PARTICLE STRUCTURE AND DOUBLE-STRANDED RNA OF RICE RAGGED STUNT VIRUS

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Introduction

The mode of insect transmission, the morphology and RNA composition of particles of rice ragged stunt virus (RRSV) suggest that it belongs to the Reoviridae[1,5,16,20,22]. However, the number and size of the genome segments are not certain, and the taxonomic position of RRSV is therefore not clear[12].

BOCCARDO and MILNE[1] reported that double-stranded RNA extracted from the enations on rice plants infected with RRSV could be separated into 8 bands by electrophoresis on 5% polyacrylamide gel and suggested that each band contained a single species of RNA. MILNE[14] described the morphology of RRSV particles obtained from the crude extract or partially purified preparations, and showed that they do not have a double-shell structure but are particles about 63 nm in diameter with broad-based spikes. Furthermore, he indicated that such a single shell structure was consistent with the number of RNA genome segments being less than those of plant reovirus subgroups 1 and 2[1,16].

So far, there have been no detailed reports on the morphology and RNA composition of RRSV particles in purifed virus preparations. In this paper, we compare the fine structure of purified RRSV particles with that of particles of rice black-streaked dwarf virus (RBSDV) which belongs to plant reovirus subgroup 2 (*Fijivirus*), and compare the RNA composition of particles from purified preparations of these viruses with that of rice dwarf virus (RDV) particles.

Materials and Methods

Viruses and virus purifications:

RRSV*; RRSV-infected rice plants were originally supplied by the late K. C. Ling, International Rice Research Institute, Philippines, and the virus was maintained in rice plants grown in a greenhouse which were inoculated using viruliferous brown planthoppers, *Nilaparvata lugens*. About 2 months after inoculation, the virus was purified according to KAWANO *et al.*[8]; Fresh leaf and sheath tissues were pulverized with a meat chopper in 2-3 vol (w/v) of 0.1 M phosphate buffer (P. B.), pH 6.0, the debris was homogenized with 1/3 vol (v/v) carbon tetrachloride and centrifuged at 3,000 rpm for 10 min. Solid 6% polyethylene glycol mol. wt. 6,000, 0.3 M NaCl and 3% Triton X-100 were added to the aqueous phase while stirring with a magnetic stirrer and centrifuged at 5,000 rpm for 20 min. The pellet was suspended in 10 mM P. B., pH 7.0, containing 5 mM ethylendiamintetraacetate (EDTA). The suspension was homogenized with 1/3 vol (v/v) fluorocarbon (Daifron solvent S3, Daikin Kogyo, Osaka) at 3,000 rpm for 5 min. The clarified aqueous phase was concentrated by one cycle of differential centrifugation at 8,000 rpm for 10 min and 22,000 rpm for 1 hour in a Hitach RP-30 rotor. The final pellet was resuspended in 10 mM P. B., pH 7.0, containing 5 mM EDTA, and centrifuged twice in 20-50% sucrose density gradient at 22,000 rpm for 2.5 hours in a Hitach RPS-25 rotor to remove virus like 35 nm diameter particles[8]. The virus was pelleted by cenrtifugation at 36,000 rpm for 1.5 hour in a Hitach RP-40 rotor and stored at −70°C.

RBSDV; RBSDV was maintained in rice plants and propagated in maize plants, which were inoculated using viruliferous planthoppers, *Laodelphax striatellus*. The plants were harvested about 2 months after inoculation. When RBSDV was purified from frozen material as descirbed by SHIKATA and KITAGAWA[21], most particles in the purified preparations were B-spiked subviral particles[15,20]. Therefore, intact RBSDV particles were partially purified from fresh maize plants by the modified procedures described by MILNE *et al.*,[15] using an additional treatment with 7% Triton X-100 for 30 min at 6°C after the fluorocarbon treatment.

RDV; RDV was maintained in rice plants inoculated using leafhopper, *Nephotettix cincticeps*. The rice plants were harvested about 1.5 month after inoculation and stored at −30°C. The virus was purified from frozen leaf tissues as described by UYEDA and SHIKATA[23].

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Electron microscopy:

The purified virus preparations were trapped on a carbonformvar coated grids, which were treated by glow discharge prior to use. The grids were washed with several grops of distilled water and negatively stained by 2% uranyl acetate or 2% potassium phosphotungstic acid at pH 5.0 and 7.0 and drained with a filter paper. Some virus preparations were fixed with 1% glutaraldehyde for 15 min before staining. The preparations were examined in a JEM-100B electron microscope and virus particles were measured using particles of tobacco mosaic virus, common strain (300 nm long), for calibration.

Extraction of viral RNA:

Double-stranded RNA was extracted as described by McCRAE and JOKLIK[13]. Purified virus was suspended in 0.1 M NaCl, 10 mM Tris-HCl, 5 mM EDTA, pH 7.4, containing 0.1% SDS, incubated at 37°C for 1 hour, and shaken with 1.5 vol of a mixture of chloroform and water-saturated phenol (1 : 10 v/v). Following centrifugation the aqueous phase was extracted twice with diethyl ether and RNA was precipitated by adding 2.5 vol of ethanol. The concentration of double-stranded RNA was determined spectrophotometrically using $E_{260}^{0.1\%}=16.7$[7].

Polyacrylamide gel electrophoresis:

Gels were 5, 7.5, 10 and 15% polyacrylamide and bisacrylamde (30 : 0.8 w/w, respectively) in slab (1.5 mm thick) form. The buffer used was L buffer (40 mM Tris, 20 mM sodium acetate and 2 mM EDTA, pH 7.4)[10], and was recirculated with a peristaltic pump while electrophoresis. From 4 to 2 μg of RNA samples were applied in 1/10-1/50 (v/v) diluted L buffer containing glycerin. Electrophoresis was at 16 mA for 43 hours at 6°C or at 20 mA for 60 hours at room temperature (~16°C). After electrophoresis, gels were fixed with 1 M acetic acid for 15 min and stained with 0.01% toluidine blue 0 in distilled water for 30 min, destained in distilled water. Molecular weights of RRSV RNA species were estimated by comparing their mobillities with those of RDV RNA species assuming the molecular weight values given by FUJII-KAWATA *et al.*[3].

Results

i) *Electron microscopy of RBSDV particles*: Purified preparations of RBSDV contained two types of particles. Most were intact particles about 70 nm in diameter, and some were smaller B-spiked subviral particles about 51-55 nm in diameter[20] (Fig. 1, A). The intact particles of RBSDV had

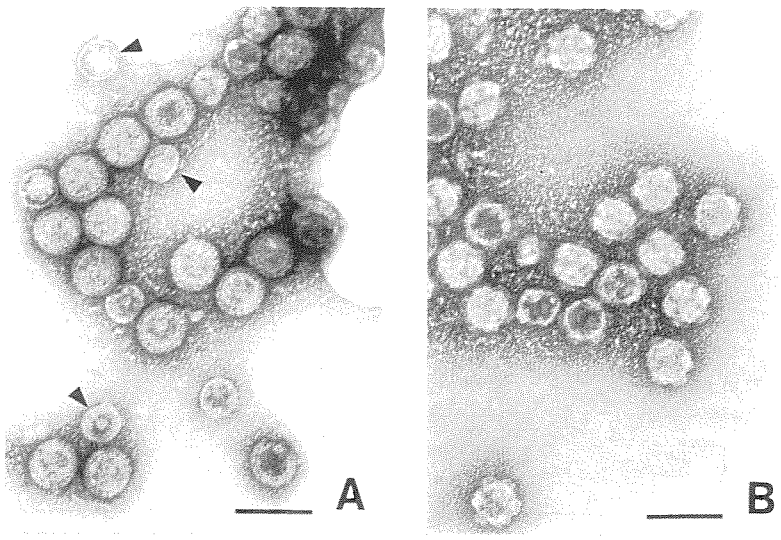

Fig. 1. Purified RBSDV (A) and RRSV (B) stained with 2% uranyl acetate. Arrows indicate B-spiked subviral particles. Bars represent 100 nm.

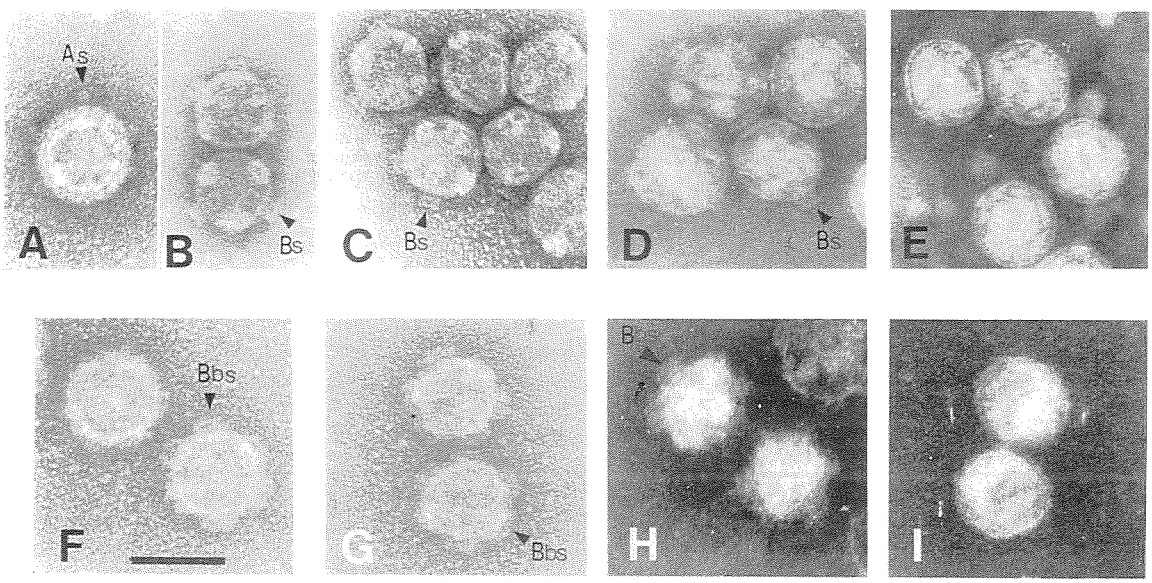

Fig. 2. Negatively stained RBSDV (A, B, C, D, E) and (F, G, H, I) particles in purified preparations. A, B and F: were stained with 2% uranyl acetate. C and G: fixed with 1% glutaraldehyde and stained with 2% uranyl acetate. D and H: same as C and G, but stained with 2% potassium phosphotungstic acid, pH 5.0. E and I: stained with 2% potassium phosphotungstic acid, pH 7.0. As: A-spike. Bs: B-spike Bbs: broad-based spike. Bar represents 50 nm.

A-spike, 10–11 nm wide and 10–11 nm long. B-spikes were 12–13 wide and 7–8 nm long (Fig. 2, A and B; Table 1).

TABLE 1. Dimensions of structures in RRSV and RBSDV particles with 2% uranyl acetate

Structure	Dimension (nm)	RRSV	RBSDV
Intact particles	Diameter	57–65	70–71
Spikes	Width	14–17 (at top)	10–11
or		23–26 (at base)	
A-spikes	Height	8–10	10–11
Core particles	Diameter		51–55
B-spikes	Width		12–13
	Height		7– 8

ii) *Electron microscopy of RRSV particles*: Purified preparations of RRSV contained only spiked particles about 63 nm in diameter (Fig. 1, B). RRSV particles stained with 2% uranyl acetate had "broad-based" 8–10 nm long spikes, apparently located on 12 vertices of the icosahedral particles, which were 14–17 nm wide at the top and 23–26 nm wide at the base (Fig. 2, F; Table 1). When the electron micrographs shown in Fig. 3, A-1, B-1, C-1, were subjected rotational superimposition[11] at n=2, 3, and 5 times (n represents the number of positions per 360° rotation), each image clearly showed fine structure. The rotated figures in Fig. 3, A-2, B-2 and C-2 show virus particles viewed down 2-fold, 3-fold and 5-fold symmetry axes, respectively. The rotated particles was 63 nm in diameter. The spikes were 10 nm long, 16 nm wide at the top and 24 nm wide at the base. As shown in Fig. 3, C-2, the rotated image of the spike is pentagonal in shape, with 16 nm sides. Views of model corresponding to the rotated images are shown in Fig. 3, A-3, B-3 and C-3.

iii) *Effect of phosphotungstate stain on particle morphology*: When purified RRSV and RBSDV particles were stained with phosphotungstate (PTA), the surface structure of the particles appeared smooth, and without spikes (Fig. 2, E and I). The diameters of smooth RRSV and RBSDV particles were about 60 nm. When the RRSV particles and B-spiked subviral particles were fixed with 1% glutaraldehyde, and then stained with PTA or uranyl acetate, the spikes well preserved, although those on RRSV particles stained with RTA were less obvious than the spikes on particles stained with uranyl acetate (Fig. 2, C, D, G and H).

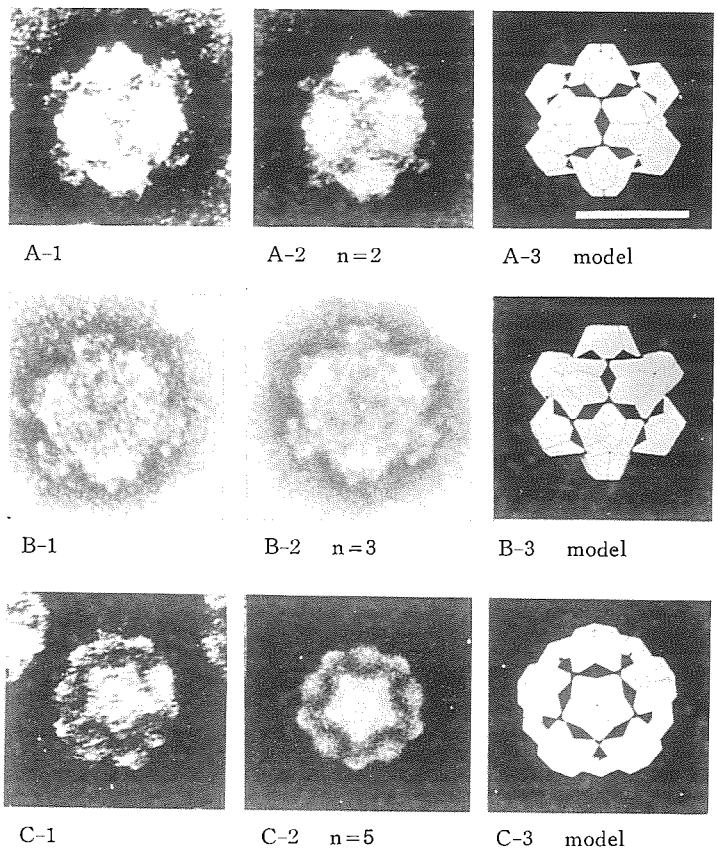

Fig. 3. Fine structure of RRSV particles. A-1, B-1 and C-1: electron micrographs of virions arranged at 2, 3 and 5-fold symmetry axes, respectively. A-2, B-2 and C-2: images of the same particles rotated by increments of 180°, 120° and 72°, respectively. A-3, B-3 and C-3: indicate a possible model viewed down 2, 3 and 5-fold symmetry axes. Bar represents 50 nm.

iv) *Polyacrylamide gel electrophoresis of RRSV RNA*: In the preliminary experiments, we confirmed that the RNA preparations obtained mostly contained double-stranded RNA, because no hyperchromic shift was observed by formaldehyde reaction and no digestion occurred by either RNase in 0.15 M NaCl, 15 mM Na citrate, pH 7.2 or DNase in 50 mM Tris-HCl, pH 7.6, containing 0.15 M NaCl and 5 mM $MgCl_2$ (data not shown). In L buffer, RRSV RNA was separated into 10 bands in 7.5% gel and 9 bands using 5, 10 and 15% gels. Whereas, 12 bands of RDV RNA was obtained in 10% gel and 11 bands in 5, 7.5 and 15% gels. RBSDV RNA was run only

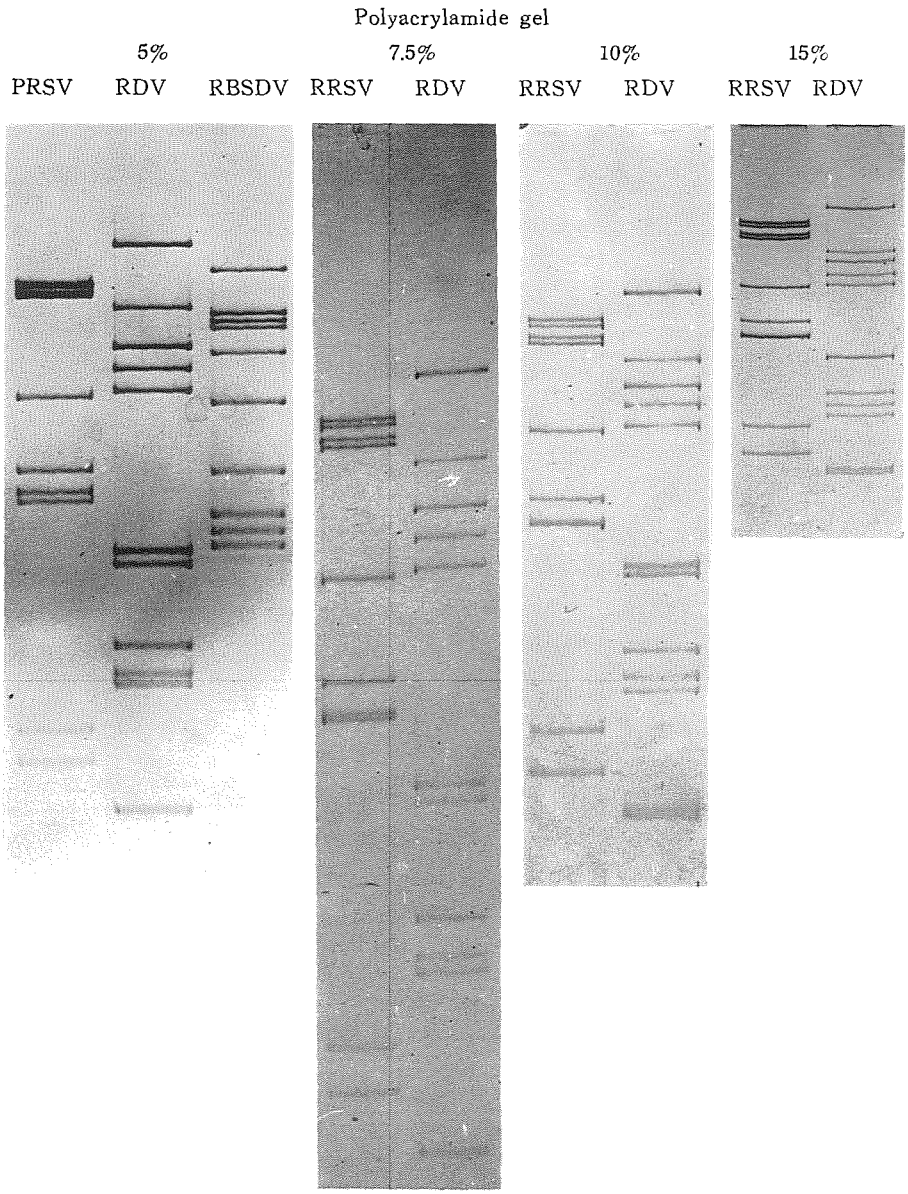

Fig. 4. Electrophoresis of RNA from RRSV, RDV and RBSDV in 5, 7.5, 10 and 15% polyacrylamide slab gel. Electrophoresis was at 16 mA for 43 hours at 6°C in a 5% polyacrylamide gel or at 20 mA for 60 hours at room temprature (~16°C) in 7.5, 10 and 15% polyacrylamide gel, stained in 0.01% toluidide blue 0 and destained in distilled water.

TABLE 2. Estimates of the molecular weights of the genome segments of RRSV, RDV and RBSDV

Segments No.	Polyacrylamide gel									RDV[c] Marker
	5%[a]			7.5%[b]		10%[b]		15%[b]		
	RRSV	RDV	RBSDV	RRSV	RDV	RRSV	RDV	RRSV	RDV	
1	2.45[d,e]	2.85	2.57	2.49	2.75	2.56	2.81	2.70	2.97	2.80
2		2.30	2.24	2.45	2.24	2.51	2.21	2.63	2.16	2.35
3	2.38	2.00	2.19	2.37	2.01	2.41	2.00	2.50	2.02	2.05
4	2.37	1.89	2.14	2.32	1.88	2.34	1.88	2.43	1.84	1.75
5	1.73	1.74	2.00	1.71	1.78	1.72	1.74	1.70	1.71	1.72
6	1.36	1.04	1.82	1.34	1.05	1.35	1.05	1.32	1.01[e]	1.03[e]
7	1.26	1.00	1.37	1.25	1.01	1.23[e]	1.02	1.19[e]		
8	1.22	0.77	1.17	1.23	0.77		0.78		0.78	0.77
9	0.59	0.71	1.11	0.57	0.70	0.59	0.72	0.63	0.72	0.71[e]
10	0.53	0.68	1.06	0.51	0.68	0.51	0.68	0.51	0.67	
11		0.45[e]			0.44[e]		0.45		0.46	0.44[e]
12							0.44		0.44	
Total Mol. wt.	16.3	15.9	17.7	16.2	15.8	16.5	15.8	16.8	15.8	15.8

a) Electrophoresis was at 16 mA for 43 hours a 6°C.
b) Electrophoresis was at 20 mA for 60 hours at room temprature (~16°C).
c) The mol. wt. were estimated using to the values for RDV RNA segments given by FUJII-KAWA *et al.*[3]
d) $\times 10^6$ daltons.
e) RNA species were not resolved.

in 5% gel obtained 10 bands (Fig. 4). Caluculated molecular weight of each RNA, using RDV RNA as marker, differed slightly depending on gel conditions. However, molecular weight of RRSV RNA species ranged between 0.5 and 2.7×10^6, totalling $16\text{–}17\times10^6$. RDV RNA species were mostly between 0.4 and 3.0×10^6, totalling 16×10^6 and those of RBSDV were between 1.1 and 2.6×10^6, totalling 17.7×10^6 (Table 2).

Discussion

Our observations of the structure of the RRSV particle and its spikes are very similar to those described by MILNE[14]. However, the spikes differed in size and shape from those on subviral particles of RBSDV. The rotated images viewed along 2-fold, 3-fold or 5-fold symmetry axes suggested a particle structure having broad-based spikes, one on each vertex of the icosahedron, and each spike seemed to be composed of 5 structural units.

BOCCARDO and MILNE[1] reported that double-stranded RNA extracted from RRSV-induced enations could be resolved into 8 bands. However, we have resolved RNA preparations from purified RRSV particles into 9 or 10 bands under the different gel conditions, and suggest that the number of genome segments of RRSV is 10. The 1st and 2nd slowest-migrating bands of BOCCARDO and MILNE[1] probably each separated into two bands in our 7.5, 10 and 15% gels. We also confirm that the number of genome segments of RBSDV and RDV are 10 and 12, respectively, as reported by REDDY *et al.*[17,18]. In addition, we showed that segments 11 and 12 of RDV not resolved previously[3,17] were clearly separated in 10 and 15% slab gels.

Because of its transmissibility by brown planthoppers, and the size and shape of its particles, RRSV was first consider to be a possible member of plant reovirus subgroup 2 (*Fijivirus*)[4,5,6,9,22]. However, our results indicate that the particle structure of RRSV differs from that of either *Phytoreovirus* or *Fijivirus* subgroups. Furthermore, as suggested by BOCCARDO and MILNE[1], the total molecular weight of RRSV RNA ($16\text{–}17\times10^6$) is smaller than that of *Fujivirus* subgroup ($18\text{–}20\times10^6$)[12].

From the results mentioned above, it is reasonable to asign RRSV to a new plant reovirus subgroup 3 as proposed by BOCCARDO and MILNE[2].

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Summary

When examined by electron microscopy after negative staining with 2% uranyl acetate, rice ragged stunt virus (RRSV) particles appeared as icosahedra, 57–65 nm in diameter, with a spike at each of the vertices; each spike was 8–10 long, 14–17 nm wide at the top, and 23–26 nm wide at the base.

Results of polyacrylamide gel electrophoresis resolved the double-stranded RNA of RRSV into 10 species. The molecular weight of the RNA species ranged from 0.5 to 2.7×10^6, a total molecular weight of about 16.5×10^6.

Thus the particle structure and RNA composition of RRSV differ from those of viruses which belong to either plant reovirus subgroup 1 (*Phytoreovirus*) or subgroup 2 (*Fijivirus*).

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