An Agent Associated With Bee-Stored Pollen That Degrades Intact Viruses

Anabel Cole and G. I. Mink

Research associate, and plant pathologist, respectively, Washington State University, Irrigated Agriculture Research and Extension Center, Prosser 99350.

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ABSTRACT

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An agent that degraded surface-borne virions of Prunus necrotic ringspot virus (NRSV) and prune dwarf virus (PDV) into serologically undetectable units was detected on and extracted from pollen taken from many honeybee hives. This agent was not found on hand-collected pollen. Leachates of bee-stored pollen degraded purified viruses and were most active when incubated at pH 5.5 and temperatures between 42 and 50 C. Activity decreased markedly as the assay pH approached neutrality and was not detected at temperatures <25 C. Degradative activity of pollen leachates was eliminated by boiling 5 min., overnight dialysis against distilled water, or addition of glucose. However, addition of fructose or sucrose stimulated virus degradation in the presence of pollen leachates. Addition of bovine serum albumin as a possible substrate competitor had no effect on virus degradation.

Additional key words: glucose oxidase, ilarviruses, inhibine.

Prunus necrotic ringspot virus (NRSV) and prune dwarf virus (PDV) are ilarviruses that infect several stone fruit species. Both are pollenborne. Earlier we reported that most, if not all, NRSV virions that are associated with pollen from infected almond [*Prunus dulcio* (Millo) D. A. Webb] and sweet cherry (*P. avium* L.) trees appear to be located on the pollen surface (2). Although the location of PDV on infected pollen has not been reported, recent studies (R. Kelley and H. R. Cammeron, *unpublished*) demonstrate that at least a portion of the PDV virions are borne internally.

Recent studies on the role of honeybees in the long-range spread of NRSV demonstrated that bee-stored pollen samples taken during bloom from hives located near NRSV-infected almond trees were uniformly contaminated with NRSV (5). We now report that essentially all NRSV detectable by enzyme-linked immunosorbent assay (ELISA) disappeared from this pollen during prolonged storage in the hives. This phenomenon could be duplicated in the laboratory simply by incubating bee-collected pollen at temperatures in or above the range of those normally found within hives. Furthermore, we could extract from the pollen of many hives an agent that degraded purified intact NRSV or PDV into units that were undetectable by ELISA (6). Some of the properties of this agent are reported here.

MATERIALS AND METHODS

Pollen sources. Samples of bee-stored pollen were taken initially from hives located in a California almond orchard where most of the trees were infected with NRSV (5). Prior to bloom, one new frame with empty comb was inserted into each of 40 hives. During full bloom, 5-g samples of freshly stored pollen were removed from each hive and stored at −20 C. After bloom, the hives were moved to Washington where each frame was then covered with Saran screen to prevent any further access by bees to the stored pollen. Pollen samples (3–5 g) were removed from the screened frames at various intervals over an 8-wk period and stored at −20 C.

Additional samples of bee-stored pollen were collected at various times each spring from hives located in Washington near blooming cherry orchards where many trees were infected with both NRSV and PDV. Most of the pollen samples taken from these hives were contaminated with both viruses. Pollen free of both viruses was collected from hives located in various parts of Washington where almond and cherry trees either did not occur or were not in bloom. All pollen samples were stored at −20 C until used.

Pollen samples collected by hand from known healthy or infected cherry trees were furnished by a local pollen collector, air-dried at 10 C for 24 hr, and stored at −20 C.

Assays for pollenborne viruses. Before use, each pollen sample was tested by ELISA for the presence of NRSV and PDV. Frozen samples were triturated in a mortar with 10 volumes (w/v) of ELISA grinding buffer (1) and tested for either NRSV or PDV in the ELISA conditions described previously (4). Antiserum against NRSV was obtained from R. W. Fulton. Antiserum against PDV was prepared locally. Gamma globulins and alkaline phosphatase conjugates were prepared according to Clark and Adams (1). To determine virus stability in situ we used pollen samples that contained one or both viruses. Pollen leachates were prepared as described below from bee-stored pollen samples that contained neither virus.

Virus stabililty in situ. Virus-contaminated pollen from selected hives or trees was divided into 0.1-g samples, placed in rubber-stoppered glass vials, and stored at selected temperatures between 4 and 50 C for various intervals. Each sample was then triturated in 1 ml of ELISA grinding buffer and assayed.

Pollen leachates. Pollen free from both viruses was dispersed in 5–10 volumes (w/v) of phosphate buffers at various molarities and pH's and which contained 0.05% sodium azide. The suspensions were stirred gently for 1–2 hr at 4 C, centrifuged at 10,000 *g* for 20 min to remove pollen grains, and the leachates were stored at −20 C.

Virus purification. NRSV was purified from *Chenopodium quinoa* Willd. and PDV was purified from squash (*Curcurbita moschata*) leaves as described earlier (3).

Assays with pollen-leachates. Standard pollen leachate assays were conducted as follows: purified virus suspension (0.1 ml) was added to 1.4 ml of 0.01 M phosphate buffer, pH 5.5, and 0.5 ml of pollen leachate to give a final volume of 2.0 ml that contained NRSV or PDV at 1 μg/ml. One aliquot (0.5 ml) was removed immediately after mixing and stored at 4 C. The remaining suspension was stored at selected temperatures between 4 and 50 C. Aliquots (0.5 ml) were removed after 1, 2, and 4 hr and stored at 4 C. Subsequently, all aliquots were tested in duplicate by ELISA for the appropriate virus.

RESULTS

In situ stability of NRSV associated with bee-stored pollen. Samples of almond pollen removed within 1–2 days of storage from

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the 40 hives that originated in California produced absorbance values ($A_{405\ nm}$) in ELISA with NRSV gamma globulins and conjugate (1-hr substrate incubation) that ranged between 1.0 and 2.5 (40-hive avg. = 1.81). In these same tests purified NRSV at concentrations of 10, 1, 0.1, 0.01, and 0 μg/ml produced $A_{405\ nm}$ values of 2.42, 2.02, 0.77, 0.08, and 0.01, respectively. Hand-collected pollen from healthy cherry trees produced $A_{405\ nm}$ values near zero.

Although every bee-stored pollen sample that we collected within 1–2 days of storage appeared to be highly contaminated with NRSV, nearly all samples taken from these same hives 7–8 wk later and tested by ELISA appeared to be free of virus. Samples that had been removed from 10 hives after hive storage intervals of 1, 15, 20, 38, 43, and 53 days were compared in a single ELISA test. The average $A_{405\ nm}$ values decreased markedly with increased storage time in the hive (Fig. 1). This decrease appeared to be a linear function of the logarithm of time.

When we incubated bee-stored pollen in dry vials at 30 C, we found that virtually all ELISA-detectable NRSV antigens disappeared within ≈ 53 hr. In contrast there was no detectable loss of NRSV antigen from hand-collected pollen (Fig. 2). These results suggested that pollen collected and stored by honeybees contained an agent not found on hand-collected pollen that was capable of degrading NRSV antigens on the pollen surface into units that were undetectable by ELISA.

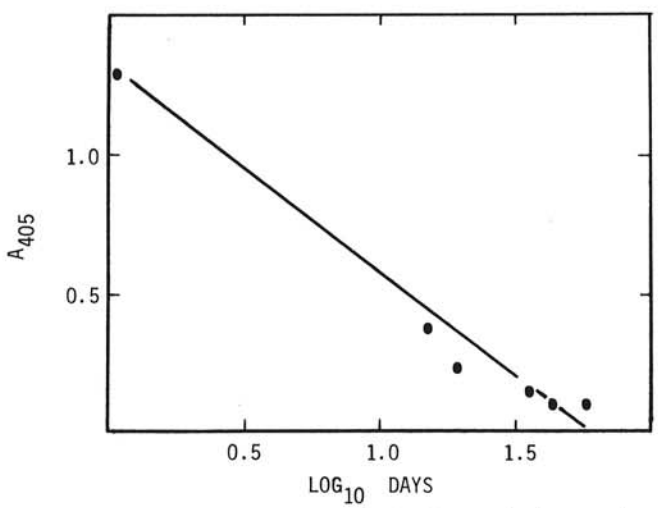

Fig. 1. Absorbance values ($A_{405\ nm}$) obtained with necrotic ringspot virus-contaminated pollen removed from bee hives at various times after bloom.

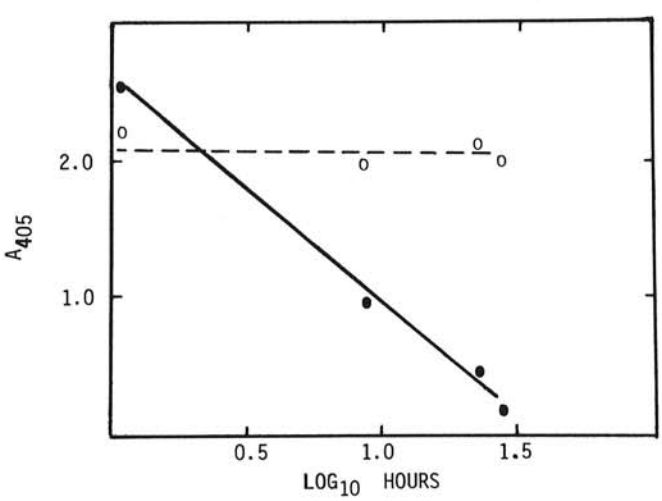

Fig. 2. Absorbance values ($A_{405\ nm}$) obtained with necrotic ringspot virus-contaminated pollen (—— bee stored; --- hand collected) incubated in dry vials at 30 C.

When NRSV-contaminated pollen samples from a single hive were incubated at temperatures between 4 and 25 C, the amount of ELISA-detectable antigen remained unchanged over a period of several days. However, as the incubation temperature was increased above 30 C, the rate at which ELISA-detectable antigens disappeared increased dramatically. In one series of tests, all detectable antigens were lost within 24 hr at 40 C (Fig. 3). In additional tests, we found that the rate of virus degradation continued to increase at temperatures up to 50 C.

The rate at which NRSV antigens disappeared at 40 C was relatively constant for all pollen samples taken from a given hive, but the rates varied greatly among samples from different hives.

Effect of pollen on purified viruses. To determine the relative susceptibility of NRSV and PDV to degradation by the agent associated with bee-stored pollen, we mixed 0.1-g samples of pollen samples known to be free from both viruses with purified, intact virus (either virus at 1 μg/ml) in 0.01 M neutral phosphate buffer. No decrease in ELISA absorbance values occurred when either virus was incubated at 37 C in the absence of pollen or when incubated at 4 C in the presence of bee-stored pollen. However, all ELISA-detectable antigens of both viruses disappeared during 4 hr of incubation at 37 C in the presence of bee-stored pollen (Table 1). In other experiments, no decrease in ELISA absorbance values occurred when either virus was incubated at 37 C with hand-collected pollen. These results further suggested that the agent associated with bee-stored pollen degraded the intact viruses into serologically undetectable units.

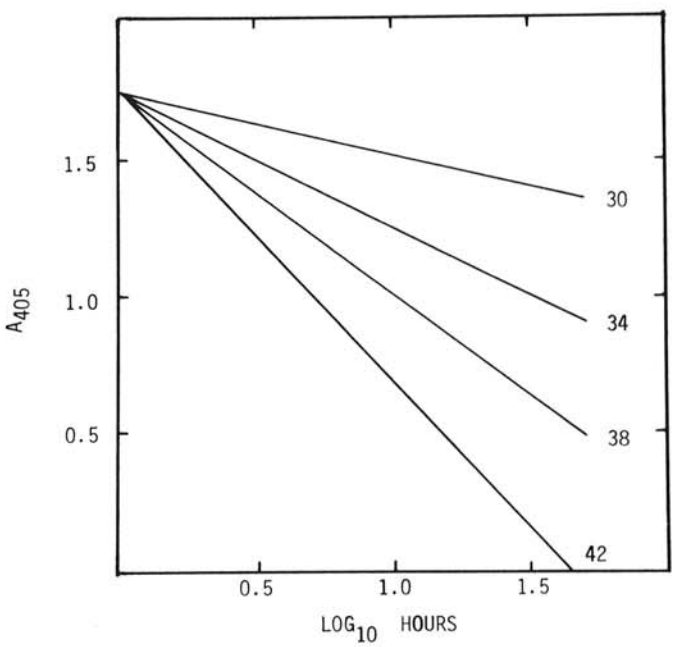

Fig. 3. Absorbance values ($A_{405\ nm}$) obtained with necrotic ringspot virus-contaminated pollen incubated in dry vials at various temperatures.

TABLE 1. Absorbance values ($A_{405\ nm}$) obtained in enzyme-linked immunosorbent assay with necrotic ringspot virus (NRSV) and prune dwarf virus (PDV) antisera[a]

Virus	Pollen added	Incubation temp (C)	Incubation time (hr) 0	1	2	4	Decrease (%) (4 hr)
NRSV	No	37	0.71	0.68	0.74	0.79	0
	Yes	4	0.50	0.55	0.48	0.63	0
	Yes	37	0.59	0.27	0.07	0.01	98
PDV	No	37	1.71	1.72	1.28	1.75	0
	Yes	4	1.59	1.38	1.45	1.77	0
	Yes	37	1.36	0.86	0.79	0.00	100

[a] Suspensions of purified virus (1 μg/ml) were incubated up to 4 hr with bee-stored pollen (0.1 g).

The effect of the pollen-borne agent on virion structure was examined as follows: Intact bee-stored pollen (0.2 g) was added to each of three tubes that contained 0.85 ml of PDV (30 μg/ml) in 0.01 M neutral phosphate buffer. One tube was incubated 4 hr at 4 C where the agent was not active; the second tube was incubated 3 hr at 4 C and then at 40 C for 1 hr; the third tube was incubated 4 hr at 40 C. After incubation, 0.5-ml aliquots of pollen-free liquid were decanted, layered on 10–40% rate zonal sucrose density gradients, and centrifuged 1.5 hr at 60,000 rpm in an SW 65 rotor. Each density gradient tube was fractionated into 0.5-ml fractions and each fraction was assayed by ELISA (Fig. 4). The sedimentation profile of ELISA-reactive antigen from tube 1 (Fig. 4A) was similar to that obtained with untreated virus, indicating that 4-hr of exposure to bee-stored pollen at 4 C had no measurable effect on sedimentation properties of the virus. After 1-hr of exposure at 40 C in the presence of bee-stored pollen, there was considerable loss of PDV antigen, and the antigen that remained sedimented more slowly than intact virus (Fig. 4B). After 4 hr at 40 C, only trace amounts of PDV antigen was detected and these were located at the top of the density gradient tube (Fig. 4C). No traces of virus aggregates were found at the bottom of the gradient tubes. The sedimentation profile of PDV incubated 4 hr at 40 C in the absence of bee-stored pollen was similar to that shown in Fig. 1A. These results suggested that PDV virions exposed at 40 C in the presence of bee-stored pollen were degraded into progressively smaller units which ultimately were undetectable by ELISA. Similar results were obtained with purified NRSV.

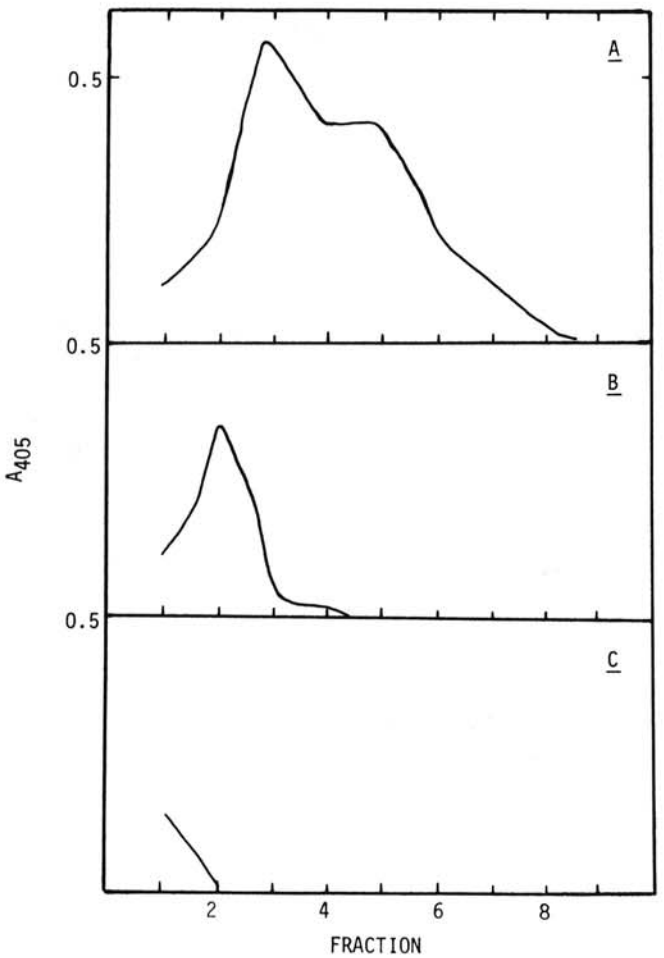

Fig. 4. Sedimentation profiles of enzyme-linked immunosorbent assay (ELISA)-reactive antigens following incubation of prune dwarf virus (30 μg/ml) with bee-stored pollen (0.2 g) for: **A,** 4 hr at 4 C; **B,** 3 hr at 4 C and 1 hr at 40 C; and **C,** 4 hr at 40 C. Each treatment was centrifuged in rate sucrose density gradients for 1.5 hr at 60,000 rpm, fractionated into 0.5-ml fractions, and assayed by ELISA. Data represent ELISA absorbance values for individual fractions.

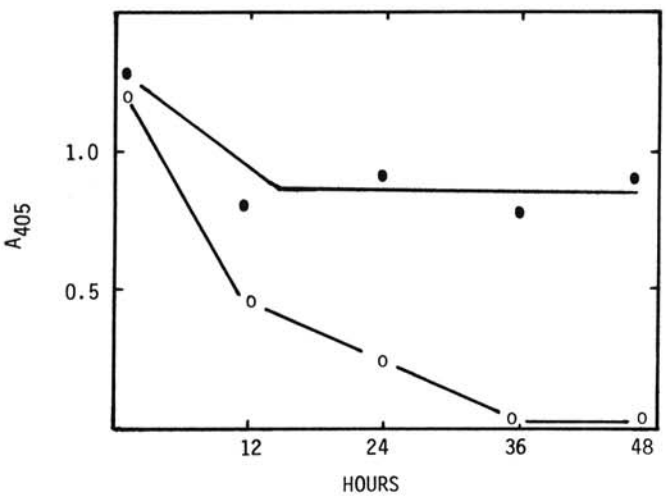

Fig. 5. Absorbance values ($A_{405\ nm}$) obtained with necrotic ringspot (o) and prune dwarf (•) virus-contaminated pollen stored at 40 C for various intervals.

Effect of virus location on in situ stability. Intact bee-stored cherry pollen that contained approximately equal amounts of NRSV and PDV was taken from a single hive and stored in dry vials at 40 C for various periods. Subsequently each sample was triturated in 10 vol of grinding buffer to release endogenous virus and tested by ELISA. All NRSV antigens disappeared within 36 hr at 40 C whereas only about 35% of the PDV antigens disappeared after 48 hr (Fig. 5). Virtually all disappearance of PDV antigen from intact pollen occurred within in the first 12 hr (Fig. 5). These results suggested that NRSV occurred primarily on the pollen surface whereas only about one-third of the PDV virions appeared to be located on the surface.

Effect of pollen leachates. Purified NRSV remained stable for >5 hr at 40 C when incubated in 0.01 M phosphate buffers between pH 5.5 and 7.5 in the absence of pollen or pollen leachates. However, in the presence of pollen leachates, viral antigens disappeared at rates that increased as the pH values decreased (Table 2). In the presence of pollen leachates at pH 5.5, nearly all antigens disappeared within 1 hr at 40 C. We detected no decrease in NRSV antigens at temperatures <30 C at pH 5.5.

Sedimentation profiles for NRSV (30 μg/ml) incubated with pollen leachates in pH 5.5 buffer and centrifuged in sucrose rate density gradients indicated that the virus remained intact when

TABLE 2. Percent decrease in enzyme-linked immunosorbent assay absorbance values ($A_{405\ nm}$) obtained with solutions of purified necrotic ringspot virus incubated with pollen leachates at various pH values

Expt.	Temp (C)	pH	Leachate	Incubation time (hr) 0	1	2	5
1	40	7.5	No	0[a]	0	0	0
			Yes	0	0	0	0
		7.0	No	0	0	0	0
			Yes	0	0	0	24
		6.5	No	0	0	0	0
			Yes	0	28	90	100
		6.0	No	0	0	0	0
			Yes	0	75	100	100
		5.5	No	0	0	0	0
			Yes	0	99	100	100
2	4	5.5	Yes	0	0	0	8
	25	5.5	Yes	0	0	0	8
	40	5.5	Yes	0	92	100	100

[a] Percent decrease in $A_{405\ nm}$ values based on 0 hr readings.

incubated at 4 C (Fig. 6A) but was progressively degraded after 1 and 4 hrs at 40 C (Figs. 6B and C). Similar results were obtained with PDV. Neither virus remained stable at pH 5.0 in the absence of pollen leachates. Consequently the activity of pollen leachates at very acidic pH's could not be determined.

In a series of experiments, we found that pollen leachates could be stored at 50 C for 15 hr or at 60 C for 4 hr without reducing their ability to degrade NRSV or PDV. However, all degradative activity was eliminated by boiling leachates for 5 min. or by overnight dialysis against distilled water.

Is glucose oxidase involved in virus degradation? Glucose oxidase, an enzyme that catalyses the formation of H_2O_2 from glucose, is known to occur in bee secretions (7) and can be found on bee-stored pollen. This enzyme has a temperature optimum between 37 and 40 C (7). In tests to determine if glucose oxidase-catalyzed H_2O_2 might be involved in virus degradation, we found that NRSV was stable for more than 4 hr in H_2O_2 concentrations up to 250 μg/ml. Furthermore, sodium cyanide at 0.001 M (reported to be a strong inhibitor of glucose oxidase) had no measurable effect on pollen leachate-induced virus degradation in our tests. However, when we added glucose to our reaction mixture, this sugar completely inhibited virus degradation as measured by ELISA (Table 3). In contrast, fructose (not a substrate for glucose oxidase) and sucrose stimulated leachate-induced virus degradation. At fructose or sucrose concentrations of 1 M, virus degradation was nearly instantaneous in the presence of pollen leachates (Table 3).

Effect of bovine serum albumin. Bovine serum albumin (BSA) was added as a possible substrate competitor to reaction mixtures of PDV or NRSV and pollen leachate. Concentrations up to 2 mg/ml had no measurable effect on the rate of virus degradation (Table 4).

DISCUSSION

The results presented here demonstrate that pollen taken from some honeybee hives contains an agent that can degrade intact

TABLE 3. Absorbance values ($A_{405\ nm}$) obtained by enzyme-linked immunosorbent assay with purified necrotic ringspot virus incubated 1 hr at 40 C with pollen leachates and various sugars

Exp.	Sugar	Conc (M)	Incubation time (hr) 0	Incubation time (hr) 1	Decrease (%) (1 hr)
1	None	0.0	0.77	0.00	100
	Glucose	0.1	1.14	0.04	96
		0.5	0.93	0.95	0
		1.0	1.02	1.19	0
		1.5	0.83	0.90	0
2	None	0.0	0.72	0.01	99
	Glucose	0.1	1.11	0.08	93
		1.5	0.79	0.78	1
	Fructose	0.1	0.72	0.02	97
		0.5	0.14	0.01	93
		1.0	0.05	0.00	100
	Sucrose	0.1	0.51	0.02	96
		0.5	0.41	0.01	98
		1.0	0.03	0.00	100

TABLE 4. Effect of bovine serum albumin (BSA) on the rate of necrotic ringspot virus degradation at 37 C in the presence of bee-stored pollen leachates

Leachate added	BSA (mg/ml)	Incubation time (hr) 0	6	12	18
No	0	1.98[a]	2.10	1.98	1.98
Yes	0	2.09	1.47	0.60	0.00
Yes	2	2.01	1.54	0.43	0.00

[a] Absorbance values at 405 nm determined by enzyme-linked immunosorbent assay.

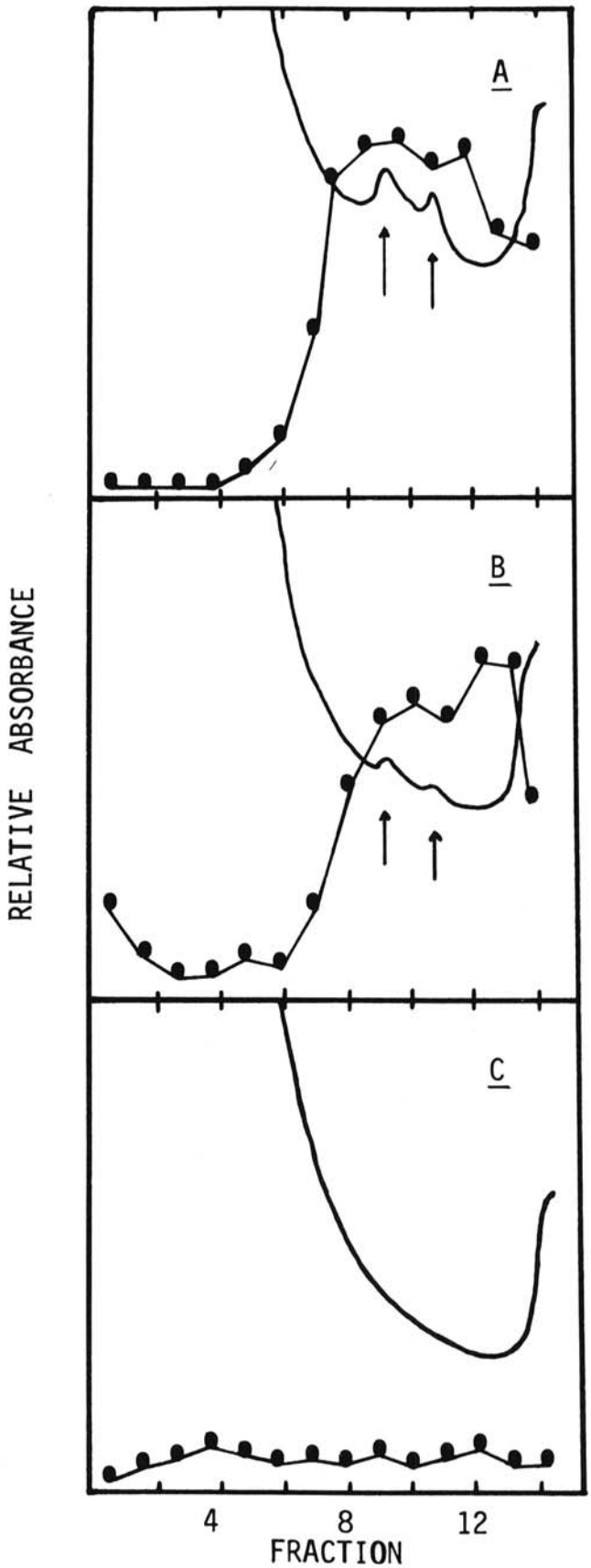

Fig. 6. Sedimentation profiles of enzyme-linked immunosorbent assay (ELISA)-reactive antigens following incubation of necrotic ringspot virus (30 μg/ml) with pollen leachates for **A,** 4 hr at 4 C; **B,** 3 hr at 4 C and 1 hr at 40 C; and **C,** 4 hr at 40 C. Each treatment was centrifuged in rate sucrose density gradients for 2 hr at 29,000 rpm. Solid line represents $A_{254\ nm}$ values obtained with an ISCO fractionator, black circles represent $A_{405\ nm}$ values obtained in ELISA. The arrows denote two UV absorbance peaks typical for intact NRSV.

virions of at least two ilarviruses. Studies in progress suggest that representative viruses of other groups may also be degraded. Degradation appeared to be unrelated to glucose oxidase (often referred to as inhibine [8]) which occurs in bee secretions and which we assumed to be present on pollen samples from some, if not all, hives.

In the presence of pollen leachates and favorable temperatures and pH, both NRSV and PDV were rapidly degraded into products that were serologically undetectable; possibly these were individual amino acids or small unreactive peptides. From this, we assume that proteolytic enzymes were responsible for at least a part of the degradation process even though the rate of virus degradation was not altered when BSA was included as a possible substrate competitor. The unusually high optimum temperature (40 C or above) and relatively low pH optimum (5.5 or below) combined with an apparent requirement for a dialyzable cofactor, suggests that at least one nonproteolytic enzyme may be involved. Total inhibition of virus degradation by glucose and the marked stimulation of degradative activity by fructose and sucrose are conditions not yet understood. However, the pronounced effect exerted by these sugars supports the hypothesis that pollen leachate-induced degradation of intact virions involves more than a single proteolytic enzyme.

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