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# CHROMATIN-LIKE ARTIFACTS FROM NUCLEAR SAP

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SUMMARY

Nuclear sap, on fixation in glutaraldehyde, forms a fibrous network that resembles chromatin in its dimensions and staining properties. This artifactual network is easily confused with true chromatin fibres in sections of nuclei. With formaldehyde a homogeneous array of beads is produced. These are approximately 10 nm in diameter – the size of nucleosomes – and are interconnected by exceedingly fine fibrils. Each fixative and buffer imposes its own distinctive, reproducible pattern on nuclear sap. The structure of nuclear sap in life cannot be deduced from this range of patterns.

## INTRODUCTION

Nuclear sap is a gelatinous fluid that is usually regarded as structureless. Although Gall (1963) suggested that nuclear sap might obscure details of the chromatin threads, it has been generally and tacitly assumed that chromatin threads can be distinguished clearly from their supposedly structureless background – the nuclear sap. That this is not so was shown by Skaer & Whytock (1976) who demonstrated that glutaraldehyde, for instance, produced a coarse artifactual network in the nuclear sap. This could produce 2 sources of confusion. The threads of this network might add on to chromatin threads and thus thicken them abnormally; and fibres observed in fixed nuclei in some cases might not be chromatin at all but artifacts of the fixation of nuclear sap.

In this paper we describe the effects of different fixatives on nuclear sap. The effects are very varied depending on the fixative and buffer used. Some fixatives do indeed produce artifactual threads in the nuclear sap that could be mistaken for chromatin fibres, particularly since the network stains in the same way as chromatin. With other fixatives the nuclear sap appears to be filled with granules or beads which may also be artifactual. Since these beads are approximately the same size as histone nucleosomes, they represent another possible source of confusion. This is of relevance to our understanding of the fine structure of intranuclear chromosomes and chromatin, since the effects of fixation of nuclear sap will normally be superimposed on the fine structure of the hereditary material.

## MATERIAL AND METHODS

Two sources of nuclear sap that is free from chromatin were studied:

(1) *Cells whose chromatin has been packed into one half of the nucleus by high-speed centrifugation.* After this treatment the centrifuged chromatin does not return into the centripetal half of the nucleus provided fixation follows rapidly after the end of centrifugation (Brenner, 1953; Pusa, 1963; Beams & Mueller, 1970). The only chromatin remaining in the centripetal half is the thin layer of heterochromatin tightly bound to the peripheral lamina lining the nuclear envelope, and occasional strands, oriented by centrifugation so they lie between the peripheral heterochromatin and the main centrifugal mass of chromatin (Brenner, 1953). Both these types of chromatin are easily recognizable and can be avoided. A chromatin-free zone of nuclear sap can thus be chosen. Slices of rat liver approximately 1 mm thick or a monolayer of an epithelial cell line isolated from pig kidneys were centrifuged as described by Brenner (1953). They were spun in Eagle's MEM at 140000 **g** for 2 h at 4 °C in an MSE Superspeed centrifuge. They were fixed immediately after centrifugation.

(2) *Cells whose nuclei contain giant polytene chromosomes.* In these nuclei all chromatin is grouped into polytene chromosomes so if one avoids the chromosomes, regions of pure nuclear sap can be observed. Salivary glands from 3rd instar larvae of *Drosophila melanogaster* or of late larvae of *Simulium equinum* L. were used.

The following *fixatives* were used for all cell types:

(*a*) 3 % glutaraldehyde. This was either redistilled as described in Skaer & Whytock (1976), or bought specially purified from Taab laboratories, Emmer Green, Reading. It was made up in either 0·1 M cacodylate buffer pH 7·4 or 6·65, or in 0·05 M HEPES buffer pH 7·4 or 6·8, and usually containing 1·25 mM $CaCl_2$.

We found, by atomic absorption spectroscopy on a Unicam SP 90 spectrophotometer that the calcium content of 0·1 M cacodylate buffer was slightly more than 1 mM. Oschman & Wall (1972) also found high levels of calcium contamination in cacodylate buffer. On the other hand, our 0·05 M HEPES buffer contained approximately $10^{-4}$ M calcium. For some experiments, therefore, 1·25 mM calcium chloride was added to the HEPES buffer so that its calcium content was comparable with that of the cacodylate buffer. Cacodylate, however, is classed as a chelating buffer (Ris, 1968). Its available calcium will depend, therefore, on the relative affinities of buffer and tissues. The significance of the presence of relatively low (but physiologically high) concentrations of calcium in the fixative cannot be assessed by these experiments, especially since when pieces of tissue are fixed, the permeability properties and calcium content of the cells will modify the composition of the fixative as it penetrates. Skaer, Peters & Emmines (1974) found that platelet dense bodies normally have a fairly constant Ca : P ratio, but that fixation, even in the presence of calcium in a non-chelating buffer, caused this ratio to vary very widely and unpredictably. Some aspects of the influence of calcium on the fixation of nuclei by glutaraldehyde are dealt with in Skaer & Whytock (1976).

(*b*) 3 % formaldehyde. This was prepared from paraformaldehyde as described in Skaer & Whytock (1976), and made up in the same buffers as glutaraldehyde, but calcium was added to the HEPES buffer at 0·67 mM.

(*c*) 1 % osmium tetroxide in 0·1 M cacodylate buffer.

All fixatives were applied for 1 h at room temperature.

After aldehyde fixation some specimens were treated with 1 % osmium tetroxide in 0·1 M cacodylate buffer for 1 h. All specimens were dehydrated in a graded series of ethanols, rinsed in propylene oxide and embedded in Spurr embedding resin.

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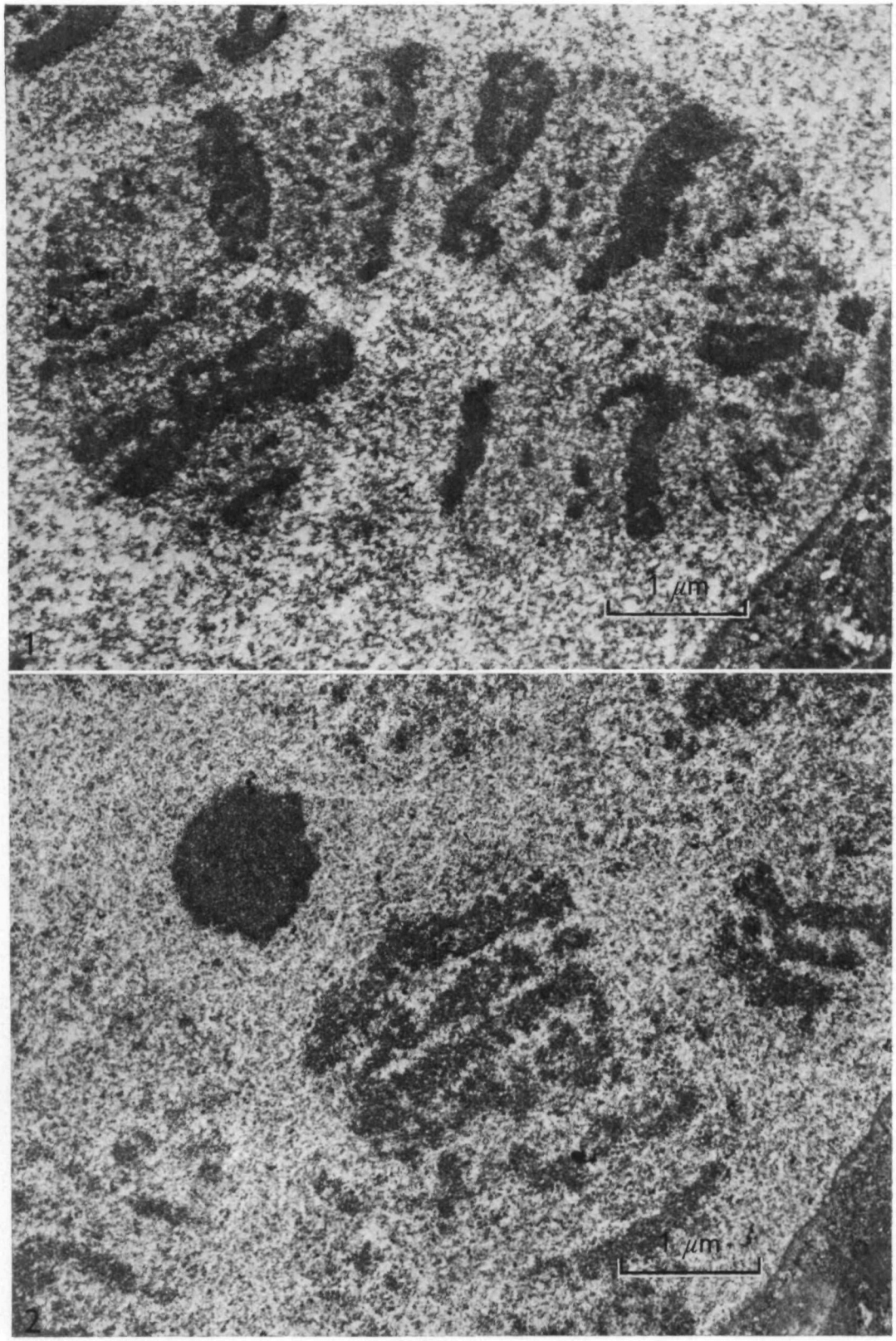
Fig. 1. Nucleus from salivary gland of *Drosophila* fixed in 3 % redistilled glutaraldehyde in 0·1 M cacodylate buffer. Not treated with osmium tetroxide. Stained with permanganate and lead citrate. The nuclear sap is coarsely fibrous. × 22000,

Fig. 2. Nucleus from salivary gland of *Drosophila* fixed in 3 % formaldehyde in 0·1 M cacodylate buffer. Treated with 1 % osmium tetroxide after fixation. Stained with permanganate and lead citrate. The nuclear sap is granular. × 22000.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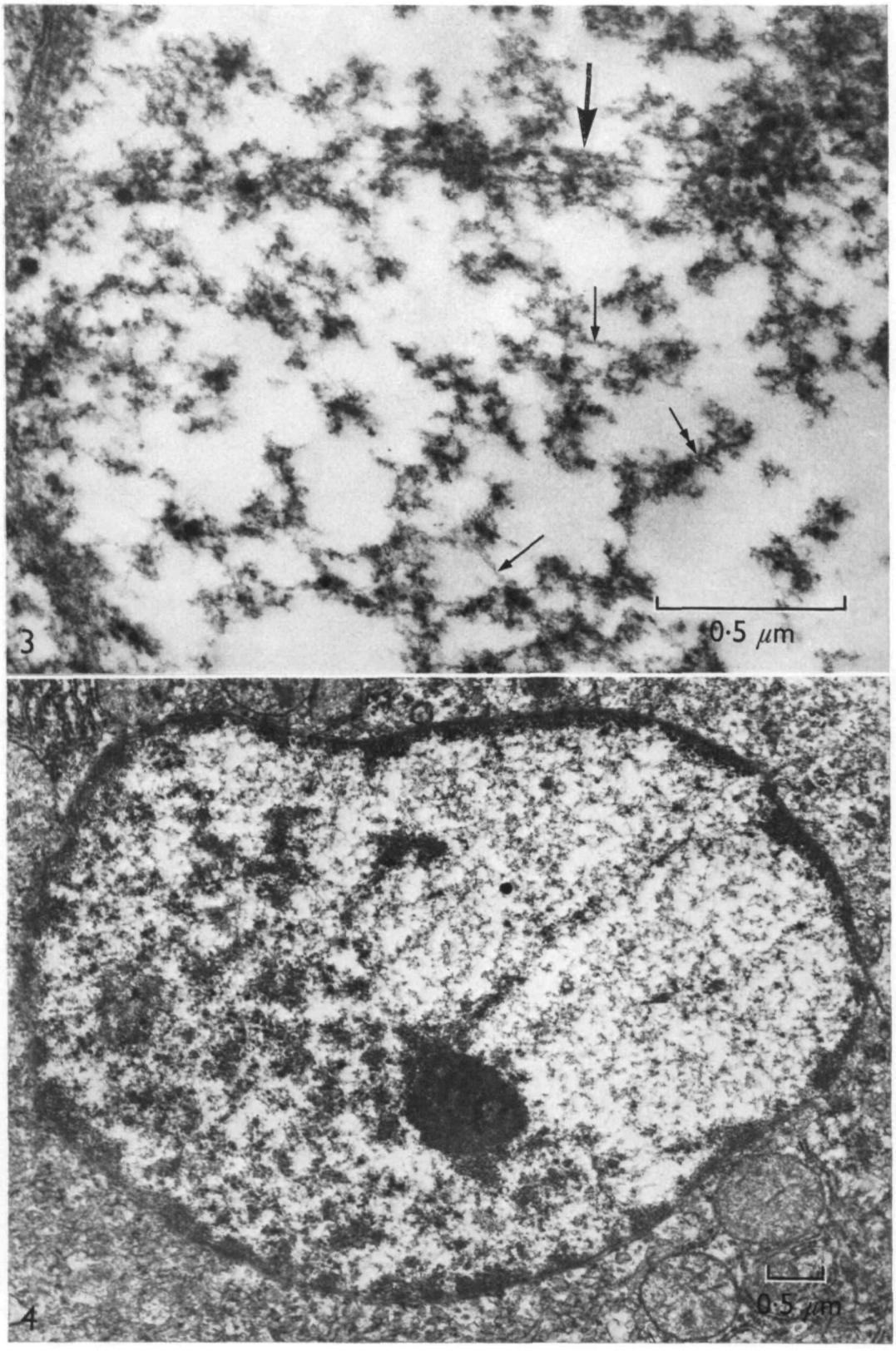
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Thin sections were stained with aqueous uranyl acetate or double stained with uranyl acetate and lead citrate. Some sections were stained with permanganate and lead citrate (Reedy, 1965). They were examined in an AEI EM6B operated at 60 kV.

The distribution of chromatin in the centrifuged nuclei was found by the osmium ammine test for DNA. This was performed on centrifuged cells fixed in glutaraldehyde but not treated with osmium tetroxide. They were embedded in Epon as recommended by Gautier & Fakan (1974). The osmium ammine reagent was synthesized and used as described in Cogliati & Gautier (1973).

## RESULTS

*Distribution of chromatin.* As suggested by the pattern of Feulgen staining of nuclei subjected to high-speed centrifugation (Brenner, 1953; Beams & Mueller, 1970), all chromatin except that attached to the nuclear envelope is spun down into the centrifugal half of the nucleus. We have confirmed this distribution at the level of fine structure by means of the osmium ammine technique. Although the technique is essentially empirical it gives a clear-cut picture of the distribution of DNA in cells (Gautier & Fakan, 1974); this specific staining does not occur if hydrolysis in 5 N HCl prior to use of the reagent is omitted. The main masses of heterochromatin are strongly electron-dense. Fine, straight strands of euchromatin in the centripetal half of the nucleus and oriented by centrifugation also take up the stain. Apart from these fine, straight strands and the peripheral heterochromatin, the centripetal half of the nucleus contains only nuclear sap. Controls processed without prior hydrolysis in 5 N HCl were completely negative.

*Staining of nuclear sap.* In all cell types examined the nuclear sap stains with permanganate and lead citrate, uranyl acetate, or uranyl acetate and lead citrate in the same way as chromatin. This is the case whether or not the tissues are treated with osmium tetroxide after fixation in aldehydes. The intensity of staining of nuclear sap is comparable with that of euchromatin but rather less than that of heterochromatin (Fig. 4).

*Glutaraldehyde.* This fixative produces a coarse network in the nuclear sap of all cell types examined. The effect varies slightly with the tissue used: in *Drosophila* the network has a rather small pore size (Fig. 1), in centrifuged liver the network is more open (Fig. 4). The effect of the buffer and calcium content is greater than these

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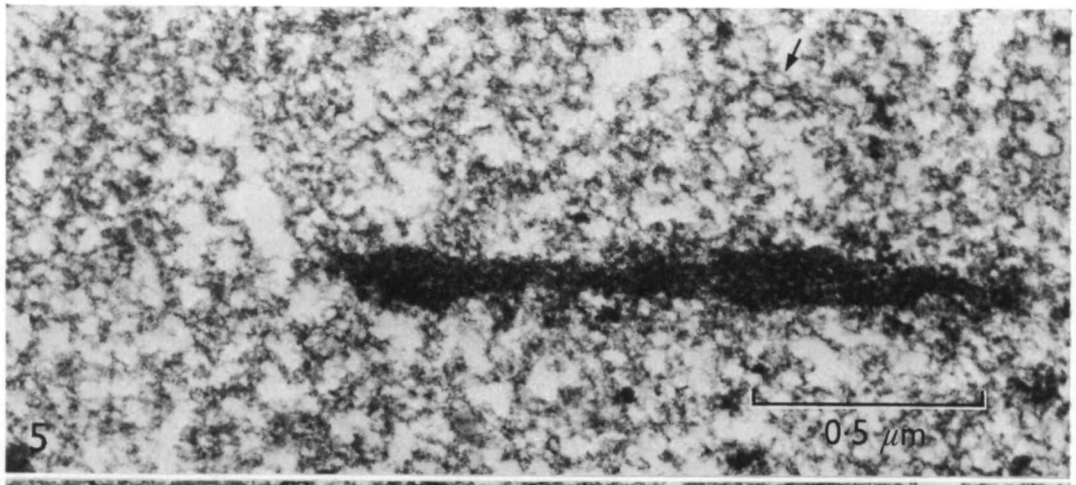
Fig. 3. Centripetal part of nucleus from centrifuged liver of rat fixed in 3 % glutaraldehyde in 0·1 M cacodylate buffer. Treated with 1 % osmium tetroxide after fixation. Stained with uranyl acetate and lead citrate. Small arrows mark long lengths of the fixation network; double-headed arrow, part of the network with fine lateral fibres; large arrow marks a chromatin thread, oriented by centrifugation and 20–25 nm thick. × 60000.

Fig. 4. Nucleus from centrifuged liver of rat fixed in 3 % glutaraldehyde in 0·1 M cacodylate buffer, from a section adjacent to Fig. 3. Shows artifactual network in centripetal half of nucleus, and main bulk of chromatin and nucleolus in centrifugal half (bottom left). Heterochromatin attached to the nuclear envelope and euchromatin threads oriented by centrifugation are present in the centripetal half of the nucleus. Stained with uranyl acetate and lead citrate. × 17250.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comparatively slight differences from tissue to tissue. With cacodylate the network is particularly pronounced. In *Drosophila* and cacodylate buffer pH 7·4 the fibres of the network range from 5 to 20 nm thick (Fig. 1). In some places it is clear that the thicker fibres are the result of lateral aggregation of thin fibres. The effect with cacodylate buffer pH 6·6 is very similar. On the other hand with HEPES buffer pH 7·4 or 6·8, each with 1·25 mM calcium, the network has a fine mesh and is made up of fibres mostly 10 nm in diameter. In centrifuged liver of rat there is a similar pattern of effects. With cacodylate buffer, individual fibres up to 100 nm long and of a more or less uniform thickness of 10–18 nm are seen (small arrows, Figs. 3, 4). Other fibres have a frayed appearance with fine lateral fibres 5–8 nm thick lying at an angle of approximately 60° to the main axis of the aggregate (double arrow, Fig. 3). In some parts of the network the nuclear sap forms a structureless coarse coagulum. Chromatin fibres, oriented by centrifugation and 20–25 nm thick are readily recognizable in the network (large arrow, Fig 3). With HEPES buffer and 1·25 mM calcium the network in the nuclear sap is of rather variable mesh size from nucleus to nucleus, but on the whole the mesh is much finer with HEPES and calcium (Figs. 5, 6) than with cacodylate (Fig. 3). The network with HEPES and calcium consists mainly of fibres 10 nm thick together with some 20 nm and some 5 nm. Up to 50-nm lengths of these fibres occur. There are very few granules present.

Treatment with osmium tetroxide after fixation does not alter any of these specific effects of glutaraldehyde on the nucleus, but merely produces a general increase in electron density in nuclear sap and chromatin as well as in cytoplasm and membranes.

*Formaldehyde.* In nuclei of *Drosophila* (Fig. 2) and of centrifuged liver cells (Figs. 7, 8) formaldehyde in cacodylate buffer produces a homogeneous array of small granules approximately 10 nm in diameter. These granules vary a little in size and intensity of staining – the smaller ones stain less intensely. This might be an effect of sectioning. When formaldehyde fixation is performed in a non-chelating buffer (HEPES) with 0·67 mM calcium added, the nuclear sap in all cell types examined forms a very fine network of anastomosing fibres approx. 10 nm thick together with branching strands 5 and 3 nm thick and a few granules 20 nm across. Treatment of formaldehyde-fixed tissue with 1 % osmium tetroxide made no difference to the morphology of nuclear sap.

*Osmium tetroxide.* Nuclear sap of both cell types fixed in osmium tetroxide consists of granules of a very wide range of size from 5–18 nm many of which are linked together by fine fibrils.

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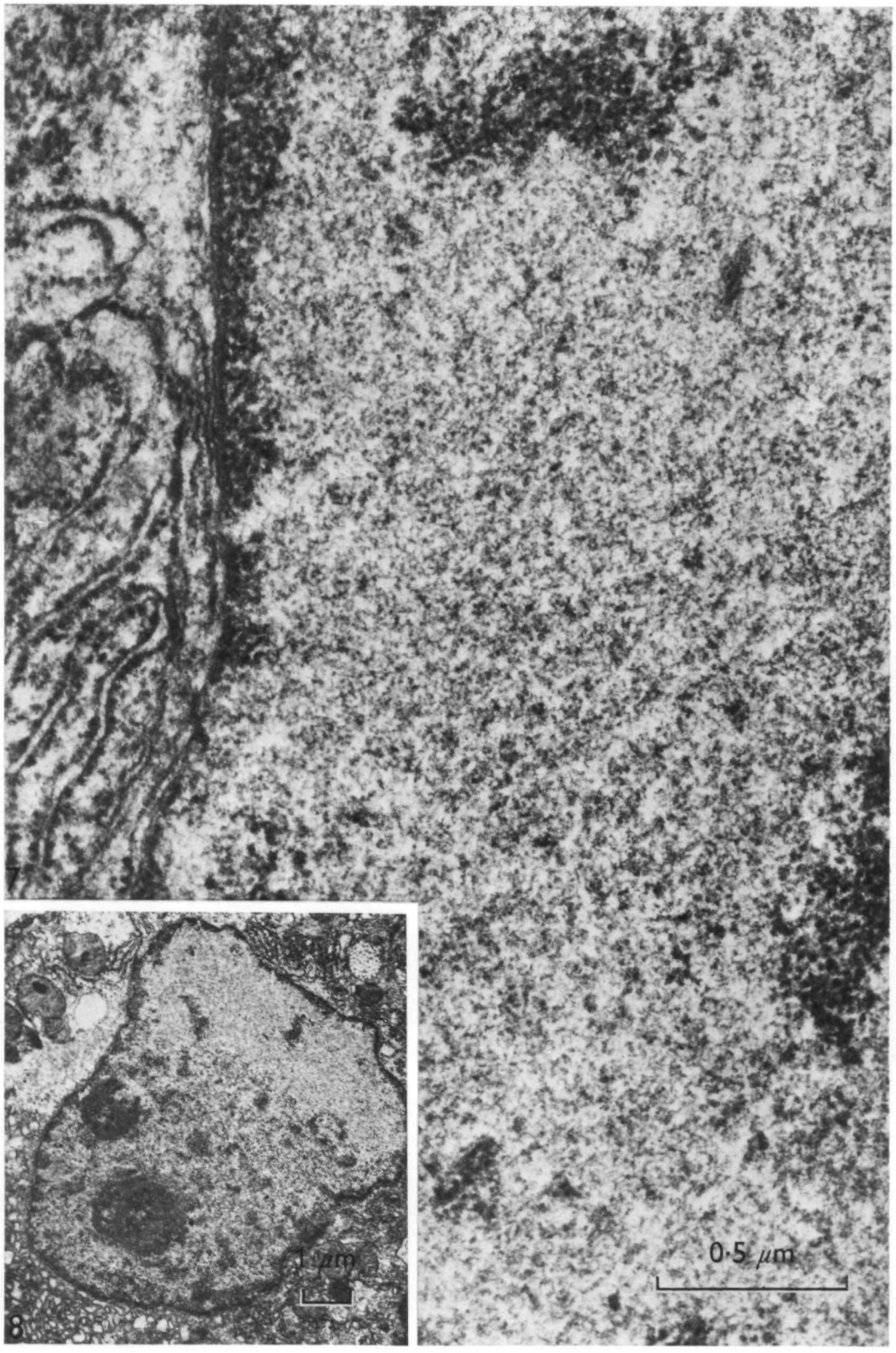
Fig. 5. Centripetal part of nucleus from centrifuged liver of rat fixed in 3 % glutaraldehyde in 0·05 M HEPES buffer with 1·25 mM calcium. The artifactual glutaraldehyde network has a relatively fine mesh. A strand of heterochromatin oriented by centrifugation lies in the centre of the picture. Not treated with osmium tetroxide after fixation. Stained with uranyl acetate and lead citrate. × 60000.

Fig. 6. Low power of the same nucleus as in Fig. 5, to show the location of the high-power picture. The nucleus demonstrates the clear cut separation of nuclear sap and chromatin that can be obtained by centrifugation. Stained with uranyl acetate and lead citrate. × 18750.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## DISCUSSION

The strikingly different effects of fixation on nuclear sap are illustrated by comparing Figs. 3, 5 and 7 and also Figs. 1 and 2. These are nuclei from the same tissue, processed in the same way except for the fixative used, and displayed at the same magnification. The glutaraldehyde network is clearly artifactual – its formation can be observed as living cells are subjected to fixation (Skaer & Whytock, 1976). This effect of glutaraldehyde calls into question the validity of measurements of the thickness of 'chromatin' fibres in nuclei fixed with glutaraldehyde (Olins & Olins, 1972; de la Torre, Sacristán-Gárate & Navarrete, 1975). The mesh size of the glutaraldehyde artifact varies with different buffers but is of the same order of size as measurable lengths of euchromatin packed in its normal convoluted form in nuclei. In normal glutaraldehyde-fixed nuclei artificial thickening of chromatin fibres at random by the network, and confusion between true chromatin threads and threads of the nuclear sap network are likely.

Berezney & Coffey (1974, 1975) subjected nuclei to a series of extractions to remove chromatin, DNA, RNA, and phospholipid. It is small wonder that the residual 'nuclear protein matrix' they have discovered retains some structural features and the shape of unextracted nuclei, since nuclear sap is a gel whose rigidity can be as high as that of a gelatinous solid (Callan, 1952; Müller, 1974). The visible framework of this residual nuclear structure may be due to Berezney and Coffey's use of glutaraldehyde to fix the nuclei.

Despite the variable mechanical properties of nuclear sap – from a thixotropic gel (Gray, 1927; Harris, 1939) to a gelatinous solid (Müller, 1974), for the cell types we have studied the range of morphology of nuclear sap appears to be more influenced by the fixative used than the tissue of origin of the nuclei. Other fixatives as well as glutaraldehyde might be expected to destroy the organization of nuclear sap. Thus Callan (1966) showed that formaldehyde would disperse the normally gelatinous nuclear sap in nuclei isolated from oocytes of the axolotl. Each different fixative and buffer imposes its own distinctive, reproducible pattern on the nuclear sap. The structure of nuclear sap in life cannot be decided from this range of pattern.

The effects of fixation of nuclear sap can be observed clearly in the interbands of polytene chromosomes (Figs. 1, 2); the image of fixed nuclear sap is superimposed on the structure of the chromosomes. Thus these effects may directly influence the fine-structural picture of fixed chromatin.

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Fig. 7. Centripetal part of nucleus from centrifuged liver of rat fixed in 3 % formaldehyde in 0·1 M cacodylate buffer. Treated with 1 % osmium tetroxide after fixation. The nuclear sap is a relatively homogenous mass of granules approximately 10 nm across with this fixative. Stained with uranyl acetate and lead citrate. × 60000.

Fig. 8. Low-power picture of the same nucleus as in Fig. 7. The region enlarged in Fig. 7 is from the upper left-hand portion of this nucleus. ×7500.

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