

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

Formaldehyde

Glutaraldehyde

Osmium tetroxide

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

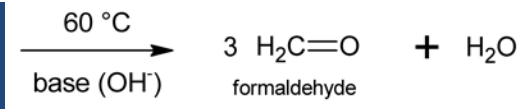
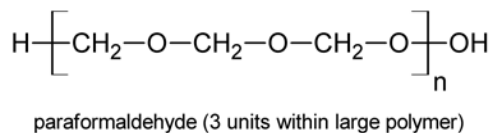
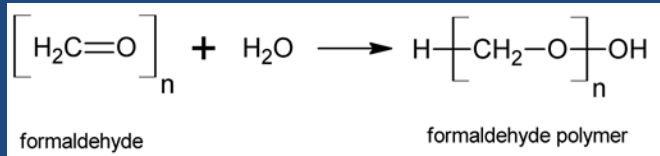
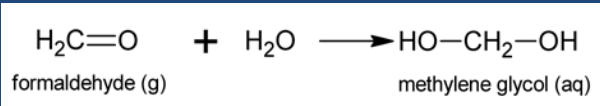
- Electron beam (high energy)
- High vacuum
- Samples must be embedded into plastic.
- Samples must be dehydrated.
- Biological tissue is not electron-opaque enough. → need heavy metal stains
- Tissue must be protected against subsequent EM processing and imaging.

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 [J Cell Biol 27:137A-138A]).
- 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

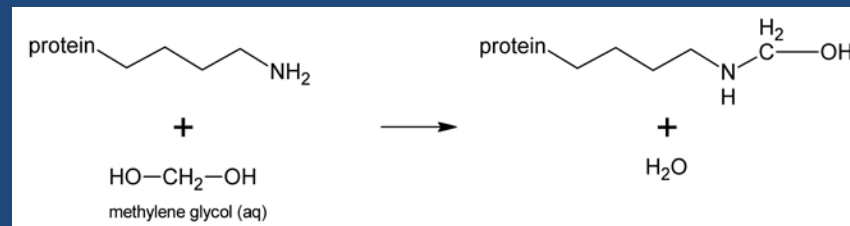
(or is it *para*formaldehyde? What is formalin, anyway?)



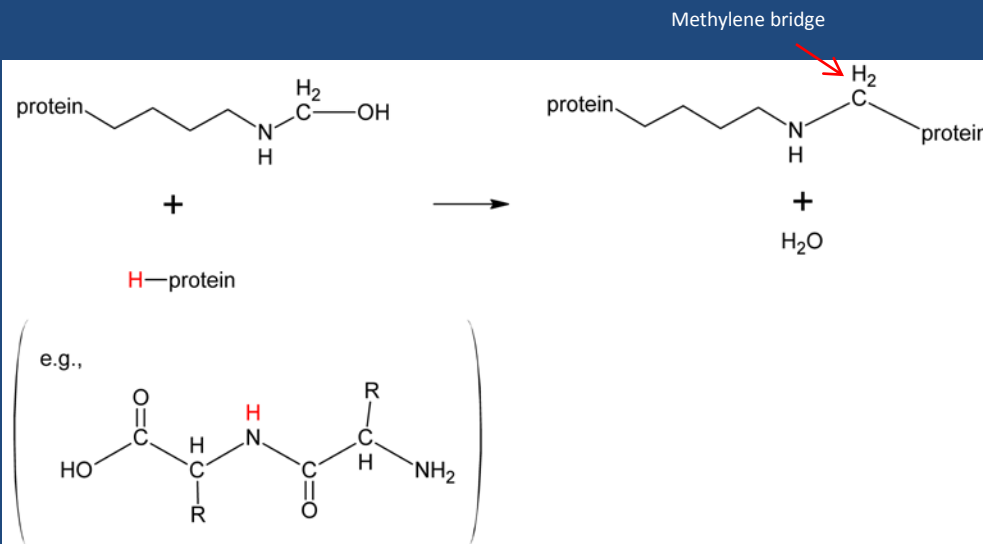
- **Formaldehyde** = water soluble gas
 - When dissolved, it forms methylene hydrate
 - Methylene hydrate molecules can react with one another to form polymers
 - Small molecule = rapid penetration into tissue
- **Formalin** = 37-40% formaldehyde (aq) with methanol (up to 15%), which prevents polymerization
- **Paraformaldehyde** = higher polymers (n = up to 100) of formaldehyde;
 - insoluble in water

Formaldehyde reacts primarily with proteins

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



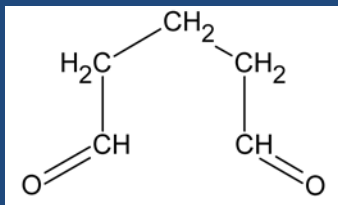
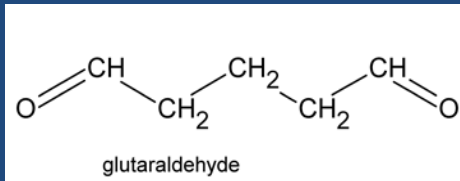
Formation of methylene bridge with a neighboring nitrogen atom (slow)



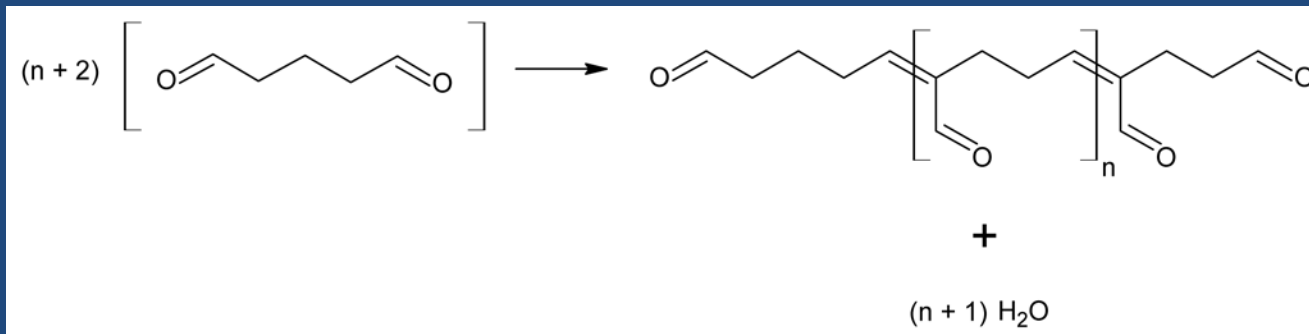
- The aldehyde group can react with nitrogen and some other atoms of proteins.
- Methylene bridge (-CH₂-) is formed between two reactive atoms in proteins that are very close together.
- Other molecules (carbohydrates, lipids, nucleic acids) are thought to be trapped in a matrix of cross-linked proteins.

Glutaraldehyde

(Quality fixation for EM since 1963)

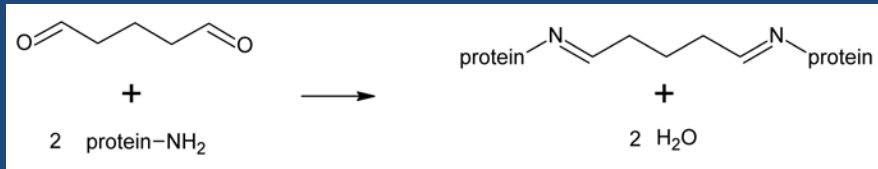


- First introduced in 1963 as a fixative for EM (Sabatini DD et al. 1963. J Cell Biol 17:19-58)
- Two aldehyde groups per molecule, with a longer, flexible hydrocarbon chain
 - More efficient cross-linking
 - Small enough to penetrate tissue (slower than formaldehyde)
- Present in aqueous solutions as monomers and polymers of variable size

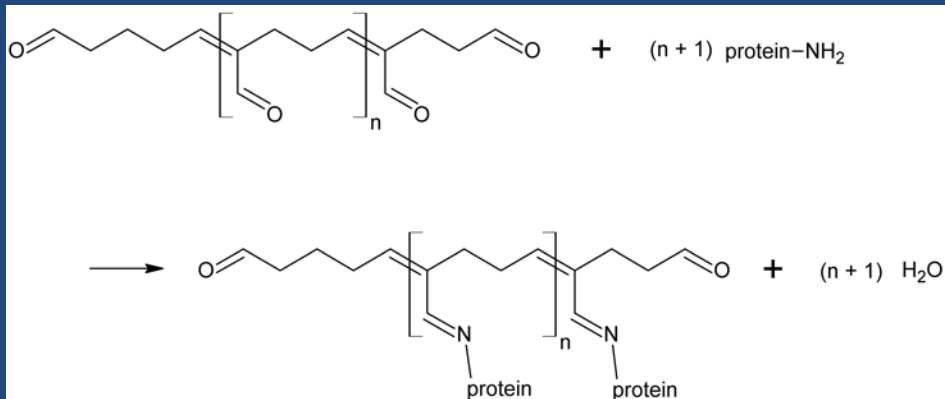


Glutaraldehyde readily cross-links proteins

Cross-linking of proteins with glutaraldehyde monomer



Cross-linking of proteins with glutaraldehyde polymer

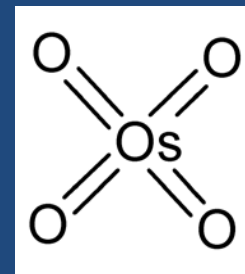


- Both aldehyde groups of a single glutaraldehyde molecule react with proteins to form cross-linkage.
- Glutaraldehyde can also react with phospholipids containing free amino groups (e.g., phosphatidylserine, phosphatidylethanolamine).
- Glutaraldehyde introduces free aldehyde groups to the fixed tissue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

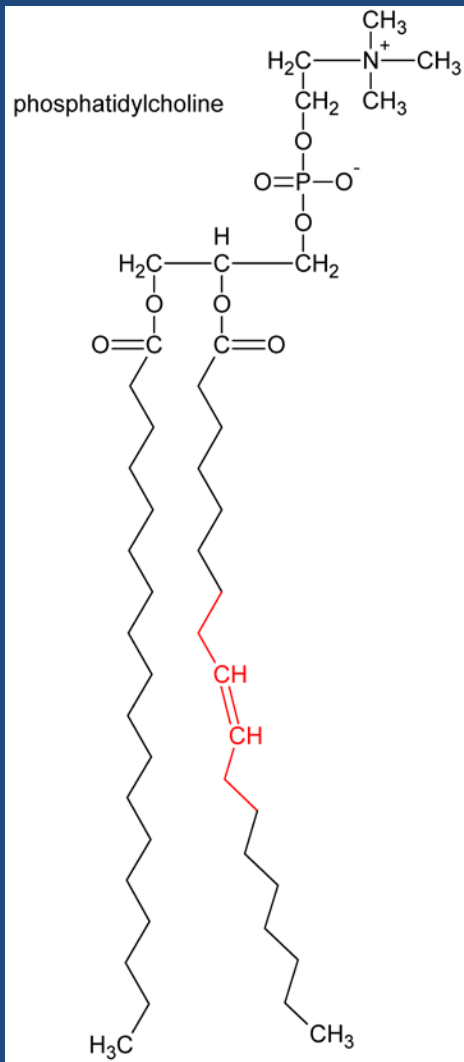
Osmium tetroxide

(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 $< 100\mu\text{m}$
- Highly toxic

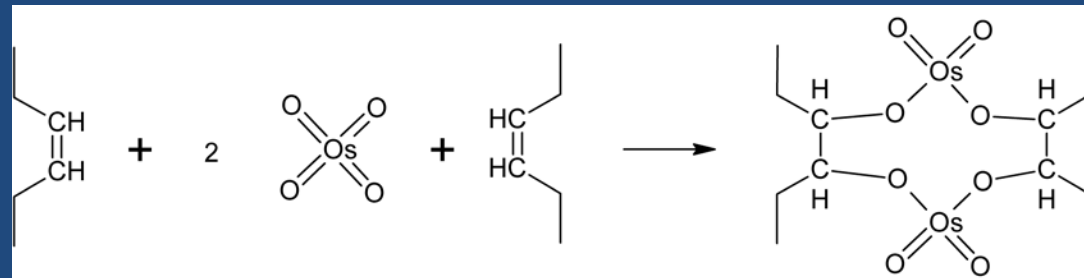


OsO₄ reacts primarily with unsaturated lipids



- OsO₄ 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.
- OsO₄ can also react with some proteins and lipoprotein complexes.

Cross-linking of unsaturated fatty acid chains with OsO₄



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 induces artifacts...
 - Minimize avoidable ones (e.g., swollen mitochondria).
 - Interpret the tissue structure in the context of the fixation and processing.