# The histochemistry of thiols and disulphides. I. The use of N-(4-aminophenyl)maleimide for demonstrating thiol groups

T. O. SIPPEL*

*Department of Anatomy, University of Dundee, Scotland*

**Synopsis.** A new bifunctional reagent, N-(4-aminophenyl)maleimide, is described. It reacts with thiol groups in tissue sections under mild conditions and with a degree of specificity equivalent to that of reliable organomercurials. The bound maleimide can be diazotized and coupled at room temperature so as to give strongly coloured mono- or polyazo derivatives without any appreciable background colour. This enables thiols in low concentrations to be detected in paraffin sections of either formalin- or Carnoy-fixed tissue.

## Introduction

Seligman *et al.* (1954) proposed the use of naphtholic maleimides for the histochemical demonstration of protein thiol groups. N-(4-Hydroxynaphthyl)maleimide gave satisfactory staining when the bound reaction product was visualized by coupling it with a diazonium salt to form an azo compound. The corresponding isomaleimide yielded a more intense colour (Tsou *et al.*, 1955) and was found to be useful for detecting thiols on chromatograms (Price & Campbell, 1957) and in tissue sections (Pearse, 1968). However, neither reagent either became as popular with histochemists as the similar disulphide (Barrnett & Seligman, 1952) or appears to be available commercially at present.

More recently a number of substituted N-phenylmaleimides have been investigated for the labelling or blocking of cysteinyl residues in native proteins (Burley & Haylett, 1959; Witter & Tuppy, 1960; Holbrook *et al.*, 1966; Kanaoka *et al.*, 1970). One of the reagents tested was N-(4-nitrophenyl)maleimide (NPM in Fig. 1) which, although unreactive toward the essential thiols of lactic and glyceraldehyde-3-phosphate dehydrogenases (Holbrook *et al.*, 1966), was found early in the present study to react with tissue sections. Sodium dithionite at pH 8 (Riordan & Sokolovsky, 1971) proved very effective in reducing the bound nitro group so that the reaction sites could be visualized by diazotization followed by coupling to a naphthol. The labelling obtained was largely

* *Permanent address:* Department of Anatomy, University of Michigan, Ann Arbor, Michigan 48104, U.S.A.

413

assignable to thiols but was not prevented entirely by pre-treatment of the section with a methylmercury salt. Upon prolonged incubation with NPM, this extraneous colouration built up enough to be detected in areas devoid of thiol groups, indicating some non-specific action of the maleimide.

A more selective and convenient reagent was sought, therefore, in the amino analogue *N*-(4-aminophenyl)maleimide (APM in Fig. 1) described in this paper. The compound has been claimed as the product of cyclicizing 4-aminomaleanilic acid in acetic anhydride at room temperature (Kojima *et al.*, 1966), but in my hands this procedure gave the expected acetylated derivatives. Cyclodehydration in hot polyphosphoric acid (Kanaoka *et al.*, 1970) is the only means that has been successful so far in transforming the maleanilic acid into the desired reagent, albeit in low yield because of hydrolysis and polymerization.
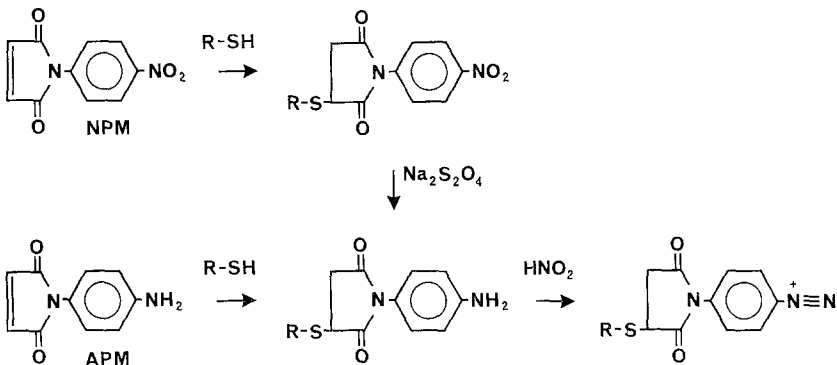


*Figure 1.* Reaction of *N*-(4-aminophenyl)maleimide (APM) with a protein thiol group to give a bound diazotizable amine, which is also formed by dithionite reduction of the reaction product from *N*-(4-nitrophenyl)maleimide (NPM).

APM applied to tissue sections for limited times reacts exclusively with thiol groups and has other advantageous properties as a histochemical reagent. Modifications of the diazotization and coupling procedures introduced by Danielli (1950) allow the reaction product to be rapidly visualized, for which a strong blue disazo label (Fig. 2) is especially suitable.
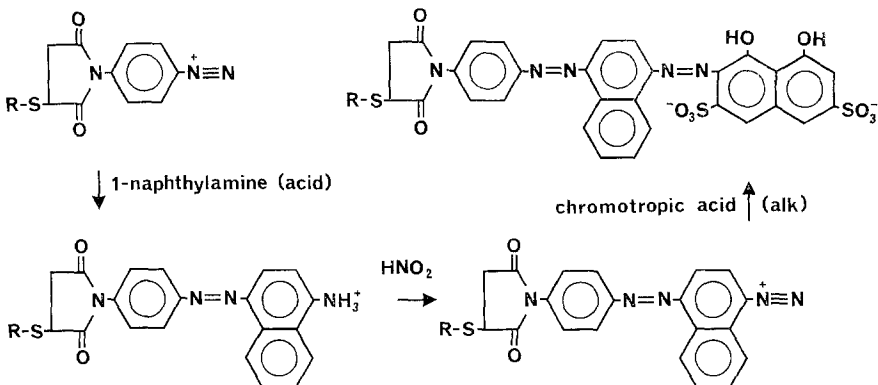


*Figure 2.* Formation of a blue disazo label by coupling the diazotized bound intermediate shown in Fig. 1 with 1-naphthylamine, rediazotization and coupling with chromotropic acid.

## Materials and methods

*Synthesis of the reagent*

*N*-(4-Aminophenyl)maleimide is now available from Koch-Light Laboratories, Coln-brook, Bucks, England. Preliminary samples of the commercial product proved equivalent to the best batches prepared as follows:

(*a*) Dry *p*-phenylenediamine (21.6 g, 0.2 mol), recrystallized twice from water, was dissolved in tetrahydrofuran (550 ml), cooled to 10°C in an ice bath and stirred rapidly during the slow addition of maleic anhydride (19.6 g, 0.2 mol) in chloroform (150 ml). The reaction mixture was stirred for 10 min in the ice bath and the precipitate then allowed to settle before the supernatant was decanted and replaced three times with toluene (100 ml each). The product was collected and air-dried to yield 39.2 g (95% of theoretical) 4-aminomaleanilic acid as a bright orange powder with traces of a yellow monohydrate.

(*b*) Polyphosphoric acid (50 g, 85% $P_2O_5$) was heated in an oil bath at 150–155°C and stirred vigorously as 4-aminomaleanilic acid (5.15 g, 0.025 mol) was added in portions. The evanescent yellow solution was maintained at 140–145°C for 30 min with occasional hand stirring, cooled to 100°C and poured onto crushed ice (100 g) in a large beaker. The mixture was stirred with water (400 ml) at room temperature until the gum decomposed and then cooled with ice (500 g) and adjusted to pH 4.3 with most of a freezing mixture of 6 N NaOH (100 ml) and ice (200 g). Acid-washed Kieselguhr (12 g) was mixed in, filtered off with suction and washed with cold 0.5 M $NaH_2PO_4$ (100 ml).

The cold filtrate was shaken with two 550 ml portions of ethyl acetate; the combined extract was washed with water, dried with $Na_2SO_4$ and boiled down on the steam bath to a crystallizing residue which was then dissolved in hot toluene (400 ml). The solution was cooled to 15°C, filtered, concentrated to 40 ml and diluted with cyclohexane (120 ml). After cooling in ice, the precipitate was collected and dried to yield 1.27 g (27% of theoretical) of fluffy dull orange crystals. Thin layer chromatography (ethyl acetate on Merck silica gel $GF_{254}$ film) with detection of diazotizable amines (modified from Ratney, 1967) showed one major component with $R_f$ 0.8 (reddish orange colour on coupling with 1-naphthol) and 5 or 6 contaminants with lower mobilities.

An analytical sample was obtained from the crude maleimide (250 mg) dissolved in warm benzene (50 ml) and applied to a 2.5 cm wide column of 60/200 mesh silica gel (100 g) poured in benzene. The first yellow fraction (85 ml) eluted with 1:1 (v/v) benzene:ethyl acetate was taken to near dryness, dissolved in toluene and precipitated with cyclohexane to yield 185 mg of yellowish orange needles, m.p. 174–175°C (uncorr.). U.v. absorption maxima (0.1 mM in dioxane): 252 and 295 nm ($\varepsilon$ 16600 and 2140, respectively). On analysis, found: C, 64.04; H, 4.31; N, 14.60%. $C_{10}H_8O_2N_2$ requires: C, 63.82; H, 4.28; N, 14.89%.

*Preparation of tissue sections*

Samples of the liver, kidney, duodenum with pancreas, tongue and cerebellum and a whole eye (fundus incised) from an adult male rat were fixed overnight at 4°C in Carnoy's fluid (6:3:1 by vol. ethanol:chloroform:acetic acid) and washed in ethanol. Duplicate pieces were fixed similarly in phosphate-buffered neutral 10% formalin, washed in tap water and dehydrated in graded ethanols. The tissues were further processed together through ethanol and toluene to paraffin wax (m.p. 61°C) in which they were infiltrated

*in vacuo* and then imbedded as a composite. Sections were cut at 6 μm, with soaking of the block face in water to save the eye lenses, and floated from a water bath at 45°C on to acid-cleaned (conc. $H_2SO_4$-$HNO_3$) glass slides. After drying for at least 1 hr at 45°C the preparations were stained immediately or stored for up to two weeks at room temperature.

In preliminary studies many of the common variations on these procedures were carried out with tissues from mice and hamsters as well as rats, usually after fixation in Carnoy's fluid for 2 hr at room temperature.

*Standard staining procedure*
Slides were deparaffined in xylene and washed in ethanol. Coating at this point with 1% celloidin in acetone-ether-ethanol prevented the later loss of inadequately dried sections but usually was unnecessary. After the slides were rinsed in 50% 2-propanol, thiol-containing sites were stained blue (Fig. 2) when the following steps were carried out at 18–22°C:

1. Treat for 30–60 min in 5 mM APM (suspend 38 mg crude maleimide in 20 ml 2-propanol and stir in 20 ml 0.05 M sodium phosphate buffer, pH 6.0; undissolved material need not be filtered off). Rinse in 50% ethanol, water and 0.1 N HCl.
2. Diazotize for 2 min in 0.05 N $HNO_2$-HCl (add 20 ml 0.1 M $NaNO_2$ to 20 ml 0.2 N HCl; discard after 30 min). Rinse in 0.1 N HCl and immerse in 0.5% sulphamic acid for 30 sec.
3. Couple for 2 min in 0.5% 1-naphthylamine in 0.1 N HCl (if pink, replace from stock). Rinse in two changes of 0.1 N HCl and repeat step 2.
4. Couple for 5 min in fresh 0.5% chromotropic acid (as disodium dihydrate) in 0.1 M sodium acetate buffer, pH 4.5. Wash well in water.
5. Counterstain, if required, for 5 min in 0.5% Safranin T or Neutral Acriflavine in 1% acetic acid and differentiate in 1% acetic acid.
6. Dehydrate in graded ethanols or, if counterstained, directly in acetone. Clear in xylene and mount in a synthetic resin.

*Variants and tests*
Naphtholic couplers tried in place of chromotropic acid (1,8-dihydroxynaphthalene-3,6-disulphonic acid) were 2-hydroxy-3-naphthoic acid, S-acid (1-amino-8-hydroxy-naphthalene-4-sulphonic acid), H-acid (1-amino-8-hydroxynaphthalene-3,6-disul-phonic acid) and *N*-acetyl-H-acid (Fierz-David & Blangey, 1949), usually obtained as sodium salts and applied at pH 4.5 to 8.5. Formation of a disazo label in one step was accomplished by coupling at pH 8.5 to disodium 1-amino-8-hydroxy-2-(4'-nitro-benzeneazo)naphthalene-3,6-disulphonate ('Nitro Red'), isolated as the monoazo intermediate of the synthesis of Naphthol Blue Black B (Fierz-David & Blangey, 1949).

Mono- and polyazo labels were obtained by the omission or repetition of couplings in the standard staining procedure (see *Results*). When the terminal coupler was 1-naphthylamine the label was also converted to the chloride by dehydration of the section in acetone containing a few drops of conc. HCl in the first change. Red labels were counterstained if required with 0.5% Methylene Blue in 1% acetic acid or with chloro-form-washed 1% aq. Methyl Green.

The reactivity of APM toward tissue sections was examined when the conditions of

its application were varied with respect to concentration, time, pH, buffer and co-solvent (see *Results*). The thiol groups in control sections were first blocked for 30 min or longer with 5 mM *N*-ethylmaleimide (NEM), applied in the manner of APM in the standard procedure, or for 5 min or longer with an organomercurial at a concn. of 0.5 mM in 0.05 M sodium phosphate buffer, pH 7.0. For the latter purpose, methyl-mercuric iodide was synthesized (Hamilton, 1960; Leach, 1965) and used to prepare the nitrate (modified from Hamilton, 1960). Commercial preparations of methylmercuric chloride and *p*-chloromercuribenzoic acid were also employed. The chloride and iodide of methylmercury were dissolved with 25% (v/v) *N,N*-dimethylformamide in the buffer. Excess blocking reagent was washed out with water and 50% 2-propanol before exposure of the sections to APM. Colour was finally developed as in the standard staining procedure.

The stability of the blue-labelled reaction product toward exogenous thiols was tested by treating the stained sections for 16 hr with 1% (v/v) 2-mercaptoethanol or 3-mercapto-1,2-propanediol (1-thioglycerol) in equal parts of 2-propanol and 0.05 M sodium barbital buffer, pH 8.0; 0.1% (w/v) dithiothreitol was applied in the same way.

## Results

### Colour development

The disazo label (Fig. 2) formed on the reaction product of APM in the standard staining procedure had a strong slightly reddish blue colour easily detected under the microscope. The few tissues such as the corneal stroma (Fig. 3) that were presumably devoid of thiol groups were colourless or sometimes very faintly grey. This background, which could tend to mask pale blue staining of keratinized structures (Fig. 4), was minimized by employing pure coupling reagents in fresh solutions. There was some evidence, how-ever, that traces of *p*-phenylenediamine in APM solutions applied for unusually long periods added a purple component to the background. *N*-Acetyl-H-acid gave almost exactly the same results as chromotropic acid. With H-acid itself, the colour was a more greenish blue but the grey background also seemed more pronounced. Other terminal couplers tried did not give satisfactory colours. Coupling to 'Nitro Red' produced a deep blue label marred by red background staining that was especially prominent in nuclei and not wholly removable, *e.g.* with 1% (v/v) triethylamine in 95% ethanol. Deliberate red (Safranin) or even yellow (Acriflavine) counterstaining of nuclei in standard pre-parations inevitably obscured some details of thiol distribution and was reserved for orienting studies.

The times specified for the colour development steps were adequate for all tissues except those containing an unusually high concentration of thiol groups in structurally dense areas. Colouration of the pre-keratin zones of the lingual papillae, for example, stopped at an orange-yellow stage through failure of chromotropic acid to couple to all diazotized sites. Prolonging the final coupling to 1 hr (at 0–4°C) could not quite com-plete the formation of the blue label in these places. In general, the anticipated break-down of diazotized intermediates at room temperature did not appear to be a problem since the results were no different when all the steps of colour development were carried out (for longer times) in iced solutions. Routinely, nevertheless, after diazotization the rinses were kept brief, bright light was avoided and acidity was maintained even into
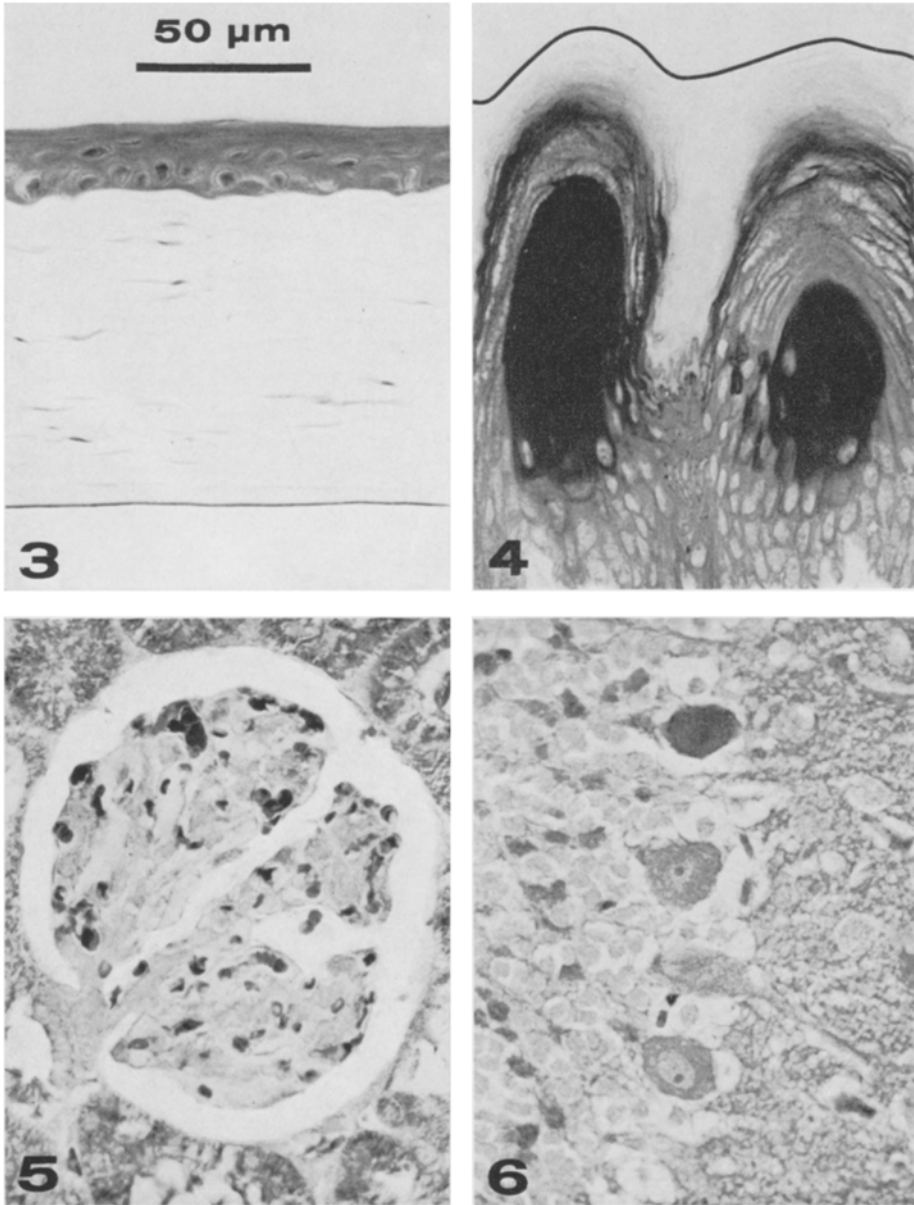
*Figures* 3–8. Rat tissues stained for thiol groups by the standard APM procedure. The micrographs were taken in red light (Corning filter 2–62) and are all at the same magnification.

*Figure* 3. Cornea (formalin fixed). The stroma and Descemet's membrane are unstained; the endothelium is strongly stained.

*Figure* 4. Tongue (fixed in formalin). Two filiform papillae cut transversely have densely stained epithelial cores and are overlain by a lightly stained cornified layer (below outline).

*Figure* 5. Kidney (fixed in formalin). Erythrocytes are strongly stained.

*Figure* 6. Cerebellum (fixed in formalin). The dark and light Purkinje cells are near the extremes of thiol staining seen in this type of neuron.
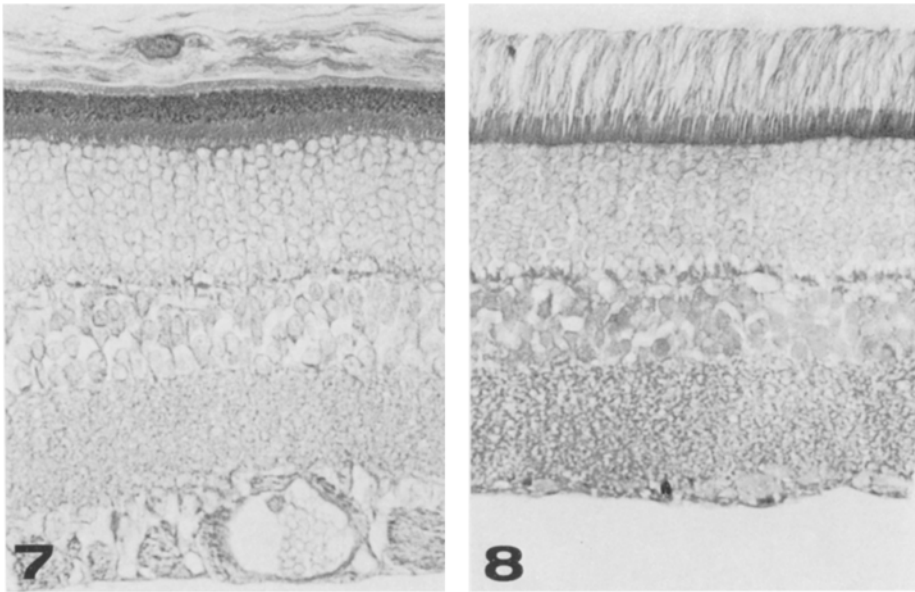
*Figure* 7. Retina (fixed in Carnoy's fluid). Rod outer segments (cut obliquely here) are more heavily stained than the inner segments; nuclei in the inner nuclear layer are faintly granular whereas those of the outer nuclear layer as well as erythrocytes (vessel at bottom) appear empty. *Figure* 8. Retina (fixed in formalin). The long outer segments are relatively pale whereas the inner segments are little different from those in Carnoy-fixed material. Staining is darker in the remaining layers.

the final naphtholic coupling. Indeed, the extent of coupling by chromotropic acid was enhanced below neutrality without any appreciable decrease in the coupling rate compared to that obtained in the conventional alkaline range.

Different arrangements in the diazotization and coupling sequence afforded some choice of final colour. Insertion of an extra intermediate reaction with 1-naphthylamine produced a greenish-blue trisazo label. Direct coupling to chromotropic acid, i.e. omission of 1-naphthylamine entirely, gave a red colour that was not as easily seen under the microscope. Much better in this range was the monoazo compound formed with 1-naphthylamine alone: although weakly orange as a free base, it could be converted to an intense bluish red chloride that was stable indefinitely in resin-covered preparations. The disazo label of this type was a dull purple convertible to a dull blue chloride.

*Reactions of APM*

When the standard procedure followed the treatment of sections with 5 mM NEM for 30 min to 2 hr, all staining was prevented except for a pale blue colouration consistently developed (even after the use of 25 mM NEM) in pre-keratin. However, if the exposure to APM was extended beyond 1 hr, an additional faint overall colouration became evident grossly (2 hr) and then microscopically (3–4 hr) in blocked sections. Methylmercuric chloride and iodide (0.5 mM) applied for 10 min were wholly effective in preventing staining by the standard procedure but similarly failed to stop the slow, apparently non-specific, reaction of APM. The remaining, ionic organomercurials also

blocked the short-term reaction of APM except in the deeper lens cortex and pre-keratin zones of the lingual papillae. The sharply delimited blue staining there was not prevented by applying these blocking reagents for 1 hr, with or without *N*,*N*-dimethyl-formamide present in the solutions.

Unblocked sections treated for 30 min with fresh 5 mM solutions of APM seemed to develop maximum colour. The intensity of staining was not appreciably increased by a 2 hr treatment and was only slightly less after 15 min. The time required for full reaction was roughly the inverse of the concentration of APM down to 1 mM, the limit of its solubility in wholly aqueous solutions. A 2 hr treatment with one standard batch kept at room temperature for 15 days and containing a precipitate of 4-aminomaleanilic acid gave acceptable staining without any non-specific reaction in methylmercury-blocked control sections. Although the useful life of APM solutions could be further extended by storage at 4°C, the unpredictable reactivity of stale solutions generally precluded their use for critical studies.

The following variations in the make-up of APM solutions were contraindicated because of reagent instability, poor reaction with thiols, or non-specific staining: pH values below 5 or above 7, citrate-HCl buffer, high chloride levels (tested with added NaCl) and the use of either ethanol or *N*,*N*-dimethylformamide as co-solvent. Ethanol was used in earlier trials but was abandoned when the u.v. spectrum of APM in 95% ethanol was found to change rapidly. This did not occur in 99% 2-propanol or in dry dioxane; the buffered reagent solutions were more stable, however, in the presence of 2-propanol.

None of the exogenous thiols applied at pH 8 for 16 hr caused noticeable fading of stained preparations.

*Preservation of thiol groups*
The stainability of most types of cells was approximately equivalent after fixation of tissues in formalin or Carnoy's fluid. Formalin had the advantage of preserving thiol-rich proteins in erythrocytes and a greater variety of cell nuclei (Figs. 5–8). Carnoy's fluid tended not only to extract these materials but to lead to misleading staining patterns because of shrinkage. The most obvious instance was the extreme shortening of the outer segments of the retinal rods (Fig. 7) so that these were among the most densely stained structures encountered in Carnoy-fixed tissues. After formalin fixation, the outer segments were long and pale (Fig. 8) whereas the inner segments were very similar in the two types of preparation. The staining of both nuclear and fibrous layers of the retina was enhanced, however, by formalin fixation.

No systematic observations were made on the effects of different ways of processing the tissues and sections. The use of high-melting paraffin, of various ribbon adhesives and of ordinary distilled water in the APM and preceding solutions did not appear to influence the staining. Usually the best results were obtained after fixation in the cold and by staining on the day the sections were cut. Whereas no obvious loss of stain-ability occurred when the mounted sections were dried at 45°C for up to 16 hr, storage of the sections in room air over a 2 week period led to progressively lighter staining. Sections subsequently taken from near the cut surface of a paraffin block similarly stored also stained poorly; the previous soaking of the block face with water may have contributed to the instability of the thiol groups, but it had no short-term effect. It may

be added that the wetted tissues swelled in the block and thus yielded sections that were somewhat thinner than those ordinarily cut at the nominal thickness of 6 μm.

## Discussion

The proposed staining method allows thiol groups to be detected in fairly low concentrations. Model experiments indicate that the recommended blue label may be detected unequivocally under the microscope at an absorbance of 0.1 against a colourless background. The molar extinction coefficient of the label (approximately $35\,000$ cm$^2$/mmol) thus provides a lower practical limit of thiol detectability of 3 nmol/cm$^2$ or, in a section 6 μm thick, 5 nmol/mm$^3$; it is assumed that all the underlying reactions are stoichiometric. In a region of ordinary protein density containing, for example, 10% (w/v) solids in the fresh state, the corresponding thiol content in the absence of subsequent shrinkage is 50 nmol/mg of dry material. This is equivalent to one cysteinyl residue in a polypeptide of mol. wt. 20000. The sensivity would be greater in thicker sections or in cells such as lens fibres and erythrocytes which have relatively high protein contents.

Realization of this potential sensitivity requires that the chromogenic reagent add exclusively to thiol groups. That this is the case with APM reacting for limited periods is implied by the complete absence of staining after thiol blockade with non-ionized methylmercury salts. Of the probable complexes formed by monofunctional alkylmercurials, only the mercaptides have significantly low dissociation constants (Hughes, 1957; and references in Leach, 1965). *p*-Chloromercuribenzoic acid is as specific for thiols and, although penetrating some tissues less well because of its charge, might be better for routine testing purposes than the hazardous methylmercury salts.

*N*-Ethylmaleimide also effectively blocked staining in the majority of tissues when followed by the standard procedure. There is considerable evidence, however, that this reagent reacts slowly with protein amino groups (Sharpless & Flavin, 1966, and references therein). It is, therefore, not ideal for testing the specificity of histochemical thiol-staining reagents and especially of the analogous and apparently more reactive APM. The faint but progressive staining obtained in blocked sections overtreated with APM probably reflects the same type of slow non-specific reaction, perhaps compounded by partial reversal of the blockades.

APM has certain advantages over some reagents used for the histochemical demonstration of thiols. The coloured anthraquinonyl derivatives of APM (Sippel, 1969, 1971) must be applied in hot solvents for adequate solubility and even then they react slowly and penetrate poorly into structurally dense tissues. At the cost of requiring the extra labour of visualization, APM is easily dissolved and acts thoroughly and rapidly at room temperature. Further, the labelling of its reaction product on the section does not introduce background colour, as happens when diazonium salts are used for the visualization of bound naphtholic reagents. Lastly, in contrast to the reversal by exogenous thiols of the binding of coloured mercurials (Pearse, 1968), naphtholic disulphides (Barrnett & Seligman, 1952) and surprisingly, naphtholic maleimides (Tsou *et al.*, 1955), the labelled reaction product of APM is stable to such reductants. Methods in which this property is made use of for the differential staining of thiol and disulphide sites on the same section are thus feasible.

Preliminary work along these lines has already shown that more assured preservation

of thiols is necessary than was obtained in the present study. The loss of thiol stain-ability noted during the storage of paraffin-imbedded tissues and of mounted sections (*cf.* also Lillie, 1965) appears to involve to a large extent the oxidation of thiol to disul-phide groups. Such transformations probably occur during the fixation and processing of tissues as well and, at present, limit the significance of the results obtained from any method for staining thiol groups.

## Acknowledgements

## References

BARRNETT, R. J. & SELIGMAN, A. M. (1952). Histochemical detection of protein-bound sulfhydryl groups. *Science, N.Y.* **116**, 323–7.

BURLEY, R. W. & HAYLETT, T. (1959). Preparation and some properties of N-(4-dimethylamino-3:5-dinitrophenyl)maleimide and the corresponding derivative of maleamic acid. *Chem. Ind.* **1959**, 1285–6.

DANIELLI, J. F. (1950). Studies on the cytochemistry of proteins. *Cold Spring Harb. Symp. quant. Biol.* **14**, 32–9.

FIERZ-DAVID, H. E. & BLANGEY, L. (1949). *Fundamental Processes of Dye Chemistry.* New York: Interscience.

HAMILTON, L. D. G. (1960). The estimation of side chain groups in the intact protein. In: *A Laboratory Manual of Analytical Methods of Protein Chemistry* (eds. P. Alexander and R. Block), Vol. 2, pp. 59–100. Oxford: Pergamon.

HOLBROOK, J. J., PFLEIDERER, G., SCHNETGER, J. & DIEMAIR, S. (1966). The importance of SH-groups for enzymic activity. IV. Preparation of the tryptic peptides containing the essen-tial cysteine residues of lactic dehydrogenase, isozymes I and V. *Biochem. Z.* **344**, 1–14.

HUGHES, W. L. (1957). A physicochemical rationale for the biological activity of mercury and its compounds. *Ann. N.Y. Acad. Sci.* **65**, 454–60.

KANAOKA, Y., MACHIDA, M., ANDO, K. & SEKINE, T. (1970). Fluorescence and structures of proteins as measured by incorporation of fluorophore. IV. Synthesis and fluorescence char-acteristics of N-(p-(2-benzimidazolyl)phenyl)maleimide. *Biochim. biophys. Acta* **207**, 269–77.

KOJIMA, K., YODA, N. & MARVEL, C. S. (1966). Base-catalyzed polymerization of maleimide and some derivatives and related unsaturated carbonamides. *J. Polymer Sci.* **4**, 1121–34.

LEACH, S. J. (1966). The estimation of thiol and disulfide groups. In: *A Laboratory Manual of Analytical Methods of Protein Chemistry* (eds. P. Alexander and H. Lundgren), Vol. 4, pp. 3–75. Oxford: Pergamon.

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

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

PRICE, C. A. & CAMPBELL, C. W. (1957). A chromatographic method of analysis for thiols. *Biochem. J.* **65**, 512–16.

RATNEY, R. S. (1967). Nitrogen dioxide as a reagent for the detection of aromatic amines on thin-layer chromatograms. *J. Chromat.* **26**, 299–300.

RIORDAN, J. F. & SOKOLOVSKY, M. (1971). Reduction of nitrotyrosyl residues in proteins. *Biochim. biophys. Acta* **236**, 161–3.

SELIGMAN, A. M., TSOU, K.-C. & BARRNETT, R. J. (1954). A new histochemical method for demonstrating protein bound sulfhydryl groups with 4-hydroxy-1-naphthyl-*N*-maleimide. *J. Histochem. Cytochem.* **2**, 484.

SHARPLESS, N. E. & FLAVIN, M. (1966). The reaction of amines and amino acids with maleimides. Structure of the reaction products deduced from infrared and nuclear magnetic resonance spectroscopy. *Biochemistry, N.Y.* **5**, 2963–71.

SIPPEL, T. O. (1969). New colored maleimides for the demonstration of bound sulfhydryl. *J. Histochem. Cytochem.* **17**, 428–30.

SIPPEL, T. O. (1971). Histochemistry of protein sulfhydryl and disulfide groups in the lens. *Exp. Eye Res.* **11**, 383–8.

TSOU, K.-C., BARRNETT, R. J. & SELIGMAN, A. M. (1955). Preparation of some *N*-(1-naphthyl)-maleimides as sulfhydryl group reagents. *J. Am. chem. Soc.* **77**, 4613–16.

WITTER, A. & TUPPY, H. (1960). *N*-(4-dimethylamino-3,5-dinitrophenyl)maleimide: a coloured sulfhydryl reagent. Isolation and investigation of cysteine-containing peptides from human and bovine serum albumin. *Biochim. biophys. Acta* **45**, 429–42.