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Ennoblement of Stainless Steel by the Manganese-Depositing Bacterium *Leptothrix discophora*

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The noble shift in open-circuit potential exhibited by microbially colonized stainless steel (ennoblement) was investigated by examining the relationship among surface colonization, manganese deposition, and open-circuit potential for stainless steel coupons exposed to batch cultures of the manganese-depositing bacterium *Leptothrix discophora*. Open-circuit potential shifted from −100 to +330 mV_{SCE} as a biofilm containing 75 nmol of MnO_x cm^{-2} formed on the coupon surface but changed little further with continued MnO_x deposition up to 270 nmol cm^{-2}. Increased open-circuit potential corresponded to decreasing Mn(II) concentration in solution and to increased MnO_x accumulation and attached cell density on the coupon surfaces. MnO_x deposition was attributable to biological activity, and Mn(II) was observed to enhance cell attachment. The experimental results support a mechanism of ennoblement in which open-circuit potential is fixed near +350 mV_{SCE} by the cathodic activity of biomineralized MnO_x.

Stainless steel and other passive metals can be corroded by exposure to biologically active natural waters (8, 15). Complex deposits of microbial cells, extracellular polymers, and organic and inorganic debris that accumulate on the metal surface accelerate corrosion by changing the electrochemical behavior of the metal (9, 31, 34). The principal electrochemical changes, which are collectively termed ennoblement, are an increase in open-circuit potential (E_{corr}) to noble values approaching +350 mV versus the saturated calomel electrode (SCE) and a 2-order-of-magnitude increase in the cathodic reaction rate at potentials above −300 mV_{SCE} (32). Elevated E_{corr} promotes corrosion initiation on type 304 and other low-molybdenum stainless steels in seawater (8), and the concurrent increase in cathodic rate stabilizes and propagates the corrosion (20, 30). The concerted effect of these two corrosive events can cause perforation failure of piping and containment vessels that are exposed to natural waters. In order to prevent increased service costs (e.g., biofouling removal and cathodic protection), decreased life expectancy, and catastrophic failure of structural materials (5, 13, 27), the petrochemical, pulp and paper, and power industries must employ increasingly expensive alloys that are able to withstand the corrosive effects of the biofilms.

A number of studies have attempted to establish a direct link between biofilm formation and ennoblement since ennoblement was first observed in the mid-1960s (7). Ennobled potential has been correlated with cell density (9) and with biological activity in the biofilms by measuring ATP accumulation (31) and electron transport activity and lipopolysaccharide content (34). These studies, coupled with studies using filter-sterilized, heat-pasteurized, and artificial seawater (8, 9) as experimental controls, support the hypothesis that biofilm formation is a prerequisite for ennoblement. However, a biological mechanism explaining ennoblement has not been established.

We have recently demonstrated that ennoblement of stainless steel coupons placed within a freshwater stream in Bozeman, Mont., was caused by the deposition of manganese-rich material on the coupon surface (10). Ennoblement could also be induced by coating the metal surface with pure manganese dioxide paste. This study established a chemical mechanism for ennoblement in which manganese dioxide acts as a galvanic cathode to elevate cathodic current and shift E_{corr} in the noble direction. The MnO_2-MnOOH redox couple ($E^{\circ\prime}$ = +335 mV_{SCE} at pH 8) was proposed as the reaction that fixes E_{corr} near +350 mV_{SCE}. Since manganese-rich deposits and manganese-oxidizing bacteria frequently occur at sites of stainless steel corrosion (24, 25, 38), this study also unified the previously separate issues of ennoblement and manganese-related corrosion.

While the origin of the manganese-rich material deposited on stainless steel coupons exposed to Bozeman streamwater was not rigorously established, mineral-encrusted bacterial sheaths characteristic of *Leptothrix* sp. (14) and mineralized toroidal capsules characteristic of *Siderocapsa treubii* (17) were abundant on the surface of the ennobled stainless steel coupons (11), and manganese-oxidizing bacteria were isolated from the manganese-rich deposits. While both genera are known to deposit manganese, principally in the sheaths and capsules, this ability is often lost during laboratory culturing, and efforts to obtain manganese-depositing axenic cultures of *S. treubii* have been unsuccessful (17). A strain of *Leptothrix discophora* that maintains its ability to form sheaths in laboratory culture has recently been described (14). This strain was chosen as a model bacterium to test the hypothesis that ennoblement of stainless steel can be induced by manganese biomineralization. We report that concomitant biofilm formation and manganese dioxide deposition by *L. discophora* induce stainless steel ennoblement. These results establish the biological basis for ennoblement and advance our ability to control this phenomenon by quantifying the surface abundance of manganese that is required to shift E_{corr} to +350 mV_{SCE}.

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MATERIALS AND METHODS

Inoculum and growth medium. Stock cultures of *L. discophora* SP-6 (14) obtained from the American Type Culture Collection (ATCC 51168; Detroit, Mich.), were maintained at 4°C on ATCC 1917 mineral salts-vitamin-pyruvate (MSVP) medium solidified by addition of 1.5% noble agar (Difco, Detroit, Mich.). The experimental medium consisted of MSVP medium amended with 35 mg of pyruvate liter^{-1} and ca. 200 μM Mn(II). Mn(II) was supplied from a filter-sterilized 0.1 M $MnSO_4$ stock solution. Colonies from the plated stock culture were incubated for 72 h at room temperature (20 to 22°C) in 100 ml of MSVP medium. An aliquot of this culture (1.0 ml) was added to 20.0 ml of MSVP medium and incubated at room temperature under static conditions for 20 h to serve as the inoculum for the experimental reactors. The cell density of the experimental inoculum after 20 h was 10^8 cells ml^{-1}.

Experimental apparatus. Type 316L stainless steel coupons (1.6-cm diameter; Metal Samples, Inc., Munford, Ala.) that were epoxy embedded in polycarbonate tubes (1.9 cm [diameter] by 10 cm) were exposed to 600 ml of the test medium in polycarbonate reaction vessels (12 cm [diameter] by 10 cm). Each reactor contained 18 coupons. The coupons were mounted in the reactors such that only one face was exposed to the test medium, and electrical connection was made to the coupons by fixing conductive copper tape to the unexposed side. The coupons were abraded and washed as previously described (10) and mounted through the reactor lids with the exposed coupon face oriented downward to prevent suspended solids from settling onto the surface. In this orientation, each coupon face was equidistant (2.5 cm) from the reactor bottom and the solution surface.

E_{corr} for each coupon was measured hourly by computer against the SCE with a high impedance (>10^{11} Ω), 64-channel, analog-to-digital converter (Keithley, Taunton, Mass.). A single SCE reference electrode was connected to each of three reactors through salt bridges filled with 1 mM Na_2SO_4 solution. The salt bridges made contact with medium in the reactors through Vycor glass tips (EG & G, Princeton, N.J.) which served as a bacterial barrier to prevent reference electrode filling solution from contaminating the reactors. The salt bridges introduced a second electrical junction in the circuit used to measure E_{corr}, producing an offset of roughly −30 mV. This offset was determined before and after each experiment and was subtracted from measured E_{corr} values to give E_{corr} versus the SCE.

The reactors were filled with 600 ml of mineral salts medium and autoclaved at 121°C and 16 lb/in^2 for 20 min. Appropriate amounts of filter-sterilized vitamin, pyruvate, and Mn(II) solutions were then added to the cooled medium in the reactors. Autoclaved salt bridges and coupons which had been soaked for 1 min in 70% ethanol and UV irradiated for 12 h were mounted in the reactors and connected to the analog-to-digital converter. The absence of bacteria in the uninoculated control experiment, determined by direct cell counts with the epifluorescence microscopy technique described below, confirmed that this treatment maintained solution sterility. Coupons were exposed to the MSVP medium for ca. 24 h to attain stable values of E_{corr} before initiation of the ennoblement experiments.

Ennoblement experiments. The ennoblement hypothesis was tested by exposing the coupons to three treatments, with each treatment performed in triplicate. Where error bars are shown or reported, they refer to 1 standard deviation about the mean of the triplicate measurements. Coupons were exposed to inoculated MSVP medium in the presence and absence of Mn (treatments 1 and 2) and to uninoculated MSVP medium containing Mn (treatment 3). For the inoculated treatments, 20 ml of inoculum was repeatedly aspirated through a 22-gauge needle attached to a 10-ml syringe to break up cell aggregates and produce a uniform cell suspension (14). Each reactor was inoculated with 6 ml of the aspirated cell suspension and stirred for 1 min to distribute the cells. Thereafter, the reactors were stirred for 1 min prior to each sampling but were otherwise left unstirred. After inoculation, or after Mn addition for the cell-free control treatment 3, E_{corr}, Mn(II), attached cell density, and surface-bound manganese abundance were measured at intervals over a 36-h period. The pH for all treatments was between 7.1 and 7.2, the temperature was 25 ± 2°C, and the initial cell densities in the reactors for treatments 1 and 2 were (1.0 ± 0.3) × 10^6 and (1.0 ± 0.1) × 10^6 cells ml^{-1}, respectively.

Dissolved Mn. The dissolved Mn(II) concentration was measured by the formaldoxime method (6). At each sampling interval, 1.5 ml of solution was removed from each reactor and filtered through a 0.2-μm-pore-size cellulose acetate filter to remove suspended solids. Mixed ammonia-formaldoxime reagent (1.5 ml) was added to 0.5 ml of each sample and incubated for 30 min, and the sample absorbance at 450 nm was measured with a Shimadzu 2101PC spectrophotometer. The spectrophotometer was calibrated with Mn(II) solutions that were previously standardized against atomic absorption standard Mn(II) (Fisher Scientific).

MnO_x deposits. The manganese concentration on the coupon surfaces, hereafter referred to as MnO_x ($x \sim 1.7$) (3), was monitored by removing a coupon from each reactor at each sampling interval and reductively dissolving the MnO_x with sodium sulfite (10). The coupon was soaked for 30 min in 5.0 ml of stirred 0.5 M Na_2SO_3 (pH 7.3), the solution was filtered (0.2-μm-pore-size cellulose acetate), and the Mn(II) concentration was determined as described above. For these measurements, analytical standards were prepared in 0.5 M Na_2SO_3 to correct for a 10 to 20% increase in sensitivity caused by the presence of sulfite. Although the reductive dissolution of manganese does not distinguish solid manganic oxides from Mn(II) adsorbed onto the oxide phase, previous work (1, 22) indicates that more than 90% of the total dissolved Mn comes from the oxide fraction. This is important in relating surface manganese abundance to the amount of MnO_x required to produce ennoblement since only manganese in oxidation states above 2 behaves as a galvanic cathode.

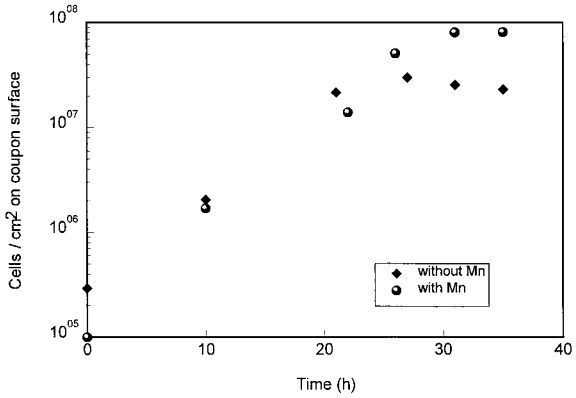

FIG. 1. Influence of Mn(II) on attached cell density for stainless steel coupons exposed to *L. discophora*. Initial Mn(II) = 200 μM.

Microbial colonization. Cells were removed from the coupon surface by scraping with a sterile razor blade, suspending the scrapings and washing the coupon surface in three 100-μl aliquots of sterile ammonium oxalate-glutaraldehyde reagent (28 g of ammonium oxalate, 15 g of oxalic acid, and 0.1 ml of glutaraldehyde per liter), and transferring the washings to a vial containing 4.8 ml of the ammonium oxalate-glutaraldehyde reagent. After extraction for at least 1 h, the solution was aspirated five times through a 22-gauge needle attached to a 10-ml syringe to suspend and break up cell aggregates. The cells were then stained with acridine orange, filtered onto 0.45-μm-pore-size polycarbonate membranes, and counted by a modification of the epifluorescence microscopy technique (19) as described previously (26).

RESULTS AND DISCUSSION

Bacterial colonization. The number of cells attached to the coupon surface in Mn(II)-containing media increased exponentially at a rate of 0.31 doublings h^{-1} during the first 31 h (Fig. 1) and reached an apparent maximum density of 8 × 10^7 cells cm^{-2} after 31 h. This attachment rate is in reasonable agreement with that reported by Adams and Ghiorse (2) (0.08 to 0.15 doublings h^{-1}) for *L. discophora* grown on glass slides. In medium without Mn(II), the rate was similar (0.26 doublings h^{-1}); however, the maximum cell density, attained after 27 h, was lower by 63%. Submillimolar Mn(II) concentrations have been previously shown to either stimulate (16) or repress (1) the maximum cell yields of *Sphaerotilus* and *Leptothrix* spp. during batch culture. No cell attachment occurred for cell-free treatment 3.

A mucilaginous biofilm was readily apparent on the coupon surfaces after 20 h of cell growth. Formation of such a biofilm, in which cathodically active MnO_x can be deposited close to the metal substratum, is considered a prerequisite for ennoblement, since electron transfer can take place only when MnO_x is in electrical contact with the metal.

Manganese uptake and deposition. After 21 h of exposure to the SP-6 culture in Mn(II)-containing medium, coupon surfaces were dark brown and were coated with a biofilm ca. 100 μm in thickness. Manganese uptake from solution and deposition on the coupons began during the exponential phase of cell growth, continued through the stationary phase, and correlated with attached cell density (Fig. 1 and 2). The Mn(II) concentration in the uninoculated control remained unchanged, and no MnO_x accumulated on the coupon surfaces. These results confirm that chemical oxidation of Mn(II) did

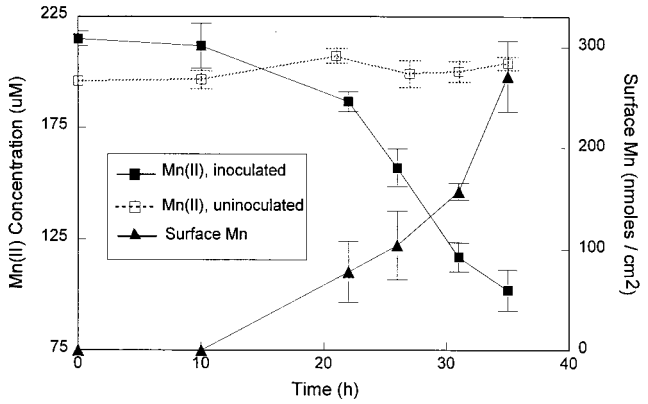

FIG. 2. Time dependence of dissolved Mn(II) concentration and surface manganese abundance on stainless steel coupons. Surface manganese for the uninoculated control treatment 3 was undetectable and is not shown.

not contribute to MnO_x deposition and that Mn mineralization was caused by the bacteria.

The Mn(II) uptake rate at the end of the experiment was 3.7 ± 0.8 μM h^{-1} [$(3.5 \pm 1.1)\%$ h^{-1} based on the final Mn(II) concentration], and the MnO_x deposition rate was 28 ± 13 nmol (Mn) cm^{-2} h^{-1}. After 36 h, 270 ± 42 nmol (Mn) cm^{-2} had accumulated on the coupons, and 68 ± 7 μmol of Mn(II) had been removed from solution.

An exact mass balance on Mn uptake and deposition requires the assumption that deposition on the polycarbonate coupon holders and reactor walls was the same as that for the stainless steel coupon surfaces. If this assumption is made, deposition [400-cm^2 reactor area by 270 nmol (Mn) cm^{-2}] agrees with loss from solution within a factor of 1.5, suggesting that MnO_x formation occurred primarily on reactor surfaces. This finding is consistent with reports that MnO_x is preferentially deposited by sessile compared with planktonic cells (33). The dark brown biofilm that covered the coupon surfaces and the reactor walls at the end of the experiment clearly indicated that much of the MnO_x had accumulated as an attached surface deposit.

Effect of bacteria and MnO_x on ennoblement. Figure 3 shows E_{corr} over time for the three experimental treatments. In inoculated media containing Mn(II), E_{corr} began to increase after 10 h, exceeded +300 mV_{SCE} after 25 h, and approached +350 mV_{SCE} at the end of the experiment. The maximum rate of increase was 48 mV h^{-1} between hours 16 and 21, after which the rate diminished rapidly to 4 mV h^{-1} for times greater than 25 h. The increase in E_{corr} began with the onset of MnO_x deposition and cell growth, and increasing E_{corr} corresponded to increases in both cell density and surface MnO_x up to potentials of ca. +300 mV_{SCE} (Fig. 4). In contrast, little change in E_{corr} was observed for coupons exposed to uninoculated medium containing Mn(II) or to inoculated medium without Mn(II). The small changes that were observed in the control treatments are of the magnitude commonly observed in abiotic medium and are usually attributed to changes in passivation current that occur as the passive film ages (15). For treatment 2 [no Mn(II)], the lower cell density cannot explain the absence of ennoblement since E_{corr} increased no more than 50 mV at a maximum cell density of 3×10^7 cells cm^{-2} while this cell density corresponded to an increase of more than 350 mV for cells grown with Mn(II).

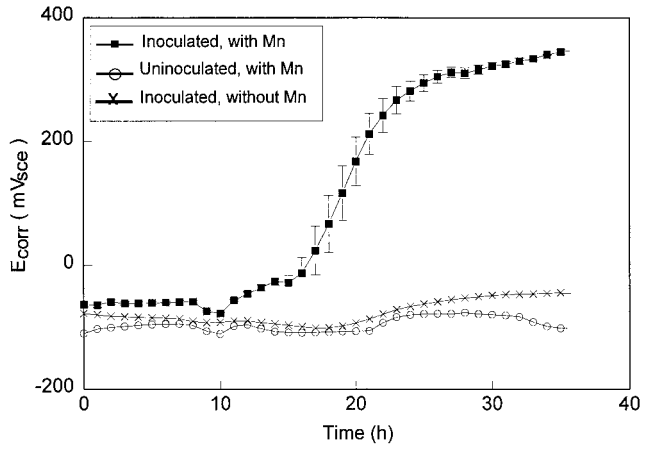

FIG. 3. Effect of *L. discophora* grown in medium containing Mn(II) on E_{corr} for stainless steel. Error bars for the lower two curves are less than symbol size.

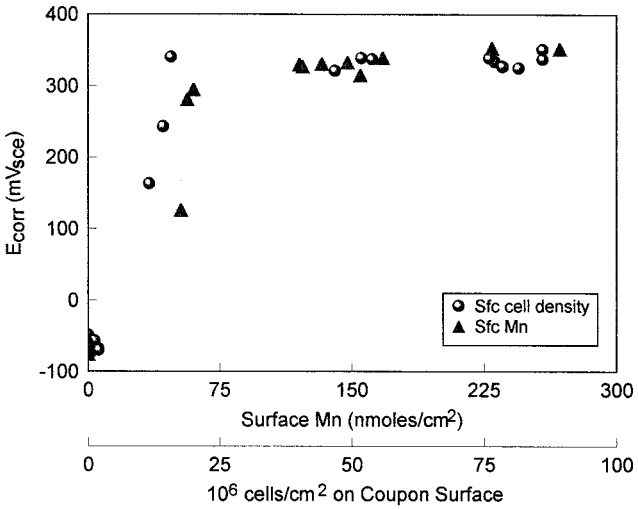

FIG. 4. Relationship among E_{corr}, surface manganese, and attached cell density for stainless steel coupons exposed to inoculated medium containing Mn(II).

Figure 4 illustrates the relationship among ennoblement, biofilm formation, and MnO_x deposition. E_{corr} increased ca. 5 mV per nmol of MnO_x cm^{-2} up to +300 mV_{SCE} and was shifted to an ennobled value of +330 mV_{SCE} by 75 nmol of MnO_x cm^{-2}. Deposition of additional MnO_x caused little further change in E_{corr}. These results are similar to findings for stainless steel exposed to Bozeman streamwater in which 15 nmol of MnO_x cm^{-2} ennobled E_{corr} to +350 mV_{SCE} while additional MnO_x deposition caused E_{corr} to increase no further (12). Variation in the amount of MnO_x required for ennoblement in the two studies may be attributable to the different characteristics of the MnO_x deposits. In Bozeman streamwater, MnO_x occurred as 3- to 4-μm-thick deposits that adhered tightly to the coupon surface. In the present work, the sheaths of SP-6 resulted in easily dislodged sessile mats in which MnO_x deposition occurred as far as 100 μm from the substratum. We speculate that a greater fraction of the MnO_x in the present study was not in electrical contact with the coupon and therefore did not influence E_{corr}.

The relationship shown in Fig. 4 supports our previous finding that surface MnO_x deposits shift E_{corr} to the reduction potential for the MnO_x phase (10). Data from Fig. 2 and 3 demonstrate that bacterial MnO_x deposition induces this pro-

cess and confirm that manganese biomineralization by *L. discophora* results in ennoblement.

The proposed half-reaction that fixes E_{corr} is

$$MnO_2 + H_2O + e^- \leftrightarrow MnOOH + OH^- \quad (1)$$

which has a reduction potential of $+335\ mV_{SCE}$ at pH 8.0 (10). At the pH used in this study (7.2), the reduction potential would be $+382\ mV_{SCE}$, ca. 30 mV higher than that observed. This difference is expected to decrease with increasing exposure time. It has been reported that the Mn oxidation state for MnO_x formed by *L. discophora* increases as the oxide ages, from a value of 3.32 after 11 h to 3.62 after 30 days (3). Such a change would increase the oxidizing power of the oxide and thus shift the reduction potential to more positive values. Figure 3 shows clearly that E_{corr} continued to increase slowly at the end of the experiment, suggesting that the final potential would have been higher than $+350\ mV_{SCE}$. Many reports on ennoblement in natural waters show an increase in E_{corr} to values greater than $+300\ mV_{SCE}$ over a period of a few days followed by a gradual increase during the ensuing few weeks (4, 20). Such findings are consistent with an increase in Mn oxidation state that would occur as microbially deposited MnO_x ages.

Relationship to ennoblement in natural waters. Partitioning of MnO_x between suspended cells and sessile cells is a key issue in determining the effectiveness of natural populations of manganese-oxidizing microorganisms in promoting ennoblement. Mn(II) uptake has been measured widely in natural waters, and microbially mediated Mn(II) oxidation has been found to be the dominant Mn(II) removal mechanism (21, 28, 36, 39). These experiments are most often carried out in acid-leached containers, and Mn(II) is recovered primarily as MnO_x on suspended particulate material. The degree to which the manganese-oxidizing activity would be concentrated at untreated metal surfaces by sessile cell growth in these same waters has not been investigated. However, the tendency for biofilms to form under these conditions is undisputed, and it has been reported elsewhere that sessile growth greatly enhances the manganese oxidation rate (33). In light of this, at least part of the manganese-oxidizing activity in natural waters would be expected to reside near the metal surface. Moffett (29) reported Mn(II) uptake rates of 0.6 to 3.2 nM h^{-1} (0.2 to 4% h^{-1}) for Mn(II) concentrations between 20 and 1,000 nM in the surface waters of Vineyard Sound, Mass., and similar rates were observed in the shallow waters of the Newport River estuary, N.C. (36). The rates indicate daily MnO_x deposition of 14 to 75 nmol $liter^{-1}$. In the shallow waters of the northern Chesapeake Bay, Md., daily rates as high as 4,000 nmol $liter^{-1}$ have been estimated (28). If even a few percent of this MnO_x deposition were to occur at the surface of metal coupons, MnO_x levels of ca. 15 to 75 nmol cm^{-2}, required to induce ennoblement, would be reached within a few hours to a few months. This time scale agrees with the period of a few days to ca. 1 month (4) required for ennoblement to develop on metals exposed to oxic natural waters.

Manganese-oxidizing bacteria are widely distributed in nature and cause a variety of industrial problems ranging from dirty drinking water (35) to fouled heat exchangers (18). Numerous reports during the past 2 decades have also associated these bacteria with stainless steel corrosion (23, 25, 37, 38), but the biological role in the corrosion process has never been established. Over roughly the same period, the ennoblement phenomenon and its corrosive consequences have become recognized (4, 15). The results presented in this paper link these separate observations to a common cause, biomineralization of MnO_x, and support the biological mechanism of ennoblement. Based on these findings, ennoblement and the associated risk of corrosion must be added to an already extensive list of industrial problems caused by manganese-oxidizing bacteria.

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