Properties of the Infectious Forms of Exocortis Virus of Citrus

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ABSTRACT

Citrus exocortis virus (CEV) can be most efficiently extracted by procedures designed to maintain the integrity of free nucleic acids. The virus is not susceptible to inactivation by DNase. Rate sedimentation experiments suggest the presence of two infectious entities, the major species with a 10-15 S and the minor form with a > 25 S. CEV eluted from methylated albumin-coated Kieselguhr columns in the region of DNA and from CF-11 cellulose in 0% ethanol as is characteristic of double-stranded RNA species. However, attempts to melt the suspected double-stranded structure as well as to observe any resistance to RNase inactivation in a high ionic strength medium have been unsuccessful. Data implicating a possible nucleoprotein-complex constituting the minor > 25 S species are also discussed. Phytopathology 60:732-736.

Exocortis virus of citrus (CEV) can be transmitted by budding or by contaminated tools, but not readily by sap inoculation (8). More recently, extension of the host range of CEV outside the family Rutaceae by sap inoculation resulted in typical symptoms of leaf epinasty and stunting of plants in a number of different families (15, 16). Partial purification of CEV has been accomplished by phenol or alkaline-high salt extraction (13, 14). The virus is susceptible to inactivation by RNase and displays an unusually low sedimentation rate, which would suggest either a low molecular-wt single-strand or double-stranded condition (14). No evidence of typical virus particles has been obtained in infectious preparations. These data are similar to those reported by Diener & Raymer (3, 4, 11) for the infectious agent of potato spindle tuber virus (PSTV), which are compatible with the suggestion that PSTV is a double-stranded free RNA virus.

This communication reports additional properties of exocortis virus, and discusses some of the possible forms in which the infectious agent may exist.

MATERIALS AND METHODS.—*Gynura aurantiaca* DC was utilized as the systemic host of CEV. Infectivity was determined by appearance of symptoms on gynura within a 30-day period after inoculation by razor-slashing the stems.

Partially purified preparations of CEV were prepared by ethanol concentration of chloroform-butanol clarified high-speed supernatants (HSS) or direct phenol extraction of infected tissue as previously described (14). After overnight dialysis of the ethanol pellet from phenol extraction against 0.01 M TKM buffer (0.01 M Tris [tris(hydroxymethyl)aminomethane], 0.01 M KCl, 10^{-4} M $MgCl_2$, pH 7.4), the preparation was centrifuged at 39,000 rpm in the Spinco No. 40 rotor for 1 hr. The supernatant was mixed with 2.5 volumes of 95% ethanol, and the precipitate collected by low-speed centrifugation. The final preparation was redissolved in TKM buffer and dialysed overnight at 4 C.

Fractionation of nucleic acid species by salt precipitation (1) was accomplished by adding 1 volume of 4 M LiCl to the final preparation and letting it stand overnight in an ice bath. After collecting the precipitate by centrifugation at 5,000 *g*, 2.5 volumes of 95% ethanol was added to the supernatant. Both the LiCl precipitate and ethanol-concentrated supernatant were resuspended in TKM buffer and dialysed overnight.

Rate sedimentation of nucleic acid preparations was accomplished by layering 0.4 ml of sample onto a 4.6 ml preformed 50-200 mg/ml linear sucrose gradient and centrifuging for 4-4.5 hr at 45,000 rpm in a precooled SW50L rotor at 1 C. The rotor was permitted to coast to a stop. After centrifugation, ultraviolet absorption patterns were obtained and samples collected with an ISCO density gradient fractionator and analyzer. Equilibrium sedimentation was performed by layering 0.1-0.2 ml of sample into 4 ml of CsCl or Cs_2SO_4 and centrifuging at 1 C in the SW50L rotor. Fractions were taken by needle puncturing the bottom of the tube and droplet collection. Samples were dialysed against TKM buffer prior to infectivity assay.

CEV preparations were fractionated on methylated albumin-coated Kieselguhr (MAK) by the method of Mandell & Hershey (9). Samples containing 2-4 mg of nucleic acid in 0.4 M NaCl, 0.05 M potassium phosphate buffer, pH 6.7, were eluted with a linear gradient formed by 300 ml each of 0.4 M and 1.2 M NaCl in phosphate buffer. Fractions were concentrated by ethanol precipitation and resuspended in TKM buffer.

Chromatography on cellulose (CF-11) was performed essentially as described by Franklin (7). Ultraviolet absorbing contaminants were removed from the cellulose by washing in 1 N KOH followed by 1 N HCl and soaking in 35% ethanol in STE buffer (0.1 M NaCl, 0.001 M EDTA, and 0.05 M Tris-HCl, pH 7.2). Samples containing 0.1-10 mg of nucleic acid were eluted from 2 × 15 cm columns stepwise with 150-200 ml each of 35%, 15%, and 0% ethanol in STE buffer.

The data presented here comprise the results of representative experiments and not the total performed.

RESULTS.—Previous reports (13, 14) have demonstrated that the infectious entity of exocortis virus of citrus can be partially purified by utilizing either a phenol extract or ethanol concentrated high-speed supernatant after alkaline-high salt extraction and chloroform-butanol clarification. The virus is relatively stable to thermal inactivation either in sap or after extraction (Table 1). Although the sensitivity of CEV to RNase

TABLE 1. Thermal inactivation of citrus exocortis virus preparations

Temp[a]	Sap	Extracted by HSS[b]	Extracted by Phenol
C			
50	5/8[c]	7/8	
60	4	7	
70	4	5	
80	6	3	3/4
85			4
90	2	3	2
95			4
100		4	2
100[d]			3

[a] 10-Min exposure.
[b] HSS = ethanol concentrated high-speed supernatant.
[c] No. plants infected/no. plants inoculated.
[d] 20-Min exposure.

TABLE 2. Effect of DNase on the infectivity of preparations from citrus exocortis virus-infected tissue

Preparation	DNase[a]	Infectivity
HSS[b]		4/5[c]
HSS	100 μg/ml	5
HSS	400 μg/ml	3
Phenol extract		5/5
Phenol extract	100 μg/ml	5
Phenol extract	400 μg/ml	5

[a] Incubated for 1 hr at 37 C.
[b] HSS = ethanol concentrated high-speed supernatant.
[c] No. plants infected/no. plants inoculated.

has been demonstrated (14), the possibility existed that the infectious entity might exist as a RNA-DNA complex, thus explaining the low sedimentation rate. Exposure of a HSS preparation and a phenol extract to RNase-free DNase did not result in any significant loss of infectivity (Table 2). Further, the sedimentation rate of the infectious moiety was also not altered (Table 3, Exp. 6).

Typical ultraviolet scanning patterns of the HSS preparations, phenol extract, and tobacco rattle virus (TRV)-RNA centrifuged under identical conditions are presented in Fig. 1. After centrifugation in a 5-20% sucrose density gradient for 4-4.5 hr at 45,000 rpm in a Spinco SW50L rotor, absorbance peaks of s-RNA (0-1 ml), DNA (1-2 ml), light r-RNA (2-3 ml), and heavy r-RNA (3-4 ml) were indicated in the phenol-extracted preparations (Fig. 1, center). The two main absorbance peaks in the lower scanning pattern of Fig. 1 conform to the RNA from the short and long rods of TRV with molecular wt of about 0.75×10^6 and 2.25×10^6, respectively (10). TRV infectivity was always associated with the bottom 2 ml of the gradient.

The distribution of CEV infectivity after similar conditions of sedimentation is presented in Table 3. When 0.5-1.0 ml samples were taken from 5 ml density-gradient columns containing either HSS or phenol-extracted preparations, the greatest CEV infectivity was localized in the top 2 ml (Table 3, experiments 1, 2). In addition, a suggestion of a second region of

TABLE 3. Distribution of citrus exocortis virus infectivity after rate sedimentation in 5-20% sucrose density-gradients for 4 hr at 45,000 rpm in the Spinco SW50L rotor at 1 C

Experiment	Preparation	Treatment	Infectivity distribution[a] →									
			0.5-ml samples									
			1	2	3	4	5	6	7	8	9	10
1	Phenol extract		2/4[b]	3	4	2	1	2	1	0	0	3
	Phenol extract		3/3	3	3	2	2	1	3	1	1	2
2	HSS		3/4	2	4	1	2	0	0	1	2	0
	HSS		1/3	2	1	3	2	1	1	0	2	2
			1.0-ml samples									
			1		2		3		4		5	
3	Phenol extract		2/5		5		3		4		2	
	Phenol extract	△ → Fast cool[c]	3/5		0		0		1		0	
4	Phenol extract	MAK Fraction[d]	3/6		2		1		0		1	
	Phenol extract	MAK Fraction △ → Fast cool	1/6		3		0		0		1	
5	Phenol extract	CF-11 Cellulose (0% CH_3CH_2OH fraction)	4/5		5		2		3		3	
6	Phenol extract	2 M LiCl supernatant	6/6		6		3		4		5	
	Phenol extract	2 M LiCl supernatant + DNase + Pronase[e]	3/3		3		1		1		0	
	Phenol extract	2 M LiCl precipitant + DNase + Pronase[e]	1/3		0		0		0		0	

[a] Arrow indicates direction of sedimentation.
[b] No. plants infected/no. plants inoculated. The number of plants inoculated remains constant within horizontal data lines.
[c] Heated to 95 C for 10 min in 0.001 M TKM, then rapidly cooled in ice bath.
[d] Fraction No. 94 from Fig. 2.
[e] 50 μg/ml DNase for 1 hr at 37 C; 5 μg/ml Pronase for 18 hr at 37 C.

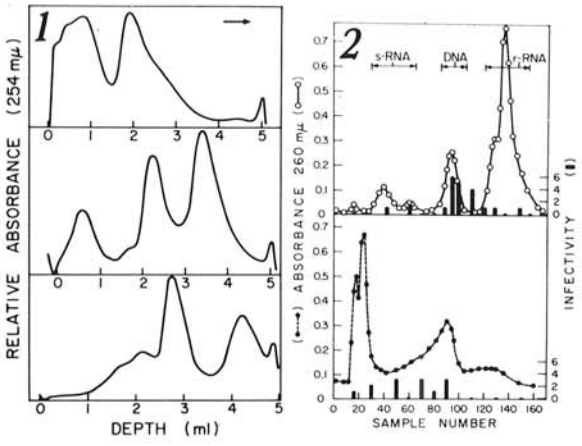

Fig. 1-2. **1)** Scanning patterns at 254 mμ of 5-20% sucrose density gradients after centrifugation in the SW50L rotor at 45,000 rpm for 4 hr at 1 C containing ethanol-concentrated high-speed supernatant (HSS) preparations (above), and phenol extracts (center) of citrus exocortis virus-infected gynura and TRV-RNA (below). **2)** Elution patterns of phenol extract (above) and ethanol-concentrated high-speed supernatant (HSS) preparation (below) from methylated albumin (MAK) columns by a 0.4-1.2 M NaCl linear gradient. Number of plants infected out of total of 6/sample from phenol extract and 5/sample from HSS preparation indicated by histogram.

infectivity can be observed in the bottom of the columns (4-5 ml) where a typical viral RNA of about 2×10^6 molecular wt would be expected to sediment. This presents the possibility that CEV might exist in two infectious forms, one a typical single-stranded RNA and the second a double-stranded form. To test this suggestion, phenol extracts as well as an infectious fraction eluted from a MAK column in the region of DNA were heated to 98 C for 10 min and rapidly cooled. This treatment was designed to "melt" the double-stranded structure, thereby separating the constituent strands and increasing the sedimentation rate of the infectious molecule. Table 3, Experiment 3 and 4, presents data which indicate that the sedimentation rate of the infectious light material was not significantly increased.

Resistance to RNase inactivation at high ionic strength buffers has been utilized to discriminate double-stranded RNA from single-stranded species (2). Both phenol extracts and HSS preparations were dialyzed against 0.01X and 2X SSC (0.15 M NaCl, 0.015 M sodium citrate, pH 7.0) prior to exposure to RNase. The concentration of RNase was varied from 0.01-1.0 μg/ml with different experiments, as were the conditions of incubations from 0-1 hr at 37 C. The relative infectivities of the different preparations varied; however, no evidence of enhanced survival after treatment in 2 SSC was observed. This would support the absence of an infectious double-stranded form of CEV.

Separation of s-RNA and double-stranded viral species (RF) from r-RNA and double-stranded viral species with regions of single strands (RI) has been accomplished by precipitation of the latter with 2 M LiCl (1). Only trace amounts of CEV of very low specific infectivity could be precipitated by this salt treatment. The ethanol-concentrated 2 M LiCl supernatant material from CEV infected gynura displayed the typical infectivity distribution in rate sedimentation. However, exposure to the proteolytic enzyme, pronase (Calbiochem Corp.), appeared to reduce the level of the rapidly sedimenting infectious material (Table 3, experiment 6).

Distribution of infectivity after equilibrium sedimentation in CsCl and Cs_2SO_4 is presented in Table 4. The bulk of the CEV either sedimented rapidly or pelleted in CsCl. The variation in the total number of samples from the 4-ml gradients was due to the sampling procedure. The main area of infectivity after Cs_2SO_4 sedimentation conformed to that of the single-stranded RNA of TRV contained in sister tubes. However, the spread of the infectious area was greater than that of TRV, and a trace of infectivity was also contained at the meniscus. These data also suggest the presence of multiple forms of infectious-free RNA or association of a portion of the single form with protein.

TABLE 4. Distribution of citrus exocortis virus after equilibrium sedimentation in cesium salt solutions in the Spinco SW50L rotor at 1 C

Experiment	Preparation	Medium	Centrifugation conditions	Infectivity distribution[a] →													
				1	2	3	4	5	6	7	8	9	10	11	12	13	Pellet
1	Phenol extract	55% CsCl	40 K 42 hr	1/3[b]	0	1	0	0	3	1	3	3					0
2	Phenol extract	55% CsCl	40 K 60 hr	0/3	0	0	0	0	0	0	0						1
	TRV-RNA	55% CsCl	40 K 60 hr	0/2	0	0	0	0	0	0							2
3	Phenol extract	50% Cs_2SO_4	35 K 66 hr	0/3	2	3	3	2	0	0	0	0					0
	2 M LiCl supernatant	50% Cs_2SO_4	35 K 66 hr	1/3	0	1	2	0	0	0	0						0
	TRV-RNA	50% Cs_2SO_4	35 K 66 hr	0/2	0	0	2	0	0	0							0
4	2 M LiCl supernatant	45% Cs_2SO_4	35 K 64 hr	1/3	0	1	2	0	2	3	2	0	0	0	0	0	0
	TRV-RNA	45% Cs_2SO_4	35 K 64 hr	0/2	0	0	0	0	0	0	2	0	0	0			0

[a] Arrow indicates direction of sedimentation.
[b] No. plants infected/no. plants inoculated. No. plants inoculated constant in horizontal lines.

Methylated albumin-coated Kieselguhr (MAK) (9) was utilized to fractionate phenol extracts from CEV infected gynura (Fig. 2, above) as well as HSS preparations (Fig. 2, below). Separation of the normal host nucleic acids is observed in the ultraviolet absorbance profile. The histogram indicates regions of relative infectivity. Samples taken from the region of DNA elution (0.7-0.8 M NaCl) of phenol extracts were most infectious. This technique, however, would not discriminate between a double-stranded molecule and a single strand of a molecular-wt intermediate to s-RNA and r-RNA. Preparations not phenolized (HSS) displayed more heterogeneity in elution of infectious material at lower salt concentrations (0.6-0.8 M NaCl).

Stepwise elution of CF-11 cellulose columns with 35%, 15%, and 0% ethanol-STE buffer is reported to fractionate s-RNA and DNA, r-RNA, and double-stranded RNA, respectively (7). The infectious moiety was predominantly eluted in 0% ethanol (Table 5). In some experiments, infectivity was recovered in the 15% ethanol. In experiment 3 (Table 5) the 0% fraction comprising a very low load (15 μg) was concentrated and recycled on CF-11 cellulose, and a portion of the infectivity was then recovered in the 35% ethanol eluent. In all cases, however, the greatest specific infectivity (infectivity/A_{260}) was characteristic of the 0% eluent.

An additional consideration which might explain the existence of two differently sedimenting forms would involve a circular RNA structure. The sedimentation properties would be markedly influenced by the secondary structure of the ring as well as either an open or closed configuration (6), both of which might be infectious, only to varying degrees. An experimental approach to test this suggestion involved exposure to the exonucleases, spleen, and venom phosphodiesterase (PDE) (7). Control preparations consisted of phenol extracts from healthy gynura with 10% A_{260} TRV-RNA added. Conditions of incubation were empirical, and both preparations were assayed on systemic hosts, gynura for CEV and *Nicotiana clevelandii* Grey for the TRV-containing controls. Under conditions where tobacco rattle virus RNA was completely inactivated either by spleen PDE or venom PDE, CEV was still infectious. The resistance of CEV to inactivation by the exonuclease was not complete, presumably due to contamination by endonuclease activity. The lack of free 3′ or 5′ hydroxyl groups at the termini could also possibly explain the resistance to PDE without the need for invoking a circular structure. These data are at best suggestive because of the difficulty involved in determining concentration of CEV in various preparations and the inadequacy of the controls.

TABLE 5. Elution of citrus exocortis virus from CF-11 cellulose columns

Experiment	Preparation	% CH_3CH_2OH 35	15	0
1	Phenol extract	0/5[c]	0	4
2	Phenol extract	0/5	2	5
3	Phenol extract[a]	0/3	2	2
	0% Fraction[b]	1/4	0	2
4	HSS	0/3	0	2
5	2 M LiCl supernatant	0/3	0	3
	2 M LiCl + DNase	0/3	0	3

[a] 10 mg total load.
[b] 15 μg total load.
[c] No. plants infected/no. plants inoculated.

DISCUSSION.—The lack of a local lesion host as well as the necessity of vegetative propagation of the assay host, gynura, coupled with the 30-day assay period present technical obstacles in these studies. Furthermore, since the CEV-RNA comprises but a small portion of the total nucleic acid extracted, the separation of viral from host nucleic acid has not been achieved. No differential ultraviolet absorbance between healthy and CEV-infected gynura could be observed following any of the various treatments reported here. Therefore, the results presented here indicate properties of CEV deduced from the effects on biological activity under various conditions. Variability is encountered because of the inconsistency in either the quantity and/or specific infectivity of CEV in the different phenol preparations. Nevertheless, by repeating experiments, an indicative trend of the effect on CEV hopefully can be discerned.

The rate sedimentation studies indicate that the infectious entity of exocortis virus is heterogeneous in size and density and may exist in two predominant forms. The major form sediments in the 10-15 S region, while the minor moiety sediments greater than 25 S. The spread of infectivity throughout some density-gradient tubes may indicate intermediate forms or simply overloading. If both forms were single-stranded structures, the expected molecular wt would be in the order of 2-5 $\times 10^5$ for the slowly sedimenting form and about 2-3 $\times 10^6$ for the rapidly sedimenting species. This inference would extend the possibility that the 10-15 S form of CEV might comprise a minimal virus approaching a monocistronic message containing only the code for the viral replicase and a sequence of nucleotides necessary for recognition.

An additional interpretation of the rate sedimentation data would involve the presence of a double-stranded conformation as the primary infectious agent similar to that reported for potato spindle tuber virus (3, 4, 7). Solubility in 2 M LiCl as well as the elution of phenol extracts from MAK columns and CF-11 cellulose support this alternative; however, it would not exclude the low molecular wt, single-stranded model. The fact that the sedimentation rate of the proposed duplex could not be increased by melting would discount the possibility of a double-stranded condition. Furthermore, no measure of resistance to inactivation by RNase was observed in high salt.

The preliminary experiments utilizing exonuclease inactivation introduces the possibility of a circular molecule similar to ϕX 174 (5). This configuration could provide some measure of protection as compared to a typical single-stranded virus, and make the existence of multiple sedimenting forms more feasible (6).

Furthermore, heating and rapid cooling does not alter the sedimentation properties of a circular structure (12).

The infectivity observed in the bottom of 5-10% sucrose gradients (> 25 S) comprises but a minor portion of the total infectivity. It may, however, represent a distinct entity. In addition to the considerations discussed above, the presence of a nucleoprotein complex cannot be unequivocally discounted. This complex might not take the structure of a typical viral nucleoprotein, but may consist of either a partially-coated form or a stable association with a host protein. The heterogeneity of infectivity distribution in rate sedimentation might reflect degrees of binding with some protein. Observation of infectivity at the meniscus of equilibrium sedimentation columns and the elution pattern of nonphenolized HSS preparations from MAK columns would support this hypothesis. The preliminary experiments in which exposure to pronase reduced the level of the rapidly sedimenting species lends credence to this interpretation.

Absolute confirmation of the structure of the infectious molecule of CEV cannot as yet be made. Nevertheless, the virus displays unusual properties that mark it as an atypical plant virus. The virus appears to exist at least in part as a free RNA species in the host. The likelihood of a double-stranded condition must at present be subordinate to either a small single-stranded or circular molecule. Finally, the existence of a portion of the infectious RNA in association with some protein remains a possibility.

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