Degradation of Ethylenediaminetetraacetic Acid by Microbial Populations from an Aerated Lagoon

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The ferric chelate of ethylenediaminetetraacetic acid (EDTA) was biologically degraded by a mixed population of microorganisms present in an aerated lagoon receiving this chemical in its feed. As determined radiorespirometrically, 28% of the acetate-2-C and 30% of the ethylene position of the ammonium ferric chelate of [14C]EDTA was recovered as 14CO₂ after 5 days. In a separate experiment using gas liquid chromatography and the sodium ferric chelate, as much as 89% disappearance of EDTA (0.1% wt/vol) was observed during a similar time period. Optimum 14CO₂ evolution was observed at a pH value between 7 and 8 and at room temperature. Degradation of NH₄Fe-[2-14C]EDTA was stimulated by the addition of either unlabeled NaFe-EDTA, nitrilotriacetic acid or ethylenediamine, and inhibited by the addition of a variety of different sugars and amino acids. Consistent with the biological nature of this degradation, little or no 14CO₂ evolution was observed after heat treatment of the microorganisms at 100°C for 10 min, or after the addition of formalin or antibiotics to the incubation mixtures. Gas-liquid chromatography and mass spectral analyses were performed to demonstrate EDTA disappearance and to identify various possible intermediates of EDTA degradation.

Ethylenediaminetetraacetic acid (EDTA) and its metal chelates are used widely in a variety of agricultural and industrial processes. The consensus expressed in earlier literature indicated that EDTA was recalcitrant to biological degradation (1, 7, 11). However, more recent studies by Tiedje and his associates (J. M. Tiedje, E. S. Perry, and T. S. Savage, Abstr. Annu. Meet. Am. Soc. Microbiol., p. 5, 1974), indicate that EDTA is degraded slowly by the microorganisms present in a variety of different agricultural soils. These workers reported that both the ethylene and acetate parts of the EDTA molecule are attacked in this degradation, and that the degradation of EDTA is strongly inhibited by microbial inhibitors.

In addition to the possible biological degradation of EDTA, several reports have indicated that the ferric chelate of EDTA is photodegraded at wavelengths between 250 and 400 nm (13, 14). Further studies by Natarajan and Endicott (16) have identified the products of this photolysis as CO₂, formaldehyde, and ferrous iron.

Because of the similarity in structure between nitrilotriacetic acid (NTA) and EDTA, insight into the mechanism of degradation of EDTA can be obtained from a review of the literature concerning the biochemistry of NTA metabolism. Such studies have shown that NTA is degraded to CO₂ and biomass with the release of the N atom as NH₄⁺ (12). Sequential induction studies have suggested that the metabolism of NTA proceeds by the successive removal of two carbon fragments to form iminodiacetic acid (IDA) and glyoxylate (20), and not by a mechanism involving decarboxylation and the formation of N-methyliminodiacetate or N-methylglycines. Recent evidence of Cripps and Noble (9) indicates that the oxidation of NTA to IDA is mediated by a nicotinamide adenine dinucleotide, reduced form, and O₂-dependent enzyme, and that growth on NTA results in increased activities of the enzymes of glycine and serine metabolism. The end products of NTA degradation reported by these workers are 2 mol of glyoxylate and 1 mol of glycine per mol of NTA degraded. Both glyoxylate and glycine are metabolized to glycerate.

In the present study, we investigated the microbial degradation of the sodium- or ammonium-ferric chelate of EDTA (Na- or NH₄Fe-EDTA) by mixed populations of microorganisms present in an aerated lagoon receiving this chemical. Degradation of acetate- or ethylene-labeled NH₄Fe-EDTA was determined
initially by a radiorespirometric technique, and confirmed by total organic carbon (TOC) and gas-liquid chromatographic (GLC) methods. Possible intermediates in the degradation of EDTA were identified by GLC and mass spectral analyses.

**MATERIALS AND METHODS**

**Biological materials.** Water samples from an aerated lagoon receiving EDTA-containing industrial effluents, located in Webster, N.Y., were collected in 5-gallon (ca. 19 liters) containers and stored overnight at 3 C. In early experiments, biological material was harvested with a table-top centrifuge at 7,000 x g for 5 min. When large quantities of cellular material were required, a refrigerated continuous centrifuge (CEPA, type 41-G) operated at 19,000 rpm was used. After centrifugation, the clear supernatant fluid was discarded, and the cell pellet was suspended in Allen basal salt medium (2) at pH 7.

**Chemicals.** Ethylenediaminemonoacetic acid (EDMA) was synthesized according to the procedure of Atherton et al. (3), and N,N'-ethylenediaminediacetic acid (N,N-EDDA) according to the procedure of Schwarzenbach et al. (18). The cobalt chelate of ethylenediaminodiacetic acid (EDDA) was synthesized by the technique of Blackmer et al. (5). For mass spectral studies, nonchelated EDTA was obtained from C. R. Flynn of the Research Laboratories, Eastman Kodak Co. All other chemicals were of the highest purity available from commercial sources.

NH₄Fe(NO₃)₂-EDTA (0.86 mM C/μg) and NH₄Fe-EDTA-[14C] (1 μCi/μg) were synthesized from either EDTA-2[14C]acetate or EDTA-[14C]-ethylene (Mallinckrodt Chemical Works, St. Louis) according to the procedure of Brintzinger et al. (6). The resultant chemicals were recrystallized in acetone-water (4:1). To test the purity of the radiochemicals, a portion of each chemical was spotted on a silica-gel chromatographic sheet without fluorescent indicator (Eastman Organic Chemicals, no. 6061) and developed with a solvent of either ethanol, NH₄OH, acetic acid (7:3:1), or ethanol, NH₄OH (2:1). Autoradiography was performed on the resultant chromatogram by means of Kodak single-coated medical X-ray film—blue sensitive. The purity of both radiochemicals was estimated to be better than 95% according to this procedure.

**Radiorespirometric incubations.** Either 1 or 2 ml of biological material was placed in a 30-ml capacity serum bottle containing 2 ml of salts solution (2) and 0.2 ml of 1 M potassium phosphate buffer, pH 7.3. The serum bottles were sealed with rubber stoppers, and either 0.1 or 0.2 ml of radioactive substrate was injected through the stopper. To prevent leakage of gas from the vial, the vials were coated with 731 RTV adhesive (Dow Corning Co.). Samples were incubated routinely in the dark at room temperature.

The reactions were stopped by the addition of 2 ml of 1 N perchloric acid to each serum vial. In addition to killing bacterial cells, the perchloric acid acidifies the reaction, releasing 14CO₂ generated into the gaseous phase.

To trap 14CO₂ produced in the reaction, both a long cannula (7.6-cm 20-gauge needle) and a short cannula (3.8-cm 16-gauge needle) were placed through the rubber stopper of the vial. Attached to the longer cannula was an air source; attached to the shorter cannula were two scintillation vials containing 12 ml of scintillation fluid and phenethylamine (19). Air was bubbled through the vials at a flow rate of about 2 bubbles/second. After 7 min, the first vial was removed from the apparatus and counted. Virtually all 14CO₂ was recovered in this first vial. The second vial served as a safety trap for 14CO₂.

Radioactive samples were counted by using a Packard TriCarb 3375 liquid scintillation spectrometer. In early studies, Aquasol Universal LSC Cocktail (New England Nuclear Corp., Boston, Mass.) was used for counting aqueous samples. However, in later studies Eastman ready-to-use II (Eastman Kodak Co., Rochester, N.Y.) scintillation fluid was used.

**Identification of intermediates in degradation.** For the identification of possible intermediates in the degradation of EDTA, 300 ml of Allen basal salts medium (2) containing 0.1% (wt/vol) NaFe-EDTA was placed in each of three 2.8-liter Fernbach flasks. The flasks were autoclaved for 15 min at 121 C. Flask 1 was not inoculated and served as an un inoculated control. Flask 2 contained cell suspension, and 30 ml of added formaldehyde solution. This flask served as a formaldehyde control. Flask 3 served as an experimental and contained 3.5 ml/mg (dry wt) (final concentration) of cell suspension. To preclude the possibility of photodegradation, the flasks were incubated in the dark at room temperature.

Five- or ten-ml samples were removed from the flasks and centrifuged at 7,000 x g for 10 min. The supernatant fluid was kept frozen until analysis.

**Total nonvolatile organic carbon analysis.** Total nonvolatile carbon analyses were performed on 1-ml samples acidified with 25 μl of 3 N HCl with an Envirotech TOC analyzer, model no. DC-50. The limit of detection for these analyses was 1 ppm of nonvolatile carbon with an accuracy and a precision of 5%.

**GLC and mass spectral analyses.** Samples for GLC analysis were acidified with HCl and stored frozen until the time of analysis. EDTA and EDTA analyses were performed by drying samples under nitrogen and then causing the residue to react with boron fluoride (Eastman Organic Chemicals, no. 3706) to form the methyl esters according to the procedure of Rudling (17). One-microliter portions were chromatographed on a Barber-Coleman 5000 gas chromatograph equipped with either a 4-ft or a 6-ft (121.92 or 188.86 cm) glass U-tube packed with 3% (wt/wt) diethylene glycol succinate on Chromosorb W. Solutions containing known concentrations of either EDTA (disodium salt) or Fe-EDTA (monosodium salt) and EDTA (cobalt chelate) were prepared, methylated, and chromatographed as standards. With flame ionization detection, the limits of detection for EDTA and EDTA were less than 0.1 nM.

Glycine, IDA, NTA, EDMA, N,N-EDDA, and N,N'-EDDA were analyzed as their n-butytri-fluoroacetyl derivatives formed by reaction of samples
evaporated to dryness under nitrogen with 3 N hydrochloric acid-butanol at 85 °C. The butylated samples were dried and then caused to react with 25% (vol/vol) trifluoroacetic anhydride in CH₂Cl₂ (4, 21). With flame ionization detection each of these compounds could be detected at less than 0.01 mM on a 5% (wt/wt) diethylene glycol adipate column. Standard addition experiments confirmed the presence of IDA, NTA, EDMA, glycine, N,N-EDDA, and N,N'-EDDA in experimental samples.

The n-butyltrifluoroacetyl derivatives of EDTA and ED3A formed in experimental samples were not used for their quantitative analysis since these derivatives eluted very late from the 5% diethylene glycol adipate column. Furthermore, standard samples of ED3A (cobalt chelate) did not react quantitatively under the mild butylation conditions used.

Mass spectra were obtained on a CEC-21-110B double-focusing mass spectrometer with Mattauch geometry. N-butyl-trifluoroacetyl derivatives were formed from standard samples of EDTA, ED3A, EDMA, NTA, IDA, N,N-EDDA, N,N'-EDDA, and glycine. In some cases impurities and reagent residues obscured their lower mass fragments. A derivatized portion from the 4-day biodegradation sample was introduced into the inlet of the mass spectrometer and several scans were taken as the inlet temperature was gradually increased. This procedure provided a partial separation of components of different volatility. Parent peaks as well as at least two other unique fragments were observed for each compound except glycine. However, peaks characteristic of glycine but not unique to this compound were observed in the biodegradation sample.

RESULTS

Initial studies demonstrating degradation of acetate- and ethylene-labeled EDTA. Degradation of NH₄Fe-EDTA was observed initially in a series of experiments in which either acetate- or ethylene-labeled NH₄Fe-[¹⁴C]EDTA was added to reaction mixtures containing buffer, a salts solution, and biological material from the aerated lagoon. After various incubation periods in the dark, the extent of ¹⁴CO₂ formation in each sample was determined radiochromatographically. As shown in Fig. 1, 27% of the initial radioactivity of the acetate-labeled NH₄Fe-[¹⁴C]EDTA was recovered as ¹⁴CO₂ after a 5-day incubation period; and in the case of ethylene-labeled compound, a 31% ¹⁴CO₂ recovery was observed after this time period. Therefore, both the ethylene backbone and the acetate side chains of the EDTA molecules are degraded in this process.

Effect of pH and temperature on NH₄Fe-[¹⁴C]EDTA degradation. The following buffers were used to determine the effect of pH on degradation: 1,4-piperazinediethanesulfonic acid at pH values between 5 and 7; N-2-hydroxyethylpiperazine - N' - 2 - ethanesulfonic acid at pH 7.5; N-tris(hydroxymethyl)methylglycine at pH 8.0; and tris(hydroxymethyl)methylaminopranesulfonic acid at pH 8.5 and 9.0. Buffers were added at a final concentration of 0.2 M and the vials incubated for 5 days at room temperature. Degradation occurred at all pH values tested between 5 and 9; optimum was between 7 and 8. Of the three temperatures tested at pH 7.3, the best for degradation was room temperature (ca. 22 °C). There was a 98% decrease in ¹⁴CO₂ evolution at 3 °C, and a 49% decrease in ¹⁴CO₂ evolution at 37 °C.

Effect of various carbon sources on NH₄Fe-EDTA degradation. It seemed possible that auxiliary carbon sources might stimulate NH₄Fe-EDTA degradation. Various carbon sources were added at a final concentration of 0.1% to a reaction mixture containing biological material from the aerated lagoon and NH₄Fe-[²-¹⁴C]EDTA. Incubation conditions are as described in Fig. 1. These experiments indicated after 5 days a 2.7-fold increase of ¹⁴CO₂ evolution in the presence of added NaFe-EDTA over control vials containing no additions. Increases also were observed when either NTA (1.8-fold) or ethylenediamine (1.2-fold) was added to the standard reaction mixtures. In contrast to the stimulation observed with the above compounds, the addition of several amino acids (e.g., L-glutamate, L-aspartate, L-serine, L-alanine, glycine) and sugars (e.g., D-fructose, D-
glucose, and glycerol) strongly inhibited degradation.

**Effect of various microbial inhibitors on degradation.** To obtain additional evidence indicating that EDTA degradation is biological, and to gain some insight into the types of microorganisms involved, a study of the effect of various antibiotics and other inhibitors on the degradation was undertaken. A strong inhibition of $^{14}$CO$_2$ evolution was observed after a 5-day incubation with a variety of antibiotics including novobiocin (98% at 1 mg/ml), chloramphenicol (86% at 11,100 U/ml), streptomycin (82% at 2,470 U/ml), cycloheximide (71% at 1 mg/ml), penicillin G (59% at 5,560 U/ml), and formaldehyde (100% at 8% vol/vol). Samples that were heated at 100°C for 10 min before the addition of the radioactive substrate demonstrated an 83% inhibition of $^{14}$CO$_2$ evolution over untreated controls.

**EDTA disappearance by TOC and GLC analyses.** Since previous evidence for EDTA degradation was based on results obtained by means of a radiorespirometric technique, we verified degradation of this compound by other techniques including total organic carbon analysis (TOC) (Fig. 2). NaFe-EDTA (0.1% wt/vol) was the sole carbon source present in the flask. Therefore, a decrease in total nonvolatile organic carbon is indicative of EDTA utilization. A reduction of 63% in total nonvolatile organic carbon was observed after 5 days.

EDTA disappearance in the flask also was measured by a GLC method quantitative for this chemical. These experiments, summarized in Fig. 3A, demonstrated a decrease in EDTA concentration from 2.6 mM to 0.29 mM during a 5-day incubation period.

**Identification of possible products and intermediates of EDTA degradation.** Various compounds in reaction mixtures containing EDTA and aerated lagoon material were identified by a GLC method quantitative for amino polycarboxylic acid (4, 21). These studies are summarized in Fig. 3A and B. Along with a decrease in EDTA concentration, there was a sizable increase in the concentrations of both ED3A, and IDA (Fig. 3A). This increase in ED3A and IDA concentrations appeared to reach a maximum at day 2, after which time there was a pronounced decrease in the levels of these possible intermediates. Such decreases presumably can be explained by a further microbial degradation of ED3A and IDA. In control samples, ED3A and IDA were present at less than 0.1 mM.

In contrast to ED3A and IDA, other possible intermediates in the degradation of EDTA were detected in rather low concentrations (less than 0.065 mM) (Fig. 3B). Included among these compounds were both isomers of ethylenediaminediacetic acid (N,N'-EDDA, and N,N'-EDDA), EDMA, NTA, and glycine. Each of these compounds was detected in 2-day-old samples with maximal concentration occurring at 3 to 5 days. After this time period, a decrease in concentration of these intermediates was observed. In control samples, these chemicals were found at levels less than 0.01 mM.

**Mass spectral analyses.** To verify the presence of the intermediates demonstrated by GLC analyses, the fragmentation patterns of the butyl ester trifluoroacetyl derivatives were determined as shown in Table 1. The spectra of these known compounds were compared with spectra observed in a similarly derivatized sample from the previous 4-day degradation study (Fig. 3A and B). Molecular ions and unique fragments were obtained for all of the compounds except glycine listed in Table 1.

**Calculated versus actual nonvolatile organic carbon.** To determine whether all intermediates of EDTA were accounted for in the previous GLC analyses, the total carbon for all intermediates detected by GLC was calculated and compared with total nonvolatile carbon analysis (TOC) of the samples. As shown in Fig. 2, consistently higher amounts of carbon were detected by TOC analysis compared to calculated carbon contents.

**DISCUSSION**

The present study is the first to demonstrate biological degradation of EDTA in an aquatic
system and to identify possible intermediates in this degradation. These results have significance not only in assessing possible accumulation of this chemical in the environment, but also in changing the consensus expressed in earlier literature indicating the recalcitrance of the EDTA molecule to microbial attack (1, 7, 11).

Because of possible underestimation of degradation with the radiorespirometric technique, both a GLC technique and a total nonvolatile carbon (TOC) analysis were used to measure the extent of breakdown of EDTA. These studies demonstrated approximately 89% degradation of EDTA after 5 days based on the GLC method, and a 63% reduction in TOC during this time. Differences between these two methods probably are attributable to the organic carbon present in various intermediates of degradation as well as to cellular carbon released as a result of lysis during the course of the experiment.

It seems clear that the degradation of NH₄Fe-EDTA reported in the present paper is biological. Several lines of evidence support this conclusion: (i) all incubations were performed in the dark, thereby precluding the possibility of photolysis; (ii) optimum degradation was observed at or near physiological temperatures and pH values; (iii) degradation was stimulated by the addition of possible intermediates of a biological degradation including NTA and ethylenediamine, and inhibited by the addition of various sugars and amino acids; (iv) controls pretreated with heat or with formaldehyde demonstrated little or no activity; (v) degradation was strongly inhibited by several antibiotics known to affect microbial protein synthesis or membrane function.

Bacterial involvement is indicated by the strong inhibition observed with chloramphenicol (86%) and streptomycin (82%). On the other hand, cycloheximide, an antibiotic primarily acting on eucaryotic cells such as algae and fungi demonstrated 71% inhibition. Because adsorption and inactivation of the antibiotics might have occurred during the long duration of our experiments, a rather high concentration of antibiotic was added. Therefore, the extent of involvement of each microbial group is difficult to determine.

Our experiments demonstrating a decrease in ¹⁴CO₂ evolution from NH₄Fe-[¹⁴C]EDTA when certain amino acids or sugars are added can be

![Graph A](image1.png)

![Graph B](image2.png)

**Fig. 3. (A and B). Possible intermediates of EDTA degradation.** Five milliliters of sample was removed from each flask at the time intervals indicated. The samples were centrifuged and the clear supernatant was kept frozen until analysis. Each experimental flask contained 3.59 mg/ml (dry wt) (final concentration) of cell suspension in addition to a basal salts solution and NaFe-EDTA. Intermediates detected by GLC analysis include EDTA, ED₃A, glycine, IDA, EDMA, NTA, N-EDDA, and N,N'-EDDA.
Table 1. Mass spectral analysis for various possible intermediates

| Compound    | Structure of derivative                                                                 | Partial fragmentation pattern (m/e) |
|-------------|----------------------------------------------------------------------------------------|-------------------------------------|
| Glycine     | \( n\text{-Bu} - O - C - CH_2 - NH - C - CF_3 \)                                      | 228, 198, 172, 154, 152, 127, 126   |
| IDA         | \( (n\text{-Bu} - O - C - CH_2)_2 - N - C - CF_3 \)                                    | 341, 267, 229, 211, 184             |
| EDMA        | \( n\text{-Bu} - O - C - CH_2 - NH - CH_2 - CH_2 - N - (C - CF_3)_2 \)                 | 366, 292, 265, 253, 240, 228, 184, 139, 126 |
| \( N,N'\text{-EDDA} \) | \( n\text{-Bu} - O - C - CH_2 - N - CH_2 - CH_2 - N - CH_2 - C - O - n\text{-bu} \) | 480, 406, 379, 310, 253, 184, 181    |
| \( N,N\text{-EDDA} \) | \( (n\text{-Bu} - O - C - CH_2)_2 - N - CH_2 - CH_2 - N - C - CF_3 \)                 | 384, 310, 283, 258                  |
| NTA         | \( N - (CH_2 - C - O - n\text{-Bu})_2 \)                                               | 359, 285, 258, 158, 144             |
| ED3A        | \( (n\text{-Bu} - O - C - CH_2)_2 - N - CH_2 - CH_2 - N - CH_2 - C - O - n\text{-bu} \) | 498, 397, 328, 258, 227, 199         |
| EDTA        | \( (n\text{-Bu} - O - C - CH_2)_2 - N - CH_2 - CH_2 - N - (CH_2 - C - O - n\text{-bu})_2 \) | 516, 442, 415, 258, 227, 144         |

*The butyl ester trifluoroacetyl derivatives of known compounds were determined on a CEC-21-110B double-focusing mass spectrometer of Mattauch geometry.

* See Fig. 3A and B for details.

* Parent peak.

interpreted in several ways. First, the presence of such organic compounds or their metabolites may repress the synthesis of one or more of the enzymes required for EDTA degradation. Second, since many of these sugars and amino acids are rapidly biodegraded, a preferential utilization of such compounds over EDTA may have occurred in reaction vials. And finally, the addition of certain sugars and amino acids may have produced population shifts in the vials resulting in a decrease in the EDTA-utilizing population.

GLC methods confirmed by mass spectral analyses were used to identify possible intermediates and end products of EDTA degradation. These analyses indicate that ED3A and IDA are probably intermediates. In addition, low levels of other possible intermediates, including \( N,N\text{-EDDA} \), \( N,N'\text{-EDDA} \), EDMA, NTA, and glycine were observed. It is reasonable that all these intermediates have resulted from the microbial degradation of EDTA, but the possibility that some of these compounds may have been formed during derivatization cannot be eliminated.

Based on the presence of all these compounds, at least two different pathways may be
postulated for the degradation of EDTA by the mixed population of microorganisms present in the aerated lagoon. One pathway shown in Fig. 4 involves metabolism of the EDTA molecule by the successive removal of 2-carbon units. Several other secondary and tertiary amines have been reported to be metabolized by a similar oxidative cleavage of the C—N bond to form amine and aldehyde products (8, 10, 15). In the case of EDTA, the analogous initial products would be ED3A and glyoxylate. ED3A then would be metabolized by successive C—N bond cleavage, ultimately resulting in ethylenediamine and glyoxylate as end products. Recent results in our laboratory (R. T. Belly, unpublished data) indicate that ethylenediamine can be metabolized to CO₂ and presumably, NH₄⁺.

Another mechanism is required to explain the occurrence of NTA and IDA in reaction mixtures. Based on reported microbial mechanisms for the cleavage of alkynitrogen bonds forming an amine and an aldehyde as products (8, 10, 15), at least three different reactions can explain the occurrence of these intermediates (Fig. 5). Whether NTA or its aldehyde is formed biologically from EDTA, ED3A, or N,N-EDDA, or all of these chemicals remains to be determined. NTA-aldehyde may be converted to NTA by either a dehydrogenase reaction or by an oxidase reaction. Tiedje et al. (20), and Cripps and Noble (9) have proposed that NTA is metabolized by an oxidative cleavage resulting in the formation of glyoxylate and IDA. IDA then undergoes a similar oxidative cleavage forming glycine and glyoxylate. Both glyoxylate and glycine are metabolized readily by a variety of microorganisms to form CO₂; and in the case of glycine, NH₄⁺ is released.

Highest concentrations of the possible intermediates of EDTA degradation were obtained between days 2 and 4. After this time, a significant decrease in the levels of many of these compounds was detected. Such a decrease can be explained by metabolism and degradation of the intermediates of EDTA degradation. If this explanation is correct, then the present study indicates that in addition to EDTA, other intermediates such as ED3A, EDDA, and EDMA can also be degraded by microorganisms

![Diagram of EDTA degradation pathways](image)

**Fig. 4. A proposed pathway for EDTA degradation.**

![Diagram of NTA-aldehyde formation](image)

**Fig. 5. Proposed mechanisms for NTA-aldehyde formation from EDTA.**
in aerated lagoons, and is consistent with findings that both the ethylene and acetate parts of the EDTA molecule are metabolized to similar extents.

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