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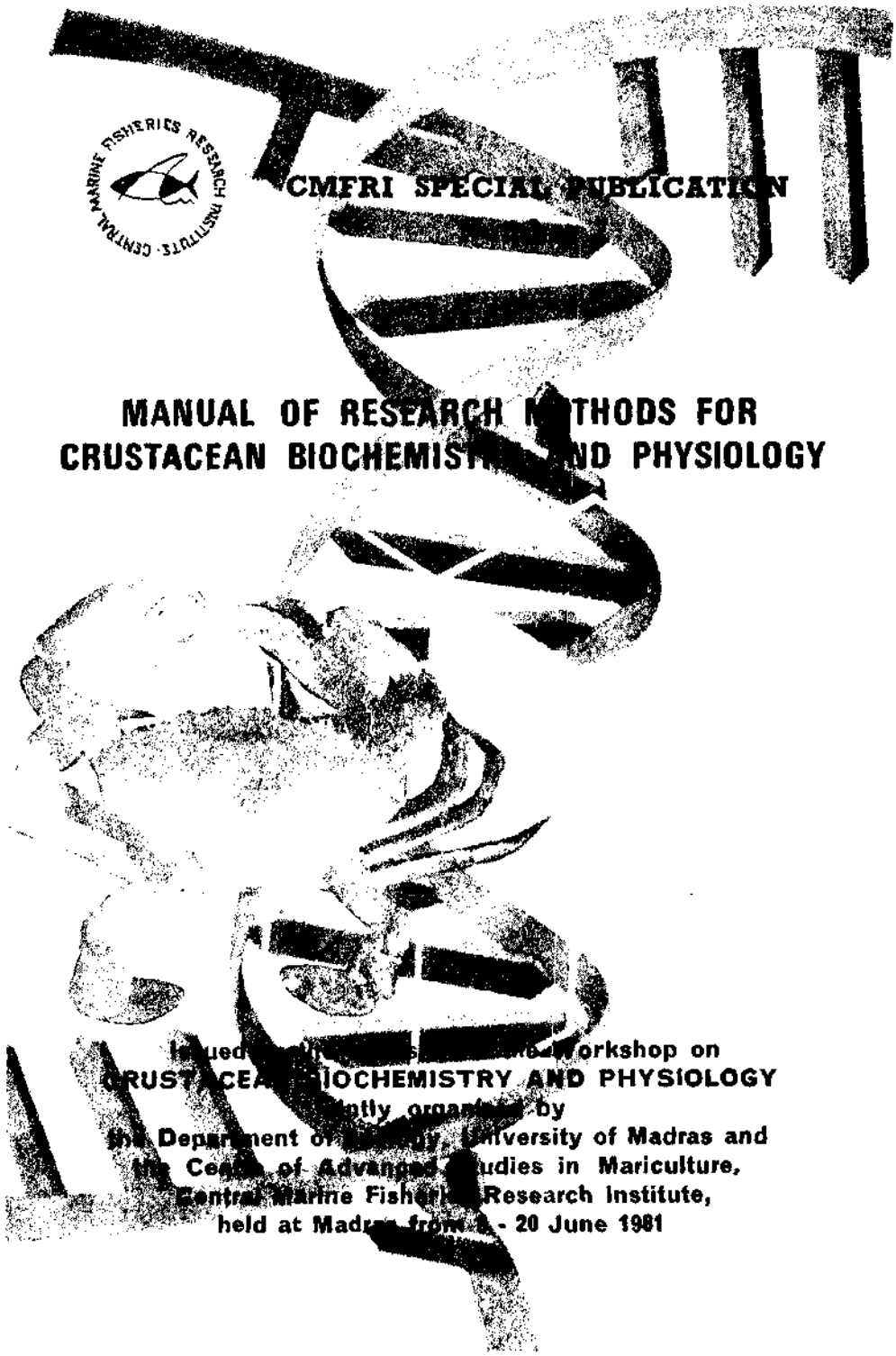
Number 7

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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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 8-30 JUNE, 1981

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6 PROTEINS *

6.1. MICRO-KJELDAHL METHOD

6.1.1. Principle

The nitrogen of the protein precipitate is converted to acid ammonium sulphate by digestion with sulphuric acid and various catalysts. On making the reaction mixture alkaline, ammonia is liberated, which is removed by steam distillation. The ammonia liberated by steam distillation is gathered in acid solution containing an indicator. Change in pH of the acid solution due to addition of ammonia is indicated by the indicator dye. This solution is back-titrated with 0.01 N HCI, and the original acidic condition indicated by the indicator dye, is taken as the end point. The amount of HCl consumed in back titration is proportional to the amount of ammonia liberated.

Percentage of nitrogen is calculated from the titre value, which is converted into gm% of protein present in sample by multiplying the percentage nitrogen with a factor 6.25. The factor 6.25 is used for conversion because in average, protein contains 16% of nitrogen.

6.1.2. Reagents

1. *Concentrated sulphuric acid.*
2. *Catalyst mixture* : 2 gm of selenium dioxide, 2 gm of copper sulphate and 8 gm of potassium sulphate-make fine powder.
3. *Sodium hydroxide-thiosulphate mixture* : 50 gm of sodium hydroxide and 5 gm of sodium thiosulphate in 100 ml of distilled water.

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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.

4. 4% *Boric acid*: Dissolve 4 gm of boric acid in 100 ml of distilled water.
5. *Indicator*: 100 mg of Methyl red and 25 mg of Methylene blue in 100 ml of 95% ethanol.
6. 0.01 *N HCl*: Dilute 0.9 ml of HCl to 100 ml with distilled water. Determine normality by titrating against alkali.

6.1.3. Procedure

1. Collect 0.05 ml of blood using a fine calibrated micro pipette and pour into 1 ml of deproteinizing agent (80% ethanol). Centrifuge at 3000 rpm for 3-5 minutes and decant the supernatant.
2. Allow the protein sample (precipitate) to be digested with 0.5 ml hot Conc. H_2SO_4 and a pinch of catalyst mixture for 4 hours at 380°C.
3. The copper and selenium of the catalyst mixture accelarates the rate of digestion.
4. After completion of digestion, cool the digested mixture and add 5 ml of distilled water.
5. Transfer the material to steam distillation unit and add 5 ml of sodium hydroxide-thiosulphate mixture.
6. Collect the ammonia that is distilled from acid ammonium sulphate in 5 ml of 4% boric acid containing one drop of indicator dye.
7. Collect the ammonia till the colour of boric acid changes to green.
8. Back titrate the green coloured boric acid with 0.01 N HCl till it revives the original colour.

6.1.4. Calculation

Calculate the percentage of nitrogen present in the sample by,

$$\frac{\text{titre value} \times 0.14008 \times 100}{\text{Amount of sample}} = \% \text{ of nitrogen.}$$

Each ml of 0.01 N HCl = 0.14008 mg of nitrogen. In order to convert the obtained % of nitrogen into gm % of protein present in sample, multiply the percentage value of nitrogen with a factor 6.25 (The factor 6.25 is used for conversion because protein contains an average of 16% of nitrogen).

6.1.5. Interpretation

This method was used by earlier crustacean investigators (Travis, 1955) for determination of blood proteins. In this procedure the nitrogen value obtained by titration was multiplied by a factor of 6.25 to convert grams of nitrogen to grams of protein. The factor is used on an assumption that the average protein contains 16% of nitrogen. As has been pointed out by Young (1963), the absolute value of nitrogen in specific protein is uncertain and varies from one protein to another. Therefore the author considers that the use of the factor 6.25 to calculate the concentration of protein in an unknown mixture such as crustacean blood must be regarded in approximation with possible error. Bailey (1967) also states 'the most accurate value for the nitrogen content of protein are obtained from samples free of lipids and polysaccharides' (p.346). The proteins in the blood of crustaceans are known to be bound with lipids and polysaccharides. These moieties associated with protein would considerably affect the % of nitrogen content and therefore the factor may not be reliable.

6.2. Biuret Method

6.2.1. Principle

Two carbamyl groups present in protein molecules combine with copper and potassium of the biuret reagent to form a blue coloured copper—potassium—biuret compound. The colour formed is proportional to the amount of carbamyl groups present in the protein (Gornall *et al.*, 1949).

6.2.2. Reagents

1. *1N NaOH*: Dissolve 4 gm of NaOH pellets in 100 ml of distilled water.

2. *Biuret reagent* : Dissolve 1.5 gm of cupric sulphate ($CuSO_4.5H_2O$) and 6.0 gm of sodium potassium tartrate ($KNaC_4H_4O_6.4H_2O$) in 500 ml of distilled water. Add 300 ml of 10% sodium hydroxide solution and make upto 1000 ml with distilled water.

6.2.3. Procedure

6.2.3.1. *Preparation of standard graph*

Dissolve 25 mg of bovine serum albumin crystals in little amount of 1N NaOH in a 5 ml standard flask and make up to 5 ml with 1N NaOH. This serves as standard protein solution.

Take known volume of protein solution containing known concentration of protein, in separate test tubes (for e.g., 5 ml of stock solution contains 25 mg of protein ; 0.2 ml of stock solution contains 1 mg of protein ; 0.4 ml of stock solution contains 2 mg of protein ; 0.6 ml of stock solution contains 3 mg of protein ; 0.8 ml contains 5 mg of protein and so on). Make up these solutions to 2 ml individually with 1N NaOH. Afterwards add 8 ml of biuret reagent, mix well and allow it to stand at room temperature. Set up the blank having 2 ml of 1N NaOH and 8 ml of biuret reagent. After 30 minutes, measure the optical density at 540 nm in a spectrophotometer. Plot the concentration of protein in X-axis and optical density at Y-axis and draw slope.

6.2.3.2. *Estimation of protein*

1. Collect 0.05 ml of blood using a fine calibrated micropipette and pour into 1 ml of deproteinizing agent (80% ethanol).
2. Centrifuge at 3000 rpm for 5 minutes, decant the supernatant and add 2 ml of 1N NaOH to dissolve the precipitate.
3. After 10 minutes, add 8 ml of biuret reagent, mix well and allow it to stand at room temperature.
4. Set up blank simultaneously having 2 ml of 1 *N NaOH* and 8 ml of biuret reagent.
5. After 30 minutes, measure the optical density in a spectrophotometer at 540 nm against the blank.
6. Refer the optical density in a standard graph and find out the protein concentration.

6.3. FOLIN-CIOCALTEU METHOD

6.3.1. Principle

The principle of this method involves two steps. The carbamyl groups of protein molecules react with copper and potassium of the reagent to give a blue coloured copper potassium-biuret complex. This complex together with tyrosine and phenolic compounds present in the protein reduce the phospho molybdate of the Folin reagent to intensify the colour of the solution (Lowry *et al.*, 1951).

6.3.2. Reagents

1. *1N NaOH* : Dissolve 4 gm of NaOH in 100 ml of distilled water.
2. *0.1N NaOH* : Dissolve 0.4 gm of NaOH in 100 ml of distilled water.
3. *Reagent A* : Dissolve 2 gm of sodium carbonate (Na_2CO_3) in 100 ml of 0.1N NaOH.
4. *Reagent B* : Dissolve 500 mg of cupric sulphate ($CuSO_4$ $5.H_2O$) in 1% sodium or potassium tartrate. Prepare afresh.
5. Mix 50 ml of reagent (A) with 1 ml of reagent (B).
6. *1N Folin reagent* : Dilute Folin-phenol reagent with equal volume of double distilled water. (Determine the normality by titrating the diluted Folin-phenol reagent with 1N NaOH using phenolpthalin as indicator).

6.3.3. Procedure

6.3.3.1. *Preparation of standard graph*

Stock and standard solutions are prepared as mentioned in 5.2.3.

Make up the volume of different concentrations of standard solutions to 1 ml with 1N NaOH individually. Add 5 ml of reagent (5) and mix well. After 10 minutes add 0.5 ml of Folin - phenol reagent and mix rapidly. Set up blank simultaneously. After 30 minutes, measure the optical density at 500 nm in a spectrophotometer.

Plot the concentration of protein in X-axis and optical density in Y-axis and draw a slope.

6.3.3.2. *Estimation of protein*

1. Collect 0.05 ml of blood using a fine graduated micropipette and pour into 1 ml of deproteinizing agent (80% ethanol.)
2. Centrifuge at 3000 rpm for 5 minutes and decant the supernatant.
3. Dissolve the precipitate in 1 ml of 1N NaOH.
4. Add 5 ml of reagent (5) and mix well.
5. After 10 minutes add 0.5 ml of Folin-phenol reagent and mix rapidly.
6. Set up blank simultaneously.
7. After 30 minutes measure the optical density of the blue colour developed by the sample at 500 nm.
8. Calculate the protein concentration by referring the O.D. obtained for the sample using the standard graph.

6.4. Interpretation

Biuret method is considered to be less sensitive than the Folin-Ciocalteu method (Young, 1963 ; Bailey, 1967). Bailey (1967) on an examination of Folin-Ciocalteu reagent has shown that ' any peptide bond will yield some colour but certain amino acid sequences not necessarily containing aromatic residues are more chromogenic than others and largely account for the colour yield of protein ' (p. 31). The author has also shown that ' a preliminary complete hydrolysis reduces the colour yielding property of albumin by more than 2/3' (p. 241). Shao-Chia & Goldstein (1960-as cited by Bailey, 1967) have shown that the cleavage of disulphide bonds in insulin by oxidation with performic acid gave a loss of about 1/3 of colour. These observations reveal that in addition to copper-potassium complex and aromatic residues, there may be other chromogenic reagents in protein to bring about colour production.

For crustacean blood the relative performance of Microkjeldahl, Biuret and Folin-Ciocalteu methods were assessed using different deproteinizing agents. The results are presented in Table 1.

1. With reference to biuret and Kjeldahl methods all precipitants behave similarly and values did not differ significantly between precipitants.

2. In Folin-Ciocalteu method the values obtained with tungstic acid as precipitant are low.

3. Comparing the methods *per se*, values obtained with the Kjeldahl method with different precipitants are significantly lower than those obtained with other methods.

4. Values obtained with the biuret and Folin-Ciocalteu methods are similar. However, taking into consideration the least coefficient of variation, the biuret method shows a lower coefficient of variation than Folin-Ciocalteu method in 8 out of 12 analyses. The biuret method appears preferable to the Folin-Ciocalteu method with 80% ethanol as protein precipitant. (Subhashini & Ravindranath 1980.)

TABLE 1. *Total protein concentration of the haemolymph of crab* Scylla serrata *determined by different methods using different protein precipitants*

Animal	*Methods*	10% TCA	80% EtOH	*Zinc Hydroxide*	*Tungstic acid*	*Statistical Remarks*
	Biuret	7.52±0.75 9.93% (5)	7.66±0.54 6.93% (5)	7.64±0.52 6.81% (5)	7.87±1.02 12.96% (5)	NS at $P>0.05$
I	Folin-Ciocalteu	7.23±1.08 14.90% (4)	7.48±1.33 17.70% (4)	7.78±0.99 12.70% (4)	5.88±0.73 12.40% (5)	NS at $P>0.05$
	Kjeldahl	5.33±0.63 11.82% (2)	5.02±0.60 11.95% (5)	4.78±1.02 21.34% (4)	3.58±0.76 21.20% (5)	NS at $P>0.05$

TABLE 1. (*Contd.*)

Animal	*Methods*	10%TCA	80%EtOH	*Zinc Hydroxide*	*Tungstic acid*	*Statistical Remarks*
	Biuret	8.96±0.66 7.37% (5)	8.96±0 86 9.60% (5)	9.29±0.69 7.43% (5)	8.75±1.19 13.60% (4)	NS at $P>0.05$
II	Folin- Ciocalteu	8.68±0.86 9.91% (5)	9.87±1.27 12.87% (4)	9.25±0.77 8.32% (5)	8.34±0.71 8.51% (4)	NS at $P>0.05$
	Kjeldahl	9.63 (1)	7.77±0.65 8.37% (5)	6.83±0.52 7.61% (2)	8.55±0.83 9.71% (4)	NS at $P>0.05$
	Biuret	5.82±0.27 4.64% (5)	7.25±0.62 8.54% (4)	6.76±0.27 3.92% (5)	5.80±0.53 9.12% (5)	NS at $P>0.01$ S at $P<0.05$
III	Folin- Ciocalteu	7.14±0.63 8.84% (5)	7.53±0.42 5.58% (5)	8.16±0.11 1.35% (5)	5.96±0.71 11.82% (5)	S at $P<0.05$ S at $P<0.01$
	Kjeldahl	5.25±0.28 5.35% (5)	5.31±0.66 12.43% (3)	5.21±0.46 8.76% (5)	4.73±1.21 25.62% (3)	NS at $P>0.05$

Values presented include : Mean±SD.
Coefficient of variation % (Sample size)
(Statistical remarks are based on analysis of variance between precipitants.)

TABLE 2. *Criteria used in the assessment of three different methods of determination of protein concentration in the blood of* Scylla serrata.

	Criteria	*Bluret*	*Folin-Ciocalteu*	*Kjeldahl*
I	Reproducibility (or) Consistency in performance (based on less co-efficient of variation)	66%*	33%*	.. (mean not comparable)
II	Simplicity			
	—No. of reagents added	one	two	more
	—No. of standard graphs needed	one (at 540 nm)	two (at 500 nm & 750 nm)	..
	—Time taken after centrifugation	35-40 mins.	45-50 mins.	3-4 hours
III	Stability (of protein - reagent complex)	Stable for more than 6 hours at 30°C.	not stable after 30 minutes at 30°C	
IV	Sensitivity (based on determination of concentration of protein with the method)	Only above 20 lamda	even to 0.2 lamda	above 1,000 lamda
V	Susceptibility (to interfering substances)	Less (due to Cu binding alone)	More (due to Cu binding and reduction of phosphomolybdate)	Less (due to variability in conversion factor due to protein, lipids & polysaccharides)
VI	Suitability (of serum albumin as standard)	suitable	Not suitable (due to other chromogenic substances in the blood proteins)	Not suitable (for reasons mentioned in V)

* Based on 12 sets of analyses (Subhashini, 1977).

An assessment was made regarding the suitability of these 3 methods in determination of blood proteins of Crustacea. The criteria used are presented in Table 2. The protein values obtained by Kjeldahl method is considerably lower than the values obtained with Folin-Ciocalteu and Biuret methods. Possibly due to the presence of lipids and polysaccharides associated with blood proteins, the percentage of nitrogen values are affected and as a result the conversion factor is higher than what has been conventionally employed. Probably that is why in Kjeldahl method the protein values are lower than the values obtained with the other two methods.

The protein values obtained with Folin-Ciocalteu method parallels with the values obtained with Biuret method. Folin-Ciocalteu method is more susceptible to interfering substances than Biuret method, possibly due to the mechanism of colour reaction. The first step in the principle of this method is very similar to the principle of Biuret method in that the copper binds with carbamyl groups of protein and gives colour. This coloured product is directly estimated in biuret method. In Folin-Ciocalteu method, the coloured copper-protein complex is made to reduce the phosphomolybdate of Folin-phenol reagent. In this method it is not only the copper-protein complex that reduce the phosphomolybdate but also the tyrosyl residues present in the protein, the amount of which differs from one protein to another. As a result the colour reaction in Folin-Ciocalteu method differs from one protein to another (Young, 1963). Bailey's (1967) observations reveal that several other reactive sites present in the proteins are also capable of yielding some colour.

6.5 References

Bailey, J. L. 1967. *Techniques in Protein chemistry*. Elsevier Publishing Company, New York.

Gornall, A. G., C. J. Bardawill & M. M. David, 1949. Determination of total serum protein by means of biuret reaction. *J. Biol. Chem.*, **177** : 751-766.

Lowry, O. H., N. J. Rosebrough, A. L. Farr & R.J. Randall, 1951. Protein measurement with folin phenol reagent. *Ibid.*, **193** : 265-275.

SUBHASHINI, M. H. 1977. Factors influencing individual variability in the haemolymph protein concentration of decapods. M. Phil. Dissertation, University of Madras. p.70.

SUBHASHINI, M. H. & M. H. RAVINDRANATH, 1980. Haemolymph protein concentration of *Scylla serrata* : assessment of quantitative methods and intra-individual variability. *Arch Int Physiol. Biochim.*, **88** : 47-51.

TRAVIS, D. F. 1955. The moulting cycle of the spiny lobster, *Panulirus argus* Latreille. III. Physiological changes which occur in the blood and urine during the normal moulting cycle. *Biol. Bull. (Woods Hole)*, **109** : 484-503.

YOUNG, E. G. 1963. Occurrence, classification, preparation and analysis of proteins. *In* '*Comprehensive Biochemistry*' Vol. 7. (Ed. Florkin, M. & E. N. Stotz) pp. 1-55. Elsevier Publishing Company, New York.

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