CMFRI SPECIAL PUBLICATION
Number 9

MANUAL OF RESEARCH METHODS FOR MARINE INVERTEBRATE REPRODUCTION

Issued on the occasion of the Workshop on
MARINE INVERTEBRATE REPRODUCTION
jointly organised by
the Department of Zoology, University of Madras and
the Centre of Advanced Studies in Mariculture,
Central Marine Fisheries Research Institute, Cochin
held at the University of Madras
from 25th October to 10th November 1982

The Centre of Advanced Studies in Mariculture was started in 1979 at the Central Marine Fisheries Research Institute, Cochin. This is one of the Sub-projects of the ICAR/UNDP project on 'Post-graduate agricultural education and research'. The main objective of the CAS in Mariculture is to catalyse research and education in mariculture which forms a definite means and prospective sector to augment fish production of the country. The main functions of the Centre are to :

—provide adequate facilities to carry out research of excellence in mariculture/coastal aquaculture ;

—improve the quality of post-graduate education in mariculture ;

—make available the modern facilities, equipments and the literature ;

—enhance the competance of professional staff ;

—develop linkages between the Centre and other Institutions in the country and overseas ;

—undertake collaboration programmes ; and

—organise seminars and workshops.

Under the programmes of the Centre, post-graduate courses leading to M.Sc. (Mariculture) and Ph.D. are offered in collaboration with the University of Cochin since 1980.

Front cover : SEM picture showing surface topography of *Streptocephalus dichotomus* egg.

Manual of Research Methods for Marine Invertebrate Reproduction

EDITED BY

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ISSUED ON THE OCCASION OF THE WORKSHOP ON MARINE INVERTEBRATE REPRODUCTION 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 THE UNIVERSITY OF MADRAS FROM 25TH OCTOBER TO 10TH NOVEMBER, 1982.

(LIMITED CIRCULATION)

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PREFACE

The technologies of controlled reproduction, induction of spawning, sex reversal, artificial fertilisation, sterilisation and preservation of gametes are increasingly applied in aquaculture to obtain quality seed, quality fish stock and better yield. In this context, researches on different aspects of reproduction, developmental biology and physiology have assumed considerable importance besides their values in understanding of the ontogeny of the organisms. Extensive researches carried out in recent years from several laboratories in the world have not only accumulated a body of information, but also broughtforth several new concepts to our understanding of the development and reproductive behaviour of finfishes and shellfishes.

In India, directed research on reproductive physiology and biology is taken up only recently and the field is still in an infant stage. In view of its emerging importance, it is identified as an area for priority research and for expertise development in the programmes of the Centre of Advanced Studies in Mariculture at the Central Marine Fisheries Research Institute, and several programmes of research are being taken up in this field with particular reference to the reproductive behaviour of the cultivable finfishes and shellfishes.

Advances made on the frontiers of invertebrate reproduction in recent years have been significant enough to organise a national workshop and to prepare a manual on research methodologies for the study of the subject. Several histological, histochemical and biochemical methods and sophisticated instruments have been introduced in these studies making it essential that the scholars who desire to work and specialise in the field are given adequate basic information on the research methods so as to enable them to appreciate and advance research to understand the problems confronted in the field.

The present manual, the third in the series, is prepared and compiled by Dr. T. Subramoniam, Leader of the ' Unit of

Invertebrate Reproduction' of the Zoology Department of the University of Madras, Tamil Nadu. During the past decade, a team of research scholars are working on different aspects of marine invertebrate reproduction including the cultivable crustaceans such as *Scylla serrata*, *Panulirus homarus* and *Macrobrachium* spp. under his leadership. Contributing to our knowledge on the subject, the research results achieved so far in these aspects by the Unit have unfolded several new concepts in oogenesis, spermatogenesis, sperm transfer strategy, fertilization and endocrine control of reproduction and gamete formation.

I wish to express my great appreciation to Dr. T. Subramoniam and his team of Scholars, who by their dedication and interest evolved a series of tested research methods and set a theme of investigation through insight and skill on marine invertebrate reproduction. I am sure that this manual will be of immense use to the research scholars and scientists who would like to specialise in the subject and cognate fields.

This is the second workshop we are organising in close collaboration with the University of Madras. I wish to express my gratitude to Dr. M. Santappa, Vice-Chancellor, University of Madras for the keen interest evinced in such collaborative programmes and for the advice. I am also indebted to Dr. K. Ramalingam, Professor and Head of the Department of Zoology, University of Madras for productive discussions, continuous support and suggestions. I wish to thank Shri P. T. Meenakshisundaram and Shri K. Rengarajan, Scientists of the Central Marine Fisheries Research Institute for their help in the preparation of this manual.

E. G. Silas,
Director, C.M.F.R.I.

II. VITELLOGENESIS

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HISTOCHEMICAL PROCEDURES FOR CHARACTERIZING THE CRUSTACEAN YOLK*

2.1. Introduction

Histochemical studies on yolk formation in crustaceans not only reveal the chemical nature of various yolk substances but also provide information on the temporal pattern of yolk accumulation and their spatial distribution. In crustaceans the accumulation of yolk material starts with the dispersion of nucleolar extrusions in the ooplasm. The yolk protein to be detected first in the ooplasm is in general glycolipoprotein in nature. This is followed by the accumulation of a glycolipoprotein substance that is presumed to be originating from extra-ovarian sources. Apart from this, discrete lipid globules have also been found to be deposited at various stages of vitellogenesis. In any study on vitellogenesis, a preliminary histochemical characterization of the sequentially deposited yolk materials is essential in view of the variability in the biochemical composition of yolk among different crustacean species. Such differences in the biochemical composition of yolk in turn reflects the nature of embryonic development. In the present experiment a battery of histochemical procedures is given to detect the major deutoplasmic substances such as the basophilic granules, (ribosomal RNA), protein, carbohydrate and lipid. The rationale of the tests is also given along with the procedure.

2.2. Materials

Paraffin and cryocut sections of the ovary of the anomuran crab, *Emerita asiatica*.

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* Prepared and verified by Sudha Varadarajan, Zoological Survey of India, Madras, M. Panneerselvam and T. Subramoniam, Unit of Invertebrate Reproduction, Department of Zoology, University of Madras, Madras-600 005.

2.3. PREPARATION OF FIXATIVES

1. *Neutral buffered formaldehyde :* Add 100 ml 40% formaldehyde to 900 ml distilled water. To the above solution, dissolve 4 gm sodium dihydrogenphosphate and 6.5 gm disodium hydrogenphosphate. Shake well.
2. *Carnoy's :* Add 60 ml ethyl alcohol to 30 ml chloroform. To this, add 10 ml glacial acetic acid.
3. *Formol-calcium :* Add 100 ml 40% formaldehye to 500 ml distilled water. Dissolve 10 gm anhydrous granular calcium chloride to the above solution. Shake well and make upto 1 litre. Store with marble chips.

2.4. TESTS FOR PROTEINS

2.4.1. Mercuric Bromophenol Blue Test for Protein

Principle

Mercuric ions of the bromophenol blue solution react with acidic, sulphydryl and aromatic residues of the protein to give blue colour.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagent

Mercuric bromophenol blue : Dissolve 1 gm mercuric chloride and 0.05 gm bromophenol blue in 100 ml 2% aqueous acetic acid.

Method

Bring sections to water. Stain in the mercuric bromophenol blue solution for 2 hours at room temperatue. Rinse sections for 5 minutes in 0.5% acetic acid. Transfer sections dirctly into tertiary butyl alcohol. Clear in xylene and mount in DPX.

Result

Proteins—deep blue colour.

2.4.2. Aqueous Bromophenol Blue Test for Basic Proteins

Principle

Bromophenol blue is an acidic dye which in aqueous medium is capable of reacting with the basic reactive groups. The acidic groups of the dye react with basic groups of the protein to give blue colour.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagent

0.1% *Bromophenol blue :* Dissolve 0.1 gm bromophenol blue in 100 ml double distilled water.

Method

Bring sections to water and stain in 0.1% bromophenol blue solution for 5 minutes at room temperature. Wash in double distilled water and observe.

Control : Deamination (*vide* section 2.7.1.).

Result

Basic proteins—blue.

2.4.3. Ninhydrin-Schiff Test for Amino Groups

Principle

In the course of oxidative deamination with ninhydrin stable tissue aldehydes are produced. These are demonstrated with Schiff's reagent.

Fixation and Section

Carnoy's ; paraffin, cryostat.

Reagents

1. 0.5% *Ninhydrin :* Dissolve 0.5 gm ninhydrin in 100 ml absolute alcohol.
2. *Schiff's reagent :* Dissolve 1 gm basic fuchsin in 200 ml boiling distilled water. Shake for 5 minutes and cool to

keep in exactly 50°C and add to the filtrate 20 ml 1 N hydrochloric acid ; cool to 25°C and add 1 gm sodium metabisulphite. Stand in the dark for 14-24 hours in frig. Add 2 gm activated charcoal and shake for 1 minute. Keep the filtrate in the dark at 0-4°C. Allow to reach room temperature before use.

Method

Bring sections to water. Treat sections with 0.5% ninhydrin solution for 16-20 hours at 37°C. Wash gently in running water, 2-5 minutes. Immerse in Schiff's reagent, 15-25 minutes. Wash in running tap water, 10 minutes. Dehydrate, clear and mount in DPX.

Control : Deamination (*vide* section 2.7.1.).

Result

Amino groups—pinkish red to magenta.

2.4.4. Toluidine Blue Test for Acidic Groups

Principle

Toluidine blue is a basic dye which in aqueous medium reacts with the acidic groups of protein to give blue colour.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagent

1% *Toluidine blue :* Dissolve 1 gm toluidine blue in 100 ml of double distilled water.

Method

Bring sections to water. Stain in 1% toluidine blue for 10 minutes. Wash in water and observe.

Control : Methylation (*vide* section 2.7.2.)

Result

Acidic group—red or pink or purple ; Nuclei—blue.

2.4.5. Ferric-Ferricyanide Method for—SH Groups

Principle

This method depends on the reduction of a fresh solution of potassium ferricyanide in acid solution at pH 2.4 by sulphydryl groups in the tissues. The resulting ferrocyanide combines with ferric ion (in ferric sulphate) to give an insoluble prussian blue precipitate.

Fixation and Section

10% neutral buffered formalin ; paraffin, cryostat.

Reagent

Ferricyanide reagent : Add 3 parts of 1% aqueous ferric sulphate to 1 part of 0.1% aqueous potassium ferricyanide and adjust to pH 2.4.

Method

Wash sections in distilled water. Immerse in 3 changes of the ferric cyanide reagent for 20-25 minutes (paraffin sections) or 10-20 minutes (fresh smears). Wash in distilled water. Dehydrate, clear and mount. Brief rinsing in 2% alkaline alcohol (2 gm NaOH in 60% alcohol) before dehydration reduces diffuse blue background staining.

Control : Mercaptide (*vide* section 2.7.4.)

Result

Sulphydryl groups—blue.

2.4.6. Thioglycollate Ferric-Ferricyanide Method for SS Groups

Principle

Unreactive disulphide groups are reduced to reactive sulphydryl groups by thioglycollate. The sulphydryl groups reduce the ferricyanide. The other reactions are as given in 2.4.5.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagent

2.5% *Sodium thioglycollate :* Dissolve 2.5 gm sodium thioglycollate in 100 ml double distilled water and adjust to pH 8.

Method

Treat 2 sets of sections simultaneously. Bring both sections to water, then immerse them for 30 minutes in 2.5% sodium thioglycollate. Wash in weakly acidified distilled water (pH 4) for 3 minutes ; wash in running tap water for 3 minutes. Rinse in distilled water. Transfer the 2nd (control) slide to phenyl mercuric chloride in butanol (48 hours). After blocking, bring back to water. Both sections should now be immersed in a fresh solution containing 10 ml freshly made 1% aqueous potassium ferrocyanide and 30 ml 1% aqueous ferric chloride (just filtered). Leave for 1 minute. Wash in 3 changes of distilled water for 10 minutes. Dehydrate, clear and mount in DPX.

Control : Thioglycollate reduction (*vide* section 2.7.7.).

Result

Disulphide groups—prussian blue.

2.4.7. Millon's Test for Tyrosine

Principle

When Millon's reagent, a mixture of mercurous and mercuric nitrates and excess of nitric acid, is added to the protein and the mixture is heated for few minutes, a white precipitate is formed which may turn yellow and then red if the reacting protein contains tyrosine. The reaction is specific for hydroxy phenyl groups unsubstituted in the meta position.

Fixation and Section

10% neutral buffered formalin ; paraffin or cryostat.

Reagent

Millon's reagent : Add 10 gm mercuric sulphate to 100 ml 10% sulphuric acid and heat until dissolved. Make up to 200 ml. Add 0.5 ml. 0.25 % aqueous sodium nitrite solution to 5 ml of the above solution.

Method

Hydrate sections, place them in a small beaker containing the reagent and leave in an oven until low boil. Bring sections to room temperature. Remove sections and wash in distilled water, three times (wash each slide for 2 minutes). Dehydrate, clear and mount in DPX. Repeat with fresh tissue smears and note the diffrrence.

Control : Iodination (*vide* section 2.7.5.).

Result

Tyrosyl groups—red or pink.

2.4.8. DMAB-Nitrite Method for Tryptophan

Principle

The aldehyde component of the p-dimethylamino-benzaldehyde (DMAB) solution reacts with the tryptophanyl reactive sites and forms a blue coloured compound called β-carboline pigment. Sodium nitrite solution is used to itensify the colour of the β-carboline pigment.

Fixation and Section

Fresh material ; cryostat.

Reagent

5% *DMAB* : Dissolve 5 gm DMAB in 100 ml hydrochloric acid.

Method

Bring sections to absolute alcohol and allow them to become just dry in the air at room temperature. Immerse sections in 5% DMAB for 1 minute. Transfer to 1% sodium nitrite solution in concentrated hydrochloric acid for a further minute. Wash in tap water for 30 seconds. Rinse in 1% acid alcohol, dehydrate, clear and mount in DPX.

Control : Formaldehyde (*vide* section 2.7.6.).

Result

Tryptophanyl groups—deep blue.

2.5.1. Periodic Acid—Schiff Technique

Principle

Periodic acid, an oxidant breaks the C-C bonds where these are available as 1,2 glycols, converts them into dialdehydes but does not oxidise the aldehyde further and these can be localised by Schiff's reagent.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagents

1. *Periodir acid*: Dissolve 0.4 gm periodic acid in 35 ml ethyl alcohol. Add 5 ml 0.2 M Sodium acetate (27.2 gm of the hydrated salt in 1000 ml) to 10 ml distilled water. Keep in dark at 17°C—22°C and use at this temperature. Discard if brown colour appears.
2. *Schiff's reagent :* (*vide* section 2.4.3.).

Method

Bring sections to water. Oxidise for 10 minutes in periodic acid. Wash in running water : 5 minutes. Immerse in Schiff's reagent : 10 minutes. Wash in running water : 5 minutes. Dehydrate and mount in DPX.

Control : Deamination, acetylation, deacetylation, chloroform/ methanol extraction, pyridine extraction and taka diastase (*vide* sections 2.7.1., 2.7.8—2.7.12.).

Result

Glycogen—deep magenta ; Other hexose containing muco substances—shades of purplish red.

2.5.2. Best's Carmine Test for Glycogen

Principle

Carminic acid at a pH on the alkaline side of its isoelectric point is negatively charged and behaves like an acid dye staining 1, 2 glycol groups, perhaps by hydrogen bonding.

Fixation and Section

Carnoy's ; paraffin.

Reagents

1. *Carmine stock solution :* Add 2 gm carmine, 1 gm potassium carbonate and 5 gm potassium chloride to 60 ml distilled water. Boil gently for 5 minutes, cool and filter. Add 20 ml ammonia (Sp. gr. 0.88) to the filtrate. This solution lasts for 3 months at 0°—4°C.
2. *Carmine staining solution :* Dilute 15 ml stock solution with 12.5 ml ammonia (Sp. gr. 0.88) and 12.5 ml methyl alcohol. This solution lasts for 2-3 hours.
3. *Best's differentiator :* To 8 ml absolute alcohol, add 4 ml methyl alcohol and 10 ml distilled water.

Method

Bring sections to absolute alcohol. Place sections in 1% celloidin in absolute alcohol/ether (equal parts) for 2 minutes. Dry in air. Down grade to water. Stain in Ehrlich's haemalum : 5 minutes. Rinse and differentiate rapidly in 1% acid alcohol. Rinse in water. Stain in Best's carmine, 15-30 minutes. Differentiate in Best's differentiator without rinsing (5-60 seconds). Wash in 80% alcohol. Dehydrate in absolute alcohol. Clear in xylene and mount in DPX.

Control : Taka diastase (*vide* section 2.7.12.).

Result

Glycogen : red ; Nuclei : dark blue.

2.5.3. Toluidine Blue at different pH for Acid Mucopolysaccharides

Principle

Toluidine blue, a basic dye reacts with acid mucopolysaccharides (AMP) at different pH. At lower pH the dye colours the sulphated AMP whereas in higher pH it stains the phosphated

AMP. The metachromasia at lower and higher pH indicates the presence of sulphated and carboxylated AMP respectively.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagents

1. *Solution A :* Dissolve 40 mg toluidine blue in 25 ml 1 N sodium acetate solution (8.25 mg sodium acetate in 100 ml distilled water)

2. *Solution B :* 1 N hydrochloric acid. (Add 90 ml of the acid to 910 ml distilled water). Prepare toluidine blue at different pH as given below.

pH	Solution A	Solution B
1.09	20 ml	28 ml
1.99	20 ml	21 ml
3.09	20 ml	19.4 ml
4.19	20 ml	15 ml

7.00. Dissolve 8 mg toluidine blue in 20 ml distilled water.

Method

Bring sections to water and stain in toluidine blue at different pH for 20 minutes, wash in distilled water and observe.

Control : Methylation (*vide* section 2.7.2.).

Result

AMP	—blue
pH 1.09	—sulphated AMP
pH 1.99	—sulphated or phosphated AMP
pH 3.09 } pH 4.19 }	—sulphated AMP
pH 7.00	—carboxylated AMP

2.5.4. Critical Electrolyte Concentration (CEC) Method for Acid Mucopolysaccharides

Principle

Both sulphated mucins and glucosaminoglucouranoglycans containing carboxyl groups will bind with alcian blue *in situ* in

the presence of low concentrations (below 0.3 M) of electrolytes whereas only sulphated mucosubstances will do so with higher concentration (above 0.8 M).

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagents

1% *Alcian blue :* Dissolve 1 gm alcian blue 8GX in 100ml 0.05 M sodium acetate buffer (410 mg sodium acetate in 100 ml distilled water) at pH 5.7.

Prepare different molar concentrations of alcian blue using the table given below :

1% Alcian blue		Magnesium chloride		Molarity
25 ml	+	0.508 gm	—	0.1 M
25 ml	+	1.016 gm	—	0.2 M
25 ml	+	2.540 gm	—	0.5 M
25 ml	+	3.048 gm	—	0.6 M
25 ml	+	4.064 gm	—	0.8 M
25 ml	+	5.080 gm	—	1.0 M

Method

Bring sections to water. Stain in alcian blue for 30 minutes at different CEC. Wash in running water for 5 minutes. Dehydrate quickly in alcohols, clear in xylene and mount in DPX.

Result

Hyaluronic acid, sialomucins and some weakly acidic sulphomucins are not stained at or above 0.1 M magnesium chloride. Most sulphated mucosubstances stain strongly at 0.2 M levels. The various sulphated mucosubstances lose alcianophilia at different levels with increasing magnesium chloride concentration.

2.5.5. Bracco-Curti's Test for Sulphated Acid Mucopolysaccharides

Principle

Benzidine in 2% boric acid reacts with the sulphate groups of AMP to form benzidine sulphate. Potassium dichromate

oxidises the benzidine sulphate to give benzidine blue colour indicating the presence of sulphated acid mucopolysaccharides.

Fixation and Section

10% neutral buffered formalin ; paraffin.

Reagents

1. *1% Benzidine :* Dissolve 1 gm benzidine in saturated aqueous solution of boric acid.
2. *2% Boric acid :* Dissolve 2 gm boric acid in 100 ml distilled water.
3. *1% Potassium dichromate :* Dissolve 1 gm potassium dichromate in 100 ml distilled water.

Method

Bring sections to water and treat in benzidine boric acid mixture for 10 minutes. Wash with 2% boric acid thrice. Treat in 1% potassium dichromate solution for 30 minutes. Wash in distilled water and observe.

Result

Sulphated AMP—benzidine blue.

2.6. Tests for Lipids

Principle of lipid staining techniques in general

Lipid histochemistry is dependent on the solubility of the dyes in the fat themselves. The commonest dyes used for this purpose are Sudan black B, Oil red 'O' and Nile blue. Staining with these dyes depends largely on the type and concentration of the fluid in which they are dissolved or suspended ; but it is imperative that the solubility of the dye in fat exceeds its solubility in the solvent. Coupled with these techniques, extraction procedures are almost always employed where, after application of known lipid solvents, lipid tests are applied and the results compared with unextracted material.

2.6.1. Sudan Black B Test for Lipid

Principle

This is a diazo dye, and being slightly basic because of its amino groups, combines with the acidic groups of compound lipids such as phospholipids.

Fixation and Section

Formol-calcium ; frozen.

Reagents

1. *Sudan black B :* Prepare saturated solution of Sudan black B in 70% ethanol. Keep aside for a week.
2. *Differentiator :* 70% alcohol.

Method

Stain in Sudan black B for 15 minutes ; differentiate in 70% alcohol until a delipidized control section appears colourless, dry and mount in glycerine jelly. Treat pyridine extracted materials in the same manner.

Control : Pyridine extraction/Chloroform: methanol extraction (*vide* sections 2.7.10 ; 2.7.11.).

Result

Bound lipids and lipids—black or dark blue.

2.6.2. Nile Blue Method for Neutral and Acidic Lipids

Principle

Neutral lipids will dissolve out of aqueous solutions of Nile blue, only the oxazone and the free base (both red). Acidic lipids will dissolve the oxazone and combine with the free base to form blue lipid-soluble compounds.

Fixation and Section

Formol—calcium ; frozen.

Reagents :

1. *Sudan black B : vide* section 2.6.1.
2. 1% *Nile blue :* Dissolve 1 gm Nile blue in 100 ml of distilled water.

3. 0.02% *Nile blue:* Dissolve 20 mg Nile blue in 100 ml distilled water.
4. 1% *Acetic acid:* Add 1 ml acetic acid to 99 ml distilled water.

Method

Stain one section (A) in Sudan Black B in 70% alcohol as a control for lipid. Stain section B in 1% Nile blue at 60°C for 5 minutes. Wash quickly in water at 60°C for 5 minutes and differentiate in 1% acetic acid at 60°C for 30 seconds.

Stain another section C as B and restain in 0.02% Nile blue at 60°C. Wash and differentiate the section (as section B). Mount all sections in glycerine jelly.

Pyridine extracted control sections are stained with Sudan black B and compared with unextracted one. If there is no difference between B and C, the first may be discarded as what will stain with 1% Nile blue will also stain in 0.02%.

Control: Pyridine extraction/Chloroform : methanol extraction (*vide* sections 2.7.10 ; 2.7.11.).

Results

Neutral lipds—red.

Acidic lipids—blue

2.6.3. Nile Blue Method for Phospholipids

Since Nile blue Principle stained phospholipids are weakly acid fast while similarly stained proteins are not, a two stage differentiation, first with acetone at 50°C followed by one with weak acid is introduced into the Nile blue method.

Fixation and Section

Formol—calcium ; cryostat.

Reagent

Nile blue sulphate: Mix 500 ml saturated aqueous Nile blue sulphate solution with 50 ml 0.5% aqueous sulphuric acid. Boil for 2 hours before use.

Method

Stain for 90 minutes at 60°C in Nile blue sulphate solution. Rinse in distilled water. Place in acetone heated to 50°C. Remove sections from source of heat but allow sections to remain in it for 30 minutes. Differentiate in 5% acetic acid for 30 minutes. Rinse in distilled water. Differentiate again in 0.5% aqueous hydrochloric acid for 3 minutes. Wash in distilled water and mount in glycerine jelly.

Control: Pyridine extraction/Chloroform : methanol extraction (*vide* sections 2.7.10 ; 2.7.11).

Result

Phospholipids—blue.

2.6.4 Oil Red 'O' Method for Neutral Lipids

Oil red 'O' is superior to the red Sudan dyes as the colour is deeper, smaller droplets are better seen and there is less tendency to the formation of dye precipitates.

Fixation and Section

10% neutral buffered formalin ; cryostat.

Reagents

1. *Stock solution:* Add 0.5 gm oil red '0' to 100 ml 98% isopropanol.
2. *Staining solution:* Dilute 6 ml of the stock solution with 4 ml of water, stand for 24 hours and filter. Use this as a stock staining solution, filtering through Whatman No. 42 paper, sufficient amounts as and when necessary.

Method

Stain frozen sections after rinsing in water and then in 60% isopropanol, in freshly filtered oil red '0' solution for 10 minutes. Differentiate briefly in 60% isopropanol. (Keep tightly stoppered or make up fresh). Wash in running water for atleast 10 minutes. Mount in glycerine jelly.

Control: Pyridine extraction/Chloroform : methanol extraction (*vide* sections 2.7.10 ; 2.7.11).

Result

Neutral lipids—red.

2.6.5. U. V. Schiff Reaction for Unsaturated Lipids

Principle

Fresh smears or frozen sections, if subjected to long and short wave (254 nm) U. V. irradiation for 3-4 hours, treated Schiff's reagent and compared with untreated intact controls, the difference in staining intensity demonstrated the number of double bonds saturated by oxidation.

Fixation and Section

Cold neutral buffered formalin; cryostat.

Reagent

Schiff's reagent: vide section 2.4.3.

Method

Fix sections for 12-18 hours in cold 10% neutral buffered formalin. Place under a source of ultraviolet light for 2-4 hours. Treat with Schiff's reagent : 15 minutes. Rinse with 3 changes of sulphurous acid water. Rinse in distilled water. Mount in glycerine jelly.

Control : Pyridine extraction (*vide* section 2.7.11.).

Result

Magenta colour absent from unirradiated control sections indicates unsaturated lipids.

2.6.6. Sudan Black ' B ' Method for Masked Lipids

Principle

Pretreatment of tissue with various organic acids (acetic, citric, oxalic) and subsequent staining with a ripened 70% alcoholic Sudan black B would demonstrate the lipids, unmasked by the pretreatment.

Fixation and Section

Formalin vapour ; cryostat.

Reagents

1. *Sudan black B : vide* section 2.6.1

2. 70% *Alcohol :* Add 70 ml alcohol to 30 ml distilled water.

Method

Use fresh smears fixed in formalin vapour for 2-5 minutes. Immerse films in 25% aqueous acetic acid. Wash thoroughly in tap water, then distilled water and allow to dry. Stain in Sudan black B (this solution should be atleast one week old). Differentiate in 70% alcohol. Blot dry and mount in glycerine jelly.

Control : Pyridine extraction (*vide* section 2.7.11).

Result

Bound lipids and lipids—black.

2.7. Blocking Procedures

The blocking procedures generally serve to prove the presence of the specific reactive group and the removal of interfering groups. Histochemical tests should therefore be carried out with suitable controls.

2.7.1. Deamination

Reagents

1. 3% *Sulphuric acid :* Add 3 ml concentrated sulphuric acid to 97 ml distilled water.

2. 1% *Sodium nitrite :* Dissolve 1 gm sodium nitrite in 100 ml 3% aqueous sulphuric acid.

Method

Immerse the hydrated sections in 1% sodium nitrite solution for 48 hours at 5°C.

2.7.2. Methylation

Reagent

I N Hydrochloric acid : Measure 91.2 ml methanol and to it add 8.8 ml concentrated hydrochloric acid.

Method

Treat the hydrated sections with 1 N hydrochloric acid for 96 hours at 37°C.

2.7.3. Demethylation

Reagent

5% *Potassium permanganate :* Dissolve 5 gm potassium permanganate in 100 ml distilled water.

Method

Treat the methylated sections with 5% potassium permanganate for 20 minutes at 37°C.

2.7.4. Mercaptide

Reagent

Mercuric chloride : Prepare a saturated solution of Mercuric chloride in distilled water. Immerse the hydrated sections in the mercuric chloride solution for 1 hour at 30°C.

2.7.5. Iodination

Reagents

1. *Iodine solution :* Dissolve 1 gm iodine and 2 gm potassium iodide in 300 ml distilled water.
2. 3% *Ammonia :* Add 3 ml ammonia with 97 ml distilled water.

Method

Treat the hydrated sections with iodination reagent for 24 hours at 30°C.

2.7.6. Formaldehyde

Treat the hydrated sections with 40% formaldehyde for 1 hour at 30°C.

2.7.7. Thioglycollate Reduction

Reagents

1. 0.5 *M Thioglycollate :* Dissolve 4.6 ml thioglycollic acid in 100 ml distilled water (adjust to pH 8 with 0.1 N sodium hydroxide).
2. 0.1 *N Sodium hydroxide :* Dissolve 400 mg sodium hydroxide in 100 ml distilled water.

Method

Treat the sections with 0.5 M thioglycollate for 4 hours at 37°C.

2.7.8. Acetylation

Reagent

Acetic anhydride and pyridine mixture : Mix equal volume of acetic anhydride and pyridine solutions.

Method

Immerse the hydrated sections in acetic anhydride and pyridine mixture for overnight at 22°C.

2.7.9. Deacetylation

Reagents

1. 70% *Alcohol :* Add 30 ml distilled water with 70 ml ethyl alcohol.
2. 1% *Potassium hydroxide :* 1 gm potassium hydroxide in 100 ml 70% alcohol.

Method

Treat the acetylated sections with 1% potassium hydroxide for 20 minutes at room temperature.

2.7.10. Chloroform Methanol Extraction

Keep the hydrated sections in chloroform: methanol (1 : 1) mixture for 18 hours at 60°C.

2.7.11. Pyridine Extraction

Reagents

1. *Bouin's fluid: vide* expt. No. 1.
2. *Dichromate—calcium:* Dissolve 5 gm potassium dichromate and 1 gm anhydrous calcium chloride in 25 ml distilled water and make it up to 100 ml.

Method

Fix in weak Bouin's fluid for 20 hours. Wash in alcohol. Immerse in pyridine at 17°—22°C for 30 minutes: immerse in pyridine at 60°C for 24 hours. Wash in running water for 2 hours. Transfer to dichromate—calcium mordant.

2.7.12. Taka Diastase Treatment

Treat the sections with taka diastase for 20 minutes at room temperature.

2.8. Observation

Tabulate the histochemical reactions for protein, carbohydrate and lipid obtained on the various yolk components of the oocytes in different stages of maturation. Also indicate the intensity of reaction.

2.9. Reference

Pearse, A. G. E. 1968. Histochemistry: Theoritical and applied. Vol. I. 3rd Edn., J & A. Churchill Ltd., pp. 758.