5.0. DISCUSSION

In an effort to evaluate simple and useful assay strategies for the screening of substances that interfere with glycation, two proteins HSA and RNase A were selected. Three sugars glucose, fructose and ribose with known differences in reactivity were used and a comparison was made of changes in the proteins induced by the sugars. The alterations studied included behavior in SDS-PAGE, intrinsic fluorescence, hyperchromicity and new fluorescence as well as adduct formation using ketoamine and borohydride-periodate assays. In case of RNase A, decrease in the ability to hydrolyze yeast RNA was also taken as a measure of glycation.

HSA is the most abundant protein in blood constituting about 40 mg/ml plasma (Shaklai et al., 1984). HSA function as a carrier of wide variety of substances including amino acids, metals, hormones, metabolites like bilirubin, hemin and fatty acids and many therapeutic compounds. HSA plays a protective role in vivo as an antioxidant (Bourdon et al., 1999). In normal individuals, nearly 10 % of the HSA is modified by glycation and the modification increases 2-3 folds under hyperglycemic condition (Guthrow et al., 1979). Glycated albumin is therefore considered a useful marker for short term monitoring (2-4 weeks) of diabetic patients. The level of glycated albumin reflects the degree of hyperglycemia in diabetes (Nakajou et al., 2002).

Glycation of protein has been shown to result in protein degradation and/or crosslinking (Singh et al., 2001; Rosca et al., 2005) and the observed alterations in the migration behavior of HSA incubated with the sugars (Fig.10) apparently results from such effect. Glucose was least reactive but additional small molecular weight bands appeared in the gel after 8 days of incubation (Fig. 10A). Interestingly, samples incubated with fructose and ribose migrated as highly diffuse bands with the increase in incubation time and showed the presence of
aggregates rather than fragments (Fig. 10B). High reactivity of the Amadori and other intermediate generated by ribose and fructose (ref) may contribute towards aggregate formation. Incubation of HSA with sugars also resulted in a time-dependent induction of carbonyl groups in the molecule which increased more rapidly in the samples incubated with ribose (Fig. 11). It is well recognized that carbonyl groups are introduced in the proteins as a consequence of glycation, and protein carbonyl content is considered as a reliable measure of glycation (Beal, 2002). Similarly, new fluorophore formation, quenching of intrinsic fluorescence, hyperchromicity at 280 nm as well as loss of tertiary structure, as evident from CD measurement were most prominent in HSA incubated with ribose. The difference in observed rates of inactivation resulting from the incubation with various sugars is attributable to their relative reactivities in glycation reactions (Seidler, 2005), which in turn dependent upon the concentration of the respective open chain form at equilibrium (Kaneto, 1994). Ketoamine and periodate assays however were contradictory showing fructose to be least reactive among the three sugars (Fig. 12, 13). The failure of these colorimetric assays to detect the Amadori product formed by the fructose may explain the observation. The Amadori product (AP) formed by fructose is an aldehyde with a carbonyl at C-1 which is unable to cyclize and hence cannot react with redox dyes such as NBT in the fructosamine assay and it also does not liberate formaldehyde in periodate assay from which the chromophores are generated.

\[
\begin{align*}
\text{Glucose-AP} & \quad \text{Fructose-AP} \\
\text{H} & \quad \text{H} \\
\text{\textbullet} & \quad \text{\textbullet} \\
\text{H-C-N} & \quad \text{H-C-N} \\
\text{C=O} & \quad \text{C=O} \\
\text{HO-C-H} & \quad \text{HO-C-H} \\
\text{H-C-OH} & \quad \text{H-C-OH} \\
\text{H-C-OH} & \quad \text{H-C-OH} \\
\text{CH_2OH} & \quad \text{CH_2OH}
\end{align*}
\]
It is now well recognized that reaction of proteins with reducing sugars can cause marked alterations in their structure and conformation (Ahmed, 2005). The resulting covalent crosslinking may induce stress in the molecule, triggering the overall structural changes as revealed by quenching in intrinsic fluorescence, hyperchromicity, generation of new fluorescence and alterations in the CD spectra (Fig. 14, 15 and 16). Loss of intrinsic fluorescence is attributed to the destruction of the tryptophan and/or modification of the tryptophan microenvironment (Davies et al., 1987). With increase in time of incubation with sugars there was a consistent increase in the peak value of emission in range 400-500 nm that suggests the formation of glycation adducts and AGEs. Increase in absorbance at 280 nm (Fig. 16) is attributed to protein unfolding and exposure of the buried chromophoric groups to the solvent (Traverso et al., 1997). CD spectra (Fig. 17) also showed alterations in the helical behaviour of HSA. The structural alterations suggest by an increase of beta structure and random coil at the expense of alpha helix. The incubation of HSA with sugars appears to promote conformational changes leading to instability in the secondary structure. A comparison of intrinsic fluorescence with far CD in a recent study confirmed that glycated HSA lost tertiary structure before losing secondary structure (Mendez et al., 2005). While the alterations in behavior of protein reported here are common to most proteins, the magnitude of glycation-induced changes may vary. It was shown earlier that while amino groups are primary targets, the extent of glycation does not directly depend on the number of glycation-prone residues but on the solvent accessible surface area of such residue (Moulick et al., 2007). These studies however suggested that fructose and ribose behave very similar to glucose but react faster with HSA leading to more rapid modification of the protein.

A number of natural or synthetic compounds and drugs as AGE inhibitors have been proposed and identified. We have studied the protective effect of some metal ion chelators, antioxidants, analgesics and other drugs that have been shown to
interfere with glycation by restricting structural alterations, protein crosslinking etc. For this study fructose was selected as a model sugar because of its high reactivity as compared to glucose and the fact that at least in some tissues in vivo, glycation with the sugar is significant under specific conditions (Kinoshita et al., 1979; Tomblinson, 1985). The inhibition of crosslinking and conformational alterations by radical scavengers and metal ion chelators DETAPAC and EDTA strongly suggest the role of free radicals in glycation. DETAPAC and EDTA are believed to act by preventing metal catalyzed oxidation of sugars (Wolff and Dean, 1988, Well-Knecht et al., 1995). Aminoguanidine on the other hand blocks the formation of sugar carbonyl and ketoamine or their derivatives by competing with nucleophilic groups on proteins (Thornalley, 2003). Aspirin protect proteins against glycation by acetylating the free amino groups (Swamy and Abraham, 1989) while analgesics like ibuprofen and paracetamol act by reducing the oxidative stress (Malik, 1996). Our experiments (Fig. 18, 19) suggested that ascorbic acid was least effective of all the inhibitors studied in preventing structural changes associated with glycation. Ascorbic acid is structurally similar to sugars therefore it is possible that it competes with fructose for sites of glycation. The protective property of ascorbic acid is also attributed to its ability to scavenge free radicals that play an important role in glycation reactions (Malik, 1996). The exact mechanism of action of these compounds needs to be further elucidated. The present study however indicates that these are powerful inhibitors which may act at multiple steps of glycation and AGE formation. This study also shows that fructose can be conveniently used as a model sugar for study of glycation inhibitors except while using the periodate and ketoamine assays.

There are reports of several plant products that show remarkable AGE inhibitory activity. These include resveratrol, a natural estrogen present in grapes (Mizutani et al., 2000), curcumin found in turmeric (Sajithlal et al., 1998), S-allylcysteine a constituent of garlic (Ahmad and Ahmed, 2006), rutin that occurs in tomatoes
(Kiho et al., 2004) and polyphenolic compounds in *Zea mays* (Farsi et al., 2008). Green tea contains tannins (flavonoids) having significant antiglycation properties (Nakagawa et al., 2002). Dearlove et al. (2008) showed polyphenolic substances in extracts of various herbs and spices inhibit fructose-mediated protein glycation.

Several flavonoids are also known to act as antioxidants (Wu and Yen, 2005). Since some antioxidants also act as inhibitors of glycation (Urios et al., 2007), we studied the effect of some flavonoid on HSA glycation by fructose. Some bioflavonoids have also been shown to reduce formation of glycated hemoglobin (Manuel et al., 1999). According to Matsuda et al. (2003), the principal structural requirement of flavonoid for the inhibition of AGE formation, is the presence of vicinyl dihydroxyl group at 3', 4', 5 and 7-position. All the flavonoids used in our study fulfil this requirement, although the magnitude of their inhibition on the glycation reaction varied remarkably.
Discussion

Our finding herein showed the reduced formation of glycation adducts in the presence of rutin and quercetin which is comparable with the protection provided by DETAPAC, EDTA and aminoguanidine. However, 10 μM rutin and quercetin produced inhibitory effect with that of 25 mM aminoguanidine or DETAPAC, which is remarkable. The chelators used in the experiment were present in excess to ensure maximum inhibition. Inhibition of free radical generation derived from protein glycation and subsequent inhibition of protein modification is one of the mechanisms of prevention of glycation (Farrar et al., 2007). The results suggest the usefulness of the assays in screening of the inhibitors of glycation and potential of some flavonoids in reducing the glycation-induced protein damage.

The data however suggest that inhibition of the formation of fluorescent adducts and AGEs presumably occurs by trapping of the ROS and carbonyl intermediates. Another parameter used for the study of protein glycation is the formation of protein crosslinks. Restriction of the crosslink formation by flavonoids suggests that their inhibitory activity is not only due to their antioxidant properties but also to some additional mechanism. In a recent study polyphenol rich extracts from the plant *Ilex paraguariensis* have been shown to inhibit the formation of AGEs (Lunceford and Gugliucci, 2005). In addition, green tea extract containing polyphenolic compounds have also shown to delay diabetic complications (Vincen and Zhang, 2005).

Our data suggest that micromolar concentration of flavonoids effectively reduce the sugar-induced protein damage. Consumption of the plant products in diet may therefore contribute to the restriction in protein glycation. Further characterization of flavonoid metabolites and metabolite structure-function relationship may lead to a better understanding of the role of these compounds in delaying progression of complications related to hyperglycemia. The study also suggests that fructose-induced glycation of HSA can be conveniently followed by
the measurement of hyperchromicity, tryptophan fluorescence, new fluorescence as well as electrophoretic behavior of protein in the presence of SDS.

Earlier studies have shown that RNase A incubated with sugars undergoes structural modification and loss in catalytic activity (Khalifah et al., 1996). As shown in Fig. 24, glucose, fructose or ribose cause marked inhibition of RNase A activity at 37°C and the loss of catalytic activity was most rapid in the presence of ribose. Incubation with glucose, fructose or ribose resulted in loss of 35, 62 and 84 percent activity respectively in eight days. The difference in observed rates of inactivation resulting from the incubation with various sugars as discussed earlier is attributable to relative reactivities of the sugars in glycation reactions (Seidler, 2005). The remarkably high reactivity of ribose towards RNase A may also be related to the structural similarity of the sugar with the enzyme substrate.

Pancreatic RNase A is an extensively studied and highly thermostable enzyme (Raines, 1998). RNase A undergoes remarkable alteration in structure and catalytic activity when incubated with glucose and other reducing sugars as discussed earlier. Lysine is a constituent of the active site of RNase A (Heinrickson, 1966), which is apparently highly reactive towards sugars and incubation with sugars resulted in a rapid inactivation of the enzyme. Since ribose is closely related to the enzymatic substrate of RNase and Lys-41 of the active site most easily form Amadori products (Watkins et al., 1985) and AGEs (Brock et al., 2003). Fig. 36 A shows that the catalytic activity of the enzyme is barely affected by incubation in the absence of sugars at 60°C for two days while the activity loss was remarkable in presence of the sugars especially ribose under the same conditions. This facilitated the assay of glycation at 60°C at which reactivity of sugar is very high which reduced the assay duration to two days. Most of the glycation assays used currently require weeks and even months. In an earlier study Matsuura et al., (2002) have used BSA at 60°C to increase the rate of glycation however albumin is known to undergo unfolding at such temperature (Takedo et al., 1993).
The loss in activity of RNase A resulting from the incubation with various sugars was comparable when either soluble or immobilized RNase A was used. This suggested that immobilization does not markedly alter the reactivity of sugars towards enzyme. It is interesting that RNase incubated in absence of sugars for two days at 60°C exhibited only minor alterations in hyperchromicity, quenching of intrinsic fluorescence and CD spectral behavior, suggesting the retention of three dimensional structure of enzyme (Fig.37, 38, 39 and 40). While it is true that ribose is not the principle sugar responsible for in vivo glycation reactions, structural alterations induced by ribose in proteins are similar to those caused by glucose. This include the cross reactivity of AGEs generated by the sugar with anti AGE antibodies raised against protein Glycated using glucose (Nagai and Horiuchi, 2003). In addition, ADP-ribose may actually cause glycation of proteins in vivo, although to a smaller degree (Cerventes-Lauren et al., 1996). We therefore felt that a combination of RNase as model protein and ribose as model sugar provides a remarkable advantage in cutting down the assay duration and facilitates the rapid screening of inhibitors of glycation.

The comparable sensitivity of immobilized RNase A with that of its soluble counterpart towards glycation induced enzyme inactivation points out to the possibility of using the later with added advantage for screening of the anti glycation principles from natural source. Immobilized RNase A, in addition to being more stable (Younus et al., 2001), offers the possibility of its rapid removal from the reaction mixture and hence elimination of components of the crude extracts that may interfere with the enzyme assay. As shown in Fig. 42, an excellent agreement between protections offered by DETAPAC, EDTA, aminoguanidine, paracetamol and ascorbic acid was observed when either soluble or immobilized RNase A was used.

Similarly, among the plant extracts that exhibited significant protective effects against ribose induced inactivation of RNase A, excellent agreement was observed
in case of ginger, garlic and bitter gourd extracts both with soluble or immobilized enzyme. The extracts like those of green tea, grapes and tomato however appeared more protective when immobilized RNase A was used as discussed earlier, due to interference in the assay by components of the coloured extracts (Fig. 43). Since the concentration of ribose used in the assay is remarkably high, interference by the amino group modifying molecules present in the extract may not be significant unless they are highly reactive.

These results suggest the usefulness of RNase-ribose assay for the rapid screening of extracts for inhibitors of glycation. Since only measurement of catalytic activity of RNase A are required, a simple spectrophotometer is adequate for the analysis. Also use of immobilized RNase A provides additional advantage of high storage stability (Younus et al., 2001) and possibility of distinguishing between early and posts Amadori inhibitors of glycation. For the study of such inhibitors, rapid removal of the glycating sugar after initial reaction with protein is required. This is normally accomplished by dilution or dialysis (Matsuura et al., 2002).

Immobilized RNase A on the other hand can be readily separated from the reaction mixture instantaneously by centrifugation and/ or filtration and freed of the sugar by washing. The immobilized enzyme will however not be reusable since part of the activity will be irreversibly lost during each assay.

A number of enzymes have been shown to undergo glycation induced loss in catalytic activity both in vivo and in vitro. Cabellero et al. (1998) and Bousova et al. (2005) suggested that the loss of catalytic activity of δ-aminolevulinic acid dehydratase and aspartate aminotransferase may be use as a measure of glycation. RNase A offers additional advantage being remarkably more stable and facilitating reaction at high temperature and thus cutting down the assay duration. Since the assay involves measurement of protection of RNase A activity, we believe it will measure only those substances that restrict the glycation reaction. Natural Inhibitor of RNase A present in the extract may contribute additionally towards
the inhibition but can be taken care of by using an appropriate control in which enzyme may be preincubated with extract for short durations. Similarly possible by activators of the enzyme and/or RNase A present in the extract may corrected by running parallel appropriate controls.

Result shown in section 4.4 shows that antiRNase A antibody support protect the bound enzyme against inactivation and other alterations induced on incubation with reducing sugars. As shown in Fig. 48 the remaining activity of RNase A immobilized on CM- cellulose and incubated for eight days with sugars was only slightly higher than that of soluble enzyme incubated under the conditions. RNase A bound to the antiRNase A antibody support however retained markedly higher activity as compared to the soluble and CM-cellulose-bound enzyme. Apparently immobilization per se is not responsible for the observed protection provided by the antibody support, since CM- cellulose bound enzyme was only moderately protected (Fig. 48). It is interesting to note that RNase A is expected to bind to CM cellulose via side chain amino groups which are potential target of glycation reaction (Ganea and Harding, 2005), yet the observed protection against sugars was small. RNase A immobilized on the antibody support was however clearly more resistant to the sugar-induced alterations and also showed minimum alteration in the electrophoretic behavior (Fig. 49).

Spectroscopic studies of the soluble RNase A, and that incubated with the sugars while remaining bound to the antibody Sepharose further revealed the protective role of the antibodies (Fig. 50, 51 and 52). Considerable evidence suggests that binding of antienzyme antibodies to enzymes improves their resistance to inactivation induced by heat and other agents (Saleemuddin, 1999). Our more recent studies have also shown that inactivation of Cu,Zn-SOD by sugars (Jabeen and Saleemuddin, 2006a), glyoxal (Jabeen et al., 2007) and methylglyoxal (Jabeen et al., 2006b) can be restricted by complexing the enzyme with specific antibodies. In addition binding with antibody/antibody fragment resulted in
restriction of enzyme aggregation as revealed by SELDI-TOF spectroscopy. Since both monomeric Fab and Fab² were protective, binding per se rather than crosslinking induced by the bifunctional antibodies fragment appears to be responsible for the observed protection (Jabeen et al., 2007). The observation that enzyme bound to the antibody support is also resistant to glycation support this argument, since crosslinking of the antibody is less likely on the antibody support.

Binding of antibodies to protein antigen results in marked lowering of the free energy and stabilization of the protein antigens because of the high association constant. Hydrogen exchange rate investigation on lysozyme and cytochrome c amide group suggested that in the complex, the exchange rates were remarkably low not only at the point of contact between the antigen and the antibody but also in the regions far removed from the epitope recognized by the antibody (Rizzo et al., 1992; Williams et al., 1996). Several enzymes complexed with monoclonal/polyclonal antibodies exhibit enhanced stability against various forms of inactivation (Saleemuddin, 1999). Our earlier studies have shown that antibodies raised against the labile region of RNase were more protective than those recognizing the N-terminal peptide (Younus et al., 2001). The labile region-specific antibodies also improved the thermal stability of a mutant RNase in which the labile region contained an altered cysteine (Younus et al., 2002). Studies with anti-lysozyme antibodies however suggest that binding of the monoclonal antibody raised against the native enzyme improves the thermal stability of the enzyme even when it do not recognize epitopes located close to the mutation site. Thus D67H mutation of the human lysozyme related to systemic amyloidosis undergoes partial unfolding and aggregation with a melting temperature 10°C lower than that of the native enzyme (Canet et al., 2002). Binding of c-Ab-HUL6 increased the melting temperature of the enzyme by 15°C, although the residue whose mutation leads to the destabilization and aggregation did not belong to the epitope and does not make any contact with the antibody (Dumoulin, et al., 2003).
It may not be therefore essential to identify the specific epitope(s) of the target protein for raising protective antibodies, although such a selection may improve the effectiveness of the antibody. Further studies are however needed to establish that nature of protection against glycation is also similar.

Several advantages of immobilization of enzyme on antibody supports have been identified (Saleemuddin, 1999) and work described in this paper suggests enzymes immobilized thus may also be resistant to glycation induced alterations. A number of enzymes that catalyze transformations of reducing sugars or those that generates reducing sugars are employed in immobilized state in biosensors and reactors for continuous operation. Such enzymes may be exposed to reducing sugars continuously over long duration. Binding to antibody support may help extend the life of such reactors and sensors.

The mechanism by which antibodies protect enzyme against glycation needs further study. Binding of the antibodies may increase the conformational rigidity of the protein antigen and restrict unfolding. The antibodies may therefore decrease the possibility of sugar reacting with additional susceptible groups of the enzyme (Jabeen et al., 2007). Some chaperones may protect the enzyme against glycation using similar mechanism (Yan and Harding, 2006). The observation that RNase A exposed to the sugars while remaining bound to the antibody support undergo fewer structural alterations supports this view.