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Electron Spin Resonance Studies on the Reaction of Peroxyphenylacetic Acid with Aminopyrine in the Presence and Absence of Catalase

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When the concentration of aminopyrine, [AMP], was greater than that of peroxyphenylacetic acid, [PPAA], the reaction of PPAA with AMP in the absence of catalase at pH 4.5 showed the electron spin resonance (ESR) spectrum of the aminopyrine cation radical (**1**), whereas that at pH 6.8 did not. On the other hand, when [AMP]≦[PPAA], a new ESR spectrum ($g=2.0053$, $A_1=16.9$ (1N), $A_2=14.5$ G (6H) which was entirely different from that of **1** was obtained at both pH 4.5 and 6.8. Spin-trapping experiments suggested that the new spectrum was derived from the phenylacetyl radical-adduct of AMP (**2**). In the presence of catalase, when [AMP]>[PPAA], the reaction of PPAA with AMP showed the ESR spectrum of **1** even at pH 6.8. The amount of benzyl alcohol formed was decreased in the presence of catalase in comparison with that in the absence of catalase. These results suggest that homolytic oxygen–oxygen bond cleavage and oxy radicals generated play no major role in the PPAA-supported oxidation of AMP by catalase.

Keywords——aminopyrine; peroxyphenylacetic acid; aminopyrine cation radical; spin-trapping; phenyl-*tert*-butylnitrone; phenylacetyl radical; benzylperoxy radical; 4-diethylamino-antipyrine; catalase; peroxidatic reaction

Catalase ($H_2O_2:H_2O_2$ oxidoreductase; EC 1.11.1.6) is a very efficient catalyst for the decomposition of H_2O_2 (catalatic reaction), but it also catalyzes the oxidation of primary alcohols, phenols, sodium nitrite, sodium azide, and hydroxylamine by H_2O_2 (peroxidatic reaction).[1] In the previous papers,[2] we reported that aminopyrine (AMP) is oxidized to the aminopyrine cation radical (**1**) by cumene hydroperoxide (CHP) or ethyl hydroperoxide (EHP) in the presence of catalase. The results of the steady-state kinetic analysis indicated that the CHP- and EHP-supported oxidations of AMP catalyzed by catalase proceed by a sequential mechanism involving the formation of a ternary complex,[2e] which is different from that generally proposed for catalase-mediated reactions.[1b]

Griffin and Ting suggested that methyl radicals derived from CHP are the oxidant in the metmyoglobin-catalyzed oxidation of AMP by CHP.[3a] Griffin *et al.* also suggested that radicals derived from CHP are the oxidant in the CHP-supported oxidation of AMP catalyzed by purified liver microsomal cytochrome P-450.[3b] However, there are some doubts regarding the hypothesis that free substrate radicals are principally formed during cytochrome P-450 catalysis.[4] Coon *et al.* used peroxyphenylacetic acid (PPAA) as a peroxide in the peroxide-supported hydroxylation of a variety of substrates catalyzed by cytochrome P-450 and found that benzyl alcohol was obtained as a product and that the source of the oxygen atom in the benzyl alcohol was PPAA.[5] From these results they proposed that the phenylacetoxy radical, formed through homolysis of the O–O bond in PPAA, is the active oxygen species.

In order to elucidate the mechanism of the peroxide-dependent oxidation of AMP catalyzed by catalase more clearly, we used PPAA as a peroxide and investigated the exact nature of the oxidant by means of electron spin resonance (ESR) spectroscopy in the absence

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and presence of catalase.

Experimental

Materials——Catalase (from bovine liver, C-40) was used as supplied by Sigma. PPAA was prepared by the method of White *et al.*[5] and recrystallized from benzene–petroleum ether. Iodometric titration indicated this material to be better than 97% pure. AMP was obtained from Aldrich Chemicals and purified by recrystallization from ligroin. 4-Diethylaminoantipyrine was prepared as described previously.[6] [*N*,*N*-Dimethyl-D_6]aminopyrine was prepared from 4-aminoantipyrine and [U-D_6]dimethylsulfate and recrystallized from hexane. Phenyl *N*-*tert*-butylnitrone (PBN) was obtained from Eastman Organic Chemicals. The buffer solutions used in this study were 0.2 M citric acid–sodium citrate (pH 4.5), 0.2 M NaH_2PO_4–Na_2HPO_4 (pH 6.8), and 0.2 M Na_2HPO_4–NaOH (pH 12.0). All other chemicals used were of reagent grade.

Methods——ESR spectra were recorded on a JES-FE 1X spectrometer, equipped with a Union-Giken MX-7 mixing device, at room temperature (25±1 °C). One reservoir was filled with aqueous solution containing 20 mM PPAA, and the other one was filled with aqueous buffer solution containing AMP at various concentrations. The spin-trapping experiments were initiated by injection of the mixture of AMP and PBN into the solution of PPAA. The aqueous solutions were deoxygenated by passing nitrogen before the mixing. The ESR spectra of the reaction solution and the benzene extract of the solution were recorded. Photolysis of dibenzyl ketone was carried out with the use of a high-pressure mercury lamp.

Spectrophotometric studies on the rate of formation of the aminopyrine cation radical were carried out by the use of a Union-Giken RA-601 stopped-flow spectrophotometer, equipped with a system 71 data processor. The concentration of the aminopyrine cation radical was determined from the absorbance at 565 nm. An extinction coefficient of $\varepsilon = 2.23\ mM^{-1}\ cm^{-1}$ was used.[3b]

Determination of Benzyl Alcohol——The residual PPAA in the reaction mixture was reduced by addition of 0.1 g of sodium bisulfite 15 min after initiating the reaction at 25 °C, and the mixture was extracted with ether. The ether was evaporated off and the residue was dissolved in 1.5 ml of 0.1 M boric acid–sodium borate buffer (pH 9.0), then 100 μl of the solution was analyzed by the use of high performance liquid chromatography (HPLC). HPLC was carried out with a reciprocating pump, model M-45 (Waters Assoc.), coupled to a variable-wavelength UV detector UVIDEC-100 (Japan Spectroscopic Co.). The detector was operated at 258 nm. The column used was a Radial-PAK cartridge packed with Bondapak C_{18} placed in a Z-module (Waters Assoc.). The mobile phase was a mixture of 0.1 M borate buffer and methanol (3 : 1).

Results

A New Radical Derived from the Reaction of PPAA with Aminopyrine

At pH 4.5 PPAA reacted with AMP fairly rapidly even in the absence of catalase. When the concentration of AMP, [AMP], was greater than that of PPAA, [PPAA], the reaction mixture turned blue-violet and showed the ESR spectrum of the aminopyrine cation radical (**1**). On the other hand, when the concentration of AMP was not greater than that of PPAA, the reaction mixture remained colorless and showed an ESR spectrum which was entirely different from that of **1** (Fig. 1). The following parameters were obtained: $g = 2.0053$, $A_1 =$ 16.9 (1N), and $A_2 = 14.5$ G (6H). In order to assign this ESR spectrum, the reaction was carried out with 4-diethylaminoantipyrine (DEA) or [U-D_6]aminopyrine (AMP-d_6) instead of AMP. The ESR spectra obtained are shown in Figs. 2 and 3. The following parameters were obtained: for DEA, $g = 2.0053$, $A_1 = 16.9$ (1N) and $A_2 = 11.7$ G (4H); for AMP-d_6, $g = 2.0053$, $A_1 = 16.9$ and $A_2 = 2.4$ G (6D). From these results the new ESR spectrum in Fig. 1 is considered to be derived from the radical **2** as shown in Chart 1. The peak-height of the ESR spectrum of **2** was plotted against time (Fig. 4). The stability of **2** suggests the presence of the pyrazolone ring in **2**. In order to clarify the structure of the radical Ẋ, spin-trapping experiments were carried out.

Spin-Trapping of the Free Radical Ẋ Generated in the Reaction of PPAA with AMP at pH 4.5

The results of spin-trapping with PBN are shown in Table I together with those of photolysis of dibenzyl ketone (DBK). An aqueous buffer solution containing PPAA and PBN at pH 4.5 gave no ESR signal. This indicates that auto-homolysis of PPAA is negligible in the

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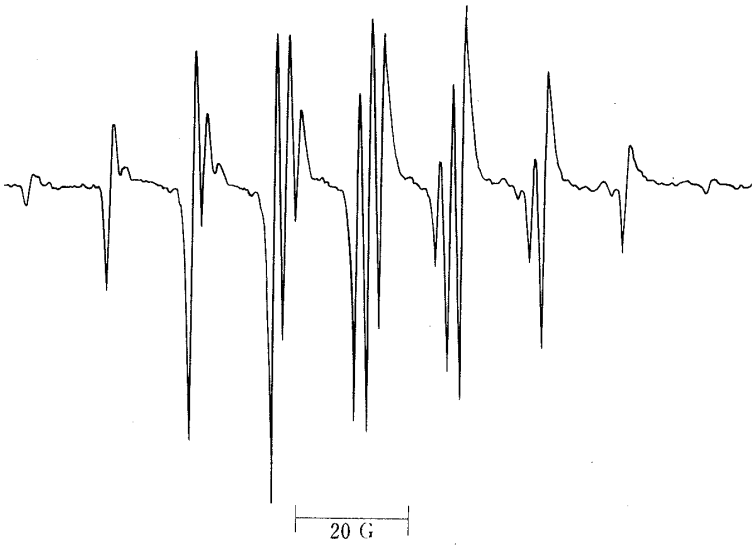


Fig. 1. ESR Spectrum of the Radical **2** Generated in the Reaction of Peroxyphenylacetic Acid with Aminopyrine

The reaction mixture contained 10 mM PPAA and 2.5 mM AMP in 0.1 M citrate buffer, pH 4.5. The ESR spectrum was recorded at room temperature (25±1°C) with the following instrumental settings: power, 10 mW; modulation amplitude, 0.5 G; scan rate, 25 G/min; time constant, 1 s; gain, 2×1000.

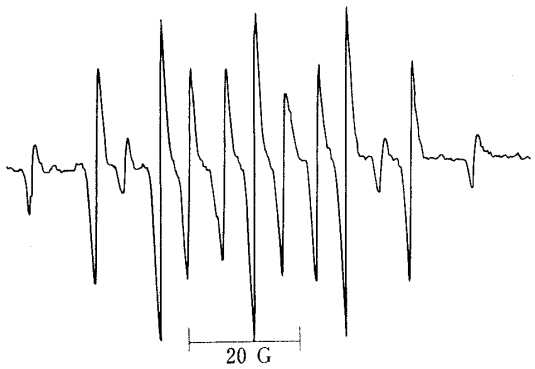


Fig. 2. ESR Spectrum of the Radical Generated in the Reaction of Peroxyphenylacetic Acid with 4-Diethylaminoantipyrine

The reaction mixture contained 10 mM PPAA and 2.5 mM DEA in 0.1 M citrate buffer, pH 4.5. ESR settings were the same as in Fig. 1.

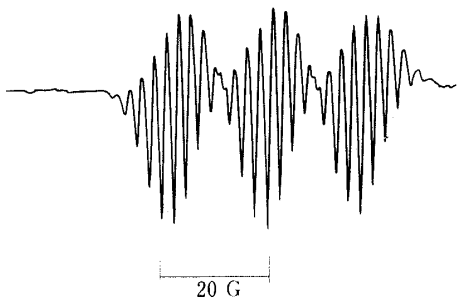


Fig. 3. ESR Spectrum of the Radical Generated in the Reaction of Peroxyphenylacetic Acid with [U-D_6]Aminopyrine

The reaction mixture contained 10 mM PPAA and 2.5 mM AMP-d_6 in 0.1 M citrate buffer, pH 4.5. ESR settings were the same as in Fig. 1.

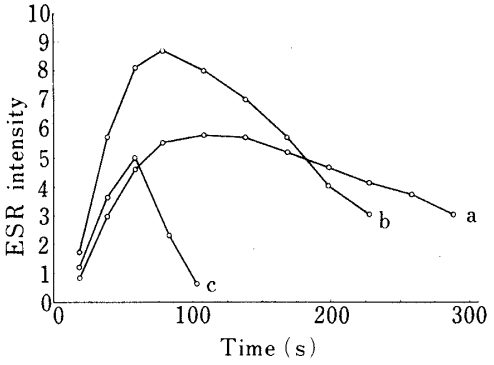
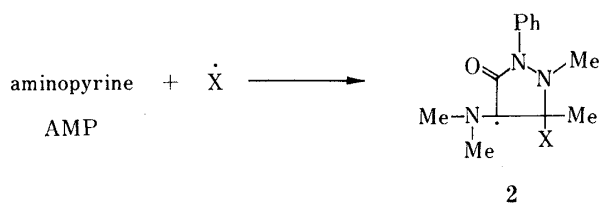


Fig. 4. Time Dependence of the ESR Signal Intensity of the Radical **2** at pH 4.5

The reaction mixture contained 10 mM PPAA and a) 2.5 mM AMP, b) 5 mM AMP, or c) 10 mM citrate buffer, pH 4.5. ESR settings were the same as in Fig. 1.



2

Ph=C_6H_5–, Me=CH_3–, Ẋ=unidentified radical

Chart 1

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TABLE I. Hyperfine Splitting Constants of the PBN Spin Adducts

Radical source	Solvent	Condition	A_N(G)	A_H(G)	Radical trapped[b]
PPAA	H_2O	Deoxygenated	—	—	None
PPAA	Benzene	Deoxygenated	14.8	7.4	H·
PPAA	Benzene		13.6	1.8	ROO·
DBK[a]	Benzene	Deoxygenated	14.4	2.3	RĊO
DBK[a]	Benzene		13.6	1.8	ROO·
PPAA+AMP	H_2O	Deoxygenated	16.1	4.3	RĊO
PPAA+AMP[c]	Benzene	Deoxygenated	14.4	2.25	RĊO

a) Irradiated with a high-pressure mercury lamp.
b) R=$C_6H_5CH_2$–.
c) The reaction was carried out in aqueous buffer solution of pH 4.5, and the solution was extracted with benzene.

solution. In a benzene solution containing PPAA and PBN without removal of oxygen, a six-line spectrum with $A_1=13.6$ (1N) and $A_2=1.8$ G (1H) was observed. This spectrum was the same as that obtained in the photolysis of a benzene solution containing DBK without removal of oxygen. It is well established that the photolysis of DBK gives phenylacetyl and benzyl radicals,[7a,b] that the phenylacetyl radical undergoes rapid decarbonylation,[7c] and that the benzyl radical reacts with oxygen to give the benzylperoxy radical.[7d] Therefore, the trapped radical can be assigned as the benzylperoxy radical. The ESR parameters obtained were similar to those reported for the α-methylbenzylperoxy radical ($A_1=13.57$ (1N) and $A_2=1.74$ G (1H))[8] and different from those reported for the benzyl radical ($A_1=13.88$ (1N) and $A_2=2.44$ G (1H)).[9] However, since Merritt *et al.* claimed that alkylperoxy radical adducts of PBN were detected only at low temperature and decomposed rapidly to alkoxy radical adducts at room temperature,[10] the observed spectrum may have been derived from the benzyloxy radical adduct formed from the benzylperoxy radical adduct.

In a degassed benzene solution containing PPAA and PBN, a seven-line spectrum with $A_1=14.8$ (1N) and $A_2=7.4$ G (2H) was observed, which was assigned to the hydrogen adduct of PBN by comparison with the known data.[11] Photolysis of a degassed benzene solution containing DBK and PBN showed a six-line spectrum with $A_1=14.4$ (1N) and $A_2=2.3$ G (1H). Although the photolysis of DBK gives both benzyl and phenylacetyl radicals, the ESR spectrum obtained can be assigned principally to the phenylacetyl radical adduct, because the ESR parameters obtained were very different from those reported for the benzyl radical adduct.[9] However, since the line-width of the spectrum was broad compared with that of other spectra, a small amount of the benzyl radical is also considered to be trapped in the system.

When the deoxygenated reaction mixture of AMP, PPAA, and PBN ([PPAA]>[AMP]) was extracted with benzene, the benzene extract showed a six-line spectrum with $A_1=14.4$ (1N) and $A_2=2.25$ G (1H), which was practically identical with that obtained from the photolysis of DBK in degassed benzene. Therefore, the radical generated in the reaction of PPAA with AMP in which the concentration of PPAA is greater than that of AMP, is assumed to be the phenylacetyl radical.

Effect of pH on the Formation of the Radical 2

At pH 6.8 neither the radical **1** nor **2** was detected in the reaction of PPAA with AMP when [AMP]>[PPAA]. This result can be interpreted on the basis of the instability of **1** in neutral and alkaline solutions.[3a] When [PPAA]≧[AMP], the ESR spectrum of **2** was observed. Although the maximum intensity of the spectrum was about half that at pH 4.5, the

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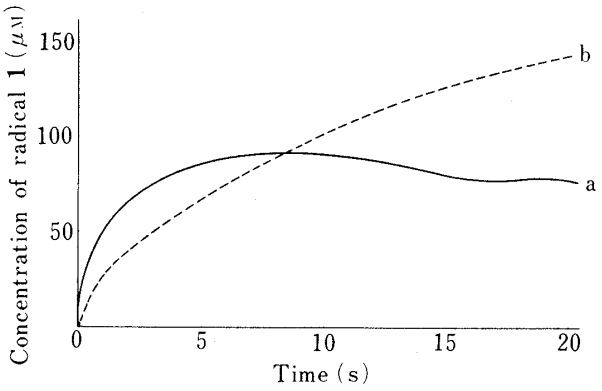

Fig. 5. Time Dependence of Aminopyrine Cation Radical Concentration in the PPAA- and CHP-Supported Oxidation of Aminopyrine in the Presence of Catalase

The radical concentration was measured by stopped-flow spectrophotometry. The reaction mixture contained 0.625 μM catalase, 6 mM AMP, and 3 mM a) PPAA or b) CHP in 0.1 M phosphate buffer (pH 6.8), at 25° C.

spectrum was observed for a longer period of time. At pH 12.0 neither the radical **1** nor **2** was detected in the reaction when [PPAA]≧[AMP] or [PPAA]<[AMP].

Effect of Catalase on the Reaction of PPAA with AMP

In the presence of catalase the reaction of PPAA with AMP at pH 6.8 when [AMP]>[PPAA] gave the radical **1**, which was observed by ESR and spectrophotometry. This result can be interpreted on the basis of an increased rate of formation of **1** caused by the catalysis of catalase. Plots of the concentration of **1** versus time are shown in Fig. 5. Although the initial rate of formation of **1** was greater than that obtained by the use of CHP instead of PPAA,[2e] the concentration of **1** soon leveled off. Addition of D-mannitol (5 times [AMP]), which is a scavenger of the hydroxyl radical, to the reaction mixture did not retard the rate. The residual PPAA was reduced by addition of sodium bisulfite 15 min after initiating the reaction at 25 °C, and the reaction mixture was analyzed by HPLC. The amount of benzyl alcohol formed was 2.5 mol% of the starting PPAA in the presence of catalase and 7.0 mol% in the absence of catalase.

When [PPAA]>[AMP], an excess of PPAA inactivated the catalase, as in the reaction with CHP,[2] and hence the effect of catalase could not be determined. Since catalase does not catalyze the peroxidatic reactions at pH 4.5, the presence of catalase did not effect the reaction of PPAA with AMP.

Discussion

The following schemes are suggested for the reaction of PPAA with AMP in the absence of catalase (Chart 2). Since the spin-trapping experiments indicated that auto-homolysis of PPAA is negligible in aqueous buffer solutions at pH 4.5 and 6.8, the reaction (1) is considered to proceed through the formation of a complex, [PPAA–AMP], as generally proposed for the reaction of peroxides with tertiary amines.[12] When [AMP]>[PPAA], the phenylacetoxy radical formed in (1) reacts immediately with AMP to give **1** and phenylacetic acid (reaction 2). On the other hand, when [PPAA]≧[AMP], the phenylacetoxy radical reacts with PPAA to give phenylacetic acid and phenylperacetoxy radical (reaction 3), and the latter loses oxygen to give the phenylacetyl radical. The phenylacetyl radical adds to AMP to give **2**. PPAA also reacts with **1**, resulting in the disappearance of the ESR spectrum of **1**. Benzyl alcohol is considered to be formed through the decarboxylation of the phenylacetoxy radical.

Although the appearance and disappearance of the ESR spectra of **1** and **2** can be explained on the basis of the schemes in Chart 2, the effect of pH on the formation of **2** is difficult to interpret unequivocally. In alkaline solution a completely different mechanism may be operating.

In the presence of catalase the amount of benzyl alcohol formed was decreased in

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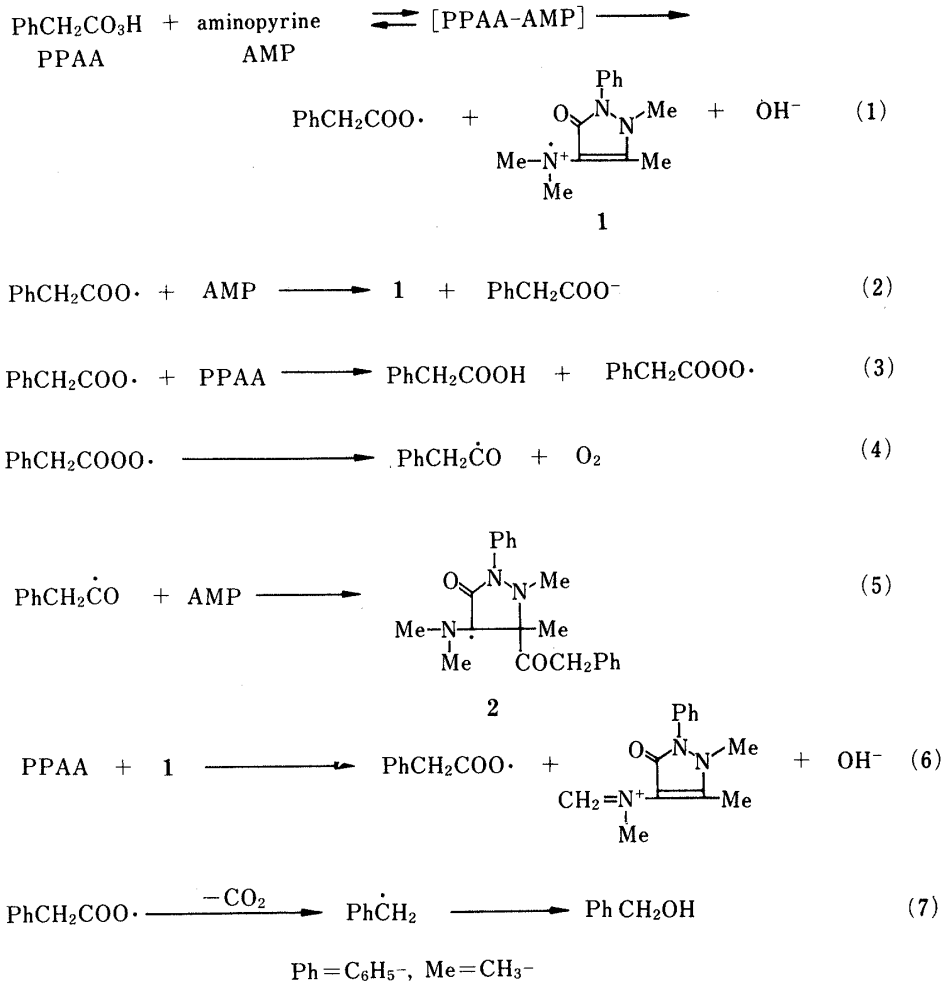

Chart 2

comparison with the result in the absence of catalase, as opposed to the results of the PPAA-supported hydroxylation catalyzed by cytochrome P-450.[5] Since the decarboxylation of peracids is diagnostic of peroxide homolysis, this result indicates that homolytic oxygen–oxygen bond cleavage and oxy radicals generated play no major role in the PPAA-supported oxidation of AMP by catalase. That is to say, although in the absence of catalase PPAA generates various radicals which react with AMP, in the presence of catalase the radicals derived from PPAA are not active oxidants. Therefore, the reaction is considered to proceed through a sequential mechanism involving the formation of a ternary complex between catalase, AMP, and PPAA in a similar manner to the CHP-supported oxidation of AMP by catalase.

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