Inhibition of the Multiplication of Bromegrass Mosaic Virus in Barley by the Antibiotic Blasticidin S

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ABSTRACT

The effect of blasticidin S (BcS) on the multiplication of bromegrass mosaic virus (BMV) in barley was investigated. Virus synthesis was evaluated (i) by spectrophotometric measurement of the virus content of the leaves; (ii) by measuring the virus-induced RNA polymerase activity (VPA) in cell-free leaf extracts, as assayed by incorporation of tritiated uridine-5′-triphosphate (UTP-^{3}H) in the presence of actinomycin D. The action of BcS on virus-induced pathological effects was also investigated.

Virus synthesis was inhibited in leaf 1, or delayed in leaf 2, when BcS was applied to one-leaf seedlings at 3 hr after inoculation with BMV; this was correlated with a similar inhibition or delay of VPA as measured in leaf extracts prepared from BcS-treated infected plants, together with a reduction of pathogenic effect. BcS added in vitro to the UTP-^{3}H-incorporating system had no effect. It is concluded (i) that BcS acts at an early phase of infection, presumably at the level of the virus-induced RNA polymerase system; and (ii) that VPA, as measured in vitro, reflects a functional step which is a prerequisite for the in vivo synthesis of BMV. Phytopathology 61:10-14.

RESUME

On a étudié l'effet de la blasticidine S (BcS) sur la multiplication du virus de la mosaïque du brome (BMV) dans les plantules d'orge. La synthèse virale a été estimée (i) par la mesure spectrophotométrique du contenu en virus des feuilles; (ii) par la mesure de l'activité de l'ARN polymérase induite par l'infection (VPA), en utilisant comme critère l'incorporation d'uridine-5′-triphosphate tritiée (UTP-^{3}H) en présence d'actinomycine D dans des extraits acellulaires de feuilles infectées. On a mesuré également l'action de la BcS sur les effets pathologiques induits par l'infection.

L'application de BcS sur les plantules d'orge 3 heures après l'inoculation par le BMV inhibe ou retarde la synthèse virale in vivo, et diminue la VPA des extraits folaires de plantes traitées. Le traitement à la BcS réduit également l'effet pathogène dû à l'infection. L'addition de BcS in vitro ne modifie pas l'activité du système d'incorporation de l'UTP-^{3}H. On conclut (i) que la BcS agit à un stade précoce de la multiplication virale, probablement au niveau du système d'ARN polymérase induit par l'infection; et (ii) que la VPA, mesurée in vitro, correspond à une étape fonctionnelle qui précède la synthèse du BMV in vivo. Phytopathology 61:10-14.

The antibiotic blasticidin S (BcS) was reported to reduce the infection of rice by rice stripe virus and to inhibit the ability of leafhoppers to transmit the virus (2). BcS also interferes with the multiplication of tobacco mosaic virus (1, 3, 4).

We have developed a technique of measuring the effect of potential inhibitors of multiplication of bromegrass mosaic virus (BMV) in barley, and have used it to study the effects of BcS.

MATERIALS AND METHODS.—Herta barley seedlings were grown in a greenhouse in 4.5-inch pots, and were inoculated at the one-leaf stage 7 days after sowing. Inoculation was made with the juice of BMV-infected barley containing about 0.5 mg of virus/ml of extract. Plants were sprayed with 25 ml of solution, using a Jet-Pak spray gun (Sprayon Products Inc., Cleveland, Ohio) operating at a distance of 1.0 m, with the pots rotating at 7 rpm. The spraying solution contained 1% glycerol, 0.1% Tween 60 (polyoxyethylene sorbitan monostearate), and BcS in water. Control pots were sprayed with 1% glycerol and 0.1% Tween 60 in water. In some experiments, the solution of BcS was painted on the upper surface of the leaves using a soft hair brush. After treatment, the inoculated plants were transferred to a growth chamber with constant light and temp (22-24 C). Estimation of BMV concn in the inoculated first leaf or in the systemically infected second leaf was performed according to Proll (5).

Leaf tissue (4-5 g for leaf 1 and 3-4 g for leaf 2) was homogenized in 0.1 M phosphate buffer at pH 5.0. One volume of leaf extract was shaken for 10 min with 0.5 volume of chloroform; after 15 min of centrifugation at 3,000 *g.*, the water phase was recovered and brought to 30% saturation with ammonium sulfate. The precipitate was discarded and the supernatant adjusted to 60% salt saturation. The insoluble material was pelleted, resuspended in phosphate buffer, and clarified by centrifugation for 5 min at 2,000 *g*. After appropriate dilution, the OD of the supernatant was read at 260 nm and 290 nm, and the virus content was calculated on the basis that one OD unit of difference between the readings at 260 and 290 nm, respectively, corresponds to a virus concn of 0.24 mg/ml with a 1-cm light path (5). Values for healthy leaves corresponded to a max overestima-

tion of 2 μg virus/leaf, and a mean overestimation of 1.2 μg virus/leaf. Due to its small size, this factor was not considered in the data. The in vitro activity of the virus-induced RNA polymerase was estimated by measuring the incorporation of UTP-^{3}H into RNA by a leaf fraction sedimenting between 1,000 *g* and 10,000 *g* (6, 7, 8).

RESULTS.—*Effects of BcS applied at different concn and with two techniques.*—Figure 1 shows the effect of painting the leaves with increasing concn of BcS at 2-3 hr after inoculation, on the virus content estimated at 6 days after inoculation. Inhibition of virus content increased rapidly with increasing BcS concn up to 2 μg/ml, and then increased at a slower rate for higher BcS concn. When the spraying technique was used instead, inhibition of virus multiplication in leaf 1 by treatment with 5 μg/ml of BcS was somewhat lower than with the painting technique (53% and 66% inhibition, respectively); both techniques gave similar results when BcS was used at 10 μg/ml (75% and 76% inhibition, respectively, for the painting and the spraying technique). A similar pattern was observed with leaf 2. In all subsequent experiments, BcS was applied as a spray at the concn of 10 μg/ml.

Treatment with BcS before or after inoculation.—BcS was sprayed at intervals before or after inoculation with BMV. Virus concn in leaf 1 and leaf 2, respectively, were estimated 4 days after inoculation. Treatment performed at 3 hr before or after inoculation resulted in a marked inhibition of the virus content of leaf 1 or leaf 2. Spraying with BcS at 24 hr after inoculation was still noticeably inhibitory. When BcS treatment was delayed for 32 hr after inoculation, inhibition was still pronounced in leaf 1, but was no more detectable in leaf 2; delaying BcS treatment for 72 hr after inoculation had no more effect in either case (Fig. 4).

Effect of BcS on the multiplication of BMV in leaf 1 or 2 of barley.—BcS was sprayed on plants 2-3 hr after inoculation with BMV, and the virus content of the inoculated leaf 1 and of the systemically infected leaf 2 were estimated at intervals. BcS reduced the virus content of leaf 1 throughout the period of rapid virus multiplication (Fig. 2-A), whereas the increase of BMV in leaf 2 was only delayed for 1-2 days by BcS treatment of leaf 1, the virus content of leaf 2 eventually reaching higher values in the BcS-treated plants than in the untreated controls (Fig. 2-B); this stimulation was also observed when virus content was expressed as mg per wt of fresh tissue.

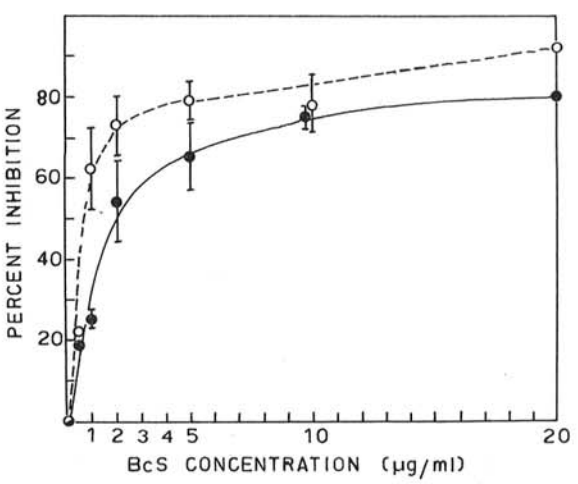

Fig. 1. Effect of blasticidin S at different concn on inhibition of virus content at barley leaves 6 days after inoculation with bromegrass mosaic virus (●——● = leaf 1; ○------○ = leaf 2). Mean values (2-5 replicates) with corresponding deviation.

Infection with BMV did not modify the length of leaf 1, whether treated with BcS or not, whereas, either virus infection or BcS application induced a slight decrease of the fresh wt of the first leaf. A more complicated pattern of effects was obtained with leaf 2 (Fig. 2-C, D). Growth was inhibited by infection, both in terms of length or wt of fresh tissue. This virus-induced inhibition was partly counteracted by BcS treatment. BcS, however, did not affect significantly the growth of leaf 2 when sprayed on healthy plants. BMV-induced symptoms were also reduced (leaf 1) or delayed (leaf 2) in plants treated with BcS.

Effect of BcS on the virus-induced RNA polymerase activity.—A virus-induced RNA polymerase activity (VPA), resistant to actinomycin D, was characterized previously in cell-free extracts of barley leaves infected with BMV (6, 7, 8). This VPA was not observed in extracts of healthy leaves. The effect of BcS on VPA was estimated in two ways. In a first set of experiments, we measured the in vitro effect of the addition of BcS to the incubation mixture, on incorporation of UTP-^{3}H. The results (Table 1) indicate that addition of BcS to the RNA-synthesizing system did not significantly affect the virus-specific, actinomycin-resistant incorporation of UTP into RNA. In a second series of experiments, VPA was measured in cell-free extracts prepared from leaves of BMV-infected barley, either untreated (control) or sprayed with BcS at 3 hr after inoculation. Results are presented in Table 2 and Fig. 4. Data of Fig. 3 are not comparable with those of Table 2 because of differences in greenhouse conditions and in preparation of the leaf homogenate. Data of Table 2 establish that the BcS-induced inhibition of BMV content of the leaves at 4 days after inoculation was associated with a lower VPA in the extracts of infected plants previously treated with the antibiotic; however, the activity of the actinomycin-sensitive cellular RNA polymerase of infected plants, as estimated by subtracting the radioactivity incorporation in the presence of actinomycin from the incorporation in controls without actinomycin, was not reduced by BcS treatment. The in vitro synthesis of cellular RNA is markedly reduced by infection, when assayed in extracts of leaf 2 at 3-6 days after inoculation (8); this reduction was not observed in extracts of leaf 2 of BcS-treated plants (Table 2) at 4 days after inoculation, the level thus obtained being comparable to that of healthy extracts.

The kinetics of the effect of BcS application on

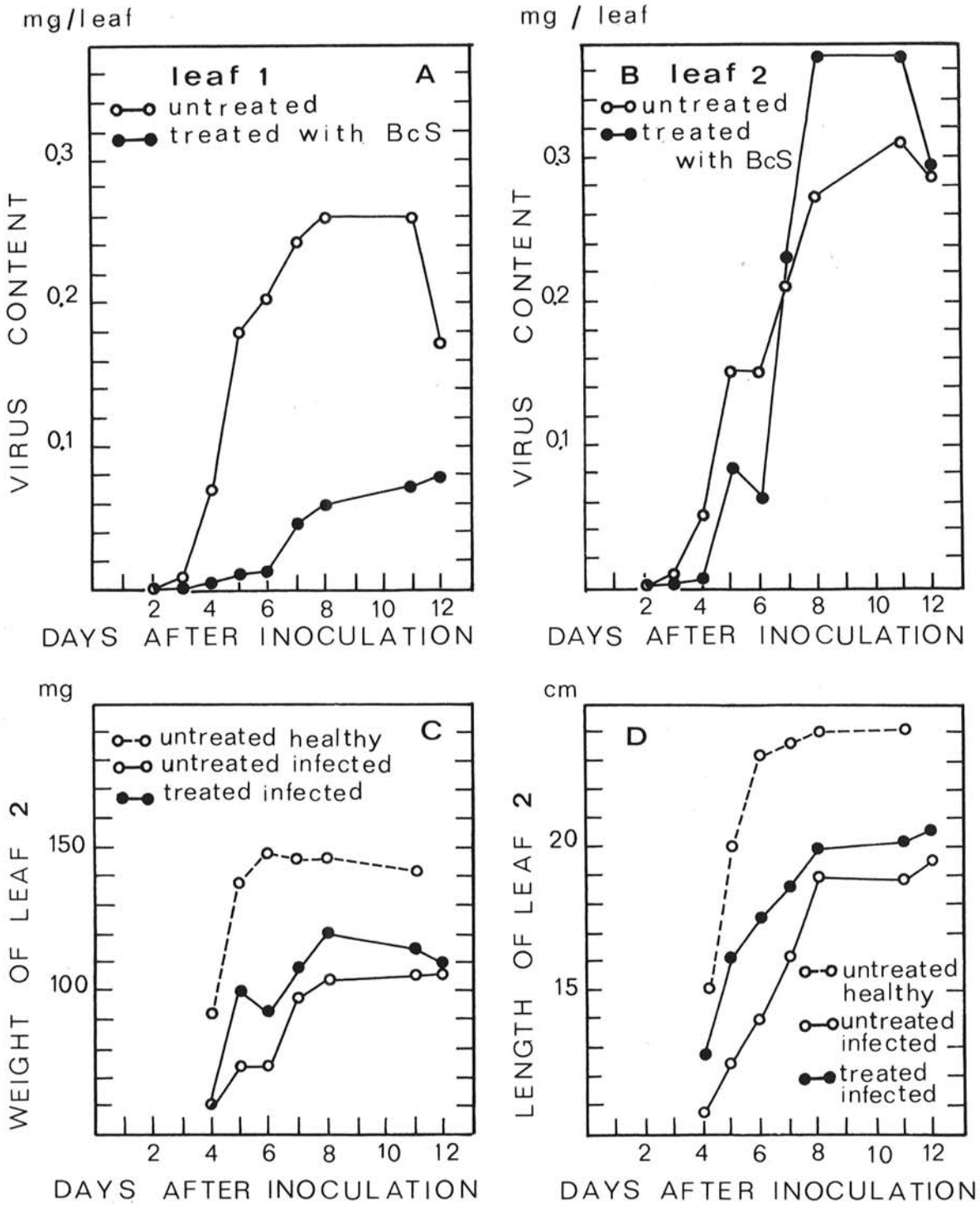

Fig. 2. Effect of blasticidin S (10 μg/ml) on virus content in barley leaves, when applied to seedlings at 2-3 hr after inoculation with bromegrass mosaic virus. Results are expressed as follows: **A, B)** virus content (mg per leaf) of leaf 1 and 2, respectively; **C)** fresh wt of leaf 2; **D)** length of leaf 2. (○———○ = infected controls; ●———● = infected seedlings sprayed with blasticidin S; ○------○ = healthy controls).

TABLE 1. Effect of blasticidin S on the incorporation of UTP-^{3}H into RNA by a cell-free extract from barley leaves infected with bromegrass mosaic virus[a]

Experiment	Days after inoculation	Concentration of blasticidin S (μg/ml)						
		0	1	10	30	100	300	1,000
1[b]	4	765 920	781 783	776 812		799 917		876
2[b]	6	288 347	284 312	253 258		275 296		287 300
3[c]	4	2,644 2,846	2,472	3,183	3,014	2,712	2,731	3,159
4[c]	4	2,192 2,392	2,234	2,947	3,123	2,989	2,905	3,567

[a] Incorporation of UTP-^{3}H (uridine-5′-triphosphate) into RNA was performed in the presence of actinomycin D (20 μg/ml) as previously described (8). Results of single or duplicate samples are expressed as acid-insoluble counts per min per assay.

[b] Leaf tissue was homogenized for 30 sec at top speed in an homogenizer (Measuring and Scientific Equipment Ltd, London, England).

[c] Leaf tissue homogenized with pestle and mortar.

VPA in leaf 2 were studied during the course of the large experiment reported in Fig. 2, using comparable plant material. The results appear in Fig. 3. In agreement with previous data (8), an early VPA peak preceded the rapid synthesis of BMV in leaf 2. Treatment of leaf 1 with BcS at 3 hr after inoculation delayed the VPA peak in leaf 2, in close correlation with the delay in virus increase shown in Fig. 2-B.

The virus-induced inhibition of the actinomycin-sensitive synthesis of cellular RNA was first observed at 5 days after inoculation in leaf 2 of BcS-treated plants, as compared to 3 days for untreated controls (8), thus showing a delay of 2 days in the induction of a virus-induced pathogenic effect in leaf 2 of BMV-infected plants sprayed with BcS.

DISCUSSION.—BcS (10 μg/ml) is a powerful inhibitor of BMV multiplication in leaf 1 of barley when it is applied to one-leaf seedlings close to the time of inoculation. Concentrations of the antibiotic which are effective against the multiplication of BMV in barley are higher than those reported for tobacco mosaic virus in floated discs of tobacco; this may result from discrepancies in the technique used. We found, however, that painting tobacco leaves with BcS at 2 μg/ml resulted in severe phytotoxicity, whereas, similar treatment of barley leaves with BcS at 10 μg/ml induced only a limited yellowing of areas covered with large drops of the solution.

Characteristics of the BcS-induced inhibition of BMV in barley are comparable to those described for

TABLE 2. Effect of spraying bromegrass mosaic virus-infected barley seedlings with blasticidin S (BcS), on the RNA polymerase activity in cell-free leaf extracts[a]

Material used for preparation of cell-free extracts	Actinomycin[b] (20 μg/ml)	Unsprayed controls		Seedlings sprayed with BcS	
		Duplicate samples	Mean	Duplicate samples	Mean
First directly inoculated leaf	—	3,081 3,108	3,095	1,801 2,202	2,002
	+	1,584 1,672	1,628	361 565	463
	Actinomycin-sensitive incorporation[c]		1,467		1,539
Second systemically infected leaf	—	2,872 3,420	3,146	1,537 1,551	1,544
	+	2,441 2,602	2,522	304 353	329
	Actinomycin-sensitive incorporation[c]		624		1,215

[a] Barley seedlings were inoculated at the one-leaf stage with bromegrass mosaic virus and sprayed with BcS (10 μg/ml) 2-3 hr after inoculation. Leaf extracts were prepared with pestle and mortar 4 days after inoculation; incorporation of UTP-^{3}H (uridine-5′-triphosphate) into RNA was performed as described previously (8). Results are expressed as acid-insoluble counts per min per assay.

[b] Actinomycin D was added to the UTP-incorporating system as indicated. The actinomycin-resistant incorporation of UTP-^{3}H represents roughly the activity of the virus-induced RNA polymerase.

[c] Actinomycin-sensitive incorporation was obtained by subtracting the actinomycin-resistant from the total incorporation. This value corresponds roughly to the incorporation into the DNA-dependent cellular RNA.

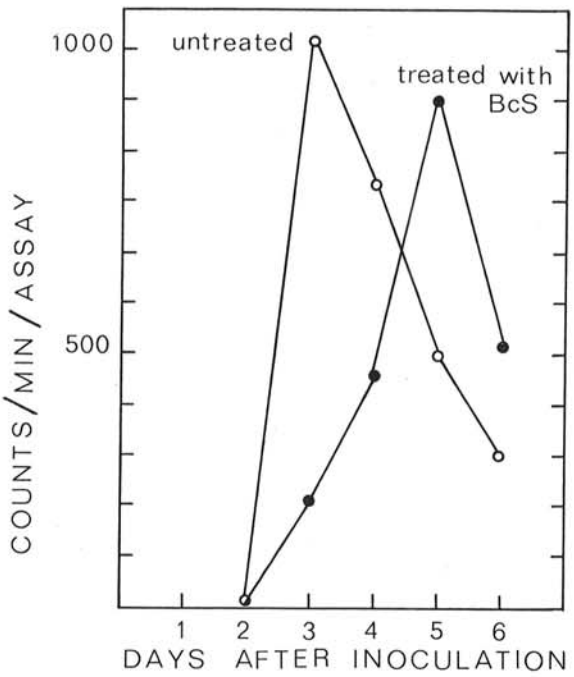

Fig. 3. Effect of blasticidin S (10 μg/ml) on the activity of the virus-induced RNA polymerase measured in extracts of leaf 2 of barley at intervals after inoculation of one-leaf seedlings with bromegrass mosaic virus. The virus-induced RNA polymerase activity is expressed as acid-insoluble counts per min per assay incorporated after 20-min incubation with uridine-5′-triphosphate-^{3}H in the presence of 20 μg/ml of actinomycin D: ●——● = seedlings sprayed with blasticidin S at 3 hr after inoculation; ○——○ = unsprayed controls.

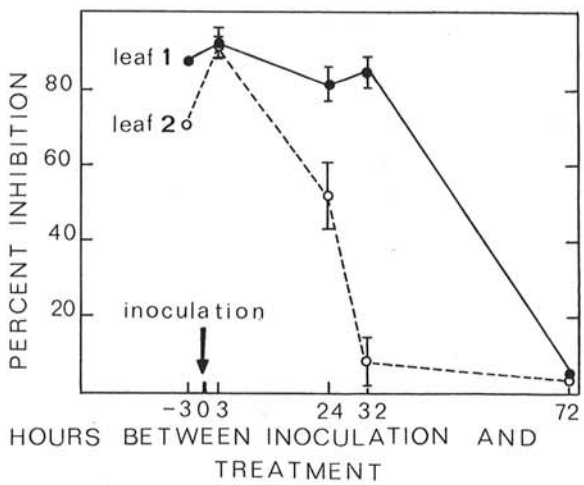

Fig. 4. Effect of blasticidin S (10 μg/ml) on virus content in barley leaves 4 days after inoculation with bromegrass mosaic virus, when applied on seedlings at different times before or after inoculation. Mean values (2-5 replicates) with corresponding deviation.

tobacco mosaic virus in tobacco, and the discussion presented for the latter system (4) does apply generally to our results with BMV. Inhibition of BMV multiplication in leaf 1 of barley was max when BcS treatment was applied close to virus inoculation. As no inhibition was observed when treatment was delayed for 72 hr (Fig. 4), it is probable that the inhibitory effect is related to an early phase of virus multiplication. Differences in penetration of BcS into the leaves at different times cannot be ruled out, however, and this aspect should be studied further. Since the lower virus content of leaf 1 treated with BcS was associated with a lower virus-induced RNA polymerase activity, as measured in vitro (Table 2), it is suggested that the latter enzyme system may be involved in the inhibitory process, as proposed also for tobacco mosaic virus (4).

The delay in virus synthesis in the systemically infected second leaf of plants whose first leaf was sprayed with BcS may be indirect, resulting from the slower movement of the infectious agent in the inoculated first leaf. The delayed virus synthesis was correlated with a retarded pathogenic effect as estimated by the following criteria: reduction of growth; development of the mosaic pattern; and inhibition of the in vitro actinomycin-sensitive synthesis of cellular RNA. The fact that the BcS-induced retarded multiplication of BMV was correlated with a similar delay in VPA development gives further support to the idea that VPA, as measured in vitro, reflects indeed a functional step which is a prerequisite for the in vivo multiplication of BMV (8).

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