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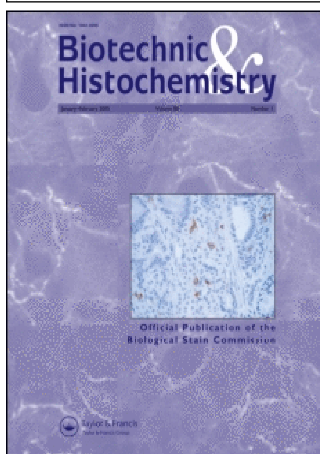

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Kinetic studies of glutaraldehyde binding in liver

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Abstract

To study the kinetics of glutaraldehyde fixation, fresh rabbit liver cubes were immersed in 3% buffered ^{3}H-glutaraldehyde for various periods of time. Following weighing and a brief rinse in water, the tissues were solubilized, and the radioactivity was measured in a scintillation counter. Binding of the isotope was half-maximal after approximately 4 h and a plateau was reached after approximately 20 h. We also investigated the reversibility of glutaraldehyde fixation. Fixed liver cubes were weighed and immersed in water for various periods of time, and after solubilization, the radioactivity was determined. After rinsing for 48 h, approximately 95% of the radioactivity was lost from the tissue specimens, indicating that fixation with glutaraldehyde is largely reversible. Light and electron microscopy of specimens rinsed for 1 and 48 h showed essentially similar morphology. Rinsing for 48 h restored some of the immunoreactivity that was absent after rinsing for only 1 h.

Key words: electron microscopy, fixation, glutaraldehyde, immunohistochemistry, kinetics, light microscopy, liver

Glutaraldehyde was introduced as a fixative for electron microscopy by Sabatini et al. in 1962, and has since enjoyed great popularity. Glutaraldehyde acts mainly by cross-linking proteins (Hayat 1981). There has been considerable discussion concerning the best conditions for fixation with glutaraldehyde (Hayat 1981) including the duration of fixation. While structural preservation is often excellent for light and electron microscopy, the loss of antigenic properties in the fixed tissues, particularly after fixation by vascular perfusion, has reduced the usefulness of glutaraldehyde as a fixative for immunohistochemistry. Several methods have been designed to restore the antigenicity of fixed tissue proteins including digestion with proteinase K or trypsin, or heating, but these methods frequently result in loss of structural details.

The aim of the study reported here was to investigate the kinetics of glutaraldehyde binding to tissues under conditions similar to those in a surgical pathology laboratory. In addition, we tested the hypothesis that glutaraldehyde binding is a reversible process.

Materials and methods

Binding of glutaraldehyde

Forty tissue specimens were cut from a fresh rabbit liver. The specimens were roughly cuboidal, a side measuring 4–6 mm. The specimens were put into a vial containing 100 ml 3% glutaraldehyde (Biological Grade, Ted Pella, Redding, CA) in 0.1 M phosphate buffer, pH 7.2. The fixative also contained 100 µCi of ^{3}H-glutaraldehyde (specific activity 5 Ci/mmol; American Radiolabeled Chemicals, St. Louis, MO). Two specimens were removed, at each time point after fixation: 15 min, 30 min and 1, 2, 4, 20, 47, 96, 166 and 214 h. Each specimen was then put into 10 ml of distilled water for 1 h to remove most of the unbound glutaraldehyde. They were then blotted on a filter paper,

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weighed, and put into a scintillation vial containing 1 ml of Solvable™ (Packard, Meriden, CT) for solubilization. For comparison, two specimens were taken after 15 min fixation and weighed without the preceding 1 h rinsing period. The dissolution of the tissues took several days and was aided by increasing the temperature to 40° C. Ten milliliters of scintillation fluid (Hiionic Fluor, Packard, Meridien, CT) was then added to 0.1 ml of the dissolved tissue, and the tritium content was determined using an LKB 1215 Rackbeta II liquid scintillation counter (Wallac, Gaithersburg, MD). Samples taken from the fixative before adding the tissue specimens and at the end of the fixation period (214 h) showed that the loss of radioactivity averaged about 9% compared to unused fixative.

Reversibility of glutaraldehyde binding

Another 40 tissue cubes were put into another vial with 40 ml fixative of the same composition as above. After 22 h, the specimens were removed, rinsed briefly in distilled water, and put into a second vial containing 50 ml water. Two of these specimens were removed immediately, blotted with filter paper and weighed. Additional specimens were removed after 1, 22, 48, 72, 168, 216 and 312 h in distilled water. At each time point, two specimens were removed, blotted and weighed.

Each tissue specimen was then put into a scintillation vial and 1 ml of Solvable™ was added. When the tissue was dissolved, 10 ml of Hiionic-Fluor was added to 0.1 ml of the dissolved tissue and the radioactivity measured.

To study the effects of rinsing on the structure of the tissue, other tissue cubes were fixed for 22 h in nonradioactive 3% glutaraldehyde as above. They were rinsed for either 1 or 48 h in distilled water, dehydrated for 30 min in 70% ethanol, 30 min in 95% ethanol and three 30 min baths in 100% ethanol, passed through two 30 min baths of xylene, and embedded in paraffin; 4 μm thick sections were picked up on glass slides and stained with hematoxylin and eosin.

Additional paraffin sections were processed for detection of immunoreactivity that might remain after glutaraldehyde fixation and rinsing. For this purpose we used antibodies against fibronectin, a protein that is abundantly present in liver (Kim et al. 1997). After removal of paraffin and rehydration, the sections were treated with 1.5% H_2O_2 in distilled water for 15 min to block endogenous peroxidase activity. They were then kept in 2% bovine serum albumin (BSA) for 30 min, followed by exposure to antibodies against rat fibronectin raised in goat (ICN/Cappel, Costa Mesa, CA) for 30–40 min. The antibody was diluted 1:50, 1:100, 1:200 and 1:300 with 1% BSA. Control sections were exposed to phosphate buffered saline (PBS) or 2% goat serum in PBS instead of the primary antibody. After rinsing in PBS, horseradish peroxidase coupled donkey anti-goat serum (ICN/Cappel) diluted 1:500 was applied for 30 min. Finally, diaminobenzidine was used for visualization of the peroxidase.

To detect ultrastructural consequences of the extended rinsing, electron microscopy studies were carried out on approximately 1 mm^3 tissue pieces removed from the periphery of the liver cubes fixed as above for 22 h, and subsequently rinsed for 1 or 48 h. The cubes were post-osmicated, dehydrated, and embedded in epoxy resin according to standard procedures. For a light microscopy control, 1 μm sections were stained with toluidine blue, pH 7. Ultrathin sections were contrasted with uranyl acetate and lead citrate.

All procedures, except the dissolution of the tissues, were carried out at room temperature and with no stirring.

Results

The binding of ^{3}H-glutaraldehyde increased up to approximately 20 h of fixation (Fig. 1). Half-maximal binding was reached after approximately 4 h.

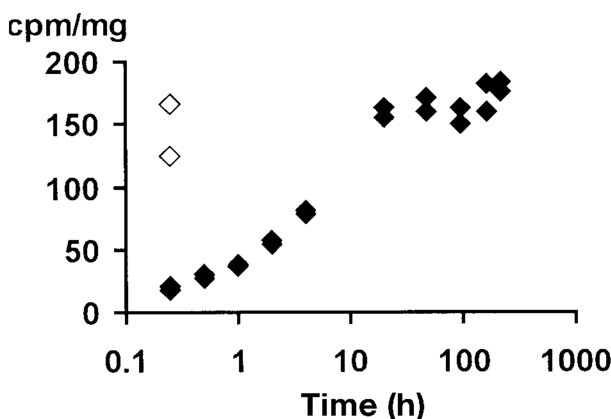

Fig. 1. Semilogarithmic diagram showing the radioactivity of rabbit liver cubes (two specimens for each time point) fixed in ^{3}H-glutaraldehyde for various periods of time. Saturation with isotope is reached after approximately 20 h fixation, with half-maximal binding after approximately 4 h. Open symbols show the radioactivity in two specimens that were fixed for 15 min, but not rinsed.

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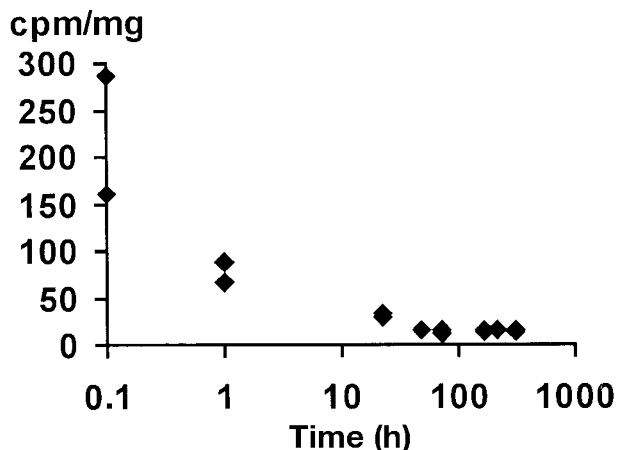

Fig. 2. Semilogarithmic diagram showing the radioactivity remaining in rabbit liver cubes fixed for 22 h and subsequently rinsed in distilled water for various periods of time. After 1 h, ~50% of the radioactivity remained. Rinsing for 48 h or more reduced the radioactivity by approximately 95%.

The isotope concentration in the tissues declined rapidly, reaching 50% of the initial level within 1 h (Fig. 2). After rinsing for 48 h, isotope concentration in the tissues was approximately 5% of the initial concentration.

Light microscopy revealed no significant differences between tissues rinsed for 1 and 48 h in either the hematoxylin and eosin stained sections (Fig. 3a, b) or toluidine blue stained epoxy sections.

Immunohistochemical staining for fibronectin showed reaction in the perisinusoidal space as described earlier (Kim et al. 1997). Immunoreactivity was moderate, but significantly stronger in specimens rinsed for 48 h than in those rinsed for 1 h (Fig. 4a,b). Immunoreactivity was generally stronger in the periphery of the tissue cubes than in the center. Antibody diluted 1:50 gave the most clear-cut results.

Under the electron microscope, tissues rinsed for 48 h showed slightly less contrast than those rinsed for 1 h (Fig. 5), but the structure of the tissues was well preserved. The outer mitochondrial membranes were sometimes lost from tissues rinsed for 48 h.

(a)

(b)

Fig. 3. Light micrographs of rabbit liver fixed for 22 h in glutaraldehyde: A) rinsed for 1 h, and B) rinsed for 48 h. There were no significant differences in the structure. Hematoxylin and eosin. × 150.

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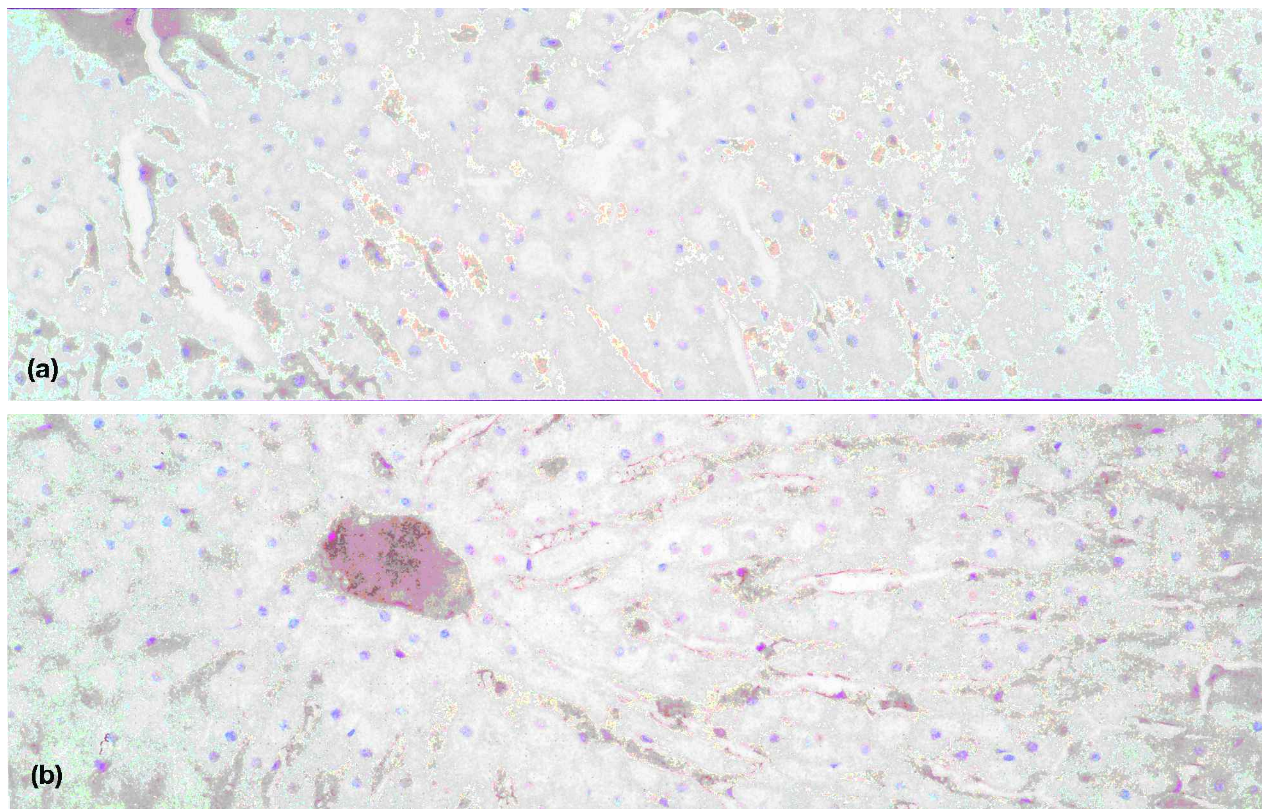

Fig. 4. Rabbit liver fixed for 22 h in glutaraldehyde: A) rinsed for 1 h, and B) rinsed for 48 h, immunostained with antibodies against fibronectin (primary antibody dilution 1:50). Immunoreactivity was observed mainly in the perisinusoidal space and was slightly greater in the specimens rinsed for 48 h. $\times$ 300.

Discussion

Ericsson and Biberfeld (1967) investigated the penetration of and fixation by glutaraldehyde in rat liver specimens. They observed a yellowish brown zone in the outer region of the specimen where tissue preservation was good, as judged by light and electron microscopy. In subjacent layers, the tissue was more whitish and the preservation of structure was poor. The thickness of the outer zone reached 1 mm after 40 min fixation and was found to be proportional to the square root of the duration of fixation. Those findings confirmed Medawar's (1941) earlier observation that fixatives obey Fick's diffusion law.

McDowell and Trump (1976) used 2 cm cubes of human liver after 1, 4 and 24 h fixation. After 24 h fixation, penetration of the fixative, judged by a color change of the tissue, averaged 6 mm. Good fixation was seen in a peripheral 3.5 mm-wide zone, whereas only the outermost 1.8 mm of tissue reacted positively to Schiff's reagent, without prior oxidation with periodic acid, indicating the presence of aldehyde groups.

Fixation can be divided into two phases: diffusion of the fixative into the tissue and the actual binding of the fixative to tissue components (e.g., cross-linking proteins). Both Ericsson and Biberfeld (1967) and Hopwood (1967) found that the diffusion rate for glutaraldehyde was slow compared to that of formaldehyde. In contrast, glutaraldehyde binding was faster.

In the present study, the rate of glutaraldehyde binding was measured using radioisotopes. Binding of ^{3}H-glutaraldehyde in 4–6 mm liver cubes reached its half-maximal level after 4 h of immersion, and saturation after approximately 20 h. A similar study of ^{14}C-formaldehyde binding kinetics (Helander 1994) demonstrated half-maximal binding levels after approximately 100 min, and saturation after approximately 24 h, confirming that formaldehyde is a faster fixative than glutaraldehyde.

It has been stated previously that glutaraldehyde, unlike formaldehyde, can be removed only partly from the fixed tissues (Høyer et al. 1997). In the present study, the reversibility of ^{3}H-glutaraldehyde binding is clearly demonstrated; only a small fraction ($\sim$5%) remained after 48 h of rinsing

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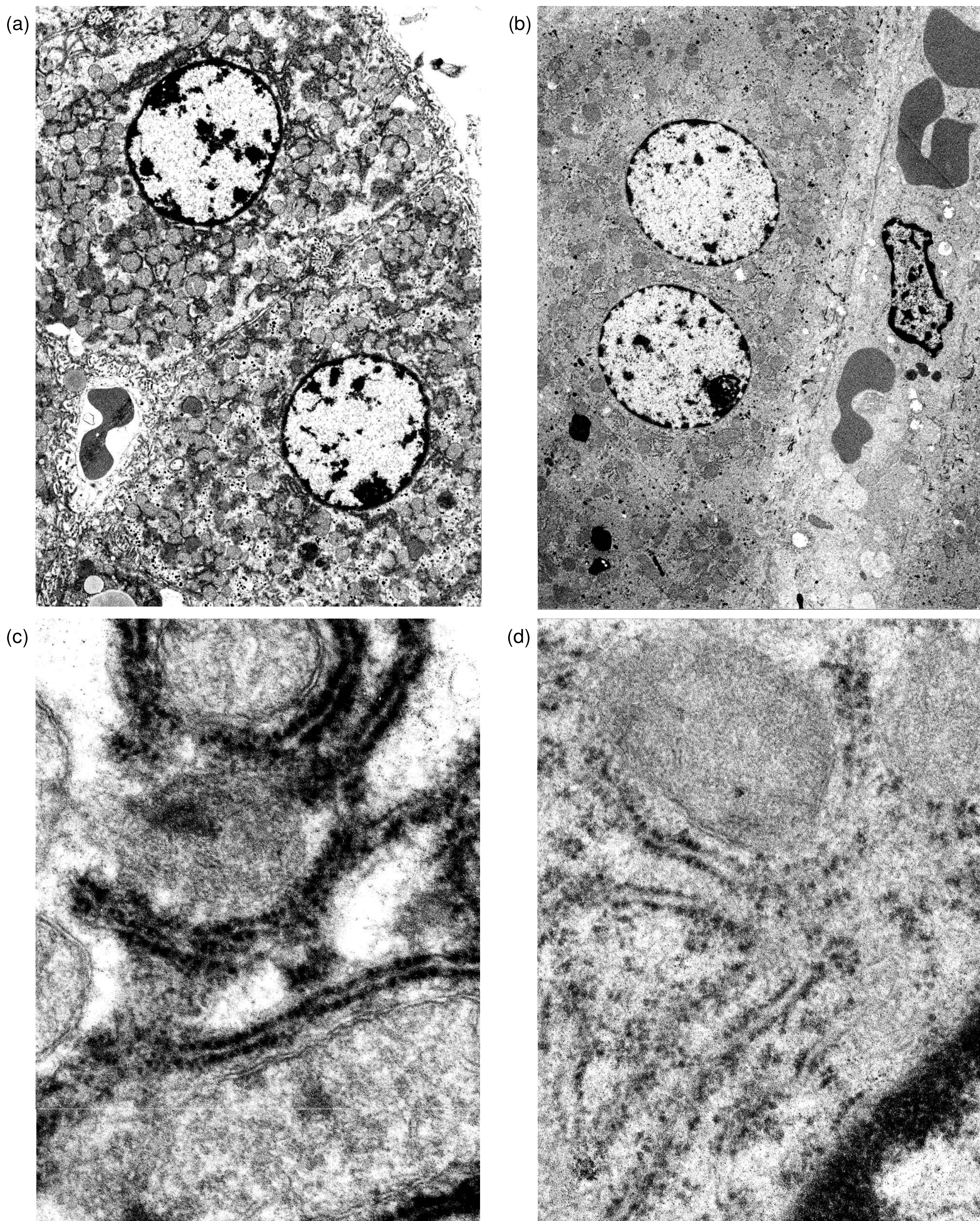

Fig. 5. Electron micrographs of rabbit liver fixed for 22 h in glutaraldehyde: A) and C) rinsed for 1 h, and B) and D) rinsed for 48 h. At low magnification (A and C; × 1300) there were no significant differences in ultrastructure, but the contrast was higher in specimens rinsed for 1 h than in those rinsed for 48 h. The same differences in contrast were observed also at high magnification (B and D, × 72 000), while the ultrastructure was similar.

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in water. Previous studies have demonstrated similar reversibility of formaldehyde fixation of rabbit liver (Helander 1994). The loss of glutaraldehyde isotope from the tissue blocks presumably reflects reversibility of the binding of glutaraldehyde to the proteins. This would also explain the apparent return of some immunoreactivity. One cannot rule out, however, that loss of glutaraldehyde–protein complexes contributes to the loss of isotope, although we presume that this accounts for a small proportion of loss of radioactivity.

Removal of the glutaraldehyde by rinsing for 48 h did not significantly affect the light microscopic structure of the tissues. By electron microscopy, a small loss of contrast was noted, but no other significant differences were observed. Immunohistochemical staining with antibodies against fibronectin was positive. Further studies are required in other tissues, with other antibodies, and with other rinsing periods.

Acknowledgments

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