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Research Article

DYES AND CHEMICALS USED IN BIOMATERIAL STUDY AS STAINS FOR INVERTEBRATES

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ABSTRACT

Most of the dyes used in histology and cytology are manufactured for use in the textile industry, printing, food, cosmetics and other colorant industries. Chemicals used in the study include dyes and stains; the stains used are eosin, acetocarmine, rose bengal, magnesium chloride, magnesium sulphate, cocaine, menthol, propylene phenoxetol, osmic mercuric chloride, mercuric chloride, acetic acid, glycerin alcohol, phenoxetol, nitric acid, potassium cyanide. The dyes and stains as chemicals are used to preserve whole mount or to anesthetize the large animals like trematodes, nematodes, cestodes, arthropods, and molluscs. The study of fresh water and marine invertebrates is not possible unless the use of above mentioned chemicals.

Keywords - Histology, Cytology, Stains, Anesthetize, Invertebrates.

INTRODUCTION

Because most tissues do not retain enough color after processing to make their components visible under a microscope, it is expedient to add colors by staining them. Correctly chosen stains aid in identifying tissues and their elements as well as in diagnosing pathological conditions. Most of the dyes used in the histology and cytology are manufactured for the use in textile, printing, food, cosmetics, drugs, and other colorant industries. The market for biomedical dyes is small, so there is little leverage when it comes to specific quality. These dyes and chemicals are evaluated for moisture content, strength of reflectance of dyeing (compatible to control a running slide), strength of transmission of dye solutions, chromatography, sensitivity to the pH, solutions stability and many other tests[1]. Environmental and safety issues became the driving force for change in the 1960's beginning in the Europe and eventually sweeping North America and Japan[2]. In case of haematoxylin, the latest shortage came about because the sole extractor of logwood simply dropped it off its production schedule to concrete on more lucrative botanical extracts for the skin care industry[3]. Participating vendors of dyes and stains submit samples of a given batch of powder to the Biological Stain Commission (BSC) Laboratory. These are put through a variety of tests that might include (depending upon the dye) absorption characteristics with a spectrophotometer to verify color, amount of colored materials in the powder (percent dye content) by physical or chemical method to assess purity and biological test on appropriate control specimens in a variety of plants, animals and microbiological procedures[4]. Ingredients other than dye itself may aid solubility, condition the tissue to be more receptive, or serve in the complex process known as ion exchange staining[5]. Such processed and potential ingredients in the form of chemicals and dyes as stains are used in the biomaterial science for staining various invertebrates, zooplankton, phytoplankton, their tissues and other processes.

Staining of benthic fauna

The monotonous procedure of separating benthic fauna from associated debris has been shortened considerably by the development of various flotation techniques[6-8], but the end result of these methods is usually a mixture of animals and organic detritus. After trying many histological stains both singly and in combination, notably gentian violet, eosin, fluorescein, malachite green, methylene blue, Gram's iodine, basic fuchsin, rhodamine B, borax carmine, lignin pink, chlorazol black, rose bengal, Leishman's stain, Lugol's iodine[9] and the xanthene stain rose bengal proved to be the best primary stains for benthic invertebrates.

The primary stain is to be added to floating benthic samples, in a ratio of about one part stain to four parts solid samples, for atleast 24 hours duration; the preservative can be either 5% formalin or 70% ethanol. After this time, the primary stain is rinsed with the tap water and just enough chlorazol black is added to cover the samples. After about 30 seconds the counter stains are rinsed out with tap water and the samples are sorted[10].

Anesthetizing and Narcotizing Agents

For the study of benthic organisms, it is very necessary to preserve and fix them with suitable chemicals. Fixatives can be applied directly to many of these organisms. However, because of their propensity to contract or ball up and pull in their tentacles or other appendages, make the whole mounts or sections practically worthless. Careful anesthetizing or narcotizing of these organisms must precede killing and fixation. Following chemicals are used as the anesthetizing and narcotizing agents[11].

Magnesium chloride or magnesium sulphate

Either one is widely and successfully used on sea anemones, corals, annelids, tunicates, and nudibranchs, to name a few. Crystalline magnesium sulphate can be tied in a bag suspended above, just touching the water surface, or a 33% aqueous solution siphoned slowly in, controlled by a screw clamp. When the organisms are anesthetized, siphon off the water until the animals are barely covered and carefully add fixative. Disturb the animals as little as possible. When partially hardened, transfer to fresh fixative[12].

Cocaine

Its possession, cultivation, and distribution are illegal for non-medicinal and non-government sanctioned purposes in virtually all parts of the world. Although its free commercialization is illegal and has been severely penalized in virtually all countries, its use worldwide remains widespread in many social, cultural, and personal settings. **Cocaine** (benzoylmethylecgonine**)** (INN) is a crystalline torpen alkaloid that is obtained from the leaves of the coca plant. It is the stimulant of the central nervous nervous system, an appetite suppressant, and a topical anesthetic. Specifically, it is a serotonin-norepinephrin-dopamine reuptake inhibitor, which mediates functionally of these neurotransmitters as an exogenous catecholamine transporter ligand. A drug, it can be used for ciliates, rotifers, bryozoans, hydras, some worms, and nudibranchs. A 1% aqueous solution is added to the water in proportions of about 1.0 ml to 100.0 ml of the water containing the animals. Eucaine hydrochloride can be used in the same manner. Check for contraction and fix[13-14].

Menthol

Sprinkle on the water surface and leave overnight. Good for sessile marine animals, coelenterates, some bryozoans, hydroids, and flukes which are difficult to narcotize. It is more efficient when combined with chloral hydrate, in proportions of 45.0 gm menthol and 55.0 gm

chloral hydrate. Grind together in a mortar with a little water. Drop on surface of the water. Fix the animals when they no longer contract. Large marine forms probably will require overnight treatment. Chloral hydrate can be used alone by sprinkling on water surface for annelids, molluscs, tunicates, bryozoans and turbellarians[15].

Chloroform

Chloroform was once widely used anesthetic. Its vapour depresses the central nervous system of the animal, allowing to perform various laboratory techniques and to proceed further for experimentation .One possible mechanism of action for chloroform is that it increases movement of potassium ions through certain types of potassium channels in nerve cells. Chloroform could also be mixed with other anaethetic agents such as ether to make C.E. mixture or ether and alcohol to make A.C.E. mixture. This can be dropped on the surface for many aquatic forms and is used in special bottles for insects and arachnids[16].

Alcohol

Alcohol in dilution or in other form is used as preservative. Ethanol is a non-additive precipitant fixative. It fixes proteins by dehydration and precipitation, the degree to which this is done being dependant on the amount of water present and the solubility of the materials in the mixture. Fixatives containing ethanol are usually, but not exclusively, water free or contain only minor amounts of water so that precipitation is a major effect[17].

Several hours for a 3 mm thick piece of tissue should be satisfactory. Thinner tissues such as fine needle biopsies will be fixed within an hour or two, bearing in mind that fixation continues during dehydration. Ethanol is rarely used alone as a fixative because of its shrinkage and hardening effects. An exception is when inadequate formalin fixation is used, and the tissue is transferred to ethanol for dehydration. Since the tissue is not properly fixed, or may be unfixed, the ethanol fixes as it dehydrates with all the hardening and shrinkage obtained when it is used alone. This is often called parched earth artifact [18].

It is compatible with formaldehyde, acetic acid, mercuric chloride, picric acid, methanol, acetone and other agents. Strong oxidising agents such as chromium trioxide should be used with caution as they may oxidise ethanol to acetaldehyde and acetic acid. No particular aftertreatment is needed. It is unusual to transfer to water after treating with ethanol, and tissues are more appropriately transferred to a clearing agent directly or to a higher concentration of ethanol if the fixative used was a mixture. These can be used by dropping on the water or alcohol can be added gradually to the water by a tube controlled with a screw clamp until the proportion of alcohol to water is approximately 10%. It is particularly good for fresh water forms and earthworms. Ether can be used like chloroform for insects[19].

Asphyxiation

It is the condition of severely deficient supply of oxygen to the body that arises from being unable to breathe normally. Asphyxia causes generalized hypoxia , which primarily affects the tissue and organs . The body creates the need to breathe from the excess carbon dioxide in the lungs ; the body has no way to detect the absence of oxygen . Many gases, though non-toxic, are classified as simple as aphyxiants in their pure form or in higher concentrations for this very reason. One form of the asphyxiation is from entering a low oxygen atmosphere or an inert atmosphere, such as in a food oil tank that has a covering blanket of nitrogen or argon to shield the oil from atmospheric oxygen. Carbon monoxide has the higher affinity than oxygen to the haemoglobin in the blood's red blood corpuscles, bonding with it tenaciously, and , in the process, displacing oxygen and preventing the blood from transporting oxygen around the body. Boil the water to remove the air and seal it in jar. It is particularly good for gastropods. Place them in the boiled water after it has cooled[20].

Hanley's solutions (Gray 1954)[21]

Water……..90.0ml

Ethyl cellosolve……10ml

Eucaine hydrochloride……..0.3gm

Add 1 drop per 10 ml of water in which animals are living. Good for rotifers and bryozoans.

Propylene phenoxetol [22-24]

Specimens of some taxa can be identified irrespective of the method of preservation used. However some taxa are unidentifiable unless specific methods and goals and organisms targeted in a given study well determined which method or methods will have to be used. A comprehensive treatment of fixation and preservatives for invertebrates can be found in only commonly used techniques. Propylene phenoxetol is widely used in studying invertebrates in different concentrations as per specifications at standard form. Introduce a large globule of the compound into the water containing the animals in an amount equally 1% of water volume, or shake vigorously 5 ml of the compound with 15-20 ml of sea water. Add to water containing animals. Good for molluscs.

Preservation and Fixation of Invertebrates

Preservation is storage of specimens in a fluid in which they are protected as much as possible from deterioration. Fixation and preservation are often confused because some solutions can be used as both fixative and preservative. Fixative fluid (often formalin) is washed from the sample in the laboratory and replaced by preservative - 70% ethanol unless otherwise specified. Ethanol is available in a 95% solution; dilution to 70% should be done with filtered tap water, or distilled water if use of tap water causes a precipitate**. (Table 1)**

Study of some specific benthic macroinvertebates with respect to stains and fixatives:

Porifera

Small forms can be dropped into osmic-mercuric chloride (water 250.0 ml; osmic acid 2.5gm; mercuric chloride 9.0 gm). Large forms are fixed better in alcoholic sublimate.

Calcareous sponges can be decalcified in 70-80% alcohol plus 3% of hydrochloric or nitric acid. Siliceous sponges can be decalcified in 80% alcohol plus 5% hydrofluoric acid added gradually. Perform the latter in a glass dish coated inside with paraffin. After a few hours, transfer to 80% alcohol. [26]

Coelenterates

Place in a small amount of water (few drops) in a shallow dish. When animals are extended, rapidly pipette warm mercuric chloride-acetic acid (95.0 ml saturated aqueous mercuric chloride, 5.0 ml acetic acid) at them. Work the fixative from base toward oral region, thereby preventing tentacles from contracting or curling. [27]

Sea anemones

Anesthetize overnight with menthol; or 30% magnesium chloride (50 to 100 ml) can be added gradually over a period of one hour; leave in the solution until there is no more contraction of the tentacles. Siphon off the solution until anemones are just barely covered. Add the fixative (Susa, Bouins, Mercuric chloride combinations. 10% formalin – sea water) slowly down the side of container. Also pipette some directly into the throats of the anemones. When the anemones are partially harden, transfer into fresh undiluted fixative [28]

Mollusca

Snails placed in boiled water or add propylene phenoxetol to the water until snails are limp, fix. For decalcifying snail shells, used a mixture of decalcified-fixative (2:1), and the concentration can be as high as 7:1 for 6 hours. RDO (p.50) is his choice for best cellular details and staining properties. This can be used for other mollusks with calcareous exoskeletons.[29]Freshwater mollusks gradually warming the water will make them extend their feet. Fix when they no longer respond to the prick of the needle. Bouins, Zenker, or Gilson are satisfactory.[30]

Table 1: Preferred fixation and preservation methods for major groups of marine invertebrates .[25]

Taxon	Fixation	Preservation	Comments
All others	4% formalin	70% ethanol	"default method"
Annelida (Leeches, Oligochaetes, Polychaetes)	4% formalin	70% ethanol	Leeches and some polychaete families are easier to identify if anaesthetised, but this is generally impractical in large benthic studies.
Brachiopoda	4% formalin	70% ethanol	
Bryozoa (=Ectoprocta)	70% ethanol	70% ethanol	
Cnidaria (others)	4% formalin	70% ethanol	
Cnidaria Octocorallia	70% ethanol	70% ethanol	Formalin will dissolve spicules and render many octocorals unidentifiable.
Cnidaria Scyphozoa	4% formalin	4% formalin	
Crustacea	4% formalin	70% ethanol	
Ctenophora	4% formalin	4% formalin	
Echinodermata	70% ethanol	70% ethanol	Formalin will render many echinoderms unidentifiable, especially holothurians.
Echiura	4% formalin	70% ethanol	Narcotise (freezing or propylene phenoxytol or MgCl2) if at all possible
Entoprocts	4% formalin	70% ethanol	
Mollusca	4% formalin	70% ethanol	
Mollusca Opisthobranchia (=nudibranchs)	4% formalin	70% ethanol	Narcotise (freezing or propylene phenoxytol or MgCl2) if at all possible; photographs recording colour in life are also very useful
Nemertea			Probably unidentifiable unless narcotised (freezing or propylene phenoxytol or MgCl2)
Phoronida	4% formalin	70% ethanol	
Platyhelminthes	4% formalin	70% ethanol	Fix living specimens on frozen 4% formalin [see safety notes above] or narcotise (freezing or propylene phenoxytol or MgCl2). Otherwise probably unidentifiable.
Porifera	70% ethanol	70% ethanol	Formalin will render most sponges unidentifiable
Pycnogonida	70% ethanol	70% ethanol	
Sipuncula	4% formalin	70% ethanol	Very difficult to identify unless first narcotised (freezing or propylene phenoxytol or MgCl2)
Tunicata	4% formalin	70% ethanol	

Annelids

If the sections of worms (aquatic or terrestrial) are desired, the intestines must be freed of grit and other tough particles.

In laboratory techniques feed earthworms on cornmeal and agar (1:1) and some chopped lettuce for 3 days, changing the food every day. Becker and Ro recommended a container with the bottom covered agar. Wash off the agar twice a day for 3-4 days. Moistened blotting paper can be used. When the animals are free from grit, place them in a flat dish with just enough water to cover them. Slowly siphon in 50% alcohol until the strength of the solution is about 10% alcohol. Chloroform can also be used for narcotizing. Fix in Bouins or mercuric chloride saturated in 80% alcohol plus 5% acetic acid. The worms may be dipped up and down in the fixative and then supported by wire through a posterior segment, hanging down in the fixative, or place in the short lengths of glass tubing in fixative to keep them straight. This is necessary if perfect saggital sections are to be cut. Embedding is probably most successful by the butyl alcohol method. After removal of mercuric chloride in iodized 80% alcohol, transfer to n- or tertiary butyl alcohol for 24 hours (change once). Transfer to butyl alcohol saturated with paraffin (in 50-60% oven): 24 hours; pure paraffin: 24 hours and embed.

Sea worms can be kept in a container of clean sea water, changed every day for 2 or 3 days, then anesthetize with chloroform and fixed, using fast penetrating fixative (Bouins or a mercuric chloride fixative).[31-32]

Arthropods

Insects and Arachnids

Ether, chloroform or potassium cyanide are used for killing. Simply place a wad of cotton with a piece of wire screen. Dampen the cotton with ether or chloroform or lay a few lumps of potassium cyanide on it before adding the screen wire. Keep tightly closed with a cork or screw cap. A piece of rubber tubing soaked in chloroform until it swells and placed under the screen wire will hold chloroform for several days. If the appendages should be spread after fixing, place it on a glass slides immediately after the insect is dead.

Glycerin……..12.0 ml

Formalin……..2.0 ml

Distilled water……100 ml

Add a few crystals of thymol, This can be injected into the body cavity of large specimens[33].

RESULT AND DISCUSSION

To shorten the time spent in hand picking of invertebrates from the soil mixtures, various staining methods have been devised[34]. Attempts were also made by researcher to improve the color contrast between animals and detritus by use of counterstaining technique. Many of the chemicals and dyes are used for staining but peculiarly out of them only eosin, acetocarmine and rose bengal are frequently used for staining small macro-benthic invertebrates. Magnesium sulphate and magnesium chloride are used as anesthetizing and narcotizing agents. Cocaine is used for ciliates, rotifers, bryozoans, hydras and some worms. [35]Menthol is used for coelenterates and hydrozoids. Chloroform, as a preservative and anesthetizing agent, can be used for arachnids and insects.[36] Hanley's solutions can be used for rotifers. Propylene phenoxetol can be used for molluscs. Osmic mercuric chloride can be used for corals. Methanol as a chemical can be used for trematodes. Small ova can also be micro-stained with resin[37]. Blood infected with microfilaria can be smeared with haematoxylin, Propylene phenoxetol can be used for molluscs. Annelids and earthworms can be feed on cornmeal and agar in 1:1 ratio to study further histological and biochemical techniques and rearing. For arthropods and insects fixation, dehydration and infiltrations with paraplast are all performed under reduced pressure[38]

CONCLUSION

Fixatives can be applied directly on many of the invertebrates, but not to others because of their propensity to contract or ball up, pull in their tentacles or other appendages, and thereby make whole mounts or these invertebrates sections practically worthless. Careful anesthetizing or narcotizing of these invertebrates must precede killing and fixation. As soon as the narcotization is complete and before death, if possible, fixation can be successful.[39]

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