# MATERIALS AND METHODS

Male albino rats, Rattus norvegicus albinus (Wistar strain) of 12.5, 25, 50, 75 and 100 weeks old were used for the study. The animals were maintained at 28 ± 2°C and 12 hr light and 12 hr dark conditions. The animals were caged in polypropylene translucent cages with a detachable stainless steel wiremesh at top and bottom. Four animals were kept in each cage with water and food (Commercial rat and mice feed, Hindustan Lever Ltd., Bombay) at ad libitum.

The rats were decapitated, the brain was excised, blotted and chilled immediately. Dissections were performed on ice-cooled glass plate. The following six regions of brain were separated as described by Glowinski and Iversen (1966): 1) hypothalamus, 2) medulla-oblongata, 3) midbrain, 4) cerebral cortex, 5) striatum and 6) cerebellum. The 'medulla oblongata' corresponds to the medulla oblongata and the pons; the 'midbrain' corresponds to the midbrain, thalamus and subthalamus (the latter structure being part of the diencephalon); the 'cortex' corresponds to the telencephalon without the striatum, it includes the white and the grey matter of the cerebral cortex; the 'striatum' contains the putamen nucleus, caudate nucleus and the globus pallidus nucleus (i.e., the basal ganglia of the telencephalon without the amygdala). The brain regions were washed in ice cold 0.9% sodium chloride solution to remove the blood clots.

## Estimation of organic solvent soluble lipofuscin

Lipofuscin pigment determination in tissue extracts was quantitated by the use of its fluorescent property. Analysis of fluorescent products was performed according to Fletcher et al. (1973). 200 mg of the tissue was homogenised and extracted with 2:1 chloroform:methanol mixture (spectral grade). The solvent to tissue ratio was 20:1 (v/w). Spectrophotofluorometric measurements were made with Perkin Elmer MPF-44 fluorescence spectrophotometer. The excitation maximum was 365 nm and the emission maximum was 445 nm. The spectrofluorometer was standardized each time with a fresh solution of quinine sulphate (Sigma). The instrument was calibrated to read 100 fluorescence units for 1 µg quinine sulphate/ml of 0.1N sulphuric acid. The lipofuscin content is expressed in terms of relative fluorescence units.

## Histochemical demonstration of lipofuscin pigments

The animals were decapitated and the brain was excised to isolate the different brain regions already mentioned. The brain tissues were immediately fixed in 10% neutral buffered formalin for 3 days. The tissues were dehydrated in graded alcohols, cleared in xylene, infiltrated with paraffin by routine procedures. Serial paraffin sections were cut and stained by the following methods as described by Pearse (1968, 1972).

a) Periodic acid-Schiff's technique

The sections were brought to water, oxidised for 10 min in 1.0% aqueous periodic acid and washed in running water. They were then immersed in Schiff's reagent for 10 min and again washed in running water. Nuclei were counterstained with Mayer's haemalum for 2-3 min and sections were washed in running water, dehydrated in alcohol, cleared in xylene and mounted in DPX.

b) Sudan black B technique

Sections were brought to 70% alcohol and stained for 3 hr at room temperature in saturated solution of Sudan black B in 70% alcohol. Excess stain was removed by rinsing quickly in 70% alcohol. The sections were washed under running water and mounted in glycerine jelly.

c) Schmorl method for lipofuscin

The sections were brought to water and immersed for 5 min in freshly prepared ferricyanide solution (3 parts of 1% ferric chloride and 1 part of 1% potassium ferricyanide). After washing in running water, the nuclei were counterstained in 1% neutral red for 3 min and the sections were dehydrated rapidly in alcohol, cleared in xylene and mounted in canada balsam.

d) Nile Blue sulphate method for distinguishing melanins from lipofuscin

The sections were brought to water, stained in Harris

hematoxylin for 10 min and washed in running water. They were then stained in freshly prepared saturated aqueous solution of Nile Blue sulphate for 30 min and rinsed in distilled water. Sections were immersed in 10% hydrogen peroxide for 24 hr, washed in running water and mounted in glycerine jelly.

Adjacent sections of the same regions were stained by different methods in order to obtain a comparative picture of the staining property of the tissue with different techniques. Sections of a particular region of various ages were stained simultaneously in order to maintain identical conditions.

Determination of the rate of lipid peroxidation

Lipid peroxide formation was determined by the thio-barbituric acid (TBA) test according to Wilbur et al. (1949). The tissue was homogenised in 0.05M phosphate buffer, pH 7.8, and centrifuged for 30 min at 6,000 RPM. One ml of the supernatant was incubated at 37°C for 1 hr and at the end of this period, the reaction was stopped by the addition of 1 ml of 35% TCA and 2 ml of 0.75% aqueous TBA. The contents of the tube were mixed well and kept in boiling water bath for 15 min, with occasional stirring. After cooling the tubes to room temperature, 2 ml of 70% TCA was added and the contents of the tube were mixed well. Turbid lecithins were dissolved by the addition of 3 ml of chloroform. After centrifugation for 15 min at 2,500 RPM, the optical density of the clear pink supernatant was read at 532 nm. The rate of lipid peroxidation

is expressed as nmoles malonaldehyde released/g fresh tissue/hr at 37°C.

## Estimation of deoxyribonucleic acid

Nucleic acids were extracted by the method of Schneider (1945). The tissues were homogenised in 5 ml of ice cold distilled water. To this, 5 ml of 10% TCA was added and kept in ice for 30 min to allow complete precipitation of proteins and nucleic acids. This was followed by centrifugation for 20 min at 5,000 RPM. The precipitate was washed with ice cold 10% TCA three times. The acid washed tissue residues were washed with 10 ml of ethanol and ether to remove lipid material. After each washing the tubes were centrifuged and the precipitates were resuspended in 5 ml of 5% TCA and kept in water bath maintained at 90°C for 15 min with occasional stirring to facilitate the quantitative separation of RNA and DNA from the precipitated proteins. The supernatant was used for the estimation of DNA after centrifugation.

DNA was estimated by measuring the intensity of the blue colour produced with diphenylamine method of Burton (1956). The extinction was measured at 660 nm against a reagent blank. Calf thymus DNA (Sigma) was used as standard. DNA content is expressed as mg DNA/g wet weight of tissue.

## Determination of lysosomal enzyme activities

The animals were decapitated, the brain was excised and washed in 0.9% sodium chloride solution to remove the blood

clots. Different regions of brain mentioned earlier were isolated and homogenised in 0.32M sucrose to give 5% (w/v) tissue suspension. Latency was abolished with the inclusion of Triton X-100 in 0.32M sucrose used for dilution to facilitate assay of total lysosomal acid hydrolase activities (Triton X-100 concentration equalled to 0.1% in final incubation mixture). All sample preparations were performed at 4°C and the samples were kept in ice until assay. Enzyme assays were performed on the supernatant fraction of the sample obtained by centrifuging the sample at 10,000 RPM for 30 min at 0°C.

a) Acid phosphatase

Acid phosphatase activity was determined according to Trouet (1974). 1 ml of the enzyme extract was incubated with 1 ml of 0.5M sodium-β-glycerophosphate (prepared in 1M acetate buffer, pH 5.0) for 1 hour at 37°C. The reaction was stopped by the addition of 10 ml of 8% TCA (w/v). Blank was run in a similar way except that the substrate was added after the termination of the reaction. The denatured proteins were filtered and the inorganic phosphate in 1 ml of the filtrate was determined by adding 1 ml of 2.5% ammonium molybdate, 1 ml of 5N sulphuric acid and 0.4 ml of 10 mM amino naphtho sulphonic acid. The optical density of the blue colour obtained was read after 10 min at 660 nm against the reagent blank. The enzyme activity is expressed as nmoles inorganic phosphate released/mg protein/hr.

b) N-Acetyl β-glucosaminidase

N-Acetyl β-glucosaminidase activity was determined according to Levy and Conchie (1966). One ml of the enzyme extract was incubated with 2 ml of 10 mM p-nitrophenyl N-acetyl β-glucosaminide (Sigma) in 0.2M citrate buffer pH 4.8 for 1 hour at 37°C. The reaction was terminated by the addition of 4 ml of 0.4M glycine-sodium hydroxide buffer pH 10.5. The mixture was centrifuged at 3,000 RPM for 20 min and the liberated nitrophenol in the supernatant was measured against a blank (in which the glycine-sodium hydroxide buffer was added before the substrate) at 430 nm. p-Nitrophenol was used as the standard. The enzyme activity is expressed as µg nitrophenol released/mg protein/hr.

c) β-Glucuronidase

β-Glucuronidase activity was determined according to Levy and Conchie (1966). One ml of the enzyme extract was incubated with 1 ml of 0.005M phenolphthalein β-glucuronide (Sigma) in 0.2M acetate buffer pH 4.5. After 1 hour incubation at 37°C, the reaction was terminated by addition of 4 ml of 0.4M glycine-sodium hydroxide buffer pH 10.7. The mixture was centrifuged at 2,000 RPM for 30 min and the extinction was measured against reagent blank (in which the glycine-sodium hydroxide buffer is added before the substrate) at 545 nm. Phenolphthalein was used as the standard. The enzyme activity is expressed as µg phenolphthalein released/mg protein/hr.

Protein content was determined by the method of Lowry et al. (1951). Bovine serum albumin (Sigma) was used as standard.

Lysosomal enzyme histochemistry

Various regions of the brain were dissected out quickly and frozen on a microtome chuck. Thick sections of 10 micra thickness were cut on a cryostat and mounted on coverslips. The enzyme activities were determined according to Pearse (1968, 1972).

a) Acid phosphatase

Modified Gomori's method was used. Frozen sections were incubated with the substrate, sodium β-glycerophosphate buffered at pH 5.0 with acetate buffer for 1 hour at 37°C. After rinsing thoroughly in distilled water, the sections were immersed in 2% acetic acid for 2 min. Following another rinse in distilled water, the sections were immersed in dilute solution of yellow ammonium sulphide for 1 min, again washed in distilled water and mounted in glycerine jelly. For 'control' sections, the substrate in the incubating medium was replaced by distilled water.

b) N-Acetyl β-glucosaminidase

Naphthol AS-BI-HPR method was used. Frozen sections were treated with cold ethanol for 2 min and rinsed with distilled water at 20°C to remove ethanol. They were then incubated in

the working solution for 1 hour at 37°C and rinsed with distilled water. The nuclei were counter stained with chloroform washed methyl green. The sections were washed in running water and mounted in glycerine jelly. For 'control' sections, Naphthol AS-BI-N-acetyl glucosaminide was replaced by distilled water in the working solution.

c) β-Glucuronidase

Naphthol AS-BI-glucuronide method was used. Frozen sections were treated with cold ethanol for 2 min and rinsed in distilled water to remove ethanol. They were then incubated in the working solution for 1 hour at 37°C and rinsed with distilled water. The nuclei were counterstained with chloroform washed methyl green for 10 min. The sections were washed in running water and mounted in glycerine jelly. For 'control' sections, Naphthol AS-BI-glucuronide in the working solution was replaced by distilled water.

Vitamin E deficiency

Vitamin E deficiency was induced by feeding the animals with vitamin E deficient diet for 10 weeks (Draper et al., 1958). The composition of the diet was 67.1% anhydrous dextrose, 20% vitamin free casein, 8% stripped corn oil, 4% salt mix (No.4164 of Draper et al., 1964), 0.5% vitamin mixture (devoid of vitamin E, ICN Pharmaceuticals, Cleveland, Ohio), 0.1% choline chloride, 0.3% DL-methionine. In addition to this 10,000 IU vitamin A and 1,000 IU vitamin D were added per kg

of diet. The composition of the diet for control animals of the same age (65 weeks) was essentially the same except that the vitamin mixture was fortified with vitamin E.

At the end of the 10 week dietary regimen, the animals were sacrificed to determine the organic solvent soluble lipofuscin content and the rate of lipid peroxidation in different regions of the brain.

Centrophenoxine administration

Rats were injected with centrophenoxine (80 mg/kg body weight) intramuscularly daily for 10 weeks. Age matched controls (65 weeks) were injected with normal saline daily for 10 weeks. At the end of this treatment period, the animals were sacrificed to determine the organic solvent soluble lipofuscin content and the rate of lipid peroxidation in different regions of the brain.