

## **Decalcification by perfusion. A new method for rapid softening of temporal bones**

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**Summary.** We describe a new technique, decalcification by perfusion, for the softening of bony tissue. The blood circulatory system was perfused in 16 rats via a cannula through the left heart ventricle with a fixative followed by New Decalc<sup>R</sup> (an acidic demineralizer) for 30-240 minutes. Perfusion decalcification for 120 minutes softened all heads and middle ear specimens could be easily sampled and prepared for studies by both light and electron microscope. For comparison, a conventional immersion technique required 72 hours of decalcification to accomplish softening. The perfusion technique considerably reduced the time needed to decalcify the tissue and preserved the morphology better than did the immersion procedure.

**Key words:** Decalcification, Perfusion, Immersion, Ear, Temporal bone, Rat

### **Introduction**

The middle and inner ear are enclosed in and protected by the temporal bone. Dissection of interesting portions in order to study their microscopical architecture in light and electron microscopes often requires softening of the tissue. Hitherto, this procedure has included immersion of the specimens in various different decalcifying agents such as both strong and weak organic or mineral acids, acidic buffers and chelating agents, e.g. EDTA (ethylenediaminetetraacetic acid), (Preece, 1965; Baird et al., 1967; Pearsxe, 1968; Kivaranta et al., 1980).

Adequate decalcification by immersion of temporal bones, e.g. from the rat, is time-consuming and requires about 3 days in acidic demineralizers and 10-14 days in chelating agents (Anniko, 1981). Furthermore, immersion for long periods in acidic demineralizers

causes distortion of tissue structures and a reduced affinity for histological stains (Preece, 1965; Kivaranta et al., 1980).

A pilot study (Albiin, 1986) indicated that administration of a decalcifier by perfusion, via the circulatory system of rats, considerably shortened the time needed for softening. The purpose of the present study was to develop this method and ascertain the optimal time needed for softening the temporal bones in the rat. The quality of the tissue morphology, including its ultrastructural appearance, and the affinity for histological stains were evaluated and compared with specimens decalcified by immersion.

### **Materials and methods**

Twenty-two healthy Sprague-Dawley rats, weighing 350-400 g, were used for the study. The animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (Mebumal<sup>R</sup>) 60 mg/kg body weight.

Through a ventral midline incision the thoracic cage was opened and a cannula inserted into the ascending aorta via the left heart ventricle. The animal was then perfused with a fixative solution containing 3% glutaraldehyde, 4% polyvinylpyrrolidone (PVP, mol wt 40,000) in 75 mM cacodylate buffer with 2 mM calcium chloride added (McDonal and Larue, 1983). The 525 mOsm solution, adjusted to pH7.3, was perfused for 30 minutes followed by a rinse with 0.9% saline for 5 minutes. The animals were then divided into two groups.

#### *1) Decalcification by perfusion (16 rats)*

After the rinsing procedure the acidic decalcifying agent New Decalc<sup>R</sup> (Bethelheim Trading Ltd, Gothenburg, Sweden) was administered through the perfusion apparatus (Hellström and Bergh, 1984). New Decalc<sup>R</sup> contains hydrochloric acid 14%, PVP 7%, aqua dest 79% and a trace surfactant. The decalcifying agent was perfused at a rate of 6 ml/min and a hydrostatic

pressure of 120 cm H<sub>2</sub>O for 30 min (n = 3), 60 min (n = 3), 90 min (n = 3), 120 min (n = 5) and 240 min (n = 2). The perfusion with New Decalc<sup>R</sup> was followed by a rinse with 0.9% saline for 5 min and the animals were then decapitated. The heads were then immersed in the fixative solution for at least 48 hours.

## 2) Decalcification by immersion (6 rats)

After fixation and rinsing by perfusion, the animals were decapitated and the heads then immersed in New Decalc<sup>R</sup> for 1 (n = 2), 2 (n = 2) or 3 (n = 2) days. The softness of the heads was checked daily by the needle test (Preece, 1965). The solution was renewed daily.

After decalcification, all heads were dissected with a razor blade and the softness of the tissue was evaluated. Sixteen left temporal bones were cut loose for embedding in paraffin wax. From seven of the right temporal bones, specimens were collected from the middle ear for embedding in an Epoxy resin and two temporal bones were prepared for studies in the scanning electron microscope.

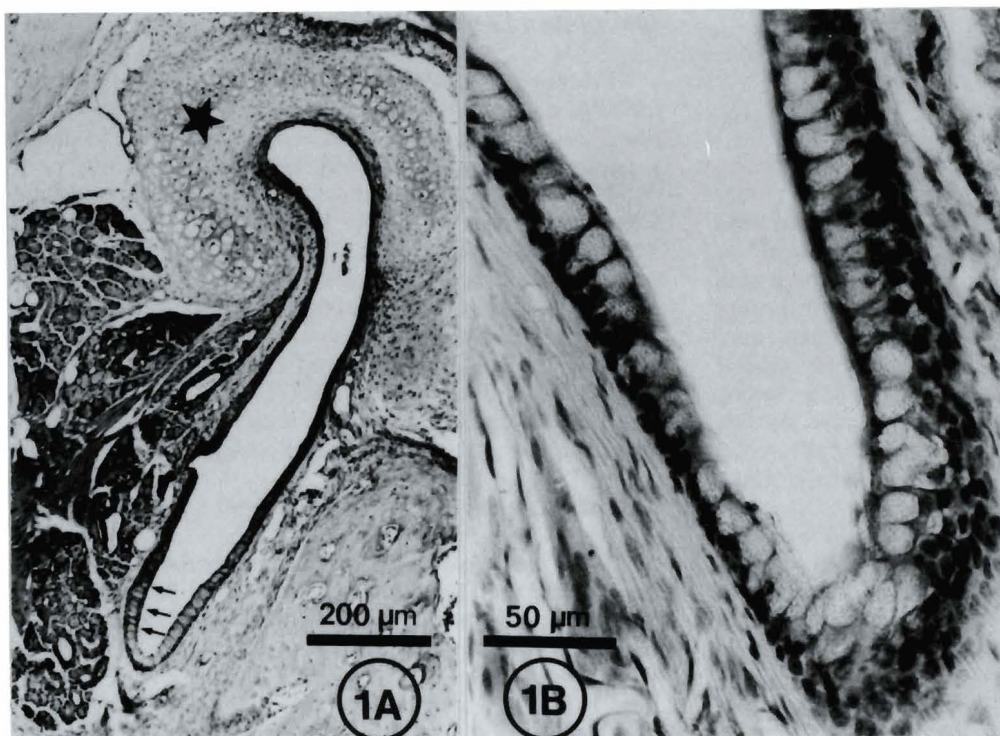
The specimens collected for paraffin embedding were rinsed under tap water for 1 hour before dehydration in graded series of ethanol. The specimens were then cleared in xylol for 30 minutes prior to infiltration and embedding in paraffin wax. The paraffin-embedded temporal bones were oriented and serially sectioned, 5 µm thick, in the frontal plane at 100 µm intervals. Also during the sectioning procedure, the softness of the bony structures was evaluated. From each time interval, sections

were stained with hematoxylin-eosin, van Gieson and periodic acid Schiff (PAS), examined and photographed in an Olympus Vanox-S light microscope.

The specimens collected for plastic embedding, i.e. the round window niche, hypotympanon, fossa nasalis and tympanal orifice of the Eustachian tube, were rinsed in 0.1 M sodium-cacodylate buffer (pH 7.4) for 30 minutes and postfixed in 1% osmium tetroxide in the same buffer overnight. After rinsing in the same buffer for 30 minutes, the specimens were dehydrated in a graded series of acetone, and embedded in an epoxy-resin. Thin (70 nm) and semithin (1 µm) sections were cut for electron and light microscopy respectively on a Porter-Blum ultramicrotome (Instrument AB Lambda, Sweden). The semithin sections were stained with toluidine blue, examined and photographed in an Olympus Vanox S light microscope. The thin sections, contrasted with uranyl acetate and lead citrate, were studied in a JEOL 100XS transmission electron microscope.

The two temporal bones for scanning electron microscopy were opened in the anteroposterior plane and the middle ear was divided into a lateral and a media half.

The specimens were dehydrated in an increasing series of ethanol and then critical-point dried with liquid carbon dioxide in a Polaron E-3000 apparatus (Polaron Equipment Ltd, UK). The dried specimens were then mounted on blocks and coated, under continuous rotation and tilting in a modified Edwards Vacuum Coating Unit E12E14 (Edwards High Vacuum, UK) at 1.3 kPa, with an approximately 20



**Fig. 1A.** Light micrograph (LM) of the rat Eustachian tube. The epithelial lining with ciliated and secretory cells (arrows) is well preserved, as are the tubal cartilage, adjacent muscles and glands. Paraffin-embedded section, Hematoxylin-eosin. Perfusion-decalcified 120 minutes,  $\times 80$ . **B.** Detail of (A) at higher magnification  $\times 340$

**Table 1.** Temporal bones decalcified with New Decalc<sup>R</sup>.

		Paraffin	Plastic	TEM	SEM	Decalcified?
*	30 (n = 6)	3				No(6)
	60 (n = 6)	3				No(6)
	90 (n = 6)	3				No(4), Yes(2)
	120 (n = 4)	4	3	3	2	Yes(10)
	240 (n = 4)	2	2	2		Yes(4)
**	24h (n = 4)					No(4)
	48h (n = 4)					No(4)
	72h (n = 4)	2	2	2		Yes(4)

The specimens were considered decalcified if 1) the heads were soft and easy to dissect, 2) the paraffin-embedded specimens could be sectioned damage to the specimen or the steel knife, 3) the plastic-embedded specimens could be sectioned in an ultramicrotome without damage to the specimen or the glass knife. \*)-PERFUSION (min) \*\*)IMMERSION

nm-thick layer of gold. The specimens were examined and photographed in an Cambridge S4 scanning electron microscope (Cambridge Scientific Instrument Ltd, UK).

The heads were considered sufficiently decalcified if they were soft and easy to dissect, the paraffin-embedded temporal bones could be sectioned without damage to the specimen or the steel knife, and the plastic-embedded specimens could be sectioned in an ultra-microtome without damage to either the specimen or the glass knife (Table 1).

## Results

The temporal bones from the rats perfused for 30 or 60 minutes with New Decalc<sup>R</sup> as well as four out of six temporal bones perfused for 90 minutes were not sufficiently softened. The heads immersed for 1 and 2 days were not softened (Table 1). Paraffin-embedded specimens from these groups could not be sectioned in a steel knife microtome without being distorted or causing damage to the blade. Therefore the temporal bones decalcified by perfusion for 90 minutes or less were not processed further.

The heads perfused 120 and 240 minutes, as well as the heads immersed for 3 days in New Decalc<sup>R</sup> were all softened. All these temporal bones could be easily dissected, specimens collected and further processed for examination in the light and electron microscopes (Table 1).

### Light microscopical findings

The stainability of sections from the decalcified plastic- and paraffin-embedded specimens was good and quite comparable to biopsies obtained from non-decalcified temporal bones (Albiin et al., 1986).

The different kinds of tissues were generally well preserved. The epithelial linings as well as the subepithelial connective tissue of the Eustachian tube and the middle ear cavity had a normal appearance.

Occasionally, small damaged areas were noted in which epithelial cells were lacking. Both ciliated and secretory cells of the mucosal lining exhibited normal characteristics (Figs. 1, 2).

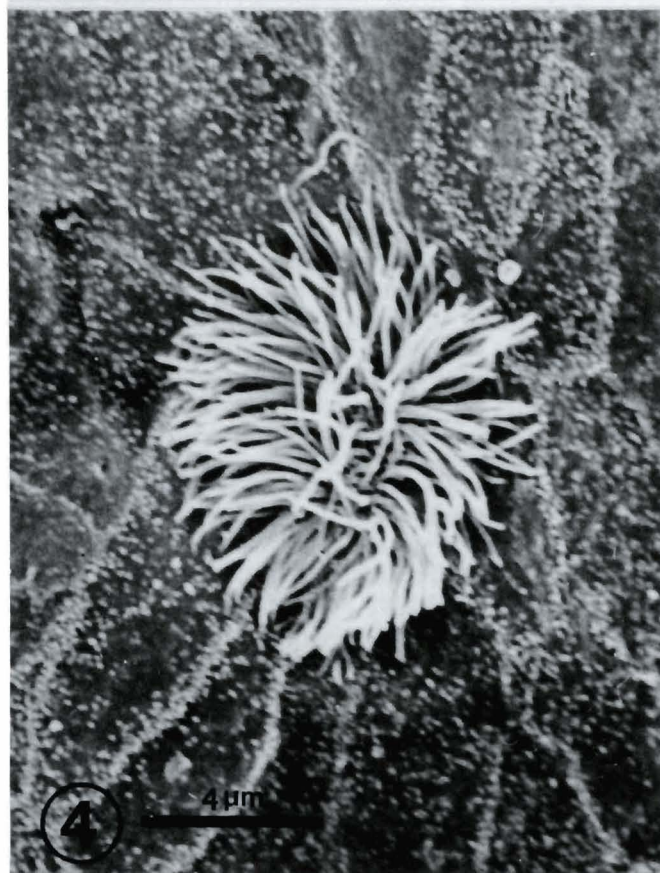
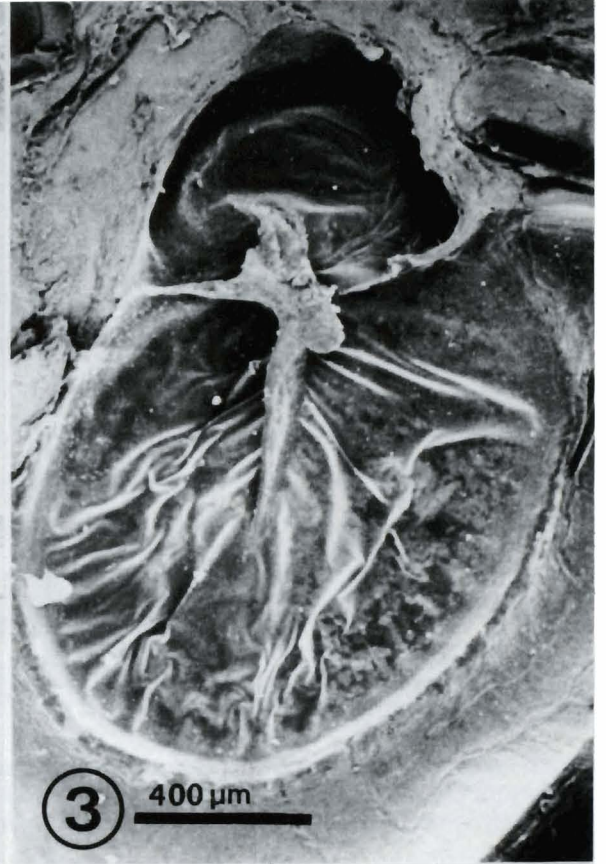
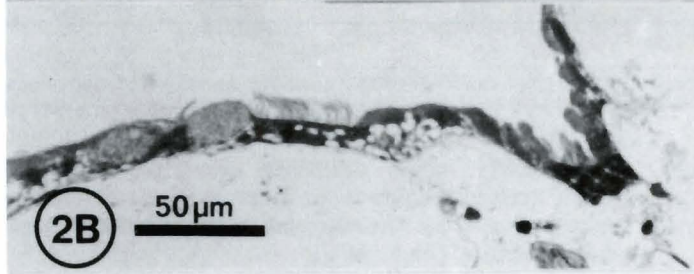
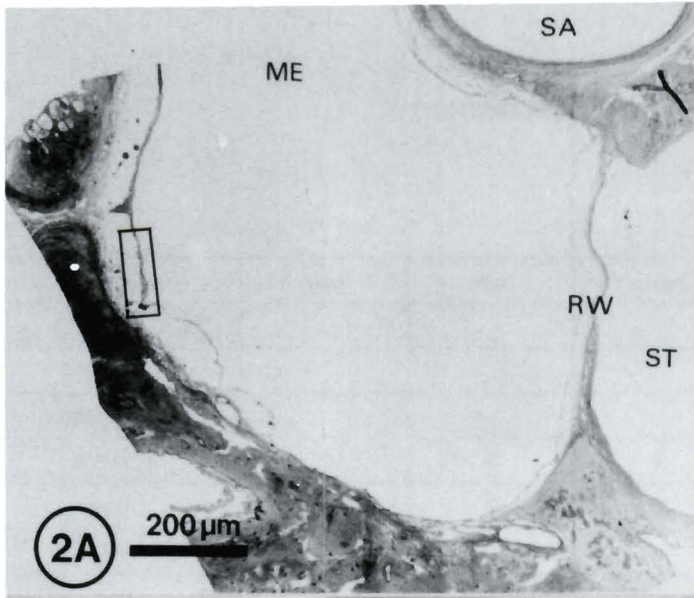
### Electron microscopical findings

Scanning electron microscopic studies revealed that the tympanic membrane (Fig. 3) lacked the ruptures commonly seen if not decalcified. The medial wall of the middle ear also lacked fissures. Mucus covered the walls, except for minor areas in which the epithelium could be studied. These epithelial surfaces exhibited nicely preserved ciliated and non-ciliated cells (Fig. 4). Furthermore, with this perfusion technique the vessels such as the stapedia artery were kept expanded throughout the preparation of the specimen (Fig. 5).

In the transmission electron microscope the epithelium of the middle ear showed minor ultrastructural damage. However, it was generally apparent that the perfusion-decalcified specimens were better preserved than those decalcified by immersion. Cell organelles, e.g. mitochondria, showed a homogeneous electron density in both the immersion- and perfusion-decalcified specimens. Widened intercellular spaces and irregular-filled secretory granulae were only present in the specimens decalcified by immersion (Figs. 6, 7).

## Discussion

Calcium carbonate and phosphate have to be removed before bony specimens can be processed and sectioned with an ordinary microtome. The principal methods to achieve decalcification includes treatment with either acid solutions or ion exchange resins. However, neither of these techniques is ideal and treatment with acid for long periods of time invariably produces distortion of the tissue (Clark, 1954; Gussen and Donahue, 1965; Preece, 1965; Bussolati, 1978; Eggert and Germin, 1979; Kiviranta et al., 1980).

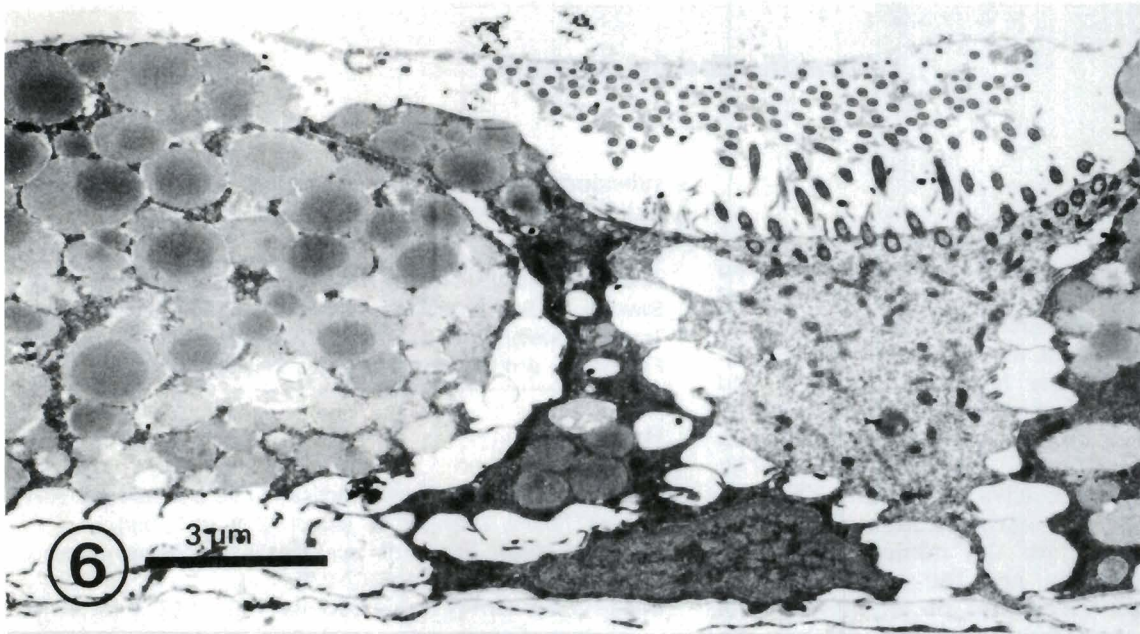


**Fig. 2A.** LM showing the round window niche. The epithelial lining changes from secretory and ciliated cells at the entrance of the niche to a squamous epithelium in the vicinity of the round window membrane (RW). SA = stapedial artery, ME = middle ear cavity, ST = scala tympani. Epon-embedded section, toluidine blue,  $\times 80$ . Perfusion-decalcified 120 minutes. **B.** Detail of (A) at higher magnification,  $\times 340$

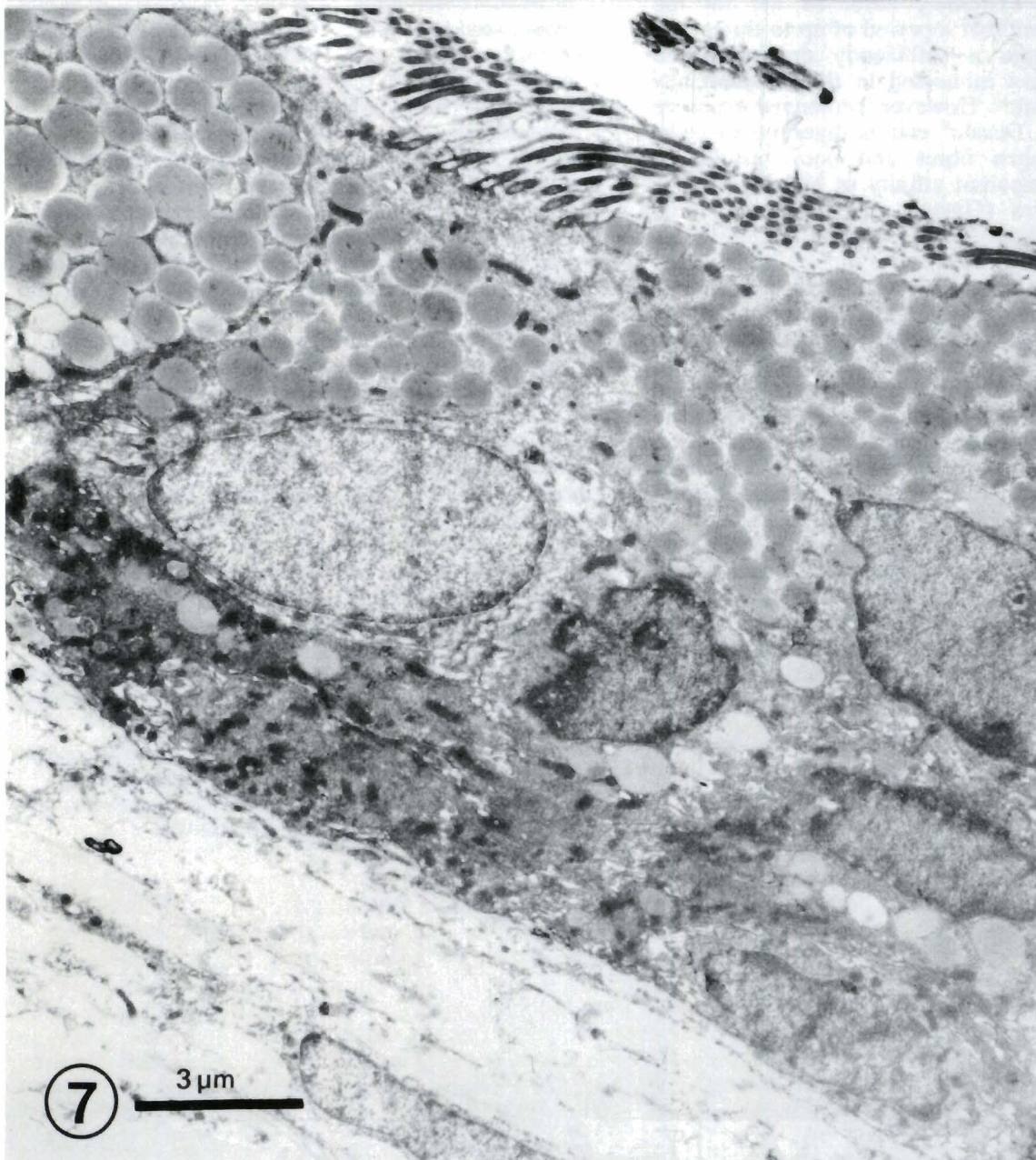
**Fig. 3.** Scanning electron (SEM) micrograph of the lateral wall of the middle ear with the tympanic membrane (TM) in its bony frame. The TM is still intact but has lost its conical shape during preparation.  $\times 60$ . Perfusion-decalcified 120 minutes.

**Fig. 4.** SEM picture from the medial wall of the middle ear (fossa nasalis) showing squamous epithelial cells and a ciliated cell.  $\times 6,000$ . Perfusion decalcified 120 minutes.

**Fig. 5.** SEM picture of the stapedial artery, the stapes and the entrance of the round window niche. Note the bulging artery which indicates that with the perfusion procedure applied, the vessel lumen has expanded throughout the preparation of the specimen. Perfusion-decalcified 120 minutes.



**Fig. 6.** Transmission electron (TEM) micrograph of epithelium in vicinity of the tympanal orifice of the Eustachian tube. Immersion-decalcified 72 hours. Note that the intercellular space is increased and that secretory granules are irregularly filled.



**Fig. 7.** TEM picture of a similar area as in Fig. 6, but with the specimen perfusion decalcified for 120 minutes. The structure is better preserved and shows less damage than in specimens decalcified by immersion.

## Discussion

Calcium carbonate and phosphate have to be removed before bony specimens can be processed and sectioned with an ordinary microtome. The principal methods to achieve decalcification includes treatment with either acid solutions or ion exchange resins. However, neither of these techniques is ideal and treatment with acid for long periods of time invariably produces distortion of the tissue (Clark, 1954; Gussen and Donahue, 1965; Preece, 1965; Bussolati, 1978; Eggert and Germin, 1979; Kiviranta et al., 1980).

The time needed for decalcification of osseous structures is dependent on the minimum diffusing distance of the specimen and the compactness or density of the bone (Kiviranta et al., 1980). The temporal bone is extremely hard. In the rat the present study showed that a period of up to three days is needed to obtain a sufficiently demineralized temporal bone when immersed in the commercially available New Decalc<sup>R</sup>. However, prolonged exposure to the acidic New Decalc<sup>R</sup> causes digestion of cells, distortion of collagen fibres and poor histological detail due to an impaired affinity of histologic stains for tissue structures (Gussen and Donahue, 1965; Eggert and Germain, 1979; Kiviranta et al., 1980; Matthews and Mason, 1984).

In attempts to obtain decalcified specimens by exposure to the decalcifying agent for shorter periods of time, we have developed the perfusion technique. By perfusion, a fresh solution is constantly flooded through the tissue. This eliminates precipitation of calcium salts within the tissue and saturation of the decalcifying solution by removed calcium, which often occur with the immersion technique (Bussolati, 1978; Eggert and Germain, 1979). After the shortest time needed for adequate softening of the tissue, 120 minutes, most structural details were well preserved at both the light microscopic and the electron microscopic level. Moreover, the specimens perfused for 240 minutes were equally well preserved, whereas the specimens immersed in New Decalc<sup>R</sup> for 72 hours exhibited artifacts, e.g. widened intercellular spaces and generally poor histological detail, especially at the ultrastructural level.

Softening by perfusion with New Decalc<sup>R</sup> is superior to the immersion technique in several ways. It is not only faster but also preserves the light microscopical morphology in a better way. The technique is also suitable for electron microscopy. Further improvement of the perfusion technique is in progress, e.g. by the use of other decalcifying

substances and in combination with microwave irradiation (Book and Kok, 1987).

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