MATERIALS AND METHODS
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1. EXPERIMENTAL ANIMALS

Female adult albino rats of the Wistar strain, weighing between 170-200 g body weight were used in all the experiments. Rats were maintained at a temperature of 24 ± 2°C in the rat colony of the School of Life Sciences. The rats were fed with standard food pellets obtained from Hindustan Lever Ltd., Bombay and given tap water ad libitum.

2. CHEMICALS

Protamine zinc insulin suspension was obtained from Boots India Ltd. All biochemicals including substrates, enzymes, cofactors, alloxan monohydrate and d,l-propranolol were obtained from Sigma Chemicals Company, St. Louis, U.S.A. All other chemicals were from British Drug House and were of Analytical Grade.

3. ADMINISTRATION OF PROPRANOLOL HYDROCHLORIDE

(a) Acute propranolol treatment

d,l-Propranolol hydrochloride (40 mg) was prepared fresh each day by dissolving in 2 ml of distilled water. The rats were injected propranolol
through the intraperitoneal route either at a dose of 30 mg/Kg body weight or 45 mg/Kg body weight according to Milmore and Taylor (1975). The control animals were injected with an equal volume of distilled water. This single injection of propranolol was referred to as the acute dose in all the experiments.

(b) **Chronic propranolol treatment**

To study the effect of chronic propranolol treatment on enzyme levels, the rats were divided into two groups. One group was injected with propranolol intraperitoneally, at a dose of 5 mg/Kg body weight every day in the morning for 14 days and the corresponding control group received an equal volume of distilled water. This dose was chosen because it is close to the therapeutic dose in man (Aglio et al., 1983). The rats of chronically treated group were sacrificed 24 hours after the last propranolol injection, whereas those of the acutely treated group were sacrificed after different time intervals (30, 60, 90 and 120 minutes) of propranolol injection.

4. **INDUCTION OF ALLOXAN DIABETES**

A group of rats was starved for 24 hours. Each rat was subsequently given a single subcutaneous injection
of alloxan monohydrate (15 mg/100 g body weight) prepared fresh in 0.154 M sodium acetate buffer (pH 4.5). The control group of rats received a corresponding volume of acetate buffer. Both the groups were given food and water ad libitum. The alloxan treated rats were administered 2 units of protamine zinc insulin (Boots Co., suspension diluted to 10 units/ml with 0.9% saline) intraperitoneally for six days. The latter procedure was found to reduce the mortality rate and the toxic effects of alloxan.

The alloxan treated rats were then divided into two groups. The first group was designated as the diabetic group and the second group receiving one unit of insulin per day was termed as the diabetic + insulin group. Blood and urine glucose were monitored daily by means of glucose detection strips (Boehringer Corp; London and Miles Laboratories, England). Only those diabetic rats showing blood sugar of over 300 mg% were included in the study. The rats were sacrificed after 3, 7, 14 and 21 days of insulin withdrawal.

Alloxan monohydrate, according to Wilson et al. (1984) causes a diabetic like disease associated with
severe \( \beta \)-cell necrosis. Moreover, Uchigata et al. (1982) have proposed that alloxan and streptozotocin induce \( \beta \)-cell damage by initiating biochemical events which lead to DNA strand breaks. As a part of the repair of these lesions, nuclear poly (ADP-ribose) synthetase is activated to form poly (ADP-ribose) utilizing NAD as a substrate. Hence, there is a critical depletion of NAD resulting in functional impairments and ultimately, cell death. Alloxan exerts its initial effects through the generation of oxygen free radicals (Uchigata et al., 1982).

5. CHRONIC PROPRANOLOL TREATMENT TO DIABETIC RATS

To study the effect of propranolol administration on diabetes, a group of diabetic rats was administered propranolol at a dose of 5 mg/Kg body weight from the 22nd day of insulin withdrawal for seven days. These rats were sacrificed 24 hours after the last injection of propranolol. Corresponding diabetic rats were maintained to serve as the control group of the propranolol treated diabetic rats.
6. PREPARATION OF TISSUE EXTRACTS AND SUB-CELLULAR FRACTIONATION.

Rats were sacrificed by cervical dislocation and the brain and heart were dissected out quickly. The tissues were excised and washed with chilled saline (0.9% NaCl). The brain was separated into three discrete regions, viz., the cerebral hemisphere, cerebellum and brain stem. The brain regions and heart were weighed separately and a 10 per cent homogenate (w/v) of the tissues was prepared using a Potter-Elvehjem type homogenizer fitted with the Teflon pestle in a homogenizing medium containing 0.25 M sucrose, 20 mM triethanolamine and 0.1 mM dithiothreitol (pH 7.6). Addition of dithiothreitol was excluded when homogenization was done for determination of acetylcholinesterase activity. All procedures were carried out at 0-4°C unless otherwise stated. The homogenate was centrifuged at 1000 x g for 10 minutes in a refrigerated centrifuge (RC-5C). The resultant pellet (P1) was discarded and the supernatant (S1) was further centrifuged at 12,000 x g for 40 minutes at 0°C. The whole homogenate, the pellet (P2) and the supernatant (S2) were taken for enzyme assays. The pellet (P2) and the supernatant (S2) used here are
referred to as the 12,000 x g pellet (particulate) fraction and the supernatant (soluble or cytosolic) fraction respectively.

7. ASSAY OF ENZYME ACTIVITY

(a) Hexokinase

Hexokinase (ATP:D-hexose-6-phosphotransferase; EC 2.7.1.1) was estimated spectrophotometrically according to the method of Sharma et al. (1963) as modified by Gumma and McLean (1972) by the coupled enzymatic reactions. Hexokinase catalyses the following reaction.

\[
\text{Hexokinase} \quad \text{Glucose} + \text{ATP} \rightarrow \text{Glucose-6-phosphate} + \text{ADP}
\]

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.

\[
\text{Glucose-6-phosphate} + \text{NADP} \xrightarrow{\text{G-6-PDH}} 6\text{-Phosphogluconate} + \text{NADPH}
\]

The reaction mixture contained the following in
a final volume of 1 ml; 0.5 ml of 0.2 M Tris-HCl, pH 7.4; 0.1 ml of 80 mM MgCl₂, pH 7.0; 0.1 ml of 4 mM NADP; 0.1 ml of 80 mM/20 mM of ATP/Mg²⁺, pH 7.2; 0.1 ml of 50 mM glucose and one unit of purified glucose-6-phosphate dehydrogenase in 0.1 ml of normal saline. All enzyme assays were carried in fresh extracts.

To solubilize the latent and bound enzyme, the 12,000 x g pellet was treated in cold with nonionic detergent, Triton X-100 (0.5% final concentration) for 45 minutes. The reaction was initiated by the addition of 20 μl of 10% supernatant and 10 μl of 10% solubilized pellet (containing about 150 μg protein). The rate of formation of NADPH was followed by measuring the increase in optical density over a period of 5 minutes in a recording spectrophotometer (Shimadzu, UV-260 model). Blank cuvettes contained the entire reaction mixture without the extract. Soluble and total particulate hexokinase activities were added up to obtain the whole homogenate hexokinase activity.

(b) Monoamine oxidase

Monoamine oxidase (MAO) (amine:oxygen oxidoreductase, deaminating, flavin containing, EC 1.4.3.4) is a mitochondrial marker enzyme which catalyses the
oxidative deamination of a variety of biogenic amines. The general reaction is represented in the following equation:

\[ R - \text{CH}_2 - \text{NH}_2 + O_2 + H_2O \xrightarrow{\text{MAO}} R - \text{CHO} + \text{NH}_3 + H_2O_2 \]

\( (R- \text{ represents a fatty aromatic or aliphatic residue}) \)

Monoamine oxidase activity was assayed according to the method of Catravas et al. (1977). The assay mixture contained 0.05 M Tris-HCl buffer (pH 7.4); 0.22 mM Kynuramine dihydrobromide; 0.08 mM MgCl\(_2\) and the appropriate enzyme preparation containing 500 to 1000 \( \mu \)g of protein. The final volume of the incubation mixture was made up to 3 ml with water. The reaction was initiated by addition of Kynuramine dihydrobromide and the incubation was carried out for 90 minutes in a water bath maintained at 37\(^\circ\)C. The reaction was stopped by the addition of 0.2 ml of 0.5 M NaOH and 0.4 ml of 10% ZnSO\(_4\). The mixture was then shaken thoroughly, heated in a boiling waterbath for 5 minutes and centrifuged at 10,000 \( \times \) g for 10 minutes in a Remi Table Top Centrifuge. The concentration of the reaction product (4-hydroxyquinoline) was determined in the supernatant by measuring the absorbance (appearance of peak) at 330 nm (Catravas
and McHale, 1974) in a Shimadzu UV-260 spectrophotometer. Corresponding blanks were prepared by replacing kynuramine with equal volume of distilled water. A stock solution of 4-hydroxyquinoline (0.001 M) was prepared by dissolving 1.45 mg in 10 ml of double distilled water. 1 ml of the stock was diluted to 10 ml and from this, different amounts were taken to prepare a standard curve for 4-hydroxyquinoline. The standard curve was obtained by measuring the absorbance at 330 nm and is shown in Fig. 10.

(c) **Acetylcholinesterase**

Acetylcholinesterase (ACHE, EC 3.1.1.7, acetylcholine hydrolase) is a neurotransmitter degrading enzyme. The essential role of acetylcholinesterase in excitable tissues seems to be the hydrolysis of acetylcholine to choline and acetic acid as given by the following reactions:

\[
N^+(CH_3)_3CH_2 - CH_2 - O - CO - CH_3 + H_2O \xrightarrow{AChe} N^+(CH_3)_3CH_2CH_2OH + CH_3COOH
\]
of the method is the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolysed. Thiocholine possesses a reactive -SH group and it reacts with 5,5-dithiobis-2-nitro-benzoate (DTNB) ion to produce the yellow anion of 5-thio-2-nitrobenzoic acid. The rate of colour production is measured by recording the increase in absorbance at 412 nm in a recording spectrophotometer.

\[
\text{Acetylthiocholine} \xrightarrow{\text{AChE}} \text{Thiocholine} + \text{Acetate}
\]

\[
\text{Thiocholine} + \text{DTNB} \rightarrow 5\text{-Thio-2-nitro-benzoic acid (yellow colour)}
\]

To release the membrane bound enzyme, the whole homogenate and the 12,000 x g pellet were treated with nonionic detergent, Triton X-100 (0.5% final concentration) for 45 minutes to 90 minutes. The activities after treatment with Triton X-100 were observed to be the same when compared to those of the non-treated fractions. Hence, enzyme activities were measured as such in the whole homogenate, pellet and the supernatant.

All determinations were carried out at 25°C in 1 ml of assay mixture containing 84 mM sodium phosphate buffer (pH 8.0); 0.32 mM dithiobisnitrobenzoate (DTNB)
prepared in 0.01 M phosphate buffer (pH 7.0); 0.48 mM acetylthiocholine iodide and the appropriately diluted enzyme preparation containing approximately 150-200 μg protein. The reaction was initiated by the addition of acetylthiocholine iodide and increase in absorbance was measured at 412 nm in a Shimadzu UV-260 spectrophotometer. All the enzymic assays were carried on fresh extracts.

8. CALCULATION OF ENZYME ACTIVITY AND DEFINITION OF UNIT

(a) Hexokinase

Enzyme activities were calculated by taking the molar extinction coefficient of NADPH as 6.22 x 10^6. An absorbance increase of 6.22 in 1 ml reaction mixture would then mean oxidation of 1 μmol of NADP, corresponding to conversion of 1 μmole of substrate. The formula for calculating units per gram per minute is given below:

\[
\text{Enzyme units per gram of tissue} = \frac{\text{Absorbance change per minute} \times \text{dilution}}{\text{Volume of extract} \times \frac{6.22}{\text{volume of assay mixture}}}
\]

One unit of activity is defined as one μmole of NADPH formed per gram of fresh weight of tissue per minute at 25°C.
(b) Monoamine oxidase

The enzyme activity was calculated using the following formula -

\[
\text{Units of activity per gram per 90 minutes} = \frac{\text{Standard value of 4-Hydroxyquinoline from graph} \times \text{Dilution X} \times \text{Volume of assay mixture}}{\text{Amount of extract taken}}
\]

\[
= \frac{\text{Standard value of 4-Hydroxyquinoline} \times 10 \times 3.6}{0.05}
\]

One unit of enzyme activity is defined as 1 μmole 4-hydroxyquinoline produced per 90 minutes per gram of tissue at 37°C.

(c) Acetylcholinesterase

The enzyme activity was calculated by taking molar extinction coefficient of the nitrobenzoate ion as 13.6.

\[
\text{Units of enzyme activity per gram of tissue/minute} = \frac{\text{Absorbance change X Dilution per minute}}{\text{Volume of extract X 13.6/ Volume of assay mixture}}
\]

One unit of enzyme activity is defined as 1 μmole of thiocholine produced per gram fresh tissue per minute.
9. DETERMINATION OF BLOOD GLUCOSE LEVEL

Blood glucose was estimated enzymatically by the method of Bergmeyer et al. (1974). Blood (50 μl) was taken into a tube containing distilled water (0.2 ml). The samples were frozen over night, thawed the next day and after centrifugation, the clear supernatant was used for the estimation of glucose by a coupled enzyme reaction.

The assay mixture contained in a final volume of 1.25 ml: Glycylglycine/Mg$^{2+}$ (50 mM/20 mM) pH 7.6; NADP (0.26 mM), ATP/Mg$^{2+}$ (8 mM/2 mM) pH 7.2, one unit of glucose-6-phosphate dehydrogenase and 20 μl of the extract. The reaction was initiated by the addition of one unit of hexokinase. The increase in optical density after obtaining the end point was taken as a measure of the glucose present in the extract. The amount of blood glucose was expressed as mg per 100 ml of blood (mg%). The same procedure was followed to determine the blood glucose levels of diabetic rats and propranolol treated rats.

10. ESTIMATION OF PROTEIN

Protein content of the different tissues was estimated according to procedure Lowry et al. (1951).
The alkaline reagent was prepared in the following manner.

(a) 2% Na$_2$CO$_3$ in 0.1 N NaOH
(b) 2% Na$^+$K$^+$ tartarate
(c) 1% CuSO$_4$

The final alkaline reagent was prepared by mixing a, b and c in the ratio of 98:1:1.

Tissue protein was dissolved in 0.1 N NaOH for 5 to 7 hours prior to estimation. Optical density was recorded after 45 minutes of addition of Folin's reagent (1N). Bovine serum albumin was used as standard. The protein values are expressed as mg per gram fresh weight of tissue.

11. STATISTICAL METHODS

The results are given as mean ± S.E.M. of four or more than four separate experiments for each value. 'p' values were calculated by student's t-test as described by Downie and Heath (1970). The 'p' values were considered significant if $p \leq 0.05$. 