DISCUSSION

GENERAL EFFECTS OF DIABETES AND VANADIUM

The anti-diabetic effect of vanadate is well established and its role as an insulin mimetic agent is being investigated in many laboratories in the world (Heylinger et al 1985a; Ramanadham et al 1989b; Saxena et al 1992a; Nechay et al 1986; Chasteen, 1990). In the present study it was observed that the blood glucose level in the vanadate treated diabetic rat was lowered to almost control level (Heylinger et al 1985a; Saxena et al 1992a). This reversal of hyperglycaemia by vanadate was much more efficient than insulin administration in diabetic rats.

This kind of improvement of diabetic condition may possibly be due to the reason that vanadate administration renders the tissues more susceptible to absorb glucose which is increased due to absence of insulin (Ramasarma, 1996). Decrease in the liver weight due to diabetes was also restored by vanadate treatment to near control values. This recovery may have been because of increased responsiveness of liver (an insulin dependent tissue) to vanadate as normoglyceamic agent.

No change in the brain regions weight was found in the diabetic rats which were treated with either insulin or vanadate. It has been shown that brain is a tissue which accumulates vanadate in least amount (Hamel and Duckworth, 1995). This might have effected the brain regions to not show any change in its weight. The kidney hypertrophy and hyperplasia was decreased when the diabetic animals were treated with vanadate. The reason of this reversal may be due to decrease in sorbitol content in kidney by vanadate as has been shown for the first
time in our laboratory (Saxena et al 1992b). The body weight loss due to diabetes could not be reversed by vanadate administration, this may have been because of mild toxicity in animals treated with vanadium (Domingo et al 1995), though the animals which were treated with insulin showed a small improvement in their body weight.

EFFECT OF DIABETES, INSULIN AND VANADIUM ON CHANGES IN THE ACTIVITY OF HEXOKINASE IN BRAIN REGIONS, LIVER AND KIDNEY

Brain regions

Hexokinase is the key regulatory enzyme of the glycolytic pathway and decides the fate of the glucose molecule entering the cell. Its activity regulates the flux of glucose through glycolytic and other related pathways. It catalyses the phosphorylation of hexoses and has a special preference for aldohexoses and has a low Km for glucose. High Km glucokinase which is specific for glucose is present in liver only, it has an important role in glucose utilization during diabetes and after high carbohydrate diet. Hexokinase isoenzymes from different tissues have been separated according to their mobility on starch gel (Katzen and Schimke, 1965; Domingo et al 1991). Out of the four isoenzymes in liver glucokinase and type II hexokinase were found to be hormone sensitive especially to insulin which was reported earlier (Katzen and Schimke, 1965; Katzen 1967; Gumma and McLean, 1972).

In the present study the activity of total hexokinase (type I & II) decreased in diabetes cerebral hemisphere, which was restored to normal value by treatment with vanadium.
Cerebellum and brain stem showed a small increase in the hexokinase activity and the pattern of change in 7 days and 21 days diabetes was the same. The vanadate treatment was found to be very effective as far as reversal of the hexokinase activity in the cerebellum was concerned. Since brain does not require insulin for uptake of glucose and the transport of glucose across the membrane is dependent on the concentration gradient of glucose, therefore brain may be taken as an insulin independent tissue. When the activity of hexokinase was compared from different regions of brain it was observed that there was a differential pattern of the changes in the activity of hexokinase in the different regions of the brain. The activity in the cerebral hemispheres showed a small decrease as compared to the control. This may be because of the change in the glucose concentration which increased in diabetes thereby effecting the normal functioning and regulation of hexokinase in brain. Vanadate treatment reversed the change in all the three regions of the brain.

The percentage of particulate or bound hexokinase is highest in brain as compared to other tissues, and this pattern was found to change diabetes (Katzen and Schimke, 1965; Sochor et al 1985; Baquer et al 1990). The change may be occurring because of entry of free fatty acid which are circulating in higher concentration in blood of a diabetic animal. This may cause the bound enzyme to be released in the soluble fraction. The bound hexokinase is not inhibited by the product of the reaction glucose-6-phosphate (Sochor et al 1985). This insensitivity of bound hexokinase to glucose-6-phosphate may be the reason for high glycolytic flux in brain. Presence of higher amount of type I hexokinase also shows that the tissues like brain and kidney are insensitive to insulin. Treatment
of vanadate restored the percentage of bound enzyme to nearer control values more effectively than the insulin in brain regions. The result once again establish the use of the vanadate as an important anti diabetic agent (see figs. 1 & 2). Since brain has predominantly type I hexokinase which is not hormone sensitive and therefore not effected in diabetes, the changes observed in the present experiment could be due to the difference in binding and release of hexokinase to the pellet fraction during the hormonal conditions measured like diabetes, insulin treatment and vanadate treatment of diabetic animals. These treatment may directly effect the enzyme activity.

Liver and kidney

Liver and kidney are severely effected during diabetes. Though the effect of diabetes is different in different tissues. Liver is an insulin dependent tissue whereas kidney is an insulin independent tissue. Kidney does not require insulin for the absorption of glucose, therefore glucose overutilization occurs in kidney under hyperglycaemic condition (Sochor et al 1979; Sochor et al 1985). The transport of glucose in kidney is not dependent on insulin and glucose is transported freely across the membrane. Since in diabetes the amount of glucose is increased several fold it is transported freely inside the kidney, thereby causing overutilization of glucose. This overutilization of glucose in diabetes is an important factor as it results in glycosylation of proteins, cataract formation, renal hypertrophy, sorbitol accumulation (Spiro, 1976; Brownlee and Cerami, 1981; Alberti and Press, 1982; Gonzalez et al 1983; Sochor et al 1985; Gonzalez et al 1986).
In present study liver showed a decrease in the (type I & II) in diabetes. This has been reported by (Katzen and Schimke, 1965; Sochor et al 1985). In animals could not restore the activity of enzyme i treatment of diabetic animals was found to be effecti of enzyme in liver (fig. 3 A).

In the kidney the activity of hexokinase (type pellet and soluble fractions in diabetes. This may increased glucose concentration in kidney as a concentration in blood (200-300 mg/dl). The transpor is increased due to higher concentration of glucose in in the substrate for the hexokinase activity. Vanadate t the activity to normal levels, which was more effective due to the lowering of blood glucose by vanadate, thei transport in the kidney (fig. 3 A).

In-vitro effect of vanadate on hexokinase

Whole homogenate was incubated with differe i.e. 5 mM and 20 mM. Different concentrations of s also added in the incubation medium. Incubation was at 37°C, after that the homogenate was centrifuged at Pellet was discarded and supernatant fraction was u enzymes.
In the incubated brain extracts it was observed that there was no significant change in the activity of hexokinase when the homogenate was treated with 20 mM glucose, equivalent to the blood glucose level in a diabetic condition, the change was not very significant even when the incubation was carried out in the presence of 2 mM and 8 mM vanadate. 4 mM vanadate addition however, showed an increase in the activity of hexokinase (fig 3 B).

The incubated extracts liver tissue showed a significant decrease in the activity of hexokinase when incubated with 5 & 20 mM glucose. Vanadate treatment completely restored the decreased activity of the total hexokinase. In kidney extracts however 4 mM vanadate was the most effective in reversing the enzyme to control levels (fig. 3 B). The results from in-vitro studies show that the effects of vanadate were not only restricted to the prereceptor level and that the vanadate may function as the enzyme regulator in-vitro also. Vanadium being a transition element has a variable oxidation state. This tendency of vanadium is very much evident when it enters the cell. Its oxidation state changes to +4 (Ramasarma and Crane, 1981; Ramasarma et al 1981; Ramasarma, 1996). This may have effect on the modulation of glycolytic flux as the redox state in the cells are an important factor in the regulation of all the enzymes (Greenbaum et al 1971; Newsholme and Start, 1973; Askar and Baquer, 1994).

Changes in the activity of pyruvate kinase with vanadium and insulin in brain regions, liver and kidney

Brain regions

It has been reported earlier from our laboratory that the activity of
pyruvate kinase increases in diabetes in brain regions due to the overutilization of glucose (Srivastava and Baquer, 1984b), this is because brain is an insulin independent tissue. In hypoglycaemic condition there was a decrease in the activity of pyruvate kinase in all the regions of the brain namely cerebral hemisphere, cerebellum and brain stem (Srivastava and Baquer, 1990). It was also reported by Srivastava and Baquer (1984) that pyruvate kinase showed a small decrease in activity in cerebral hemisphere of diabetic rat, as has been shown in the present study also.

In the present study the changes in the activity of another key enzyme of glycolysis namely pyruvate kinase was measured under diabetic condition. The activity of this enzyme was very high in brain compared to other peripheral tissues as it is known that brain has a very high rate of glycolysis (McIlwain and Bachelard, 1971). As reported earlier also from our laboratory (Srivastava and Baquer, 1984b). In the present series of experiments it was also found that the pyruvate kinase activity is decreased in cerebral hemisphere in diabetes. Whereas it was found to increase in the other regions namely cerebellum and brain stem (figs. 4 & 5). The increase in the activity of pyruvate kinase might have occurred due to the high concentration of glucose in the blood thereby increased amount of glucose must be entering the neural cells and increasing the activity of glycolytic enzyme as was for hexokinase also (fig 1 & 2). Vanadate treatment reversed the effect of diabetes in the brain regions. From the present results it seems that the role of vanadate is not only in maintaining a normoglycaemia alone as reported earlier (Heylinger et al 1985a) but also for the modulation of enzyme activity as has been shown in the present work using in-vitro studies (figs.
4 & 5). More significant changes in the pyruvate kinase activity in diabetes was observed in the cerebral hemisphere out of the three brain regions taken up for the study. The other two regions, although showing the same pattern of change showed changes with diabetes which were not so significant, one reason for this could be that the cerebral hemispheres are metabolically more active than the other two regions of the brain (McIlwain and Bachelard, 1971). A similar regional variation in the activity of pyruvate kinase was also seen as reported earlier (Srivastava and Baquer, 1984b; Srivastava and Baquer, 1990; Baquer et al 1990).

Liver and kidney

The activity of pyruvate kinase in liver and kidney is effected but differentially (Sochor et al 1985; Saxena et al 1992a). This difference in response of the liver and kidney is due the dependence of liver on insulin for the transport of glucose (Brownlee and Cerami, 1981; Sochor et al 1985). Pyruvate kinase showed a significant decrease in the activity in diabetic liver (Sochor et al 1985), whereas there was a significant increase in the activity of the enzyme in diabetic kidney (Sochor et al 1985; Saxena et al 1992a). Fructose induces L-type pyruvate kinase mRNA in the liver, kidney and small intestine of rats (Matsuda et al 1990). Glucose induces the genes coding for L type of pyruvate kinase in pancreatic beta cell line (INS-1) (Roche et al 1997).

Normalization of the activity of pyruvate kinase in liver and kidney has been reported earlier from our laboratory (Saxena et al 1992a). In the present study also the activity of pyruvate kinase decreased in liver in diabetes. In kidney
the activity of the enzyme was found to increase. Vanadate treatment of diabetic rats restored the activity of pyruvate kinase to the normal levels in both liver and kidney (fig. 6 A). There has been earlier report of other enzymes changes in diabetes to return to normal values by vanadate treatment (Saxena et al 1992a; Saxena et al 1992b; Saxena et al 1993, Khandelwal and Pugazenthi, 1995). Vanadate could be modulating the enzyme activity by altering the concentration of metabolite in the liver and kidney like, PEP, ADP, FDP and the energy state of the cells of liver and kidney as reviewed by Ramasarma (1996) as well as the redox state of the cell (Ramasarma et al 1981), like insulin administration changes the concentration of these metabolites in tissues of diabetic animal (Greenbaum et al 1971). This in-vivo results can be corroborated by the in-vitro studies in which it has been shown that the activity of the enzyme is modulated by incubation of homogenate using the tissue extracts.

**In-vitro effect of vanadate on the activity of pyruvate kinase**

Whole homogenate was incubated with different concentration of glucose i.e. 5 mM and 20 mM. Different concentrations of sodium ortho vanadate was also added in the incubation medium. Incubation was carried out for 30 minutes at 37°C, after that the homogenate was centrifuged at 10,000 rpm for 30 minutes. Pellet was discarded and supernatant fraction was used for the assay of all the enzymes.

Treatment of insulin independent tissues like brain and kidney with high concentration of glucose increased the activity of pyruvate kinase in the supernatant fraction. Whereas the activity decreased in insulin dependent tissue
namely liver with high glucose concentration. Incubation with different concentration of vanadate showed that 2 and 4 mM vanadate in the incubation medium was most effective in restoring the activity to normal values as was found in the in-vivo experiment under diabetic condition (fig. 6 A). At 8 mM vanadate concentration the enzyme activity decreased indicating the toxic effect of vanadate on the pyruvate kinase activity (fig. 6 B) (Domingo et al, 1995).

CHANGES IN THE ACTIVITY OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE WITH VANADIUM AND INSULIN IN BRAIN REGIONS, LIVER AND KIDNEY

Glucose-6-phosphate dehydrogenase (G6PDH) is the first enzyme of hexose monophosphate (HMP) shunt which catalyses the conversion of glucose-6-phosphate to 6 phosphogluconate. In the process it reduces NADP⁺ to NADPH. HMP shunt is important for the production of pentoses which are required in the synthesis of nucleic acid and secondly it produces NADPH which is the reduced coenzyme used as an energy donor in many biosynthetic pathways including fatty acid synthesis and other hydroxylation reactions. The oxidative segment of the pentose phosphate pathway comprising the NADP⁺-dependent glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase supply NADPH for the reductive processes involved in myelination and neurotransmission to various events connected with neuronal function (McIlwain and Bachelard, 1971; Baquer et al 1975; Baquer et al 1977; Hothersall et al 1982; Baquer et al 1988).

Activity of G6PDH in various tissues is increased by the injection of glucocorticoids (Bernadier et al 1976; Wurdeman et al 1978) and decreased by
glucagon (Garcia and Holten, 1975). The effect of glucocorticoids in increasing G6PDH activity may have been due to their role in amplifying the effect of insulin on the enzyme (Kelley and Kletzien, 1984). Deficiency of thyroid hormone renders the tissue insensitive to insulin (Baquer et al 1976). It was also reported that an increase in protein synthesis accounts for the insulin induced increase in hepatic G6PDH (Nakamura et al 1982). The activity of G6PDH is also regulated by thyroid hormone (Glock and McLean, 1955; Baquer et al 1976; Miksicek and Towle, 1982). Not enough data is available on the effect of hormone in brain except for few reports, where it had been shown that the effect of thyroid hormone in neonatal brain (Hostetler et al 1970). The work from our laboratory has also shown that the activity of G6PDH is altered in diabetes in brain and choroid plexus and return to normal level with insulin administration (Ansari et al 1993).

**Brain regions**

In the present study the activity of G6PDH was found to decrease in cerebral hemisphere of diabetic rats. This decrease may be because the activity of hexokinase is also decreased in this region of the brain of diabetic rats. As the substrate of G6PDH is glucose-6-phosphate, which is the product of hexokinase catalyzed reaction, the activity of G6PDH is dependent on the activity of hexokinase, which in the case of cerebral hemisphere of diabetic rat was decreased, (figs. 7 & 8). Since brain is highly compartmentalized tissue the availability of the substrate G6P is limited to a single compartment and may be it is unable to go out of the cell into the other compartments as phosphorylated
intermediate are known not to be transported across the cell membrane, thereby making the substrate unavailable for the reaction in the different compartment. Vanadate treatment reversed the effect of diabetes on G6PDH. In cerebellum of 21 days and brain stem of 7 days diabetic rats the activity of G6PDH increased (figs. 7 & 8). This behaviour of G6PDH was again in consonance with the activity of hexokinase, which was higher in diabetes in cerebellum of diabetic rats. Treatment with vanadate partially reversed the activity. Whereas, in cerebellum of 7 days and brain stem of 21 days of diabetic rats the activity decreased, which was restored by the treatment with vanadate (figs. 7 & 8).

Liver and Kidney

Glucose-6-phosphate dehydrogenase in liver has been shown to be regulated through complex interaction of many hormones (Wurdeman et al 1978). Insulin also restores the decreased activity of G6PDH in diabetic rat RBC (Nehal and Baquer, 1989). Mononuclear leucocytes from obese patients with Type II diabetes have reduced activity of G6PDH (Muggeo et al 1993).

In the present work it was found that the activity of G6PDH decreased in liver and increased in kidney of diabetic rats as reported earlier (Sochor et al 1985) (fig. 9 A). This behaviour of G6PDH can again be explained in the light of changes in hexokinase activity studied in present experiment which showed a decrease in liver and increase in kidney of diabetic rats. The decrease in the activity of hexokinase as well as G6PDH may be because liver is dependent on insulin for transport of glucose into hepatic cells. Insulin is deficient in diabetic rats making the entry of glucose difficult in hepatic cells. Leading to a decreased
level of glucose inside the hepatic cells thereby decreasing the activity of hexokinase and as a result of that the activity of G6PDH also decreased in the liver due to decreased availability of the substrate G6P. Since there is also change in energy state of the cell, the amount of ATP (the other substrate of hexokinase) is also in short supply, probably making it unavailable for the enzyme activity (Greenbaum et al 1971). Leading to decrease in concentration of product which may be lower than its Km value. Therefore, liver may also be classified as ‘insulin dependent tissue’. The activity was restored by vanadate treatment in liver which may be acting by altering substrate concentration (G6P) and energy state of the cell.

In kidney the activity of G6PDH increased in diabetic animals. Kidney is an insulin independent tissue, therefore the glucose transport in the kidney is not dependent on insulin concentration in the blood. The increase in the activity of G6PDH could be attributed to the increased availability of substrate G6P as a result of increased activity of hexokinase in diabetes. Vanadate treatment once again was able to partially reverse the effect of diabetes on kidney of the affected animals (fig. 9 A).

In-vitro effect of vanadate on the activity of glucose-6-phosphate dehydrogenase

Whole homogenate was incubated with different concentration of glucose i.e. 5 mM and 20 mM. Different concentrations of sodium ortho vanadate was also added in the incubation medium. Incubation was carried out for 30 minutes at 37°C, after that the homogenate was centrifuged at 10,000 rpm for 30 minutes. Pellet was discarded and supernatant fraction was used for the assay of all the
enzymes.

The activity of G6PDH did not change in brain of control animals when its homogenate was incubated with 5 mM glucose. There was a small increase in the activity of brain G6PDH when the concentration of glucose was increased to 20 mM. Presence of 4 mM vanadate in the incubation medium had the most reversal effect on increased activity of G6PDH in presence of 20 mM glucose, whereas 2 and 8 mM vanadate concentration were not as effective (fig. 9B).

In liver supernatant fraction the activity of G6PDH increased in presence of 5 and 20 mM glucose. This increase was contrary to the effect of diabetes on liver. The reason for the opposite effect in the in-vitro model may have been because of rupture of the cell membrane assembly during homogenization of the liver tissue, which causes the high glucose presence in the incubation medium to undergo reaction to form glucose-6-phosphate which is the substrate of G6PDH. Hence the activity of G6PDH increased with increasing concentration of glucose in the medium. Vanadate treatment reversed the activity to less than the untreated value (fig. 9B). The activity of G6PDH decreased in kidney with high glucose concentration though there was some reversal when vanadate was present in the incubation medium (fig. 9B), this decrease in the activity of G6PDH can be explained with the fact that percentage of bound G6PDH is high in kidney, the solublization of the enzyme in diabetes may render it more susceptible to inactivation in the in-vitro system. Therefore causing decreased level of enzyme as measured in in-vivo system.
CHANGES IN THE ACTIVITY OF SORBITOL DEHYDROGENASE WITH VANADIUM AND INSULIN IN BRAIN REGIONS, LIVER AND KIDNEY

Brain regions

Sorbitol dehydrogenase is the second enzyme of polyol pathway that converts sorbitol to fructose by a reversible reaction and requires NAD+ as its coenzyme, the direction of the reaction is determined by the substrate and the concentration of the cofactor present. The product fructose which is formed as a result of reduction of sorbitol can enter glycolytic pathway once again after phosphorylation. As there is requirement of NAD+ as a cofactor for the reaction this competes with glycolysis at the glyceraldehyde 3 phosphate dehydrogenase step for NAD+ (Gonzalez et al 1986; Taylor and Agius, 1988). An increase in the NADH/NAD+ ratio favours increase conversion of dihydroxyacetone phosphate to glycerol-3-phosphate (Gonzalez et al 1983). The schwann cell sheath synthesize unphosphorylated fructose with the help of this enzyme for various metabolic purposes (Brownlee and Cerami, 1981). During diabetes, unlike liver and muscle, the two enzymes of the sorbitol pathway activities and metabolite levels are found to be enhanced in insulin-independent tissues (Spiro, 1976; Alberti and Press, 1982). There is an activation of polyol pathway and accumulation of sorbitol in these tissues like kidney and lens (Gonzalez et al 1986; Raskin and Rosenstock, 1987).

In the present study it has been shown for the first time that there was an increase in the activity of SDH in cerebral hemisphere and cerebellum of diabetic rats. This increase may be attributed to a probable increase in concentration of sorbitol in specific regions of brain as a result of hyperglycaemia. There was a
small decrease in the activity of SDH in brain stem of diabetic rat. In the three regions studied it was observed that the change in the activity of sorbitol dehydrogenase was restored by vanadate administration to diabetic animals (fig. 10).

**Liver and Kidney**

The role of sorbitol dehydrogenase in insulin dependent tissue like liver is not clear (Bagnasco *et al* 1986; Bagnasco *et al* 1987). Changes in the activity of sorbitol pathway enzymes have been reported in NOD mice (Sochor *et al* 1991), where it was shown to increase. From our laboratory it has been shown earlier that the accumulation of sorbitol in the control animals RBC when it was incubated with high concentration of glucose and its reversal by vanadate (Baquer *et al* 1995). Sorbitol was found to be accumulated in localized manner in kidney, where it was found to be in high concentration in medulla as compared to cortex, and there was a two fold increase in sorbitol concentration in medulla in diabetes (Saxena *et al* 1992b). There was a small increase in the activity of sorbitol dehydrogenase in liver during diabetes (fig. 11A). This may have been because of increased accumulation of sorbitol in the cells due to increased activity of aldose reductase which has also been reported earlier in experimental diabetes (Saxena *et al* 1992b). Treatment of diabetic animals with insulin and vanadate restored the increase in the activity of sorbitol dehydrogenase to almost control levels (fig. 11 A).

In kidney the enzyme is mainly localized in the cortical region while its activity was reported to be very low in the medullary region (Chauncey *et al*
1988; Saxena et al 1992b). In the present result in kidney the activity of the enzyme in pellet fraction increased significantly though there was a small increase in the total activity. The increase in the activity of sorbitol dehydrogenase can be explained as an effect of increased accumulation of sorbitol in kidney cells. Vanadate and insulin treatment of diabetic animals restored the activity of sorbitol dehydrogenase, in different subcellular fractions of kidney to almost normal values (fig. 11 A).

**In-vitro effect of vanadate on the activity of sorbitol dehydrogenase**

Whole homogenate was incubated with different concentration of glucose i.e. 5 and 20 mM. Different concentrations of sodium ortho vanadate was also added in the incubation medium. Incubation was carried out for 30 minutes at 37°C, after that the homogenate was centrifuged at 10,000 rpm for 30 minutes. Pellet was discarded and supernatant fraction was used for the assay of all the enzymes.

A general decrease in the activity of sorbitol dehydrogenase activity was found when homogenate of whole brain from control animals was incubated with different concentrations of glucose in the medium. The activity could not be restored when 2 or 4 mM vanadate was supplemented in the medium, 8 mM vanadate in the incubation medium however, restored the activity to those obtained in the untreated values (fig. 11 B). In liver tissue also the activity of sorbitol dehydrogenase decreased in presence of 20 mM glucose in the incubation in in-vitro system. Which was restored when 4 mM vanadate was supplemented in the incubation medium (fig. 11 B). The decrease in the activity of brain and
liver sorbitol dehydrogenase *in-vitro* is similar to that obtained for the aldose reductase as shown in figure 13 B. As a result of this decrease the concentration of sorbitol in the incubation medium could have decreased causing a lowered sorbitol dehydrogenase activity as measured in *in-vitro* system. As shown in RBC from earlier results (Baquer et al 1995)

A decrease in the activity of sorbitol dehydrogenase was observed when the kidney homogenate of control animal was incubated with 20 mM glucose in the incubation medium. This decrease in the activity of sorbitol dehydrogenase was restored by the inclusion of 2 mM vanadate in the incubation medium. 4 mM concentration of vanadate in the incubation medium was also effective but 8 mM vanadate was unable to restore the activity of sorbitol dehydrogenase to the untreated levels (fig 11 B), this may have been due to changed oxidation reduction status (at 8 mM vanadate concentration) of the kidney homogenate causing the change in the pH of the incubation medium resulting in the inactivation of the enzyme activity

**CHANGES IN THE ACTIVITY OF ALDOSE REDUCTASE WITH VANADIUM AND INSULIN IN BRAIN REGIONS, LIVER AND KIDNEY**

**Brain regions**

Aldose reductase is an NADPH specific oxido-reductase and exhibits a broad substrate specificity, reducing a number of aldoses such as glucose, galactose and aldehydes to their corresponding polyols and alcohols (Varma and Kinoshita, 1974; Crabbe et al 1980; O'Brein and Schofield, 1980). Moonsammy and Stewart in 1967 demonstrated the existence of a second NADPH-specific
aldose reducing enzyme with properties other than aldose reductase in brain tissue. On the basis of previous reports, 3 major forms of aldo-keto reductase have been recognized in mammalian tissues: aldehyde reductase I, aldehyde reductase II (hexonate dehydrogenase) and aldose reductase III based on substrate and inhibitor specificity of individual enzymes and immunological characterizations (Das and Srivastava, 1985b; Das and Srivastava, 1985b; Ao et al 1991).

A few mammalian tissues like liver and kidney contain multiple forms of NADPH-linked aldo-keto reductases (Das and Srivastava, 1985b; Das and Srivastava, 1985a; Ao et al 1991), thus it may be possible that a multimolecular form of aldose reductase is present in insulin dependent tissues such as liver performing some other functions. Brain and kidney are the insulin-independent tissues where hexose entry is neither rate limiting for metabolism nor primarily modulated by insulin (Spiro, 1976; Alberti and Press, 1982; Sochor et al 1985). Since aldose reductase, the rate limiting enzyme of polyol pathway, has low substrate affinity to glucose, the activity of polyol pathway is very low at normal physiological glucose concentrations (Raskin and Rosenstock, 1987; Ao et al 1991).

Polyol pathway is a minor pathway of glucose metabolism when glucose is under normal levels. This can be understood by the fact that under normal conditions the percentage of glucose metabolizing through this pathway is less than five percent in lens (Gonzalez et al 1983; Gonzalez et al 1986; Taylor and Agius 1988). In diabetes this goes up to around four to five fold (Taylor and Agius, 1988). When there is a chronic hyperglycaemic condition this pathway is
activated, the high concentration of polyol which is synthesized through this pathway accumulates leading to cataract formation and other diabetic complications (Taylor and Agius, 1988).

The first enzyme of this pathway is aldose reductase. Aldose reductase is a broad specificity enzyme and has high Km for glucose. It reduces glucose an aldose sugar to sorbitol using NADPH as a coenzyme. Increased glucose concentration may causing an increase in the formation of NADPH from other reactions. Since NADPH is a substrate (cofactor) for the enzyme, this could also be one of the reason for increased activity of aldose reductase by increasing the availability and decreased utilization in other reactions of NADPH in different types of brain cells in-vivo. Since there is no dependence of insulin for the transport of glucose in different types of cell in brain like ganglia, astrocytes, glial cells and neuronal cells. The high concentration of sorbitol is implicated in various complications which results due to diabetes. Spontaneous glycosylation of proteins including haemoglobin and cataract formation, there are reports that aldose reductase activity is increased prior to the formation of cataract in diabetic rat lens (Gonzales et al 1983; Sochor et al 1985; Kojima, 1990).

It has been reported that aldose reductase activity decreases significantly in the lens of the diabetic rats treated with insulin (Nishigami, 1990). In the present study using brain regions it has been shown for the first time that the activity of aldose reductase is increased in diabetes. This was expected because brain is an insulin independent tissue. Hyperglycaemia may lead to increased concentration of glucose in brain which may lead to activation of aldose reductase, which has a high Km (low affinity) for glucose. Similar results were
also found in kidney which is another insulin dependent tissue. Administration of insulin and vanadate to the diabetic animals were able to partially reverse the effect of diabetes. Brain stem however did not respond in the same manner (fig. 12). The activity of aldose reductase was very low in brain as has been reported earlier (Saxena et al 1992a), as this enzyme responds to diabetic condition, the changes in its activity in diabetes and vanadate administration was followed. The changes were not very significant as can be seen in Table 18.

Liver and Kidney

The liver is dependent on insulin for glucose absorption and therefore deficiency of insulin leads to glucose starvation in hepatic cells which in turn may effect the activity of aldose reductase, glucose being its substrate. A small increase in the activity of aldose reductase in liver in diabetes was observed. As it is well documented (Brownlee and Cerami, 1981; Taylor and Agius, 1988) that in diabetes the rate of gluconeogenesis i.e. formation of glucose from precursors like amino acids (alanine) and lactate is increased almost 2-3 folds in both liver and kidney. The glucose for aldose reductase may be coming from these feeder pathway, thereby showing an increase in the activity of the enzyme in diabetic liver. Insulin and vanadate treatment reverses the effect of diabetes in liver (fig.13 A), which may be because of lowering of gluconeogenic pathway and hence less glucose is available in the liver cells.

In kidney also the activity of aldose reductase increased significantly to more than control value and since kidney is independent of insulin for the transport of glucose, its concentration increases in kidney cells in hyperglycaemia
added to it is the high rate of gluconeogenesis in diabetic kidney, therefore both factors contribute to high glucose content in the diabetic kidney. This leads to excess accumulation of glucose in kidney which may increase the activity of aldose reductase. Insulin and vanadate treatment controlled the increase in activity of aldose reductase as a result of diabetes by regulating the substrate (glucose) level (fig. 13 A).

**In-vitro effect of vanadate on the activity of aldose reductase**

Whole homogenate was incubated with different concentration of glucose i.e. 5 mM and 20 mM. Different concentrations of sodium ortho vanadate was also added in the incubation medium. Incubation was carried out for 30 minutes at 37°C, after that the homogenate was centrifuged at 10,000 rpm for 30 minutes. Pellet was discarded and supernatant fraction was used for the assay of all the enzymes.

Very low activity of aldose reductase was observed in control brain tissue and an *in-vitro* incubation of the homogenate to see the effect on the isolated enzyme seemed to further inactivate the enzyme. In liver there was a decrease in the activity when the homogenate was incubated with 5 and 20 mM glucose. The reason of the decrease in the activity of aldose reductase *in-vitro* is unclear, one of the possibility is the increased removal/utilization of glucose through glycolytic pathway (fig. 13 B). Aldose reductase activity remained unchanged when it was incubated with 5 mM glucose but drastically decreased when the concentration of glucose was increased to 20 mM in the incubation medium. The decrease in the activity remains unclear. When 2 and 4 mM vanadate was added in the
incubation medium the activity was partially restored. 8 mM vanadate was unable to restore the activity of the enzyme (fig 13 B).

_in-vitro_ experiments in liver and kidney enzymes also showed the same pattern as in the case of brain enzyme. The inactivation of the enzyme in the _in-vitro_ incubated system may be partially explained by the dilution of metabolites like ATP and other coenzymes, which were not added in the incubation medium are usually present in the _in-vivo_ system and this dilution may also inactivate the enzyme irreversibly which would not show the normal activity when the substrate and cofactors are added in excess or in higher concentration for the assay of the enzyme _in-vitro_ (Table 20).

**EFFECT OF SUBSTRATE CONCENTRATION ON THE ACTIVITY OF PYRUVATE KINASE, GLUCOSE-6-PHOSPHATE DEHYDROGENASE AND SORBITOL DEHYDROGENASE IN CONTROL AND DIABETIC ANIMALS**

The kinetic experiments were carried out in the supernatant fractions of brain, liver and kidney of control and diabetic animals. Supernatant fractions were isolated as described in Materials and Methods.

**Effect of substrate concentration, inhibitors and activators on pyruvate kinase activity**

Pyruvate kinase is one of the most important allosteric regulatory enzyme in glycolytic reactions and pyruvate is an important metabolite diverting the substrate to a pathway depending upon the requirement of the cell.

The product of the reaction, pyruvate is at the crucial position in metabolism and it feeds to various pathways. The main pathway of this flux is
tricarboxylic acid cycle apart from other routes of its metabolism.

Purified pyruvate kinase is a homotetramer and exhibits a broad specificity for the nucleotide substrate. It may utilize GDP, IDP and UDP apart from ADP which is the physiological substrate with different affinities. The enzyme shows a high specificity towards the other substrate, phosphoenolpyruvate (PEP) in liver (Seubert and Schoner, 1971; Harada et al 1978).

There are at least three isoforms of pyruvate kinase known to exist, in higher animals. These are designated as L-type, M1-type and M2-type of A-type (Wilkinson, 1970; Imamura and Tanaka, 1972; Harada et al 1978; Denton et al 1979). M1 form is present in brain and muscle whereas L form is present in liver and kidney. M type of pyruvate kinase is insensitive to activation by FDP and inhibition to alanine. L type of pyruvate kinase gets activated by FDP and is inhibited by alanine. Both L and M type of pyruvate kinase are inhibited by ATP (Srivastava and Baquer, 1984b; Engstrom L et al 1987). In liver, adipose tissue and kidney pyruvate kinase isoenzymes may occur in interconvertible forms with different catalytic properties (Bailey et al 1968). The interconversion is dependent on temperature and the concentration of the effector, fructose diphosphate (FDP).

Pyruvate kinase can be subjected to regulation under physiological conditions by a number of effectors like alanine, PEP, FDP, redox state and intracellular pH in order to prevent 'futile cycle', which may take up ATP as a result of simultaneous cycle of glycolysis and gluconeogenesis (Seubert and Schoner, 1971; Newsholme and Start, 1973). The intracellular increase in concentration of ATP and alanine are sufficient to cause a complete block in the activity of liver pyruvate kinase (Engstrom L et al 1987). Fructose diphosphate
effectively counteracts the negative control by ATP and alanine, and therefore, the activity of pyruvate kinase in liver is very sensitive to the changes in the level of this glycolytic intermediate (Seubert and Schoner, 1971; Denton et al 1979).

Brain

In the present series of experiments in brain supernatant fractions of control and diabetic rats there was a decrease in the Km of pyruvate kinase when the experiments were carried out in presence of 4 mM vanadate. Vmax however, in both the cases remained unchanged (fig. ). This behaviour showed that the affinity of PEP to pyruvate kinase increased in presence of vanadate. This is a type of positive modulation though the Vmax remained unchanged thereby the activity of the enzyme also remained unchanged. This type of positive modulation of brain pyruvate kinase must be of quite an interest in the future work. Modulation of the enzyme by vanadate could be occurring due to the conformational change in the subunit structure of pyruvate kinase, as is the case with K+. Potassium ion is essential for the activation of pyruvate kinase (Engstrom, 1987). Since K+ is already present in the reaction mix, vanadate may be acting at a site different from K+ site thereby further increasing the Km’, by modulation of the subunits of the enzyme. Although pyruvate kinase is activated by other alkali metals like Cs and Rb, K+ is known to be the physiological endogenous activator of the enzyme.
Liver

The effect of vanadate on liver pyruvate kinase was contrary to the effect on brain pyruvate kinase. In liver Km for PEP is increased in the presence of vanadate by two fold without much change in the Vmax. This type of competitive inhibition was shown by the enzyme present in liver of both control and diabetic animals. In general it has been reported earlier that the levels of glycolytic enzymes are decreased in diabetic condition, this may irreversibly inactivate the pyruvate kinase activity which could not be restored to normal values even when PEP was added in excess in reaction medium (Greenbaum et al 1971; Srivastava and Baquer, 1984; 1990). Therefore the measured activity of the enzyme in diabetic liver was also found to decrease (Table 9).

It may be postulated that vanadium effects the change in the kinetic profile of PK at the active site in liver and brain of rats.

Kidney

The enzyme activity in kidney supernatant fraction from the control rats also showed a positive modulation of PK by vanadate. But in the case of kidney there was no change in the Km with PEP to pyruvate kinase in presence and in absence of vanadate. There was a significant increase in the Vmax of PK in presence of vanadate. Therefore, it can be said that the vanadate presence increases the activity of pyruvate kinase without involvement of active site in the process. In diabetic rat kidney vanadate did not show significant effect on the activity of the enzyme.
Hence, it can be said that vanadate effects the activity of PK in a tissue specific manner.

**Effect of substrate concentration, inhibitors and activators on glucose-6-phosphate dehydrogenase activity in control and diabetic rats**

The behaviour of the enzyme glucose-6-phosphate dehydrogenase, the key regulatory enzyme in HMP shunt supplying NADPH and pentoses for biosynthetic reaction is influenced by the various hormonal and nutritional variations in the cell, enzyme quaternary structure and the changes in various metabolites including ATP, ADP, spermine and palmitoyl-CoA. (Glock and McLean, 1955; Eggleston and Krebs, 1974; Kanji et al 1976).

In brain adenosine triphosphate (ATP) at some specific concentration *in-vitro* has been known to inhibit glucose-6-phosphate dehydrogenase (Askar et al 1996). Magnesium ions show a stimulating effect in glycyglycine buffer which is not evident in dilute phosphate buffer. Glucose-6-phosphate dehydrogenase is very specific for NADP⁺ and gets inhibited by NADPH (Eggleston and Krebs, 1974; Baquer et al 1988; Askar et al 1996). Although glucose-6-phosphate dehydrogenase is very specific with respect to cofactor NADP⁺. Some earlier reports also suggests about 10-15% activity could be measured in the presence of NAD⁺ (Wilkinson, 1970). NADPH is the most powerful inhibitor of purified glucose-6-phosphate dehydrogenase and 6 phosphogluconate dehydrogenase from brain (Eggleston and Krebs, 1974; Askar et al 1996). Inhibition of glucose-6-phosphate dehydrogenase by NADPH has been reported earlier from several mammalian sources like rat liver, human erythrocytes and platelets (Kosow,
1974; Eggleston and Krebs, 1974; Holten et al. 1976; Thompson et al. 1976; Baquer et al. 1988; Yoshida and Lin, 1995). The activity of glucose-6-phosphate dehydrogenase is dependent on NADP+/NADPH ratio. The inhibition by NADPH has been found to reverse by either increasing the concentration of NADP⁺ or by the use of artificial electron acceptor like phenazine methosulphate (PMS) and methylene blue, which oxidizes the reduced coenzyme to NADP⁺. No endogenous physiological electron acceptor has been reported for this effect except oxidized glutathione (Eggleston and Krebs, 1974), which reverses the inhibition of the enzyme by NADPH by oxidizing it (Eggleston and Krebs, 1974; Holten et al. 1976; Baquer et al. 1988).

Glucose-6-phosphate dehydrogenase is the first enzyme of HMP shunt which is involved in the generation of NADPH through oxidative segment of the reaction which is utilized for fatty acid biosynthesis and other hydroxylation reactions apart from generation of pentose through the non-oxidative segment of the pathway. The kinetic study of G6PDH was carried out in presence and absence of 4 mM vanadate in the reaction mixture. The concentration of the substrate of G6P was varied in the reaction mixture.

**Brain**

Glucose-6-phosphate dehydrogenase was inhibited in a non-competitive fashion by vanadate in the brain supernatant fraction from the control animals. Though there was not much change in the Km of G6P with and without vanadate, there was a significant decrease however, in the Vmax values in presence of vanadate (fig. ). In diabetic brain supernatant fraction, vanadate
could be acting as competitive type of inhibitor of G6PDH, probably by modulating the active site of the enzyme altering (increasing) the \( \text{Km} \) value in the presence of vanadate. Using purified glucose-6-phosphate dehydrogenase from the rat brain it has been recently reported that ATP shows a non-competitive type of inhibition with respect to G6P (Askar et al 1996), this was based on two assumptions i) There are two different binding sites in the enzyme, the active site and the regulatory site which modifies the property of the active site; ii) both the sites are capable of binding ATP and D-glucose-6-phosphate (Askar et al 1996).

In the case of vanadate D-glucose-6-phosphate could be modulating the activity by binding at the active site or metal binding site showing homo/heterototropic type of allosteric modulation at a site away from active site.

Liver and kidney

The activity of liver glucose-6-phosphate dehydrogenase from control supernatant fraction showed an upregulation (increase) in presence of vanadate. A significant decrease in the \( \text{Km} \) value was observed in the presence of vanadate with no change in the \( \text{Vmax} \), the increase in the activity of glucose-6-phosphate dehydrogenase was also observed when the diabetic animals were given vanadate ad libitum as has been shown \textit{in-vivo} (Table 13). In diabetic rat liver supernatant fraction also, there was an activation which was evident with the decrease in the \( \text{Km} \) value of G6PDH in the presence of vanadate. This decrease in the \( \text{Km} \) value was similar to the change in the \( \text{Km} \) value of the control supernatant fraction. Again it can be suggested that vanadate may be acting by allosterically modulating the active site of the enzyme, thereby affecting the binding of
substrate and coenzyme.

In the kidney supernatant fraction there was not much change in the activity of glucose-6-phosphate dehydrogenase in control animals. A mixed type of inhibition of glucose-6-phosphate dehydrogenase was observed with 4 mM vanadate, in supernatant fraction of kidney from control animals. There was an increase in the Km as well as Vmax in presence of vanadate as compared to the values obtained in the absence of vanadate. It may be presumed that the action of vanadate at the cellular level may be multi-factorial depending upon the charge distribution, pH, concentration of other metabolites and various other regulatory factors like change in energy status and redox state of the cell in-vivo. Therefore it can be said that effect of vanadate is pleotropic and tissue specific. Earlier work using trace metals like manganese and iron had indirectly shown an activation of glucose-6-phosphate dehydrogenase and other enzymes from adipose tissues, adrenals, liver and kidney (Baquer et al 1982; Sochor et al 1982).

Effect of substrate concentration, inhibitors and activators on sorbitol dehydrogenase activity in control and diabetic rats

Sorbitol dehydrogenase is the second enzyme of polyol pathway that converts sorbitol to fructose by a reversible reaction. This enzyme helps in removing sorbitol which is accumulated as a result of reduction of glucose in hyperglycaemia due to increase in activity of aldose reductase, which has a low affinity towards glucose (Srivastava et al 1984; Taylor and Agius, 1988). This effect is more prominent in uncontrolled diabetes, especially in kidney and peripheral nerves, where most of the long term diabetic complications are seen
(Gonzalez et al. 1983; Gonzalez et al. 1986). It has a broad substrate specificity for many sugar alcohols (Gabbay, 1973). Conversion of sorbitol to fructose is coupled to reduction of NAD$^+$ to NADH and this competes with glycolysis at the glyceraldehyde 3-phosphate dehydrogenase step for NAD$^+$ (Gonzalez et al. 1986; Taylor and Agius, 1988). An increase in the NADH/NAD$^+$ ratio favours increased conversion of dihydroxy-acetone phosphate to glycerol-3-phosphate (Gonzalez et al. 1983).

The presence of this enzyme has been shown in kidney, liver, adrenal gland and brain (Gabbay, 1973; Leissintg and McGuinness, 1982; Gonzalez et al. 1986). Schwann cell sheath synthesizes free fructose with the help of this enzyme for various metabolic routes (Brownlee and Cerami, 1981). However, the role of this enzyme in insulin-dependent tissues like liver, adipose, muscle is not well elucidated (Bagnasco et al. 1986; Bagnasco et al. 1987). In kidney, the enzyme is mainly localized in the cortical region while its activity is very low in the medullary region (Chauncey et al. 1988; Saxena et al. 1992b). This causes several fold accumulation of sorbitol in medullary region, and the increase in the activity of sorbitol dehydrogenase in diabetes was restored to near normal levels with vanadate administration (Saxena et al. 1992b).

The kinetic study of the enzyme sorbitol dehydrogenase from the supernatant fraction was carried in presence and absence of vanadate. The concentration of substrate namely fructose was varied keeping all other factors constant.
Brain

The effect of vanadate addition on the activity of sorbitol dehydrogenase was measured and it was seen that vanadate caused a decrease in the Km value with a small change in Vmax of sorbitol dehydrogenase in the brain of both control and diabetic rats (fig.). A small increase in the Km' of fructose due to presence of vanadate in diabetes was observed, with no change in the Vmax value. The changes in the kinetic behaviour of the enzyme from brain cannot be given much significance as the measured enzyme activity was very low as has been reported earlier also (Saxena et al 1992b) and reiterated in the present work (table 15).

Liver

In the liver supernatant fraction of control and diabetic rats vanadium was found to act as a competitive inhibitor of the sorbitol dehydrogenase. There was a significant increase in Km' of fructose in the presence of vanadium with no change in the Vmax value (fig.). This again is different to the type of modulation shown by vanadate in the brain, which showed change in both Km as well as Vmax. The increase in the Km with vanadate in both control and diabetic liver supernatant fraction could be due to the in-vitro inhibition of the isolated soluble enzyme, which does not occur when vanadate is administered in-vivo to the diabetic animals. May be the oxidation state and concentration of vanadate is changing in an in-vitro system.
Kidney

The activity of sorbitol dehydrogenase showed a non-competitive type of inhibition in presence of vanadate. There was a significant decrease in the \( V_{\text{max}} \) in presence of vanadate as compared to that \( V_{\text{max}} \) in absence of vanadate. No significant change in the \( K_{\text{m}} \) value was observed using fructose as a substrate, both in the presence and absence of vanadate, although a decrease in \( V_{\text{max}} \) with vanadate was observed. Changes were seen in both the \( K_{\text{m}} \) and \( V_{\text{max}} \) values in the presence and absence of vanadate in diabetes, where both the \( K_{\text{m}} \) and \( V_{\text{max}} \) values increased. The results were slightly different from those obtained in the case of kidney from control animals (fig.), in which the vanadate administration seemed to be more effective in restoring the parameters towards the control value of the supernatant fraction of kidney.

The effect of vanadate on the kinetic study of enzymes of various carbohydrate metabolic pathway in several tissues was pleotropic. The action was tissue specific. The pattern of effect of vanadate on enzyme behaviour in some of the tissues is opposite/inverse to that of the results in \textit{in-vitro} studies. This change may have been because of the time period of the exposure of homogenate to the vanadate. Another reason could be that the measurements carried out in the subcellular fraction which were exposed to vanadate, at a much higher concentration when added in incubation medium \textit{in-vitro} than that present in \textit{in-vivo} tissue, when the animals are administered vanadate through drinking water.