Histochemical Journal, **10** (1978), 597–609

The histochemistry of thiols and disulphides. III. Staining patterns in rat tissues

T. O. SIPPEL

Department of Anatomy, University of Michigan, Ann Arbor, Michigan 48109, USA

Received 23 February 1978

Synopsis. With the aid of new staining methods, thiol groups produced by the reduction of disulphide bonds were positively distinguished from pre-existing groups in paraffin sections of several organs of the rat. Good preservation of structures in which the natural thiol-disulphide balance had been maintained was sought by fixing the tissues in neutral formalin containing an organomercurial. After dissociation of the resulting mercaptide bonds that protected the native thiols, these were shown in one colour and then disulphide sites in another within the same sections. Intracellular granules and extracellular membranes rich in disulphides thereby stood out in red against the predominantly blue labelling of the cellular ground plasm. Intimate mixtures of the two forms in some places and the presumed transformation of thiols to disulphides in others, notably the keratinizing epithelium of the tongue, were readily seen. Supplemented by separate visualization of thiols and disulphides along with suitable controls for specificity of staining, the results obtained diverged in some major respects from those of previous investigations.

Introduction

Sulphur of cysteine exists in proteins in either reduced form as the thiol groups of cysteinyl residues proper, or oxidized as the disulphide bonds of cystinyl. The very different properties conferred by the high reactivity of thiols in contrast to the relative inertness of disulphides call for careful distinction between the two. In the past, however, histochemical studies such as that by Montero (1972) have had to rely on the localization of disulphides by more or less insensitive oxidative methods and by comparisons of the thiol staining of reduced and unreduced sections, sometimes with different fixatives used for each (for example, Barrnett *et al.*, 1955).

Work reported in the preceding paper in this series (Sippel, 1978) indicated the feasibility of selectively visualizing disulphide bonds. Conditions were established for their essentially quantitative reduction by tri-*n*-butylphosphine (TBP) which had the great advantage of hardly reversing the prior blockade of native thiol groups imposed,

in turn, most conveniently by N-ethylmaleimide (NEM) among various alkylating agents. Nonetheless, the 're-reduction' procedure underlying those findings was shown to be appropriate for revealing reversal and related sources of non-specificity in the staining of disulphide sites. At the same time, variables in the labelling of both native and generated thiol groups with N-(4-aminophenyl)maleimide (APM) were considered in detail. The resulting routine procedures presented below demonstrate thiols and disulphides not only separately (single staining) but also differentiated from each other in the same section (dual staining); even triple staining has been found possible.

The kinds of results obtainable with these methods are illustrated on paraffin sections of several organs of the rat. The tissues were fixed in a formalin–organomercurial mixture to preserve as much protein as possible while preventing the autoxidation of their thiols to disulphides prior to staining. Mechanisms of this and other ways of achieving protective fixation will be considered in a subsequent communication.

Materials and methods

REAGENTS

Sources or syntheses of the staining reagents have been given (Sippel, 1978). APM now is available from Koch-Light Laboratories (Colnbrook, Bucks., England) and, together with Nitro Red, from Polysciences, Inc. (Warrington, Pennsylvania, USA); Nitro Red is also offered by Aldrich Chemical Co. (Milwaukee, Wisconsin, USA). Sodium Mersalyl was obtained from K & K Laboratories (Plainview, New York, USA).

TISSUE SECTIONS

Young adult female rats deeply anaesthetized with chloral hydrate (4%, 10 ml/kg intraperitoneal) were perfused with 0.9% NaCl followed by phosphate buffered neutral formalin (Lillie, 1965). Samples of seven organs (cf. Sippel, 1978) then were removed to this fixative in which 1% Mersalyl had been freshly dissolved (final pH 7.3) and remained in it for 16–24 h at room temperature. After washing in several changes of distilled water for at least 4 h, the tissues were processed to paraffin in the ordinary way. Composite sections cut serially at 6 μm were thoroughly dried after being floated onto acid-cleaned slides coated with chrome-gelatin (Rogers, 1973); brain tended to loosen during the staining of sections floated from 0.1% gelatin and Mayer's albumen adhesive was ineffective.

STAINING PROCEDURES

Dual staining

After deparaffined sections were brought to ethanol, the means for producing blue labelling of thiols and red of disulphide sites was to:

(1) Dissociate mercaptides for 5 min in acidified mercaptoethanol (0.1 ml in 20 ml 0.2 N HCl and 20 ml PrOH, that is, 99% or absolute 2-propanol). Rinse in two changes of 50% PrOH.

(2) React thiols for 60 min in APM (40 mg in 40 ml premixed 1:1 PrOH and 0.05 M

sodium phosphate buffer, pH 6). Rinse in two changes of 50% PrOH and wash with water.
(3) Diazotize for 2 min in HNO_2 (20 ml 0.1 M $NaNO_2$ and 20 ml 0.2 N HCl). Rinse in two changes of 0.01 N HCl.
(4) Couple for 2 min to Nitro Red (0.4 g in 40 ml 0.1 M sodium phosphate buffer, pH 7). After several changes of water, differentiate for 5 min in 1% (v/v) triethylamine in 70% ethanol and rise in 70% ethanol.
(5) Reduce disulphides for 30 min with TBP (0.1 ml in 20 ml PrOH followed with rapid stirring by 20 ml 0.05 M sodium barbital-HCl buffer, pH 8.5). Rinse in two changes of 50% PrOH and repeat steps 2 & 3.
(6) Couple for 2 min to chromotropic acid (0.4 g of the disodium dihydrate in 40 ml 0.1 M sodium acetate-HCl buffer, pH 5).
(7) Complete differentiation for 5 min in 1% (v/v) concentrated HCl in 70% ethanol. Dehydrate in ethanol, clear in xylene and mount in a synthetic resin.

All steps were carried out at 23–25°C in solutions renewed once a week except for HNO_2 and TBP (both fresh daily) and Nitro Red (monthly); dissolved APM was kept at 5°C between uses. To prepare a control slide showing red staining of non-specific origin, an adjacent section was submitted to the re-reduction procedure by inserting a preliminary reduction, cf. (5), between (1) & (2).

Single staining
Only native thiols were shown by omitting (5) & (6) of the dual staining procedure. Non-specific staining was revealed on an adjacent section when (1) was followed by blocking of the thiol groups for 5 min in fresh 1% formamidine disulphide dihydrochloride in 0.1 N HCl with subsequent rinses in 0.1 N HCl, water and 50% PrOH.

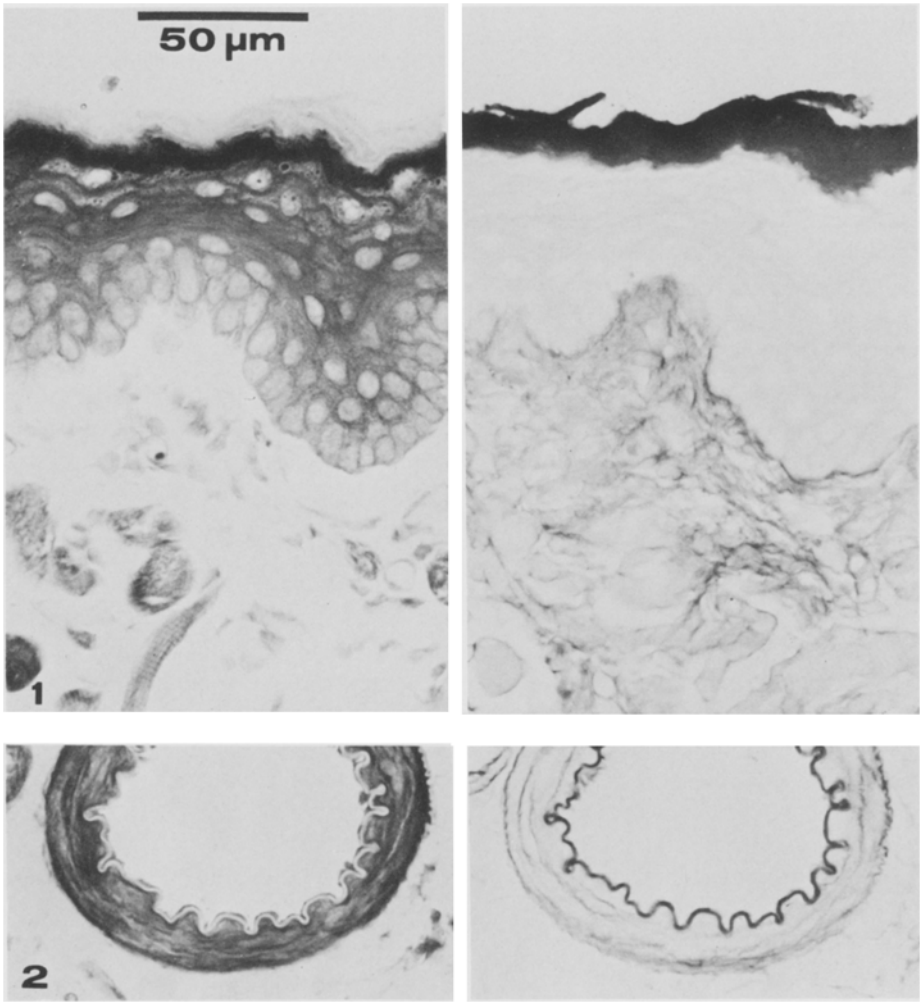
For visualizing disulphides alone, the APM in the first application of (2) of dual staining was replaced by 10 mM NEM (fresh weekly) in which the sections remained overnight; blue labelling was obtained by proceeding with the sequence of (5), (2)–(4) & (7). Non-specific staining was revealed as in dual staining.

To stain both thiols and disulphides (blue), (1) was followed directly by (5) etc. as above.

Feulgen counterstaining
Just before the final step of dual staining, sections were hydrolysed for 15 min in 4 N HCl at 30°C (cf. Fand, 1970) and then stained for 30 min in 40 ml 0.01% Neutral Acriflavin into which 0.1 ml $SOCl_2$ was stirred a few hours before use.

Results

DUAL STAINING
Red-stained disulphide sites could not be photographed accurately in preparations containing the blue label since this, although appearing pure to the eye, significantly absorbed throughout the spectrum of the red label (see Fig. 11). However, by mentally superposing in appropriate colours the pairs of singly stained fields shown in Figs. 1–10, the appearances of dually stained sections may be approximated



Figures 1–10. Adjacent sections of rat tissues singly stained for thiols (left) and disulphides (right) and photographed in red light (Corning filter 2–62); all figures are to the same scale.

Figure 1. Undersurface of tongue. Thiol staining is strong in the outermost keratohyaline granules of which some are intranuclear; also pronounced in the keratogenous layer, it is abruptly replaced by equally intense disulphide staining of the cornified layer.

Figure 2. Small artery of the kidney. Smooth muscle and endothelial cells are rich in thiols, whereas disulphides are prominent in elastic fibres and the internal elastic membrane.

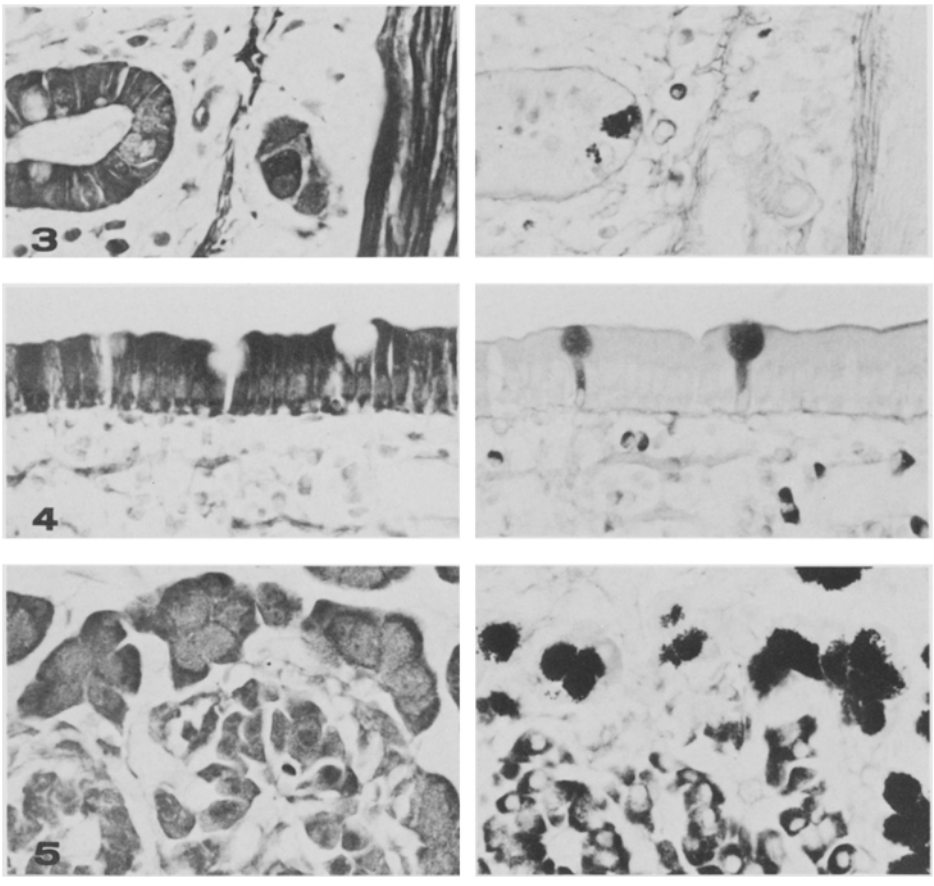


Figure 3. Duodenal wall. Thiol staining is evident in smooth muscle fibres as well as epithelial, ganglion and some free cells. One type of the last also contains disulphides which seem solely present in other free cells, Paneth cell granules and connective tissue sheaths of muscle fibres.
Figure 4. Duodenal villus. Strong thiol staining in the epithelial cells coexists with disulphide staining that is weak in the luminal cytoplasm but stronger in the cuticle. Intracytoplasmic granules of the goblet cells and the mucus elaborated from them contain only disulphides.
Figure 5. Pancreas. Disulphide-rich granules fill the apical portions of acinar cells (above) and, without clear distinctions among cell types, the perivascular sides of islet cells (below).

reasonably well. At least four colour classes were distinguishable in various elements of the actual sections.

Clear blue

The presence of thiols mixed with at most very low proportions of disulphides was inferred in structures having a blue colour similar to that contained in virtually all

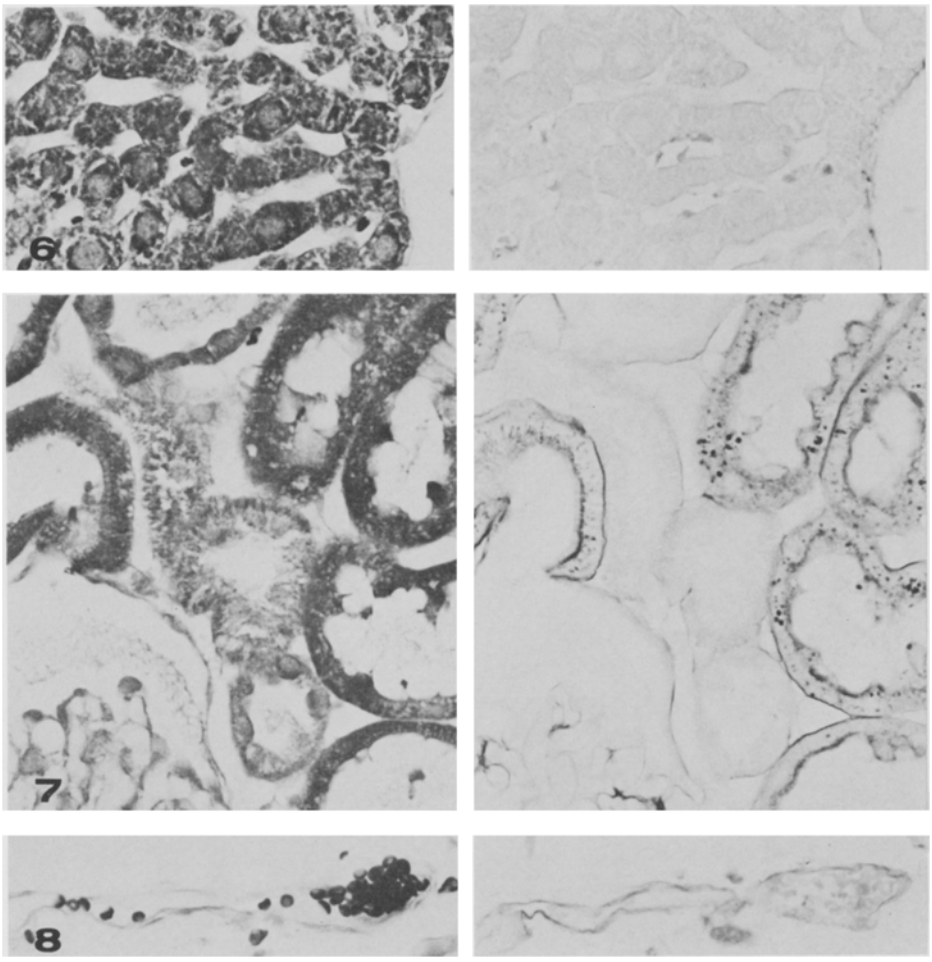


Figure 6. Liver at a central vein. Nucleoplasm and nucleoli contain only thiols, whereas the rather particulate thiol staining of the cytoplasm is mixed with a significant level of disulphide staining.
Figure 7. Kidney cortex. Proximal convoluted tubules seen near the left and right margins of the photos are rich in thiols but have pronounced disulphide staining in their intracellular granules and basement membranes. Distal tubules stain more weakly for only thiols.
Figure 8. Erythrocytes in a pancreatic vein. Thiol staining is very strong, disulphide staining questionable. Disulphide-rich blood plasma was flushed from this vessel during perfusion.

parts of the control section submitted to the re-reduction procedure. In no case was it quite the greenish-blue of single staining because of red shading consequent to slight reversal of the blue label during reduction (cf. Sippel, 1978); incomplete blue labelling of the thiols in the lens nucleus and especially the prekeratin of lingual papillae was

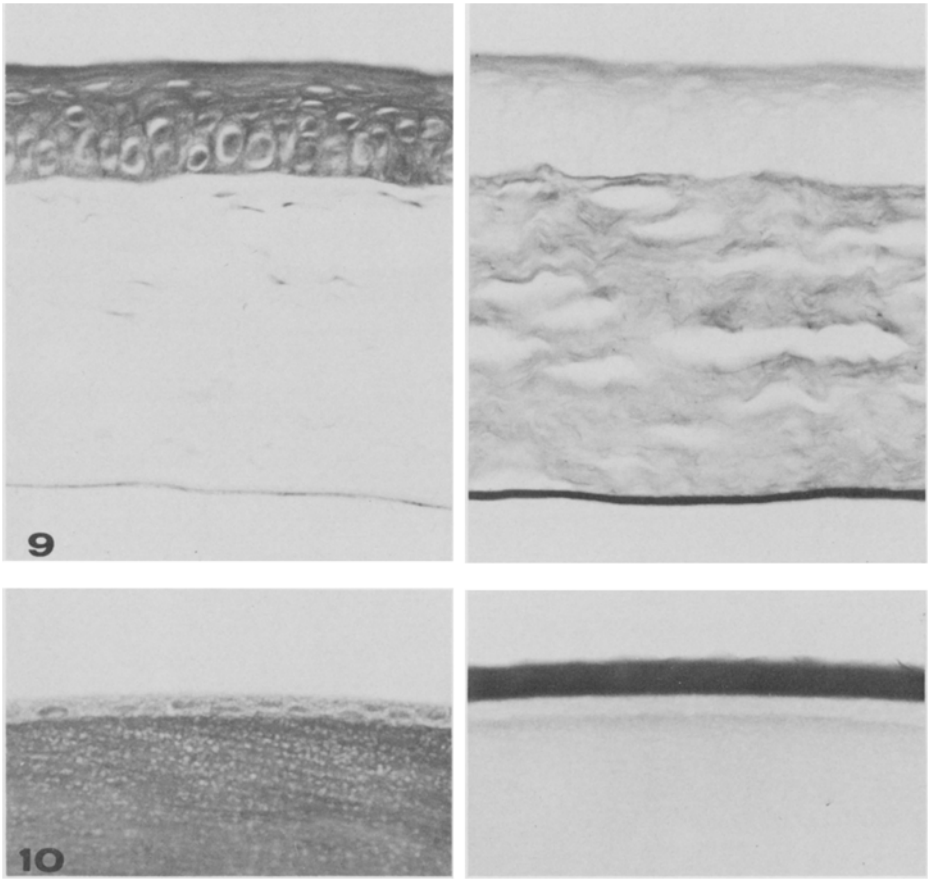


Figure 9. Cornea. Thiol staining is strong in the epithelial, stromal and endothelial cells (top to bottom) with evidence of some disulphides in the cornified surface. Disulphide staining is very intense in Descemet's membrane but only moderate in the stroma, although individual fibrils may be darkly stained.
Figure 10. Anterior surface of the lens. Thiol staining is stronger in the lens fibres and epithelial nuclei than in epithelial cytoplasm. The capsule is very rich in disulphides and lacks thiols.

indicated by their red colour in controls, hence the staining of these structures could not be interpreted.

This least altered shade of blue was particularly strong in the cell envelopes in the keratogenous layer (prekeratin) of the tongue (Fig. 1) and in erythrocytes (Fig. 8). It was nearly as intense in those keratohyaline granules lying superficially in the granular layer of the lower epithelium (Fig. 1) and between papillae on the dorsum; however, the smaller granules in the deeper cells of the undersurface resembled the ones located at the base of each papilla in being a pale blue barely distinguishable from the surrounding cytoplasm. The strongly staining lens cortex had the same hue but not always at the immediately subepithelial surface (Fig. 10). Clear blue nucleoli often stood out against a similarly coloured nucleoplasm which commonly stained with

moderate intensity (cf. Figs. 3–7) but could be very pale (Fig. 1), or quite dark as in the nuclear bow of the lens.

Possibly somewhat less greenish staining occurred in the inner segments of retinal rods, smooth muscle fibres (Figs. 2 & 3) and the light bands of striated muscle. It was seen also in the cytoplasm of cells of the lingual epithelium (Fig. 1), kidney tubules (Fig. 7) and periportal areas of the liver; although distributed throughout the epithelial cells lining the duodenal crypts (Fig. 3), this shade was present in only the basal cytoplasm of those covering the villi (Fig. 4). At least one type of free cell located in the duodenal connective tissue showed blue granules that were, however, poorly defined.

Dull blue

Staining less clear in hue than the preceding and grading toward reddish blue suggested the presence of significant levels of disulphides amongst predominating thiols. It seemed to occur in the dark bands of striated muscle fibres, but was definite in the luminal cytoplasm of cells on the duodenal villi (Fig. 4), in the basal cytoplasm of pancreatic acincar cells (Fig. 5), and throughout hepatocytes located near central veins (Fig. 6).

Mixed colours

Colours ranging from reddish-blue to bluish-red and indicating approximately equal concentrations of thiols and disulphides (literally, half-disulphides) occurred where thiols undergo natural oxidation. The best example was in the transition from prekeratin to keratin over the span of two or three cell layers in the lingual epithelium (Fig. 1). The much less extreme shift at the surface of the corneal epithelium in some specimens (Fig. 9) suggested its mild cornification. These colours occurring at intermediate depths of the lens were sharply demarcated from the blue of the outer cortex, below which staining was unreliable; the intense violet, nearly black, staining seen in the prekeratin of the lingual papillae likewise was an artifact.

Staining in mixed colours was characteristic of highly membranous structures. Thus, the thick brush borders of straight tubules in the renal medulla were purple; the thinner linings of proximal convoluted tubules (Fig. 7) and cuticles of the epithelial cells on the duodenal villi (Fig. 4) were somewhat bluer. Reddish-blue staining also occurred in the coverings of nerve fibres seen in the tongue, in myelinated tracts of the brain and in the outer segments of retinal rods; all three would have been still redder had an agent been used, reducing their particular disulphides more rapidly than TBP (cf. Sippel, 1978). That the staining of these structures represented thiols and disulphides compartmentalized on a submicroscopic scale was suggested by the fact that the similar colouring of the muscularis mucosae (Fig. 3) viewed at a low magnification could be resolved under the oil immersion lens into a mixture of blue muscle and red collagenous fibrils.

In only two instances were intracellular structures stained in this colour range. One type of free cell common in the duodenal connective tissue but present also in hepatic and extraocular tissues stood out because of its intensely violet contents; they were not distinctly granular and did not stain with Thionin. These were the same cells noted in the previous study to be uniquely resistant to alkaline extraction of their

acidophilia. In addition, among the large and strongly blue interpapillarly keratohyaline granules were a very few that seemed to be nearly black or to have black inclusions.

Clear red

Although many structures were stained red without any discernible blue shading, at least two were shown by single staining to contain appreciable concentrations of thiols (see below). Therefore, only a great preponderance of disulphides could be interpreted from the strong red colour of fully keratinized cells (Fig. 1), connective tissue fibres (Figs. 1, 3 & 9), elastic membranes and fibres (Fig. 2), various representations of basement membranes (Figs. 7, 9 & 10) and goblet cell mucin (Fig. 4). In an occasional unperfused part of the kidney, the vessels were filled with red plasma in addition to the expected blue erythrocytes.

Intracellular granules commonly were stained red. Examples found in the duodenum were the Paneth cell granules (Fig. 3) and smaller bodies in at least two kinds of free cell of which one probably was an eosinophil leukocyte. In the pancreas (Fig. 5) were both the zymogen granules of the acinar cells (the coalesced secretion filling the ducts was similarly coloured) as well as, again, smaller granules located in cells distributed throughout the islets. Cells of the proximal convoluted tubules of the kidney (Fig. 7) contained an array of small red bodies in the position of the terminal web besides those of generally larger but more varied size nearer the nucleus. In only two possibly pathological specimens were comparable structures found in some liver cells. However, sparse red granules were always just visible in the cytoplasm of Purkinje cells and other large neurons of the brain, and large clearly defined red granules filled the mural cells (pericytes) on its vessels. Lastly, the contents of lingual mast cells were strongly red, although the metachromatic (thionin) material spilling from them was only faintly stained for disulphides.

SINGLE STAINING

Thiols

The main point in demonstrating thiols alone was the detection of low levels that might be obscured by dual staining. In fact, among the various structures just described as being rich in disulphides, only the keratinized cells of the lingual epithelium and the secretion in the pancreatic ducts proved to have low but unmistakable thiol staining. Both were unstained in control sections in which the thiols had been specifically blocked by formamidine disulphide. Of the other structures of concern, corneal stroma, Descemet's membrane, lens capsule, internal elastic laminae of arteries and duodenal mucin were all negative for thiols. Finer membranes or fibres tended to be too refractile, and intercellular granules in general too intimately involved with the surrounding cytoplasm, for their levels of thiol staining to be judged.

Disulphides

Single staining of disulphides confirmed their presence in every structure noted to be coloured dull blue or redder by the dual staining procedure. Of the structures rendered clear blue by that method, erythrocytes had questionable staining for disulphides, and

nuclei and nucleoli showed none. Although large interpapillary keratohyaline granules were stained lightly (with rare exceptions), it was non-specific as shown by their comparable colour in control sections submitted to the re-reduction procedure.

Thiols plus disulphides

Combined staining of thiols and disulphides seemed to be the sum of their separate staining. The concentration of total thiol equivalents was highest in erythrocytes, cell envelopes in both the keratogenous and keratinized layers of the tongue, and virtually all granules and membranes (particularly Descemet's and the posterior lens capsule) that had been found to be rich in disulphides. It may be noted that the intense staining of a given structure could reflect as much its unusually high protein content as the relative enrichment of cyst(e)ine residues in its proteins.

NUCLEAR COUNTERSTAINING

Application of the yellow Feulgen procedure to a few dually stained preparations resulted in nuclei coloured mostly yellowish shades of green. The retina happened to show the widest range, from bluish-green in its ganglion cell nuclei to distinctly yellow in the outer nuclear layer. The elongated nuclei of the lens bow were bizarre in having their yellowish-green staining applied to the lateral aspects of the principal blue thiol staining.

Mitotic chromosomes that were not at all obvious without counterstaining showed up as bright yellow. A more careful search of duodenal crypts after dual staining alone revealed what appeared to be metaphase figures as uncoloured or possibly very pale blue refactile bodies. Single staining for disulphides gave no hint of their presence in these structures.

Discussion

By differentiating thiols from disulphides in primary colours within the same section, dual staining gives results that are as informative as they are attractive. Structures having a preponderance of either form are very readily identified and, since the underlying reactions approach unit stoichiometry (Sippel, 1978), semi-quantitative judgements of the thiol-disulphide ratios in others can be made at a glance. Success of the method depends to a large extent on the colouristic purity of the red and blue labels (B and D, respectively, in Fig. 11). Even though the latter has a relatively broad absorption spectrum, it is not as bad in this regard as the reddish blue label (C), built by two successive couplings (Sippel, 1973), that gives muddy coloured blends with all lighter coloured labels tested. Besides red, these were a strong yellow formed by coupling to 2,6-diaminopyridine in 1% acetic acid (A in Fig. 11) and a paler shade from resorcinol at pH 5. Both have been found useful in providing lightly coloured backgrounds of thiol staining against which blue stained disulphide-rich structures stand out sharply. The effect is gained nearly as well simply by interchanging the coupling solutions of the standard staining procedure (inverted dual staining).

Separate visualizations of thiols and disulphides remain important for detecting low levels of each, however, not to mention black-and-white photography. Accompanied by appropriate controls for specificity, such procedures also should be as suitable for

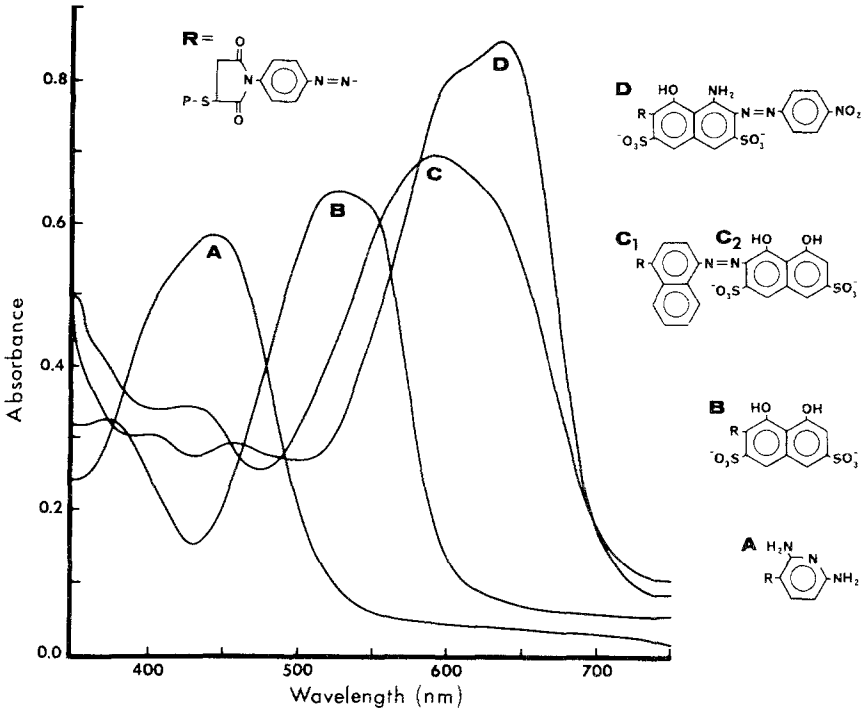


Figure 11. Absorption spectra of the labels formed by coupling of diazotized bound APM to (A) 2,6-diaminopyridine, (B) chromotropic acid, (C) 1-naphthylamine, then diazotized and coupled to chromotropic acid, and (D) Nitro Red. Liver sections cut at 15 μm and stained for thiols were scanned in a Beckman DB-G recording spectrophotometer.

densitometric analysis as those revealing disulphides by differences between thiol and total staining. In either case, the drawback to the use of Nitro Red to form a blue label is that it couples best at neutrality where diazotized bound APM is not especially stable. Consequently, any of the other labels shown in Fig. 11 might be more suitable on these grounds. However, studies in progress indicate that coupling to N-naphthylethylenediamine at pH 1, already employed for a different purpose (Sippel, 1978), is better if measurements are intended.

The results produced by both single and dual staining procedures emphasize the largely exclusive distributions of thiols and disulphides at the level of discrimination allowed by light microscopy: thiols dominate the cellular ground plasm whereas disulphides tend to be restricted to intracellular granules and extracellular membranes. Apart from minor differences in staining patterns presumably attributable to the use of other methods for fixing and staining, the same generalization could be reached from earlier surveys (Barrnett, 1953; Barrnett & Seligman, 1954; Adams, 1955; Engel & Zerlotti, 1964) supplemented by almost countless more specialized studies.

Nevertheless, there are interesting discrepancies in at least three areas. For instance, the clear indication of thiol groups in keratohyaline granules is contrary to results obtained with the 'DDD' staining reagent (Matoltsy & Matoltsy, 1962, and references

therein) yet supports biochemical, ultrastructural and some early histochemical evidence of their role in keratinization. Secondly, the finding that condensed chromatin contains at most traces of thiols and disulphides differs in one or both respects from virtually all previous histochemical data (for example, Sandritter & Krygier, 1959; Kawamura, 1960; Hyde, 1961), indirect evidence (Sumner, 1973) and biochemical analyses (reviews by Wilhelm *et al.*, 1971, and Spelsberg *et al.*, 1972). Finally, the concentration of disulphide bonds in what well may be lysosomes in brain and kidney cells does not seem to have been noted before, although some indirect evidence exists in the case of kidney. All these aspects deserve further histochemical study with particular emphasis on the undoubtedly critical factor of fixation.

Acknowledgement

These studies were initiated with the aid of Research Grant EY00235 and Special Fellowship EY52557 from the National Eye Institute of the U.S. Public Health Service.

References

ADAMS, C. W. M. (1955). A stricter interpretation of the ferric ferricyanide reaction with particular reference to the demonstration of protein-bound sulphydryl and di-sulphide groups. *J. Histochem. Cytochem.* **4**, 23–5.

BARRNETT, R. J. (1953). The histochemical distribution of protein-bound sulfhydryl groups. *J. natn. Cancer Inst.* **13**, 905–25.

BARRNETT, R. R., MARSHALL, R. B. & SELIGMAN, A. M. (1955). Histochemical demonstration of insulin in the islets of Langerhans. *Endrocrinology* **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.

ENGEL, M. B. & ZERLOTTI, E. (1966). The histochemical visualization of protein-bound sulfhydryl groups with an azomercurial reagent. *J. Histochem. Cytochem.* **12**, 156–63.

FAND, S. B. (1970). Environmental conditions for optimal Feulgen hydrolysis. In: *Introduction to Quantitative Cytochemistry – II* (ed. G. L. Wied), pp. 209–21. New York: Academic Press.

HYDE, B. B. (1961). An evaluation of the Barrnett and Seligman procedure when used to determine changes in the ratio of nuclear protein-bound disulfide to sulfhydryl groups during mitosis. *J. Histochem. Cytochem.* **9**, 640–6.

KAWAMURA, N. (1960). Cytochemical and quantitative study of protein-bound sulfhydryl and disulfide groups in eggs of *Arbacia* during the first cleavage. *Expl Cell Res.* **20**, 127–38.

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

MATOLTSY, A. G. & MATOLTSY, M. (1962). A study of morphological and chemical properties of keratohyaline granules. *J. invest. Derm.* **38**, 237–47.

MONTERO, C. (1972). Histochemistry of protein-bound disulphide groups in the duct secretory granules of the rat submandibular gland. *Histochem.* **4**, 259–66.

ROGERS, A. W. (1973). *Techniques of Autoradiography*, 2nd Edn. Amsterdam: Elsevier.

SANDRITTER, W. & KRYGIER, A. (1959). Cytophotometrische Bestimmungen von proteingebundenen Thiolen in der Mitose und Interphase von HeLa-Zellen. *Z. Krebsforsch.* **62**, 596–610.

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

SPELSBERG, T. C., WILHELM, J. A. & HNILICA, L. S. (1972). Nuclear proteins in genetic restriction. II. The nonhistone proteins in chromatin. *Sub-Cell. Biochem.* **1**, 107–45.

SUMNER, A. T. (1973). Involvement of protein disulphides and sulphydryls in chromosome banding. *Expl Cell Res.* **83**, 438–42.

WILHELM, J. A., SPELSBERG, T. C. & HNILICA, L. S. (1971). Nuclear proteins in genetic restriction. I. The histones. *Sub-Cell Biochem.* **1**, 39–65.