Journal of Histotechnology

ISSN: 0147-8885 (Print) 2046-0236 (Online) Journal homepage: http://www.tandfonline.com/loi/yhis20

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To cite this article: Isam Eltoum, Jerry Fredenburgh, Russell B. Myers & William E. Grizzle (2001) Introduction to the Theory and Practice of Fixation of Tissues, Journal of Histotechnology, 24:3, 173-190

To link to this article: http://dx.doi.org/10.1179/his.2001.24.3.173

Published online: 18 Jul 2013.

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Introduction to the Theory and Practice of Fixation of Tissues

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Abstract

Many approaches to fixation and types of fixatives have been developed and tested over the last century. The mechanisms by which fixatives act to harden and preserve tissues fall into broad categories, including dehydrants, heat effects, cross-linkers, effects of acids, and combinations of these categories. Each fixative has advantages and disadvantages, including specific molecules retained within "fixed" tissues, swelling or shrinkage of fixed tissues, variations in the quality of histochemical and immunohistochemical staining, and varying capabilities to maintain the structures of cellular organelles. One of the major problems with formaldehyde type (cross-linking) fixatives has been the loss of antigen immunorecognition; correcting this usually requires some method of antigen recovery. Similarly, the extraction of mRNA and DNA from formalin fixed tissue in paraffin blocks is problematic. All widely used fixatives are selected by compromise—good aspects are balanced against less desirable features. This article discusses the basics of fixation and provides the formulas for the fixatives currently used in pathology, histology, and anatomy and discusses good and bad aspects of specific fixatives. (*The J Histotechnol* 24:173, 2001)

Key words: antigen recovery, cross-linkage, dehydration, formaldehyde, heat artifacts

Introduction

From embalming to electron microscopy, the major objective of fixation has been to maintain excellent morphological features. The use and development of specific fixatives usually have been empirical, and in biological sciences have borrowed fixation information and techniques from such industries as leather tanning and vaccine production. In order to understand visually the microanatomy of tissues, stained microscopic tissue sections must maintain the microscopic relationships among cells, cellular components (eg, cytoplasm, nuclei), and extracellular material, with little disruption of the organization of the tissue and with maintenance of the tissue's local chemical composition. Because many components of tissues are soluble in aqueous or other liquid environments, a reliable picture of the microanatomy and microenvironment of the tissue requires that soluble components not be lost from stained tissue sections. For example, if soluble components are lost from the cytoplasm of cells, the color of the cytoplasm on hematoxylin and eosin (H&E) staining will be reduced or modified and aspects of the appearance of the microanatomy of the tissue will be lost or changed.

Almost any method of fixation induces shrinkage, swelling, and hardening of tissues, and color variations with various histochemical stains (1–5). To maintain the microarchitecture of tissue along with as much of the soluble components of tissue as practicable, a "fixative" is used to minimize the loss of cellular components, including peptides, proteins, lipids, mRNA, and DNA and to prevent the destruction of macromolecular structures such as cytoplasmic membranes, rough endoplasmic reticulum, smooth endoplasmic reticulum, nuclear membranes, mitochondria, and lysosomes. By minimizing the loss or enzymatic destruction of cellular and extracellular molecules and by maintaining macromolecular structures and protecting tissues from destruction by microorganisms, the fixative acts to "fix" *at a point in time* one view of a dynamically changing, viable tissue. A fixative should also prevent the subsequent breakdown of the tissue by enzymatic activity and/or by microorganisms during long-term storage.

A fixative not only interacts initially with the tissue in the tissue's aqueous environment, but subsequently the fixative and the chemical modifications induced by the fixative have additional activity and change the molecular features of the tissue during all phases of tissue processing and staining, from dehydration of the tissue to staining of tissue sections using histochemical or immunohistochemical stains. Fixation, tissue processing, and staining protocols used in the preparation of stained slides produce a compromised depiction of features that represent the original living tissue. Each fixative and tissue processing protocol maintains some

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molecular and macromolecular aspects of the tissue better than some other fixative-processing combinations. Presently, a universal or ideal fixative has not been identified; fixatives are selected currently on the basis of producing a final product needed to demonstrate a specific feature of a tissue.

The most important aspect of a fixative is its ability to prevent short- and long-term destruction of the microarchitecture of the tissue. Fixation prevents the short- and long-term destruction of the microarchitecture by stopping the activity of catabolic enzymes (stop autolysis), stopping the activity of microorganisms (stop tissue degradation, and minimizing the diffusion of soluble substances from their original locations. Other important characteristics of a good fixative include the destruction of infectious agents, the maintenance of tissue and cellular integrity, and possession of a good toxicological and flammability profile that permits the safe use of the chemical (6). The advent of new biological methods, the increased understanding of the human genome, and the need to evaluate rapidly the biology of disease processes have placed new demands on the old fixation processes. Fixatives used in academic environments should permit the recovery of macromolecules, including proteins, mRNA, and DNA without extensive biochemical modifications. Another important characteristic of an ideal fixative is the versatility to be used with a wide variety of tissue from humans as well as many other different species of animals. It is equally important that the ideal fixative penetrate both small and large specimens rapidly and preserve fixed tissue in paraffin blocks for at least a decade. Fixative compatibility with all automatic tissue processors is a must as well as the ability to support excellent microtomy of paraffin blocks (7). The ideal fixative should promote excellent staining with H&E and also allow histochemical, immunohistochemical, and in-situ hybridization stains and procedures. Stability and disposability are also important issues with a fixative. The ideal fixative should have a shelf life of at least 1 yr and be readily disposable or recyclable (7). Many textbooks discuss fixation; 3 of the better textbooks for practical fixation are by Kiernan, Sheehan and Hrapchak, and Carson (1–3). Theoretical aspects of fixation are discussed by Horobin (4).

Types of Fixation

Tissue fixation can be accomplished by physical or chemical methods. Physical means such as heating, microwaving, and freeze-drying as independent processes are rarely used in the routine practice of medical or veterinary pathology with the exception of dry heat which is used for fixation of microorganisms prior to the Gram stain. Most methods of fixation used in processing of tissue for medical or veterinary diagnoses rely on chemical fixation carried out by liquid fixatives. The major requirement of fixatives used for diagnostic pathology is the reproducibility over time of the microscopic appearances of specific tissues. Methods of fixation used in research protocols may be more varied including fixation using vapors and fixation of whole animals by perfusing the animal's vascular system with a fixative (8).

Several biochemical approaches can accomplish many of the stated goals of fixation and several chemicals or combinations of chemicals can act as good fixatives. Approaches to fixation include the use of agents which form covalent cross-links between proteins, between individual protein moieties, and between nucleic acids and proteins. The best examples of such cross-linking fixatives are formaldehyde and glutaraldehyde. Another biochemical approach to fixation is the use of agents that remove free water from tissues and hence precipitate and coagulate proteins. Examples of such fixatives include dehydrants such as ethanol, methanol, and acetone. Other fixatives may rely on denaturing proteins and nucleic acids through changes in pH or via salt formation. Examples of such fixatives are acetic acid, trichloroacetic acid, and zinc acetate. Some complex fixatives are mixtures of several types of fixatives. For example, alcoholic formalin acts to fix tissues by cross-linking as well as by dehydration.

Physical Methods of Fixation

Heat Fixation

Probably, the simplest form of fixation is "heat." When we boil or poach an egg, we use heat to precipitate proteins within the egg, so when cutting the egg we can identify the yolk and egg white separately. Each of these components is less soluble in water after heat fixation than they are as components of a fresh egg. Picking up a frozen section on a warm microscope slide attaches the section to the slide, partially fixes (by heat) the tissue section, and partially dehydrates the tissue section. Although reasonable morphology could be obtained by boiling tissue in normal saline, in histopathology, heat fixation alone is not used frequently; rather heat is used to accelerate other forms of fixation as well as tissue processing.

Microwave Fixation

Microwave heating is used to speed fixation reducing the time required for fixation of gross specimens and histological sections from more than 12 hr to less than 20 min for some specimens (9,10). Microwaving tissue in formalin results in the production of large amounts of dangerous vapors so in the absence of a hood for fixation, microwave fixation using formalin may be unsuitable. Recently, commercial glyoxal based fixatives, which do not form vapors when heated at 55°C, have been introduced as an efficient method of microwave fixation. Such fixatives have the potential of reducing processing time from 24 hr to 2 hr, especially for small specimens.

Freeze Drying

Freeze drying is very useful in studying highly soluble materials, particularly small molecules. Specimens, not more than 2 mm thick, are immersed in nitrogen cooled isopentane, then transferred and kept in a vacuum chamber at –40°C. Complete dehydration without loss of morphological detail occurs through sublimation (9,11). Freeze-dried tissue can then be post-fixed at the vapor phase of formaldehyde. In freeze substitution, which is not entirely a method of physical fixation, specimens are immersed in cold (–40°C) fixatives, such as acetone or alcohol, which slowly remove water through dissolution of ice crystals. At –40°C proteins are not denatured; however, bringing the temperature gradually to 4°C will complete the fixation process through protein denaturation (9,11).

Chemical Fixation

Chemical fixation involves the use of organic or non-organic solutions to maintain adequate morphological preservation. There are 2 major categories of chemical fixatives; coagulant and non-coagulant (cross-linking) fixatives.

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Coagulant Fixatives

Both organic and non-organic solutions may coagulate proteins and render then insoluble. Because the architecture of tissue is maintained primarily by lipoproteins (1 of the major components of plasma membranes), and by fibrous proteins (such as collagen and nucleoproteins), coagulating these proteins maintains tissue histomorphology at the light microscopic level fairly well. However, coagulant fixatives result in cytoplasmic flocculation as well as poor preservation of mitochondria and secretory granules.

Coagulant Fixatives of Dehydrant Types

The most commonly used coagulating fixatives are dehydrants such as alcohols and acetone. Removal and replacement of free water from tissue has several potential effects on proteins within the tissue. If water molecules surround hydrophobic areas of proteins, the water forces, by repulsion, hydrophobic moieties into closer contact with each other, stabilizing hydrophobic bonding; thus, removal of the water destabilizes hydrophobic bonding because the hydrophobic areas are released from the repulsion of water and become free to occupy a greater area. Similarly, water molecules participate in hydrogen bonding in hydrophilic areas of proteins; therefore, the removal of water destabilizes hydrogen bonding in hydrophilic areas. Together, these changes act to disrupt the tertiary structure of proteins.

Proteins that are normally soluble in an aqueous environment have primarily hydrophilic groups exposed on the outside of the protein, ie, that part of the protein adjacent to the aqueous environment. When alcohols or acetone replace water in the tissue environment, these organics pull hydrophobic areas of peptides/proteins toward the new organic environment which now comprises the external environment surrounding the protein.

Ultimately the structure of the protein may become partially reversed with hydrophobic groups forming the outside surface of the protein (Figure 1) (4). The reversed structure would be a less ordered state than the state which permitted the protein to be soluble in water. Once the tertiary structure of a soluble protein has been destroyed, the rate of reversal to a more ordered soluble state is very slow and most proteins after coagulation remain insoluble even if returned to an aqueous environment. Methanol (CH_3OH) is closer to the structure of water than ethanol, so ethanol (CH_2CH_2OH) competes more strongly than methanol to interact with hydrophobic areas. Thus, coagulant fixation begins at a concentration of 50 to 60% for ethanol but coagulation of proteins requires a concentration of 80% or more for methanol (12).

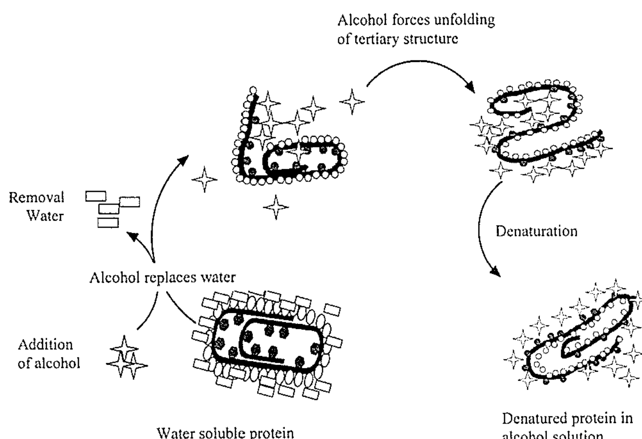


Figure 1. Denaturation of proteins after a coagulant fixative such as alcohol. Hydrophilic group ⬭, Hydrophobic group ● Water ▭, Alcohol ✧

Disruption of the tertiary structure of proteins (ie, denaturation) changes the physical properties of proteins, mainly causing insolubility and loss of function. Even though most proteins become less soluble in these organic environments, up to 13% of protein is lost with acetone fixation and 8% is lost with fixation in (4). Factors that influence the solubility of macromolecules include: (1) temperature, pressure, and pH; (2) ionic strength of the solute, which depends on the concentration and valence of different ions present in the solute; (3) the salting-in constant which expresses the contribution of the electrostatic interactions; (4) the salting in constant which reflects the net balance between salting-out through hydrophobic interactions and salting-in through electrostatic interaction of dipolar macromolecules like proteins; and (5) the type of denaturing reagent (4,13–15). For example, ethanol denatures proteins > phenols > water and polyhydric alcohols > monocarboxylic acids > dicarboxylic acids (16). Therefore, theoretically alcohol denatures protein differently depending on the choice and concentration of alcohol, the presence of organic and non-organic substances, and the pH and temperature of fixation.

Other Types of Coagulant Fixatives

Strong acids coagulants such as picric acid and trichloroacetic acid change the charges on the ionizable sidechains (eg, ($-NH_3^+$) and (–COOH)) of proteins and disrupt electrostatic and hydrogen bonding. These acids also may insert a lipophilic anion into a hydrophilic region and disrupt tertiary structures of proteins (4). Acetic acid coagulates nucleic acids but in general does not fix or precipitate proteins. Acetic acid is added to other fixatives to prevent the loss of nucleic acids. Trichloroacetic acid (UCl_3–CCOOH) can penetrate hydrophobic domains of proteins and the anion produced ($-C-COO^-$) reacts with charged amine groups. This interaction precipitates proteins and extracts nucleic acids. Picric acid (trinitrophenol) slightly dissolves in water to form a weak acid solution (pH 2.0). In reactions, it forms salts with basic groups of proteins, causing the proteins to coagulate. If the solution is neutralized, precipitated protein may redissolve. Picric acid fixation produces brighter staining, but the low pH solutions of picric acid may cause hydrolysis of nucleic acids.

Non-Coagulant Fixatives of Cross-Linking Type

Several chemicals are used as fixatives secondary to their actions of forming cross-links in and between proteins, in and between nucleic acids and between nucleic acids and proteins. Examples include: aldehydes such as formaldehyde, glutaraldehyde, chloral hydrate, and glyoxal; metal salts such as mercuric chloride, zinc chloride and other metallic compounds such as osmium tetroxide. Aldehyde groups $\left(\text{ie, } -\overset{H}{\underset{O}{C}}\right)$ are chemically and biologically reactive and are responsible for many of the histochemical reactions in histochemistry, as in the example of free aldehyde groups responsible for argentaffin reactions (17).

Formaldehyde Fixation

Formaldehyde in its 10% neutral buffered form (NBF) is the most common fixative used in diagnostic pathology. Formaldehyde is a vapor which when completely dissolved in water forms a solution containing about 37–40% formaldehyde. The aqueous solution of formaldehyde is referred to as formalin. The usual "10% formalin" used in fixation of tissues is a 10% solution of formalin v/v (it actually contains 4% formaldehyde w/v). Reactions of formaldehyde with macromolecules are numerous and complex. Fraenkel-Conrat and his colleagues meticulously and with simple chemistry identified most of the reactions of amino acids and proteins (18–23). In aqueous solutions, formaldehyde forms methylene hydrate, a methylene glycol (16).

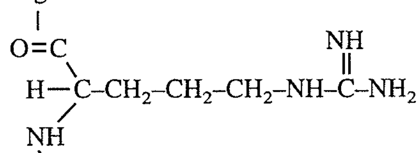
$$H_2C = O + H_2O \rightarrow HOCH_2\,OH$$

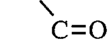
Methylene hydrate reacts with several side chains of proteins to form reactive hydroxymethyl side groups.

Formaldehyde also reacts with nuclear proteins and nucleic acids. Formaldehyde penetrates between nucleic acids and proteins and stabilizes the nucleic acid-protein shell (24–28). It modifies nucleotides by reacting with free amino groups of nucleotides, as in proteins. In naked and free DNA, the cross-linking reactions are believed to start at adenine-thymidine (A-T) rich regions, and cross-linking increases with increasing temperature (24–27). Also, formaldehyde reacts with C = C and –SH bonds in unsaturated lipids however; it doesn't interact with carbohydrates (23,29).

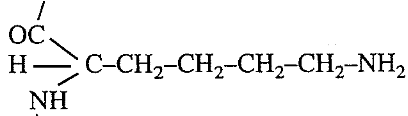
The side chains of peptides or proteins that are reactive with methylene hydrate and have the highest affinity for formaldehyde include the following: cysteine, lysine, histadine, and tyrosine (30).

Arginine side chains

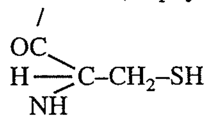




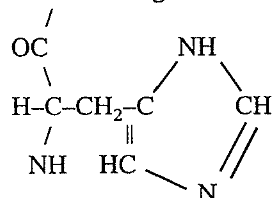
N terminal amino acids such as lysine



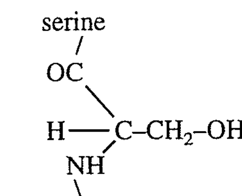
The thiol (sulphydryl) group of cysteine

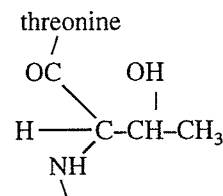


Histidine–ringed amine

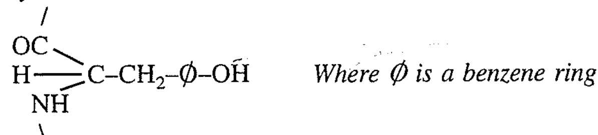


The active hydroxyl groups of serine, threonine, and especially tyrosine:





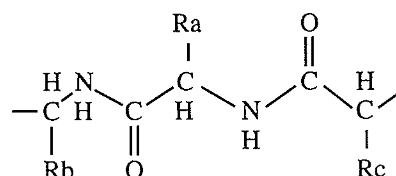
tyrosine



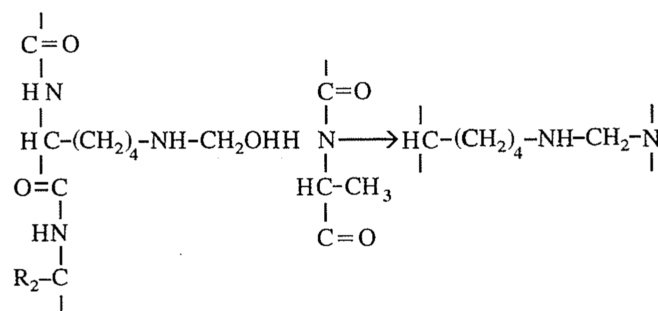
Several past and current books of histotechnology and histochemical stains indicate that 1 of the most important cross-links in "overfixation," ie, in tanning, is that between lysine and the amide group of the protein backbone. This concept is based on the book on tanning by Gustavson and is demonstrated below (31).

The amide nitrogen at peptide groups which may be accessible to cross-linking

amide groups



Cross-linking at the amide group of backbone may be as follows:



Water is lost (circle) forming the cross chain between lysine and amide group of protein backbone

In contrast to the above reaction, our review of the literature indicates that studies of overfixation (eg, tanning) as well as studies of fixation for shorter periods have seldom identified important cross-links between amine containing side chains of proteins (eg, lysine) and the backbone of a protein (18–23,30,31). The concept of direct linking to the protein backbone at amide groups via lysine may have risen as a theoretical mechanism of cross-linking, and although it

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is not supported by current studies of fixation, it continues to be propagated.

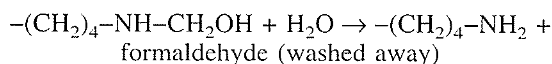
Of 6 potential types of reactive sites, most of the literature indicates that the lysine sites and histadine, an N-terminal cyclic amino group, are important in fixation reactions which are "overfixed" such as the tanning of collagen (18–23,30,31). Note that in acid environments $-NH_2$ groups may become $-NH_3^+$ groups which are less reactive with methylene glycol.

Reactions with 5 of these 6 general types of groups with methylene glycol ($OH{-}CH_2{-}OH$) occur forming the following reactive groups plus water:

lysine–	$-(CH_2)_4{-}NHCH_2\ OH$	$+H_2O$
arginine–	$-(CH_2)_3N{-}C{-}NHCH_2\ OH$	$+H_2O$
cysteine–	$-CH_2{-}S{-}CH_2\ OH$	$+H_2O$
tyrosine, theronine, serine–	$-V^*{-}O{-}CH_2\ OH$	$+H_2O$
glutamine–	$-(CH_3)_2{-}CO{-}NH\ CH_2OH$	$+H_2O$

V* = variable groups

Reversibility of Formaldehyde—Macromolecular Reactions: The reactive groups may combine with each other forming methylene bridges or with hydrogen groups. If the formalin is washed away, reactive groups rapidly are returned to their original groups, but bridging which has already occurred remains.



$$-(CH_2)_4{-}NH{-}CH_2OH + H_2O \rightarrow -(CH_2)_4{-}NH_2 + \text{formaldehyde (washed away)}$$

After 24 hr of washing, 50% of reactive groups can be removed and after 4 wk of washing 90% can be removed (32). The likelihood of this reaction is uncertain. On long-term storage in formalin the reactive groups may be oxidized to the more stable groups (eg, acids–NH–COOH) which are not easily removed by washing in water or alcohol. Thus, after the initial period of fixation, returning the specimen to water or alcohol reduces further fixation of the specimen because the reactive groups, induced by the initial reaction with formalin, are removed. Besides simple washing under running water, over-fixed tissue may be partially corrected, by soaking the tissue in concentrated ammonia plus 20% chloral hydrate (33). Fraenkel-Conrat and his colleagues frequently noted that the addition and the condensation reactions of formaldehyde with amino acids and proteins were unstable and could be easily reversed by dilution or dialysis (18–22). The details of such reversibility are discussed in the companion article in this issue "*Advanced Concepts in Fixation: Effects of Fixation on Immunohistochemistry and Histochemistry, Reversibility of Fixation and Recovery of Proteins, Nucleic Acids, and Other Molecules from Fixed and Processed Tissues, Special Methods of Fixation*" (8).

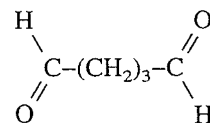
It is generally believed that it is the cross-linking that is important for fixation of tissue for biological uses and not the formation of reactive hydroxymethyl adducts; however, it is possible that formation of these hydroxyl methyl adducts may denature macromolecules and render them insoluble. The principal type of cross-link in short-term fixation is said to be between the hydroxymethyl group on lysine side chains and arginine (through secondary amino group), aspargine, glutamine (through secondary amide group), or tyrosine (through hydroxyl group) (34). For example, a lysine hydroxyl amine group can react with an arginine group to form a lysine–CH_2–arginine cross link; similarly a tyrosine hydroxyl amino group can bind with a cysteine group to form a tyrosine–CH_2–cysteine cross link. Each of these cross-links between macromolecules has varying degrees of stability, which can be modified by the temperature, pH, and type of environment surrounding and permeating the tissue. The stability of cross-links and their potential reversibility are also discussed more extensively in the companion article on advanced concepts of fixation (8).

The time of saturation of human and animal tissues with active groups by formalin is about 24 hr, but actual fixation may continue for many weeks (32).

Formaldehyde primarily preserves peptides—proteins and the general structure of cellular organelles. It can interact with nucleic acids but has little effect on carbohydrates. Formaldehyde preserves lipids if the solutions contain calcium (35).

Glutaraldehyde

Because glutaraldehyde is not used as widely in biological applications as formaldehyde, less is known about its biological reactions and effects. Glutaraldehyde is a bifunctional aldehyde that combines with the same reactive groups as does formaldehyde. In aqueous solutions glutaraldehyde polymerizes forming cyclic and oligomeric compounds and is also oxidized to glutaric acid (36). To aid in stability, it requires storage at 4°C and a pH around 5 (37).



Unlike formaldehyde, glutaraldehyde has an aldehyde group on both ends of the molecule. Thus, with each reaction, an unreacted aldehyde group is introduced into the protein and these aldehyde groups can act to further cross-link the protein. Alternatively, the aldehyde groups may react in a wide range of histochemical reagents including antibodies, enzymes or proteins. The reaction of glutaraldehyde with an isolated protein such as BSA is maximum at pH 6–7, is faster than formaldehyde, and results in more cross-linking than formaldehyde (38,39). Cross-linking is irreversible and withstands acids, urea, semicarbazide, and heat (29). Like formaldehyde, reactions with lysine are the most important in cross-linking.

Unless the aldehyde groups are blocked, increased background staining will result if several histochemical methods are used on sections from tissues fixed in glutaraldehyde (17).

Although the extensive cross-linking by glutaraldehyde results in better preservation of ultrastructure, this method of fixation negatively affects immunohistochemical methods and slows the penetration by the fixative. Thus, any tissue fixed in glutaraldehyde must be very small (1 mm maximum). Glutaraldehyde does not react with lipids unless they contain free amino groups as are found in some phospholipids (29). At room temperature (RT) glutaraldehyde doesn't cross-link nucleic acids in the absence of nucleohistones but it may react with nucleic acids at 45°C and above (29).

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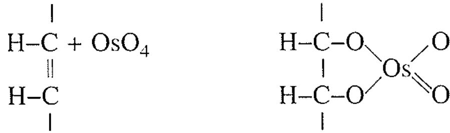
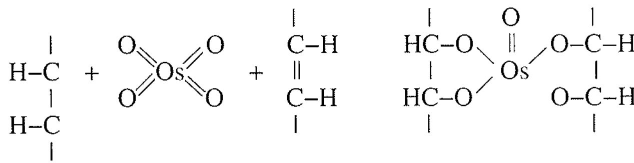
Fixation with Osmium Tetroxide

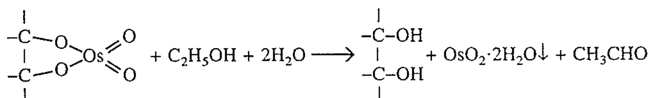
This toxic solid is soluble in water as well as non-polar solvents and can react with hydrophilic and hydrophobic sites. Osmium tetroxide (OsO_4) reacts with the side chains of proteins causing cross-linking (39). The reactive groups include sulphydryl, disulfide, phenolic, hydroxyl, carboxyl, amide, and heterocyclic groups. OsO_4 is known to interact with nucleic acid; specifically, it reacts with 2,3-glycol moiety in terminal ribose groups and with 5,6-double bonds of thymine residues. Nuclei fixed in OsO_4 and dehydrated with alcohol may show prominent clumping of DNA; this unacceptable artifact can be prevented by prefixation with potassium permanganate ($KMnO_4$), postfixation with uranyl acetate, or by adding Ca^{++} and tryptophan during fixation (29). The reaction of OsO_4 with carbohydrate is uncertain (29). Large proportions of proteins and carbohydrates are lost from tissues during osmium fixation. Some of this loss may be due to the superficial limited penetration of osmium tetroxide (ie, <1 mm) into tissues or to its slow rates of reaction. Thus, osmium tetroxide fixation works only for very small pieces of tissue. The most characterized reaction with osmium is its reaction with unsaturated bonds within lipids and phospholipids.



In this reaction, osmium in its +8 valence state converts to a +6 valence state which is colorless. If 2 unsaturated bonds are close together there may be cross-linking by osmium tetroxide:



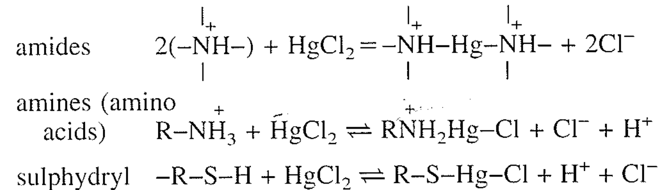
Again at the light level, this complex is colorless. For the typical black staining of membranes expected from fixation with osmium, osmium dioxide ($OsO_2 \cdot 2H_2O$) must be produced. Osmium dioxide is insoluble in aqueous solution, black, and electron dense, and it precipitates as the above unstable compounds break down, thus depositing and coating cellular membranes. The breakdown of osmium +6 valence complexes to osmium dioxide (+4 valence state) is facilitated by a reaction with solutions of ethanol.



Besides its use as a secondary fixative for electron microscopic examinations, osmium tetroxides also can be used to stain lipids in frozen sections. Osmium tetroxide fixation causes tissue swelling which is mostly reversed during dehydration steps. Swelling also can be minimized by adding $CaCl_2$ or NaCl to osmium containing fixatives (29).

Mercuric Chloride

The chemistry of fixation using mercuric chloride is not understood. However, it is known that mercuric chloride reacts with ammonium salts, amines, amides, amino acids, and sulphydryl groups, and acts to harden tissues. It is especially reactive with cysteine. Examples are the following:



The mercury-based fixatives described below have some similar characteristics:

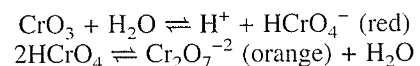
- they are very toxic and should be handled with great care
- these solutions should not be permitted to contact metal including metal forceps, containers, etc
- distilled water (DW) must be used to prevent the precipitation of mercury salts
- these fixatives penetrate slowly so specimens should be thin
- mercury and acid formaldehyde hematein pigments may deposit in tissue after fixation in these solutions.

Additional metal-mercury pigments may be deposited if metals come into contact with these fixatives. Mercural fixative, for all these reasons and others, are no longer used routinely except by some laboratories for fixing hematopoietic tissues (especially B5) (40). Some authors have reported that zinc formaldehyde, like mercuric chloride in B5, gives better nuclear detail than formaldehyde alone. It also has been proposed that zinc salts improve the penetration by formaldehyde (3).

Special Fixatives

Dichromate and Chromic Acid Fixation

Chromium trioxide ($O{=}\overset{+6}{Cr}(=O){=}O$) dissolves in water to produce an acid solution, chromic acid with a pH of 0.85. Chromic acid is a powerful oxidizing agent which produces aldehyde from the 1,2-diglycol residues of polysaccharides. Such aldehydes can react in histochemical stains (PAS and argentaffin/argyrophal stains) and could increase the background of immunohistochemical stains.



$CrO_3 + H_2O \rightleftharpoons H^+ + HCrO_4^-$ (red)
$2HCrO_4 \rightleftharpoons Cr_2O_7^{-2}$ (orange) $+ H_2O$

The pH of the solution determines the ionic ratio of $HCrO_4$ to $Cr_2O_7^{-2}$ and the strength of an unbuffered solution determines the pH (ie, 1% pH = 4.10; 2.5% pH = 4.05). It should be noted that ions of chromic acid are in the +6 valence state so "chromic" is somewhat a misnomer. Chro-

mic acid can be prepared by using hydrochloric acid to acidify a solution of potassium dichromate and most chromium based fixatives actually used potassium dichromate which is less dangerous than chromium trioxide. Actual chromic salts (ie, chromium ions in +3 valence state) destroy animal tissues and should not be used in fixation (1). Similarly, the alkaline solutions should not be used for fixation; alkaline solutions of chromate turn yellow secondary to the formation of CrO_4^{-2}. Chromium ions in their +6 state can be used as fixatives to coagulate proteins and nucleic acids. The fixation reactions are not understood completely but probably involve oxidation of proteins which varies in strength depending upon the pH of the fixative, plus the interaction of the reduced chromate ions directly in cross-linking proteins. Chromium ions specifically interact with carboxyl and hydroxyl side chains of proteins (11). Chromic acid also interacts with disulfide bridges and attacks lipophytic residues such as tyrosine and methionine (4).

Fixation with chromium salts was one of the initial methods of tanning, but its exact mechanisms of hardening tissues are uncertain. Fixatives containing chromate at a pH of 3.5 to 5.0 have been reported to be quite good fixatives that make proteins insoluble without coagulation. Chromate is reported to make unsaturated (but not saturated) lipids insoluble upon prolonged (>48 hr) fixation. This is given as a good reason for why mitochondria are well preserved by dichromate fixatives.

In histochemistry, the dichromate containing fixatives have been used primarily to prepare endocrine tissues for staining, especially the adrenal medulla and tumors of the adrenal medulla. However, many of these stains (such as the chromaffin stain used to identify chromaffin granules) are not used frequently, are unreliable, and require controls seldom available; therefore, these stains have been replaced by immunohistochemical stains such as neuron specific enolase, chromagranin, and synaptophysin (17,41)

Metallic Ions as a Fixative Supplement

Multiple metallic ions have been used as aids in fixation, including Hg^{2+}, Pb^{2+}, Co^{2+}, Cu^{2+}, Cd^{2+}, $[UO_2]^{2+}$, $[PtCl_6]^{2-}$, and Zn^{2+}. Of these, mercury, lead, and zinc are used most commonly in current fixatives. For example, zinc containing formaldehyde is touted as a better fixative than formaldehyde alone; however, this depends upon the pH of the formaldehyde as well as the zinc formaldehyde (8,42).

Fixatives for Electronmicroscopy

The requirements to preserve carefully cell organelles such as cytoplasmic and nuclear membranes, mitochondria, membrane bound secretory granules, and smooth and rough endoplasmic reticulum are not met by many fixatives, especially fixatives with dehydrant components which extract lipids. Because membranes and other lipid containing structures are very important in ultrastructural examinations, fixatives that do not solubilize lipids are necessary. The preferred fixative is a strong cross-linking fixative such as glutaraldehyde or a combination of glutaraldehyde and formaldehyde followed by post-fixation in an agent that further stabilizes and emphasizes membranes such as osmium tetroxide.

Infrequently Used Fixatives

Numerous substances have been used as fixatives but have not proven to be very useful. These include solution of iodine (Lugol's solution), mineral acids, sodium periodate, acadrine orange, satrinine 0, cyanuric chloride, and quinones.

Combination Fixatives

For pathologists, formaldehyde based fixatives are used commonly to produce specific, reproducible histomorphometric patterns. Other agents may be added to formaldehyde to produce specific effects not possible in a fixative that cross-links. ie, the dehydrant ethanol can be added to formaldehyde to produce alcoholic formalin. This combination preserves molecules like glycogen and results in less shrinkage and hardening than dehydrants.

Combination fixatives may be especially useful for specific tissues. Alcoholic formalin is useful for fixation of fatty tissues such as breast in which preservation of the lipid is not important. Similarly, fixation of gross specimens in alcoholic formalin may aid in identifying lymph nodes embedded in fat. Also some combined fixatives are good for preserving antigen immunorecognition; however, non-specific staining or increased background staining in immunohistochemical procedures can be increased by some fixatives (43–46). Two examples, are: (1) unreacted aldehyde groups, glutaraldehyde-formaldehyde fixation causing increased background staining and (2) alcoholic formalin causing non-specific staining of myelinated nerves (43–46).

Factors Affecting the Quality of Fixation

Buffers and pH

The effect of pH on formaldehyde fixation is profound and should be considered during fixation. In an acid environment, the primary amine target groups ($-NH_2$) attract hydrogen ions ($-NH_3^+$) and become unreactive to the hydrated formaldehyde (methylene hydrate or methylene glycol). Similarly carboxyl groups $(COO)^-$ will lose their charges (–COOH) which may also affect protein structure. The hydroxyl groups of alcohols (–OH), including serine and threonine, may become less reactive in an acid environment. Because the major methylene cross links are between lysine and the free amino group on side chains, the extent of cross-linking would be expected to be reduced in unbuffered 4% formaldehyde, which is slightly acidic, pKa value of 12.79 – 12.87 (23,30). With NBF (pH = 7.2 – 7.4) all amine and alcohol groups are more reactive with methylene glycol. These results support a decrease in the effectiveness of formaldehyde fixation in the acid environment and the decreased cross linking of proteins supports observation that unbuffered formalin is a better fixative than NBF with respect to immunorecognition of many antigens (8). The choice of optimum pH depends on the type of fixation and the type of tissue. For example, tissues of the gastric mucosa or the adrenal gland are fixed best in 10% formalin at acidic pH, and fixation in 10% formalin at an acid pH is best for immunorecognition for most antigens (42).

At the acidic pH of an unbuffered formaldehyde, hemoglobin is chemically modified to form a brown insoluble crystalline birefringent pigment. The pigment forms at a pH of less than 5.7 and the extent of pigment formation increases in the pH range of 3.0 to 5.0. Formalin pigment is recognized with ease and should not affect diagnoses and the pigment is removed easily with an alcoholic solution of picric acid. Nevertheless, in order to avoid the formation of formalin pigment, pathologists choose NBF as the preferred

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formaldehyde based fixative. Acetic acids and other acids work mainly through lowering pH and disrupting the tertiary structure of proteins.

Buffers are used to maintain pH at optimum. The choice of specific buffers depends on the type of fixative and the type of the analyte. The commonly used buffers are phosphate, cacodylate, bicarbonate, Tris, and acetate.

Duration of Fixation and Size of Specimens

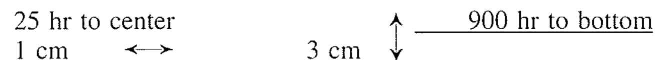
The factors which govern diffusion of fixative into tissue were investigated by Medivan (47). He found that the depth reached by a fixative is directly proportional to the square root of duration of fixation and re-expressed this relation as:

$$d = k\sqrt{t}$$

Where d = depth of penetration
k = coefficient of diffusion; specific for fixative
t = time

Thus, the time taken to penetrate a given depth of tissue depends on the co-efficient of diffusability (k) which is 0.79 for 10% formaldehyde, 1—2 for 4% acetic acid, 1.0 for 100% ethanol, and 1.33 for 3% potassium dichromate (37).

For most fixatives, the time of fixation is approximately equal to the $(\text{distance})^2$ the fixative must penetrate. Most fixatives such as NBF will penetrate tissue to the depth of approximately 1 mm in 1 hr. Thus, for a 1 cm sphere, the center of the sphere will not "see" fixative until after $(5)^2$ or 25 hr of fixation.



For a sphere 4 cm in diameter, the center will not be reached until 400 hr; thus, large pieces of tissue may take long times for adequate fixation. For a 3 cm thick, flat piece of tissue on the bottom of a container (eg, a 3 cm section of liver) penetration of formalin will be primarily from the top and it may take up to 900 hr to fix the bottom of the tissue. Thick gross specimens (≥ 1 cm), should not rest on the bottom of a container of fixative, but should be separated from the bottom by wadded paper to permit penetration of fixative or processing fluids from both the bottom and the top. Also, unfixed gross specimens should not be cut thicker than 0.5 cm if they are to be stored in fixative prior to processing.

For surgical specimens that are to be processed to paraffin blocks, the time of penetration is more critical. The piece of tissue should be small enough in length and width (1 cm × 1 cm) to permit a stable ribbon to be cut from the paraffin block. The thickness of the specimen should be less than the thickness of a histological cassette (less than 4 mm in thickness requires less than 4 hr for penetration). Thicker specimens, especially those that extend through the holes of cassettes, will block the flow of fixative or processing fluids through the cassette during tissue processing.

Penetration of the fixative is not equivalent to fixation. As discussed, fixation proceeds as a series of steps. In formalin fixation, the first step is the formation of a reactive hydroxyl methyl group with the reactive species (eg, lysine, tyrosine). Then 1 reactive group must cross link with another reactive side chain. This occurs relatively rapidly with most carbon 14 labeled formaldehyde having been added to tissue within 24 hr (32).

The time frame between the formation of reactive hydroxymethyl groups and the formation of a significant number of cross links is unknown; however, since 90% of reactive groups can be removed by 4 wk of washing, it is clear that cross linking is not a rapid process and that cross linking may require weeks for completion of potential bonds. This is mirrored by the observation that identifying antigens by immunohistochemistry in tissue fixed for months may not be possible even with the most vigorous approaches to antigen recovery, and that long periods of fixation also result in poor histochemical staining (32).

Fixatives are inactivated by proteins and may be especially inactivated (used up) by blood or bloody fluids. Bloody gross specimens should be washed with saline prior to being put in fixatives. If specimens are not bloody, the fixative volume should be ×20 the volume of the tissue specimen for optimal, rapid fixation.

Today, thin specimens may be fixed in 10% NBF for only 5 to 6 hr including the 2 hr of fixation on typical processors (8). The process that constitutes "fixation" in today's specimens with respect to the formation of cross-links is uncertain. The formaldehyde fixation of today at 6 or less hours given the rapid push for quick turn-around times for diagnostic specimens does not parallel the 24 hr of fixation of the 1980's or early 1990's. The rapid fixation of the 21st century may be secondary to the formation of very few cross-links with most changes of formaldehyde fixation being due to the addition of hydroxymethyl groups. As long as our histochemical staining continues to be adequate, rapid fixation is acceptable; in fact, our immunohistochemistry and other molecular techniques are likely to be improved by shorter times of fixation using strong aldehyde based fixation.

Temperature of Fixation

The diffusion of molecules (solutions) increases with increasing temperature due to increased movement and vibration of molecules. Thus, the rate of penetration of a tissue by formaldehyde is increased at higher temperatures. Microwaves have been used to speed formaldehyde fixation by increasing the temperature, and the resulting molecular movements (note—increased vapors are a safety problem (6)). Not only does the rate of penetration of formaldehyde increase, because most chemical reactions occur more rapidly at higher temperatures, formaldehyde reacts more rapidly with proteins at higher temperatures (36).

Concentration of Fixative

Cost, effectiveness, and solubility determine the appropriate, concentration of fixatives. Concentrations of formalin above 10% tend to cause increased hardening and shrinkage, whereas ethanol concentration of below 70% did not remove free water from tissues efficiently (5).

Osmolality of Fixatives and Ionic Composition

Osmolality of vehicle buffer is very important. Hypertonic and hypotonic solutions lead to shrinkage and swelling, respectively. The best results are obtained with solutions that are slightly hypertonic (400–450 mOsm). Various ions (Na^+, K^+, Ca^{++}, Mg^{++}) can affect cell shape and structure regardless of the osmotic effect. It is believed that the ionic composition of fluids should be as close as possible to physiological composition.

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Table 1. Action of Major Single or Combination Fixatives

	Dehydrants	*Aldehyde crosslinkers*	*Combination mercuric chloride with formaldehyde or acetic acid*	*Osmium tetroxide*	*Picric acid plus formalin and acetic acid*	*Combination alcohol plus formalin*
Examples of Category	Ethanol Methanol Acetone	Formaldehyde Glutaraldehyde	Zenker's B5	Post fixation after glutaraldehyde	Bouin's	Alcoholic formalin
Effect on Proteins	Precipitates without chemical addition	Crosslinkers: Adds active hydroxyl methyl groups to amines, amides, reactive alcohols, and sulphydryl groups; cross-links amine/amide or sulphydryl side chains of proteins	Additive plus coagulation	Additive cross-links; some extraction; some destruction	Additive and non-additive coagulant; some extraction	Additive plus precipitation
mRNA/DNA	Very slight	Slowly cross-links, slightly extracts	Coagulation	Slight extraction	None	Very slight
Lipids	Very extensive extraction	None	None	Made insoluble by cross-links at double bonds	None	Extensive extraction
Carbohydrates	None	None on pure carbohydrates; cross linking of glycoproteins	None	Slight oxidation	None	None
Quality of Staining	Satisfactory	Good	Good	Poor	Good	Good
Effect on Ultrastructure (organelles)	Destroys Ultrastructure	Good (NBF) to excellent preservation (glutaraldehyde)	Good preservation	Used for visualization of membranes	Poor	Poor
Usual Formulation	70% to 100% solution or in combination with other types of fixatives	Formaldehyde (37%)–10% aqueous solution buffered with phosphates to 7.2–7.4. Glutaraldehyde–2% buffered to 7.4	Mercuric chloride combined either with acetic acid plus dichromate or formaldehyde plus acetate	1% solution buffered to 7.4	Aqueous picric acid, formalin, glacial acetic acid	10% formaldehyde (37%) with 90% ethanol
Important Variables/ Issues	Time, specimen thickness—should only be used for very small or thin specimens	Time, temperature, pH, concentration/specimen thickness	Very toxic	Very toxic	Mitochondria and integrity of nuclear membrane destroyed; not appropriate for some stains; mordant	Time, specimen dimensions
Special Uses	Preserves small non-lipid molecules such as glycogen; preserve enzymatic activity	General all around fixative; best for ultrastructure if used with osmium tetroxide post-fixation	Excellent for hematopoietic tissues	Ultrastructural visualization of membranes; lipids on frozen sections	Mordant for connective tissue stains (trichrome)	Good general fixative; good for specific immunohistochemical reactions and good to detect lymph nodes in fatty tissue; removes fats from tissue

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Table 2. Incompatible Stains and Fixatives (modified from reference 2)

Target of special stain	*Type of special stain*	*Do not use this fixative*	*Required or best fixative*
Amebas	Best's carmine	Aqueous fixative	Alcohol or alcoholic formalin
Cholesterol and Cholesterol Esters	Schultz's method	Bouin's; Zenker's	10% NBF (frozen section)
	Digitonin	Bouin's; Zenker's	10% NBF (frozen section)
Chromaffin Granules	Ferric ferricyanide reduction test		Orth's; Möller's
	Gomori-Butner methenamine silver	Avoid glutaraldehyde	Orth's; Möller's
	Periodic acid—Schiff —(PAS)	Avoid glutaraldehyde	
	Mallory's aniline blue collagen stain	Dichromate and alcohol bases	10% NBF; Bouin's; Heiden hain's mercuric chloride
Connective Tissue	Wilder's reticulum	No picric acid Fixatives	10% NBF; Zenker's; Helly's
	Masson's trichrome	NBF tissues must be treated with mordant (Bouin's)	Bouin's
	Mallory's analine blue collagen stain	All except	Zenker's
Copper	Mallory's stain	Formalin	Alcohol based fixatives
Degenerating Myelin	Marchi's method	All except	Orth's for 48 hr 10% NBF
Elastic Fibers	Gomori's aldehyde fuchsin	No chromates	10% NBF
Fats/Lipids	Nile blue sulfate	All except	Formal calcium
	Osmic acid (Frozen section)	All except	10% NBF
	Oil red O (Frozen section)	Zenker's; Helly's	10% NBF
	Sudan black B (Frozen section)	Zenker's; Helly's	10% NBF
Fibrin	Mallory's phosphotungstic acid hematoxylin	Bouin's	Zenker's
	Weigert's stain for fibrin	Bouin's	Absolute ethanol; Carnoy's; alcoholic formalin
Glycogen	Bauer-Feulgen	Aqueous fixative	Carnoy's or Gendre's
	PAS	Aqueous fixative	Acid alcoholic formalin
	Best's carmine	Aqueous fixative	Absolute alcohol; Carnoy's
Hemoglobin	Lepehne's (frozen section)	Zenker's	Short time in 10% NBF
	Dunn-Thompson	Bouin's, Zenker's, Helly's	10% NBF
Hepatitis B Surface Antigen	Orcein	No chromates	
	Aldehyde fuchsin	No chromates	

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Table 2. Continued

Target of special stain	*Type of special stain*	*Do not use this fixative*	*Required or best fixative*
Iron	Mallory's stain	Formalin	Alcohol based fixatives
Juxtaglomerular Cells of Kidney	Bowie's stain	All except	Helly's
Melanin Pigments	DOPA oxidase	All except	See procedure
Mucoproteins Glycoproteins	PAS Müller—Mowry, colloidal iron	Glutaraldehyde Chromates	Alcoholic formalin Carnoy's
Pancreas α, β, & δ cells	Trichrome—PAS	Zenker's; Bouin's	10% NBF or Helly's
Peripheral Nerve Elements	Bielschowski's for neurofibrils and axis cylinders	All except	3 to 6 weeks in 10% NBF
	Bodian's for myelinated and non-myelinated nerve fibers	All except	9 parts ethanol 1 part formalin
	Nonidez's for neurofibrils and axis cylinders	All except	100 ml of 50% ethanol plus 25 gm chloral hydrate
	Rio-Hortega for neutrofibrils	All except	10% NBF
Phospholipids	Smith-Dietrich (frozen section)	All except	Formal calcium
	Baker's acid hematin (frozen section)	All except	10% NBF
Pituitary Beta Cells	Congo red for beta cells		10% NBF
	Gomori's aldehyde fuchsin for β cells	NBF requires mordant	Bouin's
Silver Stains	Fontana Masson Grimelius	Glutaraldehyde	
Spirochetes	Giemsa	Bouin's; Zenker's	
	Gram's technique	Bouin's; Zenker's	
	Levaditi	Bouin's; Zenker's	
	Warthin-Starry	All except	10% NBF
Uric Acid Crystals	Gomori's methenamine, silver for urate	All except	Absolute ethanol
	Gomori's chrome alum hematoxylin phloxine	Avoid chromates	Bouin's

Additives

It is known that the addition of electrolytes and non-electrolytes to fixatives improves fixation. Such substances include calcium chloride, potassium thiocyanate, ammonium sulfate, and potassium dihydrogen phosphate. The electrolytes may react directly with proteins causing denaturation, or they may react with fixatives on one hand and cellular constituents on the other (29). Addition of non-electrolyte substances such as sucrose, dextran, and detergent, has also been reported to improve fixation (29).

Selecting or Avoiding Specific Fixatives

We have emphasized that the choice of a fixative is a compromise—a balance between beneficial and detrimental effects; the fixative is matched with its use. Table 1 is a brief review of the actions of fixatives to aid in the selection of the best fixative; it is modified from a table developed by Kiernan (1).

For many uses, however, specific fixatives are unacceptable, and these fixatives should be avoided. The main problems with fixatives related to histological staining is the loss

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(by solution/extraction) of molecules that are targets of specific histochemical methods. Typically, some molecules are soluble in aqueous fixatives (eg, glycogen), while others are soluble in fixatives that are non-polar solvents (eg, lipids). Also, some fixatives may chemically modify targets of histochemical staining, thus affecting the quality of special stains (eg, glutaraldehyde for silver stains). This includes modification of staining secondary to changes in pH induced by fixation. One of the best correlations of the effects of fixation on histochemistry can be found in a table in Chapter 2 of *Theory and Practice of Histotechnology* (2).

We have modified this table so that harmful methods of fixation can be identified rapidly (Table 2).

Useful Formulas For Fixatives:

The book by Gray lists over 600 formulations for various fixatives (48). The following is a list of the fixatives and formulas used most commonly by histotechnologists/anatomists. Many of these formulas are based on those presented in standard textbooks of histochemistry (1–4).

Formalin Fixatives

For routine diagnosis, 10% NBF, is used most frequently for initial fixation and for the initial stations on tissue processors. NBF is usually composed of a 10% v/v solution of phosphate buffered 37 to 40% formaldehyde. For simplification, 37–40% formaldehyde is referred to as 37% formaldehyde in subsequent formulas. Typical formulas for NBF are as follows:

Neutral Buffered 10% Formalin

water	900 ml
formalin (37% formaldehyde solution)	100 ml
sodium phosphate, monobasic, monohydrate	4.0 gm
sodium phosphate, dibasic, anhydrous	6.5 gm

pH should be 6.8–7.4.

Carson's Modified Millonig's Phosphate Buffered Formalin

formaldehyde (37–40%)	10 ml	
tap water	90 ml	*Another formula for NBF*
sodium phosphate monobasic	1.86 gm	
sodium hydroxide	0.42 gm	

Deionized water can be used if tap water is hard and/or contains solids.

This formula is better for ultrastructural preservation than NBF.

There are other formulations of NBF and similar fixatives. In fact, NBF purchased from commercial companies may vary widely and commercial companies may add material such as methanol or other agents to stabilize NBF preparations (5).

Sometimes the term "formal" is used to refer to 10% formalin or 4% formaldehyde

Formal, calcium

water	900 ml	*A fixature that can*
formaldehyde (37%)	100 ml	*be used for preservation*
calcium acetate	20 gm	*of lipids*

Formal, saline

water	900 ml
formaldehyde (37%)	100 ml
sodium chloride	9 gm

Formal, zinc

		Unbuffered zinc
water	900 ml	*formaldehyde is touted*
formaldehyde (37%)	100 ml	*to be an excellent*
sodium chloride	4.5 gm	*fixative for*
zinc chloride or zinc sulfate	1.6 g or (3.6 gm)	*immunohistochemistry*

Formalin, buffered saline

water	900 ml
formaldehyde (37%)	100 ml
sodium chloride	9 gm
sodium phosphate, dibasic	12 gm

Formalin, buffered zinc

10% Neutral Buffered Formalin	1,000 ml
zinc chloride	1.6 gm

Mercuric Fixatives

Zenker's Solution

distilled water	250 ml	*Good fixative for bloody*
mercuric chloride	12.5 gm	*(congested) specimens.*
potassium dichromate	6.3 gm	*Excellent fixative for PTAH*
sodium sulfate	2.5 gm	*stain and trichrome stains*

Just before use add 5 ml of glacial acidic acid to 95 ml of above solution.

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Helly's Solution

distilled water (DW)	250 ml	*Excellent for bone marrow, extramedullary hematopoiesis, intercalated discs.*
mercuric chloride	12.5 gm	
potassium dichromate	6.3 gm	
sodium sulfate	2.5 gm	

Just before use add 5 ml of 37% formaldehyde to 95 ml of above solution.

Schaudinn's Solution

DW	50 ml
mercuric chloride	3.5 gm
absolute ethanol	25 ml

Ohlmacher's Solution

absolute ethanol	32 ml	
chloroform	6 ml	*Ohlmacher's and Carnoy-Lebrun fixatives penetrate very rapidly.*
glacial acetic acid	2 ml	
mercuric chloride	8 gm	

Carnoy-Lebrun Solution

absolute ethanol	15 ml
chloroform	15 ml
glacial acetic acid	15 ml
mercuric chloride	8 gm

B-5 Fixative

stock solution

mercuric chloride	12 gm	*Frequently used for bone marrow, lymph nodes, spleen and other hematopoietic tissues.*
sodium acetate	2.5 gm	
DW	200 ml	

Add 2 ml of formaldehyde (37%) to 20 ml of stock solution just before use

One of the problems with fixation in mercury solutions is that several types of pigments may form especially pigments with mercury. These pigments are typically removed using iodine treatment followed by thiosulfate to remove iodine.

Picric Acid Fixatives

Many picric acid fixatives require a saturated aqueous solution of picric acid. The following table provides easy conversions:

Saturated Aqueous Solutions of Picric Acid

1500 ml DW	31.5 gm picric acid
1000 ml DW	21 gm picric acid
150 ml DW	3.15 gm picric acid
100 ml DW	2.1 gm picric acid
100 ml absolute ethanol	5 gm picric acid

Bouin's Solution

saturated aqueous solution of picric acid	1500 ml
formaldehyde (37%)	500 ml
glacial acetic acid	100 ml

Bouin's solution is an excellent general fixative especially for connective tissue that is to be stained with trichrome. The yellow color can be removed with 70% ethanol or bluing reagent separately or during the staining sequence.

Hollande's Solution

DW	1000 ml	*Useful in gastrointestinal biopsies and the fixation of endocrine tissue.*
formaldehyde 37%	100 ml	
acetic acid	15 ml	
picric acid	40 gm	
copper acetate	25 gm	

Specimen must be washed before exposure to NBF.

Dehydrant Fixatives

Dehydrant fixatives act as tissue dehydrants—removing bound water and hence changing the tertiary structure of proteins so that proteins precipitate. Nucleic acids remain relatively unchanged. Histopathology is fair to good but not as good as

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NBF. Ultrastructure is destroyed by any of these 4 dehydrants due to the extraction of lipids and each may cause excessive shrinking of tissue components after more than 3 to 4 hr of fixation. Each of these fixatives can be modified by adding other chemicals to produce specific effects.

1. ethanol absolute
2. ethanol, 95%
3. methanol, 100%
4. acetone, 100%

Acetone fixation should be short (1 hr) at 4°C only on small specimens. Acetone produces extensive shrinkage and hardening and results in microscopic distortion. It is used for immunohistochemistry, enzyme studies, and to detect rabies. Methanol is especially useful for touch preparations, smears, and especially blood preparations.

Many alcohol mixtures may undergo slow reactions among ingredients upon long term storage; thus, in general, most alcohol based fixatives should be prepared no more than 1 to 2 days before use. Commercial formulations are stabilized by "trade secret" ingredients.

Clarke's Solution

absolute ethanol	60 ml
glacial acetic acid	20 ml

Clarke's solution produces tissue that can be stained to yield good general histological results (eg, H & E stains). It has the advantage of preservation of nucleic acids in fixed tissues. Lipids are extracted. A short term fixation is preferable with specimens transferred to 95% ethanol.

Carnoy's Fixative

acetic acid	10 ml	*Useful in cytology*
absolute ethanol	60 ml	*to clear bloody specimens.*
chloroform	30 ml	

Required fixative for RNA stains—methyl green pyronine. Good for glycogen preservation. Shrinks and hardens tissue. Hemolyzes red blood cells and may destroy staining of acid fast bacilli.

Methacarn

acetic acid	10 ml	*Same pattern of staining,*
100% methanol	60 ml	*but less hardening and less*
chloroform	30 ml	*shrinkage than Carnoy's.*

Dehydrant—Cross-linking Fixatives

Mixed fixatives with both dehydrant and cross-linking actions include an alcohol-formalin mixture. These produce excellent results in the immunohistochemical identification of specific antigens (42). However, in some situations the results may be too good. For example, the Herceptin Test developed by DAKO to identify the membrane expression of $p185^{erbB-2}$ depends upon the paraffin embedded tissue being fixed in NBF. This test is used to identify patients whose tumors (eg, breast) are likely to respond to therapy with the monoclonal antibody therapy, Herceptin. Fixation in alcoholic formalin will produce a stronger membrane pattern of staining than in tissues fixed in NBF. The mechanism of this is unknown, but may involve less immunorecognition of cytoplasmic $p185^{erbB-2}$ antigens in tissues fixed in ethanol, together with increased immunorecognition of $p185^{erbB-2}$ on membranes (42). Thus some breast tissue should be fixed in NBF. One would expect that post fixation in alcoholic formalin would not produce false positive Herceptin tests.

Alcohol-formalin fixation or post-fixation is advantageous in large specimens with extensive fat (eg, breast specimens). Lymph nodes can be detected much more easily in specimens with alcohol formalin fixation due to the extraction of lipids and due to texture differences compared to tissues fixed in NBF.

When water is being added to ethanol fixatives, 95% ethanol can be used instead of absolute ethanol to save money. Just add 5% to quantity of ethanol and subtract the same amount from water that is added.

Alcoholic Formalin

ethanol (95%)	105 ml
water	895 ml
formaldehyde	100 ml

Alcoholic Formalin (buffered)

ethanol (95%)	105 ml
NBF	895 ml

Alcohol-Formalin-Acetic Acid Fixative

ethanol (95%)	85 ml
formaldehyde (37%)	10 ml
glacial acetic acid	5 ml

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Methanol may be substituted for ethanol; similarly various mixtures of ethanol/acetic acid/ and formalin may be used. For example a fixative for small specimens is:

Davidson's (Hartmann's) Solution	
ethanol (95%)	60 ml
formaldehyde (37%)	40 ml
glacial acetic acid	20 ml
water (tap)	60 ml

Two large drops of eosin may be added to the above solution to better identify small specimens after paraffin processing.

Alcoholic Bouin's (Gendre's Solution)

This fixative is similar to Bouin's except it is less aqueous and there is better retention in tissues of some carbohydrates (eg, glycogen). Fixation should be between 4 hr and overnight followed by washing in 70% ethanol followed by 95% ethanol (several changes). This is the one alcoholic fixative which improves upon aging (12).

Gendre's Solution	
95% ethanol saturated with picric acid (5gm per 100 ml)	800 ml
formaldehyde (37%)	150 ml
glacial acetic acid	50 ml

To increase the effectiveness of Alcoholic Bouin's if there is no time for aging, the following formula has been recommended (49):

Aged Alcoholic Bouin's	
picric acid	0.5 gm
formaldehyde	15 ml
95% ethanol	25 ml
glacial acetic acid	5 ml
ethyl acetate	25 ml
water	30 ml

Another alcoholic form of Bouin's solution is as follows:

stock Bouin's solution	75 ml
95% ethanol	25 ml

This solution is excellent for lymph nodes (24 hr) and for fatty tissue (48 hr).
A closely related fixative is Rossman's Solution.

Dichromate Fixatives

Miller's Solution	
potassium dichromate	2.5 gm
sodium sulfate	1 gm
DW	100 ml

Möller's or Regaud's Solution	
potassium dichromate	3 gm
distilled water	80 ml
at time of use add:	
formaldehyde (37%)	20 ml

Orth's Solution	
potassium dichromate	2.5 gm
sodium sulfate	1 gm
DW	100 ml
at time of use add	
formaldehyde (37%)	10 ml

Time of fixation (24 hr) is critical for dichromate fixatives; tissue should be washed after fixation and transferred to 70% ethanol. Without washing pigments may be precipitated. Very extensive shrinkage occurs when tissues are processed to paraffin blocks.

Lead Fixatives (See Special Fixatives)

Fixatives for Electronmicroscopy: Initial Fixatives

Probably the most commonly used initial fixatives for electron microscopy (EM) are formalin plus glutaraldehyde or 2% glutaraldehyde. Solutions of glutaraldehyde are susceptible to oxidation on storage. Convenient stock concentrations of glutaraldehyde (25%, 50%) can be prepared at pH 7.0 and stored in vial under a nitrogen blanket to prevent oxidation.

Formalin—Glutaraldehyde	
25% aqueous glutaraldehyde	10 ml
10% NBF	90 ml

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2% Glutaraldehyde

1) Prepare 0.2 M cacodylate buffer: dissolve 2.76 gm cacodylic acid (poisonous) in 70 ml DW. Add 0.1 N HCl to adjust pH between 7.2 and 7.4. Mix and add DW to make a final solution of 100 ml.
2) Prepare 50% aqueous solution of glutaraldehyde with DW
3) Prepare pH 7.3, 2% glutaraldehyde:

DW	46 ml
50% glutaraldehyde	4 ml
0.2 M cacodylate buffer	50 ml

Zamboni's PAF fixative

1) Prepare 150 ml saturated aqueous solution of picric acid using DW (3.15 gm picric acid to 150 ml) Filter twice just prior to use.
2) Prepare phosphate buffer:

water	1,000 ml
$Na_2HPO_4 \cdot 7H_2O$	33.77 gm
(or Na_2HPO_4 anhydrous)	(17.88 gm)
$NaH_2PO_4 \cdot H_2O$	3.31 gm

3) saturated aqueous — 150 ml
   paraformaldehyde — 20 gm

   Add the 20 gm of paraformaldehyde to 150 ml aqueous solution of picric acid (saturated) and heat to 60°, mix until paraformaldehyde dissolves. Add drops of 2.52% NaOH until pH is ≥7.0. Cool, filter.

4) Add phosphate buffer to solution of formaldehyde (produced by paraformaldehyde) and picric acid so that final volume is 100 ml.

A second step frequently listed in the fixation of specimens for EM examination is the use of osmium tetroxide. A phosphate-buffered saline solution is useful in preparing a solution of osmium tetroxide.

Phosphate Buffered Saline

DW	1,000 ml
monobasic sodium phosphate ($NaH_2PO_4 \cdot H_2O$)	0.663 gm
dibasic sodium phosphate ($Na_2HPO_4 \cdot 7H_2O$)	4.04 gm
sodium chloride	8.78 gm

Adjust pH to 7.4 with 1N HCl or 1N sodium hydroxide

Stock Solution of 2% Osmium Tetroxide

Obtain a sealed vial containing 1 gm of osmium tetroxide and wash it in DW under a hood; dispose of the wash water. Put the vial in a beaker of DW in a hood and boil it for 10 to 15 min. Under the hood, fill a second 250 ml very clean beaker with 50 ml of hot DW. Break vial with melted osmium tetroxide in second beaker and using plastic or Teflon coated instruments, wash osmium tetroxide from pieces of vial. Drain the 2% solution of osmium tetroxide into a glass stoppered brown bottle. Cover top of bottle with parafilm and store in refrigerator in a 1000 ml beaker sealed with parafilm.

No more than 2 days before the fixation of tissue in osmium tetroxide, add equal amounts (as needed) of phosphate buffered saline and 2% osmium tetroxide to prepare a working 1% solution of osmium tetroxide.

Prior to post-fixing in osmium tetroxide, wash the glutaraldehyde fixed tissue in phosphate buffered saline (PBS). Remove tissue and in a hood, add the tissue to fresh buffered 1% osmium tetroxide solution and fix for 1 hr. Rinse in PBS before embedding.

Special Fixatives

Lillie's Alcoholic Lead Nitrate Formalin

For connective tissue mucins and umbilical cord

DW	10 ml
formaldehyde (37%)	10 ml
absolute ethanol	80 ml
lead nitrate	8 gm

Fix for 24 hr at RT

For Metabolic Bone Disease

1) Prepare phosphate buffer:
   DW — 1,000 ml

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$NaH_2PO_4 \cdot H_2O$	1.104 gm
$NaHPO_4$ (anhydrous)	4.675 gm

2) Prepare fixative:

phosphate buffer	900 ml
formaldehyde (37%)	100 ml
adjust pH to 7.35	

For decalcification Bouin's Decalcifying Solution

saturated aqueous solution of picric acid (10.5 gm per 500 ml)	500 ml
formaldehyde (37%)	167 ml
formic acid	33 ml

For Fat Tissue

Bouin's solution	75 ml
95% ethanol	25 ml

May require up to 48 hr for good sections of lipomas or well differentiated liposarcomas

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