Hydroxyl radical formation from Cu(II)-Trolox mixtures: insights into the pro-oxidant properties of α-tocopherol

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Abstract Although widely recognised as the most important chain-breaking antioxidant of the lipid phase, α-tocopherol has also been reported to exert pro-oxidant activity, particularly during the Cu(II)-stimulated oxidation of low density lipoproteins (LDL). In the present communication, we demonstrate that hydroxyl radicals are generated following the interaction of Cu(II) with the α-tocopherol model compound Trolox, involving the reduction of Cu(II) by Trolox and the subsequent reduction of molecular oxygen by Cu(I). We suggest, therefore, that the hydroxyl radical may be the species responsible for the initiation of fatty acid oxidation during the Cu(II)-stimulated oxidation of hydroperoxide-free LDL.

Key words: Copper; ESR Spin trapping; Hydroxyl radical; Low density lipoprotein oxidation; Tocopherol-mediated peroxidation; Vitamin E

1. Introduction

Oxidation of low density lipoproteins (LDL) in the endothelial wall is now widely recognised to be an important event in atherosclerosis [1–3] Although the mechanism(s) of LDL oxidation remain unclear, lipid peroxidation is believed to be involved and this has stimulated extensive research into the effects of α-tocopherol, the primary chain-breaking antioxidant of the lipid phase, on the process [4–11]. When LDL oxidation is stimulated in vitro by incubation with Cu(II) ions, a lag period occurs before a significant level of lipid peroxidation can be detected, which is believed to reflect the scavenging of peroxyl radicals by the endogenous antioxidant [4,5,12]. Under certain conditions, however, α-tocopherol has been shown to *promote* LDL oxidation [7–11]. For example, in the presence of peroxyl radicals generated from an azo initiator, lipid peroxidation in LDL is faster in the presence of α-tocopherol than in its absence [8]. Similarly, the rate of Cu(II)-induced peroxidation of phospholipids in a detergent dispersion has been reported to be dramatically stimulated by α-tocopherol [13].

The pro-oxidant behaviour of α-tocopherol has been attributed to the ability of its phenoxyl radical, α-Toc$^{\cdot}$, to abstract a hydrogen atom from the bisallylic methylene groups of polyunsaturated fatty acids, albeit rather slowly [7–10]: Ingold et al. calculated the rate constant for this reaction to be ca. $10^{-1} \pm 0.05$ $M^{-1} \cdot s^{-1}$ at 37° [9]. 'Tocopherol-mediated peroxidation', as the process has been described by Bowry and Stocker, has been studied largely in systems employing azo initiators to induce LDL oxidation [8,9]. Copper-stimulated LDL oxidation, however, is believed to involve the reduction of Cu(II) to Cu(I), followed by its reaction with a lipid hydroperoxide to generate an alkoxyl radical, which can abstract a hydrogen atom from a polyunsaturated fatty acid and thereby initiate peroxidation [5]. LDL preparations usually contain lipid hydroperoxides [3], which may be introduced into LDL by endothelial lipoxygenase [14]. Hence, the mechanism may be physiologically relevant. Esterbauer et al. proposed that the Cu(I) needed to generate alkoxyl radicals from lipid hydroperoxides could be generated via the reaction of Cu(II) with α-tocopherol (Reaction 1) [5].

$$\alpha\text{-TocH} + Cu^{2+} \rightarrow \alpha\text{-Toc}^{\bullet} + Cu^{+} + H^{+} \quad (1)$$

Although there is evidence to suggest that copper-stimulated LDL oxidation has an absolute requirement for pre-existing lipid hydroperoxides [3,5,15], the more recent findings of Lynch and Frei do not support this [16,17]. These authors suggested that, in the absence of lipid hydroperoxides, copper-stimulated LDL oxidation may involve the oxidation of fatty acids by the α-tocopheroxyl radical [17].

The combination of Cu(II), oxygen and ascorbic acid produces a species (probably the hydroxyl radical, $^{\bullet}OH$) that can hydroxylate organic substrates [18]. In this system, ascorbic acid reduces Cu(II) to Cu(I), which in turn reduces oxygen to the hydroxyl radical (Reactions 2 to 5).

$$Cu^{+} + O_2 \rightarrow Cu^{2+} + O_2^{\bullet -} \quad (2)$$

$$Cu^{+} + O_2^{\bullet -} + 2\,H^{+} \rightarrow Cu^{2+} + H_2O_2 \quad (3)$$

$$2\,O_2^{\bullet -} + 2\,H^{+} \rightarrow H_2O_2 + O_2 \quad (4)$$

$$Cu^{+} + H_2O_2 \rightarrow Cu^{2+} + {}^{\bullet}OH + H^{+} \quad (5)$$

Since ascorbic acid serves only to reduce Cu(II), and α-tocopherol can also do this [13], we decided to explore the possibility that the oxidant generated from the interaction of Cu(II) with α-tocopherol in the absence of pre-existing hydroperoxides is also the hydroxyl radical. Using Trolox, the α-tocopherol model compound, we present evidence that $^{\bullet}OH$ is indeed generated via these reactions and thereby provide a mechanism by which α-tocopherol may promote LDL oxidation by Cu(II).

2. Materials and methods

2.1. Reagents

Bathocuproinedisulfonic acid (disodium salt), *N*-*t*-butyl-α-phenylni-

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Abbreviations: BC, bathocuproinedisulfonic acid; DMSO, dimethyl sulphoxide; EDTA, ethylenediaminetetraacetic acid; ESR, electron spin resonance; LDL, low density lipoprotein; MNP 2-methyl-2-nitrosopropane; MOPS, 3-(*N*-morpholino)-propanesulfonic acid; PBN, *N*-*t*-butyl-α-phenylnitrone.

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trone, catalase (C-40), chelating resin (Chelex 100), cupric chloride (dihydrate), dimethyl sulphoxide, ethylenediaminetetraacetic acid (disodium salt) and 3-(*N*-morpholino)propanesulfonic acid and were from Sigma Chemical Co. (Poole, UK). (*S*)-(−)-Trolox, possessing the chiral centre of α-tocopherol, was from Aldrich (Gillingham, UK). Millipore-filtered de-ionised water was used throughout. MOPS buffer stock solution (0.1 M, pH 7.0) was treated with Chelex 100 using the batch method [19].

2.2. Oxidation of Trolox by Cu(II)

The oxidation of Trolox to its phenoxyl radical by Cu(II) was investigated using ESR spectroscopy. Reactions contained 1 mM $CuCl_2$, 5 mM Trolox, 5% (v/v) DMSO and 50 mM MOPS (pH 7.0). Following the final addition of the $CuCl_2$, reaction mixtures were transferred immediately to a quartz ESR flat-cell positioned and pre-tuned within the cavity of a Bruker ECS 106 spectrometer using a rapid delivery device [20] and recording commenced using the following instrument settings: modulation frequency, 100 kHz; centre field, 3471.5 G; sweep width, 50 G; modulation amplitude, 1 G; receiver gain, 6.3×10^4; scan time, 20.97 s; time constant, 10.24 s; power, 20 mW; number of successive scans accumulated and averaged, 15. Spectra were simulated using the SIMEPR program [21]. The apparent second-order rate constant for the reaction of Cu(II) with Trolox was estimated spectrophotometrically, essentially as described by Yoshida et al. [22]. Reaction mixtures contained 0.1 mM $CuCl_2$, 0.3 mM Trolox, 10% DMSO and 50 mM MOPS (pH 7.0) and were carried out at 25°. Where indicated, reactions were performed in anaerobic cells (Hellma GmbH & Co., Mülheim, Germany). Air was removed from solutions using a vacuum pump and replaced with nitrogen before the addition of $CuCl_2$. Trolox oxidation

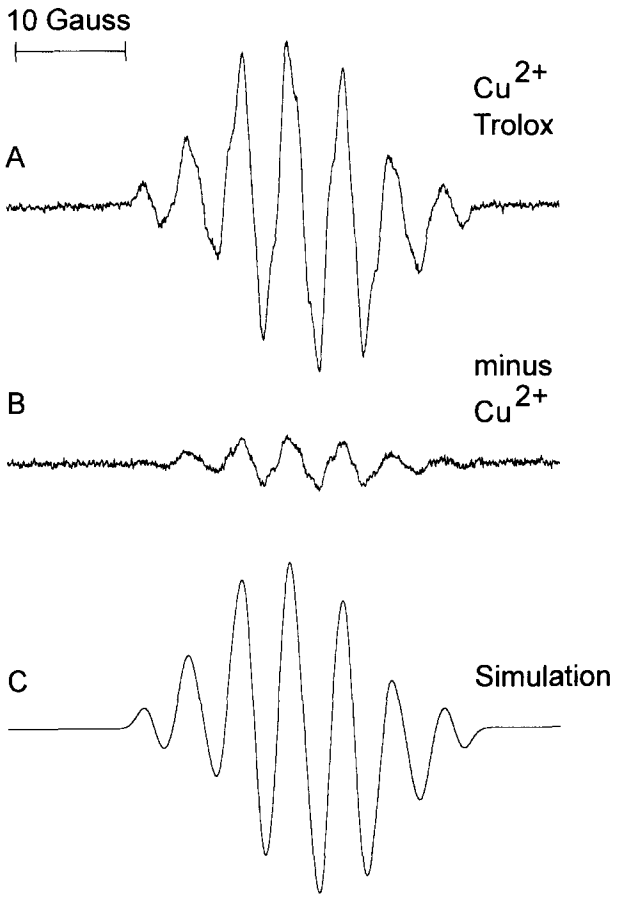

Fig. 1. ESR detection of the Trolox phenoxyl radical generated by the oxidation of Trolox with Cu(II). (A) The complete reaction mixture containing 1 mM $CuCl_2$, 5 mM Trolox, 5% (v/v) DMSO and 50 mM MOPS (pH 7.0). (B) As in A, but without $CuCl_2$. (C) Computer simulation of spectrum A obtained using the hyperfine splitting constants: a^H (CH_3) = 5.21 G; a^H (CH_3) = 3.80 G; a^H (CH_3) = 0.23 G; a^H (CH_2) = 0.37 G; $a^{H'}$(CH_2) = 0.76 G.

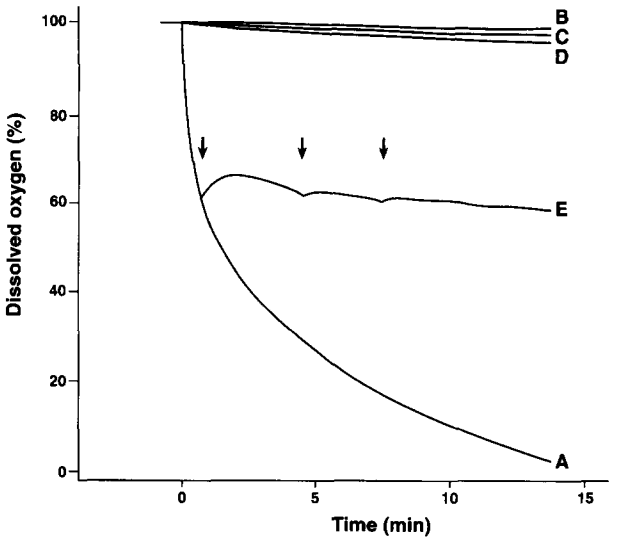

Fig. 2. Oxygen consumption resulting from the interaction of Cu(II) with Trolox. At time zero, 1 mM $CuCl_2$ was introduced into the chamber of a Clarke oxygen electrode containing an equilibrated solution of 5 mM Trolox and 10% (v/v) DMSO in 50 mM MOPS buffer, pH 7 (Trace A). When indicated, the $CuCl_2$ was replaced with an equivalent volume of water (Trace B), Trolox was omitted (Trace C) or 3 mM BC was present in the equilibrated solution before the $CuCl_2$ addition (Trace D). As indicated by the three arrows, catalase (14,165 units · ml^{-1} per addition) was added following the detection of oxygen consumption (Trace E).

was monitored at 290 nm (ε = 2980 $M^{-1} \cdot cm^{-1}$). Values given are means ± 1 S.D. (n=3).

2.3. Measurement of oxygen consumption during the interaction of Cu(II) with Trolox

Reactions were initiated by the injection of a 25 μl aliquot of 200 mM $CuCl_2$ into the chamber of a Clarke oxygen electrode (YSI Model 5300, Yellow Springs Instrument Co., Inc., Ohio) containing an equilibrated solution of 5 mM Trolox in 50 mM MOPS, pH 7.0 at 25° (5 ml final volume). The final concentration of DMSO (from the Trolox stock solution) was 10% (v/v). When indicated, 3 mM BC was included in reactions. The presence of hydrogen peroxide was demonstrated by the addition of 14,165 units · ml^{-1} catalase following the detection of oxygen consumption.

2.4. Detection of hydroxyl radical formation using ESR spin trapping

A secondary spin-trapping technique was employed in which hydroxyl radicals react with DMSO to generate methyl radicals ($^{\bullet}CH_3$). Methyl radicals then react with PBN to form the relatively stable methyl radical adduct, PBN/$^{\bullet}CH_3$, which has a characteristic six-line ESR spectrum [23]. Reactions mixtures contained 0.1 M PBN, 10% DMSO (v/v), 5 mM Trolox, 1 mM $CuCl_2$ and 50 mM MOPS, pH 7.0. When indicated, either catalase (14,177 units · ml^{-1}), BC or EDTA (each 3 mM) were included in reactions. Following the final addition of the $CuCl_2$, reaction mixtures were transferred immediately to an ESR flat-cell positioned and pre-tuned within the spectrometer cavity, as described above. Spectra were recorded using the instrument settings given above, but with a sweep width of 85 G and the successive accumulation of 50 scans.

3. Results

3.1. Reaction of Cu(II) with Trolox

As shown in Fig. 1A, a prominent ESR signal from the Trolox phenoxyl radical was observed following the addition of $CuCl_2$ to Trolox. A weak signal was observed in the absence of added $CuCl_2$ (Fig. 1B). A computer simulation of the spec-

trum shown in Fig. 1A is presented in Fig. 1C, obtained using the hyperfine splitting constants given in the legend. The reaction between Cu(II) and Trolox was also monitored spectrophotometrically [22], and found to proceed with second order rate constant of 0.31 ± 0.03 $M^{-1} \cdot s^{-1}$ at 25° (data not shown). When the reaction was carried out anaerobically, a lower rate constant of 0.18 ± 0.02 $M^{-1} \cdot s^{-1}$ was obtained, indicating that the value obtained in the presence of air represents an overall rate constant for a more complex process involving oxygen.

3.2. Oxygen consumption measurements

When Cu(II) was added to Trolox at pH 7.0, extensive oxygen uptake occurred (Fig. 2A). Oxygen uptake was dependent on the presence of both Cu(II) and Trolox, but was prevented by the Cu(I)-stabilising reagent BC (Fig. 2B, C and D, respectively). Catalase inhibited oxygen uptake when added during the course of reactions (Fig. 2E).

3.3. ESR detection of hydroxyl radicals

When Cu(II) was added to Trolox in the presence of DMSO and PBN, a prominent six-line ESR signal from the PBN/$^{\bullet}CH_3$ radical adduct was observed, confirming the generation of hydroxyl radicals (Fig. 3A). In addition to PBN/$^{\bullet}CH_3$, the spectrum contained weaker signals from other radicals. Computer

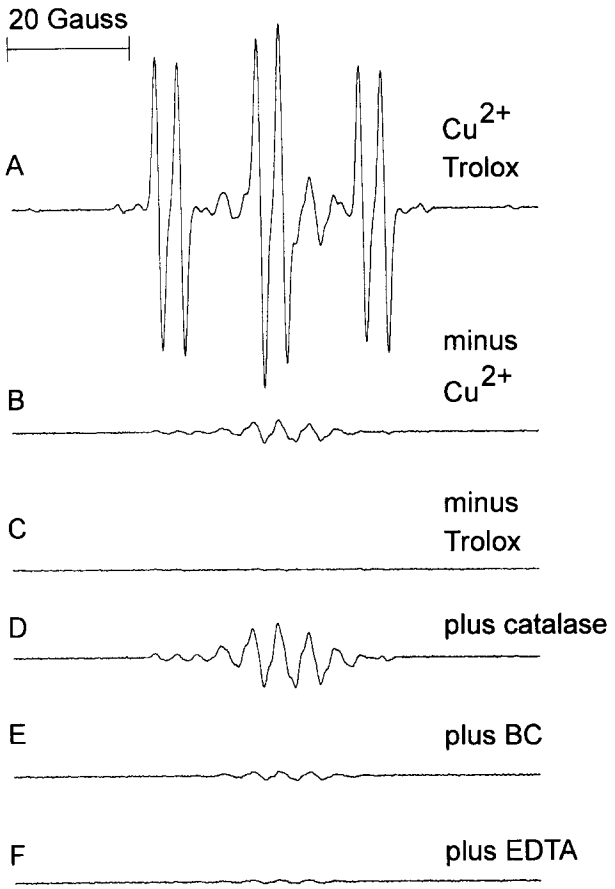

Fig. 3. ESR spectra observed during the interaction of Cu(II) with Trolox in the presence of PBN and DMSO. Reactions mixtures contained 0.1 M PBN, 10% DMSO (v/v), 5 mM Trolox, 1 mM $CuCl_2$ and 50 mM MOPS, pH 7.0. (A) The complete reaction system. (B) Complete system minus $CuCl_2$. (C) Complete system minus Trolox. (D) Complete system plus catalase (14,177 units · ml^{-1}). (E) Complete reaction system plus 3 mM BC. (F) Complete system plus 3 mM EDTA.

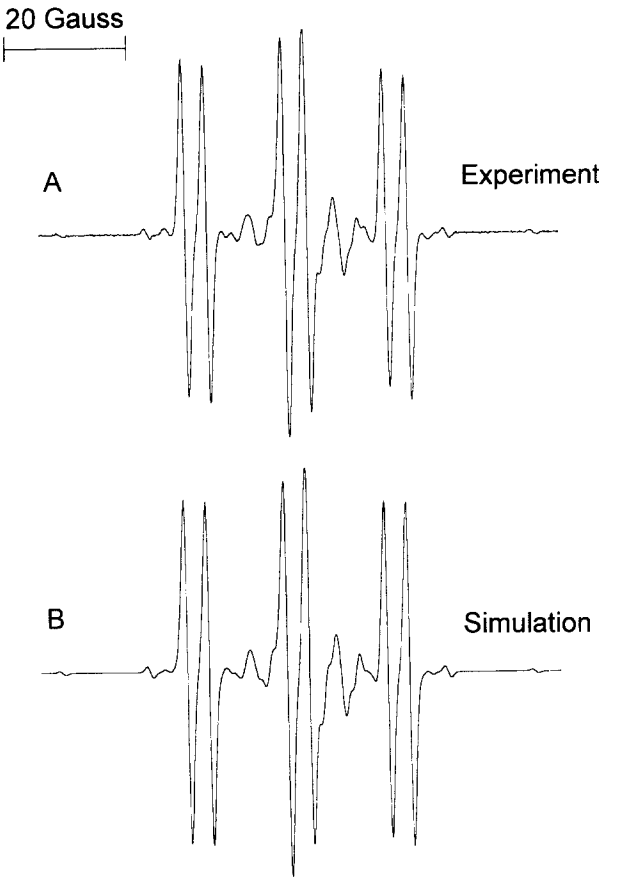

Fig. 4. Computer simulation of the ESR spectrum observed following the interaction of Cu(II) with Trolox. (A) Experimental spectrum, taken from Fig. 3A. (B) Simulated spectrum obtained using the three species: PBN/$^{\bullet}CH_3$ [a^N = 16.38 G; a^H_β = 3.36 G], MNP/$^{\bullet}CH_3$ [a^N = 17.16 G; a^H_β (3H) = 14.28 G] and the Trolox phenoxyl radical [a^H (CH_3) = 5.21 G; a^H (CH_3) = 3.80 G; a^H (CH_3) = 0.23 G; a^H (CH_2) = 0.37 G; $a^{H'}$(CH_2) = 0.76 G] with relative concentrations of 0.72, 0.03 and 0.25, respectively.

simulation confirmed the presence of the Trolox phenoxyl radical spectrum and a 12-line spectrum corresponding to the methyl radical adduct of the spin trap 2-methyl-2-nitrosopropane, MNP/$^{\bullet}CH_3$ (Fig 4B). Hydroxyl radical formation did not occur in the absence of Cu(II), with only a weak signal from the Trolox phenoxyl radical being detectable (Fig. 3B). No radicals were detected in the absence of Trolox (Fig. 3C). Catalase prevented detection of the PBN/$^{\bullet}CH_3$ and MNP/$^{\bullet}CH_3$ radical adducts, but not the Trolox phenoxyl radical (Fig. 3D). The Cu(I)-stabilising reagent BC and the Cu(II)-stabilising reagent EDTA each prevented PBN/$^{\bullet}CH_3$ and MNP/$^{\bullet}CH_3$ formation (Fig. 3E and 3F, respectively).

4. Discussion

The above findings demonstrate that hydroxyl radicals are generated following the interaction of Cu(II) with Trolox, the vitamin E model compound. This is believed to involve the reduction of the metal ion by Trolox, followed by the reduction of oxygen to $^{\bullet}OH$. The second order rate constant for the reaction of Cu(II) with Trolox was found to be 0.18 ± 0.02 $M^{-1} \cdot s^{-1}$ at 25°. Under aerobic conditions a higher

value was obtained (0.31 ± 0.03 $M^{-1} \cdot s^{-1}$), which is believed to represent an apparent rate constant for a series of complex reactions involving the formation of reactive oxygen species that also promote oxidation of the substrate.

The oxygen-electrode studies confirmed that oxygen uptake occurs during the reaction of Cu(II) with Trolox. The failure to observe oxygen uptake in the presence of BC, a Cu(I)-stabilising reagent, confirms that reaction with Cu(I) is responsible for oxygen uptake. Oxygen evolution upon the addition of catalase is good evidence for the presence of hydrogen peroxide. Similar observations have been reported following the interaction of Cu(II) with a hydroquinone [24]. The observation that catalase, which can recover only half of the molecular oxygen incorporated into H_2O_2, blocks oxygen uptake completely suggests that a reaction scheme involving Cu(II) reduction by Trolox followed by Reactions 2 to 5 may represent an over simplification of the process. Previous studies on Fe(II) autoxidation suggest that, following the initial reduction of molecular oxygen, a slower phase of oxygen uptake occurs that involves redox cycling of the metal ion by organic radicals and oxygen addition to carbon-centred radicals [25]. In the Cu(II)-Trolox system, it appears that the oxygen liberated from accumulated H_2O_2 following the addition of catalase balances the subsequent phase of slower oxygen uptake involving complex peroxidation reactions.

The ESR spin-trapping experiments confirmed $^{\bullet}OH$ formation. In addition to PBN/$^{\bullet}CH_3$, the methyl radical adduct to the spin trap MNP was also detected. The hydroxyl radical adduct of PBN decomposes to give the corresponding hydrogen atom adduct, MNP/$^{\bullet}H$ [26], which will air-oxidise to form MNP. Decomposition of the hydroxyl radical adduct of the related spin trap α-(4-pyridyl-1-oxide)-*N-tert*-butylnitrone (4-POBN) to MNP/$^{\bullet}H$ is believed to be catalysed by Cu(I) [27], which is consistent with the occurrence of a similar mechanism of MNP generation in the system reported here. The finding that catalase prevents the detection of PBN/$^{\bullet}CH_3$ (and MNP/$^{\bullet}CH_3$) demonstrates the H_2O_2-dependence of $^{\bullet}OH$ formation. Similarly, the prevention of $^{\bullet}OH$ formation by BC, which stabilises Cu(I) and EDTA, which stabilises Cu(II), proves that copper mediates formation of the oxidant from the Cu(II)-Trolox reaction system.

The conclusions drawn from this study have important implications regarding the mechanism(s) by which α-tocopherol has been proposed to act as a pro-oxidant, particularly during the copper-dependent oxidation of hydroperoxide-free LDL. It is often suggested in the literature that Cu(I) cannot initiate LDL oxidation in the absence of pre-existing hydroperoxides [3,5,11,17] and, consequently, that the α-tocopheroxyl radical may be the initiator of LDL oxidation induced by Cu(II) under such conditions [8,11,17]. Whilst we fully accept that the α-tocopheroxyl radical can oxidise polyunsaturated fatty acids, and that this may occur during the oxidation of LDL when initiated by azo compounds [8,9], we propose that the hydroxyl radical is the most likely initiator of Cu(II)-stimulated, α-tocopherol dependent LDL oxidation in the absence of pre-existing lipid hydroperoxides.

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