CMFRI SPECIAL PUBLICATION
Number 14

MANUAL OF RESEARCH METHODS FOR INVERTEBRATE ENDOCRINOLOGY

ISSUED ON THE OCCASION OF THE
WORKSHOP ON INVERTEBRATE ENDOCRINOLOGY
JOINTLY ORGANISED BY
THE CENTRE OF ADVANCED STUDIES IN MARICULTURE,
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE, COCHIN
AND THE DEPARTMENT OF ZOOLOGY,
MARATHWADA UNIVERSITY, AURANGABAD
HELD AT COCHIN IN OCTOBER 1983

The CENTRE OF ADVANCED STUDIES IN MARICULTURE was started in 1979 at the Central Marine Fisheries Research Institute, Cochin. This is one of the Sub-projects of the ICAR/UNDP project on 'Post-graduate Agricultural Education and Research'. The main objective of the CAS in Mariculture is to catalyse research and education in mariculture which forms a definite means and prospective sector to augment fish production of the country. The main functions of the Centre are to:

— provide adequate facilities to carry out research of excellence in mariculture/coastal aquaculture;

— improve the quality of post-graduate education in mariculture;

— make available the modern facilities, equipments and the literature;

— enhance the competence of professional staff;

— develop linkages between the Centre and other Institutions in the country and overseas;

— undertake collaboration programmes; and

— organise seminars and workshops.

Under the programmes of the Centre, Post-graduate courses leading to M.Sc. (Mariculture) and Ph.D. are offered in collaboration with the University of Cochin since 1980.

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STAINING PROCEDURES FOR NEUROSECRETORY CELLS IN CRUSTACEANS

1.1 Introduction

The neurosecretory cells *i.e.* hormone secreting cells, are widely distributed in eyestalk, brain and thoracic ganglion. Our knowledge of neurosecretion has been advanced due to large number of morphological and physiological experimental investigations which have been carried out in combination with the application of the most modern staining techniques. Neurosecretion which is not detectable by general histological methods, can be readily demonstrated by staining with chrome-haematoxylin, Mallory's Triple and Aldehyde fuchsin. The elaboration of staining techniques (histochemically) was introduced by Pearse (1966).

1.2 Staining Procedure

Mallory's Triple (1944), chrome-haematoxylin phloxine, CHP (Bargmans, 1949) and Aldehyde fuchsin (Ewen, 1962) are commonly used staining procedures for neurosecretory cells.

1.3 Materials

Eyestalk, brain and thoracic ganglion of the prawn.

1.4 Processing of Tissue

1. Dissect the prawns in fresh/sea water (depending upon their habitat).
2. Cut out the eyestalks, with fine scissors and take out brain and thoracic ganglion with the help of fine forceps.
3. Fix them in Bouin's fluid for 24 hours.
4. Dehydrate in alcohol series

   30% alcohol 2 hrs
   50% alcohol 2 hrs
   70% alcohol overnight

   remove cuticle of the eyestalks before passing them into 90% alcohol.

   90% alcohol 1 hr
   100% alcohol 1 hr
5. Treat with xylene (saturated with paraffin wax) for 1/2 hr.
6. Pour out xylene and pour melted wax in tissue tubes.
7. Keep the tissue for 1 hour in oven before preparation of blocks.
8. Section the tissue longitudinally (eyestalks) or transversely (brain and thoracic ganglion) at 6–7 microns and mount sections on clear microscope slides using egg albumin.

1.5 Mallory's Triple (1944) Stain for Neurosecretion in Paraffin Sectioned Tissue

1.5.1 Solutions

a. Preliminary mordant – saturated $HgCl_2$ in water + 5% acetic acid.

b. Acid fuchsin – 1% in distilled water.

c. Phosphomolybdic acid – 1% in distilled water.

1. Mallory's stain – Aniline blue 0.5 gm (water soluble)

Orange G	–	2 gm
Oxalic acid	–	2 gm
distilled water	–	100 ml

1.5.2 Procedure

1. Bring slides to water through alcohol grades.
2. Mordant in $HgCl_2$ – acetic acid – 10 minutes.
3. Rinse in distilled water.
4. Acid fuchsin – 15–30 seconds.
5. Differentiate by washing in distilled water. 10 seconds or more as required.
6. Phosphomolybdic acid – 60 seconds (avoid contact with metal forceps, protect these with collodin or paraffin).
7. Wash with distilled water – 10 seconds.
8. Mallory's stain – 75 seconds. Drain and wipe the back of the slide.
9. Distilled water – 10 sec. Drain and wipe.
10. Differentiate Aniline Blue in 90% alcohol – 10 seconds or more as required.
11. First alcohol (absolute) – 10 seconds.
12. Second alcohol (absolute) – 10 seconds.
13 Clear in xylene
14 Mount in DPX.

1.5.3 Result

Neurosecretory material – red to orange; nuclei – red.

1.5.4 Principle of the method

Acid fuchsin is very soluble in water, particularly if slightly alkaline as in tap water. It is fast in neutral alcohol. Aniline Blue rapidly dissolves in water and more slowly in 90% alcohol. The phosphomolybdate intensifies the staining of the fuchsin in some elements and helps to decolourise the collagen. The oxalic acid intensifies the Aniline Blue staining.

1.6 Chrome–haematoxylin Phloxine, CHP (Bargmann, 1949)

Please see section 14.5 of Chapter 14 "Staining methods for neurosecretory system in crustaceans" by M. Panneerselvam and T. Subramoniam in the *Manual of Research Methods for Marine Invertebrate Reproduction*. CMFRI Special Publication 9 (1982) pages 125-131.

1.6.1 Principle of the method

Chrome-haematoxylin is a basic dye commonly used for the staining of neurosecretory material (NSM). It shows affinity for the acidic groups, appearing after oxidation of NSM with oxidizing agent potassium permanganate.

1.7 Aldehyde Fuchsin (Ewen, 1962) Staining Method

1.7.1 Solutions

1. *Gomori's fluid* – Prepare 2.5% $KMnO_4$ solution and keep separately in jar. Prepare 5% concentrated H_2SO_4 solution and keep in another jar.
2. *Sodium metabisulphite* – Dissolve 2.5 gm of Sodium metabisulphite in 100 ml of distilled water.
3. *Gabe's Aldehyde fuchsin* – Add 1 gm of basic fuchsin to 200 ml of distilled water taken in a flask at 50°C. Then heat it to boiling point, allow to boil for one minute, cool to room temperature, then add 2 ml of HCl (concentrated) and 2 ml

of paraldehyde. Plug the neck of the flask with cotton wool and leave to stand for 4 days until the red colour of the fuschin has disappeared and no further precipitate appears at the bottom. Filter the precipitate and discard the liquid, wash the precipitate on the filter paper, drain and dry the filter paper (temperature not exceeding 80°C) in an oven. Remove the dried precipitate from the filter paper and store in reagent bottle. Dissolve 0.25 gm of the above crystal in 35 ml of absolute alcohol and add 15 ml of distilled water. The solution can be used upto 6 months.

4. *Halmi's Mixture* : Light green – 0.2 gm dissolve in 2 ml distilled water. Orange G – 1 gm dissolve in 20 ml distilled water. Phototungstic acid – 5 gm in 5 ml distilled water. To the total volume of 27 ml add 75 ml of 1.3% glacial acetic acid. The final volume will be 102 ml.

5. *Solution 'E'* : Alcohol 96% – 2.5 ml
   Glacial acetic acid – 1 drop

1.7.2 Procedure

1. Bring sections to distilled water.
2. Oxidise in Gomori's fluid for 1 minute. First 30 seconds in $KMnO_4$ and next 30 seconds in concentrated H_2SO_4 (which are kept in separate jars).
3. Rinse in 2.5% Sodium metabisulphite solution until all permanganate stain is removed (a few seconds).
4. Wash in distilled water.
5. Dehydrate upto 70% alcohol.
6. Immerse in aldehyde stain for 2–10 minutes.
7. Rinse in 95% alcohol (2 changes) until no more aldehyde fuchsin comes away.
8. Hydrate sections down to water.

9. Counter stain with Halmi's mixture for 20 to 30 seconds.
10. Wipe the back of the slide and differentiate in solution 'E'
11. Rinse in 95% alcohol.
12. Dehydrate in alcohol (2 changes).
13. Clear in xylene and mount in DPX.

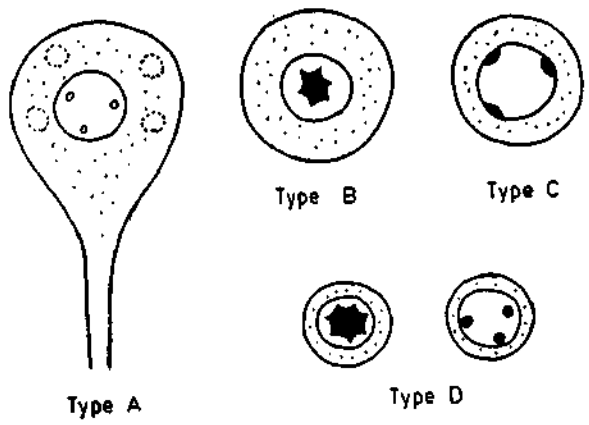

Fig. 1. Diagrammatic representation of the neurosecretory cell types present in brain, thoracic ganglion and eyestalk of the freshwater prawn *M. kistnensis*.

1.7.3 Results

Cystine, cysteine (NSM) take dark blue colour with yellow-white nucleus. Nuclear material takes violet colour.

1.7.4 Principle of the method

The aldehyde fuschsin is a basic dye. Shows affinity for acidic groups which are formed due to oxidation of NSM with oxidizing agents such as $KMnO_4$. The specific oxidation of the NSM involves the formation of cysteic acid from both cysteine and cystine which are in enormous quantity in NSM of crustaceans (Lake, 1970). The same oxidation process produces free aldehyde groups, to which the basic stains can bind (Gabe, 1953).

1.8 Types of Neurosecretory Cells

The neurosecretory cells are classified according to their size, shape and staining properties. The morphology of neurosecretory cells is highly variable in crustaceans and they are species specific (Figs. 1, 2 and Table 1).

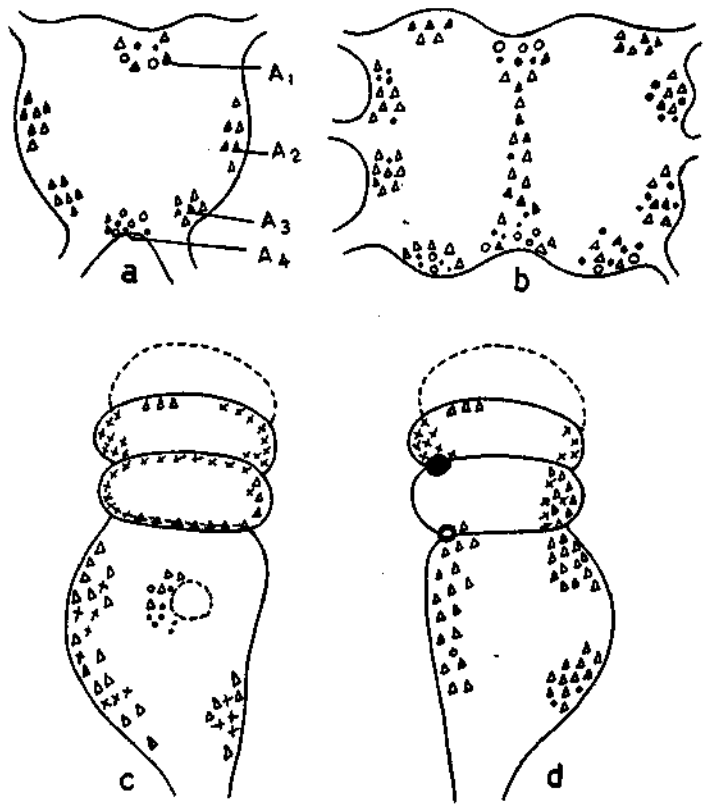

Fig. 2. Diagrammatic representation of the brain, thoracic ganglion and eyestalk of freshwater prawn *M. kistnensis* indicating the locations of neurosecretory cell groups and distribution. O – A cell, △ – B cell, • – C cell, o – D cell, ○ – Giant neuron and ● – Sinus gland.

TABLE 1. *Histological description of the cell types in the brain, thoracic ganglion and optic ganglion of the freshwater prawn* M. kistnensis

Cell type	Cell diameter range (μ)	Shape of the cell	Nuclear diameter range (μ)	Shape of the nucleus	Staining	
					Mallory's triple	AF
A	33 – 56	Oblong or round with or without axons	10 – 22	Round 2 – 3 nucleoli	Pinkish violet	Violet
B	19 – 32	Spherical	10 – 15	Nucleus round with irregular shaped nucleolus	Pinkish	Violet
C	15 – 17	Spherical	9 – 14	Round with 2 – 3 nucleoli or irregular nucleolus	Pinkish	Violet
D	9 – 12.5	Spherical	8 – 10	Round with 2 – 3 nucleoli or irregular nucleolus	Pinkish	Yellowish purple

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2

MOULTING AND MOULT HORMONES IN CRUSTACEANS

2.1 Introduction

The epidermis and cuticle are the regions of extreme physiological activity. Shedding off old exoskeleton or cuticle, formation and hardening of new cuticle is a physiological process, controlled by hormones in crustaceans. Drach (1939) described a series of morphological changes that occurred in between two intermoults and divided the moult cycle into A, B, C, D and E stages. Carlisle and Dorhn (1953) divided the moult stages into

i. **Pre-ecdysis** - during this time the animal is preparing to moult;

ii. **Ecdysis** - the splitting and shedding off the old exoskeleton;

iii. **Metecdysis** - a period of rapid redeposition of chitin and inorganic salts to produce a new exoskeleton and,

iv. **Intermoult** - a period in which the exoskeleton is hardened and calcification is maximal.

The intermoult period is of two types:

i. **Anecdysis** - is a long period of rest between the conclusion of one metecdysis and begining of the next pre-ecdysis and

ii. **Diecdysis** - is a period during which metecdysis passes imperceptibly into the succeeding pre-ecdysis.

2.2 Material

Freshwater/marine prawns.

2.3 Methods of Moult Stage Detection

The prawns collected from the fields can be maintained in glass troughs, provided with aeration. Detect the moulting stages (morphologically) under light microscope, mounting the exopodite of single pleopod of each animal in water. Observe external changes in pigmentation of the exoskeleton and later correlate with morphological changes within the pleopod.

2.3.1 Morphological changes

The stages A, B (postmoult), C (intermoult) and late D (premoult) can be readily distinguished from one another by the degree of hardness of the skeleton. The transitional stages are more difficult and can only be identified by histological techniques (Stevenson *et al.*, 1968; Aiken, 1973). Other noticeable characteristics which can be used to identify the transitional stages are for example, animals in stage A cannot raise their chelae if held out of water, their rostrum is easily bent and the setae within the pleopods are lacking an internal cone (Fig. 3 a) which can only begins to appear in stage B (Fig. 3 b). In stage B the chelae are operative, the rostrum cannot be bent and the exoskeleton is pliable. In both moult stages A and B, pigment retraction from the margins of the rostrum and abdominal plates has not started. Animals in stage C have a hard exoskeleton and little pigment retraction. Most of the setae in the pleopod have an internal cone (early C) and there is no epidermal pigment retraction from the exoskeleton. Pigment retraction begins in stage C and is first observed at the margins of the dorsal surface of the abdominal segments. The onset of stage D is characterised by epidermal retraction in the pleopods. This is known as apolysis (Jenkins and Hinton, 1966), and marks the beginning of D_0 stage

(Drach and Techernigovtzeff, 1967); extensive pigment retraction at the lateral margins of the exoskeletal segments in the abdomen becomes noticeable in early premoult (D_0, D_1) and D is indentified by complete pigment retraction at the margin of the lateral abdominal segments (Fig. 3 c).

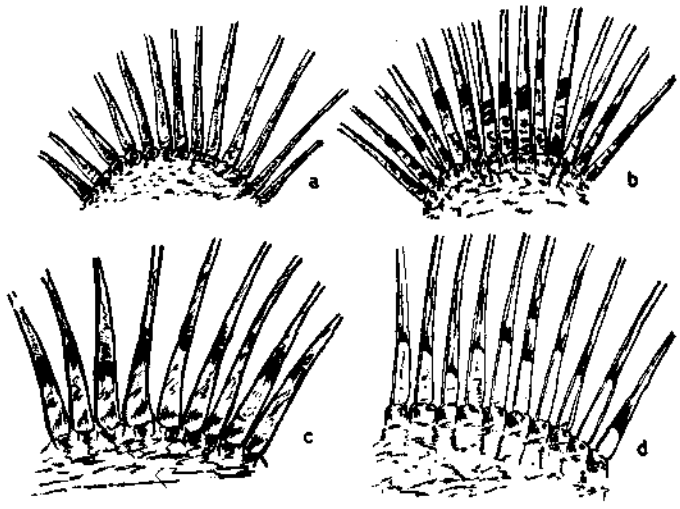

Fig. 3 a. The apical setae of a uropod in moult stage A (Note lack of internal cones and evagination of new setae) b. The apical setae of uropod in moult stage B (Note the developing internal cones), c. The apical setae of an uropod in moult stage D. Neo setogenesis initiation and d. The apical setae of uropod in moult stage D_3 and D_4 (Note new developing setae protruding into the base of old setae).

Pigment retraction in early premoult is not completed in the margins of all dorsal abdominal segments. Thus, identification of substages D_1^{I-III} and D_2^{I-III} must be made through microscopic examination of unstained and stained pleopods. Microscopic examination of the pleopod is also necessary to distinguish stage D_0 from stage D_1, as pigment retraction is variable and somewhat dependent upon the degree of pigmentation *i.e.* some individuals are lightly pigmented and it is difficult to determine the extent of retraction.

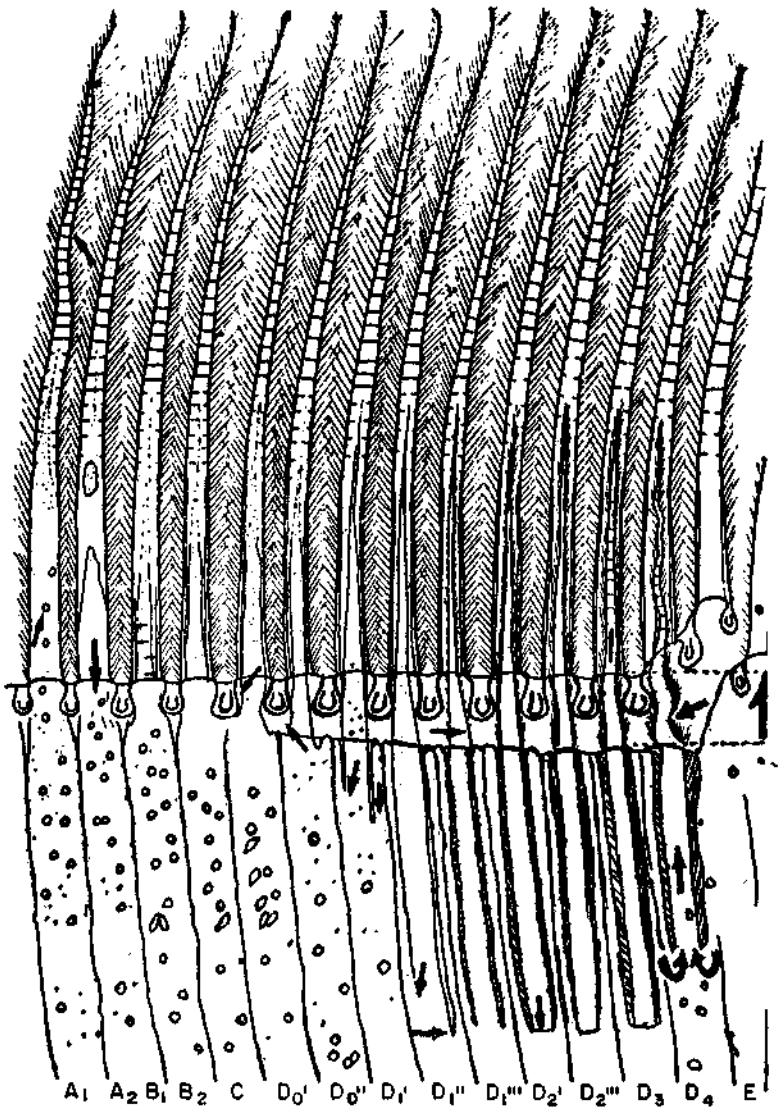

Fig. 4. Diagram showing all the moult stages (consolidated).

In stage D_1 new setae start to develop. In early D_1 (D_1') surface irregularities are apparent on the epidermis. Later (D_1''), developing setae can be seen at the epidermal surface and reorganisation appears to be ongoing within the epidermis as the setae are invaginating. In D_1''' the epidermal reorganisation is complete. The new setae can be seen and appear to be in "a tube within a tube" (Stevenson *et al.*, 1968). This structure which began to form in D_1' or D_1'' is probably similar to Aiken's setal organ (Aiken, 1973). The secretion of a new cuticle begins in D_2. This stage may be impossible to determine in the living animal (Stevenson *et al.*, 1968). Aiken (1973) suggested on the "Universal criteria for D_2'' the point in pre-ecdysis when there is histological evidence of a new pigmented layer adjacent to the midline in the gastric region of the carapace.

In *Macrobrachium rosenbergii* (Peebles, 1927) D_2 is probably best characterised by two traits in the living animal:

i. extensive pigment retraction as both lateral and dorsal surfaces in the abdomen and

ii. no visible setal development within the antennal scale.

Microscopic examination of the pleopod in early D_3' shows (i) some barbel development on the setae and (ii) a layer (possibly epicuticle and exocuticle) covering the epidermis (Fig. 3 d). In live *M. rosenbergii* D_3' is characterised by setal development in the antennal scale (Peebles, 1977). This can be seen easily by holding the antennal scale up to any bright light source and inspecting the margin for density changes in the tissues. The margin scatters light and appears denser than the centre of the antennal scale (Fig. 3 d). In stage D_3', the pigment begins to retract from the margin of the dorsal surface of the rostrum and the exoskeleton becomes flexible in D_3''.

The moult related changes which are described above can be easily remembered, are given in short in Table 1.

TABLE 1. *Changes in external and internal morphology in* M. rosenbergii (*Peebles*, 1977)

Moult stage	Development characteristics	duration in days
A	Exoskeleton soft, rostrum deflectable, no internal cones in setae.	1
B	Exoskeleton plible, rostrum hard, no pigment retraction in abdomen, internal cones forming in setae.	3–5
C	Exoskeleton hard, pigment retraction starts in margins of dorsal surface of abdomen, internal cones formed. *	29–79
D_o	Pigment retraction complete in margins of lateral surface of abdomen, apolysis apparent in pleopod tips.	**
D_1'	Surface irregularities apparent on the epidermis of the pleopod.	3–5
D_2''	New setae invaginating.	
D_1'''	Epidermal reorganization complete, setae invaginated into a 'setal organ'.	
D_2	Extensive pigment retraction on both dorsal and lateral surfaces of the abdomen and no visible setal development within the antennal scale.	
D_3'	Setal development in antennal scale.	2–3
D_3''	Pigment retraction from margin of dorsal surface of the rostrum.	2–3
D_3'''	Exoskeleton becomes flexible.	1–2

* Data from Wickins and Beard (1974) and adjusted for upper and lower limits of postmoult and premoult.

** Some crustaceans tend to remain in D_o for varying lengths of time. Presently it is not known if *M. rosenbergii* exhibits this trait.

2.3.2 Histological changes of cuticle

For the histological studies of integument, pieces of tissues were removed from the carapace and the abdomen of the prawn (*Caridina weberi*), approximately eight days, three days, twelve

hours and immediately after moult (Nagabhushanam and Chinnayya, 1972). All the integumental tissues were fixed in Bouin's fluid and sectioned at 8 μ, sections stained with Mallory's triple stain (Chapter 1).

2.3.3 Structure of the integument (Fig. 5)

The thickness of the cuticle of prawns in the intermoult period (Stage C) is 80 μ. The section of the cuticle shows a thin epicuticle and a thick procuticle.

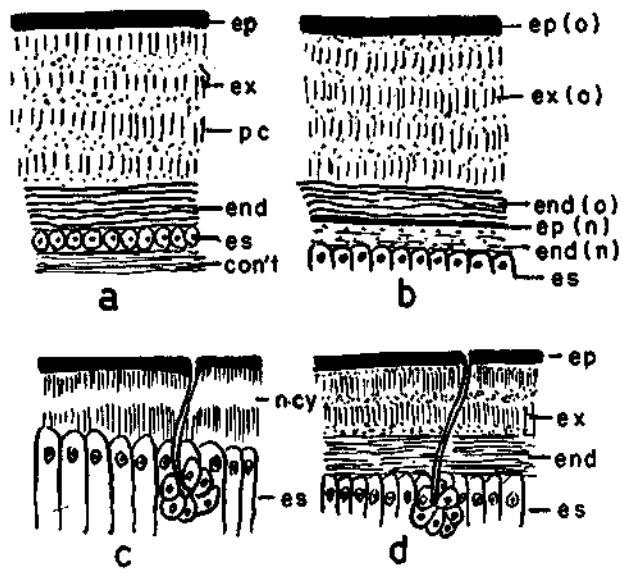

Fig. 5 a. T. S. through cuticle (stage C) – con't : connective tissue; end: endocuticle; ep: epicuticle; es: epidermis; ex: exocuticle; P.c: Pore canals, b. T. S. through cuticle (Stage D) - end (n): new endocuticle; end (o) old endocuticle; ep (n): new epicuticle; ep (o): old epicuticle; ex (o): old exocuticle, c. T. S. through cuticle (stage A) - n. cy: new cuticle; and d. T. S. through cuticle (stage B). Tegumental gland is shown c and d.

Epicuticle : It is a very thin layer and measures about a micron in thickness.

Procuticle : The procuticle is 79 μ in thickness and is subdivided into an outer exocuticle and an inner endocuticle. The exocuticle stains deeper blue than the endocuticle in Mallory's triple stain. Pore canals are seen clearly in the exocuticle. They are minutevertical ducts. Each pore canal is a discontinuous duct.

Epidermis : It is ill-defined and measures about 10 μ. The cells are cuboid in section; nuclei are round in shape.

Stage A (Fig. 5 c) : This stage follows immediately after the moult. The new cuticle is very thin and 3 μ in thickness. The epidermal cell are tall with round nuclei. Tegumental glands are sunk in the dermis The gland measures about 40 μ in length and 15 μ in width.

Stage B (Fig. 5 d) : At this stage the integument starts hardening. The rostrum and spines are firm but not brittle. In the new cuticle, two parts – epicuticle and procuticle are differentiated. The epidermal cells are less taller than the cells of the preceding stage.

Stage C (Fig. 5 a) : The cuticle is very thick and differentiated into three parts – epicuticle, exocuticle and endocuticle. The epidermis is ill-defined.

Stage D (Fig. 5 b) : This is the premoult period. This stage is further divided into the following stages:

D_1'– The integument is very thin, firm, resists to moderate pressure and breaks when bent.

D_1''– Resorption of inner layers of old integument begins.

D_2 – During this period complete resorption of old exoskeleton takes place. The new cuticle makes its appearance below the old one.

D_3 – This stage is immediately before moult. The integument is very soft and the old exoskeleton splits in preparation for moulting.

2.4 Eyestalk Ablation and Moulting

The first direct evidence for hormonal control of moult was given by Brown and Cunningham (1939). They found that removal of both eyestalks from the crayfish *Orconectes virilis*, caused an acceleration of moulting. When the contents of eyestalks were implanted into the body of an eyestalkless animal, moulting activity was delayed. This investigation was supported by many workers (Carlisle, 1953; Adiyodi and Adiyodi, 1970). Carlisle (1957) reported that Y-organs show changes during moulting. Later, Echalier (1959) found that bilateral extirpation of Y-organs caused a definite blockage of growth and moulting in the crab *Carcinus maenas*. Adiyodi and Adiyodi (1970) correlated the changes in eyestalk neurosecretory cells and Y-organ in crab *Paratelphusa hydrodromous*.

2.4.2 Eyestalk ablation technique

Before removing the eyestalks keep the prawns in ice-cold water for 15–20 seconds. Then by holding the prawns in the left hand and with the help of fine scissors cut the eyestalks at the base and release the prawns into the aquarium. Putting them into ice-cold water before ablation, reduces the flow of body fluid and ultimately the rate of mortality.

2.4.3 Effect of eyestalk ablation on moulting

Select prawns of medium size and maintain them in aerated water. Keep the intermoult prawns, confirm the stage by using Table 1 individually in 500 ml jars with sufficient water. Keep first jar as a control one and remove both the eyestalks of the prawns from other jars. Note down the day of the ablation and day of shedding the exoskeleton. Repeat the above experiment once more using different prawns. Compare the results of experimental prawns with control prawns.

2.5 Moulting Hormone (MH) and Moult Inhibiting Hormone (MIH)

Subsequent experiments of eyestalk ablation in crustaceans showed that if it is done in intermoult period, it results in earlier

initiation of proecdysis, but their removal during proecdysis has no effect. This suggests that eyestalk has a hormone which inhibit the moulting. The implantation of eyestalk neurosecretory system or injection experiment fulfilled the idea of production of MIH within the eyestalks. Later, the selective destruction of either the sinus gland or the X-organ, confirmed that the eyestalk neurosecretory system produces a moult inhibiting hormone. But discovery of Y-organ [moult accelerating hormone (MAH) secreting gland] revealed that moult inibiting hormone did not act directly upon the tissues to delay proecdysis. The Y-organ removal and implantation experiments established the fact of production of moulting hormone. Thus in crustaceans MIH inhibits the activity of the Y-organ to produce MH or MAH, during the intermoult period and its absence before proecdysis allows the Y-organs to secrete.

2.5.1 Moult inhibiting hormone (MIH)

Remove the eyestalks of the prawns as described before. Triturate 50 eyestalks in van Harreveld's solution.

Van Harreveld's physiological saline

380 ml of NaCl (0.54 M)	–	31.6 gm/lr
10 ml of KCl (0.54 M)	–	40.23 gm/lr
39 ml of $CaCl_2$ (0.36 M)	–	78.84 gm/lr
7.2 ml of $MgCl_2$ (0.36 M)	–	73.19 gm/lr
4.4 ml of $NaHCO_3$ (0.54)	–	48.6 gm/lr

Dilute it upto 3 litres with distilled water.

The supernatant should be centrifuged and used for injection purpose, 0.02 ml/prawn/2 Es.

2.5.1.1 Procedure

Take 40 prawns Keep 10 prawns (intact) separately in one jar. Remove the eyestalks of remaining 30 prawns and divide

them into three groups of 10 each. Keep first group as a control and inject other two groups with 0.02 ml/2 eyestalk/animal and 0.02 ml/prawn saline respectively. Keep individual prawns in separate jars. Note the time of eyestalk ablation. Observe the moulting in each group and compare the results.

2.5.3 Moulting hormone (MH)

For this experiment use crabs.

2.5.4 Procedure

1. Hold the specimen immobile on a dissection board with the face uppermost under a dissection microscope.
2. Place the dissection board in a tray containing 0.9% saline (0.9 gm sodium chloride in 100 ml distilled water).
3. Cut a 2.5 mm square piece of the pterygostomian region of the exoskeleton, bordering on the ventral edge of the suborbital region and directly below the cornea of the stalked eyes by means of a dental drill.
4. Incise the epidermis on three edges of the hole and lift back carefully, revealing the Y-organ which is attached to the epidermis (The Y-organ appears yellowish against the bluish-white colour of the surrounding muscle and connective tissue).
5. Remove the Y-organ with sharpened watch-makers tweezers.
6. Replace the epidermis in the original position and seal with molten paraffin wax.
7. Note down the time and day of Y-organ removal.
8. Compare the moulting period with control (intact crabs).

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3

CHROMATOPHORES AND COLOUR CHANGES IN CRUSTACEANS

3.1 Introduction

The chromatophores, the effectors situated beneath the epidermis or scattered in the deeper tissues of the body, are responsible for colour change mechanism in crustaceans ((Highnam and Hill, 1977). The crustacean chromatophore is a much branched cell whose overall shape remains unchanged during colour change (Fig. 6 a).

Crustacean chromatophores may contain one (monochromatic), two (dichromatic) or several (polychromatic) pigments. Red, blue, yellow, white and black pigments are commonly found in crustacean chromatophores.

3.2 Staging of the Chromatophores

The chromatophores can be staged according to dispersion of pigment within it. Hogben and Slome (1931) using stereoscopic dissecting microscope and transmitted light staged the chromatophores into five stages (Fig. 6 b_{1-5}).

3.3 Effect of Background on Colour Change

The morphological colour change is observed or evoked by keeping the animal on specific background. Such responses have

been demonstrated in *Ocypode ceratopthalma* and *Uca pugnax* (Green, 1964 a, b) and *Carcinus maenas* (Powell, 1962 a) and *Caridina weberi* (Vasantha, 1970). Other various coloured background response of a species found to be variable, probably depending upon the habitat.

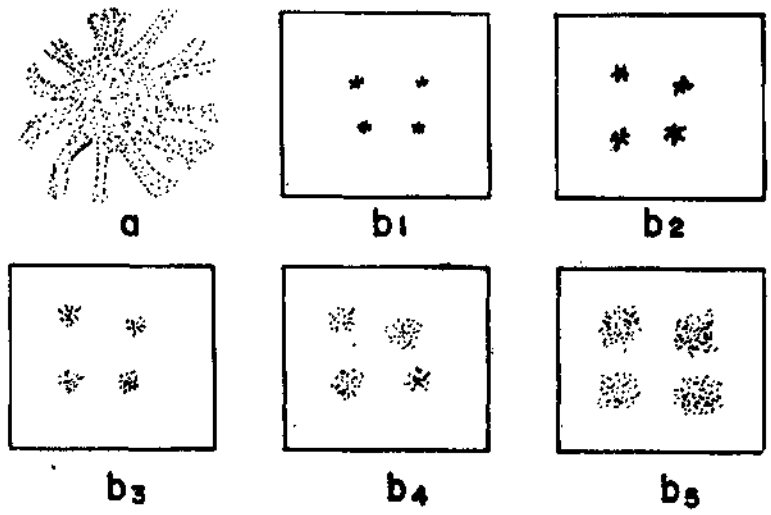

Fig. 6 a. Diagram of a chromatophore from the uropod of the prawn *Leander serratus* (Highnam and Hill, 1977) and b. *Stages* 1-5: b_1. Pigment is fully contracted "punctate stage", b_2. Progressive pigment dispersion "punctate stellate stage", b_3. Progressive pigment "Stellate stage", b_4. Progressive pigment dispersion "Stellate reticulate stage" and b_5. Pigment fully expanded or dispersed "reticulate stage".

3.4 Determination of Effect of Background on Colour Changes

3.4.1 Materials

Prawns, stereoscopic microscope, white pans, black pans.

3.4.2 Procedure

Collect the prawns and acclimate to the laboratory condition, 24 hours prior to start of the experiment. Select the adult, intermoult and intact prawns from the stock aquarium. Place them

in white and black enamel pans (10 in each pan). Place the pans under an illumination of 100 ft.c. light intensity for 2 hours. Determine the chromatophore stage using Hogben and Slome (1931) system. Then interchange the backgrounds, *i.e.* keep white background adapted prawns in black background and vice versa. Determine the chromatophore index of the prawns in each pan at interval of 15, 30, 45 and 60 minutes after the interchange of the backgrounds. Repeat the experiment once and plot the graph against chromatophore stage and minutes.

3.4.3 Inference

In freshwater prawn *Caridina weberi*, it has been observed that on a white background the white pigment became maximally dispersed and the red pigment maximally concentrated. On a black background the situation was reversed, the white pigment being fully concentrated and the red fully dispersed (Fig. 7).

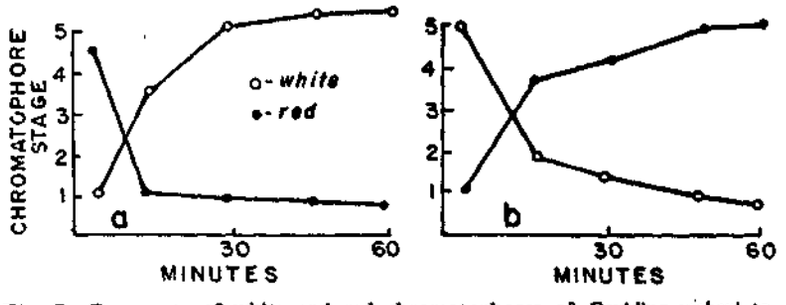

Fig. 7. Responses of white and red chromatophores of *Caridina weberi* to background changes: a. prawns on a black background changed to a white background and b. prawns on a white background changed to a black background.

3.5. Influence of Light on Colour Change Mechanism

The crustacean chromatophores respond to the environmental factors such as light and temperature. Response of chromatophores to light may be divided into two categories primary and secondary. A primary response of chromatophores to light,

involves the dispersion of pigments with increase of illumination, such response always occur through routes other than eyes. Secondary response depends on the nature of the background and the degree of pigment dispersion is calculated by the ratio of the amount of light directly incident on the eye to the quantity falling on the eyes after reflection from the background. This is known as the "Albedo Ratio" or secondary response. Primary and secondary responses have been studied in some natantians like *Hippolyte* (Keeble and Gamble, 1900; Kleinholz and Welsh, 1937); *Palaemonetes* (Fingerman and Tinkle, 1956); and *Caridina weberi* (Vasantha, 1970).

Light plays a direct role in crustacean chromatophoral activity. Hence, eyestalks which possess the sinus glands, were removed in the experimental prawns and were subjected to the varying degree of illumination. Brown (1948) reported that in the crabs which were not exposed to high intensities of light, the black pigment concentrated maximally after eyestalk removal. Fingerman (1957, 1958) observed the effect of eyestalk ablation in natantians and brachyurans.

3.6 Relation Between Light Intensity and Background Adaptation

3.6.1. Materials

Prawns, stereoscopic microscope, white and black enamelled pans, 100 watts bulb.

Calculation of light intensity

The detailed light intensities (2, 10, 100 and higher intensity) can be obtained by placing the pans at appropriate distance from the light source available in the laboratory. For 2 ft. c. light intensity pan should be placed at 6.32 5' from the source of light, for 10 and 100 ft. c. pan should be at 2. 8 and 4 to 4.5" from the source.

3.6.2 Procedure

Take 4 black pans and keep 10 prawns in each pan. Similarly keep 10 prawns in four white pans. Place one black and one white pan under 2, 10, 100 ft. c. light and sunlight. The water of the pan placed in sunlight should be changed at frequent intervals to prevent heating of the prawns. After two hours of exposure, determine the average chromatophore index of prawns in each pan. Repeat the experiment and plot the average chromatophore index against the logarithm of the incident of illumination.

3.6.3 Inference

From the results of the experiment performed on *Caridina weberi*, it can be inferred that dispersion of white pigment in the specimens on white and black background increased with increase in light intensity. But dispersion of white pigment was always more on the white background than on a black background at all intensities. The red pigment concentrated at all intensities of light on the white background. The red pigment in the specimens on black background at 2 ft. c. light intensity was at stage 5.0 and the pigment concentrated to stage 2.6 at high light intensity (Fig. 8 a, b).

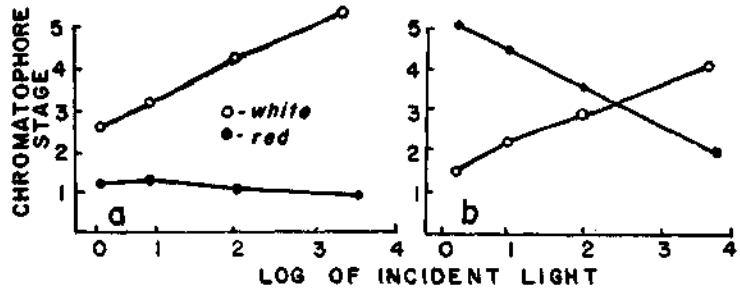

Fig. 8. Relationship between the average red and white chromatophore indices and the logarithm of the incident light intensity of prawns : a. white background and b. black background.

3.7 Response of Chromatophores to Temperature

The physiological colour changes are evoked by environmental factors and temperature is one of them. The responses to

temperature are not as uniform as light responses. Smith (1930) found dispersion of the melanophores in *Macrobrachium* with the increase of temperature, whereas in the fiddler crab *Uca* black pigment was concentrated with the rise of temperature (Brown and Sandeen, 1948). Similar observations were made by Fingerman (1956 b) on *Calinectes sapidus*. The responses of black, red and white chromatophores to temperature were also studied by Nagabhushanam (1963, 1964, 1966) in *Gelasimus annulipes* and *Metopograpsus messor* and Rangarao and Nagabhushanam (1967) *Uca annulipes*.

3.7.1 Materials

Prawns, white pans, stereoscopic microscope.

3.7.2 Procedure

Take 40 prawns, divide them into four groups and place them in 4 white pans. Add sufficient water to each pan to cover the prawns and place the pans in hot water baths. The temperature of the four water baths should be maintained at 15°C, 20°C, 25°C and 30°C respectively. After two hours the temperature of water in pans will reach to the equillibrium with the water baths. Then determine the average chromatophore stage and plot a graph against temperature.

3.7.3 Inference

The experiment performed on *Barytelphusa cunicularis* explained that the black pigment was concentrated and the red pigment dispersed with the increase in temperature.

3.8 Endocrine Control of Chromatophores and Colour Change

The chromatophorotropins, hormones which control the colour physiology, are reported to be present in crude extracts of the nervous system of various malacostracans. Perkins (1928)

described the eyestalks as the source of a hormonal substance which concentrated the red pigment of the prawn *Palaemonetes vulgaris*. Enami (1951) working on *Sesarma* reported that the black pigment dispersed maximally after eyestalk ablation. Later it was confirmed that there are both, pigment concentrating and dispersing hormones in eyestalks and central nervous tissues of crustaceans (Nagabhushanam, 1963, 1964, 1966).

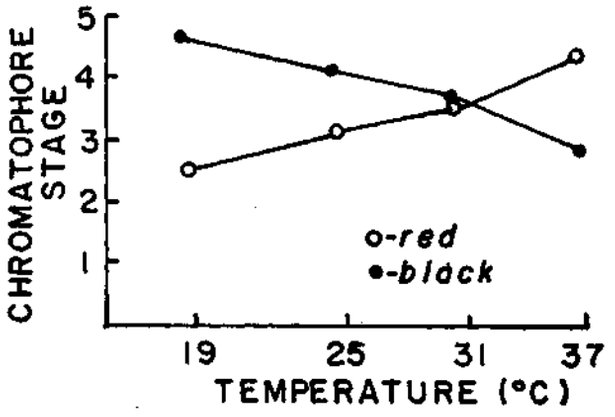

Fig. 9. Relation between the average black and red chromatophores indices and temperature in *Barytelphusa*.

3.9 Effect of Eyestalk Ablation and Injection on Chromatophores

3.9.1 Materials

Prawns, white and black pans, stereoscopic microscope, scissors.

3.9.2 Procedure

Remove both the eyestalks of twenty prawns (refer Chapter 1, method 2.4.2) and stage chromatophore stages at intervals of 15, 30, 45 and 60 minutes from both backgrounds. Plot graph of chromatophore index against time interval.

To see the effect of eyestalk extract on chromatophores keep the eyestalk ablated prawns on white and black background for two hours, then inject the eyestalk extract (see Chapter 2 method 2.5.1, 0.2 ml/prawn) at abdominal region. Stage the chromatophores using Hogben and Slome (1931) system 15, 30, 45 and 60 minutes after injection. Plot graph of chromatophore index against time interval.

3.9.3 Inference

After eyestalk ablation the white chromatophores were fully dispersed on white background in *Caridina weberi*. The minimum time for dispersion required was 45 minutes. In black background adapted prawns the red chromatophores were fully dispersed within 30 minutes.

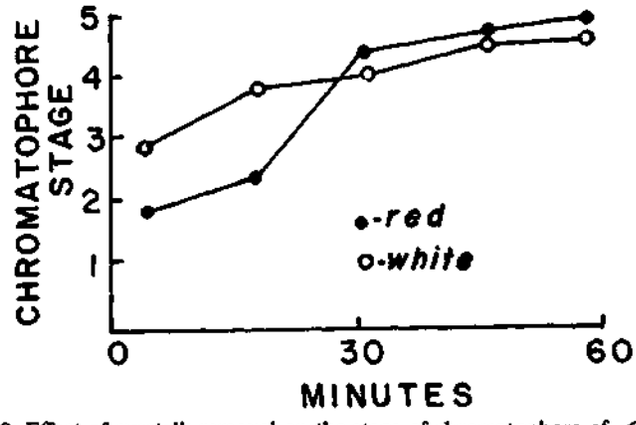

Fig. 10. Effect of eyestalk removal on the stage of chromatophore of *Caridina weberi*.

After eyestalk extract injection it was observed that the dispersed pigments get concentrated to some extent, *i.e.* the extract restored the original condition of the pigments in chromatophores to some extent. It is evident from these experiment that eyestalk has some endocrine activity which controls the chromatophore activity in crustaceans (Fig. 10).

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4

EFFECT OF EYESTALK ABLATION ON BLOOD SUGAR IN CRUSTACEANS

4.1 INTRODUCTION

The crustacean eyestalk is the source of one or more hormones which control blood glucose levels. One of these hormones called the hyperglycemic hormone (HGC, diabetogenic hormone), is able to elevate the level of blood sugar, resembling thus glucagon in vertebrates. Abramowitz *et al.* (1944) for the first time reported the relation between the eyestalk principle and carbohydrate metabolism in crustaceans. They identified a definite structure in the eyestalk of blue crab *Callinectes sapidus* which was reponsible for the elaboration of a diabetogenic factor. Rangnekar *et al.* (1961) found that the ablation of eyestalks led to rise in the blood sugar level of *Paratelphusa jacquemontii* and injection of eyestalk extract into both destalked and intact crabs produced hypoglycemia, suggesting the presence of hypoglycemic factor in the eyestalks. On the contrary Menon and Sivadas (1967) reported in *Scylla serrata* that the eyestalks has a hyperglycemic factor and removal of it resulted in hypoglycemia. From these evidences it is clear that crustacean eyestalk has a hyperglycemic hormone which control the blood sugar level. So experiment can be done in the laboratory on crabs or prawns to see the effect of eyestalk ablation on blood sugar level and to interpret the presence of hypo- or hyperglycemic hormones in eyestalks.

4.2 Methods for Blood Sugar Estimation

To determine the reducing sugar or total free sugar in blood, use method given in Section 4.2 and 4.3 of Chapter 4 "Total free sugars, Reducing Sugars and Glucose" by T. S. Saravanan and M. H. Ravindranath in the *Manual of Research Methods for Crustacean Biochemistry and physiology.* **CMFRI Special Publication 7 (1981) pages 17-21.**

4.2.1 Procedure

1. Select the intermoult prawns of same size and sex.
2. Divide them into three groups.
3. The first group will serve as the control one.
4. Remove the eyestalks of the remaining two groups. (Method given in Chapter 2, method 2.4.2).
5. Determine or estimate the blood sugar level from normal control prawns. Take out 0.2 ml of blood with the help of hypodermic syringe and 27 gauze needle.
6. Also draw the blood from eyestalk ablated prawns (Group two) at 1, 2, 4, 6 and 24 hours interval and determine the blood sugar level in order to get idea of the changes in the level of blood sugar.
7. For third group, eyestalk ablated, keep them for at least 24 hours undisturbed. After 24 hrs remove the eyestalks of fresh prawns and prepare the extract of it (refer Chapter 2, method 2.5.1) and inject 0.02 ml/2 ES/prawn and then estimate the blood sugar level at intervals of 1, 2, 4, 6 and 24 hrs respectively.
8. Calculate the blood sugar level in three groups and compare your results.

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5

ENDOCRINE CONTROL OF RESPIRATION IN CRUSTACEANS

5.1 Introduction

The oxygen uptake influenced by many extrinsic and intrinsic factors. All these factors individually and collectively affect the respiratory metabolism in animals (Sarojini and Nagabhushanam, 1968; Diwan and Nagabhushanam, 1972) and is under neuroendocrine control (Bliss, 1951; Nagabhushanam and Kulkarni, 1978; Sarojini *et al.*, 1981).

5.2 Method for Determination of Oxygen Consumption

The Winkler's method can be used for determination of oxygen uptake by prawns or crabs.

5.2.1 Principle

$MnCl_2$ when added to known volume of water, containing dissolved oxygen, get converted into $Mn(OH)_2$. This precipitate of $Mn(OH)_2$ is then dissolved into non-oxidizing acid such as HCl and made to react with KI. An equivalent quantity of iodine is then liberated which is titrated against sodiumthiosulphate using strach as an indicator.

5.2.2 Apparatus

Reagent bottles (capacity 300 ml), burette, pipette, glass jars (2 litre capacity), conical flasks, burette stand and measuring cylinder.

5.2.3 Reagents

1. *Winkler's 'A' solution* : Dissolve 40 gm of $MnCl_2$ in 100 ml of distilled water.
2. *Winkler's 'B' solution* : Dissolve 33 gm of sodium hydroxide and 10 gm of potassium iodide (KI) in 100 ml of distilled water.
3. *Sodium thiosulphate solution* (N/80) : Dissolve 3.1 gm of sodium thiosulphate in 1 litre of distilled water.
4. Concentrated HCl.
5. *Starch solution* 1% : Weigh 1 gm of starch and measure 100 ml of distilled water. Prepare a paste of starch in some amount of water and keep the remaining water for boiling. After boiling starts, add starch paste and stir constantly with the help of a glass rod. When solution starts boiling take it down and cool to room temperature. Then use for the experiment as an indicator.
6. Liquid Paraffin.

5.2.4 Procedure

1. Collect a sample of normal water for analysis in a reagent bottle. This is best done with a siphon, letting water run over the top of the bottle so that the water will have as little contact with air as possible. Insert the stopper so as to force out some of the water. Trap no air, an air bubble will ruin the analysis. The volume of the bottle should be measured previously.
2. Now add, with the tip of the pipette just below the surface, 1 ml of Winkler's 'A' solution, shake vigorously and add 1 ml

of Winkler's 'B' solution. Stopper, making sure no air is trapped. Shake thoroughly and let the precipitate settle.

3. Remove the stopper and add few drops of concentrated HCl till the precipitate dissolves. But do not add more than enough HCl to dissolve the precipitate, it may reduce oxygen in solution.
4. Take out 100 ml of solution from the reagent bottle into a conical flask.
5. Add 2 to 3 drops of starch. The colour becomes blue.
6. The solution is then titrated against the sodium thiosulphate till it becomes colourless.
7. Note down the initial and final readings of sodium thiosulphate in burette.

5.2.5 Calculations

$$\text{Amount of oxygen present in sample} = \frac{V \times 70}{S}$$

where, V = Burette reading *i.e.* volume of sodium thiosulphate used

S = Volume of sample used (100 ml).

5.3 Effect of Eyestalk Removal and Injection on Oxygen Consumption

5.3.1 Procedure

1. Collect prawns and maintain them in the laboratory for at least 24 hrs, prior to starting of the experiment.
2. Select intermoult prawns of same size and divide them into three groups.
3. First group will serve as intact control.
4. Ablate the eyestalks of other two groups (see Chapter 2, method 2.4.2).
5. Keep second group as it is.

6. Inject the third group prawns with 0.02 ml of eyestalk extract/ 2 ES/prawn (see Chapter 2, method, 2.5.1).
7. Keep these three groups in separate 2 litre jars and pour one litre water in the jar and cover the surface with liquid paraffin. Keep for 1 hour.
8. Estimate the oxygen content of the water of the experimental jars (see method given above 5.2).

5.3.3 Calculations

Calculate amount of oxygen by the formula given in 5.2.

A. Amount of oxygen present in normal water/lr.

B. Amount of oxygen present in intact prawns/lr.

C. Amount of oxygen present in eyestalk ablated prawn/lr.

D. Amount of oxygen present in eyestalk ablated extract injected prawns/lr.

Oxygen consumed by normal prawns = A–B = X. Oxygen consumed by eyestalk ablated prawns = A–C = Y. Oxygen consumed by eyestalk ablated + extract injected prawn = A–D = Z

By comparing the results X, Y, Z one can interpret the role of eyestalk hormone in oxygen consumption.

5.4 Reefrences

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6

ENDOCRINE CONTROL OF OSMOREGULATION IN CRUSTACEANS

6.1 Introduction

Osmoregulation can be understood in a better manner by studying the neuroendocrine control of chloride concentration. Scudamore (1947) demonstrated that the disturbed ecdysial water metabolism of eyestalkless crayfish was due to absence of sinus gland. Kamemoto *et al.* (1966) have given evidence for neuroendocrine regulation of salt water homeostasis in *Procambarus clarkii* and *Metapograpsus messor*. Venkatachari *et al.* (1979) working on *Barytelphusa querini* demonstrated that the eyestalk removal decreased blood chloride content while it was recovered to normal level after eyestalk extract injections. In crustaceans the neuroendocrine control of osmoregulation can be studied with the help of two experiments :

i. changes in body weight after eyestalk ablation and

ii. blood chloride estimation at different salt concentration.

6.2 Changes in Body Weight after Eyestalk Ablation

6.2.1 Principle

It is known that salt and water balance is controlled at least in part, by hormones secreted by X-organ of eyestalk which is

stored in sinus gland. Extirpating the source as well as storage site (*i.e.* eyestalk ablation) will affect the salt-water balance. There will be influx outflow of water of the body depending upon the medium in which animals are placed.

6.2.2 Material

Crabs or prawns.

6.2.3 Procedure

1. Collect the specimens and maintain them in laboratory for 24 – 48 hrs before starting of the experiment.
2. Select the prawns of intermoult stage, having the same size.
3. Divide them into two groups of 10 each.
4. Weigh each animal before the experiment starts.
5. First group will serve as intact control. Remove the eyestalk of prawns of the second group, which will be experimental one.
6. Place animals individualy in 500 ml beaker and note the weight at intervals of 1, 2, 3, 4, 6 and 24 hours.
7. Compare the results of normal prawns with eyestalkless prawns.

6.2.4 Inference

It will be observed that there is an increase in weight of eyestalkless prawns due to uptake of water.

6.3 Effect of Eyestalk Ablation on Blood Chloride Content

When the eyestalk ablated prawns are acclimatized to different salt concentrations, there will be some mechanism by which prawns will maintain the salt water homeostasis and this can be supported by the increase in blood chloride content. For the estimation of blood chloride, please refer Chapter 14 "Chloride" by K. Kannan and M. Arumugam in the *Manual of Research Methods for Crustacean Biochemistry and Physiology*. CMFRI Special Publication 7 (1981) pages 97 and 98.

6.4 Blood Chloride Estimation in Eyestalk Ablated Prawns

6.4.1 Material

Prawns or crabs.

6.4.2 Procedure

1. Remove the eyestalks from the second and third groups (see procedure in Chapter 2, method 2.4.2).
2. Determine the blood chloride contents in all the groups by method given in 6.3.1.
3. Take the readings at 1, 4, 6, 24 hrs interval for all the groups.
4. After 24 hours reading was taken inject eyestalk extract into the third group of prawns (0.02 ml/2/ES/prawn) (for preparation of extract see Chapter 2, method 2.5).
5. After injections take reading at 1, 4, 6 and 24 hrs interval.
6. Calculate the blood chloride content and compare the results in all groups.

6.5 References

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7

RETINAL PIGMENT MIGRATION IN CRUSTACEANS

7.1 Introduction

The crustacean compound eye adjusts to light intensity by means of pigmentary effector cells which are capable of altering their positions. Kleinholz (1936) was first who reported that distal retinal pigment moves towards the fully light-adapted position after injection of eyestalk extracts. This had been confirmed by Welsh (1939) in *Cambarus bartoni*, Fingerman *et al.* (1960) in *Crangon septemspinosus* and Nagabhushanam and Sarojini (1969) in *Diogenes bicristmanus*. Like other physiological and metabolic activities, the retinal pigment migration in crustaceans is also under hormonal control.

7.2 Study of Eye Pigment Position

In order to understand the pigment movements in compound eyes, it is necessary to know the general structure of the eye and the position of the light sensitive rhabdomes. It is advantageous to have a set of histological slides of light and dark adapted eyestalks of specimens. Vertical longitudinal sections are the most useful.

7.2.1 Material

Prawns, microscope, ocular micrometer.

7.2.2 Procedure

1. To obtain the extreme light adapted position of retinal pigments, place animals in bright, diffused day light and after 2-3 hours immobilize the pigments by dipping the animals in water at 90° C for 2 minutes (Fingerman and Nagabhushanam, 1963).
2. To obtain the extreme dark-adapted position, place animals in complete darkness for 3–4 hours and fix retinal pigment by keeping them in hot water as described above.
3. Then remove the eyestalks and fix them in Zenker's fluid (2.5% $K_2 Cr_2 O_7$ – 100 ml add 5 gm of $HgCl_2$, 5 ml of formalin (40%) and 5 ml acetic acid).
4. Dehydrate, embed in parffin wax and section longitudinally at 7–8 μ.
5. After sectioning observe the light and dark-adapted positions of the pigments. (Fig. 11).
6. In light-adapted situation retinal pigments are massed about the sensitive rhabdome at the inner end of each ommatidium. In this state, only light rays passing down the axis of the ommatidium reach the receptive structure.
7. In darkness, part of the pigment may retreat and part advance to a position near the cornea, leaving the sensitive rhabdome exposed to light from any angle. This shift often exposes a reflecting layer so that a dark adapted eye shows a striking red 'glow' in reflected light (Parker, 1932).
8. Further to express the positions of retinal pigments in quantitative terms, three measurements should be made with the aid of ocular micrometer under the compound microscope.
9. The technique of measurement is similar employed by De Bruin and Crisp (1957).
   a. Cornea to distal edge of the distal pigment,
   b. cornea to distal edge of the proximal pigment and
   c. cornea to the basement membrane, whose position is fixed.

The ratio of a/c and b/c are called as distal and proximal pigment indices respectively.

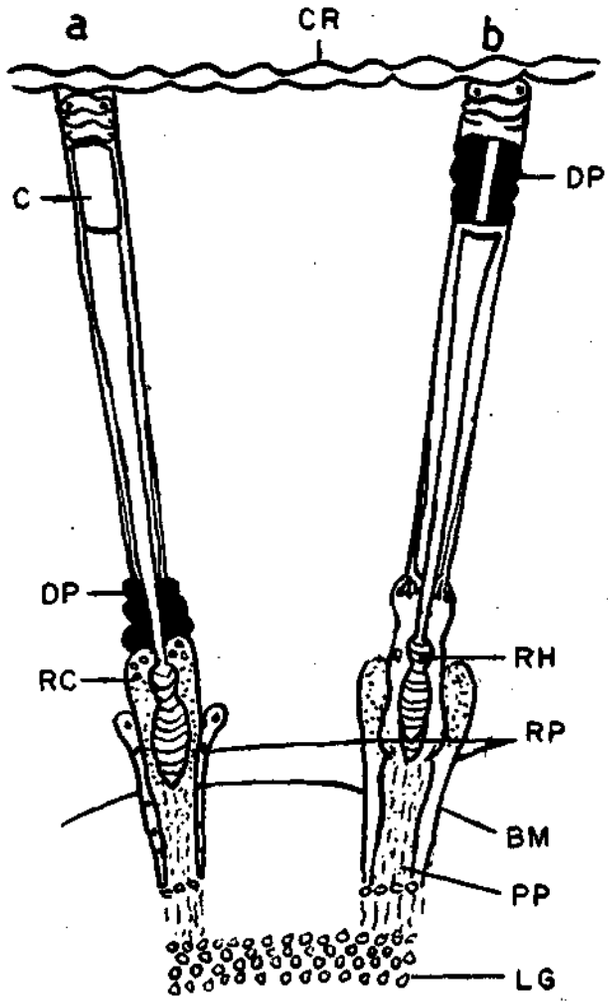

Fig. 11. Position of pigments around an ommatidium of a crustacean eye: a. Light adapted ommatidium and b. Dark adapted ommatidium (CR: Cornea, C. cone, DP: Distal pigment, RC: Retinular cell, RH: Rhabdome ,RP: Reflecting pigment, BM: Basement membrane, PP: Proximal pigment and LG: Lamina ganglionaris).

7.3 Response of Proximal Pigment to Eyestalk Extract

7.3.1 Material

Prawns or crabs, compound microscope, ocular micrometer.

7.3.2 Procedure

1. Collect the prawns and keep them in aerated aquaria.
2. Select the intact prawns and divide them into 9 groups of 10 each.
3. Place the pans (white) with prawns and sufficient water, under an illumination of 2 ft. c. for 2 hours. At the end of the period, fix the eyestalk to note the position of the proximal pigment.
4. Treat the remaining 8 groups for eyestalk extract injection experiment.
5. Inject 0.02 ml/eyestalk extract / prawn in one group and to another group injected 0.02 ml physiological saline (refer Chapter 2, method 2.4.2 and 2.5.1 for extract preparation).
6. Sacrifice the prawns from both the treatments at 15, 30, 45 and 60 minutes.
7. Fix the eyestalks and after proper processing determine the pigment position, use the method given above (7.2.1).
8. Plot the time course and pigment indices graph (Fig. 12 a).

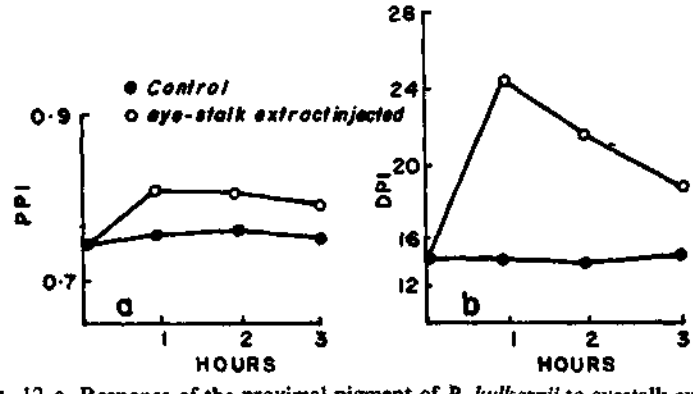

Fig. 12 a. Response of the proximal pigment of *P. kulkarnii* to eyestalk extract and b. Response of the distal retinal pigment of *P. kulkarnii* to eyestalk extract.

7.4 Response of Distal Pigment Migration to Eyestalk Extract

7.4.1 Material

Prawns or crabs, pans, compound microscope and ocular micrometer.

7.4.2 Procedure

1. Collect the prawns and maintain them in the laboratory.
2. Select healthy prawns and divide them into 9 groups of 10 each.
3. Adapt all groups for 2 hours in darkness.
4. After two hours, sacrifice prawns from one group and fix the eyestalks to study the position of distal pigments.
5. Prepare eyestalk extract (see Chapter 2) and inject it into 4 groups (0.02 ml/prawn). Inject 0.02 ml of physiological saline into the remaining four groups.
6. Sacrifice the prawns from all the groups after 15, 30, 45 and 60 minutes. Fix the eyestalks and determine the position of distal pigments.
7. Plot the graph, time against distal pigment index.
8. Compare the results with previous experiments of proximal pigment movement (Fig. 12 b).

7.5 References

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8

PERICARDIAL ORGANS IN CRUSTACEANS

8.1 Introduction

The pericardial organ lies across the opening of the branchiocardiac veins. It is also observed that nerves of pericardial organ contain neurosecretory granules. The position of pericardial organ and presence of neurosecretory granules, suggest a role in regulating the heart best (Fig. 13).

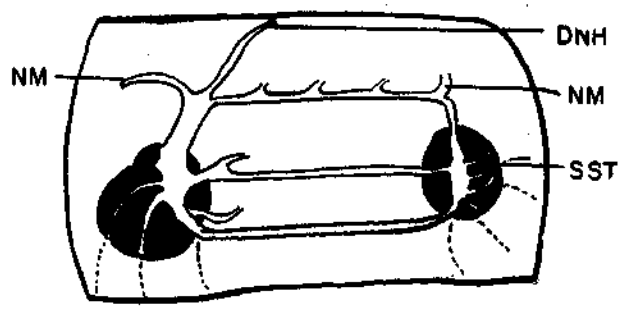

Fig. 13. Pericardial organs of the right side of the Spider crab *Maja squinado* (DNH: Dorsal nerve of the heart, NM: Nerve to muscle and SST: Strands suspending the trunks).

There are neurohaemal organs, lying within the pericardial cavity and bathed in blood moving towards the heart. The organs are shown in place on the inside of the lateral pericardial wall.

The nerves from the central nervous system which enter the pericardial organs are shown as dotted lines and the points at which the two anterior nerves enter the lumen of the veins are shown as crosses (After Carlisle and Knowles, 1959). Maynard (1961) studied the histology and location of neurons, contributing to the neurohaemal structures. The histology of the terminations in the pericardial organ was described by Maynard and Maynard (1962).

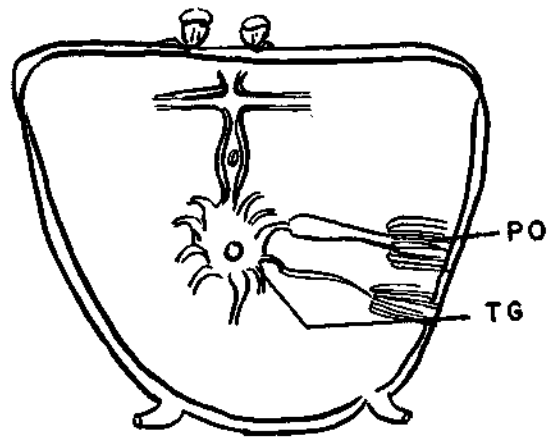

Fig. 14. The position of thoracic ganglio-pericardial organ in *Barytelphusa cunicularis* (PO: Pericardial organ and TG: Thoracic ganglion).

Devararoo (1981) examined and did the comparative study of the pericardial organs in freshwater crab *Barytelphusa cunicularis* and marine crab *Scylla serrata*. It is suggested that pericardial organs release peptide neurosecreory products designed to initiate the action of a neurotransmitter acting upon the neurons of the cardiac ganglion.

8.2 Material

Prawns or crabs.

8.3 Procedure

1. Collect the crabs from the field and maintain them in the laboratory.
2. Select healthy intermoult crabs of same size.

3. Locate the thoracic ganglion and trace its nerves going to the pericardial organs (Fig. 14 and Fig. 15).

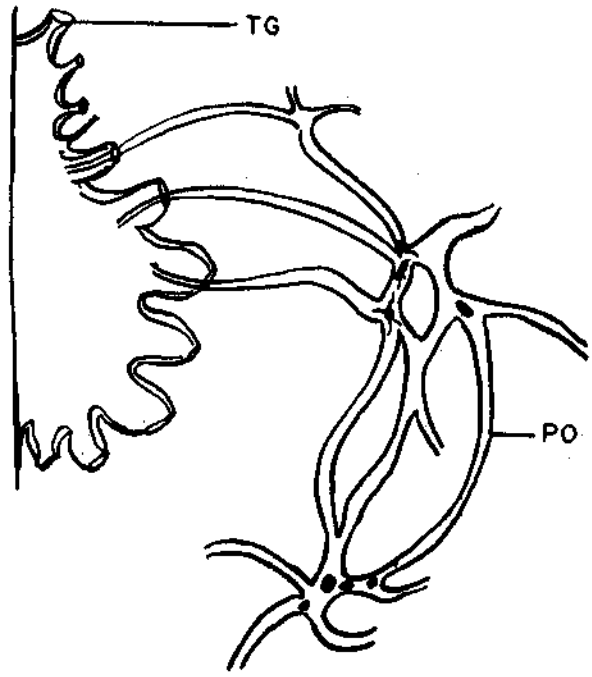

Fig. 15. The nerve connections between thoracic ganglia and pericardial organ (PO: Pericardial organ and TG: Thoracic ganglion).

4. Fix pericardial organs for 24 hours in aqueous Bouin's fluid.
5. Dehydrate, do paraffin embedding and section at 5 – 6 μ.
6. Stain the sections of pericardial organs with Heidenhan's Azan technique (Pearse, 1966).

8.4 Heindenhan's Azan Technique

8.4.1 Reagents

A. Azocarmine B – 0.5 gm.
Distilled water – 100 ml.

Glacial acetic acid – 1 ml.
Dissolve by warming, cool and filter.

B. 96% alcohol – 100 ml.
Aniline oil – 1 ml.

C. 95% alcohol – 99 ml.
Glacial acetic acid – 1 ml.

D. Phosphotungstic acid – 5 gm.
Distilled water – 75 ml.
Methanol – 25 ml.

E. Aniline Blue (Aq) – 0.5 ml.
Orange G – 2 gm.
Glacial acetic acid – 8 ml.
Distilled water – 100 ml.

Dissolve by warming, cool and filter, for staining purpose dilute 1 volume of this solution with 3 volumes of distilled water.

8.4.2 Procedure

1. Cut paraffin sections at 7 – 8 μ.
2. Bring down grade section to water.
3. Stain for 45 to 60 minutes in solution 'A' at 55°C. and then at room temperature for 6 to 10 minutes.
4. Wash in distilled water, then differentiate in solution 'B' until the cytoplasm is pale pink and nuclei red colour.
5. Rinse for one to two minutes in solution 'C'.
6. Transfer to solution 'D' for about 1 to 3 hours, until the connective tissue is completely decolourised. Wash quickly in distilled water.
7. Stain for 1 to 2 hours in a dilute solution of 'E' examining at 10 to 15 minutes interval to prevent overstaining.
8. Wash in distilled water.
9. Differentiate in 95% alcohol.

10. Followed by absolute alcohol.

11. Clear and mount.

8.4.4 Results

Collagen – Deep blue.
Reticulum – Deep blue.
Chromation – Red.
Muscle tissue – reddish to orange.
Erythrocytes – red.
Neuroglia – red.
Mucin – Blue.

8.5 References

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9

IDENTIFICATION OF STEROIDS

9.1 Introduction

The term chromatography was first introduced in 1906 by the Russian botanist Michael Tswett. He used a column of calcium carbonate to separate the components of petroleum ether and extract of chlorophyll. In recent years, chromatographic techniques have been responsible for many of the spectacular advances in biochemistry and physiology. The paper chromatography, column chromatography, thin layer chromatography (TLC) and gas liquid chromatography (GLC) are widely used in biochemistry.

For identification of steroids or steroid hormones the TLC is the best method. In thin layer chromatography a thin layer of silica gel is dried on glass plates; this offer distinct advantage in speed, sharpness of separation and the possibility of using corrosive acids (which would destroy paper) for the purpose of indentifying solute molecules (Stahl, 1965).

9.2 Extraction of Crude Steroid from Fresh Tissue

9.2.1 Material

Ovary or testis of mature prawns or crabs.

9.2.2 Procedure

1. Weigh the tissue.
2. Homogenize the tissue in a small volume of kreb's buffer at pH 7.4. Use about 5 ml of buffer per gm of tissue.
3. Add about three volumes of acetone (*i.e.* 3 x volume of homogenate) to precipitate the proteins, shake the mixture and let stand for five minutes.
4. Centrifuge and pour the supernatant into a small Erlenmeyer flask.
5. Repeat the procedure with another three volumes of acetone, centrifuge and discard the protein precipitate. Pool the acetone extracts.
6. Evaporate the acetone down to about 2 ml under a gentle stream of nitrogen directed on to the surface with a pasteur pipette attached by rubber tubing to a nitrogen cylinder. This process may be speeded up by placing the container in a warm bath but the temperature must never exceed 45°C.
7. Now transfer the acetone extract to a separating funnel (no smaller than 250 ml) add nine volumes (18 ml) chloroform gently, shake several times.
8. Wash the chloroform extract by shaking with 0.1 volume (2 ml of 0.1 N NaOH. Drain off the NaOH, wash solution, retaining the chloroform fraction in the separatory funnel. In the same way wash the chloroform extract with 0.1 volume (2 ml) of 0.1 N HCl, to neutralize any remaining NaOH. Finally add 0.1 volume (2 ml) of distilled water to remove any NaOH or HCl left in the chloroform fraction. Transfer chloroform fraction to an Erlenmeyer flask.
9. To remove water, add about 2.5 gm anhydrous Na_2 SO_4, shake well and let stand for 10 minutes.
10. Filter the mixture, rinse the filter with several ml of chloroform and add this wash to the filtrate.

11. Evaporate the chloroform down to 1–2 ml using a rotary flash evaporator (< 45°C).

12. Using a pasteur pipette, carefully transfer the remnant to a clean test tube. Rinse the flask with an approximately equivalent amount of chloroform, let stand for 3 minutes and then add to test tubes.

13. Evaporate to dryness at room temperature under nitrogen, label carefully and store the test tube in refrigerator until ready for spotting.

Note : An extraction with other solvents such as ethyl acetate ($CH_3COO.C_2H_5$), dichloromethane (CH_2,Cl_2)or cyclo hexane (C_6H_{12}) may yield satisfactory results.

9.3 TLC Techniques

Standard TLC Plates (8" x 8") may be procured from dealers in chromatographic supplies and should be available for this exercise. In addition, procedures are outlined to introduce the techniques by utilizing familiar glass microscope slides (1.5" x 3").

9.4 Preparation of Microslides for TLS

1. Clean 60 glass slides with soap water, wipe with a soft tissue paper wetted with acetone, rinse with distilled water and dry.

2. Arrange the slides on a smooth table top in six rows of ten slides each, covering an area of 9″ x 30″.

3. Carefully tape both of the 30″ edges and one of the 9″ edges of this enlarged sheet to the table top with masking tape (1.5–2 cm wide). Then apply a second thickness of tape on to the first (Fig. 16).

4. Prepare the slurry using 40 gm Silica gel G (Stahl, 1965 – Appendix I) and 80 ml of distilled water. Shake the mixture in an Erlenmeyer flask for about one minute (no longer than

100 Sec). The mixture should be smooth and uniform without lumps or bubbles.

5. Quickly pour the slurry on the plates and use a stiff glass rod (about 14″ long) to spread it evenly to a thickness of the two masking tape layers. To do this, begin at one end; bring the glass by resting the rod on the runners of tape and move smoothly to the other end, discarding any excess slurry.

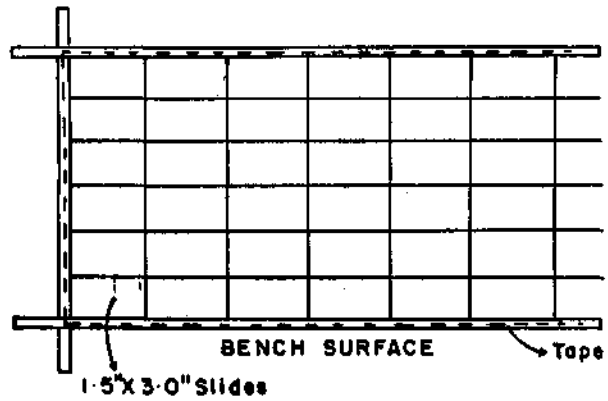

Fig. 16

6. Allow the plates to dry for 30 minutes at room temperature. Then remove the masking tape and put the slides in an oven to activate them by heating at 110°C for another 30 minutes.
7. Store the coated plates in a desiccator until ready for use.

9.5 Preparation of Standard TLC Plates

Essentially the same procedure may be used for preparing five standard 8″ x 8″ TLC plates. The plates are aligned on a flat surface in one row so that edges about two thickness of masking tape are used to tape down the two sides. The slurry is prepared and a portion is poured onto one end of each plate; the stiff glass rod is pushed with a steady motion while resting on the taped edges. The coated plates are dried, activated and stored as outlined above.

9.6 Preparation of Chromatography Chambers

Slender dishes with ground glass cover (62 x 99 mm)(Wheaton Glass Co.) will serve as chromatography chambers for the 3″ x 1.5″ slides. If 3″ x 1.0″ slides are employed, standard coplin jars are suitable. For the large plates use the standard chambers available from TLC suppliers lined on three sides with filter paper.

For this excercise a double phase system will be used, composed of chloroform, ethanol in the ratio of 9:1. For TLC, as for paper chromatography, it is very important that the system be equilibrated *i.e.* the chamber is saturated with the vapour phase of the solvents. The chambers should be set beginning of the laboratory period to permit equilibrium while the compounds are being spotted. Place 200 ml of the chloroform-ethanol mixture in the large chamber and enough into the small one to cover the bottom for about 0.5 cm

9.7 Preparation of Spotting Pipettes

Prepare an adequate number of spotting pipettes. A particular spotting pipette is used for one solution only and is discarded after use. The pipettes are prepared from open and melting point, capillary tubes (length 100 mm, inside diameter 0.1 mm). Flame the centre portion of the tube and draw-out. Break the tip to an appropriate length. The bore of the tip should be about 0.2 – 0.3 mm inside diameter. **Do not prepare pipettes near flammable solvents and their vapours.**

9.8 Spotting and Chromatographing the Extracts and Standards

Add several drops of chloroform-methanol (1:1) solution to the tube containing the dried extract and allow to dissolve. Steroid standards are similarly prepared by dissolving several mg of each in a chloroform-methanol solution.

Take up a small portion of the extract with the draw-out capillary tube and spot very cautiously about 1.5 cm from bottom edge

on the mid-line of the coated microslide. To do this, lightly touch the area where the spot is to be made, withdraw immediately to allow the solvent to evaporate and repeat until the pipette is empty. This is done to ensure a very compact spot for sharper separation. Repeat this two or three times on the same spot.

Now employ the same procedure with the standards indicated for each particular colour reaction under "Detection and identification of steroids" using separate pipettes for each steroid. Place the standard 1.0 cm to either side of the extract and 1.5 cm from the bottom edge of the microslide. Record the position of the particular standards and extract in your notes.

Place the slide in the chromatography chamber; cover and allow the solvent to attain a height of about 7 cm. Then remove slide, allow to air dry and spray with appropriate reagents as outlined below under "Detection and identification of steroids."

Similarly, using a spotting template, spot the extract and all the appropriate standard steroids on one large chromatoplate, 2.5 cm from the bottom edge and atleast 1.0 cm apart. Make a record of the position of each spot. Mark the edge of the chromatoplate 10 cm from the origin; then allow the solvent to rise to this point. Remove from chamber, air dry and spray with O-phosphoric acid solution and follow instructions for this reaction as outlined for the microslides. Calculate Rf values. A clear glass plate may be placed over the chromatoplate to give a semi-permanent record.

9.9 Detection and Identification of Steroids

In each of the following spot the extract and the indicated standard steroids on a microslide run in the solvent system; air dry and then spray lightly with the particular reagent. The general reagents merely detect the presence of steroids, the specific reagents reveal something about the structure of the steroid studied.

9.9.1 General reagents

1. *Kreb's buffer solution*

   a. Sodium chloride (0.154) M – 100 parts.

   b. Potassium chloride (0.154) M – 4 parts.

   c. Calcium chloride (0.110) M – 3 parts.

   d. Magnesium sulphate (0.154) M – 1 part.

   e. Potassium phosphate buffer (0.1) – 20 parts.

2. Vanillin-phosphoric acid (spot extract any two standard steroids on a microslide). Lightly spray the chromatoplate with a freshly prepared solution containing 1.0 gm vanillin dissolved in 100 ml of 50% aqueous solution of O-phosphoric acid. Heat at 110°C for 10–15 mintues. Record time of heating and observe colours.

3. Phosphoric acid (spot extract cholesterol and estradiol on one microslide and extract, pregnenolone and testosterone on another). Develop plates by spraying with a solution containing one volume of O–phosphoric acid diluted with one volume of water. Heat at 110°C for 8 minutes and record colours in daylight and under U.V. light. Then heat for another 7 minutes at 110-120°C and again record colours in daylight and U.V.

The following colour changes are suggested by Stahl (1965). Cholesterol and its esters under U.V. light appear pink red after initial heating, later turn rust-red. *Estrogens* - changed from brilliant yellow to orange to orange-red. *Pregnenolone* and *delyandrosterone* turn brilliant violet. *Testosterone* and *dehydrotestosterone* appear bright blue, change to dark green blue and finally remain dark bluegray in daylight. Steroids which do not show up distinctly after reacting with phosphoric acid treatment may be located by subsequent slight spraying with freshly prepared solution at 1.5 gm phosphomolybdic acid in 100 ml ethanol, followed by heating for about 5 minutes.

9.9.2 Specific Reagents

A. *Reactions for ketones*

NaOH for Δ^4–3 ketosteroids. [Spot extract, cholesterol (or estriol, estradiol) and testosterone (or progesterone) on a microslide]. Spray with 10 aqueous solution of NaOH and heat.

9.9.3 Results

Δ^4–3 ketosteroids fluorescein filtered U.V. light.

9.10 Antimony Trichloride Reaction for 3-OH – Δ^5 Steroids

(Spot extract, estriol or estradiol and cholesterol or progesterone on a microslide). Spray with a solution of chloroform saturated with antimony tricholoride).

9.10.1 Results

3 β–OH–Δ^5 steroids develop a pink colour some minutes after spraying and change to lilac after heating.

9.11 Zimmermann–reaction

(This requires the presence of an active methylene group; spot extract, estrone and testosterone, on a microslide). Spray plates with a freshly-prepared mixture of equal volumes of 2 solution of m-dinitrobenzene in absolute ethanol and 1.25 N KOH in absolute ethanol and dry in hot air.

9.11.1 Results

3-oxosteroids appear immediately as blue spots. 17-oxosteroids not substituted at position 16 give the classic violet colour after 3 to 6 min. 7-oxo- and 11-oxo-groups give no colour. Estrone 16-oxo-estrone and 16-oxo-estradiol give violet colour, 6-oxo-estradiol - brown colour. 4-OH-testosterone - red brown, 4-OH-androstenedione - first lilac then red brown, 2-OH-estradiol - yellow brown. 4-OH-estradiol - red brown.

B. *Phenoles*

(Spot extract, estradiol or estrone and testosterone on a microslide). Expose the spots to ammonia vapour and spray immediately with a 0.05 aqueous solution of stable diazo salt of p-nitroaniline (Fast Red Salt GG). Allow the vapour to air-dry and record any colours. After 30 minutes spray the plates with a saturated aqueous solution of sodium carbonate and allow to dry. Record colours.

9.12 Reference

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10

REGENERATION IN CRUSTACEA AND ANNELIDA

10.1 Introduction

Certain crustaceans can autotomize limbs under adequate stimulus; that is, they can cast off limbs at a special breakage or autotomy plane located in the basi-ischium where there is a double membrane of folded hypodermis. The region immediately proximal to the autotomy plane seems to have a high potential for limb regeneration. The regenerate develops first as an undifferentiated papilla. When it has reached the end of the plateau stage of limb development, it is already well developed (Hodge, 1956). Limb regeneration depends on several factors including the stage of animal in the intermoult cycle, environmental conditions and the number of limbs lost (Weis, 1976 a, b). Bilateral ablation of eyestalks, a major source of molt-inhibiting principle, causes precocious ecdysis (Zeleny, 1905) and acceleration of proecdysial limb regeneration in fiddler crabs (Passano, 1960). Such studies were also carried out by Durand (1960) in crayfish and Ranga Rao (1978) in crab.

In annelids, an ability to regenerate lost parts of the body, including specialised sensory or reproductive organs is of great advantage. The caudal regeneration is a wide spread phenomenon, the posterior part of the body are lost under natural conditions.

When segments are lost, the open wound is sealed by the contraction of the body wall muscles and then a blastema is formed.

Blastema produces the ecto and mesodermal parts by proliferating the cells. The growth is stimulated in both local and systematic influence of wound hormones (unspecified chemicals released from damaged cells).

10.2 Regeneration in Crustacea

10.2.1 Material

Crabs, fine scissors.

10.2.2 Procedure

1. Collect the crabs from field and maintain them in the laboratory.
2. Select healthy specimens of intermoult stage.
3. Autotomize the right third walking leg by pinching the merus joint with forceps from 10 crabs.
4. The day on which autotomy is performed is noted.
5. Make the observations, with the aid of a micrometer and a dissecting microscope at three day intervals upto ecdysis and/ or to the termination of the experiment
6 The degree of limb regeneration is expressed as = Regeneration index R (values)

$$\frac{\text{length of limb bud in mm}}{\text{carapace width in mm}} \times 100$$

This formula is comparable to that used for studies of limb regeneration in *Gecarcinus lateralis* (Bliss, 1956).

10.3 Effect of Eyestalk Ablation on Limb Regeneration

1. Collect the crabs and maintain them in the laboratory.
2. Select the crabs of equal size and weight and divide them into four groups (5 in each).
3. Autotomize the right third walking leg of each crab by pinching the merus with the help of forceps.

4. The first group will serve as control.
5. The second group (eyestalk ablated) will receive 0.5 ml of van Harevald's solution (refer Chapter 2, method 2.5.1).
6. Keep the third group (eyestalk ablated) without giving injections.
7. Give 0.05 ml/2/ES/crab eyestalk extract to group fourth crabs.
8. Make the observations three times per week with the aid of micrometer and a dissecting microscope.
9. Calculate regeneration index for each group and compare the results.

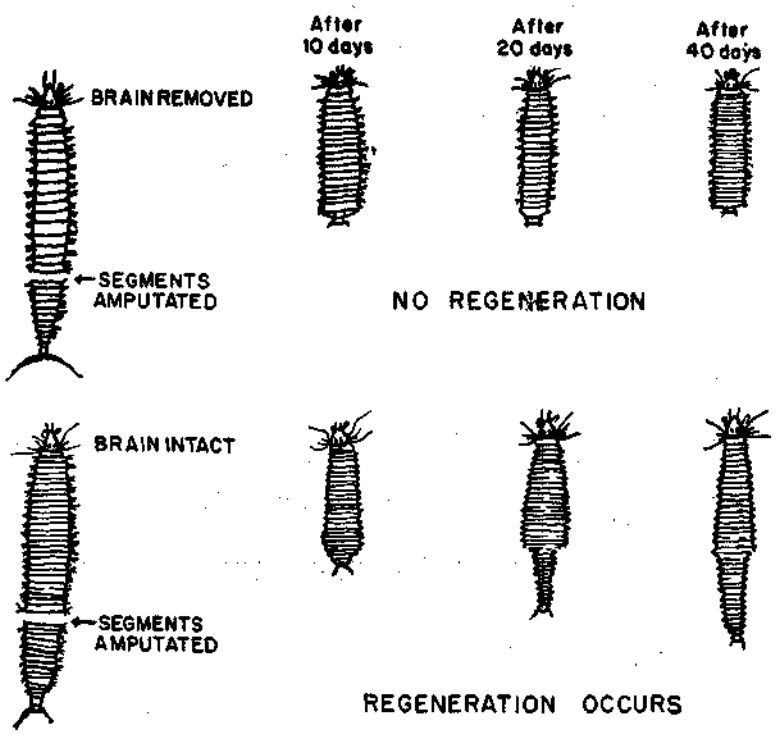

Fig. 17. Effect upon caudal regeneration of removing the brain from a growing *Nereis diversicolor*. Amputed posterior segments are regenerated as long as the brain remains *in situ;* when amputation is accompanied by brain removal, segment regeneration is prevented (Highnam and Hill, 1978).

10.4 Regeneration in Annelida

10.4.1 Material

Nereis sp.

10.4.2 Procedure

1. Collect *Nereis* and maintain them in laboratory.
2. Ampute the segments from posterior end and count the number of segments.
3. Divide them into three groups of 5 each.
4. The 1st group will serve as control.
5. From the second group remove the brain.
6. In the third group, make sham operation.
7. Observe each group for caudal regeneration and note the results (Fig. 17).

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11

DEMONSTRATION OF NEUROSECRETORY SYSTEM IN COCKROACH

11.1 Introduction

The insect endocrine system has four major groups of neurosecretory cells: (i) brain (ii) corpora cardiaca (iii) corpora allata (iv) thoracic glands. The corpora allata and thoracic glands arise as epithelial endocrine glands whereas, the corpora cardiaca develop embryonically as invagination of the foregut. The cerebral neurosecretory cells are in two groups in each half of the pars intercerebralis of the forebrain. The axons of those neurosecretory cells run directly into the corpora cardiaca.

The corpora cardiaca produce their own intrinsic hormones, but their major function in most insects is to store and release neurosecretory hormones from the cerebral neurosecretory cells.

The corpora allata are source of juvenile hormone, which tends to promote larval development and maintenance of the status quo during an insect larval stages.

The thoracic glands or the ecdysial glands are the source of ecdysone or prothoracic gland hormone. Regulation of the secretion of the hormone is by the brain hormone released from the corpora cardiaca. Ecdysone is a growth hormone of insect

development and it promotes both growth towards adult characters and moulting.

11.2 Material

Cockroaches (last instar nymphs of cockroaches), dissection tray specially prepared by putting paraffin in petridish, fine scissors, forceps, pins, etc. Methylene blue stain.

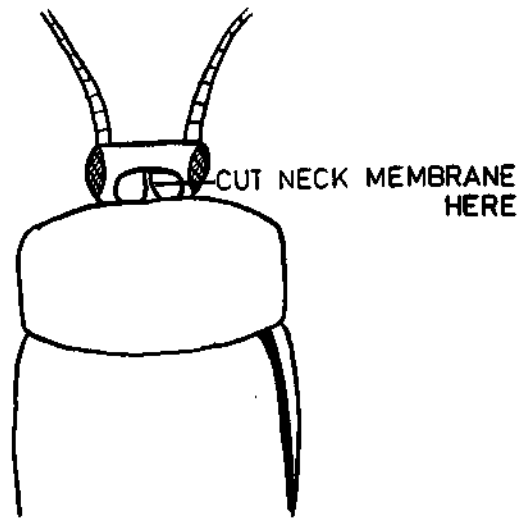

Fig. 18. Diagram showing exposed neck of cockroach and where to cut neck membrane. The head has been pushed down and forward.

11.3 Procedure

1. Put the cockroaches in soap water for 10 minutes.
2. Take one animal and pin it on the dissecting tray with the dorsal side facing up.
3. With a fine scissors cut the neck membrane at the margin of the head capsule as shown in Fig. 18.
4. This exposes two large trachea which can be pulled out with forceps.

5. Wipe blood away with cotton.

6. Bend the head slightly downward so that one can recognize the bluish white corpora cardiaca in the head capsule. The general shape of this organ is shown in Fig. 19.

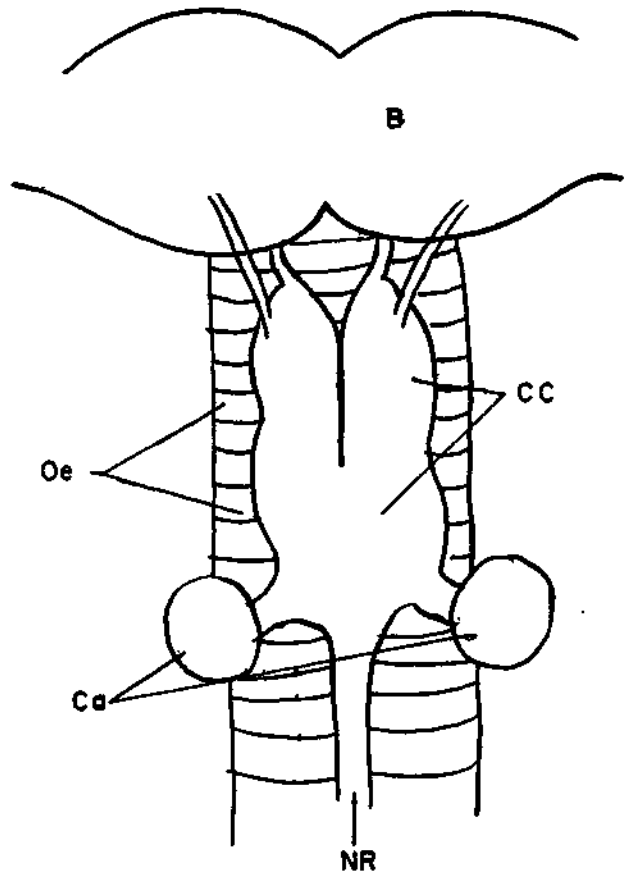

Fig. 19. Diagram showing structural relations of the corpora allata of a cockroach (B: Brain, Oe: Oesophagus, CC: Corpora cardiaca, Ca: Corpora allata and NR: Nervous recurrens).

7. To observe the corpora allata on a slide, grasp the lateral caudal part of the corpus cardiaca and pull it gently away;

the tissue separate easily leaving the larger part of the corpora cardiaca *in situ*.

8. Place the removed tissue on a slide and observe under low power of the microscope.
9. The thoracic glands in cockroaches are located primarily in the prothorax. They consist of two distinct bands which cross is an 'x' shaped fashion (Fig. 20).

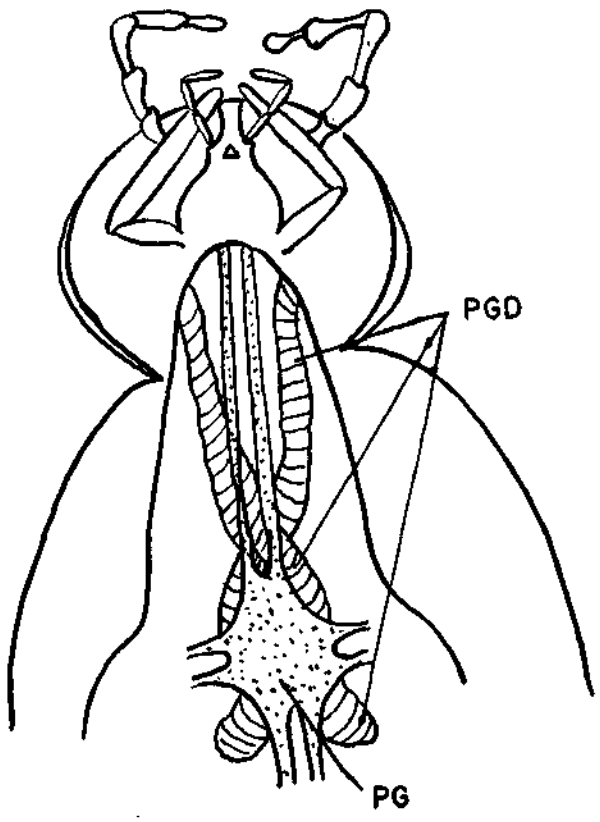

Fig. 20. Ventral dissection of a cockroach showing the prothoracic glands (PGD: Prothoracic gland and PG: Prothoracic ganglion).

10. Fasten last instar nymph (a mid instar is best), ventral side up, in a dissecting tray.

11. Remove the legs and bend the head upwards with insect pins. Carefully remove the sclerite which is located above the prothoracic ganglion (in part in between prothoracic legs).

12. The ganglia and the connective tissue should remain undisturbed.

13. Place a few drops or crystals of methylene blue on to the area between prothoracic ganglion and head.

    This should make the prothoracic gland visible within a few minutes.

14. Carefully remove the prothoracic ganglion and the connectives leading from the suboesophageal ganglion. This will further expose the glands.

11.4 Results

The corpora allata appear transparent and the corpora cardiaca are bluish white in colour. With methylene blue the prothoracic glands appear clear when compared with surrounding tissues.

11.5 Reference

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12

STAINING OF NEUROSECRETORY CELLS IN INSECTS

12.1 Introduction

Paraldehyde fuchsin (PF) following permanganate oxidation readily stains large accumulations of neurosecretory material (NSM) in the vertebrate brain and in the neurosecretory systems of invertebrates. While several PF staining techniques have been used on insects, some appear to be less sensitive than others for tracing the neurosecretory axons. For example, Johnson (1963) was able to trace the neurosecretory system of the aphid using the method of Dawson (1953). Meola (1970) was able to distinguish the neurosecretory cells in the mosquitoes using the paraldehyde fuchsin.

12.2 Material

Cockroaches, Bouins fluid, dissecting tray and instruments.

12.2.1 Preparation of PF stain (Rosa, 1953)

Stain solution

Basic fuchsin – 1 gm
70% alcohol – 200 ml

Mix well and add

Concentrated HCl – 2 ml
Paraldehyde – 2 ml

Ripen this solution at room temperature for 3–4 days.

Extraction solution

Add together in a separating funnel stain solution – 200 ml.

Chloroform – 100 ml.
Distilled water – 400 ml.

Shake briefly and gently, allow precipitate to settle (at least 30 minutes), drain off precipitate and filter this through a paper filter without suction. Dry the precipitate in the oven at 50°C. Store the PF crystals in a stoppered bottle.

Paraldehyde fuchsin working solution

PF crystals – 1 gm.
70% ethanol – 200 ml.
HCl concentration – 2 ml.

Ripen this solution a week and filter once prior to use to remove excess stain crystals and filter paper fibres. Stain at pH 1.6 to 1.7. Above or below this pH the stain does not differentiate well.

12.2.2 Staining procedure

1. Deparaffinize in two changes of xylene – 5 minutes each.
2. Absolute alcohol – 2 minutes.
3. 0.5% collodion in ether: absolute alcohol (1:1) 1 minute, drain on paper towel and dry in air for 30 seconds.
4. 70% alcohol – 1 minute.
5. Wash in running water – 5 minutes.
6. Oxidize in an aqueous solution of 0.3% $KMnO_4$ and 0.3% H_2SO_2 – 1 minute.
7. Rinse in distilled water.
8. Decolourize in 1.5% aqueous potassium metabisulfite until sections are clear – 2 to 3 minutes.

9. Wash in running water – 5 minutes.
10. Rinse in distilled water.
11. 70% alcohol – 2 minutes.
12. Paraldehyde fuchsin – 1 minute quickly on paper towelling.
13. Wash in 95% alcohol – 2 minutes.
14. Differentiate in 95% alcohol – 5 minutes.
15. 70% alcohol – 2 minutes.
16. Distilled water – 2 minutes.
17. Counter stain in Halmi's mixture (see Chapter 1 for preparation of stain) 1 minute, drain quickly on towelling.
18. Differentiate on 0.2% acetic acid in 95% alcohol – 2 to 3 minutes.
19. Rinse briefly in 95% alcohol – 15 seconds.
20. Dehydrate in two changes of absolute alcohol – 2 minutes.
21. Clear in two changes of xylene and mount in DPX.

12.2.3 Results

Neurosecretory material in both the perikarya and the axon tracts stains intense bluish-purple. Non-neurosecretory perikarya and the neuropile stain green or pale lilac. The cytoplasm of non-neural tissue stain green while the nucleoli will be bright red. Acidophilic granules in the cytoplasm will be orange.

12.3 References

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13

DEMONSTRATION OF NEUROSECRETORY CENTRES OF OYSTER AND FRESHWATER MUSSELS

13.1 Introduction

Neurosecretory cells (NSC) have been demonstrated in the nerve centres of many lamellibranchs. The cerebral and visceral ganglion of the oyster *Crassostrea virginica* have two types of NSC. NSC were found to be distributed along the dorsal surface of the cerebral, pedal and visceral ganglion of the bivalve *Meretrix casta.*

13.2 Material

Oysters, freshwater mussels, dissecting tray, scissors, forceps, pins, etc.

13.3 Procedure

A. Oyster

1. Take the oysters and remove one valve of the shell.
2. Dissect to show the cerebral and visceral ganglion.
3. The pedal ganglion of the lamellibranch do not appear because of the absence of foot.

4. The two cerebral ganglia are embedded in the loose connective tissue between the bases of the labial palp. Since the cerebral ganglia are so minute, they can be observed with the aid of stereoscopic dissecting microscope.

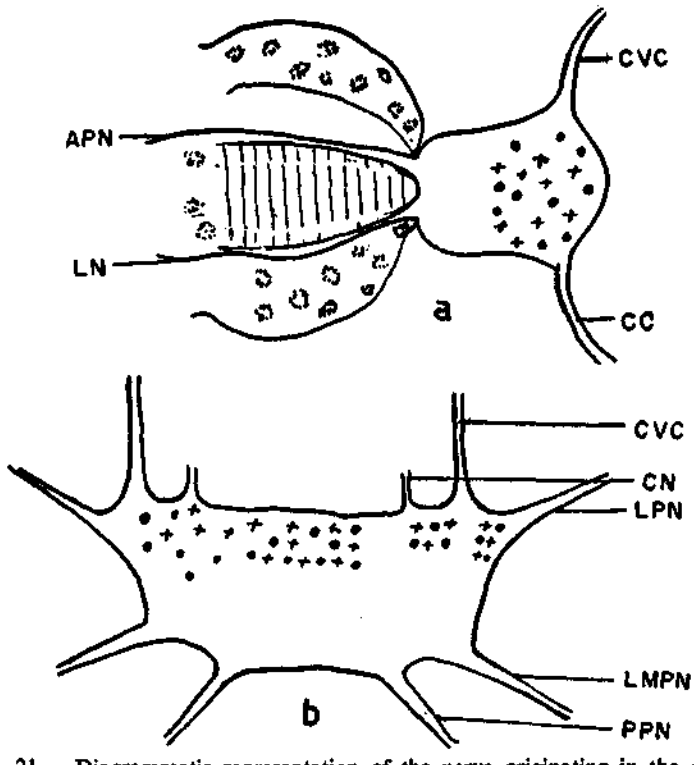

Fig. 21. Diagrammatic representation of the nerve originating in the cerebral visceral ganglia of the oyster and the distribution of neurosecretoy cells in these ganglia. a. Cerebral ganglia (lateral view) and b. Visceral ganglia (dorsal view). CC : Cerebral commissure; CVC : Cerebrovisceral connective; APN : anterior pallial nerve; LN : Labial neve; CN : Ctenidial nerve, LPN : Lateral pallial nerve; LMPN : Lateromedian pallial nerve; PPN : Posterior pallial nerve.

5. The visceral ganglion lie in a slight depression on the anteroventral surface of the adductor muscle close to the ventral end of the right kidney. The two ganglia are fused to form one large mass. The position of the ganglia is shown in Fig. 21.

B. Freshwater Mussel

1. Take the freshwater mussel and remove the two shells.
2. Dissect it with fine scissors and forceps, so as to expose the cerebral, visceral and pedal ganglion. The cerebral ganglia are paired in number and embedded under loose connective

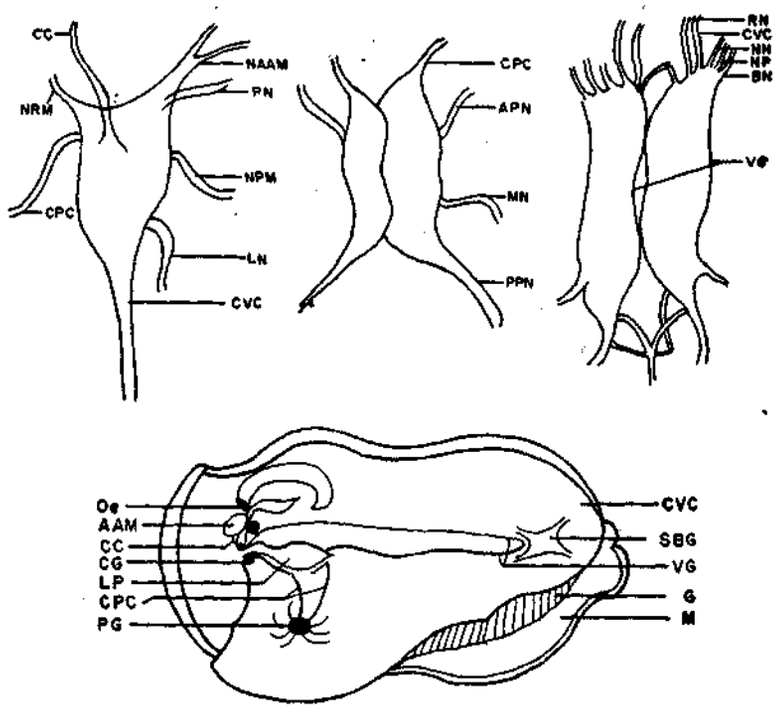

Fig. 22.

(Oe : Oesophagus; AAM : Anterior adductor muscle; CC : Cerebral commissure; CG : Cerebral ganglion; LP : Labial palp; CPC : Cerebro-pedal connectives; PG : Pedal ganglion; CVC : Cerebro-visceral connectives; SBG : Supra-branchial chamber; VG : Visceral ganglia; G : Gill; M : Mantle; NAAM : Nerve to anterior adductor muscle; PN : Pallial nerve; NRM : Nerve to the retractor muscle; NPM: Nerve to protractor muscle; LN: Labial nerve; APN : Anterior pedal nerve; MN : Median nerve; PPN : Posterior pedal nerve; RN : Renal nerve; NH : Nerve to the heart, NP : Nerve to the pericardium; BN : Branchial nerve)

tissue at the base of the labial palp, ventroposterior to the anterior adducter muscle.

3. The paired visceral ganglia are fused together and lie on the anteroventral surface of the posterior adductor muscle.
4. Like the visceral ganglia, the pedal ganglia are also fused together. The pedal ganglionic mass is situated on the ventral side of the visceral mass at the junction of the musculature of the foot, nearly one third from the anterior side (Fig. 22).

13.4 Results

Due to the lack of a locomotary organ, the foot, there are no pedal ganglia in the oysters. Because of this the cerebral ganglion and the visceral ganglion act as the neurosecretory centres. In freshwater mussel the cerebral ganglion are orange red in colour, triangular in shape. The visceral ganglion is reddish brown in colour and pedal ganglion is 'H' shaped with an incomplete vertical furrow.

14

STAGING OF THE REPRODUCTIVE CYCLE

14.1 Introduction

Several schemes for classifying the gonadal condition in mollusca have been described. These schemes provide a simple, yet precise method for distinguishing the onset and duration of the reproductive cycle (Nagabhushanam and Mane, 1975).

14.2 Material

Mytilus, dissection instruments, microscopes and slides.

14.3 Procedure

1. Take out the gonads and observe under binocular microscope.
2. The condition of gonads for the study of the reproductive cycle has been divided into six stages of maturity, based on the gross microscopic appearance of the gonads.

Stage A (spent) : Gonad considerably shrunken in volume, visible differentiation of mature egg and foamy sperm mass. Loop of the alimentary canal, usually markedly seen.

Stage B (recovery) : First recognizable stage after spawning. Loop of alimentary canal some times visible, but not perfectly

identified. Gonad is increasing in size. The thin body wall if pierced, fluid exists in portion of the follicle walls and body wall.

Stage C (early growing) : Gonad shape increasing, making digestive gland to have a restricted area, male pastry white in general appearance, loop of the alimentary canal not usually visible, but seen only in area close to the body wall.

Stage C_t (late growing) : Gonad increases in size.

Stage D (Mature) : Gonad packed with sperms and eggs, become round in shape, contains no free body fluid, if pierced gametes come out.

Stage E (Spawning) : is partially split; gonad size starts collapsing, very little amount of fluid with few gamete cells come out if pierced.

14.4 Results

The reproductive cycle can be divided into six stages depending upon the gross microscopic appearance of the gonads. Both the males and females followed the same pattern of the development at a time and hence the description is common for them.

14.5 Reference

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15

GASTROPOD NEUROSECRETORY CELLS (NSC) AND REPRODUCTIVE ORGANS

15.1 Introduction

On the basis of staining properties of neurons the presence of NSC have been reported for several molluscs. NSC were identified by using any one of the following stains. (i) Mallory's triple stain, (ii) Gomori's chrome-haematoxylin-phloxin (iii) Paraldehyde fuchsin.

15.2 Material

Pilla sp., dissecting tray, scissors, forceps, Bouin's fluid and any one of the stains mentioned in the Introduction.

15.3 Procedure

1. Dissect the animal and remove the cerebral, buccal, pleuropedal and visceral ganglia.
2. Fix the tissues in Bouin's fluid for 24 hrs. Follow the procedure given in Chapter 1.
3. Stain with one of the stains given in Introduction. Observe the slides under the microscope for cell identification.

15.4 Results

Cell type A (Fig. 23 a) : This type of cells are pyriform and measure about 83 to 133 μ in length. The nuclei are long in comparison with the size of cell. The nuclei are 70 to 100 μ in length. Normally, nucleus is spherical but it may be kidney-shaped. The cytoplasm is weakly stained with Gomori's and Mallory's stain.

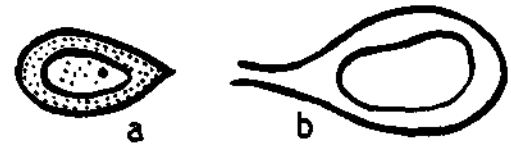

Fig. 23. The neurosecretory cell types (A and B) in *Pila globosa.*

The granules present in the cytoplasm are stained blue black with Gomori's stain and deep blue with Mallory's triple stain. Generally 10 to 15 A-cells are noticed on the postero-ventral sides of cerebral, pleural and visceral ganglia. *Cell type* B (Fig 23 b) are oval in shape ranging from 49 to 66 μ in diameter and characterised by intensive staining of the cytoplasm. The granules within cytoplasm are stained red with Gomori's as well as with mallory's stain. The nucleus of these cells varies from 25 to 40 μ in diameter. The nucleoli number varies form 1 to 10 and stain red with both stains. These cells are more in number and found throughout the periphery of cerebral, pleural, pedal and visceral ganglia.

16

NEUROENDOCRINE CONTROL OF OSMOREGULATION IN MOLLUSCA

16.1 Introduction

The variation in the salinity is an important factor which affects osmoregulation in marine animals. The neurosecretory hormones of the invertebrates are found to be controlling the osmoregulation.

16.2 Material

Any marine gastropod or a bivalve, sea water, distilled water, glass container of one litre capacity dissecting trays, forceps and scissors.

16.3 Procedure

1. Keep the animals in water and allow them to acclimate themselves for 24 hrs (control).
2. Dilute the sea water with distilled water so as to obtain 18, 12, 8 and 6 parts/lr. Keep five experimental animals in each one of the glass (salinity) containers of one litre capacity. Changee water daily.
3. After 6 days remove the animal, dissect out their neurosecretory centres and study the neurosecretory material present in the NSC.

16.4 Results

The NSM will be depleted in the NSC, becuase the neurosecretory hormones are released into the bloodstream to overcome the osmoregulatory stress caused by the change in the medium.

17

NERVOUS SYSTEM OF ECHINODERMS

17.1 Introduction

In Echinoderms the nervous system is of a simple and primitive type, there being no central ganglion. It is essentially a nerve net closely associated with the epidermis and consisting of nerve fibres with some ganglionic cells. The nervous system is difficult to see except in certain places where an aggregation of nervous tissue forms distinct nerve cord.

17.2 Material

Starfishes, dissecting tray and dissection instruments.

17.3 Procedure

1. Take the starfishes in the dissecting tray and put sufficient water.
2. Take a fine cut at dorsal surface and remove the skin.
3. The nervous system is conventionally divided into three interrelated parts as follows.

i. *Oral or ectoneural system* : It is also termed superficial or epidermal system as it is situated just beneath the epidermis. It forms the main part of the nervous system and is sensory in function. It includes a central pentagonal circumoral nerve ring

(Fig. 24), situated in the periphery of the peristomial membrane around the mouth. From this nerve ring arise five radial nerve cords, each running into one arm between the rows of tube feet in the ambulacral groove and ending in a sensory cushion above the terminal tentacle. In a cross section radial nerve cord appears as a V-shaped thickened mass (Figs. 25 and 26) separated from the

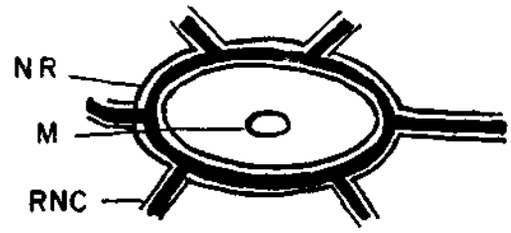

Fig. 24. The central pentagonal circumoral nerve ring of starfish (NR: Nerve ring, M: Mouth and RNC: Radial nerve cords).

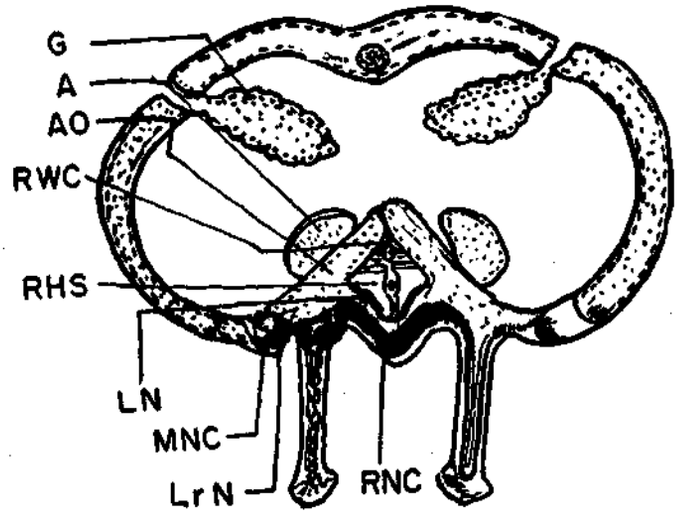

Fig. 25. Section through the arm of a starfish to show its nervous system (G: Gonad; A: Ampulla; AO: Ambulacral ossicle; RWC: Radial water canal; RHS: Radial hyponeural sinus; LN: Lange's nerve; MNC: Marginal nerve cord; Lr. N: Lateral nerve and RNC: Radial nerve cord).

hyponeural sinus by a thin dermis and coelomic epithelium covered on its outer side by epidermis. The radial nerve cords give off branches to the tube feet and are also continuous with a general subepidermal plexus of the body-wall and its appendages.

ii. *Deep or hyponeural system*: It is developed from the mesoderm and is primarily motor in function. Like ectoneural system, the deep nervous system also forms a central pentagon which in this case is double and lies aboral to the main nerve-ring.

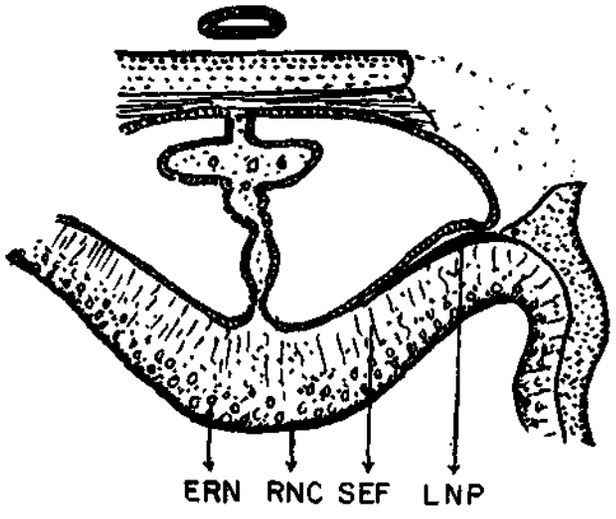

Fig. 26. Section through the lower part of an arm to show the radial nerve and the associated parts (LNP: Lange's nerve of deep hyponeural nervous system; SEF: Supporting epidermal fibres of radial nerve; ERN: Epidermis of radial nerve and RNC: Radial nerve cord).

In each arm, it gives off a pair of lateral cords called Lange's nerves after their discoverer (Figs. 25 and 26). Each Lange's nerve is a plate of nervous tissue rather than a nerve and lies in the outer oral wall of the radial hyponeural sinus separated from the radial nerve thickening only by a thin layer of dermal connective tissue. The Lange's nerve gives branches to muscles of the arm.

iii. *Aboral or coelomic system* : The subepidermal plexus is thickened at the outer margins of an ambulacral groove into marginal nerve cords extending through the whole length of the arm. From these lateral nerves ascend between ambulacral and adambulacral ossicles and form a plexus beneath the whole of the coelomic lining. The aboral system is also mesodermal and motor in function. It is also termed the ectoneural system and innervates the muscles of the body wall and gonads.

17.4 References

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