MATERIALS AND METHODS

I EXPERIMENTAL ANIMALS: - Adult male albino rats of Wistar strain, weighing between 180-230 g were used in all the experiments. The animals were maintained at a temperature of 25 ± 2° C in the Animal house of the Jawaharlal Nehru University. The rats were fed the standard laboratory diets obtained as pellets from Hindustan Lever Ltd., Bombay. Food and water were given ad libitum.

II CHEMICALS: - All the substrates and coenzymes, 3,3',5'-L-triiodothyronine, alloxan monohydrate, p-chloromercuribenzoate, bovine serum albumin (fatty acid free), methylbenzethonium hydroxide, DL-dithiothreitol, glutathione reduced, PPO and POPOP were obtained from Sigma Chemical Co. St.Louis, U.S.A. Acrylamide and N,N,methylene bisacrylamide were obtained from Sisco Research Laboratories, Bombay. Protamine zinc insulin was obtained from Boots Co., (India) Ltd.

Chromatographic products: - Phosphocellulose and Sephadex G-25 were obtained from Sigma Chemical Co., U.S.A. and DEAE-cellulose was obtained from Sisco Research Laboratories, Bombay.
Radiochemicals:- [1-\textsuperscript{14}C] glucose (specific activity 36 mCi/mmol) and [6-\textsuperscript{14}C] glucose (specific activity 24 mCi/mmol) were obtained from BARC, Bombay. [2-\textsuperscript{14}C] - glucose (specific activity 59 mCi/mmol) was obtained from Radiochemicals Division, Amersham, U.K. All the other chemicals were from BDH and were of analytical grade.

III. PREPARATION OF HOMOGENATES AND SUBCELLULAR FRACTIONATION

1. Preparation of homogenates: The animals were sacrificed by cervical dislocation and the whole brain or regions, namely, cerebral hemispheres, cerebellum and brain stem were quickly dissected out and weighed. Tissue homogenates (10\% w/v) were prepared in a Potter - Elvehjem type of homogeniser fitted with a teflon plunger. The homogenising medium was 0.32 M sucrose buffered with 20 mM triethanolamine to pH 7.6 and containing 0.1 mM dithiothreitol. All the steps were carried out at 4\textdegree C unless otherwise stated.

2. Subcellular Fractionation: Subcellular fractionation of the brain homogenates were performed by a combination of the methods of Hess and Brand (1974) and Appel and Parrot (1970), as outlined in the flow diagram (Fig. E).
Whole homogenate

Nuclear fraction ($P_1$)

Supernatant ($S_1$)

Crude mitochondrial fraction ($P_2$)

Supernatant ($S_2$)

Microsomal fraction ($P_3$)

Cytosolic fraction ($S_3$)

6.5\% Ficoll

13.0\% Ficoll

Myelin

Synaptosomes

Mitochondria

**FIG. E**: FLOW SHEET OF THE SUBCELLULAR FRACTIONATION.
(i) Differential Centrifugation: The homogenate was centrifuged at 1000xg for 10 min in a refrigerated Sorvall RC-5 centrifuge to remove the crude nuclear fraction ($P_1$). The resulting supernatant was centrifuged at 12,000xg for 30 min to obtain the crude mitochondrial pellet ($P_2$) and the supernatant ($S_2$). The supernatant ($S_2$) was centrifuged at 1,05,000xg for 60 min to obtain the microsomal fraction ($P_3$) and the cytosolic fraction ($S_3$).

(ii) Fractionation of the Crude Mitochondrial Fraction ($P_2$) by Discontinuous-Density Gradient Centrifugation:

The crude mitochondrial fraction ($P_2$) containing myelin, synaptosomes and mitochondria were fractionated essentially by the method of Appel and Parrot (1970): A discontinuous Ficoll density gradient was prepared by layering 3.5 ml of 6.5% (w/v) Ficoll in 0.32 M isotonic sucrose containing 20 mM triethanolamine pH 7.6, over a 13.0% (w/v) Ficoll in sucrose. The gradient was allowed to equilibrate for 1-2 hours at 4°C. The crude mitochondrial pellet ($P_2$) was washed twice in the homogenising medium and layered as a suspension (2.5 ml) on the gradient and centrifuged in a Beckman L 870 M ultracentrifuge at 4°C in a SW.41Ti rotor at 1,50,000xg for 75 minutes. Myelin at the homogenate-6.5% Ficoll interface and the synaptosome
fraction at 6.5% - 13.0% Ficoll interface were collected. Mitochondria was obtained as a pellet. All the fractions were washed twice in the buffered isotonic 0.32 M sucrose solution to remove any Ficoll present and resuspended in the same solution. For the in vitro studies on the flux of \(^{14}C\) glucose through alternative metabolic pathways, the synaptosomal fraction was resuspended in freshly prepared Krebs-Ringer bicarbonate medium, pH 7.4. All the fractions were kept at 0°C prior to use and were utilised as soon as possible after isolation.

IV DETERMINATION OF ENZYME ACTIVITIES

1. Assay of Enzyme Activities: The enzyme activities were determined in the 12,000xg particulate fractions containing mitochondria, myelin and synaptosomes and in the 12,000xg supernatant fractions. The particulate fractions were washed once in the homogenising medium and were treated with Triton-X100 (0.2% final concentration) to release the total bound enzymes.

(i) Glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate : NADP\(^+\), 1-oxidoreductase, EC.1.1.1.49) :-

Activities of glucose-6-phosphate dehydrogenase were determined by the method of Baquer et al. (1973), modified
to contain the following in final concentration: 100 mM Tris-HCl, pH 7.8; 2.5 mM D-glucose-6-phosphate; 0.13 mM NADP⁺ and 5.0 mM magnesium chloride in a total reaction volume of 1.0 ml. The reaction was initiated by the addition of 20 μl of the supernatant or 50 μl of 0.2% Triton-X100 treated particulate fractions. The reaction was followed for 5-7 min by measuring the increase in absorbance at 340 nM due to the reduction of NADP⁺. The activities were determined in a double beam Shimadzu-UV 150 spectrophotometer.

The activities of glucose-6-phosphate dehydrogenase at later stages of purification were determined after appropriate dilution of the enzyme with buffer to obtain linear increase in absorbance for a period of 5-7 min. Kinetic studies of the purified enzyme were carried out at a constant temperature of 32°C in a Shimadzu-UV 3000 spectrophotometer equipped with a thermostatic control.

(ii) 6-Phosphogluconate dehydrogenase (6-phospho-D-gluconate :NADP⁺ 2-oxidoreductase (decarboxylating) EC.1.1.1.44):

The activities of 6-phosphogluconate dehydrogenase were determined by the method of Baquer et al. (1973). The assay mixture contained the following in final concentration: 100 mM Tris-HCl, pH 7.8; 2.5 mM 6-phospho-D-gluconate; 0.13 mM
NADP\(^+\) and 5.0 mM magnesium chloride in a total reaction volume of 1.0 ml. The reaction was initiated by the addition of 50 \(\mu l\) of the supernatant or 50 \(\mu l\) of the 0.2\% triton treated particulate fraction. The enzyme activities were assayed for 5 min by following the reduction of NADP\(^+\) at 340 nm in a double beam Shimadzu UV 150 spectrophotometer.

(iii) Malic Enzyme (L-Malate : NADP\(^+\) oxidoreductase (oxaloacetate - decarboxylating) EC.1.1.1.40):

The activities of NADP\(^+\) dependent malate dehydrogenase (malic enzyme) were determined by the method of Wood et al. (1969). The reaction mixture of 1.0 ml contained the following in final concentration: 200 mM Tris-HCl, pH 8.0; 2.5 mM L-malate; 0.13 mM NADP\(^+\) and 2.5 mM manganese chloride (pH 7.4). The reaction was initiated by the addition of 50 \(\mu l\) of the soluble fraction or 20 \(\mu l\) of 0.2\% Triton-X100 treated particulate fraction. The reaction was followed for 5 min by measuring the reduction of NADP\(^+\) at 340 nm in a Shimadzu UV 150 spectrophotometer.

(iv) Isocitrate dehydrogenase (threo-D\(_s\)-Isocitrate : NADP\(^+\) oxidoreductase (decarboxylating) EC.1.1.1.42):

The activities of NADP\(^+\)-linked isocitrate dehydrogenase were determined by the method of Luine and Kauffman (1971).
with modifications. The reaction mixture contained the following in final concentration: 67.0 mM Tris-HCl, pH 7.4; 2.5 mM D-isocitrate; 0.13 mM NADP\(^+\) and 2.5 mM manganese chloride (pH 7.4), in a total reaction volume of 1.0 ml. The reaction was started by the addition of 50 \(\mu\)l of the soluble fraction or 20 \(\mu\)l of 0.2% Triton-X100 treated particulate fraction. The reduction of NADP\(^+\) at 340 nm was followed for 5 min in a Shimadzu UV 150 spectrophotometer as a measure of enzyme activity.

2. Calculation of enzyme activities: - The activities of all the four NADP\(^+\)-dependent dehydrogenases are expressed as units/g tissue wet weight per minute at 25\(^\circ\)C.

One unit of enzyme activity is defined as the amount required to form 1 \(\mu\)mol of NADPH/g tissue wet weight/min at 25\(^\circ\)C.

Enzyme activities were calculated by taking the molar extinction coefficient of NADPH\(_2\) AS \(6.22 \times 10^6\) cm\(^2\)/M. An absorbance increase of 6.22 in 1.0 ml reaction mixture and 1.0 cm light path at 340 nm would mean the reduction of 1 \(\mu\)mol of NADP\(^+\), corresponding to the conversion of 1 \(\mu\)mol of the substrate. The formula for calculating units/g tissue/min is given as follows:
Absorbance change/min x dilution
Volume of sample x 6.22 x total reaction volume

V

IN VITRO STUDIES ON THE FLUX OF DIFFERENTIALLY LABELLED $^{14}$CGLUCOSE THROUGH ALTERNATIVE METABOLIC PATHWAYS:

The use of $^{14}$C-glucose labelled on different carbon atoms namely C-1, C-2 and C-6 have been used to evaluate the flux of glucose through the alternative metabolic routes in the synaptosomal and cytosolic fractions.

1. Experimental procedure: All the incubations were performed in a shaking water bath at 37°C essentially according to the method of Hothersall et al. (1982), with slight modifications. The incubation medium was Krebs-Ringer bicarbonate pH 7.4, containing half the normal calcium. The Krebs-Ringer bicarbonate medium was prepared fresh before the experiment and was gassed with a mixture of gas containing 95% O$_2$ and 5% CO$_2$ just prior to use. Freshly prepared D-glucose was added to a final concentration of 10.0 mM. The total reaction volume was 1.0 ml. All the tubes were pre-incubated at 37°C for 5 min, prior to the addition of the labelled substrates. To each flask was then added, 0.1 uCi of either $[1^{-14}C]$, $[2^{-14}C]$, or $[6^{-14}C]$ glucose and the reactions were initiated by the addition of
0.5 ml (approximately 0.60 - 0.80 mg) of synaptosomal protein or (1.5 - 1.7 mg) of the cytosolic protein. The tubes with detachable polyethylene centre wells containing 0.2 ml methylbenzethonium hydroxide (to trap the $^{14}$CO$_2$), were fitted immediately with rubber caps and incubated at 37°C for 15 min in a shaking water bath. Incubation was terminated by injecting 0.2 ml of 5 N HCl through the rubber caps into the reaction mixture, taking care to prevent any gas leakage. The tubes were then shaken for additional 90 min to trap all the $^{14}$CO$_2$ evolved. At the end of incubation, the plastic wells were withdrawn and the contents were directly transferred into scintillation vials after 2-3 washings of 0.5 ml each with methanol. 10.0 ml of toluene based scintillation fluid containing 0.4% PPO and 0.025% POPOP was added to each vial and the vials were counted for two minutes each in d.p.m. in the $^{14}$C channel of the LKB Rack beta scintillation counter.

In all the experiments, tubes without extracts served as blanks and the minimal amount of $^{14}$CO$_2$ in blanks were subtracted from values obtained for tubes with extracts. 10 ul of the incubation medium from the blanks containing the labelled substrates were counted to obtain an external standard.
2. **Calculation of the Radiochemical Yields:** Specific activity of the standards was expressed in counts (d.p.m.) per umol of the substrate in the medium.

The yield of $^{14}\text{CO}_2$ in nmol/g tissue/15 min of incubation from the synaptosomal and cytosolic fractions was calculated as follows:

$$^{14}\text{CO}_2 \text{ yield in } \frac{\text{nmol}}{\text{g}}/15 \text{ min} = \frac{\text{Counts (d.p.m.)} \times \text{dilution} \times 1000}{\text{Specific activity} \times \text{time of reaction} \times \frac{\text{volume total of the labelled substrate}}{\text{reaction volume}} \times \frac{\text{reaction volume}}{\text{sample volume}}}$$

The yield of $^{14}\text{CO}_2$ in nmol/mg protein/15 min was obtained by dividing $^{14}\text{CO}_2$ yield/g tissue by mg protein/g of tissue.

3. **Evaluation of the radiochemical yields from differentially labelled glucose molecules:** Analysis of the $^{14}\text{CO}_2$ yields from glucose labelled on carbons 1, 2 or 6 gives an approximation of the relative contribution of the various pathways. It has been calculated on a system described by Hothersall et al. (1981b) to obtain a relationship for the flux of glucose through the alternative metabolic routes.
$^{14}$CO$_2$ YIELDS:

$[1-^{14}C]$glucose: Oxidative steps of the pentose phosphate pathway and metabolism of glucose via the glycolytic route and the TCA cycle.

$[6-^{14}C]$Glucose: Metabolism via the glycolytic route and the TCA cycle.

$[2-^{14}C]$Glucose: Metabolism via the glutamate-GABA pathway and the TCA cycle.

$[1-^{14}C]-[6-^{14}C]$Glucose: Approximates to the yield of $^{14}$CO$_2$ from the oxidative steps of the pentose phosphate pathway.

$[2-^{14}C]-[6-^{14}C]$Glucose: Approximates to the yield of $^{14}$CO$_2$ from the glutamate-GABA pathway.

VI  INDUCTION OF HORMONAL CONDITIONS

1. THYROIDECTOMY: Thyroidectomy was performed according to the method of Zarrow et al. (1964). The rats were starved for 24 hours prior to the operation. Two lobes of the thyroid glands, lying on either side of the trachea were surgically removed under light ether anaesthesia. After thyroidectomy, rats were divided into 2 groups. The first group of rats was designated the thyroidectomized ($T_x$)
To study the reversal of thyroidectomy, a second group of thyroidectomised rats was injected 3,3',5'-L-triiodothyronine (T₃) dissolved in 0.005 N NaOH at a dose of 15 μg/100 g body weight intraperitoneally for 3 consecutive days prior to sacrifice. This group was designated the T₃ treated thyroidectomised (Tx+T₃) group. The sham operated control rats were injected with the same volume of the vehicle. The animals were sacrificed at 7, 14, 21 and 28 days after thyroidectomy. One percent calcium lactate was given in drinking water to the thyroidectomised and T₃ treated group of rats to maintain calcium homeostasis due to the loss of parathyroid glands during thyroidectomy. Food and water were given ad libitum.

2. HYPERTHYROIDISM: A group of control rats were rendered hyperthyroid by four intraperitoneal injections of 3,3',5'-L-triiodothyronine in 0.005 N NaOH at a dose of 50 μg/100 g body weight on alternate days. The control animals were injected with the same volume of the vehicle. The animals were sacrificed 24 hours after the last injection.

3. ALLOXAN INDUCED DIABETES: A group of rats of the same age and body weight were used for the induction of diabetes. The animals were starved for 24 hrs. Then, a single subcutaneous injection of alloxan monohydrate prepared fresh
in 0.154 M sodium acetate buffer, pH 5.0 was injected intraperitoneally to each animal at a dose of 15 mg/kg body weight. Control rats were injected with the same volume of the vehicle. From the next day, all the rats were given intraperitoneal injections of 2 units of protamine zinc insulin, daily for 6 days. This treatment greatly reduced the toxic effects of alloxan and decreased the mortality rate. The control rats were injected with the same volume of saline.

The alloxan treated rats were divided into two groups. The first group was administered 0.1 ml of saline daily and was designated the 'Diabetic' (D) group. The second group was injected 1 unit insulin per day and was designated the 'Diabetic + Insulin' (D+I) group. Blood and urine glucose were monitored daily by means of glucose detection strips. The rats were sacrificed at a fixed hour, after 7 and 21 days of insulin withdrawal.

VII PURIFICATION OF GLUCOSE-6-PHOSPHATE DEHYDROGENASE FROM RAT BRAIN:

Glucose-6-phosphate dehydrogenase was purified from rat brain cytosolic fraction by procedures involving fractional precipitation and column chromatography.
1. **Preparation of the tissue homogenates:** Whole brain tissue (about 48-50 g) was homogenised as described earlier, in five volumes of homogenising medium containing 0.32M sucrose, 20 mM triethanolamine, pH 7.6 and 0.1 mM dithiothreitol. The homogenate was centrifuged at 40,000xg for 60 min and the precipitate was discarded. The enzyme was purified from the supernatant fraction.

2. **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 dithiothreitol and 10 μM NADP⁺. The dialysis tubes were prepared by boiling once in a solution containing 4% sodium bicarbonate and 10 mM EDTA, pH 7.6. The tubes were then rinsed and boiled in distilled water before use. Recrystallised ammonium sulphate of analytical grade was used for precipitation. The fractions obtained from the columns were routinely screened for protein by measuring the absorbance at 280 nm.

(i) **Ammonium sulphate fractionation:** Solid ammonium sulphate was added slowly with stirring to the supernatant upto 40% saturation. The precipitate was centrifuged at 23,000xg for 20 min and the pellet discarded. The supernatant was brought to 55% saturation with further addition of solid ammonium sulphate, stirred for an
additional 30 min and centrifuged as above. The precipitate was dissolved in 8.0 ml of 50 mM potassium phosphate buffer, pH 6.4 and dialysed for 12 hours against 100 volumes and with two changes of the same buffer.

(ii) **DEAE-cellulose column chromatography:** The dialysate was centrifuged to remove a small insoluble precipitate and was then loaded on a DEAE-cellulose column (1.8 x 17 cms) that had previously been equilibrated with 50 mM potassium phosphate buffer, pH 7.4. The column was washed extensively with a solution of 0.1 M KCl in the same buffer until the absorbance at 280 nm was less than 0.05. The enzyme was then eluted stepwise, with 50 ml each of 0.125 M, 0.15 M, 0.175 M, 0.20 M, 0.225 M and 0.250 M KCl in the same buffer. The flow rate was 22 ml per hour and 4.0 ml fractions were collected. Fractions containing enzyme activity were pooled and precipitated by 60% saturation with solid ammonium sulphate. The precipitate was dialysed for 12 hours against 50 mM potassium phosphate buffer, pH 6.2.

(iii) **Phosphocellulose column chromatography:** The dialysate was applied to a column of phosphocellulose (1.5 x 8.0 cms) previously equilibrated with 50 mM potassium phosphate buffer, pH 6.2. The column was washed with 0.1 M KCl in buffer until no protein eluted. The enzyme was
eluted stepwise with 25.0 ml each of 0.125 M, 0.150 M, 0.175 M, 0.20M and 0.225 M KCl in the same buffer. The flow rate was 22.0 ml per hour and 4.0 ml fractions were collected. The fractions containing enzyme activity were pooled and concentrated by precipitation with solid ammonium sulphate up to 60% saturation. The precipitate was dialysed against the same buffer for 10 hours.

(iv) Second phosphocellulose column chromatography

The dialysate was applied to a second 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 several column volumes of 15 mM KCl in buffer until no more protein was eluted. The enzyme was then eluted in a single step with 7 mM D-glucose-6-phosphate and 15 mM KCl in the same buffer. The fractions containing enzyme activity were immediately desalted on a Sephadex G-25 column (1.7 x 22 cms) previously equilibrated with 50 mM Tris-HCl buffer, pH 7.8, containing 25 mM KCl, thus effecting a buffer exchange.

The eluate from the sephadex G-25 column free of D-glucose-6-phosphate and having very low protein concentration per ml was immediately concentrated by passing through a small column (2.0 ml bed volume) of DEAE cellulose previously equilibrated with 50 mM Tris-HCl buffer, pH 7.8.
The column was washed with 0.1 M KCl solution in the same buffer and the enzyme was eluted in a single step with the buffer containing 0.2 M KCl.

VIII SDS-POLYACRYLAMIDE GEL ELECTROPHORESIS

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) of the purified glucose-6-phosphate dehydrogenase was carried out according to the method of Laemmli (1970) with some modifications.

The separation gels containing 7.5% acrylamide were prepared by mixing the following in a final volume of 50 ml: 12.45 ml solution of 30% acrylamide and 0.8% N,N'-methylene bisacrylamide; 18.75 ml of Tris-HCl buffer, pH 8.8 (0.375 M final concentration); 10 ul of TEMED and 11.57 ml of distilled water and the solution was degassed for 10 minutes just before use. Then, 0.50 ml of a 10% solution of ammonium persulphate (prepared fresh) was added to a final concentration of 0.1% and mixed thoroughly. The solution was immediately cast to a gel of 1.5 - 2.0 mm thickness between two glass plates (8.0 cms x 8.0 cms) and allowed to polymerise for 2-3 hours at room temperature. The samples for electrophoresis were prepared by mixing 50 ul of the sample with 50 ul of the sample buffer to obtain a final concentration of 0.12 M Tris-HCl, pH 8.0, 4% SDS, 10% β-
concentration of 0.12 M Tris-HCl, pH 8.0, 4% SDS, 10% B-mercaptopoethanol, 20% glycerol and 0.2% bromophenol blue. The standard proteins, phosphorylase b (subunit molecular size 94,000 daltons), bovine serum albumin (67,000 daltons), ovalbumin (43,000 daltons), carbonic anhydrase (30,000 daltons), soyabean trypsin inhibitor (20,000 daltons) and α-lactalbumin (14,000 daltons) were prepared in the same way. All the protein samples were immersed in boiling water for three minutes to allow for the complete dissociation of the subunits.

Samples of 25 μl volume containing approximately 2-5 μg protein were loaded on the separating gels previously equilibrated for 30 minutes with Tris-glycine buffer (0.05 M Tris, 0.4 M glycine, pH 8.8, containing 0.1% SDS). Electrophoresis was carried out at 120-150 volts and between 20-40 mA current at room temperature for about 3 hours till the marker dye reached 1 cm from the bottom of the gel. The gels were fixed in 10% TCA and stained for 12-16 hours with 0.25% Coomassie Brilliant Blue R-250 (dissolved in methanol: acetic acid and water in a proportion of 5:1:5). The gels were destained in a solution of 7% acetic acid and 7% methanol with repeated changes until the background was clear.
IX MOLECULAR WEIGHT DETERMINATION

The molecular size of the subunit of the purified glucose-6-phosphate dehydrogenase was determined by SDS-PAGE electrophoresis on 12.5% acrylamide gels as described above from a set of standard marker proteins of known molecular weight.

The molecular weight of the native enzyme was determined by High Performance Liquid Chromatography, on a 2135-360 TSK G 3000 SW gel filtration column. The column was equilibrated at a flow rate of 0.5 ml per minute for 3 hours with 50 mM potassium phosphate buffer, pH 6.2, containing 0.1 mM EDTA, 0.1 M KCl and 0.1 mM dithiothreitol. The buffer and the samples were passed through a millipore filter to remove any particulate matter before applying on the column. The buffer was degassed before use. Then, 200 ul of the sample containing approximately 20-30 µg protein was loaded on the column and the column was washed with the equilibrating buffer at a flow rate of 0.1 ml per minute. The absorbance was set at 0.01 unit and elution profile of the protein sample was monitored by the recorder. After one hour of loading the sample, 1.0 ml fractions were collected and the time of the protein peak was noted. The standard marker proteins of known molecular weight, namely, ferritin (440 KD); catalase (232 KD), aldolase (158 KD) and bovine
serum albumin (67 KD) were run under identical conditions, and the molecular weight of the purified glucose-6-phosphate dehydrogenase was calculated from a plot of log molecular weight (in KD) v/s retention volume of the standard proteins.

X OTHER DETERMINATIONS

1. **Blood glucose**: Blood glucose was determined by the method of Bergmeyer et al. (1974), using a coupled assay system linked to hexokinase and glucose-6-phosphate dehydrogenase based on the following principle:

\[
\text{Hexokinase} \\
D\text{-glucose} + \text{ATP} \rightarrow D\text{-glucose-6-phosphate} + \text{ADP}
\]

\[
\text{Glucose-6-phosphate} \\
D\text{-glucose-6-phosphate} + \text{NADP}^+ \rightarrow 6\text{-phospho-dehydrogenase} \rightarrow D\text{-glucose} + \text{NADPH}
\]

The formation of NADPH, as measured by the change in extinction at 340 nm, is proportional to the amount of glucose present. The assay mixture contained the following in final concentration: 100 mM Tris-HCl pH 7.8; 0.26 mM NADP\(^+\), 8.0 mM ATP; 5.0 mM magnesium chloride and one unit of purified yeast glucose-6-phosphate dehydrogenase. The reaction was allowed to proceed for 2-3 min until there was no change in the absorbance. Then, one unit of hexokinase was added and
the change in the absorbance due to the formation of NADPH was recorded till the completion of the reaction and taken as a measure of glucose present in the sample. The values are expressed in mmol/litre.

2. **Protein estimation:** Protein was estimated by the Coomassie Brilliant Blue G-50 dye binding method as described by Bradford (1976). Bovine serum albumin was used as the standard. During enzyme purification, all the fractions obtained from the columns were routinely assayed for protein by measuring the absorbance at 280 nm.

XI **STATISTICAL EVALUATION OF THE DATA:** The data were evaluated statistically by Student's 't' test and significant differences between the Means (calculated as P values) are shown. The differences were considered statistically significant only when the P values were less than 0.05.