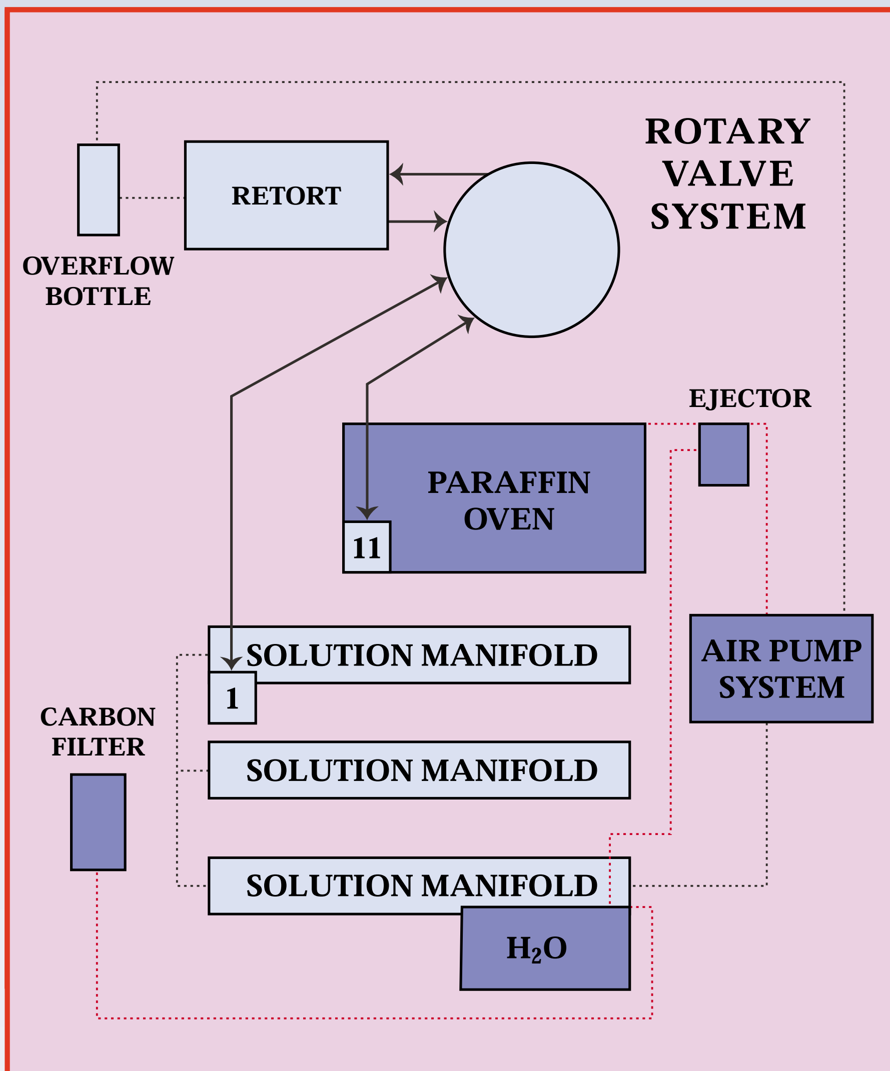

paraffin oven. The air and solution pathways are shown in the flow diagram, dashed lines representing air flow, solid arrows representing fluid flow. Colored components are the ones involved with the paraffin degassing cycle.

To prolong the useful life of the paraffin, the Tissue-Tek® VIP™ air pump applies pressure through the oven ejector (formerly called aspirator in older VIP models). As air is forced across the ejector, a vacuum is developed in the tube that connects to the oven. The clearing agent gases are therefore ejected out of the oven and channeled through the water filter and to the activated carbon filter. Depending on the operating modes, paraffin cleansing is performed at different times during every automatic processing run. For example, degassing occurs at the end of every pump-in or every time a P/V cycle is completed. This ensures that clearing reagent vapors are removed periodically and thus, do not have the time to recondense and return into the paraffin. Since the process is automatic, there is no need to perform an off-line degassing procedure and the associated extra maintenance.

Formaldehyde: Past, Present & Future

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For more than a century, formaldehyde has been the most widely used fixative in all fields of histology except electron microscopy. In part, this has been because formalin, the aqueous solution of formaldehyde, has been the simplest, least expensive option. So-called "special" fixatives like Zenker's, Helly's, Bouin's, and many others contain expensive components, have short shelf lives, require limited exposure times for the specimens, or necessitate extra steps in the staining protocol to remove unwanted precipitate. Never mind that almost any of these produce striking morphological patterns or more vibrant colors. Cost and simplicity always win, whether today or a hundred years ago.

Keep in mind that before about 1980, the pace of clinical labs was far more relaxed than it is today. Specimens arriving in the morning were fixed all day and part of the night, while those arriving later in the day simply were held over until the following evening's run on the processor. Many labs followed recommendations in the leading textbooks for 24-48 hours minimum fixation.[1,2] There was little rush for a diagnosis; when time was critical, frozen sections were the norm.

Formalin was king because it worked so well. In the times then allotted for fixation, beautiful preparations were routinely achieved on a consistent basis; in fact, they were expected by the "old masters" like Ham, Maximow, Bloom, and Fawcett, authors of standard textbooks of histology. Nuclear chromatin patterns depicted in their books were clearly defined and delicately speckled. Counterstain colors varied in shade

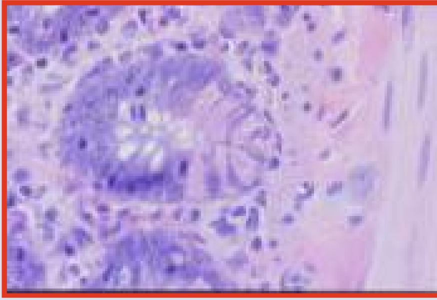
Fig. 1. Small intestine, NBF. 50x

according to tissue type (Fig. 1). Trichromes were really superfluous for many cases, but time was available and such special stains were ordered on nearly everything (or so it seemed).

Fig. 2. Formaldehyde. Carbon is gray, hydrogens are black, and oxygen is green.

Chemically speaking, formaldehyde's virtue is that it complexes with so many end groups on most tissue macromolecules. This is due to its reactivity. An aldehyde group (double-bonded oxygen) on a molecule with only one carbon atom (Fig. 2) is unstable (has high energy) and will attach to any other group that will lower that energy. Specifically, formaldehyde binds to sites containing a reactive hydrogen atom, including amides, amines, hydroxyls, peptides, sulfhydryls, and possibly carboxyls.[2]

Hydrogens can share their lone electron with other atoms. Carbon shares the electron equally with hydrogen, which is to say that the electron spends half its time around each atom. However, nitrogen, sulfur, or oxygen do not share that electron equally, with the result that hydrogen acquires a partial charge of about +0.3. This is a reactive hydrogen. It can be pulled off completely during covalent bonding, or can remain in place to engage in hydrogen bonding.

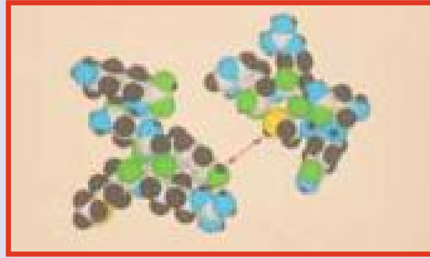
Fig. 3. Two polypeptides representing tissue macromolecules. Nitrogen is blue and sulfur is yellow. The double-faced arrow marks the points of attack by formaldehyde in subsequent modeling.

I have followed the course of fixation by formaldehyde through the use of molecular modeling software.[3] For simplicity's sake here, we'll model the fixation and cross-linking of small polypeptides which will serve to represent adjacent tissue macromolecules (Fig. 3). While both have numerous potential reaction sites (active hydrogen atoms), we will use only the two marked with arrows. Each sphere indicates the size of the electron cloud around each atom. Note that hydrogen atoms attached to nitrogen, oxygen, and sulfur are smaller than those attached to carbon: these are active hydrogens. In subsequent figures, colors will be dimmed except for atoms germane to the discussion.

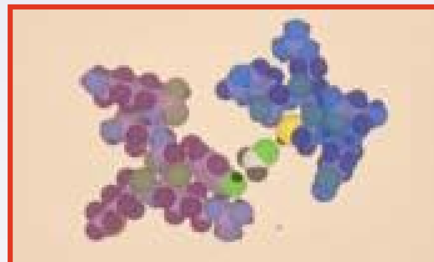
Fig. 4. Penetration of formaldehyde into the tissue. Only those atoms important to the rest of the discussion retain their original color coding; others are dimmed, although each polypeptide will be identifiable.

Step 1 in fixation is penetration (Fig. 4). Formaldehyde then attaches to an active site, a hydroxyl (-OH) group (Fig. 5). In a musical chairs sequence, formaldehyde's carbon replaces the active hydrogen which then joins formaldehyde's oxygen. This newly created hydroxyl group on formaldehyde's carbon is also reactive (note how small the hydrogen atom appears), and will combine with a second reactive hydrogen if one is conveniently available (Fig. 6).

We now have crosslinked molecules. In this final bridging reaction, the hydroxyl group of the former formaldehyde molecule and the reactive hydrogen on the sulfur atom split off to form a water molecule. What remains of the original fixative is a single carbon atom (with two hydrogens, only one of which is visible in the model) bridging the gap between the tissue molecules. This is called a methylene bridge (methyl denotes one carbon atom).

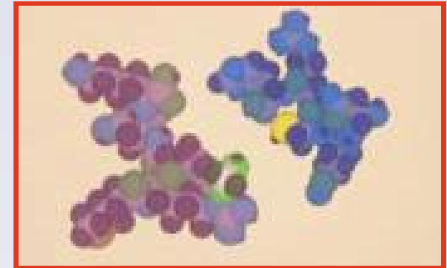
Fig. 5. Simple addition of formaldehyde onto a hydroxyl group in the first polypeptide. A hydrogen atom is transferred from the hydroxyl to formaldehyde's oxygen.

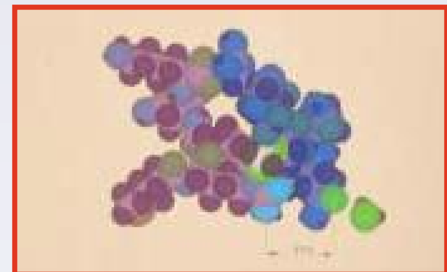
Fig. 6. Crosslinking. A water molecule is given off in this reaction (lower right). Note the spacing between the marked amino (blue and black) and carbonyl (green) groups.

The software lets me draw the structures any way I want, even if the conformation in three-dimensional space is not natural. The computer has been programmed with preferred lengths and angles for each type of bond, however, and can correct any shortcomings in my depictions. As initially drawn, some atoms may be in the way of each other. Neighboring atoms may have like charges that set up repelling forces or opposing charges that cause attractions. The computer considers all of these spatial and electrical needs, balancing them with optimal bond angles and lengths. Through a complex iterative process, it finds the most comfortable position for the population of atoms within the

molecule. In the end, calculations arrive at the best conformation, e.g., the one most likely to exist in nature.

Fig. 7. Molecular rearrangement to minimize energy. Compare the position of the amino and carbonyl here and in Fig. 6.

To appreciate this in our example, carefully compare the position of the blue polypeptide in Figs. 6 and 7. I have kept the violet polypeptide stationary. Figure 6 depicts the crosslinked polypeptides before optimizing the conformation. Below the methylene bridge are two primary amines on the violet polypeptide, each consisting of a nitrogen (blue) with two reactive hydrogens. The latter bear a +0.3 charge. Farther to the right is a carbonyl, an oxygen atom (green) that is double-bonded to the blue polypeptide. It carries a partial charge of -0.5. The distance between the farther nitrogen and the oxygen is 6.3. Charges and distances from atomic centers are determined by the software.

Now look at Fig. 7. Attractive forces between the negative carbonyl and positive amines have drawn the polypeptides together, closing the gap to 2.8. This produces several subtle, but important changes. First, the partial charges at the aforementioned sites have been essentially neutralized. This alters the charge characteristics of the molecule. Second, two hydrogen bonds have formed. Although these are weak, they do supplement the methylene crossbridge and thus, strengthen the overall connection joining the formerly independent polypeptides. The molecule has become more rigid. Third, shrinkage of the overall tissue occurs as parts of molecules move together. Finally, the former polypeptides have changed shape, and not simply by joining together. Portions are twisted relative to the original conformations. Because microbial and endogenous enzymes depend upon key-in-lock fits to their substrates, decay and autolysis are inhibited. In short, the specimen has been denatured, or fixed.

In reality, fixed molecules are joined by many crossbridges and even greater numbers of other types of bonds (ionic, hydrogen, and van der Waal's attractions) than I have shown in this simple polypeptide model. This produces denaturation on a grand scale. Keep in mind, however, that crosslinking is a slow process, and there are many other ways to effect denaturation. Formalin-induced denaturation is considered good for morphology, but bad for histochemistry involving enzymes and immunologic reagents. Heat and solvents like alcohol are also effective denaturants, but they change molecular shape differently, which usually results in poor morphological patterns.

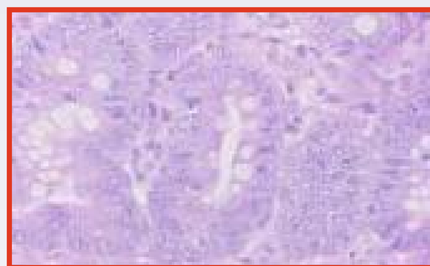

Fig. 8. Bubbling artifact in both nuclei and cytoplasm due to mildly inadequate fixation in NBF. Colon. 50x

Tissue molecules fixed in formalin for 24-48 hours acquire so many crosslinks and other connections that they are unable to be altered by other chemical or physical agents normally encountered in processing and staining. In contrast, weakly fixed specimens can be re-denatured by alcohol and heat, which is why so many tissues today show striking artifacts. Nuclear and cytoplasmic bubbling (Fig. 8) occurs when chromatin is moderately fixed (7-24 hours) in formalin, then further denatured during the rest of processing. Less well-fixed nuclei (0-7 hours in formalin) suffer greater alteration from alcohol and heat: chromatin patterns are lost altogether, with some nuclei taking on odd colors (Fig. 9). Because the daily run of specimens in a lab may include those with little, if any, fixation as well as tissues with a day or more of formalin exposure, variation in the appearance of the slides is rampant through no fault of the stains or the technicians.

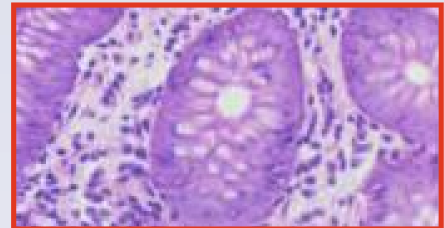

Fig. 9. More severe damage from inadequate fixation in formalin. Intestinal biopsy. 50x

Today there is an intense push for faster turnaround of specimens. Laboratories have sacrificed fixation time to gain this speed in utter disregard for the chemistry of formalin fixation. Unfortunately, the reality is that few histopathology facilities have the luxury of 24-48 hour fixation. Clearly, neutral buffered formalin is no longer suitable for our needs from that standpoint alone, never mind safety issues and immunohistochemistry.

Formalin is one of the worst fixatives for immunohistochemistry. True, most immunostaining is performed on formalin-fixed tissue, but look at the lengths to which we go to get results. The literature on reclaiming antigenicity is overwhelming, with enzymes and heat vying for preferred status. Even the method of heating and the attendant retrieval solutions are highly diverse. No one method is best for everything. Different antibodies require different techniques, and different labs often have to use different methods for the same antibody because of local variations in fixation. I have always wondered why we spend so much time trying to save a ruined specimen when it is so easy to get excellent structural preservation and retain full immunoreactivity. Said another way, why compromise a specimen with formalin fixation just

because we have procedures that might retrieve the antigens? We use these techniques to avoid false negative reactions but sometimes we create false positives instead.[4]

When OSHA first initiated the Formaldehyde Standard in the United States, all attention seemed to be riveted on the carcinogenicity of this chemical. It is a human carcinogen, let no one doubt that. The Preamble to the Standard details the evidence. However, that is not the health risk that histotechnologists need to be most worried about. Formaldehyde is a potent sensitizer, whether through inhalation exposure or skin contact. Did you know that 79% of histotechs have dermatological and respiratory symptoms attributable to formaldehyde exposure?[5] They get colds more frequently, and that last longer, than non-exposed individuals. Their incidence of asthma is higher. They suffer from dermatitis. Sensitization is an immunological condition that lasts for life. In a world where formaldehyde vapors come out of carpets, wall coverings, draperies, furniture, and many building materials, the sensitized individual is going to have a rough time.

Are there satisfactory substitutes for formalin? Definitely yes! Any of the zinc formalin solutions eliminate the technical problems, although the safety concerns are still present. Immunoreactivity is fully preserved even after months of storage in zinc formalin. Homemade and commercial products abound, with unbuffered, buffered, and alcoholic buffered versions available. Zinc formalin in some form or another has been used widely since the early 1980s, and there is an impressive body of literature on the subject.[6]

Finding a completely different alternative to formaldehyde has been more challenging. Ideally, the new fixative should create morphological patterns similar to those seen with excellent formalin fixation, but immunoreactivity should be retained. Human and environmental safety factors must be favorable. If you scan the list of fixative components in any textbook of histotechnology or histochemistry you will find none that meet these criteria. Clearly a major breakthrough was needed to bring histological fixation into the twenty-first century. In actuality, the breakthrough was made several times with a variety of unrelated chemicals, all but one contained in proprietary mixtures. Some of these do not create fixation patterns quite like formalin because they are not aldehyde and may not be fixatives in the true sense, as much as they are effective transport solutions. Since the identity of their ingredients remains secret, we cannot divine their modes of action nor really understand their limitations.

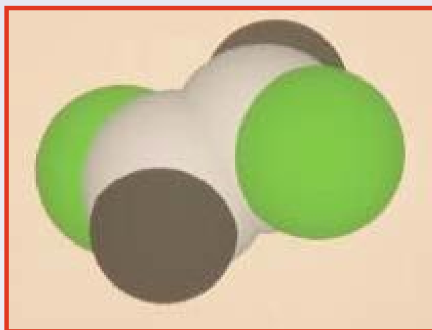

Fig. 10. Glyoxal.

The chemical that has surfaced as the one most likely to replace formaldehyde is glyoxal, a very close relative containing 2 carbon atoms and 2 aldehyde groups (Fig. 10). It adds onto the same reactive tissue groups but with greater speed. Crosslinking is either prevented despite the second aldehyde, or the 2-carbon crossbridge is too long to block immunoreactivity. Curiously, glyoxal essentially has no vapor pressure, which is why it does not evaporate or give off fumes. Inhalation risk is therefore reduced to zero, certainly below any known conditions of use in histology, including microwave fixation. Drain disposal is almost always permitted by local authorities because of glyoxal's low level of ecotoxicity. A number of vendors offer both ready-to-use solutions and concentrates. After being on the market for 8 years, glyoxal fixatives have created an impressive record. Deservedly, they have become the most widespread alternatives to formalin in the United States.

Short fixation times with glyoxal do not produce the artifacts that are so common with formalin. Biopsies can be fixed quite adequately in 45-60 minutes[7] and superbly in 2 hours. Surgicals take 4-6 hours after grossing.[8] Alcoholic versions, available from some vendors, work even faster.

For the ultimate in speed, consider microwave fixation in an appropriately vented and temperature-controlled appliance, using either the conventional glyoxal fixatives or those created especially for microwave applications. Initial fixation at the point of specimen origin can be in any common fixative, including one of the glyoxal solutions. Non-glyoxal fixatives should be rinsed out by soaking grossed specimens for 20-30 minutes in the microwave fixative at room temperature. Microwave in the glyoxal solution for 4-16 minutes and you are ready to process.[9] Talk about reducing turnaround time!

Formaldehyde has had a glorious past but a troubled present. It now faces a rather dim future. Change comes slowly, especially in our field, but it is time to move on. This is just one more example of how dynamic histotechnology is, and why it is exciting to be a part of it.

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