DISCUSSION
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1. GENERAL

The major characteristic of diabetes is hyperglycemia, which results from a decrease in glucose utilization by insulin dependent tissue and an increase in production of glucose by liver (Hue, 1987). These alterations in metabolic processes lead to an increased plasma glucose level, which is responsible for diabetic complications. Liver is one of the main organs involved in glucose metabolism and is greatly affected in diabetes because of the imbalance in production (gluconeogenesis) and utilization (glycolysis) of glucose. Increased levels of glucose in plasma as well as in interstitial fluid leads to glycation of a wide variety of proteins which are responsible for modification in a number of tissues (Strowing et al., 1992). Kidney, apart from liver, showed microvascular complications indicated by thickening of capillary basement membrane and the persistent proteinuria. The catabolism of protein increased in diabetes and the carbon skeleton is used for the synthesis of glucose (via gluconeogenesis). The amino groups get converted into urea which is excreted out via Urea cycle. AspAT, AlaAT, GLDH and arginase play significant roles in the amino acid catabolism in both liver and kidney.

Experimental diabetes was induced by alloxan. Alloxan has been used extensively to induce experimental diabetes in animals, as it exhibits highly selective cytotoxicity for the pancreatic β-cells (Malaisse, 1982). Several investigators suggest that the diabetogenic action of alloxan is mediated by the generation of toxic oxygen free radicals (Oberley et al., 1985). The rapid uptake of alloxan and an exquisite sensitivity to peroxides due to poor protection against free radicals are unique features of pancreatic β-cells (Malaisse, 1982). Alloxan toxicity
is attributed to the formation of hydrogen peroxides and of superoxide radicals, in the redox cycling of alloxan and to the formation of hydroxyl radicals (Winterbourn and Munday, 1989).

Several studies have established that vanadate compounds mimic many of the insulin like effects. Recently, much interest was focused on elucidation and exploring the beneficial antidiabetic effects of vanadate in ameliorating the metabolic disorders associated with diabetes. In earlier studies on the *in vitro* effects of vanadate, stimulation of glucose oxidation and transport were shown in hepatocytes, adipocytes and skeletal muscle (Tolman *et al.*, 1979 and Brichard *et al.*, 1990). These reports had formed the basis for further studies and it was suggested that vanadate may have a therapeutic role in regulating glucose metabolism *in vivo*. For the first time Heylinger *et al.* (1985) demonstrated that oral administration of sodium orthovanadate to diabetic rats normalized the elevated blood glucose level and improved the altered cardiac performance. Since then, several investigators have been further exploring this therapeutic aspect of vanadate with respect to diabetes (Pederson *et al.*, 1989 and McNeill *et al.*, 1982).

2. **AMINO ACID METABOLISM IN DIABETIC LIVER AND KIDNEY**

The various metabolic pathways in kidney and liver under experimental diabetic condition have been investigated by many researchers/authors (Greenbaum *et al.*, 1971; Sochor *et al.*, 1985 and Dhanakoti *et al.*, 1992.) but their amino acid metabolism has not been studied in depth. Kidney liver and muscles are severely affected in Type I diabetes and kidney failure is one of the common causes of death in uncontrolled diabetes in humans.
The present experiments were planned and carried out in the liver and kidney tissues, taken from experimentally induced diabetic rats. Both of these organs are major sites for gluconeogenesis and amino acid metabolism. In diabetes, glucose entry into the insulin dependent tissues mainly liver, adipose and muscle is decreased and energy requirement is fulfilled by the mobilization of non-carbohydrate sources. Lipids and proteins are sometimes used as energy sources in diabetes. Protein degradation, which increases in diabetes, leads to an increase in the amino acids pool. These excess amino acids get deaminated to generate carbon skeleton, which is used as an energy source, and ammonium ions which get excreted through Urea cycle. Alanine aminotransferase (AlaAT), aspartate aminotransferase (AspAT), glutamate dehydrogenase (GLDH) and arginase have been studied in experimental diabetes because these enzymes play a significant role in the catabolism of amino acids. The reversal effects on the altered activities of these enzymes using antidiabetic agents like insulin, vanadate, lithium with vanadate and Memordica charantia extract were also studied.

Except GLDH, all of the enzymes studied are present in two subcellular fractions namely cytosolic and mitochondrial. The levels of these enzymes were found to be different in liver and kidney and the pattern of distribution between cytosol and mitochondria, was also variable in these organs as reported earlier (Sochor et al., 1985).

A. CHANGES IN THE ACTIVITY OF CYTOSOLIC ENZYMES

i. Alanine aminotransferase (AlaAT)

AlaAT catalyses the interconversion of alanine and 2-oxoglutarate to glutamate and pyruvate. Liver exhibits the highest activity of these enzymes, whereas kidney contains about 10-15% of the liver enzyme activity (Schepart,
1973). Physico-chemical and kinetic studies have shown the existence of two isoforms of this enzyme: one is present in the mitochondria and the other in the cytosol (DeRosa and Swick, 1975). It has been suggested that the soluble enzyme is mainly associated with the conversion of pyruvate to alanine, whereas the mitochondrial enzyme catalyses the formation of pyruvate from alanine (Dieterle et al., 1978). The activity of AlaAT was measured in both cytosolic as well as mitochondrial fractions of kidney from the age matched control rats. An increase in the activity was observed with an increase in age, from seven to ninety days, similar results in liver have been were shown earlier by Dieterle et. al., (1978).

The distribution of AlaAT was also studied in the control animals and it was found that the activity of AlaAT in the cytosolic fractions was higher as compared to the mitochondrial fractions in the seven day controls, whereas this pattern of distribution got reversed in the ninety day control rats. DeRosa et. al., (1975) reported that cytosolic AlaAT predominates in the tissues which shows a higher glycolytic rate, whereas the mitochondrial form is relatively enriched in gluconeogenic tissues. Therefore, it may be derived from the above observation that the kidney behaves as a glycolytic tissue in younger animals and as a gluconeogenic tissue in older ones (Kanter, 1976).

The activity of the cytosolic AlaAT in diabetic rats increased on all the subsequent days i.e. 7th, 21st and 90th. The increased activity of the cytosolic AlaAT in kidney results in the enhancement of the Glucose-Alanine cycle in short term diabetes (7 and 21 days) (Marliss et al., 1971), whereas in long term diabetes (90 days), increased activity may be ascribed to enhanced gluconeogenesis and amino acid metabolism (Dolkart et al., 1964). Muscle proteolysis also increases in diabetes (Sherwin et al., 1975), which may contribute to the increase in AlaAT activity.
AlaAT was also studied in the twenty one day diabetic liver which showed an increase in the activity of this enzyme. This increase in activity could be ascribed to the enhanced gluconeogenic activity in diabetic liver as also shown earlier by other workers (DeRosa and Swick, 1975).

ii. Aspartate aminotransferase (AspAT)

AspAT catalyses the interconversions of aspartate and 2-oxoglutarate with glutamate and oxaloacetate. AspAT isolated from various tissues used pyridoxal phosphate as coenzyme, where two molecules of coenzymes were tightly bound to one molecule of the enzyme. Heart muscle has the highest activity of AspAT followed by liver and kidney (Hertzfeld and Greengard, 1971) (Table A). In brain and liver, AspAT exists in two isoforms namely cytosolic and mitochondrial (Benuck and Lajtha, 1974) and the cytosolic isoenzyme has greater affinity for the keto acid substrates, whereas the mitochondrial isoenzymes prefers the amino acid substrates (Braunstein, 1973). Kidney enzyme, like that of heart and muscle, appears to be predominantly localized in the cytosol, whereas mitochondrial form predominates in liver (Hertzfeld and Greengard, 1971).

The increase in the activity of the cytosolic AspAT in kidney was relatively small on the 7th day as compared to the 21st and 90th days after induction of diabetes. The higher change in activity in later days (21st and 90th) could be due to high ratio of NADH/NAD⁺ where NADH works as a positive modulator on the cytosolic enzyme. Liver also showed an increase in the activity of this enzyme in diabetic rats. Being an important enzyme of the amino acid metabolism, the activity of AspAT increases with the enhanced level of amino acid catabolism and replenishes the substrate like 2-oxoglutarate to meet the higher demand in gluconeogenesis during diabetes.
The ratio of cytosolic and mitochondrial AspAT in kidney showed that the enzyme was predominant in the cytosolic fraction (60:40). The activity of the cytosolic AspAT increased in diabetic kidney, may be due to the higher NADH/NAD⁺ ratio in the cytosol as shown earlier for brain (Ruderman, 1974). In diabetic kidney, the enhanced gluconeogenesis requires high level of oxaloacetate in the cytoplasm for its subsequent conversion to phosphoenolpyruvate. Oxaloacetate can not directly cross into, and out of, mitochondria because of the selective permeability of the membrane (Haslam and Krebs, 1968). However, aspartate and 2-oxoglutarate are permeable to mitochondrial membrane (Brand and Chappell, 1974). Therefore, during diabetes the enhanced substrate formation in mitochondria would lead to increased substrate availability for the soluble AspAT, explaining the increased AspAT activity in kidney (Table 4 and Figure 2).

iii. Arginase

Arginase is the last enzyme of the Urea cycle which catalyses the conversion of arginine into urea and ornithine. In liver, arginase plays a major role in Urea cycle whereas, in extra-hepatic tissue like kidney, it also participates in the metabolism of arginine to proline or glutamine apart from Urea cycle (VanElsem and Leroy, 1975). Different isoforms of arginase were identified (A1, A2, A3 & A4), liver contain A1 and A3 and kidney contain A1 and A4 forms (Porembska, 1973).

The activity of kidney cytosolic arginase was measured in the 7th, 21st and 90th days diabetes, an increase in the activity was observed with maximum change on 21st day after induction of diabetes. The increase in the excretion of nitrogenous compounds during diabetes (Chaikoff, 1950) may be because of increase in arginase activity. Apart from the ammonium ions, concentration of lactate, which increases in diabetes, is a major factor and has a direct effect on the increased activity of arginase (Briggs et al., 1976).
There was a significant increase in the activity of cytosolic arginase in liver. Due to the enhanced level of amino acid catabolism during diabetes, ammonium ion concentration increases, which is excreted out by the increased activity of all the enzymes of the Urea cycle including arginase (Takiguchi and Mori, 1995).

The distribution pattern of arginase in cytosol and mitochondria was found to be different in kidney and liver as reported earlier (Kaysan et al., 1973). The cytosolic arginase is the main form of the enzyme in liver whereas in kidney, substantial amount of arginase activity was found to be associated with the mitochondria as shown earlier by Kaysan et al., (1973) and in the present work also. The ratio of cytosolic and mitochondrial arginase changes in diabetes, favouring the mitochondrial form, may be because of the excess requirement of proline and glutamic acid for the catabolic reaction (Sadasivandu et al., 1976).

From the above observations it could be concluded that the cytosolic isoforms of the enzymes studied showed an increase in activity during diabetes. A major portion of the energy required by the body in long term diabetes is provided by the protein catabolism, leading to protein deficiency and shortage of substrates for biosynthesis of macromolecules like nucleic acid (Hoogenraad et al., 1985).

B. CHANGES IN ACTIVITY OF MITOCHONDRIAL ENZYMES

Changes in the activity of mitochondrial isoforms of the enzymes, transaminases, arginase and glutamate dehydrogenase were also estimated under diabetic conditions and with insulin and vanadate treatment to diabetic animals.

i. Alanine aminotransferase (AlaAT)

The activity of AlaAT in the mitochondria from different tissues is mainly localized in the matrix, together with a substantial part of glutamate dehydrogenase (Orlicky et al., 1981). It has been proposed that mitochondrial AlaAT catalyses the
formation of pyruvate from alanine (Dieterle et al., 1978). The occurrence of mitochondrial alanine aminotransferase in matrix, together with glutamate dehydrogenase can be related to ammonia production and NADH re-oxidation by joint reactions (Fahien et al., 1971). Furthermore, the alanine and 2-oxoglutarate produced from these joint reactions may take part in the translocation of glutamate equivalents from mitochondria to extra mitochondrial space (Rusacak et al., 1982). Fahien et al. (1971) reported that the mitochondrial AlaAT, together with glutamate dehydrogenase, can contribute to an alanine dehydrogenase system which would permit the recovery of reducing equivalent from alanine and the fixation of ammonia in liver. Mitochondrial alanine aminotransferase and glutamate dehydrogenase are located in the mitochondrial fraction of kidney homogenate. Therefore, during diabetes the increased levels of glutamate and NADH/NAD ratio may be responsible for the increase in AlaAT activity in short term diabetic kidney (Sherwin et al., 1975). However, in long term diabetes, the proteolysis appears to be diminished due to enhanced level of keto acids which may be leading to the observed decrease in AlaAT activity in diabetes (Greenbaum et al., 1971).

Liver also showed an increase in the activity of mitochondrial AlaAT during diabetes. Due to the increase in gluconeogenesis in both kidney and liver, a substrate like pyruvate is required in higher concentration, which can be provided through the anaplerotic reaction catalyzed by AlaAT (Dieterle et al., 1978).

ii. Aspartate aminotransferase (AspAT)

AspAT has been extensively studied in various pathological conditions. The enzyme exists in two different isoforms which differ in their physico-chemical properties as well as different localization in the cell. The mitochondrial AspAT showed an increase in activity in kidney. During diabetes the metabolic fate of pyruvate is favoured towards carboxylation to oxaloacetate rather than
decarboxylation to acetyl-CoA due to an increased content of free fatty acid, acetyl-CoA and a more reduced state of mitochondria in brain and liver (Ruderman et al., 1974 and Greenbaum, 1971). This shift would increase the levels of oxaloacetate. However, since the high levels of fatty acid, fatty acyl-CoA and NADH are known to inhibit the citrate synthase (Srere and Matsuoda, 1972), the oxaloacetate may be channelized for AspAT reaction, thereby increasing its activity in the mitochondrial fraction of kidney. During diabetes, the enhanced gluconeogenesis requires high level of oxaloacetate in the cytoplasm for its subsequent conversion to phosphoenolpyruvate.

Due to the selective permeability of mitochondrial membrane, the oxaloacetate cannot directly cross into and out of the mitochondria. However, aspartate and 2-oxoglutarate are permeable to mitochondrial membrane (Brand and Chapell, 1974). Therefore, increased aspartate formation in mitochondria may increase the substrate availability for mitochondrial AspAT. The activity of the mitochondrial AspAT also increased in the diabetic liver. Due to the more reduced state of the mitochondria in diabetes (Greenbaum et al., 1971), the formation of oxaloacetate from pyruvate is favoured. As this reaction is catalyzed by mitochondrial AspAT, the activity of the enzyme was also found to increase in the present work.

iii. Arginase

Arginase is present in a number of extra hepatic tissues like kidney and brain, probably carrying out functions different from the ones in the liver (Herzfeld et al., 1976). The lack of reactivity of kidney arginase with antisera prepared against liver arginase indicates the difference between these two isoforms (Kaysan et al., 1973). In kidney, besides the cytosolic isoform arginase is also present in the mitochondria. The presence of arginase in the particulate fractions could be contributing to the
formation of glutamic acid, as the enzyme concerned with conversion of ornithine to glutamic acid is localized in mitochondrial fraction (Kaysan et al., 1973). In mitochondria, the ornithine formed from the reaction catalyzed by arginase is transported to the cytosol where it is used for the synthesis of proline and polyamines through ornithine decarboxylase pathway (Baquer et al., 1976). The increase in the level of mitochondrial arginase may contribute to ornithine decarboxylase pathway, though the data is not available in this support is not sufficient.

A very low activity of arginase was detected in the mitochondrial fractions of liver. More than the 90% activity of the arginase is present in the cytosolic fractions of liver.

iv. Glutamate dehydrogenase (GLDH)

The activity of glutamate dehydrogenase is very widely distributed in different tissues. Liver is its richest source followed by kidney and brain (Eisnberg et al., 1976). There are two isoforms of glutamate dehydrogenase one uses NAD and the other NADP. The NAD specific GLDH was studied in the present work. The activity of GLDH has been presently held as a mitochondrial marker enzyme. This enzyme catalyses the reversible reaction of glutamate to 2-oxoglutarate and ammonia.

The liver glutamate dehydrogenase activity is dependent on intra mitochondrial ATP/ADP ratio, and its activity increases with decreasing ATP/ADP ratios (Lenartowicz, 1979). Leucine and ammonium ion, also act as activators of glutamate dehydrogenase activity (Shepartz, 1973). It has been reported that a high protein diet and glucocorticoids increase the activity of this enzyme in liver (Shepartz, 1973).
The activity of NAD linked glutamate dehydrogenase (GLDH) was found to increase in diabetic kidney. During diabetes, an increase in NADH/NAD ratio would favour the oxidative deamination of glutamate together with increased pyruvate oxidation, may be increasing the GLDH activity in kidney. Furthermore, ammonium ions and ADP level also increases in diabetes, which may also contribute to the increase in GLDH activity. Liver GLDH also showed an increase in diabetes. Being an integral enzyme of the amino acid metabolism, the activity of GLDH should show an increase with increase in amino acid metabolism.

3. EFFECT OF ANTIDIABETIC AGENTS ON ENZYME ACTIVITIES

Insulin, vanadate and *Memordica charantia* extract were used as antidiabetic agents and their effects were studied on the activities of AlaAT, AspAT, arginase and GLDH. Vanadate has been shown as an insulin mimetic agent and its effect has been seen earlier as an antidiabetic agent (Heylinger *et al.*, 1985). In recent years, the effect of *Memordica charantia* on enzyme activities in diabetes have been studied by a number of investigators (Shibib *et al.*, 1993; Mukherjee *et al.*, 1992 and Khan *et al.*, 1992). The effects of these antidiabetic agents on selected enzymes of amino acid metabolism are discussed both in cytosolic and mitochondrial fractions of liver and kidney in the present work.

A. Effects of Insulin

The biological action of insulin ranges from acute metabolic changes (stimulation of glucose and amino acid metabolism) to chronic alterations like the induction of enzyme or stimulation of DNA synthesis and cell growth (Granner and Piklis, 1990 and O'Brien and Granner, 1991). The effects of insulin are mediated, through a cascade mechanism which involves phosphorylation and
dephosphorylation of various proteins like IRS-1 & IRS-2 (Myers et al., 1995) by activated insulin receptors. Many trans-acting factors also get activated by the phosphorylation-dephosphorylation cycles and effect the expression of several genes at the DNA level (Takiguchi et al., 1995). The reversal effects of insulin on the activities of the enzymes of gluconeogenesis and glycolysis have been studied in diabetic conditions (Granner et al., 1990 and Morel et al., 1986.). In the present work the changes in activities of the enzymes like AlaAT, AspAT and arginases in subcellular fractions of diabetic rat kidney and liver were studied.

Insulin treatment to diabetic animals reversed the enhanced level of cytosolic AlaAT in kidney (Table 5). The reversal effects of insulin observed were almost same on all the days after induction of diabetes and reversal was found to be almost to the normal values as shown by earlier workers (Kazmi et al., 1982). Insulin treatment favours increase in the Alanine-Glucose cycle as well as increase in glucose transport in kidney which may be restoring the AlaAT activity in insulin treated diabetic rats. (Felig, 1973). The reversal effects of insulin on cytosolic AspAT was almost the same as on cytosolic AlaAT. The activity of AspAT was reversed nearly to the control values, possibly due to the normalization of NADH/NAD ratio, 2-oxoglutarate, acetyl-CoA and fatty acid levels to almost control values. The mechanism of action of these two transaminases, may be the same in kidney and liver. The cytosolic arginase activity in the kidney is very low as compared to the mitochondrial one (Table 4).

The reversal effects of insulin were not observed on the enzymes from mitochondrial fraction, and probably higher doses of insulin would be required to normalize these alterations in diabetic kidney. Ammonium ions have a stimulatory effect on kidney arginase and it was found that the levels of ammonium ions in kidney do not significantly change in insulin treated diabetic animals. This probably
is the reason that insulin could not restore the enhanced level of arginase in diabetic animals.

B. Effects of Vanadate

Vanadate is a trace metal shown to have insulin mimetic properties. Heylinger et al. (1985) found that vanadate treatment may reverse the hyperglycemic condition in diabetes. This report raised the hope that vanadate could be developed as an antidiabetic agent. Since then many groups are working on various aspects of mechanism of action of vanadate under *in vitro* and *in vivo* conditions. The exact mechanism of action of vanadate is still not well understood.

It was reported that vanadate acts at the insulin receptor level leading to the autophosphorylation of the receptors (Brichard and Henquin, 1995). These autophosphorylated receptors showed the tyrosine kinase activity which is responsible for the phosphorylation of IRS-I and IRS-2 (insulin receptor substrate). IRS-I and IRS-2 play an important role in the cascade mechanism, (Myers et al., 1994) which contribute to the change in expression of various genes at the RNA and/or DNA level.

Earlier reports showed that vanadate works at the post receptor level (Green, 1986 and Elberg and Shechter, 1994). In diabetes, when insulin levels are very low, the phosphorylation of receptor is insufficient to respond to the higher glucose level in the blood using vanadate treatment, which inhibits the phosphatases (Brichard et al., 1993), all the receptors remain in active form (phosphorylated) for a longer time, allowing the continuation of insulin action (Bollen et al., 1990). Tamura et al., 1983 showed that vanadate does not have its role at the receptor level in signal transduction pathway rather it facilitates the insulin action. Many reports suggested various actions of vanadate, like its effect at MAP Kinases and activation of transacting factors (D'Onofrio et al., 1994 and Elberg and Shechter, 1994). There are
many missing inter-links which need to be further researched into to understand the mechanism of action of insulin and of vanadate.

The effects of vanadate on the enzymes of protein catabolism \( \text{AlaAT} \) and GLDH and Urea cycle enzyme arginase were studied in both the cytosolic and mitochondrial fractions of kidney and liver. It was found that the treatment of vanadate could reverse the activity of these enzymes probably by regulating the glucose entry into the cell and reversing the rate of gluconeogenesis nearly to the normal values in both the organs. It can be concluded from the present study that vanadate could restore the increased activity of all the enzymes studied in the cytosolic fractions only. The activities of the mitochondrial enzymes could not be reversed to the normal values by vanadate administration to diabetic animals. The effects of vanadate as compared to insulin were more wide. The present study raised certain queries like whether the mitochondrial enzyme changes in diabetic kidney were permanent or not since they could not be restored to normal levels by any of the antidiabetic agents. It also raised doubts about the efficacy of insulin treatment as a panacea for diabetes. It was shown in the present study that vanadate is not only an insulin mimetic agent but it also has wider reversal effects than insulin.

C. Effect of Vanadate together with Lithium

Earlier reports showed that lithium can partially (~50%) restore the elevated blood glucose level in diabetic rats (Rosetti, 1989). A few contradictory results have also been reported. In the present study it was shown that lithium with vanadate reverses the enzyme activity to the same extent as that with vanadate and insulin separately. Since the toxicity of vanadate is known at the higher concentration (>0.6 mg/ml), it is important to reduce the dosage concentration with the same potency so that vanadate can effectively work as an antidiabetic agent. In this direction, lithium with vanadate treatment has shown encouraging results. 0.05 mg/ml of
sodium orthovanadate with lithium proved to be as effective as 0.6 mg/ml sodium orthovanadate on the enzymes like GSH, GSSG AND CAT as shown earlier in our laboratory (Srivastava et al., 1993). The effects of the combined administration of vanadate and lithium were studied on all the enzymes in cytosolic and mitochondrial fractions of kidney and the results were almost the same as with vanadate alone. The effects of vanadate with lithium were also studied in liver which also showed the same results with vanadate alone as those in kidney (Tables 8 and 13). It may be due to the complex formation between lithium and sodium orthovanadate ions. Since lithium and sodium belong to the same group (IA) of elements, it may be the reason of similar action of lithium as that of sodium orthovanadate. A more detailed study however is required before using this as an effective complex for diabetic treatment.

D. Effects of Memordica charantia Extract

Extracts of a variety of plant materials including Memordica charantia (Karela), showing hypoglycemic effects have been known for a very long time (Pabrai and Sehra, 1962; Shibib et al., 1993 and Pugazhenthi and Murthy, 1996). In most cases the preparations showed no toxicity and karela is a commonly used vegetable in the Indian diet. Administration of Memordica charantia extracts showed that it can be used, as mentioned in earlier papers (Hosain et al., 1992 and Shibib et al., 1993), as an oral antidiabetic drug. The Memordica charantia extracts have been used in many studies showing an insulin mimetic effect (Leatherdale et al., 1981). The effects of Memordica charantia extract have not been studied earlier on the enzymes of amino acid metabolism and Urea cycle. In the present study, effects of Memordica charantia on the activities of AlaAT, AspAT, GLDH and Arginase were studied in kidneys of the three week old diabetic rats.
Memordica charantia extract was prepared in aqueous medium as described in methods section (pp. 38) and orally fed to the diabetic and control animals. The effect of *Memordica charantia* on the activity of cytosolic forms of AlaAT, AspAT and arginase in kidney of the treated animals were studied. It was found that the reversal effects of *Memordica charantia* extract on these enzyme activities were almost the same as of the insulin and vanadate treated animals (Table 8). The underlying mechanism of action of *Memordica charantia* is not well understood though preliminary studies have established its antidiabetic nature including hypoglycemic effects (Ali et al., 1993). The hypoglycemic effects of orally administered extracts of *Memordica charantia* showed that it lowers the glycemic response, without altering the insulin levels (Day et al., 1990). A number of compounds have been isolated from the seeds and fruits of *Memordica charantia* and their effects at the level of DNA and RNA synthesis has been reported earlier. (Zhu et al., 1990). The effect of *Memordica charantia* was also studied in control rats and no significant changes were seen on the activity of any of the four enzymes (results are not shown). The *Memordica charantia* treatment on the diabetic animals showed the decrease in the activity of the enzymes like glucose-6-phosphatase and fructose-1,6 bisphosphatase liver and RBC (Shibib et al., 1993) and reversing the altered metabolic state towards normal, which may be as its outcome and eliciting the restoration of amino acid metabolism in reversing the enzyme activities to the control values.

4. **In Vitro effects of orthovanadate, pervanadate and *Memordica charantia* extract**

The *in vitro* effects of varying concentration of orthovanadate, pervanadate and *Memordica charantia* extract were studied in liver and kidney homogenate of
control and diabetic animals. The effects were studied on the various enzyme activities as have been described in the methods section. Pervanadate solution was prepared by mixing sodium orthovanadate with $H_2O_2$ (100 mM) as described by Posner et al., 1994, and its effects were studied on the activity of AspAT and arginase. The activities of AspAT and arginase were decreased in the samples of control animals supplemented with vanadate (V3 & V4) as compared to the control (without vanadate). These results showed that vanadate in higher concentration inhibits the activity of AspAT and arginase. Pervanadate also showed an inhibitory effect, as of orthovanadate on the activity of AspAT and arginase. The extent of inhibition by one millimolar pervanadate was however the same as that of four millimolar orthovanadate, showing that pervanadate is 4-5 times more potent inhibitor of enzyme activity than orthovanadate (Table 18). The extract of *Memordica charantia* was prepared in the aqueous medium by using fresh vegetable and its *in vitro* effects were also studied on the activities of AspAT and arginase in the kidney homogenates of control rats. Administration of *Memordica charantia* extracts also showed an inhibitory effects on the activity of these enzymes (Table 20).

The effects of orthovanadate, pervanadate and *Memordica charantia* were also studied in the twenty one day diabetic animals. The results showed that the activities of AspAT and arginase increased significantly in diabetic conditions as described in the results section. The increased activity was reversed by the treatment with both the forms of vanadate and *Memordica charantia* extract *in vitro*. This restoration could be seen as inhibitory effects of these antidiabetic agents on the activity of AspAT and arginase. Earlier studies have reported that kidney is the organ which showed the highest an accumulation of vanadate (Nechay, 1984). The higher concentration of vanadate in kidney and serum might be responsible for
the restoration (inhibition) of the activities of the above enzymes. There might be similar element or/s like orthovanadate/pervanadate in Memordica charantia extract causing inhibitory effect on enzyme activities in in vitro experiments. It is important that the effects of pervanadate and Memordica charantia should be studied in detail to delineate the mechanism of action, before using it as an antidiabetic agents.

5. ENZYMES OF AMINO ACID METABOLISM IN RETICULOCYTES

Reticulocytes are the cells which come into the blood circulation from bone marrow and remain in the circulation for one or two days (William et al., 1983). These cells are metabolically very active and oxidize seven times more glucose than normal mature red cells (Rapport et al., 1974). The reticulocytes are not only important for the studies on development and ageing but they can also be used as a model to establish the relationship between ageing and diabetes. The effects of insulin and vanadate were studied on AlaAT, AspAT and arginase in reticulocytes of three weeks diabetic rats. Reticulocytes were isolated from control, diabetic, insulin treated diabetic and vanadate treated diabetic animals and fractionated on Percoll gradient into three groups namely young (Y), middle (M) and old (O) aged cells as described in methods section (Rapport et al., 1974).

The activity of AlaAT was shown to increase only in the young reticulocytes isolated from the twenty one day old diabetic animals (Dubiel, 1989). This increase of AlaAT activity can be explained in terms of higher amino acid degradation and the production of carbon skeletons for energy generation in diabetic condition. The activity of AlaAT was not significantly different in any of the fractions (Y, M or O) of reticulocytes in control rats, showing that probably AlaAT does not play any important role in the aging of reticulocytes (Table 21).
The activity of AspAT had significantly increased in all the fractions of reticulocytes in diabetic animals. AspAT is the main enzyme of the amino acid catabolism and generates carbon skeletons for the energy production in diabetic reticulocytes (Merrick, 1990). There is an increase in the amino acid catabolism in diabetes leading to the increase in the activity of AspAT. The activity of AspAT was shown to increase from young to older cells of control animals. It may be concluded that the increased activity in older cells was due to its participation in protein degradation. The higher rate of protein degradation in older cells can be concluded in terms of ageing (Merrick, 1990). The higher activity of AspAT in diabetic animals may also explain that diabetes is a hastened process of ageing.

Owing to the increase in amino acid metabolism in diabetes, the concentrations of ammonium ions also increase (Kaysan et al., 1973). The activity of arginase was found to increase in diabetes may be due to the higher substrate concentrations and stimulation by the excess concentration of ammonium ions. Kanungo et al. (1973) showed that the activity of arginase increases with age and after thyroidectomy in diabetes (Murthy et al., 1980; Sochor et al., 1981 and Salimuddin et al., 1996), therefore it can be concluded that the process of ageing gets accelerated in diabetes.

The administration of insulin to the diabetic animals was found to restore the enhanced activity of these enzymes. Insulin is known to stimulate the protein synthesis in reticulocytes by increasing the synthesis of mRNA (Brichard et al., 1993). Insulin facilitates glucose transport in the reticulocytes. The increased amount of glucose in reticulocytes and higher production of pyruvate by the glycolytic cycle would decrease the amino acid degradation (Hamada et al., 1991) leading to the reversal in the activity of AlaAT, AspAT and arginase in insulin treated diabetic conditions. The reversal effects were different in these fractions.
This may have been because of the difference in the metabolic activity of cells of these fractions.

The effect of vanadate was also studied on the activity of AlaAT, AspAT and arginase in reticulocytes of diabetic rat. Vanadate treatment could restore the activity of these enzymes in all the fractions almost to the control values. The levels of insulin were not changed in vanadate treated diabetic animals and this fact established that vanadate was working independent of insulin. Hence, vanadate and insulin may have different mechanism of action at cellular level to restore the activities of these enzymes. The number of receptors for insulin were different in Y, M, and O reticulocytes (Rapport et al., 1974 and Azam et al., 1990), that is why insulin may be affecting differently in different fractions whereas vanadate action is independent of insulin receptors or it is at post receptor level and works uniformly in all the fractions of reticulocytes. It is also evident from these results that the effects of vanadate are more widespread as compared to insulin.

6. ENZYME LINKED IMMUNOSORBANT ASSAY OF ARGINASE

The present results show that the increase in the activity of arginase was highest on the 21st day after induction of diabetes. The per cent change, compared to the control, was also maximum in this enzyme as compared to the other enzymes studied like GLDH and AlaAT in 21st day diabetic rats. Arginase activity was therefore taken for investigation in more details. The rat liver arginase has an 86 per cent homology with human liver arginase (Takiguchi et al., 1994). In the present study, the human liver arginase antibodies have been found to react well with rat liver arginase. ELISA was done by using whole homogenate of liver and kidney tissues. It is known (Porembksa, 1984) that the antibodies against liver arginase
poorly react with kidney arginase and this was also confirmed in the present study. The results show that the number of molecules of arginase increase significantly in diabetic conditions and these results are consistent with the increase in enzyme activities. The restoration of enzyme activities, following treatments with insulin and vanadate, was confirmed by ELISA and it was shown that the number of molecules of arginase decreased almost to the control values. The cytosolic form of kidney arginase could react with the antibodies against liver arginase but the mitochondrial form, which is a major component in kidney, did not react with these antibodies. The ELISA results suggest that the enzyme arginase is probably regulated at the transcriptional level and in order to investigate this possibility, arginase specific transcript levels were assayed by slot blot analysis.

7. CHANGES IN THE LEVELS OF ASPAT AND ARGINASE SPECIFIC mRNA

The increase in the specific transcripts show a strong correlation with the increase in the enzyme activity and the protein levels obtained by ELISA. The level of arginase specific mRNA is higher in the diabetic rats consistent with the increase in enzyme activity. The results suggest that the gene for arginase is regulated at the level of transcription and insulin and vanadate, which restore the enzyme activity to normal levels possibly exert their effect by modulating the transcription. The arginase gene has already been shown to be regulated at the transcriptional level by other hormones like glucocorticoids and glucagon (Takiguchi et al., 1995 and Aggerbeck et al., 1993). The present experiments show that vanadate also acts at the transcriptional level and possibly with a similar mechanism of regulation as that of glucocorticoids and glucagon. Similar results as that of arginase were also obtained with the enzyme AspAT. The regulation of cytosolic AspAT and
mitochondrial AspAT has been studied extensively by Feilleux-Duche et al. (1993). These authors demonstrated that insulin, glucagon and corticoids act at the transcriptional level. The upstream regulatory elements of genes of both the isoforms of AspAT have been identified and found that the insulin responsible elements are present only in 5'-upstream region of the cytoplasmic AspAT gene. The increase in the activity of AspAT in the diabetic rats is apparently due to the transcriptional activation of AspAT gene which is being reflected by an increase in AspAT specific transcripts (Figure 20), consistent with the increase in enzyme activities. The restoration of the transcripts level to control values by insulin and vanadate indicates that the vanadate may be acting in a similar pattern as that of insulin, probably regulating mRNA levels by the same mechanism as that of insulin. The slot blot assay does not differentiate between mAspAT and cAspAT mRNAs. Therefore, the varying effects of insulin and vanadate on the cytoslic and mitochondrial isoforms of the enzyme could not be determined. In order to discriminate between the two isoforms, gene specific probes have to be used. It may be concluded from this study that probably the mitochondrial forms of the enzymes are modified or altered in diabetes causing irreparable damage due to the lack of insulin responsive elements in the 5'-upstream regions of these genes.