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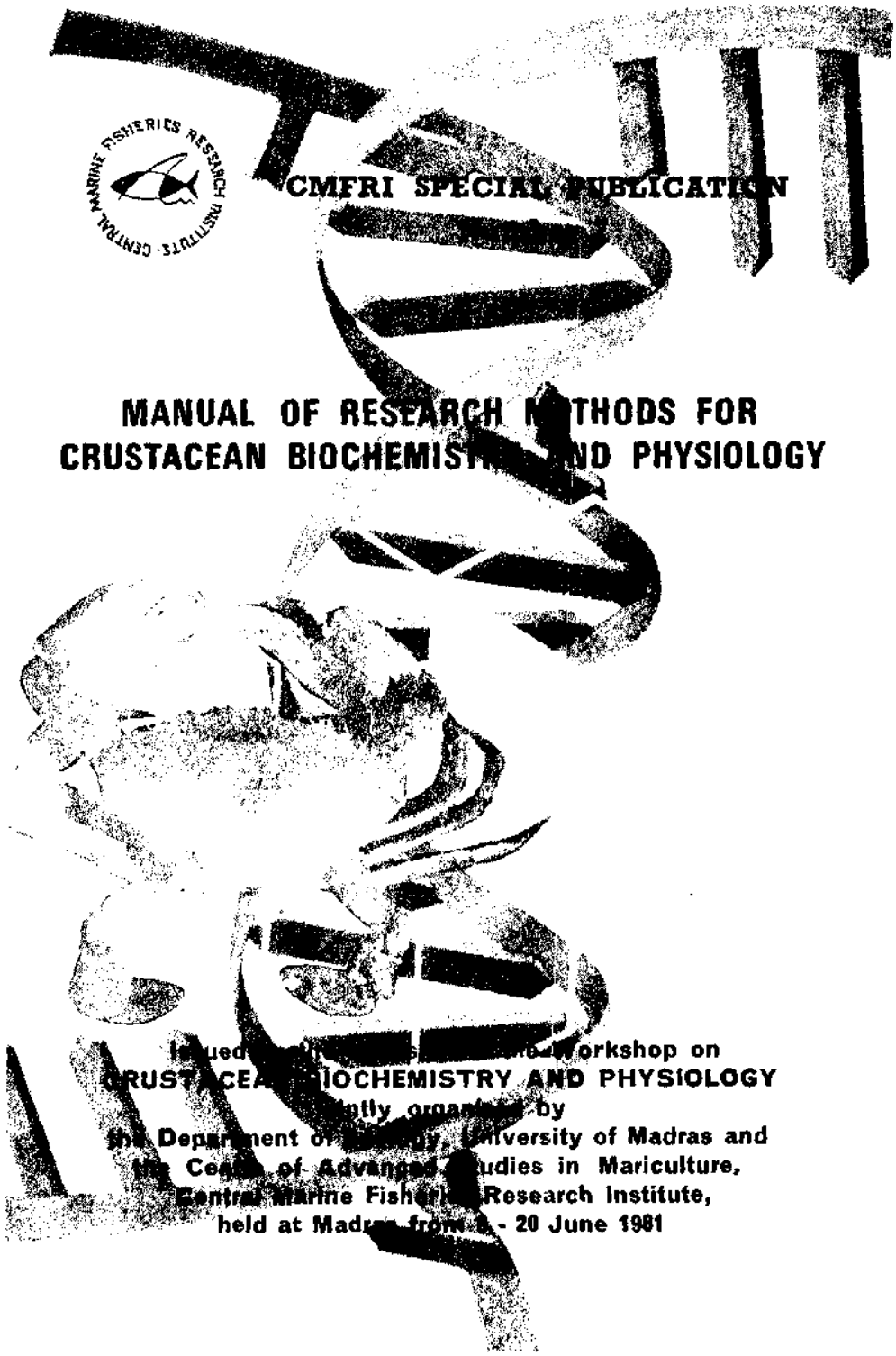
Number 7

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

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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.

For your own notes