<u>Technical Overview</u>

Cross-linking fixatives: What they are, what they do, and why we use them

Focus on: Formaldehyde, Glutaraldehyde, and Osmium tetroxide

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EM processing and imaging demands better tissue fixation

LM

- Visible light
- No vacuum (1 atm)
- Live cells/tissue can be imaged.
- Samples can contain water.
- Biological tissue has sufficient contrast.
- Formaldehyde fixation is often sufficient to preserve the tissue/cell.

EM

- Electron beam (high energy)
- High vacuum
- Samples must be embedded into plastic.
- Samples must be dehydrated.
- Biological tissue is not electron-opaque enough. → need heavy metal stains
- Tissue must be protected against subsequent EM processing and imaging.

Commonly used chemical fixatives for microscopy

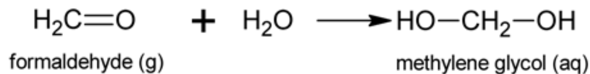
- Cross-linking (additive) fixatives: e.g., formaldehyde, glutaraldehyde, acrolein, osmium tetroxide
  - Fixative molecules form cross-linkage with their targets.
  - Formaldehyde-glutaraldehyde mixture is the most commonly used primary fixative for EM (introduced by Karnovsky in 1965).

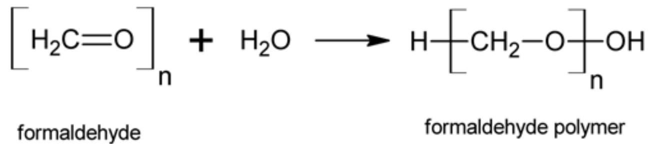
- Coagulants: e.g., EtOH, MeOH
  - Coagulate and/or precipitate proteins
  - Do not fix carbohydrates and lipids
  - LM only

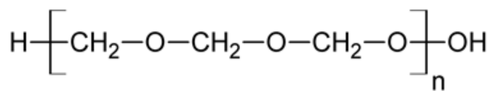
- Acids: e.g., acetic acid, picric acid
  - precipitate proteins
  - Do not fix carbohydrates and lipids
  - LM only

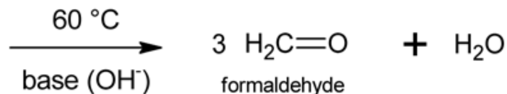
Formaldehyde

(or is it *para*formaldehyde? What is formalin, anyway?)









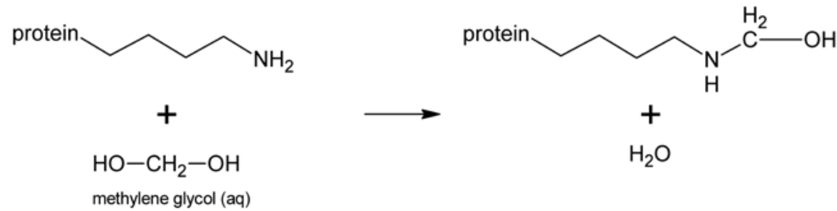
- **Formaldehyde** = water soluble gas
  - When dissolved, it forms methylene hydrate
  - Methylene hydrate molecules can react with one another to form polymers
  - Small molecule = rapid penetration into tissue

- **Formalin** = 37-40% formaldehyde (aq) with methanol (up to 15%), which prevents polymerization

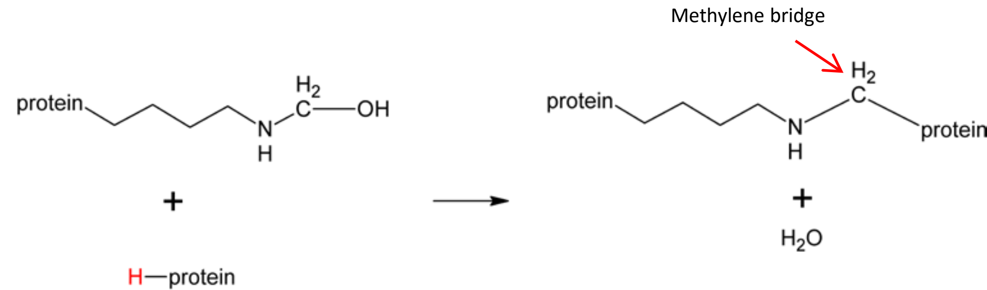
- **Paraformaldehyde** = higher polymers (n = up to 100) of formaldehyde;
  - insoluble in water
  - requires heat and high pH to de-polymerize in water.

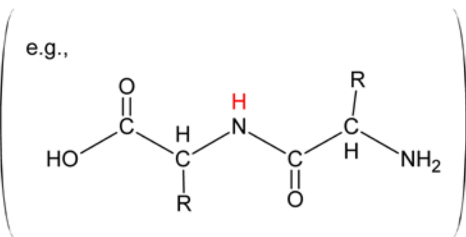
Formaldehyde reacts primarily with proteins

Addition of formaldehyde (methylene glycol) to a lysine side-chain (fast)



Formation of methylene bridge with a neighboring nitrogen atom (slow)

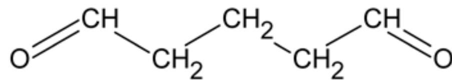


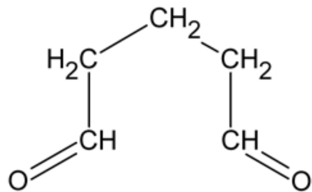


- The aldehyde group can react with nitrogen and some other atoms of proteins.
- Methylene bridge (-CH_2-) is formed between two reactive atoms in proteins that are very close together.
- Other molecules (carbohydrates, lipids, nucleic acids) are thought to be trapped in a matrix of cross-linked proteins.

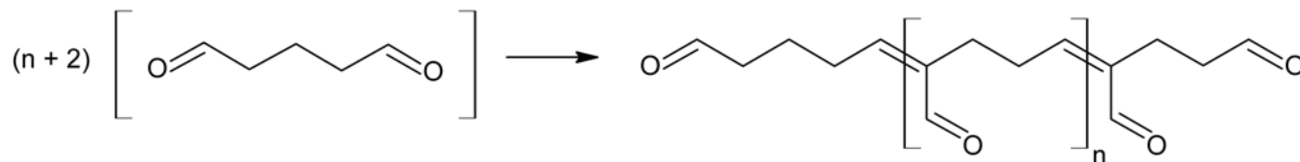
Glutaraldehyde

(Quality fixation for EM since 1963)





- First introduced by Sabatini et al. in 1963 as a fixative for EM
- Two aldehyde groups per molecule, with a longer, flexible hyrocarbon chain
  - More efficient cross-linking
  - Small enough to penetrate tissue (slower than formaldehyde)
- Present in aqueous solutions as monomers and polymers of variable size



Glutaraldehyde readily cross-links proteins

Cross-linking of proteins with glutaraldehyde monomer

O O + 2 protein–NH_2 → protein N N protein + 2 H_2O

Cross-linking of proteins with glutaraldehyde polymer

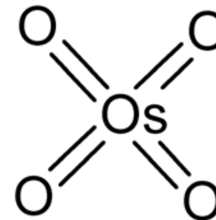
O O O O n + (n + 1) protein–NH_2

→ O O N N n protein protein + (n + 1) H_2O

- Both aldehyde groups of a single glutaraldehyde molecule react with proteins to form cross-linkage.
- Glutaraldehyde can also react with phospholipids containing free amino groups (e.g., phosphatidylserine, phosphatidylethanolamine).
- Glutaraldehyde introduces free aldehyde groups to the fixed tissue
  - Can pose problems for immunolabeling

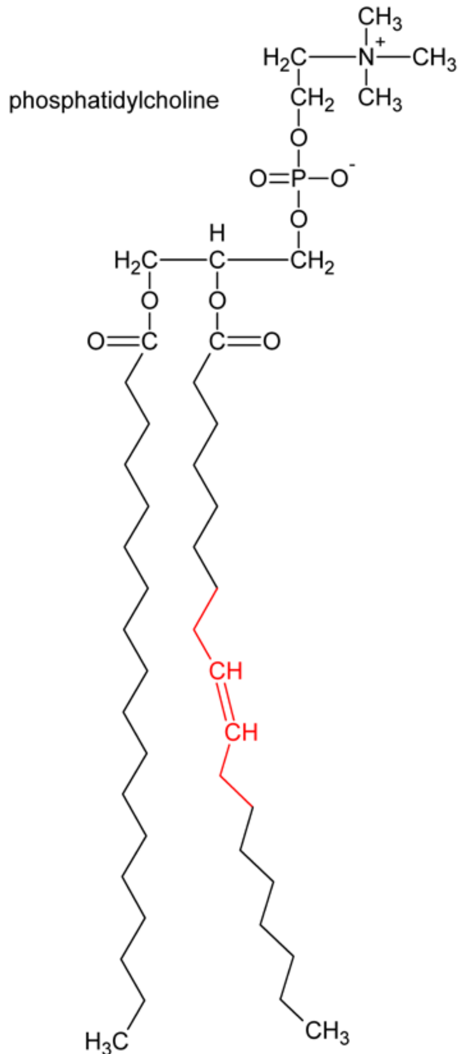
Osmium tetroxide
(the multi-tasker)

- Introduced in 1948 by Claude
- Os can exist in nine oxidative states, five of which are reasonably stable.
  - Many potential chemical reaction pathways with many substrates.
- OsO_4 is soluble in both polar (aqueous) and non-polar media.
  - Penetrate into, and react with, hydrophobic regions of tissue/cell (e.g., membrane phospholipids)
  - Water solubility: ~ 7% at RT
- Os is electron opaque.
  - Works as a stain, as well as a fixative
- OsO_4 also acts as a mordant.
  - Enhancement of lead staining



- Limited penetration into tissue
  - Limit tissue section thickness to < 100µm
- Highly toxic

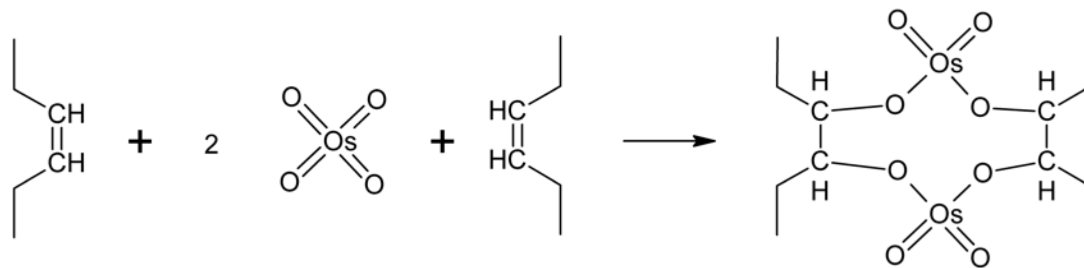
OsO_4 reacts primarily with unsaturated lipids



- OsO_4 reacts with C=C in unsaturated fatty acid chains of phospholipids
  - Reduction of Os during cross-linking reaction produces dark brown color in the processed tissue.

- OsO_4 can also react with some proteins and lipoprotein complexes.

Cross-linking of unsaturated fatty acid chains with OsO_4



Ideal vs. practice of chemical fixation

- Ideally, a good fixation method should preserve the cell/tissue as a whole.
- In practice, a chemical fixative is usually selective (e.g., aldehydes → proteins).
  - Choose a fixative for molecule/structure of your interest, OR,
  - Use a combination of fixatives, OR,
  - Use a physical fixation method (i.e., rapid freezing)

- Ideally, a good fixation method should preserve the cell structure with minimum change from the living state (volume, morphology, localization of macromolecules and organelles, etc.).
- In practice, fixation and tissue processing usually induce artifacts...
  - Minimize avoidable ones (e.g., swollen mitochondria).
  - Interpret the tissue structure in the context of the fixation and processing.

References and Notes

- Claude A (1948) Studies on cells: morphology, chemical constitution of and distribution of biochemical functions. Harvey Lecture 43:1921-1964.
- Karnovsky MJ (1965) A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy. J Cell Biol 27:137A-138A.
- Sabatini DD et al. (1963) Cytochemistry and electron microscopy. J Cell Biol 17:19-58.

- Much of the information presented here can be found in “Fixation for Electron Microscopy” by M. A. Hayat (1981, Academic Press) and “Electron Microscopy: Principles and techniques for biologists” by J. J. Bozzola and L. D. Russell (1992, Jones and Bartlett Publishers).
- This technical overview was originally created by Masaaki Kuwajima in 2011 as a short summary presentation for the Harris lab at The University of Texas at Austin.
- This technical overview is available on SynapseWeb (http://synapses.clm.utexas.edu/), maintained by Kristen M. Harris, Ph.D. (Principal Investigator)
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