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THE FIXATION OF NUCLEI AND CHROMOSOMES

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SUMMARY

Chromosomes in the nuclei of living salivary glands of *Simulium* have been observed with Nomarski optics and polarized light before and during irrigation with various fixatives. All cause loss of chromosomal organization to some degree. The best fixative is redistilled glutaraldehyde; even so, although it leaves the nucleus visually unaltered, a network forms in the nuclear sap and birefringence is lost. Calcium ions in the fixative cause chromosomal movements. The fixatives may alter nucleoli and may dissolve them completely. New fixatives are needed for reliable fine-structural studies of chromosomal organization.

INTRODUCTION

The fixatives used for nuclei and chromosomes are of two types. There are those that have been found to give good fixation for cytoplasm and are assumed, therefore, to be good for nuclei and chromosomes (Stevens & Swift, 1966; Berendes, 1970). On the other hand, there are variants on mixtures of acetic acid and methanol that fix by precipitation and, despite their very poor preservation of cytoplasmic organization, are assumed to fix chromosomes particularly well, even for electron microscopy (Sorsa, 1974).

Nuclei in the living salivary glands of *Simulium* contain large polytene chromosomes. Moreover, the cytoplasm of the cells is transparent, so that a clear image of the chromosomes can be obtained (Skaer & Whytock, 1975). They are, therefore, good material on which to observe the effects of fixatives.

MATERIAL AND METHODS

Salivary glands from late larvae of *Simulium equinum* L. and *S. argyreatum* (Meig.) were mounted in a small amount of haemolymph under a supported coverslip and observed with Nomarski optics. They were rapidly photographed before degenerative changes occurred through contact of the air with haemolymph. Fixatives at room temperature were then perfused under the coverslip. Nuclei were photographed at intervals up to 1 h in the fixative. Parallel observations were made with polarized light.

Fixatives

2 % *osmium tetroxide.* Some samples were brought to 400 mOsmol with sucrose.

0·5 *and* 3 % *formaldehyde.* This was made up from paraformaldehyde (Burgos, Vitale-Calpe & Téllez de Iñon, 1967).

1 % *acrolein*. This was used either alone or together with 1 % formaldehyde (Burgos *et al.* 1967).

3 % *glutaraldehyde*. Three preparations were used:

(*a*) Highly polymeric with a very large absorption peak at 235 nm and a very small absorption peak at 280 nm. It was made up from 25 % commercial stock solution (Koch-Light) several years old.

(*b*) Fairly polymeric with a large absorption peak at 235 nm and a small absorption peak at 280 nm. This was made up from a fresher 25 % commercial stock solution (Taab Laboratories, Emmer Green, Reading) described as specially purified for electron microscopy.

(*c*) Monomeric. This was prepared by saturating a 25 % solution of purified glutaraldehyde (Taab) with NaCl and extracting 4 times with an equal volume of ether. The ether was removed from the ethereal solution of glutaraldehyde in a rotatory evaporator and the glutaraldehyde immediately redistilled under reduced pressure and a nitrogen atmosphere. The fraction distilling at 71–73 °C at 10 mm Hg (1·333 kN m^{-2}) was collected and immediately dissolved in distilled water to a 10 % stock solution. This had no absorption peak at 235 nm; its absorption peak was at 280 nm. This is substantially the method devised by Dr P. M. Hardy (personal communication).

10 % *ethyl* 3 (3-*methyl aminopropyl*) *carbodiimide* (*Ethyl CDI*). (Kendall, Polak & Pearse, 1970). This was dissolved immediately before use in 0·1 M phosphate buffer pH 7·4.

3 : 1 *methanol : acetic acid*. This was freshly prepared and used as a non-aqueous fixative.

Buffers

Aqueous fixatives were normally made up in 0·1 M cacodylate buffer pH 7·4 osmolarity 140 mOsmol, or in 0·05 M HEPES buffer pH 7·4 and 139 mOsmol. The HEPES was used as a non-chelating buffer (Ris, 1968) so the effect of known quantities of calcium, added to the fixative as the chloride, could be assessed.

RESULTS

Osmium tetroxide

On immersion in this fixative the nucleus shrinks and the chromosomes become progressively less visible (Figs. 1–3). After 1 h the chromosomes have almost completely disappeared: all that can be seen are traces of the most prominent bands. Virtually all birefringence is lost from the chromosomes. The chromosomes also disappear when fixed in osmium tetroxide made up to 400 mOsmol with sucrose. The nucleolus remains readily visible, although its shape alters considerably on fixation. These effects of osmium tetroxide fixation do not occur when it is used after fixation in some other fixative, e.g. ethyl CDI (Fig. 16). Under these conditions there is a slight general increase in contrast of the chromosomes, but the observable effects are those of the primary fixative. It appears to make no difference to the fixation with osmium tetroxide whether cacodylate or HEPES is used to buffer the solution.

Formaldehyde

Changes are visible as soon as the fixative reaches the nucleus. The nucleus does not shrink or swell but the chromosomes become progressively less and less visible and the nucleolus becomes more prominent (Figs. 4–6). Under polarized light the slight birefringence of the bands in living nuclei (negative with respect to the long axis of the chromosome, Schmidt, 1937, 1939) disappears completely. Rather more contrast is retained in the chromosomes and bands when they are fixed in 3 % rather

than 0·5 % formaldehyde. However, there is no improvement in the image obtained with polarized light.

Acrolein

The effects of this fixative (Fig. 15) are visually very similar to the effects of formaldehyde: the chromosomes disappear completely and the nucleolus becomes prominent, but its shape alters during fixation. Mixtures of acrolein and formaldehyde recommended as giving the best fixation of cytoplasm (Burgos *et al.* 1967) have effects on nuclei very similar to those produced by the individual components of the mixture.

Glutaraldehyde

All forms of glutaraldehyde precipitate a coarse meshwork from the nuclear sap (Figs. 8, 10). The chromosomes retain their visibility under Nomarski optics. The polymeric forms of the fixative alter the structure of the nucleus considerably: many bands disappear and considerable movement of the chromosomes occurs (Figs. 7, 8). Nucleoli become rather more prominent and change their shape. The slight birefringence of the chromosome bands is lost (Figs. 13, 14). In redistilled glutaraldehyde, on the other hand, all the chromosome bands remain, with very little loss of clarity or contrast with Nomarski optics. There is, however, fairly considerable loss of birefringence of the bands. The chromosomes do not move relative to each other, nor does the nucleus shrink or swell (Figs. 9, 10).

Since commercial glutaraldehyde may contain calcium as a contaminant (Oschman & Wall, 1972), not all the effects of commercial glutaraldehyde are necessarily due to glutaraldehyde polymers. To test this effect of calcium on glutaraldehyde fixation, 1·25 mM calcium chloride was added to redistilled glutaraldehyde in HEPES buffer. In this fixative very considerable movement of the chromosomes occurs (Figs. 11, 12). There is a general slight loss of contrast due to an increase in light scattering, perhaps through coacervation of protoplasm, but it would appear that most of the chromosome bands remain visible in this fixative.

The chromosomes retain slightly greater sharpness and clarity if cacodylate buffer is used rather than HEPES.

Ethyl CDI

This causes an instantaneous and drastic change in the optical properties of the whole gland due to the increase in refractive index and enlargement of a faint deposit that occurs on the surface of the gland and to some extent between the cells. This surface material appears as short, highly refractile streaks – like an enlarged fingerprint; it dominates the Nomarski image, causing considerable loss of contrast even at the level of the nucleus. Prior fixation for a few minutes in 3 % glutaraldehyde does not prevent the development of this surface effect on fixation with ethyl CDI. Interference by this surface patterning cannot entirely account for the loss of contrast of the nucleus; there is also some loss of contrast in the chromosomes themselves. Apart from this the chromosomes appear well preserved, with distinct bands (Fig. 16)

and little shrinkage. The nucleolus, on the other hand, is rapidly and completely dissolved in the fixative. The degree of birefringence of the chromosome bands cannot be estimated owing to interference by the refractile surface patterning on the gland.

Acetic methanol

This immediately produces a coarse opaque coagulum throughout the gland so that details of the fixed nuclei cannot be seen properly. Considerable lateral shrinkage of the chromosomes, however, can be detected; longitudinal shrinkage, if any, cannot be estimated since markers, such as bands, are not visible without subsequent processing. No assessment of the degree of retention of birefringence can be made.

DISCUSSION

All fixatives tried so far cause a profound loss of organization of the chromosomes. This is shown by loss of birefringence. There is often a loss of contrast in the Nomarski image of the chromosomes and also a loss of visibility of certain or all of the bands. Berendes (1970) in fact, found fewer bands in region X, 1–4 E of *Drosophila melanogaster* by electron microscopy of glutaraldehyde-fixed material than could be seen in the light microscope after acetic methanol fixation. Considerable movement of the chromosomes relative to each other occurs if calcium ions are present in the fixative. Some fixatives create specific artifacts – glutaraldehyde, for example, changes the nuclear sap into a coarse network easily visible in the light microscope, and ethyl CDI dissolves the nucleolus.

The best fixative for structural studies is redistilled glutaraldehyde in cacodylate buffer. This leaves the nucleus visually unchanged compared with its appearance in life, apart from coagulation of the nuclear sap. Even with this fixative, however, much of the birefringence of the bands is lost. This loss of birefringence may be due to the precipitant effect of the fixative on nuclear sap, or it may be a direct effect on the chromosomes.

It seems unlikely that the coagulum revealed in the nuclear sap by glutaraldehyde fixation is a normal component of the nucleus, rendered invisible in the living nucleus by an identity of refractive index between the network and the fluid in its meshes. Other fixatives do not reveal such a network with Nomarski optics, or in the electron microscope (Stevens & Swift, 1966). Alterations in the chromatin ultrastructure, due to this precipitant effect of glutaraldehyde on nuclear sap will be particularly pernicious when normal nuclei are fixed, for here the chromatin threads and nuclear sap are even more intimately associated than in the case of nuclei containing polytene chromosomes.

Formaldehyde causes very extensive loss of organization in the nucleus. This fixative is known to cause liquefaction of the nuclear sap in groups (e.g. Anura) that have gelatinous nuclear sap; it is used to disperse the sap of oocyte nuclei in the isolation of lampbrush chromosomes (Müller, 1974). Isolated polytene chromosomes preserve a fair degree of structural integrity in appropriate saline solutions (Lezzi & Robert, 1972), so the nuclear sap, therefore, cannot be essential for maintaining the

gross organization of chromosomes. We do not know, however, to what extent nuclear sap and chromosomes can be regarded as structurally independent, particularly at the fine-structural level.

Various carbodiimides are known to modify some of the bases in RNA (Ho & Gilham, 1967; Rhodes, 1975), but it is surprising that ethyl CDI should rapidly and completely dissolve the nucleolus, particularly since carbodiimides have a strong fixative action on proteins.

It is not clear what is lost from the chromosomes as a whole or from specific bands when, on fixation, they become invisible with Nomarski optics and polarized light. Polytene chromosomes fixed in osmium tetroxide clearly do not dissolve completely in the fixative, for they and their bands are visible in the electron microscope (Stevens & Swift, 1966). It is clear, however, that none of the fixatives tested so far is capable of preserving fully the organization of chromosomes and this without the destructive forces of subsequent processing. Since loss of organization of nuclei and chromosomes can be detected even by light microscopy during fixation, such a loss may be severe when these fixatives are used for electron microscopy. A search for new fixatives for nuclei and chromosomes is indicated.

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All photographs were taken with Normarski optics, unless designated otherwise.

Fig. 1. *S. equinum*. Living nucleus. × 1260.

Fig. 2. Same nucleus fixed 5 min OsO_4, 2 % in cacodylate buffer. × 1260.

Fig. 3. Same nucleus fixed 30 min OsO_4, 2 % in cacodylate buffer. × 1260.

Fig. 4. *S. equinum*. Living nucleus. × 1365.

Fig. 5. Same nucleus fixed 15 min 0·5 % paraformaldehyde in cacodylate buffer. × 1365.

Fig. 6. Same nucleus fixed 25 min 0·5 % paraformaldehyde in cacodylate buffer. × 1365.

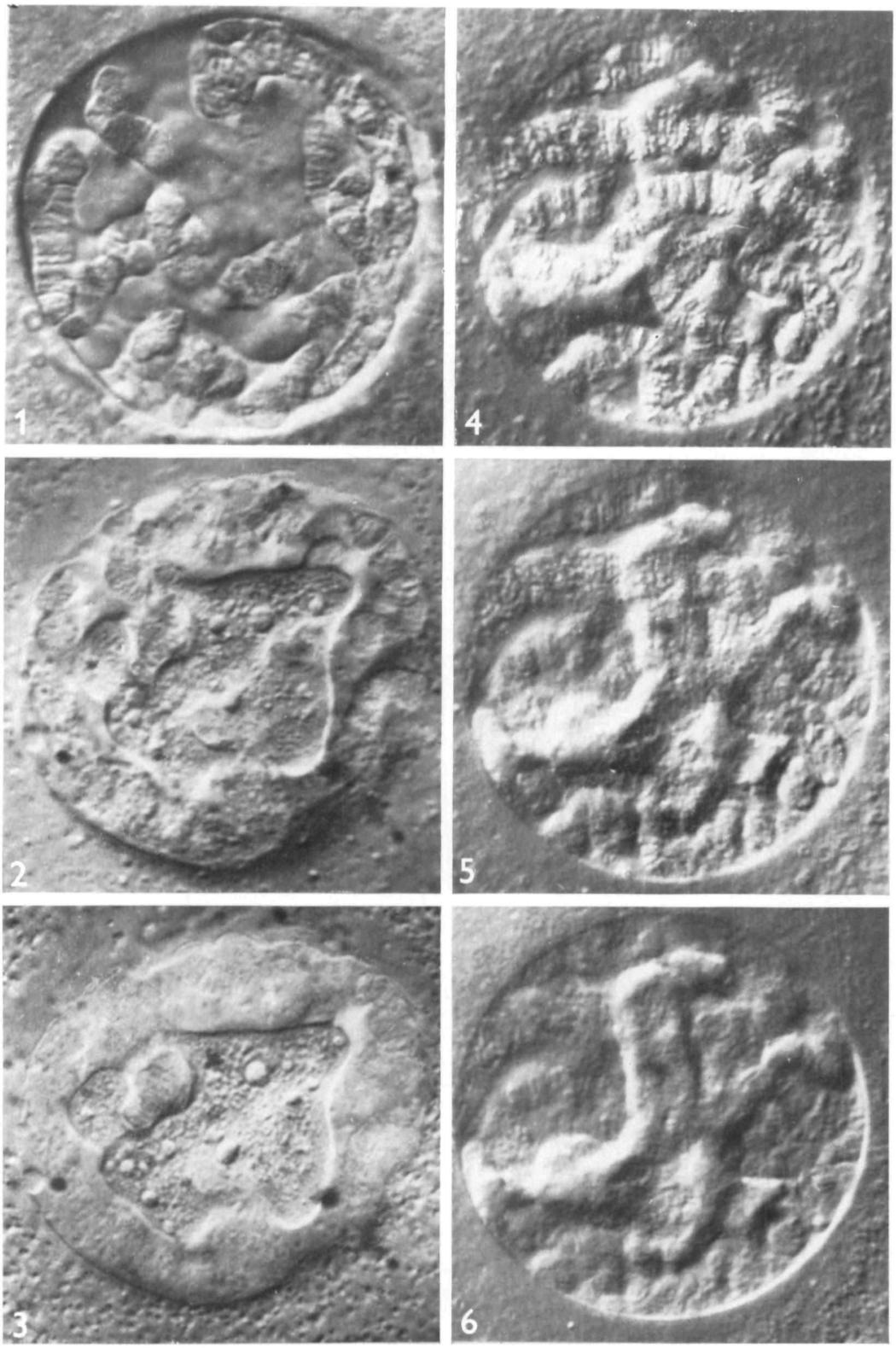
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Fig. 7. *S. argyreatum*. Living nucleus. × 1890.

Fig. 8. Same nucleus fixed 1 h 3 % polymeric glutaraldehyde in cacodylate buffer. × 1890.

Fig. 9. *S. argyreatum*. Living nucleus. × 1890.

Fig. 10. Same nucleus fixed 1 h 3 % redistilled glutaraldehyde in cacodylate buffer. × 1890.

Fig. 11. *S. equinum*. Living nucleus. × 1470.

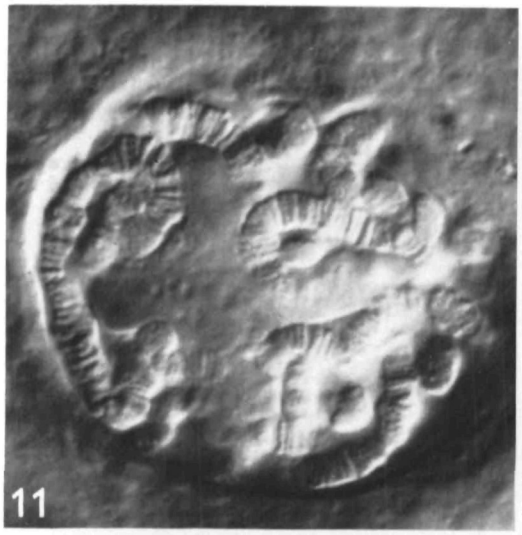
Fig. 12. Same nucleus fixed 1 min 3 % redistilled glutaraldehyde in HEPES buffer with 1·25 mM Ca^{2+}. × 1470.

Fig. 13. *S. equinum*. Living nucleus. Polarized light. × 1470.

Fig. 14. *S. equinum*. Polarized light. Fixed 1 h 3 % glutaraldehyde in cacodylate buffer. × 1470.

Fig. 15. *Simulium* nucleus fixed 1 h 1 % acrolein in 0·1 M cacodylate buffer. × 1470.

Fig. 16. *S. equinum* fixed 20 min in 10 % CDI followed by 2 % OsO_4 in cacodylate buffer for 30 min. × 1365.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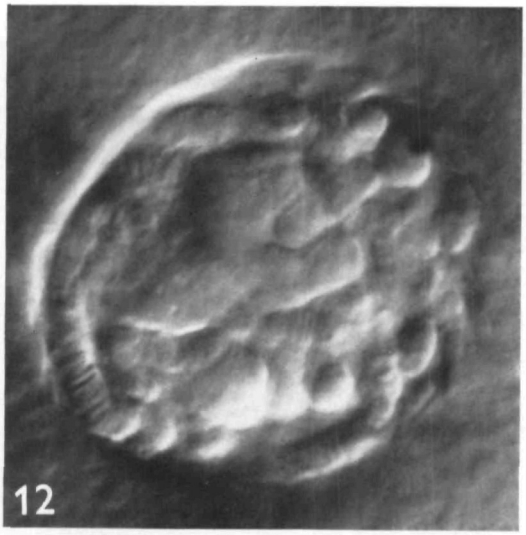
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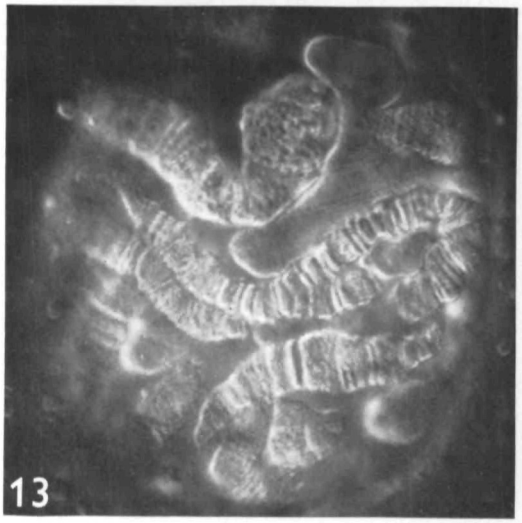
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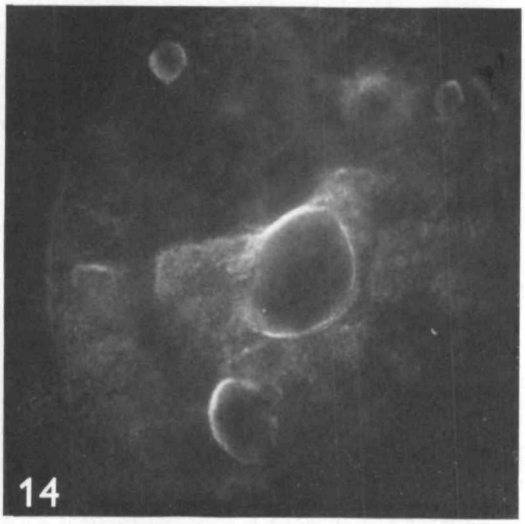
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