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A Brief Overview of the Role and Function of Fixative and Fixation: Review

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Abstract

Fixation is a physical chemical process that fixes tissues or cells chemically. Fixatives have many functions, one of which is to stop tissue putrefaction and autolysis. Formaldehyde, glutaraldehyde, glacial acetic acid, alcohol, mercuric chloride, potassium dichromate, osmium tetroxide, glyoxal, picric acid, Zenker,s solutions, Bouin,s solution and other fixative chemicals are used. A thorough search of the databases in Google Scholar, research gut, and Scopus revealed few papers on "fixation" and "fixative." A thorough overview of fixation and fixatives was written with this fact in mind. This review's main objective is to acquaint pathologists and lab personnel with the core ideas and different categories of fixatives.

Key words: Fixation, simple Fixatives, compound fixative, Formaldehyde, glutaraldehyde, , osmium tetroxide, picric acid, Review.

Introduction

Fixation is a physical chemical process that chemically fixes cells or tissues. The tissue or cell can therefore endure numerous chemical treatments with little morphological change. The perfect fixation includes a complex series of chemical processes. An excellent fixative is thought to impart tissue's mechanical toughness, preventing it from being destroyed by subsequent processing stages. It halts tissue autolysis, putrefaction, and deterioration of tissue constituents (1 and 2). Fixation should be able to maintain the tissue's architecture and cellular composition in an essentially lifelike manner.

Functional of fixatives

According to (1, and 2), a fixative's basic roles are to:

(a) preserve the tissue. (b) prevent diffusion. (c) protect the tissue from subsequent treatment. (d) The preservation of the connection between cells and extracellular molecules is another crucial function. Fixatives make the cell components intractable in order to maintain the cellular and tissue structure in a lifelike state. This lessens the alteration brought on by following therapy and protects the tissue from osmotic damage, which can induce swelling or shrinkage. (e) stiffening tissue to make gross cutting considerably simpler.

(f) accentuating the differences in refractive indices, which helps to make different tissue components more visible, and this helps to make the tissue more readily permeable for following reagents.

Since all tissues degenerate without this procedure, it is reasonable to conclude that appropriate tissue fixation for histological evaluation is essential to all histology studies. (3). When selecting a fixative, Each fixative's benefits and drawbacks must be considered in proportion. These include cellular organelle stability, molecular alterations or losses from "fixed" tissues, tissue swelling or shrinkage, variations in the caliber of histochemical and immunohistochemistry staining, the impact on biochemical tests, and variations in staining quality. Fixation's major objective in pathology is to keep morphological traits that are distinct and constant. (4). Generally, according to (5) the fixatives can be categorized in several ways, depending of the following:

1- Chemical compositions (Table 1).

2- number of fixed structures (Table.

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3- types of fixed structures (Table 3).

Table (1): lists fixatives according to their chemical composition.

No	Types of fixatives	Examples
1.	Agents of physical nature compounds	Microwaves and heat
2.	Aldehydes compounds	Formaldehyde, acrolein, glutaraldehyde
3.	Coagulants compounds	Acetic acid, ethyl alcohol, and methyl alcohol
4.	Compounds of Oxidizing agents	Osmium tetroxide
5.	Unrelated substances	Potassium dichromate, Picric acid, mercuric chloride

Table (2): classifies fixatives according to the number of structures they have fixed.

	fixatives	**es**
1.	Fixatives that are simple	Formaldehyde, picric acid, and osmium tetroxide, for instance
2.	Fixatives that are compound	such as Zenker's fluid, formol saline, and Bouin's fluid

Table 3 classifies fixatives according to the types of structures they fix.

No	Types of fixatives	Examples
1.	Histochemistry stabilizing agents	Vapor fixatives, glutaraldehyde, and formaldehyde
2.	Microanatomy stabilizing agents	Zenker's fixative, formol calcium, Bouin's fixative, 10% formalin, Helly's fluid, and Rossman's fixative
3.	Cellular stabilizing agents	Alcohol, formal saline, glacial acetic acid, Champy's fluid Fluids Carnoy's fixative, Clarke's fixative, Newcomer's, and Flemming's fixatives

To choose the right stabilizer, many factors must be taken into account, the chosen fixative works by reducing cellular and extracellular molecule loss or enzymatic degradation, protecting tissues from microbial damage and preserving macromolecular structures. This gives rise to one perspective of a living, dynamic tissue (5). To study the microanatomy of stained tissue slices, it is necessary to preserve the original microscopic connections between cells, cellular components (such as the cytoplasm and nucleus), and extracellular material with little harm to the tissue's organization. Additionally, it's important to preserve the tissue's local chemical profile (6). In order to adequately observe the microanatomy and microenvironment of these tissues, it is crucial to maintain the many tissue components that are soluble in aqueous solutions or other liquid environments during fixation and tissue processing, this is an important consideration. (7). Also In order to prevent the deterioration of macromolecular structures like cytoplasmic membranes, smooth endoplasmic reticulum, roughendoplasmic reticulum, nuclear

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membranes, lysosomes, and mitochondria, it is important to minimize the loss of cellular components such as large proteins, small peptides, mRNA, DNA, and lipids (8). When paired with the tissue processing methodology, Each fixative preserves various molecular and macromolecular characteristics of the cell well compared to other fixative/processing combinations. (9). Generally, If soluble components from the cytoplasm of the cells are lost, the color of the cytoplasm on hematoxylin and eosin (H&E) staining will be reduced or altered, and characteristics of the look of the microanatomy of the tissue, such as mitochondria, will be lost or damaged. Structure and function immunohistochemical evaluations may also be compromised or destroyed. (10). In addition to its initial interactions with the tissue in its aqueous environment, a fixative has ongoing reactivity with any unreacted fixative as well as the chemically altered tissues. Fixation affects all stages of processing and staining, including dehydrating tissue slices and staining them with histochemical, enzymatic, or immunohistochemical stains (11). As a result, any stained tissue section that is created after precise fixation and tissue processing has fixed tissue alterations, that were created from the original living tissue.

Fixation and Fixatives: Influential Factors

a- *Fixation time duration:* Depending on the size of the tissue, different time spans are required for fixation (Thinner tissues take less time to process than heavier tissues). If the fixing time frame is extended, the tissue shall become brittle owing to cross-linking. Shortening the fixation time results in decreased penetration of fixatives into tissues and no cross-linking. For maximum tissue samples, overnight fixing is preferred.

b- *level of fixative concentration:* The optimal concentration of various fixatives is found empirically. Low fixative concentrations cause fixation to take longer, and high fixative concentrations cause fixation to take longer and damage cellular structures and destroy enzyme functions.

c- *The degree of osmolarity:* If the osmolarity of the fixative and the tissue are the same, it will prevent tissue swelling or shrinkage.

d- *Ttemperature in the laboratory:* The rate of fixation is increased when the temperature is raised to an optimum (37 - 45 C^0), otherwise, autolysis occurs. When the temperature is reduced, the diffusion rate decreases, resulting in a longer penetration time.

e- *Size of specimens:* One of the key elements in fixing is tissue thickness. For optimal penetration, a specimen should be between 4 and 6 mm thick. An excessively large sample size prevents a fixative from penetrating the tissue's deeper layers, which causes autolysis. (12).

Types of fixatives for Histology and Histopathology:

a-Fixatives that are simple structure:

1- Formaldehyde or Formalin (10%): Butlerov discovered formaldehyde in 1859. Ferdinand Blum realized in 1892 that formalin could be beneficial when employed as a fixative(13 and 14). Formaldehyde is the most often used fixative in pathology in a 10% neutral buffered form. The interaction between tissue macromolecules and formaldehyde tends to be complex (15). By weight, formalin contains (37- 40)% of formaldehyde and (60-63)% of tap water. After long durations of continuous storage, white deposits accumulate in the solution. These are paraformaldehyde precipitates. It is possible to prevent these white deposits by keeping formalin at moderate temperatures. In order to slow down the polymerization reaction that leads to the paraformaldehyde precipitate, 10% methanol can be added to the formalin. There are also a few formate ions present. The outcomes of the Cannizzaro reaction are as follows, two molecules of formaldehyde react together in this process. One molecule condenses to make methanol, while the other is oxidized to form formic acid.14 Because of formic acid, the solution is acidic in reaction, although the acidic nature of the solution can be balanced (16 and 17).

2-Methanol and Ethanol: The coagulants methanol (CH3OH) and ethanol (CH3CH2OH) are thought to denature proteins. They act as a substitute for water in the tissue environment, disrupting hydrophobic and hydrogen bonds in the process, exposing the internal hydrophobic groups of proteins, altering their tertiary structure, and changing how soluble they are in water. Generally, Methanol interacts with hydrophobic regions less strongly than ethanol because it is more similar to the structure of water. While fixation begins at a concentration of 50–60% for ethanol, and does so at >80% for methanol. Although ethanol can occasionally be used to keep glycogen in place, it will change cytoplasmic and nuclear information. Usually, both alcohols coupled with additional reagents when used as

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fixatives for tissue specimens, however, 95% ethanol and methanol are often used as fixatives for cytology smears and blood films, respectively. When used in conjunction with other fixatives, such as Carnoy's fixative, they penetrate slowly, harden and shrink the tissue effectively, and penetrate quickly when another fixative is present. Because it maintains particular proteins in a condition that is largely undenatured, ethanol is used to identify specific enzymes so that they can be employed in immunofluorescence or other histochemical techniques. Because it is a good fat solvent, ethanol dissolves fats and lipids. Methyl alcohol is used to repair bone marrow and blood film stains (18, 19 and 20).

3- Acetone: Acetone (CH3COCH3), which acts similarly to alcohol and has been used in tissue processing as a fixative and dehydrator, is especially useful for small specimens that need to be processed quickly by hand. For fixation as part of the histochemical demonstration of enzymes, it is frequently used cold (4 °C) and is highly advised for this application. It is a quick-acting, strong lipid solvent that can make tissues brittle. Due to its high volatility and flammability, it is normally not used in mechanized tissue processors (19 and 20).

4- Mercuric Chloride (HgCl2): Additionally, mercuric chloride can be employed to fix tissues for histopathology. Tissue hardness is the result of its main reaction with cysteine as well as reactions with amines, amides, sulfhydryl groups, and ammonium salts. Additionally, it reacts with nucleic acid phosphate residues and fixes the nucleoproteins appropriately. These factors lead to the observation that mercuric fixatives make up the majority of several fixatives, including Helly's fixative and b-5 fixatives (2 and 18).

5- Picric acid: Trinitro phenol, often known as picric acid, is a yellow, crystalline substance that should be moistened with water before storage to prevent the risk of explosion. It is always combined with additional agents, such as Bouin and Hollande's solutions, for fixation. In various stains, such as Van Gieson's solution is used to stain muscle and as an acid dye. since of its acidic nature, residual picric acid should be eliminated from tissues with 70% ethanol before processing since it causes tissues to turn yellow after fixation. As a coagulant fixative, it modifies the charges on proteins' ionizable side chains and disrupts electrostatic and hydrogen connections. It coagulates by generating salts (picrates) with basic groups of proteins. It does not repair lipids or the majority of carbohydrates, but it does repair granulose of glycogen. Picric acid should be avoided when determining DNA or RNA since it hydrolyzes nucleic acids. t is not used alone because shrinkage happens when processing tissues fixed with chemicals that contain picric acid. It penetrates nicely and binds quickly. It precipitates proteins, which it then mixes with to produce picrates. Because some picrates are water-soluble, they must be treated with alcohol before being used in regions where tissue will come into contact with them. (21).

6- Potassium dichromate: Potassium dichromate is a noncoagulant fixative as well, but it transforms into a coagulant fixative when mixed with an acid solution because chromate ions will link with certain lipids and leave them insoluble. Chromium appears to react with both hydroxyland carboxyl groups. The affinity of tissues for eosin staining rises as the percentage of reactive basic groups increases. It maintains mitochondria but destroys DNA. Tissues that have been treated using chromate fixatives should be thoroughly washed in water before further processing. This step is critical because it prevents the formation of insoluble chromate suboxide (4).

7- Osmium tetroxide: Osmium tetroxide is a fixative that is soluble in nonpolar solvents as well as water. Osmium tetroxide seems to interact with the side chains of proteins to cause cross-linking. Disulfide, carboxyl, hydroxyl, sulfhydryl, amide, and other groups are among the reactive groups of osmium tetroxide compound. During osmium tetroxide obsessive, large amounts of carbs and proteins are excreted, due to the slow rate of reaction or the osmium tetroxide's insufficient tissue penetration. Osmium tetroxide is used in electron microscopic study as a secondary fixative. It functions as well as a stain and adds contrast when viewed through an electron microscope. Lipids in frozen sections can also be stained with osmium tetroxide. It has been discovered that osmium fixation. It is typically sold as a solid that is crystalline encased in a glass ampule. Osmium tetroxide crystals can be seen transitioning from a solid to a vapor state. Continued prolonged exposure to osmium tetroxide fumes can cause deposition within the cornea, which can lead to blindness. Osmium tetroxide (OsO4) is a very toxic, crystalline substance that dissolves in both polar and non-polar liquids. It is one of a few fixatives that fixes lipids and fats, such as Myelin, by binding to them with unsaturated lipid and phospholipid linkages. It is converted to oxides during the fixation process, which are stored in tissues as insoluble black particles, specific to membranes. Being a heavy metal, osmium compound scatters electrons and so adds electrons. Additionally, it might be utilized being an en bloc stain for light microscope-level demonstrations of lipids, particularly myelinated nerve fibers (22 and 23).

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8- Acid Acetic: A non-coagulative fixative agent is acetic acid. It works by triggering the nuclear protein coagulation. Additionally, It stabilizes and aids in the prevention of nucleic acid loss. When used in conjunction with ethanol, acetic acid works effectively as a cytological fixative that assists in nucleic acid preservation, but, when used alone, it causes cell swelling. Because acetic acid penetrates tissues more quickly than other chemicals, the time required for acetic acid stabilization is less time-consuming. (24).

9- Glutaraldehyde (C5H8O2): Glutaraldehyde was first identified in 1963 by Sabatini et al. as a special fixative for ultrastructural research. The two aldehyde groups that jointly make up glutaraldehyde are connected by three methylene bridges. Although glutaraldehyde's penetration rate is reported to be slower than formaldehyde's, In addition to inhibiting enzyme function, compared to formaldehyde, glutaraldehyde is a more effective cross-linker for proteins. (25). Glutaraldehyde in an aqueous solution polymerizes to generate oligomeric and cyclic molecules as well as glutaric acid by oxidation. It needs to be stored at 4°C and have a pH of 5 to be stable (26). The nucleic acids cannot be cross-linked by glutaraldehydes while they are at room temperature. Due to its limited absorption and possibilities of overhardening, glutaraldehyde isn't employed as a tissue fixative regarding light microscopy, despite the fact that Since it protects the tissue's ultrastructure, it is employed in studies involving electron microscopy. Glutaraldehyde becomes unstable when exposed to air and degrades with a drop in pH. When exposed, glutaraldehyde can serve as a sensitizer and irritate the digestive, respiratory, and skin systems (2).

b- Fixatives that are compound structure: These are blends of various fixatives blended in a certain ratio such that the drawback of one fixative is offset by the use of another fixative and requires less time to fix. Each of these compound fixatives has benefits and drawbacks of its own. The three main categories of compound fixatives are as follows. Fixatives for cytological, histochemical, and microscopic anatomy (27).

1- Cytological fixations: The components of the cells are preserved using cytological fixatives. They can be further split into two groups based on their mode of action, as shown in (12):

1-a- Nuclear fixatives: Fixatives that function primarily on the nuclear structure of the cell are known as nuclear fixatives. Examples include Carnoy's fluid, Clarke's fluid, etc.

1-a-1- Carnoy's fixative: It has good nuclear preservation, acts quickly, and keeps glycogen in place. Glycogen and Nissl's granules are still present. In addition to dissolving lipids and lysing erythrocytes, it can also cause excessive hardness and shrinking. As for chemical composition: (60ml) Absolute ethanol, (30ml) Chloroform solvent, and (10ml) Glacial acetic acid.

1-a-2- New Comer's fixative: For the preservation of chromatin, Newcomer's fixative, which includes dioxane, propionic acid, and isopropanol, proposed is a substitute to Fixation by Carnoy's. Additionally, it helps polysaccharides be fixed. Small tissue pieces should be utilized for mending. Fixation is finished within 12 to 18 hours. As for chemical composition: (60ml) Isopropanol, (40ml) Propionic acid, (10 ml) Petroleum ether, (10ml) Acetone, and (10ml) Dioxane (28).

1-a-3- Clarke's fixative: has been applied to smears and frozen parts. After normal processing, can yield acceptable results if fixation time is kept to a minimum. conserves nucleic acids yet extracts lipids. rapid, effective nuclear fixing, and effective cytoplasmic element preservation. It is fantastic for cell culture smear or cover slip preparation, as well as chromosomal analysis. Transferring tissues into 95% ethanol is possible. It takes 3–4 hours to fix. As for chemical composition: (75 ml) Absolute alcohol, and (25ml) Glacial acetic acid (12).

1-b- Fixatives for the cytoplasm: They are the fixing agents which concentrate on fixing the cytoplasmic components of the cell. For instance, Zenker's fixative, Champy's fluid, etc.

1-b-1- Champy's solution: For a while, the fluid is dependable and stable. When cytological preservation is the primary concern, it is appropriate for usage. Because this fixative cannot be stored, it must be made fresh. The lipids and fat in the mitochondria are preserved. It has a weak and inconsistent penetration power. The thickness of the tissues must not exceed 3 mm, and they should ideally be no more than 1-2 mm. Pieces longer than 2 millimeters should be fixed within 24 hours after being fixed overnight. To get rid of chromium and osmium compounds, After treatment, the specimen must be rinsed in running tap water for a whole night. The chemical make-up is: (1g) Chromium trioxide, (3g) Potassium Dichromate, (1g) Osmium tetroxide, and (250ml) Distilled water (29).

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1-b-2-Formal saline fixative: Standard saline Before the development of phosphate-buffered formalin, this formaldehyde in isotonic saline solution was frequently employed for standard histology. Formalin pigment is often produced. Generally, fixation lasts between 12 and twenty four hours. chemical composition is: (100ml) 40% Formaldehyde, (9g) Sodium Chloride, and (100ml) Distilled water (30).

1-b-3- Formal Calcium fixative: Recommendable for preserving lipids, particularly phospholipids. Fixation lasts between 12 and 24 hours. As for chemical composition: (100 ml) 40% of formaldehyde, (10g) Calcium Chloride, and (900 ml) distilled water (3).

2- Histochemical fixatives: They are utilized in histochemical examinations of tissues when it is necessary to make little to no modifications to the components that need to be proven. For instance, buffered formalin or vapor fixatives like acetone, acrolein, and formaldehyde vapour (21).

2-1- Acetone: Mention the details of this stabilizer in the paragraph of the stabilizers with simple installation above.

2-2- Acrolein /chromyl chloride fixative: It is employed for about two hours at 37 °C. As a major fixative agent, Luft invented acrolein, a three-carbon unsaturated monoaldehyde. Acrolein offers outstanding structural detail retention and maintains viral antigenicity. It also goes by the name acrylic aldehyde. Reversible cross-links are created as a result of their reaction with macromolecules. Acrolein is not widely used since it is unstable at alkaline pH and produces insoluble polymers. Due to its strong reactivity, acrolein is shown to quickly permeate tissues. The main application of acrolein fixatives in enzyme histochemistry (31).

2-3- Formaldehyde Vapour fixative: Paraformaldehyde is heated to a temperature of between 50 and 80 °C to produce of Formaldehyde Vapour. Sections take between one and two hours, whereas tissue blocks take between three and five (31).

3- Micro anatomical fixatives: are intended to maintain the proper anatomical structure of the tissues as well as the link between the tissue layer and big cell aggregations. For everyday work of normal and histopathological examinations, these fixatives are employed. For instance, Zenker's fluid, Bouin's fluid, buffered formalin, etc.

3-1- Buffered formalin fixative: The fixative works well as a standard fixative. Proteins and aldehydes are thought to fixate and form a gel that keeps biological elements interacting with one another in vivo. Once the tissue has been properly fixed, it should be able to withstand the future stages of tissue processing or staining. It has been used to fix tissues as a common all-purpose histology fixative. Its chemical make-up is as follows: 10ml formalin, 0.4g monohydrate acid sodium phosphate, 0.65g anhydrous disodium phosphate, and 100ml water (32).

3-2- Bouin's fixative: Noncoagulant picrate fixative solution is what it is also known as, and Pol Andre Bouin first described it in 1897. Bouin's fixative is regarded to be an effective fixative for preserving delicate and soft tissue structures. Picric acid makes up the majority of Bouin's fixative, with only a small amount of acetic acid and formaldehyde. Bouin's solution cannot be used in samples where in situ hybridization is required since it lessens the intensity of the hybridization. Bouin's fixative has the following chemical components: a) 75 ml of saturated aqueous picric acid; b) 25 ml of formalin; and c) 5 ml of glacial acetic acid (33).

3-3- Fixative of Heidenhain Susa: they are a decalcifying chemical solution and have a quick penetration that is regarded as a good all-purpose fixative. It provides excellent staining and cytological detail and is a great fixative for routine biopsy procedures. provides quick results, uniform penetration, and minimal shrinking. It must have tissue in it for longer than 24 hours. If there are any imperfections in the mercury pigment, the tissue should be treated with iodine. The chemical makeup is as follows: 100 ml of distilled water, 2.0 g of trichloroacetic acid, 2.0 g of sodium chloride, 4.0 ml of acetic acid, and 4.5 g of mercury chloride.

3-4- Zenker's fluid fixative: a fixative solution made of sodium sulfate, glacial acetic acid, corrosive mercuric bichloride, potassium bichromate, and water. If an iodine solution isn't used to dissolve the mercury precipitate, it will remain in the tissues and cause problems for the inexperienced microscopist. The modifications made to this fixative according to Maximow, Helly, and Custer, in which solution of formalin is employed instead of acetic acid, are the most popular. It provides reasonably rapid and even penetration and is a decent routine fixative. Because acetic acid (or formalin) does not stabilize it, it should only be applied right before use. To get rid of extra dichromate, tissues need to be washed under running water. The chemical makeup is the following: 5 g mercuric

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chloride, 2.5 g potassium dichromate, 1 g sodium sulfate, 100 ml distilled water, and mix well before use. 5 mL acetic acid, glacial (3).

3-5- Gendre's fixative fluid: It alcohol-based Bouin solution seems to get better over time. It is highly suggested for glycogen and other carbohydrate preservation. Following fixation, the tissue is immersed in 70% ethanol. Before staining, the residual yellow hue should be wiped away. Chemical composition contain of (80ml) of saturated picric acid in (95%) alcohol, (5ml) of Glacial acetic acid. And (15ml) of Formalin.

Conclusion: In histology and histopathology, fixation is an essential stage. Each fixative has its own set of advantages and disadvantages. Numerous fixatives serve specialized functions, and a variety of variables, such as size, concentration, temperature, and osmolarity, have a direct impact on the fixation process.

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