Interaction between long and short particles of tobacco rattle virus

H. Huttinga

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Abstract

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Tobacco rattle virus is a rod-shaped multiparticle virus.

Short particles alone are not infectious, long ones are but give rise to the formation of incomplete virus. Mixtures of long and short particles induce the formation of complete virus. The interaction between long and short particles is not strain-specific: if long and short particles of different strains are inoculated together complete virus is also formed. These new strains have properties of both parent strains.

The interaction between heterologous long and short particles explains why there are so many different tobacco rattle virus isolates and why no correlation can be found between classifications based on different characteristics.

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STELLINGEN

I

De waarneming van Kassanis en Govier, dat bij gebruik van kleine aantallen bladluizen per plant een groter percentage hiervan aardappelaucubamozaïekvirus zou overbrengen dan bij gebruik van grote aantallen, is niet te verklaren uit de meer of mindere zorgvuldigheid waarmee kleine resp. grote aantallen bladluizen in dergelijke proeven worden behandeld.

Kassanis, B. & D. A. Govier, 1971. The role of the helper virus in aphid transmission of potato aucuba mosaic virus and potato virus C. J. gen. Virol. 13: 221–228.

II

Bij het bepalen van afmetingen van virusdeeltjes aan de hand van indooppreparaten moet meer aandacht worden besteed aan het indoopmedium dan tot nu toe gebruikelijk was.

Govier, D. A. & R. D. Woods, 1971. Changes induced by magnesium ions in the morphology of some plant viruses with filamentous particles. J. gen. Virol. 13: 127–132.

III

Er zijn voldoende argumenten om het kersebladrolvirus niet tot de groep van de Nepovirussen te rekenen.

Harrison, B. D., J. T. Finch, A. J. Gibbs, M. Hollings, R. J. Shepherd, V. Valenta & C. Wetter, 1971. Sixteen groups of plant viruses. Virology 41: 356–363.

IV

Het kweken van een aardappelras dat behalve vroegrijpheid een hoge graad van horizontale resistentie tegen *Phytophthora infestans* bezit is niet mogelijk.

V

Anderson's theorie dat de evolutie grotendeels afhangt van overdracht van genetisch materiaal door transductie met behulp van virussen gaat niet op voor planten.

Anderson, N. G., 1970. Evolutionary significance of virus. Nature 227: 1346–1347.

VI

Het gebruik van pentachloornitrobenzeen voor het bestrijden van *Streptomyces scabies* in de pootaardappelteelt dient te worden afgeraden.

Labruyère, R. E., 1971. Common scab and its control in seed-potato crops. Proefschrift Wageningen.

VII

Het bepalen van drempelwaarden voor O_3 en SO_2 met betrekking tot het veroorzaken van schade aan planten, zoals dat door Macdowall & Cole is gedaan, heeft geen zin.

Macdowall, F. D. H. & A. F. W. Cole, 1971. Threshold and synergistic damage to tobacco by ozone and sulfur dioxide. Atmospheric environment 5: 553–559.

VIII

Van de Pol's conclusie dat het bloeihormoon van *Silene armeria* L. buiten de plant onmiddellijk wordt geïnactiveerd, wordt onvoldoende gesteund door zijn experimenten.

Pol, P. A. van de, 1972. Floral induction, floral hormones and flowering. Proefschrift Wageningen

IX

De tegenspraak, die bestaat tussen de resultaten van Raccah et al. en die van Mittler & Kleinjan, met betrekking tot de vleugelvorming van *Myzus persicae*, is te wijten aan een niet-gerechtvaardigde proefopzet van de eerstgenoemde auteurs.

Mittler, T. E. & J. E. Kleinjan, 1970. Effect of artificial diet composition on wing-production by the aphid *Myzus persicae*. J. Insect Physiol. 16: 833–850.
Raccah, B., A. S. Tahori & S. W. Applebaum, 1971. Effect of nutritional factors in synthetic diet on increase of alate forms in *Myzus persicae*. J. Insect Physiol. 17: 1385–1390.

X

Teneinde kinderen een grotere bewegingsvrijheid binnenshuis te kunnen geven, verdient het aanbeveling reeds bij de bouw in kamers, die door de ontwerpers tot kinderkamers werden bestemd en in vertrekken die van het daglicht zijn afgesloten, zoals in sommige gevallen badkamers en toiletten, voor de electrische verlichting trekschakelaars in plaats van tuimelschakelaars te installeren.

XI

Jaarlijks worden de straten en de openbare groenvoorzieningen van Wageningen verontreinigd door ongeveer 42 ton faecaliën van honden.

Proefschrift van Harm Huttinga
Wageningen, 7 december 1972

Woord vooraf

Graag wil ik een aantal personen bedanken, die hebben bijgedragen tot het ontstaan van dit proefschrift.

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Curriculum vitae

Harm Huttinga werd op 23 augustus 1941 geboren te Erica. Hij bezocht na de lagere school het Gemeentelijk Lyceum te Emmen en behaalde in 1959 het einddiploma H.B.S.-B. In hetzelfde jaar ging hij studeren aan de Landbouwhogeschool te Wageningen en in 1963 deed hij kandidaatsexamen richting Planteziektenkunde. In 1966 behaalde hij het ingenieursdiploma (hoofdvak virologie; bijvakken biochemie, entomologie en planteveredeling). Vanaf augustus 1966 is hij als wetenschappelijk ambtenaar verbonden aan het Instituut voor Plantenziektenkundig Onderzoek te Wageningen.

Samenvatting

In dit proefschrift wordt de interactie tussen homologe en heterologe lange en korte deeltjes van 5 isolaten van tabaksratelvirus (TRV) en een isolaat van het vroegeverbruiningsvirus van de erwt (PEBV) beschreven.

Uit de introductie blijkt dat het onderzoek er op was gericht een antwoord te vinden op de vraag waarom er zoveel verschillende isolaten van beide virussen voorkomen en waarom er geen correlatie is te vinden tussen groeperingen van isolaten die respectievelijk gebaseerd zijn op symptomatologie, serologische eigenschappen en deeltjeslengten.

In hoofdstuk 2 wordt een overzicht van de literatuur over het onderzoek aan TRV en PEBV gegeven.

De methoden en het gebruikte materiaal zijn beschreven in hoofdstuk 3.

In hoofdstuk 4 werden de TRV-isolaten, Lisse, F12a, F15, F7 en F9, en het PEBV-isolaat Dik Trom 5 gekarakteriseerd. TRV-Lisse, F12a en F15 en het PEBV-isolaat zijn zogenaamde complete isolaten; ze hebben lange en korte nucleoproteïne deeltjes. F7 en F9 zijn incomplete isolaten, ze komen als vrij nucleïnezuur in de plant voor. Van deze isolaten werden de symptomen op *Nicotiana tabacum* 'White Burley', *N. tabacum* 'Xanthi', *N. rustica* en *Phaseolus vulgaris* 'Bataaf' beschreven. Op grond daarvan was het mogelijk onderscheid te maken enerzijds tussen de TRV-isolaten en PEBV-Dik Trom 5, anderzijds tussen de complete en incomplete isolaten van TRV. Binnen de groep van de complete en van de incomplete isolaten was onderscheid op grond van symptomen niet mogelijk. De serologische eigenschappen konden worden gebruikt om TRV en PEBV van elkaar te onderscheiden. De serologische verschillen tussen de complete isolaten van TRV waren echter te klein. Het aantonen ervan werd bovendien belemmerd doordat de micro-precipitatiemethode alleen maar op gezuiverd TRV kan worden toegepast .De agar-geldiffusiemethode, die normaliter een gevoelige toets is voor het aantonen van virus in ruw sap, kon niet worden gebruikt omdat de lange staafvormige TRV-deeltjes niet in de gel binnendringen. Pogingen om deze toets gevoeliger te maken voor TRV door de virusdeeltjes in fragmenten te breken door ultrasone trillingen of door inwerking van een detergens mislukten. Door ultrasone trillingen braken de lange deeltjes in stukken die ongeveer even groot zijn als de kleine deeltjes. De kleine deeltjes zelf bleven onaangetast. Het bleek dat de toets hierdoor twee keer zo gevoelig kon worden. Stukbreken van de deeltjes o.i.v. een detergens had een onaangenaam bijverschijnsel. Het antiserum vlokte nl. door het gebruikte middel, Leonil SA, spontaan uit.

Als de bentoniet-uitvlokkingstoets werd toegepast was het mogelijk om de aan-

wezigheid van TRV in lokale vlekken aan te tonen. Dit lukte niet met PEBV.

In hoofdstuk 4 wordt ook de zuivering van compleet TRV beschreven. Het bleek dat een klaring van het perssap met een ether-tetra mengsel gevolgd door differentieel centrifugeren de beste resultaten gaf. Voor de zuivering van de incomplete isolaten gebruikten we een fenol-methode voor de extractie van het totale nucleïnezuurgehalte uit het blad.

Het bleek dat compleet TRV-Lisse vijf dagen na de inoculatie in *N. rustica* een maximale concentratie bereikte. De incomplete vorm bereikte dit maximum twee dagen na de inoculatie.

In hoofdstuk 5 zijn de resultaten vermeld, die bereikt werden met de verschillende methoden om de virusdeeltjes van TRV en van PEBV van elkaar te scheiden. Moleculair zeven op kolommen gevuld met agarbolletjes bleek weinig succes op te leveren. De scheiding was slecht en de opbrengst was laag. Dit kon verklaard worden door het feit dat TRV-deeltjes staafvormig zijn. Lange en korte deeltjes konden beide binnendringen in poriën met diameters die iets groter zijn dan de lengte van de korte deeltjes en bedoeld waren om alleen de korte deeltjes door te laten. Dit bemoeilijkte de scheiding. Doordat de deeltjes waarschijnlijk in de lengterichting binnendrongen in poriën die een te kleine diameter hadden, kwamen ze daarin vast te zitten hetgeen de opbrengst ongunstig beïnvloedde.

Door gebruik te maken van het polyethyleenglycol (PEG)-NaCl systeem konden lange en korte deeltjes van TRV wel gescheiden worden neergeslagen. De lange deeltjes sloegen neer bij 3% PEG en 0,1 M NaCl. De korte bij veel hogere PEG-concentraties (bijv. 8–10%). Dit systeem kon ook in omgekeerde richting worden gebruikt. Als met 8% PEG en 0,08 M NaCl neergeslagen TRV-Lisse gedurende 20 min bij 10000 *g* werd gecentrifugeerd op een 10–40% suikergradiënt met daarin een daaraan tegengestelde 1–8% PEG-gradiënt, gingen de virusaggregaten bewegen tot ze aan een PEG-concentratie kwamen, waarbij ze in oplossing gingen. Op die plaats bleven ze dan hangen omdat de toegepaste centrifugaalkracht niet voldoende was om losse virusdeeltjes door de suikergradiënt te doen bewegen.

Centrifugeren op een dichtheidsgradiënt bleek de beste en de eenvoudigste methode te zijn om de korte en lange virusdeeltjes in redelijke hoeveelheden van elkaar te scheiden. Centrifugeren op een suikergradiënt kon zowel in een rotor met uitzwaaiende buizen als in een zonerotor met succes worden toegepast. Met de zonerotor was het mogelijk om hoeveelheden tot 100 mg per run te scheiden. Gebruikten we meer materiaal dan werd de scheiding minder goed en de opbrengst geringer omdat de pieken elkaar gingen overdekken. Meestal gebruikten we suikergradiënten die lineair waren bij het inpompen in de rotor. In een aantal gevallen pasten we een isokinetische gradiënt toe. Alhoewel deze gradiënt niet geheel was aangepast aan de vorm van het TRV gaf ze toch betere resultaten dan de lineaire gradiënten.

Hoofdstuk 6 beschrijft hoe de zuiverheid van gezuiverde viruspreparaten op verschillende manieren kon worden getoetst. Met behulp van de elektronenmicroscoop konden we snel de samenstelling van een viruspreparaat leren kennen. In preparaten van lange deeltjes vonden we echter vaak kortere deeltjes en het was erg moeilijk om

na te gaan of men hier te doen had met de korte deeltjes dan wel met fragmenten van de lange.

Door gebruik te maken van de analytische ultracentrifuge omzeilt men een gedeelte van deze problemen. Analytisch ultracentrifugeren was een snelle methode en al het materiaal kon worden teruggewonnen. Een nadeel ervan was de hoge drempelwaarde (0,01 %) van de Schlieren optiek. Verontreinigingen tot 5% werden daarmee niet opgemerkt, hetgeen ontoelaatbaar was omdat bleek dat 1,3% korte deeltjes in een lange deeltjes fractie maakte dat in 84% van de vlekken compleet virus ontstond.

De beste toets op de zuiverheid van de deeltjes was de biologische, waarbij we gebruik maakten van het feit dat de korte deeltjes niet infectieus zijn en de lange wel, en dat alleen lange èn korte deeltjes tezamen compleet virus kunnen vormen. De aard van het gevormde virus moest dan onderzocht worden door voldoende lokale vlekken te toetsen met de bentoniet-overdrachtstoets. Om er zeker van te zijn dat in de inocula geen inactieve brokstukken aanwezig waren, werden de preparaten vlak voor de inoculatie nog eens gecentrifugeerd op een suikergradiënt in een rotor met uitzwaaiende buizen.

Hoofdstuk 7 handelt over de methodes waarmee men onderscheid kan maken tussen complete en incomplete vormen van TRV en PEBV. Dit probleem kwam in feite neer op het onderscheiden tussen virus-nucleoproteïnedeeltjes en vrij virus -RNA. Dit kon door de te karakteriseren vorm wel of niet een behandeling, die de werking van ribonucleases remt, te geven. Wij verkozen toevoegen van bentoniet, boven bijvoorbeeld een behandeling met fenol of vloeibare stikstof, welke een te omslachtige procedure eisten. De bentoniet-overdrachtstoets bleek bijzonder geschikt om te bepalen welke vorm van het virus voorkwam in lokale vlekken in de plant. De ene helft van zo'n vlek werd vermalen in buffer, de andere in buffer waaraan 25 mg bentoniet/ml was toegevoegd. Bentoniet bond niet alleen ribonucleases waardoor het de overdracht van vrij virus-RNA bevorderde, het kon ook het manteleiwit van TRV binden, waardoor de overdracht van compleet virus werd geremd. Dankzij dit karakteristieke gedrag van complete en incomplete vormen met betrekking tot de overdracht met en zonder bentoniet, waren deze vormen erg goed van elkaar te onderscheiden. Als men met systemisch geïnfecteerde planten te maken had kon men op grond van symptomen beoordelen met welke vorm van het virus ze besmet waren.

In hoofdstuk 8 beschrijven we de biologische activiteit van de verschillende virusdeeltjes. Als korte deeltjes van TRV isolaten alléén werden geïnoculeerd bleken ze niet infectieus te zijn. Lange deeltjes waren dat wel maar ze gaven in dit geval aanleiding tot de vorming van incomplete virusvormen. Als lange en korte deeltjes tezamen werden geïnoculeerd, bleek dit mengsel infectieus te zijn en het was in staat om compleet virus te vormen. Voor de deeltjes van het PEBV isolaat gold hetzelfde.

De componenten van TRV-Lisse behoefden niet tegelijk te worden geïnoculeerd om de cel tot de vorming van compleet virus aan te zetten. Als de korte deeltjes eerst werden geïnoculeerd en we wachtten zeven uur met de inoculatie met de lange deeltjes, dan werd nog compleet virus gevormd. Als we echter veel langer wachtten, bijvoorbeeld 24 uur, dan werd incompleet virus gevormd. Wanneer de lange deeltjes het eerst

werden geïnoculeerd, konden de korte deeltjes dagen later worden geïnoculeerd, mits er voor gezorgd werd dat op een tijdstip werd geïnoculeerd dat de plant nog actief aan de virussynthese bezig was. Het bleek dat de grootste hoeveelheid compleet virus echter werd gevormd als de korte deeltjes twee dagen na de lange werden geïnoculeerd.

Als combinaties van lange en korte deeltjes van verschillende TRV-isolaten werden geïnoculeerd, werd ook compleet virus gevormd. Incomplete isolaten konden worden gecomplementeerd door het toevoegen van korte deeltjes van de complete isolaten. Een combinatie van homologe lange en korte deeltjes was met het oog op de produktie van compleet virus niet altijd beter dan een combinatie van heterologe deeltjes.

Ook een combinatie van componenten van TRV-Lisse en PEBV-Dik Trom 5 kon resulteren in de vorming van compleet virus. Dit zou er op kunnen wijzen dat deze twee virussen in werkelijkheid twee typen van hetzelfde virus zijn.

Met de gegevens uit hoofdstuk 8 in gedachte, was het niet moeilijk om te verklaren waarom er zoveel verschillende TRV isolaten worden gevonden. Telkens als een lang en een kort deeltje van verschillende isolaten tezamen een plant binnendringen wordt in feite een nieuwe stam gevormd, die voor een deel de eigenschappen bevat van het isolaat dat het korte deeltje leverde, zoals de serologische eigenschappen, en voor het andere deel de eigenschappen van het isolaat dat het lange deeltje leverde, zoals symptomen.

Bovendien verklaart dit waarom men geen correlatie vindt tussen groeperingen van TRV-stammen gebaseerd op kenmerken zoals serologische eigenschappen enerzijds, en symptomen of deeltjeslengten anderzijds. Dit zijn immers kenmerken die gecodeerd liggen in verschillende deeltjes, nl. lange en korte, die kunnen samenwerken ongeacht de aard van het virus-isolaat waaruit ze werden verkregen.

Contents

Abbreviations

A^{1cm}_{260nm}	absorbance at 260 nm, optical path 1 cm
AMV	alfalfa mosaic virus
EDTA	ethylenediaminetetraacetic acid
PCA buffer	0.18 M phosphate-citric acid buffer pH 7
PEBV	pea early-browning virus
PEG	polyethylene glycol 6000
PVP	polyvinylpyrrolidon
RNA	ribonucleic acid
RNase	ribonuclease
S°_{20}	sedimentation coefficient at infinite dilution and 20 °C
TMV	tobacco mosaic virus
TRV	tobacco rattle virus
UV	ultraviolet

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1 Introduction

Tobacco rattle virus (TRV) is a rod-shaped multiparticle virus. Many strains of it are known (Paul & Bode, 1955; Cadman & Harrison, 1959; Sänger, 1960 and 1961; van Hoof, 1964c; van Hoof et al., 1966, 1967; Harrison & Woods, 1966). Several attempts have been made to group these strains on the basis of symptomatology, particle length and serology (Harrison & Woods, 1966; van Hoof et al., 1966, 1967). It has not been possible, however, to correlate the grouping based on serology, and that based on symptoms or particle length.

Lister (1966) stated that only the long particles were infectious, and gave rise to the formation of what was called unstable virus, and consisted of free ribonucleic acid (RNA). Short particles were not infectious. Long and short particles together induced stable virus, consisting of nucleoprotein particles. Lister's explanation of these facts was that the code for the coat protein was in the RNA of the short particles. Frost et al. (1967) confirmed Lister's results and concluded that a strain-specific symbiotic interaction exists between the two particles of TRV.

If this interaction of TRV components is not strictly strain-specific, it should be possible to induce 'new' strains by inoculating mixtures of heterologous long and short particles. Such a strain would have properties of both parent strains. This type of interaction would explain why there are so many strains. It would also clarify the lack of correlation between groupings based on symptomatology, serology or particle length if these were characteristics coded on different particles. There was some indication in the article of Harrison & Woods (1966) that interaction might occur. These authors found a correlation between the length of the short particle and the serotype. This is illustrated in Table 1 which is composed from their data. It is possible that the strains within one serotype all have the same short particle but different long particles. The data of Harrison & Woods (1966) also strongly suggest that the genetic code for the coat protein is located in the short particle RNA. This was proved in elegant experiments by Sänger (1968a and 1968b) in which interaction between heterologous long and short particles of two isolates was demonstrated.

The aim of my work was to find out why there are so many different TRV isolates and why no correlation can be found between the above mentioned characteristics. To study interaction between virus particles, it is absolutely necessary to separate the particles satisfactorily, so considerable time and effort was spent in testing separation methods. Furthermore I looked for a quick and reliable test to differentiate between virus in free RNA form, which hereafter will be called incomplete virus, and virus appearing as nucleoprotein particles (complete virus).

Table 1. Serotypes and short particle lengths of TRV isolates (data by Harrison & Woods (1966)).

Virus isolate	Serotype	Length of the short particle
CAM	A	52 nm
PRN	B	78 nm
FLA	B	81 nm
ORE	B	79 nm
VH	C	55 nm
SAL	D	88 nm
PMK2	D	89 nm
BEL	D	92 nm
SP2	E	100 nm
SP1	F	114 nm
PMK1	F	106 nm

The separated particles of different isolates were tested for their biological activities and I looked for interaction between long and short particles of various isolates of TRV and also between particles of TRV and pea early-browning virus (PEBV). Moreover I tried to initiate an interaction between incomplete isolates and the short particles of complete isolates.

2 Review of the literature

2.1 TRV

TRV was originally described as the causal agent of the 'ratelziekte' of tobacco and so the virus got its name (Quanjer, 1943). Nowadays it is known that TRV has an enormous host range. It infects many cultivated plants (Rozendaal & van der Want, 1948; van der Want, 1951; Uschdraweit & Valentin, 1956; Schmelzer & Pop, 1957; van Slogteren, 1958; Gold et al., 1963; Paulus et al., 1963; Cremer & Kooistra, 1964; Corbett, 1967; Hakkaart, 1968; Komura et al., 1970). Many weeds can be infected too (Schmelzer, 1955b, 1957; Noordam, 1956).

TRV is a soilborne virus. In nature, it is dispersed mainly by nematodes (Sol et al., 1960), especially *Trichodorus* spp. (van Hoof, 1964a, 1964b and 1968). According to van Hoof (1968) a high specific relationship exists between the isolate of the nematode vector and the virus isolate it is able to transmit. Taylor & Robertson (1970) suggested that this specific relationship is determined by a specific adsorption of the virus to the cuticle of the digestive tract. Another natural way in which TRV is spread is by seed (Lister & Murant, 1967). Dissemination of nematode-borne viruses in seed could explain why these viruses, despite having vectors that seem unable to transport them over a distance, are widespread, although scattered here and there (Murant & Lister, 1967). TRV is also readily spread mechanically with homogenates from infected plants or infected nematodes (Sänger et al., 1962) and it can be transmitted by dodder (Schmelzer, 1955a; van der Want, 1955), and by grafting (Rozendaal & van der Want, 1948).

With the electron microscope van der Want & Rozendaal (1948) saw that TRV was rod-shaped and had a bimodal length distribution. Paul & Bode (1955) confirmed this and reported main lengths of 70 and 180 nm. Köhler (1956b) explained the existence of the two particles by assuming that TRV was continuously formed as a long rod, breaking at preconditioned points. It also has been suggested that the short particles may be degradation products of the long particles or additional products of the infected cell (Harrison & Nixon, 1959; Sänger, 1960, 1961). There is, however, no simple correlation between the different lengths of the virus particles that could explain that the short particles are fragments of the longer ones. Furthermore TRV occurs as tubular particles with different lengths in thin sections of infected leaf tissue (de Zoeten, 1966). Harrison & Woods (1966) even listed TRV isolates with 2, 3 and 4 modal lengths. Both long and short particles contain 5% RNA, both are serologically identical and both are always found together (Harrison & Nixon, 1959). TRV

must be considered as a multiparticle virus (Bancroft, 1968; Kassanis, 1968; Sänger 1968b; Matthews, 1970).

Besides the peculiar length distribution, TRV showed a strange behaviour with respect to infectivity. Köhler (1956a) was the first to report isolates with aberrant symptoms which he called 'Wintertyp' isolates. Similar isolates were reported by Brandenburg et al. (1959), Cadman (1959), Cadman & Harrison (1959), Eibner (1959) and Sänger (1960, 1961). Such isolates were poorly transmissible and no virus particles could be found with electron microscopy. These isolates could be obtained by subculturing poorly infective local lesions produced with dilute TRV suspensions. Undiluted inocula produced lesions containing normal TRV. The dilution effect was a unique feature among plant viruses.

The poor transmissibility of these isolates could be related to the instability of their infectious agent, which is rapidly inactivated in sap. Sänger & Brandenburg (1961) tried direct phenol extraction of plants with 'Wintertyp' symptoms. From these experiments it was concluded that 'Wintertyp'-TRV occurred in the plants as free RNA, lacking coat protein. Cadman (1962) confirmed these results.

There have been many speculations about the origin of the aberrant forms of TRV. Köhler (1956a) first explained the formation of aberrant symptoms, by assuming a reversible, season-induced change in virulence of normal TRV. Later on he considered the two particles as two viruses, that each could inhibit the other by premunition (Köhler, 1960). Cadman & Harrison (1959) tried to explain the poor transmissibility of the aberrant form by assuming a quantitative difference in virus synthesis. They distinguished between 'multiplying' (M) forms which could readily be transmitted, and 'non-multiplying' (NM) forms that could only be transmitted with great difficulty. Sänger (1960, 1961) assumed that unstable TRV is the progeny of what we would now call coat-protein mutants. This suggestion would imply an unusually high mutation rate of the parent TRV. Cadman (1962) supposed that both types of TRV were multiplied at equal rates in the cell nuclei, but that the aberrant form could not escape from the nuclei ('non-escaping' virus). However, the occurrence of aberrant TRV is based on the unique feature of its multiparticle character. Brandenburg et al. (1959) were the first to recognize this. They assumed that normal TRV infections were the result of the two TRV particles, and that the 'Wintertyp' should be effected by one particle: the short one. However, Harrison & Nixon (1959) after having separated the particles on sucrose gradients, found that only the long particles are infectious, whilst the short ones are not. The number of local lesions is not influenced by the number of short particles in the inoculum. Sänger (1960) confirmed that only the long particles are infectious.

In 1964, Bawden put forward the hypothesis that infectivity and ability to synthesize a given protein are properties conferred by different parts of the longer particle, with the shorter rod containing the part able to code for the protein but lacking the part that confers infectivity. Lister (1966) was the first to suggest a functional heterogeneity between the two particles. He found that inocula containing purified long particles

predominantly produce lesions containing unstable TRV, whereas lesions containing stable TRV were obtained only when both long and non-infectious short particles were involved in infections. He stated that '...the simplest explanation of this system would be that the RNA of the long particle of the TRV type is deficient in the information required for some stage of the process leading to the enrobement of the viral RNA with virus protein; possibly the coding for the virus protein itself'. Frost et al. (1967) tested this interpretation. Their results provided quantitative support for both the findings and hypothesis of Lister (1966), and the hypothesis of Bawden (1964). They concluded that 'each isolate of tobacco rattle virus seems to be a system of two or more pieces of infective nucleic acid interacting specifically in a symbiotic manner' (Frost et al., 1967). Sänger (1968a, 1968b) proved that heterologous long and short particles can interact and that the code of the coat protein in fact is located in the short particle RNA. Sänger used two TRV isolates, TRV-USA and TRV-GER. Long particles of these isolates gave rise to the formation of unstable virus which appeared as free RNA. This form will be referred to as incomplete virus. Short particles alone were not infectious. Long and short particles together gave rise to normal TRV, with the characteristic long and short nucleoprotein particles. This will be called complete virus. Mixtures of heterologous long and short particles also induced the formation of complete virus. Because the serological properties of the virus induced by a mixture of heterologous long and short particles, were the same as those of the isolate that furnished the short particles, it could be concluded that the code for the coat protein is located in the short particle RNA. Similar results were reported by Semancik & Kajiyama (1968), Lister (1969) and Lister & Bracker (1969).

2.2 PEBV

The pea early-browning disease was first described in the Netherlands (Bos & van der Want, 1962). Some years later it was reported from Britain (Gibbs & Harrison, 1964) and Belgium (Verhoyen & Goethals, 1967). The disease is caused by a soilborne and seedborne virus: pea early-browning virus. Besides the pea, PEBV can infect many other plants, among them cultivated crops, ornamental plants and weeds (Bos & van der Want, 1962; Bos, 1963; Hubbeling & Kooistra, 1963; Gibbs & Harrison, 1964; Harrison, 1966).

PEBV, as a soilborne virus, is transmitted by *Trichodorus* spp. (van Hoof, 1962). It can also be transmitted by seed and mechanically.

PEBV closely resembles TRV in many characteristics. Bos & van der Want (1962) demonstrated that most of its biological properties are similar to that of TRV. They also found that PEBV has two tubular particles, with lengths of 105 and 210 nm. So PEBV and TRV differ in absolute lengths and in the ratio average length of short particles to average length of long particles. This ratio is 1:2 and 1:2.5, respectively. The same authors found no serological relationship between PEBV and TRV strains. This was confirmed by Gibbs & Harrison (1964). However, Maat (1963) demonstrated a distant serological relationship. Allen (1967) came to the same conclusion. The

PEBV particles have a diameter of about 20 nm (Gibbs & Harrison, 1964).

From PEBV also incomplete and complete forms are known (Gibbs & Harrison, 1964). The incomplete forms can not readily be transmitted with sap as inoculum. But in extracts made from leaves with water-saturated phenol or bentonite PEBV can be transmitted easily. The sap of these isolates contains no tubular particles, in contrast with sap of normal isolates. As in the case with TRV, only the long particles of PEBV are infectious, the short particles are not. Only an infection with both long and short particles can give rise to formation of normal virus particles (Lister, 1966; Sänger, 1968a; Huttinga 1969). Furthermore Lister (1967) found that the symptoms of PEBV on beans are determined by information present in the RNA of particles of different lengths. Huttinga (1969) postulated that between the particles of PEBV there exists the same interaction as between particles of TRV.

3 Materials and methods

In this chapter only materials and methods will be described which were used frequently in routine procedures. Other methods will be given in the appropriate chapters.

3.1 Plant material

Phaseolus vulgaris 'Bataaf' and *Pisum sativum* 'Koroza' were sown in pots and used after 10 to 20 days without any further treatment.

Nicotiana rustica L., *Nicotiana tabacum* 'Xanthi', and *Nicotiana tabacum* 'White Burley', were first sown in seed pans. After about three weeks the seedlings were pricked off into boxes and after another three weeks the plants were potted. Seven to nine weeks after sowing the plants were used. I do not know the name of the *N. rustica* L. variety used. The variety has been cultured for many years in the greenhouses. However, it is closely related to *N. rustica pumila* described by Setchell (1912) and Goodspeed (1954).

All plants were grown in a potting soil for floriculture consisting of a mixture of pre-frozen peat and clay (mixed in the ratio 7:3 (v/v)). This soil was obtained from Trio, Vroomshoop. It is marketed as Trio 17 Special.

During the whole growing period the plants were kept in a greenhouse, at an air temperature of about 20 °C. The atmospheric humidity was about 70%. In summer the greenhouse was shaded. In winter, additional light was given for 16 h a day, by mounting 6 Philips TFL 33 lamps of 65 W 50 cm above the bench on which the plants were grown.

3.2 Virus isolates

Five isolates of TRV and one isolate of PEBV were used. One isolate, TRV-Lisse, was originally obtained from a sandy soil at Lisse. From this soil *Trichodorus pachydermis* Seinhorst were extracted and these nematodes were able to infect healthy 'White Burley' plants (Sol & Seinhorst, 1961). Since 1961 this isolate has been maintained on tobacco by mechanical transmission in the greenhouse. The other TRV isolates, TRV-F7, TRV-F9, TRV-F12a, and TRV-F15, were obtained from Dr H. A. van Hoof, who isolated them from soil collected from potato fields in the north-west of France (No. 7, 9, and 12a) and in Belgium (No. 15), by planting bait seedlings in these soils (van Hoof et al., 1967). The PEBV isolate, designated Dik Trom 5, was

also obtained from Dr H. A. van Hoof, who isolated it from a dried seed of the pea cultivar Dik Trom (van Hoof, 1969).

3.2.1 Virus cultures

All isolates used were progapated on *N. rustica*. They were transmitted mechanically. The complete isolates were transmitted by grinding some infected material in the same amount (w/v) of 0.18 M phosphate-citric acid buffer pH 7 (PCA buffer) and rubbing this homogenate on plants dusted with 500-mesh carborundum. For the incomplete isolates it was necessary to grind the infected material in an equal amount (w/v) of buffer to which 25 mg bentonite/ml was added. The homogenate was then used as inoculum as described above. After inoculation the leaves were rinsed with tap water to remove cell debris and excess carborundum powder.

Virus assays were done on the local lesion host *P. vulgaris* 'Bataaf'. Primary leaves that just were fully expanded, appeared to be optimally sensitive.

3.3 Virus purification

3.3.1 Purification of complete virus

Multiplication of the virus to be purified took always place in *N. rustica* unless otherwise stated.

Ether-tetra method. The method described by Maat (1963) was slightly modified. The infected leaf material was ground immediately after harvesting in a Waring blendor in a mixture of half the amount (v/w) of PCA buffer containing 0.1 % thioglycollic acid, and a quarter of the amount (v/w) diethyl ether plus a quarter of the amount (v/w) carbon tetrachloride. The homogenate was centrifuged for 20 min at 4000 × *g*. The upper phase was decanted and centrifuged for 1½ h at 105000 × *g*. The pellets were resuspended in a quarter of the original sap volume of PCA buffer, and left for one night at 4 °C. After centrifuging again at low and high speeds (20 min 4000 × *g* and 1½ h 105000 × *g*), the virus was resuspended in 1/100 of the original sap volume of PCA buffer.

Polyethylene glycol method. This purification method, based on specific precipitation using polyethylene glycol 6000 (PEG), was first described by Hebert (1963). I used the specific precipitation with PEG for the separation of the long and short virus particles too (see 5.2). Infected tobacco leaf material was ground, immediately after harvesting, in an equal amount (v/w) of PCA buffer to which 0.1 % thioglycollic acid was added. The homogenate was centrifuged for 10 min at 12100 × *g*. To the supernatant PEG and NaCl were added to a concentration of 8 % and 0.2 M, respectively. The precipitate was collected by centrifuging for 15 min at 12100 × *g*. The pellet was resuspended in 1/10 of the original amount (v/w) of PCA buffer and after about half

an hour centrifuged for 10 min at 12100 × *g*. The pellet was resuspended another time in 1/10 of the original amount (v/w) of PCA buffer. After centrifuging again, the two supernatants, containing the virus, were combined. The suspension was still green. By adding PEG and NaCl to 8% and 0.2 M, respectively, centrifuging for 15 min at 12100 × *g*, and resuspending the pellet in PCA buffer, this suspension was concentrated to about 1/100 of the original amount of leaf material (v/w). The concentrated suspension remained greenish.

3.3.2 Isolation of incomplete virus

The incomplete virus was isolated with methods for the extraction of the total nucleic acid content of tobacco leaves.

Phenol-bentonite method. 100 g of infected leaves were homogenized in 100 ml buffer A and 200 ml water-saturated phenol. Buffer A contained 0.1 M glycine-NaOH pH 9.5, 0.1 M NaCl, 0.005 M Na_3EDTA, 1% sodium dodecylsulphate and 1% sodium bentonite. The bentonite suspension was prepared as described in section 3.8.1. The leaves were homogenized for 2 min in a Waring blendor at low speed. The components of the emulsion were separated by centrifuging (5 min at 6000 × *g*). The aqueous phase was stored for a while and the phenol phase and interphase again extracted with 100 ml buffer A as described. The two aqueous phases were combined and extracted twice with an equal volume of water-saturated phenol. The aqueous phase was then freed from phenol by three extractions with diethyl ether. Excess ether was removed by passing nitrogen through the solution. After two hours of dialysis against distilled water, the dialysed solution was treated in one of the following two ways:
1. Two volumes of cold ethanol were added. The mixture was stored for some time in a freezer. The precipitate was collected by centrifuging and resuspended in 0.01 M phosphate buffer pH 7 containing 0.005 M Na_3EDTA.
2. One volume 0.2 M sodium acetate and one volume 1% cetyltrimethylammonium-bromide were added. The mixture was kept on ice for 3 min. The precipitate was collected by centrifuging and by repeated (3×) washing with 0.1 M sodium acetate in 70% alcohol converted into the sodium salt.

Phenol-bentonite-diethylpyrocarbonate method. This method is almost the same as that described by van Griensven & van Kammen (1969). The only difference is that 3% diethylpyrocarbonate was added to the buffer solution in which the 15000 × *g* pellet was resuspended. Polysaccharides were removed using 2-methoxy methanol according to Ralph & Bellamy (1964).

3.4 Determination of virus concentrations

In this study all virus concentrations were determined by spectrophotometry. $A_{260\ nm}^{1\ cm} = 2.15$ corresponded with 1 mg virus/ml. This coefficient was determined as

follows. A TRV-Lisse suspension, purified by the ether-tetra method, was given an additional purification by density-gradient centrifuging in the zonal rotor (see 5.3.1). The virus particles were collected and concentrated by centrifuging for 3 h at 105000 $\times$ *g*. The pellets were resuspended in a small volume of PCA buffer and dialysed overnight against PCA buffer. From the dialysed virus suspension which had an A_{280}/A_{260} value of 0.86, 1 ml portions were dried and weighed, keeping the amount of buffer constituents in consideration. Dilutions were made of the virus suspension and from these dilutions $A\ {}^{1\,cm}_{260\,nm}$ was measured using the dialysis buffer as a standard. In Fig. 1 $A\ {}^{1\,cm}_{260\,nm}$ is plotted against the virus concentration. From the slope of the line it can be seen that at a concentration of 1 mg virus/ml $A\ {}^{1\,cm}_{260\,nm} = 2.15$. I assumed that this value could be used for all TRV isolates. This assumption was based on the fact that all TRV isolates investigated sofar, contain about 5% RNA (Harrison & Nixon, 1959; Semancik, 1966).

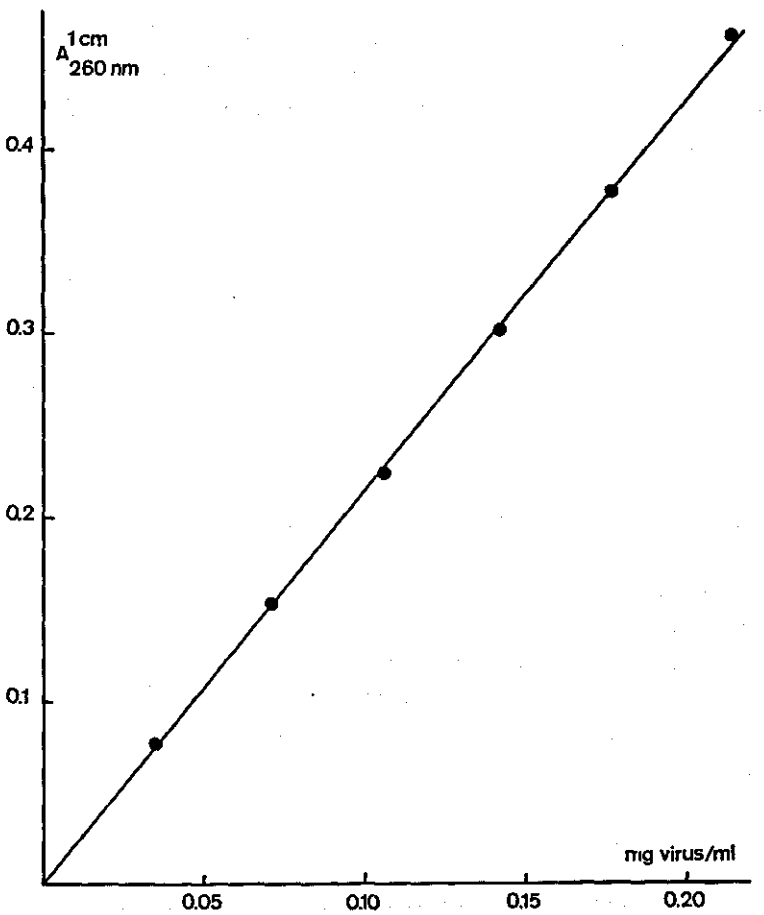

Fig. 1. Relationship between $A_{260}\ {}^{1\,cm}_{nm}$ and the concentration of a TRV-Lisse suspension.

3.5 Determination of sedimentation coefficients

Sedimentation coefficients were determined with an analytical ultracentrifuge Spinco Model E, with Schlieren optics. The viruses were suspended in PCA buffer. The rotor temperature was kept at 20 °C. After measuring the displacement of the peaks on the photographs I used the graphical method of Markham (1960) to determine the S values.

At first a series of concentrations of each virus was spun and from these the sedimentation coefficient at infinite dilution S° was found by extrapolation to concentration zero. As it was found that at concentrations below 10 mg/ml the S values were independent of concentration (see 4.6), they were always determined with a suspension containing 2–3 mg virus/ml.

3.6 Electron microscopy

Electron microscopy was used to measure the particle lengths and to determine whether a virus fraction was pure or not. The copper grids I used had a grating of 150 mesh and were covered with Formvar film. The preparations were examined with a Siemens Elmiskop I electron microscope.

3.6.1 Preparation of specimens for electron microscopy

Small drops of virus suspension were placed on the grids, and dried at room temperature. If the virus had been suspended in buffer solution the buffer salts were removed by floating the grids upside down on de-ionized water for 10 min. After drying at room temperature, the specimens were shadow-cast with palladium at an angle of 30°.

Apart from shadow-casting negative staining was also used. Then a drop of a virus suspension in water was mixed on the grid with a drop of 2% potassium phosphotungstate pH 7. Excess liquid was removed and the preparations were examined immediately afterwards.

Sometimes I used the dip method described by Brandes (1957) to prepare the specimens. In this case the preparations were shadow-cast as described above. In a few cases I used a modification of the dip method of Hitchborn & Hills (1965). Freshly cut leaf-tissue was then dipped in 2% potassium phosphotungstate pH 7 to obtain negatively stained preparations. After removing excess liquid these preparations were examined immediately.

3.6.2 Measurement of particle lengths

If particle lengths of different isolates had to be compared pictures were taken without changing the magnification factor of the electron microscope. Tobacco mosaic virus (TMV) particles were added as an internal standard. Measurements were made

directly from the negatives obtained by the electron-microscope camera, using a binocular microscope equiped with a measuring ocular. In this way the length of the particles could be determined on the negatives with an accuracy of 0.1 mm. The total measuring accuracy therefore was 5 nm.

3.7 Density-gradient centrifuging in cesium chloride

Tubes of the Spinco SW 39 L rotor were filled with 2.25 ml of a CsCl solution (6.23 g CsCl/10 ml water), 0.75 ml water, 0.1 ml of a virus suspension, and 2 ml paraffin oil. The tubes were spun overnight at 110000 $\times$ *g* at 5°C. The virus bands were visualized by light scattering. The position of the virus bands in the tube was determined by measuring the distance from the middle of the band to the bottom of the tube.

3.8 The preparation of bentonite suspensions

3.8.1 Bentonite suspensions used in the RNA purification methods

Three grams of bentonite (Technical bentonite powder; British Drug Houses) were suspended in 100 ml 0.01 M sodium phosphate buffer pH 7, using a Waring blendor. The suspension was centrifuged at 2000 $\times$ *g* for 10 min. The pellet was discarded. The supernatant was centrifuged at 10000 $\times$ *g*. The pellet was resuspended in 0.01 M sodium phosphate buffer pH 7. This differential centrifuging was repeated twice. At last the pellet was resuspended in the buffer to make a 3–4% (w/v) solution. The bentonite concentration was measured by determining the dry weight of 1 ml samples.

3.8.2 Bentonite suspensions used in the transmission of virus

Technical bentonite was, without any pretreatment, suspended in PCA buffer, using a Waring blendor. The suspension was stirred just before use. Unless otherwise stated a bentonite concentration of 25 mg/ml was used.

4 Characterization of the virus isolates

The viruses and the virus isolates already mentioned in section 3.2 will be described in more detail in this chapter.

The PEBV isolate, Dik Trom 5, could readily be transmitted in buffer, and showed the characteristic long and short nucleoprotein particles in the electron microscope. Thus it can be described as a complete PEBV isolate.

TRV-Lisse, F12a, and F15 were also easy to transfer mechanically. In plants, infected by these viruses, nucleoprotein particles could be demonstrated and serology was possible. Therefore these isolates are also complete.

TRV-F7 and F9 could only be transmitted mechanically with a phenol extract of infected material (3.3.2) or an inoculum prepared in buffer containing bentonite (3.2.1). When both isolates were investigated with the electron microscope no nucleoprotein particles could be found. These isolates thus have to be considered as incomplete. They occur as free nucleic acid in the host plant.

4.1 Symptomatology

The symptoms of PEBV and TRV on a large number of hosts have been described by Bos & van der Want (1962). The most striking difference between symptoms of TRV-Lisse and PEBV-Dik Trom 5 were those on *Phaseolus vulgaris* and *Pisum sativum*. TRV-Lisse caused a local reaction on the primary leaves of bean, resulting in small necrotic black-brownish lesions, while PEBV-Dik Trom 5 caused necrotic brown rings with a diameter of 2–3 mm on these leaves (Fig. 2). Furthermore bean became systemically infected by PEBV, the secondary leaves first showing a light green mottling. When the leaves expanded the spots spread and an irregular pattern of light and dark green areas was formed. The infected leaves showed distortion (Fig. 3). TRV-Lisse did not become systemic in bean.

TRV-Lisse only caused local symptoms on pea, which consisted of small brown lesions, usually surrounded by a light green area. PEBV caused large necrotic rings and the plants became systemically infected showing necrosis all over. The growth of the infected plants was severely retarded and their tops were stunted and distorted. The symptoms of F12a, F15, F7 and F9 on pea were the same as described for TRV-Lisse.

The symptoms of all five TRV isolates on bean were the same. They all caused small necrotic local lesions. There was no difference in symptoms on bean between complete and incomplete isolates.

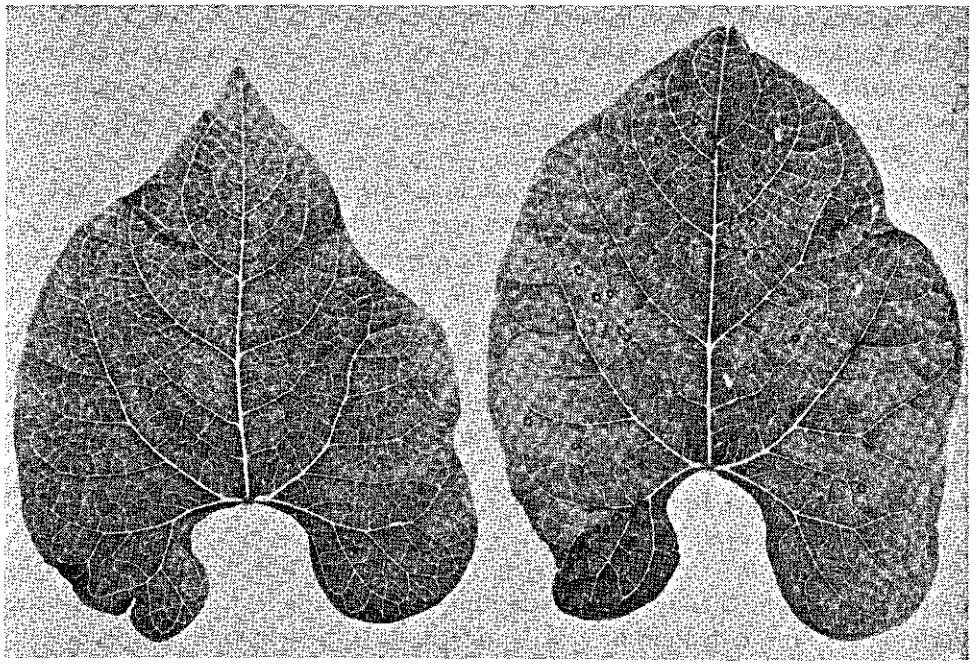

Fig. 2. Symptoms of TRV-Lisse (left) and PEBV-Dik Trom 5 (right) on primary leaves of *P. vulgaris* 'Bataaf'.

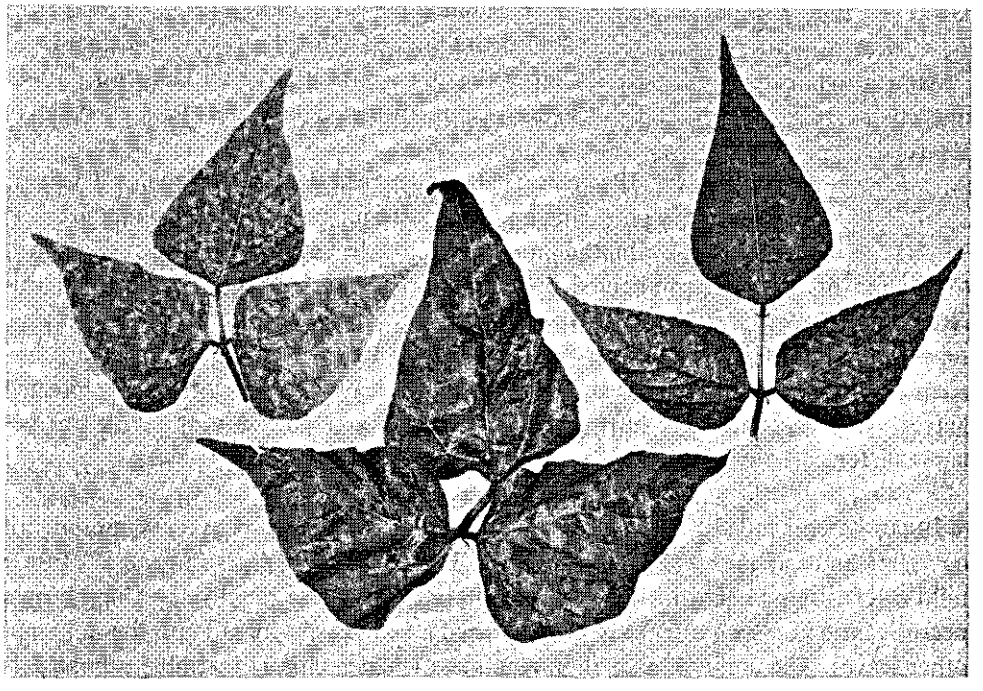

Fig. 3. Symptoms of PEBV-Dik Trom 5 on secondary leaves of *P. vulgaris* 'Bataaf'.

Symptoms caused by complete and incomplete isolates on *N. tabacum* 'Xanthi' and 'White Burley' and *N. rustica* differed considerably. The symptoms of the incomplete isolates, F7 and F9, and the complete ones, F12a and F15 on these hosts will be described in detail.

Incomplete isolates, F7 and F9. The symptoms of F9 closely resembled those of F7.
N. rustica. On the inoculated leaves brown necrotic spots, at the beginning small and with an irregular shape, appeared 3–5 days after inoculation. These lesions spread rather rapidly and if they reached a vein, they readily spread along it. Via the stem, which sometimes showed brown stripes, other leaves could be infected. In these leaves the necrosis spread from the veins. The leaves became distorted. Younger leaves sometimes escaped infection.
N. tabacum 'White Burley'. Three to five days after inoculation large necrotic spots, which spread irregularly preferentially along the veins, appeared on the inoculated leaves. Via the stem younger leaves could be infected on which large necrotic regions were also formed.
N. tabacum 'Xanthi'. The inoculated leaves showed small clear spots, which later on became necrotic. The symptoms spread irregularly as in 'White Burley' tobacco, however, they proceeded much slower. Younger leaves were not always systemically infected.

Complete isolates, F12a and F15. The symptoms of F15 resembled those of F12a. However, they were less violent and appeared somewhat later.
N. rustica. A light brown pattern of rings and lines developed on both inoculated and systemically infected leaves. On the latter this pattern occurred after chlorosis. Subsequently necrosis occurred, the leaves became distorted and growth was strongly retarded.
N. tabacum 'White Burley'. 'Partridge pattern' appeared on the inoculated leaves: small light brown necrotic spots, ring and line pattern, sometimes also white necrotic rings (2–3 mm diameter). On the non-inoculated leaves yellow rings developed (5–10 mm diameter) accompanied by a light brown necrotic pattern. Growth was retarded.
N. tabacum 'Xanthi'. Fine brown pattern of rings, but also white necrotic rings surrounded by brown rings appeared on the inoculated leaves. In the systemically infected leaves white necrotic rings surrounding dark green areas developed. The leaves became distorted.

It can be concluded that there is a characteristic difference between symptoms of complete and incomplete TRV on tobacco. The complete isolates in general cause a mild chlorosis and necrosis, while the incomplete ones cause rapidly spreading brown necrotic lesions, which after they have reached a vein, easily spread into the stem and other leaves. The symptoms of the incomplete isolates closely resembled those described by Köhler (1956a) as 'Wintertyp'.

The symptoms are greatly influenced by the climatic conditions under which the plants are grown. Characteristic symptoms are developed for both TRV and PEBV isolates, at temperatures ranging from 15–20 °C, and a relatively high light intensity.

Due to the fluctuations in symptoms caused by differences in growing conditions of the plants, it was not possible to differentiate between the complete TRV isolates. The same holds for the incomplete ones.

4.2 The multiplication of complete TRV and PEBV

In order to determine the growth curve of complete TRV, I carried out purifications at different times after inoculation.

From *N. rustica* all leaves were inoculated with TRV-Lisse. At different times after inoculation I harvested the leaves of 20 plants and purified the virus according to the ether-tetra method (see section 3.3.1). Virus concentrations were determined by spectrophotometry (3.4). The results of this experiment are shown in Fig. 4. It can be seen that although the amount of leaf material kept increasing in the period tested, the amount of virus that could be isolated was optimum 5 days after inoculation. In this particular case an optimum of 21.8 mg virus/100 g infected leaf material was reached. After the sixth day there was a fast decrease in the virus concentration in the plant.

The maximum virus concentration was reached just after the first symptoms had appeared, about 4–5 days after inoculation. The amount of virus that could be isolated from a plant decreased rapidly when the leaves became necrotic. So in general the infected leaves were harvested a day before they could be expected to become necrotic.

During routine experiments I obtained 10–20 mg virus/100 g leaf material with TRV isolates. Although this amount depended on the quality of the plants and the environmental conditions. Young, well growing plants gave higher yields than older ones. In winter the yields were low due to the low light intensity. In summer the temperature in the greenhouses was too high. Highest yields were obtained in spring and somewhat less in autumn. The yields of TRV purification found in literature are generally lower. De Zoeten & Shalla (1966) obtained 50 mg virus/kg leaves, Harrison & Nixon (1959) 50 mg virus/l sap, and with an improved method Harrison & Woods (1966) obtained 20–160 mg virus/l sap. So it can be concluded that my purification procedure was very efficient for the isolation of TRV.

When the virus was purified using PEG and NaCl and compared with the results of the ether-tetra method, it was found that when *N. rustica* was infected with TRV-Lisse the yields of the PEG-NaCl method were 1/8 of that of the ether-tetra method. However, in this particular experiment, the value of A_{280}/A_{260} for virus purified by PEG precipitation was 0.81 and that for virus obtained by the ether-tetra method was 0.62. According to Semancik (1966) this coefficient should be 0.87 for a virus containing 5% RNA.

The multiplication of TRV-F15 closely resembled that of TRV-Lisse. There was no difference in yields between Lisse and F15. With TRV-F12a 15–20% higher yields

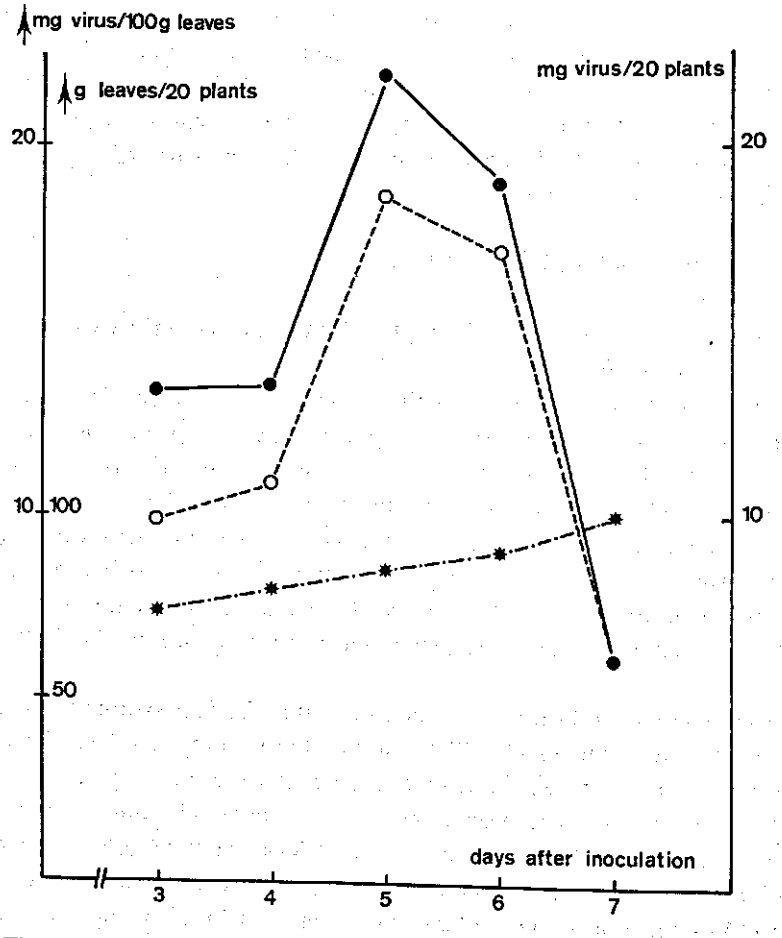

Fig. 4. Yields of purified virus from *N. rustica* infected with TRV-Lisse obtained at different times after inoculation.
——yields in mg virus/100 g leaves
– – – yields in mg virus/leaves of 20 plants
-·-·- g leaves/20 plants

were obtained and the maximum of the growth curve was reached one day earlier.

With PEBV-Dik Trom 5 the first symptoms appeared 10–14 days after inoculation. If the virus was purified according to the ether-tetra method just after the symptoms had appeared the yields varied between 5 and 8 mg virus/100 g leaf material.

4.3 The multiplication of incomplete TRV

To determine the growth curve of unstable virus I did the following experiment. At t = 0, 120 *N. rustica* plants were inoculated with long particles of TRV-Lisse (0.02 mg/ml) in PCA buffer. At t = 4, 28, 52, 76, 100, and 172 h, 20 g of leaves were

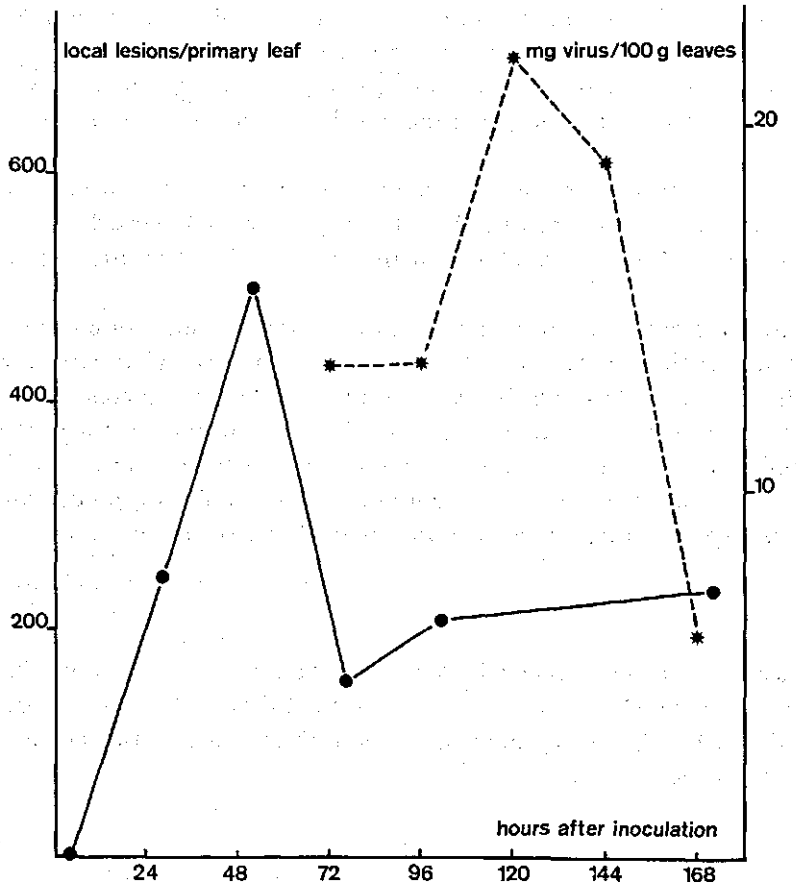

Fig. 5. Growth curves of complete and incomplete TRV-Lisse in *N. rustica*.
—— incomplete TRV-Lisse (local lesions/primary leaf). From 20 g of leaves the total RNA was extracted and dissolved in 2 ml buffer. The primary leaves of bean were inoculated uniformly with this solution.
- - - - complete TRV-Lisse (mg virus/100 g leaves). The complete virus was purified according to the ether-tetra method and concentrations were determined by spectrophotometry.

harvested at random from 20 plants and total RNA was purified from it according to Semancik & Odening (1969). With a Waring blendor 20 g of leaf material were homogenized in 2 ml 5% sodium lauryl sulphate, 8 ml 0.01 M tris, pH 7.4, 2 drops 0.1 M EDTA, pH 7.0, and 40 ml water-saturated phenol. After low-speed centrifuging, the aqueous phase was re-extracted with phenol, centrifuged, and 2 volumes of ice-cold 96% ethanol were added to the aqueous phase. The pellets from low-speed centrifuging were each dissolved in 2 ml of 0.01 M tris buffer, pH 7.4, containing 0.01 M KCl and 10^{-4} M $MgCl_2$, and dialysed overnight. The preparations were stored at —4°C for 24 h. After thawing and centrifuging for 10 min at 10000 × *g*, the samples

were tested for infectivity by inoculating them, without further dilution, on primary leaves of *P. vulgaris* 'Bataaf'. Inoculation was done very carefully so that the leaves were uniformly covered with inoculum. The extraction of total RNA was done in duplicate. Tests on bean were done in quadruplicate. The results of the local lesion test are given in Fig. 5.

From a very small quantity at 4 h after inoculation, the total amount of incomplete virus rapidly increased to a maximum after about two days. Then it decreased quickly until it became constant. The first symptoms usually appeared 3–5 days after inoculation.

In Fig. 5 I plotted the results together with the growth curve obtained for complete TRV-Lisse (see 4.2). Although the amounts of incomplete and complete virus are expressed in different parameters and the experiments were done at different times, they may be compared directly, because there is a linear correlation between the amount of complete virus in an inoculum and the number of lesions caused by it (Sänger, 1968a). It is evident that the maximum in complete TRV-Lisse production was reached three days later than the peak in the incomplete virus production.

4.4 Serology

The antisera used were kindly supplied by Mr D. Z. Maat from his collection. The TRV antiserum was prepared against TRV-Lisse and the PEBV antiserum was prepared against PEBV-E116. In the homologous reactions these antisera originally had titres of 4096 and 2048 respectively.

4.4.1 Micro precipitin test under paraffin oil

This test was as originally described by van Slogteren (1955). The micro preciptin test can not be used for TRV and PEBV in crude sap, therefore I had to use purified virus. Series of antisera dilutions were tested against series of dilutions of virus suspension (17 mg/ml).

I could differentiate between TRV-Lisse and PEBV-Dik Trom 5 with this method. The titre of the TRV-Lisse antiserum against TRV-Lisse and PEBV-Dik Trom 5 was > 256 and 16 respectively, and that of the PEBV-E116 antiserum against PEBV-Dik Trom 5 and TRV-Lisse was 64 and 8, respectively.

With the same antisera it was not possible to differentiate between the three complete TRV isolates, Lisse, F12a and F15 with the micro precipitin test.

4.4.2 Agar gel diffusion test

With purified samples of TRV-Lisse, TRV-F12a and TRV-F15 (concentration 10 mg/ml) I tried to differentiate between these isolates with the antisera. In agar plates (1% agar, 0.9% NaCl and 0.05% NaN_3), 4 holes with a diameter of 3 mm were punched around a central hole of the same diameter. The distance to the central hole

Table 2. Results of agar gel diffusion tests with three TRV isolates.

Antigen tested	Antigen dilution[1]	TRV antiserum dilution[1]									
		4	8	16	32	64	128	256	512	1024	2048
TRV-Lisse[2]	16	−[3]	−	+[4]	+	+	−	−	−	−	−
	64	−	−	+	+	+	−	−	−	−	−
	256	−	−	−	−	−	−	−	−	−	−
	1024	−	−	−	−	−	−	−	−	−	−
TRV-F12a[2]	16	−	−	−	+	+	−	−	−	−	−
	64	−	−	−	−	−	−	−	−	−	−
	256	−	−	−	−	−	−	−	−	−	−
	1024	−	−	−	−	−	−	−	−	−	−
TRV-F15[2]	16	−	−	+	+	+	−	−	−	−	−
	64	−	−	−	+	+	−	−	−	−	−
	256	−	−	−	−	−	−	−	−	−	−
	1024	−	−	−	−	−	−	−	−	−	−

1. Dilutions are given as reciprocals.
2. The concentration of the undiluted antigen suspension was 10 mg/ml.
3. − = negative reaction.
4. + = positive reaction.

was 0.7 cm. The outer holes were filled with 4 virus dilutions, the central hole with an antiserum dilution. The results of the tests against different TRV antiserum dilutions are recorded in Table 2. The three isolates did not react with the PEBV antiserum.

It is evident that although there are small differences between antigen and antisera titres in this particular experiment, these can not be used to identify the three isolates. Other experiments, in which specific antisera against the three isolates were prepared, also revealed a very close serological relationship between the TRV isolates.

Although the agar gel diffusion test is easy to handle and sensitive, only the short particles of TRV and PEBV are small enough to penetrate the gel and can react (Maat, pers. commun.). Because in the infected plant the ratio of long particle to short particle virus material is 1:2 (see Fig. 11), this implies that about one third of the virus material does not react. With concentrated virus suspensions this test is acceptable. However, I wanted to determine whether PEBV or TRV was present in clarified sap of local lesions, in which virus concentrations are low. So I looked for a method that would involve the long particles in the reaction. I therefore tried two methods to break the long particles into smaller units.

The first one was ultrasonic treatment, for which 18 mg/ml virus solutions and a Kerry's Vibrason, ultrasonic general purpose applicator were used. The probe (diameter 0.9 cm) was set with its tip just beneath the meniscus and driven at an output of 50 watts. To prevent overheating the virus suspensions, the treatment was done

Table 3. Results of the bentonite-flocculation test using purified TRV-Lisse (18 mg/ml).

Dilutions[1] of TRV	Bentonite test					Normal test			
	dilution of the TRV antiserum globulin fraction					dilution of the TRV antiserum globulin fraction			
	4	16	64	256	1024	4	16	64	256
1	±[2]	±	−	−	−	+	+	+	−
4	±	+	±	−	−	+	+	±	−
16	+[3]	+	+	±	−	+	+	±	−
64	+	+	+	+	±	+	+	−	−
256	+	+	+	+	+	+	±	−	−
1024	+	+	+	+	+	−	−	−	−
4096	−[4]	±	+	+	+	−	−	−	−
16384	−	−	±	±	−	−	−	−	−
65536	−	−	−	−	−	−	−	−	−

1. Dilutions are given as reciprocals.
2. ± = questionable reaction.
3. + = positive reaction.
4. − = negative reaction.

intermittantly, with intervals of 1 min. Furthermore the virus suspensions were cooled in melting crushed ice. The preparations were treated by ultrasound for 0, 5, 10, or 20 min, and than centrifuged for 1½ h at 22500 rev/min in an SW 25.1 rotor on a gradient of 10–40% sucrose. Untreated preparations showed bands at 41–43 and 49–51 mm from the bottom of the tube. Virus treated for 10 min did not show the 41–43 band, the 49–51 band became more dense and a new diffuse band had appeared at 46–49 mm from the bottom. Virus treated for 20 min showed the same pattern. These preparations, when tested on beans, did not show infectivity anymore. From these results it can be concluded that the long particles were broken by this ultrasonic treatment into two pieces, one about the same length as the short particle, the other somewhat longer. So in a TRV-Lisse preparation, in which the ratio of long and short particles normally is 1:5 an ultrasonic treatment increases the number of particles that can penetrate the gel by 40–50%. I found that in a test the titre for virus treated by ultrasound was double that for untreated virus.

Another way of breaking particles into smaller pieces is to use a detergent (Hamilton, 1964). I used Leonil SA (sodium dibutylnaphthalenesulphonate) for this purpose. I tested this compound in concentrations ranging from 0.2 to 5%, by adding it to the virus suspensions. In all cases, however, an aspecific line pattern was obtained, because Leonil SA diffused faster through the agar than the virus and precipitated all antibodies very close to the antiserum holes.

Table 4. Results of the bentonite-flocculation test using clarified sap of leaves systemically infected with TRV-Lisse.

	Dilution[1] of clarified sap	Bentonite test				Normal test			
		dilution of the TRV antiserum globulin fraction				dilution of the TRV antiserum globulin fraction			
		4	16	64	256	4	16	64	256
Infected material	1	+[2]	+	+	+	+	+	−	−
	4	+	+	+	+	+	+	±	−
	16	+	+	+	+	+	+	±	−
	64	+	+	+	+	±	±	±	−
	256	−[3]	+	+	+	−	−	−	−
	1024	−	−	±[4]	+	−	−	−	−
Virus-free material	1	−	−	−	−	−	−	−	−
	4	−	−	−	−	−	−	−	−
	16	−	−	−	−	−	−	−	−
	64	−	−	−	−	−	−	−	−
	256	−	−	−	−	−	−	−	−
	1024	−	−	−	−	−	−	−	−

1. Dilutions are given as reciprocals.
2. + = positive reaction.
3. − = negative reaction.
4. ± = questionable reaction.

4.4.3 Bentonite-flocculation test

The bentonite suspension was prepared according to Maat (1970). The final bentonite concentration was adjusted to 0.4–0.6 mg/ml. Antiserum globulin fractions were prepared by mixing 1 ml antiserum, 1 ml saline solution, pH 7.3 (Bozicevich et al., 1963) and 8 ml saturated ammonium sulphate solution. After 15 min the precipitate was collected by centrifuging for 10 min at 10000 × *g* and resuspended in 8 ml saline solution, pH 7.3. After centrifuging for 10 min at 10000 × *g*, the supernatant was used to sensitize the bentonite particles. For this mainly the procedure of Bercks (1967) was followed. However, centrifuging was at 7000 × *g* instead of 700 × *g*, and to the buffered NaCl solution used for washing the sensitized bentonite 0.02% polyvinylpyrrolidon (PVP) was added and the 'Cialit' was replaced by NaN_3. Dilutions were made with 0.05 M tris-HCl buffer pH 7.2 containing 0.02% PVP.

The test was done: with concentrated virus, or with clarified sap (homogenate 10 min 5000 × *g*) of leaf halves which were systemically infected, or with clarified sap of leaf discs (diameter 12 mm) containing one local lesion. The results of these experiments with TRV are given in tables 3, 4 and 5, respectively. It is evident that with TRV

Table 5. Results of the bentonite-flocculation test using clarified sap of single local lesions caused on 'White Burley' by TRV-Lisse.

Number of leaf disc	Bentonite test				Normal test[4]
	dilution[1] of the TRV antiserum globulin fraction				dilution of the TRV antiserum globulin fraction
	4	16	64	256	4
1[2]	+[5]	+	−	−	+
2	+	+	+	−	+
3	+	+	−	−	+
4	+	+	+	−	+
5	−[6]	+	−	−	+
6	+	+	±[7]	−	+
7	+	+	−	−	+
8	+	+	−	−	+
9	+	+	−	−	+
10	+	+	−	−	+
11[3]	−	−	−	−	−
12	−	−	−	−	−
13	−	−	−	−	−
14	−	−	−	−	−

1. Dilutions are given as reciprocals.
2. The numbers 1–10 were discs with a local lesion.
3. The numbers 11–14 were discs from healthy plants.
4. The precipitates in this test were not conclusive, they may have been caused by aspecific flocculation.
5. + = positive reaction.
6. − = negative reaction.
7. ± = questionable reaction.

the bentonite-flocculation test is 16 times more sensitive than the normal test both for purified virus and clarified sap. In the case of PEBV the bentonite-flocculation test could only be used with concentrated virus. No virus could be detected using clarified sap of infected leaf material.

4.4.4 Conclusion and discussion

With the micro precipitin test I could easily differentiate between TRV and PEBV, when concentrated virus was used. In clarified sap of infected leaf material TRV could hardly be demonstrated and PEBV not at all. When the bentonite-flocculation test was used, TRV could be demonstrated in clarified sap of local lesions, but this could not be done with PEBV. This may be explained by the fact that the concentration of PEBV in the infected plant is less than half that of TRV.

My findings on the three isolates, Lisse, F12a, and F15, indicated that these isolates

were very closely related. The small differences found were within experimental error. So I could not use serology to distinguish between the three complete TRV isolates.

The agar gel diffusion test which is very suitable for viruses in sap, could not be used successfully with TRV and PEBV, because the concentration of these viruses, especially PEBV, in sap is too low, and because the long particles could not diffuse into the gel. If the long particles were broken by ultrasonic treatment, pieces were obtained with lengths about equal to that of the short particles. Thus the diffusion test would at best be twice as sensitive for treated virus than untreated virus. However, this increase in sensitivity was still not enough to demonstrate TRV and PEBV in clarified sap. In another test we used Leonil SA to induce breakage of the particles. This detergent, however, caused an aspecific precipitation of the antisera.

4.5 Particle lengths

Using the leaf dip method described in 3.6.1 to prepare the grids, the particle lengths of the TRV isolates were determined. The results of these measurements and those from purified TRV and PEBV were recorded in histograms with intervals of 5 nm. From these histograms the modal lengths presented in Table 6 could be determined. The measurements on dip preparations showed that 65% of the particles had lengths that differed 5 nm or less from the modal length. From the purified virus 65% of the particles had lengths that differed 10 nm or less from the modal length. This greater spreading can be explained by breakage of particles during the purification and concentration procedures.

Table 6. Particle lengths of the TRV isolates and PEBV-Dik Trom 5.

Virus isolate	Modal lengths (nm)			
	Dip preparations		Purified virus	
PEBV-Dik Trom 5			87.5	225
TRV-Lisse	65	185	67.5	185
TRV-F12a	65	180	65	190
TRV-F15	62.5	177.5	50	190

4.6 Sedimentation coefficients

Sedimentation coefficients were measured according to the procedure described in 3.5.

Different virus concentrations were spun and after the S values had been plotted against concentrations, S^0_{20} was determined by extrapolation to zero concentration (see figs 6 and 7 for TRV-Lisse and PEBV-Dik Trom 5, respectively).

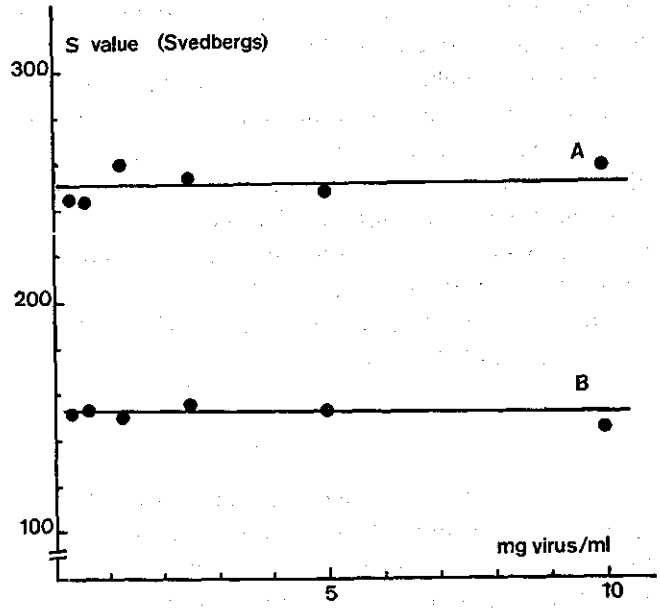

Fig. 6. Concentration-dependence of the sedimentation coefficient of TRV-Lisse. A = long particles, B = short particles.

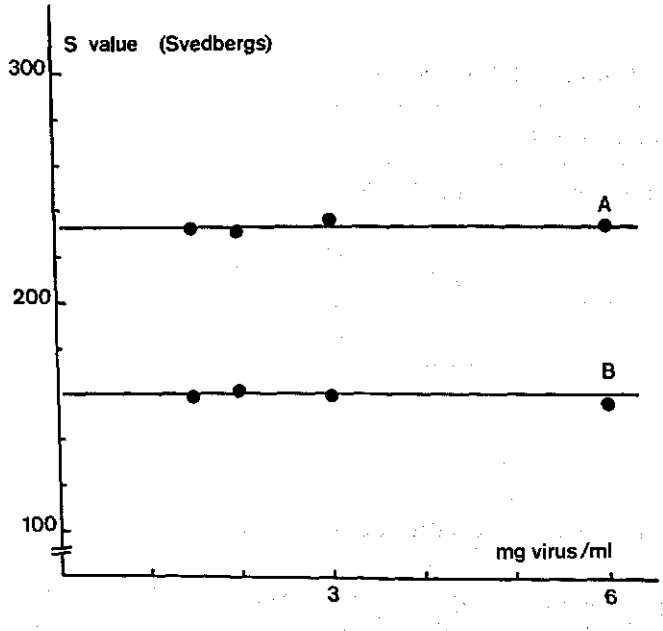

Fig. 7. Concentration-dependence of the sedimentation coefficient of PEBV-Dik Trom 5. A = long particles, B = short particles.

It is evident from figs 6 and 7 that at concentrations below 10 mg/ml the sedimentation coefficient is independent of concentration. Therefore all routine determinations were done at concentrations of 2–3 mg/ml. The experimental error in these determinations was less then 5%.

The S values obtained for the virus isolates used are given in Table 7.

Table 7. *Sedimentation coefficients of the* virus isolates.

Virus isolate	S^0_{20} (Svedbergs)
PEBV-Dik Trom 5	160 and 232
TRV-Lisse	153 and 251
TRV-F12a	145 and 236
TRV-F15	138 and 238

4.7 Density-gradient centrifuging in cesium chloride

When TRV and PEBV were submitted to density-gradient centrifuging as described in section 3.7, it was found that both viruses reached equilibrium in a run of 24 h in a single band. TRV reached equilibrium at a slightly higher density than PEBV. The bands of TRV and PEBV appeared at 18 and 20 mm from the bottom of the tube, respectively. It therefore was possible to differentiate between these two viruses with this technique. The different isolates of TRV could not be distinguished in this way.

4.8 Conclusion

The TRV isolates which were used could be divided into two groups. The complete isolates, existing as nucleoprotein particles and the incomplete ones occurring as free nucleic acid.

The symptoms of the complete isolates greatly differed from those of the incomplete. Within both groups, however, no differentiation could be made because the differences, which I detected between the three isolates, could have been due to the fluctuations of the symptoms within an isolate brought about by the environmental conditions.

The growth curves of complete and incomplete TRV showed that maximum production of complete virus was reached 5 days after inoculation, which was about 3 days later than in the case of incomplete virus, where it was reached 2 days after inoculation. These results agreed very well with what has been found for the 'c' isolate of TRV (Semancik & Kajiyama, 1967; Semancik & Odening, 1969). However, both maxima for incomplete and complete TRV production were reached one day earlier

in my experiments. Perhaps because the authors mentioned used another host plant, i.e. *N. clevelandii* Gray.

Incomplete isolates cannot be detected serologically. In the case of the complete isolates it was necessary to purify and concentrate the virus for the micro precipitin tests. With this test it was not possible to differentiate between Lisse, F12a and F15, with an antiserum against TRV-Lisse and an antiserum against PEBV-E116. In the agar gel diffusion test also no significant differences were found between Lisse, F12a and F15. The agar gel diffusion test could not be used for PEBV. With TRV the bentonite-flocculation test was 16 times more sensitive than the normal test both for purified virus and clarified sap.

Many times I examined purified preparations of the complete isolates in the electron microscope and in the analytical ultracentrifuge. In almost all cases I found the particle-length distribution characteristic for TRV. In only one preparation did I find, using the analytical ultracentrifuge, virus particles in a reasonable amount, with lengths of 300 nm (calculated from their S value) (Harrison & Klug, 1966). There are also data in literature indicating that TRV sometimes appears as longer rods with lengths up to 325 nm in plants. De Zoeten (1966) found such long particles in recently infected *N. tabacum* 'Xanthi-nc' mesophyll cells arranged in what he called virus crystals. He explained their extraordinary lengths by assuming end-to-end aggregation. Mosch (pers. commun.) found in 1964 long particles of 320 nm without short particles in a TRV preparation which he purified using the PEG-NaCl precipitation method as described by Venekamp & Mosch (1964). Attempts to repeat these experiments were unsuccessful. Köhler (1956b) stated that TRV is synthesized as a long rod and that from this rod the normal long and short particle would originate by breakage at previously determined places. In the few cases where I found some extra long particles, their existence could be attributed to end-to-end aggregation. In some experiments I tried to isolate extra long particles by working very fast and by adding bentonite to the extraction buffer to prevent any breakage due to RNase activity, but I never found any evidence for the synthesis of longer particles.

The particle lenghts of the three complete isolates did not differ in such a way that they could be used for differentiation. There was a rather striking discrepancy between the two types of particle-length distributions, especially if the lengths of the long particles were considered. The purified long particles were much longer than those in leaf dip preparations. The same effect has been described by de Zoeten (1966) for a Californian strain of TRV. Here too purified virus (107 and 196 nm) was much longer than intracellular virus (90 and 163 nm). That the short particles of F15 in purified preparations were much shorter than in the leaf dip preparations, probably may be explained by degradation.

The sedimentation coefficients varied more than was expected from the difference in particle lengths. Especially the S values of the short particles of Lisse and F12a differed too much. I can not explain this.

I could not differentiate between Lisse, F12a and F15 with density-gradient centrifuging in cesium chloride. In all cases I only found one band. Cooper & Harrison

(1967) found three bands for the CAM strain of TRV, with a sucrose or a CsCl gradient. The two major bands formed at densities differing by about 0.02 g/ml, and the upper of these appeared to contain the short particles. These results probably indicate that the run was ended before equilibrium was reached.

PEBV-Dik Trom 5 could easily be distinguished from the TRV isolates. There were clear differences in symptoms, serological properties, particle lengths and S values. There was also a difference in buoyant density between TRV and PEBV.

From the results in this chapter it may be concluded that the complete TRV isolates can easily be distinguished from the incomplete isolates of TRV. Within the group of the complete isolates, however, there were no differences in symptoms and serology. There were minor differences in particle length and S values, but these were insufficient to identify the isolates without doubt. With respect to virus multiplication TRV-F12a could be distinguished from Lisse and F15, because its yields were 15–20% higher. Moreover, the maximum of the growth curve was reached one day earlier. Within the group of incomplete isolates no differentiation was possible. My results on characterization of TRV isolates disagree with those of van Hoof et al. (1967).

The virus isolates which were characterized in this chapter enabled me to study three types of interaction: 1. interaction between particles of related complete isolates, 2. interaction between incomplete isolates and short particles of the complete isolates, 3. interaction between particles of TRV isolates and the PEBV isolate.

5 The separation of virus particles

For the study of the characteristics and the properties of TRV particles, it is important to prepare substantial amounts of the individual nucleoprotein components in a highly purified form. I investigated three different ways to achieve this: molecular sieving on agar colomns, specific precipitation by PEG and density-gradient centrifuging.

5.1 Molecular sieving in agar

If molecules and particles differ in size they can be separated by molecular sieving in agar columns (Hjertén, 1962). There are two systems. The first is with columns of block-condensed agar. Such a gel column has pores of a distinct diameter and only particles with dimensions smaller than the pore diameter can pass through. The larger particles cannot go through and are retained on top of the column. In the second system the column is filled with sphere-condensed agar or with small particles of block-condensed agar. In such a column the smaller particles can either go through the cavities between the agar particles or through the pores in the agar. In the latter case the particles will be retarded. In a long column the way of the small particles will be a mixture of both possibilities. Larger particles can only go through the cavities between the particles of the agar gel so that they leave the column before the smaller particles.

5.1.1 Molecular sieving on block-condensed agar

This method was first described by Polson (1956). By putting a dilute gel over a more concentrated gel, an osmotic pressure gradient can be established across the dilute gel. If a buffer solution containing virus particles is placed on top of the dilute gel, the fluid will be drawn into the gel, and depending on their size the virus particles will follow the fluid or remain on the gel surface.

In my experiments I used for the dilute gels 1.2%, 1%, 0.9% and 0.8% agar gels. 2.5 ml portions of one of these gels were placed on 13 ml of 6% agar in a Spinco SW 25.1 tube. According to Polson (1956) the pores in the dilute gels would have diameters of 58, 70, 78, and 88 nm. On top of the dilute gels 0.25 ml TRV-Lisse suspension was placed. The tubes were sealed with Parafilm to prevent loss of liquid by evaporation, and kept at 4°C.

After four days the liquid of the virus suspension had been drawn into the gels

The surfaces of the gels were rinsed several times very carefully to remove virus particles from the top of the gels. Then the 2.5 ml of the dilute gels were removed and homogenized in 10 ml PCA buffer. The homogenates were centrifuged for 10 min at 12000 × *g*. The supernatants were centrifuged for 1 h at 161000 × *g*. The pellets were resuspended in 1 ml PCA buffer and tested in the analytical ultracentrifuge.

The analytical ultracentrifuge patterns were the same for all four agar concentrations used. They were equal to the pattern of a normal TRV-Lisse suspension. So it was evident that the long particles of TRV could also penetrate into the agar gels of the concentrations used. Separation of long and short particles was not possible in this way.

5.1.2 Molecular sieving in a column of particles of block-condensed agarose

Using molecular sieving Taniguchi (1966) separated a mixture of TMV and ribonuclease. Steere & Ackers (1966) separated a long rod-shaped virus, TMV, from a small spherical virus, southern bean mosaic virus.

The 1% agarose gel (w/v) was made in water. The gel was crushed in a household sieve. The particles were then pressed through another sieve with a pore diameter of 0.5 mm. From these particles all those with a diameter between 0.25 and 0.5 mm were suspended in water and evacuated to remove air bubbles from the gel. With this agarose suspension a column with a diameter of 3.5 cm and a height of 60 cm was built. The column was successively rinsed with 1000 ml 0.02 M EDTA pH 8 and 1600 ml PCA buffer. The flow rate during the rinsing procedure was 4.2 ml/h/cm^2. It was kept constant by using an LKB Varioperpex 12000 peristaltic pump. The column was loaded with 2.5 ml virus suspension (9.7 mg/ml) and then eluted with PCA buffer at a flow rate of 2.1 ml/h/cm^2. The UV absorption of the column effluent was monitored with an LKB UV absorption meter. The elution pattern obtained is presented in Fig. 8. Three fractions of the effluent (in Fig. 8 marked as I, II, and III) were collected,

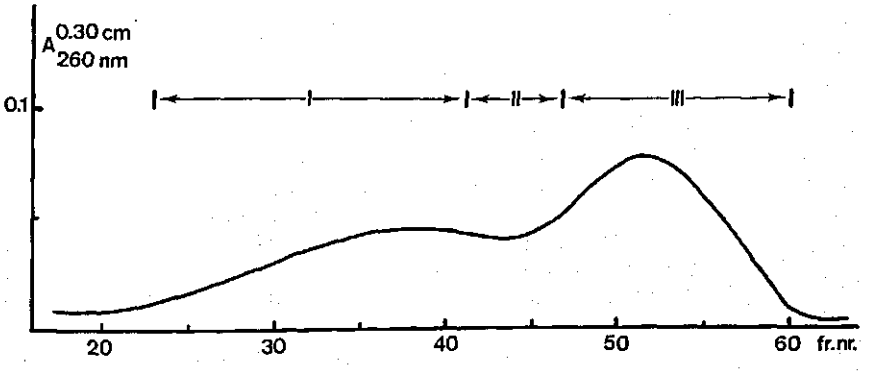

Fig. 8. UV absorption pattern of the effluent of a granular 1% agarose column, loaded with TRV and eluted with PCA buffer. Flow rate during elution 2.1 ml/h/cm^2.

concentrated by centrifuging for 1½ h at 105000 *g* and the pellets were resuspended in 1 ml PCA buffer each. These concentrated fractions were tested in the analytical ultracentrifuge. From Fig. 9 it can be seen that Fraction I has more long particles than short ones. In an unfractionated preparation of TRV one finds more short particles than long particles. In Fraction II there were relatively more short particles than in a normal preparation. Fraction III contained the same components as Fraction II but the concentration was much lower. The high UV absorption in Fraction III found in Fig. 8, therefore must be caused by material of low molecular weight in the virus preparation.

It could be concluded from these experiments that the separation of TRV components on this type of agarose column was very poor. One could hardly speak of a

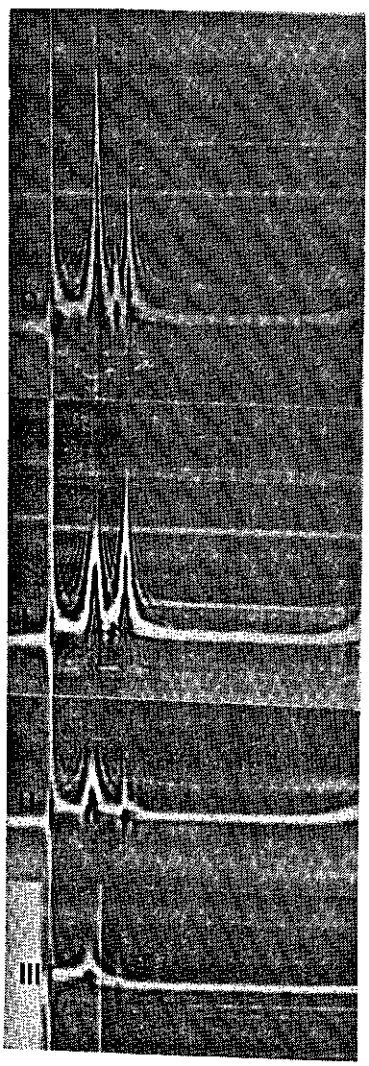

Fig. 9. Analytical ultracentrifuge patterns of:
0. unfractionated TRV-Lisse,
I. virus in Fraction I of Fig. 8,
II. virus in Fraction II of Fig. 8,
III. virus in Fraction III of Fig. 8.
Sedimentation is from left to right. The pictures were made 10 min after the selected rotor speed, 21740 rev/min was reached. The rotor temperature was 20°C. The unfractionated preparation shows an additional peak of particles with intermediate lengths. These are fragments of long particles, which always occur in older preparations. They leave the column in Fraction I and can be seen as a shoulder left of the long particle peak.

separation. Only the ratio of long to short particles was changed somewhat. The method had two other disadvantages: it took at least two days to rinse and to elute the column; the recovery was very low. In my experiments the recovery was not more than 15%. The low recovery could be explained by the entanglement of the rigid tubular particles of TRV in the pores of the agarose. From data of Steere (1963), who separated TMV fragments on agar columns one can conclude that the recovery was also very low in his experiments.

From Fig. 8 it can be seen that with this method it was only possible to separate virus particles from smaller contaminating components.

5.2. Specfic precipitation with PEG and NaCl

In 1963 Hebert found that differently shaped plant viruses could be precipitated by using various concentrations of PEG. Precipitation of two rod-shaped viruses, TMV and wheat mosaic virus, occurred at 4% PEG. In the case of spherical viruses e.g. tobacco ringspot virus and bean pod mottle virus, Hebert (1963) found that they precipitated only at 8% PEG.

Therefore I tried to separate the different particles of TRV by precipitating them at different PEG concentrations. The long particle of TRV, which is about 185 nm long and has a diameter of about 25 nm, would behave more like the rod-shaped viruses in Hebert's experiment, than the short one, which is 67.5 nm long and also has a diameter of 25 nm. The latter would possibly behave more like the spherical viruses in Hebert's experiment.

To find a combination of PEG and NaCl at which only the long particles would precipitate, I added PEG and NaCl in concentrations ranging from 3 to 5% and 0.1 to 0.5 M, respectively, to a TRV-Lisse suspension containing 2 mg/ml. In each case the precipitate was collected by centrifuging for 15 min at 12000 $\times$ *g*. The pellet was resuspended in PCA buffer of an equal volume as the initial suspension. Subsequently the concentration of PEG and NaCl in the supernatant was increased to 10% and 0.25 M, respectively. The resulting precipitate was collected by centrifuging for 15 min at 12000 $\times$ *g* and resuspended in an equal volume of PCA buffer. The fractions were tested in an analytical ultracentrifuge. With 3% PEG and 0.1 M NaCl only the long particles precipitated (see Fig. 10). When the PEG or the NaCl concentration was increased the short particles also started to precipitate.

These results indicated that it was possible to separate virus particles of different length by selective precipitation with PEG. Our results confirm those of Clark (1968), who was able to sort the particles of alfalfa mosiac virus (AMV) into three groups according to particle length. He therefore eluted a column, loaded with virus precipitated by 10% PEG, with a decreasing gradient of PEG.

Clark & Lister (1971) used PEG solubility-concentration gradients to sort particles of multiparticle viruses. In this case the viruses were precipitated with 8–10% PEG and layered on a sucrose gradient in an SW 25.1 tube with a gradient of PEG opposite to the sucrose gradient. The tubes were spun for 30 min at about 10000 $\times$ *g*. The virus

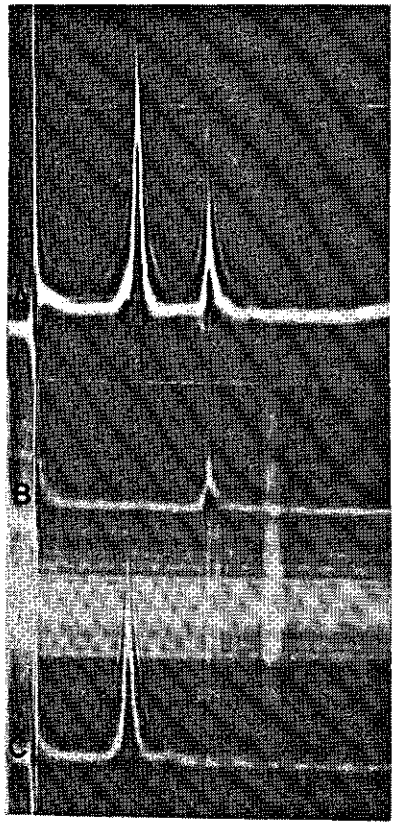

Fig. 10. Analytical ultracentrifuge patterns of:
A. TRV-Lisse,
B. long particles of TRV-Lisse precipitated at 3% PEG and 0.1 M NaCl from a normal TRV suspension (precipitate resuspended in PCA buffer),
C. short particles of TRV-Lisse precipitated at 10% PEG and 0.25 M NaCl from a solution out of which the long particles had been removed by precipitation at 3% PEG and 0.1 M NaCl (precipitate resuspended in PCA buffer). Sedimentation is from left to right. The pictures were made 10 min after the selected rotor speed, 21740 rev/min, was reached. The rotor temperature was 20 °C.

aggregates then moved through the gradient towards smaller PEG concentrations until they reached a concentration at which they dissolved again. At that site in the tube the virus was immobilized, because the applied centrifugal force was too low to move single virus particles through the sucrose gradient. Clark & Lister (1971) were able to sort particles of AMV and TRV according to lengths. I tried this procedure for TRV-Lisse. The virus was precipitated with 8% PEG and 0.08 M NaCl. The precipitate was spun for 30 min at 10000 × *g* on a 10–30% sucrose gradient in an SW 25.1 tube with a gradient of PEG opposite to the sucrose gradient. This gradient was obtained by layering from bottom to top: 4.5 ml 30% sucrose solution containing 1% PEG, 4.5 ml 26% sucrose solution containing 2.4% PEG, 4.5 ml 22% sucrose solution containing 3.8% PEG, 4.5 ml 18% sucrose solution containing 5.2% PEG, 4.5 ml 14% sucrose solution containing 6.6% PEG and 4.5 ml 10% sucrose solution containing 8% PEG. All solutions were prepared in PCA buffer containing 0.08 M NaCl. The gradients could be used 24 h after they were prepared. Three virus bands were obtained, which proved to consist, from top to bottom, of extra short, short and long particles, respectively. Separation especially between short and extra short particles, was better than with normal sucrose-gradient centrifuging. In the latter case these two types of particles appear in one band.

5.3 Sucrose-gradient centrifuging

5.3.1 Sucrose-gradient centrifuging in zonal rotors

During the experiments three types of zonal rotors were used: Spinco BIV, Spinco Ti-14, and the MSE BXIV A1. As the principle of all three rotors is the same, I shall only describe an experiment in one of them, the Spinco Ti-14. The Spinco Ti-14 rotor gives better separations than the MSE BXIV A1 rotor because it can be spun at higher speed, so that running times are shorter and there is less distortion of the separation pattern by diffusion.

A typical zonal run was as follows. The empty rotor was set to run at 3000 rev/min. At this speed the feed head was attached. The rotor was then filled with 500 ml gradient of 10–40% sucrose (w/v) in PCA buffer and 170 ml 40% sucrose (w/v) in PCA buffer. The gradient was formed by a Beckman High Capacity Gradient Pump that was programmed in such a way that the gradient was linear against volume as it was pumped into the rotor. The rotor was filled through a feeding line leading to the edge of the rotor, the 10% sucrose solution was pumped in first. Then the rotor was loaded with 10 ml of a virus suspension in PCA buffer containing 2% sucrose

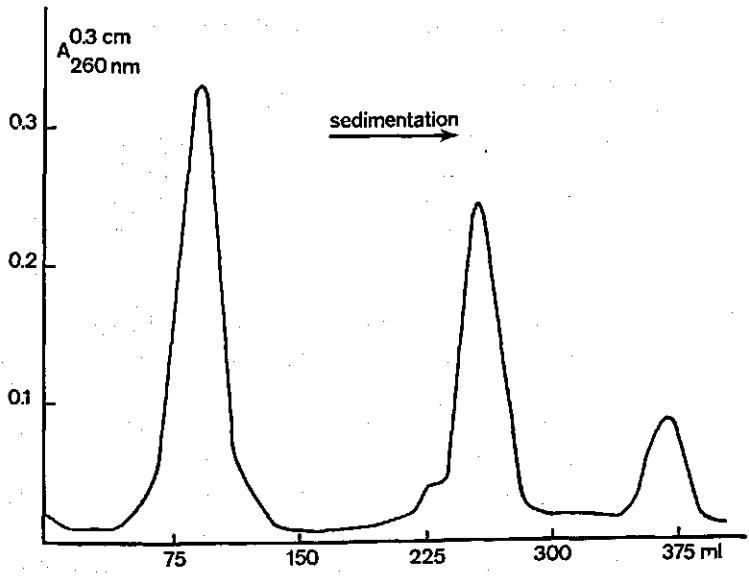

Fig. 11. Separation of TRV-Lisse particles by centrifuging in a sucrose gradient in a zonal rotor. The gradient of 10–40% sucrose (w/v) was linear with volume as it was pumped into the rotor. The gradient was loaded with 10 ml virus suspension (2.5 mg/ml) containing 2% sucrose. Overlayer 100 ml PCA buffer. Separation was for 1 h at 125000 × *g*. Temperature 4°C. From left to right; material of low molecular weight, short particles and long particles. Left to the peak representing the short particles a shoulder is caused by even smaller particles.

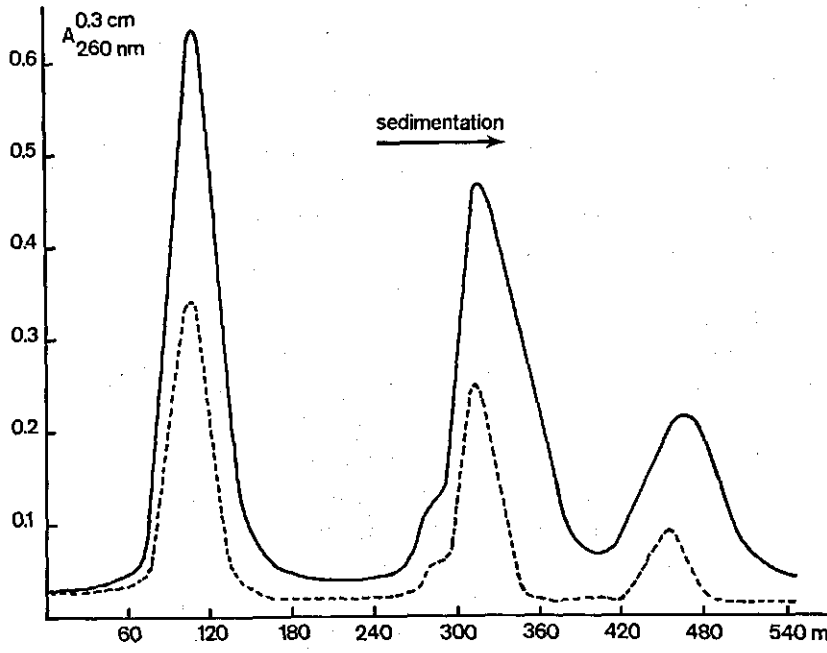

Fig. 12. Separation of TRV-Lisse particles by centrifuging in a sucrose gradient in a zonal rotor.

—— gradient loaded with 10 ml suspension containing 100 mg virus

----- gradient loaded with 10 ml suspension containing 25 mg virus

The gradients of 10–40% (w/v) sucrose were linear with volume as they were pumped into the rotor. Overlayer 100 ml PCA buffer. Separation was for 1 h at 125000 × *g*. Temperature 4 °C. From left to right: material of low molecular weight, short particles and long particles. On the left side of the peak caused by the short particles a shoulder caused by even smaller particles can be seen.

(w/v). The virus was introduced via the feeding line into the centre of the rotor. An overlayer of 100 ml PCA buffer was then introduced into the centre of the rotor. The overlayer solution removed the virus solution from the feeding lines and positioned the sample in a thin layer of even thickness at a sufficient distance from the rotor axis to give the particles a reasonable starting velocity. When the rotor was finally filled with gradient, virus, and overlayer, the feed head was removed and the rotor sealed. The rotor was accelerated to 40000 rev/min (119000 × *g*) and spun for 1 h. It was then decelerated to 3000 rev/min and unloaded by pumping a solution of 45% sucrose (w/v) in PCA buffer through the feeding line to the edge of the rotor. The rotor contents were led through an LKB UV absorption meter and collected in fractions of 15 ml. The UV absorption was registered with a Beckman Ten Inch Recorder. The fractions containing long particles were pooled, diluted 1:1 with buffer, and centrifuged for 3 h at 105000 × *g*, as were fractions with short particles. This treatment concentrated the fractions and removed most of the sugar so that degradation of the virus components was decreased during storage at 4 °C.

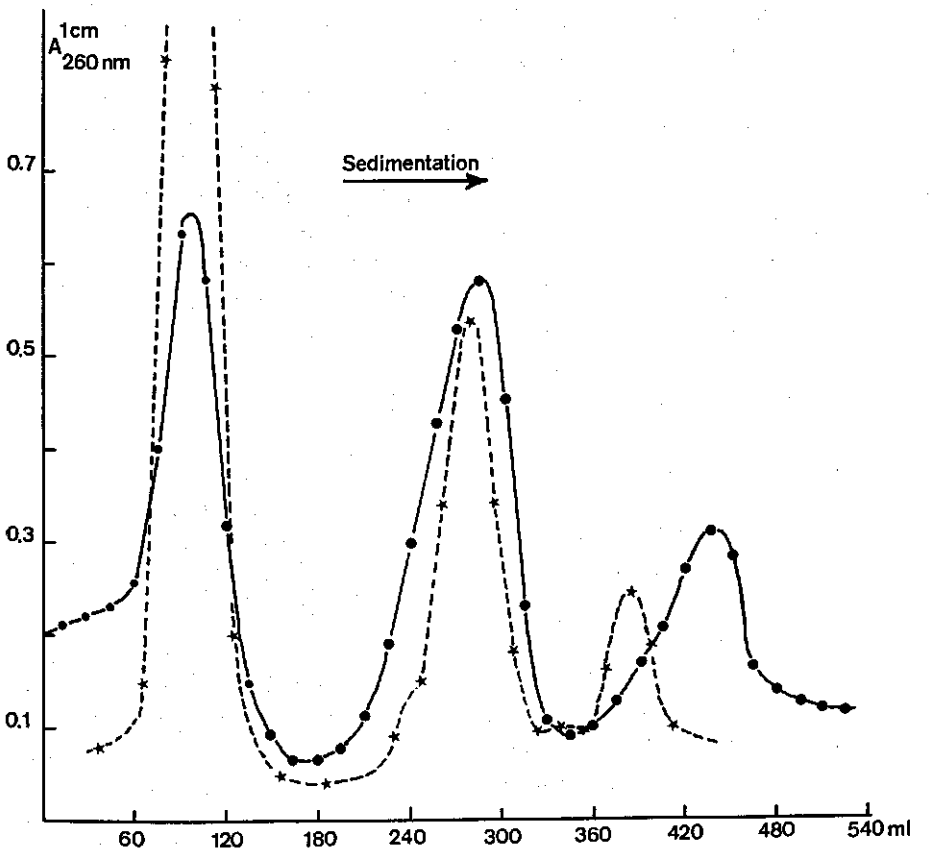

Fig. 13. Separation of TRV particles in a zonal rotor with differently shaped sucrose gradients.
—— A run with an iso-kinetic sucrose gradient (10–40%) for 1½ h at 67000 × *g*, in an MSE BXIV Al rotor.
----- A run with a gradient (10–40%) which was linear against volume for 1 h at 125000 × *g*, in a Spinco Ti-14 rotor.
In both runs the rotors were loaded with 10 ml TRV-Lisse suspension (12.5 mg/ml). Overlayer 100 ml PCA buffer. Temperature 5 °C. From left to right: material of low molecular weight, short particles and long particles. In the Spinco Ti-14 run a shoulder caused by even smaller particles can be seen on the left side of the peak caused by the short particles.

Fig. 11 shows an example of a separation obtained in the zonal rotor. The rotor was loaded with about 25 mg virus. From left to right there is a meniscus peak caused by material of low molecular weight and peaks of short and long particles, respectively. Left of the peak representing the short particles, is a shoulder caused by even smaller particles. The separation between material of low molecular weight and the virus peaks and between the two virus peaks themselves is very good.

In Fig. 12 two runs with a Spinco Ti-14 are presented. Both gradients were linear with volume. One was heavily loaded with about 100 mg TRV-Lisse, the other was loaded with about 25 mg TRV-Lisse. Although a good separation between long and

short particles could still be obtained with 100 mg virus in one run, the separation was far better if smaller quantities per run were used.

In some cases I used iso-kinetic gradients. In Fig. 13 a run of 1½ h at 67000 × *g* in an MSE BXIV A1 rotor with a 10–40% (w/v) iso-kinetic sucrose gradient is compared with a run of 1 h at 125000 × *g* in a Spinco Ti-14 rotor loaded with a gradient of 10–40% (w/v) sucrose which was linear with volume as it was pumped into the rotor. In the run with the iso-kinetic gradient the peaks of the long and short particles are better separated from each other than in the run with the linear gradient. No shoulder of extra short particles is seen in the run with the MSE rotor, because of the diffusion due to the longer centrifuging time necessary with this rotor.

5.3.2 Sucrose-gradient centrifuging in swinging bucket rotors

To complete the separation of virus components, which had been separated to a large extent by sucrose-gradient centrifuging in a zonal rotor, sucrose-gradient centrifuging in the Spinco SW 25.1 rotor was used. The gradients were prepared by mixing 15 ml of 10% and 15 ml of 40% sucrose in PCA buffer with a device described by Britten & Roberts (1960). The gradients were loaded with 0.1 or 0.2 ml of a virus suspension (2–3 mg/ml) and spun for 1½ h at 83000 × *g*.

The components were isolated from the sucrose gradients as described by van Kammen (1967). Carbon tetrachloride was forced into the tube through a hollow needle inserted into the wall near the bottom. To prevent leakage along the needle, the tube was first partially covered with a thin layer of silicone rubber paste, and a small piece of thin rubber. The carbon tetrachloride was driven by hydrostatic pressure. Later an LKB Varioperpex 12000 peristaltic pump was used to obtain a constant flow rate. The carbon tetrachloride was then replaced by 50% sucrose in PCA buffer. The tube was first closed with a cap ground conically on the under side. The tube contents were pushed out through a needle inserted in the middle of the cap and led to an LKB UV absorption meter equipped with a Beckman Ten Inch Recorder. The zones containing the virus components were collected and dialysed for one night against PCA buffer. Such preparations appeared homogeneous in biological tests and could be used directly for interaction experiments (see Table 14).

5.4 Conclusion and discussion

Of the methods I tested to separate TRV and PEBV components, the molecular sieving methods were the most laborious, the most time consuming and the ones that gave the poorest results. These methods can satisfactorily separate two spherical particles with some difference in diameter, or a mixture of rod-shaped and small spherical particles. However, for mixtures of rod-shaped particles with different lengths but the same diameter, molecular sieving was less useful. Separation was unsatisfactory and recovery was low. In my experiments the recovery was as low as 15%.

Specific precipitation by PEG and NaCl was a quick method with good results.

Sometimes considerable amounts of virus were lost with this method: if after centrifuging the supernatant containing the PEG was not carefully removed from the pellet and from the walls of the tube. The inside of the tube should be cleaned very carefully to remove any droplet containing PEG. If not, the pellet will not resolve completely and much virus will be lost as aggregates caused by retained PEG during the next low speed centrifugation. With spherical viruses there is no problem, because they do not form such strong aggregates, as rod-shaped viruses at relatively low PEG concentrations.

Sucrose-gradient centrifuging in the zonal rotor was a fast method to separate virus from normal plant proteins, and to separate the virus components from each other. Both separations could often be done in the same run. As is generally known the advantages of the zonal rotor over the SW 25.1 rotor are (1) it can handle larger amounts of material to be separated; (2) there is less disturbance during loading and unloading and (3) there are no wall effects. Wall effects may produce clumping, premature precipitation of particles, and convective disturbances (Anderson, 1966). The only disadvantage is the great radial dilution effect. Therefore the virus components have to be concentrated after separation, starting from a rather viscous sucrose solution which requires long periods (up to 3 h at 105000 $\times$ g) of centrifuging at high speed. This treatment was not very harmful to TRV but it was to PEBV, because this virus was easily broken during pelleting and resuspending. Moreover the concentration took time. Concentrating is less troublesome with spherical viruses. Then one can easily use precipitation by PEG and NaCl (van Kammen, 1967).

The effectivity of separating on sucrose gradients largely depends on the speed of the rotor, the shape of the gradient, and the amount of virus material put on top of the gradient. The higher the rotor speed the shorter the running time can be, and consequently the smaller the negative effects due to diffusion. In most cases the gradients I used were linear with volume as they were pumped into the rotor. This type of sucrose gradient has a serious disadvantage. The particles to be separated slow down as they move away from the axis of rotation, because the viscous drag of the medium increases more rapidly than the centrifugal force. Therefore the resolving power of the method is seriously limited. In most gradients the separation between two components does not improve beyond a third to a half of the entire available path length (Noll, 1967). Noll therefore constructed a so-called iso-kinetic gradient for a swinging bucket rotor. In this gradient the particles keep moving at a constant speed independent of their distance to the axis of rotation. McCarty et al. (1968) used a computer to construct the gradients for a series of swinging bucket rotors, various initial concentrations of sucrose, and various temperatures. Van Kammen (pers. commun.) applied the method of McCarty to achieve an iso-kinetic gradient for the MSE B XIV rotor. I used the results of this work in some experiments (Fig. 13). It appeared that the separation between long and short particles with an iso-kinetic gradient was better than with a linear gradient. It must be noted, however, that in the experiment shown in Fig. 13 the negative effects of diffusion due to the low maximum rotor speed were greatest in the run with the iso-kinetic gradient. If both runs had been done in the fast

Spinco Ti-14 rotor at the same speed the results of the iso-kinetic gradient would have been even better. Moreover, it must be noted that the formules used to calculate the gradient were based on two assumptions (Noll, 1967): the particle shape is unchanged by the gradient and the particles are spherical. In my experiments the particles were tubular and the density of TRV was smaller than that of the spherical viruses. So this gradient did not create exact iso-kinetic conditions for TRV.

If a gradient was too heavily loaded it became more difficult to separate the TRV nucleoprotein particles completely in one run. Quantities up to 100 mg could be separated in one run, but the results were far better with for example 25 mg per run. In the latter case the peaks did not overlap at all, and according to tests in the analytical ultracentrifuge homogeneous preparations could be isolated.

Usually I used density-gradient centrifuging in the zonal rotor for the first separation between long and short particles. However, it is clear from the results described in this chapter that the separation in the zonal rotor may be replaced by specific precipitation by PEG and NaCl which will also induce a good separation. A great advantage of the latter method is that relatively simple equipment is needed. The same holds for the PEG solubility-concentration gradient technique. An additional advantage of the latter method is the better separation between short and extra short particles.

6 Purity of separated virus particle preparations

To test the purity of the virus particle preparations I used three methods: electron microscopy, analytical ultracentrifuging, and infectivity tests on tobacco and bean plants. The value and the limitations of these methods will be discussed.

6.1 Electron microscopy

Fig. 14 gives the particle-length distributions of a purified unfractionated TRV-Lisse preparation and of long and short particles separated by sucrose-gradient centrifuging in a zonal rotor. In the separation the extra short particles were discarded. The figure shows that the preparations of the short particles are more homogeneous than the long particle fractions. This result was always found with preparations obtained by density-gradient centrifuging in a zonal rotor and is caused by two factors:

1. in the fractionating procedure (see 5.3.1) the short particles always come first. So the line from the zonal rotor to the fractionating device will be contaminated by short particles as the long particles come through. From the histograms of Fig. 14 it can be calculated that 1.3 % of the virus material in the long particle preparation was present in the region where the short particles should occur. That indeed biologically active short particles were present was proved by infectivity experiments.
2. the particles tend to break during preparation and drying of the grids. Moreover concentration of density-gradient fractions is often necessary before electron microscopy can be applied and this also damages the rod-shaped particles. Because long particles are more easily broken than short ones, there will be a greater heterogeneity of the long particle fraction.

Fig. 15 represents the particle-length distributions of long and short particles after density-gradient centrifuging in a zonal rotor followed by density-gradient centrifuging in an SW 25.1 rotor. If the data of figs 14 and 15 are compared it is clear that centrifuging in the SW 25.1 rotor considerably increased the purity of the preparation, especially for the long particle preparation. After density-gradient centrifuging in the SW 25.1 rotor, the virus preparations were not concentrated. Thus from the different fragmentation of the preparations shown in figs 14B and C and figs 15A and B, I concluded that most of the fragmentation in figs 14B and C is induced by the concentration via centrifuging and that the breakage during preparation and drying of the grids for electron microscopy is of minor importance.

In Fig. 16, electron micrographs of long and short particle preparations obtained after density-gradient centrifuging in a zonal rotor and an SW 25.1 rotor, are presented.

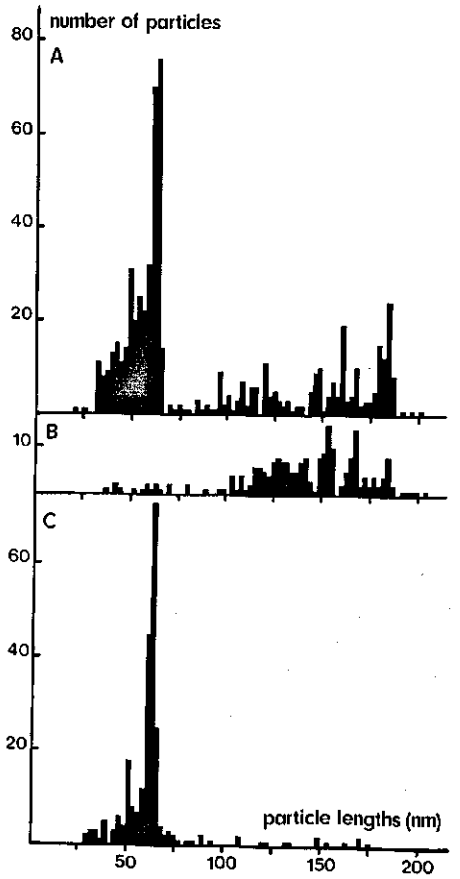

Fig. 14. Particle-length distributions of unfractionated TRV-Lisse (A), and of long (B) and short (C) particles of TRV-Lisse separated by centrifuging in a sucrose gradient (10–40%) in a zonal rotor for 1 h at 119000 × *g*.

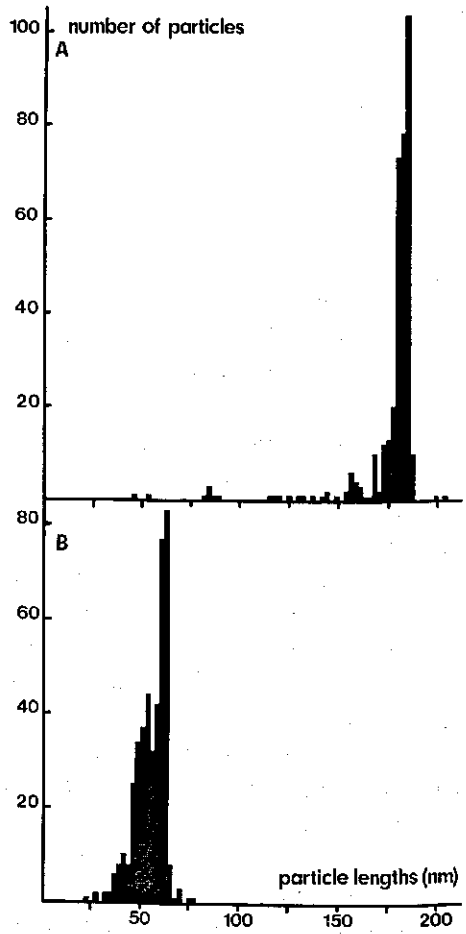

Fig. 15. Particle-length distributions of long (A) and short (B) particles of TRV-Lisse, separated by centrifuging in a sucrose gradient in a zonal rotor for 1 h at 119000 × *g* followed by centrifuging in a sucrose gradient (10–40%) in a SW 25.1 rotor for 1½ h at 83000 × *g*.

6.2 Analytical ultracentrifuging

The analytical ultracentrifuge was a very useful tool in determining the composition of virus preparations. It worked very fast and nearly all material used for the test could be recovered. However, if small amounts of contaminating particles had to be found the method had serious limitations: the lower limit of solute concentration visible with Schlieren optics is about 0.01%. Therefore in my tests, in which suspensions with about 2–3 mg virus/ml were used, components, that occured in concentrations up to 0.1 mg/ml, representing 3–5% of the material, could not be detected.

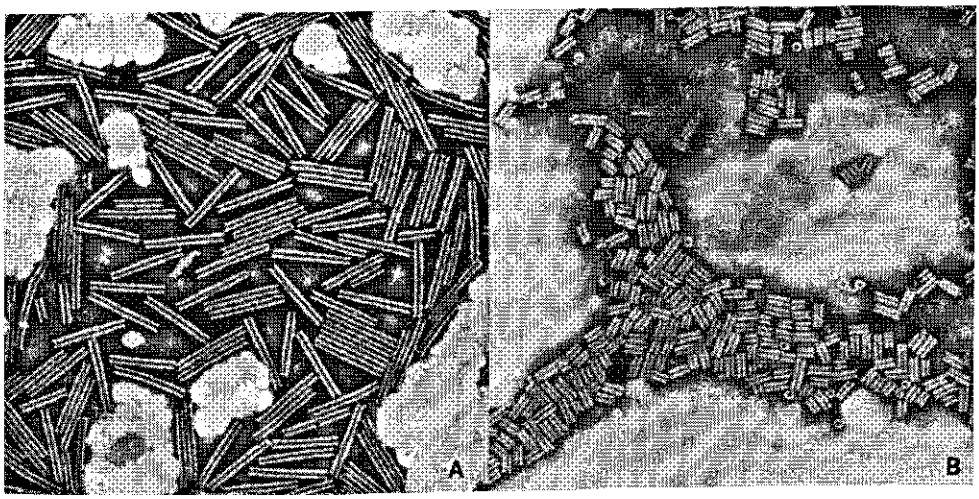

Fig. 16. TRV-Lisse fractions obtained after centrifuging in a sucrose gradient (10–40%) in a zonal rotor for 1 h at 119000 × *g*, followed by centrifuging in a sucrose gradient (10–40%) in a SW 25.1 rotor for 1½ h at 83000 × *g*. A. long particles, B. short particles.

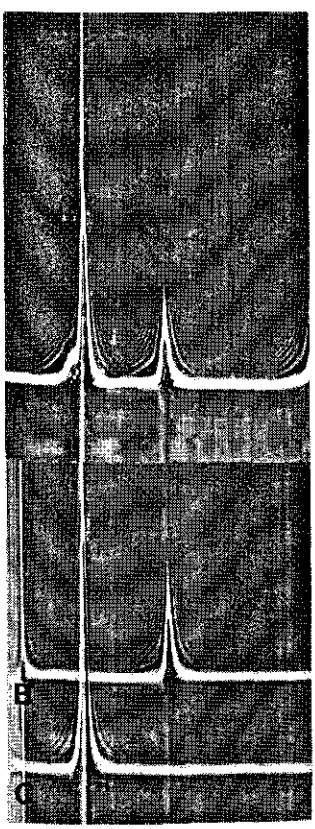

Fig. 17. Analytical ultracentrifuge patterns of unfractionated TRV-Lisse (A) and of long (B) and short (C) particles preparations of TRV-Lisse obtained by one centrifuging in a sucrose gradient (10–40%) in a zonal rotor for 1 h at 119000 × *g*. Sedimentation is from left to right. The pictures were made about 12 min after the selected rotor speed, 21740 rev/min, was reached. The rotor temperature was 20 °C.

In Fig. 17, the analytical ultracentrifuge patterns are presented of unfractionated virus and of long and short particle preparations obtained by density-gradient centrifuging in a zonal rotor. These patterns were obtained from the preparations whose particle-length distributions are in Fig. 14. In the analytical ultracentrifuge the long and short particle preparations appeared homogeneous. This was in complete agreement with what was expected from the results obtained in the electron microscope and knowledge about the lower detection limit of the ultracentrifuge. This limit is 5% and the contamination of short particles in the long particle preparation was 1.3%.

6.3 Tests with plants

Short particles of TRV and PEBV are not infectious. Long particles, however, can infect plants. So a short particle preparation should be inoculated on plants to test its purity. A short particle preparation, obtained by density-gradient centrifuging in a zonal rotor, appeared homogeneous in the analytical ultracentrifuge (fig. 17C), and had a particle-length distribution as in Fig. 14C. It did not cause local lesions on *P. vulgaris* 'Bataaf' but did cause a considerable amount of primary lesions on the systemically reacting hosts *N. tabacum* 'Xanthi' and 'White Burley' and *N. rustica* if it was inoculated in a concentration of 0.01 mg/ml. Most primary lesions were caused on 'Xanthi' tobacco. When this short particle preparation was subjected to density-gradient centrifuging in an SW 25.1 rotor only one opalescent zone was obtained (Fig. 18C). This particle zone was isolated and after dialysis against PCA buffer, the particle-length distribution was determined (Fig. 15B), and it was inoculated at a

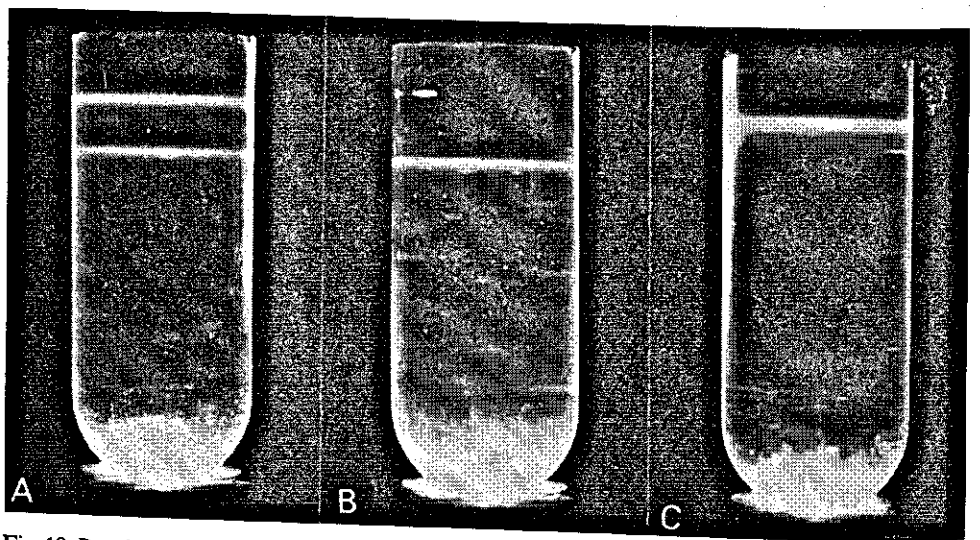

Fig. 18. Results of centrifuging in a sucrose gradient (10–40%) in a SW 25.1 rotor for 1½ h at 83000 × *g* of unfractionated TRV-Lisse (A), and of long (B) and short (C) particles of TRV-Lisse separated by centrifuging in a sucrose gradient (10–40%) in a zonal rotor for 1 h at 119000 × *g*.

concentration of 0.01 mg/ml on *P. vulgaris* 'Bataaf', 'Xanthi' and 'White Burley' tobacco and *N. rustica*. In this case no reaction was obtained on bean, *N. rustica* and *N. tabacum* 'White Burley'. Only a few infections were obtained on *N. tabacum* 'Xanthi'.

To verify whether the short particles were still biologically active, there was a test in which long and short particles of one isolate were combined. This mixed inoculum was checked for the production of complete virus.

Long particles alone can cause infection but no normal virus particles will be formed because only the RNA of the long particle is multiplied. So to determine whether a preparation contained only long particles, it had to be inoculated on tobacco plants and a sufficient number of lesions tested as described in 7.4 to make sure that they contained only incomplete virus. In the experiment, already mentioned in 6.1 and 6.2, in which virus was first fractionated in a zonal rotor followed by density-gradient centrifuging in the SW 25.1 rotor I inoculated the preparations at a concentration of 0.01 mg/ml to 'Xanthi' and 'White Burley' tobacco and *N. rustica*. Then primary infections were tested to see what kind of virus they contained. With unfractionated virus 96% of the lesions contained complete virus. For the long particle preparation, obtained after the run in the zonal rotor, this percentage was 84%. If such a preparation was centrifuged in a density-gradient in the SW 25.1 rotor, only one opalescent zone was obtained (Fig. 18B). An inoculum prepared from this zone, containing less than 0.1% short particles as determined from the particle-length distribution (Fig. 15A), induced in 12% of the lesions on 'Xanthi' and 'White Burley' tobacco and *N. rustica* complete virus.

6.4 Conclusion and discussion

Electron microscopy and analytical ultracentrifuging were fast and very suitable methods to determine the composition of virus preparations. However, neither could be used to detect small impurities. With the electron microscope fragments are found because during preparation and drying of the grids some breakage will occur. It is not always possible to differentiate between these fragments and those which already occurred in the preparation. The lower detection limit of the analytical ultracentrifuge prevents the detection of impurities which account for 5% of the material used in the test. Therefore it was possible that a preparation of long particles, which appeared homogeneous in the analytical ultracentrifuge was not pure at all. According to electron microscopic measurements, the preparation contained 1.3% short particles and produced complete virus in 84% of the primary infections on tobacco.

The test on plants was superior to both electron microscopy and analytical ultracentrifuging. The test was simple for short particles. For long particles it was somewhat more elaborate. The tobacco plants, which become systemically infected, were better test plants than the local lesion host *P. vulgaris* 'Bataaf' probably because a very small primary lesion, which could not easily be detected, ultimately resulted in symptoms all over the plant.

From the results of the three methods it became clear that density-gradient centrifuging in an SW 25.1 rotor removed not only many particle fragments induced by concentration procedures necessary after density-gradient centrifuging in a zonal rotor but also long and short particles from the short and long particle preparations, respectively. I therefore concluded that it was sufficient to separate long and short particles by only one desity-gradient centrifuging in the zonal rotor followed by density-gradient centrifuging in the SW 25.1 rotor. However, it was only possible to obtain preparations that appeared pure in the biological test if the zonal rotor was loaded with a relatively small amount of virus material.

7 Methods to distinguish between complete and incomplete TRV

If long and short particles of different isolates are mixed and inoculated, there are two possibilities. Either long and short particles interact and give rise to the formation of complete virus particles, or they do not interact in which case incomplete virus is formed. So to see whether my interaction experiments were successful or not I apparently needed a test to differentiate between virus nucleoprotein particles and free virus RNA. Lister (1966) distinguished between complete and incomplete TRV by freezing infected material for at least 48 h at —5 °C. If the material after thawing and grinding still contained infectious virus this was called stable virus; it must have consisted of nucleoprotein particles. If the material did not show any infectivity after thawing, the virus it contained was called unstable virus. Lister (1966) did not explain the mechanism by which unstable virus is destroyed by freezing and thawing in leaf material, but probably these treatments promote conditions for the breakdown of RNA by RNase. However, this can also be achieved by grinding.

There are four methods to prevent degradation of virus RNA by RNase: (1) grinding the infected material in a buffer with a pH above that at which leaf RNases are active (Babos & Kassanis, 1962; Diener, 1972); (2) grinding infected material frozen with liquid nitrogen in a mortar and, after adding some buffer, inoculating the still frozen powder with a damp brush (Sänger & Gold, 1962); (3) extraction of infected material with phenol (Schlegel, 1960; Sänger & Brandenburg, 1961; Cadman, 1962); (4) grinding the infected material in buffer containing bentonite (Singer & Fraenkel-Conrat, 1961).

The method I used to transmit incomplete TRV and PEBV in my routine experiments was grinding the infected material in buffer containing bentonite. I decided to do so because it is a simple and safe method by which large numbers of local lesions can be tested in a short time.

7.1 The use of bentonite

Bentonite effectively inhibits RNase activity, because the RNase adheres to the clay (Brownhill et al., 1959). The affinity for the clay is very high for pancreas and yeast RNase but is somewhat less for plant RNase (Singer & Fraenkel-Conrat, 1961). Bentonite has been used for the transmission of unstable forms of different viruses (Singer & Fraenkel-Conrat, 1961; Kassanis & Welkie, 1963; Yarwood, 1966; Sänger, 1968a). Bentonite adsorbs all kinds of protein including some virus proteins so that it sometimes reduces the transmission of virus. The effect of bentonite on the transmission depends on the virus, the donor host, and the age of infection (Yarwood,

1966). As we will see, bentonite reduces the transmission of complete TRV.

7.1.1 The effect of bentonite on the transmission of incomplete TRV

In literature there are very few data on the optimum concentration to be used. Sänger (1968a) used a solution with 20 mg bentonite /ml but did not mention the amount used for 1 g of infected material. Kassanis & Welkie (1963) ground 1 g of infected material in 6 ml buffer containing 25 mg bentonite/ml. To verify whether these concentrations were optimum for *N. rustica* and the *N. tabacum* varieties 'Xanthi' and 'White Burley' I carried out the following experiment.

N. tabacum 'Xanthi' and 'White Burley' and *N. rustica* plants were infected with the incomplete form of TRV-Lisse. After about a week, samples of the infected leaves were taken with a corkborer with an inner diameter of 10 mm. The leaf discs were cut in two and the halves ground separately in 0.5 ml PCA buffer, containing varying amounts of bentonite: 0, 5, 10, 15, 20, 25, 30, 40, and 50 mg/ml buffer, respectively. After grinding, the homogenate was poured from the mortars into small test tubes and then stored at —4°C (see 7.2) until the test plants were available. After thawing at room temperature the preparations were tested on primary leaves of *P. vulgaris* 'Bataaf'. The effect of each bentonite concentration was tested on 18 primary leaves. Two concentrations were compared on one plant according to Table 8. Three to seven days after inoculation the local lesions on the primary leaves of *P. vulgaris* 'Bataaf' were counted. In Table 9 the average numbers of local lesions per leaf caused by the different inocula are given.

If no bentonite was used there was no transmission. When only 5 mg bentonite/ml of inoculum was added transmission was greatly increased. One might get the impression that for *N. tabacum* 'Xanthi' and 'White Burley' there was a general tendency for the inocula to become more and more infectious as the amount of bentonite

Table 8. List of treatments applied to discs taken from leaves infected with the incomplete form of TRV-Lisse.

R 0– 0	W 5– 0	X 10– 0	R 15– 0	W 20– 0	X 25– 0	R 30– 0	W 40– 0	X 50– 0
R 0– 5	W 5– 5	X 10– 5	R 15– 5	W 20– 5	X 25– 5	R 30– 5	W 40– 5	X 50– 5
R 0–10	W 5–10	X 10–10	R 15–10	W 20–10	X 25–10	R 30–10	W 40–10	X 50–10
W 0–15	X 5–15	R 10–15	W 15–15	X 20–15	R 25–15	W 30–15	X 40–15	R 50–15
W 0–20	X 5–20	R 10–20	W 15–20	X 20–20	R 25–20	W 30–20	X 40–20	R 50–20
W 0–25	X 5–25	R 10–25	W 15–25	X 20–25	R 25–25	W 30–25	X 40–25	R 50–25
X 0–30	R 5–30	W 10–30	X 15–30	R 20–30	W 25–30	X 30–30	R 40–30	W 50–30
X 0–40	R 5–40	W 10–40	X 15–40	R 20–40	W 25–40	X 30–40	R 40–40	W 50–40
X 0–50	R 5–50	W 10–50	X 15–50	R 20–50	W 25–50	X 30–50	R 40–50	W 50–50

The pairs of figures refer to the amount of bentonite (mg/ml) added to corresponding halves of a leaf disc. R, W, and X indicate that the discs were taken from *N. rustica*, *N. tabacum* 'White Burley', or *N. tabacum* 'Xanthi', respectively.

Table 9. Average number of local lesions on primary leaves of *P. vulgaris* 'Bataaf' caused by inocula of the incomplete form of TRV-Lisse prepared with different amounts of bentonite. 0.5 ml of a bentonite suspension was used to grind half a leaf disc with a diameter of 10 mm. Tests were done in sixfold. Within brackets the extremes found are presented.

mg bentonite/ml	Average number of local lesions caused by inocula from		
	N. tabacum 'Xanthi'	*N. tabacum* 'White Burley'	*N. rustica*
0	0 (0, 0)	0 (0, 0)	1 (0, 1)
5	7 (0, 33)	65 (10, 137)	171 (68, 291)
10	9 (3, 24)	30 (0, 97)	345 (87, 672)
15	7 (0, 24)	36 (1, 155)	125 (10, 295)
20	4 (0, 8)	35 (1, 149)	108 (47, 197)
25	7 (1, 27)	53 (0, 144)	144 (19, 432)
30	36 (0, 74)	74 (3, 260)	215 (40, 641)
40	14 (0, 37)	27 (0, 84)	116 (34, 229)
50	26 (0, 145)	85 (2, 129)	63 (14, 114)

increased. With *N. rustica* the same effect seemed to occur with bentonite concentrations up to 10 mg/ml. Higher concentrations seemed to decrease the infectivity of the inoculum. However, due to fluctuations within groups with the same treatment, these effects were not statistically significant.

Similar results were obtained for incomplete TRV-F12a and TRV-F15.

From the data in Table 9 I concluded that bentonite was necessary for the transmission of incomplete virus. It was not possible to select an optimum bentonite concentration, because the effect of bentonite addition already reached a constant level at low bentonite concentrations. I decided to use a concentration of 25 mg/ml in my routine experiments just to make sure that enough bentonite was always present.

7.1.2 The effect of bentonite on the transmission of complete TRV

To test the effect of a bentonite suspension on the transmission of complete virus the following experiment was done: discs with a diameter of 12 mm were taken from *N. rustica* leaves infected with TRV-Lisse. The discs were cut in two, one half was ground in 0.5 ml PCA buffer, the other half in 0.5 ml PCA buffer containing 25 mg bentonite/ml. The obtained inocula were tested on *P. vulgaris* 'Bataaf'. Corresponding halves of discs were inoculated on opposite primary leaves. Tests were made in duplicate. The resulting numbers of local lesions are presented in Table 10.

Therefore when a bentonite suspension is used to prepare inocula of complete TRV-Lisse, it has a negative effect, compared with inocula prepared with only PCA buffer. Additional experiments revealed that with TRV-F12a, TRV-F15, and PEBV effects are similar. The number of local lesions decreases because bentonite adsorbs TRV nucleoprotein particles.

Table 10. Infectivity of inocula of complete TRV-Lisse prepared with and without bentonite. The different inocula were tested on opposite primary leaves of *P. vulgaris* 'Bataaf'. Tests were made in duplicate.

Virus	Number of local lesions/primary leaf	
	with bentonite	without bentonite
Complete TRV-Lisse	184	399
	300	343

7.1.3 The effect of bentonite and carborundum on leaves of test plants, upon transmission of complete and incomplete TRV

In a number of experiments, I investigated whether bentonite dusted on leaves of test plants has any effect on the transmission of complete and incomplete virus and what influence carborundum powder has on transmission.

Inocula were prepared as follows. For incomplete TRV-Lisse discs with a diameter of 12 mm were ground in 1 ml PCA buffer containing 25 mg bentonite/ml. For complete TRV-Lisse the leaf discs were ground in 1 ml PCA buffer. The macerate was poured into small test tubes and stored for one night at —4°C. (In 7.2 we will see that this treatment was not harmful to infectivity.) After thawing at room temperature I centrifuged all preparations separately for 10 min at 12500 × *g*. The supernatants were used without further treatment.

The inocula obtained were applied to primary leaves of *P. vulgaris* 'Bataaf', treated in four different ways: they were dusted with bentonite, with carborundum, with carborundum and bentonite, or they were not dusted at all. The number of local lesions on the leaves are in Table 11, which also gives details of the scheme in which the tests were done.

Table 11. The effect of dusting the primary leaves of *P. vulgaris* 'Bataaf' with bentonite and/or carborundum before inoculation, upon the number of local lesions.

Virus	Number of local lesions/20 leaves dusted with				Significant
	nothing	bentonite	carborundum	carborundum + bentonite	P = 0.05
Incomplete TRV-Lisse			340	298	no
Incomplete TRV-Lisse	11		496		yes
Incomplete TRV-Lisse	2	13			yes
Complete TRV-Lisse			2034	141	yes
Complete TRV-Lisse	14		766		yes
Complete TRV-Lisse	5	22			yes

The results indicated that with complete and incomplete TRV-Lisse carborundum had a large positive effect which can be explained by the abrasive nature of carborundum powder. It greatly increases the number of infection sites (Rawlins & Tompkins, 1936).

The effect of bentonite without carborundum was slightly positive compared with the control because the bentonite powder was then the only abrasive on the surface.

The effect of bentonite in combination with carborundum was negative, i.e. it decreased the number of local lesions, for complete TRV-Lisse. Carborundum was used in all routine tests for the transmission of complete TRV-Lisse. For incomplete TRV-Lisse there was no significant effect of bentonite in combination with carborundum. There are two possible explanations for this. The first is that negligible amounts of RNase were released during the rubbing of the inocula onto the leaves. The second is that not all the bentonite was removed from the inocula by the low speed centrifuging so that this residue of bentonite adsorbed all the RNase released at the inoculation.

With complete TRV-Lisse the negative effect of bentonite could be predicted from the high affinity of the clay for the nucleoprotein particles.

7.2 The effect of freezing on the preparation of the inocula

For the interaction experiments many leaf discs had to be tested for complete or incomplete virus. Therefore it was necessary to store these discs if possible so that I could do extensive experiments at one time and then carry out the tests at a later date.

Discs with a diameter of 12 mm were taken from *N. rustica* infected with either complete or incomplete TRV-Lisse. They were treated in three different ways:

A. the discs were cut in two, one half was ground in 0.5 ml PCA buffer, the other half in 0.5 ml PCA buffer containing 25 mg bentonite/ml. The obtained inocula were tested 10 min after grinding.

B. as under A, but after grinding the inocula were poured into small test tubes and stored for one night at —4 °C. After thawing at room temperature the inocula were tested.

C. the discs were divided into halves and then stored for one night in a mortar at —4 °C in 0.5 ml PCA buffer or in 0.5 ml PCA buffer containing 25 mg bentonite/ml. After thawing at room temperature the discs were ground and tested for infectivity.

All inocula were tested on *P. vulgaris* 'Bataaf'. Corresponding halves of discs were tested on opposite primary leaves. Tests were made in duplicate. The resulting numbers of local lesions are given in Table 12.

These results indicated that storing the inocula for one night at —4 °C (Treatment B) is not harmful to the infectivity of incomplete TRV-Lisse when bentonite was added. For incomplete virus the difference between grinding with and without bentonite was greater with storage at —4 °C than without storage at that temperature. For complete TRV-Lisse Treatment B was less favourable.

Treatment C was very harmful to both complete and incomplete TRV-Lisse, pro-

Table 12. Inefectivity of inocula prepared from infected *N. rustica* in three different ways as indicated in the text. The local lesion tests were done on *P. vulgaris* 'Bataaf'

Virus	Treatment	Number of local lesions/primary leaf	
		with bentonite	without bentonite
Complete TRV-Lisse	A	184	399
		300	343
Incomplete TRV-Lisse	A	438	0
		182	6
Complete TRV-Lisse	B	182	84
		119	120
Incomplete TRV-Lisse	B	286	0
		448	0
Complete TRV-Lisse	C	21	158
		11	84
Incomplete TRV-Lisse	C	42	0
		68	0

bably because of the long time necessary to reach —4°C and then to reach room temperature again, in the large mass of the mortar.

A storage period could be introduced without harm if the discs were homogenized, quickly frozen and stored at —4°C.

After this experiment I tried to find the right moment to store the material during the inoculum preparation. In principle there were two possibilities. Either the discs could be frozen, stored, ground after thawing and then inoculated, or ground, frozen, stored and inoculated after thawing. To choose between these alternatives I did the following experiment.

A batch of *N. rustica* plants was inoculated with TRV-Lisse and an equal batch with the incomplete form of TRV-Lisse. Four to five days after inoculation, 60 discs with a diameter of 12 mm, each containing one primary lesion, were taken from leaves of each batch. From every 60 discs, 30 were frozen in a Petri dish and stored for one night at —4°C. The next morning each disc after thawing at room temperature was cut in two. One half was ground in 0.5 ml PCA buffer, the other was ground in 0.5 ml PCA buffer containing 25 mg bentonite/ml. Corresponding halves were tested on opposite primary leaves of *P. vulgaris* 'Bataaf'. Each of remaining 30 discs was immediately cut in two. One half was ground in 0.5 ml PCA buffer, the other was ground in 0.5 ml PCA buffer containing 25 mg bentonite/ml. All inocula obtained were poured into small test tubes and stored for one night at —4°C. The next morning the inocula were thawed at room temperature and inocula of corresponding halves were tested on opposite primary leaves of *P. vulgaris* 'Bataaf'. When the lesions on *P. vulgaris* 'Bataaf' were well developed, usually within 3–4 days, the numbers of local lesions were counted. The results of the experiment are summarized in Table 13.

It is evident that grinding incomplete TRV-Lisse in PCA buffer containing 25 mg

Table 13. The effect of storage at –4 °C for one night at different steps in the preparation of inocula from *N. rustica* leaves infected with complete and incomplete TRV-Lisse. Local lesions tests were done on *P. vulgaris* 'Bataaf'.

Virus	Sequence of treatments				Total number of local lesions/30 discs	Significant P = 0.05
	freezing and thawing	grinding		freezing and thawing		
		with bentonite	without bentonite			
Complete	+[1]	+	–[2]	–	504	yes
TRV-Lisse	+	–	+	–	11,935	
Complete	–	+	–	+	549	yes
TRV-Lisse	–	–	+	+	5,753	
Incomplete	+	+	–	–	184	yes
TRV-Lisse	+	–	+	–	1	
Incomplete	–	+	–	+	2,435	yes
TRV-Lisse	–	–	+	+	0	

1. + = treatment applied. 2. – = treatment not applied.

bentonite/ml gave a far better inoculum than grinding in PCA buffer. This result agreed with other findings described in 7.1.1. Freezing before grinding in a solution containing bentonite almost completely destroyed the infectivity. This result could be expected because freezing would have disturbed the internal organization of the cells and during freezing and thawing the plant RNases could destroy the free TRV-Lisse RNA.

Grinding material infected with complete TRV-Lisse in a buffer containing 25 mg bentonite/ml was harmful to the infectivity of the obtained inoculum. I found less local lesions because the TRV nucleoprotein particles were adsorbed by the bentonite and therefore were probably rendered incapable of infecting the test plant. Freezing before grinding increased the infectivity of the inoculum, probably because the cells desintegrated more easily by this sequence of treatments.

Because the two forms of TRV-Lisse react very differently to the described treatments, it is possible to distinguish between these two forms. This will be discussed in 7.4.

An additional experiment indicated that if the period of storage at –4 °C was prolonged to a week, the infectivity of the preparations of complete and incomplete TRV-Lisse did not decrease noticeably. To keep possible detrimental effects of prolonged storage, i.e. the loss of infectivity, to a minimum, I always inoculated the preparations within a week after the beginning of the storage period. To make sure that the whole mass of leaf material and buffer was frozen through, storage at –4 °C was never shorter than 15 h.

7.3 Differences in symptoms between complete and incomplete TRV infections

Differences in symptoms caused by complete and incomplete TRV could be recognized on some hosts, that become systemically infected, such as tobacco varieties 'Xanthi' and 'White Burley' and *N. rustica*. On the local lesion host, *P. vulgaris* 'Bataaf', no difference between lesions containing complete or incomplete virus could be seen.

As *N. rustica* was the test plant commonly used, I shall describe the symptoms on this species in detail. In the two *N. tabacum* varieties the differences were similar to those described for *N. rustica*. On *N. rustica* complete TRV-Lisse caused chlorotic to necrotic lesions on the inoculated leaves. If a concentrated inoculum was used this caused a 'partridge pattern', consisting of numerous small brown necrotic lesions. As the virus moved on in the plant, the younger leaves showed a chlorotic and necrotic flecking in variable amounts and the leaves often became distorted. In older plants younger leaves often remained without symptoms, although virus may have been present. Incomplete TRV-Lisse, and long particles of TRV-Lisse caused large brownish necrotic lesions that spread rapidly in the inoculated leaves. These lesions were nearly circular, and remained so until they spread to a vein. Then the symptoms spread rapidly along the vein. Via the stem, which showed brown stripes in older infections, the virus moved into the younger leaves where it caused brown necrosis along the veins. If young plants were used they died shortly afterwards. Older plants reacted less violently. Fig. 19 shows symptoms on *N. rustica* leaves inoculated with complete and incomplete TRV-Lisse.

According to Lister (1967) the complete and incomplete forms of PEBV show different symptoms on *P. vulgaris* 'Prince'. We did not find different symptoms for complete and incomplete PEBV-Dik Trom 5 on *P. vulgaris* 'Bataaf'.

7.4 Conclusion and discussion

In this chapter I described two methods to distinguish between complete and incomplete TRV. To discover whether a whole plant is infected with complete or incomplete virus the difference in symptoms is sufficient. When the symptoms develop the symptom descriptions given in 7.3 could be used to decide what type of virus is present. This test could be used for TRV if *N. rustica* and *N. tabacum* 'Xanthi' and 'White Burley' were used as test plants. Different symptoms caused by complete and incomplete forms of PEBV on *P. vulgaris* 'Prince' were reported by Lister (1967). We did not detect such a difference with *P. vulgaris* 'Bataaf'.

To determine what kind of virus is present in individual lesions, the bentonite-transmission test should be used. This test clearly distinguished between complete and incomplete forms, whatever their origin, because bentonite had a totally different influence on their transmission. In the case of complete virus the transmission with bentonite was less efficient than the transmission without bentonite. With incomplete

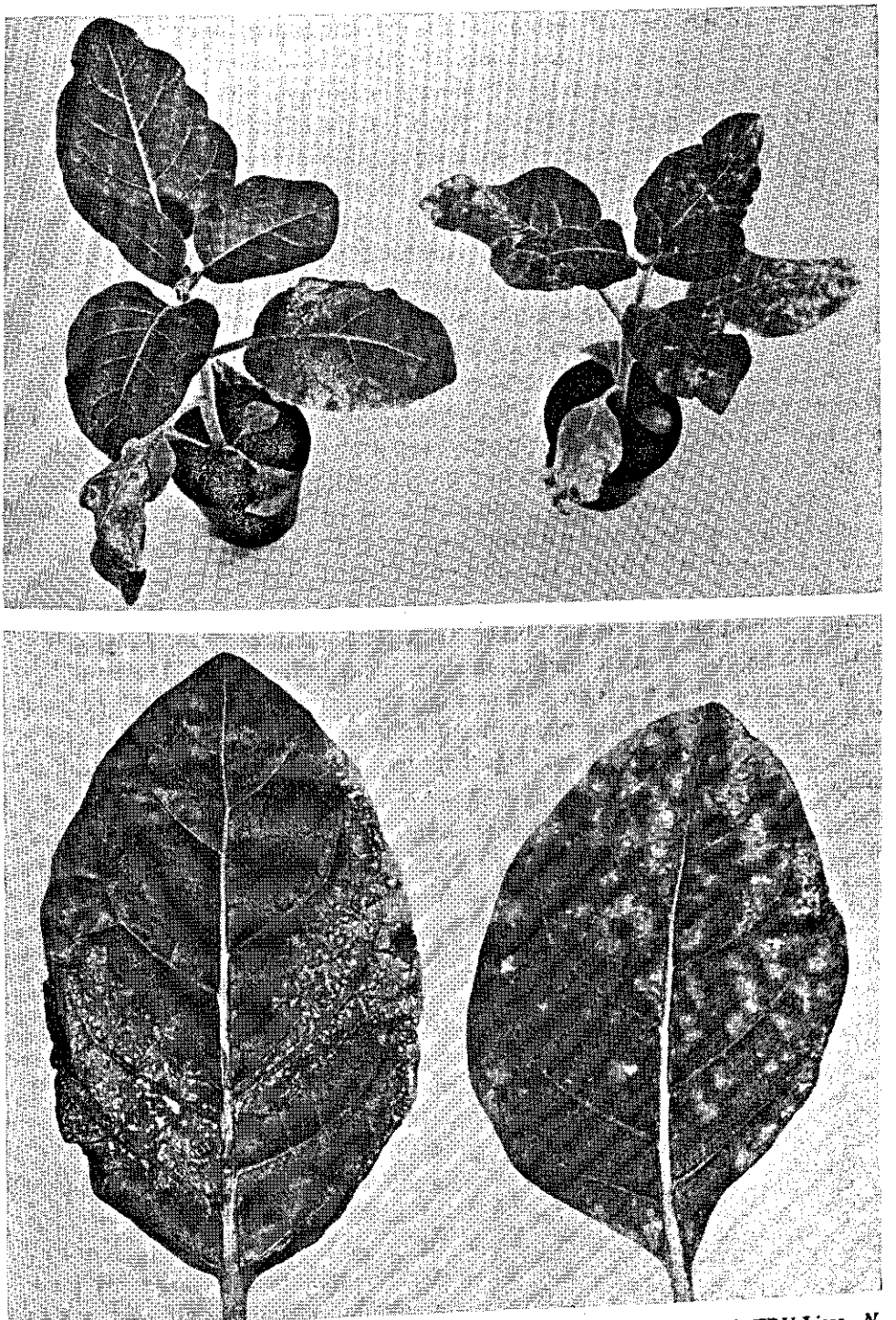

Fig. 19. *N. rustica* plants inoculated with incomplete (left) and complete (right) TRV-Lisse. *N. rustica* leaves with symptoms of incomplete (left) and complete (right) TRV-Lisse.

virus the transmission with bentonite was far better than transmission without bentonite. The test itself was very simple. The halves of a lesion were macerated with and without bentonite. After at least 15 min the infectivity of the obtained inocula was tested on primary leaves of *P. vulgaris* 'Bataaf'. If the infectivity test had to be postponed so that the test plants were in optimum condition, the inocula could be kept for at least a week at —4 °C and subsequently used without any detrimental effects. 'Bataaf' leaves could be inoculated in the normal way with carborundum as an abrasive. As seen in 7.1.3 bentonite dusted on the leaves before inoculation had a negative effect on the number of local lesions for incomplete virus. On the amount of bentonite/ml to be used there may be some discussion. From the experiments described in 7.1 I concluded that 25 mg/ml was a concentration suitable for the tobacco plants I used.

The bentonite-transmission test seems very similar to the test used by Lister (1966). However, Lister did not use bentonite, but only froze whole lesions at —5 °C for 48 h and then thawed them so that there was no control at all whether the lesions tested contained virus or not, as after freezing and thawing no infectivity could be detected.

Phenol can be used instead of bentonite in the extractions. It will give the same results. However, the phenol extractions are very laborious and one has to be very careful in handling the phenol. Therefore only a limited number of tests can be made per day.

Another method to distinguish between complete and incomplete TRV and PEBV is the presence or absence of nucleoprotein particles in the lesions. There will be no viral nucleoprotein particles present in the incomplete virus. But it can only be decided that a lesion contained incomplete virus, if negative electron microscopic results are combined with positive results of infectivity tests. This method, however, is far less efficient than the bentonite-transmission test.

8 Interaction experiments

As one of the important aims of my research was to find out if long and short particles of different TRV isolates could interact, I studied whether the particles could induce the formation of complete virus, if applied on the same plant.

I started with interaction experiments with homologous long and short particles of both TRV-Lisse and PEBV-Dik Trom 5. Then I studied the effect of inoculating the long and short particles at different times.

The last part of this chapter deals with (a) interaction between particles of different complete TRV isolates; (b) interaction between incomplete TRV isolates and short particles of complete TRV isolates; and (c) interaction between particles of TRV-Lisse and PEBV-Dik Trom 5.

8.1 Interaction between homologous particles

8.1.1 Particles inoculated at the same time

TRV. Three complete TRV isolates, Lisse, F12a, and F15, were purified according to the ether-tetra method. Starting from these preparations the long and short particles were separated by density-gradient centrifuging (5.3.1) followed by additional centrifuging on a sucrose gradient in an SW 25.1 rotor (5.3.2), just before the particles had to be used. All preparations were tested as follows. They were inoculated on *N. rustica*, *N. tabacum* 'Xanthi' and *N. tabacum* 'White Burley' in a concentration of 0.02 or 0.04 mg/ml. From these plants, 10 leaf discs of 12 mm diameter each containing one local lesion were taken, and tested with the bentonite-transmission test (7.4) to see whether they contained complete virus, incomplete virus, or no virus at all. The results are in Table 14.

It is obvious that separate short particles are not infectious. Long particles are, but give rise to the formation of incomplete virus. The mixture of homologous long and short particles is infectious and induces the formation of complete virus. In this particular case the concentration of long and short particles was 0.02 and 0.04 mg/ml, resp., a long to short particle ratio of 1 to 5. The same ratio was found in infected plants (Fig. 11).

For mixtures of long and short particles, the average number of local lesions formed after transmission without bentonite was lower than that with bentonite. The first though less important explanation for this result is that I found interaction in less than 100% of the primary lesions. In the experiment presented in Table 14, complete

Table 14. The formation of virus by homologous mixtures of long and short particles of different isolates of TRV. The concentration of the long and short particles in the inocula was 0.02 and 0.04 mg/ml, respectively, as they were inoculated on *N. rustica*, *N. tabacum* 'White Burley' and 'Xanthi'. Ten leaf discs containing one primary lesion were each tested with the bentonite-transmission test to decide what kind of virus they contained.

Tobacco plants inoculated with	Average number of local lesions on bean after transmission in		Percentage of primary lesions on tobacco with complete virus
	buffer	buffer with bentonite	
Lisse-L[1]	0	272	0
F12a-L	0	214	0
F15-L	0	73	0
Lisse-S[2]	0	0	no lesions
F12a-S	0	0	no lesions
F15-S	0	0	no lesions
Lisse-L and Lisse-S	7.3	245	50
F12a-L and F12a-S	17	196	90
F15-L and F15-S	6.7	138	40

1. L = long particles. 2. S = short particles.

virus was found for Lisse-L and Lisse-S; F12a-L and F12a-S; and F15-L and F15-S in 50, 90, and 40% of the infections, respectively. For each of the three isolates these percentages varied considerably. In most of the experiments percentages varied between 40 and 80. In comparable experiments Sänger (1969) found complete virus in 52–72% of the primary lesions. At relatively low concentrations of virus it cannot be expected that all lesions contain complete virus, because the chance that a long and a short particle invade the same infection site diminishes rapidly with decreasing concentration (Sänger, 1968b).

Another explanation for the fact that where interaction occurred transmission with bentonite was better than without bentonite could be that the leaf discs contained two infections: one with complete and one with incomplete virus. However, I excluded this possibility by taking the leaf discs early (four to five days) after inoculation to prevent the infections spreading and by selecting isolated infections. I therefore could conclude that in primary lesions containing complete TRV still a lot of the infectivity is present as free RNA.

PEBV. These experiments, done with the isolate of PEBV designated Dik Trom 5, were described in detail earlier (Huttinga, 1969), so only the results will be mentioned (Table 15).

Short particles are not infectious, long particles are but give rise to the formation of incomplete virus. If long and short particles are inoculated together complete virus is formed.

It was remarkable that lesions caused by PEBV contained far less virus than those caused by TRV.

Table 15. Results of inoculation experiments with long and short particles of PEBV-Dik Trom 5. The concentration of long and short particles in the inocula was 0.04 mg/ml as they were inoculated on *N. rustica*. Ten leaf discs containing one primary lesion were each tested with the bentonite-transmission test to decide what kind of virus they contained.

N. rustica plants inoculated with	Average number of local lesions on bean after transmission in		Percentage of primary lesions on *N. rustica* with complete virus
	buffer	buffer with bentonite	
Long particles	0	5.7	0
Short particles	0	0	no lesions
Long and short particles	3.1	7.5	43

8.1.2 *Particles inoculated at different times*

As was demonstrated in 8.1.1 complete virus is produced after inoculation with a mixture of long and short particles. In those experiments long and short particles were inoculated at the same time. In the following experiments long and short particles were inoculated at different times.

Long particles inoculated before short particles. At t = 0 120 *N. rustica* plants were inoculated with long particles of TRV-Lisse (0.02 mg/ml) in PCA buffer. After some minutes the leaves were rinsed to remove the excess particles. At t = 0, 24, 48, 72, 96, and 168 h groups of 20 plants were again inoculated, but now with short particles of TRV-Lisse (0.02 mg/ml). The plants were grown under normal greenhouse conditions and seven days after the inoculation of the short particles complete virus was isolated according to the ether-tetra method (3.3.1) and suspended in $1\frac{1}{2}$ ml PCA buffer. The resulting preparations were each tested in proper dilutions on primary leaves of *P. vulgaris* 'Bataaf'. For each test 6 primary leaves were used. The results of these tests are given in Fig. 20, where the average number of local lesions on bean multiplied by the dilution factor and converted to 100 g of leaves is plotted against the time that has passed between the inoculation of long and short particles.

It is evident that inoculating long and short particles shortly after each other is not the best way to initiate a high production of complete virus. The best results were obtained when short particles were inoculated two days after the long particles.

Short particles inoculated before long particles. N. rustica plants were inoculated with short particles of TRV-Lisse (0.01 mg/ml). After some minutes the leaves were rinsed with running tap water. 1, 2, 7 and 24 h later batches of these plants were inoculated with long particles of TRV-Lisse (0.01 mg/ml). In control experiments, plants were inoculated with either short or long particles. Four days after the inoculation of the long particles leaves of the different groups of plants were harvested and treated according to the ether-tetra method in order to isolate complete TRV. The prepara-

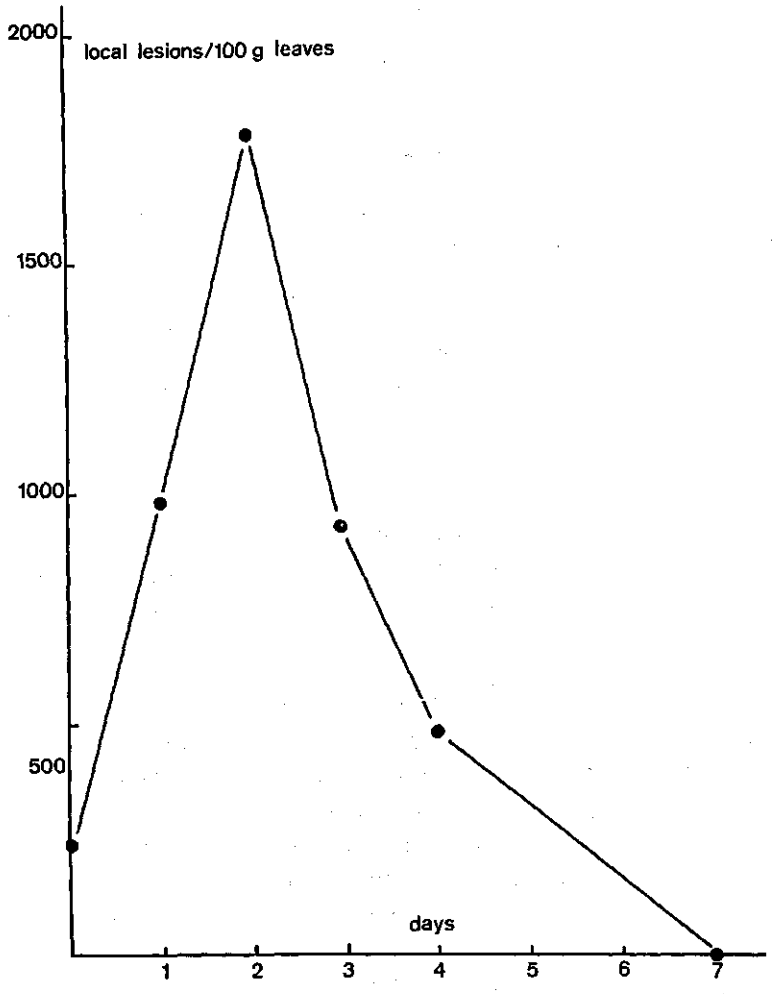

Fig. 20. Formation of complete virus in leaves of *N. rustica* inoculated with long and short particles (0.02 mg/ml) at different intervals. Seven days after the inoculation of the short particles, complete virus was isolated with the ether-tetra method, dissolved in 1½ ml buffer, and tested in proper dilutions on 6 primary leaves of *P. vulgaris* 'Bataaf'. The average number of local lesions multiplied by the dilution factor and converted to 100 g of leaves is plotted on the ordinate. On the abscissus the number of days that elapsed between the inoculation of the long and that of the short particles is plotted.

tions obtained were each tested for complete virus on 8 primary leaves of bean. The results are presented in Table 16.

When the time between inoculation of short and long particles was 1, 2, or 7 h, complete virus was still formed; after 24 h, complete virus was no longer formed.

Table 16. Formation of complete virus when the short particles are inoculated before the long ones. Inoculations were done with purified long and short particle preparations (0.01 mg/ml) of TRV-Lisse on *N. rustica*. Four days after the inoculation of the long particles the inoculated leaves were harvested and attempts were made to isolate complete TRV according to the ether-tetra method. The preparations obtained were tested for complete virus on 8 primary leaves of bean.

N. rustica inoculated with		Kind of virus formed
Lisse-S[1] at t (h) =	Lisse-L[2] at t (h) =	
0		no virus
	0	incomplete
0	1	complete
0	2	complete
0	7	complete
0	24	incomplete

1. S = short particles. 2. L = long particles.

8.2 Interaction between heterologous particles

8.2.1 Interaction between particles of different complete TRV isolates

Three TRV isolates Lisse, F12a, and F15, were purified according to the ether-tetra method and their particles separated by density-gradient centrifuging. The particle preparations were tested for purity by inoculating them in a concentration of 0.01 or 0.02 mg/ml on *N. rustica*, *N. tabacum* 'White Burley' and *N. tabacum* 'Xanthi'. Homologous and heterologous mixtures of long and short particles (concentration of long and short particles 0.01 or 0.02 mg/ml) were also inoculated on these plants. The primary infections of the plants were tested with the bentonite-transmission test to see whether they contained complete or incomplete virus. Thirty lesions per treatment were tested. The results are in Table 17.

The short particles of all three isolates were not infectious. All three kinds of long particles were, but only gave rise to the formation of incomplete virus. The combinations of homologous long and short particles induced formation of complete virus, not only for TRV-Lisse, but also with F12a and F15. These results complemented those described in 8.1.1.

In the case of heterologous combinations of long and short particles interaction also took place, leading to the formation of complete virus. A combination of a long particle with its homologous short particle was not always superior with respect to the formation of complete virus, to combinations with heterologous short particles. I tested this phenomenon several times and always found the same effect.

Table 17. Results of interaction experiments with particles of different complete TRV isolates. A. Concentration of both long and short particles was 0.01 mg/ml. B. Concentration of long and short particles was 0.01 and 0.02 mg/ml, respectively. *N. rustica*, *N. tabacum* 'White Burley' and 'Xanthi' were used as test plants. Per treatment 30 primary lesions were tested with the bentonite-transmission test to see what kind of virus they contained. In the controls ten lesions were tested.

Inoculum	Number of primary lesions on tobacco with					
	A			B		
	complete virus	incomplete virus	no virus detected	complete virus	incomplete virus	no virus detected
Lisse-L[1]				0	10	0
Lisse-S[2]				no lesions		
F12a-L				0	10	0
F12a-S				no lesions		
F15-L				0	10	0
F15-S				no lesions		
Lisse-L and Lisse-S	6 (24)[3]	19	5	14 (54)	12	4
Lisse-L and F12a-S	21 (70)	9	0	21 (75)	7	2
Lisse-L and F15-S	14 (52)	13	3	7 (32)	15	8
F12a-L and Lisse-S	5 (24)	16	9	8 (42)	11	11
F12a-L and F12a-S	16 (55)	13	1	13 (76)	4	13
F12a-L and F15-S	8 (28)	21	1	9 (33)	18	3
F15-L and Lisse-S	6 (27)	16	8	13 (54)	11	6
F15-L and F12a-S	6 (30)	14	10	15 (75)	5	10
F15-L and F15-S	1 (8)	12	17	8 (33)	16	6

1. L = long particles.
2. S = short particles.
3. number of primary lesions containing complete virus expressed as a percentage of the total number of lesions in which virus was detected.

None of the short particles of any isolate used, showed a specific superiority in all combinations over other short particles.

In Experiment A of Table 17 the concentration of both long and short particles was 0.01 mg/ml. Doubling the concentration of short particles (Experiment B), generally led to a higher number of primary lesions with complete virus; in some cases the number of lesions was doubled. In a few cases, however, no increase in the number of lesions with complete virus, and once even a lower percentage, was found. The increase of the percentage of lesions with complete virus due to doubling the amount of short particles may be explained by the greater chance that long and short particles invade the same infection site and interact.

In the interaction experiments with long and short particles of the complete isolates Lisse, F12a, and F15, I noticed that if only the long particles were inoculated on tobacco, the symptoms of incomplete virus were formed. If the long particles were

inoculated with homologous particles the symptoms of complete virus were induced. The same result was obtained if long particles were combined with heterologous short particles. Because the symptoms of Lisse, F12a, and F15 could not be distinguished, it was not possible to differentiate between symptoms induced by homologous and heterologous mixtures of long and short particles.

The viruses induced by inoculating *N. rustica* plants with a combination of heterologous long and short particles of TRV-Lisse and F15 were purified according to the ether-tetra method and particle lengths measured. The results are presented in Table 18.

Table 18. Particle lengths of virus induced in *N. rustica* by heterologous combinations of long and short particles of TRV-Lisse and F15. Grids were prepared with virus purified according to the ether-tetra method.

N. rustica inoculated with	Particle length (nm)
Complete Lisse	67.5 and 185
Complete F15	50 and 190
Lisse-L[1] and F15-S[2]	52.5 and 187.5
F15-L and Lisse-S	72.5 and 202.5

1. L = long particles. 2. S = short particles.

It is evident that in the heterologous combinations the viruses have properties of both 'parent' isolates with respect to particle length. These results agree with those of Sänger (1968a), who reported accordingly for TRV-GER and TRV-USA.

8.2.2 Interaction between the incomplete isolates F7 and F9, and the short particles of Lisse, F12a, and F15

The two incomplete isolates F7 and F9 were purified with methods for the extraction of the total nucleic acid content from 'White Burley' leaves. Plants infected with F7 were treated according to the phenol-bentonite-diethylpyrocarbonate method (3.3.2). F9 was purified by the phenol-bentonite method (3.3.2). The former method yielded preparations which were about ten times as infectious as the latter. Therefore different concentrations were used to inoculate *N. rustica*, *N. tabacum* 'White Burley' and *N. tabacum* 'Xanthi' (dilutions of 1:2 and 1:20 for F9 and F7, respectively).

The short particles of Lisse, F12a, and F15 were prepared by density-gradient centrifuging. Their concentration in the controls and the interaction experiments was 0.02 mg/ml. The results of the experiments are in Table 19.

Table 19. Results of interaction experiments with the incomplete isolates F7 and F9, and the short particles of Lisse, F12a, and F15. *N. rustica*, *N. tabacum* 'White Burley' and 'Xanthi' were used as host plants. The short particles were used in concentrations of 0.02 mg/ml. Per treatment 10 or 30 primary lesions of tobacco were tested to decide what kind of virus they contained.

Inoculum	Number of primary lesions with		
	complete virus	incomplete virus	no virus detected
F7	0	30	0
F9	0	10	0
Lisse-S[1]	no lesions		
F12a-S	no lesions		
F15-S	no lesions		
F7 and Lisse-S	2 (8)[2]	24	4
F7 and F12a-S	3 (16)	16	11
F7 and F15-S	3 (12)	22	5
F9 and Lisse-S	5 (50)	5	0
F9 and F12a-S	5 (56)	4	1
F9 and F15-S	4 (40)	6	0

1. S = short particles.
2. number of local lesions with stable virus expressed as a percentage of the total number of lesions in which virus was detected.

It is evident that the incomplete strains F7 and F9, which normally produce incomplete virus, can be stabilized by adding non-infectious short particles of Lisse, F12a, and F15.

If short particles of the complete isolates were added to the incomplete isolates, the symptoms induced on tobacco were those typical for complete virus, and these could be distinguished from the normal symptoms of F7 and F9.

By using the electron microscope, nucleoprotein particles could be demonstrated in tobacco plants inoculated with a mixture of an incomplete isolate and short particles of one of the complete isolates.

8.2.3 Interaction between particles of TRV-Lisse and PEBV-Dik Trom 5

Long and short particles of both viruses, separated by density-gradient centrifuging, were inoculated on *N. tabacum* 'White Burley' and 'Xanthi' (concentration 0.02 and 0.04 mg/ml for TRV and PEBV particles, respectively). From the tobacco plants primary lesions were tested with the bentonite-transmission test to determine what kind of virus they contained. For each treatment 10 lesions were taken from one or both of the tobacco varieties. Local lesion tests on bean were done in duplicate.

The results (Table 20) of the control experiments indicate that the different particle preparations were pure. In the heterologous combinations, TRV-L + PEBV-S gave rise to a high percentage of lesions with complete virus. On 'White Burley' in 90% of

Table 20. Results of interaction experiments between particles of TRV-Lisse and PEBV-Dik Trom 5. The particles were inoculated on *N. tabacum* 'White Burley' and 'Xanthi' in concentrations of 0.02 and 0.04 mg/ml for the TRV and the PEBV isolate, respectively. From one or both of the tobacco varieties 10 primary lesions were tested with the bentonite-transmission test to decide what kind of virus they contained.

Inoculum	Host plant	Number of primary lesions with		
		complete virus	incomplete virus	no virus detected
TRV-L[1]	Xanthi	0	10	0
TRV-L and TRV-S[2]	Xanthi	10	0	0
TRV-S	Xanthi	no lesions		
	White Burley	no lesions		
PEBV-L	White Burley	0	8	2
PEBV-L and PEBV-S	White Burley	3	4	3
PEBV-S	White Burley	no lesions		
	Xanthi	no lesions		
TRV-L and PEBV-S	White Burley	9	1	0
	Xanthi	3	7	0
PEBV-L and TRV-S	White Burley	1	8	1

1. L = long particles. 2. S = short particles.

the lesions complete virus was found. The combination PEBV-L + TRV-S gave a much lower percentage of lesions containing complete virus.

This discrepancy could be explained by the difference in biological activity of both types of virus. From Table 21 it can be seen that TRV-Lisse multiplied to a greater extent in the tobacco varieties than PEBV-Dik Trom 5, because in the bentonite-transmission test many more local lesions were found on bean for TRV than for PEBV. So in the case of TRV-L + PEBV-S, the short particles were added to long particles that were able to stimulate the plant to a high production of virus material. The short particles could be replicated immediately at a high rate and therefore many lesions with complete virus could be found. In the case of the combination PEBV-L and TRV-S the long particles hardly induced virus synthesis in the 'White Burley' plants (Table 21), so in the case of this combination less virus of both types was produced. I cannot explain the observed difference in virus production between 'Xanthi' and 'White Burley'.

A mixture of long particles of TRV-Lisse and short particles of PEBV-Dik Trom 5 induced on bean the symptoms typical for TRV. In the reciprocal combination the symptoms were typical for PEBV. On tobacco plants it was seen that interaction occurred because the plants showed symptoms of the complete virus type. These symptoms were identical to those of the parent that provided the long particles.

Table 21. Results of the bentonite-transmission test in interaction experiments between particles of TRV-Lisse and PEBV-Dik Trom 5. The particles were inoculated on *N. tabacum* 'White Burley' and 'Xanthi' in concentrations of 0.02 and 0.04 mg/ml for the TRV and the PEBV isolate, respectively. From one or from both of the tobacco varieties 10 primary lesions were tested.

Inoculum	Host plant	Average number of local lesions on bean after transmission of the primary lesions from the tobacco varieties in	
		buffer	buffer with bentonite
TRV-L[1]	Xanthi	0.4	96
TRV-L and TRV-S[2]	Xanthi	334	276
TRV-S	Xanthi	no lesions on tobacco	
	White Burley	no lesions on tobacco	
PEBV-L	White Burley	0.1	5.7
PEBV-L and PEBV-S	White Burley	3.1	7.5
PEBV-S	White Burley	no lesions on tobacco	
	Xanthi	no lesions on tobacco	
TRV-L and PEBV-S	White Burley	14.7	318
	Xanthi	1.4	235
PEBV-L and TRV-S	White Burley	4.2	3.9

1. L = long particles. 2. S = short particles.

8.3 Conclusion and discussion

My findings that long particles of TRV-Lisse are infectious and short ones are not, agreed with similar results obtained by many others (Harrison & Nixon, 1959; Sänger, 1960; Lister, 1966, 1968; Frost et al., 1967). Lister (1966, 1967, 1968), Frost et al. (1967) and Sänger (1968a) reported earlier that if long particles are inoculated together with the non-infectious short particles, stable virus is formed.

The results I obtained with the particles of the PEBV isolate confirmed those reported by Lister (1967) and Sänger (1969).

It was not necessary for the formation of complete TRV-Lisse to introduce the long and the short particles at the same time onto the host plant. If short particles were inoculated first, one could wait 7 h before the long ones had to be inoculated. If long particles were inoculated 24 h after the short ones, no complete virus was formed indicating that the short particles were inactivated in that period.

If short particles were inoculated after the long ones, one could expect that the inoculation of the short particles could be postponed as long as there is TRV polymerase activity and protein synthesizing activity left in the leaves to be inoculated. In my experiments this period appeared to be 7 days in the inoculated leaves. It was determined by the fact that incomplete virus infections gave rise to symptoms (6.3), which killed the leaves.

The amount of complete virus was maximum if short particles were inoculated two days after the inoculation of the long ones. At this time the amount of incomplete virus was maximum in plants inoculated with long particles only as seen in 4.3. Apparently at this time the whole mechanism responsible for the multiplication of TRV-RNA works at maximum capacity, so the short particle RNA will be multiplied readily after invading the plant. Consequently the synthesis of virus protein will start and virus assembly can take place.

In the interaction with homologous long and short particles I found that in primary lesions of tobacco containing complete virus still a large part of the infectivity was present as free RNA. This result agrees very well with that of Cadman (1962), who showed that for tobacco leaves infected with the potato ring necrosis culture of TRV, 95% of the virus was unstable virus, i.e. free RNA. Only 5% of the virus was complete virus, i.e. RNA coated with protein.

The interaction between long and short particles of different TRV isolates has been a subject of research for many virologists. Frost et al. (1967) reported experiments with both nucleoprotein particles and RNA, but they did not find interaction. Their conclusion was that interaction between long and short particles was strictly isolate-specific. Sänger (1968a, b), however, described the interaction between particles of two different isolates. When he extended his work in 1969 and included two more TRV isolates and a PEBV isolate, he found that only both reciprocal combinations between TRV-GER and TRV-USA could interact; the others were not able to do so. At the same time Lister (1968) reported the interaction between unstable variants of TRV and short particles of strains that belonged to the same serotype (Harrison & Woods, 1966). One year later Lister (1969) described the interaction between nucleoprotein particles of three TRV strains. Semancik & Kajiyama (1968) reported that heterologous short particles enhanced the number of stable form infections of TRV.

My findings with respect to interaction between long and short particles of the complete isolates, Lisse, F12a, and F15, and between the incomplete isolates F7 and F9 and the short particles of the complete isolates, agree very well with most of the findings of the authors mentioned. They only disagree with those of Frost et al. (1967).

A combination of a long particle with its homologous short particle is not always superior with respect to the formation of complete virus, to combinations with heterologous short particles. As already mentioned, it also is not necessary that long and short particles are inoculated at the same time. These two factors facilitate the formation of new TRV isolates that have intermediate characteristics compared with the parent isolates with respect to particle length and have the symptoms of the complete form of the parent isolates.

Interaction between long particles of TRV-Lisse and short particles of PEBV-Dik Trom 5 could be found to a large extent in 'White Burley' tobacco. The reciprocal combination was less effective with respect to interaction. Other authors have also tried to induce interaction between particles of a TRV isolate and a PEBV isolate (Lister, 1966; Sänger, 1969). They could not detect any interaction. Sänger (1969) explained this by assuming an inability of the proteins to coat the heterologous RNA,

due to the difference in diameter he thought existed between the virus particles. I tried to check this theory in reconstitution experiments, but I was not able to isolate native protein of TRV, with either the acetic acid method (Fraenkel-Conrat, 1957) or with the $CaCl_2$ method (van Regenmortel, 1967). However, Semancik & Reynolds (1969) managed to isolate native protein and described the reconstitution of TRV-RNA and TRV-protein. A year later Semancik (1970) reported the reconstitution of nucleoprotein particles, with typical biological activities from heterologous mixtures of extracted protein and RNA of two TRV isolates.

From the results of the interaction experiments with respect to symptoms, it could be concluded that short particles had an influence on the type of symptoms that were formed on tobacco. Lister described similar results for TRV (Lister, 1969) and for PEBV (Lister, 1967). From such results, however, it can not be concluded, that the RNA of the short particles contains a genetic code for part of the symptoms. These symptoms may be a direct effect of the coat-protein synthesis that is induced by the short particle RNA.

Until now the relationship between TRV and PEBV was based on both viruses having a bimodal particle-length distribution (Bos & van der Want, 1962) and being serologically related (Maat, 1963; Allen, 1967). The fact that long and short particles of TRV and PEBV have the same biological activities, i.e. the long particles are infectious, the short particles are not and complete virus is formed only if long and short particles are inoculated together, is another piece of evidence that TRV and PEBV are related.

The interaction between heterologous long and short particles of TRV and PEBV suggests even more strongly that these viruses are very closely related. In my opinion they may even be considered as two strains of one virus. The interaction, which is only possible if a close similarity exists in the mode of replication of the two particles, can be considered to be a better argument for this supposition than the arguments until now used against it. These latter arguments were differences in symptoms on pea and bean and the differences in particle-length distributions. PEBV generally has longer particles and the ratio of long particle length to short particle length is 2 instead of 2.5 as is found for TRV particles. However, the last difference is also found within the different TRV isolates. Sänger (1968a) described an isolate (TRV-USA) with particle lengths closely resembling those of PEBV. Especially the short particles (105 nm) of this isolate had exactly the same length as short particles of PEBV. Moreover the long particles were 195 nm long, so the ratio of long particle length to short particle length was almost equal to that typical for PEBV.

9 General conclusion

In the introduction I stated that the aim of my work was to explain why there are so many different TRV isolates and why no correlation can be found between classifications based on different characteristics. I can now answer these questions.

In Chapter 8 in all combinations tested, interaction was possible between long and short particles of different complete TRV isolates. Incomplete isolates could be completed by short particles of complete isolates. Even heterologous long and short particles of TRV-Lisse and PEBV-Dik Trom 5 could interact. However, several authors have reported that not all the combinations they tested were able to interact. Lister (1969) even reported that the combination of long particles of TRV-BRAZ and short particles of TRV-YEL could interact, but that the reciprocal combination failed to do so. Nevertheless it is obvious why so many TRV strains are found. Every time a heterologous combination of long and short particles interacts, a new strain is formed. The fact that long and short particles do not have to be inoculated at the same time increases the chance that new strains are formed. Moreover a combination of a long particle with the short particle found to accompany it at the isolation is not always superior with respect to the formation of complete virus, to combinations with other short particles.

In the new combinations genetic information of both parent strains is present. The long particles introduce the code for all functions necessary to start infection and the replication of their own RNA and determine the symptoms on bean. The short particles contain the code for the coat protein (Sänger, 1968a). Long and short particles together determine the symptoms on tobacco, and both RNAs determine the length of their nucleoprotein particles.

Because it is easy to form new combinations of long and short particles, capable of inducing complete virus, it is simple to explain why no correlation has been found between properties like serology, symptomatology and particle-length distribution. These are properties which are coded by different particles which can interact in many combinations.

Interaction between particles of different strains and formation of new strains is also reported for AMV (van Vloten-Doting, 1968) and tobacco streak virus (Fulton, 1970). De Jager & van Kammen (1970) described it for a mutant and its parent strain of cowpea mosaic virus.

Summary

In this thesis the interaction between homologous and heterologous long and short particles of 5 isolates of tobacco rattle virus (TRV) and one isolate of pea early-browning virus (PEBV) is described.

In the introduction the aim of the research is explained.

In chapters 2 and 3 a review of the literature is given and methods are described.

The different TRV isolates, Lisse, F12a, F15, F7 and F9, and the PEBV isolate Dik Trom 5 are characterized in Chapter 4. TRV-Lisse, F12a, and F15 and PEBV-Dik Trom 5 are called 'complete' isolates. F7 and F9 are called 'incomplete' isolates.

It was easy to differentiate between TRV and PEBV by symptomatology, serology and particle-length distribution. Complete isolates of TRV could be distinguished from incomplete ones. Differentiation within the groups of complete and incomplete isolates, however, was not possible. The differences in symptomatology were small and often the differences due to fluctuations in environmental conditions were greater. The differences in serological properties of complete isolates were minimal. These differences were hard to detect because the micro precipitin test can only be used for purified TRV and PEBV. The agar gel diffusion test which normally is a sensitive test for viruses in crude sap was not very useful for TRV and PEBV, because the long particles of these viruses did not penetrate the gel and the virus concentrations in the plants were low. Attempts to make the test more useful for TRV and PEBV by breaking the particles ultrasonically or by adding a detergent, were disappointing. Ultrasonic treatment broke the long particles into two pieces with about the length of the short particle and left the short particles intact, so that the test became twice as sensitive. Adding the detergent Leonil SA caused aspecific precipitation of the antisera. With the bentonite-flocculation test it was possible to demonstrate the presence of TRV in sap of local lesions, but this could not be done with PEBV.

Chapter 4 also includes the multiplication and purification of complete and incomplete TRV-Lisse. It was found that the amount of complete TRV-Lisse that can be extracted from *N. rustica* plants is maximum five days after inoculation. For incomplete virus the maximum was reached about two days after inoculation. These results agree very well with those of Semancik & Kajiyama (1967a) and Semancik & Odening (1969).

Chapter 5 gives results of different methods to separate the components of TRV and PEBV. Molecular sieving in block-condensed agar or in a column of particles of block-condensed agar, was unsatisfactory. The separation was bad and the yields were very low because the particles of TRV are rod-shaped. Therefore both long and

short particles can penetrate into pores with diameters larger than the particle diameter. It is impossible to make a gel with pores into which only short particles can penetrate and long particles can not. The low yields were caused by the long and short particles penetrating into pores with a diameter smaller than the length of the short particle and then getting partially fixed in the gel. According to Steere (1964) rod-shaped particles should behave like spheres with a diameter equal to the length of the particle if the elution speed is low enough. However, the results of our molecular sieving experiments did not support this hypothesis.

The present study indicated that with specific precipitation by polyethylene glycol (PEG) and NaCl it was possible to separate long and short particles of TRV-Lisse. Long particles could be precipitated specifically from a solution containing 2 mg virus/ml by adding 3% PEG and 0.1 M NaCl. Long and short particles could also be separated with PEG-solubility concentration gradients as described by Clark & Lister (1971). Furthermore separation between short and extra short particles was better than with other methods.

The easiest way to separate TRV particles in reasonable amounts was by density-gradient centrifuging. Sucrose-gradient centrifuging in an SW 25.1 rotor as well as in a zonal rotor was successful. In the zonal rotor large quantities up to 100 mg of virus components could be separated in one run. If more virus was introduced per run, separation became less satisfactorily due to overlapping of the peaks. In most of my experiments I used gradients which were linear with volume as they were pumped into the rotor. In a few cases I used a so-called iso-kinetic gradient. Although this gradient was not optimally adapted to my material, it still gave better results than the linear gradient.

To make sure that the inocula contained only intact particles, sucrose-gradient centrifuging in a SW 25.1 rotor was used as a last step to purify small quantities of virus particles just before they had to be used in biological tests.

The purity of the virus particles after they had been separated was tested in different ways (Chapter 6). Electron microscopy was a very quick method to obtain information about the composition of a virus preparation. A disadvantage was that if in preparations of long particles shorter particles were seen, it could not always be decided whether these were normal short particles or just fragments of long particles due to the particular technique.

Analytical ultracentrifuging was also a good method to test the composition of preparations in which all components were present in reasonable amounts. It was fast and nearly all material could be recovered. However, if small amounts of contaminating material had to be detected the method had serious limitations, for the lower limit of solute concentration visible with Schlieren optics is about 0.01%. Thus long particle preparations could contain 5% short particles without being detected.

The best way to see whether a preparation of long or short particles was contaminated by their supplements, was to inoculate them on test plants. Short particles are not infectious, long ones are and give rise to incomplete virus. Long and short particles together induce complete virus. The test was rather elaborate because a suffi-

cient number of local lesions had to be tested to see what kind of virus they contained.

Chapter 7 deals with methods to distinguish between complete and incomplete TRV and PEBV. One of these methods, the bentonite-transmission test originally reported by Sänger (1968a), was described in detail and all parts of it were studied separately. The test was especially useful to see what kind of virus, complete or incomplete, was present in local lesions. Therefore the lesions were punched out of the leaf and the discs were cut in two. One half was ground in buffer, the other in buffer containing 25 mg bentonite/ml. Bentonite furthered the transmission of free virus RNA, but it bound TRV protein. So the transmission of complete TRV was reduced by bentonite. Because of their characteristic behaviour towards bentonite it was easy to differentiate between complete and incomplete virus. Making extracts with and without phenol gives the same results, but this method is far more laborious.

If plants were systemically infected with one type of virus, complete and incomplete forms could be distinguished by differences in symptoms. The complete isolates in general caused a mild chlorosis and necrosis, while the incomplete ones caused rapidly spreading brown necrotic lesions.

Chapter 8 deals with the biological characteristics of the particles of TRV and PEBV and with the interaction between homologous and heterologous long and short particles. To see whether complete or incomplete virus was formed in my experiments I always used the bentonite-transmission test.

Separate long particles of the complete TRV isolates were infectious, but gave rise to the formation of incomplete virus. Short particles alone were not infectious. If long and short particles were inoculated together this led to the formation of complete virus.

It was not necessary to inoculate long and short particles at the same time. If short particles were inoculated first one could wait 7 h before inoculating the long particles and still complete virus was formed. If the interval between inoculation of short and long particles was extended to 24 h complete virus was no longer formed. If the long particles were inoculated first short particles could be inoculated any time, as long as the leaves were still active in synthesizing virus material, and still complete virus was formed. However, it appeared that when the short particles were inoculated two days after the long ones, a maximum amount of complete virus was formed. I found that at that stage of infection the amount of free virus RNA in plants inoculated with the incomplete form was maximum.

Short particles of PEBV were not infectious, long particles were but gave rise to incomplete virus. If they were inoculated together complete virus was formed.

If heterologous long and short particles of the different TRV isolates were inoculated together complete virus was formed. In the combinations tested the lengths of the induced virus particles were more or less equal to those of the comparable particles of the parent strains. If short particles of the complete isolates were added to the incomplete ones, complete virus was formed, giving rise to typical symptoms in tobacco. All combinations of long and short particles of isolates I tested were able to produce complete virus. In other reports on interaction between particles of different TRV

strains, it was always mentioned that only certain combinations were able to interact (Lister, 1968; Semancik & Kajiyama, 1968; Lister & Bracker, 1969; Sänger, 1969).

A combination of a long particle with its normally accompanying short particle was not always superior with respect to the formation of complete virus, to combinations with other short particles.

Even when heterologous long and short particles of TRV-Lisse and PEBV-Dik Trom 5 were inoculated together, complete virus was formed. The symptoms of such virus were the same as those caused by the parent virus that provided the long particle. This interaction indicates that these viruses probably are two strains of the same virus.

Therefore it was easy to explain why there are so many different TRV isolates and why no correlation could be established between groupings based on properties such as symptoms on the one hand and particle length or serological characteristics on the other.

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