AN ABSTRACT OF THE THESIS OF

Sukhbir Bhullor for the PhD in Plant Pathology
(Name) (Degree) (Major)

Date thesis is presented July 16, 1964

Title Methods of Separating Viruses from the Inhibitors Present in Chenopodium amaranticolor

Abstract approved Redacted for Privacy
(Major professor)

The infectivity of plant viruses was suppressed by inhibitory substances (IS) in the sap of Chenopodium. Various methods were attempted to find an efficient method which would allow direct transmission of viruses from Chenopodium to other plants. Viruses used in these experiments were alfalfa mosaic virus (AMV), tobacco mosaic virus (TMV) and tobacco ring spot virus (TRSV).

The infectivity of AMV-containing Chenopodium extract increased with dilution. There seemed to be an apparent dissociation of the virus and the IS at 1:1000 dilution. The AMV extract mixed with equal parts of IS was completely noninfectious at all dilutions. Dilution of TMV and IS mixture resulted in a gradual increase in infectivity. The infectivity of TMV was comparable to the water control at 1:1000 dilution, but both were in low titre as was AMV at this dilution.

AMV and IS could be separated when heated together at 60°C, but very few lesions developed. There was no virus activity with temperatures below and above 60°C. IS started inactivating at 60°C when only IS of a Chenopodium extract was heated, cooled and then added to AMV. The inactivation of IS increased as the temperature increased. TMV and IS were not separated to the same degree as were AMV and IS when heated together in a mixture. The only infectivity was present after the 60°C treatment. There was no virus activity with temperatures below and above 60°C. IS started inactivating at 60°C when only IS of Chenopodium extract was heated, cooled and then added to TMV. The inactivation of IS increased as temperature increased.

Liquid-nitrogen increased the infectivity of AMV three to seven fold, but AMV was still in low titre. There was no TRSV infectivity after adsorption with hydrated calcium phosphate. Partial separation of AMV and IS could be accomplished by high speed centrifugation.

When Chenopodium extracts were layered on 30-50 g of sucrose/100 ml of 0.033 M phosphate buffer, pH 7 density-gradient columns and centrifuged, most of the IS remained in the aqueous layer, not sedimenting into the sucrose. Consequently, all samples removed from the sucrose gradient and mixed with TMV allowed lesion formation on Pinto bean leaves, in contrast to the samples withdrawn from the aqueous layer. In other tests, TRSV and AMV from Chenopodium each sedimented into sucrose during centrifugation, concentrating in the 35 g sucrose region. When 2 ml of clarified extract of TRSV- or AMV-infected

Chenopodium was layered over 3 ml of the 35 g sucrose solution and centrifuged, both viruses were recovered in high concentration from the sucrose. Controls not centrifuged or just ground in sucrose were not infective. An attempt to recover TRSV with this method from an old infection in Chenopodium was not successful. However, some infection was obtained from old Peach yellow bud mosaic (PYBV) infected strawberry leaves with this method.

It was concluded that the most efficient method of separating viruses from IS in Chenopodium was layering extracts over 35 g sucrose followed by centrifugation.

METHODS OF SEPARATING VIRUSES FROM THE INHIBITORS PRESENT IN CHENOPODIUM AMARANTICOLOR

by

SUKHBIR BHULLOR

A THESIS

submitted to

OREGON STATE UNIVERSITY

in partial fulfillment of
the requirements for the
degree of

DOCTOR OF PHILOSOPHY

August 1964

APPROVED:

Redacted for Privacy

Professor of Botany and Plant Pathology

In Charge of Major

Redacted for Privacy

Head of Department of Botany and Plant Pathology

Redacted for Privacy

Dean of Graduate School

Date thesis is presented July 16, 1964

Typed by Penny A. Self

ACKNOWLEDGEMENTS

The author wishes to thank Dr. J.A. Milbrath for his assistance during the course of this work and in preparation of the manuscript.

Sincerest gratitude is expressed to Dr. T.C. Allen, Jr., for his helpful guidance, criticism and untiring inspiration during the course of this investigation and in preparation of the manuscript. Thanks are also given to Dr. R.A. Young.

Lastly, the author wishes to thank her family and friends, especially Dr. J.N. Singh for their constant encouragement throughout the period of this work.

TABLE OF CONTENTS

	Page
INTRODUCTION	1
REVIEW OF LITERATURE	3
Inhibitors of Infection Found in Plants	3
The Nature of Inhibitors	5
The Mechanism of Inhibition of Infection	9
MATERIALS AND METHODS	10
Virus Source	10
Plant Culture	10
Plant Inoculation	11
Thermal Inactivation	12
Density-Gradient Centrifugation	12
EXPERIMENTAL PROCEDURE AND RESULTS	14
Separation of Viruses from Inhibitory Substances (IS) in *Chenopodium* by dilution	14
Effect of Dilution on IS in *Chenopodium* Infected with AMV	15
Effect of Dilution on IS in a Mixture of IS from *Chenopodium* and AMV from tobacco	15
Effect of Dilution on IS in a Mixture of IS from *Chenopodium* and TMV from tobacco	19
The Use of Heat to Separate Viruses from IS in *Chenopodium*	21
Effect of Heat on IS in a Mixture of IS from *Chenopodium* and AMV from tobacco	22
Effect of Temperature on IS in a Mixture of IS from *Chenopodium* and TMV from tobacco	25
The Use of Liquid Nitrogen to Separate Viruses from IS in *Chenopodium*	28
Separation of Viruses from the IS Present in *Chenopodium*	29
Use of hydrated calcium phosphate (HCP) and centrifugation to separate TRSV and IS	29
Use of Centrifugation to separate AMV and IS	31
Use of Sucrose Density-Gradient Columns to Separate Viruses from IS in *Chenopodium*	32
Location of TRSV and AMV from *Chenopodium* in density-gradient columns	33
Location of IS in density-gradient columns	37
Separation of AMV from IS in an abbreviated density-gradient column	39
Use of One Layer of Sucrose to Separate Viruses from IS	41
Separation of TRSV and IS from infected *Chenopodium*	42

TABLE OF CONTENTS (Continued)

Page

Location of IS from TRSV infected Chenopodium extract 44
Attempted recovery of TRSV from an old infection in Chenopodium 46
Attempted recovery of PYBV from Strawberry 46
The effect of sucrose extraction on AMV in Chenopodium 48

DISCUSSION AND CONCLUSIONS 49

SUMMARY 55

BIBLIOGRAPHY 58

LIST OF FIGURES

Figure		Page
1	The effect of dilution on extracts of AMV infected Chenopodium	17
2	Effect of dilution on IS from a mixture of IS from Chenopodium and TMV from tobacco	20
3	The effect of temperature on IS and AMV when heated alone or together	24
4	The effect of temperature on IS and TMV when heated alone or together	26
5	Schematic diagrams of centrifuge tubes with 50 to 30 g sucrose layers and TRSV or AMV layered on top	34
6	Schematic diagrams of centrifuge tubes with 45 to 25 g sucrose layers and AMV layered on top	36
7	Schematic diagrams of centrifuge tubes with 40 and 35 g sucrose layers and AMV layered on top	40
8	Schematic diagrams of centrifuge tubes with 35 g sucrose and TRSV strains A, B and C layers	43
9	Schematic diagrams of centrifuge tubes with layer of 35 g sucrose and PYBV from strawberry	47

LIST OF TABLES

Table		
1	Average number of lesions induced on black cowpeas by AMV-infected extracts of Chenopodium at different dilutions	16
2	Effect of dilution on IS in a mixture of IS from Chenopodium and AMV from tobacco	18
3	Effect of dilution on IS in a mixture of IS from Chenopodium and TMV from tobacco	21
4	The effect of temperature on IS and AMV when treated alone and together	23
5	The effect of temperature on IS in a mixture of IS from uninoculated Chenopodium and TMV from tobacco	27

LIST OF TABLES (Continued)

Table		Page
6	The effect of liquid nitrogen on IS from AMV-infected Chenopodium	29
7	Results of three experiments using ultra-centrifugation to separate AMV from IS in Chenopodium	31
8	Infectivity of the layers removed from the 50 to 30 g sucrose density-gradient columns originally layered with TRSV and AMV. The layers were bioassayed on black cowpea	35
9	Infectivity of the layers removed from the 45 to 25 g sucrose density-gradient column originally layered with AMV. The layers were bioassayed on black cowpea	37
10	The effect on TMV infectivity on Pinto bean of various layers from a sucrose density-gradient column with uninoculated Chenopodium mixed with TMV	38
11	Infectivity of layers removed from the centrifuged extract-35 g sucrose tubes with TRSV strains A, B and C. The layers were bioassayed on black cowpea	44
12	The effect of various layers from a TRSV strain A-infected Chenopodium 35 g sucrose column when mixed with TRSV strain A. Black cowpea was the assay host	45

METHODS OF SEPARATING VIRUSES FROM THE INHIBITORS PRESENT IN CHENOPODIUM AMARANTICOLOR

INTRODUCTION

A wide variety of substances when mixed with plant viruses inhibit infection (3, p. 32-35; 2). Of particular interest to virologists are naturally occurring virus inhibitors in host plants. These inhibitors prevent the transfer of virus from host to host and interfere with the determination of virus content by bioassay. Species of Chenopodium contain sufficient amounts of these inhibitors to prevent transferring active virus from these plants to other susceptible hosts.

Chenopodium amaranticolor Coste and Reyn. has become a widely used test plant by virologists. It reacts to give local lesions and / or systemic infections which denote presence and often identity of a virus. Hollings (17, p. 58-59) reported the reaction of Chenopodium amaranticolor to mechanical inoculation with 39 different plant viruses and he observed that 24 of these viruses induced symptoms which in many cases were of diagnostic or quantitative use. However, some of these viruses were restricted to one or two other species of plants and often they failed to infect the more commonly used test plants.

In many cases the lesions on Chenopodium amaranticolor were distinctive and diagnostic of a virus. Often, Chenopodium may show distinctive reaction to different viruses thus enabling

identification to be made. Because of its habit of growth, young susceptible leaves are produced over a long period of time, or until active growth stops. Some viruses produce distinct local lesions that can be counted for virus concentration studies, especially with those viruses which do not produce distinct local lesions on other commonly used hosts.

The inhibitory effect on viruses in extracts of Chenopodium has been the major factor in limiting its use where active virus was to be recovered. There may be no transmission of a virus from Chenopodium to hosts other than Chenopodium. The inhibitory substances in the sap prevents transmission of virus from Chenopodium to other species such as tobacco or French bean but allows transmission of virus from Chenopodium to Chenopodium.

The purpose of this study was to develop techniques whereby the reaction between virus and inhibitor could be prevented or stopped in order that the virus could be readily transferred to other species of plants. If an efficient method were found to allow direct transmission of viruses from Chenopodium to other plants, this would save workers considerable time, would expand research on viruses that are usually recovered from this plant, and would greatly increase the usefulness of this species in virus research.

REVIEW OF LITERATURE

The presence of virus inhibitors in normal plant sap has been reported by many workers. There have been three major considerations in the study of these naturally occurring virus inhibitors; (a) the presence and effect of these inhibitors on active virus in extracted plant juice, (b) the nature of these inhibitors and (c) the mechanism of inhibition of plant infection.

Inhibitors of Infection Found in Plants

The first report of inhibitors in plant sap was made by Allard (1, p. 51). He could infect healthy pokeweed plants (Phytolacca decandra L.) with juice from pokeweed infected with a mosaic virus, but he could not infect tobacco plants, which were also hosts for this disease. Duggar and Armstrong (10, p. 359-360) reported that the crude extract of pokeweed markedly inhibited the infectivity of common tobacco mosaic virus (TMV). Doolittle and Walker (9, p. 38-40) were unable to transmit mechanically the common cucumber mosaic virus (CMV) from infected pokeweed plants to different plant species due to the presence of inhibitors in pokeweed. However, they could readily transmit the virus from pokeweed by the aphid vector. Johnson (20, p. 694) observed that TMV 1 could be inactivated rapidly and nearly completely by mixing it with pokeweed juice. Fulton (11, p. 679) noted that TMV is not permanently inactivated by the juice of pokeweed, i.e. virus infectivity was reversible. He found that the percentage of TMV

inactivated was greatest when the mixture with the inactivator was most concentrated and least when the mixture was most dilute.

Several reports (3, p. 32-35; 2) have indicated that the extracts of pokeweed as well as a number of other species were inhibitive to certain viruses. Robbins (27, p. 363) and Jones (18, p. 9-13) were unable to mechanically transmit sugar beet mosaic virus from infected sugat beet plants to different plant species due to the presence of inhibitors in sugar beet (Beta vulgaris L.). Grant (15, p. 323-328) demonstrated that sugar beet, swiss chard (Beta vulgaris var. cicla L.), and spinach (Spinacea oleracea L.) extracts contain inhibitors. These species were susceptible to TMV, but the extracts from healthy plants of the species greatly reduced the infectivity of the virus in juice extracts from infected tobacco plants. This inhibitive property was reduced greatly by dilution as well as by heating the plant extracts when the dilution end point and the thermal inactivation end point of the inhibitor was lower than the virus. Johnson and Grant (19, p. 744-752) noted irregularity in the amount of infection with spinach juice containing the tobacco ringspot virus (TRSV) due to the presence of inhibitors in spinach. Kuntz and Walker (23, p. 564-566) reported that when spinach extract was mixed in equal parts with plant juices containing TRSV, necrotic potato ringspot virus and CMV, the infectivity of those juices was completely or nearly completely inhibited. The action of the spinach extract was instantaneous and infectivity did not increase with time. The extract retained its inhibitive effect at least 15 months at room temperature. Benda (5, p. 441-442)

observed an inhibitory effect of the juice of New Zealand spinach (Tetragonia expansa Murr.) on the infection of cowpeas (Vigna sinensis Endl.), by TRSV.

Ragetli (26, p. 330-338) made an extensive study of the inhibitory substances in carnation (Dianthus caryophyllus L.), but was unable to characterize it. He observed the inhibitor from carnation to be active against various viruses in tests on many different plant species. The presence of an inhibitory substance was noted in cucumber plants (29, p. 32). When sap from cucumber plants infected with CMV was inoculated to black cowpeas, few local lesions were formed due to the presence of inhibitors in cucumber. When the same virus from tobacco was assayed, many lesions resulted due to the absence of inhibitors in tobacco. Sill and Walker (30, p. 351) studied the relationship of the inhibitors present in cucumber to resistance to CMV . They indicated, "While it was not possible to determine the relation of the inhibitor to resistance, it was significant that in diseased plants there was a rough inverse correlation between the amount of chlorotic tissue, in which the inhibitors content was low and virus concentration high, and the degree of resistance of the variety."

The Nature of Inhibitors

The nature of all the virus inhibitors in higher plants has not been determined. Kuntz and Walker (23, p. 570-575) showed that the spinach sap contains two inhibitors. One of them was a stable substance that withstood 125°C for 15 minutes while the

other was an unstable substance which was destroyed by heating at 70°C for ten minutes. The stable inhibitory substances withstood exposure to 95% ethanol while the unstable inhibitory substance was destroyed at this concentration of ethanol. The stable substance diffused while the unstable substance did not diffuse through cellophane. Benda (5, p. 442-452) showed that the juice of New Zealand spinach also contains two materials. One was an inhibitor which separately decreased and the other was an augmenter which separately increased the number of lesions produced by TRSV. He observed that the addition of New Zealand spinach juice to a TRSV inoculum delayed the appearance of lesions. The addition of juice heated to 100°C for ten minutes increased the total number of lesions without any apparent delay in their appearance. The addition of dialyzed juice to the inoculum suppressed lesion formation. The thermal inactivation end point of the inhibitor was found to be 80°C for ten minutes, and the augmenter was heat stable and was lost by dialysis.

Kahn et al. (21, p. 848) found that the water extracts of rice yield inhibitors that prevent the infection of various susceptible plants by several viruses tested. Their thermal inactivation studies of water extracts of rice suggested the presence of two or more inhibitors. One inhibitor was labile above 60°C and the other stable at 100°C. Zaitlin and Siegel (34, p. 225) isolated an inhibitor from tobacco (Nicotiana tabacum L.) which inhibited TMV infections. They have characterized this inhibitor as a heat stable protein. Several workers (6, p. 787; 23, p. 570-572; 24, p. 896)

obtained increased infectivity of virus after heating juice of infected hosts. The inhibitor was destroyed by heating from 50°C to 100°C for ten minutes. Weintraub and Willison (33, p. 330) found that the inhibitor of stone fruit viruses in cucumber hosts was inactivated by a ten minute exposure at 40°-45°C.

Dilution has proven to be successful in increasing the infectivity of a non-infectious mixture of virus and inhibitor. Kuntz and Walker (23, p. 566-568) observed that the inhibitive effect upon the TMV and upon the cabbage-mosaic virus was reduced by dilution of the spinach extract. Blaszczak et al. (6, p. 786-787) reported that the effect of the inhibitory activity of the infectious juices of Chenopodium album L. and Capsicum frutescens L., disappeared at dilutions of 1:100 and 1:500 respectively. Weintraub and Gilpatrick (32, p. 553) found the dilution end point of the inhibitor in Sweet William (Dianthus barbatus L.) to be between 10^{-2} and 10^{-3}. Several other workers (21, p. 848; 32, p. 553) also reported that relative inhibition decreased as reciprocal of dilution increased.

The use of high speed centrifugation to partially separate the virus from a virus-inhibitor mixture has been reported (32, p. 554). Zaitlin and Siegel (34, p. 225) reported that the inhibitor in tissue homogenates of tobacco was mostly confined to a fraction of the homogenate which would not sediment at 10,000 g. Centrifugation at 140,000 g of this active supernatant fluid, yielded a pellet and supernatant solution, both with a reduced capability of producing an inhibitory effect. When these two fractions were recombined, the full potentiality of the inhibitor was restored.

Most of the naturally occurring virus inhibitors in higher plants have no inhibitory effect on infection of the species from which they come. The inhibitory substances in the sap of Chenopodium prevents transmission of virus from Chenopodium to tobacco but allows transmission from Chenopodium to another Chenopodium. Gendron and Kassanis (14, p. 185-187) observed from their work with several viruses in different plant species that inhibitors were largely ineffective in preventing infection of the species which contain them. They concluded that the extent to which inhibitors of infection decrease the number of local lesions was independent of the virus used, but depends only upon the species to which inoculations were made.

Most virus diseases of rosaceous plants have been transmitted only by grafting or by insect vectors. Presumably, rosaceous plant extracts inhibit the transmission of the viruses. Bawden and Kleczkowski (4, p. 3-5) found that tannins in leaf extracts of strawberry precipitated the protein in the extracts. Such tannin-containing extracts also inactivated TMV and tobacco necrosis virus. van der Want (31, p. 74) suggested that tannins prevent infection of all plants and that such substances sould be called 'absolute' inhibitors to distinguish them from 'relative' inhibitors, whose action depends on the species of the plant to which inoculation is made. He distinguished 'absolute' inhibitors such as tannins in dahlia, which interfere with the transmission of the dahlia mosaic virus from dahlia to dahlia and other host plants. He called the inhibitors present in carnation sap 'relative'inhibitors.

These inhibitors prevented the mechanical transmission of carnation mosaic virus from carnation to tobacco and French bean. On the other hand, the inhibitor apparently had no effect on the activity of the carnation mosaic virus when transmitted from carnation to carnation.

The Mechanism of Inhibition of Infection

Some inhibitors apparently act on the host and others act on the virus. Kuntz and Walker (23, p. 565-572) found that a component present in an extract of spinach leaves was capable of inhibiting infection of cabbage mosaic virus on Nicotiana glutinosa L. but not of TMV on the same host. They concluded that the effect of the inhibitor was on the host. Kleczkowski (22, p. 167) showed that the ability to combine with and precipitate TMV was not correlated with ability to inhibit infection. According to him, combination with virus was not important in preventing infection unless the combination occurs with a specific group on the virus particle responsible for activity. Several reports (32, p. 556; 16, p. 50) however, have indicated that the inhibitor was due to a loose, easily broken, molecular union between virus and inhibitor. The infectivity of virus in a mixture with inhibitor could be partially regained by high speed centrifugation. The binding energy of a virus-inhibitor complex would need to be extremely weak to be broken by simple high speed centrifugation.

MATERIALS AND METHODS

Virus Source

Viruses were established and maintained in systemically-infected host plants kept in the greenhouse. The greenhouse was maintained at a day temperature of 70-75°F and a night temperature of 65°F. The following viruses were supplied by J. A. Milbrath and maintained in the following hosts; Alfalfa mosaic virus (AMV strain 7) maintained in Necrotic Turk tobacco (Nicotiana tabacum L. x N. glutinosa L.); TMV maintained in Samsun tobacco (Nicotiana tabacum L. var. Sam), three strains, designated A, B, and C of TRSV* maintained in Vinca rosea; and Peach yellow bud mosaic virus (PYBV) maintained in strawberry.

Plant Culture

The plants used for virus concentration tests were grown from seed. They were sown in loam-peat moss mixture in number ten cans. Uniform, vigorous plants were selected for the experiments. Seedlings of Chenopodium amaranticolor Coste and Reyn. were transplanted three to four weeks after germination and were large enough to use after an additional three to four weeks. However, older plants could be used as long

---

* Strain A was supplied by R. G. Grogan, Dept. of Plant Pathology, University of California at Davis, California. Strains B and C were Oregon strains, maintained at O. S. U., Corvallis, Oregon.
** Supplied by P. W. Miller, U.S.D.A., O.S.U., Corvallis, Oregon.

as the new growth was vigorous. Chenopodium was a short day plant and during the winter months supplementary light was provided to prevent flowering. The plants were maintained in the same greenhouse as the virus sources.

Plant Inoculation

The juice was extracted from the test plants by grinding 1 g of leaf tissue in 10 ml of 0.5% Na_2HPO_4, pH 7.5. The juice was extracted from the pulp by squeezing it through four layers of cheesecloth.

Juice from normal Chenopodium containing the inhibitors was extracted in the same manner. Unless otherwise stated, 1 g of tissue was macerated in 10 ml of liquid. Carborundum powder (600 mesh) was lightly and evenly dusted over each leaf surface with a De Vilbiss No. 119 Powder insufflator. The leaf to be inoculated was supported with a pad made from a paper towel and the inoculum was rubbed on the leaf gently and uniformly with the moist forefinger on each side of the midrib. Ten minutes after inoculation, the excess carborundun and inoculum was washed off the leaf with tap water. When lesion development was sufficiently advanced, detached leaves were placed on an electrically lighted counting box and the lesions counted by the aid of a needle so as to avoid duplication.

Thermal Inactivation

Thermal inactivation studies of IS (inhibitory substances) in Chenopodium were conducted with modification of the procedure outlined by Bas, Hagedorn, and Quantz (7 , p. 334-335). The juice was extracted in a mortar containing the buffer at the rate of 1 g of leaf tissue to 10 ml of 0.5% Na_2HPO_4, pH 7.5. Four ml of the juice were pipetted into thin walled glass tubes. The tubes were placed in a water bath at the desired temperature. A Blue Magni Whirl Utility water bath with a constant temperature control and mechanical agitation was used for all experiments. The tubes containing the inoculum were heated for ten minutes at the desired temperature, then cooled under water. Unheated controls were used for every experiment.

Density-Gradient Centrifugation

Density-gradient centrifugation was performed using the method of Brakke (8) with modifications. Different concentrations of aqueous sucrose were made by dissolving 25, 30, 35, 40, 45 and 50 g of sucrose each in 100 ml of 0.033 M phosphate buffer, pH 7.0. Five ml layers of each density were formed in plastic tubes by flowing one slowly on top of another with a pipette. The heaviest solution was put in first, followed by successively lighter ones. The tubes were left overnight in the refrigerator to equilibrate. Sap of diseased plants was extracted by grinding leaves in 0.033 M phosphate buffer, pH 7.0. These crude extracts were clarified by low

speed centrifugation of 12,100 g for ten minutes. The clarified juice was layered on top of the gradient column. Centrifugation followed immediately since if allowed to stand, droplet sedimentation could have developed and the virus would soon be distributed throughout the column (8, p. 275). Gradient columns were centrifuged for four hours at 53,500 g. Gradient columns were handled very gently after centrifugation to prevent mixing. Zones were observed in darkness except for a small microscope lamp, the beam of which was focused into the column from above. Zones were apparent if the beam was small so that light did not impinge on the sides of the tube. Samples for infectivity assays were removed from the centrifuged gradient tubes with a hypodermic syringe. This was fitted with a needle with a bent tip which could be inserted down into the top of the column. The syringe was washed out in distilled water each time a new layer was removed in order to eliminate any chances of contamination between the layers. Samples for infectivity assays were inoculated on assay hosts immediately.

EXPERIMENTAL PROCEDURE AND RESULTS

Separation of Viruses from Inhibitory Substances (IS) in *Chenopodium* by dilution

Extracts of virus-infected *Chenopodium* have been reported to become more infective with dilution (6, p. 786-787). Experiments were conducted to determine whether dilution could be used to obtain viruses in high titre from *Chenopodium*. AMV was selected as one test virus since it became systemic in *Chenopodium*. This strain of AMV also incited distinct necrotic lesions on black cowpea (*Vigna sinensis* Endl). The AMV culture was maintained in systemically infected Necrotic Turk tobacco. Two experiments with AMV were conducted. One experiment was to find the effect of dilution on IS in *Chenopodium* infected with AMV. The second experiment was to find the effect of dilution on IS when mixed with an AMV extract from systemically infected tobacco.

TMV was selected as another test virus. It did not produce systemic symptoms in *Chenopodium* but did so in Samsun tobacco. One experiment was conducted to find the effect of dilution on IS from a mixture of IS from *Chenopodium* and a TMV extract from systemically infected tobacco.

Extracts were obtained from systemically infected *Chenopodium*, healthy *Chenopodium*, and systemically infected tobacco by grinding 1 g of leaf tissue in 10 ml of 0.5% Na_2HPO_4.

Effect of Dilution on IS in Chenopodium Infected with AMV. Juice from leaves systemically infected with AMV was extracted by grinding 1 g of leaf tissue in 10 ml of buffer. This extract was diluted from 1:20 to 1:1000 with water (Table 1). The primary leaves of 10 cowpea plants were inoculated with each dilution.

The average of the four experiments is represented graphically in Figure 1. As seen, the average number of lesions increased as the dilution increased. The IS completely inactivated all AMV at the original 1:10 dilution and at 1:20. There was a slight infection at the 1:40 dilution in two of the four experiments. The effect of IS at 1:40 or more steadily decreased. Therefore, there was a gradual increase of infection up to the 1:1000 dilution, where infection was the highest. There seemed to be an apparent dissociation of the virus and the inhibitor at 1:1000 dilution.

Decreased infectivity would probably occur above 1:1000 dilution as noted in other experiments due to the effect of dilution on the concentration of the virus. This demonstrated that IS was diluted out before AMV. Therefore, the infectivity of AMV-infected but non-infectious Chenopodium extract could be restored by dilution, although not in very high titre.

Effect of Dilution on IS in a Mixture of IS from Chenopodium and AMV from Tobacco. Experiments were conducted to determine whether the activity of an extract of AMV which was inactivated by Chenopodium sap could be restored by dilution. Sap from leaves of N.Turk tobacco systemically infected with AMV and

Table 1. Average number of lesions induced on black cowpeas by AMV-infected extracts from Chenopodium at different dilutions.

	Av. no. of local lesions/leaf				
	Experiments				
Dilution	I	II	III	IV	Total Av.
1:10	0.0	0.0	0.0	0.0	0.0
1:20	0.0	0.0	0.0	0.0	0.0
1:40	1.0	11.2	0.0	0.0	3.0
1:60	2.3	10.5	2.4	0.8	4.0
1:80	2.9	7.5	2.8	1.1	3.5
1:100	3.0	7.8	2.8	2.8	4.1
1:120	3.0	9.4	5.1	4.0	5.3
1:140	6.6	9.9	5.8	5.8	7.0
1:160	6.4	10.8	7.0	6.0	7.5
1:180	6.9	12.4	7.3	6.8	8.4
1:500	12.4	15.1	8.0	7.3	10.7
1:1000	40.1	26.6	8.5	30.2	23.8

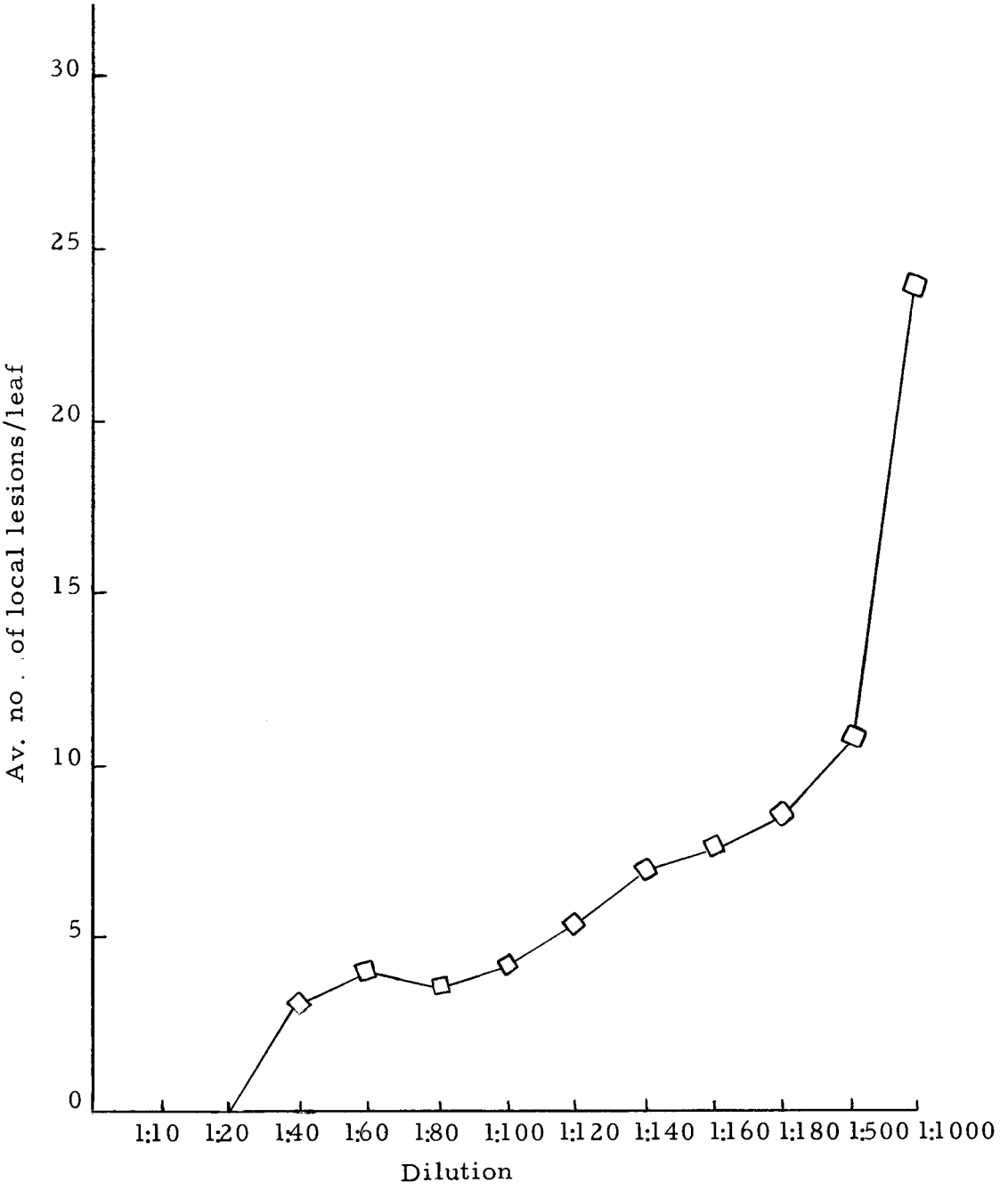

Figure 1. The effect of dilution on extracts of AMV infected Chenopodium as indicated by local lesion development on black cowpea.

normal Chenopodium was extracted by grinding 1 g of leaf tissue in 10 ml of buffer. The extract of AMV was divided into two portions. An equal amount of juice from normal Chenopodium was added to one portion and water was added to the other. The stock solutions were then diluted 1:10, 1:100, 1:1000 and 1:10,000. The primary leaves of ten black cowpea plants were inoculated with each treatment.

The results showed (Table 2) that the AMV extract mixed with equal parts of water was infectious, and infectivity decreased as dilution increased. The AMV extract mixed with equal parts of IS was completely non-infectious at all dilutions, even when the virus titre was very high, as indicated by the high number of lesions incited by the water-diluted inoculum. The results indicated that dilution was not always a reliable means of separating IS and virus.

Table 2. Effect of dilution on IS in a mixture of IS from Chenopodium and AMV from tobacco as indicated by local lesion development on black cowpea.

Dilution	Av. no. local lesions/leaf					
	AMV Experiments					
	I		II		III	
	IS	H_2O	IS	H_2O	IS	H_2O
1:1	0.0	∞	0.0	107.4	0.0	∞
1:10	0.0	220.9	0.0	57.3	0.0	198.4
1:100	0.0	148.5	0.0	60.1	0.0	150.4
1:1000	0.0	0.8	0.0	4.2	0.0	2.5
1:10,000	0.0	0.0	0.0	0.0	0.0	0.0

Effect of Dilution on IS in a Mixture of IS from Chenopodium and TMV from Tobacco. An experiment was conducted to determine if infectivity could be restored by dilution of mixtures of extracts of TMV and from Chenopodium. Sap from Samsun tobacco leaves systemically infected with TMV and normal Chenopodium was extracted by grinding 1 g of leaf tissue in 10 ml of buffer. The infectious extract of TMV was divided into two portions. An equal amount of juice from normal Chenopodium was added to one portion and water was added to the other. The stock solutions were then diluted 1:10, 1:100, 1:1000 and 1:10,000. The primary leaves of ten Pinto bean plants were inoculated with each treatment.

The results showed (Table 3, Figure 2) that the TMV extract mixed with an equal part of water was highly infectious, while the extract mixed with an equal part of Chenopodium juice was non-infectious. Dilution of the TMV and IS mixture resulted in a gradual increase in infectivity. At the 1:1000 dilution, there appeared to be complete dissociation of the virus and IS. The dilution end point of the IS was 1:1000 and the dilution end point of TMV was beyond 1:10,000. This virus appeared to be concentrated enough to be freed of the IS, but only in a relatively low titre.

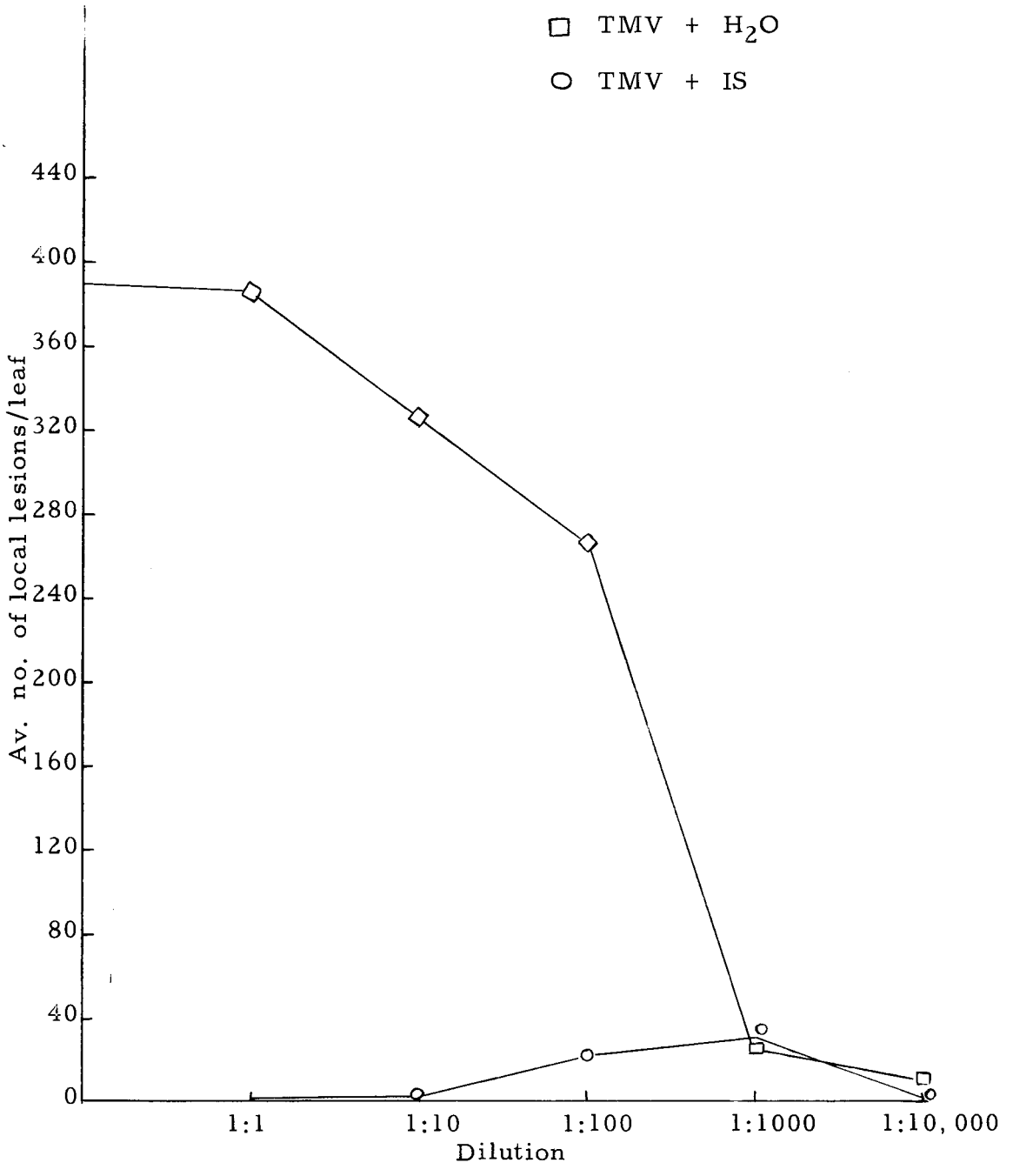

Figure 2. Effect of dilution on IS from a mixture of IS from Chenopodium and TMV from tobacco as indicated by local lesion development on Pinto bean.

Table 3. Effect of dilution on IS in a mixture of IS from Chenopodium and TMV from tobacco as indicated by local lesion development on Pinto bean.

	Av. no. local lesions/leaf	
Dilution	TMV + IS	TMV + H_2O
1:1	0.0	385.5
1:10	0.8	324.3
1:100	20.6	265.2
1:1000	31.3	27.7
1:10,000	0.4	10.0

The Use of Heat to Separate Viruses from IS in Chenopodium

Heat has been reported to increase infectivity of virus-containing Chenopodium sap (6, p. 787). Trials again were made using AMV and TMV as the test viruses and Chenopodium as the source of IS. The juice was extracted from uninoculated Chenopodium and systemically infected tobacco by grinding 1 g of leaf tissue in 10 ml of 0.5% Na_2HPO_4. Equal amounts of TMV extract were mixed with Chenopodium sap in the same manner as in the dilution experiment.

Aliquots of 5 ml of each treatment were left at room temperature, and the others were heated in test tubes at various temperatures. The test tubes were immersed in a Blue Magni Whirl Utility water bath for ten minute intervals at various temperatures. The primary leaves of ten bioassay hosts were inoculated with each treatment.

Effect of Heat on IS in a Mixture of IS from Chenopodium and AMV from tobacco. Sap from N. Turk tobacco leaves systemically infected with AMV and sap from uninoculated Chenopodium were extracted by grinding 1 g of leaf tissue in 10 ml of 0.5% Na_2HPO_4. The AMV extract from tobacco was divided into three portions. Equal amounts of sap from Chenopodium were added to one portion and water was added to the second. These were then heated from 40° to 90°C for ten minutes. Aliquots of healthy Chenopodium were similarly heated, cooled and then mixed equally with the third portion of the infectious extract of AMV. Then the primary leaves of ten black cowpea plants were inoculated with each treatment.

The experiment was repeated four times and the results are shown in Table 4 and Figure 3. The untreated AMV extract showed a high degree of infectivity at room temperature and 40°C. The average number of lesions ran as high as 410.8/leaf. There was considerable decrease in AMV infectivity at 50°C, but still some virus activity at 60°C. According to Milbrath (25, p. 1038), major virus activity of most strains of AMV was destroyed near 48°C for ten minutes but occasional particles may remain infectious up to 60°C or even at a few degrees higher.

AMV and IS could be separated when heated together at 60°C, but very few lesions developed. There was no virus activity with temperatures below and above 60°C. At temperatures below 60°C the IS was not inactivated and therefore suppressed infectivity. The heat inactivated AMV at temperatures higher than 60°C.

Table 4. The effect of temperature on IS and AMV when treated alone and together as indicated by local lesion development on black cowpea..

	Av. no. local lesions/leaf											
	Experiments											
	I			II			III			Total Av.		
°C/ 10 min.	AMV	AMV + IS	IS*	AMV	AMV + IS	IS*	AMV	AMV + IS	IS*	AMV	AMV + IS	IS*
Room	454. 8	0. 0	0. 0	439. 4	0. 0	0. 0	338. 2	0. 0	0. 0	410. 8	0. 0	0. 0
40°C	435. 6	0. 0	0. 0	424. 5	0. 0	0. 0	310. 9	0. 0	0. 0	393. 6	0. 0	0. 0
50°C	200. 1	0. 0	0. 0	198. 2	0. 0	0. 0	140. 4	0. 0	0. 0	166. 2	0. 0	0. 0
60°C	0. 0	0. 3	3. 0	2. 4	1. 2	4. 2	0. 0	0. 8	2. 0	0. 8	0. 7	3. 0
70°C	0. 0	0. 0	2. 0	0. 0	0. 0	4. 9	0. 0	0. 0	4. 9	0. 0	0. 0	3. 9
80°C	0. 0	0. 0	29. 5	0. 0	0. 0	35.4	0. 0	0. 0	27. 3	0. 0	0. 0	30. 7
90°C	0. 0	0. 0	166. 5	0. 0	0. 0	170. 4	0. 0	0. 0	180. 1	0. 0	0. 0	172. 3

* AMV added after heating

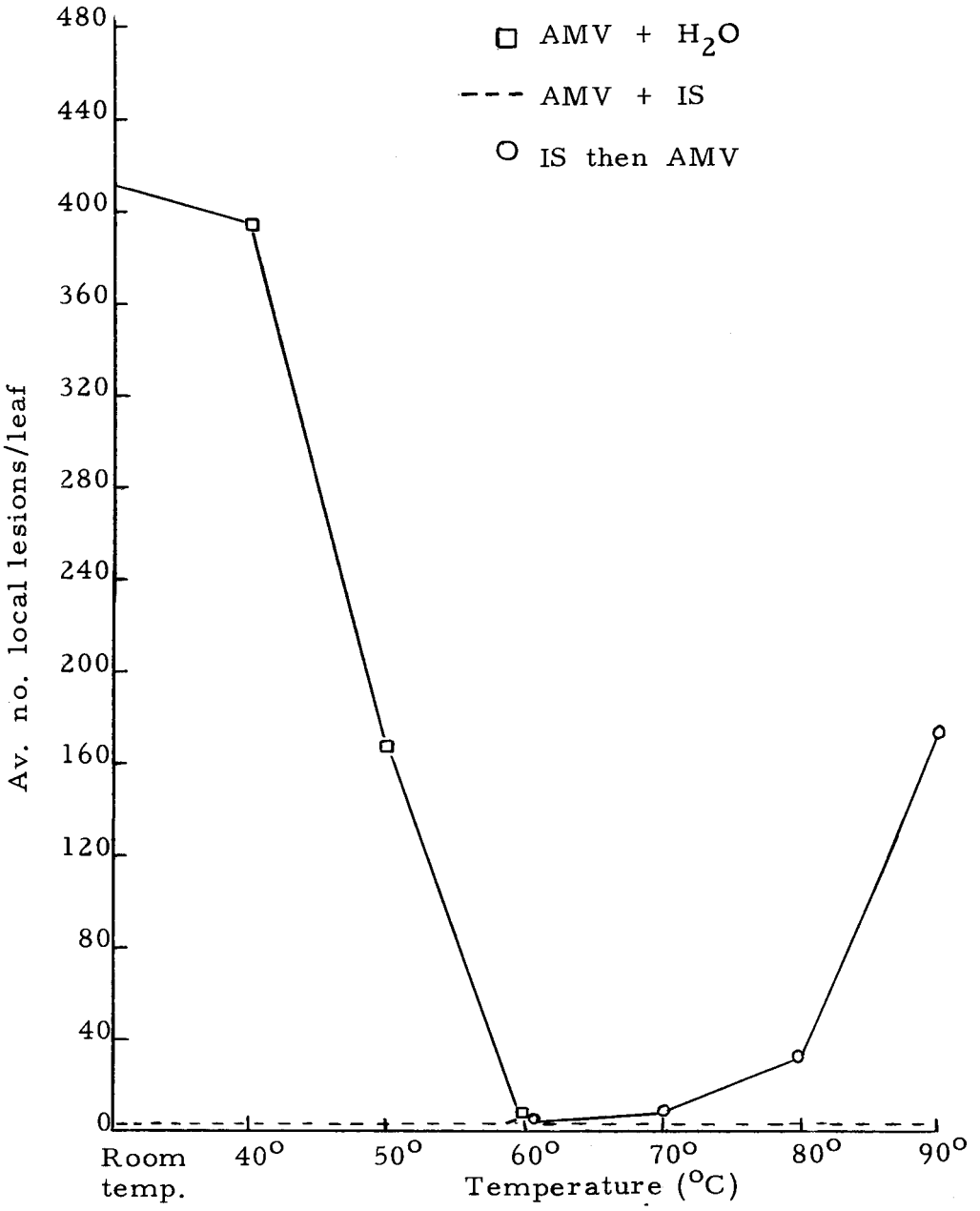

Figure 3. The effect of temperature on IS and AMV when heated alone or together as indicated by local lesion development on black cowpea.

IS started inactivating at 60°C when only the Chenopodium extract was heated, cooled and then added to AMV (Table 4). The inactivation of IS increased as the temperature increased, resulting in increased infectivity of the virus. At 90°C, IS were not completely inactivated, but were inactivated to a level that allowed an average of 172.3 lesions/leaf as compared to 410.8 for the AMV sap + H_2O at room temperature. The inhibition of AMV was 58% at 90°C.

IS started inactivating at 60°C when the mixture of AMV and IS was heated. IS also was inactivated at 60°C when only IS was heated, cooled and then added to AMV. This demonstrated that heat can be used as a method of IS-virus separation only for those viruses which have a thermal inactivation end point greater than 60°C.

Effect of Temperature on IS in a Mixture of IS from Chenopodium and TMV from tobacco. Sap from Samsun tobacco leaves systemically infected with TMV and uninoculated Chenopodium was extracted by grinding 1 g of leaf tissue in 10 ml of buffer. The same treatments and procedure were followed as in the previous temperature experiment with AMV. The primary leaves of ten Pinto bean plants were inoculated with each treatment.

One trial of the experiment was conducted and the results are shown in Table 5 and Figure 4. The untreated TMV extract showed a high degree of infectivity at room temperature and at 40°C. With the untreated infectious extract the average number of

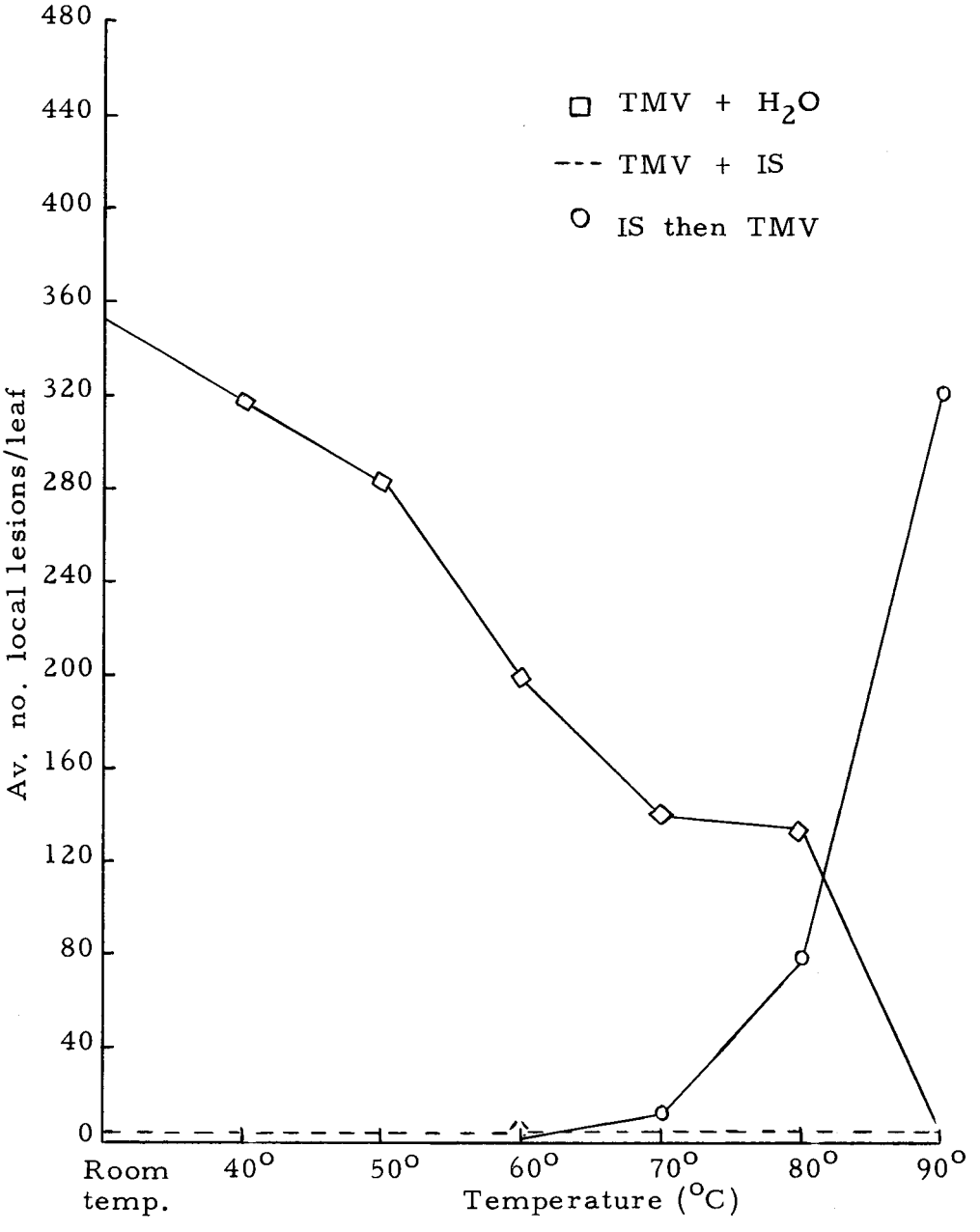

Figure 4. The effect of temperature on IS. and TMV heated alone or together as indicated by infectivity on Pinto bean leaves.

Table 5. The effect of temperature on IS in a mixture of IS from uninoculated Chenopodium and TMV from tobacco, as indicated by infectivity on Pinto bean leaves.

	Av. no. local lesion/leaf		
°C/10 min.	TMV + H_2O	TMV + IS	IS heated then TMV added
Room temperature	354.0	0.0	0.0
40°C	316.7	0.0	0.0
50°C	283.7	0.0	0.0
60°C	198.2	0.2	0.4
70°C	140.2	0.0	5.5
80°C	135.6	0.0	79.1
90°C	5.6	0.0	322.6

lesions/leaf, followed a descending straight line curve due to the increase of temperature. There was still some infectivity at 90°C demonstrating that the thermal inactivation end point of TMV was greater than 90°C.

TMV and IS were not separated to the same degree when heated together, as was the AMV and IS mixture. The only infectivity was present after the treatment at 60°C. There was no virus activity with temperatures below and above 60°C. At temperatures below 60°C the IS was not inactivated and therefore suppressed infectivity of TMV. At temperatures above 60°C, there was no virus infectivity, although the thermal inactivation end point of TMV was higher than 90°C. It is possible that one IS was inactivated and another was activated at 60°C, when TMV and IS were

heated together.

One of the IS started inactivating at 60°C when only the IS of Chenopodium extract was heated, cooled and then added to TMV. The inactivation of IS increased as the temperature increased, resulting in an increased infectivity of the virus. The inhibition of IS was 10% at 90°C.

From these results we see that there may be two IS involved. One of the IS started inactivation at 60°C and the second of the IS started activation at 60°C, when the mixture of TMV and IS was heated.

The Use of Liquid Nitrogen to Separate Viruses from IS in Chenopodium

Sänger and Gold (28) reported transmission of an unstable form of tobacco rattle virus with liquid nitrogen. Experiments were conducted to determine the effect of nitrogen on IS in Chenopodium.

Chenopodium leaves systemically infected with AMV were removed and cut into fine strips. One gram of the strips was ground in a mortar containing 10 ml of 0.5% Na_2HPO_4. This was used as a control. Another gram of strips were frozen in 40 ml of liquid nitrogen and ground in a mortar in the presence of 10 ml of buffer to form an ice powder. The ice powder was painted on ten Carborundum dusted primary leaves of black cow-peas with a moist brush.

The average number of lesions in each of three experiments are shown in Table 6. Liquid nitrogen increased the infectivity of AMV three to seven fold.

Table 6. The effect of liquid nitrogen on IS from AMV-infected Chenopodium on infectivity of AMV on cowpea.

	Av. no. local lesions/leaf		
Experiment	With liq. N.	Without liq. N.	% Inhibition
I	405.8	103.0	76.5
II	268.6	74.5	72.2
III	59.1	7.8	86.8

Separation of Viruses from the IS Present in Chenopodium

Use of hydrated calcium phosphate (HCP) and centrifugation to separate TRSV and IS. A modification of the technique, reported by Fulton(12;13) for the clarification of tissue macerates infected with virus was attempted to separate virus from IS present in Chenopodium.

The two strains, designated A and B, of TRSV were selected because they became systemic in Chenopodium and caused distinct local lesions on black cowpea. Vigorously growing plants of Chenopodium were inoculated with these TRSV strains. One gram of the systemically infected leaf tissue was ground in a mortar with 1:10 (w/v) of 0.02 M phosphate buffer, pH 7.5. Some of the extract was used to test its infectivity on

Chenopodium.

The juice from the remaining infectious tissue was extracted in the same manner as the control. Buffer of the same formulation and dilution was used throughout the remainder of the procedure. The extract was centrifuged for ten minutes at 3,300 g. Six tenths of a gram of freshly prepared HCP was added to the extract for each gram of original tissue. The mixture was homogenized with a magnetic stirrer in a beaker. Mixing was augmented by drawing and expelling the homogenate through a 30 ml syringe. Then this homogenate was centrifuged for ten minutes at 1,020 g. The supernatant was again adsorbed as described above. The clear supernatant then was centrifuged for 15 minutes at 6,870 g to remove remaining traces of HCP. A portion of this supernatant was assayed for infectivity on Chenopodium and black cowpea.

Ultra-centrifugation for 120 minutes at 105,400 g yielded a dark green pellet and a clear supernatant. The pellet was resuspended in buffer and centrifuged for 15 minutes at 6,870 g. This supernatant was assayed for infectivity on Chenopodium and black cowpea.

The control had 20 and 50 local lesions incited by TRSV strains A and B, respectively, but there was no infectivity after adsorption with HCP. According to Fulton (12, p. 521) most of the chloroplastic material and cell debris adsorbed to the gel-like HCP and sedimented readily on low speed centrifugation. The viruses remained in the nearly water-clear supernatant and retained their infectivity for several days. However, the results

in this experiment cannot be conclusive because there was only one trial carried out. The initial virus titre may have been too low and the virus may have been adsorbed along with the IS by HCP.

Use of Centrifugation to separate AMV and IS. Experiments were made to determine whether or not AMV infectivity could be increased by high speed centrifugation. AMV was selected because it became systemic in Chenopodium and caused distinct local lesions on black cowpea. One gram of the systemically infected Chenopodium leaf tissue was ground in a mortar of 1:10 (w/v) of 0.033 M phosphate buffer, pH 7.0. The control was uncentrifuged. The remaining extract was centrifuged at 39,000 rpm (Spinco No. 40 rotor). The water-clear supernatant was decanted. The pellet was resuspended in buffer and inoculated to 20 fully expanded primary leaves of black cowpea. The experiment was repeated three times. The results (Table 7) showed that ultra-centrifugation increased virus infectivity seven fold. Therefore, partial separation of AMV and IS could be accomplished by high speed centrifugation.

Table 7. Results of three experiments using ultra-centrifugation to separate AMV from IS in Chenopodium as indicated by local lesion development on black cowpea.

	Av. no. of local lesions/leaf			
	Experiments			
Treatment	I	II	III	Total Av.
Centrifuged	8.2	14.9	12.3	11.8
Not Centrifuged	2.3	1.7	1.2	1.7

Use of Sucrose Density-Gradient Columns to Separate Viruses from IS in Chenopodium

Experiments were conducted to find out if AMV and TRSV could be separated from IS in Chenopodium by using the sucrose density-gradient method (8). The TRSV and AMV strains selected became systemic in Chenopodium and caused distinct local lesions on black cowpea. Vigorously growing plants of Chenopodium were inoculated with TRSV or AMV. Two grams of young systemically infected leaves were removed 30 days after inoculation. Twenty fully expanded primary leaves of black cowpea were used to bio-assay infectivity for each treatment. The infected leaves of Chenopodium were cut into fine strips and mixed well. The strips were ground in the mortar containing the extraction fluid (5 ml/g leaf) of 0.033 phosphate buffer, pH 7.0. Then the juice was squeezed through four layers of cheese cloth. A portion of this was used as one control. The remaining extract was first centrifuged at 12,100 g for ten minutes for preliminary clarification of normal plant constituents. A portion of this was used as a second control to assay for virus infectivity. Healthy Chenopodium was extracted in a similar manner as those containing virus.

Gradient columns for rate zonal centrifugation were made by layering 5 ml portions of solution, containing 50, 45, 40, 35 and 30 g sucrose per 100 ml of 0.033 M phosphate buffer, pH 7.0 in 1 x 3" plastic centrifuge tubes at least one day before use. After a layer of 5 ml of partially purified virus solution had been

floated on each of the gradient columns (Figure 5A) they were centrifuged at 25,000 rpm (Spinco No. 25.1 rotor) for 240 minutes. Then the layers were removed from the gradient column with a syringe fitted with a 4-inch needle with a bent tip, which was slowly inserted into the column from the top.

Location of TRSV and AMV from Chenopodium in density-gradient columns. The virus layers could not be seen, so they were located by infectivity assays of samples removed from different depths. As seen in Figure 5B and Table 8 there was no TRSV infectivity in the top four layers appearing red, pink, light pink and diffused, respectively. Infectivity was found in the 5th and 6th layers; each consisting of 5 ml of sucrose solution. The 5th layer incited an average of 123.0 local lesions as compared to 76.9 lesions for the 6th layer. There was no infectivity in the 7th layer, consisting of 8 ml of the sucrose solution. Therefore, infectivity of the TRSV lies within the 35, 40 and 45 g sucrose layers with the highest amount of infectivity in the 35 and 40 g concentrations of sucrose. There was no infectivity in the crude extract and partially purified extract.

In the AMV gradient column (Figure 5C; Table 8) there was no infectivity in the top three layers which were red, pink, and light pink. There was infectivity in the 4th, 5th, 6th and 7th layers. The highest amount of infectivity was found in the 5th layer. The 5th layer produced an average of 30.9 local lesions as compared to 11.7 for the 6th layer and 7.1 for the 4th layer. The

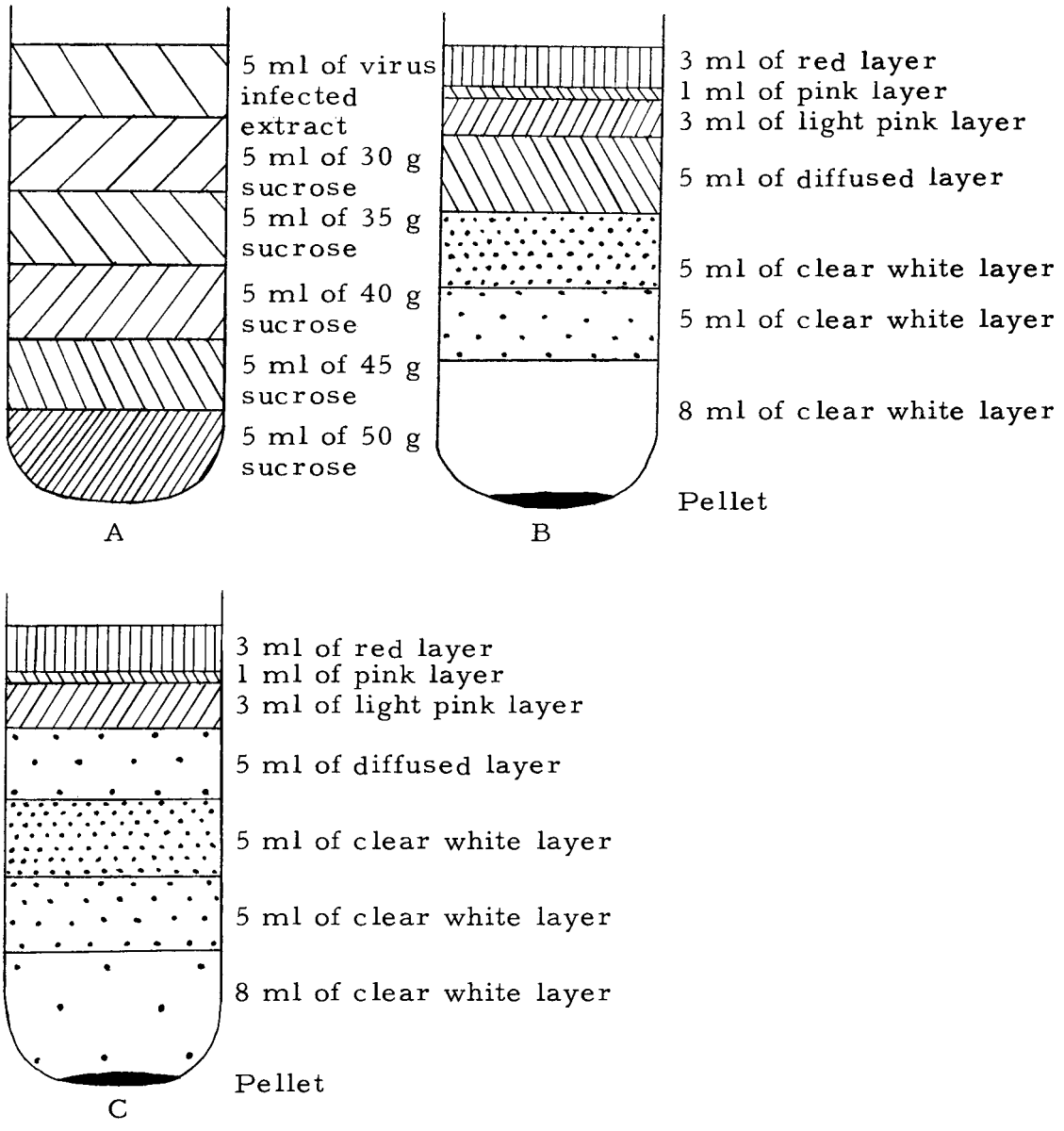

Figure 5. Schematic diagrams of centrifuge tubes with 50 to 30 g sucrose layers and TRSV or AMV layered on top.

A. Uncentrifuged sucrose density-gradient column layered with virus extract.

B. TRSV-sucrose density-gradient column after centrifugation.

C. AMV-sucrose density-gradient column after centrifugation.

Table 8. Infectivity of the layers removed from the 50 to 30 g sucrose density-gradient columns originally layered with TRSV and AMV. The layers were bioassayed on black cowpea.

Extract treatment	Av. no. local lesions/leaf	
	TRSV	AMV
Crude sap	0.0	0.0
Clarified sap	0.0	0.0
Layers		
1	0.0	0.0
2	0.0	0.0
3	0.0	0.0
4	0.0	7.1
5	123.0	30.9
6	76.9	11.7
7	0.0	0.3

7th layer produced an average of 0.3 local lesions. Since the infectivity of AMV spreads from 30 to the 50 g concentration of sucrose, it is apparent that particles of AMV are of variable size. As with TRSV, the highest infectivity of AMV lies in the 40 and 35 g concentration of sucrose. The crude extract and the partially purified extract were non-infective.

Another AMV gradient column was prepared with 5 ml layers of solutions containing 45, 40, 35, 30 and 25 g concentrations of sucrose (Figure 6A). As seen in Figure 6B and Table 9, there was no infectivity in the top three layers consisting of red, pink and light pink coloration. Infectivity was observed in the 4th, 5th, 6th and 7th layers, consisting of 3, 5, 5 and 5 ml of sucrose, respectively. The highest infectivity was found in the 6th layer which showed an average of 83.1 local lesions as compared

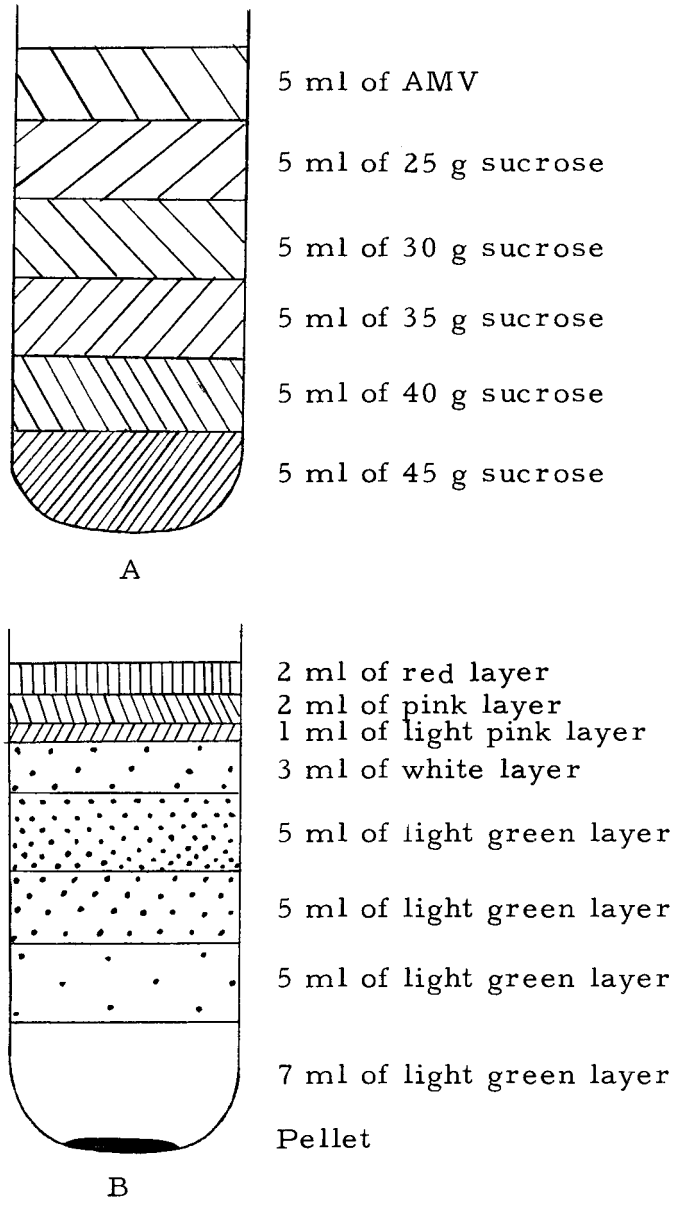

Figure 6. Schematic diagrams of centrifuge tubes with 45 to 25 g sucrose layers and AMV layered on top.

A. Uncentrifuged AMV and sucrose layers.

B. AMV and sucrose after centrifugation.

Table 9. Infectivity of the layers removed from the 45 to 25 g sucrose density-gradient column originally layered with AMV. The layers were bioassayed on black cowpea.

Extract treatment	Av. no. local lesions/leaf AMV
Crude sap	0.0
Clarified sap	0.0
Layers	
1	0.0
2	0.0
3	0.0
4	10.1
5	78.8
6	83.1
7	6.8
8	0.0
Pellet	0.0

to 78.8 for the 5th layer and 10.1 for the 4th layer. The 7th layer produced an average of 6.8 local lesions. The highest amount of infectivity again lies in the 35 and 30 g concentrations of sucrose. The pellet was resuspended in the original buffer and was non-infective. The crude extract and partially purified extract also were non-infective.

Location of IS in density-gradient columns. An experiment was conducted (Table 10) to determine the presence of IS by combining TMV with different layers from the gradient column of healthy Chenopodium extract. Pinto bean was used to bioassay infectivity since it produces local lesions in response to TMV infection. Samples of the different layers from the healthy Chenopodium column were mixed in equal proportions with the crude extract of

TMV from tobacco. Pinto beans inoculated with the crude extract of TMV served as the control. The top two layers caused 100% inhibition of TMV lesion formation. The 3rd and the 4th layer showed 95.0 and 37.3% inhibition, respectively. There was no inhibition in the 5th layer of 35 g sucrose. There was a slight inhibition of 18% in the 6th layer. The pellet,when resuspended in the original buffer,demonstrated 70% inhibition of TMV. It could be concluded from these results (Table 10) that most of the IS remained in the aqueous solution and no IS was present in the 35 g concentration of sucrose. The pellet contained some IS, probably due to the sedimentation of larger portions of plant tissue.

Table 10. The effect on TMV infectivity on Pinto bean of various layers from a sucrose density-gradient column with uninoculated Chenopodium mixed with TMV as indicated by local lesion development on Pinto bean.

Extract treatment	Av. no. local lesions/leaf	% inhibition
Crude TMV	137.2	0.0
Layers		
1	0.0	100.0
2	0.0	100.0
3	6.4	95.0
4	86.4	37.3
5	135.6	0.0
6	112.0	18.3
Pellet	41.6	70.0

It was assumed that the viruses would sediment into different layers than the IS. Therefore, these experiments were designed to see if TRSV and AMV could be freed from IS. Both TRSV and

AMV infectivity was highest in 35 g sucrose and there was no infectivity in the aqueous layers of the gradient columns (Figures 5B, C and 6B; Tables 8 and 9). Most of the IS remained in the aqueous layer. The inhibitor decreased as the sucrose density increased. There was no inhibition at the 35 g concentration of sucrose. To check this, samples were removed from the healthy Chenopodium sucrose gradient and mixed with TMV. All allowed lesion formation on Pinto beans, in contrast to the samples withdrawn from the aqueous layer. There was some inhibition in the pellet which was due to the sedimentation of the normal plant constituents (Table 10). This showed that these viruses and IS could be separated with sucrose and centrifugation.

Separation of AMV from IS in an abbreviated density-gradient column. Since the viruses were freed from inhibition near the 35 g sucrose layer, an AMV gradient column was prepared by layering 5 ml of a 34 day old AMV inoculum on 5 ml each of a 40 and 35 g concentration of sucrose (Figure 7A). There was no infectivity in the top three layers (Figure 7B). Infectivity was found in the 9 ml in the greenish yellow sucrose portion. The average number of local lesions per leaf was 38.7 in this portion of the column. It was apparent that the inhibitor was separated from the virus particles by the 35 and 40 g concentrations of sucrose. The crude extract and partially purified extract were non-infective.

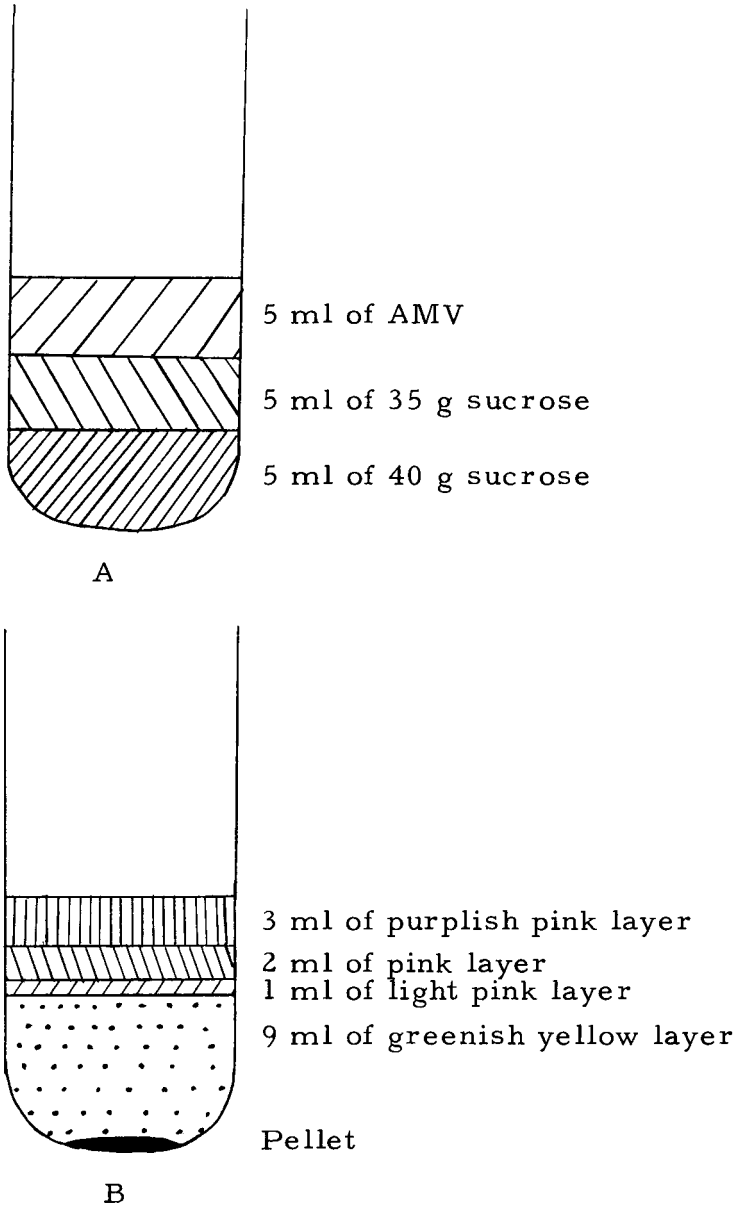

Figure 7. Schematic diagrams of centrifuge tubes with 40 and 35 g sucrose layers and AMV layered on top.

A. Uncentrifuged AMV and sucrose layers.

B. Layers after centrifugation.

Use of One Layer of Sucrose to Separate Viruses from IS

One layer of a 35 g concentration of sucrose was used because the previous experiments demonstrated that the viruses migrated into the sucrose during centrifugation and concentrated in the 35 g sucrose region. Strains A, B and C of TRSV in Chenopodium were used in these experiments (Table 11). They were selected because they became systemic in Chenopodium and caused distinct local lesion on black cowpea. After inoculation, Chenopodium became systemically infected as indicated by chlorotic veins and mottle. Two grams of young systemically infected leaves of Chenopodium were removed 15 days after inoculation. Twenty fully expanded primary leaves of black cowpea were used to bio-assay infectivity for each treatment. The infected leaves of Chenopodium were cut into fine strips, mixed well, and ground in the mortar containing 5 ml of 0.033 M phosphate buffer, pH 7.0/ g leaf. The infective juice was extracted through four layers of cheesecloth, and a portion of this was used as a control. The same buffer was used throughout the remainder of the procedure. The second control was ground in 5 ml of the 35 g sucrose solution in 100 ml of buffer/g leaf tissue. The third control was the crude extract that had been clarified at 12,100 g for ten minutes.

Gradient columns were prepared by layering 2 ml of extract over 3 ml of the 35 g concentration of sucrose in small plastic centrifuge tubes (Figure 8A). The tubes were centrifuged immediately at 121,000 g/1 hr in the SW39L rotor of the

Spinco model L ultra centrifuge. Layers were removed from the tubes as previously described and the pellet was resuspended in the original buffer and all were assayed for infectivity.

Separation of TRSV and IS from infected Chenopodium. As seen in Figure 8B and Table 11 there was no infectivity in the top four layers of TRSV strain A layered over the sucrose. The top four layers were purplish pink, light pink, rosy pink and orangy pink, respectively. Virus particles had sedimented into the 35 g concentration of sucrose, as evidenced by the high infectivity in the 3 ml of yellowish green 35 g sucrose. Local lesions were so numerous that they could not be counted. The pellet and the three controls were non-infective (Table 11).

In the TRSV strain A systemically infected tissue removed 30 days after inoculation (Table 11), there was no infectivity in the top four layers. The top five layers were cloudy white, clear white, cloudy pink, clear white and cloudy green, respectively. There was an average of 12.3 local lesions in the 5th layer and 130.2 in the 6th layer.

There was no infectivity in the top five layers of the TRSV strain B layered over sucrose (Figure 8C). They were purplish pink, light pink, rosy pink, light pink and orangy pink, respectively. Virus particles had sedimented into the 35 g sucrose. The average number of local lesions was 92.4 in the 2 ml of yellowish green 35 g sucrose. The pellet and the three controls were non-infective (Figure 8C and Table 11).

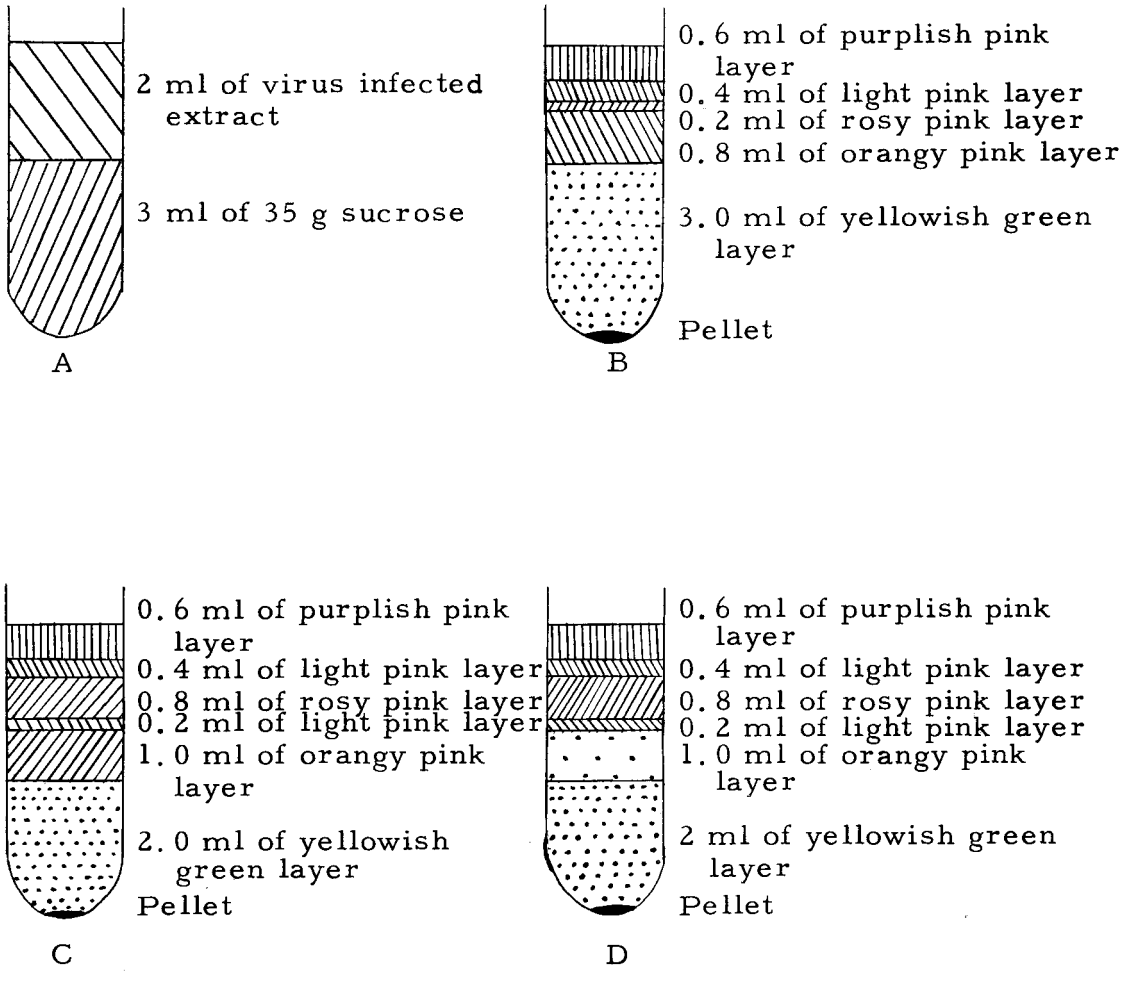

Figure 8. Schematic diagrams of centrifuge tubes with 35 g sucrose and TRSV strains A, B and C layers.

A. Uncentrifuged virus extract layered over 35 g sucrose.

B. Strain A - 35 g sucrose tube after centrifugation.

C. Strain B - 35 g sucrose tube after centrifugation.

D. Strain C - 35 g sucrose tube after centrifugation.

There was no infectivity in the top four layers of TRSV strain C (Figure 8D). The top five layers were purplish pink, light pink, rosy pink, light pink and orangy pink. There was an average of 21.8 local lesions in the 1 ml orangy pink 5th layer and high infectivity in the 2 ml of yellowish green 35 g sucrose. The numerous local lesions could not be counted. The pellet and the three controls were non-infective (Figure 8D and Table 11).

Table 11. Infectivity of layers removed from the centrifuged extract-35 g sucrose tubes with TRSV strains A, B and C. The layers were bioassayed on black cowpea.

Extract treatment	Av. no. local lesions/leaf			
	A	A	B	C
Crude sap	0.0	0.0	0.0	0.0
Crude sap in 35 g sucrose	0.0	0.0	0.0	0.0
Clarified sap	0.0	0.0	0.0	0.0
Layers				
1	0.0	0.0	0.0	0.0
2	0.0	0.0	0.0	0.0
3	0.0	0.0	0.0	0.0
4	0.0	0.0	0.0	0.0
5	∞	12.3	0.0	21.8
6	---	130.2	92.4	∞
Pellet	0.0	0.0	0.0	0.0

It is apparent from these experiments that the IS did not sediment into the 35 g concentration of sucrose while the virus particles sedimented into this concentration of sucrose.

Location of IS from TRSV infected Chenopodium extract. An experiment was conducted to locate the IS when TRSV strain A-infected Chenopodium extract was layered over 35 g sucrose. After

centrifugation, layers were mixed in equal proportions with the crude extract of TRSV strain A in black cowpea and assayed on black cowpea. One control was the inoculation of the crude virus extract on black cowpea. Lesions were too numerous to count.

The top four layers when mixed in equal proportions with the infectious juice showed no lesions. The 5th layer incited an average of 1.2 local lesions. In the 6th layer, the local lesions were too numerous to count. The pellet incited an average of 8.8 lesions. From the results (Table 12) it was clear that the IS remained in the top four layers, completely inhibiting virus activity. The IS were present to a lesser degree in the 5th layer. In the 6th layer, which was the 35 g sucrose, there were no IS present. There also was some inhibition from the pellet. In conclusion it could be said that the IS were smaller than the virus particles and that the virus particles could be separated from the IS with centrifugation over a 35 g concentration of sucrose.

Table 12. The effect of various layers from a TRSV strain A-infected Chenopodium 35 g sucrose column when mixed with TRSV strain A. Black cowpea was the assay host.

Extract treatment	Av. No. local lesions/leaf
TRSV extract from black cowpea	∞
Layers	
1	0.0
2	0.0
3	0.0
4	0.0
5	1.2
6	∞
Pellet	8.8

Attempted recovery of TRSV from an old infection in Chenopodium. An experiment was conducted to determine if TRSV strains A, B, and C could be recovered from Chenopodium after the 'shock' reaction had occurred. The above strains produced a local lesion 'shock' reaction followed by a bushy 'rosette type' of growth. Inoculum was 52 days old. When extracted, the pellet and the uncentrifuged control for each strain were found to be non-infective, in fact, there was no infectivity in any of the layers from the tube with each strain. Probably the virus titre was very low and the virus was lost in the process of purification.

Attempted recovery of PYBV from Strawberry. Experiments were conducted to determine if PYBV in strawberry could be recovered from expanded old, young and very young unfolding leaves of this plant. Since extracts of leaves, stems or roots of strawberry plants contain tannins, enough tannin can be released during maceration to precipitate the virus and prevent it from infecting other plants (4, p.3-5). It was hoped that centrifugation over 35 g sucrose could release the virus from the tannin (Figure 9A). There was no infectivity in the top three layers of the column of old strawberry leaves. The layers were white, dirty brown, and yellowish brown, respectively. The 3 ml 4th layer was found to have an average of 0.7 local lesions (Figure 9B). There was no infectivity observed from any of the layers in the columns of the young and young unfolding leaves (Figure 9C, D). The crude sap,

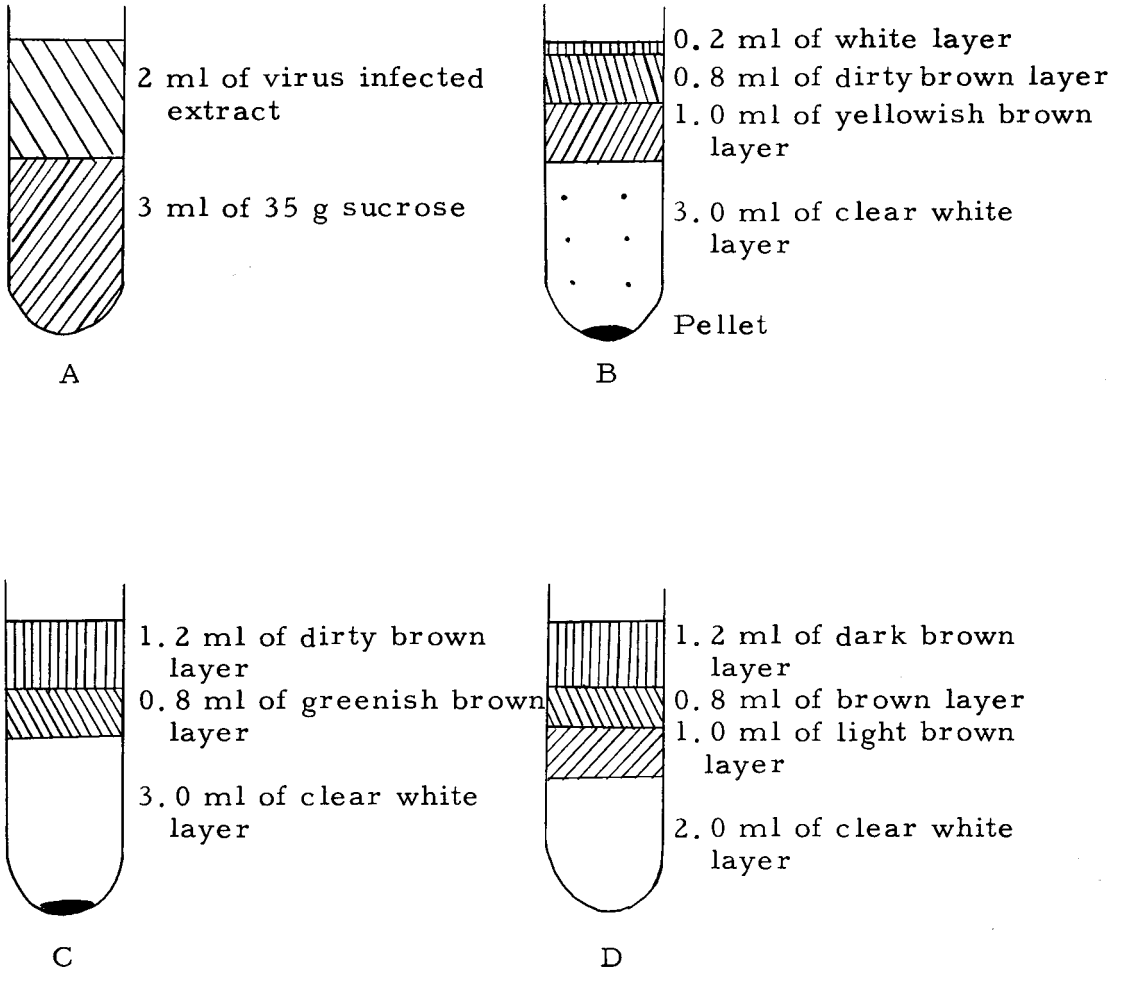

Figure 9. Schematic diagrams of centrifuge tubes with layer of 35 g sucrose and PYBV from strawberry.

A. Virus layered over 35 g sucrose.

B. 35 g sucrose with PYBV from old strawberry leaves after centrifugation.

C. 35 g sucrose with PYBV from young strawberry leaves after centrifugation.

D. 35 g sucrose with PYBV from very young strawberry leaves after centrifugation.

the sucrose treated crude sap, the clarified sap and the pellets in every case were found to be non-infectious.

It appeared that virus particles could be separated from the tannins in strawberry by this method, since some infection was obtained in the 4th layer from the column of the old strawberry leaves. Additional tests will be required for a definite answer on the release of PYBV from the tannins in strawberries.

The effect of sucrose extraction on AMV in Chenopodium

Since TRSV and AMV consistently sedimented into sucrose, an experiment was conducted to determine the effect on virus activity when virus infected Chenopodium was merely ground with different concentrations of sucrose. Six grams of young Chenopodium leaves which were systemically infected with AMV were cut into fine strips and mixed well. The extracts for different treatments were prepared by dissolving 30, 35, 40, 45 and 50 g sucrose each in 100 ml of 0.033 M phosphate buffer, pH 7.0. The infected strips were ground in a mortar containing the sucrose solutions (5 ml-g leaf). Each treatment was bioassayed for infectivity on 20 primary leaves of black cowpea. According to the results there was no infectivity after grinding the infected leaf tissue directly in the different concentrations of sucrose. Three conclusions could be drawn from the results; (a) direct grinding in sucrose did not separate the IS from the two viruses, (b) sucrose did not inactivate the IS, and (c) sucrose did not increase the activity of either virus.

DISCUSSION AND CONCLUSIONS

The infectivity of plant viruses was suppressed by IS in the sap of Chenopodium. As in previous work (21, p. 848; 32, p. 552), dilution separated viruses from IS in most of the trials. This apparent dissociation occurred at 1:1000 dilution in both AMV and TMV trials. Dilution end-point of the IS in Chenopodium also was found to be 1:1000. The infectivity of AMV-containing Chenopodium extract increased with dilution. Decreased infectivity occurred above 1:1000 dilution as noted in other experiments due to the effect of dilution on the concentration of the virus. The AMV extract mixed with equal parts of IS was completely noninfectious at all dilutions, even when the virus titre was very high, as indicated by the high number of lesions incited by the water diluted inoculum. Dilution of the TMV and IS mixture resulted in a gradual increase in infectivity. At the 1:1000 dilution, there was a complete dissociation of the virus and IS. Decreased infectivity occurred above the 1:1000 dilution due to the effect of dilution on the concentration of the virus. The dilution end point of the IS was 1:1000 and the dilution end-point of TMV was beyond 1:10,000. Therefore a virus that is concentrated enough can be freed of the IS, but only in a relatively low titre.

It was evident from previous reports (6, p. 287; 23, p. 570-572; 24, p. 896) that heat treatment of inocula removed inhibitory activity. The present studies showed that heat treatments removed inhibitory activity in some cases and not in others.

AMV and IS could be separated when heated together at 60°C, but very few lesions developed. There was no virus activity with temperatures below and above 60°C. At temperatures below 60°C the IS were not inactivated and therefore suppressed infectivity. The heat inactivated AMV at temperatures higher than 60°C. IS also were inactivated at 60°C when only the IS were heated, cooled and then added to AMV. The degree of inactivation of the IS increased as the temperature increased, resulting in increased infectivity of the virus.

TMV and IS were not separated to the same degree as was the AMV and IS mixture when heated together. The only infectivity present was observed after the treatment at 60°C. There was no virus activity with temperatures below and above 60°C. At temperatures below 60°C the IS was not inactivated and therefore suppressed infectivity of TMV. At temperatures above 60°C, there was no virus infectivity, although the thermal inactivation end-point of TMV was higher than 90°C. It is possible that one of the IS was inactivated and the other was activated at 60°C, when TMV and IS were heated together. One of the IS started inactivation at 60°C when only the IS of Chenopodium extract was heated, cooled and then added to TMV. The inactivation of the IS increased as the temperature increased, resulting in an increased infectivity of the virus.

It could be concluded that there were two IS involved. One of the IS started inactivation at 60°C and the second of the IS started activation at 60°C, when the noninfectious mixture of TMV

and IS was heated. Blaszczak et al. (6 , p. 787) reported that after ten minutes in boiling water the juice of Chenopodium lost a slight amount of inhibitory activity to PVX. Kuntz and Walker (23, p. 570-575) reported two inhibitors in spinach juice. One was an inhibitor of TMV and was destroyed by 12 minutes at 70°C. The other was an inhibitor of cabbage mosaic virus and was not destroyed by 15 minutes at 125°C.

AMV was transmitted from Chenopodium to black cowpeas, using Sanger and Golds (28) liquid-nitrogen transmission technique. These workers had suggested that enzymatic destruction of virus infectivity could be inhibited by low temperature. Liquid-nitrogen transmission increased the infectivity of AMV in Chenopodium seven fold, but the titre was very low.

A modification of Fulton's HCP technique (12, 13) was used to separate AMV from IS present in Chenopodium. He stated that most of the chloroplastic material and cell debris adsorbed to the gel-like HCP and sedimented readily with low speed centrifugation. In his tests, the viruses remained in the nearly water-clear supernatant and retained their infectivity for several days. My results with AMV in Chenopodium were negative after AMV adsorption with HCP. The results cannot be conclusive because only one trial was conducted. The initial virus titre may have been too low and the virus could have been adsorbed along with the IS by HCP.

In previous work (32, p. 554) high speed centrifugation proved effective in separating the IS from the virus particles. These workers suggested that the virus and inhibitor are in a loose

reversible, molecular union. The binding energy of such a virus-inhibitor complex would be extremely weak if broken by simple high speed centrifugation. The results in this study showed that ultra-centrifugation increased virus infectivity seven fold, but still in low titre. Therefore, partial separation of AMV and IS could be accomplished by high speed centrifugation.

TRSV and AMV were separated from IS in Chenopodium with the sucrose density-gradient method of Brakke (8, p. 275). He reported the separation of particles according to their sedimentation rates. It was assumed that the viruses would sediment into different layers of sucrose than the IS. Both TRSV and AMV infectivity was highest in 35 g sucrose and there was no infectivity in the aqueous layers of the gradient columns. Most of the IS remained in the aqueous layers. IS decreased as the sucrose density increased. There was no inhibition in the 35 g concentration of sucrose, and viruses were recovered in high titre.

One layer of 35 g concentration of sucrose was used to separate viruses from IS because the previous experiments demonstrated that the viruses sedimented into this concentration of sucrose during centrifugation. Also the use of one layer would allow a much faster method of obtaining viruses from Chenopodium. It was concluded from the experiments that the IS did not sediment into the 35 g concentration of sucrose while the virus particles of TRSV and AMV sedimented into this concentration of sucrose in high titre.

There was no recovery of TRSV from an old infection in Chenopodium when centrifuged over a layer of a 35 g concentration of sucrose. Probably the virus titre was very low and the virus was lost in the process of purification.

It was reported (4, p. 3-5) that extracts of leaves, stems or roots of strawberry plants contain tannins, and that enough tannin can be released during maceration to precipitate the virus and prevent it from infecting other plants. It was hoped that centrifugation over 35 g sucrose could release the virus from the tannin. There was no recovery of PYBV from young leaves of strawberry when centrifuged over a layer of a 35 g concentration of sucrose. There was some PYBV infectivity from old strawberry leaves layered over 35 g sucrose. It appeared that virus particles could be separated from the tannins in strawberry by this method. Since some infection was obtained in the 35 g sucrose from old strawberry leaves additional tests will be required for a definite answer on the release of PYBV from the tannins in strawberries.

Previous experiments indicated that TRSV and AMV consistently sedimented into sucrose. To test the direct effect of sucrose on IS and virus, infected leaf tissue of Chenopodium was ground in different concentrations of sucrose. No infectivity was observed. Three conclusions could be drawn from the results; (a) direct grinding in sucrose did not separate the IS from the two viruses, (b) sucrose did not inactivate the IS and (c) sucrose did not increase the activity of either virus.

In conclusion it can be said that the most efficient method of separating viruses from IS in Chenopodium was layering extracts over 35 g sucrose followed by centrifugation. The use of one layer allowed a much faster method of obtaining viruses in high titre from Chenopodium.

SUMMARY

1. The infectivity of AMV-infected but noninfectious Chenopodium extract could be restored by dilution of 1:1000, although in low titre.

2. The AMV extract mixed with equal parts of IS was completely noninfectious at all dilutions, even when the virus titre was very high, as indicated by the high number of lesions incited by the water diluted inoculum.

3. Dilution of the TMV and IS mixture resulted in a gradual increase in infectivity. At the 1:1000 dilution, there was a complete dissociation of the virus titre and IS, but the titre was relatively low.

4. IS started inactivating at 60°C when the mixture of AMV and IS was heated. There was no virus activity at temperatures below and above 60°C. IS also were inactivated at 60°C when only IS were heated, cooled and then added to AMV. The inactivation of IS increased as the temperature increased, resulting in some increased infectivity of the virus.

5. TMV and IS were not separated to the same degree as were AMV and IS when heated together in a mixture. There was no virus activity at temperatures below and above 60°C, although the thermal inactivation point of TMV was higher than 90°C. There may be two IS involved. One of the IS started inactivation at 60°C and the second of the IS started activation at 60°C, when the noninfectious mixture of TMV and IS was heated.

6. Liquid nitrogen increased the infectivity of AMV three to seven fold. The final virus titre was quite low.

7. There was no infectivity of TRSV after adsorption with HCP. This was not conclusive because only one trial was conducted.

8. Partial separation of AMV and IS could be accomplished by high speed centrifugation.

9. Both TRSV and AMV infectivity was highest in 35 g sucrose when different concentrations of sucrose were used in a sucrose density-gradient column.

10. Most of the IS remained in the aqueous layers of the gradient columns. IS decreased as the sucrose density increased.

11. Viruses sedimented into one layer of 35 g concentration of sucrose in high titre.

12. IS did not sediment into the 35 g concentration of sucrose.

13. There was no recovery of TRSV from an old infection in Chenopodium when centrifuged over a layer of a 35 g concentration of sucrose.

14. There was no recovery of PYBV from young leaves of strawberry layered over a 35 g concentration of sucrose. There was some PYBV infectivity from old strawberry leaves layered over 35 g sucrose.

15. No infectivity was observed with TRSV and AMV after grinding the infected leaf tissue directly in the different concentrations of sucrose.

16. The most efficient method of separating viruses from IS in Chenopodium was layering extracts over 35 g sucrose followed by centrifugation.

BIBLIOGRAPHY

1. Allard, H.A. The mosaic diseases of Phytolacca decandra. Phytopathology 8:51-54. 1918.

2. Allen, T.C., Jr., and R.P. Kahn. Tobacco mosaic virus inhibition by rice extracts. (Abstract) Phytopathology 47:515. 1957.

3. Bawden, F.C. Inhibitors and plant viruses. Advances in Virus Research 2:31-57. 1954.

4. Bawden, F.C. and A. Kleczkowski. Protein precipitation and virus inactivation by extracts of strawberry plants. Journal of Pomology and Horticultural Science 21:2-7. 1945.

5. Benda, G.T.A. The effect of New Zealand spinach juice on the infection of cowpeas by tobacco ringspot virus. Virology 2:438-454. 1956.

6. Blaszczak, W., A.F. Ross and R.H. Larson. The inhibitory activity of plant juices on the infectivity of potato virus X. Phytopathology 49:784-791. 1959.

7. Bos, L., D.J. Hagedorn and L. Quantz. Suggested procedures for international identification of legume viruses. Tijdschrift over Plantenziekten 66:328-343. 1960.

8. Brakke, M.K. Zonal separations by density-gradient centrifugation. Archives of Biochemistry and Biophysics 45:275-290. 1953.

9. Doolittle, S.P. and M.N. Walker. Further studies on the overwintering and dissemination of cucurbit mosaic. Journal of Agricultural Research 31:1-58. 1925.

10. Duggar, B.M. and J.K. Armstrong. The effect of treating the virus of tobacco mosaic with the juices of various plants. Annals of Missouri Botanical Gardens 12:359-366. 1925.

11. Fulton, R.W. The sensitivity of plant viruses to certain inactivators. Phytopathology 33:674-682. 1943.

12. Fulton, R.W. A rapid method for partial purification of some unstable plant viruses. (Abstract) Phytopathology 47:521. 1957.

13. Fulton, R.W. Purification of sour cherry necrotic ringspot and prune dwarf viruses. Virology 9:522-535. 1959.

14. Gendron, Y. and B. Kassanis. The importance of the host species in determining the action of virus inhibitors. Annals of Applied Biology 41:183-188. 1954.

15. Grant, T.J. The host range and behavior of the ordinary tobacco mosaic virus. Phytopathology 24:311-336. 1934.

16. Gupta, B.M. and W.C. Price. Mechanism of inhibition of plant virus infection by fungal growth products. Phytopathology 42:45-51. 1952.

17. Hollings, M. Chenopodium amaranticolor as a test plant for plant viruses. Plant Pathology 5:57-60. 1956.

18. Jones, L.K. The mosaic disease of beets. Pullman, 1931. p. 3-16. (Washington, Agricultural Experiment Station. Bulleton 250).

19. Johnson, J. and T.J. Grant. The properties of plant viruses from different host species. Phytopathology 22:741-757. 1932.

20. Johnson, J. Chemical inactivation and the reactivation of a plant virus. Phytopathology 31:679. 1941.

21. Kahn, R.P., T.C. Allen, Jr. and W.J. Zaumeyer. Characteristics of plant-virus inhibitors in rice, Oryza sativa. Phytopathology 50:847-851. 1960.

22. Kleczkowski, A. Combination of potato virus X and tobacco mosaic virus with pepsin and trypsin. Biochemical Journal 38:160-167. 1944.

23. Kuntz, J.E. and J.C. Walker. Virus inhibition by extracts of spinach. Phytopathology 37:561. 1947.

24. McKeen, C.D. The inhibitory activity of extract of Capsicum frutescens on plant virus infections. Canadian Journal of Botany 34:891-903. 1956.

25. Milbrath, J.A. An investigation of thermal inactivation of alfalfa mosaic virus. Phytopathology 53:1036-1040. 1963.

26. Ragetli, H.W.J. Onderzoekingen over een Virusremstof voor komend in Dianthus caryophyllus L. Tijdschrift over Plantenziekten 63:245-344. 1957.

27. Robbins, W.W. Mosaic disease of sugar beets. Phytopathology 11:349-365. 1921.

28. Sänger, H. L. and A. H. Gold. Transmission of unstable viruses from liquid-nitrogen-frozen plant tissue. (Abstract) Phytopathology 52:750. 1962.

29. Sill, W. H., Jr. Some characteristics of a virus inhibitor in cucumber. (Abstract) Phytopathology 41:32. 1951.

30. Sill, W. H., Jr. and J. C. Walker. A virus inhibitor in cucumber in relation to mosaic resistance. Phytopathology 42:349-352. 1952.

31. van der Want, J. P. H. Onderzoekingen over Anjermozaiek. Tijdschrift over Plantenziekten 57:72-74. 1951.

32. Weintraub, M. and J. D. Gilpatrick. An inhibitor in a new host of tobacco ringspot virus. Canadian Journal of Botany 30:549-557. 1952.

33. Weintraub, M. and R. S. Willison. Studies on stone fruit viruses in Cucurbit hosts III. The effect of cucurbit extracts on infectivity. Phytopathology 43:328-332. 1953.

34. Zaitlin, M. and A. Siegel. A virus inhibition from tobacco. Phytopathology 53:224-227. 1963.