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A REVIEW ON FIXATIVES AND PRESERVATIVES

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Following the deterioration noticed by a few specialists in zooplankton taxa collected during the International Indian Ocean Expedition, SCOR/UNESCO/WG-23 was established in 1968 to study the problems involved in detail and recommend necessary action. The experiments designed by the Working Group were replicated at the Indian Ocean Biological Centre to study the effect of climatic variations on the various fixatives and preservatives. This necessitated a review on the existing techniques in this field of study.

Fixation involves killing, fixing and hardening of living tissues or of whole organisms. By killing, a fixative destroys the life either slowly after narcotization or instantaneously. Narcotization is of particular value for keeping the contractile animals in extended condition. These are narcotized first and then fixed instantaneously to avoid any post mortem changes in shape.

Methods of narcotization are published by Tregouboff and Rose (1957), Wagstaffe and Fidler (1955) and Marr (1963). Narcotics used in general are the following: (1) Chloroform, (2) Chlorotone 0.005 to 0.05%, (3) Cocain, (4) Ice, (5) Carbon dioxide or soda water, (6) M. S. S. 222, (7) Benzamine hydrochloride, (8) Ether vapour for Halobates, (9) 2% Chloral hydrate for hydroids and nemer-

tians, (10) Nicotin or Tobacco smoke for ciliates, (11) 10% Alcohol for fresh water animals, (12) Ethyl urethane for *Sabella* and *Lumbricus*, (13) Magnesium chloride, 7.5% for marine animals and sessile actinozoa, (14) Menthol for marine and fresh water sessile forms, Polyzoa etc., (15) Saturated solution of Magnesium sulphate added in small quantities to the water for most marine and freshwater forms, (16) Hanley's solution—90 ml water and 10 ml Ethyl cellosolve with 0.3 gm eucane HCl—one drop in 10 ml for Rotifers and Bryozoans to be added at 10 minutes interval and (17) Gray's mixture—lighter than water is made by mixing 52 gm Chloral hydrate and 48 gm Menthol—for coelenterates.

Purpose of fixation is (a) to preserve the actual form of tissue elements avoiding as far as possible post mortem changes in the gross physical structure of the material preventing bacterial decay and autolysis, (b) to produce the optical differences in structure, or to affect the tissues in such a way that structural difference will be brought out through subsequent treatment with stains etc.

The fixatives should have the following properties: (1) kill the tissues instantaneously so that structural chan-

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ges taking place are at a minimum, (2) lessen subsequent shrinkage and distortion or distension and harden the material, (3) render the cell substances insoluble by denaturation, i.e., change of the nature of the protein without adding any new kind of atoms, (4) penetrate all parts equally well, (5) often change the refractive index of various parts of the cell and thus make them more visible and (6) render the parts of the cell easily stainable.

Purpose of preservation is (a) to remove the nutrient for bacterial growth, (b) to interfere with their metabolism by adjusting the physical environment beyond the tolerance of bacterial cell by dehydration, pH, osmosis etc. or (c) to stop microbial invasion by chemical or physical attack. This is because most materials are either nutrient or carry enough nutrient to render them subject to microbial invasion. For a preservative to be satisfactory it must be (1) colourless, (2) odourless, pleasant to handle (non-pungent), (3) non-volatile, (4) non-transparent, (5) non-poisonous, (6) able to preserve the natural colour of the organism, (7) bactericidal and fungicidal, (8) low in price, (9) able to maintain good condition of specimens prolonging the duration of their shelf-life and (10) able to maintain weight and volume unaltered.

Types of Fixatives : Microanatomical fixatives include (1) Heidenhain's iron-haematoxylin, (2) Bouin, (3) Duboscq-Brosil alcoholic Bouin and (4) Zenker's fluid. Cytoplasmic fixatives are (1) Gatenby's Flemming without acetate, (2) Formalin and

(3) Baker's formaldehyde calcium for cell lipids and golgi bodies. For yolk laden forms Smith formol bichromate is the best.

The chemicals most frequently used as fixatives are (1) Ethanol, (2) 40% Formaldehyde, (3) Acetic acid, (4) Picric acid, (5) 1% Chromic acid, (6) Saturated solution of Potassium bichromate, (7) Saturated solution of Mercuric chloride, (8) Cadmium chloride and (9) 2% Osmium tetroxide.

The commonest preservative in use for plankton are (1) Formaldehyde, (2) Paraformaldehyde, (3) Ethanol, (4) Isopropanol, (5) Zephiran, (6) Ethylene glycol, (7) Phenoxetol, (8) Dowicil 100 and (9) M.D.M.H.

Since centuries it was customary to fix and preserve animals and plants in dilute formalin or aqueous solution of ethyl alcohol. The same methods were used for the study of marine life also. Soon it was noted that a 70% aqueous solution of ethyl alcohol or a 37% solution of isopropanol produces a white precipitate with sea water. In addition to cost being high it volatilises very rapidly and instead of maintaining the volume and weight constant, causes shrinkage and thus causing gross morphological change. Hence, in spite of its pleasant smelling and non-poisonous nature, use of ethyl alcohol and isopropyl alcohol was restricted. Hot 70% alcohol is used for fixing insects and nematodes. Alcohol fixation in most instances is found unsatisfactory due to the necessity for frequent changes for the first three days. Glacial acetic acid

is used with alcohol to increase penetration and to counteract its tendency to cause shrinkages of tissues. This produces good optical differentiation as well.

2-4% formaldehyde (Walker, 1944 and 1964), though a good fixative and preservative in several ways has some disadvantages due to the unpleasant pungent odour, volatile, poisonous nature, absence of ability to preserve colour etc. Specimens should not be kept in formalin for long duration if decalcification is to be avoided, unless an effective buffer is maintained in order to remove the formic acid. Formaldehyde neither destroys nor preserves fat but is good cytoplasmic fixative. It is not a fixative for carbohydrates, but preserves proteins in such a way that glycogen is held by it and not easily dissolved by water. Hence it is difficult to fix fatty crustaceans in formaldehyde. Because of formalin's tendency to swell brain tissue, King (1913) pronounced it unfit for cytological work on brain but found that nerve tracts are not adversely affected by it. Formaldehyde is used for fixing and preserving when frozen sections are to be made. Material which has been in formalin may be rendered more pliable and harmless to the skin, while dissecting, by soaking in a 30% solution of Carbonic acid. Poriferans are preserved in alcohol after 40% formaldehyde fixation as is done for echinoderms. Miller (1952) discussed unfavourable effects of acidity resulting from long term preservation in formalin and presented several methods of alkalization. Addition of a thin layer of liquid

paraffin on the top of the preserving fluid in the container can prevent oxidation of formaldehyde into formic acid.

Paraformaldehyde, $\text{HO}(\text{CH}_2\text{O})\text{NH}$ (Walker, 1953) known as paraform containing 90-98% formaldehyde was used as concentrated dry fixative-cum-preservative whenever economy in the bulk and weight of supply is important and when purity of formaldehyde is desired. A method for preparation of formalin from paraform is published by Taube (1962) and Steedman (1968). 2 gm of paraform dissolved in 100 ml sea water at 70C will give 2% formaldehyde in 20 minutes. Paraform produces a precipitate and as it is poisonous its use is restricted. Fish preserved in this had an increased slipperiness and acquired translucent property (Taube, 1965).

Beers (1968) made plankton fixation in glutaraldehyde and subsequent preservation in 70% ethanol. Glutaraldehyde was used in combination with phosphate buffer in fixation of foraminifera. Flugal (1968) suggested fixation of micro-organisms in glutaraldehyde and post fixation in a Dowacide solution. Experiments are in progress to test the fixation properties of esters over aldehydes and alcohols.

Acetic acid being anomalous is not a fixative of globulin or albumin, but a powerful precipitant fixative of nucleoprotein (chromatic fixer). It penetrates well, but destroys, mitochondria, golgi apparatus, and fats and lipoids in general. Osmic acid, though a bad nucleus fixative and a poor penetrator, is the best cytoplasmic fixative. Chromic

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acid, Osmic acid and corrosive sublimate enter into chemical combination with certain of the tissue elements. Alcohol, Picric acid and Nitric acid act by coagulating or precipitating various constituents of the tissue. Hardening necessary to bring the tissues to the proper consistency for cutting sections is completed in alcohol. While Acetic acid and Potassium bichromate swells the material, Chromic acid 1%, alcohol and Mercuric chloride tends to shrink it and may lead to overhardening. Mercuric chloride in saturated solution is good for preserving calcareous structures. Bouin's fluid gives a very delicate fixative not satisfactory for kidney or cells containing mucin. Bouin's fluid decalcify molluscs and so neutral formaldehyde is good for molluscs. Coelenterates can be best fixed by the addition of hot Bouin's fluid after anesthetization. For platyhelminthes Zenker's fluid is preferred over Bouin's and is one of the best general fixing agents known, particularly for vertebrate histological work.

The fixative and preservative qualities of Ethylene glycol (glycol, ethane-diol), at first used as an additive to preservatives to prevent desiccation, was tested by Williamson (1965). Ethylene glycol has the advantage that its osmotic reactions are less violent and stronger solutions may be used without the danger of distorting delicate organisms as *Sagitta*, *Tomopterus* etc. Though pigment loss is noticed, it is less compared to formalin. Due to clumping of cytoplasm, Ethylene glycol is not found to be a good cytoplasmic fixative. Its usefulness in the storage of samples in small jars and bottles is worth mentioning due to less rapid evaporation. In this

case periodic topping up is not required and the common practice of immersing tubes of specimens in a jar full of preservative becomes unnecessary. Added to that it is less costly than alcohol and does not form any precipitate with water.

Sykes (1965) introduced use of Zephiran-alkyl dimethyl benzyl ammonium chloride—as a preservative due to its bactericidal activity in spite of its high toxicity. This odourless, non-staining chemical is used in the dilution 1:1000. This is an invert soap having a positively charged organic part as distinct from ordinary soaps having a negatively charged organic part. It is used in surgery and food industry, and as a preservative in paper and textile industries. At Indian Ocean Biological Centre use of Zephiran as a preservative for plankton is being tested.

Phenoxetol or Glycol monophenyl ether (B-phenoxyethyl alcohol) which was put into market in 1949 was introduced in the field of preservation first by Owen and Steedman (1956, 1958). Phenoxetol is a colourless viscous fluid which takes up the pH of the water used to make the aqueous solution. A 1% solution, preserves zooplankton better than formalin. Euphausiids and Hyperiids retained colour, but the shells of animals dissolved and disintegrated after 6 months. The use of phenoxetol as a post fixation preservative in the absence of irritating and unpleasant vapours (mild carbolic smell) and non-irritant, colourless nature is found to be of great importance. Phenoxetol was used by Rankin (1953) as a cure for fin rot and other diseases of the fishes. Owen (1955) used Propylene phenoxetol as a

relaxing agent for lamellibranchs. Owen and Steedman (1956, 1958) found phenoxetol in 5 to 10% glycerol retain colour and keep tissues soft and flexible. Boem (1957) noted the favourable effect of the simultaneous use of a combination of one or more of the esters of Hydroxybenzoic acid as preservative. Nipa esters are found to be narcotic as well as bactericidal.

In addition Steedman (1968) suggested usage of two new chemicals, Dowicil 100 and Monomethylol dimethyl Hydan-toin (M D M H) which are supposed to be formaldehyde donors, as fixatives and preservatives of plankton. These are being tested at Indian Ocean Biological Centre.

No ideal single fixative has been discovered which can meet all requirements. Hence it is usual to combine two or more reagents which individually

posses certain of these desirable qualities. All the best fixing reagents are mixtures, different mixtures for different purposes. The experimental results obtained so far showed that 2% formaldehyde is the most suitable for fixation of marine zooplankton at the pH between 6.5 and 7.5. Of the various preservatives tested for preservation of zooplankton for general purposes 1% formaldehyde and 1% Propylene phenoxetol were found the best.

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