CMFRI SPECIAL PUBLICATION
Number 41

A MANUAL FOR HORMONE ISOLATION AND ASSAY

ISSUED ON THE OCCASION OF THE WORKSHOP ON
HORMONE ISOLATION AND ASSAY
ORGANISED BY
THE CENTRE OF ADVANCED STUDIES IN MARICULTURE,
CENTRAL MARINE FISHERIES RESEARCH INSTITUTE, COCHIN
Indian Council of Agricultural Research
HELD AT COCHIN FROM 25TH TO 28TH JUNE 1986

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A MANUAL FOR HORMONE ISOLATION AND ASSAY

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PREFACE

The objective of this manual is to help scientists and research students who pursue endocrinological investigations in fishes and shellfishes. In 1950s and early 1960s, scientific research methodologies were limited to classical endocrinological techniques and hormonal functions were mainly deduced from indirect evidence. But with the advent of more sophisticated and precise analytical techniques in recent years, it is possible now to show the exact site of synthesis of few of the hormones and isolate, purify and characterize them and show their physiological specificity. While an earlier manual has given basic research methodologies employed in invertebrate endocrinological investigations, the present one lays emphasis on modern analytical techniques currently employed in neuroendocrine research of vertebrates and invertebrates. The manual provides guidelines to scientists in selecting the appropriate analytical techniques depending upon the material in hand.

Neuroendocrinology has assumed great significance, in view of its established role in controlling growth and reproduction. Commercially important cultivable fishes and crustaceans were induced to breed in captivity by manipulating the hormones, which are secreted by various endocrine centres. Few of the hormones which were already isolated, purified and synthesised are being used as tools to study specific physiological functions in various organisms. Yet some such as Moult Inhibiting Hormone (MIH) and Gonad Inhibiting Hormone (GIH) are yet to be isolated and purified. Research is in progress on this line and once purified, monoclonal antibodies could be developed which can block the action of these hormones resulting in fast growing or more fecund specimens. Some of the research programmes of the Central Marine Fisheries Research Institute are specifically oriented towards achieving these goals.

The manual is prepared by Professor (Dr.) Milton Fingerman, Senior Professor of Biology, Tulane University, U.S.A. and Shri E. V. Radhakrishnan, Scientist, CMFRI. Prof. Fingerman was an expert consultant in Fish and Shellfish Endocrinology at the Centre of Advanced studies in Mariculture, CMFRI, Cochin in June–July, 1986.

I express my sincere thanks to Prof. Fingerman for conducting the seminars and workshops during his consultancy and for preparing this Manual. I also thank Shri E. V. Radhakrishnan for assisting the consultant in the preparation of the manual and other assistance during the consultancy.

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CONTENTS

1

CHROMATOGRAPHY

Chromatography is a general method of analysis in which substances are separated by differential migration or elution from a sorptive medium. This technique has been used in the purification of several crustacean neurohormones. It can be used on a small scale, *e.g.* to partially purify colour change hormones from a small number of eyestalks in an effort to demonstrate the presence and actions of such hormones (Fingerman, 1970). However, the techniques can also be used with large batches of tissues where the aim is to purify enough hormone to homogeneity to allow for composition studies (Fernlund and Josefsson, 1968, 1972).

A. Gel Filtration Chromatography

The principle of gel filtration chromatography is that substances are separated on the basis of molecular size. As the solute passes down a bed of the chromatographic gel its movement depends on the flow of the solvent (mobile phase) and the Brownian movement of the solute molecules into and out of the gel beads (stationary phase). Separation depends on the ability of molecules to enter pores in the beads. Very large molecules can not enter and thus pass through the chromatographic bed fastest. Smaller molecules enter the gel pores and move more slowly along the column. Substances are eluted from the stationary phase in order of decreasing molecular size. The gels used in gel filtration chromatography that have proven useful with crustacean neurohormones are Bio-Gel P-type and Sephadex G-type. As will be described below, the gel chosen for use is prepared by allowing it hydrate in the selected solvent. Distilled water alone has been used effectively as have buffers, such as acetate.

The particular Sephadex or Bio-Gel used will depend upon the hormone to be studied. For smaller peptides Bio-Gel P–6 and Sephadex G-25 are very commonly used. The molecular weight

operating range for each gel is readily available from the manufacturers, as are pamphlets detailing their use such as Gel Filtration Theory and Practice from Pharmacia Fine Chemicals, the supplier of Sephadex. Table 1 provides such data for Bio-Gel P-type and Sephadex G-type gels. The gels are manufactured with different mesh sizes, such as fine, medium and coarse. The fine

TABLE 1. *Technical information for selected Bio-Gel P-type and Sephadex G - type gels*

Gels	Fractionation range (Daltons) for peptides and globular proteins	Hydrated hed volume (ml/g dry gel)
Bio-Gel P-2	100– 1,800	3.5
Bio-Gel P-6	1,000– 6,000	7
Bio-Gel P-10	1,500–20,000	9
Bio-Gel P-30	2,500–40,000	11
Bio-Gel P-60	3,000–60,000	14
Sephadex G-25	100– 5,000	4-6
Sephadex G-50	500–10,000	9–11

particles pack better than coarse ones, give less zone broadening and therefore, better resolution. With gel filtration, as stated above, the smaller molecules enter the gel particles whereas larger substances are excluded. Then, as solvent passes through the column, the first material to be washed through consists of all the substances with molecular weights larger than the maximum size substances the gel particles will allow to enter them (Fig. 1). The volume of fluid that is required to wash a substance out of the column is called the elution volume. When small samples are applied the peak maximum in the elution diagram should be taken as the elution volume. The elution volume of molecules which are distributed in the mobile phase because they are too large to enter the gel pores is called the void volume, Thus, the solute molecules in the void column are eluted ahead of the molecules that are small enough to be retained by the gel. The void volume can be determined once the column is packed by passing through it a coloured protein that is too large to enter the gel beads (see below). With continued addition of solvent the materials are eluted from the gel, with the larger ones coming off the column ahead of the smaller.

a. Column Preparation

1. Add dry Bio-Gel P or Sephadex G to distilled water or the buffer to be used in a beaker and stir. The amount of gel needed to pack a column of known volume can be estimated from the Hydrated Bed Volume given on the container of the gel (Table 1). Use twice as much fluid as the expected gel volume.

2. Allow the gel to hydrate 4 hours at room temperature. After an initial uniform suspension of the gel beads, additional stirring is not necessary during the hydration.

3. After hydration is complete, decant half the supernatant. Deaerate by aspirating at reduced pressure.

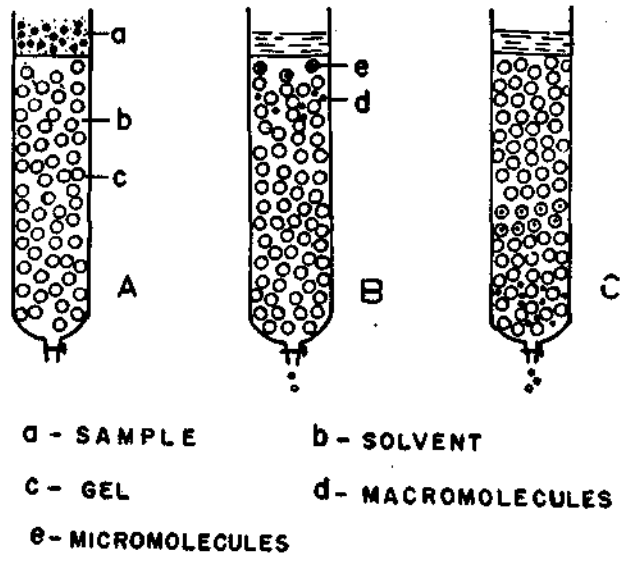

Fig. 1. Diagrammatic representation of gel filtration chromatography. A. Layering of sample onto gel; B. Early stage of filtration and C. Separation of large from small molecules.

4. Add enough of the fluid in which the gel swelled (water or buffer) to fill 20% of the column, with the column exit closed.

5. Stir the gel and begin adding it to the column.

6. When a 2–5 cm bed has been formed, allow the column to flow.

7. Keep adding gel until the desired bed height is reached. Then pass fluid alone through the column; the amount of fluid should be at least twice the volume of the gel in the column. When the column is not in use, leave some fluid above the top of the gel in the column. Before using the column, pass two or three column volumes of eluant through the column to stabilize the bed.

8. When ready to apply the sample to be separated, drain the fluid down to the level of the gel. Layer the sample carefully on to the gel surface and allow it to drain into the gel. Immediately start adding fluid (eluant). Never let the fluid level run below the surface of the gel. The sample size should not exceed 5% of the bed volume.

9. Collect the fractions for analysis and if appropriate equipment is available, monitor, *e.g.* with ultraviolet light, the eluant.

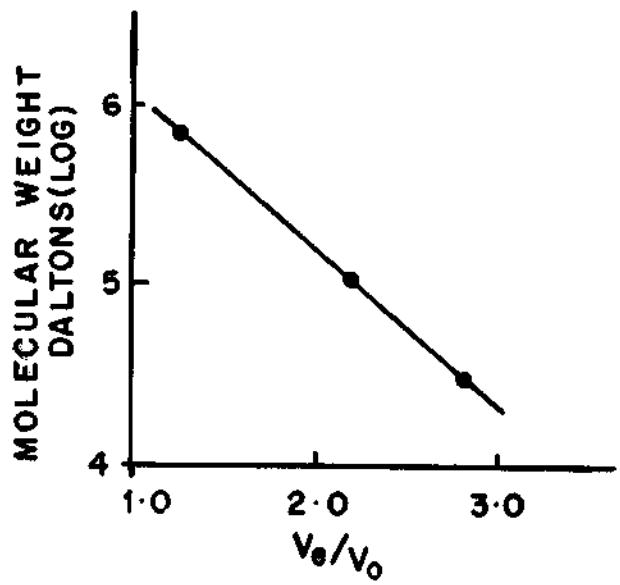

Fig. 2. Molecular weight versus elution volume/void volume (Ve/Vo) for gel filtration of a set of molecular weight markers.

b. Void Volume Determination

As stated above, the void volume (V_o) of the column is equal to the elution volume (V_e) required to wash out the material excluded from the gel. Coloured proteins such as hemoglobin or ferritin are commonly used to determine V_o. These coloured substances also enable the investigator to determine if the column is uniformly packed. If non-ideal behaviour (uneven migration or skewing of the band) is observed it should be corrected before applying a sample. This usually involves repacking the column. With Bio-Gel P the average void volume is 33% of the total gel bed volume.

c. Molecular Weight Determinations

Bio-Gel P columns can be used with a set of standards of known molecular weight to calibrate the column and allow a good estimation of the molecular weight of a substance retained by the gel. The standards, such as Bacitracin (1,400 daltons), ACTH (3,500

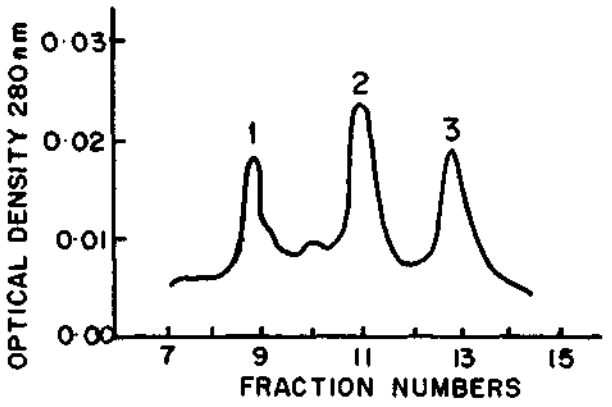

Fig. 3. Gel filtration of three proteins that have different molecular weights.

daltons) and cytochrome c (13,000 daltons) are run through the column, and their elution is followed by ultraviolet spectroscopy; 280 nm is a commonly used wavelength with proteins (Fig. 3). Plot a graph of log molecular weight versus elution volume (V_e) divided by the void volume (V_o), which gives a straight line (Fig. 2).

d. Desalting with Bio-Gel P-6DG

Bio-Gel P-6DG desalting gel gives results equivalent to those obtained by exhaustive dialysis, but in much less time. This gel is excellent for desalting proteins. It consists of 90–180 nm beads, has an exclusion limit of 6,000 daltons and a hydrated bed volume (ml per g dry gel) of 7. Consequently, if the substance to be desalted has a molecular weight greater than 6,000, it will be completely excluded from the gel, coming out in the void volume and desalted.

e. Fractionation of Eyestalk Extracts on Bio-Gel P-6

Crustacean eyestalks contain hormones that produce dispersion (pigment dispersing hormone) and aggregation (pigment concentrating hormone) of chromatophoric pigments. These antagonistically acting hormone can be readily separated from one another because of their differences in molecular weight by

using the technique of gel filtration chromatography. To accomplish such a separation Bio-Gel P-6 that has been allowed to swell in distilled water and the eluant was also distilled water has proven very effective (Fingerman *et al.*, 1971). To demonstrate these hormones in a eyestalk extract, follow the procedure given below.

1. Homogenize preweighed eyestalks (10–50 eyestalks, depending on their size) in 0.3 ml distilled water or buffer.

2. Centrifuge the extract in a table top centrifuge at top speed for 3 minutes.

3. Carefully layer the supernatant on top of a packed column (0.8 x 30 cm). Allow this extract to drain into the gel by slowly starting the eluant flow (flow rate 0.5 ml/minute).

4. Immediately allow eluant to flow through the column.

5. As soon as you begin to add eluant, start to collect 1 ml fractions. The void volume of this column is approximately the first 6 fractions collected.

6. Measure the optical density at 225 nm.

7. Plot the fraction numbers against the optical density to get the protein and peptide peaks.

8. Bioassay the fractions for the hormone activity.

9. Pigment dispersing hormone should appear in fractions 9–11 and pigment concentrating hormone in fractions 18–20.

Before injecting the fractions into test organisms, the fractions must be made isosmotic with the blood of the test species. This is most conveniently done by adding 1/3 ml of 400% crustacean saline (Pantin, 1934) to each 1 ml fraction. The methods used to assay for pigment dispersing and concentrating activities are presented below in detail. In assays for dispersing activity, the fractions are injected into specimens initially having maximally concentrated pigment. In contrast, to assay for concentrating activity, specimens whose pigment is initially maximally dispersed are used.

B. Ion Exchange Chromatography

Proteins and peptides that have similar molecular weights can be separated by ion exchange chromatography if they have different electrical properties. This type of chromatography has been used

in the purification of crustacean distal retinal pigment light adapting hormone (Fernlund, 1971). Ion exchangers are insoluble substances (*e.g.* cellulose resins, dextrans) that have chemically bound charged groups and mobile counter ions that can be exchanged with ions having the same charge as the material to be separated or purified. exchangers have mobile negatively charged ions; cation exchangers have mobile postively charged ions (Fig. 4). The substances to

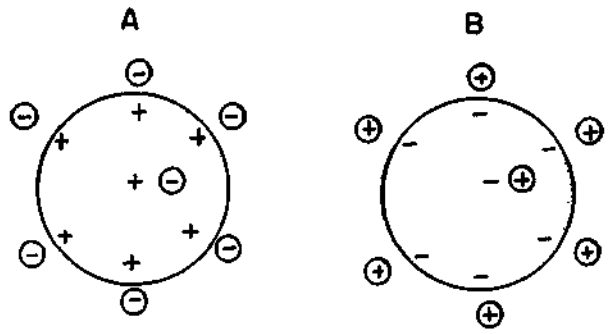

Fig. 4. Ion exchangers. A. anion exchanger with exchangeable negatively charged counter ions and B. cation exchanger with exchangeable positively charged counter ions.

be separated are added to the column and become bound to the exchanger because of their charge (Fig. 5). Binding is dependent on the amount of charge of the substance. The greater the charge the stronger the binding. The substances are then removed from the ion exchangers, ideally one at a time. This is usually accomplished by eluting the material with pH gradients of solutions having gradients of ionic strength. However, occasionally the substances will elute with use of the starting buffer alone, *i.e.* the column is developed by the starting condition procedure alone.

a. Choice of the Ion Exchanger

Because substances became adsorbed to the ion exchanger if they have a charge opposite to the bound (fixed) charge of the exchanger (*i.e.* the adsorbed substances have the same charge as the mobile counter ions), the choice of the ion exchanger depends upon the net charge of the substance to be separated out. Peptide and protein hormones, being amphoteric, have a net positive charge at low pH (thus becoming adsorbed to a cation exchanger) and at high pH are negatively charged (thus becoming adsorbed by an anion exchanger). At their isoelectric point they have no net charge and do not become adsorbed to either type of ion exchanger. An important consideration in the decision of whether to use a

cation or anion exchanger with such substances whose charge is dependent upon the pH of the solvent is the 'range of stability' of the substance; that is the pH range in which it does not become denatured. Whether use of an anion or cation exchanger is preferable for use with the peptide or protein to be separated out can be determined simply by preparing a small amount of each type of exchanger (50 mg in 30 ml buffer of 0.1 ionic strength, pH 5 for cation exchangers, pH 8 for anion exchangers), equilibrate the exchangers by two changes of buffer, allow the exchangers to settle, remove supernatant until you have 10 ml of exchanger plus remaining supernatant, and then add equal amounts of the material to be separated to each exchanger, shake, allow to settle and then by measuring the optical densities with a spectrophotometer, determine in comparison with a standard solution of the protein or peptide which exchanger removed more activity from the solution. It is a general rule that cationic buffers, such as Tris, should be used with anion exchangers and anionic buffers, such as acetate, with cation exchangers.

b. Capacity of the Ion Exchanger

The amount of ion exchanger that should be used depends upon two factors, (i) the total capacity of the exchanger and (ii) the amount of material to be chromatographed. The total capacity, *i.e.* amount of charged and potentially charged groups, is given by the manufacturer of the gel and is expressed as milliequivalents of exchangeable ions that can be taken up per gram of dry exchanger or per millilitre of hydrated exchanger. In contrast, the available capacity is the capacity available under the specific separation conditions, and not more than 20% of this should be used. Available capacity is dependent on such factors as the concentration and ionic strength of the eluant

The amount of ion exchanger needed depends upon the quantity of material to be chromatographed and can be determined by putting 50 mg of the exchanger into each of several tubes, equilibrating as was done when testing which exchanger to use and to the tubes containing the 10 ml of gel plus supernatant add varied amounts of the sample to be separated, *e.g.* 1 mg, 5 mg, etc. Then stir, allow the gel to sediment and assay each supernatant for the hormone, or determine their optical densities. The tube with the largest amount of sample in which the supernatant has no hormonal activity or the lowest optical density reveals the available capacity of the exchangers, *i.e.* the amount needed to bind all the hormone in the sample. For your separations you should then use a factor of 5 to determine the amount of exchanger needed for the weight of your sample, this factor of 5 being derived from the fact that you should not use more than 20% of the available capacity.

c. Preparation of the Column

Ion exchangers should be swollen at the pH that will be used in the experiment. At room temperature complete swelling will occur in 1–2 days. The column is filled in the same way as for gel filtration.

d. Column Elution

The sample to be purified is dissolved in a small amount of the starting buffer and applied to the top of the column. Allow the material to enter the exchanger and then begin immediately to elute by changing the pH or increasing the ionic strength. Ionic strength is increased by either increasing the molarity of the buffer or adding sodium chloride to it. The pH or ionic strength gradients may be continuous or stepwise. With an anion exchanger the pH gradient should be a decreasing one, toward the isoelectric point; and with a cation exchanger the pH gradient should be an increasing one, likewise toward the isoelectric point. However, regardless of the type of an ion exchanger used, ionic strength gradients are always increasing ones. As with gel filtration, the effluent can be monitored by ultraviolet light absorption and the fractions assayed for biological activity.

C. High Performance Liquid Chromatography (HPLC)

The gel filtration chromatography and ion exchange chromatography described in sections A and B are now classified as 'classical column chromatography' to differentiate them from the newer, increasingly popular HPLC, which is sometimes also referred to as high pressure liquid chromatography. This method allows rapid separations to be performed with very small samples, because the columns have narrow bores usually around 4 mm internal diameter, are short (typically 20 cm) and have column packings of smaller size (5–15 μm) than in classical gels. The high pressure is required to force the eluant through the column HPLC columns are operated with linear flow rates of the mobile phase of typically 30 cm per minute in contrast to the 0.1 cm per minute that is typical in classical column chromatography.

Elution is accomplished by use of an unchanging (isocratic) solvent (the mobile phase) or by gradient elution in which the composition of the mobile phase is varied. Three major types of columns are available for HPLC separations, (a) gel filtration *(i.e.* by molecular weight), (b) ion exchange and (c) reversed phase. The gel filtration columns are excellent for separation of low molecular weight proteins and peptides, the ion exchange columns are

used for proteins having a net opposite charge to the resin, and the reversed phase columns are used mostly for low molecular weight peptides, drugs, lipids, nucleotides and nucleosides.

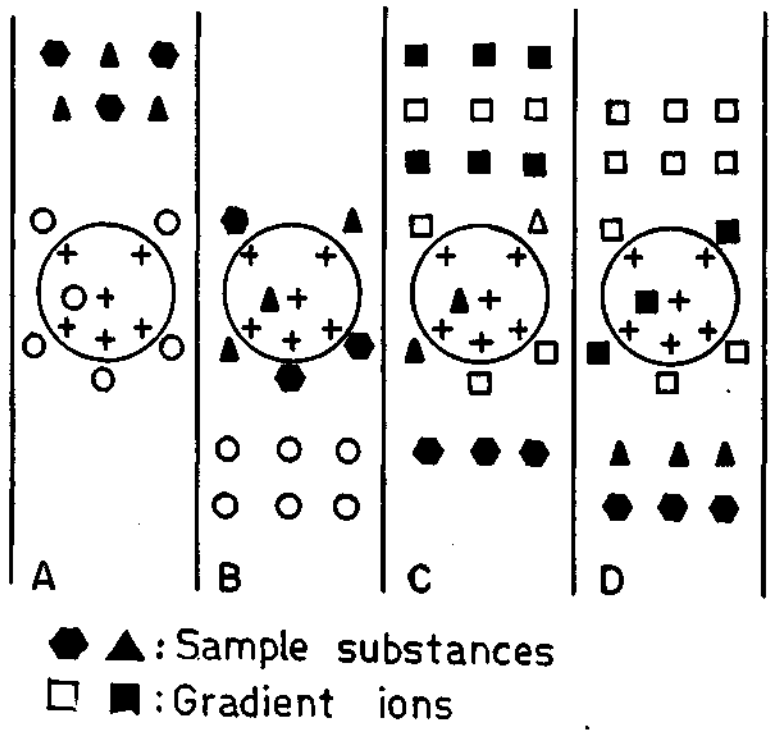

Fig. 5. Ion exchange chromatography by gradient ions: A. starting conditions with an anion exchanger. Sample substances are about to penetrate into the ion exchanger bed; B. Exchange of counter ions for the sample substances; C. Desorption of one of the substances in the sample in exchange for the eluting gradient ions and D. Desorption of a second sample substance by gradient ions. Redrawn from *'Sephadex Ion Exchangers'* (Pharmacia Fine Chemicals, Inc.) and Kleinholz (1985).

a. The Apparatus

HPLC equipment is readily available from several sources, such as Beckman, Waters and Bio-Rad. The basic system (Fig. 6) consists of a pump that will deliver high pressures with very little variation in the pressure they generate, an injection valve to force a precise amount of material into the column, the column itself, a detector to monitor the output (*e.g.* UV detector for peptide or

electrochemical detector for catacholamines) and a strip chart recorder to record the detector findings. In addition, one commonly also adds a fraction collector for the effluent, a microprocessor to program the gradient when one is used and a guard column to protect the main column from contaminants.

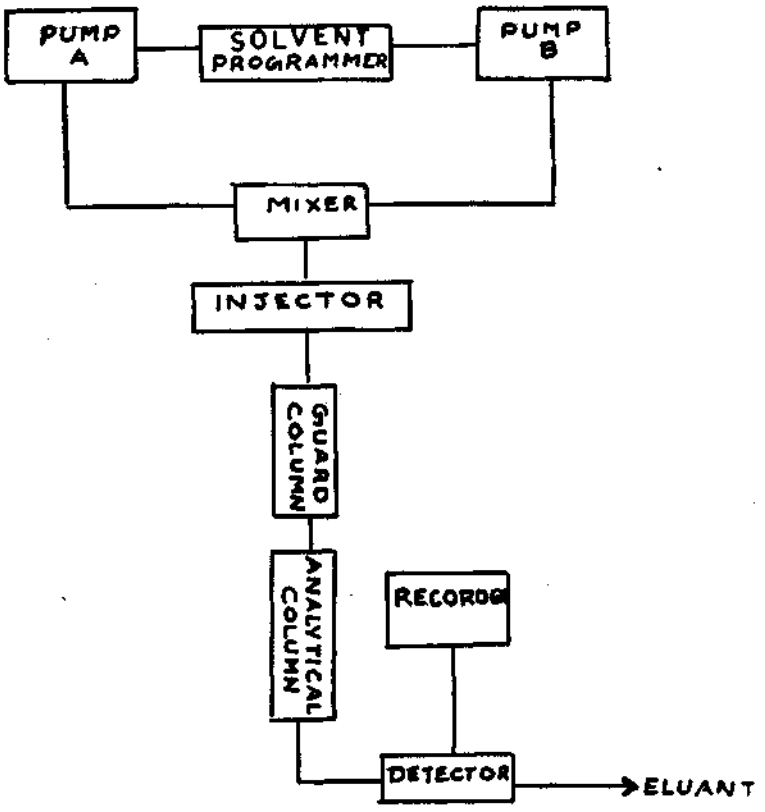

Fig. 6. Diagram of an HPLC system.

b. Reversed Phase Chromatography

Reversed phase chromatography is the most versatile mode of HPLC and is used in about 65% of the applications of HPLC. The packings are highly stable, retention times are highly reproducible and the packings can handle a wide range of polar, nonpolar, and ionized solutes. The term 'reversed phase' refers to the fact

that in classical chromatography the non-polar solvent is the mobile phase and in reversed phase chromatography the non-polar substance is the stationary phase, hydrocarbon chains being covalently bound to packing material. Reversed phase chromatography presents some very significant advantages in the separation of ecdysteroids the arthropod moulting hormones. These highly hydroxylated steroids are more soluble in alcohol and water-alcohol solutions than in chloroform. Very polar impurities found in extracts, such as sugars and glycosides, are eluted from the column before the less polar ecdysteroids under these conditions. An additional attractive feature of the analysis is that the column can be washed with a strong eluant such as (80% methyl alcohol) and then equilibrated with a weaker eluant.

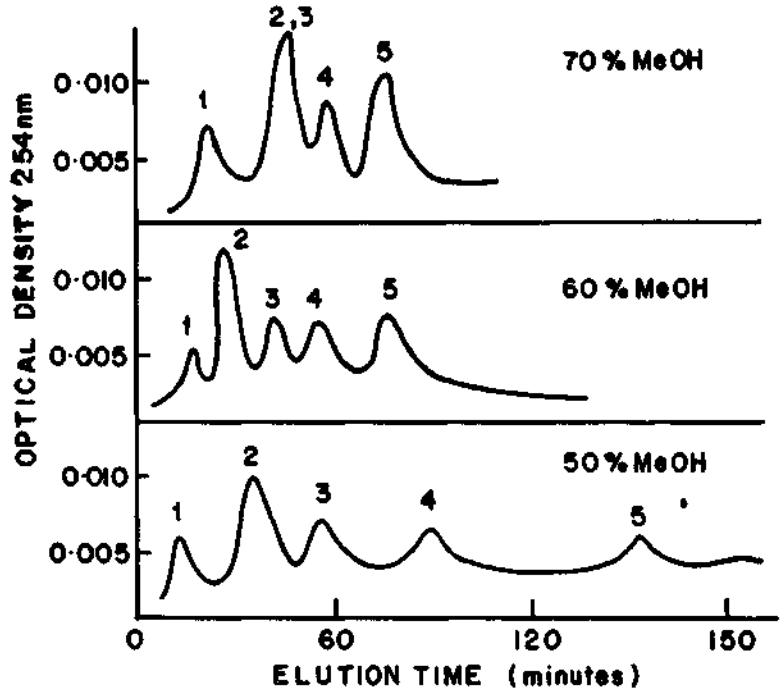

Fig. 7. Isocratic elution of ecdysteroids by reversed phase HPLC. Note that the lower the concentration of methanol, the better the separation. 1. Impurity, 2. β-ecdysone, 3. α—ecdysone, 4 and 5. phytosteroids. Flow rate 3 ml/minute.

A reversed phase column, such as the poragel PN column, can readily be used for separation of ecdysteroids. This column can be used with absolute methanol over a long period without apparent

damage. Occasional washing of the column, which is necessary to remove compounds less polar than the ecdysteroids is more effective when done with 100% methanol than with 80% methanol.

c. Isocratic Elution

As stated above, elution with an unchanging mobile phase is called "isocratic elution". The separation of a mixture of four ecdysteroids with aqueous methanol of various concentrations is shown in Fig. 7.

d. Gradient Elution

Although gradient elution may be time consuming for repeated analysis, it is certainly the best way to chromatograph mixtures of highly varied polarities. The gradient elution of ecdysteroids

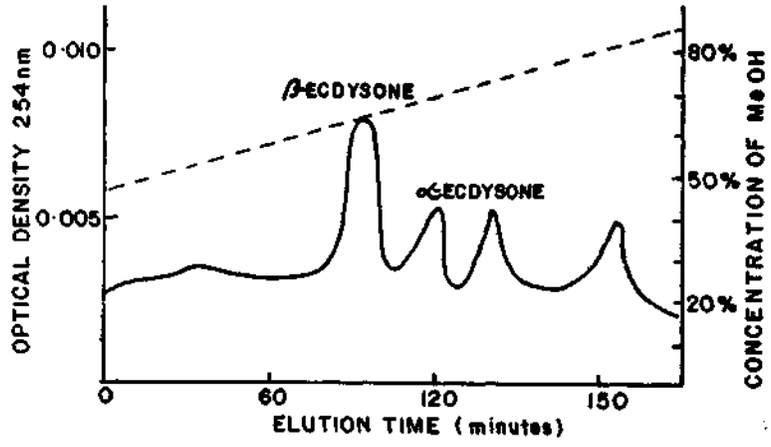

Fig. 8. Gradient elution of ecdysteroids by reversed phase HPLC. The dashed line shows the gradient. Same composition as in Fig. 7.

illustrated in Fig. 8 was performed with 240 ml of 20% aqueous methanol in the first chamber and 200 ml of 80% aqueous methanol in the second chamber. The separation was complete in 3 hours.

e. HPLC of Crustacean Red Pigment Concentrating Hormone (RPCH)

A set of two volumes dealing with the HPLC of amino acids, peptides and proteins has been published (Hancock, 1984). These books provide extremely useful information. With increasing frequent publications detailing the isolation of crustacean neurohormones by HPLC are appearing (Jaffe *et al.*, 1982, 1984; Jaffe and Hayes, 1983; Newcomb, 1983; Keller and Kegel, 1984). Because of the sensitivity of HPLC systems, RPCH has been isolated by HPLC from as few as 20 eyestalks of the prawn *Palaemonetes pugio*, in nanogram quantities (Jaffe *et al.*, 1982) whereas classical chromatographic techniques have generally required thousands of eyestalks to accomplish complete purification. The 20 eyestalks were homogenized by use of a sonicator in 1 ml chilled methanol: water: acetic acid (90:9: 1 by volume) in a 1.5 ml polyethylene centrifuge tube and centrifuged at 5°C for 30 min at 4000 rpm. The supernatant was then transferred to another polyethylene tube and reduced to minimal volume under a stream of nitrogen. The sample was then put into 1 ml of 0.1% trifluoroacetic acid and extracted three times with ethyl acetate. The ethyl acetate was discarded and any residual ethyl acetate was removed under a stream of nitrogen. The material was then filtered a final time with a centrifugal filtration apparatus (Rainin) equipped with a 0.45 Nylon-66 membrane filter, at 1500 rpm for 10 min at 5°C.

The sample was then injected into the HPLC apparatus. A concave gradient (90 to 40% aqueous buffer) of 0.1% trifluoroacetic acid versus 0.1% trifluoroacetic acid in acetonitrile was used in conjunction with a Supelcosil LC-18DB (Supelco) column. The elution was monitored at both 210 and 254 nm. This method permitted the detection of less than 5 ng of RPCH. Synthetic RPCH can be purchased from Peninsula Laboratories, 611 Taylor Way, Belmont, California 94002. By chromatographing the pure hormone first, its retention time on the column and its UV absorption characteristics can be determined and will help to identify the fraction in which the material which is being purified will be expected to be found.

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2

SDS POLYACRYLAMIDE GEL ELECTROPHORESIS

Electrophoresis in polyacrylamide gels containing the anionic detergent sodium dodecyl sulfate (SDS) is used widely for the separation of protein subunits and the determination of their molecular weights. The molecular weight of a protein can be determined by comparison of its electrophoretic mobility with the mobilities of proteins of known molecular weight. A linear relationship is observed when the logarithms of the molecular weights of standard proteins are plotted against their electrophoretic mobility. The procedure described below is based on Sigma Chemical Company Technical Bulletin No. MWS-877, which is a modification of the methods of Weber and Osborne (1969) and Davies and Stark (1970). Eastman-Reks and Fingerman (1985) used this procedure to separate vitellin subunits from ovaries of the fiddeler crab *Uca pugilator*.

Preparation of Reagents for SDS Polyacrylamide Gel Electrophoresis

A tube gel electrophoresis system is required for these analyses. Several companies, such as Bio-Rad, sell this apparatus.

The following reagent solutions must be prepared:

A. Sample buffer
Prepare the solution by combining:

Sodium phosphate, anhydrous, monobasic (NaH_2PO_4)	0.34 g
Sodium phosphate, anhydrous, dibasic (Na_2HPO_4)	1.02 g
Sodium dodecyl sulfate (SDS, Lauryl sulfate)	1.00 g
2-Mercaptoethanol	1.00 ml
Bromophenol Blue	0.015 g
Urea	36.00 g

Dissolve and dilute to 100 ml with water. The pH should be approximately 7.0 at 25°C. Keep tightly capped. The solution is stable for at least 2 weeks when stored in a refrigerator at 0-5°C.

B. Gel buffer

Prepare the solution by combining:

Sodium phosphate, anhydrous, monobasic (NaH_2PO_4)	6.80 g
Sodium phosphate, anhydrous, dibasic (Na_2HPO_4)	20.45 g
Sodium dodecyl sulfate (SDS, Lauryl sulfate)	2.00 g

Dissolve and dilute to 1 litre with water. The pH should be approximately 7.0 at 25°C. Store tightly capped in a refrigerator at 0-5°C. Suitable for use as long as there is no visible microbial growth.

C. Acrylamide gels

a. 10% Gel - for use with proteins having molecular weights of 14,000-70,000 daltons.

To prepare gel mix:

Acrylamide	22.2 g
N, N' - Methylenebisacrylamide	0.6 g

Dissolve and dilute to 100 ml with water. Remove insoluble material by filtration. Solution is stable for at least 1 month when stored in a dark bottle in a refrigerator at 0–5°C

b. 5% Gel - for use with proteins having molecular weights of 70,000 280,000 daltons.

To prepare gel, mix:

Acrylamide	11.1 g
N, N' - Methylenebisacrylamide	0.3 g

Dissolve and dilute to 100 ml with water. Remove insoluble material by filtration. Solution is stable for at least 1 month when stored in a dark bottle in a refrigerator at 0-5°C.

D. N, N, N' N' - Tetramethylethylenediamine (TMEDA)
Store at room temperature.

E. Ammonium persulfate solution
Prepare fresh daily
Dissolve approximately 100 mg in 15 ml water.

F. Fixative solution
Prepare solution by combining:

Methanol	400 ml
Glacial acetic acid	70 ml
Water	530 ml

G. Staining reagent
Prepare solution by dissolving:
Brilliant Blue R (Coomassie Brilliant Blue R)
in 500 ml of Reagent F. 1.25 g
The staining reagent may be used for approximately 12 sets of gels.
Store tightly capped at room temperature.
The solution is stable for several months.

H. Destaining reagent
Prepare solution by combining:

Methanol	50 ml
Glacial acetic acid	75 ml

Dilute to 1 litre with water.
Solution should be used only once and then discarded.
Store unused solution tighly capped at room temperature.

Preparation of Sample

The unknown proteins are prepared by weighing out samples so that the concentrations are about 1 mg protein per 1 ml of sample buffer (Reagent A).

Preparation of SDS Molecular Weight Markers

Prepare the standards in sample buffer (Reagent A). The concentration of the standard that should be used varies with the protein standard you are using. The following Table gives the recommended concentrations for a variety of commonly used standards.

Protein	Optimum concentration (mg/ml)	Molecular weight
Albumin, bovine plasma	1.5	66,000
Albumin, egg	1.5	45,000
Carbonic anhydrase	1.0	29,000
Lysozyme, egg white	1.0	14,300
Pepsin, porcine stomach mucosa	5.0	34,700

Preparation of electrophoresis gels

The gel tubes should have an inner diameter of 0.5 cm and be long enough to hold a 10 cm gel. The gel solidifies in 10–20 minutes. To prepare 12 gels:

1. Mix 15 ml gel buffer (Reagent B), 13.5 ml of acrylamide gel (Reagent C, the 5% or 10% gel). Deaerate for one minute with a water aspirator.

2. Deaerate 1.5 ml of freshly prepared ammonium persulfate solution (Reagent E) for about 15 seconds and add to above.

3. Add 0.05 ml TMEDA (Reagent D).

4. Mix solution carefully to avoid introducing air.

5. Carefully dispense 2 ml of solution into each gel tube.

6. Before the gel hardens, carefully layer a few drops of water on top of each gel solution.

Electrophoresis

Electrophoresis should be carried out at constant current of 8 milliampeers per gel with the positive electrode in the lower chamber.

1. Dilute 500 ml gel buffer (Reagent B) with 1000 ml water.

2. Decant water from top of gel and layer buffer solution on top of each gel to fill tube.

3. Underlay of 10 µl sample on gel.

4. Fill compartments of electrophoresis apparatus with diluted gel buffer.

5. Apply constant current at 8 milliamperes per gel until marker dye Bromphenol Blue is 1 cm from the anodic end of the gel. This will require about 5 hours.

6. Remove gels from tubes by squirting water from a syringe between the gel and glass wall. Then use a pipet ball to exert pressure.

Staining and destaining

1. Mark the center of the Bromphenol Blue dye front with a piece of fine wire.

2. Immerse gels in fixative solution (Reagent F) for at least 10 hours. Several changes of the fixative solution should be made.

3. Stain the gels in staining reagent (Reagent G) for at least 2 hours, but overnight staining is preferred.

4. Destain. If an electrophoretic destainer is available, destain in destaining solution (Reagent H). Follow instructions of the manufacturer of the instrument. If such an instrument is not available the gels can be destained by diffusion against several changes of the fixative solution (Reagent F). With Reagent F the destaining time should be no less than 15 hours and no longer than 25 hours, then continue, destaining with Reagent H. Destaining with Reagent F for longer than 25 hours will lead to decolourization of some of the protein bands.

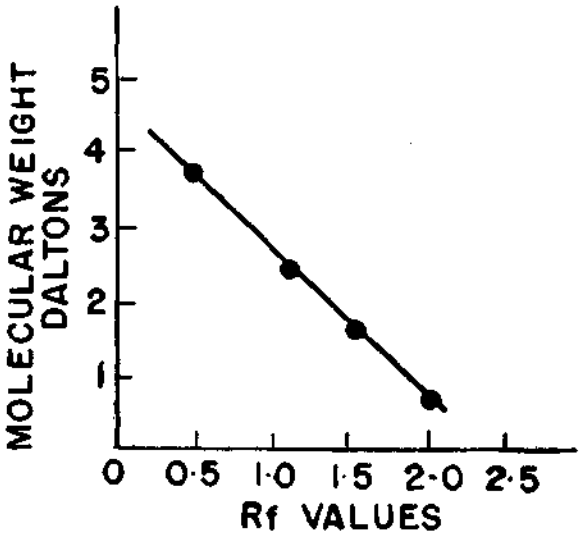

Fig. 9. Relationship between molecular weight and relative mobility (R_f). The curve is derived by using molecular weight protein standards.

5. Record the migration distance of the Bromphenol Blue tracking dye and of each blue protein zone from the top of the gel.
6. Store the gels in the destaining solution (Reagent H).

Calculation of Results

To determine the relative mobility R_f of a protein, divide its migration distance from the top of the gel to the center of the protein band by the migration distance of the Bromphenol Blue tracking dye from the top of the gel.

$$R_f = \frac{\text{distance of protein migration}}{\text{distance of tracking dye migration}}$$

The R_f values of each band are plotted along the abscissa against the known molecular weights on the ordinate on semilogarithmic paper, as in Fig. 9. The molecular weights are plotted on the logarithmic scale of the semilogarithmic graph paper. From this calibration curve you can estimate the molecular weight of each unknown.

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3

IMMUNOCYTOCHEMISTRY

Immunocytochemical studies with tissue from the crustacean nervous system are being done with increasing frequency as specific antibodies to neuropeptides and neurohormones are becoming available. There have been, in particular, several studies recently showing the distribution in the eyestalk of neuropeptides that were first discovered in vertebrates. Some pertinent references that reveal the extent of such studies are the following: Mancillas *et al.*. 1981; Gorgels-Kallen *et al.*, 1982; Jacobs and Van Herp, 1984; Jaros *et al.*, 1985; Van Deijnen *et al.*, 1985; Fingerman *et al.*, 1985,

Procedure

The tissues are fixed in 4% paraformaldehyde in phosphate buffer (0.1M, pH 7.3) or Bouin's fixative for 48 hours. The tissue may then be embedded in paraffin after routine dehydration and cleared in xylene. However, frozen sections give the best preservation of antigenic reactivity. Paraffin embedded tissue is sectioned at 5 μm, frozen material at 15 μm and then affixed to glass slides. The sections are then processed for immunocytochemistry using the Sternberger peroxidase-antiperoxidase (PAP) procedure (Sternberger *et al.*, 1970) and a modified diaminobenzidine (DAB) reaction at low pH (Vacca *et al.*, 1978). The method is diagrammatically illustrated in Fig. 10.

The following is the procedure for the immunocytochemical localization of peptides.

1. Rinse the tissue sections in phosphate buffered saline (0.01 M, pH 7.2); 2 changes, 15 minutes each with shaking.
2. Incubate half of the tissue in the detergent Triton X-100 (0.05%) in Tris-HCL saline (0.05 M, pH 7.6) for 30 minutes with gentle shaking. Rinse thoroughly with buffer; 2 changes, 15 minutes each with shaking.

3. Incubate all the sections in normal goat serum (1:30 dilution); with 30 minutes shaking. The serum is diluted with the Tris-HCL saline. This step reduces nonspecific binding of the antibody.

4. Rinse thoroughly with the buffered saline; 2 changes, 8 minutes each with shaking.

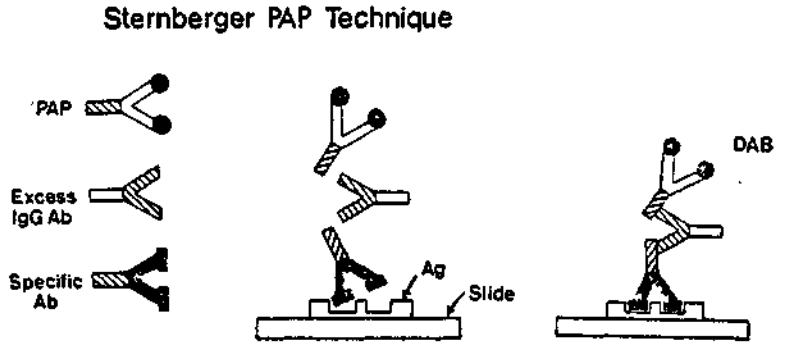

Fig. 10. Schematic drawing of the peroxidase anti-peroxidase (PAP) method of Sternberger for localization of tissue antigens (A_g). Excess IgG antibody (Ab) is applied in the second layer to form a sandwich between the first layer (specific Ab) and the third layer, PAP. The peroxidase molecules are then visualized using diaminobenzidine (DAB).

5. Incubate in rabbit antibody (1:100-1:4000); 1 hour on shaker and then overnight at 4°C. Normal rabbit serum (1:100) is used for control incubations.

6. Rinse thoroughly with the buffered saline, 2 changes, 8 minutes each with shaking.

7. Incubate in goat anti-rabbit IgG serum (1:50) for 30 minutes with shaking.

8. Rinse in the buffered saline; 2 changes, 8 minutes each with shaking.

9. Incubate in rabbit PAP (1:100) for 30 minutes with shaking.

10. Rinse in the buffered saline; 2 changes, 8 minutes each with shaking.

11. React in solution containing both 3, 3′-diaminobenzidine (DAB) tetrahydrochloride (44 mg%) and 0.003% hydrogen peroxide in ammonium acetate-citric acid buffer, 0.05 M, pH 5.5; 30 minutes in the dark with shaking.

12. Rinse in distilled water; 2 changes, 8 minutes each with shaking.

13. Dehydrate in graded ethanols, clear, and apply coverslip for microscopic observation.

Some antisera for immunocytochemistry are available from Peninsula Laboratories, 611 Taylor Way, Belmont, California 94002. Triton X-100 and the DAB are available from Sigma Chemical Company, P.O. Box 14508, St. Louis, Missouri 63178. Rabbit anti-goat IgG can be purchased from Miles Laboratories, 2000 North Aurora Blvd., Naperville, Illinois 60566. The rabbit PAP serum is available from the DAKO Corporation, 22 North Milpas St., Santa Barbara, California 93103.

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4

ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

The ELISA is a highly sensitive technique (Voller *et al.*, 1979; Butler, 1981) that has been adapted to measure hormone concentrations. To use an ELISA, antibodies against the hormone to be measured must be available. If these antibodies cannot be purchased then they must be prepared by injections of purified hormone (antigen) into rabbits. ELISA has advantages over RIA in that with ELISA no radioactive material is used and the end point is colorimetric. The ELISA depends upon two assumptions. 1. That an antigen can be linked to an insoluble carrier surface and will retain activity. 2. That an enzyme marker can be attached to an antibody and retain immunological and enzymatic activity. Both assumptions hold true.

The general principle of ELISA (Fig. 11) is: (a) an antigen is linked to a carrier surface, (b) the sensitized carrier surface captures the corresponding antibody, (c) an enzyme labelled antiglobulin attaches to the antigen-antibody complex, (d) the complex is detected by the enzyme label changing the colour of an added substrate, and (e) the optical density of the final colour is directly proportional to the amount of antigen bound to the carrier surface. Quackenbush and Fingerman (1985) devised an ELISA to determine concentrations of black pigment dispersing hormone (BPDH) in the fiddler crab *Uca pugilator*. This is described here in detail.

A. Isolation of Black Pigment Dispersing Hormone (BPDH)

The hormone was obtained by extracting 2.5 g of lyophilized eyestalks in 48 ml distilled water. The extract was boiled for 3 minutes and then centrifuged at 10,000 x at 4°C for 30 minutes. The supernatant was lyophilized, then resuspended in 50% methanol and passed through a Waters Sep-Pak (Waters Chromatography Division, Millipore Corp., 34 Maple St., Milford, Massachusetts 01757) to achieve some initial purification. Sep-Pak cartridges are disposable cartridges that contain about 1 ml of C18 reversed

phase packing and are very useful for sample preparations. The next step in the purification was to apply the material to a 15% sodium dodecyl sulfate (SDS) polyacryamide gel (0.75 x 140 mm) for electrophoresis at 50 mA constant current. After the tracking dye reached the bottom of the gel, the gel slab was cut into 31 segments (4 mm each). Each segment was extracted overnight in 500 µl of 0.05 M ammonium sulfate (pH 7.2) at 4°C. Aliquots of each gel segment extract were then made isosmotic to the crab's blood by adding 400% crustacean saline (Cooke *et al.*, 1977) and then bioassayed for BPDH. Fraction 28 had 92% of the total BPDH activity and contained 0.5 mg protein. Its estimated molecular

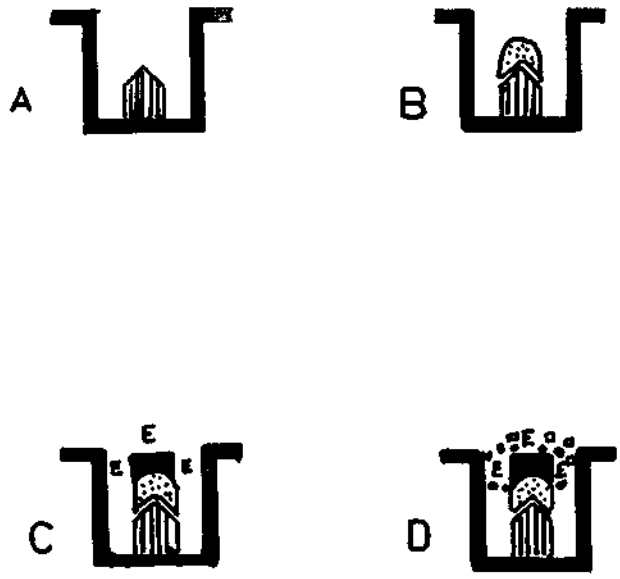

Fig. 11. Diagram of an ELISA: A. Antigen is coated on the inside of the plate wells; B. serum is added and any specific antibody attaches to the antigen; C. enzyme-labelled antiglobulin is attached, which attaches to the antibody-antigen complex and D. enzyme substrate (squares) is added which is hydrolyzed by the captured enzyme and gives a coloured product (dots), the optic density of which is directly proportional to the amount of unknown antibody.

weight was 1800 daltons and it had no dispersing activity on the red chromatophores. The BPDH in this fraction was then used in the immunization described in the following section. The protein determinations were made with the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories, 2200 Wright Ave., Richmond, California 94804). The peptide and protein bands were visualized by a highly

sensitive silver stain (Merrill *et al.*, 1982). Bio-Rad has a silver stain kit. Silver staining is 10–50 times more sensitive than Coomassie Brilliant Blue for proteins. The optical density pattern of stained gels can be determined with precision by using a soft laser scanning densitometer (Biomed Instruments, 1020 South Raymond Ave., Fullerton, California 92631). A laser densitometer permits finer resolution of stained bands than is possible with a densitometer that has only a conventional tungsten light source.

B. Immunization and Immunoprecipitation of BPDH

The immunogen mixture consisted of 34 μg BPDH, 2 ml of 0.1% methylated bovine serum albumin and 2 ml of Freund's complete adjuvant (Benoit *et al.*, 1982). Female white rabbits were each immunized with 80 intradermal injections (Vaitukaitis, 1981). No booster immunizations were given. Bleedings (10 ml) were begun one week prior to immunization and continued weekly after immunization.

To test for the formation of antibodies against the BPDH, three dilutions (1:100, 1:1,000, 1:1,500) of preimmune and immune serum were mixed with aqueous extracts of sinus glands (1:100, 1:1000, 1:10,000 1:100,000) in 0.01 M phosphate buffered saline (PBS) (0.85% NaCl, pH 7.2) and incubated at room temperature for 2 hrs. After a 10 min centrifugation (8727 g, Eppendorf Model 5414 microcentrifuge), the supernatant was made isosmotic by adding 400% saline and then assayed for BPDH activity. Preimmune serum had no effect on the amount of black pigment dispersion induced by the sinus gland extracts. However, the immune serum had a high affinity for BPDH and significantly reduced the BPDH activity. One hundred μl of immune serum and serial concentrations of BPDH (0.33–6.0 μg) in 300 μl PBS were mixed and incubated for 2 hours at room temperature. After a 10 min centrifugation, the pellet was washed 4 times with cold PBS (500 μl) and then dissolved in 0.1 N NaOH, and the absorbance was measured at 280 nm. A traditional immunoprecipitation curve was obtained. The plot of optical density versus BPDH concentration showed that 1.8 μg BPDH and 100 μl of the serum produced the most precipitate.

C. The ELISA

The ELISA is performed by running a set of standards having known amounts of BPDH along with the samples whose BPDH content is to be determined. Fifty μl samples, for example 50 μl of blood, are added to 100 μl carbonate buffer (0.03 M $NaHCO_3$, 0.03 M Na_2CO_3, pH 9.6) and then incubated 18 hours at 4°C in

polystyrene wells in Immulon 1 microtiter plates (Dynatech Laboratories, 900 Slater Lane, Alexandria, Virginia 22314). The BPDH in the samples is adsorbed to the wells in these polystyrene plates. Additional adsorption sites are then blocked by filling the wells with 5% ovalbumin for one hour at 37°C. One hundred and fifty µl of 1:8,000 diluted immune serum in 5% ovalbumin in PBS are then added to each well and incubated at 4°C for 24 hours. At a dilution of 1:25,000 this serum produced about 50% binding. A serum dilution of 1:8,000 was chosen for the BPDH assays because this dilution was the most dilute one that produced maximal binding. Rinse the plates. The wash solution contains 0.02% Tween-20, a wetting agent that minimizes non-specific binding to the plates. To detect bound rabbit IgG a second antibody, goat antirabbit antibody, conjugated with peroxidase (Kirkegaard and Perry Laboratories, Gaithersburg, Maryland 20879) is used. The goat antibody is diluted 1:800 with 5% ovalbumin in PBS. The plates are then incubated for 2 hours at 22° C to allow the goat antibody to bind to the rabbit antibody. Rinse the plates again. The substrate for the peroxidase, 2,2'–azino-di [(3-ethyl-benzthiazoline sulfonate (6)], in buffered H_2O_2 (Kirkegaard and Perry Laboratories, 2 Cessna Court), is then added. The optical density of the blue-green reaction product is then read at 420 nm. Determining the optical densities of the samples in numerous wells is facilitated by the use of a microprocessor-based plate reading spectrophotometer (Bio-Tel Instruments, 1 Mill Street, Burlington, Vermont 05401). A standard curve of optical density versus purified BPDH concentration can then be drawn and used to determine the BPDH concentration in blood samples or tissue extracts. Every assay should have three controls. One, a negative 1 background control in which this is a well with no sample. Any colour development in this well is due to non–specific binding of the conjugate to the plate, or interaction with the ovalbumin blocking solution. Two, a threshold control where a well contains a known low positive reference sample to define the cut-off value of a positive. Three, a positive control where a well contains a known high positive reference sample that defines the maximal linear signal of the assay.

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5

RADIOIMMUNOASSAY

A conventional radioimmunoassay (RIA) (Yalow and Berson, 1960) may be described as a contest between a fixed amount of radiolabelled antigen (Ag*) and a variable amount of test antigen (Ag) for the specific antigen-antibody sites of a limited number of antibody molecules (Ab)

Thus

$$\begin{matrix} Ag^* \\ +Ab \end{matrix} \rightleftharpoons \begin{matrix} Ag^*\ Ab \\ + \\ Ag \quad\ Ab \end{matrix}$$

Customarily 30 to 50% of labelled antigen is bound to antibody in the absence of other antigen and is termed the B_o value, *i.e.* the contest is a walk-over. This fraction bound decreases as the numerical strength of the opposition increases, and consequently from an experimentally derived standard calibration curve, test sample concentrations can be interpolated either manually or by a suitably programmed calculator, provided that the bound fractions can be satisfactorily measured.

RIA is one of the most specialized techniques used for measuring the circulating hormones in the blood of vertebrates and invertebrates. The major advantages of the technique over other assays are the high sensitivity (because of the use of isotopes), high specificity (use of immunogenic reactions) and high reproducibility.

A. Components Required

1. Pure antigen [Follicle Stimulating Hormone, (FSH), β ecdysone]. Follicle Stimulating Hormone (FSH) is a vertebrate glycoprotein hormone that has been commonly used in radioimmunoassays (Moudgal *et al.*, 1979) whereas β ecdysone is an arthropod steroid hormone that has also been measured by this technique (Chang and O'Connor, 1979; O'Connor, 1985).

2. Specific antisera (Rabbit), 1:20,000 dilution.

3. Radiolabelled antigen. ^{125}I labelled FSH and ^{3}H labelled ecdysones are available from NEN Research Products, 549 Albany St., Boston, Massachusetts 02118.

4. Standards - (for preparation of a standard graph) Pure FSH or β-ecdysone.

5. Quality control sera (Serum standards).

6. Sample for assay (Blood serum or haemolymph).

7. Gamma counter (^{125}I) with word processor and computer or Liquid Scintillation Counter $^{3}(H)$.

8. Refrigerated centrifuge.

9. Vortex mixer.

10. Micropipettes.

11. Labelled vials.

B. Preparation of Antigen

It is well known that compounds with a molecular weight of less than 1000 daltons do not readily elicit the production of antibodies, unless they are linked co-valently to a carrier molecule of relatively high molecular weight. While proteins are immunogenic in rabbits, steroids having smaller molecular weights (ecdysones) are not. Steroids to be conjugated to a protein are generally called 'haptens'. The carrier molecules that have been used most frequently are proteins with molecular weights from 50,000 to 1,000,000 and the linkage is a peptide bond between a carboxyl group on the steroid derivative and an amino group or certain diaminocarboxylic amino acids (usually lysine) in the protein. If bovine serum albumin (BSA) is used as the carrier, then 60 amine groups are present per molecule and usually from 20 to 30 of these will be joined to a hapten. The specificity of the antiserum depends mainly on the position at which the hapten is joined to the carrier molecule.

The conjugation of the steroid and BSA can be achieved by:

1. Hemisuccinate reaction - OH
2. O-Carboxyl methoxyl oxime reaction = O

For details refer Borst and O'Connor (1972,1974); Chang and O'Connor (1979); Soumoff *et al.*, (1981).

C. Immunization and Preparation of Antisera

3.0 mg of lyophilised conjugate is suspended in 300 µl water and briefly sonicated. This solution is then diluted with two volumes of incomplete adjuvant as shown in Fig. 12 and mixed vigrously. Each of five Newzealand rabbits is then injected subcutaneously along the back with 0.8 to 1.0 mg of the ecdysone-thyroglobulin conjugate. Booster injections consisting of 0.4 mg of conjugate in incomplete Freund's adjuvant are administered to two of the

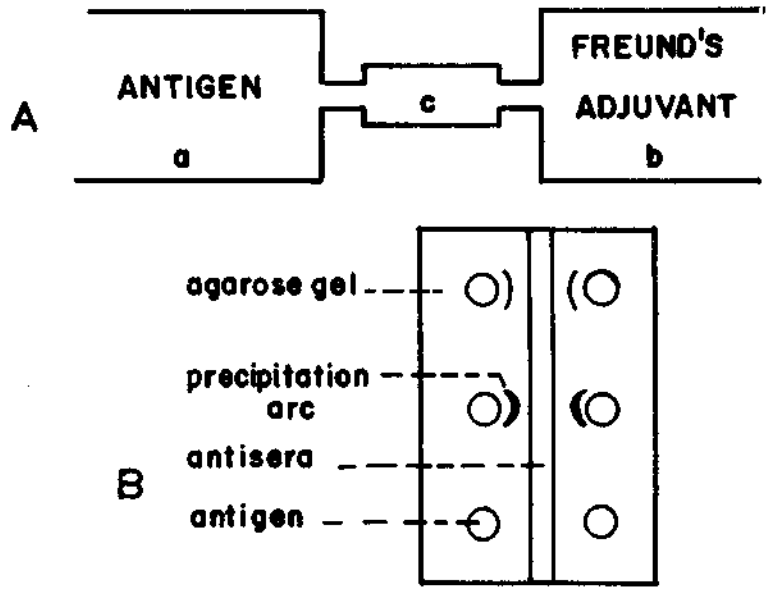

Fig. 12. A. Mixing of antigen (a) with Freund's incomplete adjuvant (b through a connecting tube (c) and B. Diagram of Ouchterlony precipitation.

rabbits 12 weeks after the initial injections. Sera from the rabbits are collected for a period of 17 weeks at approximately ten-day intervals. Blood is taken from the ear by puncturing the vein and after initial coagulation at room temperature, they were stored at 4°C to allow for adequate clot retraction. The supernatants are then removed, Merthiolate (1:100,000 dilution) is added as an antibacterial agent and the sera are stored in 2.0 ml aliquots at 0° C. The antiserum which is produced contains immunoglobulins. It is tested for antibody titre, specificity and avidity.

Simple Ouchterlony Technique

One ml of antiserum is diluted to the following dilutions.

1: 100
1: 200
1: 2,000
1: 10,000
1: 20,000

100 µl of the diluted antiserum is added in the groove of an agarose gel plate and 50 µl antigen (FSH or β-ecdysone) is added in the wells on both sides of the groove. Strong precipitation will occur wherever the specific antiserum reacts against the antigen. The specificity of an antiserum will depend not only upon the structure of the antigen, but also upon the species of animals used and the number of immunizations. Accordingly, it is essential that every batch of antiserum be evaluated in detail.

D. Procedure for the Optimization of Standard Curves

1. Obtain the most specific antiserum with high titre and avidity.

2. Select the labelled derivative and from the specific activity calculate the mass which may be added to give reasonable counting time and errors. Commercially available ^3H ∝ ecdysone has a specific activity of 68 CI/mMole and the lower limit of sensitivity is 25 pg of β-ecdysone.

3. Determine the approximate dilution of antiserum that may be used (as shown above) and confirm the ability of the pure steroid to compete with or displace the labelled derivatives under defined conditions. For example, the antiserum of Borst and O'Connor (1972) required 1.5 ng unlabelled β-ecdysone and 4.8 ng unlabelled ∝-ecdysone to achieve 50% inhibition of bound radioactivity in the ammonium sulphate pellet.

4. Determine the optimum dilution of antiserum that gives maximum displacement under the conditions selected.

The optimum dilution of antiserum for the maximum displacement of labelled material may be determined by incubating the radiolabelled derivative (100 µl) and labelled derivative plus 100 µg of pure steroid hormone with serial dilutions of the antiserum in phosphate buffer of known pH and incubate them for 8–10 hours. Three components are formed after the reaction.

1. Antiserum not bound
2. ^{125}I FSH/^{3}H-β-ecdysone not bound
3. ^{125}I FSH/^{3}H-β-ecdysone bound

The bound and free labelled hormone components are separated using either of the following methods.

a) Double antibody (antirabbit gammaglobulin) method,
b) Dextran coated charcoal method and
c) Precipitation by saturated ammonium sulphate method

To each reaction tube add 0.1 ml of antirabbit gammaglobulin and mix thoroughly. Keep the tubes at room temperature. All the free FSH will be bound to the second antibody. Centrifuge at 2500 rpm. The supernatant contains the free hormone and the precipitate contains the bound hormone. The gamma radiation emitted from the precipitate is counted as counts per minute. The amount of labelled material bound to the antiserum is plotted on the ordinate against the inverse of the dilution of the antiserum on the abcissa (log scale). If the total count from the pure labelled hormone is 90,000 cpm and that of bound 40,000 cpm, the per cent bound will be 44.4%. The dilution at which binding values go above 40% is adequate for RIA.

E. Protocol of RIA (FSH)

			Tube Nos.
1.	Blank tubes containing buffer or empty tubes		(1 & 2)
2.	Total activity - 0.1 ml of ^{125}I alone		(3 & 4)
3.	Reference (B_o) 'O' standard buffer alone		(5 & 6)
4.	Non Specific Binding (NSB) *i.e.* any accidental contamination of the buffer. Contains buffer and labelled hormone.		(7 & 8)
5.	Standards	1.5 mIU/ml	(9 & 10)
		5 mIU/ml	(11 & 12)
		10 mIU/ml	(13 & 14)
		20 mIU/ml	(15 & 16)
		40 mIU/ml	(17 & 18)
		80 mIU/ml	(19 & 20)
6.	Quality control, sero test	2 tubes for high	(21 & 22)
		2 tubes for low	(23 & 24)
7.	Experimental sample (unknown)	1st sample	(25 & 26)
		2nd sample	(27 & 28)

Flow Sheet

Reagent/tubes	Blank	Sample	B_0	NSB	Standard	Quality Control Serotest
Sample	—	0.1	—	—	—	0.1
Buffer	0.3	—	0.1	0.2	—	—
Standards (1 to 6)	—	—	—	—	0.1	—
Antiserum	—	0.1	0.1	—	0.1	0.1
^{125}I FSH	—	0.1	0.1	0.1	0.1	0.1
	0.3	0.3	0.3	0.3	0.3	0.3

This method is called 'saturation analysis'. The sample tube contains the test blood serum containing FSH (cold), FSH antiserum and labelled FSH (hot). The antigen-antibody reaction commences immediately and both cold and hot hormone compete for binding sites on the antibody. The antiserum is provided in such a way that after some time it will be saturated. Keep aside the blank and total activity tubes. After gentle vortexing, incubate the tubes in a refrigerator for 18–24 hours. After incubation, the following products will be present.

FSH
FSH + Ab
FSH*
FSH* + Ab
Ab

Bring the tubes to room temperature. To separate the FSH*Ab from the other products, 0.1 ml of a second antibody (goat antirabbit antiserum) is added to tubes from 9 to 10 onwards. Precipitation occurs. The second antibody will not react with the FSH* as this will not recognize the antigen.

After addition of the second antibody, incubate the tubes for 1 hour. Centrifuge for 15 minutes at 2,500 rpm. Decant the supernatant. Place them in the counting rack of the gamma counter and count the radioactivity for 60 seconds.

F. Sample Calculation

Total radioactivity	28,500 cpm
Blank	120 cpm
NSB (Non-specific binding)	260 cpm (below 1%)
B_0	16,815 cpm (58.9%)

Standards (mIU/ml)	1.5	15,722 – 93.5%
	5.0	14,122 – 84.0%
	10.0	12,443 – 74.0%
	20.0	10,000 – 59.5%
	40.0	7,398 – 44.0%
	80.0	4,972 – 28.5%
Sample		11,434 – 68.0%

Using semilogarithmic graph paper, plot the relative binding of each standard on the ordinate against the known concentration of the hormone on the abscissa (Fig. 13).

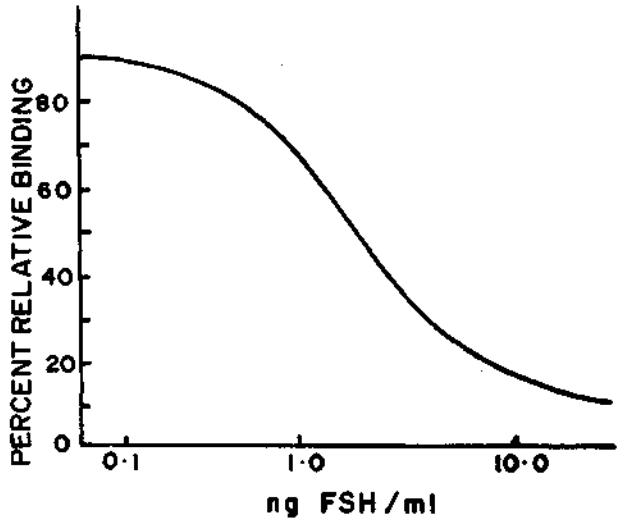

Fig. 13. Standard radioimmunoassay curve.

$$\frac{\text{Mean counts (Standard, sample or serotest)}}{B_0 \text{ Mean counts}} \times 100 = \text{Percent relative binding}$$

By interpolating the per cent relative binding of the sample values on the standard curve, the hormone concentration in the sample can be determined. Concentration of the hormone and radioactive counts are inversely proportional. For diluted samples, it is necessary to multiply the value read on standard curve by the dilution factor.

G. Protocol of RIA (Ecdysones)

1. Add unknown (10 µl of fresh crustacean haemolymph) to assay tube and dry if necessary under nitrogen. Isolated tissues or whole organisms should be extracted in a final concentration of 80% ethanol (v/v). The insoluble material is removed by filtration or centrifugation. The supernatant is added to the incubation tube and the solvent is removed under a stream of nitrogen.

2. Add radiolabelled ∝-ecdysone in 100 µl borate buffer (Label aliquot contains approximately 3600 cpms/100 µl).

3 Vortex tubes well to take up dried unknown.

4. Add 100 µl antisera solution - this step is always second.

5. Thoroughly vortex incubation mixture. This can be done after adding antisera to all assay tubes.

6. Incubate the tubes overnight at 4°C or at room temperature for 2½ - 4 hours.

7. First precipitation - Precipitate with saturated ammonium sulphate. Perform precipitation at 4° C. Take extra care to mix tubes within one minute after ammonium sulphate/ addition *i.e.*, usually do two at a time and then vortex. Allow tubes to sit in cold for 20 minutes. Centrifuge at 5000 rpm for 15 minutes and remove the supernatant.

8. Second precipitation – Rinse the pellet with 400 µl of 50% ammonium sulphate (made with original saturated ammonium sulphate and borate buffer). The buffer can be added to all tubes and then vortex pellets. Allow the tubes to sit in cold for 15 minutes. Centrifuge and then remove the supernatant.

9. Counting procedure – Dissolve pellet in 25 µl of distilled water. Vortex tubes to take up pellet. Count with 600 µl of Aquasol. Allow vials to set with counting solution for 5–6 hours, so that pellet settles and counting efficiency stabilizes.

Preparation of standard curve

A standard curve is prepared by adding to a series of incubation tubes concentrations of β-ecdysone from 50 pg to 8.0 ng. The concentration of the standards are determined by appropriate

dilutions of a stock (normally 50 ng/ml) whose concentration is routinely determined spectrophotometrically at 242 nm. The various standards are added to the incubation tubes in ethanol and the solvent is removed under a stream of nitrogen. In addition, a standard blank (with control serum) is incubated, so that background may be substracted.

50 pg/1	µl
100 pg/2	µl
250 pg/5	µl
500 pg/10	µl
1000 pg/20	µl
2000 pg/40	µl
4000 pg/80	µl
8000 pg/160	µl

Optimal sensitivity range of assay with ^{3}H-α-ecdysone: 68 CI/mM is approximately 300 pg - 1.5 ngs.

Commercially available labelled ecdysone is ^{3}H-α-ecdysone. The antiserum described by Borst and O'Connor, 1974 (β-ecdysone-CMO antiserum) had similar affinities for α and β-ecdysones. So use of these two different forms of ecdysones will not pose any problem for the measurement of the circulating ecdysteroids.

Buffers and RIA solutions

a. RIA Borate buffer:

Dissolve 6.184 g Boric acid, 9.536 g Sodium tetraborate and 4.384 g Sodium chloride in I litre of distilled water and bring to pH 8.4.

b. Saturated ammonium sulphate solution:

Prepare this with borate buffer.

c. 50% ammonium sulphate:

Dilute saturated ammonium sulphate with equal volume of RIA borate buffer.

d. ^{3}H-α-ecdysone (68 CI/mM) - borate buffer solution should have approximately 3600-4000 cpm per assay tube or 100 µl volume. Count 25 µl of the label solution in duplicate in 600 µl of Aquasol for each RIA. This gives an idea of binding at zero concentration for each assay.

e. Antisera solutions:

The diluted antisera solution should give binding at zero concentration of ecdysone in the range of 40–50% of total counts present. Therefore, the standard curve should have a range of 2000–3000 cpm. A final sera concentration of 3–10% is successfully used. By using higher total sera concentration there will be only very little variation with duplicates. Merthiolate is added to the antisera in a 1:10,000 dilution. After diluting the antisera, it may be needed to add additional merthiolate to bring the final concentration to 1:10,000 again. It may also be needed to make up a control sera solution of final total sera concentration equal to the antisera assay solutions. Then run two tubes with this control sera solution for background determination. The background cpm should run in the range of 50–100 cpm.

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6

ASSAYS FOR CRUSTACEAN HORMONES

A. Chromatophorotropic Hormones

These hormones are assayed by observing the responses of the pigment cells, the chromatophores, that they affect (Rao and Fingerman, 1970; Fingerman, 1985). The chromatophores are in turn staged by use of the system of Hogben and Slome (1931) with the aid of a dissecting microscope. Hogben and Slome divided chromatophore responses into five stages. Stage 1 represents maximal concentration (aggregation) of the pigment, stage 5 is the maximally dispersed condition, and stages 2, 3 and 4 represent the intermediate conditions. When the pigment is in stage 1, the pigment is maximally balled up in the center of the pigment cell. The pigment mass shows no sign of branches. In stage 2, the pigment mass has a rough edge, suggestive of developing branches. Stage 3 is characterized by the first appearance of distinct branches. Stage 4 shows many branches, but there is still a distinct central pigment spot, and stage 5 is when the pigment has spread into the branches so completely that there is no or virtually no central dot of pigment. The electron microscope has revealed that the branches of crustacean chromatophores are fixed in position and the pigment merely migrates into and out of the branches. Ideally, each extract or fraction to be tested should be injected into at least 5 specimens, but as few as 3 have been used in some experiments. One group of specimens serves as the control group, receiving saline alone. Crustaceans weighing 3 or more grams can tolerate an injected volume of 50 μl, smaller specimens usually receive 20 μl. With crabs the hormone is injected into the soft membrane at the base of a walking leg; in shrimps it is injected into the underside of the abdomen, on either side of the ventral nerve cord, taking care not to damage the nerve cord with the needle.

The chromatophores are staged at the time of injection and at least 15, 30, 60, 90 and 120 minutes thereafter. Often the readings are continued every 30 minutes until the response has terminated.

e. Antisera solutions:

The diluted antisera solution should give binding at zero concentration of ecdysone in the range of 40–50% of total counts present. Therefore, the standard curve should have a range of 2000–3000 cpm. A final sera concentration of 3–10% is successfully used. By using higher total sera concentration there will be only very little variation with duplicates. Merthiolate is added to the antisera in a 1:10,000 dilution. After diluting the antisera, it may be needed to add additional merthiolate to bring the final concentration to 1:10,000 again. It may also be needed to make up a control sera solution of final total sera concentration equal to the antisera assay solutions. Then run two tubes with this control sera solution for background determination. The background cpm should run in the range of 50–100 cpm.

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That occurs when the mean chromatophore stages of the experimental and control groups are the same, both likewise having identical means at the start of the experiment. Calculate the mean of each reading and draw a graph of the mean chromatophore stages versus time as in Fig. 14. The data can be converted to activity

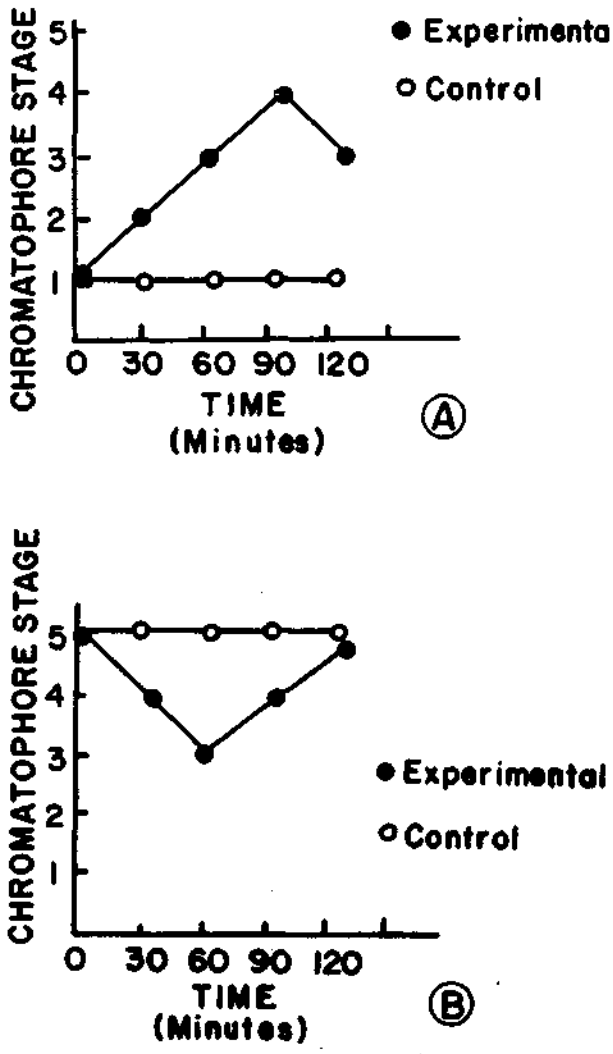

Fig. 14. Plots of chromatophore stage versus time: A. Pigment dispersing hormone and B. pigment concentrating hormone.

values in the manner that will be described below. If assaying fractions from a column, plot 'activity' versus 'fraction number' or 'effluent volume.'

a. Assay for a Pigment Dispersing Hormone

To assay for a pigment dispersing hormone inject into animals whose chromatophores (*e.g.* red chromatophores) have maximally concentrated pigment (*i.e.* stage 1) at the outset. This is accomplished by placing intact specimens in white containers for at least 2 hours and then selecting for use specimens having stage 1 chromatophores. A white background fosters pigment concentration, at least of the darker pigments. In some species, however, a white background fosters dispersion of the white pigment.

After all the data (chromatophore stages) have been collected, plot the mean chromatophore stages versus time for the experimentals and controls. These data also can be converted into a single activity value by subtracting the sum of the mean chromatophore stages of the controls for the entire experiment from the sum for the experimental group. When dealing with a pigment dispersing hormone, the sum of the chromatophore stages of the experimental specimens will become greater than that of the controls, so we substract the control sum from the experimental sum. These activity values give a number that represents an integration of the amplitude and duration of the response.

b. Assay for a Pigment Concentrating Hormone

This assay is similar to the assay for a pigment dispersing hormone, except that specimens initially having maximally dispersed pigment are used. Usually a black background favours pigment dispersion, especially of black and red pigments.

To calculate activity values for a pigment concentrating hormone, substract the sum of the mean chromatophore stages of the experimental group from that of the controls because with a pigment concentrating hormone the sum of the chromatophore stages of the experimental specimens become less than that of the controls.

B. Moult Inhibiting Hormone (MIH)

MIH is present in the crustacean sinus gland (Brown and Cunningham, 1939). Bilateral eyestalk ablation will cause the animal to precociously enter the moulting process (Rao *et al.*, 1973) The first stage of the process, called premoult on proecdysis, is evidenced visually by separation of the old cuticle from the underlying epidermis. This separation process is called apolysis.

At the onset of premoult there is a release of the moulting hormone, an ecdysteroid. In some species there is a gradual increase of this hormone in the blood until a peak is reached shortly before ecdysis, the actual shedding of the old exoskeleton. But in other species, there is a small peak of ecdysteriod in the blood at the start of premoult and a second much larger peak just prior to ecdysis (McCarthy and Skinner, 1977; Hopkins, 1983).

MIH can be assayed for in three ways. One is to inject samples suspected of containing MIH into eyestalkless specimens, controls receiving saline alone and then determine the time until ecdysis (Couch *et al.*, 1976). Of course, the specimens that received MIH should take a longer period for ecdysis to occur. A second method is actually a refinement of the first one. Here, use is made of the stages and substages for the moulting cycle described by Drach and Tehernigovtzeff (1967). They called premoult 'stage D.' This stage has several substages and some have even put forth subsubstages. These are based on the amount of epidermal retraction from the old cuticle and stages in the formation of new setae for the new cuticle that is being formed. By use of this staging system the experimental and control specimens can be followed daily or less frequently, such as every other day, and the substages or further divisions of the premoult stage for each specimen can be determined. The percentage of the experimental and control specimens in each phase as they approach ecdysis (stage E) can then be plotted. With the fiddler crab *Uca pugilator*, ecdysis will occur about 18 days after both eyestalks have been removed.

The third method makes use of the radioimmunoassay (RIA) for ecdysteroids described elsewhere in this manual. MIH is known to inhibit ecdysteroid synthesis and release from the Y-organ, the source of the moulting hormone (Mattson and Spaziani, 1985). Consequently MIH will reduce the increase in blood ecdysteroid level that normally occurs after both eyestalks have been ablated and this inhibitory effect can be detected by the RIA.

C. Gonad Inhibiting Hormone (GIH)

GIH is present in the sinus gland (Panouse, 1943, 1944). Its effect can be demonstrated by removal of both eyestalks and then observing the growth of the ovary. The control group will consist of intact specimens.

Select three groups of animals: Group I, the intact control; Group II, bilaterally eyestalk ablated receiving saline injection; Group III, bilaterally eyestalk ablated receiving injections of a

crude extract of eyestalks or eyestalk fractions eluted from a chromatographic column. This fractionation is accomplished as described herein in the section on gel chromatography. The bilaterally eyestalk ablated specimens should give sufficient time for initiation of precocious growth of these gonads. Twenty to 50 μl of the hormone fractions are injected into the animals every alternate day for 15 days, the control receiving an equal amount of physiological saline. After the experiment, the gonad indices (GI) of all animals are determined. The GI is calculated as follows:

$$\text{Gonad index for brachyurans} = \frac{\text{Wet weight of gonad x 100}}{\text{Carapace width}}$$

(Eastman-Reks and Fingerman, 1984)

$$\text{Gonad index for natantians and macrurans} = \frac{\text{Wet weight of gonad x 100}}{\text{Carapace length}}$$

(Quackenbush and Herrnkind, 1981) of

$$= \frac{\text{Wet weight of gonad x 100}}{\text{Body wet weight}}$$

(Klek-Kawinska and Bomirski, 1975)

GIH activity can be expressed as the degree (percent) of ovarian inhibition and can be calculated from the following formula provided by Bomirski *et al.* (1981).

$$\text{Degree of gonad inhibition in percent} = \frac{GI^A - GI^T}{GI^A - GI^B} X\ 100$$

where GI^B is the gonad index of intact control specimens, GI^T is the gonad index of test eyestalkless specimens and GI^A is the gonad index of control eyestalkless individuals.

D. Crustacean Hyperglycemic Hormone (CHH)

CHH produces an elevation in the blood glucose concentration (Abramowitz *et al.*, 1944). It is found in the sinus gland and is a polypeptide with a molecular weight of about 7,000 daltons (Keller *et al.*, 1985)

When injected, the maximum response to this hormone occurs about 2 hours later. The assay for this hormone described herein is based on that described by Kleinholz and Keller (1973). Crude extracts of eyestalks can be assayed or fractions obtained from a gel chromatography column. If such fractions are to be used those that correspond to protein peaks, as monitored by UV, should be

assayed for this hormone. Inject 20–50 μl from each fraction to be tested into test specimens that had not been fed during the previous 24 hours and were kept at room temperature. Larger test specimens can better tolerate the larger dose. Control specimens should receive saline. Two hours after the injection 50 μl of blood are withdrawn and mixed with glucose oxidase-chromagen. A kit for enzymatic colorimetric determination of glucose in whole blood, plasma or serum is available from Sigma Chemical Company, P.O. Box 14508. St. Louis, Missouri 63178. After 45 minutes of incubation at room temperature, using a colorimeter determine the absorbance (optical density) of the blank and the samples, at 425–475 nm of using the Sigma kit. A standard curve based on glucose solutions of known concentration can be prepared by plotting the concentration of the standards on the abscissa and the absorbance of each of the standards on the ordinate. A line drawn through the points should be linear and pass through the origin.

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* 2. Manual of research methods for fish and shellfish nutrition. CMFRI Special Publication No. 8, 1981, 125 pp.
3. Manual of research methods for marine invertebrate reproduction. CMFRI Special Publication No. 9, 1982, 214 pp.
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