4.0. GENERAL MATERIALS AND METHODS

4.1.0. Collection and maintenance of rats

Purebred albino Wistar rats, *Rattus norvegicus* weighing 130 to 150 g. were procured from Fredrick Institute of Plant Protection And Toxicology (FIPPAT) Padappai, near Chennai. They were carefully brought to the Laboratory and kept in 18” x 12” x 6” sized galvanized cages. The animals were acclimatized to the laboratory conditions detailed by Behringer (1973). The animal room and cages were cleaned daily. Rats showing symptoms of disease or poor health were discarded immediately. They were maintained at an ambient temperature of 25°C ± 2°C, under 12 hr night and 12 hr day cycle. The animal room was provided with good ventilation. They were fed with food pellets (supplied by Lipton India Ltd., Bangalore) and clean drinking water *ad libitum*. In addition, they were fed with soaked Bengal grams and fresh carrots. The food pellets contain crude protein 20%, ether extract 4%, ash 8%, Calcium 1%, Phosphorous 0.6%, and nitrogen free extract 55% and had metabolizable energy of 3,600 K cal/Kg. The feed was also enriched by stabilized vitamins, A, D3, E, K, B12, thiamine, riboflavin, pantothenic acid, niacin, choline chloride, folicacid and all minerals and trace elements.

4.2.0. Organophosphate pesticide

Technical grade monocrotrophos pesticide (90% purity), an organophosphate pesticide was obtained from Fredrick Institute of Plant Protection And Toxicology (FIPPT) Padappai, near Chennai, India. It is
chemically known as Dimethylphosphate of 3, Hydroxyl-N-methyl-ciscrotonamide and commercially it is known as azodrin. It is reported to be an effective pesticide in controlling wide range of insects. Some of the notable physical proportions are: (i) it is reddish brown solid (ii) melting point ranges between 25°C and 30°C (iii) it has a molecular weight of 223.16. It is miscible with water, soluble in acetone and ethanol.

STRUCTURE OF MONOCROTOPHOS

\[ \text{CH}_3\text{O} \quad \text{O} \quad \text{CH}_3 \quad \text{P-OC = CHCONHCH}_3 \]

\[ \text{CH}_3\text{O} \]

4.3.0. Calculation of LD\textsubscript{50} for monocrotophos

The LD\textsubscript{50} value of monocrotophos was calculated by following the method of Weil (1952). LD\textsubscript{50} refers to a statistically derived expression of a single dose of a substance that could kill 50% of the animals. Female rats were given intraperitoneal injection of monocrotophos using acetone as the carrier solvent. The range finding study was conducted and a narrow range was found out. After ascertaining the narrow range, four doses of monocrotophos was injected intraperitoneally in geometric progression. Multiple groups of four animals were maintained. The mortality data were collected and compared with the table prepared by Weil (1952).
The formula used for the calculation of LD₅₀ value by Weil method is:

\[ \log^m = \log^{Da} + d \ (f + 1) \] for \( K = 3 \) \( n=1 \)

Where,

- \( \log^{Da} \) = the log of the lowest of the four dosage levels used.
- \( d \) = the log of constant ratio between dosage levels.
- \( f \) = the values given in the table for corresponding \( r \) values.
- \( n \) = the number of rats dosed at each dose level and
- \( k \) = Constant 3 at four dosage level.

This method is followed because it is simple and reliable to estimate LD₅₀ value. For the present study, intraperitoneal injections were given to the animals, as it has been reported that the signs of intoxication in the CNS would be more pronounced by this route than by other routes of entry such as oral or dermal (Jager, 1970).

The LD₅₀ value was arrived at 25 mg / kg body weight.

4.4.0. Experimental procedure

Healthy mature female wistar rats, weighting 130 to 150 g. were used for the present investigation. Vaginal smears were examined every day and the periods of oestrous cycle were determined based on the vaginal smear. Female wistar rats showing a constant oestrous cycle of 96 hr was chosen for the present study. Animals showing wide fluctuations in oestrous cycle were discarded.
Vaginal smear was examined by the method of Long and Evans (1922), Mandal and Anitha (1951) and Watanabe and Sasamoto (1990). One or two drops of distilled water were carefully introduced into the vagina by means of Pasteur pipette with long and narrow snout. Care was taken not to disturb or injure the vaginal wall. The sample was then smeared on microscopic slides in the wet phase. Whenever necessary, the smear was stained in eosin and haematoxylin to favour clear identification of the cells. The characteristic cell types found during the different periods of oestrous cycle of rats are given below:

Proestrous - small, round nucleated cells only, 12 hr.

Estrous - cornified, squamous (scaly, irregular) cells, 9 – 15 hr.

Metestrous - leucocytes and few cornified cells, 8 - 14 hr.

Diestrous - leucocytes and epithelial cells, 60 - 70 hr.

4.4.1. Monocrotophos treatment

Multiple groups of female rats were mated and successful mating was confirmed by observing the vaginal plug and vaginal smear. Once pregnancy was ascertained, they were housed individually for the daily monitoring of general health and body weight changes.

The monocrotophos solution used for intraperitoneal injection was prepared by dissolving appropriate quantity of technical grade monocrotophos in acetone, which served as carrier solvent for the pesticide. The healthy pregnant female rats were divided into two groups, each
containing 10 animals. At the seventh day of pregnancy one group was given intraperitoneal injection of 0.02ml acetone containing 1mg of monocrotophos pesticide and another group was simultaneously given 0.02ml of acetone which served as control animals. Intraperitoneal injection of monocrotophos pesticide and acetone were given on every alternate day, beginning with 7th day, 9th day, 11th day, 13th day and 15th day of pregnancy to experimental and control animals respectively (Fig. 1). Multiple groups of control and experimental animals were maintained simultaneously. Disposable hypodermic needles and syringes were used during each time of injection. The animals were allowed to give birth to young ones. Immediately after parturition, the pups were separated from the mother and the male pups were separated.

4.4.2. Brain dissection technique

Immediately after parturition of young pups, the male pups were separated from the mother. Only the pups, which were born between 7.00 a.m. and 9.00 a.m., were chosen for the experiment. The pups were decapitated and the brains were removed quickly under chilled condition. The neural tissues were separated into five regions (Fig. 2) by following the procedures of Glowinski and Iverson (1966). The separated regions were differentiated as, cerebral hemispheres (CH), diencephalon (DIEN), cerebellum (CER), medulla oblongata (MOB) and spinal cord (SPC). The isolated regions were washed in rat physiological saline to remove the surface moisture. The tissues were weighed in an electric monopan balance. The various biochemical analyses were immediately carried out. The animals were killed between 9:00 a.m. and
10:00 a.m. to avoid diurnal variations in the metabolite level of tissues (Dunlap, 1969).

4.5.0. Estimation of Total proteins

Total protein content was determined following the colorimetric procedure of Lowry et. al., (1951).

4.5.1. Principle

The carbamyl groups of protein molecules react with copper in alkaline solution to form a blue coloured copper–biurette complex. This complex along with tyrosine and phenolic compounds present in the protein reduces the phosphomolybdate of the Folin-phenol (Ciocaltau) reagent and intensify the colour of the solution.

4.5.2. Reagents

2% sodium bicarbonate in 0.1 N sodium hydroxide.

0.5% cupric sulphate in 1% sodium tartarate.

50 ml of reagent ‘A’ was mixed with 1 ml of reagent ‘B’.

1 N sodium hydroxide.

1 N Folin phenol ciocaltau reagent (BDH, Glaxo).

For preparing the standard, 5 mg of bovine serum albumin (Sigma, USA) was dissolved in 5 ml of 1 N sodium hydroxide.
4.5.3. Procedure

The tissues were washed with physiological saline and weighed. The tissues were homogenized in 80% ethanol (10 mg/ml) and centrifuged for 5 min. at 2500 rpm. The supernatant was discarded and the residue was dissolved in known volume of 1 N sodium hydroxide. To 0.5 ml of sample, 5ml of reagent 'C' was added. After 10-min. 0.5 ml of 1 N Folin phenol reagent was added. Blank with 0.5 ml of 1N sodium hydroxide and standard with 0.5 ml (0.5 mg) of bovine serum albumin were prepared as that of sample simultaneously. After 30 min. of reaction, the O.D. was measured at 500 nm in spectronic-21 spectrophotometer. The quantity of the protein in the sample was calculated by comparing O.D. of sample with O.D. of standard. The values were expressed as mg protein / g tissue.

4.6.0. Charged characteristics (Electrophoretic characterization) of proteins

4.6.1. Principle

The principle of electrophoresis is that a charged ion or group when placed in an electric field will migrate towards one of the electrode. Any substance can migrate only if it carried a charge. If the ionization is weakly charged, it would remain at the origin when the pH is equal to or below isoelectric point. The ion or charged group will move to the anode at pH above isoelectric point. The rate of its migration is proportional to degree of
ionization and hence, higher the pH faster the levels. The converse is true for weak bases.

The separation of protein is based not only on net charge but also based on difference in the molecular mass of proteins. Proteins with the same net charge but in different molecular mass could be separated electrophoretically.

4.6.2. Reagents

Small pore buffer (pH 8.9): The buffer was prepared by dissolving 36.6 g of Tris salt in 48 ml of 1N HCl and the solution was made up to 100 ml with distilled water. 0.23 ml of TEMED was added and finally the pH of the buffer was adjusted to pH 8.9.

7% Monomer: Prepared by dissolving 287 g of acrylamide and 0.735 g of bis-acrylamide in 100 ml of distilled water and filtered through the filter paper.

0.14% Ammonium persulfate.

10% Trichloroacetic acid.

Staining solution: 0.25% of Coomassie brilliant blue was prepared by dissolving the 250 mg of CBB R-250 in 100 ml of methanol, acetic acid and distilled water in the ratio of 5:1:4 (V/V/V).
Destaining solution: Prepared by mixing methanol, acetic acid and distilled water in the ratio of 5:1:4 (V/V/V).

7% acetic acid.

Tris – HCl buffer (pH 8.3).

0.1% Aqueous bromophenol blue.

4.6.3. Procedure

4.6.3.1. Preparation of sample

200 mg of sample was homogenised with 1 ml of 40% sucrose solution and centrifuged for 15 min. at 10,000 rpm. The supernatant was used for electrophoretic analysis.

4.6.3.2. Preparation of running gel

The glassplate sandwiches were assembled using two clean glass plates with 0.75mm thick spacers and vacuum grease. The sandwich was locked using the casting stand.

The stock solutions refrigerated at 4°C were brought to the room temperature. The running gel solution was prepared by mixing small pore buffer, 7% monomer, DDW and ammonium persulfate solution in the ratio of 1:2:1:4. The solution was mixed well. Then the solution was transferred to the center of the sandwich along the edge of one of the spacers using the Pasteur pipette until the height of the solution in the sandwich. Then the comb (7 well)
was introduced into the glass plate sandwich. The solution was allowed to polymerise for 20-30 min. After the polymerisation, the comb was removed and the wells were washed with distilled water.

The clamps, casting stand and the bottom spacer over the sandwich were removed. Lower tank of the electrophoresis unit was filled with the diluted tank buffer (20 ml tank buffer diluted to 200 ml with DDW) and the sandwich was fixed in the unit using the metal clambs. 50 μl of the sample was loaded on to the well using a micropipette. The remaining portion of the well was filled with the tank buffer. Then few drops of bromophenol blue were placed with upper tank. The upper tank was also filled with the same buffer without tilting the apparatus to avoid the mixing of samples in the adjacent wells.

The power supply was connected to the well and run at 10 mA of constant current until the tracking dye enters the well. Then it was increased to 15 mA. After the Bromophenol blue has reached the bottom of the separating gel, the power supply was disconnected. The total run time was 2 hr.

After the run, the sandwich from the gel unit was disassembled. Spacers from the edge of the sandwich were carefully removed.

The gel was removed from the gel plates. The gel was stained with silver nitrate stain.
4.6.3.3. Preparation of stock solution for silver staining

1. 50% acetone: 50 ml of acetone made up to 100 ml with DDW.

2. 50% TCA

3. 20% AgNO₃: 200 mg of silver nitrate dissolved in 1 ml of DDW (Prepared freshly).

4. 10% Sodium thiosulphate: 1 g dissolved in 10 ml of DDW.

The gels were destained and stored. They were photographed and electropherogram were drawn and analysed.


4.7.1. Principle

Activity levels of alanineaminotransferase (AlAT) and aspartateaminotransferase (AAT) were studied by colorimetric method given by Reitman and Frankel (1957).

4.7.2. Procedure

To 0.5 ml of substrate, 0.01 ml of 10% homogenate was added and incubated at 37°C for one hr. 0.5 ml of 2, 4, dinitrophenyl hydrazine solution (0.198g in 1000 ml of 1N HCl) was added and kept for 15 min at room temperature. Later, 5 ml of 0.4N sodium hydroxide was added, mixed well and kept it for 20 min and read the OD at 540 nm.
Incubating substrate for alanineaminotransferase.

About 1000ml of phosphate buffer contains 0.2920 of \( \alpha \) ketoglutaric acid, 17.8 g. DL-Alanine, pH was brought to 7.4 with 1N sodium hydroxide.

Incubating substrate for aspartateaminotransferase

About 1000ml of phosphate buffer (pH 7.4) contains 0.292 of \( \alpha \) Ketoglutaric acid, 26.6 g. DL-Aspartic acid, adjusted the pH to 7.4 with 1N NaOH.

4.8.0. Estimation of Ribonucleic acid (RNA)

The RNA content in control and experimental samples was estimated by the method of Searchy and MacInnis (1970 a).

4.8.1. Principle

The pentoses form furfural, when they are heated with concentrated hydrochloric acid. Orcinol reacts with furfural in the presence of ferric chloride, a catalyst, to give a green coloured complex.
4.8.2. Reagents

0.5 N Perchloric acid.

Dische – Orcinol reagent: 33 mg of ferric chloride was dissolved in concentrated hydrochloric acid with 3.5 ml of 6% orcinol in absolute ethanol and made upto 100 ml with concentrated hydrochloric acid.

Standard: 5 mg of standard RNA from yeast (Sigma, U.S.A.), was dissolved 5 ml of 0.5 N perchloric acid.

4.8.3. Procedure

The sample preparation for RNA was similar to that of DNA analysis. To each of 2 ml of the supernatant, 4 ml of Dische – Orcinol reagent was added. They were placed in waterbath at 90°C for 15 min and then cooled. 2ml of 0.5 N perchloric acid was served as blank. 2ml of standard RNA solution was also treated similar to sample. The intensity of the green colour developed was read at 655 nm in spectronic-21 spectrophotometer. The concentration of RNA in the samples was calculated by comparing the O.D. of the sample with that of standard. The values were expressed as mg RNA / g tissue.

Then various biochemical analyses were immediately carried out. The animals were killed between 9.00 am and 10.00 am to avoid diurnal variations in the metabolic level of tissues (Dunlap, 1969).
4.9.0. Determination of acetylcholinesterase activity (Acetylcholine acetylhydrolase (AChE), E.C. 3.1.1.7)

The activity of Acetylcholinesterase (AChE) was estimated following the procedure of Ellman et. al., (1961).

4.9.1. Principle

The principle of this method is the measurement of the rate of production of thiocholine as acetylthiocholine is hydrolysed. This is accompanied by the continuous reaction of the thiol with 5-5-dithiobis-2-nitrobenzoate ion to produce the yellow anion of 5-thio-2-nitro benzoic acid. The rate of color production is measured spectrophotometrically.

4.9.2. Reagents

1. 0.1 M Phosphate buffer (pH 8.0)

2. Dithiobis nitrobenzoic acid (DTNB) reagent: 39.6 mg of DTNB was dissolved in 10 ml of 0.1-M phosphate buffer (pH 7.0) and 15 mg of sodium bicarbonate was added.

3. Substrate: 0.075 M Acetylthiocholine iodide (SigmaChemicals, U.S.A.) in distilled water.

4. Inhibitor: $10^{-4}$ M Eserine sulphate in 0.1 M phosphate buffer (pH 8.0).
4.9.3. Procedure

Saline cleaned, weighed tissues were homogenized in 0.1 M phosphate buffer (pH 8.0) at concentration of 10 mg/ml and centrifuged at 1500 rpm for 5 min. 0.4 ml of supernatant was pipetted into a cuvette containing 2.6 ml of 0.1 M phosphate buffer (pH 8.0). To this cuvette, 0.1 ml of DTNB reagent was added and mixed well. The cuvette was placed in spectronic-21 (Bausch and Lomb) and the absorbance at 412 nm of the suspension was set at zero. 0.02 ml of substrate was added and the changes in the absorbance were recorded for ten minutes and the average activity for a minute was calculated. To determine non-specific esterases, 0.1 ml of eserine sulphate (10⁻⁴ M), specific inhibitor for AChE, was added to another cuvette, containing 0.4 ml of homogenate, 2.5 ml of 0.1 M phosphate buffer (pH 8.0) and 0.1 ml of DTNB reagent. The changes in the absorbance after adding 0.02 ml of substrate was recorded as described earlier. The rate of change of activity of the suspension with eserine was substracted from that of the suspension without eserine. The enzyme activity was expressed as micromoles of substrate hydrolysed / g tissue / minute.

The rate of enzyme activity was calculated as follows:

\[
R = \frac{\Delta A}{1.36 \times 10^4} \times \frac{1}{(400/3120) \ Co} = 5.74 \times 10^{-4} \ \frac{\Delta A}{Co}
\]

Where,
\[ R = \text{Rate of enzyme activity in mole substrate hydrolysed} \div \text{g tissue} \div \text{minute.} \]

\[ A = \text{Change in absorbance per minute} \]

\[ C_0 = \text{Original concentration of tissue (mg/ml).} \]

4.10.0. Estimation of Na\(^+\)-K\(^+\) ATPase

4.10.1. Na\(^+\)-K\(^+\) ATPase

Total ATPase activities were determined by the method of Matsumura and Narahashi (1971) as modified by Yamaguchi et al., (1979). The final volume of ATPase assay was 1.5ml. This included 500μg of homogenate and 3mM Tris ATP (sigma). The total ATPase activity was measured in an incubation medium containing 25mM Tris HCl, pH 7.4, 100mM NaCl, 300mM Quabain. The assays were carried out at 37\(^\circ\)C. after 5 min of pre incubation the reaction was initiated by adding tris ATP. The reaction was stopped after 12 min by adding 0.5ml of ice-cold 10% TCA. The amount of Pi liberated was determined by the method of Fisher and Subba Row as described by Oscher (1976).

Quabain sensitive Na\(^+\)K\(^+\)-ATPase was taken as the difference in total inorganic phosphate released in the absence of quabain and inorganic phosphate released in the presence of 3mM quabain. The specific activity of ATPase was expressed as μ moles of Pi liberated / mg of protein / min.
4.10.2. Total inorganic phosphate

Total phosphate was determined in the different regions of brain and spinal cord of control and pesticide administered rat pups by the method of Fisher and Subba Row as described by Oser (1976). Total phosphate was expressed as μ moles of phosphate / g tissue.

4.11.0. Determination of free aminoacids by two dimensional chromatography

4.11.1. Principle

In unidirectional chromatogram compounds, which exhibit identical physico-chemical properties with reference to a particular solvent system, may not be resolved. In such cases, two-dimensional chromatography is very useful. In the case of the two dimensional chromatography the two different solvent systems are used in order to obtain a better resolution of the closely related chemical components (Smith and Seakins, 1976).

4.11.2. Reagents

80% ethanol: It is prepared by diluting 80 ml of absolute ethanol into 100 ml with double distilled water.

Chloroform BDH

Solvent systems:

Solvent system I: (Butanol: Acetic acid: Water). It is prepared by mixing butanol, acetic acid and water in the ratio of 12:3:5.
**Solvent system II:** (Phenol: ammonia 200:1). It is prepared by dissolving 160 g of phenol in 40 ml of distilled water with one ml of ammonia.

6N HCl: It is prepared by diluting 54 ml of Conc. Hydrochloric acid into 100 ml of distilled water.

10% Isopropanol:

Locating reagents:

0.2% Ninhydrin in acetone: It is prepared by dissolving 200 mg of ninhydrin in 100 ml of acetone.

4.11.3. Application of the sample

A Whatmann No.1 chromatography paper of size 28×28 cm is taken and noted down the flow direction.

A horizontal line is drawn from two cm above the lower margin and a vertical line leaving two-cm on the left side of the paper.

The sample is spotted at the corner where two lines meet and the spotting is repeated till getting the required concentration of amino acids.

4.11.4. Separation

60 ml of solvent is taken in a glass container.

The chromatogram paper is folded and the two ends of the paper are joined (in a hollow cylinder form) with the cellophane tape.
The paper is kept inside the glass container carefully and care is taken that it should not touch the sides of the glass container.

The chromatogram is allowed to run.

After completion of the first run, the paper is taken out and dried.

The paper is once again folded in the perpendicular direction to the first one and is kept in the solvent system II.

After the second run, the paper is taken out and dried in air.

4.11.5. Localisation and identification of amino acids:

The chromatogram is sprayed with 0.2% Ninhydrin and dried.

All the spots are marked and analysed.

4.12.0. Estimation of Succinic dehydrogenase (SDH)

To determine the activity of succinic dehydrogenase (SDH) Nachlas et al., (1960) colorimetric method was employed.

4.12.1. Principle

The principle is based on the reduction of iodophenyl nitrophenol tetrazolium in the presence of the enzyme SDH and its substrate succinate.

4.12.2. Reagents

0.25 M Sucrose
40 mM sodium succinate

100 mM phosphate buffer (pH 7.4)

2 mM Iodophenyl nitrophenol tetrazolium (chloride salts) (INT)

Glacial acetic acid.

Toluene

4.12.3. Procedure

Cleaned weighed tissues were homogenized in 0.25-M sucrose (15 mg/ml) and centrifuged for 5 min at 2500 rpm. The supernatant was used as sample. 0.4 ml of 40mm sodium succinate, 0.5 ml of 100mM phosphate buffer (pH 7.4) and 0.5 ml of INT were added to each test tube. The volume was made upto 2 ml by adding 0.6 ml of water. Then 0.5 ml of tissue extract was added and incubated at 37° C for 30 min. At the end of the incubation, 5ml of glacial acetic acid was added, followed by 5 ml of toluene. Then the test tubes were kept in refrigerator for the development of the colour. The toluene layer was separated and read against toluene blank at 495 nm in spectronic 21 spectrophotometer. The values were expressed as ml unit of activity / mg tissue / min.

4.13.0. Estimation of Lipid peroxidation

4.13.1. Method for estimation of MDA level (Melonaldehyde)

The rate of lipid peroxidation was determined by thiobarbutyric acid (TBA) test according to Wilbur et. al., (1949).
10% homogenate was prepared in 15% ice cold TCA by using a potter-Eloehjen type homogenizer fitted with a pestle. 1ml of 40% TCA made in concentrated H₂SO₄ and 2ml of 0.67% TBA were added to 1ml of homogenate and boiled for 10 min. The mixture was centrifuged and the intensity of the colour in the supernatent measured at 535 nm. The concentration of TBA reactive substance (melondialdehyde) was calculated using the formula,

\[ \frac{O_{\text{t}ical \ density \ of \ unknown} \times Volume \ in \ cuvette}{Extinction \ co-efficient} \]

Extinction co-efficient = 1.56×10⁵m⁻¹

4.13.2. Estimation of phospholipids

Content of total phospholipid was estimated by the method of Zilversmith and Davis (1950).

Approximately 50mg of tissue was homoginized in 3 ml of 10% TCA and centrifuged at 1,500 rpm for 10 min. The supernatent was discarded, 1ml of 60% TCA was added to the residue and the digestion was continued on a heating mantle to obtain a clear and colourless solution. The contents were cooled and approxmiately 5-6ml of distilled water was added to each tube. 1ml of 4% (W/V) ammonium molybdate was added and mixed well. Finally, 1ml of ANSA reagent was added and the total content was made upto 10ml with distilled water. The time of addition of ANSA was noted. The colour developed was read within 10 min at 660 nm. Quantitative determination was made by using a standard graph. Standard contains 0.01 mg of PO₄/ml.
4.13.3. Estimation of catalase (E.C. 1.11.1.6.)

Catalase activity in 10% homogenate was measured by the colorimetric procedure of Bonicksen et. al., as given by Kolowick and Kaplanj (1957).

1 g of tissue was homogenised in phosphate buffer (pH 6.8). 2 ml of homogenate was pipetted into a conical flask. 10ml of 0.01N H₂O₂ was added and incubated for 3 min. 5 ml of 10% H₂SO₄ was added to stop the reaction. Continued by the addition of 5 ml of 5% potassium iodide and 2 ml of saturated molybdic acid. Shaken well and kept inside for 3 min. The contents in the flask were titrated against 0.1N Na₂S₂O₃ using starch as indicator. A blank was run by adding same quantity of concentrated H₂SO₄ to the homogenate, prior to the addition of substrate.

The rate constant K was calculated using the equation:

\[ K = \frac{1}{t} \log \frac{X₀}{X₁} \]

The enzyme activity K at f was calculated using the equation:

\[ \frac{K}{\text{Wt. of tissue in mg.}} \]
4.14.0. Estimation of glucose, glycogen and pyruvic acid


The method of Pfluger (1905) and modified by Good et. al., (1933) is followed.

4.14.2. Reagents

30% KOH
95% ethanol
60% ethanol
0.6 N HCl or H₂SO₄
Saturated sodium sulfate
95% H₂SO₄

0.2% anthrone solution: This reagent is made by dissolving 0.2 g of anthrone in 100 ml. of 95% sulphuric acid. The reagent is not stable in solution and should be kept in the refrigerator. It should be prepared freshly every 2 days.

A standard glucose solution containing 20 γ of glucose per milliliter.

Photoelectric colorimeter.

4.14.3. Procedure

Approximately 1 g of animal tissue is dropped into a previously weighed 15 ml. Pyrex centrifuge tube containing 3 ml. of 30% potassium hydroxide solution. After delivery of the sample, the tube and contents are
reweighed and the weight of sample is determined by difference. The tissue is then digested by heating the tube in a boiling water bath for about 20 to 30 min. When the tissue is dissolved, 0.5 ml. of saturated sodium sulfate is added and the glycogen is precipitated by the addition of 1.1 to 1.2 vol. of 95% ethanol. The contents are stirred with stirring rod, and the rod washed with a small quantity of 60% ethanol then cooled and centrifuged at 3000 rpm the mother liquor is decanted and the test tube is allowed to drain. The remaining adhering alcohol may be expelled by heating the tube in a boiling water bath. The precipitated glycogen is redissolved in 2 ml. of distilled water and reprecipitated with 2.5 ml. of 95% ethanol, the alcoholic supernatant liquid decanted, and the tube drained as before. The glycogen is treated with the anthrone reagent and determined colorimetrically as glucose.

The digest is cooled, transferred quantitatively to a 50 ml. volumetric flask, and diluted to the mark with water. The contents of the flask are thoroughly mixed, and a measured aliquot is then further diluted with water in a second volumetric flask so as to yield a solution of glycogen concentration of approximately 3 to 30 γ/ml. The determination is then carried out as follows.

A 5 ml aliquot of the solution containing an amount of carbohydrate equivalent to 15 to 150 γ of glucose is transferred to a colrimetric tube. Into a second tube is introduced 5 ml of distilled water, which serves as a blank. The tubes are submerged in cold water, 10 ml of the anthrone reagent is
added to each test tube from a fast-flowing pipet or buret and the reactants are mixed by swirling the tubes. The cold tubes are covered with glass marbles and heated for 10 min. in a boiling water bath. They are then immediately cooled in a bath containing cold water and read in the colorimeter at 620 m\(\mu\) after the galvanometer has been set at 100 with the blank. With a colorimeter, the amount of glycogen in the aliquot used in calculated from the following equation:

\[
\gamma \text{ of glycogen in aliquot} = \frac{100 \times U}{1.11 \times S}
\]

Where, \(U\) = the optical density of the unknown test solution.

\(S\) = the optical density of the 100 \(\gamma\) glucose standard.

1.11 = the factor determined by Morris (1948) for the conversion of glucose to glycogen, with this equation.

4.15.0. Determination of Glutamate dehydrogenase (GDH)

4.15.1. Principle

The activity levels of Glutamate dehydrogenase (GDH) was measured following the modified method of Nachlas et. al. (1960) as described by Govindappa and Swamy (1965).

4.15.2. Procedure

10% (W/V) homogenates were prepared in 0.25 M cold sucrose using glass homogenates and centrifuged at 3,000 rpm for 15 min. The supernatant was used for the assay of enzyme activities. The incubation
mixture consisted of 0.5ml each of 0.09M sodium succinate, 0.27M sodium glutamate and 0.1M sodium pyruvate as substrates for estimation of glutamate dehydrogenase respectively. 0.5 ml of sodium phosphate buffer of 0.1M, 0.5 ml of triphenyl tetrazolium chloride (0.2% solution at neutral pH) and 0.1ml of the 10% homogenate. The incubation was carried out at 37°C for 15 min after which 6 ml of glacial CH₃COOH and 6ml of toluene were added and kept in the refrigerator for overnight. The toluene layer was extracted and optical density was read at 505 nm in spectrophotometer.

4.16.0. Determination of tissue respiration

The rate of respiration of cortical slices of the brain is studied following Warburg’s manometric technique (Umbreit et. al., 1959).

Manometric technique is based on the ideal gas law, \( PV = RT \) where \( P \) is the pressure of the gas; \( V \) is the volume; \( R \) is the gas constant and \( T \) is temperature. At constant temperature and volume, any change in the gas phase of the gas chamber can be measured by the changes in pressure. Here, the same principle is applied to determine the rate of respiration based on the pressure change.

4.16.1. Calibration

To calculate the gas consumed from the manometer recordings under specific conditions, it is necessary to know the entire volume of gas phase
in the reaction flask and free space of manometer down to level of zero. Prior to set up the apparatus, the entire volume of the gas phase was measured by filling the space with mercury. The weight of the filled mercury was determined. The volume of the gas phase is:

\[
\text{Volume (V) (ml)} = \frac{\text{Mass of mercury}}{\text{Density of mercury}}
\]

The ‘k’ (constant) for each manometer was calculated using the following formula (Umbreit et.al., 1959).

\[
K_{O_2} = \frac{V_g \frac{273}{273+T} + V_f \times aO_2}{10,000}
\]

Where,

\[
V_g = \text{Volume of gas phase in the flask} \quad (V_g = V - V_f)
\]

\[
V_f = \text{Volume of the fluid in the flask}
\]

\[
T = \text{Temperature}
\]

\[
10,000 = \text{Pressure of Brodie’s fluid}
\]

4.16.2. Reagents

(a) Manometric fluid

Brodie’s fluid was used as manometric fluid. This was prepared by dissolving 11.5 g of sodium chloride, 2.5 g of sodium choleate and 50 mg of
Evan's blue in 250 ml distilled water. The density of the solution was adjusted to 1.033.

(b) Kreb's ringer solution

This was prepared by mixing the following solutions (Kreb, 1950).

- 0.9% sodium chloride - 95 parts
- 1.15% potassium chloride - 4 parts
- 0.11 M Calcium chloride - 3 parts
- 2.11% potassium dihydrogen-phosphate - 1 part
- 3.82% Magnesium sulphate - 1 part
- 1.3% sodium bicarbonate - 3 parts

This solution was used for preparation of brain homogenate.

4.16.3. Medium

To above kreb's ringer solution, 120-mM glycyl glycine and 36-mM glucose were added and the pH of the solution was adjusted to 7.4 with 1 N sodium hydroxide (Ghosh and Guha, 1980).

20% Potassium hydroxide.

4.16.4. Procedure

The cleaned and weighed tissues were homogenized gently with ice cold kreb's ringer solution (100 mg / ml) in a glass homogenizer at 4°C. 2 ml
of the medium was pipetted into the outer surface of the central well of the reaction flask.

Then 1 ml of brain homogenate was added. 0.2 ml of 20% potassium hydroxide was added in the central well of the reaction flask and a filter paper wick was inserted to avoid spilling of potassium hydroxide during agitation of the manometer, which had already filled with manometric fluid. The fittings were made airtight by using Metro ark silica gel. The manometric fluid was set at zero. A thermobarometer, which contained distilled water instead of homogenate, was run simultaneously. The manometers were fixed to shaking apparatus in which the reaction flask was made to immerse in the water. The apparatus was switched on. The rate of oscillation of the apparatus was 120 / min. The manometers were left undisturbed for about 15 min for acclimatization. Then the manometers were closed completely and again the fluid level was adjusted to zero so as to take the initial readings on the left arm of the manometer. After this, the apparatus was again switched on and the readings were taken at 15 min interval for one hr. The recordings of the tissues from control animals were also made simultaneously.

At the end of the experiment, the rate of tissue respiration was calculated using the following formula (Ghosh and Guha, 1980)

\[ \text{Manometric readings} \times \text{Flask constant} \times 10 \]

The values were expressed as μ moles of oxygen consumed / g tissue / hr.
Fig. 1.

WORKING PLAN

Day of Mating → Days of Injection ← Day of assays

Organogenesis ←
Fig. 2.
ANATOMICAL MAP OF RAT BRAIN
(GLOWINSKI AND IVERSEN, 1966)

CH - Cerebral hemisphere
DIEN - Diencephalon
CER - Cerebellum
MOB - Medulla oblongata
SPC - Spinal cord