FINDING A CURE FOR HAZARDOUS COLLECTIONS: THE ROAD TO ZERO FORMALDEHYDE AND ETHANOL

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Introduction

In 2016 the European Commission adopted the reclassification of formaldehyde as a category 1B carcinogen. This means that marketing of manufactured products containing formaldehyde will only be authorized in the European Union (EU) if there are no better alternatives. In their Biocidal Products Regulation (ECHA 2012), the EU Parliament and the EU Council stipulate the conditions regulating the use of biocidal products and their availability on the market in the EU. The use of formaldehyde as a biocide used for disinfection, veterinary hygiene, embalming, and taxidermy is also currently being reviewed. Furthermore, it is well known that ethanol, being the most used biocide for the preservation and storage of natural history collections, is a highly flammable liquid. Because of the health and safety issues linked with the usage of formaldehyde and ethanol, it is advisable to investigate whether alternatives exist and what the impact of pursuing the road to zero formaldehyde and ethanol might be for managing fluid preserved collections.

In light of the above, four alternatives to formaldehyde and ethanol proposed in the conservation literature are discussed in relation to occupational health and safety, stability, preservation quality, and the implications for collection care (e.g., for anatomical preparations or storage of larger and smaller vertebrates in natural history collections). Isopropanol (Pope 1928) and phenoxyethanol (Steedman 1976) found their way as preservatives in fluid collections in the first half and second half of the 20th century, respectively. DMDM-hydantoin was introduced in the beginning of this century (van Dam 2003). The last and oldest candidate is glycerol, which was introduced in the late 19th century in pathology to preserve the preparations of disease-affected organs in their natural colors (Kaiserling 1896). It should be noted that these alternatives are only compared and discussed for the purpose of long-term preservation of the specimens, not for the purpose of the initial fixation (arresting autolysis), which is mostly done in the field when collecting specimens. For this essential initial fixation, neutral buffered formaldehyde 4% (in some countries referred to as 10% formalin) remains the standard fixative due to its high efficacy and the lack of suitable alternatives.

Hazard Classifications, Chemical Stability, and Preservation Quality

Isopropanol, phenoxyethanol, DMDM-hydantoin, and glycerol are compared with formaldehyde and ethanol in relation to hazard statements, stability, and preservation quality (Table 1). It seems that glycerol has many favorable aspects when compared with the other active substances used in fluid preservation.

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Table 1. Hazard labels, hazard statements, and most important properties relating to stability and preservation quality of formaldehyde, ethanol, isopropanol, phenoxyethanol, DMDM-hydantoin, and glycerol.

Active substance	Hazard statements	Stability	Preservation quality
Formaldehyde 	· Acute toxicity · Corrosive · Carcinogenic · Suspected to be mutagenic · Skin sensitizing	· Under 18°C formaldehyde in water partly polymerizes and precipitates as paraformaldehyde. · Formaldehyde in aqueous solution slowly oxidizes to formic acid. · Risk of release of dangerous concentrations of formaldehyde in air if containers are not tightly closed.	· Phosphate buffered formaldehyde 4% is the international standard for the fixation of biological tissue because of its superb fixation properties. · As a long-term preservation fluid, it is less suitable due to its poor stability causing tissue corrosion, decalcification, and bleaching.
Ethanol 	· Highly flammable liquid and vapor · Carcinogenic · Toxic if swallowed · Toxic in contact with skin · Toxic if inhaled · Causes damage to organs · Causes serious eye damage · Causes skin irritation	· Risk of ignition. Vapors can form explosive mixtures with air. · Stable when kept in tight containers under normal conditions.	· Ethanol 70-75% is the standard preservation fluid for most natural history collections. · Due to its high vapor pressure, fluid loss and concentration loss are considered the main problems in collection care. · Ethanol bleaches the tissue and can cause lipid leaching and yellowing of the preservation fluid.
Isopropanol 	· Highly flammable liquid and vapor · Causes serious eye irritation · May cause drowsiness or dizziness	· Isopropanol autoxidizes to acetone and hydrogen peroxide. · Risk of ignition. Vapors can form explosive mixtures with air. · Stable when kept in tight containers under normal conditions.	· Isopropanol 50-60% is advocated as alternative for ethanol to avoid ethanol taxation. · Due to its high vapor pressure, fluid loss and concentration loss are considered the main problems in collection care. · Isopropanol bleaches the tissue and can cause lipid leaching and yellowing of the preservation fluid. · As a long-term preservation fluid, it is less suitable due to its poor stability causing tissue corrosion and bleaching.
Phenoxyethanol 	· Harmful if swallowed · Causes serious eye irritation	· Stable under normal conditions.	· Phenoxyethanol 2% is effectively used for the preservation of zooplankton. · Phenoxyethanol dissolves better in lipids and oils than in water (max. 2.5% at 20°C) and therefore tends to migrate to the fat tissue and thus losing its antiseptic strength in the aqueous solution which makes it less suitable for long-term preservation of lipid containing specimens. · May cause lipid leaching and yellowing of the preservation fluid.
DMDM-Hydantoin 	· Harmful if swallowed · Causes serious eye irritation · Causes skin irritation · May cause cancer · May cause respiratory irritation	· In aqueous solution it releases a small amount of formaldehyde (<1%). · Under 18°C in aqueous solution the formaldehyde partly polymerizes and precipitates as paraformaldehyde. · As a solid, in pure form, stable under normal conditions.	· DMDM-Hydantoin is a formaldehyde releasing agent. At a concentration of 5 to 10%, it can be effectively used for specimen preservation. · Due to the low amount of free formaldehyde, it seems to be less effective against yeasts and molds. · A great advantage over formalin is that it is pH neutral and does not need a buffer. · Long-term preservation quality is not known due to insufficient data.
Glycerol	· No hazards have been classified	· Stable under wide range of conditions.	· For more than a century, glycerol 65% has been successfully used as preservation fluid in pathology collections. · Increases protein stability. · Glycerol is almost non-volatile which slows down fluid loss and simplifies the maintenance routine (top up with water). · Under 60% sensitive for mold growth.

Discussion

Isopropanol

Isopropanol is not a completely harmless preservative when it concerns occupational health and safety. It can cause dizziness and drowsiness and is also highly flammable, as

ethanol is. With regard to stability, it is readily oxidized into acetone and hydrogen peroxide when it comes into contact with air (Sax and Lewis 1989).

Phenoxyethanol

Phenoxyethanol is mainly used in combination with propylene glycol for the preservation of zooplankton (Steedman 1976). The real caveat of phenoxyethanol is its low water solubility. It is lipophilic and therefore tends to migrate from the aqueous solution to the lipid-rich tissue specimens. Subsequently, it loses antiseptic strength in the aqueous solution surrounding the specimen (van Dam 2003). Crimmen (1989) concluded that phenoxyethanol is an unsatisfactory preservative for fishes and does not recommend its use for similar vertebrate collections. It therefore fails to be a good overall candidate.

DMDM-hydantoin

DMDM-hydantoin in aqueous solution is a suspected mutagen and carcinogen because it donates formaldehyde to the water. It is a so-called formaldehyde-releasing agent. When DMDM-hydantoin is dissolved in water, the main part of the available formaldehyde stays chemically attached to the larger DMDM-hydantoin molecule, and a small part will dissociate into the aqueous solution. For example, if the formaldehyde is consumed by oxidizing to formic acid, polymerizing to paraformaldehyde, or binding with proteins, the formaldehyde releaser supplies new formaldehyde. The action of formaldehyde-releasing agents is therefore based on the continual attainment of this equilibrium (Emeis et al. 2007). At a concentration of about 5–10% DMDM-hydantoin, it can be effectively used for specimen preservation (van Dam 2003, Carter 2012). The concentration of free formaldehyde in the solution will be at least 10 times lower when compared with an aqueous solution of 4% formaldehyde (Emeis et al. 2007). The DMDM-hydantoin solution is pH neutral and does not need a buffer or stabilizer like formalin solutions does. Despite the reduced exposure risk to formaldehyde, proper safety precautions still have to be taken when working with this preservative.

Glycerol

For glycerol, no hazards have been classified. It is nontoxic, nonflammable, and very stable under a wide range of conditions. It binds water, increases protein stability, and maintains tissue color, shape, and texture. It is practically nonvolatile (vapor pressure <0.0002 mm Hg at 25°C), which means that, in the case of glycerol–water solutions, only water will evaporate. A negative aspect of glycerol is its susceptibility for mold growth at low and medium concentrations. By looking at the relative humidity (RH) over glycerol–water solutions, this risk can be quantified (Table 2). Above 64% glycerol, the RH over the solution will be lower than 70%. Molds can start to manifest and grow only above 75% relative humidity. This means that there is no risk of fungal growth when the glycerol concentration is maintained at 65% or above and the RH in the storage room is kept below 75% (Block 1953). Since glycerol is also known as a cryoprotectant, it is interesting to look at the freezing points of glycerol–water solutions (Table 3). Between 60% and 70% the freezing point is below −30°C. This makes it very suitable for cold storage of tissue samples, which can then be used for DNA analysis without sacrificing tissue morphology (Schaudien et al. 2007).

Table 2. Relative humidity over glycerol–water mixtures at 25°C (Miner et al. 1953).

Relative humidity (%)	Glycerol by weight (%)	Specific gravity
10	95	1.245
20	92	1.237
30	89	1.229
40	84	1.216
50	79	1.203
60	72	1.184
70	64	1.162
80	51	1.127
90	33	1.079

Table 3. Freezing points of glycerol–water mixtures (Lane 1925).

Glycerol by weight (%)	Freezing point (°C)
90	−2
80	−20
70	−39
65	−43
60	−35
50	−23
40	−15
30	−10
20	−5
10	−2

Impact on Collection Care: Glycerol versus Ethanol

Since the majority of wet natural history collections are preserved in ethanol, it is interesting to know what the implications are for collection care when wanting or having to consider glycerol preservation over ethanol preservation.

Fluid Loss/Topping Up

Ethanol has a five times higher vapor pressure than water and consequently will evaporate faster, leading to a decrease in ethanol concentration. Due to the increase of the water component in this azeotrope, the antiseptic strength diminishes, resulting in a higher risk of microbial attack and finally desiccation of the specimen. Therefore, ethanol-preserved collections have to be periodically monitored for fluid level and concentration. To bring back the solution to its original strength, topping up has to be done with a higher concentration of ethanol, which can be calculated from the recorded volume loss and concentration loss (Sendall and Hughes 1997). During topping up, the surrounding solution becomes hypertonic, which means that the fluid outside the specimen contains less water than inside the specimen. Water molecules will leave the cells at a faster rate than ethanol molecules will replace them, leading to a negative osmotic pressure in the cells. This means that collapsed structures will not recover. In fact, every time specimen jars have to be topped up, there is a risk of additional tissue shrinkage.

In the case of specimens preserved in 65% glycerol, fluid loss will be slower since it is mostly the water that evaporates due to the extreme low vapor pressure of glycerol (0.000168 mm Hg at 25°C), which is 141,667 times lower than that of water (23.8 mm Hg

at 25°C). Consequently, the glycerol concentration increases, water activity decreases, thus raising the antiseptic strength. Since glycerol is a humectant (hygroscopic), there will only be a limited amount of water loss, which thus causes no threat to the conservation quality of the specimens. The topping up procedure is incredibly simple; just add water. A positive side effect is that during topping up a hypotonic solution is created around the specimen, which means that the water concentration is higher outside than inside the specimen. Water molecules will travel faster through the cell membranes into the cells than glycerol molecules leave the cells. This creates a slight temporary build-up of osmotic pressure inside the cells. During this process, collapsed structures are able to reverse to their original shape. With regard to cell hydration, glycerol has so-called regenerative properties (Macleod and van Dam 2011).

Specimen Transfer

Ethanol and glycerol are both hygroscopic substances. When transferring specimens from an aqueous solution to glycerol 65% or ethanol 75%, this could lead to tissue shrinkage and cell collapse due to osmotic pressure. Therefore, the transfer of specimens should always be done in steps of increased concentration (e.g., for glycerol 30%–50%–70% to reach an end concentration of about 65%).

Storage Conditions

Ethanol is a highly flammable fluid and vapor. Therefore, the storage facilities containing ethanol-preserved collections have to be equipped with several costly safety precautions to meet the regulations for handling and storing large amounts of flammable liquids. For the storage and handling of glycerol, no specific safety precautions are needed according to Regulation No. 1907/2006 of the European Parliament and Council.

Conclusion

With regard to hazard risks, stability, and preservation quality, glycerol 65% seems to be the best alternative to ethanol and formalin as a long-term preservation fluid. When looking at the impact on collection management and care, switching to glycerol preservation can lead to an enormous reduction in facility and maintenance costs due to its nonhazardous and nonvolatile nature.

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