TIJDSCHRIFT OVER PLANTEZIEKTEN

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SOME APPLICATIONS OF FREEZE-DRYING IN VIROLOGICAL RESEARCH[1]

Met een samenvatting: Enkele toepassingen van het drogen door sublimatie bij het virologische onderzoek

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INTRODUCTION

Several precautions are required to store biological material without loss in activity. Often low temperatures are used for this purpose. Sometimes this procedure is not effective in preserving the infectious and antigenic properties of viruses. Thus infectivity and antigenicity of virus S are readily lost in frozen press juice of potato leaves (VAN SLOGTEREN, 1955).

Many changes can be prevented by dehydration, so that enzymatic and chemical processes are completely inhibited. The dehydration should be done under such conditions that no undesired alterations are involved. The only method to be considered then is freeze-drying, in which water is removed from the frozen product in a vacuum.

In recent decades this process has been used for several purposes; however, little information is known about its use in plant virological research. In view of this lack of knowledge, we think it desirable to describe some experiences with the method.

METHODS

The method is based on the principle that water vapour from a frozen product sublimes to the place with the lowest water vapour pressure, which means also, the place with the lowest temperature. The drying process can be made more rapid by application of heat to the frozen product, to compensate for the latent heat of sublimation.

The equipment used for drying is shown schematically in figure 1. It consists of a refrigerator (5) connected with two condensers at a temperature of –55 °C.

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For freezing and transfer of heat to the material, alcohol can be pumped through the plate (6) and the ring-compartment (7) (both hollow). The heat exchangers are supplied with a heating element as well as a connection with the refrigerator to enable the alcohol to be maintained, at a given temperature.

For drying leaves the unit (2), with the ring-compartment, has several advantages. As compared with the unit (1) several layers of leaves can be dried simultaneously, by placing aluminum strips with holes between the leaves. In this way the drying area is increased. Thick layers of leaves should not be dried, because diffusion is greatly diminished in this way and consequently long drying times are necessary.

The vacuum during drying depends on the material to be dried. With antisera it starts with about 0.2 mm Hg pressure, this is reduced to 0.07 mm at the end of the dehydration process. For leaf material these values are about two or three times higher.

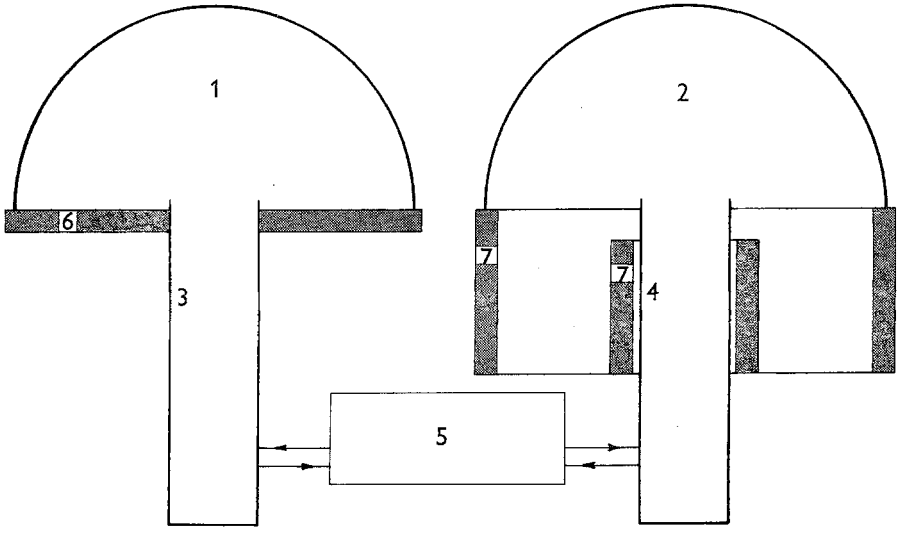

FIGURE 1. Diagram of the apparatus used for freeze-drying. Condensors (3) and (4) are cooled by refrigerator (5). Plate (6) and ring (7) (both hollow) are cooled or heated by alcohol, which is pumped through a heat exchanger. Heat exchangers and vacuum pumps are not drawn.

Schema van de apparatuur voor het drogen door sublimeren. De condensors 3 en 4 worden gekoeld door koelmachine 5. Plaat 6 en ring 7 (beide hol) kunnen gekoeld of verwarmd worden door alcohol, welke via een warmtewisselaar rondgepompt wordt. Warmtewisselaars en vacuumpompen zijn niet getekend.

Weekly changes of vacuum oil are recommended after drying potato leaves as the oil becomes contaminated by non condensable products from the leaves. These substances form combinations with the vacuum oil giving rise to a poor vacuum and poor drying.

There are two possibilities in freezing material before dehydration; either it can be done in the units or in a separate refrigerator at –30 °C. To prevent alterations during freezing and to make use of the maximum capacity of the equipment we used the latter method. In this way the drying capacity of unit (1)

is 1 litre of serum or 400 grams of leaves and for unit (2): 1000–1500 gram of leaves in 24 hours.

Applications of freeze-drying

Particular attention was given to applications closely related to virological and serological research. These concerned: 1. drying of antisera; 2. drying of diseased leaf material to preserve the activity of a number of plant viruses or, if healthy leaves, storing them for absorption of new antisera; 3. drying of leaf material for extraction of inhibiting substances in virus purification.

1. *Drying of antisera*

Serological techniques have proved extremely useful for virus identification and for routine testing of plants for freedom from virus infection. Therefore, the demand for and exchange of antisera has grown substantially in recent years (van Slogteren & van Slogteren, 1957).

In view of the need for big quantities of antisera against potato viruses by The General Netherlands Inspection Service "N.A.K.", we started in 1954 with freeze-drying these antisera.

This opened possibilities for the storage of antisera absorbed with the sap of healthy plants, with the aim of removing antibodies against normal plant proteins. In a few cases an improvement was seen when dried antiserum was compared with the same serum stored at –30 °C, even after a short time of storage. For example this was found with absorbed antiserum against sugar beet yellows virus after three days. High vacuum prevents possibly oxydation processes, which benefits the quality.

Results in table 1 indicate that the titre of dried antiserum against virus X is not greatly affected at high storage temperatures. However, this is not without danger with other antisera. A decrease in antibody content seems to be responsible for the reduction of the titre of antiserum against virus S, stored at high temperatures, and with low concentrations of antigen. This is very important for routine testing of plant material with a low concentration of virus.

In addition to the antisera against the viruses mentioned, good results were obtained by drying absorbed antisera against the following viruses: virus Y, virus M, *Cucumis virus 1* st. Chr., *Phaseolus virus 2*, tomato aspermy virus, mosaic virus and grey virus of daffodils, iris mosaic virus and hyacinth mosaic virus.

The properties of these viruses are described as follows:

hyacinth mosaic virus	(Klinkowski, 1958)
iris mosaic virus	(Brierley & McWhorter, 1936)
mosaic virus and grey virus of daffodils	(van Slogteren & de Bruyn Ouboter, 1941)
rattle virus	(van der Want & Rozendaal, 1948)
tomato aspermy virus	(Blencowe & Caldwell, 1949)
virus M	(Rozendaal & van Slogteren, 1958)
virus S	(Rozendaal & Brust, 1955)
virus Y	(Klinkowski, 1958)
Cucumis virus 1 st. Chr.	(Noordam, 1952)
Phaseolus virus 2	(Klinkowski, 1958)

Storage under atmospheres of different inert gases had no effect on the quality of the antiserum (table 1). It is noteworthy that closing the flasks or ampoules containing the dried material with rubber stoppers was not satis-

TABLE 1. Effect of different gases and temperatures on the titre of dried absorbed antisera.
Invloed van verschillende gassen en temperaturen op de titer van gedroogde verzadigde antisera.

Antiserum	Antigen *Antigeen*	Titre of dried antiserum stored *Titer van gedroogd antiserum na bewaren*					
		in *onder*	during days *gedurende dagen*	at *bij*			
				−15 °C	+9 °C	+20 °C	+35 °C
Absorbed antiserum against virus X (1 : 10) *Verzadigd antiserum tegen X-virus (1 : 10)*	Press juice of tobacco leaves infected with virus X *Perssap van tabaksblad geïnfecteerd met X-virus*	Air *Lucht*	0	1/480	–	1/480	1/480
		Id.	80	1/480	–	1/480	1/480
		Id.	140	1/480	–	1/480	1/480
		Id.	380	1/480	–	1/480	1/480
Absorbed antiserum against virus S (1 : 5) *Verzadigd antiserum tegen S-virus (1:5)*	Press juice of potato leaves with virus S + 4 parts of sap of healthy potato leaves *Perssap van aardappelblad met S-virus + 4 delen sap van gezond aardappelblad*	Air *Lucht*	90	1/320	1/320	1/160	–
		CO_2	90	1/320	1/320	1/160	–
		N_2	90	1/320	1/160	1/160	–
		H_2	90	1/320	1/320	1/160	–

factory. It was concluded that diffusion of water vapour through the stoppers will raise the moisture content above a point were damage could occur. According to our experience only paraffining the stoppers or sealing of the glass is satisfactory.

The serum to be dried should be frozen very well, otherwise there will be foaming in the drying process. The residues of these sera do not redissolve completely afterwards, though, serological activity does not seem to be greatly diminished.

2. *Drying of diseased leaf material to preserve the activity of a number of plant viruses or, if healthy leaves, storing for absorption of new antisera*

There are many differences between drying of antiserum and leaves. In the first process water is removed from a homogeneous frozen product. The time of drying depends largely on three factors: temperature of the condenser, the vacuum and transfer of heat to the frozen product. With leaves it is more complicated as water vapour from the cell must first pass the cell membranes and cell walls. So the procedure is much more time consuming. Depending on the process of freezing these cell walls or membranes are more or less damaged and this affects the rate of diffusion.

By rapid freezing at low temperatures the size of the ice crystals is reduced, as compared with the crystals resulting from slow freezing at higher temperatures. With smaller ice crystals disruption of cell walls is usually avoided (FLOSDORF, 1950b). Quikly frozen tissues should be permitted to warm only very slowly to the dehydration temperature, otherwise ice crystals will grow too fast and morphological changes will be produced. Though freezing and drying at low temperatures would be quite possible, there are practical reasons why it would rarely be done. The vapour pressure of ice becomes very low at these temperatures and consequently it would take too much time to dehydrate the material. So a choice has to be made between several different possibilities.

A good method turned out to be, to freeze the leaves in a refrigerator at –30 °C and put them afterwards directly in the drying unit. (The temperature of the plate or ringcompartment was first held at –20 °C, but in later experiments the circulation pump was not used.) Heat is then supplied by radiation from outside. Care should be taken not to heat the leaves too much, as thin leaves are easily thawed. After drying, leaves are put in flasks (Weck) and stored under vacuum. This is done by evacuating the flask with a clamp on it, in the drying equipment.

Leaves with a thick layer of wax are especially difficult to dehydrate. Tobacco leaves dry much more quickly than sugar beet or hyacinth leaves. Some volatile substances removed from potato leaves during drying are not condensed, but move into the vacuumpump. Consequently the vacuum is affected unfavourably.

ROZENDAAL & VAN SLOGTEREN (1958) used freeze-dried potato leaves infected with virus S and virus M for the production of antisera. No influence of the drying procedure was found. The same holds for other viruses as will be described later on.

More interesting is the infectivity of viruses stored in dried tobacco leaves. Suspensions obtained by grinding freeze-dried tobacco leaves infected with virus Y, rattle virus or *Cucumis virus 1* st. Chr., with 9 volumes of phosphate buffer pH 7.1, produced the well known symptoms on White Burley tobacco.

These leaves had been stored for 9 months at 7 °C. Freeze-dried expressed juice of tobacco leaves infected with virus Y was also infectious, when stored under the same conditions.

The freeze-drying technique offers good prospects of preserving leaves of healthy plants, so that uniform material can be collected. Use can also be made of it for research studies or absorption of antisera.

3. *Drying of leaf material for extraction of inhibiting substances in virus purification*

Cytoplasm of plant cells sometimes contains substances interfering with the serological or biological properties of the virus. Tannins especially are known for their inhibiting effect on infectivity. These substances may be removed by extraction with organic solvents. Several viruses are able to resist extraction with chloroform, but there is always a possibility that denaturation will occur (STEERE, 1959).

ROZENDAAL & VAN SLOGTEREN (1958) described a purification method for virus S and virus M, in which leaves were extracted with chloroform, acetone and ether. This method proved to be of value also in the purification of the following plant viruses: virus Y, rattle virus, tomato aspermy virus, *Cucumis virus 1* st. Chr. mosaic and grey virus of daffodils, hyacinth mosaic virus and iris mosaic virus.

Some fractions obtained in this purification process were examined more closely (scheme 1). In fractions I, II and III one or more ultraviolet light absorbing substances could be detected with chromatography, probably all tannins. Chlorophyll is mainly found in fraction I. Physico-chemical studies of virus solution VI revealed proteins with sedimentation constants of 19S and 80S. These constituents are also found in higher concentrations in undried plants. The reduction in concentration of the 80S component during purification is very striking. Inoculations with the fractions IV and VI, obtained from tobacco leaves infected with virus Y and *Cucumis virus 1* st. Chr., stored for 10 months at 7 °C., were successful. Some sediments such as those from fractions IV and VI were also infective, but this seemed to be due to adsorption of virus on to cell constituents. There are indications that such adsorption may be lower when fresh material is used, but results were not consistent.

DISCUSSION

Freeze-drying of biological material offers many advantages. Labile substances can often be preserved without loss in activity. The absence of water prevents harmful reactions, such as may occur with chilled or frozen products. For example, antigenicity and infectivity of virus S are readily lost at low temperatures (VAN SLOGTEREN, 1955).

The results of this work indicate the beneficial effect of freeze-drying on the keeping qualities of antisera against plant viruses. This is important for distribution and exchange of these sera. The advantages are also obvious for antisera absorbed with proteins of healthy plants. This procedure may now be carried out whenever good plant material is available.

Dried antisera should not be stored at high temperatures or loss in activity will occur. Replacement of air in the ampoules, with inert gases does not improve the results. Probably oxygen may be absorbed by the highly porous

matter and can not be completely swept out afterwards (FLOSDORF, 1950a, b). Ampoules should be preferably sealed by fusion of glass as diffusion of water vapour through rubber stoppers will decrease the keeping qualities. Further work will be necessary to study the effect of secondary drying with phosphorus pentoxide on the properties of dried antisera.

Promising results were obtained with viruses stored in dried leaves. Although changes in the cell during freezing cannot be completely avoided, infectivity is partly maintained. The same phenomenon has been observed with animal viruses. Further experiments are necessary to decide how far this property is maintained. However, some loss in infectivity has only a limited significance for infecting healthy plants. Freeze-drying could thus be of value for the maintenance of stock cultures of plant viruses. Biological activity of virus Y is also preserved in dried expressed sap. This method is not suitable when the virus is inactivated by substances in the sap.

Use can be made of dehydrated material in the purification of plant viruses. Some cell constituents are inhibitors of infectivity, others are poisonous and cause difficulties in the preparation of antisera (VAN SLOGTEREN, 1955). The aim of purification is the removal of these substances. Extraction with organic solvents, such as chloroform, removes most of this material. Several viruses will withstand this treatment (STEERE, 1959), although denaturation is always a possibility.

For infectivity experiments inhibitors should be removed before reaction with the virus can take place. This is possible only with unaltered freeze-dried material. Tannins can be extracted with alcohol, and in this way biological and serological activity of virus X and tobacco mosaic virus are not reduced (VAUGHAN, 1956). Antigenic properties of virus S and virus M are unaffected by extraction with chloroform, acetone and ether, but chlorophyll, tannins and alkaloids are removed by the treatment (ROZENDAAL & VAN SLOGTEREN, 1958). This applies also for the following viruses: virus Y, rattle virus, tomato aspermy virus, mosaic virus and grey virus of daffodils, hyacinth mosaic virus and iris mosaic virus.

With some viruses infectivity is unaffected, even partially. With others further quantitative experiments are necessary to decide how far infectivity is maintained.

To prevent changes during freeze-drying several precautions are necessary. Attention should be given to the following points, already described for animal tissues. Firstly, separation of solvent and dissolved components in the cell must be prevented. Secondly, the size of the ice crystals should be as small as possible to reduce damage to the cell structure. Both conditions are fulfilled by freezing and drying at low temperatures. Unfortunately in these low temperature ranges the vapour pressure of ice becomes very low and consequently dehydration takes too long. In our experiments the temperature of the product has been held at –20 °C and this seems to have had no adverse effect on the properties of the viruses. Other proteins were affected to greater or less degree such as the 80S component.

There are big differences in the procedure needed for drying of antisera and leaves. When drying leaves more attention must be paid to the diffusion of water from the frozen product, because of the obstruction of the cell walls. Removal of evaporated water is of great importance, so it is valuable to have a

good vacuum and a short distance to the condenser. According to MERYMAN (1959) dehydration of animal tissue can be achieved by conducting a continuous stream of dry air over the frozen material held at a low temperature. As it is difficult to establish a good contact between big quantities of leaves and the stream of air this method does not seem suitable for our work.

In spite of some defects freeze-drying is preferable to collect quantities of homogeneous plant material. The method is especially useful in the purification of viruses, that are present in plants only in low concentrations.

SUMMARY

Storage of antisera against plant viruses and leaf material from virus infected plants is simplified by application of freeze-drying. Dehydrated plant material is of great value for the removal of virus inhibiting substances by organic solvents. Several plant viruses are unaffected by this drying procedure.

SAMENVATTING

Biologische stoffen kunnen na drogen door sublimatie, zonder verlies aan activiteit, op eenvoudige wijze lange tijd bewaard worden. Goede resultaten werden geboekt bij het bewaren van verzadigde antisera tegen de volgende plantevirussen: X-virus, Y-virus, M-virus, *Cucumis virus 1* st. Chr., tomaataspermyvirus, narcissemozaïekvirus, narcissegrijsvirus, *Phaseolus virus 2*, irisvirus en hyacintevirus. In tabel 1 wordt de invloed van enkele bewaartemperaturen en gassen op de serologische activiteit weergegeven. Uit deze resultaten blijkt een nadelige invloed van hogere bewaartemperaturen op de hoeveelheid antibodies. Vooral voor het diagnostische onderzoek is dit van groot belang.

Ook voor het conserveren van bladmateriaal, al dan niet geïnfecteerd met plantevirus, opent deze methode belangrijke perspectieven. Niet alleen de antigeniteit maar ook het infectievermogen van Y-virus, ratelvirus en *Cucumis virus 1* st. Chr. uit gedroogd tabaksblad blijkt na 9 maanden bewaren bij 7 °C nog grotendeels aanwezig te zijn. Bij deze experimenten werd White Burley tabak geïnfecteerd met een suspensie die verkregen wordt door gedroogd blad fijn te wrijven met 9 delen fosfaatbuffer pH 7,0. Nader onderzoek zal moeten leren in hoeverre het infectievermogen ook kwantitatief onaangetast blijft.

Gedroogd materiaal is van belang voor het verwijderen van verbindingen die remmend werken op eigenschappen van een virus. VAUGHAN (1956) gebruikte extractie met alcohol voor de verwijdering van tanninen, terwijl ROZENDAAL & VAN SLOGTEREN (1958) bij de zuivering van S- en M-virus voor antiserumbereiding een extractie met chloroform, aceton en ether toepasten. Deze methode bleek ook goede resultaten op te leveren bij de bereiding van antisera tegen: X-virus, Y-virus, narcissemozaïek en narcissegrijsvirus, hyacintevirus, irisvirus, *Cucumis virus 1* st. Chr. en tomaataspermyvirus.

De eigenschappen van deze virussen zijn beschreven in de volgende literatuur:

hyacintegrijsvirus	(KLINKOWSKI, 1958)
tomaataspermyvirus	(BLENCOWE & CALDWELL, 1949)
irismozaïekvirus	(BRIERLEY & MCWHORTER, 1936)
mozaïekvirus en grijsvirus van narcis	(VAN SLOGTEREN & DE BRUYN OUBOTER, 1941)
ratelvirus	(VAN DER WANT & ROZENDAAL, 1948)
M-virus	(ROZENDAAL & VAN SLOGTEREN, 1958)

S-virus . . . . . (ROZENDAAL & BRUST, 1955)
Y-virus . . . . . (KLINKOWSKI, 1958)
Cucumis virus 1 st. Chr. . . . . (NOORDAM, 1952)
Phaseolus virus 2 . . . . . (KLINKOWSKI, 1958)

Bij genoemde extractie worden, volgens chromatografisch onderzoek, zowel giftige stoffen (alkaloïden) als stoffen die de virusactiviteit remmen (tanninen) verwijderd. Zelfs blijken de fracties IV en VI (schema 1) in het geval van Y-virus en *Cucumis virus 1* st. Chr. nog sterk infectieus. Genoemde virussen werden 10 maanden bij 7 °C in tabaksblad bewaard.

Volgens fysisch-chemisch onderzoek treedt vooral een sterke vermindering op van de 80S component in de fractie VI na zuivering.

De apparatuur, welke gebruikt werd, is schematisch weergegeven in fig. 1. Condensors worden door koelmachine op –55 °C gehouden. Materiaal liefst invriezen bij –35 °C in diepvrieskast. Het vacuüm is sterk afhankelijk van het materiaal dat gedroogd wordt. Bij serum bedraagt dit aan het begin 0,2 mm kwikdruk, welke waarde daalt tot 0,07 mm kwikdruk; voor blad liggen deze waarden twee- tot driemaal zo hoog.

In de discussie worden de verschillende voorwaarden, waarmee men bij het drogen door sublimatie rekening moet houden, nader besproken.

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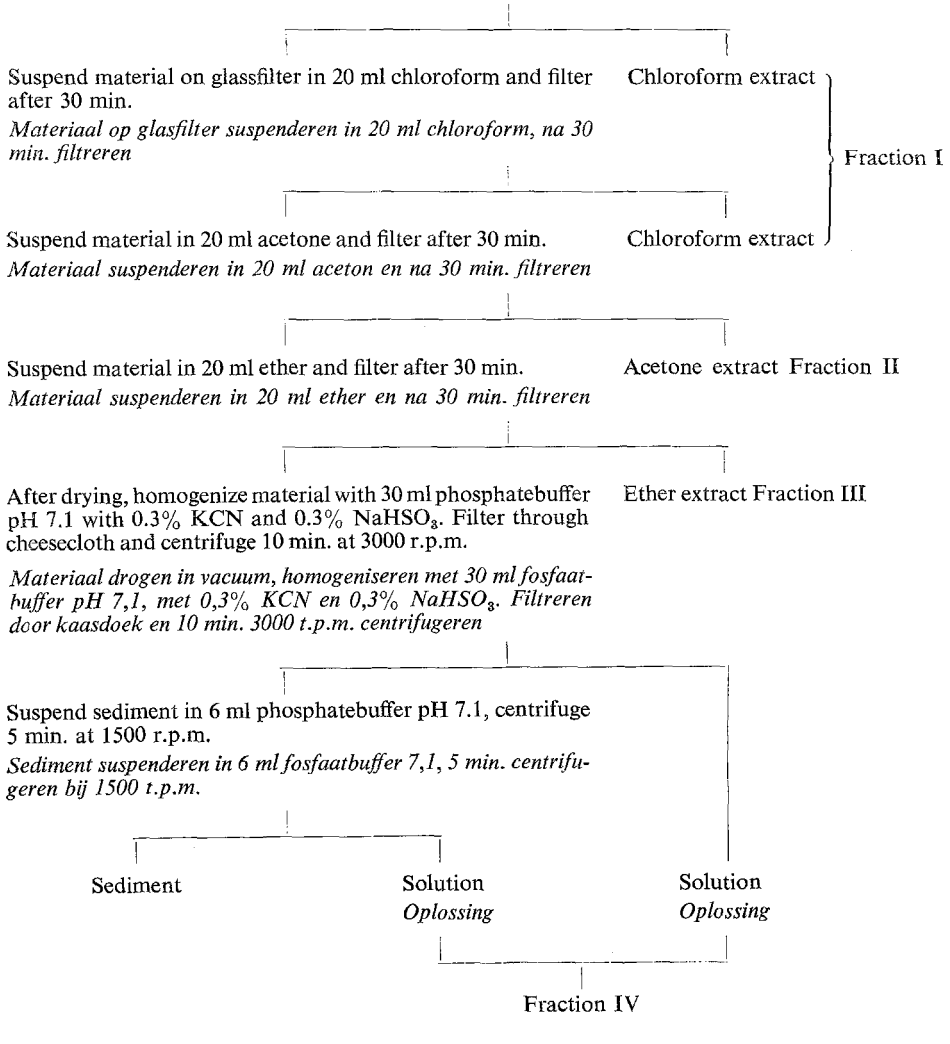
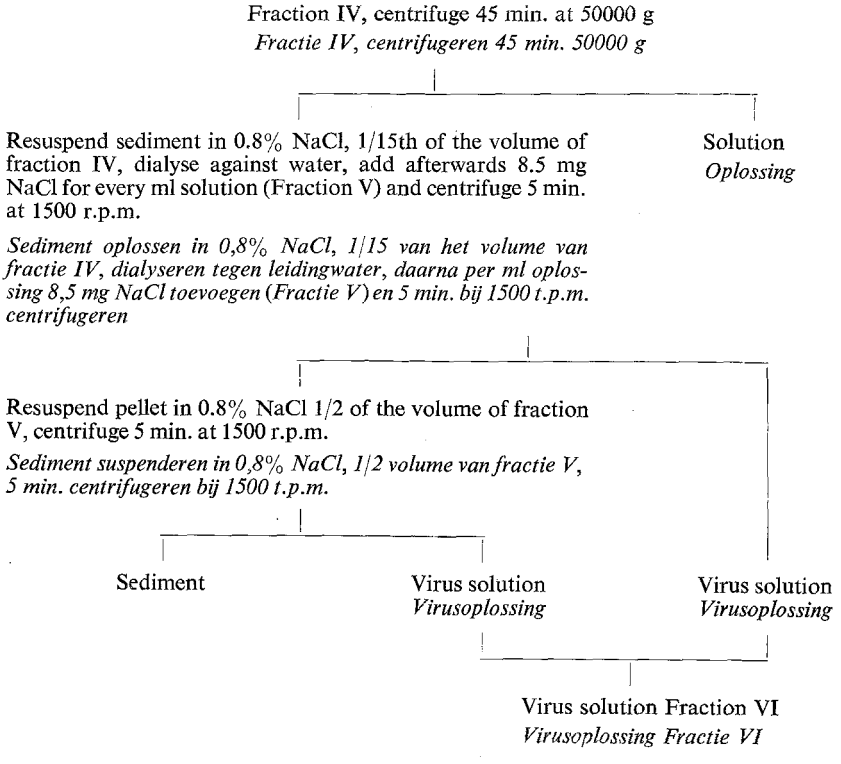
SCHEME 1. Purification of plant viruses from dried leaves for antisera production.
Zuivering van plantevirussen uit gedroogd blad voor bereiding van antisera.

Homogenize 1 gram of freeze-dried leaves with 40 ml chloroform, filter through sintered glassfilter.
1 gram gedroogd blad homogeniseren met 40 ml chloroform, filtreren door glazen filterkroes.




Fraction IV, centrifuge 45 min. at 50000 g
Fractie IV, centrifugeren 45 min. 50000 g
Resuspend sediment in 0.8% NaCl, 1/15th of the volume of fraction IV, dialyse against water, add afterwards 8.5 mg NaCl for every ml solution (Fraction V) and centrifuge 5 min. at 1500 r.p.m.
Sediment oplossen in 0,8% NaCl, 1/15 van het volume van fractie IV, dialyseren tegen leidingwater, daarna per ml oplossing 8,5 mg NaCl toevoegen (Fractie V) en 5 min. bij 1500 t.p.m. centrifugeren
Solution
Oplossing
Resuspend pellet in 0.8% NaCl 1/2 of the volume of fraction V, centrifuge 5 min. at 1500 r.p.m.
Sediment suspenderen in 0,8% NaCl, 1/2 volume van fractie V, 5 min. centrifugeren bij 1500 t.p.m.
Sediment
Virus solution
Virusoplossing
Virus solution
Virusoplossing
Virus solution Fraction VI
Virusoplossing Fractie VI