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Anti- and Prooxidative Properties of Gallic Acid in Fenton-Type Systems

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The anti- and prooxidative properties of gallic acid in Fenton-type systems containing H_2O_2 and Fe(III) were examined in pH 3−10 reaction media and at reaction temperatures of 20−50 °C. Although it is a free radical scavenger, gallic acid may exhibit prooxidative properties, as it promotes the production of hydroxyl radicals due to iron chelation. The overall effect is prooxidative if the ratio of the concentrations of gallic acid and Fe(III) in the reaction medium is smaller than 2. If the ratio is greater than 2, the overall effect of gallic acid presence is antioxidative due to free radical scavenging properties. The dependence of rates and of apparent activation energies of gallic acid consumption on pH in Fenton-type systems was also examined, and it is concluded that the rate-determining steps in acidic and alkaline media are different, the overall rate of gallic acid consumption being lowest at pH 7.

KEYWORDS: Phenolic antioxidants; transition metals; iron chelation; free radicals; Fenton reaction

INTRODUCTION

Gallic acid (GA, 3,4,5-trihydroxybenzoic acid) is a natural product of hydrolysis of tannins. It is present in food of plant origin, and since it was found to exhibit antioxidative properties, it has attracted considerable interest. Prevention of food and drug oxidation and in vivo antioxidative properties are the focus of many studies (*1*−*5*), especially since phenolic compounds are found in abundance in red wines and teas. In addition, gallic acid is an effective antimicrobial compound (*2*), and novel antioxidative and antimicrobial food additives are being developed using gallic acid as a starting compound (*6*). Since iron is the most abundant transition metal in the human body and most of the dietary intake of iron is in the ferric form (*7*), the interactions between Fe(III) and GA are of primary importance. Due to the blue-colored complex formed with iron, gallic acid was also used as a primary component of writing inks, the so-called iron gall inks (*8*, *9*), and its antioxidative properties are thus of interest also in the area of polymer (i.e., cellulose) degradation.

The reaction system containing Fe(III) and GA in the presence of atmospheric O_2 is a complex one. Stability constants for several possible complexes obtained under nitrogen atmosphere at pH 3−11 (*10*), and the rate of complex formation at pH 4−5 (atmosphere not specified), were reported (*11*). At pH 4.4−5.4, the molar ratio Fe:GA 1:3 in the complex was found (*12*), and a subsequent rearrangement and esterification reaction was postulated. In media of pH <2, oxidation of GA with Fe(III) should also be taken into account (*13*). The rate constants for an intramolecular electron transfer between Fe(III) and GA were recently provided (*7*).

At pH >7, gallic acid and its analogues are rapidly oxidized by atmospheric oxygen, as shown in a spectrophotometric study by Friedman and Jürgens (*2*), although kinetic data are given for gallic acid analogues only. In a study of the antioxidant capacity of eight phenolic and related compounds, Galato et al. (*4*) have shown that the greater the number of hydroxyls linked to the aromatic ring, the greater the antioxidant activity of the compound. In an electrochemical study (*3*) of GA and its derivatives in the pH range 2−7, the authors found that oxidation is irreversible, and a two-electron oxidation scheme leading to the production of quinoid structures in acidic media was postulated.

The presence of transition metals may greatly influence the antioxidant activity of GA, involving inhibition or promotion of free radical production (*14*) due to metal chelation. Kumamoto et al. (*5*) determined the antioxidant activity of several catechins in the pH range 1−13 and in the presence of 13 metal ions by measuring oxygen consumption. The antioxidant activity of GA was more pronounced in the pH region 6−12, and the addition of Fe(II) or Fe(III) had a slight prooxidative effect. The presence of H_2O_2 in reaction systems containing a transition metal in the reduced form (Fenton-type systems) may lead to the production of a strong oxidant, hydroxyl radical, $HO^{\bullet}$:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + HO^- + HO^{\bullet} \qquad (1)$$

Fenton chemistry is an extensively researched subject due to its importance in biochemistry and medicine (*15*, *16*), food (*17*) and environmental chemistry (*18*, *19*), and material research

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(*20*, *21*), and several reviews (*22*−*24*) are available on the subject. The hydroxyl radical scavenging activity of GA may be used to assess its antioxidative activity. In a study using chemiluminescence detection, GA was shown to exhibit such activity in the absence of iron (*25*), while in its presence, GA stimulated the production of $HO^{\bullet}$ if other iron chelators were present, e.g., EDTA (*26*, *27*), which led to the conclusion that the reducing property of GA leading to the production of Fe(II), capable of catalyzing reaction 1, is an important aspect.

Even in the system Fe(III)/H_2O_2, a steady-state concentration of Fe(II) is rapidly attained (*28*):

$$Fe^{3+} + H_2O_2 \rightarrow Fe^{2+} + HO_2^{\bullet} + H^+ \quad (2)$$

$$Fe^{3+} + HO_2^{\bullet} \rightarrow Fe^{2+} + O_2 + H^+ \quad (3)$$

Therefore, the system Fe(III)/H_2O_2/GA was used in our study, to more closely approximate real conditions, in which a higher proportion of Fe(III) is usually present due to the presence of atmospheric oxygen.

For the detection of $HO^{\bullet}$, a number of methods are in use. Effective radical scavengers are often used, as they react with hydroxyl radicals at a diffusion-controlled rate and are used in an excess concentration, so that the kinetics of the hydroxyl radical production can be assumed to be scavenger-concentration-independent. The *N*,*N*′-(5-nitro-1,3-phenylene)bisglutaramide (NPG) method (*29*, *30*) has many advantages by comparison, since neither the reactant nor the hydroxylated derivatives are strong iron chelators and the derivatives are colored, which enables spectrophotometric determination. Besides, NPG is reasonably stable at higher temperatures (*31*). Namely, to be able to estimate the anti- and prooxidative properties at various temperatures (important, e.g., in food processing), the temperature dependence of the rates of oxidation (consumption) of antioxidants is also highly interesting.

In the present study, the anti- and prooxidative activity of GA is studied at pH 3−9 and 20−50 °C in the presence of $FeCl_3$ and H_2O_2. In addition, a comparative study of antioxidative properties of NPG and GA at pH 7 is performed to reveal the mode of action of GA as it is being consumed during the reaction and as the molar ratio GA/Fe(III) in the solution approaches zero.

MATERIALS AND METHODS

Oxidation of GA. Purified deionized water (MilliQ water purification system, Millipore, Molsheim, France, producing ultrapure water with resistivity <18.2 MΩ cm at 25 °C) was used to prepare all solutions.

In the reaction solutions, gallic acid (p.a., Carlo Erba, Milano, Italy), $FeCl_3 \cdot 6H_2O$ (p.a., Fluka, Buchs, Switzerland), H_2O_2 (p.a., ∼30%, nonstabilized, Fluka), and buffer solutions (0.1 mol L^{-1}), obtained by mixing appropriate parts of KH_2PO_4, Na_2HPO_4, and Na_3PO_4 (p.a., Fluka), were used. The stock solution of H_2O_2 was titrimetrically standardized against $KMnO_4$ solution of known concentration, on a weekly basis. The reaction mixture typically contained 20 mmol L^{-1} phosphate buffer, 0.2 mmol L^{-1} GA, 0.1 mmol L^{-1} $FeCl_3$, and 20 mmol L^{-1} H_2O_2, although in certain experiments the concentration of GA was higher (0.5, 1, or 2 mmol L^{-1}).

Aliquots of the reaction mixture (1 mL) were taken periodically, mixed with 1 mL of catalase solution in phosphate buffer (0.1 mol L^{-1}, pH 7), containing 0.2 μL catalase suspension (1 300 000 u mg^{-1}, Merck, Darmstadt, Germany) to decompose the remaining H_2O_2, and shaken vigorously for 1 min.

Oxidation of GA with O_2 proceeded in the following way: 1.0 mmol L^{-1} solutions of GA in phosphate buffers, prepared as described above, were purged with O_2 (in control experiments with Ar) for 5 min, and the content of GA was followed periodically for 10 h.

All reaction mixtures (1 mL) were diluted with 4 mL of 0.5% trifluoroacetic acid solution (TFA, Fluka) prior to chromatographic determination of GA.

NPG Hydroxylation. For the experiments with *N*,*N*′-(5-nitro-1,3-phenylene)bisglutaramide (NPG), the chemical was synthesized and purified according to the literature (*29*) to obtain product of ∼99.5% purity. Hydroxylated derivatives [*N*,*N*′-(5-nitro-2-hydroxy-1,3-phenylene)bisglutaramide and *N*,*N*′-(5-nitro-4-hydroxy-1,3-phenylene)bisglutaramide] were obtained as described in (*30*) and used for the construction of the chromatographic calibration curves.

The reaction mixture typically contained 20 mmol L^{-1} phosphate buffer, 0.2 mmol L^{-1} GA, 0.1 mmol L^{-1} $FeCl_3$, 1 mmol L^{-1} NPG, and 20 mmol L^{-1} H_2O_2. Again, in some experiments, the concentration of GA was higher (0.5, 1, or 2 mmol L^{-1}). To decompose the remaining H_2O_2, 1 mL of the reaction mixture was added to 1 mL of the above-described catalase solution and vigorously shaken. The resulting mixture was used for determination of the hydroxylated derivatives of NPG, while it was acidified as above for GA determination.

Instrumentation. GA was determined chromatographically using an Agilent Series 1100 (Agilent, Palo Alto, CA) chromatographic system consisting of a binary pump, an automated injector, a reversed-phase column (Hypersil ODS, Agilent), and a diode-array detector. The volume of injected solutions was 50 μL. The eluent used was 97% TFA (0.1% v/v) and 3% acetonitrile (HPLC gradient grade, Rathburn, Walkerburn, UK) with isocratic elution. Detection was performed at 271 nm.

Hydroxylated NPG derivatives (given above) were determined with the same system; however, gradient elution was used, consisting of 3−9% acetonitrile and 97−91% phosphate buffer (pH 7, 20 mmol L^{-1}) in the first 6 min. The column was then flushed with 30% acetonitrile for 3 min and conditioned with 3% acetonitrile for 3 min prior to the next injection. The detection was performed at 232 (for the ortho-hydroxylated product) and 222 nm (for the para-hydroxylated product).

For spectrophotometric measurements, a Perkin-Elmer Lambda 2 UV−vis spectrophotometer (Perkin-Elmer, Überlingen, Germany) with a 1-cm quartz cuvette was used.

RESULTS AND DISCUSSION

Oxidation with O_2. The stability of phenolic compounds in media with pH >7 is well known to be limited (*2*). Oxidation of GA with dissolved O_2 was first studied for the purpose of comparison with systems containing transition metals and H_2O_2 (Fenton-type systems). In reaction mixtures containing no oxidant (purged with Ar), there was no significant decrease of GA concentration. In systems purged with O_2, the content of GA initially decreased in an exponential manner, so the oxidation reaction may be assumed to be first order with respect to GA concentration. The first-order GA degradation constants increase with increasing pH, as shown in **Figure 1**, while in media of pH <6, the consumption of GA due to oxidation was negligible.

Gallic Acid: Pro- or Antioxidant? Following the experiments with oxygen as the oxidant, the mode of action of GA in systems containing Fe(III) was examined. While Jovanovic et al. (*32*) have shown several Job plots for gallocatechins in order to estimate the stoichiometry of the complex Fe/ligand at pH 7, such a plot for GA is shown in **Figure 2**, clearly indicating that two molecules of GA coordinate the Fe(III) ion under the conditions used. GA forms a stable complex: Jovanovic et al. (*32*) derived the value $\log K \approx 27$ for the formation of Fe(III)-bis(gallocatechin) at pH 7, and a similar value for gallic acid, $\log K = 34$, was given by Loginova et al. (*10*).

The molar ratio GA:Fe(III) 2:1 was therefore chosen as the lower limit of GA concentrations used in Fenton-type systems. The consumption of GA due to oxidation was followed at

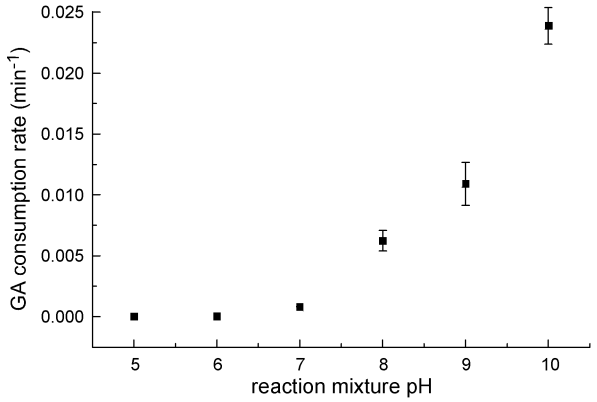

Figure 1. Rates of GA consumption during oxidation with O_2 in reaction mixtures buffered with phosphate buffers with pH as indicated, $T = 20$ °C, error bars indicating the standard deviation for triplicate determinations.

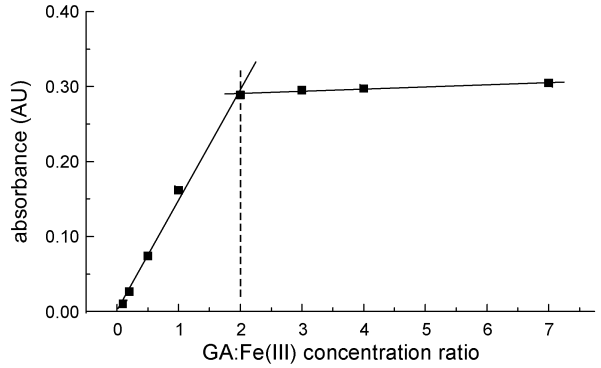

Figure 2. Absorbance at 546 nm of solutions of $FeCl_3$, $c = 4$ mmol L^{-1}, and GA (in molar ratios as indicated) at pH 7 (phosphate buffer, 20 mmol L^{-1}).

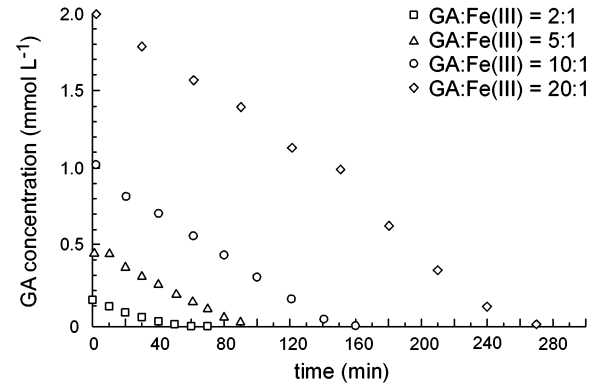

Figure 3. Decrease of GA concentration with reaction time during experiments in Fenton-type systems using different initial molar ratios GA:Fe(III), as indicated, phosphate buffer pH 7, $T = 50$ °C.

several other molar ratios up to 20:1, as shown in **Figure 3**. A linear model can be fitted to the data, and the reaction rates can be expressed as $d[GA]/dt = -kt$; the rate of consumption of GA is therefore independent of its concentration. In case of the initial molar ratio GA:Fe(III) 2:1, the linear model is initially valid, but as GA is being consumed, the zero-order approximation can no longer be used.

To study the mode of action of GA, it is of interest to estimate the quantity of hydroxyl radicals available for reaction with other oxidizable species. As NPG is a potent HO• scavenger but not an iron chelator (see Introduction), the concentration of hydroxylated NPG derivatives formed in the system Fe^{3+}/GA may be used for such estimations (**Figure 4**). The rate of consumption

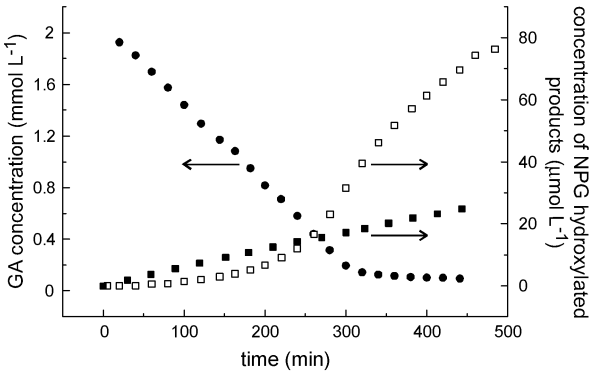

Figure 4. Decrease of GA (●) and simultaneous increase of NPG hydroxylated products (□) concentration with reaction time in Fenton-type systems with initial molar ratio GA:Fe(III) 20:1, phosphate buffer pH 7, $T = 50$ °C. (■) Production of hydroxylated HPG derivatives in the reference reaction system without GA.

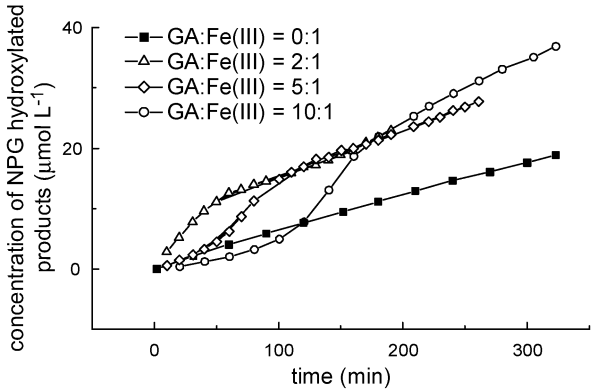

Figure 5. Increase of concentration of NPG hydroxylated products with reaction time in Fenton-type systems with initial molar ratios GA:Fe(III) as indicated, phosphate buffer pH 7, $T = 50$ °C, and in the reference system without GA.

of GA in this system is the same as that in the prior experiment (**Figure 3**), indicating that HO• production is not significantly influenced by the addition of NPG. The additional curve in **Figure 4** describing NPG hydroxylation in the absence of GA provides a reference value for the rate of HO• production.

The distinctly sigmoidal shape of the curve describing production of hydroxylated NPG derivatives in the presence of GA (**Figure 4**) leads to several conclusions. Initially, when GA is still present in considerable excess of the concentration of Fe(III), the rate of NPG hydroxylation is lower than that in the reference system. The proportion of HO• available to oxidize species other than GA is therefore comparably low, and the overall effect of GA is antioxidative. It can be presumed that GA present in the nearest vicinity to the site of HO• production, i.e., GA forming the chelate, is the most probable to be oxidized, given the high log K values discussed above.

In **Figure 5**, several additional experiments are shown with different starting molar ratios GA:Fe(III). Only hydroxylation of NPG is followed and compared with the reference reaction system. In all curves, as the concentration of GA approaches 0.2 mmol L^{-1}, i.e., the molar ratio GA:Fe 2:1, the rate of NPG hydroxylation increases and becomes higher than the rate in the reference system without GA. This leads to the conclusion that GA in fact promotes HO• production in iron-containing systems. This conclusion is in agreement with some studies (*27*). The increased rate of NPG hydroxylation continues beyond the point of consumption of all GA (**Figure 4**), so it can also be

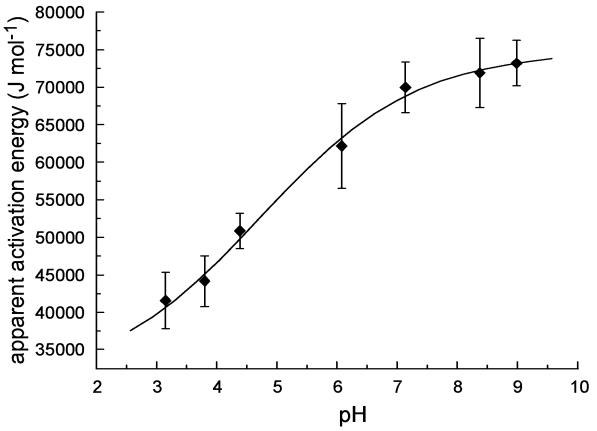

Figure 6. Apparent activation energies of GA consumption in Fenton-type systems buffered with phosphate buffers with pH as indicated, error bars representing the standard deviation of the regression line slope: $\ln(k) = -K/T + \text{const.}$, multiplied by R. Initial molar ratio GA:Fe(III) 2:1.

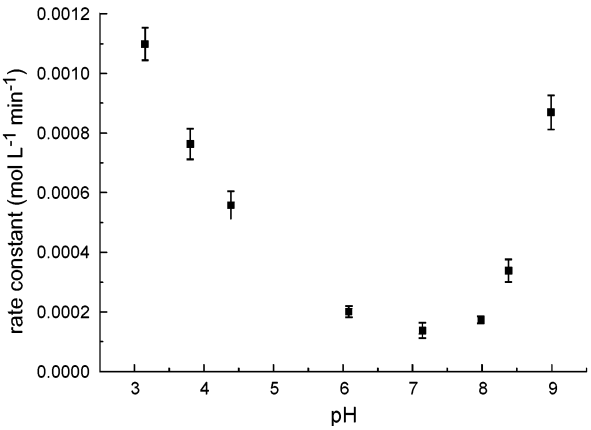

Figure 7. Rate constants for GA consumption in Fenton-type systems buffered with phosphate buffers with pH as indicated, $T = 20$ °C, error bars representing the standard deviation for triplicate determinations. Initial molar ratio GA:Fe(III) 2:1.

concluded that the intermediate products of GA oxidation in Fenton-type systems also have prooxidative properties.

From the experiments shown, it can be assumed that if the molar ratio of GA:Fe is <2, the overall effect of GA is prooxidative. It is well known that iron chelators may either promote or inhibit the production of hydroxyl radicals, depending on the values of stability constants with Fe(II) and Fe(III), and depending on the possibility of smaller ligands, such as H_2O_2, to approach iron and occupy a free orbital (*33*). In systems with the molar ratio GA:Fe > 2, GA still promotes the production of hydroxyl radicals; however, since it is also a potent scavenger, the overall effect of GA is antioxidative. From **Figure 5**, it can additionally be concluded that, unlike the intermediate oxidation products, the final degradation products do not have a pronounced effect on the rate of $HO^\bullet$ production, as the final slopes of curves describing the production of hydroxylated NPG derivatives are similar to that of the reference system.

Temperature and pH Dependence of Oxidation in Fenton-Type Systems. In Fenton-type systems containing GA, Fe(III), and H_2O_2 only, the initial rate of consumption of GA is zero-order with respect to the concentration of GA (**Figure 3**). The rates (k) obtained at different temperatures (T, in K) of the reaction mixture (20−50 °C) can be plotted in Arrhenius's coordinates, i.e., $\ln(k) = -K/T + \text{const.}$, where K represents E_a/R, R being the universal gas constant (8.314 J moL^{-1} K^{-1}) and E_a the apparent activation energy for the reaction system, representing the temperature dependence of the GA consumption rate (**Figure 6**). Using the apparent activation energy, rates of GA consumption can therefore be estimated at various temperatures from Arrhenius's equation.

The rates of GA consumption in Fenton-type systems at 20 °C in the pH region 3−9 are shown in **Figure 7**. The curve shape at pH >7 may reflect the increasing oxidizability of GA with increasing pH, as noticed in GA oxidation experiments with dissolved O_2, described above (**Figure 1**).

As it was shown in a pulse radiolysis study on the reaction of gallic acid with hydroxyl radicals (*34*), the phenoxyl radicals formed are quite stable, thus making GA a good antioxidant in neutral and alkaline solutions. The increasing rate of GA consumption as the reaction solution becomes more acidic (**Figure 7**) may reflect the pH dependence of another reaction or reaction system, possibly the production of $HO^\bullet$ (*35*, *36*). This conclusion is further supported by the pH dependence of the apparent activation energy for GA consumption, as shown in **Figure 6**.

CONCLUSIONS

At pH 7, the molar ratio of GA:Fe(III) in the complex was shown to be 2:1. The data presented indicate that in Fenton-like systems containing Fe(III)/H_2O_2:

If the molar ratio GA:Fe < 2:1, gallic acid has a prooxidative role due to promotion of $HO^\bullet$ production.

If the molar ratio GA:Fe > 2:1, the overall role is antioxidative due to the pronounced $HO^\bullet$ scavenging properties of GA. Although $HO^\bullet$ production is still promoted, GA in the immediate vicinity of the site of hydroxyl radical production, i.e., in the complex, is probably oxidized first.

The immediate degradation products increase the rate of $HO^\bullet$ production, while the end products of GA oxidation have no influence on the rate.

The rates of GA consumption in the presence of Fe were shown to be pH dependent, the lowest being at neutral pH of the reaction medium.

The dependence of apparent activation energy for GA consumption on pH was demonstrated: from 41 kJ mol^{-1} at pH 3 to 72 kJ mol^{-1} at pH 9.

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