

Benzene Induces a Dose-responsive Increase in the Frequency of Micronucleated Cells in Rat Zymbal Glands

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The Zymbal gland, a sebaceous tissue associated with the ear duct of certain rodent species, is a principal target for carcinogenesis by benzene. To investigate the mechanism of induction of tumors in the rat Zymbal gland, we have developed a procedure for primary culture of epithelial cells from Zymbal gland explants so that cytogenetic analysis can be performed on this target tissue following an *in vivo* exposure to benzene. Cytogenetic analysis performed 45 hr after *in vivo* oral dosing with benzene revealed chromosome damage that occurred as a result of acute, subchronic, and chronic dosing. This damage, expressed as a dose-related increase in the frequency of micronucleated cells, was observed in Sprague-Dawley female rats over a range of benzene doses from 12.5 to 250 mg/kg/day, and in male Fischer 344 rats at doses ranging from 1 to 200 mg/kg/day. These results are consistent with the known clastogenicity of benzene in mouse bone marrow, which is also a target tissue. This study is the first report of a genotoxic effect of benzene in the rat Zymbal gland and shows that micronucleus formation may be used as a correlate for carcinogenesis induced by benzene in this target tissue. — Environ Health Perspect 104(Suppl 6):1331–1336 (1996)

Key words: benzene, Zymbal gland, micronucleus, target tissue, genotoxicity, serum-free cell culture

Introduction

The biological effects of exposure to the aromatic hydrocarbon benzene have been studied in human and animal models. Human epidemiological studies have implicated benzene as a potential myelotoxic and leukemogenic agent for workers exposed to high levels (1–3). Other toxic effects of benzene exposure in humans are bone marrow hypoplasia and aplasia. Also, an increased level of chromosome damage in lymphocytes has been reported for humans exposed to benzene (4,5). Mechanistic studies have shown that benzene is not the actual toxic species but must be metabolized in order to exert its carcinogenic and

toxic effects (6–9). The major metabolism of benzene *in vivo* takes place in the liver, and the main metabolites are phenol, hydroquinone, catechol, 1,2,4-benzenetriol, and *trans-trans*-muconic acid (10–12). The phenolic metabolites may then be oxidized to highly reactive quinones by peroxidase enzymes present in target tissues (13–15). However, the metabolite(s) responsible for the leukemia attributable to benzene has not been determined.

Extensive *in vitro* tests have indicated that benzene is weakly mutagenic or non-mutagenic in standard mutation assays (16,17) but does cause chromosome aberrations (16–18) and induces sister chromatid exchanges (SCEs) (16,19) under appropriate assay conditions, although at a substantially reduced level compared to benzene metabolites. Numerous *in vivo* cytogenetic studies have demonstrated that benzene exposure causes structural chromosome aberrations (20,21), induces micronuclei formation (22), and increases the frequency of SCEs in bone marrow cells of mice or rats (23,24). The benzene metabolite hydroquinone has been shown to induce chromosome aberrations and micronuclei in bone marrow cells of mice,

and the micronuclei result from both clastogenicity and a spindle impairment mechanism (25,26).

Although animal studies have shown that benzene exposure causes chromosome changes in bone marrow cells, and leukemia in humans is associated with chromosome abnormalities (27), no convenient animal model for benzene-induced leukemia exists (2,3). However, benzene induces a dose-dependent formation of solid tumors in various tissues. Large-scale, chronic exposure studies by Maltoni et al. (28) performed on rats using inhalation or oral administration of benzene resulted in the appearance of tumors of the Zymbal glands, specialized sebaceous glands located in the region of the external ear canal of rodents (29,30), and at other sites including oral and nasal cavities and liver (28). The finding of Zymbal gland tumors was confirmed in a National Toxicology Program (NTP) study of orally treated rats and mice (20,31).

Because of the relatively high incidence of tumors induced in Zymbal glands in the Maltoni (28) and NTP (31) studies, we chose this target tissue for our study of the genotoxic effects of benzene. The Zymbal gland possesses both cytochrome P450 (32) and peroxidase (33) activities and is a target for tumor formation by several classes of carcinogens, including aromatic amines and polycyclic aromatics (29,34).

Although the chronic oncogenicity studies using high exposure levels clearly indicated dose-responsive induction of Zymbal gland tumors by benzene (28,31), they could not provide information on tumor formation at very low-level exposures because of the prohibitively large number of animals needed for such studies. The identification of a genotoxicity end point in the Zymbal gland that would serve as a dose-response correlate for carcinogenicity in that tissue could provide an estimate of expected carcinogenicity at low levels of benzene exposure. If the genotoxicity response at low doses departed from a linear dose response based on high concentration effects, a case could be made for an analogous departure from linearity in the dose-response curve for tumor formation.

The small size of the Zymbal gland and the limited mitotic activity present in the gland (30) make it difficult to perform genotoxicity assays on direct preparations of this tissue. The present study describes the development of a method for measuring chromosome damage, as evidenced by

This paper was presented at Benzene '95: An International Conference on the Toxicity, Carcinogenesis, and Epidemiology of Benzene held 17–20 June 1995 in Piscataway, New Jersey. Manuscript received 16 January 1996; manuscript accepted 14 June 1996.

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Abbreviations used: EG, epidermal growth factor; EGTA, ethylene glycol bis (B-aminoethyl ether) *N,N,N',N'*-tetraacetate; HBSS, Hank's balanced salt solution; NTP, National Toxicology Program; SCE, sister chromatid exchange.

micronuclei formation, in Zymbal gland cells of rats exposed to benzene.

Micronuclei result from acentric chromosomal fragments or whole chromosomes that are not incorporated into daughter nuclei during cell division, and are indicators of chromosome breakage or damage to the spindle apparatus. Since completion of mitosis is necessary for micronucleus formation, a micronucleus assay must ensure that cell division has occurred during or after exposure to the test material and before cell fixation.

The protocol used in this study involves an *in vivo* exposure to benzene, explant culture of Zymbal gland cells, and analysis for micronuclei in cells that have undergone one or more post-treatment mitoses. Similar methodologies have been used to detect chromosome damage after an *in vivo* exposure to a suspected xenobiotic agent in short-term cultures of human (35,36) or rodent blood lymphocytes (37,38), mouse skin keratinocytes (39), and rodent lung cells (40).

Using the Zymbal gland cell micronucleus assay, we determined the dose-response relationship between benzene exposure and chromosome damage in Zymbal glands of rats treated at dose ranges similar to those used in the oncogenicity studies as well as at lower exposure levels.

Methods

Animals and Dosing

Female Sprague-Dawley rats, Crl: CD(SD)BR, obtained from Charles Laboratories (Wilmington, MA) were approximately 16 weeks of age at time of dosing. Male Fischer rats, F344/NTacFBR, obtained from Taconic Laboratory Animals and Services (Germantown, NY) were approximately 9 weeks of age. The rats were housed individually in stainless steel wire cages, and were maintained at 19 to 22°C with a 12-hr day-night lighting cycle. Purina Lab Chow and water were provided *ad libitum*.

Solutions of benzene (American Burdick and Jackson, Muskegon, MI) were prepared in olive oil (Sigma Chemical Co., St. Louis, MO). Rats were weighed on the first day of dosing and were dosed by oral gavage at the rate of 3 ml/kg bw. Control rats were gavaged with olive oil only at the same rate. Dosing occurred once a day in the morning on 3 consecutive days unless stated otherwise. Animals were sacrificed approximately 24 hr after delivery of the last dose.

Preparing Zymbal Glands for Cell Culture

Rats were killed by CO₂ asphyxiation. Sterile instruments were used, and the surface of each animal was washed with antiseptic and ethanol before making an initial incision. A skin incision was made below the ear and pulled open to expose the ear canal and jaw muscle. Two prominent tendons below the ear were cut, exposing the Zymbal gland. The gland was held by forceps and excised using a scalpel. Both Zymbal glands were removed from each rat. The glands were placed in sterile, ice-chilled L15 medium until they were processed for cell culture. All media and solutions used to prepare and culture the Zymbal glands were sterile, and aseptic techniques were routine.

The excised glands were washed with Ham's F12 medium, minced, and placed in 15 ml of a digestion solution consisting of 0.5% (w/v) collagenase type I, 0.3% (w/v) hyaluronidase, 50 µg/ml gentamicin, 1.5 µg/ml amphotericin B, and 0.5% fetal bovine serum in Ham's F12 medium. The minced glands were treated overnight at 37°C on a rotary apparatus, which resulted in the digestion of adhering connective tissue and removed stromal cells from the gland pieces. The glandular fragments were collected onto a 53-mm mesh nylon screen by gravity filtration. They were washed with Hank's balanced salt solution (HBSS), followed by a small volume (1–2 ml) of the culture medium. The fragments were removed from the screen with a pipette using a minimal volume of medium and placed on the surface of a coated coverslip in a multiwell plate. Approximately 2 ml of culture medium was added to the wells, and the plates were placed in a humidified incubator at 37°C with an atmosphere of 5% CO₂ in air.

The enzymatic digestion procedure, described above and used by Stampfer (41) to obtain pure epithelial cell cultures from human mammary tissues, was used to remove stromal cells and connective tissue components from the glandular portion of the Zymbal tissue. The inclusion of this step eliminated the contamination of fibroblastlike cells from the cultures and also improved the rate of success for initiating cellular outgrowths from the attached explant material.

Growth Surface Preparation

Because many epithelial cells display enhanced growth on surfaces coated with components of the basement membrane, we precoated the growth surfaces with

collagen and fibronectin using a method developed by Lechner and LaVeck (42) for culturing bronchial epithelial cells. Thermanox plastic tissue culture coverslips (Lux #5412, 24×30 mm) were placed in the wells of an eight-well tissue culture plate (Lux #5218, 26×33 mm) and were coated with a solution containing a mixture of fibronectin (10 µg/ml), collagen (Vitrogen 100; 60 µg/ml) and bovine serum albumin (0.1 mg/ml) dissolved in Ham's F12 medium. Approximately 1.5 ml of the solution was added to each well. The multiwell plates were incubated for at least 2 hr at 37°C. The coating solution was removed from the wells by vacuum aspiration, and the coverslips were washed with culture medium before seeding tissue explants for culture initiation.

Cell Culture

Culture medium consisted of MCDB 201 base (Sigma Chemical Co.) supplemented with 5 µg/ml insulin, 10 ng/ml epidermal growth factor (EGF), 1.4 µg/ml hydrocortisone, 20 µg/ml transferrin, 70 µg/ml bovine pituitary extract (43), 0.5 µM phosphoethanolamine, 0.5 µM ethanolamine, 6.5 ng/ml triiodothyronine, 0.1 µg/ml retinoic acid, 0.5 mM sodium pyruvate, 2 mM L-glutamine, 50 µg/ml gentamicin, and 1.5 µg/ml amphotericin B. The medium was also supplemented with a trace elements solution (42) obtained from Biofluids Inc. (Rockville, MD) so that the culture medium contained 1 nM nickel, 1 nM molybdenum, 30 nM selenium, 5 nM vanadium, 0.5 nM tin, and 0.5 µM silicon. Stock solutions for additives to the base medium were prepared as described by Lechner and LaVeck (42). Source of media components and enzymes was Sigma Chemical Co. unless otherwise noted.

A serum-free medium was chosen because our preliminary attempts to obtain cell outgrowths from Zymbal gland tissue explants using fetal bovine supplemented media proved unsuccessful. The serum-free growth medium selected for use in the experiments described in the present study differs only slightly from MCDB 170 medium, which was developed for serum-free culture of human mammary epithelial cells (44). Medium MCDB 170 also provides good growth for Zymbal gland cells using the explant technique.

Description of Cells in Outgrowths in Serum-free Medium

Of the explanted Zymbal tissue that attached to the growth surface, cell migration from

the explants was observed as early as 6 hr after plating. After 20 hr in culture, the beginning of a monolayer of epithelial cells was seen radiating from the explanted tissue that had successfully attached, with some mitotic cells present at this time. After 45 hr in culture, a monolayer of epithelial-like cells, ranging from about 500 to 3000 cells, appeared around the explants that attached, with many mitotic cells clearly visible using low power magnification. If maintained in culture, the monolayers continued to expand, and confluent cultures have been obtained after several more days in culture without the appearance of fibroblastlike cells.

Staining of a representative primary culture with Oil Red stain (45) after 96 hr in culture resulted in a strong staining reaction in the remaining explanted material, indicating that the monolayer of epithelial cells had grown out of a lipid-rich tissue, consistent with Zymbal gland as the source for the monolayer of cells. Adding colcemid after 40 hr in culture and fixing cells *in situ* 4 hr later resulted in the finding that 20% of the cells were at the metaphase stage of mitosis, indicating that a large proportion of the cells in the monolayer were actively dividing at that time.

Cell Fixation and Staining

The fixation procedure was performed *in situ* on the coverslips so that all the cell outgrowths were available for analysis. After approximately 45 hr of incubation, the medium was removed, the coverslips were washed with HBSS, and approximately 2 ml of Ca²⁺, Mg²⁺-free HBSS containing trypsin (0.2 mg/ml), EGTA (0.2 mg/ml), and polyvinylpyrrolidone (10 mg/ml) were added to each well, which caused the cells to separate slightly from each other but remain attached to the coverslip. After approximately 5 min, the solution was removed and replaced with an equal volume of 0.075 M KCl. After 1 min, 0.5 ml of fixative (methanol:acetic acid, 3:1, v/v) was added to each well. The liquid was then removed and replaced with about 2 ml of fixative. The fixative was added and removed 2 more times, after which the coverslips were air dried. The coverslips were stained with 4% Giemsa in H₂O for 10 min and dried. The coverslips were placed sequentially in acetone, then acetone:xylene (1:1), and finally xylene before being mounted with PermOUNT onto a glass slide.

Analysis for Micronucleated Cells

Cells were scanned for the presence of micronuclei by microscopic examination

using a 400× objective lens and analyzed using a 630× objective lens. Only cells in the monolayer outgrowths from the explants were analyzed. A body was scored as a micronucleus by meeting the following criteria: a) color intensity similar to main nucleus; b) diameter between 1/20 and 1/4 that of the main nucleus; c) resides within the cytoplasm; d) roundish in shape with distinctly recognizable border; and e) completely separable from main nucleus and not a nuclear bleb. Degenerating cells with nuclear fragmentation were excluded from evaluation. A minimum of 1000 cells per animal were scored for the presence of micronuclei except where noted. Frequency of micronucleated cells is expressed as micronucleated cells per 1000 cells analyzed.

Results

The Zymbal gland primary cell culture method was used to determine if an *in vivo* exposure to benzene would result in an increased level of cytogenetic damage in cells growing out of the Zymbal gland explants. Because cytogenetic damage can be “diluted-out” after the first or second cell division, a fixation time of 45 hr in culture was chosen. This time allows a sufficient number of cells to migrate out of the explants and be amenable for cytogenetic analysis but restricts these cells to two cell divisions after excision. Initially, we attempted to analyze for structural chromosome aberrations in colcemid-arrested metaphase cells, but we found it difficult to locate sufficient good quality cells for definitive analysis. However, we did notice that some of the cells contained micronuclear bodies, and since an assay for micronucleus induction is not limited to metaphase cells, we decided to use this cytogenetic end point rather than structural chromosome aberrations as the indicator of benzene-induced genetic damage.

In the initial evaluation of the test system, the explant culture technique was

used to determine if a single *in vivo* exposure to a high dose of benzene would increase the frequency of micronucleated cells in the initial monolayer outgrowth from cultured Zymbal gland tissue. A single oral dose of benzene, 1000 mg/kg, was delivered to two Sprague-Dawley female rats, Zymbal glands were removed 24 hr later, cultured for 45 hr, and evaluated for the frequency of micronucleated cells. The results are presented in Table 1 and show that a higher frequency of micronucleated cells was present in the benzene-treated rats than in olive oil-treated control rats. A second experiment was performed in which female Sprague-Dawley rats were dosed orally with 250 mg/kg/day benzene on 3 consecutive days. Zymbal glands were removed approximately 24 hr after the last dose, cultured, and analyzed for micronucleated cells. As shown in Table 1, again, a higher frequency of micronucleated cells was found after benzene exposure compared to vehicle controls.

Assays were also conducted on chronically treated rats. Sprague-Dawley female rats were dosed orally with 250, 25, and 1.5 mg/kg/day benzene or with olive oil only, once a day, 5 days per week for 26 weeks. Zymbal glands were excised from one rat in each dose group. The results of the micronucleus assay are shown in Table 2. Zymbal gland cells from the benzene-treated animals had an elevated frequency of micronuclei compared to those of the control animals, although at the lowest dose, 1.5 mg/kg, the increase was statistically significant only at the *p* = 0.054 level (normal test, one-tail).

Having established that *in vivo* benzene treatment produces an elevated incidence of micronucleated cells in cultures of Zymbal gland tissues, we designed an experiment to determine the nature of the dose response for this induction. Two Sprague-Dawley female rats per dose group were treated on 3 consecutive days, and the dose range

Table 1. Frequency of micronucleated cells in 45-hr Zymbal gland explant cell cultures from female Sprague-Dawley rats.

Treatment	No. of animals	No. of cells analyzed	No. of micronucleated cells	Frequency of micronucleated cells/1000
Single dose experiment				
Benzene, 1000 mg/kg	2	2852	102	36
Olive oil, vehicle control	2	4079	42	10
Three daily dose experiment				
Benzene, 250 mg/kg/day	2	3128	57	18
Olive oil, vehicle control	2	1845*	10	5

*One of the animals had less than 1000 cells analyzed.

Table 2. Frequency of micronucleated cells in 45-hr Zymbal gland cell explant cultures from female Sprague-Dawley rats treated 5 days per week for 26 weeks (single animal/dose).

Treatment	No. of cells analyzed	No. of micronucleated cells	Frequency of micronucleated cells/1000
Benzene, 250 mg/kg/day	2013	40	20**
Benzene, 25 mg/kg/day	2035	40	20**
Benzene, 1.5 mg/kg/day	2024	17	8*
Olive oil, vehicle control	2053	9	4

** $p < 0.001$ (normal test, one-sided); * $p = 0.054$ (normal test, one-sided).

covered 0 to 200 mg/kg/day. The results of this micronucleus experiment are shown in Figure 1 and indicate that a dose-responsive increase in the frequency of micronucleated cells occurred over this dose range. Figure 1 also displays data from the oncogenicity study of Maltoni et al. (28) for the incidence of Zymbal gland tumors in female Sprague-Dawley rats in the 250, 50, and 0 mg/kg/day benzene dose groups.

The nature of the dose response was also examined in male rats using the strain (Fischer 344/N) used in the NTP carcinogenesis study of benzene (17,31). Two rats per dose group were administered oral doses of benzene ranging from 0 to 200 mg/kg/day on 3 consecutive days. The results of the micronucleus analysis are shown in Figure 2. A dose-responsive increase was again seen. The assay was repeated at doses of 1 and 10 mg benzene/kg/day to determine if the sensitivity of the method is sufficient to detect differences in the responses between these doses and a vehicle control (olive oil-only treatment). The results of this assay are presented in Table 3 and indicate that a statistically significant difference was found between the 10 mg/kg/day dose

group and the vehicle control but not between the 1 mg/kg/day and the vehicle control groups.

Discussion

The development of a method for obtaining primary cultures of rat Zymbal gland cells has made it possible to measure the genotoxic effects of an *in vivo* benzene exposure in a tissue known to develop solid tumors as a result of chronic exposure to this chemical under laboratory conditions. In developing the methodology, the choice of a growth medium was crucial to its success, and in the case of rat Zymbal gland cells, a serum-free medium was necessary for good cell growth. The growth medium that was developed is similar in composition to MCDB 170, a medium that was developed for culturing epithelial cells of the mammary gland (41,44) which, at least superficially, the Zymbal gland resembles. In developing the Zymbal gland growth medium, the absolute requirement of each component in the growth medium for cell growth was not established, nor were optimal concentrations of each of the components determined. However, the medium and culture conditions were

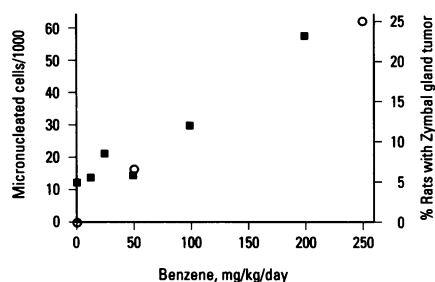
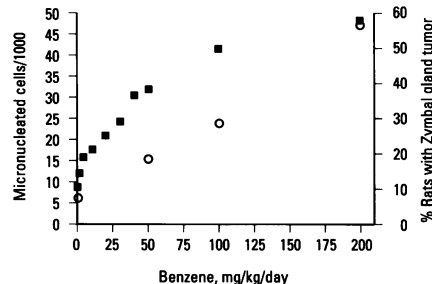
Table 3. Frequency of micronucleated Zymbal gland cells from male Fischer rats dosed with benzene.

Daily dose of benzene	Frequency of micronucleated cells/1000	Mean frequency of micronucleated cells/1000 for dose group
100 mg/kg/day	29	28
	26	
	27	
	29	
10 mg/kg/day	19	18
	20	
	17	
	18	
	17	
1 mg/kg/day	8	9
	10	
	9	
	8	
	9	
0 mg/kg/day	9	9
	8	
	7	
	11	
	9	

shown to be adequate for the purpose of providing a population of early dividing cells from Zymbal gland tissue of treated rats so that the micronucleus assay could be performed.

Because of the high frequency of mitotic cells observed in the monolayers fixed after 45 hr in culture, a cytokinesis-block step using cytochalasin B was not needed to identify dividing cells, although this step was used in the micronucleus assays developed for lymphocytes (36), mouse skin keratinocytes (39), and Chinese hamster lung fibroblasts (40).

It is generally desirable in a micronucleus assay to examine cells that have undergone only one cell division during the period between chemical exposure and cell fixation. The cells in our experiments may not have been restricted to one division but probably underwent no more than two divisions after placement in culture. This conclusion is based on the observation that the majority of cells forming the outgrowths appeared in the monolayer during the 24 hr before fixation. The sensitivity of the Zymbal gland micronucleus assay might be improved by modifications to limit the analysis to only cells that have completed one division in culture. However, the assay, as performed in the study, provided sufficient sensitivity to detect a response from high and low benzene exposures and from cyclophosphamide (data not presented), a known clastogen.

**Figure 1.** Dose response for micronucleus formation (solid squares) in Zymbal gland cells from female Sprague-Dawley rats after three daily oral administrations of benzene. Each square is an average for two animals. At least 1000 cells were analyzed per animal. Also shown (open circles) is the percentage of female Sprague-Dawley rats bearing Zymbal gland carcinomas after chronic exposure to doses of benzene reported by Maltoni et al. (28).**Figure 2.** Dose response for micronucleus formation (solid squares) in Zymbal gland cells from male Fischer 344 rats after three daily oral administrations of benzene. Each square is an average for two animals. At least 1000 cells were analyzed per animal. Also shown (open circles) is the percentage of male Fischer 344 rats bearing Zymbal gland carcinomas after chronic exposure to doses of benzene from the report of NTP study (31).

The findings in this study of an increased frequency of micronucleated cells proliferating from Zymbal glands of rats treated with single or multiple doses of benzene is, to our knowledge, the first report of a genotoxic effect in this target tissue by *in vivo* exposure to benzene or any other xenobiotic. Reddy et al. (30) have reported that DNA adducts are formed in this organ following an *in vitro* but not an *in vivo* exposure to benzene.

The data from the present experiments show a consistent induction of micronuclei following either a single dose, three consecutive daily doses, or chronic (26 weeks) dosing with benzene. Sprague-Dawley

female and Fischer male F344/N rats, both susceptible to Zymbal gland carcinogenesis, appear to be equally susceptible to micronucleus induction in this tissue. The near linear induction of micronuclei over the dose range of 12.5 to 200 mg/kg/day dose response observed in female Sprague-Dawley rats was similar to the pattern for tumor induction (28).

In the case of the male Fischer F344/N rats, the micronucleus induction pattern appeared nonlinear, with a greater slope at the lower concentrations, whereas the tumor response seen in the NTP study was linear (20,31). Because only two rats were used per dose level in the micronucleus

assay, the observed differences may not be significant. However, in the range from 50 to 200 mg/kg/day, the induction patterns in both female and males are similar. Based on the data acquired in this study of Zymbal glands, the dose-response curve for micronucleus induction follows a pattern of decline with decreasing benzene dose, similar to the response for carcinogenesis. The data obtained in our study with male Fischer F344/N rats indicate that the micronucleus end point has sufficient sensitivity to investigate a dose response for benzene in rats at or below the 10 mg/kg/day level in Zymbal gland cells.

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