

# In vitro cytotoxicity of metallic ions released from dental alloys

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**Abstract** The cytotoxicity of a dental alloy depends on, but is not limited to, the extent of its corrosion behavior. Individual ions may have effects on cell viability that are different from metals interacting within the alloy structure. We aimed to investigate the cytotoxicity of individual metal ions in concentrations similar to those reported to be released from Pd-based dental alloys on mouse fibroblast cells. Metal salts were used to prepare seven solutions (concentration range 100 ppm–1 ppb) of the transition metals, such as Ni(II), Pd(II), Cu(II), and Ag(I), and the metals, such as Ga(III), In(III), and Sn(II). Cytotoxicity on mouse fibroblasts L929 was evaluated using the MTT assay. Ni, Cu, and Ag are cytotoxic at 10 ppm, Pd and Ga at 100 ppm. Sn and In were not able to induce cytotoxicity at the tested concentrations. Transition metals were able to induce cytotoxic effects in concentrations similar to those reported to be released from Pd-based dental alloys. Ni, Cu, and Ag were the most cytotoxic followed by Pd and Ga; Sn and In were not cytotoxic. Cytotoxic reactions might be considered in the etiopathogenesis of clinically observed local adverse reactions.

**Keywords** Cytotoxicity · MTT · Metal salts · Dental alloys · Sodium tetrachloropalladate · Nickel · Palladium

## Introduction

Palladium (Pd)-based alloys used for dental applications can be subdivided based on their most important alloying metals, such as gold (Au), copper (Cu), and silver (Ag), referred to as Pd–Au, Pd–Cu, and Pd–Ag alloys, respectively. To increase the mechanical or handling properties of the alloys, other metal elements are also included. Indium (In), gallium (Ga), and tin (Sn) are added to the cast alloys to increase its strength and hardness, and they also contribute to porcelain-bonding by metal-oxide formation [1]. Zinc is generally added to prevent porosity by acting as an oxygen scavenger. Ruthenium, iridium, and rhenium are included as grain refiners, improving the characteristics of the alloys [2, 3].

Corrosion with subsequent metallic ion release is a complex phenomenon that affects all alloys. In case of dental alloys, patients are exposed orally to these constituents, which may lead to adverse reactions, such as gingival swelling, pain, erythema, burning mouth, lichenoid reactions, and chronic urticaria [4, 5]. Also, non-plaque related gingivitis due to long-term contact of the metallic restorations with sulcular tissues has been reported [6]. These adverse reactions may result from local cytotoxic effects and/or immunologic responses. Interestingly, Pd, like nickel (Ni), is able to activate the innate immune system via Toll-Like receptor 4 (TLR-4) indicating that next to allergic reactions, basic (innate) immune responses may be responsible for adverse reactions [7]. Furthermore, it has been shown that palladium allergy prevalence might be underestimated due to the low sensitivity of the

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commonly used test allergen applied for allergy diagnostics [8]. Sodium tetrachloropalladate ( $\text{Na}_2\text{PdCl}_4$ ) was found to be more sensitive compared with the commonly used palladium dichloride ( $\text{PdCl}_2$ ). The difference in sensitivity was explained by the poor solubility of  $\text{PdCl}_2$  polymer in water compared with that of  $\text{Na}_2\text{PdCl}_4$ , which is a monomer and well soluble in water. This may also imply that adverse reactions to palladium-based alloys may be underestimated [9].

Besides immune responses, cytotoxicity may also be responsible for the development of local reactions. Previous cytotoxicity studies analyzed the effects of metal ions on the gingival tissues using various cell lines and testing methods with eluates of submersed dental alloys or aqueous solutions of metal salts [10–20]. In general, the cytotoxicity of palladium is claimed to be low, but one should realize these experiments were carried out with, nearly insoluble,  $\text{PdCl}_2$  salt.

In this study, we focused on the cytotoxic effects of metal ions in concentrations that are reported to be released from the palladium-based alloys, such as Pd–Cu and Pd–Ag [15, 21–32]. Ni-containing base-metals were investigated for comparison. Furthermore, we attempted to rationalize whether these concentrations are clinically relevant.

## Materials and methods

We investigated the cytotoxic effects of the transition metals such as Ni(II), Pd(II), Cu(II), and Ag(I), and the metals such as Ga(III), In(III), and Sn(II) in a concentration range from 100 ppm to 1 ppb, on mouse fibroblast cells. These concentrations were chosen to simulate the range of concentrations expected to be released from dental alloys. Pd–Cu alloys contain approximately 80wt% Pd, 10wt% Cu and 10wt% Ga, and Pd–Ag alloys contain approximately 50wt% Pd, 40wt% Ag, and 10wt% Sn; trace elements of In and Au are also included. Au was excluded because of its limited solubility, and Ni was investigated for comparison.

### Preparation of the salts solution

Stock solutions (2000 ppm) of seven different metal salts were made from  $\text{Ni}^{2+}$  [ $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$ ] (Wako Pure Chemical Industries, Ltd. Osaka, Japan),  $\text{Ga}^{3+}$  [ $\text{Ga}(\text{NO}_3)_3 \cdot n\text{H}_2\text{O}$ ],  $\text{Cu}^{2+}$  [ $\text{CuCl}_2$ ],  $\text{Sn}^{2+}$  [ $\text{SnCl}_2$ ],  $\text{In}^{3+}$  [ $\text{InCl}_3$ ],  $\text{Ag}^+$  [ $\text{AgNO}_3$ ], and  $\text{Pd}^{2+}$  [ $\text{Na}_2\text{PdCl}_4$ ] (Sigma Aldrich Co. LLC, Missouri, USA). The metal salts were diluted with distilled water (stock solutions, 2000 ppm), purified by a filter membrane (Millex® GS Filter unit 0.22  $\mu\text{m}$ , MF-Millipore MCE Membrane, Carrigtwohill, Co. Cork, Ireland) and further diluted with culture medium to

make working solutions of six different concentrations: 100, 10, 1, 0.1, 0.01, and 0.001 ppm. These are hereafter referred as metal salt solutions.

### Cytotoxicity test

A colorimetric assay for assessing cell viability, the MTT assay, was used to determine the cytotoxicity of the metal ion solutions. This test is a basic vital staining technique (tetrazolium dye, MTT) used to rapidly monitor changes in metabolic activity of cells when in contact with an agent. Eight replicas and one control (no metal salt) for each concentration per salt were carried out. The whole experiment was carried out in duplicate according to the ISO standard for cytotoxicity testing-research, ISO 10995-5, and the results were averaged [33]. The cells used in this study were mouse fibroblasts cell line 929 (CCL-1, ATCC Lot 58928277), according to ISO 10995-5. Previously to the experiment the cells were cultured in E-MEM culture medium with 1 % P/S and 10 % horse serum. The cells were harvested and counted under phase contrast microscope (ELWD 0.3, Diaphot, Nikon, Japan, 100 $\times$ ) with the help of an hemocytometer (Burker-Turk deep 1/10 mm, Erma Tokyo 5843), after which they were resuspended in cell culture medium and diluted to a cell density of 50000 cells/ml with medium. 100  $\mu\text{l}$  of this suspension were added to each well (14700 cells/ $\text{cm}^2$ ) of a sterile 96-well cell culture plate (Cellstar, cat-no. 655180, Greiner bio-one) and the plates were incubated at 37 °C in 5 %  $\text{CO}_2$ , for 24 h in a humidified incubator (air jacket type incubator, Astec). Hereafter, the culture medium was discarded and 100  $\mu\text{l}$  of each metal salt solution was added to the incubated cells. The metal salts solutions were centrifuged and resuspended to eliminate precipitation of the metals. The cells were then incubated for another 24 h. Finally, MTT stock solution (5 mg/ml in PBS(–)) (3-[4,5-dimethyl-2thiazolyl]-2,5-diphenyl-2H-tetrazolium bromide,  $\text{C}_{18}\text{H}_{16}\text{BrN}_5\text{S}$ , Dotite MTT, Dojindo, Japan. Lot no. BK061) was diluted 10 times with E-MEM with 10 % horse serum and 1 % P/S. 100  $\mu\text{l}$  of this MTT solution was added to each well, and the cells were incubated at 37 °C in 5 %  $\text{CO}_2$  for 3 h. After discard of the cell culture medium, the cells were washed with 100  $\mu\text{l}$  PBS(–) after which 50  $\mu\text{l}$  DMSO were added and the spectrophotometric absorbance at 570 nm was read in a microplate reader (Biorad, model 680). The mean absorbance values were calculated as percentages of control, according to the formula:

$$\text{Relative cell viability (\%)} = 100 \times (A/B)$$

in which *A* are the viable cells in the experimental well and *B* are the viable cells in the control.

## Statistical analysis

The data were analyzed with one-way ANOVA and Tukey post hoc test with a significance set at  $P < 0.05$  using SPSS (IBM SPSS Statistics 21).

## Results

The effect of solution concentration for each ion on cell viability is shown in Figs. 1 and 2. Considering that a viability of less than 70 % of the blank expresses the potential cytotoxic effect of an agent [34], Ni, Cu, and Ag are cytotoxic at 10, whereas Pd at a concentration of 100 ppm. The metals of Ga, In, and Sn behaved completely different; Sn and In did not have any negative effect on cell viability in the concentrations studied. Interestingly, Ga and In showed increased viability around 0.1–1 ppm, a decrease in cell viability in 1–10 ppb region, and a cytotoxic effect at 100 ppm for Ga. The exact cell viability at different concentrations and the statistical analysis are summarized in Table 1.

Table 2 shows the reported ion release from Pd-based dental alloys and Ni-containing alloys [15, 21–32]. The cytotoxic concentrations in mouse fibroblasts found in the present study are also presented for each metal. These concentrations are within the range of reported release for each considered metal.

## Discussion

The present study aimed at investigating the cytotoxicity of metal ions in concentrations that are reported to be released from palladium-based alloys (Pd–Cu and Pd–Ag) and Ni-containing base-metal dental alloys. Several studies report the ion release from dental alloys with different results [15, 21–32]. This is due to the different testing method, pH, and composition of the analyzing solutions, but also the composition, structure, and surface area of the sample of the alloy itself. As a result, comparison among the studies is difficult and no reference values can be considered. For this reason, we attempted to summarize the reported ion release from dental alloys having Pd, Ag, Ga, Cu, and Ni in its

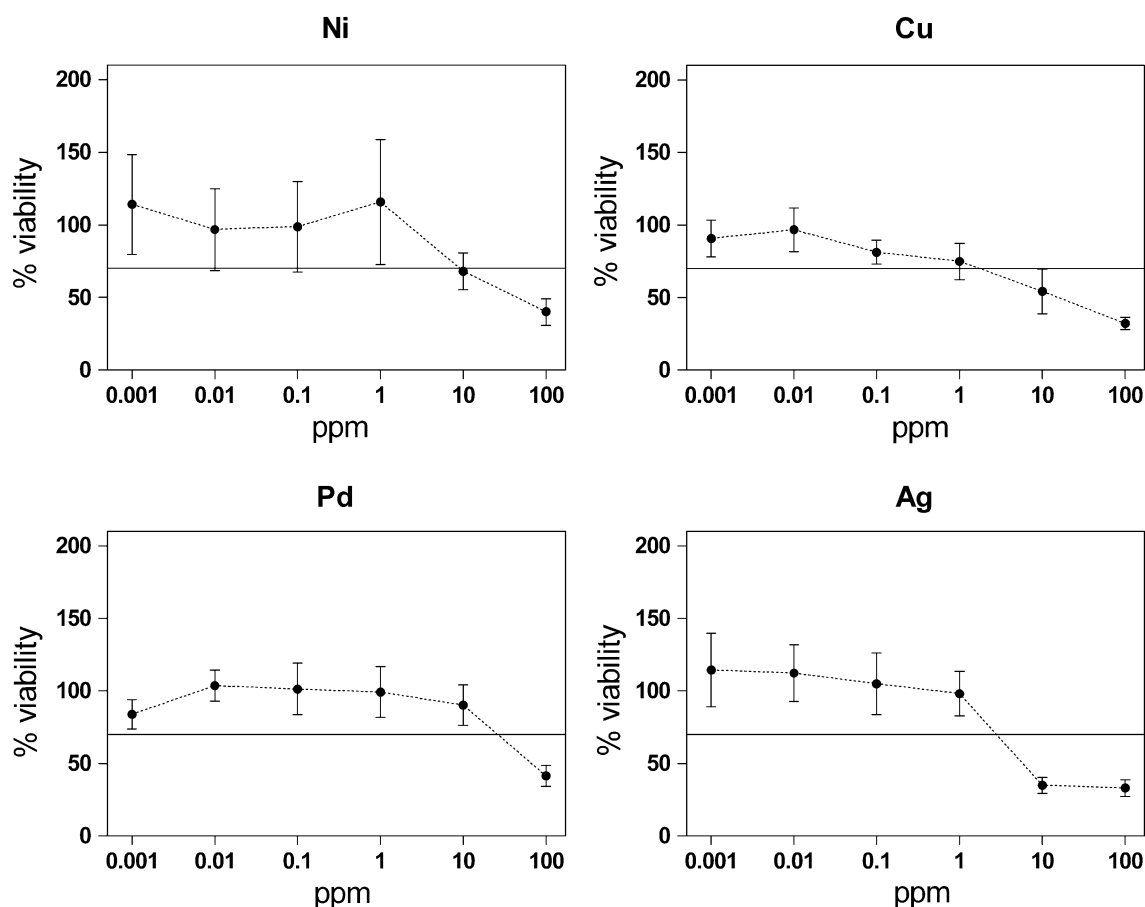


**Fig. 1** Cell viability (%) induced by the different concentrations of the transition metals tested

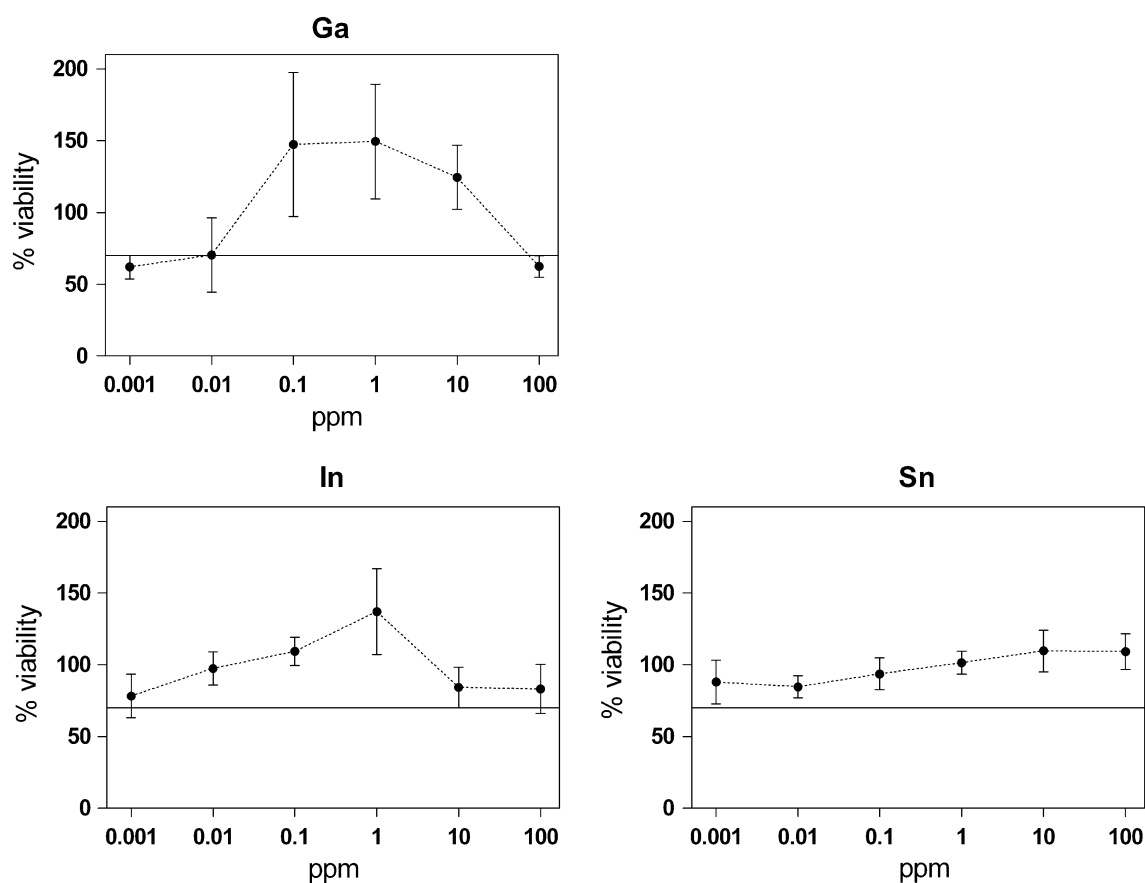


**Fig. 2** Cell viability (%) induced by the different concentrations of the metals tested

**Table 1** Cell viability in % [mean (SD)] of different metal ions depending on concentration in ppm

|    | 0.001                     | 0.01                       | 0.1                    | 1                      | 10                           | 100                       |
|----|---------------------------|----------------------------|------------------------|------------------------|------------------------------|---------------------------|
| Ni | 114 (34) <sup>c</sup>     | 96 (28) <sup>b,c</sup>     | 98 (31) <sup>b,c</sup> | 116 (43) <sup>c</sup>  | <b>68 (13)<sup>a,b</sup></b> | <b>40 (9)<sup>a</sup></b> |
| Pd | 84 (10) <sup>b</sup>      | 104 (11) <sup>c</sup>      | 101 (18) <sup>c</sup>  | 99 (17) <sup>b,c</sup> | 90 (14) <sup>b,c</sup>       | <b>41 (7)<sup>a</sup></b> |
| Cu | 91 (13) <sup>d,e</sup>    | 97 (15) <sup>e</sup>       | 81 (8) <sup>c,d</sup>  | 75 (13) <sup>c</sup>   | <b>54 (16)<sup>b</sup></b>   | <b>32 (4)<sup>a</sup></b> |
| Ag | 114 (25) <sup>b</sup>     | 112 (20) <sup>b</sup>      | 105 (21) <sup>b</sup>  | 98 (15) <sup>b</sup>   | <b>35 (5)<sup>a</sup></b>    | <b>33 (6)<sup>a</sup></b> |
| Ga | <b>62 (8)<sup>a</sup></b> | <b>70 (26)<sup>a</sup></b> | 147 (50) <sup>b</sup>  | 149 (40) <sup>b</sup>  | 124 (22) <sup>b</sup>        | <b>62 (8)<sup>a</sup></b> |
| In | 78 (15) <sup>a</sup>      | 97 (11) <sup>a,b</sup>     | 109 (10) <sup>b</sup>  | 137 (30) <sup>c</sup>  | 84 (14) <sup>a</sup>         | 83 (17) <sup>a</sup>      |
| Sn | 88 (15) <sup>c</sup>      | 85 (8) <sup>b,c</sup>      | 94 (11) <sup>b,c</sup> | 101 (8) <sup>a,b</sup> | 110 (14) <sup>a</sup>        | 109 (13) <sup>a</sup>     |

Values in bold are considered cytotoxic and values quoted with the same letter are not significantly different ( $P < 0.05$ )

**Table 2** Average range of the reported metal ion release from dental alloys (ppm/day)

|    | Reported release (ppm/day) | Cytotoxic concentration in the present study (ppm) |
|----|----------------------------|----------------------------------------------------|
| Ni | 0.006–54                   | 10                                                 |
| Pd | 0.00006–59                 | 100                                                |
| Cu | 0.045–98                   | 10                                                 |
| Ag | 0.003–19                   | 10                                                 |
| Ga | 3–27                       | 100                                                |
| In | 0.00008–50                 | –                                                  |
| Sn | 0.00008–8875               | –                                                  |

Please note that the original data were directly converted to ppm/day. The corresponding cytotoxic concentration in our study is also presented

main constituents, and base the concentration range of the metal salts solutions used on those values. The reports were considered when the authors provided the surface area, time of immersion and ion release. The original data reported were further converted into ppm/day and are summarized in Table 2.

The transition metals, Cu, Ag, Pd, and Ni, were able to affect cell viability in a toxic way in concentrations as low as 10 ppm for Ni, Cu, and Ag, and 100 ppm for Pd. Sn and In were unable to cause cytotoxic effects, and Ga had a non-straightforward behavior with cytotoxic potential at 100 ppm. The cytotoxic concentrations of the transition metals are within the range of reported concentrations of ion release due to corrosion of dental alloys and therefore, it may be expected that they potentially can cause cytotoxic effects (Table 2), while Sn, In, and Ga are most probably not cytotoxic in dental applications. In our previous studies with Pd–Cu, Pd–Ag (results submitted for publication), and Ni-containing orthodontic retention wires [35], the release of Pd, Ga, and Ni ranged from 0.1 ppm to 1 ppb under similar circumstances. Based on these results it is unlikely that these metals are able to induce direct cytotoxicity.

The adverse reactions to Pd-based alloys can in principle result from local cytotoxicity or an immunologic reaction that may be part of both the innate immune system via a TLR-4 induction [7] and the adaptive immune system in terms of an allergic reaction [36–38]. Cytotoxic effects of metal cations are in general dose-dependent and different effects in distinct cell types are observed [11]. The testing method, the cell line used, and the number of target cells influence the evaluation of cytotoxicity and explain the discrepancy of the results. Notwithstanding, there is consensus in ranking Ag has having high potency followed by Pd, Ni, and Ga [11, 39]. The ranking of In is contradictory, being considered either highly potent cytotoxic [5, 11] or the least potent cation [10, 12]. Schmalz et al. [17] reported Cu to have a dramatic effect on cell viability, and Pd and Sn to lack toxicity. In our study, Ni, Ag, and Cu seemed to have the most cytotoxic potential, followed by Pd and Ga. Sn and In did not show cytotoxic potential. Reports on the cytotoxicity of gallium are also contradictory; studies with Ga-containing alloys or Ga salts support the non-cytotoxic effects found in our study [40, 41], whereas other cytotoxicity assays with Ga alloys showed that they have cytotoxic potential [29, 42].

Individual ions may have effects on cell viability that are different from metals interacting within the alloy structure. Caution should be taken in the assessment of cytotoxicity using metal salt solutions since it might be a too simplistic approach. Medium extracts of dental alloys were reportedly non-cytotoxic on mouse fibroblasts, but the

amount of elements found in those extracts were weakly cytotoxic when prepared as salt solutions [15]. Thus, ions released from dental alloys might be below the accepted cytotoxic levels, but because of the inherent interaction between metals within the alloy's composition, the resultant alloy may be cytotoxic [15, 43]. Furthermore, the phase-structure of the dental alloy and electrochemical changes due to rearrangement of the alloy's microstructure [44–48] during the casting and manufacturing process [49] may influence both the ion release and the cytotoxicity of the alloy. The correlation of ion release and cytotoxicity is not straightforward [50] which accounts for the complexity of the evaluation of cytotoxicity of the dental alloys.

In our study, Pd showed considerable cytotoxicity in contrast to other studies where it was considered non-cytotoxic [11, 12, 51]. This difference in cytotoxicity found for Pd is probably best explained by the different Pd salt used in the current study. Here we used  $\text{Na}_2\text{PdCl}_4$  which is, in contrast to  $\text{PdCl}_2$ , well soluble in water due to its monomeric structure. Furthermore, it is interesting to note that the concentrations used to assess Pd and Ni allergy in the in vitro LTT tests [38] and for the local immunological reaction via a TLR-4 [7] is approximately 24–75 ppm with the same number of monocyte-derived dendritic cells (MoDC) cells per experiment [7]. This shows that the concentrations in the latter tests, which are suboptimal conditions, are relatively high and very close to cytotoxicity, at least for fibroblasts cells in the conditions of the present study.

The in vivo situation is difficult to mimic and to evaluate. Metallic ions from dental alloys are most likely to contact with the mucosal epithelial cells at first, and thus the choice of the cell line in our study might not have been the most adequate to mimic the oral situation. However, dental alloys are likely remaining in the mouth for decades, with a continuous low dose of metallic ion release eventually reaching the deeper layers of the oral mucosa and being clinically able to induce adverse reactions. Also, fibroblasts are the principal cells of the periodontal ligament. Considering the gingival sulcus, a privileged microenvironment for accumulation of metal ions, the role of local cytotoxicity induced by metal ions released from dental alloys in the etiopathogenesis of periodontitis might be hypothesized and deserves further studies.

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**Conflict of interest** The authors declare that they have no conflict of interest.

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