Partial Characterization of a Virus Associated with Citrus Ringspot

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ABSTRACT

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Infectivity of citrus ringspot virus (CRSV) was associated with two components that were readily separated by sucrose density gradient centrifugation. An antiserum, prepared to a partially purified preparation of the virus, was used to assay gradient fractions and crude extracts by serologically specific electron microscopy (SSEM). A mixture of short (300–500 nm) and long (1,500-2,500 nm), extremely flexible, filamentous particles was observed in extracts of infected tissue. Examination of the top and bottom fractions from gradient centrifugation that contained the infectious components revealed the short particles were concentrated in the top fractions, and the long particles were in the bottom fractions. The short and long particles were approximately 10 nm in diameter and appeared identical, except for difference in length; conceivably, they each contain a portion of the CRSV genome. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of preparations of CRSV, partially purified by gradient centrifugation and agarose gel electrophoresis, were found to contain a protein with a molecular mass of about 48 kilodaltons (kd) that was not found in comparable preparations from noninfected leaves. Because the 48-kd protein was associated with fractions that contained the infectious components and the short and long particles and was not present in preparations of healthy tissue, it appears to be viral and may function as a coat protein.

Citrus ringspot (CRS) was first described in 1968 (7) and has since been reported from many areas (6). In addition, CRS may be associated with citrus psorosis (6,7), an economically important disease of unknown etiology that was described as probably being caused by a virus in 1933 (3). Because the causal agent of CRS is mechanically transmissible to a wide range of herbaceous plants (4), it has been presumed to be a virus and is referred to as citrus ringspot virus (CRSV). The infectivity associated with CRSV can readily be monitored by inoculation to *Chenopodium quinoa* Willd. (4). CRSV produces local lesions on the inoculated leaves but does not develop a systemic infection. Infectivity can be recovered from inoculated leaves of *C. quinoa*, and leaves with a large number of lesions can be used as a source of tissue for purification attempts.

Efforts to characterize CRSV have been hampered by the rapid loss of infectivity in extracts and apparent loss of infectivity following fractionation by sucrose density gradient centrifugation. Infectivity of crude extracts of CRSV in phosphate buffer, as measured on *C. quinoa*, is lost within 1 hr (4). Infectivity is stabilized to a degree by making extracts in Tris buffer, pH 8.0, containing 0.5% 2-mercaptoethanol (2-ME), but in these preparations more than 60% of the infectivity is still lost within 24 hr at 4 C (4).

A number of preliminary experiments designed to purify and characterize CRSV have been done over a period of several years before the work reported here. Much of the information from these experiments has not been published, but it has proven to be valuable to this effort. For example, it has been established that the infectivity associated with CRSV can be concentrated by high-speed centrifugation or precipitation with polyethylene glycol, but when these concentrated preparations are subjected to fractionation by sucrose density gradient centrifugation, the infectivity appears to be lost. In addition, the infectivity of CRSV preparations has been shown to be stable when treated with trichlorotrifluoroethane (Freon 113) and 0.1% Triton X-100, but is diminished by chloroform and eliminated by *n*-butanol.

We report here the partial characterization of a virus associated with CRS and show that at least two components, which are readily separated by sucrose density gradient centrifugation, are required for infectivity. A preliminary report has been published (2).

MATERIALS AND METHODS

Virus isolate and hosts. The CRSV isolate used in this study was CRSV-4 that had been through several successive single lesion transfers on *C. quinoa* followed by transfer to *Gomphrena globosa* L. and subsequently to grapefruit (*Citrus paradisi* Macfad. 'Duncan') (4). Infectivity assays, extractions, and sucrose density gradients were done using 0.05 M Tris, 0.1% ascorbic acid, 0.1% L-cysteine, and 0.5% 2-ME, adjusted to pH 8.0 with HCl (TACM).

Partial purification and infectivity assays. Extracts for infectivity assays and purification trials were prepared from young leaves of Duncan grapefruit with ringspot symptoms or inoculated leaves of *C. quinoa*. Leaf tissue was pulverized in liquid nitrogen. The resulting frozen powder was transferred to a mortar and pestle at room temperature and ground with 2 or 7 volumes of TACM per gram of tissue. All subsequent purification steps were done at 4 C. The extracts were filtered through cheesecloth, stirred with a half volume of Freon 113 for 1 min, and clarified by centrifugation for 10 min at 12,000 g. Linear gradients of 10–40% sucrose in TACM were prepared in Beckman SW 41 rotor tubes. Clarified extracts prepared using 2 volumes of TACM per gram of tissue were applied to gradients without concentration. Extracts in 7 volumes of TACM were subjected to one or more cycles of differential centrifugation (12,000 g for 10 min followed by 300,000 g for 1 hr), and the resulting pellets were suspended in TACM (110 ml/6 g of starting tissue). In experiments where more than one cycle of differential centrifugation was done, the pellets from the first high-speed centrifugation were suspended in TACM containing 0.1% Triton X-100. One milliliter of the preparation was layered on each gradient tube, and the gradients were centrifuged for 2.5 hr at 38,000 rpm. Gradients were fractionated from the top into 0.6-ml fractions using an ISCO gradient fractionator. Extracts and fractions from gradients were assayed for infectivity on *C. quinoa*.

Electrophoresis of partially purified virus. Selected fractions from density gradient centrifugation were pooled and concentrated by centrifugation at 250,000 g for 1 hr. The resulting pellets were resuspended in 0.04 M Tris-acetate, 0.002 M EDTA,

pH 8.0, (TAE) containing 0.5% 2-ME and subjected to electrophoresis at 80 V for 4 hr at 4 C on 0.5% agarose gels in TAE containing 0.5% 2-ME. The gels were stained overnight with ethidium bromide. Gel lanes were cut into 0.5-cm segments and analyzed for proteins by SDS-PAGE.

Electrophoresis of proteins. Selected sucrose density gradient fractions and segments of agarose gels were boiled with running buffer containing 1.0% SDS and 2.0% 2-ME for 5 min. Samples were analyzed by electrophoresis on 12% polyacrylamide gels using the discontinuous slab gel system (5) and silver nitrate staining.

Serology. Sucrose density gradient fractions 6, 7, and 8 from a preparation from CRSV-infected grapefruit, which contained the top infectious component, were concentrated by centrifugation and stored at −20 C until used for antiserum production. A sample of the preparation was allowed to thaw, emulsified with Freund's complete adjuvant, and injected into the peritoneal cavity of mice. Four injections were given over a 3-mo period, and each injection contained material from 0.25 g of infected tissue.

Extracts of CRSV-infected and noninfected leaves and selected fractions from gradient centrifugations were assayed using SSEM as previously described (1). Extracts were prepared by crushing 1 g of tissue with 1.0 ml of 0.05 M Tris, pH 8.4, containing 0.15 M NaCl and 0.4 M sucrose. The resulting pulp was transferred to a microcentrifuge tube and centrifuged before assay. Virus particles were given a positive stain by floating grids on 2% aqueous uranyl acetate, pH 5.0, for 10 min and removing the stain from the grids by blotting thoroughly.

RESULTS

There was no infectivity associated with any of the individual fractions from sucrose density gradient centrifugation of a clarified extract of CRSV-infected grapefruit (Table 1, Exp. 1). The material that was applied to the gradient in this experiment, when assayed 8 hr after initiation of extraction, produced five lesions when 50 μl was used to inoculate a leaf of *C. quinoa*. As expected, since it has been shown that dilution of concentrated crude extracts of CRSV-infected tissue increases infectivity, the infectivity increased (to 87 lesions/50 μl) when the preparation was assayed at a final dilution of 1/10. After 24 hr at 4 C, the diluted preparation produced 20 lesions/50 μl.

A preparation, partially purified and concentrated by differential centrifugation, produced 136 lesions/50 μl after 8 hr and 24 lesions/50 μl when assayed after being kept at 4 C for 24 hr. Some infectivity was found in individual fractions following sucrose density gradient centrifugation of this preparation (Table 1, Exp. 2), but most of the infectivity appeared to be lost.

When fractions from sucrose density gradient centrifugation were combined and assayed, infectivity was found with mixtures of fractions 6, 7, or 8 plus 10, 11, or 12 (Table 1). Although a large number of lesions were obtained by using mixed fractions from a partially purified preparation, when assays were made 8 hr after initiation of the experiment, most of the infectivity was lost when the fractions were kept at 4 C and assayed after 24 hr.

Some unusual filamentous particles were observed when CRSV preparations were examined by SSEM (Fig. 1). The particles were observed only in preparations from CRSV-infected tissue assayed on grids prepared using CRSV antiserum (Fig. 1A, D, and E). They were not seen on control grids prepared using extracts of infected tissue and normal serum (Fig. 1B) or extracts of healthy tissue and CRSV antiserum (Fig. 1C). A number of both short particles (300–500 nm) and long particles (1,500–2,500 nm) were observed in crude extracts (Fig. 1A). When fractions from sucrose density gradient centrifugation that contained the infectious components were assayed by SSEM, the short particles were found in the top fractions (Fig. 1D), and the long particles were found in the bottom fractions (Fig. 1E).

Analysis by SDS-PAGE of proteins present in sucrose density gradient fractions revealed a 48-kilodalton (kd) species in fractions 6 through 12 in preparations from CRSV-infected tissue (Fig. 2, lanes a–g). This protein was not present in comparable preparations from healthy tissue (Fig. 2, lanes i–o). Additional purification of pooled fractions 6, 7, and 8 by a second cycle of gradient centrifugation followed by concentration by centrifugation and concentration of pooled fractions 10, 11, and 12 by centrifugation demonstrated that the 48-kd protein was the major protein associated with preparations from infected tissue (Fig. 2, lanes p–s).

The 48-kd protein was essentially the only protein detected by SDS-PAGE when sucrose density gradient fractions 6, 7, and 8 and 10, 11, and 12 of CRSV-infected grapefruit were subjected to agarose gel electrophoresis (Fig. 3). There were no specific bands observed in preparations from either infected or healthy tissue when the agarose gels were stained with ethidium bromide or Coomassie Blue (not shown), but the 48-kd protein was readily detected in preparations from infected tissue in subsequent SDS-PAGE. The 48-kd protein was concentrated in the second 0.5-cm segment of the agarose gel lane containing fractions 6, 7, and 8 (Fig. 3, lane c) and in the first 0.5-cm segment of the lane containing fractions 10, 11, and 12 (Fig. 3, lane f). The agarose gel electrophoresis procedure was also used to compare the 48-kd protein in density gradient fractions 6, 7, and 8 and 10, 11, and 12 in preparations from infected *C. quinoa* and grapefruit (Fig. 4). In some SDS-PAGE gels, proteins larger than 48 kd were observed in all lanes (Figs. 3 and 4). The origin of these apparent proteins is not known, but they may be associated with one of the components used in the assay since they are in all lanes of the gel.

DISCUSSION

The apparent loss of infectivity after sucrose gradient centrifugation has been observed many times, both in this study and by previous workers. Because the infectivity of CRSV was known to be unstable, it seemed logical that the virus was becoming inactivated during the centrifugation step. This was reinforced by observing that in some experiments low levels of infectivity could be found in individual gradient fractions as shown in Table 1, Exp. 2. The infectivity in individual fractions is now considered to be due to loss of resolution of the infectious components caused by overloading, but, previously, it was taken as evidence that in careful experiments some infectivity could be found in individual fractions. Thus, much of the work on CRSV

TABLE 1. Infectivity of fractions of citrus ringspot virus following sucrose density gradient centrifugation

Individual fractions			Combined fractions			
Fraction	Exp. 1[a] 8 hr[c]	Exp. 2[b] 8 hr	Fraction	Exp. 1 8 hr	Exp. 2 8 hr	Exp. 2 24 hr
1	0[d]	0	6 + 9	0[e]	6	0
2	0	0	7 + 9	0	1	0
3	0	0	8 + 9	0	4	0
4	0	0	6 + 10	0	4	0
5	0	0	7 + 10	0	32	2
6	0	0	8 + 10	0	29	2
7	0	1	6 + 11	6	92	2
8	0	0	7 + 11	5	71	7
9	0	8	8 + 11	12	87	22
10	0	19	6 + 12	1	5	0
11	0	17	7 + 12	8	12	1
12	0	1	8 + 12	26	23	5
13	0	0	6 + 13	0	0	0
14	0	0	7 + 13	1	3	0
15	0	0	8 + 13	1	7	0
16	0	0	6 + 14	0	1	0
			7 + 14	0	0	0
			8 + 14	1	1	0

[a] From a clarified extract representing 0.33 g of tissue per milliliter.
[b] From a preparation concentrated by differential centrifugation representing 6 g of tissue per milliliter.
[c] Fractions were assayed 8 or 24 hr after tissue extraction.
[d] Number of local lesions/leaf inoculated with 50 μl of individual fractions.
[e] Number of local lesions/leaf inoculated with a mixture of 25 μl each of two selected fractions.

focused on finding ways to prevent inactivation of what appeared to be a very unstable virus. The observation that a mixture of all the fractions from a gradient was infectious demonstrated that the infectivity was not lost by gradient centrifugation and led to the experiments where selected fractions were combined and assayed for infectivity. In various experiments, infectivity was always associated with mixtures of fractions 6, 7, or 8 plus 10, 11, or 12 (Table 1). These experiments indicated that at least two components are required for infectivity of CRSV and provided a means to partially purify the virus. Unfortunately, as with crude extracts and preparations concentrated by centrifugation, most of the infectivity associated with the gradient fractions was lost after 24 hr at 4 C. This indicated that purification schemes requiring more than 1 day probably could not be monitored by infectivity.

Electron microscopy was used routinely in this study to examine leaf-dip and partially purified preparations. Filamentous virus particles were never observed in any of the preparations, which led to the suggestion that CRSV might be an isometric particle. Filamentous particles were only observed after the production of an antiserum to a partially purified preparation and analysis by SSEM (Fig. 1). The particles were only seen when given a positive stain with uranyl acetate, and it was necessary to completely remove the stain solution from the grids by repeated blotting with

the edge of a piece of filter paper. Particles were not observed on SSEM grids, which should have had particles attached, that were negatively stained with either phosphotungstage or uranyl acetate. Apparently the high ionic strength involved in negative staining degrades the particles; this could account for repeated failure to observe the particles in leaf-dip or partially purified preparations applied directly to grids, and the need to completely remove the stain solution by blotting when applying a positive stain. The long and short particles were about 10 nm in diameter and appeared identical except for differences in length. Because they were found in regions of gradient tubes that contained the infectious components, they conceivably contain portions of the CRSV genome that were required for infectivity.

The agarose gel assays were done before the filamentous particles were detected. It has not been determined if the intact filamentous particles, or merely fragments, are migrating in the agarose gels, but the apparent slower migration from fractions 10, 11, and 12 (Fig. 2) is as would be expected for a larger particle. Because the 48-kd protein was found in two infected hosts, but not in comparable healthy tissue, and because it was found in fractions that contained the infectious components and the filamentous particles, it appears to be viral and may serve as a coat protein.

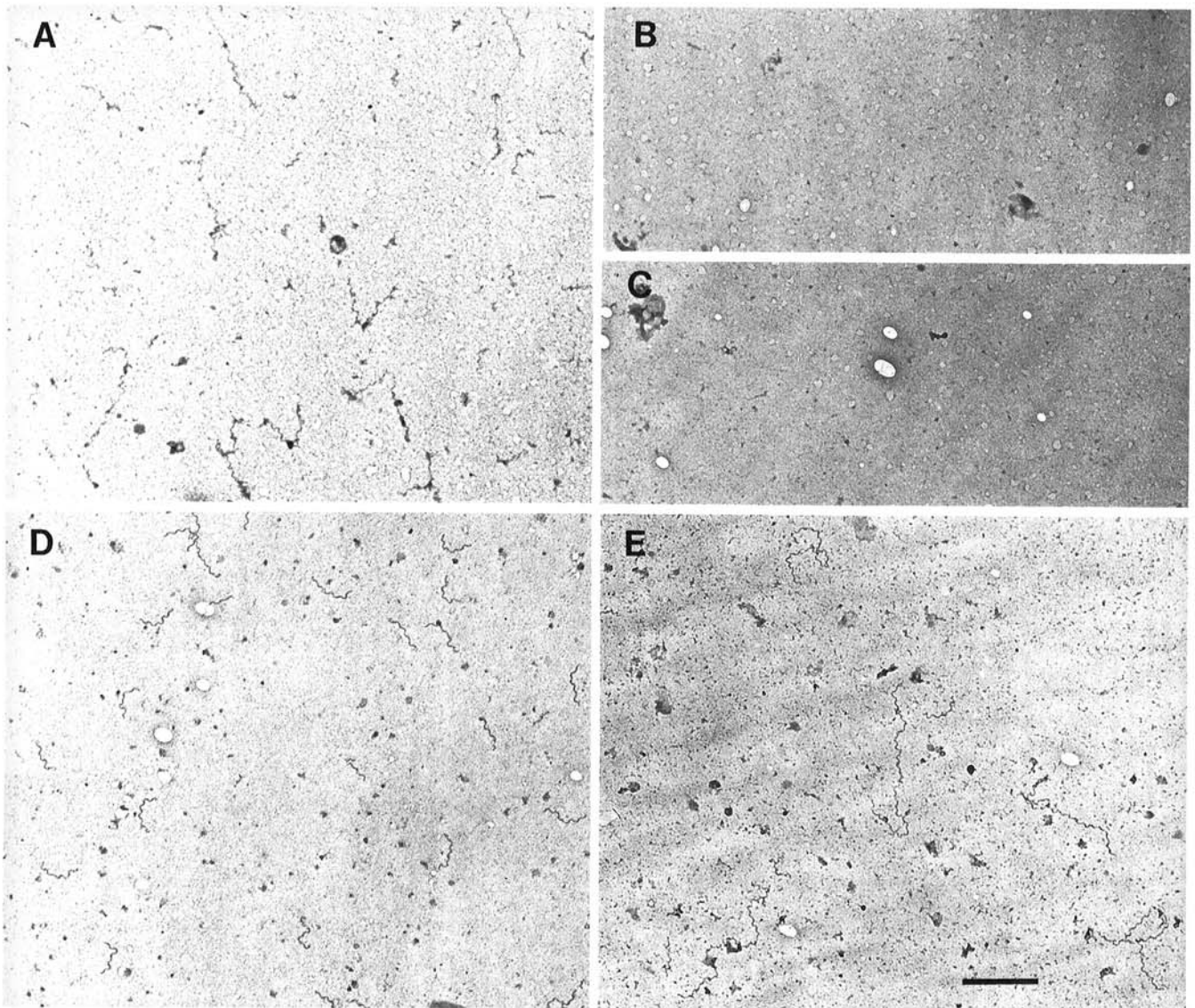The partial characterization of CRSV reported here suggests it

Fig. 1. Electron micrographs of serologically specific electron microscopy (SSEM) assays of preparations from citrus ringspot virus (CRSV)-infected and healthy tissue. Crude extracts of CRSV-infected, **A and C,** and healthy, **B,** *Chenopodium quinoa* on grids prepared using antiserum to CRSV (A and B) and normal serum (C); Fractions 7, **D,** and 11, **E,** from sucrose density gradient centrifugation of a preparation from 6 g of CRSV-infected grapefruit on grids prepared using antiserum to CRSV. The scale bar represents 500 nm.
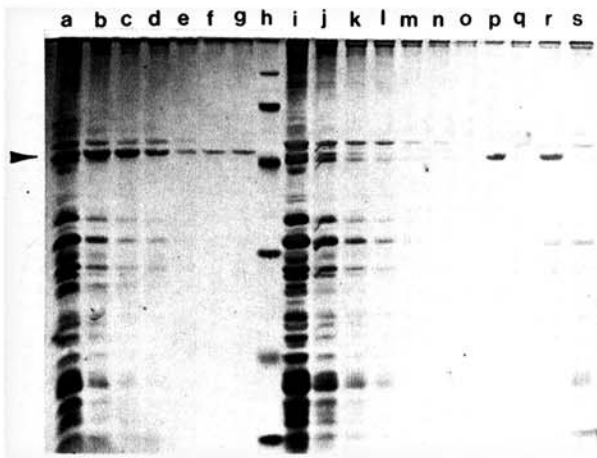
Fig. 2. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of proteins from partially purified preparations of citrus ringspot virus (CRSV)-infected and healthy *Chenopodium quinoa*. Fractions 6 through 12 from sucrose density gradient centrifugation of preparations, subjected to two cycles of differential centrifugation and treatment with Triton X-100, from 6 g of infected (lanes a through g) and healthy (lanes i through o) leaves. Fractions 6, 7, and 8 pooled and concentrated by high-speed centrifugation and subjected to a second sucrose density gradient centrifugation from infected (lane p) and healthy (lane q) tissue. Fractions 10, 11, and 12 pooled and concentrated by high-speed centrifugation from infected (lane r) and healthy (lane s) tissue. Marker proteins (molecular masses in parenthesis): phosphorylase B (94,000), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), soybean trypsin inhibitor (21,000), and lysozyme (14,300) (lane h). Forty-eight kilodalton protein identified by arrowhead.
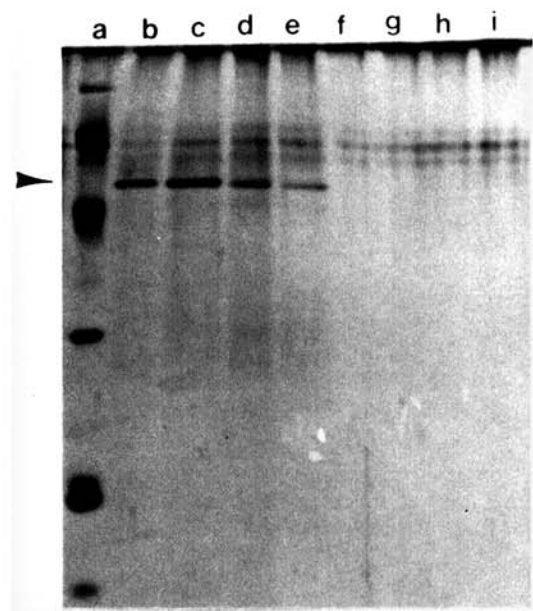

Fig. 3. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins from preparations of citrus ringspot virus-infected and healthy grapefruit after sucrose density gradient centrifugation and fractionation by agarose gel electrophoresis. Lanes from agarose gels were cut into four 0.5-cm segments, with the first segment starting at the origin, and each segment was analyzed by SDS-PAGE. Gel segments (1–4) from gradient fractions 6, 7, and 8 of infected (lanes b–e) and healthy (lanes j–m) and gradient fractions 10, 11, and 12 from infected (lanes f–i) and healthy (lanes n–q) grapefruit. Marker proteins as in Figure 2 (lane a). Forty-eight kilodalton protein identified by arrowhead.

belongs to a yet to be described group of plant viruses. The putative capsid protein, which is larger than expected for a filamentous

Fig. 4. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins from preparations of citrus ringspot virus-infected and healthy grapefruit and *Chenopodium quinoa* after sucrose density gradient centrifugation and fractionation by agarose gel electrophoresis. The top 1.0-cm segment of lanes from agarose gels were subjected to analysis by SDS-PAGE. Gel segments from gradient fractions 6, 7, and 8 of infected *C. quinoa* (lane b) and grapefruit (lane d) and fractions 10, 11, and 12 of infected *C. quinoa* (lane c) and grapefruit (lane e). Lanes f–i are from appropriate healthy tissue for comparison with lanes b–e, respectively. Marker proteins as in Figure 2 (lane a). Forty-eight kildalton protein identified by arrowhead.
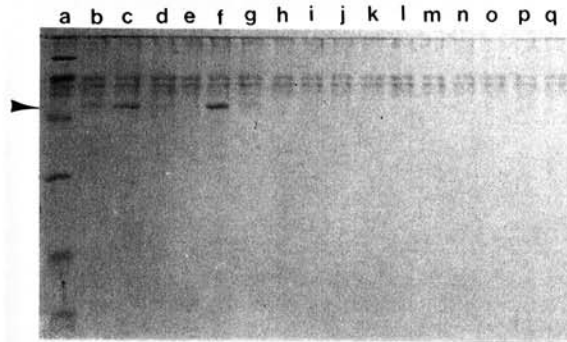
particle, and the very flexible filamentous particles that appear to contain a split genome are not characteristic of any known group of viruses.

LITERATURE CITED

1. Derrick, K. S., and Brlansky, R. H. 1976. Assay for viruses and mycoplasmas using serologically specific electron microscopy. Phytopathology 66:815-820.
2. Derrick, K. S., Brlansky R. H., Lee R. F., Timmer L. W., and Nguyen, T. K. 1986. Two components associated with citrus ringspot virus. (Abstr.) Phytopathology 76:1072.
3. Fawcett, H. S. 1933. New symptoms of psorosis, indicating a virus disease of citrus. (Abstr.) Phytopathology 23:930.
4. Garnsey, S. M., and Timmer, L. W. 1980. Mechanical transmissibility of citrus ringspot virus isolates from Florida, Texas, and California. Pages 174-179 in: Proc. 8th Conf. Int. Org. Citrus Virol. E. C. Calavan, S. M. Garnsey, and L. W. Timmer, eds. IOCV, Riverside, CA.
5. Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature (London) 227:680-685.
6. Timmer, L. W., and Benatena, H. N. 1970. Comparison of psorosis and other viruses causing leaf flecking in citrus. Proc. Int. Soc. Citriculture 3:930-935.
7. Wallace, J. M., and Drake, R. J. 1968. Citrange stunt and ringspot, two previously undescribed virus diseases of citrus. Pages 177-183 in: Proc. 4th Conf. Int. Org. Citrus Virol. J. F. L. Childs, ed. Florida Press, Gainesville.