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An *in vitro* demonstration of Pro-oxidant effect of Ascorbic acid in sheep erythrocyte hemolysate and purified hemoglobin preparations

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Abstract: *In vitro* studies have demonstrated pro-oxidant effect of ascorbic acid on sheep erythrocyte hemolysate and on purified ruminant hemoglobin preparations. Methemoglobin formation was concentration dependent over ca. 3.4 through 34 µmole ascorbic acid with sheep hemolysate ($r=0.97$, $p<0.01$, $n= 3$ each) and over ca. 5.68 through 22.7 µmole ascorbic acid with purified ruminant Hb preparations ($r >0.99$, $p<0.01$, $n=6$ each). In contrast, copper acetate was very potent showing linear increase in methemoglobin formation in Hb preparations over 1 through 5 µmole ($r\pm S.E. = 0.998\pm0.002$). Methemoglobin formation due to ascorbic acid or copper acetate has occurred only at alkaline pH and failed to occur at acidic pH. Comparative evaluation of regression estimates of copper acetate and ascorbic acid revealed that on molar basis ascorbic acid was nearly $1/11^{th}$ as potent as copper in inducing methemoglobin formation. The effect of ascorbic acid occurred even in presence of copper when the copper ions got reduced and precipitated out by ascorbic acid at alkaline test conditions. Increase in methemoglobin formation with combined copper and ascorbic acid was almost equal to that of copper alone and nearly three folds more than that due to ascorbic acid alone.
Key-words: Ascorbic acid, Sheep hemolysate, Ruminant hemoglobin, Pro-oxidant, Methemoglobin.

Introduction

Ascorbic acid has been recognized as a powerful anti-oxidant vitamin capable of protecting cellular systems from oxidant insult from hazardous oxidant moieties in hydrophilic medium as opposed to alpha-tocopherol and beta-carotene those being well- suited to check oxidants occurring in lipophilic medium. Over the decades, the opposite observations have been made implicating pro-oxidant role to ascorbic acid particularly at higher concentrations. Pro-oxidant effect of ascorbic acid has been reported in diverse systems including erythrocyte preparations[1-3], chicken supplementation experiments[4], while food processing[5] and other observations involving humans and other systems [6-7.]

These observations motivated to undertake present investigations. The experiments were designed to investigate effect of ascorbic acid on methemoglobin formation in sheep erythrocyte hemolysate. Following encouraging observations, the subsequent experiments were conducted on purified ruminant erythrocyte hemoglobin (Hb) preparations to demonstrate potential of ascorbic acid in affecting methemoglobin formation.

Experimental

The experiments were carried out at an ambient temperature of 27.7±0.5 0C. The chemicals used were of standard purity and quality obtained from reputed sources in India. Unless otherwise indicated, centrifugation of test samples was done at 8000 rpm for 5 minutes. Spectrophtometric measurements were made with UV-Visible Spectrophotometer, Model UVmini-1240 (Shimadzu Corporation, Japan). The samples were analyzed for pH by a pocket pH meter (pH Scan 3, Eutech Instruments, Malaysia).

Sheep Hemolysate: Sheep blood was collected from butcher's shop in sodium citrate containing glass bottles (5 mg sodium citrate per mL); centrifuged to separate out plasma; PCV was washed thrice with normal saline; and 2.5 mL of loosely packed erythrocytes were made 100 mL with distilled water and allowed to stand 30 minutes at room temperature to get 2.5% suspension hemolysate of erythrocytes in water.

Purified ruminant erythrocyte hemoglobin solution: Purified hemoglobin derived from ruminant erythrocytes (Hi-Media) was dissolved in a solution to prepare 0.3 % (w/v) in 0.1 % $NaHCO_3$ and 0.05 M NaOH.

Sodium bicarbonate solution: Appropriate dilutions and solutions were made by using stock solution containing 2.5% (w/v) $NaHCO_3$ in distilled water.

Ascorbic acid solutions: Stock solution was prepared as 2 %(w/v) in water. Working solutions were made by dilution in water.

Copper acetate monohydrate: Stock solution contained 2 % (w/v) copper salt in 0.01 M acetic acid providing 100 µmole copper mL^{-1}. Working solutions were made by dilution in overall 0.001 M acetic acid.

Diluents: Alkaline diluent contained 0.1 % $NaHCO_3$ and 0.05 M NaOH in water. Acetic acid diluent contained 0.01 M acetic acid. Ascorbic acid diluent was prepared by adjusting pH of 0.1 % HCl in water with 0.1 M NaOH to provide pH (3.64) comparable to that provided by 0.4 % ascorbic acid solution (pH 3.66).

Standard iodine solution: Working solution of iodine 5 µmole mL^{-1} water was prepared freshly form stock solution of 0.1 M iodine (standardized spectrophotometrically against ascorbic acid).

Dilute HCl solution: 10 % (v/v) HCl in water.

Dilute ammonia solution: 5% ammonia in water, estimated 2.7 M.

Response of sheep erythrocyte hemolysate to ascorbic acid

Each milliliter of sheep erythrocyte hemolysate was added varying masses of ascorbic acid, 0 and 0.1 through 10 mg, in 4 mL water. The samples were allowed standing at room temperature for about 30 minutes and monitored for methemoglobin formation at 630 nm against untreated control hemolysate solution as 100 % T.

Response of purified ruminant erythrocyte hemoglobin to ascorbic acid

Two milliliter hemoglobin solution was added 3 mL water containing ascorbic acid 0, 0.5 1, 2, 4, 6, 8 and 10 mg. The samples were allowed to stand at room temperature for 30 to 40 minutes, and then monitored for methemoglobin formation by recording absorbance at 630 nm.

In a separate experiment, effect of varying acidity on response to 4 mg of ascorbic acid was conducted to find effect of acidity on ascorbic acid-mediated methemoglobin formation. For this purpose, two milliliter hemoglobin solution was added 4 mg ascorbic acid in 1 mL pH matched diluent. The samples were added 3 mL water containing none and varying volumes of 1 % HCl. The standard samples contained 2 mL Hb solution and 3 mL water. Each sample included its own control containing appropriately matched reagents added to 2 mL alkaline diluent. The samples were allowed to stand at room temperature for 40 to 50 minutes, and then centrifuged. The supernatants were monitored for methemoglobin formation by taking absorbance measurements at 630 nm.

Copper-acetate induced methemoglobin formation

Two milliliter hemoglobin solution was added 0.5 mL acetic acid diluent containing varying masses of copper acetate as 0, 1, 2, 3, 4 and 5 µmole and volume made 5 mL with water. The corresponding controls contained 0.5 mL acetic acid diluent containing matching masses of copper in total 5 mL volume. The samples were allowed standing for 30 to 40 minutes and then monitored at 630 nm for methemoglobin formation. A separate experiment was also conducted using varying masses of 1 % HCl with 3 µmole copper to check effect of acidity on copper-induced methemoglobin formation.

Combined effect of copper and ascorbic acid on Hb

An experiment was designed to study effect of copper, ascorbic acid alone and in combination on Hb. The experiment included 5 sets of samples. Each set included 2 mL Hb solution. Set A contained 3 mL matched solvents. Set B contained copper acetate 3 µmole. Set C contained ascorbic acid 4 mg (equivalent to 22.7 µmole). Sets D and E contained both copper and ascorbic acid, in set D ascorbic acid was added together with copper before addition of Hb solution while in set E, copper was added before Hb addition and ascorbic acid was added exactly 10 minutes after addition of Hb. Each set included its own control containing exactly all test reagents and substituting Hb solution by water. The samples were allowed standing for about 30 to 40 minutes, and then assayed for absorbance at 630 nm.

Analysis of test samples for reduced form of ascorbic acid

Simulation experiments revealed that in alkaline medium copper 3 µmole when added ascorbic acid 4 mg developed pale yellow color that got precipitated out. The reaction did not occur if the medium was acidic, but occurred in presence of alkalinity. The samples containing precipitated copper were subjected to centrifugation. The supernatants were colorless and the residue adhering to the centrifuge tubes pale yellow suggesting reduced copper. The supernatants were analysed for mass of reduced ascorbic acid by iodometry while residues were reconstituted in 1 mL dilute ammonia solution and analyzed for precipitated copper by copperamine assay improvised for the purpose.

Ascorbic acid assay: The assay included standard assay set containing 1 mL iodine solution (5 µmole mL^{-1}) added 0.5 mL dilute HCl solution and ascorbic acid in 3 mL water as 0, 80 through 320 µg. Test samples contained measured volume of ascorbic acid without copper and supernatants from centrifuged copper + ascorbic acid samples as indicated above with matched iodine and acidity. The samples were allowed to stand for 15 to 20 minutes at room temperature and then monitored for absorbance at 430 nm.

Simple copper assay: The assay included 1 mL dilute ammonia containing 0, 1, 3, 5 and 10 µmole of copper acetate added 2 mL of water. Test samples included residues reconstituted in 1 mL dilute ammonia added 2 mL water while reference standard included 3 µmole copper in 1 mL dilute ammonia added 2 mL water. All samples were allowed standing at room temperature for 10-15 minutes, and then monitored at 570 nm for estimation of copper reduced by ascorbic acid.

Results and Discussion

The investigations were executed keeping in view the observations that ascorbic acid exerts oxidizing potential. Ascorbic acid in solution is readily oxidized to dehydro-ascorbic acid (which retains biological activity in physiological systems). The reaction is facilitated by many agents including metallic ions of copper (II), iron (III) and silver[8]. Oxidation of iron (II) of Hb to iron (III) forming methemoglobin, monitorable[9] at about 635 nm, provides a useful model to assess pro-oxidant effect of ascorbic acid. Initial observations were made while using sheep hemolysate preparations. The studies were then extended to purified Hb preparations commercially available from ruminant erythrocyte source (Hi-Media). The experiments on sheep hemolysate contained hemolysate in 0.1% sodium bicarbonate. The dissolution of purified Hb in 0.1 % sodium bicarbonate solution has required presence of 0.05 M NaOH for ease of making solution.

Ascorbic acid induced methemoglobin formation in sheep hemolysate

As evident from Table 1, 100 and 200 µg ascorbic acid failed to produce any discernible effect. In fact, absorbance values decreased below control showing decrease in basal metHb formation with mean ± S.E, correspondingly recorded as, -0.014± 0.002 and -0.016 ±0.002. Higher concentrations of ascorbic acid (0.6 through 6 mg) have caused quantitative increase in methemoglobin formation. The observations revealed pro-oxidant effect of ascorbic acid at higher concentration range is demonstratable in sheep erythrocyte hemolysate preparation. No significant difference exists between 6 and 10 mg concentrations (p>0.1, n=3 each).

Table 1 Pro-oxidant effect of ascorbic acid on sheep erythrocyte hemolysate

Ascorbic acid, mg	0.1	0.2	0.6	2.0	6.0	10.0
Absorbance	No effect[a]	No effect[a]	0.060 ± 0.001	0.131 ± 0.004	0.213[b] ± 0.001	0.211[b] ± 0.001
Statistical analysis: Linear range (0.6 through 6 mg)						
r± S.E	0.97 ± 0.03					
b± S.E	0.027 ± 0.004					

The values are mean ± S.E. of three observations each.
[a] The absorbance in fact showed decrease with respect to basal untreated.
[b] No significant difference (p>0.1).

Ascorbic acid induced methemoglobin formation in ruminant Hb preparation

As evident from Table 2, ascorbic acid 1 through 4 mg has caused concentration related increase in absorbance with perfect linearity. The effect produced by 0.5 mg ascorbic acid did not differ from control, and similarly the effect produced beyond 4 mg ascorbic acid did not differ from the effect of 4 mg (p>0.1) while 1 , 2 and 4 mg caused mean per cent increase in absorbance correspondingly as 5.6, 11.2 and 24. 2 (r ± S.E. = 0.9997±0.0003, b ±S.E. = 6.04±0.09).

Table 2 Pro-oxidant effect of ascorbic acid on purified ruminant hemoglobin

Treatment, Ascorbic acid, mg	Absorbance	Mean pH	Mean increase in absorbance
0	0.124 ± 0.001[a]	11.8	-
0.5	0.123 ± 0.001[a]	11.9	-
1.0	0.131 ± 0.001[b]	11.8	0.007
2.0	0.138 ± 0.001[b]	11.7	0.014
4.0	0.154 ± 0.001[b]	11.5	0.030
6.0	0.156 ± 0.001[c]	11.2	0.032
8.0	0.153 ± 0.001[c]	10.9	0.029
10.0	0.157 ± 0.001[c]	10.5	0.033
Statistical analysis: Linear range (1-4 mg)			
r± S.E	0.9996±0.0005		
b± S.E	0.0075±0.0001		

The values are mean ± S.E. of six observations each.
[a] p>0.1, [b] p<0.01 (with respect to control), [c] p>0.1 (with respect to 4 mg ascorbic acid)

Trial investigations revealed that in acidic medium, ascorbic acid failed to affect methemoglobin formation. To investigate the observation, simulation experiments revealed that under test conditions addition of varying volumes of 1 % HCl were capable of providing range of pH conditions to evaluate influence of pH on ascorbic acid mediated methemoglobin formation. As indicated in Table 3, ascorbic acid effect was prominent at pH

range 10.5 through 12.1 eliciting percent increase in absorbance correspondingly as 16.8 and 22.7 ($p<0.01$). The response decreased at lower pH values showing 17.6 % decrease at pH 8.9 ($p<0.01$) and about 5 % decrease at pH 4.3. The supernatant was almost colorless as all the Hb in the solution got precipitated out at neutral pH (6.9) registering 93.3 % decrease in absorbance ($p<0.01$). These observations were in conformity with linear curve for ascorbic acid (Table 2) over 1 through 4 mg ascorbic acid that retained pH within the suitable range 11.5 through 11.9.

Table 3 Effect of pH on ascorbic acid –mediated methemoglobin formation

Mean pH	Absorbance	% change
12.1	0.119 ± 0.001	Untreated control
11.8	0.139 ± 0.001[b]	16.8 ↑
10.5	0.146 ± 0.001[b]	22.7 ↑
8.9	0.098 ± 0.001[b]	17.6 ↓
6.9	0.008 ± 0.001[b]	93.3 ↓
4.3	0.113 ± 0.001[b]	5.0 ↓

The values are mean ± S.E. of five observations each.
[a] Ascorbic acid, 4 mg equivalent to 22.7 µmole
[b] $p<0.01$ with respect to control

Copper-acetate induced methemoglobin formation

Copper at 0.2 and 0.6 µmole failed to produce any methemoglobin formation under test conditions ($p>0.1$, n= 5 each) while concentrations over the range 1 through 5 µmole showed perfect linearity with most steep response over 3 to 5 µmole concentration (table 4). Higher concentrations of copper were observed to cause opalescence and precipitate out hemoglobin. The chosen range, 1 through 5 µmole copper provided transparent solutions. The pH was found to be in the range 11.1 through 12.2 (12.17 ±0.01, n = 12). Consistent with effect of acidity on ascorbic acid induced methemoglobin formation, copper failed to produce any effect in acidic medium. At alkaline pH, 10.5 to 11.0, 3 µmole copper produced 41.7 % increase in methemoglobin formation ($p<0.01$, n=5 each) while absorbance values at acidic pH, about 2.7, 0.118 ±0.001 did not differ from control absorbance value 0.116 ± 0.001 ($p>0.1$, n= 5 each). Thus effect of copper as well as that of ascorbic acid occurs only in alkaline medium under test in vitro conditions.

Table 4 Copper acetate induced methemoglobin formation

Copper, µmole	Absorbance	Absorbance increase, mean	Percent increase, mean
0	0.126 ± 0.001	-	-
1	0.136 ± 0.001	0.010	7.9
2	0.150 ± 0.001	0.024	19.0
3	0.163± 0.001	0.037	29.4
4	0.181± 0.001	0.055	43.7
5	0.198 ± 0.001	0.072	57.1
Statistical analysis			
r ± S.E		0.998±0.002	
b± S.E[a]		0.0155±0.0005	12.31±0.02

The values are mean ± S.E. of six observations each.
The pH over the assay: 11.8 ±0.1 (range 11.7 to 11.9).
[a] The steep regression is noticed at 3 to 5 µmole (r± S.E., 0.9998 ± 0.0002, b± S.E., as 0.0.0175 ± 0.0001 for absorbance increase and 13.9±0.2 for mean per cent increase in methemoglobin formation with respect to control.

Time optimization studies

Time optimization studies using 22.7 µmole ascorbic acid and 3 µmole copper acetate separately have revealed no difference in methemoglbin formation following incubations at 30 minute versus 60 minute. With ascorbic acid increase in mean absorbance values at 30 and 60 minute incubation have been respectively as 0.012 ± 0.001 and 0.011 ± 0.001 ($p > 0.1$, n= 5 each) while with copper the corresponding mean increase in absorbance has been 0.041 ± 0.001 and 0.039 ± 0.001 ($p > 0.1$, n= 5 each) with respect to untreated control mean values. Thus, incubation can be conducted for 30 to 60 minutes under test conditions.

Table 5 Copper acetate and ascorbic acid (AA) interaction study for methemoglobin formation

Experiment No	Absorbance			
	Control	Cu(II)	AA	Cu (II) + AA[a]
1	0.120 ± 0.001	0.156 ± 0.001	0.127 ± 0.001	0.154 ± 0.001
2	0.125 ± 0.002	0.164 ± 0.002	0.136 ± 0.002	0.158 ± 0.002
3	0.118± 0.001	0.158 ± 0.001	0.132 ± 0.001	0.153 ± 0.001
Overall mean	0.121 ± 0.001	0.159 ± 0.001	0.132 ± 0.001	0.155 ± 0.001

The values of each experiment are absorbance values of mean ± S.E. of six observations each. Cu (II), 3 µmole and AA 22.7 µmole alone and in combinations.
[a]Simulation experiment indicated copper was getting reduced by ascorbic acid forming pale yellow solution and precipitate at alkaline experimental conditions.

Effect of ascorbic acid on copper-acetate induced methemoglobin formation

Both copper and ascorbic acid induced significant methemoglobin formation under test conditions. The mean absorbance values with copper, ascorbic acid and in combination were respectively found as 0.157 ±0.001, 0.132 ±0.001 and 0.154±0.001 (ascorbic acid added along with copper) and 0.153 ± 0.001 (ascorbic acid added 10 minutes following addition of Hb and copper) indicating percent increase in absorbance respectively as 31.9, 10.9, 29.4 and 28.6 ($p < 0.01$, n= 6 each) compared to untreated control value 0.119 ±0.001. There was no significant difference whether ascorbic acid was added along with copper or when added following action of copper under test conditions ($p > 0.1$, n= 6 each). Methemoglobin formation in presence of 3 µmole copper has been as good as that observed when copper 3 µmole and ascorbic acid 22.7 3 µmole were present together ($p > 0.1$, n= 6 each). The observations showed similar pattern whenever executed on different occasions with overall per cent increase in absorbance by ascorbic acid with copper, 28.1, nearly 3 times that of ascorbic acid alone, 9.1, ($p < 0.01$ each) but as good as with copper, 31.4, alone ($p > 0.1$) (Table 5). The effect in presence of ascorbic acid and copper is not attributable to copper because ascorbic acid was seen, in simulation experiments, to reduce copper(II) to copper (I) oxide which appears as a pale yellow precipitate, and got removed by centrifugation. Analysis of copper reduced by ascorbic acid and precipitated out at alkaline pH showed content equivalent of 100 per cent recovery, the mean absorbance with standard 3 µmole copper, 0.052 ±0.001, matched that due to reconstituted residue 0.054 ±0.001($p > 0.1$, n= 3 each). Thus, whole copper has been reduced by ascorbic acid and precipitated out. Copper amine assay was found to provide linear estimate of copper over 1 through 10 µmole with perfect linearity (r ± S.E. = 0.9999 ± 0.0001; b ± S.E. = 0.0155 ± 0.0001). This implied that an equivalent mass of ascorbic acid, to that of precipitated copper, has been oxidized during the process. Alkaline pH and copper ions are known to favor and hasten oxidation of ascorbic acid to its oxidation products like dehydroascorbic acid (DHAA) and diketogulonic acid (DKGA)[8, 15]. Therefore, it may rule out participation of oxidized copper in mediating methemoglobin formation in presence of ascorbic acid. This appears to be in conformity with analysis of supernatant for ascorbic acid by iodometry. In principle, iodine decolorization would estimate reduced ascorbic acid and not its oxidized forms viz., DHAA and DKGA which are unable to reduce and decolorize iodine. Therefore, mass of reduced ascorbic acid is anticipated to decrease in presence of copper. Estimated reduced ascorbic acid in copper treated samples was significantly lower than untreated samples. Estimated ascorbic acid in absence of copper was found to be about 3.91 ± 0.07 mg showing 98 ± 2 % recovery while in presence of copper it was estimated as 2.73 ± 0.06 mg suggesting 32 ± 2 per cent loss (68 ± 2 % recovery) . Iodometry assay was linear for ascorbic acid over the range 80 through 320 µg with perfect linearity (r ± S.E. = 0.9993 ± 0.0007; b ± S.E. = 0.002015 ± 0.000001). Further

experiments are required to identify the roles of ascorbic acid metabolites including its oxidized forms viz., DHAA and DKGA and other Fenton reaction intermediates in the process particularly in presence of alkalinity and copper ions.

A comparison of regression coefficients of copper (b= 0.0155 absorbance basis, b= 12.3 on per cent increase basis) and ascorbic acid (b= 0.00136 absorbance basis, b= 1.1 on per cent increase basis)over respective linear ranges on molar basis has revealed that ascorbic acid is about 11 times less potent than copper acetate in inducing methemoglobin formation. A comparative evaluation of pooled data (7 to 8 experiments conducted on different occasions) has indicated that 4 mg of ascorbic acid (equivalent to 22.7 µmole) has increased mean absorbance by 0.012 ±0.001 (n=7) over control compared to increase of 0.039 ±0.001 (n=8) with 3 µmole copper . Thus, on molar basis ascorbic acid in masses nearly seven times that of copper has been at least about 1/3rd as active as copper in increasing methemoglobin formation.

Foregoing observations are in conformity to the reports that ascorbic acid has pro-oxidant effect. Ascorbic acid shows ease of reversible oxidation-reduction[8]. It has failed to antagonize nitrite induced methemoglobin formation in sheep[1], increased oxidative stress in human erythrocytic membrane by decreasing GSH levels[2] and has increased copper acetate-induced methemoglobin formation and increased oxidative stress in sheep and human erythrocytes[3]. Similar pro-oxidant observations have been recorded in other systems. Chicken fed ascorbic acid supplementations have experienced an increased oxidative stress as indicated by accumulation of select bio-markers in various parts of the body causing imbalance between pro-oxidant and anti-oxidant molecules and caused suppression in innate immunity with rise in its oxidized form dehydroascorbic acid (DHAA) in hepatic cells[4]. Ascorbic acid has shown its pro-oxidant effect in dough prepared from wheat flour[5]. Consistent with our observations on copper induced methemoglobin formation, copper (II) ions have been rated as one of the top ranking agents causing methemoglobin formation in sheep erythrocyte model[10]. Similarly, positive genotoxicity results have tended to occur in vitro when vitamin C was tested in the presence of metal ions such as iron and copper, which may be related to its reduction of the metal followed by the formation of highly reactive hydroxyl radicals via a Fenton reaction[11]. Alarming concerns have been evoked by observing pro-oxidant effects of ascorbic acid in human models[7, 12- 15].

Pro-oxidant effects of ascorbic acid may be related to one or multiple reasons: its oxidant metabolites dehydro-ascorbic acid and DKGA[4, 8], decrease in reduced glutathione in erythrocytes[2, 3, 13], generation of hydrogen peroxide and other ROS[14], formation of pro-oxidant ascorbyl radical[7] and in presence of metallic ions such as copper and iron to produce a Fenton type reaction with generation of hydroxyl radicals[11.]

Conclusions

In Vitro studies using sheep erythrocyte hemolysate and purified ruminant Hb have revealed concentration-related increase in methemoglobin formation due to ascorbic acid. The study has demonstrated pro-oxidant effect of ascorbic acid in ruminant hemoglobin in vitro assay model. Methemoglobin formation potential of ascorbic acid has been nearly 1/11th that of copper acetate on molar basis. The effect of ascorbic acid in presence of copper is much higher than in absence of copper while simulation experiments revealed all copper was getting reduced and precipitated out under test conditions in presence of ascorbic acid. Thus copper is incapacitated by ascorbic acid to express its oxidant effect. This would indicate some subtle biochemical reaction being responsible for ascorbic acid mediated methemoglobin formation. Further studies are required to assess the mechanism of ascorbic acid mediated effect particularly in presence of copper.

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