

# The Physiological Significance of Phenylacetic Acid in Abscising Cotton Cotyledons

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## ABSTRACT

The physiological role of phenylacetic acid (PAA) as an endogenous regulator of cotyledon abscission was examined using cotton (*Gossypium hirsutum* L. cv LG 102) seedlings. Application of 100 micromolar or more PAA to leafless cotyledon abscission-zone explants resulted in the retardation of petiole abscission and a decrease in the rise of ethylene evolution that normally accompanies aging of these explants *in vitro*. The partial inhibition of ethylene evolution in these explants by PAA was indirect since application of this compound stimulated short-term (<24 hours) ethylene production. PAA treatment partially suppressed the stimulation of petiole abscission elicited by either ethylene or abscisic acid. Both free and an acid-labile, bound form of PAA were identified in extracts prepared from cotyledons. No discernible pattern of changes in free or bound PAA was found during the course of ethylene-induced cotyledon abscission. Unlike indole-3-acetic acid, transport of PAA in isolated petiole segments was limited and exhibited little polarity. On the whole, these results are not consistent with the direct participation of PAA in the endogenous regulation of cotyledon abscission.

Abscission is a correlative phenomenon, wherein events occurring primarily within the leaf blade determine the initiation and subsequent rate of biochemical changes within the abscission zone itself. Recent studies examining the action of a commercial defoliant have also demonstrated the pivotal role of the leaf blade in determining the timing and extent of chemically induced leaf abscission (11). Phytohormones are currently thought to be the principal signalling agents involved in this long-distance communication between the leaf blade and the zone of cell separation (for review, see Addicott [2]). Thus, a more thorough understanding of the alterations in phytohormone activity (titer and action) that attend leaf abscission should provide additional clues to the rational design of future chemical defoliants.

While ethylene and ABA are known promoters of leaf abscission, auxins are potent inhibitors of this process (9). Early work on the role of auxins as abscission inhibitors relied totally on chemically undefined preparations obtained from natural materials such as orchid pollen or leaf diffusates (7). With the identification of IAA as an endogenous auxin (6), almost all subsequent research has focused on the effects of IAA.

Besides IAA, plants contain another, nonindolic auxin, PAA<sup>1</sup> (15). In certain species PAA can account for up to one-half of the total bio-assayable auxin activity in plant extracts (14). Depending on the assay system, PAA can elicit responses ranging

from little activity to activity equal to that of IAA itself (13; see Wightman *et al.* [16] for discussion). To our knowledge, the possible role of PAA as an endogenous regulator in leaf abscission has not been examined. This research was undertaken to explore this possibility. Portions of these studies have been presented in abstract form (12).

## MATERIALS AND METHODS

**Plant Material and Experimental Procedure.** Cotton (*Gossypium hirsutum* L. cv LG 102) seeds were sown in plastic pots containing vermiculite and were grown as described previously (11). Seedlings were watered daily with one-third strength, modified Hoagland solution (3). Seedlings were used when the cotyledons reached full maturity as indicated by cessation of petiole elongation and cotyledon expansion (18-27 d after sowing).

All experiments described in this paper were conducted a minimum of three times. Whenever possible, each treatment within an experiment was replicated ( $n = 2$  or  $3$ ). Due to the nature of some of the experiments, replication within an experiment was not feasible. Data from typical experiments are presented.

**Chemicals.** [ $1-^{14}\text{C}$ ]PAA (24 mCi/mmol) was obtained from Research Products International Corp.,<sup>2</sup> [ $1-^{14}\text{C}$ ]IAA (50 mCi/mmol) was obtained from Amersham Corp. All other chemicals and solvents employed in these studies were of the highest purity available and were obtained from commercial supply houses.

**Effect of PAA on Abscission Rates in Cotyledon Abscission-Zone Explants.** Cotyledonary-node abscission zone explants were obtained from cotton seedlings and were surface sterilized as described previously (11). Explants (10/treatment) used for the experiments examining PAA action on spontaneous or basal abscission rate and endogenous ethylene evolution were handled aseptically throughout the entire experiment. Treatment with PAA or other test chemicals, scoring for abscission, and the determination of endogenous ethylene evolution were conducted as previously described (11).

Experiments examining the effect of various concentrations of PAA or 100  $\mu\text{M}$  IAA on ethylene-induced abscission were conducted in the following manner: after exposure of the explants (10/treatment) to the treatment solution for 4 h the explants were blotted dry and placed in plastic holders. These holders were then placed inside a glass desiccator lined with water-saturated paper toweling and containing a  $\text{CO}_2$  trap (filter paper impregnated with a saturated solution of  $\text{Ba}(\text{OH})_2$  in 1 N NaOH), ethylene was added to the sealed desiccators to a final concentra-

<sup>1</sup> Abbreviations: PAA, phenylacetic acid; NPA, *N*-1-naphthylphthalamic acid.

<sup>2</sup> Mention of trademark or proprietary product does not constitute a guarantee or warranty of the product by the United States Department of Agriculture and does not imply its approval to the exclusion of other products that may also be suitable.

tion of about 25  $\mu\text{L/L}$ , and the desiccator was placed in a dark incubator ( $25 \pm 1^\circ\text{C}$ ). Periodically, the desiccator was opened and abscission was scored. The desiccator was sealed and ethylene was added as before. Ethylene levels were monitored by GC.

The effects of PAA, its hydroxylated analogs, or IAA on ABA-induced abscission were conducted similarly. After treatment with ABA or ABA plus PAA or IAA (4 h), explants (10/treatment) were placed in plastic holders, the holders were placed in chambers that were lined with water-saturated toweling, and contained a  $\text{CO}_2$  trap (see above). These containers were then incubated as described above and abscission was scored periodically thereafter.

**Effects on Ethylene Evolution.** PAA or IAA solutions were prepared in 10 mM MES-KOH buffer (pH 5.7) containing 50  $\mu\text{g/ml}$  chloramphenicol. Abscission-zone explants were prepared as described above. Leaf discs (8–9 mm) were punched out from cotyledons using a sharp cork borer. Petiole segments (2.5 cm long) were excised from the middle of each petiole (1 segment/petiole). The tissues were placed in flasks containing the treatment solutions (10 cotyledon discs in 50 ml flasks plus 5 ml solution; 3 explants or 5 petiole segments in 25 ml flasks plus 3.5 ml solution). The flasks were sealed and incubated in the dark ( $25 \pm 1^\circ\text{C}$ ). The ethylene content of the headspace was determined after 24 h by GC.

**Isolation, Identification, and Quantitation of PAA.** Following the appropriate experimental treatment, cotton cotyledons were excised at the cotyledon-petiole junction, weighed, and quickly frozen with liquid  $\text{N}_2$ . The frozen tissue was pulverized and homogenized in 80% aqueous acetone (5 ml/g fresh weight). An internal standard of [ $^{14}\text{C}$ ]PAA was added to the homogenates which were then filtered. The residue was extracted twice more and the filtrates were combined and the acetone removed under partial vacuum ( $35^\circ\text{C}$ ). The remaining aqueous phase was adjusted to pH 8 and was partitioned three times against diethyl ether. The aqueous phase was divided in half; one-half being used for the determination of free PAA and the other half for the determination of total PAA after acid hydrolysis. Hydrolysis of bound PAA was accomplished by adjusting the aqueous phase to 1 N with HCl and refluxing for 3 h. Following hydrolysis, the acidic aqueous solution was partitioned against diethyl ether yielding the total PAA fraction. The original alkaline aqueous solution containing any free PAA was adjusted to pH 2.5 and was partitioned similarly. From this point on both fractions (free and total PAA) were treated identically. The acidic ether phase was taken to dryness and was redissolved in a small volume of acetone that was then clarified by filtration through a 0.45  $\mu\text{m}$  filter.

The acetone filtrates were fractionated by HPLC using a  $\text{C}_{18}$ -reverse phase column (8  $\times$  10 cm, Nova column, Waters Associates, Inc.). Fractionation was achieved using a 15 min linear gradient (2 ml/min) of 20 to 100%  $\text{CH}_3\text{CN}$  in water (both solvents contained 1% [v/v] acetic acid). The PAA containing fraction was detected using an in-line radioactivity flow monitor and the entire zone of radioactivity was collected. These column eluates were reduced to the aqueous phase and then adjusted to pH 2.5 with HCl. They were then partitioned (3 $\times$ ) against diethyl ether and dried over anhydrous  $\text{Na}_2\text{SO}_4$ . An aliquot was then removed for the determination of recovery of the internal standard. The ether fraction was taken to dryness, redissolved in acetone, transferred to reaction vials, again taken to dryness, and the vials sealed. Methylation was accomplished using diazomethane. Free and total PAA were quantitated by GLC-FID using a SP-2100 column operated isothermally at  $90^\circ\text{C}$ . Bound PAA was inferred from the difference between these values. GC-MS analyses to confirm the identity of PAA methyl ester in both free and total PAA extracts were carried out using a H.P. 5992 capillary GC-MS operated at 70 eV. Characteristic ion fragments

(m/z) with their relative abundance in parentheses were as follows: standard PAA: 91(100), 150(23), 65(20), 63(8); free PAA: 91(100), 150(28), 65(18), 63(7); and total PAA: 91(100), 150(21), 65(23), 63(12).

**Transport Studies.** The capacity for polar transport of [ $^{14}\text{C}$ ]PAA or [ $^{14}\text{C}$ ]IAA was examined using 5 mm petiole segments excised from the center of mature, nonsenescent cotyledon petioles. The segments were placed on a disc of 1.5% (w/v) agar (6  $\times$  2 mm). Donor blocks (5  $\times$  2 mm) were prepared by adding the radioactive hormone (final concentration: 10  $\mu\text{M}$ ) to molten agar just prior to gelling. Both donor and receiver blocks contained 50 mM K-phosphate buffer (pH 6.1). Transport studies were conducted in humid chambers maintained in the dark ( $27 \pm 1^\circ\text{C}$ ). Transport was allowed to proceed for 4 h. Receiver blocks were placed in scintillation vials containing 10 ml of scintillation cocktail. After 24 h, radioactivity in the receivers was determined by scintillation spectroscopy.

## RESULTS

By analogy with the proposed role of IAA in regulating the process of abscission (9), a series of experiments were undertaken to assess the participation of PAA in the correlative control of petiole abscission by leaf-derived factors. Initially, the pharmacological effects of exogenous PAA on petiole abscission of leafless explants prepared from cotyledonary nodes of cotton seedlings were studied in comparison with the effects elicited by IAA. Under the conditions employed in these studies, petiole abscission from untreated explants could first be observed 3 d after excision and reached 50% abscission (*i.e.* 10 abscissions) by 5 d (Fig. 1). During this time, ethylene evolution from these explants rose from an initial rate of about 0.1 nl/h to a value of about 0.5 nl/h by d 3. Treatment of these explants with 0.1 mM

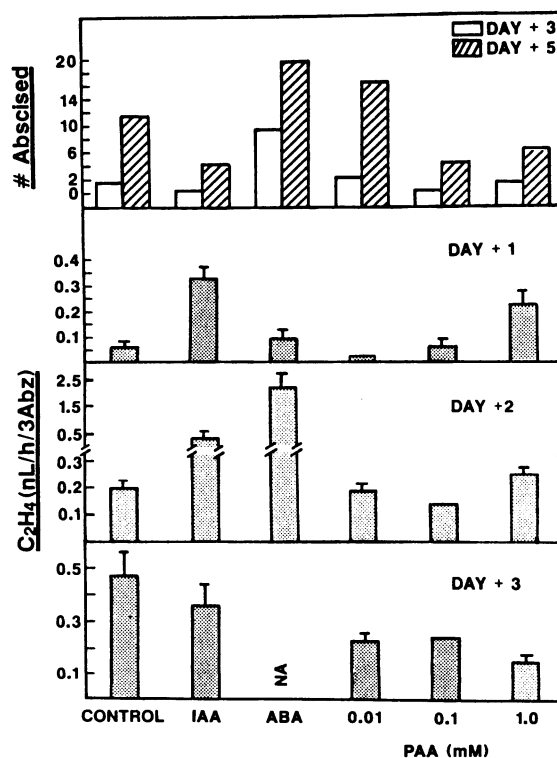


FIG. 1. Effect of various concentrations of PAA (0.01, 0.1, or 1.0 mM) IAA (0.1 mM), or ABA (0.1 mM) on ethylene evolution (lower 3 panels) and cumulative petiole abscission (upper panel) in sterile, leafless explants of cotton seedlings. Explants were prepared from the cotyledonary node, treated with the test chemicals for 4 h, and monitored thereafter. 20 abscissions = 100%. Bars indicate SE ( $n = 3$ ).

ABA resulted in accelerated petiole abscission (45% or 9 abscissions on d 3) and elicited a substantial increase in ethylene evolution by d 2. Treatment with 0.1 mM IAA resulted in an enhancement of ethylene evolution (persisting for 2 d) but the degree of petiole abscission was reduced for the duration of the experiment. Like IAA, treatment with PAA at 0.1 mM or greater resulted in a reduction in the rate of abscission. Unlike IAA, PAA treatment also suppressed the increase in ethylene evolution that normally occurred during these incubations.

Continuous exposure of cotton seedling tissues to solutions of PAA of 0.1 mM or greater resulted in a marked stimulation of ethylene evolution (Fig. 2). Even at 1 mM, PAA was far less effective than 0.05 mM IAA in this regard. These results indicate that PAA has no direct inhibitory effect on ethylene biosynthesis in these tissues (see "Discussion").

On the other hand, pretreatment of explants with 1 mM PAA resulted in a transient inhibition of ethylene-induced petiole abscission (Fig. 3). Lower concentrations of PAA were ineffective in reducing ethylene-inducing abscission (not shown). Similarly, 1 mM PAA transiently suppressed ABA-induced petiole abscission (Table I). This effect was also seen (albeit to a lesser extent) at PAA concentrations of 10 and 100  $\mu$ M (not shown). IAA (0.1 mM) was far more effective in reducing both ethylene and ABA

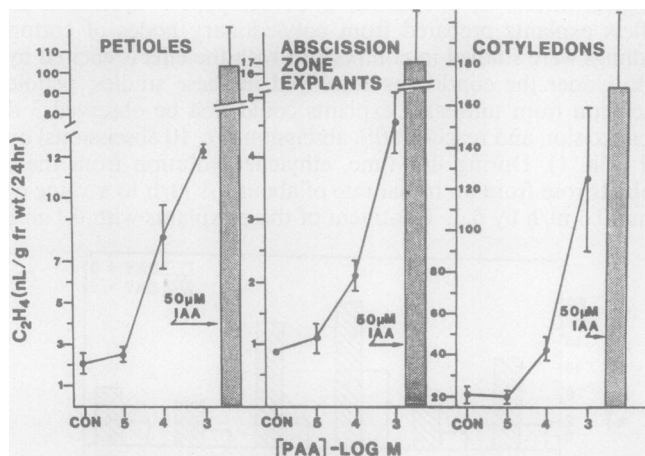


FIG. 2. Effect of various concentrations of PAA or IAA (50  $\mu$ M) on short-term ethylene evolution from various cotton seedling tissues. Tissues were floated on the test solutions and ethylene evolution was determined after 24 h dark incubation ( $25 \pm 1^\circ\text{C}$ ). Bars indicate SE ( $n = 3$ ).

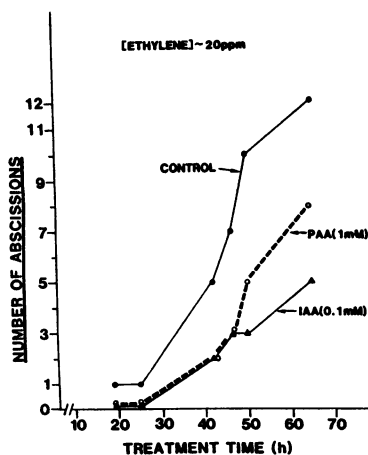


FIG. 3. Effect of PAA or IAA on ethylene-induced petiole abscission in leafless explants of cotton. Explants were maintained at  $25 \pm 1^\circ\text{C}$ . 20 abscissions = 100%.

Table I. Effect of IAA, PAA, or Hydroxy-PAA Derivatives on ABA-Induced Abscission in Cotyledonary-Node, Abscission-Zone Explants of Cotton Seedlings

All explants were treated with 0.1 mM ABA with or without IAA or one of the PAA derivatives. PAA and its hydroxy analogs were all tested at 1 mM, and IAA was applied at 0.1 mM. Explants were maintained at  $25^\circ\text{C}$ ; 100% abscission = 20.

Treatment	Cumulative Abscission <sup>a</sup> at:		
	23	41	48 h
CON (+ABA)	14	20 <sup>b</sup>	
IAA	1	6	6
PAA	2	7	14
<i>m</i> -Hydroxy-PAA	1	19	20
<i>o</i> -Hydroxy-PAA	1	8	9
<i>p</i> -Hydroxy-PAA	1	17	17

<sup>a</sup> Hours after treatment. <sup>b</sup> Explants not exposed to ABA had 3, 10, and 13 abscissions at 23, 41, and 48 h, respectively.

induced petiole abscission.

The specificity of the response to PAA was examined by testing the ability of various monohydroxylated derivatives of PAA to retard ABA-induced petiole abscission. Initially (23 h post-treatment) all compounds tested were equally effective (Table I). However, with time their effectiveness declined, but at different rates. By 41 h posttreatment both the meta and para substituted analogs were without effect and by 48 h only the ortho-hydroxy compound still exhibited biological activity.

Having established that PAA possesses significant auxin-like activity in these abscission assays, we next examined the possible physiological role of PAA in the regulation of cotton cotyledon abscission. Both free PAA and an unidentified acid-labile conjugated form of PAA (bound PAA) were identified by GC-MS in extracts prepared from cotton cotyledons (see "Materials and Methods" section for details). When expressed on a per g fresh weight basis, the endogenous levels of PAA were: free PAA, 21 to 216 ng; bound PAA, 122 to 405 ng. The effect of a known abscission accelerator (ethylene) on the endogenous levels of both free and bound PAA was examined next.

Exposure of these seedlings to ethylene (30–60  $\mu$ L/L) for 22 h resulted in roughly 20% abscission (not shown). During this treatment period levels of free PAA rose considerably while levels of bound PAA declined somewhat (Table II). Fumigation of these seedlings for an additional 26 h (*i.e.* 48 h total fumigation time) resulted in complete cotyledon abscission. At this time endogenous levels of free PAA exhibited considerable variation (being higher than controls in one replicate, lower in others). Total PAA levels (free and bound) were found to be elevated in ethylene treated seedlings. Over the course of at least seven independent determinations of endogenous PAA levels no consistent changes were found with respect to ethylene-induced abscission.

Over the years, much experimental evidence was accumulated that suggests that the sustained operation of the polar auxin transport system is essential in deferring the initiation of leaf abscission. The ability of PAA to move in the polar auxin transport system was examined by using the classical donor/receiver agar block technique. As expected, considerable polar transport of IAA was found in isolated petiole segments (Table III). The transport of PAA in these segments was limited and exhibited little, if any, polarity. Inclusion of the auxin transport inhibitor NPA in the receiver blocks completely inhibited basipetal IAA transport but had no effect on PAA transport (not shown).

## DISCUSSION

The ability of the leaf blade to inhibit the requisite biochemical alterations that underlie cell separation in the abscission zone is

Table II. Effect of Ethylene Fumigation (30 to 60  $\mu\text{l/L}$ ) on Free and Bound PAA Levels in Cotton Cotyledons

Treatment	Duration	PAA Levels		Total
		Free	Bound	
<i>h</i>				
<i>ng/cotyledon</i>				
Experiment I				
Rep. A <sup>a</sup>				
None		29	346	375
Ethylene	22	74	266	340
Rep. B				
None		88	158	246
Ethylene	22	167	143	310
Experiment II				
Rep. A				
None		78	303	381
Ethylene	48	117	344	461
Rep. B				
None		241	194	435
Ethylene	48	140	462	602

<sup>a</sup> Reps A and B refer to two independent replications within each experiment.

Table III. Movement of [<sup>14</sup>C]IAA or [<sup>14</sup>C]PAA in Isolated Cotyledon Petiole Segments

Petiole segments (5 mm) were excised from mature, nonsenescent cotton cotyledons. Movement of radioactive hormones was examined using the donor/receiver technique. Final donor concentration: 10  $\mu\text{M}$ . Transport period was 4 h, temperature was  $27 \pm 1^\circ\text{C}$  of ( $n = 6$ ).

Compound	Transport Direction	Receiver Blocks
		<i>pmol/receiver</i> $\pm$ SE
IAA	Basipetal	5.53 $\pm$ 0.97
	Acropetal	0
PAA	Basipetal	1.49 $\pm$ 0.22
	Acropetal	1.20 $\pm$ 0.26

well-known and constitutes an excellent example of the correlative control of plant development. Equally well documented is the ability of exogenous auxin-like compounds to mimic the action of the leaf blade in this system (2). The results presented in this study demonstrate that PAA, like IAA, can if provided directly to the abscission zone and in sufficient quantity, defer the initiation of petiole abscission in leafless explants. While active in this regard, the necessary concentration of PAA required for an observable effect is quite high (about 0.1–1 mM; see Table I; Figs. 1, 3). In addition, the retardation of petiole abscission elicited by PAA treatment is transient (Fig. 3; Table I). Generally, PAA was effective only when supplied at concentrations of 0.1 mM or more. This observation is in agreement with data presented by others using other assay systems (4, 15).

Treatment of leafless explants with PAA results in the partial suppression of petiole abscission and also reduces the increase in ethylene production normally observed as these explants age (Fig. 1). It can be argued that the reduced rate of abscission is a result of the impairment of ethylene production. This hypothesis appears unlikely in view of the ability of PAA to stimulate short-term (*i.e.* <24 h) ethylene production by all cotton tissues examined (Fig. 2) and to inhibit the abscission-inducing actions of both ABA (Table I) and exogenous ethylene (Fig. 3). It is possible that PAA is somehow slowing the process of accelerated aging induced by tissue excision and that the above-mentioned ability is merely a reflection of this type of action. PAA has been shown to be a moderately effective stimulator of ethylene pro-

duction in other plant tissues such as wheat coleoptiles (8).

The ability to retard ABA-induced petiole abscission is shared, at least to some degree, by all monohydroxylated derivatives of PAA (Table I). Of these *p*-hydroxy PAA has been identified as a natural-occurring compound (1). Although all derivatives exhibited equal initial potency, their effectiveness declined rapidly with time. After 48 h only the ortho derivative still exhibited biological activity. Interestingly, this same hierarchy of biological activity has been observed by others using different assay systems (4).

Both PAA and an acid-labile conjugate of PAA were identified in extracts of cotton cotyledons (Table II). The identity of this conjugated form was not determined; however it may be an amide-linked conjugate of PAA. *N*-Phenylacetylaspargate has been identified as a natural constituent of developing seeds of *Pisum* (5). Endogenous levels of free or bound PAA did not decline during ethylene induced abscission. In fact, over the course of numerous independent determinations, no consistent pattern of changes in PAA content with respect to ethylene-induced cotyledon abscission could be discerned. When expressed on a fresh weight basis, the levels of free PAA found in cotyledon extracts are similar to those found in other dicotyledonous species such as sunflower or tomato (15).

If PAA functions as an endogenous leaf-derived inhibitor of the abscission process, then it seems likely that in order for abscission to proceed, the levels of PAA reaching the abscission zone should decline prior to or during the course of leaf fall. This decline could be achieved by a reduction in the endogenous level of PAA within the cotyledon or via reduced transport of PAA to the abscission zone. The results discussed above suggest that a simple reduction in the titer of PAA does not occur during ethylene induced abscission.

Unlike IAA, the transport of PAA in isolated petiole segments is limited and exhibits little, if any, polarity (Table III). The observation that compounds such as NPA, known to interfere fairly specifically with the polar auxin transport system, can potentiate the action of subthreshold levels of ethylene in inducing abscission suggests that any leaf-derived factors involved in the deferral of abscission must be transported via this system (10). In other studies the inclusion of NPA in the receiver blocks resulted in total inhibition of basipetal IAA movement but had no effect on PAA transport (not shown). The limited basipetal movement of PAA in petioles is in accordance with the observation that direct application of PAA to the abscission zone of debladed cotton petioles completely suppressed abscission induced by leaf removal while application of PAA to the distal petiole stump had no effect (not shown). IAA effectively suppressed abscission in either case.

Taken as a whole, these results suggest that PAA is not functioning as part of the long-distance communication between the leaf blade and the abscission zone. These results do not discount the possibility that PAA functions within the leaf blade itself, perhaps by interacting with endogenous abscission inhibitors or accelerators, thereby playing a more discrete role in the endogenous regulation of leaf abscission. These possibilities may warrant future studies.

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