Purification and Properties of a Tymovirus from Abelia

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ABSTRACT

A tymovirus was isolated from *Abelia grandiflora* on the grounds of the station by ultracentrifugation of clarified flower extracts. Although the virus was latent in the source abelia, it was isolated several times during the 2-year study. In host range and symptomatology, the virus was similar to the Andean potato latent strain of eggplant mosaic virus. High yields of the purified virus and top component mixture were obtained from fresh *Datura stramonium* leaves when clarified with bentonite or with chloroform. Antiserum produced in rabbits reached a titer of 1:4,096. RNA content of the bottom component was 38 to 40%, determined by nitrogen-phosphorus ratio and by dry weight-optical density-phosphorus content procedures. The ratio of bases was A = 23.8%; U = 29.1%; G = 13.6%; and C = 33.5%. Sedimentation coefficients were $S_{20,w}$ = 54S and 114S for the top and bottom components, respectively. Serologically, the virus was closely related, but not identical with, eggplant mosaic or Andean potato latent viruses. More distant serological relationships were found with belladonna mottle, physalis mottle, dulcamara mottle, ononis yellow mosaic, wild cucumber mosaic, and cacao yellow mosaic viruses. Serological properties suggest that the virus should be regarded as Abelia latent strain of eggplant mosaic virus.

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Additional key words: immunoelectrophoresis, ribonucleic acid, specific extinction coefficient, RNase, classification.

We isolated a virus from the petals of apparently healthy *Abelia grandiflora* Rehd. located on the grounds of the Plant Introduction Station during routine indexing tests. This report gives a simple purification procedure and information on serological relationships of the virus and describes some of the physical and chemical properties of the virus and its ribonucleic acid (RNA). Because of many similarities with the type strain of eggplant mosaic virus (EMV) and with Andean potato latent virus, recently considered to be a strain of EMV (EMV-APLV) (4), we propose the name Abelia latent strain of eggplant mosaic (EMV-AL).

MATERIALS AND METHODS.—Abelia latent strain of EMV was extracted by blending fresh abelia flowers in 0.1 M K_2HPO_4 buffer containing 0.02 M 2-mercaptoethanol and chloroform in the ratio of 1:5:0.2, centrifuging at 8,000 *g* to clarify, and then at 100,000 *g* to concentrate the virus. Resuspended pellets were rubbed onto *Chenopodium quinoa* Willd. along with buffer controls. Antisera to tymoviruses were kindly supplied by R. Woods, dulcamara mottle (DMV) (5); L. Givord, okra mosaic (OkMV) (6); H. Moline, physalis mottle (PhMV) (14); A. Granett, plantago mottle (PlMV) (7); or they were from our own stock. Antisera to wild cucumber mosaic (WCuMV), Desmodium yellow mottle (DesYMV) (16), and turnip yellow mosaic viruses (TYMV) were obtained from the American Type Culture Collection (ATCC) as nos. 9, 12, and 24, respectively. Twenty-two other antisera used in this study were obtained from D. Walkey, B. Kassanis, M. Hollings (England); R. Bercks, C. Wetter (Germany); D. Maat (Holland); or the ATCC; or they were produced in our laboratory.

Sources of pancreatic ribonuclease, anion exchange resin for base ratio studies, other specialized chemicals, and descriptions of equipment used in this study are the same as reported (9, 18).

EMV-AL was maintained in *Datura stramonium* L. and *Gomphrena globosa* L. An experimental host range and some properties of EMV-AL were determined by described techniques (18). The source of virus for host range studies was infectious sap of *Petunia hybrida* Vilm., *C. quinoa*, or *Physalis peruviana* L. Inoculated symptomless experimental species were back-indexed on *C. quinoa*. The source of EMV-AL for thermal inactivation, dilution end point, and aging in-vitro experiments was sap-extracted from *D. stramonium* inoculated 2-3 weeks earlier. Treatments were bioassayed on *C. quinoa*.

Purification.—Fresh leaf tissue of *D. stramonium* plants inoculated 2-3 weeks earlier with EMV-AL was blended in 0.025 M sodium phosphate buffer (pH 7.2) containing 0.02 M 2-mercaptoethanol and cold chloroform at the ratio of 1:3:0.3. The slurry was centrifuged at 12,000 *g* for 10 minutes. The clear yellow supernatant fluid was then centrifuged at 105,000 *g* for 75 minutes.

Infectious *D. stramonium* sap was also clarified by Mg-treated bentonite (18) instead of chloroform. Usually 8 ml/100 g of leaf tissue, added after the first low-speed centrifugation, were required to remove most of the green color upon recentrifugation. The faint yellow supernatant was then centrifuged at 105,000 *g*, to pellet the virus.

The high-speed-pelleted virus of both procedures was covered with 0.01 M disodium ethylenediaminetetraacetate (EDTA) (pH 6.0) and held at 4 C overnight. After resuspension the bluish opalescent preparation was centrifuged at 10,000 *g* to remove plant materials. The virus was further purified, and the two components were separated by the Spinco rate zonal rotor TI 14. Three hundred to 500 mg of partially purified virus were layered onto 550 ml of a 10% to 30% equivolumetric sucrose gradient (17), along with 100 ml 0.02 M potassium phosphate buffer overlay (pH 7.0) and centrifuged for 2 hours at 45,000 rpm. Fractions containing only the bottom component were combined, most of the sucrose was removed by dialysis in 0.02 M potassium phosphate, and then the fractions were centrifuged at 105,000 *g* 4 hours to reconcentrate the virus. The final pellets were resuspended in 0.02 M acetate buffer (pH 6.0) or in 0.01 M EDTA (pH 6), dialyzed again to remove traces of sucrose, and used in phosphorus and nitrogen determinations, for dry weight-optical density studies or for RNA extraction.

Immunization and serology.—For antiserum to EMV-AL, we immunized a rabbit once a week with 2-4 mg virus in 1 ml 0.02 M potassium phosphate buffer emulsified with 1 ml Freund's incomplete adjuvant. The rabbit received four intramuscular injections. Bleedings began with the third injection. Serological tests were performed by the gel double-diffusion method, in plates with wells 5 mm apart in 0.75% Ionagar no. 2 in water containing 0.02% sodium azide. Immunoelectrophoresis was done with 1% agarose gel containing 230 μM sodium *p*-chloromercuribenzoate and 0.05 M tris(hydroxymethyl)aminomethane-HCl buffer (pH 8.0) (11).

RNA content determined from extinction coefficient and phosphorus content.—These values were obtained as one of the means of calculating the percentage of RNA in EMV-AL. The specific extinction coefficient (i.e., OD_{260} of 1% virus solution) of EMV-AL was determined with dry-weight optical-density data from three experiments on the purified bottom component as previously described (18). Likewise, the phosphorus content was established on the basis of eight experiments, with only the bottom component of carefully determined optical density (18), by the method of Knight and Woody (10).

RNA content by determination of total nitrogen and phosphorus.—Knowledge of the ratio of N to P in virus provides another method for calculating percentage of RNA (8). Nitrogen was determined by the micro-Kjeldahl method (8) and phosphorus by Knight and Woody's procedure (10). Multiple determinations of P and N were made simultaneously on a stock solution of 1.5 to 2.5 mg/ml bottom component in 0.02 M acetate buffer, along with solutions of known content of inorganic phosphorus. Cucumber mosaic virus and TYMV, each with known RNA contents, were also included in some experiments as controls.

Electron microscopy.—Homogeneity of purified preparation or of separated components was ascertained with the electron microscope. Samples in 0.01 M EDTA were placed on Formvar-coated grids and stained with 2% phosphotungstic acid adjusted to pH 6.8 KOH. Particles were measured and the sizes were calculated.

RNA extraction and infectivity studies.—RNA was extracted with an ice bath, cold chemicals, and rotors throughout the procedure. The virus, at 10 to 20 mg/ml, was mixed with a final concentration of 0.05 M neutral sodium phosphate buffer and 3% sodium dodecylsulfate (SDS) containing a trace of bentonite. The rest of this phenol extraction procedure has been described (13, 18). The final RNA pellet was dissolved in water, read in the spectrophotometer to ascertain freedom of phenol and to

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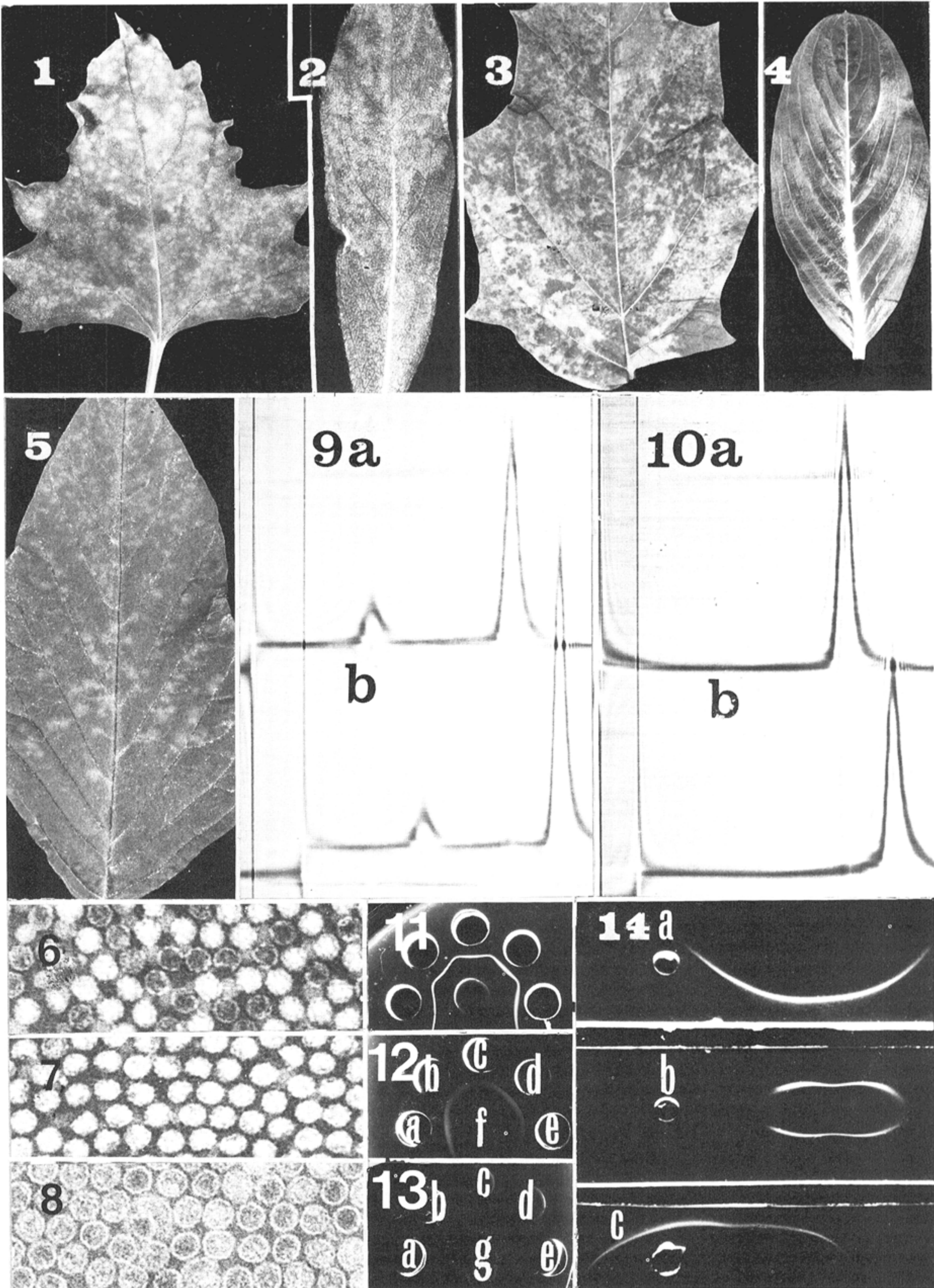
Fig. 1-14. Abelia latent strain of eggplant mosaic virus **1-2)** in inoculated leaf of **1)** *Chenopodium quinoa* and **2)** *Gomphrena globosa*; **3-4)** in systemically infected leaf of **3)** *Datura stramonium*, and **4)** *Vinca rosea*; and **5)** in inoculated leaf of *Amaranthus caudatus*. **6-8)** Particles of EMV-AL × 181,000 from **6)** top and bottom components of density gradient preparations, and **7-8)** separated components, **7)** bottom and **8)** top. **9-10)** Purified (a) EMV-AL and (b) turnip yellow mosaic virus showing **9)** both components and **10)** bottom component only. Sedimentation left to right 12 minutes after analytical centrifuge attained 29,500 rpm. Concentration of viruses is 0.3% in 0.02 M potassium phosphate buffer (pH 7.0) at 20 C. **11-13)** Agar gel diffusion showing **11)** single serological band between EMV-AL antiserum, center well, and homologous virus at five concentrations (5 to 0.25 mg/ml) in peripheral wells. **12-13)** Serological reaction between antisera to a) EMV-AL, b) PhMV, c) BelMV, d) EMV, e) EMV-APLV, and f) antigen EMV-AL in *D. stramonium* sap and g) virus-free *D. stramonium* sap. The intensity and irregular curvature of the band in Fig. 12 suggests differences in relationships among the five viruses. **14(a to c)** Immunoelectrophoresis of **a)** EMV-APLV, **b)** EMV-AL, and **c)** EMV in 1% agarose gels containing 230 μM *p*-mercuribenzoic acid and 0.05 M Tris HCl buffer (pH 8.0). Serum channels contained antiserum to EMV-AL. Anode, left; cathode, right.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determine concentration of RNA ($E^{0.01}_{260} = 250$), and then stored frozen.

We determined the infectivity of the extracted RNA as a function of its concentration compared with an equal weight of RNA in intact virus. Hence, each of the virus dilutions in the tenfold series contained about 2.5 times the weight of the corresponding RNA dilution. Dilutions were made in 0.025 M potassium phosphate buffer. To study sensitivity to RNase, we mixed the enzyme in a final concentration of 0.1 μg/ml with RNA and virus, incubated it in an ice bath, and bioassayed it at 10-minute intervals. *C. quinoa* was the most sensitive assay host by systemic infection.

Base ratio.—RNA (1-2 mg) was digested, the nucleotide mixture was layered onto a column of anion exchange resin, and was separated by elution as described (18). The eluent was monitored with an ISCO (Instrumentation Specialties Company, 4700 Superior, Lincoln, NB 68504) system. The bases were collected separately, and the concentrations were determined with the spectrophotometer. The percentage of phosphorus in the RNA was calculated from this data. RNA of broad bean mottle virus was used as a control.

RESULTS.—*Symptoms and host range.* —We saw no obvious virus-induced symptoms in the source plant *Abelia* during the six years.

The most successful procedure for isolating EMV-AL from the original plant involved ultracentrifugation. Petal tissue was blended in five volumes of 0.1 M K_2HPO_4 and centrifuged at 8,000 *g*, and the supernatant was recentrifuged at 105,000 *g* to concentrate the virus. In three such trials, five of six, three of four, and no *C. quinoa* plants developed symptoms within 10 days. All control plants, rubbed only with buffer, remained healthy.

The experimental host range and symptoms incited by EMV-AL were as follows: There were chlorotic local lesions and systemic mosaic in inoculated plants of *C. amaranticolor* Coste & Reyn., *C. quinoa* (Fig. 1), *G. globosa* (Fig. 2), *Nicotiana clevelandii* Gray and *N. glutinosa* L. *Petunia hybrida* and *Acer palmatum* Thunb. developed mild chlorotic veinbanding. EMV-AL incited systemic mosaic without local symptoms in *Chenopodium murale* L., *Celosia argentea* var. *cristata* (L.) Ktze., *D. stramonium* (Fig. 3), *D. metel* L., *Glycine max* (L.) Merr., *Lycopersicon esculentum* Mill., *Nicotiana rustica* L. *Physalis peruviana*, *Sesamum indicum* L., *Torenia fournieri* Lind., and *Vinca rosea* L. (Fig. 4). Chlorotic local lesions without systemic symptoms were observed in *Amaranthus caudatus* L. (Fig. 5), *Momordica balsamina* L., *Gossypium hirsutum* L., *Hibiscus coccineus* Walt., and *Tetragonia tetragonioides* (Pall.) Ktze. (*T. expansa* Thunb.). Local and systemic infection without symptoms occurred in *Atropa belladonna* L., *Ageratum houstonianum* Mill., *Brassica pekinensis* (Lour.) Rupr., *Coleus blumei* Benth., *Hordeum vulgare* L., *Lobelia erinus* L., *Scrophularia marilandica* L., *Sida rhombifolia* L., *Solanum tuberosum* L. 'Saco' and 'A-6', *S. melongena* L., *Tithonia rotundifolia* (Mill.) S. F. Blake, *Vicea faba* L., and *Rhododendron* sp. (Azalea).

EMV-AL did not infect *Antirrhinum majus* L., *Beta vulgaris* L., *Brassica rapa* L., *Capsicum frutescens* L., *Calendula officinalis* L., *Coreopsis grandiflora* Hogg, *Cucumis sativus* L., *Cucurbita maxima* Duchesne, *C. pepo* L., *Cynoglossum amabile* Stapf & Drumm., *Dahlia rosea* Cav., *Dianthus barbatus* L., *Dioscorea composita* Hemsl., *Ipomoea purpurea* (L.) Roth., *Pelargonium zonale* (L.) L'Herit. ex Ait., *Phaseolus vulgaris* L., *Sesbania exaltata* (Raf.) Cory., *Spinacia oleracea* L., *Tagetes tenuifolia* Cav. (*T. signata* Bartl.), *Theobroma cacao* L., *Vigna unguiculata* (L.) Walp. cv. Blackeye, *Zea mays* L., or *Zinnia elegans* Jacq.

The most distinctive symptoms occurred in *C. quinoa* and in *D. stramonium* 2-4 weeks after inoculation in the form of bright yellow-gold chlorosis in scattered *C. quinoa* leaves (Fig. 1) and uniform yellow mosaic in *D. stramonium* leaves (Fig. 3) progressively developing from the leaf base. None of 10 abelia plants, inoculated when 15-cm tall, developed virus symptoms during the subsequent eight months, compared with uninoculated plants that remained symptomless. Virus was recovered with difficulty from four of these plants. We included the less-commonly-used species (in our laboratory) *Atropa belladonna*, to aid in differentiating EMV-AL from belladonna mottle virus (BelMV) (15); *Brassica pekinensis*, for TYMV; *Cucurbita pepo*, for WCuMV; *Hibiscus coccineus*, for OkMV (6); *Physalis peruviana*, for PhMV (14); *Scrophularia marilandica*, for Scrophularia mottle virus (ScrMV) (2); *Solanum melongena*, for EMV; *S. tuberosum*, for APLV (5); and *Theobroma cacao*, for cacao yellow mosaic (CaYMV).

Symptoms incited in *N. clevelandii* by EMV-AL were compared in simultaneous tests with those incited by the type strain and by EMV-APLV at Braunschweig. EMV-AL incited symptoms of moderate severity while EMV incited severe and EMV-APLV incited mild symptoms.

Stability of EMV-AL in vitro.—Sap extracted from *D. stramonium* and assayed on *C. quinoa* remained infectious when heated to 74 C for 10 minutes, incubated at room temperature for 2 months in 0.025 M phosphate buffer and 0.2% NaN_3, or diluted 10^{-8}. However, heating to 78 C or diluting to 10^{-9} resulted in no infection. Purified virus incited a few lesions in concentrations as low as 1.3×10^{-4} μg/ml.

Purification and physiochemical properties.—Each of the clarification procedures resulted in high yields of infectious virus containing intact and RNA-devoid particles (Fig. 6). Rate-zonal gradient centrifugation produced two well-separated bands. Figures 7 and 8 show particles of the separated components, whose particles averaged 25 nm. Yields of purified virus ranged from 0.75 mg to 2.6 mg/g tissue when sap was clarified with bentonite and from 3.1 to 4.2 mg/g tissues when chloroform was used. Yields were estimated with the specific extinction coefficient $E^{0.01}_{260} = 90$ OD and adjusted upward to account for the top component. The average 260:240 absorbency ratio of purified mixed components was 1.42 and the 260:280 ratio was 1.61.

The ultraviolet-light-absorbing spectrum of the reconcentrated top component was typical of that for protein, with maximum absorption at 280 and minimum at 250. That of the bottom component averaged a 260:240 ratio of 1.52 and a 260:280 ratio of 1.71. Sedimentation behavior of the combined components was compared with those of turnip yellow mosaic virus simultaneously in the analytical ultracentrifuge, by the use of Schlieren optics. Sedimentation patterns of the two viruses were

TABLE 1. Serological relationships between abelia latent strain of eggplant mosaic virus and four other tymoviruses[a]

Antiserum to[c]	Antigen[b]				
	EMV-AL	EMV	EMV-APLV	BelMV	PhMV
EMV-AL	2,048	512	256	32	. . .
EMV-AL	1,024	256	128	64	. . .
EMV-type strain	64	256	16	2	. . .
EMV-type strain	64	128	16	4	. . .
EMV-APLV	16	8	256	2	. . .
EMV-APLV	2,048	256	8,192	8	. . .
BelMV	32	16	64	512	. . .
PhMV	64	. . .	. . .	. . .	512

[a] = Reciprocal values of antisera dilutions.
[b] = EMV = eggplant mosaic type strain, EMV-AL = strain from abelia, EMV-APLV = Andean potato latent strain, BelMV = belladonna mottle, PhMV = physalis mottle.
[c] = Two sources of each EMV antisera were selected because of their capacity to differentiate between closely related viruses.

virtually identical (Fig. 9, 10). Sedimentation coefficients of EMV-AL were $S_{20,w}$ (top) = 54 S and (bottom) = 114 S, and those for TYMV were 54 S and 113 S, both at a concentration of about 3 mg virus/ml.

Serology.—Antiserum to EMV-AL collected 2 weeks after the first injection of virus had an antibody titer of 1:128 and rose to 1:4,096 by the 8th week (Fig. 11). Antiserum diluted more than 1:4 did not react with healthy plant juice. Serology proved to be a rapid and reliable technique to detect presence of EMV-AL in crude sap of herbaceous experimental plant species. However, no reactions were obtained with extracted abelia leaf juice in gel diffusion tests.

Purified preparations of EMV-AL at concentrations from 1-8 mg virus/ml reacted with antisera to several viruses in the tymovirus group; i.e., EMV (type strain), EMV-APLV, BelMV, PhMV (Figs. 12, 13), DMV, WCuMV, CaYMV, and ononis yellow mosaic virus(es). The reactions with antisera to the latter four viruses were weak. No reactions were observed with antisera to TYMV, DesYMV, PlMV or OkMV, with antisera to viruses in the comovirus group; i.e., squash mosaic, cowpea mosaic, radish mosaic, and bean pod mottle viruses, and to 24 other common isometric viruses. Many of these observations were confirmed by A. Brunt in England.

Comparison of the homologous and heterologous titers of antisera to EMV-AL, EMV, EMV-APLV, BelMV, and PhMV (Table 1) indicated that EMV-AL was not identical with any of the other isolates, although the relationship with EMV type strain and EMV-APLV is close. The antisera to EMV and EMV-APLV were selected from previous studies (12) by their capacity to differentiate especially well between these two closely related forms. The data of Table 1 indicate that EMV-AL serologically should be grouped between the type strain of EMV and EMV-APLV.

EMV and EMV-APLV can be differentiated by immunoelectrophoresis after treatment with *p*-chloromeribenzoic acid (*p*-CMB) (11). The migration of EMV-AL after treatment with *p*-CMB resembled more that of EMV-APLV than that of EMV (Fig. 14).

Properties of bottom component.—By use of the average of several OD_{260} and dry-weight data, the specific extinction coefficient of bottom component was determined to be $E_{260}^{0.01} = 90.0$ (1-cm light path). Average phosphorus content of virus from eight determinations was 3.81%. Since all of the P is in the RNA, percentage of RNA was determined by the formula:

$$\% \text{ RNA} = \frac{\% \text{ P in virus}}{\% \text{ P in RNA}} = \frac{3.81}{9.78} = 39.0\%$$

Percentage of P in the RNA was calculated from the average base ratio data of six experiments: A = 23.8%, U = 29.1%, G = 13.6%, and C = 33.5%. The purine/pyrimidine ratio was 0.52.

Percentage of RNA, also obtained from the ratio of nitrogen to phosphorus, averaged 4.022 from three determinations. With this value, the percentage of RNA was calculated by the formula of Kaper and Litjens (8). Since the amino acid composition of EMV-AL protein is unknown, we assumed that its nitrogen content could range from 15% to 15.7%. When each of these values was used in the formula, a range of 38% to 40% RNA was obtained for EMV-AL.

Ribonucleic acid.—EMV-AL RNA produced a typical ultraviolet light absorption curve with a 260:280 ratio of 2.06. Yields of extracted RNA ranged from 60% to 75% of the total calculated nucleic acid in the source samples. When SDS was not used, yields ranged considerably lower. When assayed on *C. quinoa*, RNA was infectious in concentrations as low as 4.6×10^{-5} μg/ml. No infectivity remained when 4.6×10^{-2} μg/ml RNA was treated with 0.1 μg/ml pancreatic ribonuclease and held at 0 C for 5 minutes. RNA without the enzyme and intact virus with RNase remained highly infectious throughout the 2 hours of the experiments.

DISCUSSION.—EMV-AL shares many properties with the other viruses in the tymovirus group; e.g., it occurs in high concentration in herbaceous host plants, can readily be purified with high yields, and is a two-component virus. Its stability in vitro, particle morphology, sedimentation behavior, RNA content, and the base composition of its RNA also relate EMV-AL to that group. By its serological properties, it should be grouped between EMV type strain and EMV-APLV strain in the classification scheme of Koenig and Givord (12). Most host reactions observed with EMV-AL were similar to those reported for EMV type strain and EMV-APLV (3). However, they differ somewhat; e.g., EMV-AL did not produce local lesions in *Datura* or tomato, whereas chlorotic lesions and systemic mosaic were incited in *Gomphrena*.

The finding of *Acer palmatum*, *Amaranthus caudatus*, *Momordica balsamina*, *Gossypium hirsutum*, *Hibiscus coccineus*, *Tetragonia tetragonioides*, *Celosia argentea*, *Glycine max*, *Sesamum indicum*, and others as hosts extends the known host range of EMV strains. It is not known whether these hosts can also be infected by the type strain of EMV and EMV-APLV.

This is the first report of an EMV strain occurring in the United States. While the origin of this abelia germ plasm is unclear, it has not been a plant introduction. Hence geographical distribution of EMV strains are no longer confined to South America, Trinidad, and the West Indies.

We do not know how widespread EMV-AL is in abelias. We know only that the virus is difficult to isolate from the source plant. While flowers were superior to leaves as a source from which to extract this highly infectious virus, our results were erratic among the trials. A precedence for this phenomenon wherein it is difficult to isolate a highly infectious virus from its natural woody host was reported for tobacco mosaic virus in grapevine (1).

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