MATERIALS AND METHODS
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ANIMALS

A group of 40-50 Wistar male rats, each between 225-275 gms and three to four months old, were used for all experiments. Rats were kept in the animal house of Jawaharlal Nehru University and were fed standard pellet diet and tap water ab libitum (unless otherwise mentioned). These rats were true breed and were devoid of any disease, at all stages of the experiment.

INDUCTION OF DIABETES

After being starved overnight each rat received a single subcutaneous injection of alloxan monohydrate in freshly prepared sodium acetate buffer (0.154 M, pH 4.5). The dose of alloxan was 20mg/100g body weight of the rats. The volume to be injected was kept between 100 μl -150 μl. Similar volume of acetate buffer was given to each control rat. On the following day a single injection of 2 units of Protamine-Zinc insulin, prepared in normal saline, was given to each alloxan treated rat. This procedure continued for 6 days, as it is known to decrease the mortality of the animals after alloxan treatment (Sochor et al 1985). Control animals were given the same volume of normal saline instead of insulin.

Six days after the insulin withdrawal, urine glucose level was examined qualitatively by diastix strips (Bayer Diagnostics India Ltd) for the selection of alloxan induced diabetic rats. Rats with glucose levels above 250 mg/dl were used as diabetic animals.
ADMINISTRATION OF VANADIUM AND GROUPING OF ANIMALS

To examine the effects of vanadate, the control and alloxan induced diabetic rats were divided into the following groups:

(i) Control rats (C): received 80 mM NaCl with drinking water.
(ii) Diabetic rats (D): received 80 mM NaCl with drinking water.
(iii) Insulin treated diabetic rats (D+I): received 80 mM NaCl with drinking water and 2 units of Prtomine-Zinc insulin till the last day of experiment.
(iv) Vanadate treated diabetic rats (D+V): received sodium orthovanadate (0.6 mg/ml) in drinking water containing 80 mM NaCl as used and standardized earlier in our laboratory (Saxena et al 1992b).

Vanadate treatment was initiated after the withdrawal of insulin from alloxan injected rats and the treatment was continued for three weeks. NaCl was added to reduce the vanadate toxicity as shown by our laboratory and others (Heylinger et al 1985). Since the rats did not like the taste of vanadate supplemented water to avoid initial aversion its pH was adjusted to 7.0 with citric acid (neutralizing with NaOH) and it was given to the untreated rats. The severity of diabetes was monitored by daily testing urinary glucose by glucose detection strips (diastix).

After the insulin withdrawal the experimental diabetic rats were used on 7th and 21st day. The animals were sacrificed by cervical dislocation and decapitation and the tissues namely kidney, liver and brain regions were dissected out immediately, weighed and kept in the corresponding homogenising buffer. Further processing of tissues were performed at 0-4°C.
PREPARATION OF TISSUE HOMOGENATE AND SEPARATION OF SUBCELLULAR FRACTIONS

Tissue homogenates were prepared in a homogenizing media consisting of 0.25 M Sucrose, 20 mM triethanolamine at pH 7.4 and 0.2 mM DTT at pH 7.4, for liver and kidney, and 0.32 M sucrose, 20 mM triethanolamine at pH 7.4 and 0.2 mM DTT at pH 7.4 for brain regions. Homogenate were prepared in the ratio of 1:10 (w/v), by using a Potter Elveihjem type of homogenizer fitted with a teflon plunger. The homogenate obtained was centrifuged at 2000 g for 20 minutes to remove the nuclear debris. The supernatant was again centrifuged at 15,000 g for 30 minutes, in a RC5B type of Sorvall refrigerated centrifuge. The pellet fraction was resuspended in the homogenizing medium. The supernatant and pellet fractions were used for assay of the enzymes as described by (Sochor et al 1985).

ENZYME ASSAYS

HEXOKINASE

(ATP:D-hexose-6-phosphotransferase: EC 2.7.1.1)

The activity of hexokinase was estimated spectrophotometrically essentially by the method of Sharma (Sharma et al 1963) as modified by Gumaa and McLean (Gumma and McLean, 1972) by the coupled enzyme reaction system where formation of NADPH was estimated in brain, liver and kidney. Hexokinase catalyses the reaction:

\[
\text{ATP} \xrightarrow{\text{Hexokinase}} \text{ADP} \xrightarrow{\text{G-6-PDH}} \text{NADP}^+ \xrightarrow{\text{NADPH}} \text{6-phosphogluconate}
\]

\text{Glucose} \rightarrow \text{Glucose-6-phosphate} \rightarrow \text{6-phosphogluconate}
Tissue Homogenate

2000 rpm, 5 min

Nuclear Fraction    | Supernatant

10,000 rpm, 40 min

Pellet (mitochondria)    | Supernatant (cytosol)

Scheme for separation of subcellular fractions
The glucose-6-phosphate, so formed, is coupled to the enzyme glucose-6-
phosphate dehydrogenase, which catalyses the conversion of glucose-6-phosphate
to 6-phosphogluconate with simultaneous reduction of NADP to NADPH. The
appearance of NADPH, thus formed, is recorded at 340 nm. The reaction mixture
contained the following in the final volume and concentrations of 1 ml; 0.05 ml
(67.5 mM Tris/HCl), 0.05 ml (6.75 mM MgCl₂), 0.1 ml of NADP, 0.1 ml (8 mM/2mM ATP/Mg⁺), pH 7.2, 0.1 ml (5 mM glucose) and one unit of purified
glucose-6-phosphate dehydrogenase (Kaur et al 1983). The enzyme reaction was
started by adding 0.75-1.0 mg protein in the reaction mixture. The concentration
of the stock solutions used were as follows: 1.35M Tris/HCl; 0.135 M MgCl₂; 80
mM/20mM ATP/Mg⁺; 50 mM glucose; NADP⁺ 2mg/ml. The hexokinase activity
measured was total hexokinase containing type I and II both.

PYRUVATE KINASE
(ATP:Pyruvate 2-O-phosphotransferase, EC 2.7.1.40)

The activity of pyruvate kinase (PK) was determined in the brain, liver
and kidney by measuring the rate of formation of NAD⁺ in an assay system
coupled with lactate dehydrogenase as described by Gutmann and Bernt (1974).

\[
\begin{align*}
\text{ADP} & \quad \text{ATP} \\
\text{Phosphoenolpyruvate} & \quad \text{Pyruvate Kinase} \\
& \quad \text{Pyruvate} \\
& \quad \text{Lactate Kinase} \\
& \quad \text{Lactate Dehydrogenase} \\
& \quad \text{NADH} \quad \text{NAD⁺}
\end{align*}
\]

The reaction mixture in a volume 1 ml contained the following in the final
concentration of 67.5 mM Tris/HCl buffer pH 7.4 (0.05 ml), 95 mM KCl (0.05
ml), 6.75 mM MgSO₄ (0.05 ml), 0.4 mm NADH (0.1 ml), 1 mM
phosphoenolpyruvate (0.05 ml), 3 mM ADP (0.05 ml) and 2 units of lactate dehydrogenase. The reaction was started by the adding of 0.02 ml of 1:50 fraction of brain (0.05 - 0.08 mg protein) of 1:10 fraction of liver and kidney (0.3 - 0.5 mg protein). The decrease in absorbance of NADH was monitored at 340 nm over a period of 5 min at 27°C using Beckman DU-68 spectrophotometer.

ALDOSE REDUCTASE

(Alcohol:NADPH oxidoreductase, EC 1.1.1.2)

Aldose reductase, the first enzyme of polyol pathway, catalyses the formation of sorbitol from glucose as shown below.

\[
\text{NADPH} \rightarrow \text{NADP}^+ \\
\text{Glucose} \xrightarrow{\text{Aldose Reductase}} \text{Sorbitol}
\]

The tissue homogenate and extracts were prepared as described earlier for other enzymes. The aldose reductase activity in the tissue fraction was assayed by the method of Pottinger (1967). The assay mixture contained the following in the final concentration: 50 mM phosphate buffer pH 7.4 (0.1 ml), 300 mM glucose (0.1 ml) and 0.2 mM NADPH (0.1 ml). The stock concentrations of the solutions were 500 mM phosphate buffer (pH 7.4), 1.5 M glucose and 2 mM NADPH. The following amounts of tissue extracts were added: 0.05 ml of 1:10 liver and kidney extracts (containing 0.5 to 0.8 mg protein), 0.1 ml of 1:10 brain extracts (containing 0.3 mg protein) to initiate the reaction. The enzyme was assayed by following the rate of the oxidation at 340 nm for 5 min at 27°C in a Beckman DU-68 spectrophotometer.
SORBITOL DEHYDROGENASE

(L-iditol;NAD* 5-oxidoreductase, EC 1.1.1.14)

Sorbitol dehydrogenase, the second enzyme of polyol pathway, catalyses the conversion of sorbitol to fructose by a reversible reaction as shown below.

\[
\text{NADH} \xrightarrow{\text{Sorbitol Dehydrogenase}} \text{Sorbitol} \quad \text{Fructose} \xleftarrow{\text{NAD}}
\]

Sorbitol dehydrogenase was assayed in the tissue fraction by the method of Gerlach and Hiby (1974). The reaction mixture of 1.0 ml contained the following in the final concentration: 0.107 M Triethanolamine buffer pH 7.4 (0.1 ml), 300 mM fructose (0.1 ml) and 0.2 mM NADH (0.1 ml). The stock solutions were of the following concentrations; 1.07 M triethanolamine buffer (pH 7.4), 1.5 M fructose and 2 mM NADH. The reaction was initiated by adding the appropriate amount of tissue extracts 0.05 ml of 1:10 liver and kidney extracts (0.6-0.8 mg protein); 0.05 ml of 1:10 brain extract (0.2 mg protein). The change in absorbance was recorded for 5 min at 27°C by following the oxidation of NADH at 340 nm in Beckman DU-68 spectrophotometer.

GLUCOSE-6-PHOSPHATE DEHYDROGENASE

(D-Glucose-6-phosphate;NADP* 1-oxidoreductase, EC 1.1.1.49)

The activity of glucose-6-phosphate dehydrogenase was determined essentially by the method of Baquer et al (1973), modified to contain the following in the final concentration: 100 mM Tris-HCl, pH 7.8 (0.1 ml); 2.5 mM D-Glucose-6-phosphate (0.1 ml); 0.13 mM NADP* (0.05 ml) and 5.0 mM MgCl₂ (0.05 ml) in a total reaction volume of 1.0 ml. The stock solutions had the

\[
\text{G-6-PDH}
\]

\[
\text{Glucose-6-phosphate} \xrightarrow{\text{G-6-PDH}} \text{6-phosphogluconate}
\]

\[
\text{NADP}^* \xrightarrow{\text{G-6-PDH}} \text{NADPH}
\]
following concentration; 1 M Tris-HCl, pH 7.8; 25 mM D-Glucose-6-phosphate; 1.3 mM NADP$^+$ and 50 mM MgCl$_2$. The reaction was initiated by the addition of 0.05 ml extract having 0.2 - 0.5 mg protein. The reaction was followed for 5-7 minutes by measuring the increase in absorbance at 340 nm due to the reduction of NADP$^+$. The activities were measured in a Beckman DU-68 Spectrophotometer.

**IN-VITRO STUDIES USING WHOLE HOMOGENATE**

For in-vitro studies, the whole homogenates were incubated with different concentrations of sodium orthovanadate (2 mM, 4 mM and 8 mM) in the presence of 20 mM glucose for 30 minutes at 37 °C. To see the effect of only glucose the whole homogenates were incubated with 5 mM and 20 mM glucose alone. One sample was kept untreated as a matched control. The incubated whole homogenates were then centrifuged at 10,000 rpm for 30 minutes and the supernatant was used for the enzyme assays.

**KINETIC STUDIES OF KEY ENZYMES**

Kinetic studies of three enzyme was performed in the presence and absence of 4 mM vanadate. These enzymes were glucose-6-phosphate dehydrogenase, pyruvate kinase and sorbitol dehydrogenase. The assays of the above enzymes were carried out according to the methods mentioned above except the concentration of substrate was varied as follows:
Published values of the enzyme activities in different tissues of rats

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Tissues</th>
<th>units/g</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hexokinase</td>
<td>Brain</td>
<td>1.90</td>
<td>(Baquer et al 1982)</td>
</tr>
<tr>
<td>(soluble)</td>
<td>Liver</td>
<td>2.22</td>
<td>(Baquer et al 1982)</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>0.87</td>
<td>(Sochor et al 1988)</td>
</tr>
<tr>
<td>Pyruvate kinase</td>
<td>Brain</td>
<td>187</td>
<td>(Hothersall et al 1982)</td>
</tr>
<tr>
<td>(soluble)</td>
<td>Liver</td>
<td>28</td>
<td>(Sochor et al 1985)</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>14</td>
<td>(Sochor et al 1985)</td>
</tr>
<tr>
<td>Glucose-6-P-dehydrogenase</td>
<td>Brain</td>
<td>1.87</td>
<td>(Baquer et al 1988)</td>
</tr>
<tr>
<td>(homogenate)</td>
<td>Liver</td>
<td>1.62</td>
<td>(Unpublished)</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>1.89</td>
<td>(Unpublished)</td>
</tr>
<tr>
<td>Aldose reductase</td>
<td>Brain</td>
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<td>(Saxena et al 1992a)</td>
</tr>
<tr>
<td>(soluble)</td>
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<td>(Saxena et al 1992a)</td>
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<tr>
<td></td>
<td>Kidney</td>
<td>0.15</td>
<td>(Sochor et al 1988)</td>
</tr>
<tr>
<td>Sorbitol dehydrogenase</td>
<td>Brain</td>
<td>0.48</td>
<td>(Saxena et al 1992a)</td>
</tr>
<tr>
<td>(homogenate)</td>
<td>Liver</td>
<td>8.5</td>
<td>(Saxena et al 1992a)</td>
</tr>
<tr>
<td></td>
<td>Kidney</td>
<td>6.2</td>
<td>(Sochor et al 1988)</td>
</tr>
</tbody>
</table>
Pyruvate kinase

Pyruvate kinase was assayed in the in-vitro supernatants as described earlier. The reaction mixture in a volume 1 ml contained the following in the final concentration of 67.5 mM Tris/HCl buffer pH 7.4, 95 mM KCl, 6.75 mM MgSO₄, 0.4 mm NADH, 3 mM ADP and 2 units of lactate dehydrogenase. The concentration of PEP was varied in the range of 0.005 mM to 1.25 mM. 4 mM vanadate was added in all the reaction mixtures when the effect of vanadate was to be studied. The reaction was started by the addition of 0.02 ml of 1:50 fraction of brain, 0.02 ml (0.05 - 0.08 mg protein) of 1:10 fraction of liver and kidney (0.3 -0.5 mg protein). The decrease in absorbance of NADH was monitored at 340 nm over a period of 5 min at 27°C using Beckman DU-68 spectrometer.

Glucose-6-phosphate dehydrogenase

Glucose-6-phosphate dehydrogenase was assayed as discussed earlier with slight modificationss. For the kinetic study of the enzyme the following were added in the final concentration: 100 mM Tris-HCl, pH 7.8; 0.13 mM NADP⁺ and 5.0 mM MgCl₂ in a total reaction volume of 1.0 ml. The stock solutions were in the following concentration; 1 M Tris-HCl, pH 7.8; 25 mM D-Glucose-6-phosphate; 1.3 mM NADP⁺ and 50 mM MgCl₂. The concentration varying between 0.01 mM and 2.5 mM of D-glucose-6-phosphate were used. Sodium orthovanadate in the final concentration of 4 mM was used in all the incubations with vanadate. The reaction was initiated by the addition of 0.05 ml of extract containing about 0.2 - 0.5 mg protein. The reaction rate was followed for 5-7 minutes by measuring the increase in absorbance at 340 nm due the reduction of
NADP⁺. The activities were measured in a Beckman DU 68 Spectrophotometer.

**Sorbitol dehydrogenase**

Kinetic study of sorbitol dehydrogenase was done in the tissue fraction essentially by the method of Gerlach and Hiby (1974) with certain modifications. The reaction mixture of 1.0 ml contained the following in the final concentration: 0.107 M triethanolamine buffer (pH 7.4), 0.2 mM NADH. The variation in the substrate taken for the kinetic study was from 6 mM to 300 mM fructose in the reaction mixture, 4 mM sodium ortho vanadate was present in all the assays with vanadate. The stock solutions had the following concentrations: 1.07 M triethanolamine buffer (pH 7.4), 1.5 M fructose and 2 mM NADH. The reaction was initiated by adding the appropriate amount of tissue extracts 0.05 ml of 1:10 liver and kidney extracts (0.6-0.8 mg protein); 0.05 ml of 1:10 brain extract (containing about 0.2 mg protein). The change in absorbance was recorded for 5 min at 27°C by following the oxidation of NADH at 340 nm in Beckman DU-68 spectrophotometer.

**DEFINITION OF AN ENZYME UNIT**

The activities of the enzymes hexokinase, pyruvate kinase, glucose-6-phosphate dehydrogenase and sorbitol dehydrogenase are given in units/g/min and one unit of activity is defined as the oxidation of one μmol of NADH/NADPH oxidized or reduced per min per gm tissue. Aldose reductase activity is given in milliunits/g/min where,
1 unit = \frac{1}{6.22} \times \frac{\Delta O.D.}{V} \times \frac{d}{T}

6.22 = Molar extinction coefficient of NADH

O.D. = Change in optical density

d = Dilution factor

V = Volume of enzyme taken for assay

T = Time interval in minutes for monitoring the rate

PROTEIN ESTIMATION

Estimation of protein was done according to the method of Lowry et al. (1951). Standard protein solution was prepared with 1 mg/ml bovine serum albumin (BSA).

GLUCOSE ESTIMATION

Glucose estimation was performed by the ENZOKIT, supplied by Ranbaxy Laboratories, India. A coupled enzyme method was followed for the estimation of glucose, according to the protocol supplied by the above laboratory. It consists of three bottles namely 1, 2 and 3. Bottle 1 contained glucose oxidase, peroxidase, aminoantipyrine and phosphate buffer. Bottle 2 was phenol and bottle 3 was a standard glucose solution. Ingredients of bottle 1 were dissolved in distilled water. At the time of experiment, mixed 1 ml of phenol in 10 ml of solution 1. Took 1 ml of above solution and added 0.01 ml of serum to it. For standard, added 0.01 ml of solution 3 and for blank added 0.01 ml of distilled water. Allowed it to
incubate for 30 minutes at room temperature, and measured the absorbance at 505 nm.

**CALCULATION**

Where,

\[
\text{Glucose concentration (mg/dl)} = \frac{AT-AB}{AS-AB} \times 100
\]

\(AT\) = absorption of sample

\(AB\) = absorption of blank

\(AS\) = absorption of standard

**CHEMICALS AND REAGENTS**

Alloxan monohydrate, NADH, BSA and various other substrate and coenzymes were purchased from Sigma Chemicals St. Louis, USA. Protamine-Zinc insulin I.P. was purchased from Boots India Ltd., India. Sucrose, was purchased from BDH, Poole, UK. Mono and dibasic phosphate salt was obtained from Qualigens Chemicals, India. All the chemicals purchased locally and used in the experiment were of analytical grade.