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CYTOLOGICAL METHODS IN ALGAE

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Abstract

Eukaryotic algae differ from other eukaryotic organisms in having zygotic, gametic and sporic meiosis. The growth of algal thallus occurs through somatic divisions in apical, intercalary and basal cells. Somatic divisions also occur in reproductive bodies of haploid thallus. The nuclear divisions in algae is influenced by physico - chemical conditions of ambiances, age and physiological nature of thallus and reproductive structures, there is great difficulty in finding the time and location of nuclear divisions. Since there is variability in the chemical constituents' algal cell walls, great care is needed in selecting suitable fixatives, softening agents and nuclear stains. The present paper not only looks into the historical background of karyological studies in algae but also gives a detailed account of the time and place of nuclear divisions, fixing fluids, softening agents, stains and staining methods and induction methods of nuclear divisions in different algal groups. The karyotypic analysis helps in determining the cyto-taxonomy of algae.

Key words: Algae, nuclear divisions, fixatives, stains, chromosomes and karyotypes

Introduction

Despite an early start of algal cytology by Timberlake (1901) considerable contributions have been made in this field only after the middle of present century. There exist many problems in algal cytology. Lack of an algal cytological literature dealing with problems such as the nature of the cell division, nature and functions of pyrenoids, chloroplasts etc. and the technical difficulties incurred in dealing with many algae especially those possessing mucilaginous or gelatinous cell walls, proper choice of materials, scarcity of nuclear divisions, the minute size of nuclei and chromosomes some of which are even inaccessible to the microscope, the absence of proper fixatives and staining methods are some of reasons or impediments in the progress in algal cytology. Despite all these studies have shown many algae are known to have the conventional type of mitosis.

Several workers have emphasized the importance of algal cytology in studies relating to life histories and in the taxonomical delimitations of particular taxa. 'The Chromosomes of Algae' edited by Godward (1966) did give an account of the historical background and progress that has been made in the field of algal cytology.

Chromosome study from living cells present considerable difficulties. The phase difference or more precisely the difference in refractive indices between cellular components being insignificant, it is difficult to resolve the components separately under light microscope. For the study of chromosomes from living cells, ultra structural pattern and quantitative estimation of chromosome components different types of microscopy are employed.

In order to study chromosome structure under light microscope, the tissue is killed and the tissue parts selectively preserved without causing any appreciable distortion of nuclear matter through a process known as *fixation*. During this process the cells are killed and dividing nuclei are fixed in particular phase of division in which they were lying at the time of treatment. Chromosome study is usually performed after subsequent washing, processing and staining in suitable dyes.

It has been observed that mere fixation alone may not prove adequate for the analysis chromosome structure. This is particularly true for nuclei having high chromosome number or very small chromosomes. In the former case, all the chromosomes form an aggregate lump, the individual structure thus being discernible. In

the latter case, on the other hand, the chromosomes may undergo too much swelling to allow any analysis of details. The non- availability of adequate method of fixation and lack of suitable schedule prior to fixation, lead to the publication of large number of papers in which the description of chromosome structure was either absent or inadequate.

As the study of chromosomes forms the basis for cytogenetics, it is important that practical methods for their display be available for all levels of teaching and research. In this review we intend to bring all those fixatives and staining methods successfully employed in algae . The paraffin embedding and staining that has been used by earlier workers have been omitted since this technique is rarely used for chromosomal studies and is well described in 'Plant Micro-technique' (Johansen, 1940). Vidyavati (1994) already gave detailed account of how to prepare algal tissues for electron microscopy.

Fixation

Fixation may be defined as the process by which tissues or their components are fixed to a desired effect. The purpose of fixation is to kill the tissues without causing any distortion of components to be studied. Suitable fixation which is absolutely essential for the study of chromosome structure has always intrigued cytologists. Even now very little is known about the exact changes undergone at an intracellular level following fixation and as such the cytologists are handicapped in having to rely partly on chances.

The term fixation was previously used in a wide sense involving principally the mere visibility of chromosome structure. With the extension of the scope of cytological study, the necessity for a differential analysis of chromosome segments were felt, fixation, as is now realized, must be critical. It should not only increase the visibility of chromosome structure and morphology but should also reveal the chromosome positions such as chromatic and heterochromatic regions, primary and secondary constrictions. In view of the limited number and inadequacy of techniques that were available in the past, chromosomes could not be studied in the desired extent in majority of the species. The inadequacy of the technique was mostly due to the fact the earlier workers did not realize the importance of critical fixation in the analysis of chromosome structure.

The study of fixation and its principles in it is a specialized line of investigation. Species differ greatly in their response to a fixing material. A fixative proved to be suitable for a species may be inadequate for other. All these facts imply that the cytoplasmic constitution of each individual is one of the principle factors controlling its response to a chemical fixative. The limitations in the analysis of the exact effect of fixation at an intercellular level are the inherent complexity of all biological objects. In every multicellular living organism so many intracellular variable factors are present making it very difficult to analyze the effect of the fixing chemical on any one of them.

All the fixing reagents possess certain common characteristics which are needed for fixative. Each fixing chemical is lethal in its action. Levan (1949) has classified the lethal chemicals into two categories: (i) compounds that cause pyknosis of chromosomes or detachment of nucleic acids from the protein thread, (ii) compounds that maintain the chromosome structure intact. Therefore, lethality must not be associated with pyknosis of chromosomes or the dissolution of the nucleic acid from the protein thread. The structural integrity of the chromosome must be maintained intact. Precipitation of the chromatin matter is an essential to render the chromosome visible and to increase its basophilic nature in staining. Under living conditions, the phase differences between different components of the cell are not enough to permit them to be observed as distinct entities. Coagulation of protein and subsequent precipitation cause a marked change in the refractive index of chromosomes helping to appear as differential bodies within the cell. All fixatives, so far used, have the property of cross-linking proteins. The primary requisite of a fixative for the study of chromosomes is therefore, is the possession of the property of precipitating chromatin. A fixative for chromosome is generally is a mixture of several compounds and at least one of its constituents must have this property.

Another important requisite of the fixative is that it should have the property of rapid penetration so that the tissue is killed instantaneously, the divisional figure being arrested at their respective phases. Immediate killing is essential as otherwise the nuclear division may proceed and attain the so called 'resting' or metabolic phase. Divisional configurations are necessary for visualizing the chromosome morphology and numbers correctly.

With the death of the cells certain changes occur that are detrimental to the preservation of chromosome structure. The most important change is the autolysis of protein. Under normal conditions of the tissue, enzymes are present which help to build up proteins. With lethality, as medium become acidic, these enzymes act in the reverse direction and cause a breakdown of the complex protein into simpler amino acids. As polypeptide form one of the principal constituents of the chromosomes, its

effect of denaturation will cause ultimate disorganization of chromosome structure. Therefore, a fixative should also be able to check autolysis of proteins. With the onset of lethality, bacterial action caused the tissues to decompose. Another prerequisite for a good fixative is to prevent this decomposition by maintaining an aseptic condition in which bacterial decay cannot take place.

As the purpose of chromosome study is to observe the minute details of chromosome morphology the staining should be perfect. A number of chemicals such as formaldehyde may affect the basophils of the chromosomes. Such chemicals when used alone cannot be recommended for chromosome fixation. A proper fixative should, in general, enhance the basophils of the chromosome.

A fixing mixture which fulfills this conditions detailed above can be considered to be a truly effective fixative for chromosome study. But since all these properties are rarely to be found within a single chemical, a fixative is generally a combination of several compatible fluids which jointly satisfy all the above requirements.

The swelling of cells and shrinkage of chromosomes are the two principal factors controlling the merit of a fixative. It has always been difficult to check the initial changes in the volume occurring during fixation. Undoubtedly, with larger molecules of fixatives the initial swelling is retarded.

The main chemicals that have been used as fixatives or more precisely as ingredients of a fixating mixture are:

a) **Non-metallic:** ethyl alcohol, methyl alcohol, acetic acid, formaldehyde. propionic acid, picric acid, chloroform etc.

b) **Metallic:** chromic acid, osmic acid, platinic chloride, mercuric chloride, uranium nitrate etc. Some of these are used in the form of vapour also.

Non-metallic fixatives, except formation in cyto-chemical work, have one advantage over metallic ones that no washing in water is needed after fixation.

Time of fixation

One of the technical difficulties in algal cytology is to fix the material at its proper time of nuclear division. Several claims have been made about the nuclear divisions in algae. Organisms which grow in defined medium nuclear divisions are found to be more when aged cultures were transferred to fresh medium or 2-3 hrs after the commencement of dark period. In uniseriate filamentous forms the nuclear divisions are mostly occurs in apical cells while those producing branches nuclear divisions start at the time of small protuberances arising from cells. A constant observation under microscope is a necessity (Jose, 1976).

Marine algae, are generally difficult to grow in defined medium and exhibit very slow growth rate in culture conditions, largely fixed from natural collections, various claims have been made about the most favourable time for nuclear divisions. Singh (1973) obtained increased nuclear divisions in marine siphonales when materials fixed 2-3 hr immediately after dark period. Yamnouchi (1906) claimed abundant nuclear divisions in *Fucus* after 1-2 hours immersion by the incoming tide. Robert (1966) reported higher nuclear divisions in young and actively growing materials of a variety of members of Fuculaes fixed immediately after exposure to the ebbing tides. In totally submerged forms meiotic divisions occur in the early hours of morning but this not true with all species. The periodic release of reproductive bodies by marine algae appears to be associated with mass nuclear maturation of the products and lunar cycle. Meristamatic tissues of brown and red algae as well as their reproductive structures are good resources for karyological studies. In many cases the duration time of nuclear division is very short and it requires fixing of materials at very short intervals.

Collection of Samples

Unicellular/colonial forms are concentrated before fixation by centrifugation. If centrifugation disrupts algal cell walls only decantation is done. Filamentous and parenchymatous forms are handled with stainless needles or forceps for immersing in fixing fluids. Prior removal of water content from filaments with blotting paper yield better results.

Zoospores and developing gametophytes are generally fixed by collecting and/or growing on cover glass. For securing zoospores petridishes were covered with black a sheath which on the bottom side was perforated to enable light to pass through. The petridishes were poured either with distilled water or with liquid culture medium and cover glasses were placed at the bottom of the petridishes coinciding with the position of the perforation. Ten to fifteen day old cultures of zoospore liberating material were placed in the above petriplates with fresh medium. The zoospores get liberated after 12-36 hours in culture chamber. The zoospores by photo-activation movement get attached to the cover glass. The cover glasses thus obtained were washed with 2-3 changes of distilled water to remove the debris and unattached zoospores. They were then transferred to fresh liquid culture solutions enabling them to develop

and divide before fixation at proper time.

Fixing Fluids

The various fixing fluids commonly used in algal cytology are given below. Sometimes a fixing fluid alone or a modification of it or in combination with others found to yield better results.

Acetic acid-alcohol-mixtures:

Carnoy's fluid-I (Carnoy 1886)

Glacial acetic acid : 1 Part
Absolute alcohol : 3 Part

So far this fixative has proved to be superior to all other fixatives and good results have been obtained for Dinoflagellates (Dodge, 1966); Green algae (Godward, 1966); Euglenoids (Leedale, 1966); Brown algae (Robert, 1966; Evans, 1966) and Red algae (Dixon, 1966).

Several modifications have been made for this fixative. Dodge (1966) replaced absolute alcohol with absolute methanol for certain dinoflagellates. Identical results also have been obtained with 95% alcohol. Fixatives in 1:2, 1:1 proportion proved to be better in marine algae, Ulotrichales (Chaudhary, 1975); Siphonales (Singh, 1973); Rhodophyceae (Dixon, 1966). The mixtures may be harmful for large celled delicate forms as it causes shrinkage although nuclei are perfectly stained. These mixtures may be avoided in the cellular identification calcified tissues as the high acid content of the mixture produce rapid formation of CO_2 shattering the tissue totally (Dixon, 1966). For smaller thalli of Rhodophyceae a few minutes immersion is required for fixation, longer duration may disintegrate the material but for cartilaginous forms 1-6 hours are required (Austin, 1959).

The strong acid content of the mixture itself will serve as a softening agent; hence further softening is not necessary in some cases. Besides these mixtures also remove rapidly the pigments which obscure the cytological details in sporangia. Austin (1959) claimed, for rhodophycean members, materials can be left in this fixative for 3-6 weeks without harm but this should be avoided as far as possible. The best alternative is to store in 70% or 50% alcohol after taking the material down through alcohol series.

Carnoy's fluid-II (Carnoy 1887)

Glacial acetic acid : 1 Part
Absolute alcohol : 1 Part
Chloroform : 1Part
Corrosive sublimate- to saturation

Period of fixation varies from 15-24 minutes at room temperature. The material should be washed out with 70% or 90% alcohol.

Propionic acid modification

Propionic acid : 100ml
95% Ab.alcohol : 100ml
Ferric hydroxide : 0.04g

This is useful for materials with smaller chromosomes.

Schaudinn's Protozoological fluid

Glacial acetic acid : 009ml
Ethyl alcohol : 100ml
Saturated aqueous-
Mercuric chloride : 200ml

Material : **Marine algae**

Cave and Pocock (1951) modification

Glacial acetic acid
Saturated with ferric acetate : 1 Part
Absolute alcohol : 3 Part

Material : **Vovocales**

Formalin-Alcohol mixtures:

Drew's Soution (1935)

Formaldehyde -40% (commercial) : 06ml
Alcohol-70% : 100ml

It is a standard fixative used by many workers for Rhodophyceae. Exposure of fixed material to strong light enhances the de-colourisation. This fixative is suitable for morphological studies, haematoxylin and brazilian staining, but little value for aceto-carmine and feulgen staining. A serious limitation is that material become very hard and brittle after storage for more than few days

Dr. Lynd Jones formula

Formaldehyde -40% (commercial) : 002ml
Alcohol-70% : 100 ml

Dr. Land's original formula

Alcohol-50% : 100 ml
Commercial formalin : 006 ml

In a modification Land adds few drops of acetic acid.

The above fixatives are rarely used algal cytology.

Formalin-acetic acid-alcohol mixture:

West Brook formula (1935)

Glacial acetic acid : 002.5 ml
40% formaldehyde : 006.5 ml
50% alcohol : 100.0 ml

Material :**Red algae**

Kumage *et al.*, solution (1960)

Glacial acetic acid : 10 ml
40% formaldehyde : 10 ml
95% alcohol : 50 ml
Distilled water : 30 ml

Material : ***Dictyota dichotoma***

Modified formula which also produced good result is:

Glacial acetic acid	: 05 ml
40% formaldehyde	: 10 ml
70% alcohol	: 85 ml

The above mixtures are mainly used in Rhodophyceae. It may cause certain amount of contraction of the nucleus (Magne, 1964). For calcified tissues, if used, the fixative should be changed at half hourly intervals until calcification is completely removed. Best results are obtained with materials used immediately after fixation. Prolonged keeping in fixatives may necessitate softening in 70% alcohol.

Cole and Akintohi fixative (1967)

Glacial acetic acid	: 007 ml
40% formaldehyde	: 040 ml
Ethanol	: 100 ml
Material	: ***Prasiola***

***Chromic acid-acetic acid mixtures*:**

Flemings's solution (stronger) (c.f. Kaushik, 1993)

1% Chromic acid	: 45ml
2% Osmic acid	: 12ml
Glacial acetic acid	: 03ml
Materials	: ***Ectocarpus, Padina, Dictyopteris, Fucus***

Flemings's solution (weaker)

Weak:

A. Soution

1% Chromic acid	: 25ml
1% Glacial acetic acid	: 10ml
Sea water	: 55ml

B. Soution

1% Osmic acid	: 10ml

Keep the two mixtures separately and add B to A just before use.

Materials : ***Dictyopteris, Fucus, Chorda***

Flemings's solution (Meyer's modification 1928)

1% Chromic acid	: 25ml
1% Glacial acetic acid	: 10ml
50% Sea water	: 55ml
1% Osmic acid	: 10ml
Material	: ***Egregia***

Flemings's solution (Newton's modification)

1% Chromic acid	: 14ml
1% Glacial acetic acid	: 0.5ml
2% Osmic acid	: 04ml
Water	: 12ml

Makay's modification of Flemings's solution (1933)

Chromic acid	: 001gm
Glacial acetic acid	: 002ml
1% Osmic acid	: 003ml
Sea water	: 100ml
Material	: ***Pterygophora***

Strasburger solution (1897)

Chromic acid	: 001gm
Glacial acetic acid	: 001ml
Sea water	: 100ml
Materials	: ***Fucus, Hesperophycus, Eisenia***

Simon's fixative (Simon 1906)

1% Chromic acid	: 25ml
1% Glacial acetic acid	: 10ml
Sea water	: 65ml
Material	: ***Sargassam***

Nirenburg solution (1910)

50% Chromic acid	: 0.5ml
98% Glacial acetic acid	: 1.0ml
Sea water	: 100ml
Material	: ***Cystoseira, Sargassam***

Chamberline fluid (1924)

Chromic acid	: 1.0gm
Glacial acetic acid	: 0.4ml
Sea water	: 400.0ml
Material	: ***Dictyota***

Tahara's fluid (1924)

2% Chromic acid	: 70.0ml
2% Osmic acid	: 5.0ml
Glacial acetic acid	: 2.5ml
Sea water	: 30.0ml
Material	: ***Coccophora***

Yabu (1964) modified this fixative by adding 40% commercial formalin to the above mixture in 2:1 ratio, just before use.

Materials : ***Agarum, Alaria***

Inoh's fluid -A

*Stock solution of

Chromic acid	: 25.0ml
2% Osmic acid	: 2.5ml
Glacial acetic acid	: 2.5ml
Sea water	: 25.0ml

**Stock solution of Chromic acid is prepared as follows*

Chromic acid	: 1.0gm
Glacial acetic acid	: 1.0ml
Water	: 100.0ml

(In some cases stock solution of Chromic acid is prepared by mixing 98ml of sea water and 2ml of saturated solution of chromic acid)

Materials : ***Pelvetia, Sargassam***

Inoh's fluid -B

Solution –A

Stock solution of	
Chromic acid	: 50.0ml
2% Osmic acid	: 5.0ml
Glacial acetic acid	: 2.5ml

Solution –B

1% Chromic acid	: 25.0ml
1% Osmic acid	: 10.0ml
1% Glacial acetic acid	: 10.0ml
Sea water	: 55.0ml

The solutions A & B are fixed in the ratio 1:1 just before use

Material : ***Brown algae***

Okabe's Solution -A (1929)

Stock solution of	
Chromic acid	: 65.0ml
2% Osmic acid	: 5.0ml
Glacial acetic acid	: 2.5ml
Sea water	: 35.0ml

Material : ***Sargassam***

Okabe's Solution -B (1930)

Stock solution of	
Chromic acid	: 50.0ml
2% Osmic acid	: 5.0ml
Glacial acetic acid	: 2.5ml
Sea water	: 50.0ml

Materials : ***Coccophora, Cystophyllum***, ***Sargassam***

Higgin's Solution (1931)

1% Chromic acid	: 25.0ml
1% acetic acid	: 20.0ml
Sea water	: 55.0ml

Material : ***Stypocaulon***

Abe's fixation fluid (1933)

Solution –A

Stock solution of	
Chromic acid (seawater 98ml+	
2ml of saturated solution of	
Chromic acid)	: 50.0ml
2% Osmic acid	: 5.0ml
Glacial acetic acid	: 2.5ml
Sea water	: 50.0ml

Solution –B

Saturated solution of	
Picric acid	: 50.0ml
Glacial acetic acid	: 5.0ml
Chromic acid	: 1.0gm
Solution –c	
Saturated solution of	
Picric acid	: 25.0ml
40% formalin	: 25.0ml
Urea	: 0.5gm

Mix the solutions A : B : C at the ratio of 2 : 1 : 1 preceding use. Sometimes solution 'A' itself will serve as a fixative.

Material : ***Undaria pinnatifolia***

Modifications of Abe's fluid (Yabu,1935)

Glacial acetic acid in 'A' & 'B' of the Abe's fluid is reduced to 1ml while the rest remain same.

Material : **Red algae**

Yabu and Imai solution (1935)

1%Chromic acid	: 5.0ml
2% Osmic acid	: 1.0ml
0.1% Glacial acetic acid	: 2.0ml
Water	: 12.0ml

Materials : ***Fucus, Pelvetia***

Papenfuss (1946) modification of Karpechnko solution

Solution –A

Chromic acid	: 1.0gm
Glacial acetic acid	: 5.0ml
Sea water	: 65.0ml

Solution –B

40% formaldehyde	: 40.0ml
Sea water	: 35.0ml

Mix equal volume of 'A' & 'B' just before use

Material : **Red algae**

Fixation and de-colourisation is rapid in chromic acid -acetic acid mixtures. Prolonged washing is required for cytological studies. The fixative should be changed every half an hour calcareous materials until no further CO_2 is evolved. For marine forms sea water may be used instead distilled water though it is not, absolutely necessary (Dixon 1966),

Other Fixing Solutions

Bouin's fluid (cf.Heine 1932)

Glacial acetic acid	: 5.0ml
Formalin	: 25.0ml
Saturated sea water solution	
of Picric acid	: 75.0ml

Material : ***Xithophora***

Allen-Bouin's fluid (cf. Heine 1932)

Glacial acetic acid	: 5.0ml
Formalin	: 15.0ml
Saturated sea water solution	
of Picric acid	: 75.0ml
Urea	: 1.0gm

Material : ***Enteromorpha***

Navaschin solution

Glacial acetic acid	: 1.0ml
1% Chromic acid	: 15.0ml
1% Formalin	: 3.0ml
Sea water	: 17.0ml

Material : ***Ulva***

Navaschin solution (Randolph's modification, 1935)

Solution –A

Glacial acetic acid	: 7.0ml
Chromic acid	: 1.0ml
Distilled water	: 92.0ml

Solution –B

Neutral formalin	: 30.0ml
Distilled water	: 70.0mlof

Mix equal 'A' and 'B' solution before use

Material : **Brown algae**

Nissenbaun solution (1953)

Saturated solution of HgCl2	: 10.0ml
Glacial acetic acid	: 2.0ml
Formalin	: 2.0ml
Tertiary butyl alcohol	: 5.0ml

Material : **Algal Flagellates**

Newcomer Fixative (1953)

Isopropyl alcohol	: 6.0Part
Propionic acid	: 3.0Part
Petroleum ether	: 1.0Part
Acetone	: 1.0Part
Dioxane	: 1.0Part

Material : **Chlorococcales**

Newcomer Fixative(Belling's Modification)

Solution –A

Chromic anhydride	: 5.0gm
Glacial acetic acid	: 50.0ml
Distilled water	: 320.0ml

Solution –B1 (for Prophase)

40% formaldehyde	: 20.0ml
Distilled water	: 175.0ml

Solution –B2 (for Metaphase)

40% formaldehyde	: 100.0ml
Distilled water	: 275.0ml

Solution 'A' is mixed with equal amounts of solution 'B1' and 'B2' just before use. The fixation period is 3-12 hours. This fixative is successfully used in the meiotic study of flower buds may be useful in algal meiosis.

Material :**Brown and Red algae**

Ishikawa's fluid(1921)

2% Osmic acid	: 1 Part
2% Glacial acetic acid	: 1 Part

Material : ***Porphyra***

Boveri's fixative (cf.Damman,1930)

Picric acid (saturated in 96% ethanol)	:50.0ml
Glacial acetic acid	: 3.0ml
Filtered sea water	:50.oml

Material : **Red algae**

Yamanouchi's fluid (1906)

2% Osmic acid	: 1.0ml
1% Chromic acid	: 5.0ml
Glacial acetic acid	: 2.0ml
Sea water	: 11.0ml

Material : **Red algae**

Cave &Pocock (1956) modification of Johnsen (1940) fixative

Iodine	: 0.5gm
Potassium iodide	: 1.0gm
Glacial acetic acid	: 4.0ml
Formalin	: 24.0ml
Distilled water	: 400.0ml

Lugol's iodine solution (Modified)

1. Potassiumiodide:20gm
2. Iodinecrystals: 10gm
3. Distilledwater: 200ml
4. Glacial acetic acid: 20ml

Glacial acetic acid : 2-3 days before use.

This fixative can be used both for preservation and cytological studies for all algae with soft cell walls

STAINS

The earlier workers (Farmer, Williams, Strasburger, Yamanouchi, etc) employed the classical method of embedding, sectioning, staining and mounting their materials (c f. Rengasamy 1982).It is a laborious and time consuming process. This method also required careful cutting or piecing of material to ensure all sections through a nucleus. Squash technique (Belling, 1926) successfully used in the investigation of root tips of higher plants and animals was used for the first time in algal cytology by Godward (1948). Since then this method has been modified by several workers and successfully used in all groups of algae. It gave an impetus in algal cytological research.

The advantage of squash preparation over section is that, they permit rapid fixation, penetration of stain, smooth handling of material and examination of single layer of cells in totality. However, the cell being released of its compactness undergoes much enlargement in volume, affording wider space for the chromosome to become scattered. The combined stain fixative is the simplest of all chromosomal treatments since cells are directly mounted and studied in the fixative. The stain can be applied to living material or to materials that has been

previously fixed. The latter method of pre-fixation of materials reduces the staining of cytoplasm and allows more absorption of the stain by the chromatin. The only disadvantage of this method is that the individual cells being released from one another shift from their original site and the original topography is altered. The term *smears* and *squashes* are often loosely used, resulting the worker often getting the impression that the two processes are identical but strictly speaking they are not so. In *smears* the cells are directly spread over a slide prior to fixation and this process no treatment is necessary to secure cell separation. But in *squashes* special treatments are needed for dissolution of the pectic salts of the middle lamellae so that separated individual cells can be obtained from a compact mass of cells and this treatment being carried out after fixation or even after staining. After passing through required steps the softened bulk material or small tissues can be neatly squashed on a slide by generally applying pressure or tapping with needle over the coverslips.

The term *smear* commonly applied to cases where cells have been spread on the slides before fixation while *squashing* on the other hand, is used for the performed after fixation or staining. Of the several stains, carmine and feulgen stains are extensively used for chromosomal studies as they require only short time. The other stains are haematoxylin, crystal violet, orcin, aniline blue, methyl green, Brazilin etc. (for details of preparation see Chamberlain, 1924; Johansen, 1940)

Carmine

Carmine is one of the most widely used dyes for chromosome staining. This dye is prepared from female bodies of cochineal insect *Coccus cacti* a tropical American Hemoptera living on plant *Opuntia coccinellifera*.

The dye is crimson coloured product by adding compounds of Aluminum to Cochineal extract. It is not truly a definite compound but a mixture of substances, the composition varies on the basis of the method of extraction. The active principle of carmine, to which staining property is due, is carmic acid. Carmic acid can be obtained by extracting cochineal extract with boiling followed by treatment with lead acetate and decomposing of lead acetate with sulphuric acid (Gatenbay and Beans 1950) This dye belongs to anthraquinone group and has the formula $C_{22}H_2OO_{13}$, the molecular weight is 492.38.

The chromophoric property is attributed to its quinoid linkage. It is soluble in water in all proportions and is dibasic acid and claimed to be nearly insoluble at its isoelectric point pH4-4.5 (Baker 1950). If it is dissolved in the acid side of its isoelectric point it acquires a positive charge, behave like a basic dye and stains chromatin, but if dissolved in alkali solution it can behave as an acid dye. Carmic acid is not used as such nuclear studies, except in the form of *carmalum* as recommended by Mayer (c.f. Baker, 1950) for animal studies.

In chromosome studies, carmine is used in solution with 45% acetic acid. It is prepared by boiling required amount of carmine (1 or 2%) with 45 ml of acetic acid and 55 ml of glass distilled water in a round bottom flask fitted with a condenser, over a low flame of spirit lamp, for two hr and filtering after cooling overnight. The stain thus prepared is known as *aceto-carmine.* In its original solution 1% carmine is used by Belling (1921) but this concentration is varied (up to 5%) depending upon the materials under study. This solution serves the double purpose of fixation and staining, as acetic acid is a good fixative of chromosomes and is rapidly penetrating fluid.

Sometimes aceto-propiono carmine (carmine, propionic acid-22.5ml, and acetic acid-22.5 ml distilled water-55 ml) also produced good chromosome staining.

The carmine staining is principally a process of adsorption and physical one. The adherence of the dye can be accelerated through the process of mordanting by applying 4% aqueous solution of ferric ammonium sulphate which in principle modifies the surface condition of the fixed chromosome. A treatment of the material with iron solution up to 30 minutes was found sufficient to stain the dividing nuclei properly. Prior to and after mordanting the materials were washed thoroughly in distilled water for removing the fixative and mordant respectively. They were then squashed in 1 or 2 % aceto-carmine stain with intermittent warming and cooling of the slides. Over stained preparations for better contrast were destained by administering 45% acetic acid followed by gentle warming of the slides.

Occasionally both plant and animal materials which present difficulties in *Feulgen staining* are mounted in aceto-carmine after Schiff's reaction (Schreiber, 1954). In such cases, the hydrolysis in N.HCl as well as the treatment with Sulphuric acid clears the cytoplasm allowing specific colouration of the chromosome.

Haematoxylin

This natural coloured product is obtained from heartwood of *Haematoxylum campechianum* a native of Mexico.It is extracted in water and evaporated by boiling. The dyeing property of haematoxylin is due to the oxidation product of haematein. ($C_{16}H_{12}O_6$) Delafield's haematoxylin, Erlich's haematoxylin, Bochmer's haematoxylin, Mayer's haem alum,

Heindenhen's iron- haematoxylin etc are used in cytology (for preparations see Chamberine, 1926, Johansen, 1940)

The stain after preparation must be allowed to ripen for several weeks or for rapid oxidation a small quantity of Sodium iodate is often added if solution has to be used immediately. Haematoxylin is entirely ineffective in staining without the use of a mordant. Commonly used mordant in haematoxylin staining are Potassium alumini um sulphate, iron alum and ammonium alum.

Brazilin

Belling (1926) and Capinin (1930) used brazilin for staining chromosomes. It is extracted from Brazil wood. Similar to Haematoxylin it is not a dye. The staining property is due to its oxidation product Brazilein.

The dye is prepared by dissolving required amount in 70% alcohol and allowing to ripen for several weeks before use. Belling (1926) used this dye for plant chromosomes but it has a defect of staining cytoplasm just as haematoxylin and the stain is much weaker. Now it is used in red algae by some workers.

Orcein

Orcein was first employed as a chromosomal stain by La Cour in 1941. The dye has a molecular weight of 500.488. The formula being $C_{28}H_{24}N_2O_7$; but its exact chemical structure is unknown. It is deep purple coloured dye obtained from the action of hydrogen peroxide and ammonia on the colourless parent substance orcinol. It is available both in natural and synthetic forms. In nature it is obtained two species of Lichens, *Rocella tinctoria* and *Lecanora parella*. Conn (1953) indicated that synthetic orcein is not as effective as natural orcein.

Orcein is soluble in water and alcohol. Under certain conditions it can act as an amphoteric dye. It has got basic properties. It is used like aceto-carmine, 1% in 45% acetic acid. It does not need any mordant. It is not good for meiotic materials when compared to carmine. It is effective where carmine fails.It is good for salivary gland chromosomes as well as the chromosomes of mosses (cf Darlington and LaCour, 1960). In various species of fungi especially ascomycetes, hydrolysis in 1N.HCl few minutes after fixation and prior to staining has been found to be very effective (Singleton, 1953; Elliot, 1956). In algae this dye has limited use.

Leuco-Basic Fuchsin(Feulgen)

Feulgen Reagent –(Fuchsin Sulphurous acid)

Materials Required

Basic fuchsin	-1gm
N.HCl	-30.0ml
Potassium metabisulphate	-3.0gm
Activated Charcoal	-0.5gm
Distilled water	-200.0ml

Preparation

Dissolve 1gm of basic fuchsin gradually in 200 ml of boiling distilled water Cool at 58°C Filter, cool the filtrate down to 26°C Add 30ml of N.Hcl and 3gm Potassium meta-bisulphate Close the mouth of the container with a stopper, seal with paraffin Wrap the container in black paper and store in a cool dark chamber. Take out the container after 24 hours. If the solution is transparent and straw coloured; it is ready for use.

If otherwise colored, add 0.5gm of activated charcoal powder, shake thoroughly and filter rapidly under suction.

Store in air tight colored bottle in the dark, preferably at 4°C.Now it is ready for use.

Alternatively, after dissolving the dye, bubble stream of SO_2 through solution; filter and store (Hikawa and Ogura,1954). Sulphur dioxide water required in this staining procedure can be obtained by dissolving 5ml N.HCl , 5ml of 100% Potassium meta bisulphate in 100ml of distilled water.

Precaution

Always keep the container sealed after use and store in cool temperature, away from light. According to Whotka and Davenport (1946) Feulgen reagent in sealed containers kept at 0-5°C retains its efficiency for 6 months.

Aniline Blue: Dissolve 1gm in 100ml of 90% alcohol. The algal material can be stained at 85% alcohol stage for 5min.

Erythrosine Bluish: Dissolve 1gm in100ml absolute alcohol. The alga can be stained after dehydrating in95% ethyl alcohol by dipping for 30sec.

Light Green: Disolve 0.2gm of stain in 100ml of 90% alcohol. The algal material stained for 30-40 sec. to stain cellulosic cell wall

Congo red: Dissolve 0.2gm of stain in 100ml of absolute alcohol. The cyanophycean

mucilagenous sheath is stained by dipping in the solution after 95% alcohol stage for 1min.

Stainting Methods

Staining Methods for Dinoflagellates

Method.I (Dodge,1966)

1. Fix material in absolute methanol or on Carnoy's fluid
2. Mordant if necessary with ferric acetate. This is done by addition of few drops of mordant (ferric acetate saturated in acetic acid) to the fixative before few hours of staining.

3. Pipette out a concentrated drop of fixed material into a drop of aceto-carmine on a slide.
4. Place a coverslip gently
5. Heat the slide over a low flame of spirit lamp up to the point of boiling.
6. Squash the material when the slide is still warm.
7. Destain, if necessary with 45% acetic acid

(For cyto-chemical staining of chromosomes of Dinoflagelates-see Dodge, 1964a, b)

Method.II. (Kubai and Ris ,1969)

Kubai and Ris (1969) fixed cells of *Gymnodinium* in 10% neutral formalin and stained in 0.5% aqueous methyl green or aceto-carmine. The encysted cells which do not permit the penetration of these stains are heated in presence of stain at 60° C for 5 min. in order to render them permissible.

Method.III (vonStoch,1973)

Materials: *Gymnodinium sp*. and *Wolzynskia*

1. Fix materials either in 3:1 methanol-formic acid or 3:1 ethanol-acetic acid
2. Stain with orecin

Staining Method for *Euglena* (Leedale,1958)

1. Concentrate the cells by centrifuging low speed (250rpm)
2. Fix in methanol or Carnoy's acetic alcohol
3. Mordant with ferric acetate
4. Stain with aceto-carmine or Feulgen in the usual way

Note: To facilitate the squashing of species with elastic periplast treat cells with a standard solution of pepsin at 25°C for overnight.

Double Fixation and Staining for Flagellates (Shyam1978)

1. Keep a concentrated drop of culture material on a slide
2. Add either Nissenbaun fluid or Johansen fixative modified by Cave and Pocock (1951)
3. As soon as the fluid evaporate and reaches the point of dryness, transfer the slides to a coplin jar containing Carnoy's acetic alcohol modified by Cave and Pocock (1951). Leave it there for 4-24 hr.
4. Following this treatment, transfer the slide to a coplin jar containing saturated aceto-carmine stain. Leave it there for 15 min. (Another transfer to fresh saturated aceto-carmine will give better staining)
5. Remove the slides from the stain, clean the sticking stain from places other than where the material was present. Put a drop of fresh carmine over the material covered with a cover slip.
6. Warm the slide gently over a low flame of a spirit lamp and apply little pressure with thumb. Drain off the excess stain with a filter paper. Sometimes slight tapping is required for proper chromosomal separation.

Staining Method for Volvocales

Carmine staining technique for colonial Volvocales (Cave and Pocock, 1951)

1. Fix the materials from actively growing cultures or from field collections; directly on the coverslip or in small vials in Carnoy's acetic-alcohol consisting of 3 parts of absolute alcohol and 1 part acetic acid saturated with iron-acetate.
2. If coverslips are used for fixing materials, pick up the material with a capillary pipette and transfer to the centre of the coverslip. Sometimes addition of a drop of concentrated iodine in Potassium iodide not only kills the material but also enhances nuclear staining
3. Accumulate enough material on coverslip and put a drop of fixative directly on the material and place the coverslip on a piece of glass or flat cork, inside a petridish. Add enough fixative to petridish to prevent evaporation. The material can be kept in this way for several days. The material can be stained after 15 min. of fixation.
4. If materials are plenty, they can be stored in small vials containing fixatives for longer time. Pipette out living colonies into vials of water containing, a drop of iodine in Potassium iodide. Allow the colonies to settle down (5 min. to 2h). Remove the supernatant solution using a pipette and replace it with fresh fixative. Materials can be stored in this way in good condition for over an year. For staining purpose pipette out settled colonies from the bottom of the vials to the center of the coverslip.
5. Before staining draw off the fixative from the coverslip and allow evaporating to the point of dryness.
6. Then place the coverslip inverted position in a drop of aceto-carmine on a slide.*(The aceto-carmine is prepared by refluxing 2gm of National Aniline Carmine in 400ml of 45% glacial acetic acid for four hours. The solution is to filtered after cooling).*
7. After few minutes, heat the slide over a spirit lamp flame to just below boiling point and squash the colonies gently or severely based on the species under study. If squashing is needed it can be achieved in a better way. Put a drop of 45% acetic acid to the edge of the coverslip, reheat and squash before cooling down. This can be repeated till desired result.

8. Slides if necessary may be made permanent in the following manner by adding the following solutions one side of the coverslip and drawing them off with filter paper from the other side
   a) 45% acetic acid
   b) 1:2, 95% absolute alcohol and 45% glacial acetic acid
   c) 2:1 absolute alcohol and glacial acetic acid
   d) Absolute alcohol
   e) Absolute alcohol
   f) Ring the coverslip either with cupral or with diaphane and place in a flat covered box, the bottom of which is covered by a filter paper wetted with few drops of ab.alcohol. After 24 hrs., dry them over overnight on top of the paraffin in oven. Clean the coverslip with ab. alcohol without slipping.

(Use of iodine brightens the nuclear stain; otherwise the chromosomes from pro-metaphase to telophase will appear as black and nucleolus as grey red)

Staining Methods for *Chlamydomonas*

Method.I (Schaechter and Delamater,1955)

1. Remove blocks of agar from culture plates and place on a glass slide with growth surface up
2. Place a No. 0 or No. 1 coverslip in contact with the organism
3. Flip of the agar block and immerse the coverslip immediately in the fixative. Fix for 5min. in Carnoy's fluid. Fixation can also be accomplished with vapours from 2% solution of Osmium tetroxide. Here expose the agar blocks to vapours for 3 min.
4. After fixation, wash the cells with distilled water and hydrolyse in 1N.HCl at 60°C for 5-8min. (Carnoy's fluid fixed preparation treated in this manner showed little staining of the cytoplasm, only faint staining of cell wall. Osmic fixed preparations showed a persistence of cytoplasmic basophila through 6-7 min. of hydrolysis).
5. After hydrolysis, stain the preparation in 0.25% Azure-A containing one drop of thionyl chloride per 10ml of dye solution for 2-4hr.
6. Then wash the preparations in distilled water
7. Place overnight in absolute ethanol chilled to and maintained at approximately -60°C with solid CO_2
8. Following this pass into ethanol at room temperature for few seconds, clear with xylol and mount in Technican mounting medium

Method.II (Buffaloe,1958)

Fixation

1. Clean the slide in acid- alcohol solution and make them dry.
2. Smear one side of each slide with egg albumin.
3. After drying, put few drops of one of the fixatives (described below) upon on the end of slide.
4. To the fixing fluid introduce the cells having been removed from culture plates by platinum loop or with the edge of a coverslip.
5. When the cells are just at the point of drying, introduce the slides in coplin jars of the same fixative into which the cells have been first introduced.

Staining

Four different techniques –the iron alum acetocarmine haematoxylin, Feulgen, Azure-A (DeLamater, 1951) and aceto-carmine-are employed in the nuclear and cell division. However, two modifications have been done for the aceto-carmine method. The first is a modification of the technique developed by Cave and Pocock (1956) is used for cellular details. The schedule is as follows:

1. Fix the material in the manner described as above in Cave and Pocock (1956) fixative.
2. Allow the slides to remain in fixative at least 15min. to about 5 days. (cells do not respond to satisfactorily to further treatment if kept in the fixative for longer period)
3. After draining off the excessive fixing fluid from the slide put two drops of aceto-carmine (prepared according to Cave and Pocock (1951b) over the area occupied by fixed material).
4. Place a coverslip gently and heat the fluid under coverslip to boil
5. Observe the slide after blotting.

(In utilizing this method great care must be taken to prevent over staining. The exact and delicate point to be reached between under staining and over staining can be learned after much trials and error)

The second modification, is the aceto-carmine method of Ray's technique of staining of *Tetrahymena,* is superior in staining chromosome. The schedule is given below.

1. Put a drop of distilled water in a previously cleaned glass slide.
2. Transfer a quantity of material to be fixed to slide and distribute them as evenly as possible with in the drop of water.
3. Put a drop of Nissenbaun's fluid upon the suspension

of cells and mix them thoroughly with a dissecting needle.

4. As soon as the evaporation reaches to the point of dryness occur, immerse the slides in a coplin jar of Carnoy's 1:3 acetic acid-ethyl alcohol.

   (Primary fixation in Carnoy's fluid did not produce satisfactory results).

5. Keep it for 12-24hrs.
6. Transfer to 70% ethyl alcohol where it can be left indefinitely.
7. Transfer to 50%, 30% and 15% ethyl alcohol series and finally to water.
8. Hydrolyse the slides in 1N.HCl in the following series: 2min. at room temperature, 6 min.at 60°C and 2 min. a second time at room temperature.
9. Wash the slides in distilled water at least five times with in a period of 5min.
10. Transfer the slides to 45% acetic acid and allow to remain there for 5-7min.
11. Following this treatment remove them to a coplin jar containing aceto-carmine stain where they are stored for at least 4hrs. to obtain adequate staining(4-8 hrs. in the stain proved to be ideal) them there for longer period will over stain the cells. However, good results can be obtained from slides that are kept in the stain for several days. But before being heated they have to be removed to 45% acetic acid for a period which has to be determined experimentally probably for a period proportionally to the time the slide have been in stain .A period of 1-8hrs.have been suggested for *Chlamydomonas*
12. Following the 4-8hrs. period of staining or after the slides have been placed in 45% acetic acid for an appropriate length of time as described above, remove them individually for heating. Wipe out excess of stain from slide and add an additional drop of aceto-carmine on the area occupied by the cells. Place a coverslip gently. Heating should not be as intense as in the previous method but gentle boiling for an instant is necessary.

The two methods when supplemented each other will produce good results. However, best results are obtained from study of freshly stained material. Preparations can be made permanent with certain degree of success following the method of Marsten (1950).

Carnoy's fluid is used as fixative for staining with Feulgen and Azure-A while Schaudinn's Protozoological fluid is employed for iron haematoxylin method. However, these methods are little in practice.

Method.III (Maguire, 1973)

1. Harvest vegetative cells at various intervals into the dark cycle from liquid culture by centrifugation and air dry on slides. (Such slides can be kept long time without apparent deterioration)
2. Fix the slides for 30 min. in 3:1 ab. alcohol-acetic acid mixture
3. Wash twice in distilled water
4. Hydrolyse for 6-8 min. 1N.HCl at 60°C
5. Stain in 0.25% Azure-A for two hrs following the method of Schaechter and Delamater (1955).

Staining Methods for *Eudorina*

Method.I (Doraisamy, 1940)

1. Fix material in suitable fixative preferably in *Fleming.s mixture* or in *Schaudin's fluid.*
2. Wash the material fixed in Fleming's mixture solution thoroughly with water and run up to 70% alcohol. Material fixed in Schaudin's fluid should be washed in several changes of 50% alcohol and finally treat with *Lugol's iodine*. solution to remove the traces of Mercuric chloride.
3. Pipette out a drop of alcohol containing the material over an albumen smeared slide and spread on it with the aid of a coverslip.
4. Allow the alcohol to evaporate and immerse the slides in a jar of 80% alcohol.
5. Bring the slides to alcohol grades to water before staining.
6. Treat 6-12 hrs. in 1% Chromic acid and wash thoroughly in running water for about 1. hr.
7. Mordant with 4% iron alum for 2hrs.
8. Stain with 0.25% haematoxylin for 2-4hrs.
9. Destain if necessary in a saturated aqueous solution of Picric acid
10. Wash in running water
11. Treat the slides 1-2 hrs with 1% aqueous solution of Sodium acetate to remove any traces of acid.
12. After dehydration, clean the slides in clove oil and xylol and mount in Canada balsm.

Steps 9-11 are followed if destaining is done. Safarnin, light green, Bendi's triple stain and Mayer's haem -alum also gave better staining. [When living materials are used for smear preparation, place a drop of water containing living material on a albumen smeared slide and then fumed over osmotic vapours before it is stained]

Method.II (Goldstein, 1964)

1. Transfer colonies from 2-4 week old culture to a watch

glass containing fresh soil water supernatant.

2. Keep the watch glass in petri-dish containing 50% $NaHCO_3$ solution and place it illumination of slow intensity before the 8hrs. dark period. Dividing nucleus are most numerous 3-4hrs after the onset of light period.
3. Pick up the colonies with pipette, concentrate by centrifugation and place in a fixative (3:1ab.alcohol – gl. acetic acid). The colonies are suitable for staining after 6hrs. of fixation but best results are obtained with material fixed at least 34 hrs.
4. Pipette out small amount of fixed material on a clean microscopic slide and allow the fixative to evaporate until the material almost dry up.
5. Add 2-3 drops of aceto-carmine solution
6. Place a coverslip carefully over the material and allowed to stain at rom temperature for 5min.
7. Heat the slide over a spirit lamp for 3min.(stain should not be boiled)
8. Ring the cover slip with wax.

Preparations are not squashed because disruption of cells obliterate chromosomes

Feulgen staining for Eudorina (Kemp and Lee, 1976)

1. Fix aliquots of material at room temperature in 3:1 alcohol-acetic acid or 3% glutraldehyde and keep overnight.
2. Wash the material thoroughly in $1.7x10^{63}M.PO_4$ buffer
3. Hydrolyse for20 min. in 3.5N.HCl at37°C.
4. Squash the material over slide after placing a coverslip.
5. Remove the coverslip after freezing
6. Stain in Schiff's reagent at room temperature
7. Bleach in bisulphite solution
8. After dehydration, make the slide permanent by mounting in Canada balsm

Iron alum Aceto-carmine Method for Algae (Godward, 1950)

This is the most widely used method algal cytology. Modifications have been made for this method by some workers, majority of which is concerned with pretreatment as well as mordanting time.(for details, see Godward, 1966). The schedule given by Godward's for *Spirogyra* is as follows:

1. Fix till all colour is removed from chloroplast:
   a) in 1:3 acetic-alcohol after pretreatment with nitric acid vapour for about 5min.
   b). in acetic- alcohol without pretreatment, 15 min or more
   c). in Navasschin 12 hrs. followed by bleaching in osmic acid.
2. Put in water for few minutes.
3. Treat with 4% iron alum solution in water for 1 or 2 min. or more
4. Wash and place in water for few minutes.
5. Mount in aceto-carmineon a slide and heat. Blot if required.
6. Allow the slide to remain warm for about 10min.
7. Allow the cover slip to fall off in 95% alcohol
8. Mount in supral or (probably better) transfer for few minutes to xylol and mount in canada balsm.

Stages 7 and 8 can be omitted in temporary preparations. Slides can be observed after ringing with rubber solution.

The Kopoeeka and Miroskv (1978) stained the nuclei of *Spirogyra* by incorporating the antibiotic Lomofungin (20-100ug/ml) the culture for few hours.

Propionic-carmine Squash Technique and Chromosme Spreading in Algae

Venkataraman and Natarajan (1959)

1. Immerse material in colchicine solution ranging from 0.01% to 0.2% about 2hrs or in 8 -hydroxy - quinoline for 2-3 hrs.
2. Wash thoroughly in distilled water and fix in ab.alcohol-propionic acid solution (3:1.ratio).
3. using carmine stain squash preparations in customary way

Staining for Oedogonium (Hoffman, 1967)

1. Fix the algal filaments with dividing cells in a fresh mixture of ab.alcohol and gl.acetic acid (3:1.ratio) for 12-24 hrs. If no staining is carried out the same day change the fixative with a fresh one and store in a freezer.
2. Hydrolyse in 1N.HCl for 17-22min.
3. First stain in Feulgen reaction followed by aceto-carmine (Hoffman, 1961)
4. Make the slide permanent according to Conger and Fairchild (1953)

Staining for Chlorococcales (Mathew, 1976, Rai, 1981)

1. Fix the material, after centrifugation, in any one of the following fixative.
   i. Carnoy's fluid (Carnoy,1886)
   ii. Cave and Pocock fixative (Cave and Pocock,1951)

iii. Newcomer fixative (Newcomer,1953)

In some cases a combination of fixatives i & ii or i & iii will yield better results. Taxa like *Ankistrodesmus, Chlorococcum, Dictyosphaerium, Neprochlamys, Oocystaenium, Oocystis* and *Schroederia* better results are obtained when materials are fixed first in acetic methanol (3:1 ratio) for 12-24 hrs, followed by second fixation in Cave and Pocock (1951) fixative for 14hrs. Taxa like *Coleastrum, Chlorochytrium, Pediastrm, Scenedesmus* and *Trebouxia* after fixing in 1:3 acetic-methanol for a period of 2hrs, keep in a warm water bath maintained at a room temperature 40°C for 30 min. and then transfer to Newcomer fixative (1953) for 12-24 hrs. The latter method is suitable for materials having more oil and fat globules.

2. Mordant the material, in 2% iron-alum after removing from the fixative for 1-30min. The mordanting time vary for different taxa. Mordanting is not needed for materials fixed in Cave and Pocock (1951) fixative.
3. Boil the material in aceto- carmine or aceto-propionocarmine (1-1.5%) in a tube.
4. Pipettee out a very small quantity of stained material to a clean slide.
5. Add a fresh drop of carmine solution and place a coverslip.
6. Warm gently and apply pressure with thumb or blunt end.
7. Seal with rubber solution

Prasad and Godward's (1966) Modification for *Zygnema*

Instead of 4% iron alum solution a saturated solution was used and the time of mordanting varied from 0.5min.to 5min. After washing thoroughly in few changes of water the materials are stained in bulk in a test tube containing aceto-carmine. The material was freely boiled in stain and washed once or twice in 45% acetic acid and mounted in the same medium, warmed before observation

Staining for Polycentric chromosmes in *Sirogonium melanosporum*

(Hershaw & Waer, 1967)

1. Fix the material in a fixative of methanol-ethanol-chloroform-acetone-propionic acid in the ratio, 4:2:2:1:1.
2. Rinse the fixed material in distilled water twice for 3-5min.
3. Hydrolyse in 1N.HCl for 8min. in an oven at 58°C-60°C.
4. Rinse the hydrolysed material twice for 3-5min. in distilled water.
5. Stain the material with Schiff's reagent (Darlington and Lacour, 1956) for 15 min.
6. Differentiate the filaments in demineralized water until they appear light pink.
7. Counter stain with propiono-carmine for approximately 2 min. to intensify the colour of the chromosome. Heating is not required.

Staining for Desmids (King, 1960)

1. Lightly centrifuge the algal sample and fix in excess of acetic acid and ab. alcohol fixative (3:1ratio), after pouring off the excess of supernatant solution. Cork the centrifuge tube and shake well.
2. Add few drops of saturated solution of ferric acetate in glacial acetic acid to the cells in fixing fluid.
3. Separate the cells by light centrifugation and wash in water.
4. Pipette out small quantity of material to a clean slide.
5. Add few drops of aceto-carmine and boil the whole gently for few seconds and allow to cool
6. Place a coverslip and blot.
8. Seal with rubber solution.

Squashing

In order to observe the polar views of the metaphase plate, it is very much necessary to extrude the plate from the cell. Maceration of the cells with enzyme *cytase* obtained from the gut of snails (Faberge, 1945) worked successfully. This has brought about the rupture of the cell wall usually at the region of the isthmus.

The enzyme complex *cytase* is prepared as follows. Kill the snail *Helix pomatia* by immersion in chloroform for few minutes. Remove the shell and cut open the mantle cavity. The 'stomach' apparently seen as a translucent tube filled with a coffee coloured liquid. Extract this fluid rich in *cytase* by pulling out and opening the digestive tract at one end

Squashing can also be brought out by producing sufficient pressure on the coverslip with a blunt point.

Staining Methods for *Cephaleuros* and *Trentepohlia*

(Jose and Chowdhary, 1977, 1978)

Both genera *Cephaleuros* and *Trentepohlia* being sub-aerial forms exhibits very feeble or no growth in liquid culture; hence they have to be grown in solid defined medium. The genus *Cephaleuros* under normal culture conditions do not produce reproductive

structures while few species of genus *Trentepohlia* produces zoosporangia in culure.

Procedure.

1. Transfer 18-20 day old culures (or when the thallus turns pale yellow) to fresh medium
2. Using sterile forceps pick up very small quantity of inoculum (vegetative thallus) from old culure plates and transfer to new plates
3. Expose the plates to 12 hrs light and dark period under normal culture conditions.
4. Observe the inoculum under microscope after 24 hrs
5. Start fixing the material in carnoy's fluid (3:1.2:1.1:1) combination when the thallus filaments produces small protuberances
6. Fix the growing culture every three hours for 72 hr in both light and dark periods
7. Change the fixative after 24 hr till the material got totally bleached
8. Wash thoroughly in ditilled water
9. Using forceps take the fixed material through very dilute HCl
10. Wash again distilled water
11. Take few algal filaments on dry slide, add aceto-carmine or aceto-propiono-carmine stain over the material (use 1%, 1.5% or 2% stain)
12. Place a cover slip over the material
12. Warm the slide with material over a low flame of spirit lamp intermittently
13. Blot the excess stain with filter paper
14. Press gently with thump or tap gently with blunt end of a rubber corked pencil
15. Observe the slide and seal with rubber solution
16. Slides can be made permanent using standard procedures

Note: Sporangial producing *Trenepohlias* exhibit mitotic divisions in sporangia

Feulgen staining for *Coleocheate* (Hopkins and McBride, 1976)

1. Fix the material in 3:1, ethanol-gl.acetic acid mixture at room temperature.
2. Wash with running distilled water for 30 min.
3. Hydrolyse in 1N.HCl at 60°C for 20 min.
4. Wash again with running distilled water for 30 min.
5. Stainin Schiff's reagent for 2hr in dark at room temperature.
6. Wash again with running distilled water for 30 min.
7. Destain in 3 changes of sulfurous acid by bleaching for 10 min. each.
8. Dehydrate with ethanol series (30, 50, 70, 95 and 100%), 15min. each.
9. Transfer to Xylene 15 min.

Embed in permount (Fischer Scientific Co.)

Staining Methods in Charales

Chromosome preparations are generally made from young antheridial filaments. The materials are fixed in a suitable fixative. Sundralingam (1946) used the following fixatives for cytological studies: Fleming's weak and strong solutions, Chrome acetic acid solution, Nawaschin fluid, Bouin's fluid as modified by Allen, Formalin-acetic-alcohol, Carnoy's fluid. Many workers have got good results with materials fixed in Carnoy's fluid followed by iron alum aceto-carmine method of Godward (1950)

Procedure for staining *Enteromorpha compressa* (L) var. *lingulata* (Ag)

(Ramanathan, 1939)

1. Fix material in any fixative preferably with original Bouin's fluid or with Allen's medications of the same*.
2. Bring down the material to water and wash in running water for 10 min.
3. Mordant in 4% iron alum(aqueous solution) for 1hr.
4. Wash in running water 10 min.
5. Rinse in distilled water
6. Transfer to 0.25% aqueous solution of Heidenhain's haematoxylin and stain for 12 hrs overnight.
7. Wash in running water for 10 min.
8. Destain in saturated aqueous solution of picric acid until proper differentiation is secured.
9. Wash in running water for 1hr.
10. Dehydrate through series of alcohol grades consisting 10, 20, 30, 40, 50, 60, 70, 85, 95 and 100% alcohol about 10 min. each.
11. Clear in clove oil and xylol and
12. Mount in neutral balsm

*What ever may be the fixative used it should be washed thoroughly. Then the matrial is either directly stained or it can be taken up through a series of alcohol and xylol, embed in paraffin and cut into sections 3-10μm thick. For direct staining open small portions of the tubular thallus with a pair of needles, dehydrate with glycerin and alcohol and finally mount in venetian turpentine or canda balsm

[Sarma (1970) employed successfully the iron alum aceto-carmine method of Godward (1950) for this genus]

Staining Method for *Codium* (Borden and Stein, 1960)

1. Fix in 3:1 ethanol-glacial acetic acid fixative
2. Mordant for 1hrin iron alum solution (Johansen, 1940).
3. Rinse several times in distilled water.
4. Boil in Bellings iron aceto-carmine (Biological Stain commission, 1960) for 10-15min.

(Chromatin stains dark, black red and nucleolei in deep rose)

Staining Method for *Bryopsis* and *Acetabularia* (Burr and West, 1971)

1. Fix the germlings in 3:1,95% ethanol-gl.acetic acid fixative
2. Rehydrate in an ethanol-water series (25% increment)
3. Mordant for 2min. in 5% Ferric ammonium sulphate (iron alum)
4. Rinse in distilled water few times
5. Stain with aceto = carmine at 60°C for 2 min.
5. Mount in 45%acetic acid

Iron alum haematoxylin staining for marine macro- algae (Subramanyan, 1992)

1. Fix macro algae in 1:3 acetic acid–alcohol at the site of collection
2. Next day wash the plant parts thoroughly 2 or 3 times in freshwater / distilled water
2. Again refix in 1:3 acetic acid–alcohol in laboratory
3. Wash thoroughly in tap or distilled water. Soften the material in 0.1 N.HCl or in con.HCl if the material is hard.
4. Rinse totally in fresh water or distilled water.
5. Put the specimen in 4% iron alum 10-15 min.
6. After washing in water, immerse in haematoxylin stain solution for 1-2 hrs. (Dissolve 4gm of haematoxylin powder in 45ml of 95% alcohol. Keep for one week. The well ripened stain will be cherry red in colour and is ready for use. In certain cases 2% is enough)
7. Wash again in water and place in 45% acetic acid for 5 minutes.
8. Squash the material on a clean slide with 45% acetic acid

Feulgen, iron-propiono-carmine staining for algal chromosomes (Hanic 1979)

This method which combines the advantages of feulgen and carmine staining has been successfully used by Hanic for the following materials: *Pyramimonas, Playmonas, Dinobryon, Urospora* and *Codiolum, Spongomorpha, Acrosiphonia, Porphyridium, Petrocelis* and *Chondrus*. The schedule given by Hanic (1979) for *Pyramimonas* is given below. However, some modifications are needed for attached germinating spores and bulk tissues.

Feulgen Stain

1.Concentrate the cells by12-15ml conical bottomed tubes. Care must be taken not to pelletize. Decant the excess of water.
2. Fix in Newcomer's (1953) solution for 24hr or more till all pigment is removed.
3. Rinse once in 1 N.HCl
4. Spin down, decant, add pre-heated 1 N.HCl (60°C) and re-suspended cells with a vortex shaker.
5. Immediately place in a water bath at 60°C for 8min.
6. Cool by adding cold water.
7. Spin down, decant and rinse with distilled water.
8. Spin down; decant all but 1-2 drops of water.
9. Disperse cells with a pipette and then spread a drop of the suspension on a slide pre-coated as thinly as possible with fresh egg albumen and dried in air
10. Place slide on warmer at low heat until just dry.
11. Stain in leuco-basic fuchsin prepared according to Darlington and LaCour (1942) for a minimum of 1hr in dark (1-2days does no harm)

Fe-propionocarmine post stain.

When applying the Fe-propionocarmine do one slide at a time until the procedure that gives the best results obtained.

12. Remove one slide from the leuco-basic fuchsin and pass through fresh SO_2 water for 2-3 min.
13. Drain until dripping ceases (a few second) and add a coverslip
14. Using a micropipette add 1-2 drops of propiono-carmine close to but not touching the coverslip.
15. Tab with small 3-cornered file pre-cleaned in con. H_2SO_4 until blue streaks appear (iron mordant), then move drop over with pipette to edge of the coverslip and draw under. The mordanted carmine stain will flow under the coverslip by itself but can be assisted by either gently lifting one corner of the coverslip near the stain or drawing the stain across with a bibulous paper applied to the other side. In either case the object is to create a gradient under coverslip.
16. Heat the slide briefly (3-5sec.) over a low flame of spirit lamp and test by applying the heated side of the slide to the back of the hand. The slide should be cool

enough to hold there for a few seconds without pain. Too much heat removes the Feulgen stain, but the proper amount enhances it. The Fe-propionocarmine brings out nucleolei, spindle, polar bodies, pyrenoid, starch grains and other cytoplasmic details with increasing contrast as Fe-propionocarmine concentration increases. To determine whether the first heating is enough, blot with bibulous paper gently without pressing and observe under 400 or 1000 magnifiction. If satisfied proceed with the next steps. If not, add more stain, mordant and warm again till suitable result is obtained.

17. Place the preparations between sheets of bibulous paper and squash either by pressing firmly and quickly with the thumb or a broad eraser or tap with a rubber tipped pencil. Monitor the results again under microscope.

Permanent mounting

18. When satisfied with stain, wipe any oil from the coverslips and place vertically in 70% ethyl alcohol until the coverslips falls off, then place in absolute alcohol for 3min.

19. Remove the slide, drain briefly and add 2-3mm drop of thick eupral; add coverslip and place on a slide warmer.

20. After 1hr or so blot the slide with bibulous paper and squash further with strong thumb pressure to remove excess eupral. Dry overnight on slide warmer.

Additional notes

1. For the fixation of materials such as *Pyramimonas amylifera* which ruptures on strong centrifugation place a drop of cell suspension on a slide pre-coated with egg albumen and fix over 20% osmic acid for 5 min. then dry the slide until adhesion occurs and fix in Newcomer's fluid.

2. Germlings and spores germinated on slides for genera such as *Chondrus* and *Urospora* do not require adhesive, if they are solidly adherent to slide and this facilitates all phases of staining procedure smoothly.

3. Bulk materials with chromatohores needed long time fixation period, a few days to a week for the total removal of pigments. For acid hydrolysis place specimens in a nylon bag with in a beaker. Before staining with *Fe-propionocarmine,* place a 1 mm^3 piece of tissue on a slide, put a coverslip and tap gently to break up the tissue to distribute cells evenly. For making permanent mounts the cover slips can be removed by "freeze separation" using dry ice or CO_2 jet applied to the back of slide. Remove the coverslip with the help of a razor. In most cases the cells adhere to the slide sufficiently well to enable dehydration by dipping in the alcohol series.

4. Materials with thick cell walls which hinder squashes can be softened by treating with cupra-ammonium. It removes polysaccharide cell wall material. Add 1-3 drops of cupra ammonium under the coverslip following Feulgen Fe-propionocarmine staining. Polysacchride compounds dissolves instantly on treatment with cupra ammonium. It leads to the expansion of cells making it very easy to squash and to make thin preparations. [Cupra ammonium may be prepared by bubbling a fine stream of air for 2hrs into 3 liters of fresh concentrated NH_4OH containing 500 gm of copper turnings.]

Staining Methods for Diatoms (Subrahmanyan, 1946, 51)

1. Fix properly cleaned material in suitable fixative preferably Allen's modification of Bouin's fluid (PFA_3), 5-12 hrs. or in Scheudinn's fluid

2. If the material is fixed in PFA_3 solution, wash first in 50% alcohol, followed by 70% alcohol. Further addition of a few drops of saturated solution of *lithium carbonate* to 70% alcohol will remove if there are any traces of picric acid. Material can be preserved in 70% alcohol after thorough washing.

3. Materials fixed in Scheudinn's fluid wash first in several changes of 50% alcohol and then 70% alcohol. Treatment with *Lugol's iodine* solution will remove traces of mercuric chloride.

4. Stain in *iron alum haematoxyline* and counter stain with *erythrosin* [As centrifuging affects cells undergoing sexual fusion washing is done by decantation]

Fixing method for Epiphytic Diatoms

Epiphytic diatoms are fixed with host alga. After fixation, the diatom chain breaks up into individual cells and also from host alga. Shake bits of host filaments in fixing fluid and later in washing fluid to free diatom from the host. The diatom will sink to the bottom of the vessel.

To separate diatom from the sediment, transfer the sediment to a clean washing fluid in a petridish and rock the dish gently and hold it obliquely. After a couple of minutes the diatoms will gather together in a line near the shallow side of the liquid in petridish. Remove them carefully with the aid of a fine pipette and transfer to another dish containing fresh washing solution. Repeat the process few times till it is clean and preserve in 70% alcohol

Alternate Method for Staining

1. Fix the material on slide previously smeared with a thin coating of Meyer's albumen according to the

proto-zoological method of MeClung (1937).

2. Wash the slides well in 70% alcohol and then bring down to alcohol grades.
3. Bleach in 30% alcohol containing 10% Hydrogen peroxide.
4. Wash thoroughly and place in 4% iron alum solution for 2hr.
5. Wash 15 min. in running water.
6. Stain in 0.25% haematoxylin for 6hr (Saturated solution of picric acid freshwater is employed for differentiating the stain).
7. After differentiation wash the slides for 3-5hrs in running water.
8. Pass up in alcohol grades, counter stain with erythrosin dissolved in 95% alcohol for 10 seconds.
9. Pass immediately in to absolute alcohol, then clove oil and clear in xylol
10. Finally, mount in neutral balsm

(In the case of material preserved in 70% alcohol, spread the material on slide previously smeared with a thin coating of Meyer's albumen and place the slides in 85% alcohol down to alcohol grades and stain as mentioned above).

Staining Method for Diatoms (Brenjina 1976)

1. Fix in Lugol's iodine solution.
2. Stain with aceto-carmine. The result of staining will be as follows

Chromotophore	: brown
Pyrenoids	: bright red
Nucleus	: red
Chromosomes	: deep red
Cytoplasm	: colourless

(This method is usable for working in the laboratory and not for permanent preparation)

Staining Methods in Brown Algae

Carmine method for *Stypocaulon* (Higgin's 1931)

1. Fix material in Higgin's fluid.
2. Treat with 1% HCl for short period to remove all traces of silica and wash well in water.
3. Place in a solution of H_2O_2 (commercial strength) for 2-4hrs. This effects an almost complete decolourisation without bringing any appreciable contraction in cell contents.
4. Wash well in water and place in a solution of aceto-carmine for four days.
5. Wash in 5% acetic acid, treat with a solution of ferric ammonium citrate for 30 min.
6. Dehydrate by passing through alcohol series to absolute alcohol.
7. Pass through aniline oil and xylol and mount in dilute Canada balsm

Aceto-carmine staining (Evans, 1965,1966)

1. Fix the material in freshly prepared 3:1 ethyl alcohol-gl. acetic acid mixture for 18-24 hrs. until the material is bleached and no longer mucilaginous.
2. Wash the material well in several changes of tap or distilled water.
3. Transfer to 1M lithium chloride solution for 15 min.
4. Transfer to tap water and leave there for 15min. No change is necessary.
5. Place the material on a clean slide, put a coverslip, and squash lightly and evenly, if possible with a chisel shaped instrument. Materials such as vegetative tips of fucoids should be dissected before squashing.
6. When material is sufficiently spread, add few drops of aceto-carmine solution (containing one drop of saturated solution of ferric acetate per 25ml of stain) around the edge of the coverslip. An earlier contact of aceto-carmine stain with material is inimical to spreading, hence it should be avoided. No separate mordanting is needed. Good penetration of stain can be achieved by raising the coverslip gently from each side, in turn while holding it in place with fingers of the other hand.
7. When the stain is evenly spread, warm the slide slightly over a low flame of spirit lamp (30sec.) with intermittent cooling. Stain should not be allowed to boil. Repeat it for 3-4 times.
8. Squash the preparation again and remove excess of stain with filter paper.
9. Seal the preparation by ringing with glycerin jelly (or rubber solution) to prevent drying.
10. In the case of over stained material (to be judged under the micro-scope) destaining is done by flooding the slide (before ringing with glycerin jelly or rubber solution) with 1:5 aceto-carmine: gl.acetic acid mixture and warming slowly up to 15 sec. or more. The cytoplasmic destaining has been found to be necessary in gametophytes and sporangial material that has been left in the fixative for longer than 24 hrs. The cytoplasmic staining can be reduced by thoroughly washing the fixed material in tap water (3-4 times) and storing in 70% alcohol at 2°C. However,the best preparations are obtained from materials used immediately after fixation.

Slides may be made permanent in the following manner

1. Remove the glycerin jelly seal by scraping with a razor blade or rubber solution by lifting with a mounting needle.
2. Mark the corners of the coverslip on the slide with a diamond mark. Cut off a small piece from one corner (possibly right top) of cover slip so that the coverslip can be returned to the original place later.
3. Flood the slide with destaining solution or with aceto-carmine if no destaining is required (to be judged according to the intensity of stain). Lift the coverslip lightly to allow the liquid to penetrate. Application of little heat enhances the penetration of destaining solution.
4. As soons the coverslip is loose, lift off and place it in 95% alcohol, together with slide if material remain attached to it.
5. After 5 min. transfer to absolute alcohol (two changes after 5 min.) and then to cupral essence (5 min.).
6. Replace the coverslip in its original place on the slide, in a drop of cupral and dry on a hot plate overnight.

Note: *This method proved good for Fucoids (Evans, 1966)*

Robert's (1960) modified aceto-carmine method for large parenchymatous forms

1. Fix material in a suitable fixative preferably in Karpechenko fixative for 12-24hrs.
2. Wash well under running tap water at least 10min.
3. After washing bleach 3-4 hrs in 20% H_2O_2 (i.e. two parts of 100 vol. solution to one part distilled water). Delicate structures such as receptacle requires shorter period than vegetative apices.
4. Remove H_2O_2 completely by washing in running water otherwise bleaching will continue and the material will be spoiled.
5. Transfer to 1:3 gl.acetic acid-ethyl alcohol mixture which contains few drops of saturated solution of ferric acetate in 45% acetic acid. (Materials can be stored satisfactorily in this solution up to one year).
6. Wash well in 70% alcohol to remove excess of acetate.
7. Make hand sections, wash in water and mount in 6% Na_2CO_3.
8. Warm the slide lightly on a hot plate, the weight of the coverslip gradually spreads the section, apply manual pressure if further spreading is required.
9. Irrigate with excess of distilled water to remove excess of carbonate.
10. Flood with aceto-carmine and boil gently.
11. Remove excess of carmine solution with filter paper.
12. Invert slide in a smearing dish of 45% acetic acid until coverslip drops of.
13. Pass slide through acetic acid-alcohol series *viz.* 3:1, 1:1, 95% alcohol.
14. Pass through cupral essence and mount in cupral

Carmine method for *Nerocystis* (Kemp and Cole,1961)

A. From Culture Material

1. Fix the fertile material from culture in 3:1 ethanol-acetic acid solution for 2hrs. [The fertile material is obtained as follows: allow the fertile parts to dry for 2-3hrs. remove the surface contamination by thoroughly rinsing in water. Place them in Erlschreiber solution (Star, 1956) for a brief period. Imbibition occurs which aids in the liberation of zoospores. Pipette out 20.c.c. of nutrient solution containing active motile spores into a slide tray containing 20 slides and 100.c.c. nutrient solution. Change the culture solution every 4-6 days. Maintain the culture in a room of 6-9°C at light intensity of 40-60 ft.candles, with 12 hrs dark and light period. The gametophyte will produce eggs and sperms with in 33 days of initiation of the culture. The zygote thus produced get attached to the slide].
2. Add few drops of Potassium iodide solution to the fixative .KI aids in fixation and also serve as decolorizing agent for chromatophore.
3. Remove the slide from fixative, rinse in tap water and place in 2% iron alum solution for 15 min. at room temperature.
4. Then incline the slide in 45° angle and flood with aceto-carmines until red grey disappear. (e rinsed in tap water immediately after iron alum treatment do not stain intensively).
5. Place the coverslip and warm over spirit lamp and seal it temporarily.

B. From field

1. Collect the sore of *Nerocystis* at various stages of development and fix in 3:1 ethanol-acetic acid solution.
2. Transfer to 70% alcohol.
3. Make hand sections or microtome sections and dehydrate by tertiary butyl alcohol method of Johansen (1940).
4. Mordant the hydrated slide for 15min. at room temperature in 2% iron alum solution.

5. Place it in preheated aceto-carmine (60°C) for 15-20 min.
6. Run stained slides through an ethanol-xylene series, place cover slip using gum damer mountant.

Modified Aceto-carmine method for *Eudesmus* (Cole, 1967)

1. Fix the gametophyte and sporophyte materials in 3:1 ethanol-gl.acetic acid solution at least for 24 hrs.
2. Wash a small piece in water, place in a watch glass containing 6 drops of aceto-carmine and 2-3 drops of 2% iron alum or saturated solution of ferric acetate in acetic acid.
3. Heat and keep at boiling point for several minutes till the material become black. Add aceto-carmine as the staining solution evaporates.
4. Place the material on a microscopic slide in a fresh drop of aceto-carmine.
5. Warm it repeatedly after flooding with aceto-carmine until the grey colour disappear from the solution.
6. Place a coverslip over the material, press down firmly on the material with thumb or tap gently and firmly with the blunt end of a dissecting instrument.
7. Seal the coverslip with paraffin gum mastic compounds. Temporary slide, if necessary, make permanent according to the standard alcohol-cupral method.

Feulgen staining for Laminaria (Walker,1954)

1. Collect the germinating spores on coverslip by placing it under the bottom of tank.
2. Fix in 3:1 absolute alcohol–acetic acid solution for 2-3 hrs.
3. Hydrolyse in 1N.HCl for 10 min. at 60°C.
4. Treat with feulgen reagent, 4-5 hrs.
5. Wash in 45% acetic acid.
6. Dehydrate through increasing concentration of alcohol to absolute alcohol.
7. Mount in cupral. Slides are lightly warmed to increase differentiation.

Feulgen staining according to Naylor (1957)

This method is found to successful for gametophyte materials of Laminarials.

The schedule is as follows.

1. Fix the coverslip on which the gametophytes are growing in 3:1 alcohol-acetic acid minimum period of 24 hrs.
2. Wash them thoroughly in several changes of tap or distilled water.
3. Hydrolyse in 1N.HCl at 60°C for 8-10 min.
4. Stop the hydrolyse by rinsing in cold water.
5. Transfer the coverslip to decolourised Schiff's reagent and leave there for 8hrs at room temperature.
6. Following this bleach them in three changes of recently prepared SO_2 water (N.HCl-5ml, 10%$K_2S_2O_3$-5ml, distilled water-100ml).
7. Wash the material, if necessary rolling with a pith.
8. Dehydrate by immersing for 10 min. in 30, 50, 70, 95% and absolute alcohol [care must be taken when flooding with 30% alcohol, as much of the material will become dislodged and lost].
9. Pass through cupral essence and finally mount in cupral. The cupral must be allowed to harden.

Feulgen staining for large parenchymatous forms (Roberts 1966)

This method has been successfully employed in vegetative apices and developing receptacles of fucales

1. Follow fixation and bleaching in carmine method.
2. Heat a of portion the material to 60°C in distilled water.
3. Hydrolyse in N.HCl at 60°C for about 7-10min. (If bleaching has been omitted, extend the hydrolyse time within the range of 15-30min.).
4. Stop hydrolysis by transferring to cold distilled water.
5. Transfer to freshly prepared Schiff's reagent f12-16 hrs.
6. Wash the material for 10min. in running water.
7. Bleach again in freshly prepared SO_2 water.
8. Make hand sections and mount in SO_2 water.
9. Dehydrate the preparations and mount in eupral.

Modifications of Paraffin method

Staining for Laminarials (Ohmori, 1967)

1. Cut into small pieces the portion of sori at various stages of development, remove the mucus around it with filter paper and immerse the material in Abe's fixative for 12-45 hrs without exposure to light.
2. Wash well in sea water and dehydrate in the following manner.
3. Sea water 90ml+95% alcohol,10ml : 30min.
4. Sea water 80ml+80% alcohol, 20ml : 30min.
5. Sea water 70ml+70% alcohol, 30ml : 30min.
6. Sea water 40ml+Distilled water, 20ml+95% alcohol, 40ml : 1hr.
7. Sea water 20ml+Distilled water, 40ml+95% alcohol, 40ml : 1-2 hrs.

8. 50% alcohol : 3-4hrs
9. 70% alcohol : 24hrs or more
10. 85% alcohol : 10-24hrs
11. 95% alcohol : 14-24hrs
12. Absolute alcohol : 14-24hrs
13. Chloroform, 1ml + ab.alcohol, 3ml :1hr
14. Chloroform, 2ml + ab.alcohol, 2ml :1hr
15. Chloroform, 3ml + ab.alcohol, 1ml :1hr
16. Pure chloroform :1hr
17. Embedded in paraffin and stain according to standard method.

Staining for Pelvetia (Subrhmanyan 1956)

1. Fix material in PFA_3.
2. Washin 50% alcohol, followed by 70% alcohol.
3. When there is no more picric acid to extract in alcohol, transfer to absolute alcohol and leave for 3-7 days changing the alcohol once in a day [If the material is receptacle or older portion of thallus leave in each change overnight].
4. Replace the absolute alcohol by thin cedar wood oil which is to be changed twice in the course of two days.
5. When the material become transparent replace the cedar wood oil by xylol, three times in the course of two days.
6. Paraffin embedding and cutting according to Johansen (1940).
7. Stain iron alum haeamtoxylin
8. Wash the slides at least in 6 hrs in running water.
9. Orange G and light green dissolved in clove oil may be used as counterstain.
10. Make permanent slide in Canada balsm

Staining Methods in Rhodophyceae

Haematoxylin, Brazlin, Aceto-carmine and Feulgen staining are used in red algal cytology. By far the aceto-carmine method yielded better results. Details of different types of haematoxylin methods which are now superseded by other stains are well described by Johansen (1940). Whole mount, serial sectioning and squash preparations are employed in staining. The former with little squashing is used for uniseriate filaments. As the serial sectioning require the embedding of material in paraffin it is now disfavoured in many cases, nevertheless used in cases where cellular identification is of critical importance. However, the squash method has yielded good results in chromosomal study. The success of staining prior or after the squash preparation is depending on the material under study. If the natural adhesion between the material and slide or coverslip is very weak much material will be lost in the processing. The use of either an albumenized or gelatinized slide or coverslip will enhance the adhesion of the material and will limit its loss incurred during squashing. The hardening of red algal materials varies considerably after fixation and certain amount of softening is desired prior to staining. Some genera can be used directly for staining without any treatment for softening.

Brazilin Staining of Drew (c.f. Friedman, 1959)

1. Fix the material in alcohol–formaldehyde fixative after removing the excess of moisture. Change the fixative after 24 hrs. and place in strong light for bleaching.
2. Wash well in 70% alcohol.
3. Mordant for 1hr. in alcoholic alum. (4% aqueous iron alum, 23ml + 90% alcohol, 77ml. For many algae this solution is useful only after 4-6 weeks after preparation).
4. Wash for 1hr. in several changes of 70% alcohol.
5. Stain in 0.5% brazilin in 70% alcohol for 8-14 hrs. [This solution takes 4-6weeks to ripen and it is convenient to have concentrated stock solutions from which dilutions can be made].
6. Wash well in 70% alcohol until no excess stain comes out.

[The time required for treatment in the following solutions varies somewhat according to the material under study but what is given form a good basis for experiment.].

7. Pass through a series of 5% grades of alcohol leaving material in each grade for 15-20 min.
8. Place in absolute alcohol for 1 hr. changing the liquid once.
9. Pass through series of seven grades of absolute alcohol–xylol mixture leaving material. 15-20 min. in each grade.
10. Place in xylol for 1hr.
11. Place in dilute balsm-xylol mixture and leave for several hours (overnight is appropriate).
12. Pass through three higher grades of balsm-xylol mixture and mount.

The strength of the mordant and length of the mordanting time will determine the depth of the stain.

Advantages

1. Both cytoplasm and cell wall are lightly stained in contrast to chromosomes which are blackish red in appearance.
2. The use of stain and mordant in 70% alcohol will eliminate to ascertain extent the swelling effects met

with haematoxylin.

3. It requires less time.

Feulgen staining (Rao. 1968)

1. Fix the material in *Westbrook's* fixative.
2. After the material is sufficiently bleached by the fixative, soften it in a mixture of alcohol and hydrochloric acid (Drew, 1943).
3. Squash piece of softened material in 70% alcohol under a albumenized cover glass.
4. Carefully heat the slide over a spirit lamp.
5. Keep the slide in an inverted position in a dish containing 70% alcohol and allow the cover glass with the material adhering to it to drop.
6. Hydrolyse in N.HCl for 6-8 hr at 60°C.
7. Stain overnight in Schiff's reagent.
8. Make the slide permanent according to Johansen (1940) using Canada balsm as mounting medium.
9. Allow the permanent squash thus prepared to set for few days. Keep in a hot air oven for 10-15min. at 60°C.
10. Cover the warm slide with one or two folds of filter paper and squash further applying pressure by pounding over the area of the cover glass with a piece of hardwood having perfectly flat surface or with blunt end of a pencil. This method flattens the nucleus and all chromosomes can be seen in one focal plane.

Aceto-carmine staining method (Rao, 1953)

1. Fix material in 3:1 absolute alcohol-acetic acid mixture for 10 min. to few hrs. till complete decolour-isation occurs. Best results are obtained when the material is stained soon after fixation.
2. Bring material down to water through 90, 70, 50 and 30% alcohols.
3. Wash (2-3 changes) with distilled water.
4. Mordant in freshly prepared 4% iron-alum solution for 5-20 min.
5. Wash again (2-3 changes) with distilled water.
6. Place the material on a clean slide, add few drops aceto-carmine solution, put a cover glass and heat to boil. (repeat the boiling twice or thrice).
7. If material required spreading squash with cloth covered finger.
8. Add fresh acetocarmine to margins of cover glass, heat again and keep the slide warm the slide for 10 min. on hot plate.
9. Make the preparations permanent according to Medintold's method recommended by Manton (1950) [Temporary preparation invariably look more elegant than permanent. Being a rapid one this method is useful in determining time of nuclear division.].

Aceto-carmine staining method(Austin,1959)

1. Fix the material in Carnoy's fluid or in acetic acid-methyl alcohol-chloroform (1:6:3 and 3:6:1) mixture. The latter should be prepared just before use to minimize their esterification. Keep more delicate filamentous species for 10-25 min., cartilaginous material for 15-24 hr; tough leathery thalli for 24-48hrs. in the fixative. Most materials can be left in fixative for 3-6 weks without harm if kept at 3-6°C but best results are obtained for materials used immediately after fixation.
2. Delicate forms after alcoholic fixation may be hydrated through 70, 50 and 30% alcohol before washing. However, in most cases the material can be transferred directly to water or 30% alcohol. Wash thoroughly for at least 15-30 min. Prolonged washing successfully softens the cartilaginous species.
3. Mordant in 0.5-5% concentration of ferric ammonium sulphate for a period from 30 sec. to 1hr. depending upon the nature of the material understudy. The most useful concentration appears to be 1%. It is advantageous to use mordanting solution after fixation than by dissolving the mordanting salt in fixative.
4. Remoe the excess of alum by washing.
5. Place the material on a clean slide in a drop of aceto-carmine made up according to Belling (1926) but with a drop of (0.03ml) of saturated ferric acetate added per 10 ml of stain solution.
6. Tease or break up the material with nickel plated needles and heat the slide repeatedly by adding drops of aceto-carmine to avoid excessive drying.
7. Place a coverslip over the material when it became purple black in colour. If over stained, remove it by adding few drops of 45% acetic acid or by flooding with 1% iron alum.
8. Heat till coverslip settles and squash the material slightly. Squash further by applying pressure through filter paper with a chisel edged bone or plastic instrument. With some tiny fragments of material such as single sporangium it is best to press the coverslip directly with squashing instrument to ensure visibility and complete spreading.
9. Seal the preparations by ringing carefully with glycerol jelly. They will remain in good condition for more than 3 months. If necessary make permanent by following usual method.

Aceto-carmine staining method of Austin (1959)

modified for *Polysiphonia* (Kapraun, 1978)

1. Fix the freshly collected material in 3:1 ethanol– acetic acid- for 8hrs.
2. After washing transfer to a mordant of 2gm hamematoxylin and 0.5gm of iron-alum in 50ml glacial acetic acid.
3. Remove the excess of mordant and soak the material in 1% HCl for 3-5min.
4. Wash and stain in 1% aceto-carmine. Heat the slide to intensify the staining and clear the cytoplasm.
5. Follow steps 6-9 given above

Modified aceto-carmine method for *Prasiola* (Friedman, 1959)

1. Fix the material in the mixture of formalin- acetic acid-alcohol.
2. Transfer to distilled water through alcohol stages.
3. Mordant for several hrs. in freshly prepared 4% iron alum solution.
4. After rinsing, heat in 4% carmine solution.
5. Leave in the stain for 5-7 days.
6. Destain the purple black thalli in 45% acetic acid if necessary by boiling.
7. Wash thoroughly in distilled water.
8. Pass through alcohol stages and xylol.
9. Mount in balsm

[The whole mount can also successfully stain in Brazilin too. Cole and Akintobi (1963) after fixing the material in formalin- acetic acid-alcohol mixture, kept the fixed material in closed bottle in an oven at 75°C for about 12 days. The fixative was changed every 4 hour until the thalli was completely bleached. They got good results with propiono-carmine method of Darlington and LaCour (1960) as the method of Friedman was more time consuming.].

Aceto-iron-haematoxylin-chloral hydrate stain for *Porphyra* (Conway and Cole, 1973)

This method is adopted from Wittmann's 1965 method. The schedule is given below.

Preparation of stain.

1. Make a stock solution containing of haematoxylin-4gm, iron alum-1gm, 45% acetic acid-100ml. (Allowing 24 hrs. ripening before use is advisable; some batches require 2-7 days).
2. Take 5ml of the above stock solution and dissolve 2gm of chloral hydrate. This solution is ready for use when the chloral hydrate dissolves. It can be kept for two weeks. No heat should be applied for hastening the dissolution of any chemical.
3. Fix the material in 3:1 absolute alcohol-glacial acetic acid fixative.
4. Place the fixed material on a slide and remove the excessive fixative.
5. Add a drop of the stain solution, if necessary the material may be crushed with flat instrument and place a coverslip over the material.
6. Heat the preparation carefully until a slight colour changes occurs.
7. Squash further applying more pressure and seal by ringing with rubber solution.

Induction of Nuclear Divisions

One of the major problems in algal cytology is the scarcity of nuclear divisions which is more acute in marine algae. While it is possible to induce nuclear divisions algae grown under culture conditions, it is not so with the naturally growing materials. Various attempts have been made by several workers and different methods have been suggested to increase the nuclear divisions artificially.

By synchronizing algal cultures it is possible to get algae at a desirable state of growth and nuclear divisions. (For methods of synchronization see-Stein, 1973).

Treatment of algae with growth promoting substances such as IAA, IBA, Indole, ICA, tryptophan, gibberlic acid etc increases growth and cell divisions in algae Effective concentration is found to be vary from species to species. The growth substances are either incorporated into the medium before the inoculation of the algae or it is sprayed on the algal cultures. The materials are fixed at different times after particular period of growth under optimal conditions. Davidson (1950) found 57% increase in apical growth of *Fucus* after the application of IAA in concentrations of 1part in 10^4, 1 part in 10^9 water.

Similarly, colchicine also at different concentrations reported to induce nuclear divisions in algae (Evans, 1962). Several methods have been suggested by Evans (1962) for the induction of nuclear division Phaeophyceae. If applied these methods may also induce divisions other groups of algae.

Softening of Fixed Materials.

The complexity of algal cell walls which almost invariably harden on fixation prevents the adequate spreading of material on squashing. This hardness has found vary with different kinds of fixation as well as with different groups of algae. For example, in red algae the acidic fixatives leave the material often softer than the natural state. According to Robert (1966) the use of a fixative containing chromic or osmic acid will obscure the staining reactions in tissues having abundant plastids.

He has suggested the use of acetic alcohol or propionic acid alcohol fixatives, as these fixatives slowly decolourises the material.

While almost all green algal material and the filamentous and the germ lings of brown and red algae can be squashed directly without the use of softening agents, the major difficulty in squashing and spreading was encountered in the vegetative parts as well as the conceptacles of brown and red algae. Several methods of softening including the modifications of fixation have been suggested by various workers.

In phaeophyceae though satisfactory fixation and staining have been obtained with 1:3 acetic acid-alcohol, other fixatives have also been tested to see its softening effects on cell walls. Evans (1963) used cold 1:3 acetic acid-alcohol for the fixation of *Fucus*. This fixative was prepared just before fixation. Fixation for 18-24hrs. with equal time of washing, bleached material very well and made it less mucilaginous. However, it did not reduce the cytoplasmic staining and granular appearance of cytoplasm but gave large round and speckled nuclei very much like those seen in the squashed preparations made immediately. Other fixatives that gave similar results are cold Carnoy's B solution and cold acetic acid, hot 1:3 acetic acid –alcohol and hot acetic acid. However, cold acetic acid caused swelling of the chromosomes and hot fixatives produced contracted nuclei and chromosomes with granular appearance. The period of washing following fixation must be proportional to the duration of fixation. Satisfactory results have also been obtained with Karpechenko fixative and 1% chrom acetic acid fixative (Johanson, 1940) (c.f. Evans. 1963). Since none of these fixatives softened the wall of the material satisfactorily, it became necessary to find a suitable post fixation agent which would render the cell walls soft and amenable to spreading without adversely affecting the nuclei or chromosomes.

The following methods may be applicable for softening various algal materials.

1. Hydrolysing the material for 3-4 hrs in 1N.HCl at approximately 60°C (cf. Evans, 1968). For red algae most investigations have recommended the use of different concentrations of HCl and NaOH. Kylin (1923) suggested the use of 1% aqueous solutions of either while Drew (1945) recommended 10% HCl in 70% alcohol. Workers like Papenfuss (1937) and Cole (1963) have used HCl in concentrations up to 50% and Norris (1957) recommended the use of 10% NaOH . The use of N.HCl and as a part of feulgen staining and preliminary application should be avoided while this procedure being adopted. Hydrolysis with HCl usually softens the material sufficiently well to permit thorough squashing. Filamentous forms can be squashed directly but with parenchymatous forms it is advisable to cut hand sections and finally bleach in SO_2 water before squashing.
2. Bleaching of fixed materials 30 min. to 4hrs in 20% H_2O_2 following chromic or osmic acid fixations. It removes the pigmentation completely but care must be taken to wash thoroughly the fixative before bleaching with H_2O_2. Bleaching should be stopped after a required period of time.

Lewis (1956) used two methods to overcome the staining difficulty in fucales. (a) treatment of fixed materials for 10-15 minutes with equal volume of saturated ammonium oxalate solution and 20% H_2O_2 solution before washing in distilled water and staining with aceto-carmine. This has been proved suitable for female gametangia (b), maceration of 50gm of fruiting thallus with 150ml of fixative in a warring blender for 4min. This is used in antheridia staining.

3. Treatment of fixed material in 6% Na_2CO_3 solution for half to 30 min. This method was used by Naylor (1957) in conjunction with the treatment with H_2O_2. No satisfactory result was obtained on material previously treated with H_2O_2. It involves the fixing of materials in acid containing fixative for 24 hrs and then washing and transferring to 6% Na_2CO_3 solution on a slide. It is then warmed gently till the material squashes under the pressure of coverslip, wash and stain with aceto-carmine solution. Sodium carbonate itself proved to be an excellent softening agent but nuclei and chromosomes are lightly stained if no mordant is used. Sodium carbonate should be washed out completely; otherwise it will interfere with staining process. Though this method has reported to produce good results in brown algae, Magne (1964) suggested the use of dilute solution of Na_2CO_3 for red algae.
4. Treatment of acetic acid alcohol material for 10-15min. in 1M. lithum chloride solution. It is then immersed for a similar period of time in excessive tap water. Similarly salts of other monovalent metal ions of low atomic weight *viz.* Na K etc are also successful but with increasing atomic weight the spread lessens. Evans (1963) successfully employed this method in four species of *Fucus*.
5. Hanic (1979) suggested treatment of algal material with cupra-ammonium removes cell wall materials followed by neutralization with propiono-carmine (or feulgen) enable thin squashes and better chromosome spreading. This is done by drawing 1-3 drops of cupra-ammonium under coverslip before staining. Cupra ammonium instantaneously dissolved polysaccharide components. Cells treated in this manner expand much

in diameter and allow very easy squashes. [Cupra-ammonium is prepared by bubbling fine streams of air for 2 hrs in 3 litres of fresh concentrated NH_4OH containing 500 gms of fine copper turnings].

Thus considerable variation exists in applicability or lack of effect of different softening agents after the use of particular fixative. The treatment to soften the walls and aid of spreading varies according to the chemical constituents of cell walls and the type of staining to be used.

Karyotype Analysis.

The importance of chromosome numbers in the delimitations of taxa, where considerable plasticity exists in morphological characters, have been emphasized by several workers (cf. Sarma, 1964). Sometimes those species with the same chromosome numbers show marked differences in their karyotypic studies. Species of *Chara* and *Nitella* have been differentiated on the basis of karyotypic analysis. The karyological characters are generally used in algae, besides the chromosome numbers, in the comparison of karyotypes of different taxa are as follows:

Length of the chromosomes
Thickness (width) of the chromosomes
Position of centromere
Number of Nucleolar Organising (N.O.) chromosomes

Length of the chromosome.

On the basis of the length of the chromosomes at metaphase stage of nuclear division Sarma (1959) suggested the classification into four groups:

1. Large size (L) : 5-10 µm and above
2. Medium size (M) : 3-5 µm
3. Small size (S) : 1-3 µm
4. Minute size (Mi) : 0.25-1 µm

Width of the chromosome.

Khan and Sarma (1967) classified into following three groups

1. Thick : more than 1 µm
2. Moderately thick : 1 µm thick
3. Slender : less than 1µm thick

Position of Centromere.

To compare the karyotype of different taxa, *idiograms* (a diagrammatic representation of chromosomes) are constructed on the basis of the relative length of the arms of the chromosomes. The scheme proposed by Levan *et al.,* (1964) has been accepted to a considerable extend, as the terminology regarding the position of centromere, in algal chromosomes. Chromosomes are classified into following four categories, assuming the total length of a chromosome to be composed of ten units. Chromosome with

1. *median centromere*(m): when the length of both the arms of a chromosome are equal.
2. *sub-median centromere*(sm): when the ratio of the two arm's length is more than one and up to three.
3. *sub-terminal centromere* (st): when the ratio between two arms length is more than three.
4. *terminal centromere* (t): when the chromosomes are rod shaped and when there is absolutely no trace of a second arm.

Karyotypic formula

In order to facilitate comparison of two or more karyotypes of different taxa, either with same or with different chromosome numbers, formula giving the notations specified above are given to characterize each karyotype.

For example: (1m 1sm) L + (1m 4sm 1t) M + 3sm 1st) S + (2st) Mi = 14.

An analysis of the above formula shows that there are 14 chromosomes, of which 2 are large size (L) with a median and sub-median centromere; 6 are medium size (M) with one median, four sub-median and one terminal centromere; 4 are small size (S) with three median and one sub-terminal centromere; 2 are minute size (Mi) with two terminal centromere.

Summary

This paper discuss the brief summary of history algal cytological work, different types fixatives and stains hitherto used in cytological studies, various techniques utilized by different workers in algae, softening methods for hard algal thallus in cytological preparations, induction methods for nuclear divisions and discuss the karyotypes with karyotype formula

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