

INHIBITION OF TOBACCO MOSAIC VIRUS INFECTION
BY PLANT EXTRACTS

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ABSTRACT

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The ability of pepper (Capsicum frutescens), geranium (Pelargonium hortorum), and Datura stramonium extracts to inhibit Tobacco Mosaic Virus (TMV), when applied to leaves in the following ways was studied: mixed with the inoculum, sprayed on lower leaf surfaces before inoculation, and sprayed on upper leaf surfaces after inoculation.

Attempts were made to relate the inhibitory behaviour of each extract to the number of inhibitors present, and to the size of the inhibitor molecules. The three extracts were inhibitory when mixed with the inoculum or sprayed onto the lower leaf surfaces. The pepper and geranium extracts were also inhibitory when applied after inoculation.

The presence of two heat-stable inhibitors in the geranium and pepper, and a heat-labile one in the D. stramonium extract, was demonstrated by the ultrafiltration experiments. Each extract contained an inhibitor with a molecular weight greater than 50,000, and geranium and pepper also contained a second inhibitor with a molecular weight greater than 1,000, but less than 50,000.

The heat-stable, large molecular weight inhibitor from pepper was shown to be a glycoprotein, and is inhibitory when applied to lower leaf surfaces.

The mechanism of inhibition of TMV by large and small molecular weight inhibitors is discussed.

TABLE OF CONTENTS

	<u>PAGE</u>
INTRODUCTION.....	1
LITERATURE REVIEW.....	3
INACTIVATORS.....	3
INHIBITORS.....	6
MATERIALS AND METHODS.....	14
SOURCE OF THE VIRUS AND PREPARATION OF THE INOCULUM.....	14
PREPARATION OF THE INHIBITORY PLANT EXTRACTS...	14
INFECTIVITY ASSAYS.....	15
HEAT TREATMENT OF THE INHIBITOR-CONTAINING PLANT EXTRACTS.....	16
ULTRAFILTRATION OF INHIBITOR-CONTAINING PLANT EXTRACTS.....	17
SEPARATION OF THE INHIBITORY SUBSTANCES IN PEPPER EXTRACT BY ION-EXCHANGE RESINS.....	18
TEST FOR THE PRESENCE OF PROTEIN AND CARBO- HYDRATES.....	18
EFFECT OF ENZYMES ON THE LARGE MOLECULAR WEIGHT INHIBITOR FROM PEPPER.....	19
HYDROLYSIS OF THE LARGE MOLECULAR WEIGHT INHI- BITOR FROM PEPPER.....	20
RESULTS.....	21
INHIBITORY EFFECT OF EXTRACTS APPLIED TO LOWER LEAF SURFACES.....	21
THE EFFECT OF TEMPERATURE.....	24
INHIBITORY EFFECT OF EXTRACTS APPLIED AFTER INOCULATION.....	27
ULTRAFILTRATION OF THE PLANT EXTRACTS.....	27

TABLE OF CONTENTS CONTINUED

	<u>PAGE</u>
EFFECT OF TEMPERATURE ON THE FRACTIONS OF PEPPER AND GERANIUM EXTRACTS SEPARATED BY ULTRAFILTRATION.....	29
PARTIAL PURIFICATION OF THE LARGE MOLECULAR WEIGHT INHIBITOR FROM PEPPER.....	33
CHARACTERIZATION OF THE LARGE MOLECULAR WEIGHT INHIBITOR FROM PEPPER.....	34
Spectrophotometric Analysis.....	35
Test for the Presence of Protein and Carbo- hydrates.....	35
Ethanol Precipitation.....	35
Effect of Enzymes on the Activity of the Inhibitor.....	35
Hydrolysis.....	36
DISCUSSION.....	37
SUMMARY.....	45
BIBLIOGRAPHY.....	47

LIST OF TABLES

<u>TABLE</u>		<u>PAGE</u>
1	Inhibition of TMV Infection by Extracts from <u>P. hortorum</u> , <u>D. stramonium</u> and <u>T. roseum</u> , Applied to the Lower Surfaces of Primary Opposite Leaves of <u>Phaseolus vulgaris</u> Var. Pinto Plants.....	23
2	Effect of Temperature on the Inhibitory Activity of <u>P. hortorum</u> , <u>C. frutescens</u> , and <u>D. stramonium</u> Extracts, Applied with TMV to the Upper Surface of Pinto Bean Leaves..	25
3	Effect of Temperature on the Inhibitory Activity of <u>P. hortorum</u> , <u>C. frutescens</u> , and <u>D. stramonium</u> Extracts when Applied Twice to the Lower Surfaces of Pinto Bean Leaves Before Inoculation.....	26
4	Inhibition of TMV Infection by Heated and Non-heated Extracts of <u>P. hortorum</u> and <u>C. frutescens</u> , Applied to the Upper Surfaces of Pinto Bean Leaves after Inoculation with TMV.....	28
5	Inhibition of TMV Infection by Extracts of <u>P. hortorum</u> , <u>C. frutescens</u> , and <u>D. stramonium</u> after Ultrafiltration in a Diaflo Cell with XM-50 Membrane.....	30
6	Inhibition of TMV Infection by Extracts from <u>P. hortorum</u> and <u>C. frutescens</u> , Applied with TMV to the Upper Surfaces of Pinto Bean Leaves, After Ultrafiltration in a Diaflo Cell with UM-2 Membrane.....	31
7	Effect of Temperature on the Inhibitory Activity of the Fractions from Extracts of <u>P. hortorum</u> , and <u>C. frutescens</u> after Ultrafiltration in a Diaflo Cell with XM-50 Membrane.....	32

INTRODUCTION

It is now well recognized that the ability to transmit viruses that are potentially transmissible by inoculating healthy plants with sap from infected ones is greatly affected by substances present in the inoculum. Certain plants contain tannins or oxidizable phenolic compounds which rapidly inactivate some viruses, but many more contain substances that inhibit infection although they are not inactivators.

The nature of most inhibitors remains unresolved and there is still much controversy on the ways in which inhibitors reduce infection. The few inhibitors that have been fully characterized vary chemically from large molecules such as proteins (24,27,33), and polysaccharides (4), to substances of small molecular weight (4,25). There are indications that the large molecular weight substances act on the first stage of the infection process, which is thought to be the absorption or penetration of the virus into the host cell (4,27,32). Small molecular weight substances could act on the first stage as well as on the later stages of the infection process, since they are capable of diffusing into the leaf and may reach the sites at which the virus multiplies in the cell (4,21).

However, plant extracts reported to contain only a large molecular weight inhibitor are capable of reducing

infection when applied to lower leaf surfaces, and after inoculation of upper leaf surfaces (27). This might be explained by the fact that a second, small molecular weight inhibitor is present in the extracts, but was not detected, or that the large molecular weight inhibitors are capable of inhibiting infection because of their effects on the metabolism of the cells (4,27).

In this study, the inhibitory behaviour of three plant extracts was compared and related to the number of inhibitors present in each extract and to the size of the inhibitor molecules. Attempts were also made to resolve the nature of the macromolecular inhibitor in the pepper extract.

LITERATURE REVIEW

Since Duggar and Armstrong (9), reported that juice from Phytolacca decandra has virus-inhibiting properties, extracts from many higher plants (3,5,7,14,23,24,25,27, 36,35,41) as well as from other organisms such as fungi and bacteria (4,6,18) have been found to interfere with virus infection. It has also been shown that the extracts exert their effect in one of two ways: by inactivating virus particles, or by altering the resistance of the host. The former are referred to as inactivators, and the latter as inhibitors. Inhibitors are more prevalent in plant extracts than inactivators, and are quite varied chemically (24,25,27), while inactivators have been found to be mainly tannins and phenolic compounds (19,30,35).

INACTIVATORS

In 1954, Bawdwen and Kassanis (3), showed that enough tannin is liberated during the maceration of strawberry leaves to precipitate all the plant protein, and that the supernatant liquid still contains about 1% tannin, enough to precipitate Tobacco Mosaic Virus (TMV) and prevent it from infecting Nicotiana glutinosa. However, inhibition can be reversed by dilution (40), and precipitation was not considered sufficient evidence to show that the tannin acted on the virus and not on the host. This was shown conclusively by Cadman (7), who found that the degree to

which virus infection is inhibited by tanning substances from raspberry depends on the virus and not on the species of test plants. The infectivity of some viruses was restored by dilution but for others inhibition was irreversible. The level of inhibition with a given virus was constant for many species of test plants. Further evidence was supplied by the fact that inactivation increases with increase in time of incubation of the raspberry tannin-virus mixtures.

Phenolic compounds are apparently innocuous in the reduced state, but oxidize readily when tissue extracts are exposed to air, and become highly inhibitory. Hampton and Fulton (19), showed that inhibition of unstable *Prunus* viruses was due to the virus inactivating action of the oxidized phenolic compounds, and that the loss of infectivity was irreversible. TMV and Potato Virus X were not sensitive or required much higher amounts for their inactivation.

Mink (28), obtained similar results with Tulare Apple Mosaic Virus (TAMV). Exposure of TAMV to substituted o-quinones caused in each case immediate and total inactivation, while the catecholic equivalents had no effect.

On the other hand Pierpoint and Harrison (30), found that Cucumber Mosaic Virus (CMV) was inactivated by tobacco leaf polyphenols only when they were being oxidized. Oxidized polyphenols from virus free leaves did not inactivate CMV when added together with copper, whereas depro-

teinized extracts from leaves crushed in an atmosphere of nitrogen did. Five relatively stable viruses were also tested and only an isolate of Tobacco Necrosis Virus (TNV) was slightly inactivated by the system.

Francki (14), also reported that the infectivity of CMV was slightly reduced by exposure to leaf extracts of cucumber or Nicotiana glutinosa plants. The nature of the inactivator is unknown, but it is partially destroyed by heating at 100°C, non-dialyzable, and removed from leaf extracts by phenol extraction. He suggested that its mode of action involves some form of aggregation of CMV, or the association of some host plant materials with the virus particles.

Another inactivator of CMV was found in sugar beet sap by Rupel (35). Inactivation was suggested by the following facts: (1) Increased infection in the same species from which phenol extracts were made. (2) Failure of dilution to restore infectivity of concentrated plant extracts. (3) Reduced infectivity of the phenol-extract sap mixtures with incubation, and (4) the ratio between sap and virus. The identity of the inactivator is unknown but it differs from the one reported by Francki (14), in that it was completely dialyzed after 48 hours. Inactivation did not seem to be due to oxidation products since infectious extracts could not be obtained by use of reducing

agents in the extracting buffer. The inactivator was apparently specific for CMV, as it was not effective against three other viruses.

The results of these investigations conclusively show that tannins contained in extracts of rosaceous plants, and polyphenols present in many plant species are capable of inactivating viruses. Their effect appears to be specific for given viruses and in the case of polyphenols only unstable viruses are inactivated.

INHIBITORS

Kuntz and Walker (25), showed that extracts of spinach, garden beet, sugar beet, and chard when mixed with TMV and Cabbage Mosaic Virus, almost completely inhibited their infectivity. The spinach extract inhibited four other viruses and its action was instantaneous and did not increase with time. They also found that two inhibitory substances occurred in spinach extracts. The first was inhibitory to TMV, destroyed by heating at 65°C for 12 minutes, non-dialyzable, precipitated by 50% alcohol, and was thought to be proteinaceous in nature. The second was not inhibitory to TMV but definitely so to Cabbage Mosaic Virus. It was thermostable at 125°C for 15 minutes, dialyzable and not precipitated by 50% alcohol. The exact nature of the inhibitors was not determined. The infectivity of a non-infectious preparation of the former inhibitor could be

restored by dilution, by heating at 70°C, or by filtering through activated charcoal, whereas the action of the second inhibitor was irreversible. The author believed that both inhibitors rendered the virus non-infective, and that the inhibitory effect was not due to modifications of the host plant. Their data however seem to indicate that only the second inhibitor acted on the virus, and that the first acted on the host.

Kassanis and Kleczkowski (24), successfully isolated and purified the inhibitory substance in Phytolacca esculenta. It contained 14-15% nitrogen and they concluded that it was probably a glycoprotein. They showed that the virus and the inhibitor could combine, however they believed that this was not essential for inhibition to occur, and suggested on the basis of the following evidence, that the inhibitor probably affects the host: (1) The substances that can combine with, and precipitate TMV, are not all equally active as inhibitors, and some such as clupein and globin have only little neutralizing power. (2) If inhibition was dependent upon, and a consequence of combination with the virus it is to be expected that for complete neutralization of infectivity, a given weight of virus would need to combine with a certain minimum quantity of inhibitor. However there is no evidence for such a neutralizing ratio. (3) The number of lesions produced is reduced if the leaves are

inoculated first with the inhibitor alone.

Gupta and Price (17), tested the filtrates of 49 species of fungi and found that 84% could inhibit infection, but less than 25% reduced infectivity as much as 80%. Further work with Trichothecium roseum Link. which caused 90% reduction in infectivity showed that the agent in the extract caused immediate loss of infectivity when mixed with a virus solution, that it was thermostable, non-dialyzable, and lyophilizable.

Bawden and Freeman (4), indicated that the extent to which infection is inhibited by T. roseum extract depends on the species of the host plant, but not on the identity of the virus. This was confirmed by Gendron and Kassanis (16), who concluded from their studies with various inhibitor, virus and host combinations, that the inhibitors are largely ineffective in preventing infection of the species which contain them, or of related species. This proved to be the most important criteria as well as the easiest to use to distinguish between inactivators and inhibitors, and in cases where it cannot be applied, as for fungi or plants that are not hosts of common viruses, all the tests mentioned previously must be conducted before a decision can be reached.

After it was learned that T. roseum extract was inhibitory when applied before or up to 30 minutes after

inoculation with the virus (17), attempts were made to determine whether a given inhibitor interferes with the first or subsequent stages of the infection process, or both. The first stage of the infection process is thought to be the absorption or penetration of the virus into the host cell, and the subsequent stages virus multiplication or movement of virus from infected cells to adjoining healthy cells.

Bawden and Freeman (4), were able to separate two inhibitory substances in the extracts of T. roseum. One is trichothecin, an antifungal substance with the molecular formula $C_{19}H_{24}O_5$, and the other a polysaccharide containing 1.1 - 1.4% nitrogen, and its predominant component sugar is D-galactose. The polysaccharide inhibits infection when sprayed over leaves before inoculation but not after, whereas trichothecin inhibits when applied to leaves a day after they have been inoculated with viruses, but is ineffective when applied two days before. The authors suggest that the differences in the behaviour of the two inhibitors are more likely attributable simply to differences in their size. The small molecules of trichothecin can presumably penetrate uninjured leaves and diffuse readily from cell to cell, whereas the non-dialyzable polysaccharide, like a virus, needs a wound through which to enter. Since leaves recover sufficiently from their injuries to prevent infection.

when they are sprayed with virus solutions within one hour after being rubbed with water (24), this probably explains the short period after inoculation during which spraying with the polysaccharide inhibits infection.

Because of the behaviour of trichothecin the authors state that there is no reason to assume that inhibitors are substances that interfere only with the first stage of infection, and they suggest that it is more reasonable to postulate that inhibitors act by temporarily altering the metabolism of leaf cells, so that introduced virus particles cannot multiply.

Two small molecular weight substances which readily diffuse into leaf cells, and are capable of inhibiting virus synthesis have been extensively studied: 2-thiouracil (11,12,29), and 8 azaguanine (26).

The effect of these substances vary with different viruses, but both are incorporated into the nucleic acid of TMV, when sprayed on infected leaves, replacing guanine and uracil (26). This would appear to provide an explanation for their inhibitory effect. However, Francki and Mathews (13), showed that thiouracil is effective in inhibiting the increase of Turnip Yellow Mosaic Virus and of TMV, but in both cases it is not incorporated in their nucleic acids.

It has also been shown that thiouracil affects the

synthesis of proteins in leaf tissues, causing an accumulation of free-amino, and -amide nitrogen (31). Thus, although these nucleic acid analogues have been extensively studied, their mode of action is not completely understood. There is as yet no evidence that they bear any relationship to the small molecular weight inhibitory substances in plant and fungal extracts.

Another small molecular weight antifungal substance, Blasticidin S, was found to inhibit TMV multiplication when applied one hour after inoculation (21).

Pepper juice was reported by Mokeen (27), to inhibit the production of local lesions by CMV when applied with the virus inoculum, or to the lower or upper surfaces of cowpea plants before inoculation. Application of the juice 1 or 2 hours after inoculation did not decrease the production of lesions. The inhibitory component was partially characterized and found to be thermolabile, to resist aging and drying in vitro, and did not pass through a cellophane membrane. He concluded that the effect of the inhibitory agent is to prevent infection by, rather than increase of, the virus. To explain the fact that the inhibitor is effective when applied to lower leaf surfaces although it is a large molecular weight substance with the characteristics of a protein, and presumably not able to pass through a protoplasmic membrane, the author suggested

among other things, that the protein may affect at the epidermal surface, a type of chain reaction which alters the cell metabolism, so that protein synthesis is diverted from virus formation. Since the water used for dialysis was not tested for inhibitory activity another possibility might be that a second, small molecular weight inhibitor is present in the pepper juice.

Rice extracts inhibited TMV infection when applied to lower leaf surfaces of Pinto bean leaves, one half to one hour before inoculation and up to 24 hours after inoculation (23). Although the results of thermal inactivation studies suggested the presence of two inhibitors, one labile above 60°C, and another stable at 100°C, no attempts were made to determine whether one or both were capable of acting when applied to lower leaf surfaces or after inoculation.

An extract from flowers of red clover inhibited Red Clover Vein Mosaic Virus when mixed with the inoculum, but not when applied to leaves after they were inoculated with the virus (10). Part of the inhibition was believed to be due to glucose, galactose, and xylose present in the extract. However, since heating at temperatures, that would have no effect on sugar, partially destroyed the inhibitory property of the extract, inhibitors other than sugars were thought to be present. Lipid but not protein was present in the extract but its effect was not investigated. The

authors concluded that the extract alters the physiology of the host cells, so that they were no longer as susceptible to the virus.

Van Kammen et al (41), studied the mechanism of inhibition of TMV by an inhibitor from carnation sap, which had been previously found to be a protein, to have no direct effect on the virus, and to be capable of inhibiting when applied up to 30 minutes after inoculation with the virus (32). They described the action of the inhibitor as blocking virus receptors on the leaf surface. The inhibitor as well as the virus, would be able to reversibly bind to the receptor, but the inhibitor would combine with the receptor to give an inactive complex.

Further evidence for this hypothesis was supplied by Ragetli (32,33), who studied the mode of action of the purified proteinaceous inhibitor from carnation, and found that free amino groups, probably the ϵ -groups of lysine were responsible for the biological activity of the molecule. They postulated that the inhibitor competed via its ϵ -amino groups with possibly similar groups in complete TMV, and certain amino groups in TMV-RNA for essential sites of the host. These sites might be of common importance to both virus and infectious RNA in the early phases of virus establishment.

MATERIALS AND METHODS

SOURCE OF THE VIRUS AND PREPARATION OF THE INOCULUM

Tobacco Mosaic Virus (TMV) type strain was maintained on systemically infected plants of Nicotiana tabacum L. var. 402 in the greenhouse. Infected leaves were macerated in a mortar with distilled water (1:10 w/v). The juice was expressed through 4 layers of cheesecloth, centrifuged at 8,000 rpm for 15 minutes, and the supernatant stored in 20 ml test tubes at approximately 4°C. A new 1:10 (w/v) virus solution was prepared every three months.

Inoculum was prepared just before each experiment by diluting the stored juice with sufficient distilled water to obtain countable numbers of local lesions (50-200 per leaf).

PREPARATION OF THE INHIBITORY PLANT EXTRACTS

Extracts of geranium Pelargonium hortorum variety Red Wing, Datura stramonium, and pepper Capsicum frutescens variety California Wonder were prepared from plants grown in the greenhouse. Leaves and young stems of well-developed plants were ground in a meat grinder and strained through 4 layers of cheesecloth. The liquid was then centrifuged at 3,000 rpm for 20 minutes, and the supernatant stored in 20 ml test tubes, at approximately 4°C. After a week of storage the extracts were filtered through Whatman No.3

filter paper to eliminate a slight precipitate which had formed, and stored at 4°C. In some cases a second centrifugation was done instead of this filtration.

Extracts of Trichothecium roseum Link. were prepared from fungus cultures grown on a corn meal culture medium. Three weeks after inoculation the mycelial mat was ground in a mortar, mixed with the liquid of the medium, and strained through 4 layers of cheesecloth. The solution was then centrifuged at 3,000 rpm for 20 minutes, and the supernatant stored in 20 ml test tubes at 4°C. After approximately three weeks a flocculate material had formed, and it was removed by filtering the extract through filter paper No.1.

INFECTIVITY ASSAYS

The quantitative local lesion assay method with TMV on bean plants was used to measure infectivity of the various virus-inhibitor preparations.

To study the mode of action of the inhibitor-containing plant extracts the following methods of inoculation were used: (1) Inoculation of the upper leaf surfaces with a mixture of equal volumes of inhibitor and virus. This method was used throughout the study unless otherwise stipulated. (2) Spraying the inhibitor or water on the underside of the leaves with a manual atomizer, 4 and 2 hours before inoculating the upper surface with the virus, and (3) Spraying

the inhibitor or water once or twice on the upper leaf surface one and two hours after inoculation with the virus.

Every treatment was given to 16 primary leaves of one set of Phaseolus vulgaris variety Pinto UI 111 bean plants, and the respective control was given to a second set of plants. All the experiments were done twice. The plants were grown in the greenhouse and were used at 10 or 11 days after seeding. This proved to be the best time for inoculation under our conditions, since their susceptibility was greater as shown by preliminary tests.

The primary leaves were dusted with Silicon Carbide (600 mesh), and inoculated with a pipe cleaner imbibed with inoculum. Every section of the leaves was rubbed four times from the midrib to the edges. After inoculation the plants were kept in the greenhouse at 30°C (\pm 3°C). The symptoms started to appear 2 to 3 days after inoculation, but the lesions were counted on the 5th or the 6th day, when they were completely developed and counting was easier.

HEAT TREATMENT OF THE INHIBITOR-CONTAINING PLANT EXTRACTS

Ten ml samples of each inhibitor in test tubes were boiled for 10 minutes in a water bath, cooled immediately under running tap water and assayed. Fractions resulting from ultrafiltration of P. hortorum and C. frutescens extracts were treated similarly.

ULTRAFILTRATION OF INHIBITOR-CONTAINING PLANT EXTRACTS

Ultrafiltration with Diaflo membranes (Amicon Corporation, Lexington, Mass.), provides a rapid technique for separating small from large molecules, and for concentrating dilute aqueous solutions.

Plant extracts prepared as described previously contained slight amounts of chlorophyll and oxidation products which could stain the gel membranes. To obtain colorless solutions the ground plant tissue from the meat grinder was received in solutions of reducing agents. Capsicum frutescens extract was prepared in a 1:5 (w/v) ratio with a 0.01 M solution of sodium diethyldithiocarbamate (DIECA), and with 0.01 M solution of thioglycolic acid. The second solution gave a translucent almost colorless extract which was used in ultrafiltration. Datura stramonium extract was similarly prepared in a 1:1 (v/v) ratio with thioglycolic acid 0.01 M. Pepper and datura extracts were fully inhibitory to TMV infection when assayed on the upper leaf surfaces, thus indicating that the reducing agents did not affect their inhibitory capacity. Pelargonium hortorum extract was prepared without antioxidants since it does not oxidize rapidly.

Each of the three extracts was filtered in the Diaflo cell model 50 with the UM-2 and XM-50 membranes, which retain solutes with molecular weights larger than 1,000 and

50,000 respectively. To ensure that all substances capable of diffusing through each membrane were removed, two 5 ml aliquots of distilled water were added to the cell when the volume of the retentate had reached two ml. The aliquots used for washing were not added to the filtrate. For the infectivity assays the retentates were restored to their original volume and the filtrates were used as such.

SEPARATION OF THE INHIBITORY SUBSTANCES IN PEPPER EXTRACT BY ION-EXCHANGE RESINS

The pepper extract was prepared with thioglycolic acid (1:5 w/v), as described previously for ultrafiltration. Thirty ml aliquots of the clear extract were passed through a 9 cm x 1 cm column of Dowex 50-X8 (H+) 200 to 400 mesh, and a column of Dowex 1X-10 (Cl) 200 to 400 mesh of similar volume, which was connected to the lower portion of the cation column. The columns were eluted with 40 ml of distilled water, and then separated. The effluent was flash evaporated to the original volume.

The cation column was eluted with 50 ml 2N HCl, and the anion column with 40 ml 4N formic acid, and 30 ml 8N formic acid. Both fractions were flash evaporated to dryness and the residues dissolved in 30 ml distilled water.

TEST FOR THE PRESENCE OF PROTEIN AND CARBOHYDRATES

The presence of protein and carbohydrates was determined

by the Folin-Ciocalteu Test (37), and the Phenol-Sulfuric acid Test (38), respectively. In the protein test the absorption was read in a PM Q II Spectrophotometer at 670 m μ . The OD value obtained was compared to a standard curve of γ -globulin (bovine) Fraction II, B grade (Calbiochem.), to get an approximate quantitative estimation of the protein present.

EFFECT OF ENZYMES ON THE LARGE MOLECULAR WEIGHT INHIBITOR FROM PEPPER

The action of the enzymes papain (Mann. Research Laboratories), α -amylase (Nutritional Biochemical Corporation), and phosphodiesterase (Snake Venom, Sigma Chem.Co.) on the activity of the inhibitor was studied.

The following enzyme preparations were added to 1 ml of inhibitor solution: (a) 0.5 ml of α -amylase (0.1 mg/ml in 0.02 M, pH 7.0 phosphate buffer; (b) 1 ml of papain (3 mg/ml in 0.01 M pH 7.0 Versene (EDTA) buffer, containing 0.0005 M Cysteine HCl); and (c) 1 ml of phosphodiesterase (20 mg/ml) in 0.04 M, pH 9.4 Tris buffer, and 0.004 M MgCl₂). The mixtures of inhibitor with α -amylase and papain were incubated 24 hours at room temperature (25°C), and that with phosphodiesterase 6 hours under similar conditions. After incubation all the enzyme-inhibitor mixtures were boiled for 10 minutes in a water bath to destroy the enzymes, cooled under running tap water and tested for inhibition

of TMV infection.

HYDROLYSIS OF THE LARGE MOLECULAR WEIGHT INHIBITOR
FROM PEPPER

The method for hydrolysis of glycoproteins described by Spiro (38) was followed to hydrolyze the inhibitor. Four ml of a fivefold concentrated inhibitor solution were made 0.05 N by adding 1N H_2SO_4 , hydrolyzed in a water bath at 80°C for an hour, neutralized with $BaCO_3$, and the precipitate separated by low speed centrifugation. The supernatant was passed through a column of Dowex 1-X10 (Cl), the effluent flash evaporated, and diluted to its original volume with distilled water. The column was eluted with 0.3 N formic acid, the effluent flash evaporated to dryness, and the residue dissolved in a volume of distilled water equal to that of the original solution. Both fractions were then tested for inhibition of TMV infection.

RESULTS

The present study was undertaken to compare the ability of pepper, geranium, and D. stramonium extracts to inhibit TMV when applied to leaves in the following ways: mixed with the inoculum, sprayed on lower leaf surfaces before inoculation, and sprayed on upper leaf surfaces after inoculation. Attempts were then made to relate the inhibitory behaviour of the extracts to the number and the molecular weight of the inhibitors present in each, as determined by temperature and ultrafiltration studies.

The results of preliminary experiments with extracts from geranium, pepper, and D. stramonium confirmed published reports (5,24,27,36), that they contain inhibitors of virus infection. The number of local lesions induced on the primary leaves of Pinto beans was reduced 99-100 per cent when each extract was mixed with equal volumes of TMV inoculum.

INHIBITORY EFFECT OF EXTRACTS APPLIED TO LOWER LEAF SURFACES

Since it has been previously reported that inhibitors applied in this fashion are able to inhibit virus development on the leaf opposite to the one receiving the inhibitor (27), two series of 16 plants were used, one for the treatment and another for the control. Each plant extract or distilled water was applied to one lower leaf surface

of each plant, and both upper surfaces were inoculated with TMV.

The extract of T. roseum was also included for comparison, because it has been reported to inhibit when applied to lower leaf surfaces (17). In preliminary experiments a 1:5 (v/v) solution of the extract reduced to about 50% the number of TMV local lesions per leaf induced in Pinto bean, but was slightly phytotoxic. However, two applications of a 1:10 (v/v) solution were not phytotoxic. In similar experiments plant extracts sprayed once to lower leaf surfaces caused little or no inhibition, whereas extracts sprayed twice were inhibitory, so that two applications were used in these experiments.

The results presented in Table 1 show that the three extracts were equally effective in inhibiting the development of local lesions on the leaf that received the inhibitor as well as on the opposite leaf. The degree of inhibition on the leaves that received the inhibitor was greater than on the opposite leaves, but less than when each extract was applied mixed with the inoculum, as this latter method causes 99-100% inhibition.

Because of the inhibitory effect on the opposite leaves, two series of plants were used in subsequent experiments instead of having the treatment and the control on

TABLE 1

Inhibition of TMV Infection by Extracts from P. hortorum
D. stramonium and T. roseum, Applied to the Lower Sur-
 faces of Primary Opposite Leaves of Phaseolus vulgaris
 Var. Pinto Plants.

Source of Extract	Experiment No.	Average No. of Local Lesions/Leaf (a)						Inhibition as % of Controls (1) (2)	
		Treatments (b)		Controls (b)					
		(1)	(2)	(1)	(2)	(1)	(2)	(1)	(2)
		Inhibitor	Virus	Nothing	Water	Virus	Nothing		
<u>P. hortorum</u>	1	6.6	11.9	21.6	20.8	69.5	42.8		
	2	15.9	18.4	43.2	42.1	63.2	56.3		
<u>D. stramonium</u>	3	5.3	10.3	15.8	15.9	66.5	35.3		
	4	4.1	8.0	17.5	16.8	76.6	52.4		
<u>T. roseum</u>	5	5.4	7.6	16.7	19.0	67.7	60.0		

(a) Each value is the average for 16 replications.
 (b) Treatments and controls were done on separate series of plants.

opposite leaves of the same plant.

THE EFFECT OF TEMPERATURE

The fact that the plant extracts are capable of inhibiting the development of local lesions when applied with the virus or sprayed on lower leaf surfaces before inoculation, might be due to the presence of more than one inhibitory substance in the extracts, as has been reported for T. roseum (4).

Since the presence of two inhibitors in the same plant extract has been demonstrated by heating the extract at increasing temperatures (23,25), extracts of geranium, pepper, and D. stramonium were boiled for ten minutes to determine the stability of the inhibitor(s), and whether one could be selectively destroyed. The activity of boiled and unboiled extracts was compared by mixing them with the inoculum, and by spraying the lower leaf surfaces.

When assayed in a mixture with the virus, boiled geranium and pepper extracts retained their complete activity, while that of D. stramonium was completely lost, (Table 2). Geranium, and pepper extracts also retained their activity when assayed by the lower leaf surface method, but that of D. stramonium extract was only reduced by about 30%, (Table 3). In the case of pepper and geranium the results are in agreement with that obtained by Blaszcak et al (5), who found that both extracts contain heat-stable inhibitors

TABLE 2

Effect of Temperature on the Inhibitory Activity of P. hortorum,
C. frutescens, and D. stramonium Extracts, Applied with TMV to
 the Upper Surfaces of Pinto Bean Leaves (a).

Source of Extract	Experiment No.	Treatment	Average No. of Local Lesions/Leaf		Inhibition as % of Control
			Virus and Inhibitor	Virus and Water	
<u>P. hortorum</u>	1	Unboiled	0.18	104.0	99.8
	2	Boiled	0.62	48.3	98.8
	3	Boiled	0.50	77.3	99.3
<u>C. frutescens</u>	4	Unboiled	0.00	26.4	100.0
	5	Boiled	0.18	42.2	99.5
	6	Boiled	0.12	31.0	99.6
<u>D. stramonium</u>	7	Unboiled	0.12	37.4	99.6
	8	Boiled	68.5	58.4	0.0
	9	Boiled	87.3	68.8	0.0

(a) Inhibitors and water were applied on separate series of plants.

(b) Each value is the average for 16 replications.

TABLE 3.

Effect of Temperature on the Inhibitory Activity of P. hortorum,
C. frutescens, and D. stramonium Extracts when Applied Twice to
the Lower Surfaces of Pinto Bean Leaves Before Inoculation (a).

Source of Extract	Experiment No.	Treatment	Average No. of Local Lesions/Leaf (b)			Inhibition as % of Control
			Inhibitor	Virus	Virus Water	
<u>P. hortorum</u>	1	Unboiled	11.3		42.6	73.5
	2	Boiled	20.8		100.1	79.3
	3	Boiled	19.9		95.3	79.2
<u>C. frutescens</u>	4	Unboiled	3.9		36.9	89.5
	5	Boiled	15.5		79.4	80.5
	6	Boiled	2.9		19.4	85.1
<u>D. stramonium</u>	7	Unboiled	69.4		183.5	62.2
	8	Boiled	75.1		113.5	33.9
	9	Boiled	49.5		91.8	46.1

(a) Inhibitors and water were applied on separate series of plants.

(b) Each value is the average for 16 replications.

of Potato Virus X infection. On the other hand, McKeen (27), reported that the single inhibitor of Cucumber Mosaic Virus in pepper extract was heat-labile.

INHIBITORY EFFECT OF EXTRACTS APPLIED AFTER INOCULATION

Preliminary experiments with geranium indicated that the unboiled extract had little effect on virus development when applied one hour after inoculation. Thus, for these experiments single and double applications were made.

A single application of boiled and unboiled geranium extract did not inhibit infection, while single applications of boiled and unboiled pepper extract caused 24.9 and 49.8 per cent inhibition respectively (Table 4). In contrast with the results of previous experiments, where boiling had no effect, the activity of the boiled geranium and pepper inhibitors was reduced by about $1/3$ to $1/2$ when double applications of the extracts were made.

ULTRAFILTRATION OF THE PLANT EXTRACTS

In an attempt to detect and separate the inhibitor(s) in each extract on the basis of possible differences in their molecular weights, extracts of geranium, pepper and D. stramonium prepared as described in the Material and Methods section, were filtered through a Diaflo ultrafiltration cell with the XM-50 and UM-2 membranes. The filtrates and retentates of each extract were adjusted to their

TABLE 4

Inhibition of TMV Infection by Boiled and Unboiled Extracts of P. hortorum and C. frutescens, Applied to the Upper Surfaces of Pinto Bean Leaves after Inoculation with TMV (a).

Source of Extract	Time of Application After Inoculation	Average No. of Local Lesions/Leaf		Inhibition as % of Control			
		Boiled		Unboiled			
		Treat.	Cont.	Treat.	Cont.		
<u>P. hortorum</u>	1 hour	202.5	188.5	176.0	188.5	0.0	6.7
	1 & 2 hours	30.4	47.0	15.4	47.0	36.0	67.3
	1 & 2 hours	71.6	107.0	53.2	107.0	33.1	50.3
<u>C. frutescens</u>	1 hour	29.9	39.9	44.0	87.6	24.9	49.8
	1 & 2 hours	13.8	25.8	10.1	25.8	46.6	60.9
	1 & 2 hours	45.8	94.5	44.8	94.5	51.6	52.6

(a) Treatment and controls were done on separate series of plants.
 (b) Each value is the average for 16 replications.

original volume, and assayed for inhibitory activity. The results presented in Table 5 indicate that the geranium and pepper extracts each contain two inhibitory substances: one which was retained by the membrane and is larger than 50,000 molecular weight, and a second which is smaller than 50,000 molecular weight and was present in the filtrate. The molecular weight of the inhibitory substance(s) in D. stramonium extract was greater than 50,000.

The inhibitory substances in geranium and pepper were retained by the UM-2 membrane indicating that the molecular weight of the smaller of the two substances is greater than 1,000 (Table 6).

EFFECT OF TEMPERATURE ON THE FRACTIONS OF PEPPER AND GERANIUM EXTRACTS SEPARATED BY ULTRAFILTRATION

Since the heat-stability of pepper and geranium extracts might be due to only one of the two inhibitory substances present, the effect of temperature was determined on both fractions of each extract. The results in Table 7 indicate that all the fractions retained their full inhibitory activity after boiling. The fact that the large molecular weight inhibitor in the pepper and geranium extracts is heat-stable suggests that it might be a polysaccharide or a glycoprotein.

TABLE 5

Inhibition of TMV Infection by Extracts of P. hortorum, C. frutescens,
and D. stramonium after Ultrafiltration in a Diaflo Cell
with XM-50 Membrane. (a)

Source of Extract	Experiment No.	Average No. of Local Lesions/Leaf (b)				Inhibition as % of Control.	
		Filtrate	Virus and Water	Retentate	Virus and Water	Filt.	Retent.
<u>P. hortorum</u>	1	16.0	60.8	2.50	118.1	73.7	97.9
	2	7.8	47.7	0.00	44.5	83.3	100.0
<u>C. frutescens</u>	3	20.6	109.4	1.12	239.6	81.2	99.5
	4	17.1	100.8	1.00	185.8	83.1	99.4
<u>D. stramonium</u>	5	108.6	102.0	--	--	0.0	--
	6	86.3	106.0	--	--	16.9	--

(a) Filtrates, retentates, and water were applied on separate series
of plants.

(b) Each value is the average for 16 replications.

TABLE 6

Inhibition of TMV Infection by Extracts from P. hortorum and C. frutescens,
 Applied with TMV to the Upper Surfaces of Pinto Bean Leaves, After
 Ultrafiltration in a Diaflo Cell with UM-2 Membrane. (a)

Source of Extract	Experiment No.	Average No. of Local Lesions/Leaf (b)		Inhibition as % of Control	
		Virus and Filtrate	Virus and Retentate	Filtrate	Retentate
<u>P. hortorum</u>	1	129.1	0.37	135.3	4.6
	2	171.0	0.18	134.5	0.0
	3	313.3	0.18	276.0	0.0
	4	188.1	0.43	105.0	0.0
<u>C. frutescens</u>	1	129.1	0.37	135.3	4.6
	2	171.0	0.18	134.5	0.0
	3	313.3	0.18	276.0	0.0
	4	188.1	0.43	105.0	0.0

(a) Filtrates, retentates, and water were applied on separate series of plants.

(b) Each value is the average for 16 replications.

TABLE 7

Effect of Temperature on the Inhibitory Activity of the Fractions from
Extracts of P. hortorum, and C. frutescens after Ultrafiltration
in a Diaflo Cell with XM-50 Membrane. (a)

Source of Extract	Experiment No.	Average No. of Local Lesions/Leaf				Inhibition as	
		Virus and Filtrate		Virus and Water		% of Control	
				Retentate	Water	Filt.	Retent.
(c)							
<u>P. hortorum</u>	1	21.4	182.3	1.18	87.5	88.3	98.7
<u>C. frutescens</u>	2	29.3	182.3	15.4	87.5	84.0	82.4

(a) Filtrates, retentates, and water were applied on separate series of plants.

(b) Each value is the average for 16 replications.

(c) Filtrates and Retentates were boiled for 10 minutes in a water bath.

PARTIAL PURIFICATION OF THE LARGE MOLECULAR WEIGHT
INHIBITOR FROM PEPPER

The fraction retained after ultrafiltration of the pepper extract with the XM-50 membrane contained other large molecular weight substances as well as one of the inhibitors. To obtain a preparation of the inhibitor which would be suitable for characterization studies, 30 ml aliquots of the pepper extract were fractionated on Dowex ion exchange resin columns. The cationic and anionic fractions were flash evaporated to dryness and the residues dissolved in 30 ml of distilled water, while the neutral fraction was flash evaporated to a volume of 30 ml.

To locate the inhibitors, the cationic and anionic fractions were assayed for inhibitory activity as such. However, since the neutral fraction was assumed to contain the heat-stable, large molecular weight inhibitor, as well as monosaccharides, the former was separated by filtration in the Diaflo cell with XM-50 membrane, before performing the assay. The final volume of the retentate was adjusted to give a tenfold concentration of the inhibitor for later use in the characterization studies.

In two experiments, inhibition as per cent of control was 98.4 and 96.2%, when the retentate was added to the inoculum, and 0.0 and 16.5% for the filtrate, strengthening the assumption that the large molecular weight inhibitor.

is a polysaccharide or a glycoprotein. The retentate was also assayed by the lower leaf surface method and caused 54.9% inhibition, indicating that the large molecular weight inhibitor by itself is capable of inhibiting when applied to the lower leaf surfaces as well as when it is mixed with the inoculum. The retentate obtained after ultrafiltration with the XM-50 membrane only, was also assayed for comparison, and caused 86.4% inhibition.

The cationic and anionic fractions were both inhibitory, causing 32.4 and 56.0% inhibition respectively. To determine which fraction contained the small molecular weight, heat-stable inhibitor detected by ultrafiltration with XM-50 membrane, both fractions were boiled and assayed. In two experiments, boiling reduced the inhibitory activity of the cationic fraction, as inhibition was 6.0 and 4.8%, but it had no effect on the anionic fraction which still caused 50.6 and 72.8% inhibition. The inhibitor in the anionic fraction, thus corresponds to the heat-stable inhibitor found in the filtrate after ultrafiltration. The significance of the heat-labile inhibitor will be discussed later.

CHARACTERIZATION OF THE LARGE MOLECULAR WEIGHT INHIBITOR FROM PEPPER

A tenfold concentration of the purified inhibitor was used in the following tests:

1. Spectrophotometric Analysis

The UV spectrum of the inhibitor solution was determined with a Perkin - Elmer 4.000A spectrophotometer, and even though a high level of non-specific absorption was observed, a slight shoulder at 280 m μ , indicated the possibility of protein being present in the sample.

2. Test for the Presence of Protein and Carbohydrates.

The inhibitor solution gave a positive reaction to the Folin-Ciocalteu Test. An optical density value of 0.575 was obtained at 670 m μ which on the basis of the standard curve of γ -globulin, indicated a content of 0.201 mg of protein per ml. The inhibitor solution gave a strong, instantaneous, positive reaction to the Phenol-Sulphuric Acid Test for carbohydrates.

3. Ethanol Precipitation.

To precipitate the polysaccharides 5.5 ml of 95% ethanol were added to one ml of inhibitor solution. No precipitate could be detected within an hour after the addition of ethanol, but a slight amount of white precipitate appeared after incubation of the mixture at room temperature (25°C) for 24 hours. The precipitate was separated by centrifugation, dissolved in 10 ml of distilled water, and assayed. This solution caused 63.8% inhibition.

4. Effect of Enzymes on the Activity of the Inhibitor.

Inhibitor solutions assayed after incubation with

papain, α -amylase, and phosphodiesterase caused 13.5, 7.5, and 81.3% inhibition respectively. The inhibitory activity was almost completely destroyed by the action of papain and α -amylase indicating that the inhibitor molecule contains a protein and a polysaccharide moiety. Since the inhibitor solution was not affected by phosphodiesterase the inhibitory activity cannot be ascribed to nucleic acids.

5. Hydrolysis.

The inhibitor solution was hydrolyzed and passed through a column of Dowex 1-X10 to separate the polysaccharide and the protein parts of the glycoprotein molecule. The combined cationic and neutral fraction presumably containing the polysaccharide, and the anionic fraction containing the protein were assayed and caused 57.0 and 96.4% inhibition respectively. To determine whether the activity of the neutral fraction might be due to unhydrolyzed glycoprotein rather than the polysaccharide, the Folin-Ciocalteu Test was performed on the neutral fraction, and a weak, positive reaction was obtained. An optical density value of 0.175 was obtained at 670 m μ , which on the basis of the standard curve of γ -globulin, indicates that the fraction still contained 0.049 mg/ml of protein. This is approximately 1/4 of the original protein content of the inhibitor preparation.

DISCUSSION

The level of inhibition obtained was greater when the pepper, geranium and D. stramonium extracts were mixed with the inoculum (99-100%), than when applied to lower leaf surfaces (63-76%)(Table 1 and 3). In preliminary experiments, however, plant extracts applied once to lower leaf surfaces caused little or no inhibition of TMV. Simons et al (36), obtained similar results with geranium and pepper sprayed on the underside of Nicotiana glutinosa leaves one day before inoculation with TMV. Blaszcak et al (5), on the other hand, reported "some" inhibition of PVX when the same extracts were applied to lower leaf surfaces of Gomphrena globosa. Although no attempts were made to determine the effect of applying the inhibitor at various times before inoculation, our results suggest that the increase in inhibition obtained with two lower leaf surface applications of the extracts, is due to the greater concentration applied.

The pepper, but not geranium extract, was inhibitory when applied once after inoculation, indicating that the inhibitory capacity of pepper extract is greater than that of geranium, (Table 4). The level of inhibition of both extracts increased with double applications, suggesting that the level of inhibition is related to concentration of the inhibitor.

The temperature experiments did not conclusively show whether more than one inhibitor was present in the plant extracts. There was no indication that inhibitory substances in pepper and geranium extracts were destroyed by boiling when the extracts were assayed mixed with the inoculum or sprayed on lower leaf surfaces (Table 2 and 3). When assayed after inoculation, however, the inhibitory activity of both extracts was reduced by boiling, and in the case of pepper, more so with one application of the inhibitor than with two (Table 4). This can be interpreted to mean that two inhibitors are present in both extracts: one which is heat-stable and highly inhibitory when applied with the virus or to lower leaf surfaces, but which is less inhibitory when applied after inoculation; and a second which is heat-labile, and whose presence is masked by the high inhibitory activity of the heat-stable inhibitor, except in the after inoculation assay. However, our assay studies were not quantitative, and the results can also be interpreted to mean that only one, partially heat-stable inhibitor is present in each extract. Both extracts are still highly inhibitory at dilutions of 1:500 (v/v) (unpublished results), so that only a relatively small quantity of the inhibitor is presumably required to cause complete inhibition of TMV when assayed mixed with the inoculum. Thus, boiling may not reduce the concentration

of the inhibitor sufficiently to produce any effect when it is assayed mixed with the inoculum. However, since, as discussed previously, the level of inhibition appears to be related to the concentration of the inhibitor in after inoculation assays, boiling, in this case might reduce the concentration of the inhibitor sufficiently to reduce the level of inhibition.

The activity of Datura extract was completely destroyed by boiling, when assayed mixed with the inoculum, but was only reduced $1/3$ to $1/2$ when assayed by the lower leaf surface method (Table 3). This suggests that Datura may contain two inhibitors: one that is heat-labile and largely responsible for the inhibitory activity of the extract when assayed mixed with the virus, and a second that is heat-stable without any effect when assayed mixed with the virus, or perhaps present in too low a concentration to be inhibitory, but capable of causing some inhibition when sprayed twice on lower leaf surfaces before inoculation. Alternately, only one, partially heat-stable inhibitor may be present, and the results obtained attributed to the greater concentration used in the lower leaf surface assay.

The following evidence indicates that the large molecular weight inhibitor from pepper is a glycoprotein: it is heat-stable, neutral, gives a positive reaction in tests

for protein and carbohydrates, is precipitated by 80% ethanol, and its activity is destroyed by papain and α -amylase.

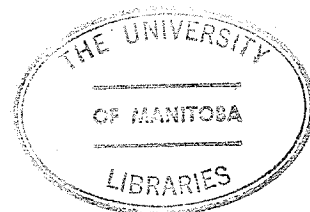
On the basis of the enzyme studies it appears that only the intact glycoprotein molecule is inhibitory. After hydrolysis, however, the fractions containing the polysaccharide and the protein parts of the glycoprotein molecule, were both inhibitory suggesting that each can prevent infection. Since hydrolysis was shown to be incomplete, the activity of the neutral fraction can be attributed to the unhydrolyzed glycoprotein remaining in this fraction. The activity of the anionic fraction might be due to the protein, but there is the possibility that some of the unhydrolyzed glycoprotein might bind to the resin and be present in this fraction also. In the enzyme studies, on the other hand, the enzymes were used as purchased and might contain impurities, or other enzymes. In view of these discrepancies it appears that only the intact glycoprotein molecule is inhibitory, but it is not possible to conclude whether the protein and polysaccharide parts possess any activity.

When assayed by the lower leaf surface method, the purified preparation of the large molecular weight inhibitor gave 54.9% inhibition as compared to 86.4% for the inhibitor preparation obtained by ultrafiltration only. The diffe-

rence in the activity of these two preparations suggests that a second, large molecular weight inhibitory substance might be present in the fraction that underwent ultrafiltration only, or that a certain amount of the inhibitor is lost during purification. The inhibitor might be partially hydrolyzed by the strong acid resin, and/or simply retained by the resins.

The heat-stable, small molecular weight inhibitor was shown to be present in the anionic fraction. The heat-labile inhibitor present in the cationic fraction, might be a third inhibitory substance detected in the pepper extract, or it might be simply a small amount of the glycoprotein inhibitor which was retained by the resin. If, as postulated previously, this inhibitor is unstable and can be partially destroyed by the boiling treatment, boiling would reduce the small amount present (as indicated by the fact that the fraction caused only 32.4% inhibition), below the minimum level required for activity. There is a possibility that this heat-labile inhibitor corresponds to that reported in pepper by McKeen (27), but more work is required with this fraction before a definite conclusion can be reached.

The role of each inhibitor in the geranium and pepper extracts, cannot be related with any degree of certainty to the inhibitory behaviour of the extracts. It was



thought, on the basis of the evidence supplied by trichothecin (4), that the presence of small molecular weight inhibitors in the extracts might provide an explanation for their ability to inhibit when applied to leaves in ways other than with the virus. However, since the molecular weight of the smallest inhibitor is greater than 1,000, its ability to diffuse into leaf cells might not be comparable to that of trichothecin, which has a molecular weight of 332.

The D. stramonium extract, however, which contains one (or two) large molecular weight inhibitor (Table 5), is inhibitory when applied to lower leaf surfaces (Table 3). This indicates that inhibition need not be dependent on the ability of the inhibitor molecules to diffuse into the cells. Further evidence that this is so, is provided by the fact that the purified pepper inhibitor is also inhibitory when applied to lower leaf surfaces. The results are in agreement with those of McKeen (27), who reported that a pepper extract containing a large molecular weight, proteinaceous inhibitor, was inhibitory to CMV when applied to lower leaf surfaces of cowpea plants. The fact that one application was inhibitory in this case, might be due to the different virus-host system used. Bawden and Freeman (4), on the other hand found that the polysaccharide from T. roseum was not inhibitory to TMV

when applied to lower leaf surfaces. Since our results indicate that concentration of the inhibitor is important in this respect, the concentration supplied by one application of the polysaccharide might not have been sufficient. The mechanism whereby large molecular weight inhibitors, such as pepper glycoprotein and the heat-labile inhibitor from D. stramonium extract inhibit virus infection when applied to lower leaf surfaces has not been resolved. There is no evidence that large molecules can penetrate uninjured leaves, and the best explanation appears to be that the inhibitors alter the metabolism of the leaf cells, so that introduced virus particles cannot multiply. This interpretation has previously been offered to explain the effects of various inhibitors, (4,10,27).

Results obtained when inhibitors are applied to lower leaf surfaces indicate that the effect induced is translocatable not only to the upper leaf surface but to the opposite leaf as well. It is thus possible that the inhibitor induces the formation of a substance which can be translocated throughout the plant.

The action of a protein inhibitor from carnation has been described as blocking or competing with, virus receptors sites on the leaf surface (34,41). The fact that the plant extracts and the purified glycoprotein inhibitor from

pepper are more inhibitory when applied with the virus than by the other two methods, appears to lend some support to this interpretation. On the other hand, our results indicate that the concentration of the inhibitor is apparently related to the level of inhibition. A similar effect might also be occurring when the inhibitor is applied with the virus, since the inhibitor would be in immediate contact with, and acting directly on, the recently infected epidermal cells, or perhaps entering the cells through the wounds created by the abrasive. The inhibitor might thus affect the metabolism of the cells rapidly and to a greater extent, than when it is applied on the lower leaf surfaces.

The present study indicates that the number of inhibitors in plant extracts, or the size of their molecules cannot be established on the basis of the inhibitory behaviour of the extracts alone.

SUMMARY

The ability of pepper, geranium, and D. stramonium extracts to inhibit TMV when applied to Pinto bean leaves in three different ways was studied. The three extracts were effective when mixed with the inoculum, or sprayed twice to lower leaf surfaces before inoculation. Pepper and geranium were also effective when applied after inoculation.

The temperature experiments indicated that the inhibitors in geranium and pepper are heat-stable, and that of D. stramonium heat-labile, but did not conclusively show whether more than one inhibitor is present in each extract.

The presence of two heat-stable inhibitors in the geranium and pepper, and one heat-labile in the D. stramonium extract, was demonstrated by the ultrafiltration experiments. Each extract contained an inhibitor with a molecular weight greater than 50,000, and geranium and pepper also contained a second inhibitor with a molecular weight greater than 1,000 but less than 50,000.

The heat-stable, large molecular weight inhibitor from pepper was purified by the use of ion-exchange resins, and ultrafiltration, and shown to be a glycoprotein. It is suggested that only the intact glycoprotein molecule is inhibitory.

The D. stramonium extract which contains a large molecular weight inhibitor, and the purified pepper glycoprotein are both inhibitory when applied to lower leaf surfaces, indicating that inhibition is not dependent on the diffusion of the inhibitors into the cells.

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