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LETTER TO THE EDITOR

Is Mercury Orange a selective stain for thiols?

In the late 1940s, Mercury Orange, an azo dye with a chloromercuri substituent, was investigated as a histochemical reagent for the demonstration of thiol groups. The remarkable specificity of Mercury Orange, or Red Sulphydryl Reagent or Bennett's Sulphydryl Reagent to give two early synonyms, was established by Bennett & Watts (1958).

Since that time Mercury Orange has remained in the histochemical armamentorium and indeed is regarded as one of the more specific staining reagents. Note for instance the opinions to be found in standard histochemical texts. Barka & Anderson (1963) stated 'The method appears to be specific'; Gabe (1976) said 'Bennett's method is attractive for its great specificity'; Lillie & Fulmer (1976) say 'the reagent appears to be specific'. Of course, similar views have been expressed by others (see, for example, Horobin, 1982).

Recently, however, Wiese (1980) has questioned the specificity of Mercury Orange, at least for metaphase chromosomes. He pointed out that the concentration of cysteinyl and cystinyl residues in the proteins of these structures is low, and is '. . . . near the limit of detectability by coloured labels'. Even his extremely sensitive fluorescence method was barely able to detect thiols plus disulphides in chromosomes. Hence, Wiese doubted if the strong Mercury Orange staining of chromosomes, previously reported, could be attributed to thiol reactivity. He suggested, instead, that such reactivity could be due to the organomercurial compounds complexing with polynucleotides. Wiese's proposal is indeed supported by the biochemical literature, and in addition to the paper he cited, there are reports of the reactivity of nucleic acid bases with mercuric chloride, and the binding of nucleic acids to certain mercurated Fluorescein dyes (for example, Yamane & Davidson, 1961; Dattagupta *et al.*, 1975; Takeuchi & Maeda, 1976). Moreover, such reactions of organo-mercurials with nucleic acids rationalizes the early observation that the staining of ribosomes by Mercury Orange and other organo-mercurials was prevented by prior extraction of the tissue by RNAse (Mundkur, 1964).

It is important that such a possible flaw of a standard histochemical method be checked experimentally. This we have endeavoured to do, by using as a model pure compounds spotted on filter paper; an appropriate system for a reactive stain such as Mercury Orange.

We applied known volumes of aqueous solutions, or suspensions, of a variety of compounds to filter paper discs. It was found that the compounds were not removed

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Table 1. The reactivities of various compounds with Mercury Orange.

Compound tested (source)	*Significance*	*Coloration with Mercury Orange*
Cystein (Sigma)	Thiol rich	Intensely orange
Histone, 11-S, calf thymus (Sigma)	Low thiol content	Very pale orange
DNA, sodium salt, calf thymus (BDH)	Thiol-free polyanion	Strongly orange
RNA, sodium salt, yeast (BDH)	Thiol-free polyanion	Strongly orange
Heprin (Evans Medical)	Sulphated polyanion	Very pale orange
Adenosin (Sigma)	Nucleotides	Orange
Cytidine (Sigma)		Orange
Guanosine (Sigma)		Orange
Thymidine (Sigma)		Orange
Uridine (Sigma)		Orange

from the paper by immersion for several hours in chloroform. The discs with the model compounds were then immersed in chloroform solutions of Mercury Orange. After reaction for up to several hours, unreacted Mercury Orange was removed from the discs by washing with chloroform. This procedure left virgin paper quite colourless. No significant reaction-rate effects were seen. All reagents were of analytical quality where available, and the identity of the Mercury Orange was checked by thin-layer chromatography. The compounds tested, and the staining resulting, are specified in Table 1.

The table shows very clearly, in keeping with Wiese's expectations, that whilst the histone protein is barely stained, the nucleic acids are strongly coloured. The intense staining of cysteine, together with the trivial staining of the sulphated polyanion and the total absence of staining of virgin paper, indicates that coloration of nucleic acids is a chemically specific phenomenon. The reactivity of the nucleotides bears this out, and is in keeping with the chemical evidence cited.

These model experiments indicate that Mercury Orange reacts with the heterocyclic bases of DNA and RNA and hence is not specific for thiol groups. However, except for studies of structures containing nucleic acids this does not invalidate the use of Mercury Orange. The reagent remains a *selective* thiol reagent.

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Dr Sippel replies

First, may I note for Dr Wiese that interphase nuclei also tend to be stained more heavily, relative to cytoplasm, by Mercuric Orange than by the azogenic maleimide used in his study. I found a similar discrepancy with samples I made of the *o*-phenetidine analogue of Mercury Orange (Szydlowska & Junikiewicz, 1972). These contained variable amounts of a compound staining bluish red and incompletely removed by repeated recrystallization from *n*-butanol; the main product, called Mercury Red below, stained more yellowish red.

Applied as saturated solutions in 80% ethanol with pH 9.5 glycine–NaOH buffer, the purest batches of Mercury Red coloured sections of epithelia (Carnoy fixation) strongly and uniformly in 1 h. But with the 24 h usually recommended for organomercurial staining, or quite quickly with heavily contaminated samples, nuclei stood out bluish red and stained so, virtually alone, in sections in which thiols had been blocked by either 5 mM *N*-(4-nitrophenyl)maleimide (pH 6.5) or the corresponding iodoacetamide (pH 8.5) for 1 h.

Evidently the Mercury Red contaminant complexes with groups unreactive toward alkylating reagents applied under non-forcing conditions. Although the extraneous binding might be to atypical thiols, the work of Horobin & Flemming allows the very strong inference that this is not the case.

I agree that Mercury Orange, perhaps supplemented by Mercury Red, deserves continued use. There seems, however, to have developed in the literature some

frustration over the choice of solvent, which probably influences specificity and, unfortunately, is not ordinarily chloroform. More importantly, verified blocking tests (not yet those above) that should have revealed the non-specificity of Mercury Orange long ago and clearly must accompany its application are sorely needed in thiol histochemistry.

Reference

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