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Diphenylamine Metabolism in 'Braeburn' Apples Stored under Conditions Conducive to the Development of Internal Browning

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Diphenylamine metabolism and ethylene action were evaluated as factors influencing the development of 'Braeburn' apple internal browning and cavitation during cold storage. Apples treated with the antioxidant diphenylamine (DPA) and/or the ethylene action inhibitor 1-methylcyclopropene (1-MCP) were held at 1 °C for up to 6 months in air or a controlled atmosphere (CA) containing 1 kPa of O_2 and 3 kPa of CO_2. Cortex tissues from fruit without disorders as well as from symptomatic and asymptomatic areas of fruit with disorders were analyzed for DPA and DPA derivative content. Internal browning and cavities developed in control and 1-MCP-treated fruit stored in CA, whereas air-stored and CA fruit treated with DPA or with DPA and 1-MCP prior to storage did not develop disorders. Depending on the storage regimen and duration, less DPA was detected in 1-MCP-treated fruit. The 4-hydroxydiphenylamine (4OHDPA) content of control fruit decreased during air storage duration but increased between 2 and 4 months in CA storage. 4OHDPA content in 1-MCP-treated fruit increased with storage duration in CA but not air. *N*-Nitrosodiphenylamine (NODPA) was detected after 2 months in control fruit stored in air or CA and in 1-MCP-treated fruit stored in CA, and NODPA content in control fruit was higher compared to that in 1-MCP-treated fruit. Accumulation of 4-methoxydiphenylamine (4MeODPA) in control fruit stored in air increased with storage duration, but 4MeODPA content did not change in 1-MCP-treated fruit stored in air or CA. 2-Nitrodiphenylamine content was reduced by prestorage treatment with 1-MCP, but storage environment and duration had no effect on its accumulation. The results indicate that CA storage increases the risk of disorder development in 'Braeburn' apples, that DPA can prevent disorder development, and that the content of DPA and DPA derivatives is influenced by storage environment and ethylene action. A clear relationship between DPA derivative formation and storage conditions that promote internal browning was not apparent.

KEYWORDS: ***Malus sylvestris*** **var.** ***domestica*** **(Borkh.) Mansf.; DPA; 1-methylcyclopropene; CO_2 injury**

INTRODUCTION

The risk of internal CO_2 injury in apple fruit during storage is influenced by a number of factors including cultivar (*1*), fruit developmental stage at harvest (*2, 3*), storage conditions (*4*), duration between harvest and CO_2 exposure (*4, 5*), and ethylene sensitivity (*6*). Management strategies that reduce injury risk focus on temperature and atmosphere control as well as delaying or avoiding the use of the ethylene action inhibitor 1-methylcyclopropene (1-MCP) with susceptible cultivars (*6, 7*).

Development of internal browning may result from oxidative stress as the presence of the diarylamine antioxidant diphenylamine (DPA) prevents injury development (*8, 9*). Biological systems in which DPA may halt oxidation cascades include control of lipid oxidation in red blood cells (*10*) and superficial scald of apple peel (*11*), most likely via abstraction of the amino hydrogen as outlined by Boozer and Hammond (*12*). Additionally, stabilization of smokeless gunpowder and propellants by DPA occurs via direct interactions with $^{\bullet}NO$ and $^{\bullet}NO_2$ produced by these materials (*13*). These interactions form various *N*-nitrosated and C-nitrated DPA derivatives, including *N*-nitrosodiphenylamine (NODPA), 2-nitrodiphenylamine (2NO2DPA), and 4-nitrodiphenylamine (4NO2DPA) (*13, 14*).

DPA derivatives that have been detected in DPA-treated apple fruit include 2-, 3-, and 4-hydroxydiphenylamine (2OHDPA, 3OHDPA, 4OHDPA, respectively), 2,4-dihydroxydiphenylamine, and indophenol (*15*), NODPA, 2NO2DPA, 4-methoxydiphenylamine (4MeODPA), and 3-methoxydiphenylamine (3MeODPA) (*16*). Glycosidic conjugates of C-hydroxylated DPA derivatives also occur in apple fruit (*15*) as well as in mammals (*18*). Mechanisms of C-hydroxylated species produc-

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tion in apples have not been characterized but may occur via DPA interaction with $^{\bullet}OH$ (*19, 20*) or catalytically by native, nonspecific oxygenases (*21*), although no unmodified cyclooxygenase has been shown to catalyze this reaction with DPA. Methoxylated derivatives form by O-methylation of hydroxylated DPA derivatives via an unresolved mechanism (*22*). Likewise, NODPA and 2NO2DPA formation may be similar to interactions between DPA and $^{\bullet}NO$ and $^{\bullet}NO_2$ reported in smokeless powder (*13, 14*).

Whether DPA prevents CO_2 injury in apple fruit directly or through interactions leading to DPA derivative production is unknown. The presence of $^{\bullet}OH$, a destructive reactive oxygen species (ROS), and $^{\bullet}NO$, a reactive nitrogen species (RNS) associated with signal mediation, are well documented in plants (*23, 24*). Hydroxyl radicals, which can react with DPA to form 4OHDPA (*20*), are widely believed to be a major initiator of lipid radical cascades in many biological systems (*25*). However, the main DPA derivative formed in peel during apple storage, 4OHDPA, and DPA provide similar levels of superficial scald control, suggesting that the hydroxylation reaction and presence of functional group in the para position is not related to the mechanism by which DPA ameliorates this disorder (*22*). Conversely, occupation of the meta, ortho, and amino positions inhibits superficial scald protection, emphasizing the relevance of amino hydrogen donation and subsequent resonance stabilization of the diphenylamidogen radical in this process (*22*).

Evaluation of DPA derivative accumulation during storage in air or in a controlled atmosphere known to enhance internal browning development may identify mechanisms provoking injury development. Our objective was to characterize the dynamics of DPA and DPA derivatives during the development of internal browning of 'Braeburn' apples as affected by storage environment, storage duration, and ethylene sensitivity.

MATERIALS AND METHODS

Analytical Standards. DPA and 2NO2DPA were purchased from Sigma-Aldrich (St. Louis, MO). NODPA, 2-, 3-, and 4OHDPA were purchased from TCI America (Portland, OR). Preparation of 4MEODPA was as previously described (*17*).

Sample Materials. Apples [*Malus sylvestris* var. *domestica* (Borkh.) Mansf. cv. Braeburn] were harvested in a commercial orchard near Manson, WA. Assessment of fruit maturity (starch index, firmness, and internal ethylene concentration) was performed the day of harvest using procedures as previously described (*26*).

Chemical Treatments and Storage Conditions. Fruit was exposed the day of harvest to 1 μL L^{-1} 1-MCP at room temperature for 12 h in a 4.7 m^3 chamber. SmartFresh (AgroFresh, Inc., Springhouse, PA) powder was used to generate 1-MCP gas in a 150 mL flask sealed with a rubber stopper. After the contents of the flask had been mixed, the flask and treatment chamber were connected using Tygon tubing and the gas in the flask pumped into the treatment chamber for 15 min. The concentration of 1-MCP in the treatment chamber was analyzed using gas chromatography using a 1-butene standard to generate a response factor for quantification (*22*).

DPA treatments to untreated and 1-MCP-treated fruit were conducted the day after harvest. An aqueous emulsion containing 2 g L^{-1} DPA was prepared using a commercial concentrate (Shield DPA, Pace International, Seattle, WA). Fruit was immersed in the emulsion for 1 min and then placed on pulp paper trays to air-dry.

Apples were stored at 0.5 °C in air or a controlled atmosphere (CA) containing 1.5 kPa of O_2 and 3 kPa of CO_2. Fruit was held at 0.5 °C for 24 h prior to the establishment of CA conditions. The CA was established and maintained using a N_2 generator (Prism Alpha, Permea Inc., St. Louis, MO), compressed air, and compressed CO_2. The gas composition of the CA chambers (0.143 m^3) was established and monitored using automated controls (TechniSystems, Chelan, WA).

Disorder Evaluation and Tissue Sample Collection. Internal breakdown incidence and severity were evaluated immediately after apples were removed from storage. Each apple ($n = 18$ per treatment) was repeatedly sliced laterally, and internal browning (1 = none, 2 = 1–33%, 3 = 34–66%, 4 = 67–100% cortex tissue brown) and collapsed or cavitated tissue incidence were visually rated. Three replicate cortex samples, each containing tissue from three apples, was collected from symptomatic or asymptomatic tissues except when symptomatic tissue quantity was insufficient for analysis. Samples were placed into paper bags, frozen using N_2 (l), and stored in large zip-lock bags at −80 °C.

Sample Pretreatment and HPLC and GC/MS Analytical Procedures. Analyses of DPA and DPA metabolites were conducted using LC-MS. Frozen tissue (10 g) was combined with 1.07 μmol of 4MeDPA (internal standard), 15 mL of saturated $CaCl_2$ solution, and 20 mL of hexanes. The mixture was homogenized for 2 min using a Tissue Tearor (Biospec Instruments Inc., Bartelsville, OK), and then the homogenate was centrifuged at 2000*g* for 10 min. Following centrifugation, the upper hexanes phase was decanted and passed through a Florisil Sep-Pak (Waters Corp., Milford, MA). The Sep-Pak was eluted in the reverse direction with 3 mL of MeOH and the MeOH evaporated under a stream of N_2 at 40 °C. The residue was resuspended in 500 μL of MeOH and passed through a 0.45 μm PTFE filter.

A series 1100 HPLC system (Agilent Technologies, Palo Alto, CA), Agilent Chemstation software (A.09.03), 5 μm Agilent Hypersil ODS (4.0 × 125 mm) reverse-phase column, G1315B diode array detector (DAD), and G1946D single quadrupole mass selective detector (MSD) using an atmospheric pressure chemical ionization (APCI) source were used for analysis. The system was operated as described previously (*17*) with the exception that elution solvents used were (A) 95:5 deionized water/formic acid and (B) 95:5 methanol/formic acid.

Compounds were identified by comparing UV–vis and/or mass spectra and retention times of extract constituents with those of authentic standards. Metabolite quantification was performed by comparing peak areas with response factors generated from external standards containing the same amount of 4MeDPA (ISTD). Metabolite losses were corrected by ISTD comparison.

Experimental Design and Statistical Analysis. The experiment was conducted using a completely random design with fruit treatments and storage duration as factors. Results were subjected to ANOVA and means separated using the Waller−Duncan *k* ratio *t* test conducted using SAS V9.1 (SAS Institute, Cary, NC).

RESULTS

Mean values at harvest for starch, firmness, and internal ethylene concentration were 2.5 ± 0.2, 86.2 ± 7.1 N, and 0.33 μL L^{-1}, respectively. In air, 1-MCP-treated fruit, but not control fruit, developed internal browning (**Table 1**). Internal browning and cavities were present in apples stored in CA with or without prestorage 1-MCP treatment. No internal browning or cavities were found in fruit treated with DPA, regardless of 1-MCP treatment or storage environment.

DPA and DPA metabolite contents were altered by 1-MCP treatment, storage regimen, and storage duration (**Table 2**). DPA residues in control fruit decreased with storage duration and were higher in fruit stored in CA compared to air, but were lower in fruit treated with 1-MCP after 2 months regardless of storage type. Control apples stored for 2 months in CA had the highest DPA content of all treatment/storage combinations, whereas fruit treated with 1-MCP and stored in air for 4 months or all air-stored fruit held for 6 months had the lowest DPA contents.

2OHDPA and 3OHDPA were not detected in any samples in this study. 4OHDPA content decreased with storage duration for control fruit stored in air, but increased in CA-stored controls between 2 and 4 months. Increased 4OHDPA content in CA-stored fruit resulted in higher values compared to air-stored fruit after 4 and 6 months. The amount of 4OHDPA in 1-MCP-

Table 1. 'Braeburn' Apple Internal Disorders Following Storage at 0.5 °C in Air or a Controlled Atmosphere (CA) of 1 kPa of O_2 and 3 kPa of CO_2[a]

months of storage	storage type	treatment	IB (1–4)	IB incidence (%)	cavity incidence (%)
2	air	control	1.0	0	0
		1-MCP	1.0	0	0
		DPA	1.0	0	0
		DPA/1-MCP	1.0	0	0
	CA	control	1.8	61	17
		1-MCP	2.0	50	6
		DPA	1.0	0	0
		DPA/1-MCP	1.0	0	0
4	air	control	1.0	0	0
		1-MCP	1.0	0	0
		DPA	1.0	0	0
		DPA/1-MCP	1.0	0	0
	CA	control	1.9	50	22
		1-MCP	1.8	44	22
		DPA	1.0	0	0
		DPA/1-MCP	1.0	0	0
6	air	control	1.0	0	0
		1-MCP	1.2	17	0
		DPA	1.0	0	0
		DPA/1-MCP	1.0	0	0
	CA	control	1.3	28	6
		1-MCP	1.7	39	33
		DPA	1.0	0	0
		DPA/1-MCP	1.0	0	0

[a] Fruit was treated the day of harvest with 0 or 1 μL L^{-1} 1-methylcyclopropene (1-MCP) for 12 h and then 0 or 2 g L^{-1} diphenylamine (DPA). Internal browning (IB) rating (1 = none; 2 = 1–33% cortex brown; 3 = 34–66% cortex brown; 4 = 67–100% cortex brown), internal browning incidence, and presence of cavities were recorded the day fruit was removed from storage.

Table 2. Content of Diphenylamine (DPA) and DPA Metabolites in 'Braeburn' Apple Cortex Tissue Following Storage at 0.5 °C in Air or a Controlled Atmosphere (CA) of 1 kPa of O_2 and 3 kPa of CO_2[a]

months of storage	storage type	treatment	nmol kg^{-1} of fresh wt			
			DPA	4OHDPA	4MEODPA	NODPA
2	air	control	1040 b	1836 bc	218 b	24 a
		1-MCP	656 de	1112 de	119 b	nd
	CA	control	1501 a	999 de	30 c	23 a
		1-MCP	774 cd	1154 de	62 c	4 b
4	air	control	550 ef	1477 cd	496 b	nd
		1-MCP	284 g	1449 cd	296 b	nd
	CA	control	957 bc	2212 b	52 c	nd
		1-MCP	786 cd	2228 b	59 c	nd
6	air	control	313 fg	660 e	2472 a	nd
		1-MCP	230 g	1369 cd	187 b	nd
	CA	control	750 cd	2234 b	220 b	nd
		1-MCP	810 cd	3259 a	356 b	nd

[a] Fruit was treated the day of harvest with 0 or 1 μL L^{-1} 1-methylcyclopropene (1-MCP) for 12 h and then 0 or 2 g L^{-1} diphenylamine (DPA). Fruit cortex tissue samples were collected immediately after fruit was removed from storage. 4OHDPA, 4-hydroxydiphenylamine; NODPA, *N*-nitrosodiphenylamine; 4MEODPA, 4-methoxydiphenylamine; 2NO2DPA, 2-nitrodiphenylamine. Means ($n = 4$) followed by different letters within a column are significantly different ($p < 0.05$), Waller−Duncan *k* ratio *t* test. nd: not detected.

Table 3. Content of 2-Nitrodiphenylamine (2NO2DPA) in 'Braeburn' Apple Cortex Tissue Following Storage at 0.5 °C in Air or a Controlled Atmosphere (CA) of 1 kPa of O_2 and 3 kPa of CO_2[a]

storage type	treatment	2NO2DPA (nmol kg^{-1} of fresh wt)
air	control	88.8 ab
	1-MCP	40.2 b
CA	control	130 a
	1-MCP	64.0 ab

[a] Fruit was treated the day of harvest with 0 or 1 μL L^{-1} 1-methylcyclopropene (1-MCP) for 12 h and then 0 or 2 g L^{-1} diphenylamine (DPA). Fruit cortex tissue samples were collected immediately after fruit was removed after 2, 4, or 6 months of storage. Means ($n = 4$) followed by different letters are significantly different ($p < 0.05$), Waller−Duncan *k* ratio *t* test.

treated fruit did not change or increased with storage duration for fruit stored in air and CA, respectively. 4MEODPA content in air-stored control fruit increased with storage duration. CA storage resulted in less accumulation of 4MEODPA in control and 1-MCP-treated fruit after 2 and 4 months, and values in 1-MCP-treated fruit stored in air were also lower than controls after 6 months. NODPA was detected only after 2 months in air-stored control fruit or in both control and 1-MCP-treated CA-stored fruit. NODPA content in air-stored fruit was higher compared with 1-MCP-treated fruit. Storage duration did not influence accumulation of 2NO2DPA; however, 1-MCP-treated fruit stored in air had lower 2NO2DPA content compared with control fruit stored in CA (**Table 3**).

DISCUSSION

Although internal browning of apple fruit induced by CO_2 can be managed by horticultural practices at and after harvest, the physiological mechanisms that initiate the disorder remain obscure. Ethylene insensitivity resulting from 1-MCP exposure can contribute to 'Braeburn' internal browning development (*6*); however, in the current study, 1-MCP was a factor contributing to injury development only for fruit stored 6 months in air. Prestorage DPA application to 'Braeburn' apples prevented disorder development in untreated and 1-MCP-treated fruit, confirming earlier reports (*9, 27*). The absence of internal browning in control fruit stored in air illustrates that 'Braeburn' susceptibility to internal browning is influenced by factors in addition to storage temperature. Injury induced in the low O_2 and high CO_2 controlled atmosphere is consistent with previous results (*4, 5, 27*).

The decrease in DPA residue occurring during air storage and the relatively slower decrease in CA-stored fruit were consistent with previous results (*15, 28*). The influence of storage atmosphere on DPA degradation may result from greater DPA conversion to free derivatives and possibly to conjugated forms (*15*) not analyzed in this study. Lower DPA content in 1-MCP-treated fruit compared to controls after 2 months regardless of atmosphere and after 4 months in air that lacked an accompanying 1-MCP treatment effect on derivative content could indicate that conversion of DPA to compounds not detected in this study may be occurring in ethylene-insensitive fruit. Reduced DPA content in 1-MCP-treated fruit compared to controls is consistent with previous work with 'Granny Smith' apple peel (*28*) but not 'Delicious' apples (*29*). Differences may have been due to cultivar, storage environment, fruit maturity at harvest, or storage environment.

Changes in DPA derivative content in 'Braeburn' cortex tissue differed from those reported for apple peel tissue (*15, 28*). Cortex DPA, 4OHDPA, and 4MEODPA contents were similar after some storage durations in contrast to higher amounts of DPA relative to derivatives in apple peel (*28*). The different DPA to DPA derivative ratios may reflect the lower amount of DPA that moves to the cortex tissues relative to peel following DPA application to whole fruit (*15, 30*). DPA in cortex tissue may also be relatively more accessible as a substrate compared to peel, and/or the interior tissue may have a relatively higher capacity to metabolize DPA. The relatively low amount of DPA present in cortex tissues is sufficient to prevent internal browning, indicating the magnitude of the initial events in the process leading to internal browning induced under the storage conditions in this study may be less than that required to induce superficial scald in peel.

Only 4OHDPA was detected in 'Braeburn' cortex tissues in contrast to 2-, 3-, and 4OHDPA all being present in 'Delicious' apples and 'Granny Smith' peel (*15, 17*). 4OHDPA was present regardless of storage duration, storage type, or ethylene sensitivity, although all of these factors influenced its concentration. Apple storage in low O_2 partial pressure reduces metabolism beyond that of low temperature alone (*31*), and enzyme-catalyzed hydroxylation reactions require O_2 (*21*). 4OHDPA is produced in apples stored in 1 kPa of O_2, indicating hypoxic conditions are sufficient for 4OHDPA synthesis if this reaction proceeds via enzymatic hydroxylation. Hypoxia can also induce ROS production in plants (*31*), resulting in the production of several reactive species including $^{\bullet}OH$ (*33, 34*). $^{\bullet}OH$ can hydroxylate substituted aryl compounds, and this reaction occurs predominately at the para position when steric hindrance is present at the ortho position (*20*). As previously suggested (*28*), DPA's aryl structures are substituted by aniline, which may favor parahydroxylation in the presence of $^{\bullet}OH$, resulting in 4OHDPA accumulation more so than 3OHDPA and 2OHDPA in 'Braeburn' apple tissues.

Accumulation of 4MEODPA in control fruit during air storage was consistent with the pattern in apple peel tissue (*28*). The increase in 4MEODPA content was accompanied by a reduction in 4OHDPA, although a similar pattern was not observed in CA-stored and/or 1-MCP treated apples. Enzymes known to catalyze O-methylation of hydroxylated aryl compounds, including phenylpropanoids, exist in plants (*35*) and could be involved in 4MEODPA synthesis from 4OHDPA in apple fruit. The lack of a consistent relationship between 4OHDPA and 4MEODPA for all treatment and storage combinations may indicate 4MEODPA synthesis capacity is reduced under nonambient atmosphere conditions and in the absence of ethylene action. 4MEOHDPA is not the only product of 4OHDPA metabolism in apple fruit; a 4OHDPA glucose conjugate was the most abundant DPA metabolite in 'Delicious' apples after 40 weeks of cold storage in air (*15*). Analysis of conjugated DPA forms was not conducted in this study, but on the basis of previous results with 'Delicious' apples (*15*), conjugate formation in 'Braeburn' is likely to occur and may, in part, explain the reduction of 4OHDPA levels that is not attributable to other DPA metabolites.

The detection of NODPA and 2NO2DPA may reflect RNS presence during 'Braeburn' ripening. Storage duration and ethylene sensitivity affected NODPA accumulation. The lack of detectable NODPA after 4 or 6 months of storage is in contrast to previous results with 'Granny Smith' peel, with which NODPA was detectable through 6 months (*22*). Differences in NODPA accumulation patterns may be related to cultivar and/or tissue type. Apple peel is more metabolically active than cortex (*36*). The lack of a storage environment effect on NODPA content after 2 months suggests NODPA is produced under low O_2 or that formation occurred after DPA treatment and prior to establishment of CA conditions. In fruit stored in air or CA, ethylene insensitivity results in reduced NODPA and 2NO2DPA production, consistent with previous results for 'Granny Smith' peel (*28*). Unlike 'Granny Smith' peel, a relationship between NODPA and 2NO2DPA contents was not apparent in 'Braeburn' cortex. 2NO2DPA was consistently detected in 'Braeburn' cortex regardless of postharvest treatment or storage environment.

NODPA and 2NO2DPA production can result from interactions between DPA and $^{\bullet}NO$ or $^{\bullet}NO_2$ (*13, 14*). Transient and consistent detection of NODPA and 2NO2DPA, respectively, may indicate that both $^{\bullet}NO$ and $^{\bullet}NO_2$ are present soon after harvest followed by a decrease in $^{\bullet}NO$ as the interval from harvest increases. Under aerobic conditions, $^{\bullet}NO$ reacts with O_2 to form $^{\bullet}NO_2$ (*37*). $^{\bullet}NO$ is present in apple fruit (*38*) and may be an inhibitor of ethylene synthesis and senescence in plant tissues (*39*). Detection of NODPA after 2 but not 4 or 6 months of storage may indicate that $^{\bullet}NO$ production decreases with ripening of 'Braeburn' apples, whereas lower levels of NODPA and 2NO2DPA following 1-MCP treatment may indicate that generation of RNS in apple fruit is reduced in the absence of ethylene action.

Relationships between DPA metabolism and CA conditions that resulted in injury during the first 2 months of storage are not readily apparent. Whereas DPA clearly ameliorates the environmental conditions that induce injury, a clear pattern of metabolite production consistent with a response to oxidative stress was not detected. In fact, DPA content was consistently higher in CA compared to air-stored fruit, suggesting that DPA metabolism is less prevalent under conditions most conducive to this disorder. Relationships between DPA metabolites and treatments that induced internal injury in fruit not treated with DPA prior to storage were limited to 4MEODPA, which accumulated to a lower amount in fruit stored in CA.

Prevention of internal browning in apples by prestorage DPA treatment confirms previous results (*8, 9, 27*). Of the DPA derivatives detected, only 4MeODPA content was related to internal browning development patterns in control fruit. Clear relationships between DPA metabolism in fruit stored under conditions that induced internal browning were lacking, suggesting, as with superficial scald, that abstraction of the amino hydrogen of DPA may provide internal browning control rather than the radical trapping model outlined for smokeless gunpowder (*13, 14*).

Internal browning induced by storing 'Braeburn' apples in a low O_2/high CO_2 CA was prevented by prestorage treatment with DPA. Although patterns of DPA and DPA derivatives were related to storage regimen, storage duration, and ethylene sensitivity, a relationship between DPA, DPA derivatives, and control of internal browning was not apparent.

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