The uptake and subcellular partitioning of benzo[a]pyrene (BaP) were examined in a rat-liver cell line (Clone 9) using confocal and multiphoton microscopy. Following a 16-h treatment, intracellular accumulation of BaP increased with increasing concentration, and cytoplasmic BaP fluorescence reached saturation at 10 μM. Analysis of the kinetics of BaP uptake at this concentration indicated that BaP is rapidly partitioned into all cytoplasmic membranes within several minutes, although saturation was not reached until 4 h. Based upon the rapid uptake of BaP into membranes, the chronology of changes in gap junction-mediated intercellular communication (GJIC), plasma membrane potential (PMP), and steady state levels of intracellular Ca²⁺ in relation to the time-course for induction of microsomal ethoxyzoresufin-0-deethylase (EROD) activity were examined. EROD activity in Clone 9 cells treated for 16 h increased with increasing concentrations of BaP and reached the highest levels at 40 μM BaP. In addition, kinetic analysis of EROD activity in Clone 9 cells treated with 10 μM BaP indicated that significant induction of EROD activity was not detected before 3 h, and it reached maximal levels by 16 h of treatment at this concentration. Both GJIC and PMP were directly affected by the partitioning of BaP into cellular membranes. The most sensitive index of BaP-induced changes in membrane function was GJIC which revealed a 25% suppression in cells exposed to 0.4 μM BaP for 16 h. Kinetic analysis revealed that suppression of GJIC occurred within 15 min of exposure of cells to 10 μM BaP, whereas significant suppression of PMP was not detected prior to 30-min exposure at this concentration. Elevation of basal Ca²⁺ level was also detected simultaneously with PMP at this dose. These data suggest that early changes in cellular membrane functions occur prior to detectable induction of EROD activity, although basal metabolic activation of BaP may contribute to these changes.

Key Words: benzo[a]pyrene; cytotoxicity; intracellular Ca²⁺ homeostasis; plasma membrane potential; gap junctional intercellular communication; rat liver cell line; Clone 9 cells; fluorescence; confocal microscopy; multiphoton microscopy.

The polycyclic aromatic hydrocarbon (PAH), benzo[a]pyrene (BaP), is an ubiquitous environmental pollutant occurring in products of incomplete combustion of fossil fuels and identified in surface water, tap water, rain water, groundwater, waste water, and sewage sludge. Following inhalation exposure, BaP is rapidly distributed to several tissues in rats, with highest levels found in the liver, esophagus, small intestine, and blood within 30 min of exposure (Sun et al., 1982; Weyand and Bevan, 1986). The metabolism of BaP is complex and includes the formation of a proposed ultimate carcinogen, benzo[a]pyrene 7,8 diol-9,10-epoxide as the result of metabolic activation by cytochrome P450 1A1, 1A2, and 1B1 enzymes (Thakker et al., 1985), which it induces upon binding to the aryl hydrocarbon receptor (AhR) and formation of the AhR–AhR nuclear translocator (ARNT) complex (reviewed in Rowlands and Gustafsson, 1997). The diol epoxide metabolite of BaP is capable of forming stable DNA-adducts leading to mutations and oxidative damage in target organs. BaP can also undergo one-electron oxidation to form depurinating adducts that are released from DNA by cleavage of the bond between the purine base and deoxyribose (Devanesan et al., 1996). The latter process may contribute over 80% of all the DNA adducts that are formed (Li et al., 1995).

Numerous epidemiologic studies have shown a clear association between exposure to various mixtures of PAHs containing BaP and increased risk of cancer and other tumors (Chang et al., 1987; Ketkar et al., 1978; McCormick et al., 1981; Neal and Rigdon, 1967; Thyssen et al., 1981; Wattenburg and Leong, 1970). BaP exposure during gestation results in decreased fertility of offspring (Mackenzie and Angevine, 1981). In addition to its mutagenic effects, BaP may also induce epigenetic effects that contribute to deregulation of growth and differentiation by altering growth-related gene expression and signal transduction (Aaronson, 1991; Parrish et al., 1998). For example, BaP has been shown to activate the c-Ha-ras proto-oncogene by a transcriptional mechanism that may involve the AhR-ARNT complex (Bral and Ramos, 1997) and/or the electrophile response element (Ramos, 1999). It has also been suggested that carcinogenic PAHs including
BaP may stimulate cellular proliferation by altering intracellular Ca\(^{2+}\) homeostasis in a variety of cell types (reviewed, Davila et al., 1995). Given the importance of intracellular Ca\(^{2+}\) in signal transduction, cellular proliferation, and gene expression (reviewed Berridge, 1997; Hardingham et al., 1998), one of the objectives of the present study was to examine the mechanisms by which BaP alters intracellular Ca\(^{2+}\) in cells. Due to the lipophilic character of BaP, it is possible that BaP could affect intracellular Ca\(^{2+}\) homeostasis through direct effects on cytoplasmic membranes. Therefore, another objective of this study was to characterize the partitioning and compartmentalization of BaP within cells using confocal and multiphoton microscopy. Fluorescence bioassays were also employed to determine the chronology of cellular changes resulting from this exposure including the changes in steady-state levels of intracellular Ca\(^{2+}\) in relation to the time-course for induction of microsomal ethoxyresorufin-0-deethylase (EROD) activity. In these studies, a well-characterized rat-liver cell line (Clone 9) was used as a model system. In addition to effects on intracellular Ca\(^{2+}\), the effects of graded doses of BaP on gap junction-mediated intercellular communication and plasma membrane potential were examined in cells treated for periods of up to 16 h.

MATERIALS AND METHODS

**Materials.** Culture media, Dulbecco’s phosphate buffered saline (PBS), serum, benzo[a]pyrene (BaP), and general chemical reagents were purchased from Sigma (St. Louis, MO). Tissue culture flasks were obtained from Corning (Oneonta, NY), LabTek chamber slides and cover glass slides were purchased from Nunc, Inc. (Naperville, IL), ER-Tracker Blue-White DPX (ER-tracker), GolgiTracker, MitoTracker Green FM, LysoTracker, 5-Carboxyfluorescein diacetate (CFDA), bis-(1,3-dibutylbarbituric acid) trimethine oxonol [DiBAC\(_4\)(3)], and Fluo-4,AM were purchased from Molecular Probes, Inc. (Eugene, OR). BaP stock was prepared in DMSO and diluted in media (<0.1% DMSO) immediately before each experiment. Stock solution of 1.0 mM Fluo-4,AM was prepared with DMSO and diluted with medium to 3.0 μM (0.3% final DMSO concentration) for loading in cultured cells. DiBAC\(_4\)(3) was prepared as 10 mM stock in ethanol and used at 5 μM (0.05% ethanol). Fluorescamine, resorufin, and resorufin ethyl ether used in EROD assays were purchased from Sigma.

**Cell culture.** The rat liver cell line, Clone 9 (ATCC, CRL 1439, passage 17) was used in experiments that were conducted within 10 passages after being received. Clone 9 cells were grown in Ham’s Nutrient Mixture F-12 containing 10% fetal bovine serum. Cells were seeded at a density of 50,000 cells/cm\(^2\) and were cultured for 24 h before experimental treatments. Cells were cultured in 2-well LabTek chamber slides for all experiments.

**EROD activity.** EROD assays were performed using the fluorometric assay described by Kennedy and Jones (1994) using a microplate fluorescence reader (Bio-tek FL600, Bio-Tek Instruments, Inc., Winooski, VT). Total protein was determined using bovine serum albumin as a protein standard. Excitation and emission wavelengths were set to 530/25 nm and 590/35 nm, respectively, for the EROD assay.

**Laser cytometry.** BaP accumulation and localization, as well as effects of BaP in Clone 9 cells were monitored with a Meridian Ultima confocal workstation (Meridian Instruments, Okemos, MI). After 24 h of culture, cells were treated with graded concentrations of BaP (0, 0.4, 4, 10, 20, and 40 μM) for at least 16 h, or with 10 μM BaP for different intervals of time. The Ultima was then used to conduct fluorescence measurements as described below.

**BaP accumulation in Clone 9 cells.** Following a 16-h treatment with varying concentrations of BaP, Clone 9 cells were washed in culture medium without serum and phenol red. Eight areas from each chamber of the 2-chamber slide and 2 slides (4 wells) per dose were scanned using UV excitation (351–360 nm), and fluorescence emission of BaP was collected (BP 405/45 nm). Due to the photosensitivity of BaP, great care was taken to optimize the signal-to-noise ratio and minimize photobleaching by adjusting laser power and scan strength to result in less than 10% photobleaching during 100 image scans. The kinetics of BaP (10 μM) accumulation in Clone 9 cells were also studied for up to ~6 h.

**BaP localization in Clone 9 cells.** To determine the subcellular distribution of BaP in Clone 9 cells, each of the fluorescent probes, including Golgi-tracker, MitoTracker Green FM, and LysoTracker, was used in BaP-treated cells. The MitoTracker is a cell-permeant mitochondria-selective dye, which passively diffuses across the plasma membrane and accumulates in active mitochondria. The MitoTracker Green FM is essentially nonfluorescent in aqueous solutions and only becomes fluorescent once it accumulates in the lipid environment of mitochondria. This probe accumulates in mitochondria regardless of mitochondrial membrane potential and produces a bright green, fluorescein-like fluorescence when excited with 488 nm wavelength. The LysoTracker probe is membrane permeant, and concentrates rapidly in lysosomes when used at low concentrations (50 nM) and short incubation time (1 to 5 min). The Golgi apparatus was selectively stained with GolgiTracker, one of the ceramide analogs NBD C6-ceramide, which associates preferentially with the trans-Golgi in live cells. The ER-tracker is a selective and photostable stain for ER in live cells, which requires UV excitation at 374 nm. However, the peak fluorescence emission is variable and ranges from 430 to 640 nm, which permits discrimination of BaP and ER signals.

Following a 16-h treatment with 10 μM BaP, Clone 9 cells were washed with serum- and phenol red-free medium. Cells were then loaded with either 50-nM MitoTracker for 15 min, 50-nM GolgiTracker for 15 min, 50-nM LysoTracker for 1–5 min or 5 μM DiBAC\(_4\)(3) for 15 min. Using 488-nm excitation wavelength, single cells were then scanned in confocal mode to determine the position of each of the fluorescent tracker probes. The same cells were then also scanned in confocal mode upon switching the laser excitation to UV, to determine the BaP position inside the cell. Images collected were then compared to determine the co-localization of BaP with each of the organelle trackers. In the experiments using ER-tracker, cells treated with BaP were loaded with 50 nM ER-tracker for 15 min and washed as above. Both BaP and ER-tracker were visualized simultaneously using UV excitation (351–360 nm) and 2 emission filters (405/45) for BaP and (590/25) for ER. Due to the photosensitivity of BaP, parallel studies were subsequently performed using multiphoton microscopy (Centonze and White, 1998) at the National Biophotonics Resource (University of Wisconsin, Madison, WI) to take advantage of high detection sensitivity and minimal fluorophore excitation volume. The Optical Workstation used in this study was designed for observing living specimens with maximum depth penetration and minimal photo-bleaching and phototoxic effects. The scanning systems and data-capture were controlled by hardware and software derived from the BioRad 1024 confocal microscope. Two ultrafast infrared pulsed-laser systems were employed (a Ti:sapphire laser tunable between 780 nm and 910 nm and a fixed wavelength 1047-nm Nd:YLF laser). A 2 or 3 photon image of BaP was first collected, followed by image capture of one of the organelle-tracker probes using 2-photon excitation.

**Plasma membrane potential in Clone 9 cells.** For measurements of plasma membrane potential (PMP), the fluorescent probe bis-(1,3-dibutylbarbituric acid) trimethine oxonol (DiBAC\(_4\)(3)) was used. This slow responding, negatively charged dye, undergoes a potential dependent distribution between the cytoplasm and the extracellular medium. Inside the cell, cytoplasmic fluorescence of DiBAC\(_4\)(3) is increased over extracellular DiBAC\(_4\)(3) due to binding to cytoplasmic membranes (Apell and Bersch, 1987). In the case of hyperpolarization of cells, efflux of the dye from the cells results in decreased fluorescence. DiBAC\(_4\)(3) was monitored with 488 nm excitation and emitted fluorescence was monitored with barrier filter (BP 530/30 nm).
After 16 h of treatment with BaP, cells were washed with phenol red-free and serum-free medium and then loaded with 5 μM DiBAC₄(3) for 15 min at 37°C. Eight areas from each well were selected and 4 wells were scanned to determine basal levels of PMP of the cells. Control experiments were performed similarly with the addition of the solvent for each BaP dose tested. The kinetics of changes in PMP was also monitored in Clone 9 cells treated with 10 μM BaP for up to 6 h.

Assessment of basal intracellular Ca²⁺ level in Clone 9 cells. Fluo-4,AM was used to monitor intracellular Ca²⁺, is a non-ratiometric visible wavelength probe which exhibits approximately a 40-fold enhancement of fluorescence intensity with Ca²⁺ binding. To minimize differences in fluo-4 from experiment to experiment, cells were seeded at the same density, all experiments were performed with the same fluo-4,AM stock, and each treatment was compared to a separate control.

Following 16 h of treatment with graded concentrations of BaP, cells were washed and loaded for 1 h with 3.0 μM fluo-4,AM in serum-free and phenol red-free medium. Cells were again washed and placed on the stage of the fluorescence microscope and 8 areas of the well were selected and scanned. For image collection, scan parameters were adjusted for maximum detection of fluorescence with minimal cellular photobleaching. Fluorescence was generated in the cells by excitation at 488 nm and fluorescence emission from scanned individual cells were collected (530 nm) by means of a photomultiplier tube. The kinetics of changes in basal Ca²⁺ were also studied in Clone 9 cells treated with 10 μM BaP for up to 6 h. In both short and long term experiments, basal Ca²⁺ was determined by averaging data collected from 8 areas per well and 4 wells per treatment group.

Analysis of gap junctional intracellular communication (GJIC). GJIC between cells was monitored by dye coupling, using the fluorescence recovery after photobleaching (FRAP) technique adapted to determine the rate constant for dye transfer as previously described (Barhoumi et al., 1993). Cells were loaded with 10 μg/ml CFDA for 15 min at 37°C, washed 3 time, and maintained in serum-free medium. Carboxyfluorescein is membrane impermeant and is subsequently transferred only between cells that are coupled by gap junctions. Gap FRAP data from control (solvent) and BaP-treated cells, were collected and analyzed to determine the rate constant for dye transfer as previously described. Rate constants presented are independent of dye leakage caused by changes in plasma membrane permeability.

Data analysis. For each fluorescence endpoint measured, data from different concentrations were compared statistically (p < 0.05) using one-way analysis of variance (ANOVA) and the Duncan multiple range test from SAS/STAT (1985).

RESULTS

Accumulation and Distribution of BaP in Clone 9 Cells

Exposure of Clone 9 cells to 0, 0.4, 4, 10, 20, and 40 μM concentrations of BaP for 16 h revealed an increase in fluorescence intensity that increased with increasing concentration of BaP up to 10 μM, where fluorescence intensity reached saturation (Fig. 1). Analysis of the kinetics of BaP uptake at this concentration indicated that BaP is rapidly partitioned into Clone 9 cells within several min although fluorescence saturation was not reached until 4 h (Fig. 1).

The rapid uptake of BaP within cells was confirmed by confocal and multiphoton imaging of Clone 9 cells using BaP as a fluorophore (Fig. 2). Cells treated with 20 or 40 μM BaP for 16 h showed some precipitation of BaP at the cell surface. Therefore, the 10 μM saturating concentration of BaP was used to further examine the subcellular distribution of the fluorescent signal within cells. Cells were co-treated with 10 μM BaP and one of a series of organelle-specific probes for Golgi, mitochondria, endoplasmic reticulum, and lysosomes and examined by both confocal and multiphoton microscopy. The organelle-specific probes indicated extensive BaP localization in Golgi, and in cytoplasmic membranes including plasma membrane, endoplasmic reticulum, and nuclear envelope (Fig. 2). There was little detectable accumulation of BaP within the nucleus, mitochondrial matrix, or within lysosomes. Interestingly, the distribution of the membrane potential-sensitive probe DiBAC₄(3) (Fig. 3) was virtually identical to the pattern seen with the ER-tracker probe. It appears, therefore, that this lipophilic toxicant is extensively distributed throughout the cytoplasmic membrane systems (Fig. 2).

Analysis of GJIC

Using a fluorescence recovery after photobleaching assay adapted to quantify the rate of dye transfer between cells, the rate constant in control Clone 9 cells (DMSO solvent only) was 0.76 ± 0.03 min⁻¹ (Fig. 4). Cells exposed to graded doses of BaP for 16 h exhibited a significant suppression of GJIC (p < 0.05) at the lowest dose tested (0.4 μM) where the rate constant was 0.58 ± 0.01 min⁻¹. The greatest suppression of GJIC was detected in cells treated with 10 μM BaP (0.43 ± 0.03 min⁻¹). As noted previously, cultures exposed to 20 or 40 μM BaP showed evidence of precipitation of BaP out of culture media at 16 h. All of the groups of cells analyzed were viable, based on the absence of dye leakage from the corresponding nonphotobleached control cells, which would be indicative of reduced plasma membrane integrity. Analysis of the kinetics of alterations in GJIC revealed that significant suppression of GJIC was detectable within 15 min of treatment with 10 μM BaP, and this suppression was sustained for at least 6 h (Fig. 4).

Analysis of PMP

Clone 9 cells treated with 0.4 μM BaP for 16 h and loaded with DiBAC₄(3) showed a significant change in PMP as indicated by the decreased fluorescence intensity of DiBAC₄(3)

![FIG. 1.](http://toxsci.oxfordjournals.org/)
when compared to control (solvent-treated) cells. An approx-
imate 50% decrease in fluorescence intensity was obtained at
10 μM BaP. Within 30 min of treatment of cells with 10 μM
BaP, DiBAC₄(3) fluorescence intensity was significantly sup-
pressed and remained suppressed for at least 6 h (Fig. 5).

Analysis of Basal Intracellular Calcium Levels

Increased basal intracellular Ca²⁺ levels were not detected at
0.4 μM BaP, but were detected at concentrations of 4 μM and
higher BaP (Fig. 6). Analysis of the kinetics of altered basal
Ca²⁺ levels induced by the 10 μM dose revealed a gradual
increase in intracellular Ca²⁺, which was significant by 30 min
and reached a plateau by 4 h (Fig. 6).

Analysis of EROD Activity

Exposure of Clone 9 cells to BaP for 16 h revealed that
EROD activity in this cell type increased with increasing
concentrations of BaP. EROD activity reached the highest
levels at 40 μM BaP (Fig. 7). In addition, kinetic analysis of
EROD activity in Clone 9 cells treated with 10 μM BaP
indicated that significant induction of EROD activity was de-
tected after 3 h and reached maximal levels by 16 h of treat-
ment at this concentration.

DISCUSSION

Analysis of the uptake and subcellular distribution of BaP in
the rat-liver cell line, Clone 9, in conjunction with simulta-
neous labeling of cells with one or two different organelle-
specific probes (Lysotracker, mitotracker, Golgitracker, ER-
tracker) revealed partitioning of BaP into cytoplasmic
membranes within min of addition of the fluorescent toxicant.
Plasma membrane, nuclear envelope, and ER labeling were
clearly identified and the accumulation of BaP was particularly
prominent in the Golgi apparatus, due to the concentrated
stacks of membrane-bounded cisternae in this membrane-traf-
ficking organelle. There was no significant accumulation of
BaP within the nuclei, mitochondria, or lysosomes during the
duration of the experiment (up to 16 h), although the mem-
branes comprising these organelles appeared to contain BaP.

The characteristic fluorescence properties of BaP have pre-
viously been exploited to monitor mixed-function oxygenase
activity in bulk cell populations by flow cytometry (Miller et
al., 1983), and in single cell basis by laser cytometry (Moore
et al., 1994), since loss of BaP fluorescence is a consequence of
BaP metabolic conversion. Elegant confocal microscopy ap-
lications have also been developed to monitor specific cyto-
chrome P450 monooxygenases in single cells using resorufin
substrates (Heinonen et al., 1996).

Due to the photosensitivity of BaP and the potential for
photobleaching due to unwanted collateral non-confocal irra-
diation, which is out of the plane of focus, parallel studies were
performed with multiphoton microscopy, in order to take ad-
vantage of high detection sensitivity and minimal fluorophore
excitation volume. Multiphoton microscopy also reduced the
likelihood of photobleaching in organelles with minimal con-
centrations of BaP. Although photobleaching of BaP was significantly reduced (as was the probability of phototoxicity), the information obtained from both vital imaging technologies was the same. The present study suggests that it may also be possible to monitor the metabolism of BaP in situ at the organelar level, using confocal microscopy in thin samples if conditions are employed to minimize photobleaching of this highly photosensitive toxicant. However, multiphoton microscopy provides an even better approach to this end due to the minimal phototoxicity characteristics of ultrafast, pulsed infrared lasers applied to cellular imaging instrumentation. Analysis of the BaP metabolism at the organelar level in metabolically activated cells is currently under way.

Both GJIC and PMP were directly affected by the partitioning of BaP into cellular membranes. The most sensitive index of cytotoxicity employed in the present study was GJIC, which revealed a 25% suppression in cells exposed to 0.4 μM BaP for 16 h. GJIC has previously been identified as a target for BaP toxicity (Budunova et al., 1990; Upham et al., 1994). Similarly, kinetic analysis revealed suppression of GJIC within 15 min of exposure of cells to 10 μM BaP, whereas significant suppression of PMP was not detected prior to 30-min exposure at this concentration. Elevation of basal Ca\(^{2+}\) level was also detected simultaneously with PMP at this dose. These data
suggest that early changes in cellular membrane functions occur prior to metabolic activation of BaP.

In human lymphocytes and lymphoid cell lines, two patterns of Ca\(^{2+}\) elevation produced by PAHs have been identified. One pattern involves a transient increase in Ca\(^{2+}\) that can be partially inhibited by a protein-tyrosine-kinase inhibitor such as genistein. PAHs can also induce a sustained elevation of Ca\(^{2+}\) (lasting for 18 h or longer), which is thought to involve cytochrome P450-mediated metabolism (Romero et al., 1997; Tannheimer et al., 1997). Metabolites, including BaP-7,8-dihydrodiol and BaP-7,8-dihydrodiol-9,10-epoxide, were more effective in elevating Ca\(^{2+}\) than the parent compound BaP, which did not stimulate significant Ca\(^{2+}\) elevation in either human B cell or mammary epithelial cell lines (Mounho and Burchiel, 1998; Romero et al., 1997; Tannheimer et al., 1997; 1999).

In the present study, the increase in basal Ca\(^{2+}\) levels was directly proportional to the intracellular accumulation of BaP and reached a plateau by approximately 4 h, as indicated by BaP fluorescence intensity. This plateau indicates either a saturation of BaP uptake or a balance between BaP uptake and metabolism. EROD activity in Clone 9 cells increased with increasing concentrations of BaP; however, significant increase in EROD activity in cells treated with 10 \(\mu\)M BaP was not detected prior to 3 h of treatment.

It is possible that alterations in Ca\(^{2+}\) homeostasis in rat-liver Clone 9 cells may also involve both cytochrome P450-independent and -dependent mechanisms. It has also been reported that BaP-induced alterations in Ca\(^{2+}\)-mediated signal transduction pathways may contribute to tumor promotion and progression through non-genotoxic mechanisms (Aaronson, 1991; Parrish et al., 1998). It should be noted that basal P450 isozyme expression in Clone 9 cells has not yet been analyzed. Because of the presence of possible basal BaP metabolic capacity, analysis of whether these effects involve P450-independent and -dependent mechanisms is currently focused on the utilization of more sensitive techniques to detect BaP metabolites, as well as the characterization of P450 enzyme expression in Clone 9 cells.

Metabolic activation of BaP leads to the formation of both stable DNA adducts formed from diol epoxide intermediates and depurinating adducts via one-electron oxidation (Li et al., 1996) resulting in genotoxicity associated with mutagenicity (Dipple, 1994). As the result of one-electron oxidation, quinone metabolites could contribute to redox cycling and thus we are unable to rule out the possibility that oxidative stress could also contribute to altered Ca\(^{2+}\) homeostasis.

Given the diversity of cellular functions that are regulated by intracellular Ca\(^{2+}\) ranging from secretion to changes in gene expression (Berridge, 1997; Dolmetsch et al., 1997 Hardingham et al., 1998; Tse et al., 1993), a better understanding of the amplitude and frequency-encoded Ca\(^{2+}\) signals, the specific contributions of the Ca\(^{2+}\) sources contributing to the signals, and the action of BaP on membrane systems that regulate intracellular Ca\(^{2+}\) release should prove useful. Studies are currently underway to define the mechanisms by which BaP alters amplitude and frequency-encoded Ca\(^{2+}\) signals in Clone 9 cells that are activated by the hormones.
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