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SPIN TRAPPING OF NITRIC OXIDE (NO·) AS AMINOXYL RADICALS BY ITS REACTION WITH TWO SPECIES OF SHORT-LIVED RADICALS DERIVED FROM AZO COMPOUNDS SUCH AS 2,2′-AZOBISISOBUTYRONITRILE AND SOME ALIPHATIC ALCOHOLS

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Aminoxyl radicals of the type $R^1N(O\cdot)R^2$ are formed in the photochemical reaction between nitric oxide (NO·) and carbon-centered radicals $R^1\cdot$ and $R^2\cdot$. $R^1\cdot$ was formed from azo compounds such as 2,2′-azobisisobutyronitrile (AIBN): $R^1\cdot$ = NC-$\dot{C}(CH_3)_2$, 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN): $R^1\cdot$ = CH_3-$CH(CH_3)$-CH_2-$\dot{C}(CN)CH_3$, or 4,4′-azobis(4-cyanovaleric acid) (ACVA): $R^1\cdot$ = HOOC-$(CH_2)_2$-$\dot{C}(CN)$ CH_3. $R^2\cdot$ was derived from aliphatic substances such as methanol, ethanol, or 2-propanol by homolytic abstraction of a hydrogen atom brought about by $R^1\cdot$ from the azo compounds.

KEY WORDS: Nitric oxide, aminoxyl radicals, spin trapping, azo compounds, photochemistry

INTRODUCTION

Nitric oxide (NO·) is a substance of general biological interest.[1-4] Therefore, its detection and quantitative estimation are of importance. NO· has an unpaired electron, and consequently it is paramagnetic. However, the direct determination of NO· in solutions by use of electron spin resonance spectroscopy (EPR spectroscopy) is not possible due to a short relaxation time which gives rise to broad absorption lines. NO· has been detected by the EPR technique after it had formed complexes with iron (II) and sulfur compounds[5,6] or with hemoglobin.[7] Spin trapping of NO· by use of nitroso or nitrone spin traps has not been successful, possibly because of the relatively low reactivity of NO·.[8] A further method based on the EPR technique has recently been described in which a nitronyl nitroxide radical is transformed by NO· to an imino nitroxide radical. The EPR spectra of nitronyl nitroxides and imino nitroxides are characteristic and distinctly different.[9]

By use of a pair of reactive radicals $R^1\cdot$ $\underline{\underline{1}}$ and $R^2\cdot$ $\underline{\underline{2}}$, carbon-centered alkyl radicals or $\cdot SO_3^-$ radicals, the low reactivity of NO· has been compensated for, so that detectable aminoxyl radicals $\underline{\underline{4}}$ are formed via the nitroso compound $\underline{\underline{3}}$ (eqns 1 and 2)

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$$\underset{\underline{\underline{1}}}{R^1\cdot} + NO\cdot \rightarrow \underset{\underline{\underline{3}}}{R^1NO} \qquad (1)$$

$$\underset{\underline{\underline{3}}}{R^1NO} + \underset{\underline{\underline{2}}}{R^2\cdot} \rightarrow \underset{\underline{\underline{4}}}{R^1N(O\cdot)\ R^2} \qquad (2)$$

Thus, the stable nitroso disulfonate anion radical $\underline{\underline{5}}$ (Fremy's radical) is formed in high yield in the reaction between NO· and two molecules of the reactive $\cdot SO_3^-$, i.e.

$$\begin{aligned} \cdot SO_3^- + NO\cdot &\rightarrow ONSO_3^- \\ \cdot SO_3^- + ONSO_3{}^- &\rightarrow \underset{\underline{\underline{5}}}{\cdot ON(SO_3^-)_2} \end{aligned} \qquad (4)$$

In this reaction, NO· was formed in the thermal cleavage of *tert*-butyl nitrite (*t*-BuONO). The $\cdot SO_3^-$ ion radicals were formed by homolytic abstraction of H· radicals from HSO_3^- by *tert*-butoxy radicals, also produced in the thermal cleavage of *t*-BuONO.[10] A similar method has been described for the detection of NO· in which a stable precursor molecule is photochemically transformed to a diradical or reactive "diradical equivalent". This substance reacts with NO· to give a stable cyclic aminoxyl radical.[11]

In this connection it should be pointed out that nitric oxide has been used for the trapping of alkyl radicals as aminoxyl radicals. The alkyl radicals were formed by photolysis of compounds containing alkyl groups or by hydrogen abstraction initiated by the triplet state of anthraquinone or by *tert*-butoxy radicals obtained from the decomposition of di-*tert*-butyl peroxide.[12]

It has now been found that aminoxyl radicals are obtained in the photochemical reaction between NO· and a number of aliphatic alcohols such as methanol, ethanol, or 2-propanol, and azo compounds such as 2,2′-azobisisobutyronitrile (AIBN), 2,2′-azobis(2,4-dimethyl-valeronitrile) (AMVN), or 4,4′-azobis(4-cyano-valeric acid) (ACVA). The photochemistry of AIBN and the reaction of 2-cyano-2-propyl radicals formed from AIBN, including reactions with NO·, have previously been studied with non-EPR methods. These reactions are rather complicated, with the formation of substances such as tetramethylsuccinonitrile, dimethyl-*N*-(2-cyano-2-propyl)ketenimine, or *N*,*N*,*O*-tris(2-cyano-2-propyl)hydroxylamine.[13,14]

EXPERIMENTAL PROCEDURES

The EPR spectra were recorded using a Varian E-9 EPR spectrometer at 20°C with a microwave power of 1–5 mW and a 100 kHz modulation amplitude of 0.01 to 0.10 mT. The samples were contained in a flat aqueous solution cell. Hyperfine splitting constants were measured by comparison with the splittings of Fremy's radical ($a_N = 1.3$ mT; 1 Gauss equals 0.10 mT). The samples were irradiated with UV light from a mercury lamp (Osram HBO-200) *in situ* in the EPR cavity. Quantitative estimation of NO· was made by measuring the relative peak-to-peak amplitudes of the 3 × 2 line spectra of the aminoxyl radicals formed in the

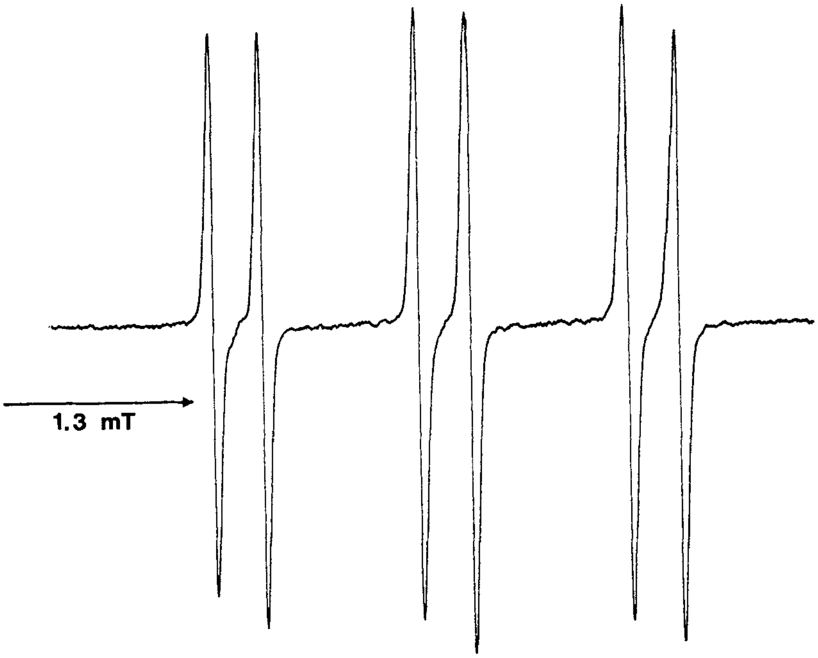

FIGURE 1 EPR spectrum of the aminoxyl radicals formed in the photochemical reaction between AIBN dissolved in ethanol bubbled for a few s with NO· gas. 2-Cyano-2-propyl radicals from AIBN form a nitroso compound with NO· A further 2-cyano-2-propyl radical abstracts a hydrogen atom from the methylene group of ethanol. This radical is trapped by the nitroso compound with the production of the aminoxyl radical NC-$(CH_3)_2CN(O\cdot)CH(CH_3)OH$.

photochemical reaction of NO· with ACVA and ethanol. The chemicals used were obtained from Aldrich, Fluka AG and Polyscience Inc. Sodium ^{15}N nitrite was from ICON Inc. Physically dissolved O_2 was removed by bubbling the solutions with N_2.

RESULTS AND DISCUSSION

Figure 1 shows the EPR spectrum of the aminoxyl radicals formed with ethanol. The reaction mixture was a solution of AIBN (~2%) in ethanol into which NO· gas was bubbled for a few seconds. The radicals appeared almost immediately after the samples had been irradiated *in situ* in the EPR cavity by UV light. By adjusting the intensity of the light, the amplitude of the spectral lines could be kept constant for at least 10 min. In the dark after irradiation, the radicals disappeared slowly, and could not be detected after about 10 min. The 3 × 2 line pattern (Figure 1) indicated an interaction of the unpaired electron with one ^{14}N nucleus and one hydrogen nucleus. The coupling constants are given in Table 1. It is believed that the following reactions take place:

TABLE I
Aminoxyl radicals $R^1N(O\cdot)R^2$ formed in the photochemical reaction between AIBN, AMVN, or ACVA, and substrate compounds containing R^2 in the presence of NO.

R^1	Substrate/R^2	NO· source	a_N(mT)	a_H (mT)
AIBN	AIBN* $NC\text{-}\dot{C}(CH_3)_2$	gas	1.44	-
AIBN	2-propanol $(CH_3)_2\dot{C}OH$	gas	1.47	-
AIBN	ethanol $CH_3\dot{C}HOH$	gas	1.44	0.34 (d)
AIBN	ethanol/H_2O 1: 1 $CH_3\dot{C}HOH$	Na nitrite acetic acid	1.44	0.31 (d)
AIBN	methanol $\dot{C}H_2OH$	gas	1.40	0.43 (t)
AMVN	DMSO $CH_3\cdot$	*t*-BuONO	1.56	1.20 (q)
AMVN	DMSO $CH_3S.$	*t*-BuONO	1.24	0.07 (q)
ACVA	ethanol/H_2O 4: 1 $CH_3\dot{C}HOH$	Na nitrite	1.44	0.34 (d)

*in benzene d = doublet, t = triplet 1: 2: 1, q = quartet 1: 3: 3: 1

A. AIBN is photochemically cleaved into 2-cyano-2-propyl radidals $\underline{\underline{6}}$ and nitrogen, (eqn 5),

$$NC\text{-}C(CH_3)_2\text{-}N = N\text{-}C(CH_3)_2\text{-}CN \xrightarrow{h\nu} 2\ \underset{\underline{\underline{6}}}{NC\text{-}\dot{C}(CH_3)_2} + N_2 \qquad (5)$$

B. The 2-cyano-2-propyl radicals $\underline{\underline{6}}$ bring about a homolytic abstraction of one of the methylene hydrogen atoms of ethanol (eqn 6),

$$\underset{\underline{\underline{6}}}{NC\text{-}\dot{C}(CH_3)_2} + CH_3CH_2OH \rightarrow \underset{\underline{\underline{7}}}{CH_3\dot{C}HOH} + NC\text{-}C(CH_3)_2H \qquad (6)$$

C. A 2-cyano-2-propyl radical $\underline{\underline{6}}$ reacts with NO· to give the nitroso compound $\underline{\underline{8}}$ (eqn 7),

$$\underset{\underline{\underline{6}}}{NC\text{-}\dot{C}(CH_3)_2} + NO\cdot \rightarrow \underset{\underline{\underline{8}}}{NC\text{-}C(CH_3)_2NO} \qquad (7)$$

D. Finally, the nitroso compound $\underline{\underline{8}}$ traps the radical $\underline{\underline{7}}$ derived from ethanol with the formation of the observed aminoxyl radical $\underline{\underline{9}}$, i.e., eqn 8

$$\underset{\underline{\underline{8}}}{NC\text{-}C(CH_3)_2NO} + \underset{\underline{\underline{7}}}{CH_3\dot{C}HOH} \rightarrow \underset{\underline{\underline{9}}}{NC\text{-}C(CH_3)_2N(O\cdot)CH(CH_3)OH} \qquad (8)$$

Evidently, the doublet splitting (Figure 1) originated from interaction with the remaining hydrogen nucleus of the methylene group of ethanol.

Similar results were obtained with methanol, or 2-propanol, (see Table 1). Almost identical results were obtained when nitrite/acetic acid, or *t*-BuONO was used as the NO· source (Table 1). Nearly the same results were also observed with the more lipid soluble azo compound AMVN, and with ACVA. In these cases the radicals derived from the azo compounds have the structures $CH_3\text{-}C(CH_3)\text{-}$

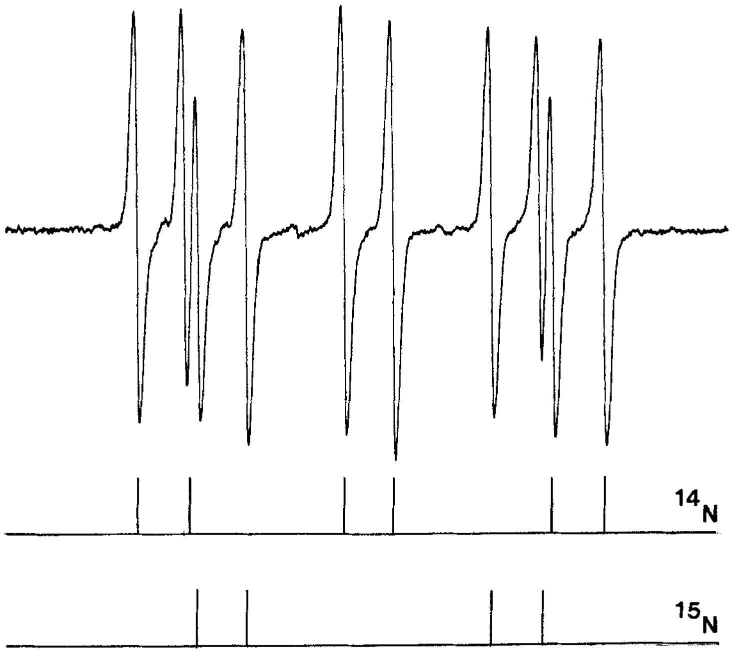

FIGURE 2 The EPR spectrum of the aminoxyl radicals NC-$(CH_3)_2CN(O\cdot)$-$CH(CH_3)OH$ formed in the photochemical reaction between AIBN dilxolved in ethanol and NO·. In this experiments, NO· was derived from sodium nitrite/acetic acid in H_2O. The sodium nitrite was a 1: 1 mixture of Na $^{14}NO_2$ and Na $^{15}NO_2$. The spectrum is a superposition of the ^{14}N and ^{15}N spectra.
a_{N14} = 1.44 mT; a_H = 0.312 mT.
a_{N15} = 2.03 mT; a_H = 0.312 mT
The reaction mixture: 400 μl of a 1% solution of AIBN in ethanol + 80 μl of 5 mg Na $^{14}NO_2$ + 5 mg Na $^{15}NO_2$ in H_2O + 20 μl of acetic acid.

-H-CH_2-$\dot{C}(CH_3)CN$ and HOOC-$(CH_2)_2$-$\dot{C}(CH_3)CN$.

When the reaction with AIBN was performed in benzene without alcohols, a 3 × 1 line pattern was obtained. The radical concentration was initially rather low, but increased slowly after some time of irradiation. It is suggested that the radical is formed in the reaction between two 2-cyano-2-propyl radicals and NO·, and has the structure $[NC\text{-}C(CH_3)_2]_2NO\cdot$.

No radicals could be detected in the photochemical reaction of a mixture of AIBN and ethanol without NO·. This finding indicated that the nitrogen atom of the nitroxide group of the aminoxyl radicals was derived from NO· and not from any of the nitrogen atoms of AIBN. This statement was confirmed by the spectrum obtained with AIBN/ethanol and $^{15}NO\cdot$, derived from Na $^{15}NO_2$. This spectrum exhibited a 2 × 2 line pattern, in which the large doublet splitting was due to an interaction with one ^{15}N nucleus: $a_{N15} = 2.03$ mT; $a_H = 0.312$ mT (cf. Figure 2). The ratio a_{N15}/a_{N14} was 1.4 in conformity with the known ratio between isotropic hyperfine coupling constants of these nuclei.

The reaction between AIBN, NO· and ethanol could also be performed in the presence of H_2O. Nearly identical coupling constants were obtained with 1 : 1 ethanol/H_2O (Table 1).

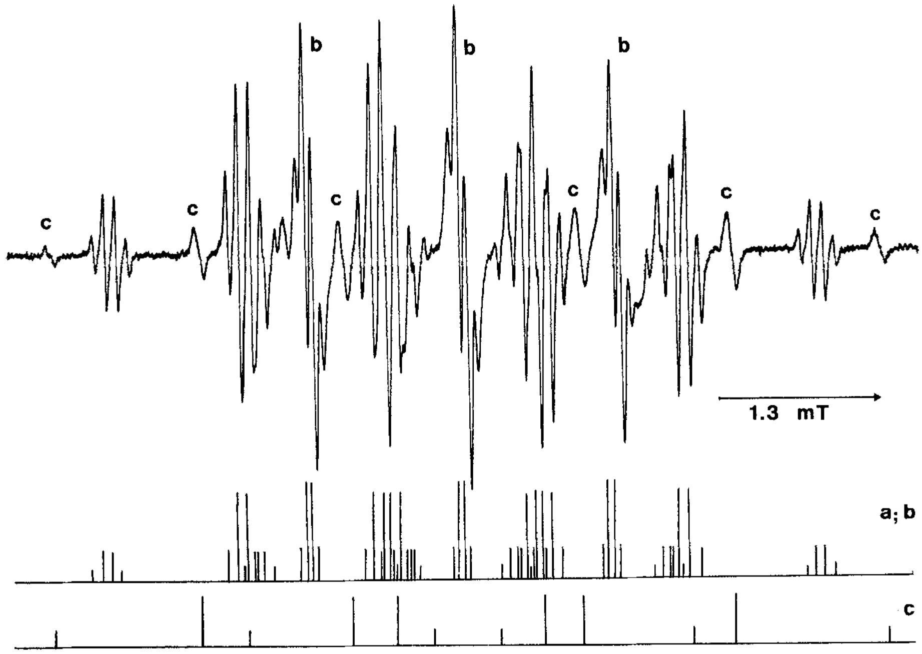

FIGURE 3 EPR spectrum of the radicals formed in the photochemical reaction of AMVN dissolved in DMSO together with a small amount of *t*-BuONO. The spectrum is a superposition of the spectra of three radical species:
a. $CH_3S(O_2)N(O\cdot)CH_3$
b. $R^1N(O\cdot)SCH_3$. The 3 main groups are indicated "b"
c. $R^1N(O\cdot)CH_3$. 6 of the 12 lines free from overlaps are indicated "c" where R^1 is the alkyl group derived from AMVN, i.e. CH_3-CH (CH_3)-CH_2-C(CH_3)CN.

The yield of aminoxyl radicals obtained without degassing of the solutions was somewhat lower as compared with the results observed after removal of dissolved O_2. The difference was considered to correspond to the amount of NO· oxidized by dissolved oxygen.

Dimethyl Sulfoxide (DMSO)

Some experiments were performed with DMSO. Figure 3 shows the EPR spectrum obtained with AMVN dissolved in DMSO together with a small amount of *t*-BuONO (no degassing) after UV irradiation of the sample for about 10 min. The spectrum is a superposition of the spectra of three radical species:

a. 6-group spectrum

The radical species that gives rise to this spectrum has previously been shown to be $CH_3S(O_2)N(O\cdot)$ CH_3 formed in the photochemical reaction between DMSO and nitrogen dioxide $(\cdot NO_2)$,[15] in the present case produced by oxidation of NO· by physically dissolved O_2. The coupling constants are: $a_N = 1.22$ mT; $a_{H1} = 1.10$ mT (quartet 1: 3: 3: 1) and $a_{H2} = 0.094$ mT (quartet 1: 3: 3: 1). This radical species was dominant in the early phase of the irradiation.

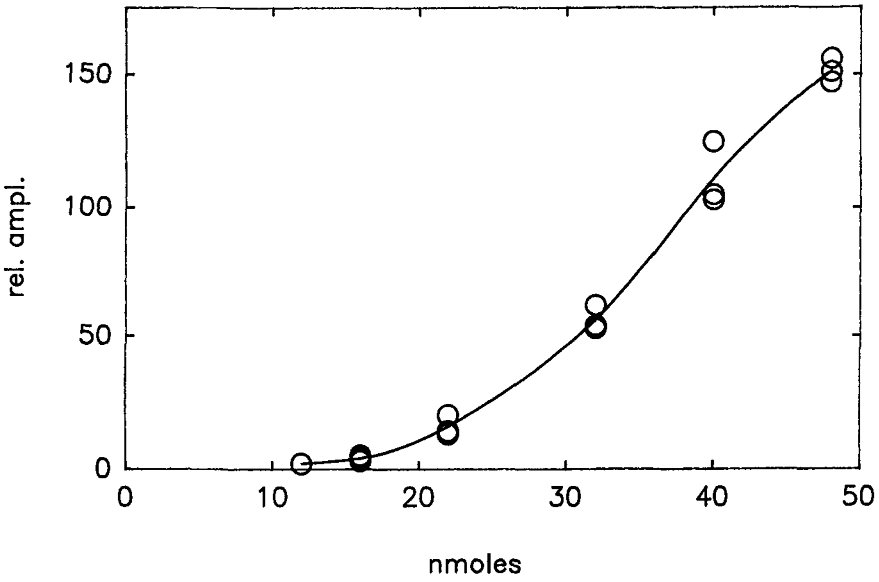

FIGURE 4 The relative amplitude of the spectrum of the aminoxyl radicals formed in the photochemical reaction between the azo compound ACVA, ethanol and NO· plotted against the amount of NO· in the EPR cuvette.

b. A 3 × 4 line spectrum

The spectral pattern indicated an interaction with one ^{14}N nucleus and three equivalent hydrogen nuclei. The coupling constants are given in Table 1. It is suggested that this spectrum originates from the radical species $R^1N(O\cdot)$-S-CH_3 formed in the reaction between the radical CH_3-$CH(CH_3)$-$CH_2\dot{C}(CH_3)CN$, ($R^1\cdot$) derived from AMVN, NO· and the radical ·S-CH_3 derived from the dissociation of DMSO.

c. A 3 × 4 line spectrum

The spectrum indicated an interaction with one ^{14}N nucleus and three equivalent hydrogen nuclei (Table 1). The structure of this species is very probably R^1 $N(O\cdot)CH_3$ where R^1 is derived from AMVN and the trapped $CH_3\cdot$ radical from DMSO.

Quantitative Estimation of NO

Some experiments were made for a quantitative estimation of NO·. Figure 4 shows the relative amplitude of the 3 × 2 pattern obtained in the photochemical reaction between ACVA, ethanol and NO· plotted against the amount of NO· (nanomoles) present in the EPR cuvette (10 μl). The NO· source was sodium nitrite with NO· produced by photoreduction. 400 μl of a 1% solution of ACVA in 4: 1 ethanol/H_2O was added to varying volumes (125, 100, 75, 50, 35, 25 μl) of a standard solution of sodium nitrite (2 μmol in 100 μl of H_2O). The reaction mixtures were transfered to the EPR cuvette and irradiated *in situ* in the cavity by UV light. The radicals appeared after about 2 min and reached a maximum after about 4–5 min. The maximum amplitudes were used as the relative measure of the radical concentration.

CONCLUSIONS

The essential features of the reaction described involve the formation of reactive alkyl radicals in the photochemical dissociation of the azo compounds AIBN, AMVN or ACVA. These alkyl radicals participate in the reaction both as abstractors of hydrogen atoms from the alcohol substrates, and as reagents that form nitroso compounds with NO·. It is also possible that NO· combines first with the radical derived from the substrate, i.e. such as $\underline{\underline{7}}$, and that the nitroso compound formed reacts in a secondary step with the alkyl radical from the azo compound.

The yield of aminoxyl radicals obtained so far seems to be too low for a quantitative determination of NO· under physiological conditions (minimun detectable ~1 mM of NO·, Figure 4). However, optimal conditions have not yet been established for the yield and stability of the aminoxyl radicals formed in the reaction. It is possible that improvements could decrease the detection limit of the method.

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