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On the chemistry of formaldehyde fixation and its effects on immunohistochemical reactions

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Summary. Formalin has been recommended as an innocuous fixative for immunohistochemistry. However, several studies demonstrated impairment or blocking of antigenic activity of certain proteins. Formalin fixation was discovered accidentally by F. Blum in 1893 and its deleterious effects on various tissue structures were discussed extensively during the following decades. More recently, some authors assumed that formaldehyde bound to tissues can be largely or completely removed by washing and dehydration. According to chemical data, formaldehyde forms highly reactive methylols with uncharged amino groups. Such methylol groups yield methylene bridges with suitably spaced amides, arginine and aromatic amino acid side-chains. Only loosely bound formaldehyde is removed by washing for several hours. Residual bound formaldehyde cannot be dislodged by washing for weeks, but some formaldehyde is gradually removed when tissues are stored in water for an extended number of years. Methylene cross-links resist treatment with high concentrations of urea, and can be broken only by drastic hydrolysis. It appears unlikely that such firmly bound formaldehyde is removed by conventional washing and dehydration procedures used in histochemistry. The superiority of methacarn, alcohol or acetone over formaldehyde fixation for immunohistochemical demonstration of prekeratin, myosin, type I and type IV collagen, laminin and fibronectin can be ascribed to the irreversible alterations of tissue proteins by formaldehyde.

Introduction

Formalin fixation is widely regarded as an innocuous, reversible procedure suitable for immunohistochemistry. But Altmannsberger et al. (1981) reported blocking of prekeratin and vimentin antigens by formalin fixation; alcohol-fixed, embedded material gave results equivalent to those obtained with unfixed frozen sections. Nadji and Morales (1983) confirmed that formaldehyde is not the best fixative for immunoperoxidase reactions. Kaku et al. (1983) recommended acetone for optimal retention of antigenic activity in embedded tissues. The major disadvantage of ethanol and acetone fixation is the considerable hardening and shrinkage of tissues. Warburton et al. (1982) and Gusterson et al. (1982, 1984) found methacarn superior to formalin fixation for immunohistochemical demonstration of prekeratin, myosin, type I and type IV collagens, laminin and fibronectin. Comparative studies confirmed extensive to complete blocking of prekeratin antigens by buffered formalin; duplicate blocks from the same organs fixed in methacarn showed intense coloration of prekeratin by peroxidase-antiperoxidase (PAP) technics (Barton et al. 1984; Meloan et al. 1984a). Yet, Sternberger (1979) recommended formalin as a relatively mild fixative for preservation of antigenic reactivity. Perusal of the literature indicated striking discrepancies between chemical and histochemical concepts of the interactions of formaldehyde with tissue proteins. We therefore reviewed historical, histochemical and chemical data on formalin fixation.

Historical review

Formaldehyde was first prepared by A.W. Hofmann in 1868 (Walker 1964) and was used as a disinfectant in the 1880's (F. Blum 1893, 1910). F. Blum (1893) placed a mouse infected with anthrax in formalin overnight and observed hardening of tissues similar to that obtained with alcohol. Further studies of liver, kidney, brain and stomach showed that sections could be stained with hematoxylin, aniline dyes, and Weigert's reaction for fibrin and microorganisms. Formaldehyde preserved erythrocytes and the natural color of tissues better than alcohol (J. Blum 1893). F. Blum (1893) therefore recommended a 10% solution of formalin (approximately 4% formaldehyde) as a fixative for histology. Eccles (1894) found it useful for rapid hardening of tissues.

The introduction of formaldehyde fixation is usually ascribed to F. Blum (1893). However, according to Langeron (1921), the ability of formaldehyde to penetrate and coagulate tissues was first reported by Trillat in 1892 (original not available).

As indicated by Dell'Isola's (1895) and Blum's (1896, 1910) reviews of the literature, formalin fixation was tested extensively. Dell'Isola (1895) considered formalin suitable for fixation of cytoplasm and nuclei, but warned that it caused considerable shrinkage and damage of muscle and connective tissue ("La formalina esercita un'azione dannosa sui tessuti connettivi e sul tessuto muscolare ..."). Lubarsch (1895) deplored the shrinkage of autopsy material and discussed the disadvantages of formalin fixation for demonstration of glycogen and fine cytoplasmic structures. In 1896 Blum demonstrated that formaldehyde forms methylene compounds with amino, amide and hydroxy groups, and thus affects the solubility and reactivity of proteins. Bound formaldehyde could no longer be demonstrated by chemical reactions. Parenthetically, Blum (1896) advised against fixation in alcoholic solutions of formaldehyde.

During the following decades formalin became popular in histology and pathology, despite its deleterious effects on the respiratory system, eyes and skin of personnel exposed to it (Romeis

1948). These health hazards are still the topic of much controversy. Romeis (1948) regarded formalin as unsuitable for nuclear structures, hematopoietic tissues, and demonstration of glycogen or iron. Roulet (1948) stressed the importance of thorough washing of formalin-fixed blocks prior to embedding, especially if tissues are intended for carmine or fat stains, special reactions for bacteria, or silver impregnation technics. The often neglected washing of blocks in running water may explain some difficulties reported with the latter technics.

Recent classification of formaldehyde as a relatively mild fixative (Sternberger 1979) may be due in part to a rather optimistic evaluation by Barka and Anderson (1963) that "In practice we assume that most of the formalin is washed out after fixation and the remainder removed by alcoholic dehydration or by the reagents employed in the histochemical procedure." However, it has long been known that formaldehyde blocks amino groups and forms methylene cross-links (Blum 1896, 1910; Zeiger 1930; Romeis 1948) and thus renders tissues more acidic (Gerngross and Bach 1923), i.e. the negative charge is increased (Zeiger 1930). Consequently, formalin-fixed tissues are more basophilic than duplicate blocks fixed in alcohol (Romeis 1948; Singer 1952; Baker 1958) and binding of anionic dyes is decreased (Langeron 1921; Zeiger 1938; Baker 1958). The blocking of amino groups by formaldehyde was confirmed by Barrnett and Roth (1958), who found that the naphthaldehyde reaction for amino groups was greatly diminished after fixation in formalin or Zenker-formol, but not after fixation in Zenker-acetic mixtures. The decreased coloration of formalin-fixed Brunner's glands by the periodic acid-Schiff (PAS) reaction (Hale 1955) suggests partial blocking of 1,2-glycol groups. Chemical aspects of formalin fixation were briefly reviewed by Pearse (1968) and are discussed in more detail below.

Chemical data

Formaldehyde has long been used in leather tanning (Gustavson 1956) and in the textile industry (Rath 1972; Peters 1975); its reactions with various compounds were described in detail by Walker (1964). A comprehensive review of the voluminous literature is beyond the scope of this report. This discussion will be limited to major data applicable to tissue fixation under conditions of histochemical and histological technics.

Reactions of formaldehyde and hydroxyl groups

In dilute aqueous solutions (4% or less) little or no formaldehyde is present in the free state. Spectroscopic studies show neither the absorption band of the C=0 group, nor the typical carbonyl frequency. The principle lines of the Raman spectrum correspond to those of methylene glycol $CH_2(OH)_2$ (Gustavson 1956). At higher concentrations polyoxymethylenes $HO(CH_2O)_nH$ appear (Walker 1964). In alcoholic solutions formaldehyde forms hemiacetals, $R \cdot OCH_2OH$, that are probably in equilibrium with higher polymers; such alcoholates of formaldehyde are more stable than the corresponding hydrates (Walker 1964). An increase in the boiling point of alcohols suggests cross-linking of alcohol molecules by formaldehyde.

According to Walker (1964), "sugars, starch and cellulose apparently react with formaldehyde in much the same manner as simpler hydroxy compounds, with the formation of unstable hemiacetals and the more stable methylene ethers or formals." In experiments with starch unstable hemiacetals predominated in the neutral and alkaline range; more stable compounds, probably formals, were obtained under acid conditions (Walker 1964). Formalin fixation up to 48 h seems to have little effect on the intensity of the PAS reaction, but binding of direct cotton dyes, e.g. in the alkaline Congo Red procedure is decreased. The exact chemical mechanism of this blocking effect has not been determined.

Reactions of formaldehyde with proteins

Amino, amide and guanidyl groups: Formaldehyde reacts with uncharged, but not with protonated ($-NH_3^+$) groups. In the first step

$$R \cdot NH_2 + CH_2O \rightarrow R \cdot NH \cdot CH_2OH$$

highly reactive methylol compounds are formed. If steric conditions are favorable, methylol groups condense with amide or other groups to yield methylene bridges, e.g.

$$R \cdot NH \cdot CH_2OH + NH_2 \cdot CO \cdot R' \rightarrow R \cdot NH \cdot CH_2 \cdot NH \cdot CO \cdot R' + H_2O$$

that cross-link polypeptide chains (Gustavson 1956). The ratio of bound formaldehyde and amino groups is nearly 1:1, this suggests that each methylene bridge links an amino group to another functional group (French and Edsall 1945). At room temperature and within the pH range 3 to 9, cross-links can be formed between aminomethylol groups and amide or guanidyl groups (Fraenkel-Conrat and Olcott 1948a; Walker 1964). Cross-links between two amino groups were not found under these conditions (Fraenkel-Conrat and Mecham 1949).

Primary amides give condensation products with primary or secondary amines over a range of pH 3.2 to 7.6; more acid or alkaline conditions catalyze the stable fixation of increasing amounts of formaldehyde by amides alone (Fraenkel-Conrat and Olcott 1948b). Proteins rich in amides but poor in amino groups were not readily cross-linked, unless divalent amino compounds were added which provided bridges between pairs of amide groups (Fraenkel-Conrat and Mecham 1949), e.g.

$$2R \cdot CO \cdot NH_2 + 2CH_2O + NH_2R' \rightarrow R \cdot CO \cdot NH \cdot CH_2 \cdot NR' \cdot CH_2 \cdot NH \cdot CO \cdot R + 2H_2O$$

It is not clear whether or not amino acids or low molecular peptides can be incorporated into such cross-links during fixation of tissue blocks. Secondary amides ($R \cdot CO \cdot NH \cdot R'$) do not participate in condensation reactions; these findings suggest that peptide bonds do not contribute to the cross-linking of proteins (Fraenkel-Conrat and Olcott 1948b). However, Gustavson (1956) considers participation of peptide linkages probable, though not at room temperature.

The importance of ε-amino groups for the uptake of formaldehyde was demonstrated in comparative studies of native and deaminated collagen. Below pH 8, native collagen bound about four times more formaldehyde than deaminated collagen (Gustavson 1956). The rapid increase in formaldehyde binding above pH 8 was ascribed to guanidyl groups. Acetylation abolished cross-linking of proteins by formaldehyde (Fraenkel-Conrat and Mecham 1949).

Aromatic amino acids: Cross-linking can occur also between aminomethylol groups and phenol, indole or imidazole side-chains by a type of Mannich reaction (Fraenkel-Conrat and Olcott 1948a). The methylene bridges between nitrogen and carbon atoms are very stable and resistant to acid hydrolysis, e.g. boiling 2N H_2SO_4 (Gustavson 1956). Clearly,

such bound formaldehyde cannot be removed by washing and dehydration procedures used in histology and histochemistry. Irreversible blocking of reactive sites of indoles and their derivatives, e.g. tryptophane, by formaldehyde was confirmed by Glenner (1957).

Carboxyl groups: In aqueous solutions carboxyl groups show little or no reaction with formaldehyde and are unimportant for fixation of proteins, except in the dry state (French and Edsall 1945).

Model experiments: Reaction of formaldehyde with amino groups within a few hours at pH 3.6 was demonstrated by the fall of the pH of the reaction mixture and by the change of optical rotation of solutions containing L-amino acids and formaldehyde (Fraenkel-Conrat and Olcott 1948b). Eightfold increases in molecular weight of soluble proteins treated with formaldehyde provided conclusive evidence for cross-linking (Mason and Griffith 1964). Insolubility of formaldehyde-fixed gelatin and other proteins was ascribed to cross-links and formation of a three-dimensional stable structure (Fraenkel-Conrat and Mecham 1949; Mason and Griffith 1964).

Effects of pH: The pH of the fixing solution is a controlling factor in the reaction of formaldehyde with proteins in aqueous media. In unbuffered formalin (approximately pH 4) many amino and guanidyl groups are protonated (Vickerstaff 1954), hence formaldehyde can react only with relatively few uncharged groups. As the reaction proceeds, other groups give off their protons and combine with formaldehyde. Consequently, fixation of tissues proceeds slowly. In buffered neutral formalin (pH 7) amino groups are discharged and react avidly with formaldehyde. For example, the amount of formaldehyde bound increases from 0.05 mmol/g collagen at pH 4 to 0.4 mmol at pH 7 to 8 (Gustavson 1956). Maximum tissue fixation, i.e. cross-linking, occurs in the pH range 4 to 5.5; no increase in tissue stabilization was observed above pH 5.5 (Gustavson 1956). The increased amount of formaldehyde bound at higher pH levels only blocks numerous reactive groups. Thus fixation in buffered neutral formalin inactivates more tissue groups than unbuffered formalin without improving fixation, i.e. cross-linking. Comparative studies of a one-step trichrome stain showed less binding of Chromotrope 2R and phosphomolybdic acid-Aniline Blue WS complex by tissues fixed in buffered neutral formalin than by material fixed in unbuffered formalin solutions (Waldrop et al. 1984). Since all tissue blocks were washed in running tap water for approximately 15–24 h, these discrepancies are apparently due to different amounts of formaldehyde bound at pH 4 and 7.

Effects of formaldehyde fixation on protein configurations: Data on structural alterations of proteins by formaldehyde could not be found in the literature available for this study. However, Lenard and Singer (1968) demonstrated a 22% to 29% loss of α-helix during glutaraldehyde fixation. Since glutaraldehyde is supposed to preserve tissue ultrastructure better than formaldehyde, it can be assumed that the latter causes at least as much alteration of protein structures as glutaraldehyde. This assumption is supported by observations that formalin-fixed tissues are unsuitable for configurational staining methods for myosins (Puchtler et al. 1969).

Removal of formaldehyde by washing: As already mentioned, it has been assumed that formaldehyde bound during fixation is largely or completely washed out. Only adsorbed and loosely bound formaldehyde is displaced by washing in running water. However, such formaldehyde is not likely to play an important role in fixation (French and Edsall 1945). After a few hours washing, wash water may give a negative test, but tissues still contain bound formaldehyde (Gustavson 1956). Bonds linking the remaining formaldehyde to tissues differ in stability. Some formaldehyde can be dislodged by prolonged treatment with water; but residual bound formaldehyde cannot be removed even by weeks of washing at room temperature (French and Edsall 1945). Upon storing tissues in water "*for an extended number of years*" more formaldehyde is gradually removed (Gustavson 1956). However, such extensive washing is impractical for histochemistry and histology.

Cross-links in formaldehyde-fixed proteins generally could not be broken by treatment with high concentrations of urea or other disaggregating solvents (Fraenkel-Conrat and Mecham 1949). Treatment of formalin-fixed gramicidin with hot aqueous sulfuric acid liberated one fifth to one third of the bound formaldehyde (Fraenkel-Conrat et al. 1947). In fixed casein, only drastic hydrolysis could break down the cross-links produced by formaldehyde (French and Edsall 1945). It would be unrealistic to expect that such firmly bound formaldehyde can be removed by conventional washing and dehydration procedures used in histology and histochemistry.

Effects of fixation on immunohistochemical reactions

According to Sternberger (1979), "... extensive cross linking impairs antigens by direct chemical effects and by structural distortions." Chemical studies discussed above demonstrated that formaldehyde produces extensive cross-linking of proteins. Furthermore, if fixatives block amino groups, then "... any antibodies to amino groups would not participate in the staining" (Sternberger 1979). The review of chemical data showed that formaldehyde reacts avidly with amino groups. The impairment or abolition of immunological reactions for prekeratin (Altmannsberger et al. 1981; Barton et al. 1984; Meloan et al. 1984a), vimentin (Altmannsberger et al. 1981) and fibronectin (Holund et al. 1981) indicate that these antigens are affected by alterations of amino groups and/or cross-linking of proteins.

Previous investigations showed that the prekeratin of current immunology is an epidermin (Meloan and Puchtler 1982), i.e. a member of the keratin-myosin-epidermin-fibrin (k-m-e-f) group of α-helical proteins that were studied extensively by chemical and x-ray diffraction technics from the 1930's to the 1950's. Methacarn fixation does not cause irreversible blocking of reactive groups and was designed for preservation of α-helical configurations of proteins (Puchtler et al. 1970). The strong reaction of methacarn-fixed tissues with PAP technics for prekeratin and configurational stains for myosins indicate that methacarn fixation does not impair these proteins (Meloan et al. 1984b; Waldrop et al. 1984). These observations are in agreement with data by Warburton et al. (1982) and Gusterson et al. (1982, 1984) who obtained optimal binding of antibodies against myosin and prekeratin in methacarn-fixed tissues. Furthermore, these authors found methacarn fixation suitable also for immunohistochemical demonstration of type I and type

IV collagens, laminin and fibronectin. Apparently, formaldehyde affects a variety of tissue antigens and cannot be considered as a mild, innocuous fixative.

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