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Bacterial sulfate production by biodesulfurization of aromatic hydrocarbons, determined by ion chromatography[1]

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Abstract

The use of bacteria to remove sulfur from crude oil or petroleum distillates is a novel concept that presents an alternative biotechnology to the current technology of hydrodesulfurization (HDS). Sulfur must be removed from crude oils prior use. The burning of fossil fuels containing sulfur releases sulfur dioxide into the atmosphere causing acid rain. The aim of this work is to determine the sulfate concentration by ion chromatography (IC), and calculate the percentage of transformation of organic bound sulfur, that is converted to sulfate, and estimate the efficiency of bacteria in desulfurization. IC is a suitable method for sulfate concentration determination. However, when chloride concentrations are significantly high, interference of the sulfate signal does occur. In this case, it could be avoided by diluting samples. A Dionex Model 2000i/SP IC system, with an anionic pre-column (Dionex AG4A), an anion separator column (Dionex AS4A), a suppresor column (Dionex AMMS-II), and a conductivity detector was used. The eluent (21 m*M* NaOH) and regenerant (electrolyzed 18 MΩ/cm water) flow-rates were 1.0 and 2.0 ml/min, respectively. The sample loop volume was 10 μl and the conductivity sensitivity was 30 μS. The diluted samples were filtered through a 0.45-μm filter before injection. The highest sulfate concentration detected was 24.10 mg/l, corresponding to a maximal conversion rate of 10% in a month. Sulfate ions were not detected in control samples. The correlation coefficient for a linear least squares fit was 0.99 ($p<0.001$). The minimal concentration that we can read was 0.02 mg/l and this concentration corresponded to the limit of detection obtained under the conditions employed in this study. IC is an economical, sensitive and accurate way to estimate the sulfate concentrations in microbiological samples.

Keywords: Petroleum; Oils; Dibenzothiophene; Sulfur compounds; Thianthrene; Sulfates; Hydrocarbons, aromatic

1. Introduction

Petroleum contains sulfur compounds that represent the predominant species in heavy oils, such as bitumen. A variety of sulfur-containing organic compounds are found in fossil fuels. Heavy oils and bitumens contain 3 to 6% sulfur. Most of the sulfur present in heavy oils is organically bound, and is found in structures such as thiol, sulfide, disulfide, thiophenic forms, substituted benzothiophenes and dibenzothiophenes and more complex molecules like thianthrene [1].

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Objectionable quantities of sulfur must be eliminated before being acceptable as refinery feedstock. The burning of fossil fuels containing sulfur releases sulfur dioxide into the atmosphere, causing acid rain. Conventional physical and chemical desulfurization technologies (hydrodesulfurization) are energy intensive, expensive and do not remove organically-bound sulfur [2].

Bacteria have been identified that can transform organosulfur compounds into lower-molecular-mass sulfur-containing products by partially consuming the carbons.

Different strains of bacteria have shown that they are capable of metabolizing dibenzothiophene, in most cases through partial degradation, since the thiophene ring remains untouched (Fig. 1) [2–4]. Other bacteria have the ability to selectively remove sulfur from organosulfur and transform it to sulfate, leaving the carbon skeleton almost intact [5,6]. Selective sulfur removal by an oxidative microbial attack on dibenzothiophene has been reported. Numerous aerobic microorganisms convert dibenzothiophene to 2-hydroxybiphenyl, 2,2′-dihydroxybiphenyl (2-phenylphenol), with the liberated sulfur converted into sulfate (Fig. 2), which can be subsequently assimilated by the microorganism, or liberated into culture medium. This latter group of organisms is able to remove sulfur from organosulfur compounds without causing a significant loss in the calorific value of the fuel material, an important economic consideration.

Studies demonstrating organic sulfur removal by dibenzothiophene-degrading bacteria have been published [7]. Many of these have shown the metabolic pathway of biodesulfurization of dibenzothiophene, but do not pay attention to sulfate quantification. In

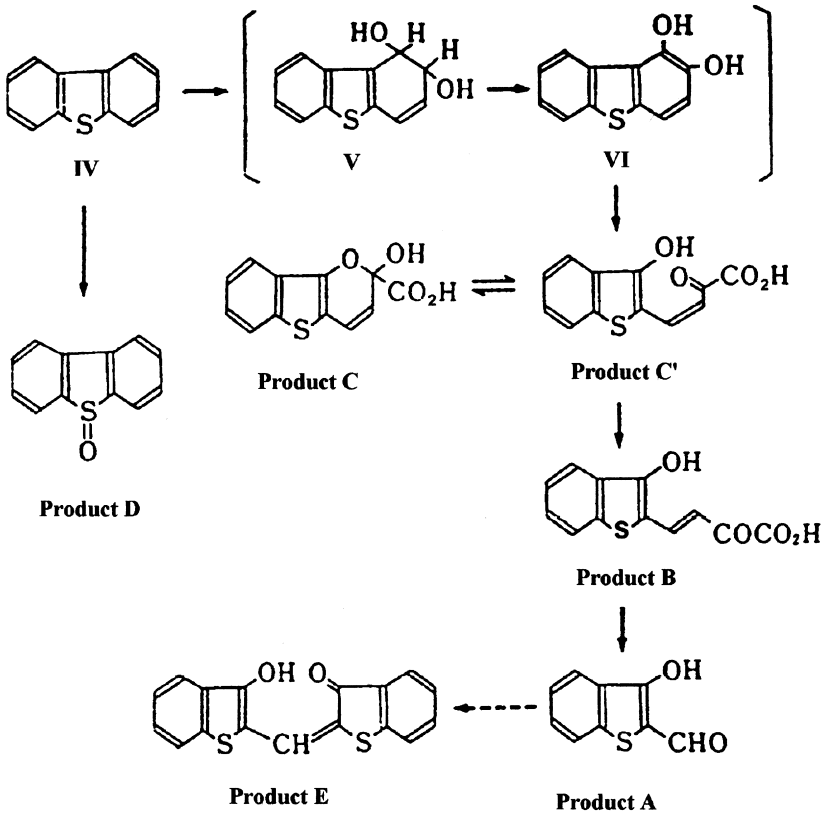

Fig. 1. Proposed pathway of dibenzothiophene metabolism [4].

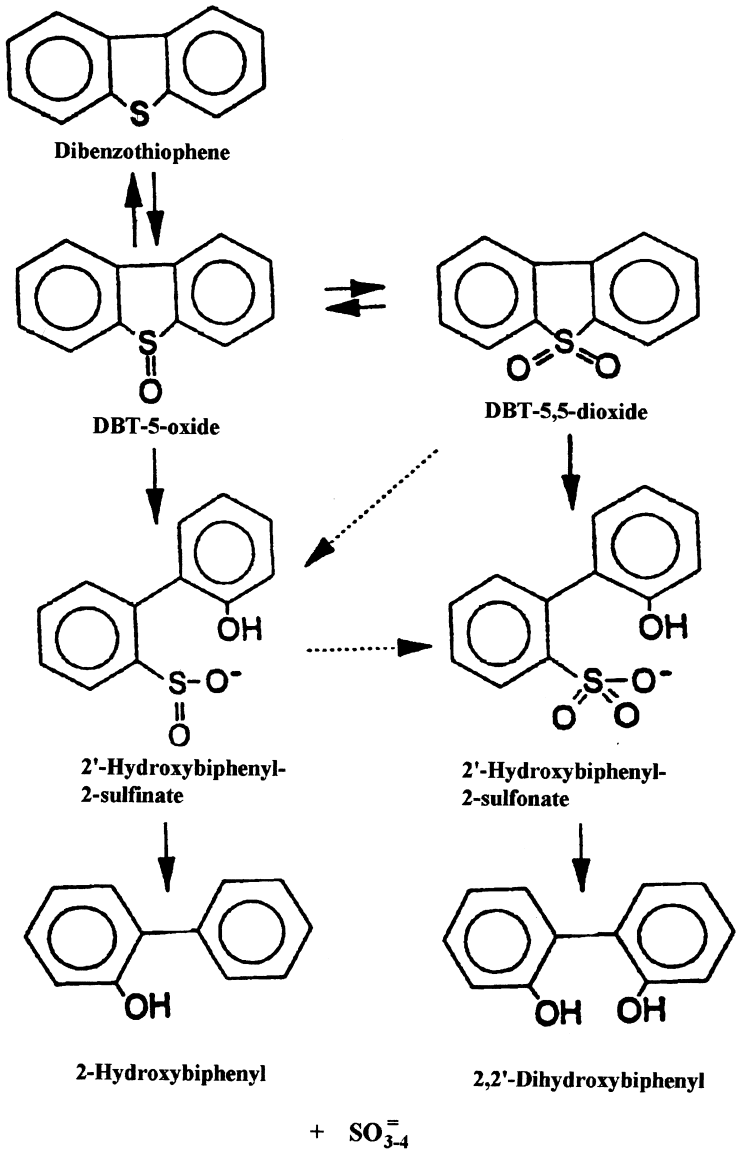

Fig. 2. Pathway of dibenzothiophene biodesulfurization [6].

other cases, the studies have underestimated the contribution (by mineralization) of the bacterial sulfate pool.

Traditionally, sulfate methods have been based on turbidimetry. However, there are several drawbacks associated with this technique, especially that of low sensitivity. Ion chromatography (IC) is particularly suitable for this purpose, because of its versatility. Its attributes include speed and low maintenance costs.

The aim of this study is to establish the presence and level of production sulfate, determined by IC, as evidence of bacterial biodesulfurization of aromatic compounds.

IC has been the method of choice for the determination of sulfate concentration but its determination, using an AS11 column, becomes almost

impossible, owing to peak overlapping in the presence of high chloride concentrations. Chloride interference can easily be reduced by the dilution step and the addition of a matrix of culture medium to standards.

2. Experimental

2.1. Enrichment and isolation of organisms

The bacteria were isolated from soils and waters contaminated with heavy crude oils. The microbial populations were selectively enriched to adapt them to organic sulfur compounds like dibenzothiophene and thianthrene. The only sources of sulfur were the dibenzothiophene and thianthrene, and the carbon sources were glucose and ethanol.

The theory of enrichment culture is simple. The compound to be metabolized is supplied as the growth-limiting, and usually sole source of essential nutrient in a culture-medium. Only the organism(s) with the necessary degradative ability will grow significantly under these conditions, and these organisms will outgrow the very large number of other organisms also added at the start of the experiment [8].

Bacteria from genuses: *Bacillus*, *Staphylococcus* and *Sporosarcina*, with the capacity to utilize the sulfur from thianthrene and dibenzothiophene and produce sulfate from biodesulfurization, were isolated from the soil.

2.2. Media and growth conditions

The medium is sulfur-free synthetic medium containing, 5 g of glucose, 0.5 g of KH_2PO_4, 4 g of K_2HPO_4, 1 g of NH_4Cl, 0.2 g of $MgCl_2 \cdot 6H_2O$, 0.02 g of $CaCl_2$, 0.01 g of NaCl, 10 ml of metal solution and 1 ml of vitamin mixture in 1000 ml of distilled water. The solution of metals contained per liter: 0.5 g $FeCl_2 \cdot 4H_2O$, 0.5 g $ZnCL_2$, 0.5 g $MnCl_2 \cdot 4H_2O$, $Na_2MoO_4 \cdot 2H_2O$, 0.05 g $CuCl_2$, 0.05 g Na_2WO_4 and 120 mmol HCl in 1000 ml of distilled water. The vitamin solution contained per liter: 400 mg of calcium pantothenate, 200 mg of inositol, 400 mg of niacin, 400 mg of pyridoxine hydrochloride, 200 mg of *p*-aminobenzoic acid and 0.5 mg of cyanocobalamin. This medium, without a sulfur source was sterilized at 121°C for 15 min [5]. The sulfur sources (dibenzothiophene and thianthrene) were dissolved with ethanol. The final concentrations of dibenzothiophene and thianthrene were 13.5 m*M* and 13.5 m*M* respectively (0.025% dibenzothiophene and 0.025% of thianthrene).

All glassware was scrupulously cleaned with HCl to prevent spurious growth on contaminated sulfur.

Chemicals used to prepare growth medium were of the highest degree of purity. High purity of chemicals is essential to prevent spurious enrichments.

The selected strains showed a positive growth in solid medium supplemented with dibenzothiophene, thianthrene, and the mixture of both compounds and they did not grow without a sulfur source.

For the biodesulfurization experiment, the bacterial strains were transferred to Erlenmeyer flasks with 20 ml of growth medium, plus the mixture of dibenzothiophene and thianthrene, and incubated at 30°C for 21 days with reciprocal shaking (100 rpm). Controls used were medium with sulfur sources and without bacteria, and medium with bacteria and without sulfur source, in order to detect the sulfate contribution from bacterial biomass (bacterial mineralization).

2.3. Chemicals and reagents

All the reagents were of analytical-reagent grade. A series of working standard SO_4^{2-} solutions was prepared by diluting of the stock standard Orion 948207 Certified traceable to NBS standard reference material 926 (0.1000 ± 0.0005 mol/l) solution in 18 MΩ/cm water (obtained using a Milli-Q system; Millipore).

Dibenzothiophene (98%) and thianthrene (99%) were obtained from Aldrich (Milwaukee, WI, USA).

2.4. Apparatus

Samples were analyzed using a Dionex 2000i/SP ion chromatograph fitted with a conductivity detector. The analytical column was an AS11, protected by an AG11 guard column. An ASRS-1 self-regenerating suppressor, current setting 1, was operated in the chemical suppression mode. All com-

ponents were obtained from Dionex. The eluent (21 m*M* NaOH) and regenerant (electrolyzed 18 MΩ/cm water) flow-rates were 1.0 and 2.0 ml/min, respectively. The sample loop volume was 10 μl and the conductivity sensitivity was 30 μS.

Samples were filtered through a 0.45-μm filter before injection.

3. Results and discussion

When chromatograms of samples containing high concentrations of chloride were compared with chromatograms of standard solutions, an increase in the retention time of sulfate was observed in the first one. At the same time, a decrease in the peak area of sulfate occurred. Co-elution of both species occurred at chloride concentrations higher than 22.62 mg/l. The appearance of this peak in the chromatogram was attributed to the co-elution of sulfate in the system, as a result of the effect of a very high concentration of chloride.

The effect of chloride concentration on the peak area of sulfate was due to the high chloride concentration in the matrix present in the culture mixture, and in the metal solution (see culture medium composition). In order to avoid peak overlap due the high chloride concentration, calibration graphs were prepared adding matrix solution to the standards under the same conditions as the samples. Fig. 3 shows a chromatogram obtained with standard sulfate and culture medium. As can be seen, there is a good resolution. The repeatability of the concentration was investigated by determining three times each level of sulfate. This resulted in an acceptable relative standard deviation (R.S.D.) lower than 3%.

Chloride interference can be reduced by the precipitation of silver chloride with silver acetate or silver oxide or by passing the sample through a column containing a cation-exchange resin in the Ag^+ form [9–13], using an eluent containing the matrix anion [14] or using the ''heart-cut'' technique [15]. However, these methods of chloride removal can result in blockage of the separation column by colloidal silver chloride and coprecipitation of sulfate, or to be a very expensive alternative. We used chloride precipitation with $AgNO_3$, to form AgCl, but under-estimations in sulfate concentrations occurred and a recovery lower than 80% was obtained.

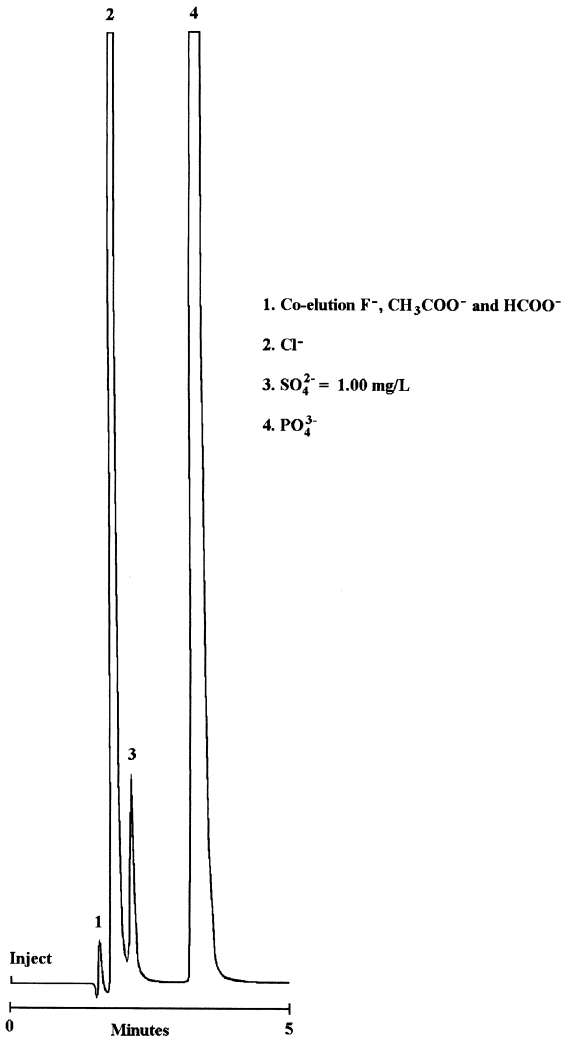

Fig. 3. Standard sulfate on culture medium (0.5 μS/cm).

In this work, the only the presence of sulfate reflects the success of the biodesulfurization process but a big transformation percentage from organic sulfur to sulfate is desirable. Quantities of sulfate with a transformation percentage very small are not significant to the biodesulfurization process, and big quantities reflect a success of the biodesulfurization process.

Simple dilution with water was chosen for sample preparation (1:50) in order to reduce the peak area of

chloride, and to obtain sulfate concentrations falling within the linear range of the calibration graphs, because high chloride concentration affects the analyte recovery and the determination of sulfur that is product of biodesulfurization of polycyclic aromatic hydrocarbons (PAHs). Fig. 4 shows two chromatograms of the same sample obtained with 1:25 and 1:50 dilution factor. It is seen that the approach described gives quite good results for SO_4^{2-} with 1:50 dilution. Consequently, all the results presented in this paper refer to a 1:50 dilution.

The possible influence of the sample matrix on the determination of sulfate was verified by the standard addition method. The calibration graphs were linear ($r=0.998$, $p<0.001$) over two-orders of magnitude of concentration for sulfate (0.100–5.000 ppm), giving good coincidence with the calibration graph for a standard. The slopes of both lines were

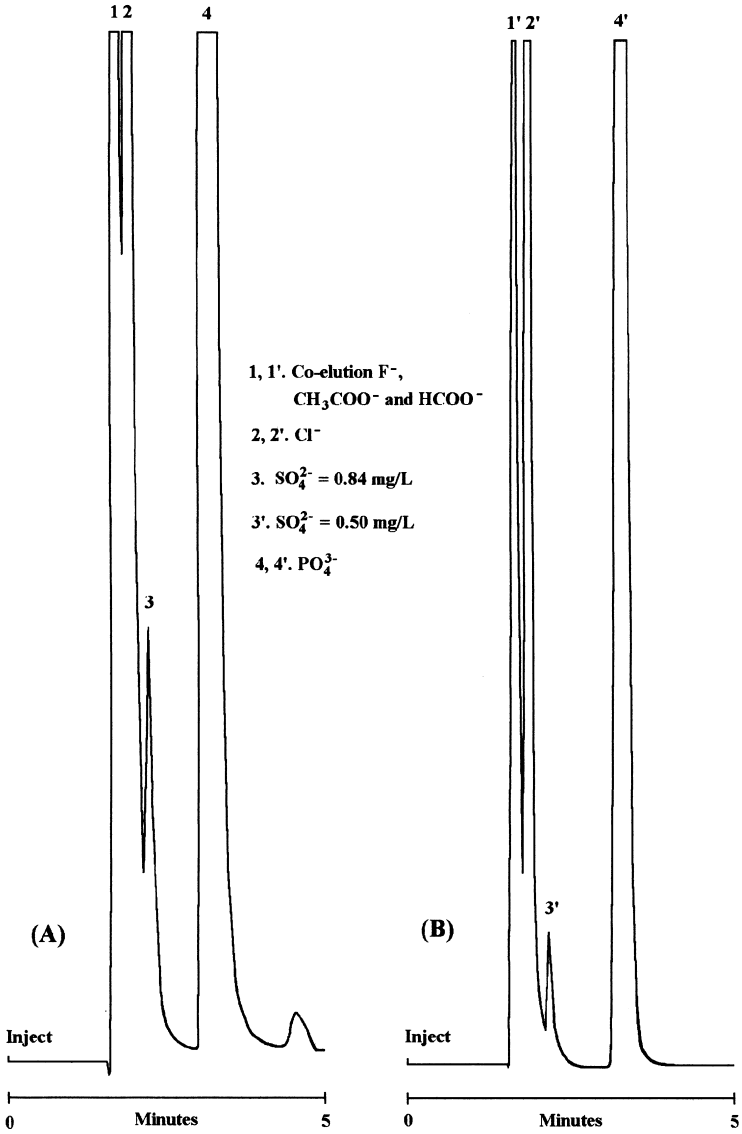

Fig. 4. Separation of sulfate on culture medium (0.5 μS/cm). (A) Dilution 1:25, (B) dilution 1:50.

Table 1
Results of standard additions of Orion sulfate solution for the determination of sulfate by ion chromatography

Sample	Sulfate concentration (ppm)		
	Taken	Added	Found
1	0.070	0.020	0.092
2	2.500	1.500	4.110
3	0.100	0.050	0.148
4	0.100	0.100	0.195

Mean recovery=97.8%.
Mean R.S.D.=4.5%.
n=3.

compared by a *t*-test. There was no significant difference at the level of 5% between slopes for either standard addition or the calibration external. These sulfate data, and the recoveries, which were better than 97%, demonstrate the negligible influence of the matrix on the sulfate responses, as can be seen in Table 1.

To investigate the reproducibility of samples sulfate concentration after dilution step, three replicates of samples and standard injections were measured. The mean R.S.D. values were below 8%, which demonstrates good reproducibility.

Statistical analysis was used to determine the regression coefficients. The correlation coefficient for a linear least squares fit was 0.99 ($p<0.001$) and the equation was: $y=x-16814.5/147381$, where y is the sulfate concentration and x is the peak height.

The minimal concentration that can be read is 0.02 mg/l, and the concentration corresponding to the limit of detection under the conditions employed in this study. It was determined as the lowest concentration corresponding to linear response using the culture matrix, and the signal obtained was sufficiently precise for quantitative purposes.

Table 2 shows the concentration of sulfate produced by different strains. As can be seen, nine (9/19) bacterial strains, corresponding to genus *Bacillus* and *Staphylococcus* produced sulfate as a biodesulfurization product. The highest sulfate concentration detected was 24.10 mg/l (obtaining taking in account the dilution factor), corresponding to a maximal conversion rate of 10% in a month. Sulfate ions were not detected in control samples.

The aerobic degradative pathway has been described, in which sulfate is liberated from dibenzothiophene, leaving 2,2′-dihydroxybiphenyl as the hydrocarbon skeleton [5]. Also Kargi and Robinson [16,17] have reported liberation of sulfate from cultures of the thermophilic organism *Sulfolobus acidocaldarius*, incubated with dibenzothiophene and similar heterocycles.

The thianthrene metabolism not has been elucidated.

4. Conclusions

Mixtures of dibenzothiophene and thianthrene, incubated with culture medium with high chloride concentration and bacteria, followed by IC, are found to be very effective for the determination of sulfate used to evaluate oil desulfurization processes.

For a successful determination to occur, a previous step of dilution is essential. For the calibration curve, the addition of matrix (culture medium) in the same

Table 2
Sulfate concentrations obtained by the strains employed in this study

Strain	Sulfate (mg/l)	Strain	Sulfate (mg/l)
Bacillus 1B12B	4.900	*Bacillus* 2B11	n.d.
Bacillus 2BA	8.270	*Bacillus* 2B41	n.d
Bacillus 1B12T	2.560	*Bacillus* 3BB	n.d
Bacillus 2A11B1	n.d.	*Bacillus* 32A	n.d
Bacillus 3BD	n.d.	*Bacillus* 32B	n.d
Bacillus 3BA	8.240	*Bacillus* 3B11	9.320
Bacillus 2A11C	n.d.	*Staphylococcus* 31AA	24.100
Bacillus 2B21	2.630	*Staphylococcus* 11B2	n.d
Bacillus 3BC	n.d.	*Sporosarcina ureae*	n.d
Bacillus 2BB	n.d.		

Mean R.S.D.=7.80%.

dilution factor as that in the samples is suitable to determine the sulfate concentration.

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