CMFRI SPECIAL PUBLICATION
Number 7

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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
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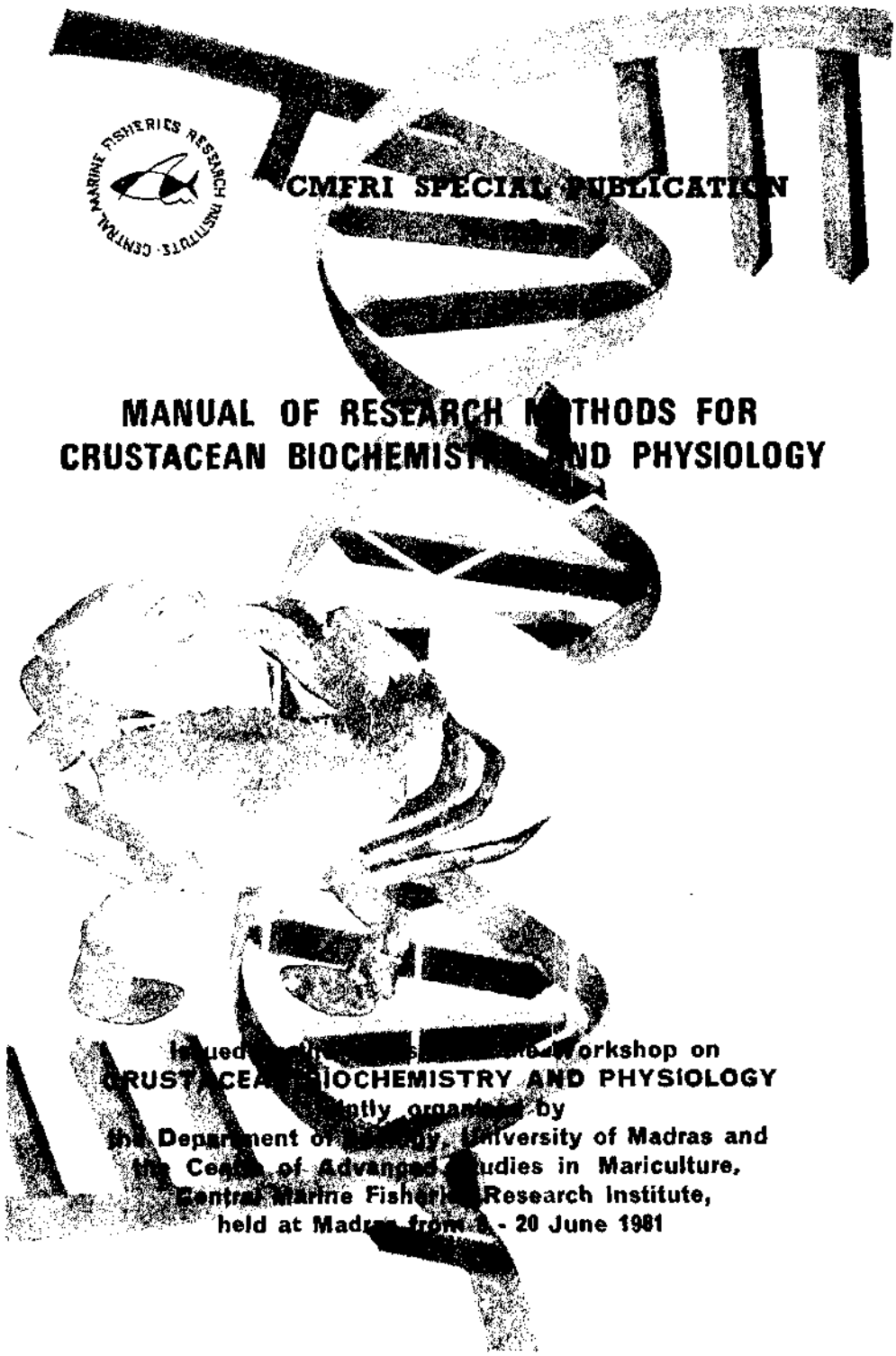

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Manual of Research Methods for Crustacean Biochemistry and Physiology

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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.

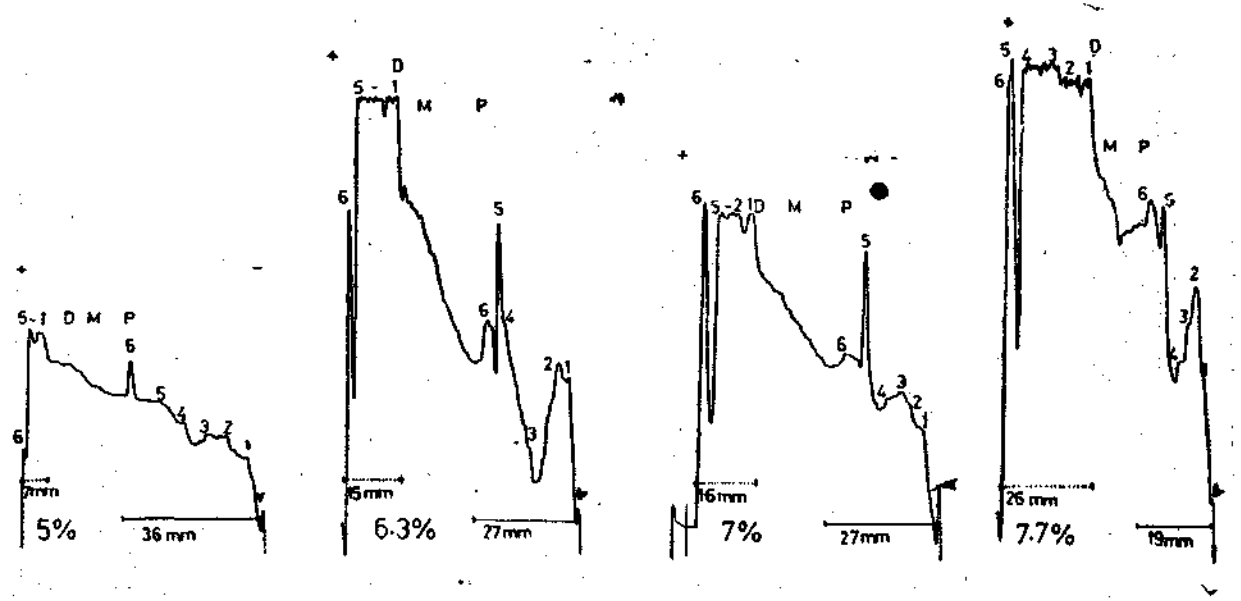

Fig. 1

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.

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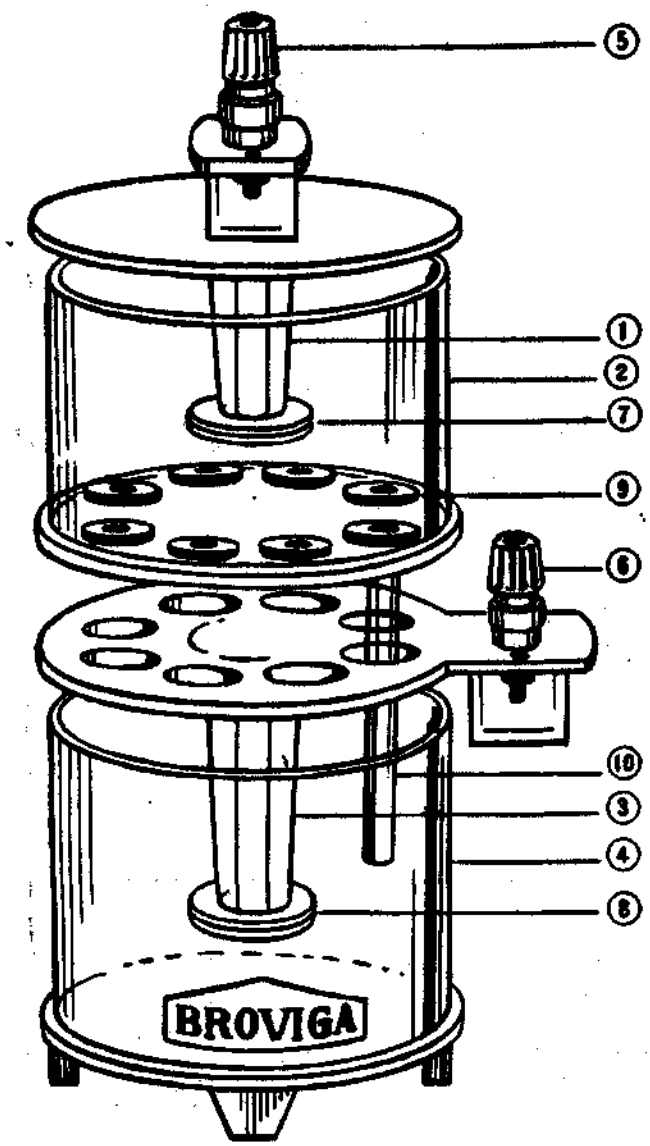

PLATE I

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).

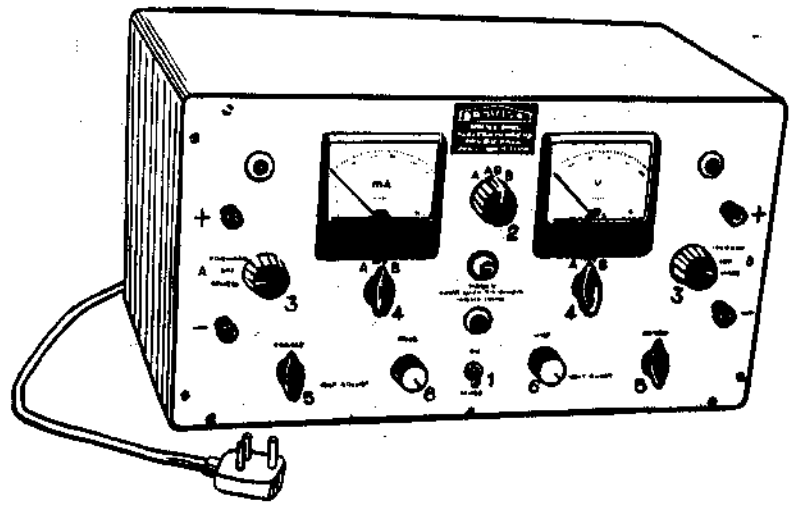

PLATE II

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

For your own notes