CHAPTER II

Materials and Methods
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Animals

Rats of specified age (Wistar strain) were obtained from the Animal house of Indian Drugs and Pharmaceuticals Limited (I.D.P.L.), Hyderabad, and also from the Animal house of this University. Rats were fed on Hindustan Lever (New Delhi) "Rat and Mice Feed", which is complete in all nutritional aspects.

Materials

Highly polymerized calf thymus DNA, yeast RNA, bovine serum albumin, 2',3'-cyclic AMP, unlabelled nucleotides dATP, dGTP, dCTP and dTTP were purchased from Sigma Chemical Company, St. Louis, MO, USA. (\(^3\)H-methyl) dTTP (Sp. act. 46 Ci/m mole) was purchased from the Radio-chemical Centre, Amersham, England, 2',3'-dideoxythymidine triphosphate (ddTTP) was purchased from P.L. Biochemicals. Aphidicolin was a gift from Dr. A.H. Todd of I.C.I. Ltd., U.K. All the other chemicals used were of analytical grade.

Separation of White and Grey Matter Regions

Animals were sacrificed by decapitation at various stages of life. Cerebral hemispheres were carefully removed from the brain (i.e., Whole brain excluding brain stem, cerebellum and optic lobes). Starting from the dorsal side, the outer grey matter was gently scraped off with a scalpel until no more grey matter could be visualized. Then the underlying white
matter portion was collected separately. The whole separation was achieved with consistency after a few trial experiments. The separation was carried out at 0-4°C. Cerebellum was taken separately for the studies.

Preparation of Homogenates

White, grey and cerebellar regions of the rat brain were separately homogenized in 9 volumes of cold distilled water with Potter-Elvehjem type homogeniser. A portion of the homogenate was taken for the extraction of nucleic acids and the other was used for the assay of acid and alkaline DNases. For the assay of DNA polymerase, the tissues were homogenized in 0.5 M Tris-HCl buffer, pH 7.5 containing 0.1 mM β-mercaptoethanol, 1 mM MgCl₂, 0.1 mM EDTA, 5% glycerol, 1% Triton X-100 and 0.5 M KCl. After the homogenization the sample was kept at 0-4°C for 1 hr and centrifuged at 1,00,000 g for 1 hr. The clear supernatant thus obtained was used as the source of DNA polymerase.

Extraction of Nucleic Acids

The nucleic acids were extracted according to the procedure of Schmidt and Thannhauser (1945) slightly modified as per the suggestions of Munro (1966).

Two millilitres of 10% homogenate (w/v) was mixed with 2.5 ml of ice cold 10% TCA and centrifuged to remove acid soluble compounds. The sediment was washed once with 2.5 ml of ice cold TCA. The final sediment remaining after removal of the acid soluble compounds was extracted twice with 5 ml of 95% ethanol and the extract was removed by centrifugation.
An alcohol-ether (3:1) wash was given to the sediment to remove any traces of lipids. The lipid free pellet was suspended in 2 ml of 1 N KOH and incubated for 2 hours at 37°C. This incubation with 1 N KOH was sufficient to hydrolyze the brain RNA using rat brain. DNA and protein were then precipitated by addition of 0.4 ml of 6 N HCl and 2.6 ml of 5% TCA and allowed to stand in ice for 10 min and centrifuged. The supernatant fraction was collected separately to estimate RNA content. The sediment was suspended in 2.5 ml of 5% TCA and boiled at 90°C for 15 min with occasional shaking. The mixture was centrifuged and the supernatant was collected in a test tube. Now, the sediment was washed with 1.5 ml of 5% TCA and both the supernatants were taken for the estimation of DNA.

Estimation of DNA and RNA

DNA was estimated by the diphenylamine method while RNA was estimated by the orcinol reaction (Burton, 1956; Schneider, 1957). The DNA and RNA were also estimated by measuring the U.V. absorption of the acid soluble fraction at 260 nm.

Preparation of standards

DNA and RNA were first dissolved in water at a concentration of 2 mg/ml. Then a portion of this solution was diluted with 5% TCA and heated for 15 min at 90°C. The volume of the solutions so obtained was made up with 5% TCA in such a way that the final concentration became 200 μg/ml.
Estimation of Protein

Protein was estimated either by Biuret Method (Gornall et al., 1949) or as per the method of Lowry et al. (1951), depending upon the concentration of the protein in the sample.

Estimation of Phosphorus

Phosphorus in the standard DNA sample was measured by the method described by Bartlett (1959).

Assay Procedure for DNases

Acid and alkaline DNases were assayed essentially as described by McDonald (1955).

Acid DNase

The 3.0 ml reaction mixture consisted of 2 mg of DNA in 1 ml of water (highly polymerized, calf thymus), 0.1 M sodium acetate buffer, pH 5.1 and brain homogenate. At the end of 2 hr incubation at 37°C in a Dubnoff metabolic shaker water bath, the reaction was terminated by adding 2 ml of 1.4 N perchloric acid followed by chilling. The whole mixture was filtered through Whatman No. 42 filter paper and the absorbance of the filtrate (acid soluble deoxynucleotides) was measured in spectrophotometer at 260 nm against an appropriate blank. The enzyme activity was also followed by estimating the acid soluble deoxyribose by the diphenylamine colour reaction according to the procedure of Burton (1956). It was found that both these methods agreed well and therefore routine assay procedure consisted of spectrophotometer measurements with occasional cross checking with the colorimetric procedure.
The enzyme activity was expressed as μg of acid soluble DNA-phosphorus (DNA-P) liberated in two hours of incubation.

Alkaline DNase

The reaction mixture volume, method of assay and expression of activity and other details are the same as in the case of acid DNase, except that the reaction mixture consisted of heat denatured DNA as substrate (2 mg in 1 ml of water), 0.05 M Tris-buffer, pH 8.25, and the enzyme.

Preparation of Substrate

Heat denatured DNA was prepared by keeping a solution of DNA (2 mg/ml) at 100°C for 10 min and cooling it rapidly in an ice bath as described by Sung (1968).

DNA Polymerase Assay

The reaction mixture contained in a total volume of 50 μl, 40 mM Tris-HCl, pH 8.0, 1 mM β-mercaptoethanol, 7.5 mM MgCl₂, 4 mM ATP, 10 μg of 'activated DNA', 0.1 mM each of dATP, dGTP, dCTP and 20μM dTTP (1 μCi). Incubation was carried out at 37°C for 20 min. At the end of the incubation, 1 mg of DNA as a carrier was added and the reaction was stopped by adding 2 ml of cold 10% TCA. The samples were kept in ice for 10 min and centrifuged at 4,000 rpm. The precipitate thus obtained was washed thrice with 5% cold TCA and thrice with 95% ethanol. The precipitate after washings was dissolved in 0.1 ml of 0.05 M NaOH and aliquots were taken into radioactive vials containing 10 ml of Bray's mixture and were counted in a Beckman LS-3133P liquid scintillation counter.
The enzyme activity was linear up to 60 µg of protein. Specific activity was expressed as picomoles of TMP incorporated into DNA per mg protein per hour.

**CNPase Enzyme Assay**

2',3'-cyclic nucleotide 3'-phosphohydrolase (CNPase) was assayed as described by Probaska et al. (1973).

**Preparation of Homogenates**

Cerebral hemispheres were removed from the brain after decapitation, white and grey matter regions were separated physically. Ten percent homogenates were prepared in distilled water. The homogenate (0.1 ml) was solubilized by adding 0.1 ml of 0.2 M Tris-HCl, pH 7.5 and 0.2 ml of 1% sodium deoxycholate solution. After 10 min at 0-4°C, approximate amounts of distilled water were added and homogenized, so that the protein was of an approximate dilution of 0.1 mg/ml.

**Final CNPase Assay Conditions**

In 0.2 ml of final volume, 5-25 µg of protein were added to 7.5 mM 2',3'-cyclic AMP, 50 mM Tris-maleate buffer, pH 6.2. The substrate initiated reaction was carried out at 30°C for 10 min. A sample containing substrate and buffer with boiled enzyme served as a reagent blank. The reaction was terminated by placing the tubes in boiling water bath for 30 sec. The mixture was returned to the 30°C water bath when 0.1 ml of 0.3 M Tris-HCl containing 21 mM MgCl₂, pH 9.0 was added along with 60 µg of E. coli alkaline phosphatase. After the inorganic phosphate was
liberated the volume was made up to 3 ml by addition of water. 5 ml of isobutanol : benzene (1:1) and 1 ml of 1% ammonium molybdate were added to the above solution with shaking for 30 sec to get the yellow chromophore into the upper layer. The phosphomolybdic acid thus formed was reduced by the addition of stannous chloride solution (0.2 ml) in presence of alcoholic sulphuric acid (7 ml) and the stable blue colour thus obtained was measured at 720 nm.

The enzyme activity was expressed as μ moles of inorganic phosphate liberated per min.