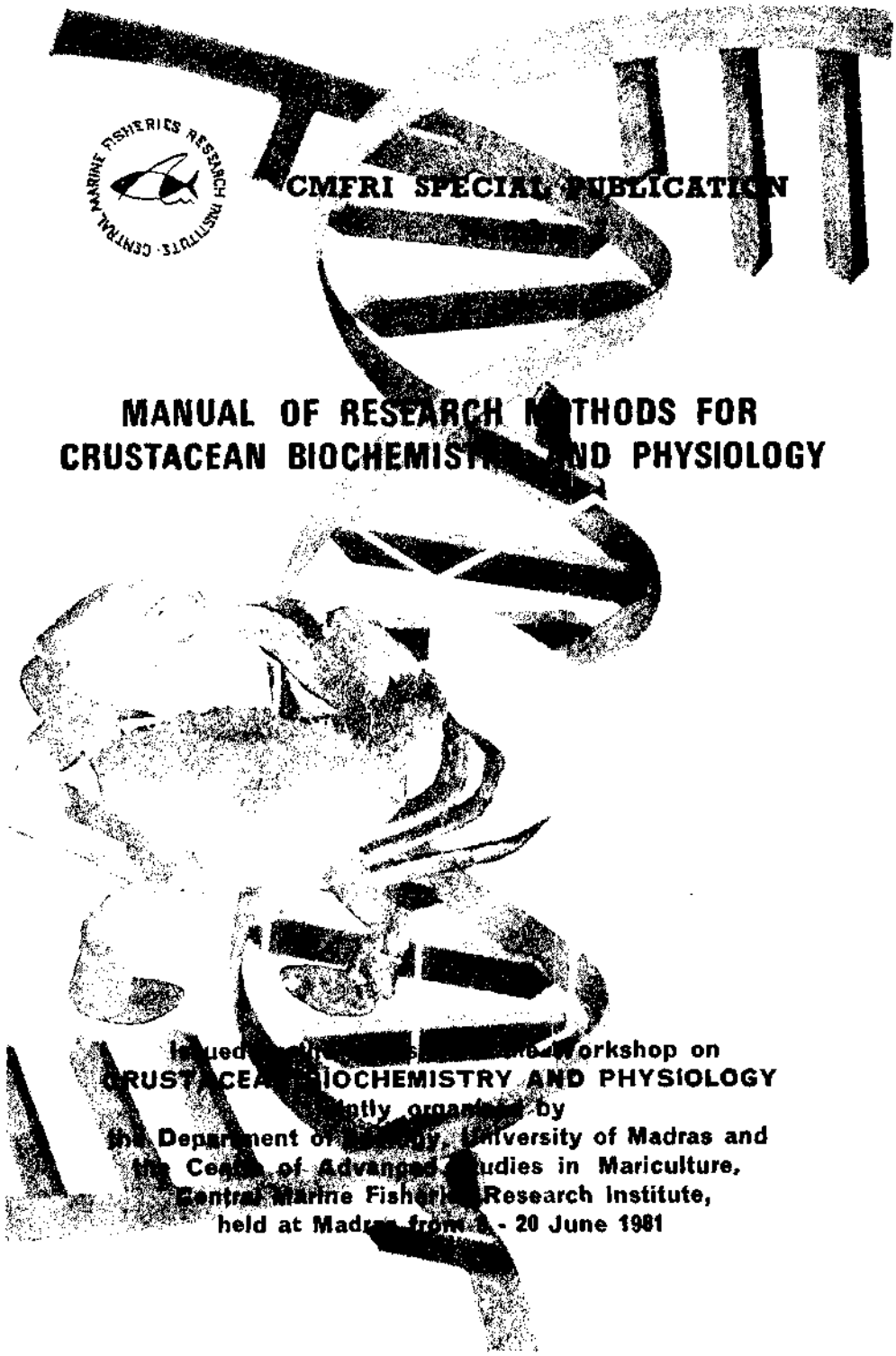
CMFRI SPECIAL PUBLICATION
Number 7

MANUAL OF RESEARCH METHODS FOR CRUSTACEAN BIOCHEMISTRY AND PHYSIOLOGY

Issued on the occasion of the Workshop on
CRUSTACEAN BIOCHEMISTRY AND PHYSIOLOGY
jointly organised by
the Department of Zoology, University of Madras and
the Centre of Advanced Studies in Mariculture,
Central Marine Fisheries Research Institute,
held at Madras from 8 - 20 June 1981



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Manual of Research Methods for Crustacean Biochemistry and Physiology

EDITED BY

M. H. RAVINDRANATH

School of Pathobiology, Department of Zoology, University of Madras, Madras 600 005

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ISSUED ON THE OCCASION OF THE WORKSHOP ON CRUSTACEAN BIOCHEMISTRY AND PHYSIOLOGY JOINTLY ORGANISED BY THE DEPARTMENT OF ZOOLOGY, UNIVERSITY OF MADRAS AND THE CENTRE OF ADVANCED STUDIES IN MARICULTURE, CENTRAL MARINE FISHERIES RESEARCH INSTITUTE HELD AT MADRAS FROM 8-30 JUNE, 1981

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PREFACE

One of the main objectives of Centre of Advanced Studies in Mariculture established at the Central Marine Fisheries Research Institute, is to enhance the competence of professional staff in the country to undertake high quality research to tackle both basic and applied problems in the field of mariculture. The Science of mariculture is new in this country, and the institution and persons engaged in the field are also few. It is only in the last decade that concerted R & D efforts to develop the sector are made.

An essential pre-requisite to establish mariculture on rational manner is to evolve suitable technologies which should be viable and adoptable to local conditions. To develop such technologies it is essential that intensive studies are undertaken on various aspects of biology, physiology and ecology of the organisms selected for culture. To facilitate such studies, particularly by the technicians and research workers entering afresh into the field, the need of practical guides describing the research techniques and methods, planning of investigations, collection of data and their interpretation need not be emphasized. It is in this context, the Centre of Advanced Studies in Mariculture proposes to issue a series of manuals on research methodologies for mariculture investigations.

The present manual of research methods for Crustacean Biochemistry and Physiology is compiled and prepared by Dr. M. H. Ravindranath, School of Pathobiology, Department of Zoology, University of Madras. Dr. Ravindranath and his associates are currently involved in intensive and extensive studies of different aspects of biochemistry and physiology of the mud crab, *Scylla serrata* in particular, and of decapods in general. The methods discussed in this manual have also been checked and verified on the candidate species by the authors to enable standardisation. I record with great appreciation

the treatment of the subject matter and concepts and the empirical approach of the methods presented in the manual by Dr. Ravindranath and his group. It is believed that this manual will be a useful one not only to the Research Scholars but also for those who desire to undertake *ad hoc* studies in the subject besides their field of specialisation.

I wish to thank Dr. K. Ramalingam, Professor & Head, Department of Zoology, University of Madras for his co-operation and valuable counsel. Thanks are also due to Shri P. T. Meenakshisundaram, Scientist, C.M.F.R.I., for the help rendered by him in preparing this manual.

E. G. SILAS,
Director, C.M.F.R.I.

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BIOCHEMICAL VARIABILITY *

A perusal of previous literature on the biochemistry of crustaceans would reveal a remarkable intra-specific variability in biochemical composition of tissues. A critical assessment of factors responsible for biochemical variability of tissues of individual species is necessary, in order to understand the physiological role of the biochemical components in question. Halberg (1973) has broadly classified the factors influencing individual biochemical variability as inter-individual and intra-individual. The inter-individual factors would include sex, size, moulting, nutritional and reproductive status in addition to pathological and environmental conditions. The intra-individual factors which may bring about biochemical variability include techniques and time of day.

Williams (1956) while studying biochemical individuality of organisms has pointed out that most of the data showing biochemical variability could be explained in terms of poor performance of methods used in collection of data. Therefore, he suggests that before interpreting any individual variation in the biochemical components, the result of repeated samples has to be analysed from the same individual.

There is always a need to estimate the reliability of the results of biochemical measurements. Two aspects of reliability are precision and accuracy (Strobel, 1965). Precision is a measure of degree of reproducibility of the biochemical measurement. This also depends on selecting a suitable method for biochemical analysis.

Often one may note that the crustacean investigators employ the quantitative methods chiefly borrowed from mammalian

* Prepared by M. H. Ravindranath, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

tissue analysis. It is now fairly evident from the existing literature that the chemical components of the crustacean tissues differ profoundly from those of mammalian tissues and that analytical methods developed for the latter are often inadequate to cope up with the complexities of crustacean tissues.

Accuracy is often defined as the measure of the closeness of a result to the 'true' or accepted value. In order to accurately assess the normal biochemical composition of an individual crustacean, one has to control suitably both inter and intra-individual variabilities. In the following chapters the suitability of a particular method for analysing the biochemical constituents of crustacean tissue is empirically assessed.

1.1 References

HALBERG, P. 1973. Laboratory techniques and rhythmometry. *In*, '*Biological aspects of circadian rhythms*' (Ed. Mills, J. M.) pp. 319. Plenum Press, New York.

STROBEL, H. A. 1965. Introduction. *In*, '*Instrumental methods of experimental biology*' (Ed. Newman, D.W.) pp. 1-12. The Macmillan Company, New York.

WILLIAMS, R. J. 1956. *Biochemical individuality* pp. 209. John Wiley & Sons Inc., New York.

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2 DRY WEIGHT, WATER AND ASH CONTENTS *

2.1 PRINCIPLE

The wet (fresh) tissue is allowed to dry by keeping it in a desiccator. The difference between the wet weight of the tissue and its dry weight gives the amount of water present in the fresh tissue (Passoneau & Williams, 1953; Mullainadhan, 1979). On heating the dry material to higher temperature (550-600°C) all the organic constituents are burnt leaving only the inorganic constituents in the form of ash.

2.2 PROCEDURE

1. Take a small amount of tissue on previously weighed plastic slip.
2. Find out the weight of the tissue (=wet weight).
3. Keep the tissue along with the plastic slip in a desiccator maintaining 0-5% R.H.
4. Dry the tissue in the desiccator till it reaches a constant weight.
5. Keep the dry material in a porecelain crucible and heat it at 550-600°C in an incinerator for 4 hours.
6. Find out the weight of the ash.

2.3. CALCULATIONS

2.3.1. Dry weight

$$\frac{\text{Dry weight}}{\text{Wet weight}} \times 100 = \quad /100 \text{ gm wet weight (gm \%)}$$

* Prepared and verified by P. Mullainadhan, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

2.3.2. Water content

$$\frac{\text{Wet weight-dry weight}}{\text{Wet weight}} \times 100 = \text{/100 gm wet weight (gm \%)}$$

2.3.3. Ash content

$$\frac{\text{Ash content}}{\text{Dry weight}} \times 100 = \text{/100 gm dry weight (gm \%)}$$

2.4 References

Mullainadhan, P. 1979. Haemolymph water, volume and tissue water in *Scylla serrata* Forskal (Crustacea : Decapoda). M. Phil. Dissertation, University of Madras. p. 67.

Passoneau, J. V. & C. M. Williams, 1953. The molting fluid of the Cecropia silkworm. *J. Exp. Biol.*, 30 : 545-560.

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3 BLOOD VOLUME *

3.1. PRINCIPLE

The principle underlying the methodology of blood volume determination involves the determination of degree of dilution of an injected dye by comparing the concentration of the injected dye and the concentration of the dye in the blood after a particular period of injection. The degree of dilution reflects blood volume (Lee, 1961).

3.2. REAGENTS

1. 0.9% *sodium chloride solution*: Dissolve 900 mg of sodium chloride in 100 ml of distilled water.
2. 0.1% *Congo red in* 0.9% *sodium chloride solution* (*stock solution*): Dissolve 100 mg of Congo red in 100 ml of 0.9% sodium chloride solution.
3. 0.001% *Congo red* (*working solution*): Take 1 ml of the stock solution and make it upto 100 ml with 0.9% sodium chloride solution.

3.3. PROCEDURE

3.3.1. Preparation of standard graph]:

1. Take 0.5,[1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5 and 5 ml of the working solution and make it upto 5 ml with 0.9% sodium chloride solution using a 5 ml standard flask. The solutions contain 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10 μg/ml respectively.

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* Prepared and verified by P. Mullainadhan & M. H. Ravindranath, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

2. Measure the optical density of these solutions at 480 nm in a spectrophotometer against the blank (5 ml of 0.9% sodium chloride solution).

3. Plot the O.D. in Y axis against the concentration in X axis and draw a slope.

3.3.2. Estimation of blood volume :

1. Withdraw 0.1 ml of blood by cutting the dactylus of the walking leg and make it up to 5 ml with 0.9% sodium chloride solution to serve as a blank.

2. Inject 0.1 ml of 0.1% Congo red through the arthrodial membrane at the base of the uropod.

3. Place the crab in a tank containing 50% sea water.

4. After 40-45 minutes, collect 0.1 ml of blood and make it upto 5 ml with 0.9% sodium chloride solution.

5. Find out the optical density of Congo red in the sample at 480 nm in spectrophotometer against the blank.

6. The optical density is referred to the standard graph to find out the concentration.

3.4. Calculation

The blood volume (V) can be calculated following the equation given by Lee (1961) :

$$V=(dg1/g2)-a$$

where $g1$ is the concentration of dye injected, $g2$ is the concentration of dye in the sample, d is the volume of the sample and a is the volume of saline injected with the dye.

3.5. Interpretation

One of the necessary prerequisites in this method is to empirically assess the time taken for the complete dilution of the injected dye. It should also be noted, as the dye is getting diluted there is a possibility of accumulation and excretion of the dye. Therefore, the dye concentration in the blood has

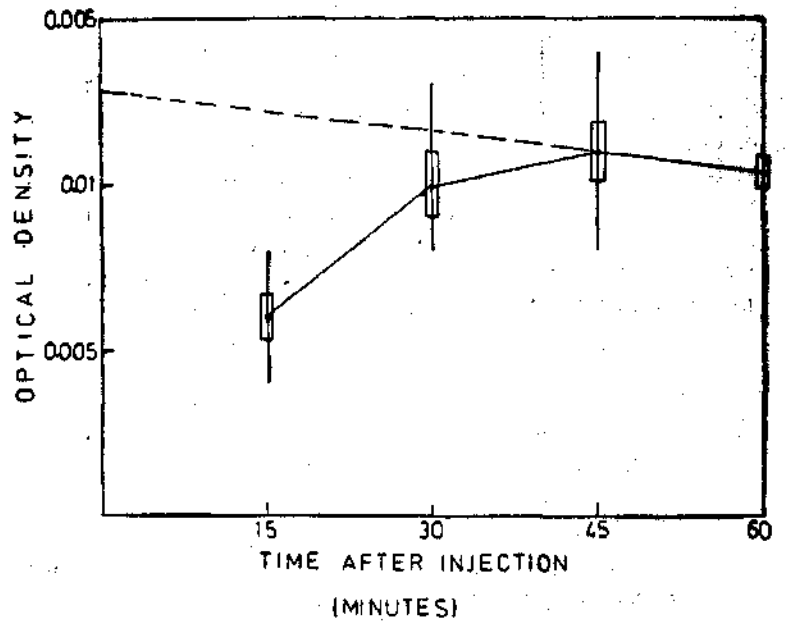

Fig. 1 : Concentration of the dye Congo red in the blood after different hours of injection in *Scylla serrata*. The extrapolation of the declining point is indicated by the dotted line.

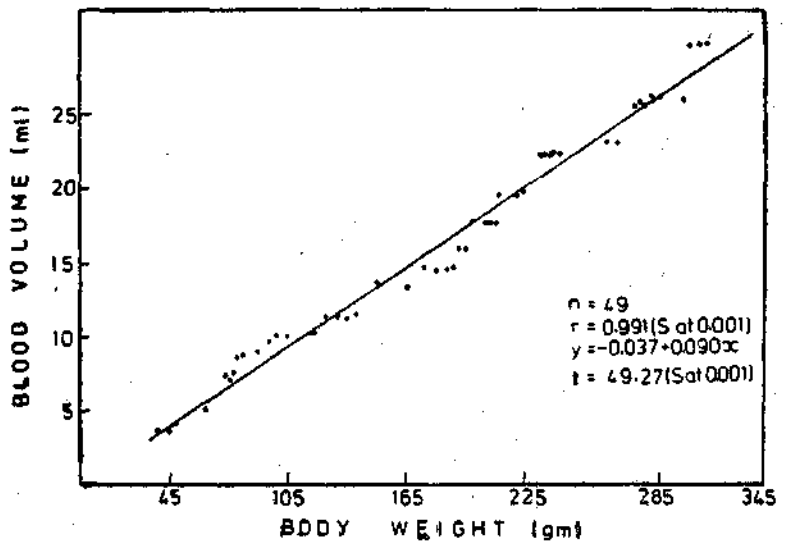

Fig. 2 : Variation in the blood volume in relation to body weight in *Scylla serrata*.

to be determined at different intervals after the injection of the dye (Mullainadhan, 1979). Figure 1 indicates the rate of dilution as well as the rate of elimination of the dye from the blood. Therefore, the accurate blood volume may be assessed by extrapolating the curve at the declining point. For crustaceans, the weight of the animal should be taken into note of (Fig. 2). During blood collection, sufficient care should be exercised to prevent loss of blood. Cut end of the dactylus may be sealed with molten wax.

3.6 References

Lee, R. M. 1961. The variation in the blood volume with age in the desert locust (*Schistocerca gregaria*). *J. Insect Physiol.*, **6** : 36-51.

Mullainadhan, P. 1979. Haemolymph water, volume and tissue water in *Scylla serrata* Forskal (Crustacea : Decapoda). M. Phil. Dissertation, University of Madras, p. 67.

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4 TOTAL FREE SUGARS, REDUCING SUGARS AND GLUCOSE *

4.1. Introduction

Carbohydrates in the tissues of crustaceans exist as free sugars and as bound with proteins (Saravanan, 1979). The free sugars in haemolymph consist of mono, di and oligosaccharides. All monosaccharides, maltose and its oligosaccharides constitute the total reducing sugars. Trehalose constitutes the non-reducing sugar fraction of the total free sugars. The total free sugars are estimated by Anthrone method and reducing sugar by Nelson-Somogyi method. The difference in the values obtained by these two methods indicates total non-reducing sugar value which is primarily trehalose in crustacean blood. The glucose can be determined by Glucose-oxidase method. The difference between values of glucose and reducing sugars would indicate the concentration of non-glucose reducing sugars.

4.2. Method for Total Free Sugars

4.2.1. Principle

Sulphuric acid in anthrone reagent hydrolyses di and oligosaccharides into monosaccharides and dehydrates all monosaccharides into furfural or furfural derivatives. These two compounds react with number of phenolic compounds and one such is anthrone which produces a complex coloured product. The intensity of which is proportional to the amount of saccharides present in the sample (Roe, 1955).

4.2.2. Reagents

1. *Anthrone reagent* : Dissolve 50 mg of anthrone and 1 gm of thiourea in 100 ml of 66% sulphuric acid (AR : 1.84 sp.gr.).

* Prepared and verified by T. S. Saravanan & M. H. Ravindranath, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

2. *Glucose standard*: Dissolve 100 mg of D-glucose, in 100 ml of saturated benzoic acid (1 ml of this solution is containing 1 mg of glucose).

3. *Deproteinizing agents*:

(*a*) 5% *Trichloro acetic acid (TCA)*: Dissolve 5 gm of TCA in 100 ml of distilled water.

(*b*) 80% *ethanol*: Dilute 80 ml of absolute ethanol to 100 ml with distilled water.

(*c*) *Tungstic acid*: Dissolve 50 gms of anhydrous sodium sulphate and 6 gms of sodium tungstate in 1 litre of distilled water. Dilute 33.3 ml of 1N H_2SO_4 to 100 ml with distilled water. Mix the former solution with latter in the ratio of 8 : 1 at the time of the experiment.

(*d*) *Zinc hydroxide*: Dissolve 9 gms of barium hydroxide in double distilled water and make upto 200 ml. Dissolve 10 gms of Zinc sulphate in distilled water and make up to 200 ml. Mix these two solutions in 1 : 1 ratio at the time of the experiment.

4.2.3. Procedure

1. Add 1.8 ml of deproteinizing agent to 0.2 ml of blood.
2. Centrifuge at 2500 rpm for 5 minutes and collect the supernatant.
3. Add 10 ml of the anthrone reagent to 1 ml of blood filterate, 1 ml of standard glucose (containing 1 mg of glucose) and 1 ml of water.
4. Heat the mixture in water bath for 10 to 15 minutes.
5. Cool in dark at room temperature for 30 minutes.
6. Determine the optical density at 620 nm.

4.2.4. Calculation

$$\frac{\text{O.D. of the unknown}}{\text{O.D. of the standard}} \times \text{conc. of the standard} \times \text{dilution factor (10)} \times 100 = \text{mg \%}$$

4.3. Method For Reducing Sugars

4.3.1. Principle

Reducing sugars convert soluble cupric hydroxide into insoluble curprous oxide which in turn reduces the molybdate. The lower oxidation products of blue colour is measured colorimetrically. The intensity of which is a measure of the amount of copper reduced to the cuprous condition and therefore of the sugars (Nelson, 1944 ; Somogyi, 1945).

4.3.2. Reagents

1. *Alkaline copper reagent* :

   Solution-A : Dissolve 50 gm of anhydrous Na_2CO_3, 50 gm of sodium potassium tartrate, 40 gm of $NaHCO_3$ and 400 gm of anhydrous Na_2SO_4 in about 1600 ml of distilled water and dilute to 2000 ml, mix and filter.

2. *Solution-B* : Dissolve 150 gm of $CuSO_4.5H_2O$ in 800 ml of distilled water and dilute to 1000 ml. Add 0.5 ml of Conc. H_2SO_4 and mix.

   Note : On the day it is to be used, mix 4 ml of solution B with 96 ml of solution A.

3. *Arseno molybdate colour reagent* : Dissolve 100 gm of ammonium molybdate in 1800 ml of distilled water. Add 84 ml of Conc. H_2SO_4 with stirring. Dissolve 12 gm of disodium orthoarsenate ($Na_2HASo_4.7H_2O$) in 100 ml of distilled water and add it with stirring to the acidified molybdate solution. Place the mixture in an incubator at 37°C for 1 to 2 days, then store it in a brown bottle.

4. *Glucose standard* : As mentioned in 4.2.2.

5. *Deproteinizing agents* : As mentioned in 4.2.2.

4.3.3. Procedure

1. To 0.2 ml of blood, add 1.8 ml of deproteinizing agent.
2. Centrifuge and collect the supernatant.
3. To 0.5 ml of blood supernatant, 0.5 ml of standard glucose solution and 0.5 ml of water add one ml of alkaline copper reagent to all the tubes.

4. Cover the top of the tubes with marbles and heat it in water bath for 20 minutes.
5. Cool by placing the tubes in water at room temperature for 1 minute. Add one ml of arsenomolybdate colour reagent to each tube.
6. Then dilute to 5 ml with distilled water.
7. Determine the optical density at 540 nm.

4.3.4. Calculation

$$\frac{\text{O.D. of the unknown}}{\text{O.D. of the standard}} \times \text{Concentration of the standard} \times \text{dilution factor (10)} \times 100 = \text{mg\%}.$$

4.4. METHOD FOR GLUCOSE

4.4.1. Principle

Glucose oxidase, oxidises the glucose to gluconic acid and hydrogen peroxide. The hydrogen peroxide in the presence of peroxidase, oxidises ortho-dianisidine or any other oxygen acceptor to give chromogenic oxidation products. The intensity of the coloured compound is proportional to the amount of glucose initially present (Hugget & Nixon, 1957).

4.4.2. Reagents

1. *Glucostat*: *A coupled glucose oxidase-peroxidase enzyme preparation* (Worthington Biochemical Corporation, Freehold, N. J.). Dissolve the reagents in 80 ml of water.
2. *Glucose standard* : As mentioned in 4.2.2.
3. *Deproteinizing agents* : As mentioned in 4.2.2.

4.4.3. Procedure

1. To 0.2 ml of blood, add 1.8 ml of deprotenizing agent, centrifuge and collect the supernatant.
2. To 1 ml of blood supernatant, 1 ml of standard glucose solution and 1 ml of distilled water add 2 ml of glucostat reagent to all the tubes.
3. After 10 minutes add 2 drops of 4 N HCl to each tube.
4. After the development of colour determine the absorbance at 450 nm.

4.4.4. Calculation

$$\frac{\text{O. D. of the unknown}}{\text{O. D. of the standard}} \times \text{Concentration of the standard} \times \text{dilution factor (10)} \times 100 = \text{mg\%}.$$

4.5. Interpretation

Saravanan (1979) has studied influence of deproteinizing agents namely TCA, zinc hydroxide, ethanol and tungstic acid on total free sugars, reducing sugars and glucose of haemolymph of *Scylla serrata*. TCA is found to be unsuitable as a deproteinizing agent for a comparative estimation of haemolymph total free sugars, reducing sugars and glucose because the reagents used in Nelson-Somogyi's method for reducing sugars and glucose oxidase method for glucose did not produce colour with TCA supernatants. Tungstic acid is also not suitable for comparative study of haemolymph sugars, because glucose oxidase method did not produce colour with tungstic acid supernatant. Both zinc hydroxide and ethanol are suitable for determining total free sugars, reducing sugars and glucose. The values obtained after deproteinization with zinc hydroxide are highly reproducible compared to ethanolic deproteinization. Therefore zinc hydroxide is recommended for Anthrone, Nelson-Somogyi's and Glucose-oxidase methods.

4.6 References

Hugget, G. St. A. & D. A. Nixon, 1957. Use of glucose peroxidase and O-dianisidine in determination of blood and urinary glucose. *Lancet*, **2** : 386.

Nelson, D. H. 1944. A photometric adaptation of the Somogyi's method for the determination of the glucose. *J. Biol. Chem.*, **153** : 373-380.

Roe, J. H. 1955. The determination of sugar in blood and spinal fluid with anthrone reagent. *Ibid.*, **212** : 335-343.

Saravanan, T. S. 1979. Studies on haemolymph carbohydrates in *Scylla serrata* Forskal (Crustacea : Decapoda). Ph. D. Thesis, University of Madras, p. 151.

Somogyi. M. 1945. Determination of blood sugar. *J. Biol. Chem.*, **160** : 69-73.

For your own notes

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5 POLYSACCHARIDES *

5.1. Introduction

Polysaccharides in crustacean tissues occur both in free state as well as bound to proteins. The free polyaccharides are invariably glycogen. The protein bound sugars may be determined after precipitating the protein (Saravanan, 1979).

5.2. Method For Glycogen

5.2.1. Principle

Sulphuric acid in the anthrone reagent hydrolyses the glycogen into glucose and then dehydrates it into furfurals. This compound reacts with anthrone to produce a complex coloured product, the intensity of which is proportional to the amount of glucose present in glycogen (Caroll *et al.*, 1956).

5.2.2. Reagents

1. *Anthrone reagent* : Dissolve 50 mg of Anthrone and 1gm of thiourea in 100 ml of 72% Conc. H_2SO_4.
2. *Glucose standard* : Dissolve 100 mg of glucose in 100 ml of water.
3. 5% *TCA* : As mentioned in 4.2.2.
4. *Absolute ethanol.*

5.2.3. Procedure

1. Add 0.1 ml of blood, to 1 ml of 5% TCA.
2. Centrifuge for few minutes at 2500 rpm and separate the supernatant.
3. Add 5 ml of absolute ethanol to 1 ml of the above supernatant.

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* Prepared and verified by T. S. Saravanan, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

4. Keep this set up undisturbed for overnight in refrigerator.
5. Then centrifuge for 15 minutes at 2500 rpm.
6. Decant the supernatant completely and then add 1 ml of water to dissolve the precipitated glycogen.
7. To 1 ml of the sample, 1 ml of standard solution and 1 ml of distilled water add 10 ml of anthrone reagent.
8. Keep them in boiling water for 10-15 minutes then cool at room temperature in dark.
9. Determine the optical density at 620 nm.

5.2.4. Calculation

$$\frac{\text{O. D. of sample}}{\text{O. D. of standard}} \times \text{Concentration of standard} \times \frac{100}{\text{Vol. of sample}}$$

= mg% of glycogen (in glucose equivalents)

5.3. Method for Protein Bound Sugars

5.3.1. Principle

Sulphuric acid hydrolyses polysaccharides bound to proteins into monosaccharides and dehydrates all monosaccharides into furfural or furfural derivatives. They combine with anthrone to form a coloured complex which is proportional to the amount of monosaccharides complexed with the proteins (Caroll *et al.*, 1956).

5.3.2. Reagents

1. *5% TCA* : As mentioned in 4.2.2.
2. *1N H_2SO_4* : Dilute 27.8 ml of Conc. H_2SO_4 to 100 ml with distilled water.
3. *Anthrone reagent* : As mentioned in 5.2.2.
4. *Glucose standard* : As mentioned in 5.2.2.

5.3.3. Procedure

1. Collect 0.1 ml of blood in 1 ml of 5% TCA and centrifuge at 2500 rpm for 5 minutes.

2. Decant the supernatant. Add 1.5 ml of 1N H_2SO_4 to the precipitate in the serum tubes.
3. Keep the tube closed with marble and place it in oven at 100°C for 12 to 14 hours for hydrolysis.
4. Add 10 ml of the anthrone reagent to the hydrolysate, 1 ml of standard glucose, containing 1 mg of glucose and 1 ml of water to be used as a blank respectively.
5. Heat the mixture in water bath for 10 to 15 minutes.
6. Cool in dark at room temperature for 30 minutes.
7. Read the optical density at 620 nm in a spectrophotometer.

5.3.4. Calculation

$$\frac{\text{O.D. of the unknown}}{\text{O.D. of the standard}} \times \text{Concentration of standard} \times \frac{100}{\text{Vol. of sample}} = \text{mg\% (in glucose equivalents)}$$

5.4. Interpretation

Observation made on *S. serrata* reveals that the glycogen occurs both in free state as well as bound with proteins (Saravanan, 1979). Electrophoretic analysis reveals that almost all protein fractions show diastase labile PAS positivity.

5.5. References

Caroll, W. V., R. W. Longley & J. H. Roe, 1956. The determination of glycogen in the liver and muscle by the use of anthrone reagent. *J. Biol. Chem.*, 220 : 583-593.

Saravanan, T. S. 1979. Studies on haemolymph carbohydrates of *Scylla serrata* Forsskal (Crustacea : Decapoda). Ph. D. Thesis, University of Madras. p. 251.

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6 PROTEINS *

6.1. MICRO-KJELDAHL METHOD

6.1.1. Principle

The nitrogen of the protein precipitate is converted to acid ammonium sulphate by digestion with sulphuric acid and various catalysts. On making the reaction mixture alkaline, ammonia is liberated, which is removed by steam distillation. The ammonia liberated by steam distillation is gathered in acid solution containing an indicator. Change in pH of the acid solution due to addition of ammonia is indicated by the indicator dye. This solution is back-titrated with 0.01 N HCI, and the original acidic condition indicated by the indicator dye, is taken as the end point. The amount of HCl consumed in back titration is proportional to the amount of ammonia liberated.

Percentage of nitrogen is calculated from the titre value, which is converted into gm% of protein present in sample by multiplying the percentage nitrogen with a factor 6.25. The factor 6.25 is used for conversion because in average, protein contains 16% of nitrogen.

6.1.2. Reagents

1. *Concentrated sulphuric acid.*
2. *Catalyst mixture* : 2 gm of selenium dioxide, 2 gm of copper sulphate and 8 gm of potassium sulphate-make fine powder.
3. *Sodium hydroxide-thiosulphate mixture* : 50 gm of sodium hydroxide and 5 gm of sodium thiosulphate in 100 ml of distilled water.

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* Prepared and verified by M. H. Subhashini & M. H. Ravindranath, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

4. 4% *Boric acid*: Dissolve 4 gm of boric acid in 100 ml of distilled water.
5. *Indicator*: 100 mg of Methyl red and 25 mg of Methylene blue in 100 ml of 95% ethanol.
6. 0.01 *N HCl*: Dilute 0.9 ml of HCl to 100 ml with distilled water. Determine normality by titrating against alkali.

6.1.3. Procedure

1. Collect 0.05 ml of blood using a fine calibrated micro pipette and pour into 1 ml of deproteinizing agent (80% ethanol). Centrifuge at 3000 rpm for 3-5 minutes and decant the supernatant.
2. Allow the protein sample (precipitate) to be digested with 0.5 ml hot Conc. H_2SO_4 and a pinch of catalyst mixture for 4 hours at 380°C.
3. The copper and selenium of the catalyst mixture accelarates the rate of digestion.
4. After completion of digestion, cool the digested mixture and add 5 ml of distilled water.
5. Transfer the material to steam distillation unit and add 5 ml of sodium hydroxide-thiosulphate mixture.
6. Collect the ammonia that is distilled from acid ammonium sulphate in 5 ml of 4% boric acid containing one drop of indicator dye.
7. Collect the ammonia till the colour of boric acid changes to green.
8. Back titrate the green coloured boric acid with 0.01 N HCl till it revives the original colour.

6.1.4. Calculation

Calculate the percentage of nitrogen present in the sample by,

$$\frac{\text{titre value} \times 0.14008 \times 100}{\text{Amount of sample}} = \% \text{ of nitrogen.}$$

Each ml of 0.01 N HCl = 0.14008 mg of nitrogen. In order to convert the obtained % of nitrogen into gm % of protein present in sample, multiply the percentage value of nitrogen with a factor 6.25 (The factor 6.25 is used for conversion because protein contains an average of 16% of nitrogen).

6.1.5. Interpretation

This method was used by earlier crustacean investigators (Travis, 1955) for determination of blood proteins. In this procedure the nitrogen value obtained by titration was multiplied by a factor of 6.25 to convert grams of nitrogen to grams of protein. The factor is used on an assumption that the average protein contains 16% of nitrogen. As has been pointed out by Young (1963), the absolute value of nitrogen in specific protein is uncertain and varies from one protein to another. Therefore the author considers that the use of the factor 6.25 to calculate the concentration of protein in an unknown mixture such as crustacean blood must be regarded in approximation with possible error. Bailey (1967) also states 'the most accurate value for the nitrogen content of protein are obtained from samples free of lipids and polysaccharides' (p.346). The proteins in the blood of crustaceans are known to be bound with lipids and polysaccharides. These moieties associated with protein would considerably affect the % of nitrogen content and therefore the factor may not be reliable.

6.2. Biuret Method

6.2.1. Principle

Two carbamyl groups present in protein molecules combine with copper and potassium of the biuret reagent to form a blue coloured copper—potassium—biuret compound. The colour formed is proportional to the amount of carbamyl groups present in the protein (Gornall *et al.*, 1949).

6.2.2. Reagents

1. *1N NaOH*: Dissolve 4 gm of NaOH pellets in 100 ml of distilled water.

2. *Biuret reagent* : Dissolve 1.5 gm of cupric sulphate ($CuSO_4.5H_2O$) and 6.0 gm of sodium potassium tartrate ($KNaC_4H_4O_6.4H_2O$) in 500 ml of distilled water. Add 300 ml of 10% sodium hydroxide solution and make upto 1000 ml with distilled water.

6.2.3. Procedure

6.2.3.1. *Preparation of standard graph*

Dissolve 25 mg of bovine serum albumin crystals in little amount of 1N NaOH in a 5 ml standard flask and make up to 5 ml with 1N NaOH. This serves as standard protein solution.

Take known volume of protein solution containing known concentration of protein, in separate test tubes (for e.g., 5 ml of stock solution contains 25 mg of protein ; 0.2 ml of stock solution contains 1 mg of protein ; 0.4 ml of stock solution contains 2 mg of protein ; 0.6 ml of stock solution contains 3 mg of protein ; 0.8 ml contains 5 mg of protein and so on). Make up these solutions to 2 ml individually with 1N NaOH. Afterwards add 8 ml of biuret reagent, mix well and allow it to stand at room temperature. Set up the blank having 2 ml of 1N NaOH and 8 ml of biuret reagent. After 30 minutes, measure the optical density at 540 nm in a spectrophotometer. Plot the concentration of protein in X-axis and optical density at Y-axis and draw slope.

6.2.3.2. *Estimation of protein*

1. Collect 0.05 ml of blood using a fine calibrated micropipette and pour into 1 ml of deproteinizing agent (80% ethanol).
2. Centrifuge at 3000 rpm for 5 minutes, decant the supernatant and add 2 ml of 1N NaOH to dissolve the precipitate.
3. After 10 minutes, add 8 ml of biuret reagent, mix well and allow it to stand at room temperature.
4. Set up blank simultaneously having 2 ml of 1 *N NaOH* and 8 ml of biuret reagent.
5. After 30 minutes, measure the optical density in a spectrophotometer at 540 nm against the blank.
6. Refer the optical density in a standard graph and find out the protein concentration.

6.3. FOLIN-CIOCALTEU METHOD

6.3.1. Principle

The principle of this method involves two steps. The carbamyl groups of protein molecules react with copper and potassium of the reagent to give a blue coloured copper potassium-biuret complex. This complex together with tyrosine and phenolic compounds present in the protein reduce the phospho molybdate of the Folin reagent to intensify the colour of the solution (Lowry *et al.*, 1951).

6.3.2. Reagents

1. *1N NaOH* : Dissolve 4 gm of NaOH in 100 ml of distilled water.
2. *0.1N NaOH* : Dissolve 0.4 gm of NaOH in 100 ml of distilled water.
3. *Reagent A* : Dissolve 2 gm of sodium carbonate (Na_2CO_3) in 100 ml of 0.1N NaOH.
4. *Reagent B* : Dissolve 500 mg of cupric sulphate ($CuSO_4$ $5.H_2O$) in 1% sodium or potassium tartrate. Prepare afresh.
5. Mix 50 ml of reagent (A) with 1 ml of reagent (B).
6. *1N Folin reagent* : Dilute Folin-phenol reagent with equal volume of double distilled water. (Determine the normality by titrating the diluted Folin-phenol reagent with 1N NaOH using phenolpthalin as indicator).

6.3.3. Procedure

6.3.3.1. *Preparation of standard graph*

Stock and standard solutions are prepared as mentioned in 5.2.3.

Make up the volume of different concentrations of standard solutions to 1 ml with 1N NaOH individually. Add 5 ml of reagent (5) and mix well. After 10 minutes add 0.5 ml of Folin - phenol reagent and mix rapidly. Set up blank simultaneously. After 30 minutes, measure the optical density at 500 nm in a spectrophotometer.

Plot the concentration of protein in X-axis and optical density in Y-axis and draw a slope.

6.3.3.2. *Estimation of protein*

1. Collect 0.05 ml of blood using a fine graduated micropipette and pour into 1 ml of deproteinizing agent (80% ethanol.)
2. Centrifuge at 3000 rpm for 5 minutes and decant the supernatant.
3. Dissolve the precipitate in 1 ml of 1N NaOH.
4. Add 5 ml of reagent (5) and mix well.
5. After 10 minutes add 0.5 ml of Folin-phenol reagent and mix rapidly.
6. Set up blank simultaneously.
7. After 30 minutes measure the optical density of the blue colour developed by the sample at 500 nm.
8. Calculate the protein concentration by referring the O.D. obtained for the sample using the standard graph.

6.4. Interpretation

Biuret method is considered to be less sensitive than the Folin-Ciocalteu method (Young, 1963 ; Bailey, 1967). Bailey (1967) on an examination of Folin-Ciocalteu reagent has shown that ' any peptide bond will yield some colour but certain amino acid sequences not necessarily containing aromatic residues are more chromogenic than others and largely account for the colour yield of protein ' (p. 31). The author has also shown that ' a preliminary complete hydrolysis reduces the colour yielding property of albumin by more than 2/3' (p. 241). Shao-Chia & Goldstein (1960-as cited by Bailey, 1967) have shown that the cleavage of disulphide bonds in insulin by oxidation with performic acid gave a loss of about 1/3 of colour. These observations reveal that in addition to copper-potassium complex and aromatic residues, there may be other chromogenic reagents in protein to bring about colour production.

For crustacean blood the relative performance of Microkjeldahl, Biuret and Folin-Ciocalteu methods were assessed using different deproteinizing agents. The results are presented in Table 1.

1. With reference to biuret and Kjeldahl methods all precipitants behave similarly and values did not differ significantly between precipitants.

2. In Folin-Ciocalteu method the values obtained with tungstic acid as precipitant are low.

3. Comparing the methods *per se*, values obtained with the Kjeldahl method with different precipitants are significantly lower than those obtained with other methods.

4. Values obtained with the biuret and Folin-Ciocalteu methods are similar. However, taking into consideration the least coefficient of variation, the biuret method shows a lower coefficient of variation than Folin-Ciocalteu method in 8 out of 12 analyses. The biuret method appears preferable to the Folin-Ciocalteu method with 80% ethanol as protein precipitant. (Subhashini & Ravindranath 1980.)

TABLE 1. *Total protein concentration of the haemolymph of crab* Scylla serrata *determined by different methods using different protein precipitants*

Animal	*Methods*	10% TCA	80% EtOH	*Zinc Hydroxide*	*Tungstic acid*	*Statistical Remarks*
	Biuret	7.52±0.75 9.93% (5)	7.66±0.54 6.93% (5)	7.64±0.52 6.81% (5)	7.87±1.02 12.96% (5)	NS at $P>0.05$
I	Folin-Ciocalteu	7.23±1.08 14.90% (4)	7.48±1.33 17.70% (4)	7.78±0.99 12.70% (4)	5.88±0.73 12.40% (5)	NS at $P>0.05$
	Kjeldahl	5.33±0.63 11.82% (2)	5.02±0.60 11.95% (5)	4.78±1.02 21.34% (4)	3.58±0.76 21.20% (5)	NS at $P>0.05$

TABLE 1. (*Contd.*)

Animal	*Methods*	10%TCA	80%EtOH	*Zinc Hydroxide*	*Tungstic acid*	*Statistical Remarks*
	Biuret	8.96±0.66 7.37% (5)	8.96±0 86 9.60% (5)	9.29±0.69 7.43% (5)	8.75±1.19 13.60% (4)	NS at $P>0.05$
II	Folin- Ciocalteu	8.68±0.86 9.91% (5)	9.87±1.27 12.87% (4)	9.25±0.77 8.32% (5)	8.34±0.71 8.51% (4)	NS at $P>0.05$
	Kjeldahl	9.63 (1)	7.77±0.65 8.37% (5)	6.83±0.52 7.61% (2)	8.55±0.83 9.71% (4)	NS at $P>0.05$
	Biuret	5.82±0.27 4.64% (5)	7.25±0.62 8.54% (4)	6.76±0.27 3.92% (5)	5.80±0.53 9.12% (5)	NS at $P>0.01$ S at $P<0.05$
III	Folin- Ciocalteu	7.14±0.63 8.84% (5)	7.53±0.42 5.58% (5)	8.16±0.11 1.35% (5)	5.96±0.71 11.82% (5)	S at $P<0.05$ S at $P<0.01$
	Kjeldahl	5.25±0.28 5.35% (5)	5.31±0.66 12.43% (3)	5.21±0.46 8.76% (5)	4.73±1.21 25.62% (3)	NS at $P>0.05$

Values presented include : Mean±SD.
Coefficient of variation % (Sample size)
(Statistical remarks are based on analysis of variance between precipitants.)

TABLE 2. *Criteria used in the assessment of three different methods of determination of protein concentration in the blood of* Scylla serrata.

	Criteria	*Bluret*	*Folin-Ciocalteu*	*Kjeldahl*
I	Reproducibility (or) Consistency in performance (based on less co-efficient of variation)	66%*	33%*	.. (mean not comparable)
II	Simplicity			
	—No. of reagents added	one	two	more
	—No. of standard graphs needed	one (at 540 nm)	two (at 500 nm & 750 nm)	..
	—Time taken after centrifugation	35-40 mins.	45-50 mins.	3-4 hours
III	Stability (of protein - reagent complex)	Stable for more than 6 hours at 30°C.	not stable after 30 minutes at 30°C	
IV	Sensitivity (based on determination of concentration of protein with the method)	Only above 20 lamda	even to 0.2 lamda	above 1,000 lamda
V	Susceptibility (to interfering substances)	Less (due to Cu binding alone)	More (due to Cu binding and reduction of phosphomolybdate)	Less (due to variability in conversion factor due to protein, lipids & polysaccharides)
VI	Suitability (of serum albumin as standard)	suitable	Not suitable (due to other chromogenic substances in the blood proteins)	Not suitable (for reasons mentioned in V)

* Based on 12 sets of analyses (Subhashini, 1977).

An assessment was made regarding the suitability of these 3 methods in determination of blood proteins of Crustacea. The criteria used are presented in Table 2. The protein values obtained by Kjeldahl method is considerably lower than the values obtained with Folin-Ciocalteu and Biuret methods. Possibly due to the presence of lipids and polysaccharides associated with blood proteins, the percentage of nitrogen values are affected and as a result the conversion factor is higher than what has been conventionally employed. Probably that is why in Kjeldahl method the protein values are lower than the values obtained with the other two methods.

The protein values obtained with Folin-Ciocalteu method parallels with the values obtained with Biuret method. Folin-Ciocalteu method is more susceptible to interfering substances than Biuret method, possibly due to the mechanism of colour reaction. The first step in the principle of this method is very similar to the principle of Biuret method in that the copper binds with carbamyl groups of protein and gives colour. This coloured product is directly estimated in biuret method. In Folin-Ciocalteu method, the coloured copper-protein complex is made to reduce the phosphomolybdate of Folin-phenol reagent. In this method it is not only the copper-protein complex that reduce the phosphomolybdate but also the tyrosyl residues present in the protein, the amount of which differs from one protein to another. As a result the colour reaction in Folin-Ciocalteu method differs from one protein to another (Young, 1963). Bailey's (1967) observations reveal that several other reactive sites present in the proteins are also capable of yielding some colour.

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7 FREE AMINO ACIDS *

7.1. PRINCIPLE

Ninhydrin deaminates amino acids liberating ammonia and gets reduced to hydrindantin. The liberated ammonia condenses with hydrindantin to form a violet coloured compound diketohydrindylidenediketohydrindamine (DYDA) at pH 5.0. Potassium cyanide prevents the oxidation of the reduced hydrindantin. The intensity of violet colour is directly proportional to the amount of amino acid (Yemm & Cocking, 1955).

7.2. REAGENTS

1. *Citrate Buffer pH* 5.0 (0.2 *M*) : Dissolve 21.008 gm of citric acid $C_6H_8O_7.H_2O$ in 200 ml of distilled water, add 200 ml of N sodium hydroxide and dilute to 500 ml ; store in the cold with little thymol.
2. *Potassium cyanide* (0.01 *M*) : Dissolve 0.1628 gm of potassium cyanide in distilled water and dilute to 250 ml. This solution is stable for at least 3 months at room temperature.
3. 60% *ethanol* : Dilute 60 ml of absolute ethanol to 100 ml with distilled water.
4. *Amino acid standard solutions* :

   Standard A : *Glycine standard* :

   Dissolve 0.268 gm of pure dry glycine in 5 ml of distilled water. Add 35 ml of N hydrochloric acid and 1 gm of sodium benzoate, dilute to 500 ml with distilled water.

* Prepared and verified by M. H. Subhashini, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

Standard B : Glutamic acid standard :

Dissolve 0.525 gm of pure dry glutamic acid in 5 ml of water. Add 35 ml of N hydrochloric acid and 1 gm of sodium benzoate and dilute to 500 ml with distilled water.

Standard C :

Mix 3 ml of standard *A* and 3 ml of standard *B* and dilute to 100 ml with distilled water. 1 ml of this solution contains 0.006 mg of amino acid nitrogen. It is stable for 1 week if kept in the cold.

5. *Solution A* : Dilute 5 ml of 0.01 M potassium cyanide to 250 ml with methyl cellosolve. This solution is stable for at least 1 month at room temperature.
6. *Solution B* : Dilute 500 mg of ninhydrin in 10 ml of methyl cellosolve. This solution is stable for at least 6 months at room temperature.
7. *Solution C* : Mix 50 ml of solution *B* with 250 ml of solution *A*. The resulting solution is first red, but soon becomes yellow. It is stable for at least one week when kept in a stoppered flask at room temperature.
8. *Deproteinizing agent* :

   80% *Ethanol* : As mentioned in 4.2.2.

7.3. PROCEDURE

1. To 0.05 ml of blood add 2 ml of deproteinizing agent.
2. Centrifuge at 5000 rpm for 5 minutes and collect the supernatant (sample).
3. Add 0.5 ml of citrate buffer pH 5.0 (0.2 M) to 1 ml of supernatant (sample), 1 ml of standard amino acid solution (standard) and 1 ml of distilled water (blank).
4. Add to each solution 1.2 ml of solution C.
5. Heat the solutions for 15 minutes at 100°C.
6. Cool in running tap water for 5 minutes.
7. Add 2.3 ml of 60% ethanol to each tube.
8. Determine the optical density at 570 nm in a spectrophotometer.

7.4. Calculation

$$\text{Amount of amino acid nitrogen present in the sample} = \frac{\text{O.D of sample}}{\text{O.D. of standard}} \times \frac{0.006}{0.025} \times 100 = \text{mg\%}$$

Where 0.006 refers to the amount of nitrogen present in the standard, 0.025 refers to the amount of blood present in one ml of sample (Oser, 1971). (0.05 ml of blood was diluted to make 2 ml with deproteinizing agent and 1 ml of this is taken for analysis.)

7.5 References

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AMMONIA * 8

8.1. PRINCIPLE

Sodium hypochlorite ($NaOCl^-$) combines with ammonia present in the sample to produce NH_2Cl and OH^- ions. Sodium nitroprusside catalyses the reaction. The NH_2Cl in the presence of 3 $0H^-$ combines with phenol and forms a quinonoid complex. This quinonoid complex combines with another phenol molecule to form indophenol, a coloured compound. The colour thus formed is directly proportional to the ammonia present in the sample (Boltz & Howel, 1978).

8.2. REAGENTS

1. *Reagent A*: Dissolve 10 gm of phenol with 50 mg of sodium nitroprusside in 500 ml of water (This solution is stable for one month if kept in stoppered amber bottle in refrigerator).
2. *Reagent B* : Dissolve 5 gm of sodium hydroxide in 10 ml of sodium hypochlorite and dilute to 500 ml of water.
3. *Ammonia standard* :
   (*a*) *Stock solution* : Dissolve 0.3819 gm of anhydrous ammonium chloride in 1 litre of water.
   (*b*) *Working solution* : Dilute 1 ml of stock solution to 1000 ml with water (1 ml=0.122 μg of NH_2=0.1 μg of N_2).
4. *Deproteinizing agent* :
   80% *ethanol* : As mentioned in 4.2.2.

8.3. PROCEDURE

1. Add 0.2 ml of blood in 2 ml of deproteinizing agent.

* Prepared and verified by M. H. Subhashini, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

2. Centrifuge it at 5000 rpm for 5 minutes and collect the supernatant.
3. Add 2.5 ml each of reagent A to 1 ml of the supernatant (sample), 1 ml of 80% ethanol (Blank) and 1 ml of standard ammonia solution (standard).
4. After five minutes, to each tube add 2.5 ml of reagent B.
5. After five minutes incubate all tubes at 37°C for 20 minutes.
6. Read the optical density after 30 minutes at 625 nm.

8.4. Calculation

$$\text{Amount of } NH_3 \text{ present in the sample} = \frac{\text{O.D. of the sample}}{\text{O.D. of the standard}} \times \frac{0.00122}{0.1} \times 100 \text{ mg\%}$$

where 0.00122 refers to the amount of NH_3 present in mg in the standard ; 0.1 refers to the amount of blood (in ml) present in the sample.

8.5 Reference

Boltz D. F. & J. A. Howel, 1978. *Colorimetric determination on non-metals* Vol. 8 Second edn., pp. 210-213. Wiley-Interscience Publication, New York.

PHENOL OXIDASE * 9

9.1. Introduction

Phenol oxidase is an enzyme which is responsible for hydroxylation of phenols and dehydrogenation of O. diphenols into quinone. It is known to be distributed in the blood as well as in the cuticle of crustaceans (Summers, 1967). The enzyme can be assayed either spectrophotometrically, by measuring the dopa chrome formed (Preston & Taylor, 1970) or monometrically by measuring the oxygen consumed during the oxidation of the substrates (Hackman & Goldberg, 1967). The spectrophotometric method of assaying the enzyme of blood from *Scylla serrata* is presented here.

9.2. Phenol Oxidase Activity

9.2.1. Principle

Phenol oxidase oxidises the Dopa (diphenol) into brown coloured dopachromes. The formation of dopachromes can be determined by reading at 420 nm in a spectrophotometer (Preston & Taylor, 1970).

9.2.2. Reagents

1. 0.01 M Dopa in 0.05 M Tris—HCl buffer at pH 7.5.
2. Sodium dodecyl sulphate.

9.2.3. Procedure

9.2.3.1. *Enzyme preparations*

The haemolymph was collected in a cold tissue homogenizer. The clotted haemolymph was homogenized and centrifuged at 4000 rpm to remove the particulate material for 10 minutes using

* Prepared and verified by K. Nellaiappan, Department of Zoology, University of Madras, Madras-600 005.

a refrigerated centrifuge. The supernatant was used as enzyme source. To activate the enzyme, few crystals of sodium dodecyl sulphate was added in the enzyme to the concentration of mg/ml.

9.2.3.2. *Assay*

1. With 2 ml of substrate, add 0.2 ml of enzyme source. The increase in O.D. of the mixture should be noted immediately at 420 nm in a spectrophotometer.
2. Note the O.D. of the same upto 3 minutes for every 30 seconds interval.
3. Prepare the control with 0.2 ml of distilled water in 2 ml of substrate.
4. Determine the protein content of the sample by Biuret method (as mentioned in 6.2).

The results can be expressed as O.D./mg protein/minute.

9.3. Interpretation

It is known that the blood phenol oxidase of most of the arthropods is existing in the proenzyme state. The enzyme can be activated by artificial activators like detergents such as sodium dodecyl sulphate, sodium oleate, etc.

Though the phenol oxidase oxidizes a number of phenols, certain phenols are oxidized more effectively. Different substrates such as tyrosine, tyramine, phenol, cresol, dopa, dopamine protocatechuic acid, catechol, methyl catechol, Hydroquinone, pyrogallol, N-acetyl dopamine adrenaline, nor adrenaline, N-acetyl nor adrenaline can be used and based on the activity of the enzyme, a possible metabolic pathway can be suggested. In insect, it is reported that N-acetyl dopamine is the immediate precursor for tanning. However, in *Uca pugilator*, it is reported that N-acetyl nor adrenaline is the immediate precursor for tanning (Vacca & Fingerman, 1975). Substrate specificity and metabolic pathway of sclerotization of most of the other crustaceans as well as characterization of the enzymes asscoiated with sclerotization other than phenol oxidase in Crustacea have received little attention. Moreover it is known that in addition to tanning, the blood phenol oxidase is involved in defense mechanism (Brunet, 1980).

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PHOSPHATASES * 10

10.1. INTRODUCTION

The phosphatases are the group of enzymes of low substrate specificity and are characterised by the ability to hydrolyse a large variety of organic phosphate esters with the formation of an alcohol and phosphate ions. This group is composed of those enzymes which attack only monoesters of orthophosphoric acid. The alcohol esterified to the orthophosphoric acid, $(HO)_2 P{=}O$, may be a simple aliphatic alcohol, a polyhydric alcohol such as sugar or any one of a variety of aromatic hydroxyl compounds such as tyrosine. The phosphatases are not one enzyme but a group of related enzymes. In crustaceans in general, two types of enzymes are recognised : alkaline phosphatase and acid phosphatase. In *Scylla serrata*, the optimal activity of acid phosphatase is at pH 5.0 and that of alkaline phosphatase at pH 9.0 (Mercy, 1979). The probable function of the phosphatases is the transfer of the phosphate group from a donor substrate to an acceptor compound containing an (OH group). If the acceptor is water, the net effect is hydrolysis.

10.2. PRINCIPLE

The phosphatases activity is determined following the procedure of Barret (1972). The enzyme is allowed to hydrolyse an organic phosphate ester. The liberated phosphate combines with ammonium molybdate. The compound thus formed combines with elon giving a blue colour which is read at 650 nm in a spectrophotometer.

* Prepared and verified by Sr. P. D. Mercy & M. H. Ravindranath, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

10.3. Reagents

1. *Substrate B-glycerophosphate* (BGP) and *p-nitrophenyl-phosphate* (PNP) : 0.5% solution is prepared with citrate buffer at pH 5.0.
2. *Ammonium molybdate* : 2.5% of ammonium molybdate is prepared by dissolving 2.5 gm of salt in 100 ml of N H_2SO_4.
3. *Elon* : 0.5 gm of elon (P-methyl amino phenol sulphate) is dissolved in 10% sodium metabisulphate solution to make 100 ml.
4. *Standard phosphate solution* : A 30 mM stock solution of KH_2 PO_4 in 5N H_2SO_4 is used as a 1 : 100 dilution containing 0.30 μM/ml.
5. *Enzyme sample* : 2.5 ml of blood is diluted 10 times with double distilled water and stored immediately in a deep freezer at -7°c.

10.4. Procedure

10.4.1. Acid phosphatase

1. Add 2.0 ml of buffered β-glycerophosphate (BGP) or p-nitrophenylphosphate (PNP) to 1 ml of enzyme sample at 37°C.
2. After 30 minutes, stop the reaction by adding 3 ml of ice-cold 10% trichloroacetic acid which results in the formation of a precipitate.
3. After 15 minutes in the cold, filter the mixture.
4. Add 0.8 ml of ammonium molybdate and 0.3 ml of elon to 2.0 ml of the filtrate, 2 ml of distilled water and 2 ml of standard solution of potassium phosphate separately to serve as sample, blank and standard respectively.
5. Maintain a suitable control by adding 10% TCA to the substrate, before the addition of enzyme source.
6. After 15 minutes, measure the colour intensity at 660 nm in a spectrophotometer against the blank.

10.4.2. Alkaline phosphatase

For alkaline phosphatase, the substrates are dissolved in boric acid-borax buffer at pH 9.0. The buffer is prepared by mixing 0.2 M boric acid and 0.5 M borax solution. The procedure for the enzyme assay is same as that of acid phosphatase.

Determine the protein concentration in the sample by the biuret method as given in 6.2.

10.5. Calculation

1. $\frac{\text{O.D. of sample-O.D. of the control}}{\text{O.D. of standard phosphate}} \times \text{concentration of standard (in mg)} = \text{mg phosphate liberated.}$

2. $\frac{\text{mg phosphate liberated}}{\text{mg protein in sample} \times 15} = \text{mg phosphate libertaed/mg protein/minute.}$

10.6 References

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COPPER * 11

11.1. Introduction

Copper is an essential element found in the blood and other tissues of Crustacea. It forms a part of cuproprotein required for oxygen transport and electron transport systems. It also forms as prosthetic group in many enzymes such as phenol oxidase and acts as activator for enzymes such as malate dehydrogenase. It is toxic in free state (Holden, 1970) and dialysable copper is absent in the blood of crustaceans (Arumugam & Ravindranath, 1980). The copper exists in blood both in the cuprous and cupric state and is linked with protein through sulphydryl groups (Klotz & Klotz, 1955). Several methods are in vogue for determination of copper. Some of them are suitable for vertebrate tissue where it is 100 times lower than the crustacean blood or other tissues. Here the suitability and consistency of 3 spectrophotometric methods were analysed for determination of crustacean blood or other tissue copper concentration.

11.2. Sodium Diethyl Dithio Carbamate Method

11.2.1. Principle

This method involves liberation of copper from protein by hydrochloric acid (6N). The protein is precipitated by 20% TCA and again washed in 5% TCA. The other interfering metals like iron, zinc, bismuth, nickel and cobalt are precipitated by 6% sodium pyrophosphate and concentrated ammonia. The freed copper binds with sodium diethyldithio carbamate and forms a yellow coloured complex called copper-diethyldithio carbamate which is extracted by amyl alcohol-ether mixture. The intensity of the colour developed is proportional to the amount of copper present in the sample (Ventura & King, 1951).

* Prepared and verified by M. Arumugam & M. H. Ravindranath, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

11.2.2. Reagents

1. 0.1 *N HCl* : Prepare by diluting 0.9 ml of concentrated HCl to 100 ml with deionized distilled water.
2. 6 *N HCl* : Prepare by diluting 54 ml of concentrated HCl to 100 ml with deionized distilled water.
3. 20% *TCA* : Prepare by dissolving 20 gm of TCA crystals into 100 ml with deionized distilled water.
4. 5% *TCA* : Prepare by dissolving 5 gms of TCA in 100 ml of deionized distilled water.
5. 6% *sodium pyrophosphate* : Prepare by dissolving 6 gm of sodium pyrophosphate in 100 ml of deionized distilled water.
6. *Concentrated ammonia solution* (*sp. gr.* 0.91).
7. 0.4% *sodium diethyldithio carbamate* : Prepare by dissolving 400 mg of sodium diethyl dithio carbamate in 100 ml of deionized distilled water.
8. *Amyl alcohol - Ether mixture* (1 : 1)
9. *Standard copper solution* : Dissolve 0.398 gm of copper sulphate pentahydrate in one litre of deionized distilled water with 0.1 ml of concentrated sulphuric acid which contains 100 μg of copper in 1 ml (Robertson & Webb, 1939).

11.2.3. Procedure

1. To 0.2 ml of blood (sample), 0.2 ml of standard and 0.2 ml of deionized water (blank), add 1.8 ml of deionized distilled water.
2. To this, add 1 ml of 0.1 N HCl and mix it well. Then heat the mixture over boiling water bath for 10 minutes.
3. To each tube, add 1.5 ml of 6 N HCl and allow it to stand.
4. After 10 minutes, add 1 ml of 20% TCA and mix it well. Then centrifuge the content at 2500 rpm for 5 minutes.
5. Transfer the supernatant to another tube and wash the precipitate in 1 ml of 5% TCA and recentrifuge for 2 minutes at 2500 rpm. Then mix the supernatants together.

6. To the supernatant add 1 ml of 6% sodium pyrophosphate, 2 ml of concentrated ammonia and 1 ml of 0.4% sodium diethyldithio carbamate.
7. Then add 5 ml of amyl alcohol-solvent ether mixtures and shake it well.
8. Allow the extract to stand at room temperature for 15 minutes.
9. Transfer the extract solution to the cuvette of the spectrophotometer and read the absorbance of standard and sample against the reagent blank at 440 nm.

11.2.4. Calculation

$$\frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times \text{concentration of standard} \times \text{conversion factor.}$$

$$= \frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times 20\ \mu g \times 5 = \mu g \text{ copper/ml}$$

11.3. 2,2' Biquinoline Method

11.3.1. Principle

In this method copper is freed from proteins by hydroxylamine hydrochloride which also reduces all the cupric ions to cuprous state. 2,2', biquionoline is a specific reagent for cuprous ions which forms a complex with cuprous ions and gives purple colour which is read at 540 nm. The intensity of the colour developed is proportional to the amount of copper present in the sample (Guest, 1953).

11.3.2. Reagents

1. *Hydroxylamine hydrochloride crystals.*
2. 0.02% 2,2' *Biquinoline in amyl alcohol*: Prepare by dissolving 20 mg of biquionoline in 100 ml of amyl alcohol.
3. *Standard copper solution* : as mentioned in 11.2.2.

11.3.3. Procedure

1. Add 0.9 ml of deionized water to 0.1 ml of blood, 0.1 ml of standard and 0.1 ml of deionized water individually.

2. To each add few crystals of hydroxylamine hydrochloride and mix it well ; allow it to stand for 10 minutes.
3. Add 10 ml of 0.02% biquinoline in amyl alcohol to each tube, shake well and centrifuge for 5 minutes at 5000 rpm.
4. Transfer the supernatants to the cuvette of the spectrophotometer and read the colour intensity of standard and sample at 540 nm against the reagent blank.

11.3.4. Calculation

$$= \frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times \text{Concentration of standard} \times \text{conversion factor}$$

$$= \frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times 10\ \mu g \times 10$$

= μg copper/ml.

11.4. Oxalyldihydrazide Method

11.4.1. Principle

In this method copper is freed from proteins by 6 N Hydrochloric acid (according to the original procedure 2NHCl). The protein is precipitated by 20% Trichloroacetic acid. The other interfering metals like iron, nickel, zinc, bismuth and cobalt are precipitated by citric acid crystals and ammonia. The freed copper binds with oxalyl dihydrazide in the presence of acetaldehyde and gives lavender colour complex which is read at 542.5 nm. The intensity of the colour developed is proportional to the amount of copper present in the sample (Rice, 1960).

11.4.2. Reagents

1. 20% *TCA* : as mentioned in 11.2.2.
2. 6 *N HCl* : as mentioned in 11.2.2.
3. 0.1% *oxalyldihydrazide in 6 N HCl* : Dissolve 100 mg of oxalyldihydrazide in 100 ml of 6 N HCl.
4. *Ammonia solution undiluted.* (Sp. gr. 9.91).

5. *50% aqueous acetaldehyde solution* : Prepared by diluting 50 ml of cold conc. acetaldehyde to 100 ml with deionized water (should be stored in the refrigerator).
6. *Ethylene diamine tetracetic acid (EDTA-disodium) crystals.*
7. *Citric acid crystals.*
8. *Standard copper solution* : as mentioned in 11.2.2.

11.4.3. Procedure

1. Add 0.8 ml of deionized distilled water to 0.2 ml of blood, 0.2 ml of standard and two 0.2 ml of deionized distilled water (as blanks) individually.
2. Add 0.7 ml of 0.10% oxalyldihydrazide in 6 N hydrochloric acid to each tube, stir well and allow to stand for 30 minutes.
3. Then add 1 ml of 20% trichloroacetic acid, mix well and allow to stand for 10 minutes and then centrifuge at 5000 rpm for 15 minutes.
4. Add a pinch of citric acid crystals with 2 ml of supernatant and a pinch of EDTA (disodium) with one blank.
5. Add in all tubes 0.5 ml of concentrated ammonium hydroxide and 0.5 ml of 50% cold acetaldehyde and allow them to stand for 30 minutes.
6. After 30 minutes, read the absorbance of blank (with EDTA), standard and sample at 542.5 nm against the blank (without EDTA).

11.4.4. Calculation

$$= \frac{\text{O.D. of sample-O.D. of blank (with EDTA)}}{\text{O.D. of standard-O. D. of blank (with EDTA)}} \times$$

Concentration of standard (2μg) $\times$ convenin factor (5).

$= \mu$g copper/ml.

11.5. INTERPRETATION

The sodium diethyl dithio carbamate (SDDC) method, the oxalyldihydrazide (ODH) method and the 2,2' biquionoline (BQ) method were compared for determination of total copper

in the haemolymph. In the first 2 methods copper was liberated from blood protein by incubating in 6 N HCl. Results presented in Table 1 reveal that the ODH method gave poor results. With the SDDC method, the absorbance after adding amyl alcohol-ether was highly variable (Table 2). The BQ method was precise consistent and reliable. This method has been previously employed by a number of crustacean hematologists. The blood copper concentration determined in 12 crabs ranged from 35.0 to 153.3 μg/ml (Arumugam & Ravindranath, 1980).

TABLE 1. *Blood copper concentration in* Scylla serrata *as determined by 3 different methods* (in μg./ml).

SDDC method	*ODH method*	*BQ method*
64.9±1.2 (9); 5.2%	57.2± 2.1 (9); 11.1%	53.5±1.6 (9); 8.6%
40.2±1.2 (9); 8.9%	62.8± 2.3 (10); 9.9%	53.6±1.3 (5); 5.5%
48.9±1.2 (9); 7.5%	66.3± 3.9 (6); 14.5%	48.1±1.7 (9); 10.7%
34.0±1.3 (9); 11.4%	100.8±10.7 (9); 32.9%	35.0±0.5(10); 4.9%
119.1±4.5 (8); 10.7%	—	108.7±1.2 (8); 3.0%

The values represent mean±SE and coefficient of variation. The number of aliquots of blood is given in parenthesis.

SDDC: Sodium diethyl dithio carbamate method
ODH: Oxalyldihydrazide method.
BQ: Biquinoline method

TABLE 2. *Variation in absorbancy (expressed in O. D.) soon after and 30 minutes after the addition of amyl alcohol: ether mixture to copper-carbamate complex in sodium diethyldithio carbamate method*

Sample Number	*Soon after adding amyl alcohol-ether mixture*	*30 minutes after adding amyl alcohol-ether mixture*
1.	0.35	0.42
2.	0.30	0.40
3.	0.34	0.44
4.	0.46	0.51
5.	0.33	0.39
6.	0.29	0.34
7.	0.47	0.51
8.	0.59	0.66
9.	0.52	0.61
10.	0.47	0.54

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For your own notes

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CALCIUM * 12

12.1. Introduction

Calcium in tissues of crustaceans exists in two states namely diffusible and non-diffusible. Diffusible calcium is also referred to as dialysable and ultra filterable calcium, which includes free calcium ions and calcium complexed with carbonate, citrate, phosphate and free acidic amino acids. Non-diffusible calcium is referred to as non-dialysable calcium and non-ultra filterable calcium. It is also commonly called as bound-calcium. In this state, calcium may be bound to proteins, lipids and acidic mucosubstances. Bound calcium is precipitable with 80% ethanol. The supernatant will contain dialysable fraction. Complete precipitation of bound calcium is achieved by diluting the tissues twenty times with ethanol (Kannan & Ravindranath, 1980).

There are several methods for determination of calcium in biological samples. The direct method to measure calcium after ashing is to read the ionic concentration in an atomic absorption spectrophotometer or in a flame photometer. In other methods, calcium is measured indirectly. One of the oldest methods of calcium determination is by gravimetric analysis in which calcium is precipitated by ammonium oxalate, which is either heated to 300°C or ignited to convert into $CaCO_3$ and CaO respectively (Hecht, 1914). The resultant ash is measured to indicate calcium concentration.

Turbidometric method is equally an old method of calcium determination in which ammonium ferrocyanide (Fiegal & Pavelka, 1924-as cited by Snell & Snell, 1959). or sodium oleate

* Prepared and verified by K. Kannan & M. H. Ravindranath, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

is made to combine with calcium in solution. The turbidity difference between the reagent-calcium complex and the reagent is measured. The difference is considered to reflect the calcium concentration in the solution.

Another classical and very widely used method of calcium determination is that of Clark & Collip's (1925) modification of Kramer & Tisdall's (1921) permanganometric titration method. In this method, calcium is precipitated as calcium oxalate and dissolved in hot sulphuric acid and this mixture is titrated against potassium permanganate to measure the oxalic acid liberated, which serves as an index of calcium. This method is not only used as a standard method for purposes of comparison when a new method is introduced or employed (Webster, 1962; Haefner, 1964) but has also been commonly employed by crustacean workers in determination of blood calcium (Robertson, 1937; Webb, 1940; Travis, 1955; Sitaramiah & Krishnan, 1964).

This method is further extended to employ manometer to measure the CO_2 liberated, on the addition of potassium permanganate to calcium oxalate-sulphuric acid mixture. The CO_2 liberated is measured manometrically which serves as the measure of calcium present in the blood (Van Slyke & Sendroy, 1929).

One of the recent methods in calcium determination is that involving spectrophotometric analysis of calcium complexed with dyes or anionic organic compounds. Calcium is initially precipitated with dyes such as Alizarin red or Eriochrome black or organic compounds such as Chloranilic acid or Ammonium purpurate or Picrolonic acid. The precipitate is washed and dissolved to liberate the dye or the organic compound. The optical density of the dye is directly measured. The organic compounds are coloured with other reagents and the O.D. is read. The colour intensity of the dye or the organic compound is proportional to the amount of calcium present in the sample.

The combination of titrimetry and spectrophotometry was found to render a good precision in obtaining the calcium values. This method is called the compleximetric method where calcium

in the tissues is precipitated and titrated with either EDTA or amino napthol sulfonic acid or titanium chloride whose end point is measured with the help of a spectrophotometer (Roe & Khan, 1929 ; Mousseron & Bouresson, 1930 as cited by Snell & Snell, 1959 ; Fales, 1953). This method is also employed by crustacean investigators in determining blood calcium (Gross, 1959, 1964 ; Haefner, 1964).

Most of these methods are employed in the determination of mammalian serum calcium which is ten to fifteen times lower than that of the blood of crustaceans (Keynes, 1966). Moreover the concentration of other cations are also very high in the blood of decapods (Robertson, 1960). They, by simulating calcium may interfere with the methods of calcium analysis. Therefore, it is felt that a highly reproducible method is necessary for the determination of tissue calcium whose sensitivity and reproducibility should also be equal in measuring diffusible and non-diffusible calcium in the tissue. Furthermore, it is felt that the method should be simple and the reagents should be stable to enable measurements of large number of samples.

In this study, it is necessary to compare the following three methods namely :

1. Flame photometric method
2. Clark and Collip's titrimetric method and
3. Webster's chloranilic acid spectrophotometric method.

12.2. Flame Photometric Method

12.2.1. Principle

Elements when heated to a high temperature emits light, each having a distinct spectrum. The many wavelengths of light created by the complexity of solution are passed through a filter which eliminates all wavelength except that emanating from the ion of interest (Ca). The light emanating is allowed to fall on a photoelectric cell. The electric response is measured on a suitable meter and is expressed as percentage transmission (Robinson & Ovenston, 1951).

12.2.2. Apparatus

Flame photometer, Gas-Cylinder of Butane or Pentane, Air Pressure Condenser.

12.2.3. Reagents

1. *Conc. Nitric acid.*
2. *Deionized distilled water.*
3. *Calcium standard* : Dissolve 250 mg of $CaCO_3$ in a minimal quantity of 1N HCl and make it upto 100 ml (1 mg/ml).

12.2.4. Procedure

1. Add 2 ml of Conc. Nitric acid to 0.2 ml of blood, 0.2 ml of deionized water and 0.2 ml of calcium standard.
2. Make up the solutions to 10 ml with deionized distilled water individually.
3. Feed them in the apparatus.

12.2.5. Apparatus Instructions

1. Switch on and unclamp the galvanometer.
2. Ignite the flame.
3. Set air pressure to 10 lb/inch^2.
4. Keep all the ten tongues of the flame of equal length.
5. Spray distilled water and set zero.
6. Spray the standard and set full scale deflection.
7. Spray the distilled water.
8. Spray the sample and note the reading.

12.2.6. Precautions

1. Gas supply should be continuous and homogeneous.
2. All the tongues of the flame should be of equal length.
3. The nozzle of the atomizer should be clean.
4. Any salts in the atomizer nozzle or the passage should be removed by spraying distilled water.
5. After feeding either sample or standard or blank, the distilled water should be sprayed in.

12.2.7. Calculation

0.2 mg in standard calcium shows transmittance of 100%.
So 100% Transmittance = 0.2 mg for 0.2 ml

$$\therefore \text{ x Transmission} = x \times \frac{0.2}{0.2} \times \frac{100}{100}$$

= : .. mg Ca/100 ml

12.3. Clark & Collip's Titrimetric Method

12.3.1. Principle

Calcium is precipitated as insoluble calcium oxalate and this is redissolved in hot sulphuric acid which liberates the oxalic acid. The oxalic acid-sulphuric acid mixture is titrated against 0.01N Potassium permanganate. The amount of oxalic acid liberated is directly proportional to the amount of calcium present.

12.3.2. Reagents

1. 4% *Ammonium oxalate* : Dissolve 4 gm of Ammonium oxalate in 100 ml of deionized water.
2. 2% *Ammonia* : Dilute 2 ml of ammonia to 100 ml with deionized water.
3. 1*N Sulphuric acid* : Dilute 27.8 ml of Conc. Sulphuric acid with deionized water and make upto 1 litre.
4. 0.1 *N Potassium permanganate stock solution* : Dissolve 3.162 gm of $KMnO_4$ in 1 litre of deionized water.
5. 0.01*N Potassium permanganate* : Take 10 ml of 0.1 *N* $KMnO_4$ and make upto 100 ml with deionized water.
6. 80% *Ethanol.*

12.3.3. Procedure

For the determination of total calcium, the blood is used directly. For the determination of ethanol soluble calcium, 4 ml of 80% ethanol is added to 0.2 ml of blood and the whole

supernatant, after centrifugation at 2500 rpm for 5 minutes is used directly.

1. Add 2 ml of deionized water to 0.2 ml of blood, 0.2 ml of deionized water.
2. Add 2 ml of deionized water and 1 ml of 4% ammonium oxalate to all the tubes and let it stand for 1 hour.
3. Centrifuge at 3000 rpm for 8 to 10 minutes, decant the supernatant and drain by keeping it inverted on a filter paper for 5 minutes. Wipe the mouth of the tubes with soft clean and dry cloth.
4. Add 3 ml of 2% ammonia and centrifuge at 3000 rpm for 3 to 5 minutes and decant as in step 3.
5. Add 2 ml of 1N sulphuric acid and keep it in a warm water bath for a minute.
6. Titrate this oxalic acid-sulphuric acid mixture against 0.01N Potassium permanganate at a temperature of 70-75°C.
7. The end point is the appearance of pink colour which lasts at least for a minute.

12.3.4. Calculation

If 1 ml of 0.01 N $KMNnO_4$ is consumed, it is equivalent to 0.2 mg of calcium (Clark & Collip, 1925).

$$\frac{(\text{Titre value of unknown} - \text{Titre value of Blank}) \times 0.2 \times 100}{\text{Vol. of sample}} = \text{mg. Ca/100 ml of blood}$$

To convert into mM/L multiply the mg% values by 10 and divide by the molecular weight of calcium (40).

12.4. Webster's Spectrophotometric Method

12.4.1. Principle

Chloranilic acid (2, 5 dichloro 3, 6 Dihydroxy P. quinone compound L 111) precipitates the calcium present in the blood

forming a calcium chloranilate complex. This precipitate is dissolved in tetra sodium EDTA which liberates the chloranilic acid. The liberated chloranilic acid combines with ferric chloride to form a coloured complex which is measured at 490 nm in a spectrophotometer. The amount of liberated chloranilic acid is directly proportional to the amount of calcium precipitated.

12.4.2. Reagents

1. *Chloranilic acid* (Baker's analysed reagent) : Dissolve 1 gm of chloranilic acid in approximately 50 ml of deionized water containing 7 ml of 1N NaOH, mix and dilute to 100 ml with deionized water. Filter before use if crystallization occurs.
2. 50% *Iso-propyl alcohol* : Mix equal volumes of isopropyl alcohol and deionized water.
3. 5% *Tetra sodium EDTA* : Dissolve 5 gm of Na_4 EDTA in 100 ml of deionized water.
4. 6% *Aqueous Ferric chloride* : Dissolve 10 gm of $FeCl_3$ $6H_2O$ in 100 ml of deionized water. Discard the solution if it turns cloudy.
5. 0.6% *Aqueous Ferric chloride* : Prepare by mixing 1 part of 6% $FeCl_3$. $6H_2O$ with 9 parts of deionized water on the day of the experiment.
6. *Calcium standard* : Dissolve 250 mg of calcium carbonate with a little amount of 1N HCl in 100 ml of deionized water. The solution contains 1mg calcium/ml.

12.4.3. Procedure

For determination of total calcium, blood is used directly. For determination of ethanol soluble calcium, 2 ml of 80% ethanol is added to 0.1 ml of blood and the whole supernatant, after centrifugation is used directly.

1. Add 0.1 ml of chloranilic acid to 0.1 ml of blood, 2 ml of ethonolic supernatant, 0.1 ml of deionized water and 0.1 ml of calcium standard solution. Mix and allow to stand for at least one hour at room temperature.

2. Centrifuge at 3000 rpm for 10 minutes. Decant the supernatant and drain by keeping it inverted on a filter paper for 5 minutes.
3. Pour in 5 ml of 50% isopropyl alcohol.
4. Centrifuge at 3000 rpm for 5 minutes and decant the supernatant as in step 2.
5. Add 2 drops of 5% tetra sodium EDTA and break up the precipitate by striking the bottom of the tube forcibly against a rubber stopper.
6. Add 5 ml of 0.6% ferric chloride solution and mix it well by agitation or inversion and keep it for 5 minutes.
7. Determine the O.D. at 490 nm in a spectrophotometer.

12.4.4. Calculation

$$\frac{\text{O.D. of unknown}}{\text{O.D. of standard}} \times \text{concentration of standard} \times \frac{100}{\text{volume of sample}}$$

= mg Ca/100 of blood

$$\frac{\text{O.D. of unknown}}{\text{O.D of standard}} \times \text{concentration of standard} \times \frac{1000}{\text{Vol. of sample}}$$

$$\frac{}{\text{Molecular weight of Ca (40)}}$$

= mM/L

Since, the volume of sample and standard is the same and concentration of the standard used is 1 mg/ ml the formula can be modified to

$$\frac{\dfrac{\text{O.D. of unknown}}{\text{O.D. of standard}} \times 1000}{\text{Molecular weight of Ca (40)}} = \text{mM/L}$$

12.5. INTERPRETATION

The mean total blood calcium values obtained with the 3 methods, do not differ much from one another (Table 1). However taking the percentage coefficient of variation into consideration, Webster's method showed the smallest coefficient of variation, indicating consistency in the performance of the method.

TABLE 1. *Blood calcium concentration in* Scylla serrata (Forsskal) *as determined by 3 different methods. Values are expressed in mg/100 ml.*

Size (mm)		*Flame photometric method*	*Permanganometric method*	*Chloranilic acid method*
97	Mean ± SE	146.44 ± 2.45 (10)	146.60 ± 4.39 (10)	146.38 ± 3.14 (9)
	Coefficient of variation (%)	9.83	9.47	6.44
134	Mean ± SE	100.00 ± 2.56	145.33 ± 4.13 (10)	142.44 ± 1.54 (10)
	Coefficient of variation (%)	16.16	8.48	3.42
119	Mean ± SE	135.20 ± 1.46 (5)	127.80 ± 4.03 (9)	147.30 ± 5.58 (10)
	Coefficient of variation (%)	2.48	9.98	10.05
111	Mean ± SE	130.20 ± 0.56 (10)	135.27 ± 2.77 (11)	152.09 ± 3.12 (9)
	Coefficient of variation (%)	1.36	6.80	6.16

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For your own notes

7

For your own notes

POTASSIUM * 13

13.1. PRINCIPLE

Potassium in the blood is precipitated as potassium cobalt nitrite and when dissolved in sodium thiosulphate it liberates cobalt. The amount of cobalt liberated is equivalent to the amount of potassium present in the sample and this cobalt concentration is determined spectrophotometrically by choline chloride and potassium ferricyanide which impart green colour to the sample (Snell & Snell, 1959).

13.2. REAGENTS

1. *Silver cobaltinitrite reagent*

   Solution A : Dissolve 25 gm of cobaltinitrite in 150 ml of distilled water and then add 12.5 ml of glacial acetic acid.

   Solution B : Dissolve 120 gm of sodium nitrite in 180 ml of water (the final volume will be 210 ml).

   Then add 210 ml of B to A and remove the nitrous fumes using a pipette.

   Store the reagent at 0°C in a refrigerator. Before use, filter 20 ml of cobaltinitrite reagent and add 1 ml of 40% silver nitrate.

2. 1% *aqueous sodium thiosulphate* : Dissolve 1 gm of sodium thiosulphate in 100 ml of distilled water.

3. 1% *aqueous choline chloride* : Dissolve 1 gm of choline chloride in 100 ml of distilled water.

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* Prepared and verified by K. Kannan & M. Arumugam, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

4. 2% *aqueous potassium ferricyanide* : Dissolve 2 gm of potassium ferricyanide in 100 ml of distilled water.
5. *Washing agent* : Prepare by mixing 95% ethanol : ether : water in 2 : 1 : 2 V/V/V.
6. *Standard K^+ Solution* : Dissolve 0.191 gm of potassium chloride already oven-dried at 110°C for over night in 100 ml of distilled water. This solution contains 1 mg of K^+ per ml of the solution.
7. 40% *silver nitrate* : Dissolve 40 gm of silver nitrate in 100 ml of distilled water.
8. *Deproteinizing agent* : 80% ethanol.
9. 2.5% *silver nitrate* : Dissolve 2.5 gm of silver nitrate in 100 ml of distilled water.

13.3. PROCEDURE

1. Add 0.8 ml of distilled water to 0.2 ml of blood and 0.2 ml of K^+ standard solution.
2. Add 4 ml of 80% ethanol to all the tubes and centrifuge at 3500 rpm for 5 minutes.
3. Add 0.5 ml of 2.5% silver nitrate to the supernatant and centrifuge at 3500 rpm for 10 minutes.
4. Add 0.4 ml of silver cobaltinitrite to the supernatant and centrifuge at 3500 rpm for 10 minutes and decant the supernatant.
5. Wash the precipitate in 2 ml of 95% ethanol : ether : water mixture twice.
6. Dissolve the precipitate in 5 ml of 1% aqueous sodium thiosulphate.
7. Add 1 ml of 1% choline chloride and 2% potassium ferricyanide.
8. Read the colour intensity developed at 430 nm in a spectrophotometer.
9. 5 ml of 1% sodium thiosulphate serves as blank.

13.4. CALCULATION

$$\frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times \frac{\text{Concentration of the standard} \times 100}{\text{Vol of sample}}$$

$$= \text{mg } K^+/100 \text{ ml of blood}$$

$$= \frac{\text{mg } K^+/100 \text{ ml of blood}}{\text{moulded weight of K (39)}}$$

$$= \text{mM/L or mEq/L}$$

13.5. PRECAUTIONS

1. The sample should be free of proteins and chlorides.
2. The washing of the precipitate should be complete. All the excess cobaltinitrite should be washed.

13.6 REFERENCE

SNELL, F. D. & C. T. SNELL, 1959. *Colorimetric methods of analysis*, Vol. II A. Von Nostrand Co. Inc., New York, pp. 463.

For your own notes

CHLORIDE * 14

14.1. PRINCIPLE

Chloride in the sample displaces thiocyanate from mercuric thiocyanate, which in turn combines with iron of ferric nitrate to become ferric thiocyanate, which is a coloured compound. The colour intensity is proportional to the iron complexed with thiocyanate which inturn depends on the amount of Cl^- ions which have displaced thiocyanate from mercuric thiocyanate (Schorenfeld & Lewellen, 1964).

14.2. REAGENTS

1. *Colour reagent*

   (*a*) *Saturated mercuric thiocyanate solution* : Dissolve 2 gm of mercuric thiocyanate ($Hg(SCN)_2$ in 1000 ml of water. Keep the solution at room temeprature for 48 hours or longer and shake frequently, filter it before use.

   (*b*) *Ferric nitrate solution* : Dissolve 13 gm of ferric nitrate in approximately 400 ml of distilled water and 1.5 ml of Conc. HNO_3. Then to the whole volume of solution b, add 500 ml of solution *a* and make upto 1000 ml. Then add 5 to 6 ml of 6% mercuric nitrate until the absorbance of 80 m Eq/L Chloride standard is between 0.07 and 0.1.

2. *Standard Cl^- solution* : Dissolve 585 mg of dry pure NaCl in 100 ml of distilled water. This standard is equivalent to 100 m Eq Cl^-/L.

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* Prepared and verified by K. Kannan & M. Arumugam, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

14.3. PROCEDURE

1. 0.1. ml of blood, 0.1 ml of distilled water (blank) and 0.1 ml of standard solution are added to 1 ml of 80% of ethanol individually and centrifuge at 3500 rpm for 5 minutes.
2. Take 0.1 ml of supernatant from all the tubes and add 3 ml of colour reagent separately.
3. After 10 minutes, find the optical density at 480 nm in a spectrophotometer.

14.4. CALCULATION

$$\frac{\text{O.D. of sample}}{\text{O.D. of standard}} \times \text{Concentration of standard} \times \frac{1000}{\text{Vol. of sample}}$$

$$= \text{m Eq/L.}$$

14.5 REFERENCE

SCHOENFELD, R. S. & C. J. LEWELLEN, 1964. Colorimetric method for determination of serum chloride. *Clin. Chem.*, **10** : 533-539.

For your own notes

For your own notes

15 ELECTROPHORETIC SEPARATION OF PROTEINS *

15.1. Introduction

There are several structural and storage proteins in the tissues of Crustacea. Some of them are metallo-proteins, some are enzymic in nature. Several electrophoretic procedures were used for the separation of these proteins. The methods used by crustacean workers include paper (Zuckerkandl, 1956; Hughes & Winkler, 1966 ; Stewart, *et al.*, 1969), agar gel (Decleir, 1961 ; Vranckx & Durlait, 1976), starch gel (Whittaker, 1959 ; Cowden & Coleman, 1962; Dall, 1964) and polyacrylamide gel electrophoresis (Dall, 1974 ; Durliat *et al.*, 1975 ; Alikhan & Akthar, 1980).

With polyacrylamide gel electrophoresis a good resolution is achieved with minimum quantity of blood sample (0.1 ml). Here polyacrylamide gel electrophoretic method of Davis (1964) is described.

15.2. Principle

The principle involved in this procedure is that charged ion or group will migrate towards one of the electrodes when placed in the electric field. A substance can migrate only when it is ionised. A weak acid will remain at the origin at a pH equal to or below its isoelectric point and move to anode above this point because it gets ionised at higher pH. The rate of migration is proportional to the degree of ionisation and hence higher the pH, the faster it travels. The converse is true for weak bases.

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* Prepared and verified by M. H. Subhashini & M. H. Ravindranath, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

In polyacrylamide gel electrophoresis separation is based not only on net charge but also based on differences in the mass of the proteins. Thus proteins with same net charge but differing mass may be separated.

15.3. Reagents

1. For running gel :

(*a*) **Small pore buffer (pH 8.9)**

1N HCl — 48 ml

Tris (hydroxymethyl)—36.6. gm

TEMED (N, N, N′, N′-Tetramethylethelene diamine) —0.23 ml

make upto 100 ml with distilled water.

(*b*) 0.14% Ammoniumpersulphate :

(*c*) *Monomer solution for different concentrations* :

(*i*) 5% *gel concentration* : Dissolve 19.0 gms of acrylamide and 1.0 gm N, N′methylenebisacrylamide in 100 ml of double distilled water.

(*ii*) 6.3% *gel concentration*: Dissolve 24.4. gm of acrylamide and 0.8 gm of N, N′methylenebisacrylamide in 100 ml of double distilled water.

(*iii*) 7.0% *gel concentration* : Dissolve 28.0 gm of acrylamide and 0.735 gm of N, N-methylenebisacrylamide in 100 ml of distilled water.

(*iv*) 7.7% *gel concentration* : Dissolve 30.0 gm of acrylamide and 1.0 gm of N, N′methylenebisacrylamide in 100 ml of distilled water.

2. For spacer gel :

(*a*) *Large pore buffer* : Dissolve 5.98 gm of Tris (hydroxymethyl) in double distilled water. Add 0.46 ml of TEMED (N, N, N′, N′-tetramethyethylenediamine) and 48 ml of 1N HCl and make upto 100 ml with distilled water. Adjust the pH of the solution to 6.7.

(*b*) ***Monomer solution*** : Dissolve 10.0 gm of acrylamide and 2.5 gm of methylenebisacrylamide in 100 ml of distilled water (The concentration of spacer gel monomer is 3%).

(*c*) Dissolve 4 mg of riboflavin in 100 ml of distilled water.

(*d*) Dissolve 40 gm of sucrose in 100 ml of distilled water.

3. **Indicator solution :** 0.1% aqueous Bromophenol blue.

4. **Tank buffer solution :** Add 6 gm of Tris and 28.8 gm of glycine in distilled water, mix well and make it upto 1 litre.

5. **Staining solution :**

(*a*) 10% Trichloroaceticacid.

(*b*) 0.25% *coomassie Brilliant Blue* : Dissolve 0.25 gm of coomassie brilliant blue in 100 ml of methanol, water and acetic acid in 5 : 5 :1 ratio.

(*c*) *Schiff's reagent* : Dissolve 1 gm of basic fuchsin in 200 ml of boiling distilled water. Shake for 5 minutes and cool exactly 50°C Filter and add to the filtrate 20 ml of 1N-HCl. Cool to 25° and add 1 gm of sodium (or potassium) metabisulphite. Allow this solution to stand in dark for 14-24 hrs. Add 2 gm of activated charcoal and shake for 1 minute. Filter. Keep the filtrate in dark at 0°-4°C. Allow it to reach room temperature before use.

6. **Destaining solution :** Methanol, water and acetic acid in the ratio of 5 : 5 :1 ratio serves as destaining solution.

7. **7% acetic acid.**

15.4. PROCEDURE

1. Bring the stock solutions and small pore solution to room temperature.
2. Fix the gel tubes in gel tube stand.
3. Prepare running gel solutions by mixing small pore buffer, monomer, double distilled water and ammonium persulphate in 1 : 2 : 1 : 4 ratio, mix well.

4. Using a syringe, pour the solution gently along the side of the gel tubes upto the first scratch mark. (Avoid air bubble while pouring the solution.)
5. Add few drops of water over the solution to avoid miniscus formation.
6. Allow it to polymerise.
7. After completion of polymerisation, remove the overlying water carefully.
8. Prepare spacer gel solution by mixing large pore buffer, monomer solution, riboflavin and sucrose in 1 : 2 : 1 : 4 ratio.
9. Add 0.15 ml of spacer gel solution over running gel. Add few drops of water over it to avoid miniscus and allow it to polymerise.
10. After the completion of polymerisation of spacer gel, the sample should be applied.
11. Collect 0.1 ml of blood and mix well in 0.5 ml of 40% sucrose.
12. Add 0.04 ml of sample over spacer gel.
13. Remove the gel tubes gently and insert into the grommets of the upper buffer tank.
14. Take 50 ml of the tank buffer and dilute to 500 ml with distilled water.
15. Take 250 ml of tank buffer in lower tank.
16. Fill the remaining space of the gel tubes gently with tank buffer and add 250 ml of the buffer in upper tank.
17. Add few drops of indicator dye (Bromophenol blue) in the upper tank buffer.
18. Connect the electrophoresis tank with the power pack and run electrophoresis at 10°C.
19. Adjust the current supply to 4mA/tube and 200-240 V.
20. When the bromophenol blue comes to the lower edge of the gel tubes, the current supply must be switched off.
21. Remove the gels from the gel tubes carefully using syringe filled with the used buffer.

15.5. Detection of Protein Fractions

After the completion of electrophoresis, remove the gels carefully from the tubes and incubate in 10% TCA for 30 minutes. Then transfer it to coomassie brilliant blue solution and keep it in dark for 10 minutes. Destain it in dark for 20 minutes in methanol, water and acetic acid mixture (5 : 5 : 1). Then store the gels in 7% acetic acid in dark. The destaining procedures are to be carried out in dark since it was noticed that during the course of staining, exposure of gels to light resulted in the fading of the fractions, possibly due to photosensitivity of coomassie brilliant blue.

15.6. Densitometric Analysis

Take the densitometric tracings of the proteins by subjecting the electropherograms to chromoscan using blue filter with aperture size 10.10/10.05.

15.7. Migration Velocity or Mobility of Each Fractions

This method involves measurement of the distance moved by each fraction from the point of application to the region of the peak of each fraction. After measuring the distance moved, calculate the migration velocity following the formula recommended by Smith (1968). The same formula was also used by Stewart *et al.* (1966) for measuring the migration velocity of serum proteins of *Homarus americanus.*

$$\text{Migration velocity} = \frac{\text{Distance travelled by a fraction} \times \text{Total length of gel}}{\text{Time of electrophoresis in seconds} \times \text{V/tube}} \times 10^{-3}$$

$$= \text{mm}^2/\text{V/sec}$$

15.8. Relative Proportions

Determine the total area by counting each square, occupying the scan pattern. After counting the total number of squares

occupied by each fraction calculate the relative proportion of each fraction as follows :

$$\text{Relative proportion (in \%)} = \frac{\text{Number of squares occupied by a particular fraction} \times 100}{\text{Total number of squares occupied by the whole scan region.}}$$

15.9. Quantification of Fractions

Based on the relative proportions, determine the concentration of the individual fractions taking into consideration the protein concentration of the blood of respective animals used for analyses.

15.10. Localisation of Polysaccharide Moiety

Polysaccharides associated with proteins were localised with periodic acid-Schiff (PAS) test, following Smith (1968), which gives a majenta colour indicative of a positive reaction.

After removing the gels from gel tubes, immerse them for 1 hr. in 1% periodic acid in 3% acetic acid. Leach them in water for one hour and treat with Schiff's reagent. Destain the gels and store them in 1% sodium metabisulphite solution.

Presence of glycogen is verified by treating the gels with taka-diastase solution before testing for PAS reactivity. Test the natural aldehydes interfering with PAS reaction with Schiff alone. For the presence of glycogen, treat the gels for Bauer-Feulgen test. For this purpose pre-oxidise the gels for 60-90 minutes with 4 % chromic acid. Then treat the gels with Schiff's reagent. The presence of glycogen is indicated by the development of purple colour. Destain the gels and store them in 1% potassium metabisulphite. Diastase treated gels have to be used as controls.

15.11. Localization of Lipid Moiety

Incubate the gels in saturated solution of Oil red 0 in 50% methanol containing 10% TCA for 2 hrs. at 60°C. Development of red colour suggests the presence of neutral lipids.

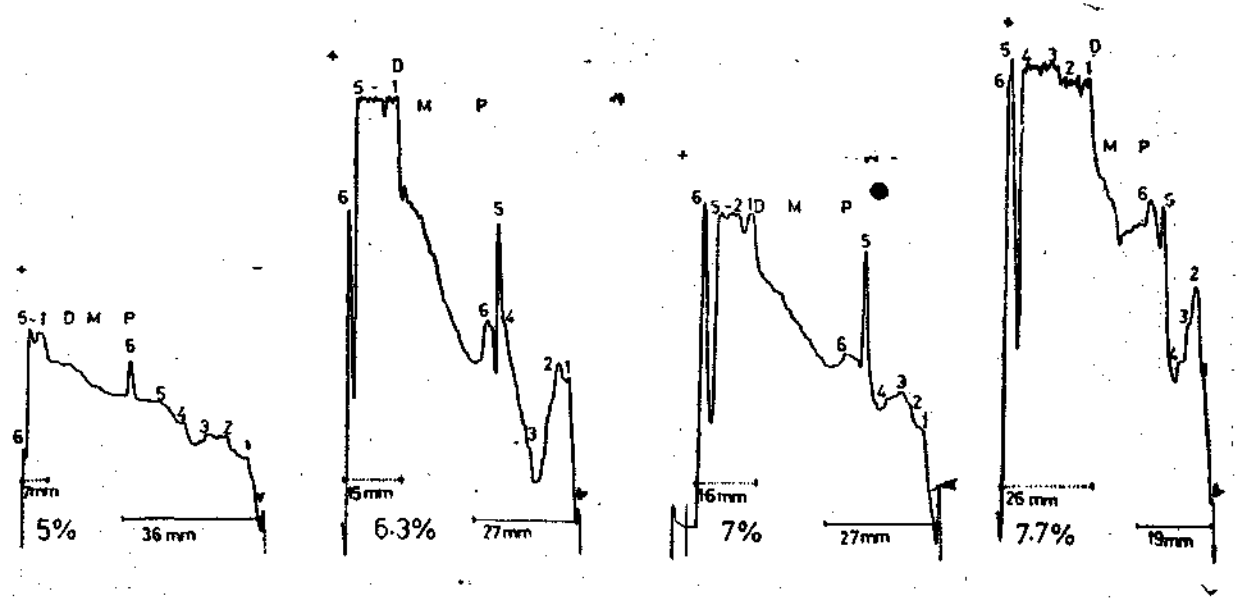

Fig. 1

The scanning patterns of the electropherogram of the blood proteins of male *Scylla serrata* showing the effect of concentration of gels on the resolution and fractionation of proteins. The concentration of gels used are given as %. Note how different zones vary with concentration of the gel.

15.12. Localisation of Copper Containing Protein Fractions

Detect the presence of copper by treating the gels for 48 hrs. at room temperature in saturated solution of rubeanic acid in methanol, acetic acid and water in the ratio of 5 : 2: 5 (V/V/V). The appearance of greenish brown colour indicates the presence of copper.

15.13. Interpretation

Electropherogram of the blood proteins of decapods can be broadly classified into 3 zones. They are mainly proximal, middle and distal. The number of protein fractions constituting each zone varies with the species. The resolution of blood proteins vary with different concentrations of running gel. (Subhashini, 1980.)

The resolution of proximal fractions and found to be better in 5% concentration of gel than in higher concentration. The distance occupied by the proximal zone decreases with the increase in the concentration of the gel. Resolution of distal fractions is better in the higher concentration (7.7%) of the running gel. The distance occupied by distal zone increases with the increase in concentration of the gel.

Observations made with and without spacer gel reveal that the resolution of fraction is better and the bands are more compact after using spacer gel. Further the distal fractions showed increased mobility in gels with spacer gel.

The optimal concentration of the protein sample for better clarity and resolution is 350-400 μg.

15.14 References

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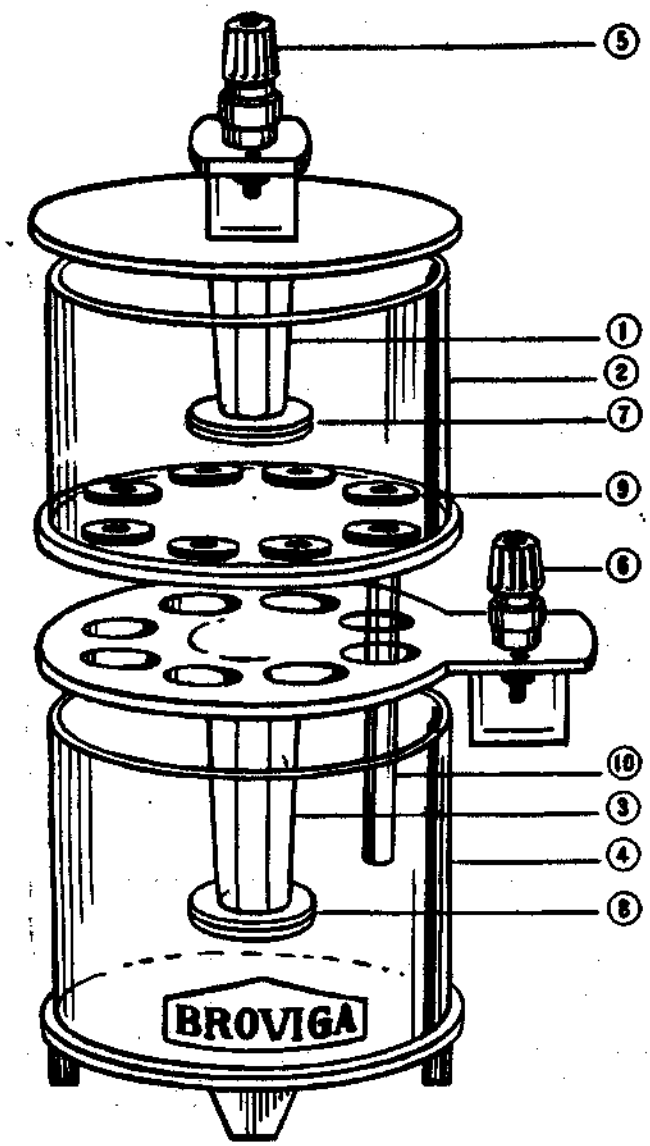

PLATE I

DISC ELECTROPHORESIS TANK

Disc electrophoresis tank consists of 2 parts, namely, Upper Buffer Tank (1, 2, 5, 7 & 9) and Lower Buffer Tank (3, 4, 6 & 8).

The Upper Buffer Tank consists of a lid (1, 5 & 7) with a platinum wire settled in a groove (7), passing through the central rod (1) and terminating at cathode terminal (5); and a tank provided with rubber grommets (9) to hold the gel tubes (10).

The Lower Buffer Tank consists of a lid (3, 4, 6 & 8) with a platinum wire in a groove (8) passing through the central rod (3) and terminating at Anode terminal (6); and a tank (4).

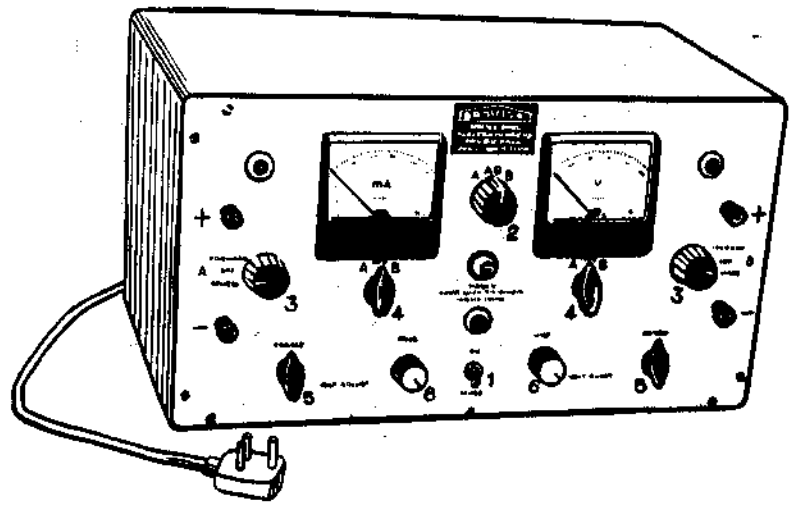

PLATE II

DISC ELECTROPHORESIS POWER PACK

This is a double channel power pack (A, B) which can be operated individually as well as simultaneously using channel selector (2). Anode (+) and Cathode (−) terminals are provided separately with both the channels. Forward/reverse direction selectors (3) are situated on either side. Meter indicators (4) to indicate mA/V are situated below the respective meter. This can be used both for A and B by suitably turning the knob. Coarse adjust (5) and fine adjust (6) of mA/V are provided separately for A & B channels. Main switch (1) is situated in the centre.

For your own notes

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CHROMATOGRAPHIC SEPARATION OF SUGARS * 16

16.1. PRINCIPLE

The principle of chromatography involves separation of a mixture on the basis of specific differences in physical and chemical properties, which result from the structural differences of the chemically related groups of compounds which are under investigation. They therefore have differential affinity for both the mobile and stationary phases of the chromatographic systems. This chromatographic separation is the resultant of propelling (mobile phase) and retarding forces (stationary phase). The stationary phase in strict sense includes the medium (paper) together with the polar solvent (water). The mobile phase or propelling force includes both polar and non-polar solvent.

The separation is brought about by continuous partition between the mobile phase (solvent flowing along the paper) and the water held in the paper and paper *per se*. Paper together with water acts as an adsorbent ; it has a strong affinity for polar molecules which are held by hydrogen bonding and vander Waals' forces (Smith & Seakins, 1976).

16.2. REAGENTS

1. 5% *TCA* : Prepare by dissolving 5 gm of TCA in 100 ml of distilled water.
2. **Pyridine undiluted.**
3. **10% Iso-Propyl alcohol :** Prepare by diluting 10 ml of isopropyl alcohol in to 100 ml with distilled water.
4. **Solvent system (Butanol : Pyridine : water) :** Prepare by mixing Butanol : Pyridine : water in the ratio of 2 : 2 : 1 (Smith & Seakins, 1976).

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* Prepared and verified by T. S. Saravanan & M. Arumugam, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

5. Alkaline silver oxide.

(*a*) *Saturated Silver nitrate in distilled water*—0.1 vol.

(*b*) *Sodium hydroxide :* Dissolve 0.5 gm NaOH in 5 ml of distilled water and dilute to 100 ml with ethanol—100 vol.

16.3. PROCEDURE

16.3.1. Preparation of sample for separation of sugars :

1. Take 0.1 ml of blood in 1 ml of 5% TCA and centrifuge at 2500 rpm for 5 minutes.
2. With 1 ml of supernatant, add 3 ml of pyridine and heat it over a boiling water bath, till the solution gets evaporated completely.
3. Dissolve the residue again in 3 ml of pyridine and evaporate it over a boiling water bath. Repeat this procedure for 4-5 times.
4. Dissolve the salt-free residue in 1 ml of 10% isopropyl alcohol.

16.3.2. Separation

1. Take a Whatman No. 1 chromatogram paper (23 × 18 cm) and note down the flow direction.
2. Draw a line two inches above the lower margin and make two spots.
3. Spot the sample and the standard on the points separately and dry it using a hair dryer.
4. Fold the paper into a hollow cylinder and join the ends with cellophane tape.
5. Take 40 ml of solvent in a glass container (1500 ml) and keep the paper inside, (care should be taken to avoid any contamination with paper and the paper should not touch the sides of the glass container) and allow it to run.
6. After the completion of the run, take out the paper carefully and dry it in air.

16.3.3. Localisation and Identification of spots

1. Dip the dried paper in silver nitrate.
2. When dried dip it in Sodium hydroxide.
3. Excess reagents to be removed by dipping in 2 M Ammonia.
4. Make out the spot and determine the Rg values and identify the different sugars.

16.4 References

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For your own notes

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17 TWO DIMENSIONAL CHROMATOGRAPHIC SEPARATION OF FREE AND BOUND AMINO ACIDS *

17.1. Principle

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

17.2. Reagents

1. **80% ethanol :** Prepare by diluting 80 ml of absolute ethanol into 100 ml with double distilled water.
2. **Chloroform**
3. **Solvent systems :**

   (*a*) *Solvent system I* : (*Butanol* : *Acetic acid* : *Water*). Prepare by mixing butanol, acetic acid and water in the ratio of 12 : 3 : 5.

   (*b*) *Solvent system II* : (Phenol : ammonia 200 : 1). Prepare by dissolving 160 gm of phenol in 40 ml of distilled water with one ml of ammonia.
4. **6N HCl :** Prepare by diluting 54 ml of Conc. hydrochloric acid into 100 ml of distilled water.
5. **10% Isopropanol :** As mentioned in 16.2.
6. **Locating reagents :**

   (*a*) 0.2% *Ninhydrin in acetone* : Prepare by dissolving 200 mg of ninhydrin in 100 ml of acetone.

* Prepared and verified by P. Ramasamy, School of Parasitology, Department of Zoology, University of Madras, Madras-600 005.

(*b*) *Sulphanilic acid* (*Pauly*) *reagent* :

(*i*) *Sulphanilic acid* : Prepare by dissolving 0.9 gm of sulphanilic acid in 9 ml of Conc. hydrochloric acid and then made upto 90 ml with distilled water.

(*ii*) 5% *sodium nitrite* : Prepare by dissolving 5 gm of sodium nitrite in 100 ml of distilled water.

(*iii*) 10% *sodium carbonate* : Prepare by dissolving 10 gm of anhydrous sodium carbonate in 100 ml of distilled water.

(When required, mix 1 volume of reagent (i) with 1 volume of reagent (ii) allow it to stand for 5 minutes at room temperature and 2 volumes of reagent (iii) carefully as the mixture effervesces vigorously).

(*c*) 0.5% *Isatin* : Prepare by dissolving 500 mg of isatin in 100 ml of acetone.

(*d*) *Folin-Ciocalteu reagent* :

(*i*) *Folin reagent* : Prepare by diluting 1 ml of folin phenol with 5 volumes of distilled water.

(*ii*) 10% *sodium carbonate* : as mentioned in 17.3.

17.3. PROCEDURE

17.3.1. Sample preparation for the separation of free amino acids.

1. Add 0.1 ml of blood in 3 ml of 80% ethanol ; mix it well, centrifuge it at 5000 rpm for 4-5 minutes.
2. With the supernatant add 3 volumes of chloroform, shake it well and allow it to stand for few minutes. An aqueous layer formed at the top can be used for spotting.

17.3.2. Sample preparation for the separation of bound amino acids.

1. Take the precipitate from 17.3.1.a. and add 5 ml of 6 N hydrochloric acid.

2. Transfer it to a standard flask and keep it for hydrolysis at 110°C for 12-15 hours.
3. After complete hydrolysis, transfer it to a porceline crucible and evoporate it over a water bath.
4. Dissolve the residue with 1 ml of water and repeat the evaporation procedure.
5. Dissolve the residue in 1 ml of 10% aqueous isopropanol and use it for spotting (Smith & Seakins, 1976).

17.3.3. Application of the sample

1. Take a Whatmann No. 1 chromatography paper of size 28×28 cms and note down the flow direction.
2. Draw a horizontal line from two cm above the lower margin and a vertical line leaving two cm on the left side of the paper.
3. Spot the samples at the corner where two lines meet and repeat the spotting till getting the required concentration of amino acids. (A hair dryer may be used to hasten the drying of spottings. Take care that the paper is kept clean and should not be touched by bare hands.)

17.3.4. Separation

1. Take 60 ml of solvent in a glass container.
2. Fold the chromatogram paper (in a hollow cylinder form) and join the two ends of the paper with the cellophane tape.
3. Keep the paper inside the glass container carefully and it should not touch the sides of the glass container and allow it to run.
4. After completion of the first run, take out the paper, and dry it.
5. Once again fold the paper in the perpendicular direction to the first one and keep it in the solvent system-II.
6. After the completion of the second run take out the paper, dry it in air.

17.3.5. Localisation and identification of amino acids

1. *For amino acids*

   (*i*) Dip the chromatogram in 0.2% Ninhydrin and dry it at 100°C for about 3 minutes.

   (*ii*) Examine it under both visible light and ultraviolet light and mark all the spots.

2. *For Imidazoles.*

   (*i*) Dip the chromatogram in sulphanilic reagent, dry it and make the spots.

3. *For proline and hydroxy proline.*

   (*i*) Dip the chromatogram in 0.5% Isatin, dry it and mark the spots.

4. *For phenolic amino acids.*

   (*i*) Dip the chromatogram in Folin phenol and dry it.

   (*ii*) make a second dip in 10% sodium carbonate and mark the spots.

17.4 References

Smith, I. & J. W. T. Seakins, 1976. *Chromatographic and electrophoretic techniques.* Volume I. Paper and thin layer chromatography, pp. 465. William Heinemann Medical Books Ltd., London.

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THIN LAYER CHROMATOGRAPHIC SEPARATION OF LIPIDS * 18

18.1. Principle

A chromatographic separation in general is a technique in which a mobile phase while passing over a stationary phase transports different substances with different velocities in the direction of flow. In the case of thin layer chromatography the stationary phase (an adsorbent such as silica gel, silicic acid or cellulose) is placed on a glass support. A processed sample is spotted onto the stationary phase and then placed into a chromatographic chamber containing a relevant solvent system (mobile phase). As the solvent rises through the adsorbent by absorption and capillary action, it tends to resolve the components of the sample. Electrostatic forces of the stationary phase act to retard the component in the sample as the mobile phase rises. This and the fact that the components have different solubilities in the mobile phase cause the individual components to move at different rates below the solvent front (Stahl, 1958).

18.2. Reagents

1. *Chloroform* : *Methanol* (2 : 1) Mix 2 volumes of chloroform to one volume of methanol.
2. *Eluting solvent* : Mix benzene : diethyl ether : ethyl acetate : acetic acid in the ratio of 80 : 10 : 10 : 0.2.
3. *Iodine crystals.*

18.3. Procedure

18.3.1. Extraction of Lipid (Johnson & Davenport, 1971)

1. Take out 300-400 mg of hepatopancreas.
2. Add 15 ml of chloroform : methanol (2 : 1) and homogenize it well for 15 minutes.

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* Prepared and verified by S. Gunasekaran, School of Membrane Biology, Department of Zoology, University of Madras, Madras-600 005.

3. Mix with water in the ratio of 1 vol. of the mixture to 0.8 vol. of water shaking vigorously.
4. Allow the whole sample to settle in a separating funnel. (The chloroform rich layer settles down which contains all the lipids).
5. Separate the chloroform layer and evaporate the solvent at room temperature.
6. Find out the weight of the lipid gravimetrically.
7. Calculate the % lipid in hepatopancreas using the known weights of the tissue taken and the lipid extracted.
8. Dissolve the lipid in minimal quantity of chloroform and make it ready for spotting.

18.3.2. Thin Layer Chromotography

18.3.2.1. *Preparation of Plates and Activation*

1. Weigh 25 gm of silica gel G.
2. Grind it in a mortar using pestle in 50 ml of water and make a slurry.
3. Transfer it to a TLC spreader.
4. Adjust the slit width to 0.35 mm.
5. Arrange TLC plates (20 cm × 20 cm) on a TLC Board so that they are continuous.
6. Spread the slurry on TLC plates.
7. Dry the plates with slurry in air.
8. Activate the plates at 120°C for 30 minutes in an oven.

18.3.2.2. *Spotting, Elution and Identification*

1. Spot the lipid extract (known quantity) in TLC plates using a micropipette. Spots are to be 1" above the base line, small but concentrated.
2. Keep the plates vertically in eluting chamber which has approximately 150-180 ml of eluting solvent and close the lid to make it air-tight.

3. Allow the solvent to run on the plate till it reaches 1/2" below the top edge of the plate.
4. Air dry the plate and transfer it to another chamber containing iodine vapour.
5. After few minutes take out the plate and mark the spots of sublimated iodine using a needle and calculate the Rf values.
6. The spots could be identified by using known Rf values of phospholipids standard, eluted under identical conditions.

$$Rf = \frac{\text{Distance travelled by the lipid spot from the origin}}{\text{Distance travelled by the solvent from the origin}}$$

18.4 References

Johnson, A. R. & J. B. Davenport, 1971. *Biochemistry and methodology of lipids.* Wiley Interscience, pp. 578.

Stahl, E. 1958. *Thin layer chromotography.* Academic Press, New York, pp. 835.

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AMMONIA EXCRETION AND UPTAKE * 19

19.1. Introduction

Ammonia is one of the major excretory products of aquatic crustaceans. The rate of ammonia excretion may reflect the activity of the animal (Subhashini, 1981). Ammonia excretion in crabs as well as in several isopods show diurnal variation (Kirby & Harbaugh, 1974 ; Subhashini, 1981). The amount of ammonia excreted by the animal can be determined by placing the animal in ammonia free artificial sea water, following the method as mentioned in 8.0. Crabs are known to take in ammonia from the medium (Mangum & Towle, 1977 ; Subhashini, 1981).

19.2. Reagents

1. *Artificial sea water* : Subow recipe as cited by Sverdrup *et al.* (1961).

   NaCl-26.518 gm ; $MgCl_2$-4.47 gm ; $MgSO_4$-3.305 gm ; $CaCl_2$ - 1.141 gm ; KCl - 0.725 gm ; $NaHCO_3$ - 0.202 gm ; NaBr - 0.083 gm.

   Dissolve in 2 litres of distilled water.

2. 10 *mM ammonium chloride in artificial sea water.*

3. Reagents for ammonia determination as mentioned in 8.2.

19.3. Procedure

19.3.1. Excretion :

1. Keep the crabs in individual clean plastic tanks holding 2 litres of 50% artificial sea water, free of ammonia. Give

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* Prepared and verified by M. H. Subhashini & M. H. Ravindranath, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

aeration throughout the experiment. A tank of artificial sea water without animal serves as control.

2. Periodically take quadruplicates of 0.1 ml of water sample, make upto 1 ml with double distilled water and analyse for the presence of ammonia following the method mentioned in 8.0.
3. Calculate the rate of ammonia excretion by calculating the amount of ammonia in 2 litres of water/gm body weight of animal/hour.

19.3.2. Uptake :

Maintain the crabs in 2 litres of 50% artificial sea water containing 10 mM of ammonium chloride. All the other conditions and procedures are same as mentioned for ammonia excretion.

Calculate ammonia uptake/excretion by substracting ammonia concentration in the medium at different hours from that found initially in the medium.

19.4 References

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EFFECTS OF THE SURGICAL EXCISION OF THE SINUS GLAND AND EYESTALK ABLATION ON OSMOTIC REGULATION *

20

20.1. Introduction

Sinus gland is a neurohaemal organ wherein hormones from different neurosecretory centres are stored. The sinus gland of *Scylla serrata* is well developed and macroscopically visible owing to its well known characteristic opacity and slightly bluish-hue. It is located at the dorsal aspect of the junction between the medulla interna and the medulla terminalis in the eyestalk. Since it is a compact structure it is possible to remove the sinus gland from the eyestalk. There are two surgical procedures for the removal of the sinus gland. The first procedure involves the removal of the retinal portion of the eye cap and the other involves surgical excision without disturbing the eye cap and thereby the vision of the crab (Kleinholz, 1947).

20.2. Removal of the Retinal Portion of the Eye Cap

20.2.1. Procedure I :

1. Prechill the crab and make it immobile ;
2. Make a sharp cut at the retinal portion of the eye cap and expose the medulla interna.
3. The gland will be visible as an opaque organ of bluish-hue dorsally at the corner of the medulla interna.
4. Using a fine pair of forceps and a needle, the sinus gland can be lifted and transferred to physiological saline (0.9% NaCl).
5. The cut end of the retinal portion may be sealed with cold paraffin wax to avoid loss of blood.

* Prepared and verified by G. Dayanithi & M. H. Ravindranath, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

20.2.2. Procedure II :

1. Animal is anesthetized as in procedure I.
2. Make a rectangular cut (length 5 mm × width 3 mm) at the posteriorad of the long axis of the eyestalk approximately in the centre of the non-retinal portion.
3. Remove the rectangular piece of the exoskeleton and the underlying hypodermis using a fine pair of forceps.
4. Place the removed exoskeleton and hypodermis in physiological saline (0.9% NaCl).
5. Remove carefully, the apparently visible bluish-hue glandular part (sinus gland) by lifting the gland from its base using a fine pair of forceps.
6. Replace the hypodermis and the exoskeleton to their respective positions.
7. Seal the groove between the margin of the opening and the rim of the replaced exoskeleton with cold paraffin wax.
8. Place the crab in a tank containing 50% sea water.

20.3. Eyestalk Ablation

Bliss *et al.* (1954) have observed that the effects of sinus gland removal is different from that of bilateral eyestalk removal. Bilateral eyestalk removal involves the removal of X-organ, sinus-gland and excision injury to the optic peduncle. Eyestalk can be removed very easily at the base and the loss of blood can be arrested by applying cold paraffin wax. The operation can be simplified if the crabs are wrapped with cloth leaving the eye exposed so that they cannot struggle and placed them on crushed ice to reduce their activity.

20.4. Influence of Eyestalk Hormones of *Scylla serrata* on Osmotic Regulation

Scylla serrata is exposed to fluctuating salinities (Joel, 1973). It is able to control to some extent its body fluid concentration. It is known that the salt and water balance is controlled at least in part, by hormone secreted by X-organ in eyestalk. The

hormone is also stored in the sinus gland. By extirpating the source of the hormone as well as the storage site (sinus gland), the osmoregulatory ability of the animal can be changed.

20.4.1. Procedure

1. Select 6 sets of intermoult, male and unautotomized crabs for this experiment.
   (*a*) *Animal* 1 : Normal, uninjured.
   (*b*) *Animal* 2 : Sinus gland removed after cutting the retinal portion of the eye cap.
   (*c*) *Animal* 3 : Sinus gland removed by simple excision without cutting the retinal portion of the eye cap.
   (*d*) *Animal* 4 : Bilateral eyestalk ablation.
   (*e*) *Animal* 5 : Eye cap covered with black paint to blind the animal.
   (*f*) *Animal* 6 : Dactylus injury as suitable control for the experiment.
2. Keep these animals immersed in separate tanks filled with water of particular salinity for two hours.
3. Determine the ammonia and chloride concentrations in the blood as well as in the media before keeping the crab in the media following the methods mentioned in 8.0 & 14.0.
4. After two hours, determine ammonia and chloride concentrations in the medium as mentioned earlier.
5. Then collect the blood from the crabs and determine ammonia and chloride concentrations.
6. Repeat the same experiment with different sets of animals with different salinities.

20.5 References

Bliss, D. E., J. B. Durand & J. H. Welsch, 1954. Neurosecretory systems in decapod Crustacea. *Z. Zellforsch.*, 39 : 520-536.

Joel, R. D. 1973. Studies on the biology and fisheries of the pulicate lake. Ph. D. Thesis, University of Madras, p. 140.

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PERCENTAGE COEFFICIENT OF VARIATION * 21

The amount of variation in population having different means is compared using the coefficient of variation.

$$\text{Coefficient of variation} = \frac{\text{Standard deviation}}{\text{Arithmetic Mean}}$$

where standard deviation is the amount of variation that can possibly deviate from the calculated arithmetic mean.

$$\therefore \text{Standard deviation} = \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{n-1}}$$

The coefficient of variation is usually quantified in terms of percentage (percentage coefficient of variation).

$$\text{Percentage coefficient of variation} = \frac{\text{Standard deviation}}{\text{Arithmetic mean}} \times 100$$

* Prepared by K. Kannan, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

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STUDENTS 't' TEST * 22

22.1 PAIRED SAMPLE 't' TEST

In 't' test for mean difference, the significance of a difference between 2 sample mean is tested. Under certain conditions 2 sets of sample values may be related to one another. Such paired data may be tested to see if the mean difference between the pair is significant by different from zero.

$$i.e.\ t = \frac{\text{mean difference between the pairs}}{\text{standard error of the mean difference}}$$

1. $x_1 - x_2 = y$
2. Σy
3. $(\Sigma y)^2$
4. Σy^2
5. $$\frac{\Sigma y^2 - \frac{(\Sigma y)^2}{n}}{n-1} = Sy$$

$$\therefore t = \frac{y}{Sy}$$

22.2. 't' TEST FOR MEAN DIFFERENCE

Whether the means of two normally distributed samples are significantly different at a particular level of probability or not may be tested with the following prerequisites.

1. Means of two samples ($\bar{x}_1$, $\bar{x}_2$), their differences, $\bar{x}_1 - \bar{x}_2$.
2. Variance (s_1^2, s_2^2) of two samples i.e.

$$\frac{\Sigma x_1^2 - \frac{(\Sigma x_1)^2}{n_1}}{n-1} = s_1^2 ; \qquad \frac{\Sigma x_2^2 - \frac{(\Sigma x_2)^2}{n_2}}{n_2 - 1} = s_2^2$$

* Prepared by M. Arumugam, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

3. The variance of means $\frac{s_1^2}{n}$, $\frac{s_2^2}{n}$ and

4. The variance of the difference between the two means 1 & 2 (sum of the variances of the samples).

5. Calculating standard error of the difference beween the variances of the means $\frac{s_1^2}{n} + \frac{s_2^2}{n}$

6. 't' is the ratio of the differences between the means and the standard error of difference between the variances of the means.

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{s_1^2}{n_1} + \frac{s_2^2}{n_2}}}$$

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CORRELATION COEFFICIENT * 23

Correlation coefficient (r) is an index of association between continuous variables. The coefficient 'r' may have values ranging from + 1 to − 1. r = + 1 corresponds to a rectilinear relationship (of the form y=a+bx) in which the two variables are positively related. r=—1 corresponds to a rectilinear relationship (of the form y=a-bx) in which the two variables are negatively related. Values of 'r' near + 1 (or—1) indicate an approach to a rectilinear relationship. But intermediate values of 'r' are more difficult to interpret (Parker, 1973). Values of 'r' near zero may arise under two conditions : 1. when there is no relationship and 2. when there is a real relationship but it is curvi-linear.

Therefore, prior to estimating 'r', a scatter diagram is prepared to assess whether the distribution of the data is bivariate normal. Although 'r' provides a measure of association between variables it does not in itself indicate the significant level of any association. When 'r' is small, there is a possibility of obtaining 'r' value deviating markedly from zero because of accidental 'covariation' in the few pairs of values involved. Therefore, it is necessary to test the significance of any deviation of the calculated value of 'r' from zero (which is given in correlation coefficient table).

$$r = \frac{\Sigma xy - \frac{(\Sigma x)\ (\Sigma y)}{n}}{\sqrt{\Sigma x^2 - \frac{(\Sigma x)^2}{n} \times \Sigma y^2 - \frac{(\Sigma y)^2}{n}}}$$

* Prepared by P. Mullainadhan, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

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REGRESSION (y=a+bx) * 24

In regression, the relationship of one variable with another is estimaed by expressing the one in terms of the linear function of the other. It is different from correlation (r) in that, in correlation the degree to which the two variables vary together is estimated. In both regression and correlation the values are continuous. The functional relationship in regression is a mathematical relationship which enables to predict the value of a variable y which corresponds to a given variable x. The relationship is determined by y=a+bx in which y is the function of x and is called the dependent variable, x the independent variable. By this formula when the independent variable (x) equals zero, the dependent variable equals 'a'. This point is the intersection of the function line with the y axis which is called as 'y-intercept', and 'b' refers to the slope or the gradient of the function y=a+bx. 'b' is called the regression coefficient and the formula is referred to as regression equation (Sokal & Rohlf, 1973).

The regression equation y=a+bx is calculated as follows :—

$$b = \frac{\Sigma xy - \frac{(\Sigma x)\ (\Sigma y)}{n}}{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}$$

$$a = \bar{y} - b\bar{x}$$

$$y = a + bx$$

* Prepared by S. Paulraj & P. Mullainadhan, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

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TESTS OF SIGNIFICANCE FOR REGRESSIONS * 25

25.1. 't' REGRESSION

This is a method of testing the significance of regression. Here we test whether the estimated value of slope (b) significantly deviates from zero. The 't' is calculated by the following formula.

$$t = b \sqrt{\frac{\Sigma x^2 - \frac{(\Sigma x)^2}{n}}{Sd^2}}$$

25.2. 'F' REGRESSION

The regression slopes of two dependent variables can be compared by F-test. The test for the difference (d) between two regression coefficients is given by the formula.

$$d = \frac{b_1 - b_2}{\sqrt{\frac{s_1^2}{\Sigma x_1^2 - \frac{(\Sigma x_1)^2}{n_1}} + \frac{s_2^2}{\Sigma x_2^2 - \frac{(\Sigma x_2)^2}{n_2}}}}$$

* Prepared by S. Paulraj & P. Mullainadhan, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

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ANALYSIS OF VARIANCE (ANOVA) * 26

In t-test, the difference between 2 sample means are tested for significance. In ANOVA the differences between means of more than 2 samples are tested for significance. This is done by examining the variation within the whole groups of sample means. It consists of a comparison between 2 estimates of the overall variation (of the complete set of measurements included in the analyses), one estimate being based on the variance of sample means about the grand mean. The other based on the variance of the individual measurements about their treatment means. The first estimate is called *treatment variance*. The second estimate is called *error variance*. If the null hypothesis is true, the ratio of these estimates would approximate 1. If, on the other hand, the sample means estimates differ from the population or group means then the ratio would exceed 1. In practice, this ratio is calculated as F and the level of probability of obtaining such a ratio is determined if the null hypothesis were to be true.

The following prerequisites are necessary for calculating the analysis of variance.

1. Different groups or treatments (T)
2. Sample size of each group (n)
3. Number of treatments (k)
4. Individual values (x)

With the above requirements the following are calculated :

1. Correction term (c) :

Correction term (c) is the square of the sum of all values of x divided by the total number of values $= (\Sigma x)^2/n$.

* Prepared by M. H. Subhashini & M. Arumugam, School of Pathobiology, Department of Zoology, University of Madras, Madras-600 005.

2. Total sum of squares (SS) :

In this, c is subtracted from the sum of squares of individual values of x.

$$\therefore \text{SS} = \Sigma x^2 - c$$

3. Sum of squares for treatments (SST) :

The sum of the squares of sum of each treatment is divided by the number of treatments and then the correction term is subtracted.

$$\text{SST} = \frac{T^2}{n} - c$$

4. Sum of squares for error (SSE) :

Sum of squares for treatments is subtracted from total sum of squares :

$$\therefore \text{SSE} = \text{SS} - \text{SST}$$

5. Degrees of freedom of SST and SSE.

6. Mean squares of SST and SSE :

This is calculated by dividing SST and SSE by its respective degrees of freedom.

7. The F- is calculated by dividing mean squares for treatments by mean squares of error.

The calculated F value is compared with the tabulated F value.

26.1 References

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