Properties of Asparagus Virus II, a New Member of the Ilarvirus Group

Ichiro Uyeda and Gaylord I. Mink

Former graduate research assistant and plant pathologist, respectively, Irrigated Agriculture Research and Extension Center, Washington State University, Prosser 99350. Present address of senior author: Department of Botany, Faculty of Agriculture, Hokkaido University, Sapporo, Japan.

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ABSTRACT

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A virus originally isolated from asparagus in Washington and provisionally designated asparagus virus C was found to be very similar, if not identical, to asparagus virus II (AV II) briefly described earlier in Germany. The virus was seed-transmitted but induced no visible symptoms in greenhouse-grown asparagus seedlings. Purified virus preparations separated into three major nucleoprotein (NP) components and occasionally a minor component on rate zonal sucrose density gradients. The major components, designated NP 1, NP 2, and NP 3, sedimented at 104S, 95S, and 90S, respectively. A minor component, NP 0, sedimented faster than NP 1 and appeared heterogeneous in quasi-equilibrium centrifugation. Maximum infectivity was obtained with the mixture NP 1 + NP 2 + NP 3; each component alone exhibited little or no infectivity. Quasi-isometric particles of NP 1, NP 2, and NP 3 had modal diameters of 32 nm, 28 nm, and 26 nm, respectively, and were serologically identical. Two types of particles were found with NP 0: monomer particles 34–36 nm in diameter and dimers of particles 28 nm in diameter. Two serotypes of AV II were found in Washington.

Three different mechanically transmissible viruses were reported in 1977 to occur in Washington asparagus fields and were provisionally designated A, B, and C (16). Virus A was identified as tobacco streak virus (16). The identity of virus B is still being investigated. This report describes properties of virus C and demonstrates that it belongs in the ilarvirus (isometric labile ringspot virus) group (18) and is closely related, if not identical, to asparagus virus II (AV II), first reported in Germany (10,21). Asparagus virus II appears to be synonymous (9) with asparagus latent virus described later in Denmark (17).

MATERIALS AND METHODS

Virus source. Two isolates of AV II obtained from asparagus plants in Washington, AV II-P and AV II-S, were used. A European isolate of AV II was kindly supplied by H. Groschel. All isolates were obtained after two to three successive single-lesion transfers on either bean (*Phaseolus vulgaris* 'Bountiful') or cowpea (*Vigna unguiculata* 'California Blackeye') and were maintained in tobacco (*Nicotiana tabacum* 'Havana 423') leaf tissue. For purification, tobacco leaves were harvested 2–4 wk after inoculation and kept at −20 C until used.

Host range and symptomatology. Tobacco leaf tissue infected with AV II was triturated in 0.1 M neutral potassium phosphate buffer containing 0.01 M sodium diethyldithiocarbamate and 0.01 M cysteine-HCl and rub-inoculated with a cotton swab to test plants that had been dusted with 600-mesh Carborundum. The test plants were grown either in a greenhouse as described by Howell and Mink (11) or in 21–24 C growth chambers with 16-hr photoperiods of 5,380–7,580 lux illumination, then placed in the dark for 1–2 days before inoculation. Symptom development was observed for 2–3 wk after inoculation, and then samples from inoculated and young tip leaves were separately back-indexed to *Chenopodium quinoa*.

Purification. Infected tobacco leaf tissue (40–60 g) was macerated in a Waring Blendor for 2 min with 0.1 M neutral phosphate buffer (2.5 ml per gram of tissue) containing 0.02 M ethylenediaminetetraacetic acid disodium salt (EDTA). Triton X-100 (5 ml per 100 ml of extract) was added, and the mixture was stirred for 15 min at room temperature. The extract was then clarified by treatment with chloroform (1 ml per 4 ml of extract) and by freezing overnight at −20 C.

The virus was concentrated into 1 ml of 0.01 M phosphate buffer containing 0.001 M EDTA, pH 7.2–7.4, by two cycles of differential ultracentrifugation in a Beckman No. 40 rotor (39,000 rpm for 90 min and 15,000 rpm for 15 min). The virus was further purified by 10–30% rate zonal sucrose density gradient centrifugation. The density gradient tubes, containing 5 ml of each of 10, 15, 20, 25, and 30% (w/v) sucrose solutions in 0.01 M potassium phosphate buffer, pH 7.2–7.4, were stored overnight at 4 C, layered with 1 ml of virus preparation, and centrifuged at 24,000 rpm for 3 hr in a Beckman SW 25.1 rotor. The purified virus was stored in 0.01 M phosphate buffer, pH 7.2–7.4, at 4 C.

Sedimentation coefficients. Sedimentation coefficients were determined by using "linear-log" sucrose density gradient centrifugation, as described by Brakke and van Pelt (3). The middle and bottom components of tobacco ringspot virus (19); potato virus X (2); Tb, M, and B components of alfalfa mosaic virus (20); and TV component (13) of Tulare apple mosaic virus (15) were used as sedimentation standards.

Quasi-equilibrium density gradient centrifugation. Quasi-equilibrium sucrose density gradient columns were prepared by layering 4, 7, 7, and 7 ml of 40, 45, 50, and 55% (w/v) sucrose solutions, respectively, in 0.01 M sodium phosphate buffer, pH 7.4. The sucrose gradient columns were stored for 2 days before use. One milliliter of the purified virus was layered on each column and centrifuged at 23,000 rpm for at least 17 hr in an SW 25.1 rotor.

Ultraviolet absorption spectra. Ultraviolet absorption spectra were determined with a Beckman DB-G spectrophotometer. An extinction coefficient E(mg/ml)/1 cm (260 nm) of 5.3 determined for citrus leaf rugose virus (4) was used to estimate the concentration of the purified AV II.

Serology. Antisera to AV II isolates P and S were produced by injecting 1 ml of purified virus (0.2–0.4 mg) intravenously into rabbits four times at 2–3 day intervals and twice at weekly intervals starting 1 wk after the fourth injection. Bleedings were made 2 days

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after the fourth injection and continued every 2–3 days for 2–3 wk.

Agar plates were prepared by pouring 10 ml of autoclaved Difco agar (0.7%) containing 0.85% NaCl and 0.04% sodium azide in 0.01 M neutral phosphate buffer into plastic petri plates 90 mm in diameter. The agar was allowed to solidify, and wells were cut just before use. Diameters of the center and peripheral wells were 7 mm and 6 mm, respectively. Distance between edges of the center and peripheral wells was 5 mm.

Ring interface precipitin tests were conducted with antisera diluted in 0.01 M neutral phosphate-buffered saline + 10% glycerin (1) and with antigens (OD_{260} = 0.1–0.3) in buffered saline.

Electron microscopy. Samples from each visible band in density gradient after three to four cycles of rate zonal centrifugation were negatively stained with 2% uranyl acetate, pH 7.0, and examined with a Zeiss EM 9 electron microscope. Particle measurements were taken from electron micrographs enlarged to the same magnification as that of a carbon-grating replica grid (22,835 lines per centimeter) or from electron micrographs containing particles of tobacco mosaic virus (assumed width 15 nm).

RESULTS

Host range and symptomatology. Symptoms induced by AV II-P on 11 plant species commonly used as virus indicator plants are listed in Table 1. The following additional species were infected and developed a systemic mottle as the principal symptom: *C. capitatum, N. augustifolia, N. glutinosa, N. sanderae,* and *Plantago virginica.* The following species showed systemic necrosis: *N. debneyi, N. nudicaulis, N. occidentalis, N. suaveolens, N. sylvestris,* and *N. trigonophylla.* Infected plants of *Ambrosia artemisiifolia, Capsicum annuum,* and *C. urbicum* developed systemic mottle and necrosis, while *C. album, C. polyspermum, Glycine max, Pisum sativum* '447', and *Vicia faba* developed local lesions only. *Anthriscus cerefolium* was infected locally but showed no symptoms. No infection was detected by back-inoculation from *Apium graveolens, Cucumis melo, Citrullus vulgaris, Daucus carota* 'I-58', or *Pastinaca sativa.*

Thus far, all attempts to infect asparagus seedlings by rub-inoculation with triturates of infected tissue or purified virus preparations have been unsuccessful.

Effect of AV II on asparagus seedlings. No symptoms were observed on asparagus seedlings grown from infected seed even though AV II could be readily transmitted from them. No growth differences were observed between healthy and AV II-infected seedlings grown in the field, greenhouse, or growth chambers for 1 yr. However, during the second growing season, some AV II-infected plants produced less growth than healthy plants and a few plants showed a nonspecific decline. Further studies are in progress to evaluate the effects of AV II under field conditions.

Seed transmission in asparagus. Seeds collected from a single field-grown asparagus plant known to be infected with an isolate similar to AV II-P were germinated in the greenhouse, and the seedlings were indexed on *C. quinoa.* Of 38 seedlings tested, 24 were infected with AV II.

Incidence in asparagus fields. A preliminary survey of 20 asparagus fields revealed that AV II was present in each field. More than half the plants tested in some fields were infected.

Sucrose density gradient centrifugation. Mink and Uyeda (16) reported that AV II produced two visible zones in 10–40% rate zonal sucrose density gradient tubes after 2-hr ultracentrifugation. However, when purified virus preparations were centrifuged on 10–30% sucrose columns for 3 hr, four closely spaced components were resolved visually. These components were designated NP 0, NP 1, NP 2, and NP 3 in order of decreasing sedimentation velocity. Components NP 1 and NP 3 were always detected as sharp and distinct peaks on ISCO ultraviolet analyzer profiles (Fig. 1), while NP 0 and NP 2 were minor peaks and not always clearly resolved. Preparations from healthy plants did not produce comparable peaks.

The four viral components were separated by three to four cycles of rate zonal density gradient centrifugation and examined for homogeneity by quasi-equilibrium density gradient centrifugation. Components NP 1 and NP 3 produced single sharp peaks, while NP 0 appeared to be heterogeneous, producing two peaks (Fig. 2). Component NP 2 appeared as a broad peak with occasional suggestions of heterogeneity.

Sedimentation coefficients. Sedimentation coefficients of NP 1 and NP 3 determined by linear-log sucrose density gradient centrifugations were 104S ± 2S and 90S ± 2S, respectively. In two

TABLE 1. Symptoms produced on 11 plant species inoculated with asparagus virus II-P

Species	Symptoms
Beta vulgaris	Primary chlorotic or white necrotic ring lesions
Chenopodium amaranticolor	Small necrotic ring local lesions
C. murale	Sunken necrotic local lesions
C. quinoa	Primary necrotic ring lesions or yellow spots on young inoculated leaves. Systemic mottle and occasional slight necrosis
Cucumis sativus	Local chlorotic spots
Cucurbita pepo	Systemic mottle
Gomphrena globosa	Primary necrotic and necrotic ring lesions; systemic mottle and necrosis
Nicotiana tabacum 'Havana 423'	Primary necrotic and necrotic ring lesions; systemic necrosis followed by recovery
N. clevelandii	Latent systemic infection in most cases; occasional systemic necrosis and mottle
Phaseolus vulgaris 'Bountiful'	Small, red, local lesions
Vigna unguiculata 'California Blackeye'	Small, red, local lesions

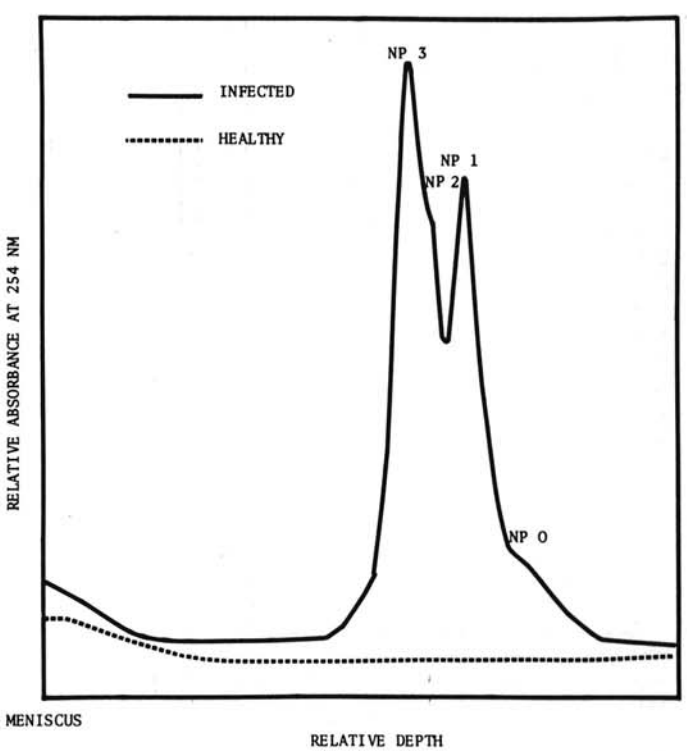

Fig. 1. Absorbance profile of asparagus virus II after 3-hr centrifugation at 24,000 rpm in 10–30% sucrose density gradients.

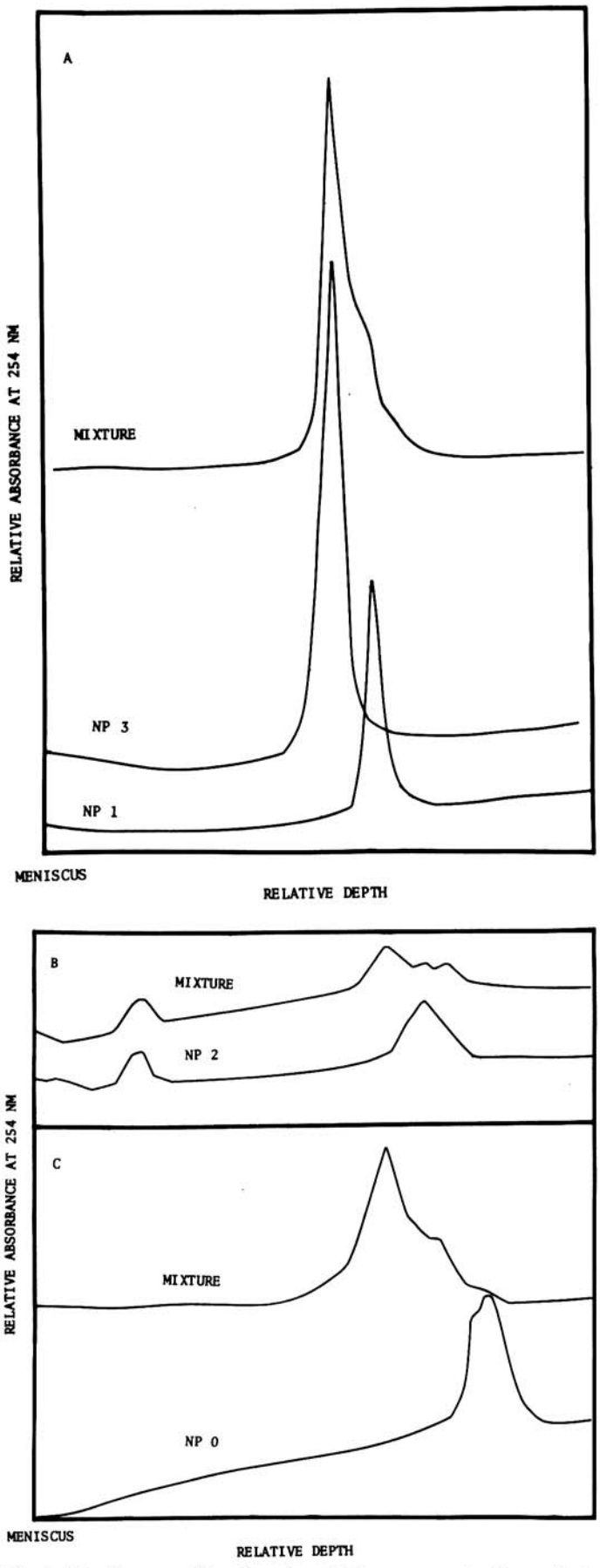

Fig. 2. Absorbance profiles of quasi-equilibrium sucrose density gradient tubes containing asparagus virus II nucleoprotein components initially separated by three to four cycles of rate sucrose density gradient centrifugation. **A,** NP 1 and NP 3. **B,** NP 2 (the peak near the meniscus of each tube was a nonvirus contaminant in the suspension buffer). **C,** NP 0.

experiments, NP 2 was resolved as a diffuse zone with a sedimentation coefficient of approximately 95S. No value was established for NP 0.

Ultraviolet absorption spectra. Component mixtures in purified preparations had ultraviolet absorption spectra typical of nucleoprotein with A260/280 ratios ranging from 1.31 to 1.36. After separation, each component had an absorption spectrum typical of a nucleoprotein, but maximum wavelength and A260/280 ratios varied slightly among components (Table 2).

Serology of nucleoprotein components. Components NP 1, NP 2, and NP 3 of AV II-P were compared in agar gel double diffusion tests with antisera prepared against the mixture of components. Each separated component and the unseparated mixture produced single precipitin lines that coalesced (Fig. 3), indicating that all components were serologically identical.

Electron microscopy. Isometric particles 22–30 nm (16,21) in diameter were associated with virus preparations, while comparable particles were not seen in preparations from healthy tobacco leaf tissue. The particles associated with each component after three to four cycles of density gradient centrifugation had modal diameters of 32, 28, and 26 nm (Fig. 4) for components NP 1, NP 2, and NP 3, respectively. Although particles associated with components NP 2 and NP 3 appeared isometric, particles associated with NP 1 frequently appeared somewhat ovate (Fig. 5).

TABLE 2. Characteristics of ultraviolet absorption spectra of separated nucleoprotein components of asparagus virus II-P[a]

Characteristics	Component			
	NP 0	NP 1	NP 2	NP 3
Maximum wavelength	262	261–262	261–262	262–263
Minimum wavelength	245	245	245	245
Maximum/minimum ratio	1.19–1.24	1.29	1.24	1.31–1.32
A260/280 ratio	1.34–1.35	1.35–1.36	1.35–1.37	1.30

[a] Values from two experiments. Not corrected for light scattering.

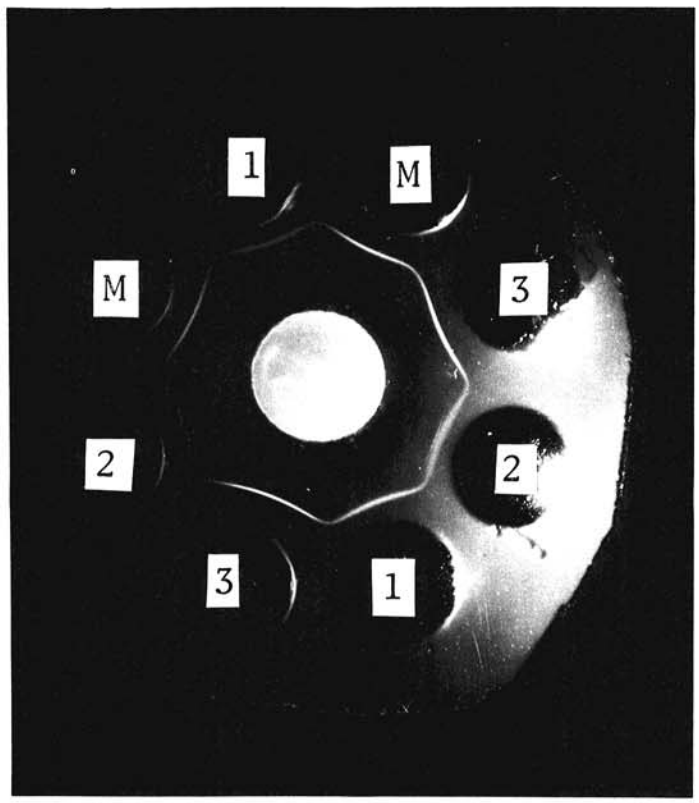

Fig. 3. Agar gel double diffusion test of separated NP 1, NP 2, and NP 3 of asparagus virus II-P. Center well was charged with undiluted antiserum against the mixture of components. Peripheral wells were charged with a mixture of components (M) and separated NP 1 (1), NP 2 (2), and NP 3 (3).

The histogram of particle diameters for NP 0 had two peaks, one at 28 nm, the other at 34–36 nm (Fig. 4); the 28-nm diameter particles invariably appeared as dimers (Fig. 5).

Infectivity of the nucleoprotein components. The four nucleoprotein components separated by three to four cycles of density gradient centrifugation were assayed on cowpea half-leaves individually and mixed (Table 3). Components NP 0 and NP 3 appeared to be noninfectious, whereas traces of infectivity were associated with components NP 1 and NP 2. This very low infectivity may have resulted from incomplete separation of NP 1 and NP 2 and suggests that each component alone was noninfectious. Any combination of two of the three components NP 1, NP 2, and NP 3 resulted in some infectivity (Table 3). Maximum infectivity levels occurred with all three components. Component NP 0 did not appear to influence infectivity.

Serotypes of AV II. Two isolates of AV II were initially differentiated by symptoms produced on *C. quinoa*: AV II-P produced a mild systemic mosaic, while AV II-S produced severe systemic necrosis. These two isolates differed serologically by spur formation in agar gel double diffusion tests (Fig. 6). However, in later field collections, we found isolates that were serologically indistinguishable from AV II-P but that produced symptoms on *C. quinoa* ranging from systemic mottle to severe necrosis of the tip leaves. Consequently, symptoms on *C. quinoa* or other hosts tested could not be used reliably to differentiate serotypes.

Comparison of AV II-P and AV II-S with European AV II. European AV II produced symptoms indistinguishable from AV II-P on *C. amaranticolor, C. murale, C. quinoa, Gomphrena globosa,* tobacco cultivar Havana 423, and cowpea cultivar

TABLE 3. Infectivity of separated and mixed nucleoprotein (NP) components of asparagus virus II

Nucleoprotein component or mixture[a]	Infectivity[b]		
	Experiment I[c]	Experiment II[d]	Experiment III[d]
0	0	...[e]	0
1	1	0	2
2	3	1	3
3	0	0	0
1+2	...	27	50
1+3	...	16	11
2+3	...	6	9
0+1+2	43	...	...
0+1+3	24	...	...
0+2+3	16	...	...
1+2+3	126	94	125
0+1+2+3	97	...	...

[a] NP 1, NP 2, and NP 3 were separated by four cycles of rate zonal sucrose density gradient centrifugations and NP 0 by three cycles.
[b] Infectivity is expressed as the average number of local lesions on six half-leaves of cowpea cultivar California Blackeye.
[c] Final concentration of each component in all preparations was A260 = 0.005.
[d] Final concentration of each component in all preparations was A260 = 0.007.
[e] ... = Not tested.

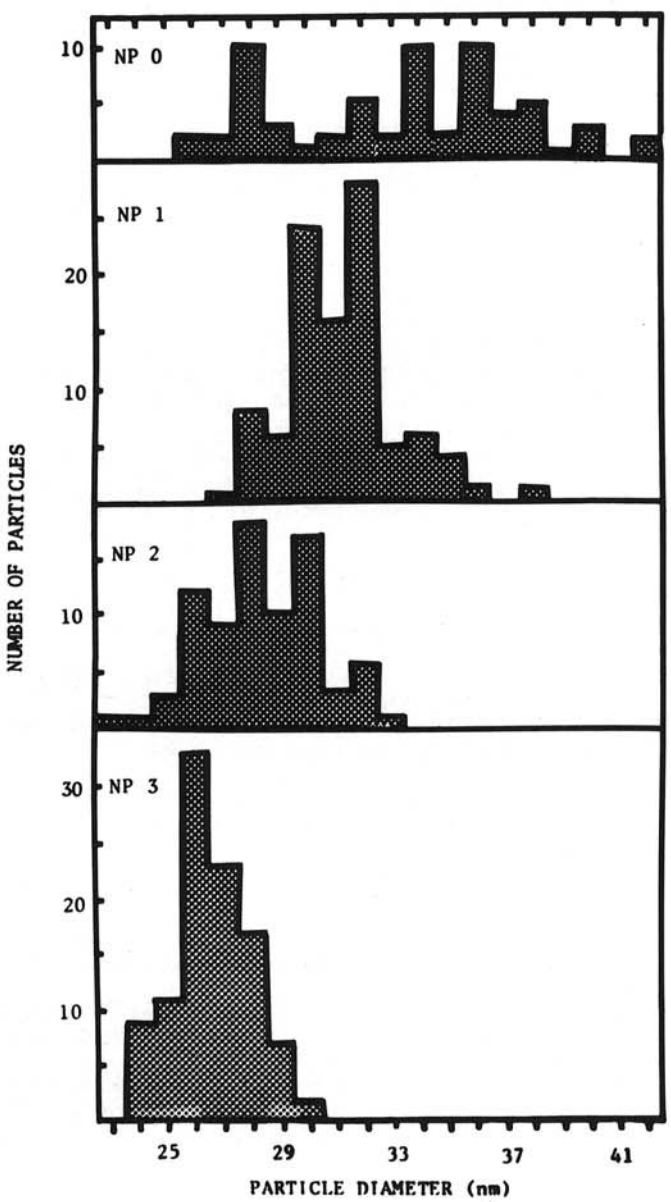

Fig. 4. Histogram of particle diameters for separated NP 0, NP 1, NP 2, and NP 3 of asparagus virus II.

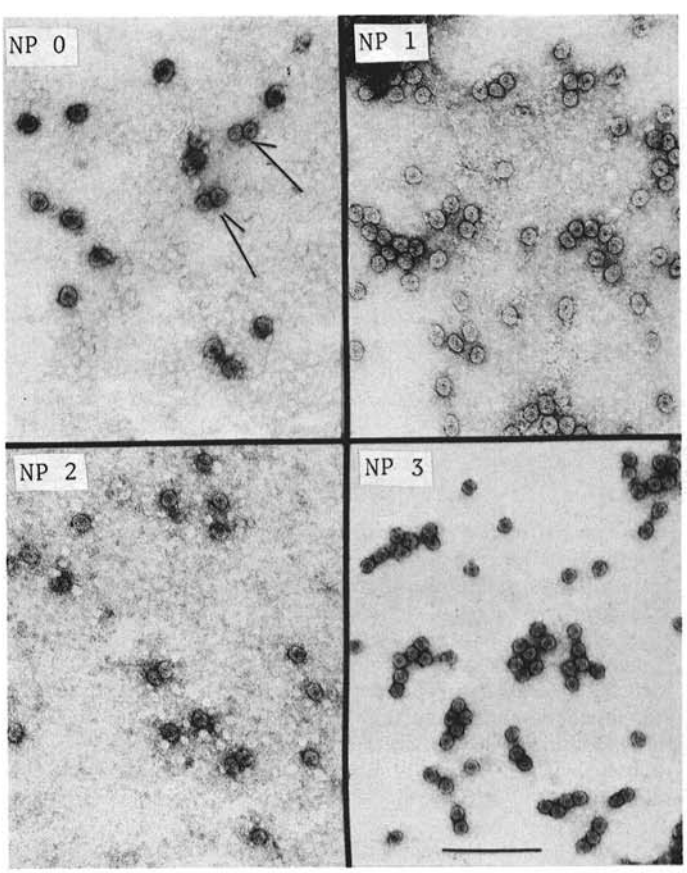

Fig. 5. Electron micrographs of separated NP 0, NP 1, NP 2, and NP 3 of asparagus virus II-P. Arrows indicate dimer particles of NP 0. Approximately ×71,600 (bar represents 200 nm).

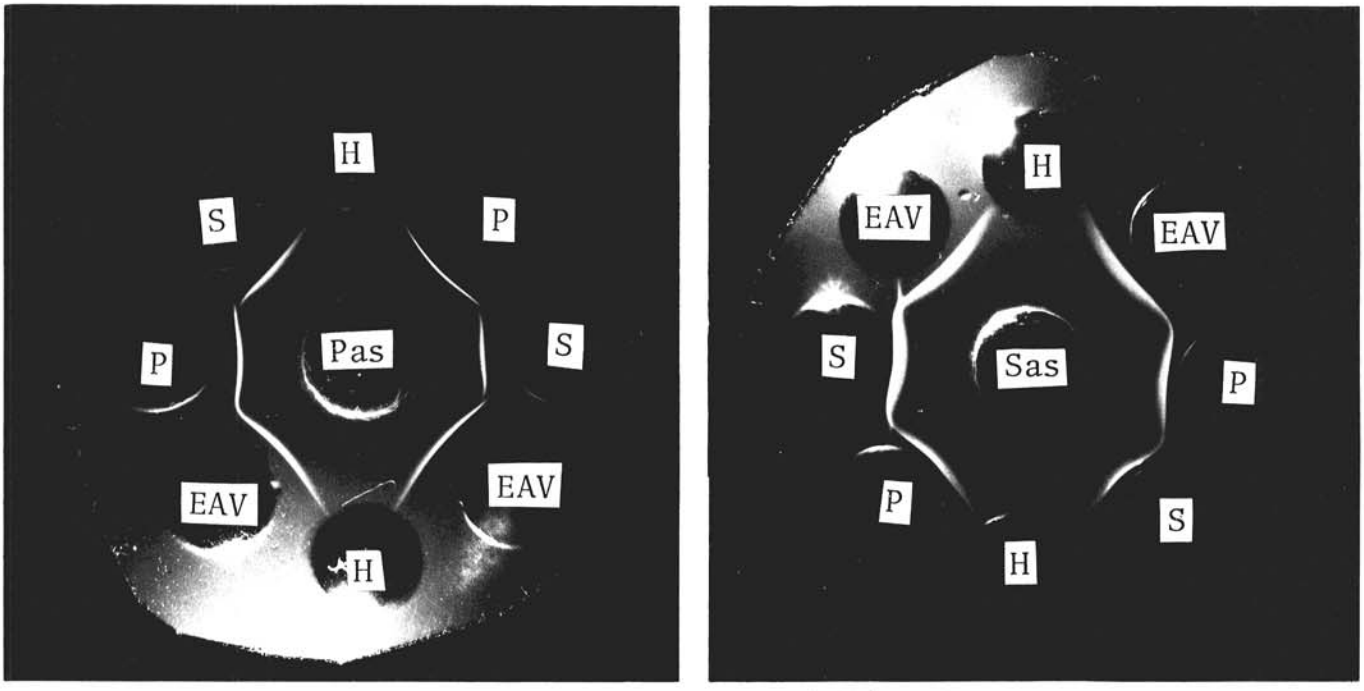

Fig. 6. Serologic relationships between asparagus virus II-P (P), asparagus virus II-S (S), and European asparagus virus II (EAV). Healthy tobacco leaf tissue (H) prepared in the same way as the viruses was used as a control antigen. Center wells were charged with undiluted antisera against asparagus virus II-P (Pas) and asparagus virus II-S (Sas).

California Blackeye. ISCO analyzer profiles after density gradient centrifugation and ultraviolet absorption spectra of purified European AV II resembled those obtained with AV II-P.

In agar gel double diffusion tests, European AV II produced a precipitin line that coalesced with that of AV II-P but formed a spur with AV II-S when it was tested against antisera to AV II-P and AV II-S (Fig. 6).

Serologic relationship of AV II to other isometric viruses. We reported that AV II did not react with antisera prepared against 12 isometric viruses (16), including two ilarviruses, *Prunus* necrotic ringspot virus and tobacco streak virus. In addition, AV II did not react with antisera prepared against prune dwarf virus or Tulare apple mosaic virus. However, in ring interface tests, AV II (40 μg/ml) did react with undiluted but not with a 1:2 dilution of elm mottle virus antiserum provided by A. T. Jones. Furthermore, AV II (40 μg/ml) reacted at final dilutions of 1:40 and 1:10, respectively, with antisera provided by S. M. Garnsey against citrus leaf rugose virus (homologous titer 1:1,260) and citrus variegation virus (homologous titer 1:80). In the same test, both AV II serotypes reacted with their homologous and heterologous antisera at dilutions up to 1:512.

DISCUSSION

The properties of AV II clearly indicate that this virus belongs in the ilarvirus group. Like other ilarviruses, purified AV II preparations contain three major isometric or quasi-isometric, serologically identical nucleoprotein components of different sizes, all of which were required for maximum infectivity. However, any combination of two major components resulted in some infectivity, with NP 1 + NP 2 showing the most infectivity of the three two-component combinations. In this respect, our results are similar to those obtained with Tulare apple mosaic virus (13), citrus leaf rugose virus (4,6), and *Prunus* necrotic ringspot virus (14) and suggest that AV II contains a multipartite genome, as do other ilarviruses (4–8, 12).

Although AV II was not related serologically to several ilarviruses, we did observe weak relationships with elm mosaic, citrus leaf rugose, and citrus variegation viruses, all viruses infecting woody plants. This apparent relationship will be examined in more detail.

Under greenhouse and growth chamber conditions, no symptoms were observed on asparagus, the principal host of AV II. However, preliminary field observations over 2 yr suggest that AV II-infected plants may be less vigorous and possibly less productive than healthy plants. A replicate field has been planted to determine the long-term effects of this virus on productivity. The fact that AV II is readily transmitted through asparagus seed and appears to be widespread in commercial plantings throughout Washington suggests that this virus may be in part associated with a general decline in asparagus yields in the state.

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