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Review Article

# Formaldehyde Fixation

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The use of formaldehyde as a biological reagent occurred rather late in the history of histological methodology. The reason for this lay not in any lack of astuteness on the part of pathologists and histologists, but rather with the late development of formaldehyde manufacture by the chemical industry. While formaldehyde was discovered by Butlerov in 1859, practical aspects of the manufacture of this, the simplest aldehyde, only emerged in 1868 when Van Hoffman, as an academic exercise, developed a practical method for synthesis from methanol, and further established its properties. First production of formaldehyde as an industrial reagent occurred after the patent issued to Trillat in 1889, who in turn licensed several firms in France and Germany for manufacture (27).

One such firm was Meister, Lucius and Brunig, located at Hoechst am Main, near Frankfurt am Main. While this firm, later to become one of the giants of chemical manufacturing under the name Hoechst, probably did not manufacture formaldehyde on any appreciable scale until 1891 (27), there was considerable interest in developing uses for formaldehyde. One likely possibility was in finding medical applications for aqueous solutions of formaldehyde. There had been reports from France that aqueous formaldehyde could be used as an antiseptic, either to treat or prevent wound infections. At that time, only a relatively few antiseptic agents were available and most of these were highly toxic and corrosive to tissues and instruments alike. The possibility that formaldehyde solutions might provide a "nontoxic sublimate" was a desirable goal that was not lost on Meister, Lucius and Brunig. In 1892 the firm approached a young physician in Frankfurt with the proposal that he test the antiseptic properties of formaldehyde.

This young man, Ferdinand Blum, was a native of Frankfurt, born 3 October 1865. He attended the Universities of Kiel, Munich, Heidelburg, and graduated in medicine from Freiburg. Following graduation, and in the fashion of the time, he worked in several clinics, including the mental hospital at Schloss Marbach and the gynecologic clinic at Freiburg. On his return to Frankfurt he began a remarkable career, beginning with the project with Meister, Lucius and Brunig (14).

His approach to this assignment was usual for the time. Formaldehyde was supplied by the manufacturer in a 40% aqueous solution, which is the concentration resulting from bubbling formaldehyde gas through water until no more will dissolve. Blum diluted the commercial solution for testing. The dilution he chose was a decimal one, or one part of commercial reagent diluted with nine parts of water to give a 4% weight/volume solution. He tested the bacteriocidal properties of this dilution against several bacterial species, including *Bacillus anthracis*, *B. typhi*, *Staphylococcus aureus*, and *Proteus* sp. The results of these experiments showed that formaldehyde was an effective but slow agent for killing bacteria (3). An incidental finding of this research was to have a far broader use and was reported by Blum in a second paper a few months later.

In his second paper on formaldehyde, Blum reported that in the process of studying disinfection, he noticed that the skin of his fingers that had come in contact with the diluted solution became hardened, much as with alcohol, then one of the commonest methods for hardening tissues for histological processing. When he examined the tissues of an anthrax infected mouse preserved in formaldehyde, he found that the tissues had the same consistency as alcohol hardened or "fixed" tissues. When tissue samples were prepared for histology after formaldehyde treatment, excellent staining results were obtained using common staining methods of the time, such as hematoxylin and the analine dyes. The famous Frankfurt histologist, Wiegert, consented to examine some of Blum's preparations of various organs and found them entirely suitable for critical use. Better still, formaldehyde produced only marginal shrinkage and distortion of tissues, unlike alcohol fixed tissues (4,5).

An account of formaldehyde would not be complete without some further information about Ferdinand Blum. After two initial papers about the use of formaldehyde, Blum was launched on a remarkable career in experimental medicine. By 1896 he was able to cite over 50 references to the use of formaldehyde by investigators in all areas of biology and medicine (6). At that time, Blum was appointed to the new Paul Ehrlich Institute where he started his life's work in endocrinology and general medicine. His work continued unabated through World War I, the economic chaos of Germany in the twenties, and the early years of National Socialism. In 1939, Blum, who had been friend and physician to most of the Jewish community of Frankfurt, including the Rothschilds and the Mertons, fled Germany at the age of 75 to begin a new career

Figure 1. Ferdinand Blum as he appeared in the 1940s. Photograph courtesy of Prof. Gerhard Siefert and the Paul Ehrlich Institut, Frankfurt a. M.

in Switzerland. He returned to his beloved Frankfurt after the war and continued to publish until his death at 94 (14). Today the Ferdinand Blum Institute for Experimental Biology is part of the famous Paul Ehrlich consortium of Frankfurt.

Although Blum's paper was extremely well received, there were from the beginning several points of confusion about nomenclature and the fixation process. Foremost is the problem of naming commercial preparations of formaldehyde, a source of confusion to the present day. English companies listed formic aldehyde, while the German manufacturers each chose a favorite name. Formol was the name taken by Meister, Lucius, and Brunig, while the Chemischen Fabrik auf Actien (later Schering) chose "formalin" for their saturated aqueous formaldehyde solution. Unfortunately American producers of formaldehyde took the name formalin for the 40% aqueous solution of commerce, despite objections to the term as early as 1896 (2).

Another source of confusion is the question of concentration of the commercial product. Blum stated that the solution with which he worked contained 40% formaldehyde; information he obtained from the producer. The producer expressed concentration as grams per 100 cc of aqueous solution (weight/volume). Other producers chose to express concentration in terms of grams per 100 grams of solution (weight/weight). In current practice, English and American producers use grams per 100 grams of solution so that what was once a 40% solution of formaldehyde has now been labeled as a 37% solution, although it still contains the same amount of formaldehyde. Fortunately, most laboratories dilute formaldehyde on a volume basis (i.e., 100 ml plus 900 ml of water) so that there is little harm done except when workers attempt to adjust for the difference between the label on the bottle and the recipe in the book (17).

A further complication occurs in that formaldehyde will undergo virtually spontaneous condensation reactions with itself when stored in a concentrated form. This reaction can be inhibited at room temperature by including modest amounts of alcohols in the solution as preservatives or inhibitors. In the manufacture of formaldehyde, the most convenient alcohol to use is methanol, from which formaldehyde is made. Usually commercial formaldehyde solutions contain about 10% methanol as a preservative, a fact that may or may not appear on the label of the bottle. In addition, some commercial formaldehyde solutions will contain alcohols such as butanol, but these rarely find their way into a histopathology laboratory (1).

Blum's original report solved a major problem in the development of histopathological technology. Aqueous formaldehyde is a cheap readily available fixative that is very forgiving, that is, it works under a broad variety of conditions, is stable, functions effectively over a fivefold or more range of concentration and is usable with almost any tissue. Better still, formaldehyde is not a coagulating fixative so that tissues fixed in formaldehyde do not contain clumps of coagulated materials nor is cellular detail distorted by formation of a coagulum. Finally, formaldehyde fixation does not produce "over fixation," that is, tissues do not become hardened unpredictably, which would require several hardnesses of paraffin for sectioning in laboratories where a variety of tissue types are processed (7).

On first inspection, formaldehyde seems the ideal example of a serendipitous discovery, one that circumstances have certainly made of universal utility. There are, however, a number of puzzling characteristics about the use of formaldehyde that have either escaped scrutiny or have been ignored because of the remarkable success of this simple reagent.

One fundamental point that has not been especially well investigated is the anomaly, long known to histologists, that can best be described as the penetration–fixation paradox. Formaldehyde has a molecular weight of only 30 and diffusion theory would predict that such a molecule would penetrate tissues more or less independently of the concentration of formaldehyde. Medawar (23) devised a clever model system using coagulated chicken plasma to measure the rates of penetration. Formaldehyde showed a relatively constant rate of penetration of the clot over a concentration range of fivefold, ranging from 40 to 8%. He used a chemical method for detecting penetration, but had no means of determining whether the clots were actually "fixed," that is, how completely the

formaldehyde had reacted with the fibrin clot. Underhill (26), ten years before, had found that formaldehyde was a very slow fixative for tissues, which gave experimental credence to the axiom that formaldehyde penetrates rapidly but fixes slowly.

Formaldehyde has a natural tendency to be oxidized, producing formic acid (27). This oxidation process occurs quite readily in the presence of atmospheric oxygen, but an unknown amount of formic acid may be present in commercial formaldehyde as the result of storage or from the manufacturing process. A symptom of the problem is in the formation of "formalin pigment" (20). When blood-rich tissue specimens are fixed in formaldehyde solutions with an excess of formic acid, a birefringent finely divided pigment precipitates in the tissue. The pigment is probably a derivative of hematin and is formed when the pH is below 6.0 in the fixative solution. Whether the formation of formalin pigment is the result of the lowered pH of the fixative is not clear, and the role played by formate ion has not been investigated.

The spontaneous formation of formic acid in dilute formaldehyde solutions has resulted in a variety of different schemes for either removing the formic acid as it is formed or neutralizing the hydrogen ions produced. One rather crude method is to store diluted formaldehyde with an excess of calcium carbonate in the form of "marble chips." In theory, formic acid, as it is formed, will react with the calcium carbonate to form a roughly neutral solution. Alternatively, a buffer solution, usually in the form of a phosphate salt, can be added to maintain the pH of the solution at a predetermined level (21). Another method for solving the problem is to pass the formaldehyde solution over a mixed bed ion-exchange resin that will remove both hydrogen and formate ions, and to do so immediately before fixing objects.

An oversight, first by Blum, but perpetuated by countless others, is the question of temperature for fixation. Some investigators reasoned that since unfixed tissues undergo autolysis and since formaldehyde is known to fix slowly, one should retard autolysis by chilling the tissues and fixative. Other investigators assume that fixation is not a chemical reaction in the usual sense and fix tissues at room temperature. The choice of temperature has probably also been affected by the problems that were assumed to occur if formaldehyde was heated much above room temperature. Certainly the easiest alternative to the question of the ideal temperature for fixation is to use that most readily accessible—ambient temperature.

The temperature problem is also linked to the problem of length of fixation. Classical sources recommended that tissues be fixed for at least 24 hours (21). The advent of automatic tissue processing machines has taken a serious toll on the quality of fixation; a situation that is compounded in hospital practice by efforts to produce a diagnosis as quickly as possible with the intention of reducing the costs of medical care by decreasing hospital stay. The result is that in many diagnostic situations tissues are exposed to 1.3 M formaldehyde at room temperature for only a few hours, or occasionally, a few minutes. Fixation then occurs in the alcohol used for dehydration of the tissue, thus taking pathologic diagnosis back to the pre-Blum days of the 1880s. Obviously tissues fixed in alcohol are adequate for histological diagnosis, but are not always comparable to classical descriptions from tissues fixed exhaustively in formaldehyde. The effects of tissues fixed in this way on the various morphological schemes of tumor grading, for instance, are unknown.

One rationalization of the fixation process has resulted in recommendations for fixation in formaldehyde solutions at reduced atmospheric pressure, as in vacuum cups on tissue processors. The obvious futility of such a step has been lost on manufacturers of tissue processors, who maintain there is a market demand for such a device. A fundamental tenet of chemistry is that reactions increase in rate with increases in pressure, and fixation of tissues with formaldehyde is no exception.

Formaldehyde, when dissolved in water, rapidly becomes hydrated to form a glycol called methylene glycol (19). While the formation of this derivative was well known to Blum in the early part of this century, the chemistry of methylene glycol has escaped most histologists interested in fixation (7). In fact, the reactivity of aqueous solutions of formaldehyde is well known to physical chemists as an example of a "clock" reaction (8,9). This means that the equilibrium between methylene glycol and formaldehyde in aqueous solution lies so far in favor of methylene glycol that the conversion of methylene glycol to formaldehyde by removal of formaldehyde can be used as a "real-time" clock, measurable in hours. When tissues are immersed in formaldehyde solutions, they are penetrated rapidly by methylene glycol and the fraction of formaldehyde present. Actual covalent chemical reaction of the fixative solution with tissue depends on the formaldehyde present being consumed after forming bonds with the tissue components and more formaldehyde forming from dissociation of methylene glycol (16,18). Leather chemists, who have long been concerned with the reaction of formaldehyde with hides, use conditions for tanning that favor the dissociation of methylene glycol, such as low pH, high concentrations, and elevated temperatures. Thus, equilibrium between formaldehyde as carbonyl formaldehyde and methylene glycol explains most of the mystery of why formaldehyde penetrates rapidly (as methylene glycol) and fixes slowly (as carbonyl formaldehyde). The molecular mechanism of tissue fixation is not well understood. Chemical studies indicate that formaldehyde is a reactive electrophilic species that reacts readily with various functional groups of biological macromolecules in a cross-linking fashion (12), such as with proteins, glycoproteins, nucleic acids, and polysaccharides. The most reactive sites are primary amines (for example, lysine) and thiols (cystein), and the subsequent cross-linking of these functional groups to less reactive groups, such as primary amides (glutamine, asparagine), guanidine groups (arginine), and tyrosine ring carbons is a favored process (13). This intra-and intermolecular cross-linking of macromolecules alters considerably the physical characteristics of tissues.

Tissue to be fixed consists of a system of membranes and structures that are to varying degrees susceptible to osmotic forces. Osmotic properties of a solution may be expressed as the moles of molecules or ions dissolved in a liter of solvent, typically, water. A "10% formalin" or 4% formaldehyde solution is 1.3 molar by definition, no matter that most of the formaldehyde has become methylene glycol. This means that

a completely unbuffered solution of formaldehyde, without methanol preservative, exerts an osmotic pressure of 1300 mO under standard conditions. By comparison, tissue culture media or physiological salt solutions have osmolarities on the order of 250–350 mO. The addition of buffer salts further increases the osmolarity of the solution so that pressures exerted on individual cells may reach extreme values. Formulators of fixatives have shown little inclination to regard the osmotic pressures exerted by formaldehyde (actually methylene glycol) as being important (21), but have recommended a variety of buffers and even saline as providing more "isotonic" conditions for fixation (22). When glutaraldehyde, a dialdehyde with a molecular weight three times that of formaldehyde, is used for fixation a much lower molar concentration is used. Glutaraldehyde has the advantage that most of the aldehyde groups in the solution are not bound up as glycols (15). A 3% solution of glutaraldehyde has a much lower osmolarity than 4% formaldehyde, and has significantly more available reactive groups for fixation.

A peculiar characteristic of formaldehyde fixation is vesiculation of cell membranes. Various concentrations of formaldehyde will cause individual cell's membranes to form fluid filled vesicles to such an extent that the membrane vesicles may be removed and purified (25). The vesicles contain some portion of the protoplasm of the cell but other membranes within the cells, such as nuclear membranes and mitochondrial membranes, are also affected. The formation of large balloon-like vesicles is not as apparent. The mechanism for vesiculation may be in part due to the presence of methylene glycol, since glutaraldehyde, which has few hydrated carbonyl groups, is also less active in forming vesicles and other aldehydes show vesicle-forming activity in proportion to their tendency to form glycols (15).

A major concern in fixation by formaldehyde, or with any fixative, is the amount of distortion produced by fixation. The usual term applied to fixation distortion is shrinkage. Most histologists have firm ideas about shrinkage and what causes it, but close inspection of the literature reveals some discrepancies. In order to describe shrinkage, one must first have dimensions before treatment. At the microscopic level, this information is not always easy to obtain, especially when dealing with tissues. Consequently, most measurements of shrinkage due to fixation have employed blocks of tissue or whole organs, or single cells (10,11). To estimate shrinkage in tissue, a piece of tissue is measured, then fixed, then measured again. This kind of estimate, while useful for anatomical measurements with whole organs, has little meaning with tissues that will be used for histology. The reason for this is that tissues that are to be processed for histology are subjected to a number of subsequent procedures that will have a more pronounced effect on the dimensions of a structure than the original fixative. For example, after immersion in a formaldehyde solution for some period of time, a tissue processed for histology will then be thoroughly dehydrated by solvent extraction, have some portion of the lipids and other alcohol soluble substances removed, will be cooked at 55–60°C for some period of time, will be frozen in wax, shattered on a wedge (microtome knife edge), and the slice stretched to its limits by surface tension at the surface of a water bath–air interface. Substitution of plastic embedding for the paraffin process is no exception, especially in the stretching phase of applying the section to the slide for final staining and mounting. Single cells, such as lymphocytes, are not an ideal model system for studying shrinkage as they consist mostly of an already condensed nucleus with very little cytoplasm. Avian erythrocytes, although preferable, do not have the diversity of cellular organelles seen in tissues. Cell lines are so frequently aneuploid that dimensions of cultured cells are unreasonably varied.

Another difficulty, well known to careful histologists, is the variation within a block of tissue produced by fixation in formaldehyde solutions. If a block of tissue is fixed in formaldehyde, cells at the extreme dimensions of the block will have different tinctorial and morphological properties from cells a few tenths of a millimeter further within the block of tissue. This is especially evident when synthetically active cells such as liver cells are fixed in a cubic block of tissue.

Morphometry, measurement of physical parameters of tissues sectioned for routine histology, has become an active field in histology since the advent of image processing computers. It would be ideal, if it were possible, to apply image processing to tissues prepared for routine diagnostic purposes. Tissue processing has considerable importance for morphometry, not only from the standpoint of shrinkage and "geometric" distortion of tissue components but for tinctorial qualities. For example, estimations of texture depend on reproducible staining properties of cells and their nuclei and the assumption that all cells within a particular sample have the same properties is not necessarily true for many formaldehyde fixed tissue samples.

## Experimental Observations

### *Fixation of Formaldehyde by Tissues*

The binding of $^{14}C$ formaldehyde to tissues is shown in Figure 2. At room temperature (25°C), formaldehyde bound to tissue sections increased with time until an equilibrium was reached. Since the tissue sections were extremely thin (16 $\mu$m), penetration was not considered a factor in the kinetics of the reaction. At 37°C, the reaction of formaldehyde is considerably faster and equilibrium is reached after 18 hr or less. The thickness of tissue that will be penetrated by formaldehyde under these conditions is far greater than that which bonded to the tissue according to the Medawar constant for aqueous formaldehyde where penetration of tissue-like substances is a function of the square root of the time of exposure. In the case of 4% formaldehyde the constant is about 5.5, which means that in 24 hr immersion, formaldehyde may penetrate 20 or more mm. The rate of binding of $^{14}C$ formaldehyde to tissues that thick was not measured. In a practical sense, this experiment shows that since covalent binding of formaldehyde into cross-links is a fundamental event in fixation, fixation with formaldehyde depends on 24 hr exposure at room temperature or 18 hr at 37°C.

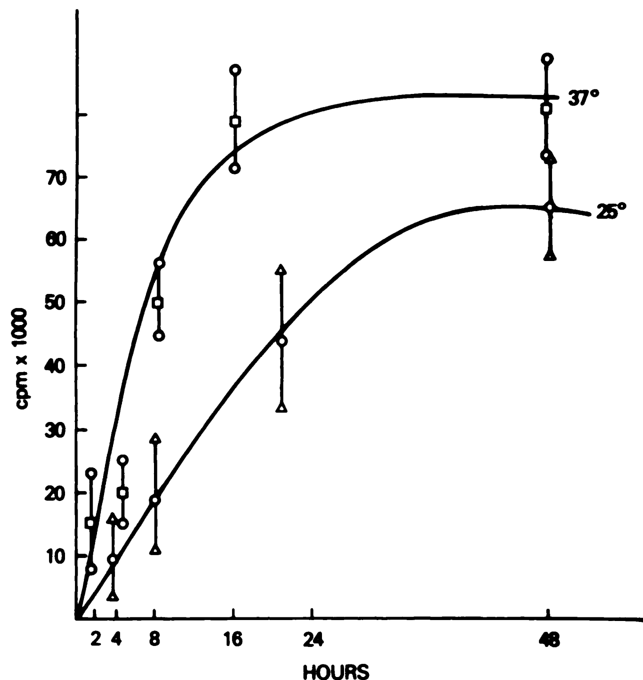


Figure 2. Fixation of $^{14}C$ formaldehyde by sections of rat kidney. Fresh rat kidneys were sectioned on a cryostat and 16 $\mu$m thick longitudinal sections were attached to microscope cover glasses. The tissue sections were fixed in labeled $^{14}C$ formaldehyde diluted with unlabeled formaldehyde to give a specific activity of about 50 $\mu$Ci per ml of 1.3 M formaldehyde. The sections were fixed for the indicated times, washed in copious amounts of water and while still attached to coverslips, dried. The dried cover glasses with tissue were counted in a liquid scintillation counter. Solubilizing the tissue sections had little effect on total counts, and was not done routinely. Fixations were performed at two different temperatures and each point represents four determinations.

## *Contaminants of Formaldehyde Reactions*

Formic acid is the most obvious contaminant of commercial or stored formaldehyde solutions. The formate ion content of some fixative formaldehyde solutions is shown in Table 1. While many of the effects of formic acid may be overcome by simply adjusting the pH of a fixative, an ideal resolution of the problem would be to prepare solutions directly from paraformaldehyde, as shown in Table 2. The effects of formate on tissues are probably more subtle than most histologists consider important, since many fixatives contain far greater amounts of cations and anions than occur in formaldehyde solutions such as picric or acetic acid. To test whether formic acid has an effect on nuclear size, pieces of rat liver were fixed in 1.3 M formaldehyde solutions that contained added formic acid. At concentrations that might occur in even badly contaminated formaldehyde there was little evidence of quantitative alterations in nuclear size.

**Table 1.** *Fixatives and formate*[a]

| No. | Formate (mM) | Phosphate (mM) |
|---|---|---|
| 1 | 1.20 | 56 |
| 2 | 0.42 | 50 |
| 3 | 0.75 | 60 |
| 4 | 0.90 | 72 |
| 5 | 1.45 | 100 |
| 6 | obscured by acetate | 3.2 |

[a]Six fixative solutions submitted to the AFIP tissue repository were examined for formate content by ion chromatography. The solutions were selected at random. In specimen 6, the formate peak was obscured by acetate, which is evidently still used as a buffer in some laboratories.

**Table 2.** *Formate (formic acid) in fixatives*[a]

| | | |
|---|---|---|
| 37% Formaldehyde (commercial formalin) | 3.7 | mM formate |
| 10% AFIP buffered formalin | 0.45 | mM formate |
| 4% Formaldehyde from paraformaldehyde 1.33 M fresh solution | 0.026 | mM formate |
| 4% Formaldehyde from paraformaldehyde after 1 month | 0.04 | mM formate |

[a]Fixative formaldehyde solutions were prepared from various sources. The last two solutions demonstrate the amounts of formate ion that may be generated per month in a flask stored with access to the atmosphere.

## *Shrinkage of Cells within Tissues*

Measuring shrinkage of cells within tissues suffers not only from the variables introduced by subsequent steps in processing the tissues but from other variables as well, such as alterations in the block of tissue introduced by the geometry of fixation where cubes of tissue may have different properties than spheres. When liver was fixed at room temperature and the number of nuclei per unit area used as an index of shrinkage (Figure 3), the differences between the corners of the tissue cubes were different at barely significant levels ($p > 0.85$).

## *Shrinkage of Tissues by Formaldehyde*

When tissues are fixed in 1.3 M formaldehyde solution for 24 hr under observation with a time-lapse video camera 1 × 1 × 8 cm strips of rat liver shrank in length only about 3% at room temperature. At 37°C the amount of shrinkage of both liver and whole rat kidney was so small that it could not be measured. Subsequent steps in the tissue processing protocol, alcohol dehydration, clearing in xylene, and infiltrating with paraffin produced as much as a 20% decrease in linear dimension of the tissues. The actual amount of processing shrinkage depends on the adequacy of the entire fixation sequence. Tissues that had been fixed for 18–24 hr at 39°C with 1.3 M formaldehyde in phosphate buffer (AFIP formaldehyde) showed the least shrinkage in the dehydration and embedding steps.

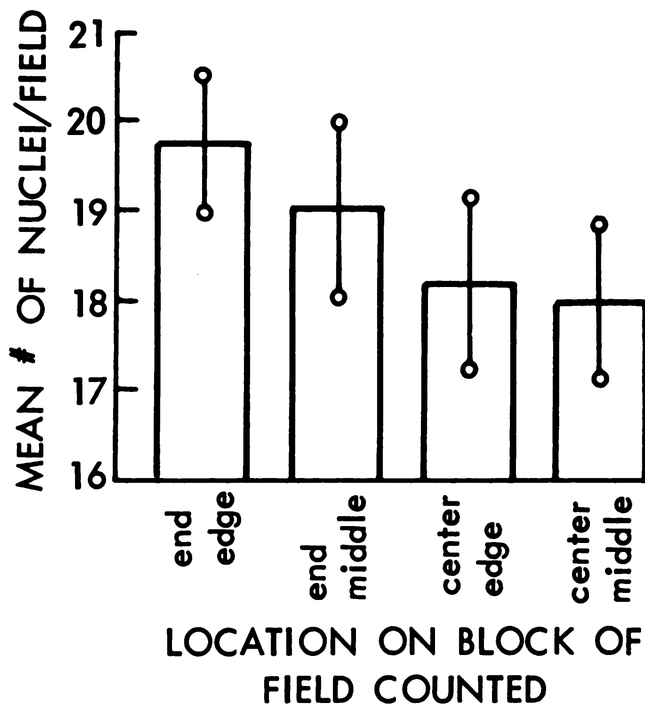


Figure 3. One centimeter blocks of rat liver were fixed in formaldehyde at room temperature for 24 hr. Sections were cut from the outside and the center of the blocks. The number of nuclei per field were counted on a video screen. The numbers of nuclei per field are an inverse measure of how much shrinkage has occurred so that the larger the number of nuclei per field, the greater the tissue shrinkage. Fifty fields were counted for each location.

## *Concentration of Formaldehyde*

The effect of varying concentration in preparing formaldehyde solutions may be measured by several criteria. A direct method is to measure volume changes in tissue. A more useful method for histomorphometric use is to determine the numbers of cells per unit of tissue by counting nuclei and to determine the changes that occur in nuclear area. Figures 4 and 5 show the alterations in size of cells and of nuclei when the concentration of formaldehyde is varied 40-fold. The extreme changes that occurred in tissues fixed in 40% formaldehyde may be in part due to the amounts of formate and/or methanol, since the solution was a commercial preparation. Over a 10-fold range, varying the amounts of formaldehyde in a fixative solution has little effect on the size of nuclei, whch seem the most resistant to fixation changes, and only a small effect on the cytoplasmic volume as measured by nuclei per unit area.

## *Methylene Glycol in Fixative Formaldehyde*

When paraformaldehyde is depolymerized in deuterium oxide buffered with deuterated phosphate salts, the molecular characteristics of the resulting species can be observed by high resolution nuclear magnetic resonance (NMR) spectroscopy. Thus, the signals in the proton NMR spectrum in Figure 6 indicate that a 1.3 M solution of formaldehyde contains chiefly the methylene glycol species and to a small extent its dimeric oligomer, but not the carbonyl formaldehyde species. That is, if the nonhydrated carbonyl form was present, it would be there in less than 0.1% abundance, considering the sensitivity of the experimental conditions. The reported equilibrium constant (24) for methylene glycol to carbonyl formaldehyde interconversion is $4 \times 10^{-4}$, showing that the hydrated form is overwhelmingly favored. While the formation of higher oligomers of methylene glycol occurs with increasing concentration, the distribution of components is not appreciably affected by temperature variations.

## *Formaldehyde and Electron Microscopy*

A variety of concentrations of formaldehyde were tested for use as fixatives for electron microscopy. Since the resulting micrographs were judged subjectively, they will not be presented here, but no concentration of formaldehyde between 0.5 and 20% produced photomicrographs comparable with those from glutaraldehyde fixed tissues. The quality of fixation could be improved somewhat by fixation at either 37 or 42°C. Increasing or decreasing the osmolarity of the formaldehyde fixative by adding salts had little effect on fixation, even when the formaldehyde concentration was reduced to isotonic levels.

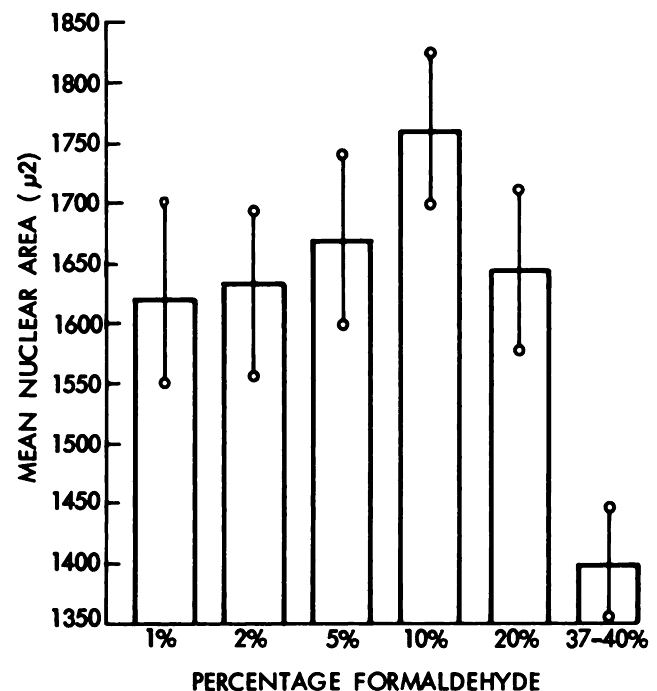


Figure 4. One centimeter blocks of rat kidney were fixed in 1, 2, 5, 10, 20, or 40% formaldehyde solutions from a commercial reagent for 24 hr. The mean nuclear area of 200 nuclei was measured with a Zeiss MOP3 electronic planimeter from each specimen. Significant changes were found only in tissues fixed in unbuffered stock commercial formaldehyde. Similar constancy of nuclear area was seen with liver treated the same way.

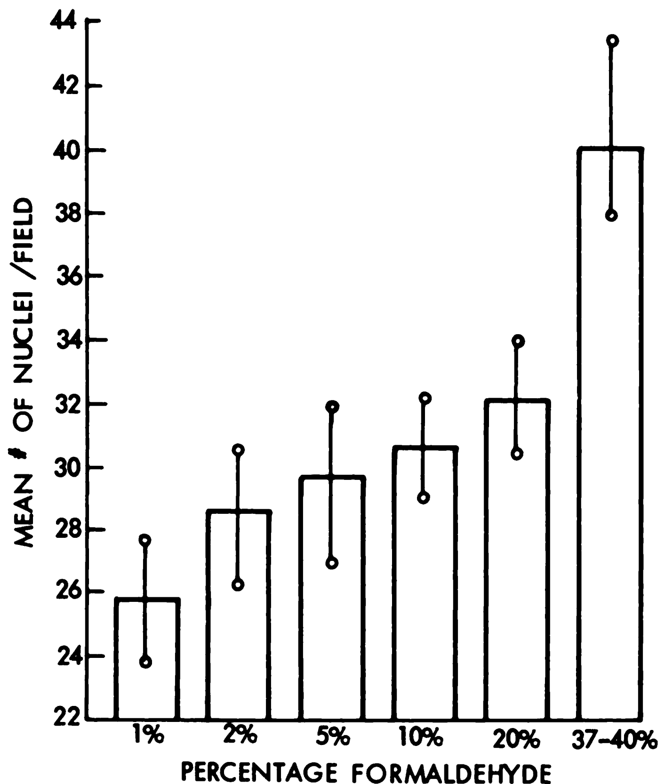


Figure 5. Kidney was fixed in either 1, 2, 5, 10, 20, or 40% formaldehyde solutions. All were buffered with 50 mM phosphate buffer except for the 40% solution, which was the commercial stock solution. Between 2 and 20% formaldehyde there was little alteration in the numbers of nuclei (and cells) per field. Only at very low or very high concentrations was there a significant difference.

### *Effects of Temperature*

Rat liver was fixed in an 4% unbuffered solution of depolymerized paraformaldehyde at 4, 25, and 37°C. A control of 4% commercial formaldehyde compounded as formolsaline was included (Figure 7). There were significantly more nuclei per unit area in tissue fixed at higher temperature. The tissues fixed at 4°C appeared to have greater intracellular spaces as though the cells had been "loosened" by the slower rates of fixation. Tissues fixed in formolsaline were comparable with those fixed in formaldehyde alone, despite the higher osmolarity of the saline formulation.

### *Fixation of Cultured Cells.*

When cells are cultured on the surface of coverslips and fixed under the microscope with 1.3 M formaldehyde solutions, remarkable changes occur within the cells. Between 5 and 30 min after the addition of the fixative solution portions of membrane on many of the cells balloon out into large round blebs. These blebs contain liquid (Figure 8). When seen through Jamin–Lebedeff interference optics the optical path difference of the blebs is considerably different from the fixative solution alone. Within the cytoplasm of the cell a variety of changes may occur. Mitochondria or the cytoplasm surrounding them may form vesicles, indentations may form in the nucleus, and vacuoles may occur anywhere in the cytoplasm. To determine whether the blebbing phenomenon is peculiar to cultured cells, frozen sections of rat kidney were fixed in a perfusion chamber under a phase microscope. Using time-lapse video, blebbing of the intracellular spaces in the tissue occurred in the same period as for cultured cells.

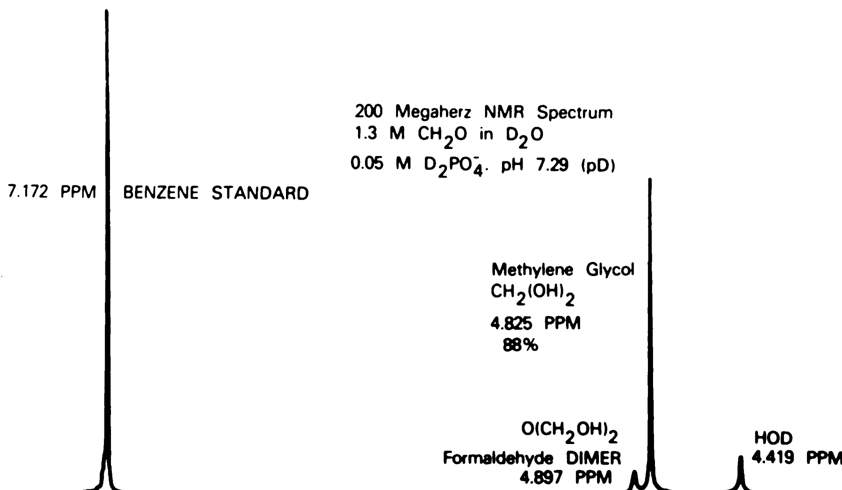


Figure 6. The presence of the hydrated forms of formaldehyde is observed at 4.825 and at 4.897 ppm in the proton NMR spectrum measured in a phosphate buffered deuterium oxide solution with a Varian XL-200 NMR spectrometer at 60°C. The chemical shifts in ppm are referenced to the benzene standard, measured at 7.172 ppm from TSPA. The benzene standard was contained in a coaxial tube within the 5 mm NMR cell. No signals could be observed that would correspond to the carbonyl aldehydic proton expected in the 9–11 ppm region (flat baseline not shown).

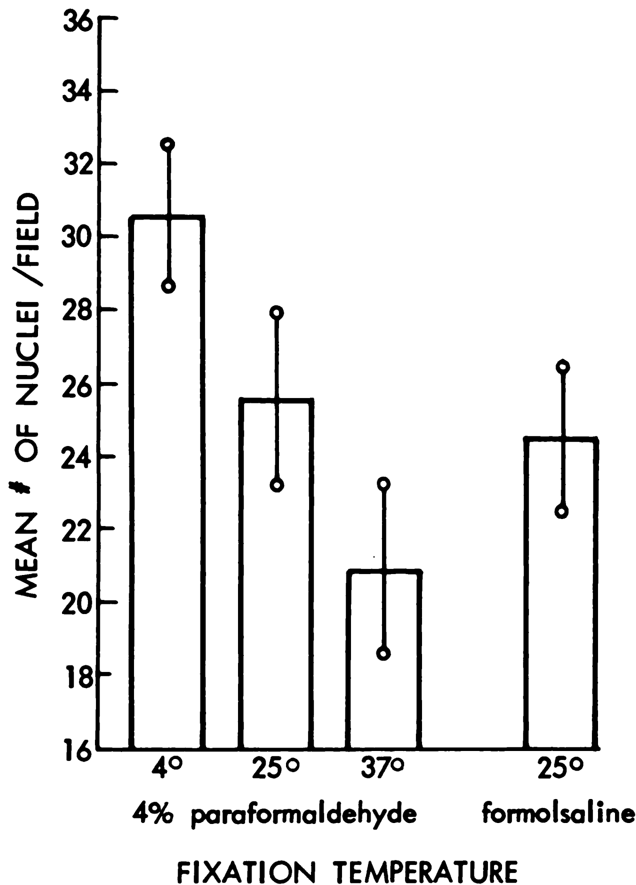


Figure 7. When rat liver was fixed at three different temperatures and the numbers of nuclei per field were measured, tissue fixed at 37°C showed the least shrinkage as compared to 4°C or 25°C. An even more hypertonic solution, formolsaline, produced similar results at the same temperature.

## Conclusion

Formaldehyde has a long and useful history for fixation of tissues. Despite this, there are aspects of the chemistry and action of formaldehyde that have not been adequately explained in the 90 years of its use. Peculiar to formaldehyde is its slow formation of covalent bonds in aqueous solution yet rapid diffusion in tissue. The results of the experiments described here indicate the following:

a) When formaldehyde is used as a fixative in aqueous solution at least 24 hr at room temperature or 16 hr at 37°C are required for the reaction to reach equilibrium.

b) While 1.3 M aqueous formaldehyde solutions are standard, this concentration is not critical to fixation, since most of the formaldehyde is present as methylene glycol.

c) Formic acid, a spontaneous oxidation product in formaldehyde solutions, seems to have little effect other than as an acid causing formation of "formalin pigment" in blood rich tissues.

d) Aqueous solutions of formaldehyde in the usual concentrations produce marked alterations in cellular membranes and in mitochondrial organization. Whether these changes are a result of the methylene glycol present or are due to some other mechanism is not clear.

e) Shrinkage of tissues is minimal in formaldehyde fixation, but becomes manifest in later steps of tissue processing. Tissues incompletely fixed in formaldehyde or fixed in formaldehyde at different temperatures may have different spatial characteristics than tissues fixed under dissimilar conditions. This property may limit morphometric measurements unless the chemistry of fixation and the limits of tissue processing are better defined.

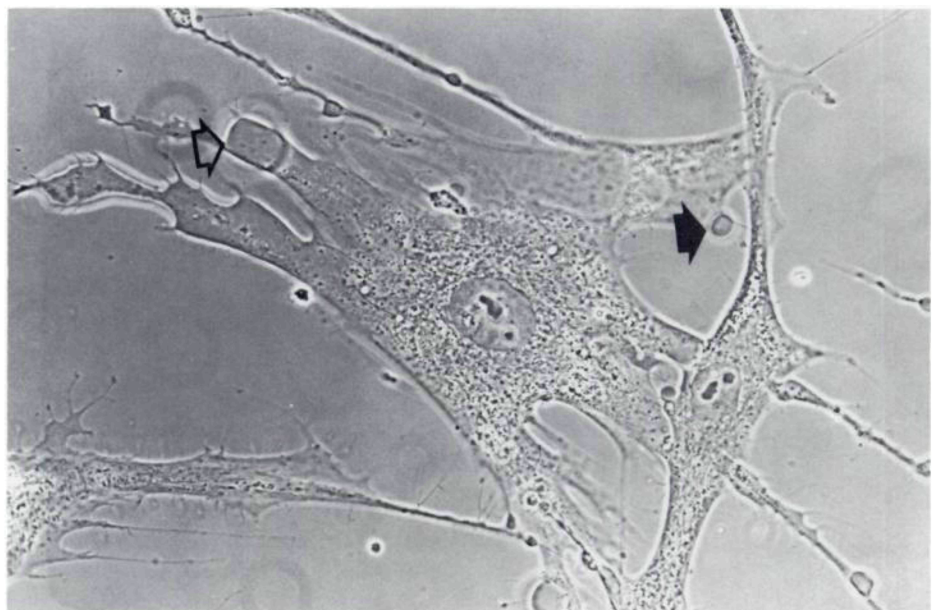

Figure 8. Membrane changes occur in most cells exposed to formaldehyde. These human fibroblasts in cell culture have been exposed to 1.3 M formaldehyde solution for 20 min. There are large blebs of cell membrane that contain cytoplasmic substances. Whether these membrane vesicles are formed by the effect of methylene glycol or by some other mechanism is not clear. Vesicular blebbing occurs in tissues as well, usually after only a few minutes immersion in formaldehyde solutions.

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