Virchows Arch. Abt. A Path. Anat. 350, 216—224 (1970)
© by Springer-Verlag 1970

A Method for Ultrastructural Study of Lesions Found in Conventional Histological Sections* **

G. L. Rossi, H. Luginbühl and D. Probst
Institute of Animal Pathology, University of Bern
(Director: Prof. Dr. H. Luginbühl)

Received February 12, 1970

Summary. Histological sections of paraffin-embedded formalin-fixed postmortem specimens have been prepared for electronmicroscopic study by a modified method of "open-face" embedding. Lesions or other features are located and photographed by the light microscope. Their locations are then marked, coverglasses removed, sections rehydrated, postfixed in OsO4, dehydrated and the study foci embedded in epoxy resin, separated from the slide, sectioned and stained for electronmicroscopy. Thus, the morphological features of disease processes may be recorded through a much greater range of magnification than hitherto available.

Zusammenfassung. Die vorliegende, modifizierte „Open-face"-Methode erlaubt, histologische Schnitte von formalinfixiertem, in Paraffin eingebettetem Autopsiematerial für die elektronenmikroskopische Untersuchung umzubetten. Pathologische Veränderungen oder andere Besonderheiten werden lichtmikroskopisch festgestellt und photographiert. Man markiert die genauen Lokalisationen von ultramikroskopisch zu untersuchenden Stellen in den histologischen Präparaten, entfernt die Deckgläser, rehydriert die Schnitte, fixiert dann in Osmiumtetroxyd und entwässert. Anschließend werden die zu untersuchenden Stellen in Epoxyresin eingebettet, vom Objektträger entfernt, mit dem Ultramikrotom geschnitten und für die elektronenmikroskopische Untersuchung gefärbt. Auf diese Weise sind viele morphologische Einzelheiten in konventionellen histologischen Schnitten der elektronenmikroskopischen Untersuchung zugänglich.

Introduction

Interpretations of ultrastructures of cells and tissues are commonly believed to require material taken either by biopsy or immediately after death from a subject that has been killed, and processed specifically for electronmicroscopy (EM). Hence, EM study of disease processes usually has been limited to biopsy specimens. Tissues from autopsy, especially after formalin fixation, are seldom considered suitable for EM study.

If, however, suitable specimens might be obtained from autopsy and processed specifically for EM study, this study might require an impossibly great expenditure of time and effort unless clinical diagnosis had indicated sites to be examined and lesions to be expected. But even with accurate clinical diagnosis, morbid changes or microorganisms may be widely scattered and difficult to locate.

Authors who attempted to reduce the time and effort needed for these studies have embedded specimens in media suitable for EM and thereafter have selected

* Supported by grant HE-4885, National Heart Institute.

** The authors are indebted to Dr. I. Gersh, School of Veterinary Medicine, University of Pennsylvania in whose laboratory part of this work was carried out, and to Mr. W. R. Schnarr and Mr. J. Di Lullo for technical assistance.

foci for EM examination by scanning with the light microscope either the embedded tissue faces, or large sections cut from these faces (Gray, 1961; De Bruijn and McGee-Russel, 1966; Grimley, 1965; Lehner *et al.*, 1966; Goricki, 1966; Seliger, 1968). When, however, autopsies are not supported by definitive clinical diagnoses, these methods are not particularly helpful.

We have overcome the deficiencies of these methods by locating and photographing lesions in conventionally prepared paraffin sections from which the study focus, even single cells or parts of cells, was then processed and examined by EM. This method permits the histopathologist to record information of disease processes through an unusually large range of magnification.

The Method

Sections processed by this method usually had been prepared from formalin-fixed autopsy materials after paraffin embedding. Fixation in Bouin's solution or embedding in carbowax have served equally well and sections cut by the freezing microtome also have been processed. Sections have ranged from six to ten microns in thickness, the thicker sections being more readily cut for EM study. None of the stains commonly used for light microscopy has interfered.

Proceed as follows:

1. Locate the Lesion and Mark the Slide

Find and center a lesion or study focus in the microscopic field, then without moving the slide, replace the objective with a "marking objective" Winkel-Zeiss (Fig. 1a) and cut a circle, one to two mm in diameter, in the cover glass; the circle centered about the study focus. Next, turn the slide face-down, center the circle in the microscopic field and again cut a circle concentric to the first on the undersurface of the slide.

EM study will be aided by selecting study foci that are located near an easily recognized tissue structure, as, for example, a bronchiole or an artery.

2. Record Study Foci by Photomicrographs

3. Remove Cover Glass and Rehydrate Section

Place slide on end in a tightly covered jar of xylene until the cover glass separates and falls to the bottom. Next, rehydrate the section by successive steps from xylene through graded alcohols to water without allowing it to become dry.

4. Post-fixation in OsO4

Rinse the rehydrated section thoroughly in acetate-veronal buffer pH 7.2. Then place the slide in a Petri dish on wooden or glass supports two to three mm in thickness, carry the dish and slide to a well ventilated hood and add OsO4 solution (Palade, 1952) in drops to the section surface until the section is covered. Allow fixative to remain on the section for five minutes, then decant and replace it with fresh fixative through four changes (twenty minutes), and rinse thoroughly in acetate-veronal buffer.

5. Dehydration, Infiltration and Embedding

Return the slide through graded alcohols to absolute ethanol, place it in a Petri dish, flood the section with a mixture of Durcupan[1] I-ethanol 1:3 and tightly cover the dish. Decant this mixture after an hour and replace it with Durcupan I-ethanol 1:1, followed by a 3:1 mixture and undiluted Durcupan I, allowing one hour for each step.

[1] Durcupan ACM, Fluka (Araldite).

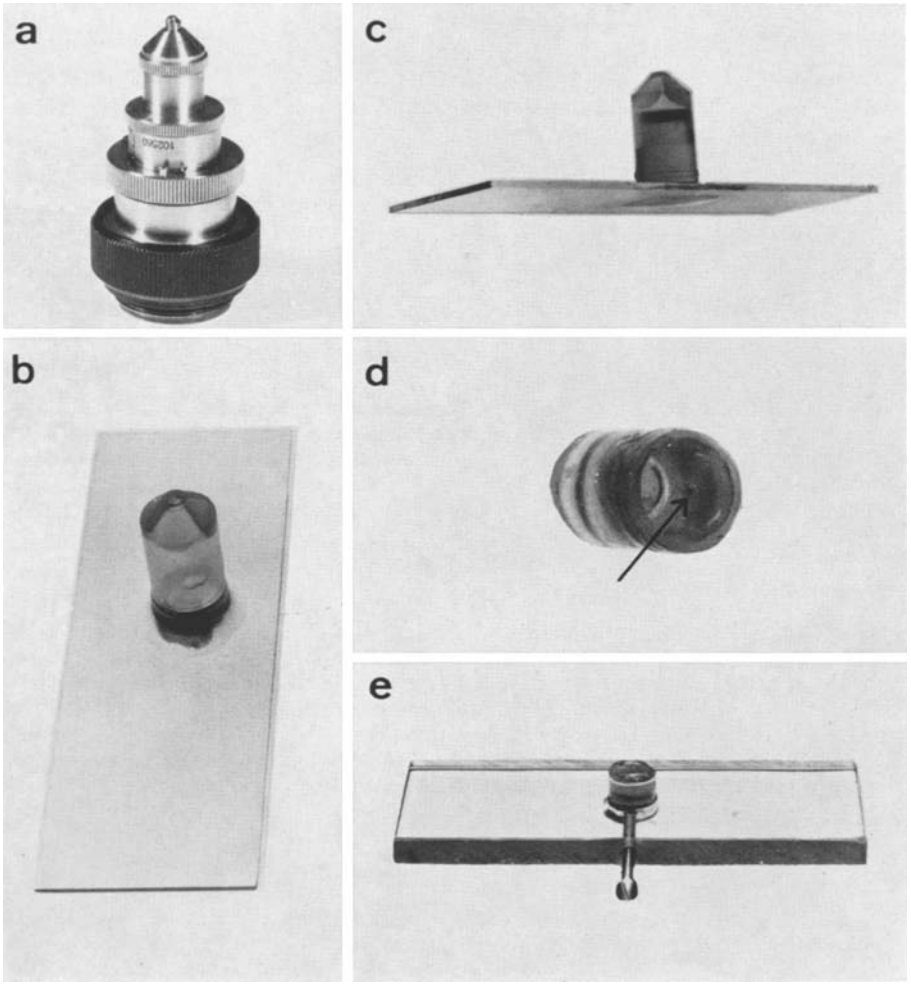

Fig. 1. a Marking objective. b Infiltration in embedding medium of an area preselected in a conventional histological section. c Hardened block attached to the glass slide. d Hardened block after separation from glass slide. Arrow points to the pre-selected area as found after embedding. e Block holder

The undiluted Durcupan (ca 0.25 ml) is contained in a plastic embedding capsule (BEEM), the open end of which is brought into contact with the section in and about the encircled focus (Fig. 1 b). After one hour, replace the capsule of Durcupan I with a capsule of Durcupan II setting the capsule orifice concentric with the circle that marks the study focus.

6. "Curing" and Separating the Durcupan Block from Slide

Transfer the slide with the capsule of Durcupan II to an incubator at 60° C for 48 hours, which hardens the Durcupan and attaches it firmly to the slide (Fig. 1 c). Next cut away the BEEM capsule from the Durcupan block and carefully scrape away with a razor blade the unwanted parts of the section and the small amount of Durcupan that may have been spilled about the base of the block.

Again, warm the slide and attached block to 60° C, then thoroughly chill by placing the undersurface of the slide on a block of "dry-ice" after which bring the slide into contact with hot water. Usually, at this point, with no difficulty the block and embedded tissue will separate cleanly from the slide (Fig. 1d) but if the first attempt fails, repeat the warming-cooling-warming cycle a second or third time until the separation will occur.

7. Hardening the Block Face, Precision Trimming, Ultrasectioning and Staining

Immediately after separation, brush a minute quantity of accelerator (Durcupan C) over the block surface previously adherent to the slide and incubate the block at 60° C for 24 hours.

Clamp the hardened block in a suitable holder (Fig. 1e), examine the central area of the face with the light microscope and identify the study site. Next, center the site in the microscopic field and cut a circle about it with the "marking objective". With this circle for orientation, trim the block face under a stereomicroscope.

Insert the block into the ultramicrotome with the block face as nearly parallel as possible to the plane of section. Collect all sections or fragments of sections since all will contain parts of the tissue. Stain the section in uranyl acetate and lead citrate (Reynolds, 1963).

8. Electronmicroscopic Examination

Examine the sections at low magnification (i.e. scanning position, Philips EM 300) and determine their exact orientation by comparing the EM image with the photomicrograph. At this point, the study focus may be examined by higher magnification and photographed.

Results and Comments

This method is based on the so-called "open-face" embedding technique by which cells from tissue culture or smears on flat surfaces of slides may be prepared for EM study (Gay, 1955; Howatson and Almeida, 1958; Heyner, 1963; Rosen, 1962; Robbins and Gonatas, 1964; Sheffield, 1965; Sparvoli *et al.*, 1965). Electronmicrographs obtained from specimens that have been processed by this method demonstrate that cell components and pathological changes may be identified even though formalin fixation and paraffin embedding may have caused some distortion.

For example, Fig. 2 a and b illustrate progressive magnification from photomicrograph to low-power electronmicrograph. The tissue is an area of small intestine from a dog that died at age eleven years. It had been fixed approximately 3 hours after death in 4% neutral formaldehyde, and embedded in paraffin. Fig. 2c illustrates that many components of the cells are recognizable, and Fig. 2d shows that even the myofilaments of a smooth muscle cell are reasonably well preserved. Fig. 3a—c are to illustrate the ultrastructural details of a section of testis from a dog that died at age three years. The tissue had been fixed in Bouin about 30 minutes after death and embedded in paraffin. These electronmicrographs do not differ widely from views obtained from conventional EM preparations.

An example of study of a lesion is given in Fig. 4a—c. The low-power electronmicrograph Fig. 4a shows the wall of a meningeal artery from a dog that died at age fourteen years. The tissue had been fixed approximately 6 hours after death in 4% neutral formaldehyde and embedded in paraffin. The photograph demonstrates that many structural details are preserved and readily visualized by the method. Fig. 4b illustrates the ultrastructural details of collagen fibers from the tunica media and 4c the typical structure of amyloid.

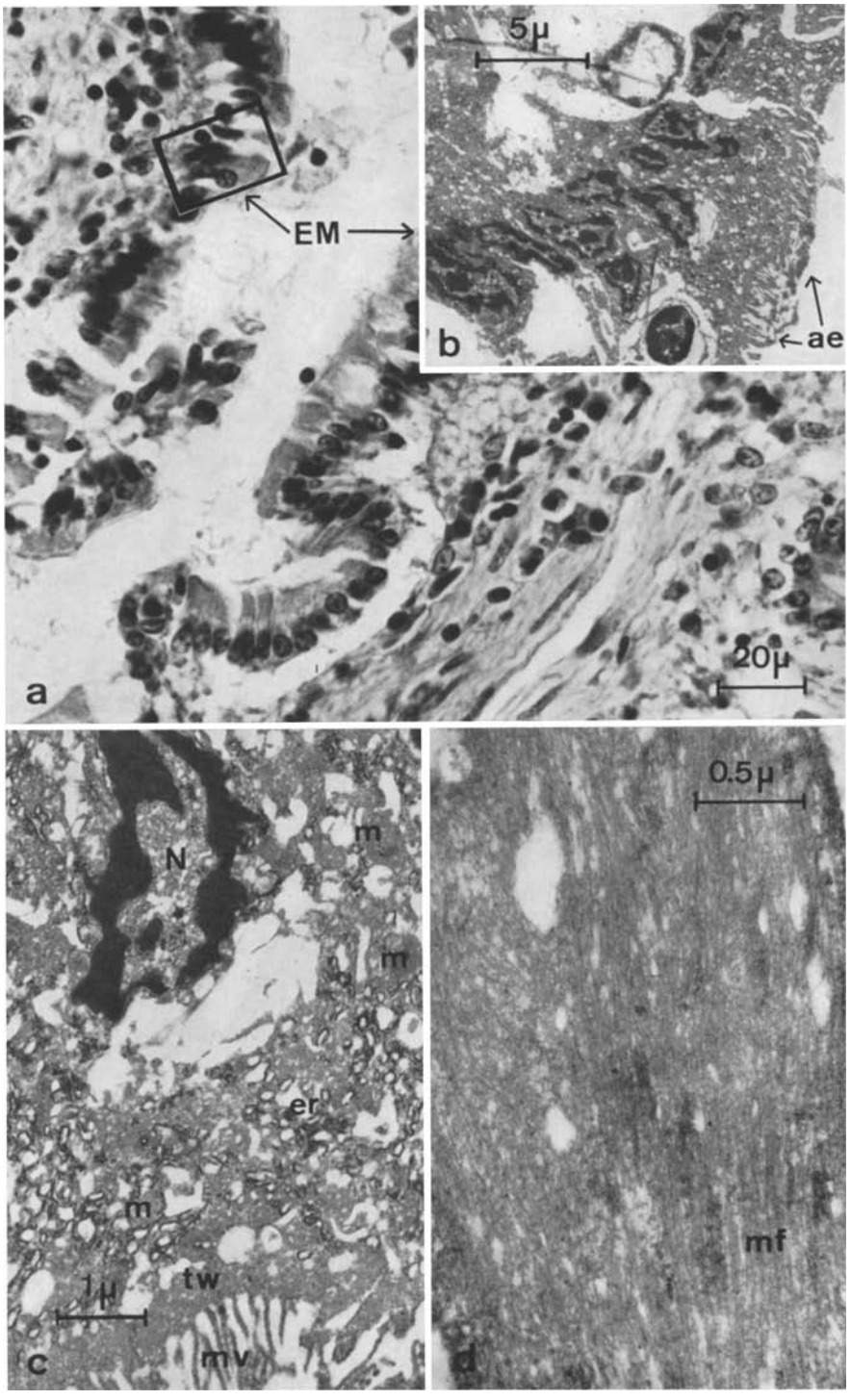

Fig. 2. a Microphotograph of a section of small intestine from a dog age 11 years. Formalin fixation, H and E staining. The area within the rectangle (*EM*) was selected for electron-

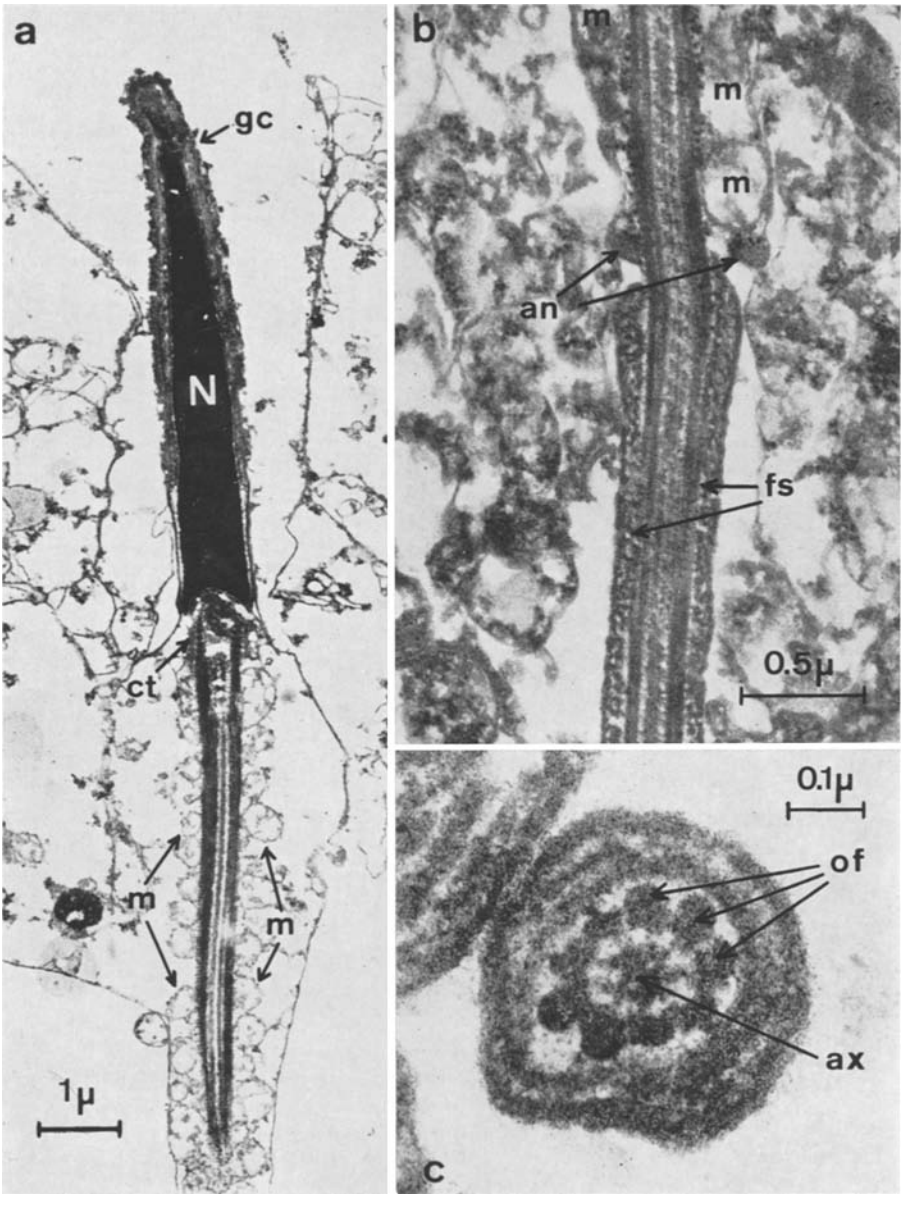

Fig. 3. a Electronmicrograph of a sperm from the testis of a dog age 3 years. The cell was pre-selected on a conventional Bouin fixed paraffin section, stained with H and E. *N* nucleus, *gc* galea capitis, *ct* centriole, *m* mitochondrion. b Detail of dog sperm. *m* mitochondrion, *an* annulus, *fs* fibrous sheath. c Detail of dog sperm. Cross section through the first portion of the principal piece of the tail. *of* outer fibers, *ax* axial filament complex

microscopy. b Dog intestine. Low power electronmicrograph of the selected area outlined in Fig. 4a. Arrow (*ae*) points to free surface of epithelium. c Details of absorptive cell (Fig. 4b, *ae*). *N* nucleus, *m* mitochondrion, *er* endoplasmic reticulum, *tw* terminal web, *mv* microvilli. d Dog intestine. Detail of smooth muscle cell from core of villus; *mf* myofilaments

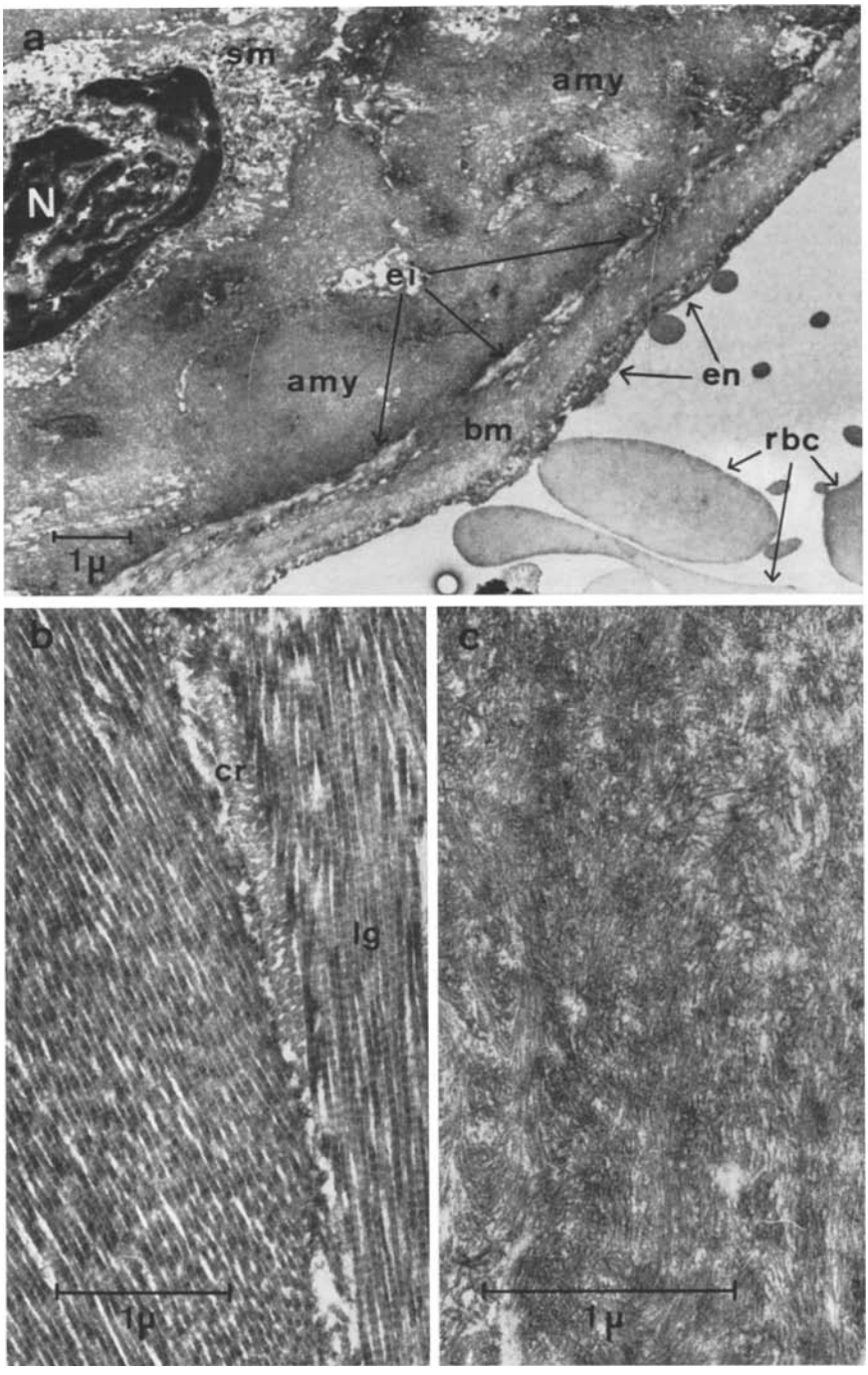

Fig. 4. a Low power electronmicrograph of the wall of a meningeal artery from a dog age 14 years. The site was pre-selected on a conventional paraffin section stained with thioflavine S. *N* nucleus, *sm* smooth muscle cytoplasm, *ei* elastica interna, *en* endothelium, *bm* basement membrane, *amy* amyloid, *rbc* erythrocytes. b Detail of dog meningeal artery. Collagen fibers from the tunica media. *cr* cross sections, *lg* longitudinal sections. c Detail of dog meningeal artery. Amyloid deposit from the tunica media

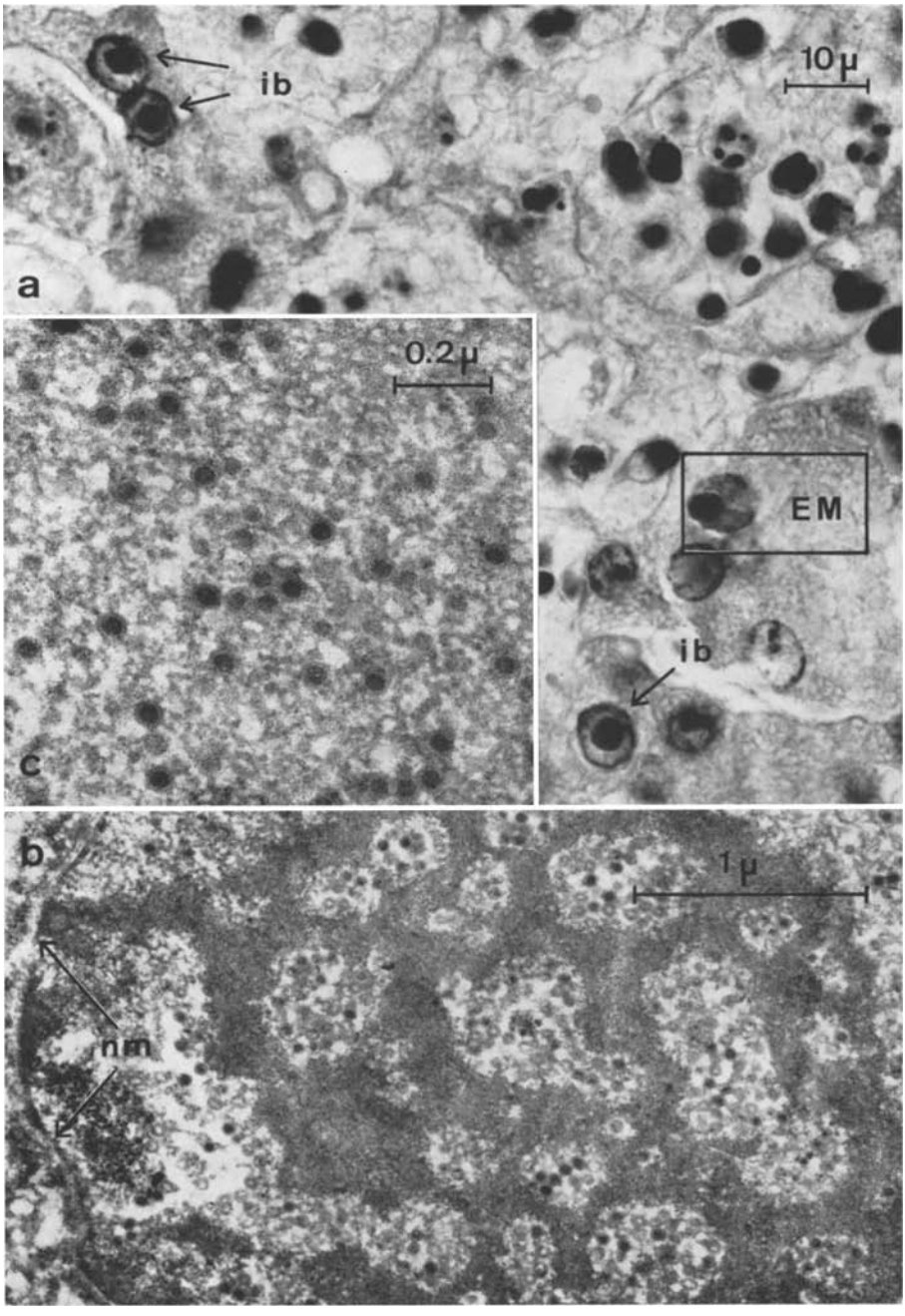

Fig. 5. a Microphotograph of a section of liver from a dog age 3 months affected by infectious canine hepatitis. Formalin fixation, H and E staining. *ib* intranuclear inclusion body. The area within the rectangle (*EM*) was selected for electronmicroscopy. b Dog liver. Electronmicrograph of the intranuclear inclusion body present in the selected area outlined in Fig. 5a. *nm* nuclear membrane. c Dog liver. Detail of viral particles present in the inclusion body

A further example of study of a lesion is given in Fig. 5a—c. The microphotograph shown in Fig. 5a was obtained from the liver of a dog that died at age three months for canine infectious hepatitis. The tissue had been fixed approximately 8 hours after death in 4% formaldehyde and embedded in paraffin. The electronmicrographs shown in Fig. 5b and c illustrate ultrastructural details of an inclusion body and the fine morphology of the virus.

This method obviously is not offered as a substitute for the conventional glutaraldehyde-osmium fixation and resin embedding in preparing material for EM study. However, its limitations do not render it invalid since it brings to electronmicroscopy material that would otherwise be lost. And certainly, it can be an aid to diagnosis and more importantly, it can provide information that may be needed to plan more detailed study of disease processes.

References

Bruijn, W. C. de, McGee-Russel, S. M.: Bridging the gap in pathology and histology. J. roy. micr. Soc. **85**, 77—80 (1966).

Gay, H.: Serial sections of smears for electron microscopy. Stain Technol. **30**, 239—242 (1955).

Goricki, M. A.: Oriented embedding of biological materials and accurate localization for ultrathin sectioning. Stain Technol. **41**, 37—42 (1966).

Gray, E. G.: Accurate localization in ultrathin sections by direct observation of the block face for trimming. Stain Technol. **36**, 42—44 (1961).

Grimley, P. H.: Selection for electron microscopy of specific areas in large epoxy tissue sections. Stain Technol. **40**, 259—263 (1965).

Heyner, S.: *In situ* embedding of cultured cells or tissue, grown on glass, in epoxy resin for electron microscopy. Stain Technol. **38**, 335—336 (1963).

Howatson, A. F., Almeida, J. D.: A method for the study of cultured cells by thin sectioning and electron microscopy. J. biophys. biochem. Cytol. **4**, 115—118 (1958).

Lehner, T., Nunn, R. E., Pearse, A. G. E.: Electron microscopy of paraffin embedded material in amyloidosis. J. Path. Bact. **91**, 297—300 (1966).

Palade, G. E.: A study of fixation for electron microscopy. J. exp. Med. **95**, 285 (1952).

Reynolds, E. S.: The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J. Cell. Biol. **17**, 208 (1963).

Robbins, E., Gonatas, U. K.: In vitro selection of the mitotic cell for subsequent electron microscopy. J. Cell Biol. **20**, 356—359 (1964).

Rosen, S. I.: Cover-glass embedding in open-end capsules for electron microscopy. Stain Technol. **37**, 195—197 (1962).

Seliger, W. G.: The productions of large, epoxy embedded, 50 μ sections by precision sawing: a preliminary to survey for ultrathin sectioning. Stain Technol. **43**, 269—272 (1968).

Sheffield, H. G.: A device for flat-face, epoxy resin embedding of tissue and coverslip cultures. Stain Technol. **40**, 143—145 (1965).

Sparvoli, E., Gay, E., Kaufmann, B. P.: Open-face, epoxy embedding of single cells for ultrathin sections. Stain Technol. **40**, 83—88 (1965).

Dr. G. L. Rossi
Institut für Tierpathologie
Länggasstraße 122
CH-3000 Bern