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Some factors controlling carotene destruction by chloroplasts in vitro

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Abstract. Loss of β carotene from moist leaf protein stored with a preservative, is closely simulated by the destruction of β carotene by acetone extracted chloroplasts (stroma) suspended in acetone containing 30% water. During this exposure, stroma lose destructive ability, but it is restored by mercaptoacetate and other reducing agents. Stroma can therefore be used repeatedly. This catalytic process is activated by removing intrinsic inhibitors, predominantly calcium, by extraction at pHs less than 4, or by alum. The objective is to find inhibitors which would be acceptable in a food. That restricts choice. Citrate, tartarate and phosphate are among the more attractive possibilities.

Introduction

Protein extracted from leaves (LP) is regularly made and eaten in more than 30 places, mainly in Central and South America (Pirie, 1989). The observed beneficial effect of this dietary supplementation is probably as often the effect of the β carotene (βc) in LP as of the protein. Whenever possible, freshly made LP should be used, but it will often be convenient to use dried or conserved moist material. Some βc is lost during drying, more is lost during storage unless air is rigidly excluded. As with other leafy material, preservation with salt increases the rate of loss: loss can be lessened by adding ascorbate, oxalate, phenolic compounds or sulfite (Pirie, 1984, 1987a). This protection is incomplete and, for various reasons, these agents have defects. With more information about the process of βc destruction, it should be possible to devise better ways for preventing it.

Destruction by lipoxidases and other enzymes is well-known. These may be the main destructive agents in living plants during the morning and in detached leaves; they are inactivated when juice is heated to make food-grade LP (Pirie, 1987b). There is no evidence that the more stable system described here is active in vivo. Nevertheless, it has considerable academic

interest. In suitable conditions and at 0 °C, especially after removing intrinsic inhibitors such as Ca, it destroys βc in minutes to an extent which requires hours in a living plant, days in detached leaves, and weeks in moist preserved LP at 34 °C. It is surprising that this latent destructive system is so thoroughly inhibited that some βc survives so long in LP as usually made. The object of this work is to lengthen survival.

Materials and methods

About 30 species are useful sources of LP (Pirie, 1987b): phenomena of the type described here have been noticed in about a third of them. Attention is restricted here to bean (*Vicia faba*), cabbage (*Brassica oleracea*), rape (*B. napus*) and wheat (*Triticum aestivum*). These species are representative and all the phenomena described were observed invariably with all of them although there were quantitative differences both between species and between different preparations from the same species.

Most of the protein and all the βc in LP was initially in chloroplasts. They are therefore the logical starting material for work on βc destruction. Plants, grown in the open by normal farm practice, were harvested in the morning, washed, pulped in a domestic mincer and pressed. After centrifuging at 1000 g for 10 min, the juice was centrifuged at 1600 g for 1 to 2 h. The sediment was suspended in water, sedimented again, and then repeatedly suspended in acetone and sedimented until the extract was colourless. Qualitatively similar material was made by extracting with ethanol, but the process was slower. This material is loosely called stroma. When preparations were given no further treatment, the term is unqualified. All preparations contained 9 to 11% N and small amounts of leaf fibre. That is inert: prolonging the first sedimentation to 30 min removed it more completely and diminished the yield of chloroplasts, but did not affect the activity of stroma. Material precipitated from chloroplast supernatants by heating or freezing had no similar reactivity with βc.

Acetone suspensions of stroma do not destroy βc in several days at room temperature. Destruction becomes significant when 5% water is added and increases steadily up to 30% water. With more water the rate becomes erratic, presumably because colloidal βc no longer penetrates stroma uniformly. Exclusion of air prevents βc destruction.

In all these comparisons between stroma samples, pretreatments or reaction conditions, 30% water was finally present. For each set of comparisons, volumes of an acetone suspension of stroma containing 15 mg (DM) were centrifuged, the sediments were allowed to drain for 1 to 2 min without

evaporation. They then contained 120 to 130 mg acetone. They were dispersed with 0.1 ml acetone containing 0.5 to 0.6 g l^{-1} βc (Sigma, from carrots); addition of 0.1 ml of whatever aqueous solution was being tested brought the water concentration to 30%. The duration of contact of stroma with βc before adding water is immaterial, but the sequence of additions is critical; βc penetrates moist stroma unevenly. No differences were noticed when βc isolated from LP was used instead of the commercial product.

Within the range 4.0 to 6.0, pH had little effect on the rate of action. Several monovalent ions e.g. K, Na, NH_4, Cl, CH_3COO, increased the rate. To establish some uniformity in conditions, the 0.1 ml of aqueous solution always contained 100 mM pH 5 acetate as well as any other components. Because of uncertainty about the extent to which aqueous acetone in a stroma suspension is available as a solvent, the stated concentrations are those of the components in the aqueous 0.1 ml added to the mixture.

After the required reaction time, usually at 0 °C, 8 ml acetone was added. Undestroyed βc was measured photometrically in the supernatant after centrifuging. That reading, subtracted from the reading given by 0.1 ml of the original βc solution in 8 ml acetone, divided by the latter reading and multiplied by 100, gives the % destruction of βc.

Duplicate measurements by this simple method of analysis differed by less than 2 µg βc, i.e. less than 10% when about half the βc is destroyed. No stroma sample was used which released perceptible colour as a result of any of the treatments. For that reason a few samples from mature rape and from elder and mustard could not be used in experiments with alum: alum liberated green material from them.

This technique of exposing βc to stroma is called Standard Conditions (SC). Sediments from which the 8 ml acetone extracts have been removed for analysis are, after draining, in a suitable state for use again in SC. Because the same sample of stroma can be used repeatedly to destroy βc, the process is called catalytic. However, fresh samples of stroma undergo antithetical modifications during exposure in SC. They gradually lose the ability to destroy βc, but this can be restored by adding reducing agents such as mercaptoacetate (Ma). They also lose inhibitors, predominantly Ca, and therefore become more active, in the presence of Ma, than they were initially. These changes proceed at different rates in different environments.

Potential inhibitors cannot always be tested by including them in the 0.1 ml aqueous component used in SC because some βc is destroyed before the inhibitor has made effective contact with the stroma. They were therefore tested either by exposing an aqueous suspension of stroma to them and extracting with acetone to restore SC conditions, or by adding the inhibitor in < 0.1 ml water to stroma suspended in > 4 ml acetone, shaking

occassionally during 1 h, and centrifuging. This relatively dry acetone pretreatment is labelled RDA.

Valid comparisons by this technique depend on starting with the same concentrations of *βc* in all tubes because, when conditions were otherwise the same, approximately the same amount of *βc* was destroyed in tubes containing different concentrations of it. Presumably, catalytic sites which were occupied by *βc* before water was added, are not always reoccupied by colloidal *βc* later. Otherwise reactions proceeded as would be expected. With each batch of stroma, the reaction rate was proportional to the amount of stroma used, and increased as temperature was increased. Each set of comparisons was made with several durations of exposure. To simplify presentation, results are given for only that set in which the duration led to destruction of about half the *βc* in some samples.

This paper aims at no more than outlining the nature of the problem, suggesting methods for inhibiting *βc* destruction in LP, and ranking stroma preparations, pretreatments, and reaction conditions in order. A system in which an insoluble catalyst, which loses and gains activity while acting, acts on a substrate which is not in true solution, is unsuited for detailed kinetic analysis.

Only phenomena which were qualitatively similar in all species are described. That restriction excludes reference to types of experiment in which results were often incomprehensively erratic. For example: results were consistent on stroma after extraction with cold aqueous acid, alum etc. and then suspended in acetone. They were also consistent when stroma were reused but kept in 90+% acetone throughout. Results became inconsistent when aqueous extraction was interposed between exposures to *βc*. The catalytic system is relatively thermostable or it would not act in LP as normally made. Aqueous stroma suspensions, heated at 100 °C for 2 to 30 min and suspended in acetone, lost some activity. But activity was restored, sometimes to the original level, on repeated reuse with Ma and alum. Destruction of *βc* by stroma is called catalytic because such behaviour is not characteristic of a conventional enzyme.

Results

Column a in Table 1 shows that *βc* was not destroyed as soon as the SC mixture was assembled, and that destruction increased as the length of exposure increased. Column b shows that reused samples, when exposed again for 60 min in SC, destroyed more *βc* the shorter their earlier exposure had been. This is also shown in column c: all samples were exposed for 1 h,

Table 1. Effect of duration of exposure and reuse on the activity of stroma

a Duration of first exposure		b Duration of second exposure		c Third exposure, all for 1 hour	d Fourth exposure, with Ma for 20 min
Percentage of β carotene destroyed					
0	0	20 min	31	42	47
0	0	60 min	57	36	53
20 min	38	20 min	25	35	47
20 min	38	60 min	42	29	50
60 min	66	60 min	33	27	50
60 min	67	120 min	55	25	47

Six 15 mg samples of cabbage stroma were exposed to 100 mM pH 5 acetate in the Standard Conditions (described in the text) at 0 °C, after the times specified in column a, they were extracted with 8 ml of acetone. The numbers are the percentages of the β carotene, which had been added to the stroma, which did not appear in the acetone extract. For columns b and c the sediments, after acetone extraction, were exposed again in the same way. For column d they were exposed yet again, but 20 mM mercaptoacetate (Ma) was included in the aqueous component of the Standard Conditions mixture.

those which had already had the shortest exposures destroyed the most βc. Column d shows that exposure to Ma for 20 min, not only restored activity but (lines 3 and 4) led to greater activity than had been shown in 20 min in column a. Reversible inactivation does not depend on the presence of βc. In tests with all four species, in which no βc was included in the 0.1 ml acetone added in SC in the first exposure, results in columns b, c and d closely resembled those in Table 1.

Destruction of βc depends on the presence of water and is increased by the presence of ions. These also increase the rate of reversible inactivation. This is shown in Table 2. The greater loss of activity with increased acetate concentration was shown consistently: the slight difference between species

Table 2. Inactivation by pretreatment with water, 0.1 M or 1.0 M acetate

Species	Untreated stroma	Water	0.1 M acetate	1.0 M acetate
	Percentage destruction of β carotene			
Bean	44	36	15	10
Cabbage	57	36	27	17
Rape	54	27	18	14
Wheat	46	32	25	16

15 mg samples of the four types of stroma were suspended in 5 ml of the specified fluids and centrifuged after 10 min at 0 °C. They were then extracted twice with acetone and exposed at 0 °C for 40 min to βc in Standard Conditions. One sample is an untreated control.

was not. As in Table 1, activity was restored to all 12 pretreated samples when they were reused in SC with 20 mM Ma in the mixture. This effect is discussed later.

Other ions caused similar reversible inactivation. More concentrated solutions caused still more inactivation but the position is confused because they make stroma gummy so that penetration of βc into the mass may be impeded. Activity is obviously not similarly lost when untreated chloroplasts are similarly exposed: they encounter such solutions when leaf juice is made. Vulnerability appears only after extraction with acetone or ethanol. However, stroma made in bulk by the method described will be partly inactivated during the phase of acetone extraction in which water is still being removed. An experiment with wheat stroma illustrates this. Sediments from an acetone suspension, containing 15 mg DM, were mixed with 0.1 ml water and kept at 0 °C for 5, 20, 60 and 1200 min before being reextracted with acetone and exposed to βc in SC. They retained 85, 66, 61 and 22% of their initial activity. Delay during the first few cycles of acetone extraction is therefore ill-advised when making stroma.

Extraction of an activating agent, for which Ma can substitute, is an obvious possible reason for this reversible loss of activity. Many samples of stroma, from all four species, were extracted with 0.1 or 0.3 M ammonium acetate. The extracts were dried in vacuo to remove the volatile salt, they contained 5 to 10% of the DM of the stroma. This contained 1 to 3% N, 0.1, to 0.3% S, 0.1 to 0.5% P and 10 to 20% carbohydrate (measured by heating with orcinol in 66% H_2SO_4 (Pirie, 1936)). Possibly relevant metals which were consistently present were Ca, Cu, Mn and Zn. Stroma extracts made thus did not share with Ma the ability to reactivate stoma. On the contrary, when included in the 0.1 ml aqueous component used in SC they regularly, but to variable extents, inhibited destruction of βc. There was more inhibition in tests by the RDA technique, but results were still inconsistent. If reversible inactivation during exposure in SC or to aqueous extraction, is the result of removal of an activating agent, the agent is unstable or its action is masked by an accompanying inhibitor.

Activation and reactivation

Reactivation by including Ma in the aqueous component of the SC mixture was shown in Table 1. Suitable concentrations of Ma invariably activate untreated stroma in a similar manner. As all stroma samples will have undergone some inactivation during preparation, there seems to be no reason to distinguish the two actions. Ascorbate and cysteine reactivate less consistently. Therefore, although Ma is less relevant biologically, results

Table 3. Effect of mercaptoacetate concentration on β carotene destruction by bean stroma

	Untreated stroma				Acid extracted stroma			
Mercaptoacetate concentration, mM	100	30	10	0	100	30	10	0
	Percentage destruction of a β carotene							
	37	65	74	17	87	87	80	0
Reuse without further Ma	64	32	17	8	44	32	16	0

15 mg samples of untreated bean stroma, or stroma which had been extracted with water at pH 1.8, were exposed for 15 min at 0 °C in Standard Conditions to the specified concentrations of Ma. After extraction with acetone for the β carotene analysis, each sediment was exposed again in the same way but without including Ma in the SC mixture.

with it are the only ones given here. Activation by Ma is not affected by adding catalase to the SC mixture.

In Table 3, the four columns of results on untreated stroma make the essential points on the action of Ma. At 10 mM it obviously activates: 74% destruction compared to 17% when destruction depended on intrinsic activation. Stronger Ma caused less destruction. Comparisons with many stroma samples, and smaller steps between Ma concentrations, showed that the optimum could be anywhere between 15 and 100 mM and that activation was occasionally perceptible with 1 mM. The lower line in Table 3 shows that, after exposure to inhibitory concentrations of Ma, stroma, in spite of acetone extraction, may retain enough of the Ma (or of its effect on stromal structure) to act strongly on reuse without again including Ma in the SC mixture. When screening stroma for activity, several Ma concentrations were therefore used and all the samples were reused.

Extraction with acid inactivates stroma more quickly and completely than extraction with salts, but, with Ma, original activity is restored or even exceeded. Unlike untreated stroma, acid-extracted stroma (A-ES) is not inhibited by 100 mM Ma. The stroma sample used in Table 3 was so active that, with 10 mM Ma, the difference between stroma and A-ES is unclear. It was obvious with the same stroma exposed for only 5 min. The upper line in Table 4 gives two examples of activation by acid extraction, it also contains a suggestion that extraction at pH 1.7 is beginning to be harmful. That suggestion was confirmed by many extractions at pHs < 1. The effect of including alum in the SC, in the lower line, is explained later. After acid extractions that do not cause irreversible loss of activity, A-ES retain 0.2 to 0.3 mg Ca/g. Untreated stroma contain 3 to 8 mg/g.

About 5% of the DM of stroma was extracted into each of a succession of aqueous extracts at pHs such as 4.5, 3.0 and 1.5. Unlike saline extracts, the material in them consistently inhibited βc destruction by both types of

Table 4. Effect of extraction at various pHs on the activation of rape stroma

Activator	pH of aqueous pretreatment of stroma			
	a	b	c	d
	Untreated control	5.0	3.0	1.7
	Percentage of β carotene destroyed at 0 °C			
20 mM mercaptoacetate	12 (20 min)	21 (20 min)	81	74
20 mM mercaptoacetate plus 20 mM alum	41	43	85	75

After extraction with water at the specified pHs, neutralisation and suspension in acetone, two 15 mg samples of each type of stroma were sedimented and, after added β carotene, exposed to 100 mM acetate containing either mercaptoacetate (Ma) or Ma + alum. Because slow action was expected in two of them, they were exposed for 20 min and the other six for ten min. All were at 0 °C.

stroma. Inhibition by these extracts is discussed later along with inhibition by other substances.

A-ES is convenient material with which to study the nature of this catalytic system. Measurements with it are not confused by the loss of the original state of activation, and of the inhibiting material discussed above, during use. In acetone suspension its activity is apparently unaffected by many months storage.

Phenolic substances are usually present in LP (Jennings et al. 1968); increasing the amount, e.g. by adding catechol or extracts of used tea (Pirie, 1987a), increased the stability of the intrinsic βc in LP. Unpublished experiments on LP containing different amounts of phenolics (a by-product of work on the effect of intrinsic phenolics on LP extractability (Butler, 1982)) showed a parellelism between phenolic content and βc stability. Caffein reverses the inhibition of several enzymes by tannins (e.g. Mejbaum-Katzenellenbogen, 1962): it had little effect on the behaviour of stroma in SC.

Alum ($KAl(SO_4)_2$) was tested as a possible sequestrator of phenolics; it increased the lability of βc in LP (Pirie, 1987a). That is in agreement with Hanczakowski and Skraba's (1985) observation (which I confirm with several species) that LP contains less βc if coagulated from lucerne juice with alum rather than by heating. Alum also restored lability to βc in LP in which it had been protected by some nonphenolic substances (Pirie, 1987a). That suggested that it might act by displacing Ca in the manner familiar from work on soil (e.g. Coulter and Talibudeen, 1968). After alum extraction, as after acid extraction, the Ca content of stroma was 0.2 to 0.3 mg/g; the Al content increased from 0.5 to 1.5 mg/g to 8 mg/g.

The lines in Table 4 show that acid pretreatment increased βc destruction with both Ma and Ma + alum in SC. The columns show that, without acid

Table 5. Inhibition of acid-extracted rape stroma by calcium, zinc and material extracted from stroma at pH 5.0

Inhibiting agent	Percentage destruction of β carotene	
	First exposure	Second exposure
None	85	80
15 mM calcium	78	75
30 mM calcium (0.12 mg Ca)	39	45
60 mM calcium	15	19
30 mM zinc	9	69
15 mM zinc	22	75
1 mg of extract (0.075 mg Ca)	30	16

15 mg samples of rape stroma, which had been extracted with water at pH 2.0 and sedimented from acetone, were suspended in 4 ml acetone; the specified inhibitors in 0.1 ml water were added. After 1 h at 0 °C with occasional shaking they were centrifuged and the sediments were exposed to βc and 20 mM Ma in SC for 20 min at 0 °C. After extraction with 8 ml acetone for analysis, the sediments were reused with β carotene and Ma in SC for 20 min at 0 °C.

pretreatment, alum increased destruction, but it had little further effect after acid pretreatment.

Stroma samples differ in the concentration of alum needed for activation. With a few, 5 mM in the SC mixture was not enough, 20 to 30 mM was always enough. Unlike the effect of strong Ma shown in Table 3, A-ES rather than untreated stroma was inhibited by alum concentrations > 70 mM. As with inhibition by strong Ma, activity was recovered on resuse.

Table 5 shows that Ca inhibits βc destruction. Activation by acid extraction can therefore be plausibly attributed to the removal of part of the Ca. Extraction with aqueous alum removes Ca; but in SC, Ca is not removed though it may be displaced. It seems unlikely that redistribution of Ca is a full explanation of activation because alum also counteracts several other inhibitors. Ca is bound by, or affects, several hundred plant and animal proteins (e.g. Heizmann & Hunziker, 1991); the effects of Al, a widely distributed but ill-understood element, on some of them could be interesting.

Inhibition

Inhibition by concentrated alum or Ma may ultimately help to explain the nature of this novel catalytic system but it has little bearing on the immediate problem of protecting βc in stored LP. To be useful in practice, an inhibitor must be cheap and, if possible, locally available. Widespread fears about toxicity, whether or not justified, must also be borne in mind. These factors eliminate feeble inhibitors such as azide, cyanide, oxalate and the more potent inhibitors EDTA, ascorbate and cysteine (Pirie, 1987a). Agents such

Table 6. The contrasted effects of alum and citrate

Pretreatment	First exposure		Second exposure		Third exposure	
	Percentage destruction of β carotene in standard conditions					
Acetate (Ac)	Ac	60	Ac	51		
Citrate (Cit)	Ac	16	Ac	7		
Citrate	Alum	77	Ac	65		
Alum	Cit	65	Ac	62		
Citrate	Ac	34	Ac	22	Alum	69
Alum	Cit	62	Cit	55	Cit	39
Ac	Ac	62	Cit	13	Alum	60

15 mg lots of bean stroma, sedimented from acetone suspension, were used in the first four lines, 15 mg lots of rape stroma in the next three. The sediments were mixed with 1 ml of aqueous 100 mM pH 5 acetate, either alone or with 10 mM citrate or 30 mM citrate or 30 mM alum. After 10 min at 0 °C they were centrifuged. The sediments were exposed for 30.min at 0 °C to Standard Conditions: the 0.1 ml aqueous components of the SC mixtures had the compositions used in the pretreatments. After extraction with 8 ml acetone for analysis, the sediments were exposed again for 50 min in SC at 0 °C to the specified agents. The three rape samples were exposed again in a similar manner.

as strong acid would be impractical in the places where there is most need for locally produced LP. Table 5 shows that zinc inhibits more strongly than Ca but it is probably irrelevant in this context. Stroma and extracts from them contain 20 times as much Ca. Stromal extracts were more inhibitory than could be accounted for by their Ca; they still inhibited after precise removal of the Ca with oxalate. Identification of the non-Ca intrinsic inhibitor(s) is unimportant practically because in, similar conditions and with equal weights, this material was $< 1/10$ as inhibitory as the inhibitors discussed in the next paragraph.

Some inhibitors, e.g. citrate, phosphate and tartarate, which all act similarly, do not make effective contact in the absence of water. Their efficacy may therefore be underestimated in tests by RDA and, when tested in SC, they may inhibit more strongly on reuse than in the first exposure. Tests were consistently successful when 1 ml of an aqueous solution of the agent was mixed with stroma sedimented from acetone suspension, sedimented again after a few minutes, and extracted with acetone as a prelude to testing with Ma in SC.

Table 6 contrasts the effects of alum and citrate. Line 1 is a control showing that pretreatment with acetate does not interfere with βc destruction in SC, and on reuse. Lines 2 and 3 show that pretreatment with citrate inhibited the action unless alum was in the SC mixture. Line 4 shows that alum pretreatment prevents citrate inhibition in both the first exposure and on reuse. Lines 5 and 6 show that citrate pretreatment persists on reuse but

is counteracted by the inclusion of alum in the SC mixture used in the third exposure, by contrast, the effect of alum pretreatment is gradually overcome by repeated exposure to citrate. EDTA is the only agent found so far which is more effective than citrate: it would, however, be unacceptable in a food.

Discussion

This is only the beginning of work on what seems to be a novel type of catalyst. An alternative to the technique used–stripping components away from the practically relevant system–would be to start by dispersing chloroplasts mechanically, and examining histologically recognised components separately for catalytic activity. Such techniques, similar to those used by Webber and Gray (1989) to show that two components of wheat photosystem II bind Ca strongly, would be interesting academically but less relevant practically. Similarly, had experiments of the type described been made on one species, grown in controlled conditions, they would probably have been more consistent quantitatively. But LP is not made from such material.

Because every batch of stroma from all four species exhibited the effects described here, the effects are probably typical of species which would be used for making LP. Unfortunately, βc destruction is easier to activate than inhibit. One reason is the presence of removable intrinsic inhibitors. Another is that acetone extraction gives βc more access to catalytic sites in the insoluble catalyst. That is probably why, in some tests, destruction was 1000 times faster than in LP. Greater access of substrate to catalyst may also explain the curious loss of βc from leaves preserved with salt.

These experiments suggest that it may be possible to devise practical ways for destroying the intrinsic activator(s), for inactivating the catalyst by prolonged heating without converting too much trans-carotene into the cis isomer, for making the use of inhibitors such as Ca or citrate more effective, and for finding better inhibitors which are acceptable in a food.

Summary

It is difficult to dry leaf protein without damaging the protein and destroying part of the β carotene. When storage is necessary, it can be kept as moist press cake with a preservative such as salt. It still gradually loses β carotene. To find out more about the nature of the destructive process, in the hope that this will suggest methods by which it can be inhibited, β carotene and

other lipids were extracted from the chloroplasts of four representative species, and the conditions in which the resulting material destroyed added β carotene was studied. Destruction depends on access of air and on an original state of activation. This activation is lost during exposure to the conditions in which β carotene is destroyed, but is restored by adding reducing agents such as mercaptoacetate. Destruction is increased by extraction with acid or alum which remove intrinsic inhibitors–predominantly calcium–if a reducing agent is added. After these treatments the material is stable for many months and can be used repeatedly. It is therefore a convenient material on which to study the potentialities of inhibitors which could be used in food, and methods for destroying the catalyst itself. It also has some properties which are interesting academically.

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