POTATO LEAFROLL VIRUS, ITS PURIFICATION FROM ITS VECTOR *MYZUS PERSICAE*

Dit proefschrift met stellingen van

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POTATO LEAFROLL VIRUS, ITS PURIFICATION FROM ITS VECTOR *MYZUS PERSICAE*

(MET EEN SAMENVATTING IN HET NEDERLANDS)

PROEFSCHRIFT
TER VERKRIJGING VAN DE GRAAD
VAN DOCTOR IN DE LANDBOUWKUNDE
OP GEZAG VAN DE RECTOR MAGNIFICUS, IR. F. HELLINGA,
HOOGLERAAR IN DE CULTUURTECHNIEK,
TE VERDEDIGEN TEGEN DE BEDENKINGEN
VAN EEN COMMISSIE UIT DE SENAAT
VAN DE LANDBOUWHOGESCHOOL TE WAGENINGEN
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H. VEENMAN & ZONEN N.V. – WAGENINGEN – 1967

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STELLINGEN

I

Het voorkomen van meer dan één persistent virus in Solanaceeën gebiedt dat de zuivering van bladrolvirus gepaard moet gaan met een biologische toetsing op de zuiverheid van het gebruikte uitgangsmateriaal.

Dit proefschrift.

II

De hoeveelheid bladrolvirus die een bladluis na het zuigen op een besmette plant bij zich draagt, is voor het grootste deel direct uit de plant opgenomen en voor een kleiner deel toe te schrijven aan virusvermeerdering in de bladluis zelf.

HARRISON, B. D., Virology 6, 265–277, 1958.

III

Dat virussen niet volgens een algemene procedure kunnen worden gezuiverd moet niet alleen worden toegeschreven aan de verschillen in de oppervlakte-eigenschappen van hun capsiden, maar ook aan de samenstelling van cel- en weefselmaceraten en de verhouding tussen de concentraties van het virus en de normale celbestanddelen.

Dit proefschrift.

IV

Op grond van onze kennis van de virusoverdracht door bladluizen moet worden verondersteld dat de overdracht van virussen die eerst in de vector circuleren, niet vermindert door een bespuiting van planten met minerale oliën.

BRADLEY, R. H. E., Can. J. Microbiol. 9, 369–380, 1963.

V

De experimentele inductie van tumoren in *Locusta migratoria* L. kan worden verklaard door de activering van een tumorverwekkend virus.

MATZ, G., J. Insect Physiol. 6, 309–313, 1961.
MATZ, G. *et al.*, J. Invert. Path. 8, 8–13, 1966.

VI

De cyclische activiteit van de prothoracale klier wordt gedurende de ontwikkeling van het insekt in belangrijke mate gereguleerd door het juveniel hormoon.

KRISHNAKUMARAN, A. en H. A. SCHNEIDERMANN, J. Insect Physiol. 11, 1517–1532, 1965.

Proefschrift van Ir. D. PETERS
Wageningen, 6 oktober 1967.

VII

Voedselpreferentie bij een fytofaag insekt is geen onveranderlijke eigenschap, zodat taxonomische differentiatie slechts gebaseerd op verschillen in voedselpreferentie, met groot voorbehoud moet worden beschouwd.

JACOB, F. H., Proc. R. ent. Soc. Lond. A. 24, 99–110, 1949.

VIII

Vegetatieve planten van *Silene armeria* L. komen niet tot bloei door passage in een entplaats van een zich autokatalytisch vermeerderend bloeihormoon, doch door die van een bloei-continuator, die tijdens de bloemknopvorming wordt geproduceerd.

WELLENSIEK, S. J., Naturwiss. 53, 411, 1966.

IX

De hypothese van Kauffmann dat de vorming van niet definieerbare produkten bij de inwerking van lithiumpiperidide op 2-chloorpyridine aan het optreden van 2, 6-dehydropyridine als tussenprodukt moet worden toegeschreven, is onvoldoende gefundeerd.

KAUFFMANN, TH. Angewandte Chemie 77, 557–571, 1965.
ZOLTEWICZ, J. A. en C. L. SMITH, J. Am Chem. Soc. 88, 4766–4767, 1966.

X

De invoering van arbeidsbesparende bereidingswijzen van Goudse Boerenkaas heeft voor de zelfkazer alleen zin, indien dit niet ten koste gaat van de uitzonderlijke kwaliteiten, die haar zo vermaard hebben gemaakt.

DIJKSTRA. H., Landb. T. 78, 312–317, 1966.

XI

De effecten van gammastraling op het substraat zijn zo onvoldoende bestudeerd dat het thans nog geen aanbeveling verdient het gebruik van voedsel te bevorderen, waarvan de houdbaarheid door deze bestraling is verlengd.

Persbericht van het Voorlichtingsbureau voor de voeding nr. 1276.

XII

Het gebruik van visuele hulpmiddelen tijdens de verdediging van een proefschrift zou het behandelde voor een groter publiek bevattelijk kunnen maken.

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CHAPTER 1

INTRODUCTION

The purification of the causal agent of leafroll disease of potato from its vector *Myzus persicae* Sulz. and its subsequent characterization will be described.

The disease was first mentioned by HOPPE in 1747 in Germany (SALAMAN, 1949). APPEL (1906) gave a detailed description of the symptoms and was able to separate leafroll as a specific disease from the complex of potato degeneration ('Abbau'). The infectious nature of potato leafroll was demonstrated by QUANJER *et al.* (1916). Shortly after this, OORTWIJN BOTJES (1920) provided evidence that the aphid *M. persicae* transmitted the causal entity from plant to plant. This was independently found by SCHULTZ and FOLSOM (1921). On the basis of the results obtained in the graft experiments the causal entity of leafroll was considered to be a virus on the analogy of other viruses (QUANJER *et al.*, 1916).

However, knowledge of the intrinsic properties of the virus was lacking because of the failure to transmit it mechanically from plant to plant and the absence of a method to purify the virus. After a technique had been developed for testing extracts for infectivity by injecting aphids (STEGWEE & PONSEN, 1958) it became possible to attempt the purification and the characterization of this virus.

In the following sections a survey of published work being of interest to the present study will be given[1]. In the last section of this chapter the scope of the study will be presented in more detail.

1.1. TRANSMISSION OF POTATO LEAFROLL VIRUS

The transmission of potato leafroll virus (PLRV) has been extensively studied by many workers. QUANJER *et al.* (1916) were able to transmit the virus from diseased plants or tubers to healthy ones by stem or tuber grafts thus demonstrating the infectious nature of the disease. These experiments do not, however, explain the transmission in the field. OORTWIJN BOTJES (1920) demonstrated in greenhouse experiments that the disease could be transmitted by the aphid *M. persicae*. He correctly assumed that this also occurred in the field. SCHULTZ & FOLSOM (1921) reported similar results with the aphids *M. persicae* and *Macrosiphum solanifolii* (Ashm.). In subsequent years this transmission was studied in detail. From the results obtained by ELZE (1927), SMITH (1929 and 1931), DAY (1955), MACCARTHY (1954), and MACKINNON (1963) it may be summarized that the aphid acquires the virus more readily in a long than in a short feeding period, that after a short acquisition feed a period exists during which the

[1] An extensive review of literature on potato leafroll has recently been published by BODE (1962).

aphid is not able to transmit the virus, and that after this period of latency the aphid is infectious for the remainder of its life. PLRV and most of the other circulative aphid transmitted viruses can not be transmitted to plants by mechanical inoculation.

Infectivity assays were, therefore, not possible till a decade ago when methods for injecting aphids were developed. HEINZE (1955), DAY (1955), HARRISON (1958), STEGWEE & PONSEN (1958), DAY & ZAITLIN (1959), STEGWEE & PETERS (1961), MUELLER & ROCHOW (1961), MUELLER & ROSS (1961) and MURAYAMA & KOJIMA (1965) were able to make aphids infective by injecting them with small quantities of haemolymph or extracts from viruliferous aphids, or extracts from infected plants. Furthermore, studies on PLRV were favoured when KIRKPATRICK (1948) discovered that *Physalis floridana* Rydb.[1] could be used as test plant for this virus. This plant species developed more pronounced symptoms ten to fourteen days after infection than potato plants did. WILLIAMS & ROSS (1957) showed that *P. floridana* was more sensitive for the virus than potato. It, therefore, became possible to test injected aphids easily and effectively for infectivity.

STEGWEE & PONSEN (1958) were able to demonstrate that PLRV multiplies in the aphid. Haemolymph of a viruliferous aphid was injected into a PLRV-free aphid, which was confined to a Chinese cabbage plant for 7 days. The haemolymph of this aphid was injected into another PLRV-free aphid. The donor-aphid was tested for infectivity on *P. floridana*. After 7 days the process was repeated, and this was carried out 15 times. The aphid injected in the last transfer proved to be infective. The authors calculated that if no multiplication had taken place the dilution would have reached 10^{-21} while the haemolymph could only be diluted to 10^{-4} to remain infective. From results obtained by testing the virus content of aphids at various periods after they had acquired the virus, HARRISON (1958), however, concluded that PLRV does not multiply in the vector.

Recently another assay method was developed for certain circulative aphid-transmitted viruses. Barley yellow dwarf virus (ROCHOW, 1960) and beet western yellows virus (DUFFUS & GOLD, 1965) could be fed to aphids from liquid preparations placed between membranes, but this procedure has not been used in the assay of PLRV.

The mechanical transmission of infectious nucleic acid extracted from PLRV infected potato plants, was claimed by BRANDENBURG (1962). This could not be confirmed by other authors (SARKAR, 1963; PETERS & DIELEMAN, 1963 and KOENIG & MUELLER, 1964). Results obtained by SPIRE (1965) and FRITZ (1966) are, however, in agreement with those of BRANDENBURG. These authors used potato plants as test plants. It should, however, be pointed out that other factors may cause symptoms on potato plants, which may be confused with those caused by a leafroll virus infection (QUANJER *et al.*, 1916; ROZENDAAL, personal comm.).

[1] Throughout this report the name *P. floridana* will be used. WATERFALL (1958) considered this plant to be a variety of the species *P. pubescens* L. referring to it as *P. pubescens* L. var. *pubescens*.

1.2. Isolation of the virus from infected plants and viruliferous aphids

The first recorded attempt to isolate PLRV was by Bawden & Nixon (1951). Sap of infected potato and *Datura stramonium* L. plants was clarified at 7000 rpm for 10 minutes. Portions of the supernatants were mounted on specimen grids for electron microscopy but no specific particles were detected. They concluded that the virus concentration would be lower than 10 mg/l if the particles were spherical and 1 mg/l if they were rod shaped.

In a similar study made by Sprau (1952) sap of infected potato plants was partially clarified in three different ways and studied by electron microscopy. Filiform particles with lengths from 150 mμ to 4250 mμ were found in one preparation, whereas the length varied from 66 mμ to 2300 mμ in another extract. He doubted, however, whether these particles were those of PLRV.

Threads with lengths of the same order were found by Heinze (1955). Particles varying in length from 337 to 5000 mμ were found in the blood of viruliferous aphids and particles varying in length from 620 to 1670 mμ were found in sap of infected *P. floridana* clarified at 25.000 rpm for 60 min. The nature of the threads was not determined in the studies of both Sprau and Heinze.

Day & Zaitlin (1959) partially clarified the sap of infected *P. floridana* plants by a cycle of differential centrifugation. The sediments formed at 140,000 *g* for one hour proved to be infectious when injected into aphids. Specific particles were not observed in the infectious preparations by electron microscopy. The virus was also not recovered with continuous electrophoresis at 2°C. They concluded that leafroll virus protein constitutes only a very small fraction of the proteins in the extracts.

Day (1955) isolated infectious extracts from viruliferous aphids. About 10 per cent of the plants became infected when the inoculated aphids were permitted to feed on them. Infectivity of aphid macerates was also demonstrated by Harrison (1958) and Murayama & Kojima (1965). Stegwee & Peters (1961) recovered infectivity from sucrose gradients after the resolution of a macerate from viruliferous aphids at 90,000 *g* for 90 minutes. Stegwee & Peters (1961) as well as Murayama & Kojima (1965) showed that infectivity could be retained when preparations were stored in vitro at 2°C for a few days.

1.3. The nature of the virus

Some excitement arose when Brandenburg (1962) reported that the infectious component of PLRV was DNA. Plant viruses usually contain RNA as the infectious component. He based his conclusions on the fact that pressed sap of infected potato plants developed a positive blue colour with the diphenylamine test for DNA of Dische. Furthermore, when a phenol extract of leaf material was inoculated mechanically onto potato seedlings, they became infected. The infectivity in phenol extracts could be destroyed by DN-ase, but not by RN-ase. Inoculation of crude sap, however, did not result in diseased plants. Brandenburg, therefore, assumed that PLRV occurs in the plant as a free DNA

molecule. PETERS & DIELEMAN (1963), SARKAR (1963) and KOENIG & MUELLER (1964) could not confirm that the infectious agent of PLRV was DNA. The development of the blue colour in sap of infected plants with the diphenylamine reaction is caused by the larger concentration of sugars in these plants (GOVIER, 1963; FRITZ *et al.*, 1963; HECHT *et al.*, 1963; PETERS & DIELEMAN, 1963; KOENIG & MUELLER, 1964).

The mechanical transmission of PLRV nucleic acid to potato plants was again claimed by SPIRE (1965) who used extracts of frozen plants in an electro-inoculation method, and by FRITZ (1966) who used phenol extracts. In these cases, as well as in the studies of BRANDENBURG potato plants were used as test plants.

1.4. ELECTRONMICROSCOPICAL STUDIES ON TISSUES OF VIRULIFEROUS APHIDS

The occurrence of PLRV in the salivary glands and the intestinal canal of viruliferous aphids has been extensively studied.

After an electronmicroscopical study on the morphology of the salivary glands of the aphid *M. persicae* two types of particles resembling viruses were found in the glands of viruliferous aphids by MOERICKE (1961) and MOERICKE & WOHLFAHRT-BOTTERMAN (1962). Some particles were about 200 mμ long and 30 mμ wide and often had a laminated structure, and others found in only one cell, were measuring 300–400 mμ in length and about 50 mμ in width. The particles were found to be solitary or arranged in bundles. They resembled the polyhedra virus of *Bombyx mori* (BERGOLD, 1958) and a virus infecting corn (HEROLD *et al.*, 1960). In further studies of MOERICKE (1963) identical structures were also found in non-viruliferous aphids in different tissues. He, therefore, concluded that they had no connection with PLRV.

FORBES (1964 b) made an electron microscopical investigation on sections of the stomach of *M. persicae*. In some cells of viruliferous aphids rod shaped particles were found, which were absent in nonviruliferous aphids (FORBES, 1964 a). However, he did not give sufficient information for a comparison to be made with the results of MOERICKE.

1.5. THE SCOPE OF THIS STUDY

The preceding sections show that very little is known about the properties and chemical constitution of potato leafroll virus and, moreover, that results are often contradictory. Some physical properties can be established using crude virus preparations, but for investigations on the chemical constitution it is necessary to use purified virus. An attempt was made to develop a purification procedure whereby virus could be purified from its vector or from one of its hosts. This seems to be justified because of the recent development of purification techniques and the method now available to test the infectivity of PLRV suspensions, which open new possibilities.

Purification experiments were carried out on viruliferous aphids because usually their extracts were infectious, whereas those of infected plants were not.

As the ribosome content of aphids is very large, it was difficult to perform a separation between the virus and the the non-viral constituents. After preliminary studies the virus could be purified in very small quantities. The biological purity of the virus isolate was tested in order to verify the causal relationship between the extracted virus particles and the disease.

Besides PLRV, particles resembling viruses could also be extracted from aphids which were reared on plants infected with one of the PLRV isolates. Some biological and physical properties of these particles referred, to as virus-like particles in this study, will be described in Chapter 8.

CHAPTER 2

MATERIALS AND METHODS

The general procedures that were followed and materials that were used will be described. In subsequent chapters relevant experimental details will be given where necessary.

2.1. Symptomatology of the isolates of potato leafroll virus used

Most experiments were carried out with the isolate of potato leafroll virus (PLRV) used by Stegwee & Ponsen (1958) in their work on the multiplication of this virus in the aphid *M. persicae*. The symptoms caused by this isolate on the potato variety Bintje, were described by Beemster (pers. comm.) as severe. It was found that this isolate caused moderate symptoms on *P. floridana*. On some leaves a strong chlorosis between the veins occurred fourteen days after inoculation by aphids, with intensification of the green colour along the veins around the chlorotic areas. The edges of the basal parts of the leaves showed a tendency towards a slight cupping. The other leaves of the plant showed a mild chlorosis over the whole leaf. The entire plant was somewhat stunted. Epinasty of the petioles, as described by Webb *et al.* (1952) for all the strains of PLRV they investigated, was hardly discernible. Twisting of the petioles was never observed. After development of the symptoms the plant recovered rapidly and could only be distinguished from the healthy ones by a lighter green colour. Over a period of 5 years during which this isolate was used, instability of symptoms as observed by MacCarthy (1963) in his PLRV isolates was never seen.

In the final experiments on the purification of PLRV an isolate was used which caused severe symptoms on potatoes as well as on *P. floridana*. This isolate was supplied by Mr. A. Rozendaal from the Laboratory of Phytopathology. Severe leaf chlorosis, leafrolling and basal cupping of the leaves, epinasty and twisting of the petioles and severe stunting of the plant were the characteristic symptoms of the infected *P. floridana* plants. Three to four weeks after inoculation the lower leaves which also showed the most severe symptoms, abscised. Webb *et al.* (1952) reported that leafdropping was followed by dying of the plant. In our case, however, death rarely occurred. The plant recovered slowly from the initial effects of the infection and there was some subsequent growth. Besides symptomatological differences, the two virus isolates also differed in another respect. Aphids colonized better on *P. floridana* plants infected with the isolate first described than on those infected with the one obtained from Rozendaal.

Most of the preliminary work on the purification was done with the first isolate. It appeared, however, that this isolate was contaminated with another, unknown, virus. Therefore, in the final purification experiments the second isolate, which proved to be free of contaminating viruses, was used. The isolates

described above and used in this study will be referred to as the moderate and severe isolate, respectively.

2.2. Cultivation of the plants

All plants were grown in soil consisting of a mixture of sand, compost, leaf-mold and farmyard manure. The soil was sterilized by steaming for two hours at 100 °C and sieved before use.

To have uniform test plants *P. floridana* seeds were sown in seed trays three times a week. The soil used was then sieved once more with a 5 to 6 mesh sieve. An even germination of the seeds was obtained after six to eight days when they were kept at a temperature of 27 °C and a high relative humidity. When the cotyledons of the seedlings were 4 to 5 mm long, the trays were put in a greenhouse at 20–22 °C. The seedlings were transplanted into 10 cm diameter pots at the time that the first true leaf had reached half the length of the cotyledons. After one or two days the plants were used for the test feeding of the aphids. The true leaf had usually not reached the size of the cotyledons by then.

P. floridana plants to be used as virus source and for colonization of aphids were infected with the moderate isolate a week after being transplanted to 15 cm pots.

The severe isolate was maintained on *D. stramonium* plants. Three weeks after sowing, seedlings of this species were transplanted to 15 cm diameter pots. The aphids were placed on these plants when the first leaves had reached a length of approximately 15 cm.

Radish plants *(Raphanus sativus* L.*)* and Chinese cabbage plants *(Brassica sinensis* L.*)* used to rear PLRV-free aphids were sown every week and after 7 days the seedlings were transplanted to pots with a diameter of 15 cm. They were allowed to germinate and grow at a temperature of 20–22 °C and a relative humidity of 65–80 per cent. Two to 4 weeks after transplantation the plants could be used for colonization of the aphids. In winter plants were exposed to additional light, but this was not sufficient to produce the same growth as in summer.

2.3. Rearing of the aphids

PLRV – free aphids *(M. persicae)* were reared on radish or Chinese cabbage plants. Four to six plants were covered by an aphid-proof cage and placed on a zinc tray. To prevent the aphids from passing the chinks between the tray and the rim of the cage, the bottom of the tray was covered with a layer of wet sand. The cages were made of a wooden frame on a strip of zinc. The top and two of the sides of each cage were closed by glass plates, and the other sides by Monodur netting cloth no. 314. Each new colony was started by placing 5 to 6 adults on fresh plants. These colonies were maintained in a separate air-conditioned greenhouse at 20–22 °C and a relative humidity of 60–80 per cent under long day conditions.

The viruliferous aphids were bred under the same conditions in another greenhouse with similar climate conditions. A continuous colony of these aphids was obtained by removing the old plants and adding fresh diseased plants each week.

2.4. Production of virus-free nymphs

The circulative aphid-borne viruses which have been studied extensively do not pass from the adults to the nymphs (Björling & Nillson, 1966; Smith, 1929). By collecting newly-born nymphs virus-free aphids can easily be obtained. A great number of nymphs of *M. persicae* can be produced in the following way. Viviparous adults are starved for 24 hours at room temperature and thereupon placed on a detached radish leaf in a petri-dish. During the next hour nymphs are born at a rate of two to three per adult. At intervals of a few minutes the newly-born nymphs are transferred to a fresh plant.

This is not a general way for aphids to react. Some species like *Megoura viciae* Bckt. and *Acyrthosiphon pisum* Harris did not give a similar response to such a long starvation period.

2.5. Collecting aphids

Aphids used for testing the infectivity of samples and extracts were cautiously collected from the plants with a paint-brush.

Viruliferous aphids used in the purification experiments were sucked from the *P. floridana* plants with an aspirator connected to a vacuum system. When the aspirator was made from a centrifuge tube closed by a double bored stopper, the skins could be easily separated from the aphids. A rubber tube was pushed onto the glass tube that reached into the tube about 2 cm beneath the stopper. Another glass tube connected to the vacuum system reached to the bottom of the stopper and was closed by a piece of cheese cloth. On collecting the aphids they were piled up at the bottom of the tube, while the skins were gathered around the closed vacuum outlet. When the vacuum was taken away the skins could be removed from the cheese cloth without contaminating the aphids.

When aphids from *D. stramonium* plants or large amounts of aphids from other plants had to be collected a washing method was used. The leaves or plants were plunged in water. The aphids were washed from the leaves with tap water collected on a sieve fixed to the outlet of the funnel in which the leaves were placed. The skins could be separated from the aphids by returning them to water: the aphids sunk and the skins floated on the surface. This method is less time consuming than collecting aphids with an aspirator, but it has the disadvantage that aphids cannot be weighed in a dry condition.

2.6. The injection of aphids

Infectivity of extracts from viruliferous aphids and infected plants was tested by injecting small parts of the preparations into aphids. This was done by means

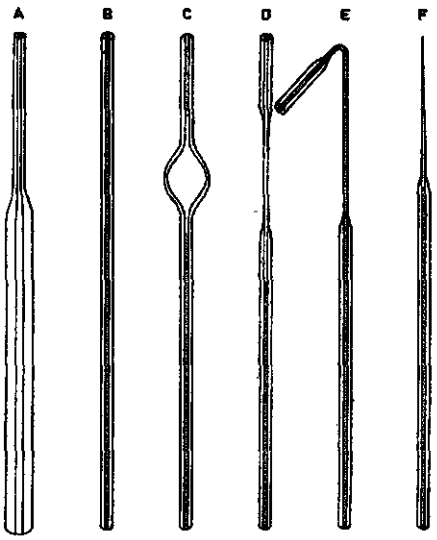


FIG. 1. Schematical representation of the method by which the injection needle is made from the capillary tube. The process is described in the text.

of a micro-injection device developed by WORST (1954) and modified by STEGWEE & PONSEN (1958). The complete procedure including the preparation of the needle, the injection apparatus and the injection process will be described.

A capillary tube is drawn from a glass tube of 6 mm o.d. and 2 mm i.d. (Fig. 1 A and B). The capillary tube is filled with both distilled and filtered water. One end is sealed on a small gas flame and upon cooling the other end is also sealed. About 2 cm from this end the tube is heated, so that the vapour pressure forms a bubble in the glass wall (C). The last sealed end is opened with a glass-knife and the bubble is drawn out to a length of one to two cm (D). This thin walled section is made into a hook and filled with water again (E). The micro-tip proper (F) is made on a micro-forge. The pulling force is provided by a small weight (7 gram) hung on the hook (Photo 1). When the filament of the micro-forge is heated, the tube is drawn out into a tip. If the tip is closed it can be opened by scraping the tip with the platinum filament of the forge.

The micro-forge (Fig. 2) is on a light microscope and stands between the stage and the body tube which are tilted into the horizontal position. The base of the micro-forge is fixed on the base of the microscope which in turn stands on a wooden table (Photo 1). The micro-forge consists of a stationary arm mounted on the base and a two-jointed movable arm attached by a spring plate to the base. The two joints of the movable arm can be moved with respect to each other by a hinge. A block of ebonite has been placed in the nick of the short joint to support and insulate two connectors to which the filament has been connected. The filament can be manipulated by two adjusting screws. The adjusting screw on the stationary arm gives the filament a horizontal motion, whereas the other screw can almost manipulate the filament in the vertical plane. It is thus possible for the heated filament to follow closely the glass tube while the tip of the needle is being drawn out.

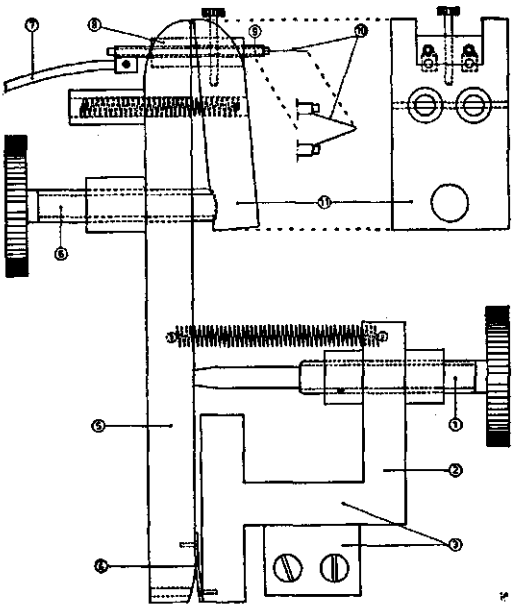


FIG. 2. Diagrammatic vertical section of the micro-forge. 1) Adjusting screw on the stationary arm. 2) Stationary arm. 3) Base of the micro-forge. 4) Spring plate. 5) Long joint of the moveable arm. 6) Adjusting screw on the moveable arm. 7) Wire to power supply. 8) Block of ebonite. 9) Connectors. 10) Platinum filament. 11) Short joint of the moveable arm.

The capillary tube can move to and fro in a holder mounted on the stage in the plane of the body tube in order to adjust the needle side by side with the filament.

Injections are made under the binocular microscope. The needle is fixed in a horizontal position. The force needed for injection is provided by thermal expansion of the water at the closed end of the needle (Fig. 3). The heat is furnished by an electric device. Upon cooling the water contracts and the fluid to be injected can be drawn into the needle. A small air bubble at the sealed end provides a continuous flow of the water column.

The aphids are anaesthetized with carbon dioxide and handled by means of a vacuum system, which consists of a glass tube with a bore just fitting an aphid's head and connected to a suction pump. The insects are gently pressed onto the needle and injected into the abdomen. The needle is inserted superficially into one of the intersegmental membranes (Photo 2). Injections are usually made at the ventral side of the abdomen, because these punctures close more easily than those on the dorsal side. The needle is cleaned at every change

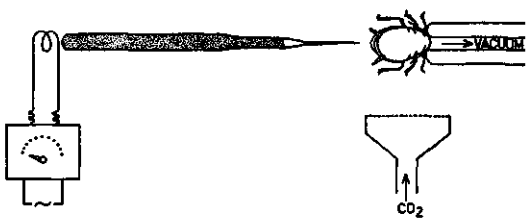


FIG. 3. Diagrammatic representation of the method by which aphids are injected.

of the sample and when necessary to prevent plugging of the needle by aphid constituents. In this way about 50 aphids can be injected per hour and 40 to 200 individuals with one needle.

2.7. Testing injected aphids for infectivity

Usually 22 aphids were injected from each sample to be tested for infectivity. The injected aphids were put on a piece of filter paper in a glass tube which is then closed with a piece of cheese cloth and stored overnight in the cold room (2 °C). After such storage the aphids appear to accept the test plant more readily. The next day the surviving aphids were caged on *P. floridana* test plants. Two aphids were confined to one seedling. The cages were made from a 13.5 cm high Perspex tube of 9 cm diameter. At 4 cm from the bottom 2 windows of 2 cm diameter were cut. The windows and the upper side were closed with Monodur netting cloth no. 314. The pieces of netting cloth were glued to the Perspex tubes with Klebelösung P.C. 10, supplied by the firm Wientjes at Roden, The Netherlands.

Observations on the mortality of the aphids were made 24 hours later. The living aphids that had left the test plants were returned to them. After an inoculation feeding of 5 days the cages were removed and the aphids killed by a Phosdrin or Lirohex (TEPP) spray. The plants were placed in an aphid proof greenhouse, which was fumigated or sprayed regularly with Lindane or Phosdrin. The test plants were checked for symptoms two to three weeks after termination of the inoculation feed. In winter additional light was given to provide a daylength of 16 hours.

2.8. Ultracentrifugal analysis

Sedimentation analysis was performed in a Spinco model E analytical ultracentrifuge, using Schlieren optics. All runs were made at a constant temperature in the range 19–21 °C in 1.2 cm cells. Sedimentation coefficients were calculated with the graphical method of Markham (1960) and corrected for a temperature of 20 °C.

CHAPTER 3

THE CHOICE OF THE VIRUS SOURCE

It is desirable to select the host most suitable as a virus source. There is only a limited choice for PLRV. It has a restricted host range and its propagation has been demonstrated in one vector only by STEGWEE & PONSEN (1958). Infectious fractions have been extracted by several workers from diseased plants as well as from viruliferous aphids as mentioned in the first chapter. It was, therefore, of considerable interest to determine whether plants or aphids were the best source in the purification of PLRV. Among the known host plants of PLRV *P. floridana* may be of considerable interest because this plant develops clear symptoms shortly after infection. Furthermore, aphids can acquire the virus more easily from this plant than from any other species that has been tested (PETERS & ASJES, unpublished). This observation may point to a higher concentration of the virus in this plant than in others. It was, therefore, decided to compare the usefulness of this plant as a source of virus with that of aphids for our study. The infectivity of clarified aphid macerates was compared with that of crude sap of *P. floridana* plants. Subsequently a test was performed to determine whether the infectivity could be recovered from macerates and sap after concentration at 100,000 *g* for 1 hour.

3.1. INFECTIVITY OF UNCONCENTRATED APHID MACERATES AND PLANT SAP

Aphid macerates were prepared in the following way. Viruliferous aphids (50–100 mg) were triturated in a glass tube with a glass rod. Buffer (0.01 M phosphate buffer pH 7.2) was added using a volume twice that of the weight of aphids used. The macerates were clarified by centrifuging at 1000 *g* for 10 minutes. The supernatant was used to inject PLRV-free aphids. The results of the infectivity assays are given in Table 1. The clarified aphid macerates which were diluted three times, were consistently infectious. In fact, infectivity could

TABLE 1. Infectivity of clarified and diluted macerates of viruliferous aphids.[1]

Experiment	Amount of aphids (mg)	Infectivity[2] Dilution of the macerate 1:3	1:30
1	60	4/10	1/10
2	100	7/10	4/10
3	80	2/10	0/10
4	80	3/10	1/10

[1] The aphids had been reared on diseased *P. floridana* plants.

[2] Two injected aphids were confined on a *P. floridana* test plant. Numerator: number of infected plants. Denominator: number of inoculated plants.

be demonstrated in macerates diluted up to thirty times in three out of four experiments.

One or two droplets of sap were pressed from leaves of diseased *P. floridana* plants inoculated two to three weeks earlier and showing clear symptoms. The sap was collected in a glass tube placed in an ice-bath. Samples of the sap were injected into a number of aphids within 7 to 15 min after preparation of the inoculum.

The results of infectivity tests and the mortality of the aphids after being on the plants for 24 hours are given in Table 2.

With the exception of the first four experiments the injected aphids were kept overnight in the cold room after injection. Each test plant was inoculated by one or two aphids as indicated in Table 2.

TABLE 2. Virus infectivity in sap pressed from diseased *P. floridana* plants.

Exp.	Time interval between preparing the inoculum and using it for the last injection (min)	Injected aphids stored overnight at 2°C	No. of aphids injected	Survival on the test plant after 24 hours	No. of aphids on each test plant	Infectivity[1]
1	7	no	38	35	2	4/19
2	7	no	22	6	2	0/11
3	7	no	32	24	2	0/16
4	7	no	36	29	2	0/18
5	7	yes	42	14	2	3/21
6	10	yes	58	51	1	9/58
7	10	yes	49	36	1	2/49
8	15	yes	77	12	1	0/77

[1] Numerator: number of infected plants. Denominator: number of inoculated plants.

Only a small number of test plants became infected. The infectivity of the leaf sap thus tested may be compared with the infectivity of macerates of viruliferous aphids diluted thirty times. This means in terms of virus concentration that the content of plant sap is about 30 times lower than in aphid macerates. Thus, aphids were preferred as a virus source. However, it may be expected that an increase in infectivity can probably be obtained by concentrating sap from diseased plants by high speed centrifugation or some other mild procedure.

3.2. EFFORTS TO CONCENTRATE THE VIRUS IN SAP FROM DISEASED PLANTS

In the experiments described in this section 4 to 6 weeks old *P. floridana* plants showing clear symptoms were used. The plants had then been infected for 2 to 4 weeks. Leaf material (5–7 gram) was ground in 8 ml 0.1 M or 0.01 M potassium phosphate buffer pH 7.2. The homogenate was filtered through cheese cloth, partially clarified at 4000 *g* for 10 min and the supernatant centrifuged

for two hours at 76,000 *g*. The pellets consisting of a large quantity of green material with a dark brown coloured core were dissolved in 0.3 to 0.5 ml 0.01 M phosphate buffer pH 7.2. The resulting suspension was clarified at 1000 *g* for 10 minutes and injected into aphids. In six experiments performed in this way none of the injected aphids was able to infect a test plant.

Concentration of the virus from the sap did not result in an increased infectivity as expected. DAY & ZAITLIN (1959) found 8 per cent of the plants were infected when a comparable fraction was used as inoculum. The difference in the results may be explained by the presence of some plant material in our inoculum. In order to prevent loss of virus, we retained the green layer of plant material overlying the high speed pellet which DAY & ZAITLIN had washed away. This may have influenced unfavourably the transmission of the virus after injecting the aphids with this material. We performed, therefore, some experiments to concentrate the virus after removal of most of the greenish plant material.

Leaves of diseased *P. floridana* plants (10–12 g) were ground with buffer containing 0.5 M sucrose, 0.005 M tris-HCl (tris (hydroxymethyl)-aminomethane-hydrochloride) and 0.01 M magnesium acetate, pH 7.0 in a mortar with a pestle and sand. The homogenate was filtered through cheese-cloth. The filtrate was centrifuged at 25,000 *g* for 15 min and the supernatant was then centrifuged at 100,000 *g* for 90 min. A brownish-green pellet without an overlying layer of green material was obtained after high speed centrifugation and resuspended in 0.2 ml of a buffer consisting of 0.005 M tris supplemented by 0.001 M magnesium acetate and adjusted to pH 7.0. This suspension was clarified at 1000 *g* for 10 min and tested for infectivity by injecting portions of it into aphids. In two out of four experiments one of ten inoculated test plants became infected, thus giving an average of 5 per cent infection. These results are comparable with those obtained by DAY & ZAITLIN, who did not include the green plant material in their inoculum. Therefore, we may tentatively conclude that some substance among the green plant material may have some inhibitory influence on the transmission of the virus.

On comparing the results using either unconcentrated or concentrated fractions from infected *P. floridana* plants similar levels of infectivity were observed. From the data in Table 2 it may be inferred that an average of 6.6% of the inoculated plants became infected. A level of 12% was found by MURAYAMA & KOJIMA (1965) in a test on the infectivity of unconcentrated *P. floridana* sap. From the infectivity levels of unconcentrated and concentrated *P. floridana* sap it may be concluded that concentration of this sap does not lead to an increase in the infectivity of the extracts.

3.3. DISCUSSION

In comparing different purification procedures it is essential to follow the virus in the various fractions which are obtained. This has to be done by infectivity tests, because other tests to detect the virus are not available. It is,

therefore, necessary to commence the purification from a source in which a fair concentration of virus could be detected. It was shown that aphids provide a better source of PLRV than *P. floridana*.

In addition to aphids, phloem sap may be a rich source of PLRV because this virus seems to be restricted to the phloem within the plant (BENNETT, 1956; ESAU, 1961). Localization of symptoms in this vascular tissue, acquisition and transmission of the virus by aphids during long feeding periods, and the the failure of mechanical inoculation are usually interpreted as evidence for such a restriction. There is, however, no experimental evidence for this assumption nor are estimates on the virus concentration in phloem sap available. Some conclusions on the virus content of phloem sap may be derived from the work of HARRISON (1958) on the behaviour of the virus within the aphid. Aphids which were allowed to acquire the virus in a period of 24 or 48 hours, were divided into three comparable groups. One group was directly tested for infectivity, whereas the other groups were transferred to turnip plants for one to four days and subsequently tested. These tests were made by injecting small portions of aphid extract into aphids. The aphids which were immediately tested after the acquisition feed yielded an infective extract, while the other groups did not. HARRISON concluded that the amount of virus acquired during brief feedings soon fell below the level needed for detection by the injection method. This may be explained by assuming that only a small part of the acquired virus has reached the haemocoel, while the majority of the virus disappears by excretion from the alimentary canal. Thus, the virus charge of an aphid which had a short acquisition feeding on a diseased plant presumably occurred in the intestinal tract. It may be concluded that the infectivity in the extracts from aphids which were fed for 24 or 48 hours originate primarily from the contents of the intestinal tract. Even though the content of the intestine tract has been diluted some times by the aphid's blood and the buffer added, infectivity could be demonstrated in the extracts (26% of the inoculated plants became infected). Assuming that the virus is not concentrated by the aphid, its concentration in the intestine would be the same as in phloem sap. The virus, therefore, occurs probably in a high concentration in the phloem sap.

Phloem tissue constitutes only a small part of the plant. Thus, virus present in it is considerably diluted upon grinding the whole plant. In addition, the virus is then under these conditions unnecessarily mixed with large amounts of plant constituents from which it has then subsequently to be separated. It is preferable, therefore, to avoid such a contamination by plant constituents. This may be done by collecting phloem exudate. Feeding aphids have been cut away from their mouthparts (MITTLER, 1957 and 1958; ZIMMERMANN, 1961; VON DEHN, 1961; and VAN SOEST & DE MEESTER-MANGER CATS, 1956). The phloem exudate flows out through the severed stylets and may be collected. Appreciable amounts of sap can be collected from woody plants, but only very small amounts have been obtained from herbaceous plants thus far. For this reason and because of the technical difficulties involved in the method, we have not explored its possibilities.

Honeydew excreted by aphids on plants infected with pea enation mosaic virus was a good inoculum to render aphids infective by injection (RICHARDSON & SYLVESTER, 1965). This source did not play a role in our study because collected honeydew was scarsely infectious.

CHAPTER 4

PROPERTIES OF THE VIRUS IN AN APHID MACERATE

Before commencing the purification of the virus it was desirable to study some properties of the virus in the crude macerate. Tests to determine the longevity in vitro, the thermal inactivation point, and the dilution end point were, therefore, conducted to determine the stability and concentration of the virus in the sap. Although these results have a restricted value (Ross, 1964), they are of importance in determining the procedure for the purification of the virus.

4.1. Thermal inactivation point

An aphid macerate in 0.01 M phosphate buffer pH 7.2 was divided in 3 equal parts of 4 ml. These aliquots were heated for 10 min at 50, 60 and 70°C respectively. Before assaying the aliquots, they were concentrated by centrifugation at 100,000 *g* for 90 min after the removal of the heat denatured material at 10,000 *g* for 10 min. The high speed pellets were dissolved in 0.2 ml phosphate buffer, and tested for infectivity by injecting aphids. The aliquots heated at 50, 60 and 70°C caused 8, 2 and 1 plants out of ten to become infected, respectively. From these data it was concluded that PLRV was inactivated at about 70°C. Murayama & Kojima (1965) came to similar conclusions.

4.2. Longevity in vitro

The longevity in vitro is clearly dependent on the storage temperature. Stegwee & Peters (1961) reported that PLRV could be stored at 3°C for 24 hours and at –16°C for three weeks without loss of infectivity. In subsequent experiments it was found by the present author that virus suspensions were still infectious after storage at 2°C for 3 days. In one experiment when 0.006 M mercaptoethanol was added to the phosphate buffer at 2°C infectivity was still demonstrable after 5 days. These values also agree with those reported by Murayama & Kojima (1965), and by Mueller & Ross (1961).

At higher temperatures the infectivity of the virus was destroyed more rapidly. Aphid macerates stored at 25°C were still infectious after 12 hours, but not after 24 hours. Macerates incubated at 37°C were still infectious after 4 hours. These experiments were not extended to longer periods.

4.3. Dilution end point

Macerates of aphids were diluted in 0.01 M phosphate buffer pH 7.2. Dilutions of 1:100 were slightly infectious in six experiments, while those of 1:1000 were only in one of the six experiments. In that experiment one out of ten

plants became infected. STEGWEE & PONSEN (1958) reported some infectivity in haemolymph diluted at 1:1000. Hence we can conclude that aphid macerates diluted 1:1000 and tested by injecting aphids, approximates the dilution end point. Again, MURAYAMA & KOJIMA (1965) obtained similar results.

4.4. DISCUSSION

The stability of a virus is usually determined by testing its longevity in vitro and its thermal inactivation point. When these tests are carried out with crude sap the results may vary with a number of factors such as the occurrence of oxidizing agents in the sap, the virus concentration, the host and its age, and the pH and the ionic activity of the sap (BAWDEN, 1950; ROSS, 1964). Therefore, the measurements carried out on the aphid homogenates are of a restricted importance. This fact can be supported by comparing the results obtained for barley yellow dwarf virus (BYDV) with those of PLRV. HEAGY & ROCHOW (1965) demonstrated that BYDV was inactivated between 65 and 70°C. They concluded from their observation that this virus was a rather stable one. ROCHOW & BRAKKE (1964) also recorded BYDV as rather stable when they found that BYDV was still infectious after storage at 3°C for at least six weeks. However, the application of these criteria for determining the stability of PLRV would lead to conflicting conclusions, viz. PLRV would then be called a stable virus on the one hand, as its thermal inactivation point is about 70°C, but a rather unstable one on the other hand as its infectivity is lost after 4–5 days at 3°C.

Since the tests on longevity were carried out on crude aphid macerates the instability of the virus should not be overemphasized, because a sap environment may favor stability less than buffer. If this were true it may be expected that the longevity in vitro would increase with the degree of purity of the virus. Such behaviour has already been demonstrated for virus Y which when stored in vitro remains infective for longer periods when it is in a purified form than when in crude sap (BAWDEN, 1950).

An important conclusion can be drawn from the longevity experiments. When purifying PLRV from an aphid macerate, allowance should be made for its rapid loss of infectivity. Thus it is essential to extract the virus as quickly as possible, and at low temperatures.

In vitro at room temperature, the virus loses its capacity to infect a plant within a day. On the other hand an aphid, after acquiring the virus, remains infective for many days and often for its whole life (ELZE, 1927; SMITH, 1929; MACCARTHY, 1954; DAY, 1955; PETERS & ASJES, unpublished). This may be attributed to the multiplication of the virus in the aphid (STEGWEE & PONSEN, 1958).

If the dilution end point is 1:1000 and assuming that 1) 0.003 μl inoculum is injected into the aphid (STEGWEE & PONSEN, 1958), and that 2) an aphid needs only one PLRV particle to render it infective, it may be calculated that the lower limit of the number of PLRV particles occurring in one gram of aphids is

3.3×10^8. As the mean weight of one adult of *Myzus persicae* is about 0.2 mg, one infective aphid would contain at least 6.6×10^4 virus particles.

The assumption that injecting only one virus particle suffices to make an aphid infective is disputable, for we do not know the efficiency of the inoculation method nor the number of virus particles needed to initiate virus multiplication in the aphid and to overcome the transmission threshold. Thus, the required number of particles to make an aphid infective may be higher. If this number were 10^4, the concentration of the virus in the aphid would still be low, viz. 3.3×10^{12} particles in one gram aphids. Assuming that PLRV has a particle weight of about 5.10^6, an amount of $\frac{3.3 \times 10^{12}}{6.3 \times 10^{23}} \times 5.10^6$ gram $= 2.6 \times 10^{-5}$ gram PLRV particles will occur in 1 gram aphids. This figure will be 2.6×10^{-9} if one particle is necessary to render an aphid infective. These calculations are speculative, but they offer some idea on the concentration of the virus in the aphid.

CHAPTER 5

STUDIES ON THE NUCLEOPROTEINS OBTAINED FROM APHIDS

In Chapter 6 preliminary studies on the purification of PLRV are described. It appeared necessary to eliminate large quantities of non-viral components from the macerates in order to obtain a pure virus preparation. Some knowledge of the nature of the impurities was essential in order to develop a suitable procedure for purifying PLRV. It was evident from spectrophotometrical studies that the impurities were mainly composed of nucleoproteins. Some properties of a nucleoprotein rich fraction isolated from aphids are described in this chapter.

5.1. Isolation of the nucleoprotein particles

Aphids were reared on radish or Chinese cabbage plants. They were collected with an aspirator, and 1 ml of 0.001 M phosphate buffer pH 7.2 was added to each gram of aphids. Alternatively, the aphids were collected by the washing method. The aphids were triturated with a mortar and pestle, and the homogenate obtained from each 1.5 gram of aphids was diluted with buffer to 10 ml. The suspension was centrifuged at 1000 *g* for 10 min and then at 25,000 *g* for 15 min. The supernatant was decanted into another tube and spun at 100,000 *g* for 75 min. After this centrifugation the supernatant was discarded and the resulting pellet was dissolved in buffer. The suspension was clarified by centrifuging at 25,000 *g* for 15 min. Again the supernatant was decanted into another tube and then centrifuged at 100,000 *g* for 75 min. The supernatant was discarded and the pellet dispersed by stirring with a glass rod. For studies with the analytical centrifuge the volume was made to 1.0 ml with 0.001 M phosphate buffer pH 7.2 for each gram of aphids used. This suspension was clarified by spinning at 10,000 *g* for 10 min. The whole procedure was carried out at 0–3 °C.

The fractionation of the nucleoprotein particles was checked with spectrophotometrical measurements. Some data of UV spectra of various fractions are represented in Table 3. The increases of the quotients of extinction at 258 and

Table 3. Spectral data of fractions obtained by differential centrifugation from macerates of the aphid *M. persicae*.

Fraction	λ max	λ min	E_{max} / E_{min}	E_{258} / E_{280}	E_{258} / E_{230}
	mμ	mμ			
First supernatant after 25,000 *g* for 15 min	258	240	1.29	1.61	0.92
First high speed pellet, resuspended in 0.001 M phosphate buffer and clarified	258	238	1.37	1.73	1.09
Second high speed pellet, resuspended in buffer and clarified	258	238	1.47	1.85	1.25

230 mμ, at 258 and 280 mμ, and at wavelengths of minimum and maximum absorption after recentrifugation indicated increasing purity of the preparations (VAN KAMMEN, 1963; LANSINK, 1964; and PETERMAN, 1964). It can be seen in Table 3 that fractionation of an aphid homogenate by differential centrifugation resulted in fairly pure preparations of nucleoproteins. This is also evident from the shift of the wavelength from 240 mμ to 238 mμ at which minimum absorption occurs. Due to the high content of nucleic acid in the aphid homogenates, this shift is not as pronounced as that found for ribosome preparations from tobacco leaves (VAN KAMMEN, 1963).

5.2. UV ABSORPTION SPECTRUM

The ultraviolet absorption spectra of the nucleoproteins agreed with those obtained for ribosome preparations from other tissues and organisms. They have a maximum absorption at 258 and a minimum absorption at 238 mμ.

To compare the UV absorption of nucleoprotein preparations with those prepared by other workers both the quotient of the maximum and the minimum extinctions as well as the extinctions at 258 and 230 mμ are given in Table 3. The ratios E_{max} / E_{min} and E_{258}/E_{280} are in agreement with those of the ribosomes of tobacco plants (VAN KAMMEN, 1963). The ratio E_{258} / E_{230} is very similar to the values reported by LANSINK (1964) for the ribosomes of *Escherichia coli*.

The extinction coefficient of nucleoprotein preparations $E_{1\%\text{-}258}^{1\,cm}$ has not been determined. To estimate their concentrations in the suspensions from aphids we used the value $E_{1\%\text{-}258}^{1\,cm} = 127$ which is the mean of a number of extinction coefficients of various sources listed by LANSINK (1964).

5.3. THE AMOUNT OF NUCLEOPROTEIN PARTICLES IN APHIDS

To determine the amount of nucleoprotein particles which occur in the aphid 0.21 grams aphids were collected with an aspirator. The nucleoprotein particles were then extracted in 36 ml of 0.001 M phosphate buffer pH 7.2 with the procedure as described in 5.1. In this particular case the low speed centrifugations were not carried out at 25,000 *g* for 15 min, but at 10,000 *g* for 15 min in order to minimize the precipitation of nucleoprotein particles in low speed pellets.

Using the value $E_{1\%\text{-}258}^{1\,cm} = 127$ we calculated that 1 gram of aphids contained at least 20 mg of nucleoprotein particles. This means that 2% of a fresh aphid or 8% of its dry weight consists of nucleoprotein particles. The dry weight of an aphid was found to be 24% of its fresh weight.

The sample contained aphids in all stages of development. It was not determined whether aphids in one stage of development contain more nucleoprotein particles than in another. The sample reflects the situation in a growing colony of aphids and may be considered as a bulk-sample. Therefore, it may be assumed that the nucleoprotein particles content of any other sample from a growing colony will also be about two per cent of their fresh weight.

5.4. Influence of magnesium ions on the nucleoprotein particles

The nucleoprotein particles of aphids, extracted in 0.001 M phosphate buffer pH 7.2 had one main peak in the analytical centrifuge with a sedimentation coefficient $S_0^{20} = 78$ (Fig. 4A). They had also four small peaks representing components with sedimentation coefficients of 115, 60, 45 and 20 *S* respectively (Fig. 4A). From the similarity in the sedimentation coefficients of ribosomes (Van Kammen, 1963; Peterman, 1964) with those obtained in the nucleoprotein preparations of aphids, we may conclude that these preparations consist mainly of ribosomes.

The effect of magnesium ions on the ribosomes was examined by analytical centrifugation of aliquots of ribosome preparations which were extracted in 0.001 M phosphate buffer pH 7.2 and dialyzed in the same buffer to which various amounts of magnesium ions were added. A concentration of 0.1 mM magnesium had a slight influence on the sedimentation diagram. It caused a small decrease in concentration of the 45 *S*, 60 *S* and 78 *S* components, and a similar small increase of the 115 *S* peak as can be seen by comparing Fig. 4B

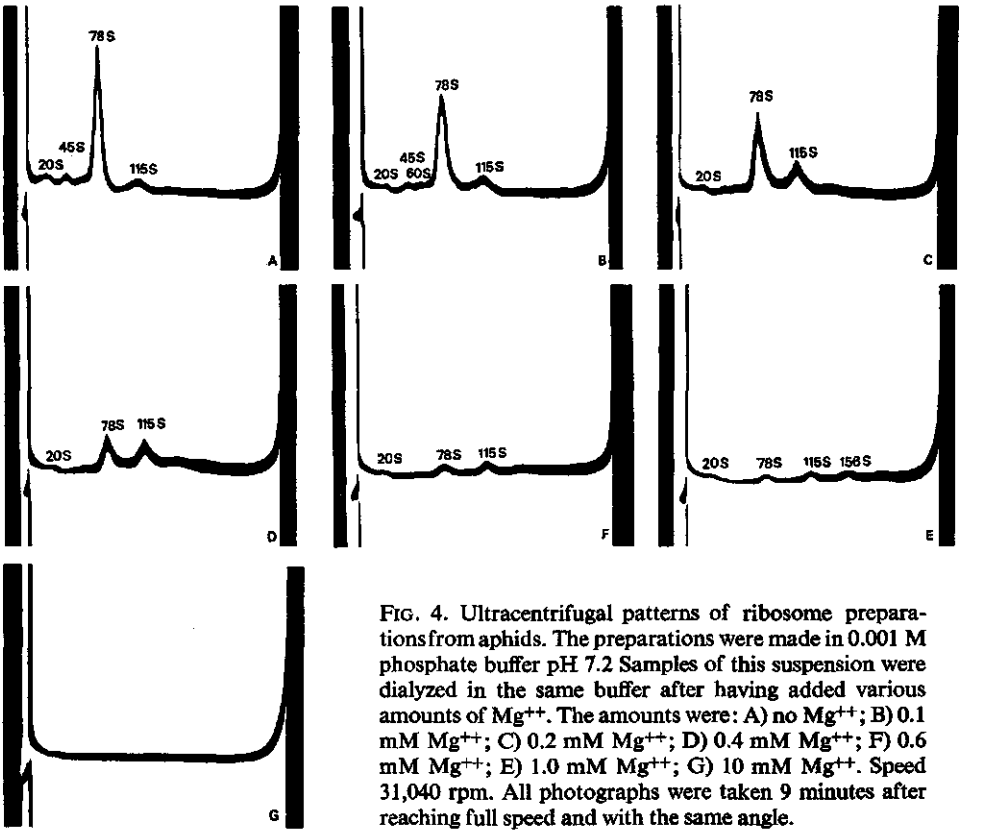


Fig. 4. Ultracentrifugal patterns of ribosome preparations from aphids. The preparations were made in 0.001 M phosphate buffer pH 7.2 Samples of this suspension were dialyzed in the same buffer after having added various amounts of Mg^{++}. The amounts were: A) no Mg^{++}; B) 0.1 mM Mg^{++}; C) 0.2 mM Mg^{++}; D) 0.4 mM Mg^{++}; F) 0.6 mM Mg^{++}; E) 1.0 mM Mg^{++}; G) 10 mM Mg^{++}. Speed 31,040 rpm. All photographs were taken 9 minutes after reaching full speed and with the same angle.

with Fig. 4A. At a concentration of 0.2 mM magnesium ions the respective decrease and increase was greater (Fig. 4C). At a concentration of 0.4–1.0 mM both the 78 *S* and the 115 *S* peak become gradually smaller, whereas the 45 *S* and the 60 *S* peaks disappeared (Fig. 4D, E, and F). A complete aggregation of the aphid ribosomes occurred in the presence of 10 mM magnesium. From the disappearance of the peaks in the sedimentation diagrams (Fig. 4G) it may be concluded that there was some aggregation of the ribosomes at magnesium concentrations of 0.1 mM and that the aggregation was complete at 10 mM.

The size of the peak formed by the 20 *S* component was not altered by changing the magnesium concentration.

The sedimentation patterns were similar whether the ribosomes were extracted in 0.001 M or 0.002 M potassium phosphate buffer (4:1) pH 7.6, or in 0.01 M tris-HCl pH 7.2. When the ribosomes were extracted with 0.01 M phosphate buffer pH 7.2 the peak of the 60 *S* component was larger than when 0.001 M phosphate buffer was used.

5.5. Discussion

Ribosomes have been extracted from different organisms and tissues, and one must assume that they are of general occurrence. The literature on ribosomes has recently been summarized in an extensive review by Peterman (1964). Isolated ribosomes of *Drosophila* larvae (the species name was not given) were used by Langridge (1963) in X-ray studies on the structure of ribosomes from various sources. Other detailed studies on the ribosomes of insects are not available as yet. Therefore, it is not possible to compare the properties of ribosome preparations from aphids with those of other insects. As the presence of magnesium seems to be unnecessarily for the integrity of the aphid ribosomes, it would be interesting to determine whether this also holds for the ribosomes of other insects.

The biochemically functional unit of ribosome preparations has sedimentation coefficients of 70 and 80 *S* depending on the source. They require different levels of magnesium ions to preserve their integrity. The 70 and 80 *S* ribosomes of plants which are restricted to chloroplasts and cytoplasm, respectively, also differ in their magnesium requirements (Brouwer & Van Kammen, 1967). Accordingly, Hsiao (1964) divided ribosomes in three groups, viz. a high magnesium group represented by ribosomes from *Escherichia coli* (Tissieres *et al.*, 1959) requiring 5–10 mM magnesium to preserve the main component; a medium magnesium group including ribosomes from yeast (Chao, 1957), and from tobacco leaves (Van Kammen, 1963) whose main components are stable in 1–2 mM; a third group represented by ribosomes from rat liver (Hamilton & Peterman, 1959) and from root tips of *Zea mays* (Hsiao, 1964) which are stable for short periods in buffers without magnesium and for at least several days in 0.5 mM magnesium. The ribosomes of the last group form aggregates in 2–5 mM magnesium. The ribosomes from root tips of maize can be extracted in low-ionic media in the absence of divalent cations and remain stable when

the sample is dialyzed for 12 hours. Magnesium concentrations of 0.5–1.0 mM have little effect on these particles. Like the ribosomes of corn, the aphid ribosomes can be extracted in media with low molarities of salts and in the absence of magnesium. Further similarities between the aphid ribosomes and those of the third group with respect to their behaviour in low magnesium concentrations were not found. The addition of 0.2–0.4 mM magnesium decreased the amount of the 80 *S* component in favour of the 115 *S* component, while the smaller components disappeared completely. It may be concluded that contrary to the behaviour of ribosomes of corn even small amounts of magnesium promote the aggregation of the aphid ribosomes. Therefore, the ribosomes of aphids do not seem to fit into one of the groups mentioned above.

The property of the ribosomes to associate in aggregates, which can be precipitated at low centrifugal forces, may permit separation of PLRV from ribosomes. This would be very useful if magnesium ions did not adversely effect the infectivity of PLRV or precipitate the virus. Therefore, a number of experiments were performed to study the precipitation of the ribosomes by magnesium ions during purification of the virus (see 6.8).

The occurrence of symbionts in aphids is a well established fact (BUCHNER, 1952). These symbionts are present in so-called mycetomes located in the abdominal cavity. The symbionts are described as yeast- and bacterium-like organisms. In view of the dimensions of the mycetomes it may be assumed that some part of the ribosomal content of the aphid will be derived from the symbionts. Because magnesium is necessary to preserve the integrity of ribosomes from yeast and bacteria it is doubtful whether the ribosomes of the symbionts are extracted simultaneously with the ribosomes from aphids. If the ribosomes of the symbionts are extractable without magnesium, however, the estimated ribosome content would be the sum of the ribosomes of the aphids and symbionts. If, on the other hand, the ribosome extraction of the symbionts must be done in the presence of magnesium, a larger part may be lost in the extraction of the aphid ribosomes.

From the fact that the RNA content amounts to about 50% of the ribosomes (PETERMAN, 1964), the RNA content of apterous aphids of *M. persicae* would be at least 1% of the fresh weight. This is more than has been reported for the alates of *Brevicoryne brassicae* L. LAMB (1964) found a content of 0.72% for these aphids.

CHAPTER 6

PRELIMINARY STUDIES ON THE PURIFICATION OF POTATO LEAFROLL VIRUS FROM APHIDS

As discussed in Chapter 3, leaf material is not the most favourable source for the purification of PLRV. On the other hand, aphids may be a more suitable source because infectivity can easily be found in both crude and clarified fractions from aphid homogenates. However, the purification of the virus from this source is severely handicapped because the concentration of the virus in the homogenates is probably low. In addition, large amounts of non-viral constituents, presumably ribosomes, which interfere strongly with the virus in the various purification procedures, are present. Differences were not observed between the homogenates from PLRV-free and viruliferous aphids by analytical centrifugation and spectrophotometry. Hence the fractionation and purification of PLRV can not be followed by any of these techniques. Also the effectiveness of the treatments could not be determined by electron microscopy because it was only possible to observe particles characteristic for PLRV-carrying aphids in highly purified preparations. A progress in the purification could also not be evaluated by means of measuring the specific virus activity, because the estimates of the virus concentrations made by injecting aphids are too inaccurate for that purpose. Therefore, a quantitative appraisal of a procedure in the purification of PLRV could hardly be made by the assay methods available.

In this chapter a number of methods will be discussed in order to estimate their effectiveness in the removal of impurities and their influence on the infectivity of the virus. The methods include mainly those that have been applied successfully to the purification of other plant and animal viruses. The purification of the virus was extensively studied by means of precipitation techniques such as iso-electric precipitation, ammonium sulphate saturation, precipitation of ribosomes by magnesium ions, and polyethylene glycol precipitation; with partition of the virus suspensions in different phase systems; and with centrifugation techniques such as differential centrifugation and density gradient centrifugation on both sucrose and CsCl columns. The problems encountered with the purification of PLRV from aphids may be clarified from the results obtained with the methods studied. Results of techniques such as the precipitation of the non-viral constituents by their antibodies, by heat treatment and by protamine sulphate; adsorption of virus to barium sulphate; filtration over Sephadex and agar columns, will be omitted because they did not help to solve the problem of the purification of PLRV from aphids.

The success of each purification step in the removal of impurities was evaluated by comparing the size and the colour of the high speed pellets and by measuring the optical density of suspensions at 260 mμ. Portions of certain fractions were injected into aphids in order to determine the effect of the purifi-

cation steps on the infectivity and to follow the distribution of the virus in the several fractions obtained. Even though elucidating results were not expected from these tests, several repetitions of each treatment were made in order to obtain a fair estimate of the effects on infectivity.

6.1. The distribution of the virus in fractions from aphid homogenates

In most of the experiments in which the purification of PLRV was studied the first step was a low speed centrifugation of the aphid homogenate at 1000 *g* or 10,000 *g* for 10 min to remove debris. This caused a partition of the homogenate into a pellet, a supernatant, and a layer of fatty material floating on the supernatant. The distribution of the virus in these fractions was studied.

The presence of PLRV in the fatty material was determined in a layer tightly packed by centrifugation. Such layers, containing very little debris, were made by centrifuging homogenates obtained from 200–400 mg aphids in 0.01 M phosphate buffer pH 7.2 at 10,000 *g* for 10 min. The fatty layer was removed from the supernatant, resuspended in buffer by means of a Potter-Elvejhem homogenization tube, and was formed again by centrifugation. Traces of aphid debris were removed from the layer by this treatment. The new layer was suspended in a mixture of equal amounts of chloroform and phosphate buffer, and shaken for 5 min. It can be assumed that the virus, if it was adhering to the fatty substance in the layer, would be suspended by the emulsification and be transferred to the water phase upon centrifugation. The aqueous phase was centrifuged at 100,000 *g* for 90 min. A slight precipitate was formed, which proved to be non-infectious. Virus particles were not observed in this fraction by electron microscopy. It is evident that no virus or only a negligible amount is adsorbed onto the substances in the floating layer.

However, some infectivity could be demonstrated in these extracts, when the debris was not removed from the fat-layers. This infectivity may be attributed to PLRV which is bound to the debris in detectable quantities as will be shown below.

Infectious material always occurred both in the pellet and in the supernatant. The supernatant was used in further experiments for purification of the virus.

Some attention was, however, paid to the presence of the virus in the pellet precipitated at low forces (1000 *g* or 10,000 *g* for 10 min). This pellet consisted of aphid debris. After decantation of the supernatant and the floating layer of fatty material the pellet was washed by suspension in a buffer as indicated in Table 4. The suspension was left for 20 to 30 min. The crude material was then precipitated at 10,000 *g* for 10 min. The new pellet was washed again with the same or, occasionally, another buffer. The supernatants were centrifuged at 100,000 *g* for 90 min to concentrate the virus into a pellet. These pellets which became gradually smaller in the subsequent washings, were dissolved in 0.2 ml ml phosphate buffer. After clarification at 1000 *g* for 10 min the resulting suspensions were assayed for infectivity by injecting aphids. The results of these

TABLE 4. Infectivity obtained in consecutive washings of crude debris of an aphid macerate.

Exp.	Quantity of aphids (mg)	Infectivity [5] obtained in the			
		first washing	second washing	third washing	fourth washing
1	300	3/10[1]	1/10[1]	–	–
2	2700	5/10[1]	8/10[1]	5/8[2]	2/10[2]
3	1500	–[3]	8/10[3]	7/9[3]	–
4	2000	2/9[1]	2/10[1]	4/10[1]	2/10[1]
4	rewashed pellets[4]	3/10[1]	4/10[1]	2/10[1]	–

[1] The debris was washed in 0.01 M phosphate buffer pH 7.2
[2] The debris was washed in 0.5 M phosphate buffer pH 7.8
[3] The debris was washed in 0.001 M phosphate buffer pH 7.2.
[4] Before the inocula of the fourth experiment were injected into aphids they were clarified at 1000 *g* for 10 min. The pellets were washed and also tested for infectivity.
[5] Numerator: number of test plants infected. Denominator: total number of test plants used.

experiments presented in Table 4. show that infectious material can be extracted from the debris even after several washings. Thus, PLRV appears to adsorb strongly to other substances. This was confirmed in the following experiment. The pellets obtained in the clarification at 1000 *g* for 10 min in the fourth experiment were suspended again in 0.2 ml 0.01 M phosphate pH 7.2. The suspensions were clarified at 1000 *g* for 10 min and the resulting supernatants tested for infectivity. They were almost as infectious as the former supernatants (Table 4, exp. 4).

It appears that PLRV is precipitated for a large part with the debris at low speed centrifugation. Furthermore, after resuspending the precipitate obtained after a high speed centrifugation of a virus suspension, virus was again lost when the resuspended material was subjected to low speed centrifugation. Thus, it may be concluded that considerable amounts of virus will be lost with the removal of the impurities from the virus suspensions.

The tendency of the virus to precipitate with other material was observed throughout the entire study and was a serious handicap for a quantitative purification of the virus.

6.2. THE EXTRACTION MEDIA

In the purification of viruses a large number of solvents are employed to promote the extraction and to preserve the infectivity of the virus. Different solvents can be used for one virus, whereas others require solvents of distinct composition (BRAKKE, 1956). A solvent had to be selected for PLRV which would prevent aggregation and allow the activity of the virus to be retained for long periods.

Few solvents have been employed for the extraction of PLRV from plant and aphid homogenates. DAY & ZAITLIN (1959) extracted some infectious material

from diseased *Physalis floridana* plants in 0.10 M phosphate buffer pH 7.0. Their final extract was resuspended in M/90 phosphate buffer pH 7.0. The virus retained its infectivity for at least 24 hours. However, DAY (personal comm.) preferred using 0.5 M potassium phosphate buffer pH 7.8 in later extractions of PLRV from aphids. MURAYAMA & KOJIMA (1965) used 0.03 M phosphate buffer pH 7.0 to extract the virus from plants and aphids. A 0.85% sodium chloride solution was applied by HARRISON (1958) to macerate aphids.

In this study infectious fractions could be obtained by using different solvents. Most of the experiments were carried out in 0.01 M potassium phosphate buffer pH 7.2. Similar infectivity results were obtained with 0.5 M potassium phosphate buffer pH 7.8, and with 0.001 M potassium phosphate buffer pH 7.2 containing 0.0002 M magnesium acetate. It is difficult to determine which of the three media was most suitable for extraction of the virus from aphids. However, aphid macerates in 0.5 M phosphate buffer pH 7.8 are jelly-like which was considered a disadvantage because of the tendency of the virus to become adsorbed to non-viral substances.

To separate the virus from ribosomes, a number of experiments were performed with the 0.001 M phosphate buffer. Ribosomes of aphids are stable and homogeneous in this solvent (Chapter 5).

Borate buffers pH 8.0 and pH 9.0 were as useful as phosphate buffers in extracting virus infectivity from aphid macerates. These buffers were made by mixing a solution containing 0.2 M boric acid and 0.05 M sodium chloride with a solution of 0.05 M borax up to the required pH. Infectivities of extracts in these buffers were compared with similar extracts in 0.01 M phosphate buffer (Table 5).

TABLE 5. Infectivity of extracts made from viruliferous aphids in phosphate and borate buffers[1].

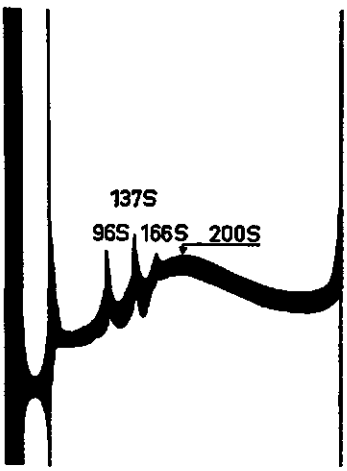
Medium	Infectivity[2]	
	Exp 1	Exp 2
0.01 M phosphate buffer pH 7.2	6/10	8/10
Borate buffer pH 8.0	4/10	11/11
Borate buffer pH 9.0	2/10	9/10

[1] Portions of 100 mg of viruliferous aphids were triturated each in one of the indicated buffers. The macerates were spun at 1000 *g* for 10 min and at 100,000 *g* for 70 min. Each final pellet was resuspended in its original buffer and tested for infectivity by injecting small volumes into healthy aphids.

[2] Numerator: number of test plants infected. Denominator: total number of test plants used.

CHAO (1957) disintegrated ribosomes from yeasts in a medium consisting of 0.002 M potassium carbonate, 0.002 M potassium bicarbonate and 0.001 M magnesium sulphate, pH 10. In one experiment a test was made to determine whether PLRV could retain its infectivity at this high pH value. About 200 mg

FIG. 5. Sedimentation diagram of an aphid extract prepared in a solution of 0.002 M potassium carbonate, 0.002 M potassium bicarbonate, and 0.001 M magnesium sulphate pH 10. The extract was made from 1.8 g of aphids by two cycles of differential centrifugation. The last high speed pellet was resuspended in the carbonate buffer and examined in the analytical centrifuge at 33,450 rpm. Photograph has been taken 5 min after the rotor reached the full speed



of viruliferous aphids were ground in 10 ml of this medium. The macerate was centrifuged at 10,000 *g* for 10 min and at 100,000 *g* for two hours. The final pellet was dissolved in 0.2 ml of this buffer and assayed for infectivity. Four out of ten plants became infected. It was demonstrated that this medium was not superior to the phosphate buffers in removing impurities from the homogenates. A large wide peak with a sedimentation coefficient of 200 *S*, and two small peaks of 137 *S* and 96 *S* were found in the sedimentation diagram of this extract (Fig. 5). A similar extract in 0.01 M phosphate buffer pH 7.2 consisted of material with an *S* value of 84 as had been discussed in Chapter 5. This aggregation in 0.002 M potassium carbonate is not sufficient to cause a quantitative separation of the ribosomes and the virus.

A chelating agent such as ethylenediaminetetra-acetic acid (EDTA) is known to cause the splitting of ribosomes. The 80 *S* particles from tobacco leaves split into components with sedimentation coefficients of about 20 *S* and 30 *S* (VAN KAMMEN, 1963). The ribosomes of aphids splitted up into particles with a sedimentation coefficient of about 40 *S* upon dialyzing overnight in 0.01 M EDTA pH 7.0.

The extraction of the virus in 0.01 M EDTA did not affect its infectivity. Experiments were not performed to test whether this splitting up of the ribosomes with EDTA had a favourable effect on the removal of the impurities from the virus suspension. Considering that the disintegrated ribosomes remain in the virus suspension it was assumed that addition of EDTA would not be helpful in the purification of PLRV.

A mixture of equal volumes of 0.02 M tris and 0.02 M EDTA adjusted to pH 7.2, was used by REES & SHORT (1965) to desaggregate or to avoid aggregation of a strain of tobacco mosaic virus. In search for solvents that could reduce the adsorption of PLRV to other substances in the aphid homogenate the influence of the tris-EDTA mixture on the infectivity was studied in two experiments. In both cases the aphids were triturated in such a mixture adjusted to

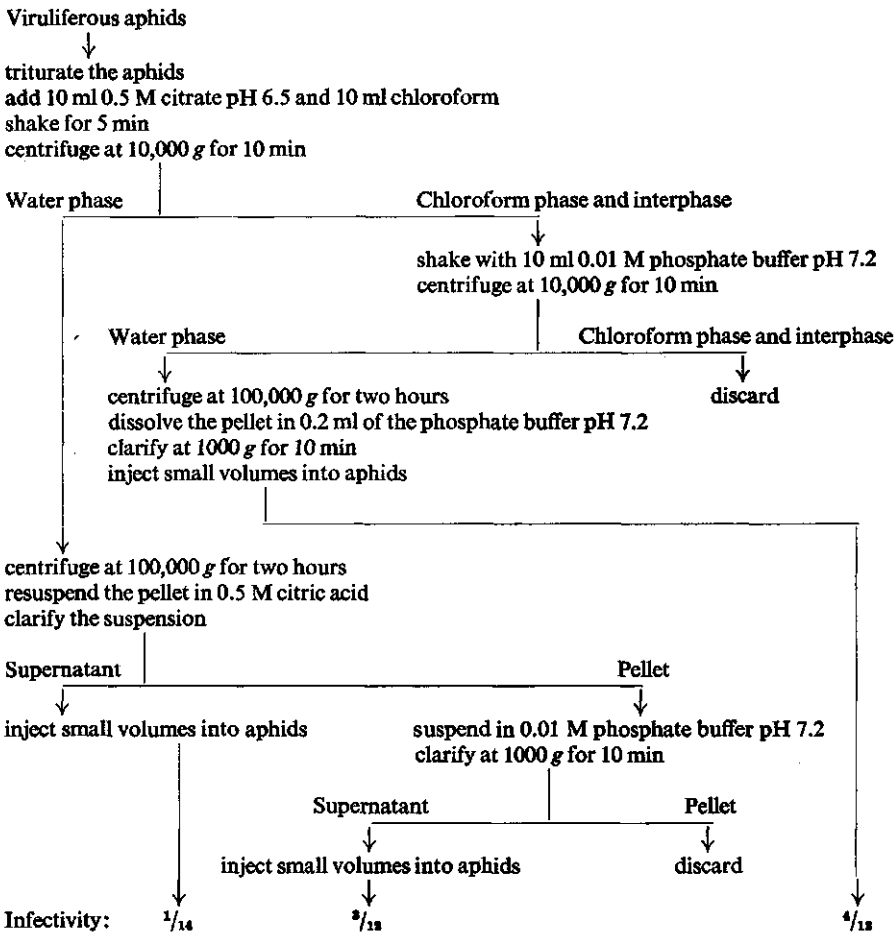
pH 7.2. The macerate was then emulsified with chloroform for 5 min. The aqueous phase obtained in the 10,000 *g* centrifugation was centrifuged at 100,000 *g* for 90 min. The pellet was suspended in 0.2 ml of the solvent and tested for infectivity by injecting 74 aphids. Out of the 37 plants which were infested with the injected aphids, only two became infected. Therefore, no further experiments were conducted with this mixture.

A buffered medium containing 0.1 M glycine and 0.01 M magnesium chloride, pH 6.1 (BRAKKE, 1956) protected the infectivity of potato yellow dwarf virus during its extraction. In the present work no infectivity could be demonstrated in extracts from viruliferous aphids using this solvent. The question whether the virus was precipitated by the low pH or by the high molarity of magnesium in the extract was not studied. Results obtained from studies on the behaviour of the virus at low pH values (section 6.5) or in solutions with a similar magnesium concentration (section 6.8) suggest that both factors may have caused the loss of the virus.

For the preservation of viruses it is often advisible to add some reducing agent or enzyme inhibitor to the extraction media. In a series of experiments the influence of 0.01 M ascorbic acid, 0.006 M mercaptoethanol, 0.01 M sodium sulphite, and 0.01 M sodium diethyl dithiocarbamate on the longevity of the virus was studied. These substances were included in 0.01 M phosphate buffer pH 7.2. Only the addition of mercaptoethanol increased the longevity of the virus as described in 4.2. In the early experiments this reducing agent was not included in the buffers.

SCOTT (1963) purified cucumber mosaic virus by homogenizing infected plant material with 0.5 M citrate buffer pH 6.5 and chloroform. Upon dialyzing the aqueous phase in 0.001 M borate buffer pH 9.0 it appeared that a much larger amount of virus could be extracted than with the earlier methods. Therefore, SCOTT's method was tried for the purification of PLRV. The behaviour of the virus in the chloroform and 0.5 M citrate buffer pH 6.5 treatment was investigated. About 500 mg of aphids were homogenized in 10 ml of the citrate buffer and 10 ml chloroform. The emulsion was broken at 10,000 *g* for 10 min. The water phase was pipetted off and centrifuged at 100,000 *g* for two hours. The pellet was then resuspended and assayed for infectivity after clarification at 1000 *g* for 10 min. The chloroform phase was washed with 0.01 M phosphate buffer pH 7.2 and assayed for infectivity. The whole procedure is outlined in Fig. 6. Washing of the chloroform phase and the interphase with the phosphate buffer yielded a preparation which infected 4 out of 12 inoculated plants, whereas the water phase provided an inoculum which infected 1 out of 14 plants. Before testing the latter inoculum it was clarified at 1000 *g* for 10 min (Fig. 6). The resulting pellet was washed in 0.2 ml phosphate buffer pH 7.2 and also tested for infectivity. Three out of 12 plants became infected using this inoculum. A parallel experiment using 0.01 M phosphate buffer pH 7.2 was made from a similar amount of viruliferous aphids. This inoculum infected 5 out of 12 plants. It may be concluded from these experiments that 0.5 M citrate buffer pH 6.5 precipitated more virus than 0.01 M phosphate buffer pH 7.2. Therefore, the ci-

FIG. 6. The extraction of potato leafroll virus from an aphid homogenate with 0.5 M citrate and the infectivity of some final fractions obtained.



trate buffer was not considered an appropriate solvent to extract PLRV from an aphid homogenate.

In efforts to feed the virus to aphids it was evident that the pellets obtained during the centrifugation of aphid macerates at 100,000 *g* for 90 min dissolved completely and rapidly in the offered diet. Therefore, it was of interest to study the value of the diet medium in the extraction of PLRV from certain precipitates. The diet medium was that used by AUCLAIR (1965). It contained about 4% amino acids, 18% sucrose and some vitamins. These substances were replaced in our extraction studies by 4% glycine adjusted to pH 7.2. Highly infectious preparations could be obtained with this solvent. It had the disadvantage, however, of dissolving more impurities than other solvents.

It has been shown that several solvents can be used in the purification of PLRV.

No conclusion can be made as to which of the solvents was most suitable for purifying the virus. This will be dependent on the eventual purification procedure selected. Moreover, the choice of the extraction media might be greatly facilitated if the virus concentration could be measured in a more accurate way.

6.3. Differential centrifugation

Virus suspensions can be clarified and concentrated by a process of alternate cycles of low and high speed centrifugations (Knight, 1963). The cell debris is removed at low speed, while the virus can be collected in a pellet by a high speed centrifugation. The virus pellet can now be dissolved in the required medium. Some material consisting of denatured and undissolved proteins, etc., can then be removed with a short centrifugation at low gravitational fields, and the virus in the supernatant can be pelleted again with a subsequent high speed centrifugation. Tobacco mosaic virus has been purified with 4 or 5 cycles of low and high speed runs (Knight, 1963).

The efficiency of this procedure was examined in the purification of PLRV from crude macerates. In one experiment 2 grams of aphids were ground in 12 ml 0.005 M K_2HPO_4-KH_2PO_4 (4:1) pH 7.4, containing 0.0005 M magnesium acetate. This magnesium concentration caused a partial aggregation of the aphid ribosomes as shown in Chapter 5. The macerate was subjected to four cycles of low (5000 *g* for 10 min) and high speed (100,000 *g* for 70 min) centrifugation. Much aphid material was removed in the first two cycles, whereas only a little material was removed in the last two cycles. The final pellet, which still contained considerable amounts of aphid material was assayed for infectivity by injecting small volumes of the suspended pellet into aphids. Twelve out of fifteen plants became infected by these aphids.

In another experiment 850 mg of viruliferous aphids were treated in a similar way. After the fourth high speed run the pellet was dissolved and stored at–18 °C overnight. After thawing, it was again centrifuged at 5000 *g* for 10 min and at 100,000 *g* for 70 min. In this low speed run a much larger pellet was obtained, but the high speed pellet still contained considerable amounts of material as could be seen from the size of the pellet. On testing this pellet for infectivity, only twenty per cent of the inoculated plants became infected.

It appeared from these experiments that differential centrifugation did not remove sufficient impurities from the aphid macerate to play an important role in the purification process of PLRV.

In some experiments it was estimated how long the homogenates had to be centrifuged to precipitate the virus quantitatively in the pellet. The aphid homogenates in 0.01 M buffer pH 7.2 were first clarified at 5000 *g* for 10 min, and divided into equal portions. Each aliquot was centrifuged at 100,000 *g* for different periods as indicated in Table 6. To test whether some virus activity could be demonstrated in the supernatants, they were carefully poured into other tubes and again centrifuged at the same speed but for a longer period. From the data given in Table 6 it appears that no infectivity remained in the

TABLE 6. Estimation of the time required to sediment quantitatively potato leafroll virus from a homogenate of viruliferous aphids at 100,000*g*.

Number of experiment	Duration of the first centrifugation	Duration of the second centrifugation	Infectivity assay[1]	Weight of the aphids (mg)
1a	55	80	7/12	1150
1b	60	80	3/11	
1c	65	80	2/11	
2a	75	120	0/10	2100
2b	85	120	0/10	
2c	95	120	0/10	
3a	60	80	1/10	1300
3b	65	80	0/10	
3c	70	80	0/10	
4a	60	120	2/10	400
4b	70	120	0/10	

[1] Numerator: number of test plants infected. Denominator: total number of test plants used.

supernatant fraction of homogenates after being centrifuged at 100,000 *g* for 70 min.

6.4. PRECIPITATION OF THE VIRUS WITH AMMONIUM SULPHATE

Proteins and viruses can be precipitated with ammonium sulphate. Tobacco mosaic virus was purified with this technique by STANLEY (1935). Since then, several other viruses have been partially purified and concentrated with precipitation by ammonium sulphate (BAWDEN, 1950; STEERE, 1959; THUNG, 1949). Complete precipitation of the viruses may usually be obtained by addition of 25 to 30 g ammonium sulphate to 100 ml of the virus solution. A remarkable effect of differences in concentration of ammonium sulphate on potato yellow dwarf virus was demonstrated by WHITCOMB (1965). This virus remained in solution after an equilibration overnight in 29% but not in 33% saturation.

The precipitation of PLRV by ammonium sulphate was studied in macerates in 0.01 M phosphate buffer pH 7.2 and clarified at 1000 *g* for 10 min. The ammonium sulphate concentration in the clarified homogenates was stepwise increased by the addition of proper amounts of a saturated salt solution. After the homogenates and the ammonium sulphate solutions were mixed thoroughly the mixture was left for 10 min. The flocculated material was collected at 1000 *g* for 10 min. The supernatant was then brought to a higher degree of saturation and treated as before. The pellets were dissolved in 0.2–0.4 ml 0.01 M phosphate buffer pH 7.2 and the suspensions were tested for infectivity by injecting aphids after they had been dialyzed against the buffer for two to four hours. The results are given in Table 7. PLRV started to precipitate at a concentration of 20% saturation. The amount of PLRV that remained in the supernatant after saturation to 40% was negligible.

The distribution of the virus over the several fractions was interpreted as

TABLE 7. Infectivity of the fractions obtained by partial ammonium saturation of aphid macerates.

Exp	Weight of aphids (mg)	Degree of ammonium sulphate saturation (percentages)	No. of aphids placed on the plants	No. of aphids alive on the plants after 24 hours	Infectivity[1]
		20	20	16	1/10
		30	21	11	0/11
1	175	40	20	18	5/10
		50	21	9	1/11
		20	20	17	4/10
		30	22	18	8/11
2	150	40	29	14	5/15
		50	22	13	0/11
		25	24	22	9/12
		33	24	20	8/12
3	170	40	22	16	4/11
		50	22	17	0/11

[1] Numerator: number of infected plants. Denominator: total number of test plants used.

follows. It might be that the presence of the virus in precipitates at low saturation degrees was due to its adsorption to the floccules formed under these conditions. At higher ammonium saturation levels the virus precipitated by being salted out.

Another explanation is that the mixture was not equilibrated sufficiently after the addition of the ammonium sulphate. The equilibrium period is usually extended for a longer period by most authors than the 10 min given to the mixtures in the experiments described above.

The effect of longer equilibrium periods was tested in an experiment in which the time was extended from 10 min to 3 hours. The results did not differ from those presented in Table 7. Therefore, it seems that longer equilibration is of minor importance.

Whilst discussing experiments in section 6.1 it was shown that the virus was easily adsorbed to cell debris and other components in the macerates. Thus floccules formed at low concentrations of ammonium sulphate could adsorb some of the virus. Thus, ammonium sulphate saturation did not appear suitable as a step in the purification.

Moreover, nearly all aphid constituents present in homogenates already centrifuged at 1000 *g* for 10 min were precipitated by the ammonium sulphate in the infectious fractions. This could be expected since most proteins are precipitated at the levels of ammonium sulphate saturation that were used. Therefore, a selective separation of the virus from the impurities can not be achieved with manipulation of ammonium sulphate concentrations. This technique may, however, be used to concentrate the virus from purified preparations.

6.5. Precipitation of PLRV by lowering of the pH

Sometimes viruses are partially purified by removing proteins from virus suspensions or by precipitating virus from its suspensions by acidification. To test whether acidification can facilitate the purification of PLRV a number of experiments were performed.

Aphid homogenates in 0.01 M phosphate buffer pH 7.2 were equally divided. Each aliquot was lowered to one of the chosen pH values by addition of 1 N acetic acid. After 10 min the floccules were collected by a low speed centrifugation. The precipitates were dissolved in 0.2 ml 0.01 M phosphate buffer pH 7.2 and assayed for infectivity. All but one of the fractions were infectious (Fig. 7).

The impurities which can be pelleted by a high speed centrifugation at 100,000 *g* for 70 min were nearly completely precipitated at pH values below 5.5. This was demonstrated by raising the pH of each aliquot to pH 7.2 after the floccules were removed. These aliquots were then centrifuged at 100,000 *g* for 70 min. Very small pellets were observed in the tubes with the aliquots which had been lowered to pH values below 5.5. Those brought to pH 5.5 and 6.0 formed somewhat larger pellets and were slightly infectious, whilst no infectivity could be demonstrated in the other aliquots.

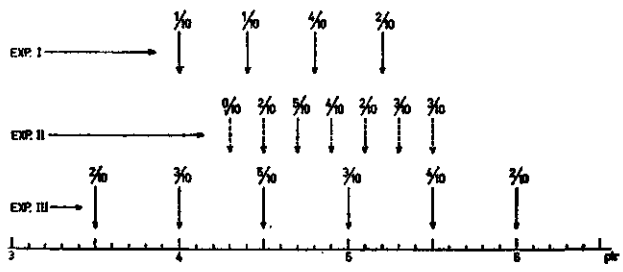


Fig. 7. The infectivity of precipitates obtained by lowering of the pH of aliquots of aphid homogenates. The arrows indicate the pH to which the aliquot was adjusted. The results of the infectivity tests are indicated. The numerator represents the number of plants infected and the denominator the number of test plants used.

The impurities which sediment at 100,000 *g* for 70 min could be precipitated at pH values below 5.5 which was verified by spectrophotometrical measurements. An aphid homogenate was subjected to two cycles of differential centrifugation, consisting of runs at 10,000 *g* and 100,000 *g* for 10 and 70 min respectively. The final pellet was dissolved and divided into a number of equal parts. Each part was adjusted to a certain pH value. After removal of the floccules at 10,000 *g* for 10 min the extinction of the supernatant at 260 mμ was measured. The influence of pH on the precipitation of the aphid material is presented in Fig. 8. A marked aggregation takes place below pH 5.5. The extinctions are also given of similarly treated aliquots of a macerate which was only clarified at 10,000 *g* for 10 min and not subjected to the differential centrifugation. The aliquots of this homogenate acidified to pH 4 and 3 have higher extinctions than the corresponding aliquots of the homogenate clarified by differential centrifugation. These higher extinctions are probably due to smaller constituents which are still pre-

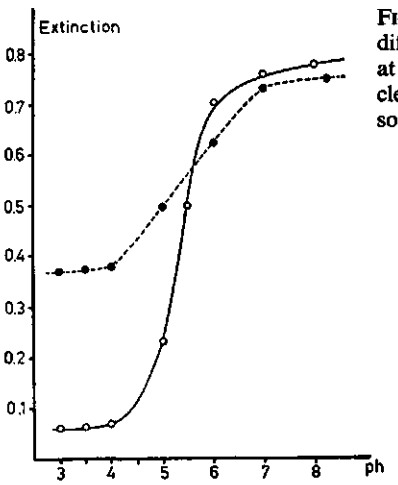


FIG. 8. Precipitation of normal host constituents at different pH values from aphid homogenates, clarified at 10,000 *g* for 10 min (● - - - ●), and clarified by two cycles of differential centrifugation (o——o). The absorbancy was measured at 260 mμ.

sent in these aliquots and which are removed with the high speed supernatants from the sample prepared by differential centrifugation.

From these experiments it was concluded that PLRV was easily precipitated on acidification, but also that nearly all the impurities were precipitated together with the virus. Therefore, a separation of the virus from the bulk of the material could not be achieved by acidification of aphid macerates.

This simultaneous precipitation may be due to a similarity in the iso-electric point of the virus and that of the majority of the impurities or to a salt-like combination between the virus and the impurities. Such salt-like combinations were studied by KLECZKOWSKI (1946). Tobacco mosaic virus was easily precipitated far from its iso-electric point after addition of ribonuclease, clupeine and globuline. These precipitates could partly be dissolved by adding moderate concentrations of salts, e.g. 2% sodium chloride.

The addition of 2% sodium chloride to aphid macerates had, however, apparently no effect on the precipitation behaviour of the aphid constituents and PLRV by lowering of the pH. The material which was precipitated at pH 5.0 was as infectious as the precipitate of a macerate to which no sodium chloride was added. It was not tested whether the supernatant with 2% sodium chloride after adjustment to pH 5.0 was infectious.

From these experiments it appeared that on acidification of the homogenate the bulk of the aphid constituents flocculated together with the virus. Whether the precipitation of PLRV was due to its own iso-electric point or to an adsorption of the virus to the flocculating impurities could not be determined. It was conceivable, however, that a partial removal of some impurities would diminish adsorption of the virus to any remaining impurities. This was investigated in the following experiments.

Aphid homogenates were made in borate buffers, pH 8.5 and were clarified as follows. The debris was removed from the homogenate with a centrifugation at 10,000 *g* for 10 min. The supernatant was emulsified with chloroform for 5 min

and the emulsion was broken at 10,000 *g* for 5 min. The water phase was then centrifuged at 100,000 *g* for 80 min. The pellet was suspended in 10 ml of the same borate buffer and the suspension was clarified at 10,000 *g* for 10 min. The pellet fraction was washed in 0.2 ml of the borate buffer and tested for infectivity. In order to remove as much impurities as possible from the supernatant it was acidified in several steps to successive pH values as indicated in Table 8. After each acidification step the flocculated material was collected into a pellet at low centrifugal forces. A brown-greenish pellet was formed at pH 6.5. The pellets obtained at lower pH values were transparent and became successively smaller. These pellets were dissolved in 0.2 ml of the borate buffer and assayed for infectivity by injecting small parts into the aphids. The results of these experiments are listed in Table 8. Most of the fractions were infectious.

TABLE 8. Infectivity of fractions obtained after a stepwise lowering of the pH of partially clarified homogenates of viruliferous aphids.

Exp	Weight of aphids (mg)	Infectivity[1]					
		pH 8.5	pH 6.5	pH 6.0	pH 5.5	pH 5.0	pH 4.0
1	700	2/10	–[2]	4/10	0/9	2/8	
2	700	4/10	6/10	6/10	8/10	3/10	
3	800	0/10	2/10	0/10	0/10	0/10	
4	700	1/10	0/10	0/10	–	1/10	
5	1100	–	2/10	2/10	1/10	–	
6	1000	–	2/10	1/10	7/10	5/10	
7	500						5/10

[1] Numerator: number of test plants infected. Denominator: total number of test plants used.
[2] – = this fraction was prepared, but not tested.

A negligible amount of aphid material was left in the supernatant after the pH was lowered to 5.0. This was determined by concentrating the suspensions by a high speed centrifugation after they had been adjusted to pH 7.0. A very small pellet was obtained which proved non-infectious in three experiments.

No specific structures could be discerned in the fractions obtained at pH 5.5 and pH 5.0, and in the concentrated pH 5.0 supernatant fraction when they were examined by electron microscopy.

Evidence has been put forward in these experiments that PLRV has been distributed over several fractions made by a stepwise acidification of a partially purified aphid homogenate. This distribution might be explained by assuming that the virus was adsorbed to the precipitating impurities. The retention of the virus in aphid debris and its distribution in several fractions obtained with ammonium sulphate saturation was explained by the same phenomenon (section 6.1 and 6.4, respectively).

The precipitation of the virus is not prevented by a partial removal of the impurities from the aphid homogenate. It was concluded that acidification of an aphid homogenate was not effective in the separation of the virus from the

impurities because the virus always precipitates together with the impurities. However, acidification can be useful in concentrating the virus or in changing the medium by precipitating the virus and resuspending it in another medium.

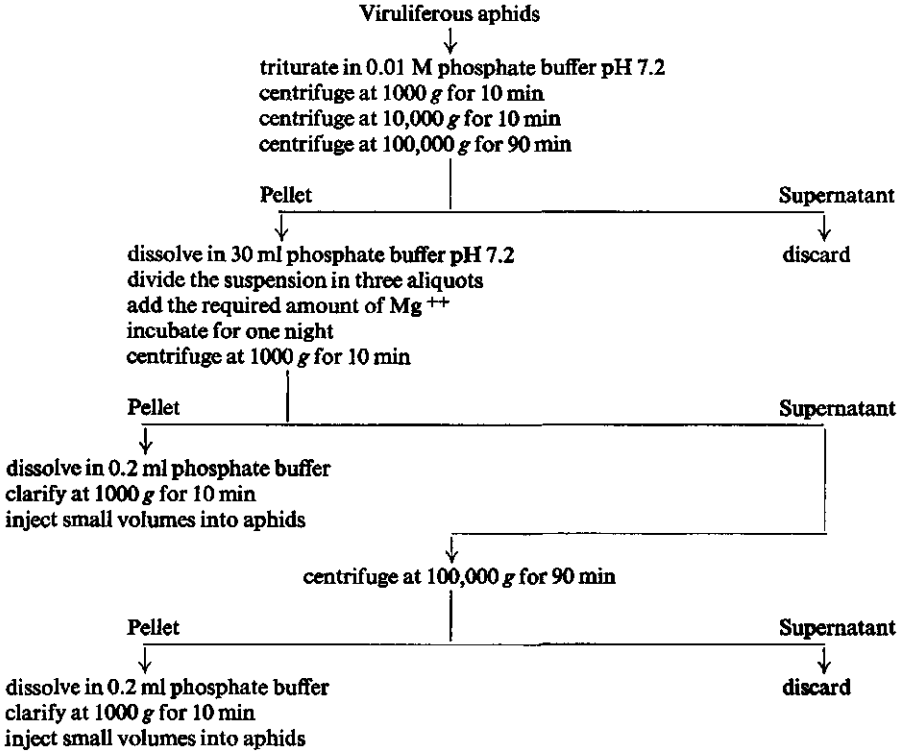
6.6. Precipitation of the ribosomes by magnesium ions

It was shown in Chapter 5 that aphid ribosomes were easily precipitated with moderate concentrations of magnesium ions. A concentration of 0.01 M magnesium caused the complete precipitation of all the ribosomes. Because it was relevant to the present study to determine whether magnesium could aid the separation of the virus from ribosomes, the following studies were made.

First of all it was necessary to investigate how the virus itself behaves in magnesium concentrations which cause the complete precipitation of the ribosomes. It was demonstrated in sections 1, 4 and 5 of this chapter that PLRV activity could be found in each precipitate that was made. Therefore, it was desirable to investigate whether the virus precipitated together with the ribosomes.

About one gram of viruliferous aphids were triturated in 10 ml of 0.01 M

Fig. 9. Extraction of the ribosomes from viruliferous aphids and the incubation of the ribosome suspension with varying amounts of Mg^{++}.



phosphate buffer pH 7.2 and treated as follows (Fig. 9). The debris were removed from the macerate at 1000 *g* for 10 min. After a centrifugation at 10,000 *g* for 10 min the supernatant was spun at 100,000 *g* for 90 min. The pellet was then resuspended in 24 ml of phosphate buffer. This suspension, referred to as the ribosome suspension, was divided into three equal parts. These aliquots were respectively incubated with 0.01 M, 0.033 M, 0.066 M magnesium overnight at 2°C. The flocculated material of each aliquot was then collected as a pellet by centrifuging at 1000 *g* for 10 min. The pellet and the supernatant will be referred to as the precipitate and the supernatant, respectively. The precipitates were dissolved in 0.2 ml of phosphate buffer. The suspensions were then clarified at 1000 *g* for 10 min and tested for infectivity by injecting small volumes into aphids. After incubations the supernatants were centrifuged at 100,000 *g* for 90 min to concentrate the virus suspension into a smaller volume. The small pellets were resuspended in 0.2 ml 0.01 M phosphate buffer pH 7.2 and also tested for infectivity. As can be seen in Table 9 infectivity was not destroyed by the magnesium. Some of the virus occurred in the precipitates, while some remained in the supernatant after incubation with magnesium. This distribution may have been due to partial adsorption of the virus on the precipitated ribosomes.

TABLE 9. Infectivity of fractions[1] derived from ribosome suspensions of viruliferous aphids which were incubated overnight at 2°C with different concentrations of Mg^{++}.

Concentration of Mg^{++} in the aliquots of the ribosome suspension	Infectivity[2]		Relative amount[3] of material present in concentrated supernatant (%)
	precipitate	supernatant	
0.01 M	0/10	1/10	16
0.033 M	5/10	4/10	4
0.066 M	4/10	2/10	4

[1] The material flocculated was collected at 1000 *g* for 10 min. The material in the supernatant fraction was concentrated at 100,000 *g* for 90 min. The ribosome suspension was extracted and suspended in 0.01 M phosphate buffer pH 7.2.

[2] Numerator: number of test plants infected. Denominator: number of test plants used.

[3] The amount of material in the resuspended pellet obtained after centrifugation of the supernatant fractions was determined spectrophotometrically. The extinction of the ribosome suspension before the addition of Mg^{++} is regarded as 100%.

The influence of lower phosphate concentrations on the co-precipitation of the virus along with the aggregated ribosomes was studied in three more experiments. The ribosomes were extracted and suspended in 0.001 M phosphate buffer pH 7.2. Infectivity occurred only in the precipitates and could not be recovered from the supernatants as shown in Table 10.

These results suggested that most of the PLRV present in ribosome preparations containing 0.001 M phosphate buffer were precipitated together with the ribosomes. The infectivities recorded in Table 10, however, are too low to make any definite conclusions on the absence of infectivity in the supernatants.

TABLE 10. Infectivity of fractions[1] derived from ribosome suspensions of viruliferous aphids which were incubated overnight at 2 °C with different amounts of Mg^{++}.

Concentration of Mg^{++} in the ribosome suspension	Infectivity[3]					
	Exp 1		Exp 2		Exp 3	
	P[2]	S[2]	P	S	P	S
0.01 M	3/10	0/10	–	–	0/10	0/10
0.033 M	0/10	0/10	2/10	0/10	1/10	0/10
0.066 M	2/10	0/10	1/10	0/10	3/10	0/10

[1] The material flocculated was collected at 1000 *g* for 10 min. The supernatant fraction was centrifuged at 100,000 for 90 min. The ribosomes were extracted and suspended in 0.001 M phosphate buffer pH 7.2.

[2] The pellet (P) and supernatant (S) fraction obtained at 1000 *g* for 10 min.

[3] Numerator: number of test plants infected. Denominator: total number of test plants used.

Comparing these results with those in Table 9 it was apparent that more infectivity could be recovered from supernatants in 0.01 M phosphate than those in 0.001 M phosphate. This might mean that PLRV is more soluble in 0.01 M phosphate buffer than in 0.001 M phosphate buffer when magnesium is included in these buffers. To make more conclusive statements on the solubility of the virus it would be necessary to repeat these experiments. The occurrence of virus in the aggregated ribosome fraction points again to the tendency of PLRV to precipitate along with other precipitating aphid constituents as demonstrated in sections 1, 4 and 5. Therefore, the virus can not be separated from the ribosomes by magnesium ions.

The optical densities at 260 mμ of the dissolved high speed pellets were compared with the optical density of the ribosome suspension. It appeared that much material was precipitated during the incubations with Mg^{++}. About 4% of the material precipitable at 100,000 *g* for 90 min, was not precipitated. This figure was higher for the sample in 0.01 M phosphate buffer which was incubated with 0.01 M magnesium ions. In this case, about 16% of the original material precipitable at 100,000 *g* for 90 min was recovered from the high speed pellet. This difference may probably be due to the different phosphate molarities used.

The absorption spectrum of the ribosome suspension is given in Fig. 10. As expected, the curve is typical for nucleoproteins. A quite different curve was obtained when the ribosomes were removed from the suspension (Fig. 10) as demonstrated by the curve, obtained from measurements on the dissolved high speed pellets. It may be assumed that this extract still contained some proteins and nucleic acids. However, this curve has no absorption maximum at 258 mμ (the maximum for nucleic acids) and 280 mμ (the maximum for proteins). It was not determined which substances other than proteins and nucleic acids played a role in determining the shape of this curve and whether proteins and nucleic acids had also contributed to it.

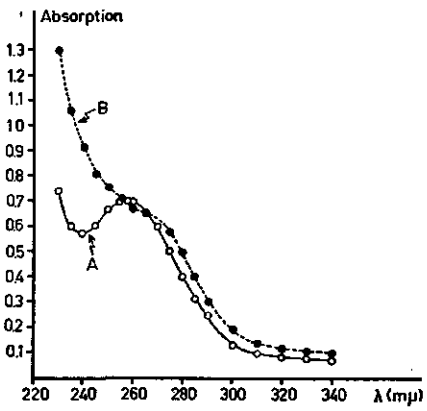


FIG 10. Ultraviolet absorption spectrum of the ribosome suspension before (A) and after (B) removal of the ribosomes by Mg^{++}.

6.7. EXTRACTION OF PLRV FROM APHID HOMOGENATES WITH ORGANIC SOLVENTS

Removal of contaminants from virus suspensions has often been achieved by the extraction of the suspensions with organic solvents. A chloroform extraction procedure was introduced by SCHNEIDER (1953) to clarify a suspension of tobacco mosaic virus. Water, Freon 112 and *n*-heptane was used by GESSLER *et al.* (1956) and by PORTER (1956) for the purification of viruses. STEERE (1956) used a mixture of equal parts of chloroform and *n*-butanol. These extraction procedures have the feature that the virus particles remain in the aqueous phase, while the impurities are transferred to the organic phase or are collected in the interphase. Actually, these phase systems act as selective denaturing agents, destroying the contaminants and leaving the particles under study intact. The number of empty capsids occurring in some virus preparations can be affected by some of these organic solvents (MARKHAM, 1959; VAN KAMMEN, 1967). In this study where the issue was the separation of PLRV from aphid components the effect of these phase systems on the infectivity of the virus and on the clarification of the virus suspension was studied.

Homogenates of viruliferous aphids, sometimes clarified by low speed centrifugation, were brought to 8 or 10 ml with 0.01 M phosphate buffer pH 7.2 and mixed with an equal part of chloroform. The mixture was shaken for 5 min and the emulsion broken at 10,000 *g* for 10 min. The water phase was consistently infectious upon its concentration by high speed centrifugation (100,000 *g*) or by salting out with ammonium sulphate. In two experiments the influence of the length of the emulsification time on the infectivity was studied. Two equal parts of a homogenate were shaken with chloroform for 5 and 15 min respectively. Even after an emulsification of 15 min PLRV does not lose its infectivity as can be seen in Table 11. However, these preparations were slightly less infectious than those emulsified for 5 min. A conclusive statement may not be made from two experiments, but the results do, however, suggest that contact of the virus with chloroform for longer periods destroys infectivity to some degree.

Appreciable amounts of contaminants were removed from the homogenate

TABLE 11. Influence of the period of emulsification with chloroform on the infectivity of potato leafroll virus in aphid macerates.

Weight of aphids (mg)	Infectivity[1] after emulsification for	
	5 min	15 min
300	7/10	4/10
450	3/10	2/10

[1] Numerator: number of test plants infected. Denominator: number of test plants used.

by the chloroform treatment. The optical density at 260 mμ was reduced by 17% in a homogenate which was initially clarified at 10,000 *g* for 10 min. The amount of material which sedimented at 100,000 *g* for 90 min was reduced by 50% in comparison with the corresponding fraction derived from an untreated aliquot. This means that material which can be sedimented at 100,000 *g* for 90 min is more affected by the chloroform than that remaining in the supernatant. Additional steps are required to obtain a pure virus preparation, for no specific particles could be observed by electron microscopy between the large amounts of amorphous debris on the grid.

Infectious preparations were also obtained when the virus suspensions were treated with a mixture of equal parts of butanol and chloroform. This system was shaken for 30 or 60 min. The treatment with this mixture diminished the infectivity more than emulsification with chloroform. Whether this was due to the organic solvents in this phase system or to the extended time of shaking was not studied.

Extraction of the virus with fluorocarbons also resulted in highly infectious fractions. The unclarified homogenates were mixed with Freon 113 for 2 or 3 min. As it was believed that identical results were obtained with chloroform emulsification, extraction of the virus with the fluorocarbon was not further used in the purification.

Another organic solvent-water phase system was developed for the purification and concentration of ECHO-virus type 7 by KITANO *et al.* (1961), who mixed the virus suspension with one vol. of a 2.5 M potassium phosphate solution pH 7.5 and 0.8 vol. of a mixture of organic solvents composed of 2 parts of 2-ethoxy-ethanol and 1 part of 2-butoxy-ethanol; after being gently mixed the system was separated in a top and bottom layer, while an interphase had been formed between them (Fig. 11). KITANO *et al.* showed that the bulk of the virus occurred in the interphase, while hardly any infectivity was found in the liquid phases.

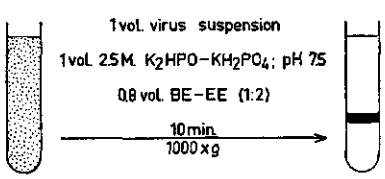


FIG. 11. Scheme for the partition of a virus suspension in a two phase system of butoxy-ethanol, ethoxy-ethanol, and 2.5 M potassium phosphate. Prior to the centrifugation the mixture is gently mixed.

On application of this procedure to an homogenate of viruliferous aphids infectious fractions were extracted from the interphase. The interphase which consists of a large amount of denaturated proteins, was suspended in 0.01 M phosphate buffer and subsequently centrifuged at 10,000 *g* for 10 min and at 100,000 *g* for 2 hours. These high speed pellets were transparent or slightly yellow. The level of their infectivity could be compared with those obtained after a chloroform emulsification of the aphid homogenates. It was demonstrated by spectrophotometrical studies that the butoxy-ethanol and ethoxy-ethanol phase system was suitable to eliminate more impurities than any other technique studied thus far. It was, however, necessary to include some additional steps to this method in order to purify successfully the virus from aphid homogenates.

In a few experiments the chloroform treatment was followed by the partition of the aqueous phase in the butoxy-ethanol and ethoxy-ethanol phase system. The resuspended interphase was clarified at 10,000 *g* for 10 min, concentrated at 100,000 *g* for 2 hours and subsequently resolved on a sucrose gradient at 90,000 *g* for 90 min. The distribution of the extracts in the gradient has been sketched in Fig. 12. When the aphids were triturated in 0.01 M phosphate buffer pH 7.2 two bands were observed at 35 and 38 mm from the meniscus of the gradient. These bands were absent in tubes with material from aphids ground in 0.5 M phosphate buffer pH 7.8. In both tubes a zone was found 10 mm beneath the meniscus. Furthermore, a faint light scattering was observed throughout each

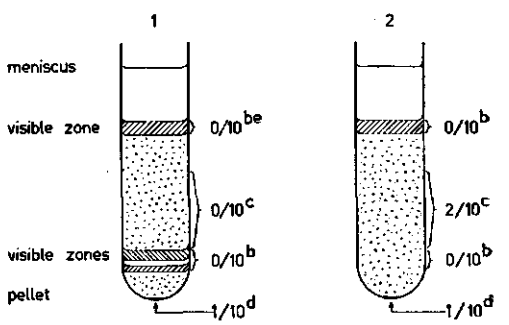


FIG. 12. Comparison of two extracts[a] from aphid macerates by density gradient centrifugation on sucrose gradients.

[a] Two samples of aphids (450 and 400 mg) were triturated in 0.01 M and 0.5 M phosphate buffer, respectively. The macerates were emulsified with chloroform. The waterphases were then partitioned with equal amounts of 2.5 M phosphate buffer pH 7.8 in a phase system of butoxy-ethanol and ethoxy-ethanol. The virus was extracted from the interphases with 0.01 M phosphate buffer pH 7.2, and successively centrifuged at 10,000 *g* for 10 min and 100,000 *g* for 120 min. The final pellet was dissolved in 0.5 ml of 0.01 M phosphate buffer and placed on top of a sucrose gradient. The tubes were centrifuged at 90,000 *g* for 90 min. The extract of the aphids macerated in 0.01 M phosphate buffer, was layered on gradient 1. The other extract on gradient 2.

[b] This fraction was withdrawn with a hypodermic syringe and tested for infectivity by injecting samples into aphids.

[c] This fraction was centrifuged at 100,000 *g* for 3 hours after its removal from the gradient. The pellet was dissolved in 0.2 ml 0.01 M phosphate buffer and tested for infectivity as described above.

[d] The pellet was dissolved in 0.2 ml 0.01 M phosphate buffer and tested for infectivity. Numerator: number of infected plants. Denominator: number of plants used.

tube. Some infectivity was found in the zone between 20 and 35 mm from the meniscus. The material in this zone was concentrated for testing the infectivity at 100,000 *g* for three hours. Virus particles in the infectious fractions could not be discerned by electron microscopy between the amorphous material on the specimen grid.

KITANO *et al.* (1961) reported that only 5% of the total protein was transferred to the interphase, while the remainder occurred in the bottom phase. The exact distribution of aphid substances over the bottom phase and the interphase was not measured but it may be assumed that most of this material was collected in the interphase. This can be considered a disadvantage because PLRV may be adsorbed to the substances occurring in the interphase. Thus it may be expected that some virus is lost with the impurities in the interphase. In one experiment it was demonstrated that some infectious material was still present in the interphase after the third extraction found.

Efforts to improve the efficiency of the butoxy-ethanol and ethoxy-ethanol phase system by a number of modifications did not favour the purification of the virus. In most of the modifications the system was altered from di-phasic to mono-phasic. Lowering the molarity of the phosphate solution resulted in a lowering of the position of the interphase in the tube. When 0.5 M potassium phosphate was added to the mixture instead of 2.5 M potassium phosphate, the bottom phase was lost and the interphase settled at the bottom of the tube as a pellet. In mixtures with this phosphate concentration or even lower ones a reasonable amount of impurities were disintegrated. Whether this happened to the same extent as in the first method used was not investigated. It can be assumed that the impurities which are transferred to the bottom phase at high concentrations of phosphate will also be present in the pellet if lower phosphate concentrations are used. Other modifications were made by replacing butoxy-ethanol with methoxy-ethanol or with mixtures of chloroform and methoxy-ethanol. Systems containing chloroform are di-phasic and those without chloroform are mono-phasic. Infectious fractions were obtained with each modification. No comparison of the relative infectivity of these fractions was made because the experiments were performed once only. None of the studied modifications removed more impurities than the original butoxy-ethanol and ethoxy-ethanol phase system.

The behaviour of tobacco mosaic virus was studied in butoxy-ethanol and ethoxy-ethanol systems in which the quantities of the solvents were varied. When the infectivity of the treated sample was compared with an untreated control, it appeared that such systems reduced the infectivity 10 to 50%. Deleterious effects of these systems on the infectivity of PLRV were not observed. Experiments were not designed to determine potentially harmful effects of the butoxy-ethanol and ethoxy-ethanol phase systems on the virus.

6.8. Fractionation of aphid homogenates with polyethylene glycol

Partition of virus suspensions in liquid phase systems of polyethylene glycol (PEG) with other polymers (Albertsson, 1960) and precipitation of viruses with PEG (Hebert, 1963; Lebermann, 1966; Van Kammen, 1967) are used to clarify and to concentrate virus suspensions. A number of plant viruses have been clarified and purified by stepwise resolution of PEG precipitates of virus suspensions on cellulose columns (Venekamp & Mosch, 1964 a and b; Venekamp *et al.*, 1964; Berg, 1964).

The partition of macromolecules in two-phase systems composed by aqueous solutions of high polymers were extensively studied by Albertsson (1960). With some of these systems the concentration and the purification of a number of animal viruses and one plant virus, viz. tobacco mosaic virus, was studied. It was not obvious from the results reported by Albertsson (1960) whether a phase system could be constructed in which the partition coefficients of ribosomes and small viruses are so different that a complete separation between these particles can be made. Moreover, the development of an efficient phase system is hampered considerably by the lack of an adequate quantitative assay method for PLRV. The fractionation of the aphid homogenate by distribution in high polymer phase systems was therefore not attempted.

6.8.1. *Precipitation of PLRV by polyethylene glycol*

Precipitation of certain proteins in low-ionic media by high amounts of PEG was reported by Albertsson (1960). The fractionation of PLRV under similar conditions was studied using a virus suspension which was partially purified in order to reduce the simultaneous precipitation of large amounts of impurities with the virus. About 1.7 grams of viruliferous aphids were triturated in a mortar. The homogenate was suspended in 12 ml 0.01 M phosphate buffer pH 7.2 and clarified at 1000 *g* and 10,000 *g* for 10 min each centrifugation. The supernatant was spun at 100,000 *g* for 75 min. The pellet was resuspended in 12 ml phosphate buffer and clarified at 10,000 *g* for 10 min. PEG was added to 3 ml portions of this supernatant as indicated in Table 12. Thirty min after dissolution of PEG the floccules were collected by centrifuging at 25,000 *g* for 10 min. The distribution of the virus in the precipitates and the supernatants was studied as follows. Each precipitate was dissolved in 0.25 ml 0.01 M phosphate buffer and tested by injecting aphids. The supernatants were made up to 12 ml with the buffer and centrifuged at 100,000 *g* for 90 min. The pellets were dissolved in 0.2 ml buffer and tested for infectivity by injecting aphids. Both the pellet and the supernatant of each treated portion was infectious (Table 12). It may be concluded that under these circumstances PEG does not quantitatively precipitate PLRV.

From Table 12 it can also be seen that by increasing the amount of PEG in the aliquots the infectivity in both fractions of each portion had decreased. A statement about the influence of PEG on the infectivity is not warranted because

TABLE 12. The distribution of PLRV in low-ionic virus suspensions on addition of different amounts of polyethylene glycol.

	Aliquots			
	1	2	3	4
Added amount of PEG in grams per 3 ml virus suspension	0.4	0.8	1.2	1.6
Infectivity of the floccules collected at 25,000 *g* for 10 min	6/10[1]	4/10	4/10	2/10
Infectivity of the supernatant obtained after centrifugation at 25,000 *g* followed by a centrifugation at 100,000 *g* for 90 min	9/10	5/10	4/10	2/10

[1] Numerator: number of infected plants. Denominator: number of inoculated plants.

this experiment has been performed once only.

HEBERT (1963) precipitated wheat mosaic virus from a suspension in 0.3 M sodium chloride with 2% PEG but higher concentrations of PEG were required to precipitate tobacco ringspot virus and bean pod mottle virus from suspensions in 0.3 M and 0.2 M NaCl.

An investigation was conducted to determine whether precipitation by PEG and NaCl could be a helpful clarification method. This time, the homogenate which was in 0.01 M phosphate buffer pH 7.2, was emulsified with chloroform for 5 min. Four per cent PEG was added to the water phase which was formed at 10,000 *g* for 10 min. NaCl was then added to portions of the water phase using concentrations as is indicated in Table 13. The mixtures were centrifuged at 10,000 *g* and 100,000 *g* for 15 min and 90 min respectively. Each pellet was resuspended in 0.2 ml of 0.01 M phosphate buffer and assayed for infectivity by injecting aphids. PLRV appeared to be precipitated with 4% PEG in 3% NaCl. This pellet contained about 38% of the total material originally present as determined by measuring the optical density at 260 mμ. Although a precipitation of PLRV with PEG will not be a powerful step in the purification of the virus, it might be of use as a first step in the clarification or to concentrate the virus in a suspension.

TABLE 13. Effect of NaCl concentration on the precipitation of potato leafroll virus from suspensions in 4% PEG.

	Aliquots			
	1	2	3	4
Concentration of NaCl in %	0	1	2	3
Infectivity of the flocculates	3/10[1]	5/10	2/10	4/10
Infectivity of the supernatants	1/10	3/10	2/10	0/10

[1] Numerator: number of infected plants. Denominator: number of inoculated plants.

6.8.2. *Fractionation of aphid homogenates on cellulose columns*

In their studies on the elimination of contaminating proteins from PEG precipitates of virus suspensions VENEKAMP & MOSCH (1964a; 1964b) developed a chromatographic procedure to separate the viruses from the impurities. To achieve a fractionation between PLRV and the impurities in PEG precipitates of aphid homogenates the efficiency of these chromatographic procedures was studied.

About 2.5 gram of viruliferous aphids were triturated in a mortar and subsequently mixed with 20 ml of a solution containing 5% PEG, 0.1% Dextran and 4.5% glucose. The aphid debris was removed from the homogenate at 1000 *g* for 10 min, and washed once more with this mixture. After combining both supernatants, NaCl was added to a concentration of 3%. The homogenate was added to the top of a column prepared by layering 2 cm sand, 5 grams of cellulose and again 2 cm sand consecutively which were suspended in a solution containing 5% PEG, 0.1% Dextran, 4.5% glucose and 3% NaCl. After an incubation period of 30 min, to allow large floccules to settle on the sand, the homogenate was allowed to flow through the column. The homogenate was eluted with the last mentioned PEG solution until the unprecipitated material had passed through. The precipitate was then eluted by the consecutive addition of 25 ml amounts of solvents containing constant amounts of PEG, Dextran and glucose, while the NaCl concentration was lowered stepwise as indicated in Fig. 13. The column was finally washed with 100 ml 0.01 M phosphate buffer pH 7.0.

The absorption at 254 mμ of the eluted material was measured with an LKB Uvicord absorption meter. An absorption diagram of the effluents from viruliferous aphids is given in Fig. 13. A similar diagram was obtained from healthy aphids. No differences in the patterns were observed between the healthy and viruliferous aphids.

The occurrence of the virus in the effluents was tested by injecting aphids with portions of the concentrated effluents. From each solvent that had been passed through the column 12 ml was taken and centrifuged at 100,000 *g* for two hours. The pellets were dissolved and tested for infectivity. The results of

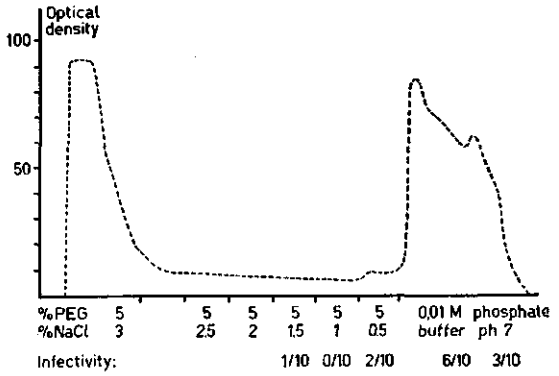


FIG. 13. Fractionation of a polyethylene glycol precipitate of a macerate of viruliferous aphids on a cellulose column. Absorption of the effluents was recorded by the LKB Uvicord absorption meter at 254 mμ. The results of the infectivity tests are indicated. The numerator represents the number of test plants infected and the denominator the number of plants inoculated.

these tests are indicated in Fig. 13, corresponding to the place where the fractions were sampled. It can be seen in Fig. 13 that PLRV was distributed over several fractions and that some of these fractions still contained a considerable amount of impurities. It was concluded that a separation of PLRV and non-viral aphid constituents could not be achieved by fractionating a PEG precipitate on a cellulose column under the above mentioned circumstances.

6.9. Density gradient centrifugation

The separation of PLRV from its contaminants was also studied with density gradient centrifugation. These studies were made both on gradient columns of sucrose and on columns of cesium chloride solutions. Different results were obtained as the substances differed from which the gradients were made. Therefore, it is desirable to discuss the experiments performed and results obtained with the sucrose and cesium chloride gradients separately.

6.9.1 *Density gradient centrifugation in sucrose columns*

Stegwee & Peters (1961) reported the centrifugation of a crude aphid homogenate on a sucrose gradient. They recovered infectivity from a fraction between 32 and 28 mm from the bottom of the tube, as well as from the sediment. In one of their experiments infectivity was also demonstrated in another fraction. This fraction was located between the former fraction and the sediment. The bulk of the virus seemed presumably to be associated with the zone between 32 and 28 mm from the bottom of the tube in their experiments.

However, when these experiments were repeated by the present author infectivity could be recovered from a wide zone in the tube. In these particular experiments about 200 mg aphids were ground in 0.5 ml 0.01 M phosphate buffer pH 7.2. After clarification at 1000 *g* for 10 min the supernatant was layered on top of the sucrose gradient which was prepared by mixing solutions of 4 and 50% sucrose in 0.01 M phosphate buffer pH 7.2 with a device described by Britten & Roberts (1960). The gradients were centrifuged at 90,000 *g* for 90 min in the SW 39 rotor of the Spinco model L ultracentrifuge. The results of two typical experiments are given in Table 14. It is evident that infectivity could be recovered in various fractions sampled from different depths in the tube using a syringe and a double-bended needle. Infectivity was also demonstrated in the sediment. The distribution of the infectivity varied from tube to tube. The failure to recover the virus from some of the fractions was perhaps due to the unreliability of the infectivity test, and not to the absence of the virus in the fraction in question.

These experiments and those carried out by Stegwee & Peters (1961) differed in the width of the infectious zone. In the present studies infectivity was found in a layer more than 20 mm wide whereas in the formerly published experiments it was found in a layer of 4 mm. This difference may be caused by the unreliability of the infectivity test, but it may also be attributed to differences in composition of the sucrose solutions and the used amount of aphids.

TABLE 14. Distribution of the aphid constituents and PLRV infectivity in sucrose columns after density gradient centrifugation at 90,000 *g* for 90 min.

Exp.	Weight of aphids	Height[1] of the zones	Appearance of the zones	Fractions tested for infectivity	Infectivity[2]
		0– 6	Very turbid	–	
		6–18	Slightly turbid	–	–
		18–25	Turbid	18–22	2/10
1	200 mg			22–25	0/10
		25–32	Very turbid	25–28	0/10
				28–32	5/10
		32–42	Clear	32–42	2/10
		Sediment			2/10
		0– 4	Very turbid	–	–
		4–10	Slightly turbid	–	–
		10–14	Clear	–	–
		14–22	Turbid	14–22	1/10
				22–26	3/10
2	125 mg	22–35	Very turbid	26–30	2/10
				30–35	1/10
		35–42	Clear	35–39	3/10
				39–42	0/10
		Sediment			3/10

[1] The depths are measured in mm from the meniscus of the gradient.
[2] Numerator: number of test plants infected. Denominator: total number of test plants used.

STEGWEE & PETERS included 0.1 M NaCl in the sucrose solutions and used about 100 mg of aphids. In the experiments of the present author NaCl was omitted and twice the amount of aphids was used.

The non-viral substances of the aphids were also distributed in the same broad zone as the virus. This poor resolution of the virus and the contaminants may be due to overloading of the tube. BRAKKE (1964) has demonstrated that the width of a zone of virus or proteins increases as their amount increases. The spread of the material in the zone was determined by their total amount or their relative concentration. Minor components will be spread into the zone of a major component, if the zone of the first component overlaps the zone of the second one. In view of the fact that no virus particles could be observed in each of the tested fractions by electron microscopy PLRV had to be considered as a minor component in density gradient studies. Its presence in a wide zone points to an overlapping of this zone by that of the major component. Thus a separation of the virus from the bulk of the aphid constituents could not be achieved by density gradient centrifugation of crude aphid macerates.

It was conceivable that a better separation of the virus and the impurities could be established after a partial purification of the aphid macerates. A number of experiments were performed with this in mind, even though an adequate procedure to clarify the macerates was not yet available. Clarifications of the

macerates were made with ammonium sulphate precipitation, chloroform emulsification, incubation at 55°C for 10 min, iso-electric precipitation and filtration over a Sephadex column. In various experiments these treatments were combined in different sequences. The gradients were made as described previously. The extracts were centrifuged at 35,000 *g* for 90 or 120 min in the SW 39 rotor of the Spinco model L.

Although the extracts layered on top of the gradient were prepared in different ways one wide zone only could be revealed by the Tyndall effect in each experiment. This zone extended from about 20 or 24 mm to 34 mm from the meniscus of the gradient. The maximum distribution of light scattering capacity within the zone varied somewhat between the different extracts, but in general it was found in the lower parts of the zone. Infectivity could be found throughout the whole zone as well as in the sediment. Its quantitative distribution over the zone and the pellet was not estimated. Particles were not found in the fractions studied by electron microscopy.

We can conclude that a better resolution of the virus and the impurities was not achieved after the partial clarification of the aphid macerates. This can be attributed to the inefficiency of the clarification procedures used to separate aphid constituents from the virus. Besides, due to adsorption of virus to aphid constituents, a loss of some virus occurs during the clarification of the macerates. Therefore, a more adequate method to clarify the aphid macerates has to be developed before density gradient centrifugation in sucrose gradients can successfully be applied in the purification of PLRV.

6.9.2. *Density gradient centrifugation in CsCl columns*

In the previous section it became clear that the rate zonal centrifugation in sucrose gradients was not efficient in our study. An investigation was made to determine whether a better separation between PLRV and the normal aphid constituents could be obtained by isopycnic centrifugation on CsCl gradients.

Centrifugation of virus suspensions in columns of concentrated solutions of CsCl have been used by different workers (MATTHEWS, 1960; AGRAWAL, 1964) in the purification and study of several viruses. A similar centrifugation in dense rubidium chloride solutions has been used by SINSHEIMER (1959) and RUECKERT *et al.* (1962) to separate impurities from partially purified preparations of the bacteriophage ø X 174.

In the first experiments the aphids were macerated in a buffer containing 0.001 M potassium phosphate and 0.0002 M magnesium acetate, pH 7.2. The macerates were partially clarified by two cycles of low and high speed centrifugations. The pellets were dissolved in 2 ml of the above mentioned buffer and layered over 3.3 ml of a CsCl solution (7.2 g CsCl was added to 10 ml of the buffer) in a 5 ml lusteroid tube and centrifuged at 35,000 rpm for 24 hours in a SW 39 rotor (Spinco L). After this centrifugation the material was distributed over the tube as sketched in Fig. 14. A large transparent pellet was formed containing mainly nucleic acids as concluded from its UV absorption spectrum. This pellet will be referred to as the CsCl pellet. At a depth of 23 to 25 mm from

FIG. 14. The distribution of aphid constituents over a CsCl (7.2 g was added to 10 ml phosphate buffer pH 7.2) gradient after 35,000 rpm for 24 hours. The zones at 9 mm and 12–14 mm from the bottom which have been found in a few experiments are also indicated (Chapter 8, section 1).

the meniscus of the tube one or several bands were found, containing only a small amount of the original material. The rest of the disintegrated material was aggregated into a thick film which floated somewhat below the original meniscus of the CsCl column and above the bands. In a few runs it was observed that a small part of the material disintegrated into small flocculates, which were suspended beneath the film in the CsCl gradient. A particular band occurring only in tubes with macerates of viruliferous aphids was not observed. The bands and the film were devoid of any infective material, whereas some infectivity could be recovered in the lower parts of the CsCl gradient and the CsCl pellet. The CsCl gradient was sampled in two fractions in order to avoid testing large numbers of fractions for infectivity. The fractions which were sampled were the opalescent bands below the floating film and the rest of the CsCl gradient. These fractions were tested as follows. The bands were sampled together from the tubes by a hypodermic syringe and a needle bent twice at right angles. Its volume was increased with buffer to 12 ml and then the fraction was centrifuged at 10,000 *g* for 10 min and 100,000 *g* for 3 hours, successively. In order to remove the CsCl from this fraction and to concentrate its content to a smaller volume, the rest of the CsCl gradient was handled in the same way as the bands, the film was homogenized in 12 ml buffer and subjected to the same cycle of centrifugation. The pellets obtained by the high speed centrifugation of these fractions had diameters of only 2 to 3 mm and contained a negligible amount of material in comparison with the original homogenate. These pellets and the CsCl pellet were all dissolved and injected into aphids. Likewise the infectivity results, the distribution of the infectious material in the CsCl tube, and the weight of the viruliferous aphids are shown in Tabel 15. A low level of infectivity was recovered from the density gradient. This can be explained in two

TABLE 15. Infectivity of a number of fractions obtained after equilibrium centrifugation of extracts from viruliferous aphids on CsCl gradients.

Exp.	Weight of aphids (mg)	Infectivity[1]			
		Pellicle	Visible bands	Rest of the CsCl gradient	CsCl pellet
1	2530	0/16[1]	0/17	1/23	2/14
2	4100	0/21	0/20	0/24	0/24
3	2200	0/14	0/13	0/13	1/14
4	2600	–	–	0/23	0/21

[1] Numerator: number of infected plants. Denominator: number of inoculated plants.

ways. Either the CsCl had a deleterious effect on the infectivity or the period required to perform the whole procedure had diminished the activity of the virus. The question also arises whether traces of CsCl can influence the transmission of the virus in an unfavourable way.

It appears from the data given in Table 15 that the infectivity had been recovered in two fractions, the CsCl pellet and the lower part of the gradient. This may have been caused by a too low density of the gradient. It should be possible to collect all the virus in the pellet or in a particular band in the gradient by an appropriate alteration of the density.

Virus-like particles were observed in pellets derived from the opalescent band(s) and in the fraction that is referred to as the rest of the CsCl column by electron microscopy. However, it appeared that identical particles also occurred in the same extracts from PLRV-free aphids, which were reared on radish and Chinese cabbage plants. These particles will be referred to as virus-like particles. A number of experiments which were carried out to establish the nature of these particles will be discussed in Chapter 8. The occurrence of these particles in aphids has already been published in a preliminary report (PETERS, 1965).

The following conclusions are drawn from the foregoing experiments. A large quantity of subcellular material of aphids could be disintegrated and separated into a pellet and a film. CsCl might have had a deleterious influence on the infectivity of the virus. The PLRV carrying aphids contained particles which were virus-like in appearance. Identical particles occurred also in PLRV-free aphids. It will be demonstrated in Chapter 8 that these particles are viruses. Therefore, the PLRV isolate used in the foregoing experiments was mixed with (an)other virus(es).

6.9.3. *Effect of CsCl on the infectivity*

The results in the foregoing section suggest that density gradient centrifugation of an extract from viruliferous aphids on a CsCl column was deleterious for the infectivity. The following experiments were performed to investigate whether a concentrated solution of CsCl had a harmful effect on the virus.

Aphids were ground in 0.01 M phosphate buffer pH 7.2, shaken with chloroform, and the emulsion broken at 10,000 *g* for 10 min. The water phase was spun at 100,000 *g* for two hours. The pellet was suspended in 1.2 ml of the phosphate buffer and divided in four equal parts. Different quantities of CsCl were added to the aliquots. The solutions were incubated at 4 °C and then dialyzed to remove the CsCl. The amounts of CsCl added, the length of the incubation and the dialyzing period, and the results of the infectivity tests are shown in Table 16.

Obvious harmful effects of CsCl on the infectivity were not demonstrated in these experiments. The results of the infectivity test were somewhat lower for the samples which were incubated with higher amounts of CsCl. This may be due to some effect of CsCl on the virus, but may also be explained by the unreliabilities of the infectivity tests. This decrease, however, can not explain the low infectivity obtained after density gradient centrifugation of the virus in

TABLE 16. The infectivity of aphid extracts after a short incubation in high concentration of CsCl[1].

Amounts of CsCl (mg) added to each 0.3 ml extract	Length of the incubation period (min)	Duration of dialysis (hours)		Infectivity[2]	
		Exp 1[3]	Exp 2	Exp 1	Exp 2
0	30	14	6	5/10	4/10
120	30	14	6	0/10	4/10
180	30	14	6	2/10	2/10
240	30	14	6	3/10	1/10

[1] The infectivity was determined by injecting small volumes of the incubated and dialyzed aliquots into aphids. The injected insects were confined in groups of 2 to *Physalis floridana* seedlings.

[2] Numerator: number of plants infected. Denominator: total number of test plants used.

[3] The weight of the viruliferous aphids was 650 mg in both experiments.

CsCl columns. The duration of the incubation was relatively short in comparison to the length of the isopycnic density centrifugation. The effect of a long incubation on the infectivity was studied in the following experiment.

An extract was made from 420 mg viruliferous aphids in the same way as described above. The final extract was suspended in 0.3 ml 0.01 M phosphate buffer and incubated for 20 hours with a similar concentration of CsCl as was used in the density gradient centrifugations. The CsCl was removed from the virus suspension by a subsequent dialysis for three hours. On testing this suspension by injecting aphids, 2 out of 10 plants became infected. Despite the fact that the control was lost in this experiment it may be concluded that even a long incubation with CsCl does not have a pronounced effect on the virus.

No obvious influence of CsCl on the virus was demonstrated in these experiments. Fairly good values were obtained in the infectivity tests irrespective of whether the virus was incubated for short or long periods with CsCl. The amounts of aphids used in the incubation experiments were about 10 times smaller than those used in the density gradient experiments. Therefore, the bad recovery of the infectivity from the centrifuged gradient column can not be attributed to some detrimental effect of high concentrations of CsCl on the virus.

It can be recalled here that the infectivity was recovered in a fraction which was obtained by dilution of a part of the CsCl gradient and subsequent centrifugation of the diluted fraction at 100,000 *g* for 3 hours (section 6.9.2). It may be assumed that the virus was not quantitatively collected by centrifugation in the pellet as convection in the tube during centrifugation interferes with the sedimentation of the virus if it is present in small quantities in a fairly pure suspension. Experiments have not been conducted thus far to test this possibility. The exact position of the virus in the centrifuged CsCl gradient may be located by analyzing the tube contents in small fractions. Progress in the purification of PLRV will be made if in the analysis of the tube contents a highly infectious fraction can be indicated.

6.10. Conclusion

In the preceding sections preliminary investigations on the purification of PLRV have been described. This purification from aphids involved two major problems. One may be defined as the separation of a small amount of virus from preponderant quantities of non-viral subcellular constituents. Whatever infectious partially purified fraction was studied by electron microscopy no particle which could be correlated with PLRV infection could be found between the amorphous debris on the specimen. The virus was not discernible in these debris. This points also to a low virus content of the aphid.

The other major problem involved, perhaps the most important one, was the precipitation of the virus with normal host material under several conditions. As was shown in the sections 1, 3 and 4 of this chapter considerable amounts of virus could be recovered from debris and aggregated material which was obtained from homogenates and virus suspensions, respectively. Infectivity was demonstrated in all fractions obtained from a macerate by stepwise addition of ammonium sulphate or lowering of the pH. Precipitation of ribosomes by magnesium ions also caused some of the virus to precipitate. The nature of the binding between the virus and the normal constituents is unknown at the present. It may be an adsorption of the virus to other material or an enclosure of the virus by the flocculating and precipitating material. This co-precipitation may, again, be due to the small amount of virus in comparison to the host components present.

Infectivity was fairly consistently demonstrated in fractions obtained from viruliferous aphid macerates using different clarification and purification procedures. However, with none of the techniques previously discussed could the virus be adequatly separated from the non-viral components. Nearly all the ribosomal material was still present in the infectious fractions obtained by techniques such as differential centrifugation, salting out with ammonium sulphate and acidification. Some of the aphid material was removed from the macerate by emulsification with chloroform or by partition of the virus suspension in a phase system of butoxy-ethanol, ethoxy-ethanol and 2.5 M potassium phosphate buffer pH 7.5. No tests were made to determine whether the specific activity in any fractions was increased using those techniques, because of the unreliability of infectivity tests in measuring the relative infectivity of the fractions. The virus could not be separated from the ribosomal material using density gradient centrifugation on sucrose gradients, if crude aphid macerates or preparations clarified with above-mentioned techniques were used. It may be useful if highly clarified virus preparations can be obtained. Resolution of a virus suspension on a CsCl gradient cause the disintegration of much non-viral host material. The recovery of the virus from this gradient was, however, poor. Perhaps, if a more reliable technique to analyse the CsCl gradient is available, that difficulty may be overcome. Prior to density gradient centrifugation in CsCl the aphid macerates also need to be clarified in a more efficient way, than has been done up to now. This is necessary for obtaining more concentrated

virus suspensions, and avoiding loss of substantial amounts of the virus during the clarification.

Particles resembling viruses were found in fractions after large amounts of impurities were removed from the macerates of viruliferous aphids (section 6.9). Similar structures could also be found in the corresponding extracts of healthy aphids. It has been established that these virus-like particles are viruses (Chapter 8). Therefore, it was obvious that the PLRV isolate used was contaminated with other viruses. When the virus-like particles were extracted form PLRV carrying aphids, they were found in a fraction of the CsCl gradient which contained also some PLRV-infectivity (Table 15). No specific particle could be found among the virus-like particles, which could be correlated with the PLRV infection. The failure to detect a specific particle may be due to a number of PLRV particles that was too low to be observed, or to a morphology resembling that of the virus-like particles. These observations demonstrated the importance of studying the purification with a PLRV-isolate which was free of any contaminating virus. A severe isolate was found free of any contaminating virus. It was tested with a procedure which will be described in the next Chapter. The aphids could be freed from the virus-like particles as described in 2.4.

CHAPTER 7

THE PURIFICATION OF PLRV FROM APHIDS

In Chapter 6 preliminary investigations on the clarification of PLRV from macerates of viruliferous aphids have been described. The inadequateness of a number of purification steps has been demonstrated. The conclusion was made that a selective procedure is required to separate the virus from a large amount of non-viral host material.

A clarification procedure yielding a well clarified virus suspension, has been developed. Emulsification of an aphid macerate at pH 5.0 with chloroform brought about considerable quantities of impurities in the aqueous phase, whereas the virus remained in the interphase. After extracting of the virus and concentrating the virus suspension, it was partitioned in a phase system of butoxy-ethanol, ethoxy-ethanol and 2.5 M phosphate buffer pH 7.8. The resulting virus suspension was then centrifuged successfully on a sucrose gradient.

In the preceding Chapter contamination of the moderate PLRV isolate with other viruses has been mentioned. To establish whether the severe isolate used in the final investigations on the purification was not contaminated by other viruses, the biological purity of PLRV was checked. The results of these tests should be presented before the description of the purification procedure. Because the purification of the virus was, however, an essential part of the procedure developed to test the biological purity of PLRV, they will be presented after the results of purification and description of properties of the virus.

Some of the results presented in this chapter have already been published (PETERS, 1967)[1].

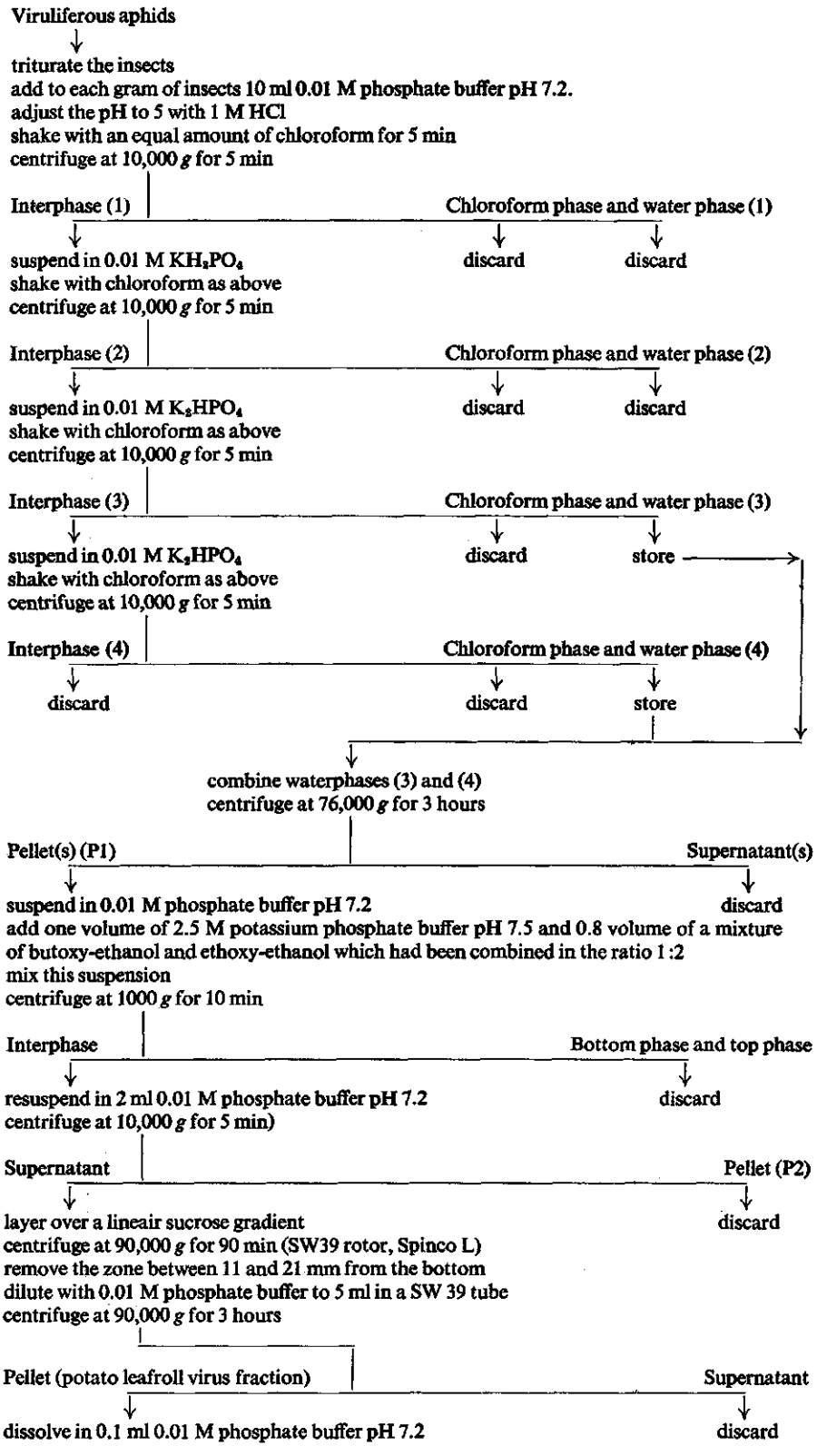
7.1. THE ADOPTED PURIFICATION PROCEDURE

The viruliferous aphids used in these purification experiments were reared on *D. stramonium*, infected with the severe isolate. This did not appear to be contaminated with other viruses as demonstrated in section 7.4. The purification procedure is outlined in Fig. 15.

Frozen or freshly collected viruliferous aphids were triturated with a mortar and pestle. To the crude macerate of each gram of aphids, 10 ml 0.01 M potassium phosphate buffer pH 7.2 was added. The ratio between the aphid material and buffer had an influence on the quantities of impurities which could be extracted from the macerate. When the ratio was small, less material was transferred to the water phases 1 and 2 (Fig. 15) and more could be found in the water phases 3 and 4 which also contained the virus. The pH of the macerate was then lowered to 5. At this pH nearly all the aphid material that interfered with the purification of the virus flocculated (section 6.5). The macerate was

[1] I am indebted to the editors of Virology for permission to reproduce Figure 15 and 16; and Table 17.

FIG. 15. The purification of potato leafroll virus from an aphid homogenate.



shaken vigourously with an equal amount of chloroform for 5 min and the phases were separated at 10,000 *g* for 5 min. A thick interphase occurred between the aqueous top phase and the chloroform phase. This treatment of reducing the pH to 5.0 caused a large amount of impurities to dissolve in the water phase, while the virus and some of the debris and impurities remained in the interphase. The pH of the water phase was also changed from 5.0 to 5.3 by this treatment. Both phases were decanted while keeping the interphase in the tubes. The interphases were emulsified three more times with chloroform as shown in Fig. 15. The first obtained interphase was resuspended in 0.01 M KH_2PO_4. Despite the pH of this solvent (4.6) the resuspended interphase did not require a correction of its hydrogen ion concentration due to the buffer activity of the impurities. In the two subsequent treatments the virus was extracted from the interphase by 0.01 M K_2HPO_4. The pH of the resulting water phases was now 7.1 and 7.6 respectively. The water phases obtained from the last two treatments, containing the virus, were collected and combined. The distribution of infectivity in the various water phases is given in Table 17. Infectivity occurred in water phases 3 and 4, but not in 1 and 2. To concentrate the virus the combined suspensions were centrifuged at 76,000 *g* for three hours. The supernatants were discarded and the pellets suspended in 5 to 10 ml 0.01 M phosphate buffer pH 7.2. The volumes used for resuspension depended upon the size and number of the pellets. The suspension was now further clarified by its partition in a phase system of butoxy-ethanol and ethoxy-ethanol. The virus suspension was added to one volume of 2.5 M potassium phosphate buffer pH 7.5 and 0.8 volume of a mixture of butoxy-ethanol and ethoxy-ethanol which were combined in the ratio 1:2. The system was gently mixed till a homogeneous solution was obtained. The phases were separated at 1000 *g* for 10 min. The bottom and top phase were carefully decanted while keeping the interphase in the tube. The last liquid traces were removed from the tube with a piece of filterpaper. The virus was extracted from the interphase by resuspending it in 2 ml 0.01 M phosphate buffer pH 7.2. The extract was clarified at 10,000 *g* for 5 min. The supernatant was now subjected to density gradient centrifugation in a sucrose gradient at 90,000 *g* for 90 min using the SW 39 rotor of the Spinco model L. Gradients were made by mixing 1.9 ml of 10 and 50% sucrose solutions with a device similar to that described by BRITTEN & ROBERTS (1960). The sucrose was dissolved in a 4% glycine solution adjusted to pH 7.2. Glycine may dissociate aggregates which might be present in the virus suspension.

After centrifugation two faintly visible zones at distances of 27–28 mm and 31–32 mm from the bottom of the tube were observed. They were situated just below the original sucrose gradient meniscus. No virus particles were discerned by electron microscopy, and virus activity could not be demonstrated by infectivity tests in these bands. Similar bands were also observed in gradients with material from non-viruliferous aphids. No other visible bands were present in these gradients.

The virus was located by fractionating the gradient in layers of 1.5 mm. For that purpose the tube was sealed with a rubber stopper through which passed

a needle serving as an air inlet. After the tube was fitted in a clamp its bottom was punctured and successive 1.5 mm layers were collected by controlling the inlet of air. The thickness of the sampled fractions was measured using a millimeter ruler attached to the clamp beside the tube. Only a few virus particles were found in each of the fractions taken from 11 to 21 mm from the bottom of the tube when checked by electron microscopy.

In order to concentrate the virus, the zone between 11 and 21 mm was sampled from the gradients by means of a hypodermic syringe with a needle bent twice at right angles. This fraction was diluted to 5 ml with 0.01 M phosphate buffer pH 7.2 and centrifuged at 90,000 *g* for three hours in a Spinco SW 39 rotor. The supernatant was discarded. A pellet could not be observed at the bottom. Even though a pellet was not discernible, a quantity of phosphate buffer equal to or less than 0.1 ml was added to the tube. The obtained suspension will be referred to as the purified virus suspension. The entire purification procedure was completed within a day and all manipulations were made in the cold at 2°C.

7.2. Infectivity of some fractions obtained during the purification procedure

The purified virus preparations were tested for infectivity by injecting aphids (Table 17). The percentage of the plants that became infected after the inoculation feeding of the injected aphids, fluctuated between 10 and 70 in the different experiments.

The water phases 1 and 2 (Fig. 15), prior to testing for infectivity, were adjusted to pH 7.2 and centrifugated at 76,000 *g* for 3 hours. The pellets were

Table 17. Infectivity of several fractions obtained in the purification procedure as represented in Fig. 15 from potato leafroll virus carrying aphids

Fraction	Infected plants[1]									
	1	2	3	4	5	6	7	8	9	10
Water phase (1)	0/10	0/10	0/10	0/10	–	–	–	–	–	–
Water phase (2)	0/10	0/10	0/10	–	–	–	–	–	–	–
Water phase (3)	5/14	4/10	3/10	4/10	–	–	–	–	–	–
Water phase (4)	2/14	2/10	1/10	1/10	–	–	–	–	–	–
Interphase (4)		0/10[2]	1/10[2]	0/10[2]	0/10[2]	1/10[2]	2/10[3]	4/12[3]	–	–
Pellet (P2)						1/10[2]	0/10[2]	3/10[3]	1/10[2]	3/10[2]
PLRV-suspension					8/11	6/13	1/10	6/10	7/10	8/22
Amount of aphids[4]	0.3	0.6	0.75	3.0	3.0	3.5	10.0	15.0	3.0	10.0

[1] Number of plants that became infected over number infested with 2 aphids *(M. persicae)* that were injected with parts of the indicated sample.
[2] Extracted with 0.01 M potassium phosphate buffer pH 7.2.
[3] Extracted with 4% glycine, adjusted to pH 7.2.
[4] Estimated weight in grams of the viruliferous aphids used in each experiment.

dissolved in 0.2 ml of 0.01 M phosphate buffer pH 7.2 and assayed by injecting small amounts into aphids. In none of these cases could any infectivity be demonstrated. In contrast, water phases 3 and 4 were infectious in each experiment. Water phase 3 was more infectious than water phase 4 (Table 17).

During the purification procedure the residual interphase (4, Fig. 15) as well as the pellet (P2, Fig. 15) were usually discarded. Upon testing for infectivity they proved to contain a small amount of infectious material. This was done by suspending them in 0.01 M phosphate or in 4% glycine pH 7.2 and centrifuging at 5000 *g* for 10 min and at 100,000 *g* for 2 hours. The pellets from the final centrifugation were suspended in 0.2 ml 0.01 M phosphate pH 7.2 and tested for infectivity. Their infectivity was slightly higher when the studied fractions were extracted in 4% glycine than when extracted in 0.01 M phosphate buffer. No conclusive statement can, however, be made because data were obtained from only a few experiments. However, 0.01 M phosphate buffer was preferred to 4% glycine for extraction of the virus from the interphases 2 and 3 (Fig. 15), because the latter caused resuspension of a considerable amount of impurities which had to be eliminated in subsequent treatments.

7.3. Electron microscopy

For electron microscopy the purified virus suspensions were dialyzed overnight against 0.001 M potassium phosphate buffer pH 7.2. Negatively stained specimens were prepared by mixing a small droplet of the virus suspension and a droplet of a 2% potassium phosphotungstate solution of pH 5.5 on a carbon-coated copper grid. The excess fluid was carefully sucked up by a piece of filter paper leaving, however, a film of fluid on the grid. After drying, the specimen was examined in a Siemens E Elmiskop 1 electron microscope. The surface tension between the membrane and the material mounted could be reduced when necessary, by the addition of 0.1 volume of 0.3% serum albumin to the supension. Another method which also gave good results was that of washing the grid with 0.001 M cetylpyridium bromide (Fixanol C) before depositing the virus solution. The Fixanol C was kindly supplied by Imperial Chemical Industries Ltd., Rotterdam. In order to prevent the disintegration of the virus particles the cetylpyridium bromide was removed by washing the grid with distilled water.

Particles were consistently found in each preparation. As shown in Photo 3 the particles are uniform in size and shape, and show a more or less hexagonal outline. They have a diameter of approximately 23 mμ (side to side). The number of particles found in the specimens was low. When 15 grams of viruliferous aphids were used, in some experiments only one to ten particles were counted on each of the randomly chosen areas of the specimen. In other experiments using similar amounts of aphids no particles were found in several areas probably due to the uneven distribution of the material deposited on these specimens. Because of the low number of particles and the uneven distribution of the particles in the specimen particles could not be counted in a reliable way.

So-called empty particles were never found in the virus preparations. In some micrographs other types of particles were observed, which are interpreted to be disrupted particles (Photo 4).

No structures or particles resembling virus were found in the noninfectious bands which were observed in the density gradient tubes at 27–28 mm and 31–32 mm from the bottom. These bands were removed from the tube with a hypodermic syringe and dialyzed against 0.001 M phosphate buffer pH 7.2 overnight prior to electron microscopical studies.

In some preparations of aphids supposedly free of PLRV and virus-like particles, a few structures which seemed to be virus particles in the electron microscopical field were occasionally found. When these particles were more closely observed they showed a diversity in shape and size (Photo 5). Therefore, it was doubtfull whether these particles were identical to those found in the purified PLRV preparations. Moreover, the number of these particles in the preparations of healthy aphids was negligible in comparison to the number of characteristic particles in the virus suspension.

The presence of virus-like particles in PLRV-carrying aphids was demonstrated in earlier experiments (section 6.10). In additional experiments (Chapter 8) these particles appeared to be other viruses contaminating the moderate PLRV isolate used. As these particles measure 24 mμ they can not be easily distinguished from PLRV by electron microscopy. However, the purified PLRV preparations differ significantly from the virus-like particle preparations with respect to the number of particles present. When the final preparations of virus-like particles and PLRV extracted from a given quantity of aphids are suspended in a similar volume, the number of virus-like particles is some 1000 times greater than the number of PLRV particles. From this ratio it may be concluded that the virus-like particles did not occur in the PLRV isolate studied in this Chapter. This does not mean that this PLRV isolate was not mixed with other viruses. However, in the next section where tests to determine the purity of the PLRV isolate will be described, it will be demonstrated that the used isolate was indeed free of any contaminating virus.

7.4. The biological purity of the purified PLRV isolate

A number of viruses exist which are transmitted circulatively by aphids and have certain host plants in common with PLRV. MacKinnon (1956) discovered a turnip latent virus (TLV) which caused a pronounced vein chlorosis on *P. floridana*. It was established that TLV was a complex of two viruses. One of them, mild chlorosis virus (MCV) caused a mild chlorosis on *P. floridana* and sometimes cupping of only the lower leaves with slight stunting of plants (MacKinnon, 1965). These symptoms could be easily mistaken for those incited by PLRV. In addition to *P. floridana* other solanaceous plants could be infected by MCV as can be seen in Table 18. Duffus (1964) pointed out that beet western yellows virus strains can also be transmitted to some solanaceous plants including *P. floridana* and *D. stramonium*. From the possible occurrence

TABLE 18. Susceptibility of selected plant species to infection by potato leafroll virus (PLRV) mild chlorosis virus (MCV), turnip latent virus complex (TLVC), 5 strains of beet western yellows virus, and the viruses referred to as virus-like particles (VLP) in this report[1].

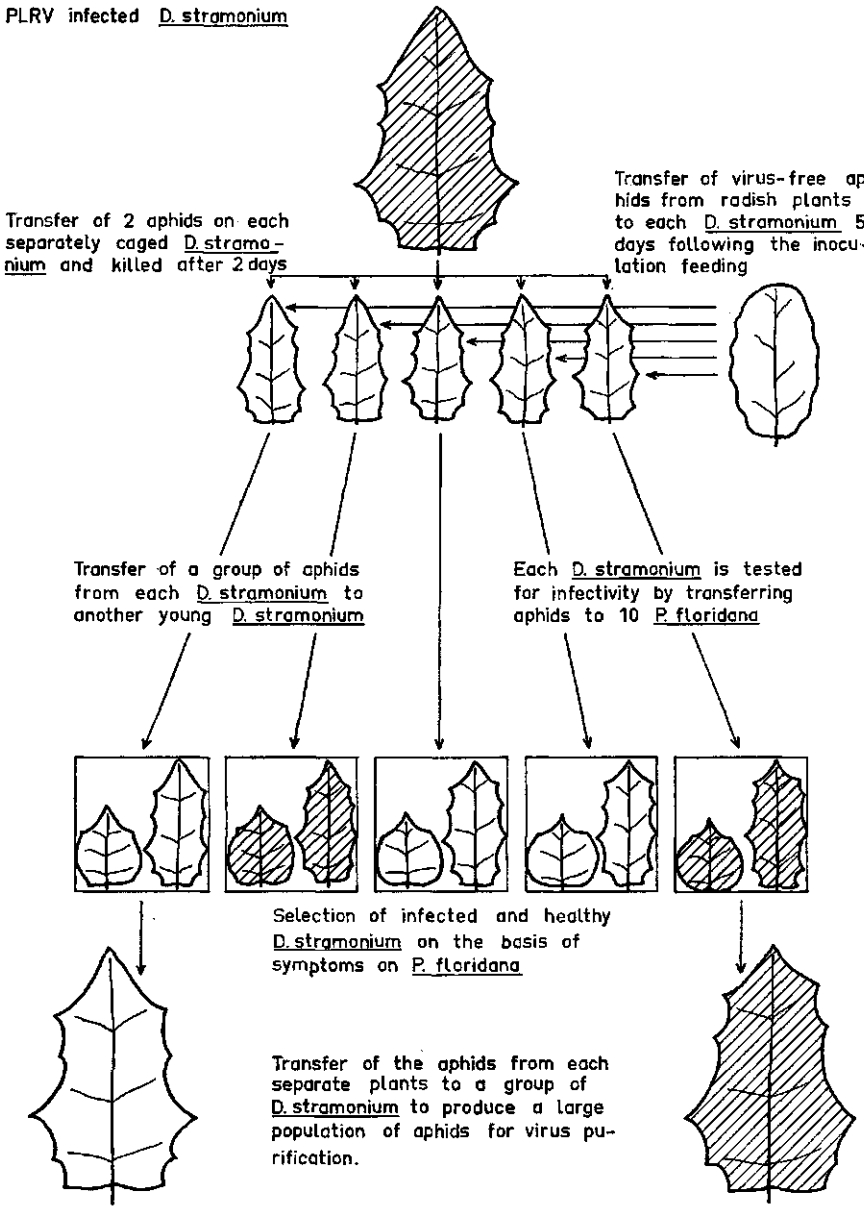
	PLRV	MCV	TLVC	Strains of beet western yellows					VLP
				1	2	3	4	7	
Amaranthaceae									
Gomphrena globosa L.	+	+	+						
Amaranthus tricolor L.				+	+	—	—	—	
Chenopodiaceae									
Beta vulgaris L. 'Detroit'	—	+	+						
Beta vulgaris 'NB1'				—	—	+	—	—	
Spinacia sp 'King of Denmark'	+	+	+						
Cruciferae									
Brassica napus L.	—	+	+						
Brassica peckinensis Rupr.	—	—	—	—	—	—	+	+	+
Brassica rapa L.	—	+	+	—	+	+	+	+	+
Raphanus sativus L.	—	+	+						+
Solanaceae									
Capsicum annuum L.	—	—	—						
Datura bernhardii Lund.	+	+	+						
Datura metel L.	—	—	—						
Datura stramonium L.	+	—	—	+	—	—	—	—	+
Datura tatula L.	+	—	—						
Lycopersicum esculentum Mill.	+	—	—						
Nicandra physaloides Gaertn.	+	+	+	+	+	—	+	+	
Nicotiana tabacum L. 'White Burley'	+	—	—						
Physalis floridana Rydb.	+	+	+	+	+	+	+	+	+
Solanum tuberosum.	+	—	—						

[1] This table has been derived from a paper reported by MACKINNON (1965) and extended with data from DUFFUS (1964) and from the present author.

of viruses other than PLRV in these plant species it may be expected that PLRV isolates could be contaminated with any of them. It was thus essential to ascertain the biological purity of the PLRV isolates which were used in purification experiments. A contamination of a PLRV isolate used in the preliminary experiments on the purification of the virus from aphids, has already been demonstrated (PETERS, 1965; and Chapter 8). The biological purity of the isolate used in the final purification was verified by the following experiments.

A number of viruliferous aphids reared on infected *D. stramonium* plants were colonized on radish plants which are immune to the PLRV. The aphids were harvested when the colony had reached maximum numbers. Since the maximum life expectancy of this species is 20 days, and the colonies were not harvested until after 25 days, it is unlikely that any of the original adults were included. The harvested aphids were subjected to the purification procedure used for the purification of PLRV from aphids as described in section 7.1. The final prepara-

FIG. 16. Diagrammatical representation of the procedure to test the biological purity of the used PLRV isolate.



tion was studied by electron microscopy and tested for infectivity. In these preparations no particles were found, and infectivity tests were also negative. From these experiments we concluded that the characteristic PLRV particles present in viruliferous aphids could not be acquired from the radish plants by aphids.

In these experiments, however, the possibility was not excluded that the PLRV isolate was contaminated with a virus which caused a latent infection in *D. stramonium*. In order to test this possibility the following experiment was performed as outlined in Fig. 16. A number of young *D. stramonium* plants were infected by placing two viruliferous aphids on separately caged plants for two days. The aphids were then killed. Under these circumstances only some of the inoculated plants became infected with PLRV. Five days later aphids free of PLRV and virus-like particles, were placed on the inoculated plants and colonized for 12 days. In order to select the healthy from the diseased plants and to avoid misreadings they were tested for infection by transmission tests to *P. floridana* seedlings, which develop symptoms more readily than *D. stramonium* on PLRV infection. For this purpose 40 aphids from each inoculated *D. stramonium* plant were transferred to ten *P. floridana* plants. The rest of the aphids on each *D. stramonium* plant were transferred to another young *D. stramonium*. This transfer was made in order to have an actively growing aphid colony by the time that symptoms occurred on the *P. floridana* plants. On the basis of these records, infected and non-infected *D. stramonium* plants could be separated by the results obtained with the corresponding *P. floridana* plants. After this selection, the aphids from each *D. stramonium* were colonized on a separately caged group of *D. stramonium* plants. When some 5 grams of aphids from each colony could be harvested, they were collected and subjected to the purification procedure. Aphids from 6 non-infected *D. stramonium* plants were tested and proved to be free from any particle. The characteristic particles were present in preparations from the aphids reared on the infected *D. stramonium* plants. So it may be concluded that the PLRV isolate in question had not been mixed with other viruses. Therefore, there appears to exist a correlation between the PLRV infected plants and the causal agent of the infection, viz. the particles in the purified preparations of viruliferous aphids.

7.5. Conclusion

From macerates of PLRV-carrying aphids a virus suspension could be extracted which contained a number of particles uniform in size and shape. Upon testing these suspensions for infectivity in the usual way the infected plants showed symptoms characteristic for PLRV. Furthermore it could be established that the PLRV-isolate used in the final purification studies was free of any contaminating virus. Thus we may draw the conclusion that the particles found represent the causal agent of the leafroll disease of potato. The infectious virus suspension contained only a small number of virus particles as may be derived from the sparse number of particles found in each area observed. This fact as

well as the absence of visible bands in the region associated in the gradient tube with infectivity suggest that very little virus has been extracted from the aphids. This in turn might mean that either the quantity of virus in the aphids is rather small or that the adopted procedure causes considerable loss in the number of particles. Some virus may be lost with the residues obtained after the last chloroform treatment and the partition in the butoxy-ethanol, ethoxy-ethanol phase system. Furthermore, these treatments may be harmful both in reducing the number of particles and their activity. This, however, needs further investigation.

The method developed to eliminate the non-viral constituents from the macerates of viruliferous aphids seems to be highly effective. This may be concluded from the facts that the final pelleted material was not observable and that impurities of any importance could not be detected on the specimen grid.

CHAPTER 8

STUDIES ON THE VIRUS-LIKE PARTICLES, CONTAMINATING ONE OF THE PLRV ISOLATES USED

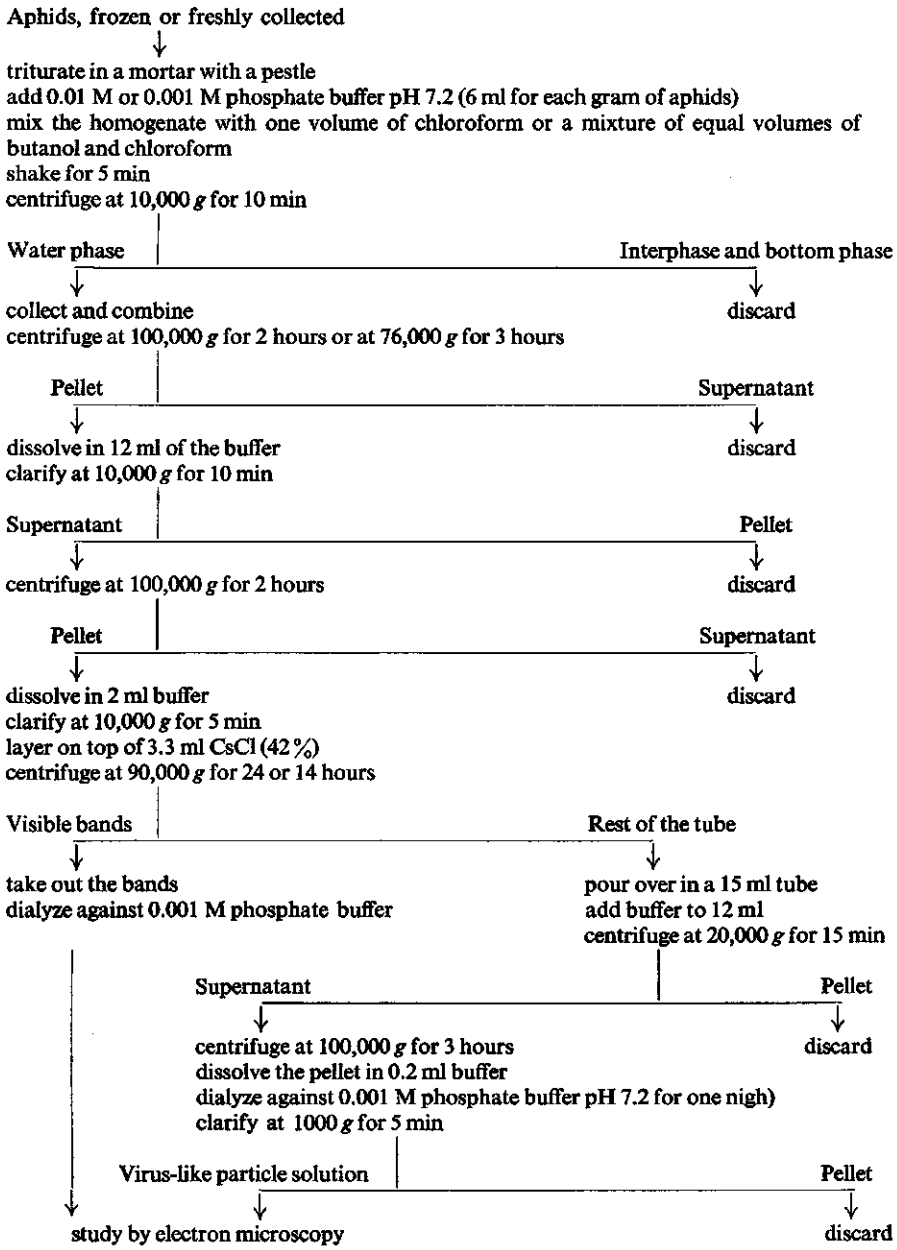
In the previous chapter the purification of PLRV and some of its morphological properties have been described. In the course of the preliminary purification studies particles resembling viruses were extracted from aphids which had been reared on *P. floridana* plants infected with the moderate isolate of PLRV. However, it appeared in subsequent studies that these particles could not be identified as PLRV, because particles with a similar appearance could be extracted when aphids from diseased *P. floridana* plants were colonised on radish plants. The occurrence of these particles in aphids reared on plants, which were either susceptible to or immune to PLRV, evoked a number of questions. The question arose whether the particles were viruses or normal constituents of aphids. The nature of these particles was established in a number of experiments which will be described in this chapter. The occurrence of virus-like particles in aphids emphasize the importance of developing a method to test the biological purity of the PLRV isolate.

8.1. The extraction of the virus-like particles from aphids and plants

The extraction of the virus-like particles from aphids by two cycles of differential centrifugation and resolution of the extract on CsCl density gradients has been described in 6.9.2 and has also been published (Peters, 1965). A modified procedure often used is outlined in Fig. 17.

Frozen or freshly collected aphids (2 grams) were triturated in a mortar with a pestle. To each gram of aphids 6 ml buffer (0.01 M or 0.001 M phosphate buffer pH 7.2) were added together with an equal volume of chloroform or a mixture of butanol and chloroform (1:1). This mixture was then emulsified for 5 min and centrifuged at 10,000 *g* for 10 min. The water phases were pooled and centrifuged at 100,000 *g* for 2 hours or at 76,000 *g* for three hours. The supernatants were discarded and the pellets suspended in 12 ml of that buffer which was added to the original homogenate. The suspension was clarified at 10,000 *g* for 10 min. The supernatant was then centrifuged at 100,000 *g* for 2 hours. The pellet obtained was resuspended in 2 ml of the buffer. This was clarified at 10,000 *g* for 5 min. The supernatant was then layered on top of 3.3 ml of a 42% CsCl solution (7.2 gram CsCl was added to 10 ml of the buffer) in a 5 ml lusteroid tube. The tubes were centrifuged at 90,000 *g* for 14 hours in a SW 39 rotor (Spinco L). The aphid material was distributed in the gradient as sketched in Fig. 14. A film consisting of denatured material occurred at a height of 25–27 mm from the bottom. This film contained more denatured material when the aphid homogenate was not emulsified with chloroform. Some

FIG. 17. Flow diagram for the extraction of the virus-like particles from aphids.



bands which blended into each other, could be observed by light scattering at 22–25 mm from the bottom. Although, virus-like particles were always found in these bands the light scattering was mainly caused by non-viral host compo-

nents, for these bands were also present with the same intensity when extracts of aphids free of virus-like particles had been similarly centrifuged. A band at 17–19 mm from the bottom of the tube was occasionally found. Two preparations exhibited faint bands at 9 mm and between 12–14 mm from the bottom of the tube. These bands were withdrawn separately by means of a hypodermic syringe with a needle bent twice at right angles. These fractions were dialyzed against 0.001 M potassium phosphate buffer pH 7.2 and studied by electron microscopy. Apparently these bands contained virus-like particles.

When the bands were not seen, the contents of the tube were poured into a 15 ml tube of Servall Type SS-1 superspeed centrifuge and diluted with buffer to 12 ml. After thoroughly mixing the suspension was clarified at 20,000 *g* for 15 min. The supernatant was then centrifuged at 100,000 *g* for three hours. A pellet with a diameter of 2–3 mm was usually obtained. This was dissolved in 0.2 ml buffer and dialyzed overnight against the same 0.001 M phosphate buffer pH 7.2. The dialyzed preparations were clarified at 1000 *g* for 5 min and the clear supernatant studied by electron microscopy.

A similar procedure was used to extract the virus-like particles from plants. Amounts of 100–150 grams of leaf material including the midribs were ground in a meat chopper. The juice was extracted by squeezing through cheese cloth and emulsified with a half volume of a mixture consisting of one part of butanol and one part of chloroform. The phases were separated at 10,000 *g* for 10 min. The top phases were pooled and centrifuged at 76,000 *g* for three hours to sediment the virus-like particles. The pellets were resuspended in 12 ml 0.01 M phosphate buffer pH 7.2, and subjected again to a cycle of differential centrifugation to remove some contaminants and to collect the material in one pellet. The pellet was dissolved, layered on a CsCl gradient and further treated in the same way as the corresponding extract from aphids. The material was distributed in the same way over the CsCl gradient as has been described for aphid material, except that the zones at 9, 12–14 and 17–19 mm from the bottom of the tube were never observed.

8.2. Electron microscopy of the virus-like particles

The purified preparations were studied by electron microscopy to determine whether virus-like particles occurred in the extracts and, if so, to determine their size and shape and to estimate their number. Negatively stained specimens were prepared as follows. A small drop of the suspension was placed on the grid together with a similarly sized drop of 2% phosphotungstic acid, adjusted to pH 5.5. The excess fluid was removed slowly with a piece of filter paper, allowing a thin liquid film to remain on the surface. Suspensions, mounted in this way, often showed an uneven distribution of the particles over the grid. The specimens were examined in a Siemens Elmiskop I electron microscope. Potassium phosphotungstic acid (PTA) used at pH 5.0; 6.0 and 7.0 did not seem to make much difference.

The measurements of the particles fall into two groups. The particles of one

group had diameters of 24–25 mμ (side to side) for full particles and diameters of 22–24 mμ (side to side) for empty ones. The other group consisted of particles with a diameter of 29 mμ (side to side). The particles will be referred to as the 24 mμ and the 29 mμ particles. Particles of 20 mμ were occasionally found in the earliest experiments, but were not observed in later ones. No explanation can be offered for the disappearance of these particles.

The general appearance of some preparations after negative staining is shown in Photo 6 and 7. Most of the particles in these micrographs show patterns which are circular. Some of the particles show a clear hexagonal symmetry. In preparations shadowed with palladium the particles cast angular shadows (Photo 8A and B) similar to those for particles with cubical symmetry. On the basis of these features and by analogy with other isometric viruses it seems that the virus-like particles possess a polyhedral shape.

Empty particles, representing the protein coat or the capsid of the particles, occurred in each preparation in variable numbers. From measurements it may be concluded that they are more prevalent in the 24 mμ group than in the 29 mμ group. Investigations were not made to determine whether they occur in the aphid before the extraction or are generated by the purification and the preparation of the specimen.

Distinct cores varying in size, shape and density can be seen within the capsids (Photo 6). The space between the core and the outer shell has apparently been filled by negative stain. The number of particles with cores is higher in old preparations than in fresh ones. Some particles of tobacco ring spot virus exhibit cores similar to ours (LISTER *et al.*, 1963). Until now the nature of these cores has not been fully understood. It is tempting to assume that particles with cores have not been entirely emptied of nucleic acid or have been only partially filled with PTA. However, another explanation could be put forward from the appearance of some particles in shadow-casted preparations. In Photo 8B particles can be seen having a differently sized dark spot in the center. These dark spots appear to be shadows formed by the particles themselves. They occur in the center of the particles and may be evoked by a dent or dimple. How these dimples arise in the capsid is not known, but if the capsid already possesses the dimple at the time of staining with PTA, less stain could enter the particle and thereby would cause a region of lower density.

Dimpling of virus particles has also been observed for the empty capsid of turnip yellow mosaic virus (COSENTINO *et al.*, 1956). Capsids in an aggregate each had a dimple, while single particles were obviously flattened. Dimpling was explained as collapsing of the capsids upon drying in air.

Particles other than the virus-like particles were sometimes found in the preparations from aphids. With respect to their shape these particles had a close resemblance to, and were similar in size to particles which have occasionally been described under different names in the literature.

Four types of particles could be distinguished in our preparations. The most commonly occurring particle was a round or ring-like structure with a diameter of approximately 12 mμ (Photo 6, 7 and 9). It was not possible to determine

with certainty how many subunits occurred in the ring. In some rings 6 or 8 subunits could be distinguished, whereas 7 were seen in most rings. The elucidation of the exact arrangement was not possible from the micrographs and needs further investigation. A second type of particles resembled to spheres with a diameter of 10 mμ (Photo 6 and 7). Rectangular particles formed by 4 parallel rows or discs of proteins were present in some preparations (Photo 6, 7, and 9). A specific structure consisting of 5 large subunits arranged in a pentagonal order was observed only in one preparation (Photo 9). All these particles were found in preparations obtained when the whole content of the CsCl tube was concentrated.

The ring-shaped particles were found in virus-free as well as in aphids carrying PLRV or virus-like particles. Since both types of particles were infrequently isolated from the aphids and then usually in low and varying numbers, experiments to discover the nature of these particles and their biological function were not conducted. Various ring-shaped particles having identical dimensions to ours have been found by different authors. We mention the fraction 1 protein from plants (REGENMORTEL, 1964; HASELKORN *et al.*, 1964), phytoferritin extracted from *Pisum sativum* by HYDE *et al.* (1963) and ring-shaped particles found in extracts from BHK 21 cells infected with Kilham rat virus by BREESE *et al.* (1964).

Pentagonal and rectangular structures similar to those described above were found in *Escherichia coli* by KISSELEV & SPIRIN (1964). The particles accumulated in the bacterial cells after inhibition of the protein synthesis with chloramphenicol. The authors referring to them as chloramphenicol particles or „CM-particles", claimed that they can be considered as metabolic and structural precursors of ribosomes.

Rectangular particles resembling the particles we found were also observed in extracts of Kilham rat virus together with numerous round or polygonal structures (BREESE *et al.*, 1964). They consider the rectangular particles as products of the rings, placed together in longitudinal arrays. Both types of particles were found only in infected cells, but not in freshly prepared BHK 21 cells.

8.3. PROPERTIES OF THE VIRUS-LIKE PARTICLES

The virus-like particles were discovered in an extract prepared from aphids which were collected from *Physalis floridana* plants infected with the moderate isolate of PLRV. Thereafter it was established that particles which are identical in their appearance under the electron microscope, occurred also in extracts from aphids reared on radish and Chinese cabbage plants which are immune to PLRV. The number of particles which was extracted from both groups of aphids was roughly of the same order. The emergence of these particles in the aphid extracts evoked a number of questions on the nature of the particles, viz.:

1. Are the particles viruses or normal constituents of aphids?
2. Do relationships exist between the particles found in aphids from *Physalis floridana* and those found in aphids from cruciferous plants?

3. Are the particles viruses transmissible to plants and can they be acquired from the plants by the aphids?
4. Do the particles cause symptoms on any plant species?

8.3.1. *The occurrence of particles in other aphid species and nymphs of Myzus persicae*

An investigation was made to check whether the particles did occur in aphids other than *Myzus persicae*. Two to 3 grams of the following aphids were collected from plants in the field: *Aphis fabae* Scop. from *Papaver rhoeas* L., *Rumex* species and *Vicia faba* L., 'Emma'; *Megoura viciae* Bckt. from *Vicia faba* (the aphids were colonized on these plants in the green house); *Rhopalosiphum padi* L. from *Prunus padus* L.; *Aphis pomi* Deg. from an undetermined *Prunus* species; *Hyalopterus pruni* Geoff. from *Phragmites communis* Trin.; and *Acyrthosiphon pisum* from *Pisum sativum* L. 'Kelvedon's Wonder'. The collected aphids were subjected to the extraction procedure as given in 8.1. The final extract was studied by electron microscopy with the same methods as used for the virus-like particles obtained from *M. persicae*. Particles with a similar appearance to those found in *M. persicae* were not found in any of the colonies. Therefore, the virus-like particles found in *M. persicae* may not be a normal component of the other aphid species.

Subsequently, it was investigated whether or not the particles were transmitted to the offspring of the aphids, which carry the virus-like particles. Nymphs were produced from these aphids in the way described in 2.7 and were then colonized on radish and *D. stramonium* plants. These colonies were isolated from other aphids. At the time that the colony had reached its maximum number the aphids were collected and examined for the presence of particles. It appeared that the extracts were free of the particles. Thus they are not transmitted to the progeny of the aphids and, accordingly, they are not normal constituents of *M. persicae*. The most plausible explanation for the occurrence of these particles in the aphids was then that they were acquired from plants. Tests on this will be described in the next section.

8.3.2. *Virus-like particles and vector-host relationships*

Experiments were conducted to establish whether the virus-like particles could be extracted from plants which were exposed to the aphids carrying the particles for one or two weeks. Extracts were made of 100 gram of leaf material with the procedure given in 8.1. for plants. Virus-like particles were found in extracts from *P. floridana* and *D. stramonium* plants. The number of particles which were found in the final extracts made from 100 grams of leaf material was about the same as those for the extracts from two grams of aphids reared on these plants. It was conspicuous that a higher number of the 29 mμ particles occurred in the extracts from *D. stramonium* (Photo 7) than in those of *P. floridana*. These observations made in 6 experiments, hold also for the extracts of aphids reared on these plants. The particles could not be found in extracts of plants kept free of aphids. Particles could also not be found in the extracts

when nymphs were put on these plants directly after birth.

The results indicate that the particles occur in the tested plants infested with aphids carrying the particles. It is concluded that the aphids transmit the particles to the plants and that subsequently the offspring of the aphids acquire them again by way of the plant. As regards this behaviour the particles bear a clear resemblance to viruses.

The particles were not found in extracts from radish and Chinese cabbage plants after they were infested with virus-like particle carrying aphids. It could not be established whether the concentration of the particles was much lower in these plants than in the solanaceous plants or whether particles were lost during the extraction. Despite the failure to extract the particles from the cruciferous plants it may be assumed that they occur in these plants because the extracts of aphids contained virus-like particles and the offspring of the aphids had to acquire the particles from the plant to become contaminated.

The particles from aphids reared on radish plants had a similar appearance in the electron microscope as those from aphids colonized on *P. floridana*. The question arises whether these particles are related or not. Virus-like particle carrying aphids of radish plants were colonized on radish and *P. floridana* plants in separate cages. Likewise aphids of *P. floridana* plants were caged on radish and *P. floridana* plants. The cages were placed in separate rooms free of aphids. After three weeks when the aphid population had approximately reached its highest number the insects were collected and subjected to the extraction procedure as described in 8.1. The final extracts were studied by electron microscopy. It appeared that all extracts contained virus-like particles and their number did not differ significantly. From these results it was clear that the particles could be transmitted from radish to *P. floridana* plants and conversely. These results suggest further that there is no specificity of the particles for the radish and *P. floridana* plants.

8.3.3. *Pathogenic character of the particles*

In the foregoing section evidence was given that the particles are probably viruses. The plants used in these studies did not develop any perceptible symptom upon infection. The studies on the virus-like particles could be facilitated considerably if a plant species could be found which developed symptoms after an inoculation feeding of the aphids.

Several species of plants were infected with aphids from radish plants. As many as 10 aphids were caged on seedlings for 7 days. The aphids were then killed by a Phosdrin or TEPP spray. None of the following plants developed perceptible symptoms: *Brassica nigra* Koch, *B. peckinensis* Rupr., *B. rapa* L., *B. incana* Döll., *B. elongata* Ehrh., *Beta vulgaris* L. 'Kühn', *Capsella bursa-pastoris* Med., *Claytonia perfoliata* Donn., *Datura stramonium* L., *Lactuva sativa* L. 'Meikoningin', *Malva neglecta* Wallr., *Nicandra physaloïdes* Gaertn. *Nicotiana clevelandii* Grey., *N. tabacum* L. 'White Burley', *Papaver somniferum* L. 'Emma', *Petunia hybrida* Vilm., *Physalis floridana* Rydb., *P. pruinosa* L., *P. pubescens* L., *Raphanus sativus* L., *Pisum sativum* L. 'Kelvedon's Wonder',

Sinapis alba L., *Solanum tuberosum* L. 'Bintje', *Spinacia oleracea* L. 'Breedblad', *Trifolium incarnatum* L., *T. repens* L., *T. pratensis* L., *Tetragonia expansa* Murr. and *Vicia faba* L. 'Emma'.

Experiments were not carried out to determine whether the aphids had transmitted the virus-like particles to these plant species. This should be tested by extracting the particles from plants. In case the procedure fails to extract the particles as has been demonstrated for radish plants, the recovery could then be studied by using aphids. They colonize in such a way on some of the plants that large quantaties can be obtained, whereas only small colonies are formed on other plants. In this latter case the aphids had to be reared on suitable host plants after acquisition feeding on the plants to be tested. In view of the time and space-consuming character of these experiments and the extensive provisions required to control the aphids, these experiments were not carried out.

8.4. Conclusion

It may be recalled here that the particles investigated in this Chapter were actually found during attempts to purify PLRV from viruliferous aphids. On the basis of their morphology the particles were regarded as viruses. When radish plants immune to PLRV were infested with these aphids, their progeny contained similar particles. The conclusion was made that the particles involved did not represent PLRV. In the preceding Chapter it was shown that PLRV isolates which were not mixed with any other virus, existed. In view of this fact there is no reason to assume that the virus-like particles mixed with the moderate isolate of PLRV are connected with PLRV infection.

These particles could not be found in extracts of the progeny of nymphs of aphids carrying virus-like particles. These nymphs were directly after birth transferred to plants, which had not previously been infested with other aphids. The particles could also not be detected in extracts of other aphids species. Therefore, these virus-like particles are not normal constituents of aphids.

The aphids are able to transmit the particles to plants but first had to acquire them in order to become contaminated with them. On account of these observations and of their morphology it was concluded that the particles are the causal agents of a latent virus infection. The particles proved to be non-pathogenic on all the plants tested. This is not necessarily in conflict with the present day concept of viruses. If defined as sub-microscopic, infective entities that multiply only intracellularly and are potentially pathogenic (Bawden, 1964) the ability of viruses to infect is a more fundamental property than pathogenicity.

The particles were referred to as virus-like particles throughout this whole report notwithstanding their apparent virus nature. In order to give them a more suitable name it is desirable to elucidate more thoroughly their properties and continue the studies on the host range of these particles.

Two types of virus-like particles could be distinguished, one measuring 24 mμ and the other 29 mμ. The occurrence of these particles in the extracts may be explained as follows:

a. They are differently sized components of one virus.
b. They form a complex in the sense that the transmission of one component occurs only if the other component is simultaneously transmitted as has been described for tobacco rosette and carrot motley dwarf virus complexes (SMITH, 1946; WATSON *et al.*, 1964).
c. They form a mixture of two unrelated viruses, which are transmitted independently by aphids as has been demonstrated for the viruses of the turnip latent virus complex (MACKINNON, 1965; MACKINNON & LAWSON 1966). The existence of possible relationships between these particles will have to be established in further investigations.

CHAPTER 9

GENERAL DISCUSSION

An isolate of PLRV causing severe symptoms on potato was purified from viruliferous aphids by a multistep procedure.

The first step of the procedure consisted of the emulsification of the macerates with chloroform at pH 5.0. This brought a considerable amount of disintegrated material into suspension, whereas the virus remained in the interphase. This treatment seems to be most suitable as an initial step in the purification. After extracting the virus from the interphase and concentrating it into a smaller volume, the remainder of the impurities could be almost completely removed by partition of the virus suspension in a phase system of butoxy-ethanol and ethoxy-ethanol. When a virus suspension prepared by other means was partitioned in this system less favourable results were obtained. The effect of the emulsification with chloroform at pH 5.0 may be attributed to a more thorough disintegration of the ribosomes than that obtained with any other procedure tried, and to suspending of denatured material in the aqueous phase.

Two interphases are formed in the course of purification, viz. the interphase obtained in the chloroform treatment at pH 5.0, and the interphase obtained in the butoxy-ethanol and ethoxy-ethanol phase system. With regard to the tendency of PLRV to become adsorbed to normal aphid constituents the formation of these interphases from which the virus has subsequently to be extracted, is a less attractive aspect of the procedure developed. Further investigations into this problem could lead to a better removal of the virus from these fractions.

Loss of some virus occurred during the high speed centrifugation of the virus suspension extracted from the chloroform interphases, and during the concentration of the zone sampled from the sucrose gradient. Precipitation by polyethylene glycol may be more effective for concentrating the virus from diluted suspensions than high speed centrifugation as found by VAN KAMMEN (1967) for cowpea mosaic virus.

Infectious preparations of the virus were found to be relatively free of debris when examined by electron microscopy. The regular structures found could not be ribosomes, because ribosomes are destroyed by chloroform treatment and the partition in the butoxy-ethanol and ethoxy-ethanol phase system. Furthermore the ribosomal structure would probably also have disintegrated on application of phosphotungstic acid to the material mounted on the specimen grid. Prior to studies by electron microscopy ribosomes have to be fixed with formaldehyde (HUXLEY & ZUBAY, 1960). The ribosomes are then characterized by a smaller density and less distinct morphology than that of viruses. In preparations of virus-free aphids some particles, were found which could have been mistaken for virus particles. However, they had a less regular structure than the PLRV particles. Moreover, the number of these particles was negligible in comparison to the number of those characteristic for the PLRV suspension. In

view of this there can be little doubt that the particles observed in the infectious suspensions by electron microscopy are those of PLRV.

The position of PLRV in the sucrose gradient could not be associated with a light scattering band. This indicates a low quantity of virus which is in agreement with the observations made by electron microscopy. After rate zonal centrifugation, the virus was distributed in the gradient column in a wide zone which can be explained by the relatively large volume of virus supension which was layered on a rather short gradient column. The zone could probably have been narrowed by using isopycnic density gradient centrifugation (BRAKKE, 1964). The prospect of a better resolution of the virus suspension on a CsCl gradient followed by an adequate analysis of the gradient as discussed in section 6.9.3 has not been explored up to now.

Characteristic particles which were found in a fraction obtained by concentration of a specific zone in a sucrose gradient, were correlated with PLRV infectivity. This correlation could be made because the PLRV isolate was not contaminated with any other virus. The particles, characteristic of PLRV could not be recovered from the progeny of viruliferous aphids colonized on plants immune to PLRV, or from aphids colonized on susceptible plants which did not become infected after inoculation with PLRV. In the latter case the conditions were chosen in such a way that only some of the inoculated plants became infected. It should be pointed out that the biological purity of any other purified virus, which is circulative in its vector, and which can not be transmitted mechanically, has to be tested by a similar procedure. Obviously this test should be made irrespective of whether the source from which the virus is purified, is plant or insect material.

MOERICKE (1961) found particles 200 mμ × 30 mμ and 300–400 mμ × 50 mμ in sections of salivary glands of aphids carrying PLRV. It appeared later that the particles were also present in aphids living on *Brassica rapa* which is immune to leafroll virus (MOERICKE, 1963). These particles may be an aphid-infecting virus or an unrecognized plant virus. It was not determined whether our aphids were contaminated with these particles. The fact that they were not observed in the preparations from aphids studied by electron microscopy, does not mean that they were not present in our aphids. They may be destroyed by the chloroform treatment of the aphid macerates like lettuce necrotic yellows virus particles (CROWLEY *et al.*, 1965; HARRISON & CROWLEY, 1965) which resemble these particles.

Viruses circulative in their aphid vectors differ widely in their forms and dimensions. One of these viruses, lettuce necrotic yellows virus, appears to be bacilliform and 230 mμ long (HARRISON & CROWLEY, 1965). PLRV appears to be one of the smallest plant viruses: a polyhedron with a diameter of approximately 23 mμ. The particle of barley yellow dwarf virus is a polyhedron of diameter 30 mμ (ROCHOW & BRAKKE, 1964), and that of pea enation mosaic virus one of diameter 26–28 mμ (GIBBS *et al.*, 1966; BOZARTH & CHOW, 1966; MAHMOOD, to be published; SHIKATA *et al.*, 1966).

The absolute amount of PLRV particles extracted from aphids could not be

determined. It was, however, estimated that 0.1 μg virus was extracted from 20 grams aphids. Assuming that 90% of the virus had been lost during the purification the aphids would contain 1 μg virus. The following calculation may give some impression of the number of particles in one aphid, and the numbers required to render an aphid infective by injection. Twenty grams of aphids equal about 10^5 insects if the mean weight of an aphid is 0.2 mg. This means that one aphid contains $10^{-5} \times 10^{-6}$ g virus. Using a particle weight of 5×10^6 g for PLRV one aphid would contain $\frac{10^{-11}}{5 \times 10^6} \times 6 \times 10^{23} = 1.2 \times 10^6$ particles. It has already been shown (section 4.4) that if one particle could render an aphid infective the aphid which had supplied the inoculum, contained 6.6×10^4 particles. From the assumed virus content of one aphid it can be calculated that $1.2 \times 10^6 / 6.6 \times 10^4 = 20$ particles had to be injected to render an aphid infective. Even though the above calculations are of a speculative nature they do give some indication of the small number of virus particles present in an aphid which may explain some of the difficulties encountered in the purification of PLRV.

The aphids used in the purification experiments were collected when they were feeding on the infected host plants. This means that the aphids contained virus acquired from the plants as well as virus propagated in the aphids themselves. Thus the purified virus suspension is a mixture of particles acquired and propagated by the aphid. With regard to this it is interesting to know which proportion of the purified particles resulted from virus multiplication in the aphid. This proportion would depend on the rate of multiplication within the aphid and the amount of virus that accumulated in the intestinal tract and haemocoel after ingestion. Owing to technical difficulties it is impossible to acquire any information on this problem at present.

It is occasionally proposed that the form of potato leafroll virus acquired by the aphid differs from that which is transmitted to the plant. KÖHLER (1964), for example, came to the conclusion that „möglicherweise dieses Virus in der Kartoffel als freie Nucleinsäure vorliegt und sich nur im Vektor zum Vollvirus ergänzt". This remark may have been made with reference to the results of BRANDENBURG (1962). The recovery of virus from macerates, made from aphids collected from infected plants (HARRISON, 1958) and from virus containing plant sap (Chapter 3) points to a higher degree of protection of virus activity than may be expected for a free nucleic acid. On the contrary, STEGWEE (1961) has proposed the possibility that the virus may be transformed within the aphid to a form less resistant to degrading agents in aphid macerates. Such a form would explain the results regarding the multiplication of the virus in the aphid (STEGWEE & PONSEN, 1958), and the decrease of the virus concentration in the aphid during its stay on an immune host as demonstrated by HARRISON (1958). This labile form of the virus formed during the multiplication might be protected in such a way in the blood that it remains infective if transferred with haemolymph from viruliferous aphids to virus-free aphids by a needle-injection

technique. The decrease of the infectivity found by HARRISON may be explained by the destruction of this labile form in the aphid macerates. The present experiments neither support nor deny the existence of a less stable form of the virus in the aphid. This could perhaps be elucidated by comparing the virus extracted from aphids which were allowed to acquire virus in a short feed to that from aphids which were transferred to immune plants for a period of several days after they had acquired virus. The assumption may be made that the virus can be extracted from these aphids in the form in which it was acquired by the aphid and multiplied in the aphid.

Thus far there is no evidence that a virus which is transmitted and multiplied by a vector, has different forms in plant and vector. Wound tumor virus appears to have particles identical in size and morphology when present in plants as well as when present in its insect host (BRAKKE *et al.*, 1954). Similar results were also obtained by FUKUSHI *et al.* (1962) for rice dwarf virus.

It is difficult to predict whether the procedure described in this study to purify PLRV from aphids will be applicable to the purification of other circulative viruses from their aphid vectors. It seems likely that the procedure may be useful for viruses which occur in small amounts in insects, and are insensitive to chloroform and the mixture of butoxy-ethanol and ethoxy-ethanol. In some preliminary studies the method appeared of value for the purification of the „virus-like particles" from aphids. Because of its sensitivity to organic solvents lettuce necrotic yellows virus may not be purified with this method (CROWLEY *et al.*, 1965). A similar conclusion may be made for pea enation mosaic virus. This virus is not precipitated completely at pH 5.0 in aphid macerates, and is sensitive to butoxy-ethanol and ethoxy-ethanol (MAHMOOD, unpublished). On the other hand the method may be useful in the purification of barley yellow dwarf virus from aphids, as it is quite a stable virus.

Because it is expected that the ribosome content of leafhoppers will be of the same order as that of aphids, some leafhopper transmitted viruses as e.g. beet curly top and aster yellows virus may with advantage be purified by the method described. Wound tumor virus and rise dwarf virus have already been extracted from their leafhopper vectors (BRAKKE *et al.*, 1954; FUKUSHI *et al.*, 1962) by far less elaborate procedures. The occurrence of ribosomes in the macerates of leafhoppers seems not to interfere with the purification of these viruses. This may be attributed to the size of these viruses and their concentration.

It was evident that nearly all the aphid constituents which can be sedimented at 100,000 *g* for 2 hours flocculated at pH 5.0. The precipitation of viruses at this pH value in aphid macerates was previously described (HUTCHINSON, 1963). In studies on the fate of turnip yellow mosaic virus in the aphid *Brevicoryne brassicae* (L.) HUTCHINSON & MATTHEWS (1963) found that virus was lost at pH 4.8 during its extraction from aphid macerates. Investigating this phenomenon further HUTCHINSON (1963) concluded that the loss of virus was caused by the aid of a specific protein with a low molecular weight. This protein was referred to as the precipitating factor. Because of its low molecular weight this factor may be lost in some cycles of differential centrifugation of aphid mace-

rates by remaining in the supernatant fraction obtained in the high speed runs. After differential centrifugation the precipitation behaviour of aphid constituents and PLRV was not altered. Therefore, PLRV and the aphid ribosomes seems to be precipitated at pH 5.0 without the help of the precipitating factor. The conclusion was made (Chapter 6) that the aphid constituents and PLRV are precipitated simultaneously at this point by the coincidence of their iso-electric points or by a co-precipitation. Such a co-precipitation may also play a role in the precipitation of turnip yellow mosaic virus at pH 4.8 in aphid macerates. This was also suggested by the results of HUTCHINSON (1963). He centrifuged aphid macerates at 100,000 *g* for 1 minute, 1 hour, and 2 hours respectively. Whilst the precipitating ability of the supernatant fraction which he obtained after each centrifugation was hardly reduced, an increase of precipitating activity was found in the pellet fraction.

It was apparent from the experiments described in 6.1 that some virus occurred in the crude aphid debris. The fourth extract made from this debris proved to be as infectious as the first extract. The assumption was, therefore, made that the virus was adsorbed to the debris. It is also conceivable that the release of virus from the debris may be partly due to breakage of some cells during washing. To be circulative it is assumed that PLRV has to pass through the wall of the intestinal tract and the salivary glands. This means that virus would occur in the cells of these tissues. The failure to demonstrate particles characteristic for PLRV in cross sections of these tissues points to a low virus content of the cells (MOERICKE, 1963; FORBES, 1964a). Thus it is doubtful whether bursting of a single tissue cell may cause a detectable amount of virus in the debris washings. This statement does not exclude the possibility that cells of other tissues may contain higher quantities of virus as could perhaps be revealed by electron microscopical examinations of thin sections.

In view of the tendency of PLRV to become adsorbed one should be careful in associating infectivity with particular cell fractions. At one time KAPLAN & MELNICK (1953) thought on the basis of such fractionation studies that poliovirus was associated with nuclear fractions, but currently it is believed that its development takes place in the cytoplasm (TYRRELL, 1963).

Aphids appeared to be a richer source of virus than *P. floridana* plants. It was concluded that the virus concentration is higher in aphids than in plants. In preliminary studies on the purification of aster yellows virus, infected plants appeared to be poorer virus sources than viruliferous insects (BLACK, 1955). Thus the occurrence of higher virus concentrations in vectors than in plants seems not to be restricted to PLRV.

SUMMARY

1. The purification of potato leafroll virus (PLRV) from its vector *Myzus persicae* was studied. Small quantities of the virus could be extracted from these aphids.

2. Two isolates of potato leafroll virus (PLRV) causing moderate and severe symptoms on potato plants, respectively, were used. The former strain, used in the preliminary studies on the purification, proved to be contaminated with other viruses which were referred to as virus-like particles throughout this whole report.

3. PLRV-carrying aphids could be reared in large numbers on infected *Physalis floridana* and *Datura stramonium* plants. The aphids could be rapidly harvested from the plants by a washing method.

4. Fractions obtained during the fractionation of aphid macerates were tested for infectivity by injecting small portions into aphids. After storage overnight at 2 °C the injected aphids were allowed to feed on the test plant *P. floridana* for 5 days.

5. In some comparative experiments the results of infectivity tests were higher for extracts from viruliferous aphids than for those from diseased *P. floridana* plants. Therefore, the aphids were regarded as a better source for virus purification.

6. PLRV in aphid macerates was found to have a thermal inactivation point between 70–80 °C and longevity in vitro of 5 days at 2 °C. The virus was still infectious at a dilution of 1:100, but when diluted at 1:1000 only one out of 60 plants became infected.

7. Some 2% of the aphid's fresh weight consists of ribosomes. The ribosomes were fractionated from the aphid macerate by differential centrifugation. Magnesium ions appeared not to be necessary for keeping the ribosomes intact. Some aggregation of the ribosomes occurred at a concentration of 0.1 mM magnesium ions whereas it was complete at 10 mM.

8. Infectious fractions could be extracted from aphid macerates by using different buffers.

9. The virus could be precipitated from the macerate by centrifugation at 100,000 *g* for 70 min, but as ribosomes were also precipitated, differential centrifugation was not suitable for purifying the virus.

10. Stepwise addition of ammonium sulphate to the macerate or lowering of its pH in subsequent steps resulted in infectious fractions. It was not evident whether the concentration of the virus was increased in any specific fraction obtained. The separation of the virus from the bulk of aphid constituents could also not be improved by the addition of appropriate amounts of magnesium ions to the macerates. It was concluded from these experiments and from

the occurrence of considerable amounts of virus in the debris, that PLRV either has a large affinity for the aphid constituents or that the virus is co-precipitated with the precipitating material. Therefore, it might not be expected that PLRV could be purified by any procedure which precipitates aphid constituents.

11. The aphid macerates could be clarified to some extent by emulsification with chloroform as infectivity of the virus was not destroyed by this treatment. It was often used as the first step in purification procedures.

12. Partition of the aphid macerates in a mixture of butoxy-ethanol, ethoxy-ethanol and 2.5 M potassium phosphate buffer pH 7.5 resulted in a considerable removal of aphid constituents. This treatment required some additional steps in order to achieve the purification of the virus.

13. Density gradient centrifugation of an aphid macerate on a sucrose gradient resulted in a zone consisting of the bulk of the aphid constituents. Clarification of the aphid macerates in different ways prior to this centrifugation did not promote the separation of the virus from the normal host components.

14. Density gradient centrifugation in CsCl resulted in the disintegration of the normal aphid constituents and the subsequent partition of this material into two fractions, viz. a pellet and a film floating on the CsCl layer. Only a small amount of infectivity could be recovered from the CsCl gradient. Even though this may suggest that CsCl has a detrimental effect on the virus, this was not found in other experiments.

15. From the preliminary studies on the purification it was evident that substantial loss of virus occurred with the removal of the normal host components from the suspension. It was, therefore, decided to approach the clarification by removing the normal host constituents from a precipitate of the aphid macerates thus leaving the virus in the precipitate.

16. The final procedure followed to purify the virus was to emulsify the aphid macerates at pH 5.0 with chloroform. The virus was subsequently extracted from the interphase. The suspension obtained after concentration by centrifugation was subjected to partition in a phase system and centrifuged in a sucrose gradient column. A fairly pure virus preparation was obtained when the material from an infectious zone of the gradient was concentrated at 90,000 *g* for 3 hours (Fig. 15).

17. The percentage of plants infected after being inoculated by aphids which were injected with portions of purified virus preparations, fluctuated between 10 and 70. Particles having a diameter of approximately 23 mμ and showing a hexagonal outline were found in each preparation extracted from aphids.

18. The biological purity of the severe PLRV isolate used was studied in order to ascertain whether the particles found were the causal agent of the leaf-roll disease. Viruliferous aphids were colonized for 2 days on *D. stramonium* plants. Then virus-free aphids were placed on the inoculated plants. Virus particles were absent from the extracts of aphids from uninfected plants, whereas

the extracts of the aphids of the infected plants contained the characteristic particles. It was emphasized that the purity of each virus circulative and propagative in aphids, has to be checked in this way.

19. It was estimated that approximately 0.1 μg virus was extracted from 20 grams of viruliferous aphids and that 90% of the virus was lost during the the purification. This means that each aphid contains about 10^{-11} g virus. Thus when aphid macerates 1:1000 remain infective, only 20 PLRV particles are required to render an aphid infective.

20. Virus-like particles were found in extracts of aphids. In studies on the nature of these particles it appeared that they could also be extracted from aphids reared on radish plants, which are immune to PLRV. These particles are regarded as viruses because a) the particles are not normal components of aphids; b) aphids had to acquire them from plants, for nymphs of infected adults appeared to be free from these particles; c) aphids could only acquire these particles from plants, which had been infected by aphids carrying virus particles. A plant species reacting with symptoms after being inoculated with the virus-like particles has not been found thus far.

21. Extracts of virus-like particles contained two types of polyhedral particles measuring 24 mμ and 29 mμ in diameter respectively. There were less 29 mμ particles in the extracts than 24 mμ particles. The number of 29 mμ particles in extracts from *D. stramonium* or from aphids reared on this plant was relatively higher than in extracts from *P. floridana* or from aphids, reared on this plant.

22. Virus-like particles with a dark spot in the center were found in shadow-cast preparations. In negatively stained preparations 24 mμ particles were found with distinct cores varying in size, shape and density. The dark spot is probably cast by a dimple in the capsid. If negatively stained, less stain can penetrate the dimpled particle and would then cause a region with a lower density.

23. Names for the viruses involved will be proposed when more is known about their host range and their properties.

SAMENVATTING

In 1916 concludeerden QUANJER *et al.* dat de verwekker van de bladrolziekte bij de aardappel een virus is. Ondanks dat sindsdien veel onderzoek aan deze ziekte en zijn verwekker is verricht, is men nog slecht geinformeerd over de intrinsieke eigenschappen van het virus, omdat geen zuivere virussuspensies konden worden bereid. Het doel van het onderhavige onderzoek was om in deze leemte te voorzien.

In het tweede hoodstuk worden de gebruikte bladrolisolaties, het kweken van de bladluizen en de benodigde planten beschreven. Het onderzoek over de zuivering van het bladrolvirus werd met twee isolaties verricht. Een isolatie bleek te zijn besmet met andere virussen, die waarschijnlijk tot nu toe onbekend waren. De andere isolatie werd gebruikt in de uiteindelijke zuivering van het virus. Voor het onderzoek waren grote aantallen bladluizen nodig. Deze konden worden gekweekt op *Physalis floridana* en *Datura stramonium*. Ze konden met een straal leidingwater van deze planten worden gespoeld en op een zeef worden verzameld. Voorts wordt het inspuiten van bladluizen en het toetsen van de ingespoten bladluizen op infectiositeit beschreven. De infectiositeit van een bladrolvirus-suspensie werd getoetst door injectie van kleine porties in bladluizen met een fijn glazen naaldje (Fig. 5). Na de injectie werden de bladluizen gedurende een nacht bij 2°C bewaard. Hiermee kon worden bereikt dat de bladluizen de toetsplanten *(P. floridana)* beter als waardplant accepteerden.

Uit de resultaten van een paar vergelijkende experimenten (Hoofdstuk 3) bleken extracten van bladluizen infectieuzer te zijn dan de overeenkomstige extracten van besmette *P. floridana*-planten. Een concentratie van plantesap door het gedurende 2 uur bij 76.000 *g* te centrifugeren verhoogde de infectiositeit van het extract niet. Op grond van deze resultaten werd besloten om de zuivering van het bladrolvirus uit bladluizen te bestuderen.

De inactiveringstemperatuur, zoals in Hoofdstuk 4 is beschreven, blijkt voor het virus in ruwe bladluismaceraten tussen 70 en 80°C te liggen; bij 2°C kon het virus 3 tot 5 dagen zijn infectiositeit behouden, terwijl de verdunningsgrens bij 10^{-3} ligt. Hoewel deze waarden een geringe betekenis hebben kan toch geconcludeerd worden dat met het virus bij lage temperaturen zal moeten worden gewerkt.

Het bleek in de zuiveringsproeven dat in bladluizen veel nucleoproteïnen aanwezig zijn. Fractionering van de bladluishomogenaten (Hoofdstuk 5) met een methode waarbij afwisselend bij hoog en laag toerental werd gecentrifugeerd, leverde een extract met nucleoproteïnen op. Het sedimentatiediagram leerde dat dit extract 5 componenten bevatte, n.l. een hoofdcomponent met een sedimentatie-coëfficiënt van 80 S en kleine hoeveelheden 20 S–, 40 S–, 60 S– en 112 S–componenten. Uit deze S–waarden mag geconcludeerd worden dat dit extract voornamelijk uit ribosomen bestaat. Magnesium-ionen bevorderen de associatie van deze ribosomen. Een geringe associatie trad op bij een concentratie van 0,1 mM magnesium-ionen, terwijl deze compleet was bij een concentratie

van 10 mM. Ongeveer 2% van het levend gewicht van bladluizen bestond uit ribosomen. De aanwezigheid van zulke grote hoeveelheden ribosomen bemoeilijkte de zuivering van het bladrolvirus uit bladluishomogenaten zeer aanzienlijk.

Om tot een zuivering van het virus uit bladluizen te komen werden allerlei technieken toegepast. De meeste echter bleken weinig effect te sorteren, omdat zij niet in staat waren een scheiding tussen de ribosomen en het virus, waarvan werd aangenomen dat het in zeer geringe concentraties in de bladluis aanwezig was, te bewerkstelligen. In Hoofdstuk 6 worden de resultaten van een aantal toegepaste technieken besproken, die een indruk geven van de problemen die aan de zuivering van het virus voorafgingen. Technieken zoals trapsgewijze uitzouting of verlaging van de pH van de homogenaten, leverden een aantal infectieuze fracties op. Het bleek dat in deze fracties ook het merendeel van de normale bladluisbestanddelen werden neergeslagen. Omdat de infectiositeitstoets meer een kwalitatieve dan een kwantitatieve waarde had, kon niet worden bepaald of de specifieke activiteit van een fractie groter was dan die van een ander. Een scheiding van het virus en de ribosomen kon evenmin bereikt worden door precipitatie van de laatste componenten met magnesium. Uit waarnemingen met deze en andere technieken kon worden geconcludeerd dat het virus een grote neiging heeft om met precipiterende bladluisbestanddelen mee te precipiteren. De aanwezigheid van aanwijsbare hoeveelheden virus in het bladluisdebris nadat dit herhaalde malen was uitgewassen, doet vermoeden dat het virus sterk aan bladluisbestanddelen is gebonden. Het is echter ook mogelijk dat het bladrolvirus door de grovere of uitvlokkende bestanddelen wordt ingesloten en dientengevolge wordt neergeslagen. Dit verschijnsel zal sterk optreden indien de concentratie van het bladrolvirus relatief zeer laag is.

De bladluishomogenaten konden enigermate gezuiverd worden door ze met chloroform te emulsificeren. De activiteit van het virus werd door deze behandeling niet vernietigd. In zeer veel zuiveringspogingen werd deze emulsificatie gebruikt als een eerste stap in een zuiveringsprocedure. Verdeling van een bladluishomogenaat in een fasen-systeem gevormd door een mengsel van butoxy-ethanol, ethoxy-ethanol en 2,5 M kaliumfosfaatbuffer pH 7,5, was effectiver. Met deze methode konden echter nog geen zuivere virussuspensies worden verkregen, zoals bleek uit bestudering van een infectieuze fractie.

Centrifugering van ruwe bladluishomogenaten of extracten van deze homogenaten in een dichtheidsgradiënt van saccharose resulteerde meestal in een brede zone bestaande uit het overgrote deel van de normale bestanddelen. Virusactiviteit kon op elke hoogte in deze zone gevonden worden. Uit de bespreking van de resultaten werd geconcludeerd dat alleen gunstige resultaten konden worden verwacht, indien deze centrifugering werd uitgevoerd met een ver gezuiverd viruspreparaat.

Meer effectief voor de verwijdering van de bladluisbestanddelen bleek een centrifugering van een virussuspensie in een CsCl-gradiënt te zijn. Het bleek dat de meeste bestanddelen gedesintegreerd werden en daarna gescheiden in twee fracties, n.l. in een sediment en in een op de CsCl-oplossing drijvende laag. Er

kon slechts een geringe hoeveelheid infectiositeit uit de CsCl-gradiënt worden teruggewonnen. Dit werd in eerste instantie uitgelegd als zou CsCl een nadelige invloed op het virus hebben, maar dit kon niet worden bevestigd wanneer een virussuspensie voor kortere of langere tijd met CsCl werd geïncubeerd. We menen nu dat het virus niet op de juiste manier uit de gradiënt werd teruggewonnen. Door een verfijnde analyse van de gradiënt zal dit waarschijnlijk kunnen worden bereikt, maar dat houdt in dat een zeer groot aantal fracties op infectiositeit getoetst moet worden. Bovendien verdient het dan ook de voorkeur om deze centrifugering eveneens uit te voeren met een ver-gezuiverd preparaat teneinde meer virus op een gradiënt te kunnen brengen.

Uit deze inleidende experimenten werd geconcludeerd dat met de reeds bekende procedures het virus niet van de normale bladluisbestanddelen kon worden gescheiden en dat het wenselijk was de zuivering van het virus op een minder conventionele manier te benaderen.

In Hoofdstuk 7 wordt een procedure besproken, die een goed gezuiverde virussuspensie opleverde. In vroegere proeven was reeds vastgesteld dat de bladluisbestanddelen en het virus bij pH 5 konden worden neergeslagen. Het bleek nu dat een emulsificatie van een bladluishomogenaat op pH 5,0 met chloroform een zeer groot gedeelte van de bladluisbestandelen in oplossing bracht, terwijl het virus in de tussenlaag, die ontstond tijdens een centrifugering bij laag toerental, terug kon worden gevonden. Vervolgens werd het virus hieruit geëxtraheerd en geconcentreerd door een centrifugering bij hoog toerental. Het sediment werd opgelost en de verkregen suspensie verdeeld in een fasen-systeem, bestaande uit butoxy-ethanol, ethoxy-ethanol en 2,5 M fosfaat buffer pH 7,5. In dit fasensysteem ontstond na toevoeging van de virussuspensie en centrifugering een tussenlaag. Hieruit werd het virus opnieuw geëxtraheerd en in een suikergradiënt gecentrifugeerd. De zone met het infectieuze materiaal werd uit de buis genomen en het virus geconcentreerd in een kleiner volume door een centrifugering bij hoog toerental. De op deze wijze verkregen virussuspensies waren steeds infectieus (Tabel 17). Bij bestudering met de elektronenmicroscoop werden in elk preparaat, zij het in kleine hoeveelheden, deeltjes gevonden met een diameter van 23 mμ en een hexagonale omtrek. Om zeker te zijn dat de gevonden deeltjes niet moesten worden toegeschreven aan een ander virus, die de betrokken bladrolisolatie besmette, werd de biologische zuiverheid van de isolatie getoetst. Wanneer bladrolvirusdragende bladluizen op radijs werden gekoloniseerd konden geen deeltjes in de nakomelingen van de bladluizen worden gevonden. Verder werden alleen deeltjes gevonden in bladluiskolonies die op besmette *D. stramonium* planten leefden, terwijl de bladluizen, die gekweekt waren op geïnoculeerde maar niet besmette *D. stramonium* planten, vrij van deeltjes waren. Hieruit wordt geconcludeerd dat de onderhavige deeltjes inderdaad het bladrolvirus vertegenwoordigen.

In extracten van bladluizen besmet met de eerst gebruikte bladrolisolatie, werden in een vroeg stadium van het onderzoek op virus gelijkende deeltjes gevonden. Deze deeltjes hadden een diameter van 24 en 29 mμ. Het bleek dat deze deeltjes (Hoofdstuk 8) geen normale bestanddelen van bladluizen waren,

maar vertegenwoordigers van twee onbekende virussen. Tot deze conclusie kwamen we omdat de bladluizen de deeltjes verwierven uit planten, want nympfen van besmette bladluizen bleken vrij van deze deeltjes te zijn, en bladluizen bleken alleen deze deeltjes op te nemen, indien op dezelfde planten reeds besmette dieren geleefd hadden. In dit verslag worden de deeltjes steeds aangeduid met de naam „virus-like particles" omdat nog te weinig gegevens beschikbaar zijn om ze met een andere naam aan te duiden.

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PHOTO 1. The tilted light microscope with the fitted micro-forge. The weight has been placed in the hook of the glass tube. The filament of the micro-forge is in a position next to the glass tube to be drawn out.

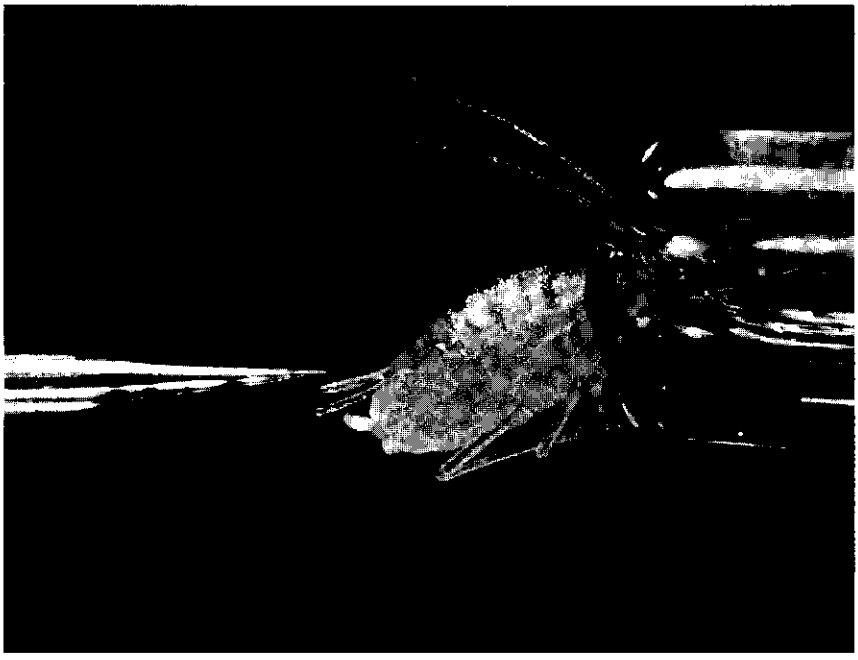


PHOTO 2. A photograph of an aphid fitted with its head in the vacuum tube before being injected.

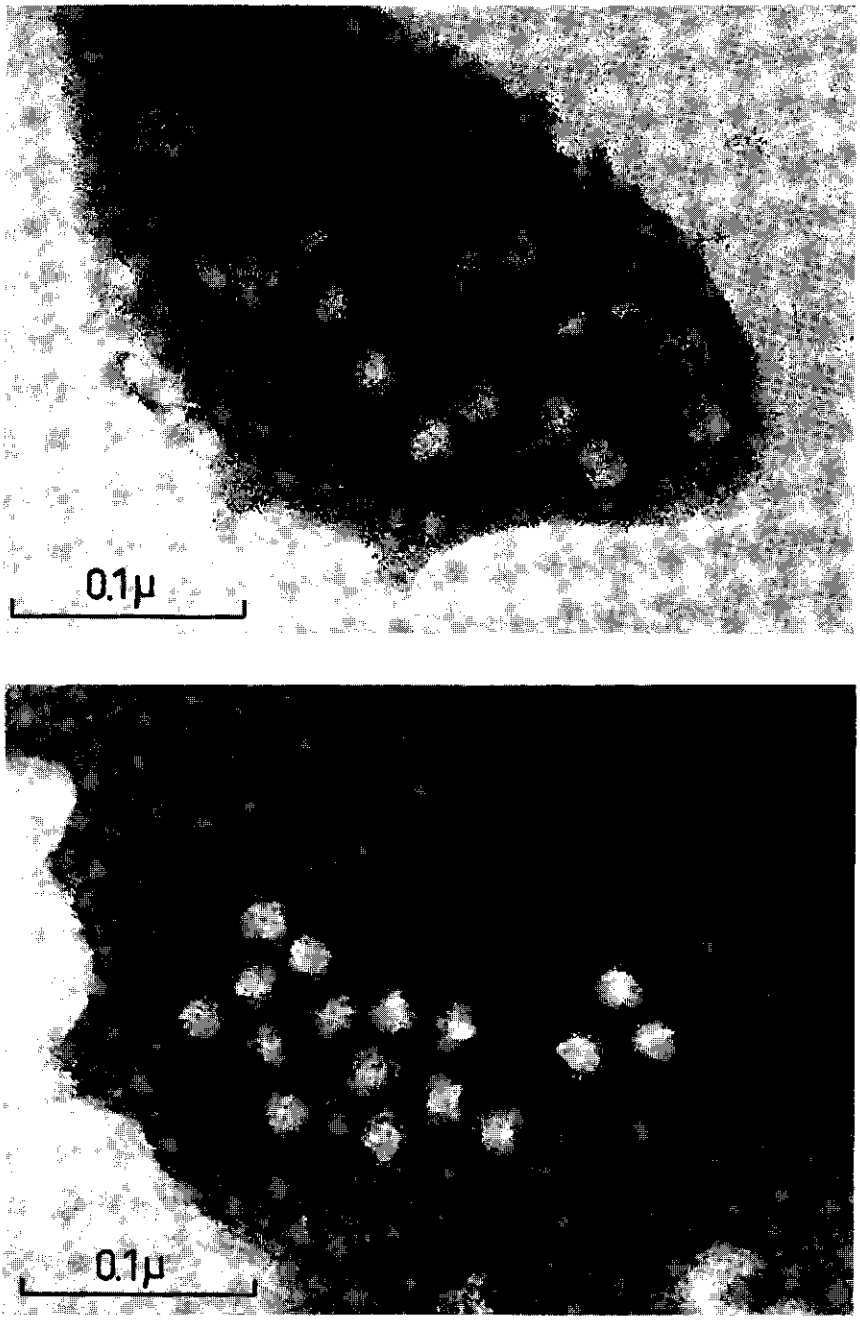


PHOTO 3. Electron micrographs of potato leafroll virus, negatively stained with potassium phosphotungstate (pH 5.5). The particles were found in a purified suspension obtained from about 15 g of viruliferous aphids. Magnification: x320,000.

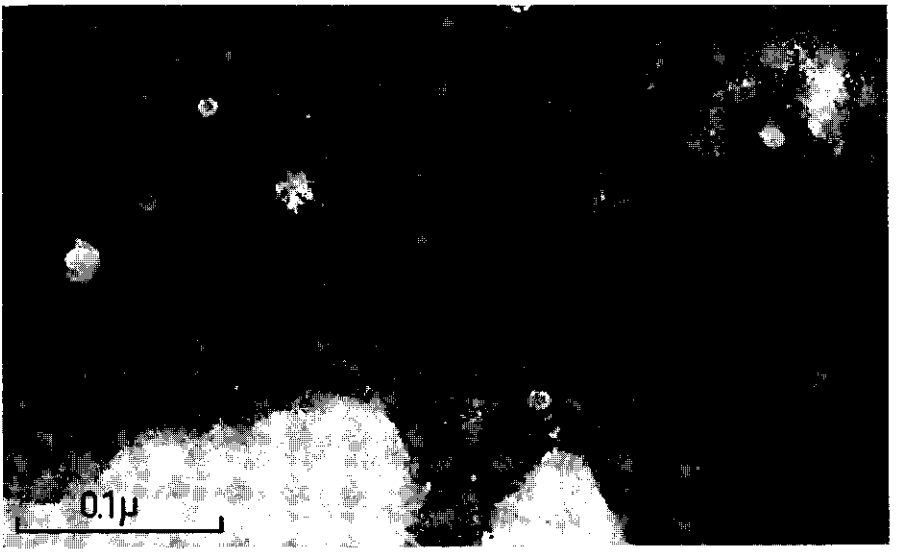


PHOTO 4. Electron micrograph of some disrupted particles found in a purified suspension of potato leafroll virus. Magnification: x320,000.

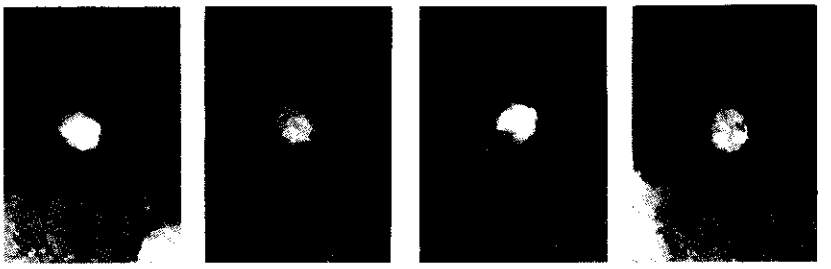


PHOTO 5. Micrographs of particles which could be mistaken for virus particles, found in purified suspensions of PLRV-free aphids. Magnification: x320,000. The four particles shown are from different experiments.

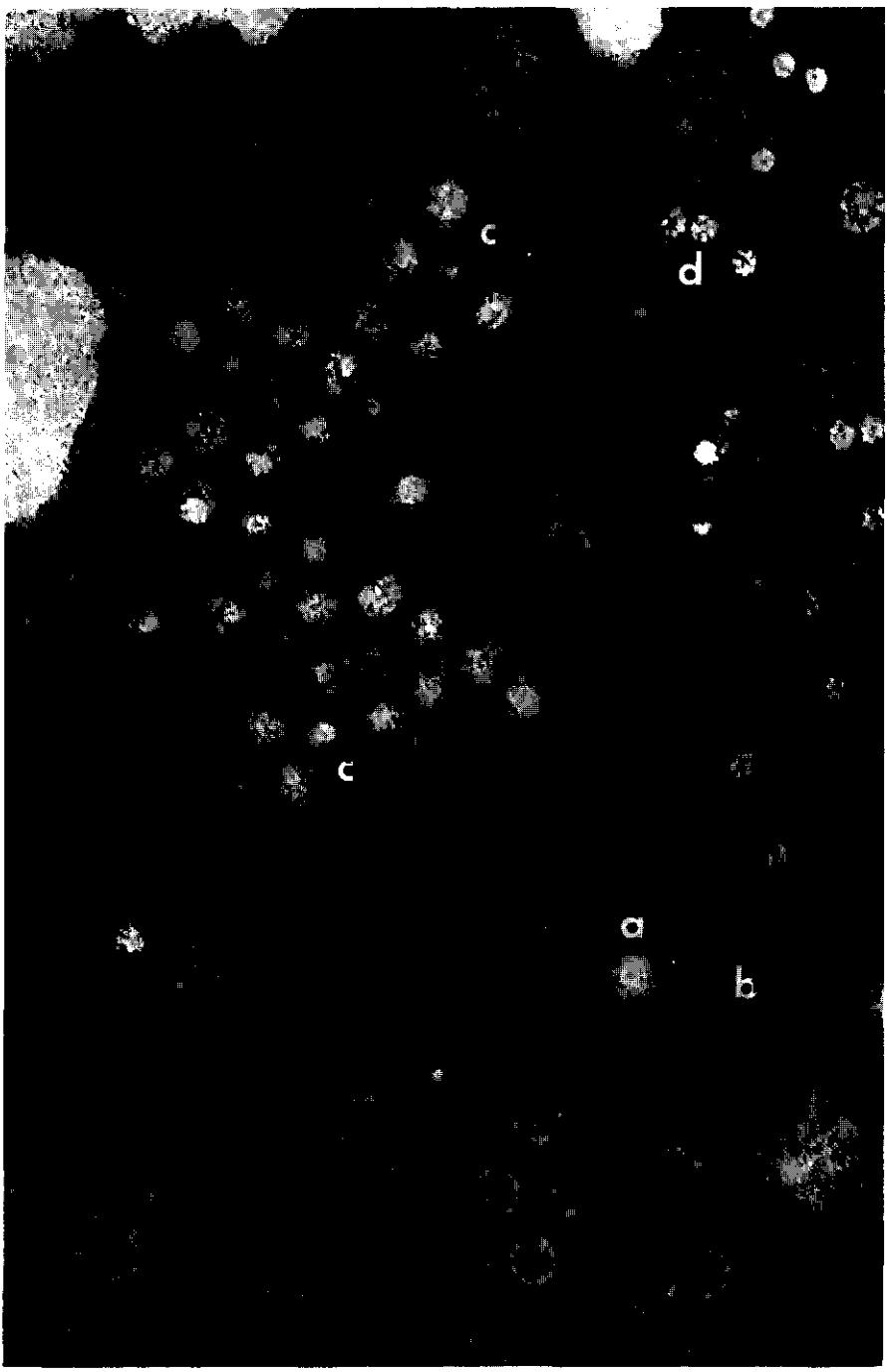


PHOTO 6. Electron micrograph of virus-like particles extracted from aphids reared on radish plants. The specimen is negatively stained with potassium phosphotungstate (pH 5.5). Particles of 24 mμ with (a) and without (b) nucleic acid can be seen. Some particles having differently sized cores can be noted (c). The ring-shaped particles of 12 mμ (d) as described in section 8.2 can also be found in this micrograph. Magnification: x320,000.

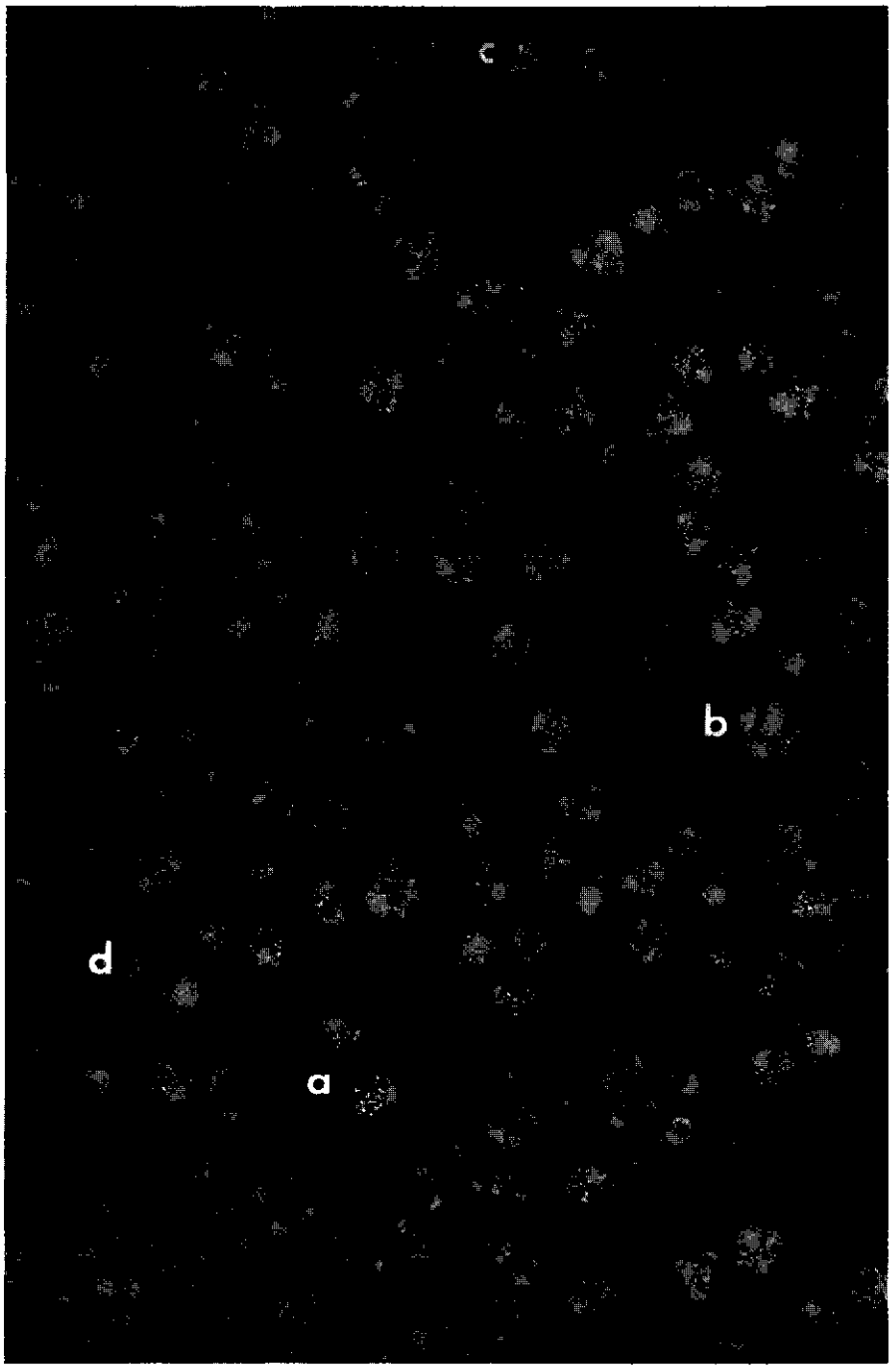
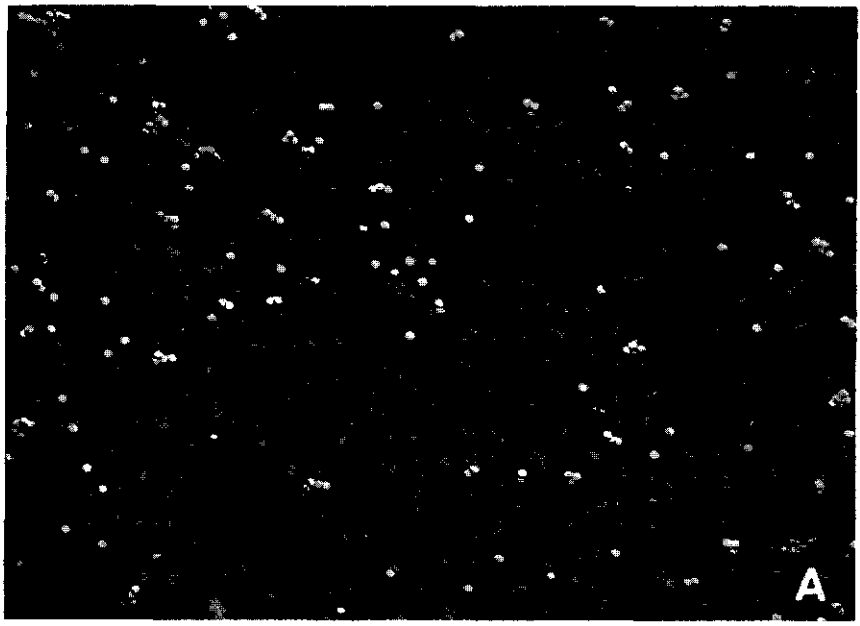


PHOTO 7. Electron micrograph of virus-like particles extracted from aphids reared on *D. stramonium* plants. Particles of 24 mμ (a) and 29 mμ (b) can be found. Besides some ring-shaped particles of 12 mμ (c) and 10 mμ (d) rectangular structures can also be found. Magnification: x320,000.



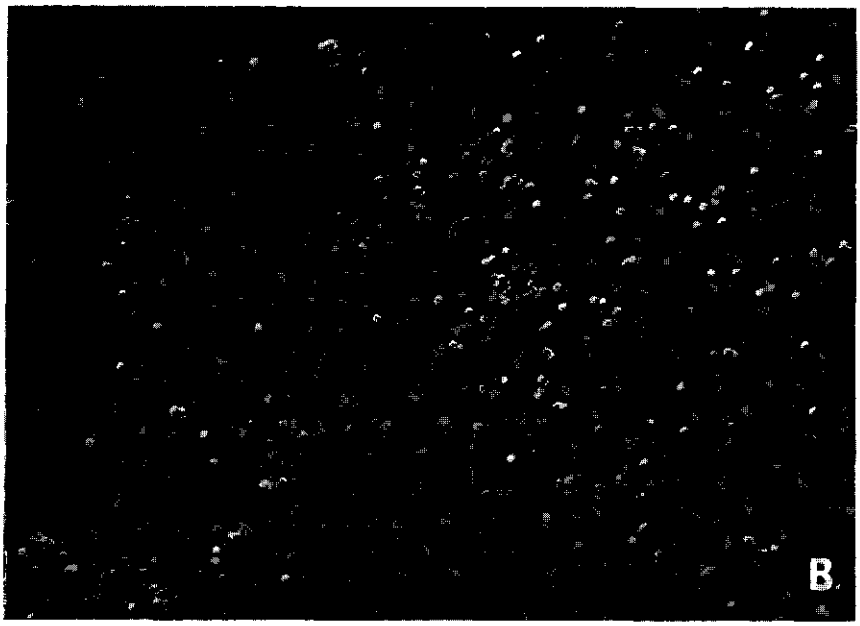


PHOTO 8. Electron micrograph of virus-like particles, shadow casted with palladium. Hexagonal contour, angularity of particles and the angular shadows can be noted. A Only few particles have a dimple. B. Most of the particles have dimples.

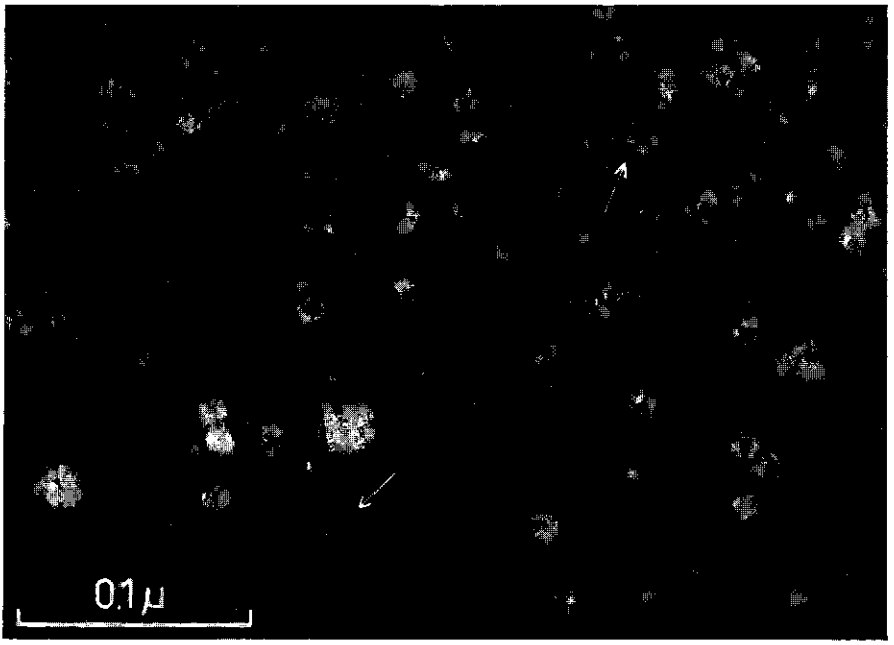


PHOTO 9. Electron micrograph of virus-like particles and a large number of small particles, negatively stained with potassium phosphotungstate (pH 5.5), Note the pentagonal particles (arrows).