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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
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Central Marine Fisheries Research Institute,
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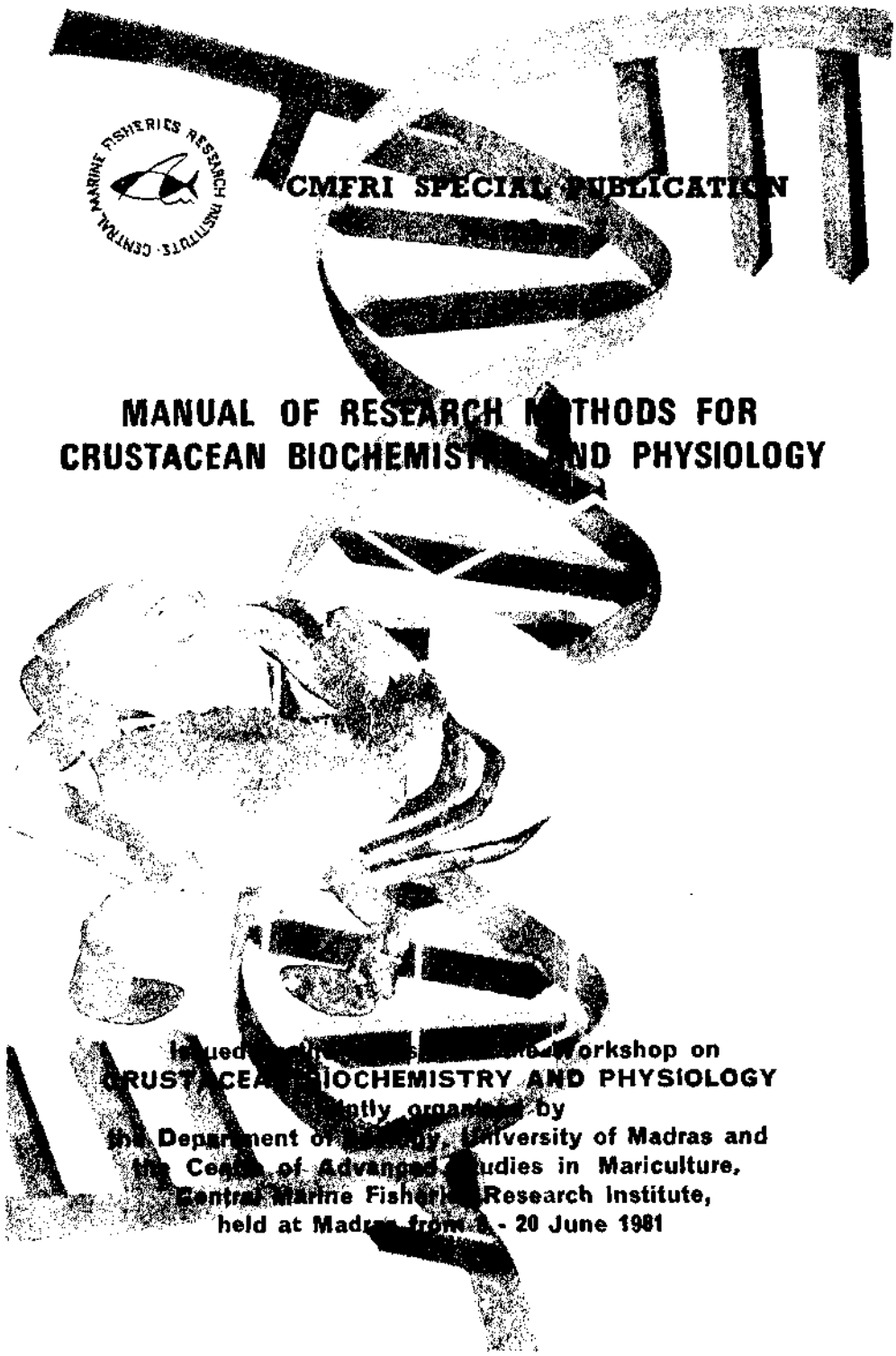

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Manual of Research Methods for Crustacean Biochemistry and Physiology

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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 chloraniliate 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

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