SUMMARY

The production of an artifactual network in the nuclear sap of the salivary glands of Drosophila has been investigated. Mechanical stress to the cells, 3 % glutaraldehyde containing more than 4 mM calcium, tannins, or cacodylate buffer or whose temperature is above 10 °C all enhance this artifactual effect. Over the range pH 6.5-7.4 there is no significant effect of pH. The inclusion of sucrose, and adenine nucleotides or other 'chelating' agents in the fixative reduces the effect, especially at low temperature (6-10 °C). These influences are additive and can abolish the artifactual effect. So too can 8 % glutaraldehyde, but this is irrespective of temperature.

INTRODUCTION

Nuclei are difficult to fix satisfactorily. It is common to see a badly fixed nucleus in well fixed cytoplasm. This has not been recognized in the past, for criteria of good fixation of nuclei have been lacking. In a previous paper (Skaer & Whytock, 1976), however, we used Nomarski optics to follow the changes that take place in the nuclei as living cells were perfused with fixatives. We used the large polytene nuclei in the salivary glands of Simulium since in these nuclei the chromosomes and the nuclear sap are to some extent visibly separate systems. The effects of fixatives on both these components can be assessed, particularly in relation to the maintenance of organization and the possible production of artifacts. We found that all fixatives caused some loss of organization. Redistilled glutaraldehyde preserved the chromosomes with least structural change, but it induced an artifactual network in the nuclear sap (Skaer & Whytock, 1976). The abolition of this artifactual network is the main concern of this paper.

MATERIALS AND METHODS

Living 3rd instar larvae of the Canton S strain of Drosophila melanogaster were cut in half transversely in the fixative. The cut passed just behind the tip of the salivary glands; muscular contraction of the front end of the larvae forced the glands into the fixative. The fat body was not removed. This form of dissection, which avoided all possibility of stretching, squashing, piercing or severing the glands was essential to give a constantly reproducible specimen for fixation. 3 % glutaraldehyde (redistilled as described in Skaer & Whytock, 1976) was used routinely together with the additives set out in Table 1 p. 17. 8 % unbuffered glutaraldehyde (ultrastructural grade) was purchased from Polaron Ltd and used undiluted. All other fixatives were made up in glass-distilled water and 0.05 M HEPES was used as buffer. The following chemicals were obtained from Sigma Chemicals Ltd: disodium adenosine 5' triphosphate.
RESULTS

Effects of stretching, squashing or transecting the gland

Slightly stretching the cells of the gland before fixation, as occurs, for example, when the fat body is dissected off the gland, causes the development of a very extensive artifactual network in the nuclear sap (Fig. 1). This occurs even when a fixative is used that is capable of excellent fixation of undamaged cells. Any mechanical abuse to the cells causes this effect. Despite the very pronounced network in the nuclear sap (Fig. 1) and loss of detailed structure in the chromosomes, the cytoplasm, in particular mitochondria, rough endoplasmic reticulum and differentiations of the plasma membrane appear reasonably well fixed (Fig. 1 and inset). Although it is difficult to give a standard mechanical stress to cells it appears that the inclusion of various additives in the fixatives – especially the addition of 5 mM EDTA, or 5 mM ATP or AMP together with 0.25 M sucrose – helps to minimize the artifactual network caused by mechanical stress.

Effects of additives to the fixative (3% glutaraldehyde)

Since cacodylate buffer enhances the network in the nuclear sap and may contain fairly large quantities of calcium as a contaminant, cacodylate buffer was avoided. HEPES buffer has been used throughout this work. Cells that are not stressed mechanically have the glutaraldehyde fixation network further suppressed by inclusion of 0.25 M sucrose and not more than 1.25 mM calcium in the fixative (Fig. 2).

In an attempt to ensure that divalent cations present in and around the tissue did
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Fig. 3. Nuclear sap and part of chromosome from a gland dissected and fixed exactly as that in Fig. 2 but in addition 5 mM ADP was present in the fixative. There is no network in the nuclear sap. The relatively homogeneous nuclear sap has a high electron density so some structures have low contrast (e.g. the perichromatin granule arrowed). × 50000.

not alter the composition of the fixative while it penetrated the tissue, some larvae were dissected in *Drosophila* Ringer modified to be free from divalent cations and with the following formula: KCl, 800 mg; glucose, 2000 mg; NaCl, 1005 mg; in 500 ml 0.05 M HEPES buffer pH 6.8. Although the cells were rinsed in this solution for only a few seconds before fixation, it caused widespread damage to the chromosomes — even the bands became diffuse or disappeared entirely. Ringer containing divalent cations must be used. However, fixation even in glutaraldehyde that contains calcium ions causes extensive and variable changes in intracellular calcium deposits (Skaer, Peters & Emmines, 1974). It is clear that some intracellular modification of the fixative solution is unavoidable. Nevertheless this effect can be minimized by agitating the tissue in a relatively large (10 ml) volume of fixative and so diluting out the high levels of divalent cations in the haemolymph and tissue.

If the calcium content of the fixative rises above 4 mM, the artifactual network is enhanced. The effect of precisely 4 mM calcium in the fixative is very variable from nucleus to nucleus. In some there is no enhancement of the nuclear sap network, in
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other nuclei there is slight enhancement of the network and the bands of chromosomes appear very distinct and strongly stained, in yet other nuclei the network is very pronounced (fig. 8 in Skaer, 1977). The effect of large amounts of magnesium on the nuclear sap is qualitatively similar to that of calcium. The critical limit for magnesium concentration has not been established, nor has it been established whether 4 mM is the critical limit for total divalent cations. It is not known whether the effects of different divalent cations on the nuclear sap network are additive or not.

Table 1. Effects of physical conditions and additives on fixation of nuclei by 3% glutaraldehyde

<table>
<thead>
<tr>
<th>Condition</th>
<th>Effect on the artifactual network</th>
</tr>
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<tbody>
<tr>
<td>0.1 M cacodylate buffer</td>
<td>+ +</td>
</tr>
<tr>
<td>4 mM calcium in HEPES buffer</td>
<td>+ + +</td>
</tr>
<tr>
<td>1.25 mM calcium or Mg in HEPES buffer</td>
<td>+</td>
</tr>
<tr>
<td>0.5 mM calcium or Mg in HEPES buffer</td>
<td>+</td>
</tr>
<tr>
<td>8% proglalin (tannin) + 1% digitonin, HEPES buffer</td>
<td>+ + +</td>
</tr>
<tr>
<td>pH range 6.7-7.4 (cacodylate buffer or HEPES buffer)</td>
<td>+</td>
</tr>
<tr>
<td>High temperature (20-37 °C)</td>
<td>+</td>
</tr>
<tr>
<td>Low temperature (below 10 °C)</td>
<td>-</td>
</tr>
<tr>
<td>0.25 or 0.4 M sucrose</td>
<td>-</td>
</tr>
<tr>
<td>4 mM EDTA (HEPES buffer)</td>
<td>-</td>
</tr>
<tr>
<td>5 mM disodium ATP, sodium ADP or AMP</td>
<td>-</td>
</tr>
<tr>
<td>5 mM sodium phosphoenolpyruvate</td>
<td>-</td>
</tr>
<tr>
<td>5 mM adenosine</td>
<td>-</td>
</tr>
<tr>
<td>0.25 M sucrose + 1.25 mM calcium + 5 mM ATP, or ADP</td>
<td>-</td>
</tr>
<tr>
<td>0.25 M sucrose + 5 mM ATP or 5 mM ADP or 4 mM EDTA</td>
<td>-</td>
</tr>
<tr>
<td>0.25 M sucrose + 1.25 mM calcium at 6 °C</td>
<td>-</td>
</tr>
<tr>
<td>8% glutaraldehyde (total concentration)</td>
<td>-</td>
</tr>
</tbody>
</table>

Addition of 4 mM EDTA, or 5 mM ATP, ADP or AMP almost completely abolished the network in the nuclear sap, particularly if 0.25 M sucrose was also added. This effect was seen even if the fixative contained 1.25 mM Ca (Table 1). The nuclear sap, then, appears very electron-dense and nearly homogeneous (Fig. 3) so that the whole nucleus lacks contrast. Addition of other compounds containing phosphorus (e.g. 5 mM phosphoenolpyruvate PEP) also help to abolish the generalized network in the nuclear sap. Phosphoenolpyruvate, however, reveals localized clusters of very fine fibres that link particular regions of the nucleus, e.g. the nucleolus to adjacent bands, parts of the chromocentre to adjacent bands and certain bands to the nuclear envelope. The significance of these fibres, which can also be seen with other treatments is not known. Varying the pH of the fixative over the range 6.65-7.4 has almost no effect, except that there is a very slight gain in contrast in nuclei fixed at the higher pH. Most fixation however was carried out at pH 6.7-6.8 since in other situations this has caused less disruption to the structure of isolated chromosomes.

No network is present in the nuclei of glands fixed in 8% glutaraldehyde whether fixation is carried out at room temperature or higher, up to 37 °C. The glands are, however, difficult to section after this fixative. The inclusion of tannin in glutaraldehyde
appears to improve the fixation of microtubules (Burton, Hinkley & Pierson, 1975). The effect of a soluble tannin of known composition (ethyl gallate) on the fixation of nuclei was therefore tried. It markedly enhanced the network in the nuclear sap. These effects are summarized in Table 1.

**Effects of temperature on fixation**

The network in the nuclear sap is less marked if fixation is carried out at low temperature (Table 1). Ice-cold fixation, however, causes the bands of the chromosomes to become very diffuse with irregular profiles. A fixative temperature in the region of 6–10 °C has been found to preserve the structure of the bands without production of a network in the nuclear sap. A higher concentration of glutaraldehyde in the fixative solution did not give fixation effects showing this temperature dependence (Table 1).

**DISCUSSION**

Fixation of nuclear sap with redistilled glutaraldehyde ranges from very poor to very good, depending partly on the composition of the fixative and partly on the physical stresses to which the cells are subjected before and during fixation. With certain regimes we find that the network can be completely abolished so that the nuclear sap appears almost homogeneous apart from ribonucleoprotein granules (Fig. 3). Whether or not nuclear sap in the living nucleus is indeed homogeneous at the fine-structural level is not clear. Unfortunately there is at present no satisfactory yardstick at the level of fine structure against which to measure the effects of fixatives on nuclei.

It is not clear whether the network that is produced in the nuclear sap after stressing the cell mechanically develops before fixation, or whether the mechanical effects predispose the nuclei to develop the artifact during fixation. Cells that have been subjected to extreme mechanical stress, or whose plasma membranes have been damaged – as occurs when nuclei are isolated in bulk – show a fibrillar network in the nucleus even after extraction of chromatin. Thus the apparently unfixed whole-mounts of nuclei prepared by Comings & Okada (1976) show fibres that they call ‘matrixin’ with a range of diameters from 3 to 40 nm arranged in a sponge-like matrix. If these matrixin fibres are artifactual, as is suggested by their apparent similarity to the network in Fig. 1, it would show that a fibrillar network can develop even in unfixed nuclei.

Callan (1952), moreover, has observed that the nuclear sap in unfixed nuclei, isolated from amphibian oocytes, undergoes coagulation, followed by flocculation and settling of a precipitate when these nuclei are treated with solutions containing 0.05–0.003 M Ca. At lower concentrations than 0.0015 M calcium the structural phase of the nuclear sap is seen first to liquefy; a floccular precipitate then appears. Gall (1966) has also observed instantaneous liquefaction of the sap when these isolated nuclei from amphibian oocytes are treated with 10⁻³ M calcium. He observed liquefaction of the sap down to 10⁻⁶ M calcium. Liquefaction of the sap is presumably an
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indication that the structure of the gel has been destroyed. Calcium, unlike magnesium, has a very adaptable coordination sphere that makes cross-linking a major feature of its solution chemistry (Williams, 1976). A gel that is rich in carboxyl groups and whose structure is based on weak cross-links that do not involve calcium may, particularly if the protein ligand becomes highly hydrated, be cross-linked by calcium in such a way that the gel structure is destroyed and the protein precipitated (Williams, 1976).

Since the network in the nuclear sap described in this and other papers (Skaer & Whytock, 1976, 1977): (a) resembles a protein precipitate, and calcium ions applied to nuclei will precipitate nuclear sap (Callan, 1952); (b) is enhanced by calcium concentrations in the fixative of more than 4 mM; and (c) is abolished by substances such as adenine nucleotides that form complexes with calcium (Manery, 1968), it is tempting to ascribe the network, directly or indirectly, to the presence of abnormally high concentrations of calcium around the nucleus. In this context ‘abnormally high’ concentrations may be in the region of $10^{-5}$ M and may need to be applied for only a short time – as for example before EDTA can penetrate into a mechanically damaged cell.

There is very little difference in the appearance of nuclei of Drosophila salivary glands fixed with glutaraldehyde containing 0.5 mM CaCl$_2$ to 3 mM CaCl$_2$. It may be that over this range of calcium concentration, the plasma membrane is effectively impermeable to calcium in the fixative. This certainly seems to be the case for vertebrate striated muscle, for, although a rise of intracellular calcium concentration around the myofibrils from approximately $10^{-8}$ to $10^{-6}$ M (half maximal ATPase activity at $10^{-6}$ M Ca$^{2+}$) (Bendall, 1969) provokes contraction, striated muscle is readily fixed in the relaxed state even if no special precautions are taken to exclude calcium from the fixative. It is, however, not true for apparently undamaged smooth muscle of guinea pig (Schoenberg, Goodford, Wolowyk & Wootton, 1973) where a sustained tension is produced if the glutaraldehyde fixative contains 2.4 mM calcium. Thus whether the significant calcium contamination of some samples of glutaraldehyde and buffers (Oschman & Wall, 1972) is likely to produce a network in the nuclear sap depends not only on the relative affinity of the buffer and nucleus for calcium (Ris, 1968, 1975) but also on whether the plasma membranes of the tissue are naturally permeable to calcium (or have been made so by prior treatment).

For Drosophila it is possible that concentrations of calcium above 4 mM in the fixative may enter the cell despite the postulated impermeability of the membrane to lower concentrations of calcium. Measurements of the calcium permeability of the living, unfixed salivary glands of Drosophila and Chironomus do suggest that the plasma membrane is very impermeable to calcium unless it is damaged (Loewenstein, 1966). Rose & Loewenstein (1976) found that the free calcium in the cytoplasm of salivary glands of Chironomus was less than $5 \times 10^{-7}$ M in the presence of an external calcium concentration of 5 mM. Although it would appear that more than 4 mM calcium in the fixative swamps the barrier properties of the plasma membrane, it must be remembered that the divalent cation content of the haemolymph around Drosophila cells is very high. Thus Schneider’s tissue culture medium for Drosophila cells (Schneider, 1964) contains rather more than 5 mM CaCl$_2$ and approximately 15 mM MgSO$_4$. 

Callan (1952, 1966) found that calcium ions and also formaldehyde (vapour) would dissolve the normally gelatinous nuclear sap of isolated nuclei from the oocytes of amphibia; he also found a similar effect with a pH above 7 (Callan, 1952). It thus seems possible that with standard electron microscopy the gel of the nuclear sap is often fixed in a state of 'explosive collapse' (Callan, 1952) due to the fixative or the treatment of the cells before fixation. It is not clear whether a pH above 7 destroys the gelatinous nature of the nuclear sap of amphibian oocytes by precipitation of proteins at their isoelectric point or by simple solution of the components of the gel. Müller (1974) mentions that although a pH of above 7 is by itself insufficient to cause detectable dispersion of the nuclear sap of *Xenopus* oocytes, when the pH is raised above 7 there is a loss of organization of the loops of isolated chromosomes that is clearly detectable even by light microscopy. In view of these effects of pH on isolated nuclei and chromosomes it is perhaps surprising that we find almost no effect of the pH of the fixative on the fine-structural organization of either nuclear sap or chromosomes over the range pH 6.5–pH 7.4. This lack of marked visible effect of pH might be due to the presence of the glutaraldehyde preventing the nuclear sap from being dissolved, or it might possibly be due to the buffering power of the cytoplasm in the early stages of fixation.

Despite the general reduction of contrast resulting from the addition of adenine nucleotides (and to a less marked extent EDTA) to glutaraldehyde, it may be useful to add one or other of these in situations where mechanical stress or damage to the cells is unavoidable — as when salivary glands are squashed to spread the chromosomes. Although chromosomes observed after destruction of the gelatinous nature of the nuclear sap of amphibian oocytes appear intact by light microscopy (Callan, 1952; Müller, 1974), so that, at this level, dispersion of nuclear sap can be separated from disintegration of the chromosomes, at the level of fine structure, chromosomal organization can be markedly influenced by dispersal of the nuclear sap. Thus Mott & Callan (1975) have shown that the chromomeres of lampbrush chromosomes undergo an extensive change in packing when the nuclear sap is dispersed. The organization of nuclear sap and chromosomal organization are clearly interrelated. The abolition of the network in the nuclear sap is a necessary step towards good fixation; other effects of fixation on nuclei — such as the loss of birefringence of the bands of polytene chromosomes (Skaer & Whytock, 1976) have still to be tackled.

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REFERENCES


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