Decline of Bean Pod Mottle Virus Specific Infectivity in Vivo Correlates with Degradation of Encapsidated RNA-1

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ABSTRACT

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The specific infectivity (SI) of bean pod mottle virus (BPMV) recovered from inoculated primary leaves of bean (*Phaseolus vulgaris* 'Pinto') and soybean (*Glycine max* 'Williams 82') at 20–25 days post inoculation was only 2% that of virus at 3–5 days post inoculation. Similarly, BPMV from systemically invaded older soybean leaves possessed a lower SI than that from younger leaves. There were no differences in either the structure or stability of high and low SI BPMV. Differences were detected in electrophoretic migration patterns of virions and in the molecular weight of the small coat protein subunit, but these did not contribute to the infectivity decline. The bottom component RNA (RNA-1) of low SI BPMV was degraded but not the middle component RNA (RNA-2). Replacing the bottom component in preparations of low SI with that from high SI restored infectivity. These results suggest that a selective in situ degradation of one of the two genomic RNAs leads to BPMV inactivation, a mechanism that appears unique among multicomponent plant viruses. Additionally, RNA-1 was degraded preferentially when BPMV was exposed briefly to high alkaline conditions, indicating that RNA-1 may be physically less stable than RNA-2.

Additional keywords: dot immunobinding assay, infectivity complementation test, protein:RNA interactions.

A progressive decline in specific infectivity (SI) with increasing duration of infection has been reported for several isometric plant viruses. For southern bean mosaic virus (SBMV), the low SI of late-progeny virions was correlated with a preferential encapsidation of noninfectious subgenomic RNAs, relative to the full-length or genomic RNA (29). The SI decline in tobacco ringspot virus (TRSV) was attributed to in situ degradation of genomic RNAs 1 and 2, accompanied by an increase in the relative proportion of bottom component particles encapsidating two copies of RNA-2 (21). Degradation of encapsidated RNA, as virions aged in vivo, was implicated in the inactivation of broad bean mottle virus (15). For cowpea chlorotic mottle virus (CCMV), in situ degradation of genomic RNAs originally was proposed to be the cause of inactivation (5,8,16); however, it was found subsequently (32) that a progressive change in coat protein structure and an increased strengthening of protein:RNA interactions were responsible for infectivity loss. In the breakdown of CCMV RNA in situ, oxygen and some other free radicals also have been implicated (28).

Bean pod mottle virus (BPMV), a comovirus, exists in two electrophoretic forms: a fast (F) mobility form of high SI, which predominates during early periods of infection, and a slow (S)

form of low SI, which dominates during later stages of infection (2,25,31). Further, RNA isolated from low SI BPMV is only one-third to one-fourth as infectious as that from high SI virus. From these observations, it was inferred (10,31) that capsid structural changes along with RNA degradation in situ were involved collectively in BPMV inactivation. The objective of the present study, to reinvestigate the basis for in vivo decline of BPMV infectivity, is based on two considerations. First, the intactness and infectivity of RNA, especially for virions where the protein:RNA interactions have undergone changes, can be greatly affected by the isolation method (12,22,27); consequently, it is important to establish that the reported (10,31) breakdown of BPMV RNAs in situ is not simply an artifact of the RNA isolation procedure. Second, in the case of cowpea mosaic virus (CPMV, type member of the comovirus group), infectivity of the two electrophoretic forms of virions was the same, and the RNAs recovered from both forms were intact and infectious. Thus, CPMV undergoes no SI decline in planta (9,20). Our results confirm that BPMV is inactivated progressively in vivo and that a selective degradation of RNA-1 was responsible for the infectivity loss. Changes in capsid organization played no apparent role in BPMV inactivation.

MATERIALS AND METHODS

The BPMV isolate used in this study was recovered during 1968 from a soybean (*Glycine max* (L.) Merr.) plant at the Agronomy Research Center, University of Missouri, Columbia. A "pure" line culture was established by five consecutive local lesion transfers through bean (*Phaseolus vulgaris* L. 'Pinto'). BPMV was propagated in soybean cultivar Williams 82 and bean cultivars Pinto and Brittle Wax. Local lesion assays were performed on the primary leaves of Pinto bean according to the protocol developed for SBMV (23). BPMV virions were diluted in 20 mM sodium phosphate buffer, pH 7.0, and RNA was diluted in 50 mM Tris-HCl buffer, pH 7.5, containing bentonite (100 μg/ml) and 0.02% diethylpyrocarbonate (DEPC). Lesions were counted 6–7 days post inoculation.

BPMV was purified (19,25) in the presence of 6 mM ascorbic acid and 0.1% β-mercaptoethanol (23,32), then maintained in 20 mM phosphate buffer, pH 7.0, containing 0.025% sodium azide. Centrifugations in sucrose gradients and cesium chloride were performed as previously described (12,24). The middle (91 S) and bottom (112 S) BPMV components were resolved on a preparative scale with at least two successive centrifugations (120,000 g, 6 hr, 4 C) in linear 15–40% sucrose gradients and then concentrated by ultracentrifugation.

BPMV coat proteins were isolated as follows. Three to four milligrams of virions was sedimented by ultracentrifugation, suspended in 2 ml of 100 mM sodium carbonate-bicarbonate buffer, pH 9.3, and maintained at 4 C for 12–15 hr. The sample was layered on a 2-ml cushion of 15% sucrose and centrifuged (145,000 g, 2 hr, 5 C) to sediment nondegraded virions, which were discarded. The buffer fraction above the sucrose cushion containing the dissociated coat proteins was recovered; the proteins were precipitated with ammonium sulfate and then dissolved in 20 mM phosphate buffer, pH 7.0. After dialysis against the same buffer, the samples were maintained at −17 C.

Virion RNAs were isolated routinely by a modification of the phenol extraction procedure (6). BPMV (1.5 mg/ml) in 100 mM Tris-borate buffer, pH 8.3, plus 100 μg of purified bentonite/ml and 0.05% sodium dodecyl sulfate (SDS) was treated with 10 μg of Proteinase K (E. Merck, Darmstadt, Germany)/ml. After 30 min at 25 C, the sample was heated at 50 C for 5 min and then chilled on ice. After extraction with phenol, RNAs were recovered by precipitation with ethanol. The sodium perchlorate-SDS method (18,30) also was used in isolating virion RNAs. An additional procedure found particularly useful for small quantities of virus was a modification of a previously described method (12). Fifty to 100 μg of virions in 300 μl of 20 mM phosphate buffer, pH 7.0, was precipitated by adding 900 μl of 95% ethanol containing 0.05% DEPC. After 4 hr at 5 C, the sample was centrifuged

(15,800 g, 10 min), and the pellet was dried under a stream of nitrogen. It then was dissolved in 100 μl of 100 mM sodium carbonate-bicarbonate buffer, pH 9.3, containing 4 mM ethylenediaminetetraacetic acid (EDTA), 1% SDS, 0.05% DEPC, and 2 μg of Proteinase K/ml. After 8–10 hr at 4 C, 200 μl of TBE (20 mM Tris-borate buffer, pH 8.3, containing 20 mM EDTA) was added, and the samples were stored at −60 C until further use.

BPMV (400 μg/ml) was treated with immobilized trypsin and/or ribonuclease (RNase, Sigma Chemical Co., St. Louis, MO) for 2 hr at 37 C, after which the enzyme(s) was removed by centrifugation. The virions were diluted in 20 mM phosphate buffer, pH 7.0, containing 0.1% DEPC, 0.01% phenylmethyl sulfonyl fluoride, and bentonite (100 μg/ml), and incubated for 30 min at 25 C. After centrifugation (8,000 g) to remove bentonite, BPMV was sedimented through a 15% sucrose cushion and suspended in 20 mM phosphate buffer, pH 7.0, containing 0.025% sodium azide.

Alkaline treatment of BPMV was conducted by the procedure of Kaper and Halperin (13). Virions (120 μg/ml in 1.0 M KCl) were exposed at pH 10.4, pH 10.8, and pH 11.1 for 8 min at 37 C. The reaction was stopped with dilution in ice-cold 20 mM phosphate buffer, pH 7.0.

Electrophoresis of virions or viral RNAs was performed in 1% low gelling Sea Plaque agarose (FMC Bioproducts, Rockland, ME). BPMV capsid proteins were examined by standard denaturing polyacrylamide slab gel electrophoresis (17). The dot immunobinding assay (DIBA, 11) for BPMV was performed as described previously (14). For quantifying BPMV antigens, membranes were treated with paraffin oil which rendered these transparent. The membranes then were scanned with a densitometer (Model GS 300, Hoeffer Scientific Instruments, San Francisco, CA) attached to a recorder. Standard curves were prepared by plotting antigen concentrations (known amounts of purified BPMV added to clarified sap, 1:100, w/v, of healthy leaves) versus peak heights and were used to estimate antigen concentration in the unknown samples. A linear regression analysis revealed a good correlation ($R^2 = 0.96$) between peak heights (a reflection of the dot intensity) and BPMV concentration (range 500 pg to 10 ng). The lower threshold amount of BPMV detectable by DIBA was between 50 and 100 pg per dot. No nonspecific reaction occurred when sap from healthy soybean or Pinto leaves was used at a dilution of >1:50 (1 g of tissue/50 ml of buffer).

Immunodiffusion tests were performed in 1% agarose using the unfractionated BPMV antiserum. Uranyl-formate-stained samples were examined with a JEOL 100 B (Japan Electron Optics Laboratory Ltd., Tokyo, Japan) electron microscope.

RESULTS

Infectivity alterations in vivo. Three different approaches were taken to obtain a reliable quantitative measure of in vivo decline of BPMV infectivity.

First, changes in SI were followed for virus recovered from the inoculated primary leaves of soybean cultivar Williams 82 and bean cultivar Pinto (Table 1). A similar trend in the loss of infectivity was observed in both of these hosts; the SI of BPMV at 25 days post inoculation was approximately 2–3% that of virus at 5 days post inoculation. It also was apparent that BPMV concentration increased rapidly (approximately 3.5-fold) between 5 and 10 days post inoculation with very little increase occurring thereafter, the decline in SI started fairly early and coincided with the period of maximal virus accumulation (5–10 days post inoculation) with greatest loss occurring between 10 and 20 days post inoculation, and BPMV attained approximately 30% higher concentration in Pinto bean leaves compared with that of soybean leaves.

Second, SI was determined for BPMV recovered from various soybean trifoliate leaves after systemic infection (Table 2). Virus from older leaves that had been infected for a longer time possessed a lower SI than that from younger leaves (shorter time of infection). For example, although BPMV concentration in the first

trifoliate leaf was comparable to that in the fourth leaf, SI of the former was only 10% that of the latter. The endogenous concentration of BPMV in second and third trifoliate leaves, which had developed only mild symptoms (3), was only 25% that in other leaves.

Third, BPMV was purified from bean cultivars Brittle Wax (a systemic host) and Pinto (a local lesion host) at various periods after inoculation, and their infectivities were compared. In both cases, BPMV recovered after a longer period of infection (20 days post inoculation) possessed a lower SI (0.02–0.04) than that from early (3–6 days post inoculation, SI = 1.00–0.86) infections.

Infectivity of enzyme-treated BPMV. Infectivity was not altered appreciably when BPMV was treated with trypsin, RNase, or trypsin followed by RNase. For example, compared with untreated BPMV (SI = 1.00), the SI of enzyme-treated virus was as follows: trypsin treated, 0.80; RNase treated, 0.82; and trypsin followed by RNase, 0.86. Similarly, infectivity remained unaltered when extracts of diseased Pinto leaves (3 days post inoculation) were maintained for 24 or 48 hr at 27 C before virion purification.

At a concentration of 0.1 μg/ml, BPMV purified after incubation of the extracted sap for 0, 24, and 48 hr induced, respectively, 75, 68, and 64 lesions per primary Pinto bean leaf.

Physicochemical characteristics of virions. When electrophoresed in 20 mM sodium barbital buffer, pH 8.3 (Fig. 1, I), BPMV isolated at 3 days post inoculation (SI = 1.00) migrated faster (F form) toward the anode than that isolated at 20 days post inoculation (SI = 0.02, S form); these virions appeared to be largely monophoretic (Fig. 1, I, A and D). BPMV isolated at 6 days post inoculation (SI = 0.86) or 10 days post inoculation (SI = 0.52) consisted of a mixture of the F and S forms (Fig. 1, I, B and C). There was a definite trend for a progressive conversion of the F to the S mobility form with increasing period of infection. Virions isolated after incubation of the extracted sap for 24 hr (SI = 0.90) or 48 hr (SI = 0.84) were mostly of the S form (Fig. 1, I, E and F). Upon trypsin treatment, BPMV isolated at 3 days post inoculation was converted from the F to the S form. Virions that were isolated at 20 days post inoculation and were of the S mobility form showed no further alteration in

TABLE 1. Infectivity of bean pod mottle virus (BPMV) recovered at different times after infection from directly inoculated leaves

			Number of		
	Days post inoculation[a]	Viral antigen (μg) per gram of tissue[b]	Local lesions[c]	Lesions per μg of viral antigen[d]	Relative infectivity[e]
Glycine max 'Williams 82'					
	5	95	104	1,095	1.00
	10	330	260	788	0.72
	15	335	63	188	0.17
	20	426	13	31	0.03
	25	511	11	22	0.02
Phaseolus vulgaris 'Pinto'					
	5	220	260	1,182	1.00
	10	680	464	682	0.58
	15	720	99	138	0.12
	20	880	51	58	0.05
	25	760	24	32	0.03

[a] Uniformly growing 10-day-old seedlings were selected. The apical meristem (trifoliate leaf and terminal bud) on each seedling was removed, and the primary leaves were allowed to expand for 2 days. Thereafter, these were rub inoculated with BPMV, and plants were maintained 25–27 C. All newly opened buds in the axils of the primary leaves were removed during the course of this experiment. At the indicated periods, leaf disks (1 cm diameter) were excised from the primary leaves, weighed, and homogenized (1 g of tissue/10 ml of buffer), and the extract was clarified by centrifugation (10,000 g, 30 min).
[b] Estimated by dot immunobinding assay using 1:100 dilution of the leaf extracts. Values include total coat antigens (that is, virions or empty capsids) and any free subunits.
[c] Average number of lesions per primary leaf of *Phaseolus vulgaris* 'Pinto.' The inocula (dilution 1:1,000) contained Celite (50 mg/ml) and were kept ice cold during the inoculations. The experiment was repeated twice using 24–28 primary leaves per treatment.
[d] Number of lesions × 1,000 (dilution factor) ÷ the amount of viral antigen.
[e] Infectivity relative to that of the virions recovered at 5 days post inoculation, which was considered 1.00.

TABLE 2. Infectivity of bean pod mottle virus recovered from trifoliate leaves of *Glycine max* 'Williams 82'[a]

Leaf number[b]	Viral antigen (μg) per gram of tissue[c]	Local lesions[d]	Lesions per μg of antigen[e]	Relative infectivity[e]
1	423	12	28	0.06
2	90	4	44	0.09
3	110	25	227	0.47
4	424	143	337	0.70
5	385	185	481	1.00

[a] The primary leaves of 10-day-old soybean seedlings were inoculated, and plants were maintained in a greenhouse at 24–27 C. At 50 days post inoculation, leaf disks (1 cm diameter) were excised from each of the 18 infected plants. One disk was obtained from every leaflet, and disks from the comparable trifoliates were pooled. These were rinsed with water, air dried, weighed, and frozen at −17 C. The tissue was homogenized (1 g/10 ml of buffer), and the sap was clarified by centrifugation (10,000 g, 20 min).
[b] The trifoliate leaves were numbered in ascending order, the lowermost (oldest) trifoliate designated as No. 1.
[c] Estimated by dot immunobinding assay using 1:100 dilution of the extracts. Values represent the average of three independent determinations.
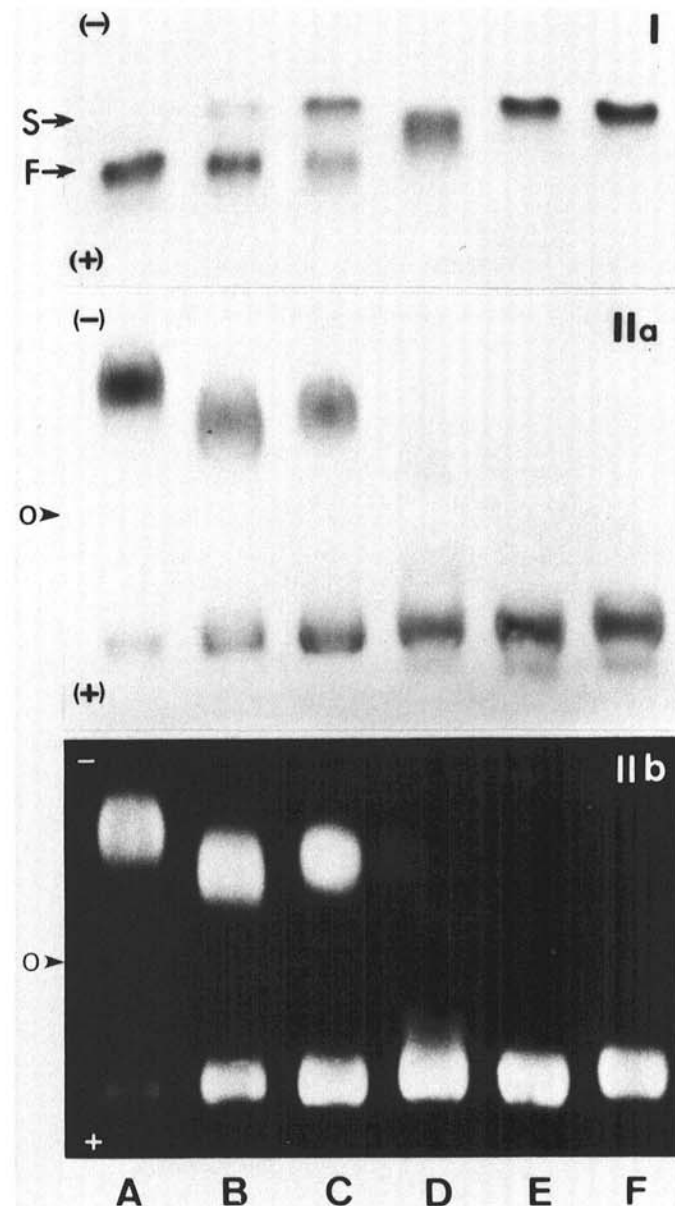[d] Average number of lesions per primary leaf of *Phaseolus vulgaris* 'Pinto.' The extract was assayed at a dilution of 1:1,000. The inocula contained Celite (50 mg/ml) and were kept ice cold during inoculations. The experiment was repeated twice, employing a total of 18–24 primary leaves per treatment.
[e] Number of lesions × 1,000 (dilution factor) ÷ the amount of viral antigen. For example, this value for trifoliate leaf No. 5 is 185 × 1,000 ÷ 385.
[f] Infectivity relative to that of the virions recovered from trioliate leaf No. 5, which was considered 1.00.

electrophoretic behavior after trypsin treatment (data not shown). The electrophoretic behavior of BPMV in 20 mM phosphate buffer, pH 7.5, was similar to that observed in 20 mM sodium barbital buffer, pH 8.3.
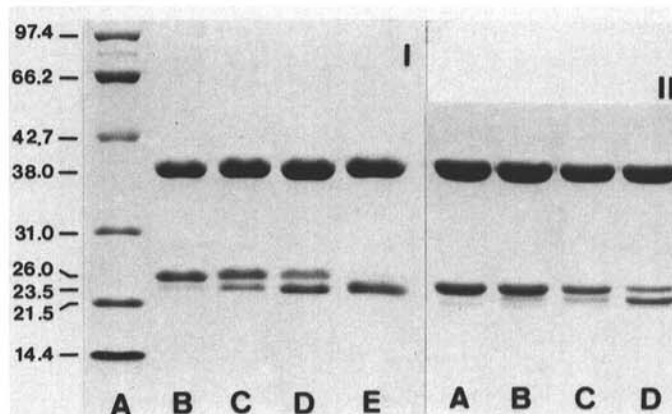
The differences in electrophoretic behavior were even more pronounced when BPMV after the various infection periods was electrophoresed in TBE buffer, pH 8.3 (Fig. 1, II). BPMV from 3-day-old infection (Fig. 1, II, A) migrated toward the cathode (cationic type), whereas that from 20 day-old infection (Fig. 1, II, D) migrated toward the anode (anionic type). Virus from 6- and 10-day-old infections consisted of a mixture of the two types (Fig. 1, II, B and C). Virions isolated after incubation of the extracted sap in vitro for 24 or 48 hr were mostly of the anionic type (Fig. 1, II, E and F). The electrophoretic migration pattern of BPMV in 100 mM Tris-HCl buffer, pH 7.5, 50 mM sodium borate-boric acid buffer, pH 8.3, or 100 mM glycine-NaOH buffer, pH 8.3, was similar to that observed in TBE buffer, pH 8.3 (data not shown).

Several other tests were conducted to establish if additional differences existed between high and low SI BPMV. There were no differences in the morphology of the negatively stained virions. Moreover, BPMV of high and low SI remained structurally stable when treated with 4 M urea, 1% formaldehyde, or 0.025% glutaraldehyde, heated at 60 C for 10 min, or submitted to three cycles of alternate freezing and thawing. Further, the relative proportion and sedimentation values of the middle (91 S) and bottom (112 S) components in BPMV of low and high SI were identical. Lastly, the banding patterns of BPMV in CsCl gradients


Fig. 1. Agarose gel electrophoresis of bean pod mottle virus. Virions were purified from inoculated primary leaves of *Phaseolus vulgaris* 'Pinto.' A, Virions isolated at 3 days; B, 6 days; C, 10 days; and D, 20 days post inoculation; E, extract from the infected leaves maintained for 1 day; and F, 2 days at 27 C before virion purification. Approximately 50 μg of virions was electrophoresed at 40 V for the first 30 min and then at 70 V for the next 3 hr at 4 C. **I,** Electrophoresis buffer was 20 mM sodium barbital, pH 8.3, and gel was stained with Coomassie Brilliant Blue. **IIa,** Electrophoresis buffer was Tris-borate-ethylene-diaminetetraacetic acid, pH 8.3, and the gel was stained with Coomassie Brilliant Blue. **IIb,** The same as IIa, but the gel was stained with ethidium bromide (1 μg/ml). S and F refer to slow- and fast-migrating virion forms. 0 indicates origin.

Fig. 2. Electrophoresis under denaturing conditions of disrupted virions of bean pod mottle virus. Virions were purified from inoculated primary leaves of *Phaseolus vulgaris* 'Pinto.' **I,** Untreated virions. A, Molecular-weight marker proteins; B, virions isolated at 3 days; C, 6 days; D, 10 days; and E, 20 days post inoculation. **II,** Virions (isolated at 3 days post inoculation) treated with trypsin. A, non-enzyme-treated control; B, trypsin, 0.02 units; C, trypsin, 0.04 units; D, trypsin, 0.08 units. The molecular-weight standards apply to panel I only.
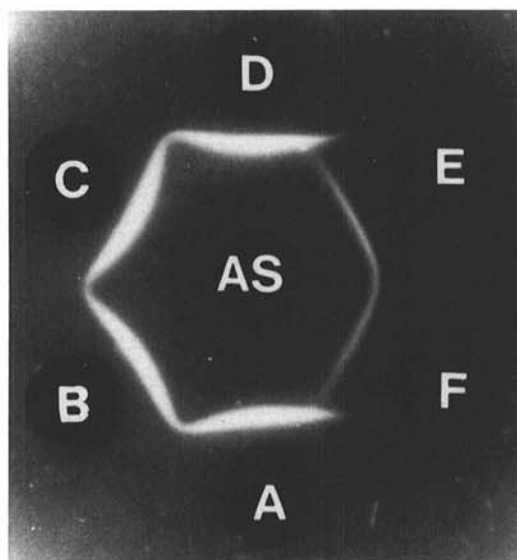
Fig. 3. Immunodiffusion test for virions of bean pod mottle virus (BPMV) and purified coat proteins. The central well contained anti-BPMV serum (dilution, 1:10). Peripheral wells: BPMV propagated in *Phaseolus vulgaris* 'Brittle Wax' for 6 days (A), and 20 days (B); BPMV propagated in *P. vulgaris* 'Pinto' for 6 days (C), and 20 days (D) post inoculation; coat proteins isolated from virus propagated in Pinto leaves for 6 days (E) and 20 days (F). Virions reacted at a concentration of 100 μg/ml, whereas the coat proteins were at 600 μg/ml.

were similar. The buoyant densities of the middle and bottom components were determined to be 1.403 and 1.421 g/ml, respectively.

Capsid protein alterations. The apparent molecular weights of large and small coat protein subunits of BPMV isolated at 3 days post inoculation were, respectively, 38 and 26 kDa (Fig. 2, I, B). With increasing time after infection, the small subunit was degraded progressively into a 23.5-kDa product, and by 20 days post inoculation, all subunits were so altered (Fig. 2, I, C–E). No change occurred in the mass of the large coat subunit. Treatment with trypsin in vitro also caused a selected proteolysis of the small coat subunit (Fig. 2, II, B–D). Virions isolated after incubation of the extracted sap for 24 or 48 hr in vitro also

showed a selective degradation of small coat subunit (data not shown). These values of the molecular weights of large coat subunit and of nondegraded or partially degraded small subunits represent averages of several independent determinations in polyacrylamide concentrations varying from 8 to 16%.

No differences were detected in the serological reactivities of high and low SI BPMV (Fig. 3). Similarly, coat proteins isolated from such virions reacted identically when tested against BPMV antiserum. The precipitin bands formed by the isolated proteins did not coalesce with those developed by virions, indicating that conformational changes occur in these proteins after dissociation from capsid.

Structural integrity and infectivity of virion RNAs. Upon denaturation with methyl mercury hydroxide and electrophoresis in agarose containing the denaturant (1), the molecular weights of the large (RNA-1) and small (RNA-2) BPMV RNAs were estimated to be 2.5×10^6 Da and 1.4×10^6 Da, respectively. These values agree with the other reports (7,25). Additionally, by using the resolved nucleoproteinaceous components, it was established that RNA-2 was encapsidated in the middle component whereas RNA-1 was contained in the bottom component, as is known in the other comoviruses (7).
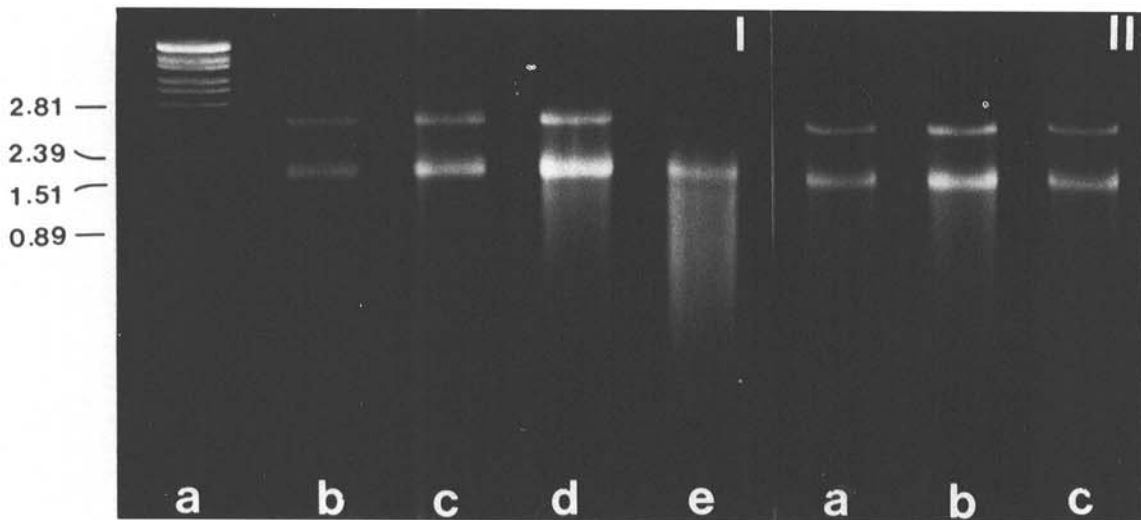
BPMV isolated at 3 days post inoculation (SI = 1.00) yielded intact RNA-1 and RNA-2 (Fig. 4A, C, and E), but for virus isolated at 20 days post inoculation (SI = 0.02), RNA-1 was mostly degraded whereas RNA-2 appeared intact (Fig. 4B, D, and F). Similar results were obtained by three different procedures for RNA isolation. This selective degradation of RNA-1 in situ was time dependent and was apparent for virus purified at 20 days post inoculation (Fig. 5, I, E); both of the RNAs appeared intact for BPMV recovered between 3–10 days post inoculation (Fig. 5, I, B–D). BPMV treated with trypsin, RNase, or trypsin followed by RNase yielded intact RNAs (Fig. 5, II, A–C). Similarly, virions isolated after incubation of the extracted sap for 24 or 48 hr at 27 C yielded intact RNAs (data not shown).

The infectivity of total, unfractionated RNA isolated by the phenol-SDS method from BPMV of low SI (0.02) was about 5% that of RNA from high SI (1.00) virus. For example, at the assay concentration of 50 µg/ml, RNA from low SI BPMV induced 2.5 lesions per primary Pinto leaf compared to 40 lesions induced by high SI virus. Comparable results were obtained with RNA isolated by the sodium perchlorate-SDS method.

Virions were purified separately from the inoculated primary leaves (5 days post inoculation, Table 1) and the various systemically invaded soybean trifoliate leaves (50 days post inocu-
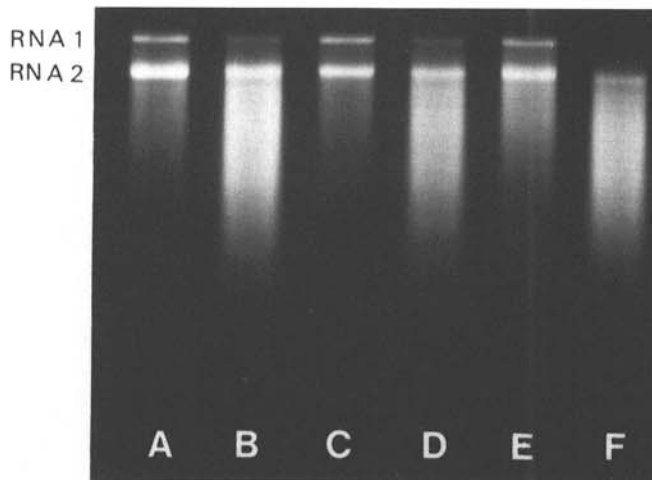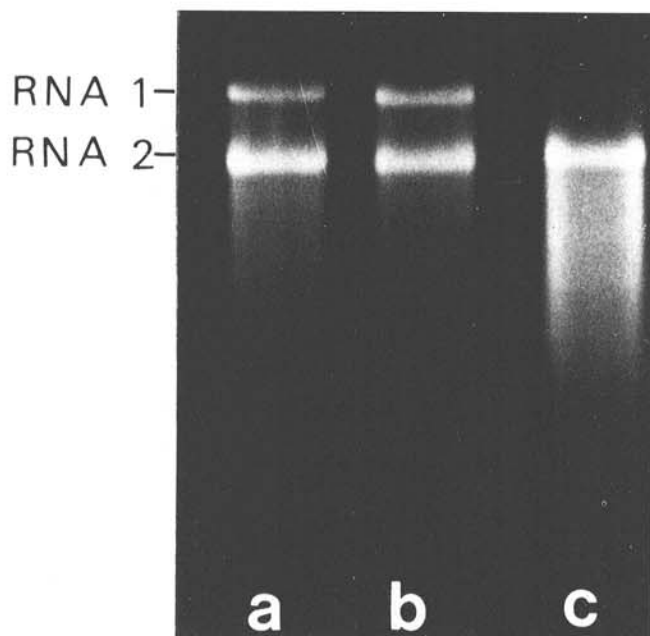

Fig. 4. Agarose gel electrophoresis of virion RNAs of bean pod mottle virus isolated by three different methods. Virions were purified from the inoculated primary leaves of *Phaseolus vulgaris* 'Pinto.' Phenol-sodium dodecyl sulfate method: A, virions recovered at 6 days, and B, 20 days post inoculation. Sodium perchlorate-sodium dodecyl sulfate treatment: C, virions recovered at 6 days, and D, 20 days post inoculation. Carbonate-bicarbonate extraction procedure: E, virions recovered at 6 days, and F, 20 days post inoculation. Approximately 0.5 µg of RNA per lane (6 days post inoculation) or 1.0 µg per lane (20 days post inoculation) was electrophoresed in 20 mM Tris-borate-ethylenediaminetetraacetic acid, buffer pH 8.3, for 2 hr at 25 C at 50 V and then stained with ethidium bromide (1 µg/ml).

Fig. 5. Gel electrophoresis of the virion RNAs of bean pod mottle virus (BPMV). **I,** Untreated virions: A, molecular-weight markers; B, virions isolated at 3 days; C, 6 days; D, 10 days; and E, 20 days post inoculation. **II,** Virions (isolated at 3 days post inoculation) treated with: A, trypsin; B, ribonuclease; C, trypsin followed by ribonuclease. BPMV was propagated in *Phaseolus vulgaris* 'Pinto,' and RNAs were isolated by the carbonate-bicarbonate method. Approximately 0.5 µg of RNA per lane (6 days post inoculation) or 1.0 µg per lane (20 days post inoculation) was electrophoresed in 20 mM Tris-borate-ethylenediaminetetraacetic acid, buffer pH 8.3, for 2 hr at 25 C at 50 V and then stained with ethidium bromide (1 µg/ml).

lation, Table 2), and their RNAs were examined by gel electrophoresis (Fig. 6). BPMV from primary leaves (SI = 1.00) and from the two top trifoliate leaves (No. 4 and 5, SI = 0.85) yielded intact RNA-1 and RNA-2 (Fig. 6A and B). However, virions from the two bottom trifoliate leaves (No. 1 and 2, SI = 0.03) yielded intact RNA-2 but degraded RNA-1 (Fig. 6C).

No breakdown of either RNA-1 or RNA-2 was observed when BPMV purified from the primary leaves of soybean (at 5 days

post inoculation) or bean cultivar Pinto (at 3 days post inoculation) was maintained for 4 wk at 5 C in 20 mM phosphate buffer, pH 7.0, containing 0.025% sodium azide.

Infectivity complementation tests. A bioassay protocol was designed in which the resolved middle and bottom nucleoprotein components of BPMV of low SI (0.02) and high SI (1.00) were mixed in homologous and heterologous combinations (Table 3). These tests show the following results. 1) The middle and bottom components per se were noninfectious. 2) A mixture of the resolved middle and bottom components of low SI virus possessed only 2% of the infectivity of a similar mixture of the components from virus of high SI; this is consistent with the infectivities of the corresponding unfractionated preparations. 3) Infectivity was enhanced markedly when the bottom component from high SI virus was mixed with the middle component of low SI virus. 4) A combination of middle component from high SI BPMV and bottom component from low SI virus was weakly infectious.

Sensitivity to high pH. With a brief exposure of BPMV virions at pH 10.4, pH 10.8, or pH 11.1, RNA-1 was degraded completely (Fig. 7). Some RNA-2 also was degraded under these conditions. Analysis by sucrose density gradient centrifugation revealed that virions exposed at pH 10.4 remained structurally intact but those treated at pH 10.8 or pH 11.1 were degraded into nonsedimenting entities. Compared to untreated virus maintained at pH 7.0 (SI = 1.00), the infectivity of treated BPMV was as follows: pH 9.3, SI = 0.88; pH 10.4, SI = 0.63; pH 10.8, SI = 0.04; pH 11.1, SI = 0.

Fig. 6. Agarose gel electrophoresis of bean pod mottle virus RNAs. Virions were purified separately from the inoculated (5 days post inoculation) primary leaves and systemically invaded (50 days post inoculation) trifoliate leaves of *Glycine max* 'Williams 82.' A, RNA from virions (specific infectivity [SI] = 1.00) recovered from the primary leaves; B, virions (SI = 0.82) recovered from the two upper or youngest (Nos. 4 and 5) trifoliate leaves; C, virions (SI = 0.07) from the two lower or oldest (Nos. 1 and 2) trifoliate leaves. Approximately 0.5 μg of RNA per lane (6 days post inoculation) or 1.0 μg per lane (20 days post inoculation) was electrophoresed in 20 mM Tris-borate-ethylenediaminetetraacetic acid, buffer pH 8.3, for 2 hr at 25 C at 50 V and then stained with ethidium bromide (1 μg/ml).

DISCUSSION

BPMV inactivation, as a function of time after infection, was closely correlated with degradation of RNA-1 in situ. This was corroborated by the observation that virus recovered between 3 and 6 days post inoculation was highly infectious and contained intact RNA-1 and RNA-2, whereas virus isolated at 20 days post inoculation possessed low SI and contained degraded RNA-1 but physically intact RNA-2. Additional evidence that in situ cleavage of RNA-1 contributed to virus inactivation was provided by the observation that, with replacing the bottom component in BPMV of low SI with that from high SI, infectivity was restored. Because BPMV of low or high SI yielded intact RNA-2 and the middle component from low SI preparations proved competent in the infectivity complementation tests, it may be inferred that RNA-2 undergoes no structural impairment as BPMV aged in planta.

TABLE 3. Infectivity complementation tests employing the resolved middle and bottom components of bean pod mottle virus (BPMV)

| | | | Number of local lesions[b] | | |
| | Concentration (μg/ml) | | Separated components or a mixture | Unfractionated | Relative |
Inocula[a]	M	B	thereof	virions	infectivity[c]
I. M (low)	10.0	0	0	8.8	0
B (low)	0	10.0	0.3	9.2	0.03
M (low) + B (low)	10.0	10.0	8.2	8.7	0.94
II. M (high)	0.1	0	0	56.7	0
B (high)	0	0.1	2.4	27.3	0.09
M (high) + B (high)	0.1	0.1	43.6	42.0	1.04
III. M (low) + B (high)	10.0	0.1	39.7	32.7	1.20
M (low) + B (high)	5.0	0.1	9.6	24.0	0.40
IV. M (high) + B (low)	0.1	10.0	2.5	35.0	0.07
M (high) + B (low)	5.0	10.0	4.2	42.1	0.10

[a] Middle (M) and bottom (B) components of BPMV preparations of high (3 days post inoculation) and low (20 days post inoculation) specific infectivity were purified by two cycles of preparative sucrose density gradient centrifugation (as described in the Materials and Methods section).

[b] Average number of lesions per primary leaf of *Phaseolus vulgaris* 'Pinto.' One primary leaf on each plant was inoculated with the resolved BPMV component or an appropriate mixture thereof, and the opposite leaf was inoculated with a comparable unfractionated preparation. The inocula contained Celite (50 mg/ml) and were kept ice cold during inoculations. The experiment was repeated twice, employing a total of 18–32 primary leaves per treatment.

[c] Infectivity relative to that of the comparable unfractionated preparation. Virions of high specific infectivity were assayed at 0.2 μg/ml, whereas those of low specific infectivity were assayed at 20 μg/ml.

The altered electrophoretic migration pattern of BPMV virions and selective proteolysis of small coat protein subunit were additional changes that accompanied BPMV aging in vivo. Because structural organization of the capsid of middle and bottom components is identical (7,25), these changes must have occurred coincidentally in both components. These alterations probably were not involved in BPMV inactivation because similar changes could be simulated in vitro (treatment with trypsin, incubating extracted sap in vitro before virion purification) with no infectivity loss. The proteolysis of small coat subunit and breakdown of RNA-1 in situ appear to be independent processes because the former occurred in both the nucleoprotein components whereas the latter was confined to the bottom component only. It appears fortuitous, however, that changes in BPMV capsid became detectable at the same time that decline in SI started. The selective proteolysis of small BPMV coat subunits is due to their arrangement as large protruding pentameric clusters at the fivefold axes and, consequently, their ready accessibility to protease attack; the large subunits form somewhat recessed clusters around the threefold axes (4). The limited proteolysis of small coat subunits caused a net loss of negative charge from capsid and transformed virions from cationic to the anionic form (Fig. 1). A segment comprised of approximately 15–20 amino acid residues was cleaved from the small coat subunit, probably from the carboxyl terminal (7,20,25), but it caused no detectable change in immunoreactivity. Furthermore, it did not impair the ability of the capsid to effectively protect the viral genome against RNase attack. The in situ degradation of RNA-1 had no effect on the buoyant density, sedimentation value, or morphology of the bottom BPMV component. Further, such particles remained structurally intact after certain physical and chemical treatments, similar to particles encapsidating intact RNA-1. These results signify that interprotein interactions play an essential role in providing stability to BPMV capsid.

For a reliable and accurate measure of SI decline as a function of time after infection, it is necessary to use virus recovered from the inoculated leaves only. This eliminates SI variations due to differences in the time of infection of systemically invaded tissue after virus transport from primary infection sites. The fact that BPMV of high SI was contained in younger tissue whereas that of low SI was contained in older leaves within the same plant underscores the importance of selecting appropriate plant tissue in conducting such studies. Investigations with CCMV (5,16), TRSV (21), and now with BPMV in the inoculated leaves demonstrate that the decline in infectivity starts fairly early (2–6 days)

after infection and is coincidental with the peak period of viral replication and/or accumulation. Thereafter, infectivity is lost rapidly, but little increase in virus concentration occurs. These results indicate that SI decline in BPMV, CCMV, and TRSV results from aging in vivo and not necessarily that virus synthesized late during infection is per se of low infectivity. Another factor contributing to the low SI of TRSV was the relative increase in the proportion of bottom component particles that had encapsidated two molecules of RNA-2 (21). Whether a similar situation exists for BPMV remains to be determined. Also, it is possible that a minor proportion of BPMV virions contains heterogenous RNAs of viral or host origin, as is known in other plant viruses (26,29).

It is interesting that for BPMV maintained in vitro for 4 wk both the RNAs remain structurally intact whereas in planta degradation of encapsidated RNA-1 begins within a few days after infection. The mechanisms involved or factors contributing to the degradation of encapsidated viral RNAs are poorly understood. In CCMV, SI decline with increasing age of infection was attributed to coat protein alterations and a strengthening of the protein:RNA bonds (32). If antioxidants were used during virus purification and genome isolation and coat protein was digested with pronase, before phenol extraction, structurally intact and infectious CCMV RNAs were obtained; in the absence of the antioxidants or when pronase treatment was omitted, all three genomic RNAs were degraded extensively. Despite the use of antioxidants and Proteinase K, highly degraded RNA-1 yet structurally intact RNA-2 were recovered from BPMV of low SI. Cross-linking of coat subunits to BPMV RNA-1 may have rendered it susceptible to breakage during phenol treatment, as has been reported for other plant viruses (12,22,27,32). This possibility appears less likely because isolation of RNA with two additional methods yielded degraded RNA-1 from BPMV of low SI; one of these methods, namely, dissociating viral capsid at pH 9.3 in the presence of SDS, had proved highly effective in releasing full-length RNA from virions with a high degree of cross-linking between coat protein and RNA (12,22). Furthermore, intact RNA-2 was recovered consistently even with the phenol extraction method from BPMV of low SI. These results indicate that the observed selective degradation of RNA-1 was not an artifact of isolation procedure. Alternatively, the preferential breakdown of encapsidated RNA-1 may have a structural basis (J. E. Johnson, *personal communication*). On the assumption that size of BPMV RNA-2 permits it to develop an optimal or effective structural association with capsid, the extra length (approximately 3,000 nucleotides) of RNA-1 may impede the generation of a stable complex; thus this part of RNA will be prone to cleavage. Our gel electrophoretic analysis, however, showed that RNA-1 had undergone random and extensive degradation without formation of any identifiable intermediate products.

The observed mode of BPMV inactivation, that is, a selective degradation of one of the two genomic RNAs in situ, is unique among plant viruses. Furthermore, it is significant that encapsidated RNA-1 is prone to breakdown under the natural conditions (that is, in planta) and upon alkali treatment. The precise basis for the observed short "life expectancy" of encapsidated RNA-1 remains to be established. Whether degradation of RNA-1 in situ is a random autocatalytic process is speculative; in any event such a process will be a self-limiting step in the viral life cycle.

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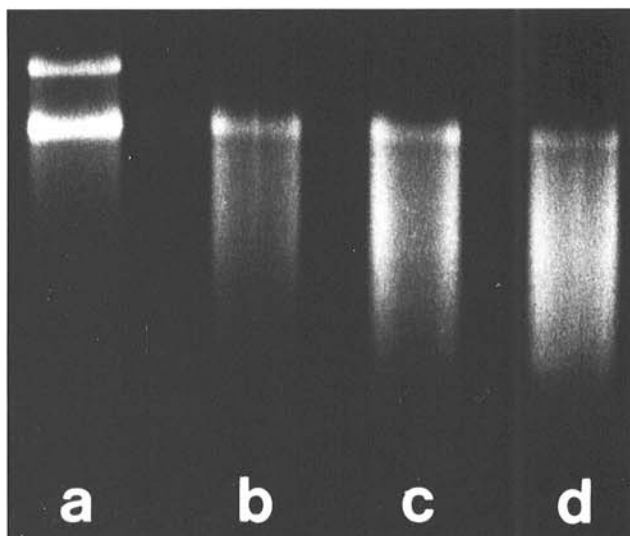4. Chen, Z., Stauffacher, C., Li, Y., Schmidt, T., Bomu, W., Kamer, G.,

Fig. 7. Preferential degradation of RNA-1 in alkali-treated bean pod mottle virus. A, Untreated control (pH 7.0); B, virions treated at pH 10.4; C, virions treated at pH 10.8; D, virions treated at pH 11.1.

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