Preservation and retrieval of antigens for immunohistochemistry – methods and mechanisms. 1. Effects of formaldehyde fixation.

J. A. Kiernan
Department of Anatomy and Cell Biology
The University of Western Ontario
London, Canada N6A 5C1

There is no ideal way to prepare all tissues for immunohistochemistry. The access of antibodies in solution to tissue-bound antigens may be enhanced or inhibited by **fixation**. Some antigens can be detected only in unfixed cryosections; most are more easily detected after fixation. Paraffin embedding is usually preferable to either plastic embedding or cutting unembedded specimens with a vibrating microtome or in a cryostat. There are, however, plenty of exceptions to this generalization. Antibodies penetrate thin sections more quickly than thick ones, but sometimes the requirements of an investigation demand thick sections or even whole-mounts for proper interpretation of the results.

The first article (this one) briefly explains the effects of formaldehyde on tissues and the antigens that they contain. The second paper of the pair will examine several techniques commonly called **antigen retrieval**. These methods, applied to sections, allow immunostaining of antigens that might not otherwise be detectable after fixation of in formaldehyde.

These two articles also have some **references**, which fall into two categories: most are ordinary books or chapters in books; a few are papers in scientific journals. I urge readers to look up and study some of these references. Immunohistochemical staining is a responsible job, and should be undertaken only by people who understand the reasons for all the steps in a particular technique. Without proper understanding it is impossible to intelligently troubleshoot a procedure that does not yield the expected results.

Preservation of structure and antigenicity by formaldehyde

The fixative solution

In histopathology laboratories the great majority of specimens are fixed in an aqueous formaldehyde solution that contains sodium phosphates, contrived to provide **buffering** (minimal pH change following addition of a small amount of strong acid or base) to pH 7.2-7.6 and an approximately **isotonic solution** (one whose osmotic pressure is the same as that of mammalian extracellular fluids). The formaldehyde is usually derived from **formalin**, which is a solution containing 37% w/w (= 40% w/v) formaldehyde in water. The working fixative is a ten-fold dilution of formalin (4 grams per 100 ml). A solution of almost identical composition may be made with **paraformaldehyde** as the starting material. Paraformaldehyde is a solid polymer that changes into formaldehyde when heated (in slightly alkaline water) to 60EC. Many people do not realize that there is no such thing as a solution of paraformaldehyde. The frequently published phrase "fixed in 4% paraformaldehyde" is a clear indication that the writer knows nothing about fixation, even though this is the preparative step that most affects the appearance of a microscopical preparation.

Most of the formaldehyde in a diluted aqueous solution is present as **methylene glycol**, which is formed by addition of a molecule of water to one of formaldehyde:

Formaldehyde   Methylene glycol
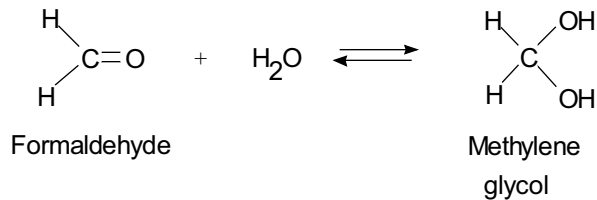
This reaction is reversible, but the equilibrium lies far to the right. The concentration of free formaldehyde in the fixative solution is, therefore, very low. Nevertheless, it is free formaldehyde, rather than methylene glycol, that enters the chemical reactions of fixation. There is always a large reservoir of methylene glycol that instantly replaces formaldehyde molecules that are removed from the solution by reaction and combination with the specimen being fixed (Pearse, 1980; Fox et al, 1985).
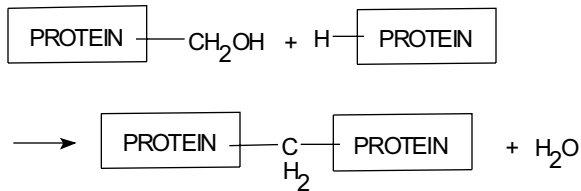
Reaction with tissue proteins

The small molecules of formaldehyde (MW 30) and methylene glycol (MW 48) penetrate quite rapidly through extracellular materials and cells, typically reaching a depth of 5 mm in about 2 hours (Baker, 1958). The chemical reactions of fixation by formaldehyde are with proteins. These reactions are slower than those of any other substance used as a fixative, and it is generally agreed that for reasonable structural preservation a specimen must remain in a formaldehyde solution for at least 24 hours (Drury & Wallington, 1967; Lillie & Fullmer, 1976). Helander (1994) found that after immersion for 24 hours, tissue-bound [^{14}C]formaldehyde could be largely removed by prolonged washing in water. Half was removed in 17 days, and 90% was removed in 3 weeks. Further studies (Helander, 1999) indicated that maximal binding of formaldehyde to brain and kidney occurred in 50 hours, but half-maximal binding required only about 4 hours. The rapidly bound formaldehyde probably stops autolysis but it does little to stabilize the fine structure of the tissue, and does not provide effective protection against disruptive effects of later treatments such as paraffin embedding. Indeed, nervous tissue immersed for 24 hours in 4% buffered formaldehyde shrinks or swells when transferred to salt solutions with higher or lower osmotic pressure than the fixative (Paljarvi et al., 1979). The structural instability is attributed to the fact that reaction of formaldehyde with proteins occurs in two stages, the first being fairly rapid (hours) and the second much slower (days).

In the first stage, formaldehyde molecules combine with various parts of protein molecules, especially the side-chain amino group of lysine and the nitrogen atoms of peptide linkages:
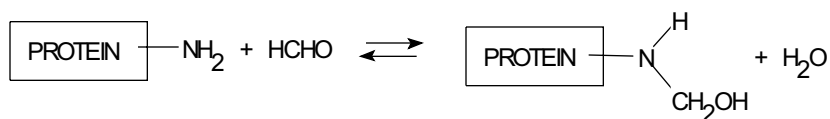
These reactions can be reversed by washing in water (or alcohol). The slow second stage is reaction of the bound hydroxymethyl groups with other nitrogen atoms of the same or adjacent protein molecules.

$$\boxed{\text{PROTEIN}} - CH_2OH \ + \ H - \boxed{\text{PROTEIN}}$$

$$\longrightarrow \boxed{\text{PROTEIN}} - \underset{H_2}{C} - \boxed{\text{PROTEIN}} \ + \ H_2O$$

The resulting cross-links, known as methylene ($-CH_2-$) bridges, are stable and account for the insolubility and rigidity of protein-containing tissues that have been fixed by formaldehyde. The reacting groups for the second stage must, of course, be close together; this condition is not met for a majority of the hydroxymethyl groups formed in the first stage, so it is possible to wash away loosely bound formaldehyde even from thoroughly fixed specimens. Knowledge of the chemistry of formaldehyde fixation has come mainly from investigations in the tanning industry, where bovine dermal collagen is converted into leather (Gustavson, 1956; Walker, 1964).

In addition to the reactions with proteins, formaldehyde may also combine with some basic lipids. These other reactions are not generally considered to participate significantly in the fixation of tissues (Pearse, 1980; Fox et al, 1985; Hopwood, 2002). The immobilization of DNA and RNA is attributed to trapping of the long nucleic acid molecules in networks of associated basic protein molecules, which are cross-linked by methylene bridges (Hopwood, 2002).

Inadequate and adequate fixation

These chemical reactions account for some of the difficulties encountered when working with formaldehyde-fixed material. Brief exposure to formaldehyde does not cause sufficient cross-linking to immobilize proteins. Instead, fixation is due to coagulation of proteins by the alcohols used to dehydrate the specimens. Alcohol alone is a poor fixative for blocks of tissue (Baker, 1958). Nuclei of cells can be greatly damaged during processing through paraffin after inadequate times in formaldehyde (Dapson, 1993). With adequate formaldehyde fixation, however, the cross-linked protein molecules form a dense network that can impair the penetration of paraffin wax. Cross-linking also impairs the penetration of large antibody molecules applied to sections of tissue as imunohistochemical reagents, so that antigen molecules of interest may be masked even if their epitopes have not been chemically modified by reaction with formaldehyde.

Cellular structure after formaldehyde fixation is often better preserved in frozen than in paraffin sections, especially when exposure to the fixative has been brief. Minimal fixation, either before or after sectioning in a cryostat, is customary in enzyme activity histochemistry because most (though not all) enzymes are inactivated by exposure to formaldehyde, organic solvents or heat. Completely unfixed cryosections can deteriorate or even disintegrate when incubated in the near-neutral aqueous solutions used in enzyme activity histochemistry and immunohistochemistry, though some protection is afforded by including a hydrophilic polymer such as polyvinyl alcohol in the medium (Chayen & Bitensky, 1991; Van Noorden & Frederiks, 1992, 2002).

Requirements for immunohistochemistry

For successful immunostaining of an antigen in a section of a tissue there must be:

1. Retention of the antigen at the sites it occupied in the living organism. This is favoured by formaldehyde fixation, which, by way of methylene bridges, can bind protein antigens to other protein molecules and trap antigens of any kind within a cross-linked protein matrix.

2. Permeability of the tissue, including cell membranes, to the large antibody molecules used as immunohistochemical reagents. A proteinaceous matrix that is tightly cross-linked by methylene bridges impedes penetration of large molecules, as do intact cell membranes. Exposure to organic solvents damages cell membranes and also distorts protein structures that have been incompletely fixed by formaldehyde. Hence, inadequate fixation and paraffin processing enhance the penetration of a tissue by antibodies, at the expense of inferior structural preservation.

3. The epitopes of the antigen must be accessible to the primary antibody. An epitope is a small part of a large molecule, such as a sequence of 3 to 10 amino acids, that specifically binds to the active site of an antibody molecule. A monoclonal antibody recognizes only one epitope.. An antiserum, on the other hand, is polyclonal, containing antibodies that recognize several different epitopes of the same antigen. Cross-linking due to formaldehyde fixation is likely to mask epitopes, leading to false negative immunostaining. This failure is more likely to occur when the primary immunoreagent is a monoclonal antibody than when a polyclonal antiserum is used.

In summary, fixation in formaldehyde can impair immunohistochemical staining, not usually by damaging or removing the antigens but by preventing contact between epitopes and antibody molecules. There are several strategies for avoiding or reversing these undesirable consequences of the otherwise desirable process of fixation. The simplest form of avoidance is to use a fixative mixture other than neutral buffered formaldehyde - one that immobilizes proteins either by coagulation or by a combination of coagulation and cross-linking (Polak & Van Noorden, 1997; Van Noorden, 2002). If neutral formaldehyde has already been used for fixation it is necessary to reverse some of its actions and re-expose the concealed epitopes. Techniques for antigen retrieval and their possible mechanisms of action will be discussed in the second article.

Featuring in the Spring edition of "The Cutting Edge"

Part 2 ~ Antigen Retrieval
"Preservation and retrieval of antigens for immunohistochemistry – methods and mechanisms"
By J. A. Kiernan

References
Preservation and retrieval of antigens for immunohistochemistry – methods and mechanisms. 1. Effects of formaldehyde fixation.
J. A. Kiernan

Baker JR (1958) *Principles of Biological Microtechnique* (Reprinted 1970, with corrections). Methuen, London.

Chayen J, Bitensky L (1991) *Practical Histochemistry*. 2nd ed. Wiley, Chichester.

Dapson RW (1993) Fixation for the 1990's: a review of needs and accomplishments. *Biotechnic & Histochemistry* 68: 75-82.

Drury RAB, Wallington EA (1980) *Carleton's Histological Technique*. 5th ed. Oxford University Press, Oxford.

Fox CH, Johnson FB, Whiting J, Roller RP (1985) Formaldehyde fixation. *Journal of Histochemistry and Cytochemistry* 33: 845-853.

Gustavson KH (1956) *The Chemistry of Tanning Processes*. Academic Press, New York.

Helander KG (1999) Formaldehyde binding in brain and kidney: A kinetic study of fixation. *Journal of Histotechnology* 22: 317-318.

Helander KG (1994) Kinetic studies of formaldehyde binding in tissue. *Biotechnic & Histochemistry* 69: 177-179.

Hopwood D (2002) Fixation and Fixatives. Ch. 5 in: JD Bancroft, M Gamble, *Theory and Practice of Histological Techniques*. Churchill Livingstone, London. pp. 63-84.

Lillie RD, Fullmer HM (1976) *Histopathologic Technic and Practical Histochemistry*. 4th ed. McGraw-Hill, New York.

Paljarvi L, Garcia JH, Kalimo H (1979) The efficiency of aldehyde fixation for electron microscopy: stabilization of rat brain tissue to withstand osmotic stress. *Histochemical Journal* 11: 267-276.

Pearse AGE (1980) *Histochemistry, Theoretical and Applied*, 4th ed. Vol. 1. Preparative and Optical Technology. Churchill-Livingstone, Edinburgh.

Polak JM, Van Noorden S (1997) *Introduction to Immunocytochemistry*. 2nd ed. BIOS Scientific Publications, Oxford.

Van Noorden CJF, Frederiks WM (2002) Metabolic mapping by enzyme histochemistry. Ch. 11 in: JA Kiernan, I Mason, *Microscopy and Histology for Molecular Biologists: A User's Guide*. Portland Press, London. pp. 277-311.

Van Noorden CJF, Frederiks WM (1992) *Enzyme Histochemistry: A Laboratory Manual of Current Methods*. Oxford University Press & Royal Microscopical Society, Oxford.

Van Noorden S (2002) Immunohistochemical methods. Ch. 9 in: JA Kiernan, I Mason, *Microscopy and Histology for Molecular Biologists: A User's Guide*. Portland Press, London. pp. 219-268.

Walker JF (1964) *Formaldehyde*. 3rd ed. Reinhold, New York.

References
Leadership Corner: Change

Bridges, W. (1991). *Managing transitions: Making the most of change*. MA: Addison-Wesley Publishing Co.

Calnan, J. (2001). *Shift: Secrets of positive change for organizations and their leaders*. ON, Canada: Creative Bound Inc.

Hayes, J. (2002). *The theory and practice of change management*. New York: Palgrave.