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2.1 Materials

ADP, Alanine, Aspartate, ATP, Bovine serum albumin, Brilliant blue-G250, Citrate, Coenzyme A, Dithiothreitol, 5-5-dithiobis-2-nitrobenzoic acid, EDTA, Glutamate, HEPES, Isocitrate dehydrogenase, Lactate dehydrogenase, Malate dehydrogenase, Nicotinamide adenine Dinucleotide, Nicotinamide adenine Dinucleotide phosphate, Nicotinamide adenine Dinucleotide phosphate reduced, Nicotinamide adenine dinucleotide reduced, Oxaloacetate, Phenazine methosulfate, Pyridoxal-5-phosphate, Sodium pyruvate, Thiamine pyrophosphate, Tris, α-ketoglutarate, malate, γ-amino butyric acid, 2,4-idophenyl-3,4-nitrophenyl-5-phenyl tetrazolium chloride, acetyl CoA, oxaloacetate, isocitrate, thiamine pyrophosphate, Dichlorophenol indophenol (DCPIP), Ubiquinone, Cytochrome c, decylubiquinol, Rotenone, Antimycin, Mannitol, SDS, o-pthalaldehyde, were purchased from Sigma chemical Co., USA. Ficoll-400 was purchased from Pharmacia Fine Chemicals, Uppsala, Sweden. Triton X -100 was procured from Koch-Light chemicals, U.K. Rest of the chemicals which are not mentioned here, were procured from the local companies and were of high quality.

2.2 Animals

Male rats (~300g) of Wistar strain were used in the present study. The animals were kept in cages (4 per cage) at 25 ± 2°C with 12h day-night cycles in the animal house facility available at the
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University of Hyderabad. Animals had free access to food (Balanced pellet diet from Hindustan Lever Ltd.,) and water.

2.3 Drug Treatment

Thioacetamide (TAA, 300 mg/kg body weight) dissolved in physiological saline was administered intraperitoneally for 2 days at a 24 h interval. Animals were killed at different time periods after the administration of the second dose. Food and water were provided to the animals ad libitum. Control rats received normal saline to serve as vehicle controls. All the rats were given a 25ml/kg body weight of supportive therapy which consisted of 5% dextrose and 0.45% saline with 20 mequiv/L of potassium chloride (Norton et al., 1997).

2.4 Preparation of Serum

Blood from normal and thioacetamide induced rats at specific intervals of time was drawn by cardiac puncture into a syringe and was then transferred into clean dry centrifuge tubes. It was allowed to stand for 30 minutes at room temperature without any disturbance and was allowed to clot. Serum was separated from the clotted blood by centrifugation at 5,000 rpm at 4°C. This was used for the estimation of glucose, urea, proteins and enzymes such as aspartate and alanine amino transferases.

2.5 Preparation of Serum for Ammonia Estimation

Serum was deproteinized by adding an equal volume of 10% perchloric acid (v/v). This was allowed to stand for 15 minutes for complete precipitation of proteins. The tubes were centrifuged at 5,000
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rpm for 10 minutes at 4°C. The supernatant was neutralized with saturated potassium carbonate till pH was 7.0. Tubes were kept in ice for 15 minutes and they were then centrifuged at 10,000 rpm for 15 minutes at 2°C to remove precipitated potassium perchlorate. The supernatant was used for the estimation of ammonia.

2.6 Preparation of Brain and Liver Extracts for Ammonia Estimation

Rats were decapitated and the head was allowed to fall into liquid nitrogen and frozen at this temperature for 10-15 minutes. Brains were chiseled out with pre-cooled (with liquid nitrogen) stainless steel chisel and powdered with stainless steel mortar and pestle at the temperature of liquid nitrogen. Liver was excised and plunged into liquid nitrogen. After 10 minutes tissue was powdered and as described above for brain. Powdered tissues were transferred into pre-weighed tubes containing 3 ml of ice-cold 10% perchloric acid and the tubes were weighed again. The powder was dispersed well and homogenized in Potter-Elvehjem glass homogenizer with Teflon pestle. Samples were allowed to stand for 15 minutes and centrifuged at 5000 rpm for 10 minutes at 4°C. The supernatant thus obtained was neutralized as described for the serum and used for the assay of ammonia.

2.7 Preparation of Liver Sample for Biochemical Estimations

Liver was excised from the normal and thioacetamide treated rats and was transferred into a beaker containing ice-cold 0.32 M
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sucrose. The tissue was gently pressed between Whatman No. 1 filter papers wetted with sucrose to remove blood present in the tissue. Tissue was cut into small pieces weighed and homogenized in 0.32M sucrose to get required percentage homogenate. This was used for the estimation of enzyme activities and protein content.

2.8 Biochemical Characterization

2.8.1 Estimation Ammonia

Ammonia was estimated in the neutralized PCA extracts of serum, liver and brain by the method of Ratnakumari and Murthy (Ratnakumari and Murthy, 1990). To 1ml of the supernatant 1.5 ml of phenol-nitroprusside reagent (containing 50g of phenol and 250mg sodium nitroprusside in 3.75 liters of water) and 2 ml of sodium hypo chlorite reagent (8.4 g sodium hydroxide, 8.92 g disodium hydrogen orthophosphate and 10ml of 5% sodium hypo chlorite per liter) were added. After 20 minutes at room temperature, the colour intensity was measured at 630 nm against distilled water blank. Ammonium chloride (0.1-1.0 mmoles) was used as a standard.

2.8.2 Estimation of Glucose and Urea

Glucose and Urea levels in serum and in liver extracts were measured by using the diagnostic kits from Glaxo. These kits were meant for the estimation in human samples. Hence it was very much necessary to standardize the same kit for the present study in the rat samples.
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Glucose estimation by the kit is based on the glucose oxidase/peroxidase (GOD/POD) method. The principle of this assay is that Glucose is oxidized by the enzyme glucose oxidase (GOD) to give D-gluconic acid and hydrogen peroxide. Hydrogen peroxide in presence of the enzyme peroxidase (POD) oxidizes phenol which combines with 4-Aminoantipyrine to produce a red coloured quinoneimine dye. The intensity of the colour developed is proportional to glucose concentration in the sample.

D-glucose + H₂O + O₂ \xrightarrow{\text{GOD}} \text{D-gluconic acid} + H₂O₂

H₂O₂ + Aminoantipyrine + Phenol \xrightarrow{\text{POD}} \text{Quinoneimine} + H₂O

1ml of the working enzyme reagent was added to the 20 μl sample. It was mixed well and incubated at 37°C for 10 minutes. Absorbance of the sample was measured against a blank (working enzyme) on a double beam Shimadzu-1601-UV-Visible spectrophotometer at 505 nm.

Urea in the serum and liver were measured by Diacetyl monoxime (DAM) method. The principle in this assay is that urea reacts with DAM in an acidic medium to produce a coloured complex. The colour is intensified by using thiosemicarbazide and a cadmium salt. The absorbance of the coloured complex is proportional to the urea concentration in the sample.

To 10 μl of the sample 1ml of urea reagent and 1ml of the DAM reagent were added and mixed well. The final volume in the tubes was made to 6 ml with de-ionized water. The tubes were kept in a water
bath at 100°C for 10 minutes, cooled under tap water and then the absorbance was measured against blank (urea reagent + DAM reagent) at 520 nm.

2.8.3 Determination of Activities of Aminotransferases

2.8.3.1 Aspartate aminotransferase (AST)

Activity of aspartate aminotransferase was measured by following the method of Bergmeyer and Brent (1974). The final reaction mixture of 1ml contained 500 µl of 160 mM of potassium phosphate buffer (pH 7.4), 25 µl of 20 mM aspartic acid (pH, 7.4), 50 µl of 18 mM α-ketoglutarate (pH, 7.4), 25 µl of 0.4 mM NADH, 5 µl malate dehydrogenase (0.5 mg protein/ ml), and 20 µl of sample. The reaction was started by the addition of α-ketoglutarate. Change in the absorbance was measured at 340 nm for 10 minutes at one minute interval. Activity of the enzyme in liver was expressed as μmol/g wet weight, h, while the serum activity was expressed as μmol/ml. h.

2.8.3.2 Alanine Aminotransferase (ALT)

Activity of alanine aminotransferase activity was measured by following the method of Bergmeyer and Brent (1974). The final reaction mixture of 1ml contained 500 µl of 160 mM of potassium phosphate buffer (pH 7.4), 25 µl of 40 mM alanine (pH, 7.4), 25 µl of 18 mM α-ketoglutarate (pH, 7.4), 25 µl of 0.4 mM NADH, 5 µl lactate dehydrogenase (0.5 mg protein/ ml), and 2 µl of sample. The reaction was started by the addition a-ketoglutarate. Change in the absorbance
was measured at 340 nm for 10 minutes at one minute interval. Activity of the enzyme in liver was expressed as mentioned above for AST activity.

2.8.4 Determination of Protein Content

Protein content in an aliquot of brain and liver preparations was estimated by the method of Lowry et al., (1951). Protein content in the serum was estimated using biuret reagent by the method described by (Varley, 1969). For the mitochondrial studies protein content was determined by the method of Bradford (1976). Bovine serum albumin was used as standard.

2.9 Preparation of Plasma

20μl of heparin was added to clean and dry test tubes. Rats were decapitated and approximately 2 ml of blood was collected into these tubes and mixed thoroughly. This was centrifuged at 5000 rpm for 10 min. The upper clear pale straw coloured supernatant, plasma, was aspirated carefully.

2.10 Determination of Prothrombin Time

Prothrombin time was determined by using the diagnostic kit, Liquiplastin, supplied by Tulip Diagnostic (P) Ltd. Liquiplastin is a ready to use calcium- thromboplastin liquid reagent.

In a tube 0.1 ml of the plasma was taken and placed in water bath at 37°C for 5 min. To this tube, 0.2 ml of Liquiplastin reagent (prewarmed at 37°C) was added, mixed the contents and
simultaneously started a stop watch. The time required for the appearance of the first fibrin strand was recorded.

2.11 Histopathology

Liver histology was studied in control and in thioacetamide treated rats at different time periods (6, 12, 18, and 24 h) after the administration of drug. Animals were anaesthetized with ether and the portal vein was cannulated with Viggo Venflon-2 I.V. cannula with an injection valve and PTFE catheter (0.8mm O.D; 22G). Leur-lock end of the catheter was connected to 0.9% (w/v) ice-cold saline reservoir while the injection valve was connected to 10% buffered formalin (pH 7.4) through a two way Teflon valve. Both saline and formalin were allowed to flow under gravitational force (35-40ml/min). Initially liver was perfused with 0.9% saline till the colour of the tissue turns to pale brown (5-8 min of perfusion). At this juncture the two way valve was opened in such a way that formaldehyde reservoir was connected and saline reservoir was disconnected. Perfusion with formalin was continued for 10 min to achieve total fixation of the tissue. Liver tissue was then excised, cut into 1mm cubes and stored in Bouin's fluid for 3-4 days. The tissue was dehydrated by passing through graded series of ethyl alcohol (30 min each in 30% and 50% alcohol; 80 min in 70% alcohol; 30 min each in 90%, 95% and 100% alcohols).

Then the tissue was transferred to 1:1 (v/v) Acetone : Alcohol mixture for 10 min and then into acetone for 5 min followed by transferring the tissue into acetone: benzene mixture(1:1; v/v) for one
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hour; to Benzene for 1 h and then to benzene: paraffin wax (1:1; v/v) for 2 h. The last step was done in an oven at 55°C and was changed after every one hour for three times. Finally, the tissue was transferred to molten wax in an oven maintained at 55°C. The tissue was then embedded in paraffin wax with in 24 h.

2.11.1 Preparation of Wax

Wax (melting point 60-62°C) was seasoned by melting (60-62°C) and cooling for at least 4-5 times. To 100 g of seasoned wax, 1 g of bee wax was added and allowed to melt. This was thoroughly mixed and stored in frozen condition. Requisite quantity of this wax was melted just before use.

2.11.2 Preparation of Tissue Sections

Sections (5-7μm) were cut with a rotary microtome. Ribbons were placed on slides pre-coated with Meyer’s albumin and spread by slight warming of the slides.

2.11.3 Staining of Sections

Slides with tissue sections were placed successively in xylene (twice-5 min each), absolute alcohol (5 min), 90% alcohol (5 min), 70%alcohol (5 min), 50% alcohol (5 min), 30% alcohol (5 min), distilled water (10-15 min), 3% iron alum (30 min), tap water (10 min), distilled water (2 min) haematoxylene (1 hour), 1% iron alum (allowed to differentiate), running tap water (30 min), distilled water(5 min), 30% alcohol (5min), 50% alcohol (5 min), 70% alcohol (5 min), alcoholic eosin (2 min), 90% alcohol (2-3 min), absolute alcohol (2-3 min), 1:1
alcohol and acetone (5 min), acetone (5 min), acetone and xylene (5 min), xylene (5 min), and finally mounted in DPX. The slides were observed under Nikon Labphot microscope and photographs of the randomly selected areas were taken by an observer not aware of the treatments.

2.12 Mitochondrial Isolation by Sucrose Density Gradient

Brain mitochondria were isolated from adult Wistar rats. Following decapitation, brains were rapidly dissected out and placed in ice-cold isolation buffer consisting of 225 mM Mannitol, 2 mM EDTA and 5 mM Hepes (pH 7.40). Brains were rinsed in the above buffer and were blotted between two filter papers to remove coagulated blood and capillaries. Brain was weighed and homogenized in the isolation buffer using Potter-Elvejhem homogenizer to obtain a 10% (w/v) homogenate.

The homogenate was centrifuged at 1,200g for 10 minutes. The pellet (P₁) was discarded and the resulting supernatant was centrifuged at 10,000g for 10 minutes. The pellet (P₂) obtained was washed with the same buffer, and centrifuged at 15,000g for 15 minutes. The final pellet (P₃) was suspended in 2 ml 0.32 M sucrose.

A discontinuous density gradient of sucrose was prepared using 1 M, 0.8 M, 0.32 M sucrose solutions. The P₃ pellet, suspended in 0.32M sucrose, was loaded on the top of the gradient. This tube was then centrifuged at 65,000g for 75 min. The mitochondria were obtained as a pellet in the 1M layer. This was suspended in 0.32 M
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Sucrose and the protein content was adjusted to 1 mg/ml. The mitochondria isolated by this method was used in the first part of the studies (to study the TCA cycle enzyme activities) where the coupling of the mitochondria was not an important factor. For the rest of the studies mitochondria was isolated by using a Ficoll density gradient.

2.13 Mitochondrial Isolation by Ficoll-400 Density Gradient

Mitochondria were also isolated from the cerebral cortex of rats according to the method of Cotman (1974) as described by Ratnakumari and Murthy (1990). Following decapitation, brains were rapidly dissected and placed in ice-cold saline. The tissue was blotted between two filter papers to remove coagulated blood and capillaries. Tissue was weighed and homogenized in 0.32 M sucrose using Potter-Elvehjem homogenizer to obtain a 10% (w/v) homogenate. The homogenate was centrifuged at 720g for 5 minutes to obtain a pellet ($P_1$) consisting of unbroken cells, debris, nuclei and capillaries. The supernatant ($S_1$) was centrifuged at 15,000g for 12 minutes to obtain a pellet ($P_2$) consisting of mitochondria, synaptosomes and myelin. The pellet ($P_2$) was resuspended in 5 ml of 0.32 M sucrose and was layered on top of a preformed discontinuous density gradient (consisting of 10ml each of 4%, 6%, and 13% Ficoll-400 in 0.32 M sucrose) and centrifuged at 63,500g for 45 minutes. This resulted in the separation of myelin in 4% Ficoll layer, synaptosomes (at the interphase of 6%-13% ficoll) and mitochondria (pellet below the 13% ficoll layer). Myelin and synaptosomes were aspirated out with a pasture pipette and discarded. The mitochondrial pellet was resuspended in
0.32M sucrose and centrifuged at 20,000g and the final pellet was used for various assays.

2.14 Assessment of Purity of the Isolated Subcellular Fractions

In order to avoid the contamination of the mitochondria with the synaptosomes and with the vesicles formed by the sheared nerve endings during the homogenization, metabolically active contamination-free nonsynaptic mitochondria from the cortex was isolated. The purity of the fractions was assessed by estimating activity of marker enzymes - Succinate dehydrogenase for mitochondria and glutamic acid decarboxylase for synaptosomes.

2.14.1 Succinate Dehydrogenase

Activity of SDH was determined by the method of Nandakumar et al., (1973). The assay mixture (1ml) containing 40 mM succinate, 100 mM phosphate buffer pH (8.0), 4 mM INT, 1.6 μM PMS, 50 μl of sample was incubated for 20 minutes at 37°C. The reaction was stopped by adding 2 ml of glacial acetic acid. The colour was extracted into 5 ml of toluene and absorbance was read at 500 nm. Formazan standards were prepared by reducing various amounts of INT with ascorbic acid in alkaline medium. These were used for calculating enzyme activity.

2.14.2 Glutamic Acid Decarboxylase (GAD)

The reaction mixture (250μl) containing 100 μl substrate mixture (3.6 mg glutamic acid in 25 μl of 1N NaOH, 7.8 mg NaH₂PO₄ in 1 ml
distilled water), 5 μl of pyridoxal 5 phosphate (2.5 mg in 10 ml), 100 μl of sample was incubated at 37°C for 20 minutes. The reaction was stopped by boiling for 10 minutes. The mixture was then centrifuged and 20 μl of supernatant was spotted on to a Whatman filter paper and developed in butanol : acetic acid: water (65:15:25). The chromatogram was sprayed with ninhydrin. GABA spot was eluted with 75% alcohol containing 0.005% CuSO₄. The colour was read at 515 nm and compared with values obtained using GABA standard.

2.15 Assay of Citric Acid Cycle Enzymes
2.15.1 Pyruvate Dehydrogenase (PDH)

The method described by Hinman and Blass (1981) was used for PDH

\[
\text{Pyruvate + CoA + NAD} \rightarrow \text{Acetyl CoA + CO + NADH + H}^+ \\
\text{NADH + H}^+ + \text{PMS + INT} \rightarrow \text{Formazan + NAD}^+ + \text{PMS}
\]

The reaction mixture consists of 50mM potassium phosphate buffer (pH. 7.8), 2.5 mM NAD⁺, 0.2 mM thiamine pyrophosphate, 100 μM CoA, 0.3 mM dithiothreotol, 5 mM pyruvate, 1mM magnesium chloride, 6.5 μM Phenazine methosulfate (PMS; intermediary electron acceptor), 300 μM 2,4-idophenyl-3,4-nitrophenyl-5-phenyl tetrazolium chloride (INT; terminal electron acceptor), 5 mM pyruvate, 0.2% Triton X-100 and 20 μg of mitochondria. After pre-incubation for 5 min at 37°C, CoA was added and the change in absorbance was read at 500 nm at 15 second intervals for 5 min. Activity was expressed as nmoles/min.mg protein.
2.15.2 Citrate Synthase

The method of Shepherd and Garland (1969) was adopted for the assay of citrate synthase.

\[
\text{Acetyl CoA + Oxaloacetate} \rightarrow \text{Citrate + CoA (SH)}
\]

Free thiol groups of CoA formed in the course of the reaction were reacted with 5,5'dithio-bis(2-nitrobenzoic acid) (DTNB) and the rate of formation of DTNB-thiol complex was measured at 412 nm.

Free thiol groups + DTNB \rightarrow DTNB-thiol coloured complex

Assay mixture consisted of 96 mM Tris-HCl buffer (pH 8.0), 97.2 µM DTNB, 0.24 mM oxaloacetate, 48 µM acetyl CoA, 0.2% Triton X-100 and 20 µg of mitochondrial protein. Reaction was initiated by the addition of oxaloacetate. Increase in the absorbance at 412 nm was recorded at 5 sec interval for 5 minutes. Enzyme activity was calculated using the molar extinction coefficient of DTNB-thiol complex (1.36 x 10^6). Activity was expressed as nmoles/min.mg protein.

2.15.3 Isocitrate Dehydrogenase (ICDH) (NAD)

This enzyme was assayed by the method of Plaut (1969)

\[
\text{Isocitrate + NAD}^+ \rightarrow 2\text{-oxoglutarate} + \text{CO}_2 + \text{NADH} + \text{H}^+
\]

\[
\text{NADH} + \text{H}^+ + \text{PMS + INT} \rightarrow \text{Formazan} + \text{NAD}^+ + \text{PMS}
\]

The reaction mixture contained 33.3 mM Tris-acetate buffer (pH 7.2), 1 mM magnesium chloride, 6.7 mM ADP, 333 µM NAD^+, 5.28 mM isocitrate, 6.52 µM PMS, 300 µM INT, 0.2% Triton X-100 and 20 µg of mitochondrial protein.
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mitochondrial protein. The reaction was initiated by the addition of isocitrate and the change in absorbance was followed at 500 nm. Activity was expressed as nmoles/min.mg protein.

2.15.4 2-Oxoglutarate Dehydrogenase (2-OGDH)

The method of Reed and Mukherjee (1969) was used for the assay of this enzyme.

\[
\begin{align*}
2\text{-oxoglutarate} + \text{CoA} + \text{NAD}^+ & \rightarrow \text{Succinyl CoA} + \text{CO}_2 + \text{NADH} + \text{H}^+ \\
\text{NADH} + \text{H}^+ + \text{PMS} + \text{INT} & \rightarrow \text{Formazan} + \text{NAD}^+ + \text{PMS}
\end{align*}
\]

The reaction mixture consists of 50 mM potassium phosphate buffer (pH 8.0), 1 mM magnesium chloride, 2 mM NAD\(^+\), 0.2 \(\mu\)M thiamine pyrophosphate, 60 \(\mu\)M coenzyme A, 1 mM 2-oxoglutarate, 6.52 \(\mu\)M PMS, 0.3 mM INT, 0.2% Triton X-100 and 20 \(\mu\)g of mitochondrial protein. The reaction was initiated by the addition of CoA and the change in absorbance was followed at 500 nm. Activity was expressed as nmoles/min.mg protein.

2.15.5 Succinate Dehydrogenase (SDH)

Succinate dehydrogenase was assayed as per the method of Veeger et al., (1969)

\[
\begin{align*}
\text{Succinate} + \text{FAD} & \rightarrow \text{Fumarate} + \text{FADH}_2 \\
\text{FADH}_2 + \text{PMS} + \text{DCPIP} \text{ (oxidized; chromobase)} & \rightarrow \text{FAD} + \text{PMS} + \text{DCPIP} \text{ (reduced; leucobase)}
\end{align*}
\]
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The assay mixture consists of 50 mM potassium phosphate buffer (pH 7.6), 40 mM succinate, 6.52 μM PMS, and 50 μM dichlorophenol indophenol (DCPIP), 0.2% Triton X -100 and 20 μg of mitochondrial protein. Reaction was initiated by the addition of succinate and the reduction of DCPIP was followed at 600 nm for 5 min at 15 sec intervals. Activity was expressed as nmoles/min.mg protein.

2.15.6 Malate Dehydrogenase (MDH)

Activity of this enzyme was studied by the method of Yoshida (1969).

\[
\text{L-Malate} + \text{NAD}^+ \rightarrow \text{Oxaloacetate} + \text{NADH} + H^+
\]

The reaction mixture in the direction of oxaloacetate formation, consisted of 83.2 mM Tris-HCl buffer (pH 8.8), 3.2 mM malate, 0.33 mM NAD\(^+\), 6.52 μM PMS, 0.3 mM INT, 0.2% Triton X -100 and 2 μg of mitochondrial protein. Reaction was initiated by the addition of malate and was followed at 500 nm. Activity was calculated by using the extinction coefficient \((ε_{622}=6.22)\) of NADH. Activity was expressed as nmoles/min.mg protein.

2.16 Electron Transport Chain Enzyme Activities

2.16.1 NADH - Ubiquinone Oxidoreductase (Complex I)

Mitochondrial respiratory complex I activity was measured according to the method of Ragan et al., (1987) The decrease in the absorbance due the oxidation of NADH at 340 nm leading to the reduction of ubiquinone (COQ1) to ubiquinol was measured. The
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reaction mixture contained 20 mM phosphate buffer, pH 7.2, 10 mM MgCl₂, 0.15 mM NADH, 1 mM KCN, 2.5 mg BSA (fatty acid free) and the mitochondrial sample. The reaction was initiated by the addition of 50 μM CoQ1 and it was run at a temperature of 30°C. After measuring the activity for 5 minutes 10 μM rotenone was added and the activity was further measured for another 5 min. The complex I activity measured was rotenone sensitive NADH-ubiquinone oxidoreductase. Activity was expressed as nmoles/min. mg protein.

2.16.2 Succinate - Ubiquinone Oxidoreductase (Complex II)

The activity of complex II was determined as per the method of Hatefi and Stiggal (1978) as described by Cardoso et al., (1999). The disappearance of the colour of the DCPIP dye due to the secondary reduction of the dye by the ubiquinol that is formed as a result of the reduction of the ubiquinone (CoQ2) compound was measured at 600nm. The final reaction mixture contained 50 mM potassium phosphate, pH 7.4, 20 mM sodium succinate, 1 mM KCN, 75 μM DCPIP dye, 0.1 mM EDTA (di-potassium salt), 10 μM rotenone and mitochondrial sample. The reaction was initiated by the addition of 50 μM CoQ₂. The activity was expressed as nmoles/min. mg protein.

2.16.3 Ubiquinone-Cytochrome-c Oxidoreductase (Complex III)

Mitochondrial complex III activity was measured according to the method of Birch-Machin et al., (1994). This enzyme donates the electrons from ubiquinol to cytochrome c, thus resulting in the reduction
of cytochrome c. This reduction of the cytochrome c was measured at 550 nm with a reference wave length of 580 nm. The final reaction mixture contained 35 mM phosphate buffer, pH 7.5, 5 mM MgCl₂, 2.5 mg BSA (fatty acid free), 60 µM decylubiquinol, 1.8 mM KCN, 50 µM rotenone and mitochondrial sample. The reaction was initiated by the addition of 125 µM cytochrome c. the reaction was run for 3 min at 30°C and then 3 µg/ml antimycin A was added and the reaction was run for another 3 min. The activity of complex III was found to be antimycin sensitive. The activity of the enzyme was expressed as nmoles/min. mg protein.

2.16.4 Cytochrome-c Oxidase (Complex IV)

The activity of complex IV was measured according to the method of Wharton and Tzagoloff (1967). The activity was determined by monitoring the decrease in the absorbance at 550 nm due to the oxidation of cytochrome c. Before the reaction was done, cytochrome c was reduced by the addition of a pinch of ascorbate. The mixture was then dialyzed for 24 h against 0.01 M phosphate buffer, pH 7.0, in a cold room maintained at 4°C. This was used for the assay of the activity of this enzyme. The reaction mixture contained 0.01 M phosphate buffer, pH 7.0, 5 µM reduced cytochrome c. The reaction was initiated by the addition of the mitochondrial sample and was run at 30°C. The activity was expressed as nmoles/min. mg protein.
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2.17 Measurement of Mitochondrial Respiration

Oxygen consumption in the nonsynaptic mitochondria was measured by using a Clark oxygen electrode (Gilson Model 5/6 Oxygraph). For this purpose mitochondria isolated by the Ficoll density gradient was suspended in a medium containing 250 mM sucrose, 1 mM EDTA, and 10 mM Tris-HCl, pH 7.4. Respiratory measurements were carried out in the medium consisting of 25 mM sucrose, 75 mM Mannitol, 5 mM KH₂PO₄, 100 mM KCl, 0.5 mM EDTA, and 10 mM Tris-HCl, pH 7.4, in the presence of the respiratory substrate containing 0.5 M succinate or 5 mM pyruvate and 2.5 mM malate. 200 nmoles of ADP/mg protein was added in order to measure the state 3 respiration and the P/O ratio. State 3 and state 4 refer to the active and resting states of the mitochondrial respiration respectively. The respiratory control ratio was calculated as the ratio of state 3 to that of state 4 respiration. The P/O ratio was calculated as the amount of ADP to that of the oxygen consumed during the state 3 respiration. The respiration measurements were performed at 27°C.

2.18 Measurement of Mitochondrial Swelling

Mitochondrial swelling was measured according to the method of Packer (1967). About 40 μg of the mitochondrial protein was suspended in a buffer containing 10 mM Tris, 50 mM sucrose, 5 mM MgCl₂, 10 mM KCl and 0.25 mM ADP in 1 ml reaction mixture. The reaction was started by the addition of succinate (5 mM/μl).
Swelling was measured by following the change in absorbance at 540 nm in a Shimadzu-1601 UV-Visible spectrophotometer.

**2.19 Preparation of Tissue for Transmission Electron Microscopy**

The cerebral cortex tissue blocks were cut into 400 μm thick sections with a vibratome. Slices were washed in cold 0.1 M sodium cocodylate buffer and kept in 2.5% glutaraldehyde in 0.1 M cocodylate buffer until further processing. When processing resumed, slices were washed in cold cocodylate buffer and were then post fixed in 1% osmium tetroxide in cold cocodylate buffer for 1 hour. After osmium tetroxide step, 400 μm thick sections were washed in 0.1% cocodylate buffer. 2x2 mm sections were cut out from the tissue slices, dehydrated in a graded series of ethanol and embedded in Spur Epon. Blocks were trimmed and semi thin 0.5 μm thick sections were cut out with an ultramicrotome stained with toluidine blue and examined by light microscope for an overall view. Ultra thin 70-90 nm thick sections were then cut, picked up on 200 mesh copper grids, double stained with uranyl acetate and lead citrate and scanned in Joel 100CX electron microscope.

**2.20 Oxidative Stress**

In order to evaluate the involvement of oxidative stress in the nonsynaptic mitochondria isolated from the cerebral cortex of rat brain in conditions of thioacetamide induced FHF, lipid peroxidation, total thiols, various antioxidant enzymes have been evaluated.
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2.20.1 Lipid Peroxidation

Malondialdehyde, a by product of lipid peroxidation, was determined by the classical thiobarbiturate assay of Ohkawa et al., (1979) as described by Kosenko et al., (2003). In brief, the brain homogenates were prepared in 1.15% KCl. Mitochondria were prepared as described earlier and later suspended in 1.15% KCl. To 0.1 ml of the mitochondrial sample, 0.2 ml of 8.1% SDS, 1.5 ml of acetic acid (20%, pH 3.5) and 1.5 ml of 0.8% thiobarbituric acid were added, and the final volume was made up to 4ml. This total mixture was incubated at 90°C for a period of one hour. The samples were then cooled and centrifuged at 1000g for 10 min at room temperature. The absorbance of the supernatant was measured at 535 nm with malondialdehyde as the standard.

2.20.2 Assay of Glutathione Peroxidase (GPx)

Activity of Glutathione peroxidase (GPx) (EC 1.11.1.9) was measured according to the method described by Lawrence and Burk (1976)

One unit of activity was defined as one nmole of NADPH oxidized per min.

\[ \text{ROOH} + 2 \text{GSH} \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{GSSG} \]

Activity was calculated according to the following equation.

\[
\text{Difference in absorbance per min} \times \text{Volume of the reaction mixture (ml)} \times e \times \text{NADPH (6.22)} \times \text{volume of the enzyme (ml)}.
\]
Specific activity was expressed as units per mg protein, where one unit is defined as one nmole of NADPH oxidized per minute.

2.20.3 Glutathione Reductase Assay (GR)

Activity of glutathione reductase (GR) (EC 1.6.4.2) was determined by following the procedure of Carlberg and Mannervik (1975). In brief, the reaction mixture (final volume 1ml) consists of 0.2M sodium phosphate buffer pH 7.0, 0.2 mM EDTA, 1 mM oxidized glutathione (GSSG) and 0.2 mM NADPH. The reaction was initiated by the addition of mitochondrial protein sample and the oxidation of NADPH was recorded as decrease in absorbance at 340 nm for five minutes. Nonspecific oxidation of NADPH was measured in the absence of added GSSG and the enzyme activity was calculated using the molar extinction coefficient of NADPH (6.22 mM$^{-1}$ cm$^{-1}$). Specific activity was expressed as units per mg protein, where one unit was defined as one nmole of NADPH oxidized per minute.

\[
\text{NADPH} + \text{H}^+ + \text{G-S-S-G} \rightarrow \text{NADP}^+ + 2\text{GSH}
\]

Activity was calculated according to the following equation.

\[
\text{Difference in absorbance per min X Volume of the reaction mixture (ml)} = \frac{\text{E NADPH} (6.22) \times \text{volume of the enzyme (ml)}}{1}
\]

2.20.4 Total Thiols

Total thiols were estimated as per the method of Sedlak and Raymond (1968). Aliquots of 0.1ml sample were mixed with 1.5 ml of 0.2 M Tris buffer, pH 8.2 and 0.1ml of 0.01M DTNB. The mixture was
made up to 10 ml with 7.9 ml of absolute methanol and it was incubated for 30 minutes. The mixture was then centrifuged at 3000 rpm for 15 minutes and the absorbance of the supernatant was read at 412 nm. The molar extinction coefficient of 13100 was used to calculate total thiols.

2.2.5 Assay of Superoxide Dismutase (SOD)

Total SOD activity was assessed according to the method of described by Beauchamp and Fridovich (1971) by measuring the degree of inhibition of the reduction of NBT in the presence of Xanthine-xanthine oxidase system. Mn-SOD activity was calculated as the difference between the total activity (Cu,Zn-SOD and Mn-SOD ) and the activity measured in the presence of Cu,Zn-SOD inhibitor cyanide. One unit of activity was defined as the amount of enzyme required to inhibit 50% NBT reduction rate.

2.2.6 Preparation of the Sample for Estimation of GSH and GSSG

For the estimation of glutathione, isolated mitochondria were homogenized in potassium phosphate buffer (100 mM, pH 7.4) containing 1 mM EDTA and 1 ml of 25% orthophosphoric acid for precipitation. The above homogenized mixture was centrifuged at 10,000 g for 30 min at 4°C. The supernatant that was obtained after the spin was used for the estimation of GSH and GSSG.

2.2.6.1 Estimation of Glutathione (GSH)

GSH was determined by following the modified method of Hissin and Hilf (1976). To 0.5 ml of the above obtained supernatant, 2 ml of
phosphate-EDTA buffer was added. The final reaction mixture of 2ml contained 20 µl of the mitochondrial supernatant, 1.8 ml of the phosphate-EDTA buffer, and 100 µl of o-pthalaldehyde (OPT) solution containing 100 µg of OPT. The contents were mixed thoroughly and incubated for 15 minutes. Fluorescence at 420nm was measured with an excitation at 350 nm.

2.20.6.2 Estimation of GSSG

To 0.5 ml of the supernatant obtained above after spinning at 10,000g for 30 min at 4°C, 200 µl of 0.04M N-ethylmaleimide (NEM) was added and incubated at room temperature for 30 minutes. To this mixture 1.8 ml of 0.1 N NaOH was added. The final reaction mixture of 2ml contained 20 µl of the mitochondrial supernatant, 1.8 ml of NaOH, and 100 µl of o-pthalaldehyde (OPT) solution containing 100 µg of OPT. The contents were mixed thoroughly and incubated for 15 minutes. Fluorescence at 420nm was measured with an excitation at 350 nm.

2.21 Statistical Analysis

The data in this thesis are reported as Mean ± SD of at least five sets of experiments. One way ANOVA was performed to evaluate the statistical differences between the groups. Comparisons between multiple groups were carried out using Newman-Keul's multiple range test (Keul, 1952). P< 0.05 was considered statistically significant.