Retrograde degeneration of neurite membrane structural integrity of nerve growth cones following *in vitro* exposure to mercury

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Inhalation of mercury vapor (Hg^0) inhibits binding of GTP to rat brain tubulin, thereby inhibiting tubulin polymerization into microtubules. A similar molecular lesion has also been observed in 80% of brains from patients with Alzheimer disease (AD) compared to age-matched controls. However the precise site and mode of action of Hg ions remain illusive. Therefore, the present study examined whether Hg ions could affect membrane dynamics of neurite growth cone morphology and behavior. Since tubulin is a highly conserved cytoskeletal protein in both vertebrates and invertebrates, we hypothesized that growth cones from animal species could be highly susceptible to Hg ions. To test this possibility, the identified, large Pedal A (PeA) neurons from the central ring ganglia of the snail *Lymnaea stagnalis* were cultured for 48 h in 2 ml brain conditioned medium (CM). Following neurite outgrowth, metal chloride solution (2 μl) of Hg, Al, Pb, Cd, or Mn (10^{-7} M) was pressure applied directly onto individual growth cones. Time-lapse images with inverted microscopy were acquired prior to, during, and after the metal ion exposure. We demonstrate that Hg ions markedly disrupted membrane structure and linear growth rates of imaged neurites in 77% of all nerve growth cones. When growth cones were stained with antibodies specific for both tubulin and actin, it was the tubulin/microtubule structure that disintegrated following Hg exposure. Moreover, some denuded neurites were also observed to form neurofibrillary aggregates. In contrast, growth cone exposure to other metal ions did not effect growth cone morphology, nor was their motility rate compromised. To determine the growth suppressive effects of Hg ions on neuronal sprouting, cells were cultured either in the presence or absence of Hg ions. We found that in the presence of Hg ions, neuronal somata failed to sprout, whereas other metalic ions did not effect growth patterns of cultured PeA cells. We conclude that this visual evidence and previous biochemical data strongly implicate Hg as a potential etiological factor in neurodegeneration. *NeuroReport* 12:733–737 © 2001 Lippincott Williams & Wilkins.

Key words: Mercury; Microtubules; Neurite growth cone; Neurodegeneration; Neurofibrillary aggregates; Tubulin

INTRODUCTION

Growth cones located at the tip of developing and regenerating neurites are responsible for neurite extension, axonal pathfinding and target cell selection in the nervous system. Actin and tubulin that comprise the bulk of growth cone cytoskeleton are highly sensitive to various environmental cues that are present in the extracellular milieu of growth cones. A growth permissive environment facilitates growth cone assembly whereas various growth inhibitory molecules dissemble microtubular structure, induce growth cone collapse and neurite retraction [1]. Microtubules, a principal protein of the cytoskeleton, are composed of polymerized tubulin dimer subunits. Brain neurons require intact microtubules for axoplasmic transport, membrane structure, and normal neurite outgrowth; the cytoskeletal architecture being dependent upon microtubular stability [2,3]. Methylmercury (MeHg) is a potent neurotoxicant, and its effects on microtubule integrity during CNS neuronal development are well documented [4].

Attention has also focused on potential CNS toxicity resulting from chronic exposure to another predominant toxic mercury species, that of mercury vapor (Hg^0); the principal source being dental amalgam tooth fillings [5]. Approximately 70% of all Hg ions in human urine originate solely from amalgam [6]. Recently, we have reported that inhalation exposure of rats to Hg^0 causes disruption of brain microtubule metabolism by inhibiting the polymerization of tubulin molecules. Such polymerization is dependent upon the ability of GTP nucleotide to bind to β-tubulin, binding that is markedly reduced by the presence of Hg ions. A similar *in vivo* molecular lesion was observed in brains of 80% of Alzheimer disease (AD) patients, but

was not seen in brains from age-matched control patients [7].

Since the amino acid sequence of tubulin from all animals brains (vertebrates and invertebrates) is highly conserved, with >97% sequence homology across animal species [8], the present investigation employs a well-established snail neuronal culture model [9] to study microtubule metabolism in the presence of Hg. The development of time-lapse imaging techniques for intact isolated neurons, using cell culture systems, has allowed the direct observation of axonal microtubule structure and protein synthesis at the neurite growth cone [10,11]. Therefore, the primary objective of the present study was to determine whether the marked inhibition in microtubule metabolism following Hg^0 exposure, as measured at the molecular level [7], could actually be directly observed by imaging the membrane dynamics of neurite growth cone activity in the presence or absence of Hg ions or other toxic heavy metals.

MATERIALS AND METHODS

Animals: An established stock of the fresh water snail *Lymnaea stagnalis* derived from that of the Department of Biology at the Free University of Amsterdam was used. Animals were maintained in an aerated, filtered pond water aquarium at room temperature in the University of Calgary Animal Resources Centre and were fed lettuce as described by Ridgway et al. [12]. In all experiments, central ring ganglia were used for neuronal cell isolation and to make brain conditioned media (CM). Snails with a shell length of 25–30 mm (3–4 months old) were used in all experiments.

Cell culture: Animals were de-shelled and anesthetized for 10 min in normal *Lymnaea* saline ((in mM): 51.3 NaCl, 1.7 KCl, 4.0 $CaCl_2$ and 1.5 $MgCl_2$; buffered in HEPES to pH 7.9) containing 10% Listerine. All primary cell culture procedures from this point forward were carried out in a laminar flow hood to prevent infection of culture samples from air-borne microorganisms. Anesthetized snails were pinned down in a dissection dish containing antibiotic saline (ABS) (autoclaved normal *Lymnaea* saline; gentamycin 150 μg/ml) and their CNS removed as described previously [9,12]. The isolated central ring ganglia were washed in plastic culture dishes (Falcon; Becton Dickinson, Meylan Cedex, France; 35 × 10 mm) containing ABS to ensure an aseptic culture [9]. Three consecutive 10–15 min washes were completed, each in a culture dish containing 3 ml ABS. Brains were then transferred into a culture dish with 3 ml defined media (DM; 50% L-15 medium with added inorganic salts (in mM): 40 NaCl, 1.7 KCl, 4.1 $CaCl_2$, 1.5 $MgCl_2$, 10 N-2 hydroethyl-piperzine-n′-2-ethanesulfonic acid, pH 7.9; and 20 μM gentamycin; (Gibco BRL, Gaithersburg, MD; special order) containing 6 mg trypsin (2 mg/ml to yield a 0.2% volume solution; Type T-4665; Sigma, St. Louis, MO), and left at room temperature (18–20°C) for 23 min. Following this enzyme treatment, the central ring ganglia were placed into a 0.2% volume trypsin inhibitor (Type I-S; Sigma)/DM solution and left for 15 min. The brains were then transferred to a dissection dish containing high osmolarity DM (750 μl of 1 M glucose added to 20 ml DM to yield a 180–190 mOsm solution) and pinned down dorsal surface up. Fine forceps were used to remove the outer and inner connective tissue sheathes surrounding each ganglion. A Sigmacote (Sigma)-treated glass capillary pipette was attached to polyethylene tubing and sterilized with 70% ethanol for 5 min. Following this sterilization, a micro-syringe (Gilmont, Model GS-1100) was connected to the tube and the pipette/tubing/syringe system rinsed thoroughly with ABS prior to being filled with high osmolarity DM. A micromanipulator was used to maneuver the pipette tip overtop a Pedal A (PeA) neuron cell body and gentle suction pressure was applied through the micro-syringe to isolate the neuron from its ganglion. This PeA neuron was then gently flushed into a poly-L-lysine coated glass coverslip/culture dish [12,13] containing brain conditioned media (CM, described below). Three to five neurons were plated ~5–10 soma diameters apart per dish and were left undisturbed overnight to allow for cell attachment and neurite outgrowth.

To prepare CM, 12 isolated central ring ganglia, washed seven times in ABS, were incubated in Sigmacote-treated glass culture dishes containing 6 ml DM for 3 days as described by Wong *et al.* [13]. These ganglia were then removed from the culture dish and the CM (first time) was discarded. The ganglia were incubated for an additional 4 days in fresh DM and removed. This medium (second time) was filtered (0.22 μm pore; Nalgene) and placed in a poly-L-lysine-coated plastic culture dish. The ganglia were added (2/ml filtered media) and the dish incubated for one additional day. These ganglia were then discarded and the culture dishes with this CM were used immediately.

Application of heavy metal solutions: Only neurons with well-developed neurites were used for experimentation to ensure a well established microtubule cytoskeletal structure. PeA cells were allowed to extend neurites for 24–48 h. after plating in CM before exposure to a heavy metal solution. Heavy metal chloride salts of mercury, aluminum, lead, cadmium, and manganese were obtained from J.T. Baker (Phillipsburg, NJ; room temperature solubilities in water respectively (g/100 ml): 6.9, 69.9, 0.99, 140, 151) to make the experimental solutions used. Stock solutions were made in 5.0 ml Falcon sterile centrifuge tubes with autoclaved normal *Lymnaea* saline at room temperature (18–20°C) to obtain a concentration of 1×10^{-3} M. This stock solution was then serially diluted, also in normal *Lymnaea* saline, to obtain a final working experimental solution concentration of 1×10^{-7} M. Mercury chloride stock and experimental solutions were made fresh every few days due to a moderate loss of ions adsorbed on the container surfaces. The 1×10^{-7} M heavy metal solutions were loaded into wide-bore, fire-polished glass microinjection pipettes and delivered via pressure ejection into the CM in a region adjacent to growth cones at 2–5 psi using an Eppendorf microinjector (Model 5242). Rather than using a pulse ejection, the holding pressure of the microinjector was set at 2 psi to deliver a constant stream of experimental solution for 20 min. The volume of metal solution delivered to the culture dish (containing 2 ml CM solution) was estimated as 2 μl. A peristaltic pump (Gilson, Model Minipuls -2) was used to provide a constant flow (400 μl/min) of sterile normal *Lymnaea* saline through the cell culture dish during heavy metal exposure. Neurons

were observed as controls for 40 min prior to heavy metal treatment and for an additional 30 min after the cessation of mercury ejection into the culture.

Imaging: Neurons were viewed with a Zeiss (Axiovert Model 135) inverted microscope using a ×40 objective. A time lapse video recording of the neurite growth cones during heavy metal exposure was captured using a CCD camera (Hitachi Denshi, Japan, Model KP-M1U) connected to a time-lapse frequency VCR (Panasonic model no. AG 6720A) set at 1 frame/s using Sony VHS SP tape. Linear growth rates for neurite growth cones were estimated using a stage micrometer scale.

A section of the video tape was converted to Betacam SP tape and a digitized edition was developed by the Advanced Media for Learning unit at the University of Calgary's Learning Commons. Tape editing was performed with a Media 100 XS System, version 4 (Media 100, Marlboro, MA) and compressed for web delivery with Media Cleaner Pro, version 4 (Terran Interactive, Los Gatos, CA). The supporting animation was created with Softimage, version 3.8 sp 2 (Avid Technology Inc., Tewksbury, MA). This digital tape is replayed at a normal VHS speed of 30 frames/s and can be accessed for web viewing at http://movies.commons. ucalgary.ca/mercury [14].

Immunostaining: RITC, Bodipy and FITC phalloidins (Molecular Probes Inc.) were used to label F-actin. Tublin was visualized with anti-β-tubulin, a mouse monoclonal antibody obtained from Boehringer-Mannheim. The secondary antibodies were obtained from Vector Labs Inc. Cultured cells were fixed for 30 min with 4% paraformaldehye in PBS containing 3 mM EGTA and 0.02% glutaraldehyde, then permeabilized in 0.5% NP-40. The preparations were subsequently rinsed in PBS and incubated for 1 h at room temperature with 25 units fluorescein phalloidin diluted with 20 µl PBS. The cells were rinsed with PBS and incubated with (1:100) β-tubulin diluted in PBS for 1 h. The cultures were then rinsed and incubated with 1:20 dilutions of either FITC or rhodamine conjugated anti-mouse IgM for 1 h. Coverslips were mounted in PBS/glycerol (15–85%) containing 1%-n-propylgalate. Growth cones were viewed under a Zeiss (Axioskop) fluorescent microscope and photographed with a 35 mm camera.

RESULTS

To test for both immediate and chronic effects of Hg ions on growth cone morphology and behavior, individually identified neurons from a homogeneous population of Pedal A cluster were isolated *in vitro* and maintained in primary cell culture. All neurons cultured in the presence of CM exhibited robust outgrowth over night. Figure 1a–c shows sequential digital photographs, without image enhancement, of typical nerve growth cones from intact neurons cultured in 2 ml media before, during and after the addition of 2 µl of a 10^{-7} M solution of $HgCl_2$. The tip of the microejection pipette is visible in Fig. 1b. Within a few minutes of Hg exposure, not only did the growth cone cease its motility but it also exhibited robust collapse and retraction (Fig. 1c). Consistent with this image (Fig. 1c) the denuded neurofibrils eventually formed neurofibrillary aggregates, an observation reflected in the enlarged bul-

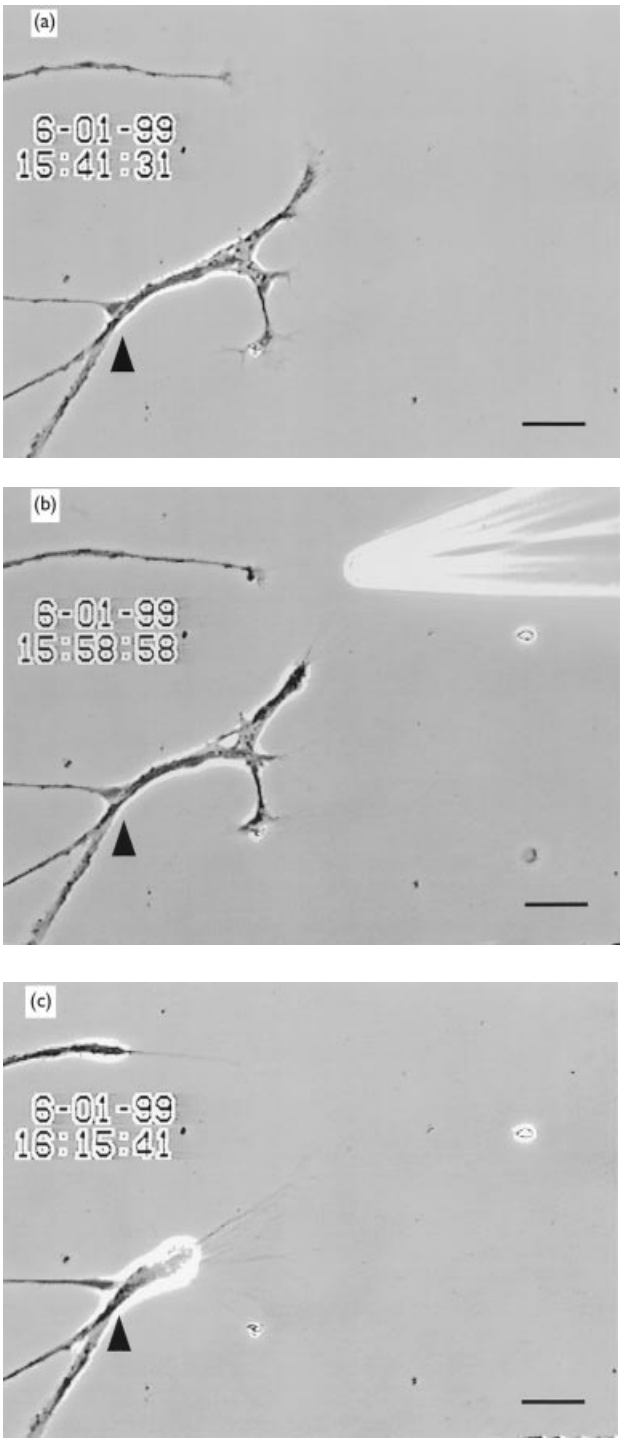

Fig. 1. Digital images of cultured nerve growth cones from identified *Lymnaea* neurons before (**a**), during (**b**) and after (**c**) mercury exposure. The arrow indicates the same reference point in all three images. Bar = 30 µm. Neurons were cultured in the presence of brain conditioned medium and allowed to exhibit outgrowth. Following 24–48 h neurite outgrowth, growth cone behavior was monitored for 40 min with time-lapse video imaging (a). Individual growth cones were subsequently subjected to Hg which was pressure applied locally under a fast perfusion system for 20 min (b). Hg exposure induced growth cone collapse within 10 min (b). Neurite retraction continued under an additional 30 min of observation (c).

bous structure that resulted from neurite retraction following growth cone collapse. This figure is from our June 1, 1999 experiment where tape frame times 15:41:31, 15:58:58, and 16:15:41 were selected. The entire film sequence, illustrating the dynamics of neurite membrane disassembly and retraction following Hg exposure, is available on the web [14]. The average linear growth rate for three of these growth cones was determined to be $+28\,\mu m/h$ before Hg exposure, compared to $-102\,\mu m/h$ during and $-146\,\mu m/h$ after Hg exposure. We have repeated this experiment with similar results for ~40 different neuron cultures under the same conditions over a 2-year period. In these cultures, on average, ~77% of all nerve growth cones were affected by Hg.

To test for the specificity of the effects of Hg ions on growth cone morphology, we next sought to determine whether other metalic ions such as Al, Pb, Cd or Mn (10^{-7} M chloride) would also alter neurite membrane integrity. Despite multiple exposure to the above ions, the growth cone morphology and behavior remained unperturbed suggesting that these ions do not affect growth cone cytoskeleton ($n = 3$ different cultures for each metal, data not shown).

Because Hg ions have previously been shown to effect tubulin polymerization, we next asked whether Hg-induced degeneration of growth cone structure involved actin/tubulin architecture of newly assembled cytoskeletal elements. Specifically, neurons were cultured and allowed to extend neurites. Following neurite outgrowth, individual growth cones were exposed to Hg ions and following collapse, these were fixed and processed for actin/tubulin immunoflurescence. We found that as compared with their control, untreated counterparts (Fig. 2a), the Hg ion treated growth cones exhibited a high degree of disintegration of tubulin/microtubule structure (Fig. 2b). These data demonstrate that Hg-induced degeneration of growth cone structure probably involves microtubular disassembly.

Taken together, the above findings demonstrate that Hg ions exert growth suppressive effects on the growth cone of PeA neurons. To test the extent of these effects, PeA cells were cultured in the presence of Hg ions and the extent of total neurite outgrowth (sprouting) was measured after 48 h. Consistent with our hypothesis we found that neurons cultured in the presence of Hg ions failed to initiate neurons ($4.6 \pm 2.4\%$ sprouting), whereas control neurons extended robust outgrowth ($93.4 \pm 3.1\%$ sprouting). These data, shown in Table 1, thus demonstrate that the effects of Hg ions are not restricted to individual growth cones, rather they prevent neurite initiation from the entire neuron.

DISCUSSION

The results of the investigation described herein clearly demonstrate that exposure to Hg ions markedly disrupts the membrane structural integrity of neurites and the growth cones of identified neurons. This phenomenon appears to be specific for Hg, since exposure to four other heavy metals had no observable effect on either growth cone morphology or individual neurites. These findings are consistent with earlier biochemical evidence demonstrating that microtubule metabolism is compromised in the presence of Hg ions because Hg inhibits GTP nucleotide binding to β-tubulin, a requisite step for tubulin polymerization in the formation of microtubules [7]. We believe that the Hg-induced disassembly of the neurite membrane, as seen in the present study, is a physical manifestation of a disrupted microtubule-tubulin polymerization cycle.

The question arises as to whether this Hg-induced retrograde degeneration of the neuron membrane is solely the result of disruption in microtubule metabolism. Previous evidence indicates that the autoradiographed 45 kDa band of rat brain cortex proteins showed no change in GTP nucleotide binding in the presence of Hg [7]. This protein

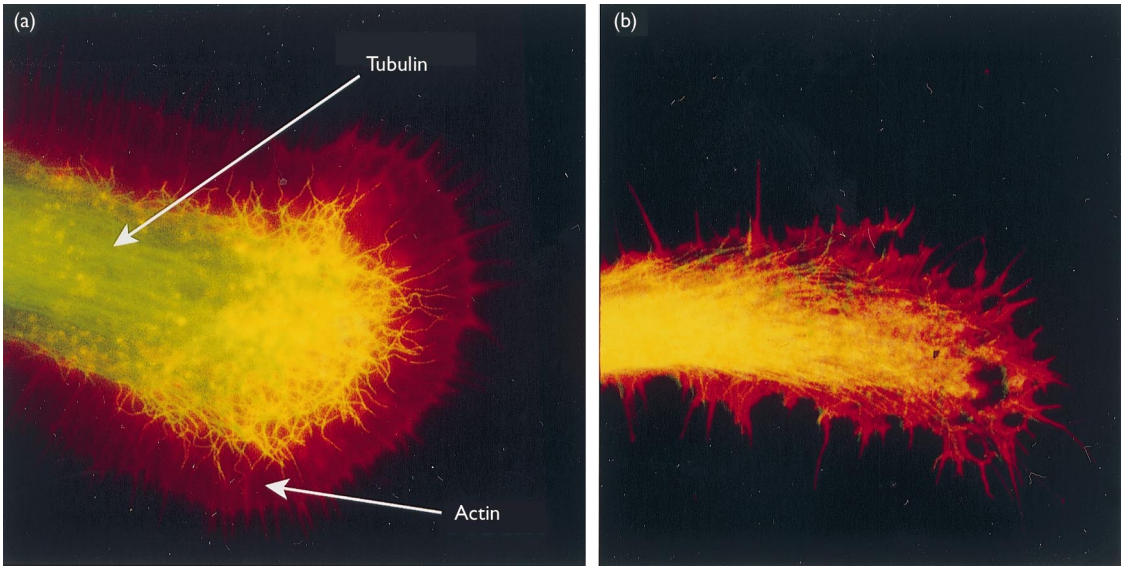

Fig. 2. Fluorescent images of cultured nerve growth cones double-stained with antibodies against actin (red-rhodamine) and tubulin (green-fluorescene) before (**a**) and after (**b**) mercury exposure.

Table 1. Sprouting assay of neurite outgrowth.

	No. cells plated	No. cells sprouted	% sprouted
CM dish			
1	10	9	90
2	13	13	100
3	8	7	87
4	9	9	100
5	10	9	90
			Average 93.4 ± 3.1%
CM + Hg dish			
1	14	1	7
2	12	0	0
3	9	1	11
4	21	1	5
5	10	0	0
			Average 4.6 ± 2.4%

band is primarily composed of actin, another cytoskeletal protein involved in growth cone motility and which is ATP nucleotide-specific, and the band also contains lesser amounts of glutamine synthetase and creatine kinase. Duhr *et al*. [15] have previously demonstrated that Hg had no effect on GTP binding to actin. This supports the interpretation in the present study that the structural disassembly of the neurite membrane, observed herein, is a direct effect of Hg on tubulin rather than actin; an interpretation confirmed by the immunostaining evidence presented in this report.

These results do not, however, rule out other neurite constituents as potential targets for Hg. For example, neuromodulin (also known as B-50 or GAP-43), present in the cytoskeleton and inner plasma membrane surface of the growth cone, also helps stabilize the neurite cell membrane and is involved in neurite outgrowth [16,17]. ADP ribosylation, an essential process in brain metabolism of cytoskeletal and growth associated proteins, is markedly inhibited after both *in vitro* and *in vivo* exposure to inorganic Hg [18].

The actual Hg concentration present in our neuronal cultures was indeed lower than 10^{-7} M because of a dilution effect in the culture media. The Hg concentrations to which these neurons were exposed were of the same order of magnitude as Hg levels reported in human and animal brains after chronic exposure to Hg^0 (reviewed in [5]).

Although more than three-quarters of all Hg-exposed growth cones that we imaged showed evidence of neurite membrane disassembly, the absence of any response by some growth cones may reflect maturational changes in microtubules. This interpretation is supported by rationale proposed by Reuhl *et al*. [4] in which they suggest that less differentiated developing neurons may be more susceptible to microtubule disruption in the presence of MeHg.

It has been claimed that microtubule assembly is defective in AD brains. However, the relationship between the paired helical filaments characteristic of neurofibrillary tangles in AD brains and microtubule instability is unclear [19]. Given the species differences between human and snail neurons, the aggregation of denuded neurofibrils observed in the present study, following Hg exposure, may not be directly analogous to lesions seen in AD brains.

Recently, Escheverria *et al*. [20] have reported a variety of neurobehavioral effects in dental personnel resulting from chronic low-level exposure to Hg^0. Their report is confirmed by the results of several other clinical investigations conducted by the same group. We suggest that the cellular findings in the present study, revealing that Hg disrupts the integrity of the neurite membrane in growth cones of intact neurons, may implicate Hg as a potential etiological factor in neurodegeneration that could ultimately be observed as altered neurobehavior.

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