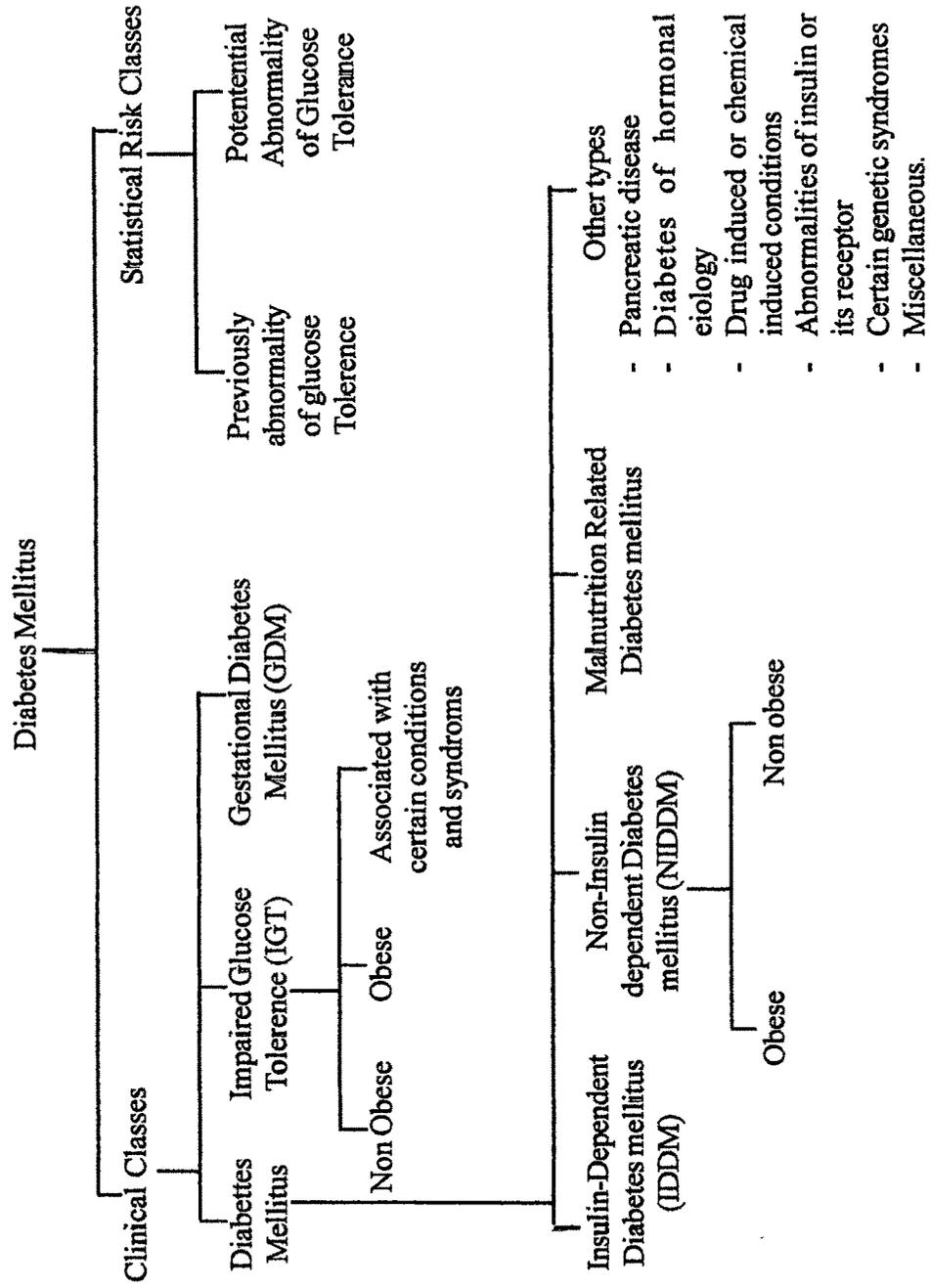# CHAPTER : 1
Introduction

Diabetes mellitus is prevalent in all populations throughout the world. Diabetes mellitus is characterized by hyperglycemia and disturbances of carbohydrate, fat and protein metabolism, that are associated with absolute or relative deficiencies in insulin action and / or in insulin secretion (1). Therefore although diabetes is an endocrine disease in origin its major manifestations are those of a metabolic disease (2).

Diabetes mellitus is broadly classified into two major groups (Figure 1).

1) Clinical classes : people of clinical classes show abnormality of glucose tolerance.

2) Statistical risk classes : These are further sub-classified into two a) Previous abnormality of glucose tolerance - people who had abnormality of glucose tolerance in the past b) potential abnormality of glucose tolerance.

Most of the patients lie in the insulin-dependent diabetes mellitus (IDDM) and non-insulin dependent diabetes mellitus (NIDDM) sub-groups. Insulin dependent diabetes mellitus is characterized by absolute insulin deficiency. These individuals require exogenous insulin to sustain life. Generally this condition is found in children and young adults and so it is also called juvenile onset or type I diabetes mellitus. The non-insulin-dependent-diabetes mellitus class includes individuals who are resistant to insulin or their beta cell wall is incompletely destroyed. It

Figure 1 Classification of Diabetes Mellitus.

Diabetes Mellitus

Clinical Classes

Statistical Risk Classes

Diabettes Mellitus

Impaired Glucose Tolerence (IGT)

Gestational Diabetes Mellitus (GDM)

Previously abnormality of glucose Tolerence

Potentential Abnormality of Glucose Tolerence

Non Obese

Obese

Associated with certain conditions and syndroms

Insulin-Dependent Diabetes mellitus (IDDM)

Non-Insulin dependent Diabetes mellitus (NIDDM)

Malnutrition Related Diabetes mellitus

Obese

Non obese

Other types
- Pancreatic disease
- Diabetes of hormonal eiology
- Drug induced or chemical induced conditions
- Abnormalities of insulin or its receptor
- Certain genetic syndromes
- Miscellaneous.

is commonly found in mature individuals so it is also called as maturity onset diabetes or type II diabetes (3).

Insulin

By observing all different types of diabetes we are able to conclude that insulin plays direct or indirect role in diabetes. The complex inter linking of different facets of metabolism requiring rapid adaptation changes in environment, particularly the changes from the fasted to the fed state, necessitates a prompt and clear signal. This signal is provided by insulin (2).

Insulin is released from the B cells of islets of Lengarhanse present in the pancreas. It originates as pre-pro-insulin. In endoplasmic reticulam 23 amino acids get separated and form pro-insulin. Pro-insulin goes to Golgi where c-peptide gets separated from A and B chains. Mature insulin is a polypeptide of 39 amino acids arranged as an A and B chain joined by one intra- and two inter-peptide disulfide bridges. The double-chain structure results from its origin as pro-insulin, when a peptide length joins the end of the A chain to the B chain. The connecting peptide chain, c peptide secreted in equimolar amounts with insulin, appears to be inert and in contrast to insulin is excreted virtually unchanged in urine (2).

During maturation of insulin C-peptide is cleaved from the protein, but has been thought to be biologically inert. Recently the beneficial effects have been demonstrated after prolonged treatment of diabetic rats with pharmacological doses of C-peptide. These effects include a restoration toward normal of the diabetes-induced decrease in cellular sodium-potassium adenosine triphosphatase (ATPase) activity and impaired nerve conduction, and reductions in the diabetes-induced increase in vascular permeability and blood flow, changes that are concomitants of the hyperglycemia associated with diabetes. It is suggested that C-peptide can prevent or slow the progression of some of the complications of this chronic often debilitating disorder (4).

The physiologic effects of insulin in mammalian system include stimulation of hexose, ion and amino acid uptake (5), modification of the activities of rate-limiting enzymes such as glycogen synthase, hormone sensitive lipase and pyruvate dehydrogenase by net dephosphorylation (6), phosphorylation of seryl residues in proteins such as ribosomal S 6, acetylcoenzyme A carboxylase and adenosine triphosphate (ATP) citrate lyase (7), regulation of gene expression for a small number of regulatory enzymes (7), redistribution of membrane proteins such as the glucose transporters and the insulin-like growth factor II (IGF-II) and transferrin receptors (8) and promotion of cell growth (9). Many of these effects are tissue or cell-specific and involve only a discrete subset of proteins. The chronology varies. Transcription of the gene

encoding phosphoenol pyruvate carboxikinase is inhibited within seconds of addition of insulin, whereas growth promotion requires hours of exposure (Table 1). Many of the rapid action of insulin, such as stimulation of hexose transport alteration and alteration of enzyme activities, do not depend on synthesis of new proteins or nucleic acids. Even this incomplete summary of the actions of insulin, however, invokes seryl and threonyl phosphorylation and dephosphorylation of cytosolic and mitochondrial proteins, membrane translocations with the likelihood of cytoskeletal protein involvement, and nuclear action. The first essential and common step in insulin action is interaction with the insulin receptor (10).

Insulin receptor has two alpha and two beta subunits. All subunits link with each other by inter- and intra-peptide disulfide bridges. Alpha subunit is present on the outer surface of the cell, while the beta subunit is a trans-membrane subunit. Alpha and beta subunits have molecular weight of 130 kd and 95 kd respectively. Binding of insulin to its receptor is followed by internalization of the hormone receptor complex. Dissociation leads to a return of receptor to the cell membrane and probable degradation of insulin (10).

Characteristic Symptoms

Table 1 Chronology of insulin action

Seconds

Binding ot receptor

Activation of receptor protein tyrosine kinase

Receptor autophosphorylation

Seconds to minutes

Changes in gene transcription

Stimulation of hexose and ion transport

Ligand-mediated receptor internalization

Alterations in intracellular enzyme activities

Seryl and threonyl phosphorylation of the receptor

Hours

Synthesis of protein, lipid and nucleic acids

Maximal down regulation of the receptor

Cell growth

Characteristic symptoms in diabetes are hyperglycemia, glycosurea, polyphagia, polydypsia and polyurea. Diabetics also have high risk of developing long-term diabetes complications, including microvascular (i.e. nephropathy, retinopathy and neuropathy) and macrovascular (i.e. cardiovascular, cerebrovascular and peripheral vascular disease) (11). The development of diabetic complications does not depend entirely on diabetes duration and control. Predisposing and aggravating factors, either constitutional or environmental, seem to play a role (12).
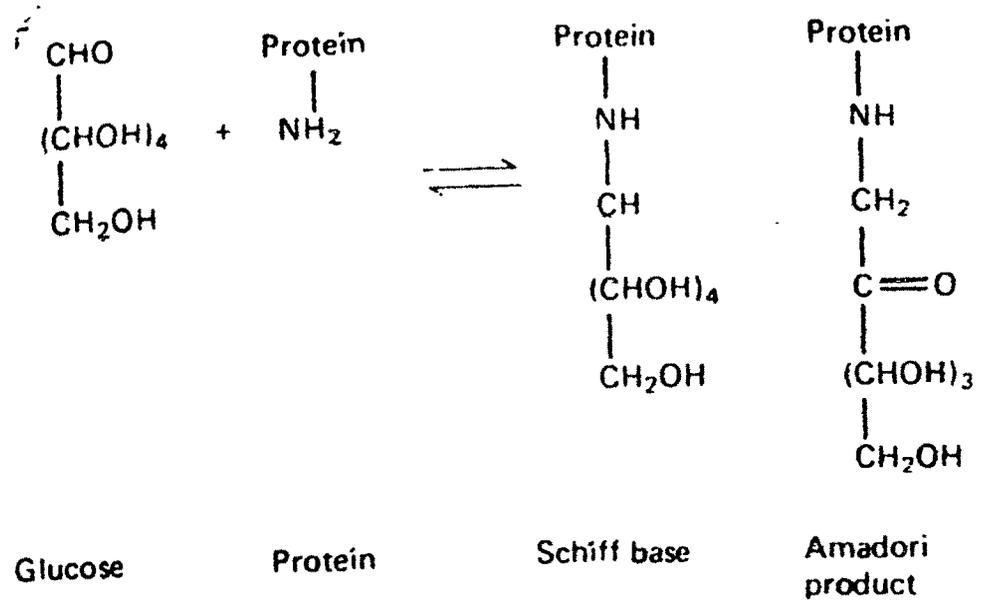
Nonenzymatic glycosylation

It is believed that protein glycosylation is a factor in the development of diabetic complications.
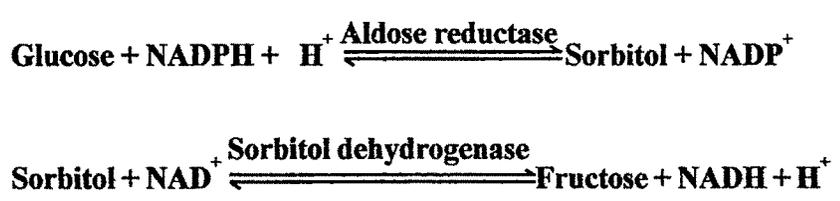
General considerations

Nonenzymatic glycation refers to the condensation reaction between glucose and free amino groups at the amino-terminus or E-amino group of lysine residues of proteins. The reaction proceeds through formation of a Schiff base between the carbonyl group of glucose and the free amino groups. The resulting aldimine linkage is stabilized by undergoing an Amadori rearrangement to form a ketoamine (13) (Figure 2).

Glycosylation of hemoglobin has been studied extensively and has entered laboratory use as a long-term marker of diabetic

Figure 2 Glycosylation of Protins

CHO   Protein   Protein   Protein

$(CHOH)_4$ + NH_2 ⇌ NH   NH

CH_2OH CH CH_2

$(CHOH)_4$ C═O

CH_2OH $(CHOH)_3$

CH_2OH

Glucose   Protein   Schiff base   Amadori product

Figure 3 Polyol pathway

$$\text{Glucose} + \text{NADPH} + H^+ \underset{}{\overset{\text{Aldose reductase}}{\rightleftharpoons}} \text{Sorbitol} + \text{NADP}^+$$

$$\text{Sorbitol} + \text{NAD}^+ \underset{}{\overset{\text{Sorbitol dehydrogenase}}{\rightleftharpoons}} \text{Fructose} + \text{NADH} + H^+$$

control.(2) It has been appreciated for some time that normal human RBC hemolysates contain several minor hemoglobin components in addition to the single major hemoglobin, Hb_{AO}. Together, these minor hemoglobin species account for five to ten percent of the total found in normal adult erythrocytes. Two to three fold elevations of three of these components Hb_{A1a}, Hb_{A1b} and Hb_{A1c} are consistently found in diabetic patients (14). Recently, Hb_{A1a} has been resolved into two separate components, Hb_{A1a1} and Hb_{A1a2} (15). All four of these negatively charged minor hemoglobins have been proposed to be glycosylated, with carbohydrate present on the beta-chain by means of a Schiff base and an Amadori rearrangement to form a 1-amino-1-deoxy fructose derivative (16). The carbohydrate moieties of the other glycohemoglobins are presumed to be attached to the protein through analogous ketoamine linkages. Recently a significant portion of Hb_{AO} has also been shown to be glycosylated (17). Glucose ketoamine linkages have been identified on the N-terminus of the alpha-chain, and on several lysine residues on both the alpha and beta chain. Results from diabetic subjects show increase comparable to the increases of Hb_{A1c} (17-19).

The amount of glycosylated protein formed reflects the prevailing degree of glycemia with length of exposure. In turn, length of exposure is a function of protein turnover rates. Proteins that may be glycosylated include lens proteins, albumin and collagen. Collagen and advanced

glycosylation end products form cross-links that disturb the function of the protein for example glycosylated basement membrane is more resistant to enzymatic digestion than when not glycosylated (2). Increased nonenzymatic glycosylation has also been reported to occur in renal glomerular proteins isolated from diabetic animals (20). Thus there is also a possible involvement of this mechanism in the development of diabetic nephropathy. Nonenzymatic glycosylation of erythrocyte membrane proteins occurs to a greater extent in diabetics (21). The nonenzymatic glycosylation of human albumin both in vitro and in vivo has been described (21), with the level in diabetic patients averaging over twice that of normal controls (22-24). Similarly, other classes of serum proteins undergo nonenzymatic glycosylation in vitro and in vivo, both in animals and in man (25, 26). The level of these adducts is increased in diabetics, and is proportional to the degree of hyperglycemia. Several other tissues in which diabetic complications occur are currently being evaluated for the presence and significance of similar glycosylated derivatives (19).

Polyol pathway

Certain mammalian tissues have the unique ability to synthesize free (non-phosphorylated) fructose for various metabolic purposes (27). This biosynthetic pathway or polyol pathway (Figure 3), termed the sorbitol pathway, consists of two reactions. Glucose is first reduced to its corresponding

sugar alcohol, sorbitol by the enzyme aldose reductase, with NADPH as the electron-donating coenzyme. The sorbitol molecule is then oxidized to fructose by the enzyme sorbitol dehydrogenase and NAD^+ (19). This latter enzyme is rate-limiting in the pathway and consequently there is an accumulation of sorbitol. Sorbitol does not diffuse readily across cell membranes and intracellular accumulation leads to osmotic effect with subsequent cell damage (2). Significantly elevated concentrations of both sorbitol and fructose have been reported in several tissues of diabetic animals and man, including lens, retina, arterial wall, erythrocytes and Schwann cell sheath (19, 27-32).

For many years after the identification of the polyol pathway and the implication that it participated in the pathogenesis of certain complications of diabetes, evidence that it might play a role in the development of diabetic nephropathy was largely inferential. With the demonstration that glomeruli contain aldose reductase activity, and that the glomerular sorbitol content is elevated in experimental diabetes (33-35), this hypothesis assumed more credence. This increase was prevented by the administration of the aldose reductase inhibitor sorbinil, indicating that the drug enters renal glomerular cells and effectively inhibits aldose reductase activity, which was presumed to reside in podocytes (13).

The "polyol pathway" hypothesis has led to clinical trials

with aldose reductase inhibitors in an attempt to reverse diabetic neuropathy (2). Results have been disappointing, perhaps because clinical diabetic neuropathy is too late stage for reversibility to be found. Alternatively, perhaps sorbitol is of no causal import or inhibition of aldose reductase is not achieved (2). The development of diabetic neuropathy follows a typical route. A patient will develop initially slight and intermittent proteinuria . After a variable length of time this proteinuria becomes both heavy and constant, and some time later the signs of declining renal function will become evident in the blood. Once creatinine concentrations begin to rise they do so inexorably. Indeed, once this stage has been reached a plot of time vs the reciprocal of the creatinine concentration can be extrapolated to date the occurrence of creatinine concentration at which death will results (2).

Diabetic Nephropathy

At least four general theories or processes have been advanced in recent years to explain the pathophysiology of diabetic nephropathy. These relates to

a) hemodynamic changes,

b) structure-function alterations resulting from nonenzymatic glycation of basement membrane macromolecules and other proteins,

c) increased flux through insulin-independent pathways for glucose utilization, particularly the polyol pathway, and

d) disturbed regulation of the synthesis or metabolism of specific basement membrane components, in particular collagen and proteoglycans (13).

Excess nonenzymatic glycation and activation of the polyol pathway, and their consequences as mechanisms are two major pathways contributing to the development of diabetic neuropathy.

Diabeic retinopathy

The pathogenesis of diabetic retinopathy is full of uncertainty. It is clear that the diabetic retina shows abnormalities of blood flow, although the precise role of these abnormalities remains in doubt. Similarly a variety of abnormalities in rheology and coagulation can be demonstrated in patients with diabetic retinopathy. Viscosity is increased, platelet sensitivity is increased, and the levels of B thromboglobulin and factor VIII are raised. But changes in blood rheology and coagulation are common in diabetics both with and without retinopathy. (2).

Diabetic cataract

Because of their avascular nature and extremely low regenerative potential, mammalian lenses are very susceptible to damage associated with metabolic disorder such as

diabetes. Increases in the concentration of polyols (36-38), lens fiber swelling, loss of intracellular metabolites (39-44), decrease in glutathione concentration (42-44), and increase in protein glycation (45-47) are the general features of the lenses in diabetes. A frequent pathological endpoint of this multifactorial degenerative process is the production of cataracts (48).

Diabetic Neuropathy

The most frequent symptoms of diabetic peripheral neuropathy are numbness and paraesthesias (19). While the pathogenesis of diabetic neuropathy remains obscure (49), certain clues have been provided by some recent studies. These include reduced energy utilization (50), increased sorbitol and decreased nerve free myo-inositol concentrations (50-51), increased intra-axonal sodium levels (52), and a reduced rate of incorporation of lipid and amino acids in myelin (53). Axonal transport rates are also reduced in experimental diabetic neuropathy (54, 55). While the link between these abnormalities and development of neuropathy is still not clear, all are energy-dependent processes that might be impaired if the nerve microenvironment were hypoxic (56). In diabetic animals, hyperglycemia and the resultant increased sorbitol pathway activity in peripheral nerve is associated with decreased oxygen uptake in this tissue (57, 58). Such impairment of tissue oxygen delivery could conceivably contribute to the development of neurological dysfunction

(19).

Macroangiopathy

Accelerated large vessel decrease in diabetes may be due in part to abnormalities in plasma lipids, and perhaps also to changes in the composition and metabolism of the arterial wall. Epidemiological data appear to show no excess risk from diabetes independent of hypertension, cigarette smoking and hyperlipidemia for a large proportion of the specific age-sex groups (59). However, relatively inaccurate and insensitive correlates of the integrated concentration of blood glucose were used to assess severity of diabetes. Insulin deficiency may well influence the progression of atherosclerosis through synergistic pathological mechanisms involving hyperlipidemia, altered platelet behavior and abnormalities in arterial function (19).

Macroangiopathy, or large vessel disease is known to occur in diabetics. Early histologic studies on coronary arteries revealed a thickening of the intima in diabetics (60) which was confirmed in a later study (61). More recently, a reduction of the lumen of extramural coronary arteries of young juvenile diabetics was shown (62). Besides these changes, histochemical studies have revealed an accumulation of hyaline substances along with deposition of cholesterol crystals, calcium and increased amounts of glycoproteins

(63). Such a deposition, which may result in hardened arteries and a narrowed arterial lumen, superimposed with atherosclerosis could easily account for the angina and myocardial infarction associated with diabetes (64).

Heart Abnormalities

Although other studies have shown that the incidences of causative factors such as large blood vessels disease (macroangiopathy) and small blood vessel disease (microangiopathy) are increased these factors can not always be definitely implicated due to their absence in a significant number of diabetic patients. In addition, the finding that heart disease can occur even in the presence of normal coronary arteries suggested that factors besides abnormal blood vessels are involved in the pathogenesis of diabetes-induced heart disease (65). Other factors which may contribute to myocardial problems in diabetes include the accumulation of glycoprotein and collagen in the myocardium. This may lead to increased myocardial stiffness, reduced compliance and increased end diastolic pressure; this could result in heart failure. Autonomic neuropathy of the cardiovascular system is another manifestation of diabetes. It is conceivable that an altered parasympathetic system (which is known to be affected before the sympathetic system in diabetes) would increase the heart rate and subsequently lead to impaired ventricular filling. The net result of all the above mentioned factors acting together, along with the

other alterations of the myocardium as described later could well account for the other alterations of the myocardial performance (66).

Cardiac muscle cells in diabetic heart showed condensation of nuclear chromatin and folding of nuclear membranes, swelling of mitochondria and clearing of mitochondrial matrix and incorporation of lysosomal membrane, to mitochondrial matrix (67). Lysosomal and lipid droplets increased. Focal areas in diabetic hearts showed contracted sarcomeres, myofibrillar degeneration and separation of the intercalated discs. Atherosclerotic plaque formation as well as structural changes in the smooth muscle or endothelial cells in the small arteries, arterioles or capillaries were not seen to accompany the structural changes in the cardiac muscle cell of the diabetic hearts (67). Chronic streptozotocin-induced diabetes in rats is associated with a significant depression of cardiovascular sodium pump activity as well as myocardial force of contraction (68). After 10 weeks of alloxan-induced diabetes heart and left ventricular weight decreases, serum and heart triglycerides and cholesterol increases, while magnesium level decreases (69).

Diabetes induced abnormalities in the myocardium

One of the leading causes of mortality in diabetics is myocardial disease. Though originally thought to occur as a

result of atherosclerosis, various studies have shown that heart disease can occur in the absence of atherosclerosis, suggesting a diabetic cardiomyopathy (64). Using diabetic animals, it has been possible to characterize diabetes-induced myocardial abnormalities. Diabetic rat hearts do not respond to conditions of high stress as well as controls (64). The functional depression is accompanied by altered cardiac enzyme systems. A decrease in myosin ATPase activity which appears to be a result of diabetes-induced hypothyroidism is seen. Also, a depression of sarcoplasmic reticular calcium ATPase, along with a depression of calcium uptake by the sarcoplasmic reticulam, is seen in diabetic rat hearts (64). Na^+,K^+ ATPase activity has also been shown to be depressed and the depression appears to correlate with depressed arterial contractility. High levels of circulating fats in diabetics may alter the integrity of membranes leading to altered enzyme activities. Insulin treatment has been relatively successful at reversing or preventing myocardial changes in the diabetic rat (64).

An acute myocardial infarction in diabetics is associated with some special features :

1) The case fatality of acute myocardial infarction is higher in diabetics than in non-diabetics (70)

2) Diabetics develops infarction with acute myocardial infarction more often intraventricular or atrioventricular condition disorder than non-diabetics (70)

3) The long-term prognosis of survivors of a myocardial infarction is less favorable in diabetics than in non-diabetics (71)

4) Diabetics have more often painless or unrecognized myocardial infarction than non-diabetics (72, 73). This phenomenon may be ascribed to subclinical autonomic neuropathy (74, 75).

Coronary heart disease (CHD) and congestive heart failure (CHF)

A number of clinical studies have shown that the incidence of coronary and congestive heart disease is much greater among diabetics. Amongst other studies, the Framingham study showed that the morbidity and mortality in diabetics was almost three times greater than in the general population. Mortality due to cardiovascular causes in diabetic women was five times greater and in diabetic men two times greater than in nondiabetics of the corresponding sex (64, 76).

Several reports are available on the incidences of CHD events among diabetics belonging to the Framingham study population (77-79). They have shown that the incidence of all types of CHD events, myocardial infarction, angina pectoris and sudden death, was increased in diabetics as compared to non-diabetics, the excess being more marked in females than in males. About 40 percent of the Framingham diabetics were

insulin-treated, most of them being diagnosed before entry. About 40 percent were treated by oral hypoglycemic agents and about 20 percent by diet only. Cardiovascular mortality risk, and this was mainly CHD mortality risk, was increased in diabetics receiving all forms of treatment, but in female diabetics treated with insulin the excess cardiovascular mortality was particularly striking (75). In the Whitehall study the most striking finding was the huge excess of CHD mortality among female diabetics treated with insulin - a finding in accordance with the Framingham study results (75).

The classic cardiovascular risk factors of high blood cholesterol, hypertension and smoking are predictors of cardiovascular mortality, not only in nondiabetic subjects but also in diabetic subjects (80). However, epidemiological data suggest that these classic cardiovascular risk factors do not account for all excess risk of CHD mortality and morbidity associated with diabetes (81, 82). High homocysteine may be a stronger risk factor for cardiovascular disease with non-insulin dependent diabetes mellitus than in nondiabetic subjects (83). In the families of IDDM patients with elevated albumin excretion rate (AER) there was a higher frequency of risk for cardiovascular disease as well as a predisposition to cardiovascular disease event (84).

Overall, 66 % of all morbidity and mortality in diabetes is due to cardiovascular disease (85).

Diabetes and membrane structure function alterations

Interest in hormone interactions with cell membranes has increased considerably during recent years. One hypothesis postulates that changes of membrane lipid structure and microviscosity may play the role of a hormonal information transducer (86, 87). Several major functions of plasma membranes, such as enzyme activities and ligand - receptor interaction depend on membrane fluidity, a concept related to movement of lipids and proteins in the plane of the membrane (88, 89).

A variety of polypeptide hormone can provoke rapid changes in phospholipid metabolism in their target tissues (90-96). Insulin acutely increases phospholipids in the phosphatidate-inositide cycle in rat adipose tissue both _in vivo_ and _in vitro_ (91, 97). Shifts in membrane phospholipid content may be important in regulating the activity of a variety of cellular enzymes (98, 99). In diabetic condition different membrane system are affected differently and severity depends on duration and control of diabetes. Myelin membrane is complex lipid containing triglyceride, sphingomyelin, cholesterol, cerebrosides and proteins. Abnormalities in nearly all of these constituents have been reported in diabetes, in association with decreased nerve conduction velocity (99). Effect of diabetes on liver and kidney membrane phospholipids and phospholipid classes are different

(99). In these studies it was shown that total phospholipid content was similar in liver and kidney plasma membranes of diabetic and control rats, while phosphatidylethenolamine content in kidney and liver plasma membranes of diabetic rats was, lower than in the control rats. Phosphatidylinositol content was higher in kidney but not in liver membranes from diabetic rats (99). Neither the molar ratios of cholesterol to phospholipids in the synaptosomal membrane nor the fatty acid composition of the synaptosomal membrane alters significantly in streptozotocin-diabetic rats (100). In other study it was shown that low brain weight is accompanied by a loss of major fatty acid components lost within the whole brain, nerve endings and mitochondrial membranes. Cholesterol levels are low in whole brain but are not significantly different from normal in the synaptosomal membrane (101). The phospholipid concentration is significantly decreased in whole brain homogenates, crude synaptosomal membranes and crude mitochondrial membranes of the (db/db) diabetic mice (101). In IDDM human patients erythrocyte membrane fluidity decreases (86). Erythrocyte, platelet and polymorphonuclear leukocyte membrane phospholipid content and composition gets affected differently in type I and type II diabetes (102). In diabetes the relative abundance of phosphatidylethenolamine increased in erythrocyte and polymorphonuclear leukocyte membrane, whereas those of sphingomyelin and phosphatidylcholine were decreased in platelets and polymorphonuclear leukocyte membrane (102). The percentage of phosphatidylserine was

reduced in erythrocytes but increased in platelet (102). The level of sphingomyelin in polymorphonuclear leukocyte membrane was significantly lower in type I than in type II diabetics (102). Moreover, the longer the duration of diabetes and poores the metabolic control, the greater the decrease in sphingomyelin (102). Membrane fluidity of platelets and polymorphonuclear leukocytes alters in both type I and type II diabetes (103). It was shown that the platelet membrane fluidity instead does not discriminate normals from diabetics, but differentiates diabetics of type I and II, while polymorphonuclear leukocyte membrane fluidity does not discriminate normals from diabetics (103). Phospholipid content and composition of heart microsomal membrane alters in diabetic rats (104). Phospholipid content of sarcoplasmic reticulam membrane increased significantly 8 weeks after treatment with streptozotocin owing to increase in phosphatidylcholine and phosphatidylethenolamine, a decrease in arachidonic acid, and an increase in docosahexaenoic acid in the early stage of diabetes (104).

From foregoing survey of literature it is clear that the diabetic condition severely affect membrane structure-function; and also affects the functions of the cardiovascular system maximally (86). Incidences of CHD and CHF are high in diabetics as compared to non diabetics (75). Alterations in membrane structure function of blood cells are also noted (102). AChE is present on erythrocyte membrane

(140), while Na^+,K^+ ATPase is integral membrane protein in erythrocyte membrane (107). In the present study affects were made to determine the kinetic properties of erythrocyte membrane Na^+,K^+ ATPase and AChE from human volunteers. To examine the effects of diabetic state on cardiac function, alloxan-diabetic rats (both males and female) were used as a model. The cardiac function was monitored using BChE as a marker. A brief review of literature on Na^+,K^+ ATPase and cholinesterases is given below.

Na^+,K^+ ATPase

Na^+,K^+ ATPase in animal cells, is a membrane bound enzyme which couples free energy contained within the ATP molecule, to the translocation of Na^+ and K^+ across the plasma membrane so that a higher concentration of Na^+ outside and of K^+ inside the cell is maintained (105). The purpose of creating such ionic distribution is for generating an ionic gradient across the membrane. Depending on the subunit composition (105) of this enzyme and also on its distribution and arrangement (106), Na^+,K^+ ATPase can use such gradient as free energy sources for a number of other processes like :

1) Formation of membrane potential.
2) Depolarization and repolarization of membrane.
3) Cell volume regulation.
4) Transport of glucose and amino-acids into cells against concentration gradient.

5) Co- and counter-transport of ions across the cell
membrane for homeostasis, and

6) Transepithelial transport in intestine, kidney and
secretory glands (107).

Properties

Na^+,K^+ ATPase is a complex of two polypeptides (107) - and a
number of lipid molecules incorporated into the lipid bilayer
of the plasma membrane. The molecular weight of alpha
subunit is 106,400 d and it has 1012 amino acids (107) while
that of beta subunit is 36,600 d with 300 amino acids (107).

Alpha is the catalytic subunit (As shown in figure 4). It has
8 membrane spanning segments and 70 % of the subunit
protrudes into the aqueous cytoplasm. It has binding sites
for Na^+ - near the amino terminus, K^+ and ouabain on the
ectoplasmic loop, the phosphorylation site which is an
aspartic acid residue and ATP binding site on large central
cytoplasmic loop. Model for path of alpha subunit in the
membrane bilayer has been shown in the Figure 4 (106).

Three isoforms of alpha subunit are known viz. alpha 1, alpha
2 and alpha 3. The three isoforms are encoded by separate
genes which exhibit tissue specific and developmental pattern
of expression. Alpha 1 is ubiquitously present, alpha 2 is
mainly present in skeletal muscle and brain and alpha 3 is
mainly found in fetal brain (108). The three isoforms have
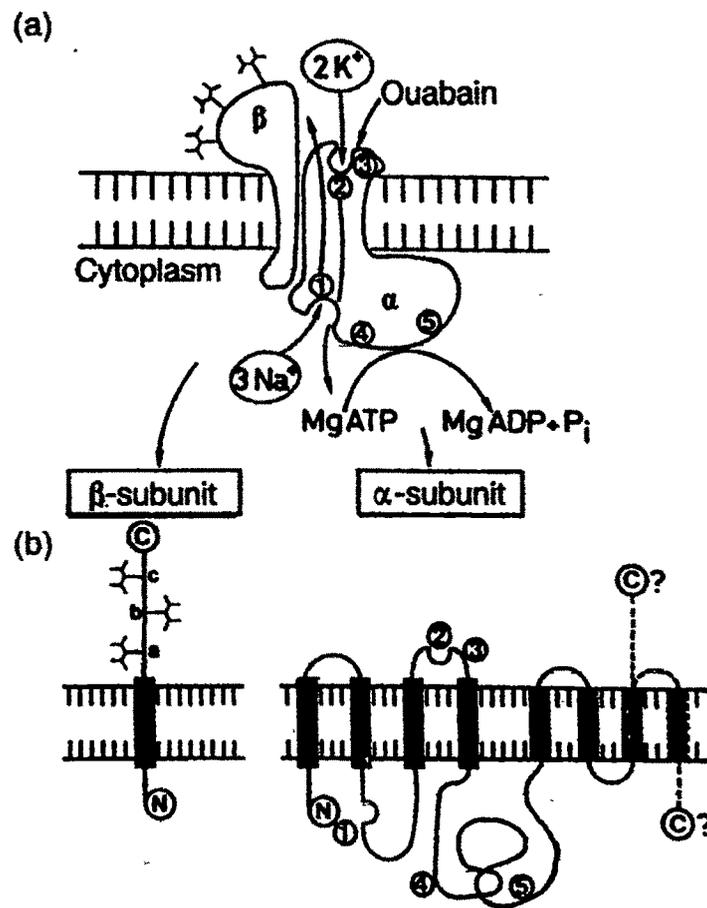
Figure 4 (A) General model for Na⁺,K⁺ ATPase : an alpha and beta heterodimer is schematically represented with five binding sites : the Na⁺ binding site (1), the K⁺ binding site (2), the oubain binding site (3), the phosphorylation site (4) and the ATP binding site (5). (B) Model for the membrane insertion of the beta subunit (left) and the alpha subunit (right).

same molecular weight but there are differences in their amino acid composition and sequence (108). The sequence identity is about 82 % with major differences in the N - terminal part.

Beta is a small molecular weight glycoprotein which exposes most of its mass to the exterior. There is one membrane spanning segment near the N terminal part of the protein, and the C-terminus is located on the extracellular side of the membrane. It plays an important role in anchoring the alpha subunit (109). For the first time, it was shown by Shyjan et al. (105) that it is due to differences in subunit compositions that Na^+,K^+ ATPase shows variable functions in different tissues.

Model

The alpha and beta subunits of this enzyme are present in 1 : 1 mole basis. The complex can function independently catalytically but coupling between ATP hydrolysis and Na^+,K^+ transport requires the formation of the oligomeric complexes. Two models have been proposed (Figure 5) (110, 111) :
1) Protomer model in which, transport of ions is seen through alpha subunit.
2) An oligomer model in which the channel for transport of ions is from between the two alpha subunits.
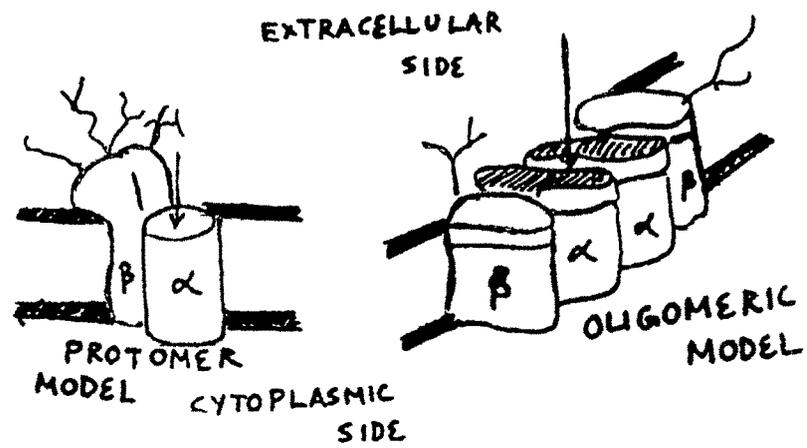
Figure 5 Models of the structure of (Na⁺,K⁺ ATPase. Left : independent function of alpha, beta unit with pathway for cations passing through the structure of the alpha-subunit. Right : redrawn from Kyte (111) to illusterate an oligomeric (alpha, beta)$_2$ structure with the channel for cations in the space between the two alpha subunit.

Hormonal regulation of Na$^+$,K$^+$ ATPase

Hormones or factors regulate the activity of the Na$^+$ pump by two distinct mechanisms (112) :

i) induction of Na$^+$,K$^+$ ATPase gene expression (113) or

ii) post-translational modifications of enzymes from a pre-existing pool.

The hormones whose action is mediated by a DNA-binding receptor including steroids (aldosterone (113, 114-119)), corticosterone, cortisol (113, 114, 119-121) and thyroid hormones (114, 122-124), mainly control the transcriptional activity of the Na$^+$,K$^+$ ATPase gene, and to a lesser extent the processing and stability of the corresponding mRNAs, and the translation, membrane assembly and cell surface expression of newly synthesized enzyme molecules. Chronic perturbation of intracellular Na$^+$ concentration which follows either ouabain treatment at low doses or partial depletion of extracellular K$^+$, triggers the up regulation of the Na$^+$ pump. Whether the effect is at the transcriptional level is not known, but protein synthesis is required (125). Regulation of such processes is slow and extends over hours or days. It represents the prototype of slow adaptation to environmental changes.

Hormones including polypeptides (insulin (126-129), glucagon (129, 130), EGF (127, 131), vasopressin (132, 133)), catecholamines (128, 134) and possibly progesterone (135)

(Table 2), act on pre-existing pumps by modulating their activity. The action of these hormones and growth factors is mediated by plasma membrane receptors and second messengers. It has been proposed that protein-kinase dependent phosphorylation of the alpha subunit modulates the activity of the Na^+ pump in transformed cells (136). Few covalent modifications induced by hormones have been identified so far. This group of hormones may also act by regulating the number of pumps expressed at the cell surface by insertion (exocytosis) of Na^+,K^+ ATPase (135, 137). Regulation which involves chemical modification of the pump or membrane insertion of pre-existing pumps is probably fast and occurs within minutes. This is the prototype for fast adaptation to environmental changes.

The precise molecular mechanisms whereby hormones and factors act on the Na^+ pump and elicit a physiological response are still poorly understood. some recent work along these lines is mentioned in Table 2.

CHOLINESTERASES

Cholinesterases are primarily responsible for the rapid elimination of acetylcholine, within one millisecond after its release at cholinergic synapses. Cholineaterases are among the fastest enzymes known, with the hydrolysis of acetylcholine by acetylcholinesterase approaching the maximal theoretical limit set by molecular diffusion of the

Table 2 Effect of different hormones on Na⁺,K⁺ ATPase activity.

Hormone	Physiological role	Target cells	Time course (Ref.)
Aldosterone	Increased trans-epithelial sodium transport in response to salt deprivation	Tight epithelia (i.e. distal part of the nephron or large intestine	Fast (hours) (113,114-119)
Cortisol Corticosterone	Increased trans-epithelial sodium transport	Tight epithelia (i.e. distal part of the nephron or large intestine	Fast to slow (113,114, 119,120, 121)
Dexamethasone	Increased sodium transport	Non-epithelial cells	(hours to days) (106)
Thyroid	Increased trans-epithelial sodium transport Premis-sive effect on aldosterone action	Tight eipthilia	slow (123)
`	Increased sodium transport driving an increased cell heat production (thermogenesis)	Epithelial and non-epithelial cell	slow (day) (114,122-124)
Progesterone	Oocyte maturation (amphibia)	Oocyte	Fast (hours) (135)

Insulin	Increased sodium transport and sodium coupled substrate cotransport	Liver, muscle fat	very fast (minutes) (126,127, 128,129)
Glucagon	Liver	fast	(hours) (129,130)
EGF	Part of the cell activation	Fibroblast fast	Very fast response (minutes) (127,131)
Vasopressin	Increased trans-epithelial sodium transport in response to water deprivation	Tight epithelia	Fast to slow (132,133)
	Increased sodium transport	Liver	Fast (144)
Catecholamines	Prevention of K^+ losses during exercise	Skeletal muscl	very fast (128,134)

substrate. Cholinesterases display an extremely rich molecular polymorphism, possessing soluble, membrane-bound and basal lamina-anchored forms (138).

The presence of acetylcholinesterase outside of cholinergic synapses and the existence of a sister enzyme, butyrylcholinesterase, whose function is not understood, suggest that cholinesterases may be involved in functions other than the hydrolysis of acetylcholine. Cholinesterase may have additional role e.g. proteolytic activity and the processing of neuropeptides (138).

Acetylcholinesterase and Butyrylcholinesterase in vertebrates

Cholinesterases are defined by the fact that they hydrolyze choline esters faster than other substrate and are inhibited by the natural carbamate alkaloid, physostigmine. Cholinesterases are serine esterases and are irreversibly inhibited by organophosphates such as di-isopropylfluorophosphate (DFP), which react with the active serine.

Vertebrates possess two cholinesterases, corresponding to two distinct genes : acetylcholinesterase (AChE, EC 3.1.1.7) and butyrylcholinesterase or non-specific cholinesterase (BChE, EC 3.1.1.8). The two enzymes are distinguished primarily on the basis of their substrate specificity : AChE hydrolyses

acetylcholine faster than other choline esters and is less active on butyrylcholine. In contrast, BChE hydrolyses butyrylcholine well, but also acetylcholine. The two enzymes may also be distinguished by their affinity for, or activity with various selective inhibitors, such as BW284C51 for AChE and ethopropazine, iso-OMPA and bambuterol for BChE. AChE can be characterized by excess substrate inhibition (138).

Properties of these two enzymes are given in Tables 3 and 4.

Molecular forms of AChE and BChE

Cholinesterases are monomers or oligomers of glycoproteic catalytic subunits, whose molecular weights are generally in the range of 70 - 80 KDa. These molecular forms, sometimes called size - isomers, are similar in their catalytic properties, but differ in their hydrodynamic parameters and ionic or hydrophobic interactions (138).

Asymmetric or Collagen - Tailed forms of Cholinesterase

Asymmetric forms of AChE and BChE are characterized by the presence of a collagen - tail, which is formed by the triple helical association of three collagenic subunits. Each subunit may be attached to one catalytic tetramer; molecules containing one, two or three catalytic tetramers are named A_4, A_8 and A_{12} forms respectively.

Table 3 Comparision of AChE and BChE differences :

Property	AChE	BChE
Preferred substrate	ACh	BCh, PCh
Selective inhibitor	BW284C51**	Iso OMPA*
		ETPZ.HCl
Substrate inhibition	At higher concentration	No inhibition
Regulation	Tissue specific	Homeostatic

* Iso OMPA   - Isotetra monoisopropyl pyrophosphotetramide

ETPZ.HCl   - Ethopropazine hydrochloride - 10-2 diethyl-1- aminopyropyl phenothiazine hydrochloride

** BW284C51 - 1,5bis (4allyldimethylammoniumphenyl) pentane-3 one di-bromide

Table 4 Comparision of AChE and BChE - Similarities (39) :

AChE	BChE
1) Glycoprotein	Glycoprotein
Torpedo AChE has 4 ASn-linked carbohydrate chains	Human BChE has 9 Asn-linked carbohydrate chains
2) Catalytic subunits	
575 amino acid in hydrophilic Torpedo AChE	574 amino acid in hydrophilic Human BChE
3) Parathion, neostigmine, eserine inhibit	same
4) Active site	same
similar to serine proteases active site Ser closer to the N - terminus than in Ser protease. The pattern of disulfide bond is different.	
5) Similar to other esterases	same
disulfide bonds on each side of the active Ser are conserved.	

They are shown in Figure 6 (139).

Globular forms of Cholinesterase

Globular forms of cholinesterases may be defined in a negative fashion, by the absence of a collagen - like tail. They constitute, in fact a heterogeneous group. Classification on the basis of hydrodynamic properties, particularly sedimentation coefficients, identify these forms as monomers G_1, demurs G_2 or tetramers G_4.

These various forms are as shown in Figure 6 (139).

Comparison of the distribution of AChE and BChE in tissue : The various cholinesterase forms are tissue-specific. Asymmetric AChE and BChE forms are found only in peripheral nerves and muscles of vertebrates. Membrane-bound G_4 AChE and G_4 BChE are found in mammalian brain and membrane-bound G_2 AChE is found in erythrocytes (140).

Distribution of AChE and BChE in various tissues is summarized in Table 5.

Enzymatic properties

Reaction kinetics : Role of this enzyme is hydrolysis of acetyl-choline to choline and acetic acid.
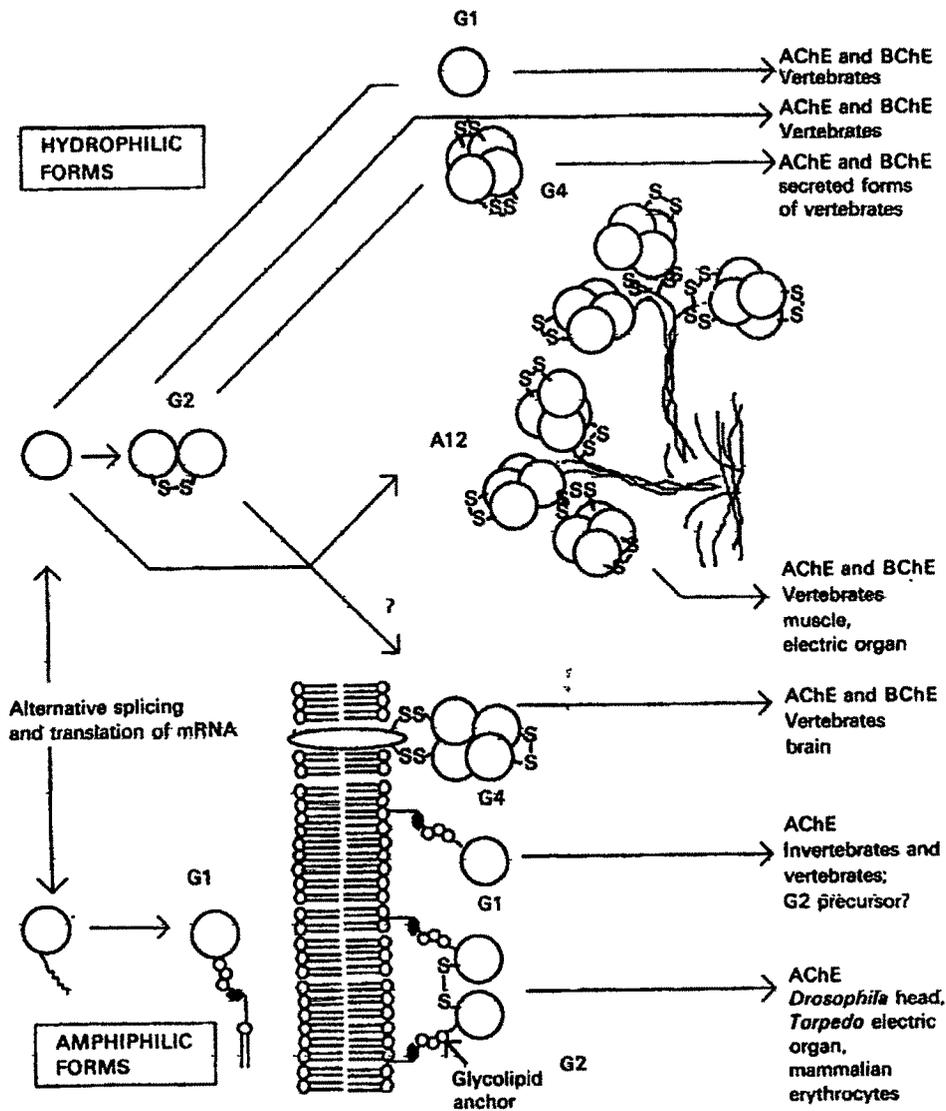
Figure 6. Schematic model of the molecular polymorphism of AChE and BChE. Open circles designate catalytic subunits. Disulphide bonds are indicated by S-S. Hydrophilic forms are G_a, G_2 and G_4 forms. The asymmetric A_{12} forms have three hydrophilic G_4 heads linked to a collagen tail via disulphide bonds. The G_4 amphiphilic forms of brain are anchored in to a phospholipid membrane through a 20 K Da anchore. The G_2 amphiphilic forms of erythrocytes have a glycolipid anchore. In *Torpedo* AChE hydrophilic forms and amphiphilic G_2 forms are produced by alternative splicing so that he proteins are identical at 535 amino acids but are nonidentical at their C-termini. (139)

Table 5 Distribution of AChE and BChE :

Tissue	AChE	BChE
Nervous system	Associated with cholinergic system	Present in places not related to AChE
	Large amount	Little amount
	Asymmetric form only in peripheral nerves	same
	Membrane bound G4 AChE found in mammalian brain	G4 BChE found in brain
Plasma	8 ng / ml	3300 ng / ml
	54 % in G4 form	98 % in G4 form
	G1, G2 origins from RBCs G4 form from neuromascular junction	Origin in liver
Heart	15 % of total ChE	85 % of total ChE
	The proportion of AChE/BChE increases from birth	
	This causes the physiological hydrolysis of ACh	The physiological Function not known
Muscle	Present at the neuromascular junction	same

This is actually a two step reaction viz. choline is released followed by the formation of acetyl - enzyme intermediate complex and then deacylation of the enzyme.

The Michaelis complex (ES) i.e. enzyme substrate complex is acetylcholine enzyme and the acetyl intermediate forms later.

Deacylation of the enzyme is the rate limiting step. The reaction is virtually irreversible as long as the concentration of the hydrolytic product is so low that it cannot effect the reaction kinetics.

In electrical eel, the turnover number of this enzyme is 1.6 $X \ 10^4$ / sec and the Km is 10^{-4} M.

The velocity substrate concentration curve deviates from a typical hyperbola in that it gives a bell shaped curve. This is because the velocity of hydrolysis decreases at substrate concentration of higher than 3 mM (141).

The catalytic Mechanism (142)

A) Substrate binding : The active site of AChE consists of two subsites, an anionic and an esteratic site. The anionic site determines specificity with respect to the alcohol moiety, and the esteratic site is involved in the actual catalytic process.

Anionic site is the locus of an electrically negative potential which attracts the quaternary ammonium head of acetylcholine. Most of the binding of ACh is accounted for by coulombic and hydrophobic interactions at the anionic site, and of these the sum of hydrophobic interactions is the larger (142).

B) Esteric site : Although pH changes may affect a protein in many ways, it was suggested that the bell-shaped curve for enzymatic hydrolysis might reflect the ionization of two essential groups in the esteratic site.

The actual catalytic process occurs at the esteratic subsite. The reaction is basically, a nucleophilic substitution. An atomic group in the esteratic site functions as a nucleophile and displaces choline from acetylcholine. The hydroxyl group of serine in the esteratic subsite is finally acetylated although neighboring functional groups are also essential for this step. Hence, choline esterases are serine hydrolases that possess a nearly identical sequence of amino acids in their active sites.

There is a charge relay system of hydrogen bonds in the active centre consisting of Ser-OH, imidazole ring of His and a COO- group of a dibasic amino acids.

Catalytic Mechanism : It is of a general acid base type and

the function of relay system is to deprotonate Ser-OH group, thus increasing its nucleophilicity and in this way, increasing acylation of the enzyme.

Activators

Some cationic ligands greatly accelerated acylation of active serine by acid transferring inhibitors containing fluoride as the leaving group.

Metal ions also have an influence on activation. Increase in concentration of mono- or divalent cations decreases apparent affinity of this enzyme for the substrate and increases maximum velocity of hydrolysis.

These effects result mostly from changes in ionic strength; divalent cations are more effective than monovalent ones.

Substrates

In contrast to butyrylcholinesterase, acetylcholinesterase is subjected to marked substrate inhibition and hydrolyses D-beta-methyl acetylcholine. Neither of the enzyme is completely specific for choline esters. For example acetylcholinesterase can hydrolyze ethyl acetate and phenyl acetate. The former is a poor substrate while the latter is good substrate.

Inhibitors

i) Inhibitors of anionic subsite : Mono and bis quaternary ammonia compounds are reversible inhibitors, eg. tetramethyl ammonia, tetraethyl ammonia, phenyl trimethyl ammonia, endorfonium, decamethonium and BW284C51.

Electrostatic and hydrophobic interactions are important for their binding. Affinity for the enzyme increases with the bulkiness of hydrophobic substituents on the quaternary nitrogen atom.

ii) Inhibitors of esteratic subunit : These usually cause irreversible inhibition. eg. organophosphorous compounds,carbamates and methne sulfonates (Figure 7).

iii) Peripheral anionic site ligands : Inhibit AChE binding to the enzyme outside its active centre and interfere with its activity by stabilizing its inactive conformation e.g. pachycurare reagents like d-tuburarine, gallamine and propidium.

iv) Substrate inhibition : This might be due to binding of a second molecule of acetylcholine to form a less efficient ternary complex.

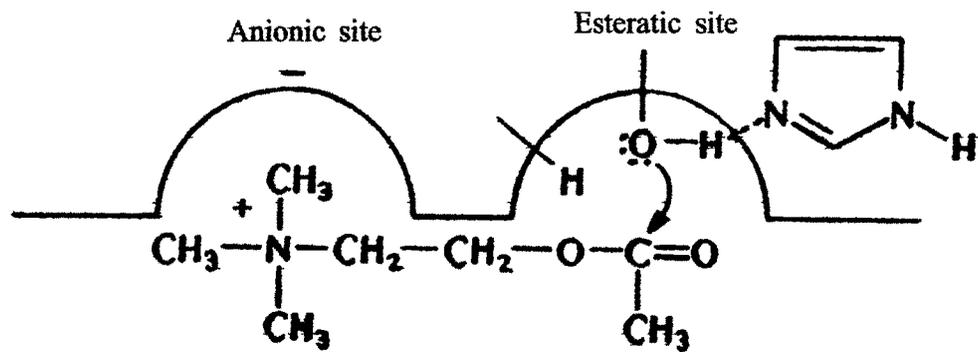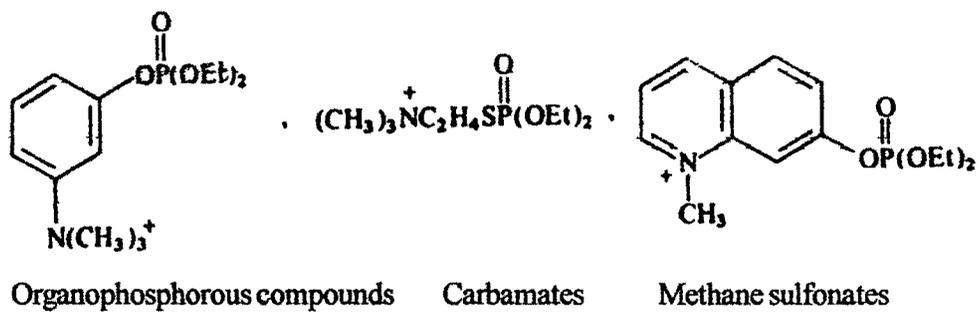Figure 6 : The anionic and esteric site of AChE

Figure 7 : Structures of inhibitors

Organophosphorous compounds   Carbamates   Methane sulfonates

Where an esterase is first acylated with release of the alcohol moiety of the substrate and later deacylates, the second substrate molecule may combine with the alcohol-binding site of the acylated enzyme, so retarding deacylation (41, 42).

Objectives of the present studies :

The present studies were under taken to illustrate aspects of protein glycosylation, membrane alterations and kinetic properties of cholinesterases as influenced by the diabetic status. The studies are divided in two parts : Human studies and Rat studies.

Human studies :

The list of area covered under human studies are given below.
1. Extent of protein glycosylation i.e. serum proteins and erythrocyte membrane protein.

2. To determine kinetic properties (i.e. substrate and temperature) of AChE from RBC membranes in human diabetic patients. These involve studies on Km, Vmax.

3. To determine BChE activity in the plasma and its kinetic properties (i.e. substrate and temperature). Parameters as in 2 above were be measured.

4. To determine the Na^+,K^+ ATPase activity in RBC membrane

and its substrate kinetic properties.

5. To determine total phospholipid and cholesterol content, contribution of different groups of phospholipids in total phospholipid and membrane fluidity.

Animal model studies

1. 1, 2, 3, 4 and 5 parameters as mentioned above in human study are studied in diabetic rats (male and female) in comparison with control and insulin treated diabetic rats.

2. To determine kinetic properties (i.e. substrate and temperature) of soluble and membrane-bound forms of BChE in hearts under these conditions (as mentioned in 1 animal study).

Detailed plan of work

Human studies

Blood samples were obtained from clinical laboratories from populations belonging to four groups

1) Control

2) Diabetic

3) Insulin-treated diabetic

4) Tablet (sulfonylurea type) treated diabetic

Animal model studies

Albino rats of Charles-Foster strain (both male and female) are made diabetic by injecting alloxan. Diabetic state was ascertained in terms of blood and urine sugar levels, polyurea, polyphagia and polydypsia. One group of the diabetic rats were treated with NPH insulin.

Blood was collected as described in human studies. Soluble and membrane-bound forms of BChE were isolated from the heart and studies on kinetic properties were carried out as described above.

It is expected that the above experiments, particularly there on cardiac BChE may through more light on basic biochemical defects underlying increased incidences of CHD and CHF in diabetic females.

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