Albino female rats of Holtzman strain obtained from the experimental facility of V.C.Chest, Institute of Delhi University were used in this investigation. The rats were housed in cages (size 8"x 12"x 5"), which were made of transparent polystyrene with covers made of stainless steel or zinc plated thin iron rods; in each cage only four to five rats were housed. The bedding, pine savings were changed twice a week. Food and water were provided ad libitum. The food consisted of commercial rat food pellets obtained from Hindustan Liver Ltd., Delhi. The temperature of the animal room was maintained at 26±3°C. The room remained lighted only during 6 A.M. to 6 P.M.

**Chemicals**: All chemicals used were of analytical grade and were purchased mostly from Sigma Chemicals Co., U.S.A. The drugs (dimethylaminoethanol and Chlorpromazine) were obtained from Sigma Chemicals Co., U.S.A. and centrophenoxine (hexerol) was obtained from Fromontal Chemische Fabrik, West Germany.

**Experimental Set-Up**: Rats of three age-groups viz., 6 months, 9 months and 12 months were employed in this study. Each age-group was divided into three subgroups: one served as control, second received one dose of the drug and third received the other dose of the drug. The details of the experimental design are summarized and presented in table 1.

**Determination of the Dose to be Used**: Drugs used in this study were centrophenoxine (CIC), dimethylaminoethanol (JMAC or Jecnom) and chlorpromazine (CPZ). Centrophenoxine has been commonly used in the range of 60-100 mg/kg for the study of reduction of lipo-fuscin and life span extension (Hany, 1968, 1976; Hochschild, 1973b
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Centrophe­noxine (CPO)</th>
<th>Dimethylami­noethanol (DMAE)</th>
<th>Chlorproma­zine (CPZ)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Number</strong></td>
<td>6-8/each</td>
<td>6-8/each</td>
<td>6-8/each</td>
<td>6-8/each</td>
</tr>
<tr>
<td><strong>age-group</strong></td>
<td></td>
<td>age-group</td>
<td>age-group</td>
<td>age-group</td>
</tr>
<tr>
<td><strong>Age</strong></td>
<td>6,9 and</td>
<td>6,9 and</td>
<td>6,9 and</td>
<td>6,9 and</td>
</tr>
<tr>
<td>(months)</td>
<td>12 months</td>
<td>12 months</td>
<td>12 months</td>
<td>12 months</td>
</tr>
<tr>
<td><strong>Treatment</strong></td>
<td>0.9% NaCl</td>
<td>80 mg/kg &amp;</td>
<td>30 mg/kg &amp;</td>
<td>5 mg/kg &amp;</td>
</tr>
<tr>
<td></td>
<td></td>
<td>120 mg/kg</td>
<td>45 mg/kg</td>
<td>10 mg/kg</td>
</tr>
<tr>
<td><strong>Administra­tion</strong></td>
<td>1 ml/Rat</td>
<td>1 ml/Rat</td>
<td>1 ml/Rat</td>
<td>1 ml/Rat</td>
</tr>
<tr>
<td></td>
<td>intraperi­toneally</td>
<td>intraperi­toneally</td>
<td>intraperi­toneally</td>
<td>intraperi­toneally</td>
</tr>
<tr>
<td><strong>Frequency</strong></td>
<td>Daily</td>
<td>Daily</td>
<td>Daily</td>
<td></td>
</tr>
<tr>
<td><strong>Treatment Period</strong></td>
<td>Six weeks</td>
<td>Six weeks</td>
<td>Six weeks</td>
<td>Six weeks</td>
</tr>
<tr>
<td><strong>(45 days)</strong></td>
<td>(45 days)</td>
<td>(45 days)</td>
<td>(45 days)</td>
<td>(45 days)</td>
</tr>
</tbody>
</table>
Riga and Riga, 1974; Spoerri and Gless, 1975; Sandy and Lal, 1977; Jamieson and Taylor, 1981). A 80 mg/kg dose of centrophenoxine has been reported as most effective for lipofuscin reduction (Sandy, 1963, 1976, 1978). In the present work, a 80 mg/kg dose of centrophenoxine and slightly higher to 30 mg/kg i.e., 120 mg/kg dose to find out whether higher dose produces adverse effect, has been used.

Centrophenoxine in aqueous solution gets hydrolysed into its constituents: dimethylaminoethanol and chlorphenoxyacetic acid in the ratio of approximately 1:3 (Feldre et al, 1962). Since our objective was to study whether DMAE moiety alone or centrophenoxine as such were responsible for lipofuscin reduction, the doses of dimethylaminoethanol (30 and 45 mg/kg) corresponding nearly to the DMAE moiety of centrophenoxine doses used has been used.

5 mg/kg and 10 mg/kg dose of chlorpromazine reported to reduce the lipofuscin deposits (Samorajski and Rolsten, 1976) has been used in the present work.

**Drug Administration**: Drugs were administered to the rats of different age-groups intraperitoneally.

Centrophenoxine was dissolved in physiological saline and two doses - 80 mg/kg and 120 mg/kg were used.

Dimethylaminoethanol was diluted in physiological saline solution and prior to the administration to the animals, its pH was adjusted to 7.0 with HCl. Two doses 30 mg/kg and 45 mg/kg were used.

Chlorpromazine was dissolved in saline solution and two doses (5 mg/kg and 10 mg/kg) were used.
One day after the administration of the last dose of the drug, the animals were sacrificed by cervical dislocation. After decapitation, the skull was rapidly dissected open and the brain was quickly taken out and kept in ice. Cerebral hemisphere, cerebellum and brain stem were excised, weighed promptly and were processed for histochemical and biochemical determinations.

1. **Histochemical Methods for demonstration of Lipofuscin**: To study the histochemical distribution and localization of lipofuscin, brain tissues were fixed in formalin and were processed for preparing sections. Following methods were employed for histochemical localization of lipofuscin in paraffin sections of various brain regions.

   **Fluorescence method**: Fluorescence microscopic demonstration of lipofuscin was performed according to the method of Sandys (1971). Tissue sections were exposed to ultraviolet light at a wavelength of 365 nm and emission range of 380-460 nm. This was done on an ERNST Leitz GMBH Fluorescence Microscope.

   **Paraaldehyde Fuchsin method**: Localization of lipofuscin was made by paraaldehyde fuchsin method (Adams, 1965; Dixon, 1955; Singh and Mukherjee, 1972).

   **Cresyl violet method**: Microscopic demonstration of lipofuscin by cresyl violet method was done according to the method given by Samorajski et al (1965).

2. **Biochemical Methods**:

   A. **Preparation of homogenate**: The tissues were homogenised in 0.25 M sucrose solution by using Potter Elvehjem type homogeniser fitted with teflon plunger. The ratio of the tissue to solution was 1:10.
B. Subfractionation: The whole homogenate was further subfractionated by differential centrifugation according to the method of Hess and Brand (1974).

(i) The homogenate was first centrifuged at 1000 x g for 15 minutes in a refrigerated centrifuge (K-24 MLW). The supernatant obtained contains mitochondria, microsomes, synaptosomes, myelin and cytosol and is called as post nuclear supernatant fraction, while the pellet contains nuclei and tissue debris. This supernatant was used for the assay of the catalase enzyme.

(ii) The pellet obtained in (i) was resuspended in homogenising medium and centrifuged at 1000 x g for 15 minutes. The resulting supernatant contains some residual mitochondria, pellet was discarded.

(iii) The supernatant obtained in (i) and (ii) were mixed together. The mixture was centrifuged at 12,000 x g for 60 minutes in K-24. The pellet obtained in this step consists of crude mitochondria, synaptosomes and myelin, the supernatant contains microsomes and cytosol.

(iv) The pellet (iii) was once washed with homogenising medium. Pellet (iv) and supernatant (iii) were used for the assay of the enzymes SOD, GR, GP, GG-PDH and 6-PGDH.

(v) The supernatant (iii) was centrifuged at 1,00,000 x g for 60 minutes in an ultracentrifuge (VAC 601). The pellet obtained in this step consists of microsomes, while the supernatant contains cytosol. Both the supernatant and pellet were used for the assay of age-related changes in GG-PDH and 6-PGDH.

Determination of Enzyme Activities:

Activation of enzyme in particulate fraction: Particulate frac-
Brain tissue

<table>
<thead>
<tr>
<th>Homogenised in 0.25 M sucrose (1:10)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Whole homogenate</td>
</tr>
<tr>
<td>1000 x g for 15 min.</td>
</tr>
<tr>
<td>Pellet I</td>
</tr>
<tr>
<td>Supernatant I</td>
</tr>
<tr>
<td>(Nuclear and tissue debris)</td>
</tr>
<tr>
<td>(Mitochondria, microsome, cytosol etc.)</td>
</tr>
<tr>
<td>1000 x g for 15 min.</td>
</tr>
<tr>
<td>Pellet II</td>
</tr>
<tr>
<td>Supernatant II</td>
</tr>
<tr>
<td>(Residual mitochondria)</td>
</tr>
<tr>
<td>Supernatant (I + II)</td>
</tr>
<tr>
<td>12,000 x g for 60 min.</td>
</tr>
<tr>
<td>Pellet III</td>
</tr>
<tr>
<td>Supernatant III</td>
</tr>
<tr>
<td>(Crude mitochondria)</td>
</tr>
<tr>
<td>(Soluble fraction containing microsome and cytosol)</td>
</tr>
<tr>
<td>1,000,000 x g for 60 min.</td>
</tr>
<tr>
<td>Pellet IV</td>
</tr>
<tr>
<td>Supernatant IV</td>
</tr>
<tr>
<td>(Microsome)</td>
</tr>
<tr>
<td>(Cytosol)</td>
</tr>
</tbody>
</table>

**Fig. 1.** SCHEME FOR SUBCELLULAR FRACTIONATION
tion (pellet separated at 12,000 x g) was treated with triton (12) for 30 minutes to achieve activation and solubilization of the enzymes.

**Activation of catalase activity**; This enzyme catalyses the following reaction -

\[
2\text{H}_2\text{O}_2 \xrightarrow{\text{Catalase}} 2\text{H}_2\text{O} + \text{O}_2
\]

Catalase activity was assayed according to the method of Aebi (1974) in the post nuclear supernatant fraction separated at 1000 x g. The assay mixture contained 0.05 M phosphate buffer; 0.2% H₂O₂ and the sample (enzyme extract) in a final volume of 3 ml. Absorbance at 240 nm was recorded against reagent blank using Shimadzu UV-240 spectrophotometer. Enzyme activity was expressed as unit/gm wet tissue.

**Activation of superoxide dismutase**; This enzyme catalyses the following reaction -

\[
2\text{O}_2^- + 2\text{H}^+ \xrightarrow{\text{SOD}} \text{H}_2\text{O}_2 + \text{O}_2
\]

Superoxide dismutase activity was assayed according to the method of Thomas et al (1976). The assay mixture contained 0.5 M carbonate buffer (pH 10.2); 10⁻⁴ M EDTA; 2.5 x 10⁻⁵M nitroblue tetrazolium; 1 x 10⁻⁴M xanthine; 25 µl of xanthine oxidase (7.6x10⁻³ unit) and 0.05 ml of enzyme extract in a final volume of 3 ml. The reduction of tetrazolium was measured against reagent blank at 560 nm using Shimadzu UV-240 spectrophotometer. The SOD content was estimated by comparison with the homogenous erythrocyte SOD. A 50% inhibition of tetrazolium reduction was obtained by 0.225 µg of the superoxide dismutase used.

**Activation of glutathione reductase**; This enzyme catalyses the
following reaction -

\[
\text{GSSG} \xrightarrow{\text{Glutathione reductase}} \text{2GSH} \\
\text{NADH} + \text{H}^+ \rightarrow \text{NAD}^+
\]

Glutathione reductase assay mixture contained 0.1 M phosphate buffer (pH 7.0); 0.5 mM EDTA; 2 mM GSSG; 0.1 mM NADPH and the enzyme extract in a final volume of 1.0 ml. Glutathione reductase was assayed according to the method of Carlberg and Mannervich (1975). Absorbance was recorded against reagent blank at 340 nm using Shimadzu UV-240 spectrophotometer. Activity was calculated as umoles GSSG reduced per minute per gm wet tissue.

Estimation of glutathione peroxidase: This enzyme catalyses the following reaction -

\[
\text{H}_2\text{O}_2 + \text{GSH} \xrightarrow{\text{Glutathione peroxidase}} 2\text{H}_2\text{O} + \text{GSSG}
\]

Glutathione peroxidase was assayed according to the method of Prohaska and Sander (1976) with some modifications. The assay mixture contained 0.1 M phosphate buffer (pH 7.0); 3 mM EDTA; 1 mM sodium azide; 0.11 mM NADH; 1 unit of glutathione reductase; 100 mM H\text{2}O\text{2} and the sample in a final volume of 1 ml. Absorbance was measured against reagent blank using Shimadzu UV-240 spectrophotometer at 340 nm. Activity of the enzyme was calculated as umoles NADH oxidized per minute per gm wet tissue.

Estimation of glucose 6-phosphate dehydrogenase: This enzyme catalyses the following reaction -

\[
\text{Glucose 6-phosphate} \xrightarrow{\text{G6-PDH}} \text{6-phosphogluconate} \\
\text{NAD}^+ \xrightarrow{\text{NADH} + \text{H}^+}
\]
The activity of glucose 6-phosphate dehydrogenase was assayed according to the method of Saquer and McLean (1972). The assay mixture (in a total volume of 1.0 ml) contained 0.1 M TrisCl (pH 7.6); 2 mg/ml NADP; 0.5 m glucose 6-phosphate; 0.1 M glycylglycine (pH 7.6) and the tissue extract. Absorbance was recorded against reagent blank at 340 nm using Shimadzu UV-240 spectrophotometer and the enzyme activity was calculated as umoles NADP reduced per minute.

**Estimation of 6-phosphogluconate dehydrogenase**: This enzyme catalyses the following reaction -

\[
\text{6-Phosphogluconate} \xrightarrow{\text{6-PGDH}} \text{Ribulose 5-phosphate}
\]

The activity of 6-phosphogluconate dehydrogenase was assayed according to the method of Lashen and Talwar (1967). The assay mixture contained 2 mM 6-phosphogluconate; 2 mg/ml NADP; 0.25 M/0.1 M glycylglycine/MgCl₂ and the tissue extract in a final volume of 1 ml. Absorbance was recorded against blank reagent at 340 nm using Shimadzu UV-240 spectrophotometer and the enzyme activity was calculated as umoles NADP reduced per minute.

**Estimation of monoamine oxidase**: This enzyme catalyses the following reaction -

\[
\text{Biogenic amine} \xrightarrow{\text{Monoamine oxidase}} \text{H}_2\text{O}_2 + \text{Aldehyde} + \text{NH}_3
\]

Monoamine oxidase was assayed according to the method of Meynil et al (1982). The assay mixture contained Tris-HCl buffer 0.05 M (pH 7.4), kynurenine dihydrobromide 0.22 mM, MgCl₂ 0.08 M and the appropriate enzyme preparation containing 0.6 to 1.0 mg protein in a final volume of 5 ml. The reaction was stopped by the
addition of 0.2 ml of 0.5 M NaOH and 0.4 ml of 10% Im3C4. The mixture was heated in a boiling water for 5 min. and centrifuged at 16,000 x g for 10 minutes. The concentration of reaction product, 4-hydroxyquinoline, was determined spectrophotometrically by measuring the increase in absorbance at 330 nm using Shimadzu UV-240 spectrophotometer. One unit of enzyme activity is defined as umoles 4-hydroxyquinoline produced per 90 min. per gm tissue.

**Estimation of acetylcholine esterase**: This enzyme catalyses the following reaction:

\[
\text{Acetylcholine} \xrightarrow{AchE} \text{Acetate} + \text{Choline}
\]

Acetylcholinesterase activity was assayed according to the method of Illman et al (1961). The assay mixture contained 0.1 M phosphate buffer (pH 8.0); 0.075 M acetylcholine iodide; 0.01 M dithionitrobenzoic acid and the enzyme extract in a final volume of 3 ml. Absorbance was recorded against reagent blank at 412 nm in a Shimadzu UV-240 nm spectrophotometer. Enzyme activity was expressed as umoles acetylcholine hydrolysed per minute.

**Estimation of lipid peroxide**: Lipid peroxide was estimated according to the method of Rehncrona et al (1980). Aliquots (0.5 ml) of the brain homogenate (from 12 month old rat) were extracted with 0.5 ml of TCA (20% w/v). After centrifugation, 0.9 ml of the supernatant was added to 1.0 ml of thiobarbituric acid (0.67%) dissolved in 0.26 M Tris-HCl buffer (pH 7.6). The sample was heated in boiling water for 10 minutes. After cooling the absorbance at 532 nm was recorded and activity of lipid peroxide was expressed as umoles per gm wet tissue.

**Biochemical estimation of lipofuscin**: To determine whether drug administration in rats resulted in the lipofuscin reduction, the
Lipofuscin content of brain was determined spectrophotometrically in experimental and control animals. Method of Tappel et al. (1973) was used for the biochemical estimation of lipofuscin. Brain tissue was homogenised in chloroform: methanol medium (2:1) in a 20:1 ratio. Homogenates were centrifuged at 1200 x g for 10 minutes. Aliquots were washed with distilled water and fluorescence emission of chloroform layer was recorded at 450 nm upon excitation at 370 nm in a spectrofluorometer. Activity was expressed as units per 0.2 g wet tissue.

3. **Electrophysiological Methods**

**Implantation of electrodes:** Animals were anaesthetized by intraperitoneal injection of nembutal (35 mg/kg) for stereotaxic implantation of the cortical electrodes according to the method given by Klemm (1964). After positioning the rat in a stereotaxic instrument (Narishige, Japan), a short mid-sagittal scalp incision exposed the calvarium. After drilling the holes in the skull bone at determined positions, the screw electrodes were implanted over parietal and occipital cortices one right and one left in each. Electrodes were fixed on the skull with the acrylic dental cement. 0.2 cc of penicillin to counteract infection was administered intramuscularly following surgery and a period of at least 5–7 days was allowed for recovery from surgical intervention.

**Recording of electrocorticographic activity:** For recording, the signals were led off for amplification from the animals through suitable connectors and cables. The eCoG was recorded in two channels (from both left and right sides) of Grass Polygraph (790 EEG Polygraph). Recordings were obtained from conscious unrestrained animals. This technique permits almost complete freedom
of movement of the animals and minimizes mechano-electrical artifacts. Activity from one pair of electrodes was also fed into RC integrator amplifier to obtain a record of integrated trace which facilitated analysis of the EEG wave form.

Recording of multiunit activity (MUA): For recording of multiunit action potential, signals from the electrodes implanted over the parietal cortex were fed to Grass Amplifier 1511 with low and high voltage cut off set at 300 Hz and 10 kHz respectively. High pass filter completely eliminated slow wave EEG signals, leaving only much higher frequency multiunit activity components (action potentials). Cut-out of amplifier was fed to a Window discriminator (Model-1211W) and through the multiplex of the discriminator, the multiunit activity was displayed on a dual beam Storage Tektronix Oscilloscope. Window discriminator circuit further eliminates the noise of amplifier (lower aperture at 4 mv) and detects the action potential by producing a standard pulse whenever signals fall within a selective voltage range (lower aperture at 4 mv and upper aperture at 16 mv). The standard pulse (undiscriminated) were displayed on the second channel of dual beam oscilloscope for direct comparison with the multiunit spikes and were also passed to a time-gated digital event counter (Tektronix) which gave a read out of the number of spikes per 10 sec. A diagram of the experimental design used for the multiunit recording is given in fig. 2.

Evaluation of the drug effects: Before obtaining the recording, rats were first allowed to accommodate to the recording set-up for 5-6 days.

a. Effect of the chronic doses of the drug: Effect of the chronic ad
Fig. 2. Experimental design of multi unit recording.
administration (45 days) of two doses of each drug was studied in the different age-groups of rats. LCoG and MUA were recorded on 8th, 15th, 22nd, 30th and 45th day of the drug administration. Recordings were carried out always at the same hours of the day. On the recording session day drug was not administered to the animals.

b. Effect of acute dose of the drug: For determining the effect of acute dose, in each recording session LCoG and MUA were recorded as follows:

(i) Base line - a 20 minutes control period record during which preinjection unit firing rate and LCoG were recorded.

(ii) Saline treatment - Recordings were obtained after saline injection for 20 min. in the same manner as given in (i).

(iii) Drug effect - Thereafter the dose of the drug to be studied was administered and recording of LCoG and MUA were obtained for about 60 minutes.

(iv) Recovery - Following the records as obtained in (iii) recordings were continued till the pre-drug records reappeared. Partial recovery was considered to have taken place and experiments was then terminated if unit activity attained 30-50% of its pre-drug firing rate. If after sixty minutes recording periods, the pre-drug activity of MUA was not achieved, 5 minutes recordings at 30 minutes interval were made until the normal activity was attained.

c. Sleep-wakefulness cycle evaluation: For the study of sleep-wakefulness cycle (SWS), the three basic vigilance states defined by Role et al (1969) were followed.

(i) Wakefulness - This was characterised by the presence of rapid
waves (fast waves) of small amplitude in electrocorticograms, rats moving about, sitting or lying down with open eyes in a state of repose.

(ii) Nonrapid eye movement sleep (NREM) - EEG with spindles or slow waves of high amplitude, rats lying down with closed eyes.

(iii) Rapid eye movement sleep (REM) - High frequency low amplitude, rats lying down with eyes closed and showing occasional muscle twitches.

The periods of three states (no attempt was made to distinguish between spindle and slow wave REM) were calculated in minutes.

The testing procedures as outlined above were used in all experiments which included recording sessions on three consecutive pre-drug days, on the day of drug administration and on the post-drug day. Intraperitoneal injection was made at 8.30 a.m. and immediately afterwards EEG were recorded continuously for 6-8 hours on five consecutive days. In the first three days, 1.0 ml saline injection was given in order to have control hypnograms.

On the fourth day the drug dissolved in saline was administered. In the fifth day possible delayed effects of the drug given on the fourth day were checked for.