CHAPTER VII

GENERAL SUMMARY
It is becoming increasingly apparent to investigators and clinicians in a variety of disciplines that saliva has many diagnostic uses and in large-scale screening and epidemiological studies. The highly sensitive test procedures that are now common place, makes it practical to quantitate enzymes despite very low concentration.

Whole saliva contains a much higher amount of distinct enzymes than pure salivary gland secretions. Some of the enzymes of whole saliva may originate from oral microorganisms. Other sources of enzymes are salivary glands, polymorphonuclear leukocytes, epithelial cells, plasma and dietary constituents.

Proteolytic enzymes are present in dental plaque and there is also enzyme activity in inflamed gingival tissue and gingival crevicular fluid. In periodontium, proteases have a multiple role in both the normal tissue and remodelling of the tissue and in pathophysiological conditions such as periodontal disease, where the destruction of tissue structure is likely to be associated with increased proteolytic activity.

During the development of periodontal disease, a number of biochemical reactions are known to occur, which both initiate and prolong inflammation. These processes require enzymes, which clearly arise from both the infecting organism and the host. Many of the hallmarks of this disease, including bleeding on probing, neutrophil accumulation, attachment loss and increased crevicular fluid flow involve proteolytic events like degradation of fibrinogen, complement pathway activation and kallikrein/kinin pathway activation.

Most studies have utilized crevicular fluid as the indicator material. β-Glucuronidase, aspartate aminotransferase, collagenase, elastase and gelatinase are some of the enzymes studied in this respect. Some studies have focussed on bacterial plaque enzymes. It is clear that site-specific periodontal disease tests involving sulcular fluid will be restricted mainly for use by specialist periodontists. However, the accurate and reproducible sampling of fluid is not
trivial. On the other hand, saliva has long been recognized as being a potentially valuable material for oral disease detection.

Protease like activities in normal human and animal blood plasma have been demonstrated with the aid of chromogenic substrates (189, 190). p-Nitroanilides of tri and tetrapeptides allow the direct determination of the activity of proteases activated in vivo. This technique allows a quantitative assay of salivary protease activity.

In this investigation, the results of three different groups of biochemical parameters in saliva that can be considered as useful in diagnosis of oral diseases are presented. First group of factors are the salivary proteolytic activity and the second group combines factors that exhibit antiprotease activities. The third group comprises total protein and protein bound neutral hexose, fucose and sialic acid.

Very low proteolytic activity was observed in normal saliva with dye-bound substrates like azocollagen, congocollagen, azocasein and orcein albumin. However, significant activities were observed with synthetic peptide-p-nitroanilide substrates.

Bovine crystalline trypsin showed relatively low activity on BAPNA, comparable action on substrates like S-2302 (H-D-prolyl-L-phenylalanyl-L-arginine-p-nitroanilide dihydrochloride, substrate for plasma kallikrein), S-2266 (substrate for glandular kallikrein, H-D-valyl-L-arginine-leucyl-L-p-nitroanilide dihydrochloride), S-2288 (H-D-isoleucyl-L-prolyl-L-arginine-p-nitroanilide dihydrochloride, broad spectrum substrate for many serine proteinases) and S-2366 (glutamyl-prolyl-arginine-p-nitroanilide hydrochloride, substrate for protein ‘C’ and factor XI). The action on S-2238( H-D-phenylalanyl-L-pipecolyl-L-arginine-p-nitroanilidedihydrochloride,substrate for thrombin) was slightly lower. Saliva had negligible action on BAPNA and the activities of saliva on S-2266, S-2288 and S-2366 were 2.3 fold higher than on S-2302 and S-2238. The present data suggest that there are more than one enzyme in significant levels
in saliva. According to reported values, plasma kallikrein activity on S-2302 is 2-7 times more than that of trypsin. The present study suggests that normal salivary S-2302 activity is contributed by kallikrein as well as other proteases. The ratios of activities on different p-nitroanilide substrates show that in addition to kallikrein, saliva contains other proteases like trypsin, thrombin and plasmin-like enzymes.

Salivary S-2302 hydrolytic activity increases slowly from pH 5.0 to 7.0, then a steep increase can be seen from pH 7.0 to 10.0. The activity was fairly heat stable. 24% of hydrolytic activity was destroyed after heating for 10 minutes at 60°C. The activity decreased with increasing time of heat treatment. Heating at 80°C for 10 minutes destroyed all activity.

The salivary hydrolytic activity with S-2302 was susceptible to different extent to inhibitors. 47.4% activity was inhibited by phenylmethylsulfonyl fluoride (PMSF). Lima bean trypsin inhibitor (LBTI) inhibited the activity around 36.6% and with soyabean trypsin inhibitor (SBTI) inhibition was 57.8%. Heparin had negligible action.

No correlation between age and S-2302 hydrolytic activity in saliva or protein concentration was noticed. No significant difference with respect to salivary S-2302 hydrolytic activity in regard to sex was observed.

Salivary S-2302 hydrolytic activity when expressed as absorbance units/ml, increased in gingivitis (1.08±0.332, Mean±S.D.), periodontitis (0.802±0.169) and diabetes (without gingival inflammation, 1.05±0.466), compared to controls (0.464±0.081). This can be explained as due to increased leakage of plasma enzymes because of vascular permeability during inflammation. The volume of gingival fluid, i.e., plasma leakage is increased because of enhanced vascular permeability (193, 194). This vascular permeability in turn, is thought to increase plasmin and kallikrein level (194, 195).
In terms of protein concentration the increase in proteolytic activity was more in periodontitis than in gingivitis. The values in diabetic samples were not statistically different from normal values. This is because in periodontitis the salivary protein increase was marginal, but in gingivitis and diabetes the salivary protein was significantly elevated. This can be explained by increased flow of gingival fluid into saliva. Increased basement membrane permeability in diabetes (1) can be considered to increase both protein level as well as S-2302 hydrolytic activity.

Thus, S-2302 hydrolytic activity when normalized to protein level can be used as a marker in differentiating periodontitis.

Salivary proteolytic activities against different chromogenic substrates were estimated and this was compared with serum activity. Maximal hydrolytic activities were observed in saliva samples with S-2366, S-2288, S-2266, (1.220-1.510, 0.940-1.17, 0.955-1.20 0.D units/ml, respectively). Moderate activities were seen with S-2302 (0.375-0.545), S-2238(0.475-0.560), S-2222, (N-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-p-nitroanilidehydrochloride, substrate for factor Xa and trypsin, 0.455-0.505) and S-2251, (H-D-valyl-L-leucyl-L-lysine-p-nitroanilide dihydrochloride, 0.375-0.450). But the elastase (0.33-0.380 0.D units per ml saliva with NMSAAPVNA, N-methoxy-succinyl-alanyl-alanyl-prolyl-valine-p-nitroanilide), chymotrypsin (0.360-0.450 0.D units per ml saliva with SAAPPNA, N-succinyl-alanyl-alanyl-prolyl-phenylalanine-p-nitroanilide) and aminopeptidase (0.245-0.560 0.D units/ml saliva with LPNA, L-leucine-p-nitroanilide) like activities were relatively lower in normal saliva.

Salivary kallikrein-like activity (S-2302 hydrolysis) is 10-20 times less than serum activity. Saliva shows 5-10 times less activity with S-2288, S-2266 and S-2238 compared to serum. Serum activity with S-2222, S2251 (substrate for plasmin) and S-2586 (3-carboxymethoxy-propionyl-L-arginyl-L-prolyl-L-lysine-p-nitroanilide hydrochloride, substrate for chymotrypsin) are about 2-3 times more than in saliva. Elastase like activity (with S-2484, L-pyroglutamyl-L-prolyl-
L-valine-p-nitroanilide) is not significantly different between serum and saliva. Comparison of the ratios of these activities in saliva and serum indicates that some of the trypsin-like activity can arise from plasma due to a high gradient.

Effect of time of incubation on hydrolytic activities with 13 model substrates was studied. In most cases, linearity with respect to time of reaction was not observed. Salivary hydrolytic activities with S-2288, S-2302, S-2238, S-2266, S-2222 and S-2251 were progressive with time but highly nonlinear. An almost linear relationship was observed with S-2366 and S-2484. Leucine aminopeptidase and chymotryptic activity (SAAPPNA hydrolysis) showed highly nonlinear pattern. Hydrolysis of elastase substrate, N-succinyl-L-alanyl-L-alanyl-L-alanine-p-nitroanilide (STANA) reached a plateau by 60 minutes. Presence of reversible inhibitors and relative lability of enzymes in the reaction medium might be the contributing factors for lack of linearity.

On exposure of salivary samples to 80°C for 10 minutes, the proteolytic activities with different model substrates were completely destroyed. By changing the duration of heat treatment and temperature it could be noticed that trypsin-like activity is more heat labile and that there are two types of elastase activities differing in heat stability.

Trypsin-like activity showed fairly sharp pH optima around pH 8.5 to 9.0 (with S-2238, S-2288 and S-2266). Chymotryptic activity (SAAPPNA hydrolysis) and elastase activity (MSAAPVNA hydrolysis) were fairly constant over the wide pH range of 5.5 to 10.0. Earlier one showed a small hump at pH 7.5 and the latter exhibited a shallow pH optimum around 8.5.

Phenylmethylsulfonylfluoride (PMSF), a nonspecific serine protease inhibitor reduced the hydrolytic activities with different synthetic substrates to varying degrees. PMSF inhibited elastase activity (MSAAPVNA hydrolysis) maximally. This is followed by inhibition of chymotryptic activity (SAAPPNA hydrolysis). The magnitude of inhibition of trypsin-like enzymes was less. High
molecular weight inhibitors like soyabean trypsin inhibitor and Limabean trypsin inhibitor inhibited elastase activity maximally. Chymotryptic activity inhibition was minimal. The trypsin-like activity was susceptible to these inhibitors. Heparin marginally activated elastase activity.

Presence of bulk of the amidolytic activities along with washings of Red Sepharose chromatography suggests that no significant proteolytic activity in saliva was associated with α2-macroglobulin (223).

It was observed that salivary trypsin-like activities (with S-2288 and S-2366) increased in a nonspecific manner in gingivitis, periodontitis and also in diabetes, indicating that they are not significant for differential diagnostic purposes. When proteolytic activity was normalized to protein level, the patterns were different. Only in periodontitis S-2288 and S-2366 hydrolytic activities were found to be significantly elevated ‘p’ and ‘t’ values were <0.01 and 5.4 for S-2288 and <0.01 and 4.73 for S-2366. In gingivitis and diabetes increased proteolytic activity is counterbalanced by increased salivary protein level. The same pattern was obtained with amino peptidase (leucine p-nitroanilide hydrolysis). Thus, measurement of amino peptidase activity is as reliable as measuring activity of trypsin-like enzymes in the diagnosis of periodontitis.

Salivary chymotryptic activity (units/ml) was found to be elevated in periodontitis (p<0.05, t =2.14), gingivitis (p<0.01, t = 2.99) and diabetes (p<0.05, t = 2.37), compared to controls (0.290 ± 0.110, Mean ± S.D.). When normalized to protein level, marginal increase was noticed in periodontitis alone. However, the increase was not significant statistically.

Increase in salivary elastase activity was significantly higher compared to activities with S-2288, S-2366, LPNA and SAAPPNA in different clinical conditions. The magnitude of increase was 5.2 fold in gingivitis compared to a 2 fold increase in S-2366 activity and a 2.35 fold increase in LPNA activity.
When elastase activity was expressed as units per mg protein the increase was statistically significant in all three conditions.

These data suggest that aminopeptidase and trypsin-like activities in saliva are of more diagnostic significance in periodontitis.

Measured antitryptic activity in saliva is very low. In gingivitis and periodontitis, the increase in trypsic activity, more than compensates for the increase in antitryptic activity if any. Statistical analysis showed that there is no difference between these groups and controls. In some salivary samples, there was an apparent activation of BAPNA hydrolytic activity on addition of saliva to bovine trypsin. Even though the activation was small, it was highly reproducible, in individual samples. Time of preincubation did not alter the magnitude of activation. This shows that bovine trypsin does not activate latent trypsin-like activity in saliva, as function of time. The alternate possibility for activation observed could be due to protection of bovine trypsin by salivary constituents against denaturation.

Salivary antielastase activity in normals was found to be low. There was no measurable antielastase activity in gingivitis. Compared to normals, in periodontitis, increase in antielastase activity was statistically highly significant. Salivary antielastase activity was significantly increased in periodontitis than in gingivitis. The difference is also significant when the values are normalized for protein levels, because protein level in saliva is higher in gingivitis than in periodontitis.

Unlike antitryptic and antielastase activities, significant antichymotryptic activity could be detected in normal salivary samples. In periodontitis and gingivitis, the values were well within the normal range.

The present studies show that measurement of antitryptic and antichymotryptic activity is not of much diagnostic significance. However,
antielastase activity was found to be significantly elevated in periodontitis but not in gingivitis.

The last part of the study deals with the measurement of salivary total neutral hexose, protein bound carbohydrate, bound fucose and sialic acid along with total protein in different conditions like gingivitis, periodontitis and caries in children.

Salivary neutral hexose was measured and expressed as mannose units. There was no significant difference between males and females. The values varied from 16.5 to 26.2 mg/dl. No difference between adults (21.90±2.71 mg/dl, mean±S.D.) and children (18.0±4.41) was observed by statistical analysis. There was no difference between healthy adults and periodontitis patients. The values were slightly lower in gingivitis than in normal adults and the difference was found to be statistically significant (p<0.01, t = 4.16). No significant difference in values between healthy children and caries children was observed when the values were expressed per unit volume of saliva. In periodontitis, gingivitis and caries salivary protein level was found to be elevated. This nonspecific increase is not of much diagnostic significance. However, when corrected to protein level neutral hexose value was found to be significantly reduced in gingivitis, periodontitis and dental caries. This is in sharp contrast to the observation in diabetes where total neutral hexose level was not altered (262).

Ethanol precipitable hexose values accounted for slightly less than 50% of the total hexose values. Compared to normal adults (8.44 ± 1.22 mg mannose units/100 ml saliva, Mean ± S.D.), there was no difference in values in different groups. When corrected to protein level, the values were drastically reduced in gingivitis (3.71 ± 1.64), periodontitis (5.91 ± 1.63) and caries (3.85 ± 1.52) compared to corresponding controls, normal adults (7.65 ± 0.86 mg mannose units/100 mg protein, Mean ± S.D.) and normal children (8.07 ± 1.78 mg mannose units/100 mg protein) respectively.
The neutral hexose in ethanol soluble fraction represents free saliva hexose (glucose) as well as glycopeptides and oligosaccharides not precipitated by ethanol. The values for ethanol soluble neutral hexose are calculated based on differences between total neutral hexose and ethanol precipitable neutral hexose. Per unit volume of saliva, the decrease in gingivitis (9.46 ± 3.33) and periodontitis (10.80 ± 3.27) compared to controls (13.50 ± 1.71 mg mannose units / 100 ml saliva, Mean ± S.D.) is statistically significant. There was no difference in the parameter between healthy children and those with dental caries (10.20 ± 2.96 and 9.15 ± 3.88 mg / 100 ml saliva, Mean ± S.D. respectively). However, when it is expressed per 100 mg protein the values were found to be drastically reduced in gingivitis (3.71 ± 1.64) and periodontitis (5.91 ± 1.63) compared to normal adults (12.20 ± 1.28 mg/100 mg protein, Mean ± S.D.) It is also decreased significantly in caries (3.85 ± 1.52) compared to normal children (11.00 ± 3.55 mg / 100 mg protein, Mean ± S.D.). This decrease in both ethanol precipitable and ethanol soluble neutral hexose levels could be attributed to the increased level of glycosidases (270) and increased utilization of released oligosaccharide units by microorganisms (271, 272) in these clinical conditions.

Trichloroacetic acid (5%) soluble fraction accounts for nearly two thirds of total hexose in saliva, which is slightly higher than ethanol soluble fraction. A significant proportion of neutral hexose represented by oligosaccharide units in saliva is not precipitated by either ethanol or trichloroacetic acid.

Earlier studies reported altered fucose:protein ratio in GCF (258) and increased level of fucose in terms of protein concentration in saliva as well as in serum of diabetics (262). The present study shows that fucose level in saliva is increased both in periodontitis (p<0.01, t = 4.74) and gingivitis (p<0.01, t = 4.72) compared to normals (3.51±0.55 mg/dl). However, when normalized to protein concentration, salivary bound fucose values did not differ in gingivitis (2.95 ± 1.59) and periodontitis (3.26 ± 0.98) unlike in diabetes (262) compared to controls (3.20 ± 0.50 mg/100 mg protein, Mean ± S.D.).
The present studies indicate that protein bound sialic acid level/100 mg protein was found to be significantly reduced in both gingivitis (p<0.001, t=10.65) and periodontitis (p<0.001, t = 8.54) compared to controls (1.92 ± 0.33 mg, Mean±S.D.). The increased sialidase activity could be the reason for the observation.