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Preservation of neuronal ultrastructure in hippocampal slices using rapid microwave-enhanced fixation

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The goal of this study was to obtain fixation as rapidly as possible and to achieve preservation of neuronal ultrastructure to a depth in hippocampal slices where electrophysiological responses are optimal. This study demonstrates that perfusion quality preservation of in vitro hippocampal slices can be achieved within seconds after removal from the incubation chamber by using microwave (MW)-enhanced immersion in mixed aldehydes. The optimal method was determined to be MW irradiation of the slice for 8–11 s, to a tissue temperature of 35–50 °C, during immersion in fixative containing 6% glutaraldehyde and 2% paraformaldehyde. Electron microscopy of these slices revealed ultrastructural preservation that was comparable to hippocampi from animals perfused with mixed aldehyde fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde. Excellent ultrastructural preservation extended to 100–175 μm from the hippocampal slice surface after MW-enhanced fixation and therefore was much deeper than the 8–20 μm that can be obtained by rapid freezing. Hippocampal slices are routinely maintained in vitro for electrophysiological or pharmacological studies. This method of MW-enhanced fixation preserves tissue within seconds after removal from incubation, and should provide good preservation of the hippocampal anatomy that might be associated with in vitro physiology.

Introduction

Hippocampal slices are widely used to study neuronal responses to electrophysiological or pharmacological manipulation (e.g. Collingridge, 1985; Smith, 1987). To examine ultrastructural effects that might be associated with these responses, preservation of hippocampal slices has usually been achieved with immersion into fixatives for hours to days (Harris et al., 1980; Lee et al., 1980; Moshkov et al., 1980; Chang and Greenough, 1984; Petukhov and Popov, 1986; Reid et al., 1988). By standard aldehyde immersion at room temperature, the rate of fixation is largely dependent upon the volume of tissue (Hopwood, 1967). At this rate, preservation by immersion fixation of a brain slice that is a few hundred microns thick can require several hours to complete.

In order to evaluate ultrastructural correlates of physiologic events, fixation should be as rapid as possible. Rapid freezing to liquid helium temperatures has been employed in several studies to avoid the delay of conventional immersion fixation (Heuser et al., 1976; Ornberg and Reese, 1979; Wallace et al., 1987). Under optimal conditions, this method can result in good preservation to a depth of 10–20 μm beneath the frozen tissue surface. Below this depth, ice crystal artifact causes severe distortion of neuronal structure. Hippocampal slices fixed by this method have intact ultrastructure for only 8–15 μm below the surface exposed to the cold (Harris, unpublished observa-

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tions; Wallace, C.S., Crang, R.E., Chang, F.L.F. and Greenough, W.T., personal communication; Wallace et al., 1987). The best electrophysiological responses are obtained at a depth of 70–150 μm below the surface of an incubated hippocampal slice (Anderson et al., 1980). Thus, the ultrastructure at this depth is not well-preserved with rapid freezing methods. In order to obtain fixation that is not only rapid but also penetrates deeper into the slice, we have refined a method of microwave (MW) irradiation during aldehyde immersion. MW irradiation has been used to enhance aldehyde fixation for light microscopy (LM) and electron microscopy (EM) (Hopwood et al., 1984; Leong et al., 1985; Login and Dvorak, 1985, 1988; Login et al., 1986, 1987; Marti et al., 1987). With this method, small blocks of tissue are well-preserved within milliseconds to seconds (Login et al., 1986). The precise mechanism of the fixation achieved with MW irradiation during aldehyde immersion is not known. This MW-enhanced preservation does not appear to be solely due to the heat generated because heat-matched control tissue immersed in heated aldehydes is less well fixed when compared to tissue that is MW irradiated to the same temperature during immersion (Login and Dvorak, 1985). MW fixation is uniform throughout tissue blocks with volumes up to 1 cm^3 (Login and Dvorak, 1985), hence an in vitro hippocampal slice receives uniform exposure to MW irradiation throughout its 400 μm thickness.

We determined an optimal method of rapid immersion fixation for hippocampal slices maintained in vitro by evaluating several variables, including MW irradiation time and resulting tissue temperature, glutaraldehyde concentration, and duration of aldehyde postfixation after MW irradiation.

Materials and Methods

Tissue

Eleven male rats of the Long Evans Hooded strain and 60–61 days of age were sacrificed by cervical dislocation followed by severing the carotid arteries. The brain was rapidly removed from the skull and the left hippocampus dissected free. The hippocampal slices of 400 μm thickness were prepared and incubated in an interface chamber for 1.5 h using methods that have previously been shown to yield satisfactory electrophysiological responses (Harris and Teyler, 1984). A total of 45 slices were evaluated by LM, and of these, 33 were sectioned and evaluated for this study by EM.

For perfusion-fixed controls, hippocampal sections from 2 rats used in previous studies were used for comparison with the MW fixed slices (Harris and Landis, 1986; Harris and Stevens, 1987). These animals were anesthetized with intraperitoneal sodium pentobarbital and perfused with mixed aldehyde fixative (2.5% glutaraldehyde and 2% paraformaldehyde in 0.1 M sodium cacodylate buffer with 2 mM $CaCl_2$) at pH 7.35, 37 °C, and 4 psi for 15–30 min. The animals were left undisturbed for 1 h and then the left hippocampus was removed and sliced transversely to its long axis into 400 μm-thick blocks. The tissue was then postfixed in osmium, stained with uranyl acetate, dehydrated, and embedded in Epon according to the same protocol described below for the hippocampal slices.

Microwave irradiation and fixation

Optimal procedure. The procedure that yielded perfusion quality preservation of hippocampal

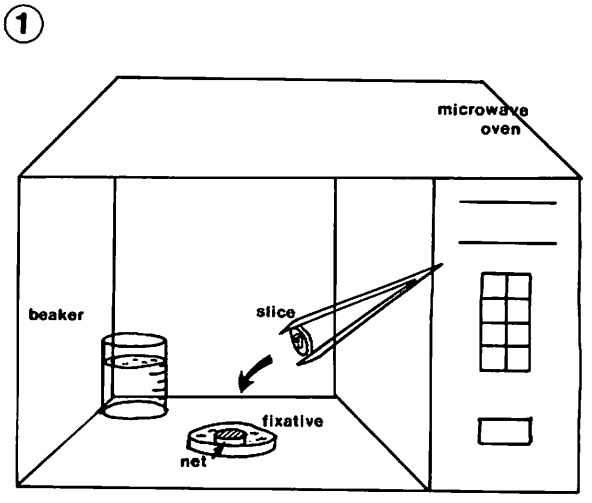


Fig. 1. Apparatus used for optimal method of MW-enhanced fixation. See text for explanation.

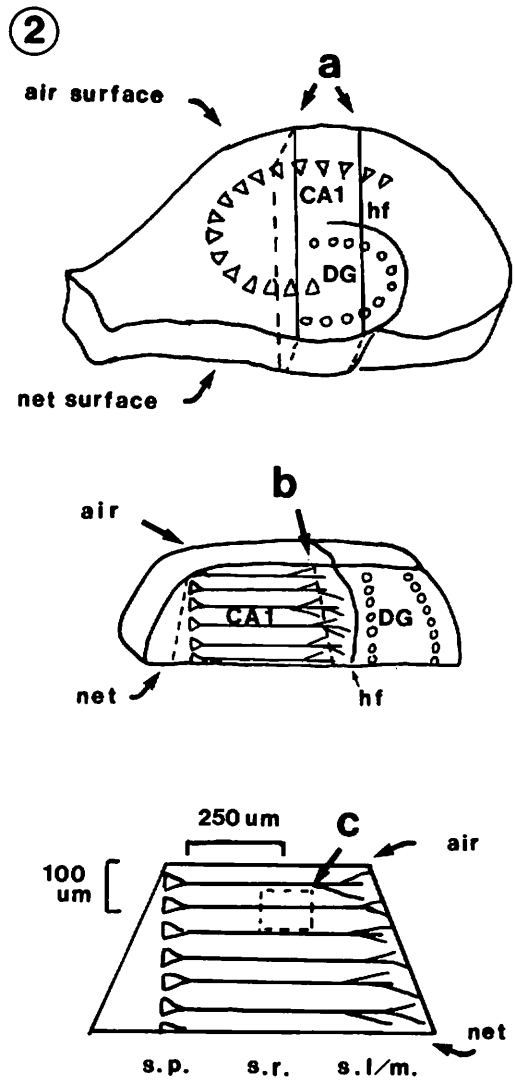


Fig. 2. Dissection of Epon-embedded slices for EM analysis. An initial cut (a) was made perpendicular to the slice surface, turned sideways, and remounted with rapid-bonding adhesive on an Epon blank so that the block face retained both the net and air surfaces. Examination of 1 μm-thick sections from this face guided the final trim (b) of the EM block face separating CA1 from the hippocampal fissure (hf) and dentate gyrus (DG). The EM face contained a part of CA1 and included stratum pyramidale (sp), stratum radiatum (sr), and stratum lacunosum/moleculare (sl/m) of area CA1. Sections of 0.07 μm thickness were cut from this surface. Areas approximately 125 μm^2 (c), located 100 μm below the air surface in stratum radiatum and 250 μm from the cell bodies, were photographed in the EM for ultrastructural and mitochondrial analysis.

slices is described here. First, it was necessary to calibrate the MW oven in order to determine the time required to bring a slice and fixative bath to specific temperatures. Irradiation of the slices was accomplished by rapidly transferring the slices, still on their nets, into a 35 mm polystyrene Petri dish located in the center of the floor of an Amana RS414 microwave oven (maximum power output 700 W at a frequency of 2450 MHz with a variable power setting). A beaker filled with 275 ml of distilled water preheated to 40–45 °C was placed in the corner of the oven as a water load to absorb reflected irradiation. A Digisense JKT thermocouple unit with type T needle probe was used to measure the temperature of the fixative before and after MW irradiation.

The Petri dish used in this procedure contained 5.5 ml of fixative preheated to a temperature of 31 °C to maintain slice temperature before fixation with MW irradiation. Optimal results were obtained from a fixative comprised of 6% glutaraldehyde and 2% paraformaldehyde and 2 mM $CaCl_2$ in 0.1 M sodium cacodylate buffer. The Petri dish contained a net platform made with a 15 mm diameter glass O-ring over which filter

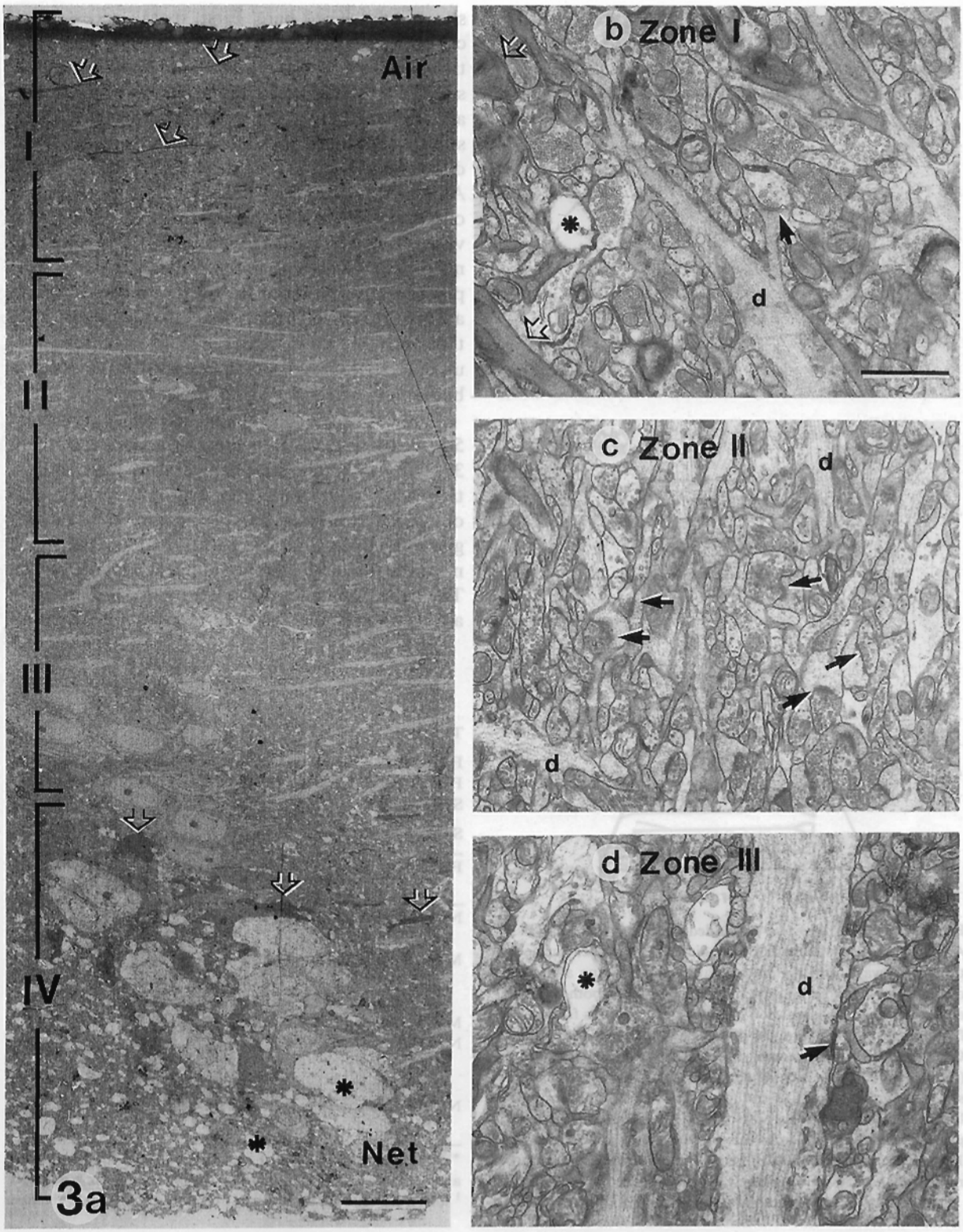
TABLE I

FIXATION CONDITIONS RANKED BY SCORING FOR FEATURES OF POOR ULTRASTRUCTURAL PRESERVATION

See text for explanation.

Fixation condition	n_A, n_s *	Quality score **
No MW, perfused with 2.5% GA fix [1] (Fig. 4)	2	0–0.2
MW irradiated to 35–50 °C in 6% GA fix, optimal procedure (Figs. 5, 6)	3, 3	0.2
MW 35–50 °C in 2.5% GA fix	1, 2	3.5
No MW, immersion in 6% GA fix ≥ 2 h (Fig. 7)	3, 3	0.8
< 2 h (Fig. 9)	1, 2	4.0
No MW, immersion in 2.5% GA fix 2 h	2, 2	4.5

* n_A, number of animals; n_s, number of slices. ** Mean score averaged from individual slices fixed by the same method. [1] %GA, percent glutaraldehyde in the mixed aldehyde fixative.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b Zone I
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c Zone II
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d Zone III
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paper (Norelco coffee filters, average hole diameter of 100 μm) was stretched and glued with a 24 h epoxy; the net platform was adhered to the bottom of the dish with silicone grease. The ring with the net holding the incubated slice was placed face down upon the net platform within the Petri dish, thereby sandwiching the slices between the 2 nets (Fig. 1). This procedure minimized mechanical trauma to the slice, and allowed equal exposure of both surfaces of a slice to the fixative solution. The time required for this transfer was 6–8 s. For optimal results, the MW oven was turned on for 8–11 s to yield a postirradiation temperature of 35–50 °C. Thus the total time from removal of the slice to completion of fixation was 14–19 s. Immediately after MW irradiation, the net containing the slice was rapidly transferred to 0.1 M sodium cacodylate buffer at room temperature.

Other fixation procedures. For small tissue blocks and hippocampal slices, immersion fixation in mixed aldehydes has usually been accomplished at room temperature (Hayat, 1981; Chang and Greenough, 1984). In this study, control slices were placed in vials containing fixative with 2.5% or 6% glutaraldehyde at room temperature for 2 h or 14–16 h (overnight). Then these slices were rinsed in cacodylate buffer for 1–2 h, and processed for EM (see below). In addition, 2 within-animal control slices were immersed in fixative containing 6% glutaraldehyde in the absence of MW irradiation for 11 s or 60 s, although there is no evidence in the literature that such short immersion times should result in optimal fixation. No heat-matched controls were prepared as heat alone has previously been reported to give suboptimal fixation compared with MW-enhanced immersion (Login and Dvorak, 1985). Non-incubated control slices from the same animals were also prepared for fixation in order to distinguish ultrastructural changes due to tissue chopping and incubation from those due to fixation artifacts.

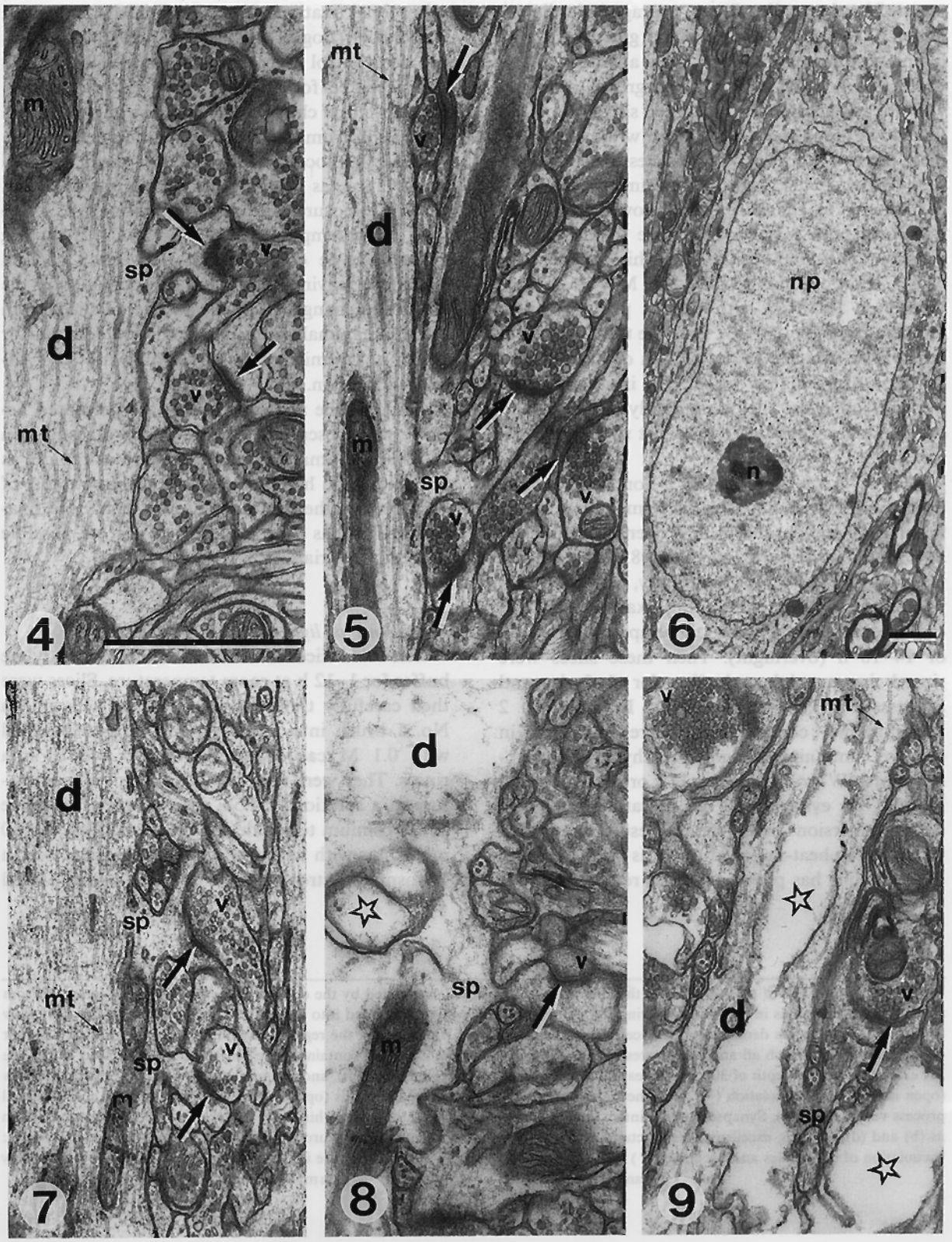
Other protocols which did not yield as good preservation as the optimal procedure included: (1) MW exposure for longer durations (18–27 s) to yield tissue temperatures of 50–92 °C; (2) varying glutaraldehyde concentration between 2.5% and 6% while leaving the other components of the fixative unchanged; and (3) additional slices from the same animals were postfixed in aldehydes for 1–2 h or overnight (14–16 h) following MW enhanced fixation.

Some of the above protocols are presented because they describe the path of our investigation to obtain optimal tissue preservation as rapidly as possible, and highlight some of the pitfalls of variations in the technique of MW-enhanced fixation. There has been no effort to test all possible procedural variations.

Processing for light and electron microscopy

The fixed slices were stored in 0.1 M cacodylate buffer for 1–12 h at room temperature. Slices were then carefully transferred from their nets with a No. 1 brush into Pyrex sample vials and rinsed with 0.1 M cacodylate buffer for five 10 min rinses. They were then postfixed at room temperature in a solution of 1.5% potassium ferrocyanide in 1% osmium tetroxide for 1 h. Following five 10 min rinses with buffer, the slices were immersed in 1% osmium tetroxide for 1 h, rinsed again, placed

Fig. 3. Low power EM of the full 400 μm thickness of a hippocampal slice, fixed by the optimal procedure and photographed from an EM face prepared as in Fig. 2c. a: varying ultrastructural preservation is divided into zones: Zone I, extending 40–60 μm below the air surface (Air) has dendrites of dark cells (open arrows); Zone II, occupying the region between 50 and 150 μm below the air surface and within which all analysis of preservation quality was done; Zone III, containing the central core of the slice; and Zone IV, extending from a depth of 300 μm beneath the air surface to the net surface (Net), and containing dark cell bodies and processes (open arrows) and vacuolation (*). b: higher power view of Zone I, where dark cells (open arrows) are frequent and an occasional process vacuolation (*). Synapses appear intact (closed arrow). d, dendrite. c: field within Zone II, taken at the same magnification as (b) and (d), showing excellent ultrastructural preservation of all elements of the neuropil, including synapses (closed arrows). d: vacuolation of cell bodies and processes (*) is most severe in Zone IV, but synapses are intact (closed arrow). Figs. b, c and d show Zones I, II, and III, respectively. Calibration bars: a, 20 μm; b-d, 1 μm.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in 50% EtOH for 10 min, then stained en bloc with 4% uranyl acetate in 70% EtOH for 1 h. The tissue was subsequently dehydrated with increasing EtOH concentrations, rinsed in propylene oxide, then placed in 50 : 50 propylene oxide : Epon and rotated overnight. Slices were then embedded in Epon and cured at 60 °C for 48 h.

The embedded tissue was trimmed and remounted to obtain a block face that was a cross-section of the 400 μm thickness including both surfaces of the slice (Fig. 2a,b). One micron-thick sections were cut and mounted on glass slides and stained with 1% alkaline Toluidine blue for light microscopy (LM). Further trimming of the block was guided by these sections to obtain a block face for electron microscopy (EM) of 0.2 mm × 0.8 mm spanning stratum pyramidale and stratum radiatum of area CA1 (Fig. 2b,c). Blocks were sectioned at 0.06–0.08 μm thickness with a sapphire knife (LKB Saphatome SS-45) on a Reichert Ultracut E ultramicrotome, placed on Formvar-coated slot grids (Synaptek), and stained with Reynold's lead citrate for 5 min. The sections were viewed and photographed at 2000–15 000 × at 100 kV on JEOL 100B, 100S, and 1200EX electron microscopes.

Evaluation of ultrastructural preservation

All slices were evaluated for ultrastructural preservation within the same region, an area centered in stratum radiatum at 200–250 μm from the CA1 pyramidal cell bodies and 100 μm below the air surface (Fig. 2c). An initial EM comparison of perfusion-fixed hippocampus with in vitro slices fixed by immersion at room temperature revealed several ultrastructural features associated with poor preservation. These included: (1) vacuolation within mitochondria; (2) vacuolation within neuronal and glial processes; (3) presynaptic vesicle clumping; (4) cytoplasmic membrane breakdown; and (5) microtubular disruption. Electron micrographs were printed at the same magnification from the perfused hippocampi and all slice fixation conditions. The micrographs were then coded and viewed without knowledge of the fixation condition. Each micrograph was evaluated for the presence of each of these 5 ultrastructural features associated with poor preservation. The evaluation

Fig. 4. A CA1 dendrite (d) in stratum radiatum from a perfused hippocampus. Continuous microtubules (mt, small arrow), mitochondria (m), and a dendritic spine (sp) are present. Synapses (large arrows) have intact membranes, postsynaptic densities, and presynaptic axonal vesicles (v). Cellular process vacuolation is not present. Calibration bar = 1 μm, for Figs. 4, 5 and 7–9.

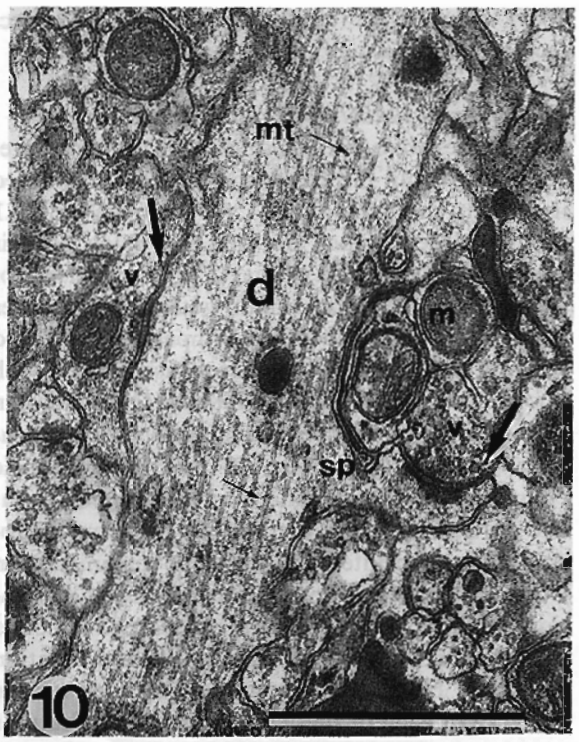
Fig. 5. A CA1 dendrite (d) in stratum radiatum from a slice fixed by the optimal MW procedure. A continuous microtubules (mt, small arrow) and intact mitochondria (m), spine (sp), and several synapses (large arrows) are labelled. Synapses reveal good preservation with intact membranes, postsynaptic densities and presynaptic axonal vesicles (v). Vacuolation is not seen within either mitochondria or cytoplasmic compartments.

Fig. 6. A CA1 pyramidal cell body from a slice fixed by the optimal MW procedure. The nucleoplasm (np) is homogeneous, and contains a distinct nucleolus (n), cytoplasmic and nuclear membranes are intact, and the cytoplasm and mitochondria are not vacuolated. Calibration bar = 1 μm.

Fig. 7. A CA1 dendrite (d) in stratum radiatum from a slice fixed by overnight immersion without MW irradiation in fixative containing 6% glutaraldehyde and 2% paraformaldehyde. Two spines (sp) protrude from the dendrite, and reveal synapses (large arrows), with intact synaptic membranes, postsynaptic densities, and vesicles (v). The microtubules (mt) are less distinct than in Figs. 4 and 5.

Fig. 8. A CA1 dendrite in stratum radiatum from a control slice fixed for 2 h in a solution containing 6% glutaraldehyde and 2% paraformaldehyde without MW irradiation. This dendrite (d) has vacuolation (star). Spine (sp) and synaptic structures (large arrow) are intact and membrane breakdown is minimal.

Fig. 9. A CA1 dendrite in stratum radiatum from a control slice immersed in fixative containing 6% glutaraldehyde and 2% paraformaldehyde for 11 s without MW irradiation. Severe vacuolation (star) is present within the dendrite (d), as well as extensive disruption of membrane integrity. A spine (sp) possesses a synapse (large arrow), with intact postsynaptic density and synaptic membranes. At another synapse, vesicles (v, upper left) are clumped inside the presynaptic terminal.



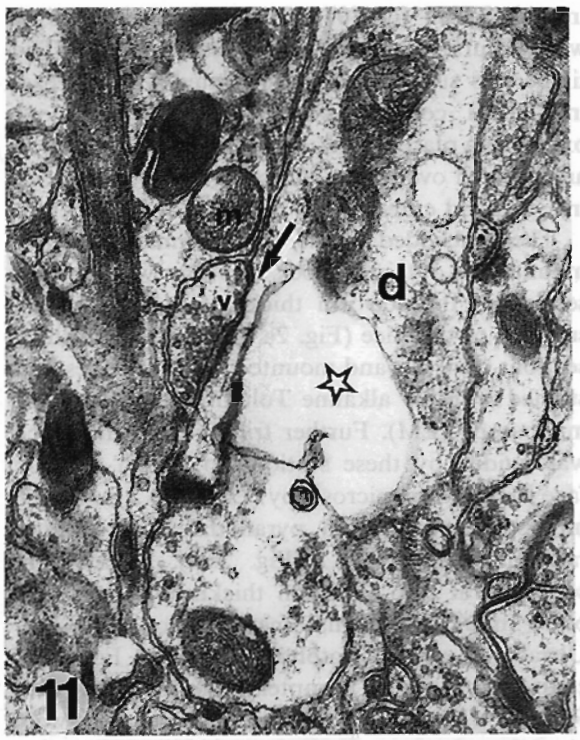


Fig. 10. A CA1 dendrite (d) with a spine (sp) from a slice fixed with MW irradiation during immersion in fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde. The field location is 100 μm below the air surface of the slice and approximately 250 μm into stratum radiatum from the pyramidal cell bodies. While fixation was variable with this method, this particular field reveals good quality preservation: membranes, mitochondria (m), microtubules (mt, small arrows), and synapses (large arrows) and their presynaptic axonal vesicles (v) are intact. Calibration bar = 1 μm.

Fig. 11. A CA1 dendrite (d) in stratum radiatum is located within the same section and in a field adjacent to that in Fig. 10. This photomicrograph demonstrates the variability that could be found in tissue fixed using fixative containing 2.5% glutaraldehyde. Vacuolation (star) is present within the dendritic cytoplasm.

was graded on a scale of 0 to 1.0, with 0 being assigned if the feature was not seen, 0.5 if it was occasionally seen, and 1.0 if it was frequently seen. The total score thus obtained ranged between 0 and 5 for an individual micrograph. An overall score was obtained for each fixation condition by calculating the average score from 1–6 micrographs from slices fixed under the same condition.

Quantitative evaluation of mitochondria for shrinkage

Mitochondrial size has been shown in other preparations to be sensitive to changes in osmolarity (Hayat, 1981). In the present study, mitochondrial cross-sectional areas were measured to assess whether the increased osmolarity of the 6% glutaraldehyde concentration used in the optimal procedure could affect tissue volume. For this analysis, areas of mitochondria cut in cross-section in hippocampi fixed by perfusion with fixative containing 2.5% glutaraldehyde concentration were compared to slices microwaved in 6% glutaraldehyde; the experimenter was blind to the fixation conditions. Perfused tissue was chosen instead of control in vitro slices fixed with 2.5% glutaraldehyde because preservation was inhomogeneous in the latter condition (see Results). A total of 20 fields, each approximately 125 μm^2 were analyzed: 5 fields per animal, from 2 animals

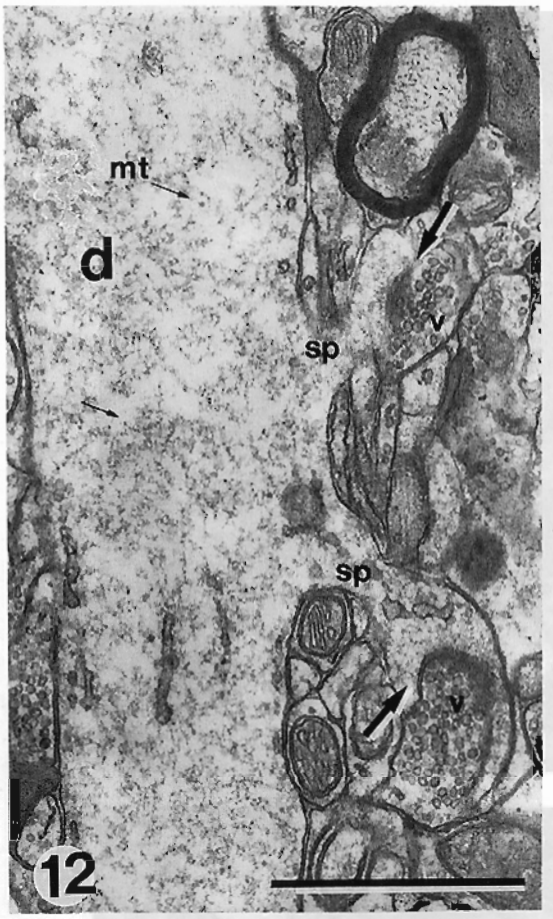


Fig. 12. A CA1 dendrite (d) from a slice irradiated to 64.8°C during immersion in fixative containing 6% glutaraldehyde and 2% paraformaldehyde. This dendrite shows disruption of microtubules (mt, small arrows). Other structures, including mitochondria, cellular membranes, spines (sp), and synapses (large arrows) were well-preserved. Calibration bar = 1 μm.

of the MW group and 2 animals that were perfused. Cross-sectioned mitochondria were identified as those which were bounded by a clear double membrane for at least 3/4 of their circumference and contained well-defined cristae membranes. Mitochondrial images from electron micrographs were captured by video camera, and viewed on a Gould image processing system interfaced to a VAX 780. Cross-sectional areas were measured using a digitizing bitpad to trace the mitochondrial outer membranes, and compute the enclosed areas (Pearlstein et al., 1986). The RS1 statistical package (Bolt, Beranek, and Neuman, Cambridge, MA) was used to calculate mean mitochondrial areas and S.D. for each group and a one-way ANOVA was performed to determine the effect of fixative concentration on mitochondrial areas.

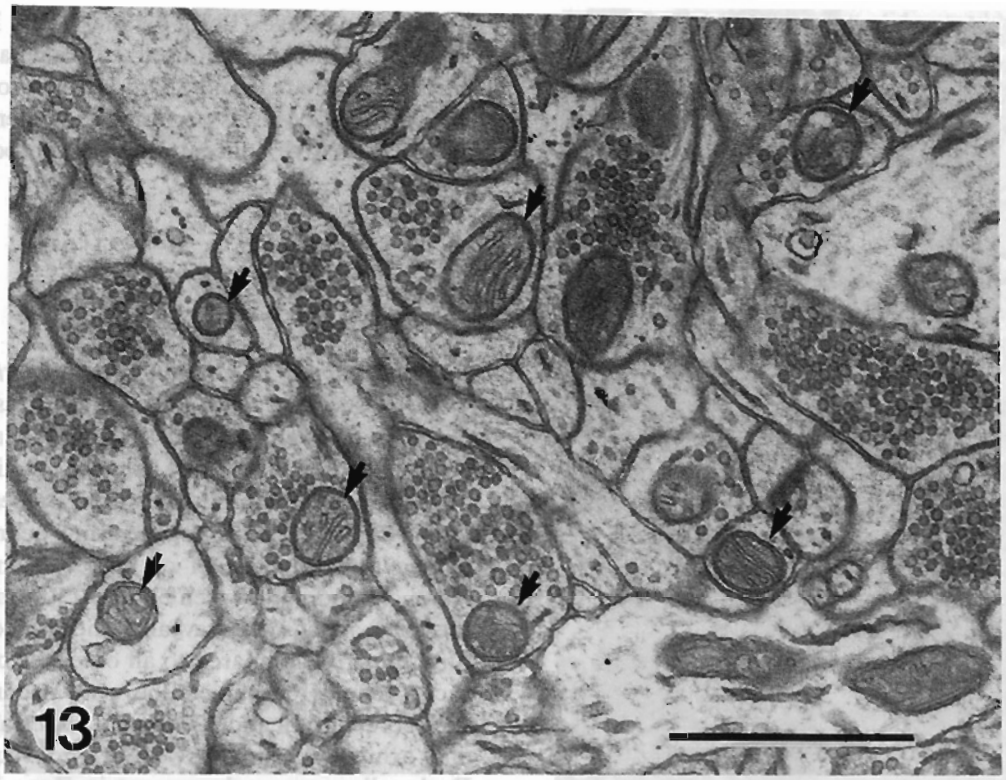
Results

EM evaluation of ultrastructural preservation

In all slices, 4 zones of varying ultrastructural preservation were present. There was a narrow layer extending 40–60 μm beneath the air surface of the slices containing somal and process vacuolation and occasional degenerating neurons with dark cytoplasm (Zone I of Fig. 3). Zone II, the region extending between 50 and 150 μm beneath the slice air surface, revealed the greatest structural integrity compared to all other regions of the slice. Zone III occupied the central core of the slice between 150 and 300 μm below the air surface and exhibited cellular swelling and vacuolation. Dark cells were rarely present in Zone III. Zone IV was continuous with Zone III and extended from 300 μm below the air surface to the net surface. Zone IV exhibited severe vacuolation and membrane breakdown as well as dark cells.

The non-incubated slices differed only in the extent of vacuolation in Zone I. In these slices, vacuolation extended to depths of 75 μm beneath the air surface, deeper than the 40–60 μm observed in the incubated slices.

Effect of microwave irradiation. Table I compares 5 of the fixation conditions with respect to the frequency of each feature of poor preservation, where a low score indicates good preservation. In hippocampal slices, the best ultrastructural preservation was obtained by immersion of the slices in fixative containing 6% glutaraldehyde during MW irradiation for 8–11 s. Ultrastructural preservation in Zone II of these slices was equivalent to that in perfused hippocampi (Figs. 4, 5 and Table I). Throughout Zone II, all slices processed according to this optimal procedure showed excellent preservation of dendritic, synaptic, and axonal structure. The cell bodies were also well-preserved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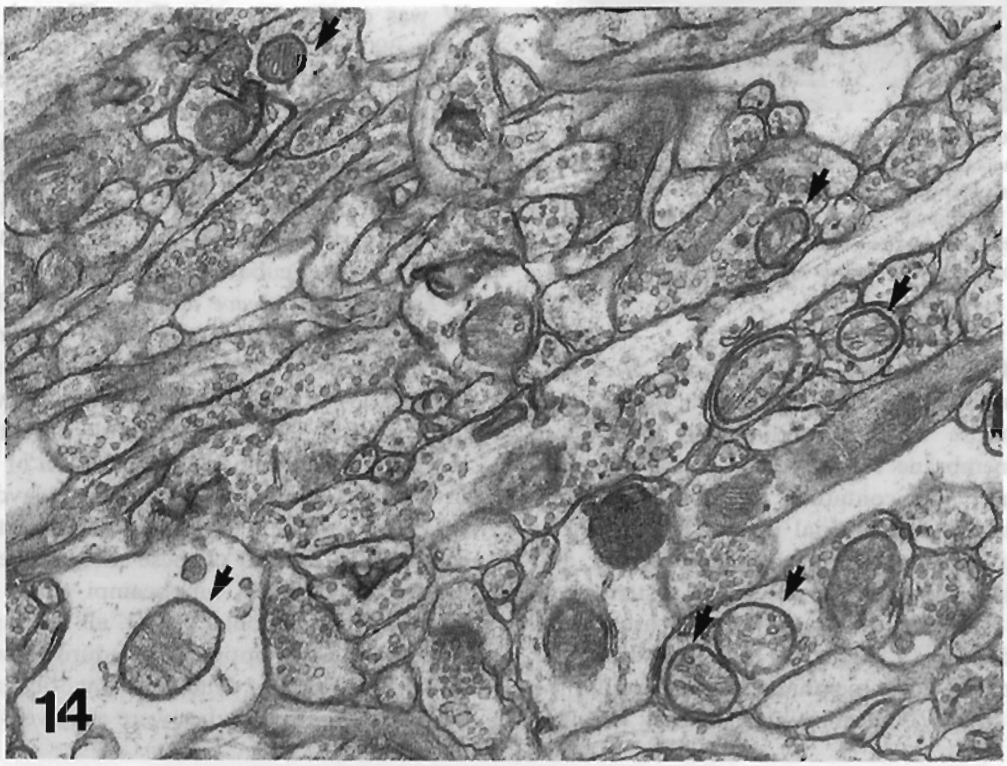
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in Zone II, with distinct nucleoli, evenly distributed chromatin, intact nuclear and cytoplasmic membranes, and no vacuolation in the perikaryal cytoplasm or mitochondria (Fig. 6).

To determine whether the MW irradiation was important for the enhanced quality of preservation, several control slices from the same animals were immersed without MW irradiation in fixative containing varying concentrations of glutaraldehyde at room temperature (Table I). Immersion in fixative containing 6% glutaraldehyde for 14–16 h (overnight) resulted in ultrastructural preservation approaching that achieved by the optimal MW enhanced procedure (Fig. 7). However, even these control slices had subtle changes in microtubule structure; the microtubules in Zone II were less distinct than those found in perfused hippocampi or slices prepared by the optimal MW procedure. In 2 slices, immersion time was only 2 h in fixative containing 6% glutaraldehyde without MW irradiation, and this resulted in more frequent vacuolation of mitochondria and cellular processes in Zone II (Fig. 8). Two control slices that were not MW-irradiated, but immersed in the same fixative at room temperature for 11 s (Fig. 9) or 60 s resulted in very poor preservation of neuronal structure, with vacuolation and severe membrane breakdown throughout all zones.

Effect of glutaraldehyde concentration. A concentration of 6% glutaraldehyde in the fixative resulted in better preservation under all conditions tested in the slice. MW irradiation of 2 slices to 35–50 °C during immersion in fixative containing a glutaraldehyde concentration of 2.5% did not yield optimal ultrastructural preservation (Table I). The main defect was inhomogeneous preservation occurring within Zone II at 75–125 μm below the air surface; some areas were well-preserved but many adjacent areas showed cytoplasmic and mitochondrial vacuolation within cell processes (Figs. 10, 11).

Effect of postfixation in aldehydes. In 4 slices fixed during MW-enhanced immersion in the 6% concentration of glutaraldehyde, aldehyde postfixation for 2 h or overnight following MW irradiation did not further enhance preservation. Moreover, microtubules from the postfixed slices were not as distinct as in the slices that were only exposed to fixative during MW irradiation. This subtle change was not reflected in the scoring, which only rated the presence or absence of microtubules. For slices fixed and MW-irradiated in a solution with 2.5% glutaraldehyde, postfixation for 1–2 h in the same fixative did result in less membrane breakdown. Despite this improvement, preservation of these postfixed slices was still inferior to the quality obtained by the optimal MW procedure in which there was no postfixation.

Effect of temperature. Increasing the time of exposure to MW irradiation, and hence the temperature obtained in the tissue, did not improve tissue preservation. At comparable glutaraldehyde concentrations, higher temperatures (50–92 °C) resulted in ultrastructural preservation that was inferior to that obtained at 35–50 °C. The principle defect in all 9 slices fixed at higher temperatures was severe disruption of microtubules (Fig. 12).

Quantitative evaluation of tissue shrinkage

Measurements of cross-sectional areas were made of 166 mitochondria from 2 animals ($n = 58$, $n = 108$) that had been perfused with fixative containing 2.5% glutaraldehyde, and of 148 mitochondria in slices from 2 animals ($n = 65$, $n = 83$) fixed with the optimal MW procedure. A portion of a field within Zone II from the perfused group is

Fig. 13. Part of one field that was used for mitochondrial area measurements from hippocampi perfused with fixative containing 2.5% glutaraldehyde and 2% paraformaldehyde. Arrows show mitochondria which are cross-sectioned and thus met criteria for inclusion in the analysis. Mitochondria that were cut obliquely were not measured. Calibration bar = 1 μm.

Fig. 14. Part of one field that was used for mitochondrial area measurements from slices fixed with the optimal MW procedure. Arrows indicate mitochondria which were cross-sectioned and included in the analysis.

shown in Fig. 13, and one from a slice in the MW group is shown in Fig. 14. The mean area for mitochondria from the perfused animals was 0.052 $\pm$ 0.02 μm^2. The mean area for the MW group was 0.048 $\pm$ 0.02 μm^2. Four swollen mitochondria, with areas greater than 0.14 μm^2, were excluded from the MW group because they occurred in neurons with dark cytoplasm or in glia. No intact mitochondria were found in dark cells. Intact mitochondria found in glia were included. No significant difference in mitochondrial cross-sectional area was found between the perfused tissue and the MW slices.

Discussion

These results demonstrate that ultrastructural preservation of in vitro hippocampal slices can be achieved by MW-enhanced immersion with much greater speed than by conventional immersion at room temperature. The process of fixation was complete within 14–19 s from the time that the slice was removed from the tissue chamber; post-fixation did not improve preservation achieved by the optimal MW procedure. MW-enhanced immersion results in perfusion quality preservation, and is more rapid than that obtained with conventional immersion. Ultrastructural preservation after MW irradiation was to depths where previous studies have shown optimal electrophysiological responses, and is deeper than that obtained by rapid freezing.

Mixed aldehydes containing a concentration of 6% glutaraldehyde resulted in better preservation deeper into the slice (Zone II) under all conditions than a 2.5% concentration. This observation suggests that the higher concentration of glutaraldehyde facilitates the penetration of fixative into the slice. Neither the higher 6% glutaraldehyde concentration nor the MW irradiation caused mitochondrial shrinkage compared to perfusion-fixed tissue. Neuronal cell body areas and total area of brain slices have previously been shown to be unaffected after immersion in 4% or 5% glutaraldehyde, similarly suggesting no shrinkage effect at higher glutaraldehyde concentrations (Hillman and Deutsch, 1978). Login and Dvorak (1985) also found no size change in mitochondria and granules in non-CNS tissue following MW enhanced immersion when compared to conventional aldehyde immersion.

A long exposure to fixative, a high concentration of glutaraldehyde, or a high tissue temperature seemed to have a damaging effect on the microtubules. Previous studies have also reported that prolonged exposure to glutaraldehyde at low temperatures between 0 and 25 °C is associated with loss of microtubules (Langenberg, 1980). We found that the best ultrastructural preservation was obtained when the temperature range after MW irradiation of hippocampal slices was kept to 35–50 °C. This is similar to the range of 50 $\pm$ 5 °C that has previously been demonstrated as optimal for MW-enhanced fixation of non-CNS tissue (Login and Dvorak, 1985).

Several ultrastructural characteristics of the slice were more likely to be caused by tissue handling prior to fixation, rather than to occur from fixation artifact. All slices showed vacuolation and dark cells adjacent to the cut surfaces of the slice (Zones I and IV), and moderate process vacuolation in the central core of the slice (Zone III). Similar neuronal pathology has been observed in other studies of in vitro slice morphology (Cohen, 1962; Bak et al., 1980). It has been suggested that the pathology in the outer layers beneath each surface of the slice may be caused by trauma to neuronal processes during chopping, since it has been shown in cerebellar slices to be reduced when the slices are hand cut (Garthwaite et al., 1979). Anoxia has been proposed to be the cause of vacuolation in the central core of the slice (Bak et al., 1980; Whittingham et al., 1984). Slices rapidly fixed with MW in the present study also had central core vacuolation, further suggesting that much of this change might be due to anoxia during slice preparation and incubation, and not due to anoxia caused by delay of fixation which was minimized to seconds. Finally, the more intact ultrastructure below the air surface (Zone I) of the incubated slices compared to the non-incubated slices prepared by the same optimal procedure suggests that some recovery took place during incubation. This observation is consistent with physiological and metabolic data showing

that recovery to steady state can take up to 2 hours after chopping (Anderson et al., 1980; Whittingham et al., 1984).

In summary, this study demonstrates that MW-enhanced fixation of hippocampal slices produces perfusion quality preservation in less than 11 seconds. This rapid preservation can minimize the introduction of artifactual changes due to autolysis or anoxia, thereby improving the opportunity for obtaining accurate quantitative measurements in the study of anatomical effects associated with in vitro hippocampal physiology.

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