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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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Central Marine Fisheries Research Institute,
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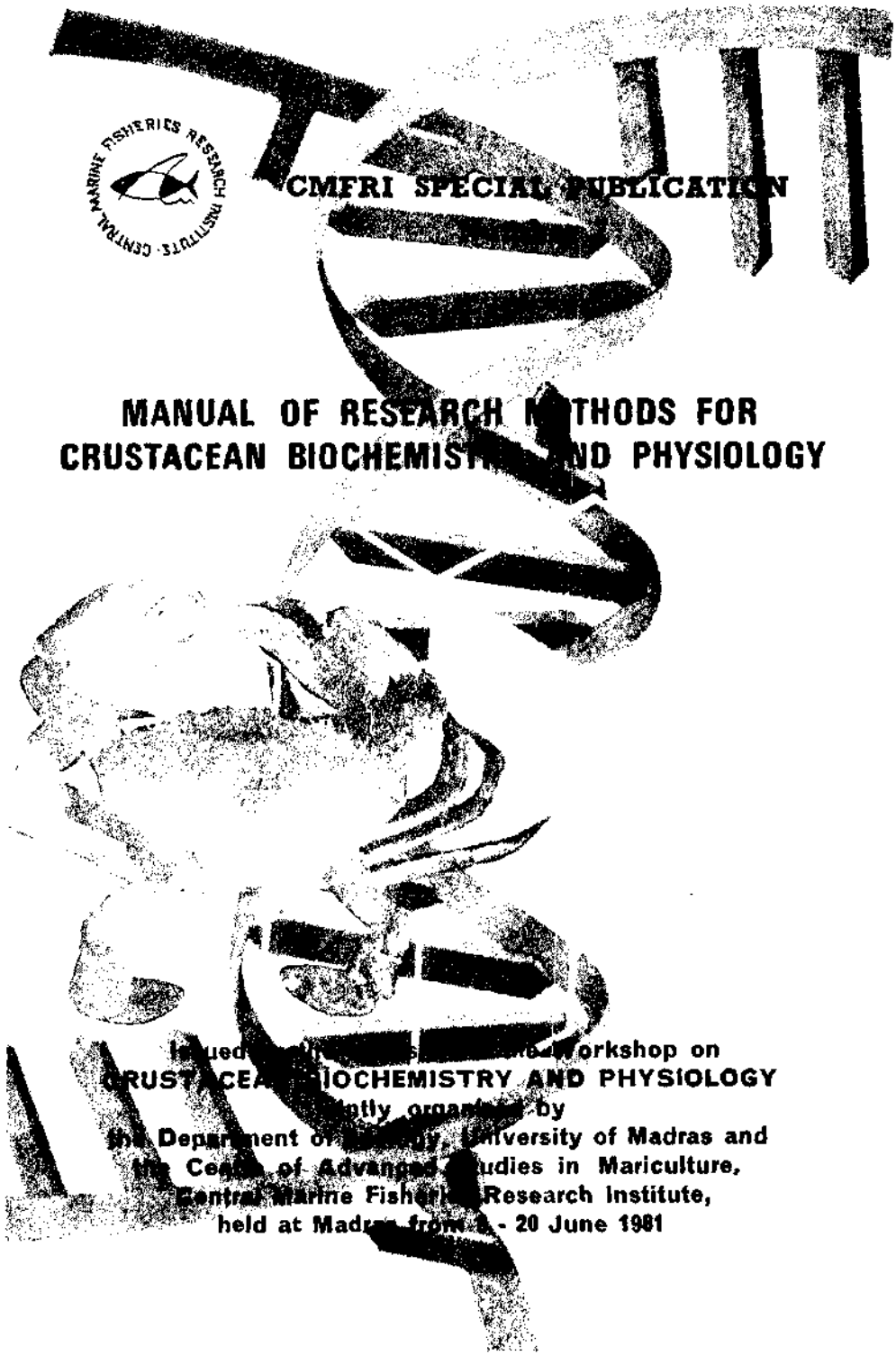

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Manual of Research Methods for Crustacean Biochemistry and Physiology

EDITED BY

M. H. RAVINDRANATH

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

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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

11.6 References

Arumugam, M. & M. H. Ravindranath, 1980. Significance of the variation in hemolymph copper-protein ratio of the crab *Scylla serrata* during different hours of the day. *Experientia* (Basel), 36 : (11) : 1306-1307.

Guest. R. J. 1953. Methods of copper in metallurgical analysis. *Anal. Chem.*, 25 : 1484-1486.

Holden, W. S. 1970. *Water treatment and examination*. J. and A. Churchil, London, pp. 513.

Klotz, I. M. & T. A. Klotz, 1955. Oxygen carrying proteins : A comparison of the oxygenation reaction in haemocyanin and hemoerythrin with that in hemoglobins. *Science* (*Wash*), 121 : 477-480.

Rice, E. W. 1960. Spectrophotometric determination of serum copper with oxalyldihydrizide. *J. Lab. Clin. Med.*, 55 : 325-328.

Robertson, J. D. & D. A. Webb, 1939. The microestimation of Na, K, Ca and SO_4 in sea water and body fluids of some marine animals. *J. Exp. Biol.*, 16 : 155-177.

Ventura, S. & S. King, 1951. Determination of copper and zinc in blood. *Biochem. J.*, 48 : 49.

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