Acquisition and Transmission of Pea Enation Mosaic Virus by the Individual Pea Aphid

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ABSTRACT

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Pea enation mosaic virus (PEMV) can be detected by enzyme-linked immunosorbent assay (ELISA) in individual nymphs or adults of *Acyrthosiphon pisum*. However, the virus concentrations of an infective aphid may be below the level (5 ng/ml, based on one aphid homogenized in 0.2 ml of phosphate-buffered saline) that is detectable by ELISA. The amount ingested during a 24-hr acquisition access period (AAP) varies widely among individual aphids, from a nondetectable level up to 40 ng/ml for nymphs and up to 200 ng/ml for adults. Circumstantial evidence suggests that this variation reflects a highly uneven distribution of PEMV in the phloem cells of the source plant. The amount accumulated in the aphids during the AAP increases rapidly up to 16 hr and then continues at a lower rate. This suggests that ingestion and excretion are approximately in equilibrium after 16 hr and that the subsequent lower rate of increase may be due to enlargement of the intestinal tract of the growing insect. Large amounts of virus could be detected in the intestinal tract and small amounts in the hemolymph. Most of the virus passed through the intestinal tract within 6 days after acquisition when aphids were feeding on healthy plants. The infectivity of groups of aphids and their average viral charge were closely related. However, the transmission efficiency of individual aphids was poorly related to the amount of virus carried. The length of the latent period of an individual aphid was not closely related to either viral charge or transmission efficiency.

Relationships between pea enation mosaic virus (PEMV) and its aphid vector *Acyrthosiphon pisum* (Harris) have been widely investigated as a model of circulative virus transmission and were recently reviewed (12,22). Virus-vector relationships such as the influence of the length of the acquisition access period (AAP) on the transmission efficiency, the length of the latent period, and measurements on the relative virus concentrations in the various tissues of the insect have been measured by bioassays (4,5,20,21). Thus far, no quantitative data are available on the amount of virus ingested and its relationship to the mechanism of virus persistence in aphids.

Recently, the enzyme-linked immunosorbent assay (ELISA) (6) has been used successfully to detect several viruses in their aphid vectors (8,9,10,25). This sensitive test offers the possibility of quantifying the virus present and studying its distribution in the insect body.

We used ELISA to study the acquisition and persistence of PEMV in its vector and infectivity assays to investigate the relationships between quantities of virus acquired and infectivity of aphids. This paper presents the results of those studies.

MATERIALS AND METHODS

Aphids, plants, and virus. All experiments were done with a clonal line of the pea aphid, *A. pisum*. Broad bean (*Vicia faba* 'Drie × Wit') plants were used for rearing virus-free aphids. The pea plant, *Pisum sativum* 'Koroza,' was used both as a virus source plant and as a test plant for all aphid transmission tests. Pea plants were infected 5–6 days after sowing and those with severe symptoms were used as source plants 12–14 days after inoculation. The PEMV isolate used was obtained from L. Bos, Wageningen, and purified virus preparations were obtained by the procedure of Mahmood and Peters (17).

Transmission tests. All experiments were done with 24- or 48-hr-old nymphs and/or with 2- to 3-day-old apterous adults. In each experiment, 84 or more aphids were successively assayed for infectivity on a series of pea seedlings. Since the efficiency of PEMV transmission by the pea aphid is similar in short and long test feedings (27), the aphids were given inoculation feeding periods of 1 hr to test transmission efficiency. After removal of the aphids the plants were sprayed with dichlorovos and placed in a growth chamber at 20.5 + 0.5 C for symptom development. The aphids were kept at 20.5 + 0.5 C during the AAP, the bridging period, and inoculation feeding period. Further relevant experimental details are given in the Results section.

ELISA. ELISA was performed as described by Clark and Adams (6). Antiserum was prepared by three subcutaneous injections of 5–10 mg of virus, which was fixed in 1% glutaraldehyde and mixed with an equal volume of Freund's incomplete adjuvant after removal of glutaraldehyde by dialysis.

Partially purified immunoglobulins (Igs) were prepared by precipitation with ammonium sulfate and dialysis in half-strength phosphate-buffered saline (PBS) pH 7.4. The Igs at 1 mg/ml ($A_{280\ nm} = 1.4$) were conjugated with alkaline phosphatase (Type VII, Sigma Chemical Co., St. Louis, MO 63178) at an enzyme: Ig ratio of 2:1 (w/w) with 0.06% glutaraldehyde, dialyzed, and stored at 4 C after addition of 5 mg/ml of bovine serum albumin.

Wells in Microelisa plates (M 129 A; Dynatech, Alexandria, VA

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22314) were coated by incubating 200 μl of unlabeled Ig diluted 1:1,000 in 0.05 M sodium carbonate, pH 9.6, at 36 C in each well for 3 hr. After incubation, the plates were rinsed three times with demineralized water. The aphids, assayed after either the AAP or inoculation feeding period, were individually homogenized in a small Elvejhem-Potter tube with 0.2 ml of PBS (0.15 M NaCl in 0.02 M phosphate buffer, pH 6.5) containing 0.05% Tween-20 and 2% polyvinylpyrrolidone (PVP; mw 44,000). The aphids used in control tests were of the same age and had access either to healthy broad bean or pea seedlings for identical feeding periods. Antigen preparations were incubated overnight at 6 C in the coated, rinsed wells and the antibody conjugate was diluted 1:1,000 in a buffer mixture containing PBS-Tween-PVP and 0.02% ovalbumin. Unreacted conjugate was removed by rinsing, and specific antibody-antigen reactions were assessed by adding *p*-nitrophenyl phosphate at 1 mg/ml in 10% diethanolamine buffer at pH 9.8. Reactions were read at 405 nm after 3 hr by using a Titertek Multiskan colorimeter (Flow Laboratories Ltd., Irvine, Scotland).

Dissection of the aphid. The hemolymph was taken up in 0.2 ml of PBS after removing the head and legs and gently pressing the aphid. The remaining body was transferred to another 0.2 ml of PBS, the cauda was removed to separate the hindgut from the skin, and the gut was forced out of the abdomen by gently pushing the body with a needle.

RESULTS

The sensitivity of ELISA for PEMV. Purified PEMV was assayed after dilution in PBS or in PBS in which aphids were homogenized (one aphid in 0.2 ml of buffer). The values of the PEMV dilutions containing aphid material were slightly lower (Fig. 1). We considered an $A_{450\ nm}$ value twice that of the background as positive. Therefore, PEMV was detected at a concentration of approximately 5 ng/ml, and 1 ng of virus could be detected in a single aphid when homogenized in 0.2 ml of buffer. The $A_{405\ nm}$ values found with virus-free nymphs or adults were approximately 0.02 and 0.04, respectively.

Virus acquisition by nymphs and adults. The acquisition of virus by nymphs and adults was tested by using 50 aphids of each stage given a 24-hr AAP. PEMV was detected by ELISA in 90% of the nymphs and 98% of the adults (Fig. 2). The percentage of viruliferous aphids in which no virus was detected decreased as the length of the AAP increased (Table 1). The $A_{405\ nm}$ values ranged from 0.02 to 0.55 for nymphs and from 0.02 to 1.1 for adults. This indicates that 24-hr nymphs and adults can acquire quantities of virus ranging from nondetectable amounts up to 40 and 200 ng, respectively. An average of four times more virus was ingested by adults than by nymphs. The amount ingested was not simply a function of size between adults and 24- to 48-hr-old nymphs because the weight ratio between these stages is 20:1, respectively (Figs. 2 and 3). In one experiment, the weight of aphids of several ages and amount of virus acquired in 24 hr was determined. There was a linear relationship between the logarithm of the body weight and the average ELISA value (Fig. 3). Some nymphs ingested at least 50 times more virus than did some adults (Fig. 2).

The rate of virus acquisition was measured by allowing groups of 84 aphids AAPs of 2, 4, 8, 16, 32, and 64 hr, respectively. Each aphid was individually tested by ELISA. A sharp increase in the average values occurred during the first 16 hr (Fig. 4). The rate of increase diminished greatly during the subsequent 48 hr, increasing only from 0.87 to 1.0. The curve suggests that after 16 hr of feeding the ingestion of virus may approximate the loss due to excretion of virus. The slight increase of virus during the 16- to 48-hr interval could be due to gut enlargement during growth.

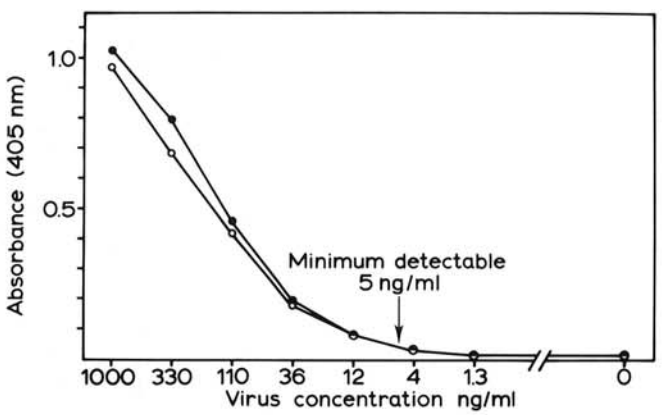

Fig. 1. ELISA absorbances (405 nm) obtained in a threefold dilution series of PEMV in buffer (●—●) and in buffer mixed with aphids in a ratio of one aphid per 0.2 ml of buffer (o—o). The dilution of the γ-globulin in the coating buffer and conjugated antiserum was 1:1,000. The results are the means of eight replicates.

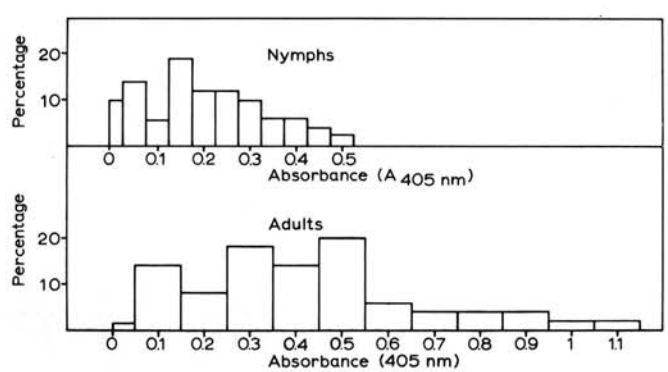

Fig. 2. Percent distribution of ELISA absorbances (405 nm) of 50 individual 24- to 48-hr-old nymphs or adults that had fed on PEMV-infected plants for 24 hr.

TABLE 1. Percentage of viruliferous aphids showing no reaction in the ELISA[a]

	Acquisition access period (hr)			
Experiment	2	4	8	16
1	37	37	21	16
2	40	···	19	···

[a] Measurements of 84 aphids were used in each sample.

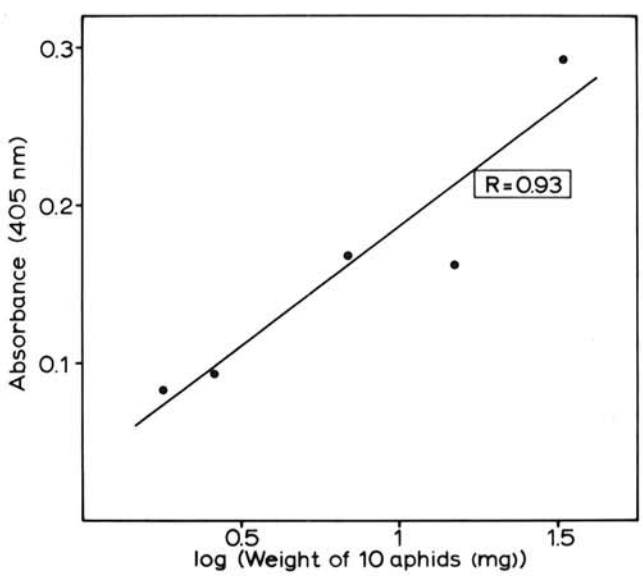

Fig. 3. Increase of ELISA absorbances (405 nm) as a function of the average body weight obtained from nymphs of various ages and 2-day-old adults after feeding for 24 hr on PEMV-infected plants. Each point is the average value for 50 aphids.

Loss of virus from aphids by excretion was studied in an experiment in which nymphs were placed on healthy broad bean plants for 0, 1, 2, 4, 6, and 10 days after an AAP of 24 hr. The aphids were transferred every 2 days to avoid uptake of virus from plants that were infected. The average $A_{405\ nm}$ values decreased rapidly in the first day (Fig. 5) and reached a constant level 6 days after the acquisition. Because most of the acquired virus was eventually lost it was concluded that it accumulates to a great extent in the intestinal tract. It is noteworthy that the infectivity of the aphids did not decrease over a period of 2 wk. Forty aphids infected 81, 82, and 80% of the test plants at 1, 7, and 14 days after the 24-hr AAP, respectively.

The virus content of hemolymph and intestinal tract. Dissection of 16 adults given access to virus for 72 hr revealed most of the virus occurred in the intestinal tract ($A_{405\ nm}$ value, 0.549). Virus, amounting to 200 ng, was detected in 15 of 16 guts analyzed. The hemolymph of 13 of 16 aphids reacted positively with an average $A_{405\ nm}$ value of 0.047, equivalent to ~1.2 ng per aphid. The amount of virus in the hemolymph was close to the detectable level. However, some virus from the intestinal tract may have been released into the hemolymph during dissection. If this occurred the values for virus found for hemolymph would be too high.

The relation between acquisition and infectivity. Infectivity tests have shown that the transmission efficiency for circulative viruses increases with the length of the AAP (3,20,23). As shown in this report, the amount of virus ingested by each aphid of a group subjected to a controlled AAP varied considerably. Therefore, it was of interest to study the relationships between the amount of virus ingested and the transmission efficiency.

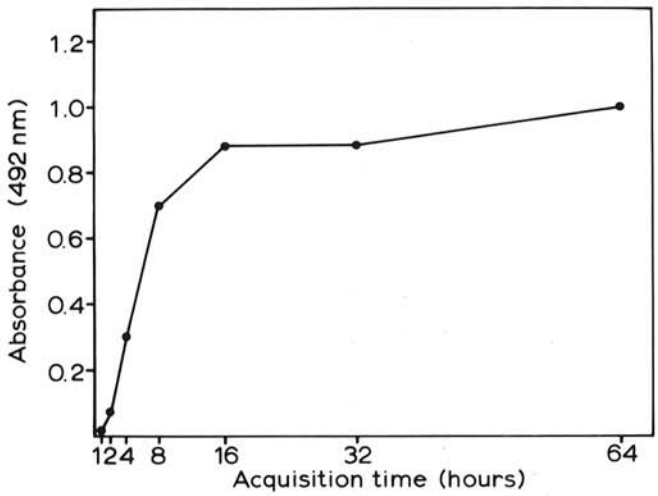

Fig. 4. Average ELISA absorbances (492 nm) of aphids that had fed in groups on PEMV-infected plants for 2, 4, 8, 16, 32, and 64 hr. Each point is the average value for 84 aphids. Horseradish peroxidase was used in this experiment.

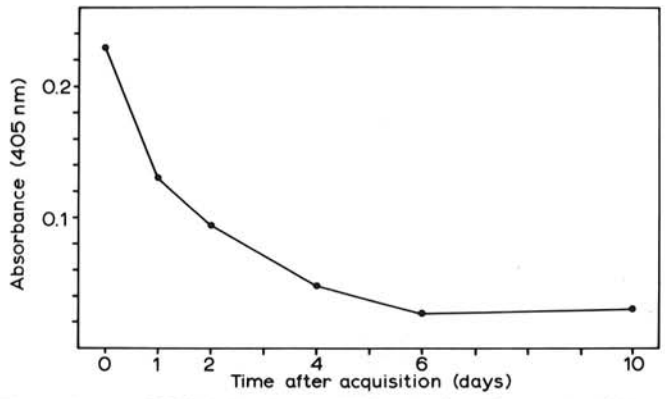

Fig. 5. Average ELISA absorbances (405 nm) of aphids that had fed on healthy plants for 1, 2, 4, 6, and 10 days after a PEMV acquisition access period of 24 hr. Each point represents the average value for 50 aphids.

In a first experiment four groups of 84 aphids were given an AAP of 2, 4, 8, or 16 hr. To allow completion of the latent period, the aphids were individually placed on pea plants until 24 hr from the start of the AAP and were then assayed hourly for infectivity by serial transfer to three pea plants. The results (Fig. 6) confirmed observations of earlier workers that the percentage of plants infected by aphids increases with the length of the AAP, which in turn determines the amount of virus ingested.

In another test, the individual transmission efficiency and the dosage acquired were tested. A group of aphids in two replicates was allowed an AAP of 8 hr. Each aphid was placed for 18 hr on a single pea plant to complete the latent period and then consecutively transferred hourly to a series of 10 pea plants. After the last inoculation access period the virus content of each aphid was measured. The results of one replicate are shown in Fig. 7. The second replicate gave similar results. Again, the quantity of virus ingested by a single aphid varied considerably, but the transmission efficiency of an aphid was not highly correlated with the amount of virus ingested. Transmission efficiency of aphids in which no virus could be detected varied from 0 to 100%. Aphids with high $A_{405\ nm}$ values invariably transmitted with efficiencies of 80–100%.

We also studied the relationships between the length of the latent period and the transmission efficiency. After AAPs of 6 to 7.5 hr the nymphs were individually transferred to a series of nine to 11 pea plants at hourly intervals to determine the time of the first successful transmission. The transmission efficiency of these aphids was tested 24 hr after the start of the AAP as described earlier. The aphids were allowed to feed individually on a pea plant to bridge

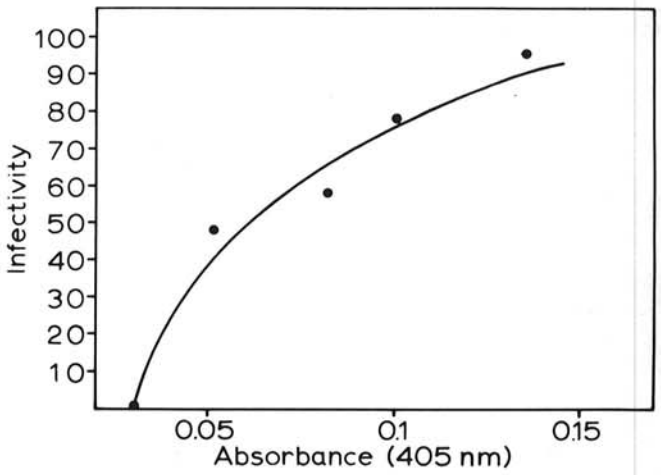

Fig. 6. The relation between the percent infectivity of 24- to 48-hr-old nymphs that acquired PEMV during acquisition access periods of 0, 2, 4, 8, and 16 hr (left to right) and the average value of the ELISA absorbance. Each group consisted of 84 aphids.

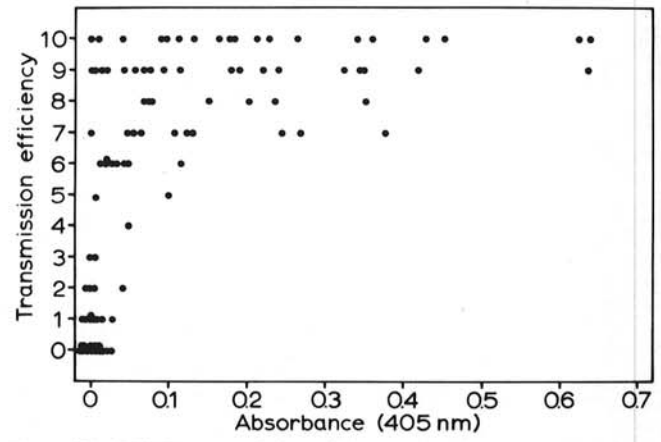

Fig. 7. The PEMV transmission efficiency of aphid nymphs individually bioassayed for infectivity on a series of 10 pea plants and tested for virus content by ELISA. Each point represents the value for a single aphid.

the period between the last transfer in the latent period test and the first transfer in the transmission efficiency test. The results of two replicates showed that the latent period in a single aphid is not clearly dependent on virus content (Fig. 8); the correlation coefficients were 0.39 and 0.41, respectively. The transmission efficiency showed the same irregular relationship with the quantity of virus ingested as in the former experiment. The length of the latent period in individual aphids was not correlated with the transmission efficiency (Fig. 9).

DISCUSSION

The sensitivity of ELISA for PEMV in our experiments is of the same range as previously reported for other viruses such as plum pox virus (6), Arabis mosaic virus, Prunus necrotic ringspot virus (26), and potato virus Y (11). We detected PEMV in individual aphids, nymphs, or adults, whereas groups of 5–30 often are required for detection of the circulative viruses such as barley yellow dwarf virus (BYDV) (9) and potato leaf roll virus (PLRV) (8). We believe that this difference does not reflect a lower sensitivity of ELISA for BYDV (16) and PLRV (24), but that the concentration of PEMV in the phloem is probably higher than that of either BYDV or PLRV.

It is often assumed that aphids acquire similar doses of virus in a given AAP. However, the amount of PEMV ingested by an aphid can vary at least by a factor of 200 for adults and 50 for nymphs after a 24-hr AAP. Since Banks and Macaulay (1,2) reported that the volume of sap ingested is approximately constant for each aphid and occurs more or less at the same rate, the wide variation in the amounts of virus ingested may reflect an unequal distribution of virus in the phloem cells. A highly uneven distribution of virus particles was found for BYDV (14) and PLRV (15) in phloem cells by electron microscopy. Tamada and Harrison (25) working with ELISA on the PLRV-*Myzus persicae* system also found large differences in the virus content of individual aphids.

Analysis of the virus content of the gut and hemolymph showed most of the ingested virus accumulated in the intestinal tract (Fig. 5). As shown for BYDV (19) and PLRV (25) the virus in the intestinal tract passed through the gut during feeding on healthy plants and the virus content in each aphid reached a constant level 6 days after acquisition. This constant level may represent virus that has been translocated from the gut into the hemolymph and other tissues plus virus still present in the gut and which is not removed by feeding on healthy plants. The low $A_{405\ nm}$ value obtained with the hemolymph fraction indicates that only small amounts of virus are absorbed from the gut. It may be the reservoir that maintains the persistent infectivity. Similarly, Tamada and Harrison (25) found that only a small proportion of the PLRV particles ingested seems to pass from gut to hemocoele.

Adults fed for 16 hr on an infected plant could contain approximately 2.5×10^{10} particles in their intestinal tract. This may account for the "enormous concentration" of particles observed by Harris and Bath (13) in this organ. The virus particles in the gut accumulate during the first 16 hr at a rapid rate, then at a slower rate (Fig. 4). This suggests that the ingestion and excretion of the virus are roughly in equilibrium and that extra accumulation may be due to an increase in size of the intestinal tract of the growing aphid.

The latent period and transmission efficiency of PEMV have been reported to be sensitive to dosage (7,19,22). The first variable declines and the second increases as the length of the AAP increases. We confirmed this in experiments with groups of aphids. As shown in Fig. 6, the average charge of virus acquired increases with the length of the AAP, and aphid infectivity was positively correlated with this charge. However, the latent period and the transmission efficiency of a single aphid were found to be poorly related to its charge. Aphids with a large virus charge invariably showed a high transmission efficiency, but aphids in which no virus was detected varied in transmission efficiency. Some transmitted with low and others with high efficiency. The cause of poor correlation between the latent period and the transmission efficiency on one hand, and the size of the charge on the other is not known. It may be affected by several highly variable factors. One of these may be a threshold charge of virus that results in infectivity. Neither the moment at which this charge is acquired nor the rate of its acquisition is known. These parameters are subject to such variables as feeding behavior (18) during the AAP and the concentration of virus in cells from which the food is ingested. Another factor that may influence aphid infectivity is the translocation of virus from the intestinal tract into hemolymph. The occurrence of low amounts of virus in the hemolymph could be due to the gut wall acting as a barrier. The increase of the latent period in older aphids (3,4,20), in spite of high amounts of virus ingested, could be caused by an increase of resistance to virus translocation. A decreased gut permeability to virus with increased age has been demonstrated in several virus-vector relationships (12). However, the possibility remains that nymphs may attain higher titers in the hemolymph than adults because they acquire more virus per unit body weight (4).

Little is known about the mechanism of the passage of virus through the gut wall and the concentration of virus in the hemolymph and salivary glands. These factors are probably of paramount importance to maintain the persistence of circulative

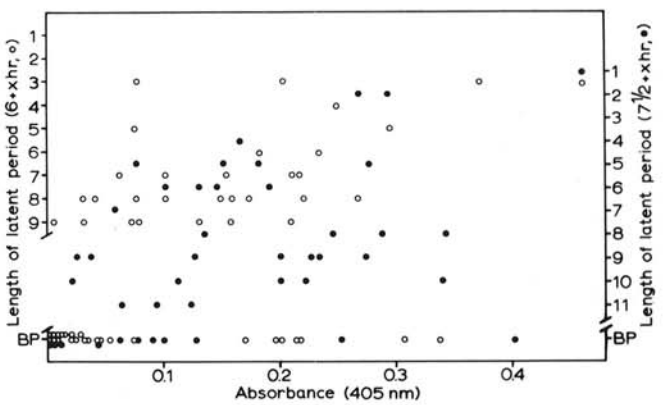

Fig. 8. The relation of the ELISA values of nymphs that had fed for 6 hr (o) or 7.5 hr (•) on PEMV-infected peas to the length of the latent period. The latent period was assayed by serial transfer of the aphids to a series of nine or 11 test plants every hour. After this assay the aphids were placed individually on pea plants for 9 or 5.5 hr on which the last aphids could complete their latent period. After this bridging period (BP) the transmission efficiency was tested in hourly serial transfers and the aphids were assayed for virus content. Each symbol represents the value obtained by measuring a single aphid.

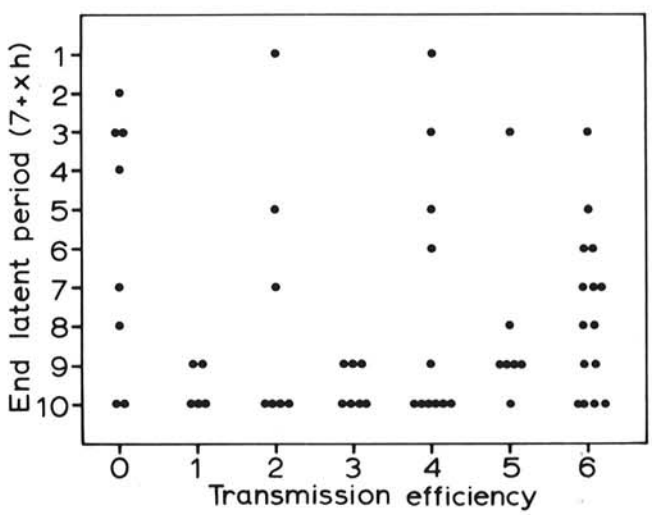

Fig. 9. The relation of the latent period and the PEMV transmission efficiency found in the third replicate in which single aphids had acquired virus in an acquisition access period of 7 hr. Each point is the value found for a single aphid. The transmission efficiency was tested on a series of six pea plants at hourly intervals.

viruses in the aphid. To study these factors a more sensitive test than ELISA is required.

An increase in the number of infectious particles after acquisition is evidence for virus multiplication in the vector. Our data do not show an increase in quantity of viral antigen after acquisition of PEMV; no evidence was obtained of virus multiplication in the vector.

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