1. GENERAL EFFECTS OF DIABETES AND VANADIUM

Diabetes mellitus is a disease which is identified by hyperglycaemia and glycosuria. It is mainly of two types. a) Type I or insulin dependent diabetes mellitus (IDDM) and b) Type II or non-insulin dependent diabetes mellitus (NIDDM). Diabetes is considered to be hereditary as well as stress related disease. Study on diabetes is becoming more relevant today as the society is moving more towards the urbanization and sedentary life style. In a third world country like India, which has a large population obviously the number of diabetes patients too is very high. It has been estimated that 50 million people with diabetes are currently living in developing countries and the number may grow to 78 million over the current decade. Nearly two third of these will be in Asia and West-Asia of which one third in South-East Asia. India will account for half of the total diabetes patients in South East Asia.

Diabetes as of today is an incurable disease. Therefore the only way out is to minimize the complications arising out of diabetes and related disorders like hyperglycaemia. There are mainly two ways of controlling it and one of it is by drugs like sulphonyl ureas and other is by exogenous insulin injection, which is isolated from various sources. Both of these methods have some limitations. The drug which is given initially may not be very effective in the long run, which is necessary for the patients suffering from diabetes. Insulin on the other hand is quite effective without side-effects, though hypoglycaemia is common in cases of overdose of insulin in the patients taking insulin injections. Apart from this, it is difficult to carry insulin in remote areas, where the storage of this hormone is a problem as refrigeration is required.

Utility of vanadate as a replacement of insulin was first reported about two decades
ago. Later the studies were carried out mainly in experimental animals, there are some reports regarding studies on some human volunteers also. In these studies vanadate was shown to be a promising insulin-mimetic agent.

In the present work vanadate was studied as an insulin mimetic agent. Wistar rats were used as a model and alloxan was used as diabetes inducing agent.

This kind of improvement of the diabetic condition by vanadium 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, which has been reported earlier also. Decrease in the liver weight due to diabetes was also restored by vanadate treatment to control values. This recovery may have been because of increased responsiveness of liver to vanadate as normoglycaemic agent. No change in the brain regions weight was found in the diabetic rats which were treated with either insulin or vanadate. The kidney hypertrophy and hyperplasia were decreased when the diabetic animals were treated with vanadate as reported earlier. The reason of this reversal may be due to decrease in sorbitol content in kidney by vanadium.

The enzyme activities which were studied were those which are involved in the glucose metabolism. The enzymes studied were *hexokinase, pyruvate kinase, glucose 6 phosphate dehydrogenase, aldose reductase and sorbitol dehydrogenase* being regulatory enzymes of respective pathways. The tissues which were studied were cerebral hemisphere, cerebellum and brain stem from brain and liver and kidney.

In-vivo and in-vitro studies were carried out on the above mentioned tissues. Kinetic studies of pyruvate kinase, glucose 6 phosphate dehydrogenase and sorbitol dehydrogenase were also carried out. Various groups which were used for the in-vivo studies are as follows;

a) Control (C)
b) Diabetic (D)
c) Diabetic treated with insulin (D+I)
d) Diabetic treated with vanadate (D+V)

For in-vitro studies different concentrations of sodium orthovanadate were used in the incubation medium which were supplemented with high glucose concentration. For kinetic studies different concentrations of substrate were used in the presence and absence of vanadate (concentration of vanadate was kept constant).

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

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. 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, but this changes in diabetes.

In present study liver showed a decrease in the activity of total hexokinase (type I & II) in diabetes. In the present study, insulin treatment to diabetic animals could not restore the activity of enzyme in liver, whereas vanadate treatment of diabetic animals was found to be effective in reversing the activity of enzyme in liver. In the kidney the activity of hexokinase
(type I & II) increased in both pellet and soluble fractions in diabetes. This may have been due to the higher concentration of glucose in the blood which increases the glucose concentration in the kidney. Vanadate treatment partially restored the activity to normal levels, which was more effective than insulin. This may be due to the lowering of blood glucose by vanadate, thereby decreasing the glucose transport inside the kidney.

In-vitro effect of vanadate on hexokinase in different tissues

In brain it was observed that there was no significant change in the activity of hexokinase when the homogenate was treated with 20 mM glucose, 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 showed an increase in the activity of hexokinase.

The liver tissue homogenate showed a significant decrease in the activity of hexokinase when incubated with 5 & 20 mM glucose. Vanadate treatment completely restored the activity of the enzyme. In kidney 4 mM vanadate was most effective in reversing the enzyme to control levels. The results from in-vitro studies show that the effects of vanadium were not only restricted to the prereceptor level and that the vanadium may function as the enzyme regulator also.

3. CHANGES IN THE ACTIVITY OF PYRUVATE KINASE WITH VANADIUM AND INSULIN IN BRAIN REGIONS, LIVER AND KIDNEY

In the present study the change 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. In the following experiment it was also found that 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. Vanadate treatment reversed the effect of diabetes in the brain regions. From the present results it seems that the role of vanadium is not only for maintaining a normoglycaemia alone but also for the modulation of enzyme activity as has been shown in the present work using in-vitro studies.

More significant changes in the pyruvate kinase activity in diabetes was observed in the cerebral hemisphere out of the three brain regions studied. The other two regions, although showing the same pattern of change, showed changes with diabetes which were not significant, one reason for this could be that the cerebral hemispheres are metabolically more active than the other two regions of the brain.

Pyruvate kinase showed a significant decrease in the activity in diabetic liver, whereas there was a significant increase in the activity of the enzyme in diabetic kidney. 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 animals restored the activity of pyruvate kinase to the near normal levels in both liver and kidney. Vanadate could be modulating the enzyme activity by altering the concentration of metabolite in the liver and kidney.

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

Treatment of insulin independent tissues (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.
4. 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. 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 also decreased in this region of the brain of diabetic rats. 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. 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.

The activity of G6PDH decreased in liver and increased in the kidney of diabetic rats. 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. Insulin is deficient in diabetic rats making the entry of glucose difficult in liver hepatic cells. 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. 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 diabetic animals.

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

No change in the activity of G6PDH was observed 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 maximum reversal effect on increased activity of G6PDH. In liver the activity of G6PDH increased in presence of 5 and 20 mM glucose. Vanadate treatment reversed the activity to less than the untreated value. The activity of G6PDH decreased in kidney with 20 mM glucose concentration though there was some reversal with vanadate.

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

In the present study using brain regions it was found that the activity of aldose reductase is increased in diabetes. This was expected because brain is an insulin independent 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. The activity of aldose reductase was very low in brain.

A small increase in the activity of aldose reductase in liver in diabetes was observed. Insulin and vanadate treatment reverses the effect of diabetes in liver. In kidney also the activity of aldose reductase increased to more than control value in diabetes. Hyperglycaemia leads to excess accumulation of glucose in kidney which may increase the activity of aldose reductase. Insulin and vanadate treatment reversed the increased activity of aldose reductase.

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

Very low activity of aldose reductase was observed in 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. When 2 and 4 mM vanadate was present in the
incubation medium the activity was partially restored.

6. CHANGES IN THE ACTIVITY OF SORBITOL DEHYDROGENASE WITH VANADIUM AND INSULIN IN BRAIN REGIONS, LIVER AND KIDNEY

In the present study there was an increase in the activity of sorbitol dehydrogenase in cerebral hemisphere and cerebellum of diabetic rats which may be attributed to a probable increase in concentration of sorbitol in specific regions of brain as a result of hyperglycaemia. The change in the activity of SDH was restored by vanadate administration to diabetic animals.

There was a small increase in the activity of SDH in liver in diabetes. This may have been because of increased accumulation of sorbitol in the cells due to increased activity of aldose reductase. Treatment of diabetic animals with insulin and vanadate restored the activity of sorbitol dehydrogenase to almost control levels. In kidney the activity in pellet fraction increased drastically though there was a small increase in the total activity, which may be the effect of increased accumulation of sorbitol in kidney cells. Vanadate and insulin treatment of diabetic animals restored the activity of sorbitol dehydrogenase.
In-vitro effect of vanadate on the activity of sorbitol dehydrogenase in different tissues

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. 8 mM vanadate in the incubation medium however, restored the activity to those obtained in the untreated values. In liver tissue also the activity of sorbitol dehydrogenase decreased in presence of 20 mM glucose. Which was restored when 4 mM vanadate was supplemented in the incubation medium. A decrease in the activity of sorbitol dehydrogenase was observed when the kidney homogenate 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.

7.EFFECT OF SUBSTRATE CONCENTRATION ON THE ACTIVITY OF PYRUVATE KINASE, GLUCOSE 6 PHOSPHATE DEHYDROGENASE AND SORBITOL DEHYDROGENASE

The kinetic quantitation of the enzyme activities were carried out in the supernatant fraction of brain, liver and kidney of control and diabetic animals.

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

In brain supernatant fractions of control and diabetic rats there was a decrease in the Km of pyruvate kinase when the experiment was carried out in presence of 4 mM vanadate. Vmax however, in both the cases remained unchanged. This behaviour showed that the affinity of PEP to pyruvate kinase increased in presence of vanadate. 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. 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 (substrate) to PK in presence and in absence of vanadate. Hence, it can be said that vanadium effects the activity of PK in a tissue specific manner.

Effect of substrate concentration, inhibitors and activators on glucose 6 phosphate dehydrogenase activity

Glucose 6 phosphate dehydrogenase was inhibited in a non-competitive fashion by vanadate in the brain supernatant fraction from the control animals. In diabetic brain supernatant fraction, vanadate could be acting as a competitive type of inhibitor of G6PDH, probably by modulating the active site of the enzyme altering the Km value in presence of vanadate. The activity of liver glucose 6 phosphate dehydrogenase from control supernatant fraction showed an increase in activity in presence of vanadate. In diabetic rat liver supernatant fraction also, there was an activation which was evident with the decrease in the Km value of G6PDH in the presence of vanadate. 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.

Effect of substrate concentration, inhibitors and activators on sorbitol dehydrogenase activity

The effect of vanadate addition on the activity of sorbitol dehydrogenase was measured and it was seen that the addition of 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. In the liver supernatant fraction of control and diabetic rats vanadate was found to act as a
competitive inhibitor of the sorbitol dehydrogenase. There was a significant increase in $K_m'$ of fructose in the presence of vanadate with no change in the $V_{max}$ value. The increase in the $K_m$ with vanadate in both control and diabetic liver supernatant fraction could be due to the in-vitro inhibition of the isolated soluble enzyme. The activity of sorbitol dehydrogenase showed a non-competitive type of inhibition in presence of vanadate. Changes were observed in both the $K_m$ and $V_{max}$ values in the presence and absence of vanadate in diabetes.

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 were inverse to that of the results obtained in in-vitro studies. This change in the in-vivo and in-vitro results could be attributed to the time period of the exposure of homogenate to vanadium in in-vitro system and its differential distribution and concentration in in-vivo system. One of the other reason may be also the subcellular fraction which were exposed to vanadate, at a much higher concentration when added in incubation medium than that present in in-vivo tissue, as discussed above.
FUTURE PLAN OF WORK

1. Purification and characterization of various allosteric enzymes effected by hormones and the effect of vanadium on these purified enzymes in-vitro.

2. Correlation of structure of trace metals like vanadium with different functions like hormone action.

3. Involvement of second messenger like cAMP and IPG and elucidation of the cascade of physiological reaction from the molecular level to the cellular and functional level.

4. Comparison of the in-vivo and in-vitro characterization of the enzyme with more emphasis on quantitative characterization.

5. Effect of other trace metals like Mn, Cr, Se, Ni and Li on the hormonally treated animals.

6. Use of plant extracts for the reversal of diabetic complications, especially the quantitation of trace metals present in these extracts.
LIST OF PUBLICATIONS


PAPERS PRESENTED AT MEETINGS

1. Second Asia Pacific Society of Neurochemists (APSN) meeting at Hyderabad, India, Sept. 14-15, 1994


Changes in the Activity of NADH-Oxidase in Rat Tissues During Experimental Diabetes.

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SUMMARY

The effects of alloxan diabetes and its reversal with insulin treatment, on NADH-oxidase (E.C.1.6.99.3) was measured in the microsomal fractions of brain, liver and kidney at different time interval after diabetes induction. A significant increase was found in the enzyme activity in brain and kidney microsomes of the diabetic animals, whereas liver showed a decrease. The decrease in the NAD+/NADH ratio in the diabetes reported earlier could be due to the changes in the enzyme activity as well as other changes in the metabolite concentration.

INTRODUCTION

Morre et al. (1) reported the presence of NADH oxidase from liver plasma membrane and its stimulation by guanine nucleotide, but in a manner different from that shown by classic trimeric protein and G-proteins. The redox state of NAD+/NADH is greatly reduced in the hepatic cytoplasm of diabetic animals (2). The increased NADH concentration diverts the pathway of glucose metabolism (3). This leads to the channelization of glucose through polyol pathway and pentose phosphate pathway (4). Decrease in the redox state of the cell (NADH oxidase) favours gluconeogensis which is harmful for a condition like diabetes. The NADH oxidase is localized on the outside of the inner-membrane of rat liver mitochondria (5), this enzyme is specific for NADH and requires oxygen as its electron acceptor. Other NADH oxidase reported in mammalian system are in kidney and liver plasma membrane (6,1) and rat liver microsomes (7). These oxidases are probably involved in the control and regulation of the redox state of the cell. Measurement of the activity of NADH oxidase in
diabetes in the microsomal fraction, therefore, will be an important parameter in determining
the redox state in insulin independent (kidney and brain) and insulin dependent (liver) tissues
of the rat. These experiments have been attempted in this communications.

MATERIALS AND METHODS

Animals and Induction of Diabetes: A group of 40-50 Wistar male rats between 160-220 gms
and 3-4 months old were used for all the experiments. Rats were starved overnight and
received a subcutaneous injection of alloxan monohydrate 20mg/100gm body weight in
freshly prepared sodium acetate buffer (0.154 M, pH 4.5). A single injection of 2 units of
protamine zinc insulin in normal saline was given to each rat, for six days, this decreases the
mortality of the animals. Controls received same volume of normal saline (8). Rats were
divided into diabetic and insulin treated diabetic group as described elsewhere (9).

Preparation of Tissue Homogenate and Separation of Fractions: Animals were sacrificed by
cervical dislocation and the tissues namely, kidney, liver and brain were taken out
immediately weighed and homogenized in a Potter Elvehjem homogenizer fitted with teflon
plunger. All procedures were carried out at 0-4 °C. The microsomes were isolated according
to the method of Kurup et al. (10).

Assay of NADH Oxidase: The activity of NADH oxidase (E.C.1.6.99.3) was measured
essentially according of the method of Morre et al. (11) and Rau et al. (7). The reaction
mixture in a final volume of 1.0 ml contained the following in final concentration, 50 mM
phosphate buffer, 1 mM NADH, 2 μM rotenone. The reaction was carried out at 37 °C,
change in absorbence was followed at 340 nm on a DU-68 recording Beckman
spectrophotometer. One unit of NADH oxidase activity is defined as the oxidation of one
nmole of NADH/minute/mg protein.

Estimation of Protein & Glucose: Protein was measured in the microsomal fraction according
to the method of Lowry et al. (12). Glucose was measured spectrophotometrically in whole
blood by double enzyme reaction using hexokinase and glucose 6 phosphate dehydrogenase
as described by Bergmeyer et al (13).

RESULTS AND DISCUSSION

The effect of alloxan diabetes on NADH oxidase activity and its reversal by insulin
has been measured in microsomal fractions form liver, kidney and brain. Rotenone was
included in the measurements to inhibit any contamination from the mitochondrial NADH
oxidase. The severity of diabetes was checked by the measurement of glucose in the blood
as described earlier (8,9).
A general decrease in the activity of NADH oxidase was found in the liver microsomal fraction of diabetic animals when compared to the control (Table 1). Maximum decrease was observed on the third day after induction of diabetes. Insulin administration had a differential effect in the reversal of the enzyme at different time intervals. The activity of the enzyme showed an increase in the activity in the diabetic state in kidney and in brain. A partial reversal of activities was observed in both kidney and brain with insulin administration.

**Table 1**: Activity of NADH Oxidase in Tissues of the Diabetic Rats

<table>
<thead>
<tr>
<th>Time After Insulin Withdrawal (Days)</th>
<th>Liver (Units/gm)</th>
<th>Kidney (Units/gm)</th>
<th>Brain (Units/gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>D</td>
<td>D + I</td>
<td>D</td>
</tr>
<tr>
<td>3</td>
<td>121.9±15</td>
<td>119.7±12</td>
<td>106.7±14</td>
</tr>
<tr>
<td>7</td>
<td>173±21</td>
<td>184±35</td>
<td>127±8</td>
</tr>
<tr>
<td>14</td>
<td>145±12</td>
<td>170±23</td>
<td>108±7</td>
</tr>
<tr>
<td>21</td>
<td>168±24</td>
<td>134±15</td>
<td>127±15</td>
</tr>
<tr>
<td>Control</td>
<td>183±19</td>
<td>102±16</td>
<td>45±4</td>
</tr>
</tbody>
</table>

Each value is a mean ± SEM of four or more separate experiments. The enzyme assay was carried out in the presence of 2 μM rotenone. D = Diabetic, D + I = Diabetic treated with insulin. a = P < 0.001, b = P < 0.005, c = P < 0.01, d = P < 0.05. Enzyme unit is defined as the oxidation of one n mole of NADH/min/mg protein. All the other details are given in Materials and Methods.
The decrease in the activity of NADH oxidase in liver of diabetic animals could be due to lack of insulin since liver is dependent on insulin for most of its metabolic pathways. NADH being a very important component of the different pathways. Earlier research had shown (2) changes in the redox potential of the liver, the redox state being more reduced (higher levels of NADH). This decrease in the redox state can be due a decrease in the activity of NADH oxidase which has been reported in this communication for the first time in a diabetic state. The activity of rat liver plasma membrane NADH oxidase is two to three folds stimulated by growth factors and hormones including insulin and pituitary extracts (14). The NADH oxidase from rat liver plasma membrane is not inhibited by common inhibitors of respiratory chain of mitochondria. Growth factor responsive NADH oxidases are however strongly inhibited by chloroquine and quinone analogues (14).

The increased activity of NADH oxidase in insulin independent tissues like brain and kidney could be due to several reasons. The increased entry of glucose into these tissues would increase the metabolism of glucose, requiring cofactors for enzymes including NAD⁺ in order to channelize the extra glucose absorbed. Besides, there is a high rate of glycolysis in these tissues, there are other pathways including polyol pathway and the pentose phosphate pathway in the kidney and lens (3, 15), GABA shunt in the brain through which excess glucose is diverted using a large proportion of NAD⁺.

The polyol pathway and pentose phosphate pathway demand that a highly oxidized NAD⁺/NADH ratio be maintained in the cytoplasm in order to maintain the flux of glycolysis in the forward direction (16). The function of NADH oxidase is to oxidize NADH to NAD⁺ (17) and to maintain the physiological ratio and the redox potential of the cell. The changes in the activity of the NADH oxidase may help to maintain and reverse the metabolic changes occurring during diabetes. Kidney is the main excretory organ of the body and therefore requirements for the energy is continuous and high. The normal functioning of the kidney, therefore is of extreme importance to maintain a homeostasis. The increase in the NADH oxidase activity during diabetes could, therefore be an important factor and may help the kidney to restore the normal functioning of the body.

The changes in the activity of NADH oxidase during diabetes focuses the involvement of this enzyme in the normal metabolism. This inverse pattern in the changes of the activity
between brain and kidney on one hand and liver on the other gives a possibility that the regulation of this enzyme may be dependent on the glucose concentration in the tissues. The activity was elevated in insulin independent tissues like kidney and brain and decreased in the insulin dependent tissue like liver. Apart from the use of NADH as a cofactor in various metabolic reactions, there are some recently reported use of NADH as a therapeutic agent in Parkinson’s disease (18), where it was reported that NADH can be used as one of the substitute drug instead of dopamine. As the patients of Parkinson’s disease may develop resistance towards dopamine after some time. Details on the mechanism of action of NADH oxidase and its regulation therefore becomes a very important aspect of physiological imbalances and needs further investigations.

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REFERENCES


Glucose-6-phosphate dehydrogenase from rat brain was purified 13,000 fold to a specific activity of 480 units/mg protein. The molecular weight was 121 kDa. The kinetics of brain glucose-6-phosphate dehydrogenase are compatible with a model involving two possible states of the enzyme with a low and high affinity for the substrate d-glucose-6-phosphate. NADP⁺ and ADP offered protection against p-chloromercuribenzoate inhibition. NADPH is a powerful competitive inhibitor with respect to NADP⁺. The apparent $K_i$ for NADPH inhibition was lower than the $K_m$ for NADP⁺. ADP inhibited the enzyme competitively with respect to NADP⁺, whereas kinetics of mixed inhibition was observed with respect to substrate d-glucose-6-phosphate. The interplay between NADP⁺ and NADPH leading to enzyme activation or inhibition according to their relative or absolute concentrations as well as the control of enzyme activity by the adenine nucleotide system may contribute a refined mechanism for the regulation of glucose-6-phosphate dehydrogenase and therefore the pentose phosphate pathway in brain.

The oxidative segment of the pentose phosphate pathway comprising the NADP⁺-dependent glucose-6-phosphate dehydrogenase (G-6-PD, EC. 1.1.1.49) and 6-phosphogluconate dehydrogenase (6-PGD, EC.1.1.1.44) supply NADPH for the reductive processes from myelination to various events connected with neuronal function. Specific activation of the pentose phosphate pathway by catecholamine neurotransmitters has been observed to provide NADPH for the synthesis and degradation of neurotransmitters. Hothersall et al. have suggested that the existence of high potential activity and correspondingly high activity of the oxidative enzyme of the pentose phosphate pathway in brain is part of a neuronal protection mechanism for the efficient removal of the products of biogenic amines arising due to monoamine degradation. Glucose-6-phosphate dehydrogenase and 6-phosphogluconate dehydrogenase play a central role in determining the flux of glucose through the pentose phosphate pathway for the generation of reducing equivalents. 6-Phosphogluconate dehydrogenase has been purified from brain and its kinetic properties have been studied. However, very little is known about the regulation and kinetic properties of brain glucose-6-phosphate dehydrogenase. Glucose-6-phosphate dehydrogenase has been purified from several mammalian sources like erythrocytes, liver, adrenal and platelet and its properties have been summarised. To elucidate the molecular mechanism and regulation of the enzyme activity and pentose phosphate pathway in brain, the enzyme was purified from the rat brain and some of its kinetic and regulatory properties were studied. The results are discussed in relation to its regulatory significance under in vivo conditions.

**Materials and Methods**

**Animals**

Adult male albino rats of Wistar strain weighing between 200-250 g were used in all the experiments.

**Chemicals**

All the substrate and coenzymes, and reduced glutathione, cysteine, DL-dithiothreitol and the chromatographic products were obtained from Sigma Chemicals Co., St. Louis, USA. β-mercaptoethanol and $N,N',N''$-tetramethylene diamine (TEMED) were obtained from E. Merck (India) Ltd. Acrylamide and $N,N'$-methylene bisacrylamide were
obtained from Sisco Research Laboratories, Bombay.

**Determination of enzyme activity**

The activity of glucose-6-phosphate dehydrogenase was determined kinetically by measuring the reduction of NADP⁺ at 340 nm. The enzyme was assayed by the modified method of Baquer et al.¹⁵, and the assay mixture contained the following concentration: 100 mM Tris-HCl pH 7.7; 5.0 mM MgCl₂; 2.5 mM d-glucose-6-phosphate and 0.13 mM NADP⁺ in a total reaction volume of 1.0 ml. One unit of enzyme activity was defined as the amount required in reducing 1 μmole of NADP⁺ per minute at 25°C.

**Protein estimation**

Protein was estimated by the method of Bradford¹⁶. Fractions obtained from the column were screened for protein by measuring the absorbance at 280 nm.

**Purification procedure**

All the steps were carried out at 0-4°C unless otherwise stated. The buffers used for purification contained 1 mM EDTA, 0.1 mM DTT and 10 mM NADP⁺.

The enzyme was purified from the cytosolic fraction of rat brain. The buffer for purification was 20 mM potassium phosphate buffer, pH 6.2. The enzyme in the cytosolic fraction was precipitated by 40-55% saturation with ammonium sulphate, dialysed and loaded on DEAE-cellulose column (1.8 x 17 cms) and eluted between 0.125 M and 0.250 M KCl in buffer. The enzyme was precipitated with 60% ammonium sulphate, dialysed and loaded on a phosphocellulose column (1.5 x 3.4 cms) previously equilibrated with 20 mM potassium phosphate buffer, pH 6.2. The column was washed extensively with the buffer containing 15 mM KCl. The enzyme was then eluted in a single step with 5 mM d-glucose-6-phosphate and 15 mM KCl in buffer. The fractions containing enzyme activity were immediately desalted on Sephadex-G 25 column (1.7 x 22 cms) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.8 thus effecting a buffer exchange. The eluate from Sephadex-G 25 column free of d-glucose-6-phosphate which was low in protein was immediately concentrated by passing through a small column of DEAE-cellulose (bed volume 2.0 ml) equilibrated with 50 mM Tris-HCl buffer pH 7.8. The column was washed with 0.1 M KCl in buffer and the enzyme was eluted in a single step with 0.02 M KCl.

**Polyacrylamide gel electrophoresis**

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) of the purified glucose-6-phosphate dehydrogenase was carried out by the method of Laemmli¹⁷.

**High performance liquid chromatography**

Molecular weight of the native glucose-6-phosphate dehydrogenase was determined by High Performance Liquid Chromatography on LKB 2135-360 TSK G-3000 SW gel filtration column. The standard marker proteins were ferritin (440 kDa), catalase (232 kDa), aldolase (158 kDa) and bovine serum albumin (67 kDa). The molecular weight was calculated from a plot of log molecular weight in (kDa) v/s retention volume of the standard proteins.

**Results**

Glucose-6-phosphate dehydrogenase was purified nearly 13,000 fold from the cytosolic fraction of rat brain with an overall recovery of 33%. The results of a typical purification procedure are summarised in Table 1. The specific activity of 428 units/mg protein is higher than the values reported for the purified enzymes from other mammalian tissues like rat liver¹¹,¹² and human platelet¹⁸ but lower than the value reported for the purified enzyme from human erythrocytes.⁹ The protein stained polyacrylamide gel electrophoresis of the final preparation contained one protein band.

NADP⁺ stabilises the enzyme. The enzyme was rapidly inactivated in the presence of its substrate, d-glucose-6-phosphate. Hence it was necessary to desalt the enzyme as quickly as possible after elution from the phosphocellulose column to minimize this inactivation. The molecular weight of the native enzyme determined by High Performance Liquid Chromatography was 121 kDa. The subunit molecular weight of glucose-6-phosphate dehydrogenase determined by SDS-PAGE was 62 kDa, which showed that the native enzyme is a dimer composed of two identical subunits. The molecular weight of the native enzyme and the subunit are in the range of molecular weights reported for the enzyme from other mammalian sources¹³,¹⁴.
Table I—Purification of glucose-6-phosphate dehydrogenase from rat brain [The enzyme was purified from the 40,000×g supernatant fraction of rat brain as described in the 'Materials and Methods' section]

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total activity μmol min⁻¹</th>
<th>Total protein mg</th>
<th>Specific activity μmol min⁻¹ mg protein</th>
<th>Recovery %</th>
<th>Purification fold</th>
</tr>
</thead>
<tbody>
<tr>
<td>40,000×g supernatant</td>
<td>39.39</td>
<td>1184.11</td>
<td>0.03</td>
<td>100</td>
<td>1.0</td>
</tr>
<tr>
<td>40-55% (NH₄)₂SO₄ precipitation</td>
<td>34.66</td>
<td>130.12</td>
<td>0.27</td>
<td>88.0</td>
<td>8.0</td>
</tr>
<tr>
<td>DEAE-Cellulose column chromatography</td>
<td>29.05</td>
<td>23.15</td>
<td>1.26</td>
<td>73.7</td>
<td>37.7</td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation (0-60%)</td>
<td>26.78</td>
<td>22.0</td>
<td>1.22</td>
<td>67.9</td>
<td>36.6</td>
</tr>
<tr>
<td>Phosphocellulose column chromatography</td>
<td>20.48</td>
<td>1.24</td>
<td>16.52</td>
<td>52.0</td>
<td>496.6</td>
</tr>
<tr>
<td>KCl elution</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(NH₄)₂SO₄ precipitation (0-70%)</td>
<td>18.0</td>
<td>1.02</td>
<td>17.65</td>
<td>44.8</td>
<td>531.0</td>
</tr>
<tr>
<td>Phosphocellulose column -affinity elution with d-glucose-6-phosphate</td>
<td>12.84</td>
<td>0.03</td>
<td>428.0</td>
<td>33.5</td>
<td>12969.0</td>
</tr>
</tbody>
</table>

Substrate and coenzyme effects

Hill plot of the data (Fig. 1) shows clear positive cooperativity in the binding of the substrate which was more significant at higher concentration of the substrate than at lower concentrations with Hill coefficients (n) of 2.2 and 1.1 respectively. This indicates the presence of two binding sites for d-glucose-6-phosphate. The purified enzyme had an apparent $K_m$ of 29 μM when determined over 10-90 μM concentration of d-glucose-6-phosphate and 0.013 mM for NADP⁺. The apparent $V_{max}$ of the enzyme was achieved at 5.0 μM concentration of the substrate with a correspondingly high activity upto 10 μM concentration. This gave an apparent $K_m$ of 41 μM for the second substrate binding site. This in all probability represents a regulatory site (it needs to be confirmed more exhaustively) which indicates that the binding of substrate d-glucose-6-phosphate at this site increases the activity of G-6-PD in a modulator independent way. The purified enzyme was specific for NADP⁺ as the coenzyme. The apparent $K_m$ for NADP⁺ was 17.4 μM when determined at 2.5 μM fixed concentration of d-glucose-6-phosphate.

Coenzyme binding studies

Incubation of the purified G-6-PD with 10 mM p-chloromercuribenzoate caused complete inhibition of the enzyme. Dithiothreitol (DTT) at 10 μM concentration caused a complete reversal of inhibition whereas cysteine and reduced glutathione caused only a partial reversal of inhibition. The results indicate the presence of thiol groups in the enzyme essential for catalytic activity. Preincubation of the enzyme with substrate d-glucose-6-phosphate did not protect the enzyme from inhibition whereas

Fig. 1—Hill plots of the data at (a) lower concentration of 0.01 mM -0.10 mM and at (b) higher concentrations of 0.1 mM -1.5 mM of the substrate [NADP⁺ was present at a fixed concentration of 0.13 mM]. The Hill coefficients (n) are given alongside the slope. Each value is a mean of 4 determinations with S.E.M. ± 5%]
NADP' provided considerable protection, indicating that the -SH group essential for catalytic activity was involved in coenzyme binding. Among the various analogues of NADP' tested, which contained portions of the coenzyme structure like NAD', NADH, ADP, 5'AMP and nicotinamide, only ADP protected the enzyme against p-CMB inhibition.

**Inhibition studies**

NADPH was the most powerful inhibitor of the purified G-6-PD from brain. Inhibition of G-6-PD by NADPH has been reported for the enzyme from other tissues. The inhibition of G-6-PD by NADPH was competitive with NADP'. When determined over a range of 10-90 μM NADP' and 2.5 mM fixed concentration of D-glucose-6-phosphate, the apparent Kᵢ for NADPH inhibition was 3.5 μM (Fig. 2). The ratio of Kᵢ/Kₘ for the reduced and oxidized form of the coenzyme was 5.4, a factor which explains the potent inhibition of G-6-PD seen in vivo conditions. This finding of the enzyme having a lower Kᵢ for NADPH than Kₘ for NADP' has not been observed for the enzyme from other sources.

The other factor besides the concentration of NADPH and the NADPH/NADP' ratio for the regulation of brain G-6-PD activity is the ATP concentration. At physiological concentrations of over 10-90 μM of D-glucose-6-phosphate, ATP inhibited the enzyme non-competitively with D-glucose-6-phosphate with an apparent Kᵢ of 0.3 mM. At higher concentration of ATP above 1 mM, kinetics of mixed inhibition was observed which reduced the Vₘₐₓ and increased the apparent Kₘ of the enzyme for D-glucose-6-phosphate (Fig. 3). This indicates the presence of a regulatory site for ATP inhibitions similar to that observed for the Ehrlich ascites tumour enzyme. ATP also inhibited the enzyme non-competitively with respect to NADP' (Fig. 4). ATP inhibition of G-6-PD was found to be reversed by the addition of Mg²⁺. Hence all the ATP inhibition studies were carried out in the absence of added MgCl₂.

**Discussion**

The purification of G-6-PD was carried out using normal procedure i.e. ammonium sulphate precipitation.

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![Fig. 2](image2.jpg)

**Fig. 2**—Dixon plot of the NADPH inhibition of purified glucose-6-phosphate dehydrogenase with respect to NADP' [The enzyme activity was assayed at different concentrations of 0.10 mM (S₁), 0.09 mM (S₂) and 0.075 mM (S₃) of NADP' and at a fixed concentration of 2.5 mM D-glucose-6-phosphate. The apparent Kᵢ for NADPH inhibition was 3.5 μM. v is expressed in mmol/min. Each value is a mean of 4 determinations with S.E.M. ± 5%]

![Fig. 3](image3.jpg)

**Fig. 3**—Inhibition of purified glucose-6-phosphate dehydrogenase by ATP with respect to glucose-6-phosphate at varying concentrations (0.02 mM-0.10 mM) of glucose-6-phosphate and 0.13 mM fixed concentration of NADP' [The enzyme activity was assayed without MgCl₂ in the absence, (○) and in the presence of 0.1 mM (●), 0.50 mM (△), 1.0 mM (▲), 2.0 mM (□); and 4.0 mM (■) final concentration of ATP. v is expressed in μmol/min. Each value is a mean of 4 determinations with S.E.M. ± 5%]

![Fig. 4](image4.jpg)

**Fig. 4**—Inhibition of purified glucose-6-phosphate dehydrogenase by ATP with respect to NADP' [The enzyme activity was determined at varying concentrations (0.01-0.10 mM) of NADP' and at a fixed concentration of 2.5 mM D-glucose-6-phosphate. The enzyme was assayed in the absence, (●) and in the presence of 0.50 mM (○), 1.0 mM (□), 2.0 mM (△) ATP (final concentration). v is expressed in μmol/min. Each value is a mean of 4 determinations with S.E.M. ± 5%]
tion, DEAE-cellulose column, phosphocellulose column and affinity column with D-glucose-6-phosphate. Ten fold purity was achieved at the step of using phosphocellulose column, which was eluted with a gradient of KCl in comparison to the ammonium sulphate precipitation (0-60%). The maximum purification was achieved in the last step when affinity chromatography was done using D-glucose-6-phosphate as the ligand, a 20 fold purification was achieved in this step. This shows the efficacy of phosphocellulose column in the purification of G-6-PD.

One of the significant observation of the kinetic properties of brain G-6-PD in the present study has been the binding at two sites with increasing concentrations of its substrate D-glucose-6-phosphate. This property has been reported for few G-6-PDs from microbial sources but has not been observed with the purified enzyme from mammalian tissues all of which show a simple Michaelis-Menten kinetics with increasing substrate concentration. The active site of the enzyme with an apparent $K_m$ of 29 $\mu M$ represents the high affinity substrate binding sites. The apparent $K_m$ of 41 $\mu M$ for the second substrate binding presents a regulatory site which indicates that the binding of D-glucose-6-phosphate at this site increases the activity of G-6-PD in a modulator independent way. Though D-glucose-6-phosphate can not be termed as a 'target' substrate for brain G-6-PD activity (since its intracellular concentration in brain is higher than the apparent $K_m$ of the enzyme for the substrate) it is very likely that the occurrence of an allosteric control on the enzyme is directed towards the distribution of the substrate through a branching or a modulated pathway, which in brain would be the pentose phosphate pathway. This would be significant in the synaptic endings, where a high proportion of glucose has been found to be specifically oxidised via the pentose phosphate pathway to provide NADPH for the synthetic and degradative reactions.

The findings of Baquer et al. and Hothersall et al. of the existence of a high and the low $K_m$ systems for $[1-14C]$ glucose oxidation (approximate pentose phosphate pathway) support these findings. Baquer et al. suggested a rate limiting step either at glucose transport/phosphorylation or the oxidative steps of the pentose phosphate pathway.

The transport of glucose into synaptosomes can not be rate limiting due to the presence of high affinity glucose transport site on the synaptic membrane with a low $K_m$ of 0.25 mM (ref. 26). Phosphorylation of glucose by hexokinase appears to be a rate limiting step in glucose oxidation in brain. Ouchi et al., and Baquer and Hothersall reported that the cytosolic hexokinase response specifically to increase in glucose supply to the organ. Even under these conditions the concentration of the D-glucose-6-phosphate in brain seldom exceeds 0.25-0.30 mM as seen in the presence of high circulating blood glucose concentrations such as in diabetes. But this increase is still sufficient to activate G-6-PD as the $K_m$ of 41 $\mu M$ for the second substrate binding site is within this range. This substrate activation of G-6-PD explains the biphasic nature of $[1-14C]$ glucose oxidation and also two $K_m$ values observed for $[1-14C]$ glucose oxidation and the regulatory site activation of G-6-PD. D-glucose-6-phosphate is at the branch point of the glycolytic route and the pentose phosphate of the glucose oxidation and the flux of the glucose through either route would depend on the activation of key regulatory enzymes. The finding of a unique regulatory site for G-6-PD activation by the substrate would therefore provide a mechanism to direct the flux of glucose through the pentose phosphate pathway as observed in the synaptic endings.

The enzyme had an apparent $K_m$ of 17 $\mu M$ for NADP which is higher than the $K_m$ values reported for the purified enzyme from other tissues like liver, muscle, adipose tissue, rat mammary gland, and human platelet. The $K_m$ value for NADP is also much higher than the intracellular concentration of 5 $\mu M$ of NADP reported in brain. These observations show that the concentration of NADPH is the limiting factor for G-6-PD activity in brain.

NADPH was the most powerful inhibitor of the purified G-6-PD from brain (present communication). Inhibition of G-6-PD by NADPH has been reported earlier from several mammalian sources like rat liver, human erythrocytes and platelet. The $K_i/K_m$ of 5.4 for the reduced and oxidised forms of the coenzyme along with the low intracellular concentration of NADPH (ref. 22) and correspondingly high redox state of the NADPH/NADP couple in brain, therefore indicate that NADPH could be the most powerful regulator of the brain G-6-PD activity. The existence of high potential activity of the pentose phosphate pathway in brain which is nearly 80-fold higher than the normal value could be due to the profound inhibition of the pathway by NADPH. Therefore, the control of the ox-
The other important factor besides the concentrations of NADPH and NADPH/NADP\(^+\) ratio for the regulation of brain G-6-PD activity is the ATP concentration. Effects of ATP on numerous G-6-PDs have been reported\(^{23,12,22}\), suggesting that it could be an important regulator of the enzyme activity in vivo. Along with the observation of kinetics with d-glucose-6-phosphate, the observation of ATP inhibition of brain G-6-PD can be explained on the basis of two assumptions: (i) There are two different binding sites on 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. At lower concentrations, ATP would compete with d-glucose-6-phosphate for the active site on the enzyme and when this is saturated by the prevailing high concentrations of the substrate, ATP inhibits the activity of G-6-PD by replacing the substrate molecules on the regulatory site. This explains the negative cooperativity in substrate, binding observed in the presence of the inhibitor. Though ATP inhibition of G-6-PD was found to be reversed by Mg\(^{2+}\), the intracellular concentration of 2.5 mM reported for ATP in brain\(^{20,38}\) is much higher than the concentration of 1 mM of free Mg\(^{2+}\) reported in brain. This indicates that ATP inhibition could still be a significant factor for G-6-PD regulation in vivo, particularly in nerve endings. Synaptosomes have been shown to maintain an ATP/ADP ratio of 5:1 (ref.36), and the role for ATP as a neurotransmitter has been postulated\(^{27,38}\).

These evidences suggest that the selective localisation of ATP in nerve terminals could be important for the regulation of G-6-PD activity in brain. The inhibition by ADP with high apparent \(K_a\) > 5 mM and its low concentration in brain of 0.5 mM (ref. 34) may not be very significant, but the ADP inhibition along with that of ATP may exert a cumulative negative influence on G-6-PD activity.

However, the relative physiological significance of the control of brain glucose-6-phosphate dehydrogenase activity by its substrate d-glucose-6-phosphate and ATP inhibition in view of the presence of a unique allosteric site on the enzyme is not very clear, as the \(K/\text{K}_{\text{on}}\) value of 5.4 for the reduced and oxidised forms of coenzymes indicates that this factor could offset any of the kinetic constants. The control of G-6-PD activity and the pentose phosphate pathway by inhibition/de-inhibition by the rates of the NADPH utilising systems provides a fine mechanism for the activation or inhibition of the pathway to the demand for reducing equivalents.

Acknowledgements

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