Penetration Rates of Formaldehyde

Bryan R. Hewlett
McMaster University Medical Centre (Retired)
bhewlett@cogeco.ca

Formaldehyde is one of the most rapidly penetrating fixatives used. Unfortunately, it is one of the slowest to fix tissue. This paradox was finally explained by Burnett[1] in 1982. An fine description of the properties of formaldehyde may be found in John Kiernan's book.[2]

The penetration rate of formaldehyde in mm/hr is a variable thing. It depends on how the data is obtained. It may also vary slightly depending on tissue type. The penetration rate of formaldehyde fixatives has been extensively studied, often with conflicting results. The penetration of non-coagulating fixatives is difficult to measure.

The original experiments of Medawar[3] utilized plasma clots with an indicator to mark depth of penetration. Medawar showed that fixatives obey the diffusion laws, that is, the depth penetrated was proportional to the square root of time. Medawar determined a coefficient of diffusibility for each fixative, the Medawar constant *K*. Using the equation $d = K\sqrt{t}$, where *d* is distance penetrated in mm, *t* is time in hours and *K* the Medawar constant for the fixative in question, it is possible to determine the penetration rate.

Medawar determined $K = 5.5$ for formaldehyde. Using this value NBF would penetrate 27.5 mm in 25 hours. Plasma clots are easier to penetrate than solid tissues, so the rate is probably less.

Baker[4] chose a gelatin/albumen gel to more closely mimic solid tissue and determined $K = 3.6$ for formaldehyde, or 18 mm in 25 hours. Baker also pointed out that the actual penetration into tissue would probably be less, possibly due to the resistance of lipid containing cell membranes. He quotes the data of Tellyesnicsky[5] who, using liver tissue samples mainly, indicated a more conservative $K = 0.78$ for formaldehyde. That would translate to 3.9 mm in 25 hours.

From $d = K\sqrt{t}$, it follows that fixatives penetrate more quickly into small samples of tissue compared to large ones. The initial rate of penetration into tissue is extremely rapid.

The first layer of cells (20 µm) takes a second or so (70 mm/hr). Using Baker's $K = 3.6$, the following examples will illustrate further;

1 hour = 3.6 mm,
4 hours = 7.2 mm, averaged to (1.8mm/hr),
9 hours = 10.8 mm (1.2mm/hr),
16 hours = 14.4 mm (0.9mm/hr),
25 hours = 18 mm (0.72mm/hr),
100 hours = 36 mm (0.36mm/hr).

So much for the penetration rate, the real issue is the fixation rate, *i.e.* penetration rate plus binding time. Fox et al.[6] used ^{14}C labeled formaldehyde to study the covalent binding time for rat kidney tissues. At a temperature of 25°C, the amount of formaldehyde bound to tissue increased with time until equilibrium was achieved at 24 hours. At 37°C the reaction was faster and equilibrium was reached at 18 hours. A later study by Helander[7] also used ^{14}C labeled formaldehyde to study binding time for the fixation of rabbit liver. At 25°C. equilibrium was achieved at 25 hours.

The correlation of results between these two studies is impressive. Particularly in view of the fact that Fox used 16µm thick sections of fresh rat kidney, whereas Helander used 4 mm cubes of fresh rabbit liver. The virtually identical equilibrium times achieved by each study indicate that penetration time is not a factor in the kinetics of the reaction. Despite the fact that thin slices of tissue will be penetrated faster than thicker cubes, it would seem that the binding time is the limiting factor for tissue stabilization. In a further study also by Helander,[8] using rat brain and kidney, equilibrium was not achieved until 50 hours. However, the tissue in Helander's latest study was twice the thickness (8mm) of the original study, a factor to be taken into account when comparing the data.

Failure to recognize the importance of formaldehyde binding time is the leading cause of the tremendous intra and inter- laboratory variability in immunohistochemical (IHC) performance. A clinical laboratory's so called "routine formaldehyde fixation," actually consists of allowing the tissue to fix for variable periods of time, dictated by the start time of the tissue processor!

Formaldehyde fixes not by coagulation, but by addition, reacting with basic amino acids (primarily lysine and arginine) to form several adducts. These reactions are readily reversible by water and alcohol. These adducts have free hydroxymethyl groups, which are capable of further reaction to form stable methylene bridges between proteins (see Kiernan[2] for more information). This type of cross-linking is responsible for the stabilization of proteins that we term fixation.

For both 1 mm thick core biopsies and 4mm thick tissue slices, the minimum stabilization time is 24-25 hours at ambient temperatures. The minimum stabilization time does not, unfortunately, denote complete fixation time. The initial cross-links are still relatively weak and easily reversible; stronger cross-linking continues to occur over time. Complete fixation is thought to take at least 7 days. Even after this time cross-links continue to form slowly.

Werner[9], quoting the two papers above, considers cross-linking complete in 24 -48 hours, but also expresses concern about the "over-fixation" due to excessive cross-linking, which may occur if fixation is allowed to exceed 24-48 hours. I agree with Werner, in that cross-linking may mask some epitopes, but in my experience this does not occur with the vast majority of antibodies in use until 5 -7 days of fixation. Even then, providing the IHC has been optimized, with the majority of antibodies, fixation up to 4 weeks is acceptable. The major strength of formaldehyde, as a fixative for IHC, lies in the fact that the cross-linking is 90% reversible. This reversibility allows the successful use of "Antigen retrieval" techniques. Far more serious is the problem of short <24 hour fixation.

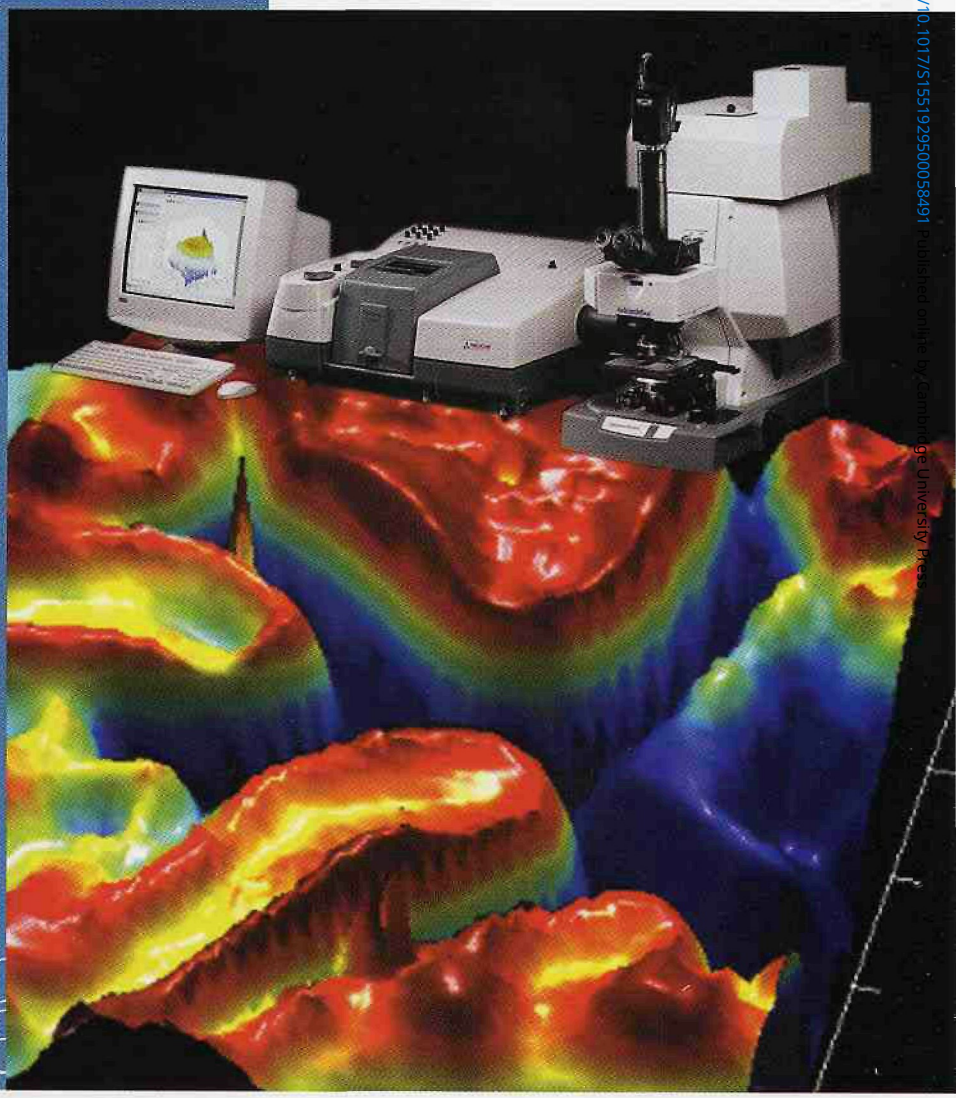
In aqueous solution, formaldehyde rapidly becomes hydrated to form methylene hydrate (methylene glycol).[1] The equilibrium of the reaction lies so far in favor of the hydrated form, that little(less than 0.1%) true formaldehyde is present.[6] The reactivity of aqueous solutions of formaldehyde is known to physicals chemists as an example of a "clock" reaction. The conversion of methylene glycol to formaldehyde by removal of the little formaldehyde present can be used as a "real-time" clock, measured in hours.[1]

Formaldehyde fixation begins at the periphery of the tissue. The initial layers of cells bind all of the available formaldehyde (<0.1%) and start the "clock." Methylene glycol continues to rapidly penetrate the tissue and, over hours, more formaldehyde is generated from methylene glycol. If this process is interrupted before completion, the formation of addition compounds will be incomplete, easily reversed and full stabilization by cross-linking will not occur. Depending upon the time of interruption, the periphery may show adequate cross-linking, whereas the remainder of the tissue is fixed by coagulant alcohol during processing. This may have disastrous effects upon IHC staining and will occur whether the tissue is a small biopsy or a 4 mm slice. ■

References

1) Burnett MG., *J Chem. educ.* 1982; **59,** 160
2) Kiernan J.A., *Histological and Histochemical Methods: Theory and Practice,* 3rd Edition, 1999. Oxford: Butterworth-Heinemann. ISBN # 0-7506-3106-6.
3) Medawar PB., *J R Microsc Soc.* 1941; **61,** 46
4) Baker JR. (1958).*Principles of Biological Microtechnique*, Methuen & Co. Ltd, pp 37-40.
5) Tellyesnicsky, K., (1926). Article on 'Fixation' in R. Krause's *Enzyklopädie der mikroskopischen Technik*, vol.2. Berlin (Urban & Schwarzenberg).
6) Fox CH., et.al., *J Histochem. Cytochem.* 1985; **33,** 845 -853
7) Helander, KG., *Biotechnique and Histochemistry.* 1994; **69,** 177 -179
8) Helander, K.G.,*The Journal of Histotechnology.* 1999; **22(4),** 317-318.
9) Werner M., et.al., *Am J Surg Pathol.* 2000; **24(7),** 1016 -1019

IR Imaging like you have never seen before.



Imagine the most advanced IR microscopy technologies – unsurpassed interferometry, focal plane array detection and imaging software – seamlessly united into one system.

But why imagine? See it now in ImageMax™. Developed specifically for imaging, ImageMax offers exceptional performance with optimized signal-to-noise. An innovative and integrated design for imaging allows the analysis of large and small samples, in transmission or reflection mode, without removing or realigning the detector.

The unparalleled sensitivity of the ImageMax is backed by two of the industry's best – Thermo Nicolet's expertise in infrared analysis and Spectral Dimension's pioneering knowledge of spectral imaging.

Best of all, imagine ImageMax at work for you. For a demonstration of the full range of capabilities, contact us today.

Thermo Nicolet, 5225 Verona Road, Madison, WI 53711-4495
Phone: 1-800-201-8132 or 1-608-276-6100.
Fax: 1-608-273-5046
E-mail: nicinfo@thermonicolet.com
www.thermonicolet.com

A Thermo Electron business

Thermo Nicolet