CHAPTER VI

GLYCOPROTEINS IN SALIVA AND SERUM IN DIABETES
INTRODUCTION

Saliva circulating in the mouth at any given time is termed as whole saliva and it comprises a mixture of secretions from the major and minor salivary glands (228, 229). The slimy, viscous properties of saliva are due to glycoproteins (mucins) which comprise about one-third carbohydrates and two-third proteins (230). The glycoproteins are characterized by containing carbohydrate side chains and are resistant to proteolytic enzymes (230). The carbohydrate protein links in the glycoproteins include a) those containing an O-glycoside linkage (i.e., o-linked), involving the hydroxyl side-chain of serine or threonine and a sugar such as N-acetylgalactosamine (GalNAc-Ser[Thre]) and b) those containing an N-glycosidic linkage (i.e., N-linked), involving the amide nitrogen of asparagine and N-acetylglucosamine (GlcNAc-Asn).

The chemical composition of the carbohydrate moieties in salivary glycoproteins has been extensively investigated (231). Sialic acid, hexosamines, fucose and neutral hexoses form part of the glyco groups. It has been proposed by several workers (232, 233) that salivary glycoproteins consist of discrete structural domains, each of which may be responsible for a particular biological function. Indeed, many of the protective function of saliva can be attributed to the physical, structural and rheological characteristics of salivary glycoproteins (232). Salivary glycoproteins are thought to play important roles in the formation of the acquired enamel pellicle (234), selective clearance and adherence of microbial flora (235), antimicrobial activity, utilization as a microbial substrate (236, 237), prevention of calculi formation and regulation of bacterial growth. It has been proposed that specific binding of salivary glycoproteins by oral bacteria constitute a mechanism to collect nutrients in the vicinity of the cell (237). It has been demonstrated that various strains of S.sanguis are capable of growth while using saliva as their sole source of nutrients. Further, the salivary glycoproteins are susceptible to enzymatic
hydrolysis and the enzymes originate mainly from oral microorganisms (238). The sugar moieties of the glycoproteins are rapidly released by enzymatic hydrolysis (238). Protein component is also susceptible to proteolytic activity (239). This would suggest that utilization of glyco groups would be faster under disease conditions even though no study has been undertaken in this direction. Oral bacteria are known to elaborate a variety of glycosidases and accumulate glycogen, dextrans and levans during growth (231).

Recently, the oral manifestations in chronic metabolic diseases are receiving a lot of attention. The effect of these diseases on saliva and its constituents, modulate the oral ecology, which has an important role to play in the cause and progress of mucosal lesions. Diabetes mellitus represents one such major chronic health problem facing the world today afflicting approximately 4% of the population (240).

The association between diabetes mellitus and changes in the oral cavity has been widely reported in both the medical and dental literature. A wide spectrum of oral manifestation of diabetes mellitus have been reported, which include advanced periodontal disease, a high rate of dental caries, biphasic dental development, sialadenosis, xerostomia, sialosis, altered salivary secretion, abnormal taste, prolonged or recurrent bacterial or fungal infections and burning mouth syndrome (241, 242).

Many investigators have demonstrated that the concentration of the glycoproteins in human serum is abnormally high in a number of physiological and pathological status (243). Significant increase in the glycoprotein content of serum (as hexose or hexosamine) has been shown to be associated with neoplastic diseases (244), tuberculosis (245), diabetes complicated by degenerative vascular diseases (246), rheumatoid arthritis (247), as well as with
various miscellaneous infections and inflammatory reactions (248). There is also a gradual increase in serum glycoprotein levels with age (249).

Salivary changes offer interesting parameters to evaluate and monitor diabetes mellitus and has been opened up new vistas of research. Many investigators have demonstrated alteration in flow rate and glucose and protein levels in saliva in poorly controlled diabetes (250, 251, 252). Increase in protein content in saliva in diabetes mellitus and other oral disease conditions led to a study on the changes in protein bound neutral hexoses. This chapter deals with the detailed study of salivary protein and protein bound neutral hexoses in normal and diabetic subjects.

MATERIALS

The present work was conducted with twenty-five confirmed diabetic patients. The patients were chosen from the outpatient clinics of the Department of Oral Medicine and General Medicine, and also from patients admitted to S.D.M. College of Dental Sciences, Sattur, Dharwad. Twenty-five healthy, non-diabetic subjects served as control. The criterion for diagnosis of diabetes was based on the recommendation of National diabetic data group.

The control subjects were apparently healthy dental students, para-dental staff and laboratory technicians. They were free from any sort of disease, and diabetes mellitus was excluded after estimation of fasting blood sugar, examination of urine for sugar and oral glucose tolerance test, wherever indicated.

Collection of saliva

Unstimulated whole saliva samples were collected around 9-10 AM by the spitting method and centrifuged at 800 g for 10 minutes. The clear
supernatant was assayed immediately for total protein, total hexose and individual protein bound neutral hexoses.

**Serum samples**

Blood samples used in this study were collected from persons admitted to or attending the outpatient clinics of the Department of Oral Medicine and General Medicine, S. D. M. College of Dental Sciences, Sattur, Dharwad. Fifteen diabetics and equal number of healthy control subjects were chosen for the study. All specimens obtained by venepuncture were collected into blood collection tubes, the blood was allowed to clot at room temperature and serum was removed after centrifugation at 1200 g for 10 minutes.

All other materials used are listed in Chapter II.

**Methods**

Unless stated otherwise, the following routine procedures were employed.

**ESTIMATION OF CARBOHYDRATES IN SALIVA**

**Total hexoses**

The method described by Rao and Pattabiraman (44) was adapted with the major modification of substituting o-cresol in place of phenol. To 0.1 ml of clear saliva, 0.9 ml of distilled water was added followed by 3.0 ml of concentrated sulfuric acid. The solution was mixed thoroughly and vortexed. After cooling for 30 minutes at room temperature, 0.1 ml of ethanolic o-cresol (10 mg) was added and mixed. The pinkish orange color formed was measured after 30 minutes at 500 nm. Total hexoses values were expressed in terms of mannose units.
Bound hexoses

In this method, salivary glycoproteins were precipitated with 95% ethyl-alcohol and the precipitate was used for the estimation of bound hexose.

The method employed is as follows: To 0.5 ml saliva, 1.2 ml 95% ethanol was added followed by 0.3 ml normal saliva. The contents were mixed thoroughly, vortexed and centrifuged in a clinical centrifuge for 15 minutes at 2000 r.p.m. and the supernatant was decanted. The precipitate was washed thrice with 1.2 ml of 95% ethyl alcohol. To the precipitate 1.0 ml of distilled water was added and mixed thoroughly. Bound hexose was estimated by the two-step o-cresol: sulfuric acid reaction as described above. Bound hexose values were also expressed as mannose units.

Protein bound fucose

The method developed by Pattabiraman (114) was adapted for the estimation of protein bound fucose. To the precipitate, obtained after ethanol treatment, 1.6 ml of distilled water was added and mixed thoroughly. To this 2.4 ml of concentrated sulfuric acid was added and mixed. The solution was allowed to cool at room temperature for 30 minutes. Freshly prepared L-cysteine hydrochloride (50 μl, 5 mg) was added followed by 0.1 ml of ethanolic o-cresol (10 mg). After 30 minutes reaction at room temperature, the chromogen formed was analyzed at 490 nm. In this experiment, the final sulfuric acid concentration was close to 60%.

The method was based on the fact that the condensation product of HMF or other hexoses with cysteine is unstable in 60% hot sulfuric acid and the partially dehydrated products from hexoses reacted very slowly with cysteine or o-cresol.
Sialic acid

The method developed by Yao and associates (253) using acidic ninhydrin reaction was used for the estimation of sialic acid in saliva. The acidic ninhydrin reagent was prepared according to Gaitonde (254).

The reagent contained 250 mg of ninhydrin in a mixture of 6.0 ml of glacial acetic acid and 4.0 ml of concentrated sulfuric acid. The contents were mixed thoroughly by using a vortex mixer for 30 minutes to get a clear solution. The reagent was prepared fresh just before use.

To the precipitate, obtained after ethanol treatment, 1.0 ml of distilled water was added, and mixed thoroughly. To this 1.0 ml of glacial acetic acid was added followed by 1.0 ml of acidic ninhydrin reagent. The reaction mixture was heated at 100°C in a boiling water bath for exactly 10 minutes. Then the mixture was rapidly cooled under tap water. The absorbance was measured at 470 nm. The salivary sialic acid was calculated by running standards. N-acetyl neuraminic acid of varying concentrations (20 to 100 µg) was used to obtain a standard graph (Figure 6.1).

Estimation of salivary proteins by bicinchoninic acid method (255)

Reagent A

One gram of bicinchoninic acid, 1.702 gram of anhydrous sodium carbonate, 160 mg of sodium potassium tartrate, 400 mg of sodium hydroxide and 950 mg of sodium bicarbonate were dissolved in 100 ml distilled water. The pH of the reagent was adjusted to 11.25.
Figure 6.1 Standard graph for the estimation of sialic acid by acid ninhydrin method.
Reagent B

400 mg of CuSO₄·5H₂O was dissolved in 100 ml distilled water and stored in a dark place.

Working bicinchoninic acid reagent was prepared by mixing 100 ml of reagent A and 2.0 ml of reagent B. Working reagent is prepared freshly every time.

To an aliquot of 0.2 ml diluted saliva (1→5 dilution with distilled water), 4.0 ml of working bicinchoninic reagent was added, gently mixed and incubated at 37°C for 30 minutes in a water bath. 0.2 ml of 0.9% sodium chloride with 4.0 ml of bicinchoninic acid serves as blank. Similarly, a series of standard protein solutions (bovine serum albumin) ranging from 40 to 200 μg in a volume of 0.2 ml were treated with 4.0 ml of bicinchoninic acid reagent. The reaction mixture was allowed to stand for 30 minutes at 37°C in a water bath. The solution was cooled at room temperature and then absorbance was measured against the blank at 562 nm in a spectrophotometer. A standard graph was constructed and the protein content of the saliva was calculated using the graph.

Estimation of glycoproteins in serum

In this method, serum glycoproteins were precipitated with 95% ethanol and the precipitate was used for the estimation of protein bound hexose and fucose.

The method employed is as under. To 0.05 ml serum, 0.2 ml normal saline was added followed by 1.75 ml 95% ethylalcohol. The contents were mixed thoroughly and centrifuged at 3000 r.p.m. for 15 minutes. The precipitate was washed three times with 1.75 ml of 95% ethanol. The precipitate was suspended
Figure 6.2 Standard graph for protein estimation by bicinchoninic acid method.
in distilled water mixed thoroughly and used for the estimation of protein bound hexose and fucose as described earlier.

RESULTS

Table 6.1 shows the mean value of salivary total protein expressed in terms of mg/100 ml saliva. For normals and diabetics, the values are 113.2 ± 16.4 (mean ± S.D.) range (85.9-134.5) and 171.0 ± 41.1 (mean ± S.D.) range (120-272) respectively. There is an increase of 1.5 fold salivary protein value in diabetics compared to normals. The increase in salivary protein level is highly significant (P<0.001, t-6.62).

Bound carbohydrates in saliva

The data obtained for total hexose, bound hexose, fucose and sialic acid are shown in Table 6.2. The mean value of salivary total hexose and protein bound hexose are expressed in terms of mannose units in mg/100 ml of saliva. The values of total hexose in normal and diabetic subjects are 21.4 ± 2.8 (mean ± S.D.) range (15.3 - 28.9) and 31.35 ± 7.5 range (20.9 - 43.2) respectively. This corresponds to a 1.46 fold increase in total hexose value and the increase is highly significant (P<0.001, t-6.309). The values of protein bound hexose in normal and diabetic subjects respectively are 7.76 ± 0.15 range (5.3 - 10.6) and 17.45 ± 4.95 range (9.24 - 24.9) corresponding to a 2.25 fold increase in protein bound hexose in diabetic subjects. The increase in this case is also highly significant (P<0.001, t-9.406).

The values of protein bound fucose in normal and diabetics expressed in mg/100 ml of saliva are 3.28 ± 0.7 and 7.40 ± 1.8 (mean ± S.D.) range (2.2 - 4.9) and (4.6 - 10.9) respectively. The mean increase is around 2.25 fold and is statistically highly significant (P<0.001, t-10.66). While the values of protein bound sialic acid in normal and diabetic subjects
### TABLE 6.1
VALUES OF SALIVARY TOTAL PROTEIN (mg/100 ml ± S.D) IN NORMAL AND DIABETIC SUBJECTS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normals mean ± S.D. (range) n=25</th>
<th>Diabetics mean ± S.D (range) n=25</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total Protein</td>
<td>113.21±16.38 (85.9-134.5)</td>
<td>171.08±41 (120-272)</td>
<td>P&lt;0.001</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(t=−6.62)</td>
</tr>
</tbody>
</table>
TABLE 6.2
VALUES OF SALIVARY TOTAL HEXOSE, BOUND HEXOSE, FUCOSE AND SIALIC ACID IN NORMAL AND DIABETIC SUBJECTS
(VALUES FOR TOTAL HEXOSE AND BOUND HEXOSE ARE EXPRESSED AS MANNOSE UNITS IN mg/100 ml SALIVA ± S.D)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normals mean ± S.D. (range) n=25</th>
<th>Diabetics mean ± S.D (range) n=25</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total hexose</td>
<td>21.37±2.83 (15.28-25.92)</td>
<td>31.35±7.47 (20.88-43.2)</td>
<td>P&lt;0.001 (t-6.30)</td>
</tr>
<tr>
<td>Protein bound hexose</td>
<td>7.76±0.15 (5.28-10.56)</td>
<td>17.45±4.95 (9.24-24.96)</td>
<td>P&lt;0.001 (t-9.40)</td>
</tr>
<tr>
<td>Protein bound Fucose, mg/100 ml</td>
<td>3.28±0.68 (2.17-4.92)</td>
<td>7.40±1.83 (4.66-10.90)</td>
<td>P&lt;0.001 (t-10.66)</td>
</tr>
<tr>
<td>Protein bound sialic acid, mg/100 ml</td>
<td>1.90±0.48 (1.01-2.61)</td>
<td>3.22±1.02 (1.65-4.95)</td>
<td>P&lt;0.001 (t-5.94)</td>
</tr>
</tbody>
</table>
are 1.9 ± 0.5 and 3.2 ± 1.0 mg/100 ml of saliva range (1.0 – 2.6) and (1.6 – 4.9) respectively. An increase of around 1.70 fold is observed and is highly significant (P<0.001, t=5.94).

The data indicate that there is significant increase in diabetics of protein bound neutral hexose, fucose and sialic acid. This is in accordance with the increase in protein in saliva of diabetic subjects. The data suggest that a variety of glycoproteins were found to be increased in diabetic subjects compared to normals.

The values of total hexose, protein bound hexose, fucose and sialic acid are also expressed as mg sugar/100 mg of protein. The data are shown in Table 6.3. The values of protein bound hexose for normal and diabetics are 7.36 ± 1.37 and 10.35 ± 2.39 respectively corresponding to an increase of 1.4 fold and the increase is also significant (P<0.001, t=5.52). The values of fucose in normal and diabetic subjects are 3.02 ± 0.59 and 4.47 ± 1.17 respectively. This corresponds to an increase of 1.48 fold and is statistically significant (P<0.01, t=5.59).

For total hexose and sialic acid when the values were expressed in mg per 100 mg of protein, there is no significant changes in the values. The values of total hexose in normal and diabetics are 19.39 ± 3.12 and 18.72 ± 3.96 respectively. The data indicate that there is no significant change in the value of total hexose. Similarly the values for sialic acid in normal and diabetic subjects expressed as mg/100 mg protein are 1.75 ± 0.43 and 1.92 ± 0.51 respectively. In this case also there is no significant change.
TABLE 6.3
VALUES OF SALIVARY TOTAL HEXOSE, BOUND HEXOSE, FUCOSE
AND SIALIC ACID IN NORMAL AND DIABETIC SUBJECTS
(VALUES ARE EXPRESSED IN mg/100 mg PROTEIN)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normals mean ± S.D. (range) n=25</th>
<th>Diabetics mean ± S.D. (range) n=25</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total hexose (as mannose)</td>
<td>19.39±3.12 (15.36-28.8)</td>
<td>18.72±3.96 (10.44-25.94)</td>
<td>P&lt;0.05 (t-0.68)</td>
</tr>
<tr>
<td>Protein bound Hexose (as mannose)</td>
<td>7.36±1.37 (5.28-12.28)</td>
<td>10.35±2.39 (5.42-14.37)</td>
<td>P&lt;0.01 (t-5.52)</td>
</tr>
<tr>
<td>Protein bound Fucose</td>
<td>3.02±0.59 (2.07-4.27)</td>
<td>4.47±1.17 (2.44-6.44)</td>
<td>P&lt;0.001 (t-10.66)</td>
</tr>
<tr>
<td>Protein bound sialic acid</td>
<td>1.75±0.43 (1.01-2.43)</td>
<td>1.92±0.51 (1.02-3.07)</td>
<td>P&lt;0.05 (t-1.37)</td>
</tr>
</tbody>
</table>
Glycoproteins in serum

The data obtained for protein bound hexose and fucose in serum of normal and diabetic subjects are shown in Table 6.4. The mean values of protein bound hexose in normal and diabetics expressed as mg of mannose/100 ml are 103.83 ± 2.55 (range 101 - 109) and 127.92 ± 5.15 (range 120 - 135) respectively. The mean increase is around 1.25 fold and the increase is statistically significant. Since the glycoproteins vary in their relative content of the four monosaccharides, the net increase obtained in the total level would also vary, depending on the monosaccharide for detection. The data obtained for protein bound hexose are in agreement with the values reported by Kennedy et al (257) in terms of fold increase. The reported values of protein bound hexose are 174.7 and 190.8 mg/100 ml for normals and diabetics respectively. Similarly, the mean values of serum protein bound fucose in normal and diabetics expressed in mg/100 ml of serum are 8.30 ± 0.67 (range 7.45 – 8.75) and 17.78 ± 0.60 (range 17 – 18.7) respectively. There is an increase of 2.10 fold in diabetics compared to normal subjects and the increase is statistically significant. The observed values are in agreement with the values reported by Mehta and Venkataraman (258). The reported values are 9.53 ± 2.3 and 23.61 ± 5.32 mg/100ml for normal and diabetics respectively.

DISCUSSION

A number of investigations have addressed salivary alterations in diabetes mellitus. However, a consensus regarding the influence of diabetes on salivary composition especially salivary total protein has not been obtained. One group of workers (250) observed that the protein concentrations in the diabetic patients and in the control group are similar. The reported value for normal and diabetic subjects are 177.2 ± 94.2 and 209.3 ± 146.9 mg/100 ml saliva respectively.
**TABLE 6.4**
VALUES OF SERUM PROTEIN BOUND HEXOSE AND FUCOSE IN NORMAL AND DIABETIC SUBJECTS
(VALUES ARE IN mg/100 ml OF SERUM)

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Normals mean ± S.D. (range) n=25</th>
<th>Diabetics mean ± S.D. (range) n=25</th>
<th>Correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein bound hexose (as mannose)</td>
<td>103.83 ± 2.55 (101–109)</td>
<td>127.92 ± 5.1 (120 – 135)</td>
<td>P&lt;0.01 (t=15.93)</td>
</tr>
<tr>
<td>Protein bound fucose</td>
<td>8.30 ± 0.67 (7.45 – 8.75)</td>
<td>17.78 ± 0.60 (17.0 – 18.7)</td>
<td>P&lt;0.01 (t=10.3)</td>
</tr>
</tbody>
</table>
However, another group of workers (251) reported an increase in salivary protein level in diabetes. Our studies show that there is an increase of 1.5 fold in salivary protein value in diabetics compared to normal subjects. The salivary protein values estimated by bicinchoninic acid method, expressed as mg/100 ml saliva (mean ± S.D.) for both normal and diabetics are 113.21 ± 16.38 and 171.08 ± 41.10 respectively. The increase in salivary protein level is highly significant. Our findings are in agreement with the values reported by Kertesz and associates (251). As proposed by Mandel (252), increased basement membrane permeability, often associated with diabetes, is one of the possibilities for the increased passage of proteins from exocrine glands into their secretions in some patients.

The values for total hexose and protein bound neutral hexose are expressed in terms of mannose units (mg/100 ml of saliva). The data indicated that there is a significant increase in diabetes, of protein bound neutral hexose, fucose and sialic acid. There is an increase of 1.46 fold in total hexose value and the increase is highly significant. Similarly an increase of 2.25 fold with respect to protein bound neutral hexose was found. The increase in this case is also highly significant. With fucose and sialic acid the fold increase is around 2.25 and 1.70 respectively for diabetics compared to normal subjects. These increases are in accordance with the increase in protein in saliva in diabetics, which suggest that a variety of glycoproteins were found in elevated amounts.

The values were also expressed as mg sugar/100 mg protein. The protein bound hexose when expressed as mg of mannose/100 mg protein, there is a 1.4 fold increase in diabetics compared to normal subjects. However, the fold increase is comparatively less unlike when expressed as mg/100 ml saliva. Similarly, there is an increase of 1.48 fold in fucose in diabetic subjects. When
the values for total hexose and sialic acid expressed as a function of protein concentration, the increase is offset by increase in total protein in saliva.

With respect to serum protein bound hexose and fucose, there is a significant increase in protein bound fucose and a marginal increase in protein bound hexose in diabetics compared to normal subjects. These data suggest that increased glycosylation of serum proteins may occur in diabetes mellitus leading to an abnormality in serum protein bound neutral hexoses and fucose.