Discussion
I. GENERAL

Studies of several investigators have established that vanadium compounds mimic many of the insulin effects. Recently, much interest has been focussed to elucidate and explore 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; Dubyak and Kleinzeller, 1980; Shechter and Karlish, 1980; Brichard et al, 1990). These reports formed the basis of further studies and it was suggested that vanadate may have a therapeutic role in regulating glucose metabolism in vivo. For the first time Heyliger et al (1985) demonstrated that oral administration of sodium orthovanadate to streptozotocin-induced 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; Ramanadham et al, 1989a, 1989b, 1990; Pugazhenthi and Khandelwal, 1990; McNeill et al, 1992).

Prior to starting the present series of experiments, preliminary studies were carried out to determine the safe, minimal effective therapeutic dose of vanadate which could be given to alloxan diabetic rats. For this sodium orthovanadate was administered in different doses of 0.4, 0.5, 0.6 and 0.8
mg/ml in the drinking water.

Vanadate administration at a dose of 0.4 mg/ml was not sufficient enough to reduce the blood glucose level to normal values even when rats were maintained for many days on this dose (Fig. 9). It was observed that the dose of 0.6 mg/ml was the minimal effective dose that brought about a stable normoglycemia within 4 days of treatment (Fig. 9 & 10). Moreover, sodium orthovanadate at a dose of 0.6 mg/ml has been reported to be non-toxic for liver and kidney functions (Gil et al, 1988; Bollen et al, 1990) though only 70% of the diabetic rats responded to it in the present experiments. Administration of vanadate at a dose of 0.8 mg/ml was also effective in causing normoglycemia (Fig. 9), however, the mortality rate of rats was found to be high which may be due to dehydration as animals did not like the bitter taste of vanadate added in high concentration in the water and thus could not drink this water with satiety. Hence, the dose of 0.6 mg/ml orthovanadate was carefully chosen for further experiments to study and characterize the insulin-mimetic/antidiabetic effects of vanadate. NaCl was included in the drinking water with vanadate to avoid the diarrhoea in vanadate treated rats that was observed when only vanadate was given in the drinking water, as has also been suggested in the previous reports (Heyliger et al, 1985; Meyerovitch et al, 1987).

Following orthovanadate administration (0.6 mg/ml) the elevated blood glucose level was restored to normal level and it continued as long as the
vanadate treatment was given to diabetic rats (Table 4; Fig. 9 & 10). The discontinuation of treatment resulted in recurrence of hyperglycemia within 48 hours in the vanadate treated diabetic rats. This recurring may be due to the progressive destruction of beta cells to a critical value before the initiation of vanadate treatment and thus the long term islet protective role of vanadate is lost (Pederson et al, 1989).

II. EFFECT OF VANADATE ON HEXOKINASE ISOZYMES, PYRUVATE KINASE AND MALIC ENZYME IN INSULIN-DEPENDENT AND -INDEPENDENT TISSUES

During diabetes, liver and kidney are severely but differently affected, as the liver is an insulin-dependent tissue whereas the kidney is an insulin-independent tissue which does not require insulin for glucose transport and which overutilizes glucose during persistent hyperglycemia (Sochor et al, 1979, 1985). Glucose overutilization is of critical importance in diabetes as exemplified by the glycosylation of proteins, cataract formation, renal hypertrophy etc. (Spiro, 1976; Alberti and Press, 1982; Brownlee and Cerami, 1981; Sochor et al, 1985).

The present study investigates the antidiabetic effects of vanadate on hexokinase isozymes, pyruvate kinase (glycolytic regulatory enzymes) and malic enzyme (a NADPH generating lipogenic enzyme) in the liver and kidney of alloxan diabetic rats. The study is of crucial importance in view of the different
alterations in insulin requirement and in glucose uptake, utilization and flux into different metabolic pathways in the two selected tissues and their responsiveness to vanadate even at a lowered insulin level.

In the present investigation, enzyme activities are expressed in total units/100 g body weight, a parameter relating biochemical activity to the functional requirement of the whole animal, which makes comparisons possible in the liver and kidney (Sochor et al, 1985; Saxena et al, 1992a). During diabetes, in contrast to the general pattern of tissue loss, the kidney increases in weight as observed here (Table 4) and by previous workers (Sochor et al, 1979, 1985). The liver requires insulin for glucose uptake, glucose phosphorylation and the entry of glucose-6-phosphate into different metabolic pathways. The kidney, on the other hand does not require insulin for the same functions. During diabetes, in the absence of insulin, the enzyme levels are altered differently in the liver and kidney. While in the liver the enzyme activities are depressed, in kidney the enzyme activities are enhanced (Sochor et al, 1979, 1985). The present work clearly demonstrates that vanadate, even in the absence of insulin, exerts antidiabetic effects on both insulin-dependent and -independent tissues, namely the liver and kidney, respectively.

The distribution and different isozymic forms of hexokinase were markedly elevated (Table 7, Fig. 13) as the total capacity of the kidney for phosphorylation seems to keep pace with kidney growth in diabetes. The increase
in total hexokinase activity reported here (Table 7, Fig. 13) is in agreement with the earlier observation of Sochor et al (1979). Vanadate therapy in the dose applied completely normalized the altered hexokinase isozyme profiles in the liver and kidney of diabetic rats (Table 6 & 7; Fig. 12 & 13). Normalization of hexokinase by vanadate is of critical importance as the enzyme regulates glucose entry in the tissue, forms glucose-6-phosphate and also regulates glucose entry into alternative metabolic routes. Upon activation during diabetes, these alternative metabolic routes may cause glycogen accumulation, thickening of renal basement membrane, cataract formation and other effects (Anderson and Stowring, 1973; Seyer-Hansen, 1983; Ledbetter et al, 1987; Taylor and Agius, 1988).

The restoration of pyruvate kinase by vanadate in the liver and kidney (Table 8, Fig. 14) suggests a probable role of vanadate in the channelling of pyruvate and glucose into the tricarboxylic acid cycle. Malic enzyme, which is an important supplier of NADPH for lipogenesis, remained unchanged in the kidney but was significantly decreased in the liver during diabetes (Table 8, Fig 14) as also shown by earlier workers (Gibson et al, 1972; Sochor et al, 1985). A marked increase in the hepatic level of malic enzyme was observed in vanadate-treated diabetic rats (Table 8, Fig. 14B), suggesting that vanadate therapy may also influence the hydrogen shuttle systems and transhydrogenase reactions of lipogenic pathways (Saxena et al, 1992a).
Despite the lowered insulin level in vanadate treated diabetic rats (Table 4), vanadate is able to act as an antidiabetic agent. This suggest that vanadate action may be independent of insulin action (Saxena et al, 1992b). In vanadate treated control rats, a significant decrease in plasma insulin level was observed (Table 4). Similar findings were also reported by earlier workers (Heyliger et al, 1985; Pugazhenthi and Khandelwal, 1990) who suggested that this decrease may be due to an increased responsiveness to insulin. The vanadium level of kidney is much higher than the liver and plasma levels (Table 5) that could be due to its greater accumulation in kidney during its administration.

The present work clearly shows that vanadate has potent antidiabetic effects and at the dose administered it restored the differently altered metabolic pathways even under conditions of depressed insulin secretion, both in insulin-dependent and - independent tissues.

III. POLYOL PATHWAY IN DIFFERENT TISSUES:

EFFECT OF DIABETES AND INSULIN TREATMENT

The polyol pathway is a minor pathway of glucose metabolism and consists of two successive dehydrogenases namely aldose reductase (AR) and sorbitol dehydrogenase (SDH) that catalyze the following reactions:
Under normal physiological conditions, there is very little glucose flux through this pathway in most tissues. However, in a diabetic hyperglycemic condition, the flux through the pathway increases dramatically in insulin-independent tissues (Spiro, 1976; Kinoshita et al, 1979; O’ Brien and Schofield, 1980).

In the present experiments, the presence of both the polyol pathway enzymes was found in all the four tissues studied namely liver, kidney, adrenals and brain (Table 10-13). High activity of these enzymes in the insulin-dependent tissue like liver is intriguing and their role in this tissue remains unclear. These enzymes in the liver and adrenals, did not change during persistent hyperglycemia in the diabetic state or with insulin treatment as shown in Table 10 and 11.

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

Aldehyde reductase I and II show no reaction with glucose at any concentration, in contrast to aldose reductase which reacts with glucose as a substrate (Petrash and Srivastava, 1982; Raskin and Rosenstock, 1987) and has a high Michaelis constant (Km, 70-150) for glucose; thus, when levels of glucose are elevated, as in diabetes with hyperglycemia, significant polyol formation can occur (Gabbay, 1973; Raskin and Rosenstock, 1987). Hence, the use of glucose in the present assays of aldose reductase was selected as the best means of distinguishing the activity of this enzyme, linked to sorbitol formation from glucose as also suggested in earlier report (Sochor et al., 1988). However, the presence of aldose reductase in liver does not seem to be an aldehyde reductase as neither of them, like aldose reductase, have been shown to possess significant activity with glucose (substrate used for aldose reductase assay in the present experiments).
The present data showed an enhancement in both aldose reductase and sorbitol dehydrogenase levels in diabetic rat brain but the increase was less significant than that seen in the diabetic kidney (Table 12 & 13). 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 (high Km), the activity of polyol pathway is very low at normal physiological glucose concentrations (Raskin and Rosenstock, 1987; Ao et al, 1991). However, when there are elevated glucose levels, as in diabetes mellitus, the concentration dependent increased glucose entry into these tissues activates the polyol pathway enzymes. This increased aldose reductase activity is of critical importance because it has been implicated in the progression of diabetic complications such as cerebral oedema and nephropathy in brain and kidney respectively (Gabbay, 1973; O’ Brien and Schofield, 1980).

In the present work, treatment of diabetic rats with insulin (4IU/day, i.p.) was observed to restore the elevated aldose reductase and sorbitol dehydrogenase activities in kidney and also in brain to some extent (Table 12 & 13). Though, on insulin-independent tissues like kidney and brain, insulin does not seem to have any direct effect, however, an indirect effect of insulin on these tissues can be produced by causing increased glucose entry into the insulin target tissues and
thereby reducing the elevated blood glucose levels during diabetes. Following this, entry of glucose in the insulin-insensitive tissues would also be decreased that would prevent the activation of polyol pathway enzymes in diabetic state.

The increase in kidney weight observed in the diabetic rats was also found to be partially prevented following the insulin treatment (Table 9).

These experiments formed the basis of the idea that vanadate, like insulin, may also have a therapeutic role and could cause improvement in the polyol metabolism and renal hypertrophy in the diabetic rats.

IV. POLYOL PATHWAY IN WHOLE KIDNEY: EFFECT OF VANADATE AND INSULIN

While previous studies showed the effects of vanadate on liver, adipose tissues and skeletal muscles (Tolman et al, 1979; Gil et al, 1988; Shechter, 1990; Strout et al, 1990), no such study had been conducted to elucidate the effect of vanadate in alloxan diabetic kidney. The present investigation examined and compared the effects of vanadate and insulin on the altered polyol pathway enzymes and metabolites in the whole kidney. Diabetes resulted in highly enhanced polyol pathway activity in kidney as observed by an increased levels of aldose reductase and sorbitol dehydrogenase (Table 15, Fig 15). The levels of metabolites, sorbitol and glucose, were also significantly elevated in the diabetic kidney when compared with their control values (Table 15, Fig. 16). These
results are in accord with the previous reports (Hutton et al, 1975; Sochor et al, 1985; Steer et al, 1985).

Present investigation reveals that vanadate therapy proved to be very effective and completely normalized the increased aldose reductase, sorbitol dehydrogenase and metabolite levels namely sorbitol and glucose in the kidney of diabetic rats (Table 15; Fig 15 & 16). This reversal effect of vanadate seems to be contributed primarily because of its glucose lowering capacity leading to stable normoglycemia (Saxena et al, 1993b). The normoglycemia once achieved by the vanadate treated diabetic rats would also normalize the enhanced glucose flux and its concentration in kidney that in turn could prevent the activation of aldose reductase and accumulation of sorbitol. Earlier reports have demonstrated that vanadate has the ability to restore the expression of a glucose transporter gene, thereby regulating the altered glucose homeostasis (Mountjoy and Flier, 1990; Strout et al, 1990).

Insulin treatment to diabetic rats caused a significant restoration of enhanced enzyme activities and metabolite levels (Table 15; Fig 15 & 16). As insulin injections to diabetic animals could not lead to stable normoglycemia most of the time, thus due to the variations in the blood glucose level (Fig. 11), a steady control of glucose flux in metabolic pathways cannot be expected. This could explain the inability of insulin therapy to completely reverse the polyol pathway activated by increased glucose concentrations in diabetes.
Oral administration of insulin is ineffective and insulin injections, due to large variability in insulin absorption among diabetics, lead to frequent variations in blood glucose levels causing various cardiovascular diseases and shortening the life expectancy of diabetic patients (Meyerovitch et al, 1987). Thus, availability of orally administered potent insulin-mimetic agents like vanadate could be of immense importance for better therapeutic interventions in the treatment of diabetes.

V. EFFECT OF VANADATE ADMINISTRATION ON POLYOL PATHWAY IN KIDNEY CORTEX AND MEDULLA OF DIABETIC RATS

Earlier reports indicated that aldose reductase and sorbitol dehydrogenase are not uniformly distributed in the two regions of kidney, the cortical and medullary regions (Bagnasco et al, 1986, 1987; Oates and Goddu, 1987; Chauncey et al, 1988) and this aspect of unequal distribution has been implicated in the diabetic renal injury (Chauncey et al, 1988; Bagnasco et al, 1991). In the present experiments the polyol pathway was, therefore, studied separately in the cortex and medulla of control and diabetic kidney together with the responsiveness of these two kidney regions for the in vivo effects of orthovanadate.

In the control rats, a higher activity of aldose reductase and a very low
activity of sorbitol dehydrogenase was found in the medullary region than in the cortical region (Table 16), thus favouring the net formation of sorbitol in the renal medulla. This is substantiated by the localization of sorbitol in several folds higher concentration in the medulla than in the cortex of control rats as shown in Table 17. The presence of high sorbitol dehydrogenase activity in the renal cortex of control rats (~58 fold higher than in medulla) may account for a very low level of sorbitol content as it may get metabolized further to fructose.

In the diabetic renal medulla, an activated aldose reductase (as seen by its 41% increased activity) due to several folds elevated tissue glucose level with no change in sorbitol dehydrogenase, could be a causative factor for a further ~2 fold increase in sorbitol level (Table 16 & 17; Fig. 17-19). On the contrary, in the kidney cortex, during diabetes, there was no elevation in sorbitol level because the increase in aldose reductase was concomitant with an increased sorbitol dehydrogenase activity that would prevent the enhancement in sorbitol formation by further metabolizing it. Thus in diabetes, during persistent hyperglycemia, the increased glucose flux in kidney results in accumulation of sorbitol in the medullary region. Similar finding was also reported earlier by Chauncey et al (1988).

It has been reported that sorbitol which is nonpermeable through most of the biological membranes, may act as one of the several osmolytes that maintain the osmotic balance of the cell (Bagnasco et al, 1986, 1987; Oates and Goddu,
Thus, renal sorbitol accumulation may lead to increase in osmotic pressure resulting in electrolyte imbalance, membrane stretching and cell death and thereby causing kidney dysfunction at a later stage of diabetes (Gabbay, 1973; Hutton et al, 1975; Sochor et al, 1988). Similar mechanisms may be responsible for the formation of cataract in diabetic lens (Kinoshita et al, 1979; Crabbe et al, 1980). Sorbitol accumulation has also been reported to be a causative factor for peripheral neuropathy and vascular complications in diabetes (Gabbay, 1973; Brownlee and Cerami, 1981).

In addition, there was a marked increase in kidney growth characterized by hypertrophy in contrast with general pattern of tissue loss in the diabetic state as observed in the present study (Table 4) and also in previous reports (Sochor et al, 1979, 1985; Steer et al, 1985). The activated polyol pathway, during diabetes, is linked with other pathways and have subsequent metabolic consequences in the following manner:

(1) Enhanced polyol metabolism, by reoxidizing the NADPH, activates the pentose phosphate pathway which supplies ribose-5-phosphate used for nucleotide and nucleic acid synthesis and NADPH for reductive biosynthesis (Fig. 3). These stimulated biosynthetic mechanisms finally cause an increase in kidney growth (Sochor et al, 1979, 1988; Gonzalez et al, 1986).

(2) Aldose reductase and sorbitol dehydrogenase require the cofactors
NADPH and NAD\(^+\), respectively. High polyol pathway activity may result in an altered reduced to oxidized pyridine nucleotide ratio (Taylor and Agius, 1988). Aldose reductase also competes with glutathione reductase for NADPH which is required for the conversion of oxidized to reduced glutathione (Fig. 3), a powerful antioxidant that protects cellular components from oxidative damage (Taylor and Agius, 1988). Thus, the enhanced polyol pathway in diabetes may contribute in the impairment of tissue antioxidative system.

**Effect of Vanadate**

Vanadate treatment to diabetic rats restored the raised enzyme activities of polyol pathway to almost control values (Table 16; Fig. 17). A near complete reversal was observed in the enhanced aldose reductase and sorbitol dehydrogenase activities in both cortical and medullary regions. The increased sorbitol level in medulla and elevated glucose content in the two regions of diabetic kidney were also effectively restored following vanadate treatment (Table 17; Fig. 18 & 19). As the polyol pathway enzymes are activated by high glucose, their reversal may primarily be due to the normalization of blood and kidney glucose levels by vanadate (Saxena et al, 1992b).

Another point of interest which emerged from this study is that the vanadate administration to diabetic rats successfully prevented the renal hypertrophy measured as the increase in weight of the kidney (Table 4). Vanadate
therapy completely reversed the increase in kidney growth to control values and a 74% reversal was found when kidney weight was expressed per 100 g body weight that could be due to a concomitant loss in body weight during diabetes (Table 4). This prevention in the increase in kidney growth may be due to the normalization of polyol pathway in diabetic rats following vanadate therapy leading to normalization of other related pathways like pentose phosphate pathway that cause increase in kidney growth (Saxena et al, 1992b).

On the other hand, insulin injections to diabetic rats only partially reversed the increase in kidney growth (Table 14) that may be due to the inability of insulin to cause stable normoglycemia (Fig. 11). From these studies, it can be concluded that oral administration of vanadate markedly improves the diabetic state and has a profound antidiabetic effect on polyol pathway accompanying renal hypertrophy.

VI. **IN VIVO STUDIES ON ERYTHROCYTE ALDOSE REDUCTASE AND SORBITOL LEVELS IN DIABETES: EFFECT OF VANADATE**

Measurement of sorbitol content in erythrocytes (an insulin-independent tissue) by Travis et al (1971), suggested the presence of aldose reductase in this tissue. Recently, erythrocyte aldose reductase was isolated and purified by Das and Srivastava (1985a) and Hamada et al (1991).
Similar to other insulin-independent tissues, in erythrocytes also the polyol metabolism gets activated during persistent hyperglycemia, resulting in an intracellular accumulation of sorbitol in diabetes as observed in the present study and in earlier reports (Srivastava et al, 1986; Robey et al, 1987). The sorbitol accumulation has been reported to be one of the factors responsible for decreased membrane deformability which has been implicated in the pathogenesis of diabetic microvascular complications (Crabbe et al, 1980; Robey et al, 1987).

In the present study, the erythrocyte aldose reductase activity isolated by DE-52 column chromatography (details given in "Materials and Methods" section) was found to be very low as compared to that in kidney and the activity was observed to be 65 % elevated from the control value in the diabetic erythrocytes (Table 18, Fig. 20). Concomitant with the enhanced aldose reductase activity, increase in sorbitol content was also found during diabetes that may possibly be due to the absence of sorbitol dehydrogenase activity which could not further metabolize sorbitol in erythrocytes.

Oral administration of vanadate was observed to effectively normalize the highly elevated aldose reductase and sorbitol levels in diabetic erythrocytes as was also seen in diabetic kidney (Table 18, Fig. 20). As also discussed earlier on page 109, the prophylactic efficacy of vanadate against increased aldose reductase and sorbitol levels seems mainly due to its ability to produce stable normoglycemia which would subsequently normalize the glucose entry and the polyol pathway in
erythrocytes of diabetic rats.

VII. IN VITRO STUDIES ON ERYTHROCYTE SORBITOL CONTENT

(A) EFFECT OF GLUCOSE CONCENTRATIONS

The incubation of erythrocytes with the increasing concentrations of glucose (100, 200, 300, 400 and 500 mg/dl final concentration) for 6 hours at 37°C caused the sorbitol content to increase almost linearly and a maximum of around 6 fold increase was observed due to the presence of 500 mg/dl glucose (Table 19; Fig. 21). In erythrocytes, entry of glucose is concentration dependent, thus the presence of high glucose in the medium could cause unregulated increased glucose flux in the erythrocytes leading to activation of aldose reductase and accumulation of sorbitol.

Hence, the amount of intracellular sorbitol may be a reflection of the surrounding glucose concentration and its flux through the polyol pathway. As the red blood cells are more readily available for sampling; red cell sorbitol, therefore, may be a useful diagnostic indicator of the severity of diabetes and of tissue sorbitol levels that participate in the pathogenesis of diabetes associated complications.
(B) EFFECT OF VANADIUM COMPOUNDS

The presence of sodium orthovanadate and vanadyl sulfate at 1 mM or above concentration in the incubation medium with high glucose concentration reversed the several fold elevated red cell sorbitol level to near control value (Table 20, Fig. 22). Vanadate seems to bring about this reduction in sorbitol content by regulating the increased glucose transport across the RBC membrane and thereby normalizing glucose flux into polyol pathway and preventing the sorbitol accumulation.

The present experiments indicated that vanadyl sulfate and sodium orthovanadate were more effective than sodium metavanadate and vanadium pentaoxide at similar concentrations, in preventing the sorbitol accumulation in erythrocytes (Table 20, Fig. 22).

Earlier, it has been reported that vanadate on entering the cell gets reduced into vanadyl ions by intracellularly present reductive compounds like GSH and form vanadyl-GSH complexes which have been suggested to be responsible for exerting the insulin-like effects of vanadate (Degani et al, 1981; Shechter and Karlish, 1980). However, the exact mechanism of action or the series of events are not yet known by which vanadate is able to exert its antidiabetic response. In contrast to vanadyl sulfate and sodium orthovanadate, sodium metavanadate and vanadium pentaoxide even when used at higher concentration of 4 mM could not completely reduce the increase in sorbitol formation (Table 20, Fig. 22). This
indicates that vanadyl sulfate and sodium orthovanadate may have a greater ability to readily get converted into reactive species (like vanadyl-GSH complexes) than the other two compounds, in order to generate a more potent and effective metabolic intracellular response.

(C) EFFECT OF INSULIN

The present study suggest that insulin does not seem to play any role in the regulation of glucose flux or glucose uptake inside the erythrocytes as indicated by an unaltered level of enhanced sorbitol formation with insulin, in the red blood cells incubated in 500 mg/dl glucose (Table 21, Fig. 23). The results of the present study add strength to the previous hypothesis of "glucose overutilization" by insulin-insensitive tissues in which insulin does not primarily modulate glucose uptake and its phosphorylation (Spiro, 1976; Sochor et al, 1985).

(D) EFFECT OF ALDOSE REDUCTASE INHIBITORS

Previous reports demonstrated that tetramethylene glutarate, hydantoin derivatives, some flavanoids and several other compounds possess aldose reductase inhibitory ability and could reduce aldose reductase implicated diabetic complications such as cataractogenesis and neuropathy (Varma and Kinoshita, 1976; Hu et al, 1983; Raskin and Rosenstock, 1987; Ao et al, 1991).

In the present set of experiments, it was observed that the presence of
aldose reductase inhibitors at an optimum/effective concentration (0.1 mM diphenylhydantoin, 0.4 mM quercetin and 1.2 mM tetramethylene glutaric acid) or above completely block the increase in sorbitol formation in red cells due to presence of high glucose in medium (Table 21, Fig. 23), by inhibiting the conversion of glucose to sorbitol. Though, tetramethylene glutaric acid is a specific aldose reductase inhibitor (Malone et al, 1980), in the present study it was found to be less effective than quercetin which is reported to be non-specific in nature (Bhatnagar et al, 1990) and a higher concentration of the former is required to reduce the elevated sorbitol content in erythrocytes.

To date, a number of aldose reductase inhibitors such as sorbinil, tolrestat and statil have been found to improve some diabetic complications in animal experiments and have been developed to clinical stages of evaluation (Raskin and Rosenstock, 1987; Sochor et al, 1988; Ao et al, 1991).

VIII. IN VITRO STUDIES ON ERYTHROCYTE GLUCOSE OXIDATION USING U14C-LABELLED GLUCOSE: EFFECTS OF VANADIUM COMPOUNDS AND INSULIN

Earlier results clearly indicate that vanadium compounds prevent sorbitol accumulation by regulating the increased glucose flux inside erythrocytes. Further to substantiate this, the present work was especially aimed to examine the possible effect of vanadium compounds in regulating the altered total glucose oxidation
in the erythrocytes due to high glucose environment. The total glucose oxidation was measured with low glucose (5 mM) and high glucose (20 mM) mimicking a diabetic condition. The total glucose oxidation was found to be about 3 fold enhanced due to the presence of high glucose in the medium (Table 22, Fig. 24). This confirms that the entry of glucose in the erythrocytes is dependent on the concentration of glucose in the surrounding environment.

The restoration of increased total glucose oxidation in the high glucose media by the presence of vanadium compounds (Table 22, Fig. 24) suggest that these compounds have the ability to effectively regulate the transport of glucose inside red blood cells. Insulin does not seem to have this ability in erythrocytes as the total glucose oxidation remained at an elevated level due to high glucose media and was not affected even at a higher concentration of insulin (Table 22, Fig 24).

These reversal actions of vanadium compounds on erythrocytes are of immense importance and suggest an upper hand of vanadate over insulin in controlling the metabolic disorders related with diabetes on the basis of following:

(i) Insulin leads to diminution in hyperglycemia by virtue of its ability to promote glucose uptake in insulin target tissues. There are now ample evidence to suggest that vanadate also possess this ability as its administration causes stable normoglycemia and in vitro it has been shown
to increase glucose uptake and its metabolism in hepatocytes, adipocytes and skeletal muscle (Tolman et al., 1979; Heyliger et al., 1985; Shechter, 1990).

However, the present in vitro experiments strongly indicate that insulin does not play any role in regulating glucose uptake and its metabolism in erythrocytes. Contrary to this, vanadium compounds effectively reverse the elevated glucose oxidation and markedly corrects the sorbitol accumulation inside red blood cells, possibly by preventing the transport of excess glucose across red cell membrane, when incubated in high glucose environment. This forms a direct evidence that vanadium compounds could regulate the altered glucose metabolism in tissues which are not targets of insulin action. The plausible explanation for this could be that vanadate may have the ability to regulate the expression of glucose transporter genes in erythrocytes. Recent studies have demonstrated that vanadate treatment restores the expression of genes for glucose transporters and for key enzymes of glucose metabolism in the diabetic rat liver (Brichard et al., 1993; Valera et al., 1993). Alternatively, the possibility of simultaneous inhibition of aldose reductase by vanadate cannot be ruled out.

(ii) Another point of interest which emerges from these studies is that vanadate seems to act at a site distal to insulin receptor, possibly by
utilizing an insulin-independent alternative pathway, as has also been suggested earlier (Green, 1986; Blondel et al., 1990; Strout et al., 1990), enabling it to perform its antidiabetic effect on insulin-insensitive tissues also.

From a pathophysiological angle, the idea of having an insulin-mimetic agent capable of utilizing an alternative pathway is very attractive. Vanadate has a future potential as an insulinomimetic drug for the treatment of diabetes since it can be taken orally and can maintain a stable longlasting normoglycemia. Contrary to this, insulin cannot be taken orally and its injection causes frequent variations in blood glucose level giving rise to several cardiovascular complications (Meyerovitch et al., 1987). Besides, the diabetics generally remain reluctant and unable to administer insulin injections themselves several times a day.

IX. EFFECT OF VANADATE ON ANTIOXIDANT STATUS OF DIABETIC RAT LIVER

(A) CHANGES IN ANTIOXIDANT STATUS DURING DIABETES

There is now ample evidence to suggest that, in certain pathological states like diabetes, the increased production and ineffective scavenging of toxic reactive oxygen species may play a crucial role in determining tissue injury (Wolff and Dean, 1987; Wohaeib and Godin, 1987; Oberley, 1988; Wolff et al., 1991).
Diabetes caused a depression in overall liver antioxidant status (Table 23-25, Fig. 25-27) making it more vulnerable to oxygen radical attack which may cause oxidative damage to membranes and alterations in subcellular organelle structural and functional integrity (Saxena et al, 1933a). The present data show that the levels of glutathione peroxidase, catalase, CuZn-superoxide dismutase, Mn-superoxide dismutase and GSH were significantly decreased in the diabetic state (Table 23-25, Fig. 25-27). This decrease in antioxidant enzymes may be due to inactivation caused by reactive oxygen species (Pigeolet and Remacle, 1991) and may also be due to the emaciation observed in diabetic rats (Wohaeib and Godin, 1987). Hydrogen peroxide may accumulate in diabetic tissues and in an earlier study it is shown to inhibit Cu-Zn superoxide dismutase (Hodgson and Fridovich, 1975). In diabetes, non-enzymatic glycation due to persistent hyperglycemia may also inactivate the antioxidant enzymes as shown previously with superoxide dismutase by Arai et al (1987). Insulin treatment to diabetic rats has been shown previously to normalize all the foregoing alterations in the antioxidant status (Wohaeib and Godin, 1987).

There may also be other possibilities as to how these enzyme activities are depressed in diabetic rat liver. Insulin might directly induce the synthesis of these antioxidant enzymes or activate a proenzyme. Likewise, the absence of insulin may in some way lead to the inhibition or increased degradation of antioxidant enzymes (Loven et al, 1982).
(B) EFFECT OF VANADATE

In view of the previous insulin-mimetic effects of vanadate, an attempt was made to study the efficacy of vanadate treatment in controlling the altered antioxidant status of the liver of diabetic rats.

Glutathione peroxidase has a key role in enzymatic defence system and acts on peroxides (H$_2$O$_2$, lipid or organic peroxides) to remove them. In the present investigation, it was observed that vanadate therapy could effectively normalize the glutathione peroxidase and Mn-superoxide dismutase level but it only partially restored the level of CuZn-superoxide dismutase (Table 23 & 24; Fig. 25 & 26). This suggests that the increased oxidative stress during diabetes (involving increased O$_2^-$ and H$_2$O$_2$ production) is controlled to some extent only. Paradoxically, it was found that instead of restoration, the decreased levels of catalase and GSH in diabetic rats declined further in vanadate-treated diabetic rats (Table 23 & 25; Fig. 27). This observation was not expected from an insulin-mimetic agent like vanadate. A similar pattern of decrease in catalase and GSH was seen in vanadate treated controls. This decrease in treated control and diabetic rats from their counterpart is of paramount importance since both catalase and GSH are crucial for the host defence and protect cellular components from oxidative damage. Degani et al (1981) showed by election paramagnetic resonance, studies that externally applied vanadate (V$^{4+}$, VO$_4$) is reduced intracellularly to vanadyl (V$^{4+}$, VO$_2$) and binds endogenously with GSH to form vanadyl-GSH
complexes which may produce its insulin-like effects. In fact, Degani et al. (1981) were able to detect two such complexes. Thus, the decrease observed in the level of GSH in treated control and diabetic rats may be due to its consumption in the formation of such vanadyl-GSH complexes (Saxena et al., 1993a). The downward trend in catalase level seen in treated control and diabetic rats as compared to their counterparts cannot be explained at this stage. Vanadate may produce some other form of free radical or factor leading to site-specific inactivation of catalase. It is also possible that vanadate administration in the dose applied has some other role in the biological system independent of its insulin-mimetic effects. This is likely as vanadium is a transition element with variable oxidation states (+3, +4, +5) and theoretically should influence the tissue redox state and, in turn, the antioxidant enzymes (Saxena et al., 1993a).

In conclusion, the present investigation clearly indicates that vanadate therapy is only partially effective in controlling the impaired antioxidative system of diabetic rats and is accompanied by some adverse effects as, unlike insulin, it fails to normalize catalase and GSH levels which are of crucial importance for the host defence. On the basis of the foregoing study, it is suggested that vanadate treatment if accompanied by some known antioxidant (e.g., flavanoids; Yuting et al., 1990) to improve the concomitant defence system disturbances, may produce better therapeutic results. More incisive studies are warranted to explore other insulin-like effects of vanadate that may also elucidate the mechanism of vanadate
action at the molecular and cellular level.

X. EFFECT OF PRETREATMENT OF VANADATE PRIOR TO DIABETES INDUCTION

The chemical agent alloxan has been used extensively as an experimental model for diabetes mellitus in animals, as it exhibits highly selective cytotoxicity for the pancreatic beta cells (Malaisse, 1982). Several investigators suggested that the diabetogenic action of alloxan is mediated by the generation of toxic oxygen free radicals (Malaisse, 1982; Oberley et al, 1988). The rapid uptake of alloxan and an exquisite sensitivity to peroxides due to poor protection against free radicals are unique features of pancreatic beta 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; Sakurai and Minura, 1980). Several studies have demonstrated that pretreatment of rats prior to diabetes induction with free radical scavengers; such as reduced glutathione, cysteine (Lararow, 1946), ethanol (Heikkila et al, 1974), dimethyl sulfoxide (Heikkila, 1977), superoxide dismutase (Grankvist et al, 1981b), alpha-tocopherol (Slonim et al, 1983) and gamma-hydroxy butyrate (Pierrefiche et al, 1991); prevents alloxan induced diabetes and its toxic manifestations namely elevated blood glucose level, thus showing a protective role against pancreatic beta cell killing.
It is now firmly established that vanadium compounds possess potent antidiabetic properties. Pederson et al (1989) have demonstrated that treatment of streptozotocin-diabetic rats with vanadyl sulfate yielded normalization of glucose tolerance and also caused protection of islets from destruction by streptozotocin. It was thus of interest to evaluate whether the pretreatment of vanadate to rats, like above mentioned agents could prevent, reduce or delay the diabetogenic action of alloxan. The present experiments were thus designed and the rats were pretreated with sodium orthovanadate in 3 different doses (0.2, 0.4 and 0.6 mg/ml) for 5 weeks prior to diabetes induction. Details of the treatment and grouping of animals are given in "Materials and Methods" section.

(A) CHANGES IN FLUID INTAKE

It was observed that inclusion of vanadate in the drinking water of control rats caused a decrease in their fluid intake that became even more prominent if the dose of vanadate is increased from 0.2 to 0.6 mg/ml (Fig. 28A). Thus, the control rats given 0.6 mg/ml vanadate drank lesser water than those given 0.2 mg/ml that may be due to the bitter taste of vanadate (Fig. 28A). The present results are consistent with earlier reports showing lesser fluid intake by vanadate treated control rats when compared to that of untreated control rats (Heyliger et al., 1985; Bollen et al, 1990).

Alloxan induced diabetes caused several folds increase in the fluid intake which is a characteristic manifestation of diabetes (Fig. 28A). In vanadate
pretreated diabetic rats, despite the pretreatment with vanadate, alloxan produced its toxic characteristic effect i.e. appearance of hyperglycemia following which the conditions of more fluid intake is expected to be produced as seen in diabetic rats due to the altered physiology. Instead, a decrease in fluid intake was observed in vanadate pretreated diabetic rats from their corresponding control values (Fig 28A). It may be possible that the pretreated diabetic rats have increased urge for fluid intake, but could not drink with satiety because of the inclusion of vanadate with its bitter taste and their inability to tolerate alloxan toxicity. This condition was marked by the signs of dehydration in later hours after alloxan injection.

(B) CHANGES IN BLOOD GLUCOSE LEVEL

It becomes evident from the above results that pretreatment of vanadate could not protect the animals against the diabetogenic action of alloxan. However, the increase in blood glucose level was observed to be at a slower rate during the first 24 hours in vanadate pretreated diabetic rats when compared to that of diabetic rats (Fig. 28B). This indicates that pretreatment with vanadate, though could not provide complete protection against induction of diabetes, however, offers some resistance against the main toxic manifestation of diabetes namely elevation of blood glucose levels.
(C) CHANGES IN THE LIPID PEROXIDE CONCENTRATION IN PANCREAS AND PLASMA

Induction of diabetes resulted in a rapid increase in the levels of lipid peroxides (TBARS; thiobarbituric acid reactive substances) that reached to several folds at 48 hours, both in pancreas and plasma. Results are shown in Table 26 and 27. Vanadate pretreatment to diabetic rats provided a similar pattern of slow response (as was observed against elevation of blood glucose level) in the enhancement of lipid peroxide concentrations only during the first 24 hours and the values reached to diabetic state level at 48 hours after alloxan injection.

The results showed that though administration of vanadate after induction of diabetes causes marked improvement in the diabetic state, however, contrary to this, administration of vanadate prior to induction of diabetes could not prevent the diabetogenic process and its toxic manifestations.