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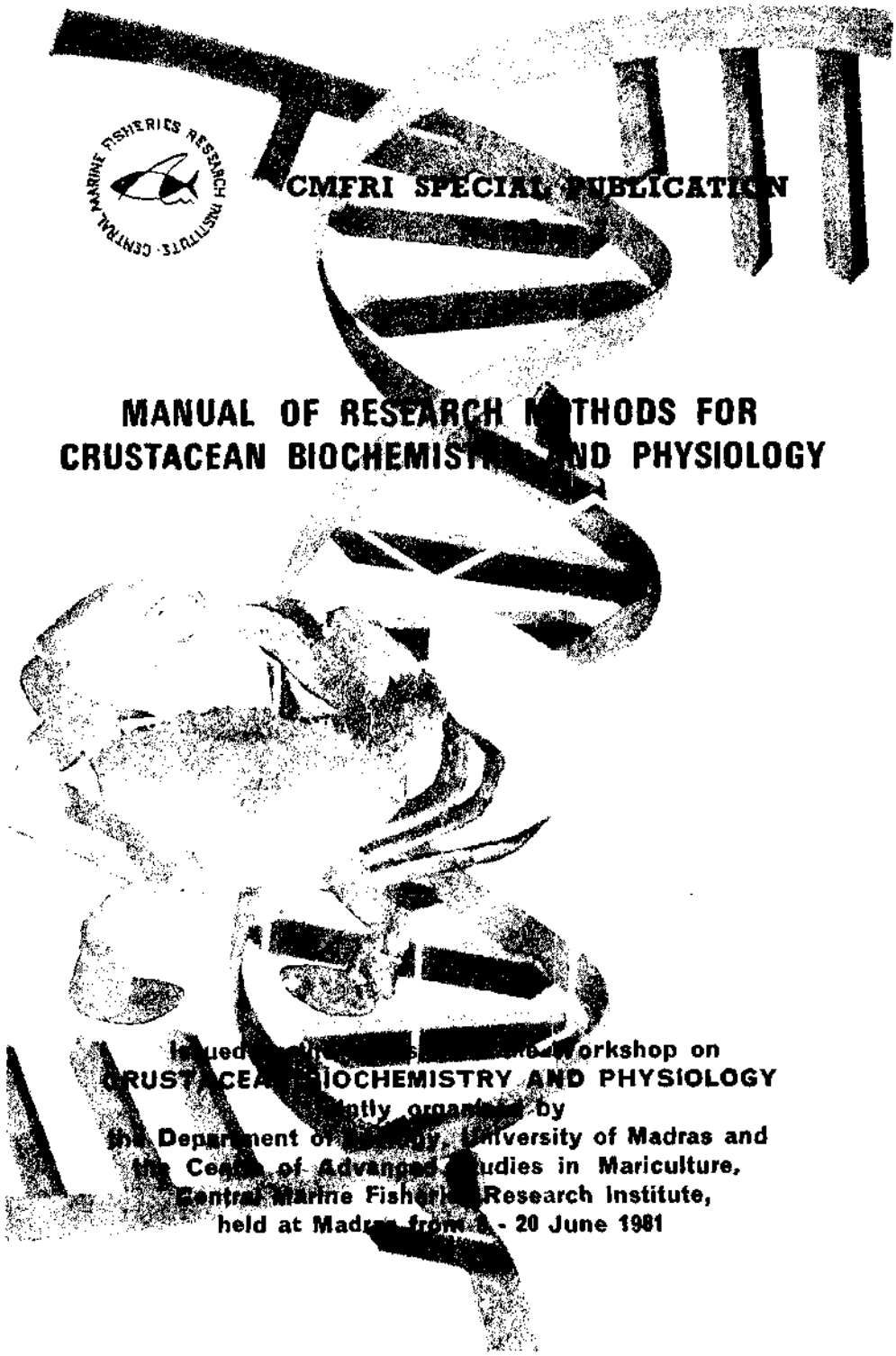
Issued on the occasion of the Workshop on
CRUSTACEAN BIOCHEMISTRY AND PHYSIOLOGY
jointly organised by
the Department of Zoology, University of Madras and
the Centre of Advanced Studies in Mariculture,
Central Marine Fisheries Research Institute,
held at Madras from 8 - 20 June 1981



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Manual of Research Methods for Crustacean Biochemistry and Physiology

EDITED BY

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(LIMITED DISTRIBUTION)

Published by : **E. G. SILAS**
Director
Central Marine Fisheries
Research Institute
Cochin 682 018

PRINTED IN INDIA
AT THE DIOCESAN PRESS, MADRAS 600 007—1981. C2375.

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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