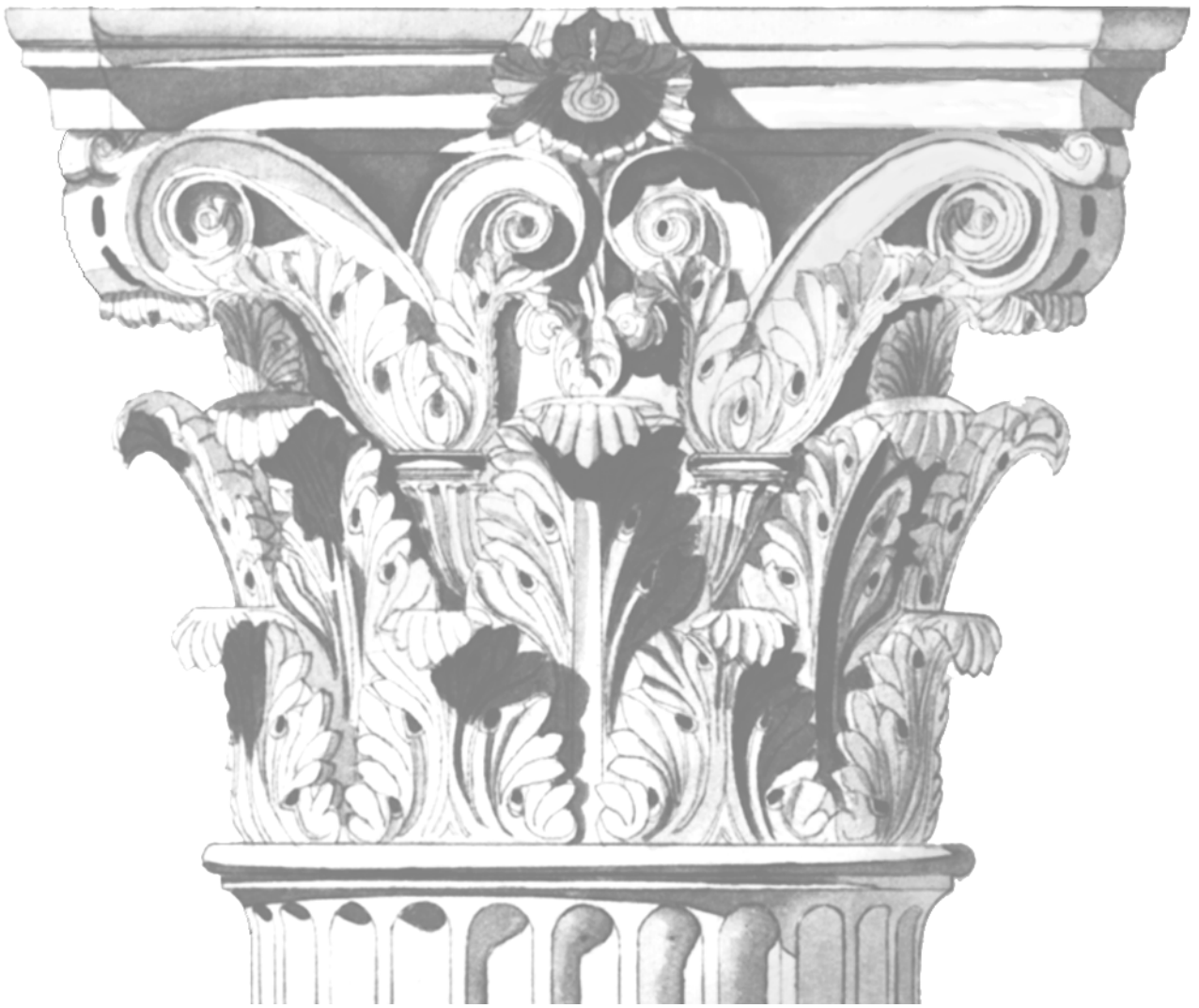
HUMAN SALIVARY CARBONIC ANHYDRASE ISOENZYME VI

Physiology and association with the experience of dental caries

JYRKI KIVELÄ

Department of Anatomy and Cell Biology

OULU 1999



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Physiology and association with the experience of dental caries

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Abstract

The carbonic anhydrases (CAs) participate in the maintenance of pH homeostasis in various tissues of the human body by catalyzing the reversible reaction $CO_2 + H_2O <=> HCO_3^- + H^+$. Carbonic anhydrase isoenzyme VI (CA VI) is secreted into the human saliva by the serous acinar cells of the parotid and submandibular glands. The present work was undertaken in order to gain an understanding of the physiological role of CA VI in the oral cavity.

CA VI concentrations were compared with other salivary characteristics and with the clinical dental status of the subjects. Saliva samples were collected under strictly controlled conditions from 209 young, healthy men and their CA VI concentrations determined by means of a specific time-resolved immunofluorometric assay. Salivary secretion rate, pH, buffering capacity, α-amylase activity level and counts of lactobacilli and mutans streptococci were also determined. Salivary CA VI concentrations showed positive correlations with salivary secretion rate ($r = 0.20$, $p = 0.003$) and amylase activity level ($r = 0.46$, $p < 0.001$), but not with pH, buffering capacity, or counts of mutans streptococci or lactobacilli. Salivary CA VI concentration, pH and buffering capacity correlated negatively with the number of decayed, missing or filled teeth (DMFT index). The correlation between salivary CA VI concentration and DMFT index was closest in the subjects with poor oral hygiene. No correlation was found between salivary secretion rate or amylase activity and the DMFT index.

The location of CA VI in the enamel pellicle, a thin layer of proteins on dental surfaces providing a protective interface between the tooth surface and the external environment, was demonstrated in samples of extracted teeth using immunostaining with anti-CA VI antibody. Immunostaining for salivary α-amylase, which was used as a positive control, produced virtually the same staining patterns. The presence of CA VI in the natural enamel pellicle was confirmed by Western blotting of pellicle proteins. Histochemical staining of enamel pellicle formed *in vitro* showed that the bound enzyme retains its CA activity.

To determine whether CA VI is transferred into the circulation, blood and saliva samples were collected from four healthy male volunteers at 3-h intervals throughout a 24-h period and assayed for CA VI concentration. CA VI was present in all the serum samples, although its concentration was about 22 times lower than in the saliva. The presence of CA VI in serum was confirmed using a sensitive Western blotting method. Western blotting also showed that serum CA VI is associated with IgG, which may protect the enzyme from proteolytic degradation or target it to sites that do not contain CA VI.

The present results suggest that salivary CA VI is not involved in regulation of the actual pH or buffering capacity of the saliva, but it does seem to have a specific role in the oral cavity. High salivary concentrations of CA VI appear to be associated with low caries experience. Since active CA VI is located in the enamel pellicle, it may function locally in the microenvironment of the dental surfaces and accelerate the neutralization of the acid metabolic products of bacterial plaque.

Keywords: buffering capacity, pH, secretion, serum

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Pähkinämäki, January 1999 Jyrki Kivelä

Abbreviations

BSA	bovine serum albumin
CA	carbonic anhydrase
CA2	carbonic anhydrase II gene
CA6	carbonic anhydrase VI gene
cAMP	cyclic adenosine 3',5' monophosphate
CAs	carbonic anhydrases
cDNA	complementary deoxyribonucleic acid
cfu	colony forming unit
CPI	community periodontal index
CV	coefficient of variation
Cys	cysteine
DAB	3,3'diaminobenzidine tetrahydrochloride
dd.	*dentes*
DELFIA	dissociation enhancement lanthanide fluorescence immunoassay
dH_2O	deionized water
DMFT	decayed, missing or filled teeth
ECL	enhanced chemiluminescence
GPI	glycosyl phosphatidylinositol
HCA	human carbonic anhydrase
HCA6	human carbonic anhydrase VI gene
IgA	immunoglobulin A
IgG	immunoglobulin G

IgM	immunoglobulin M
kDa	kiloDaltons
LB	lactobacillus, lactobacilli
mRNA	messenger ribonucleic acid
NRS	normal rabbit serum
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate-buffered saline
PBST	phosphate-buffered saline with Tween-20
P-type	pancreatic type
PVDF	polyvinylidene fluoride
SD	standard deviation
SDS	sodium dodecyl sulphate
sEGF	salivary epidermal growth factor
SEM	standard error of mean
sIgA	secretory immunoglobulin A
SM	mutans streptococci
S-type	salivary type
TBST	Tris-buffered saline with Tween-20
TR-IFMA	time-resolved immunofluorometric assay

List of original publications

This thesis is based on the following original articles, which are referred to in the text by Roman numerals I-IV:

I Kivelä J, Parkkila S, Metteri J, Parkkila A-K, Toivanen A & Rajaniemi H (1997) Salivary carbonic anhydrase VI concentration and its relation to basic characteristics of saliva in young men. Acta Physiol Scand 161:221-225.

II Kivelä J, Parkkila S, Waheed A, Parkkila A-K, Sly WS & Rajaniemi H (1997) Secretory carbonic anhydrase isoenzyme (CA VI) in human serum. Clin Chem 43:2318-2322.

III Kivelä J, Parkkila S, Parkkila A-K & Rajaniemi H (1999) A low concentration of carbonic anhydrase isoenzyme VI in whole saliva is associated with caries prevalence. Caries Res, in press.

IV Leinonen J, Kivelä J, Parkkila S, Parkkila A-K & Rajaniemi H (1999) Salivary carbonic anhydrase isoenzyme VI is located in the human enamel pellicle. Caries Res, in press.

Contents

1. Introduction

The carbonic anhydrases (CA; EC 4.2.1.1) are evolutionarily old enzymes, expressed in most organs of the human body (Maren 1967, Hewett-Emmet & Tashian 1991, Sly & Hu 1995a). They participate in a variety of physiological processes involving pH regulation, CO_2 and HCO_3^- transport, ion transport, and water and electrolyte balance (Tashian 1992, Sly & Hu 1995a). In the mammalian body, CAs maintain pH homeostasis in various tissues and biological fluids by catalyzing the reversible hydration of carbon dioxide, $CO_2 + H_2O <=> HCO_3^- + H^+$ (Maren 1967, Tashian 1989, Brown *et al.* 1990, Swenson 1991, Tashian 1992, Sly and Hu 1995b, Kaunisto *et al.* 1995, Parkkila S and Parkkila A-K 1996, Lai *et al.* 1998). Eight isoenzymes with CA activity have been identified in mammals to date, and all of them are expressed in the alimentary tract (Lönnerholm *et al*. 1985, Parkkila S *et al.* 1994, Fleming *et al*. 1995, Parkkila S & Parkkila A-K 1996, Pastoreková *et al*. 1997, Türeci *et al*. 1998). Two isoenzymes are known to be expressed in the salivary glands, cytoplasmic CA II, a high-activity isoenzyme that is not secreted into the saliva but may catalyze the production of salivary bicarbonate (Case *et al.* 1982, Parkkila S *et al.* 1990), and secretory CA VI, which is produced by the serous acinar cells of the parotid and submandibular glands (Fernley *et al*. 1979, Feldstein & Silverman 1984, Murakami & Sly 1987, Parkkila S *et al*. 1990, Ogawa *et al*. 1992, 1993, Parkkila S *et al*. 1994). The presence of CA activity in human saliva was reported almost 60 years ago (Becks & Wainwright 1939, Rapp 1946), but its physiological role has remained undefined. It has been proposed that CA VI may serve to regulate the pH in saliva by utilizing the bicarbonate provided by CA II in the major salivary glands (Feldstein & Silverman 1984, Kadoya *et al.* 1987, Sly & Hu 1995a). Salivary CA VI may also have a protective effect against excess acidity on the mucosa of the oesophagus and stomach (Rees & Turnberg 1982, Parkkila S *et al*. 1994, 1997).

Saliva is responsible for the maintenance of homeostasis on oral surfaces (Ericson & Mäkinen 1986, Mandel 1989, van Houte 1994), and its importance for dental health is demonstrated by the rampant caries seen in patients with grave salivary hypofunction (Dreizen & Brown 1976, Mandel 1989). Saliva contains inorganic compounds and multiple proteins that affect conditions in the oral cavity and on the tooth surfaces (Tenovuo 1989). One essential factor in maintaining oral homeostasis is salivary buffering capacity (Kleinberg & Jenkins 1964, Jensen 1986, Mandel 1987, van Houte 1994). Three major buffer systems contribute to the total buffering capacity of saliva: a

carbonic acid/bicarbonate system, a phosphate system, and a system based on proteins (Leung 1951, Lilienthal 1955, Leung 1961, Izutsu & Madden 1978, Helm *et al*. 1982, Ericson & Mäkinen 1986, Birkhed & Heintze 1989). The carbonic acid/bicarbonate system, based on the equilibrium $CO_2 + H_2O <=> HCO_3^- + H^+$, is physiologically the most important buffer system in the oral cavity (Leung 1951, Lilienthal 1955, Leung 1961, Izutsu 1981, Helm *et al*. 1982, Birkhed & Heintze 1989).

The human oral cavity, as the entrance to the alimentary canal, is exposed to widely varying conditions due to the differing physical and chemical properties of the ingested food and drinks. The metabolism of the abundant microbial flora also increases acidity on dental surfaces, mainly in the form of lactic, acetic, formic and propionic acids (Muntz 1943, Stephan 1944, Kleinberg 1970, Sandham & Kleinberg 1970, Geddes 1975, 1981, Abelson & Mandel 1981). Increased acidity on dental surfaces will lead to demineralization if the removal of excess acid is not rapid enough. Taken together, the mechanisms responsible for oral homeostasis, particularly pH homeostasis, are of vital importance to the protection of the oral cavity from pathological conditions. Knowing that CA isoenzymes serve to maintain pH homeostasis in various biological fluids and that CA VI is abundantly secreted into the saliva, we undertook this study to explore the possible physiological role of CA VI in the human oral cavity.

2. Review of the literature

2.1. Historical aspects

Saliva has been a disregarded substance throughout history, even though human life without saliva is a misery. Until the end of the 17th century, the salivary glands were regarded as emunctories, sieving excrementous substances from the blood, particularly the evil spirits of the brain (Garrett 1975), and up to the early 19th century physicians who practiced according to the premise that all diseases were due to disorders of the four "principal humours" (phlegm, blood, yellow bile and black bile) would prescribe doses of calomel, mercurous chloride, to cause massive salivation, probably to cleanse the system from noxious substances. Only the strongest survived both illness and cure, and preventive medicine meant keeping out of the hands of physicians. (Mandel 1987.) In the course of the present century salivary research has attracted an ever-increasing interest, and the importance of saliva for the physiology of the mouth and upper alimentary canal is now well established.

The first notion of the existence of carbonic anhydrases arose in the late 1920's, when a red cell substance catalyzing the reversible hydration of carbon dioxide, $CO_2 + H_2O <=> HCO_3^- + H^+$, was recognized in studies on the rate of escape of carbon dioxide from haemolyzed blood (Henriques 1928). A few years later the discharge of CO_2 from the lung capillaries and its uptake in tissues was found to proceed much more rapidly than was calculated from the rates of the non-catalyzed reactions, and the substance responsible for this was shown to be an enzyme and named carbonic anhydrase (Meldrum & Roughton 1932, Edsall 1968, Carter 1972). The enzyme was soon isolated and partially purified (Meldrum & Roughton 1933), and in 1939 it was found to have a molecular weight of about 30 kDa and to contain one zinc ion per molecule (Keilin & Mann 1939).

Although CA activity was likewise observed in human saliva for the first time almost 60 years ago (Becks & Wainwright 1939, Rapp 1946), only few studies have been carried out on the physiological role of salivary CA. In 1974, Szabó reported higher mean salivary CA activity levels in caries-free children than in children with active caries. Great advances were achieved in the late 1970's when Fernley *et al.* described a novel CA expressed in the ovine parotid gland (Fernley *et al.* 1979). Over the following

10 years, the enzyme was first purified from rat saliva by Feldstein and Silverman (1984) and later from human saliva by Murakami and Sly (1987) and Kadoya *et al.* (1987), and designated CA VI. In 1991, Aldred *et al.* cloned and characterized the cDNA encoding for HCA VI. The next major step in research into salivary CA was the development of specific immunofluorometric and radioimmunoassays for human salivary CA VI (Parkkila S *et al.* 1993b, Fernley *et al.* 1995), which allowed accurate quantification of CA VI in difficult matrices such as saliva and serum.

2.2. Carbonic anhydrases

2.2.1. General aspects

Eight isoenzymes with CA activity have been identified and characterized in mammals (CAs I-VI, IX, and XII). The cDNAs encoding for the known human isoenzymes have been cloned and characterized (Henderson *et al.* 1973, Lin & Deutsch 1973, Butterworth *et al.* 1991, Edwards 1991, Aldred *et al.* 1991, Okuyama *et al.* 1992, Nagao *et al.* 1993, Pastoreková *et al.* 1997, Türeci *et al.* 1998), and the crystallographic structures of isoenzymes CA I-V have been determined (Eriksson & Liljas 1991, Stams *et al.* 1996, Boriack-Sjödin *et al.* 1995). CAs are formed of a single polypeptide, and in the native form the molecule contains one tightly bound Zn^{2+}, which is essential for catalytic activity (Lindskog 1982). The refined structures of the cytoplasmic isoenzymes (CA I, II and III), as determined crystallographically, appear to be quite homologous (Eriksson & Liljas 1991), having a 10-stranded β sheet that bisects the molecule. The zinc ion is located near the centre of the molecule, at the bottom of a cavity 15 Å wide and 15 Å deep which forms the active site of the enzyme. Three histidine residues ligate the zinc ion to the β sheet structure, the fourth and fifth ligand sites of the zinc ion probably being occupied by a water molecule and a hydroxyl ion (Kannan *et al.* 1977).

The carbonic anhydrases catalyze the reversible hydration of carbon dioxide, $CO_2 + H_2O <=> HCO_3^- + H^+$, and participate in various biological processes involving the maintenance of pH homeostasis, CO_2 transfer and ion exchange (Tashian 1989, 1992). CAs can also act upon a large variety of other substances which undergo hydration of aldehydes (Pocker & Meany 1965, 1967) or hydrolysis of aromatic esters (Schneider & Liefländer 1963). The physiological significance of functions other than those associated with the interconversion of CO_2 and HCO_3^- has nevertheless remained undefined. CAs are found in almost all organisms, from algae and bacteria to mammals. In addition to this wide expression among different species, they are distinctive because of their great diversity in tissue distribution, cellular and subcellular location (Table 1), and in biological functions (Tashian 1989, 1992).

Table 1. Summary of the subcellular location of CA isoenzymes.

Isoenzyme	Subcellular location
CA I	cytoplasmic
CA II	cytoplasmic
CA III	cytoplasmic
CA IV	membrane-associated
CA V	mitochondrial
CA VI	secreted
CA IX	transmembrane protein
CA XII	transmembrane protein

2.2.2. Carbonic anhydrase isoenzyme II

CA II is one of the most efficient enzymes known, having a turnover number of 1.3-1.9 $\times 10^6$ /s under physiological conditions (Khalifah 1971, Sanyal & Maren 1981, Wistrand 1981). The human CA2 gene is 17 kb long and is located on chromosome 8, like the genes for CA I and III (Nakai *et al.* 1987, Tashian 1989). CA II is the most widely distributed isoenzyme of the CA gene family, being present in virtually every human tissue or organ (Tashian 1992). It was first found in erythrocytes, where it is abundantly expressed and is involved in the hydration of CO_2 (Meldrum & Roughton 1932, 1933, Wistrand 1981).

CA II is expressed in many organs of the digestive system (Parkkila S & Parkkila A-K 1996). It is located in the serous acinar cells of the parotid and submandibular glands and thought to release bicarbonate ions into the saliva (Parkkila S *et al.* 1990, 1991b, Ogawa *et al.* 1993, Parkkila S *et al.* 1994). The squamous epithelium of the oesophagus appears to contain CA II, and it may participate in endogenous bicarbonate production in the oesophagus (Meyers & Orlando 1992, Parkkila S *et al.* 1994). In the stomach, it is expressed in the parietal and surface epithelial cells of the gastric mucosa, where it regulates the acidity of the gastric juice (Davenport & Fisher 1938, Davenport 1939, O'Brien *et al.* 1977, Parkkila S *et al.* 1994, Parkkila S & Parkkila A-K 1996). The parietal cells of the gastric glands secrete protons to acidify the gastric juice (Sato *et al.* 1980, Kumpulainen 1981, Lönnerholm *et al.* 1985, Swenson 1991, Sasaki *et al.* 1993), and the gastroduodenal surface epithelial cells secrete bicarbonate below the mucous gel layer covering the epithelia to protect the epithelium from being itself digested (Richardson 1985, Allen & Carrol 1988, Swenson 1991, Parkkila S & Parkkila A-K 1996, Takeda *et al.* 1997). CA II has been found in the non-goblet epithelial cells of the mammalian colon (Lönnerholm *et al.* 1985, Parkkila S *et al.* 1994), in which it is implicated in the regulation of NaCl reabsorption (Binder *et al.* 1987, Goldfarb *et al.* 1988, Charney & Egnor 1989, Swenson 1991).

In the liver, CA II has been demonstrated in the hepatocytes and the epithelium of the bile ducts (Dodgson *et al.* 1984, Carter *et al.* 1989, Parkkila S *et al.* 1994), its best-

known physiological function being to produce HCO_3^- for alkalization of the bile (Swenson 1991). It is also expressed in the epithelial cells of the gallbladder, where it is involved in bile concentration and acidification (Juvonen *et al.* 1994, Parkkila S & Parkkila A-K 1996), and in the pancreas, where it is located in the epithelial duct cells. Its role in the secretion of bicarbonate into the pancreatic juice is well established (Kumpulainen & Jalovaara 1981, Spicer *et al.* 1982, Kumpulainen 1984, Swenson 1991).

CA II is well documented in renal tubular cells, where it contributes to urinary acidification (Wistrand 1980, Wåhlstrand & Wistrand 1980, Kumpulainen 1984, Sly & Hu 1995a,b, Lai *et al.* 1998), and it has an essential role in the bone resorption, to the extent that hereditary CA II deficiency causes osteopetrosis, renal tubular acidosis and cerebral calcification (Sly *et al.* 1983, 1985, Sly & Hu 1995a,b). CA II is expressed in the central nervous system and is involved in control of the production of cerebrospinal fluid (Maren 1967, Maren & Broder 1970, Parkkila A-K *et al.* 1994, 1995, 1997, Catala 1997). Recent immunohistochemical studies have shown that it is also expressed in some endocrine cells of the human pituitary and adrenal glands (Parkkila A-K *et al.* 1993, Sasano *et al.* 1994, Parkkila A-K *et al.* 1996). In addition to the locations listed above, CA II has been demonstrated in the type II pneumocytes of the lung (Fleming *et al.* 1994), various cells in the tissues of the male reproductive tract (Kaunisto *et al.* 1990, Parkkila S *et al.* 1991a, Kaunisto *et al.* 1995) and in the human placenta and foetal membranes (Mühlhauser *et al.* 1994).

2.2.3. Carbonic anhydrase isoenzyme VI

CA VI is the only known secreted isoenzyme of the CA gene family, and has several properties that distinguish it from the well-characterized cytoplasmic isoenzymes. Its reported molecular weight is 39-46 kDa (Feldstein & Silverman 1984, Kadoya *et al.* 1987, Murakami & Sly 1987, Fernley 1991a,b, Parkkila S *et al.* 1991b, Ogawa *et al.* 1992). The enzyme molecule has two N-linked oligosaccharide chains, which can be cleaved by endo-###-N-acetylglucosaminidase F but not by endo-###-N-acetylglucosaminidase H, indicating that the oligosaccharides are of a complex type (Murakami & Sly 1987). Neuraminidase has no effect on the endo-###-N-acetylglucosaminidase F-digested protein, suggesting that HCA VI has no O-linked oligosaccharide which contains neuraminidase-sensitive sialic acid residues (Murakami & Sly 1987). The complete amino acid sequence of ovine CA VI was determined by Fernley *et al.* (1988), and the complete nucleotide sequence for human CA6 cDNA by Aldred *et al.* (1991). The HCA6 gene is located on chromosome 1 (Sutherland *et al.* 1989, Aldred *et al.* 1991). The HCA VI protein has a sequence identity of 35 % to HCA II, while residues involved at the active site of the enzyme are conserved. HCA VI has three potential N-glycosylation sites and two cysteine residues (Cys25 and Cys207) (Aldred *et al.* 1991), the latter presumably forming a disulphide bond, as in the ovine enzyme (Fernley *et al.* 1988).

Immunohistochemical studies have demonstrated the expression of CA VI exclusively in the acinar cells of the mammalian parotid and submandibular glands

(Kadoya *et al.* 1987, Parkkila S *et al.* 1991b, Ogawa *et al.* 1992), where it is secreted into the saliva. Salivary concentrations of ovine and human CA VI have been investigated using a radioimmunoassay (Fernley *et al.* 1991, 1995) and a time-resolved immunofluorometric assay (TR-IFMA) (Parkkila S *et al.* 1993b, 1995). Radioimmunoassay of ovine CA VI showed that its mean ± SD concentration in sheep parotid saliva is 5.61 ± 3.01 mg/l in the normal conscious animal, while feeding increased the concentration to 33.0 ± 19.0 mg/l (Fernley *et al.* 1991, Fernley 1991a). Nerve stimulation and cholinergic drug administration have been used to demonstrate that both parasympathetic and sympathetic pathways may control the CA VI concentration in the sheep saliva (Fernley *et al.* 1991). Radioimmunoassay methods have shown the mean ± SD concentration of CA VI in human parotid saliva to be 47.0 ± 39.2 mg/l, in which case it represented about 3 % of total protein in the parotid saliva (Fernley *et al.* 1995). The mean ± SD concentration of CA VI in human whole saliva, measured by TR-IFMA, was 6.8 ± 4.3 mg/l (Parkkila S *et al.* 1993b). In addition, secretion of CA VI into the saliva was observed to follow a circadian periodicity, its concentrations being very low during sleep and rising rapidly to the daytime level after awakening and breakfast (Parkkila S *et al.* 1995).

The physiological function of CA VI has remained undefined, but it is thought to have a specialized role in the maintenance of bicarbonate levels in the saliva (Feldstein & Silverman 1984, Kadoya *et al.* 1987, Fernley 1988, Sly & Hu 1995a). This suggestion is in line with an earlier finding that children suffering from caries have lower salivary CA activity compared to caries-free children (Szabó 1974). A new insight into CA VI research has been provided by the recent observations that CA VI probably maintains its activity in the harsh environment of the gastric lumen and that patients with verified oesophagitis or oesophageal, gastric or duodenal ulcers have reduced salivary CA VI concentrations relative to patients with a non-acid peptic disease (Parkkila S *et al.* 1997). Parkkila S *et al.* (1990, 1994, 1997) have proposed that CA VI and CA II may form a mutually complementary system for the regulation of pH homeostasis on the epithelial surfaces of the upper alimentary canal.

CA VI also appears to be involved in the physiology of taste function. In a recent study Thatcher *et al.* (1998) identified gustin, a salivary protein associated with the function of taste buds, as human CA VI by protein sequencing, activity profiles and other physical data.

2.2.4. Other carbonic anhydrase isoenzymes

CA I is a well characterized cytoplasmic enzyme with a molecular weight of about 30 kDa (Bundy 1977, Lindskog *et al.* 1984). It is a low-activity isoenzyme expressed in mammalian erythrocytes, the epithelium of the colon, the capillary endothelium, the corneal endothelium, the lens of the eye, the islets of Langerhans, the placenta and foetal membranes (Lönnerholm *et al.* 1985, Venta *et al.* 1987, Sasaki *et al.* 1993, Parkkila S *et al.* 1994, Mühlhauser *et al.* 1994). A striking feature of this isoenzyme is that although it is one of the most abundant proteins in mammalian red cells, no haematological abnormalities have emerged in its absence as the result of a mutation, and it is not

expressed in the red cells of certain species, e.g., ruminants and felids, making the assignment of a physiological role in erythrocytes problematic (Tashian *et al.* 1971, Kendall & Tashian 1977, Tashian *et al.* 1980, Tashian 1992).

CA III is a cytoplasmic, very low-activity isoenzyme expressed primarily in the type I fibres of the skeletal muscle (Tipler *et al.* 1978, Holmes 1976), where its exact physiological function has remained undefined. Smaller amounts of CA III have been detected in the human uterus, urine bladder, lung, cardiac muscle (Jeffery *et al.* 1980), human myoepithelial cells (Väänänen & Autio-Harmainen 1987), equine thymus (Nishita & Matsushita 1989), guinea pig salivary glands and mouse colon (Spicer *et al.* 1990). CA III is the second isoenzyme to be demonstrated in the rodent liver (Carter & Jeffery 1985, Spicer *et al.* 1990), but only traces of this hormonally regulated CA isoenzyme have been found in the adult human liver (Jeffery *et al.* 1980, Carter *et al.* 1984). The presence of CA III in hepatocytes has aroused interest in its specific function. Cabiscol & Levine (1995) have demonstrated that it functions in an oxidizing environment and that it is the most oxidatively modified protein in the liver known so far.

CA IV was the first membrane-associated CA isoenzyme to be described. It is thought to facilitate the reversible hydration of CO_2 at sites where CO_2 and HCO_3^- flux across membranes needs to be very rapid (Sly & Hu 1995a, Parkkila S & Parkkila A-K 1996). HCA IV contains a 27-amino acid COOH-terminal extension which may serve as a recognition signal for cleavage and transfer to a glycosyl phosphatidylinositol (GPI) link, through which CA IV molecules are anchored to the plasma membrane (Zhu & Sly 1990, Ghandour *et al.* 1992, Waheed *et al.* 1992a, Sly & Hu 1995a, Parkkila S & Parkkila A-K 1996). CA IV has been purified from bovine lung microsomes (Whitney & Briggle 1982) and human kidney (Wistrand & Knuuttila 1989), and in a catalytically active form from human lung and kidney (Zhu & Sly 1990) and rat lung (Waheed *et al.* 1992a). It has been found to function in the plasma membranes of the proximal convoluted tubule and thick ascending limb of Henle in the rat kidney (Brown *et al.* 1990, Rector *et al.* 1998), and it is present in the endothelial cells of the choriocapillaries of the human eye (Hageman *et al.* 1991) and in the endothelial cells of rat brain capillaries (Ghandour *et al.* 1992). CA IV is expressed on the apical surface of epithelial cells in the colon (Fleming *et al.* 1995), in the luminal plasma membrane of the human gallbladder epithelium (Parkkila S *et al.* 1996), and on the plasma face of endothelial cells of the pulmonary microvasculature (Fleming *et al.* 1993). Waheed *et al.* (1992b) have demonstrated that the membrane-associated CA in skeletal muscle is CA IV. This same isoenzyme is also expressed on the apical surface of the epididymal duct, where it is known to play a major role in the acidification of the epididymal fluid (Parkkila S *et al.* 1993a). It has been demonstrated that the expression of CA IV in the epididymis is regulated by androgens and oestrogen (Caflisch 1990, Caflisch & DuBose 1990, Kaunisto 1998). The gene for CA IV is located on chromosome 17 (Okuyama *et al.* 1992, 1993).

CA V is the mitochondrial CA isoenzyme, uniquely located in the mitochondrial matrix. Its presence has been established in mammalian liver and pancreas (Nagao *et al.* 1993, Sly & Hu 1995a, Parkkila A-K *et al.* 1998). The cDNA for human mitochondrial CA V has been cloned from a human liver cDNA library, and its gene has been localized to chromosome 16 (Nagao *et al.* 1993). CA V is thought to participate in two metabolic

processes in the mitochondria of hepatocytes: ureagenesis and gluconeogenesis, supplying bicarbonate for the first urea cycle enzyme, carbamyl phosphate synthetase I in ureagenesis and for pyruvate carboxylase in gluconeogenesis (Dodgson 1991). CA inhibitors have been observed to retard both of these processes in the livers of guinea pigs and rats (Dodgson *et al.* 1983, Metcalfe *et al.* 1985, Dodgson 1991). The expression of CA V in pancreatic β-cells and the observation that the CA inhibitor acetazolamide inhibits glucose-stimulated insulin secretion have led to a proposal that CA V may have a role in the regulation of insulin secretion (Parkkila A-K *et al.* 1998).

CA VII is a putative isoenzyme, the gene for which has been isolated from a human genomic library (Montgomery *et al.* 1991, Tashian 1992, Sly & Hu 1995a). The gene is about 10 kb long and located on chromosome 16, and the predicted amino acid frequency shows 50, 56, 49, and 37 % homology with human CA I, II, III, and VI, respectively.

CA IX is an integral transmembrane isoenzyme expressed in the human gastric mucosa, the cryptal enterocytes of the duodenum and jejunum, the gallbladder mucosa and the bile ducts (Pastorek *et al.* 1994, Pastoreková *et al.* 1997, Saarnio *et al.* 1998b). CA IX has also been found in human tumours derived from cervix uteri, kidney, colon, oesophagus and other organs (Závada *et al.* 1993, Liao *et al.* 1994, 1997, McKiernan *et al.* 1997, Turner *et al.* 1997, Saarnio *et al.* 1998a). Its subcellular location in the basolateral membranes of cells and its intense expression in proliferative cells have led to the suggestion that it may be involved in intercellular communication and/or cell proliferation (Pastoreková *et al.* 1997, Saarnio *et al.* 1998b). The active site domain of the CAs is completely conserved in CA IX, suggesting that it could also participate in carbon dioxide/bicarbonate homeostasis (Opavský *et al.* 1996).

CA XII was the second transmembrane CA isoenzyme to be described, having recently been identified in a human renal cell carcinoma. Northern blot analysis of normal tissues has demonstrated CA XII mRNA only in the kidney and intestine. Türeci *et al.* (1998) have shown that in 10 % of patients with renal cell carcinoma the CA XII transcript was expressed at higher levels in the tumour than in the surrounding normal kidney tissue, suggesting that it is the second catalytically active membrane-associated CA isoenzyme that is overexpressed in certain cancers. The cDNA encoding for CA XII has been cloned and characterized, and its gene has been mapped to chromosome 15. (Türeci *et al.* 1998.)

2.3. Salivary homeostasis

2.3.1. Composition and secretion of whole saliva

Whole saliva is a mixture of the secretions of the parotid, submandibular, sublingual and minor salivary glands and gingival crevicular fluid. An outline of the secretions of the individual salivary glands is shown in Table 2. Saliva contains inorganic compounds and multiple proteins that affect conditions in the oral cavity and locally on the tooth surfaces. It is involved in the clearance of food debris and provides inorganic ions for the

neutralization of the acid and alkaline metabolic products of oral bacteria and for the remineralization of the enamel. It also brings various defence mechanisms, including leukocytes, secretory IgA (sIgA), agglutinating proteins and a number of enzymes, to the actual sites of microbial growth on the tooth and mucosal surfaces (Tenovuo 1989, Lamkin and Oppenheim 1993, Johnsson *et al.* 1993, Lagerlöf and Oliveby 1994, Edgar *et al.* 1994, Wolinsky 1994).

Table 2. Types of secretion produced by the salivary glands (Ross et al. *1989).*

	Secretion type	
Salivary glands	serous	mucous
Parotid	+++	–
Submandibular	++	+
Sublingual	+	++
Minor	–(+)	+++

In addition to the secretions from the salivary glands, several other factors, including oral bacteria, desquamated epithelial cells, crevicular fluid and leukocytes, contribute to the composition of whole saliva (Söderling 1989). The majority of the salivary IgG is passively diffused from the serum, mainly through the gingival crevices (Challacombe *et al*. 1978, Grönblad & Lindholm 1987), while many innate proteins, e.g. lysozyme, lactoferrin and myeloperoxidase, are partly derived from degenerating leukocytes migrating into the oral cavity again predominantly via the gingival crevices (Schiött & Löe 1970, Raeste 1972, 1976, Bennett & Kokocinski 1978, Friedman *et al.* 1983, Kowolik & Grant 1983). Furthermore, monocytes and macrophages actively secrete lysozyme, which may be blended with saliva via the crevicular fluid (Nord *et al.* 1971, Gordon *et al.* 1974, Bennett & Kokocinski 1978).

Salivary secretion can be assessed under resting and stimulated conditions, and a significant correlation has been reported between the flow rates of chewing-stimulated and unstimulated saliva (White 1977, Heintze *et al.* 1983). Normal values for the salivary secretion rate stimulated by chewing paraffin wax have been estimated to be 1-3 ml/min, and values below 0.7 ml/min have been considered to indicate hyposalivation. For resting salivary flow, these values are 0.25-0.35 ml/min and < 0.1 ml/min, respectively. (Ericsson & Hardwick 1978, Lagerlöf & Tenovuo 1994.) Stimulated salivary flow rate is slightly higher in men than in women, probably due to the larger size of the salivary glands in men (Parvinen & Larmas 1982). Ageing does not seem to affect the flow rate of stimulated whole saliva in healthy, unmedicated men, whereas a postmenopausal decline can often be observed in women (Kullander & Sonesson 1965, Baum 1981, Parvinen & Larmas 1982, Heintze *et al.* 1983). (Tenovuo 1992.) The concentrations of most salivary components depend on the secretion rate. At increased salivary secretion rates the concentrations of sodium, calcium, chloride and bicarbonate increase while potassium and fluoride concentrations remain unchanged and phosphate and iodide concentrations decrease (Dawes 1969, Shannon 1973, Dawes 1974, Ferguson

1989). The total protein concentration in saliva also increases with increased flow rate (Dawes 1969, Shannon 1973). Salivary immunoglobulins show deviating responses to stimulation (Söderling 1989), IgA correlating negatively with salivary secretion rate whereas IgG has been reported to be fairly independent of stimulation (Grönblad 1982, Brandtzaeg 1989).

Salivation is initiated by the salivary centres in the medulla oblongata, which receive afferent signals from the sensory terminals of the oral and nasal cavities and from the higher centres in the brain (Garrett 1987). The secretion of saliva is regulated by the autonomic nervous system (Asking & Gjörstrup 1980, Helm *et al*. 1982, Garrett 1987, Olsen *et al*. 1988, Calvert *et al*. 1998), and its composition follows circadian rhythms (Dawes 1972, 1975, Ferguson & Botchway 1979, Parkkila S *et al*. 1995). Water and electrolyte secretion are mainly controlled by parasympathetic activity, whereas protein synthesis and exocytosis are mainly controlled by sympathetic activity (Garrett 1987, Jensen *et al*. 1991, Nederfors & Dahlöf 1992, Nederfors *et al*. 1994, Nederfors & Dahlöf 1996). Two basic stimulus-response coupling pathways are thought to be involved in the secretion of saliva (Putney 1986). The Ca^{2+} pathway regulates ion and water flux, and to some extent protein secretion, while the other pathway, involving cyclic adenosine 3',5' monophosphate (cAMP), controls primarily enzyme secretion (Putney 1986). β-adrenoceptor activation has been shown to stimulate the exocytotic discharge of α-amylase in the rat parotid gland by a mechanism in which cAMP functions as a messenger (Rasmussen & Tenenhouse 1968, Schramm & Selinger 1975).

2.3.2. Salivary buffering capacity and pH

Salivary buffering capacity is a factor of primary importance in maintaining oral homeostasis (Kleinberg & Jenkins 1964, Jensen 1986, Mandel 1987, Birkhed & Heintze 1989). The main buffer systems known to contribute to the total buffering capacity of saliva are the bicarbonate and phosphate systems and those based on proteins (Leung 1951, Lilienthal 1955, Leung 1961, Izutsu & Madden 1978, Helm *et al*. 1982, Ericson & Mäkinen 1986, Mandel 1987). These systems have different pH ranges of maximal buffering capacity, the phosphate and bicarbonate systems having pK values of 6.8-7.0 and 6.1-6.3, respectively, whereas the proteins contribute to the salivary buffering capacity at very low pH values only (Ericson & Mäkinen 1986). Most of the salivary buffering capacity operative during food intake and mastication is due to the bicarbonate system, which is based on the equilibrium $CO_2 + H_2O <=> HCO_3^- + H^+$ (Leung 1951, Ericsson 1959, Lilienthal 1955, Leung 1961, Izutsu 1981, Helm *et al*. 1982, Birkhed & Heintze 1989, Lagerlöf & Oliveby 1994). The concentration of bicarbonate in the saliva is greatly increased at increased flow rates (Dawes 1969, Shannon 1973, Dawes 1974, Abelson & Mandel 1981, Ferguson 1989, Söderling 1989). Another essential feature of this buffer system under the conditions prevailing in the oral cavity is the phase conversion of carbon dioxide from a dissolved state into a volatile gas. When acid is added, this phase conversion considerably increases the efficacy of the neutralization reaction, as there is no accumulation of the end products but complete removal of the

acid (Birkhed & Heintze 1989), a phenomenon referred to as "phase buffering" (Ericson & Mäkinen 1986, Birkhed & Heintze 1989).

Phosphate makes a minor contribution to the total salivary buffering capacity relative to bicarbonate. Its system is in principle analogous to that of bicarbonate but without the important phase buffering effect. Within the pH range of the oral cavity, the phosphate buffer is based on the reversible reaction $H_2PO_4^- <=> HPO_4^{2-} + H^+$ (Ericson & Mäkinen 1986). The concentration of HPO_4^{2-} in saliva is relatively independent of the salivary secretion rate, and thus the capacity of the phosphate buffering system does not increase during food intake or mastication.

Evaluation of the salivary buffering effect based on proteins has produced controversial results, but in general the effect has been regarded as insignificant, or at least of minor importance (Lilienthal 1955, Ericson & Mäkinen 1986, Birkhed & Heintze 1989), although data suggesting a deviating conclusion have also been presented (Leung 1961, Izutsu & Madden 1978).

2.3.3. Role of saliva in protecting the epithelial surfaces of the upper alimentary canal

The epithelia of the human oral cavity and oesophagus are exposed to widely varying conditions due to the differing physical and chemical properties of the ingested food, in addition to which the epithelium of the oesophagus is challenged by acid reflux from the stomach. The saliva is responsible for the luminal defence of the epithelia of the upper alimentary canal. A number of salivary proteins are known to bind to the epithelial surfaces of the oral cavity, including salivary mucins, amylase, salivary cystatins and acidic proline-rich proteins (Bradway *et al.* 1989, 1992). This epithelial pellicle provides a lubricatory film and an effective barrier against desiccation and environmental factors, and it is also thought to protect the epithelial cells from proteases emanating from bacteria attached to the mucosal surfaces and from degenerating polymorphonuclear leukocytes (Mandel 1987, 1989, Vaahtoniemi *et al.* 1992).

The role of immunoglobulins in saliva is far from clear. Evidence has been presented for binding of salivary immunoglobulins IgA and IgM to the mucosal epithelial cells (Boackle & Suddick 1980). sIgA is the primary immunoglobulin in saliva, and it may promote microbial aggregation (Liljemark *et al.* 1979, Cohen & Levine 1989), while specific sIgA antibodies may inhibit microbial enzymes involved in colonization or pathogenic processes (Tomasi 1983, Gregory *et al.* 1990, Rudney 1995).

It has been suggested that salivary epidermal growth factor (sEGF) and prostaglandin E2 may play essential roles in maintaining the integrity of the mucosa of the upper alimentary canal (Li *et al.* 1993, Rourk *et al.* 1994, Wu-Wang *et al.* 1995, Yang *et al.* 1996, Sarosiek *et al.* 1996, Namiot *et al.* 1997), and even that the influence of sEGF may extend to the mucosa of the ileum (Rao *et al.* 1997).

Salivary bicarbonate secretion is known to be of vital importance for the maintenance of oesophageal pH homeostasis (Rees & Turnberg 1982, Helm *et al.* 1982, 1984, Sarosiek & McCallum 1995, Sarosiek *et al.* 1996), and recent findings suggest that CAs are also involved in this process. It has been proposed that salivary CA VI catalyzes the neutralization of excess acid in the mucous layer covering the oesophageal and gastric

epithelial cells (Parkkila S & Parkkila A-K 1996, Parkkila S *et al.* 1997). Three cytosolic isoenzymes (CA I, II, and III) and one membrane bound isoenzyme (CA IV) have been identified in human oesophageal epithelial cells, where they probably play a significant part in protecting the oesophageal mucosa from acid injury (Christie *et al.* 1997).

2.3.4. Role of saliva in protecting the dental hard tissues

Dental enamel is the hardest tissue in the human body, and the main challenge to it comes from acidic conditions in the oral cavity, which can cause dissolving of the mineral contained in it, i.e. dental caries or erosion. The metabolism of the microbial flora on the dental surfaces produces considerable amounts of acid, mainly in the form of lactic, acetic, formic and propionic acids (Clarke 1924, Muntz 1943, Stephan 1944, Fitzgerald & Keyes 1960, Kleinberg 1970, Sandham & Kleinberg 1970, Geddes 1975, Geddes 1981). Moreover, various foods and drinks add to the acid charge on these surfaces. Saliva can be considered the oral tissue fluid of the enamel, and the maintenance of homeostasis on the dental surfaces is totally dependent on salivary factors, including inorganic compounds and multiple proteins (Kleinberg & Jenkins 1964, Abelson & Mandel 1981, Jensen 1986, Ericson & Mäkinen 1986, Mandel 1987, 1989, Mäkinen 1989, Tenovuo 1989, van Houte 1994, Edgar & Higham 1995, Hall *et al.* 1997). The importance of saliva for dental health is demonstrated by the rampant caries seen in patients with grave salivary hypofunction (Dreizen & Brown 1976, Birkhed & Heintze 1989, Mandel 1989, Peeters *et al.* 1998). Saliva is involved in the clearance of food debris, detached epithelial cells and microbes. It also provides inorganic ions for the neutralization of acid microbial metabolic products and for remineralization of the enamel, and brings various defence mechanisms, including bicarbonate ions, leukocytes, sIgA, agglutinating proteins and a number of enzymes, to the actual sites of microbial adherence and growth on the tooth surfaces (Edgar 1976, Boackle & Suddick 1980, Cohen & Levine 1989, Hay & Moreno 1989, Lamkin & Oppenheim 1993, Johnsson *et al.* 1993, Lagerlöf & Oliveby 1994, Edgar *et al.* 1994, Wolinsky 1994). Despite numerous clinical studies, no distinct correlations have been found between the concentrations of any particular salivary proteins, alone or in combination, and the prevalence of caries (Gråhn *et al.* 1988, Rudney 1995, Kirstilä *et al.* 1998, Dodds *et al.* 1997). (Tenovuo 1989.)

2.4. The enamel pellicle

The enamel pellicle is a thin layer of proteins covering the enamel, the formation of which is initiated by the adsorption of specific salivary proteins to the hydroxyapatite surface (Kousvelari *et al.* 1980, Al-Hashimi & Levine 1989, Cohen & Levine 1989, Lamkin & Oppenheim 1993, Lamkin *et al.* 1996). This adsorption is assumed to be dependent on the chemical characteristics of the surface as well as the properties of the particular proteins (Moreno *et al.* 1984, Al- Hashimi & Levine 1989, Rykke *et al.* 1990,

Jensen *et al.* 1992, Raj *et al.* 1992, Johnsson *et al.* 1993, Lamkin & Oppenheim 1993, Skjorland *et al.* 1995, Lamkin *et al.* 1996). The accumulation of additional organic material on the initially formed basal layer is thought to constitute the subsequent phase of pellicle development (Lamkin *et al.* 1996, Hannig 1997).

The enamel pellicle evidently prevents demineralization of the surface hydroxyapatite, increasing its acid resistance, although the mechanisms responsible for this effect have remained elusive (Zahradnik *et al.* 1976, 1977, 1978, Kousvelari *et al.* 1980, Meurman & Frank 1991, Featherstone *et al.* 1993, Wolinsky 1994). It is also thought to prevent calculus formation on the dental surfaces by controlling the precipitation of calcium phosphate from supersaturated saliva (Hay & Moreno 1989).

3. Aims of the research

The overall aim of this research was to elucidate the physiological role of salivary CA VI in the oral cavity. The specific goals were:

- to examine the possible correlations between the CA VI concentration and some basic salivary characteristics,
- to examine the influence of smoking habits on salivary CA VI concentration,
- to investigate the possible correlation between salivary CA VI concentration and caries experience,
- to determine whether CA VI binds to the enamel pellicle, and
- to find out whether CA VI is transferred into the blood circulation.

4. Materials and methods

4.1. Subjects (I, III)

The voluntary subjects were selected at a health examination for conscripts at Parolannummi Garrison Hospital in January 1996. All the subjects were in good health and were not taking any medication. A conscious effort was made to select cases in which the subjects had adequate oral hygiene with average or poor dental status, or poor oral hygiene with comparatively good dental status. The final group of subjects consisted of 209 healthy men ranging from 18 to 24 years of age (mean 19.8 years).

Smoking habits were ascertained on a 5-grade scale: non-smoker, less than 5 cigarettes per day, 5-9 cigarettes per day, 10-20 cigarettes per day, and more than 20 cigarettes per day.

The research was approved by the ethical committee of the Finnish Defence Forces, and informed consent was obtained from each subject.

4.2. Clinical examination (III)

The clinical examinations were carried out by the author. The cariological status of each subject was recorded in terms of the DMFT index (number of decayed, missing or filled teeth: Klein *et al.* 1938, Burt 1981). All the permanent teeth were examined. Only cavitated lesions were included in the D component. Teeth filled because of fractures, unerupted teeth and teeth extracted for orthodontic reasons were excluded from the DMFT index. The robust DMFT index was considered appropriate for the measurement of total lifetime caries experience in this cross-sectional study. It is relatively insensitive to the influence of behavioural differences in seeking dental treatment and to possible differences in the caries therapy received. Periodontal status was recorded in terms of the Community Periodontal Index (CPI), in which each dental sextant (*dd.* 18-14, 13-23, 24-28, 38-34, 33-43 and 44-48) is examined for gingival bleeding and calculus (Ainamo *et al.* 1982). The code CPI = 0 indicates a healthy periodontium, and CPI = 1 and CPI = 2 indicate gingival bleeding and calculus, respectively, in at least one sextant.

4.3. Collection of saliva samples (I, III)

The saliva samples were collected from groups of 15-20 volunteers per morning. The subjects did not eat or smoke during the 8-h period before sampling. The first sample was collected 30 min after awakening, between 6.00 and 6.30. Saliva secretion was stimulated by chewing paraffin wax for seven minutes. During the first two minutes the saliva was swallowed, and the rest was collected by spitting into 10-ml tubes containing 200 µl of 0.2-M benzamidine in dH_2O to prevent proteolysis, and subjected immediately to microbial tests and measurement of the secretion rate, pH and buffering capacity. After having breakfast, the subjects returned to the Garrison Hospital, and another saliva sample was collected in a similar fashion as the first, between 7.00 and 7.30. These samples were subjected to the same tests as the first ones, except for the microbial tests.

4.4. Microbial tests (III)

The Dentocult LB test was used to obtain a salivary lactobacillus count. A high LB count reflects sugar in the diet and acidic, cariogenic conditions in the mouth (Larmas 1975, 1985, Bratthall & Carlsson 1989). The Dentocult SM Strip mutans test was used to assess mutans streptococci in the saliva (Jensen & Bratthall 1989, Bratthall & Carlsson 1989), providing an estimate of the intensity of cariogenic infection on the dental surfaces (Clarke 1924, Fitzgerald & Keyes 1960, van Houte & Green 1974, Schaeken *et al*. 1987, Lindquist *et al*. 1989). The LB and SM tests were performed and the values expressed according to the manufacturer's instructions (Orion Diagnostica, Espoo, Finland).

4.5. Tests for salivary pH, buffering capacity and secretion rate (I, III)

Immediately after sampling, salivary secretion rate in ml/min was calculated, and pH was measured using a pH meter (Schott model CG 837). Buffering capacity was determined by a slight modification of the method of Ericsson (1959). In brief, 1 ml of fresh saliva was added to a tube containing 3 ml of 5-mM HCl, shaken, and measured for pH. After these tests, the saliva samples were frozen and stored at -20°C until the enzyme assays were performed.

4.6. Tissue samples and collection of the enamel pellicle (IV)

To investigate the presence of CA VI in the enamel pellicle, permanent non-carious human teeth were obtained from the Department of Oral Surgery, University of Oulu, and Parolannummi Garrison Hospital. These had been extracted on orthodontic

indications and stored in sterile saline at 4°C until used for the experiments. The teeth were cut using the Exakt Standard Cutting Grinding System (Exakt, Norderstedt, Germany). Prior to extraction, the teeth examined for *in vivo*-formed enamel pellicle had been polished with pumice and the pellicle allowed to reform for 2 h, during which time no ingestion of food or liquids (except water) had been permitted.

Samples of the enamel pellicle were collected from two volunteers into Eppendorf tubes containing 300 µl of 0.01-M EDTA, pH 7.5, and 3 µl of 0.2-M benzamidine. The samples were stored at -80°C until used. Paraffin-stimulated saliva samples for immunoblotting were collected from the same two volunteers, centrifuged at 16,000 × *g* for 10 min at room temperature and the supernatants stored at -80°C until used for electrophoresis.

4.7. Collection of serum (II)

To find out whether CA VI is transferred into the circulation, saliva and serum samples were collected from four healthy male volunteers at 3-h intervals throughout a 24-h period. The protocol involved meals at 9.00, 12.30 and 18.30, and sleep from 0.10 to 9.00. During sleep, the subjects were woken up only for collection of the samples, still at 3-h intervals. The saliva samples were collected, frozen and stored as described in section 4.3. After collecting the saliva samples, 1-ml blood samples were obtained through an intravenous catheter from the cephalic vein in the cubital fossa. These samples were centrifuged at 2000 × *g* for 20 min, after which the serum was frozen and stored at -20°C until assayed.

The samples were collected after informed consent, and all procedures were in accordance with the Helsinki Declaration of 1975 (as revised in 1983).

4.8. Purification of CA VI. Antisera and immunoreagents (I-IV)

The affinity chromatography purification of human CA VI and production of a rabbit anti-CA VI serum are described by Parkkila S *et al*. (1990, 1991).

Reagents for sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transblotting were obtained from Bio-Rad Laboratories (Richmond, CA). For the immunoblots of saliva and pellicle proteins (IV), polyclonal rabbit antibody to human salivary α-amylase, purified human salivary α-amylase, biotin-conjugated swine anti-rabbit IgG and peroxidase-conjugated streptavidin were purchased from Sigma Chemical Company (St. Louis, MO), and peroxidase-conjugated donkey anti-rabbit IgG from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Purified human IgG was obtained from the Sigma Chemical Company (St. Louis, MO) (II).

4.9. SDS-PAGE and Western blots (II, IV)

To confirm the presence of CA VI in the *in vivo*-formed enamel pellicle (IV), samples of saliva and pellicle protein were subjected to SDS-PAGE under non-reducing conditions using 4 % concentrating and 10 % separating polyacrylamide gels (Laemmli 1970). Parallel experiments were performed for α-amylase, as a positive control. The separated proteins were transferred to nitrocellulose sheets as described earlier (Parkkila S *et al*. 1990), and these were briefly washed with 0.1 % Tween-20 in phosphate-buffered saline (PBST) and blocked with 20 % goat colostrum in PBST for 16 h at 4°C. After washing in PBST, the sheets were incubated with the primary antibody for 3 h at room temperature. Anti-CA VI serum and normal rabbit serum (NRS) were both diluted 1:2500 in 3 % bovine serum albumin (BSA) in PBST, and antibody to salivary α-amylase 1:15,000. The sheets were then washed extensively with PBST, treated again with 20 % goat colostrum in PBST for 1 h at room temperature, washed briefly with PBST, and incubated with peroxidase-conjugated anti-rabbit IgG diluted 1:20,000 for 1 h at room temperature. After extensive washing of the sheets in PBST, the bands were visualized by the enhanced chemiluminescence (ECL) method (Amersham International plc, Aylesbury, UK) according to the manufacturer's instructions.

To confirm the presence of CA VI in serum (II), aliquots of 5 µl of saliva, 0.5 µl of serum and 0.5 µg of purified human IgG were subjected to SDS-PAGE under non-reducing or reducing conditions. The electrophoreses were performed according to Laemmli (1970), using 4 % concentrating and 9 % separating polyacrylamide gels. The proteins were electrophoretically transferred from the gel onto a polyvinylidene fluoride (PVDF) membrane (Millipore Corporation, Bedford, MA) at a constant voltage of 30 V for 1.5 h in a Xcell II Mini-Cell apparatus (Novex, San Diego, CA). After the transblotting the PVDF membranes were stained with Coomassie brilliant blue R-250 and the lanes containing the molecular weight standards were cut out. The sample lanes were destained in methanol, rinsed in TBST buffer containing 10-mM Tris-HCl, pH 7.5, 150-mM NaCl and 0.05 % Tween 20 and incubated for 30 min with 1:10 diluted cow colostrum in TBST buffer. The sheets were then incubated for 1 h with anti-CA VI serum or normal rabbit serum diluted 1:5000 in TBST buffer, washed five times for 5 min with TBST buffer and incubated for 1 h with peroxidase-conjugated goat anti-rabbit IgG (Sigma) diluted 1:1000 in TBST buffer. After washing four times for 5 min in TBST buffer, the polypeptide bands were visualized using a chemiluminescent reagent (Leong & Fox 1988).

4.10. Isolation of the immunoglobulin-CA VI complex from serum (II)

A 1-ml human blood sample from the cephalic vein was centrifuged at 4000 × *g* for 10 min at 4°C and 5 µl of the serum was added to 45 µl of phosphate-buffered saline (PBS) and mixed for 2 h at 4°C with 50 µl of protein A immobilized on Sepharose (Sigma). After centrifugation at 15,000 × *g* for 2 min, the supernatant was saved as an unbound fraction and the protein A-Sepharose conjugate was washed twice with 400 µl of PBS.

Finally, the bound proteins were eluted with 400 µl of a 0.1-M glycine-HCl solution, pH 2.5, and the eluted material was mixed appropriately with 1-M Tris base to neutralize the pH of the solution. The eluted proteins were concentrated in Centricon P-10 tubes (Amicon, Beverly, MA) to a volume of 100 µl, and analyzed by SDS-PAGE followed by Western blotting.

4.11. Immunohistochemistry (IV)

To demonstrate CA VI in the *in vivo*-formed enamel pellicle, the halves of the crowns of the extracted teeth were immunostained by a biotin-streptavidin complex method as follows (Sternberger 1970, Guesdon *et al.* 1979):

1. Rinsing in 0.1-M PBS.
2. Pre-treatment with goat colostrum for 40 min to block the non-specific binding of the first antibody, and rinsing in PBS.
3. Incubation for 1 h with the primary rabbit antibody or NRS, both diluted 1:70 in 1 % BSA-PBS.
4. Treatment with goat colostrum for 40 min and rinsing in PBS.
5. Incubation for 1 h with biotin-conjugated swine anti-rabbit IgG diluted 1:300 in 1 % BSA-PBS.
6. Treatment with goat colostrum for 5 min and rinsing in PBS.
7. Incubation for 30 min with peroxidase-conjugated streptavidin diluted 1:600 in PBS.
8. Incubation for 5 min in 3,3'-diaminobenzidine tetrahydrochloride (DAB) (9 mg DAB in 15 ml of PBS plus 10 µl of 30 % H_2O_2).

The halves of the crowns were washed three times for 10 min in PBS after steps 3, 5 and 7. The entire procedure was carried out at room temperature.

The immunostaining was repeated using samples of the *in vitro*-formed enamel pellicle. The halves of the crowns were polished with pumice and incubated in paraffin-stimulated saliva (300 µl / crown half), in purified human CA VI (3 or 9 µg/ml in PBS), or in PBS in a rotary shaker for 2 h at room temperature. The samples were immunostained for CA VI as described above.

To compare the results with those for amylase which is known to be present in the enamel pellicle, parallel samples were immunostained using α-amylase antibody in conjunction with the biotin-streptavidin complex method as described above.

4.12. Demonstration of CA activity on the enamel surface (IV)

To examine the activity of the enamel-bound CA VI, the halves of the crowns of the extracted teeth were stained for CA activity by a slight modification of the histochemical method of Hansson (1967). The samples were polished with pumice and incubated either in paraffin-stimulated saliva or with purified human CA VI (30 µg/ml in PBS) in a rotary shaker for 2 h at room temperature. The staining was performed as follows:

1. Incubation for 1 min with a washing buffer solution containing 9 ml of 67-mM KH_2PO_4 and 1 ml of 67-mM Na_2HPO_4 in one litre of physiological saline.
2. Rinsing for 8 min with Hansson's medium, produced by adding a freshly prepared solution of 0.75 g of $NaHCO_3$ in 40 ml of dH_2O to a solution containing 1 ml of 0.2-M $CoSO_4$, 6 ml of 0.5-M H_2SO_4 and 10 ml of 67-mM KH_2PO_4. The samples were repeatedly flushed with the medium to promote air contact, and carbon dioxide was blown over the surface of the medium for 10 min prior to the addition of the $NaHCO_3$ solution and during the flushing.
3. Incubation for 1 min with the washing buffer.
4. Incubation for 5 s with a freshly prepared solution of 1 % $(NH_4)_2S$ in dH_2O.
5. Incubation for 1 min with the washing buffer.

Control experiments were performed using samples incubated in PBS in the absence of CA VI and saliva and stained as above, and using samples incubated in saliva or CA VI and stained with Hansson's medium in the presence of the CA inhibitor sodium acetazolamide (Diamox, Lederle Parenterals, Carolina, Puerto Rico, USA) at a final concentration of 50 mM.

4.13. Antigen labelling and fluoroimmunoassay procedure for CA VI (I-III)

After measuring the pH and buffering capacity, the samples were frozen, stored at -20 °C and thawed just before the measurement of CA VI concentration and amylase activity. A 1-ml aliquot from each sample was centrifuged at 15,000 × *g* for 10 min at 4 °C and the supernatant assayed for CA VI concentration and amylase activity. Both enzyme assays were performed without knowing the clinical data on the subjects.

Purified CA VI (Parkkila S *et al*. 1990) was labelled with 0.12 mg of Eu labelling reagent and the fluoroimmunoassay procedure was performed as described earlier (Parkkila S *et al*. 1993b). The fluorescence was measured with a 1234 DELFIA research fluorometer (Wallac, Turku, Finland). In the series discussed in papers I and III, the mean intra-assay coefficient of variation (CV) was 6.2 %, and the inter-assay CV, determined in 17 assays, was 10.3 %. In the paper II the mean intra-assay CV was 4.6 %, and the inter-assay CV between 3 assays, was 10.1 %. This assay based on determining the CA VI concentration was considered to be superior to the previously used CA activity assay (Krebs & Roughton 1948, Gloster 1955, Szabó 1974), which does not distinguish CA VI from CA II derived from contaminating red blood cells.

4.14. Determination of amylase activity (I, III)

The saliva samples were assayed for amylase activity using the α-amylase EPS-test (Boehringer Mannheim, Mannheim, Germany) according to the manufacturer's instructions. The measurements were performed using a BM/Hitachi 911 Automatic

Analyzer (Naka Works, Hitachi Ltd., Ibaraki-Ken, Japan), and the values were expressed as U/l. The inter-assay CV determined in 10 assays was 1.4 %.

4.15. Statistical methods (I, III)

The statistical evaluations were performed by linear regression analysis, one-way analysis of variance and non-parametric analysis of variance. Statistically significant differences were accepted at the $p < 0.05$ level. All reported p-values were calculated using the t-test. The SAS package (Statistical Analysis System, Cary, NC) was used to perform the statistical analyses.

5. Results

5.1. Correlation of salivary CA VI concentration with the basic characteristics of human whole saliva (I)

The mean (± SEM) rate of saliva secretion, determined for 209 young, healthy men, was 1.8 ± 0.0 ml/min (range 0.6-4.4 ml/min), the mean pH of the samples 7.3 ± 0.0 (6.1-7.7) and the mean buffering capacity 4.1 ± 0.1 (2.7-6.3). The secretion rates correlated positively with pH ($r = 0.38$, $p < 0.001$) and buffering capacity ($r = 0.43$, $p < 0.001$), and a close correlation was found between salivary pH and buffering capacity ($r = 0.67$, $p < 0.001$).

CA VI concentrations in the saliva samples collected before and after breakfast were in the range 0.1-10.0 mg/l and 0.6-16.0 mg/l, respectively, the mean concentrations being 1.8 ± 0.1 mg/l and 5.0 ± 0.2 mg/l. Since a close positive correlation was found between the enzyme levels in the two successive samples ($r = 0.71$, $p < 0.001$), only the second samples were subjected to further analyses. A positive correlation was observed between the CA VI concentrations and salivary secretion rates ($r = 0.20$, $p = 0.003$), whereas no significant correlation was found between CA VI levels and pH or buffering capacity.

The most abundant enzyme in human whole saliva, α-amylase (Mason & Chisholm 1975), was measured as a control enzyme, and its activity levels in the second saliva samples were also assayed and correlated with the basic salivary characteristics and CA VI levels. The mean amylase activity was 225 ± 10 × 1000 U/l (1-818 × 1000 U/l). No significant correlation was found between amylase activity and salivary secretion rate, pH, or buffering capacity. On the contrary, amylase activity did correlate positively with CA VI concentrations ($r = 0.46$, $p < 0.001$).

5.2. Effect of smoking on salivary characteristics (I)

According to their own declarations, 97 subjects did not smoke, 19 smoked less than 5 cigarettes per day, 29 smoked 5-9 cigarettes per day, 61 smoked 10-20 cigarettes per day, and 3 smoked more than 20 cigarettes per day. Since no significant differences emerged among the subgroups of the subjects, only smokers versus non-smokers were evaluated. The smokers (n = 112) had a slightly lower average salivary pH, 7.2 ± 0.0 (6.1-7.6) versus 7.3 ± 0.0 (6.3-7.7), p = 0.007, and buffering capacity, 3.9 ± 0.1 (2.7-6.1) versus 4.2 ± 0.1 (2.9-6.3), p = 0.035, than the non-smokers (n = 97), whereas no significant differences were seen in mean salivary secretion rate, CA VI and amylase levels.

5.3. CA VI in human serum (II)

The presence of CA VI in serum was demonstrated using blood samples collected from four subjects at 3-h intervals throughout the 24-h period. The serum concentrations of CA VI were measured using a time-resolved immunofluorometric assay (Parkkila S *et al*. 1993b), and the values were compared with the CA VI concentrations measured in the corresponding saliva samples. The serum was found to contain detectable amounts of CA VI, although the enzyme concentrations were only about 1/22 of those of the saliva samples. After breakfast, the mean (± SEM) concentration of CA VI was 0.20 ± 0.02 mg/l in serum and 4.29 ± 0.57 mg/l in saliva. Although salivary CA VI concentrations varied greatly among the subjects, they were very low during the sleeping period in all cases and increased rapidly in the morning after waking up and having breakfast. Much intra-individual variation was seen in the serum enzyme levels, although the circadian rhythm was less evident than in the saliva.

The presence of CA VI in the serum was confirmed by Western blotting. Under non-reducing conditions, anti-CAVI antibody detected a high molecular weight band of greater than 130 kDa in all the serum samples studied, suggesting that CA VI is associated with some other serum protein(s). After reduction, two major polypeptide bands of 51 kDa and 42 kDa were identified in the serum samples, the latter band being antibody-specific and corresponding to the molecular weight of the monomeric CA VI (Murakami & Sly 1987, Aldred *et al*. 1991). The 51-kDa band appeared to be a non-specific IgG heavy chain reaction, since NRS identified a polypeptide of the same molecular weight in a Western blot of purified human IgG. By contrast, the anti-CA VI antibody identified a strong 42-kDa polypeptide band in Western blots of the saliva samples under non-reducing conditions. In addition, the minor, differentially deglycosylated forms of CA VI were occasionally seen in the saliva blots, as in earlier studies (Murakami & Sly 1987, Parkkila S *et al*. 1990).

The results of the Western blots implied that CA VI may be associated with IgG in serum. To confirm this association, a protein A-Sepharose conjugate was used to isolate the IgG-CA VI complex from the serum. After electrophoretic transfer, Coomassie brilliant blue R-250 staining of the PVDF membrane showed a strong 51-kDa polypeptide band corresponding to the IgG heavy chain. In the same bound fraction,

anti-CA VI serum identified 42-kDa and 36-kDa polypeptide bands, corresponding to the monomeric and deglycosylated forms of CA VI, respectively (Murakami & Sly 1987). These results indicate that CA VI is associated with IgG in serum, whereas it mainly occurs as a monomeric enzyme in saliva.

5.4. Association between salivary CA VI concentration and dental caries (III)

5.4.1. Clinical and microbial findings

The DMFT index was determined as an indication of the caries experience of each subject. The mean index was 7.3 ± 0.4 (0-28) and the median 7.0. A healthy periodontium (CPI = 0) was found in 106 subjects, and gingival bleeding (CPI = 1), a clinical indicator of poor oral hygiene, in 50. Calculus (CPI = 2) was found in 53 subjects.

The counts of salivary lactobacilli, indicating cariogenicity of the diet, and the counts of mutans streptococci, reflecting the intensity of cariogenic infection, were determined using commercially available tests. Both the mean LB and SM counts were between 10^5 and 10^6 cfu/ml. The distribution of frequencies for LB was < 10^4 cfu/ml 35.4%, 10^4 - 10^5 cfu/ml 23.0%, 10^5 - 10^6 cfu/ml 26.3%, and > 10^6 cfu/ml 15.3%, and that for SM 24.4%, 11.0%, 20.1% and 44.5%, respectively. No significant correlations were found between salivary CA VI concentrations and LB or SM counts. As expected, the subjects with high microbial counts had higher caries experience. The LB counts displayed a higher correlation in linear regression analysis than the SM counts, the r values being 0.50 and 0.32, respectively ($p < 0.001$ in both cases).

5.4.2. Correlations between clinical findings and analytical salivary characteristics

The correlations between the DMFT index and salivary characteristics are shown in Table 3. The data obtained from the immunofluorometric assay supported the initial hypothesis that CA VI may have a protective effect against dental caries, since the DMFT index and CA VI concentration showed a statistically significant negative correlation ($r = -0.22$, $p = 0.001$), which was increased in the group of subjects with CPI = 1 ($r = -0.43$, $p = 0.002$). In contrast, no statistically significant correlation was found between the DMFT index and amylase activity.

Table 3. Correlations between salivary variables and DMFT indices in linear regression analysis.

	Group of subjects					
	All, n = 209		CPI = 0, n = 103		CPI = 1, n = 50	
Variable	r	p	r	p	r	p
CA VI concentration	-0.22	0.001	-0.11	0.288	-0.43	0.002
Buffering capacity	-0.24	< 0.001	-0.23	0.017	-0.29	0.039
pH	-0.21	0.002	-0.17	0.083	-0.25	0.081
Rate of saliva secretion	-0.09	0.215	-0.01	0.897	-0.21	0.136
Amylase activity	-0.11	0.120	-0.03	0.791	-0.16	0.272

To widen the analysis, the samples collected after breakfast were classified into three CA VI concentration categories: the 66 subjects with the lowest CA VI levels (< 3.0 mg/l), the 83 subjects with moderate levels (3.0-6.0 mg/l), and the 60 subjects with the highest levels (> 6.0 mg/l). The mean DMFT values in these categories are shown in Fig. 1a. To compare the results with those for another salivary enzyme, amylase activity was determined in the same saliva samples, which were classified correspondingly into three categories: 70 subjects with amylase activities of < 150 × 1000 U/l, 70 with activities of 150-250 × 1000 U/l, and 69 with activities of > 250 × 1000 U/l. The mean DMFT values in these categories are shown in Fig. 1b.

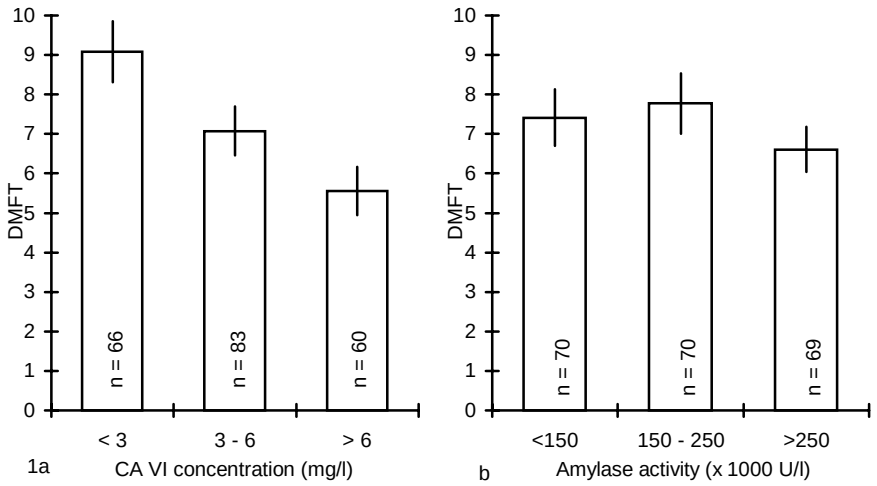

Fig. 1. DMFT values (mean ± SEM) in categories classified according to (a) CA VI concentrations, p = 0.002; (b) amylase activity levels, p = 0.5.

Two groups of samples were taken for further analyses, the 52 with low LB and SM counts (both < 10^5 cfu/ml) and the 65 with high counts (both > 10^5 cfu/ml). Statistically significant differences in the mean DMFT indices among the subjects grouped according to their CA VI levels were seen in both groups (low microbial counts, p = 0.018, and high microbial counts, p = 0.011). Subjects with low microbial counts and low CA VI concentrations displayed a mean DMFT index value (7.4 ± 1.8) close to that for the subjects with high microbial counts and high CA VI concentrations (8.5 ± 1.0). Without any classification according to the CA VI concentration, the mean DMFT index values in the groups of low and high microbial counts were 4.2 ± 0.7 and 11.4 ± 0.6, respectively.

To extend the analysis, two groups of subjects were distinguished on the basis of the CPI values: the 103 subjects with a healthy periodontium (CPI = 0), and the 50 individuals who had gingival bleeding but no calculus (CPI = 1). When the samples in these groups were classified according to CA VI concentration, as before, significant differences in the mean DMFT indices were found only in the second group, suggesting a greater protective action of CA VI in subjects with neglected oral hygiene (Fig. 2). In this group (CPI = 1) linear regression analysis revealed a close negative correlation between salivary CA VI concentration and DMFT index, whereas no correlation was found in the group with CPI = 0 (Table 3).

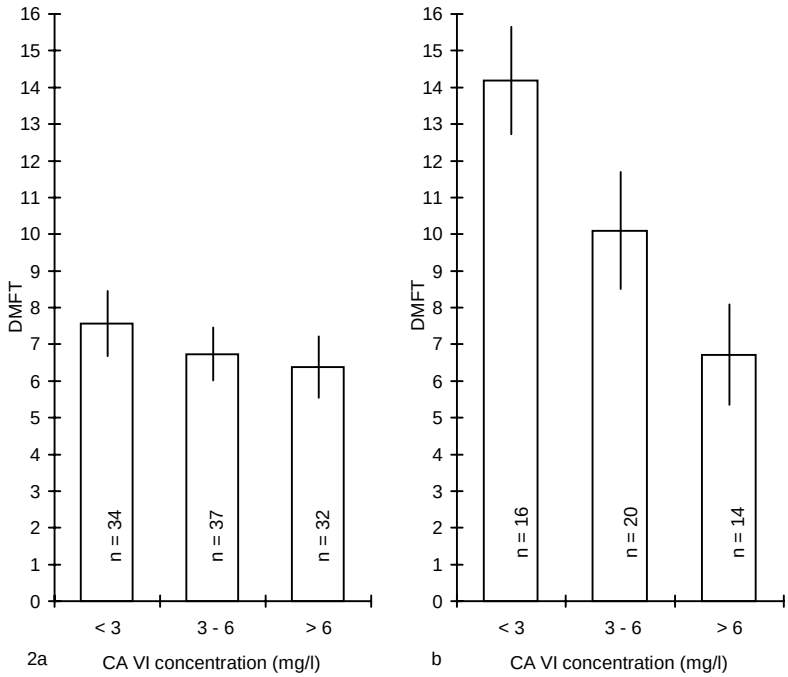

Fig. 2. DMFT values (mean ± SEM) in categories classified according to CA VI concentrations. (a) Healthy periodontium (CPI = 0), p = 0.6, n = 103; (b) Gingival bleeding, no calculus (CPI = 1), p = 0.008, n = 50.

5.5. CA VI in the enamel pellicle (IV)

5.5.1. Presence of CA VI in the enamel pellicle formed in vivo

To determine whether CA VI is naturally associated with the pellicle, the halves of the crowns of the extracted teeth having enamel pellicle that had formed *in vivo* were immunostained for CA VI. Parallel samples were immunostained for salivary α-amylase as a positive control, since amylase is known to be present in the pellicle (Al-Hashimi & Levine 1989). Both enzymes showed virtually identical staining patterns on the dental surfaces, indicating that CA VI is present in the enamel pellicle in the same manner as α-amylase. Control staining using NRS showed no positive reaction.

Immunoblotting of the pellicle proteins confirmed the presence of CA VI in the enamel pellicle formed *in vivo*. Anti-CA VI antibody recognized the major 39-kDa polypeptide band of CA VI in the pellicle proteins, salivary proteins and purified CA VI preparation. The minor bands of CA VI, 37 kDa and 34 kDa, which represent the differentially glycosylated forms of the enzyme (Murakami & Sly 1987), were seen in the immunoblot of purified CA VI. Antibody to salivary α-amylase recognized a doublet 57/60-kDa polypeptide band in the pellicle proteins, salivary proteins and purified salivary α-amylase preparations.

5.5.2. Binding of CA VI to enamel in vitro

To examine the ability of CA VI to bind to the enamel surface, polished crown quarters were incubated with purified CA VI *in vitro* and immunostained using anti-CA VI antibody. A specific CA VI immunoreaction was observed on the dental surface, suggesting that the enzyme can indeed bind to the enamel. Two concentrations of purified CA VI were used, 3 mg/l and 9 mg/l, and the intensity of the immunostaining was seen to be dependent on the CA VI concentration during the incubation. Salivary α-amylase again served as a positive control protein, giving similar staining results. No immunoreaction was seen in the samples incubated with CA VI or salivary α-amylase and immunostained using NRS, nor in the samples incubated in PBS and immunostained using CA VI or salivary α-amylase antibodies.

To confirm that salivary CA VI can bind to the enamel *in vitro*, polished crown halves were incubated in whole saliva, washed to remove all unbound proteins, and immunostained using anti-CA VI serum. A positive signal for CA VI was detected on the enamel surface. Anti-α-amylase antibody served as a positive control, as before. Polished crown halves incubated in whole saliva and immunostained using NRS showed no positive immunoreaction.

5.5.3. CA activity on the enamel surface

To determine whether CA VI bound to enamel retains its enzymatic activity, polished crown halves were incubated *in vitro* either in saliva or with purified CA VI. Both specimens showed positive histochemical staining for CA activity. No staining was detected in the control samples incubated in PBS. When acetazolamide, a CA inhibitor, was added to Hansson's medium, a marked decrease was observed in the intensity of the staining reaction.

6. Discussion

The carbonic anhydrases form a family of zinc metalloenzymes that participate in controlling the ion, fluid and acid base balance in various organs. The only known secreted isoenzyme is CA VI, several milligrams of which are secreted daily and pass into the gastrointestinal canal (Parkkila S *et al.* 1993b). Its physiological role has remained unclear, however. To explore the role of CA VI in the oral cavity, we determined its concentrations in human whole saliva and correlated them with basic salivary characteristics (I). A highly sensitive and specific time-resolved immunofluorometric assay (Parkkila S *et al.* 1993b) was used to determine the salivary CA VI concentrations. This method was considered to be superior to the previously used CA activity assay (Szabó 1974), which does not distinguish CA VI activity from the CA II activity present in any contaminating red blood cells. Because the CA VI concentrations in saliva follow a circadian periodicity and are under the control of the autonomic nervous system (Parkkila S *et al.* 1995), it is of utmost importance that quantitative studies should be carefully standardized regarding the sleeping period and meal times, and that the time of collecting the saliva samples is fixed and properly related to the meals. The appropriate time for sample collection appears to be in the morning, immediately after breakfast. Since the investigation reported in papers I and III was carried out among young male soldiers, the daily rhythm and other factors that might influence CA VI secretion could be well standardized. The soldiers had spent 4 weeks in the garrison prior to collection of the saliva samples and had thus adapted to the regular daily rhythm. In women, the phase of the menstrual cycle may cause variation in the composition of saliva, as the female sex steroids are known to influence salivary pH and buffering capacity during pregnancy (Laine *et al.* 1988).

The results showed that CA VI levels increased markedly after the first sampling (I), suggesting that reflexes related to the meal had enhanced enzyme secretion. Despite the careful standardization, salivary CA VI concentrations showed a high inter-individual variation, which probably reflects differences in enzyme expression. This conclusion is supported by the finding of a significant positive correlation between the enzyme concentrations in the first and second samples. These differences cannot be explained by proteolysis of the enzyme, as a protease inhibitor was used and all the samples were collected and handled identically. In addition, CA VI seems to be a very stable enzyme

which can withstand the harsh conditions in the alimentary canal, and non-degraded enzyme can be detected even in the gastric juice (Parkkila S *et al.* 1997).

A significant positive correlation was observed between salivary amylase activity levels and CA VI concentrations (I). Combined with an earlier report that salivary amylase activity levels and CA VI concentrations follow a parallel circadian periodicity (Parkkila S *et al.* 1995), this finding suggests that both enzymes are secreted via similar mechanisms and may possibly be present in the same secretory granules. Since the role of the autonomic nervous system in amylase secretion is well established, it is conceivable that the parasympathetic/sympathetic pathways also control CA VI secretion (Asking & Gjörstrup 1980, Olsen *et al.* 1988, Nederfors & Dahlöf 1992, Parkkila S *et al.* 1995).

To find out whether CA VI is transferred to the circulation, we collected serum and saliva samples from four healthy male volunteers throughout the 24-h period (II). Small amounts of CA VI were detected in serum using the previously mentioned fluoroimmunoassay. In view of its high expression and secretion in the salivary glands, CA VI may be leaked from the salivary glands or absorbed from the alimentary canal into the bloodstream. By analogy to the salivary CA VI concentrations, serum levels also showed a marked intra-individual variation during the 24-h period. These changes in serum CA VI concentrations may be linked both with the periodicity in expression and with rapid clearance from the bloodstream, the latter being supported by the recent findings that CA VI contains unique Asn-linked oligosaccharides terminating in GalNAc-4-SO_4, which is known to enhance the clearance of lutropin from the circulation via a specific receptor in the liver (Fiete *et al.* 1991, Hooper *et al.* 1995).

The presence of CA VI in serum was confirmed using a sensitive Western blotting method (II). Under non-reducing conditions, the anti-CA VI antibody identified only a single high molecular weight band of greater than 130 kDa, but after reduction, the high molecular weight polypeptide was reduced to two polypeptides of 42 and 51 kDa, corresponding to monomeric CA VI and the immunoreactive IgG heavy chain, respectively. The Western blotting results therefore suggest that the protein associated with CA VI could be IgG. This observation was further confirmed by isolating the IgG-CA VI complex from the serum using protein A-affinity resin. Western blotting of the unbound and bound fractions revealed that CA VI was indeed associated with IgG, which may protect the enzyme from proteolytic degradation or target it to cells which do not express CA VI.

Laboratory testing for serum α-amylase is commonly used in the diagnosis of diseases of the pancreas and in the investigation of pancreatic function (Lott *et al.* 1976, Salt & Schenker 1976). As most amylase assay methods are based on determination of the enzyme activity and cannot differentiate P-type and S-type amylase isoenzymes, more specific methods have been developed (Rauscher & Gerber 1989). Since both CA VI and S-type amylase are produced in the serous elements of the salivary glands and probably share the same secretory pathways, it will be a challenging prospect to study whether CA VI measurements can help to assess the contribution of the salivary glands to elevated amylase levels. The present results (II) provide a basis for further investigations to determine serum CA VI concentrations in patients with different salivary gland disorders.

In the study of salivary CA VI concentrations and their correlation with basic salivary characteristics (I), over half of the subjects examined were smokers (112 out of 209). Our findings were in accordance with earlier reports that there is no significant difference in salivary secretion rate between non-smokers and smokers, but that smokers have markedly decreased salivary pH and buffering capacity (Heintze 1984, Parvinen 1984, Wikner & Söder 1995). Previous research has shown that smoking reduces the secretion of epidermal growth factor into the saliva (Jones *et al.* 1992). We did not find that smoking influenced the secretion of either CA VI or amylase. This confirms an earlier observation that smoking has no effect on amylase activity levels in saliva (Nagaya & Okuno 1993), although the present data do not justify any conclusions regarding the long-term effects of smoking on salivary enzyme secretion, due to the young age of the subjects.

The observation, that no correlation exists between salivary CA VI concentration and pH or buffering capacity (I), suggests that CA VI is not involved in the regulation of the actual salivary pH. A weak positive correlation was observed between salivary secretion rate and CA VI concentration, but this is not specific to CA VI since the overall protein content of saliva is known to increase with an increased salivary secretion rate (Shannon 1973). The correlation between salivary secretion rates and amylase activity levels did not reach statistical significance, however, suggesting that CA VI concentration and salivary secretion are more specifically interrelated.

The large inter-individual variation in salivary CA VI levels is an important finding from the clinical point of view. In addition, evidence has been presented to show that the relative levels of many salivary constituents remain unchanged over time (Rudney *et al.* 1985, Wu *et al.* 1993). To determine the possible clinical effects of CA VI in the oral cavity, we compared its concentrations in whole saliva with the dental status of the subjects (III). The results were in accordance with the well-established notion that salivary pH and buffering capacity are negatively correlated with the subjects' caries experience as measured using the DMFT index and counts of salivary lactobacilli and mutans streptococci positively correlated with caries experience. Our data were also in line with previous observations of no correlation between the salivary secretion rate and the DMFT index (Birkhed & Heintze 1989, Russell *et al.* 1990, 1991). A novel and interesting finding was that the salivary CA VI concentration exhibits a negative correlation with the DMFT index. This is of special interest as previous research has revealed no distinct correlation between the concentration of any particular salivary protein and the prevalence or incidence of caries (Rudney 1995, Dodds *et al.* 1997, Kirstilä *et al.* 1998). In the present research we found no correlation here between salivary amylase activity and the DMFT index, suggesting that CA VI plays a specific role in the natural defence systems against dental caries. Interestingly, this protective effect emerged mainly in subjects with gingival bleeding without dental calculus (CPI = 1), the group that can be considered to have neglected their oral hygiene. Figure 2a demonstrated that adequate oral hygiene can compensate for a low salivary CA VI concentration, and conversely, poor oral hygiene does not inevitably lead to high caries experience provided that the salivary CA VI concentration is high (Fig. 2b). Poor oral hygiene combined with a low salivary CA VI concentration can result in extensive caries experience (Fig. 2b).

The rate of dissolution of dental hard tissues as a consequence of caries is dependent on the extent and duration of the decrease in pH on the dental surfaces. Salivary pH and buffering capacity are known to be central factors protecting the teeth from caries (Birkhed & Heintze 1989, Russell *et al.* 1990, 1991, Wolinsky 1994), and our results suggest that, salivary CA VI concentration is an equally important factor in this respect (III). However, our results did not confirm the earlier proposal (Feldstein & Silverman 1984) that CA VI may control salivary pH or buffering capacity (I). An interesting alternative is that it may attach to the enamel pellicle, like several other salivary proteins (Kousvelari *et al.* 1980, Al-Hashimi & Levine 1989, Cohen & Levine 1989, Lamkin & Oppenheim 1993, Lamkin *et al.* 1996) and function as a local pH regulator. In the microenvironment of dental surfaces, it could accelerate the neutralization of excess acidity locally by catalyzing the reaction $H^+ + HCO_3^- \Rightarrow H_2O + CO_2$, which constitutes the main buffering system in the saliva (Lilienthal 1955, Birkhed & Heintze 1989, Wolinsky 1994) (Fig. 3). To test this hypothesis, we explored the location and activity of CA VI in the enamel pellicle, the thin layer of salivary proteins associated with the hydroxyapatite of the enamel. Our results demonstrated that CA VI is indeed a natural component of the pellicle (IV). Its binding to and activity on the enamel surface was directly confirmed by incubating pieces of enamel in saliva or in solutions of purified CA VI in vitro. In the enamel pellicle CA VI is located at the optimal site to catalyze the conversion of salivary bicarbonate and microbe-delivered hydrogen ions to carbon dioxide and water. The present results indicate that active CA VI is located in the enamel pellicle, suggesting that it may accelerate the removal of acid from the local microenvironment of the tooth surface.

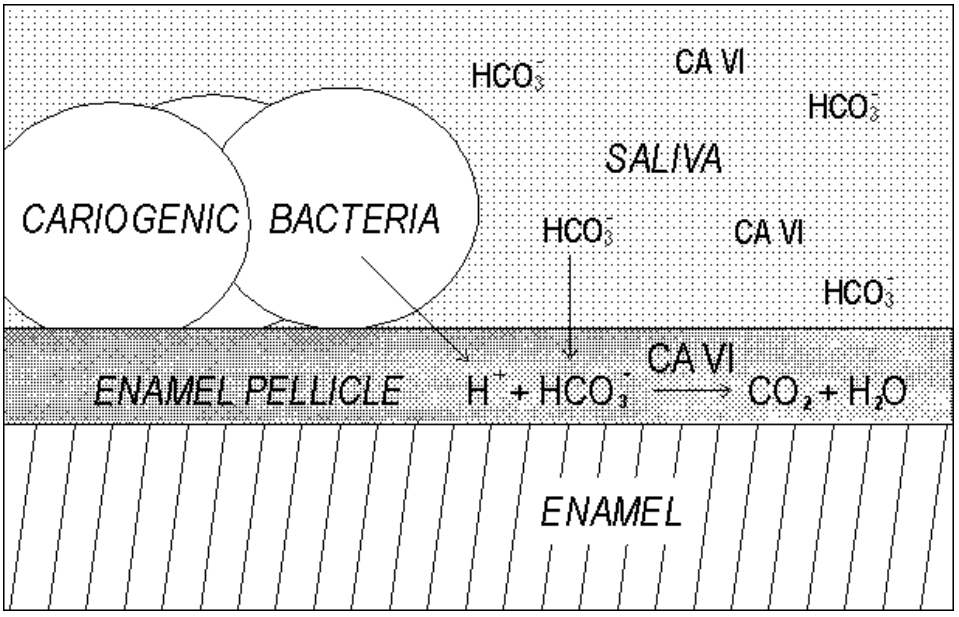

Fig. 3. Hypothetical model for the function of CA VI on dental surfaces.

CA VI possesses several properties that make it compatible for functioning in the microenvironment of dental surfaces. First, it is a very stable enzyme, retaining its activity in an acidic environment (Parkkila *et al.* 1997). Its well-established stability may be attributable to its structural properties. Sheep CA VI contains an intramolecular disulphide bond linking Cys 25 and Cys 207 (Fernley *et al.* 1988), and these two cysteines are conserved in the human enzyme, in which they presumably also form a disulphide bond (Aldred *et al.* 1991). Such intramolecular disulphide bonds are a common feature of secreted proteins, in which they stabilize the molecular conformation. Second, CA VI is a glycoprotein, possessing two sites of N-linked glycosylation (Murakami & Sly 1987, Aldred *et al.* 1991). The exact function of these oligosaccharide side chains is not known, but it has also been suggested that they may participate in stabilizing the functional conformation of the enzyme (Fernley 1991a). The oligosaccharide side chains of CA VI may also be involved in protein-protein and/or protein-hydroxyapatite interaction, linking the enzyme to the enamel pellicle. The third unique property of CA VI is that its secretion follows a circadian periodicity (Parkkila *et al.* 1995). Its salivary levels are very low during sleep and rise rapidly after awakening. The increase in salivary CA VI concentration during the daytime obviously increases the amount of pellicle-bound CA VI, as our results suggest (IV), ensuring that the excess acidity arising from increased bacterial metabolism during the daytime can rapidly be neutralized on the dental surfaces.

Several factors, including diet, oral hygiene and genetic properties of the saliva and teeth, play a role in the aetiology of dental caries (Birkhed & Heintze 1989, Holbrook 1993, van Palenstein Helderman *et al.* 1996, Watson *et al.* 1997). The importance of salivary factors in the host defence against caries is salient among patients suffering from severe salivary gland dysfunction. Previous investigations have shown that the saliva brings various defence mechanisms to the actual site of microbial adherence and growth on the dental surfaces (Tenovuo 1989, Lamkin and Oppenheim 1993, Johnsson *et al.* 1993, Lagerlöf and Oliveby 1994, Edgar *et al.* 1994, Wolinsky 1994). Taken together, our results indicate that salivary CA VI also plays an essential role in the oral cavity. Its role does not appear to be antibacterial, but rather protective, contributing to conservation of the dental hard tissues under the unfavourable influence of microbial metabolism.

7. Conclusions

- The mean (± SEM) salivary concentration of CA VI in young men, measured after breakfast, 1.5 h after awakening, is 5.0 ± 0.2 mg/l.
- Salivary CA VI concentration is positively correlated with salivary α-amylase activity. A weak positive correlation also exists with salivary secretion rate.
- CA VI is not directly involved in the regulation of actual salivary pH or buffering capacity, nor does it have any association with counts of mutans streptococci or lactobacilli in the saliva.
- Smoking habits have no influence on salivary CA VI levels in young men.
- High salivary CA VI concentrations are associated with lower caries experience, particularly in subjects with neglected oral hygiene.
- Enzymatically active CA VI is present in the enamel pellicle, suggesting that, in the local microenvironment of the dental surface, it may reduce demineralization of the dental hard tissues by catalyzing the neutralization of excess acidity through the reaction $H^+ + HCO_3^- => H_2O + CO_2$.
- Low levels of CA VI are present in human serum, where the enzyme is associated with IgG.

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