Procurement of experimental materials

Adult specimens of the slug, *Leucoglaucia alba* were collected from moist, grassy areas in and around Tirupati. They were reared in wooden boxes containing mud that was wetted once in two days. The animals were fed daily on croton leaves.

The slugs are hermaphrodites and hence the influence of sex on the rhythmic activity does not arise. To avoid influences of feeding habits (Wolfgang, 1974), a fixed feeding time schedule was maintained.

Recording of locomotor activity

The aktographic method of Naylor (1958) for recording the locomotor activity of the slug under different experimental conditions was adopted. Transparent perspex boxes (14x7x4 cms) with a lever attached at one end, were used as animal chambers. The boxes were suspended about their median transverse axis with the tip of the lever touching the smoked paper. As the animal creeps along the length of the chamber, the tilt to one side results in registering a nearly vertical mark or line on the smoked paper. The activity records on the smoked paper were fixed with varnish and terpentine mixture in 1:2 proportion. This activity was later analysed on the basis of the known speed of the kymograph.
The number of flicks recorded in an hour was considered proportional to one hour locomotor activity of the animal. Thus the hourly activity of the animal was measured by counting the vertical marks and from this data histograms were plotted. The animals were starved during the course of recording locomotor activity. To avoid influences of lunar cycle (Brown, 1959), recording of the activity was avoided five days before and after the full moon day. The locomotor rhythms were recorded at room temperatures (25-30°C) under the following four light conditions:

**Locomotor rhythms in normal day and night (LD) conditions:**

The recording set up was placed in a room which was spacious and well ventilated. One side of the room fitted with transparent glass shutters and ventilators was always kept open. Thus the laboratory will also be exposed to the outside atmospheric variations in the abiotic factors like light, temperature and humidity of natural day-night variations. Under these diurnal variations of light conditions the locomotor activity of the animal was recorded.

**Locomotor rhythms in reversed light (RL) conditions:**

Light dark cycles of the day were reversed by making daytime dark and illuminating night time with 60 W bulb. Thus, the regimen of 12 hours light characterising artificial day alternated with a dark 12 hours artificial light.
Locomotor activity under constant conditions:

(1) **Constant light (LL) condition**: A 60 W bulb as light source for illuminating the animals constantly was arranged 200 cm above and 100 to 150 cm away. The light source was directed to the animal chamber and the locomotor activity was recorded as described earlier.

(2) **Constant dark (DD) condition**: The activity was recorded in a dark room. Temperature variations in the room were ±1.0 to 1.5°C over normal conditions.

Prior to placing the slugs in animal chambers, they were acclimated to the four regimens of light conditions for a period of fifteen to twenty days in the laboratory. The activity of the animals thus acclimated only were recorded in all cases. Recordings were made continuously for five to seven days and these were analysed and represented by histograms with activity plotted against time of the day.

**Estimation of biochemical constituents:**

Preliminary experiments to determine the basic protocol of the reaction mixture for the estimation of different biochemical constituents were conducted. In studying the diel variations in the biochemical constituents six timings viz., 08.00, 12.00, 16.00, 20.00, 00.00
and 04.00 were chosen to cover the 24 hour period. The three time periods covering the day light, of these six periods, viz., 08.00 to 16.00 hours, and the rest of the periods viz., 20.00 to 04.00 hours were considered as "day light" and "night dark" hours of the animals. This broad division of 24 hour day selected for convenience, was used in the text. ACh and AChE were estimated in the nervous system, including all the ganglia, and foot muscle. The other biochemical constituents were estimated in the nervous system, foot muscle and midgut gland. As for the suggestion of Van Weel (1974) the term "midgut gland" was used in the text in place of conventional usage "hepatopancreas". All the biochemical constituents were estimated in animals that were acclimatized to normal (LD), reversed (DL), LL and DD conditions. In all the experiments adult specimens in the weight range of 7.0 to 9.0 gms were used. Further, in all the experiments, tissues isolated from minimum of three individuals each time were pooled.

Estimation of Acetylcholine (ACh) content:

ACh content was estimated by the method of Westrin as given by Augustinsson (1957).

The tissues isolated and pooled into a single sample, were immediately transferred into prechilled tubes and their weights were accurately determined by single pan
sartorius balance. The samples were kept in boiling water bath for 5 minutes to inactivate the enzyme \( \text{AChE} \) and to release bound \( \text{ACh} \), as described by Vasantha et al., (1975). The tubes were cooled and the contents were homogenized in two ml of double distilled water. Two ml of alkaline hydroxylamine hydrochloride and 1.0 ml of HCl (1:1 HCl:H₂O) were added to the homogenate. To 2.5 ml of centrifuged clear supernatant, 0.5 ml of ferric chloride was added. The amount of \( \text{ACh} \) present in the sample was measured at 540 nm in Kausch and Lomb Spectronic 20 colorimeter. The content was expressed as \( \mu \) moles of \( \text{ACh} \)/gm wet weight of fresh tissue.

**Estimation of acetylcholinesterase (\( \text{AChE} \)) (Acetylcholine acetylhydrolase: EC 3.1.1.7) activity:**

1% (w/v) homogenates of the tissues was made in 0.25M ice-cold sucrose solution. The \( \text{AChE} \) activity of the un-centrifuged homogenates was estimated by the method of \( \text{AChE} \) (1951).

The incubation mixture contained 8 \( \mu \) moles of acetylcholine chloride, 100 \( \mu \) moles of phosphate buffer (pH 7.8), and 0.2 ml of tissue homogenate. After 30 minutes of incubation at 37°C, the reaction was arrested by the addition of 2.0 ml of alkaline hydroxylamine hydrochloride and 1.0 ml of HCl (1:1 HCl:H₂O). The contents were thoroughly
shaken and centrifuged at 3000 rpm for 15 minutes. To 2.5 ml of clear supernatant, 0.5 ml of ferric chloride solution was added and the developed color was measured against a blank at 540 μm in Bausch and Lomb Spectronic 20 colorimeter. The blank contained 1.0 ml of buffer solution instead of buffer-substrate mixture.

The enzyme activity was expressed as μ moles of ACh hydrolysed/mg protein/hour.

**Estimation of Butyrylcholinesterase (BuChE EC 3.1.1.8) activity:**

BuChE activity in the tissue homogenates was determined by spot check study at 12.00 noon and 04.00 hours of 24 hour period. The amount of BuChE in the tissue homogenates was estimated by the method described for AChE, as suggested by Bojji Reddy (1970). The reaction mixture contained 8 μ moles of BuCh in place of ACh. The enzyme activity was expressed as μ moles of BuCh hydrolysed/mg protein/hour.

**Estimation of succinate dehydrogenase (SDH) (succinate: (acceptor) oxidoreductase, EC 1.3.99.1) activity:**

SDH activity in the tissue homogenates was estimated by the method of Srikantan and Krishna Murthy (1965) as modified by Govindaappa and Swami (1965). INT (2-p-iiodophenyl)-
3-(p-nitrophenyl)-3-phenyl tetrazolium chloride was used as the terminal electron acceptor. 1% (w/v) homogenates of the tissues were prepared in ice-cold 0.25 M sucrose solution. The homogenates were centrifuged at 3000 rpm for 15 minutes to remove the cell debris and the clear supernatant was used for the assay.

The assay mixture contained 100 μ moles of phosphate buffer (pH 7.2), 30 μ moles of sodium succinate and 4.0 μ moles of INT in a final volume of 2.5 ml. The reaction was initiated by the addition of 0.8 ml of homogenate. The tubes with the contents were incubated for 30 minutes at 37°C. The reaction was arrested by 5.0 ml of acetic acid and the formazan formed was extracted overnight at 5°C in 5.0 ml of toluene. The color was measured against toluene at 495 nm in Bausch and Lomb Spectronic 20. The enzyme activity was expressed as μ moles of formazan formed/mg protein/hour.

**Assay for aspartate aminotransferase (AAT) (L-aspartate: 2-oxoglutarate aminotransferase : EC 2.6.1.1) and alanine aminotransferase (ALAT (L-alanine : 2-oxoglutarate aminotransferase : EC 2.6.1.2) activity:**

1% (w/v) homogenates of the tissues were made in 0.25 M ice-cold sucrose solution. The AAT and ALAT activities were determined by using the colorimetric procedure
of Reitman and Frankel (1957) as given by Bergmeyer (1963). The incubation mixture contained: 100 μ moles of phosphate buffer (pH 7.2); 50 μ moles of L-aspartic acid; 2.5 μ moles of α-ketoglutaric acid for AAT; 100 μ moles of phosphate buffer (pH 7.2); 25 μ moles of DL-alanine; 2.5 μ moles of α-ketoglutaric acid for ALAT and 0.2 ml of centrifuged clear homogenate. The contents were mixed by inversion and incubated at 37°C in thermostatic water bath exactly for 30 minutes for ALAT and one hour for AAT, since they represented the initial velocities. The reaction was stopped with 1.0 ml of 2,4-dinitrophenyl hydrazine in 0.1 n HCl, and allowed to stand for 15 minutes at room temperature after the incubation was over. For each experimental tube a blank was run to which the homogenate was added after the addition of 2,4-dinitrophenyl hydrazine solution. 10.0 ml of sodium hydroxide (0.4 N) were added to the tubes and the developed color was read at 546 μm in the Bausch and Lomb Spectronic 20 colorimeter. The enzyme activity was expressed as μ moles of sodium pyruvate formed/mg protein/hour.

*Estimation of total amino acids:*

Total amino acid level in the tissues was estimated by the method of Moore and Stein (1954). 1% (w/v) homogenates of tissues were prepared in 10% trichloroacetic acid.
(TCA) and centrifuged at 3000 rpm for 15 minutes. To 0.5 ml of the above supernatant, 2.0 ml of ninhydrin reagent was added and kept in boiling water for six and half minutes and immediately cooled. It was made upto 10.0 ml and the bluish pink color was read at 570 nm in Bausch and Lomb Spectronic 20 colorimeter. Standard graph was prepared by using tyrosine and the amino acid content was expressed as µgms tyrosine per g wet weight of fresh tissue.

Estimation of total carbohydrate levels:

Following the method of Carrol et al., (1956), total carbohydrate levels in the homogenates of tissues were estimated.

1% (w/v) homogenates of tissues were made in 10% trichloroacetic acid (TCA) solution. To 0.5 ml of the centrifuged clear supernatant, 5.0 ml of anthrone reagent was added and boiled for 15 minutes in water bath. The tubes with the contents were immediately cooled. A standard sample containing known quantities of analar glucose solution was always run along with the experimental samples. The colour was measured at 620 nm in Bausch and Lomb Spectronic 20 against a reagent blank. The level of the content was expressed as µgms per gram wet weight of fresh tissue.
Saturation of proteins:

Protein levels in the tissues were determined by the Folin phenol method as given by Lowry et al., (1951).

Validity of experimental procedures:

(a) General:

Aliquots for assay:

Aliquots were selected for the assay such that the initial rates are approximated as nearly as possible, yet providing sufficient product to fall in a convenient range for colorimetric determination.

Enzyme units:

Enzyme activities are expressed in standard units i.e., /μM of product formed or substrate cleaved per mg protein per hour.

Substrate requirements:

All the enzyme assays were made under the conditions following zero order kinetics.

Lambert-Beer Law:

Almost all the products of the reactions are measured by calorimetric procedures, in which the optical density (absorbance) of the resulting coloured complexes are proportional to the concentration of the reaction product.
(b) Assay of dehydrogenases using INT:

The advantage of using tetrazolium salts as electron acceptors are (1) the tetrazolium salts give a stable colour on reduction, (2) they are highly soluble in aqueous solutions, (3) they can be reduced both aerobically and anaerobically, (4) they have high redox potential which makes the reduction easier, (5) they are freely permeable through membranes.

Various tetrazolium salts receive electrons from various sites of electron transport system (Oda, 1958; Nachlas et al., 1960). This is due to the inherent difference in redox potentials of various tetrazoliums. The introduction of P-nitrophenyl group in N₂ phenyl ring was observed to increase the efficiency of the dye by increasing its redox potential. Karmarkar et al., (1959) reported that INT was superior to most of the tetrazolium salts as an electron acceptor for the assay of succinate dehydrogenase and other dehydrogenases.

(c) Statistical treatment of the data:

Standard deviation and probability test i.e., t-test were calculated following the method of Pillai and Sinha. All the values of t, below 5% level are designated as significant and those falling below 1% level are designated as highly significant and those values above 5% level are designated as not significant.