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Fixation: an overview

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Fixation and preservation are commonly used terms when dealing with fluid preserved material. The terms are used separately or as synonyms, but in general we can consider the terms as;

- Fixation is aimed to arrest the physical and chemical changes that would occur upon the death a biological tissue, and consequently preserve its gross form.
- Preservation is considered to be the method of preserving the fixed state of the specimen by protecting from decay and deterioration, giving the specimen a 'normal' appearance and affording mechanical protection.

So why the need to carry out fixation? It is important to remember that biological tissues are a whole range of reactive chemicals that are held in status by the regulatory control mechanisms of the living organism. On death these processes break down and autolytic decay occurs. Chemically treating biological tissue can prevent autolysis and coagulate cell contents into insoluble substances. A secondary function of fixation is to sterilise the specimen and thus prevent biological decay of the specimen. The process of fixation is extremely important in many research areas, notably histological studies, electron microscopy and immunocytochemistry.

Ideally fixation should preserve a specimen so that it remains unchanged. However in reality this is not possible. To prevent biological tissues from degradation some significant chemical changes need to occur. The result is that these reactions can cause significant chemical change with subsequent effects such as colour change, and shrinkage or swelling on the morphological form of the fixed specimen. It is also important to remember that fixation is only effective with certain chemical compounds in biological tissue, causing incomplete fixation of all the cellular components.

Fixative treatments can be considered as two types;

- 'True' fixatives involve the formation of chemical cross-links. These

chemical reactions tend to be directed at proteins due to the abundance of chemical groups available for such reactions e.g. amines; amides; carboxyl's; hydroxyls etc.

- 'Pseudo' fixatives involve the coagulation or denaturation of the molecules that form the biological tissue. With proteins this involves an unwinding or disordering of the steric structure, altering patterns of steric bonding.

A whole range of fixative chemicals and 'recipes' exist, especially for use in specialist studies (see further reading). As natural science collection conservators we are only likely to come across a few of these fixative chemicals and their associated recipes. By far the most commonly used fixative is the aldehyde formaldehyde, HCHO. This is a non-functional aldehyde that is considered to act as a polymeric fixative that converts protein into an insoluble macromolecular network. Other aldehyde fixatives are also available such as Glutaraldehyde and Acrolein. These are bifunctional aldehydes and are more effective as histological fixatives. However formaldehyde remains the better 'general purpose' fixative due to its small molecular size and subsequent better rates of infusion into the specimen.

Formaldehyde fixation reactions tend to focus on the production of inter and intra-molecular crosslinks, especially with protein molecules as these have the largest number of side groups such as amines and amides etc. It is with such groups that formaldehyde can effectively form crosslinks with. Other cellular components are also potentially fixed but to a lesser extent. Nucleic acids can have crosslinks induced with histones, nucleoproteins and other cellular components, whilst unsaturated fatty acids tend to be converted to glycols and then to other, irreversible products. Lipids are thus lost or become highly modified so that their localisation and histological chemical reactions in biological samples remains of doubtful veracity. Small molecules such as carbohydrates tend to be lost to the preservation fluid.

When in its hydrated form, formaldehyde can form methylene glycol. With protonation this can lead to the formation of the reactive electrophile (figure 1). It is this carbonium ion that is believed to be responsible for the fixative effect of formaldehyde as it is capable of nucleophilic attack with

electron rich areas such as the free electron pairs of nitrogen, oxygen and sulphur or unsaturated bonds. The lower the pH, the more the carbonium ion is formed. A 'typical' reaction pathway could be through an addition reaction between a compound containing a reactive hydrogen and the carbonium ion, forming a hydroxymethyl derivative. A further condensation reaction with another hydrogen atom to form a methylene bridge (figure 2). However this methylene bridge is considered to be reversible, and thus further, more permanent cross-linking reactions are occurring. A possible example of this is shown in figure 3.

Other non-aldehyde fixatives also exist. Examples of these are organic acids such as acetic acid; metal salts such as cadmium and lead; and osmium tetroxide. However these tend only to be suitable as specialist fixatives and can be more hazardous to work with.

It is important to remember that how a fixative is used can have a great effect. The osmotic strength of the fixative solution can effect how well a fixative penetrates a biological sample. The more isotonic the fixative solution, the lower is the osmotic stress on the sample. The pH of the fixative solution can also have a significant effect. At an alkaline pH the reactions with protein groups is different to those occurring at an acid pH.

So far we have discussed 'true' chemical fixation. However a good example of a 'pseudo' fixative is the use of ethanol or Industrial Methylated Spirits (IMS – 95% ethanol with 5% methanol). Ethanol is a clear flammable liquid, usually considered as a preservative rather than a fixative. However it can be considered as a 'dehydrating pseudo fixative' that is a non-additive, denaturing coagulant of proteins. It does this by altering the stereochemistry of the protein molecules. Proteins can be considered to have a number of steric structures depending on the molecular bonding occurring. The covalent bonding holds the primary structure between the amino acids that form the peptide chain of the protein. The secondary structure is determined by the hydrogen bonding between the components of the peptide chain itself, whilst the tertiary structure is considered to be the proteins total structure in three dimensions. A series of bonding types; hydrogen bonds; ionic bonds; and hydrophobic bonds determine the tertiary structure. Both hydrogen and hydrophobic bonds are very weak, but have a significant effect due to large numbers of these bonds occurring in

the protein molecule. Ethanol has the effect of disrupting the hydrophobic bonds in the protein molecule, thus causing the loss of the tertiary structure but not the secondary structure. The result is that ethanol fixation will leave the reactive groups on enzymes and proteins in a near original state as a result of stereochemical changes. Ethanol will also precipitate nucleoproteins but will not 'fix' them. However ethanol will dissolve lipids and precipitate or dissolve carbohydrates, as well as cause morphological changes due to shrinkage.

A fortunate result of the use of ethanol as a 'pseudo' fixative and preservative is that it can preserve DNA well. The ability to extract and use DNA from natural science collection specimens is becoming of increasing importance. Table 1 summarises current knowledge on this subject.

Mode of fixation	Subsequent preservation	External morphology	Histology	Internal anatomy	DNA
Cryo preservation	Freezer at – 70°C or below.	Can be good.	poor	Fair to good	good
Absolute ethanol	Absolute ethanol	Poor to good	Poor to fair	Poor to fair	good
70-80% IMS	70-80% IMS	Fair to good	Fair to good	Fair to Good	Fair - good
70-80% IMS	CPD or HMDS drying	Good	Poor	Good	Fair - good
70-80% IMS	Air drying	Good for certain groups.	Poor	Variable	Fair
4% Formaldehyde	70-80% IMS	Fair to Good	Fair	Fair	Poor – Fair
4% Formaldehyde	4% Formaldehyde	Good	Fair to good	Good	Poor
Ethyl acetate	Air dried	Fair to Good	?	Variable	Very Poor
Formaldehyde based histological.	Same	Fair to good	Good	Good	Very Poor to Poor.
Mercury based histological.	Same	Fair to Good	Good	Good	None or Very Poor

Table 1: Summary of the effects of various preservation protocols on invertebrate specimens (after Thomas, 1994; Dillon *et al.*, 1996; Quicke *et al.*, 1999).

Figure 1: Possible mechanisms for the production of the carbonium ion responsible for the main fixation reactions of formaldehyde.

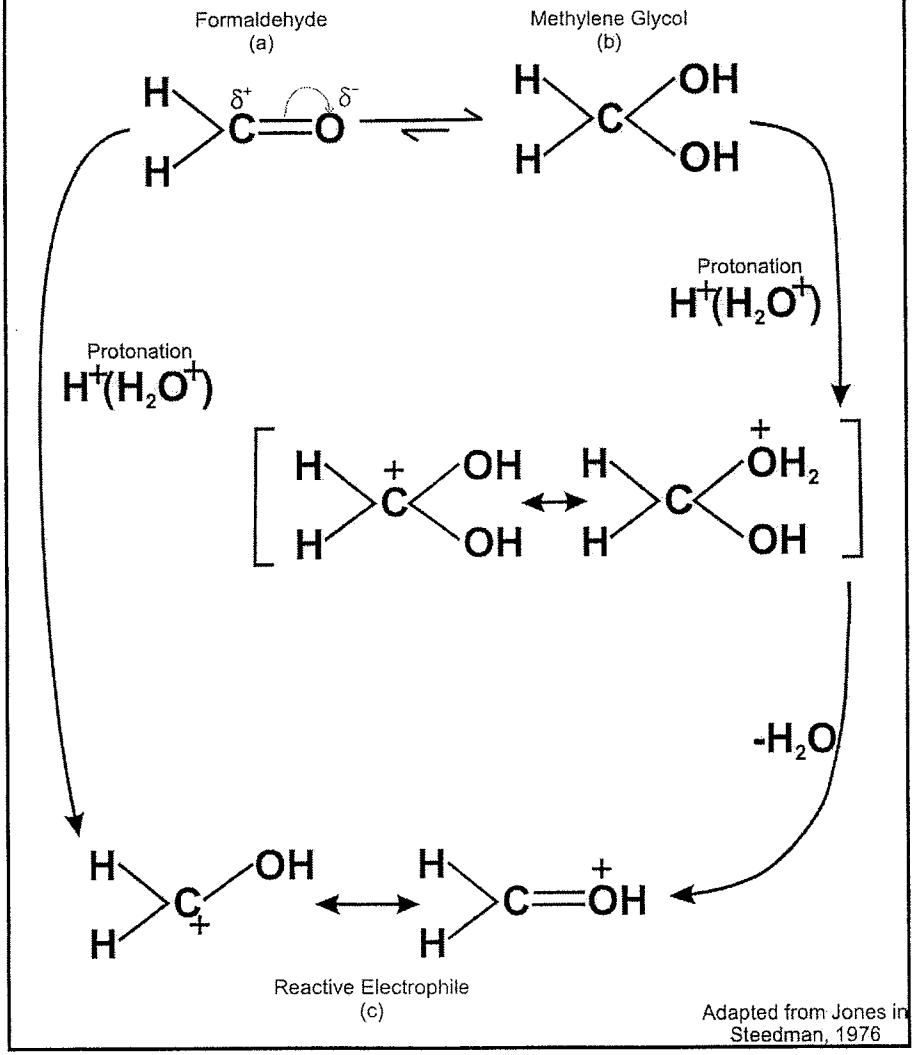


Figure 2: 'Typical' reaction pathway for methylene bridge formation.

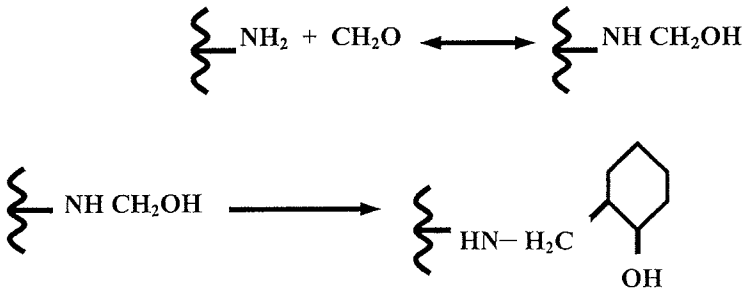
1. Addition reaction to a compound containing a reactive hydrogen;

$$R.H + CH_2O \longleftrightarrow R.CH_2(OH)$$

2. Condensation reaction with a further hydrogen atom to form a methylene bridge ($-CH^2-$);

$$R.CH_2(OH) + HR' \longleftrightarrow R-CH_2-R' + H_2O$$

Figure 3: example of possible cross-linking reactions involving amino acids and formaldehyde;



Further reading

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