CHAPTER VIII

GENERAL SUMMARY AND DISCUSSION
The first part of the work presented in this thesis deals with the effect of cysteine on chromogen formation from hydroxymethylfuraldehyde (HMF) and furaldehyde in o-cresol: sulfuric acid reaction. It also deals with the differential behavior of hexoses and pentoses in o-cresol: sulfuric acid reaction in presence of cysteine. Subsequent to this, a study on the effect of sulfuric acid concentration on chromogen formation from furaldehydes and some monosaccharides in the absence and in the presence of cysteine is presented. Based on the differential behavior of hexoses and pentoses in presence of cysteine in o-cresol: sulfuric acid reactions, conditions were standardized for the development of a method for the estimation of neutral hexoses in presence of pentoses. The second part of the work deals with the development of a simple, reliable, non-invasive colorimetric method for the estimation of glyco groups attached non-enzymatically to proteins in hair and nail samples for the detection and management of long-standing diabetes mellitus. The last part of the work deals with the study of serum and salivary glycoproteins in normal and diabetic subjects. This thesis also includes a study on salivary total protein in normal and diabetic individuals. Further, a colorimetric method has been described for the estimation of salivary fructosamine, which can be used for the short-term assessment of glycemic control in diabetic individuals.

Since, HMF and furaldehyde considered to be the major products formed during the degradation of hexoses and pentoses respectively (53, 38) in acidic medium, preliminary studies were carried out with HMF and 2-furaldehyde. Among the hexoses, glucose, fructose, mannose and galactose were chosen for the following reasons. Fructose and glucose are known to form comparable amount of HMF in 75% sulfuric acid, whereas mannose and galactose yield 50% of HMF under similar conditions (58, 59 and 63). Among the pentoses, xylose, ribose and arabinose were chosen for the study. Xylose is known to quantitatively degrade to 2-furaldehyde while ribose and arabinose form considerably more of alternative products than 2-furaldehyde (61).
The studies with HMF/furaldehyde were carried out under two different reaction conditions namely, the one-step and two-step o-cresol (cysteine): sulfuric acid reactions. In the one-step method, both furaldehyde and developer were exposed to a temperature of 115-118°C caused by the heat of dilution of sulfuric acid. In the two-step method, only furaldehydes were exposed to 115-118°C and the developer was added to the reaction mixture after it cooled down to room temperature. The two-step reaction was carried out based on a report by Rao and Pattabiraman (44) regarding the sulfonation of phenol occurring in the high temperature conditions of the one-step method, leading to a decrease in the effective concentration of free phenol required for the condensation reaction with furaldehyde.

Analysis of the absorbance values of the condensation products formed by HMF/furaldehyde with cysteine indicated that the condensation product formed by furaldehyde with cysteine in 75% sulfuric acid, in one-step assay, is heat labile compared to the product formed by HMF. In the two-step method, both HMF and furaldehyde reacted efficiently at low cysteine level. Higher values with furaldehyde can be attributed to the stability of the condensation product (thiazolidine) at room temperature. The chromogen yield from HMF did not vary significantly with varying cysteine concentration. The color yield from furaldehyde was reduced drastically at high cysteine level.

Analysis of the absorption spectra of HMF in presence of cysteine in o-cresol: sulfuric acid reaction showed a major peak at 500 nm and a hump at 410 nm. Similarly, furaldehyde under similar conditions had a major peak at 485 nm and a minor peak at 395 nm. Hump around 410 nm for HMF and a minor peak at 395 nm for furaldehyde provide evidence for thiazolidine formation in the overall reaction.

Cysteine when included in the assay system significantly enhanced chromogen formation from HMF and furaldehyde in the one-step
o-cresol: sulfuric acid reaction. This is similar to the observation of Halliwell et al. (112) who showed that cysteine enhanced reaction of some hexoses and pentoses in phenol: sulfuric acid reaction. The increase in color intensity by cysteine is due to faster reaction of thiazolidines with o-cresol than with the parent furaldehydes. Cysteine was found to protect degradation of furaldehydes especially HMF in presence of hot 75% sulfuric acid.

The chromogen formation by furaldehydes in the two-stage assay with o-cresol was better than that in the one-stage assay. This can be attributed as indicated by Rao and Pattabiraman (44) to the rapid sulfonation of phenols and effective reduction of the concentration of prochromogens. This appears to be partially reversed by cysteine.

Differential behavior of HMF and furaldehyde in a reaction with o-cresol in presence of cysteine, in two-step assay, is interesting. The patterns were different depending on the compounds and on the fact, whether cysteine was included in the first-stage (during heat of dilution) or in the second-stage (along with o-cresol). The condensation product formed by furaldehyde with cysteine (thiazolidines) is less stable in acid medium than furaldehyde per se. Furaldehyde cysteine adducts is also less stable than the product formed from HMF and cysteine. Further, cysteine was found to significantly reduce chromogen formation of furaldehyde with o-cresol, when the thiol was used at high concentration. This is especially significant when furaldehyde and cysteine were exposed to hot 75% sulfuric acid.

Fructose and mannose behaved similar to HMF in the two-step assay with cysteine. Ribose showed a pattern similar to furaldehyde. Since the chromogens formed from hexoses had absorption maxima similar to that of HMF and since ribose showed a pattern similar to that of furaldehyde, it is obvious that furaldehydes formed from sugars are responsible for the condensation products formed with cysteine. Higher concentration of cysteine marginally inhibits
chromogen formation from hexoses, but significantly from ribose. Color yield from mannose is much lower than from fructose. This is due to the fact that yield of HMF from mannose is low.

In the one-step o-cresol: 75% sulfuric acid assay, cysteine significantly increased chromogen formation for fructose ($\lambda_{max}$ 495 nm. absorbance values for 20 $\mu$g sugar are 0.33 without cysteine, 0.799 with 5 mg cysteine and 0.950 with 30 mg cysteine). This is quantitatively similar to the effect of cysteine on chromogen formation by HMF with o-cresol. Effect of cysteine on mannose was marginal (corresponding $\lambda_{max}$ 495 nm. absorbance values are 0.564, 0.754 and 0.760). No significant effect was seen with ribose also ($\lambda_{max}$ 485 nm. absorbance values for 20 $\mu$g ribose are 0.645, 0.700 and 0.635 respectively). The behavior of cysteine with ribose is in sharp contrast to the behavior with furaldehyde. This is probably due to the fact that ribose forms considerably more of the alternative products than furaldehyde when exposed to aqueous acid solution at an elevated temperature. The spectra of chromogens formed by both the hexoses with o-cresol in presence of cysteine were almost identical to the spectra in the absence of cysteine. Further, no absorption maxima in the region, 390-410 nm were observed indicating that there is no significant residual thiazolidines in the system.

When the acid concentration was 60%, cysteine had no significant effect on chromogen formation by these sugars with o-cresol. Fructose reacted efficiently with o-cresol in 60% sulfuric acid medium compared to 75% acid concentration. This is very much similar to the observation made by Mallya and Pattabiraman (110) who showed optimal sulfuric acid concentration for fructose in condensation reaction with 2,6-dimethylphenol is 57.5%.

The behavior of hexoses and pentoses in the two-step o-cresol: sulfuric acid assay in presence of cysteine is quite different. A significant observation was, cysteine powerfully inhibited chromogen formation from ribose when
included in the first-stage. A similar observation was made with reference to furaldehyde earlier. It can be concluded that the thiazolidine of furaldehyde with cysteine is highly unstable to hot sulfuric acid condition, whereas the corresponding products from hexoses are heat stable. Another observation was that, cysteine significantly enhances chromogen formation from fructose and mannose when included in the first-stage. Presence of cysteine marginally shifted \( \lambda \) max of the chromogens in two-stage method. Behavior of fructose and mannose are similar to that of HMF.

When additional heating at 100°C for 10 minutes was included in the assay system, color intensity of mannose decreased by 14%, whereas that of ribose decreased by 75%. When color intensity was measured after 24 hours, ribose showed no color at all, whereas mannose showed a decrease of only 5%. This differential behavior of hexoses and pentoses towards cysteine in o-cresol: sulfuric acid reaction with a provision of additional heating for 10 minutes and measurement of absorbance after 30 minutes provides an opportunity of estimating differentially hexoses and pentoses in biological fluids.

Based on the effect of cysteine in o-cresol: sulfuric acid reaction, a colorimetric method has been developed for the estimation of hexoses in presence of pentoses. This is based on the observation that the condensation product of furaldehyde with cysteine is highly labile to hot 75% sulfuric acid and on standing for 24 hours.

The one-step and two-step o-cresol: 75% sulfuric acid assay methods were applied for the estimation of glycated proteins in hair and nail in diabetics and normal individuals, so as to check its utility for the long-term assessment of glycemic status.

Preliminary studies showed that hair and nail samples reacted with o-cresol in sulfuric acid medium to form chromogens, which were somewhat similar in spectral characteristics to those formed from HMF and fructose. Nail samples
showed maximum color intensity around 65% sulfuric acid concentration in direct one-step assay. In the two-step method, optimum sulfuric acid concentration for nail samples was 75% as was with fructose. Time taken for maximal color development was found to be 10 minutes for nails. Optimal concentration of o-cresol required, was higher (20-30mg) for the estimation of glyco groups in nail samples. Hair samples could not be subjected to direct assay, since pigments interfered in the color development.

Since the pigments of hair samples interfered in the color development in the direct method, the following procedure was devised to overcome the problem. The hair or nail samples were subjected to prior acid hydrolysis with 1.33 M HCl for varying time intervals. The proteins along with the pigments were precipitated by the addition of equal amount of 20% trichloroacetic acid. The clear supernatant (1.0 ml) was subjected to two-step o-cresol: sulfuric acid reaction. With the hair and nail samples, the maximal time of prior hydrolysis with 1.33 M hydrochloric acid was 4 hours at 100°C. The chromogens formed by hair and nail samples were identical to that of HMF in o-cresol: sulfuric acid reaction. Inclusion of cysteine in the assay system did not enhance chromogen formation.

Glyco group contents in hair, thumb and toe nail samples were compared. It was found that the extent of glycosylation of nail samples is significantly more than the hair samples.

The mean values of glycosylation of hair in normal and diabetic subjects estimated by the two-step o-cresol: sulfuric acid assays method, after prior hydrolysis with 1.33 M HCl are 0.089 ± 0.006 (mean ± S.D.) and 0.173 ± 0.056 µg fructose/mg sample respectively. There is an increase of 1.94 fold in diabetics and the increase is highly significant. The values for thumb nails in terms of µg fructose/mg sample for normal and diabetic subjects are 0.237 ± 0.026 (mean ± S.D.) and 0.403 ± 0.088 respectively. The increase is around 1.7 fold, which is highly significant. Raghavendra and Pattabiraman (45)
reported a 1.45 fold increase in glycated hemoglobin values in diabetics compared to normal subjects using o-cresol: sulfuric acid method. Similarly the data also agree with the values obtained by Paisey et al (137) by thiobarbituric acid method. They reported a 1.79 fold increases in hair glycosylation level in diabetics compared to normal subjects.

The present studies show that extent of glycosylation is about 14 times lower in hair proteins than in hemoglobin in normal subjects. However, an increase in glycosylation in hair is found to be slightly more than in hemoglobin in diabetics.

Glycosylation values obtained by the direct method for nail samples are found to be significantly increased in diabetics. The increase is around 1.7 fold. The values obtained by direct two-step method are comparable with the values obtained by prior hydrolysis with mild acid in terms of fold increase in glycosylation. The method described is non-invasive, reliable and therefore can be used routinely for the assessment of long-term glycemic control in diabetic individuals.

The protein bound hexose and fucose was estimated in serum and saliva. There is a significant increase in serum protein bound neutral hexoses in diabetics compared to normal subjects. The mean values ± S.D. expressed as mannose units (mg/100 ml) for normal and diabetics are 103.83 ± 2.55 mg/100 ml (range 101-109 mg/100 ml) and 127.92 ± 5.1 mg/100 ml (range 120-135 mg/100 ml) respectively. The mean value of serum protein bound fucose expressed as mg/100 ml (mean ± S.D.) in normal and diabetic subjects are 8.30 ± 0.67 (range 7.45 – 8.75) and 17.78 ± 0.60 (range 17.0 –18.7) respectively. There is an increase of 2.1 fold in diabetics compared to normals and the increase is statistically significant.
Similarly, total protein, total hexose and glycoproteins present in the saliva were measured under normal and diabetic conditions. Studies with diabetic patients indicated that there is a significant increase in the total protein value compared to normal subjects. The mean value of total salivary protein expressed as mg/100 ml ± S.D. in normal and diabetic subjects are 113.2 ± 16.4 (range 85.9-135) and 171.0 ± 41 (range 120-272), respectively. The increase is statistically significant. The increase in salivary proteins in the diabetics could be attributed to the increase in basement membrane permeability allowing easy and increased passage of serum proteins into the whole saliva via the salivary glands and gingival crevices. The salivary total hexose, protein bound neutral hexose, fucose and sialic acid were measured in normal and diabetic subjects. The value of total hexose expressed as mannose units in mg/100 ml of saliva in normal and diabetics are 21.4 ± 2.9 (mean ± S.D.) range (15.3 - 26) and 31.6 ± 7.5 range (20.9-43) respectively. The values of protein bound neutral hexose expressed as mannose units mg/100 ml ± S.D. in normal and diabetics are 7.75 ± 0.15 and 17.45 ± 4.95 respectively. There is an increase of 2.24 fold in diabetics compared to normal individuals. The values of protein bound fucose in normal and diabetics are 3.28 ± 0.68 and 7.40 ± 1.83 mg/100 ml respectively. This corresponds to an increase of 2.25 fold in diabetic subjects. Similarly, the values of protein bound sialic acid in normal and diabetic subjects are 1.90 ± 0.48 and 3.22 ± 1.02 mg/100ml saliva respectively. This is in accordance with the increase in protein in saliva in diabetics, which suggests that a variety of glycoproteins were found in elevated amounts. The values for protein bound individual neutral hexose were also expressed as mg sugar/100 mg protein. The values of total hexose expressed as mannose units in mg/100 mg protein (mean ± S.D.) in normal and diabetics are 19.4 ± 3.1 (range 15.4 – 28.9) and 18.7 ± 4.0 (range 10.5 – 26.0) respectively. The values of protein bound hexose expressed as mg mannose/100 mg protein (mean ± S.D.) in normal and diabetics are 7.4 ± 1.4 (range 5.3 – 12.3) and 10.4 ± 2.4 (range 5.4 –14.4) respectively.
Similarly the values of fucose in normal and diabetics are 3.0 ± 0.6 mg/100 mg protein (range 2.07 – 4.3) and 4.5 ± 1.2 (range 2.4 – 6.4) respectively. The values of protein bound sialic acid expressed as a function of protein in normal and diabetics are 1.75 ± 0.40 mg/100 mg protein (range 1.01 – 2.4) and 1.92 ± 0.50 (range 1.0 – 3.1) respectively. There is a marginal increase in protein bound neutral hexose and fucose. No change in the values with respect to total hexose and protein bound sialic acid were noticed.

Lastly, serum and salivary fructosamine were measured in normal and diabetic subjects so as to check its utility for the assessment of short-term glycemic control. Serum fructosamine was measured by the method described by Johnson et al. (185) for salivary fructosamine estimation after modifications. The modification involved was increase in the volume of saliva in assay conditions to 1.0 ml. Concentration of NBT used was also increased to 0.75 mmol/L. Further, reaction rate timing was increased from the conventional 10 to 15 minutes to the modified 10 to 60 minutes. The mean value of serum fructosamine expressed in terms of µ mole DMF/ml for normal and diabetic subjects are respectively 1.69±0.124 (range 1.45-1.95) and 2.29±0.399 (range 2.99-3.82). An increase of 1.76 fold was observed in diabetics compared to normal individuals and the increase was statistically significant. Similarly, salivary fructosamine was determined by the modified method. The data showed a significant increase in diabetics compared to normal. The observed values for normal and diabetics are 0.034 ± 0.002 (range 0.032-0.040) and 0.050 ± 0.008 (range 0.038-0.074) respectively in terms of µ mole DMF/ml. When the values of salivary fructosamine were compared to serum fructosamine, a 50-fold decrease in the value of salivary fructosamine was observed. Such a low value of salivary fructosamine is probably due to low concentration of protein in saliva. Further there is little time for glycosylation of protein in saliva unlike in serum and bulk of the fructosamine appears to originate from serum. When the values were
expressed as a function of protein, the increase is offset by increase in total protein in saliva.

To conclude, based on the effect of cysteine in o-cresol: sulfuric acid reaction, a new colorimetric method has been developed for the estimation of hexoses in presence of pentoses. A non-invasive simple and reliable technique for the assessment of long-term glycemic control in diabetes has been developed. This involves estimation of glyco groups bound non-enzymatically to proteins in hair and nail samples. In this procedure, samples of hair and nails are treated with 1.33 M hydrochloric acid at elevated temperature. The dehydrated products released are condensed with o-cresol in sulfuric acid medium to form characteristic chromogen, which is quantitated. Protein bound neutral individual hexoses, fucose and sialic acid were fond to be increased in saliva, in diabetes in terms of units/100 ml of saliva suggesting alteration of glycoprotein levels in diabetes mellitus. Finally, a simple colorimetric method has been developed for the estimation of salivary fructosamine, which can be used as an adjunct to other parameters, for the short-term assessment of glycemic control in diabetic subjects.