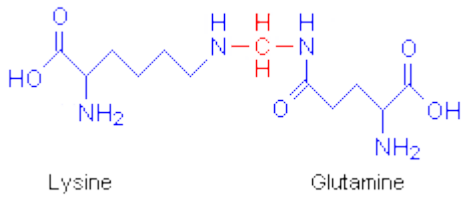
Strong formalin

The formaldehyde in strong formalin does not exist as the monomer but as short polymers. When diluted these revert to the monomer, so solutions of formalin should be made at least a day ahead to allow for this to happen. On standing the concentrated solution may develop a white precipitate. This is paraformaldehyde, a polymer of formaldehyde. Heating the solution can reverse the polymerisation, but heating strong formalin is a potentially dangerous practice – see the safety article referenced above. It is far safer and more practical to ignore it and either filter the diluted solution or allow it to settle. There will be a very slight drop in concentration as a consequence, but it is insignificant and not enough to affect fixation in the slightest.

How it fixes

Protein



Formaldehyde causes proteins to cross link in a meshwork, stabilising the protein mass and preserving morphology. Baker points out that although it is a strong reducing agent it fixes by an oxidative reaction, forming methylene bridges between the side amino groups of lysine and glutamine on different protein chains. In the diagram the methylene bridge is red.

This cross linking takes some time, and tissues should be treated with simple formaldehyde solutions for a minimum of 48 hours for it to take place. Tissues left for shorter periods than this may show inferior fixation with the likelihood of damage to the preservation by reagents applied after the formalin. In particular, ethanol, used as a dehydrant, may be a problem as it is a fixative in its own right, and may fix proteins which formalin has not fully protected. Unfortunately, ethanol is a poor morphological fixative.

Carbohydrate

There are no particular chemical reactions with carbohydrates that would fix them, although the protein part of glycoproteins will be fixed, of course. The crosslinking brought about by formaldehyde does effectively trap glycogen, even though it remains unfixed. It is quite effective at this, although not quite as effective as picric acid. Simple carbohydrate is unaffected.

Lipid

Generally, triglycerides also remain unfixed. Most lipids dissolve during treatment with xylene or other clearant, during paraffin processing, and are not detectable in finished sections. Some phospholipids, however, resist removal by the paraffin process and can be demonstrated. The protein part of lipoprotein may well be fixed, and these too may resist extraction by solvents.

Morphology

Morphological preservation is fair to good. Some shrinkage occurs, and spaces may be seen between elements, but this is not obtrusive. Morphological preservation is at its best when fixation is extended to several days.

Histochemistry

Nucleic acids are not fixed and may be reduced in amount. Most enzymes are inactivated, except for a few if it is used for a short period at 4°C.

Time

Formalin fixes quite slowly, and days are required for fixation to be completed, if it truly ever is. Certainly, 24 hours should be considered minimal. Formalin penetrates tissue fairly quickly but the chemical reaction involved is much slower. This means that penetration by formalin does **not** indicate the tissue is fixed, *i.e.* a change in colour of the tissue is not a reliable indicator of fixation. This is an important point, since a 1 mm

diameter needle biopsy of tissue will be penetrated by formalin within an hour or two and likely change colour, but adequate fixation will still require overnight or longer. It is strongly recommended that if rapid fixation for surgical tissue be required, a fixative designed to fix within a short period be used so that processing artifacts can be avoided.

The shortcomings of simple formalin mixtures may be overcome to a limited extent by increasing the temperature, but doing so will reduce the quality of preservation. Elevating the temperature of formalin mixtures moderately can accelerate fixation enough to enable routine overnight processing with generally acceptable results, but doing so may affect special stains and immunohistochemistry. Also, if the temperature is increased too much, heat fixation (cooking) takes place and quality drastically diminishes. In the past, dropping a 1 mm slice of tissue into boiling formalin for 1 minute was often used to fix breast tissue for rapid frozen sections. Some technologists used to leave out the formalin, using plain tap water for the purpose, with much the same results. The point is that too high a temperature is counterproductive.

Formal saline

By itself 10% formalin is adequate, but does have some disadvantages. It is not isotonic, and it lyses erythrocytes. To overcome this, it is common to add sodium chloride to it at a concentration of 0.9% w/v. The resulting solution is known as formal saline, and is a standard mixture.

Acid free formalin

On storage formalin solutions can deteriorate and produce formic acid. This is a problem with bloody tissues, because the formic acid can react with hemoglobin to produce acid formaldehyde hematin, more usually known as formalin pigment. It appears as a brown, doubly refractile, granular deposit dispersed throughout the tissue. Although more common with bloody tissues, in which it can form within a day or so, it is also seen in most tissues stored for a long time in plain formalin mixtures. Removing the acid largely inhibits its formation, and in the past this was done by storing the formalin over marble chips. However, when the marble is removed the acid is produced again, so tissue stored in the formalin will likely develop formalin pigment over time. This is not usually a problem for tissues which are processed within a day or so. If formalin pigment is present, it may be removed before staining.

Neutral buffered formalin

Perhaps the most popular simple formalin solution is Neutral Buffered Formalin, otherwise known as NBF. It is 10% formalin buffered with sodium dihydrogen phosphate and disodium hydrogen phosphate to pH 7.0, *i.e.* with Sorenson's buffer. Using this solution ensures that the pH of the fixative remains constant before and during fixation and, for most purposes, eliminates the problem of formalin pigment except for extremely bloody tissues. It is still possible for it to form during very long term storage, but changing the fixative every six months or so will restrict that.

Methanol free formalin

Commercial strong formalin contains some methanol to inhibit polymerisation of formaldehyde to paraformaldehyde. This does not usually interfere with fixation, and in the vast majority of applications 10% formalin made by dilution of commercial strong formalin is completely satisfactory. On those very rare occasions when methanol free formalin is necessary, it may be made directly from the polymer, paraformaldehyde, which is available commercially as a white powder. To make 100 mL methanol free 10% formalin, combine 4 grams paraformaldehyde, 100 mL distilled water and a small amount of a base, then raise the temperature above 60°C until the solution clears. This should be done under a fume hood. When it has cooled other materials, such as buffer salts, may be added.

Aftertreatment

Formalin requires no particular aftertreatment, and fixed tissues may be placed directly into the dehydrant. If using NBF, it is recommended that an alcohol concentration of about 60% or so be used first. The phosphates used to buffer the formalin may precipitate at higher ethanol concentrations and the lower concentration

provides an opportunity for the salts to be washed out. Washing formalin fixed tissues can eliminate this problem and, provided the tissue is properly fixed, does no harm. In a surgical service laboratory, however, fixation may not be complete enough for the tissue to withstand extensive washing and nothing more than a quick rinse should be given.

Properly fixed tissues (48 hours or longer) can withstand extensive washing, and doing so can remove much of the formalin attached to the proteins. The tissue never becomes unfixed, of course, but this may be of use for ”unmasking“ proteins for histochemical methods. It has no practical application for dye staining methods, and is not necessary for most procedures.

Colour restoration

One of the observed effects of formalin fixation is that the colour changes, and the life-like appearance of fresh tissue is lost. Formalin fixation permits this colour to be restored, but only once. This makes formal saline or NBF the fixatives of choice for tissues to be photographed or for those to be preserved and displayed as medical specimens in glass or methacrylate containers.

For photography, the procedure is to first wash and clean the specimen. It is then soaked in an excess of 60% ethanol until the colour has been restored satisfactorily. This takes a little time and the tissue should be checked every five minutes or so until the colour is satisfactory. The specimen is them patted dry to remove liquid which may cause reflections, and photographed. When replaced into fixative or if left in the 60% ethanol for too long the colour changes again and cannot be restored a second time.

For mounted specimens, the colour is restored in the final mounting fluid (Kaiserling III) by the addition of a small amount of sodium hydrosulphite. If the container is properly sealed, the colour restoration is then permanent.

Reference

Baker, John R., (1958)
Principles of biological microtechnique