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Microwave fixation provides excellent preservation of tissue, cells and antigens for light and electron microscopy

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Summary

It was demonstrated that microwave energy used simultaneously in combination with low concentrations of glutaraldehyde (0.05%) and formaldehyde (2.0%) rapidly preserved light microscopic histology and excellent fine structural details, as well as a variety of cytoplasmic and membrane-bound antigens. Specimen blocks up to 1 cm^3 can be fixed in as brief a time as 26 ms using a specially designed microwave device (ultrafast microwave fixation method). The fast microwave fixation method, using a commercially available device, was successfully used to preserve granule-bound rat mast cell chymase which was subsequently detected by a postembedding immunogold procedure. Control of the following parameters is important to the microwave fixation method: (1) specimens with one dimension less than 1 cm; (2) irradiation temperatures lower than 50° C; (3) irradiation times less than 50 s; (4) immediate replacement of the postirradiation solution with cold storage buffer; (5) fixing the specimen within 15 min after it is removed from its blood supply.

Introduction

Microwave energy has recently been used for (1) rapid fixation of tissues for examination by light microscopy (Mayers, 1970; Zimmerman & Raney, 1972; Bernard, 1974; Gordon & Daniel, 1974; Login, 1978; Hopwood *et al.*, 1984; Leong *et al.*, 1985; Login & Dvorak, 1985; Kok *et al.*, 1986; Login *et al.*, 1986, 1987b, 1988), electron microscopy (Bernard, 1974; Chew *et al.*, 1983; Hopwood *et al.*, 1984; Leong *et al.*, 1985; Login & Dvorak, 1985; Login *et al.*, 1986, 1987a, 1988) and biochemical analyses (Schmidt *et al.*, 1971; Ruoff, 1977; Stavinoha, 1983); (2) preservation of intra- and extracellular antigens (Patterson & Bulard, 1980; Hopwood *et al.*, 1984; Leong *et al.*, 1985; Leong & Milios, 1986; Login *et al.*, 1987a, 1987b, 1988); (3) acceleration of the time required for a variety of staining procedures (Brinn, 1983; Estrada *et al.*, 1985; Hafiz *et al.*, 1985; Kok, 1986; Leong & Milios, 1986; Chiu & Chan, 1987; Tovar *et al.*, 1987); and (4) acceleration of the time required for tissue processing (i.e. dehydration, clearing and paraffin infiltration) (Boon *et al.*, 1986).

We describe herein microwave fixation methods which are capable of rapidly preserving specimens for examination by light and electron microscopy as well as being sufficiently gentle to demonstrate a wide variety of antigens using immunohistochemical and immunocytochemical techniques.

Materials and methods

Specimens

Animals were cared for in accordance with guidelines established by the Beth Israel Hospital's Committee on Animal Research and those prepared by the Committee on the Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (DHHS publication no. 86–23, revised 1985). The Beth Israel Hospital's Animal Research Facility is fully accredited by AAALAC.

Fresh human tissue was collected during evaluation of specimens for routine surgical pathology. Fresh mouse (CD–1) male and female (Charles River Breeding Labs, Boston, Massachusetts, USA) visceral tissues including pancreas, salivary gland, and liver were also collected. Specimens used in the microwave method were rapidly trimmed to 0.2–1 cm on a side in 0.1 M sodium cacodylate buffer and placed in plastic tissue cassettes (3 cm × 2.5 cm × 0.6 cm height) or glass vials (1.7 cm diameter × 4.5 cm height). Rat peritoneal mast cells were isolated according to a slight modification (Login *et al.*, 1987a) of the method of Enerbäck and Svensson (1980). In brief, lavage fluid consisting of 0.1% bovine serum albumin

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in Hanks' balanced salt solution (HBS–BSA) was recovered from the peritoneal cavities of male retired breeder rats (CD–1, Charles River Breeding Labs). A gradient was made with 3.7 ml physiologic Percoll (Sigma Chemical Co., St Louis, Missouri, USA) and mixed with 0.7 ml of the concentrated cell suspension in HBS–BSA (2000 000 cells/ml), followed by careful layering of 0.5 ml HBS–BSA. The gradient was centrifuged at 1500 *g* for 15 min at 4° C. Mast cells were recovered from the bottom of the tube and washed in HBS–BSA prior to fixation.

Fast microwave fixation method

A conventional microwave oven (Amana Radarange RR–8B, Amana Refrigeration Company, Amana, Iowa, USA) was used without modification. It has a maximum power output of 750 W, an operating frequency of 2.45 GHz, a thermometer sensitivity of 5° C in the 35–90° C operating range, and a magnetron warm-up time for delivery of peak power ranging between 2 and 4 s.

A glass dish (dimensions: 15 cm diameter × 2 cm height) was centred on the floor of the microwave oven and filled with 200 ml of a modified Karnovsky's fixative (i.e. 0.05% glutaraldehyde, 2% formaldehyde, described in detail below). The final height of solution in the dish was 1 cm. The microwave temperature probe was immersed in the solution. A total of 10 standard, plastic tissue cassettes containing the tissue samples were submerged in the aldehyde solution such that each cassette was in contact with the bottom of the dish (Fig. 1). The microwave oven was preset on maximum power and programmed to shut off when the final solution temperature reached 45° C ± 5° C. Specimen cassettes were immediately removed from the warm solution and either processed directly after microwave fixation or were stored in 0.1 M sodium cacodylate buffer, pH 7.4, 4° C, prior to processing.

Single specimens were fixed in 5–9s by the previously described fast microwave method (Login *et al.*, 1985). In brief, a sample vial containing 2 ml of the aldehyde mixture was located centrally in the microwave chamber and positioned 1.2 cm above the floor on a polystyrene block (Bernard, 1974). A 400 ml beaker (11 cm height × 8 cm diameter) containing a 300 ml distilled water load was located in the left rear corner of the oven. The temperature probe automated shut-off of the microwave power when the final solution temperature (50° C) was reached. Specimens were immediately removed from the warm solution and either stored in 0.1 M sodium cacodylate buffer with 0.02% sodium azide up to 2 weeks or immediately processed.

Ultrafast microwave fixation method

The ultrafast microwave fixation device used for these experiments has been previously described (Jones and Stavinoha, 1979; Login *et al.*, 1986, 1988). In brief, the device can deliver a maximum power of 7.3 kW at a frequency of 2.45 GHz. It produces continuous power up to a 2s interval, and has a 0.5ms rise time to peak power. The duration of microwave irradiation can be adjusted within 1ms intervals. The maximum specimen dimensions tested were 0.2 cm in length. In these experiments, tissue samples were irradiated in Karnovsky's fixative with 5.4 kW of microwave power for a duration ranging between 26 and 98 ms. Final solution temperatures were measured immediately after microwave irradiation with a copper–constantan thermocouple and Digisense thermometer (Cole-Palmer Instrument Company, Chicago, Illinois, USA) and ranged between 31° C and 47° C ± 2° C. Samples were immediately removed from the warm, irradiated solution and stored directly in 4° C, 0.1 M sodium cacodylate buffer until processing.

Controls

Specimens fixed by the microwave methods were compared to specimens fixed by (a) immersion in our standard fixation control for electron microscopy: standard Karnovsky's fixative for 2 h at room temperature, which consisted of 2% formaldehyde made from paraformaldehyde, 2.5% glutaraldehyde, 0.025% calcium chloride in 0.1 M sodium cacodylate buffer, pH 7.4 (Karnovsky, 1965; Login & Dvorak, 1985); (b) immersion in standard Karnovsky's fixative for 1–30s at 25° C or at 50° C; (c) immersion in our standard fixation control for light microscopy: 10% unbuffered formalin for 2–8 h at room temperature; (d) microwave irradiation of specimens immersed in modified Karnovsky's mixtures with glutaraldehyde and formaldehyde concentrations ranging between 0.05% and 10%; (e) microwave irradiation of specimens immersed in physiological saline or distilled water. Control specimens for electron microscopy were sectioned to 1–2 mm cubes prior to immersion in the control fixatives.

Processing for light and electron microscopy studies

Processing for light microscopy employed an automated tissue processor (Tissue Tek VIP, Miles Scientific, Naperville, Illinois, USA) and consisted of a 6 h protocol including dehydration in graded ethanols, clearing in xylol, and infiltration with paraffin (Login *et al.*, 1987b). Sections 4 μm thick were stained with Haematoxylin and Eosin.

Processing for electron microscopy consisted of immersing 1 mm^3 tissue blocks and cell pellets in solidified agar blocks in 2%, 0.2 M sym-collidine buffered osmium tetroxide for 2 h (1 h for cell pellets) at 25° C, staining en bloc for 2 h with a 2% solution of uranyl acetate (1 h for cell pellets), dehydration in a graded series of ethanols and propylene oxide, and embedding in Epon. Sections 1 μm thick were stained with alkaline-Giemsa and examined by light microscopy. Sections (50–70 nm) were stained with dilute lead citrate and examined with a Philips 300 electron microscope at 60 kV (North American Philips, Mahwah, New Jersey, USA) (Login & Dvorak, 1985).

Immunohistochemical studies

Paraffin sections 4–5 μm in thickness were conventionally stained with monoclonal antibodies to epithelial membrane antigen (1 : 10–1 : 100, Dako Corporation, Santa Barbara, California, USA), leucocyte common antigen (1 : 25, Dako) a broad spectrum of keratins (1 : 100, AE1 and AE3, Hybritech Incorporated, San Diego, California, USA), and chromogranin-A (1 : 800, Hybritech) for 1 h at 25° C and 100% humidity using an avidin–biotin complex technique described previously (Schnitt & Vogel, 1986; Login *et al.*, 1987b). Sections were also conventionally stained with polyclonal antisera to S–100 protein (1 : 500–1 : 1000, Dako), carcinoembryonic antigen (1 : 200, Dako), and factor VIII-related antigen

(1:250, Dako) for 1 h at 25° C and 100% humidity using a peroxidase–antiperoxidase technique previously described (Schnitt & Vogel, 1986; Login *et al.*, 1987b). The need for preliminary trypsinization was also tested (0.25 mg ml^{-1} porcine pancreas type II trypsin, Sigma Chemical Company, St Louis, Missouri, USA). Negative controls included substitution of mouse myeloma protein for monoclonal antibodies and normal rabbit serum for polyclonal antisera. Known positive control specimens for each antibody were tested. 3,3'-Diaminobenzidine was used as the chromogenic agent to detect antibody binding sites. Sections were lightly counterstained with Haematoxylin.

Immunocytochemical studies

The postembedding immunogold labelling technique to demonstrate rat mast cell chymase involved floating nickel or gold grids (Pelco, Tustin, California, USA) section-side down on 50 µl drops of the following reagents at 25° C: a saturated solution of sodium metaperiodate, 1 h (Sigma Chemical Company) (Bendayan & Zollinger, 1983); 5% normal goat serum, 20 min; 1:10–1:1000 dilutions (5 mg ml^{-1}) of goat anti-rat mast cell chymase IgG, 1 h (gift of Dr Lawrence Schwartz, Department of Immunology and Connective Tissue Diseases, Medical College of Virginia, Richmond, Virginia, USA) (Schick *et al.*, 1984); 1:20 dilution of rabbit anti-goat IgG conjugated to 5 nm gold particles, 1 h (E. Y. Laboratories, Incorporated, San Mateo, California, USA) (Login *et al.*, 1987a).

Controls for the specificity of the chymase binding reaction were tested in competition experiments by preincubating the grids of mast cells with and without 0.17 mg ml^{-1} purified rat mast cell chymase (0.17 mg ml^{-1}) (Wintroub *et al.*, 1984) (gift of Dr Lawrence Schwartz) followed by addition of the anti-chymase antibody(1:600). The competition experiments were performed with and without soybean trypsin inhibitor (0.70 mg ml^{-1}) during incubation with chymase antibody to control for possible digestion of the sections by chymase (Login *et al.*, 1987a). Additional controls for non-specific binding of the secondary gold-labelled antibody to the primary antibody and to the grid sections involved substitution of non-immune goat serum for the primary antichymase antibody and omission of the primary antibody, respectively.

Results

Light and electron microscopy

The best microwave fixation results were obtained in specimens immersed in aldehyde mixtures and irradiated for 4–8 s between final solution temperatures of 40–50° C ± 5° C by the fast microwave method and for 26–90 ms to final solution temperatures between 34 and 42° C ± 2° C by the ultrafast method. Specimens fixed by the fast and ultrafast microwave methods and evaluated by light microscopy (Figs 2(a)–(e), 3(a)) and electron microscopy (Figs 4(a), 5(a), 6(a), 7(a), 8) displayed excellent morphological preservation compared to their standard immersion fixation controls (Figs 3(b), 4(b), 5(b)). Fatty tissues (e.g. breast, Fig. 2(c)) were preserved as well as their standard formalin-fixed counterparts (not shown). Erythrocytes in

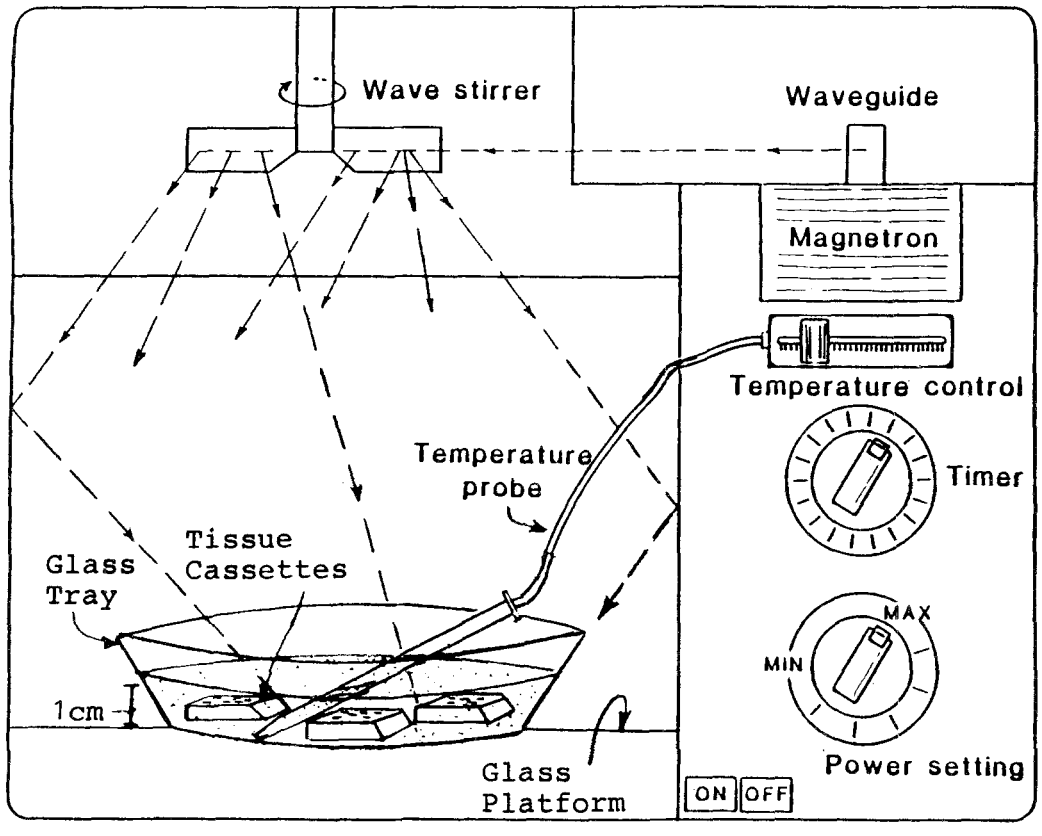


Fig. 1. Diagram of batch cassette loading in conventional microwave oven for fast fixation.

tissue blocks (Fig. 3(a)) and cells in suspension (Fig. 8) were very well preserved in samples fixed by the microwave methods. Delicate membranes of fixation-sensitive organelles such as mitochondria (Figs 4(a), 5(a), 6(a), 7(a)) and granules in salivary gland (Fig. 3(a)) and pancreas (Fig. 4(a)) were uniformly well preserved by the microwave methods.

Specimens immersed in modified Karnovsky's mixture with glutaraldehyde concentrations as low as 0.05% and fixed in seconds by the fast microwave method demonstrated excellent fine structural detail of mitochondrial and nuclear membranes when compared to control specimens immersed in the same mixture for 2 h at 25° C (Fig. 7(a),(b)). Cisternae of some of the endoplasmic reticulum in specimens fixed by this microwave method and the control showed mild dilatation. Electron-lucent areas are glycogen deposits not rendered electron-dense by the processing methods used.

Microwave-fixed specimens did infrequently have discrete areas of poor preservation which did not appear to be clustered spatially in the 1 cm^3 specimens studied (not shown). These focal areas demonstrated histology consistent with inadequate fixation (cold spots); however, some of these areas within a sample displayed pyknotic nuclei and intensely eosinophilic cytoplasm, an artifact we interpreted as 'hot spots'. Microwave irradiation of specimens immersed in solutions other than the aldehyde mixtures (e.g. physiological saline or distilled water) showed vacuolated, swollen cells evident in 1 μm Giemsa-stained light microscopy sections (Fig. 3(c)) and in electron microscopy sections (Fig. 4(d)). However, microwave irradiation alone does result in some degree of ultrastructural preservation (Fig. 4(d)). Specimens exposed to the aldehyde for seconds at room temperature (Fig. 6(b)) or between 35 and 55° C (Figs 3(d),4(c)) without microwave irradiation demonstrated loss of granules and uneven staining by light microscopy (Fig. 3(d)) and loss of detail of the mitochondria and clumping of nuclear chromatin by electron microscopy (Figs 4(c), 6(b)). Specimens irradiated by microwave energy while immersed in low concentrations of formaldehyde alone (i.e. 0.2–1%) displayed swollen membranes and numerous vesicles in the cytoplasm while high concentrations of formaldehyde (i.e. 5–10%) resulted in discontinuities of organelle and cellular membranes (not shown).

Immunohistochemistry

Tissue blocks fixed by the fast microwave method demonstrated intense specific staining of the seven antigens tested (Fig. 9). Epithelial membrane antigen (Fig. 9(a)), evaluated on tissue fixed by the fast microwave method, revealed optimal staining characteristics even when the concentration of the primary antibody was diluted ten-fold compared to its formalin-fixed controls. Immunohistochemical staining by factor VIII, carcinoembryonic antigen, and keratin AE1/3 antibodies required no trypsin pretreatment on samples fixed by the microwave method (Figs 9(b), (c), 10(a), respectively). Formalin-fixed controls did require pretrypsinization for optimal staining of factor VIII and carcinoembryonic antigen (not shown). No staining was observed in the epithelium of formalin-fixed skin using a 'cocktail' of keratin antibodies AE1/3, regardless of the use of trypsin digestion (Fig. 10(c), (d)). Specimens fixed by the microwave method were sensitive to trypsin digestion, although some specific staining was discernible (Fig. 10(b)).

Immunocytochemistry

Granules in rat mast cells showed specific labelling of chymase. Labelling density averaged 125, 5–nm immunogold particles per granule (Fig. 11(a)). Other sites in these cells were negative. The competition control demonstrated that free chymase competed with granule-associated chymase for the primary goat anti-chymase IgG. The frequency of the immunogold label in the competition experiments was approximately 10–15 gold particles per granule (Fig. 11(b)). Soybean trypsin inhibitor effectively blocked protease digestion of the grid section by the free chymase. In a separate control, we observed that the soybean trypsin inhibitor alone did not qualitatively diminish the intensity of the gold label on the granules (not shown). Negative controls in which non-immune goat serum was substituted for the primary antibody (Fig. 11(c)), or in which the primary antibody was omitted (not shown), demonstrated no non-specific staining.

Discussion

Microwave irradiation used in conjunction with aldehyde mixtures can provide morphological preservation of specimens in as brief a time as 26 ms (ultrafast technique). The fast microwave method (seconds), using a commercially available microwave oven simultaneously with an aldehyde mixture containing 0.05% glutaraldehyde and 2% formaldehyde, preserves a variety of intracellular and membrane

Fig. 2. Light micrographs of haematoxylin and eosin-stained paraffin sections of human tissues fixed by the fast microwave method in 5–8 s to a final solution temperature of 45° C ± 5° C. Solution used during irradiation: modified Karnovsky's mixture containing 0.05% glutaraldehyde and 2% formaldehyde. (a) Skin, (b) cervix, (c) breast, (d) colon, (e) endometrium. The general tissue architecture and nuclear and cytoplasmic details are well preserved. Original magnifications (a)–(d), × 330; (e), × 665.

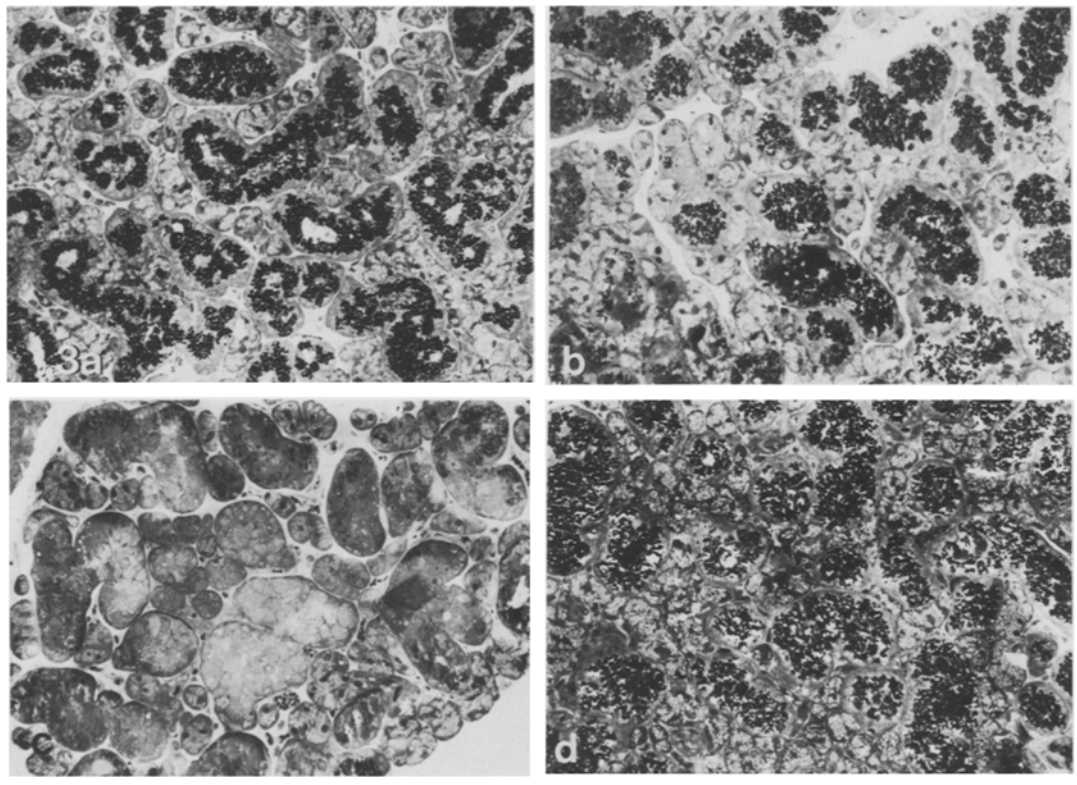
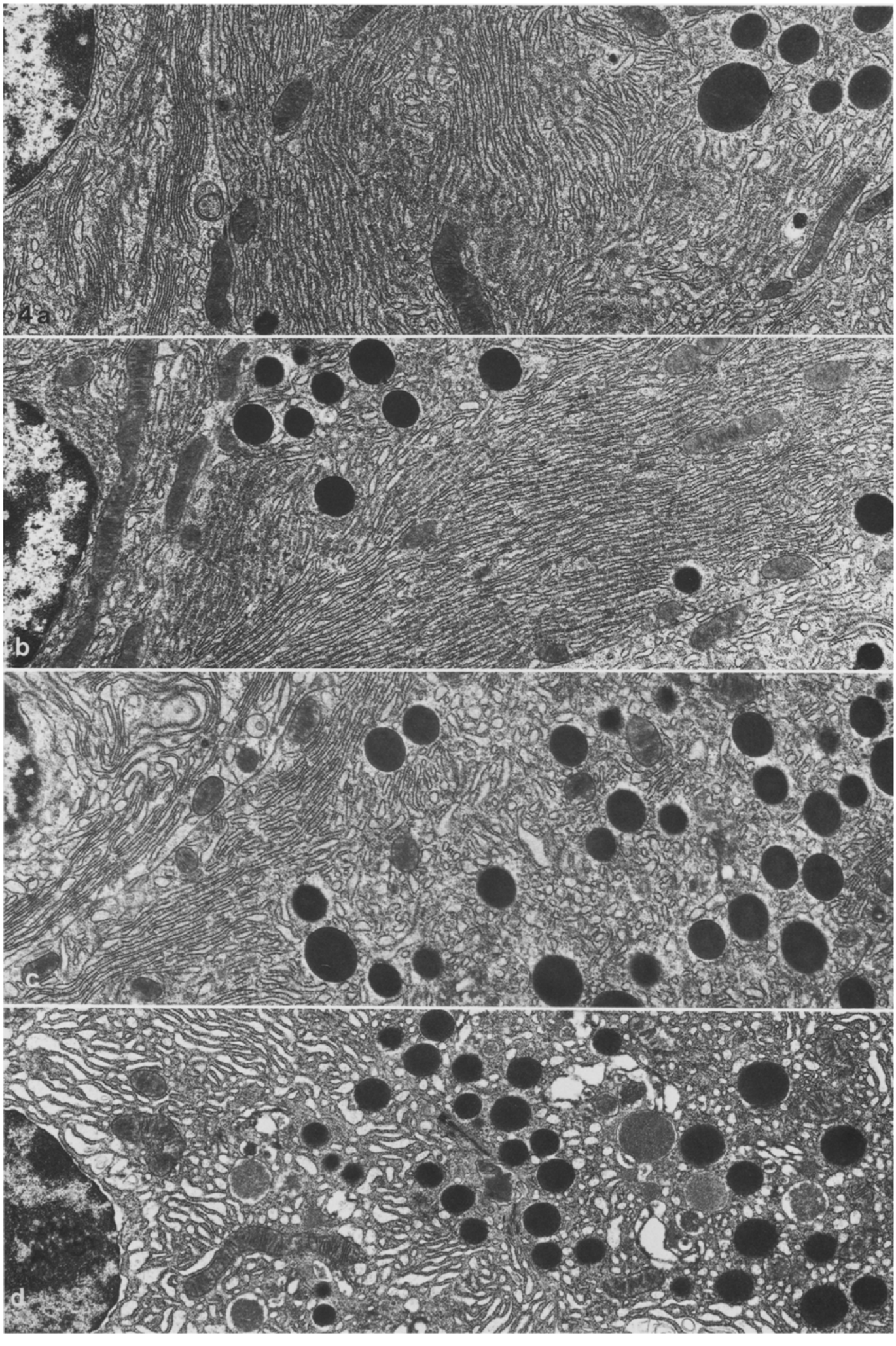


Fig. 3. Light micrographs of alkaline Giemsa-stained 1 μm Epon sections of mouse salivary gland. (a) Fixed by the fast microwave method in 9 s to a final solution temperature of 50° C. Solution used during irradiation: standard Karnovsky's mixture containing 2.5% glutaraldehyde and 2% formaldehyde. (b) Fixed by immersion in standard Karnovsky's mixture for 2 h at 25° C. (c) Fixed by microwave control method in 15 s to a final solution temperature of 50° C. Solution used during irradiation: distilled water. (d) Fixed by the conventional heat control method, i.e., immersion for 20 s in standard Karnovsky's mixture heated to 50° C in a water-bath. Glandular architecture, granules and erythrocytes are well preserved in (a) and (b). In (c), glandular cells are swollen and granules are absent; however, erythrocytes are present. In (d), glandular architecture is discernible; granules have coalesced into lakes; staining is uneven. Original magnification (a)–(d), × 110. Reprinted with permission from Login, G. R. and Dvorak, A. M. *Amer. J. Pathol. (1985)* **120**, 230–43.

antigens as well as fine structure. Our controls demonstrate the importance of this simultaneous microwave–aldehyde combination. Specimens fixed by the fast microwave method in seconds appear qualitatively similar to controls fixed by immersion for 2 h in standard Karnovsky's mixture, corroborating our previously reported stereological measurements on liver mitochondria and salivary gland granules (Login & Dvorak, 1985). Microwave irradiation of specimens in non-aldehyde solutions or immersion of control samples in aldehyde without microwave irradiation for brief times regardless of the solution temperature do not yield fine structural results equivalent to the microwave methods (Hopwood *et al.*, 1984; Leong *et al.*, 1985; Login *et al.*, 1986; Login & Dvorak, 1985).

While the precise physicochemical events responsible for the rapid fixation of specimens exposed to microwave irradiation are not known, temperature elevation of the solution is a convenient endpoint for monitoring fixation in both the fast and ultrafast microwave methods. The end point microwave fixation temperature varies with the microwave irradiation chamber design and the magnitude of the

Fig. 4. Transmission electron micrographs of mouse pancreas. (a) Fixed by the fast microwave method in 9 s to a final solution temperature of 50° C. Solution used during irradiation: standard Karnovsky's mixture containing 2.5% glutaraldehyde and 2% formaldehyde. (b) Fixed by immersion in standard Karnovsky's mixture for 2 h at 25° C. (c) Fixed by the conventional heat control method, i.e. immersion for 20 s in standard Karnovsky's mixture heated to 50° C in a water-bath. (d) Fixed by microwave control method in 15 s to a final solution temperature of 50° C. Solution used during irradiation: distilled water. (a) and (b) show well-preserved nuclear chromatin, rough endoplasmic reticulum, perinuclear membranes and mitochondria. (c), The conventional heat control shows dilated rough endoplasmic reticulum, and poorly imaged mitochondrial cristae. (d), The microwave control, shows marked dilation of the cisternae of the rough endoplasmic reticulum and the perinuclear membranes, however, mitochondrial cristae are well preserved. (a) × 10370; (b) × 10500; (c) × 9710; (d) × 9310.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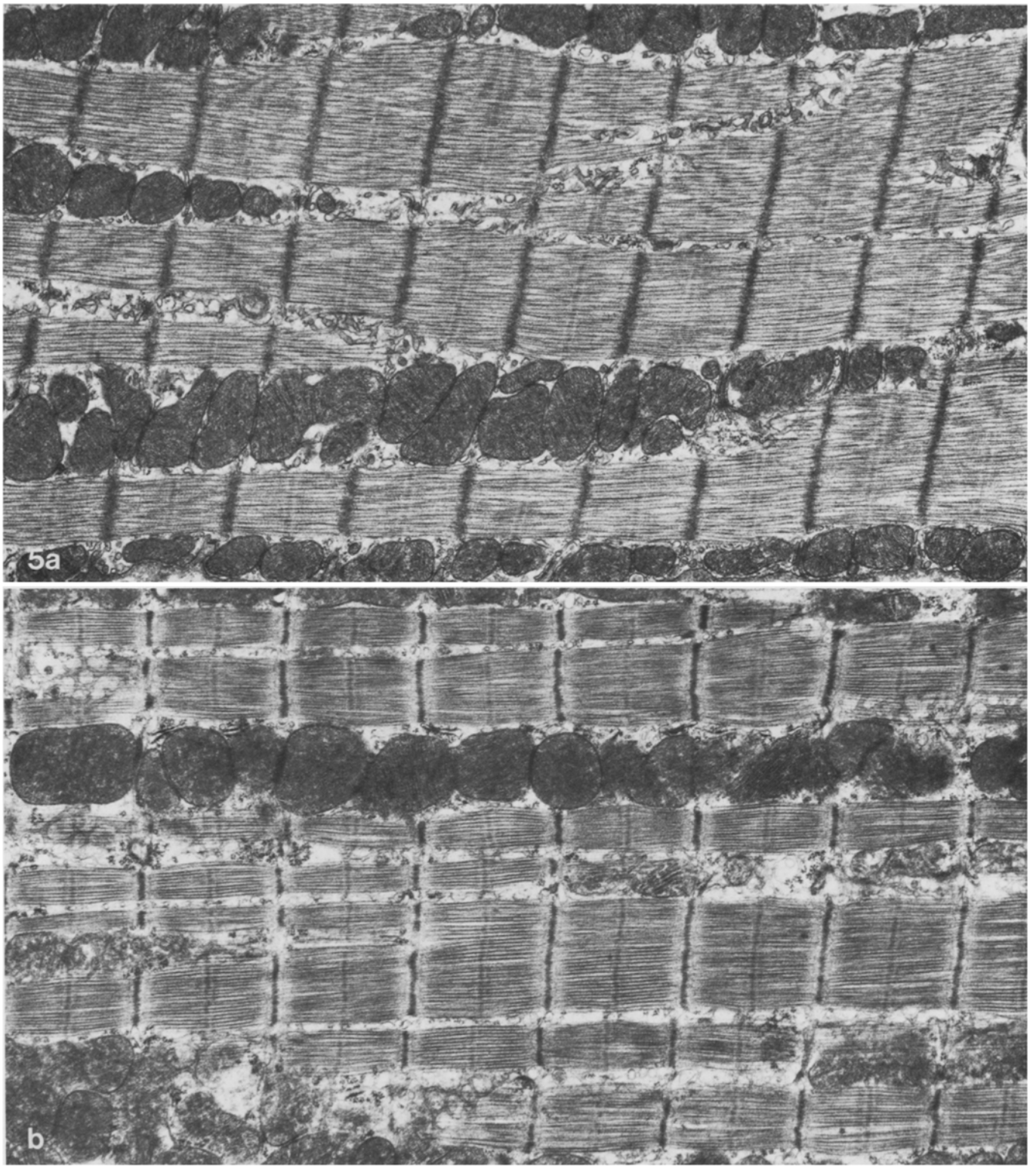


Fig. 5. Transmission electron micrographs of mouse heart. (a) Fixed by the ultrafast microwave method in 26 ms to a final solution temperature of 32° C. Solution used during irradiation: standard Karnovsky's mixture. (b) Fixed by immersion in standard Karnovsky's mixture for 2 h at 25° C. Myofilaments, sarcoplasmic reticulum, T tubules and mitochondria are equally well preserved in both preparations. (a) × 14820; (b) × 12350.

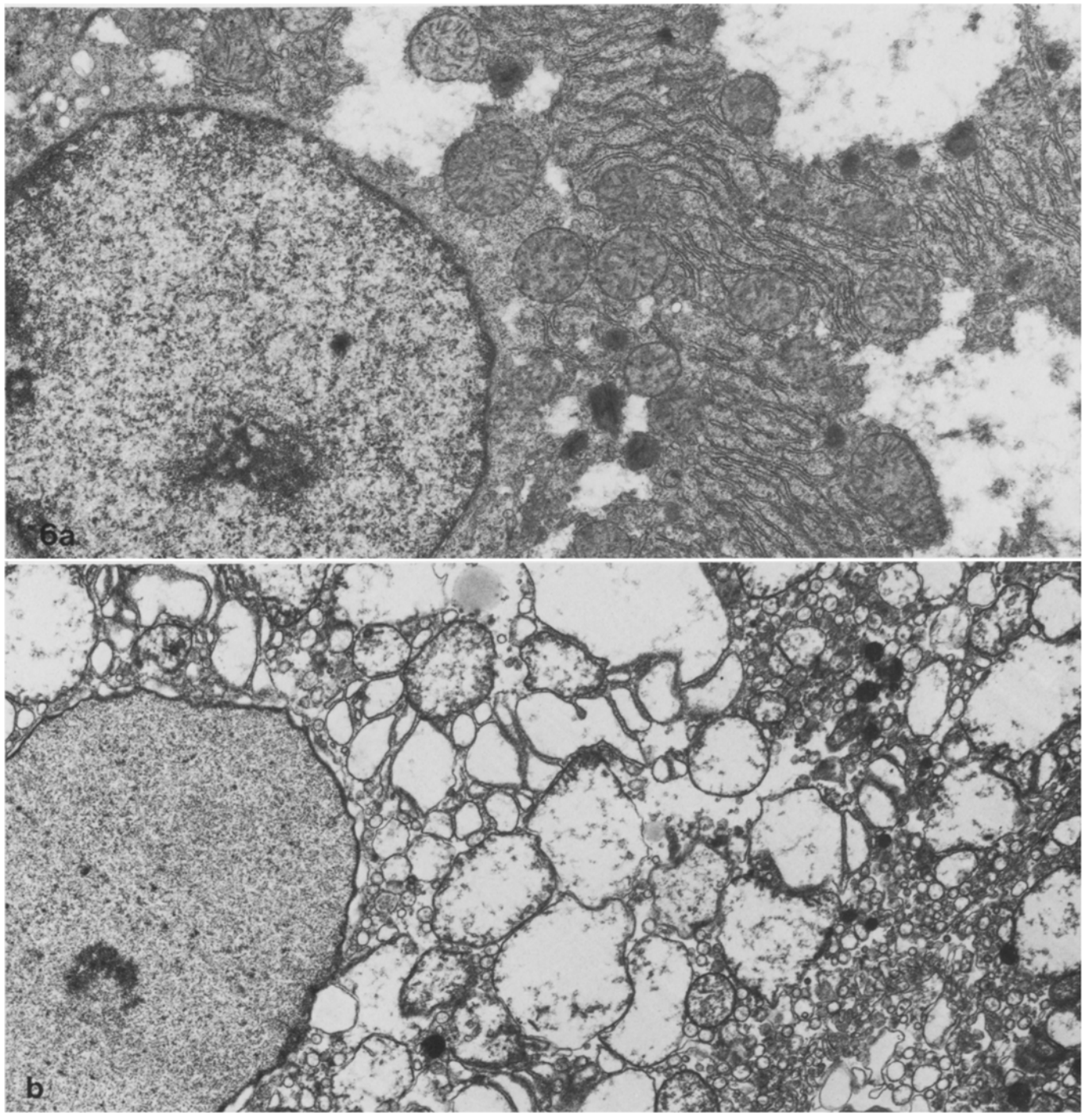


Fig. 6. Transmission electron micrographs of mouse liver. (a) Fixed by the ultrafast microwave method in 43 ms to a final solution temperature of 34° C. Solution used during irradiation: standard Karnovsky's mixture. (b) Fixed by the brief immersion control, i.e. immersion for 30 s in standard Karnovsky's mixture at 25° C. (a) Shows excellent nuclear and organelle preservation. Electronlucent areas are glycogen aggregates not rendered electrondense by the processing methods used. (b) Shows that brief exposure (seconds) to aldehyde at room temperature results in insufficient fixation with disruption and vesiculation of cytoplasmic membranes and swollen mitochondria evident in the micrograph. (a) × 10450; (b) × 7315.

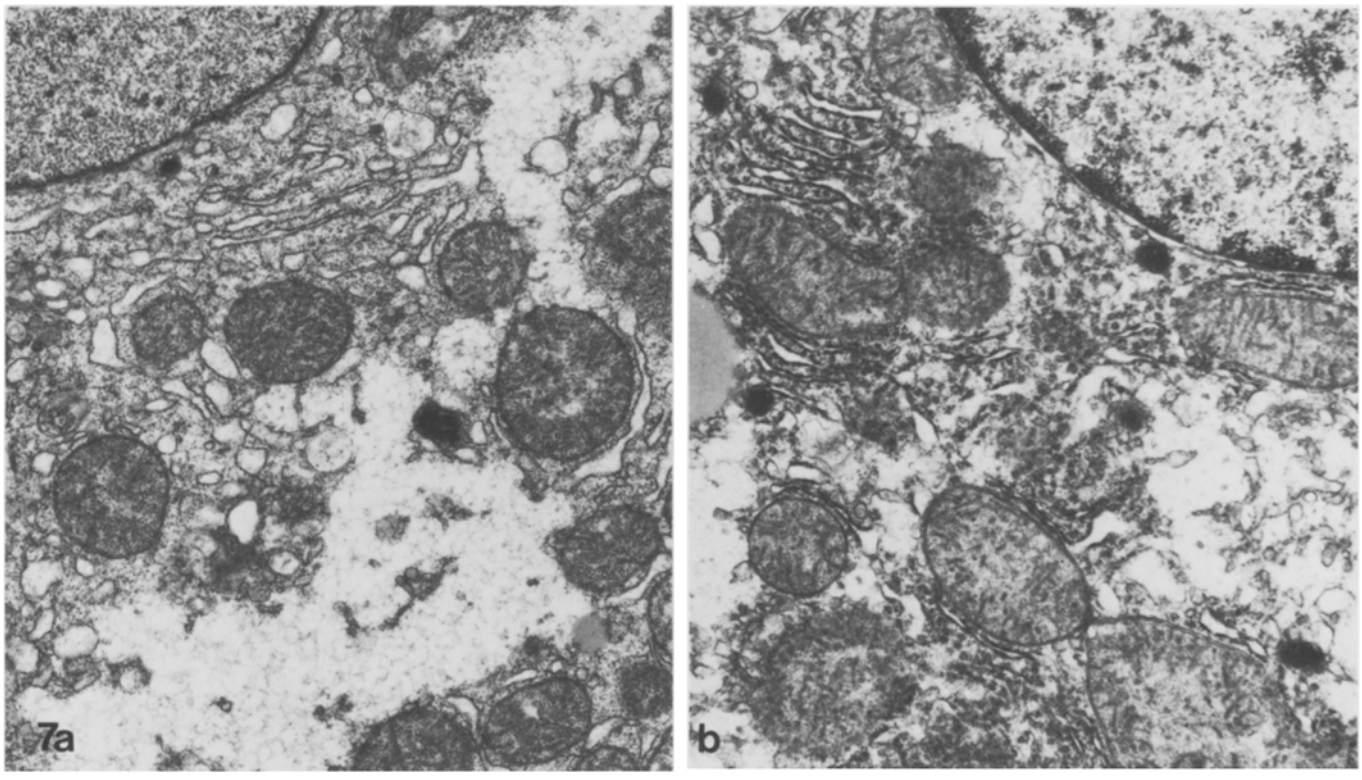


Fig. 7. Transmission electron micrographs of mouse liver. (a) Fixed by the fast microwave method in 6 s to a final solution temperature of 50° C. Solution used during irradiation: modified Karnovsky's mixture containing 0.05% glutaraldehyde and 2% formaldehyde. (b) Fixed by immersion in modified Karnovsky's mixture for 2 h at 25° C. Nuclear chromatin, perinuclear membranes, and mitochondria are well preserved in both preparations. Mild dilatation of the rough endoplasmic reticulum is seen in (a) and (b). Electronlucent areas are glycogen aggregates not rendered electrondense by the processing methods used. (a) × 14000; (b) × 15000.

microwave power. We empirically learned that the ideal final fixation temperature of the irradiated solution for the ultrafast device (5400 W) was on average 15° C lower than the final solution temperature necessary for the fast microwave (700 W) device. In a high power microwave field, intracellular water bounded by membranes may heat faster than the aqueous environment surrounding the specimen (McClean *et al.*, 1981). More precise information regarding the endpoint temperature for microwave fixation may in the future be obtained from intracellular temperature detection methods not currently available.

Several investigators previously reported eosinophilia of connective tissue, pyknotic, nuclei, and the loss of erythrocytes in specimens fixed by or in conjunction with microwave irradiation (Mayers, 1970; Bernard, 1974; Hopwood *et al.*, 1984; Leong *et al.*, 1985). Our results indicate that these observed tissue changes may be the result of the destructive effects of conductive heat in specimens irradiated for more than 60 s or in samples which are allowed to stand in the postirradiated warmed solution for more than a few seconds.

The concentration of the aldehyde used during irradiation is a more critical factor in retaining antigen activity and obtaining ideal morphology than is the final irradiation temperature (45° C) (Login *et al.*, 1988). Our observations are indirectly supported by the findings of biochemists who report that aldehyde concentrations as low as 0.1% can inactivate a variety of enzymes (Ellar *et al.*, 1971; Wakabayashi *et al.*, 1975) and antigens (Clements & Beitz, 1985), and neuropharmacologists who report microwave fixation methods in which final irradiation temperatures of 75–95° C are required to inactivate many neurochemical enzymes (Schmidt *et al.*, 1971; Merritt & Frazer, 1977; Stavinoha, 1983; Ruoff, 1977). In addition, we previously demonstrated by immunofluorescence microscopy that preservation of fibrinogen/fibrin, laminin, type IV collagen and fibronectin in frozen sections of guinea-pig skin by the fast microwave method required the use of glutaraldehyde concentrations less than 0.25% and formaldehyde concentrations less than 5% (Login *et al.*, 1988).

Using the fast microwave fixation method, a post-embedding immunogold technique, and transmission electron microscopy, we have confirmed histochemical (Gomori, 1953; Seppa *et al.*, 1979) and biochemical (Lagunoff & Benditt, 1963) evidence localizing chymase to the granule matrix of unstimulated rat peritoneal mast cells. We chose to apply our

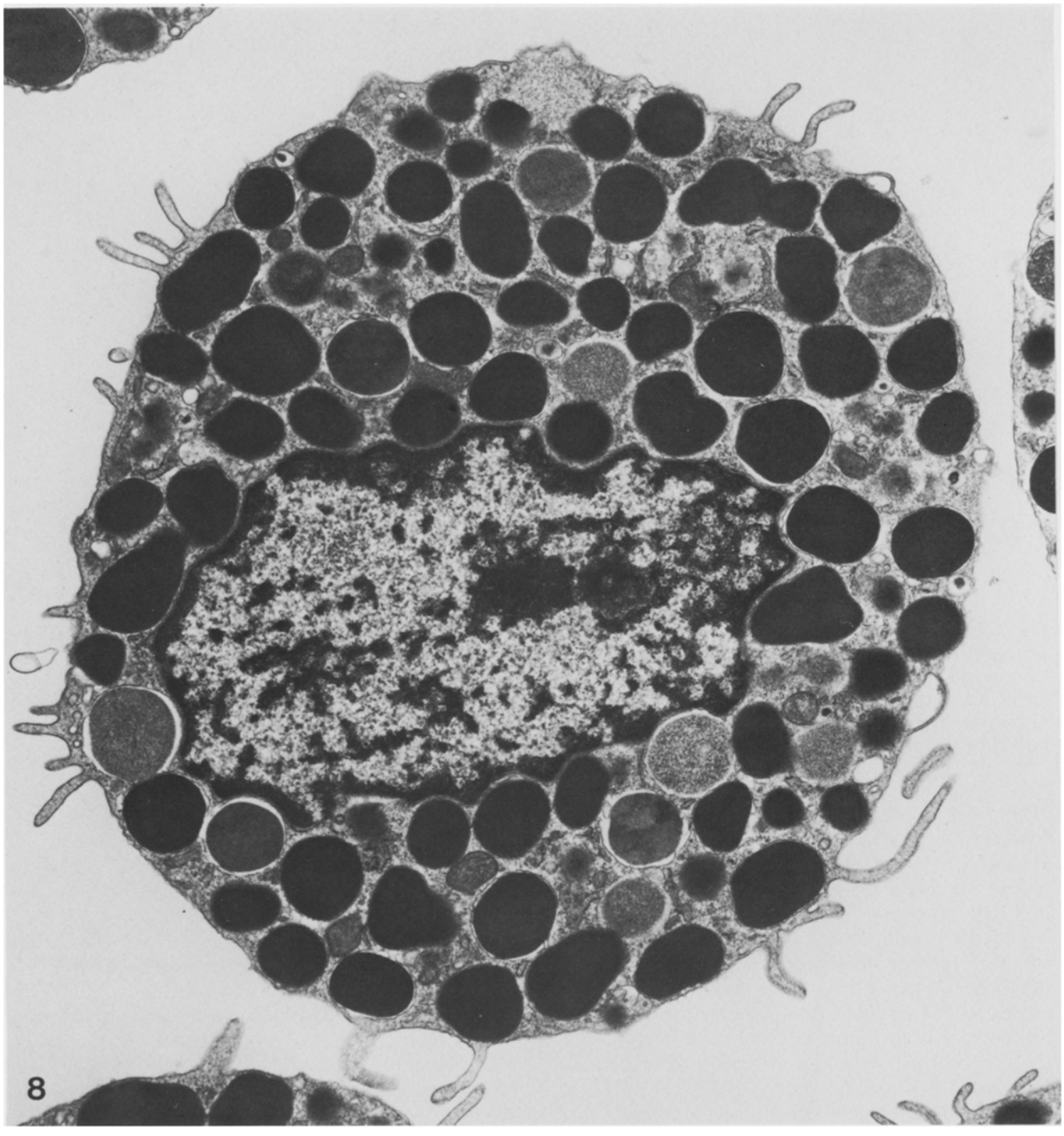


Fig. 8. Transmission electron micrograph of a rat peritoneal mast cell fixed by the fast microwave method in 7 s to a final solution temperature of 45° C. Solution used during irradiation: modified Karnovsky's mixture containing 0.05% glutaraldehyde and 2% formaldehyde. The cell exhibits excellent preservation of cytoplasmic granules, membranes, mitochondria and the nucleus. × 14 800.

Fig. 9. Light micrographs of immunoperoxidase-stained and haematoxylin and eosin-counterstained paraffin sections of human tissues fixed by the fast microwave method in 5–8 s, final solution temperature of 45° C ± 5° C. Solution used during irradiation: modified Karnovsky's mixture containing 0.05% glutaraldehyde and 2% formaldehyde. (a) Surfaces of breast glandular epithelial cells are positive for epithelial membrane antigen (1 : 100); (b) factor VIII stains endothelial cells in the endometrium (1 : 250); (c) carcinoembryonic antigen (1 : 200) stains the surface of glandular epithelial cells in the endometrium; (d) leucocytes are positive for leucocyte common antigen (1 : 25) in the endometrium; (e) S–100 protein (1 : 1000) stains a nerve in the cervix; (f) Chromogranin (1 : 800) -positive neuroendocrine cells in the colon are present among glandular epithelium. No trypsin pretreatment was used. (a)–(f) × 330.

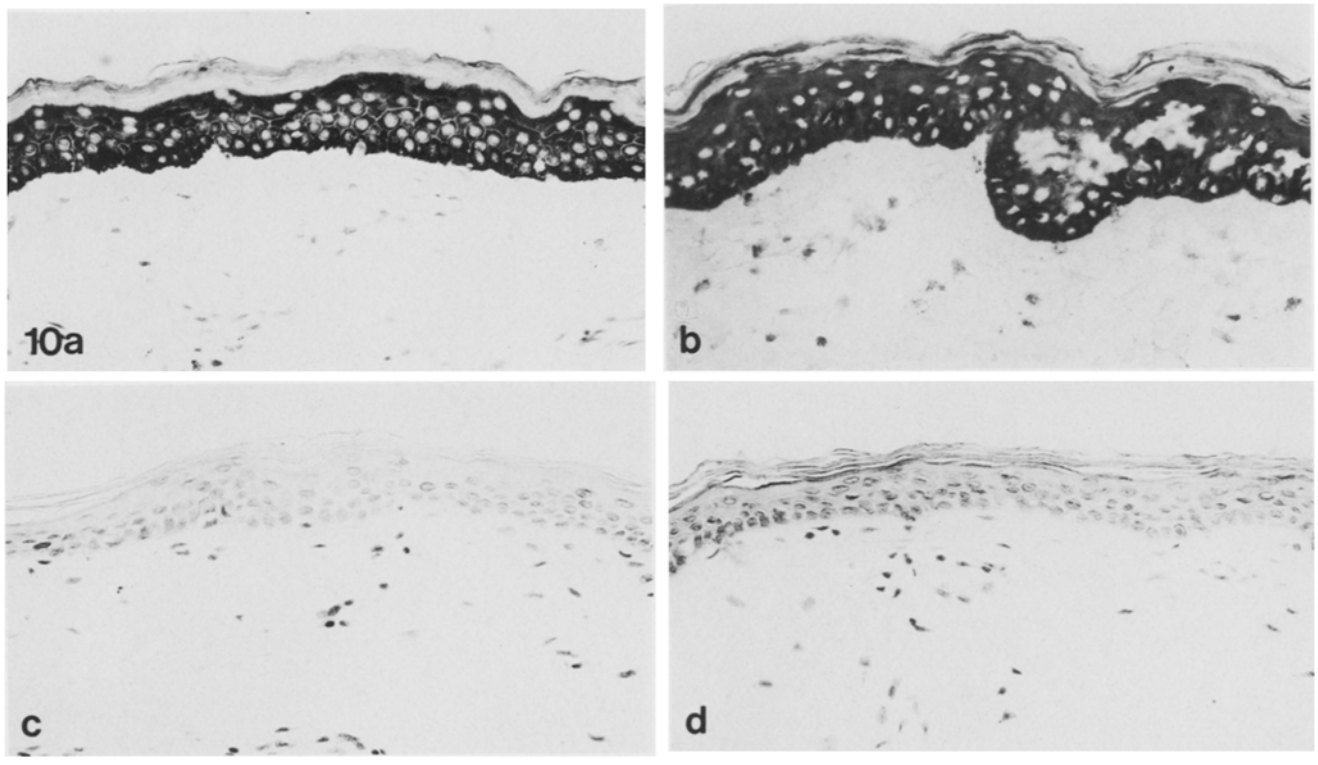


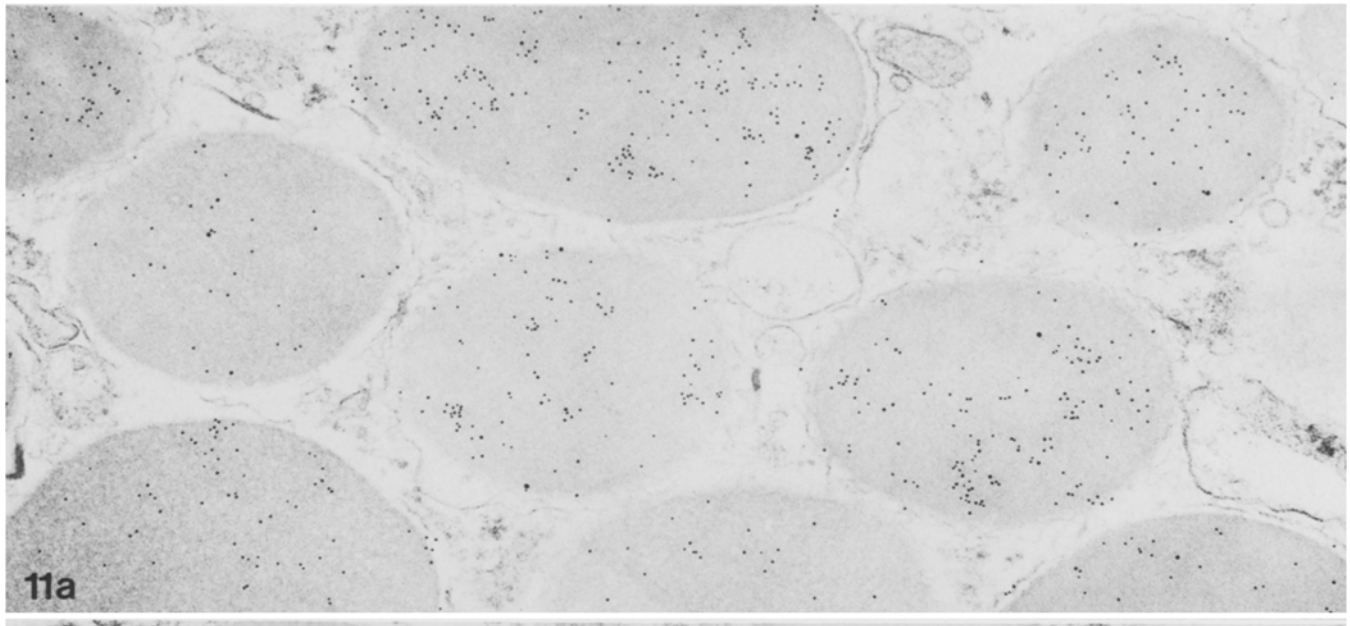
Fig. 10. Light micrographs of keratin AE1/3- (1:100) immunoperoxidase-stained and Haematoxylin and Eosin-counterstained paraffin sections of human skin. (a) Fixed by the fast microwave method in 5 s to a final solution (modified Karnovsky's mixture) temperature of 45° C without trypsin pretreatment. (b) As in (a), with trypsin pretreatment. (c) Fixed by immersion in 10% unbuffered formalin for 6 h at 25° C, no trypsin pretreatment. (d) As in (c), and with trypsin pretreatment. (a) Specimens fixed by the microwave method show specific, intense staining of epidermal keratins; (b) demonstrates some trypsin digestion and diminished specific staining; (c) and (d) show that formalin-fixed sections do not stain with this keratin antibody preparation, regardless of the use of trypsin. (a)–(d) × 350.

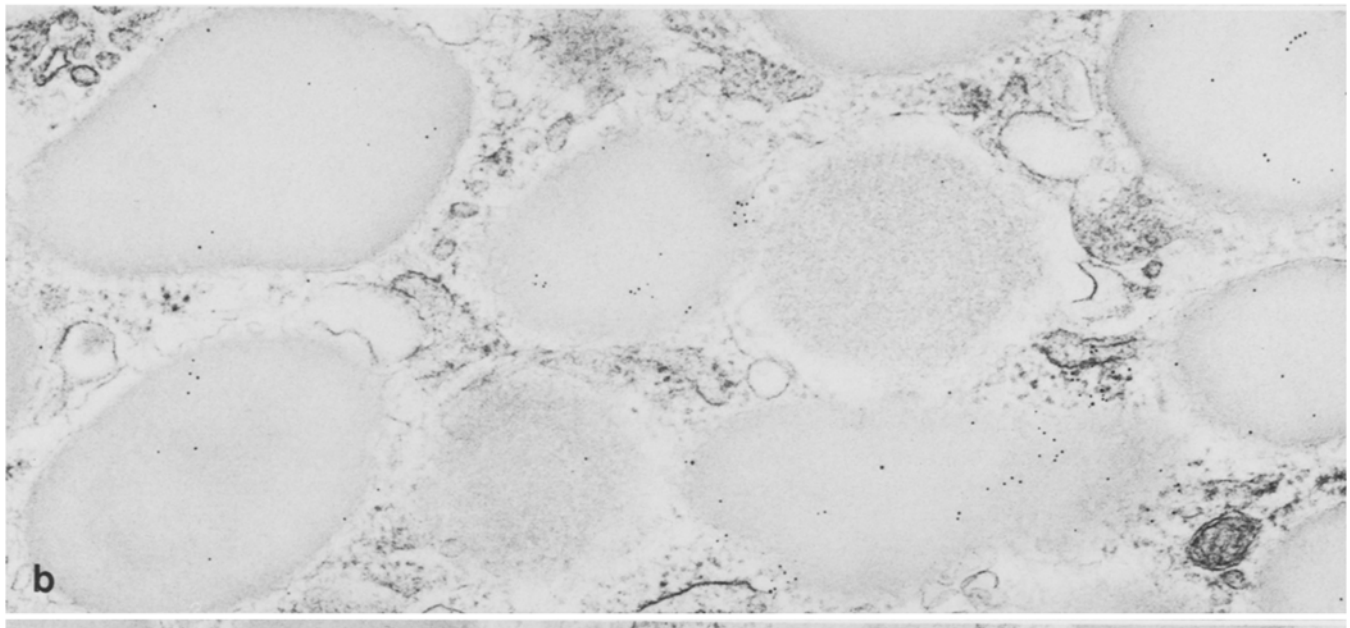
fast microwave method to this immunocytochemical investigation for the following three reasons. First, intact rat mast cells can spontaneously degranulate, thereby representing a challenging fixation model for the microwave method. Second, chymase is a hardy enzyme, resistant to aldehyde inactivation (Benditt & Arase, 1959) and tightly bound to insoluble heparin (Yurt & Austen, 1977), thus we could use routine electron microscopic methods to verify our microwave fixation results. Third, we are investigating fast intracellular processes (Dvorak *et al.*, 1985a, 1985b) in the mast cell models.

The finding that microwave-fixed skin is susceptible to trypsin digestion may provide insight into the mechanism of the microwave fixation method. The time required to unmask keratin antigens by trypsin incubation is directly proportional to the specimen's exposure time in the aldehyde fixative (Hautzer *et al.*, 1980; Mepham, 1982; Pinkus *et al.*, 1985; Battifora & Kopinski, 1986). Specimens fixed by the fast microwave method are limited to seconds of aldehyde exposure. We postulate that the degree of crosslinking of specimens immersed in the dilute aldehyde mixture (0.05% glutaraldehyde, 2% formaldehyde) during microwave irradiation results in limited but sufficient stabilization of proteins for excellent microscopic images.

We considered the possibility that ethyl alcohol exposure during processing may be responsible for fixation. We controlled for this by storing some of our specimens fixed by the microwave method (without additional aldehyde exposure) in physiological saline up to 2 weeks at 4° C. The morphology and immunoperoxidase staining results were equivalent to specimens immediately processed. Ethanol fixation for light microscopy generally requires 12–24 h (two to four times longer than our processing protocols), and results in considerable shrinkage, 20% or greater, compared to formalin-fixed tissue (Fox *et al.*, 1985; Battifora & Kopinski, 1986).

An important practical consideration of the microwave methods described herein is the environment in which samples are irradiated by microwave energy. Biological samples must be immersed in a solution to prevent desiccation (Gordon & Daniel, 1974) and loss of antigenicity (Chiu & Chan, 1986; G. R. Login, unpublished data) during irradiation. Microwave energy at a frequency of 2.45 GHz can penetrate approximately 1.5–2 cm into physiological salt solutions and biological specimens (Johnson & Guy, 1972;


11a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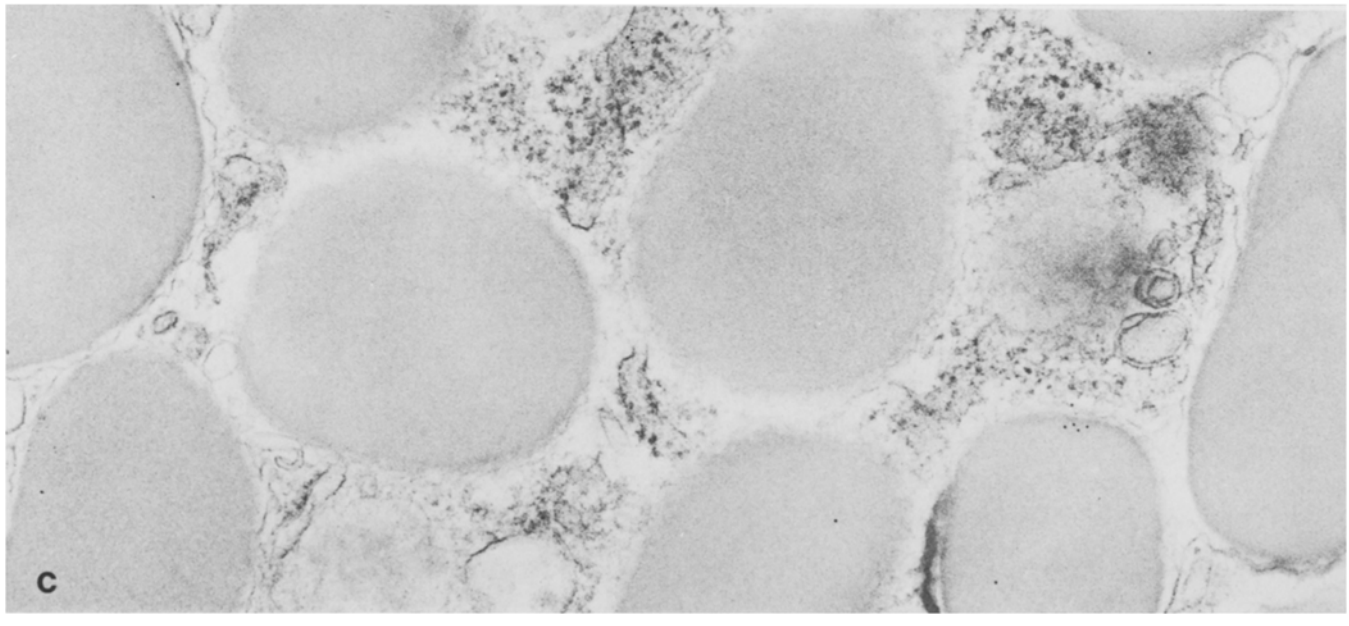
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McLees & Finch, 1973; Kok, 1986). Hand (1977) measured an 87% loss of microwave power in the centre of a tissue density sphere with a diameter of 17 mm. Therefore, techniques using containers or specimen blocks which exceed 1–2 cm in one dimension are probably being warmed slowly and unevenly by conductive heat. We use a glass container with overall dimensions of 15 cm in diameter by 2 cm in height for microwave fixation of batches of specimens in standard plastic tissue cassettes for light microscopy (Fig. 1).

Our microwave fixation methods enable specimens to be exposed to uniform maximum microwave power during irradiation. The temperature probe semiautomates the microwave fixation process. A manual method for determining the final irradiation temperature of the solution utilizes a heating curve and is described by Login (1978). The use of a rotating table during microwave irradiation has been suggested (Estrada *et al.*, 1985; Leong *et al.*, 1985). We have not found this apparatus necessary. The rotational speed of the platforms we tested may be too slow when compared to the brief irradiation/fixation times we recommend. The microwave units used in these studies have yielded reproducible fixation results over a period of years (Login, 1978; Login and Dvorak, 1985; Login *et al.*, 1986, 1987a,b).

The fast microwave fixation technique is modified for preservation of single samples by placing a beaker containing 300 ml of distilled water in the left rear corner of the irradiation chamber. If a small sample load (2 ml) is irradiated for 4 s in a microwave oven chamber without the water load, it can unpredictably reach a final solution temperature ranging between 25 and 50° C, depending on the warm-up time of the magnetron. The warm-up time of magnetron tubes in household grade microwave ovens is variable, between 2 and 4 s. Therefore, the water load increases the irradiation/fixation time by a few seconds but improves the reproductibility of the desired final solution temperature for a preselected irradiation time.

In conclusion, the microwave fixation methods described herein are simple techniques which are easy to perform. By virtue of the enhanced speed of fixation, these methods hold great promise for the demonstration of dynamic intracellular processes that have previously been undetectable by standard chemical and physical fixation methods. We have shown that the microwave methods preserve excellent architecture of tissue blocks and cells in suspension for study by light and electron microscopy. We have broadened the application of our fast method by demonstrating that it also preserves diagnostically useful membrane and intracellular antigens for detection by immunohistochemical techniques. In our chymase study, we demonstrated that our fast microwave fixation method, combined with immunocytochemistry, provides a powerful new approach for the subcellular localization of antigens.

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Fig. 11. Transmission electron micrographs of rat peritoneal mast cells fixed by the fast microwave method as in Fig. 8. (a) 5 nm immunogold labelling of antichymase antibody (1:600). (b) Competition experiment to demonstrate specificity of the immunogold technique. Thin sections of rat mast cells were incubated with free chymase (0.17 mg ml^{-1}) for 1 h at 4° C, followed by adding free chymase, the antibody to chymase (both diluted 1 : 600), and soybean trypsin inhibitor (0.70 mg ml^{-1}) for 24 h at 4° C. (c) As in (a) with substitution of the antibody to chymase with non-immune goat serum (1:600). In (a), immunogold labelling is specific to the granules. In (b), the competition study shows a marked reduction in specific label. (c) Demonstrates that non-specific sticking of the secondary immunogold reagent is negligible. (a)–(c) × 42 750. (Taken with permission from Login, G.R. *et al.*, *Lab Invest.* (1987) **57**, 592–599.)

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