Histochemistry 70, 29–32 (1980)

Histochemistry

A Histochemical Study of Protein-Bound SH and SS in Chromosomes of Hyacinth and Fava Bean Root Tips

D. A. Wiese

Department of Anatomy, The University of Michigan Medical School, Ann Arbor, Michigan 48109, USA

Summary. Root tips were fixed in ethanol-acetic acid and in neutral formalin containing a mercaptide-forming agent to protect thiols from autoxidation. Serial paraffin sections 2 μm thick were labelled for thiols, disulfides and both together with the azogenic maleimide 'APM'. Nuclei were stained somewhat lighter for thiols, and nucleoli much darker, than was the surrounding cytoplasm; disulfide staining was paler and more evenly distributed. However, regardless of the fixative employed, mitotic chromosomes (precisely localized by fluorescent Feulgen counterstaining) contained negligible amounts of either thiols or disulfides. Although published biochemical studies also have indicated low concentrations of cyst(e)inyl residues in chromosomal proteins, the present findings contradict most previous histochemical reports.

Introduction

There are several histochemical studies indicating high protein thiol or disulfide levels in mitotic chromosomes of cells preserved with acidic fixatives (Kawamura and Dan 1958; Kawamura 1960; Sandritter and Krygier 1959; Hyde and Paliwal 1959; Hyde 1961). However, when the azogenic thiol reagent *N*-(4-aminophenyl) maleimide or 'APM' (Sippel 1973) was used to stain sections of rat organs protectively fixed in neutral formalin, figures in the duodenal crypts seemed to contain neither form of cysteine (Sippel 1978). The present observations on the relatively large root tip chromosomes of two plant species confirm the virtual absence of protein thiols and disulfides irrespective of the fixative employed.

Materials and Methods

Actively growing root tips from commercial hyacinth bulbs (*Hyacinthus* sp.) and germinated fava beans (*Vicia faba*) were fixed for 2 h in ethanol-acetic acid (3:1) or for 4 h in 10% phosphate-buffered neutral formalin containing 1% sodium Mersalyl (Sippel 1978). Paraffin sections cut

0301-5564/80/0070/0029/$01.00

at 2 μm were floated onto formalin-gelatin coated slides (Pearse 1968) and dried overnight at 45° C.

The sections were brought through xylene to ethanol, and mercaptides in the formalin-fixed material were dissociated in acidified mercaptoethanol; thiols, disulfides and both together were demonstrated in serial sections by the 'APM' method, with the final coupling in all cases to Nitro Red (Sippel 1978). After the resultant blue labelling was photographed with a Zeiss Photomicroscope II, the coverslips were removed and the sections hydrolyzed (1 N HCl at 60° C for 8 min) for fluorescent Feulgen staining for 1 h in an acriflavin Schiff-type reagent (0.01% aqueous neutral acriflavin to which 0.25% v/v $SOCl_2$ was added 2 h before use). Following three rinses in sulfite water (Ruch 1966), the sections were dehydrated, cleared and mounted in a non-fluorescent medium. Fluorescence photomicrographs were taken with an American Optical Corp. Series 10 microscope equipped with a Model 2070 epi-illuminator using an excitation filter of 400 nm (BG12) and a barrier filter of 515 nm (OG515).

Results

In the sections of protectively-fixed roots, thiol staining generally was moderate in the cytoplasm, weaker but more variable in the nucleoplasm, and darkest in the nucleoli. Disulfide staining was much paler and more uniform. Comparable staining patterns were seen in the acid-fixed material in which, however, nuclear staining was relatively weak.

After either fixative, chromosomes always appeared as very lightly stained structures on a darker background, whether thiols and disulfides were shown together (Figs. 1, A and C) or separately. Thus neither form appears to be

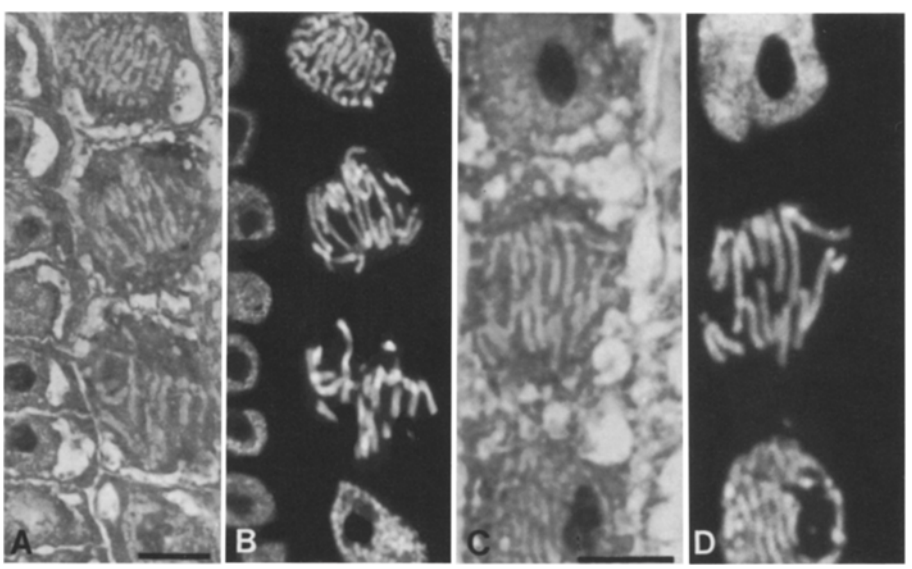

Fig. 1. A and **C**. Sections of hyacinth root tip stained for thiols plus disulfides. (Formalin-Mersalyl fixation.) Calibration bar = 5 μm. **B** and **D**. Each of the preceding sections after fluorescent Feulgen counterstaining

present in significant amounts in the chromosomes of dividing cells. The blue label was retained during the subsequent fluorescent Feulgen procedure but did not interfere with the localization of DNA (Figs. 1, B and D).

Discussion

Amino acid analyses of nuclear proteins associated with chromatin from a variety of species have indicated the presence of only low levels of cysteine or cystine in the H3 histone fraction (DeLange and Smith 1975) and in the major classes of non-histone protein (Elgin and Stumph 1975). Nonetheless, Kawamura and Dan (1958) and Kawamura (1960) obtained intense thiol staining of chromosomes in sections of acid-fixed fertilized sea urchin eggs labelled with the mercaptide-forming agent, mercury orange; the staining was blocked by p-chloromercuribenzoate. It has been found since then, however, that organomercurials complex with polynucleotides (Vallee and Ulmer 1972) and that staining by mercury orange is sometimes hardly affected by prior blocking with iodoacetate (Bachmann and Cowden 1965).

It is less easy to account for the often deep thiol staining of chromosomes by the widely used 'DDD' method in similarly fixed HeLa cells (Sandritter and Krygier 1959) and onion root tips (Hyde and Paliwal 1959); chromosomes in the latter also were reported to contain significant amounts of disulfides (Hyde 1961). Although 'DDD' has been found to react non-specifically with disulfide bonds (Sippel 1977; Nöhammer et al. 1977) and with presumably aldehydic components in elastic tissue (Barnett and Seligman 1952), neither of these mechanisms would be expected to explain the intensity of the reported chromosomal staining. Since 'DDD' gave negligible staining of chromosomes in human bone marrow smears fixed by formalin vapor (Ackerman and Sneeringer 1960), it might appear that acidic fixatives, which extract histones and some non-histones (Sivak and Wolman 1974), unmask thiols or cause chromosomes to adsorb nucleoplasmic proteins. However, neither thiols nor disulfides could be demonstrated in significant amounts in acid-fixed material in the present study. Furthermore, in my own preliminary tests, the 'DDD' procedure applied to protectively-fixed hyacinth root tips gave extremely variable results: chromosomal staining in sections labelled for both thiols and disulfides ranged from pale to intense.

Totally consistent patterns were given by the 'APM' method which involves first the reaction of *N*-(4-aminophenyl)maleimide with thiol groups and then visualization of the bound reagent as, in the present instance, a strongly colored disazo label. The staining is free of background coloration, and the sensitivity, specificity and stoichiometry of the label have been found satisfactory on various objects including isolated sperm heads (Wiese 1979); there is no evidence that the procedure gives false negative results. It is inferred, therefore, that although cyst(e)inyl residues are probably significant in chromosomal structure and function (Sumner 1973; Hilton and Stocken 1966), their levels are near the limit of detectability by colored labels.

Acknowledgements. From a doctoral dissertation submitted to the University of Michigan, and supported in part by a grant from the University of Michigan Cancer Research Institute. I thank Professor Theodore Sippel for helpful discussions.

References

Ackerman G, Sneeringer S (1960) Histochemical localization of sulfhydryl groups in the cells of normal human blood and bone marrow. Lab Invest 9:356–363

Bachmann K, Cowden R (1965) Quantitative cytophotometric studies on polyploid liver cell nuclei of frog and rat. Chromosoma 17:181–193

Barrnett R, Seligman A (1952) Histochemical detection of protein-bound sulfhydryl groups. Science 116:323–327

DeLange R, Smith E (1975) Histone function and evolution as viewed by sequence studies. In: Fitzsimons D, Wolstenholme G (eds) The structure and function of chromatin, Ciba Foundation Symposium 28 (new Series). Associated Scientific Publishers, New York, pp 59–76

Elgin S, Stumph W (1975) Chemistry of the non-histone chromosomal proteins. In: Fitzsimons D, Wolstenholme G (eds) The structure and function of chromatin, Ciba Foundation Symposium 28 (new Series). Associated Scientific Publishers, New York, pp 113–130

Hilton J, Stocken L (1966) The role of thiol groups in the modification of the template activity of histone-deoxyribonucleic acid complexes. Biochem J 100:21c

Hyde 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 9:640–646

Hyde B, Paliwal R (1959) The Barrnett-Seligman sulfhydryl reaction for plant meristems. Stain Technol 34:175–186

Kawamura N (1960) Cytochemical and quantitative study of protein-bound sulfhydryl and disulfide groups in eggs of *Arbacia* during the first cleavage. Exp Cell Res 20:127–138

Kawamura N, Dan K (1958) Cytochemical study of protein-bound sulfhydryl groups of sea urchin eggs during the first cleavage. J Biophys Biochem Cytol 4:615–625

Nöhammer G, Schaunstein E, Bajardi F, Unger-Ullman C (1977) Microphotometrical quantification of protein thiols in morphologically intact cells of the cervical epithelium I. Basic results with superficial cells of healthy women. Acta Cytol 21:341–344

Pearse AGE (1968) Histochemistry, vol 1, 3rd edn. Churchill Livingstone, London, p 728

Ruch F (1966) Determination of DNA content by microfluorometry. In: Wied G (ed) Introduction to quantitative cytochemistry, vol 1. Academic Press, New York, pp 281–294

Sandritter W, Krygier A (1959) Cytophotometrische Bestimmung von proteingebundenen Thiolen in der Mitose und Interphase von HeLa Zellen. Z Krebsforsch 62:595–610

Sippel T (1973) The histochemistry of thiols and disulphides I. The use of *N*-(4-aminophenyl) maleimide for demonstrating thiol groups. Histochem J 5:413–423

Sippel T (1977) Nonspecific staining in the demonstration of thiols with aryl disulfide reagents. J Histochem Cytochem 25:236

Sippel T (1978) The histochemistry of thiols and disulphides II. Staining patterns in rat tissues. Histochem J 10:597–609

Sivak A, Wolman S (1974) Chromosomal proteins in fixed metaphase cells. Histochemistry 42:345–349

Sumner A (1973) Involvement of protein disulphides and sulfhydryls in chromosome banding. Exp Cell Res 83:438–442

Vallee B, Ulmer D (1972) Biochemical effects of mercury, cadmium and lead. Annu Rev Biochem 41:91–128

Wiese D (1979) Histochemical analysis of nuclear protein thiols and disulfides. Ph. D. dissertation. The University of Michigan, Ann Arbor, Michigan

Received September 2, 1980