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The histochemistry of thiols and disulphides. IV. Protective fixation by organomercurial-formalin mixtures

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Summary

Formation of mercaptides as the result of adding organomercuric salts to neutral formalin used for fixation was found to protect protein thiols from autoxidation, provided the tissues were washed in distilled and not tap water. Such blocking, in contrast to that given by $HgCl_2$, could be reversed quantitatively by mercaptoethanol made strongly acid to keep it from reducing disulphides. However, some cleavage of disulphides by the mercurials themselves caused slight artifactual thiol staining in a limited number of sites. Three of the nine compounds tested are sufficiently soluble to penetrate tissues with reasonable speed, stable enough to preclude more than incidental mercurial deposits and currently available commercially. Of them, the diuretic mercurial Mersalyl is at present the protecting agent of choice since methyl- and ethylmercuric chlorides are too toxic to recommend for routine use.

Introduction

The simple acidic fixatives usually favoured for demonstrating thiol groups in tissue sections not only extract several proteins of interest but also fail to give lasting protection against autoxidation of the thiols to disulphides (Gomori, 1956; Lillie, 1965). Although generally good thiol staining has been obtained after overnight fixation in neutral formalin (Barrnett & Seligman, 1954; Sippel, 1973), the virtue of formaldehyde in retaining proteins is offset by mercaptal formation which sooner or later abolishes thiol staining altogether (Rudall, 1952; Lillie, 1965). Obviously neither type of fixative would be suitable before staining methods designed to differentiate thiols from disulphides (Sippel, 1978b).

Instead of attempting to discriminate directly between the two forms, some workers chose to visualize both together (Barrnett *et al.*, 1955; Gomori, 1956). They, therefore, could take advantage of the superior morphological preservation afforded by formalin–$HgCl_2$ mixtures since, just before staining, thiols were liberated from

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their mercuric mercaptides by the alkaline mercaptan solutions used to reduce the disulphide bonds. The starting point of the present study was the inference that the mercaptides might be selectively dissociable by a mercaptan solution that was sufficiently acidic to prevent its reducing action.

Unfortunately, in preliminary trials with fixatives containing formalin and $HgCl_2$ at pH 6–7, little thiol staining was recovered upon treating the sections with acidified mercaptoethanol. Fixatives of lower pH gave somewhat better results but, with the exception of the strongly acid formulation of Romeis (1968) considered in detail below, they dissolved out several types of intracellular granule. Meanwhile, seemingly acceptable staining patterns obtained after fixation in neutral formalin containing methylmercuric chloride prompted further examination of this organomercuric salt along with others of a less toxic nature. Among the best of the latter was a mercurial diuretic compound, Mersalyl, already employed in the preceding studies in this series (Sippel, 1978a, b).

Materials and methods

Reagents

Sources of mercaptoethanol, 1-thioglycerol, 2,3-dimercaptopropanol (BAL) and 1,4-dithiobutane have been given elsewhere (Sippel, 1978a). Except for hydroxymercuritoluene sulphonic acid (Whitmore & Ehrenfeld, 1926), the organomercurials listed in Table 1 were obtained from K & K Laboratories (Plainview, New York, U.S.A.). After methoxyethylmercuric chloride and sodium Mersalyl became unavailable, the latter was replaced with identical results by neutralized Mersalyl acid (mol. wt. 483.9) from Sigma Chemical Co. (St. Louis, Missouri, U.S.A.). A portion of the phenylmercuric acetate was recrystallized from ethanol.*

Neutral formalin solutions

The mercurials were freshly dissolved in phosphate-buffered neutral formalin (Lillie, 1965) at the concentrations shown in Table 1. Sodium Mersalyl was freely soluble and allowed to remain at pH 7.3, whereas Mersalyl acid had to be dissolved first in an excess of N NaOH and, after dilution, readjusted carefully (unstable in acid) to pH 7; hydroxymercuritoluene sulphonic acid was handled in the same way. $HgCl_2$ soon turned cloudy in neutral formalin which was left at pH 6.9. The remaining compounds were rubbed in a few drops of N,N-dimethylformamide (DMF) before dilution to volume with rapid stirring.

To avoid repeated weighings during extensive testing of the slightly volatile methylmercuric chloride, 0.2 ml aliquots of a 500 mM refrigerated stock in DMF were diluted as needed with 20 ml neutral formalin. Spent fixative was decomposed by strong HCl to $HgCl_2$ before discarding.

Survey of properties

Wedges of liver from a rat killed by cervical section were used to compare the penetrabilities of the various mercurials and dissociabilities of the resulting mercaptides. The pieces were fixed

*Mercurials in general when dissolved in organic solvents can cause severe burns and possibly systemic poisoning. Handling of especially alkylmercurials with disposable gloves and syringes under adequate ventilation is advisable (see Discussion).

for 5 h with constant agitation, washed in running tap water or many changes of distilled water and processed to paraffin in the usual way. Sections cut at 6 μm were stored for different periods before and after being floated from distilled water onto slides coated with formalin–gelatin (Pearse, 1968) or chrome–gelatin (Rogers, 1973).

Just before the application of single staining methods for thiols, disulphides and both together (Sippel, 1978b), the de-waxed sections were exposed to an acidified solution of a mercaptan (see Results); this step was omitted when just unblocked thiols were to be shown. Either ethanol or 2-propanol, required to dissolve BAL and dithiobutane, regularly was included as co-solvent; only 2-propanol (50%) was used for rinsing to minimize breakdown of the staining solution that followed (Sippel, 1973).

Practical trials

Composite paraffin sections of formalin-perfused rat organs were prepared as before (Sippel, 1978a, b) with the nature of the mercurial and fixing conditions varied (see Results). Sets of fresh samples also were fixed directly in neutral formalin, certain of the mercurial formalin mixtures, Carnoy's ethanol–chloroform–acetic fixative and a solution of 2% trichloroacetic acid and 5% $HgCl_2$ in 10% formalin (essentially the fluid of Romeis, 1968, endpaper chart). Fixation for 16–72 h in the first two and 4–5 h in the others was followed by appropriate washes avoiding the use of tap water. Thiols and disulphides were visualized by both single and dual staining procedures (Sippel, 1978b).

Chemical analysis

Approximately 5 mm cubes of liver were taken from rats that had been anaesthetized with chloral hydrate and perfused for 3 min with 0.9% NaCl. As half started fixing in methylmercuric formalin, three batches of the remaining pieces were homogenized in nine volumes of cold 2.2% sulphosalicylic acid; non-protein thiol was measured on the supernatants by Ellman's (1959) method. Each precipitate, washed by and resuspended in ethanol, was divided into two parts of which one was redispersed directly into cold 5 mM disodium EDTA; this was sampled for duplicate determinations of protein thiol (filtered after colour development) and dry weight (corrected for EDTA). Before measurement of total protein thiol and dry weight in the other portion it first was suspended for 1 h in 0.25% (v/v) tri-*n*-butylphosphine (in 60% ethanol containing 0.02 M sodium barbital-HCl buffer at pH 8.5), reprecipitated by cold sulphosalicylic acid and washed well in ethanol.

After samples that had fixed overnight were washed several hours in distilled water and stored for one day in 70% ethanol, they were homogenized in 50% ethanol containing 0.2% (v/v) mercaptoethanol and 0.1 N HCl. The precipitates were washed repeatedly in ethanol before being suspended in cold sulphosalicylic acid to begin the above analyses.

Results

Entry of mercurials

In sections of liver fixed in dilute mercurial formalin solutions for only 5 h, mercaptide formation was confined to an outer shell of tissue (Fig. 1A). Measurement of the depths reached by the different compounds (Table 1) indicated that $HgCl_2$ and, apart from Chlormerodrin, the uncharged organomercurials (the salts themselves are essentially non-ionic) had the greatest penetrability, that is, entry relative to concentration. All of these except tolylmercuric chloride could be made up

Table 1. Penetration of mercurials into rat liver during fixation in neutral formalin for 5 h.

Compound (mol. wt.)	*Approx. concn. of satd. soln.* (mM)	*Concn. used* (mM)	*Approx. depth of blocking* (mm)	*Penetrability* (mm/mM)
Group A				
Methylmercuric chloride (251.1)	30	5	1.0	0.20
Ethylmercuric chloride (265.1)	5	5	0.9	0.18
Mercuric chloride (271.5)	sol.	5	0.7	0.14
2-Methoxyethylmercuric chloride (295.2)	5	5	0.6	0.12
Phenylmercuric acetate (336.8)	5	satd.	1.3	0.26
Sodium Mersalyl* (505.9)	sol.	20	0.6	0.03
Group B				
4-Tolylmercuric chloride (327.2)	0.5	satd.	0.15	0.30
Chlormerodrin* (367.2)	10	10	0.35	0.03
Sodium 4-chloromercuribenzoate (379.1)	0.5	satd.	0.05	0.10
2-Hydroxymercuritoluene-4-sulphonic acid (388.8)	2.5	2.5	0.1	0.04

*Synonyms, structures and properties are given in the Merck Index (Stecher, 1968).

to at least 5 mM levels and comprised the majority of compounds diffusing 0.6 mm or further in 5 h (Group A). Mersalyl, despite low penetrability consequent to its negative charge and high molecular weight, could be made as effective by raising its concentration to 20 mM.

Organomercurials seemed to penetrate about twice as fast as might be predicted when they were applied with an undissolved excess present, either as a matter of convenience or in the case of phenylmercuric acetate to compensate for its conversion by tissue salts to the much less soluble chloride. Nonetheless, tolylmercuric chloride and especially chloromercuribenzoate did not enter liver at a practical rate; though fairly soluble, hydroxymercuritoluene sulphonate was not likely to do so even if it were applied in a saturated solution. Chlormerodrin fell in this same class (Group B) because of its unexpectedly low penetrability which, together with its being much less soluble than reported (Stecher, 1968), suggested that the sample was not authentic or had decomposed.

When a variety of rat organs was fixed in Group A solutions for 16–24 h with occasional stirring, all compounds except Mersalyl blocked thiol staining throughout the duodenum and pancreas, up to 4–5 mm thick pieces of liver and kidney, and hemi-sected samples of brain and tongue. Mersalyl quite often failed to reach the centres of brain pieces until 36–48 h and even then did not block all thiols in the lingual papillae. (It alone among organomercurials also appreciably extracted hemes as tissues were fixing). Neither 5 mM $HgCl_2$ nor any organomercurial fully penetrated the eye lens within 72 h whereas Romeis' fluid (184 mM $HgCl_2$) completely blocked thiols in all tissues by 4 h.

Dissociability of mercaptides. Mercurial artifacts

Free thiol groups were regenerated (Fig. 1B) when organomercurial-protected sections were exposed for 1–2 min to 0.1–0.2% (v/v) mercaptoethanol or thioglycerol acidified with 0.1 N HCl. Treatment with stronger solutions for up to 30 min did not increase the staining intensity which was independent of the mercury compound used and uniform (provided the tissues were washed in distilled water; see below) throughout the protected zones; the latter, however, did not extend quite as deeply as blocking (cf. Fig. 1A). Staining in the unblocked centres also became somewhat darker. Both of these areas (and sections of tissues fixed in formalin alone) were stained with slightly greater intensity after exposure to comparable solutions of BAL or dithiobutane, probably because of thiolation (Sippel, 1978a). None of the four mercaptans applied in the presence of HCl for up to 16 h reduced disulphide bonds, but when more weakly acidified by 0.1 N acetic acid they induced considerable staining in essentially thiol-free structures such as the lens capsule.

It was consistent with the known lability of aklylmercuric mercaptides to halogen acids that virtually all of the thiol staining of tissues fixed in the presence of methyl- or ethylmercuric chloride could be regenerated by 0.1 N HCl alone. However, to regain thiols in the outermost 50–100 μm layer a mercaptan such as mercaptoethanol

had to be added. The inference that the mercaptides initially formed there had been transformed by traces of mercuric ion to mercuric mercaptides was supported by experiment: only mercaptans were effective in regenerating any staining after such sections were treated as briefly as 1 min with 5 mM $HgCl_2$ at pH 7 (phosphate buffer).

When the above mercuration was extended to 1 h, however, even exposure to mercaptoethanol failed to regenerate some of the thiol groups. Similarly in liver fixed for 5 h in neutral formalin with 5 mM $HgCl_2$, the staining regenerable by mercaptoethanol was diminished in the superficial 2–3 cell layers; a few branched clumps of black granules also were present in this zone. Both defects extended farther into tissues fixed for longer times, especially when 50 mM $HgCl_2$ was used, and no thiol staining at all could be regained about each of the deeper lying deposits. Although BAL was more effective than mercaptoethanol, it too did not return staining fully.

Small spherical clumps of granules were numerous in all organs but particularly brain after fixation in neutral formalin containing phenylmercuric acetate, whether or not it had been recrystallized. Thiols, though not disulphides, were demonstrable in their immediate vicinities. The same type of artifact appeared occasionally in tissues fixed in the presence of Mersalyl or methoxyethylmercuric chloride.

Action of formaldehyde

Compared to peripheral areas in which mercaptides had been formed, the central parts of liver sections reached by just formalin stained more weakly for thiols (Fig. 1B) and more strongly for disulphides (Fig. 1C). The major factor undoubtedly was autoxidation of the unprotected thiol groups. However, the deficiency of thiol plus disulphide staining (Fig. 1D) in the centres indicated the presence of some products resistant to dissociation by mercaptoethanol as well as to reduction by tri-*n*-butylphosphine. It was not established whether they were thiols irreversibly complexed with formaldehyde or oxidized beyond the disulphide level to sulphur acids.

Evidence of temporary protection of thiols through mercaptalization came indirectly from varying the onset of mercaptide formation. After it became the routine practice to pre-fix tissues by perfusing anaesthetized rats with neutral formalin for 5 min (after flushing the circulatory system with 0.9% NaCl for 2 min) definitive fixation in the presence of a mercurial usually ensued immediately. However, the tissues could be left in neutral formalin for at least three days without any diminution in their thiol–disulphide ratios shown by dual staining. The opposite extreme of including the mercurial in one or both of the perfusing solutions conversely gave no improvement. It may be added that vasodilation by amyl nitrite, use of depolymerized paraformaldehyde in place of stock formalin, addition of sucrose to the fixative and fixation in the cold likewise had no effect on the quality of staining.

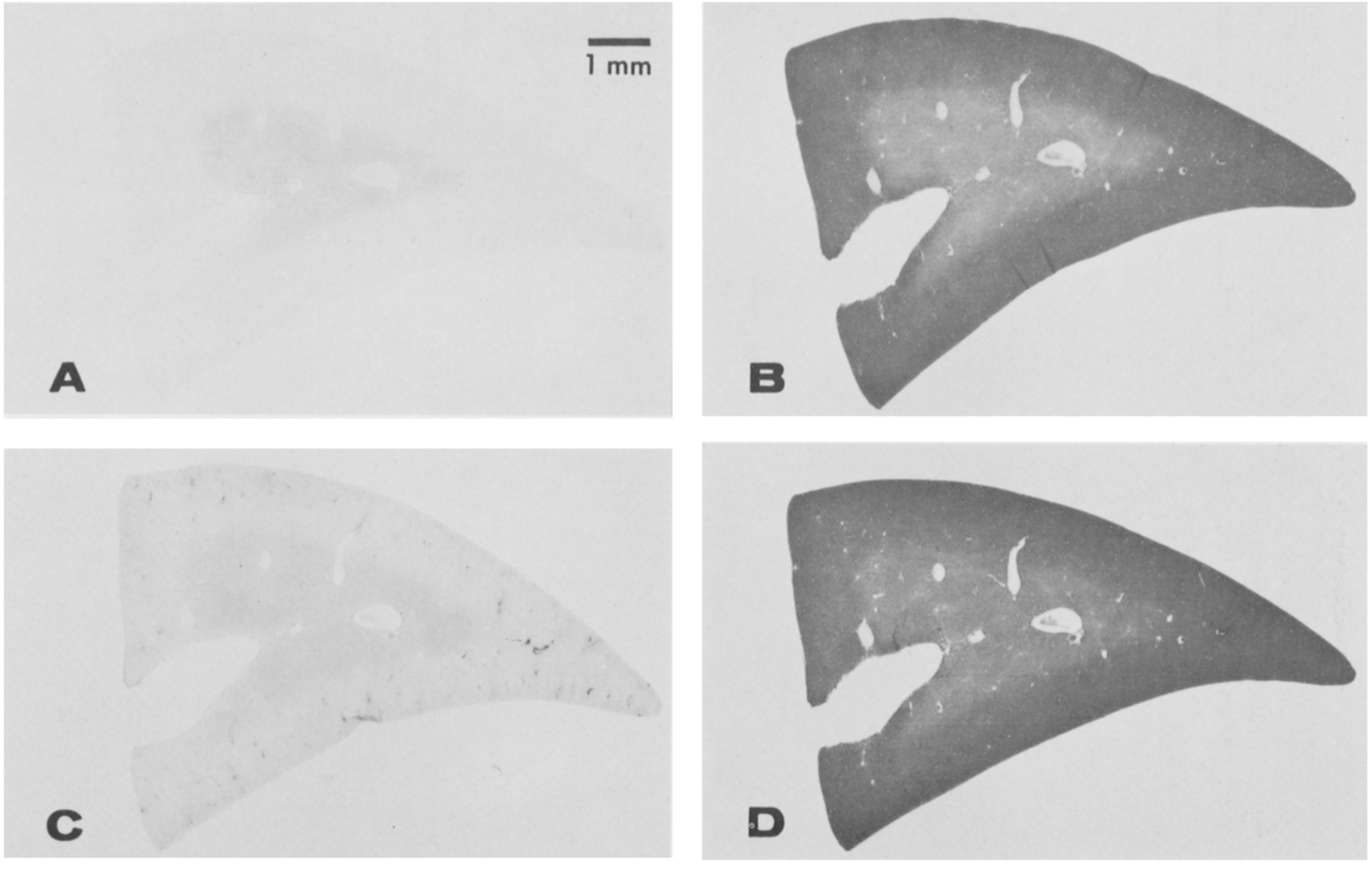

Fig. 1. Rat liver fixed 5 h in neutral formalin saturated with phenylmercuric acetate. Native thiols are shown (A) before and (B) after dissociation of mercaptides; disulphides are shown (C) alone and (D) together with thiols.

Dual staining patterns of nearly all freshly removed organs fixed directly in Group A organomercurial formalin solutions were indistinguishable from those of perfused material. The one exception noted was the tongue in which the disulphide level was elevated slightly in the basal layers of the epithelium. Presumably entry of not only the mercurial but more importantly formaldehyde was impeded by the overlying keratin.

Efficiency and stability of protection

Chemical analyses of liver fixed in methylmercuric formalin showed that mercaptide formation and its subsequent reversal by excess mercaptoethanol preserved the natural protein thiol–disulphide ratio (Table 2). The exact agreement was somewhat fortuitous in view of the modest precision of the dry weight measurements, made because colorimetric protein analysis (for example, biuret) was impossible on formalin-fixed tissue and Kjeldahl nitrogen determination was not practical at the time. The increase in thiol concentration in fixed tissues by 10–15 nmol/mg was thought to reflect extraction of some acid-precipitable component of the dry weight rather than cross-linking of glutathione to protein.

The original levels of thiol stainability were maintained for over four years in both free and mounted paraffin sections of methylmercury-protected tissues. No differences were noted between sections mounted on slides coated with formalin–gelatin and chrome–gelatin. Fixed and washed tissues could be made into frozen sections or stored in 70% ethanol for several weeks before paraffin embedding without any change in thiol stainability. However, if the washing was carried out overnight in tap instead of distilled water, mercaptides in the outermost cell layer were converted to disulphides to a depth of 5–10 μm.

Mercuration of disulphides

Sections of tissues fixed in Romeis' fluid were, upon dual staining (thiols blue, disulphides red), uniformly bluer than after organomercurial formalin fixation.

Table 2. Stabilization by protective fixation of the protein thiol-disulphide ratio in rat liver.

Sample		*Non-protein thiol* (nmol/mg)*	*Protein thiol* (nmol/mg)*		
			Native (unreduced)	*Total (reduced)*	%
Rat 1	Fresh	31 (28–34)	98 (95–102)	111 (109–112)	88
	Fixed†	0	111 (111–112)	126 (122–133)	88
Rat 2	Fresh	38 (36–40)	90 (86–94)	99 (94–103)	91
	Fixed†	0	100 (95–103)	110 (107–112)	91

*Average (range) of duplicate determinations on three sub-samples.
†Methylmercuric formalin for 16 h; mercaptides dissociated by mercaptoethanol-HCl.

Amongst structures containing mostly disulphides, only connective tissue fibres and blood plasma were stained clear red. Duodenal goblet cell mucin and the somewhat erratically preserved granules of the pancreatic acini and duodenal Paneth cells were bluish red; the lens capsule, Descemet's membrane and lingual keratin were distinctly purple in contrast to their brilliant red coloration after fixation either in Romeis' fluid lacking $HgCl_2$ or in Carnoy's fluid.

This evidence of cleavage of some disulphide bonds by mercuric ion suggested the need for closer study of disulphide-rich structures fixed in organomercurial formalin. However, only the pancreatic secretion and lingual keratin showed unmistakable traces of thiol staining (cf. also Sippel, 1978b). Although their absence after fixation in neutral formalin alone could have come about through lack of protection, it is taken provisionally to indicate the artifactual origin of these thiols.

Discussion

Evidently $HgCl_2$ at neutrality slowly forms thiol complexes resisting dissociation by acidified monothiols. In opposition to earlier views (Pearse, 1968; Hopwood, 1972), it seems not unlikely that they are mercuric bis-mercaptides, but the presence of formalin complicates the issue by depositing mercurous chloride and elemental mercury (Lillie, 1965). Whatever the nature of the products they are only partly susceptible to an avid chelator like BAL; furthermore the use of a dithiol is countermanded for critical work by its potential for thiolating (Sippel, 1978a). It is clear that, if thiols are to be regenerated selectively, $HgCl_2$ must be applied in strongly acid media typified by Romeis' fluid to form what are probably mercuric mono-mercaptides.

The severity of the accompanying cleavage of disulphide bonds, wherein three-quarters of the products also end up as mercaptides (Jocelyn, 1972), might be lessened by decreasing the $HgCl_2$ concentration. This line was not pursued because Romeis' fluid as formulated is already unreliable for preserving some disulphide-rich organelles. Moreover, recent trials with Carnoy's fluid have indicated that solutions of $HgCl_2$ as weak as 10 mM, though giving the wanted type of thiol protection, still cleave disulphides significantly; $HgCl_2$ has the further drawback in this fixative of forming mercurial deposits. However, phenylmercuric acetate showed neither defect and would seem a useful additive for situations in which extraction of proteins by a non-formalin acidic fixative is acceptable.

Monofunctional mercury compounds represented by the organomercuric salts, while causing at most limited and not wholly anticipated disulphide cleavage (cf. Jocelyn, 1972), protect thiols without sacrificing the advantage of fixation in neutral formalin. The underlying reactions (Fig. 2) are almost certainly indirect inasmuch as the more rapidly diffusing formaldehyde would convert the thiols first to at least hemimercaptals. Whether some of these go on as illustrated to form more stable full mercaptals is debated (Lillie, 1965; Pearse, 1968; Jocelyn, 1972), yet strongly

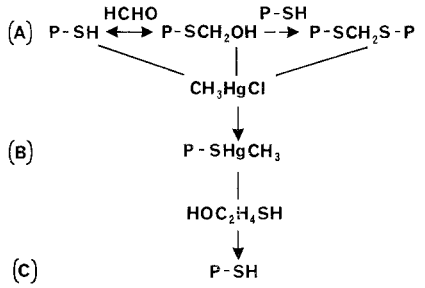

Fig. 2. Reactions of protein thiol with formaldehyde (A) and, together with the products, with methylmercuric chloride to form mercaptides (B) subsequently dissociable by mercaptoethanol to regenerate free thiols (C).

suggested by the present observations. Nonetheless, all formaldehyde adducts evidently are convertible to highly stable organomercuric mercaptides in reactions analogous to those undergone with mercuric ion (Makarova & Nesmeyanov, 1967; Elleder & Lojda, 1972). Unless washing to tap water causes the most superficial ones to be supplanted by autoxidizable transition-metal mercaptides (cf. Friedman, 1973), the protecting groups remain intact until removed by an excess of a mercaptan (Fig. 2).

Action within the day or so typically allowed for fixation in neutral formalin requires that the organomercurial be reasonably soluble, especially if it is negatively charged. Five of the nine compounds tested satisfy, but further selection among them involves considering two opposing aspects of their stability.

Alkylmercury salts in general are sufficiently stable under neutral conditions that methyl- and ethylmercuric chloride give no detectable artifacts arising from the reduction of mercuric ion by formaldehyde. However, in the body this stability, compounded by a tendency to dissolve in lipids, greatly delays breakdown to the more rapidly excreted and (in that sense only) less toxic mercuric ion. Methylmercury is notoriously neurotoxic if ingested at a sustained rate of 0.3 mg (as Hg) per day by a 70 kg man (Nelson *et al.*, 1971); it may be noted that 20 ml of 5 mM methylmercuric chloride contains the equivalent of 20 mg Hg.

Arylmercurials are much less stable and widely regarded as not so hazardous (Goldwater, 1972), although the unusual lability of phenylmercuric acetate makes this particular one nearly useless as a thiol protecting agent in neutral formalin. That leaves the alkoxyalkylmercurials (for example, Mersalyl and methoxyethylmercuric chloride if available) which, as the basis of their diuretic action and relatively low toxicity, readily decompose in mild acidity and especially when complexed to thiols (Friedman, 1973). But as they create very few mercurial deposits in neutral formalin, such compounds are suitable for routine use.

Because mercurial diuretics are no longer prescribed, even the free acid form of Mersalyl soon may become unavailable so that further screening of mercury

compounds seems warranted. Casual observations already have suggested that fluorescein mercuric acetate can meet the requirements established in this study.

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References

BARRNETT, R. J., MARSHALL, R. B. & SELIGMAN, A. M. (1955) Histochemical demonstration of insulin in the islets of Langerhans. *Endocrinology* **57**, 419–38.

BARRNETT, R. J. & SELIGMAN, A. M. (1954) Histochemical demonstration of sulfhydryl and disulfide groups of protein. *J. natn. Cancer Inst.* **14**, 769–803.

ELLEDER, M. & LOJDA, Z. (1972) Studies in lipid histochemistry. VII. Interference of protein-bound formaldehyde in the plasmal and some other reactions using Schiff's reagent. *Histochemie* **30**, 325–8.

ELLMAN, G. L. (1959) Tissue sulfhydryl groups. *Archs Biochem. Biophys.* **82**, 70–7.

FRIEDMAN, M. (1973) *The Chemistry and Biochemistry of the Sulfhydryl Group in Amino Acids, Peptides and Proteins.* Oxford: Pergamon.

GOLDWATER, L. J. (1972) *Mercury*. p. 172. Baltimore: York Press.

GOMORI, G. (1956) Histochemical methods for protein-bound sulfhydryl and disulfide groups. *Q. Jl microsc. Sci.* **97**, 1–9.

HOPWOOD, D. (1972) Fixation with mercury compounds. *Prog. Histochem. Cytochem.* **4**, 193–234.

JOCELYN, P. C. (1972) *Biochemistry of the SH Group.* London: Academic Press.

LILLIE, R. D. (1965) *Histologic Technic and Practical Histochemistry*. 3rd edn. New York: McGraw-Hill.

MAKAROVA, L. G. & NESMEYANOV, A. N. (1967) The organic compounds of mercury, Vol. 4. In *Methods of Elemento-organic Chemistry* (edited by NESMEYANOV, A. N. and KOCHESHKOV, K. A.). Amsterdam: North-Holland.

NELSON, N., BYERLY, T., KOLBYE, A., KURLAND, L., SHAPIRO, R., SHIBKO, S., STICKEL, W., VAN DER BERG, L. & WEISSLER, A. (1971) Hazards of mercury. *Envl Res.* **4**, 1–69.

PEARSE, A. G. E. (1968) *Histochemistry, Theoretical and Applied*, 3rd edn., Vol. 1. Boston: Little, Brown.

ROGERS, A. W. (1973) *Techniques of Autoradiography*, 2nd edn. New York: McGraw-Hill.

ROMEIS, B. (1968) *Mikroskopische Technik*, 16th edn. Munich: Oldenbourg.

RUDALL, K. M. (1952) The proteins of the mammalian epidermis. *Adv. Protein Chem.* **7**, 253–90.

SIPPEL, T. O. (1973) The histochemistry of thiols and disulphides. I. The use of N-(4-aminophenyl)maleimide for demonstrating thiol groups. *Histochem. J.* **5**, 413–23.

SIPPEL, T. O. (1978a) The histochemistry of thiols and disulphides. II. Methodology of differential staining. *Histochem. J.* **10**, 585–95.

SIPPEL, T. O. (1978b) The histochemistry of thiols and disulphides. III. Staining patterns in rat tissues. *Histochem. J.* **10**, 597–609.

STECHER, P. G., ed. (1968) *The Merck Index*, 8th Edn. Rahway: Merck & Co.

WHITMORE, F. C. & EHRENFELD, L. (1926) The mercuration of aromatic sulfonic acids. *J. Am. chem. Soc.* **48**, 789–91.