Conversion of Comovirus Electrophoretic Forms by Leaf-Feeding Beetles

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ABSTRACT

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In vitro and in vivo conversion of comovirus electrophoretic forms by regurgitant of leaf-feeding beetles was demonstrated by agarose gel electrophoresis of virus particles. Converted and unconverted electrophoretic forms of three comoviruses–bean pod mottle, cowpea mosaic, or squash mosaic virus–consistently occurred in either infected tissue or purified virus preparations. Incubation of each virus with regurgitant from Mexican bean beetles, bean leaf beetles, or spotted cucumber beetles changed the form of each virus. Only the converted form was found in regurgitant from beetles that were fed on infected tissue or on purified bean pod mottle virus in 5% sucrose. This conversion is similar to the change that occurs when the virus is incubated with protease solutions. Beetle regurgitant contained approximately ten times more protease activity than bean sap. Bean leaf beetle regurgitant contained more protease than that of the Mexican bean beetle or spotted cucumber beetle.

Additional keywords: beetle transmission of plant viruses.

Regurgitant from leaf-feeding beetles, principally its ribonuclease component, differentiates between beetle-transmitted and non-beetle-transmitted viruses by limiting the availability of infection sites near the area wounded by feeding (8,9,16). Beetle-transmitted viruses are able to translocate to plant tissue that is unaffected by ribonuclease and to establish primary infection sites in unwounded cells (7). Vectors, in general, are not known to alter the physical properties of the viruses which they transmit.

Comoviruses, a major group of beetle-transmitted viruses, contain two forms with differing electrophoretic mobilities. Using polyacrylamide gel electrophoresis of virus particles, Bancroft (4) demonstrated two electrophoretic forms, slow and fast, in preparations of bean pod mottle virus (BPMV). Agrawal (1) also found two forms of cowpea mosaic virus (CPMV). Differences in the forms reflect postassembly cleavage of the capsid protein. Niblett and Semancik (18) demonstrated in vitro proteolytic conversion of one form of CPMV or BPMV to the other form. The slow form of CPMV was converted to the fast form, and the fast form of BPMV was converted to the slow by proteolysis. Geelen et al (6) found that the smaller capsid protein (S) of the CPMV fast form is 2.5 kDa (approximately 22–25 amino acids) smaller than the S protein of the slow form and that the loss of amino acids correlates with conversion of slow to fast.

Association of vectors and viruses depends on the interaction of virus-coded proteins with vector surfaces (11). This interaction

may lead to passage through vector membranes, as proposed for luteoviruses (10), or the attachment and release of virus particles on vector surfaces, as found in nematode-transmitted viruses (11). Regardless of the mechanism, virus surface properties are important. Comoviruses with two forms differing in surface charge provide a unique system to study the interaction of the virus with the beetle and with regurgitant.

The objectives of this research were to study the electrophoretic forms of three comoviruses, BPMV, CPMV, and squash mosaic virus (SqMV) by agarose gel electrophoresis of virus particles (19) to analyze the in vitro effect of beetle regurgitant on the electrophoretic forms of comoviruses, to measure the proteolytic activity of regurgitant, and to determine in vivo the effect of retention by the beetle on comovirus electrophoretic forms. This study presents evidence of modification of viral properties during its retention by the beetle.

MATERIALS AND METHODS

Beetles and regurgitant collection. Mexican bean beetles, *Epilachna varivestis* Mulsant, were reared in the laboratory on *Phaseolus vulgaris* L. 'Pinto'. Spotted cucumber beetles, *Diabrotica undecimpunctata howardii* Barber, and bean leaf beetles, *Cerotoma trifurcata* (Förster) were collected from the field and maintained on healthy Pinto bean for a minimum of 5 days to ensure that they were nonviruliferous. Beetles were induced to regurgitate by holding them between the thumb and forefinger and teasing their mouthparts with a glass capillary tube into which the emitted regurgitant was collected. Collected regurgitant was pooled, chilled in ice water during collection, and used immediately.

Viruses and virus purification. Three comoviruses, BPMV, CPMV, and SqMV, were studied. BPMV and CPMV were purified from *P. vulgaris* L. 'Black Valentine' 10–14 days after inoculation. SqMV was propagated in *Cucurbita pepo* L. 'Early Prolific Straightneck'. Viruses were purified by chloroform-butanol extraction followed by two to three cycles of differential centrifugation. High-speed pellets were resuspended in 0.01 M phosphate buffer (pH 7.2) except for BPMV, which was resuspended in 0.1 M phosphate buffer (pH 7.2).

Separation of electrophoretic forms by agarose gel electrophoresis. BPMV or CPMV forms were separated by electrophoresis using the method of Serwer et al (19) in a 6.5 × 10.2 cm submerged mini-slab gel apparatus (Bio-Rad Laboratories, Richmond, CA) at 2 V/cm for 7 hr or 1 V/cm for 14 hr. Separation of SqMV was carried out at 2 V/cm for 16 hr. To separate large quantities of BPMV or CPMV for recovery of electrophoretic forms from agarose gels, a 20 × 25 cm horizontal, submerged gel apparatus (Bethesda Research Laboratories, Gaithersburg, MD) was utilized at 1 V/cm for 18 hr.

Virus bands were visualized by staining for 1 hr in ethidium bromide (0.5 μg/ml in 0.05 M sodium phosphate buffer, pH 7.2, containing 1 mM ethylenediaminetetraacetic acid) followed by destaining in distilled water for 2 hr. Gels were photographed on an ultraviolet transilluminator (302 nm) with both a 23A and 9 Wratten filter (Eastman Kodak Company, Rochester, NY) using Polaroid Type 655 film. Proteins were visualized by the Coomassie Brilliant Blue method of Serwer et al (19).

Recovery of separated electrophoretic forms of BPMV and CPMV from agarose gels. Unstained virus-containing bands were cut from agarose gels and macerated. Gel pieces were suspended in electrophoresis buffer and agitated overnight. The supernatant was separated from the agarose pieces by filtration through Miracloth. The viruses were concentrated by one or two cycles of differential centrifugation. The final pellets of the electrophoretic forms were resuspended in the appropriate buffer, and the integrity and purity of the extracted electrophoretic forms were confirmed by electron microscopy of negatively stained preparations and by agarose gel electrophoresis. The separated forms of BPMV were tested for infectivity by mechanical inoculation on Carborundum-dusted primary leaves of Pinto bean, and infectivity of both forms during simulated beetle feeding was determined by gross-wound inoculation (8) of Black Valentine bean.

In vitro conversion of virus particles. Purified virus (10 mg/ml) was combined with an equal volume of either regurgitant, buffer, or protease [trypsin (EC 3.4.21.4) or chymotrypsin (EC 3.4.21.1) (Sigma Chemical Company) at 125 μg/ml]. After incubation for 2 hr at 37 C, an equal volume of sample buffer (50 mM sodium phosphate buffer, pH 7.2, containing 1 mM magnesium chloride, 8% sucrose, and 400 μg/ml bromophenol blue) was added. Treated preparations were analyzed by agarose gel electrophoresis as described above.

In vivo conversion of virus particles by beetles. Because in vitro conversion may not accurately reflect events occurring within the beetle, regurgitant from beetles fed on infected bean or squash leaves was analyzed electrophoretically. Regurgitant from beetles fed on healthy leaves served as a control. BPMV- or CPMV-infected bean tissue served as a virus source for Mexican bean beetles or bean leaf beetles. SqMV-infected squash tissue was tested only with spotted cucumber beetles since other beetles would not feed on squash. After 24 hr, regurgitant from 50–100 beetles was collected, pooled, and immediately analyzed by electrophoresis. Virus purified from the same source of infected plants on which the beetles fed served as a control to determine the electrophoretic forms present in the source tissue.

To eliminate the possible conversion by ingested plant enzymes, beetles were fed with purified virus (drop-drink method). For this method, 50 μl of BPMV (5 mg/ml in 5% sucrose and 0.1 M phosphate buffer, pH 7.2) was placed in a petri dish with five bean leaf beetles that had been starved for 24 hr. Control beetles were fed a solution of 5% sucrose in 0.1 M phosphate buffer (pH 7.2). Beetles had access to the solution for 24 hr prior

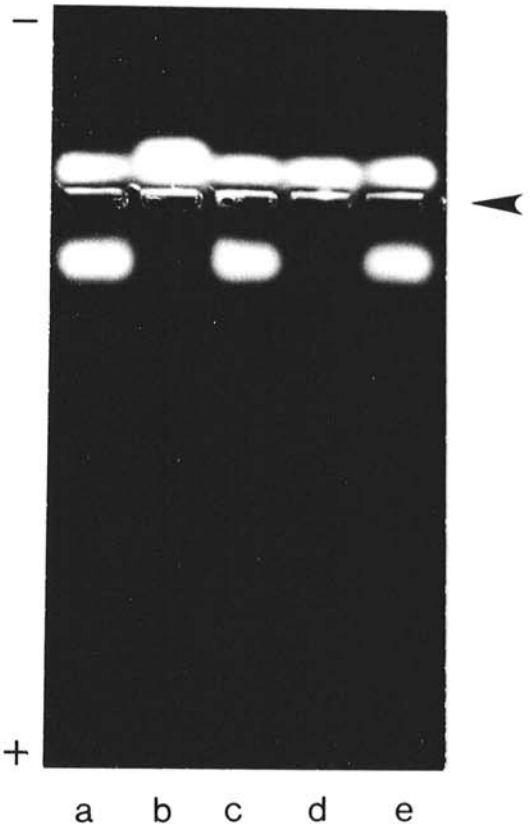

Fig. 1. Conversion of bean pod mottle virus (BPMV) electrophoretic forms by proteases or by Mexican bean beetle regurgitant. BPMV was incubated for 2 hr at 37 C after mixing with an equal volume of the following solutions: Lane a, 0.1 M sodium phosphate buffer (pH 7.2); lane b, trypsin, 125 μg/ml; lane c, chymotrypsin, 125 μg/ml; lane d, Mexican bean beetle regurgitant; and lane e, 0.1 M sodium phosphate buffer (pH 7.2). Each sample (10 μl) was analyzed by whole particle electrophoresis for the number of electrophoretic forms present. Arrowhead indicates the origin of electrophoresis.

to regurgitation. Pooled regurgitant and purified virus from the original feeding solution were immediately analyzed by electrophoresis.

Protease activity in beetle regurgitant. Since the conversion of electrophoretic forms by beetle regurgitant is similar to the proteolytic conversion (18), total proteolytic activity in beetle regurgitant was measured by a modified casein digestion method (15). Pooled beetle regurgitant was diluted with 50 mM Tris-HCl (pH 8.0), and 350 μl aliquots were warmed at 37 C for 5 min. Prewarmed 1% casein (350 μl) was added, and the reaction was terminated after 5 min by adding 10% trichloroacetic acid (700 μl). Reaction mixtures were clarified by filtration, and the absorbance of the filtrate at 280 nm was determined. One unit of protease activity was defined as a change of 0.001 absorbance units per min in 100 μl of undiluted sample. Five separate determinations were completed for each type of regurgitant. Solutions of Pronase*R* (Calbiochem Biochemicals, San Diego, CA), at concentrations of 200, 100, 50, and 25 μg/ml were used to establish the linearity and range of the assay.

Trypsinlike activity was measured by monitoring the hydrolysis of the artificial protease substrate, N*a*-p-tosyl-L-arginine methyl ester, (Sigma Chemical Company) as described by Hummel (12). Assays were conducted as follows: 5 μl aliquots of regurgitant were added to 1.0 ml of 1 mM substrate in 50 mM Tris-HCl (pH 8.0), and the change in absorbance at 247 nm was recorded for 10 min. One unit of trypsinlike activity was defined as a change of 0.001 absorbance units per min at 247 nm divided by 540. Three separate determinations with 3 replicates per determination were done for each type of regurgitant, and activities were calculated using the initial linear portion of the curve.

RESULTS

Agarose gel electrophoresis of virus particles. Agarose gel electrophoresis clearly separated the two electrophoretic forms of each comovirus. Both slow and fast electrophoretic forms of CPMV and SqMV migrated toward the anode; however, only the fast form of BPMV migrated in that direction. The second BPMV electrophoretic form (slow) moved toward the cathode side of the origin (Fig. 1). The electrophoretic forms of CPMV and BPMV were widely separated, but those of SqMV migrated closely together (Fig. 2). A minimum virus concentration of 400 ng per lane could be visualized as bands.

Intact virus particles of BPMV (Fig. 3A and 3B) and CPMV electrophoretic forms were isolated from preparative gels. The forms were pure when analyzed by electrophoresis (Fig. 3C). No detectable differences in infectivity were observed between the two forms of BPMV when inoculated mechanically or by gross wounding.

In vitro conversion of virus particles. When equal volumes of beetle regurgitant and purified virus were incubated for 2 hr at 37 C, only one electrophoretic form could be detected after electrophoresis. This was the same electrophoretic form observed after incubation with the appropriate protease for conversion (Fig. 1). To study conversion, each virus preparation was incubated with regurgitant (Fig. 2). The fast forms of BPMV and SqMV were converted to the slow forms, and the slow form of CPMV was converted to the fast form. This change was also observed when isolated unconverted virus was incubated with regurgitant. Regurgitant from each of the three beetle species used in this study converted each of the viruses, as shown for CPMV in Fig. 4. Regurgitant from the bean leaf beetle and the spotted cucumber beetle was not as effective as regurgitant from the Mexican bean beetle. CPMV was completely converted by Mexican bean beetle regurgitant up to a dilution of 1:2, and the change in form occurred gradually through a number of intermediate steps with increasing dilution (Fig. 5). Two bands which migrated closely together were found in trypsin digests of CPMV. The conversion of BPMV was accomplished by dilutions of Mexican bean beetle regurgitant up to 1:100, but no intermediate forms were observed. This type

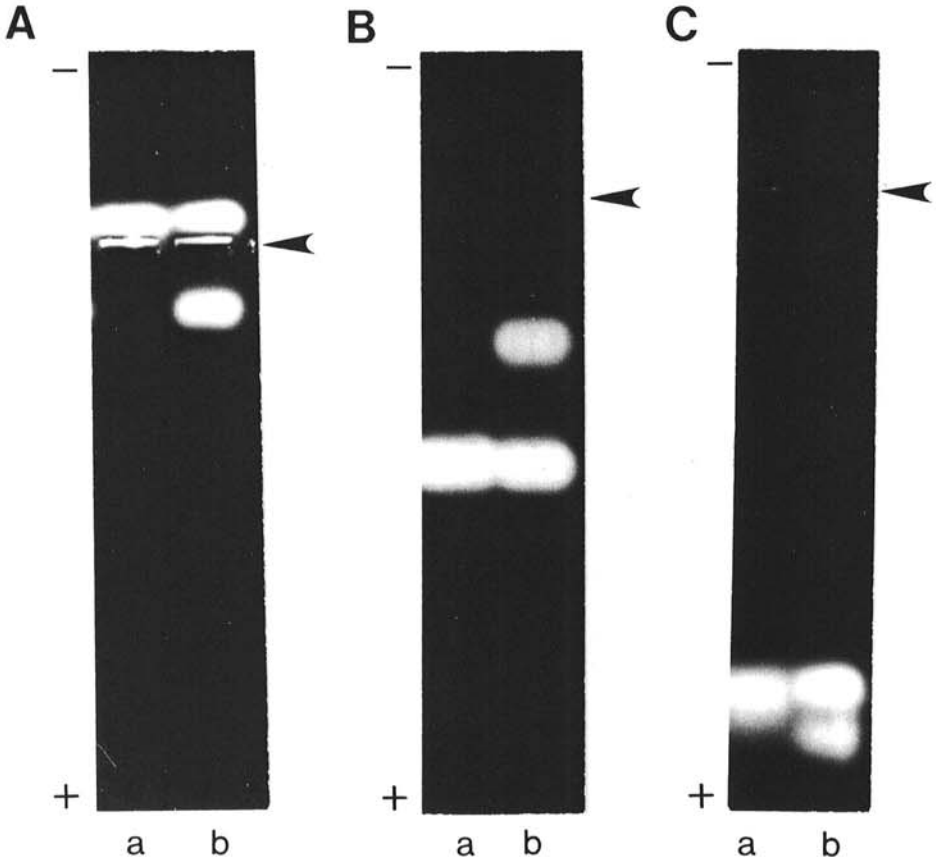

Fig. 2. In vitro conversion of the electrophoretic forms of three comoviruses by Mexican bean beetle regurgitant. **A,** bean pod mottle virus, **B,** cowpea mosaic virus, or **C,** squash mosaic virus was incubated for 2 hr at 37 C with an equal volume of Mexican bean beetle regurgitant (lane a) or phosphate buffer (pH 7.2) (lane b). Ten-μl samples were analyzed for electrophoretic forms. Arrowheads indicate the origins of electrophoresis.

of conversion is similar to the conversion induced by trypsin. No further changes in the converted form of BPMV were observed after incubation with regurgitant for more than 8 hr.

In vivo conversion of virus particles by beetles. Regurgitant from beetles fed on infected plants was analyzed by gel electrophoresis to determine which electrophoretic forms of the virus were present. The regurgitant contained abundant virus. The virus band formed by 10 μl of regurgitant from naturally fed beetles was as intense as the band formed by 10 μl of a 5 mg/ml solution of purified virus. In all combinations tested, beetle regurgitant contained only the converted electrophoretic form of the virus, although the source tissue contained both forms (Fig. 6).

Regurgitant samples from some beetles fed on both healthy and infected tissue produced extraneous nucleoprotein bands (Fig. 6C). Viruslike particles were visualized by electron microscopy of negatively stained regurgitant from beetles fed only on healthy tissue and were also detected by electrophoresis of the regurgitant (Fig. 7). The particles are assumed to be of insect origin, similiar to those reported by Kim (13) and Kim and Scott (14).

When beetles were prestarved and fed purified virus by the drop-drink method to eliminate plant enzymes, the regurgitant contained only the converted form of the virus (not shown). The original virus preparation contained both virus forms.

Proteolytic activity of beetle regurgitant. The total proteolytic activity was generally higher in the regurgitant of the bean leaf beetle (1,600 units) than in that of the Mexican bean beetle (900 units) or the spotted cucumber beetle (1,000 units). Pronase at 2 mg/ml had an activity of 1,000 units. The activity in regurgitant was approximately ten times that of bean plant sap (100 units), which served as the food source for the beetles. Trypsinlike activity was higher in the regurgitant of the spotted cucumber beetle (3.0 units) than in Mexican bean beetle (0.6 units) or bean leaf beetle (2.6 units) regurgitant.

DISCUSSION

Beetle transmission of plant viruses depends on the interaction of virus, vector, and host. One major factor in this interaction is capsid surface, which is the portion of the virus that is exposed to the microenvironment during the transmission process. Comoviruses occur as two electrophoretic forms with different average surface charges. Regurgitant from leaf-feeding beetles contained only the converted electrophoretic form of comoviruses, although the beetles were fed on infected tissue that contained both virus forms. Conversion also occurred when regurgitant was

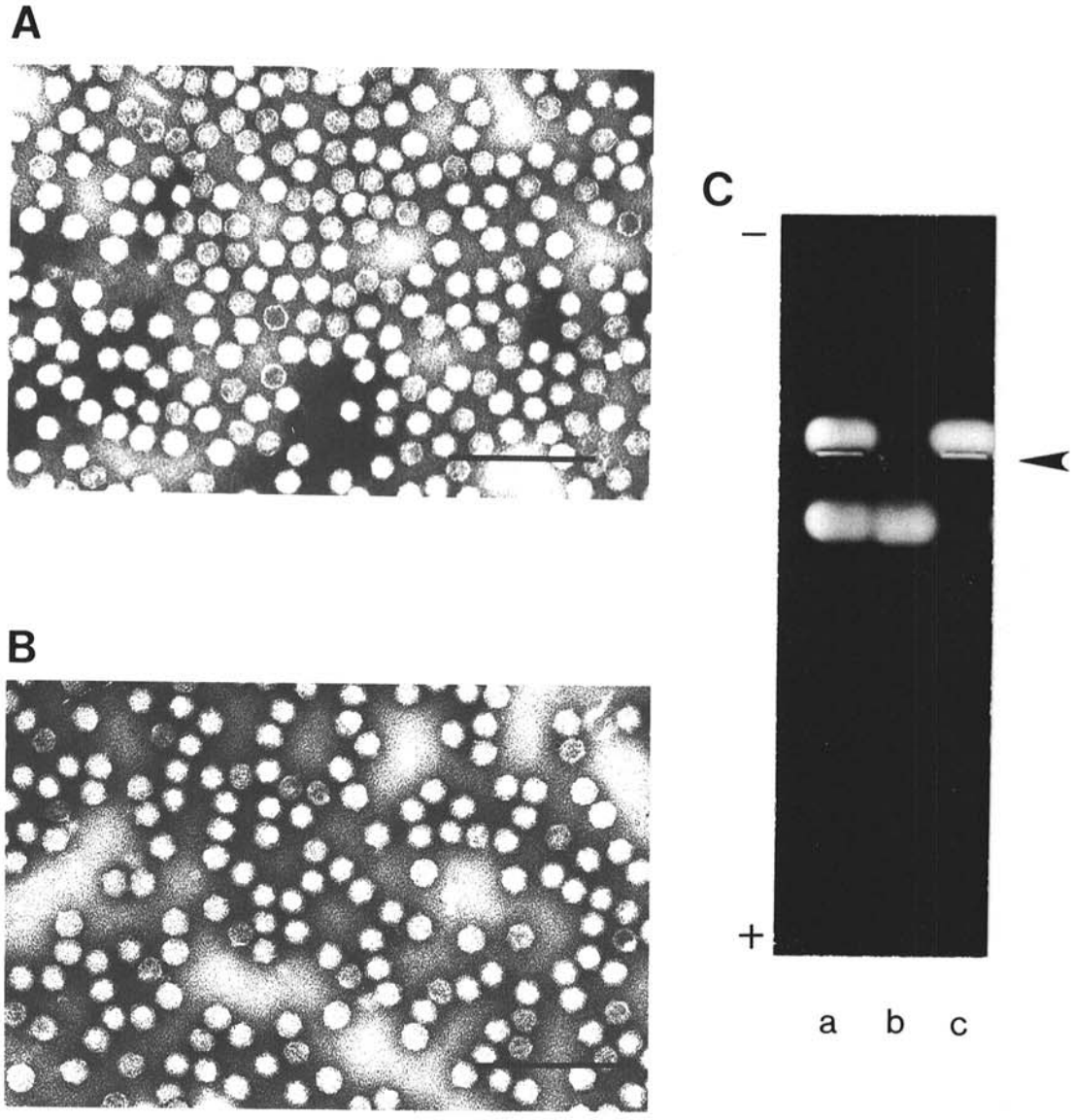

Fig. 3. Analysis of bean pod mottle virus (BPMV) isolated from agarose gels after electrophoresis to separate electrophoretic forms. **A,** Electron microscopy of BPMV isolated from the slow electrophoretic form and **B,** the fast electrophoretic form negatively stained with 2% phosphotungstic acid (pH 7.1) was done to determine the integrity of virus particles. The bars represent 150 nm. These same preparations were also analyzed by **C,** agarose gel electrophoresis. The original solution used in the initial separation is in lane a, the virus isolated from the fast band is in lane b, and the virus isolated from the slow band is in lane c. The origin of electrophoresis is indicated by the arrowhead.

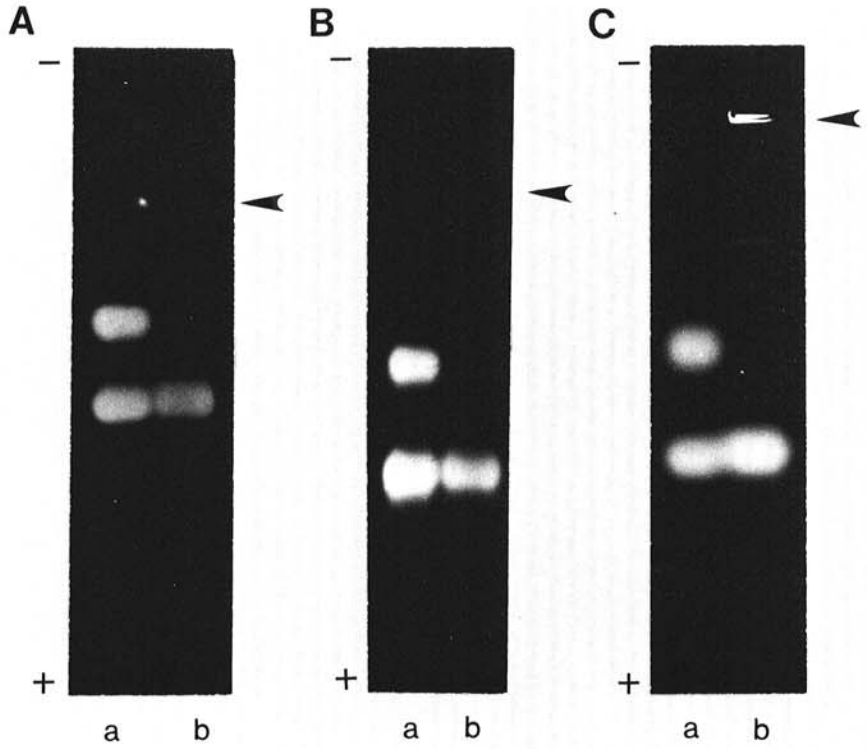

Fig. 4. In vitro conversion of cowpea mosaic virus (CPMV) by regurgitant from **A,** Mexican bean beetles, **B,** bean leaf beetles, and **C,** spotted cucumber beetles. CPMV was incubated for 2 hr at 37 C with an equal volume of either 0.01 M phosphate buffer (pH 7.2) (lane a) or regurgitant (lane b). Ten-μl samples were analyzed by agarose electrophoresis. Gel A was run at 20 V for 7 hr, gel B at 40 V for 4 hr, and gel C at 10 V for 17 hr. The origins of electrophoresis are indicated by the arrowheads.

incubated with purified virus in vitro or when beetles were fed virus mixed with sucrose. This phenomenon was observed with three comoviruses treated with regurgitant from three different leaf-feeding beetles. We conclude that only the converted form initiates infection during beetle transmission of comoviruses. However, separated slow and fast forms were both infectious when mechanically inoculated, as observed in earlier research (5), or when inoculated by gross wounding. Preinoculation conversion is, therefore, not essential for infectivity, although the possibility of postinoculation conversion could not be eliminated.

The proteolytic digestion of comoviruses is restricted to the carboxyl terminal portion of the S protein (18). The bulk of peptide bonds are inaccessible to proteases as shown by the stability of the converted form for long periods in beetle regurgitant. Intermediate charge forms found in CPMV may result from the stepwise digestion of S proteins or from the increasing number of digested S proteins per virus particle. Infectious CPMV transcripts which produce S proteins with altered C-termini may facilitate clarification of conversion and its potential biological roles.

The digestive physiology of Coleoptera is not well characterized. Research on the digestive proteases, which has primarily focused on the activity of gut homogenates or washings, is centered on determining if serine or cysteine proteinase is responsible for the majority of this activity. Baker (2) found that trypsinlike and chymotrypsinlike proteases constituted the majority of proteinase activity in midgut homogenates of black carpet beetle larvae, *Attagenus megatoma* (F.); and Baker (3) also isolated six trypsinlike, two chymotrypsinlike, one aminopeptidase, and one carboxypeptidase from the larval midgut of the hide beetle, *Dormestes maculatus* De Geer. In contrast, Murdock et al (17) suggest that cysteine proteases are the major proteinases involved in digestion since high levels of cysteine protease were found in midgut homogenates of 10 Coleoptera species, including *Epilachna varivestis* larvae and adult *Diabrotica* sp. Although the relationship between midgut homogenates and regurgitant is unclear, beetle regurgitant contained strong protease activity,

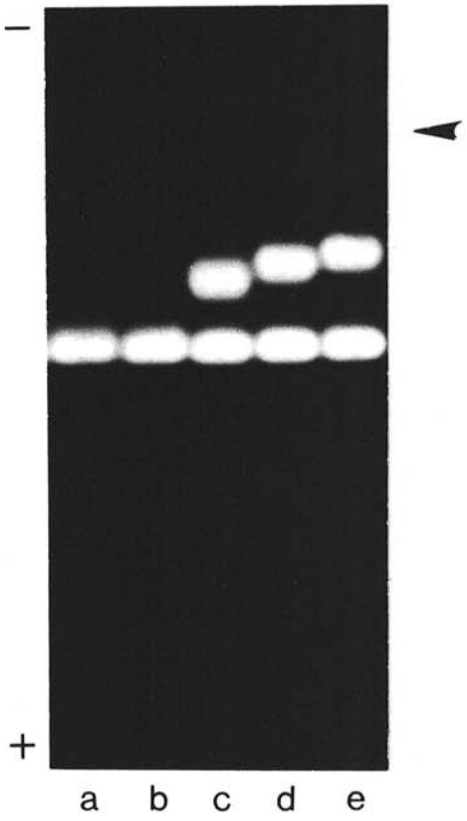

Fig. 5. The stepwise conversion of cowpea mosaic virus when incubated for 2 hr at 37 C with dilutions of Mexican bean beetle regurgitant. The virus was mixed with equal volumes of regurgitant at the following dilutions: undiluted (lane a), 1:2 (lane b), 1:25 (lane c), 1:50 (lane d), and 1:100 (lane e). The buffer control exhibited the same pattern of electrophoretic components seen in lane e. The arrowhead indicates the origin of electrophoresis.

and a portion of this activity was trypsinlike. Trypsin is one of the enzymes that converts comovirus electrophoretic forms (18). Although this trypsinlike activity functions in the conversion of comoviruses, other proteases in regurgitant probably are also involved since regurgitant converts CPMV more completely than trypsin alone. The role of other regurgitant proteases in comovirus conversion could be clarified by blocking trypsinlike proteases with inhibitors.

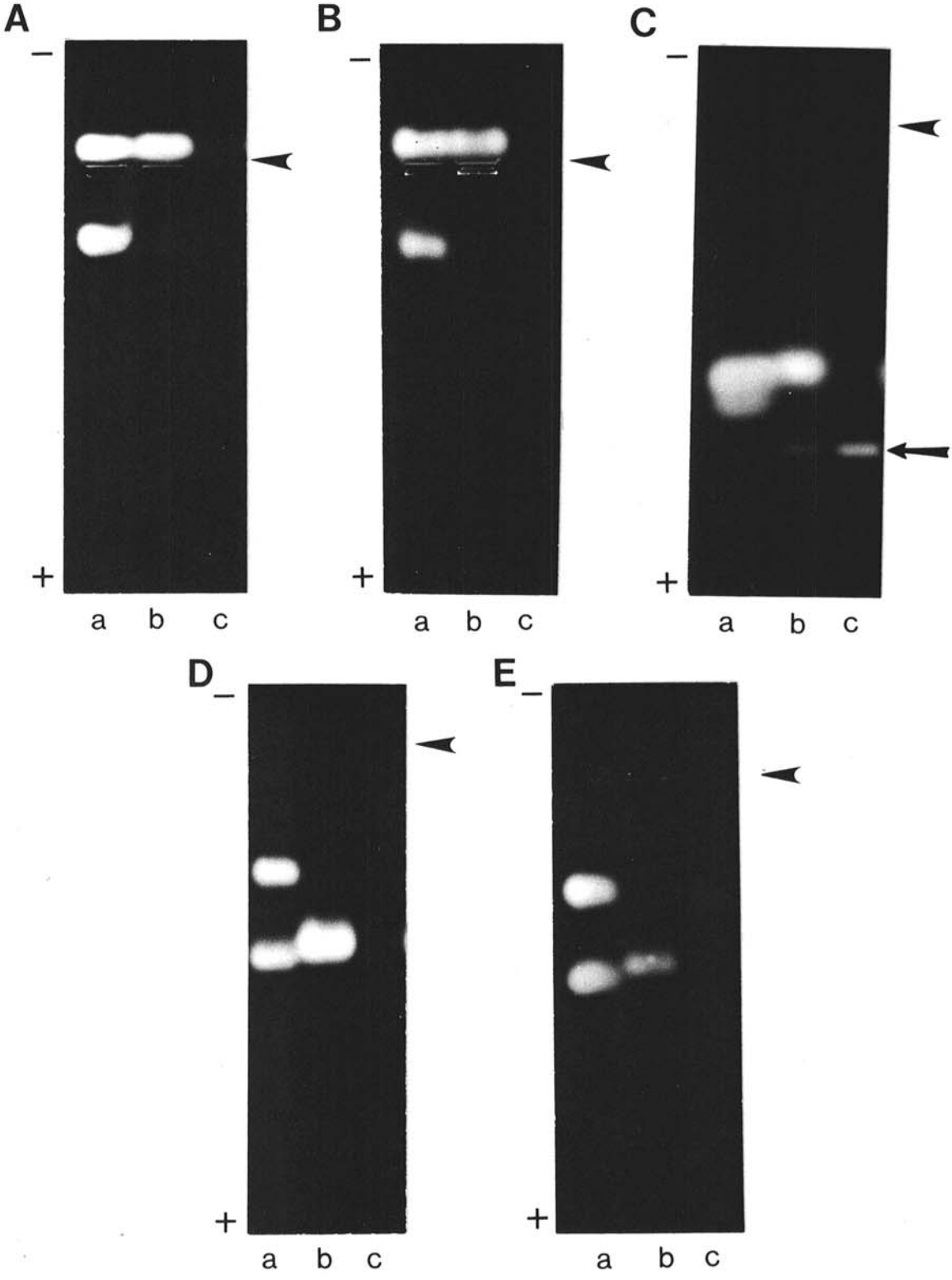

Fig. 6. In vivo conversion of comoviruses by leaf-feeding beetles that were fed on infected or healthy tissue. In all cases, lane a contains virus that was purified from the same source of infected tissue, lane b contains 10 μl of regurgitant from beetles fed on infected tissue, and lane c contains 10 μl of regurgitant from beetles fed on healthy tissue. Black Valentine bean tissue infected with bean pod mottle virus was the feeding source for **A,** Mexican bean beetles or **B,** bean leaf beetles. Early Prolific Straightneck squash infected with squash mosaic virus was the feeding source for **C,** spotted cucumber beetles. Extraneous nucleoprotein bands (arrow) were observed in the regurgitant from beetles fed on both healthy (lane c) and infected (not shown) material. Cowpea mosaic virus-infected bean tissue was the feeding source for **D,** Mexican bean beetles or **E,** bean leaf beetles. The origins of electrophoresis are indicated by the arrowheads.

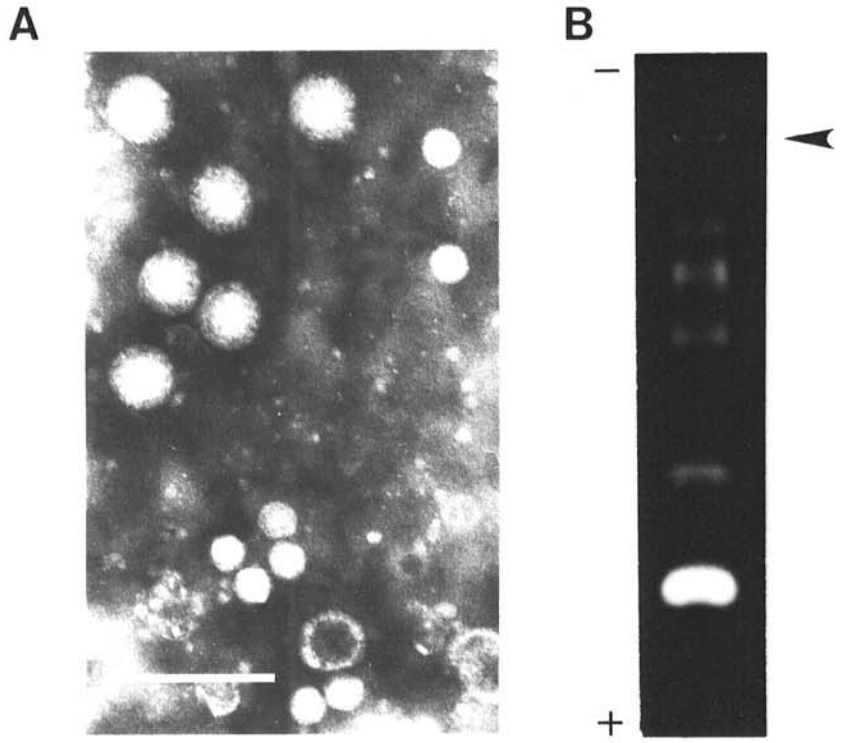

Fig. 7. Analysis of regurgitant from spotted cucumber beetles maintained on healthy Pinto beans. The regurgitant sample was analyzed by **A,** electron microscopy and **B,** whole particle electrophoresis to demonstrate some of the viruslike particles found in field-collected beetles after extended laboratory maintenance. Negative staining of regurgitant was done with 2% phosphotungstic acid (pH 7.1). The bar is equal to 150 nm. Regurgitant (10 μl) was analyzed by agarose gel electrophoresis, and the arrowhead indicates the origin of electrophoresis.

Regurgitant from leaf-feeding beetles is a complex mixture that includes several enzymes such as RNase (9), DNase, cellulase (*unpublished*), and proteases. Differentiation between beetle-transmissible and nontransmissible viruses has been attributed to RNase activity in regurgitant (9). However, the remaining enzymatic activities in regurgitant may also influence transmission.

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