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Thiruvanamalai Palaniswamy Sivakumar

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Expression of Secretory Protein in Saliva and
Gingival Crevicular Fluid of Children

Thiruvanamalai Palaniswamy Sivakumar

B.D.S., Madras Dental College, 1988

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Master of Dental Science Thesis

EXPRESSION OF SECRETORY PROTEIN IN SALIVA AND GINGIVAL CREVICULAR FLUID OF CHILDREN

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INTRODUCTION

In the early part of the 20th century, several attempts were made to identify and analyze ions, chemicals and other molecules found in saliva (Kirk, 1903; Michaels, 1901). These efforts served as proof of principle for saliva to become a diagnostic fluid in the years to follow. Improved technology has made it possible to identify, measure and monitor the level of ions, chemicals and macromolecules in saliva (Mayhall, 1975; Mandel and Wotman, 1976). Rapid advances in pharmacology have translated into precise methods of monitoring saliva to measure drugs and substances of abuse (Edgar, 1992; Beeley, 1991).

The traditional biological samples for the qualitative and quantitative measurement of most drugs are blood, plasma and urine. Many substances and their metabolites are present in different concentrations in these media. Blood or plasma provides an estimate of the current circulating concentration of the analyte of interest. Urine permits measurement of the accumulated concentration of analytes since the last void of the bladder. Even though it has been convincingly demonstrated that for many drugs, the monitoring of saliva is a real alternative for determining plasma levels its adoption has been slow. Saliva lacks "the drama of blood, the sincerity of sweat and the emotional appeal of tears" (Mandel, 1990). Collection of saliva and gingival crevicular fluid (GCF) is noninvasive and painless. Therefore, samples can be easily obtained, stored and analyzed using biochemical and molecular reactions (Malamud, 1992). In recent years saliva has attracted considerable attention, in particular among people interested in the determination of drug concentrations, who suggest that saliva might be substituted for plasma in the areas of pharmacokinetic studies and drug monitoring.
Background

Saliva

Saliva is a complex fluid that is produced by a number of specialized glands that discharge into the oral cavity of mammals. A small contribution is made by the numerous small labial, buccal and palatal glands, which line the mouth (Van Dam and Van Loenen, 1978; Vining and McGinley, 1985). The salivary glandular tissue is comprised of specialized groups of cells called acinar cells arranged as endpieces surrounding a small central lumen that opens into a narrow intercalated duct. The intercalated duct leads from the secretory endpieces to the striated ducts, which in turn drain into the excretory ducts, that join to form a single main excretory duct, which drains into the oral cavity. The salivary glands are lobulated, the parenchyma consisting of discrete lobules of closely packed secretory endpieces and intralobular ducts. The lobules are separated by interlobular connective tissue septae. The secretory endpieces are supported by cells called myoepithelial cells that are contractile in nature and contain filaments of actin and myosin. Since these cells lie within the basal lamina that envelops the endpiece, they are considered a part of the endpiece. These cells have an important, though indirect role to play in salivary secretion. They provide support for the endpiece, preventing its distention during the secretory process, and in special cases, provide a propulsive force to aid in the expulsion of especially viscous secretions (Young and Van Lennep, 1978).

The parotid glands are serous glands, for their acini contain only serous-secreting cells, and their secretions are devoid of mucin. The secretory end-pieces of the submandibular and sublingual glands, on the contrary, contain both serous and mucin-
secreting cells (Dawes, 1966; Davenport, 1977). The viscosity of the submandibular saliva usually decreases with increasing flow rate since the serous cells have a greater response to stimulation than the mucin-secreting cells. The sublingual glands contain predominantly mucin-secreting cells and thus their secretion has a thick, viscous nature (Vining and McGinley, 1985).

A recent review (Edgar, 1992) states that salivary glands are unique among the digestive tract glands in that they are under nervous control. Salivary secretion is (a reflex response) controlled by both parasympathetic and sympathetic secretomotor nerves. Stimulation of sympathetic fibers to all glands causes vasoconstriction, and in man, stimulation of the sympathetic trunk in the neck, or injection of epinephrine, causes secretion by the submandibular but not the parotid glands. Parasympathomimetic drugs cause high salivary flow rates (Davenport, 1977; Mazariegos et al, 1984).

An adult produces typically between 500–1500ml of saliva each day (Lentner, 1981). The volume of saliva secreted appears to be entirely a function of the activity of the secretory endpiece, the ductal system neither adding nor reabsorbing more water (Young and Van Lennep, 1979). It is a very dilute fluid, consisting of 99% water. Salivary proteins comprise approximately 200mg/100ml (Edgar, 1992). Mixed saliva, which is the most accessible and most frequently used for analysis, contains primarily the secretions of the submandibular (65%), parotid (23%) and sublingual (4%) glands, the remaining 8% being produced by the numerous minor salivary glands (Dawes and Wood, 1973; Caddy, 1984). These proportions are a function of the type, intensity and duration of stimulation. It has been demonstrated that saliva contains various proteins and glycoproteins (Carlson, 1988), especially proline-rich proteins (PRPs) that account for
nearly 70% of protein in saliva and are mainly found in parotid saliva (Bennick, 1982). The presence of other salivary proteins in very low concentrations has made characterization of these proteins difficult in the past (Ritschel and Thompson, 1983). Recently, however, technology has advanced sufficiently, that even a small quantity of saliva or other fluid can be characterized with confidence (Mandel and Wotman, 1976).

Saliva is known play an important role in the oral cavity in various ways. In addition to assisting in the dilution and clearance of food, the alkaline nature of saliva acts as a buffer against acids in food and plaque. The functions of salivary proteins were recognized by Mayhall (1975), and more recently, Edgar (1992). The \( \alpha \)-amylase in saliva initiates digestion of starches, the mucins provide lubrication of oral tissues enabling physiological functions such as speech, mastication and deglutition. In addition, the PRP’s may play an important anti-microbial role along with several other salivary proteins such as lysozyme, lactoferrin, peroxidases and immunoglobulins such as IgA, the presence of which has been demonstrated in human saliva (Ellison et al, 1960; Tomasi et al, 1963). The partial or complete absence of saliva has been shown to result in significant dysfunction, including impaired speech and mastication, increased susceptibility to infection and inflammation of oral tissues and increased incidence of dental caries and periodontal diseases (Fox et al, 1985).

As saliva is essential to the maintenance of oral health, proper digestion and nutrition, alterations in its composition may provide clues to an individual’s susceptibility to various types of oral infections and also help serve as a marker in the progression of a local or systemic disease or disorder. Environmental and pharmacological stimuli have been shown to change cellular and molecular reactions in tissues and body fluids (Seigel,
1993; Mednieks et al, 1993). Therefore, secretory protein levels in saliva may be used as an index of metabolic responses of salivary exocrine cells to systemic stimuli (Mednieks and Hand, 1982). This is achieved by studying events mediated by cyclic AMP (cAMP), through cellular events associated with changes in the cAMP receptor proteins.

This study was carried out to measure levels of secretory proteins in saliva and gingival crevicular fluid of children, the presence of the cAMP receptor proteins (cARP) in oral fluid, the ratio of total protein to cARP, and to determine if developmental stages influence their expression.

Techniques for collection of saliva

Many of the advantages of analyzing saliva relate to the non-invasive nature of the collection procedure. For the collection of samples from children, mixed whole saliva is the only practical alternative. Several methods have been described for the collection of mixed saliva.

In healthy subjects, gingival crevicular fluid (from the tooth/gum margin) may constitute up to 0.5% of the volume of mixed saliva. This proportion may markedly increase in patients with gingivitis (Cimasoni, 1974). Plasma exuding from minor abrasions in the mouth may also contribute to saliva. The protein content of gingival fluid is similar to that of plasma. Therefore, it is usually recommended that subjects should not brush their teeth or practice any other methods of oral hygiene for several hours before collecting a saliva sample.

Although most patients prefer donating saliva rather than blood, a substantial social barrier exists to "spitting". For this and other reasons, subjects often experience
decreased salivary secretion (dry mouth) if asked to provide a sample. Many researchers have found it advantageous to further stimulate salivation and a number of stimuli have been used.

Chewing paraffin wax, Parafilm (American National Can., Chicago, IL), rubber bands, pieces of Teflon or chewing gum (Dawes and Macpherson, 1992) will usually elicit a flow of 1 to 3 ml/min. Mucklow (1982) recommends that, when these types of stimuli are used, the subject should allow saliva to accumulate in the mouth until the desire to swallow occurs, at which time the fluid can be expelled smoothly into a vessel. Expectorations should be avoided since this introduces bubbles, which may result in changes in pH and contaminate saliva with secretions of respiratory epithelium leading to errors in interpretation of the saliva/plasma concentration ratio (S/P ratio). The use of acid lemon drops or a few drops of 0.5 mol/l citric acid are among the most potent of taste stimuli and will generally induce a maximal secretion of 5 to 10 ml/min (Vining and McGinley, 1985). In general, the secretion rate increases with the size of the bolus and the pressure required to chew it. If chewing is unilateral, then the glands on the active side may secrete copiously while those on the inactive side secrete very little. For studies requiring high saliva flow rates for extended periods of time, secretion-stimulating drugs, such as the parasympathomimetic drug pilocarpine, have sometimes been used orally, subcutaneously, or intravenously. Administration of 2.0 mg/kg pilocarpine resulted in sufficient flow to collect (by capillary tube) 5 ml of saliva per rat.
Special devices

Within the last few years, much research has been done to develop a method that solves many of the existing problems in using saliva for the quantitative determination of the "free" (= non-protein bound) component of drugs. Cooper et al (1981) and May et al (1978) were the first to use a dental cotton roll to collect saliva in order to monitor desipramine. Over the years their method has undergone some improvements, and the dental cotton roll is currently available as the Salivette®. The advantage of the Salivette® over many other sampling devices is that it reliably absorbs a relatively large volume of saliva (1.5 ml) in a short time (Höld et al, 1995). The OraSure®, another collection device, absorbs only 1.0 ml and, moreover, collects a mixture of gingival crevicular fluid and saliva rather than saliva alone, since the pad is placed between cheek and gums. The term "oral sample" is used rather than saliva when the OraSure® device is used (Thieme et al, 1992).

An Oral-Diffusion-Sink (ODS) device is used for the in situ collection of an ultrafiltrate of saliva (Wade, 1992; Wade and Haegele, 1991). The ODS device, a variation on an earlier design developed for time-integrated measurement of corticosteroids in interstitial fluid (Wade, 1984), is worn in the mouth and continuously accumulates the compounds of interest as they diffuse into the device along a concentration gradient.

Schramm and coworkers (1990) developed a device for the in situ collection of an ultrafiltrate of saliva. The collecting device is based on the principle of an osmotic pump. A semi-permeable membrane encloses an osmotically active substance (sucrose),
that within a few minutes of being put into a patient's mouth, draws an ultrafiltrate of saliva into the device (1 ml of ultrafiltrate after 5 min). The drawback to all these devices is that they all adsorb proteins from a solution to a greater or lesser extent and are therefore unsuitable for studying protein expression.

**Gingival crevicular fluid**

Gingival crevicular fluid (GCF) is found within the gingival crevices that encircle the teeth and has components that point to serum as its origin. In healthy individuals, the GCF is separated from the whole saliva by the free marginal gingiva. The composition of GCF, therefore, is considerably different from that of the other oral fluids. The proteins present in GCF have been studied by a variety of techniques. Sueda et al (1966) demonstrated histochemically that crevicular fluid contains proteins similar to those found in serum. However, the concentration of proteins in GCF is significantly lower than that of serum (Marcus et al, 1985).

Since the flow rate of GCF has been shown to increase with inflammation, it has been suggested that GCF may be useful in the diagnosis of inflammatory diseases within the oral cavity. Its measurement makes it possible to treat periodontitis in its earliest stages, before irreversible changes occur in the supporting periodontal tissues. Additionally, GCF may be useful in the diagnosis of systemic diseases. For example, it has been demonstrated that both GCF and blood glucose levels are elevated in diabetic patients (Kleinberg and Golub, 1985).
**Current techniques in GCF collection and quantification**

Since the amount of GCF obtainable from clinically healthy gingival sulci is extremely small, the collection of sufficient GCF for analysis poses particular problems. Most investigative procedures require that samples from different sites be combined in order to provide sufficient GCF for analysis. Currently, there are only a few widely accepted techniques for the collection of GCF. The most common method of GCF collection is described by Offenbacher et al (1986). In this technique, paper strips (Perio Paper, Harco; Tustin, CA) are inserted about 1mm into the gingival crevice. An alternative method is to use glass capillary tubes. In this technique, a thin glass capillary tube is inserted under the free gingival margin and the GCF is allowed to enter the tube for a period of time (Marcus et al, 1985). In each technique, there is considerable variability in the suggested amount of time needed for collection. Some investigators suggest collecting for a little as 20 seconds, while others suggest as much as 90 minutes (Cimasoni, 1974, 1983). Egelberg (1966) noted, however, that during the lengthier collection periods, the filter paper or capillary tubes might cause local irritation, resulting in an inflammatory response, which interferes with the normal tissue fluid dynamics in that area.

The analysis of individual proteins in GCF can be accomplished with many different techniques. In immunoelectrophoresis, the sample is placed in a gel containing antibodies to the particular protein being quantified, and an electric field is applied to the gel. The protein of interest would be identified using a dye that would stain the antibody-antigen complexes (Marcus et al, 1985). Radioimmunoassays are also employed in the quantification of individual proteins. The principle of radioimmunoassay is that proteins
in the GCF sample compete with radioactively labeled proteins for a limited number of sites on antibodies to the protein (Grieve et al, 1994). Enzyme linked immunosorbent assay (ELISA) kits are also popular in the quantification of proteins in GCF, but the experiments are complex and time consuming.

**Cyclic AMP-dependent Protein Kinase (PKA)**

Cyclic AMP dependent protein kinase is also known as protein kinase A (PKA). PKA participates in the regulation of various cellular activities (Flockhart and Corbin, 1982), and is able to catalyze phosphorylation of a number of cellular proteins. PKA is influenced in response to either hormonal or β-adrenergic stimulation. Cyclic 3', 5'‐adenosine monophosphate (cAMP) is responsible for the activation of PKA in a series of steps. First, a neurotransmitter arrives at a cell membrane and binds to a transmembrane receptor. This bound transmembrane receptor causes conformational changes in G-proteins on the inner cell membrane, activating adenylate cyclase. Adenylate cyclase catalyzes the formation of cyclic AMP from adenosine triphosphate (ATP), and finally, PKA is activated by cyclic AMP (Lehninger et al, 1993).

The metabolic effect of elevated intracellular cAMP, therefore, is the phosphorylation of serine or threonine residues of specific proteins by PKA. However, changes in the levels of cAMP due to hormonal or pharmacological stimulation are only transient. The binding of cAMP to regulatory (R) subunits of PKA can be measured reliably and is a useful index of the stimulatory effects of PKA (Hoyers et al, 1980; DeCamilli et al, 1986). Two isozymic forms of PKA were identified by their differing R subunits, type I, termed RI and type II, termed RII (Scott et al, 1987; Taylor et al, 1990).
The activity of PKA is dependent on the dissociation of an inactive holoenzyme tetramer in the presence of cAMP. This dissociation can be represented in the following equation,

\[ R_2C_2 + 4 \text{cAMP} \rightarrow 2R\text{-cAMP}_2 + 2C \]

where, \( R_2C_2 \) is the inactive holoenzyme with two regulatory (R), and two catalytic (C) subunits. In the presence of cAMP, the regulatory subunits are occupied (R-cAMP\(_2\)) and are referred to as cARP; and the catalytic subunits are free to catalyze phosphorylation (Flockhart and Corbin, 1982; Mednieks et al, 1997).

**Distribution of Cyclic AMP-dependent Protein kinase**

Within cells, PKA is present in both the nucleus and the cytoplasm (Steiner et al, 1978). It was documented by DeCamilli et al (1986) that after appropriate stimulation, both R and C subunits might translocate from one cellular compartment to another. The regulatory subunit of type II PKA (RII) is known to be present in both human and animal saliva in relatively large quantities (Mednieks and Hand, 1984; Mednieks et al, 1994). Mednieks et al (1987) used immunocytochemical labeling to show the distribution of RII in rat parotid acinar cells. In this study, a polyclonal antibody to RII was utilized to determine the distribution of RII, which was shown to be present in the nuclei, cytoplasm, rough endoplasmic reticulum, Golgi apparatus and secretory granules, but generally not in mitochondria. This study confirmed the localization of PKA within parotid cell nuclei and established the secretory granules as the source of the PKA regulatory subunits in saliva.

Further work has shown RII to be present in several other types of exocrine and endocrine cells of the rat such as the pancreas, seminal vesicles, pituitary gland cells and
intestinal cells. This widespread occurrence of the PKA regulatory subunits in secretory granules suggests, that in addition to its role in intracellular regulatory functions such as secretory events, RII may also function extracellularly (Mednieks et al, 1989).

**Changes in PKA levels in response to environmental stimuli**

Previous studies indicate that environmental and pharmacological stimuli may lead to changes in the amount and distribution of PKA in secretory cells. An *in vitro* study showed that rat parotid acinar cells were responsive to isoproterenol, an analog of norepinephrine, and a strong β-adrenergic agonist. Incubation of rat parotid gland pieces with $10^{-6}$ M isoproterenol resulted in significant changes in the localization of PKA proteins within the parotid acinar cells (Mednieks et al, 1982).

It was shown that RII levels in rat saliva were sensitive to pharmacologic stimulation *in vivo*. Saliva was collected from a group of rats injected with 30mg/kg isoproterenol. Each sample was labeled for identification of R proteins and the proteins were separated and quantified by polyacrylamide gel electrophoresis. This study showed that the saliva of rats injected with isoproterenol contained high levels of RII, indicating that R proteins are released during exocytosis (Mednieks and Hand, 1984).

Environmental stimuli also have been shown to correspond with changes in PKA levels. Experimental animals subjected to periods of microgravity had significant changes in R subunit levels within heart muscle cells (Mednieks et al, 1991).

It has also been demonstrated, that levels of R subunits in human saliva are affected by auditory stimulation. Volunteers were subjected to high-decibel sound for a brief period of time. Salivary levels rose sharply immediately after the auditory stress was
initiated. Forty-eight hours after the cessation of the high-decibel sound, the R levels returned to original baseline levels (Mednieks et al, 1994).

Most recently, work from our laboratory indicates that the levels of salivary R subunits can be easily and reproducibly determined using samples from a variety of human subjects (Sivakumar et al, 1999; Burke et al, 2000).

**Saliva as a diagnostic fluid**

Saliva is increasingly used for a variety of diagnostic purposes. Michaels (1901) and Kirk, (1903) were perhaps the first to examine the usefulness of saliva in diagnosis by attempting to use saliva in the recognition of certain abnormal states and as an index of faulty metabolism. Since drugs and their metabolites appear in saliva, the fluid is useful in monitoring of drugs. Pharmacologically active drugs are usually unbound. Saliva differs from plasma in that it contains only unbound drug. Therefore, saliva may in some instances, reflect therapeutically active drug concentration more closely than plasma (Siegel, 1993). Cone (1993) showed that many drugs of abuse, including alcohol, are easily demonstrated in saliva.

Katz and Shannon (1969) demonstrated the feasibility of assaying steroids in saliva. They showed that steroids in saliva could be measured with adequate precision. The lipid-soluble unconjugated steroids pass readily into saliva and their concentrations are proportional to plasma concentrations. Since this discovery, collection of saliva for estimation of steroid concentrations has been part of routine monitoring of persons for such conditions as delayed puberty, adrenal hyperplasia and Addison’s disease (Malamud, 1992).
Mandel, in a review (1993), described the changes in salivary composition that are closely associated with cystic fibrosis. These patients have markedly increased levels of calcium, phosphate and proteins in their submandibular saliva. Doering et al (1977) also found that children with cystic fibrosis exhibited unique electrophoretic salivary banding patterns, indicating that this disease appears to permanently alter the expression of secretory proteins.
OBJECTIVES

Working Hypothesis

Since secretory proteins in saliva show heterogeneity, varying from person to person, their expression is apparently individually regulated.

Null Hypothesis

There is no difference in the expression of secretory protein present in whole saliva and gingival crevicular fluid of children and adults.

Experimental Design

In order to carry out the stated objectives, it was necessary to devise reliable procedures for the collection and analysis of secretory proteins in saliva and gingival crevicular fluid. The experimental design entailed developing collection methods of oral fluids (whole saliva and GCF) that were feasible for clinic use. The first criterion was that the method should be rapid and reproducible since it involves children. The next major consideration was that it should be harmless, non-invasive.

The analytical phase required refinement of existing techniques of electrophoresis and immunoblotting. The design involved miniaturization of the electrophoretic separation methods to successfully run gels with small amounts of samples.
Specific Objectives

1. To examine the expression of salivary protein and measure the level of cARP in children’s saliva.

2. To provide information about similarities or differences in specific salivary proteins between children and adults.
MATERIALS AND METHODS

Patient Selection Criteria

Adult and children volunteer patients between the ages of six and fifteen at the pediatric dental clinics in the Connecticut Children’s Medical Center, Burgdorf dental clinic and a private dental office (Children’s Dental Associates), who were healthy and not taking any medications, were eligible for inclusion in this study. Individuals volunteered to participate in the study and were not compensated in any manner. All samples were collected from cooperative patients, who were able to follow instructions. In addition, all participants in the study were required to have a healthy periodontium as indicated by a lack of plaque and bleeding on probing with either a periodontal probe (in adults) or Perio Paper®. Whole saliva and GCF were collected from patients before recall procedures to avoid any contamination from topical fluoride application. Samples from patients found to have new carious lesions or gingivitis were not used. All patients meeting the above criteria were used regardless of race or gender.

A total of 49 samples were included in the study. GCF and whole saliva were collected after obtaining informed consent prepared in accordance with NIH and the University of Connecticut guidelines (IRB reference # 98-177) involving human subjects. Signatures were obtained from both the parent and the patient in accordance with the protocol. The samples were coded, to protect the privacy of the donors. The IRB protocol, a sample Informed Consent form and the IRB approval letter are included in Appendix 1.
Sample collection

Subjects with clinically normal gingiva were used in the study. The examination and sample collection were performed in a dental chair with good lighting. Sample collection always preceded clinical evaluation and probing which prevented any contamination with blood. Samples of GCF were collected from the gingival crevices around four teeth using medium paper points (Mynol®). The collection sites were isolated with cotton rolls and gently dried with an air syringe to minimize the contamination of saliva. The mesial and distal surfaces of either upper or lower incisors and canines were used. Canines were the preferred teeth, but if not present, incisors were used. A total of eight paper points were inserted in these sulci for approximately 5-10 seconds or until the paper points were wetted. Care was taken to avoid any mechanical irritation of the gingival crevice. The paper points were then removed and inspected visually to make sure that they were ‘wetted’ sufficiently and that they had no blood on them (evidence of mechanical injury). Only the very tip (2-3 mm) of the points were expected to be wetted by the GCF.

A portion of each paper point containing GCF was then clipped with a sharp sterile scissors approximately 1 mm above the point of ‘wetting’ and immediately inserted into a vial containing 25 μl of sample buffer (20% glycerol, 1% SDS, 0.2% β mercaptoethanol and 0.1% bromophenolblue) and protease inhibitors (5 μl phenylmethyl sulfonyl fluoride).

An illustration of the GCF collection method is shown in Figure 1. In our pilot studies we used Perio Paper® to collect GCF, but discovered that it was very difficult to recover the sample from the paper. We then tried paper points, and this
approach improved collection and recovery, but was not sufficient to load the gel with confidence. Finally, we cut the paper point to 1 mm above the wetted area. This avoided absorption of most of the sample buffer by the dry end of the paper point allowing more proteins to be eluted into the buffer and a more concentrated sample for analysis. The other advantages of collection of GCF with paper points are that the wetted area can clearly be visualized and once it is cut, the loss of sample in the paper point is minimal. Our experience shows that as much as 20-22 μl (out of a total of 25 μl) of the sample can be recovered using this method. The sample buffer and the protease inhibitor prevented the breakdown of the proteins.

The vial containing the sample buffer and the eight paper points was boiled in the clinic for five minutes and stored in ice until the end of the day. The samples were later transported to the lab and stored at -20°C.

Whole saliva was collected in a sterile cup. The subject was advised to swallow, take a drink of water (2-3 ounces), and then thoroughly rinse the mouth with cool water. After rinsing, the subject was given a collecting container and saliva was allowed to accumulate by bending the head forward and allowing it to drain into the cup. The saliva was collected passively with no active expectoration. The purpose of the above procedure was to avoid any upper respiratory contamination of the sample. Using a mechanical pipette, 25 μl of the sample was immediately transferred to a vial containing an equal amount of sample buffer and a protease inhibitor (5 μl of PMSF). The sample was then boiled in the clinic and stored in ice, while the remaining salivary sample was transferred to another vial and stored similarly.

Each sample was then transported to the laboratory and given a numerical code without using identifiers linked to the subject. The samples were then stored at -20°C.
until the time of laboratory analysis. Laboratory analysis of the sample was generally performed within 72 hours.

**Sample preparation**

The samples were removed from -20°C, thawed at 4°C, and boiled for approximately 2-3 minutes. During the subsequent preparative steps, the samples were maintained in ice. After centrifugation of both the saliva and GCF samples for 15 minutes, 3 µl of EDTA was added to the saliva sample to chelate calcium ions (this reduced the viscosity and allowed for easier and more consistent loading of the sample in the gel). The samples were then vortexed for approximately 15 seconds to achieve a consistent mixture and then placed on ice.

**Determination of protein concentration**

The protein concentration of each sample collected was determined to compare samples reliably. The samples were removed from ice, and 1.5 µl of each salivary and GCF sample was "dot blotted" onto a nitrocellulose membrane. Proteins and complexes containing proteins readily and reversibly bind to nitrocellulose membranes. Additionally, 1.5 µl each of various concentrations bovine serum albumin (BSA) were "dot blotted" on the nitrocellulose membrane as controls (0.1, 0.25, 0.50, 1.0, 2.5, 5.0 and 10 mg/ml). This nitrocellulose membrane was allowed to dry and then immersed in 2% Ponceau S (Ponceau solution: Ponceau-S 2 g (2%), Trichloroacetic acid 30 g (30%), Sulfosalicylic acid 30 g (30%), deionized water to 100 ml) red dye for five minutes.

The Ponceau S red dye was selected for use to stain proteins instead of the
traditional Coomassie stain because it can be removed by washing and the same membrane later used for Western blotting. This avoids the necessity to create a sister gel which would require dividing the small GCF sample between two gels. Also, Ponceau S stains certain salivary proteins, i.e., the proline-rich proteins, that fail to adequately stain with Coomassie blue. Each of the samples stained red with different intensities. The density was directly correlated to the total amount of protein in the sample.

Separation of proteins by SDS-PAGE

Sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE) is widely employed in the analysis of proteins. Traditional SDS-PAGE methods were adapted to study human salivary secretions. We used denaturing SDS-PAGE gels under reducing conditions by a modification of a previously reported method (Mednieks et al, 1994). SDS-PAGE can resolve complex mixtures into distinct bands on a gel. The position of the protein band along the lane gives a good approximation of its size, and after staining the band density is a rough indicator of the amount present in the sample. The standards and reagents used in this and the subsequent analysis were purchased from Sigma Chemical Company.

Mini gels were prepared according to the vendor’s specifications (BioRad Corp). The dimensions of the gels were approximately 10 cm × 7 cm × 1.5 mm. A glass plate sandwich, assembled using two clean plates and two 0.75 mm thick spacers on each side, placed one on top of the other, was locked to a casting stand. This allowed for the creation of a 1.5 mm spacer and a 1.5 mm gel. A 10% running gel solution was prepared and pipetted into the glass plate sandwich to a height of approximately 10 mm. This acted as a stop or a plug, over which the running gel was poured. The 10% running gel
(prepared with the following mixture 40% acryl/Bis 2.5ml, 1.5M Tris-HCl, pH 8.8 2.5ml, 10% SDS 100µl, deionized water 4.83 ml, tetramethylethylenediamine (TEMED) 10µl, ammonium persulphate (APS) 83µl) was pipetted between the glass plate another 7 cm. This was overlayed with water to create a sharp interface.

The gel takes approximately 15-20 minutes to fully polymerize and once it has polymerized, the overlay of water is removed and a stacking gel (40% acryl/Bis 125 µl 0.5 M Tris-HCl, pH 6.8 315 µl, 10% SDS 13 µl, deionized water 795 µl, TEMED 1 µl, APS 8 µl) is prepared and loaded another 1 cm. A 1.5 mm comb with 12 teeth was then inserted in between the glass plates, taking care not to trap any bubbles below the teeth of the comb. The gel was allowed to sit for at least an hour before it was wrapped in a cellophane sheet and stored in the refrigerator overnight at 4°C.

The whole saliva and GCF samples were briefly vortexed and loaded in the wells with a micro-pipette. Additionally, 3.0 µl of a protein standard marker (Rainbow Standard®, Amersham, Glenview, IL) was added to the first well for the estimation of protein sizes within a set of samples. The combs were removed after 24 hours and the wells were rinsed with de-ionized water. The sample containing equal amounts of sample buffer was then loaded depending on the density of the dot blotting (see below). On average, 20 µl of GCF and 25 µl of salivary sample were loaded in each well. The salivary and GCF samples of the same patient were loaded side by side to have a visual comparison of the banding patterns of the two fluids. The polymerized gel sandwich was then attached to an upper buffer chamber using the manufacturer’s instructions. The upper buffer chamber was filled with 1x SDS electrophoresis buffer, and the attached gel sandwich was placed in a lower buffer chamber also filled with the same buffer. After the samples were loaded, electrophoresis was carried out at 30 mA for 3 hours.
The SDS PAGE gel was then prepared for transblotting in order to transfer the electrophoretically separated proteins from the polyacrylamide gel to a nitrocellulose membrane. An electric current was applied perpendicular to the gel causing the proteins in the gel to migrate horizontally onto the nitrocellulose membrane (Towbin et al, 1979; Mogi et al, 1986). Care was taken to avoid trapping of air bubbles between the gel and the nitrocellulose by rolling a clean test tube over the membrane. The assembled transblotting cartridge was submerged in transblotting buffer (10% 1M Tris without SDS, pH 7.6, 20% ethanol, 70% water), connected to power supply and run at 100 mA for 2 hours. Generally the proteins are negatively charged and will transfer from the cathode to the anode.

At the completion of transblotting, the nitrocellulose membrane was removed from the cartridge and gently rinsed in distilled water. The membrane was then placed in a 2% Ponceau S red dye for 5 minutes. The excess dye was rinsed away and the protein-banding pattern could be visualized. The presence of visible Rainbow standard remaining on the gel indicates that a small amount of protein may not have been transferred. These gels were stored in a box containing Coomassie blue solution (0.025% Coomassie brilliant blue R 250, 40% methanol, 7% acetic acid) as a reference for identification of the original banding patterns.

**Densitometry**

Densitometric analysis of the dot blots was accomplished by using a flat bed color scanner and NIH Image software (developed at the U.S. National Institutes of Health and available on the internet at http://rsb.info.nih.gov/nih-image/). This allowed us
to estimate the amount of protein in the sample by creating a standard curve of known BSA concentrations.

Sample concentrations can be determined using optical, electronic, and for our purposes, a computer based imaging technique. Densitometry science was described originally by Bouguer and Lambert who noted loss of radiation (or incident light/transmitted light) in passing through a medium. Later, Beer found the radiation loss in a medium was a function of the substance's molarity or concentration. According to Beer's law, concentration is proportional to optical density (OD). According to Beer's law, the density of a point is the log ratio of incident light upon and transmitted light through, represented by the formula

$$\text{OD} = \log_{10} \left( \frac{I_0}{I_1} \right)$$

The NIH Image densitometric software conforms to Beer’s law and estimates the total protein content of "dot blots". The salivary and GCF lanes on the nitrocellulose membranes were analyzed and plot profiles of every band in the lane were produced in the form of a graph. Densitometric values for a particular band or a lane were obtained using the formula

$$(A \times \text{BKG}) + \text{ID} = \text{Av. Dens}$$

where, A = Area, BKG = Background, ID = Integrated density and AV. Dens = Average density. Since the value of total protein is known, the ratio of cARP/total protein can be obtained using the same methods.

The nitrocellulose membranes containing the dot blots of both the samples and the known BSA standards were scanned and the image was saved on the computer hard drive. Next, a density profile of the BSA dot blot was produced. This was repeated for each different concentration of the BSA dot blots. The computer-generated density
profile produced a graphic representation as well as a numeric value for the average
density of the BSA. Next, the average density values of each different concentration of
BSA were graphed to produce a standard curve. This standard curve was then used to
estimate the protein concentration of each individual sample by visually comparing the
intensity of each sample stained. The total proteins of the salivary and GCF samples
were then loaded for electrophoresis based on the calculations using the value in the
linear portion of the standard curve.

**Western blotting**

The nitrocellulose membranes containing the total protein dot blots as well as the
proteins separated PAGE were Western blotted for identification of the R subunit of
PKA. The nitrocellulose membranes were washed with 0.015 M phosphate buffer
solution and distilled water for about 5 minutes to remove the Ponceau S red dye. The
membranes were incubated in 5X TeloBlot (non-mammalian proteins 4% w/v, sodium
azide 10^-6 M, 0.5% Tween 20 detergent) for 1 hour to block the non-specific binding sites.
The secretory proteins on the membrane were identified with Western blotting by a
modified method of Szczepanski et al (1998). Briefly, the membranes were incubated in
a solution of rabbit anti-rat RII (1:500 dilution) in phosphate buffered saline solution
(PBS) and 0.5X TeloBlot at room temperature for 1 hour and then at 4°C overnight. The
membranes were washed with 3 changes of PBS to remove non-binding primary
antibody. A secondary antibody (goat-anti rabbit IgG) conjugated with horseradish
peroxidase (HRP) diluted in PBS-0.5X TeloBlot was added and the membrane incubated
for 1 hour at room temperature. The membrane was washed with 3 changes of PBS.
Diaminobenzidine-H_2O_2 (6mg DAB, 10ml 50mM Tris [pH 7.6], 15 μl 30% hydrogen
peroxide) was added to the membrane and incubated at room temperature for 1-5
minutes. A brown reaction product identified the specific protein on the membrane. The membrane was then removed from the solution and rinsed with distilled water and dried. The Western blots were scanned into a computer file and densitometric analyses were performed as described previously.
RESULTS

The results of this study are in two main categories. The first part reflects the ratio
determination of total proteins to that of cARP as measured by “dot blotting”. Next, the
relationship of total protein banding patterns and cARP was determined by
electrophoresis and Western blotting.

Protein concentration of whole saliva and GCF

A standard curve of protein concentration was developed using measured amounts
of bovine serum albumin and their numerical values were plotted as shown in Fig 2.
These results indicate that the obtained values were in the linear range and can be used to
calculate protein concentrations of the samples. Fig 3 shows dot blotting of whole saliva
samples (panel A) and immunoblotting with anti RII antibody (panel B). Note that
samples 14 and 15 are BSA and α-amylase, respectively, and do not show in panel B.

The comparison of traditional Coomassie blue dye staining method with that of
Ponceau S used in the study is presented in Fig 4. Although the signal in the membrane
using Ponceau S is apparently weaker, the banding pattern analysis (panel C) shows that
the majority of the peaks are present, indicating that this method can be used without loss
of information.

The procedures shown in Figs 3 and 4 were applied and digital files of the dot
blots of whole saliva and GCF samples were subjected to densitometric analysis using
OFOTO and NIH Image software. The average density of each sample was calculated.
Since the average density of each dot blot was directly correlated with the protein
concentration of the sample, calculations of the protein concentrations were made from these average density values. The total concentration of protein in 49 whole saliva samples is shown in Figs 5 and 6. The average density was in the range of 0.14-2.81, in arbitrary units. This corresponded to actual salivary protein concentrations of 2.5-5.0 ml/mg.

Twenty-five samples of GCF were analyzed for total protein content (Fig 7). The protein concentration ranged from 0.24-1.82 in arbitrary units. The range was comparable to that of saliva. The dilution factor, however, is 10 fold for GCF, therefore the protein content of GCF is 10 times greater than that of saliva (unpublished observation, M Mednieks and J Burke, Chicago, IL).

The mean values of proteins in saliva (n=49) and in GCF (n=25) were obtained and the standard deviations were calculated. Table 1 shows the mean value of total proteins in saliva (0.885) and that of GCF (0.636) in arbitrary units. Their standard deviations were calculated to be 0.61 and 0.37 (Fig 8), respectively.

Immunoblotting was carried out using the dot blots to identify total proteins and quantify cARP (Figs 9,10). The values are compared in Figure 11. In all the samples tested, cARP was consistently present. The percentage of cARP to total protein was reasonably consistent with a mean percentage of 2.69 (Fig 12). The range of cARP in samples varied from 0.010-0.48 in arbitrary units, whereas the values for total protein in the same samples of saliva ranged from 0.46-2.62 (arbitrary units). The amount of cARP in total protein varies from 0.5-3.0% of total protein.

The total protein concentration of whole saliva of male and female subjects was compared. The range for male children was 0.24-2.62 (Fig 13) and that for females was
0.19-2.69 (Fig 14) in arbitrary units. The mean value for males (n=22) was 1.017 and for females (n=27), 0.792 (Table 2). The standard deviations were 0.662 and 0.574 (Fig 15), respectively. A two-tailed t-test was done and the difference in values for males and females was not statistically significant (p > 0.05).

The difference in concentration of proteins between the three ethnic groups in the study is shown in Figs 16-18. Protein concentrations of Caucasian children with European background (EC) were in a range of 0.2-2.69. Protein concentrations of Hispanic/Latino (H/L) background children were in the range of 0.14-1.11, and those of African American (AA) background children, 0.19-1.11 in arbitrary units. The mean values for each of these ethnic groups were EC (n=23) 1.13, H/L (n=15) 0.681 and AA (n=11) 0.559 (Table 3). The standard deviations for these groups were 0.755, 0.330 and 0.302, respectively (Fig 19). Analysis of variance was performed and there was a statistically significant difference in the concentration of proteins between the Caucasian group and the other two groups (p < 0.05).

Samples were divided into two age groups (6-9 and 10-15) based on their stage of mixed dentition. The values for the 6-9 age group ranged from 0.16-2.69 (Fig 20). The 10-15 age group had a range of 0.2-2.62 (Fig 21). The mean values for the 6-9 age group (n=29) was 0.762 and for the 10-15 age group (n=20) was 0.995 (Table 4); their standard deviations were 0.537 and 0.698, respectively (Fig 22). A two-tailed t-test revealed that the difference between the two groups was not statistically significant (p> 0.05).

**General Protein Banding Patterns**

Saliva and GCF samples from each individual were collected and electrophoretically separated. A densitometry profile for each lane of the SDS gels was
produced with NIH Image software. Protein banding patterns of children’s saliva samples were analyzed using densitometry and a PAGE pattern is shown in Fig 23. Protein banding patterns and Western blots of different adult human saliva have shown that the banding pattern is varied for each individual donor. Thus, the expression of salivary secretory salivary proteins appears to be unique in each individual.

Panel B shows the same membrane after immunoblotting for cARP. RI is more consistently seen in all samples of children’s saliva. This differs from adult samples, which show predominantly RII (Sivakumar et al, 2000).

Densitometric analysis of the Ponceau S stained PAGE samples (Fig 24) shows the consistent presence of a peak at 90 kD, which was not seen in adult samples. Also in these graphs is a peak in the 45-50 kD region, which is the relative mobility of RI. Some adult samples did not have a peak in the 55 kD areas (Sivakumar et al, 2000) which represents the relative mobility of α-amylase, whereas virtually all children’s samples had α-amylase.

Except for the differences noted above, banding patterns in children presented a similar pattern to that of adults. There were no consistent, qualitative differences seen among different age, sex or ethnic groups.

In panel A of Fig 25 the PAGE patterns for 5 children’s whole saliva and GCF samples are seen. The first and the last lane are Rainbow standards. Lanes 1-10 represent alternating banding patterns of children’s whole saliva and GCF. The whole saliva lanes consistently showed a band with the relative mobility of α-amylase. This band was consistently absent in GCF. A slower moving band similar to serum albumin was consistently present in GCF, but absent in whole saliva. These results demonstrate the
integrity of the loading technique. More importantly, these results show the segregation of children’s oral fluid (saliva and GCF) in fully separate compartments. Densitometric analysis of five whole saliva and five GCF samples is shown in Fig 26. The presence of a peak at the relative mobility of α-amylase is seen in all saliva samples but not in GCF.

Panel B, Fig 25, shows the Western blot for cARP of alternate samples of whole saliva and GCF. A consistent pattern of RI was seen in whole saliva whereas various combinations of RI and RII were seen in GCF, as would be expected in serum. Densitometric analysis done on the Western blot of these samples (Fig 27) clearly demonstrates the presence of only RI in whole saliva of children.

Summary

Secretory protein expression in children's saliva showed notable differences to that of adults.

1. Qualitatively, a low mobility (~90 kD) band was seen in the majority of saliva samples from children and only in fewer than 20% of the adults. This difference may be due either to a larger proportion of submandibular/sublingual saliva (which contains more high molecular weight glycoproteins than parotid saliva) or to less proteolytic degradation in children's saliva than in that of adults.

2. Adult samples had variability in the presence of α-amylase whereas all children's saliva had a band consistent with the mobility of α-amylase.

3. Most notably, children's saliva contained predominantly RI which is different from adult saliva which contained predominantly RII.
DISCUSSION

The majority of early studies on salivary glands and saliva has been carried out using tissues from laboratory animals or pooled human saliva. The resulting protein profiles in humans appear to be similar to those found in other species (Beely, 1991; Bennick, 1982). This study shows that protein expression in humans may be quite complex, regulated by catecholamine hormones via the cyclic AMP pathway with developmentally distinguishing features significantly different from those of rodents.

In recent studies it has been demonstrated that developing salivary glands undergo extensive morphological and functional differentiation (Sivakumar et al, 1998; reviewed in Denny et al, 1997). The secretion of proteins, regulated by beta adrenergic responses via cyclic AMP, can serve as an index to differentiate acute from chronic stress responses: namely, the expression of cAMP receptor proteins reflect the responses in an apparent proportional manner (Mednieks et al, 1982, 1991). Moreover, immediate responses such as mechanical stress are also mediated via the cAMP pathway (Uematsu et al, 1997; Burke et al, 2000). It remains to be determined in future studies if secretory responses to pharmacological or environmental stress in children are comparable to those of adults.

As little data are available regarding the protein profiles of GCF in general and the expression of secretory proteins in children, the basic aims of the study were to investigate these parameters. The findings were unexpected in that adult and children’s saliva exhibited unique individual protein profiles. Moreover, a major salivary protein, alpha amylase, varies extensively, being entirely absent in some adults. In children, the
protein appears more stable as indicated by the consistent finding of a protein with the relative mobility of alpha amylase. The prevalence of large molecular weight proteins in the saliva of children may indicate that there are differences in either the expression or activation of proteases. This is supported by apparent stability of amylase in saliva of children compared to that of adults.

Notable differences in the expression of another secretory protein in children were observed, a large (~90 kD) band in the majority of saliva samples from children and only in fewer than 20% of adults. This may be due to developmental differences in expression of protein, a larger proportion of submandibular/sublingual gland secretion (which contains more large kD glycoproteins than parotid saliva) in children’s saliva, or there is less proteolytic degradation in children’s saliva than that of adults.

By performing densitometry and analyzing the total protein content of saliva and GCF, it was found that there was no difference between males and females and the different age groups (divided based on stage of mixed dentition). However, there was a small, but statistically significant difference in the concentration of protein between Caucasians and the other two groups (Hispanics and African Americans). This, however, has to be substantiated by testing larger sample populations.

Interestingly, and very significantly, children’s saliva expressed predominantly RI, which is different from adult saliva, which expressed RII predominantly. These developmental changes may reflect that whole saliva of children has a different composition (more submandibular/sublingual) than adults. It may also indicate that children’s salivary glands may be undergoing developmental changes even between 6-15 years of age.
Effectiveness of paper point collection method

In pilot studies Perio Paper (Harco, Tustin CA) was used to collect GCF. It was, however, very difficult to recover the sample from the Perio Paper. Paper points were then tried and this approach improved collection and recovery, but the recovered protein was not sufficient to load the gel with confidence. Finally, we cut the paper point to 1 mm above the wetted area (Fig 1) limiting diffusion of GCF to a small paper area which could be extracted with 25 µl of buffer. The use of sterile paper points for the collection of GCF has many advantages over the traditional collection methods. The paper points are narrow, cylindrical and flexible. They are also available in many different sizes. Typically, the tip of the paper point will have a diameter of 1 mm, which is much smaller than the Perio Paper strip. The small tip of the paper point allows it to be easily introduced into the gingival sulcus without disturbing the adjacent gingival tissue. For the same reason, they can easily be negotiated between teeth and the line angles of teeth. It is especially useful in clinically healthy mouths where the gingiva is more closely adapted to the teeth. By using this method we were able to consistently load higher amounts of protein for electrophoretic analysis. An average of 20 µl was confidently loaded each time.

The technique for collecting GCF with paper points was compared with the more traditional method of using Perio Paper. Samples of GCF collected with Perio Paper strips, as well as GCF collected using the paper point method described were dot blotted and the average densities were calculated. Collection of GCF with paper points yielded more consistent and significantly higher concentrations (3-6 fold)
of recovered salivary proteins. These results indicate that the proteins could be recovered more efficiently and reliably from the clipped paper point tips than by the traditional Perio Paper method.

The collection of GCF is technique sensitive. The amount of GCF obtainable from healthy gingival sulci is extremely small. It is necessary to collect enough GCF in order to reasonably quantify the protein composition. Paper strips and glass capillary tubes are the most common methods to collect GCF. However, the insertion of these relatively large and inflexible objects into the gingival sulcus may cause mechanical irritation and create an inflammatory response. A local inflammatory response will more than likely alter the protein composition of the GCF and render any quantification techniques useless. Once the GCF is collected on the paper strips it must be thoroughly extracted and suspended in a buffer solution for analysis.

A commercially available Perio-Paper® strip commonly used to collect GCF measures 2mm X 2mm. The large strips can cause problems in the collection of GCF since they are not easily guided into the sulci of teeth and around the line angles where again GCF is present. GCF absorbs into the large surface area of Perio-Paper® and is difficult to displace with a buffer solution. The Perio-Paper strip also absorbs much of the buffer solution during storage and ultimately requires a greater volume of the buffer, which decreases the final concentration of proteins in the sample. Recovery of the GCF was relatively easy when a paper point is used. When the GCF was collected the paper point was immediately cut above the point of ‘wetting’. This prevents the GCF from further absorbing in to the remaining portion of the paper point. The ‘wetted’ portion of the paper point is more saturated with GCF since it absorbs less sample buffer solution.
during storage. The technique used to collect is simple and easy to learn and is similar to that of collection with Perio-Paper®. Collection of GCF with glass capillary tubes also presents problems since they are extremely technique sensitive and it takes some time to learn the technique. The glass tubes are not flexible and can easily cause mechanical irritation to the gingival sulcus. The tubes are also a source of anxiety as they appear sharp and needle-like. In contrast, paper points present a minimal threat to children and they can be invited to inspect and visualize the flexible piece of paper.

The development of a new collection and analytical technique provided the opportunity to successfully study the molecular composition of GCF and calculate the concentration of specific proteins. In both children and adults different protein patterns were seen in GCF compared to those of whole saliva. These data indicate that GCF and saliva constitute separate compartments with distinct protein compositions.

It was determined that total banding patterns were individually unique. While a general pattern of previously identified major proteins was seen in all subjects tested, a pleomorphy of discrete bands made a distinct individual profile. If these findings are substantiated by further studies, then a number of potential applications can be envisioned. For example, a unique individual secretory protein profile could serve as an alternative or additional identification instrument to finger printing and may have application in forensics.

Analyses of general protein profiles and comparison with specific components, e.g. total proteins vs. cARP, may provide a means for measuring physiological responses (e.g. mechanical stress or inflammation). Developing a clinical device may eventually be useful for applications in diagnosis.
Previous studies (Mednicks et al, 1991, 1994) have shown that the level of cARP rises in acutely stressful situations and falls below normal in chronic stress. There are no objective ways to evaluate the stress level of a child. Though multiple scales are available for indicating the behavior of a child, they are all subjective. If a child coming to the dental clinic is anxious, it would be extremely useful for a provider to know the stress level of a juvenile patient objectively, to determine the extent of treatment which can be accomplished in that visit. If a chair-side biochemical test is made available which can instantly determine the level of cARP in a patient, it may serve as a tool to accomplish treatment in a more humane and efficient manner.

Alternatively, if the level of cARP, which has a constant ratio to total protein in normal subject, differs, then these changes may signify present or potential disease. Such predictive instruments, on a molecular level, are not presently available. The development of biochemical tests for diagnosis of potential susceptibility to oral or systemic diseases, especially in children, may have future applications.
FIGURE LEGENDS

Fig 1  Diagram of collection method used for GCF:

The panel on the left shows the introduction of a paper point into the gingival crevice of a tooth. In the middle panel the wetted area is shown. The paper point was cut just above the wetted area and transferred to the plastic tube (shown in the right panel), which contains the sample buffer.

Fig 2  Developing a standard curve:

Protein concentrations were determined by developing a standard curve using increasing concentrations of bovine serum albumin (BSA). The Ponceau S stained dot blot is shown below, and the corresponding optical densities vs the BSA concentrations are plotted in the graph.

Fig 3  Dot blots on nitrocellulose membrane:

Shown here are 1.5 μl dot blots on a nitrocellulose membrane with various salivary samples stained with Ponceau S red dye. Panel A shows the membrane as prepared for densitometric analysis of total protein content in saliva. Samples 1-13 represent 13 salivary samples and samples 14 and 15 are BSA and α-amylase, respectively. Panel B shows the same nitrocellulose membrane after immunoblotting for cARP. Samples 14 and 15 are not recognized by the antibody.
Fig 4  Comparison of staining methods:

Comparison of protein banding patterns on wet polyacrylamide gels stained with Coomassie blue dye and on nitrocellulose paper stained with Ponceau S red dye. Panel A shows an acrylamide gel stained with Coomassie blue dye and dried for scanning. Panel B shows a transblot onto nitrocellulose paper of an identical sample stained with Ponceau S red dye. Panel C shows densitometric peak displays of the respective banding patterns. The ordinate in panels A and B, and the abscissa in panel C, shows relative molecular size in kilo Daltons (kD). There are fewer signals in the banding pattern with the Ponceau S red dye; nevertheless, all major peaks are visualized. Panels A and B are displayed in pseudocolor to provide a visual comparison.

Fig 5  Total protein concentration of saliva (samples 1-25):

Graphical representation of total protein concentration of saliva in 25 children is shown. The samples are shown on the X-axis and the density of total proteins is presented on the Y-axis in arbitrary units. The values have a range of 0.2-2.81 (arbitrary units).

Fig 6  Total protein concentration of saliva (samples 26-49):

Graphical representation of total protein concentration of saliva in the next 24 children’s samples is shown. The sample numbers are shown on the X-axis and the density of total proteins is on the Y-axis, in arbitrary units. The values have a range of 0.14-1.1 (arbitrary units).
Fig 7  Total protein concentration of GCF:

Graphical representation of total protein concentration of GCF in 25 children’s samples is shown. The sample numbers are shown on the X-axis and the density of total proteins is on the Y-axis, in arbitrary units. The values have a range of 0.24-1.82 (arbitrary units).

Fig 8  Mean values of saliva and GCF:

Graphical representation of the mean values of both saliva (n=49) and GCF (n=25) is shown here. The Y-axis shows the density in arbitrary units. The mean values are 0.885 and 0.636, respectively. The standard deviation for salivary samples is 0.61 and that for GCF is 0.37. All values shown are arbitrary.

Fig 9  Total protein concentration of saliva in 28 samples:

The total concentration of proteins is shown in the graph for 28 samples of children’s saliva. The X-axis shows the sample number and the Y-axis the density in arbitrary units. The concentration of proteins in each individual is varied and unique.

Fig 10  Concentration of cARP in saliva of 28 samples:

The concentration of cARP in the same 28 samples is shown in the form of a graph. The X-axis shows the sample number and the Y-axis the density in arbitrary units.
Fig 11  Mean values of cARP and total proteins for 28 samples:

Graphical representation of the mean values of both the total protein and cARP concentrations in whole saliva is shown. The Y-axis shows the average density in arbitrary units. The range for total protein is 0.46-2.62 and that of cARP is 0.010-0.48. Protein lanes in the graph (blue) represent the concentration total proteins and cARP lanes in the graph (red) represent the concentration of cARP.

Fig 12  Comparison of cARP/total protein in saliva:

The comparison of cARP to total proteins present in the salivary samples of the 28 children is shown here. The X-axis shows the sample number and the Y-axis the density in arbitrary units. Light gray bars represents the concentration total proteins and dark gray bars, the concentration of cARP. Though the concentration of cARP differs in each individual the percentage of cARP to total protein falls within the same range.

Fig 13  Total protein concentration of salivary samples in male subjects:

Graphical representation of total protein concentration of saliva in 22 male children’s samples is shown. The sample numbers are shown on the X-axis and the density of total proteins is on the Y-axis, in arbitrary units. The values have a range of 0.24-2.62 (arbitrary units).

Fig 14  Total protein concentration of salivary samples in female subjects:

Graphical representation of total protein concentration of saliva in 27 female children’s samples is shown. The samples are shown on the X-axis and the density of
total proteins is presented on the Y-axis in arbitrary units. The values have a range of 0.19-2.69 (arbitrary units).

Fig 15 Mean values of salivary samples of male and female subjects:

Graphical representation of the mean values of salivary samples in male \( (n=22) \) and female \( (n=27) \) children is shown here. The Y-axis shows the average density in arbitrary units. The mean values are 1.017 and 0.792, respectively. The standard deviation for male salivary samples is 0.662 and that for females is 0.57. All values shown are in arbitrary units.

Fig 16 Total protein concentration of salivary samples in Caucasian children:

Graphical representation of total protein concentration of saliva in 23 Caucasian children with European background (EC) samples is shown. The sample numbers are shown on the X-axis and the density of total proteins is on the Y-axis, in arbitrary units. The values have a range of 0.2-2.69 (arbitrary units).

Fig 17 Total protein concentration of salivary samples in Hispanic children:

Graphical representation of total protein concentration of saliva in 15 Hispanic/Latino (H/L) children’s samples is shown. The sample numbers are shown on the X-axis and the density of total proteins is on the Y-axis, in arbitrary units. The values have a range of 0.14-1.11 (arbitrary units).
Fig 18 Total protein concentration of salivary samples in African American children:

Graphical representation of total protein concentration of saliva in 11 African American children's samples is shown. The sample numbers are shown on the X-axis and the density of total proteins is on the Y-axis, in arbitrary units. The values have a range of 0.19-1.11 (arbitrary units).

Fig 19 Mean values of salivary samples of EC, H/L and AA children:

Graphical representation of the mean values of salivary samples in European Caucasians (EC), Hispanics (H/L) and African Americans (AA) are shown. The n values are 23, 15 and 11, respectively. The Y-axis shows the average density in arbitrary units. The mean values are 1.15, 0.681 and 0.559, respectively. The standard deviation for EC salivary samples is 0.755, H/L is 0.33, and that for AA is 0.30 in arbitrary units. The difference between EC and the other two groups is statistically significant (p < 0.05).

Fig 20 Total protein concentration of saliva in children in an age group of 6-9 years:

Graphical representation of total protein concentration of saliva in 29 children in an age group of 6-9 years is shown. The sample numbers are shown on the X-axis and the density of total proteins is on the Y-axis, in arbitrary units. The values have a range of 0.16-2.69 (arbitrary units).

Fig 21 Total protein concentration of saliva in children in an age group of 10-15 years:

Graphical representation of total protein concentration of saliva in 20 children in an age group of 10-15 years is shown. The sample numbers are shown on the X-axis and
the density of total proteins is on the Y-axis, in arbitrary units. The values have a range of 0.2-2.62 (arbitrary units).

Fig 22  Mean values of salivary samples for 2 different age groups:

Graphical representation of the mean values of salivary samples in two different age groups, viz 6-9 and 10-15, are shown. The n values are 20 and 29, respectively. The Y-axis shows the average density in arbitrary units. The mean values are 0.762 and 0.995, respectively. The standard deviation for the 6-9 age group salivary samples is 0.537 and that for the 10-15 age group is 0.698 in arbitrary units. There is no significant difference between the two groups (p > 0.05).

Fig 23  Protein banding patterns and Western blotting of children’s saliva:

Panel A shows the PAGE protein banding patterns after transblotting onto a nitrocellulose membrane and staining with Ponceau S red dye. The first four lanes are various standards purchased commercially. Lane M represents the Rainbow standard. Lanes number one and two show RI (Sigma Chemical Company) and RII (Sigma Chemical Company), respectively. Lane three was a preparation of α-amylase purchased from a commercial vendor (Sigma Chemical Corporation). Lanes 4-8 represent the banding patterns of five children’s whole saliva samples. In Panel B the same membrane is seen after immunoblotting for cARP. Lanes 4-8 show the presence of RI only in all of the saliva samples. This differs from that of adult samples where RII was more commonly seen.
Fig 24 Densitometric analysis of total proteins and cARP in children’s saliva:

Panel A shows the densitometric analysis of the protein banding patterns from Fig 23. Note the consistent presence of a peak at the 90 kD areas in all five samples. Panel B shows the densitometric analysis of the same samples after immunoblotting for cARP. There is a consistent presence of a protein of the relative mobility of RI. Note that the values on the X-axis vary for panels A and B.

Fig 25 Protein banding pattern and Western blotting of children’s whole saliva and GCF:

Shown here are the protein banding pattern and Western blot of children’s whole saliva (lanes 1,3,5,7 and 9) and GCF (lanes 2,4,6,8 and 10). Relative molecular size, kD, is shown by bands from a commercial protein standard marker (lanes M1 and M2) solution on the polyacrylamide gel electrophoretic pattern (PAGE), shown in panel A. Lanes 1-10 represent alternating whole saliva and GCF samples of 5 children. Panel B shows the Western blot (WB) of the nitrocellulose membrane from panel A, with antibody to RII/cARP. Predominantly RI is present in saliva, while both RI and RII are present in GCF.

Fig 26 Densitometric analysis of children’s whole saliva and GCF:

Shown here is the densitometric analysis of the PAGE patterns in panel A of Fig 25. It shows various protein peaks represented graphically and distinctions between whole saliva and GCF. Peaks seen in each saliva sample consistent with the relative mobility of α-amylase are generally absent in GCF.
Fig 27 Western blotting and densitometry of proteins shown in Fig 25:

Immunoblotting of the above membrane reveals the consistent presence of R1 in all children's saliva samples (Lanes 1,3,5,7 and 9), whereas both R1 and RII were seen in GCF samples (Lanes 2,4,6,8 and 10). Densitometric analyses performed on all 10 samples are shown.
GCF Collection

Paper point → cut → sample
BSA standard curve
Dot blots on nitrocellulose membrane

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Comparison of staining methods

A = Coumassie Blue stained PAGE
B = Ponceau S stained electroblot
Total protein concentration in whole saliva

![Graph showing total protein concentration in whole saliva for samples 1-25. The y-axis represents density in arbitrary units, ranging from 0 to 3, and the x-axis represents samples 1 to 25.]
Total protein concentration in whole saliva

Density in arbitrary units

Samples (26-49)
Total protein concentration in gingival crevicular fluid
Total Protein Concentration In Oral Fluid

Average density in arbitrary units

SD=0.61  n=49
SD=0.37  n=25

SALIVA  GCF
Total protein in saliva

Density in arbitrary units

Sample Number
Mean values of total protein and RI

![Graph showing mean values of total protein and RI](Image)
Comparison of RI and total protein in saliva

Sample Number

Density in arbitrary units

□ Total protein
■ RI
Total salivary protein concentration in males

Density in arbitrary units

SAMPLES (1-22)
Total protein concentration in females

Density in arbitrary units

Samples (1-27)
Mean total protein concentration in saliva

![Graph showing mean total protein concentration in saliva for males and females. The graph indicates that the mean total protein concentration for males is higher than for females, with standard deviations of 0.662 and 0.574, respectively. The number of samples for males is 22, and for females is 27.](image-url)
Total protein concentration in Caucasian children
Total protein concentration in Hispanic children
Total protein concentration in African American children

Samples (1-11)

Density in arbitrary units
Total protein concentration in saliva

Average density in arbitrary units

EC
H/L
AA

n=23
n=15
n=11

0.755
0.33
0.30

n=23
n=15
n=11
Total salivary protein concentration in age group (10-15)
Mean total protein concentration in saliva

Average density in arbitrary units

- **SD = 0.537**
- n = 20
- **SD = 0.698**
- n = 29
Protein banding patterns and Western blotting of children’s saliva
Densitometric analysis of total protein and cARP in children’s saliva

Relative mobility

A

B

Relative peak heights in arbitrary units
Protein banding pattern and Western blotting of children’s whole saliva and GCF
Densitometric analysis of total protein and cARP in children’s saliva and GCF

Relative Peak Heights In Arbitrary Units

Relative Mobility

[Graph showing relative peak heights and mobility for samples 1 to 10]
Western blotting and densitometric analysis of children’s whole saliva and GCF
Table 1  Total protein concentration in saliva and GCF

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<tr>
<th>Group</th>
<th>Mean</th>
<th>SD</th>
<th>n</th>
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<tr>
<td>Saliva</td>
<td>0.887</td>
<td>0.610</td>
<td>49</td>
</tr>
<tr>
<td>GCF</td>
<td>0.636</td>
<td>0.337</td>
<td>25</td>
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</table>

The difference in protein concentration of whole saliva and gingival crevicular fluid (GCF) is presented. There is a 10 fold dilution factor for GCF and hence the value of GCF is approximately 10 times higher than what is shown here.
The difference in concentration of salivary protein in whole saliva between males and females was determined. The difference was not statistically significant.
<table>
<thead>
<tr>
<th>Subject group</th>
<th>Mean</th>
<th>SD</th>
<th>n</th>
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</thead>
<tbody>
<tr>
<td>EC</td>
<td>1.15</td>
<td>0.755</td>
<td>23</td>
</tr>
<tr>
<td>H</td>
<td>0.681</td>
<td>0.330</td>
<td>15</td>
</tr>
<tr>
<td>AA</td>
<td>0.559</td>
<td>0.302</td>
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</table>

The children are divided into European background Caucasians (EC), Hispanic (H) and African Americans (AA). The sample means, the standard deviations (SD) and the number of samples (n) are shown in columns 1-3 respectively. The difference in concentration of salivary proteins between EC and that of H and AA was statistically significant.
Table 4  Total protein concentration in two different age groups

<table>
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<th>Age group</th>
<th>Mean</th>
<th>SD</th>
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<tr>
<td>6-9 years</td>
<td>0.762</td>
<td>0.537</td>
<td>29</td>
</tr>
<tr>
<td>10-15 years</td>
<td>0.995</td>
<td>0.698</td>
<td>20</td>
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</table>

The salivary samples were divided into two age groups within the mixed dentition. The difference in concentration of salivary protein between the two age group (6-9 and 10-15) is not statistically significant.
REFERENCES


APPENDIX 1

1. Study Protocol
2. Consent Form
3. Institutional Review Board Letter
**Application for Project Approval**

**INSTITUTIONAL REVIEW BOARD**

**The University of Connecticut Health Center**

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<table>
<thead>
<tr>
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<tbody>
<tr>
<td>Investigator:</td>
<td>Arthur R. Hand</td>
</tr>
<tr>
<td>Department:</td>
<td>Pediatric Dentistry</td>
</tr>
<tr>
<td>Phone Number:</td>
<td>X4085</td>
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<tr>
<td>Mail Code:</td>
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<tr>
<td>Co-Investigators:</td>
<td>Maija Mednieks</td>
</tr>
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<td>02/01/00</td>
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<tr>
<td>Project Title:</td>
<td>Secretory Protein Expression in Human Saliva</td>
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<tr>
<td>Funding Agency or Research Sponsor</td>
<td>University of Illinois, and HCRAC</td>
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**Type of Review:**

- [X] Full Board
- [ ] Expedited
- [ ] Exempt

**Type of Project:**

- [X] New
- [ ] Continuation*
- [ ] Duplicate Submission*
- [X] Modification/Addendum *
- [ ] Training/Center

(*Include: Title/IRB#/Last Approval Date: Secretory Protein Expression in Human Saliva/98-177/1/12/99)

**I. Project Location:**

Revised 10/98
X Uconn Health Center
VA Medical Center
Other: ________________

II. Cooperating Institutions: ________________

III. Type of Human Subjects to be Used:

X Children/Adolescents
X Males
X Females
Inpatients
X Outpatients
Pregnant Women
Mentally/Physically Handicapped
Fetuses
Abortuses

Number of Subjects: 25 Age Range: 08-80

IV. Project Involves the Use of An Investigational New Drug:

X No

(If yes, list IND by name and number, inform the Director of Pharmacy Service at John Dempsey Hospital.)

IND Name: N/A #: N/A Sponsor: N/A

V. Project Involves the Use of an Investigational Device:

X No

IDE #: N/A Sponsor: N/A

Revised 10/98
VI. Project involves the Use of Radiation or Radioisotopes:

X No

(If yes, inform the Radiation Safety Office prior to IRB submission. Approval from the Committee of Research Safety and Environmental Health (CRSEH) may be necessary.)

VII. Signatures: Applicant (and Faculty Sponsor if Applicant is Non-Faculty)

Signature: [Signature]
Date: 3/9/99

Revised 10/98
This abstract should contain a clear and succinct description of the long-term objectives and specific aims of this project with specific reference to its health relatedness. It should also include an accurate description of the experimental design and methods of achieving these goals. This abstract is meant to serve as a complete description of the proposed study. DO NOT EXCEED THE SPACE PROVIDED.

The purpose of this study is to determine the expression of secretory proteins in two types of oral fluid: whole saliva and in gingival crevicular fluid. Individual protein patterns of each person are unique and may be used for identification purposes. Additionally, elevation or decrease below basal levels of specific proteins may be an index of specific disorders or may serve as indices of susceptibility to some disorder.

Samples of saliva will be collected from normal, healthy individuals by allowing a small amount to flow into a sterile tube or by touching a sterile paper strip to the area between the canine teeth and the gingiva. All procedures will be carried out using full infection control precautions and following UCHC and NIH guidelines.

Proteins which will be studied include secretory proteins such as salivary enzymes such as alpha amylase, salivary immunoglobulins such as IgA, and cellular regulatory proteins such as cyclic AMP receptor proteins (cARP). Standard methods for polyacrylamide electrophoresis and Western Blotting will be employed.

Collection of saliva is a noninvasive procedure that poses no danger to either the subject or the investigator, takes very little time and can be carried out either in the clinic or the laboratory. This investigation is intended to develop a potentially useful system for the identification of an individual as well as detecting existing or potential disorders, most likely those related to stress.

In the space below, provide a flow diagram or outline of the experimental design of the investigation focusing on those aspects that involve human subjects and emphasizing the specific time sequence of the procedures to be performed.

1) Subject selection. Only healthy individuals able to understand the protocol as described on the Consent Form will be asked if they would be interested in participating in this study.
2) Sample collection. Two types of oral fluid will be collected: whole saliva from the oral cavity will be collected by asking the subject to allow a small quantity (1-2 ml) to flow into a sterile tube. Gingival crevicular fluid (GCF) will be collected by touching a sterile paper strip to the crevice between a tooth and the gingiva. The sample size obtained from this area is approximately 1-2 micro liters. The saliva samples will be treated with a preservative solution and stored at -40°C until use.
3) Electrophoresis: The collected whole saliva will be heated with a denaturing solution and the proteins will be separated by polyacrylamide gel electrophoresis (PAGE). The proteins will then be transferred from the acrylamide gel to a nitrocellulose membrane by electroblotting and stained to visualize the total protein pattern. This pattern will be recorded by a digitized scanning process and stored as a computer file.
4) Western Blotting: To localize specific proteins on the general protein patterns, the membranes will be incubated with antibody solutions specific for an individual protein and compared to controls. These blots can be run on duplicate gels and several individual protein patterns can be determined.
5) Densitometry: Quantification of the total proteins determined by PAGE will be carried out by a densitometric analysis of the banding patterns. Determination of the specific proteins will be done by densitometric analysis of the Western blotting bands. The quantification will be normalized by calculating ratios of amounts of each specific protein to the amount of the total proteins.
6) Data Analysis and Applications. By carrying out these analyses an array of total protein patterns can be established for each individual tested. Secondly, the expression of specific proteins can be determined and compared to an established baseline. For example, a ratio of approximately 0.03 has been found for cARP to total proteins. In the case of persistent environmental stress this ratio drops significantly whereas under short-term acute stress, the ratio rises and then returns to normal.

Revised 10/98
1. List specific eligibility requirements for subjects, including those criteria which would exclude otherwise acceptable subjects.

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<td>a. in good health; not on medication</td>
<td>a. physical or mental disorders</td>
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<td>b. able to understand the protocol</td>
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<td>c. participate without compensation</td>
<td>c. require compensation</td>
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2. a.) How will subjects be recruited? b.) Explain process of obtaining consent.

a) Patients at UCHC dental clinics and UCHC staff and students will be approached and asked if they would be willing to participate in a study. The study would then be explained and the sample collection would be described. b) Consent will be obtained following standard procedures in use at UCHC. The subjects will be informed that there will be no negative consequences if they do not wish to participate in the study. The subjects will be informed that they can stop participating at any time and that this will have no effect on their treatment, their academic progress or their work situation.

3. List all procedures (both experimental and non-experimental) to be performed on human subjects. Also list alternate procedures available to the subject.

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<th>Alternate procedures/treatments:</th>
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4. State the potential risks (e.g. psychological, social, legal, drug toxicities) associated with the proposed procedures.

The research to be conducted poses no risk to the patient.

5. Describe procedures to protect against or minimize potential risks and to assure confidentiality.

<table>
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<th>Potential Risks:</th>
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Confidentiality: Individual samples are identified only by a coded number

Revised 10/98
6. What benefit, if any, is to be gained by the subject? In the event of monetary gain, include all payment arrangements, including reimbursement of expenses, free medication, etc.

None

7. Describe any costs related to the research procedures that are over and above those incurred by standard treatment, and indicate who will be responsible for them.

None

8. What new information may arise from this work?

Knowledge of the expression of various proteins in saliva

INFORMED CONSENT CHECKLIST
Informed Consent is a representation of the interaction between the subject and the researcher. The checklist covers the required elements of informed consent, and must be completed by the investigator as part of this application. If a submission contains an informed consent statement which does not address each of the elements, it will be returned to the investigator for revision, possibly resulting in a delay of the review process. Assistance in preparation of the informed consent form is available by calling the IRB Office, 679-2142. A sample consent form is available.

IRB - Informed Consent Checklist

__X__1. Use wording understandable to the subject population, and exclude any statements which may be considered coercive (unduly encourage subjects to participate).

__X__2. At the beginning of the consent form give an explanation of the procedures to be followed including: a) purpose of research; b) procedures which are experimental; c) expected duration of the subject's participation.

_N/A_3. Description of the risks, discomforts, and side effects.

__X__4. Description of the safeguards to be used to protect the subject.

_N/A_5. If project involves experimental therapy, discuss alternate treatment procedures available (i.e. the subject has to know what is available to him/her if the choice is made not to participate).

_N/A_6. Description of benefits to be expected.

__X__7. An offer to answer any inquiries concerning the procedures.

__X__8. Tell subjects that participation is voluntary and they may refuse to participate.

__X__9. State that the subjects may withdraw, or be withdrawn from the study at any time without jeopardizing present or future care. describe anticipated circumstances under which the subject’s participation may be terminated by the investigator without regard to the subject’s consents.

_N/A_10. State the terms of subject compensation for participation, if any.

_N/A_11. State clearly what costs the subject will incur by participating in the study, and what will be paid for as part of the study.

__X__12. State that every effort will be made to maintain confidentiality.

Revised 10/98
a. In research involving FDA regulated products, the consent form must contain a statement that the FDA and the drug company may inspect the subject's research and medical records. b. For VA studies involving an INDIIDE the consent form must have the following language verbatim: "I have been informed that because this study involves articles regulated by the FDA (Food and Drug Administration), the FDA may choose to inspect records (including my medical records) identifying me as a subject in this investigation."

---

_X__13. a) The name and telephone number of the principal investigator or other responsible individual who can be contacted if the subject experiences problems or an adverse effect during the research study. b) An explanation of whom to contact for answers to questions about the research. c) For questions about the rights as a research subject use the following language: "If you have questions regarding your rights as a research subject you may contact an IRB Representative at 860/679-3054".

INCLUDE #14-17, if appropriate

---

_N/A_14. A statement that the particular treatment or procedure may involve currently unforeseeable risks to the subject.

_N/A_15. The procedures for orderly termination of participation by the subject (i.e., removal of a device or discontinuation of study drug).

_N/A_16. A statement that significant new findings that arise during the course of research, and which may relate to the subject's willingness to continue participation, will be provided to the subject.

_N/A_17. The approximate number of subjects involved in the study at this facility compared to overall subject population.

---

_X__18. Include a statement of consent to participate in the study.

_X__19. Include a statement that the subject has received a copy of the consent form.

_X__20. Inclusion of the appropriate policy statement regarding compensation in the event of a research related injury for projects involving more than minimal risk:

a. For Projects Conducted at UConn Health Center include the following:

The University of Connecticut Health Center (UCHC) does not provide insurance coverage to compensate me if I am injured during this research. However, I may still be eligible for compensation. If I am injured, I can file a claim against the state of Connecticut seeking compensation. For a description of this process, or available compensation options, I may contact a representative of the UCHC Institutional Review Board (IRB) at 860/679-3054.

The UCHC does not offer free care. However, treatment for a research related injury may be obtained at UCHC for the usual fee.

If I need additional information, I may contact the IRB at 860/679-3054 who can review the matter with me, identify the resources that may be available, and provide me with further information as to how to proceed.

b. For VA projects include the following:

Revised 10/98
If you are physically injured as a result of taking part in this study, all necessary and appropriate care will be provided if you are eligible for medical care as a veteran. In the event of such injury, compensation may or may not be payable under federal law. Further information regarding eligibility for medical care and compensation under federal law in the event of injury, may be obtained from the Chief of Medical Administration Service at the Newington, VA, telephone number 666-6951.

__X__ 21. Include a signature and date line for each of the following:

Subject
Investigator or Person Obtaining consent
Witness (must be someone other than individual obtaining consent).

__X__ 22. For Projects Involving Subjects Under the Age of 18 Years:

a. If the subject is 12 years of age or older, the subject signs the consent form and a parent or guardian also signs the consent form. No assent statement is required.

b. If the subject is between 7-12 years of age, and the study is a therapeutic trial*:

the subject sign nothing; and the parent which must be included at the end of the consent form after the signature lines.

I am satisfied that Dr._________ has discussed these procedures with my child in a manner and to an extent that is appropriate for his/her capacity to understand at the present time. My child has been informed of the procedures that will be performed, the reason for such treatment, and the associated risks. In addition, all of the alternative procedures and the voluntary nature of their participation have been described.

As the parent (or guardian) of__________(subject's name), I am signing this consent form and authorizing the above procedures to be performed because in my judgment, my child is not mature enough to fully understand the complexities of the procedures involved with his/her treatment. (Include signature and date lines for Parent, Person Obtaining consent, and Witness).

OR

The study is not therapeutic trial*:

the parent or guardian signs the consent form; and the subject signs the following assent statement which must be included at the end of the consent form after the signature lines.

My (include appropriate person, doctor, dentist, psychologist, researcher, etc.) and parents have talked to me about being part of a study of (explain study in child-appropriate language). I understand the reason for the study and why I am being asked to take part in it. I have been told that as a subject in the study I will have the following performed: (list procedures to be followed in child-appropriate language—the statement need not be as detailed as information provided to an adult). I understand that these procedures may cause (list potential harmful effects in child-appropriate language). I know that I can ask questions about this study at any time. I also know that I can decide not to be in this study, or after entering the study I can decide that I want to be taken out of it.

Whatever I decide to do, I know my (include appropriate person as above) will not be angry with me and continue to treat me as his/her patient.

(Include signature and date lines for subject, Person Obtaining Consent, and Witness)

Revised 10/98
c. If the subject is less than 7 years of age, the parent or guardian signs the consent form, the subject signs nothing. No assent statement is required.

*Note: If you have any questions regarding what qualifies as a therapeutic or non-therapeutic trial, please call the IRB Office"
CONSENT FORM

Study Title: “Secretory Protein Expression in Human Saliva”

Principal Investigator: Dr. Arthur R. Hand
Department of Pediatric Dentistry
(860) 679-4085

Human Subjects Oversight Committee Approval: IRB# 98-177

Description and Purpose of the Study:
In this study enzymes and other proteins of human oral fluid (saliva from the mouth) will be measured and identified so that changes that take place during human development and growth can be identified.

You will be asked to make a donation of a small amount (about a teaspoon full) of saliva and a small amount (less than a drop) of fluid from the crevice between the gums and the teeth. These procedures do not cause any pain or do any harm. The total time will not be more than 10 minutes. The length of this study will be approximately one year.

The samples will be collected by:
1) allowing saliva to run from the mouth into a small sterile cup
2) by touching an absorbent sterile strip to the inside of the cheek or under the tongue
3) by touching a narrow sterile strip of absorbent material to the crevice between the teeth and gums.

To protect your privacy, your name will not be used. The samples will be identified by a number code. Your participation or withdrawal from the study will not affect your treatment or medical outcome. You will not receive payment or other benefits from volunteering. It is hoped that the results of this study may help in diagnosis or treatment of some diseases.

If you agree to participate and have understood this information, please sign this statement: “I have been informed of the nature of this study and understand that I can stop participating at any time.”

______________________________  ________________
Signature of subject or parent/guardian  date

______________________________  ________________
Signature of doctor  date

______________________________  ________________
Signature of witness  date

The University of Connecticut Health Center does not provide free care. If there are any questions regarding this protocol, Ms. Joanne R. Lamothe, Executive Secretary of the IRB, may be contacted at (860) 679-3054 or by e-mail, lamothe@nso.uchc.edu
CONSENT FORM cont’d.

Non-Therapeutic Study Consent Form For Subjects Between Ages 7-12 Years Old.

Dr. ______________________ and my parents have talked to me about being in this study.
I will give a small sample of my saliva for the doctor to use for laboratory experiments. I understand that this will not cause me any pain or harm and that I can ask questions to the doctor at any time. I also know that I can decide not to be in this study at any time and that Dr. ______________________ will not be angry with me and will continue to treat me as his/her patient.

Signature of subject   date

Signature of doctor   date

Signature of witness   date

For Projects at the University of Connecticut Health Center Involving More Than Minimal Risk: (This Project Poses No Risk)

The University of Connecticut Health Center (UCHC) does not provide insurance coverage to compensate me if I am injured during this research. However, I may still be eligible for compensation. If I am injured, I can file a claim against the State of Connecticut seeking compensation. For a description of this process, or available compensation options, I may contact Ms. Joanne R. Lamothe, Executive Secretary of the IRB at the UCHC, at (860) 679-3054 or by e-mail, lamothe@nso.uchc.edu.

The UCHC does not provide free care. However, treatment for a research-related injury may be obtained at UCHC for the usual fee.

If I need additional information, I may contact Ms. Joanne R. Lamothe, Executive Secretary of the IRB at the UCHC, at (860) 679-3054 or by e-mail, lamothe@nso.uchc.edu. The Executive Secretary can review that matter with me, identify the resources that may be available, and provide me with further information as to how to proceed.

APPROVED ON 4/17/2001

APPROVED UCHC IRB
VALID THRU 4/30/2001
Principal Investigator: Dr. Arthur Hand  
Pediatric Dentistry 1610

Co-Investigators: Maija Mednieks

Reference Number: 98-177

Progress Report Due: 2/28/01

Type of Review: Modification/Continuation Expedited

IRB Meeting Date: 4/17/00

Proposal: Secretory Protein Expression in Human Saliva

- Approved for continuation on 4/17/2000. Study determined to qualify for expedited review under the revised Expedited Federal Regulation (rev. 11/98) Title 45 Code of Federal Regulation Part 46, section 46.110(3). Please note that since this study had lapsed on December 31, 1999, you are not authorized to enroll any new subjects while a study is not approved. The IRB Office requests that future continuations be submitted in a timely fashion. The PI is required to use the attached IRB approved consent form valid through 4/30/2001.

- IRB approval is valid for one additional year through 4/30/01.

- With approval of a proposal, the committee requests that should any untoward effects occur, the committee be informed immediately. In addition, should there be any modifications or changes contemplated, it is necessary that the committee be informed in writing, for review and approval prior to their inception.

- It is the committees’ understanding that this proposal will be terminated as 4/30/01 unless the investigator notifies the committee that the study will be continued.

Health And Human Services Multiple Project Assurance Number 1345-02.

Scott H. Kurtzman, MD, Chair, Institutional Review Board

cc: Department Chair, Pediatric Dentistry  
 ORSP, MC 5355