INTRODUCTION
As the name indicates proteins are of paramount importance for biological systems. Out of total dry body weight, \( \frac{3}{4} \) is made up of proteins. Proteins are like building blocks i.e. all the major structure and functional aspects of the body are carried out by protein molecules. All proteins are polymers of amino acids. Commonly occurring amino acids are 20 in number.

It is almost impossible to correctly classify all proteins. However, proteins may be divided into three major groups; simple, conjugated and derived. Simple protein includes albumin, globulin, protamines, lectins and scleroproteins. Conjugated protein contains glycoproteins, lipoproteins, chromoproteins, phosphoproteins and metalloproteins. Derived proteins are degraded products of the native proteins (Vasudevan and Sreekumari, 2000).

**Human serum albumin**

Serum albumin belongs to a multigene family of proteins that includes \( \alpha \)-fetoprotein (AFP) and human group-specific component (GC). It is relatively large multi-domain protein, which, as the major soluble protein constituents of the circulatory system, has many physiological functions and has a half life of about 20 days (Ancell, 1939).

Originally, the protein was referred to as “albumen” derived from latin word *albus* meaning “white”, after the white colour of flocculant precipitate produced by various proteins. About 40% of the total albumin is found in the circulatory plasma (Peters, 1970) where as of the remaining 60%, about half resides in viscera and half in muscle and skin (Rabilloud et al., 1988). Albumin also occurs in milk (Phillippy and McCarty, 1979), amniotic fluid (Bala et al., 1987), semen (Blumsohn et al., 1991) and mammary cyst (Balbin et al., 1991).

Albumin concentration in plasma declines slightly with age (Cooper and Gardner, 1989) but it is lower in new borns (Cartlidge and Ruter, 1986) and as low as 20.0 g/l in premature infants (Reading et al., 1990). Albumin is produced by the liver at the rate of 0.7 mg/g liver per hr (Peters, 1985). About 4-5% of albumin is daily replaced by hepatic synthesis (Olufemi et al., 1990). Albumin concentration in plasma is maintained through transcriptional control of the albumin gene by the anabolic hormones-insulin and somatotrophin (Hudson et
Albumin synthesis is also highly dependent upon the supply of dietary amino acids (Kaysen et al., 1989).

The first crystal structure of HSA at low resolution was reported by Carter and coworkers in 1989 (Carter et al., 1989; Carter and He, 1990), and its refined structure at 2.8 Å resolution was published by the same group. The crystal structure of HSA shows that it is a three-domain, each domain contain two subdomains, heart shaped molecule that is predominantly composed of helical structure, with the remaining polypeptides in turns and extended or flexible regions that connect subdomains. Each domain is composed of two sub-domains that are stabilized by internal disulphide bridges (He and Carter, 1992). Albumin have 585 amino acids, this globular Mr 65 kDa protein contains 18 tyrosines, six methionines, one tryptophan, 17 disulphide bridges, and only one free cysteine, Cys34 (Sugio et al., 1999). Roughly 67% of HSA is helical, with the remainder in turns and extended polypeptide. The disulphide pairings in the primary structure of HSA predicted by Brown (Brown et al., 1989) (Fig. 1).

**Functions of human serum albumin**

Albumin contributes to about 80% of colloid blood pressure (Lundsgaard, 1986) and 100% of protein effect in the acid base balance of plasma (Figge et al., 1991). It acts as a carrier for long chain fatty acid (Brodersen et al., 1991; Cistola and Small, 1991), their acyl-coenzyme esters (Richards et al., 1990) and monoacetyl phospholipids (Robinson et al., 1989) and affects the activity of lipase, esterases (Posner et al., 1987) and carnitine acyl transferase (Richards, 1991). Albumin binds to polyunsaturated fatty acids (Anel et al., 1989) and influences the stability (Haeggstrom et al., 1983), biosynthesis (Heinsohn et al., 1987) and transformations of prostaglandins (Dieter et al., 1990). It binds weakly to cholesterol (Deliconstantinos et al., 1986), bile acids (Malavolti et al., 1989), corticoid hormones (Watanabe et al., 1991; Mendel et al., 1990) sex hormone (Padridge, 1988), thyroxine (Petitpas et al., 2003) and is involved in transport of thyroid hormones (Mendel et al., 1990). Albumin also helps in the transport of
Fig. 1. Schematic drawing of the HSA molecule. Each subdomain is marked with a different color (yellow for subdomain Ia; green, Ib; red, Ila; magenta, IIB; blue, IIIa; and cyan, IIIb). N- and C-termini are marked as N and C, respectively. Arginine 117, lysine 351 and lysine 475, which may be binding sites for long-chain fatty acids, are colored white (Sugio et al., 1999).
pyridoxal phosphate (Fonda et al., 1991), cystein and glutathione (Joshi et al., 1987) by forming a covalent bond with these ligands.

Albumin is also responsible for the transport and store housing of many therapeutic drugs in the blood stream (Bhattacharya et al., 2000). It is an important constituent of tissue culture media (Barnes and Sato, 1980) and serves as a medium to support the growth of bacteria, fungi and yeast (Callister et al., 1990; Morrill et al., 1990).

Albumin is also proposed to serve high affinity to metals such as Cu$^{2+}$ and Zn$^{2+}$ and act as an antioxidant function in the vascular compartment because of its scavenging of reactive oxygen and nitrogen species that are generated by basal aerobic metabolism and can be produced at increased rates during inflammation (Halliwell, 1988; Halliwell and Gutteridge, 1990).

The anticoagulant and antithrombotic effects of albumin are poorly understood, this may be due to binding nitric oxide radicals inhibiting inactivation and permitting a more prolonged anti-aggregatory effect.

It is possible that albumin has a role in limiting the leakage from capillary beds during stress that increases capillary permeability. This can be related to the ability of endothelial cells to control the permeability of their walls, and the spaces between them (Jakus and Rietbrock, 2004).

**Antigenecity of human serum albumin**

Albumin is highly antigenic giving high antibody titers in experimental animals due to which it was considered a popular source of antigen by immunologists’ way before the development of precipitation reaction demonstrating molar ratio of bovine serum albumin (BSA) to antibody at equivalence point of 4:1 (Heidelberger, 1938). Recent studies, suggests that there are 13 major antigenic sites on HSA which are recognized by 19 monoclonal antibodies (Doyen et al., 1985) which is consistent with the maximum number of sites predicted by precipitation analysis thus indicating a third of the surface of albumin being antigenic. The synthetic antigenic sites localized with rabbit antisera against bovine serum albumin, were shown to bind considerable amounts
of specific mouse 125 I-labelled antibodies against bovine serum albumin, this
showed that recognition of the antigenic sites of serum albumin is independent of
the immunized species and is inherent in their structural and conformational
uniqueness (Sakate and Atassi, 1980). Albumin-specific antibodies could also be
used for albumin quantifications not only in urine, but also in other biological
fluids. Anti-albumin monoclonal antibodies could be used for the preparation of
immunosorbents to deplete albumin from serum, which is a necessary stage of
proteomic studies of blood proteins. A-HAlb/98 hybridoma secreting monoclonal
antibody that specifically recognizes HSA, the ability of these antibodies is to
quantitate urinary albumin in nanogram ranges (Aybay et al., 1999; Aybay and
Karakus, 2003).

IgG reactivity towards glycated HSA was significantly higher in diabetic
than in control sera (Vay et al., 2000). Certain sera, IgG, IgM, IgA and IgE
antibodies against formaldehyde-HSA could be measured. In the highest titered
sera, it was shown that the IgG antibody was not directed against formaldehyde
(F) alone or F-lysine but against an antigenic grouping of F-HSA. Some sera
from dialysis patients had antibody activity against HSA. Two individuals with a
history of F-induced asthma had no IgG antibodies but did have IgE antibodies
against F-HSA and HSA (Fiehn et al., 2004).

HSA specific polyclonal rabbit immunoglobulin was labelled with biotin
and then used as the tracer antibody makes it a favourable candidate for
utilization in diagnostic applications as well as in research studies (Aybay and
Karakus, 2003).

**Non-enzymatic glycation of proteins**

Non-enzymatic glycation is a process by which sugar is chemically bound
to free ε-amino groups of proteins but without the help of enzymes. However, in
the recent years extensive investigations have been made on the glycation of
proteins exposed directly to high glucose concentrations, e.g. lens crystalline
proteins (Stevvens et al., 1978), insulin (Dolhofer and Wieland, 1979), proteins
of erythrocyte membrane (Miller et al., 1980), bovine serum albumin (Arakawa
and Timasheff, 1982), human serum albumin (Shaklai et al., 1984). enzymes
(Coradello et al., 1984), high and low-density lipoproteins (Krittein et al., 1990), peripheral nerve myelin (Green, 1980), elastin (Baydanoff et al., 1994) and immunoglobulin G (Newkirk et al., 2003).

It is a classical covalent reaction in which, by means of N-glycoside bonding, the sugar-protein complex is formed through a series of chemical reactions described by a chemist Maillard (Singh et al., 2001). Maillard reactions are complex and multilayer, and can be analyzed in three steps. (i) The sugar-protein complex is formed first (Amadori rearrangement), an early product of non-enzymatic glycation lead to an intermediary products which is a precursor of all later compounds. (ii) It includes the formation of numerous intermediary products, some of which are very reactive and continue with glycation reaction. (iii) Final phase consists of polymerization reaction of the complex products formed in the second step, whereby heterogeneous structures named advanced glycation end products (AGE) are formed (Fig. 2). It was believed that the primary role in Maillard reactions was exclusively played by high glucose concentration. However, recent data show that, in spite of the fact that sugars are the main precursors of AGE compounds, numerous intermediary metabolites, i.e. α-oxoaldehydes, also creatively participate in non-enzymatic glycation reactions. Such intermediary products are generated during glycolysis (methylglyoxal) or along the polyol pathway, and can also be formed by autoxidation of carbohydrates (glyoxal).

**Advanced glycation end products**

Protein modification with AGE is irreversible, as there are no enzymes in the body that would be able to hydrolyze AGE compounds. These structures then accumulate during the lifespan of the protein on which they have been formed. Examples include all types of collagen, albumin, basic myelin protein, eye lens proteins, lipoproteins, and nucleic acid. It is now well documented that AGE change the function of many proteins, thus contributing to various late complications of diabetes mellitus (Turk, 1997). The major biological effects of excessive glycation include: inhibition of regulatory molecule binding, crosslinking of glycated proteins, trapping of soluble proteins by glycated
Fig. 2. Schematic representation of potential pathway leading to AGE formation. The abbreviations given above are represented as, GLO=glyoxal; MGO methylglyoxal; 3-DG 3-deoxygluicosone; CML=carboxymethyl-lysine (Turk, 2001).
extracellular matrix, decreased susceptibility to proteolysis, inactivation of enzymes, abnormalities of nucleic acid function, and increased immunogenicity in relation to immune complex formation (Turk, 2001).

**Glycotoxins (AGE peptides)**

In process of glycation, AGE peptides are released as degradation products, which partly occur through proteolysis of the matrix component commonly named glycotoxins. Glycotoxins (AGE peptides) are very reactive on entering blood circulation. In case they have not been eliminated through the kidneys, recirculating AGE peptides can generate new AGE products that react with other plasma or tissue components. At this stage, glycation becomes an autonomic process, which significantly accelerates the progress of the complication (Turk, 2001).

**Glycation of human serum albumin**

Serum albumin non-enzymatically reacts with glucose yielding a stable glycated form of albumin (Garlick and Mazer, 1983; Shaklai et al., 1984), which is elevated in human diabetics (Dolhofer and Wieland, 1980). The incubation of HSA with glucose results in its non-enzymatic glycoxidation in a concentration, incubation period and temperature dependant manner (Bayness et al., 1984). The principal site of glycation of HSA is lys-525, but the lysine residues in positions 199, 281 and 439 are also susceptible to glycation. In addition there are six more residues that glycate less efficiently (Shaklai et al., 1984).

The *in vitro* exposure of protein to glucose results in the non-enzymatic covalent attachment of glucose to lysine side chains in a manner that observed *in vivo* (Brownlee et al., 1987). This process also occurs in individuals with normal control of plasma glucose concentrations, but HSA is typically three times more glycated than the rest of the population in conditions of hyperglycemia (Bourdon et al., 1999) where, during periods of poor control of plasma glucose concentration, circulating levels of 25 mM glucose have been recorded. The interaction of HSA with glucose showed two sets of binding site. The first set
consists of two sites with cooperativity and the second set consists of nine identical non-cooperative sites (Mohamadi-Nejad et al., 2002). The non-enzymatic glycation of HSA induces refolding of globular proteins, accompanied by formation of cross-β structure that may lead glycated albumin into fibrous or amorphous aggregates (Bouma, et al., 2003) which may change its secondary and tertiary structure (Coussons, et al., 1997), alter its normal functions (Shaklai et al., 1984; Iberg and Fluckiger, 1986). Compared to the original albumin, AGE molecules are not only larger in size but also have lower isoelectric points and carry more negative charges. Both the size and the negative charges of AGEs continue to increase over time during incubation (Wu, et al., 1996) and this may also changes the recognition of proteins (Bitensky et al., 1989). Abnormalities in protein recognition may contribute to the pathological impact of glycation (Bitensky et al., 1989).

**Free radical biochemistry**

It is believed that life originated as a result of free radical reactions (FRRs), selected FRRs play major metabolic roles, causing repeated mutation, and death, thereby assuring evolution. Further, life span evolved in parallel with the ability of organisms to cope with damaging free radical reactions. In short, the origin and evolution of life may be due to free radical reactions and, in particular, to their ability to induce random change (Harman, 2001).

Oxygen free radicals are believed to be generated by a number of processes in vivo, including the ‘respiratory burst’ of phagocytic cells, metal catalyzed substrate autoxidations, mitochondrial electron transfer and the reduction of hydroperoxides (Halliwell and Gutteridge, 1984).

Regardless of how and where free radicals are generated (exogenously or intracellularly), a rise in the oxidant level has two important effects: damage to various cell components and triggering the activation of specific signaling pathways NF-κB, ERK, JNK, NAPK, and PKC isoforms (Idris, et al., 2001). Reactive oxygen species (ROS) generated during various metabolic and biochemical reactions having multifunctional effects that include oxidative damage to DNA leading to various human degeneration and autoimmune
diseases (Hasan et al., 2003), moreover, also responsible for the elimination of invading pathogens (Khan et al., 2005)

ROS encompasses a variety of diverse chemical species including superoxide anions (O$_2^-$), hydrogen peroxide (H$_2$O$_2$), alkoxyl (RO'), peroxyl (ROO'), hydroxyl radicals (·OH), and hydrochlorous acid (HOCl) (Jackus, 2000). ROS are highly reactive and have extremely short half-lives. Most estimates suggest that the majority of intracellular ROS production is derived from the mitochondria. There are numerous mechanisms for generation of ROS in vivo (Emerit et al., 1990).

**Superoxide anion radical**

The uptake of one electron by molecular oxygen result in the formation of the superoxide anion radical (O$_2^-$) (Florence, 1990; Harris, 1992). Superoxide radical is a by-product of many of detoxification reactions (Beckman and Ames, 1998). First, superoxide is a strong base and can therefore abstract protons from a variety of the compounds. Second, O$_2^-$ is a potent reducing agent; it can reduce quinines reversibly to semiquinones and transition metal ions such as Fe$^{3+}$ and Cu$^{2+}$ into their reduced forms (Fe$^{2+}$ and Cu$^+$). Third the superoxide ion is a nucleophile and may thus readily interact with a number of electrophilic agents. System generating O$_2^-$ has been observed to kill bacteria, inactivate viruses, damage enzymes and membrane and destroy animal cell culture (Fridovich, 1978; Halliwell, 1981).

The superoxide radical is generated within aerobic biological systems during both enzymatic and non-enzymatic oxidation. It is eliminated by conversion to H$_2$O$_2$ and O$_2$ by superoxide dismutase (Fridovich, 1983; 1986). The finding that O$_2^-$ is produced by some enzymes and is efficiently scavenged by others (McCord and Fridovich, 1968; 1969) led to the view that O$_2^-$ is an agent of oxygen toxicity. In this view the superoxide dismutases (SODs), which catalytically scavenge O$_2^-$, serve a defensive role (McCord et al., 1971).
Hydrogen peroxide and the hydroxyl radical

Hydrogen peroxide is ubiquitous in the biological systems, formed by the divalent reduction of dioxygen or by dismutation.

During the stepwise one-electron reduction of molecular oxygen, hydrogen peroxide is the second intermediate. It may however also be generated directly via a two-electron reduction of molecular oxygen. Hydrogen peroxide is a stable molecule that is generated as an end product of a variety of oxidative reactions in living cells. It may, in fact, act as both as oxidizing and as a reducing agent. In presence of transition metals, H₂O₂ is thought to give rise to peroxyl radicals and extreme reactive hydroxyl radicals via the Fenton reaction (O’Brein, 1969)

\[
H_2O_2 + \text{Fe}^{2+}/\text{Cu}^+ \rightarrow \text{Fe}^{3+}/\text{Cu}^{2+} + \cdot \text{OH} + \text{OH}^-
\]

Or through reaction with a multiplicity of other agents (e.g. the air pollutants NO or NO₂)

\[
\text{NO} + H_2O_2 \rightarrow \cdot \text{OH} + \text{HONO}
\]
\[
\text{NO}_2 + H_2O_2 \rightarrow \cdot \text{OH} + \text{HONO}_2
\]

The hydroxyl radicals are a highly reactive oxidizing agent that can react with a wide variety of organic molecules. It can abstract a hydrogen atom from essentially any hydrogen–carbon bond and undergo addition reaction with aromatic systems at a reaction rate close to diffusion limit.

Photolysis is another important way to produce hydroxyl radical. UV irradiation is responsible for this reaction that can directly split H₂O₂ into two OH (Masaki et al., 1995; Mazellier et al., 2004).

\[
H_2O_2 \xrightarrow{\text{hv}} \cdot \text{OH} + \cdot \text{OH}
\]
Singlet molecular oxygen

Another radical derived from oxygen is singlet oxygen, designated as $^1O_2$. This is an excited form of oxygen in which one of the electrons jumps to a superior orbital following absorption of energy. Ever since its discovery (Kantsky and deBruijn, 1931), its production by photosensitization reactions (including those involving endogenous sensitizer) has been intensively investigated but more recently attention has been drawn by studies showing that $^1O_2$ can also be generated in the absence of light for example by a number of enzymatic reaction (Gille and Joenje, 1991) or by interaction between superoxide and reduced glutathione (Wefers and Sics, 1983). Singlet oxygen is also produced during photoxidation of variety of biological compounds and xenobiotics (Krinsky, 1977; Krasnovsky, 1991). It has been established that human leukocytes can generates $^1O_2$ (Kanofsky et al., 1988). $^1O_2$ is relatively long lived, with half time in the range of 4-50 $\mu$s, so that the diffusion of singlet oxygen is possible within a radius of estimated to be in the range of hundred Å° (Schnuriger and Bourdon, 1968; Moan, 1990). Reactions of singlet oxygen are physical and/or chemical (Kasha and Khan, 1970; Krasnovsky, 1979), as physical reactivity is characterized by photoemissive decay, while the chemical reaction are many fold, including, additions to olefins, forming dioxytanes, allylic hydroperoxide, ('ene reaction') and endoperoxide, as well as oxide of sulphide or phenol to form sulphoxide or hydroperoxidienones (Aubry, 1991). This chemical reactivity is the bases of biological damage inflicted by singlet oxygen.

Autoxidation and glycation

Glycation is a major source of ROS that is generated by oxidative pathways of glycation (Rahbar and Figarola, 2003). Several studies support the idea that glycation and oxidation are closely linked processes, glucose autoxidation plays an essential role in non-enzymatic glycation of protein.

In vitro glycated serum albumin showed possible interactions between glycation and oxidation (Traverso et al., 1997). Contents of the glycated serum albumin are an index of oxidative modification during glycation (Fu et al., 1992).
Glycation mediated modifications induced free radicals can cause conformational changes which alter its antioxidant properties (Bourdon et al., 1999).

Glucose exists in equilibrium with their enediol, which can undergo autoxidation to form an enediol radical. This radical reduces molecular oxygen to generate the superoxide radical and becomes oxidized itself to a dicarbonyl ketoaldehyde that reacts with protein amino groups forming a ketoamine. Ketoamine are similar to, although more reactive, than Amadori products and participate in AGE formation (Ahmed, 2005).

In hyperglycemic conditions, most of the carbonyl compounds generated by glycation need oxidative steps in their formation. The protein dicarbonyl compounds can participate in AGE formation and referred to as glycoxidative products (Liggins and Furth, 1997).

Free radicals and glycation (also called glycosylation) are central to chronic diseases, degeneration and aging. One important source of free radicals is advanced glycation end products (AGEs) resulting from non-enzymatic glycation and oxidation of proteins and lipids (Thomas et al., 2005).

There is ample evidence that ageing, and thus life span, correlate with free radical generation and antioxidative defense as well as with advanced glycation end products. Most chronic diseases are also associated with free radicals and AGEs (Harman, 1984). Overproduction of free radicals accelerates cell ageing and is counteracted by antioxidants. The analysis of the mechanism generating free radicals and of the reaction of AGEs with cellular metabolism opens new avenues for the delaying of the development of chronic diseases such as atherosclerosis and neurodegenerative disease (Giardino et al., 1998).

For a number of age-associated diseases, there is evidence of an association with free radicals and AGEs. Table 1 lists these diseases (Stachelin, 1997).

**Table 1: Age-associated diseases thought to be related to free radicals and AGEs**

- Atherosclerosis
- Cancer
- Complications of diabetes
- Cataract
• Alzheimer's disease
• Parkinson's disease
• Amyotrophic lateral sclerosis

Diabetic individuals may exhibit elevated levels of iron and free copper ions (Cutler, 1978; Mateo et al., 1978), which in the presence of glycated proteins in vitro have been shown to generate free radicals (Hunt, 1994). The accumulation of glycated material in tissues that contain free copper ion, contribute to the generation of free radical mediated damage. These highly reactive species are capable of causing oxidative degradation of protein in vitro (Hunt et al., 1994; Smith et al., 1992). The formation of α-dicarbonyl compounds is known to be an essential step for the cross-linking of proteins and subsequent free radical generation (Rahbar and Figarola, 2003). Methylglyoxal is increased 5-6 fold; in adult onset, non-insulin dependent diabetes mellitus, 2-3 fold. In the presence of oxidative stress, glycation of proteins by methylglyoxal is enhanced. This may underlie the link of glycation and oxidative stress with diabetic complications, and may also contribute to pathological processes of ageing (Harman, 1998).

**Reactive oxygen species and protein oxidation**

Organic free radicals and related electrophilic metabolite can bind covalently to cellular proteins. Amino acids can undergo oxidative damage if they interact with oxygen free radicals. Even peptide bonds can be subject to oxidative modification by reactive oxygen species. The consequence of such oxidative protein modification, in general, is a loss of physiological function, as in the case of covalent binding of reactive organic metabolites to proteins (Bradford and Allen, 1997). As free radicals are capable of initiating chain reactions, cross-linking of soluble and/or membrane-bound proteins, yielding larger aggregates. The direct consequence is a change in higher order structure of the protein (e.g., in the quaternary structure), which, in turn, results in a loss of biological function (Adams et al., 1999; Schoonover, 2001; Dhall et al., 2000).

As another extracellular event that may mark protein for complete intracellular proteolysis, we have studied the action of autoxidizing sugars on
some soluble proteins. Partly consequent on radical generation, proteins are glycosylated. Protein fragmentation takes place, with BSA as target protein, using glucose and glyceraldehyde autoxidizing for one to eight days (Wolff and Dean, 1987).

Elevated radical flux lead to an increased rate of proteolysis of bulk of long half-life proteins in cultured cells (Vince and Dean, 1987) and of mitochondrially synthesized proteins (Dean and Pollak, 1985). A greater acceleration of intracellular protein degradation after radical attack can be observed in erythrocytes (Davis, 1986).

Thus protein damage by radicals is critical in many biological processes. This damage may lead to functional inactivation (Willson, 1983), but usually the inactivated proteins are degraded, so that proteolysis forms a secondary defence. However, when the target proteins are critical for rapid homeostatic mechanisms (as in the case of transport proteins) or when the proteolytic defence and/or other antioxidant defences are overwhelmed, toxic events may ensue.

**Antioxidants**

Antioxidants are key line of defence capable of scavenging free radicals by preventing radical formation, intercepting radicals from further activity (Cotgreave *et al.*, 1998). Antioxidant defences are primary and secondary. The defences that directly scavenge, H$_2$O$_2$ and 'OH are known as primary antioxidant defence. Secondary antioxidant defences consist of the repair mechanisms that act on biomolecules that have undergone oxidative damage.

The major endogenous antioxidants are: 1) superoxide dismutase (SOD) which removes O$_2$$^-$, catalase that converts H$_2$O$_2$ to water (H$_2$O) and O$_2$, and glutathione peroxidase, which helps with H$_2$O$_2$ removal and prevents hydroxyl radical ('OH) formation (Fig. 3) (Halliwell and Gutteridge, 1999). Lipoic acid has the unique ability to regenerate several other antioxidants such as vitamin E, vitamin C (Packer and Coleman, 1999). Vitamin C, the major plasma antioxidant, is a scavenger of many ROS and reactive nitrogen species, and is capable of
Fig. 3. Schematic representation of antioxidant activity of SOD, catalase and glutathione (Halliwell and Gutteridge, 1999).
regenerating tocopherol from its radical (Halliwell and Gutteridge, 1999; Maxwell, 1995). Metal chelators DETAPAC and EDTA sequester the trace amounts of transition metal and hence inhibiting oxidation (Wolff and Dean, 1987). Benzoic acid is also a hydroxyl radical scavenger, which inhibits protein fragmentation (Hunt et al., 1988). There are numerous other dietary sources of antioxidants (McDermott, 2000).

**Autoimmunity**

The lack of an immune response to self when responses to environmental antigens are retained is due to immunological tolerance. The role of tolerance, or lack of tolerance, is important to the understanding of autoimmune diseases and transplantation immunobiology (Goldsby et al., 2003). A loss of natural tolerance (to self) underlies all autoimmune diseases. Many more individuals develop autoimmune phenomena than autoimmune disease. Immune-mediated (Type I) diabetes results from an organ-specific autoimmune-mediated loss of insulin-secreting β cells. This chronic destruction process involves both cellular and hormonal components detectable in the peripheral blood, months or even years, before the onset of clinical diabetes (Kukreja and Maclaren, 1999).

In order to elicit an immune response, a molecule must be recognized as nonself by the biological system. When an antigen is introduced into an organism, the degree of immunogenicity depends on the degree of its foreignness, generally the greater the phylogenetic distance between two species, the greater the structural disparity between them. Bovine serum albumin is strongly immunogenic when injected into rabbit (Goldsby et al., 2003).

Anti-HSA antibodies have been observed in diabetes (Eilat et al., 1981), a five fold greater occurrence than in nondiabetic persons (Gregor et al., 1986). Proteins containing AGEs are highly immunogenic and CML is one of the major epitopes recognised by anti-AGE antibodies (Reddy et al., 1995; Ikeda et al., 1996). The presence of AGE-antibodies in the serum of streptozotocin-diabetic rats as well as in a small number of diabetic patients have been reported (Shibayama et al., 1999) AGE can exert their immunogenicity, demonstrate that presence of AGEs-immune complexes (AGE-IC) in the diabetic patients that may
play a role in the artherogenesis (Turk et al., 2001). Interactions of AGE autoantibodies with AGES as a continuously produced antigen result in the formation of AGE-ICs that may play role in diabetic complications (Jakus and Rietbrock, 2004). The analysis of the frequency distribution profile shows that 14% of the diabetic subjects display significant antibody binding to AGE-HSA than the control subjects (Vay et al., 2000).

**Diabetes mellitus**

Diabetes is growing worldwide at an ever-increasing pace. The latest WHO estimate for the number of people with diabetes worldwide in the year 2000 was 177 million, which is expected to increase to 300 million by 2025. Around 4 million deaths per year are caused due to conditions directly or indirectly associated with diabetes.

Diabetes mellitus (DM) is a group of metabolic diseases characterized by hyperglycemia resulting from defects in insulin secretion, insulin action or both. In DM low insulin levels prevent cells from absorbing glucose. As a result, glucose builds up in the blood. When glucose-laden blood passes through the kidneys, all the excess glucose cannot be absorbed. This excess glucose spills into the urine, accompanied by water and electrolytes; ions required by cells to regulate the electric charge and flow of water molecules across the cell membrane. This causes frequent urination to get rid of the additional water drawn into the urine (polyurea); excessive thirst to trigger replacement of lost water (polydipsia); and hunger to replace the glucose lost in urination (polyphagia) (Alvin, 2005).

A wide spectrum of pathogenic processes is involved in the development of diabetes mellitus, which may range from autoimmune destruction of beta cells to the development of insulin resistance. The major risk factors of diabetes mellitus are age, obesity, lack of exercise, along with a strong genetic predisposition. A strong HLA association with some forms of the disease along with some specific antibodies has also been identified (Alvin, 2005).
Diabetes mellitus was broadly classified into two major types.

**Type 1 diabetes mellitus**

Type 1 or insulin dependent diabetes mellitus, also known as juvenile onset diabetes mellitus is characterized by an absolute deficiency of insulin where the patient is totally dependent on an exogenous source of human insulin. In over 90% of the cases Type 1 diabetes mellitus is immune mediated and in less than 10% of the cases it is idiopathic. It usually occurs in younger age group with highest incidence in 10-14 years age group. Improper glucose metabolism causes an increase in the blood levels of ketone bodies making the patient very prone to ketosis. The incidence of Type 1 diabetes shows little bias upto 15 years of age, but more males are diagnosed in early adult life. The lower prevalence of IAA in adolescent females implies sex specific modulation of the autoimmune process during puberty (Williams *et al.*, 2003).

Immune mediated diabetes results from a cellular mediated destruction of the \( \beta \)-cells of the islets of langerhans of the pancreas (Atkinson and McLaren, 1994). A number of antibodies have been detected as the markers of autoimmune diabetes. These include islet cell autoantibodies (ICA), insulin autoantibodies (IAA) (Atkinson *et al.*, 1992) GAD\(_{65}\) autoantibodies (Jun *et al.*, 2002), antibodies to tyrosine phosphatases IA-2 and IA-2\(\beta\) (Lan *et al.*, 1996; Lu *et al.*, 1996).

Even in the presence of the above predisposing factors the pathogenic process might not get initiated. Detailed studies have shown that a toxic or infectious insult is required for triggering the autoimmune destruction of \( \beta \)-cells of pancreas. Coxsackie-B virus, mumps virus and congenital rubella and cytomegalovirus have been shown to be related to Type 1 diabetes in this regard (Forrest *et al.*, 1971; King *et al.*, 1983; Karjalainen *et al.*, 1998; Pak *et al.*, 1988). Molecular mimicry has been closely linked to Type 1 diabetes mellitus in this respect where an autoimmune response is developed either against pancreatic antigen or against molecules of B cells resembling the viral protein.
Type II diabetes mellitus

Type II or non-insulin dependent diabetes mellitus, also known, as adult onset diabetes mellitus is the most common form of the diabetes mellitus. It is characterized by hyperglycemia secondary to insulin resistance and insulin levels that may be normal or even raised (Defronzo et al., 1979; Turner et al., 1979; Koltermann et al., 1981). A heterogeneous group of factors is involved in its pathogenesis. Obesity has been a strong predisposing factor for the development of insulin resistance and hence Type 2 diabetes mellitus (Bogardus et al., 1985; Olefsky et al., 1982). Moreover, visceral obesity and not subcutaneous disposition of fat has been held responsible for giving rise to insulin resistance (Kissebah et al., 1982). Lack of physical activity and increasing age has also been the precursors of this form of disease. Genetic predisposition is also a strong predisposing factor (Herman et al., 1994; Byrne et al., 1996).

Type II DM has a strong genetic component, major gene that predisposes yet to be identified, but it is clear that inheritance is polygenic and multifactorial. The concordance of Type II DM in identical twins is between 70 - 90%. Individual with a parent with Type II DM have raised risk of diabetes; if both parents with Type II DM, the risk is approximately 40%.

Environmental factors (examples, nutrition and physical activity) further modulate phenotypic expression of the disease. Genetic defect in insulin secretion or action may not manifest itself unless an environmental event of another genetic defect such as obesity is superimposed (Sherwin, 2001; Alan, 1998).

Type II DM is characterized three pathophysiologic abnormalities:
1- Impaired insulin secretion
2- Peripheral insulin resistance
3- Excessive hepatic glucose production

In early stages β-cells compensate by increase in insulin production. So glucose tolerance remains normal despite insulin resistance. Insulin resistance and compensatory hyperinsulinemia progresses, the β-cells in certain individuals are unable to sustain the hyperinsulinemic state. A further decline in insulin secretion and an increase in hepatic glucose production leads to overt diabetes is
a predominant feature and results from combination of genetic susceptibility and obesity.

The pathogenesis of insulin resistance is currently focused on PI-3 kinase signaling defect, which reduces translocation of GLUT 4 to the plasma membrane. Another emerging theory proposes that increased levels of free fatty acids, a common feature of obesity, may contribute to pathogenesis. Free fatty acids can impair glucose utilization in skeletal muscles, promote glucose production by the liver and impair β-cell function.

The diabetes preventive programme demonstrated that intensive changes in lifestyle (diet and exercise for 30 min/day five times in a week) in individual with IGT prevented or delayed the development of Type II diabetes by 58 percent (Alvin, 2005).

**Albumin in diabetes mellitus**

In fact, only a small number of factors are known to result in the variation in serum albumin. The alteration in the structure of albumin due to uncontrolled hyperglycemia causes vascular complication (Bourdon *et al.*, 1999). Its level decreases about 30-40% in diabetes mellitus (Woodside *et al.*, 1998). Moreover, in diabetes the detection of an increase in albumin urinary excretion is a marker of some disease and dysfunction of kidney (Keen and Chiouverkis, 1963: Howthorne, 1989). Alteration of redox state of HSA in patients with diabetes mellitus may be the important parameter for the pathogenesis of the disease, as oxidized form increased in diabetic patients (Suzuki *et al.*, 1992: Oettl *et al.*, 2005). Glycated HSA increases in the diabetic sera which is transported into renal glomerulus, induce an increase in Type IV collagen production and a decrease in proliferate capacity by mesangial cells (Wolff *et al.*, 1991).

**Advanced glycation end-products in the pathogenesis of diabetic complications**

In summary, AGE products are important mediators of diabetic secondary complications and age-related diseases.
Diabetic retinopathy

It is characterized by abnormal vessel development leading to haemorrhages, ischaemia and infections. Specific morphological and functional changes include basement membrane thickening, loss of pericytes and increased permeability (Chappey et al., 1997). Accumulation of AGE might contribute to this state of vasculopathy by increasing retinal endothelial cell permeability resulting in vascular leakage (Vlassara et al., 1994).

A study comparing post-mortem human retinas between diabetic subjects with background and proliferative retinopathy and non-diabetic retinas (Murata et al., 1997), none of the control subjects showed CML immunoreactivity. In vitro studies have showed that AGE modification of retinal basement membrane results in reduced retinal pericycle proliferation but increased retinal endothelial cell proliferation. These effects are observed in diabetes and with AGE effects on nitric oxide depletion, reactive oxygen production and changes in vessel elasticity, could explain AGE effects on microvascular structure and function (Brownlee, 2000).

Diabetic nephropathy

Persistent hyperglycemia has a central role in the development of diabetic nephropathy that is clinically manifested by proteinuria progressing to renal insufficiency, and histopathologically by mesangial expansion and glomerular basement membrane thickening. A possible link between elevated glucose level and diabetic nephropathy resides in the glycation process producing AGEs. This modification can impair the original function of either protein and may affect normal processes of turnover and clearance (Monnier et al., 1992; Berg et al., 1997).

Circulating serum AGE level is markedly increased in patients with diabetes and renal insufficiency. Serum AGEs include both serum proteins that have been modified by advanced glycation and low molecular weight AGE peptides (Makita et al., 1994). Using specific immunoassay, serum AGE peptide levels have been found to correlate with renal function. In normal controls, AGE peptide clearance has been estimated to be 0.72 ml/min. Diabetic persons with
normal glomerular filtration rate can clear AGE peptides at the same rate. However, progressive loss of renal function is associated with increasing circulating AGE peptide levels. In these patients, AGE peptides persist at up to 8-fold normal level (Turk, 2001).

**Diabetic atherosclerosis**

It has also been well documented that lipoproteins are deeply involved in the atherogenic process. Diabetes can lead to several lipoprotein modifications that can affect their interaction with arterial wall cells, thereby contributing to the increased risk of atherosclerosis. Glycation of apolipoproteins in all classes of circulating lipoproteins has been found in diabetes. Approximately 2% to 5% of apo B in the plasma of diabetic persons are glycated, compared with about 1% in the plasma from nondiabetic control subjects (Turk, 2001). Reactive AGE peptides have been experimentally shown to rapidly promote AGE-LDL formation *in vitro*, indicating that glucose may not be the most proximal reactant capable of producing AGE-LDL *in vivo*. AGE lipoproteins like other advanced glycation modified proteins, bind to specific receptors on macrophages and other cell types, and can stimulate the release of cytokines and growth factors which may play a role in atherogenesis (Lopes-Virella *et al.*, 1988; Vlassara, 1996). Thus, a reduction in the level of glycation of lipoproteins as well as of the arterial wall extracellular matrix might alter the interaction of lipoproteins with the matrix and reduce their retention in the arterial wall where they are able to exert their atherogenic damage.

**ROS in diabetes mellitus**

The putative role of ROS in the development of diabetic complications has been investigated for several decades (Baynes and Thorpe, 1999; Oberley, 1988; Ceriello *et al.*, 2000). The level of ROS is increased in hyperglycemic condition, which may be closely associated with diabetic complications (Palm *et al.*, 2003). It is also described that the increased release of superoxide anions (O$_2^-$) from the uterine arteries of diabetic patients due to enhanced O$_2^-$ production in smooth muscle cell than the endothelial cells. The overproduction of ROS lowered
antioxidant defense and alterations of enzymatic pathways in humans with poorly
controlled diabetes mellitus (Jakus, 2000).

Rheumatoid arthritis

Rheumatoid arthritis (RA) is inflammation of the joints along with the
production of a number of autoantibodies (Newkirk et al., 2003). In many part of
the world the disease is called rheumatism. It is named after the town where it
first appeared. It can be caused by bacteria, virus, by a disruption of body
chemistry or by high stress. A predominant feature of synovitis in rheumatoid
arthritis (RA) is the development of a hypertrophic, oedematous and highly
vascularized so-called pannus-like tissue that progressively invades and degrades
adjacent cartilage and bone (Gay et al., 1993; Firestein, 1996; Pap et al., 2000).
This tissue, which originates from the synovial lining layer of affected joints,
consists of synovial fibroblasts (SFs), synovial macrophages and various
infiltrating inflammatory cells such as activated T and B lymphocytes. The joint
destruction is mediated by matrix-degrading enzymes released predominantly by
activated SFs similar to mechanisms observed in malignant disease (Muller-
Ladner et al., 1998).

Long term outcome predictors, such as the presence of rheumatoid factors
(Eberhardt et al., 1990; Van Schaardenburg et al., 1993) and the QKRAA shared
epitope on the HLA-DRβ chain (Wagner et al., 1997; Weyand et al., 1992).

Albumin in rheumatoid arthritis

Albumin is the largest transportable source of nitrogen and energy in the
body and can be used to carry drugs to cells with a high nitrogen and energy
demand such as the cells involved in the synovial inflammation of RA (Wunder
et al., 2003). Recently shown that methotrexate-HSA (MTX-HSA) is more
effective than equivalent concentrations of MTX in preventing CIA (Wunder et
al., 2003). In addition to synovial fibroblasts, which we previously identified as
target cells for albumin-mediated drug delivery in RA, monocytes, granulocytes
and T and B lymphocytes, which all were shown to play a significant role in the
pathophysiology of this disease, might be further targets of this novel treatment approach. (Fiehn et al., 2004). Albumin conjugates are internalized into cells by endocytosis.

The potential use of albumin as a drug carrier in RA is favoured by the facts that the permeability of the blood–joint barrier for albumin in inflamed joints is markedly increased and patients with active RA, similar to cachectic cancer patients. A complex of human serum albumin and rabbit IgG anti-HSA antibodies is used as antigen for RF (Kleveland et al., 1988). Moreover, MTX-HSA is superior to MTX in the suppression of tumour growth and development of arthritis in rodent models (Fiehn et al., 2004).

**Glycation and rheumatoid arthritis**

Rheumatoid arthritis (RA) is characterized by persistent articular and systemic inflammation, along with the production of a number of autoantibodies. Antibodies directed toward the Fc portion of the IgG molecule or rheumatoid factor (RF) are detected in approximately 70% of patients with RA.

During inflammation proteins can be damaged by non-enzymatic glycation (Singh et al., 2001; Ulrich and Cerami, 2001). AGE-damaged proteins are increasingly being implicated in other diseases such as atherosclerosis, amyloidosis, aging (in particular, cartilage and the lens of the eye) (Ulrich and Cerami, 2001), also in RA (Chen et al., 1998; Furumitsu et al., 2000; Miyata et al., 1998) and osteoarthritis (Torchiana et al., 1998; Pokharna et al., 1995). The cross-links that form in cartilage due to pentosidine, which cause the typical yellowing (Monnier et al., 1984), are thought to contribute directly to the joint pathology and increase in urinary AGE detected in patients with osteoarthritis or RA. Such increases may also reflect cartilage degradation.

Not only cartilage but also antibodies can be damaged during inflammation. Previous studies have shown that AGE-damaged IgG can be detected in patients with arthritis of long duration (Ligier et al., 1998; Lucey et al., 2000; Newkirk et al., 1998). AGE can be detected on both the heavy and light chains of IgG (Ligier et al., 1998; Tai and Newkrik, 2000; Lapolla et al., 2000). Studies indicated not only that IgG-AGE could be detected in patients with RA, but also that
approximately 30–40% of RF-positive patients mounted an immune response to IgG-AGE. One possible explanation for the origins of these antibodies and the link to RFs is that RF-positive B-cells could act as antigen-presenting cells for the damaged IgG and thus stimulate the anti-IgG-AGE response (analogous to epitope spreading) by other antigen-selected B-cells that express a surface immunoglobulin specific for the IgG-AGE. In a previous study of RA patients with long standing disease (Lucey et al., 2000), the anti-IgG-AGE antibodies were found to correlate significantly with measures of disease activity whereas RFs did not.

Patients with early disease, anti-IgG-AGE was found to correlate significantly with the swollen joint count and thus appears to be a marker of disease activity.

**ROS in Rheumatoid arthritis**

Recent studies indicated that increased oxidative stress and/or defective antioxidant status contribute to the pathology of rheumatoid arthritis (RA) (Karatas et al., 2003). The study showed raised levels of malondialdehyde and low levels of endogenous antioxidants in patients of rheumatoid arthritis. Plasma catalase had also been reported to be significantly lower in patients with RA (Kamanli et al., 2004). Another study reported impaired glutathione reductase activity in synovial fluid in rheumatoid arthritis (Bazzichi et al., 2002). In active RA (De Leo et al., 2002) and juvenile idiopathic arthritis (Gotia et al., 2001), increased oxidative stress and decreased levels of antioxidants have been reported. An epidemiological study (Knekt et al., 2002) suggested that low selenium status may be a risk factor for rheumatoid factor-negative RA and low α-tocopherol status may be a risk factor for RA independently of rheumatoid factor status. Another study (Kerimova et al., 2002) suggested that ROS generation could be decreased via inhibition of an enzyme (thioredoxin reductase) by gold thioglucose in RA. Overall it is well evident that there is increased state of oxidative stress in RA, which proposes the use of antioxidant supplementation in such patients.
Objective of the present study

Advance glycation end products produced by covalent interaction between sugar and free amino groups of proteins along with free radical production are known to be involved in the pathogenesis of several diseases like diabetes and rheumatoid arthritis. These free radicals and glycation end products are known to cause severe protein damage resulting in major structural alterations, which is implicated either through their functional disability or these being highly immunogenic.

Thus based on earlier observations the present study was planned to study the effect of glucose and oxygen reactive oxygen species (OH) on HSA, as it being most abundant protein in human blood. It was first modified with glucose and the glycated HSA was then subjected to hydroxyl radical (OH), generated by UV irradiation in presence of hydrogen peroxide. Native and modified HSA were then characterized by various electrophoretic, spectral, circular dichroism spectropolarimetry, colorimetric and thermal denaturation studies.

Furthermore, antigenecity of native and modified HSA was probed by inducing antibodies in rabbits. Both native and modified HSA induced high titer antibodies. However, glycated HSA and ROS-glycated HSA were found to be more immunogenic in comparison to its native form, as assessed by direct binding ELISA. The specificity of induced antibodies was evaluated by competition ELISA and gel retardation assay.

The serum of patients with diabetes and rheumatoid arthritis contains a bewildering variety of autoantibodies that react with a variety of protein and nuclear antigens. In order to assess the possible role of glycated HSA and ROS-glycated HSA epitopes in the aetiology these diseases, sera from diabetic and rheumatoid arthritic patients were investigated for the presence of antibodies to native and modified HSA. The polyclonal anti-native and modified HSA antibodies could be used as probe to detect and quantitate protein modified with glucose and hydroxyl radical in healthy and disease.

More sensitive methods of quantitation of HSA modified with glucose and/or OH might serve as a better diagnostic and prognostic marker of disease process.