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The action of chromium(III) in fixation of animal tissues

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Summary

Chromic salts have been studied as fixatives of mammalian tissues for light microscopy, and the binding of the metal has been examined histochemically. Tissues bind chromium(III) from aqueous solutions less acid than pH 2.5; the metal attaches mainly to collagen and basement membranes. Solutions containing chromium(III) as the only active ingredient cannot be used as fixatives because they destroy cytoplasm and cause great structural distortion. When mixed with other fixative agents, however, chromic salts can bring about considerable improvement in structural preservation. In aqueous mixtures more acid than pH 2, and in aqueous–methanolic solutions in the pH* range 4.0–5.3, a chromic salt provides only a nonspecific osmotic effect: little or no metal is bound to the tissue, and an aluminium or a sodium salt can be effectively substituted. In less acid (pH 2.3–3.2) aqueous mixtures, the beneficial action of chromium(III) cannot be imitated by aluminium or sodium ions.

Chromium(III) forms coordinate bonds that cross-link ionized carboxyl groups of macromolecules. The reaction occurs so slowly that such cross-links can internally strengthen a tissue only after the structure has been stabilized by rapidly acting fixative agents. Thus, a valuable future use of chromic salts may be in a post-fixation treatment to protect specimens against the adverse effects of embedding in paraffin wax. Chromium(III) might also be useful for enhancing the opacity of collagen fibrils in electron microscopy.

Introduction

Salts of chromium(III), also called chromic salts, are used in the tanning of leather and in the hardening of gelatin for photographic emulsions. In both processes the only significant reaction of chromium(III) cations is the formation of coordinate bonds with ionized carboxyl groups, which become cross-linked. The chemistry of the process has been thoroughly studied (see Britton, 1956; Gustavson, 1956; Pouradier & Burness, 1966; Hopwood, 1969; Thorstensen, 1969; Hormann, 1974; Burness & Pouradier, 1977; Pouradier, 1977). It is surprising that very few histological fixatives have been devised that contain chromium(III). Many valued fixatives contain chromium(VI), introduced as

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the dichromate anion. Some of these mixtures also contain reducing agents such as ethanol or formaldehyde, so that chromium(III) is formed as the solutions age. Even with stable solutions containing chromium(VI) there may be local reduction to chromium(III) within the tissues being fixed. Thus, cross-linking of protein molecules by chromium(III) may contribute to the stabilization of structure by the dichromate ion (Zirkle, 1928; Casselman, 1955a,b; Baker, 1958; Pearse, 1980; Horobin, 1981).

It is necessary to know whether chromium(III) is able to bring about fixation in tissues, alone or in combination with other substances. In this study the fixative properties of several solutions containing chromic ions are examined and compared with those of similar solutions lacking chromium. In order to assess the merits of these mixtures, a new system of standards is developed for the evaluation of fixatives for light microscopy. The sites of binding of chromium and the distribution of protein-bound carboxyl groups are also examined histochemically within the fixed tissues.

Materials and methods

FIXATION AND PROCESSING OF TISSUES

Pieces of kidney, brain (cerebral hemisphere), shaved abdominal skin, and duodenum with attached pancreas were taken from normal adult male albino Wistar rats. After trimming away adherent fat, slices no more than 2 mm thick were immersed in at least 100 times their own volumes of fixing fluid, for 18 h (overnight) at 20–25° C.

The fixatives tested were made up 45–60 min before use. The pH of each fluid was measured immediately before adding the specimens and immediately after removing them. Four groups of fluids were examined:

1. Well-known mixtures (Table 1), most of which had been critically studied by Baker (1958). These served as standards to establish the validity of the system of numerical criteria for judgement of the quality of fixation.
2. Solutions in which chromium(III) or aluminium ions were the only active ingredients (Table 2). Alumimium was of interest because its salts, like those of chromium(III) are used to tan collagen and to harden photographic gelatin.
3. Solutions based on Perenyi's fluid (Table 3) with ethanol, an acid and other ingredients in addition to chromium. Perenyi's fluid was of special interest because the reduction of dichromate by other components of this fixative is completed within a few minutes of mixing (Kiernan, 1981). Chromium(VI) cannot, therefore, be involved in the process of fixation.
4. The fixatives of Ammerman (1950), Masson (published by Paquin & Goddard, 1947) and Waterman (1934), and some modifications of these mixtures (Table 4). These are among the few fixatives in which chromic salts have been intentionally included as active components.

After fixation in aqueous liquids, the specimens were washed in six changes of distilled water, each for 30 min (until no more orange colour flowed from the pieces of tissue that had been fixed in dichromate-containing fluids), then transferred to 70% ethanol until the following morning. Specimens from alcohol-containing fixatives were moved directly into 70% ethanol. Dehydration was completed chemically in two changes of acidified 2,2-dimethoxypropane, each of 15 min (Prentø, 1978). The specimens were cleared in terpineol and embedded in paraffin wax according to the schedule of Kiernan (1981, p 30).

Table 1. Evaluation of some common fixatives according to the scoring system described in this paper. The grades allocated by Baker (1958) are shown for comparison. (Baker's scale from I [best] to V [worst] was based on paraffin sections of mouse kidney and testis.)

	Evaluation		
Fixative	*Microanatomy*	*Cytology*	*Baker's grade*
Allen's 'B.15' (Allen, 1916)	21	20	I
Altmann's fluid (Kiernan, 1981, p. 23)	15	16	IV–V
Bouin's fluid (Kiernan, 1981, p. 21)	15	18	III–IV
Chrome–acetic, strong (Clark, 1981, p. 16)	11	11	(Not examined by Baker, 1958)
Clarke's fixative (Culling, 1974, p. 50)	21	20	I
Formaldehyde (4%, from paraformaldehyde in 0.1 M sodium phosphate buffer, pH 7.2)	15	18	III (for unbuffered formalin–saline)
Heidenhain's SUSA (Kiernan, 1981, p. 21)	19	19	III
Helly's fluid (Kiernan, 1981, p. 21)	20	22	I–II
Müller's fluid (Gray, 1954, p. 232)	11	11	V (For $K_2Cr_2O_7$ without added Na_2SO_4)
Phosphate-buffered formaldehyde–picrate (Stefanini *et al.*, 1967)	17	22	(Not examined by Baker, 1958)
Zenker's fluid (Kiernan, 1981, p. 22)	21	21	I
Zenker without acetic (Kiernan, 1981, p. 21)	19	21	I

STAINING METHODS

Sections 7 μm thick, mounted on slides, were de-waxed and hydrated. When appropriate, mercury deposits were removed with 0.5% iodine in 70% ethanol, followed by 0.2 M aqueous sodium thiosulphate. Tissues darkened by osmium were bleached with 0.03 M potassium permanganate, followed by 0.2 M sodium thiosulphate. The sections were stained with iron–chromoxane cyanine R and eosin Y (Hogg & Simpson, 1975, as modified by Kiernan, 1984). Other sections were stained for 4 h in 0.01% fresh aqueous Haematoxylin to demonstrate bound metals (Lillie & Fullmer, 1976) or by the chrome azurol S-acetylacetone method of Suzuki *et al.* (1978) for selective staining of chromium. Other sections were stained by the acid anhydride method (Barrnett & Seligman, 1958) for side-chain carboxyl groups of proteins.

Table 2. Mixtures with chromium(III) or aluminium as the only protein fixing ingredient. The chromic salt used was chrome alum, $CrK(SO_4)_2.12H_2O$, mol. wt 499.4. The aluminium salt was potassium alum, $AlK(SO_4)_2.12H_2O$, mol. wt 474.2.

Name of solution	Composition	Molarity of Cr^{3+} (or Al^{3+})	pH		Evaluation	
			Before fixation	After fixation	Microanatomy	Cytology
Chrome alum hardener (Mack & Martin, 1939)	Chrome alum 30 g, water to 1000 cm^3	0.06	3.0	3.0	10	12
Chrome alum in saline	Chrome alum 20 g, NaCl 7.2 g, water to 1000 cm^3	0.04	2.6	2.8	13	13
Chrome alum with acetate	Chrome alum 20 g, water 200 ml; add 0.1 M CH_3COONa until pH is 5; water to 1000 cm^3	0.04	5.0	4.5	11	13
Chrome alum with acetic acid	Chrome alum 12.46 g, acetic acid 5 cm^3 water to 1000 cm^3	0.025	2.8	2.7	12	12
Chrome alum with cacodylate	Chrome alum 20 g, water 200 ml; add 0.1 M sodium cacodylate–HCl buffer (pH 7.4) until pH is 5, then water to 1000 cm^3	0.04	5.0	3.7	11	9
Chrome alum with borax	Chrome alum 20 g, water 200 ml; add 0.05 M sodium tetraborate until pH is 5; then water to 1000 cm^3	0.04	5.0	3.7	11	10
3% alum	$AlK(SO_4)_2.12H_2O$ 30 g, water to 1000 cm^3	0.06	3.3	3.4	8	10

Table 3. Composition of Perenyi's fixative and of four related mixtures. For the mixtures made from chromium trioxide, the composition before and after reaction with acid and ethanol is stated. The change in colour from orange to pale blue is complete in 10–15 min. The pH* for these partly non-aqueous solutions is calculated from the reading taken with an ordinary pH meter, following Perrin & Dempsey (1974).

	Composition (moles per litre)								
Name and description of solution	CrO_3	Cr^{3+}	C_2H_5OH	CH_3CHO	H^+	K^+	NO_3^-	SO_4^{2-}	*pH**
Perenyi's (1882) Fluid									
Fresh	0.015	0	5.10	0	0.65	0	0.65	0	
Mature	0	0.015	5.08	0.02	0.60	0	0.65	0	0.94
Perenyi with sulphuric instead of nitric acid, and added K^+									
Fresh	0.015	0	5.10	0	0.65	0.015	0	0.35	
Mature	0	0.015	5.08	0.02	0.60	0.015	0	0.35	1.04
Modified Perenyi's fluid made with chrome alum	0	0.015	5.05	0.02	0.60	0.03	0	0.40	1.04
Modified Perenyi, made with chrome alum but without acetaldehyde	0	0.015	5.05	0	0.60	0.03	0	0.40	1.04
Perenyi (1882) but with no chromium compound	0	0	5.10	0	0.65	0	0.65	0	0.84

Table 4. Compositions of the fixatives of Ammerman, Masson and Waterman, and several related mixtures. The pH* values for the last five items in the table (solutions in 50% [v/v] methanol) are calculated according to Perrin and Dempsey (1974). The source of chromium(III) in Waterman's fixative and related mixtures was a blue methanolic solution of chromic acetate, freshly prepared from chrome alum and potassium acetate as described by Waterman (1934).

Name and description of solution	*Composition* (moles per litre)										*pH**	
	Cr^{3+}	Al^{3+}	Na^+ *or* K^+	SO_4^{2+}	*Acetate (from salts)*	*Acetic acid*	*Picric acid*	CH_3OH	*HCHO*	I_2	*Before fixation*	*After fixation*
Ammerman's (1950) fixative	0.02	0	0.02	0.04	0	0.13	0	0	1.48	0	2.8	2.6
Ammerman without chromium	0	0	0	0	0	0.13	0	0	1.48	0	2.8	2.3
Ammerman with aluminium	0	0.02	0.02	0.04	0	0.13	0	0	1.48	0	2.9	2.9
Ammerman with sodium	0	0	0.30	0.15	0	1.48	0	0	1.48	0	3.2	3.2
Masson's fixative (Paquin & Goddard, 1947)	0.06	0	0.06	0.12	0	0	0.04	0	3.50	0	1.9	1.9
Masson without chromium	0	0	0	0	0	0	0.04	0	3.50	0	1.9	1.9
Masson with aluminium	0	0.06	0.06	0.12	0	0	0.04	0	3.50	0	1.9	1.9
Masson with sodium	0	0	0.30	0.15	0	0	0.04	0	3.50	0	2.1	2.1
Waterman's (1934) fixative	0.05	0	0	0	0.15	0.87	0	12.5	0	0.05	4.19	4.17
Waterman without chromium	0	0	0.10	0	0.10	0.87	0	12.5	0	0.05	4.39	4.33
Waterman without chromium or iodine	0	0	0.10	0	0.10	0.87	0	12.5	0	0	5.37	5.30
Waterman without iodine	0.05	0	0	0	0.15	0.87	0	12.5	0	0	4.07	4.00
Waterman without iodine or acetic acid	0.05	0	0	0	0.15	0	0	12.5	0	0	5.31	5.25

ASSESSMENT OF FIXATION

Conspicuous and consistent differences among the effects of the various fixatives were seen only in the cortex of the kidney and in the brain, so the quantitative evaluation was based only on sections of these two organs. Code numbers from a table of random digits were assigned by the technician to all the slides stained with iron–chromoxane cyanine R and eosin, so that I did not know their identities when assessing the quality of fixation. Two sections of brain and two of a kidney from each fixing fluid were included in the randomly numbered series. The numerical scoring was confined to the best fixed area of each section. For each structure evaluated, the score increased with the quality of preservation. Microanatomical preservation (distortion and differential shrinkage of tissue) and cytological preservation (internal structure of nuclei and cytoplasm) were assessed separately.

Microanatomical fixation

In the kidney, a score of 0.5, 1.0 or 1.5 was recorded for each of the following: glomeruli; Bowman's capsule; external form of tubules in the cortex; lumina of proximal and distal tubules. In the brain, a score of 1.0, 2.0 or 3.0 was awarded for lack of shrinkage spaces around capillary blood vessels and the somata of neurons. When the code was broken, the total scores for the two sections of each tissue from each fixative were added, so that the minimum possible score for microanatomical preservation was 8 (worst) and the maximum was 24 (best).

Cytological fixation

In the kidney, a score of 0.7, 1.3 or 2.0 was awarded for each of the following: nuclei (chromatin patterns of all cell-types); cytoplasm (proximal and distal tubules); and erythrocytes (shape, distortion, lysis). In the brain, a score of 1.0, 2.0 or 3.0 was awarded for nuclei (neurons, astrocytes, oligodendrocytes), cytoplasm (perikarya and dendrites of large neurons). As in the case of microanatomical fixation, the cytological scores were added together. The possible scores ranged from 8 (worst) to 24 (best).

All total scores were rounded off to the nearest whole number. Examples to illustrate details of the scoring system are shown in Figs. 1 and 2.

Results and discussion

Validity of the procedure for assessment of fixatives

The scoring system was based on criteria of lifelike preservation previously determined in studies of fixation for light and electron microscopy of cultured cells and of renal and central nervous tissue (Strangeways & Canti, 1927; Patek, 1944; Policard *et al.*, 1952; Woollam & Millen, 1954; Baker, 1958; Trump & Ericsson, 1965; Hayat, 1981). The scores for common fixatives are listed in Table 1 and compared with the grades given by Baker (1958). Unfortunately, Baker's scheme was not described in enough detail to allow its repetition by another investigator. Table 1 shows general agreement about the quality of fixation as assessed by the two methods. The only conspicuous discrepancy arises with Heidenhain's SUSA. Most authors other than Baker have considered this an excellent fixative (Gabe, 1976; Humason, 1979), an opinion supported by the present quantitative evaluation.

Solutions with chromium(III) or aluminium as the only active ingredient

Six solutions containing 0.025–0.06 M chromic ion, at pH 2.5–5.5, were tested (Table 2).

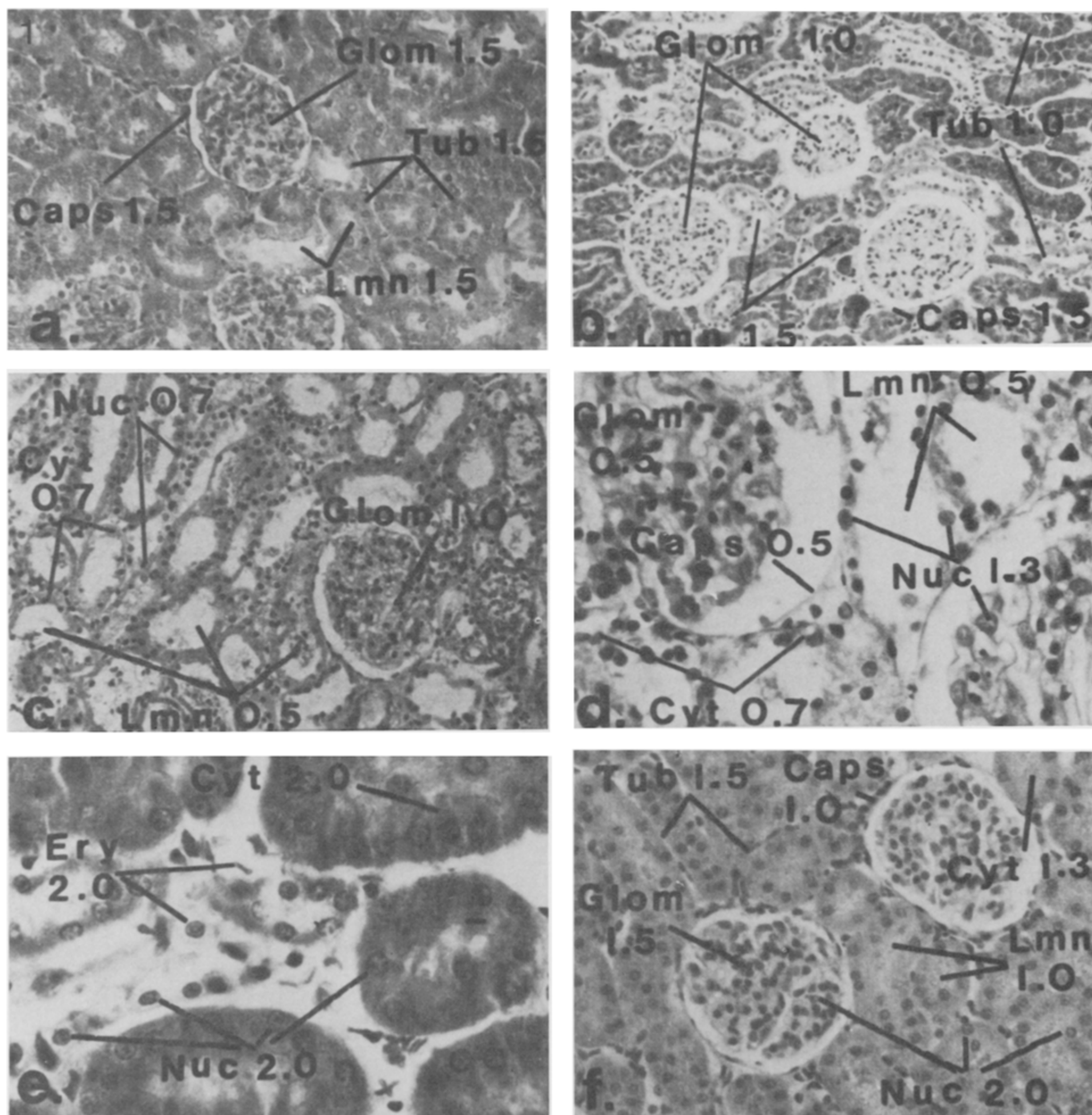


Fig. 1. Rat kidney after fixation in various mixtures, showing scores for microanatomical and cytological fixation. (The small size of the lumen of the proximal tubule is due to anoxia. This change is unavoidable with any fixation by immersion; it allows the proximal tubules to be distinguished from the more open distal tubules.) Glom, glomerulus; Caps, Bowman's capsule; Tub, tubules, external form; Lmn, lumen; Nuc, nuclei; Cyt, cytoplasm; Ery, erythrocytes. Fixatives used (a) Allen's 'B.15', × 300; (b) Zenker without acetic, × 190; (c) Perenyi's fluid, × 190; (d) Perenyi's fluid, × 475; (e) Zenker without acetic, × 750; (f) Clark's fixative, × 300.

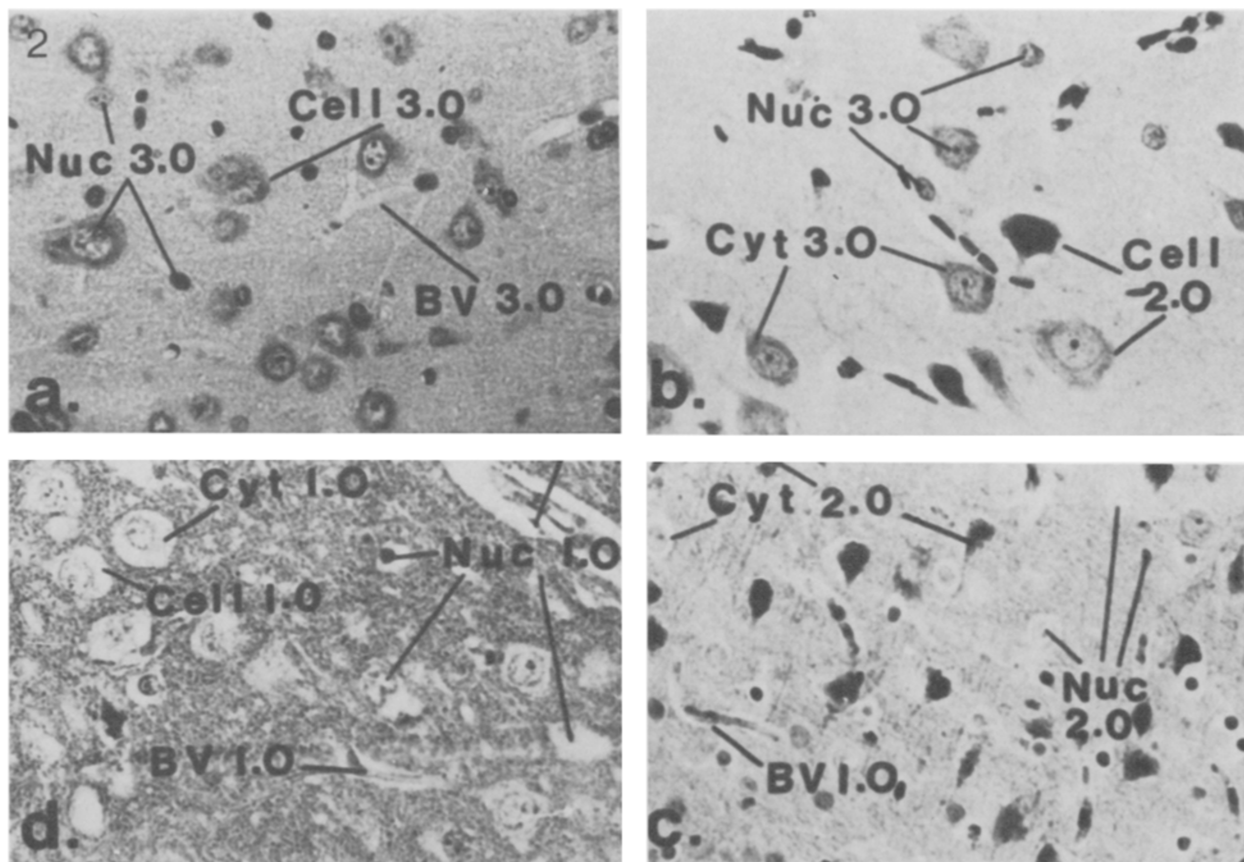


Fig. 2. Rat brain (cerebral cortex) after fixation in various mixtures, showing scores for microanatomical and cytological fixation. BV, blood vessel, to show perivascular space or its absence; Cell, large neuron, to show presence or absence of pericellular space; Nuc, nuclei; Cyt, cytoplasm. Fixatives used were (a) Clark's fixative, × 475; (b) Neutral, buffered formaldehyde–picrate, × 475; (c) Altmann's fluid, × 475; (d) Chrome–acetic fluid, strong, × 475.

None provided acceptable microanatomical or cytological fixation. The greatest destructive effect was upon cytoplasm, which was reduced to a few granules in a large space around the nucleus. Nuclei were intact but vesicular, containing only the nucleoli and scanty particles of chromatin. Extracellular structures were less extensively damaged than cells. Thus, the basement membranes of the renal glomeruli and tubules were largely intact, though there were large artefactual spaces between adjacent tubules. The solutions in Table 2 all had similar effects on the tissues. The least offensive solution was chrome alum in saline, which caused less differential shrinkage than the others.

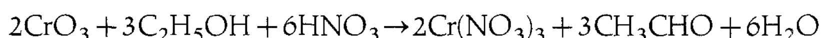
Substitution of aluminium for the chromic ion resulted in the worst microanatomical

distortion seen with any of the liquids tested. Cellular destruction was similar to that caused by solutions of chrome alum.

Perenyi's fluid and variants

Two different solutions containing nitric acid, chromium trioxide and ethanol were introduced by Perenyi (1882, 1888) for the eggs of amphibians, fishes and reptiles. If his *'Salpetersäure'* was the concentrated (70% w/w) nitric acid of modern laboratories, the 1882 mixture is closer to the Perenyi's fluid of current texts (e.g. Culling, 1974; Humason, 1979). The modification of Perenyi (1888) contained 50% more nitric acid, 33% more ethanol and 38 times as much chromium trioxide as the 1882 mixture. The last quantity seems ridiculous, and the *'19%ige Chromsäure'* of the original account was surely an error, perhaps for 1%. Only the mixture of 1882 was studied in this investigation.

Perenyi's fluid was orange when mixed, but changed to pale blue in less than 15 minutes. This change was presumed to be due to the reaction:



$$2CrO_3 + 3C_2H_5OH + 6HNO_3 \rightarrow 2Cr(NO_3)_3 + 3CH_3CHO + 6H_2O$$

in which all the chromium(VI) was reduced to chromium(III), with production of acetaldehyde. Only minute proportions of the ethanol and acid are consumed in this reaction. Solutions with the same concentration of chromium(III) as matured Perenyi were prepared using chrome alum. Acetaldehyde was introduced as paraldehyde, which depolymerizes in acidic media. Other solutions allowed for effects of the potassium and sulphate introduced with the chrome alum. One solution contained no chromium (Table 3).

These fixatives were superior to those with chromium(III) alone, but they were nevertheless too poor to have any value for the kidney or brain (Table 5). The fixative properties were unaffected by the presence or absence of acetaldehyde.

The fixatives of Ammerman, Masson and Waterman, and variants

A detailed study was made of the fixatives of Ammerman (1950), Masson (after Paquin & Goddard, 1947) and Waterman (1934), in which chromic ions are mixed with other commonly used fixative agents. The fluids of Williamson & Pearse (1923) and of Zirkle (1928, p 225, numbers 1, 2 and 7) were not examined because they were devised for specialized purposes and contain unusual ingredients in addition to chromium salts. The compositions of the fixatives of Ammerman, Masson and Waterman and of the variants also studied are given in Table 4.

The results of the evaluation are shown in Table 6. All three mixtures were excellent for microanatomical and cytological purposes, comparing favourably with the best of the well known fixatives in Table 1. Omission of chrome alum from the fluids of Ammerman and Masson resulted in marked deterioration in microanatomical preservation, but cellular structure was only slightly affected. Aluminium could be substituted for chromium(III) in Masson's fluid, but not in Ammerman's. The metal salts cannot have

Table 5. Evaluation of Perenyi's (1882) fixative and related mixtures. The composition of each solution is given in Table 3.

Mixture	*Score for:* Microanatomical fixation	Cytological fixation
Perenyi's (1882) fluid	15	12
Perenyi with sulphuric instead of nitric acid, and added K^+	16	16
Modified Perenyi's fluid made with chrome alum	15	13
Modified Perenyi made with chrome alum, but without acetaldehyde	16	15
Perenyi (1882) but with no chromium compound	18	15

Table 6. Evaluation of the fixatives of Ammerman, Masson and Waterman, and related mixtures. The composition of each solution is given in Table 4.

Mixture	*Score for:* Microanatomical fixation	Cytological fixation
Ammerman's (1950) fixative	19	20
Ammerman without chromium	15	19
Ammerman with aluminium	16	17
Ammerman with sodium	12	13
Masson's fixative (Paquin & Goddard, 1947)	19	21
Masson without chromium	16	19
Masson with aluminium	18	21
Masson with sodium	17	22
Waterman's (1934) fixative	19	20
Waterman without chromium	18	18
Waterman without chromium or iodine	11	14
Waterman without iodine	14	14
Waterman without iodine or acetic acid	12	13

served only to provide osmotic pressure, because their replacement by isotonic sodium sulphate did not restore the quality of fixation (Table 6).

The omission of chromium(III) from Waterman's fixative resulted in slightly inferior fixation. Much more severe effects followed omission of the other active ingredients. Waterman's blue solution of chromic acetate in 50% methanol was no better than

aqueous chrome alum with added acetate (Table 2). Evidently chromium makes only a small contribution to fixation by Waterman's mixture.

Distribution of bound chromium

Dilute Haematoxylin (Lillie & Fullmer, 1976) gave a brownish blue colour in sections of tissues fixed in solutions containing chromium(III) or chromium(VI), and pure blue if the fixative had contained an aluminium or a mercury salt. In the absence of metals there was only a light yellowish background stain. Chrome azurol S, used as directed by Suzuki *et al.* (1978), gave a blue colour only after fixation in chromium-containing fluids. This dye also imparted a pink background to all components of the tissue. The demonstrated distribution of chromium was identical with both methods. In sections from the same block, Haematoxylin gave a stronger colour than chrome azurol S. The former dye is therefore a more sensitive though less specific histochemical reagent for chromium.

After fixation in aqueous solutions containing chromium(III) as the sole active ingredient (Table 2), the metal was localized principally in collagen and basement membranes. Weaker staining was seen in occasional nuclei and in cytoplasm. The strongest staining for chromium was seen in material fixed at pH 2.6–3.0. Somewhat less chromium was bound from the less acidic (pH 3.7–4.5) solutions. No chromium could be detected histochemically in sections of tissue fixed in 0.05 M chromic acetate in 50% methanol (pH* 5.3). Perenyi's fluid and its variants (Table 3) were the most acidic fixatives examined (pH* 0.9–1.0). Specimens fixed in these liquids exhibited very weak staining for chromium, confined to collagen and basement membranes.

In tissues fixed in Ammerman's fluid (0.022 M Cr^{3+}, pH 2.6–2.8), moderately strong staining for chromium was observed in collagen. There was less intense colouration of most nuclei, and weak staining of the cytoplasm of some cells, notably large neurons. After fixation in Masson's fluid (0.059 M Cr^{3+}, pH 1.9), staining for chromium was weak, and of about equal intensity in nuclei and collagen. Chromium could not be detected in tissues fixed in Waterman's mixture (0.05 M Cr^{3+}, pH* 4.2).

Table 1 includes seven fixatives (Allen's 'B. 15', Altmann, chrome–acetic strong, Helly, Müller, Zenker, Zenker without acetic) that contain chromium(VI). Chromium was detected histochemically after fixation in some of these fluids, but the distribution of the metal was not the same as that seen after fixation in solutions of comparable pH containing chromium(III). Fixation by chromium(VI) will be the subject of another investigation. When aluminium ions were included in fixing fluids (Tables 2 and 4), their localization as revealed by Haematoxylin was quite different from that of chromium(III). Aluminium was detected principally in the nuclei of cells. Weak staining was observed in some cytoplasms, but collagen was unstained.

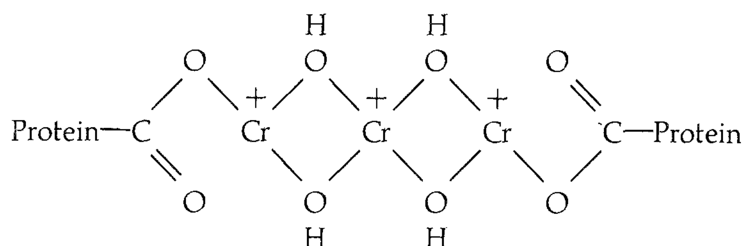
Distribution of carboxyl groups of proteins

With the acid anhydride method, a purple colour indicated side-chain carboxyl groups of proteins (Karnovsky & Mann, 1961; Stoward & Burns, 1971). These were demonstrable in all parts of all the tissues. The reaction was more intense in cytoplasm than in collagen

and nuclei. The intensity and distribution of the purple colour were the same after all the fixatives tested, whether they contained chromium or not.

Chemical reactivity of solutions containing chromium(III)

Chromic ions (and also aluminium ions) are positively charged and may therefore be attracted to anionic materials such as nuclear DNA. Ionic binding is unlikely to cause stabilization of structure, however, so that the only reactions of chromium(III) likely to be involved in fixation are the formation of coordinate bonds with carboxylate anions and with other hydrated chromic ions. These reactions occur slowly, by displacement of coordinately bound water from the blue $[Cr(H_2O)_6]^{3+}$ ion. The cross links have such forms as:



(Coordinated water and other ligands not involved in the cross-links are omitted. The number of chromium atoms per cross-link is variable.) The pH is critical: if it is too low, the carboxyl groups will not be ionized, but if it is 5.3 or higher, a green polymeric 'chromic hydroxide' will be precipitated. In chrome tanning, the chromium(III) penetrates the hide most rapidly as monomeric ions, at pH 3, but cross-linking occurs optimally at pH 4 to 5. The pH is therefore slowly raised during the course of the tanning process (Britton, 1956; Gustavson, 1956; Thorstensen, 1969). Simple solutions of chrome alum (pH 3) suffice to insolubilize photographic gelatin (Burness & Pouradier, 1977). Binding of chromium(III) from histological fixatives occurred in the range of pH 2.6 to 3.0.

Most of the bound chromium in tissues fixed at suitable pH was localized in collagenous structures, whereas protein carboxyl groups were demonstrable by the acid anhydride method in all parts of the tissues. Indeed, the prior binding of chromium(III) did not visibly reduce the intensity of staining by the acid anhydride method, even in collagen. It is probable, therefore, that only a small proportion of the potentially available carboxyl groups were involved in chromium-containing cross-links.

Structural changes caused by chromium(III)

Even under the most favourable conditions of binding, chromic ions alone were unable to fix kidney or brain tissue. This observation confirms the findings of Sheinin & Davenport (1931), who sought fixative properties in a wide range of metal salts including 0.5 M aqueous chromic acetate, chloride, nitrate and sulphate (pH from 2.6 to 5.8). When other fixative agents were present, however, as in the mixtures of Ammerman and Masson, the

chromic salt contributed significantly to the quality of fixation, for its omission resulted in inferior preservation of structure (Table 6). With Masson's fluid the action may have been a nonspecific one. This fixative was quite strongly acid (pH 1.9) and did not introduce much chromium into the tissue. Furthermore, the effects of omitting chrome alum from Masson's fixative were largely corrected by substitution of either an aluminium or a sodium salt.

With Ammerman's fixative (pH 2.6–2.8), the chromic ions contributed significantly and specifically to the quality of fixation (see Table 6). Evidently the beneficial actions of chromium(III) in Ammerman's fluid are principally manifest in microanatomical rather than in cytological fixation. Such an effect may be due to cross-linking of collagen fibrils in connective tissues and basement membranes, the sites in which the bound chromium was detected.

The future of chromium(III) as an ingredient of fixatives

This investigation has revealed that chromium(III) attaches to tissue, mainly to collagen, but that it can make a significant contribution to the stabilization of structure only when used in conjunction with other fixative agents at a pH higher than about 2.5. The coordination reactions of chromium(III) are notable for their slowness (see Cotton & Wilkinson, 1972; Rollinson, 1973). It is probable, therefore, that the chromic ions in a fixative such as Ammerman's work by forming cross-links in collagen only after the tissue has been fixed by the other ingredients. The cross-links involving chromium can be expected to impart extra physical strength to the extracellular framework of the specimen, thus protecting its internal structure against disruption during the process of embedding in paraffin wax. Most other fixatives that contain chromic ions are too acid to allow the coordination of the metal with carboxyl groups (e.g. Perenyi's and Masson's fluids), or contain other ingredients that prevent the reaction (as in Waterman's fixative).

On the basis of the present observations, two uses of chromic salts in histology can be foreseen. The first is as a post-fixation treatment to protect tissues against the damaging effects of embedding in paraffin. This would be potentially valuable for specimens fixed in formaldehyde or glutaraldehyde, in which it is known that initially excellent fixation is severely marred by subsequent processing into paraffin. The second use might be as a post-fixation 'block staining' to introduce chromium into collagen and perhaps other proteins, providing selectively enhanced contrast for electron microscopy. These applications are being investigated.

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