INHIBITION OF LIPID OXIDATION BY PHENOLIC ANTIOXIDANTS IN RELATION TO THEIR PHYSICOCHEMICAL PROPERTIES

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Summary

The aim of this study was to correlate the lipid peroxidation inhibition capacity (LPIC) of phenolic antioxidants to their physicochemical properties. The antioxidant activity of phenolic compounds is thought to be related to their physicochemical properties such as the phase partition coefficient (log P), the half-wave potential ($E_{p/2}$) and the bond dissociation enthalpy (BDE). The LPIC assay uses liposomes to simulate a cell membrane and can measure the ability of both lipophilic and hydrophilic antioxidants to protect a lipophilic fluorescent probe (C_{11}-BODIPY) from free radical attacks. BDE was calculated by the semi-empirical quantum chemical method (PM3), $E_{p/2}$ was taken from published research and log P was determined by RP-HPLC. A quantitative structure-activity relationship (QSAR) was derived between LPIC activities of twenty phenolic compounds and their BDE, $E_{p/2}$ and log P ($r^2 = 0.82$). This work demonstrated that the LPIC activities of phenolic antioxidants had strong correlation with their physicochemical properties. Therefore, it is feasible to estimate LPIC activity of phenolic antioxidant from their physicochemical properties. This method can be used in estimating the potential biological activities of phenolic antioxidants and the optimization of structures.

Keywords: Antioxidant; Lipid oxidation; LPIC; Lipophilic fluorescent probe; Liposome; Partition coefficient; Phenolic antioxidants; Physicochemical property.

Phenolic compounds (ArOH) are widely distributed in fruit and vegetables and form part of the human diet. These compounds are thought to be a contributing factor to the health benefits of fruit and vegetables in part because of their antioxidant activities.

From a chemical point of view, the antioxidant activity of phenolic compounds is based on an electron transfer followed by transfer of an hydroxylic hydrogen to a chain-promoting radical and on the relative stability of the phenolic free radical formed (1). In lipid oxidation, the lipid hydroxyperoxyl radical is converted to a relatively stable hydroperoxide (2). One reason that phenolic compounds are considered antioxidants is their ability to quench oxidative radical chain-reactions by transfer of a hydroxylic hydrogen to a chain-promoting radical. In lipid oxidation, the lipid hydroxyperoxyl radical is converted to a relatively stable hydroperoxide. The bond dissociation enthalpies (BDE) in ArOH have been considered to provide the quantitative measure of the stabilities of the radicals formed ($ArO^{\bullet}$) (3). The properties of the ArO-H bond appear to be essential to understanding the chemical and biochemical behaviour of phenolic compounds. As phenolic compounds are usually chain-breaking antioxidants, the H-abstraction mechanism plays the most important role in terms of antioxidant activity (4). The electron-donating ability of phenolic compounds is determined by the one-electron oxidation potential of the parent antioxidants (5).

The redox potential is a key thermodynamic property to predict the direction of a possible reaction (6). The half-wave potential ($E_{p/2}$) is the potential of a polarographic or voltammetric indicator electrode at the point, on the rising part of a polarographic or voltammetric wave, where the difference between the total current and the residual current is equal to one-half of the limiting current (7). The $E_{p/2}$ reflects the specific reducing power of a component (or components with similar potential) and is thus a suitable single parameter to describe antioxidant power (7, 8). In this study, the half-wave potentials of phenolic antioxidants were taken from the literature and limited to cyclic voltammetric values.

Lipophilicity of compounds of bioactive interest is also an important parameter to account for as bioactive potential is influenced by transport processes across biological barriers (9). The lipophilicity of phenolic compounds, as indicated by the base ten logarithm of the octanol/water partition coefficient (log P), is used as a parameter in chemical toxicology as it can indicate metabolic fate, biological transport properties and intrinsic biological activity (10). Uptake of most organic chemicals to the site of action is by passive diffusion and is best modeled by lipophilicity (11). It is possible to quantify the degree to which an antioxidant′s action is moderated by its ability to enter the locus of autoxidation (12, 13).

An *in vitro* antioxidant assay has been recently developed to better reflect the *in vivo* conditions of antioxidants interacting with lipid membranes and thus better predict their ability to contribute to human health. . The lipid peroxidation inhibition capacity (LPIC) assay (14) employed the lipid-like fluorescent probe C_{11}-BODIPY ((4, 4-difluoro-5-(4-phenyl-1,3-butadienyl)-4-bora-3a,4a-diaza-s-indacene-3-undecanoic acid), which is incorporated into 100 nm DOPC liposomes. Various antioxidant compounds were either incorporated into liposomes ($LPIC_{Inco}$) or mixed with preformed liposomes ($LPIC_{Mixed}$). The azo initiator AAPH (a peroxyl radical generator, 2,2′-azobis(2-amidinopropane)hydrochloride) was used to trigger lipid peroxidation and effects on integrated C_{11}-BODIPY red fluorescence intensity (600 nm) were tracked. Inhibition of fluorescence decay (reflecting oxidation of probe) indicated antioxidant potency. The apparent advantage of this approach is that it tested antioxidants in lipid membrane settings instead of homogeneous solution used for many existing assays. In addition, the LPIC can be used to measure the activities of both lipophilic and hydrophilic antioxidants by using membrane lipids as the carrier.

In this study, the relationship between LPIC activities and these three physicochemical properties (BDE(ArO-H), $E_{p/2}$ and log P) of antioxidants was investigated and the contribution of each property to its LPIC activity was evaluated.

Materials and Methods

Materials

The fatty acid fluorescent probe C_{11}-BODIPY was obtained from Molecular Probes (Eugene, OR). All phenolic compounds and other chemicals were purchased from Sigma (St. Louis, MO).

Methods

The lipid peroxidation inhibition capacity (LPIC) assay was performed as previously described (14). The $LPIC_{Inco}$ version (the antioxidant was incorporated into the liposomes when prepared) of the LPIC assay was used in this study. The partition coefficients (log P) of phenolic antioxidants were calculated from their HPLC retention times (t_R). The retention times of antioxidants were determined by RP-HPLC as described previously (14). The semi-empirical quantum chemical method PM3 (parameterization method 3) was employed to calculate theoretical parameters, including the heat of formation of a parent phenolic molecule (H_{fp}) and that of its phenoxyl radical (H_{fr}). Calculation of these parameters was performed by using the ChemOffice 2002 ultra program (http://www.camsoft.com/). Energy was minimized by using the PM3 method at restricted Hartree-Fock (RHF). After the calculation of the H_{fp}, the phenolic H was removed and a restricted Hartree-Fock optimization was performed on the phenoxyl radical. The BDE(O-H) can be approximated by the difference (ΔH_f) in calculated heat of formation between parent phenolic molecule (H_{fp}) and its phenoxyl radical (H_{fr}). Calculations for the heat of formations of large flavonoid molecules are computationally intensive, and therefore, the ΔH_f of some flavonoids was obtained from the literature with similar calculation settings. The cyclic voltammetric half-wave potentials of phenolic antioxidants were taken from the literature.

Statistical analysis

The method of multiple linear regressions was adopted since more than one set of independent variables needed to be analyzed. All the stepwise multiple linear regressions were preformed by Microsoft Excel® 2000.

Results and Discussion

Thirteen flavonoids, four hydroxycinnamic acids, two synthetic antioxidants (BHT and BHA) and α-tocopherol were used in this study. These compounds were selected based on the availability of the $E_{p/2}$ values in the literature. The $LPIC_{Inco}$ along with their ΔH_f, $E_{p/2}$ and log P values are shown in Table 1.

Table 1. $LPIC_{Inco}$, ΔH_f, $E_{p/2}$, and log P values of flavonoids, hydroxycinnamic acids, synthetic antioxidants (BHT and BHA) and α-tocopherol.

Selected Compounds	**$LPIC_{Inco}$ (μmole Trolox)**	**ΔH_f (KJ/mole)**	**$E_{p/2}$ (mV)**	**log P**
Quercetin	4.34	112.3 (15)[1]	60 (16)	2.29
Rutin	3.62	125.5 (15)	180 (17)	1.53
Catechin	3.11	125.7 (18)	160 (19)	1.04
Naringin	0.29	155.4	1000 (19)	1.23
Apigenin	0.36	153.2 (18)	1000 (19)	2.85
Morin	1.78	142.1	340 (20)	1.93
Kaempherol	1.58	150.8 (18)	390 (21)	2.77
Chlorogenic acid	2.57	134.2 (15)	370 (21)	0.49
Naringenin	0.41	149.6 (18)	760 (21)	2.05
Hesperitin	1.43	125.8 (18)	590 (21)	2.18
Sinapic acid	2.31	135.4 (22)	605 (23)	0.96
Ferulic acid	2.23	138.6 (22)	775 (23)	1.09
Caffeic acid	3.92	113.0 (22)	333 (24)	1.08
Phloridzin	0.80	151.3	786 (24)	1.40
Phloretin	1.04	146.5	540 (25)	2.22
Myricetin	2.16	130.4 (18)	-30 (25)	1.22
Hesperidin	1.14	140.5	440 (25)	1.28
α-Tocopherol	1.61	124.0 (15)	859 (26)	12.05
BHA	1.72	145.7	328 (27)	2.60
BHT	3.00	136.4 (15)	276 (27)	3.86

Notes[1]: Numbers in brackets are literature citations.

Correlation between O-H bond dissociation enthalpy (BDE) and $LPIC_{Inco}$

All heat of formations were calculated or selected by the PM3 semi-empirical method, for energy-optimized species. The ΔH_f of a given compound represents the difference between the parent compound and the appropriate radical, which was constructed by an abstraction of a hydrogen atom from assigned hydroxyl moiety. This value may represent the relative stability of a radical with respect to its parent compound, and it enables a comparison to be made between the stabilization achieved by hydrogen abstraction (toward radical formation) (15, 28-30). A summary of calculated and selected ΔH_f for the H-abstraction from hydroxyl groups in all the phenolic compounds tested is shown in Table 1.

Generally speaking, the smaller the ΔH_f, the more stable the phenoxyl radical and the weaker the O-H bond in the molecule, so the more active is the antioxidant (8). There was good correlation between the $LPIC_{Inco}$ values and the lowest ΔH_f value of each compound tested. Moreover, for the antioxidants evaluated, the correlation between $LPIC_{Inco}$ and ΔH_f showed linearity (Figure 1, r^2 = 0.71).

As shown in a previous work, flavonoids with a catechol group in the B-ring are the most active free radical scavengers (14). It appears that the rest of the hydroxyl groups of the flavonoid are of little importance to the antioxidant activity, except for quercetin and its derivatives, in which the combination of the catechol moiety with a 2,3-double bond at the C-ring and a 3-hydroxyl group results in an extremely active scavenger. Therefore, the ΔH_f values were calculated from the O-H bond in the B-ring, and only the most stable phenoxyl radical is considered as shown in Table 1.

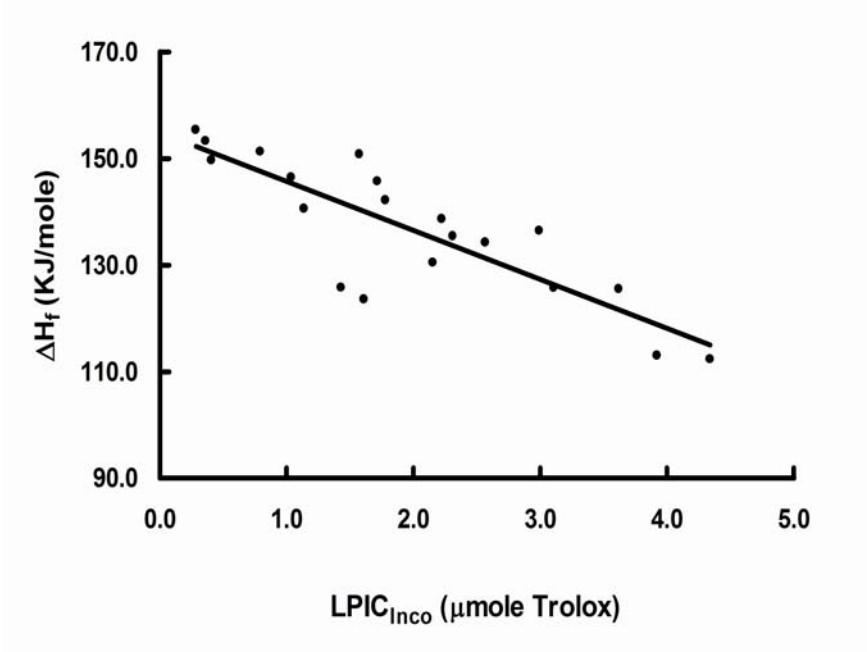

Figure 1. Correlation between $LPIC_{Inco}$ (lipid peroxidation inhibition capacity of phenolic compounds) and ΔH_f (the lowest heat of formation of ArO-H bond of phenolic compounds) ($r^2 = 0.71$, n=20)

Correlation between half-wave potential ($E_{p/2}$) and $LPIC_{Inco}$

Another parameter thought to govern the biological activity of phenolic compounds is their redox property. The tendency of an antioxidant to undergo electron transfer may relate directly to its free radical scavenging capacity (25, 31, 32). The half-wave potential of compounds tested is listed in Table 1. All the data was obtained from literature as indicated. The data are relative to SCE (saturated calomel reference electrode). There was some correlation between the $LPIC_{Inco}$ values and the $E_{p/2}$ value of each compound tested (Figure 2, $r^2 = 0.55$) and the $LPIC_{Inco}$ activity of antioxidants increased with the decreasing half-wave potential.

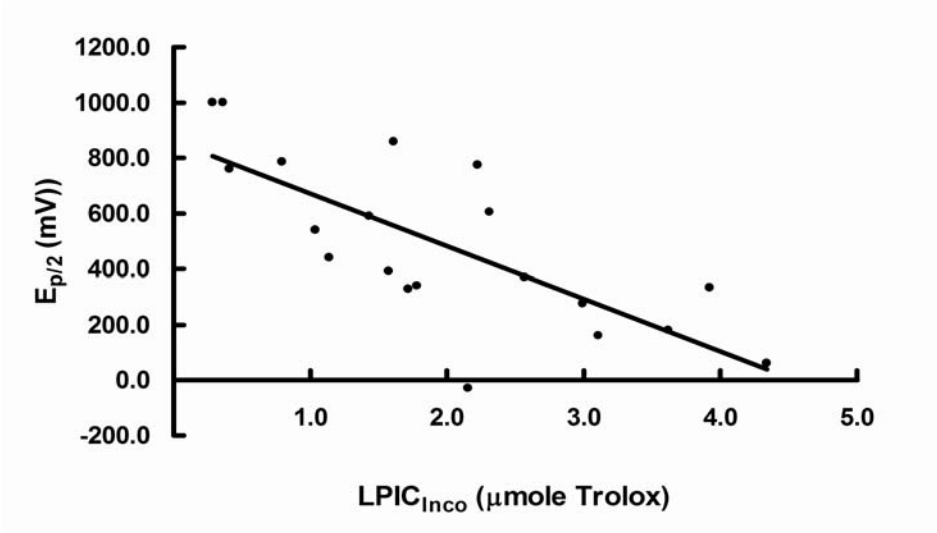

Figure 2. Correlation between $LPIC_{Inco}$ (lipid peroxidation inhibition capacity of phenolic compounds) and $E_{p/2}$ (cyclic voltammetric half-wave potentials of phenolic antioxidants) ($r^2 = 0.55$, n=20).

There was a wide range of half-wave oxidation potentials of the investigated antioxidants. They spread from very easily oxidized (-30 mV for myricetin) to not oxidizable (greater than 1000 mV for naringin and apigenin). There was much more variation than with the ΔH_f values. To be a good antioxidant, the $E_{p/2}$ should be less than 350 mV. There was an exception to this rule in that myricetin was much less active than expected from the $E_{p/2.}$ This indicated that there must be other factors that govern the antioxidant activity, such as chemical structural features and solubility.

Correlation between partition coefficient (log P) and $LPIC_{Inco}$

There was no correlation between the partition coefficient (log P values) and the $LPIC_{Inco}$ activities (Figure 3) in contrast to the expectation that $LPIC_{Inco}$ should be related to lipid solubility.

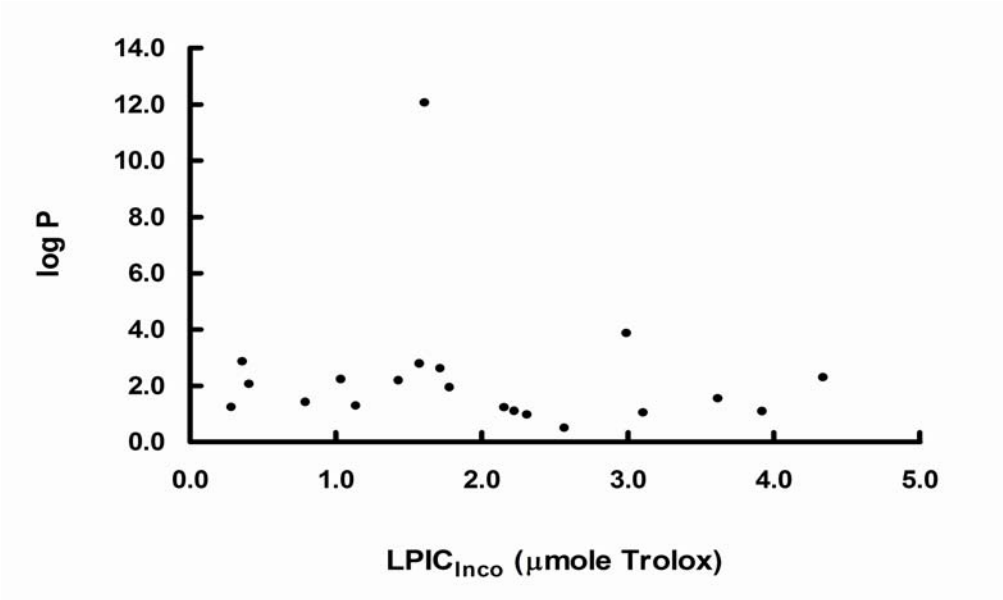

Figure 3. Correlation between $LPIC_{Inco}$ (lipid peroxidation inhibition capacity of phenolic compounds) and log P (partitioning coefficients of phenolic compounds measured by RP-HPLC ($r^2 = 0.02$, n = 20).

This result demonstrated that the antioxidant activity of phenolic compounds is not solely dependant on their partition coefficient. It has long been recognized that for a chemical to be biologically active, it must first be transported from its site of administration to its site of action and then it must bind to or react with its receptor or target, i.e. biological activity is a function of partition and reactivity (33). It should be noted that the effect of membrane partitioning is not necessarily a direct relationship with lipophilicity. Beyeler and coworkers (34), for example, reported that the effects of cianidanols on rat hepatic monooxygenase increased with lipophilicity, reached a plateau, decreased and then leveled off for the most lipophilic compounds.

Therefore, the balance of lipophilicity and lipophobicity allowing concentration at the interface is an important factor in the estimation of the antioxidant activity of phenolic compounds. This is presumably because a) at high values of log P, the antioxidant is dispersed in a lipid phase and not located at the lipid-water interface, b) at low values of log P, the antioxidant is located in an aqueous phase and has insufficient solubility in the lipid phase. This can also be important in terms of paracellular transport of phenolic compounds and the ability to enter the cell to participate in *in vivo* protection from oxidative damage.

Quantitative structure-activity relationship model

The quantitative structure-activity relationships (QSAR) paradigm has been useful in elucidating the mechanisms of chemical-biological interactions in various biomolecules, particularly enzymes, membranes, organelles and cells (28, 35, 36). The description of quantitative structure-activity relationships (QSARs) has been undertaken in order to find predictive models and/or mechanistic explanations for chemical as well as biological activities (37). The underlying premise of SARs and QSARs is that the properties of a chemical are implicit in its molecular structure (37) and the behavior of chemical compounds is dominated by their physicochemical properties (10). If a QSAR model is deficient in modeling either partition or reactivity, only a partial correlation with the *in vivo* response is likely to be observed. The ΔH_f, which represents the stability of the free radical formed after H-abstraction, and the half-wave oxidation potentials, which describe the electron transfer property, could be used to describe the activity of antioxidants. Experimentally determined half-wave potential ($E_{p/2}$) has been used as a direct measure of the antioxidant property by several authors (7, 38, 39). Log P is also an important parameter in chemical toxicology as it can indicate metabolic fate and biological transport properties.

In total, twenty phenolic compounds were selected based on the availability of the $E_{p/2}$ values in the literature. The $LPIC_{Inco}$ along with their log P, $E_{p/2}$, and ΔH_f values are shown in Table 1. Here, a quantitative structure-activity relationship is modeled by starting from log P values and then incorporating other reactivity parameters (ΔH_f and $E_{p/2}$).

The relationship between $LPIC_{Inco}$ and log P can be described by the Equation 1.

$LPIC_{Inco} = -0.0525 (\pm 0.11) \log P + 2.091 (\pm 0.38)$ [1]
$n = 20, r^2 = 0.0117, p = 0.650$

A two-parameter equation was derived using the lipophilicity (log P) and the ease of oxidation ($E_{p/2}$) in the lipid membrane system by using multiple regressions as in Equation 2.

$LPIC_{Inco} = 0.0486 (\pm 0.081) \log P - 0.0030 (\pm 0.00066) E_{p/2} + 3.326 (\pm 0.37)$ [2]
$n = 20, r^2 = 0.5468, p = 0.000971$

The introduction of the new parameter ($E_{p/2}$) to Equation 1 increased the correlation coefficient r^2-value from 0.0117 to 0.5486. With the introduction of ΔH_f (ease of H-abstraction) rather than $E_{p/2}$ into Equation 1, it can also be used to derive a two-parameter predictive model in the lipid membrane system as shown in Equation 3.

$LPIC_{Inco} = -0.116 (\pm 0.058) \log P - 0.0080 (\pm 0.011) \Delta H_f + 13.23 (\pm 1.53)$ [3]
$n = 20, r^2 = 0.7637, p = 0.00000473$

The introduction of ΔH_f increased the correlation coefficient r^2-value from 0.0117 to 0.7637. Compared with Equation 2, the $LPIC_{Inco}$ activity can be more precisely predicted by using the lipophilicity and ΔH_f than using lipophilicity and the half-wave oxidation potential.

A three-parameter model was obtained by the introduction of the ΔH_f (ease of H-abstraction) into the Equation 2 or the $E_{p/2}$ (ease of electron transfer) into the Equation 3. Therefore the addition of $E_{p/2}$ or ΔH_f to their corresponding two-parameter model increased the correlation coefficient r^2-value but the order of addition has no effect on the three-parameter model.

$LPIC_{Inco}$ = -0.058 (±0.057) log P–0.0013(±0.00056) $E_{p/2}$–0.062(±0.013) ΔH_f +11.18(±1.62)
n = 20, r^2 = 0.8228, p = 0.00000298 < 0.05 [4]

The use of the new parameter (ΔH_f or $E_{p/2}$) increased the correlation coefficient r^2-value to 0.8228. The significant increase in correlation coefficient upon the introduction of ΔH_f confirmed the importance of O-H bond strength (bond dissociation energy approximated by ΔH_f), which contributed most to the model. A QSAR model was derived from the lipid peroxidation inhibition capacity and calculated and/or measured theoretical parameters (enthalpy of hemolytic O-H bond cleavage ΔH_f, half-wave oxidation potential, and the partition coefficient). It demonstrated that the H-abstraction was not the sole mechanism responsible for the reaction between phenolic antioxidants and the lipid peroxyl radicals. Although its relative contribution of half-wave oxidation potential was less than that of ΔH_f, it significantly influenced the reactivity of phenolic antioxidants. It seems that the relative contribution of lipophilicity (log P) is much smaller than that of ΔH_f and $E_{p/2}$. However, the balance of lipophilicity and lipophobicity is still critical in the selection of phenolic antioxidants.

Conclusions

In this study, we carried out a theoretical investigation on the possible mechanisms governing the antioxidant activity of a series of phenolic compounds by computational chemistry, and explored the correlation between experimentally determined lipid peroxidation inhibition capacity and physicochemical properties. Judging from the improvement in the correlation coefficient in the stepwise multiple-linear regression, we can conclude that more precise the mechanistic information included in the QSAR model, the better the coefficient of relation that is obtained. It is reasonable to conclude that multiple mechanisms regulate the antioxidant actions of phenolic compounds although they contribute to antioxidant activity to different degrees.

In summary, it is feasible to estimate the $LPIC_{Inco}$ activities of a phenolic compound from the lipophilicity, half-wave oxidation potential and the difference of heat of formations. These results suggest the possibility of predicting the degree of contribution of different physicochemical factors among phenolic compounds to their *in vitro* inhibition of lipid peroxidation in liposomal system.

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