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The future of fluid preservation - fixation in the balance: the case for using formalin

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Abstract
Despite much research into safer alternatives, ten percent aqueous formalin is still the most effective fixing agent for biological specimens. Many countries are trying to ban its use, based largely on its carcinogenic property. This article lists possible substitutes and alternatives that are safer to use, reviews other traditional fixing and preserving agents that have been mixed with other compounds and briefly discusses the problems in posting loans of fluid-preserved material.

Introduction
No matter how we may view collections of fluid-preserved natural science specimens (largely biological), with the coming of DNA analysis and molecular-based research, scepticism about the need to keep hazardous and high-maintenance collections of whole specimens, mooted by Peake (1989), seems to be reaching a zenith. A good example of this is the case for banning formaldehyde as a fixative as it is deemed to be too hazardous. Firstly there are many reasons why fluid collections need to be maintained and it isn't all because we need the DNA to unravel the mysteries of biological sciences in the future.

Ever since first Butlerov and then von Hoffman identified and isolated formaldehyde in the 19th century and the discovery of its antiseptic properties in 1893 (34 years later) it has been used as one of the most effective bactericides in the medical and biological worlds. Even in diluted solution formaldehyde is rather user unfriendly, provoking lachrymation and irritation of mucous membranes plus it is toxic, dematitic and can initiate cancer, yet there are many who have used it carefully for decades (including myself) without obvious ill effects (the carcinogenic properties of a substance are not dose dependent).
At lower temperatures (below 10° C) formalin starts to polymerise, forming a mixture of meta- and paraformaldehyde molecules creating a white mist effect which rapidly becomes much denser and can eventually solidify!

Considerable research has been carried out, especially over the past 20 years, to finding an equally-effective or better, fixing agent that is safer and yet formaldehyde's track record is still unrivalled, despite frequent attempts to discredit its use on health grounds, implying careless usage. The main problem, I suspect in these litigious days, is that many are turning their backs on formaldehyde, to the detriment of specimens, due to the risk of being sued.

Fixatives
A fixative is defined as a chemical compound which initiates tissue preservation by precipitating or chemically combining with cellular proteins to prevent decay, lysis or osmotic collapse (Moore, 1999).
Stoddart (1989) takes it to a molecular level. True fixation involves the formation of permanent covalent or coordinate bonds which link together the molecules composing a tissue, so that they are unable to undergo rearrangement or extraction.

There are several primary fixing agents:

- **Formalin** (37.5% formaldehyde gas dissolved in water – a saturated solution diluted to 3.75 ≈ 4% for fixation).
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- **Osmic acid** (a solution of osmium tetroxide) used primarily for transmission electron microscopy tissue fixation but also useful and effective for small and delicate biological organisms even though it does stain the internal organs black and it's also very toxic and hazardous to use. It has also been compounded with another cytological fixative - glutaraldehyde and buffered with sodium cacodylate.

- **Picric acid** must be kept moist as when it crystallizes it becomes explosive it also stains (almost) everything yellow – it is used as a compounding fixative and often combined with formalin in Bouin's fluid with or alcohol in Dubosq-Brasil's fluid.

- **Acetic Acid** – also used as a compounding fixative and often combined with formalin (again) and alcohol in FAA, formol-acetic-alcohol: it's used as a tissue swelling agent and can chemically 'unzip' chromosomes, so its use is fairly restricted.

- **'Dowicil'** is a formaldehyde release agent when combined with protein and was used widely during the late 60s and early 70s. It came in powder form and was used at 10% strength (in water) as a fixative. I have not heard of its use as a gross tissue fixative outside the boundaries of histology and I suspect it was used without injection into body cavities and eventually disfavoured due to its poor penetrative qualities (Fig.1).

Secondary fixatives (or pseudo fixatives) include alcohol (ethanol or IMS), mercuric chloride (very toxic and will attach mercury atoms to tissues), potassium dichromate (powerful oxidising agent) and cobalt nitrate - there are many more.

Additives can either raise or lower the pH, or function as humectants: lower glycols and glycerol, are well-reputed to prevent total desiccation in the event of a container's seal collapsing. Note how many of the above are 'acidic' in nature, having a relatively low pH but in fact are in fact, just more liberal with H+ ions.

Today we are obsessed with fluid neutrality and yet the author has tested many fluids in which specimens were preserved in perfect equilibrium and with a pH between 4 and 5. Organic acids (acetic, citric, picric) have been used in fixatives (FAA –formol-acetic-alcohol) or Bouin's fluid (formalin, picric acid and acetic acid). Many of these are used in skin pickles or taws. While it is true that specimens start to denature if the pH drops below 4, this would not necessarily be due to acidic corrosion but the high concentration of H+ ions in solution would form a cocktail of highly preservative-unfriendly compounds. Conversely, an overdose of OH- ions can just as easily lead to protein hydrolysis if the pH rises to even a tad above 7.5.

Compound fixatives

These are used more by histologists to facilitate or enhance the preservation of tissue elements and many contain highly toxic compounds such as mercuric chloride (cf. Heidenhain's Susa and Zenker's fixative). These are rarely used in the preservation of scientific or museum collections of whole organisms. One store I worked in contained two jars of birds preserved in something called Serventy's fluid. The Serventy family were Australian ornithologists and Vincent Serventy produced a fluid for conserving bird feather pigmentation *circa* the 1950s; the formula is similar to Kew mixture for plants, containing formalin, alcohol and glycerine (70% alcohol - 90 parts, 40% formalin - 5 parts, glycerine - 5 parts). Then there have been effective dual and triple-stage methods for colour preservation (Kaiserling 1922 and Wentworth 1938) - all of these contain formalin, mainly for the fixation stage.

Recent advances

Although we may not have advanced much technologically since the antiseptic qualities of formalin were revealed, several fixatives have been put forward for testing more recently.

Steedman (pronounced Stedman) produced fixing and preserving solutions for marine zooplankton for a UNESCO-funded book in 1976. The fixative contained formalin, propylene glycol as a humectant and propylene phenoxetol (or 2-phenoxyethanol, an embalming fluid). This gave excellent results both for the zooplankton and for larger tissues, providing a more osmotically friendly solution than previously and with results that showed a slight swelling of tissues rather than the usual shrinkage. Given this apparent panacea (since it is also slightly safer to use than buffered 10% formalin and more user friendly) it was greeted with enthusiasm and important collections were rather-too-rapidly transferred over without a proper test-of-time. Bearing in mind that this and the post-fixation-preservative (less the formalin), were intended for zooplankton, putting large specimens into the fixative seemed OK somehow. The problem with denser fluids is that they are poor penetrants for large and gross tissues and following some awkward moments over the ensuing years, Steedman's fluids were largely abandoned. Once again, a lack of fully understanding about fixation and preservation processes seems to have brought this about!

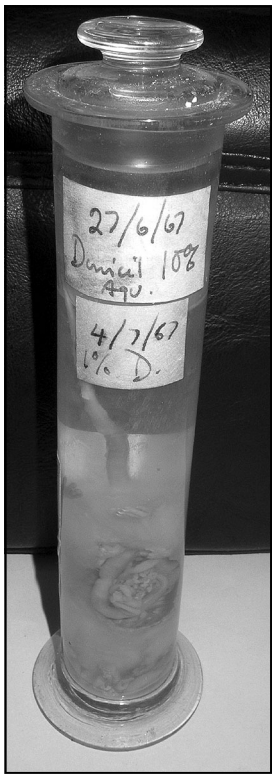

Fig 1. Eviscerated mouse fixed in 10% Dowicil and subsequently preserved in 1%, July 1967, preservative renewed just over 40 years later! (photographed in 2008).

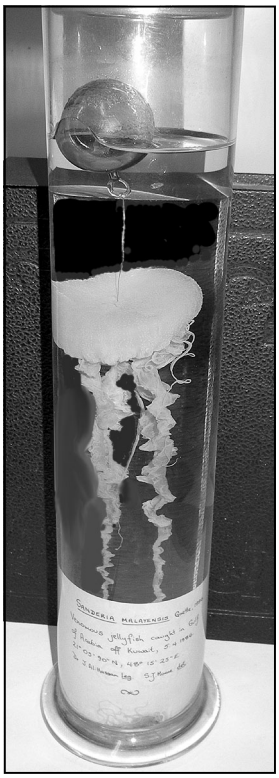

Fig 2. Jellyfish fixed in Steedman's fixative (1986), then into PFP. Note the good state of preservation of each specimen. (photographed in 2008).

Since then people have been more cautious about proposing alternatives to formalin and Simmons (unpublished MS in prep.) will review these. In an in-depth attempt to remedy the situation, the formaldehyde-releasing bactericide DMDM-Hydantoin was put forward by van Dam (2001) but was found to crosslink with certain protein groups leading to their denaturation. Once again formaldehyde is at the root of fresh tissue fixation.

Preservatives

The text book definition of a preservative is a compound that maintains a sample in a fixed (stable) state both at macroscopic and microscopic levels. Many new preservatives have been tested but few have found to be effective in the long-term (over 20 years). There is often some regularly-occurring deterioration of a specimen (or part) somewhere during a specimen's preservation history.

Bactericides and even some plant fungicides (Chinosol – 8-hydroxyquinoline sulphate) have been tested as both fixatives and/or preservatives, many are highly beneficial to specimens but only in the short-term (up to five years). Crimmen (1989) records how large fish specimens can eventually rot internally. Moore (1997) puts this down to the poor penetrative properties of many preserving agents, especially those that are glycol-related or of slightly higher OP (osmotic pressure) than water.

Most workers now fix in formalin and then transfer to 70-80% IMS (methylated ethanol). This works well since the tissues are stabilised by the formalin and then preserved by the IMS. However, IMS has its own set of problems particularly solvency, osmotic pressure differential (related to that of water and formalin), evaporation, rapid dehydration, extraction of proteins and of course, flammability. The author has always maintained that keeping a collection in IMS is creating a rod for the back – some jar seals and especially greases are affected by its solvency and often end up as an unsightly layer of stickiness in the bottom of a jar with the specimen sitting right on it. Solvency also dissolves out lipids both discolouring and eventually acidifying the preservative, especially when the dissolved lipids oxidise into fatty acids. Osmotic pressure

can affect tissues if IMS is employed as an initial preservative or if specimens are transferred directly from aqueous solutions (formalin included) directly into IMS. The effect causes cells to shrivel (syneresis) and tissues to distort or even tear. Histological samples of rat liver have shown this effect (Moore 1999). Evaporation occurs slowly in a well-sealed jar and the IMS auto-dilutes since alcohol and water are not a true solution but a binary azeotrope; by 30% strength IMS can become infected with fungi which rapidly aid in specimen lysis! Dehydration means that specimens (especially delicates) have to be brought up a ladder of increasing IMS concentrations until preserving strength is both achieved and equilibrated for the specimen. Flammability brings with it a whole raft of safety regulations.

We are still prepared to take on this tranche of problems as alcohol has been tried and tested as a preservative (and pseudo-fixative) for many centuries and it's good for preserving DNA. Many will leave formalin-fixed specimens in the fixative solution indefinitely and which will denature the DNA sequence. Half-strength formalin can be used as a more user-friendly preservative but this dilution will still not effect DNA deterioration.

A small group of museum-related conservators have been trying to rectify the fixative situation: John Simmons (USA), Andries van Dam (NL), Jules Carter and Simon Moore (UK) and although several fixatives and preservatives have been mooted and tested there is still no substitute for formalin.
There is also the slightly lesser question of finding something that will fix lipids – formalin will only preserve them which is why these can emerge from specimens that have already undergone fixation.

More recently the Smithsonian (Washington DC) has entered the 'preservation arena' by testing a new preservative called Novec. This comprises a mix of hydrofluoroethers forming a bactericidal envelope around the specimen. So far results are promising and it is being tested on a giant squid specimen; the colours have been well preserved and this author will be interested in observing the outcome. Novec is a dense fluid (1.5 Sp. Gr.) and I doubt that it will penetrate the interior of the specimen sufficiently to fulfil its preservative role on the tissues initially fixed by formalin (note!), longer than about 10 years. Small 'biopsy' samples are taken periodically to check for any deterioration. The fluid has the disadvantages of being quite expensive and dense causing freshly-fixed material to float so that it initially requires weighing down. It also has a lower boiling point than ethanol requiring a special jar/container seal to prevent evaporation (Drahl, 2008, via Simmons, pers. comm.).

Postal loans
Specimens have been safely sent in the post around the world using either deionised water or IMS as moistening (note) agents but now many countries' postal systems are banning the use of IMS due to its evaporative and flammable properties even though IMS has not been shown to pose a danger in the concentrations used for museum specimens. In the USA museums, initiated by Andy Bentley (2007), are having a say and modifying these regulations to a sensible level. Some museums are substituting IMS with glycol-related preservatives, often without prior permission of the loaning institutions! For delicate specimens this can be deteriorative since the osmotic pressures of IMS and glycol-based preservatives are very different and such an OP reversal can cause microscopic tearing through cellular swelling and bursting.

The future?
Firstly, IMS needs to be tested to determine whether or not it really poses a problem in the postage system. Secondly, and this is the difficult part, with easier access to litigation, increasing paranoia with formalin and an apparently-increasing sensitivity to its effects on humans, we do need to find an effective long-term and safer substitute for formaldehyde solutions. Perhaps regulating bodies might consider funding a research project into finding a fixation panacea that will effectively replace formalin in the long-term, before banning its use.

Conclusions
Despite many years of research, trial and error, there is still nothing as effective as 10% formalin as a primary fixative. The author strongly suspects that John Simmon's unpublished MS will also similarly conclude. Despite efforts to shift formalin out of the fluid-preservation sector this will ultimately result in deterioration to many specimens in our care.

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Science and Heritage Programme

Researching Ivory: Integrating Scientific Analyses, Historical Data, Artefact Studies and Conservation Needs

AHRC/EPSRC Science and Heritage Programme

April 23rd 2009
10am until 4.30pm

Session 1 - The what, where, when and why of ivory studies
Session 2 - Sampling and analysis - problems and potentials
Session 3 Conservation & curation of ivories and approaches to questions problems

This workshop is intended for anyone with active research interests and/or curatorial or conservation responsibilities for objects made from ivory – whether from elephants or other species – who would like to know more about current research in British universities and museums on their identification, origins, working, uses, history, exhibition, curation and conservation, and the potential for future multi-disciplinary studies. The workshop will also appeal to individuals responsible for monitoring and controlling the illegal contemporary trade in ivory and ivory products.

Attendance is limited to a maximum of 50 participants. Modest travel grants are available for UK-based curatorial staff, conservators and researchers who have relevant responsibilities or interests.

For more information about the workshop, including speakers, venue, and travel grants please contact Dr Sonia O'Connor via e-mail S.Oconnor@Bradford.ac.uk or by post at Division of Archaeological, Geographical and Environmental Sciences, University of Bradford, Bradford, West Yorkshire, BD7 1DP, UK, Phone: +44 (0)1274 236498, Fax 01274 235190.