

Preservation and retrieval of antigens for immunohistochemistry – methods and mechanisms Part 2. Retrieving masked antigens

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The mechanisms of fixation by formaldehyde were described in a previous article (*The Cutting Edge*, January 2005, pp. 5-9), and it was noted that the cross-linking of protein molecules can render the epitopes (antibody-binding regions) of antigens inaccessible to large molecules, preventing detection by immunohistochemical methods. The lipoprotein membranes that enclose cells and organelles constitute another barrier to penetration of tissue by large molecules, especially when processing has not involved passage through organic solvents.

Despite having been available for many years (Coons et al, 1942) immunostaining was still a “new” method in the 1960s and 1970s. The deleterious effects of fixation on enzymatic activity were well known, and it was widely assumed that fixation also destroyed the chemical basis of antigenicity. Cryostat sections of unfixed or minimally fixed tissue were commonly used for immunohistochemistry (e.g. Nairn, 1976). Some fixatives, including alcohol-based mixtures, Bouin’s fluid and periodate-lysine-formaldehyde (McLean & Nakane, 1974), were said to “preserve” the antigenicity of certain peptides and proteins. Since about 1980 it has been recognized that distortion of macromolecular architecture by a fixative may either expose or conceal epitopes. Although antigens respond differently to fixatives it is generally true to say that epitopes are exposed by coagulation of proteins and masked by cross-linking. In terms of making antigens accessible to immunoreagents, neutral buffered formaldehyde was the worst of seven fixatives compared by Arnold et al (1996).

Nevertheless, neutral formaldehyde is the most frequently used fixative. Fortunately there are several ways to improve the access of antibodies to tissue antigens that have been masked by formaldehyde fixation.

Proteolytic enzymes

Probably the earliest way of unmasking formaldehyde-fixed antigens was to incubate the preparation, before exposing to the primary antibody, in a solution of a proteolytic enzyme (see Bullock & Petrusz, 1982). Usually an inexpensive grade of porcine trypsin (containing some chymotrypsin) is used at a concentration of 0.1% in 0.1 M aqueous CaCl_2 , adjusted to pH 7.8 with TRIS or a few drops of 0.1M NaOH. An optimum incubation (in the range 10 to 60 minutes, at room temperature or 37°C) must be found for each tissue and antigen. The rationale of using a proteolytic enzyme is that breaking some peptide bonds will make holes in the matrix of cross-linked proteins, allowing the entry of antibody molecules (Fig. 1). Enzymes other than trypsin have been used in much the same way, including pronase and pepsin (Hume & Keat, 1990). Endogenous proteolytic enzymes, released from cells damaged by the microtome knife, have been shown by Mori et al (1992) to unmask certain epitopes of extracellular proteins.

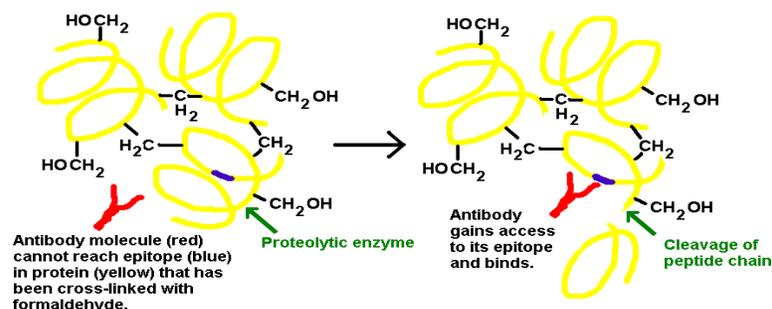


Fig.1. Unmasking of an epitope by the action of a proteolytic enzyme

The chief disadvantage of proteolytic enzymes is their propensity to digest the tissue, including the antigen one is attempting to demonstrate. A duration of exposure to the enzyme solution must be found that is just right for the job at hand (see Hayat, 2002). This requirement makes enzymatic treatment rather too labour-intensive for routine use.

Heat induced antigen retrieval

Most of the formaldehyde bound to a fixed tissue can be removed by prolonged washing (two to three weeks) in cold water (Helander, 1994; see also my previous article in *The Cutting Edge*). One may reasonably suspect that removal of formaldehyde would be accomplished more rapidly at high temperatures. Water alone, however, is seldom used for heat induced antigen retrieval. Other substances are nearly always dissolved in the water, and the reasons for trying the various solutes are not explained by the authors of most publications in this field. The first hot solutions to be used (Shi et al., 1991) contained either zinc sulfate or lead thiocyanate. The slides, bearing hydrated sections, were brought to 100°C in these solutions. The sensitivity of immunohistochemical staining was usefully increased for most of the 52 antibodies tested, and lead thiocyanate was generally more effective than zinc sulfate.

Subsequent studies of antigen retrieval in the 1990s focused especially on the pH of the hot water, and the type and duration of heating. The general consensus is that for most antigens pH 6 (nearly always obtained with a citrate buffer) is suitable. It is also generally agreed that a minority of antigens require either more acidic (pH 1) or more alkaline (pH 9) retrieval solutions. The source of heat may be a microwave oven, a boiling water bath or an autoclave. Some have argued that microwave heating may do more than simply raise the temperature, but the arguments are not convincing (see Hayat, 2002 for references and discussion). Effects of temperature have also been examined. It is evident that higher temperatures permit shorter times in an antigen retrieval solution. An overnight immersion in citrate buffer (pH 6) at 80°C is as effective as immersion for less than one hour in the same solution at 100°C (Koopal et al., 1998). With autoclaving (about 120°C), antigen retrieval is accomplished in about 10 minutes (Bankfalvi et al., 1994; Hunt et al., 1996), but much additional time is taken up waiting for the autoclave to cool without releasing the pressure. The usual procedure of decompression followed by closing the air intake valve causes boiling of the water in the jar containing the slides and detachment of all the sections (Kiernan – unpublished observation that should have been anticipated). A domestic pressure cooker is better suited to antigen retrieval than an institutional autoclave (Pileri et al., 1997). After 10 minutes at full steam remove it from the source of heat but do not release the pressure valve or cool the outside of the pressure cooker with cold water.

Ingredients of antigen retrieval solutions

No-one doubts the importance of pH (Shi et al., 1995; Boon, 1996), but ingredients other than buffer salts can also contribute to the efficacy of solutions for antigen retrieval. Before the advent of methods involving heating, techniques to improve immunostaining included treating sections at room temperature with 5M urea (Hausen & Dreyer, 1982) or with detergents (see Feldmann et al., 1983). Table 1 shows some of the substances that have been included in solutions for heat induced antigen retrieval.

Table 1. Possible functions of substances other than water in some solutions used for heat induced antigen retrieval. The formulations are listed in order of date of publication. The references should be consulted for exact details of composition of the solutions.

Solute	Possible function	Reference for formulation	Comments
Zinc sulfate [ZnSO ₄], 1%	Protein coagulant cation	Shi et al., 1991, 1992	Shi et al. (1991) examined retrieval of 52 antigens. ZnSO ₄ was generally less effective than Pb(SCN) ₂

Lead thiocyanate [Pb(SCN) ₂], 1%	Protein coagulant cation with chaotropic anion	Shi et al., 1991; Takahashi et al., 1993	Takahashi et al. found that heating with Pb (SCN) ₂ improved immunostaining of Bouin-fixed tissue. Methacarn (a non aqueous fixative that does not contain formaldehyde) provided for even better immunostaining
Citrate buffer, 0.1M, pH 6	pH control; chelation of Ca ²⁺	Shi et al., 1993, 1994	Generally more effective than Pb(SCN) ₂
Urea, 0.8M (5%)	Non-ionic chaotrope	Shi et al., 1994	Concentration lower than those used by other investigators
Glycine-HCl buffer, pH 3.5	Acidic medium	Shi et al., 1994	Less background immunostaining than after 0.8M urea
Aluminum chloride ₃ , 4% (pH2.5) or HCl, 0.1M (pH 1.0)	Acidic protein coagulants	Evers & Uylings, 1994	Vibratome sections of brain that had been in formaldehyde for more than a year.
Citrate buffers, pH 2.5, 4.5, 6.0	pH control; chelation of Ca ²⁺ (at higher pH)	Evers & Uylings, 1994	Vibratome sections of brain that had been in formaldehyde for more than a year.
Citrate buffer, 0.1M, pH 6.0	pH control; chelation of Ca ²⁺	Beckstead, 1994	Applicable to most of the antigens tested
Urea, 3M (18%)	Chaotrope	Beckstead, 1994	Applicable to most of the antigens tested
Tris buffer, 0.05M, pH 10	Alkaline medium	Beckstead, 1994	Retrieved some antigens that could not be immunostained after pH 6 buffer or 3M urea
No solutes	Distilled water	Umemura et al., 1995	More effective than buffers for autoclave retrieval of Bcl-2 protein
EDTA, 0.001M, pH 8	Chelation of Ca ²⁺ ; pH control	Balaton et al., 1995	1.5 minutes in pressure cooker
Citrate buffer, 0.01M, pH 6	pH control; chelation of Ca ²⁺	Man & Tavassoli, 1996	70-80°C (oven)
Urea, 0.8M (5%) in Tris buffer, pH 9.5	Non-ionic chaotrope at high pH	Shi et al., 1996	Effective with 32 of 34 antigens examined
EDTA, 0.001M, pH 8	Chelation of Ca ²⁺ ; pH control	Pileri et al., 1997	2 minutes in pressure cooker. Tested with 61 antibodies, and found generally superior to citrate pH 6, Tris pH 9.5 or a proteolytic enzyme
Tris buffer, pH9.0	Alkaline medium	Koopal et al., 1998	Used for 16 antigens; overnight at 80°C
Formic acid, 19M (88%) followed by guanidine thiocyanate, 4M	Acid followed by chaotropic cation and anion	Everbroek et al., 1999	For unmasking prion protein. The sections were autoclaved before exposure to the reagents.
EDTA, 0.01M, pH 8	Chelation of Ca ²⁺ ; pH control	Gown & Willingham, 2002	Higher concentration of EDTA than in earlier studies; 10 minutes at 100°C

Citraconic anhydride, 4.5mM, pH 7.4	May reverse formaldehyde fixation	Namimatsu et al., 2005	Equal or superior to two other retrieval solutions, for 62 antigens tested
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Mechanisms

At least four features of antigen retrieval solutions may be involved in their actions on formaldehyde-fixed tissue: hydrolysis of bonds to bound formaldehyde, actions of metal cations, chaotropic effects, and chelation of calcium ions.

Hydrolysis of bonds to bound formaldehyde

The possible action of hot water in undoing some of the cross-linking of protein molecules has already been mentioned, but this putative mechanism (Fig. 2) had not, until recently, been tested experimentally. Montero (2003) noted that prolonged fixation in formaldehyde results in poor stainability of tissue proteins by eosin, and that eosinophilia was restored by hot solutions used to retrieve antigens. Stronger evidence supporting the breaking of cross-links comes from the work of Yamashita & Okada (2005), who used SDS gel electrophoresis to study proteins that had reacted in solution with formaldehyde. Cross-linking resulted in the formation of dimers, trimers and other polymers; subsequent heating restored the original monomers. Eosin anions are electrostatically attracted to the basic side chains of proteins – the ones to which formaldehyde molecules bind covalently. Hot water may also alter the conformations of protein molecules. The latter process (cooking) can be expected to expose antigenic sites in much the same way as a coagulant fixative.

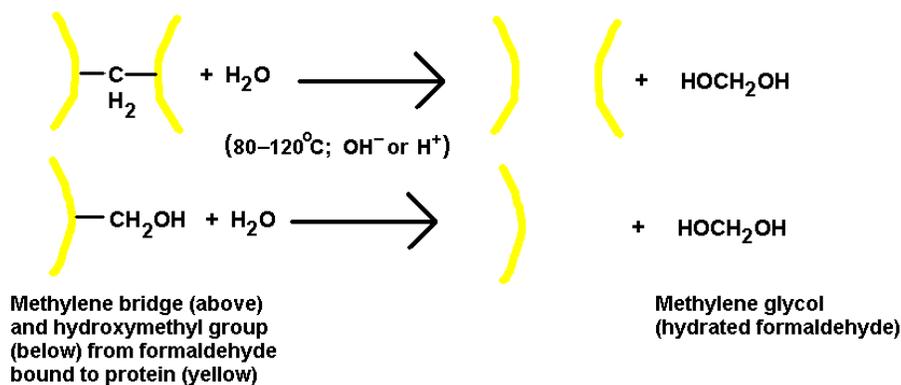


Fig. 2. Removal of bound formaldehyde and undoing of cross-links by base- or acid-catalyzed hydrolysis.

The effects of pH on different formaldehyde-fixed epitopes may be due to their different constituent amino acids, with some linkages to formaldehyde being more easily broken by hydrolysis in an acidic or alkaline medium. Shi et al. (1997) noted that some antigens could be heat-retrieved over a wide range of pH whereas others required an alkaline medium and yet others were retrievable at low or high but not at neutral pH.

Recently heating in an aqueous solution of citraconic anhydride (0.05%, pH 7.4, 45 minutes) has been proposed as a "universal antigen retrieval method" (Namimatsu et al., 2005). The procedure was equal or superior to citrate (pH 6.0) or Tris-HCl-5% urea (pH 9.0) for all 62 antibodies tested. The authors suggest that citraconic anhydride, which can combine reversibly with amino groups, attacked the bonds between formaldehyde-derived carbon atoms and protein nitrogens, thereby breaking the cross-links and removing the cross-links and giving antibodies access to epitopes.

Effects of metal cations

Solutions of the metal salts used in antigen retrieval solutions acidify the water. For aqueous solutions the pH is about 2 for 1% zinc sulfate, 5 for 1% lead thiocyanate, and 2.5 for 4% aluminum chloride. Zinc, lead and aluminum ions also coagulate proteins. The first are included in several modern fixatives, and the second in some older mixtures (see Gray, 1954). Solutions of zinc, lead and aluminum salts are traditional astringent lotions, which coagulate blood and proteinaceous exudates on inflamed skin or mucous membranes. Aluminum ions are not used in fixatives but they are used to harden the gelatin in photographic emulsions. Coagulant metal cations are not now considered major ingredients of antigen retrieval solutions, though they may be necessary for some particular antigens. The cations may cause changes in the conformations of protein molecules, especially in tissue that has not been adequately fixed by formaldehyde. Associated anions may have related effects; these will be discussed next.

Chaotropic effects

Water molecules occur in clusters of 280 molecules that can flip between an expanded and a collapsed structure (Fig. 3).

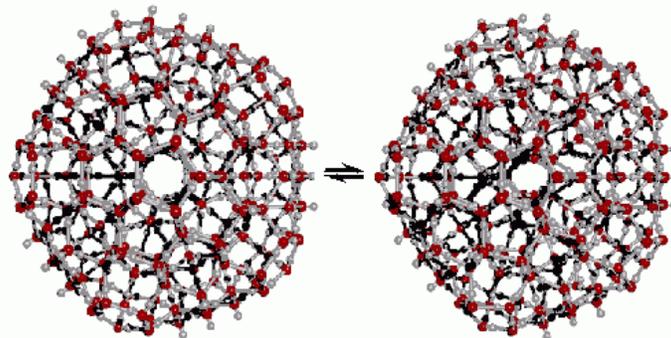


Fig. 3. Expanded (left) and collapsed (right) structures of water clusters. [Reproduced with permission from Chaplin (2004)]

Large molecules such as proteins dissolve by occupying the spaces between clusters. These spaces become wider when clusters change from the expanded to the collapsed form. The latter can therefore hold more macromolecules in solution. Smaller molecules or ions dissolved in water can alter the equilibrium between expanded and collapsed structures. Solutes that favor the collapsed structure are called chaotropes. Chaotropic ions, which include guanidinium, and thiocyanate, make the spaces bigger by inducing water clusters to flip to the collapsed state, making more room for dissolved macromolecules. Urea, a non-ionic compound, is a chaotrope when dissolved at high concentrations. Chaotropes are included in a number of solutions used for antigen retrieval (Table 1) but their modes of action have not been investigated. It is possible that these substances modify some proteins in fixed tissue to make them resemble proteins in solution, with more of their epitopes exposed.

Chelation of calcium

The most popular ingredient of antigen retrieval solutions is the citrate anion. This is a component of the buffer system that stabilizes the pH, but citrate ions can also form soluble complexes with calcium ions. Indeed, sodium or ammonium citrate is an ingredient of at least five solutions used for decalcifying bony specimens (see Lillie & Fullmer, 1976). A more powerful chelator of calcium is the EDTA anion, which is also widely used for decalcification. EDTA is included in several recently published antigen retrieval solutions (see Table 1 for a few examples), in which it serves to buffer the pH and to remove Ca^{2+} from the tissue. A chelating agent reacts with a metal ion, which becomes one of a ring of covalently bonded atoms in a stable, soluble, unreactive compound. Chelation reactions remove metal ions from liquid or solid materials.

A Ca^{2+} ion can form 4 coordinate bonds to other atoms such as oxygen or nitrogen. A coordinate bond is a covalent (strong) bond in which both electrons are donated by the O or N atom. Such a bond is often represented in a structural formula by an arrow; the head of the arrow pointing towards the metal atom indicates the electron donation. Some antigen-antibody reactions in solution are known to be inhibited in the presence of Ca^{2+} and facilitated by EDTA, presumably because the conformations of the proteins are changed by coordinate bonding between calcium and their amino, hydroxyl or carboxyl groups. Simple experiments have shown, for a few antigens, that addition of a calcium salt can impair immunostaining of sections of formaldehyde-fixed tissue. Heating in a Na_2EDTA buffer effectively retrieved these masked antigens, but a CaEDTA solution with the same pH was ineffective (Shi et al, 1999). It has been suggested that coordinate bonding of tissue-derived calcium occur with protein side-chains and also with bound hydroxymethylene groups derived from formaldehyde (Jasani et al., 1997; see also Hayat, 2002 for references and diagrams).

More recently, however, Yamashita & Okada (2005) have used SDS gel electrophoresis to examine the effects of some heat induced antigen retrieval procedures on five proteins in solution. This analytical procedure separates protein molecules according to size. Treatment with formaldehyde caused aggregation of protein molecules into dimers and trimers. Heating restored four of the proteins to their unfixed, predominantly monomeric, conditions. (The fifth protein was degraded by the heating procedure, yielding molecules smaller than the original monomers.) No effects of added calcium ions or of EDTA were detected, indicating that cross linking and deformation of antigens by calcium ions is not a major mechanism of epitope masking in formaldehyde-fixed proteins.

Conclusions

The large variety of ingredients in solutions for high temperature antigen retrieval indicates that more than one mechanism is probably involved. There is experimental evidence for temperature-dependent chemical reactions of water with formaldehyde-protein linkages, with breaking of cross-links. Most antigens can be retrieved at near-neutral pH, but a more alkaline medium is needed for some. In a few cases bonds to tissue-bound calcium ions may mask epitopes, necessitating removal of the metal ions by chelation. Other ingredients of retrieval solutions include heavy metal ions, which may expose epitopes by a coagulation-like action on proteins, and chaotropic substances which may modify the shapes of proteins by changing the structures of clusters of water molecules. Most recently, a hot citraconic anhydride solution has been introduced as a reagent to undo the fixation of proteins by formaldehyde (Namimatsu et al., 2005). Further work will be needed to determine if this is truly a universal antigen retrieval method as claimed, and to clarify the mechanism of action.

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