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The acquisition and transmission of pea enation mosaic virus by the pea aphid, studied by Elisa and bioassays.

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ABSTRACT

Pea enation mosaic virus (PEMV) may be detected by enzyme linked immunosorbent assay (ELISA) in individual nymphs or adults of *Acyrthosiphon pisum*. However, the concentration that makes an aphid infectious is below that which (5 ng/ml) can be detected by ELISA. The charge ingested varies widely among individual aphids; from a non-detectable level up to 40 ng for nymphs and up to 200 ng for adults after a 24 hr acquisition period. We obtained indirect evidence that this variation reflects a highly uneven distribution of PEMV in the phloem cells of the source plant. The amount ingested rapidly increases with the length of the acquisition access period up to 16 hr, and later at a lower rate. This suggests that ingestion and excretion are roughly in equilibrium and the low rate of increase may be due to the growth of the intestinal tract. We did not find any significant effect of a preacquisition period on the amount of virus ingested.

Large amounts of virus could be detected in the intestinal tract, and small amounts in the hemolymph. Most of the virus is flushed out of the intestinal tract within 6 days after acquisition when aphids are fed on healthy plants.

Infectivity of groups of aphids and their average charge were closely related. However, the transmission efficiency was poorly related to the charge of virus carried by individual aphids. The length of the latent period of an individual aphid is not closely related to its virus charge or transmission efficiency.

RESUME

Le virus de la mosaïque énation du pois (VMEP) peut être détecté dans son puceron vecteur *Acyrthosiphon pisum* grace à une technique immunoenzymatique (ELISA). Il suffit d'un seul puceron, larve ou adulte, pour détecter le virus. Cependant, la charge minimale nécessaire pour réaliser une transmission aphidienne est inférieure au seuil de détection de la méthode ELISA (5 ng/ml). Après une période d'acquisition de 24 hr, la charge ingérée varie considérablement entre les pucerons; du seuil de détection jusqu'à 40 ng dans le cas des larves, et jusqu'à 200 ng dans le cas des adultes. Il semble probable que cette variation est liée à la distribution irrégulière du VMEP dans les vaisseaux du phloème de la plante source. Dans une première phase (0-16 hr), la quantité de virus ingérée croit linéairement en fonction du temps. La deuxième phase (16-64 hr) est marquée par un ralentissement de l'accumulation du virus. Cette inflexion de la courbe d'acquisition peut s'expliquer par un équilibre entre l'ingestion de sève et l'excrétion de miéllat. Le faible taux d'accumulation caractéristique de la deuxième phase serait alors dû à l'augmentation du volume du tractus intestinal. Un temps de jeûne précédant l'acquisition n'affecte pas significativement la quantité de virus ingérée.

La quantité de virus détectée dans le tractus intestinal est très supérieure à celle que l'on mesure dans l'hémolymphe. L'essentiel de la charge ingérée est finalement éliminé du tractus intestinal et rejeté avec le miéllat.

L'étude des relations entre la charge ingérée par le puceron et diverses caractéristiques de la transmission aphidienne a été réalisée. Ainsi l'infectivité et la concentration moyenne de VMEP sont étroitement liés. Cependant, en considérant chaque puceron pris individuellement la charge ingérée ne permet de rendre compte ni du temps de latence, ni de la capacité de transmission.

INTRODUCTION

Foreword. This report describes a research program conducted from October 1980 to July 1981, during a period of research and training at the Department of Virology of the Agricultural University of Wageningen, The Netherlands. From this work, I have presented a communication to the Netherlands Plant Pathologists (May 6, 1981), a colloquium to the Department of Virology of the Agricultural University of Wageningen (June 6, 1981), a communication and an abstract to the Fifth International Congress for Virology (August 3, 1981) held at Strasbroug. It has also been submitted for publication to Phytopathology (July 6, 1981), and a poster will be presented to the Symposium on Insects to be held in Wageningen in September 1981.

The primary objective of this research program was to investigate various features of the acquisition and transmission of pea enation mosaic virus (PEMV) by its aphid vector *Acyrthosiphon pisum*. Virus vector relationships have received much attention over the past decades, as a key factor in the understanding of virus ecology and epidemiology. Several systems of classification have been subsequently proposed to account for the different patterns of transmission. They are described in the first part of the introduction. PEMV is one of the best studied circulative virus transmitted by aphids. Its intrinsic properties are reviewed. An extensive survey of the literature regarding the PEMV-*A. pisum* relationships was conducted. As most of the research done so far was carried out by bioassays, we consider the perspective opened by the application of a new and more sensitive serological technique, the ELISA test. Ultimately, we lay down two lines of research on transmission of PEMV which we want to exploit.

Importance of aphid borne viruses and their virus vector relationships. The biology, feeding behaviour and worldwide distribution of aphids make them ideally suited for transmitting plant viruses (Harris and Maramorosch, 1977). Aphids (Aphidoidea) with 192 vector species, transmit as we know

today 164 viruses and possibly three mycoplasma like organisms and they account for more than 86% of all sternorrhynchous vectors. Indeed, the aphids alone are responsible for the transmission of c. 60% of all insect-borne plant pathogens. The total number of described aphid species ranges over 3700, whereas only 300 species have been tested as vectors of c. 300 different viruses in about the same number of plant species (Harris, 1977). This suggests that the role of aphids as virus vectors could actually still be underestimated.

Two classification systems have been used to describe virus transmission, in particular by aphids. One is based on how long vectors retain virus, the other on where and how virus is carried by the vector. The former system, the elder of the two, was proposed by Watson and Roberts in 1939. Transmissions and, later by common usage, the viruses are classified as non-persistent, semipersistent and persistent depending on whether virus is retained for minutes, for several hours or some days, or for their whole life, respectively. This system is based on a readily and testable characteristic, the retention. However, experimental conditions used are not always comparable, and are known to affect the length of the retention time. In addition, distinctions between the three categories are sometimes blurred, with some viruses not fitting neatly into any category (Pirone and Harris, 1977).

The second and newer system was proposed by Kennedy *et al.* in 1962. They classified viruses as stylet borne or circulative. The term stylet borne replaces nonpersistent and gives recognition to the research of Bradley and Ganong (1955a; 1955b) who demonstrated that treating the stylets of viruliferous aphids with certain antiviral agents rendered the insects non-viruliferous. The term circulative replaces persistent and refers to transmission in which virus is acquired via the maxillary food canal, absorbed from the intestinal tract into the hemocoele, translocated to the salivary glands and inoculated into plants via virus-laden salivary: an

ingestion-salivation mechanism. Circulative viruses which also multiply in their vector are circulative propagative.

Recently (Harris, 1977), the nonpersistent and semipersistent aphid borne viruses were categorized as non-circulative . Circulative is used in the traditional sense (Black, 1954). The term non-circulative does not only apply to stylet borne transmission but at the same time to recent findings that non-circulative viruses can be acquired (ingested) from and inoculated (egested) into plants via the food canal in the maxillary stylets: an ingestion-egestion mechanism (Fig. 1 and 2). The majority of the 164 aphid-borne viruses is non-circulative (101 nonpersistent and 8 semipersistent), 38 are circulative and the status of 17 is uncertain (Eastop, 1977).

Among the circulative viruses, PEMV (along with barley yellow dwarf virus, potato leaf roll virus and beet western yellow virus) has received major and continuing research emphasis. Its widely investigated transmission pattern provides much of the available knowledge about the circulative model.

Pea enation mosaic virus disease. The International Committee on Taxonomy of Viruses (ICTV) has placed PEMV in a monotypic group (Shepherd *et al.*, 1977) host range includes in addition to a number of legumineous hosts also species from other families. Local lesion, chenopodiaceous, assay species include *Chenopodium album*, *C. amaranticolor* and *C. quinoa* (Ruppel and Hagedorn, 1963 ; Hagedorn *et al.*, 1964; Izadpanah and Shepherd, 1966). PEMV causes one of the most serious diseases of garden pea, *Pisum sativum*, broad bean, *Vicia faba*, and sweet pea, *Lathyrus odoratus*. It is widely distributed in northern temperate regions (Chaudhuri, 1950; Klinkowski, 1958; Werner, 1967). Occasional oubreaks of economic importance have been reported in New York State, Oregon, California and Wisconsin (Harris, 1979).

Systematically infected leaves show conspicuous hyaline transluant spots, mosaic patterns and frequently small necrotic flecks and streaks.

Chronically infected plants generally develop enations considered to be of special diagnostic value, associated with the veins undersides the leaves. Often the plants become severely stunted and in very young seedlings the first symptom may be an abrupt bending down of the petiole and of the first leaves. Anatomical studies by McWorther (1949, 1965) revealed nuclear changes in cells of infected plants. Hyperplasia and hypertrophy of vascular bundles as well as necrosis of the mesophyll were frequently observed. On the basis of electron microscopic observations it was concluded that PEMV first invades and multiplies in cell nuclei from which it is then released into the cytoplasm (Shikata *et al.*, 1966; Shikata and Maramorosch, 1966).

Purification and intrinsic properties of PEMV. PEMV has a diffusion coefficient (D20, w) of c. 1.89 10^{-7} cm^2/sec (Bozarth and Chow, 1966), a buoyant density in CsCl of 1.42 g/cm^3, an absorbance of 7.5 at 260 nm (1 mg/ml, 1 cm lightpath) (Shepherd, 1970), a thermal inactivation point (10 min) of c. 65°C, a dilution end point of c. 10^{-4}, and a longevity *in vitro* at 20°C of 3-13 days depending on the isolate (Pierce, 1935; Osborn, 1935; Ruppel and Hagedorn, 1963). For some isolates, yields as high as 0.1-0.3 mg of virus per gram of leaf material are not unusual when infected young pea are harvested 10-12 days after inoculation. Numerous purification schemes have been reviewed by Hull (1977). In most cases, PEMV has been purified from infected plants, usually garden pea. However, purifications from tobacco protoplasts (Motoyoshi and Hull, 1974), and aphids (French *et al.*, 1973) have also been reported.

PEMV is an RNA-containing virus with isometric particles. Estimates in the literature of particle diameter range from 20-36 nm. Size estimates seem to be largely dependent on the treatment of virions prior to measurements and the medium in which they are measured (Shikata *et al.*, 1966; Harris and Bath, 1972; Harris *et al.*, 1975; Farro and Vanderveken, 1969; Farro and Russel, 1971). Purified preparations of PEMV sediment as two nucleprotein components, a faster sedimenting bottom component and a slower sedimenting

middle one (Hull and Lane, 1973). The ratio of bottom to middle component varies with the virus strain of variant studied (Hull, 1977). Estimates in the literature of the sedimentation coefficients (S20, w) range from 106 to 90 S for middle component and from 122 to 107 S for the bottom component. Purified PEMV preparations contain about 72% protein and 28% RNA (single stranded) with a base ratio of about 26% guanylic acid, 24% of adenylic acid, 24% cytidylic acid, and 26% uridylic acid (Shepherd *et al.*1968; Shepherd, 1970; Hull, 1977). Middle and bottom component have essentially the same percent nucleic acid content. An analysis of the available data on the infectivity of the two nucleoprotein components has led Hull (1977) to the conclusion that the genome of PEMV is divided between two RNA species; RNA 1 is associated with the bottom component and RNA 2 with the middle component. Unlike other divided genome viruses, the information for PEMV coat protein is found on the larger piece of nucleic acid (Hull, 1977). The type PEMV gives two bands on SDS polyacrylamide gels. The major band has a molecular weight of c. 22,000 and consists of 199 amino acid residues (Shepherd *et al.*, 1968; Hill and Shepherd, 1972; Hull and Lane, 1973; Hull, 1977). The relative molar ratios of the 18 amino acids represented have been determined (Shepherd *et al.*, 1968). Possible models for the distribution of protein subunits in middle and bottom components have been reviewed by Hull (1977). A second protein found in the coat determines whether the virus can be transmitted by aphids.

PEMV relationships with its aphid vectors.

The model. Like other circulative viruses, PEMV is accessible in phloem tissues of infective plants, although inoculation may occur in nonvascular tissue (Nault and Gyrisco, 1966). Acquisition by aphids is expected to begin with ingestion of phloem sap. Virus accumulates in the gut as feeding on a source plant continues. Some of the ingested virus is actively moved through the gut wall into the hemocoel, and some is excreted. Once in the

hemocoel, virus can be transported in the hemolymph and may accumulate in specific tissues, including phagocytic cells. The virus is moved through the salivary system and introduced into plants with salivary material during stylet penetration and the establishment of a feeding site. If effective movement of virus to the salivary system has not occurred before the aphid leaves the source, then a short, but variable latent period occurs before the aphid begins to transmit.

Comparative vector competency. Six species of aphids, *Macrosiphum euphorbiae* Thos., *Myzus persicae* Sulz., *M. ornatus* (Laing), *Acyrthosiphon solani* (Kalt), *A. pisum* (Harris) and *Aulacorthum solani* (Kalt) have all been shown to be experimental vectors of PEMV. Like most circulative viruses, PEMV is acquired with varying degrees of efficiency by the various vector species. *A. pisum* was reported to be a more efficient transmitter than *M. persicae* and *M. euphorbiae* (Bath and Chapman, 1966). Within species, variations exist in the transmission of PEMV by *M. euphorbiae* , the green and yellow biotypes of *A. pisum* (Hinz, 1963) and other biotypes of this species (Thottappilly *et al.*, 1972; Jurik *et al.*, 1980). Other specificities of transmission are known. Bath and Chapman (1967) showed that a strain of *A. pisum* did not transmit one PEMV isolate but did transmit another isolate, whereas a Californian strain of the aphid transmitted both virus isolates.

Recent studies on PEMV indicate that the vector-virus specificity phenomena associated with circulative transmission is mediated by salivary gland-virus coat protein interaction. It appears that reciprocity between recognition sites on virus coat proteins and salivary gland membranes is required for passage of virus through the salivary system. Slight variation in either the coat protein of virus, virus strains or variants, or in the salivary membrane systems of vector species or biotypes could affect virus transmissibility (Bath and Chapman, 1967; Clark and Bath, 1977). Data in support of the salivary gland-PEMV capsid interaction hypothesis have been extensively reviewed by Harris (1979).

Acquisition. Because of the lack of a sensitive method there was no direct information about the charge of virus in the aphids. Then there is very little information about the quantitative aspects of the PEMV acquisition. Frequently, researchers have tested the effect of increasing acquisition access time on the number of aphids that subsequently transmit the virus. Actually the acquisition of PEMV begins about the time one would expect ingestion from phloem tissue to occur, viz. somewhat longer than 10 min after the beginning of penetration (Nault and Gyrisco, 1966). There are ample data to show that, like other circulative viruses, PEMV acquisition efficiency increases with increasing length of the acquisition access period (Simons, 1954; Bath and Chapman, 1966; Bath and Chapman, 1969).

Inoculation. Unlike most circulative viruses PEMV is sap transmissible and occurs in both superficial and deep tissues of infected plants. The broader range of susceptible tissues in the plant is reflected by unusually short inoculation thresholds. Bath and Chapman (1964), Nault and Gyrisco (1966) and Peters and Lebbink (1973) reported that PEMV was efficiently transmitted in inoculation access periods of some seconds or minutes. These observations show that aphids can inoculate PEMV into epidermal cells during brief probes.

Virus release is a periodic event associated with salivation and probably not with ingestion (Sylvester, 1980). Saliva is secreted in superficial layers, parenchyma and non-sap conduction phloem cells (phloem parenchyma, companion cells, or other deep seated tissue such as bundle sheath cells). Once the feeding site (sieve elements) is reached, all secretions terminate (McLean and Kinsey, 1965). Actually, Toros *et al.* (1978) showed that PEMV is transmitted at high rates during short inoculation access periods (probing and phloem seeking activities). The rates do not increase significantly when longer inoculation access periods (feeding activity) are used. These findings have an important bearing on the method used to measure the parameters of PEMV transmission and possibly for other circulative viruses. To obtain good estimates of the infectivity, the transmission

efficiency or the length of the latent period, the aphids have to be transferred frequently.

Latent period. The aphid is not readily infectious after the short acquisition feeding periods. This delay in the development of full transmission potential is called the latent period. The latent period of PEMV in *A. pisum* has been widely investigated. As the latent period varies widely among individual aphids, authors primarily emphasized the use of the terms minimum, maximum, range, mean or medium in reporting the data (Osborn, 1935; Simons, 1954; Chaudhuri, 1950; McEwen *et al.*, 1957; Heinze, 1959). The workers probably used different combinations of pea aphid biotypes, PEMV isolates, host plants, conditions of environment (temperature..), allowing different acquisition and inoculation periods. All these factors are known to influence the latent period of PEMV, and this would account for the different values reported (Tsai *et al.*, 1972; Bath and Chapman, 1966; Bath and Chapman, 1967; Sylvester and Richardson, 1966b; Tsai and Bath, 1970). Sylvester (1965) defined a new measure, the average latent period, the time at which 50% of the infectious aphids had completed latency. He advocated the use of the log-probit transformation (LP 50) to get a better approximation. Toros *et al.* (1978) substituted short acquisition and inoculation access period to the traditional 12-14 hr long feeding time, and reported an LP 50 of 10 hr for 12 hr old nymphs. There is a general agreement that young nymphs have a shorter latent period and are better transmitters than adults (Simons, 1954; Bath and Chapman, 1964; Sylvester, 1965; Sylvester, 1967). Bath and Chapman (1968) found a latency gradient from short to long periods in first instars to adults. The length of the latent period has been reported to be dependent on the dose of inoculum (Sylvester, 1965; McLean and Kinsey, 1969; Clark and Bath, 1973).

Retention. Viruliferous aphids remain infectious following ecdysis (transstadial passage). Retention time varies depending on the vector species, virus isolate, vector age at the time of acquisition. length of acquisition feed, ambient temperature, number of molts following acquisition

(Osborn, 1935; Chaudhuri, 1950; Simons, 1954; Heinze, 1959; Nault *et al.*, 1964; Ehrhardt and Schutterer, 1965; Sylvester and Richardson, 1966b; Sylvester, 1967). In general, *A. pisum* may retain inoculativity for from a few days to as long as 4 weeks, depending on the experimental design and conditions. With one strain of PEMV Sylvester and Richardson (1966a) found that aphids declining in their rate of transmission could have their inoculative potential at least partially restored if given an additional acquisition access feeding on infected plants. With another isolate that was retained for a considerable period of time, the declining transmission rate was directly correlated with the declining feeding rate of older insects as measured by the rate of excretion (Sylvester, 1967).

Multiplication. The problem of a possible multiplication of PEMV in its aphid vector is often discussed. Arguments for and against propagation have been reviewed extensively by Sylvester (1969) and Harris (1979). Transstadial passage, the presence of a latent period, long persistence of vector inoculativity, and the fact that ambient temperature exerts an effect on both retention and duration of the latent period have sometimes been interpreted as suggesting PEMV multiplication in *A. pisum*. Transmission data not favoring multiplication include the fact that vectors can be "recharged" by additional acquisition access feeding periods (Sylvester and Richardson, 1966a). Vector inoculativity cannot be maintained by serial passage of hemolymph from one insect to another. Failure to maintain inoculativity through serial passage is perhaps the strongest argument against multiplication. Nevertheless it cannot preclude a limited multiplication in selected tissues such as gut epithelium. Harris (1979) observed by electron microscopy high concentrations of virions in the gut lamina of aphids fed for 5-6 days on healthy plants. He reported also that virions occur in high titer in the guts of PEMV-injected aphids fed for 2-5 days on virus free artificial feeding medium (Harris *et al.*, 1975). He then suggested that virus may have multiplied in the aphids during the holding period.

The enzyme linked immunosorbent assay. Immunological reactions are used for assays because they can give high levels of specificity and sensitivity. Antibodies or antigens labeled with various markers such as fluorescent dyes and radioisotopes have been found to particularly enhance the sensitivity of the reaction. The heterogeneous enzyme immunoassay overcomes many of the disadvantages of other methods (Voller *et al.*, 1979). Heterogeneous enzyme immunoassays depend on the assumption that either an antigen or antibody can be linked to an enzyme whilst retaining both immunological and enzymatic activity in the resultant conjugate. The next stage in the development of enzyme immunoassays was the linkage of soluble antigens or antibody to an insoluble solid phase in such a way that the reactivity of the immunological component was retained. This was the basis for techniques known as ELISA. Various types of ELISA assays have been applied. They were reviewed by Voller *et al.* (1979). For plant viruses, the double-antibody sandwich method has exclusively been applied.

Fig. 3 illustrates the various steps of the double antibody sandwich method. 1) Immunoglobulins specific to the antigen to be measured is attached to the solid phase. 2) The test solution thought to contain antigen is then incubated with the sensitive solid phase. 3) Enzyme-labeled specific antibody to the antigen is then incubated with the solid phase. 4) Enzyme substrate is added. The colour change is function of the antigen amount in the tested solution.

The microplate ELISA based on the double antibody sandwich method was first described for plant viruses by Voller *et al.* (1976). It combines the outstanding advantages of sensitivity, strain specificity, reliability on one hand, and practibility, easiness and quickness in the handling of the technique, on the other hand. The initial studies on arabis mosaic virus and plumpox virus (Clark and Adams, 1977) indicated that 1-10 ng/ml of virus could be detected. Since then, the ELISA test has been successfully applied to a large number of viruses in a broad range of aspects of virology (Voller *et al.*, 1979). It has widely been used as a routine test for field

surveys, plant certification, breeding programs. Recently, the ELISA test has been successfully applied to detect virus particles in aphids. Cucumber mosaic virus (Gera *et al.*, 1978), barley yellow dwarf virus (Déméchère *et al.*, 1980), potato leaf roll virus (Clark *et al.*, 1980; Tamada and Harrison, 1981), and pea enation mosaic virus (Jenniskens, 1979) were detected in *Aphis gossypii, Rhopalosiphum padi, M. persicae* and *A. pisum*, respectively. Jenniskens (1979) reported that it was possible to detect PEMV in individual aphids, 12 hr old nymphs and adults, after acquisition periods as short as 2 hr.

Scope of the study. As reviewed above, a lot of information is available about the relationships of PEMV with *A. pisum*. However, there are considerable gaps in our knowledge on acquisition and transmission of PEMV. Actually, because of the lack of a sensitive technique to assay the virus, the transmission could not directly be related to the amount of virus acquired by the aphids. The ELISA test opened the possibilities to study this relation. We could quantify the charge of virus ingested by an aphid. We followed the content of virus after various acquisition access periods, we tested the influence of a preacquisition starvation period on the amount of virus ingested and we studied the relationship between the age of the aphids and the charge of virus acquired. We followed the translocation of virus particles by assessing the virus content of the intestinal tract and the hemolymph of exposed aphids. Ultimately we followed the excretion of virus from the gut lumen of *A. pisum*.

Parameters by which the pattern of circulative transmission is characterized are transmission efficiency, length of the latent period and retention time. There are no studies made on the relation between the virus content of the aphid and these parameters, mainly because the charge could not be directly quantified. We applied ELISA tests to study the relationships between the fate of PEMV in *A. pisum* and the charge ingested.

MATERIAL AND METHODS

Purification of pea enation mosaic virus. The isolate E154 of PEMV used was obtained from Dr. L. Bos (Research Institute for Plant Protection, Wageningen). It was maintained in peas (*Pisum sativum*, var. "Koroza") through aphid transmission. Plants were grown in a greenhouse at a temperature of 20-30°C and inoculated 6 days after sowing. As the virus content appeared to be considerably lower in the symptomless parts of the plants, only those parts showing symptoms were harvested 11 to 14 days after inoculation and used for purification.

The following procedure to purify the virus was adapted as a suitable and rapid method (Mahmood and Peters, 1973). Harvested plant material was homogenized in chloroform and 0.15 M sodium acetate, pH 6.1, containing 0.015 M magnesium chloride, using 1 ml and 2 ml of these liquids per gram of tissue. The homogenate was filtered through cheesecloth and centrifuged at 10,000 rpm for 15 min. The supernatant was adjusted to pH 5.3 with 1 N acetic acid and allowed to stand for 60 min. The flocculated material was removed by means of centrifugation at 10,000 rpm for 10 min. After readjusting the pH to 6.1 with 2 N NaOH, 6% (w/w) polyethylene glycol was added and the extract was allowed to stand overnight at 4°C before being centrifuged at 10,000 rpm for 10 min. The pellet was dissolved in 4-5 ml 0.1 M Na acetate, pH 6.1. The virus suspension was now centrifuged in a gradient consisting of 5-30% sucrose in acetate buffer. The gradients were centrifuged at 22,500 rpm for 2½ hr. The virus band was removed, diluted to 20-30 ml and centrifuged 2 hr at 40,000 rpm. The pellet was dissolved in 2 ml 0.1 acetate buffer and kept in a cold room (4°C).

Preparation of immunoglobulins and ELISA procedure.

Composition of buffers used. The coating buffer (pH = 9.6) consisted of 1.5 g Na_2CO_3, 2.93 g $NaHCO_3$ and 0.2 g NaN_3 in one liter of distilled water.

Phosphate buffered saline (PBS; pH ≠ 7.4) containing 0.05% Tween and 2% polyvinyl pyrrolidone (PSTP) adjusted to pH 6.5 with acetic acid 2N was used to homogenize the aphids. Conjugate buffer consisted of 97 ml of diethanolamine in 800 ml H_2O. The pH was adjusted to 9.8 with hydrochloric acid.

Production of antiserum. The 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 adjuvant after removing glutaraldehyde by dialysis.

Purification of γ-globulins. After thawing, the antiserum was centrifuged 5 min at 10,000 *g*. The resulting supernatant was divided in samples of 1 ml. The γ-globulins were precipitated with 10 ml saturated ammonium sulfate solution (22 g in 25 ml) to antiserum which was diluted 1 : 10. The ammonium sulfate was droplet-wise added while stirring. After 1 hr at room temperature, the solution was centrifuged, the precipitate collected and dissolved in 2 ml ½ strength PBS, and dialysed three times against 500 ml ½ strength PBS. The strength was then adjusted to 1 mg/ml (OD_{280} = 1.4).

Conjugating the γ-globulins with enzyme. A sample of 0.2 ml alkaline phosphatase (5 mg/ml) was centrifuged for 5 min at 8000 *g* and the supernatant (solution of ammonium sulfate) discarded. The precipitate is directly dissolved in 0.5 ml in the γ-globulin solution (1 mg/ml). This mixture was dialysed 3 times against 500 ml PBS to remove the ammonium sulfate and to equilibrate with the buffer. The next day fresh glutaraldehyde to a concentration of 0.06% was added and incubated 4 hr at room temperature. A pale yellow color should develop. This mixture was dialysed 3 times against 500 ml of PBS to remove the glutaraldehyde. The conjugate was stored at 4 to 6°C after the addition of bovine serum albumin (BSA) to a concentration of 1%.

Preparation of aphid and plant samples to be assayed with ELISA. Aphids were collected individually and ground with 0.2 ml extraction buffer (PSTP) in a small Elvejhem Potter tube. On one plate 60 aphids were titrated. Plant extracts were prepared in a mortar with acetate buffer in a ratio of 1 to

2 (g/ml).

The ELISA procedure. The ELISA procedure was essentially carried out as described by Clark and Adams (1977). The wells of polystyrene microELISA plates (No. 1.233.29) were coated by pipetting 200 µl of unlabeled Ig diluted in coating buffer. Peripheral wells were not used, but were kept filled with water to stabilize the temperature. After incubation of 3 hr, they were rinsed 3 times with demineralized water. Antigen preparations, aphid or plant extracts, were incubated overnight at 6°C in the coated, rinsed wells to react with the bound Ig. After another rinsing to remove the unbound antigens, the enzyme-conjugated Ig diluted in conjugate buffer was added to react with bound antigen for 3 hr at 37°C. Finally, unreacted conjugate was rinsed away, and antibody-antigen reaction was assayed adding 200 µl p-nitrophenyl phosphate at 1 mg/ml in substrate buffer. Reactions were assayed 3 hr later by reading absorbances (A 405 nm) of well contents by means of a Titertek Multiscan.

Rearing of the aphids. Aphids are highly specialized plant parasites and changes in hosts, or in hosts conditions are reflected in the reproduction rate and morphogenesis. Offspring originating from plants in poor conditions could have their vectors characteristics modified. In general, these conditions that favor host plant growth, also favor the development of vector cultures. The key factors when setting up an aphid culture are the choice of the host, its age and conditions of environment, especially temperature and photoperiodism (Swenson, 1967).

A. pisum was grown on broad beans, *Vicia faba*, var. "Drie x Wit". Actually, broad bean is reported to be the host on which this aphid took the least time to become adult and on which it survived at a high rate (McLean, 1971). Colonies were obtained from a single parthenogenetically reproducing viviparous female. Cultures conditions were such that no alatae were produced. Temperature in the rearing chamber was set at 22°C under daily photoperiodism of 16 hr. Virus-free adults were placed on broad bean plants to deposit nymphs.

After 24 hr, the adults were removed and, after a further 24 hr, the nymphs were collected and given access to source plants.

Virus sources.

Source plants. Pea plants were infected by aphid inoculation 4 to 5 days after sowing. These peas showing severe symptoms 12 to 14 days after inoculation were chosen as source plants, as the virus titer and infectivity reaches then a maximum and declines thereafter very rapidly (Izadpanah and Shepherd, 1966; French *et al.*, 1974). Aphids are placed at random on the pea plants and allowed to feed. Each plant was covered by a plastic cage to prevent the escape of the aphid. For each group of 80 aphids which were allowed to acquire virus, 6 to 10 source plants were used in most experiments.

Membrane feeding systems (Fig. 4). Pieces of Parafilm (Parafilm M) are stretched into a thinner membrane (I). This should be done in two directions at right angles. Pieces of Parafilm were draped over plastic cylinders (Diam. 2.6 cm) (2). A sample of purified PEMV solution containing 20% sucrose was placed on this membrane (3, 4), and covered by another piece of stretched Parafilm (5). Then, the droplet of virus solution spread over the whole surface of the first membrane (6, 7). So the sachet obtained is ready to be used. Aphids are then placed beneath the lower membrane and allowed to feed. Stylet insertion can be checked by a simple glance to the upper membrane.

Transmission trials. Certain terms used below are defined following the definitions of Sinha (1963) and Paliwal and Sinha (1970).

Virus-free aphids are aphids which were never confined to a virus source.

Exposed aphids are apnids that had been confined to a virus source.

Infectivity is the ability of aphids to transmit the virus (inoculativity).

Infectious aphids are aphids that transmitted the virus to at least one plant when tested for infectivity (inoculative aphid; viruliferous aphid).

Infectivity of a group of aphids is the percentage of infectious aphids in a group of exposed aphids.

Transmission efficiency per aphid is the number of transmissions made by an aphid when given a number of inoculation feeding periods on a series of successive test plants.

All our experiments were done with pea seedlings in the preleaf stage. Pea seedlings are much more susceptible to PEMV infection than plants in older stages (Tsai and Bath, 1970). The tests were carried out in a growth chamber at 20 $\pm$ 0.5°C. Aphids, adults or nymphs, were transferred from plant to plant with a small moistered brush. The plants with aphids were covered by small plastic cages to prevent the aphids from escaping. Fig. 5 illustrates the sequence of operations applied in the nymph production, transmission trials and ELISA test.

Transmission trials to assay the infectivity of the aphids. To assay their infectivity, aphids were first allowed to complete their latent period after the acquisition period. Nymphs were tested for infectivity 24 hr and adults 3-4 days after the start of the acquisition access period. They were individually transferred 3 times onto test plants, one transfer every hour. Aphids which infect at least one plant were considered to be infectious.

Transmission trials to assay the transmission efficiency per aphid. After an 8 hr long acquisition access period, nymphs were allowed to complete their latent period. Then, they were individually transferred to a series of 10 successive test plants, the transfers were made every hour. The number of plants showing symptoms gave an estimate of the transmission per aphid (ranging from 0 to 10).

Transmission trials to assay the length of the latent period. Young nymphs, after an acquisition access period of 6 to 8 hr, were individually transferred 10 to 12 times, one transfer every hour. The first plant showing symptoms gives an estimate of the end of the latent period. It was found that adults required longer acquisition access period to become as infectious

as nymphs. In addition, their latent period ranged over a much longer period of time than nymphs. Therefore, transfers at every 6 to 8 hr would be more convenient. It should be reminded that the length of the latent period always includes the acquisition time.

RESULTS

Characteristics of the Igs and procedures related to the ELISA test.

Absorbance of a purified PEMV solution. Fig. 6 illustrates the absorbance of PEMV between 320 and 200 nm obtained by means of a double-beam spectrophotometer (Gilford 2400-2). This curve shows typical characteristics of a nucleoprotein with a minimal and maximal absorbance at 240 nm and 260 nm, respectively. It provides the basis for determining the virus concentration of purified solutions from the extinction obtained at 260 nm, using the value of 7.5 for a preparation of 1 mg/ml (Shepherd, 1970). Diluted purified solutions were used in the membrane feeding systems experiments and to assay the sensitivity of ELISA.

Optimum concentration of the γ-globulins suspension. Fig. 7 illustrates results obtained in tests involving dilutions of the globulin suspension in coating buffer at 1:250 and 1:2000 (approximately 4 and 0.5 µg/ml) and conjugated globulins in conjugate buffer at 1:250, 1:500, 1:1000, 1:2000 (approximately 4, 2, 1 and 0.5 µg/ml). The sensitivity of the reaction increased with the conjugate concentration. On the contrary, the sensitivity was approximately identical for each γ-globulin concentration used in the coating, ranging from 0.5 to 4 µg/ml. One possible explanation is that at low concentrations, antibody molecules are optimally distributed over the polystyrene well surface. At higher concentrations steric hindrance of antigen-antibody binding would occur due to an "overcrowding" of antibody molecules which are then not evenly distributed over the polystyrene well surface. Therefore, as noticed by Lister and Rochow (1979), higher concentration of γ-globulins in the coating does not increase and could even decrease the sensitivity of the reaction.

The effect of the incubation period of substrate. Fig. 8 illustrates results

obtained in tests using various lengths of substrate incubation. The sensitivity of the reaction increased with the length of the incubation over the period tested. We could obtain a reasonable compromise combining sensitivity and economy in the use of coating and conjugated globulins at 1:1000 (1 µg/ml), and by allowing a 3 hr-long incubation with substrate instead of the traditionally used 0.5 hr.

The sensitivity of ELISA for PEMV. Purified PEMV diluted either in PBS or in PBS in which 24-48 hr old nymphs were homogenized in a ratio of one aphid/0.2 ml of buffer, was assayed. The dilutions give identical A 405 readings except that the dilutions containing aphid material were slightly lower (Fig. 9). We considered a reading of two times higher than the background as positive. This means that an amount of one ng of virus could be detected in a single aphid when homogenized in 0.2 ml buffer. The A 405 readings found with virus-free nymphs and adults were approximately 0.02 and 0.04 respectively when using PSTP and PSTP + 1% ovalbumin as extraction and conjugate buffer, respectively (see "Composition of buffer used"). With these buffers a significant lower background was obtained than with the use of PBS as extraction and conjugate buffer. Readings of 0.1 and 0.3 were then found for nymphs and adults, respectively.

Virus source systems.

Source plants. Pea seedlings were inoculated with viruliferous nymphs. Some inoculated peas escaped infection. The others show symptoms from mild to severe. We tested the PEMV titer of 12 severely infected peas with ELISA. Results indicated that the absorbances ranged from 0.6 to 0.8 among the various pea plants. Despite standard inoculation techniques, virus concentration is not identical in the source plants.

It is often assumed that viruses which move systematically become fairly

evenly distributed throughout the plant. But as reviewed by Matthews (1970) this hardly occurs, and several factors, such as virus translocation, cell sensibility, tissue resistance, can lead to a sharp unequal distribution within the infected plant. We tested the distribution of PEMV in a single plant by removing disk samples from various leaves, and sections from the stem. Fig. 10 illustrates that the virus concentration varies from one leaf to another, or from one part of the leaf to another.

Membrane feeding systems. With membrane feeding systems we could accurately control the PEMV concentration of the virus source. However, throughout all experiments we carried out, we got erratic , not replicable results. The behaviour of the aphid was affected. *A. pisum*, starved or unstarved, spent much time walking around before initiating a probe. After short feeding periods they usually move around again. We therefore stopped the attempts to use artificial feeding. To get significant and reliable results we increased the number of source plants up to 10, and the number of aphids up to 84 per group in our experiments.

Acquisition of virus by aphids.

Virus content of exposed A. pisum, nymphs and adults. The acquisition of PEMV by nymphs and adults was tested in experiments in which 50 aphids of each stage were given a 24 hr acquisition access period. Analysis of these aphids with ELISA revealed that PEMV can be detected in 90% of the nymphs and 98% of the adults (Fig. 11) after a 24 hr long acquisition. The A 405 nm readings ranged from 0.02 to 0.55 for nymphs and from 0.03 to 1.1 for adults indicating that nymphs can acquire virus over a range from non detectable amounts (< 1 ng) up to 40 ng; and adults up to 200 ng. The average amount ingested by adults was 4 times higher than by nymphs. This cannot be explained by the differences in size between nymphs and adults

as their weight ratio is 1 to 20. It is also evident (Fig. 11) that some nymphs can ingest at least 50 times more virus than some adults do.

In one experiment we determined the weight of aphids at various stages of development and the amount of virus ingested in 24 hr by these aphids. We found a linear relationship between the logarithm of the body weight and the average value of the ELISA readings. Fig. 12 indicates that with the size of the aphid the amount of virus ingested increases.

Influence of a preacquisition starvation period on the amount of virus ingested. In one experiment we tested whether starvation had an effect on the amount of virus to be acquired. A group of 60 nymphs which were 24-48 hr old were collected, directly transferred to infected peas and given an 8 hr long acquisition access period. A second group of aphids was starved for 3 hr before being exposed for a similar period on source plant. The aphids were then collected and individually assayed for the virus content with ELISA. Table 1 indicates the average A 405 value for 120 aphids obtained in two replicates and shows that there is a slight difference. This difference, however, is not significant at a level of 0.01 (Student's test). This corroborates results of Jenniskens (1979) who found that starved aphids did not acquire a significantly higher amount of virus.

Relationships between the acquisition access period and virus content. The rate of virus acquisition was measured by allowing groups of 84 aphids to acquire virus in periods of 2, 4, 8, 16, 32 and 64 hr, respectively. Each aphid was individually tested by ELISA in which peroxidase was used. Fig. 13 shows a sharp increase in the average A 492 nm readings within the first 16 hr. Afterwards the rate of increase greatly diminished during the following 48 hr. During this period the average A 492 nm readings increased from 0.87 to 1.0. The course of the curve suggests that after 16 hr of feeding the ingestion of virus approximately equals the excretion and that the slight increases of virus noticed follow the growth of the aphid.

Loss of virus from aphids feeding on healthy plants. The loss of virus from the aphid 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 acquisition access period of 24 hr. The aphids were transferred every two days to avoid uptake of virus from the plants which were infected during the period of infestation by the viruliferous aphids. The average A 405 absorbances decreased rapidly in the first day (Fig. 14) and reached a constant level 6 days after acquisition. As most of the acquired virus can be flushed away it can be 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 weeks. Forty aphids infected 81, 82 and 80% of the test plants at 1, 7 and 14 days after the acquisition access period of 24 hr, respectively.

The virus content of the hemolymph and the intestinal tract. Dissection of 16 adults which had acquired virus revealed that most of the virus present occurred in the intestinal tract (Table 2). Virus amounting up to 200 ng, was detected in 15 out of 16 guts analysed. The hemolymph of 13 out of 16 aphids reacted positively with an average absorbance (405 nm) value of 0.047, equivalent of approx. 1.2 ng virus per aphid. This indicates that the amount of virus present in the hemolymph is near to the level that can be detected. However, it cannot be excluded that during dissection some virus present in the intestinal tract may have been released with hemolymph which diffused into the fluid. If this occurred the values found for the hemolymph are too high.

Relationship between charge of virus and transmission pattern.

As shown in this report, the amount of virus ingested by a single aphid in a group, subjected to a controlled acquisition, varies considerably. Therefore,

it was of interest to study the relationships between the amount of virus ingested and the transmission efficiency. Exposed aphids were submitted to transmission trials, and then assayed for their virus content by ELISA.

Charge of virus and infectivity. In a first series of experiments four groups of 84 12-24 hr old nymphs were given acquisition access periods of 2, 4, 8 and 16 hr. To complete their latent period, the aphids were individually placed on a pea plant until 24 hr from the start of the acquisition access period, and then assayed for infectivity by serial transfer to 3 pea plants. In Table 3 we summarize the percentage of viruliferous aphids which did not give positive readings. The percentage of viruliferous aphids in which no virus can be detected decreases when the length of the acquisition periods increases. Fig. 15 illustrates the relationship between the average charge ingested and the infectivity. These results confirm the observations of earlier workers (Simons, 1954; Bath and Chapman, 1966; Sylvester and Richardson, 1966b) that the percentage of plants infected by aphids increases with the length of the acquisition access period, which is thought to be correlated with the amount of virus ingested.

Charge of virus and transmission efficiency per aphid. The results of the former experiments are the average of a whole group of aphids and they do not refer to the activity of a single aphid. The individual transmission efficiency per aphid was tested in relation to the dosage acquired as measured by ELISA. A group of aphids in two replicates was given an acquisition access period of 8 hr. Each aphid was placed for 16 hr on a single pea plant to complete the latent period and tested for their infectivity in series of transfers of one hr to 10 test plants. After the last feeding period the virus content of each aphid was measured. The transmission efficiency was plotted against the virus content of each aphid and the results of one of the replicates which gave similar results are shown in Fig. 16. Again, the

quantity of virus ingested by a single aphid varied considerably. The test revealed that the transmission efficiency of an aphid is not clearly correlated with the amount of virus ingested. Aphids in which no virus could be detected varied in their efficiency from non-transmitters with an efficiency of 100%. Aphids with high A 405 readings invariably transmitted with efficiencies of 80 to 100%.

Charge of virus per aphid and length of the latent period. In two experiments we studied the relationships between the length of the latent period and the transmission efficiency. After the acquisition access period of 6 or 7.5 hr the nymphs were individually transferred to pea plants for a feeding period of one hr to determine the moment at which the aphids made their first successful transmission. Fig. 17 illustrates that the latent period ranges from less than 6 hr to more than 18 hr in aphids. Results also show that the latent period in a single aphid is not clearly dependent on dosage. The correlation coefficient for the association between the dosage detected and the length of the latent period is 0.39 in one experiment and 0.41 in the other. This indicates that only 16% of the variation of the latent period can be explained by the differences in amount of virus ingested.

Length of latent period and transmission efficiency. In one experiment we studied the possible relationships between the length of the latent period and the transmission capacity per aphid. Aphids were transferred as described above to determine when they have completed their latent period. After an additional 16 hr on a single pea, the aphids were individually transferred 6 times, one transfer every hour, to assess their transmission efficiency. The length of the latent period plotted against the transmission efficiency of each individual aphid (Fig. 18) shows that these two parameters are not correlated.

DISCUSSION

Much attention has been devoted so far to the detection of virus particles in their insect vector, in particular for possible epidemiological application. A serological test, sensitive enough, could serve as a substitute for time consuming transmission trials (Lapierre, 1980; Plumb, 1980). In virus-vector relationships much of the information available is drawn from experiments involving bioassays. Bioassays to assess the virus content of an aphid can only be done by indirect, and therefore imprecise techniques. The new serological test, the ELISA test, provided the required sensitivity to quantify the amount of virus particles in the aphid.

Jenniskens (1979) found that it was possible to detect PEMV with ELISA in individual nymphs and adults after an acquisition access period as short as two hr. In our experiments we were able to find PEMV in 90% of the 24-48 hr old nymphs, and 98% of the adults exposed for 24 hr. So far, Kojima *et al.* (1979), Déméchère *et al.* (1980) could detect barley yellow dwarf virus (BYDV) and potato leaf roll virus (PLRV) with ELISA only in groups of 5 to 30 aphids exposed for 24 hr and crushed together. With this serological test we were able to detect 5 ng of PEMV per ml. Our results can be interpreted as such that PEMV occurs in a higher concentration in the phloem than PLRV and BYDV. *A. pisum* feeding on PEMV infected plants can ingest a higher amount of virus in these circumstances due to the higher titer of PEMV in the sap. A finding that does not surprise us when one considers the yield of virus obtained in the purification (5-100 mg per kg leaf material for PEMV against 100 µg for BYDV) and the results obtained by electron microscopy of infected plants (Shikata and Maramorosch, 1966).

The amount of virus acquired could reach 40 ng in 24-48 hr old nymphs and 200 ng in adults exposed 24 hr. This indicates a number of particles of approx. 2.5×10^{10}. This would account for the "enormous concentration" of particles detected by electron microscopy in the guts of viruliferous *A. pisum*

(Harris, 1979). It is sometimes assumed that aphids, when given acquisition access period of a certain length on source plants, would ingest similar amounts of virus. Our results, however, show that there is a wide variation in the charge of PEMV ingested. The ratio between maximum and minimum virus content is beyond 40 for nymphs and 200 for adults, when they are exposed 24 hr. The amount of virus ingested depends on the volume of sap intaken and the virus titer of the sap. Banks and Macaulay (1963, 1965) studied the rate of sap ingestion of *Aphis fabae* by counting the number of droplets of honeydew released from the hindgut. McLean and Kinsey (1964, 1965) devised a technique for recording the electrical potential associated with aphid salivation and ingestion. From these experiments it can be safely stated that the amount of sap ingested is approximately similar among aphids of a given stage. It therefore suggests that the wide variation in the charge of virus ingested would reflect a highly uneven distribution of virus particles in the phloem bundles. For two other circulative virus, BYDV and PLRV, electron microscopy clearly reported that some phloem bundles contained a high concentration of virus particles, others very few, and some others not at all (Jensen, 1969; Kojima *et al.*, 1968). Then, aphids feeding on highly infected cells would ingest a high amount of virus, whereas others feeding on phloem bundles containing no or small amounts of particles would acquire PEMV in non detectable amounts.

Mittler and Dad (1962) predicted "the practicability of the artificial feeding method for investigations of virus which would be facilitated greatly by prolonged maintenance of aphids divorced from their host plants". To further investigate the mechanism of virus acquisition it was tempting to use membrane systems to control the virus titer of the source. Simple artificial diets, which do not duplicate the chemical constituents of phloem sap, like our sucrose solution, are known to be a poorly efficient tool for studying food uptake, salivation... (Hunkel, 1977). Thottappilly *et al.* (1972), French *et al.* (1972) reported that behaviour of *A. pisum* is closely affected by the

method of purification. Slight modification of the chemical composition of the diet could affect the feeding activities of the aphid. McLean (1971) showed that the behaviour of aphids given access to a diet differs from that of aphids on plants in the feeding and salivation process. Aphids on diet easily reach the food source by simply piercing the Parafilm membrane. On plants, however, they must first penetrate a "considerable" distance through paremchyma tissue to reach the phloem (Nault and Gyrisco, 1966). All these findings could explain why in our experiments we could not closely simulate the situation on the host plant with membrane feeding systems. All our experiments were therefore carried out using PEMV infected peas as virus source for aphids.

We dissected the intestinal tract and collected droplets of hemolymph from 72 hr-exposed aphids. We found that most of the virus is located in the gut lumen whereas only small amounts, around the detectable level, could be detected in the hemolymph. Our results suggest that most of the virus remains located in the intestinal tract, and that only a very low percentage of the virus content passes through the gut wall into the hemocoele and shows that the gut wall would act as a relative barrier. Paliwal and Sinha (1970), Richardson and Sylvester (1965), Ponsen (1972) have studied the fate of BYDV, PEMV and PLRV respectively, in their aphid vector. They used an infectivity bioassay procedure in which whole bodies as well as various organs, tissues and fluids of viruliferous aphids were used to prepare inocula for injection into virus free aphids. Injected aphids were then placed on test plants to check for infectivity. Our results corroborate their findings, that in terms of virus titer, guts were shown to be the best source of inocula.

As was the case of two leafhopper-borne circulative-propagative viruses, wound tumor and wheat striate mosaic viruses (Shikata and Maramorosch, 1967 ; Paliwal and Sinha, 1970) no BYDV was detected in the guts of abdominally inoculated aphids, thus suggesting that gut permeability to BYDV is uni-directional. On the contrary, PEMV has been observed by electron microscopy

in the stomach of hemocoelically injected pea aphids (Harris *et al.*, 1975). From our experiments we could not get any evidence to state whether the gut permeability is unidirectional or bidirectional. The possible effects of the gut permeability on the pattern of PEMV transmission are discussed below.

The amount of virus in the aphid increased with the acquisition access period. The longer the aphids can feed, the higher the amount of virus they acquire. The increase is rapid within the first 16 hr. Then, there is a sharp slowdown in the increase. This suggests that excretion and ingestion are roughly in equilibrium in young nymphs exposed for more than 16 hr. Further increases in the amount ingested are likely to reflect increases in the volume of the intestinal tract. The bigger the aphid, the higher the amount of virus acquired. It is of interest to note that adults can ingest an amount of PEMV around 4 times as high as 24-48 hr old nymphs can, but that their weight ratio is beyond 20 to 1. This corroborates previous findings (Bath and Chapman, 1964; 1968) that virus titer defined as the ratio between the charge ingested to the weight of the aphid is higher for nymphs than for adults. We were not able to show, although suggested by McLean and Kinsey (1969), that starved aphids when exposed on a virus source would acquire a significantly higher charge of PEMV per unit of time than unstarved ones. Our results confirm the belief accepted by many workers (Sylvester, 1962) that a preacquisition starvation period of vectors of circulative viruses does not enhance the ability of aphids to ingest virus particles. As we did not study the infectivity efficiency of the starved and unstarved aphids, a possible effect of a preacquisition starvation period on the infectivity cannot be ruled out. Also we did not study the effect of preinoculation starvation on the transmission.

To study the loss of virus by aphids, viruliferous aphids were allowed to feed on healthy plants. The virus titer decreases regularly within the first six days. These results and those summarized in Table 2 suggest that most of the virus is flushed out the intestinal tract. This would account for the

excellence of the honeydew as inoculum for injection of non-viruliferous aphids (Richardson and Sylvester, 1965). Similar conclusions on the release of ingested circulative viruses were drawn for BYDV (Sinha and Paliwal, 1970) and PLRV (Ponsen, 1972). We found that some detectable amount of PEMV is retained in *A. pisum* up to 14 days after acquisition. This finding could reflect the small charge stored in the hemolymph or some virus which is bound to the gut wall and which has not been flushed out. It is of interest to note that the infectivity did not decline over a period of these two weeks. It is tempting to assume from these observations that the hemolymph would act as a virus reservoir which makes the aphid infectious and maintains its infectivity.

Very little is known about the key processes which determine the pattern of transmission of circulative viruses such as the passage of particles through the gut wall, the distribution of virions in the insect body, the fate of virus particles in salivary glands and the exact mechanism of inoculation. However, several workers studied the possible relationships between the acquisition of virus and its transmission. Several characteristics are reported to be dosage dependent. They include infectivity, transmission efficiency per aphid, and the length of the latent period (Sylvester, 1965; Clark and Bath, 1973; McLean and Kinsey, 1969). However, in all these experiments they could not control the amount of virus biologically ingested by the exposed aphids. With the application of acquisition access period of various lengths one assumes that the charge ingested would be proportional to the time of feeding. This is an imprecise assumption as demonstrated above (Fig.13). On the other hand, some research workers have controlled the amount of virus ingested in the hemolymph by micro-injection of PEMV purified solutions. In this way the gut wall has been bypassed. In our experiments however, we could get a direct quantification of the amount of virus ingested without interfering in the normal biological process of transmission. Transmission trials and ELISA test combined would provide meaningful results on the relationships between the virus content of the aphid and transmission

characteristics.

Throughout all our experiments we could find aphids which were infectious and in which no PEMV could be detected with ELISA. However all the aphids giving a positive reaction with the serological test turned out to be viruliferous. This suggests that the level which makes an aphid infectious lies beyond the level detected by ELISA, i.e. 1 ng. With large groups of aphids (84 per group) we showed that the infectivity was closely related to the charge ingested. The higher the amount acquired, the higher the infectivity. The transmission efficiency per aphid, on the other hand, was not directly correlated with the virus content. We found that some nymphs with a non-detectable amount of virus were very efficient transmitters (80%); better transmitters than some with a 405 nm reading in the range of 0.1-0.2. Aphids with a charge larger than 0.3 were all efficient transmitters. The length of the latent period was not closely related to the amount of PEMV ingested (correlation coefficient of 0.4). Some aphids with a high charge had a long latent period (over 18 hr), whereas some with a low charge had a short latent period (less than 8 hr).

These results altogether suggest that the transmission pattern of PEMV in *A. pisum* is not clearly dependent on the charge of virus detected. It could be argued that the virus content of aphids, determined at the end of a 24 hr long inoculation access period may bear little relation to that at the start of this period, and only a weak relation between transmission of PEMV and the subsequent virus content of individual aphids is to be expected. More probably, our results are an indication that factors other than virus content have an important influence on the pattern of transmission of the virus by the aphid vector. Then, on the basis of our experiments we could rule out a "mechanistic" approach of circulative virus transmission in which the aphid would only act as a syringe, a passive transmitter of virus particles. Actually, very little is known about the key processes involved which are likely to cause variation, and which are not necessarily

dosage dependent. Among the biological processes which may be involved, we can mention the pattern of ingestion, the passage of virus particles from the intestinal tract through the gut wall into the haemocoele, and the efficiency of passage of virus particles from hemolymph to saliva.

For example, if we are correct in supposing that the reservoir of PEMV that maintains the infectivity of the aphid is mainly located in the hemolymph, then this must constitute a reservoir that is only depleted slowly, as the infectivity of groups of aphid does not vary over a period of two weeks. The key to transmission seems then to be the efficiency of transfer of virus particles from hemolymph to saliva. Consecutively, the relation between the amount of virus acquired, located mainly in the intestinal tract, and the transmission capacity per aphid, is likely to be poor.

When we investigated the relationships between charge and transmission through different aphid stages, we could confirm that there is a positive gradient between the amount of virus ingested and the weight of the insects. The bigger the aphid is, the higher the amount ingested. It is, however, steadily established that the infectivity decreases and the latent period increases with the age of the aphids (Bath and Chapman, 1968). Some authors (Bath and Chapman, 1964) proposed a "mechanistic" explanation and assumed that it is due to the higher virus titer in nymphs which would induce altogether a shorter latent period and a higher infectivity. We are more bent to think that the explanation has to be searched among the biological processes involved quoted above. For example, a decreased ability of virus particles to pass from gut to hemolymph and/or from hemolymph to saliva with older aphids, could account for their lower infectivity and their longer latent period. It is of interest, then, to note that a decreased gut permeability to virus with vector age (leafhopper) has been demonstrated (Sinha, 1963; Sinha, 1967).

We did not find in our experiments any results which cannot be explained by a circulative, non-propagative model. High virus titers in gut (up to

100 ng) were detected after acquisition access period of 8 hr. We found that some 24-48 hr old nymphs with a latent period as short as 6 hr. One might assume that multiplication of virus in an aphid in several tissues before transmission can occur, would take more time. A strong evidence for multiplication would be given if we could detect an increase of charge in the aphids after removal from a source plant. We found on the contrary a regular decrease of virus content in aphids feeding on healthy plants. To our opinion these results tilt the balance in favor of a non-propagative model for PEMV in *A. pisum*. However, we cannot rule out a limited multiplication in certain tissues which would occur to a small extent relative to the total virus content of the aphids.

The experiments conducted during this period of research provided information on the virus content of the aphids and the possible consequences on the pattern of transmission. As reported above, some transmission characteristics are poorly related to the amount of virus in the aphid. Factors, others than the virus charge, must play a key role and influence the various parameters of the circulative transmission, such as infectivity, transmission capacity, latent period... We are inclined to think that much attention should then be devoted to the biological processes involved in the transmission such as the timing and rate of feeding and excretion, the passage of virus particles through the gut wall into the hemocoele, and from the hemolymph into the salivary glands. Two new approaches have to be applied soon at the Department of Virology of the Agricultural University of Wageningen. By counting the number of droplets of honeydew released from the intestinal tract, a technique developed by Banks and MacAulay (1963, 1965), and assaying them by ELISA we could gain additional information about acquisition and excretion of virus by aphids. By enhancing the sensitivity of ELISA (Butler *et al.*, 1981), or by developing a more sensitive technique which would lower the threshold of virus detection, we could quantify smaller amounts of virus particles in the hemolymph and salivary glands. This information would help to go further in the knowledge of virus-vector relationships.

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Table 1. Comparison by ELISA of the average virus content of groups of 24-48 hr old nymphs, each group consisting of 120 aphids. 1 = Starved 3 hr before acquisition; 2 = Unstarved.

Treatment	1	2
After 8 hr acquisition	0.130	0.120
Aphids on healthy plants	0	0

Table 2. Comparison by ELISA of the average virus content in the gut and hemolymph of 16 aphids expressed as absorbance (405 nm).

Treatment	gut	hemolymph
After 72 hr acquisition	0.594	0.047
Aphids from healthy palnts	0	0

Table 3. Percent of viruliferous aphids showing no reaction in the ELISA-test.

Experiment	Acquisition access period (hours)			
	2	4	8	16
1	37	37	21	16
2	40	--	19	--

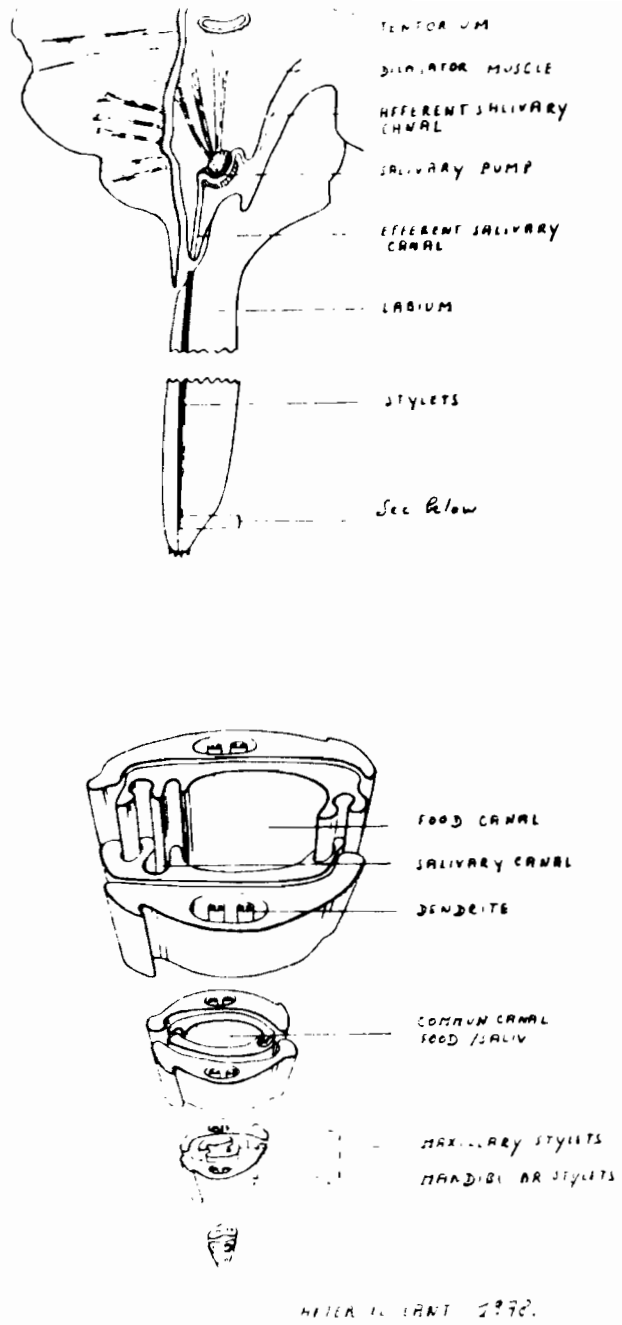

Fig. 1. Mouthparts of aphids.

The mouthparts of aphids consist, as all the hemipterous, of two pairs of stylets. Two maxillary stylets and two innerved mandibular stylets which enclosed the formers. The stylets lay in a dorsal groove of the labium. At the tips of the labium are sensory cones (mechanoreceptors). The stylets of aphids are thin and flexible. Except at their base, the maxillary stylets are interlocked by a series of ridges and grooves to form two longitudinal canals between their opposed inner surfaces. The anterior one connected to an alimentory suction pump and to the intestinal tract, for the food ingestion; the posterior one, thinner, for emission of saliva produced by two pairs of salivary glands. The rate of emission is regulated by a salivary pump.

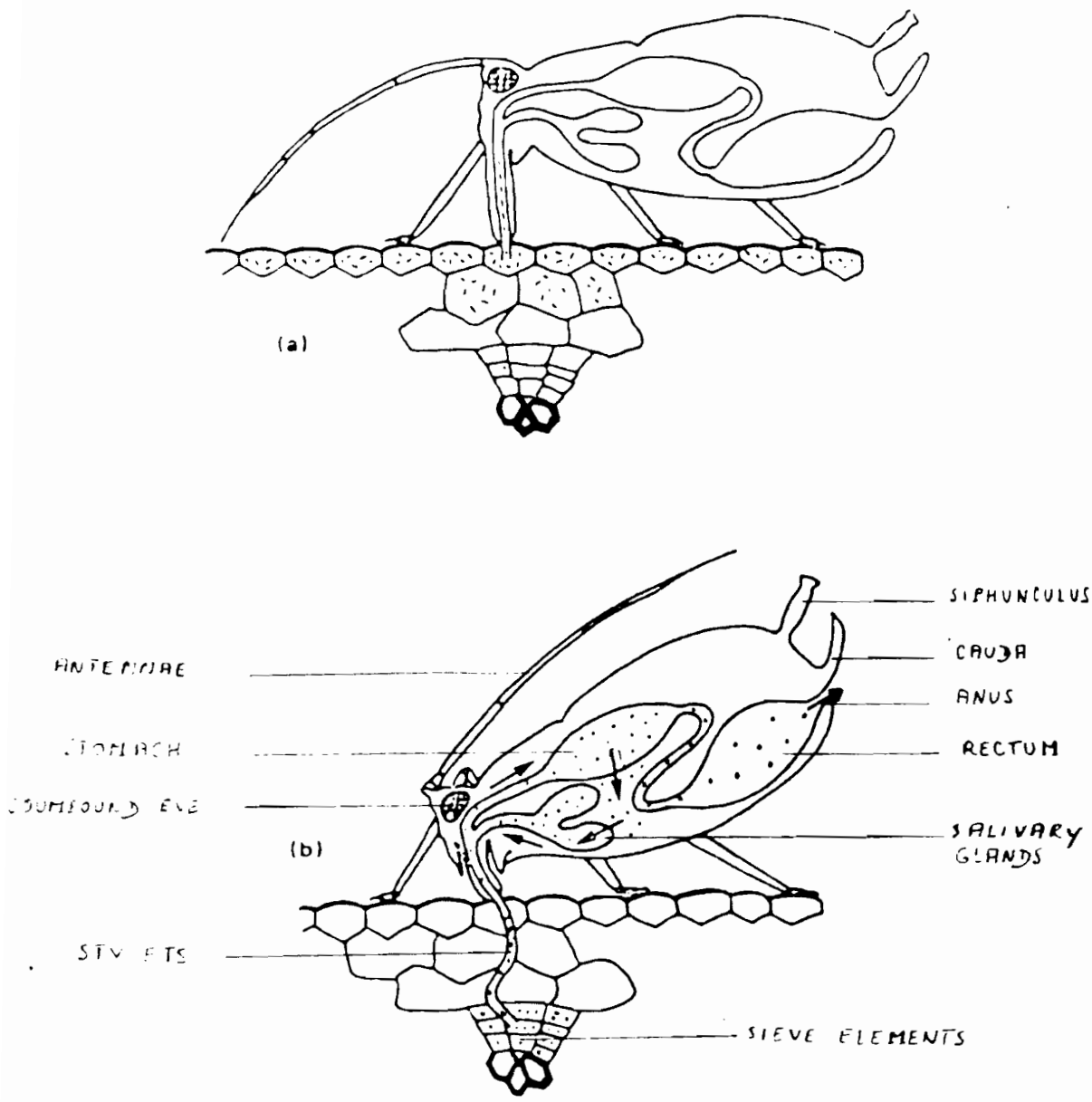

Fig. 2. Diagram showing the mechanism of acquisition of viruses by aphids (After Dixon, 1973).

a) Stylet borne viruses acquired when aphids probe the epidermal tissue of infected plants.

b) Circulative viruses acquired when aphids feed on the phloem tissue of infected plants.

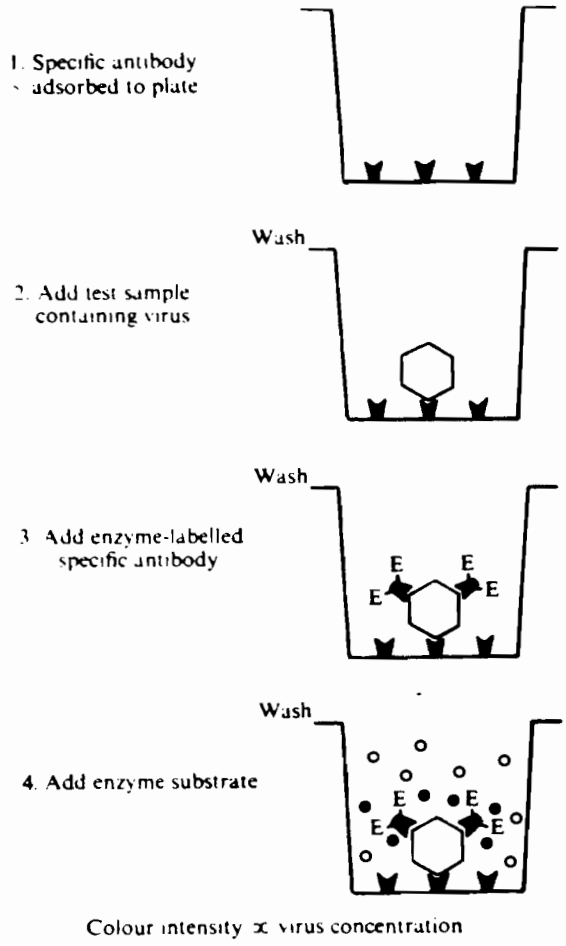

Fig. 1. Principle of the ELISA technique for plant viruses.

Fig. 3. Principle of the ELISA technique for plant viruses.

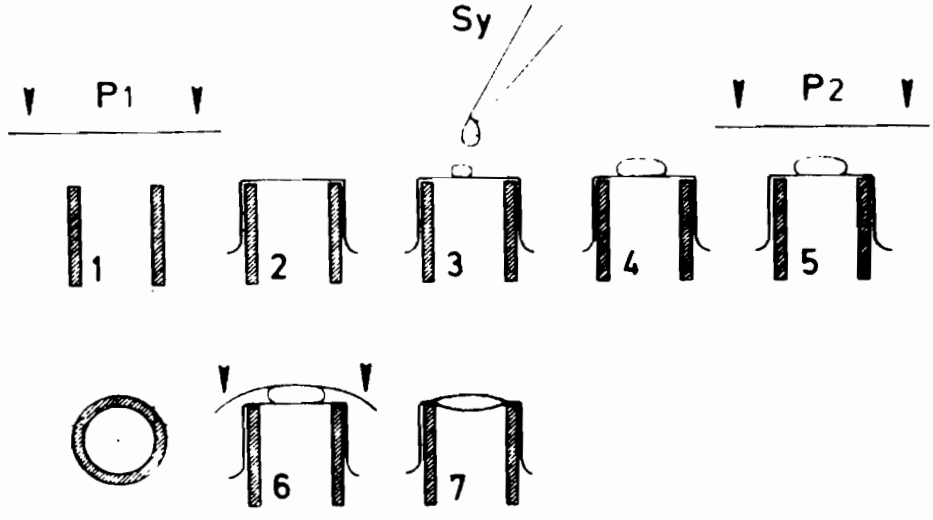

Fig. 4. Schematism of sachet preparation. See section "Virus sources" (p. 15) for explanation.

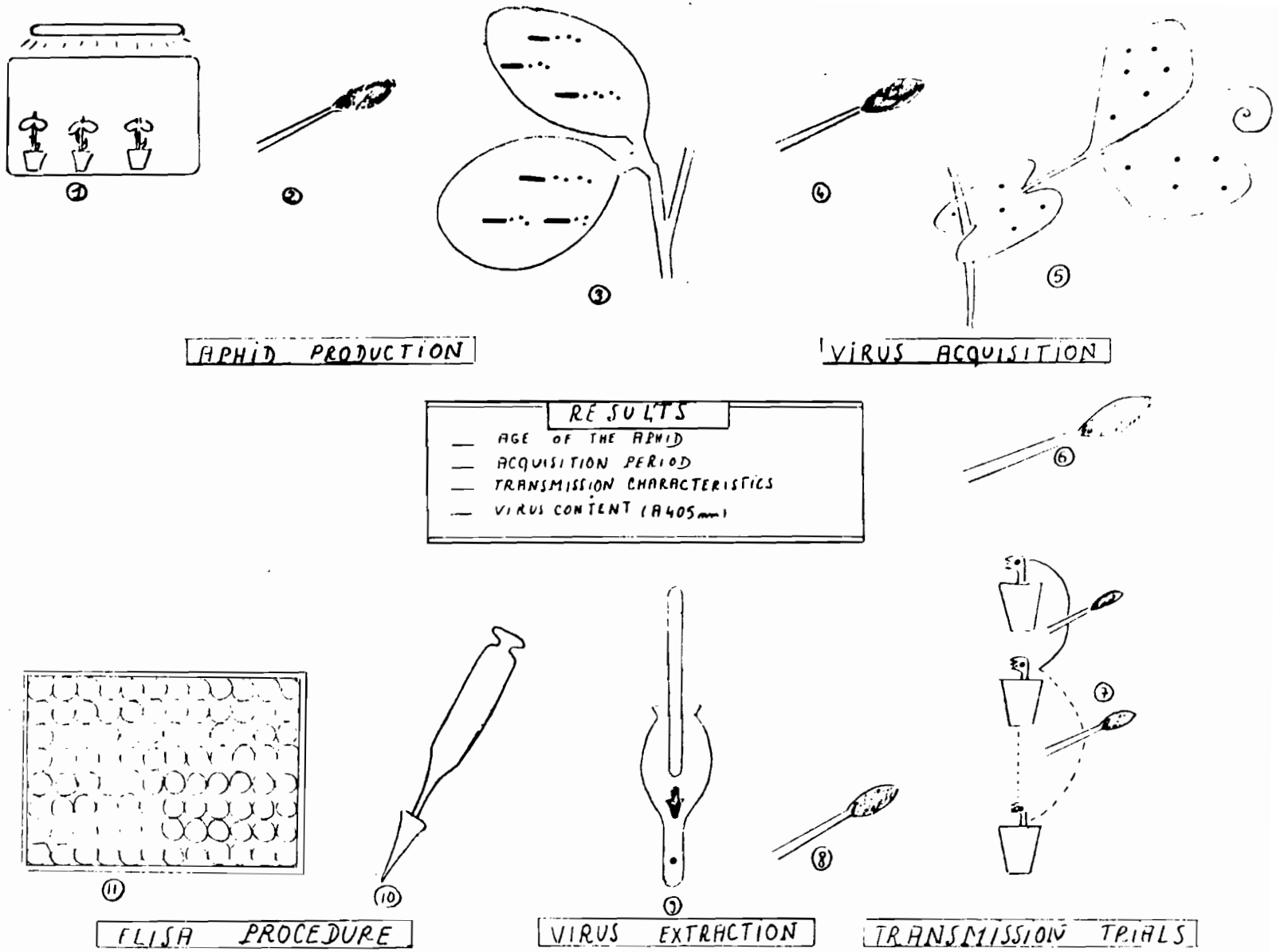

Fig 5:see text next page.

Fig. 5. Summary of techniques used in our virus-vector relationships.

1) *A. pisum* is produced on broad bean (*Vicia faba*) in a rearing chamber.
2) Virus free adults are collected and transferred onto broad beans.
3) Adults are allowed to deposit nymphs 24 hr and are removed.
4) After the appropriate timing, nymphs are collected and transferred to source plants.
5) Nymphs are allowed to acquire PEMV for a given period.
6) Each nymph is individually collected and transferred to test plants.
7) Each nymph is serially transferred on pea plants the appropriate number of time.
8) Each aphid is collected and transferred in the bottom of an Elvejhem Potter tube.
9) Each aphid is ground in 0.2 ml of extraction buffer.
10) The solution (0.2 ml extraction buffer + a crushed aphid) is transferred in a single well of an ELISA plate.
11) Peripheral wells are filled with water. Then up to 60 wells are filled with "aphid solution". Each well is read at A 405 nm by means of a Titertek Multiskan.

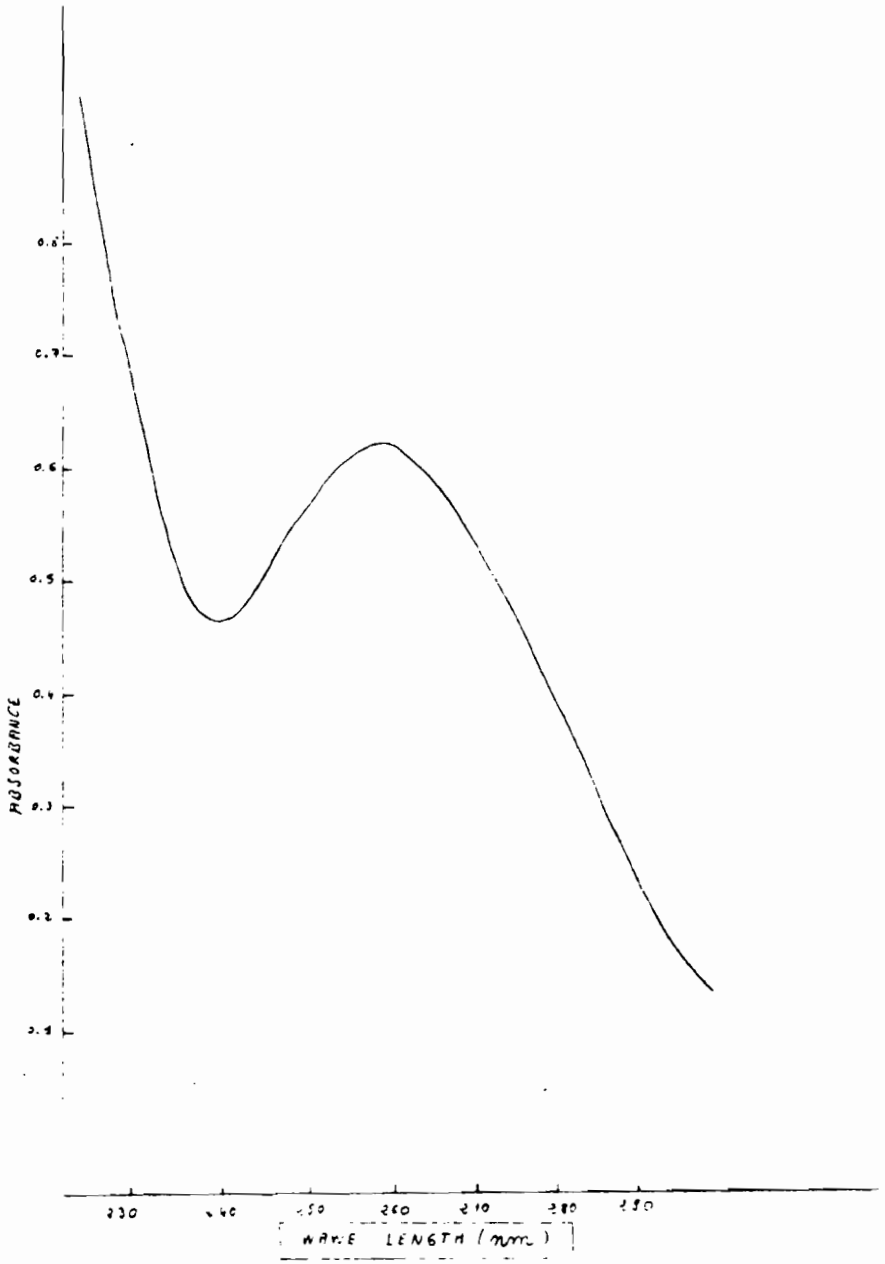

Fig. 6. Absorbance curve of a purified PEMV solution.

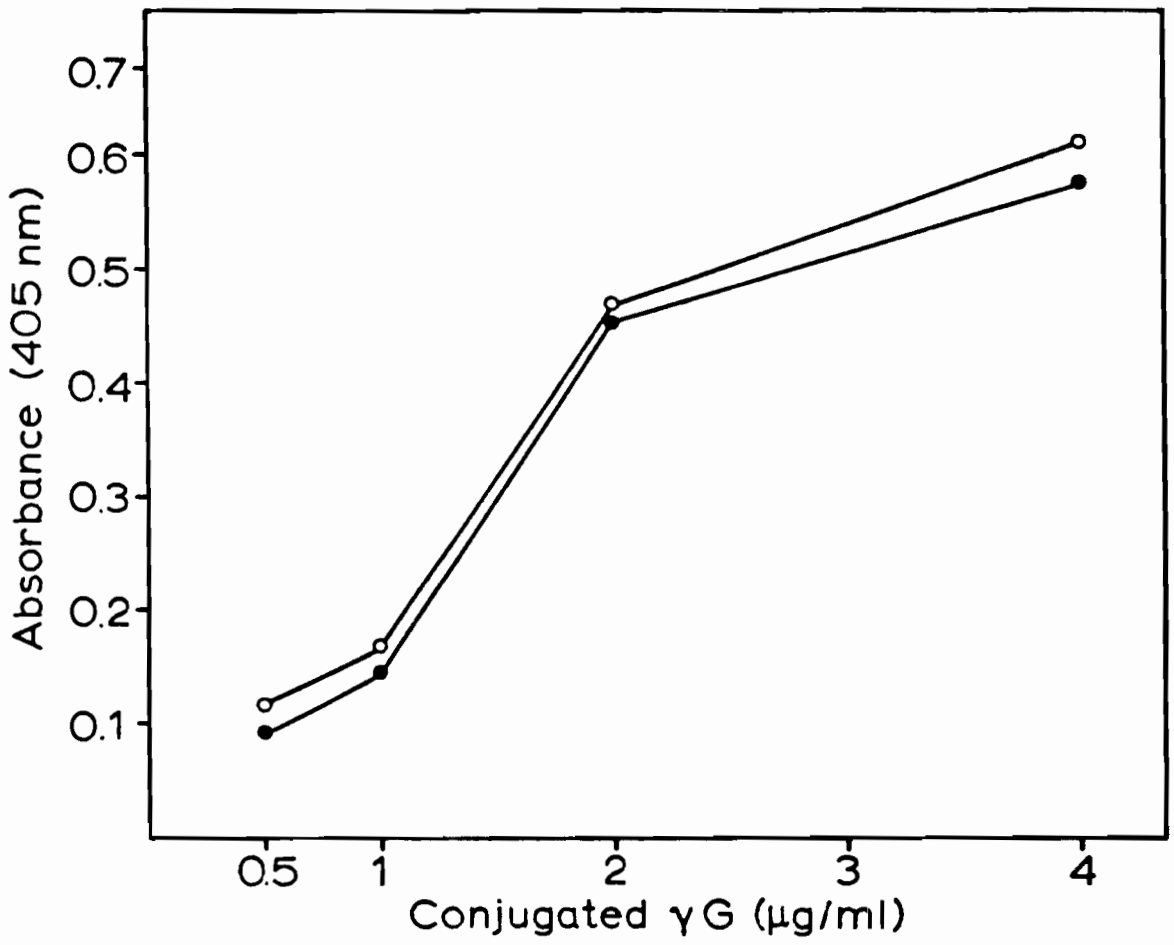

Fig. 7. ELISA absorbances of PEMV purified solutions with different concentrations of conjugated globulins in conjugate buffer. The γ-globulin concentrations for coating are 1:250 (o) and 1:2000 (●).

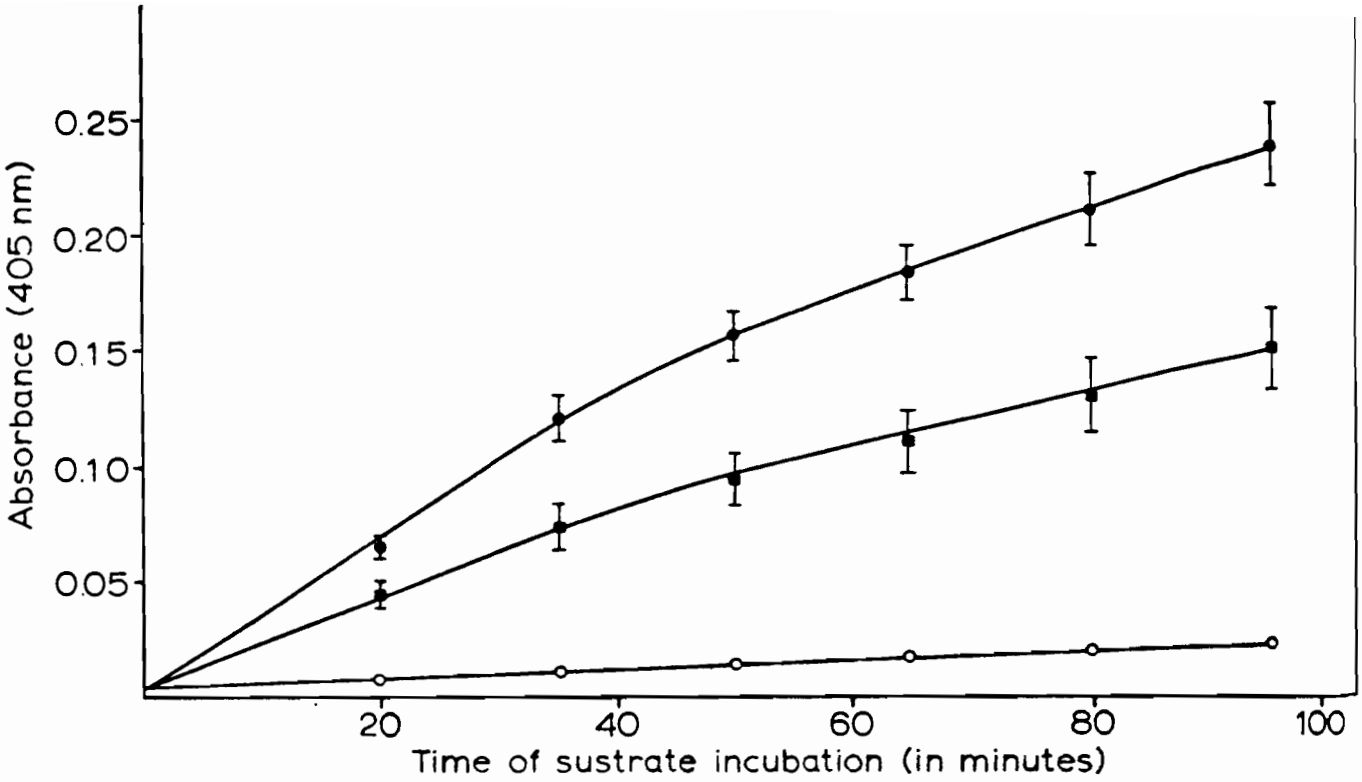

Fig. 8. ELISA absorbances of a PEMV purified solution with different incubation periods. The used conjugated γ-globulin concentrations in conjugate buffer are 1 µg/ml (●) and 0.5 µg/ml (■).

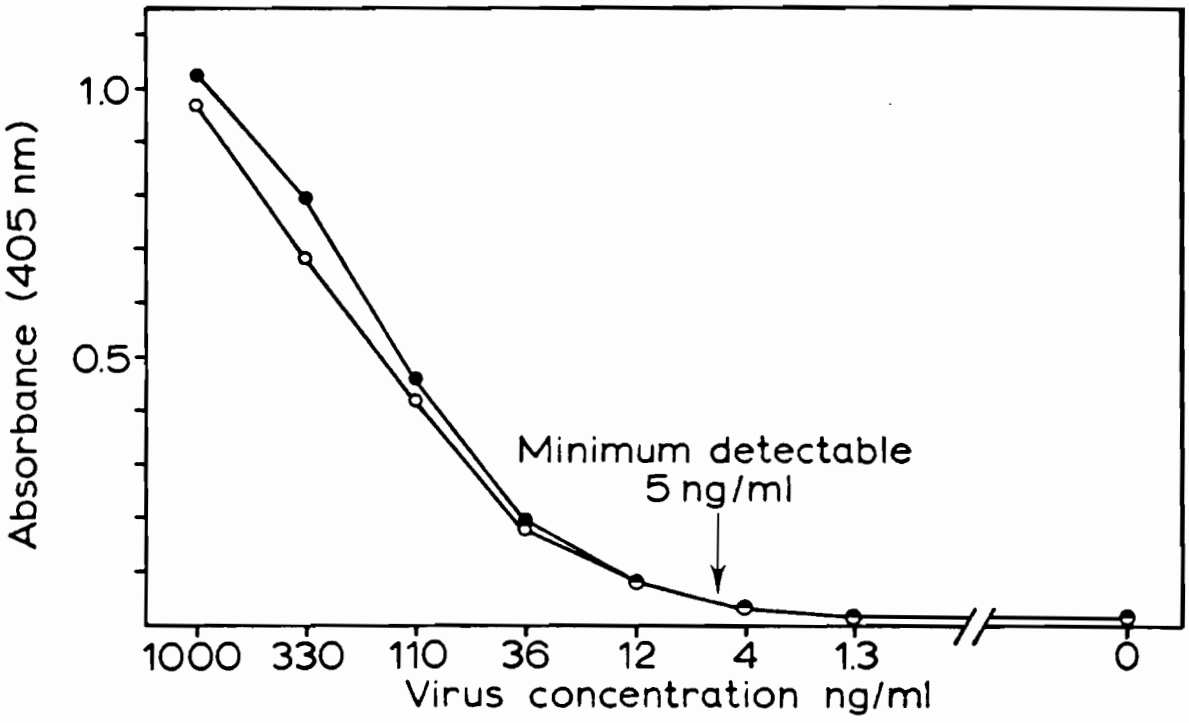

Fig. 9. ELISA absorbance (405 nm) obtained in titrations of three-fold dilution series of PEMV in buffer (●——●) and in buffer mixed with aphids in a ratio of 1 aphid/0.2 ml buffer (o——o). The dilution of the γ-globulin in the coating buffer and conjugated antiserum was 1:1000. The results are the means of eight replicates.

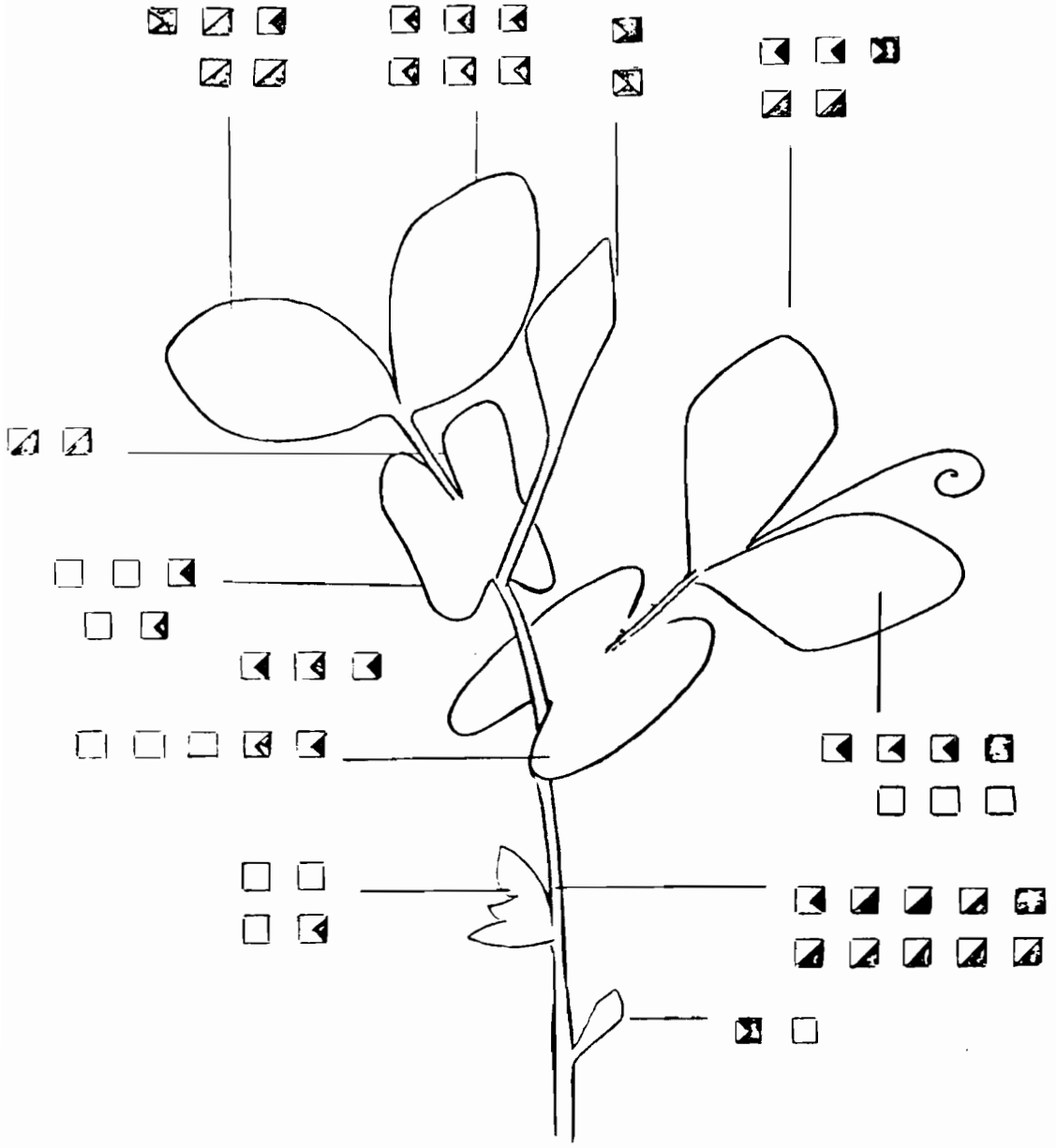

Fig. 10. ELISA absorbances of leaf disks and thin stem sections for various locations of the plants. The results are illustrated as follows: A 405 < 0.02 □, 0.02 < A 405 < 0.1 ◥, 0.1 < A 405 < 0.2 ◢, 0.2 < A 405 < 0.3 ◣, 0.3 < A 405 ■.

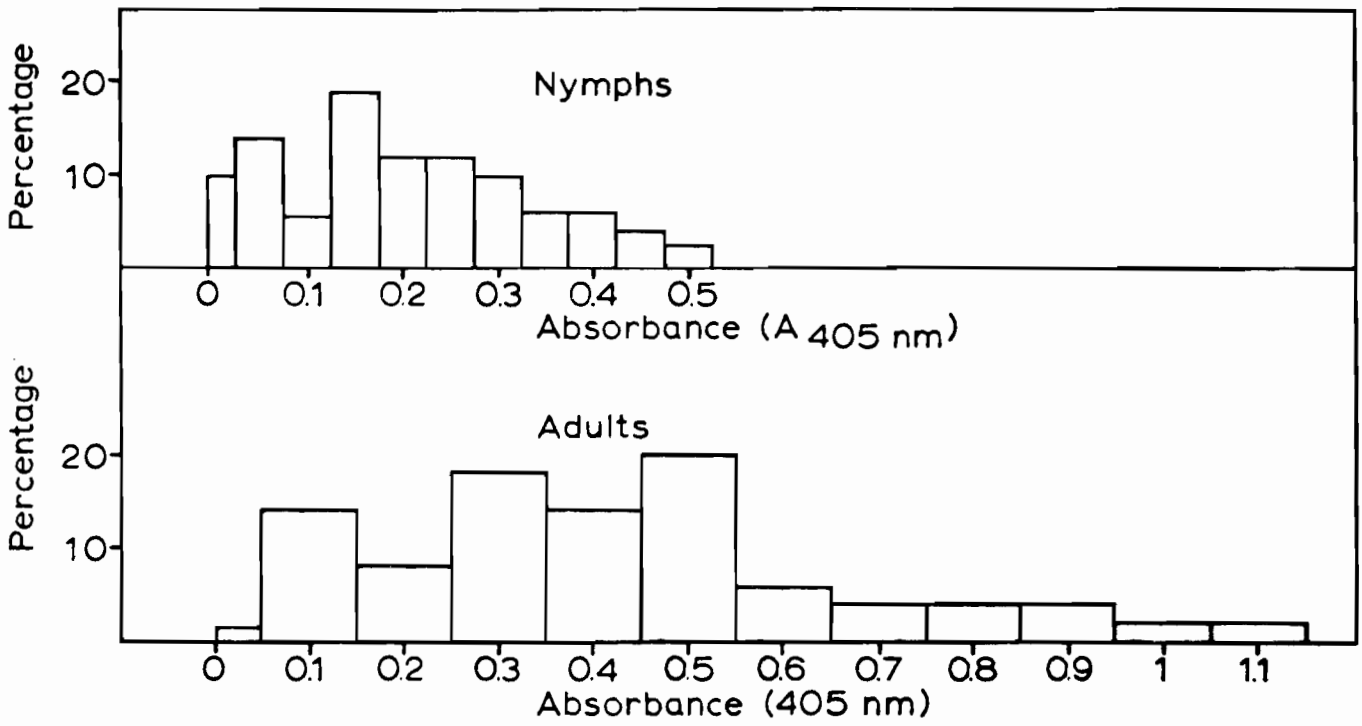

Fig. 11. ELISA absorbance (405 nm) obtained in titration of 50 individual 24-48 hr old nymphs and adults which fed on PEMV infected plants for 24 hr.

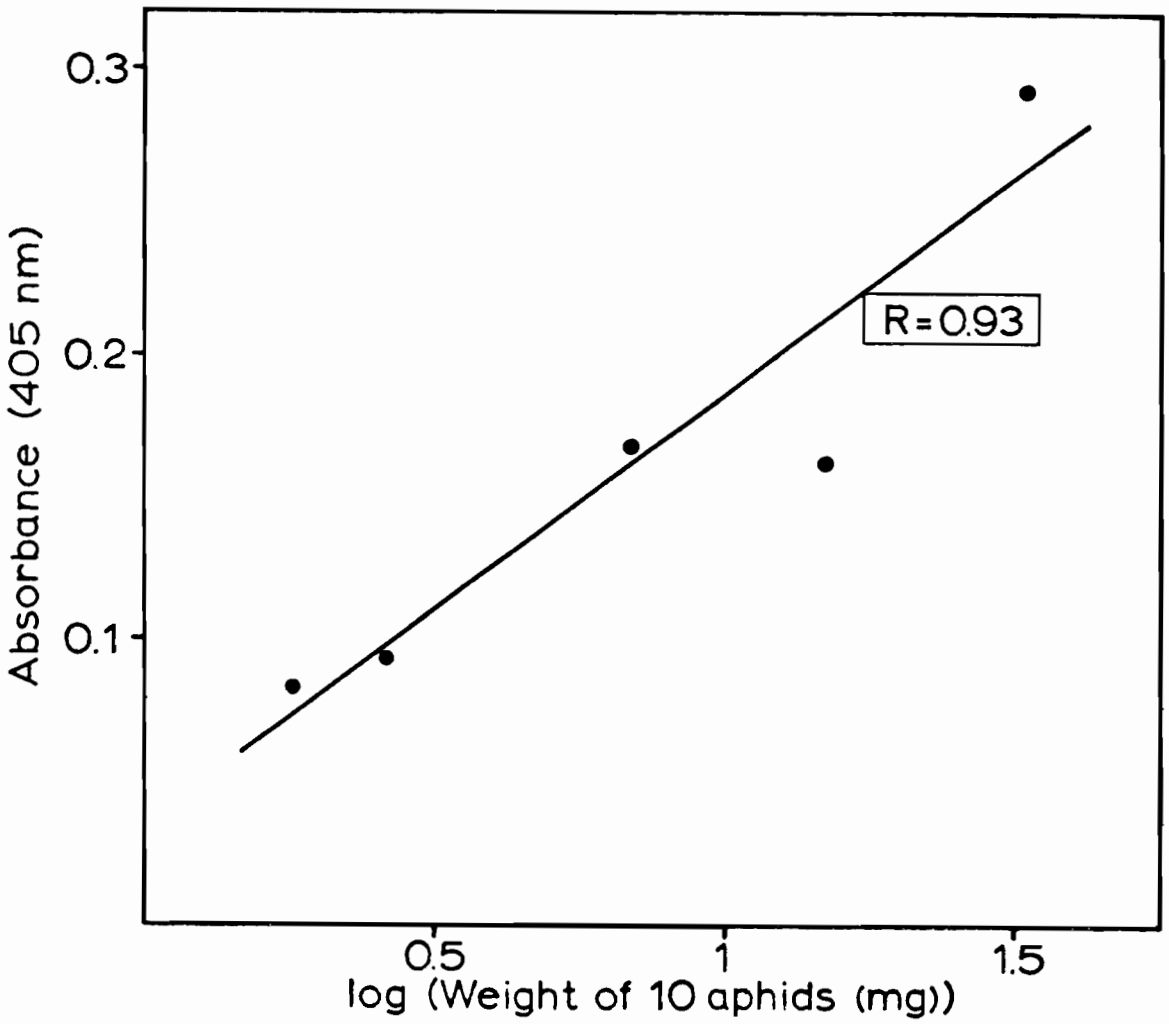

Fig. 12. Increase of ELISA absorbances (405 nm) as a function of the average body weight obtained from nymphs of various ages and adults of two days old after feeding for 24 hr on infected plants. Each point is the average value for 50 aphids.

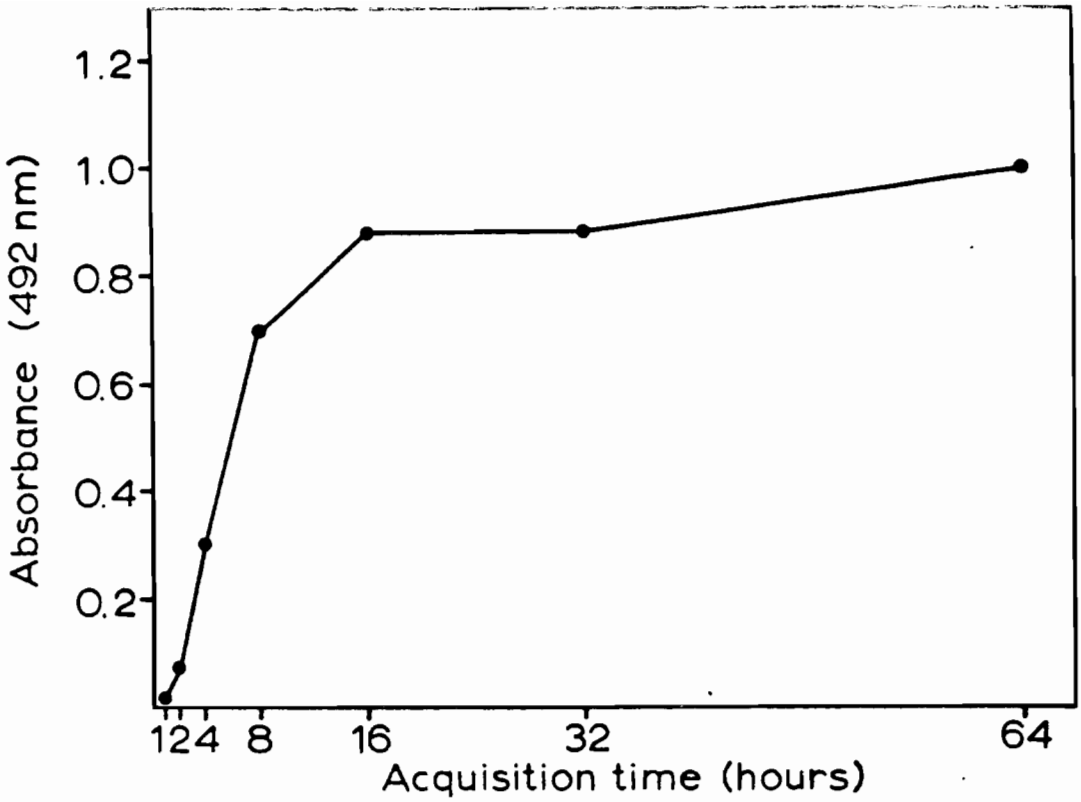

Fig. 13. Average ELISA absorbance (492 nm) obtained in titration of aphids which had fed in groups on PEMV infected plants for 2, 4, 8, 16, 32 and 64 hr. Each point is the average value for 84 aphids.

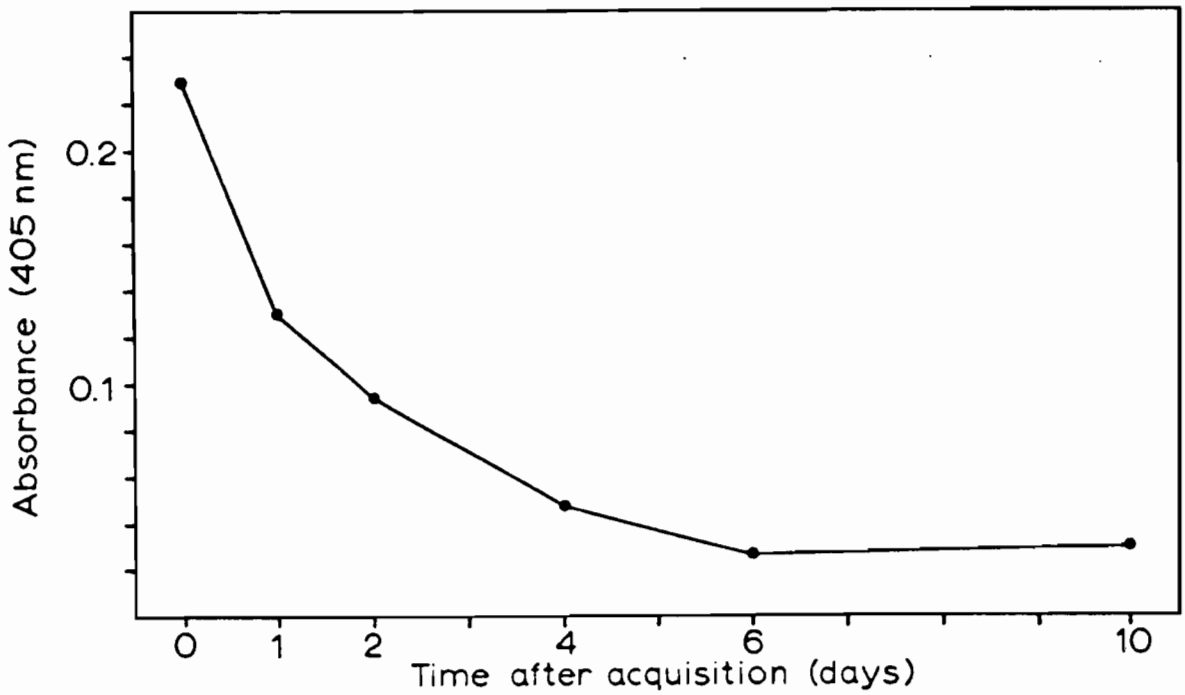

Fig. 14. Average ELISA absorbance (405 nm) obtained in titration of aphids which had fed on healthy plants for 1, 2, 4, 6 and 10 days after an acquisition access period of 24 hr. Each point represents the average absorbance found for 50 aphids.

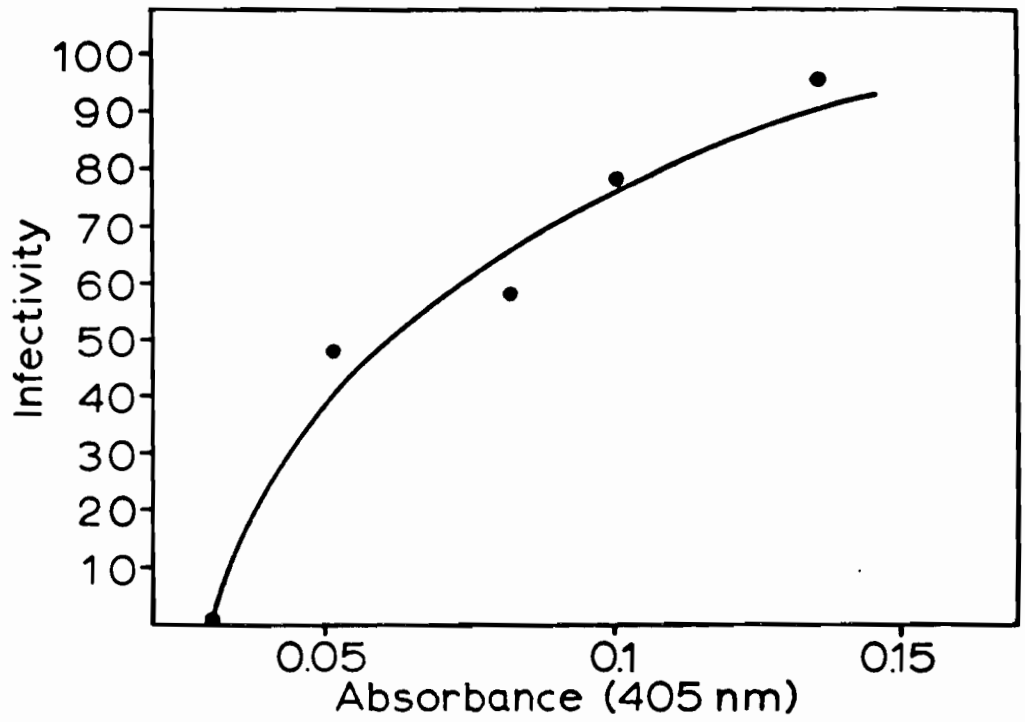

Fig. 15. The relation between the infectivity (%) of 24 - 48 hr old nymphs which acquired virus in periods of 2, 4, 8 and 16 hr, and the average value of the ELISA absorbance. Each group consisted of 84 aphids.

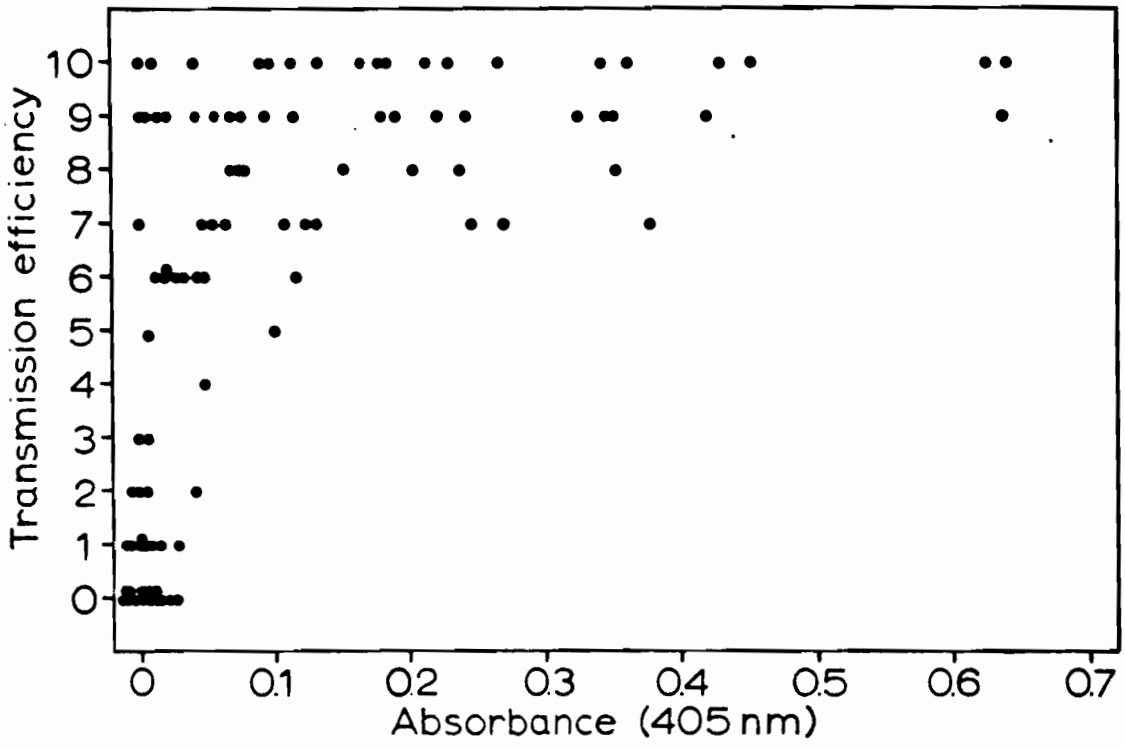

Fig. 16. The transmission efficiency of nymphs individually bioassayed for infectivity on a series of ten pea plants, and titrated for their virus content by ELISA. Each point represents the value for a single aphid.

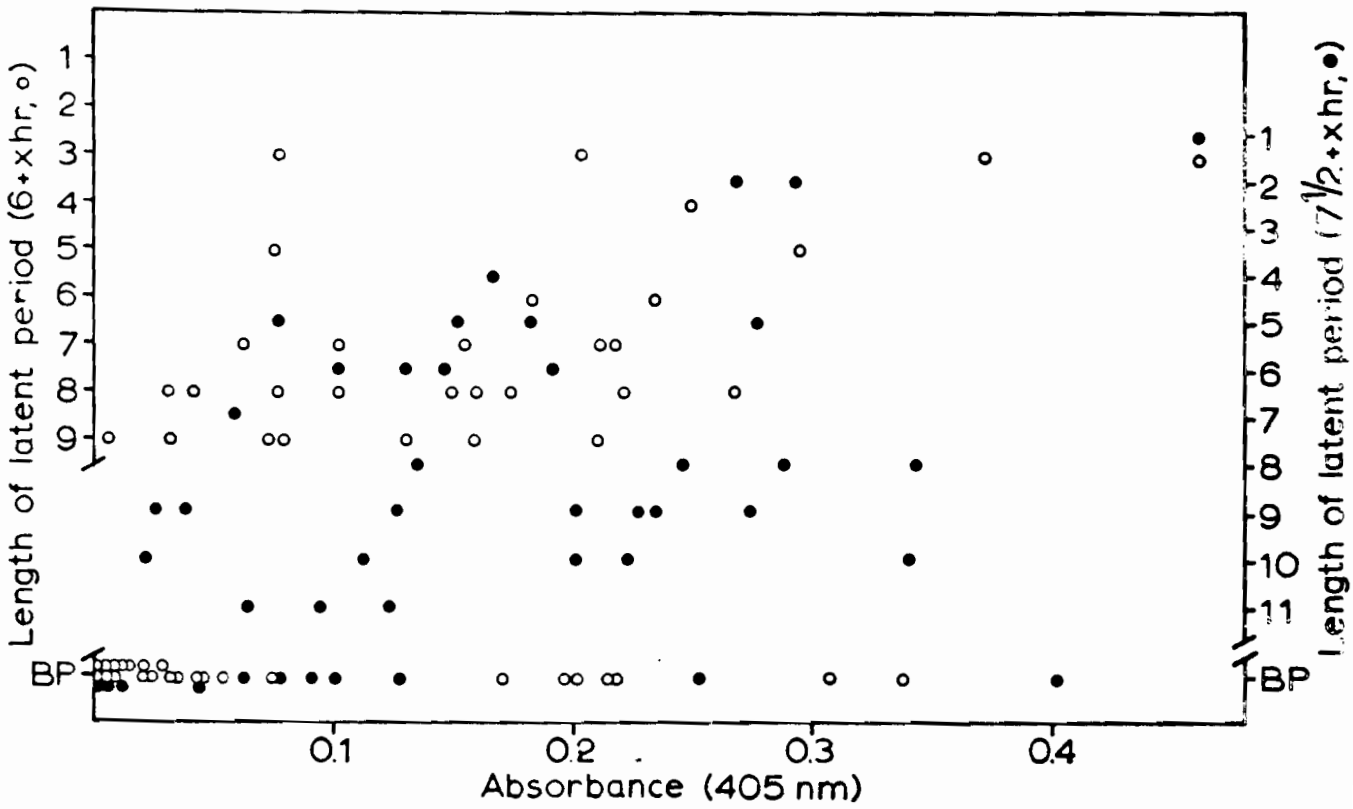

Fig. 17. The latent period of PEMV in nymphs which were allowed acquisition access periods of 6 hr (o) or 7½ hr (•), and titrated for their virus content after bioassay to measure their transmission efficiency. The latent period was assayed by serial transfer to pea plants every hour. The aphids completing the latent period in the interval on pea plants before the test on the transmission efficiency, are shown by BP (bridging period). Each symbol represents the value obtained with a single aphid.

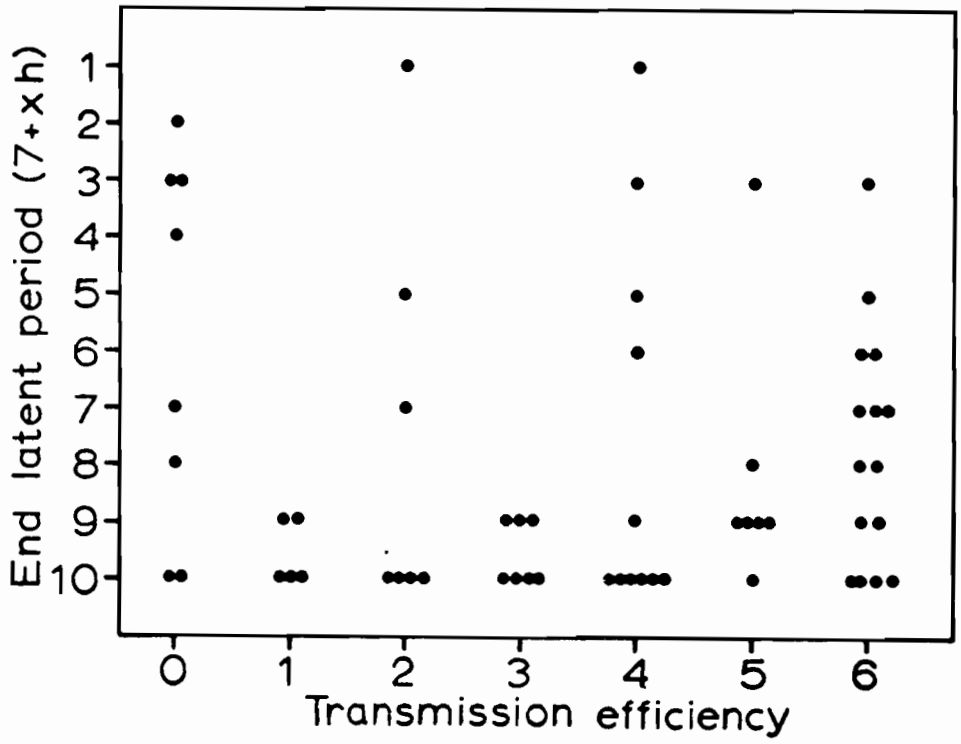

Fig. 18. The relation of the latent period and the transmission efficiency of single aphids after acquisition of virus in access periods of 6 hr (see also Fig. 8). Each point is the value found for a single aphid.