

## Effect of cadmium-induced oxidative stress on antioxidative enzymes in mitochondria and cytoplasm of CRL-1439 rat liver cells

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**Abstract.** Cadmium affects human health through occupational and environmental exposure. In this report, we present the response of mitochondrial and cytoplasmic antioxidant enzymes of CRL-1439 cells exposed to different concentrations (0-150  $\mu$ M) of CdCl<sub>2</sub> for 24 h at 37°C. Exposure of liver cells to 50  $\mu$ M CdCl<sub>2</sub> increased mitochondrial catalase and glutathione reductase (GR) activities more than the cytoplasmic enzymes. Although the mitochondrial selenium-dependent glutathione peroxidase (Se-GPx) showed less enzymatic activity than the cytoplasmic enzyme, the mitochondrial selenium-independent glutathione peroxidase (non-Se-GPx) showed a slight increase in activity over its cytoplasmic counterpart compared to untreated controls. With 100  $\mu$ M CdCl<sub>2</sub>, catalase maintained an increase in specific activity in mitochondria over the cytoplasmic enzyme compared to the controls. The level of GR was higher in the cytoplasm than in the mitochondria. However, the activity of Se-GPx and non-Se-GPx decreased slightly in the mitochondria compared to their cytoplasmic counterparts. Exposure of cells to 150  $\mu$ M CdCl<sub>2</sub> decreased all antioxidant enzyme activities compared to the 100  $\mu$ M CdCl<sub>2</sub>-treated samples due to toxic effect. Each antioxidant enzyme exhibited its own pattern of activation or inhibition upon exposure to different concentrations of cadmium, with more oxidative stress observed in the mitochondria.

### Introduction

Cadmium, a heavy toxic metal that is widely used in industry, affects human health through occupational and environmental

exposure. It is present in soils, sediments, air and water and is listed by the US Environmental Protection Agency as one of 126 priority pollutants (1). Today, the main uses for this metal are for nickel-cadmium battery manufacture, pigments and plastic stabilizers. Anthropogenic sources of cadmium in the environment are from refining, copper and nickel smelting, and fossil fuel combustion. Natural sources of cadmium in the atmosphere are from volcanic activity, forest fires and wind-borne transport of soil particles. Irwin *et al* (2) reported that anthropogenic sources add 3-10 times more cadmium to the atmosphere than natural sources. Major occupational exposure occurs in non-ferrous smelters, from the production and processing of cadmium, its alloys and compounds, and increasingly in the recycling of electronic waste. Non-occupational exposure is mainly from cigarette smoke that contains high concentrations of cadmium. For non-smokers who are not occupationally exposed, diet is the main route of exposure to cadmium.

The half-life of cadmium in humans is estimated to be between 15 and 20 years (3). It can cause osteoporosis, non-hypertrophic emphysema, irreversible renal tubular injury, anemia, eosinophilia, anosmia and chronic rhinitis (4). Cadmium has been classified as a category I carcinogen (human carcinogen) by the International Agency for Research on Cancer, (5) and the National Toxicology Program, (6). It can cause cancer in the lung, the prostate, the pancreas and the kidney.

The basic mechanisms involved in cadmium carcinogenesis are gene regulation of proto-oncogenes (7), oxidative stress (8-12), disruption of cadherins, inhibition of DNA repair and interference with apoptosis (13). Recently, we studied the response of antioxidant enzymes and redox metabolites in CRL-1439 normal rat liver cells due to cadmium-induced oxidative stress (12). Enzymes involved in the detoxification of oxygen radicals are present in two organelles, peroxisomes and mitochondria. It is well-documented that oxygen reduction occurs in the mitochondria, generating reactive oxygen species (ROS) such as superoxide (O<sub>2</sub><sup>-</sup>) and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) which accumulate as a result of either autooxidation of ubiquinone of the cytochrome bc<sub>1</sub> complex or by spontaneous and/or enzymatic dismutation of O<sub>2</sub><sup>-</sup> (14,15). The main scavengers of O<sub>2</sub><sup>-</sup> and H<sub>2</sub>O<sub>2</sub> in the mitochondria are superoxide

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dismutase (Mn-SOD), catalase, glutathione peroxidase, and peroxiredoxins (16-20). Since these enzymes are present both in the cytoplasm and in the mitochondria of the cells, the response of antioxidant enzymes to different concentrations of cadmium was investigated in the present study with a view to compare these enzyme activities.

## Materials and methods

**Maintenance of cell line.** Rat normal liver epithelial cell line was purchased from the American Type Culture Collection (ATCC) catalog no. CRL-1439 at the passage number 17 stage. The supplied frozen cells were cultured according to ATCC instructions. Cells were grown in F12K medium containing 2 mM L-glutamine, supplemented with 10% fetal bovine serum, 100 units/ml penicillin, and 100 µg/ml streptomycin in T-75 mm<sup>2</sup> flasks (Greener Labortechnik) in a humidified chamber with 5% CO<sub>2</sub> in air at 37°C in an incubator (Nuiare Co).

**Preparation of cytoplasmic and mitochondrial enzyme extracts.** Approximately 1x10<sup>7</sup> cells grown in T-75 mm<sup>2</sup> flasks were used for preparing cytoplasmic and mitochondrial enzyme extracts. The cells were treated with 0, 50, 100 and 150 µM CdCl<sub>2</sub> and incubated for 24 h at 37°C in a 5% CO<sub>2</sub> incubator. At the end of incubation, the cells were trypsinized and pelleted by centrifuging at 3,500 rpm for 10 min. The cell pellets from two flasks were pooled and washed once with cold PBS. Mitochondria were isolated by the method of Guerra (21). In brief, each cell pellet was suspended in 1 ml of ice-cold isolation solution (0.33 M sucrose, 0.25 mM Na<sub>2</sub>EDTA, 15 mM Tris-HCl (pH 7.4) and homogenized in a vial for 1 min at intervals of 15 sec, using a Polytron homogenizer. The homogenate was transferred to an Eppendorf tube and centrifuged at 3,000 rpm for 10 min at 4°C to remove the lysed cell membrane debris. The supernatant was transferred into different tubes and again centrifuged at 10,000 rpm for 10 min. The obtained supernatant was the source of cytoplasmic fraction utilized in enzyme assay studies. The pellet was suspended in 500 µl of 0.33 M sucrose and centrifuged at 10,000 rpm for 10 min. The pellet containing the mitochondria was suspended in 500 µl of 0.33 M sucrose. The remaining supernatant was pooled with the cytoplasmic fraction. Both fractions were stored at 4°C till further use.

### Enzyme assays

**Catalase.** Catalase activity was assayed according to the method of Aebi (22). The assay volume (900 µl) contained 50 µl cell extract and 550 µl of 50 mM phosphate buffer pH 7.0. The reaction at 37°C was started by the addition of 300 µl of 30 mM H<sub>2</sub>O<sub>2</sub>. The decrease in absorbance at 240 nm was monitored for 60 sec in a Beckman DU 7500 spectrophotometer. The enzyme activity was calculated using the extinction coefficient of 0.00394 l mmol<sup>-1</sup> mm<sup>-1</sup> and the unit of enzyme activity was expressed as mmoles H<sub>2</sub>O<sub>2</sub> decomposed per minute.

**Glutathione reductase.** Glutathione reductase activity was assayed according to Smith *et al* (23). This assay is based on the following reactions:

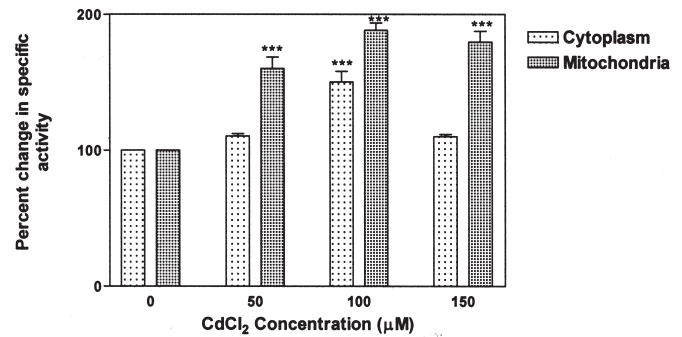
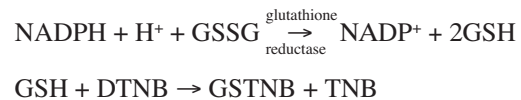


Figure 1. Effect of CdCl<sub>2</sub> on catalase in cytoplasm and mitochondria of rat normal liver cells. Approximately 10x10<sup>6</sup> cells were treated with 0, 50, 100 and 150 µM CdCl<sub>2</sub> and incubated for 24 h at 37°C in a 5% CO<sub>2</sub> incubator. The cells were trypsinized and pelleted by centrifuging at 3,500 rpm for 10 min. The mitochondrial and cytoplasmic extracts were prepared according to the method of Guerra (21). Fifty microliters of the extracts was used in enzyme assays. Data are expressed as mean ± SD, n=3. \*\*\*p<0.01 was considered highly significant.



The change in absorbance at 412 nm due to the formation of TNB was measured. The specificity of this assay allows quantification of glutathione reductase without purification. The assay volume (2 ml) contained 1 ml of 0.2 M potassium phosphate with 1 mM EDTA buffer pH 7.5, 500 µl of 3 mM DTNB [5'-dithiobis (2-nitrobenzoic acid)] in 0.01 M phosphate buffer pH 7.0, 250 µl distilled water, 100 µl of 2 mM NADPH in water, 50 µl cell extract and 100 µl of 20 mM oxidized glutathione. The increase in absorbance at 412 nm was monitored for 3 min in a Beckman DU 7500 spectrophotometer at 24°C. The enzyme activity was calculated using the extinction coefficient of TNB to be (E<sub>412</sub> = 13.6 l mmol<sup>-1</sup> cm<sup>-1</sup>). One unit of enzyme activity is defined as the amount of enzyme that catalyzes the oxidation of 1 µmole NADPH per minute.

**Glutathione peroxidase (GPx).** Glutathione peroxidase activity was assayed according to the method of Yang *et al* (24). Briefly, the reaction mixture (0.5 ml) contained 3.2 mM GSH, 0.32 mM NADPH, 1 unit glutathione reductase (GR), 1 mM sodium azide and 0.82 mM EDTA in 0.16 M Tris-HCl, pH 7.0. When H<sub>2</sub>O<sub>2</sub> was used as a substrate, 1 mM sodium azide was added to the reaction mixture to inhibit endogenous catalase activity. The reaction mixture was pre-incubated with 50 µl of sample at 37°C for 5 min. The reaction was started by addition of cumene hydroperoxide and H<sub>2</sub>O<sub>2</sub> or H<sub>2</sub>O<sub>2</sub> alone at a final concentration of 100 µM in the reaction mixture. The rate of NADPH consumption was monitored at 340 nm for 3 min. One unit of GPx activity was defined as the amount of enzyme that catalyzes the oxidation of 1 µmol of NADPH/min in the coupled assay. Se-dependent GPx was calculated from the change in absorbance using only H<sub>2</sub>O<sub>2</sub> as substrate. Se-independent GPx was calculated by subtracting the change in absorbance at 340 nm/min with H<sub>2</sub>O<sub>2</sub> from the absorbance at 340 nm/min with cumene hydroperoxide and H<sub>2</sub>O<sub>2</sub> substrates in the reaction.

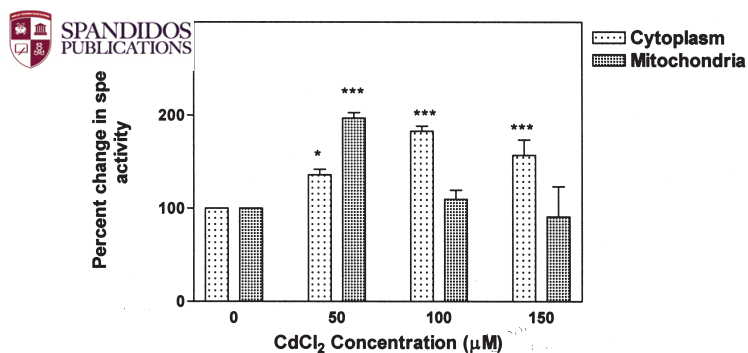


Figure 2. Effect of CdCl<sub>2</sub> on glutathione reductase in cytoplasm and mitochondria of rat normal liver cells. Approximately 10x10<sup>6</sup> cells were treated with 0, 50, 100 and 150 μM CdCl<sub>2</sub> and incubated for 24 h at 37°C in a 5% CO<sub>2</sub> incubator. The cells were trypsinized and pelleted by centrifuging at 3,500 rpm for 10 min. The mitochondrial and cytoplasmic extracts were prepared according to the method of Guerra (21). Fifty microliters of the extracts were used in the enzyme assays. Data are expressed as mean ± SD, n=3. \*p<0.05 and \*\*\*p<0.01 were considered significant and highly significant respectively.

**Protein estimation.** The protein contents of the enzyme extracts were measured using a unique method that combines the reduction of Cu<sup>+1</sup> by proteins in an alkaline medium (Biuret reaction) with the highly-sensitive and selective colorimetric detection of cuprous cation (Cu<sup>+1</sup>) using reagent containing bicinchonic acid (25,26). Protein contents were used to calculate specific activities of the enzymes and subsequently to estimate percent increase/decrease in enzyme activities at different concentrations of CdCl<sub>2</sub>.

**Statistical analysis.** The experimental results were presented as mean ± SD (n=3). The data were analyzed for significance by one-way ANOVA, and then compared by Dunnett's multiple comparison test, using GraphPad Prism Software, version 3.00 (San Diego, CA). The test values p<0.05 and p<0.01 were considered significant and highly significant respectively in comparison to the respective untreated control.

## Results

**Comparison of the catalase activity in the mitochondria and cytoplasm due to cadmium-induced oxidative stress.** The effects of cadmium-induced ROS production on mitochondrial (M-catalase) and cytoplasmic (C-catalase) catalase activities were investigated. For this purpose, normal rat liver epithelial cells were treated with cadmium at 50, 100 and 150 μM for 24 h. The data are presented in Fig. 1. The results clearly indicate that cadmium at 50, 100 and 150 μM increased the M-catalase activity by 60.3%, 88.0% and 80%, respectively, in comparison to untreated control. In the case of C-catalase, cadmium treatments (50 and 100 μM) caused increased activity of 10.4% and 50.5%, respectively, when compared to the untreated control. Further increase in cadmium concentration (150 μM) decreased (40%) the C-catalase activity to the level of control. Comparison of M-catalase and C-catalase revealed that the M-catalase showed higher enzyme activity (30-50%) than the C-catalase. The significance of increased activity may be correlated to increased ROS production in mitochondria due to cadmium treatment.

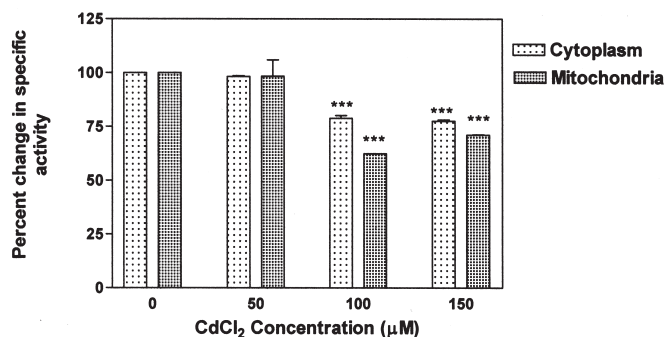


Figure 3. Effect of CdCl<sub>2</sub> on selenium-dependent glutathione peroxidase activity in the cytoplasm and in the mitochondria of rat normal liver cells. Approximately 10x10<sup>6</sup> cells were treated with 0, 50, 100 and 150 μM CdCl<sub>2</sub> and incubated for 24 h at 37°C in a 5% CO<sub>2</sub> incubator. The cells were trypsinized and pelleted by centrifuging at 3,500 rpm for 10 min. The mitochondrial and cytoplasmic extracts were prepared according to the method of Guerra (21). Fifty microliters of the extracts were used in enzyme assays. Data are expressed as mean ± SD, n=3. \*\*\*p<0.01 was considered highly significant.

**Comparison of the glutathione reductase (GR) activity in the mitochondria and cytoplasm due to cadmium-induced oxidative stress.** To examine the effect of oxidation stress on the GR enzyme, which was present in the cytoplasm (C-GR) and mitochondria (M-GR), rat liver cells were exposed to various concentrations of cadmium (50, 100 and 150 μM) for 24 h at 37°C, and the activities in the cytoplasm and mitochondria were determined. The data are shown in Fig. 2. Exposure to 50 μM CdCl<sub>2</sub> increased the GR activity by 35.8% and 96.8% in the cytoplasm and the mitochondria respectively, when compared to the untreated control. However, further increase in cadmium concentrations (100 and 150 μM) caused a drastic decrease in the M-GR activity (~90%) in comparison to its activity at 50 μM. This level of activity is about the same as that of untreated control. In contrast, the C-GR activity was found to be the highest at 100 μM cadmium treatment, whereas only a slight decrease (16%) in C-GR activity was observed in cells treated with 150 μM CdCl<sub>2</sub>. The high initial increase, followed by sudden decrease (~90%) in M-GR activity may indicate that this enzyme was denatured by high ROS production.

**Comparison of the selenium-dependent glutathione peroxidase (Se-GPx) activity in the mitochondria and cytoplasm due to cadmium-induced oxidative stress.** The Se-GPx activity in the mitochondria (M-Se-GPx) and cytoplasm (C-Se-GPx) of the liver cells exposed to various concentrations of cadmium (50, 100 and 150 μM) for 24 h at 37°C are shown in Fig. 3. The Se-GPx activity in the cytoplasm and in the mitochondria at 50 μM cadmium treatment remained the same as control. However, at 100 μM CdCl<sub>2</sub> treatment, the activity decreased in the cytoplasm and mitochondria. Further increase in cadmium concentration (150 μM) did not decrease the enzyme activity significantly in the cytoplasm, while a small but insignificant increase (8.6%) in the activity was noticed in the mitochondrial enzyme when compared to 100 μM CdCl<sub>2</sub> exposed cells.

**Comparison of the selenium-independent glutathione peroxidase (non-Se-GPx) activity in the mitochondria and**

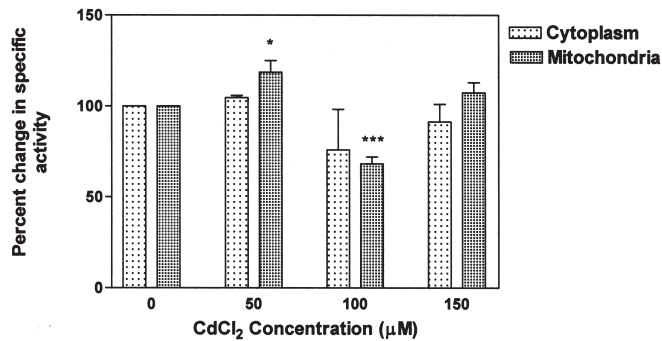


Figure 4. Effect of CdCl<sub>2</sub> on selenium-independent glutathione peroxidase activity in the cytoplasm and in the mitochondria of rat normal liver cells. Approximately 10x10<sup>6</sup> cells were treated with 0, 50, 100 and 150 μM CdCl<sub>2</sub> and incubated for 24 h at 37°C in a 5% CO<sub>2</sub> incubator. The cells were trypsinized and pelleted by centrifuging at 3,500 rpm for 10 min. The mitochondrial and cytoplasmic extracts were prepared according to the method of Guerra (21). Fifty microliters of the extracts were used in enzyme assays. Data are expressed as mean ± SD, n=3. \*p<0.05 and \*\*\*p<0.01 were considered significant and highly significant respectively.

*cytoplasm due to cadmium-induced oxidative stress.* The non-Se-GPx activity in the cytoplasm (C-non-Se-GPx) and mitochondria (M-non-se-GPx) of the liver cells treated with various concentrations of cadmium (50, 100 and 150 μM) for 24 h at 37°C are shown in Fig. 4. Exposure of liver cells to 50 μM CdCl<sub>2</sub> reduced the non-Se-GPx activity to the same level observed in the untreated controls, while an increase of 18.7% was detected in the mitochondria when compared to the untreated control. Interestingly, treatment with 100 μM CdCl<sub>2</sub> caused a decrease in enzymatic activity of 24.2% in the cytoplasm and 31.8% in the mitochondria. But at 150 μM CdCl<sub>2</sub>, enzymatic activity increased again in both the cytoplasm and mitochondria when compared to the untreated controls.

## Discussion

It is well documented that reactive oxygen species (ROS) play contrasting roles, beneficial and harmful, in living organisms (27). ROS can be generated by both exogenous and endogenous sources. Cadmium is one of the exogenous sources shown to indirectly produce ROS in various cell lines (28-30). The production and accumulation of ROS inhibit the electron transfer chain in mitochondria (31). In general, the accumulated ROS consists of various amounts of hydrogen peroxide, hydroxyl ions, singlet oxygen, superoxide anions, lipid hydroperoxides, phospholipid hydroperoxides etc.

Excessive production of ROS disturbs the balance between the ROS and antioxidant agents (enzymes and antioxidant substances) in the cells. Hydrogen peroxide is the common substrate for catalase and GPx enzymes in the cells. While catalase decomposes H<sub>2</sub>O<sub>2</sub> into water and oxygen, GPx oxidizes GSH to GSSG by utilizing H<sub>2</sub>O<sub>2</sub>. Another enzyme that is required for the antioxidant defense mechanism is glutathione reductase (GR). It reduces GSSG into GSH. Both GPx and GR work in tandem in the cells in order to maintain the GSH/GSSG ratio at a steady state level. When the cells are under oxidative stress, catalase, GR and GPx respond by altering their activities.

Since the isolation procedure of mitochondria yields 95% pure mitochondria with only 5% contamination of peroxisomes (21), the high catalase activity in the mitochondrial fraction indicates the presence of catalase in rat mitochondria in the current study. The presence of catalase in rat liver mitochondria (32) and rat heart mitochondria (14) were reported previously. Since catalase decomposes hydrogen peroxide specifically, the dose-dependent increase in M-catalase activity in this study initiated by increasing doses of cadmium (50 and 100 μM) suggests that hydrogen peroxide is produced in increasing amounts with increasing concentrations of cadmium. The steep increase of M-GR activity at 50 μM cadmium indicates accumulation of more GSH in the cells. The high levels of GSH help protect the cells against oxidative stress by quenching different types of radicals of the ROS (33).

Interestingly, the M-Se-GPx activity at 50 μM cadmium remained the same as the untreated control. Since GPx competes with catalase for hydrogen peroxide under normal conditions in the cells, the unchanged M-Se-GPx activity at 50 μM cadmium in comparison to untreated control indicates that it competed with catalase for hydrogen peroxide at 50 μM cadmium treatment. However, treatment with 100 and 150 μM cadmium decreased the enzymatic activities of M-GR and M-Se-GPx significantly. At this point, the GSH levels were the same as that of the control, while the GSSG levels were significantly lower than the control. The lower levels of GSSG in comparison to untreated control may also indicate that treatment with 100 and 150 μM CdCl<sub>2</sub> reduced the ability of M-Se-GPx to compete with catalase for hydrogen peroxide. Since GSH/GSSG ratio is taken as a good measure of the oxidative stress in cells (34), the high levels of GSH compared to the observed levels of GSSG at such cadmium treatments indicate that the cells were not extremely stressed due to treatment with cadmium.

Overall, it appears that when hydrogen peroxide levels in ROS are low, catalase, GR and Se-GPx work in tandem in the cells without disturbing the GSH/GSSG ratio to counteract hydrogen peroxide and various radicals of the ROS. On the other hand, when the production of hydrogen peroxide is high due to high oxidative stress, the situation alters the above ratio and results in increased catalase activity and decreased GR and Se-GPx activities. The altered enzyme activities ensure the efficient removal of ROS in order to maintain the integrity of cell membranes, proteins, DNA etc.

When normal rat liver cells were treated with varying concentrations of cadmium, the mitochondrial enzymes were more effective in reducing various ROS than their cytoplasmic counterparts. This observation may not be surprising in view of the fact that most oxido-reduction reactions take place in the mitochondria, leading to the formation of several ROS (35). As less ROS are produced in the cytoplasm, the activities of antioxidant enzymes in the cytoplasm were not as high as the mitochondrial enzymes with cadmium treatments. Nevertheless, the cytoplasmic enzymes also exhibited similar patterns of activities with cadmium treatments and are thus not discussed separately.

In conclusion, more oxidative stress was observed in the mitochondria than in the cytoplasm. Each antioxidant enzyme shows its own pattern of activation or inhibition upon exposure of cells to different concentrations of cadmium.

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