

SYNOPSIS

Diabetes mellitus, the single most important metabolic disease affects more than 150 million people in the world. Diabetes mellitus is the condition characterized by defective glucose metabolism. It affects nearly every organ/system in the body in a differential manner and to different extents. Thus the kidneys, heart, pancreas and eyes etc. are the most severely affected systems whereas the peripheral nervous system and the brain are affected last; the liver is the least affected tissue. The complications include nephropathy, retinopathy, macro- and micro- angiopathy and increased incidences of cardiovascular diseases (CVDs). The peripheral and autonomic nervous system is affected initially and with time the central nervous system (CNS) also gets affected.

It has been reported that, the factors responsible for structure-function alterations include excessive polyol flux, advanced glycation end products (AGEs), increased accumulation of reactive carbonyl compounds (RCOs), impaired essential fatty acids metabolism and oxidative stress. These alterations result in damaged proximal tubules cells, thickening of glomerular basement membrane (GBM) and podocyte apoptosis which leads to altered filtration process and renal failure. Also, in the diabetic patients, the sensory neurons are the primary target and their involvement accounts for prominent sensory loss and pain. Patient with diabetes mellitus have a greater than 3 folds increased risk of coronary ischemic events and congestive heart failure. Diabetes is associated with profound changes in cardiac metabolism, characterized by diminished glucose utilization, diminished rates of lactate oxidation and increased use of fatty acids. Involvement of reactive oxygen species (ROS) in etiologies in diabetes is now being increasingly

recognized. Hyperglycemia in diabetes leads to excessive production of: ROS, lipid peroxidation (LPO) and protein glycation.

Earlier reports suggest that, insulin status significantly altered the oxidative energy metabolism and the insulin treatment was ineffective or only partially effective in restoring the function to normality in rat liver, kidney, brain and heart mitochondria. Thus, it is possible that the diabetic state could also influence the functional aspects of the enzymes of electron transport chain (ETC). In the liver of diabetic rats, the redox status of the endoplasmic reticulum and its redox chaperons altered significantly which contributes to defective protein secretion.

In brain microsomes, Ca^{2+} ATPase activity was significantly reduced in diabetes. However, there are no reports available on effects of insulin status on kinetic properties of Na^+ , K^+ -ATPase and glucose-6-phosphatase (G6Pase) in microsomes.

The present thesis addresses this question by evaluating effects of alloxan-diabetes and subsequent insulin treatment on the kinetic properties of FoF_1 ATPase, succinate oxidase (SO) and cytochrome oxidase in mitochondria and Na^+ , K^+ -ATPase and G6Pase in microsomes from rat liver, kidney and brain. In parallel studies the insulin-status-dependent changes on lipid/phospholipid profiles in mitochondria and microsomes from rat liver, kidney and brain were also examined.

Chapter 1 of the thesis embodies “Introduction” which gives a general overview of diabetes, classification of the diabetes classes and population distribution in diabetes. A brief overview of possible cause, symptoms and complication of diabetes are given in the “Introduction” Chapter. Since, the effects of insulin on different parameters are also evaluated; details of insulin structure, mechanism of action etc are included in this Chapter. A brief account of mitochondria and microsomes and their enzymes are also detailed. Since studies on ROS parameters were also carried out simultaneously, a detailed account of ROS, ROS generation, ROS action and ROS defense system are included in the “Introduction” Chapter.

Chapter 2 summarizes the results on early and late effects of alloxan-diabetes and subsequent insulin treatment on the kinetic properties of FoF₁ ATPase and lipid/phospholipid profile in rat liver mitochondria. Thus in the diabetic animals the body weight decreased by 14 and 34 % at the end of one week and one month respectively. Insulin treatment had partial restorative effects in one week group whereas complete restorative effect was noted for the one month diabetic animals. Liver weight decreased progressively in diabetes and insulin treatment brought about significant increase in the liver weight, beyond controls. Polyuria, glucosurea and hyperglycemia were evident in diabetic condition; insulin treatment restored these parameters to normality.

The FoF₁ ATPase activity decreased significantly in one month diabetic group; insulin treatment resulted in hyper-stimulation. Diabetic stage increased the basal activity whereas Mg²⁺- and DNP- stimulated activities decreased. The total i.e. (Mg²⁺ + DNP)-

stimulated activity decreased only in one month diabetic animals. Insulin treatment was effective only in restoring the total activity to normality. Substrate kinetics studies showed that the mitochondrial FoF₁ ATPase activity resolved in three kinetic components. In one week diabetic group, both K_m and V_{max} of component I increased while K_m of component II decreased. Insulin treatment restored the K_m of component I and decreased the V_{max} of component I. For component II both K_m and V_{max} decreased. Long term diabetic state had a general V_{max} lowering effects and the K_m of component I and II decreased. Treatment with insulin in one month diabetic group restored V_{max} values, in general, near normality while K_m of component I increased by 2 fold and that of component II was restored. The efficiency of the enzyme in terms of K_{cat}/K_m suggests that, at late diabetic state the enzyme efficiency increased whereas upon insulin treatment it decreased. Analysis of substrate kinetics data by Hill plots indicated that up to 1.0 mM ATP concentration one ATP molecule was bound while beyond this concentration of substrate two molecules of ATP were bound under all experimental conditions. From the temperature kinetic analysis it was noted that in diabetic animals the energy of activation in low temperature ranges (E_L) decreased. Insulin treatment was ineffective in restoring the E_L. Also, upon insulin treatment the energies of activation in high temperature ranges (E_H) decreased compared to the controls. The phase transition temperature (T_i) increased in diabetic animals and insulin treatment had no effect. Studies on lipid/phospholipid profile indicated that, in one month diabetic group the total phospholipids (TPL) content decreased (32 % decrease) and cholesterol (CHL) content increased by 51%. This was reflected in terms of decreased molar ratio of the two entities. Insulin treatment restored CHL content but had marginal

effect on TPL content. The diabetic state had a generalized effect of increasing lysophospholipid (Lyso), sphingomyelin (SPM), phosphatidylinositol (PI) and phosphatidylserine (PS) components and a tendency to decrease phosphatidylcholine (PC) and phosphatidylethanolamine (PE) components. Insulin treatment could correct the Lyso component and partially correct the PC and PE composition. The computed contents of the individual phospholipid classes were generally consistent with the above changes. The membrane fluidity decreased in diabetic animals; insulin treatment in one week diabetic animals completely restored the fluidity whereas in one month diabetic animals, it had only marginal effect.

Parameters similar to those described above were checked in kidney mitochondria. These results are summarized in **Chapter 3**. Kidney weight increased by 12 and 30 % in one week and one month diabetic animals respectively. FoF₁ ATPase decreased in diabetic animals which upon insulin treatment increased significantly beyond control. In both the diabetic groups, the basal activity increased whereas the DNP stimulated and total activity decreased. Even after insulin treatment the activity, in general, remained elevated. The substrate kinetics data revealed that as in the case of liver, the kidney enzyme also resolved in three kinetic components. However, in both diabetic groups component III and in both insulin treated diabetic groups component II were absent. In one week diabetic animals Km of component I increased whereas in one month diabetic animals it decreased and Km of component II increased. Upon insulin treatment, in one week diabetic group Km of component I increased whereas that of component III decreased, whereas in one month diabetic group the Km of both the components was

high. V_{max} of all the components remained high in both the diabetic stages as well as upon insulin treatment. The enzyme efficiency increased in diabetes; insulin treatment was somewhat effective in restoring normality. Hill analysis of the data suggested that up to 0.9 mM ATP concentration one ATP molecule was bound while beyond this concentration two molecules of ATP were bound under all experimental conditions. Temperature kinetics data indicated that in contrast with the liver, the E_L for the kidney enzyme increased in both the diabetic groups. Insulin treatment in one month diabetic animals reversed the pattern of Arrhenius plots. In one week diabetic animals insulin treatment partially restored the E_L whereas in one month diabetic animals the E_H increased and the E_L decreased. The CHL content increased by 16% in one week diabetic group; insulin treatment restored the CHL value. In one month diabetic group the TPL content decreased by 52 % and CHL content increased by 63 %. Insulin treatment partially restored the TPL and CHL contents. The TPL/CHL (mole : mole) ratios decreased in the diabetic groups and insulin treatment was effective in normalizing it only in early stage. The membrane fluidity decreased in diabetic animals and insulin treatment fluidized the membrane in one week diabetic animals. The proportion of Lyso, SPM, PC, PI and PS increased and PE and diphosphatidylglycerol (DPG) decreased in diabetic state. Insulin treatment partially corrected the Lyso and PS composition in both the diabetic groups. Insulin treatment could not correct the PI and PE components in one week group and elevated the latter component in the one month group. The computed contents of the individual phospholipid classes were generally consistent with the above data.

Chapter 4 includes the results of similar studies on the brain mitochondria. At late stage of diabetes brain weight decreased by 17 % and was only marginally restored by insulin treatment. Also, at late stage FoF₁ activity decreased by about 15 % and insulin treatment resulted in hyper-stimulation. In one month diabetic group, basal, Mg²⁺- and DNP-stimulated and total FoF₁ ATPase activities decreased; insulin treatment increased the activities beyond controls except for the DNP-stimulated activity which was almost comparable to the control values. Substrate kinetics data revealed that, the brain mitochondrial enzyme in control animal showed sigmoidal substrate saturation curve i.e. allosteric pattern and corresponding Eadie-Hofstee plots confirm the allosteric behavior. In one month diabetic and insulin treated diabetic groups the typical Michaelis-Menten pattern was observed and corresponding Eadie-Hofstee plots displayed two component system. In one month diabetic group treated with insulin K_m values of both the components were low while V_{max} values increased compared with the corresponding diabetic groups. Hill plot analysis indicated that the Hill coefficients n₁ and n₂ decreased in late diabetic state and insulin treatment was ineffective in normalization. Up to 0.35 mM ATP concentration one ATP molecule was bound while beyond this concentration two ATP molecules were bound to the enzyme under all the experimental conditions. From the temperature kinetics analysis it was noted that in control animals the enzyme activity displayed two phase transition temperatures (T_H and T_L) and three values for energy of activation (E_H, E_I and E_L), whereas in both diabetic and insulin treated diabetic groups only one T_t and two values for energies of activation (E_H and E_L) were noted. In one week diabetic group the TPL and the CHL content decreased significantly whereas no change was observed in one month diabetic group. Insulin treatment in one week

diabetic animals had only marginal effects on the TPL and no corrective effects on the CHL. The TPL and the CHL content decreased in one month diabetic animals after insulin treatment. The phospholipid composition remained practically unchanged except in the one week diabetic animals where Lyso and DPG decreased while in one month diabetic group PE decreased. Insulin treatment in one week diabetic animals decreased Lyso and PS whereas DPG and SPM increased. In one month diabetic animals insulin treatment resulted in significant reduction in PI and PS composition and almost 2 fold increase in the DPG.

The forgoing results thus indicate that alloxan-diabetes significantly altered the kinetic properties of FoF₁ ATPase and lipid/phospholipid profile in a tissue-specific manner and that insulin treatment, in general, was ineffective in restoring these parameters to normality.

In the next set of experiments, effects of alloxan-diabetes and subsequent insulin treatment on kinetic properties of another important enzyme of ETC i.e. succinate oxidase (SO) were examined. The results are summarized in **Chapters 5 and 6**. Thus in the liver mitochondria from the diabetic animals the SO activity decreased significantly; insulin treatment in one week diabetic animals almost restored the activity whereas in one month diabetic group activity decreased further. The temperature kinetics studies revealed that in diabetic stage E_H and E_L decreased; insulin treatment in one diabetic week animals completely restored the E_H with partial restoration in E_L whereas in one

month diabetic animals insulin treatment was ineffective. The T_t was lowered in all the experimental conditions compared to the corresponding control values.

Chapter 6 summarizes the results of similar studies on kidney mitochondria. In contrast with the liver, the SO activity in kidney mitochondria increased significantly in both the diabetic groups. Insulin treatment restored the activity. Also, the temperature kinetics analysis indicated that, the pattern of Arrhenius plot in control was reversed compared to the liver enzyme i.e. high E_H and low E_L . Diabetic states reverse the Arrhenius pattern. Insulin treatment in one month diabetic animals normalized the Arrhenius pattern. In diabetic groups the E_H increased whereas E_L decreased. Insulin treatment was generally effective in one month diabetic groups. Diabetic stage decreased the T_t ; insulin treatment in one week diabetic animals had marginal restorative effects whereas in one month diabetic animals it increased T_t beyond control values.

From the forgoing results it may be concluded that the diabetic state had opposite effects on the temperature kinetics properties of liver and kidney enzyme and insulin treatment once again in general, was ineffective in restoring the parameters to normality.

The results on the effects of alloxan-diabetes and subsequent insulin treatment on temperature kinetic properties of cytochrome oxidase from brain mitochondria are summarized in **Chapter 7**. The enzyme activity was unchanged in one week diabetic

group whereas in one month diabetic animals the activity decreased by 22 %. Insulin treatment increased the activity almost by 2 fold in one week diabetic group but was ineffective in one month diabetic groups. The Arrhenius pattern revealed that, like brain mitochondrial ATPase, the cytochrome oxidase activity displayed two phase transition temperatures and three values for energies of activation. In controls, the values for E_H was highest followed by E_L and the values of E_I was lowest. The pattern changed only in one week diabetic group where only E_H and E_L were present; insulin treatment restored the Arrhenius pattern to normality but the values for E_I was highest followed by E_H and the values of E_L was lowest.

Since the microsomal enzyme systems are also affected in diabetes, experiments were carried out to evaluate the effects of alloxan-diabetes and subsequent insulin treatment on kinetic properties of microsomal membrane-bound enzymes viz. Na^+ , K^+ -ATPase and G6Pase and lipid/phospholipid profile in rat liver, kidney and brain.

The results relating to liver microsomes are summarized in **Chapter 8**. Thus at the early stage of diabetes the microsomal Na^+ , K^+ -ATPase activity increased by about 18 % whereas an opposite effect was noted at late stage (28 % decrease). Insulin treatment restored the activity only in one month diabetic group. Substrate kinetics studies revealed that the enzyme activity resolved in two kinetic components. Diabetic state in general increased the values for K_m ; insulin treatment was effective only at the late stage of diabetes. In one week diabetic animals the V_{max} of component II increased. By contrast

in one month diabetic group V_{max} of both the components decreased; insulin treatment once again was effective only at the late stage. The Hill plot analysis of substrate kinetics data revealed that up to 0.95 mM ATP concentration one ATP molecule was bound while beyond this concentration two ATP molecules were bound to the enzyme under all the experimental conditions. The temperature kinetics data revealed that the E_H and E_L values decreased significantly in diabetic animals; insulin treatment restored the E_H and partially corrected the E_L value only at late stage. No change was noted in T_i under any experimental conditions.

The G6Pase activity increased significantly with time in diabetic state; insulin treatment had marginal corrective effect only at late stage. From the substrate kinetics studies it can be noted that the G6Pase activity displayed a two kinetic component systems under all the experimental conditions. The K_m of component I decreased significantly in one week diabetic animals; in one month group K_m of both the components decreased. Insulin treatment was ineffective at early stage whereas it restored the K_m of component II while partially correcting the K_m of component I at the late stage. Hill plots analysis of the substrate kinetics data revealed that up to 2 mM glucose-6-phosphate (G6P) concentration one G6P molecule was bound while beyond this concentration two G6P molecules were bound to the enzyme under all the experimental conditions. The Arrhenus pattern was reversed in one week diabetic as well as insulin treated diabetic animals. E_H increased in diabetic groups; insulin treatment was ineffective. E_L decreased at early stage of diabetes whereas increased at the late stage; insulin treatment again was ineffective. No change was observed in T_i under all the experimental conditions. In one

week diabetic animals the TPL and CHL contents increased by 23 and 36 % respectively; insulin treatment restored only TPL content. In one month diabetic group the CHL content increased almost by 2.5 fold which was partially brought back by insulin treatment. The diabetic states resulted in increased Lyso, PC and PI whereas SPM decreased. Phosphatidic acid (PA) and PS decreased only at early stage and PE at late stage of diabetes. Insulin treatment was effective only in restoring the PI and PS at early stage. Diabetic states decreased the membrane fluidity; insulin treatment increased the fluidity beyond control in one week diabetic group whereas in one month diabetic group there was further decrease.

Chapter 9 summarizes the results on kidney microsomes. Thus Na^+ , K^+ -ATPase activity increased by 16 % at the early stage but decreased by 48 % late stage of diabetes. Insulin treatment caused hyper-stimulation of the Na^+ , K^+ -ATPase activity. In all the experimental groups the kidney microsomal Na^+ , K^+ -ATPase resolved in two kinetic components. Diabetic state, in general, lowered the K_m and V_{max} values and the effects were more pronounced at late stage. Insulin treatment only marginally corrected the K_m values in one month group; V_{max} in general increased beyond control values upon insulin treatment. The data from Hill plot analysis showed that up to 0.85 mM ATP concentration one ATP molecule was bound while beyond this concentration two ATP molecules were bound to the enzyme under all the experimental conditions. In one week diabetic animals E_H decreased and upon insulin treatment it was restored. Insulin treatment in one week diabetic animals significantly lowered the E_L value. T_t increased at late stage of diabetes and also in one week diabetic group treated with insulin.

The G6Pase activity increased in diabetes with the magnitude being greater in the one month group. Insulin treatment was somewhat effective in restoring the activity to normality. As in the case of liver, the kidney enzyme displayed two kinetic components. The diabetic state increased the K_m as well as V_{max} values. Insulin treatment restored the K_m of component I in both the diabetic groups whereas marginally restored the K_m of component II only in one week diabetic group. V_{max} values in general were partially restored upon insulin treatment. Hill plot analysis suggested that up to 2.2 mM G6P concentration one G6P molecule was bound while beyond this concentration two G6P molecules were bound to the enzyme under all the experimental conditions. The temperature kinetic analysis revealed that the early diabetic state as well as insulin treatment in early and late state reversed the Arrhenius pattern. The value for E_H increased in early diabetic state but decreased at the late stage. E_L values decreased in both the diabetic groups. Insulin treatment restored the E_H but not the E_L values. No change was observed in T_i under any of the experimental conditions. Early diabetic state resulted in increased TPL and CHL contents (28 and 47 % increase respectively). The TPL content was unaltered in the late diabetic stage but the CHL content increased by 59 %. Insulin treatment was effective only in restoring the CHL value at late stage. The membrane fluidity decreased in diabetic animals and treatment with insulin significantly fluidized the membrane in one week diabetic animals; opposite effect was seen in the one month diabetic animals. In one week diabetic group, Lyso, PC and PI increased whereas SPM, PE, PS and PA decreased; insulin treatment had no effect on PC component which remained elevated. PE increased beyond control level after insulin treatment while there

was a further lowering of the other phospholipid classes. In one month diabetic animals Lyso and PC increased whereas PI, PS, PE and PA decreased; insulin treatment completely restored PE and PI with partial restoration of PS; Lyso was further elevated while opposite effect was noted for PA component.

In the brain the G6Pase is a mitochondrial enzyme. Hence, studies could be carried out only on Na^+ , K^+ -ATPase and lipid/phospholipid profiles and the results are summarized in **Chapter 10**. The Na^+ , K^+ -ATPase activity decreased in diabetic groups. Insulin treatment in one week diabetic animals increased the values beyond control whereas in one month diabetic group partial restoration was noted. The brain microsomal Na^+ , K^+ -ATPase also displayed two kinetic components in all the experimental conditions. Thus, the K_m of component I increased whereas that of component II decreased in one week diabetic group; insulin treatment had no effect on the K_m of component I but resulted in elevating the K_m of component II. In one month diabetic group K_m of component I increased which was corrected following insulin treatment. In early diabetic stage, V_{\max} of component I increased whereas that of component II decreased. Insulin treatment resulted in significant increase in the V_{\max} of component II. At late stage of diabetes V_{\max} of component II decreased which was marginally restored by insulin treatment. The Hill plot analysis suggested that up to 0.7 mM ATP concentration one ATP molecule was bound while beyond this concentration two ATP molecules were bound to the enzyme under all the experimental conditions. Temperature kinetics studies revealed that late diabetic stage as well as insulin treatment reversed the Arrhenus pattern. In diabetic animals E_H increased which was restored by insulin treatment only at early stage. E_L

decreased significantly (63 % decrease) at late stage of diabetes and insulin treatment was ineffective in correcting the same. T_i increased significantly in one month diabetic animals and insulin treatment was again ineffective in restoring the values to normality. The lipid/phospholipids analysis showed that in early diabetic stage the TPL content decreased by 19 %. By contrast the CHL content increased by 50 %. Insulin treatment restored the TPL content while CHL content increased further. At the late stage of diabetes decrease in TPL content amounted to 21 %. By contrast, the CHL content increased substantially by 2.4 fold. Insulin treatment had no effect on TPL content but marginally lowered the CHL content. The membrane fluidity decreased in diabetic animals and treatment with insulin significantly fluidized the membrane in one week diabetic animals; no appreciable effect was seen in the one month diabetic animals. In one week diabetic group SPM increased and PI and PS decreased whereas in one month diabetic animals PI, PS and PE increased while PC decreased. Insulin treatment in one week diabetic animals decreased the proportion of Lyso, PI, PS and PA components while PE increased. In one month diabetic animals, insulin treatment caused significant decrease in Lyso, SPM, PI, PS and PA; PC and PE increased.

The forgoing results (Chapter 8-10) suggested that alloxan-diabetes significantly altered the kinetic properties of the microsomal enzymes. The effects were tissue-specific and insulin treatment in general once again was unable to correct the defects caused by the experimental diabetic condition.

It has been reported that, the incidence of myocardial infarction, CHF and CHD are significantly high in diabetic patients. Interestingly, the incidence of CVDs is higher in diabetic women than in diabetic men. Involvement of reactive oxygen species (ROS) in etiologies of several disease conditions is now being increasingly recognized. Hence the studies were also carried out to evaluate the role of alloxan-diabetes and subsequent insulin treatment on ROS related parameters in mitochondrial and post-mitochondrial fractions of liver and heart from male and female rats. The results related to the studies in the liver are included in **Chapter 11**. In male rats, in one week diabetic group, xanthine oxidase (XO), mitochondrial glutathione peroxidase (GPox), glucose-6-phosphate dehydrogenase (G6PDH), and reduced glutathione (GSH) in post-mitochondrial fraction decreased while mitochondrial superoxide dismutase (SOD) and GPox in post-mitochondrial fraction increased. At the end of one month, XO, mitochondrial and post-mitochondrial SOD, catalase, G6PDH and GSH in mitochondrial as well as post-mitochondrial fractions decreased while GPox in post-mitochondrial fraction increased significantly. Insulin treatment, in general was ineffective in restoring XO, mitochondrial and post-mitochondrial SOD and catalase activities or GSH content in mitochondrial as well as post-mitochondrial fractions in one month group. Our results suggest that GPox activity may be a major route of eliminating ROS in the liver of diabetic and insulin-treated diabetic rats. The pattern in female rats was comparable to that in the male rats.

Chapter 12 summarizes the results on ROS parameters in the heart. The heart tissue was characterized by very low XO activity. However, the diabetic state caused significant raise in the XO activity with the magnitude of increase being higher in the females.

Paradoxically, insulin treatment resulted in further increase in the XO activity. The G6PDH and catalase activities decreased and the reduced GSH content in mitochondria was completely depleted in diabetic state with significant decrease in the GSH levels in the post-mitochondrial fraction. The effect was more pronounced in the females. The SOD and GPox activities increased in the diabetic state to a greater extent in male rats. Insulin treatment had restorative action only on some parameters.

In conclusion, the results suggest that compared to the liver, the ROS defense system of the heart is weak. The results also suggest that the diabetic state may further compromise the weak ROS defense systems in the heart thus initiating a lesion at the level of mitochondria which ultimately leads to cardiomyopathy. The results also point out that insulin treatment was ineffective in restoring ROS related parameters. Also, the effects were more prominent in the females, which may correlate with higher incidence of CVDs in female diabetics.

In the initial stages of the work, attempts were made to standardize methods for determining the kinetic properties of cytochrome oxidase, pH sensitivity of tissue SOD, and for improving color stability and sensitivity of inorganic phosphorus (Pi) estimation. These experiments are described in following three **Supplementary Chapters** of the thesis.

Substrate for cytochrome oxidase is reduced cytochrome c (which is not cost effective) and the reaction follows a first order kinetics rather than a linear kinetics. The latter

parameter makes data presentation cumbersome. To obviate these two problems a polarographic method was standardized which uses ascorbate + N,N,N',N'-tetramethyl-p-phenylenediamine (TMPD) as the electron donor system. **Chapter 13** summarizes these results. These studies revealed that the cytochrome oxidase activity was highest in the heart mitochondria followed by kidney, brain and liver mitochondria. Analysis of the substrate kinetics data revealed tissue-specific expression of kinetic components exhibiting differences with respect to K_m , V_{max} and K_{cat}/K_m values, the latter parameter being the index of enzyme efficiency. Liver, kidney and heart enzyme displayed presence of two kinetic components whereas brain enzyme displayed three kinetic components. Regression analysis of substrate kinetics data with phospholipid composition suggest that the enzyme activity may be regulated in a tissue-specific manner.

SOD purified from bovine erythrocyte is used as a standard source for delineate methods for SOD assay. The enzyme is unusually stable even at highly alkaline pH, a condition conducive for generating superoxide (O_2^-) radical. However, measurements at around physiological pH only can give correct estimates of the true SOD activity in the tissues. Thus the estimation of SOD activities in the human and rat RBCs and rat liver, kidney, brain and heart mitochondria as well as cytosolic fractions were determined by the pyrogallol assay procedure with slight modifications. Measurements were carried out in 0.1 M potassium phosphate buffer pH 8.0 and 9.2 to assess the pH stability of the SODs from various systems. Under these conditions the SODs from different systems including RBCs exhibited differential pH stability i.e. they displayed differential susceptibility at

pH 9.2. Even in a given tissue, the mitochondrial and cytosolic SODs were inactivated differentially at pH 9.2. It was observed that the total mitochondrial and cytosolic SOD contents show a tissue-specific pattern. The results also suggest that measurements carried out at pH 8.0 may give more realistic estimates of SOD activities in the tissues/subcellular fractions. These results are detailed in **Chapter 14**.

The conventional colorimetric method of estimation of inorganic phosphorus (Pi) has the inherent problem of color instability. Attempts were made to improve the color stability as well as sensitivity in the routine assay and the results are summarized in **Chapter 15**. To this end, studies were carried out where the content of H₂SO₄ used ranged from 0.5 to 1.0 N and the intensity of the molybdenum blue color was determined for up to 24 h. It was found that the highest concentration of H₂SO₄ was most effective in improving both the parameters. The sensitivity improved by 1.5 to 2.2 fold with a shift in absorbance maximum to around 820 nm. Presence of sugars (1-10 mM), NaCl and KCl (5-100 mM), MgCl₂ (1-10 mM) or BSA (up to 500 µg) did not interfere in color development. The extent of ATP hydrolysis was 1.8 to 3.4 % for up to 1h. Only negligible hydrolysis of glucose-6-phosphate (G6P) was noted under these conditions. Thus the method is suitable even for Pi analysis in enzyme assays in which substrate containing labile phosphate is used. The present modification, besides producing stable color and improving sensitivity also allows batch operation of a large number of samples.

In conclusion, the overall results of the present studies show that alloxan-diabetes significantly altered the structure-function relationship in mitochondria as well as

microsomes in rat brain, kidney and liver. The effects were tissues/subcellular organelle-specific and that the treatment with insulin was, in general, ineffective in normalizing the altered structure-function relationship. Additionally, methods were standardized for Cytochrome oxidase, SOD and Pi assays.

Signature of the Candidate

Date

Signature of Guiding Teacher

Date