GENERAL MATERIALS AND METHODS
MATERIALS

1. Chemicals
Caffeine was purchased from Fluca-Chemica-Biochemica (Switzerland). γ-Aminobutyric acid (GABA), 3-methyl-2-benzothiazolinone hydrazone (MBTH), adenosine triphosphate (ATP), α-ketoglutaric acid (α-KG), pyridoxal-5-phosphate (PALPO), ninhydrin, L-glutamic acid, catalase (CAT, 2000-5000 U / mg protein), glutathione reductase (100-300 U / mg protein), adenosine diphosphate (ADP), pyrogallol, reduced glutathione (GSH), reduced nicotinamide adenine dinucleotide phosphate (NADPH), corticosterone, ascorbic acid used in the experiments were purchased from Sigma Chemical Co., St. Louis, MO, USA. \(^{3} \text{H}\)-GABA (Sp. activity 60 Ci / mmole) was bought from New England Nuclear, Boston, USA. \(^{3} \text{H}\)-thymidine (Sp. activity 6.5 Ci / mmole) was purchased from BARC, Bombay, India. Scintillation fluid (Cocktail 'O') used was from Spectrochem Pvt. Ltd., Bombay, India. Sodium hydroxide (NaOH), potassium permanganate, potassium dihydrogen phosphate (KH$_2$PO$_4$), hydrochloric acid (HCl), sucrose, sodium dihydrogen phosphate (NaH$_2$PO$_4$), disodium hydrogen phosphate (Na$_2$HPO$_4$), sodium carbonate (Na$_2$CO$_3$), sodium bicarbonate (NaHCO$_3$), tartaric acid, copper sulphate (CuSO$_4$), sodium-potassium tartarate, sodium chloride (NaCl), potassium chloride (KCl), acetone, alcohol, magnesium chloride (MgCl$_2$), trichloroacetic acid (TCA), sulfuric acid (H$_2$SO$_4$), ammonium molybdate, magnesium sulphate (MgSO$_4$), Triton-X-100, ferric chloride (FeCl$_3$), 2,4-dinitrophenyl hydrazine (DNPH), chloroform, mercaptopoethanol, potassium hydroxide (KOH), ethylene diamine tetraacetic acid (EDTA), boric acid, ethanol, meta-phosphoric acid, imidazole, thiobarbituric acid (TBA), 2, 6-dichlorophenol indophenol (DCPI), hydrogen peroxide (H$_2$O$_2$) were purchased from BDH Chemicals Ltd., Poole, England. All other reagents used in the present investigation were of analytical grade.

2. Animals
Adult female swiss mice (body weight 20 - 25 gm) were used for the present investigation.
(a) Care of animals

i) All the mice kept in a cage [usually 12 mice per cage (10" × 6" × 5")], were maintained in a room temperature (28° ± 0.5°C) and at constant relative humidity (85 ± 5%). Animals were maintained in 12 h dark / 12 h light cycle and were feed with standard laboratory diet and water *ad libitum*.

ii) The body weight of the animals and their food and water intake were checked every alternate day.

(b) Composition of laboratory animals diet

Composition of the laboratory animals diet is given in Table 3.

**Table 3**: Composition of diet for laboratory animals.

<table>
<thead>
<tr>
<th>Component</th>
<th>Percentage (%)</th>
<th>Per Kilogram (gm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Carbohydrate (wheat flour)</td>
<td>66</td>
<td>660</td>
</tr>
<tr>
<td>Protein (casein)</td>
<td>20</td>
<td>200</td>
</tr>
<tr>
<td>Fat (ground nut oil)</td>
<td>8</td>
<td>80</td>
</tr>
<tr>
<td>Standard vitamin mixture*</td>
<td>2</td>
<td>20</td>
</tr>
<tr>
<td>Standard salt mixture**</td>
<td>4</td>
<td>40</td>
</tr>
</tbody>
</table>

*Standard vitamin mixture (Thiamine - 0.6 mg, Riboflavin - 1.2 mg, Pyridoxin - 0.4 mg, Niacin - 5 mg, Ca-pantathionate - 4 mg, Inositol - 100 mg, Vitamin B₁₂ - 1 μg, Paraaminobenzoic acid - 2.5 mg, Vitamin A - 200 I.U., Vitamin D - 20 I. U., Vitamin E - 12 mg, Vitamin K - 0.01 mg, Vitamin C - 45 mg, Biotin - 0.02 mg and Folic acid - 0.09 mg)
**Standard salt mixture in gm (NaCl - 4.2, KCl - 4.8, KH₂PO₄ - 12.5, Ca₃(PO₄)₂ - 4.1, CuSO₄ - 0.0121, ZnSO₄ - 0.06, KI - 0.0015, MgSO₄ - 1.807, FeSO₄ - 0.00008, NaF - 0.0028, K₂H₃(SO₄)₃·24H₂O - 0.0136 and CaCO₃ - 0.084).**

**METHODS**

1. **Experimental Design**

   (a) **Transplantation of Ehrlich ascites carcinoma (EAC) cells**

   The EAC cells were transplanted into recipient mice by intraperitoneal inoculation with 0.2 ml of ascites fluid containing 10⁷ cells (approximately). This cell concentration of ascites fluid was made up with sterile normal saline.

   (b) **Treatment of caffeine in mice with or without EAC cell for the measurement of locomotor activity (LA)**

   Mice were divided into 6 groups, each group containing 8-10 animals. Mice of group 2 were treated with caffeine at a dose of 20 mg / kg / day, (p.o maximum response observed, please see Table 4) in a volume of 0.2 ml for 1, 2, 4, 6, 12, 15, 18, 21, 24, 27 or 30 consecutive days. Animals of group 1 were treated with an equal volume of vehicle (water) of caffeine through the same route for the same period under similar conditions and were considered as control of the corresponding experimental group 2. Animals of group 4 were inoculated intraperitoneally (i.p) with EAC cells (0.2 ml) and they were allowed to develop EAC cells for 3, 6, 9, 12, 15 or 18 days. Mice of group 3 were treated with 0.2 ml of saline through the same route (i.p) under similar conditions and they were considered as control of the corresponding experimental group 4. Animals of group 6 were inoculated intraperitoneally (i.p) with EAC cells after 12 consecutive days of caffeine treatment and the caffeine treatment was continued for another
Table 4: Dose response effect of caffeine on locomotor activity (LA) of mice

<table>
<thead>
<tr>
<th>Dose of caffeine (mg / kg, p.o)</th>
<th>LA (arbitrary unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>17.34 ± 1.42</td>
</tr>
<tr>
<td>5</td>
<td>14.61 ± 1.41</td>
</tr>
<tr>
<td>10</td>
<td>27.69 ± 1.64&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>15</td>
<td>32.75 ± 2.28&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>20</td>
<td>35.10 ± 1.13&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>30</td>
<td>37.17 ± 1.76&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>40</td>
<td>36.04 ± 2.21&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM of 8-10 separate observations. All the experimental groups were treated with caffeine (5-40 mg / kg p.o) in a volume of 0.2 ml. Control groups corresponding to experimental groups were treated with vehicle (water) of caffeine in a same volume through the same route. No significant difference between the LA of the control groups of the corresponding experimental condition was observed. The value of LA presented in the table against 0 mg / kg caffeine is the average of all the above controls. Significantly different from control ap < 0.001; bp < 0.01
of group 5 were treated (i.p) with 0.2 ml saline (as vehicle of EAC cells) after treatment with an equal volume of vehicle (water) of caffeine through p.o (the route of caffeine treatment) for 12 consecutive days and then these vehicle of caffeine treatment was continued for another 3, 6, 9, 12, 15 or 18 consecutive days and were considered as control of the corresponding experimental group 6. The LA was measured after 30 min (the optimum time for response, please see Table 5) of the last caffeine treatment.

(c) Treatment of caffeine in mice with or without EAC cells for biochemical studies

Mice were divided into 7 groups. Each group was divided into three subgroups a, b and c. Each subgroups contained 4-6 animals. Animals of group 2a, 2b and 2c were treated with caffeine (20 mg / kg / day p.o.) in a volume of 0.2 ml for 24, 27 and 30 consecutive days respectively. Animals of subgroups 1a, 1b and 1c were treated with an equal volume of vehicle (water) of caffeine through the same route for the same period under similar conditions as described in animals of groups 2a - 2c. The subgroups 1a, 1b and 1c were considered as control of subgroups of experimental animals 2a, 2b and 2c respectively. Animals of subgroups 4a, 4b and 4c were the recipient of EAC cells. The EAC cells (0.2 ml) were transplanted intraperitoneally (i.p) to these three subgroups (4a, 4b and 4c) of mice and they were allowed to develop EAC cells for 12, 15 and 18 days respectively. Animals of subgroups 3a, 3b and 3c were treated with 0.2 ml of saline through the same route (i.p) under similar conditions as described in animals of groups 4a - 4c and they were considered as control of subgroups 4a, 4b and 4c respectively. The animals of subgroups 6a, 6b and 6c were inoculated intraperitoneally with EAC cells after 12 consecutive days of treatment with vehicle (water) of caffeine and treatment was continued for another 12, 15 and 18 consecutive days respectively. Animals of subgroups 7a, 7b and 7c were inoculated intraperitoneally with EAC cells after 12 consecutive days of caffeine treatment and the
Table 5: Time response effect of caffeine (20 mg / kg, p.o) on the locomotor activity (LA) of mice

<table>
<thead>
<tr>
<th>Time of treatment with caffeine (min)</th>
<th>LA (arbitrary unit)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>24.10 ± 2.10</td>
</tr>
<tr>
<td>10</td>
<td>38.27 ± 3.50(^b)</td>
</tr>
<tr>
<td>20</td>
<td>41.17 ± 4.31(^b)</td>
</tr>
<tr>
<td>30</td>
<td>51.81 ± 4.90(^a)</td>
</tr>
<tr>
<td>40</td>
<td>39.56 ± 3.86(^b)</td>
</tr>
<tr>
<td>60</td>
<td>26.67 ± 3.99</td>
</tr>
</tbody>
</table>

Results are expressed as mean ± SEM of 8-10 separate observations. No significant difference between the LA of the control groups of the corresponding experimental groups was observed. The results (LA) of the '0' h presented in this table is the average of LA of all the controls (not shown).

Significantly different from control \(a_p < 0.001; b_p < 0.01\)
caffeine treatment of the subgroups 7a, 7b and 7c was continued for another 12, 15 and 18 consecutive days respectively. Animals of subgroups 5a, 5b and 5c were treated (i.p) with 0.2 ml saline (as vehicle of EAC cells) after treatment with an equal volume of vehicle (water) of caffeine (p.o) for 12 consecutive days and then these vehicle of caffeine treatment was continued for another 12, 15 and 18 consecutive days as did in experimental groups (7a - 7c) and were considered as control of corresponding experimental subgroups 6a, 6b and 6c respectively as well as subgroups 7a, 7b and 7c respectively. The treatment of caffeine under above mentioned condition in presence and absence of EAC cells is also presented in a flowsheet (Fig. 5).

2. Collection of cells and tissues

At the end of the treatment, EAC cells were collected from peritoneum cavity. Mice of both control and experimental groups were killed by cervical dislocation. Caffeine treated animals were killed between 10.00 a.m. to 12 noon, after 30 min of the last caffeine administration and the whole brains were immediately either immersed in liquid nitrogen (within 30 sec of sacrifice) for the estimation of GABA levels or kept at ice cold condition (0°-4°C) for the assay of enzyme activities and the GABA receptor binding studies. Adrenal glands free from adhering fat tissue were collected either in 0.1 (N) HCl for the estimation of corticosterone or in 5% meta-phosphoric acid for the estimation of ascorbic acid. Liver was perfused immediately with cold 0.15 M KCl, containing 2 mM EDTA (pH 7.4), minced with scissors and tissues were collected under ice-cold condition (0°-4°C) for the measurement of antioxidant defense system. Blood was collected with heparin as an anticoagulant and plasma was prepared according to the method described by Talwar (279).
Group 1 (Control) treated with vehicle (0.2 ml water / day p.o) of caffeine for Caffeine treatment

Group 2 (Experimental) treated with caffeine (20 mg / kg / day p.o) for

Group 3 (Control) treated with saline (0.2 ml i. p) and experiments were carried out after inoculated with EAC cells

Group 4 (experimental) treated with EAC cells (0.2 ml i.p) and allowed to develop for

Group 5 (Control) treated with vehicle (0.2 ml p.o) of caffeine for 12 consecutive days prior to treatment with vehicle (0.2 ml of saline) of EAC cells and mice were kept for

Group 6 (Experimental) treated with vehicle (0.2 ml p.o) of caffeine for 12 consecutive days prior to the EAC cells inoculation (i.p) and EAC cells were allowed to develop for

Group 7 (Experimental) treated with caffeine (20mg/kg/day p.o) for 12 consecutive days prior to the EAC cells inoculation (i.p.) and EAC cells were allowed to develop for

During these periods the vehicle (0.2 ml water, p.o) of caffeine treatment was continued

During these periods the vehicle (0.2 ml water, p.o) of caffeine treatment was continued

During these periods the caffeine treatment (20mg/kg/day p.o) was continued

Fig. 5: Treatment of caffeine in mice with or without EAC cells
3. Preparation of hepatic microsomes

Liver microsome was prepared according to the method as described by Appel et al. (280). Liver tissue of mice was minced in ice-cold (0°-4°C) 0.25 M sucrose solution and homogenized with a Teflon glass homogenizer in the same sucrose solution to prepare a 10% (w/v) liver homogenate. The homogenate was centrifuged at 20,000 g for 20 min under cold condition (0°-4°C) to remove cell debris, nuclear fraction, mitochondria and lysosomal particles as pellet. This supernatant was then centrifuged under ice cold condition (0°-4°C) at 1,05,000 g for 60 min in an ultracentrifuge (Hitachi SCP70H) to obtain the desired microsomal fraction as pellet. This microsomal pellet was used for biochemical assay of enzymes.

4. Preparation of plasma from blood of mice

Trunk blood was collected with heparin as an anticoagulant and plasma was prepared according to the method described by Talwar (279). Blood was centrifuged at 4,000 rpm for 5 min. This supernatant known as plasma, was collected and used in the present study.

5. Parameters Studied

(a) Measurement of LA

The vertical rearing frequency of each animal was measured between 8.00 - 12.00 h during 5 min observation period to monitor the LA (281). LA of both control and experimental mice was measured 30 min after the last caffeine or its vehicle administration. After the treatment, the animals were gently placed back into their home cages for a period depending on the drug administered and then transferred to a transparent plastic chamber (24 x 24 x 20 cm³) illuminated
with an electrical lamp at the top. The vertical rearing frequency was measured by an electrical device based on the capacitance change proportional to the distance between the animal's head and the probe as described by Keenan and Johnson (282).

(b) **Parameters for Ehrlich ascites carcinoma (EAC) cell**

(i) **Estimation of cell viability of EAC cells**

Cell viability of EAC cells was estimated according to the method of Bekesi et al. (283). EAC cells were collected from peritoneum cavity. The cells were initially diluted and washed with normal saline and the pellets were finally washed and suspended in PBS (phosphate buffered saline containing 40 mM NaCl, 2.7 mM KCl, 6.5 mM Na₂HPO₄, 1.5 mM KH₂PO₄, pH was finally adjusted to 7.3 with 0.01 (N) NaOH solution) buffer. The 1 ml of cells suspension in PBS was added to 1 ml of trypan blue solution (0.1% w/v in saline) and this mixture was loaded into a hemocytometer and the number of unstained cells were counted separately. Viable cells excluded the dye, while non viable cells appeared blue in colour. Cell viability was expressed as \( x \times 10^6 \) cell /ml (where \( x \) = any number).

(ii) **Measurement of oxygen consumption of EAC cells**

Oxygen consumption of EAC cells was measured according to the method of Ray et al. (284). Oxygen consumption was measured in an oxygraph (Gilson, Villiers le Bel, France) equipped with a clark electrode. The 2 ml of the reaction mixture contained PBS, and \( 10^7 \) (approx.) EAC cells. Oxygen consumption was expressed as natom oxygen consumed / sec / ml.

(iii) **Measurement of \(^{3}H\)- thymidine incorporation in EAC cells**

\(^{3}H\) - thymidine incorporation to the EAC cells was measured following
the method of Kupka et al. (285). EAC cells were collected from peritoneum cavity. The cells were initially diluted and washed with normal saline and the pellets were finally washed and resuspended in PBS buffer and was used for the estimation of $[^{3}H]$ - thymidine incorporation. Cell suspension was incubated in a total volume of 1 ml containing PBS, 0.1 mg (8.5 units) heparin and 1 mg glucose with 0.1 $\mu$Ci $[^{3}H]$- thymidine (Sp. activity 6.5 Ci / mmole) for 30 min at 37°C. The cells were immediately centrifuged at 12,000 g in a microcentrifuge (REMI model no. RM/2C microcentrifuge) under 0°-4°C and the pellets were suspended in 1 ml of 5% TCA and collected on cellulose nitrate filter. Then radioactive count was taken in a liquid scintillation counter (model no. 1209 Rackbeta). The amount of $[^{3}H]$ - thymidine incorporated was expressed as pmole / $10^{7}$ cells / ml.

(c) Measurement of corticosterone level

(i) Measurement of corticosterone level in adrenal gland

corticosterone level in mice adrenal gland was estimated spectrofluorometrically following the method of Purves et al. and Vemikoss - Danielliis et al. (286, 287). After sacrifice the mice, adrenal glands free from adhering fat tissue were immediately taken out and collected in 0.1 (N) HCl. The tissue samples were homogenized (15% w/v) in a Teflon glass homogenizer (25 mm clearance, 12 stokes / tissue sample) in 0.1 (N) HCl and then centrifuged at 8,000 rpm for 10 min in a cold centrifuge. The supernatant was taken for the measurement of corticosterone. In 0.1 ml of adrenal supernatant, 0.2 ml of distilled water was added to make the total volume 0.3 ml and 4.5 ml of chloroform was added. After shaking for 15 sec, the mixture was centrifuged for 5 min in 2,500 rpm. 3 ml aliquot from chloroform layer was taken and mixed with 0.45 ml 0.1 (N) NaOH. This mixture was vortexed for 15 sec and centrifuged for 5 min in 2,500 rpm. 2 ml chloroform layer was taken and 2 ml $H_{2}SO_{4}$ reagent (2.4 volume of concentrated $H_{2}SO_{4}$ + 1.0 volume of 50% aqueous ethanol) was added. After shaking for 15 sec, centrifuged for 5 min in 2,500 rpm. Acid layer was taken and
fluorescence was measured at excitation and emission wavelength 470 nm and 520 nm respectively in spectrophotofluorometer (Hitachi model F3010). The concentration of corticosterone was expressed as µg corticosterone / mg protein.

(ii) **Measurement of corticosterone level in blood plasma**

Blood was collected with heparin as an anticoagulant and plasma was prepared according to the method described by Talwar (279). The method has been described in detail previously [5 (c) (i)]. Then corticosterone level was measured following the method of Purves et al. and Vernikoss - Daniellis et al. (286, 287). The method is same as described in the method for the estimation of corticosterone in adrenal gland. The concentration of plasma corticosterone was expressed as µg / ml.

(d) **Measurement of adrenal ascorbic acid level**

(i) **Measurement of total ascorbic acid level**

Level of total ascorbic acid in adrenal gland of mice was estimated spectrophotometrically following the method of Roe and Kuether (288). After sacrifice the mice, the adrenal glands free from adhering fat tissue were immediately collected in 5% meta-phosphoric acid. The tissue samples were homogenized in 0.5 ml meta-phosphoric acid and centrifuged at 4,000 rpm for 10 min. To 0.2 ml of this supernatant, 1 drop bromine water was added and kept for 10 min at room temperature. Then 0.5 ml DNPH was added and incubated for 3 hr at 37°C. In this reaction mixture, 2 ml 85% H₂SO₄ was added and incubated for 30 min and reading was taken in spectrophotometer (Hitachi U2000) at 540 nm. The concentration of total ascorbic acid was expressed as µg ascorbic acid / mg adrenal tissue protein.
(ii) *Estimation of reduced ascorbic acid level*

Reduced vitamin C in mice adrenal gland was estimated according to the method of Glick (289). Immediately after sacrifice the mice, the adrenal glands free from adhering fat tissue were taken out and collected in 5% metaphosphoric acid. The tissue samples were then homogenized in 2 ml metaphosphoric acid and centrifuged at 4,000 rpm for 10 min. 1 ml of this supernatant was titrated with standard solution (1 mg/ml) of DCPI (dye) until a faint pink colour appearing at the end point was obtained. The concentration of DCPI was obtained by using standard reduced ascorbic acid (0.5 mg/ml in metaphosphoric acid) stock solution. The unit of reduced ascorbic acid was expressed as μg ascorbic acid / mg adrenal tissue protein.

(e) *Parameters for hepatic antioxidant defense system*

(i) *Measurement of hepatic catalase (CAT) activity*

The activity of CAT (EC 1.11.1.6) in hepatic microsomal preparation was measured according to the method of Cohen *et al.* (290). Microsomal pellet was suspended in 0.5 ml of 0.05 M phosphate buffer, pH 7.0. 1% Triton X-100 was added in this solution. 30 mM H₂O₂ was added and reading was taken at 240 nm after 2 mins incubation in Hitachi spectrophotometer (U2000). The activity of CAT was expressed as mole of H₂O₂ / mg protein / min.

(ii) *Measurement of glutathione peroxidase (GPx) activity*

The microsomal glutathione peroxidase (GPx) (EC 1.11.1.9) activity of mice liver was measured following the method of Pierce and Tappel (291). The microsomal pellet was suspended in 0.1 M K-phosphate buffer (pH 7.0) contained 1 mM EDTA. Reaction was carried with 100 μl sodium azide (1 mM), glutathione reductase (2.4 U/ml in 0.1 M phosphate buffer, pH 7.0) 100 μl, reduced
glutathione (10 mM) 100 µl, 550 µl of 0.1 M phosphate buffer (pH 7.0) and 50 µl enzyme preparation in a total volume of 0.9 ml. The mixture was preincubated for 10 min at 37°C. Thereafter, 100 µl NADPH (1.5 mM in 0.1% NaHCO₃) solution was added. The overall reaction was started by adding 100 µl of prewarmed H₂O₂ (1.5 mM in 0.1 M phosphate buffer, pH 7.0) and decreased in absorbance at 340 nm was monitored for about 5 min in a spectrophotometer (Hitachi U2000). GPx activity was expressed as mole NADPH oxidized / mg protein / min.

(iii) Estimation of superoxide dismutase (SOD) activity

Liver tissue of mice was minced in ice-cold (0°-4°C) 0.25 M sucrose solution and homogenized with a Teflon glass homogenizer in the same sucrose solution to prepare a 10% (w/v) liver homogenate. The homogenate was centrifuged at 20,000 g for 20 min under cold condition (0°-4°C) to remove cell debris, nuclear fraction, mitochondria and lysosomal particles as pellet. The supernatant known as post mitochondrial fraction was used for SOD assay. The mice liver SOD (EC 1.15.1.1) activity was estimated according to the method of Marklund and Marklund (292) and Nandi et al. (293). In the supernatant of mice liver post mitochondrial fraction, 1% Triton X-100 was added. Reaction was carried in 50 mM Tris-HCl buffer (pH 8.5) 0.77 µl, CAT (20 µg) 0.025 ml, pyrogallol (0.13 mM) 0.1 ml, EDTA (1 mM) 0.1 ml and 5 ml enzyme preparation in a total volume of 1 ml. O.D at 420 nm was taken after 2 mins incubation in a Hitachi spectrophotometer (U2000). The activity of the enzyme (SOD) was expressed as unit / mg protein / min. 1 unit of SOD is described as the amount of enzyme required to cause 50% inhibition of pyrogallol auto-oxidation per 1 ml of assay mixture.

(iv) Determination of hepatic microsomal lipid peroxidation (LP)

Microsome of mice liver was prepared following the method of
Appel et al. (280) which has been described previously. Microsomal LP was estimated according to the method of Sinnhuber et al. (294). Microsomal pellet was suspended in 1 ml of 0.15 M KCl solution. The LP was estimated by incubating the reaction mixture containing Tris-HCl buffer (50 mM, pH 7.4), ADP-Fe (0.05 ml), NADPH (135 µg), KCl (50 mM) and 1 mg microsomal protein at 37°C for 30 min. The reaction was terminated by adding 1 ml 20% TCA and allowed to stand for 30 min for complete precipitation. Then the mixture was centrifuged for 10 min at 1,000 g. 2 ml of the supernatant was mixed with 2 ml of 0.67% TBA dissolved in 0.1 (N) HCl and then placed in boiling water bath for 15 min for developing the colour and cooled to room temperature. Intensity of the colour was measured spectrophotometrically at 543 nm. LP was expressed as mole of TBA reactive substances / mg protein / h.

(f) Parameters for whole brain GABA system

(i) Steady state level of whole brain γ-aminobutyric acid (GABA)

GABA level of mice whole brain was estimated according to the method of Lowe et al. (295). The frozen tissue samples were homogenized (10% w/v) in a Teflon glass homogenizer in 10% TCA and then centrifuged at 4,000 rpm for 10 min in a cold centrifuge. The supernatant was neutralized by 2 M KOH. 1 µl aliquot of this neutralized supernatant fluid was mixed with 2 µl of ninhydrin reagent (14 mM ninhydrin in 0.5 M carbonate buffer of pH 9.95) and was heated at 60°C for 30 min and then 50 µl copper tartarate (0.16% Na₂CO₃, 0.03% CuSO₄ and 0.032% tartaric acid) was added. After 15 min, fluorescence was measured at excitation and emission wavelength 377 nm and 451 nm respectively in spectrophotofluorometer (Hitachi model no. F3010). The concentration of whole brain GABA was expressed as µg / mg protein.
(ii) Estimation of glutamic acid decarboxylase (GAD) activity

The activity of GAD (EC 4.1.1.15) was determined by the method of MacDonnel and Greengard (296). Mice whole brain tissue was homogenized in ice-cold (0°-4°C) 0.32 M sucrose solution and homogenate was centrifuged in Hitachi 20 PR model cold centrifuge at 1,000 g for 10 min. The supernatant obtained was recentrifuged at 20,000 g for 20 min. The resulting pellet (P₂) was suspended in 0.16 M Na-phosphate buffer (pH 6.2) and used for the enzyme assay. Prior to the assay of enzyme, pellet (P₂) suspension was incubated with 0.5% Triton X-100 for 30 min at 0°C. The GAD activity was measured by incubating the reaction mixture containing 25 mM Na-L-glutamate, 0.5 mM PALPO and 1-2 mg enzyme protein in a total volume of 1 ml (volume was adjusted with 80 mM phosphate buffer, pH 6.2) at 37°C for 30 min. The reaction was stopped with 1 ml ice-cold (0°-4°C) 10% TCA and then centrifuged at 5,000 g for 10 min. From the supernatant, 1 μl was taken and 2 μl ninhydrin reagent (14 mM ninhydrin in 0.5 M carbonate buffer of pH 9.95) was added and the mixture was heated at 60°C for 30 min and then 50 μl of copper tartarate reagent (0.16% Na₂CO₃, 0.03% CuSO₄ and 0.032% tartaric acid) was added and after 15 min fluorescence was measured at excitation 377 nm and emission 451 nm in spectrophotofluorometer (Hitachi model no.F3010). GAD activity was expressed in terms of μg GABA formed / mg protein / h.

(iii) Measurement of GABA-transaminase (GABA-T) activity

Estimation of GABA-T (EC 2.6.1.19) activity was determined by the method of Sytinsky et al. (297). Mice whole brain tissue was homogenized in ice-cold 0.32M sucrose solution and the homogenate was centrifuged in a Hitachi 20 PR model refrigerated centrifuge at 1,000 g for 10 min. The supernatant obtained was recentrifuged at 20,000 g for 20 min. The resulting pellet (P₂) was suspended in 25 mM Tris-HCL buffer (pH 8.6) and used for the enzyme assay. Prior to assay of the enzyme, the pellet P₂ suspension was incubated with Triton-X-100 (final concentration 0.5%) for 30 min at 0°-4°C. The activity of GABA-T was assayed
by incubating the reaction mixture containing 10 μmole of α-KG and 10 μmoles in 1 ml of 0.25 M Tris-HCl buffer (pH 8.6), 5 μg PALPO and 1-2 mg enzyme protein at 37°C for 30 min. The reaction was terminated by adding 0.5 ml ice-cold 20% TCA. Then the mixture was centrifuged in a Hitachi 20 PR model cold centrifuged at 5,000 g for 10 min. Then 0.1 ml supernatant was taken and 0.5 ml 1% MBTH was added and kept in boiling water bath for 3 min and cooled to room temperature and then 1 ml 0.25% FeCl₃ (pH 2.0) was added. Just after 3 min, 4.0 ml acetone was added and reading was taken immediately in spectrophotometer (Hitachi U2000) at 660 nm. The activity of GABA-T was expressed as Δ₆₆₀ nm / mg protein / h.

(iv) Measurement of in vitro [³H] - GABA binding to its receptors

[³H] - GABA binding was measured according to the method of Ticku (298). Membrane protein was isolated as pellet according to the method of Ticku (298). Whole brain tissue of mice was homogenized with ice-cold (0°-4°C) 0.32 M sucrose using a Teflon pestle and glass homogenizer. The homogenate was centrifuged at 1,000 g for 10 min and the supernatant was centrifuged at 14,000 g in a Hitachi 20 PR model cold centrifuged for 45 min to obtain crude mitochondrial and microsomal (P₂+P₃) fraction. The (P₂+P₃) fraction was osmotically shocked with ice-cold water, centrifuged twice, resuspended in 0.05 M Tris-citrate buffer and frozen overnight. The tissue was then thawed, pelleted, and washed twice in the above buffer and frozen, thawed and washed and resuspended in the buffer in a dilution of 100 μg per 0.1 ml. Then 0.1 ml of membrane protein fraction was taken and mixed with buffer and water in presence of [³H] - GABA (320 nM) and cold GABA (0.1 mM) in a total volume of 1 ml and incubated at 0°-4°C for 10 min and centrifuged at 48,000 g for 15 min. The pellet obtained was washed with 4 ml ice-cold 0.05 M Tris-citrate buffer (pH 7.1) and then pellet was dissolved in 0.25 ml 0.2 (N) NaOH and neutralized with 0.25 ml 0.2 (N) HCl. From this suspension, 0.3 ml aliquot was taken and 4 ml scintillation fluid (cocktail 'O'
Spectrochem Pvt. Ltd., Bombay, India) was added and reading was taken in liquid scintillation counter (1209-Rack beta). \[^{3}H\] - GABA binding was expressed as fmole / mg protein.

**(g) Estimation of protein**

Protein content of the tissue was measured according to the method of Lowry et al. (299) using bovine serum albumin (BSA) as a standard. 25-250 μg protein was taken in 1 ml and mixed with 5 ml protein reagent (alkaline CuSO\(_4\) solution obtained by mixing 98 ml 2% Na\(_2\)CO\(_3\) in 0.1 (N) NaOH and 2 ml 0.5% CuSO\(_4\).5H\(_2\)O in 1% Na-K-tartarate) and kept for 10 min at room temperature. 0.5 ml 1 (N) Folin reagent was added to this and mixed quickly and was kept for 30 min at 37°C. Readings were taken in a Hitachi spectrophotometer (U2000) at 660 nm against a reagent blank which contained all the above mentioned reagents except protein.

6. **Statistical Analysis**

The statistical significance between the mean values was assessed by analysis of variance (ANOVA) using Tukey test (300) unless otherwise stated.