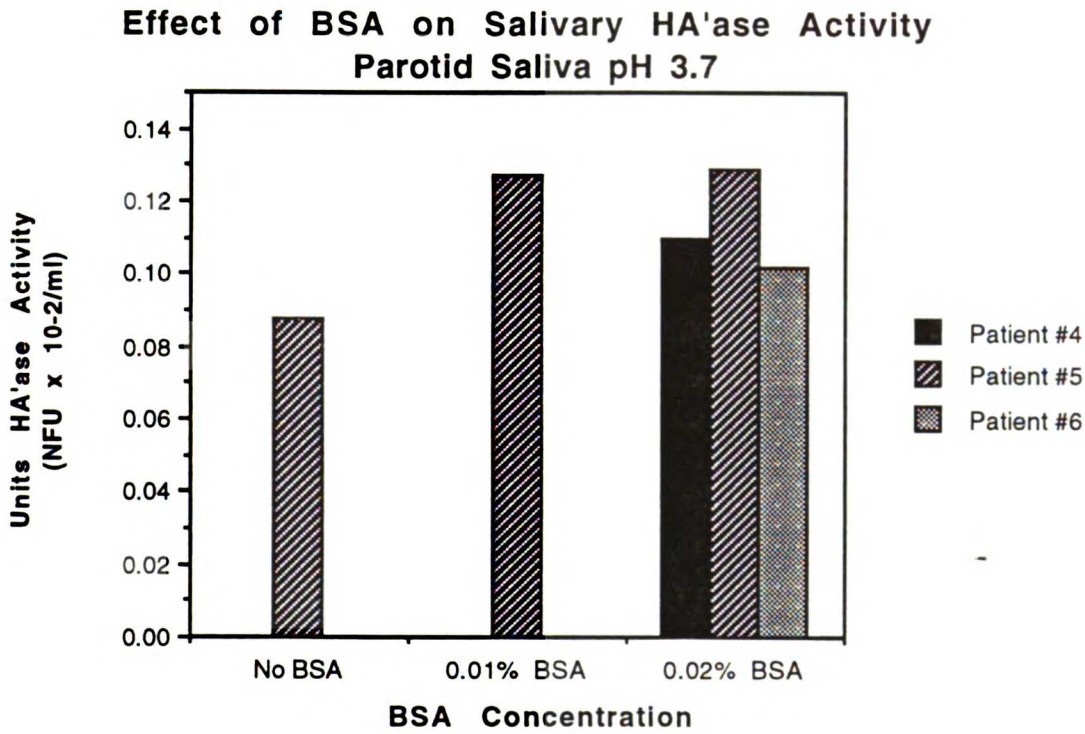



**Figure 3-12: Parotid saliva HA'ase activity in the presence of various concentrations of BSA. Assay conditions for unstimulated parotid saliva consisted of citrate-phosphate buffer, 0.5 M NaCl, pH 3.7. Samples from 3 subjects are shown. Enzyme activity appeared to be greatest with 0.02% BSA.**

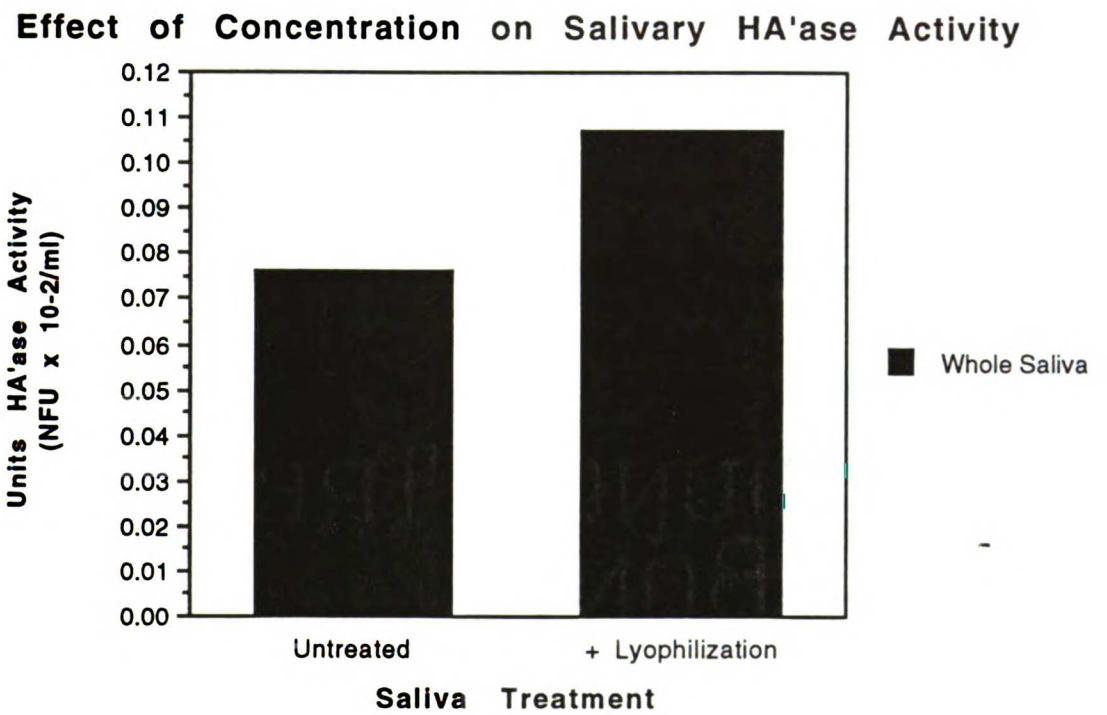



**Figure 3-13: Comparison of HA'ase activity between untreated and concentrated samples. Unstimulated whole saliva was lyophilized, concentrated 10-fold, and assayed for neutral HA'ase activity. Less than a 2-fold increase in activity was apparent following concentration of saliva.**

activity in parotid and mixed saliva, the activity levels were determined for 10 individuals in both unstimulated and stimulated samples. As shown in Figure 3-14, acidic enzyme activity was detected in unstimulated parotid saliva in 4 subjects of whom 2 subjects also exhibited activity in stimulated samples. Unstimulated and stimulated whole saliva samples were assayed at both pH optima and revealed HA'ase activity as indicated in Figure 3-15. Unstimulated whole saliva from 6 subjects demonstrated HA'ase activity at pH 4.0 whereas only 3 subjects displayed activity at pH 6.8. Although neutral enzymatic activity was not present in stimulated whole saliva, analysis showed 2 subjects with acidic HA'ase activity. Similar to parotid saliva, the HA'ase activity present in stimulated whole saliva samples was detected in the same subjects displaying activity in unstimulated samples.

The mean HA'ase activities for unstimulated and stimulated parotid and whole saliva samples displaying levels greater than zero are reported in Table 3-1. Stimulated parotid saliva exhibited slightly increased total levels of HA'ase activity (NFU/ml) as compared to unstimulated samples. However, the relative HA'ase activity, reported in NFU/gm protein, demonstrated decreased levels in stimulated parotid saliva compared to unstimulated samples. Although relative HA'ase activity increased in stimulated samples, the total

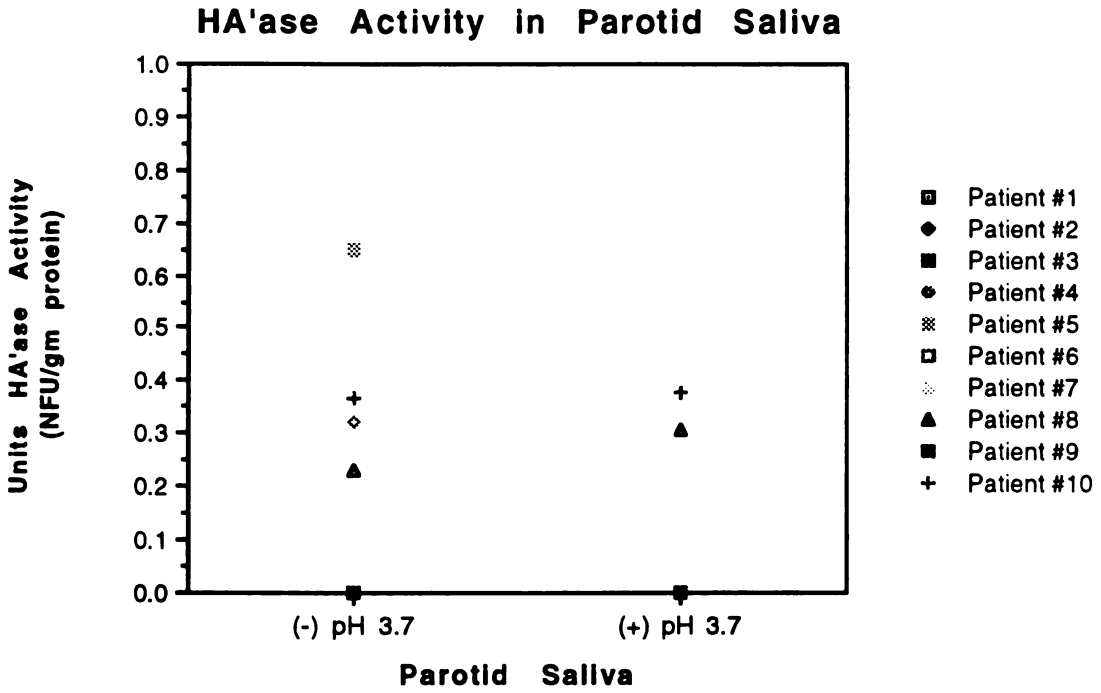



**Figure 3-14: Distribution of HA'ase activity in unstimulated and stimulated parotid saliva. The mean relative HA'ase activity (NFU/gm protein) was determined in 10 subjects utilizing the optimally defined buffer which contained 0.05 M citrate, 0.1 M phosphate, 0.5 M NaCl, 0.02% BSA, 1 mM saccharic acid 1,4-lactone, pH 3.7. The mean relative HA'ase activity in unstimulated saliva samples displaying detectable levels tended to be slightly higher than in stimulated samples.**

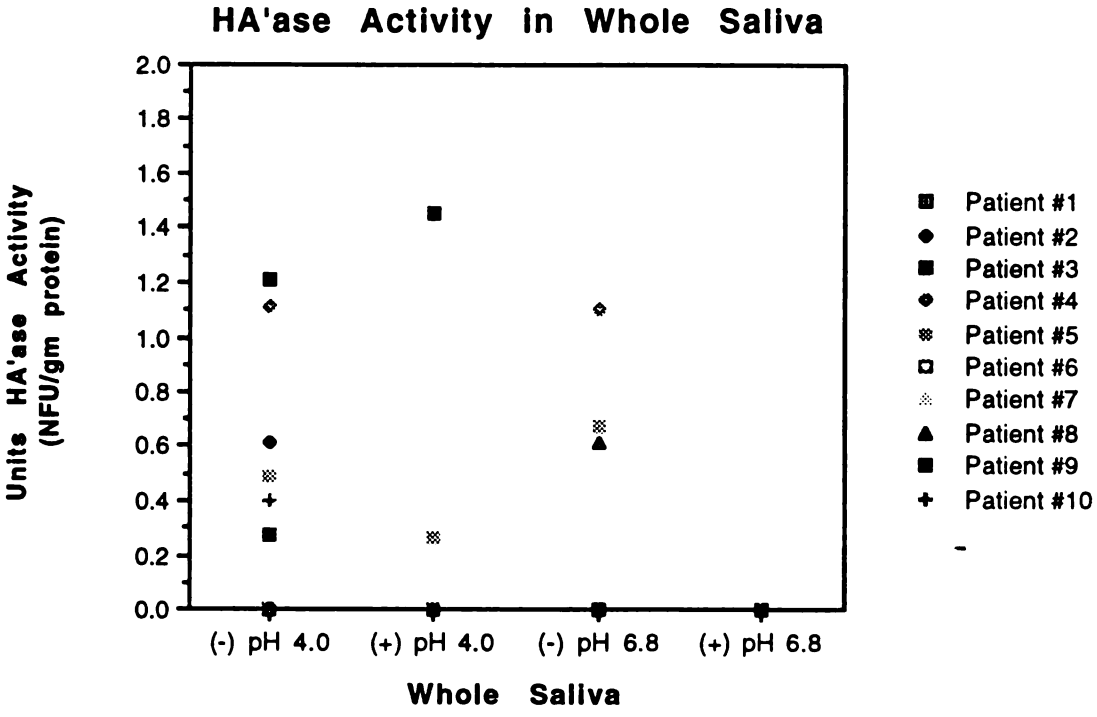



Figure 3-15: Distribution of HA'ase activity in unstimulated and stimulated whole saliva. The mean relative HA'ase activity (NFU/gm protein) was determined in 10 subjects utilizing the optimally defined buffers which contained either 0.05 M citrate, 0.1 M phosphate, 0.05 M NaCl, 0.02% BSA, pH 4.0 or 0.1 M phosphate, 0.05 M NaCl, pH 6.8. Each buffer included 1 mM saccharic acid 1,4-lactone. Although the acid-active enzyme was detected in more subjects, the neutral-active HA'ase appeared to exhibit an elevated mean relative HA'ase activity in the samples in which it was detected.

| | UNSTIMULATED | | STIMULATED | |
|---|---|---|---|---|
| HA'ase ACTIVITY | Total NFU x 10-3/ml | Relative NFU/gm protein | Total NFU x 10-3/ml | Relative NFU/gm protein |
| PAROTID pH 3.7 | 0.051 ± 0.017 | 0.393 ± 0.181 | 0.059 ± 0.004 | 0.341 ± 0.050 |
| WHOLE pH 4.0 | 0.056 ± 0.025 | 0.682 ± 0.389 | 0.056 ± 0.041 | 0.855 ± 0.842 |
| WHOLE pH 6.8 | 0.068 ± 0.002 | 0.796 ± 0.265 | 0 | 0 |

Table 3-1 : Hyaluronidase Activity in Saliva
Reported values represent the mean ± standard deviation HA'ase activity of the saliva samples in which the enzyme was detected. Total and relative HA'ase activity are given in NFU/ml and NFU/gm protein, respectively.

levels of acidic enzyme activity in unstimulated and stimulated whole saliva were comparable. Interestingly, neutral HA'ase activity was detected only in unstimulated whole saliva. In both cases, total and relative HA'ase activity in unstimulated whole saliva tended to show increased neutral HA'ase activity relative to the acidic enzyme activity.

The presence of endogenous HA'ase inhibitors was investigated by measurement of commercially purified *Streptomyces* HA'ase activity with the addition of increasing quantities of saliva sample. Figure 3-16 demonstrates the presence of HA'ase inhibitors in both parotid saliva and whole saliva which were capable of masking the activity of the purified *Streptomyces* HA'ase. Using a 1:10 dilution of saliva as was utilized in the ELISA-like HA'ase assays, parotid saliva and whole saliva inhibited *Streptomyces* HA'ase activity by a mean value of 64% for all samples. With the exception of one sample, saliva secretions exhibiting HA'ase activity demonstrated decreased levels of inhibition than those samples lacking enzymatic activity (Table 3-2).

**VI. Dot Blot Analysis**

Immunorecognition of salivary HA'ase was achieved using polyclonal antibodies against purified bovine testicular HA'ase as indicated in Figure 3-17. All unstimulated and

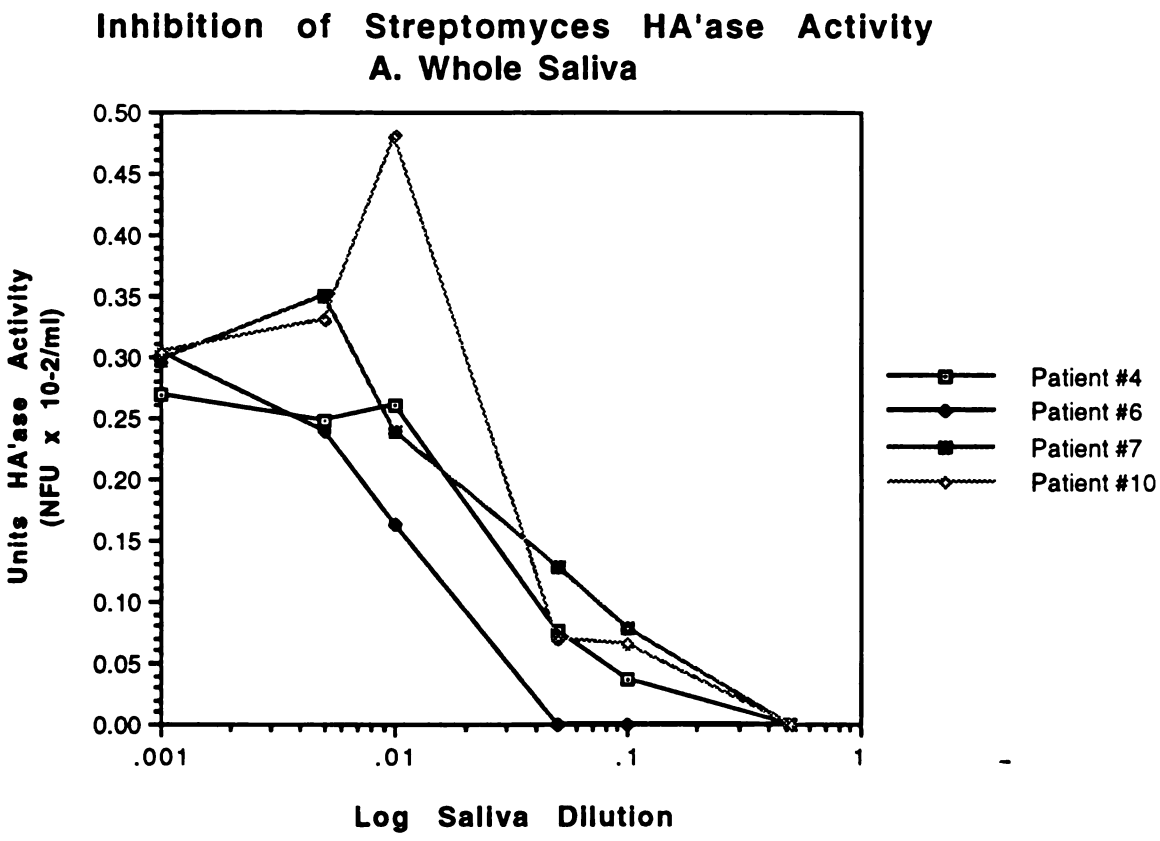
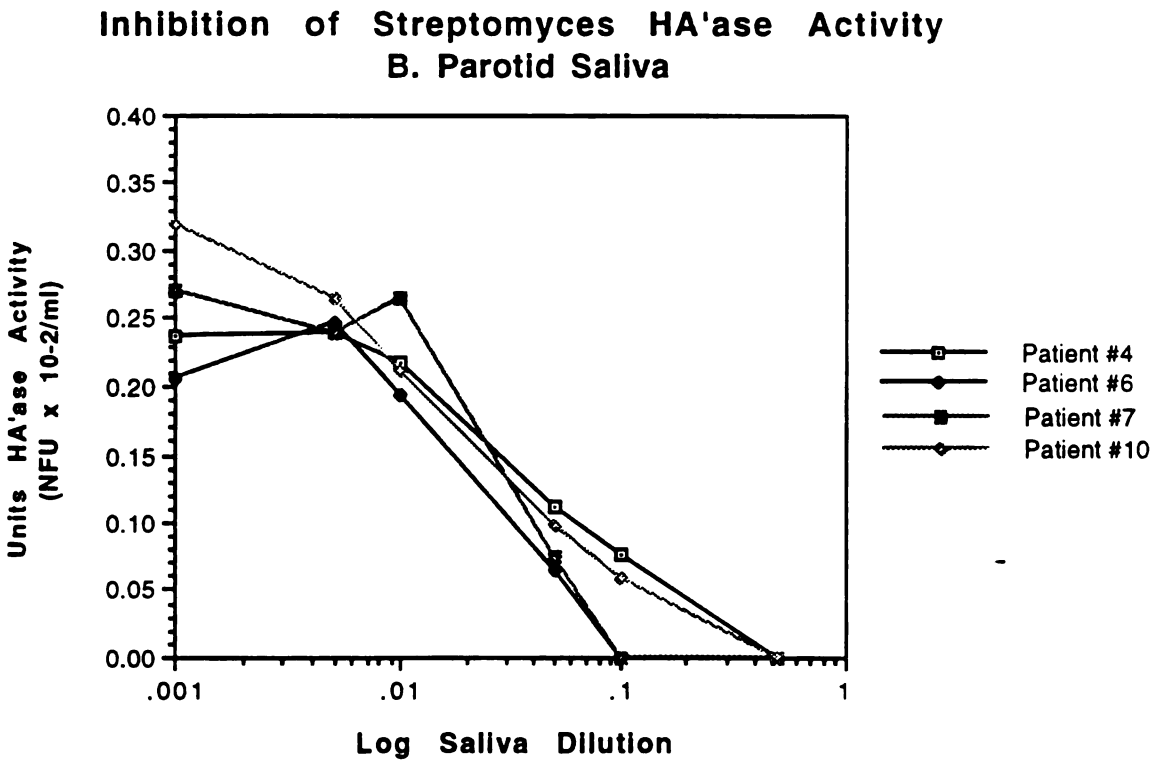



Figure 3-16: Demonstration of HA'ase inhibitors in saliva. Increasing amounts of unstimulated whole saliva (A) and parotid saliva (B) samples were assayed for their ability to inhibit activity of commercially purified *Streptomyces* HA'ase. The incubation buffer consisted of 0.5 M acetate, 0.05 M NaCl, 0.02% BSA, pH 5.0. Samples from 4 subjects exhibiting either positive or negative HA'ase activity were analyzed. Inhibition of *Streptomyces* HA'ase activity was apparent in both whole and parotid saliva samples in which HA'ase activity had or had not been detected previously by the ELISA-like assay for HA'ase.


Inhibition of Streptomyces HA'ase Activity
B. Parotid Saliva
Units HA'ase Activity
(NFU x 10-2/ml)
0.40
0.35
0.30
0.25
0.20
0.15
0.10
0.05
0.00
.001
.01
.1
1
Log Saliva Dilution
Patient #4
Patient #6
Patient #7
Patient #10

| PATIENT | PAROTID pH 3.7 | | WHOLE pH 4.0 | WHOLE pH 6.8 | |
|---|---|---|---|---|---|
| | HA'ase | %I | HA'ase | HA'ase | %I |
| 4 | + | 43.4 | + | + | 66.1 |
| 6 | - | 82.9 | - | - | 88.9 |
| 7 | - | 72.9 | - | - | 47.4 |
| 10 | + | 56.6 | + | - | 53.4 |

Table 3-2 : Inhibition of *Streptomyces* HA'ase Activity

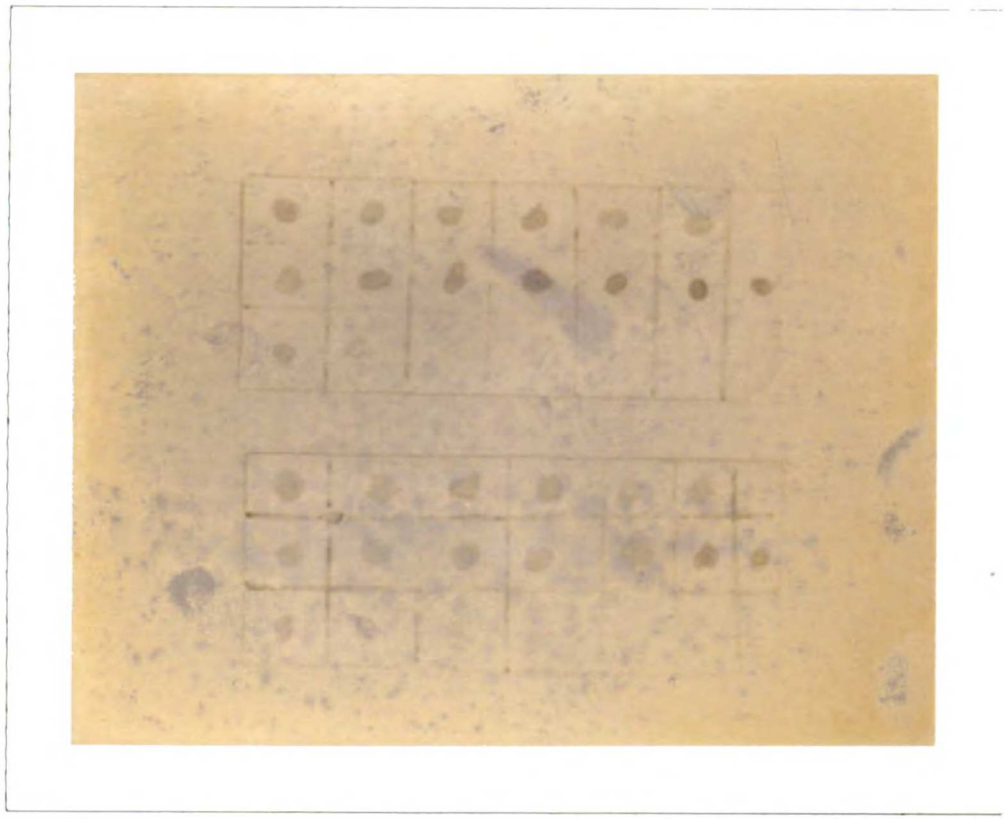
Enzymatic assay results of 4 individuals in which HA'ase activity was positively (+) or negatively (-) detected are reported with the corresponding percent inhibition (%I) of $2.5 \times 10^{-4}$ NFU/ml *Streptomyces* HA'ase activity in the presence of a 1:10 dilution of saliva. Most saliva samples containing HA'ase activity tended to show decreased inhibition levels as compared to samples lacking enzymatic activity.

| | | | | | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 4 | 5 | (-) |
| 6 | 7 | 8 | 9 | 10 | (+) | 1:2 |
| 1:4 | 1:8 | 1:16 | 1:32 | 1:64 | 1:128 | |

**KEY**

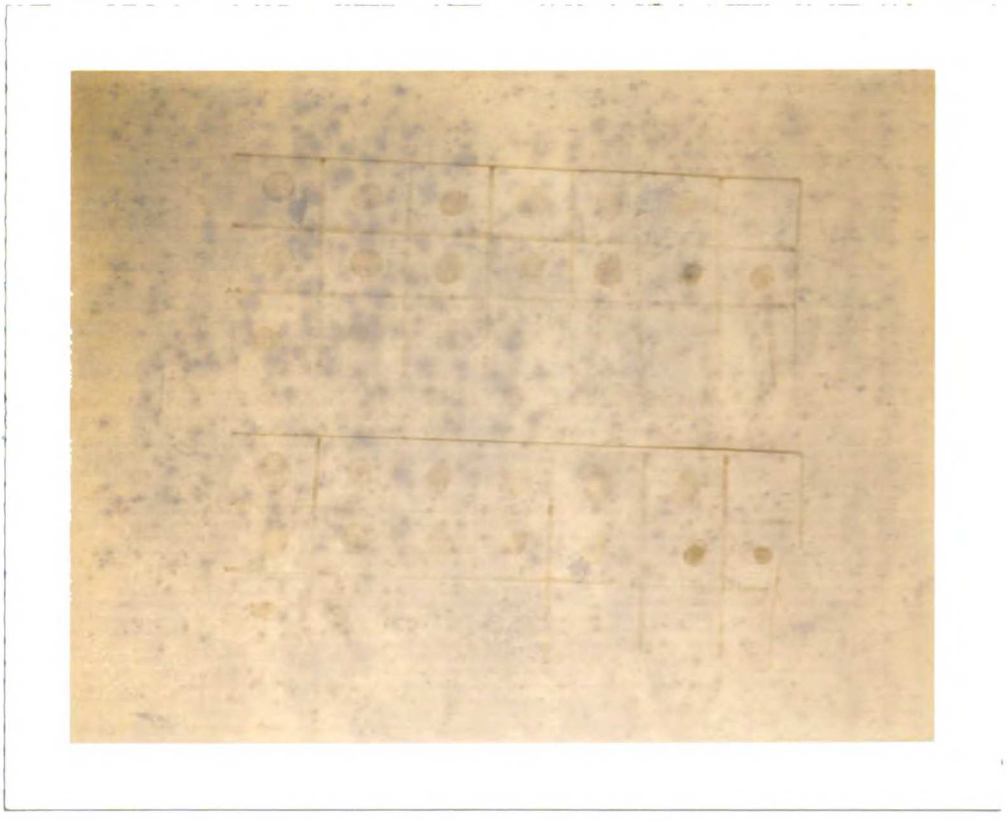
Patient # 1 - 10 = saliva sample
(-) = Negative control: 3% BSA
(+) = Positive control: 150 NFU/ml bovine testicular HA'ase
1:2 - 1:128 = Serial 1:2 dilutions of bovine testicular HA'ase

**Figure 3-17: Dot blot analysis for detection of salivary HA'ase. Polyclonal antibodies against purified bovine testicular HA'ase were utilized to detect HA'ase in unstimulated (-) and stimulated (+) parotid saliva (P) and whole (W) saliva of all 10 subjects. 3% BSA and serial 1:2 dilutions of purified bovine testicular HA'ase were included as negative and positive controls, respectively. All saliva samples reacted positively with the anti-HA'ase antibody indicating HA'ase was present in each saliva sample, independent of whether enzyme activity was detected or not.**



-W

+W



-P

+P

stimulated parotid and whole saliva samples reacted positively with this polyclonal antibody. In an attempt to quantitate the salivary HA'ase, a comparison of reaction intensities to serial dilutions of bovine testicular HA'ase permitted an estimation. Unstimulated parotid saliva and whole saliva samples reacted with similar intensity to the 37.5- 75 NFU/ml range for bovine testicular HA'ase. Although more difficult to estimate definitively, samples of stimulated saliva displayed a slight decrease in reactivity within the range of 18.8 - 37.5 NFU/ml of bovine testicular HA'ase.

# CHAPTER 4: DISCUSSION

As an essential macromolecule of the ECM, HA functions not only as a structural moiety, but also participates in several fundamental biological processes including cellular growth, development, and repair of tissues. In particular, HA levels escalate whenever rapid tissue proliferation, regeneration, and repair occur (Longaker and Adzick, 1991). Furthermore, the catabolism of HA by its degradative enzyme HA'ase is critically important because of its role in signalling a change from cellular proliferation to cellular differentiation in morphogenesis as well as in healing wounds (Toole et al., 1972; Bertolami and Donoff, 1978; 1982; Alexander and Donoff, 1979; Thet et al., 1983). Hence, the regulation of HA metabolism may represent an intricate balance between factors which direct HA production (Decker et al., 1989) and those responsible for its degradation by HA'ases.

The healing properties of saliva have been recognized by many investigators (Hutson et al., 1979; Li et al., 1980; Niall et al., 1982; Hart and Powell, 1990; Bodner, 1991). However, little study has been devoted to the presence and possible roles of HA and HA'ase in intraoral wound healing. This study establishes baseline levels of HA in normal adult human pure parotid saliva and mixed whole saliva. Additionally, although HA'ases of both tissue and microbial origin have been reported

in whole saliva (Hopps and Prout, 1972), identification of HA'ase activity in parotid saliva has not been described previously. Moreover, in this study HA'ase was detected in both whole and parotid saliva samples by a polyclonal HA'ase antibody further supporting the presence of salivary HA'ase, particularly in parotid secretions.

**I. Hyaluronan Levels in Saliva**

With the advent of new techniques with increased sensitivity and specificity, detection of HA has become possible in several tissues and body fluids including human umbilical cord, synovial fluid, vitreous body, dermis, lymph, urine, and serum (Laurent and Fraser, 1986). Based on a high affinity biotinylated HA-binding peptide of proteoglycan core protein derived from bovine nasal cartilage, an ELISA-like assay (Fosang et al., 1990) was utilized to determine HA levels in saliva samples. Unstimulated whole saliva and parotid saliva (459.2 ng/ml and 98.3 ng/ml, respectively) contained HA at concentrations that were comparable to levels reported in other human bodily fluids. For example, HA concentrations in urine range from 100 - 500 ng/ml whereas human serum HA levels are somewhat lower ranging from 10 - 100 ng/ml (Laurent and Fraser, 1986). The increased quantity of HA in whole saliva may be attributed to the HA contributions from all salivary sources since whole saliva, often referred to as mixed saliva, consists of output from the three major salivary glands, the

parotid, submandibular, and sublingual, as well as the numerous minor salivary glands.

By its physical properties alone, HA functions to maintain the integrity of connective tissues. In aqueous solution, HA displays an immense capacity to hold water and small molecules (Sweeney and Weiss, 1992) resulting in an increased viscosity. Demonstrating strong shear-dependence and visco-elastic behavior, HA serves as a excellent lubricant of joints and tissues (Laurent and Fraser, 1992). Although the HA content in saliva is relatively low compared to other fluids such as human synovial fluid (1420 -3600 mg/L) (Laurent and Fraser, 1986), its mere presence in saliva may contribute to the functional quality of saliva. The major function of saliva is to protect the oral cavity through its lubricating properties, selective permeability, buffering capacity, role in taste recognition of noxious substances, and antibacterial characteristics (Ten Cate, 1985; Herrera et al., 1988). The high glycoprotein content and lubricating properties of saliva serve to protect against microbial toxins, noxious stimuli, and minor trauma. This protective capacity may be enhanced by HA since it also functions as a lubricant (Laurent and Fraser, 1992). Alternatively, HA in saliva may result from localized turnover in the salivary gland tissues which is subsequently secreted. Preliminary attempts to concentrate HA in parotid saliva by Centricon-10 ultrafiltration techniques (molecular

weight cut off = 10,000) resulted in over 50% of the original HA sample as low molecular weight components which may represent secreted oligosaccharide cleavage products (data not shown). Further investigation is necessary to confirm this observation which may be possible by molecular-sieve chromatography using a CL4B sepharose column.

**II. Hyaluronidase Activity of Tissue and Bacterial Origin**

Several HA'ases have been detected in both mammalian and non-mammalian tissues and fluids. Mammalian enzymatic activity has been observed in the kidney, liver, lung, placenta, synovial fluid, serum, and urine; however, the most abundant source has been the testis (Bollet et al., 1963; Yamada et al., 1977; Sahu, 1981; Gold, 1982; Fenger, 1982). Each of these enzymes, with the exception of testicular HA'ase, is acid-active and thus, is presumed to originate from the lysosome. Interestingly, the testicular enzyme (Yang and Srivistava, 1975) and the more recently discovered cultured human dermal fibroblast HA'ase (Stair, 1993) display neutral activity. Other sources of neutral-active enzymes include prokaryotes (Abramson, 1967; Greiling et al., 1975; Fitzgerald and Repesh, 1987; Hynes and Ferretti, 1989; Sting et al, 1990) and non-mammalian organisms such as the bee, leech, and snake (Richman and Baer, 1980; Fiszer-Szafarz, 1984; Yuki and Fishman, 1963; Budds et al., 1987; Pukrittayakamee, 1988).

In the oral cavity, HA'ases of both tissue and microbial origin have been reported to exist. For example, normal buccal mucosa of rabbits exhibited an intrinsic HA'ase activity with an acidic pH optimum indicating a lysosomal enzyme (Bertolami and Ellis, 1985). In contrast, neutral HA'ase activity associated with several species of oral bacteria in dental plaque has been confirmed by many investigators (Schultz-Haudt and Scherp, 1955; Soder and Nord, 1969; Kjellman et al., 1971; Tam and Chan, 1985; Tipler and Embery, 1985).

**III. Hyaluronidase and Periodontal Disease**

Considerable attention has been focused on the role of various enzymes such as collagenase and HA'ase in connective tissue alterations evident in gingivitis and periodontal disease. Increased breakdown of collagen, a major structural component of the periodontal ECM and ECM in general, has been reported as evidenced by elevated levels of collagenase in periodontal patients (Ando, 1980).

Several analogies between collagenase and HA'ase can be made because of their contribution to localized remodeling of the ECM. As a member of the matrix metalloproteinase (MMP) family, collagenase participates in the intricate balance of cell division, matrix synthesis, and matrix degradation to maintain the integrity of surrounding tissues in a similar

manner to HA'ase. Regulation of collagenase activity is imparted by MMP-specific inhibitors, collectively called tissue-inhibitors of metalloproteinases (TIMPS) (Docherty et al., 1992). Hence, HA'ase inhibitors also may be present, particularly in saliva or gingival tissues. Furthermore, collagenase contains a hemopexin-like domain which functions in the specific binding of matrix substrate and is not believed to be required for catalysis (Hunt et al., 1987; Murphy et al., 1992; Clark and Cawston, 1989). Two similar hemopexin-like domains have been recently identified in pig liver HA'ase (Stern, unpublished data). One of these domains contains two HA-binding regions resembling RHAMM (Turley, 1992) and the highly specific peptide sequence of eight basic amino acids out of twelve (Hope et al., 1993). This evidence supports the notion that HA'ase also functions as a HABP (Tapper et al., 1993).

HA'ase activity in human gingiva has been identified although findings have differed as to the origin of the enzyme. D'Allaird and colleagues (1969) reported the presence of bacterial HA'ase activity in periodontal tissues whereas Goggins and coworkers (1968a) provided evidence for an enzyme of lysosomal origin. Additionally, gingival crevicular fluid contained bacterial HA'ase activity and has been associated with increased gingival inflammation (Schultz-Haudt et al., 1954; van Palenstein-Helderman and Hoogeveen, 1976; Last and

Embery, 1987).

**IV. Hyaluronidase Activity in Human Saliva**

Until recently, rapid, convenient, and sensitive assays for the detection of HA'ase were lacking although many have been described (Dorfman, 1948; Dorfman and Ott, 1948; Reissig et al., 1955; Benchetrit et al., 1977; Richman and Baer, 1980; Delpech et al., 1987; Nakamura et al., 1990). Utilizing a novel ELISA-like assay for HA'ase with increased sensitivity (Stern and Stern, 1992), unique HA'ase activity profiles for whole saliva and parotid saliva are described in the present study. However, enzymatic activity is not detected in all individuals and hence, preliminary evidence suggesting the presence of endogenous HA'ase inhibitors is reported. Besides the advantages described above, this ELISA-like assay permits the analysis of multiple samples, detection of HA'ase activity in small sample volumes (particularly important for the limited quantity of pure parotid saliva), and determination of HA'ase inhibitors with only minor assay modifications.

Quantitation of protein in saliva samples using the BioRad Protein Microassay permitted determination of the relative HA'ase activity in NFU/gm protein. The mean salivary protein concentrations were slightly lower than those reported elsewhere (Mason and Chisholm, 1975). It has been recognized that Coomassie Brilliant Blue reacts poorly with proline-rich

proteins which are abundant in saliva (Nakamura and Slots, 1983). Therefore, the salivary protein content may be somewhat underestimated. However, the protein values obtained appear useful for the purpose of this investigation.

Limited studies on salivary HA'ases have been conducted to date. The presence of bacterial HA'ase in human whole saliva has been reported in the literature (Mahler and Lisanti, 1952; Rolvstead et al., 1958; Hopps and Prout, 1972). However, this observation was anticipated since salivary enzymes originate from a variety of sources including oral microorganisms and the gingival crevicular fluid (Chauncey, 1961). This investigation reveals the presence of both acid-active (pH optimum = 4.0) and neutral-active (pH optimum = 6.8) HA'ase activities in human whole saliva indicating enzymes of tissue and bacterial origin, respectively. The detection of an acid-active enzyme coincides with the HA'ase reported by Hopps and Prout (1972) in which the optimum activity ranged from pH 3.5 - pH 3.9; however, a discrepancy in the pH optimum for the neutral-active HA'ase is evident. These investigators demonstrated optimum bacterial activity to be between pH 4.7 - pH 5.3 despite not evaluating saliva samples above pH 6.0. Other pH optimum values for bacterial HA'ases quoted in the literature have varied between pH 5.5 and pH 6.1 (Rogers, 1961). Moreover, bacterial plaque HA'ase exhibits pH optima ranging from pH 5.0 and pH 6.6 (Soder and Nord, 1969; Nord et

al., 1970). Therefore, given the diversity of oral microorganisms in saliva as well as variations in assay techniques and buffers used (Rogers, 1961), the neutral-active HA'ase shown in the present study is considered to be of bacterial origin.

Tissue (lysosomal) HA'ases are characterized by a low pH optimum (Yamada et al., 1977; Hopps and Prout, 1972). In addition to a narrow acidic pH range of activity, Orkin and Toole (1980) have described typical non-bacterial, non-testicular lysosomal HA'ases to possess an endoglycosidic mode of action and distinct substrate preference for HA. In the present study, the acidic peaks of HA'ase activity observed in parotid saliva (pH optimum = 3.7) and whole saliva (pH optimum = 4.0) conform with values of other mammalian and non-mammalian tissue HA'ases (excluding testis). Since whole saliva demonstrates an enhanced acidic HA'ase level and a more broad pH range of activity, it is reasonable to speculate that additional enzyme sources such as the gingival crevicular fluid and other major and minor salivary glands contribute to this activity. Preliminary analysis of pure submandibular/sublingual salivary gland secretions also indicates the presence of an acidic HA'ase enzyme; however, additional research is necessary to determine the HA and HA'ase contributions of the other major and minor salivary glands.

Previously, detection of HA'ase activity in cannulated parotid saliva failed (Mahler and Lisanti, 1952) perhaps as a result of the decreased sensitivity of the viscosimetric assay. The ELISA-like assay for HA'ase employed in this study is advantageous over the more commonly utilized Reissig assay (1955) because of its simplicity and increased sensitivity (Stern and Stern, 1992). Moreover, action by the exoglycosidic ß-glucuronidase may obscure actual endoglycosidic HA'ase activity using the Reissig assay since both enzymes generate new reducing N-acetylglucosamine termini which are measured by this technique. Thus, higher, inaccurate activity values may be obtained. In this study precaution was exercised to exclude non-specific exoglycosidase activity by the addition of saccharo-1, 4-lactone monohydrate to reaction buffers (Levvy and Marsh, 1959; Levvy and Conchie, 1966). However, further investigation is necessary to confirm an endoglycosidic mode of action which may be possible by cellulose acetate electrophoresis and paper chromatography to determine N-acetylhexosamine-containing oligosaccharide cleavage products (Bertolami and Ellis, 1985).

Some lysosomal HA'ases are believed to also catabolize different GAGs such as chondroitin-4-sulfate and chondroitin-6-sulfate (Fenger, 1982) whereas other enzymes demonstrate activity specifically against HA (Bertolami and Ellis, 1985).

The substrate specificity of the observed acidic salivary HA'ase is yet to be determined although it may be possible with the addition of competing GAG substrates to the reaction mixture.

In general, salivary enzymes originate from several sources including oral microorganisms, gingival crevicular fluid, salivary glands, epithelial cells, polymorphonuclear leukocytes, and dietary constituents (Chauncey, 1961). Despite several possibilities, the origins of oral tissue HA'ases are not well characterized. Tissue HA'ases are postulated to originate from epithelium (Squier and Waterhouse, 1970), macrophages (Goggins et al., 1968b), and polymorphonuclear leukocytes (Tynelius-Bratthall and Attstrom, 1972; Last and Embery, 1987). Hence, Hopps and Prout (1972) suggested that the tissue HA'ase observed in whole saliva arose from sloughed epithelial cells, gingival crevicular leukocytes, or from oral mucosal leukocytes which had migrated into saliva.

The origin of the tissue HA'ase detected in parotid saliva is less certain. Similar to the ECM elsewhere, the connective tissue of salivary glands consists of fibroblasts, macrophages, mast cells, plasma cells, and adipose cells embedded in a mixture of collagen, GAGs, and PGs (Ten Cate, 1985). Tan and Bowness (1968) identified a HA'ase from canine

submandibular gland homogenate and provided evidence for an enzyme of lysosomal origin by pH optimum, product formation, substrate specificity, and substrate inhibition. Present in sedimentable and soluble supernatant fractions, this acid-active enzyme resembled lysosomal HA'ase from rat liver. Therefore, the presence of a parotid salivary gland HA'ase for endogenous HA turnover and catabolism is possible although its release into salivary secretions remains in question. Since the HA'ase present in parotid saliva strongly displays characteristics of a non-bacterial, non-testicular tissue enzyme, this implies a lysosomal or other acidic site of action (Bertolami and Ellis, 1985). In addition, the lack of parotid saliva HA'ase activity in a higher pH range provides further evidence that this enzyme most likely is not active in the more neutral salivary secretion. More specifically, the pH values of whole saliva and parotid saliva are pH 7.08 and pH 5.92, respectively (Mason and Chisholm, 1975) which are not amenable environments for the acid-active salivary enzyme.

**V. Characterization of Hyaluronidase Activity in Human Saliva**

Characterization of the optimal salivary HA'ase activity in whole saliva and parotid saliva required several modifications of the assay conditions as described by Stern and Stern (1992). Typically, enzymatic buffers for detection of HA'ase activity contain salt near physiological concentrations. For example, both tissue and bacterial HA'ase activities of

various oral components have been reported at 0.15 M NaCl by several investigators (Goggins et al., 1968a; Hopps and Prout, 1972; Bertolami and Ellis, 1985, 1986; Last and Embery, 1987). In the present study, the parotid salivary HA'ase required high salt concentrations (0.5 M NaCl) for enzymatic activity suggesting elevated ionic conditions were necessary to dissociate putative enzyme-inhibitor complexes. This distinct requisite also was characteristic of the HA'ase activity recently identified in cultured human dermal fibroblasts (Stair, 1993). In contrast, whole saliva displayed increased acidic HA'ase activity at low salt concentrations although some activity was detected also at higher ionic strengths. Presumably, the other acidic HA'ase enzymes in whole saliva, active at lower salt concentrations, originate from previously identified tissue HA'ase sources (Hopps and Prout, 1972). However, since the normal salivary environment is not likely to be at elevated salt concentrations, it is assumed that the tissue HA'ase in parotid saliva is inactive until dissociated from the inhibitor complex by a yet undefined mechanism. The neutral enzyme showed little influence of ionic strength which corroborates the known enzyme activity of bacterial origin in whole saliva.

The interaction of albumin and HA has been well documented (Johnston, 1955; Gramling et al., 1963; Davies et al., 1963; Niedemayer et al., 1966; Gold, 1980) and thus, has been

classified as a HABP, or hyaladherin (Toole, 1990). The structural and cell surface-associated hyaladherins are known to contain homologous domains identified as HA receptors which contribute to the physiochemical capacities of HA and its role in regulation of cell behavior (Toole, 1990). Although the role of albumin in HA metabolism is not understood, previous studies in this laboratory and others (Afify et al., 1993; Stern and Stern ,1992; Stair, 1993) have included routinely BSA in enzyme reaction cocktails. Furthermore, loss of the HA and BSA complex formation is the basis for an early turbidimetric assay for HA'ase activity at acidic pH (Dorfman and Ott, 1948).

The enhanced acidic HA'ase activity observed in whole saliva and parotid saliva in the presence of 0.02% BSA suggests the requirement of albumin to function as a carrier protein for association of HA as a suitable substrate for the tissue HA'ase reaction. Gold (1980) has described the formation of albumin-HA complexes that caused conformational changes in the albumin molecules. He postulates that the conformation of HA is altered when associated with albumin. Therefore, it is hypothesized that this is necessary for substrate binding to the tissue HA'ase enzyme in saliva. In addition, the presence of increased ionic strength (greater than 0.02 M NaCl) has been shown to disrupt the albumin-HA complex (Gold, 1980) which may explain the need for exogenous BSA for the acid-

active HA'ase to function under the elevated salt assay conditions. Parotid saliva normally contains relatively low albumin concentrations (stimulated saliva [albumin] = <1 - 8 mg/L) (Mason and Chisholm, 1975). In comparison, the data suggest bacterial HA'ases obviate the need for exogenous albumin to provide conformational alterations of substrate for enzymatic activity to proceed. This phenomenon may serve as a further measure for distinguishing bacterial and tissue HA'ases in saliva.

Determination of salivary HA'ase activity under the optimally defined conditions mandated dilution of samples in the appropriate enzyme buffer cocktail. The highly sensitive ELISA-like assay was effective in demonstrating the dose-dependent activity of salivary HA'ase. Utilizing a 10-fold sample dilution, HA'ase activity in parotid saliva, presumably of tissue origin, was identified for the first time. This low level of HA'ase activity may be explained by the presence of endogenous HA and HA'ase inhibitors which were demonstrated also in both parotid saliva and whole saliva. An attempt to enhance salivary HA'ase activity by lyophilization and 10-fold concentration of sample yielded less than a 2-fold increase in activity. In the same manner as concentrating the enzyme of interest, endogenous HA and HA'ase inhibitors inadvertently may have been concentrated also and thus, may have obscured the anticipated increase in HA'ase activity.

Obstacles in accurate determination of baseline salivary HA'ase activity levels were incurred during this investigation. Initial evaluation of salivary HA'ase stability indicated the enzyme was capable of maintaining HA'ase activity following harsh sequential freeze-thaw cycles. Furthermore, addition of protease inhibitors appeared to slightly decrease activity rather than preserve it and hence, was omitted from the reaction mixtures. As the investigation progressed, however, the HA'ase activity appeared to decrease over time although most saliva samples were stored at - 20°C, thawed, and assayed only once. Unfortunately, it was difficult to make a direct comparison of activity levels of saliva samples since the majority of the study involved manipulating assay conditions to characterize the enzymes. Several investigators have noted a characteristic lability of other HA'ases during manipulative procedures (Rautela and Abramson, 1973; Gold, 1982; Fenger, 1982; Bertolami and Ellis, 1985). Therefore, further investigation is necessary to confirm a loss of activity in salivary HA'ase within an extended period under standardized conditions. To avoid this uncertainty, however, saliva samples should be collected and assayed immediately.

Definitive measurements of salivary HA'ase activity were further complicated by the presence of endogenous HA in saliva samples because it could serve as a competitive substrate in

the reaction mixture. Therefore, actual HA'ase activity levels may have been higher than recorded. To circumvent this problem, endogenous HA in salivary samples can be removed by FPLC chromatography using a Mono-Q column (Pharmacia) which effectively separates molecules based on charge. Furthermore, an additional benefit of this chromatographic procedure is that it would permit partial purification of the HA'ase enzymes from the complex salivary milieu.

Another limitation of the ELISA-like assay for HA'ase activity is that it is a solid phase assay. Thus, although a fixed amount of exogenous HA is used to coat the wells, precise quantification of HA adsorption to the microtiter plate is not possible and as a result, enzyme kinetics cannot be determined. Such kinetics utilize precise substrate concentrations. The Reissig assay (1955) is advantageous in this respect; however, this assay is 10,000-fold less sensitive than the ELISA-like assay used in this study and may not be sufficient to detect the relatively low levels of salivary HA'ase activity.

**VI. Hyaluronidase Inhibitors in Human Saliva**

Despite their obvious biological significance, HA'ase inhibitors have not been well characterized to date. HA'ase inhibitors have been identified in serum (Mathews and Dorfman, 1955) and have been implicated to play a role in tumor

progression by slowing tumor spread (Fiszer-Szafarz, 1968). One of the advantages of this ELISA-like assay is its capacity to be modified for assay of HA'ase inhibitors. Preliminary evidence suggests the presence of HA'ase inhibitors in both whole saliva and parotid saliva samples in which HA'ase activity was observed, as well as in samples in which it was lacking. Similar observations of potent HA'ase inhibitors have been shown in human serum (Afify et al., 1993) and in the media of cultured human dermal fibroblasts (Stair, 1993) utilizing this assay technique. It may be postulated that in the absence of injury HA'ase inhibitors are normally present in saliva in order to limit substrate degradation and to permit HA to confer its protective role in saliva.

Further characterization of the salivary HA'ases and their inhibitors is essential for understanding the intricate mechanisms of HA metabolism. This may be possible utilizing the recently developed substrate-gel assay for HA'ase activity (Guntenhoner et al., 1992). In brief, the HA'ase enzyme can be separated from its inhibitors via electrophoresis utilizing a polyacrylamide gel containing the HA substrate. Following enzymatic degradation of HA embedded in the gel and differential staining techniques, pertinent information is gained including the molecular weight determination of the isolated HA'ase and other major proteins, particularly HA'ase inhibitors. However, because of the complexity of salivary

secretions, including the abundance of proline-rich proteins in ductal saliva (Bennick, 1987), specific detection procedures are necessary for resolving the numerous major salivary protein constituents (Beeley et al., 1991). In addition, utilizing a spectrophotometer for gel scanning, the substrate-gel assay can be an effective quantitative tool for determination of HA'ase activity (Guntenhoner et al., 1992).

## VII. Possible Roles of Hyaluronidase in Human Saliva

Mammalian buccal mucosal HA'ases from normal (uninjured) and granulation wound tissue have been identified by Bertolami and colleagues (1985, 1986). Interestingly, these enzymes demonstrated characteristics typical of lysosomal HA'ases including a mechanism of endoglycosidic cleavage and distinct substrate preference for HA; however, each enzyme exhibited a unique pH profile. Normal buccal mucosa HA'ase was found to be optimally active at pH 3.5 while mucosal wound HA'ase displayed peak activity at pH 4.5. The slightly elevated pH observed in buccal mucosal wounds coincided with observations made in skin wound granulation tissue (Ruggiero et al., 1987). As a result, it was concluded that different HA'ases exist to function in normal HA metabolism of uninjured tissues and under conditions requiring rapid tissue proliferation and regeneration. In fact, the term "hyaluronidase" refers to a large family of enzymes whose members can occur also in multiple isoenzyme forms (IUPAC-IUB Commission Report, 1977).

Therefore, HA'ases are grouped according to their mechanism of action. The most distinguishable characteristic among different HA'ases is their pH optimum for enzymatic activity (Hopps and Prout, 1972; Fiszer-Szarfarz, 1984). Enzymes exhibiting an acidic range of HA'ase activity such as lysosomal and serum HA'ase (pH optimum = 3.5) typically are involved in the regulation of baseline metabolism of endogenous GAGs (Fiszer-Szarfarz, 1984). In comparison, other HA'ases, active during developmental or reparative processes in which GAG turnover is elevated, demonstrate a broad range of activity near neutral optimum pH (Polansky et al. 1974; Bertolami and Donoff, 1982; Fiszer-Szarfarz, 1984), and may degrade their substrate to intermediate oligosaccharide oligomers rather than to the limit sugar.

Several hypotheses can be formulated regarding the contribution of saliva to GAG processing during intraoral wound healing. The observation that intraoral wounds clinically resemble scarless fetal wounds suggests similar mechanisms of healing by regeneration rather than by scarring and fibrosis (Peacock, 1984; Adzick and Longaker, 1991). Although distinct HA'ases have been identified in parotid saliva (pH optima = 3.7), whole saliva (pH optima = 4.0 and 6.8), and buccal mucosal wounds (pH optima = 4.5) (Bertolami and Ellis, 1986), these enzymes may be regulated by potent HA'ase inhibitors in saliva. Therefore, since intraoral

wounds are continually bathed in saliva, these inhibitors are readily available to promote a prolonged presence of HA. Additionally, elevated levels of HA'ase inhibitors in saliva may be induced in the event of a wound. As regenerative healing progresses, perhaps other regulatory factors appear to create a more permissive environment for endogenous HA'ase activity later in the healing stages and thus, cellular differentiation may be accelerated in a similar manner to extraoral wound healing. Clearly, more investigation is needed to delineate the presence of HA'ases and HA'ase inhibitors and their role in modulating wound healing.

The importance of precise regulation of HA metabolism during embryogenesis and tissue repair is evident. However, the details of these complex mechanisms have not been fully elucidated. Obviously, the major factors which regulate HA synthesis and its degradation must be delicately balanced in both a quantitative and temporal manner. In particular, the appearance of HA'ase during the transition from the morphogenetic phase to the cytodifferentiative phase may require careful modulation between the enzyme and its inhibitors which have yet to be well characterized.

# CHAPTER 5: CONCLUSIONS

Understanding the mechanisms of mammalian wound healing requires delineation of the intricacies of cellular growth, differentiation, and remodeling. As a major component of the ECM, HA is believed to play a central role during fetal tissue repair in which healing occurs without scar formation (Longaker and Adzick, 1991). The hypothesis that salivary constituents contribute to intraoral wound healing in a similar fashion to components implicated in scarless fetal wound repair is supported by the presence of key factors in saliva which are involved in HA metabolism. Most likely, regulation of HA metabolism is imparted partially by factors which are associated with its breakdown.

This investigation has established baseline levels of HA in whole saliva and parotid saliva of normal human adults at levels comparable to those observed in serum and urine. Analysis of whole saliva and parotid saliva for HA'ase activity revealed distinct conditions for optimal activity. An acid-active enzyme was detected in parotid saliva whereas whole saliva displayed both acid and neutral enzyme activities. Therefore, the different acid and neutral HA'ase activities observed in saliva may indicate enzymes of tissue and bacterial origin, respectively. Additional evidence was provided by immunorecognition of HA'ase in all saliva samples

although it was not possible to distinguish the enzyme origin by this technique. Furthermore, preliminary results suggested HA'ase inhibitors were responsible for the regulation of salivary HA'ase since enzyme activity was not detected in all individuals although HA'ase was confirmed to be present by immunological means.

Additional investigation is necessary to further characterize and isolate salivary HA'ases as well as their inhibitors. Western blot analysis and substrate gel electrophoresis for HA'ase activity may provide insight about the enzyme's molecular weight as a preliminary step toward its purification. A series of differential column chromatographic techniques based upon charge, binding specificity, and size would assist in enzyme purification. With the detection of salivary HA'ase activity in the normal (uninjured) state, much work remains to determine if HA synthesis and catabolism are modulated in saliva during the wounded condition. If this is the case, identification and elucidation of the roles of these HA and HA'ase modulators during wound healing are necessary. Moreover, analysis for HA stimulatig activity in mixed saliva and pure saliva under normal and injured conditions may reveal additional information to determine if intraoral wound healing mimics fetal repair processes in regards to HA processing.

The significance of unraveling the mechanisms of HA, HA'ase,

and HA'ase inhibitors in normal salivary physiology and their possible role in modulating wound repair is indisputable. If modulation of salivary GAGs does indeed promote intraoral wound repair in a similar manner to that observed in the fetus, this knowledge will enhance efforts to prevent scar formation in general, which can create a debilitating cosmetic or functional problem.

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