Chapter 1

INTRODUCTION

Carbohydrates together with proteins, lipids and nucleic acids constitute the four classes of macromolecules in living matter. Some carbohydrate polymers of higher organisms except glycogen and nucleic acids contain amino sugars and were referred to as mucosubstance, the prefix ‘muco’ denoting the viscous property of such substances. The Committee on protein nomenclature of the American Society of Biochemists in 1908 has defined Glycoproteins as “compounds of the protein molecules with a substance or substances containing a carbohydrate group, other than nucleic acids” [1].

Glycoproteins are differentiated by the mucopolysaccharides present in them. The latter include well known compounds of connective tissue such as hyaluronic acid, chondroitin sulphates, heparin etc. and are acidic in nature because of the presence of hexuronic acids and/or sulphate esters. They are linked to protein by ionic or labile covalent bonds. This complex of mucopolysaccharide and protein may be referred to as a mucoprotein. But, in the glycoproteins, carbohydrates are linked by a firm covalent bond to a protein. While the mucoproteins can easily be dissociated by mild procedures like a change in pH or salt concentration, it is not possible to separate the carbohydrates and peptide portions of the glycoprotein without any drastic degradation of the complex. Further, the glycoproteins do not contain hexuronic acids. Their usual sugar constituents are the amino sugars (glucosamine and
galactosamine in their N-acetyl form), the neutral sugars (mannose, galactose and fucose) and the N-acetyl or N-glycolyl form of neuraminic acid (acidic nine carbon amino sugar derivatives collectively called Sialic acids). Glucose is seldom present in mammalian glycoproteins.

Montreuil in France coined the term \textit{glycoconjuges} (glycoconjugates) to designate compounds having a covalent linkage between a carbohydrate moiety (the glycan fraction) and another (the aglycan) moiety which we know may be a protein, a peptide, an amino acid or a lipid which may have both protein and lipid constituents [2]. The primary structure of glycans has been detailed by Montreuil [1] along with the Concepts and Rules involved in structure elucidation. The three dimensional structure of N-glycans and N-glycosyl proteins and their biosynthesis have also been narrated.

Glycoproteins can be isolated by the classical method of ammonium sulphate gradient from one-third saturation to saturation or a pH decreasing gradient from 7 to 3. These fractions are subjected to more recent techniques like gel filtration, ion exchange and affinity chromatography on lectins. Glycopeptides can be broken by Pronase and the carbohydrate moieties of glycoproteins can be liberated chemically or enzymatically. For analysis of monosaccharide composition, the glycans are subjected to methanolysis followed by a re-N-acetylation, trimethyl sialylation and Gas Liquid Chromatography (GLC) or Gas Liquid Chromatography-Mass Spectrometry (GLC-MS). Much progress has been attained by the application of Mass Spectroscopy, particularly Fast Atom Bombardment Mass Spectroscopy (FAB-MS), Electronic Spray Mass Spectroscopy (ESMS) and Matric Assisted Laser Desorption Time Of Filigat Mass Spectrometry (MALDI-TOF-MS) and Nuclear Magnetic Resonance (NMR) Spectrometry.
1.1. STRUCTURE OF GLYCANS

1.1.1. Monosaccharide Constituents

The “classical monosaccharides” identified a long time ago as constituents of glycoconjugate glycans include D-galactose, D-mannose, D-glucose, D-xylose, L-fucose, L-arabinose, N-acetyl D glucosamine and galactosamine, D-glucuronic and L-iduronic acids and some Sialic acids. With the improvement of analytical methods and the extension of research from higher animals to all other living organisms, some new monosaccharides were also identified as constituents of glycans. These are called “Orphan monosaccharides” [3]. The diversity of glycan structures increase with the addition of new monosaccharides.

1.1.2. Glycan-Protein linkages

Two types of primary covalent linkage exist among glycoproteins: O-glycosyl and N-glycosyl linkages. Accordingly, two kinds of glycoproteins are identified: O-glycosyl proteins and N-glycosyl proteins. The hydroxyl-amino acids (Serine or Threonine or Lysine) in the protein core can link with a glycan forming the O-glycosyl protein whereas the amino acid L-Asparagine links with an N-acetyl β-glucosamine residue of the glycan to form N-glycosyl proteins. In some bacteria, the N-acetyl glucosamine residue is replaced by glucose or L-rhamnose residues. The N-acetyl glucosamine-asparagine linkage is considered the most primitive one and the other amino acids involved in O-glycosyl linkages may have taken the place of asparagines by a single mutation of the original codons.
AAU and AAC into AGU and AGC for serine or into ACU and ACC for threonine or into AAA and AAG for lysine [1].

1.1.3. Common ‘Inner Core’ of glycans

The glycans of N-and O-glycosyl proteins possess oligosaccharide structures common to numerous glycans. These are consequently non-specific. They are conjugated to the peptide chain and hence form the most internal part of glycans. Montreuil has designated this common, nonspecific fraction as core (or inner core) or invariant fraction (inv fraction)

\[
\text{Gal (}\beta_{1-3}\text{) Gal NAc (}\alpha_{1-0}\text{) Ser/Thr} : A
\]

\[
\text{Gal (}\beta_{1-3}\text{) Gal (}\beta_{1-4}\text{) Xyl (}\beta_{1-0}\text{) Ser} : B
\]

\[
\text{Man (}\alpha_{1-6}\text{)} \\
\text{Man (}\beta_{1-4}\text{) GlcNAc (}\beta_{1-4}\text{) GlcNAc(}\beta_{1-N}\text{)Asn} : C
\]

Core A exists in all the O-glycosyl proteins in which the glycan-protein linkage is of GalNAc (\(\alpha_{1-0}\))Ser/Thr type, the mucin type.

Core B constitutes the terminal sequence of almost all the glycosaminoglycan of proteoglycans.

(Proteoglycan is defined as a hybrid macromolecule consisting of a heteropolysaccharide joined to a polypeptide. The polysaccharide is the major component. In Glycoproteins, core protein is the major component. Glycosaminoglycan is constituted by a heteropolysaccharide of two alternating units, one invariably either N-acetyl glucosamine or N-acetylgalactosamine and the other a uronic acid.)
Core C is common to all N-glycosyl proteins where the linkage between glycan and protein is GlcNAc (β1-N):Asn type.

1.1.4. The ‘Antenna’ of glycans

The glycan structures arising from the substitution of the inv inner core by a variety of glycosidic structures confer specificity to the glycans. It thus becomes the variable fraction (var fraction) of a glycan. On the basis of the special conformation of such glycans, their mobility in space and their role as signals, Montreuil has called these outer branches of inner core as ‘Antenna’.

1.1.5. Microheterogeneity and Central heterogeneity

In spite of the genetic variation exposed in polypeptide chains, almost all glycoproteins reveal another form of polymorphism associated with their carbohydrate residues. A given glycan located at a given amino acid in a glycoprotein shows structural heterogeneity called Microheterogeneity or peripheral heterogeneity. This involves the number and position of the most external monosaccharides in the glycan. The existence of different types of structure or linkage in the chain of glycans carried as the same peptide offers another type of diversity called Central heterogeneity because it is located in the core of carbohydrate groups. These variants are called glycoforms. The presence of di-, tri- and tetra antennary glycans at different glycoprotein sites in a polypeptide intensifies the complexity of the variant. Moreover, fucosylation and sialylation at different levels increase the microheterogeneity too. The significance of this phenomenon is not obvious. Isolation of each glycoform and the pattern of its glycoprotein is of interest in the field of recombinant glycoproteins.
1.2. DISTRIBUTION OF GLYCOPROTEINS

Glycoproteins (GPs) occur in most organisms, bacteria, fungi, plants and animals. Many viruses also contain GPs some of which have been much investigated in biosynthetic studies. Some of the more important glycoproteins formed in mammalian tissues are listed below:
1. Plasma glycoproteins

Orosomucoid, fetuin, haptoglobins, transferrin, ceruloplasmin, fibrinogen, prothrombin, low molecular weight $\alpha_2$-glycoproteins, $\alpha_2$-macroglobulins, gamma globulins.

2. Urine

Glycoprotein of Tamm and Horsfall.

3. Blood Group substances

4. Glycoproteins of mucous secretions

Submaxillary, cervical, bronchial, gastric.

5. Gonadotrophins

Interstitial cell stimulating hormone, follicle stimulating hormone, human chorionic gonadotrophin, pregnant mare’s serum gonadotrophin.

6. Glycoprotein relating to thyroid

Thyrotropic hormone, thyroglobulin.

7. Glycoproteins of the connective tissue

Reticulin, collagen, basement membranes, lens capsules, Descemet’s membrane, soluble glycoproteins.

Human serum proteins have been separated and analyzed for carbohydrate contents. The average value for total protein-bound carbohydrate in serum of normal human has been reported to be 273 mg per 100 ml with 121 mg per 100 ml represented by Hexoses in the form of galactose and mannose; 83 mg per 100 ml present as Hexosamines almost in the form of N-acetyl glucosamine; 60 mg per 100 ml occurring as sialic acid present as N-Acetyl Neuraminic acid and 9 mg per 100 ml represented by Fucose in the form of 5 methyl pentose. It is apparent
that the concentration of protein-bound carbohydrates amounts approximately three times that of free glucose in the normal blood.

1.3. FUNCTIONS OF GLYCANS

Glycans in glycoconjugates play various roles in the cells. A few are listed below.

1. Glycans help to increase the solubility of glycoproteins. They modulate the viscosity, charge, conformation and denaturation of protein to which they are linked.

2. They act by masking the non-polar amino acid sequences, thus preventing protein-protein interaction and protein aggregation.

3. Glycans are involved in the folding of proteins during their synthesis. They promote the formation of protein oligomers and secretion by the cell.

4. Glycans protect the protein (glycoprotein) against proteolysis. Eg: Mucins of the digestive tract are resistant to proteases. Deglycosylation (esp. desialylation) leads to loss of their resistance.

5. The proteolysis of some protein precursors leading to inactive proteins or peptides is controlled by glycans. Proteolysis of non glycosylated proteins results in a random degradation when compared to that of their glycosylated counterparts.

6. The permeability of cell membrane is controlled by glycans. This effect is related to the relative number of highly hydrophilic sialic acid residues and of the relatively hydrophobic fucose moieties.

(Any modification to glycan may lead to metabolic disorders as observed in cancer cells).
7. Glycans produce immune response in two ways.
   i. Glycoprotein Antigenicity:- Some glycans may be recognized by antibodies. For eg. A family of IgA is specific for the Gal (α1-3)-Gal (β1-4) GlcAc epitope. The ABO blood group determinants are antigens recognized by concerned antibodies.
   
   \[
   \text{GalNAc (α1-3) Fuc (α1-2) Gal (β1-) : A antigen} \\
   \text{Gal (α1-3) Fuc (α1-2) Gal (β1-) : B antigen} \\
   \text{Fuc (α1-2) Gal (β1-) : O (H) antigen}
   \]

   ii. Glycoprotein immunogenicity:- Oligosaccharides attached to proteins elicit an immune response, the exact mechanism of which is not clear. These are seen in cancer cells and are called ‘neoglycans’. These Tumour Associated Antigens (TAAs) with carbohydrate epitopes produce antibodies in the plasma of cancer patients.

8. It has been proved that desialylation of glycoproteins of circulating cells lead to the removal of such cells by a capture in the spleen or liver by a β-galactolectin present in the membrane of resident macrophages. Glycans thus help in phagocytosis of aged cells.

9. Glycoconjugates in the membrane take part in cell-cell recognition, cell adhesion, cell differentiation and development and cell-contact inhibition. Glycans thus intervene with the social life of cells.

10. Membrane glycoconjugates serve as epitopes of tissue antigens of bacteria, fungi, parasitic envelopes and tumour cells. Immunodiagnosis and Immunotherapy of cancer rely on this fact.
11. Glycoproteins are important in fertilization. The **zona pellucida** which is a thick, transparent, non cellular envelope of the oocyte contains three glycoproteins ZP1, ZP2, and ZP3. Of these ZP3 functions as a receptor of the sperm. A protein on the sperm surface, possibly a galactosyl transferase, interacts with the oligosaccharide chains of ZP3. This interaction, by transmembrane signaling, induces the **acrosomal reaction** in which proteases and hyaluronidase are released from the sperm. These enzymes digest the zone pellucida enabling the sperm to pass through and reach the plasma membrane of ovum.

12. Glycoproteins called Selectin play key roles in inflammation and in lymphocyte homing.

A few more functions of glycoprotein are listed in the table below.

**Table 1.1. Functions of Glycoproteins** [4]

<table>
<thead>
<tr>
<th>Functions</th>
<th>Glycoproteins</th>
</tr>
</thead>
<tbody>
<tr>
<td>Structural molecule</td>
<td>Collagens</td>
</tr>
<tr>
<td>Lubricant, Protective agent</td>
<td>Mucins</td>
</tr>
<tr>
<td>Transport molecule</td>
<td>Transferrin, Ceruloplasmin</td>
</tr>
<tr>
<td>Immunologic molecule</td>
<td>Immunoglobulins, Histocompatibility antigens</td>
</tr>
<tr>
<td>Hormones</td>
<td>Chorionic gonadotropin, Thyroid Stimulating Hormone</td>
</tr>
<tr>
<td>Enzymes</td>
<td>Various (eg. Alkaline phosphatase)</td>
</tr>
<tr>
<td>Cell attachment recognition sites</td>
<td>Various proteins involved in cell-cell, virus-cell, bacterium-cell interactions</td>
</tr>
<tr>
<td>Antifreeze</td>
<td>Some Plasma proteins of cold water fish</td>
</tr>
<tr>
<td>Interaction with specific carbohydrates</td>
<td>Lectins, Selectins, Antibodies</td>
</tr>
</tbody>
</table>
1.4. BIOSYNTHESIS OF GLYCOPROTEINS

Tremendous amount of information has been gathered in the field of glycoprotein biosynthesis. The reasons include:

a) a better knowledge of glycan primary structure,

b) the observation that dramatic changes occur in the structure of membrane glycoconjugates in cancer cells,

c) discovery of new diseases due to failures in glycosyltransferase activity and

d) more recent development of the industry of recombinant glycoproteins of therapeutic interest.

Protein glycosylation is the most important post-translational event because of the crucial biological and physiological role played by glycans. The specific category of enzymes called glycosyltransferases is actively involved in this process. Glycosyltransferases are enzymes which transfer monosaccharides from activated sugars to various acceptors. The general reaction can be schematically represented as

\[ \text{Sugar-Donor} + \text{Acceptor} \xrightarrow{\text{Glycosyltransferase}} \text{Glycosylated acceptor} + \text{deglycosylated donor} \]

1.4.1. Activated Sugar Donors

Activated sugar donors are phosphorylated forms of carbohydrate moieties. A phosphate or pyrophosphate is attached to the anomeric carbon atom of a sugar. It is then linked to a nucleoside moiety to form a nucleotide sugar, also called glycosyl nucleotide, or to a polyprenol lipid in the case of lipid intermediates.
Monosaccharide $-1$ phosphate + Nucleoside triphosphate $\rightarrow$ \textit{Pyrophosphorylase} \rightarrow Nucleoside diphosphate – monosaccharide + Pyrophosphate

**a) Glycosyl nucleotides**

Nucleotide sugars appear to be found in the cytosol since the enzyme catalyzing their formation is mostly cytoplasmic. (An exception to this is the formation of CMP-NeuAc, which occurs in the nucleic acid for which no real explanation is available). The glycosyl nucleotides implicated in glycan synthesis include:

i. UDP-$\alpha$-Gal, -$\alpha$-Glc, -$\alpha$-GlcNAc, -$\alpha$-GalNAc, -$\alpha$-Xyl, and – $\alpha$-GlcU.

ii. GDP-$\alpha$-Man and – $\beta$-L-Fucose.

iii. CMP-$\beta$-NeuAc.

**b) Lipid intermediates**

The lipid intermediates called Dolichol monophosphate (Dol [P]) or Dolichol pyrophosphate (Dol [PP]) act as another type of sugar donors. Dolichol, the longest naturally occurring hydrocarbon next to rubber, consists of long chain of isoprenyl alcohols, containing 17-20 isoprene units. Dolichol is first phosphorylated to form Dolichol phosphate (Dol-P) in a reaction catalyzed by Dolichol kinase and using ATP. Oligosaccharide-P-P-Dol is synthesized in the membranes of endoplasmic reticulum from Dol-P and UDP-GlcNAc in the following reaction catalyzed by GlcNAc-P transferase.

\[
\text{Dol-P} + \text{UDP-GlcNAc} \xrightarrow{\text{GlcNAc-P transferase}} \text{GlcNAc-P} + \text{Dol} + \text{UMP}
\]

**1.4.2. Glycosyltransferases**

These enzymes are monomeric glycoproteins with a molecular weight ranging between 30 - 60 kDa [5]. All of them share a common domain structure
with a short amino-terminal cytosolic tail of 9-11 amino acids, a single transmembrane domain of 16-20 amino acids, an extended stem region of about 80 amino acids and a large luminal carboxy-terminal catalytic domain.

**Fig: 1.2 Biosynthesis of O-Glycan innercores.** A, core 1β1-3-gal-T; B, core 2 β 1-6 – GlcNAc-T; C, core 3 β 1-3 GlcNAc-T; D, core 4 β 1-6 GlcNAc-T; E, core 5 α 1-3 GalNAc – T; F, core 6 β1-6-GlcNAc-T; G, core 7 α 1-6- GalNAc-T; H, core 1β 1-3 Gal-T; T, transferase; R, (α 1-0) Ser/Thr.

They are classified as type II transmembrane proteins. Proteolysis of membrane leads to the release of glycosyltransferases lacking the transmembrane domain into the blood. The determination of this enzyme in blood is of great interest for the diagnosis or prognosis of diseases like cancer.

Glycosyl transferases are highly specific to the nucleotide sugars and their specificity has led to the concept of “one monosaccharide-one glycosyltransferase” which later was complemented by “one linkage-one
glycosyltransferase”. One glycosyltransferase expression seems to be regulated at the level of transcription and therefore most glycosyltransferases are constitutively expressed at very low levels in mammalian somatic cells, thus said to be showing the ‘housekeeping’ activity. However, the activity of some glycosyltransferases is shown to be enhanced in a number of tumour cells and other diseases.

Cellular glycosyltransferases are distributed in rough endoplasmic reticulum (RER), smooth endoplasmic reticulum (SER) and also in plasma membrane. But, they are not uniformly distributed and are located in specific territories.

i. Mannosyl-, glucosyl-, N-acetyl glucosamine phosphoryl transferase, and a part of N-acetyl glucosaminyltransferases in Rough Endoplasmic Reticulum.


It can therefore be inferred that

a. the synthesis of O-glycosyl proteins occur entirely in the Golgi apparatus and is a post-translational event and

b. on the contrary, biosynthesis of N-glycosyl protein starts in Rough Endoplasmic Reticulum and terminates in the Golgi apparatus revealing that it is first a co-translational and then a post-translational event. Thus the inner core of N-glycosyl protein is synthesized in the RER and the antennae in Golgi apparatus.
1.4.3. Biosynthesis of O-Glycosyl proteins

O-glycosyl proteins are synthesized in the Golgi complex by the sequential addition of one sugar at a time without preassembly processing on a dolichol derivative [6]. Addition of the first sugar to the peptide (N-acetyl galactosamine in mucus and xylose in proteoglycans) occurs mainly in cis-Golgi without requiring a specific amino acid sequon as in the case of N-glycosyl proteins.

A. Inner Core Biosynthesis

The synthesis of the glycan inner core regulates the expression of functional terminal sugar sequences of O-glycans. Control at early steps of synthesis has a great influence on the primary structure of O-glycans and consequently on their functions.

B. Elongation process

Elongation of O-glycans involves

i. The addition of N-acetyl lactosamine residues forming linear chains

ii. Branching of glycan chain with the formation of mannosidic $\beta$1-6 linkages between GlcNAc and GalNAc.

C. Termination of Glycans

Terminal structures include $\alpha$ 2,3- and $\alpha$ 2,6-sialyl; $\alpha$ 1,3- and $\alpha$ 1,4 fucosyl; $\alpha$ 1,3- or $\alpha$ 1,6-N-acetyl galactosaminyl; $\beta$ 1,4-N-acetyl glucosaminyl, $\alpha$ 1,4-N-acetyl glucosaminyl, $\alpha$ 1,3 galactosyl; and sulphated residues. Many of these structures are blood group substances and tissue antigens.
1.4.4. Biosynthesis of N-Glycosyl proteins

Unlike O-glycosyl proteins, N-glycosyl protein glycans are not synthesized directly in their definitive form, but pass through the following intermediate stages [5].

A. Assembly of dolichol-pyrophosphate-oligosaccharide, Glc₃Man₉GlcNAc₂-P-P-Dol

B. Transfer ‘en block’ of the megaloglycan to an asparagine residue of nascent polypeptide.

C. Trimming and processing of the oligosaccharide within the lumen of Golgi apparatus leading to glycans of the oligo type.

D. Elongation and formation of antennae.

A. Assembly of Dolichol-Pyrophosphate oligosaccharide

The precursor oligosaccharide Glc₃Man₉GlcNAc₂ is assembled in the RER by stepwise addition of monosaccharide residues on to dolichol-phosphate in two different cell compartments: cytosol and the lumen of RER [7].

1. In Cytosol:-

a) A GlcNAc residue is added to Dolichol + P using UDP-GlcNAc as the donor. The reaction is catalyzed by P-Dolichol GlcNAc-1-phosphate transferase (This enzyme is inhibited by tunicamycin which therefore blocks the N-glycan synthesis) [Step 1 of diagram A].

b) A second GlcNAc residue is added to the first again using UDP-GlcNAc as the donor. Enzyme involved is GlcNAc-P-P-dolichol GlcNAc transferase [Step 1 of A].
c) Five Mannose (Man) residues are added using GDP Mannose as the donor. Reaction is catalyzed by α mannosyltransferases [Step 2 of A].

Fig: 1.3 A, Dolichol-phosphate cycle in the endoplasmic reticulum. B, Trimming and processing of N-linked oligosaccharides in the Golgi apparatus. The structures are represented by shorthand symbolic notation. Δ, D-glucose; •, D-Glc NAc; ♦, D-Man; ●, D-Gal; ◊, D-GalNAc; ○ D-Sia
2. In RER:-

‘Glucomegalaglycan’ is synthesized in the lumen of RER in two steps.

d) Four additional Man residues are added using Dol-P-Man as the donor in presence of $\alpha$-mannosyltransferases. [Step 4 of A]. Dol-P-Man is formed by the reaction

$$\text{Dol} - \text{P} + \text{GDP} - \text{Man} \xrightarrow{\text{Mannosyltransferase}} \text{Dol} - \text{P} - \text{Man} + \text{GDP}$$

e) Finally, three peripheral glucose residues donated by Dol-P-Glc are catalytically added using $\alpha$-glycosyltransferases [Step 6 of A]. Dol-P-Glc is formed by the reaction

$$\text{Dol} - \text{P} + \text{UDP} - \text{Glc} \xrightarrow{\text{Glycosyltransferase}} \text{Dol} - \text{P} - \text{Glc} + \text{UDP}$$

The net result is the assembly of the compound $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$-$\text{P}$-$\text{Dol}$.

B. Transfer ‘en block’ of $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ onto the Nascent Peptide chain

Glycosylation is the signal for the transfer of the glucosyl oligosaccharide to an asparagine residue of the nascent polypeptide chain that emerges from the luminal surface of the RER [Step 7 of A].

C. Trimming and Processing of N-linked Megalooligosaccharide to $\text{Man}_5\text{GlcNAc}_2$-Protein

i. RER Trimming:- After transfer to the nascent peptide chain, the oligosaccharide $\text{Glc}_3\text{Man}_9\text{GlcNAc}_2$ is trimmed in the RER to $\text{Man}_8\text{GlcNAc}_2$ [Step 8 of A] by the successive action of the following glycosidases.

a. Glucosidase I removes the first $\alpha$ 1-2 linked terminal glucose residue.

Glucosidase II removes the next two $\alpha$-1-3 linked glucose residues. (In high mannose glycoproteins, the process may stop here).
b. ER α- mannosidase removes one α-1-2 linked mannose residue [Step 9 of A].

The glycoprotein now moves from the RER into the cis-cisterna of the Golgi apparatus by a sequential membrane budding and fusion mechanism [Step 10 of A & Step 1 of B].

ii. Golgian Trimming:- The cis-Golgi mannosidase I cleaves the three remaining α 1,2 linked mannose residues, generating Man5GlcNAc2 structure. [Step2 of B]

D. Elongation and Formation of Antennae

The Man5GlcNAc2 glycan may be converted to the hybrid type or the complex type (N-acetyl lactosamine type). Formation of antennae is initiated by the addition of N-acetyl glucosamine residues to the inner core of N-glycan in the medial Golgi. These GlcNAc residues are elongated and terminated by the addition of variable number of GlcNAc, Gal, GalNAc, Fucose and Sialic acid residues in an ordered way. Sulphates, Phosphates or other non-carbohydrate groups can also be added.

Enzymes involved in Elongation and Biosynthesis of Antennae

a) Enzymes of Medial Golgi:- Three enzymes are involved in the following order.

1. An N-acetyl glucosaminytransferase I (Gn T-1) that transfers a GlcNAc residue β1-2 linked to the α 1,3 Mannose residue from UDP-GlcNAc [Step 5 of B]. This gives signal to remove two additional Mannose residue by α mannosidase II.

2. α mannosidase II removes the α 1-6 mannose residue of the penta saccharide inner core [Step 6 of B]. This allows the action of another N-acetyl glucosaminytransferase II.
3. N-acetyl glucosaminyl transferase II (Gn T-II): This transfers a second β1-2 linked GlcNAc to the α 1-6 residue [Step 7 of B]

b) Enzymes of Trans Golgi:- The involvement of six β N-acetyl glucosaminyl transferases (Gn T-I to Gn T-VI) are depicted below.

**Fig: 1.4 β-N-Acetylg glucosaminyltransferases (GnTs) responsible for the addition of N-Acetylg glucosamine residues to the trimannosyl core**

### 1.5. CATABOLISM OF GLYCOPROTEINS

The normal catabolism of glycoproteins results from the action of two types of specific hydrolases called glycosidases. They are

1. Exoglycosidases which bring about stepwise degradation of glycan starting from the non reducing terminus Eg. α-Mannosidase, β-Galactosidase, α-Fucosidase, β-Hexosaminidase, β-Glucuronidase, Neuraminidase etc..

2. Endoglycosidases which split glycosidic bonds inside the glycan structure. Eg: Endo-N-acetyl β D glucosaminidase.

Both these glycosidases are located in all of the cell territories like nucleus, cytosol, endoplasmic reticulum, plasma membrane and lysosomes. It is believed that the α and β exoglycosidases present in the lysosomes viz.,
mannosidases, galactosidases, hexosaminidases, neuraminidases etc. are responsible for the degradation of glycoproteins. Accumulation of certain carbohydrate materials in times and the subsequent excretion through urine in diseases called glycoproteinoses or glycanoses are now explained due to the lack of various lysosomal glycosidases [8].

The complete degradation of N glycans is not at all a random mechanism, but an ordered bi-directional pathway. Two catabolic pathways hypothesized for N-glycan degradation are given below.

![Diagram](image)

**Fig: 1.5 Two catabolic pathways hypothesized for the degradation of N-glycosylprotein glycans.** R, N-acetylactosaminic or oligomannosidic structures.

It is hypothesized that stepwise modification of tri-dimensional structure of oligosaccharides occurs and that each removal of a mannose residue modifies the spatial conformation of the oligosaccharide formed. The next mannose to be removed is then well presented to the next α-mannosidase.
1.6. GLYCOSIDASES

Synthesis of glycoproteins is a complex process mediated by glycosyl-transferases that results in the formation of Glc₃ Man₉ GlcNAc₂ – PP-dolichol. It is then transferred to Glc₃Man₉GlcNAc₂ oligosaccharide which is attached to asparagine residues of nascent proteins in the Endoplasmic Reticulum (ER). Shortly after transfer, this oligosaccharide is processed by $\beta$-glucosidases I and II which sequentially trim the three glucose units in the ER. This is followed by the removal of at least one mannose residue by $\alpha$-mannosidase in ER and/or a few mannose residues in Golgi cisternae depending on the organism [1, 9]. Hydrolysis of $\alpha$ 1,2, linked mannose residues gives rise to hybrid and high-mannose N-glycan, whereas removal of $\alpha$ 1,3 and $\alpha$ 1,6 linked mannose leads to the formation of complex N glycans [9]. These glycosaminoglycans have multiple functions in the extracellular matrix as well as the surface which depend on their sequence structure [10]. They are degraded in the lysosomes by the concerted action of a number of exohydrolase activities following partial catabolism by endoenzymes (endoglycosidases, hyaluronidases, heparases and endosulfatases). These endo and exo enzyme activities maintain highly conserved substrate structure specialties. Endohydrolysis of glycosaminoglycans release oligosaccharides which are acted upon by about 13 lysosomal exoenzymes reducing them to monosaccharides and inorganic sulphate to enable exit from lysosomes. A deficiency of any one of these exoenzyme activities may result in lysosomal storage of the GAG substrates and consequently clinical symptoms broadly classified as ‘mucopolysaccharidoses’ appear [11].

Lysosomal glycosidases catalyze the hydrolysis of terminal glycosidically linked monosaccharide constituents of oligosaccharide moieties of glycoproteins, glycolipids and glycosaminoglycans. These glycosidases are synthesized as
prepropolypeptides that are subject to N-glycosylation and proteolytic processing. Addition of mannose-6-phosphate to the N-linked oligosaccharides of lysosomal enzymes serves to generate the recognition marker that mediates interaction with mannose-6-phosphate receptors and ultimately directs intracellular targeting to the lysosome [12]. The major lysosomal exoglycosidasces are β-N acetyl glucosaminidase, β-Nacetyl galactosaminidase (both together called N-acetyl hexosaminidases), β-galactosidase, β-glucosidase, α-mannosidase, α-fucosidase and β-glucuronidase. These enzymes have been detected in and isolated from different types of plant and animal tissues.

Touster [13] had reported the isolation of these enzymes from lysosomes, Golgi or cytosol of liver, testes, certain reproductive glands, kidney, brain etc. of mammals; detailed the procedures for purification and had studied the properties too. Later, different workers had studied one or the other glycosidase in serum [14-18], in seminal fluid [19], skeletal muscles, articular cartilage and platelets [20], eye lens [21, 22] etc.

α-mannosidase degrades mannose containing glycopolymers. It has been purified from human and rat liver, rat brain, rat epididymis, hog kidney etc. [13]. Its pH range is 4.6 – 5.5. Mannosidases are grouped into glycosylhydrolase families 47 and 38 which includes Class I α1,2-mannosidases and Class II α1,2-, α1,3- and α1,6-mannosidases respectively [24]. Of these, family 47 includes 2 types of α-mannosidases: i) those residing in ER of yeast and mammalian cells capable of eliminating one mannose from Man9GlcNAc2 (M9 form) to Man8GlcNAc2 (Man8 form) and ii) others residing in Golgi which release the four α1,2 linked mannose from M9 to produce M5 form.
β-D-glucosidase (EC 3.2.1.21) releases terminal β-D-glucose moiety of glycoproteins and related compounds. This enzyme has an optimum pH range of 5.0 - 5.5.

β-D-galactosidase (EC 3.3.1.23) of mammalian tissues has a molecular weight ranging from 63,000 to 85,000. The optimal pH was found to be 4.0. This enzyme hydrolyses terminal non-reducing galactose residues from desialised glycoproteins, glycolipids, gangliosides and other glycosaminoglycan derivatives. The pH optimum is 4.3 (for bovine testicular β-galactosidase) with a range of 3.6 to 4.5.

α L-fucosidase (EC 3.2.1.51) catalyzes the hydrolysis of terminal α L-fucosyl residues from oligosaccharides and glycopeptides. Seen in every mammalian tissue, this enzyme has been purified from pig kidney, human placenta, rat epididymis, as well as human and rat liver lysosomes [13]. This enzyme has a M.W. of 40 – 60 kDa. The active form is a tetramer. It has a broad pH range with significant activity in acidic and neutral pH. The pH optimum lies between 5.3 and 5.5. Rat liver α-fucosidase has four consensus sites for N-glycosylation and its calculated Mol.wt. is 50.5 kDa [12].

N-acetyl β-D-Hexosaminidases (EC 3.2.1.52) catalyze the hydrolysis of terminal, non-reducing N-acetyl β-D-glucosamine and N-acetyl β-D-galactosamine residues in glycoproteins, gangliosides and glycosaminoglycans. It has been purified from numerous plant, animal and microbial sources [13]. Human hexosaminidase has two major isoenzymes; Hexosaminidase A (Hex.A) and Hexosaminidase B (Hex.B) [23]. Hex. A is a heterodimer composed of α and β subunits whereas Hex. B is a homodimer consisting of two β subunits. Due to this difference Hex. A can hydrolyze both neutral and negative charge
Introduction

substrates, mainly sulphated substrates while Hex. B can only hydrolyze neutral substrates. The optimum pH range is 4.5.

Glycosidases in normal conditions regulate the size and amount of proteoglycans in extra cellular matrix as well as in the cells by a conditioned degradation. Enzymatic glycosylation leading to the formation of functional glycoconjugates and deglycosylation (degradation by glycosidases) of glycanic chains constitute complex pathways in glycoprotein metabolism. Alterations in these functional glycoconjugates could induce abnormal cellular behaviour as is noticed in diabetes and inflammatory diseases. The activity of glycosidases are affected in many kinds of diseases and disorders concerned with cell adhesion, cell – cell interactions or cell signaling. Significant changes in the activity of serum glycohydrolases have been detected in altered physiological states such as pregnancy and in several diseases [25-30]. Deranged glycosidase activities have been noticed in the fatal cerebral degenerative disorder, Tay-Sachs disease [14], in cataract [22], in diabetes causing retinal changes of the eye [31] and in inflammatory diseases of the joints [32-34].

1.7. GLYCOPATHOLOGY

The first described storage disease due to the lack of lysosomal glycosidases concerned the mucopolysaccharides of proteoglycans and of glycolipids. These were called mucopolysaccharidosis and mucolipidosis respectively. A long list of diseases is now available which are explained due to the lack of lysosomal glycosidases. The diagnosis of glycoproteinoses and the discovery of new types of glycoproteinoses are based on the analysis of the material accumulated in urine of patients and in amniotic fluid at antenatal diagnosis. In this way, fucosidosis (lack of α fucosidases), α-mannosidosis
(deficiency of α-neuraminidase and β-galactosidase), β-mannosidosis etc. have been described and correlated to respective inheritance patterns.

A number of diseases involving abnormalities in the synthesis and degradation of glycoproteins have been recognized. Certain changes in the pattern and contents of glycopeptides occur in the membrane of cancer cells. Defects in synthesis of glycoproteins appear to be implicated in Carbohydrate Deficient Glycoprotein Syndrome (CDGS) and Hereditary Erythroblastic Multinuclearity with a Positive Acidified test (HEMPAs) and Paroxysmal nocturnal haemoglobinemia. Some diseases are due to genetic deficiencies in the activities of specific glycoprotein lysosomal hydrolase. These include α and β-mannosidosis, fucosidosis, sialidosis, aspartyl glycosaminuria etc. Glycoproteins are also involved in diseases including influenza, AIDS, Diabetes and rheumatoid arthritis. It is hoped that basic studies of glycoproteins and other glycoconjugates will lead to effective treatments of diseases in which they are involved.

It is accepted that the structural alterations in glycoconjugate structures are the result of anomalous biosynthetic or degradative pathways. In other words, disturbed activities of either glycosyltransferases (biosynthetic enzymes) or glycosidases (degradative enzymes) bring changes in these complex substances.

Certain glycoconjugates are used in clinical practice as markers of some types of cancer. Eg: Carcino Embryonic Antigen (CEA) and α-feto protein are glycoprotein markers in colorectal cancer; glycoprotein GP120 for HIV with a high level of carbohydrate content etc.
1.8. GLYCOTHERAPEUTICS

Some glycoconjugates are now being investigated as potential therapeutic agents.

1. Fructose sulphate shows anticonvulsive action.

2. Some glycoconjugates are used as antigens, vaccines or anti-tumoural agents.

3. Oligosaccharide preparations with antimicrobial activity have been prepared.

4. Use of a structural analogue of glucose to interfere with the metabolism of HIV envelope carbohydrates seems to reduce the capacity of this virus to infect new cells.

5. Work is underway on glycoforms that control processes related to asthma, rheumatoid arthritis etc.

6. Preparation of ‘glycomimetics’, glycosylation of antibodies and modification of glycoconjugates by replacement of Sialic acid components are directed towards a glycotherapy.

7. Drug targeting by coupling adequate oligosaccharides to the active drugs has been found successful.

8. Some neoglycan conjugates are found effective in creating competitive inhibition of metastasis.

1.9. GLYCOTECHNOLOGY

Glycoconjugates have spread into the field of genetic engineering of human glycoproteins of therapeutic interest. Since the proteins require proper
glycosylation for therapeutic effectiveness and safety, eukaryotic cells have been engineered to produce recombinant glycoproteins which strictly conform to the native glycoproteins. The glycosaminoglycan chains account for many of the functions and properties of proteoglycans. The development of proteoglycan glycotechnology to exploit the functionality of glycosaminoglycan chains is an extremely important aspect of glycobiology. Yamaguchi et al. [8] described an efficient and widely applicable method for chemoenzymatic synthesis of conjugate compounds comprising intact long chondroitin sulfate (ChS) chains. This method enables highly efficient introduction of ChS into target materials. The ChS-introduced compounds have marked stability against proteolysis. The chemically linked ChS chain also contributed to the stability of these core compounds. Such methods will contribute to the development of proteoglycan glycobiology and technology.

1.10. GLYCOPROTEINS IN INFLAMMATION

Inflammation is defined as the local response of living mammalian tissue to injury due to any agent. It is a body defense reaction in order to eliminate or limit the spread of injurious agents as well as to remove the consequent necrosed cells and tissues. The agents causing inflammation may be physical (heat, cold, radiation, trauma), chemical (organic and inorganic poisons), biological (bacteria, viruses and their toxins) or immunological (cell mediated and antigen-antiobody reactions). Signs of inflammation are heat (calor), swelling or oedema (tumor), redness (rubor), pain (dolor) and loss of function [35].

Depending on the defense capacity, inflammation can be classified as: 1) Acute inflammation of short duration representing early body reactions that are repairable and 2) Chronic inflammation of longer duration leading to
complex reactions challenging the repair. The changes in acute inflammation include vascular events like transient vasoconstriction of arterioles followed by persistent vasodilatation, elevation of local hydrostatic pressure, slowing of microcirculation, and leucocytic margination. The cellular events include exudation of leucocytes, changes in the formed elements of blood, rolling of leucocytes and their adhesion with endothelial cells mediated by the adhesion molecules like selectins, integrins and some intercellular adhesion molecules (ICAM-1,2) etc. [35]. Chronic inflammation follows acute inflammation and is characterized by infiltration of mononuclear cells (macrophages, lymphocytes and plasma cells), proliferation of fibroblasts, fibrotic changes in connective tissue and tissue destruction.

The chemical mediators of inflammation are either cell-derived or plasma-derived. The former category includes amines (histamine, 5-hydroxytryptamine), arachidonic acid, cytokines (IL-1, TNF-α, IF-γ), and enzymes like acid proteases, glycohydrolases, collagenase, elastase etc. Some of these agents are glycoprotein-based and are related to glycoprotein metabolism. Glycotherapy of inflammatory diseases have originated from the investigations on altered glycosylation in such states.

1.11. FREE RADICALS IN INFLAMMATION

Involvement of oxygen-derived free radicals like H₂O₂, superoxide (*O₂⁻) and hydroxyl radicals (*OH⁻) in the pathology of inflammation has been established [35]. They are supposed to cause endothelial cell damage leading to increased vascular permeability, inactivation of anti-proteases resulting in unopposed protease activity and injury to various cell types. Oxygen metabolites are detoxified by antioxidants which include the serum glycoproteins namely, ceruloplasmin and transferrin, and enzymes such as
superoxide dismutase, catalase, glutathione peroxidase etc. The net effects on tissue injury of oxygen free radicals depend on the balance between their production and inactivation. Free radicals play a major role in the degradation of cell/tissue structure and hence affect their functions [36].

Free radicals are very reactive atoms or molecules possessing one or more unpaired electrons in its valence shell and are capable of independent existence. Each free radical contains an odd number of electrons which makes it unstable, short lived and highly reactive. It reacts quickly with other compounds to capture the needed electrons for gaining stability. The attached molecule then looses its electrons and become a free radical itself, beginning a chain of reaction cascade disrupting the living cell. Free radicals are produced continuously in cells either as byproducts of metabolism or by leakage from mitochondrial respiration. The most important reactions of free radicals in aerobic cells involve molecular oxygen and its radical derivatives like superoxide anions (\(\cdot\text{O}_2^-\)), hydroxyl radicals (\(\cdot\text{OH}\)), lipid peroxyl (\(\cdot\text{LO}^-\)), lipid alkoyl (\(\cdot\text{LOO}^-\)) and lipid peroxide (\(\cdot\text{LOOH}\)) as well as non radical derivatives like hydrogen peroxide (\(\text{H}_2\text{O}_2\)) and singlet oxygen (\(\cdot\text{O}_2\)) that are collectively called Reactive Oxygen Species (ROS). These free radicals and reactive oxygen species create an oxidative stress to all important cellular components like proteins, DNA and membrane lipids and damage them leading to cellular death.

1. 11.1. Generation of Reactive Oxygen Species

The initial step in the formation of ROS involves the generation of \(\cdot\text{O}_2^-\) by a single electron donation to molecular oxygen (\(\text{O}_2\)), resulting in a very reactive unpaired electron state. This non-enzymatic process can occur during the passage of electrons through mitochondrial electron transport system. Also,
*O$_2^-$ can be produced enzymatically by various cellular oxidases like xanthin oxidase, NADPH oxidase, nitric oxide synthetase etc. H$_2$O$_2$ is formed when two molecules of *O$_2^-$ dismutate either enzymatically or spontaneously to produce di oxygen and H$_2$O$_2$. Thus generation of *O$_2^-$ leads to H$_2$O$_2$ production. Adding one electron to the oxygen bond of H$_2$O$_2$ results in cleavage of the bond and subsequent release of a hydroxide anion and the very reactive species *OH$. This electron addition is catalyzed by transition metal cations such as ferrous iron (Fe$^{+2}$) in which the iron is converted from the ferrous to ferric (Fe$^{+3}$) state. The following figure illustrates those pathways of ROS formation and the antioxidant enzymes involved in their detoxification.

1.1.2. Sources of Antioxidants

Four endogenous sources have been accepted for production of oxidants by cells. They are:

1. Consumption of O$_2$ by mitochondria in aerobic cells and its reduction to H$_2$O. The byproducts of this process are *O$_2^-$, H$_2$O$_2$ and *OH$. Leakage of partially reduced oxygen account for 2% of oxidant production.

2. Phagocytic cells destroy bacteria or virus infected cells into an oxidative burst of nitric oxide (NO), *O$_2^-$, H$_2$O$_2$ and *OH$. Chronic infection leads to an excess of oxidative species.

3. Peroxisomes which are responsible for degrading fatty acids and other molecules produce H$_2$O$_2$ as a byproduct which is then degraded by catalase. Some of the peroxide escapes degradation, enter into other compartments of the cell and cause damage to DNA.

4. The induction of cytochrome P 450 enzymes which constitute one of the primary defense systems against natural toxins (from plants) prevents
acute toxic effects from foreign chemicals, but also results in oxidant byproducts that damage DNA.

1.11.3. Antioxidant defense system

Cells have developed a comprehensive set of antioxidant defense mechanism to prevent the formation of free radicals and ROS or to limit their damaging effect. Normally there is a balance between a free radical / ROS formation and endogenous antioxidant defense mechanism. If this balance is disturbed, it creates oxidative stress and subsequent damages to the cell due to oxidation of biomolecules. These protective mechanisms include enzymes to inactivate peroxides, proteins etc., to sequester transition metals and a range of compounds to scavenge or remove the free radicals. The antioxidant defense system can be subdivided into:

1. Enzymatic antioxidant system including superoxide dismutase, catalase, glutathione peroxidase etc.
2. Non enzymatic antioxidant system comprising the nutrient antioxidants (ascorbic acid, α-tocopherol, β-carotene etc.) and metabolic antioxidants (glutathione, ceruloplasmin, albumin, bilirubin, ferritin, lactoferrin, transferrin etc.)

Antioxidants are thus defined as compounds of exogenous or endogenous nature which either prevent the generation of toxic oxidants or intercept any that are generated and inactivate them thereby blocking the propagation of the chain reaction produced by these oxidants [37].
1.11.4. Oxidative Stress Indicators

1.11.4.1. Antioxidant Enzymes

1. Superoxide dismutase (SOD)

Superoxide dismutase is produced by highly conserved genes and can convert the reactive oxygen (*O₂⁻) into less toxic H₂O₂. This molecule is also toxic to cells and is broken down to release hydroxyl radical (*OH⁻), a reactive species more damaging to cells than either (*O₂⁻) or H₂O₂. Enzymes responsible for detoxifying H₂O₂, namely catalase and glutathione peroxidase prevent the formation of *OH radicals by converting H₂O₂ to other less harmful products. Thus this enzyme family acts in a sequential fashion to ‘dismutase’ one toxic oxygen species to another, which then can be rapidly broken down into non toxic byproducts.

The ‘dismutases’ include Cu Zn-SOD, Mn-SOD, Fe-SOD and EC-SOD. The Copper Zinc superoxide dismutase is a dimer containing copper for its catalytic action and zinc as a protein stabilizer. This enzyme may be localized in cytoplasm and nuclear matrix or in peroxisomes. Cu Zn-SOD reduces (*O₂⁻) in the reaction:

\[
\text{SOD} - \text{Cu}^{2+} + *\text{O}_2^- \rightarrow \text{SOD} - \text{Cu}^{1+} + \text{O}_2
\]

\[
\text{SOD} - \text{Cu}^{1+} + *\text{O}_2^- + 2\text{H}^+ \rightarrow \text{SOD} - \text{Cu}^{2+} + \text{H}_2\text{O}_2
\]

Manganese superoxide dismutase (Mn-SOD) is the mitochondrial from of the dismutases. It is a protein tetramer containing manganese (Mn) for its catalytic activity and reduces (*O₂⁻). It is compartmentalized in the mitochondria presumably to detoxify (*O₂⁻) generated by the Electron transport system.
Iron superoxide dismutase (Fe-SOD) is primarily a prokaryotic SOD, but seen in several plant families. It contains iron in its catalytic site. It is more closely related to Mn-SOD than Cu Zn-SOD. It may co-exist with Mn-SOD in certain bacteria.

Extracellular superoxide dismutase (EC-SOD) is the predominant SOD found in the extracellular space. It contains copper and zinc and has affinity for heparin sulphate and proteoglycans located on endothelial and other cell surfaces.

SOD acts as an endogenous cellular defense system to degrade superoxide (*O$_2^-$) into oxygen and hydrogen peroxide. SOD, thus, has been suggested as a potentially useful therapeutic agent for treatment of inflammatory disorders. Exogenously added SOD induces Neutrophil apoptosis controlling the tissue injury.

2. Catalase

Catalase, a 240,000 dalton (M.W). protein tetramer is seen in cytosolic peroxisomes. It functions to detoxify H$_2$O$_2$ to oxygen and water in the reaction:

$$2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$$

3. Glutathione reductase (GPx)

GPx is also a tetramer, with M.W. 85,000 dalton containing selenium. It uses glutathione as a co-substrate. It is a cytosolic enzyme and eliminates H$_2$O$_2$. In comparison to catalase, GPx has a wider range of substrates including lipid peroxides. It shows greater affinity to H$_2$O$_2$ than that shown by catalase. GPx primarily functions to detoxify low levels of H$_2$O$_2$ in the cell.
Antioxidant enzymes may act in a coordinate manner to protect living tissue from oxidative damage. Two of these enzymes detoxify $*\text{O}_2^-$ and the other two catalyze $\text{H}_2\text{O}_2$. Since the enzymes which reduce $*\text{O}_2^-$ are inactivated by $\text{H}_2\text{O}_2$, and the enzymes catalyzing $\text{H}_2\text{O}_2$ are inactivated by $*\text{O}_2^-$, one enzyme system complements the other by detoxifying its corresponding inactivating oxidant. As a further benefit, catabolism of $\text{H}_2\text{O}_2$ by GPx and catalase inhibits the formation of $*\text{OH}^-$. This defensive interplay may be critical for cell survival under oxidant stress.

Other enzymes newly recognized as responsive to oxidative stress include haeme oxygenase, metallothionein, heat shock proteins etc. they are not exclusively regarded as antioxidants. SOD and Catalase were taken as the markers of reactive oxygen scavenging in this study. Reduced Glutathione level was also measured as an additional parameter. Reduced Glutathione (GSH) plays an important role in the protection of cells and tissue structures. Its role includes detoxification of xenobiotics, free radicals, peroxides and regulation of immune function. It can scavenge various free radicals directly as well as being a substrate for Glutathione peroxidase enzymes.

$\alpha$- Tocopherol is an important free radical scavenger within membranes. Attack of reactive species like $\text{OH}^-$ upon membranes can damage them by setting off a free radical chain reaction leading to lipid peroxidation. $\alpha$- Tocopherol inhibits this by scavenging peroxyl radicals in the following way:

$$\alpha \text{ TH} + \text{ LOO}^- \rightarrow \alpha \text{ T}^* + \text{ LOOH}$$

However, the tocopherol thereby becomes a radical, $\alpha \text{ T}^*$. 
1.11.4.2. Lipid peroxidation

Polyunsaturated Fatty Acids (PUFA) which carry the potential to regulate serum triglyceride and cholesterol levels are vulnerable to attack by Reactive Oxygen Species. This results in the formation of lipid peroxides. Lipid peroxides are deleterious to health as they include cross-linking of proteins, DNA damage and activation of inflammatory pathways.

Lipid peroxides are further metabolized in the cell with the formation of a wide variety of products including alkenes and carbonyl compounds. Some of these products (eg. hydroxyl alkenals) are toxic by themselves and may serve as second messengers for radical damage. Products resulting from lipid peroxidation are thus attractive parameters to monitor radical damage. Two parameters selected in this study are Malondialdehyde (MDA), the most widely used index of lipid peroxidation and Conjugated Dienes (CD). An elevation in the levels of these compounds indicate increased rate of lipid peroxidation.

1.11.5. Reactive Oxygen Species in diseases

It has recently been established that Free Radicals are involved in the pathogenesis of many diseases including Rheumatoid Arthritis [38-42]. Owing to the short life times of these species and also due to lack of sufficiently sensitive methodology to detect these radicals directly in biological systems, much of the evidence is circumstantial. Therefore, it is not still clear whether free radicals are the sole cause of the injury leading to the disease or are formed as a result of the disease [43]. Reactive Oxygen Species has directly or indirectly been associated with various clinical disorders like atherosclerosis; Diabetes mellitus; lung disorders like Asbestosis and Adult respiratory syndrome; inflammatory disorders like Asthma, Rheumatoid Arthritis; Skin
diseases, brain disorders like Anoxia, Parkinson’s disease, Alzheimer’s disease, Down Syndrome, Multiple sclerosis; problems due to iron overload like Thalassaemia, Idiopathic haemochromatosis; Cancer; toxic states caused by xenobiotics, metal ions etc. and in injuries due to reperfusion and radiation [44-49]. Alterations in antioxidant enzymes have also been reported due to hyperoxia, alcohol, smoking and exposure to ozone [50]. Aging in mammals is explained due to deranged antioxidant system [38].

Lipid peroxidation, one of the best known manifestations of oxidative cell injury has been correlated to deficiency of Vitamin E and selenium [51,52], toxicological factors like CCL$_4$ [53], ethanol [54], paraquat [55] and to a wide variety of clinical disorders. Increased lipid peroxidation was reported in patients with RA [56], Acute Myocardial infarction [57, 58], Multiple sclerosis [59], Alcoholic liver disease experimentally introduced in rats [54], Atherosclerosis [60], Diabetes mellitus [61-63], Alzheimer’s disease [64], Congenital heart failure [65] etc.

1.12. GLYCOPROTEINS IN CONNECTIVE TISSUE DISORDERS

Connective tissue comprises thin layers of cells separated by extracellular matrices. These matrices contain proteoglycans consisting of glycosaminoglycans (GAGs) covalently linked to tissue specific protein. GAGs consist of repeating disaccharides and these in the connective tissue include hyaluronic acid (N-acetyl glucosamine and glucuronic acid), Chondroitin sulphate A (CSA), B (CSB) and C (CSC) containing galactosamine and glucuronic acid; Heparin (Glucosamine and Iduronic acid); Heparin sulphate (Sulphated glucosamine and Iduronic acid) and keratin (Glucosamine and galactose). Elevated levels of GAGs in the blood and synovial fluid have been observed in
the diseases of articular cartilage [66 – 68]. The destruction of involved joints in RA patients had been correlated positively with high GAG levels in synovial fluid [69].

Cartilage contains large amounts of extracellular matrices consisting mainly of proteoglycans and type II collagen. They play an important role in the regulation of proliferation and differentiation of chondrocytes and maintain specific physical properties of the cartilage. In growth plates, chondrocytes proliferate and produce proteoglycans and type II collagen. The cells then turn hypertrophic, induce calcium deposition in the matrix and the calcified matrix gets finally replaced by new bone. The series of changes occurring in growth plates during ossification are essential for elongation of bones. In articular cartilage, the biochemical properties of the matrices are tuned for smooth joint movement and dispersion of compressive load.

Shapses *et al.* [70] found that the role of proteoglycan synthesis equaled the rate of its breakdown in normal tissues. This balance was lost in cartilage diseases with a drop in the rate of synthesis while the rate of breakdown continued. Alternatively, an enhanced rate of GAGs with a constant rate of synthesis can not be ruled out. The GAGs derived from the degradation of proteoglycans in the extracellular matrix of connective tissues are released into synovial fluid from where it reaches urine through blood. Changes in the level of GAGs in these fluids reflect the metabolism of proteoglycans in connective tissue.

The auto antigenic reactivity has been localized to the constant region (C₂ domains) of IgG. There was no evidence for a polypeptide determinant. But carbohydrate changes have been reported by Parekh *et al.*[71]. They compared the N-glycosylation pattern of serum IgG isolated from normal individuals and
from patients with RA and primary osteoarthritis. The results showed that IgG of normal individuals and the patients contained different distributions of asparagine-linked, bi antennary complex-type glycans. No new oligosaccharide structures were detected. The relative extent of galactosylation were compared with that of normal individuals. One or both the arms of IgG molecules from patients terminated in N-acetyl glucosamine revealing a failure in galactosylation. These two were considered as glycosylation diseases which reflected changes in the intracellular processing or post-secretory degradation of N-linked oligosaccharides.

Recently, Wang and Roehrl [69] proposed a pathogenic model for the role of GAGs in connective tissue diseases. According to them, the circulating or locally released GAGs induce the clonal expansion of various GAG-binding cells (T and B cells and macrophages). These cells migrate and adhere to connective tissue where GAGs are abundant. Excessive and prolonged accumulation of these abnormal cells leads to pathological symptoms including joint cartilage and bone erosion. Because GAGs can bind to many type of cell surface receptors as well as cytokines and other soluble protein messengers and also because of these polysaccharides are capable of cross-linking multiple receptors on a single cell or multiple cells, GAGs could act as “Super antigens” and provide signals to promote the expansion of GAG-binding cells. Wang and Roehrl [69] speculated that the disease development is an intrinsic abnormal homeostasis caused by GAGs and not as a consequence of antigen recognition by GAG-binding cells.

Self antigenic GAGs and their correlation with cellular activity and disease prevalence serve as a model system for the discovery and development of drugs against autoimmune connective tissue diseases. Also the inhibition of
abnormal growth or adhesion of immune cells reactive to GAGs may open new therapeutic avenues for the treatment of RA and related diseases. Variations in the activity of certain glycohydrolases may be accepted as markers in the diagnosis of the disease. The effect of drugs for the alleviation of the symptoms may be attributed to their capacity to stabilize lysosomal enzymes. Also, drugs can be designed targeting the enzyme release that would prove beneficial to the organism. Use of inhibitors of glycosidases to prevent or revert glycoprotein degradation offers a new strategy of treatment. Recently, exoglycosidases have been employed for the structural elucidation of O-linked glycoconjugates.

1.13. GLYCOBIOLOGY OF RHEMATOID ARTHRITIS

Rheumatoid Arthritis (RA) is a chronic inflammatory condition which affects the entire body and especially the joints of hand, wrist, feet, ankles and knees. It begins with fatigue, weakness, stiffness of joints, vague joint pain and mild fever. Within weeks, the disease worsens with swellings and severe pain at the joints accompanied by the appearance of a ruddy purplish skin over the joints later developing into nodules. As the disease progresses, deformities develop in the joints of hand and feet. Rheumatoid Arthritis further leads to permanent damage of bones, cartilage and joints. It may even hurt organs like heart, kidney, lungs, eyes and nervous system. The cause of the disease is characterized by periods of progression and remission.

In order to decide the requirements of therapy, it is essential to identify the biochemical abnormalities that are early indicators of the disease. The disease mainly affects the extra cellular matrix of the cartilage. Cartilage is made up of proteoglycan aggregates constrained within a net work of collagen fibres. Aggrecan, the major cartilage proteoglycan, consists of one or more
glycosaminoglycan chains covalently attached to a protein core by their reducing terminals. Cartilage is thus broken down by proteases (metalloproteases, acid proteases) and glycosidases. The polysaccharide chains of glycosaminoglycans are broken down by endoglycosidases, exoglycosidases and suphatases. Hyaluronidase is a widely distributed endoglycosidase that cleaves hexosaminidic linkage. Exoglycosidases include β-glucuronidase, β-galactosidase, β-glucosidase, α-mannosidase, α L-fucosidase, α L-iduronidase and N-acetyl β-D hexosaminidases. N-acetyl glucosamine is abundant in glycosaminoglycans of cartilage and the terminal β-linked GlcNAc residues are released by N-acetyl β-D hexosaminidases (NAHases). Glycosidases take part in the degradation of glycoproteins, glycolipids and glycosaminoglycans. However, the role of these enzymes in cartilage matrix homeostasis and physiopathology of many diseases is yet to be known. This is partly due to the lack of systematic studies on analysis of glycosaminoglycan degrading glycosidases.

Collagen Induced Arthritis (CIA) is a well known model for RA because it resembles the latter in a number of pathological, histological, biochemical and immunological aspects. Features of CIA include chronic synovitis with inflammatory cell infiltration, pannus formation, destruction of cartilage and bone erosion [72-75]. Humoral and cellular immunity is involved in CIA as well as RA. The similarities in the joint pathology between CIA and RA are widely used for studying the pathogenesis of RA and for screening of new drugs against this disease [76, 77].

No detailed studies have been conducted on tissue specific variations in the carbohydrate components of glycoproteins in relation to the respective glycosidase in rheumatoid arthritis and during its treatment using nutritional supplements like vitamins and spices. Also, the involvement of free radicals in
RA and the role of antioxidants in the treatment of this disease are not elaborated enough. Hence this work has been taken up with the following objectives.

1. To study the normal profile of glycoproteins in serum, liver, heart, kidney, testis and brain of rats.

2. To study the variations in oligosaccharides of glycoproteins in Collagen Induced Arthritis (CIA) in rats.

3. To estimate the activity of glycosidases in tissues of normal and CIA rats.

4. To investigate the curative effects of vitamin C, vitamin E, ginger, turmeric, sallaki and glucosamine against arthritis.

5. To evaluate the extent of inflammation induced by Collagen in rats.

6. To examine the anti-inflammatory effect of vitamins C and E, extracts of the spices ginger and turmeric, extract of the plant sallaki and of glucosamine in Collagen Induced Arthritis.

7. To investigate the changes in antioxidant defense system in different tissues of CIA rats.

8. To establish a relationship between anti-inflammatory and antioxidant roles of selected vitamins, spices, sallaki and glucosamine in CIA rats.

9. To establish the anti-rheumatoid effect of selected vitamins, spices, plant extract and glucosamine in CIA rat model.