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Antioxidant Activity of Resveratrol Compared with Common Food Additives

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ABSTRACT

Resveratrol is a phenolic compound of the stilbene family present in wines and various parts of the grape, including the skin. In this study, the antioxidant and prooxidant activities of resveratrol were compared with other antioxidants (butylated hydroxytoluene [BHT], butylated hydroxyacetone [BHA], phenol, propyl gallate [PG], sodium tripolyphosphate [TPP], α-tocopherol, and vanillin) widely used in foods. The ability of these compounds to inhibit lipid peroxidation was as follows: BHA > resveratrol > PG > tripolyphosphate > vanillin > phenol > BHT > α-tocopherol, the first three inhibiting the peroxidation in a concentration-dependent manner. The order of $OH^{\bullet}$ scavenger activity of the tested compounds was BHA > TPP > BHT. Resveratrol and vanillin produced between 10 to 7% and 16 to 10% inhibition of the deoxyribose attack, respectively, but they do not scavenge $OH^{\bullet}$. Neither the resveratrol analyzed nor PG or the rest of compounds reacted with H_2O_2 and must be considered inefficient in catalyzing any subsequent oxidation. The ability to scavenge HOCl was, in decreasing order, PG > resveratrol > α-tocopherol > phenol. The other compounds did not scavenge HOCl.

An antioxidant may be defined as "a substance that when present at low concentrations compared with those of an oxidizable substrate such as fats, proteins, carbohydrates, or DNA, significantly delays or prevents the oxidation of the substrate" *(1)*.

Antioxidants are of interest to food scientists, polymer chemists, radiation chemists, and food manufacturers *(5)*. Oxidation of the lipid components in food by the free radical chain reaction of lipid peroxidation is a major problem for food manufacturers. The extent to which oxidation of fatty acids and their esters occurs in foods depends on the chemical structure of the fatty acid, the nature of food processing, and the temperature at which the foods are stored and/or cooked, and the minor constituent antioxidants *(2)*.

Many food antioxidants (butylated hydroxyanisole [BHA], butylated hydroxytoluene [BHT], and propyl gallate [PG]) are chain-breaking inhibitors of lipid peroxidation that act through a mechanism that produces a reaction between the radicals and proteins or fatty-acid side-chains *(21)*. The use of these compounds is increasingly limited out of safety considerations, and their replacement by natural antioxidants is widely advocated *(1)*. Also, food manufacturers are very interested in natural antioxidants to act as replacements for the synthetic antioxidants currently used by the industry to combat the formation of off-flavors and rancidity, particularly in fat-based products *(1, 15)*.

There is an emerging consensus that the micronutrients and nonnutrient components of fruits and vegetables play a preventive role in the development of chronic diseases. Epidemiological studies have found that diets high in fruits, vegetables, herbs, and spices correlate with low incidences of cancer and heart disease *(29, 30)*. Plant extracts that have been proposed to have antioxidant capacities include tea, olives, grapes, apple, clove, *Vanilla planifolia,* among others. The flavonoids and other polyphenols found in some of these extracts have been widely discussed as potential antioxidant prophylactics *(3)*. The role of flavonoids in the diet is receiving increasing interest and the biological activity of these polyphenolic components is of great importance for understanding their mode of action in vivo. Interest has focused on the health benefits derived from a diet rich in flavonoids *(3)*.

Among the most frequently used antioxidants in food is sodium tripolyphosphate (TPP, E-452) that is used as a preservative, metal ion sequestrant, and texturizer in meat and fish products *(26, 27)*. BHA (E-320) and BHT (E-321) have been used as food antioxidants because the end-products of lipid peroxidation are toxic and can impart a rancidity or off-flavor to foods like cakes, cookies, vegetable oils, and dehydrated soups *(5)*. Propyl gallate (E-310) has been used as a food antioxidant, e.g., in vegetable oils, margarine, instant mashed potato, and breakfast cereals. Vanillin is also a widely used flavoring agent, e.g., in ice cream, cakes, syrups, and chocolate *(4, 11)*. α-Tocopherol (E-307) is the most important natural antioxidant found in vegetable oil-derived foods. It has a widely extended use in fats and oils *(23)*. α-Tocopherol contains a phenolic structure that scavenges lipid and oxygen radicals *(28)*.

Resveratrol (3,4′,5-trihydroxystilbene) is a phenolic compound of the stilbene family together with other hydroxylate compounds. Resveratrol is found in wines and in various parts of the grape, including the skin *(38)*. Wines from various parts of the world have been shown to vary widely in their resveratrol content, on account of the influence of soil and climate factors *(12)*. A number of research-

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ers have measured the resveratrol concentration of wines *(9, 13)* and discussed the differences in terms of the various grape varieties used *(14, 19, 34)*. The phenolic content of red wine is about 20 to 50 times higher than that of white wine *(22)*.

In recent years, many papers have been published on resveratrol in grapes and wines, reflecting the widespread interest this compound has aroused in connection with pharmacological properties that would seem to help prevent cardiovascular disease. Resveratrol would seem to have antiplatelet aggregating properties and facilitate an increase in high-density lipoprotein cholesterol. Moreover some authors have recently reported that resveratrol delays the onset of tumors in animals. It is known that the moderate consumption of red wine reduces ADP-induced platelet aggregation *(12)*. Frankel and coworkers *(18)* and Ector et al. *(16)* attributed the positive benefits of red wine to the ability of phenolic substances to prevent oxidation of low-density lipoprotein, a critical event in the process of atherogenesis *(22)*. In addition, investigators concluded that the beneficial effects of the beverages, such as beer and red wine, are partially due to components other than the alcohol *(37)*. Nevertheless it should always be taken into account that the problems of alcoholism might be a greater problem than any of the benefits derived from the mentioned alcoholic beverages.

The results of epidemiological studies based on World Health Organization data indicate that the consumption of wine, especially red wine, may reduce the risk of coronary heart disease by 40% *(33)*.

Resveratrol is a natural product that has received attention for use as a possible food antioxidant. The aim of this study is to compare the in vitro effect of resveratrol with other widely used antioxidants, such as BHT, BHA, phenol, PG, TPP, α-tocopherol, and vanillin to better understand the nature of the beneficial effect of resveratrol.

MATERIALS AND METHODS

Chemicals were of the highest quality available and were purchased from Sigma Chemical Co. (Poole, Dorset). Resveratrol, BHT, BHA, phenol, PG, TPP, α-tocopherol, and vanillin were also from Sigma Chemical Co.

Peroxidation of phospholipid liposomes. The ability of compounds to inhibit lipid peroxidation at pH 7.4 was tested using ox brain phospholipid liposomes, essentially as described in Quinlan et al. *(32)* and in Aruoma et al. *(8)*. The experiments were conducted in phosphate-buffered saline (3.4 mM Na_2HPO_4-NaH_2PO_4, 0.15 M NaCl), pH 7.4. In a final volume of 1 ml the assay mixtures were made up with phosphate-buffered saline, 0.5 mg/ml phospholipid liposomes, 100 μM $FeCl_3$, varying concentrations of resveratrol, and the rest of the compounds analyzed (BHT, BHA, phenol, PG, TPP, α-tocopherol, and vanillin) at 0.05, 0.125, 0.25, and 0.5 mM dissolved either in water or in ethanol or in methanol (neither ethanol nor methanol affect the outcome of the lipid peroxidation assay; BHT and α-tocopherol are not fully soluble in aqueous solution, and their emulsions are not homogeneous). To overcome this problem it was necessary to use deionized water, with conductivity not more than 4 μS/cm, to dissolve them *(35)*, and 100 μM ascorbate (added last to start the reaction). Incubations were at 37°C for 60 min. At the end of this incubation period, 0.1 ml of 2% (wt/vol) BHT was added to each mixture followed by addition of 1 ml each of 1% (wt/vol) thiobarbituric acid (TBA) and 2.8% (wt/vol) trichloroacetic acid. The solutions were heated in a water bath at 80°C for 20 min to develop the malondialdehyde thiobarbituric adduct ($[TBA]_2$-MDA). The addition of BHT to the reaction mixtures minimizes erroneous increases in color due to iron ion-dependent hydroperoxide decomposition during the acid heating stage *(29)*. The $(TBA)_2$-MDA chromogen was extracted into 2 ml of butan-1-ol and the extent of peroxidation measured in the organic layer as absorbance at 532 nm.

This TBA test measures not only peroxidation occurring in the experiment itself but also peroxidation taking place during the acid-heating stage of the assay. In order to avoid any interference, the TBA test was performed in the presence of the antioxidant BHT to inhibit peroxidation during the assay itself *(6)*.

Hydroxyl radical scavenging. In a final volume of 1.2 ml, reaction mixtures contained the following reagents at the final concentration stated of 10 mM KH_2PO_4-KOH buffer (pH 7.4), 2.8 mM H_2O_2, 2.8 mM deoxyribose (where used), 50 μM $FeCl_3$ premixed with 100 μM EDTA before addition to the reaction mixture, and 0.05, 0.125, 0.25, and 0.5 mM of resveratrol and the rest of the compounds tested dissolved in water. Ascorbate (100 μM), where used, was added to start the reaction. The tubes were incubated at 37°C for 1 h. The products of hydroxyl radical ($OH^{\bullet}$) attack upon deoxyribose were measured as described in Aruoma et al. *(7)*.

Scavenging of hydrogen peroxide. Resveratrol, PG, and the different components to be tested for their reaction with H_2O_2 were incubated at 0.05, 0.125, 0.25, and 0.5 mM with 6.72 mM H_2O_2 for 10 min at 25°C. Aliquots of these compounds were then taken and assayed for remaining H_2O_2 by using the peroxidase system *(6)*. The remaining H_2O_2 was measured as the formation of a brown color (recorded at 436 nm) in reaction mixtures containing, in a final volume of 1 ml, 0.15 M KH_2PO_4-KOH buffer, pH 7.4, 50 μl guaiacol solution (made by adding 100 μl of pure liquid to 100 ml water), and 10 μl of Sigma type IV horseradish peroxidase (5 mg/ml in the same phosphate buffer).

Reactions with hypochlorous acid. The hypochlorous acid (HOCl) reaction was studied using the elastase assay, essentially as described by Aruoma et al. *(7)*. For the assay, 68 μM HOCl (produced immediately before use by adjusting NaOCl to pH 6.2 with dilute H_2SO_4) and the compounds to be tested (0.5 mM) were incubated for 20 min in a final volume of 1.0 ml in phosphate-buffered saline, pH 7.4, containing 140 mM NaCl, 2.7 mM KCl, 16 mM Na_2HPO_4, and 2.9 mM KH_2PO_4. α_1-Antiproteinase (2 mg/ml) were added to the reaction mixture. This allows any HOCl remaining to inactivate α_1-antiproteinase. After a further 20-min incubation, 0.05 ml of 2.5 mg/ml elastase was added. The mixture was allowed to stand for 30 min more before the 2 ml of phosphate-buffered saline was added. The remaining elastase activity was then measured by adding elastase substrate (5 mg/ml, *N*-succinyltriala-*p*-nitroanilide), which is hydrolyzed by elastase, resulting in an increase in A_{410}.

Data analysis. The resulting data were analyzed using Statistical Package for the Social Sciences Windows 9.0. An analysis of variance was carried out after triplicate experiments, calculating the significance level by using a proportions test.

RESULTS AND DISCUSSION

Inhibition of phospholipid peroxidation. Lipid peroxidation is sometimes a major mechanism of cell injury

TABLE 1. *Inhibition of peroxidation by resveratrol in the lipid system using ox brain phospholipids compared with the activity of different compounds frequently used as food additives*[a]

Addition to reaction mixtures[b]	Concentration (mM)	% inhibition
Resveratrol	0.5	62
	0.25	35
	0.125	24
	0.05	22
PG	0.5	51
	0.25	47
	0.125	39
	0.05	20
BHA	0.5	71
	0.25	62
	0.125	36
	0.05	26
BHT	0.5	22
	0.25	24
	0.125	22
	0.05	24
Phenol	0.5	25
	0.25	21
	0.125	20
	0.05	18
TPP	0.5	26
	0.25	25
	0.125	22
	0.05	21
Vanillin	0.5	26
	0.25	22
	0.125	21
	0.05	20
α-Tocopherol	0.5	15
	0.25	13
	0.125	10
	0.05	6

[a] Statistical differences were analyzed by analysis of variance ($P \leq 0.05$).

[b] Compounds in aqueous medium.

in organisms subjected to oxidative stress, although it is by no means the only mechanism of injury *(6).* Ox brain phospholipid liposomes undergo rapid nonenzymatic peroxidation when incubated with $FeCl_3$ and ascorbic acid at pH 7.4 *(1).* At low concentrations, ascorbate accelerates lipid peroxidation through its ability to reduce iron into the active ferrous state while, at high concentrations, ascorbate inhibits lipid oxidation by inactivating free radicals *(15).* The ability of resveratrol, BHT, BHA, phenol, PG, TPP, α-tocopherol, and vanillin to inhibit phospholipid liposome peroxidation was tested. All these compounds scavenge the peroxyl radical when they are dissolved in water. The inhibition percentage of peroxidation recorded was in the following decreasing order: BHA > resveratrol > PG > TPP > vanillin > phenol > BHT > α-tocopherol ($P \leq 0.05$) (Table 1). Resveratrol, BHA, and PG inhibited peroxidation in a concentration-dependent manner. BHA, resveratrol, and PG performed the best % inhibition up to 50%. TPP, vanillin, phenol, and BHT worked best at 25% inhibition. α-Tocopherol was less effective than PG with poor inhibition capacity of peroxidation. BHT showed increasing antioxidant activity in ethanol or in methanol. However, α-tocopherol did not show a significant difference ($P \leq 0.05$) in % peroxyl radical inhibition by change in solvent.

Many of the tested compounds are organic. In fact some of them like resveratrol *(20)* and α-tocopherol *(36, 40)* have been reported to incorporate into model membranes and biomembranes. The experiment was carried out with the compounds dissolved in ethanol or methanol, and the results were almost identical. Also, these compounds were observed to have a similar antioxidant capacity when they were dissolved in water. The results showed a gradual increase in lipid peroxidation inhibition with increasing concentrations of resveratrol, PG, and BHA ($P \leq 0.05$). We observed that resveratrol dissolved in ethanol produced an inhibition that varied from 57% to 3% at the concentrations tested; the BHA results varied from 70 to 17% inhibition for concentrations of 0.5 to 0.05 mM. PG showed values from 57 to 3% inhibition and BHT in ethanol showed a considerable increase in its inhibitory activity that varied from 64 to 1% inhibition. The results obtained for phenol, vanillin, and α-tocopherol dissolved in ethanol were similar to those obtained in aqueous medium. These compounds could be used as preservers of fat or oil foods. TPP was a poor inhibitor of peroxidation in ethanol.

Our results are in accord with Aruoma et al. *(4),* who showed that BHT, BHA, PG, and vanillin dissolved in ethanol inhibited lipid peroxidation, although rat liver microsomes were used in this experiment. Several reports have demonstrated the protective action of α-tocopherol against lipid peroxidation *(17).* Also, Decker and Xu *(15)* showed that BHA, BHT, and PG inhibit lipid oxidation by free radical scavenging.

Brand-Williams et al. *(10)* determined the efficiency of monophenols (phenol, vanillin) and polyphenols as antiradicals. Camire and Dougherty *(11)* concluded that vanillin protects against lipid oxidation better than BHT in snacks-type foods. Free radical termination, rather than iron binding, is the probable mechanism for this retardation of oxidation.

Assessing the antioxidant action of resveratrol in the deoxyribose assay. The deoxyribose method evaluates the ability to damage carbohydrates. Hydroxyl radicals damage the sugar deoxyribose. Highly reactive hydroxyl radicals ($OH^{\bullet}$) are generated by a mixture of ascorbate and $FeCl_3$-EDTA at pH 7.4 *(1).* The deoxyribose is broken down into fragments and, on heating with TBA at low pH, generates a pink chromogen. The addition of ascorbic acid greatly increases the rate of $OH^{\bullet}$ generation by reducing iron and maintaining the supply of Fe^{2+} *(2).*

The effects of increasing concentrations of resveratrol on the deoxyribose attack by $OH^{\bullet}$ radical compared with different compounds frequently used as food additives (BHA, BHT, phenol, PG, TPP, vanillin, and α-tocopherol) are shown in Table 2. The results can be divided into several classes. The first, comprised of those compounds that

TABLE 2. *Desoxyribose attack by OH•* radical in the presence of resveratrol compared with the activity of different compounds frequently used as food additives[a]

Addition to reaction mixtures[b]	Damage to deoxyribose A_{532nm}[c]			
	mM	RM + DR	% inhibition	Omit ASC
Resveratrol	0.5	1.21	10	0.51
	0.25	1.22	9	0.50
	0.125	1.24	8	0.49
	0.05	1.24	7	0.48
PG	0.5	1.71	—	0.83
	0.25	1.56	—	0.76
	0.125	1.42	—	0.70
	0.05	1.36	—	0.65
BHA	0.5	1.00	25	0.09
	0.25	1.10	18	0.10
	0.125	1.12	16	0.12
	0.05	1.19	11	0.14
BHT	0.5	1.22	9	0.25
	0.25	1.30	3	0.27
	0.125	1.31	2	0.32
	0.05	1.33	1	0.36
Phenol	0.5	1.58	—	0.16
	0.25	1.43	—	0.18
	0.125	1.33	—	0.22
	0.05	1.25	7	0.27
TPP	0.5	1.18	12	0.25
	0.25	1.20	10	0.35
	0.125	1.26	6	0.24
	0.05	1.35	—	0.33
Vanillin	0.5	1.13	16	0.55
	0.25	1.15	14	0.45
	0.125	1.19	11	0.34
	0.05	1.20	10	0.26
α-Tocopherol	0.5	1.31	2	0.38
	0.25	1.30	3	0.46
	0.125	1.26	6	0.52
	0.05	1.11	17	0.82

[a] Statistical differences were analyzed by analysis of variance ($P \leq 0.05$).

[b] Compounds in aqueous medium.

[c] When deoxyribose is omitted the range of values oscilated from 0.001 to 0.006 absorbance units. RM, reaction mixtures; DR, deoxyribose; ASC, ascorbate; —, no % inhibition detected.

show moderate (<25%) inhibition as a direct result of OH• scavenging activity of the antioxidants, was BHA > TPP > BHT ($P \leq 0.05$) (Table 2). Only BHA increases until 25% inhibition on deoxyribose attack, and the % inhibition decreased with the concentration.

The second, comprised of those that react with ascorbate, resveratrol, and vanillin, produced similar % inhibition to TPP and BHT. But they did not scavenge OH•, because when ascorbate was omitted, the level of the pink chromogen exceeded that of the control. Probably these compounds react with ascorbate, decreasing the amount of OH• generated.

Another class is comprised of those that show prooxidative activity. PG was prooxidant at the concentrations tested because it has a synergic effect with ascorbate and stimulates deoxyribose degradation, as Aruoma et al. *(7)* found. This property has been adopted as one measure of prooxidant capacity *(24).* An OH• scavenger will inhibit deoxyribose damage, whereas a prooxidant molecule will accelerate the damage *(4).*

The last class is comprised of compounds that scavenge low levels of OH• radicals. The results also show that α-tocopherol and phenol, in the presence of ascorbate, may only act as antioxidant at low and medium concentrations. These compounds were shown to have at least a potential as antioxidants within a certain range of concentrations. As their concentration increases, the antioxidative effect reaches a maximum and may, at higher concentrations, be reversed into a prooxidative effect *(25).*

According to our results, other authors have described phenol as a poor antiradical compound for hydroxyl groups using the free radical 2,2-diphenyl-1-picryl hydrazyl. Polyphenols have a higher antioxidant activity than monophenols due to effective hydrogen donation accompanied by the effective delocalization of an unpaired electron. The double bond in the C-ring in conjugation with the carbonyl im-

proves electron delocalization that stabilizes the antioxidant radical. The poor efficiency of phenol is due to the absence of any electron-donating group *(10, 28)*.

BHA and phenol are very good scavengers of OH• radicals in the absence of added ascorbic acid (Table 2). When ascorbate was omitted from the deoxyribose reaction mixture, absorbance fell substantially because of the slow rate of OH• generation. BHT, TPP, and α-tocopherol showed low scavenging ability. In fact, Aruoma et al. *(4)* explained that α-tocopherol and other phenolic compounds are capable of binding iron (III) and reducing it to iron (II) that may result in a prooxidant action under certain circumstances.

This assay was only developed with the compounds dissolved in water because organic solvents such as ethanol are themselves good scavengers of OH• *(4)*.

Hydrogen peroxide scavenging. The generation of hydrogen peroxide by activated phagocytes is known to play an important part in the killing of several bacterial and fungal strains. Additionally, hydrogen peroxide is generated in vivo by several oxidase enzymes *(21)*. There is increasing evidence that H_2O_2, directly or indirectly via its reduction product OH', acts as a messenger molecule in the synthesis and activation of inflammatory mediators *(39)*.

The scavenging of hydrogen peroxide activity is easily and sensitively measured by using peroxidase-based assay systems, when one looks for a decrease in the absorption spectrum after the compound is added to peroxidase-H_2O_2 mixtures. In our study, neither the resveratrol analyzed nor PG or the rest of the compounds reacted with H_2O_2 and must be considered inefficient catalysts of any subsequent oxidation.

Scavenging of HOCl. HOCl is produced by the neutrophil-derived enzyme, myeloperoxidase, at inflammation sites and when activated neutrophils infiltrate reoxygenated tissue. One of the important targets attacked by HOCl in vivo is α_1-antiproteinase, the major circulating inhibitor of proteolytic enzymes such as elastase *(5)*. Thus, a good test for physiologically relevant HOCl scavenging activity by a compound is to see whether that compound, in the concentrations achieved in vivo, can protect α_1-antiproteinase against inactivation by HOCl *(31)*.

In Table 3 the scavenging of HOCl by resveratrol using the elastase assay is compared with the capacity of different compounds frequently used as food additives. α_1-Antiproteinase inhibited the activity of elastase in vitro. After incubation of 68 μM HOCl with α_1-antiproteinase, which is very rapidly inactivated by HOCl, α_1-antiproteinase loses its elastase-inhibitory capacity.

Resveratrol scavenges HOCl, so that α_1-antiproteinase acts by inhibiting elastase activity. The ability to scavenge HOCl ($P \leq 0.05$) in decreasing order was PG > resveratrol > α-tocopherol > phenol. The percentage of inhibition was 86, 82, 45, and 29%, respectively. The other compounds (BHA, BHT, TPP, and vanillin) did not have this capacity.

When the tested compounds were dissolved in ethanol, resveratrol (54% inhibition) lost its HOCl scavenging capacity compared with its capacity in water. The same occurred with PG that showed 62% inhibition. BHT, BHA, vanillin, and TPP showed a greater capacity to scavenge in HOCl than in water, 51, 48, 44, and 27% inhibition, respectively. The phenol (83% inhibition) was a better HOCl scavenger in ethanol. The α-tocopherol (48% inhibition) showed similar abilities in both water and ethanol. None of the compounds tested interfered with the ability of α_1-antiproteinase to inhibit elastase nor did they inhibit elastase directly under our assay conditions.

TABLE 3. *Scavenging of HOCl by resveratrol using the elastase assay compared with the capacity of different compounds frequently used as food additives*[a]

Addition to first reaction mixture[b]	Elastase activity in final reaction mixture (Δ A_{410}/min)
Elastase only	1.174
α_1-AP	0.022
HOCl + α_1-AP	0.597
HOCl + 0.5 mM PG + α_1-AP	0.162
HOCl + 0.5 mM Resveratrol + α_1-AP	0.213
HOCl + 0.5 mM BHA + α_1AP	1.759
HOCl + 0.5 mM BHT + α_1-AP	1.488
HOCl + 0.5 mM Phenol + α_1-AP	0.834
HOCl + 0.5 mM TP + α_1-AP	1.856
HOCl + 0.5 mM α-tocopherol + α_1-AP	0.649
HOCl + 0.5 mM vanillin + α_1-AP	1.257

[a] Statistical differences were analyzed by analysis of variance ($P \leq 0.05$).

[b] Compounds in aqueous medium.

An important observation from the present work is that the different compounds analyzed have different antioxidant properties in the different assays used. Each assay measured a different parameter. Resveratrol extract is shown to be a good natural antioxidant that could easily be incorporated in the human diet.

The consumption of vine products (e.g., wine, unfiltered juice, and whole berries without seeds, and especially, products made with pomace puree) could be a means of incorporating a significant quantity of resveratrol in the diet *(16)*.

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