1. INTRODUCTION
1.1. Diabetes mellitus - a historical perspective

Greek physician Aretaeus of Cappadocia first coined the name diabetes in the second century AD. He described the disease "a melting down of the flesh and limbs in the urine. Life is short, unpleasant and painful. Restlessness and burning thirst affect the patients and within a short time, they expire".

The association of polyuria with sweet tasting substances in the urine was first reported in Sanskrit literature from 5th to 6th century AD by Sushruta. The urine of certain polyuric patients was described as testing like honey (madhumeha), being sticky to the touch and strongly attracting ants. Even in those days, they described two forms of diabetes, one affecting older, fatter people and the other affecting thin people who did not survive long. There is obvious but possibly accidental parallels with present day subdivision of diabetes into two types - insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM).

Avicenna, an Arabic physician (960-1037A.D) described accurately the clinical features of diabetes and mentioned two specific complications of the disease, namely, gangrene and 'collapse of sexual functions'.

In 1869, Paul Langerhans identified β cells of pancreatic islets. Opie in 1900 noted that β cells in the islets to be damaged in humans with uncontrolled diabetes. Discovery of insulin in 1921 by Benting and Best was a golden landmark in the history of diabetes mellitus.

In 1969, Dorothy Hodgkin determined three-dimensional structure of insulin. In 1993, Diabetes Control and Complication Trial Study reported that
strict glycemic control reduces the risk of diabetic microvascular complications in
IDDM. Table-1.1 and Table-1.2 present several milestones in the discovery of
diabetes mellitus in ancient and 20th century, respectively [1].
Fig.-1.1.: Occurrence of Fibro Calculous Pancreatic Diabetes (FCPD) in India. Southern India, situated within the tropical belt, has higher frequency of FCPD than northern India. The south western Kerela, Tamilnadu has the highest known prevalence of FCPD in the world (From Text Book Of Diabetes, Vol.-I, 2nd Edn., Ed., J. Pickup and G. Williams, 1997).
<table>
<thead>
<tr>
<th>Date</th>
<th>Source</th>
<th>Observation</th>
</tr>
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<tbody>
<tr>
<td>15th Century B.C. to 2nd Century A.D.</td>
<td>Ebers papyrus (Egypt)</td>
<td>Clinical descriptions of polyuric conditions resembling diabetes mellitus.</td>
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<tr>
<td></td>
<td>Galeu (Rome)</td>
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<td></td>
<td>Aretaeus (Cappadocia)</td>
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<tr>
<td>5th Century</td>
<td>Sushruta and Charuka (India)</td>
<td>Clinical description including sugary urine; obese and thin patients distinguished</td>
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<td>7th Century</td>
<td>Chen Chuan (China)</td>
<td>Clinical description including sugary urine</td>
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<tr>
<td>10th Century</td>
<td>Avicenna (Arabia)</td>
<td>Clinical Descriptions in including sugary urine</td>
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<tr>
<td>17th Century</td>
<td>Thomas Willis (England)</td>
<td>Diabetic urine contains sugar</td>
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<td>18th Century</td>
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<td>1776</td>
<td>Matthew Dobson (England)</td>
<td>Diabetic serum contains sugar</td>
</tr>
<tr>
<td>1788</td>
<td>Thomas Cowley (England)</td>
<td>Pancreatic calculi cause diabetes</td>
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<td>19th Century</td>
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<tr>
<td>1815</td>
<td>Michel Chevrenl (France)</td>
<td>Excess sugar in diabetes is glucose</td>
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<tr>
<td>1849-55</td>
<td>Claude Bernard (France)</td>
<td>Glucose stored in liver as glycogen</td>
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<td>1857</td>
<td>Wilheld Petter (Germany)</td>
<td>Acetone found in diabetic urine</td>
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<tr>
<td>1869</td>
<td>Paul Langerhans (Germany)</td>
<td>Pancreatic islets identified</td>
</tr>
<tr>
<td>1889</td>
<td>Oskar Minkowski and Josef Von Mering</td>
<td>Pancreatectomy causes diabetes in dogs.</td>
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### Table-1.2.: Milestones in the scientific understanding of diabetes mellitus—20th Century

<table>
<thead>
<tr>
<th>Date</th>
<th>Source</th>
<th>Observation</th>
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<tbody>
<tr>
<td>1900</td>
<td>E.L. Opie (USA)</td>
<td>Hyaline Degeneration (amyloid deposition) of islets</td>
</tr>
<tr>
<td>1907</td>
<td>Jean de Meyer (Belgium)</td>
<td>Hypothetical glucose lowering islet hormone named insulin</td>
</tr>
<tr>
<td>1907</td>
<td>George L. Zuelzer (Germany)</td>
<td>Isolated Pancreatic extract with hypoglycemic activity</td>
</tr>
<tr>
<td>1019</td>
<td>Israel Kliener (USA)</td>
<td></td>
</tr>
<tr>
<td>1921</td>
<td>Nicholas Paulesco (Romania)</td>
<td></td>
</tr>
<tr>
<td>1921</td>
<td>Frederick G. Benting (USA)</td>
<td>Isolation and first clinical use of insulin</td>
</tr>
<tr>
<td></td>
<td>Charles H. Best (USA)</td>
<td></td>
</tr>
<tr>
<td>1936</td>
<td>H.P. Hims Worth (England)</td>
<td>Distinguished insulin sensitivity and insulin resistant type</td>
</tr>
<tr>
<td>1955</td>
<td>F. Sangar (England)</td>
<td>Determined sequence of insulin</td>
</tr>
<tr>
<td>1969</td>
<td>Dorothy Hodgkin (England)</td>
<td>Determined three dimensional structure of insulin</td>
</tr>
<tr>
<td>1971</td>
<td>Pierre Freychet (USA)</td>
<td>Identified insulin receptor</td>
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<tr>
<td>1980</td>
<td>World Health Organization</td>
<td>Agreed diagnostic blood glucose limits for diabetes</td>
</tr>
<tr>
<td>1993</td>
<td>Diabetic Control and Complication Trial (USA)</td>
<td>Strict glycemic control reduces the risk of microvascular complication in IDDM</td>
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1.2. Definition, classification, complications and biochemical basis for pathogenesis of diabetes mellitus

1.2.1. Definition of diabetes mellitus

Diabetes mellitus is a syndrome characterized by chronic hyperglycemia and disturbances of carbohydrate, protein and fat metabolism associated with absolute or relative deficiencies in insulin secretion and / or insulin action [2].

Diabetes mellitus may be suspected or recognized clinically by the presence of characteristic symptoms, such as, excessive thirst, otherwise unexplained weight loss or one or more of the many complications associated with the disease. The central identifying feature of diabetes mellitus is chronic and substantial elevation of blood glucose level. According to WHO criteria [3], a random venous plasma glucose level that exceeds 11.1 mmol/L or a fasting value of ≥ 7.8 mmol/L establishes the diagnosis.

1.2.2. Classification of diabetes mellitus

Two major types of diabetes mellitus are recognized - insulin dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM). The latter type is again subclassified as obese and non-obese group. Another type, which is commonly found in developing and underdeveloped countries is classified as malnutrition related diabetes mellitus, is characterized by onset in youth emaciation and heavy glycosuria accompanied by little or no ketouria.

Insulin dependent diabetes mellitus (IDDM)

IDDM is classified as life threatening form of diabetes mellitus, which has been treatable only after the discovery of insulin. The term 'insulin dependent' is
crucially important as it emphasizes the fact that these patients depend on insulin replacement for their survival and die unless it is administered. This reflects the severity of insulin deficiencies in those patients, which is so profound that triglycerides in fat are broken down and resulting free fatty acids are metabolized to yield ketone bodies and form diabetic ketoacidosis, the biochemical identification of IDDM.

The commonest cause of IDDM is autoimmune destruction of β cells of islets of Langerhans of pancreas, which synthesize and secrete insulin. There is evidence that in some areas the incidence of IDDM in childhood is increasing as much as two folds each decade [4]. This increase may be related to the relative affluence, as IDDM seems to be associated with high socio-economic class [5], although some outbreaks suggest that a localized epidemic of an infectious agent which could precipitate the disease [6]. Intercurrent infections can also explain the seasonal variation in the presentation of IDDM, commonly in autumn and winter when viral and other infections are prevalent [7]. IDDM is prevalent most commonly in children and adolescents, the peak of age of onset being 11-13 years. However, it can appear at any age and a significant proportion (10%) of elderly diabetic patients over 65 years of age requires insulin [8]. For this reason, the term 'juvenile onset diabetes' has been suggested to be abandoned. Genetic influence is important in IDDM. Genes in class II region of HLA system on chromosome 6 are associated with IDDM. However, IDDM is partly due to non-genetically determined factors. Attention has been focused on virus and toxins. Wild virus exhibiting specific β-cell tropism and diabetogenic potential has been
reported in animals and diabetes is frequently found in patients with congenital rubella syndrome [9].

**Non insulin dependent diabetes mellitus (NIDDM)**

Non-insulin dependent diabetes mellitus, as its name implies, identifies diabetic patients, who do not depend on exogenous insulin treatment to remain alive. By definition, NIDDM is taken to exclude the autoimmune destruction of \( \beta \) cells.

NIDDM was previously known as "mature onset" diabetes to acknowledge the fact that the disease is diagnosed in the middle-aged people. However, it is now clear that NIDDM may also develop in young adults (Mature Onset Diabetes mellitus on Young adults or MODY). Despite the presence of hyperglycemia, the level of ketone bodies in the blood and urine are low in NIDDM. Insulin treatment is not necessary to maintain life or prevent ketosis, although patients may need it to attain adequate blood glucose control. Most forms of NIDDM are associated with a positive family history of the disease. NIDDM is commonly associated with obesity. Specific insulin receptor gene mutation has now been identified in some persons with NIDDM [10]. Mutation in the glucokinase gene in chromosome 7p constitutes the primary genetic defect in at least 50% of pedigrees of maturity onset diabetes of the young (MODY). Identified mutations include both missense and nonsense, which interfere with the enzymatic activity of glucokinase impairing the ability of the \( \beta \)-cells to secrete insulin in response to glucose and resulting in relative insulin deficiencies [11]. Mutation of mitocondrial DNA encoding leucine t-RNA is associated with the
rare syndrome, myopathy, encephalopathy, lactic acidosis and stroke like episodes (MELAS), in which diabetes may occur. The inheritance of mitochondrial DNA, unlike nuclear DNA, is exclusively maternal, and mitochondrial defects have been associated with certain rare neurological syndrome, some accompanied by NIDDM. An adenine to guanine point mutation at nucleotide 3246 of mitochondrial DNA, which encodes a leucine t-RNA, was first noted in patients with MELAS syndrome [12].

Islet amyloid polypeptide (IAPP), 37 amino acid monomeric polypeptide that forms the amyloid deposits is commonly found in islet of Langerhans of NIDDM patients [13]. Since IAPP inhibits glucose uptake in insulin stimulated muscle, it has been considered as a possible factor in the etiology of insulin resistance in NIDDM [14]. NIDDM accounts for about 90-95%, while IDDM accounts for 5-10% of cases of all types of diabetes mellitus.

**Malnutrition related diabetes mellitus**

In developing tropical countries throughout the world, the association of certain forms of diabetes with malnutrition has been recognized for several decades. In 1985, WHO study group on diabetes mellitus identified malnutrition-related diabetes mellitus (MRDM) as a separate type of diabetes mellitus.

Two subgroups of MRDM have been described, one group resulting from pancreatic damage inflicted by chronic pancreatitis, in which large pancreatic stones commonly occur. This type is called fibro-calculous pancreatic diabetes (FCPD). The second group is called protein deficient diabetes mellitus (PDDM) developed in severely malnourished people and clinically resembles classical
IDDM except that ketoacidosis does not develop, if insulin treatment is withdrawn. They are distinguished from FCPD by the absence of pancreatic pathology such as pancreatic calculous or other feature of chronic pancreatitis [15]. In India, FCPD is more common in the southern than in the northern part of the country. In southwest India, particularly Kerala probably has the highest prevalence of FCPD in the world [16]. Fig.-1.1 shows distribution of different types of malnutrition related diabetes mellitus patients in India. India has the largest diabetes population in the world (around 30 million diabetes or about a quarter of world's diabetic population). However, a vast majority of them remains oblivious that they suffer from diabetes.

Urban prevalence of diabetes in India has gone up from 11.8% in 1998 to 13.2% in 2000, according to the latest urban diabetes study by Diabetes Epidemiology Study group in India and Novo Nordisk Education Foundation. The study covered six big cities of India - Delhi, Mumbai, Chennai, Bangalore, Hyderabad and Kolkata. According to Novo Nordisk Education Study group, currently there are 151 million people worldwide with diabetes but of them, only 50% are aware of it.
1.2.3. Common complications of diabetes mellitus

Most identifying feature of diabetes mellitus is hyperglycemic condition. Although hyperglycemia can be regulated by diet, insulin injection or oral hypoglycemic agent therapy, standard treatment is not always effective in preventing the development of chronic complications affecting eye, kidney, nerves, and arteries. In the eye, retinal capillary damage leading to edema, new vessel formation, and hemorrhage causing blindness is 25 times more common among diabetics and is leading cause of blindness (Diabetic retinopathy) [17]. Capillary in glomerulous associated with basement thickening makes chronic renal failure with albuminuria, which is 17 times more common in diabetic patients (Diabetic nephropathy) [18]. In the diabetic peripheral nerves, axonal dwindling and segmental demyelination are associated with very high prevalence of motor, sensory and autonomic dysfunction including impotence, which affect approximately 40% of diabetic patients (Diabetic neuropathy) [19]. Increased atheromata in medium and large arteries makes coronary artery disease and strokes are as twice as common among diabetic patients. Gangrene leading to amputation is at least 5 times more frequent among diabetics [20]. The average life expectancy of diabetes mellitus patients is only two thirds of the general population [21]. Until recently, knowledge about diabetic complications was in large part limited to clinical description. In recent years information from physiological and biochemical investigations of diabetic complications has been accumulating rapidly.
1.2.4. Biochemical basis for pathogenesis of diabetes mellitus

From several investigations, it has been suggested that causal factor primarily responsible for the development of diabetic complications is prolonged exposure to hyperglycemia. Hyperglycemia appears to damage tissues by causing acute reversible and irreversible alterations in stable macromolecules. The acute metabolic reversible changes that result from hyperglycemia include polyol pathway activity [22], increased NADH/NAD$^+$ ratio [23], decreased intracellular myoinositol concentration [24], increased de novo synthesis of diacyl glycerol [25], stimulation of protein kinase [22,25] and enhanced formation of early glycation products [26].

The first and rate limiting step of polyol pathway (Fig.-1.2) is governed by aldol reductase, which is found in insulin independent tissues, such as nerve, retina, lens, glomerulous and blood vessel wall. As the $K_m$ of aldol reductase is much higher, normally the pathway is inactive in normoglycemic condition. In hyperglycemia, glucose concentration rises parallel in insulin independent cells and thus sorbitol is produced from glucose and then subsequently oxidised to fructose by specific dehydrogenase using NAD$^+$ as cofactor (Fig.-1.2). Sorbitol does not diffuse across the cell membranes and may accumulate sufficiently within certain cells to cause osmotic damage and swelling, an example of which is the lens during development of diabetic cataracts.

In streptozotocin-induced diabetes in wistar rats, 66% loss of glutathione level occurs after 1 week of diabetes, two weeks after which morphological manifestation of cataracts takes place. The lens eventually becomes cloudy due to
Diabetes Mellitus (High Glucose)

GLYCOLYTIC PATHWAY

Glucose → Glucose 6-P → Fructose 6-P

↑ Glycation → Glyceraldehyde 3-P → NAD⁺ → NADH

1,3 Diphosphoglycerate → Pyruvate → Lactate

POLYOL PATHWAY

Glucose → Aldol reductase → Sorbitol → Fructose

NAD⁺ → NADH

DIACYL GLYCEROL PATHWAY

Dihydroxyacetone → α-Glycerol-P → Diacylglycerol

Protein Kinase C Activation

Fig. 1.2: Interactions between polyol (sorbitol) and diacyl pathways. Increased flux through the polyol pathway may increase the nonenzymatic glycation and diacyl glycerol synthesis by increasing the NADH/NAD⁺ ratio (from Text book of Diabetes, Vol.-1, 2nd Edn., edited by J. Pickup and G. Williams, 1997).
Figure 1.3: Schematic representation of the protein glycation. (British Medical Bulletin, vol. 45, edited by R.D.G. Leslie, 1989)
increased level of high molecular weight disulfide linked aggregates of crystalline protein. This suggests that in addition to osmotic stress, lens is damaged by metabolic changes, including formation of reactive oxygen species (ROS) or decreased antioxidant capacity [27].

In other tissues such as peripheral nerve, sorbitol level in diabetes is probably too low to cause osmotic damage, but yet altered redox state i.e., increased level of NADH/NAD⁺ as a result of sorbitol oxidation would raise the intracellular levels of highly reactive glycating sugar (methylglyoxal, acetol), by inhibiting glyceraldehyde-3-phosphate dehydrogenase [28] and it would also accelerate the reduction of dihydroxyacetone phosphate to α-glycerol-3-phosphate, the precursor of diacyl glycerol that stimulates protein kinase C activity. Protein kinase C activity has been implicated in many processes relevant to diabetic complications, including regulation of vascular permeability, blood flow, growth factor effects and basement membrane synthesis [29]. An interaction of polyol and diacyl glycerol pathway is shown in Fig.-1.2. Another significant change induced by hyperglycemia is excessive production of early glycation products [26]. This is normally formed continuously both outside and inside cells, as glucose rapidly attaches to amino groups of proteins through nonenzymatic process of nucleophilic addition, to form Schiff base adducts, which then by Amadori rearrangement form more stable early glycation products (Fig.-1.3). Excessive formation of these early glycated products may adversely affect several functions leading to diabetic complications [30,31,79].
Some early glycation products dissociate or are degraded but those formed on collagen, DNA and other long lived macromolecules slowly undergo further complex formation by irreversible chemical rearrangements and form advanced glycated end products or AGE [32].

The AGE forming reactions appear to follow two patterns [33,34]. One type closely resembles the heterocyclic imidazole derivative, 2-furyl-4(5)-(2-furanyl)-1-H-imidazole derivative (FFI), a yellow compound with a fluorescence spectrum characteristics of AGE proteins. This type of AGE appears to form from condensation of two Amadori products. The other route of AGE formation apparently involves a reaction of the Amadori products with a variety of sugar fragmentation product, particularly the highly reactive dicarbonyl compounds like methylglyoxal or 3-dioxy-glucosone [28,35]. Fig.-1.4 shows formation of AGE from early non-enzymatic glycation products in hyperglycemic condition. The AGEs are stable. They, therefore, accumulate throughout the life of the tissue and vessel wall and their levels do not return to normal, even if hyperglycemia is corrected. The formation of AGE could contribute to the diabetic tissue damage in many ways, including effects of extracellular matrix proteins, interaction with specific AGE receptors on various cells and interaction with intracellular macromolecules. Various AGEs can form covalent bonds with nearby amino groups on other proteins and nucleotides, resulting in glucose derived crosslinks. Increased formation of glycated proteins (both early and advanced) in insulin independent tissues could play a role in pathogenesis of diabetes mellitus, few examples may be cited. Glycation of lens crystalline protein leads to opacity of
the protein matrix mimicking the effect of diabetic cataract [27,36]. Glycation of serum albumin not only causes aggregation, but also decreases its capacity to bind drugs and bilirubin [37]. Thickening of glomerular basement membrane due to accumulation of glycated collagen protein has been suggested as one of the reasons of kidney disease (diabetic nephropathy) in diabetic patients [38]. Glycation of low-density lipoproteins (LDL) impairs its ability to stimulate cholesterol ester synthesis by fibroblast and peritoneal macrophages [39,40]. Spectrin, an important protein of erythrocyte membrane is glycated and oxidatively damaged in diabetes [41]. Hematological abnormalities including erythrocyte aggregation with increased microviscosity [42] and decreased elasticity causing deformation [43] have been reported in diabetes mellitus. Thus, protein glycation may be directly or indirectly related to different complications of diabetes. Even though several body proteins are known to be significantly glycated in diabetes, hemoglobin glycation is very important and it is routinely estimated for evaluating the extent of hyperglycemic status.
1.3. Hemoglobin and its non-enzymatic glycation

1.3.1. Hemoglobin - a brief review on structure and function

"Its 10,000 atoms are assembled into four chains each a helix with several bends. The molecule has one shape when ferrying oxygen molecules and a slightly different shape when it is not." - M. F. Perutz.

Large organism has a high respiratory demand that cannot be satisfied by diffusion of oxygen from environment. Hence, they have been forced in the evolutionary sense, to develop special transporter of oxygen from outer environment to the tissues. These systems are represented by the so-called respiratory proteins-hemoglobin, erythrocruorin, hemocyanin, hemerythrins, which differ greatly in the nature of prosthetic group and the protein moiety.

In human and other vertebrates, the clinical basis for oxygen transport is represented by hemoglobin (Fig.-1.5), which is packaged into specialized cells (erythrocytes) sufficiently pliable to withstand mechanical stress during their circulation in the cardiovascular system. Every milliliter of blood has approximately 5 billion erythrocytes and each erythrocyte is packed with 280 millions hemoglobin molecules and the molecular weight of hemoglobin is 68,800 and is made up of 4 subunits containing about 10000 atoms of hydrogen, carbon, nitrogen, oxygen and sulfur plus four atoms of iron [44,46]. Each iron atom lies at the center of the group of atoms that form the pigment called heme [Fig.-1.6], which gives blood its red colour and its ability to combine with oxygen. Each heme group is enfolded in one of the four chains of amino acid units that collectively constitute the protein part of the molecule called globins. The four chains of globin consist of two identical pairs. The members of one pair
Fig. 1.5.: Hemoglobin molecule, as deduced from X-ray diffraction studies. The drawing follows the representation scheme used in three-dimensional model. The molecule is built up from four subunits; two α-chains (light block) and two β-chains (dark block). [From M.F. Perutz, Scientific American, Inc., 1973].
Fig.-1.6.: Heme: The pyrole rings and methylene bridge carbons are co-planer and the iron atom (Fe\(^{2+}\)) resides almost in the same plane. [From Harper's Biochemistry, 24th Edn., 1996].
are called α-chains and those of other called β-chains. Different helices of the β-chains of hemoglobin have been shown in Fig.-1.7. Although almost similar in overall length, the α (141-residues) and β (146-residues) polypeptides of adult hemoglobin (HbA-α₂β₂) are encoded by different genes and have different primary structures.

In both subunits of adult hemoglobin (HbA), apart from two histidyl residues (E₇ and F₈) that function in oxygen binding, non-polar residues e.g. Leu, Val, Phe and Met are at the internal part of the molecule. The hydrophilic residues are surface feature of both α and β subunits [45].

In absence of an oxygen carrier, a liter of arterial blood at body temperature could dissolve and transport only 3 milliliters of oxygen. However, the presence of hemoglobin increases this quantity to 70 times. Without hemoglobin large animals could not get enough oxygen to survive. Similarly, hemoglobin is responsible for carrying more than 90% of carbon dioxide transported by venous blood. Each of four atoms of iron in the hemoglobin can take up one molecule of oxygen. The reaction is reversible in the sense that oxygen is taken up where it is plentiful as in the lungs, and released where it is scarce as in the tissues. The reaction is accompanied by a change in color. Hemoglobin containing oxygen, known as oxyhemoglobin makes arterial blood scarlet, deoxy or oxygen free hemoglobin makes venous blood purple [45,46].

The oxygenated and deoxygenated hemoglobin are in the same electronic condition i.e., ferrous state. They become oxidised to trivalent or ferric state (methemoglobin), if hemoglobin is treated with ferricyanide or removed from the
red cells and exposed to air. Oxidation of hemoglobin is also found in certain blood diseases. Under these conditions hemoglobin turns brown as methemoglobin (cyanosis).

Ferrous iron acquires its capacity for binding molecular oxygen only through its combination with heme and globin. Heme alone does not bind with oxygen in aqueous solution. If it binds, it rapidly oxidises the ferrous heme to ferric heme, which can not bind oxygen. A complex of oxygen, sandwiched between two hemes, is an intermediate in this reaction. The formation of this sandwich is sterically blocked by the distal histidine and other residues surrounding the sixth co-ordination position of iron [47]. Thus, the specific chemical environment of the globin chain makes the binding of oxygen possible with ferrous heme iron (Fig.-1.7). The function of globin, however, goes further. It enables the four iron atoms within each molecule to interact in a physiologically advantageous manner. The combination of any three of the iron atoms with oxygen accelerates the combination with oxygen of the fourth. Similarly, release of oxygen by three of the iron atoms makes easier for the fourth iron atom to cast off its oxygen. Hemoglobin, thus, exhibits cooperative binding kinetics, a property that permits it to bind a maximum quantity of oxygen at the respiratory organ and to deliver a maximum quantity of oxygen at the prevailing pO₂ of peripheral tissues. Uptake and delivery of oxygen, a vital process is carried out by hemoglobin molecule has been carefully engineered to display a fine tuning of its oxygen carrying properties characterized by the presence of both homotropic and heterotropic
Fig.-1.7.: A model of β-chain of hemoglobin. Proximal histidine ($F_8$) of globin chain binds with 5th coordination position of heme iron and distal histidine ($E_7$) is closely situated at free 6th coordination position of iron [From M.F. Perutz, Scientific American, Inc., 1973].
Fig.-1.8.: Oxygen binding curves of hemoglobin and myoglobin [From Harper's Biochemistry, 24th Edn., 1996].
Fig.-1.9.: Salt links between different subunits in deoxyhemoglobin. These non-covalent, electrostatic interactions are disrupted on oxygenation. [From Stryer L., Biochemistry, 3rd Edn., 1988].
interaction, in order to ensure an adequate delivery of oxygen in response to the physiological demands of the living species.

Heme-heme or homotropic interactions are responsible for sigmoid shape of the oxygenation curve (Fig.-1.8), whose steep middle portion indicates release suitably sensitive to small drop of oxygen pressure. Equally important are the environmental factors, namely, chloride ion, hydrogen ion, carbon dioxide and intra-erythrocyte 2,3-bisphosphoglycerate (2,3 BPG), whose interactions with the protein moiety (i.e. heterotropic interactions) control the position of the curve, along the pO$_2$ axis or overall oxygen affinity. An increase in the concentration of any of the above-mentioned effectors lowers the oxygen affinity of the hemoglobin, and this change is reversible.

This fine-tuning of the hemoglobin molecule is based on the ligand-linked conformational change in a multisubunit structure. This phenomenon is often described in terms of two state allosteric model, proposed by Monod et al [48] and determined by X-ray crystallography by Liddington et al in 1992 [49]. In such a mechanism, two alternative quaternary structure (low affinity T state and high affinity R state) having different affinities for oxygen are in equilibrium with each other at all stages of saturation and the binding of ligand swings the equilibrium towards the high affinity form (R state). Binding of oxygen accompanies the rupture of salt bonds between carboxyl termini of all four subunits (Fig.-1.9). Subsequent oxygen binding is facilitated, since it involves rupture of only fewer salt bonds [50]
Fig. 1.10.: The iron atom moves into the plane of the heme on oxygenation. The proximal histidine $F_8$ is pulled along with the iron atom and become less tilted. [From Stryer, L., Biochemistry, 3rd Edn., 1988].
The iron atom of deoxyhemoglobin lies about 0.06 nm beyond the plane of the heme ring. On oxygenation, it moves into the plane of the heme ring (Fig. 1.10). This movement is transmitted to the proximal (F₈) histidine, which moves toward plane of the ring and to residues attached to the histidine.

In addition to transporting oxygen from lung to peripheral tissues, hemoglobin also plays an important role in bearing CO₂ from the tissues back to lungs for exhalation. But this gas is not borne by the iron atoms, and only 15% of it is bound directly to the globin molecule and most part is taken up by red cells and the non cellular fluid of the blood in the form of bicarbonate. The carbonic anhydrase in erythrocytes catalyses the formation of carbonic acid.

\[
\text{CO}_2 + \text{H}_2\text{O} \xrightarrow{\text{Carbonic anhydrase}} \text{H}_2\text{CO}_3 \xrightarrow{\text{H}^+} \text{H}^+ + \text{HCO}_3^-
\]

Carbonic acid rapidly dissociates into bicarbonate and proton. To avoid the danger of increasing acidity of the blood, hemoglobin molecule binds two protons for every four oxygen molecules are lost and so contributes significantly to the buffering capacity of blood. The re-appearance of the proton when oxygen is taken up in the lungs set in motion a series of chemical reactions that leads to discharge of CO₂, i.e., as oxygen binds to deoxygenated hemoglobin, protons are released and combined with bicarbonate, forming carbonic acid which forms CO₂ by carbonic anhydrase and CO₂ is exhaled. Thus, binding of oxygen forces the exhalation of CO₂. This reversible phenomenon was first described by Christin Bohr and known as Bohr effect. The Bohr effect is a property of tetrameric
hemoglobin and is dependent upon its homotropic (heme-heme interaction) or cooperative effects. The proton responsible for Bohr effect is generated by breaking salt bonds during the binding of oxygen to T structure. The protons are released from β-chain histidine residues. These protons drive bicarbonate towards carbonic acid, which is then released as CO₂ in alveolar blood. Conversely, upon the release of oxygen, the T structure and its saltbridges are reformed, requiring protons to bind to the β chain. Thus, the presence of protons in peripheral tissues favors the formation of salt bridges by protonating the terminal histidine residues of β subunits. Reformation of salt bridges facilitates the release of oxygen from oxygenated (R-form) hemoglobin. Overall, an increase of protons favors oxygen release, while an increase in oxygen causes proton release. In peripheral tissues, an oxygen storage causes an increased accumulation of 2,3 BPG [51], which stabilizes the T or deoxygenated form of hemoglobin by crosslinking the β chains and contributing additional salt bridges that must be broken for the T form to click into R form.

1.3.2. Non-enzymatic glycation of hemoglobin

In normal adult human, the major hemoglobin protein (90-95%) is HbA₀, which has a subunit structure α₂β₂ (i.e. without any modification). Minor components that make up the remaining 5-10% are HbA₂ (2-5%), HbF (0.5%), HbA₁₈ (0.2%), HbA₁₉ (0.4%) and HbA₁₆ (3-5%). Last three species are glycated at the N-terminal Valine residues of the β-chains as follows:

HbA₁₈: β-N-Fructose-1,6-diphosphate (HbA₁₈a) and
β-N-Glucose-6-phosphate (HbA₁₈b)
HbA_1b: β-N-Carbohydrate

HbA_1c: β-N-Glucose

A small fraction of hemoglobin may also be glycated at ε_{139} residues of α or β chain. The separation by cation exchange chromatography of the negatively charged minor components of normal adult hemoglobin, designated HbA_{1a}, HbA_{1b} and HbA_{1c}, collectively known as HbA_1 was demonstrated in 1958 by Allen et al [52]. Four years latter, Huisman and Dozy [53] observed that the HbA_1 fraction was increased in diabetic patients and over the next decade several studies confirmed that HbA_{1c} levels were generally two to three times higher than normal in diabetes.

In 1975, Fluckiger et al [54] showed that HbA_{1c} could be formed in vitro by incubating purified hemoglobin in the presence of glucose at 37°C, suggesting that the action was not mediated by enzyme. The difference in ionic change between HbA_{1c} and ordinary adult hemoglobin HbA_0 is due to the adduction of glucose molecule to the amino terminal valine of the β chain via an aldimine linkage, which then undergoes an Amadori rearrangement to form a more stable and virtually irreversible ketoamine product [55]. The logical culmination of these findings was the hypothesis that in diabetes the level of the glycated hemoglobin (HbA_{1c}) would be proportional to the integrated or time averaged blood glucose level into previous seven to eight weeks- a period approximating to the half life of the average red blood cell. By early 1980s, confirmations of usefulness of glycated hemoglobin measurement as an objective and retrospective index of glycemia had been provided in a number of studies and many diabetic clinics.
included the test as a regular and routine part of management strategy as an index of long term glycemia [56].

The study of hemoglobin glycation has served as a model system, because it is now clear that other proteins undergo similar non-enzymatic reaction. Fig.-1.3 is a diagrammatic representation of the non-enzymatic glycation of proteins in general. In HbA\textsubscript{1c}, glucose reacts with hemoglobin forming an intermediate Schiff base which then undergoes Amadori rearrangement to form more stable and virtually irreversible 1-amino-1-deoxy-fructose derivative [55,57].

1.3.3. Properties of glycated hemoglobins

Although glycation of hemoglobin is well known, available information on structural and functional properties of glycated hemoglobin is strangely very scantily. Only few reports are available in this regard. Of all glycated hemoglobin species, HbA\textsubscript{1c} has received considerable attention, because its concentration is proportionately increased in diabetic patients with ambient hyperglycemia and reflects the extent as well as management of diabetic condition [58]. HbA\textsubscript{1c} has attracted attention not only for its clinical importance in diabetes mellitus, but also for a basic interest that it represents a rare occasion of natural hemoglobins with altered oxygen affinity present in normal human blood [59].

When a chemical modification is introduced in the primary structure of hemoglobin, functional changes might be induced in two ways. In human HbA\textsubscript{1c}, for example, formation of the sugar adducts with the $\beta$-chain N-terminal valine results in:
(1) abolishment of the reactivity of the termini [60], which have been shown to be a part of the binding sites for organic phosphates and molecular CO$_2$, which is accumulated within the tissue and

(2) a dampened R-T transition of the quaternary structure upon oxygenation [61].

The array of positively charged residues between $\beta$-chains in the central cavity of hemoglobin (3-Val, 2-His, 82-Lys, 143-His) have been considered to repel each other in a low anionic concentration (Fig.-1.11) and thus destabilizes T-state quaternary structure of deoxyhemoglobin relative to R-state conformation of oxyhemoglobin [62]. Glycation-induced elimination of positive charge of N-terminal Valine due to ketoamine linkage may be a cause of slight reduction of oxygen affinity of HbA$_{1c}$.

Even in the presence of high concentration of 2,3-BPG, glycated hemoglobin (HbA$_{1c}$) was found to be still 50% saturated with oxygen at partial pressures where HbA$_0$ gave up most of its oxygen [59,63]. Thus, compared to HbA$_0$, HbA$_{1c}$ exhibits moderately high oxygen affinity.

Using ESR spectroscopic study, Watla et al [64] reported the decreased mobility of the Lysine residue in glycated hemoglobin and suggested a change of conformation of the molecule.

Nagai et al [61] studied EPR spectra of different types of glycated hemoglobins and found an increased stability of low affinity quaternary structure (T) and decreased susceptibility to organic phosphate.
Fig. 1.11.: Mode of binding of BPG to human deoxyhemoglobin. BPG interacts with three positively charged groups on each β-chain. [From Stryer, L., Biochemistry, 3rd Edn., 1988].
Hemoglobin molecule possesses peroxidative properties. Compared to nonglycated hemoglobin, a reduced peroxidase activity of glycated hemoglobin (HbAic) was reported by Khoo et al [65]. They also suggested a modulation mechanism linked to structural change of the protein due to glycation.

Oxidative stress and free radical mediated cellular injuries have been suggested as the major causes of pathophysiological complications in diabetes mellitus. However, very little is known about the mechanism of formation of such free radicals in this disorder.
1.4. Oxidative stress and free radical injuries in diabetes mellitus

1.4.1. Free radicals and reactive oxygen species

Free radical is defined as any species capable of independent existence and contains one or more unpaired electrons [66]. Although reactive oxygen species (ROS) does not contain any unpaired electron, but under certain conditions they have potential to enter free radical reactions. Hydrogen peroxide ($\text{H}_2\text{O}_2$), peroxy radical (HOO') etc are examples of reactive oxygen species.

Free radicals are constantly formed in lysosome, peroxysome, endoplasmic reticulum, plasma membrane and cytosol. One electron reduction of oxygen produces superoxide radical ($\text{O}_2^-$), which is comparatively inactive among free radicals [66].

Hydroxyl radical (OH) is produced when water is exposed to high ionizing radiation [67]. Most of the OH generated in vitro comes from the transition metal dependent breakdown of $\text{H}_2\text{O}_2$ and is often called Fenton reaction [66]. OH is highly active, so it reacts in vivo close to its site of formation. Thus, the type of damage would depend on its site of function e.g., production of OH close to DNA could lead to modification of purine or pyrimidine or to strand breakage [68]. Further reaction of OH with biomolecules produces other radicals usually of lower reactivity. Such less reactive radicals cause different side effects [69]. The best example of such secondary radical in vitro is peroxy radical, which initiates lipid peroxidation.
1.4.2. Antioxidant enzymes and antioxidants

There are natural antioxidant enzymes present in the biological systems that take care of free radical mediated damage. These are superoxide dismutase (SOD), catalase, glutathione reductase etc. Some of the body metabolites like uric acid [69], bilirubin [70], glutathione [71], estrogen [72] protect the organisms by their strong antioxidant properties. Some iron binding proteins namely, transferrin, lactoferrin and heme binding proteins haptoglobin or hemopexin and ceruloplasmin also protect the cells by removing reactive metals from the reaction site.

Fat soluble vitamins, like vitamin E or α-tocopherol [73] vitamin A or β-carotene [74] and water soluble vitamin C or ascorbic acid [75] are antioxidant vitamins and their presence strengthen the antioxidant defence mechanism within the living system.

Vitamin E, a fat-soluble component, protects the membranes, mainly by breaking of fatty acid radical chains [73]. As a chain breaking antioxidant in most biological membranes, it reacts primarily with peroxyl radicals, which are the principal chain carrying species in biological lipid peroxidation.

\[ \alpha-\text{TH} + \text{LOO}^\cdot \rightarrow \alpha-\text{T}^\cdot + \text{LOOH} \]  
\( \alpha-\text{TH} = \text{vitamin E}; \text{LOO}^\cdot = \text{Fatty acyl radical} \)

The reaction yields the tocopheroxyl radical, a resonance stabilized phenoxy radical that does not readily propagate radical chain reaction. The \( \alpha\text{T}^\cdot \) radical that does not complete redox cycle may react with a second phenyl radical to form other products. \( \alpha\text{T}^\cdot + \text{LOO}^\cdot \rightarrow \text{Product} \)
This reaction removes $\alpha T$ from the system, which leads to $\alpha$-tocopherol oxidative turnover and yields marker products for $\alpha$-tocopherol function in biological system.

Vitamin A, a fat-soluble vitamin also possesses peroxyl radical scavenging activity [74]. It also quenches the singlet oxygen and traps organic radicals [76]. Vitamin C, an aqua soluble vitamin; protects free radicals like superoxide ($O_2^-$) and singlet oxygen in cytosol and traps organic radicals [77]. The mechanism of their antioxidant activity is not yet clearly known. Cerebrospinal fluid (CSF) contains very little catalase or SOD. Thus, antioxidant vitamin C, present in such extracellular media, helps the antioxidant defence mechanism. Living species, at every moment of their survival, encounter free radical mediated insults and when these overwhelm body's natural defence, these are referred as oxidative stress conditions.

1.4.3. Oxidative stress in diabetes mellitus

Overload of reactive oxygen species that exceeds the capacity of normal antioxidant system, induces oxidative stress and it has been associated with a number of pathological conditions including inflammation, carcinogenesis, aging, atherosclerosis and reperfusion injury. Oxidative stress may play a significant role in diabetes mellitus, because prolonged exposure to hyperglycemia induces nonenzymic glycation of proteins through Maillard's reaction. The resulting products such as Schiff base and Amadori products can lead to the production of ROS [78]. The toxicities of many xenobiotics are also associated with formation of free radicals or ROS such as superoxide ($O_2^-$), hydroxyl radical ($\cdot OH$) or $H_2O_2$, ...
which may be responsible for tissue damaging effect as lipid peroxidation as well as DNA and protein degradation. Increasing evidence indicates that oxidative stress associated with production of ROS is involved with the pathophysiology of aging and various age related disorders including cataracts [36], retinopathy [17], nephropathy [18], neuropathy [19,20] and atherosclerosis [79], which are commonly associated with uncontrolled diabetes mellitus. The reasons for such pathological disorders are not yet known, but prevailing evidence suggests that the levels of antioxidants and antioxidant enzymes are reduced in diabetes mellitus.

Several studies have reported that the level of antioxidant vitamins like vitamin E, vitamin C [80-82] or antioxidant enzymes like glutathione reductase, superoxide dismutase and catalase reduce in diabetes mellitus [83,87]. These are the major scavenging systems to protect cellular constituents from free radicals. It has been reported that when lens homogenate is incubated in hyperglycemic condition (5.5 M glucose), increased ESR signals are observed indicating generation of free radicals by metal derived Fenton type of reaction [84]. In streptozotocin-induced diabetic neuropathy, involvement of oxygen free radical generation and its activity in the nerves are associated with the diabetic state [85].

Kashiwagi et al [86] have reported that when cultured human umbilical vein are exposed to high glucose condition, the activation of pentose phosphate pathway (PPP) is reduced by 50%, which is consistent with the decrease in NADPH content. Moreover, activation of polyol pathway was less marked in high glucose control cells than nonglucose control cells. Thus, polyol pathway is not
responsible for the decrease in intracellular NADPH content in hyperglycemic condition. Activation of the PPP and NADPH formation are impaired in high glucose cells exposed to \( \text{H}_2\text{O}_2 \), which may result in oxidative stress to endothelial cells. Blakynty et al [87] have claimed that in diabetes mellitus inactivation of glutathione synthesis and thiol transport increase the sensitivity of the cells to oxidative stresses and these effects may lead to the development of some complications in diabetes mellitus.
1.5. Heme Iron

1.5.1. Heme iron - an oxidant 'Trojan Horse' in biological systems

It has been observed that patients suffering from excessive vascular shearing forces show an increased mechanical erythrocyte damage near turbulent vascular locals for example, bifurcation and stenoses, which are very common sites of abnormal atheroma formation. Hemoglobin released from such fractured cells insinuates into juxtaposed endothelium, resulting intracellular accumulation of iron after heme degradation by heme oxygenase. Free intracellular heme induces production of heme oxygenase that cleaves the porphyrine ring forming bilirubin, carbon monoxide and free iron. The bilirubin has strong antioxidant properties and thus its formation protects the cell from free radical mediated insult. Relative insolubility of bilirubin assists in keeping local concentration higher where heme is being degraded [88].

Heme can be thought of a domesticated form of iron in which highly reactive and potentially hazardous metal atom is tamed within a planar cage formed by nitrogen atom of four pyrole groups of porphyrin molecule. Thus, when iron is released from porphyrin cage of heme after its degradation, accumulated iron might act as 'Trojan Horse' sensitizing the endothelium to oxidant damage. Further, this free iron, in turn, specifically and potentially stimulates ferritine formation at a very high level as a defence mechanism. This ferritine, a chelator of free iron is a frontier protectant that challenges metal mediated oxidant damage [89].
Abundant evidence show that this oxidant injury is markedly amplified in the presence of transition metal ions. The well known Haber-Weiss reaction generates potentially toxic hydroxyl radicals (\(\cdot\)OH) from less reactive substrates H\(_2\)O\(_2\) and O\(_2^−\) [90].

\[
\begin{align*}
O_2^− + Fe^{3+} & \rightleftharpoons O_2 + Fe^{2+} \\
2O_2^− + 2H^+ & \rightarrow O_2 + H_2O_2 \\
Fe^{2+} + H_2O_2 & \rightarrow \cdotOH + OH^- + Fe^{3+}
\end{align*}
\]

The possibility that iron-loading endothelium might make it vulnerable to inflammatory cell mediated oxidant injuries is supported by Varani et al [91]. They reported that endothelium cells with prolonged pretreatment with desferrioxamine, an iron chelator become highly resistant to activated polymorphonuclear leukocyte assault. The studies of Bala et al [89] indicate that exogenous heme can sensitize cells to oxidant damage. Recent findings of Mark Paller et al [92] demonstrate that breakdown of endogenous heme-protein, cytochrome P450 can also generate deleterious oxidant free iron. Cytochrome P450 is also found to be labile and under certain pathophysiological conditions it is degraded and releases iron. In cultured renal tubule cells, it is found that three distinct cytochrome P450 ligand could inhibit \(\cdot\)OH radical formation. In vitro studies confirmed the protective effect of cytochrome-P450 stabilization, that is glomerulus filtration is better preserved in rats subject to renal artery occlusion-reperfusion, if they are pretreated with cytochrome P450 ligand-cimetidine.
Paul Cutler [93] reported a considerable improvement of diabetic complications, when desferrioxamine is injected (10 mg/Kg i.v.) to high ferritine rich non-insulin dependent diabetic patients. Blood glucose, triglyceride and HbA1c levels are improved. These studies demonstrate that iron metabolism has some correlation with diabetic complications.

1.5.2. Hemoglobin - a large reservoir of iron

In normal physiological state, iron is found almost exclusively bound to protein molecule in specific subcellular compartments and concentration of free or labile iron is very tightly controlled. The iron within the protoporphyrin ring becomes domesticated and tamed to form heme moiety. However, when it is decompartmentalised and liberated out of the heme cage, it can contribute to development of pathophysiological states through various mechanisms [94].

Ferrous iron with six co-ordination states is bound in heme pocket of hemoglobin by four pyrole nitrogen of protoporphyrin moieties, forming a tetradentate chelate with iron. The imidazole of the proximal histidine (F8) of heme pocket occupies the fifth coordination position of iron and sixth co-ordination position is free to bind ligand distal to heme moiety (Fig.-1). To function as redox couple, iron must have at least one free co-ordination position, which is the case of hemoglobin bound iron. Consequently, it was hypothesized that hemoglobin bound iron can catalyze the Haber-Weiss reaction. However, other studies have suggested that redox-reactive species is either free iron or heme outside hemoglobin [95]. But despite the fact that iron is bound tightly to hemoglobin, it can be liberated under specific circumstances yielding a source of
'free reactive iron', which is ligated to other part of the hemoglobin moiety, perhaps distal histidine in the heme pocket. Once outside heme pocket, iron can be translocated to other binding sites, i.e. inorganic chelators, lipids, other proteins or DNA, where it can catalyse Haber-Weiss reaction [94,95].
1.6. Trifluoperazine - a brief review

Phenothiazine compounds have antihistaminic and sedative effects. The central action becomes known as ataractic or neuroleptic effects. According to Delay and Deniker [96], these drugs achieve symptomatic relief of agitation and anxiety, and that it has an ameliorative effect among psychic processes with diverse symptomatology.

The basic background structure of all phenothiazine drugs is more or less same. It has three ring structures in which sulphur and nitrogen atoms link two benzene rings, i.e., all phenothiazines consist of tricyclic rings which differ only in their side chains (Fig.-1.12). Fig.-1.13 shows structures of two potent phenothiazine drugs chlorpromazine (CPZ) and trifluoperazine (TFZ). Trifluoperazine is 2-trifluoro methyl 10-[3′-(1-methyl-4 piperazinyl) propyl] phenothiazine, which is most potent and well-known psychoactive drug [97].

The biochemical effects of TFZ with respect to different biomolecules have been studied. It inhibits DNA synthesis by blocking one or more events involved in triggering DNA replication [98]. TFZ has been found to alter conformation of calmodulin and thereby inactivating its biological activity [99].

In pancreatic β-cells of Langerhans, calcium dependent calmodulin regulates glucose-induced insulin release. TFZ antagonises the activity of calmodulin in this respect and inhibits insulin secretion [100]. CPZ and TFZ are known to induce hyperglycemia and can inhibit insulin secretion both in normal and in patients with latent diabetes mellitus who are administered with high dose of the drugs [100]. Bhattacharyya and Das have recently reported that the drug causes insulin aggregation possibly by reduction of disulfide bonds and thereby inhibiting insulin function and causing hyperglycemic condition [101].

Hemoglobin is a major component of red blood cells. As an allosteric protein, it has an important role in carrying oxygen from lungs to different tissues. Both CPZ and TFZ can penetrate through cell membranes of erythrocytes and
Fig.-1.12.: General structure of phenothiazine drugs.
Fig. 1.13.: Structure of CPZ and TFZ.
The interaction of hemoglobin with CPZ and TFZ has been thoroughly studied in our laboratory by Bhattacharyya et al [103-105]. They have observed that binding of CPZ or TFZ induces oxygen release from oxygenated hemoglobin. The percentage of oxygen release due to drug binding is cooperative in nature. Moreover, a change in conformation of hemoglobin occurs due to drug interaction in such a way that tryptophan moieties of the protein molecule are more exposed to the polar region. They have also reported that tryptophan residues are in or near the possible binding site for hemoglobin and myoglobin interacting with CPZ and TFZ [103].