# FIXATION BY MEANS OF GLUTARALDEHYDE–HYDROGEN PEROXIDE REACTION PRODUCTS

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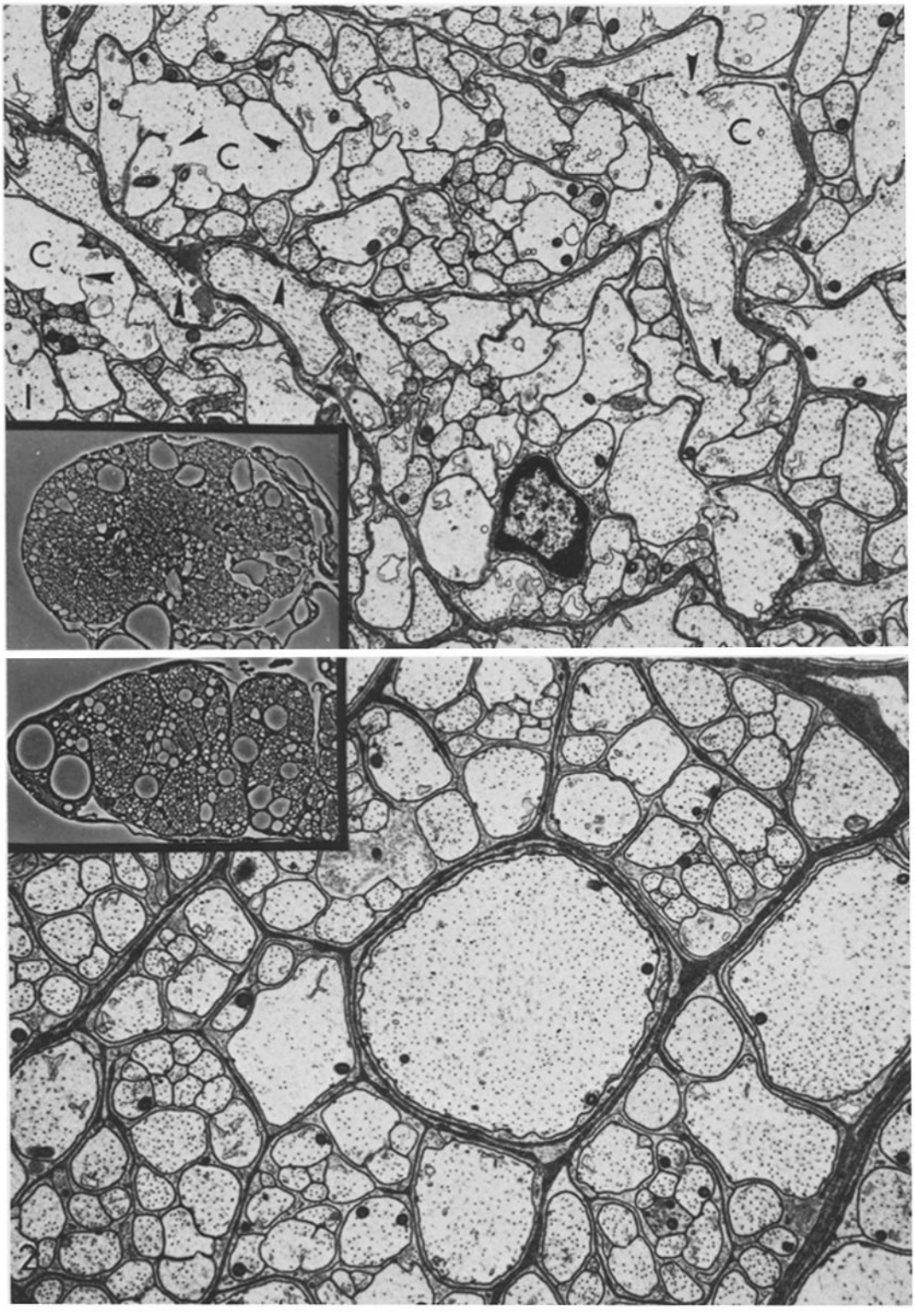
Improvements in fixation were previously observed in specimens treated either by a weak $H_2O_2$ solution followed by glutaraldehyde (1) or by glutaraldehyde-$H_2O_2$ mixtures (2). The effectiveness of the $H_2O_2$ treatment was presumed to depend on the activity of reaction products of $H_2O_2$ and glutaral-

FIGURE 1 Control fixation. Cross-section through a root of the sixth abdominal ganglion of crayfish Shrinkage is apparent, primarily in central areas of the root (*inset*, phase-contrast microscopy). The profile of the axons is distorted. Microtubules are poorly preserved and several axon-surface membranes are broken (arrowheads). Large cisterns (*C*) result from the rupture of small contiguous axons into common cavities. × 8700. *Inset*, × 270.

FIGURE 2 Glutaraldehyde-$H_2O_2$ fixation. Cross-section through a root from the sixth abdominal ganglion of crayfish. The general architecture of the root is well preserved also in central areas where even the smallest axons can be resolved (*inset*, phase-contrast microscopy). Most of the axons have a circular profile. The microtubules are homogeneously distributed and broken membranes are rarely seen. × 8700. *Inset*, × 270.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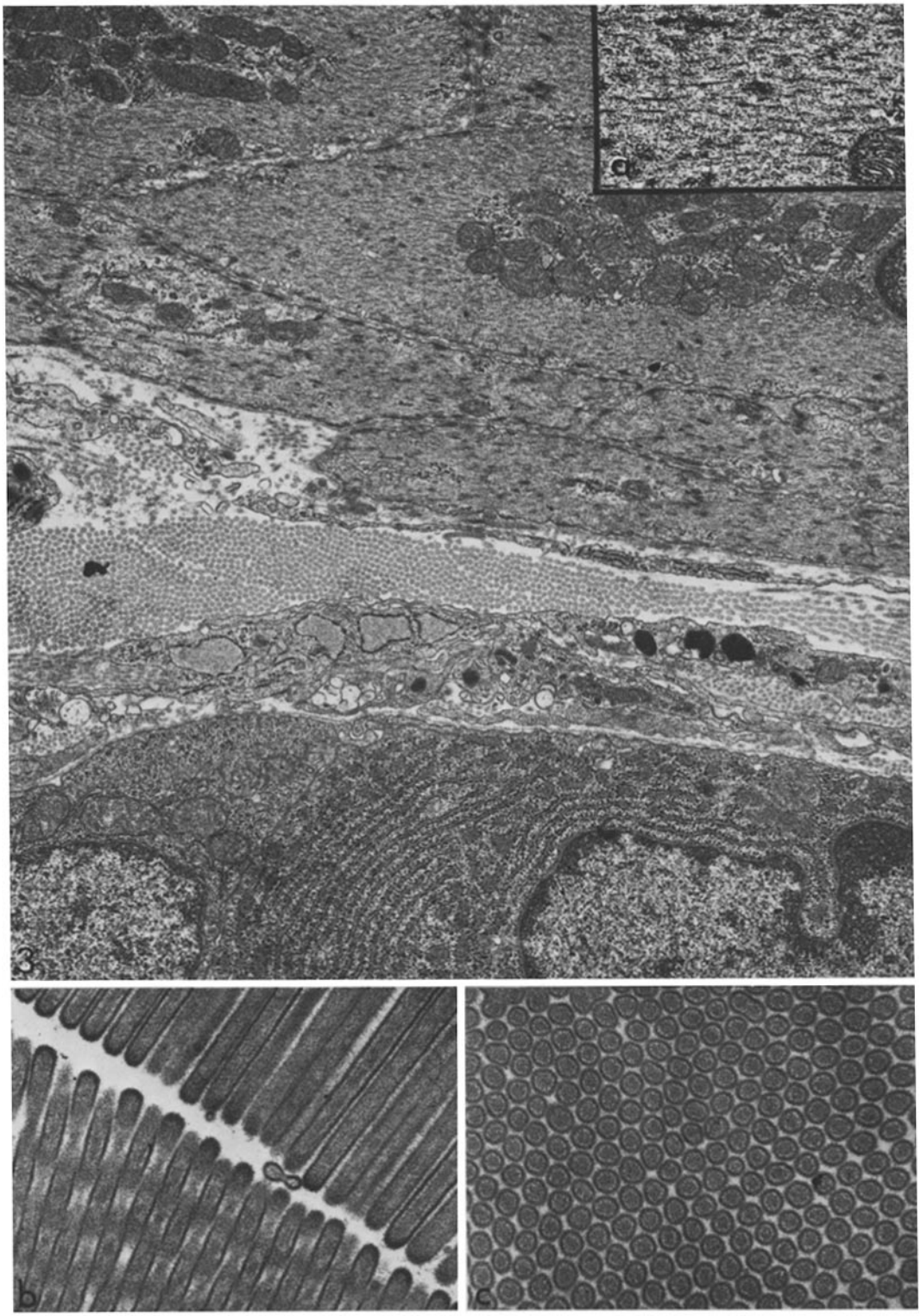



FIGURE 3 Glutaraldehyde-$H_2O_2$ fixation. Rat jejunum. Notice the good preservation of different tissues such as epithelium and connective and smooth muscle. In smooth muscle cells, thick filaments (~120 A in diameter) are seen, obliquely cut, organized in a quite regular fashion (*inset a*). Intestinal microvilli are shown in longitudinal (*inset b*) and cross-section (*inset c*). × 11,800. *Inset a*, × 26,200; *Insets b, c*, × 22,500.

dehyde (2). This note describes the new method in more detail.

Glutaraldehyde-$H_2O_2$ fixative is prepared as follows. 50% glutaraldehyde (Fisher Scientific Co., Pittsburgh, Pa.) is diluted to a 3–6% solution buffered to pH 7.4 with 0.1 M (Na, K) phosphate or Na cacodylate at room temperature. 30% $H_2O_2$ (Fisher Scientific Co.) is added to it, with continuous stirring, in the amount of 1–5 drops per each ml of 50% glutaraldehyde. The fixation is carried on for 1–2 hr at room temperature or 3–4 hr at 4°C. In the mixture the specimens reach a pink-orange color and a few bubbles may form at their surface.

The specimens are then passed for 1 hr into 3–6% glutaraldehyde (pH 7.4) at room temperature, washed for 0.5–1 hr in buffer with three to four changes, and postfixed for 2 hr in 2% osmium tetroxide at room temperature. During osmium tetroxide treatment, the specimens do not blacken as much as after the usual glutaraldehyde fixation.

In certain experiments, after glutaraldehyde-$H_2O_2$ treatment the specimens are transferred directly into the buffer wash and then into $OsO_4$. The specimens are dehydrated in alcohol and embedded in Epon. The sections are stained by immersion for 15 min in uranyl acetate in 50% ethanol followed by immersion for 3 min in 3% lead salts (3). Glutaraldehyde-$H_2O_2$ mixtures are always handled under the hood, with rubber gloves, because the compound irritates the skin and could be carcinogenic.

Control specimens are fixed for 2–3 hr in 3–6% glutarladehyde (pH 7.4). Buffer wash and osmium tetroxide postfixation, dehydration, embedding, and staining of sections are carried out as described in the glutaraldehyde-$H_2O_2$ procedure.

Figs. 1 and 2 compare control and glutaraldehyde-$H_2O_2$–fixed axons from a root of the sixth abdominal ganglion of crayfish. In control, cross-sectioned roots (Fig. 1), the axons are clearly distorted and shrunken. The phenomenon is more pronounced in central areas of the root (Fig. 1, inset). In some axons the preservation of microtubules is poor, and several axon-surface and Schwann cell membranes are broken. Frequently, large cisterns are seen, which presumably result from the rupture of adjacent single axons. Roots fixed by glutaraldehyde-$H_2O_2$ are not distorted even in central areas (Fig. 2, *inset*). The profile of the axons is mostly round, and broken membranes are rarely seen. Microtubules are well preserved and regularly distributed (Fig. 2).

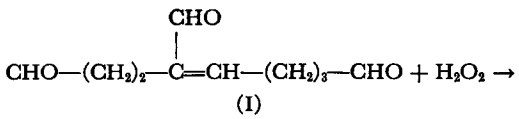
Various mammalian specimens have also been tested. In Fig. 3 a portion of rat jejunum is seen. Notice the good preservation of the general architecture as well as of specific structures such as mitochondria, endoplasmic reticulum, collagen, and microvilli (Fig. 3, insets *b*, *c*). In smooth muscle cells, thick filaments ($\sim$ 120 A in diameter) are clearly visible (inset *a*). In all the specimens tested, the preservation of glycogen and basement membranes was always greatly improved.

Various mixtures of glutaraldehyde-$H_2O_2$ have been tested. Those containing less than 1 drop of $H_2O_2$ per ml of glutaraldehyde are less effective. Mixtures containing more than 5 drops frequently damage the specimens as a result of excessive bubbling around and within the tissue, primarily where blood is present. In the same experiments, a drop in the pH of the mixture occurs within a few hours.

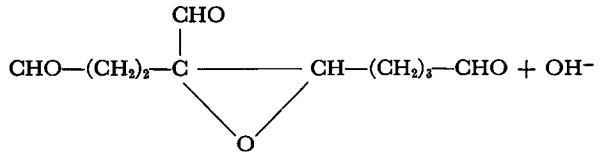
Regarding the chemical nature of the reaction products of glutaraldehyde and $H_2O_2$, it was presumed at first that mainly hydroxyalkyl peroxides are synthetized (2) as a result of reactions between $H_2O_2$ and carbonyl groups of glutaraldehyde (4). However, preliminary experiments with NMR spectroscopy[1] on diluted glutaraldehyde-$H_2O_2$ mixtures suggest that primarily epoxides are synthetized. The epoxy groups could result from a reaction between $H_2O_2$ and double bonds of $\alpha$-$\beta$-unsaturated aldehydes. These aldehydes, products of aldol condensation of glutaraldehyde, are always present in commercial glutaraldehydes and are considered very active in protein cross-linking (5–8). Epoxy aldehydes (II) could, therefore, be synthetized from $H_2O_2$ and unsaturated aldehydes (I) (9) (as follows on top of page 238). The addition of epoxy groups to the unsaturated polymers of glutaraldehyde may enhance their reactivity. Epoxides, in fact, are unstable groups which would readily react and bind at least to amino, imino, hydroxyl, and mercapto groups (9). The high reactivity between epoxy and hydroxyl groups may explain the improved preservation of polysaccharides. In this reaction, $\beta$-hydroxy ethers are formed.

The decreased blackening observed during osmication in specimens fixed by glutaraldehyde-$H_2O_2$ might depend on the low reactivity between

[1] Aldridge, W. G. Personal communication.



(I)



(II)

glutaraldehyde-$H_2O_2$ and $OsO_4$. The presumed decrease in double bonds of glutaraldehyde-$H_2O_2$ as a result of epoxide synthesis may explain this phenomenon, since osmium tetroxide reacts with double bonds (10) but not with epoxides.

The use of glutaraldehyde-$H_2O_2$ fixation for cytochemical study of specimens has not been tested in our work. However, a recent study on nucleoside diphosphatase and thiamine pyrophosphatase activities in hepatocytes and other cells of rat suggests that glutaraldehyde-$H_2O_2$ preserves enzyme activity better than conventional glutaraldehyde (11). Improvements in the tissue preservation as well as in the speed and depth of fixative penetration are also reported in the same study.

The fixation properties of other compounds containing epoxy and carbonyl groups together should be explored. For this purpose we propose to test mixtures of $H_2O_2$ with various unsaturated aldehydes. However, attempts to mix formaldehyde with $H_2O_2$ should be discouraged, first of all, because explosive compounds may be synthetized in the mixture (12) and, secondly, because $\alpha$-$\beta$-unsaturated aldehydes are not likely to be present in formaldehyde solutions.

In conclusion, improvements in fixation occur with the use of glutaraldehyde-$H_2O_2$ mixtures rather than conventional glutaraldehyde. The active compounds in the mixture could be $\alpha$-$\beta$-epoxyaldehydes.

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