Chemical effects of biofilm colonization on 304 stainless steel

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Changes in the surface concentrations of the main alloying elements of an as-received 304 stainless steel, exposed to a mixed culture of biofilm-forming bacteria under flowing conditions, were observed using Auger electron spectroscopy. In the oxide film close to the bulk stainless steel, there was an enrichment in the relative concentration of Cr with a corresponding decrease in the relative Fe concentration as compared to a control coupon exposed only to sterile media. There were no changes observed in the relative Ni concentration.

I. INTRODUCTION

Accumulation of microorganisms and their metabolic products on metal surfaces has been proposed to contribute to localized attack or pitting corrosion under certain conditions.[1–3] The mechanisms through which microorganisms participate in pitting corrosion, however, remain obscure. Much of the chemical evidence linking pitting corrosion to microbial processes is based on data obtained from surface deposits that have accumulated long after microorganisms exert their initial effects. Biofilm-forming bacteria are likely to induce subtle effects on the underlying metal surfaces during early stages of colonization and biofilm formation.

Detection of near-surface chemical changes mediated by biofilms requires surface-sensitive analytical techniques. Auger electron spectroscopy (AES) obtains elemental information from the top 1–6 nm of the metal surface where biofilm microbial processes are likely to have their greatest influence. Few studies have combined AES and other surface spectroscopic analyses with biological analyses. Bruemmer[4] used AES to characterize grain boundary composition of the bulk. Geesey *et al.*[5] showed that some film-forming bacteria selectively colonized the surface oxide of channels of 316L stainless steel (described below) using AES. Compositional changes of the main alloying elements (Fe, Cr, and Ni) in the passive film of the 316L stainless steel have been studied in a borate–boric acid buffer. Depending on the conditions in which the oxide layer was formed on the surface, enrichment or depletion of Cr and a corresponding depletion or enrichment of Fe have been reported.[6]

The objective of this work is to determine the role that the bacteria play on the initial stages of biocorrosion involving surface Fe, Cr, and Ni concentrations of as-received stainless steel.

II. METHODS AND MATERIALS

A. Sample preparation and treatment

Two unpolished 304 stainless steel coupons (6.4×12.7 mm^2, 0.5 mm thick) were cut from the same sheet metal of 20 gauge (0.5 mm) with a 2B finish (''as-received''). The sheet metal was obtained from Metal Samples, Munford, Alabama. The manufacturing procedure of the sheet metal is as described in the article by Geesey *et al.*[5] The surface topography of these as-received coupons is highly nonisotropic as seen from the atomic force microscopy (AFM) image in Fig. 1. Here we will refer to the flat surfaces in the figure as ''grains'' and to the deep boundaries surrounding each grain as ''channels.'' These are the features seen on the surface. The AFM line scan showed that the channels have a V-shaped cross section with about 1–1.5 μm depth and width. The surface topography is a result of the manufacturing process of the stainless steel sheet metal.

A line pattern was marked on the surface of the two coupons in order to facilitate the identification, study, and moni-

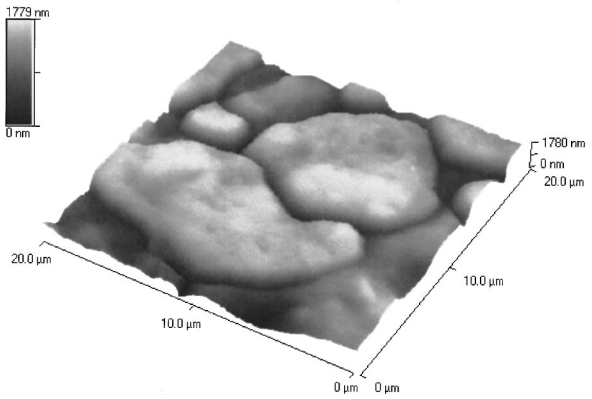

FIG. 1. Three-dimensional atomic force microscope (AFM) image and of an as-received 304 stainless steel showing the grains and channels. The depth and width of the channels are about 1–1.5 μm.

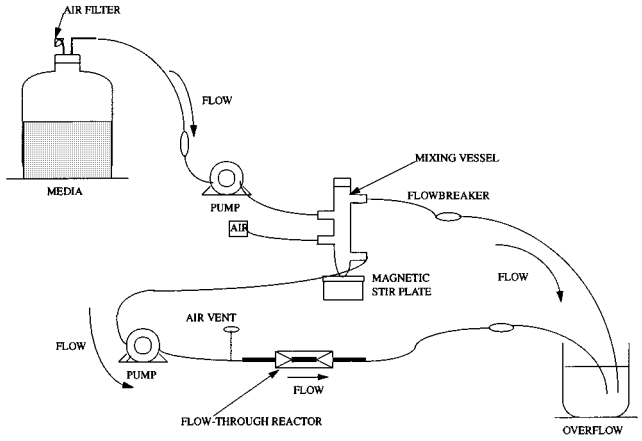

FIG. 2. Schematic of the experimental setup of the flow-through reactor system.

toring of common areas by different microscopy techniques. The two coupons were subjected to ultrasonic cleaning using acetone, after which Auger analysis was performed on preselected areas (100×100 μm^2, large enough for Auger analyses to be done as well as to be identified using the confocal microscope). One of the coupons was exposed to sterile abiotic medium, henceforth referred to as the ''control coupon,'' and the other coupon was exposed to medium containing a defined mixed culture of bacteria (described below), henceforth referred to as the ''biocoupon.''

After initial Auger analysis, the biocoupon was glued (using Torr Seal epoxy) in the flow-through reactor (Fig. 2, described below) and exposed to bacteria and medium for 18 days. Each experiment was accompanied by a control run, where the control coupon was exposed to sterile medium for 18 days. The coupons were transported in air to the reactor for the control and biofilm experiments. At the end of each experiment the biocoupon was cleaned using a cotton swab to remove the biofilm from the surface of the coupon and then rinsed with distilled water. The same treatment was applied to the control coupon.

B. Flow-through reactor

The flow-through reactor consisted of a polycarbonate housing (5 mm wide, 1 mm deep, and 24 mm long) with a viewing window that consisted of a rectangular coverslip (60×24 mm^2). The coverslip sealed the housing by tight contact with the flat face of the polycarbonate holder. The reactor was steam sterilized for 20 min at 121 °C. After sterilization and assembling the reactor system shown in Fig. 2, sterile medium were pumped through this reactor using a Masterflex pump at a flow rate of 1.6 ml/min. The approximate residence time of the system was maintained at 15 min in order to minimize suspended cell growth. The inoculum was injected into an aerated mixing vessel located upstream from the flow-through reactor.

C. Inoculum and media

A mixed culture consisting of *Pseudomonas aeruginosa*, *Pseudomonas fluorescens*, and *Klebsiella pneumoniae* that had been previously isolated from a corrosive biofilm[7] was used in all experiments. The bacteria were inoculated into the system from 24 h batch cultures grown in a minimal salts medium [4.0 mM K_2HPO_4, 2.2 mM KH_2PO_4, 0.76 mM $(NH_4)_2SO_4$, 0.041 mM $MgSO_4$, and 2.2 mM glucose]. Medium flow was initiated 24 h after inoculation so as to allow initial bacterial growth and attachment.

D. Biofilm imaging with confocal scanning laser microscopy

Confocal scanning laser microscopy (Bio-Rad MRC 600) was used to follow the colonization and biofilm formation of mixed bacterial populations on various surfaces.[7] The technique offers reflected white and/or red light capabilities to visualize surface features of opaque substrata such as metals under developing biofilms.

Biofilm growth and development in the preselected areas on the coupons was monitored daily with the confocal microscope. Images were captured and analyzed using the Bio-Rad Cosmos software. Biofilm growth was monitored until the coverslip became opaque due to thick biofilm growth. At this point (after 18 days) the biofilm experiment was terminated and the coverslip was replaced with a new one so as to record areas colonized by the biofilm clearly. The areas that were not colonized by biofilm were marked for analysis and for comparison with those areas colonized by biofilm.

E. Surface analysis using atomic force microscopy

The surface of the as-received coupons was analyzed by AFM (Topometrix TMX 2000) in the contact mode to establish the exact surface morphology of the grains and channels.

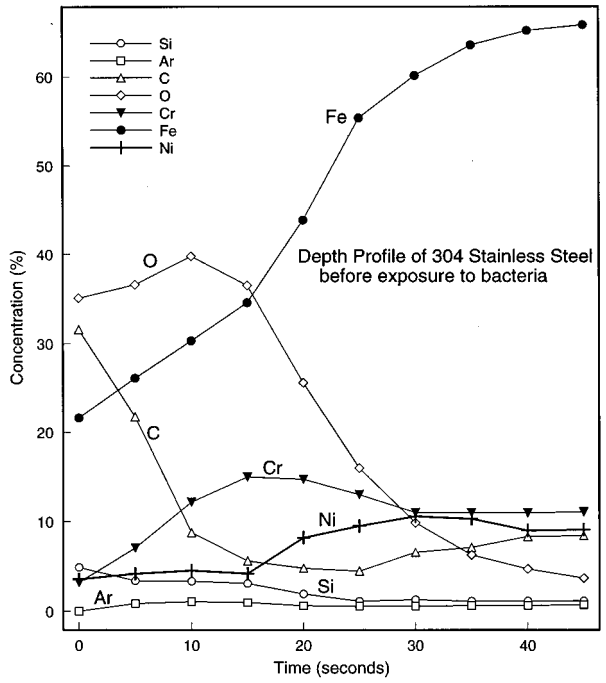

FIG. 3. Depth profile of an as-received 304 stainless steel coupon showing the distribution of the various elements on the coupon.

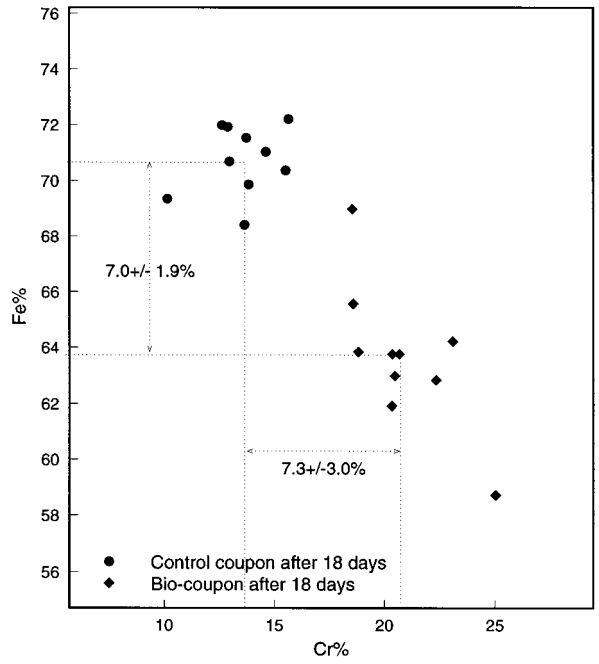

FIG. 4. Normalized Fe concentration vs the normalized Cr concentration for control coupon and biocoupon after 18 days of exposure. The dotted lines indicate the mean relative concentration for that group of data.

F. Surface analysis using Auger electron spectroscopy

Preselected areas on the biocoupon and on the control coupon were analyzed using AES (PHI 595), before exposure to bacteria or sterile medium (i.e., in the as-received condition). The coupons were initially sputtered in short time intervals using the Ar-ion sputtering technique. This was done to remove the surface contamination due to exposure to air and to remove part of the protective oxide layer until approximately 5%–14% oxygen was left on the surface in order to determine the chemical changes within the oxide layer close to the bulk stainless steel. After each sputtering an area-averaged spectrum from a 100×100 μm^2 area was taken so as to obtain depth profiles of the various elements on the surface as shown in Fig. 3. Elemental chemical maps and scanning electron microscope (SEM) images were also taken of these areas. Ten grains on the control coupon were analyzed before and after exposure to the sterile medium. The positions of these grains were noted on a SEM picture of the area. Individual grains were also analyzed on the biocoupon before and after exposure to the biofilm.

III. EXPERIMENTAL RESULTS AND DISCUSSION

Experimental results will be presented in two major groups: the first group of data were obtained from the as-received stainless steel coupons and the second group of results were obtained when the coupons were exposed for a period of 18 days to medium with and without bacteria.

Concentrations of the elements are calculated from the peak-to-peak height of each line and the sensitivity factors of each element as given in the PHI-Auger manual.[8] The concentrations of Fe, Cr, and Ni are normalized so that Fe %+Cr %+Ni %=100%; these have been referred to as relative percentages or relative concentrations.

A. As-received coupons

The mean relative concentrations of Fe, Ni, and Cr, respectively, before exposure to either bacteria or sterile medium, were 74.9%±2.4%, 12.6%±1.5%, and 12.5%±1.5%. The distribution of these concentrations varied somewhat from sample to sample and grain to grain but the values stayed within the expected statistical uncertainty in the data.

B. The 18-day exposure

Figures 4 and 5 show, respectively, the relative Fe % versus the relative Cr % and the relative Fe % versus the relative Ni % for the control coupon and biocoupon. Note that Fig. 4 shows a distinct separation between the two groups of data. There was an overall increase in the relative percentage of carbon on the biocoupon and control coupon after 18 days of exposure as compared to that before exposure. There were marked differences in the Cr and Fe relative concentrations between the biocoupon and the control coupon. Mean relative Cr concentration increased by 7.2%±1.7% in the biocoupon as compared to that of the control coupon. Correspondingly, a 7.0%±1.9% decrease was observed in the mean relative Fe concentration in the biocoupon as compared to the control coupon. There were no differences observed in the mean relative Ni concentrations between the two coupons, as seen in Fig. 5; the mean relative concentrations for the two groups are also shown.

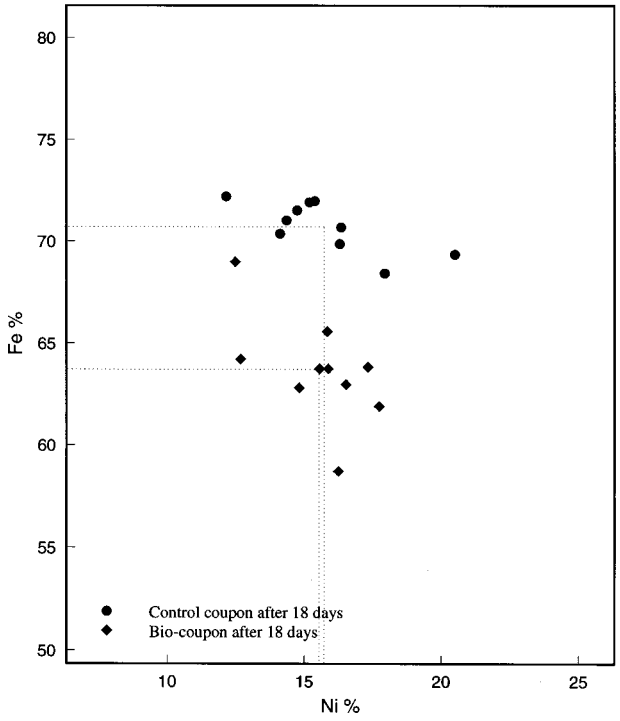

FIG. 5. Normalized Fe concentration vs the normalized Ni concentration for the control coupon and biocoupon after 18 days of exposure. The dotted lines indicate the mean relative concentration for that group of data.

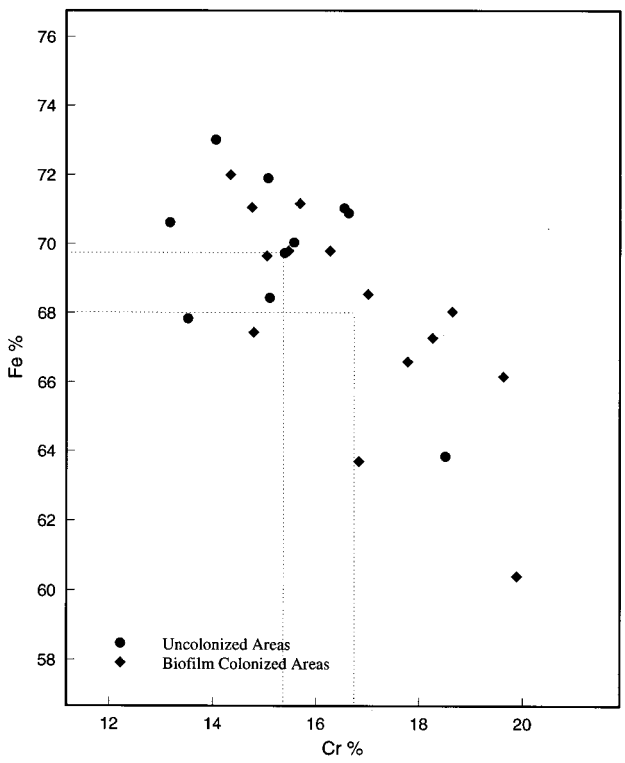

FIG. 6. Normalized Fe concentration vs the normalized Cr concentration for biofilm colonized and uncolonized areas on the biocoupon. The dotted lines indicate the mean relative concentration for that group of data.

Figure 6 shows the relative Fe % versus relative Cr % for colonized and uncolonized areas on the biocoupon selected using the confocal microscope. This comparison is similar to that done in Fig. 4, but the separation of the data groups is not as distinct as in Fig. 4.

One difficulty of analyzing the data in terms of the main components of the stainless steel was the question of how to handle the other elements such as C, O, Si, N, and P. Let us keep in mind that the main emphasis here is to determine the statistically meaningful alterations in Fe, Cr, and Ni concentrations in the oxide layer of the surface due to bacteria. This objective was difficult to achieve due to variation in the elemental composition of the surface from grain to grain and from time to time. There are several reasons for this variation. One reason was the variation in the amount of the other elements (mentioned above) present. The other reason is the noise in the spectra; particularly at high kinetic energy (Ni region) noise causes fluctuations in the Ni concentrations. Another reason is the uncertainty associated in assessing exact peak-to-peak determinations. For example, Cr and O peaks are very near each other, which causes difficulty in determining the exact peak locations.

Let X denote the sum of the absolute concentrations of the rest of the elements (Si+N+O+Ar+P+S) except for Fe, Cr, and Ni present on the surface. The absolute Fe concentration versus X for the control coupon and the biocoupon, before and after the 18-day exposure, is shown in Fig. 7. The data points fall on two distinct (dotted) lines, the top line in which no bacteria was involved and the bottom line in which the coupon was exposed to the bacteria. The spread in concentrations is due to inhomogeneities in the distribution of various elements from grain to grain and time to time, on the area of interest. These (dotted) lines are the least-square fit to the data points. These lines indicate the linear dependence of the absolute Fe concentrations on X. The same dependence on X was also observed in the case of Cr and Ni (not shown).

The linear dependence of the Fe, Cr, and Ni on X suggests that the relative concentrations of Fe, Cr, and Ni do not change with the accumulation of other elements on the surface. This implies that X can be eliminated from the analysis by simply considering the relative concentration of Fe, Cr, and Ni. Thus, these concentrations are normalized so that Fe %+Cr %+Ni %=100%; they have been referred to as relative percentages and are shown in Figs. 4, 5, and 6.

In order to determine whether the differences observed in the mean relative percentages of Fe, Cr, and Ni on the biocoupon and the control coupon (Fig. 4) were statistically significant, a bivariate analysis of variance test[9] was conducted. The calculations were accomplished using the statistical analysis system (SAS) procedures PROC MANOVA and PROC DISCRIM.[10] The same statistical analysis was applied to the data for the colonized and uncolonized areas on the biocoupon. The main result of the test is a probability value (p). A small p value indicates that the data contradict the null hypothesis that the true mean of the relative concentrations (of the alloying elements) on the control coupon is identical to the true mean of the relative concentrations on the biocou-

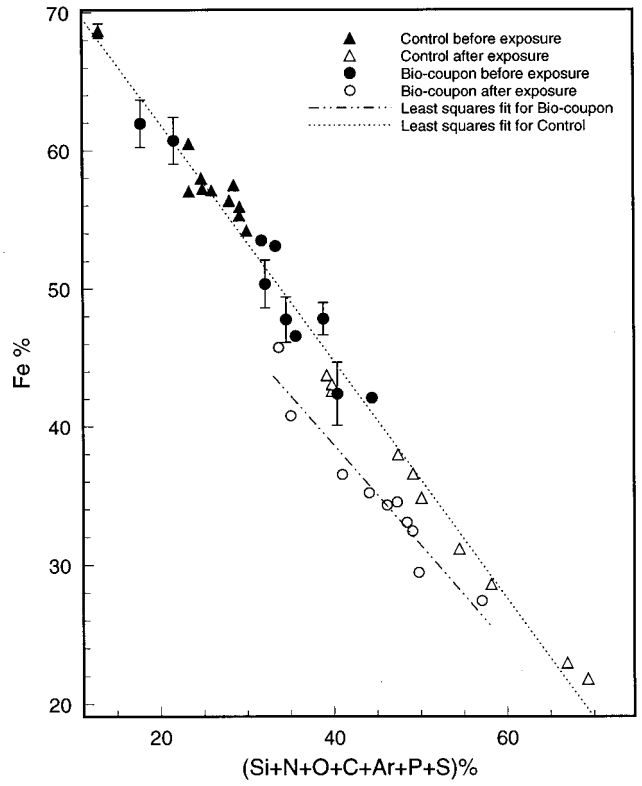

FIG. 7. Absolute Fe concentrations vs the sum of the absolute concentrations of all other elements (except Cr and Ni) present on the control coupon and biocoupon. The dotted lines indicate the linear least-square fit to the data.

pon. The true mean is the conceptual mean of the relative concentration if relative concentrations were measured on an infinite number of identically treated grains. For a 95% confidence on the test, the value of p should be less than 0.05 to conclude that there is a statistically significant difference between the means being compared.

Table I shows the mean relative concentrations for the control coupon and the biocoupon, after 18 days and the corresponding p value ($p=0.001$). We can conclude that there is a statistically significant difference between the Fe and Cr concentrations on the control coupon and the biocoupon after 18 days. The mean concentrations of Ni between the two coupons are not significantly different. The mean relative percentages of colonized and uncolonized areas on the biocoupon after 18 days and the corresponding p value ($p=0.17$) for this comparison are shown in Table II. We can conclude that there is no significant difference between the colonized and uncolonized areas on the biocoupon. The reasons for this may be that these areas are several 100 μm outside the area of interest. Hence, the sputtering during the preanalysis (before exposure) will be inadvertently less in those areas, creating different levels of removal of the passive layer present on the surface. This will give rise to variations in the passive layer present on the surface, creating a different environment for the bacteria on the various areas.

TABLE I. Comparison of mean relative concentrations of Fe, Cr, and Ni between the control coupon and biocoupon after 18 days; $p=0.001$.

Element	Control coupon after 18 days	Biocoupon after 18 days
Mean Fe (%)	70.7±1.2	63.7±1.7
Mean Cr (%)	13.6±1.5	20.8±2.0
Mean Ni (%)	15.7±2.2	15.5±1.7

TABLE II. Comparison of mean relative concentrations of Fe, Cr, and Ni between the colonized and uncolonized areas on the biocoupon after 18 days; $p=0.17$.

Element	Uncolonized area after 18 days	Colonized area after 18 days
Mean Fe (%)	69.7±2.6	67.9±3.0
Mean Cr (%)	15.4±1.6	16.9±1.8
Mean Ni (%)	14.9±2.3	15.2±2.1

Another reason for the lack of differences between Fe, Cr, and Ni relative concentrations in Table II could be that the bacteria may have initially attached to an area on the biocoupon and then subsequently detached from it. This could lead to the idea that a certain area was uncolonized when viewed (under the microscope) after this detachment process. The presence of the bacteria earlier would have lead to chemical changes on the so-called uncolonized areas.

The presence of excess carbon on both samples after the 18-day exposure could be partly due to the bacteria and organic molecules present in the media and partly due to some contamination from air. The mechanisms of Cr enrichment and Fe depletion are under investigation. One speculation is that Fe may have been carried away by the flowing media and that Cr compounds may have been redeposited on the surface.

IV. CONCLUSIONS

Biofilm causes chemical changes on 304 stainless steel surface in the oxide film close to the bulk. These changes can be summarized as follows: in a normalized scale where relative concentrations of Cr, Fe, and Ni add up to 100%, the concentration of Cr is enriched by 7.2%±1.7% relative to that of the control coupon. The relative concentration of iron is depleted by 7.0%±1.9%. The relative Ni concentration showed no noticeable change (0.2%±1.6%). No statistically significant differences were observed in the relative Cr, Fe, and Ni concentrations between the colonized and uncolonized areas on the biocoupon. We are currently investigating these issues further using time of flight secondary ion mass spectrometry, x-ray photoelectron spectroscopy, and powder x-ray diffraction spectroscopy with thin film analysis capabilities.

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