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PLANT VIRUS INHIBITORS EXTRACTED FROM THE CARYOPHYLLACEAE

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By

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To

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and

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ABSTRACT

Thirty species from the family Caryophyllaceae were found to contain inhibitors of local lesion production by TNV. The crude extracts of 20 species totally eliminated lesion production. Dilution experiments revealed the presence of at least two materials in the extracts of some species, an inhibitor which decreased and an augmenter which increases the number of lesions. Heat treatment decreases inhibitory activity in some species and not in other.

Two species were studied in detail, Gypsophila paniculata which showed powerful inhibitory activity was partially thermostable and Minuratia capillacea which showed weak thermolabile inhibition.

The constituents of Gp extracts acted as virus inhibitors rather than inactivators, survived at room temperature for 60 days, and were stable to a wide range of pH. Dialysis experiments confirmed that the inhibitory activity resided in both high and low molecular weight compounds. Ethanol or ammonium sulphate precipitation, and disc electrophoresis suggested that the inhibitors are composed in part, of protein and glycoprotein. Furthermore, inhibitory activity could not be attributed to RNA, but remained unchanged after incubation with each of three proteolytic enzymes,

Sephadex G-100 gel filtration showed that Gp extract inhibitors to have a molecular weights of about 12,600 - 27,500 and 2,000 for the materials which eluted slowly. Ion exchange column chromatography on CM-52 cellulose showed that extracts contained at least three virus inhibitor fractions, one neutral and two basic in nature. The low molecular weight fraction, dialysable part, contained 17 free amino acids most of which had inhibitory activity against TNV, and 3 free sugars all of which stimulated local lesion production.

Aqueous extracts prepared from G.paniculata and M.capillacea each inhibit local lesion production by TNV, TMV and PVX viruses. The G.paniculata inhibitors seem to have direct effects on TNV and some was precipitated on centrifugation. Minuratia inhibitors are not precipitated by centrifugation. Inhibition in both extracts influences host plants and acts either by altering the susceptibility of the cells to virus attachment, or perhaps allow penetration and prevent early events of virus replication.

The crude extracts from Gp induced local and systemic resistance in untreated parts of the plant. Systemic resistance was induced on French bean leaves when Gp extracts were applied to their roots. The sample taken from resistant leaves contained an inhibitor of virus infection and showed 3 additional protein bands with molecular weights of 105, 94 & 40 $x10^{-3}$daltons on SDS polyacrylamide gel electrophoresis.

Gp = Gypsophila paniculata

TNV = tobacco necrosis virus

TMV = tobacco mosaic virus

PVX = potato virus X

CONTENTS

CHAPTER I

GENERAL INTRODUCTION

Viruses invade and damage a very wide range of plants including a number of economically important crops. Control of virus diseases is confined largely to control of vectors. The direct control of viruses by chemical treatment is only moderately successful and in general can not be used for large scale field disease prevention.

The chemical that have been used to control viruses are very diverse, the most successful being analogous of nucleic acid bases. Many other pure chemicals have virus inhibitory properties these include: dyes (Orlob, 1967), RNA (Gicherman and Loebenstein, 1968). Polyelectrolytes, both basic (Polylysine) and acidic (Polyglutamic acid), inhibit TMV (Stahmann and Gothoskar, 1958). Proteins such as bovine lactoglobulin, rabbit serum, and TMV-protein also have been reported to inhibit virus infection (Santilli et al., 1961). Enzymes, for example, trypsin, papain, and ribonuclease (RNase) are known to reduce infectivity of TMV (Nene, 1960). The growth regulators, α-naphthaleneacetic acid (NAA) and gibberellic acid also show effects on virus symptom production (Upreti et al., 1964), and sometimes growth retardants such as N-dimethylaminosuccinamic acid inhibit virus (Karas et al., 1964). The divalent cations Ba^{+2}, Ca^{+2}, and Mg^{+2} at suitable concentrations also inhibit virus (Rossouw and Fulton, 1963). Milk and various extracts from plants are natural substances also known to bring about the inhibition of virus activity.

The term inhibition refers to instances whereby virus infection or replication is prevented by a nonmultiplying substance that does

not inactivate the virus *in vitro*. The first suggestion of a plant virus inhibitor in plant sap occurs in the work of Allard (1914, 1918). It is now well known that numerous plants contain potent inhibitors of virus infection, and the visible effect of their action in reducing symptoms is uniform, the mechanism by which this is achieved may be diverse.

There are two theories about the way in which inhibitors of infection act. One theory suggests that inhibitors affect the resistance of the inoculated host plant, and the other they inhibit by acting directly on the virus particles *in vivo*. Since the action of the latter inhibitors results in chemically modified inactive viruses, it has been suggested that these compounds be classified as virus inactivators (Fulton, 1943). Several substances have been reported to inactivate viruses, such as true tannins (Cheo and Lindner; 1964; Ragetli and Weintraub, 1974), naphthoquinone derivatives or benzoquinones formed from natural (poly) phenolic precursors through oxidase action (Best, 1937; Hampton and Fulton, 1961). Saponins may affect membrane bound complex viruses such as tomato spotted wilt and rhabdo viruses. Enzymes, too, may affect the intergity of viruses or their nucleic acids (Sänger and Brandenburg, 1961).

Inhibitors have been divided by Bawden (1954) into two categories; inhibitors of infection and inhibitors of virus replication. Inhibitors of infection are defined as "those substances that, when inoculated to leaves simultaneously with viruses, prevent infection from occurring". Inhibitors of virus multiplication are defined as "substances that, when applied to leaves already infected, retard the rate at which the infective viruses multiply". These definitions

depend on the mode of action and not on the chemical nature of the inhibitor. Thiouracil, for example, may both inhibit infection and affect multiplication (Commoner and Mercer, 1951; Bawden, 1964a).

It was noticed that crude extracts prepared from virus infected plants often failed to produced infection in other susceptible plants. This apparent resistance might be due to many factors, such as insufficient concentration of virus in the inoculum, or the instability of the virus during the process of transmission because normal infection may occur via insect vectors rather than by mechanical inoculation. Alternatively, absence of infection may be due either to the physical incompatibility of the host plant to mechanical virus infection or the presence of inhibitor (s) in plant sap. Such inhibitory substance may be by -products of virus multiplication or they may occur naturally in the tissue of healthy plants and be subsequently released from the cells during sap extraction.

Although inhibitor compounds have been found in extracts of fungi, lichens, bacteria and insects, of particular important is the possibility that extracts of higher plants may contain substances that inhibit virus infection either by influencing the virus or by acting on the plant being tested. Considerable interest has been paid to the possible presence of virus inhibitor compounds in the vegetative parts of a wide range of plants.

Various inhibitor materials have been extracted from the vegetative tissue of over sixty different families of plants (Bawden, 1954; Hajj, 1976). However, studies of the chemical nature and mode of action of the virus inhibitor materials have tended to be concentrated on relatively few families of flowering plants particularly the Chenopodiaceae,

Phytolaccaceae, Rosaceae, Solanaceae, and a few members of the Caryophyllaceae. A brief summary of virus inhibitor studies from each of these families is given below.

CHENOPODIACEAE

Many inhibitors of virus infection are known to occur in the family Chenopodiaceae. Kuntz and Walker (1947), for example, described two inhibitors in spinach juice (Spinacia oleracea L.), one which was absorbed by charcoal and which was considered to be a high molecular weight protein, the other was an oxalate and was removed by adding KCl. Other oxalates were isolated from New Zealand spinach sap by Benda (1956) who thought them to be virus-enhancing compounds. Later, Ebrahim-Nesbat (1971) Ebrahim-Nesbat and Nienhaus (1972) isolated and partially purified an inhibitor fraction from spinach sap. He studied the mode of action of the inhibitors by electron microscopy, and he found that the addition of the inhibitor to a suspension of TMV caused an aggregation of virus particles resulting in loss of infectivity. He concluded that the virus inhibitor from spinach leaves was a protein.

Thomson and Peddie(1965) found that sap extracted from Chenopodium amaranticolor leaves, inhibited infection by TMV of Nicotiana glutinosa L., but had only slight effects on species from which the sap was extracted. They also found that most inhibitor remained in the supernatant fluid after centrifuging sap at 40,000 rpm for 4 hrs. Kimmins (1969), on the other hand, found that crude extract from C.amaranticolor leaves can inhibit TNV on French bean plants, and the inhibitory activity was found to reside in the RNA-portion. Furthermore, Smookler (1971) examined 29 species of the Chenopodiales (an order including some members of the Chenopodiaceae and the Amaranthaceae)

and found that leaf extracts inhibited the infection of Phaseolus vulgaris by TNV. He also examined the nature and properties of inhibitors extracted from C.amaranticolor, Atriplex nitens, Chenopodium album and Amaranthus caudatus. Ion exchange column chromatography on CM sephadex C-25 showed that most inhibitory activity were eluted by 0.4 M sodium acetate buffer, the inhibitors being proteinaceous in nature having molecular weights of 25,000-38,000. In contrast, Alberghina (1976), found the inhibitor from C.amaranticolor to be an highly active against TNV on C.amaranticolor leaves, it was not destroyed by protease, was thermostable, and although largely not dialysable it seems not to be a protein.

Recently, Taniguchi and Goto (1979) further purified the inhibitor from C.amaranticolor leaves by Sephadex G-100, G-50 and DEAE-Sephadex ion exchange column chromatography. They found the inhibitor was a protein which may consist of a single polypeptide chain with a molecular weight of 32,000. Taniguchi et al. (1974) also isolated two fractions from leaf extract of C.album by ECTEOLA cellulose column-chromatography. Both fractions showed inhibitory activity against TMV. The inhibitor consisted of particles of various sizes, and their activity was destroyed by phenol but not by RNase, pronase or chloroform.

PHYTOLACCACEAE

The first indication that substances present in higher plants were inhibitory to plant virus infection was obtained by Allard (1914, 1918) using extracts of Phytolacca decandra L.(pokeweed). Allard found that mosaic virus could be transmitted by sap-inoculation from pokeweed to pokeweed but not to tobacco, and he therefore suggested that pokeweed sap might contain substances inhibitory to pokeweed mosaic

virus. A few years later, Duggar and Armstrong (1925) ascertained the presence of an inhibitor in pokeweed that interfered with mechanical transmission of TMV. Since then many workers have investigated several other species belonging to the same family, and in some cases, it has been possible to purify and characterize the inhibitor.

Kassanis and Kleczkowski (1948) isolated an inhibitor from the sap of Phytolacca esculenta, which was identified as a glycoprotein containing 14-15 % nitrogen and 8-12 % carbohydrate. Gupta (1964) also isolated an inhibitor from Phytolacca acinosa. The inhibitor was found to be a glycoprotein containing glucose. Marchoux (1967, 1970), on the other hand, proved that extracts prepared from pokeweed leaves had ribonuclease activity (RNase) which suggests that proteins may be involved in the inhibitory activity of the extracts.

Wyatt and Shepherd (1969) purified the inhibitor isolated from Phytolacca americana by column chromatography using CM-Sephadex, the inhibitor was found to be a protein of 116 amino acids residues, giving a molecular weight of 13,000. Later, Misawa et al. (1975) isolated an inhibitor with high activity from both callus tissue and suspension cultured cells of P.americana. This inhibitor was found to be similar to inhibitor substances isolated by Wyatt and Shepherd (1969) from an intact plant. Recently, Grasso and Shepherd (1978) isolated a pure virus protein from P.americana by adsorption to, and elution from, the cation exchange CM-Sephadex followed by passage through DEAE-Sephadex. They used the same procedures to examine 14 other plant species reported to contain virus inhibitors, including several species of the order Centrospermae which includes the Phytolaccaceae.

Similar biologically active proteins were obtained in every case. The possible mechanism of inhibition of plant viruses by P.americana extracts has been studied by Owens et al. (1973). They suggested that the inhibitor acts in vivo by blocking the messenger function of a potentially infective virus RNA.

More recent studies have shown that extracts from P.americana contain several virus inhibitor fractions (PAP, PAP-II & PAP-S) each with the ability to inhibit protein synthesis as well as virus replication (Irvin et al., 1980; Barbieri et al. 1982).

ROSACEAE

Tannic substances were found to be responsible, at least in part, for the virus inhibitor activity of some plant species. For example, extracts prepared from Fragaria vesca L. (strawberry) leaves, contained tannins which were sufficient to precipitate TMV and prevent it from infecting N.glutinosa, even at 1 % concentration (Bawden and Kleczkowski, 1945). Other workers have reported that tannic substances have a direct effect on viruses (inactivators) rather than on the host plants (Best, 1937; Hampton and Fulton, 1961; Cheo and Lindner, 1964).

It was suggested by Van der Want (1951) that tannins could prevent infection in all plants, and such substances should be called "absolute inhibitors" to distinguish them from "relative inhibitors" where their action depends on the species of plant into which inoculations are made.

SOLANACEAE

The possible occurrence of inhibitory substances in plants from the Solanaceae has been investigated by a number of workers.

Zaitlin and Siegel (1963), for example, extracted an inhibitor from Nicotiana tabacum L. var. Turkish Samsun which inhibited TMV on several host plants. This inhibitor was a protein having a molecular weight greater than 40,000. Further fractionation of such extracts was carried out by Wolffgang (1970) using column chromatography. He found both high and low molecular weight fractions were inhibitory against TMV.

Palm (1967) showed that extracts from Nicotiana glutinosa L. caused 60 % inhibition when mixed with an equal volume of TNV. The physical and chemical tests of such extracts indicated that the inhibitor was a protein of the globulin type. Kimmins (1969), on the other hand, fractionated crude extracts from N.glutinosa into protein and RNA, and suggested that the inhibitory activity was retained in RNA portion.

Extracts prepared from Capsicum annuum L. showed inhibitory activity against TMV and cucumber mosaic virus (CMV) and were found to contain proteins with ribonuclease activity (RNase) (Marchoux, 1967 and 1970). A similar finding was reported by Fischer and Nienhaus (1973) who found that inhibitory power of C.annuum extract was connected with a protein and a phenolic substance. After polyacrylamide gel-electrophoresis the protein was located in the zone with the greatest ribonuclease activity. The phenolic substance was identified as a flavone, isoflavone or flavonone compound. In 1972, Apablaza and Bernier, separated the inhibitory materials of Capsicum frutescens into two fractions: one with a molecular weight greater than 50,000 and one with a molecular weight within the range 1000 - 50,000. They also found a high molecular weight fraction from

such extracts was inhibitory when applied to the underside of the leaf.

CARYOPHYLLACEAE

The occurrence of an active principle in the genus Dianthus that interferes with the mechanical transmission of virus was discovered some years ago. It was shown independently in Holland and in Canada that sap from both carnation; Dianthus caryophyllus L.; (Van der Want, 1951) and sweet william; Dianthus barbatus L.; (Weintraub and Gilpatrick, 1952) upon mixing with viruses such as TMV and tobacco ring spot, suppresses or greatly reduces the development of symptoms on their respective hosts. Later it was shown (Ragetli, 1957) that the carnation inhibitor probably acts via the host plant in the early stages of virus establishment and is active in combination with at least 14 viruses and 20 different plant species. Early attempts aimed at isolating and characterizing the inhibitor from D.caryophyllus by ultracentrifugation, paper electrophoresis, and ammonium sulphate precipitation. These techniques revealed that the active agent in carnation extracts had a molecular weight of 10,000 and showed no active electrophoretic movement in the pH range 5.4 - 8.1, and required high salt concentrations (50 % ammonium sulphate saturation) for precipitation (Ragetli and Weintraub, 1962a). In later experiments, Ragetli and Weintraub (1962a) passed carnation inhibitor through an anion exchange column (DEAE), followed by column chromatography over a cation exchanger (carboxymethyl cellulose) and hydroxylapatite. The fractions produced were tested for inhibitory activity and the nature of the inhibitor was studied. Ragetli and Weintraub (1962b) concluded that the virus inhibitor from carnation was a protein which upon acid hydrolysis yielded 14 amino acids. However, before

being clearly recognized, these properties had led to some confusion regarding the nature of the active agent from Dianthus, which was originally considered to be a mixture, comprising a dialysable and a non-dialysable components (Van der Want, 1953).

Nart (1972) confirmed the Ragetli and Weintraub results by showing that sap from carnation inhibited the number of lesions induced by three different viruses TMV, alfalfa mosaic (AMV) and barley stripe mosaic virus (BSMV) on five different local lesion hosts. The inhibitor was identified as a protein by various methods such as low speed centrifugation, phenol extraction and ethanol precipitation of the sap. Moreover, Fantes and O'Neill (1964) found that material isolated by Ragetli and Weintraub from apparently healthy carnation plants seemed to possess marked similarities to chick interferon.

Recently, Stirpe et al. (1981) isolated two proteins (Dianthin 30 and Dianthin 32) from the leaves of carnation, with molecular weights of 29,500 and 31,700 for Dianthin 30 and 32 respectively. Both Dianthins are glycoprotein containing mannose and they act by damaging ribosomes, so preventing protein synthesis in a manner similar to that described for PAP (Irvin et al., 1980; Barbieri et al., 1982).

This literature review records that inhibitors extracted from vegetative parts of several plant species have been variously identified as proteins, glycoproteins, tannins and RNA. Whether such compounds act in preventing or diminishing virus multiplication in vivo is not understood.

Some crude plant extracts have very little effect on viruses, this may be because inhibitory effects are counterbalanced by the

virus enhancing effects of other substances. Plant extracts have been reported to contain virus-enhancing compounds. Benda (1956), for example, found that sap extracted from New Zealand spinach (Tetragonia expansa), family Aizoaceae, when mixed with tobacco ring spot virus and inoculated onto cowpea (Vigna sinensis) leaves, caused a delay in the appearance of the primary virus symptoms. On further analysis, New Zealand spinach appeared to contain two active fractions, one an inhibitor which decreased the number of lesions, and the other an augmenter, identified indirectly as a soluble oxalate salt which increased the number of lesions. Later, enhancement of virus activity was described for plant extracts (Blaszczak et al., 1959), seed extracts (Stevens, 1970, Hajj and Stevens, 1979) and for various sugars (Kongsvik and Santilli, 1970). The mode of action of such virus enhancing compounds is not yet understood. This phenomenon will be described and discussed where relevant in the later chapters.

It seems also from the review that the inhibitory substances from only a few species of higher plants have been isolated and characterized, and fewer yet have been investigated for their mode of action. However, the current opinions regarding possible mechanism by which inhibitors prevent infection can be summarized as follows: 1- aggregation of virus particles, thereby decreasing the number of separate particles. 2- preventation of attachment of the virus to receptors, due to complexing of the inhibitor with the virus particles. 3- competition of the inhibitory substance with the virus for receptors. 4- alterations in the physiology of the host, so preventing infection.

The inhibitor from carnation, for example, fully inhibits infection

on N.glutinosa when applied before inoculation with TMV. No significant inhibition was obtained when the inhibitor was applied later than 30 minutes after inoculation (Ragetli, 1957). Ragetli and Weintraub (1962) purified this substance and presented evidence that it is a protein. It has been suggested that this inhibitor acts by competing with the virus for receptor sites at the beginning of the infection process (Van Kammen et al., 1961). The interaction between virus and receptor leading to infection, or the interaction between inhibitor and receptor leading to inhibition, is in dynamic equilibrium and depends on the concentrations of virus and inhibitor. Increasing the virus concentration at a given inhibitor concentration decreases the inhibition, and vice versa-increasing the amount of inhibitor at a given virus concentration increases the degree of inhibition (Van Kammen et al., 1961; Gicherman and Loebenstein, 1968).

Applying the inhibitory substance several days before inoculation is also generally ineffective. RNase (Nene, 1960), however, and heat killed cells of Pseudomonas syringae (Loebenstein and Lovrekovich, 1966) strongly inhibit infection when applied seven days before inoculation. An inhibitor from rice has been reported to be effective when applied to bean leaves 1-3 days prior to TMV inoculation (Jones et al., 1959). Regarding heat-killed bacteria, however, it has been suggested that an induced interference mechanism is involved (Loebenstein and Lovrekovich, 1966). Interference is defined as that phenomenon whereby the infection process of one virus interferes with the multiplication of another virus or strain; or whereby a nonmultiplying substance interferes via a host-mediated process, requiring the transcription mechanism of the cell, with the multiplication of

the virus.

Acquired resistance is a distinct type of interference, developing in noninvaded host tissue after viral or fungal infection of other parts of the plant; these resistant tissues are less able to support virus multiplication than non resistant controls. The first observation that infection in one part of the plant confers resistance in other noninvaded tissues was reported by Gilpatrick and Weintraub (1952). In local infections it is thought a product of the infected cells is translocated ahead of infection and confers resistance on yet uninfected cells, thus confining the spread of the viral infection.

It was found that the tissue around the virus-induced local lesions is resistant to subsequent virus infection (Yarwood, 1953; Ross, 1961a). Such resistance is called local aquired resistance (LAR). In addition, distant parts of the virus-localizing plant are partially resistant (Ross, 1961b), this resistance is called systemic acquired resistance (SAR).

Yarwood (1953) reported the presence of LAR in the tissue surrounding local lesions induced by TMV on bean. Ross (1961a & b) attributed the inability of virus to move from living cells around necrotic lesions in N.tabacum cv. Samsun NN or Xanthi inoculated with TMV, to an alteration of cells in advance of the virus, the tissue around the lesions becoming highly resistant. He also added that, not only the healthy tissue around the local lesions, but also the uninoculated opposite half leaves and the leaves above and below the inoculated one became resistant to further infection.

Loebenstein and Ross (1963) showed that juice from virus free

Datura stramonium produced very little inhibition to TMV infection of the test plants, N.glutinosa, N.tabacum var. Samsun NN or D.stramonium. Juices, extracted from uninfected halves of Datura leaves previously inoculated on their basal halves with TMV or TNV were found to inhibit TMV. Such extracts inhibited lesion number by an average of 70 %. They suggested that infection of basal halves either induced the formation in apical halves of an interferon-like agent (s) not found in healthy leaves, or stimulated the production of an inhibitor (s) normally found in low concentration in Datura leaves.

It has been reported that extracts prepared from some healthy plants can induce resistance (LAR & SAR) similar to that induced by viruses. McKeen (1956) found that inhibitor from pepper when applied to the lower surface of one half-leaf was able to exert an inhibitive effect on the upper leaf surface of the entire leaf. Similar observation was reported by Apablaza and Bernier, 1972; Fischer and Nienhaus, 1973. McKeen explained this fact as an influence of a readily transportable inhibitive agent. Fischer and Nienhaus (1973), who also studied the pepper inhibitor, attributed this phenomenon to the production of a chemical effect which was translocated through cells to the upper epidermis.

Verma and Awasthi (1979) found that the inhibitor root extracts of Boerhaavia diffusa when applied onto two basal or two apical leaves of C.amaranticolor; a systemic host, and N.glutinosa, a hypersensitive host, of TMV; induced resistance in untreated tissues. This suggests that inhibitory substances had been transmitted to unprotected leaves. This was confirmed when extracts from these untreated leaves were

shown to inhibit virus, whereas normal sap was non inhibitory. Furthermore, Noronha et al. (1980) found the extracts taken from each of five species of the Caryophyllales showed inhibitory activity not only on the treated half-leaves, but also on the half-leaves opposite to those to which they were applied. They suggested that the inhibitory materials aretranslocated across leaves and between leaves through the leaf blade.

Application of virus, or chemicals to leaves produced changes in leaf proteins and these may be associated with resistance. Kassanis et al. (1974) found that induced resistance of infected leaves when challenged with a second virus was correlated with the appearance of three proteins not present in healthy leaves. Later, Gianinazzi et al. (1977) demonstrated the appearance of four new leaf protein components in the living tissue around the local lesion produced during the hypersensitive reaction of N.tabacum var. Xanthi to TMV infection. These new proteins not found in the healthy tissue. Such proteins could also be induced by polyacrylic acids (Gianinazzi and Kassanis, 1974), salicylic acid and benzoic acid (White, 1978). These same chemicals induced resistance to virus infection.

It seems likely that the proteins induced either by virus invasion of tissues, or by treatment of tissues with chemicals or plant extracts are similar, if not identical, to the antiviral factors first described by Sela and Applebaum (1962). These workers reported for the first time the presence of antiviral activity in clarified virus-free sap from virus infected leaves. They called the factor responsible for this activity as antiviral factor (AVF).

It has not been clearly established that AVF is a protein, although Mozes et al. (1978) isolated AVF from TMV-infected leaves of Nicotiana glutinosa, and identified it as a phosphoglycoprotein with a molecular weight of about 22,000. AVF has been found following TMV infection (Antignus et al., 1977) only in plants that carry the N-gene. This gene is responsible for TMV localization in Nicotiana. Previous studies of induced virus inhibition or antiviral factors has caused authors to draw analogies between AVF and interferon (Baron, 1963; Atanasoff, 1964; Sela et al., 1965; Gianinazzi and Kassanis, 1974; Mozes et al., 1978; Sela, 1981).

To summarize briefly it may be concluded that resistance of plants to virus can be induced by the virus itself, by chemicals, or by virus inhibitory plant extracts. Resistance is often associated with the appearance of new proteins. These proteins may be described as antiviral factors and may have similarities to interferon. The role of virus inhibitor plant extracts may therefore involve amongst other things, the induction of AVF.

There is some information on inhibitors extracted from plants in a wide variety of species. The need is for more intensive study of a few species. Within the Caryophyllaceae there are well documented accounts of inhibitor fractions from Dianthus caryophyllus and also some information on Dianthus barbatus. No other species within this family appears to have been studied for antiviral properties in spite of the possible relationship of the Dianthus inhibitor to interferon (Fantes and O'Neill, 1964).

The work described in this thesis is designed to:

(1) Examine a selection of species from the family Caryophyllaceae for inhibitor activity against plant viruses (Chapter III).

(2) Study the effect of heat and other physical factors on the inhibitors with a view to establishing some details of the characteristics of the compounds reponsible for inhibition using Gypsophila paniculata as a selected example (Chapter IV & V).

(3) Study the nature of the inhibitor fractions of G.paniculata with a view to gaining information about the chemical identity of the inhibitor (Chater VI).

(4) Gain some ideas of the possible mode of action of virus inhibitors (Chapter VII).

(5) Study the possible induction of virus resistance by the G.paniculata extracts (Chapter VIII).

CHAPTER II

MATERIALS AND METHODS

1. Growth of assay plants

a) Glass house conditions

In summer, all test plants were grown in natural light, the range of temperature was maintained between 20 - 30 °C. High temperature was reduced by the use of a fan, and by watering the plants twice a day. The relative humidity was kept high and further cooling achieved by damping the floor with water.

In winter, the natural light was supplemented with illumination from mercury vapour lamps to give 16 light hours in every day, the range of temperature was maintained between 20 - 25 °C by thermostatically controlled tubular heaters.

b) Growth of Phaseolus vulgaris L. var. The Prince (French bean)

Seeds of P.vulgaris var. The Prince were sown in 12 cm plastic pots containing John Innes No.2 (JI2) compost. Six plants were grown in each pot. Only obviously healthy plants of uniform appearance were used for assay. French beans were usually ready for inoculation 10-13 days after planting.

c) Growth of Nicotiana tabacum var. Xanthi (Tobacco)

Seeds of N.tabacum var. Xanthi were initially sown in plastic seeds trays containing compost (JI2). After three weeks, individual healthy seedlings were transferred to 6 cm plastic pots containing (JI2) compost, one plant in each pot.

d) Growth of Gomphrena globosa

Seeds of G.globosa were sown and grown as for N.tabacum var. Xanthi

2. Preparation and infectivity assay of plant viruses

a) Tobacco Necrosis Virus (TNV)

French bean leaves infected with tobacco necrosis virus strain D were stored at -25 °C and used as the source of virus. Frozen leaves were ground using a pestle and mortar with a little acid washed sand and distilled water (1:2 W/V). The bulk of the leaf debris and sand was removed by squeezing the pulp through three layers of muslin. The extract was centrifuged at 2000 xg for 15 minutes, and the supernatant decanted and kept at room temperature over night to precipitate any proteinaceous virus inhibitor present in the leaf sap (Bawden, 1954). The supernatant was clarified by centrifugation at 2000 xg for 15 minutes. Ten ml aliquots of the clarified supernatant were stored in glass vials at -25 °C. When required, clarified extracts were thawed and diluted with 0.06 M phosphate buffer pH 7.0 to a concentration giving approximately 40 - 100 local lesions per leaf.

Phaseolus vulgaris var. The Prince was used as local lesion host to quantitatively measure the activity of TNV (D). The apical bud was removed from test plants since Youden and Beale (1934) have shown that inherent variability among leaves may be reduced by removal, before inoculation, of the growing tip and all leaves except those to be inoculated.

For virus assay comparisons, sets of ten primary leaves of 10-13 days old french beans were inoculated with the test solution and virus, another set was inoculated with control inoculum of TNV in water or phosphate buffer pH 7.0. Carborundum 300 mesh was used as an abrasive (Beraha et al., 1955). The inoculation was made by dipping

the forefinger in inoculum and rubbing once over the upper surface of the leaf.

The relative percentage inhibition of the extract was estimated by using the following calculation:

$$\% \text{ Inhibition} = 100 - \frac{A}{B} \times 100$$

Where A = Number of lesions on treated leaves

B = Number of lesions on control leaves

The difference between control and test treatment was analysed statistically. Details of the statistical methods, together with worked examples are given in the Appendix 1 & 2

b) Tobacco Mosaic Virus (TMV)

Tobacco mosaic virus (TMV) was maintained in P.vulgaris var. The Prince. Systemically infected leaves, showed clear symptoms after 4 - 5 weeks from inoculation (Figure 1). Such leaves were ground with distilled water (1:2 W/V) using a pestle and mortar. Crude TMV was extracted and stored using the same procedures as described above for TNV. Infectivity assay was carried out on the young expanded leaves of 7-9 weeks old N.tabacum var. Xanthi. The lower older leaves and the growing points were removed before inoculation.

c) Potato Virus X (PVX)

Potato Virus X (PVX) was prepared from systemically infected leaves of N.tabacum var. Xanthi. Virus was extracted and stored using the same procedures as for TNV. Infectivity assay of PVX was measured by the local lesion method on the young fully expanded leaves of Gomphrena globosa L.

Figure 1 : Effect of TMV on Phaseolus vulgaris plants

A B

A = Water treated plant (control),after five weeks

B = A systemically infected plant, after five weeks

3. Preparation of extracts from Caryophyllaceae

Plants from the family Caryophyllaceae were obtained from the Botanical Supply Unit, University of London.

a) Preparation of crude extracts

Crude extracts were prepared from each of thirty Caryophyllaceae species by grinding the leaves and stems in distilled water (1:2 W/V). The pulp was squeezed through four layers of muslin and the fluid centrifuged at 2000 xg for 15 minutes. The supernatant was decanted and used as the test solution.

Crude extracts of Gypsophila paniculata and Minuratia capillacea were examined in more details. In order to achieve uniform samples, large volumes of extracts were prepared from these two species and lyophilized (freeze dried).

b) Preparation of lyophilized plant extracts

100 g leaves and stems of G.paniculata and M.capillacea were each ground in 200 ml distilled water, the pulp was squeezed through four layers of muslin and the fluid extract centrifuged at 2000 xg for 15 minutes. The clear supernatant was lyophilized over night. The yield of lyophilized material was weighed and the quantity required to make up a concentration identical to that of fresh crude extract was calculated. The lyophilized extracts were stored in screw-topped glass bottles at -25 °C. The effect of lyophilized and fresh crude extracts of G.paniculata and M.capillacea were compared as shown in (Table 1). The results show that lyophilization has no effect on their inhibitory activity compared to freshly prepared extracts.

Table 1 : Comparison between the fresh and lyophilized extracts on local lesion production by TNV

Source of plant extract	Mean number of lesions *		% Inhibition
	TNV + Water	TNV + Extract	
G.paniculata (fresh)	80.2	0	100
G.paniculata (lyophilized)	136.9	0	100
M.capillacea (fresh)	71.7	12.8	82
M.capillacea (lyophilized)	77.0	13.3	83

* Each figure represents the mean number of lesions for ten replication

4. Estimation of protein

Protein concentrations were measured with the Folin-phenol reagent alkaline copper treatment by the method of Lowry et al.(1951). This Folin technique seems to be 10 - 20 times more sensitive than measurment by the Ultraviolet absorption at 280 nm. A calibration curve was obtained using different concentrations of Bovine serum albumin.

5. Estimation of carbohydrate

Molish's test was used to indicate the presence of a carbohydrate in the inhibitor extracts. The test is a general reaction for all carbohydrates and substances containing a carbohydrate radical. Quantitative and qualitative estimations of carbohydrate by Thin Layer Chromatography (TLC) will be given in the appropriate section.

6. Estimation of amino acid

Free amino acids were detected using 0.5 % ninhydrin. Details of amino acids in G.paniculata extracts were chromatographically determined

using an automatic amino acid analyser. Quantitative and qualitative estimation of amino acids will be given in the appropriate section.

7. Disc electrophoresis

Polyacrylamide gel electrophoresis is one of the most effective methods for the separation of ionic components of a mixture. The method of Ornstein (1964) and Davis (1964) was used. Gels of 7 % acrylamide were run using Tris-glycine buffer, pH 8.3, containing bromophenol blue (Smith, 1968) as marker. Samples of 0.2 ml were carfully layered on to the top of the acrylamide gels in siliconized glass tubes (7.5 x 0.5 cm). The marker separation was achieved using a power supply of 5 milliamps per tube until the sample had completely entered the gel. The current was then reduced to 2 mA per tube. Separation was stopped after 3-4 hours when the marker had moved 4.5 cm into the gel. At the end of electrophoresis, the gels were extracted from the tubes by rimming with the use of stainless steel needle. Gels were then immersed in fixing solution of methyl alcohol (150 ml), sulphosalicylic acid (17.2 g), trichloroacetic acid (57.5 g), and distilled water (350 ml) for two hours. Proteins were stained by immersing the gel in 1 % Coomassie brilliant blue R. in a water bath at 60 ^{o}C for one hour. The gels were destained by immersing in destaining solution (ethanol 500 ml, glacial acetic acid 160 ml, and water 2 liters) for 2-3 days, and stored in 7 % acetic acid in glass vials.

Glycoproteins were located with periodic acid Schiff (PAS) reagent. Gels were fixed in oxidizing solution of 1 % periodic acid dissolved in 3 % glacial acetic acid for one hour, they were then rinsed in running water for one hour. Gels were stained in Schiff's reagent.

Within one hour, sharp red bands developed, indicating the position of glycoproteins. After staining, the gels were stored in 1 % sodium metabisulfite (Zacharius et al., 1969).

A quite successful technique for the analysis of the proteins in crude extracts by polyacrylamide slab electrophoresis has also been used. The details of this technique and other special techniques are given in their appropriate sections.

CHAPTER III

EFFECTS OF CRUDE AND DILUTED EXTRACTS FROM THE CARYOPHYLLACEAE ON LOCAL LESION PRODUCTION BY TNV

Introduction

In this study, a survey has been made of the effects of extracts from thirty species of the family Caryophyllaceae on TNV infection of Phaseolus vulgaris.

In previous studies, extracts from Dianthus caryophyllus L. and Dianthus barbatus L. have been reported to inhibit the infection of plants by viruses (Van der Want, 1951; Ragetli and Weintraub, 1962a). No other species within the family Caryophyllaceae appear to have been studied.

In order to see whether extracts contain compounds that inactivate virus, or inhibit their activity, many workers have undertaken dilution studies. Kuntz and Walker (1947) found that dilution of spinach extract to 10^{-3} with distilled water reduced its inhibitory activity against TMV to 45 %. Kassanis and Kleczkowski (1948) found that sap isolated from Phytolacca esculenta was a very strong inhibitor against tobacco mosaic virus (TMV) on Nicotiana glutinosa, on the dilution of the extracts virus infectivity was restored. Allen and Kahn (1957) observed that extracts from rice polish were active at dilution up to 1:6,000. Blaszczak et al. (1959) found that tenfold dilution of extracts from 28 species or varieties of plants, in most cases, removed the inhibition or decreased it markedly.

A study of the extracts prepared from Chenopodium quinoa was carried out by Saksena and Mink (1969) who reported that when the

extract was diluted with phosphate buffer to 10^{-4}, it produced 9 % inhibition against apple chlorotic leaf spot virus (CLSV) on P.vulgaris var. Kinghorn (Kinghorn bean) compared to 100 % with the crude preparation. On the other hand, Marchoux (1967) reported that pepper leaf extract (Capsicum annuum)were still active, even when dilution reached 10^{-4} giving 32 % inhibition. Takagi and Sugimura (1977) found that the inhibition of TMV by extracts from the fruit-bodies of the fungus Lentinus edodes was linearly decreased with dilution, it reached zero at 2 x 10^{-3} dilution.

Various reports have shown that most of the inhibitory activity of Chenopodium album and Chenopodium amaranticolor extracts against TMV infection of N.glutinosa was lost at a dilution of 10^{-3} with distilled water (Thomson and Peddie, 1965; Yoshizaki and Murayama, 1966). However, Blaszczak et al. (1959) reported that sap extracted from C.album failed to inhibit PVX infection on Gomphrena globosa when diluted to 10^{-2} with distilled water. Smookler (1971) found that sap preparation from 29 species of Chenopodiales were strongly inhibitory (more than 90 %) at dilution 2 x 10^{-1} , but at 10^{-4} only eight species inhibited. Furthermore, in studies carried out by Vicente et al. (1977) it was observed that the percentage of inhibition by C.amaranticolor leaves extract was gradually decreased with the increases of dilution until it reached 14 % and 0 % at dilution 10^{-3} & 10^{-4} respectively. Recently, Noronha et al. (1980) reported that leaf juice from some members of the Caryophyllales were inhibitory to TMV infection on N.glutinosa, it was also observed that the higher dilution of inhibitory extract the lower percentage of

inhibition. At dilution of 5 x 10^{-2} of Alternanthera ficoidea and Chenopodium ambrosioides lost their inhibitory action, while Amaranthus deflexus, Bougainvillea spectabilis and Mirabilis jalapa showed an inhibition of 47 %, 50 % and 76 % respectively.

A few reports have been made concerning the effect of dilution of seed extracts on plant virus infection. Verma et al. (1975) showed that seed extract of Lawsonia alba was quite potent inhibitor against TMV on N.glutinosa, it was effective up to a dilution of 1:50, whereas at higher dilution 10^{-2} the inhibitory activity was completely absent. Hajj and Stevens (1979) reported that seeds of 15 species of legume inhibited the infection of plant by virus and lost their inhibitory activity on dilution.

Some workers have studied the effect of dilution on the enhancement of virus activity. Benda (1956) obtained evidence of an augmenter that increased virus activity in the juice of New Zealand spinach, this augmenter was identified indirectly as a soluble oxalate salt. It was reported that with a constant concentration of sodium oxalate (augmenter) and virus (tobacco ring spot) mixed with progressive dilutionsof inhibitor, there was an increased number of lesions until eventually more were produced than on controls. Blaszczak et al. (1959) showed that dilute plant extracts caused an increase in virus symptoms compared to controls, and suggested that dilution revealed the presence of augmenters of PVX infection in juices of G.globosa, Cucumis sativus, Lycoperiscon esculentum, N.tabacum, N.debneyi and P.vulgaris. It was also reported that diluting juices 1:10 with distilled water removed the inhibitory action partially or completely in most cases. Dilution of other juices, not only removed their

inhibitory action, but markedly increased PVX infection e.g. 55 % with tomato juice and 143 % with G.globosa juice. Stevens (1970) found that extracts prepared from the seeds of Lactuca sativa, Vicia faba, and Phaseolus aureus caused enhancement (augmentation) of virus activity and suggested that seeds contain both inhibitor and compounds favouring virus activity (augmenters).

Reduced inhibitor activity brought about by dilution suggests that extracts contain compounds that inhibit rather than inactivate virus. Thus virus and inhibitor must interact in such a way that the ability of the virus either to replicate, or to produce symptoms, is impaired. Dilution also reduces the chances of virus and inhibitor interacting. It appears also from the literature that plant extracts or some fractions of extracts may enhance virus activity. Enhancement often appears on dilution of plant sap.

Dilution of inhibitor extracts can therefore provide information regarding the activity of inhibitor compounds and may also reveal the presence of compounds favourable to virus activity. For this reason dilute extracts of Caryophyllaceae were tested against virus as well as the crude undiluted extracts.

Experimental procedures and results

Crude extracts were prepared and lyophilized as described earlier (Materials and Methods). The dried material was reconstituted by adding distilled water. Dilution of crude extracts were made with distilled water as follows:

1 : 10 , 1 : 100 , 1 : 1000

The final dilutions were x 2 of that above after adding an equal

volume of TNV. Controls consisted of distilled water instead of extract. Virus assay was made by counting local lesion production 3-5 days after inoculation on P.vulgaris leaves. Each treatment was replicated ten times on ten primary leaves, opposite leaves on each plant acting as controls. Each experiment was performed 2-3 times.

a) Effect of crude extracts

Results in Tables 2,3, 4 & 5 and Figures 2, 3, 4 & 5 show that all the species examined inhibited to some degree local lesion production by TNV on P.vulgaris. Undiluted crude extracts of twenty species totally eliminated lesion production giving 100 % inhibition. Some other species were less effective but still inhibitory. For example, crude extracts of Silene coeli-rosa, Gypsophila elegans, Cerastium biebersteinii, and Silene alpestris gave 56, 66, 69 and 71 % inhibition respectively.

b) Effect of dilution on the inhibitory activity

Following dilutions the extracts could be divided into two groups:

Group 1- Extracts in which inhibitory activity decreased on dilution

This group includes 18 species as shown in Tables 2 & 3 and Figure 2 & 3. Results show that all the species are affected by dilution and that with the decrease in inhibitor concentrations the inhibitory activity decreased, except in Cerastium tomentosum, Minuratia capillacea and Silene alpestris. With these extracts the initial dilution 10^{-1} produced a drop in percentage of inhibition, but further dilutions reversed the slope of the graphs.

The most powerful inhibitor extracts included D.caryophyllus, G.paniculata and S.ocymoides. These extracts still showed 50, 38 and

57 % inhibition respectively at dilution of 10^{-3} as compared to controls. Dilution of these extracts (Group 1) have established that they are acting as inhibitors and not inactivators.

Group 2- Extracts which enhanced virus activity on dilution

This group includes 12 species as shown in Tables 4 & 5 and Figures 4 & 5. Undiluted crude extracts of these species were very strong inhibitors against TNV. The percentage of inhibition reached about 100 % with these crude extracts, except G.elegans which gave 66 % inhibition. On dilution, these crude extracts showed virus enhancing activity at dilutions of 10^{-2} and 10^{-3} . Dilution of extracts from D.gratianopolitanus, D.monspessulanus and L.viscaria showed extremely high enhancement at dilution of 10^{-2} and 10^{-3} . The mean number of lesions were 212.1, 177.5 and 366.6 respectively at a dilution 10^{-3} as compared with their controls which gave 77.3, 58.1 and 82.5 lesions respectively.

It would appear that dilution of these extracts results in enhancing or augmenter compounds becoming evident because the inhibitor compounds have been diluted beyond their effective concentrations. It seems also that dilution of these extracts has revealed the presence of compounds enhancing virus activity, and the effect of such compounds oppos es the effect of inhibitors.

Discussion

These results suggest that virus-inhibitor compounds are present in the crude extracts of all the plants tested, but in varying quantities. However, the level of inhibitory activity in the majority of species was very high (twenty species showed total inhibition).

Table 2 : Effect of crude and diluted extracts from various species of (Group 1) Carophyllaceae on the infection of P.vulgaris by TNV

Source of extract	Mean number of lesions *					% Inhibition			
	TNV + water (comtrol)	Extract dilution							
		0	10^{-1}	10^{-2}	10^{-3}	0	10^{-1}	10^{-2}	10^{-3}
1- Cerastium biebersteinii Dc.	60.3	18.4	28.8	39.9	36.7	69	52	34	39
2- Cerastium tomentosum L.	67.7	3.8	53.3	39.8	39.8	94	21	41	41
3- Cucubalus baccifer L.	71.8	0	15.0	69.8	72.2	100	79	3	0
4- Dianthus arenarius L.	131.7	0	6.5	67.8	95.9	100	95	48	27
5- Dianthus caryophyllus L.	110.3	0	0	13.7	55.1	100	100	88	50
6-Gypsophila paniculata L.	136.9	0	0	19.4	84.9	100	100	86	38
7- Lychnis chalcedonica L.	83.9	0	7.5	66.8	67.3	100	91	20	20
8- Lychnis coronaria Desr.	79.0	0	21.5	59.2	79.1	100	73	25	0
9- Lychnis flos-jovis Desr.	80.1	0	0.6	26.4	51.7	100	99	67	35

* Each figure represents the mean number of lesions for ten leaves

Table 3 : Effect of crude and diluted extracts from various species of Caryophyllaceae on th infection of P.vulgaris by TNV

(Group 1)

Source of Extract	Mean number of lesions*					% Inhibition			
	TNV + water (control)	Extract dilution ·							
		0	10^{-1}	10^{-2}	10^{-3}	0	10^{-1}	10^{-2}	10^{-3}
10- Minuratia capillacea Graebn.	71.1	12.8	67.2	60.6	53.9	82	5	15	24
11- Saponaria ocymoides L.	53.3	0	0	11.9	23.0	100	100	78	57
12- Silene alpestris Jacq.	35.3	10.2	30.5	21.2	13.4	71	14	40	62
13- Silene coeli-rosa Godron.	90.3	39.5	52.2	90.2	79.1	56	42	0	12
14- Silene maritima With.	117.0	0	53.0	89.0	90.6	100	55	24	23
15- Silene saxifraga L.	111.8	1.7	27.3	66.0	76.3	98	76	41	32
16- Silene schafta Gmel.	82.2	5.3	45.0	60.3	59.4	94	45	27	28
17- Telephium imperati L.	131.7	0	32.4	67.3	102.3	100	75	49	22
18- Vaccaria pyramidata Medic.	57.2	0	1.2	18.2	58.0	100	98	68	0

* Each figure represents the mean number of lesions for ten leaves

Figure 2 : Effect of crude and diluted extracts from various species of Caryophyllaceae on the infection of P.vulgaris by TNV

Group 1 (see text)

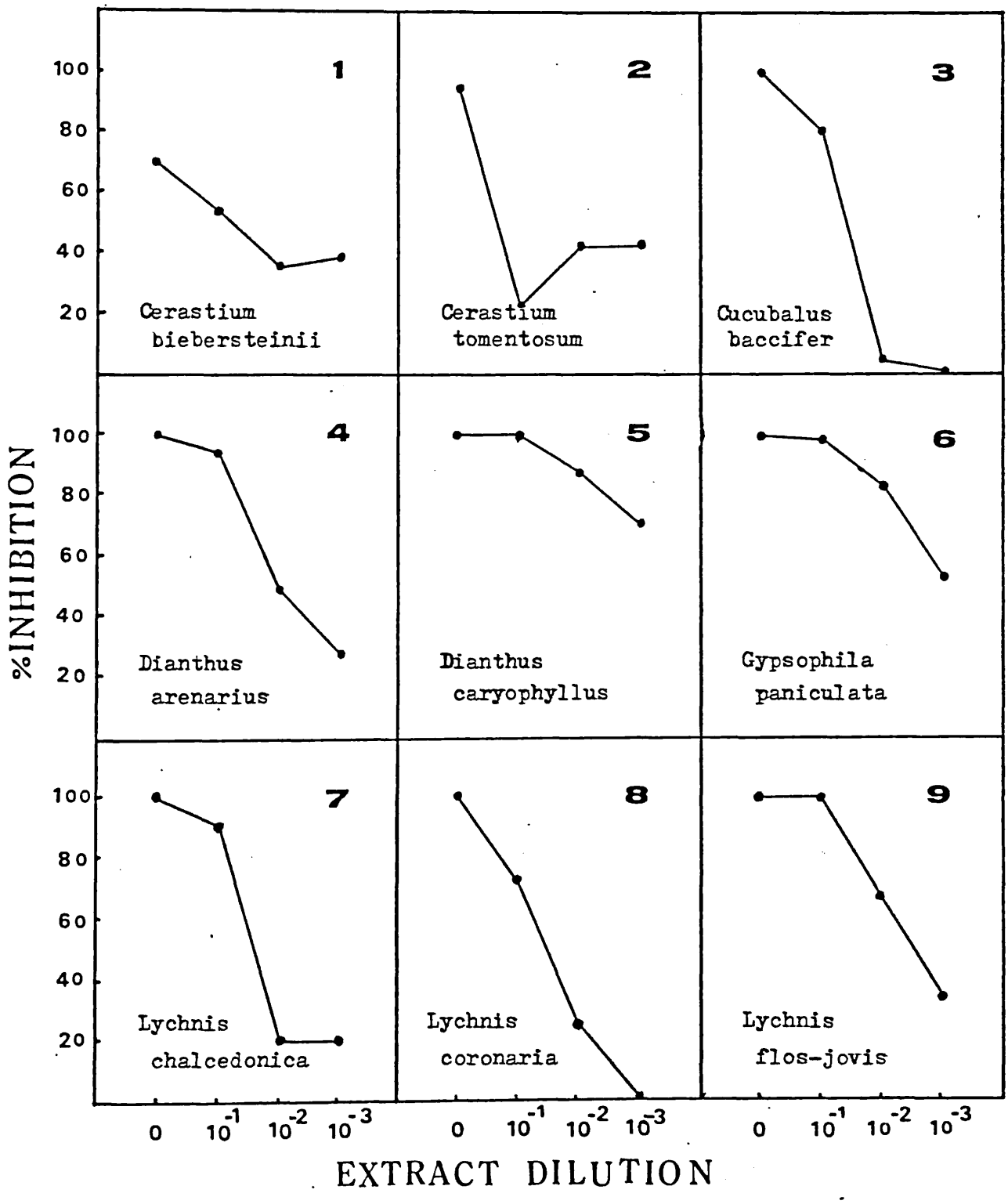


Figure 3 : Effect of crude and diluted extracts from various species of Caryophyllaceae on the infection of P.vulgaris by TNV

Group 1 (see text)

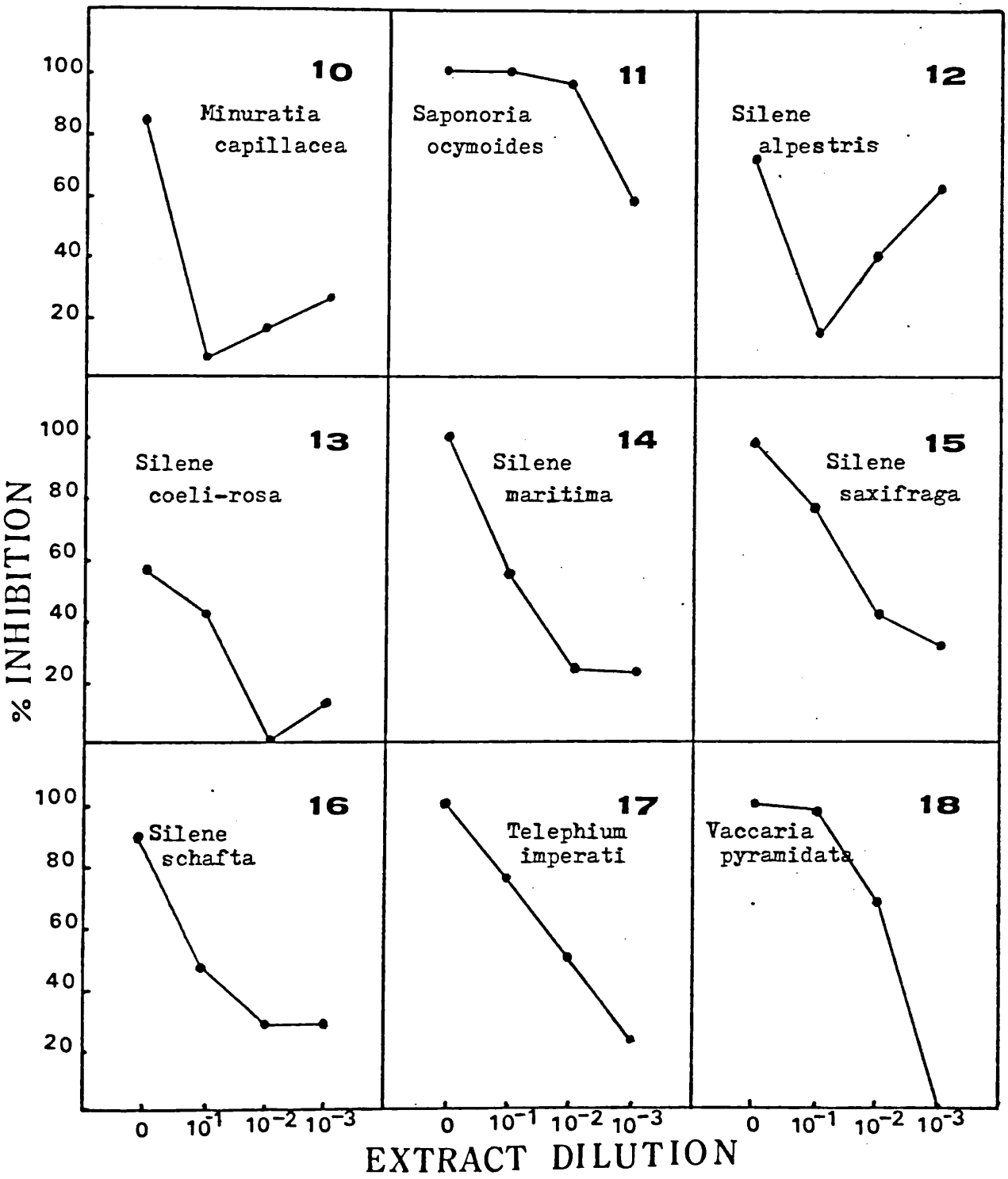


Table 4 : Effect of crude and diluted extracts from various species of Caryophyllaceae on the infection of P.vulgaris by TNV

(Group 2)

	Source of extract	Mean number of lesions *					% Inhibition			
		TNV + water (control)	Extract dilution							
			0	10^{-1}	10^{-2}	10^{-3}	0	10^{-1}	10^{-2}	10^{-3}
1	Arenaria balearica L.	41.8	0	22.0	45.6	59.2	100	47	-9	-42
2	Dianthus campestris Bieb.	25.6	0.4	9.8	28.9	33.2	98	62	-13	-30
3	Dianthus gratianopolitanus Vill.	77.3	0	52.1	157.7	212.1	100	33	-104	-174
4	Dianthus knappii Aschers.	112.8	0	4.9	66.1	203.1	100	96	41	-80
5	Dianthus monspessulanus L.	58.1	0	24.4	125.2	177.5	100	58	-115	-206
6	Dianthus petraeus Waldst. & Kit.	75.6	1.0	20.0	39.7	77.9	99	74	47	-3

* Each figure represents the mean number of lesions for ten leaves

Negative values of % inhibition = enhancement

Table 5 : Effect of crude and diluted extract from various species of Caryophyllaceae on the infection of P.vulgaris by TNV (Group 2)

	Source of extract	Mean number of lesions *					% Inhibition			
		TNV + water (control)	Extract dilution							
			1 0	10^{-1}	10^{-2}	10^{-3}	0	10^{-1}	10^{-2}	10^{-3}
7	Dianthus plumarius L.	105.7	0	28.5	90.1	147.5	100	73	15	-40
8	Gypsophila elegans Bieb.	53.8	18.3	80.3	55.2	74.3	66	-49	-3	-38
9	Herniaria glabra L.	98.1	0	36.8	66.3	115.0	100	62	32	-17
10	Lychnis viscaria L.	82.5	0	50.1	89.9	336.6	100	39	-9	-308
11	Petrorhagia saxifraga Link.	55.8	0	18.5	62.8	33.2	100	67	-13	41
12	Silene dioica Clairv.	23.6	0	6.0	28.1	28.6	100	75	-19	-21

* Each figure represents the mean number of lesions for ten leaves

Negative values of % inhibition = enhancement

Figure 4 : Effect of crude and diluted extracts from various species of Caryophyllaceae on the infection of P.vulgaris by TNV

Group 2 (see text)

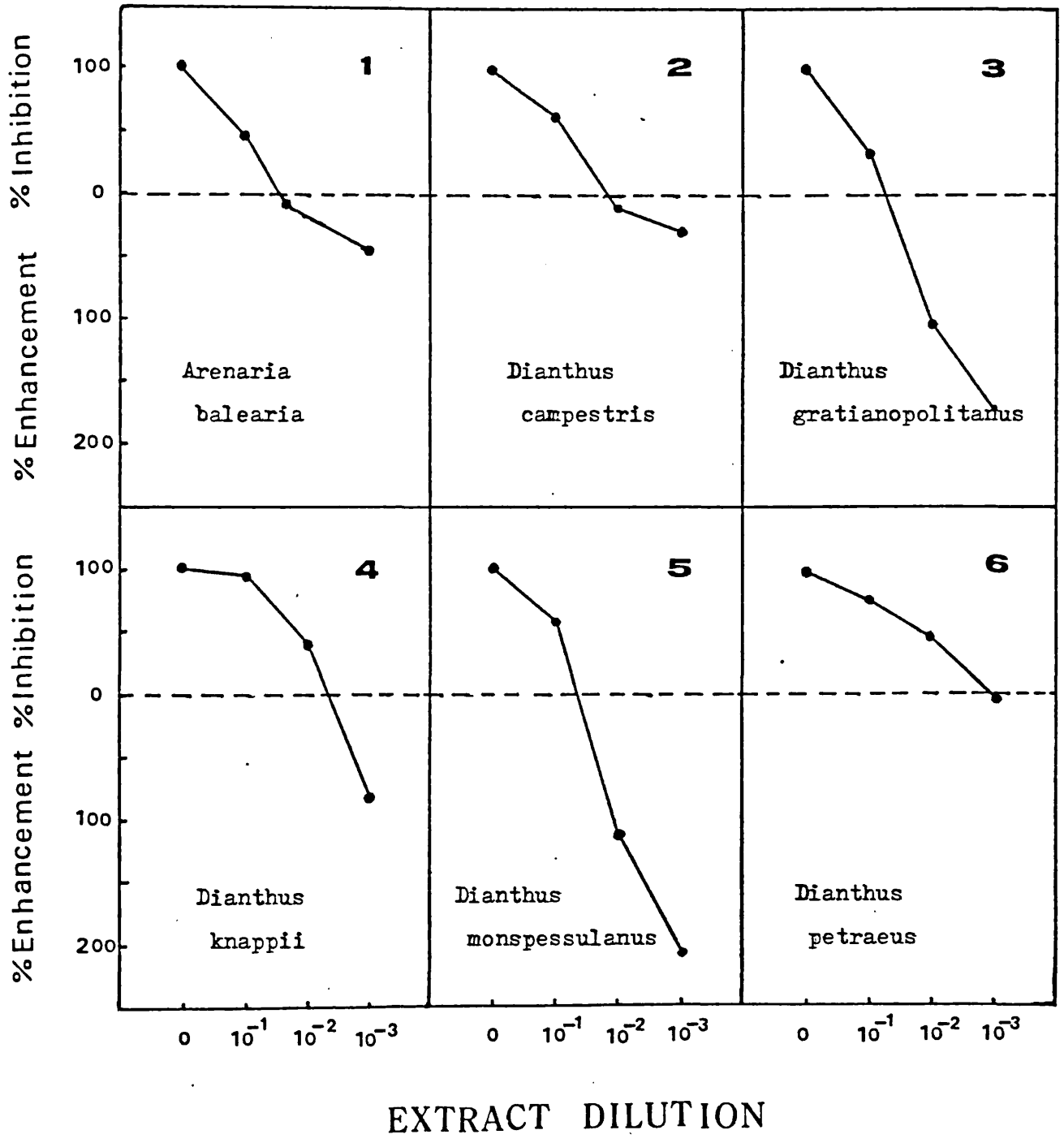
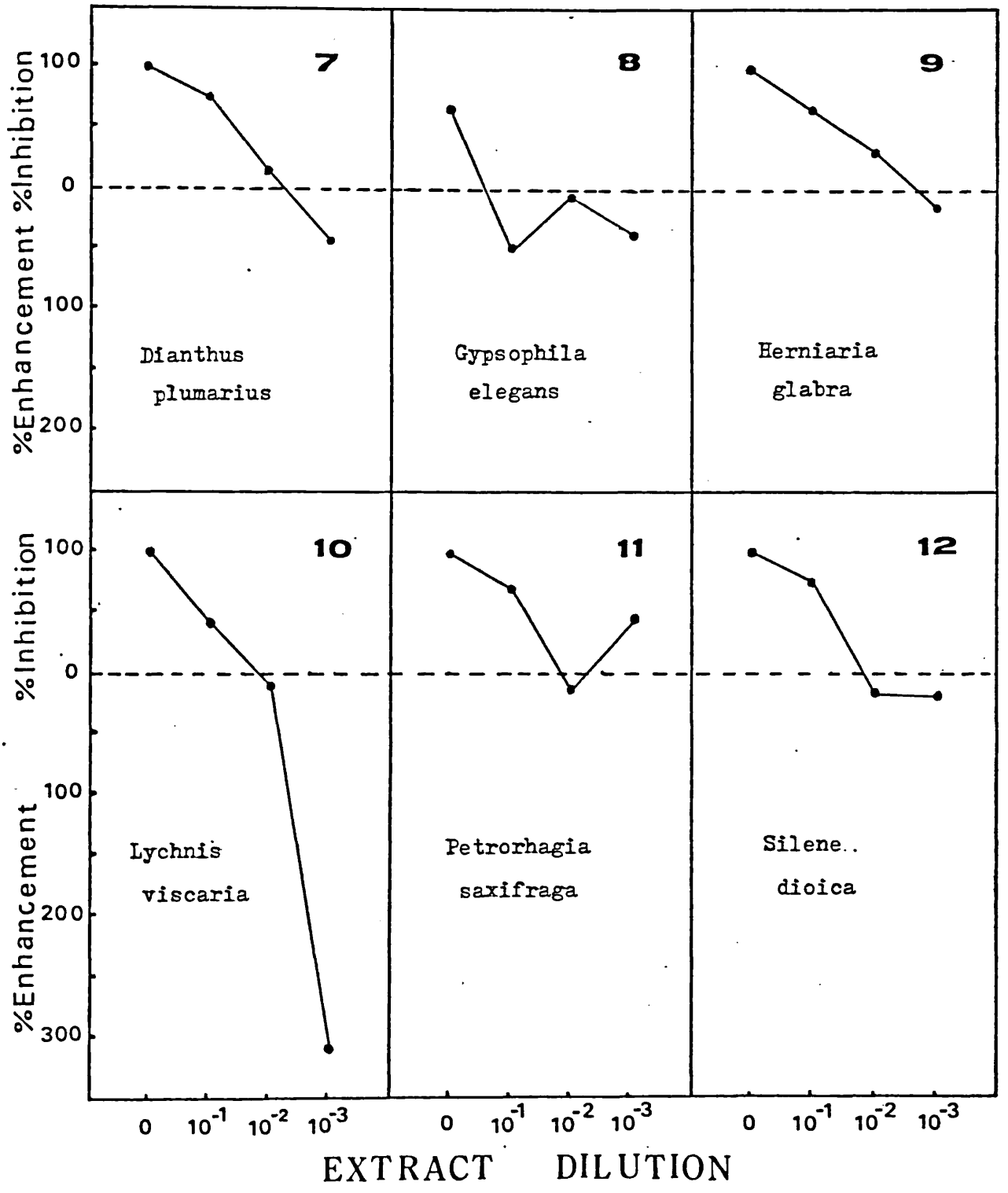


Figure 5 : Effect of crude and diluted extracts from various species of Caryophyllaceae on the infection of P.vulgaris by TNV

Group 2 (see text)



In extracts showing weak inhibitory activity, the effect of inhibitor compounds may either be poor, because the inhibitors are present in low concentrations, or their effect is reduced as a result of the antagonism of enhancing compounds. In only 4 out of 18 species, in the first group, in which dilution reduced the inhibitory activity, could it be shown that dilution to 10^{-3} with distilled water reduced the level of inhibition to zero.

Twelve of the thirty species examined gave extracts which on dilution produced enhancement of virus activity. These extracts presumably contain both inhibitors and virus-enhancing or augmenter compounds. The effect of virus-enhancing compounds can only be seen on dilution, since undiluted crude extracts of 11 out of 12 species (Group 2) showed total inhibition.

These results suggest that there are, at least, two materials in the extracts of some species, an inhibitor which decreases, and an augmenter which increases the number of lesions. The effect of these augmenter compounds seems to become apparent on dilution, and overcomes the effect of inhibitors which have been diluted beyond their effective concentration.

Compounds enhancing virus activity have previously been described from plants (see earlier). Various sugars((Kongsvik and Santilli, 1970), other compounds including DEAE-dextran (Hull, 1971) and alkali (Helms and Zaitlin, 1970) can also enhance virus activity,

The evidence produced in this survey showed that twenty of the species tested contain inhibitors equally, as powerful as, that from D.caryophyllus (Van der Want, 1951; Ragetli, 1957; Van Kammen et al.1961; Ragetli and Weintraub, 1962a and Nart, 1972).

CHAPTER IV

EFFECTS OF HEAT ON CARYOPHYLLACEAE EXTRACTS

Introduction

The effect of temperature on the inhibitory activity of plant extracts has been studied by many workers. Some reports indicate that heat treatment completely removes inhibitory activity, or reduces it, other indicate that heat may enhance virus activity.

One of the earliest reports by Johnson (1941) showed that Phytolacca rigida inhibitor extract was inactivated by boiling. Kuntz and Walker (1947) reported that heating spinach juice at 70 °C for 12 minutes, completely inactivated the inhibitor of TMV infection of Nicotiana glutinosa, although this juice resists dilution. However, this same inhibitor when used against cabbage mosaic virus was not destroyed by heating the extract at 125 °C for 15 minutes. Similarly, Takagi and Sugimura (1977) reported that the inhibitory activity of extracts of the fruit-bodies of Lentinus edodes was maintained after heat treatment at 120 °C for 20 minutes when tested against TMV.

A number of workers have tested the effect of temperature on Chenopodium species. Manil (1949) found that juice from Chenopodium bonus-henricus, which was inhibitory to TMV infection of P.vulgaris was inactivated by boiling for 4 minutes, but was still inhibitory after heating at 60 °C for 15 minutes. Alberghina (1976) showed that inhibitory activity of Chenopodium amaranticolor leaf extract to TNV on C.amaranticolor was thermostable to treatment at 60 °C for 10 min., although treatment at 80 °C reduced it action by about one half. Blaszczak et al. (1959) found that boiling the juices of spinach and

C.amaranticolor for 10 minutes had little effect on their inhibitory activity against PVX infection of Gomphrena globosa. On the other hand, Thomson and Peddie(1965); Yoshizaki and Murayama (1966) and Yoshii and Sako (1967) showed that boiling the sap from C.album for 10 minutes completely destroyed its inhibitory activity on N.glutinosa against TMV. However, Yoshii and Sako (1967) showed that the inhibitory activity of sap from C.album on daikon mosaic virus infection of White Burley tobacco was reduced by only 50 % after boiling for 10 minutes. Apablaza and Bernier (1972) demonstrated that the inhibitory activity of Jimsonweed extracts to TMV on Pinto bean leaves was not completely lost on boiling, but only reduced by about 35 % .

The effect of moderately high temperature on the inhibitory activity of plant extracts has been reported by several workers. Thomson and Peddie (1965) found no significant reduction in the effectiveness of C.album inhibitor after heating sap for 10 minutes at 55 °C, although no inhibitory activity remained in the sap which was boiled at 100 °C for 10 minutes. Similarly, Saksena and Mink (1969) found that the inhibitory activity of sap from C.quinoa was not affected by exposure to 70 °C, but at higher temperature the inhibitory activity decreased linearly. Inhibition was totally destroyed by boiling for 5 minutes. Yoshizaki and Murayama (1966); Yoshii and Sako (1967) showed that heating C.album extract for 10 minutes at 60 °C had no effect, but at 80 °C produced a slight decrease in inhibitory activity.

Smookler (1971) classified extracts from members of the Chenopodiales into two main types in relation to their thermal inactivation point: those which lost most of their activity after exposure to 60 °C - 80 °C

for 10 minutes (Beta nana, Halimione portulacoides and Amaranthus caudatus); and those which lost it only after 80 °C - 100 °C treatment (Chenopodium spp., Atriplex calotheca, Basella rubra, Hablitzia tamnoides, Salsola kali and Celosia plumosa). More recently, Noronha et al. (1980) found that the inhibitory activity in five Caryophyllales species varied when sap was mixed with TMV and inoculated onto N.glutinosa. Some lost their inhibitory action at 60 °C, others lost activity at 90 °C and others at 100 °C.

Zaitlin and Siegel (1963) showed that an inhibitor from the leaves of N.tabacum var. Turkish samsun was not inactivated by boiling. Similarly, Crowley (1955) reported that the inhibitor from Blue Prior tobacco seeds was heat stable. Taniguchi (1974) noted that inhibitor fractions of crude french bean extracts were heat stable at 100 °C for 6 minutes when inoculated onto P.vulgaris, N.glutinosa and C.album together with TMV. Ragetli (1957) reported that the inhibitor from Dianthus caryophyllus was thermolabile above 80 °C. Similar result was reported by Weintraub and Gilpatrick (1952) for D.barbatus. However, Ragetli and Weintraub (1962b) found that the purified inhibitor from D.caryophyllus was largely inactivated by heating at 70-80 °C for 10 minutes at pH 6.6 .

Some conflicting results have been obtained on the effect of temperature on the inhibitory activity of extracts from pepper. McKeen (1956); Blaszczak et al. (1959) and Marchoux (1967) found that the inhibitory activity from Capsicum annuum was thermolabile. Conversely, Feldman (1963) and Apablaza and Bernier (1972) found that it was thermostable.

The effects of temperature on seed extracts have been studied by Verma et al. (1975) who noted that diluted seed extracts of Lawsonia alba (1 : 10) when heated at 100 °C for 10 minutes were thermostable when tested against TMV on N.glutinosa. Stevens (1970) examined the effect of some legume seed extracts which enhanced virus activity. Such enhancement was reduced, but not completely eliminated by heating these seed extracts to 100 °C for 10 minutes. Recently, Hajj and Stevens (1979) divided 15 species of legume seed extracts into two groups; those in which inhibitory activity was reduced on heating, and those in which inhibitory activity was increased on heating.

In order to establish whether the Caryophyllaceae extracts contain compounds sensitive to heat (thermolabile), or insensitive to heat (thermostable) a series of experiments were performed on the effects of heat on the inhibitory activity of crude and diluted extracts. Heating of inhibitor extracts either undiluted or diluted were also undertaken to gain some information regarding the activity of inhibitor compounds.

Experimental procedures and results

Samples of lyophilized extracts of each species were reconstituted by adding 2 ml distilled water. Each was heated for 10 minutes in a water bath at 100 °C, and immediately cooled to about 25 °C with tap water. Extracts were mixed with an equal volume of standard TNV, and inoculated onto P.vulgaris.

Dilutions were made of extracts as above using distilled water as follows: 1 : 10 , 1 : 100 , 1 : 1000

2 ml of each dilution was heated by using the same method as described

above. An equal volume of standard TNV preparation was added to each tube. Controls consisted of water instead of extract. Each treatment was replicated ten times on ten primary leaves of P.vulgaris.

a) Effect of heat on crude extracts

Tne results shown in Tables 6 & 7, in terms of percentage inhibition, fall into four main groups:

Group 1- Extracts in which percentage inhibition was decreased by heating (thermolabile). This group includes 20 species (Table 6). The most marked decrease in the inhibitory activity by heating was shown by Dianthus campestris, D.petraeus, D.plumarius, Silene dioica, and Telephium imperati. Unheated crude extracts of these plants gave almost total inhibition. On heating, the percentage inhibition decreased to 50, 12, 52, 53, and 45 % inhibition respectively. On the other hand, unheated crude extracts from Gypsophila paniculata and Vaccaria pyramidata gave 100 % inhibition, but on heating their inhibitory activity was reduced to 94 & 91 % inhibition respectively.

Group 2- Extracts in which inhibitory activity was unaffected by heating (thermostable). This group includes 6 species (Table 7). Crude extracts of these species gave maximum inhibition (98-100 %) of local lesion production before and after heating. These results suggest that compounds other than protein molecules are responsible for inhibition of local lesion production by TNV.

Group 3- Extracts in which virus enhancement was obtained on heating

This group includes 3 species L.viscaria, D.knappii and L.coronaria (Table 7). Unheated extracts of these plants gave total inhibition. Heating the extracts produced enhancement of virus activity to 12, 28

Table 6 : Effect of heat on the virus inhibitor activity of crude extracts from various species of Caryophyllaceae

Source of extract (Group 1)	% Inhibition		Source of extract (Group 1)	% Inhibition	
	Unheated extract	Heated extract		Unheated extract	Heated extract
Cerastium biebersteinii Dc.	69	41	Cerastium tomentosum L.	94	69
Cucubalus baccifer L.	100	91	Dianthus arenarius L.	100	77
Dianthus caryophyllus L.	100	64	Dianthus campestris Bieb.	98	50
Dianthus gratianopolitanus Vill.	100	80	Dianthus monspessulanus L.	100	49
Dianthus petraeus Waldst.& Kit.	99	12	Dianthus plumarius L.	100	52
Gypsophila elegans Bieb.	66	25	Gypsophila paniculata L.	100	86
Silene alpestris Jacq.	71	58	Silene coeli-rosa Godron.	56	20
Silene dioica Clairv.	100	53	Silene maritima With.	100	91
Silene saxifraga L.	98	91	Silene schafta Gmel.	94	83
Telephium imperati L.	160	45	Vaccaria pyramidata Medic.	100	91

Table 7 : Effect of heat on the virus inhibitor activity of crude extracts from various species of Caryophyllaceae

	Source of extracts	% Inhibition		Source of extract	% Inhibition	
		Unheated extract	Heated extract		Unheated extract	Heated extract
Group 2 *	Arenaria balearica L.	100	98	Hernaria glabra L.	100	100
	Lychnis chalcedonica L.	100	100	Lychnis flos-jovis Desr.	100	99
	Petroorhagia saxifraga Link.	100	100	Saponaria ocymoides L.	100	100
G.3 *	Dianthus knappii Aschers.	100	-28	Lychnis coronaria Desr.	100	-112
	Lychnis viscaria L.	100	-12			
G.4 *	Minuratia capillacea Graebn.	82	95			

Negative values of % inhibition = enhancement

* See text

and 112 % more lesions respectively as compared with their controls. It seems that heating these extracts destroyed all of their inhibitory properties and the effect of enhancing compounds start to appear.

Group 4- Extracts in which the percentage inhibition was increased by heating. This group includes only one species (Minuratia capillacea). Unheated crude extract gave 82 % inhibition, heating the extract increased the inhibition to 95 %. Therefore, heat reveals the presence of an inhibitor which is stable to boiling at 100 °C for 10 minutes. It seems that the extract of this species contains an inhibitor and compounds which mask in some way its activity. On heating, this masking compound (s) is destroyed or inactivated resulting in greater inhibition by the thermostable inhibitor.

b) Effect of heat on dilute extracts

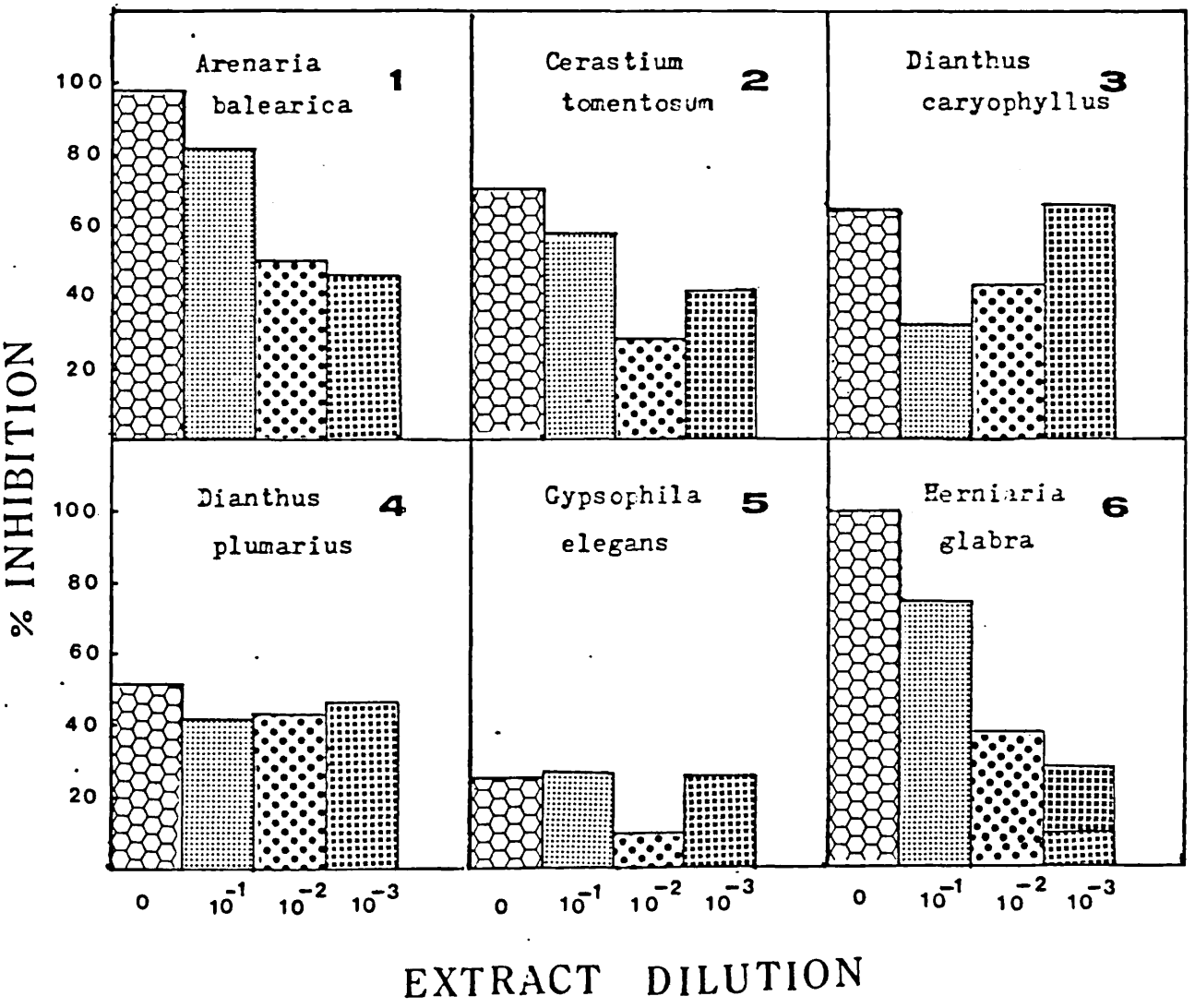
Few, if any, reports have previously been described concerning the effect of heat on the inhibitory activity of a progressive dilutions of plant extracts.

From the results in Chapter III, dilute extracts could be divided into two different groups. In group 1 (Tables 2 & 3) dilution decreased the inhibitory activity. In group 2 (Tables 4 & 5) inhibitory activity was completely destroyed by dilution, and at the same time the activity of some augmenters or enhancing compounds started to appear.

In this section, the effect of two factors together (dilution and heat) on the inhibitory activity of extracts is considered. From the results the extracts can be divided into two groups:

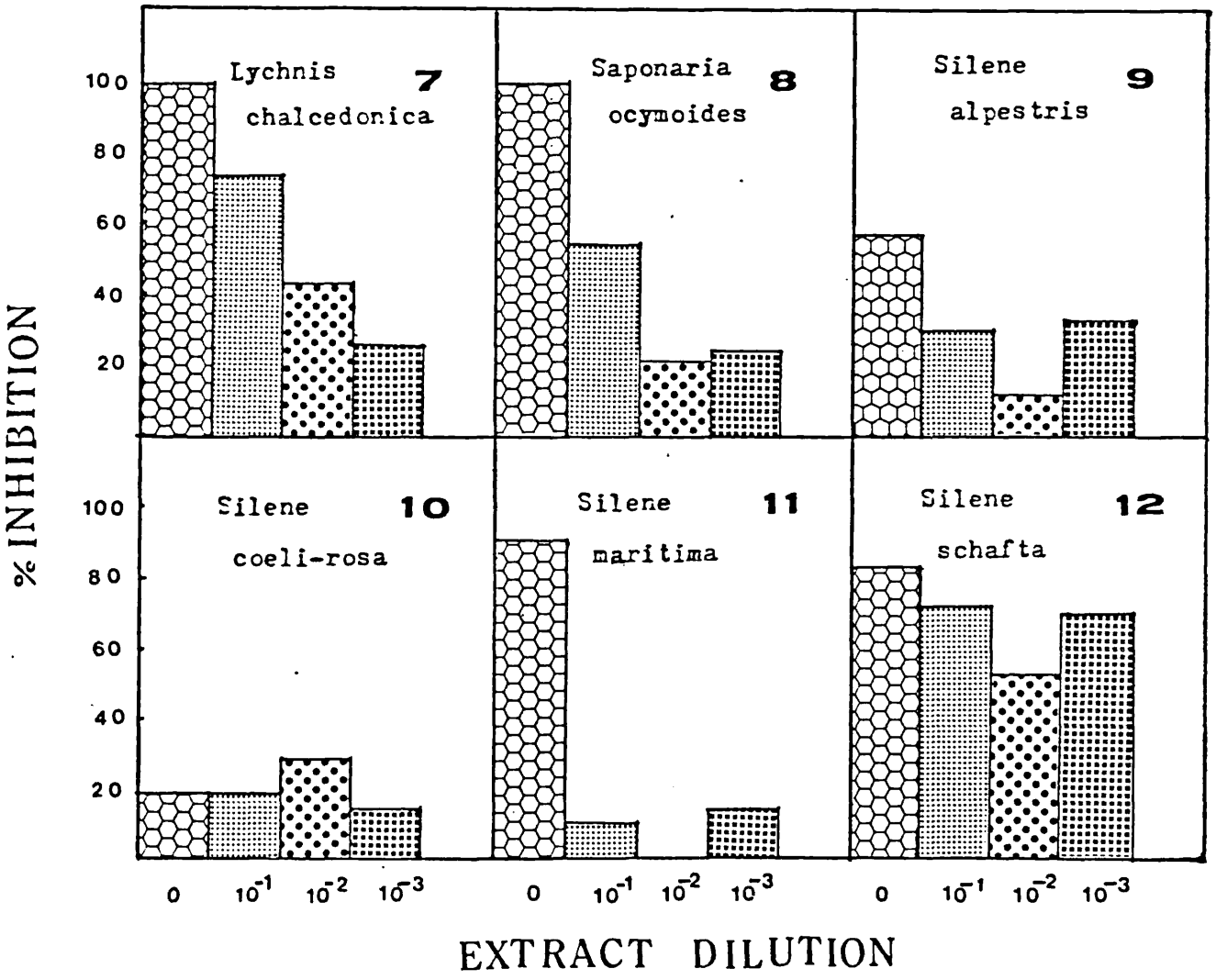
1) Diluted extracts in which inhibitory activity was further decreased by heating. The results in Figures 6 and 7 show that this group

Figure 6 : Effect of heat on the virus inhibitor activity of crude and diluted extracts from various species of Caryophyllaceae



Histograms show effect of extracts on local lesion production by TNV on P.vulgaris as a % of controls

Figure 7 : Effect of heat on the virus inhibitor activity of crude and diluted extracts from various species of Caryophyllaceae



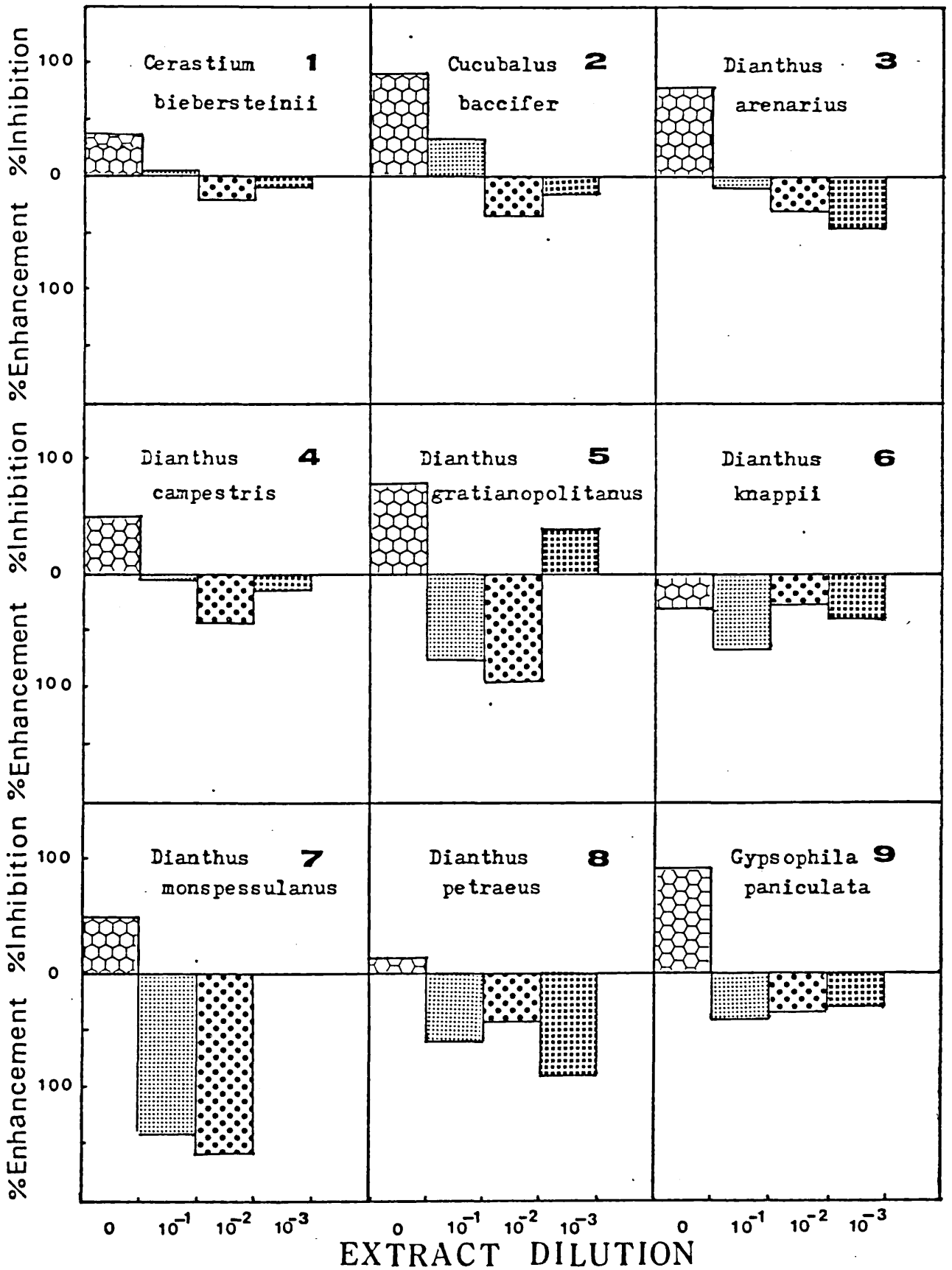
Histograms show effect of extracts on local lesion production by TNV on P.vulgaris as a % of controls

includes 12 species. In eight species, inhibitory activity decreased with dilution. Heat had slight effect on the inhibitory activity of diluted extracts of these species. In the other four species, A.balearica, D.plumarius, G.elegans and H.glabra, a 10^{-3} dilution of their extracts produced virus enhancing solutions (Tables 4 & 5), this shows that the inhibitor has been diluted beyond its effective concentration. On heating, a 10^{-3} dilution of these extracts produced considerable reduction in lesion numbers giving 46, 47, 26 and 31 % inhibition respectively. This shows that the augmenter is thermolabile, but at this concentration the inhibitors should have been too dilute to operate. Why then are these extracts inhibitory ? Possible answers include : 1- The breakdown product from the augmenter is inhibitory. 2- The inhibitors are modified by heating, for example, some inhibitor molecules may themselves be covered by masking compounds which are removed only by heating at dilute concentrations.

2) Diluted extracts which enhance virus activity on heating

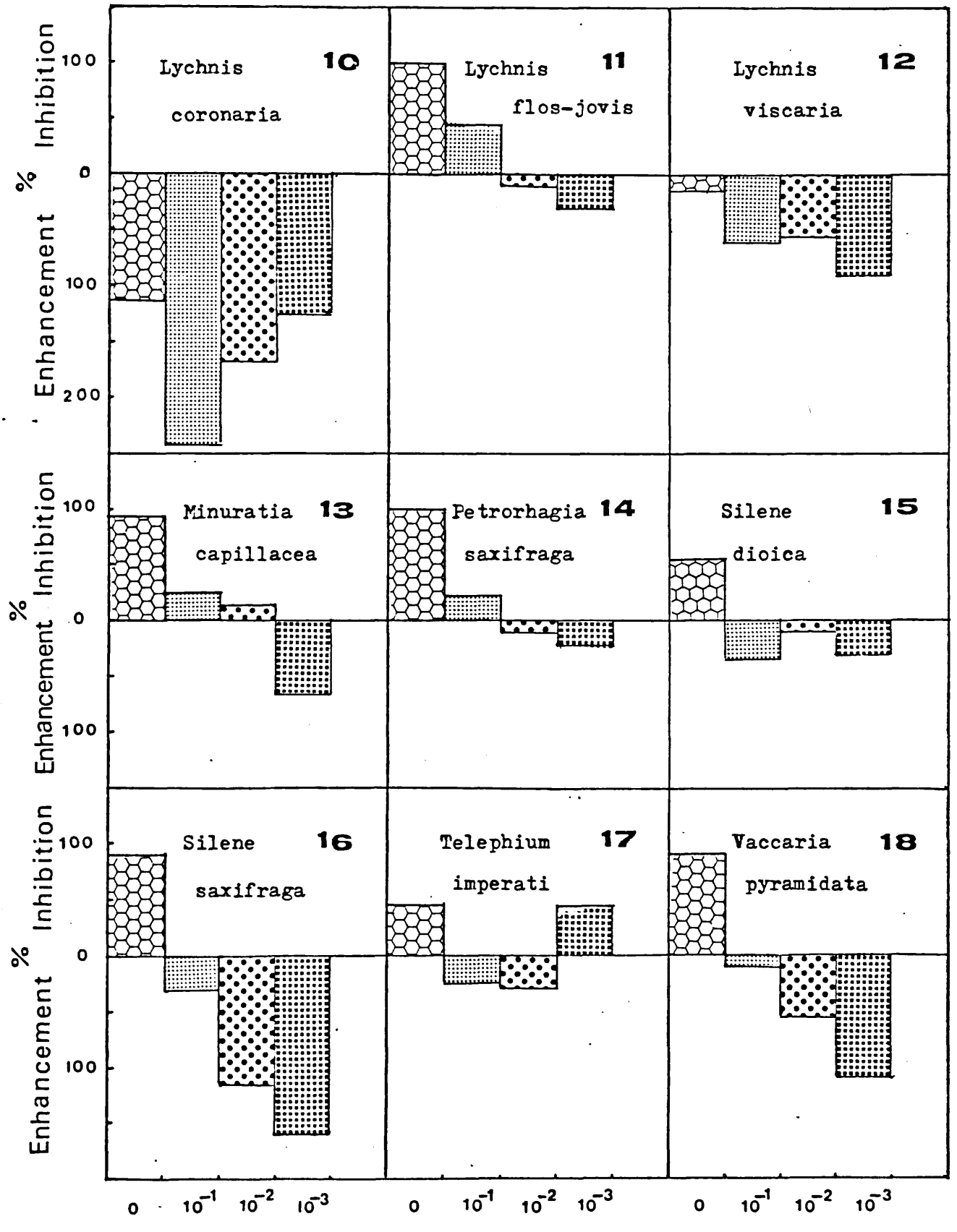
This group includes 18 species as shown in Figures 8 & 9. Eight of them on dilution already showed virus enhancing activity. In most of these species heat and dilution slightly decreased their activity. The virus enhancing properties of D.gratianopolitanus and D.monspessulanus were completely lost on heating a 10^{-3} dilution (Table 8). D.petraeus gave greater enhancement on dilution and heating. This was similar to the 10 remaining species (Table 9), where dilution decreased inhibitory activity, producing enhancement when heated. Telephium was unusual in that the enhancement of 10^{-1} & 10^{-2} heated extracts was lost on further dilution.

Figure 8 : Effect of heat on the virus inhibitor activity of crude and diluted extracts from various species of Caryophyllaceae



Histograms show effect of extracts on local lesion production by TNV on P.vulgaris as a % of controls

Figure 9 : Effect of heat on the virus inhibitor activity of crude and diluted extracts from various species of Caryophyllaceaa



Histograms show effect of extracts on local lesion production by TNV on P.vulgaris as a % of controls

Table 8 : Effect of heat on the virus inhibitor activity of diluted extracts from various species of Caryophyllaceae

Source of extract	% Inhibition					
	Unheated extract dilutions			Heated extract dilutions		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
Dianthus campestris	62	-13	-30	-6	-43	-13
Dianthus gratianopolitanus	33	-104	-174	-72	-93	41
Dianthus knappii	96	41	-80	-57	-24	-40
Dianthus monspessulanus	58	-115	-206	-138	-159	0
Dianthus petraeus	74	47	-3	-60	-41	-82
Lychnis viscaria	39	-9	-308	-66	-53	-88
Petrorhagia saxifraga	67	-13	41	22	-8	-21
Silene dioica	95	-19	-21	-36	-13	-28

Negative values of % inhibition = enhancement

Table 9 : Effect of heat on the virus inhibitor activity of diluted extracts from various species of Caryophyllaceae

Source of extract	% Inhibition					
	Unheated extract dilutions			Heated extract dilutions		
	10^{-1}	10^{-2}	10^{-3}	10^{-1}	10^{-2}	10^{-3}
Cerastium biebersteinii	52	34	39	5	-20	-10
Cucubalus baccifer	79	3	0	32	-38	-16
Dianthus arenarius	95	48	27	-9	-31	-46
Gypsophila paniculata	100	86	38	-38	-33	-30
Lychnis coronaria	73	25	0	-244	-167	-126
Lychnis flos-jovis	99	67	35	46	-10	-30
Minuratia capillacea	5	15	24	25	17	-65
Silene saxifraga	76	41	32	-28	-112	-167
Telephium imperati	75	49	22	-26	-29	46
Vaccaria pyramidata	98	68	0	-11	-55	-111

Negative values of % inhibition = enhancement

Discussion

The results described in this investigation indicate that the plant extracts examined contain different types of inhibitors. These results, as well as, those from other reports show that heat treatment decreases inhibitory activity in some cases and not in other. Some inhibitors are apparently thermolabile and other thermostable.

It appears that some of tested species contain both inhibitors and virus enhancing or augmenter compounds. The effect of heating crude or diluted extracts has established that there are augmenter compounds in some plant extracts. Compounds enhancing or augmenting virus multiplication have been reported by a number of workers (Benda, 1956; Blaszczak et al.,1959; Simons et al.,1963 and Stevens, 1970).

Our results are in agreement with Benda(1956) who obtained evidence of an augmenter in the juice of New Zealand spinach that had been boiled to remove inhibitors. He explained that the effect of augmenter might be due to: 1- An decrease in the viscosity of the outer protoplasm which might permit a more ready movement of the virus into the cell. 2- A delay in surface precipitation reactions, which might permit a more thorough mixing of inoculum and protoplasm. Lastly, 3- an increase in the permeability of the wounded cell membrane, so permitting easier entrance of virus into the cell.

Blaszczak et al. (1959) found that milder heat treatment did not reveal augmenters in the juices he tested, but some evidence of augmenters was obtained in tests with juices diluted sufficiently to remove their inhibitors.

Enhancement might come about by effects on the virus, on the host plant, or by neutralising the effects of virus inhibitors. This latter suggestion is supported, in part, by the observation of Benda (1956) who found that New Zealand spinach leaf extracts contain two active fractions, a virus inhibitor and an augmenter. It is not clear however, whether augmenters act by neutralization of inhibitors.

The conclusion from these experiments on the effects of heat on Caryophyllaceae extracts show that at least part of inhibitory activity probably resides in protein or glycoprotein because they are heat-sensitive (thermolabile). In those extracts, insensitive to heat (thermostable), the inhibitor may well be either polysaccharides or other non-proteinaceous substances.

Results in Chapters III & IV show that all thirty species examined inhibit to some degree local lesion production by TNV. However, time allowed a detailed study to be made of only two species Gypsophila paniculata and Minuratia capillacea. G.paniculata extracts showed powerful inhibitory activity which was slightly reduced on heating, and resisted dilution. In contrast, M.capillacea extract showed weak inhibition which increased on heating and was completely removed by dilution. These differences between the two extracts may be due to different activities of inhibitors, or to the presence in each of different inhibitor compounds.

The following chapters of this thesis describe attempts to study the general properties and chemical nature of G.paniculata extracts. The final chapter describes the induction of antiviral factors by these extracts. A comparison between the mode of action of G.paniculata and that of M.capillacea is also described.

CHAPTER V

GENERAL PROPERTIES OF GYPSOPHILA PANICULATA EXTRACTS

In this chapter experiments carried out to establish some general ideas about the properties of G.paniculata inhibitor extracts are described. Inhibitor extracts from Gypsophila paniculata will be referred to as Gp extracts and Gp inhibitor. For convenience, the results are described and discussed in two separate sections, A and B. In section A, studies were made of the effect of various dilutions of inhibitor extracts and of various dilutions of virus, as well as, the effects of storage, heat, pH and dialysis. The results from this section indicated that at least part of Gp inhibitory activity resides in protein. In section B, the effect of the protein precipitants ethanol and ammonium sulphate on inhibitors were studied, extracts were also examined by disc electrophoresis and subjected to treatment with proteinase enzymes.

SECTION A

PHYSICAL PROPERTIES

In order to establish whether the Gp extracts act as inhibitors or inactivators, the effect of progressive dilutions of such extracts on a constant concentration of virus, as well as, the effect of a fixed concentration of Gp extracts on different concentrations of virus have been studied.

1. Effect of progressive dilutions of Gp extracts on constant concentration of virus

In more extensive studies of Gp extracts the finding described in chapter III have been extended using a wide range of dilutions.

Experimental procedure

Lyophilized extracts, after reconstitution, were diluted with distilled water in a tenfold dilution series as described earlier (chapter III). Each dilution was mixed with an equal volume of TNV. Controls consisted of TNV inoculum and distilled water (1 : 1) .

Results

Dilutions of 10^{-1} and 10^{-2} gave highly significant inhibition (see Table 10, Figure 10 and Appendix). The 22 % inhibition shown by Gp extracts at 10^{-3} dilution was not significant and marks the dilution end point of the inhibitor, since further dilution gave 34 & 78 % more lesions. This enhancement of lesion numbers proved significant at P = 0.05 and 0.01 respectively.

2. Effect of Gp extracts on progressive virus dilutions

Caldwell (1935) showed that substances inhibiting plant infection by a virus could be distinguished from substances inactivating a virus because inhibitors have their greatest effect on concentrated inocula, whereas inactivators have their greatest effect on diluted inocula. For example, Crowley (1955) reported that a reduction in lesion numbers was consistently found to be greatest with concentrated inocula of cucumber mosaic virus when mixed with constant concentration of cucumber embryo extract, indicating that the extracts act as inhibitors. Similarly, Gupta and Raychaudhuri (1972) found that extracts of Callistemon lanceolata and Syzygium cuminii each showed significantly less inhibition when mixed with concentrated extract (1:1) of the virus giving 30 and 16 % inhibition respectively compared to 97 and 99 % inhibition with dilute (10 %) virus.

Table 10 : The effects of various dilutions of G.paniculata extracts on local lesion production in P.vulgaris by TNV

Extract dilutions	Mean number of lesions *		% Inhibition
	TNV + Water (control)	TNV + Extract (treated)	
" 0	96.3	0	100 "
10^{-1}	112.8	3.0	97
10^{-2}	102.6	24.7	76
10^{-3}	75.4	58.9	22
10^{-4}	84.8	113.7	-34
10^{-5}	127.8	227.6	-78

* Each figure represents the mean number of lesions for ten leaves

Negative values of % inhibition = enhancement

Full statistical treatment for this table is givin in the appendix 1

" Figures in brackets not included in statistical analysis "

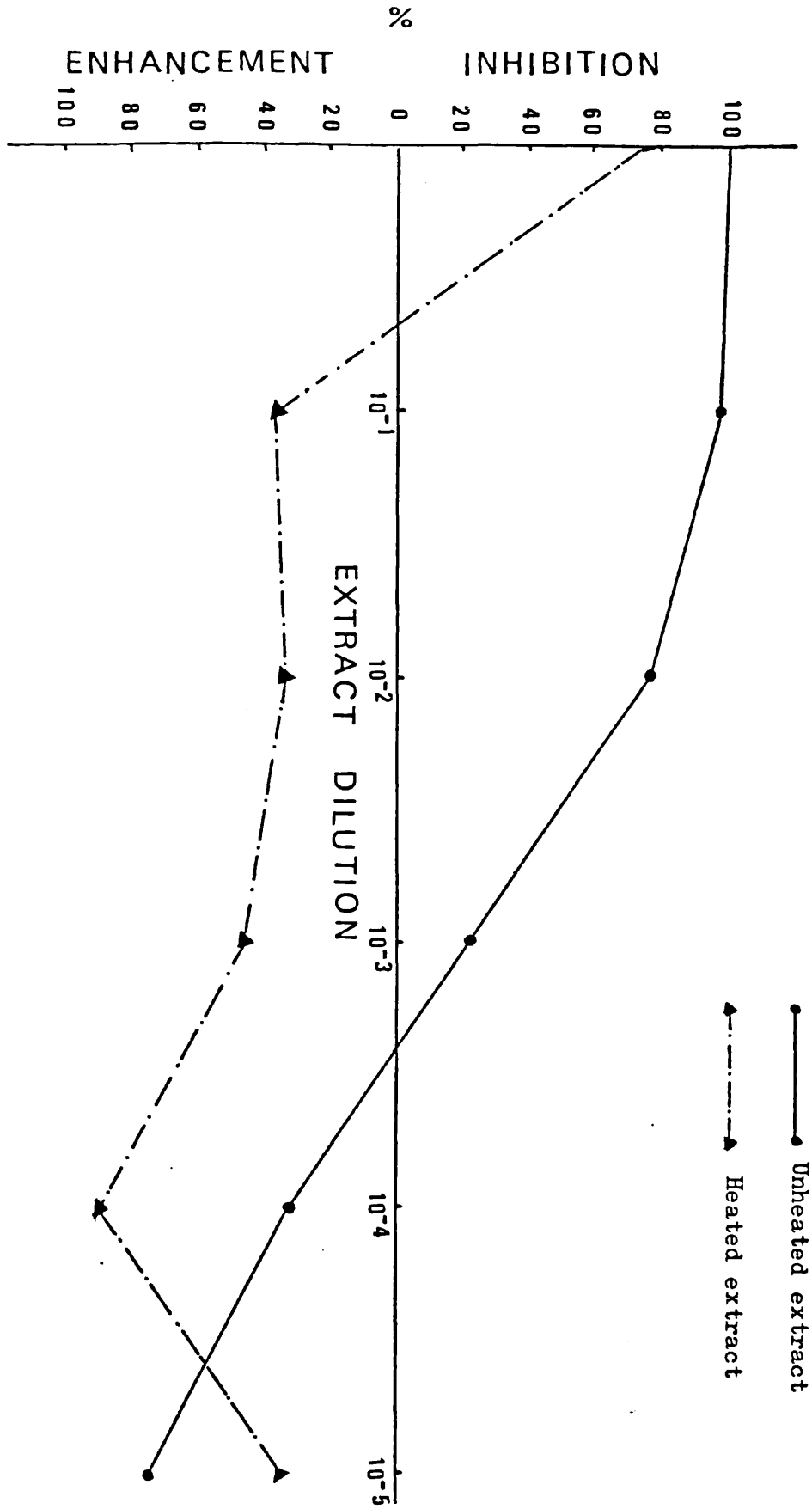


Figure 10: Effect of dilution on the inhibitory activity of G.paniculata extracts

Vicente et al. (1977) found that the inhibitor prepared from Chenopodium amaranticolor leaves acted on all inoculum concentrations of TMV. Similar results have been reported by Noronha et al. (1980) who showed percentage inhibition of Bougainvillea spectabilis and Mirabilis jalapa was the same in all virus dilutions used. However, the inhibitory action of Alternanthera ficoidea, Amaranthus deflexus and Chenopodium ambrosioides increased slightly with the increase of virus dilutions.

It has been reported that the inhibitors from some plant extracts are virus inactivators being more effective at higher dilutions of virus. Fischer and Nienhaus (1973) reported that the inhibitory activity of Capsicum annuum crude leaf extract, applied to variously aged leaves of mature Xanthi tobacco plants, was markedly increased when mixed with lower concentrations of TMV. However, when leaves of similar aged from young Xanthi tobacco plants were used, there was no effect on the inhibitor in any of the virus dilutions. Verma et al. (1975) found that seed extracts of Lawsonia alba when mixed with different dilutions of TMV showed the greatest inhibition at higher dilutions of virus.

Experimental procedure

Constant amounts of diluted Gp extracts (either 1:10 or 1:100) were added to serial dilutions of crude TNV in phosphate buffer pH 7.0 as follows: $0.5x10^{-2}$, $1.0x10^{-2}$, $1.5x10^{-2}$, $2.0x10^{-2}$, $2.5x10^{-2}$ and $3.0x10^{-2}$. In controls, Gp extract was replaced by water.

Results

Results in Table 11 show that the inhibitory activity of extracts acted in all dilutions of TNV. At higher concentrations ($2.5x10^{-2}$ and

Table 11 : The effect of dilute (1:10) G.paniculata extracts on local lesion production by various dilutions of TNV

Virus dilutions	Mean number of lesions*		% Inhibition
	TNV + Water (control)	TNV + Extract (treated)	
$0.5x10^{-2}$	19.4	0.2	99
$1.0x10^{-2}$	59.5	0.6	99
$1.5x10^{-2}$	95.7	2.7	97
$2.0x10^{-2}$	102.4	2.2	98
$2.5x10^{-2}$	155.8	9.3	94
$3.0x10^{-2}$	198.5	10.3	95

* Each figure represents the mean number of lesions for ten leaves

$3x10^{-3}$) there was no significant reduction in lesion numbers, this might be due to the extract being too concentrated. In further experiments, when Gp extracts were diluted 1:100 and then mixed with a series of virus dilutions, the results (Table 12 and Figure 11) showed that the reduction in lesion numbers was greatest at highest concentrations of virus, suggesting that Gp extracts act as virus inhibitors rather than virus inactivators.

3. Effect of storage

In order to examine the stability and highly potent inhibitory activity of Gp extracts, it was necessary to prepare bulk samples. These samples were stored at room temperature for several weeks for two reasons; one was to establish information regarding the stability of the active ingredients, and the second was to avoid exposing the extracts to changing temperature which would have resulted from storage at low temperature followed by thawing to room temperature.

Longevity and storage investigation of inhibitor extracts at room temperature have been reported by some workers. Kuntz and Walker((1947) found that the juice of spinach leaves did not lose its ability to inhibit any of several viruses during 15 months storage at room temperature. In contrast, Weintraub and Willison (1953) reported that juices from 4 species of Cucurbita lost their ability to inhibit certain stone-fruit viruses within 4-8 hours at the same temperature. Verma and Awasthi (1979) found that Boerhaavia diffusa root extracts survived at 20 °C ± 6 for 20 days. Similarly, Thomson and Peddie (1965) reported that the Chenopodium album inhibitor did not lose its activity during storage at 20 °C for 6 days. Saksena and Mink (1969) showed also the inhibitory activity from Chenopodium quinoa was

Table 12 : The effect of dilute (1:100) G.paniculata extracts on local lesion production by various dilutions of TNV

Virus dilutions	Mean number of lesions *		% Inhibition
	TNV + Water (control)	TNV + Extract (treated)	
$0.5x10^{-2}$	25.0	8.0	68
$1.0x10^{-2}$	54.9	19.3	65
$1.5x10^{-2}$	83.4	33.8	59
$2.0x10^{-2}$	108.2	41.8	61
$2.5x10^{-2}$	145.8	65.8	55
$3.0x10^{-2}$	182.6	89.0	51

* Each figure represents the mean number of lesions for ten leaves

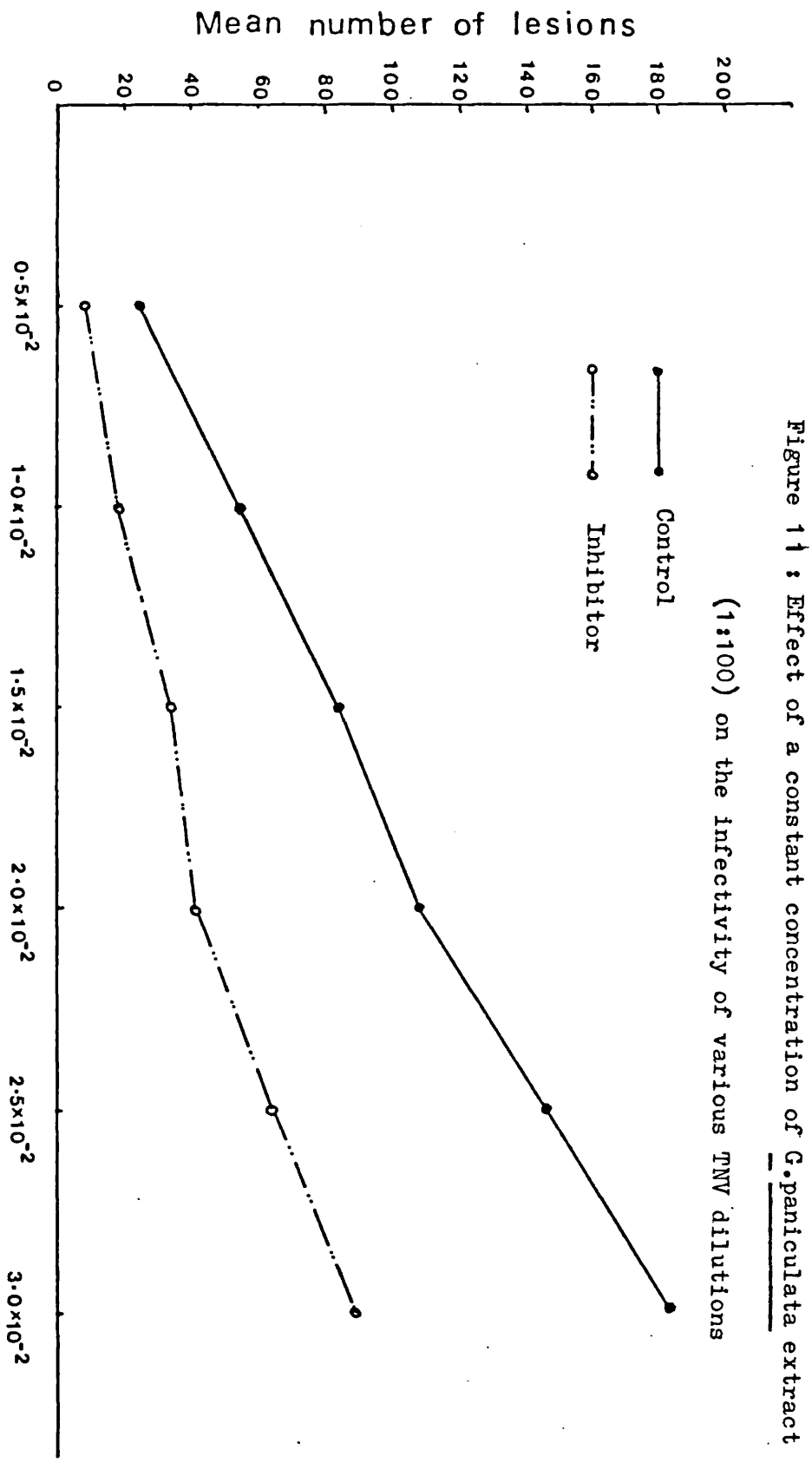


Figure 11 : Effect of a constant concentration of G.paniculata extract (1:100) on the infectivity of various TNV dilutions

unaffected by storage for 120 hours at room temperature.

Effect of lower temperature on the stability of some plant virus inhibitors has been reported by Blaszczak et al. (1959) who found most of 26 plant inhibitors retained their activity against PVX after several weeks storage at 4 °C, however, Datura stramonium extracts lost some inhibitory activity after storage for 48 days. Noronha et al. (1980) found that the inhibitory activity of Caryophyllales species only changed after 4 months at 5 °C. However, extracts from B.spectabilis, A.ficoidea and M.jalapa still presented a high inhibitory activity after 12 months. Smookler (1971), on the other hand, reported that the inhibitory activity of sap from C.album, Atriplex nitens, Beta trigyna, Amaranthus aureus and A.caudatus was not lost by storage for 56 days at either 4 °C or -25 °C. However, sap of Chenopodium botrys lost at least 50 % of its activity on storage under the same conditions.

Experimental procedure

Gypsophila extracts were stored in sterilized glass vials at room temperature(25 °C ± 5)for periods of 2, 7, 14, 21, 28, 35 and 60 days. After the appropriate time intervals equal volumes of the extracts and TNV were mixed and inoculated onto French bean leaves. Controls consisted of TNV mixed with distilled water.

Results

Crude undiluted G.paniculata extracts did not lose any of their ability to inhibit TNV on storage for two months at room temperature (Table 13). These results suggest that the extracts are very stable even after 60 days storage at room temperature.

Table 13 : Effect of storage at room temperature on the inhibitory activity of G.paniculata extracts upon the infectivity of TNV on P.vulgaris

Period of storage (days)	Mean number of lesions *		% Inhibition
	TNV + Water (control)	TNV + Extract (treated)	
2	68.3	0	100
7	117.9	0	100
14	93.6	0	100
21	78.3	0	100
28	46.4	0	100
35	49.0	0	100
60	79.7	0	100

* Each figure represents the mean number of lesions for ten leaves

4. Effect of heat

Many workers have been studied the effect of heat on the inhibitory activity of plant extracts (see introduction; chapter IV). Few reports, if any, have previously described the effects of heat on the inhibitory activity of progressive dilutions of plant extracts. Such experiments may be of particular value where a number of compounds are influencing virus replication. If, for example, extracts contain a variety of inhibitors; some sensitive to heat and other insensitive, then heating dilute extracts reveal heat sensitive inhibitors. Similarly, dilution may substantiate the idea given by earlier experiments that extracts contain augmenter compounds; heating dilute extracts will show whether such augmenters are thermostable.

Experimental procedure

Tenfold dilutions of Gp extracts were prepared and their effect on local lesion production compared with similar dilutions heated to 100 ^{o}C for 10 minutes using methods described previously(see Figure 10).

Results

Results in Table 14 and Figure 10 show that the inhibitory activity of crude Gp extracts was decreased to 79 % by heating, compared to the 100 % inhibition given by unheated crude extracts. This leads to the conclusion that Gp extract is in part thermolabile. Heating the progessive dilutions of extracts revealed the presence of thermostable enhancing compounds. Enhancement of virus activity was shown at all the dilutions used. At 10^{-4} dilution the effect of virus enhancing compounds reached the maximum value giving 90 % more lesions compared to its control.

Table 14 : Effect of heat on the virus inhibitor activity of crude and diluted extracts from G.paniculata

Extract dilutions	Mean number of lesions *		% Inhibition
	TNV + Water (control)	TNV + Extract (treated)	
0	92.3	19.8	79
10^{-1}	112.8	156.2	-38
10^{-2}	110.5	147.2	-33
10^{-3}	127.8	186.8	-46
10^{-4}	84.8	160.7	-90
10^{-5}	93.6	125.6	-34

* Each figure represents the mean number of lesions for ten leaves

Negative values of % inhibition = enhancement

5. Effect of pH

Studies of the effect of pH on the inhibitory activity of some plant extracts have been reported by a number of authors. Yoshizaki and Murayama (1966) reported that the inhibitor extracted from C.album was stable in solutions between pH 3.0 - 10.0, it gave a constant degree of inhibition over this wide pH range. Similar results were reported by Singh and Gupta (1970) who found that the inhibitor from Psidium guaiava bark was not affected over the pH range 4.0 - 10.5. Gupta and Raychaudhuri (1972) found that the inhibitor from Callistemon lanceolatus and Acacia arabica was not affected over the same pH range. Demski and Chalkley (1977) also noted that the inhibitor isolated from watermelon remained active over a pH range 2.0 - 12.0.

Verma et al. (1975) found that the inhibitory activity from Lawsonia alba extract was very pronounced at acidic or neutral pH, but at pH 10 the virus activity was completely restored. Similarly, Fukaya and Taniguchi (1979) found that the inhibitor extracted from Phytolacca americana was completely destroyed by alkaline treatment but not by acidic treatment. In contrast, Verma and Awasthi (1979) reported that inhibitor from Boerhaavia diffusa root extracts was not destroyed at pH 10, but inactivated at pH 4.0.

Change in pH may, however, have direct influence on the virus. Some previous reports indicate that pre-inoculation treatment of the virus with alkali reduces infectivity (Schramm et al., 1955; Diener and Desjardins, 1966). Other reports indicate that alkali treatment

increases virus infectivity (Bawden, 1950; Kleczkowski, 1957; Lippincott, 1958; Helms and Zaitlin, 1970).

In view of these interesting reports and to gain more information about the nature of Gp inhibitor the effect of pH on the inhibitory activity of extracts from Gypsophila was studied. Phosphate buffer only was used since buffers containing phosphate increase in some way the number of local lesion produced by viruses (Yarwood, 1952), and components in other buffers often show adverse effects on local lesion production.

Experimental procedure

Lyophilized extracts of Gp were dissolved in Sorensen phosphate (0.06 M) buffer at pHs 5.4, 6.0, 7.0 and 8.0. These extracts were then diluted 1:10 and 1:100 in more buffer. Each extract dilution was mixed with TNV and inoculated onto P.vulgaris. Controls consisted of 2 ml of TNV and 2 ml of corresponding phosphate buffer.

Results

Results in Table 15 show that there was no effect on the inhibitory activity of the extracts (diluted 1:10) over the pH range 5.4 - 8.0, each gave about 100 % inhibition compared to controls. On the other hand, when Gp extract was diluted 1:100, there was little change in the inhibitory activity between pH 5.4 - 8.0 (Table 16).

Increased pH caused an increase in local lesion production for both controls and experimental treatments in a similar manner to that described by Helms and Zaitlin (1970). However, the percentage reduction of lesions by the Gp extracts remained the same at each pH suggesting that there was no effect of pH on Gp inhibitory activity.

Table 15 : Effect of pH on the inhibitory activity of G.paniculata extracts diluted (1:10) with phosphate buffer

Buffer pH	Mean number of lesions *		% Inhibition
	TNV + Buffer (control)	TNV + buffered diluted extract	
5.4	54.8	0	100
6.0	45.1	0	100
7.0	124.1	0.5	99
8.0	188.6	1.8	99

* Each figure represents the mean number of lesions for ten leaves

Table 16 : Effect of pH on the inhibitory activity of G.paniculata extracts diluted (1:100) with phosphate buffer

Buffer pH	Mean number of lesions *		% Inhibition
	TNV + Buffer (control)	TNV + buffered diluted extract	
5.4	18.5	6.1	67
6.0	33.2	9.5	71
7.0	36.5	10.8	70
8.0	52.7	18.9	64

* Each figure represents the mean number of lesions for ten leaves

6. Effect of dialysis

Some reports have been made on the effect of dialysis on inhibitor activity in order to give some information regarding the types and sizes of molecules involved in inhibitor extracts.

Kuntz and Walker (1947) reported that the inhibitor of TMV extracted from spinach leaves appears to be non-dialysable. On the other hand, the inhibitor of cabbage mosaic virus was completely dialysable. Takagi and Sugimura (1977) found that the inhibitory activity of Lentinus edodes extract to TMV infection was diminished by dialization, and the activity was detected in the external water around the dializing bag. Verma and Verma (1965) also reported the presence of a dialysable inhibitor in wheat seed extracts.

Apablaza and Bernier (1972) separated the extracts from pepper and geranium into two fractions; one with molecular weight greater than 50,000 which was retained by the dialysis membrane, and a second with a molecular weight range of 1,000 - 50,000 which was present in the filterate. They found both residue and filterate from extracts of these two plants inhibitory to TMV. These authors also showed the inhibitor from jimsonweed to be present in an high molecular weight fraction.

Many reports describe inhibitor extracts to be non-dialysable, including the inhibitors from Cucumis sativus and Blue prior tobacco seeds (Crowley, 1955), Pepper (McKeen, 1956; Marchoux, 1967), Dianthus caryophyllus (Ragetli, 1957), Rice (Feldman, 1963), and Chenopodium amaranticolor leaves (Thomson and Peddie, 1965). Other workers have also shown that inhibitors from C.album (Yoshizaki and Murayama, 1966), and C.quinoa (Saksena and Mink, 1969) are not dialysable. Inhibitors

studies of 13 species from the family Chenopodiales, showed that dialysis had virtually no effect on the inhibitors present (Smookler, 1971). Recently, Verma and Awasthi (1979) reported that the inhibitory principle in extracts of Boerhaavia diffusa root was also non-dialysable.

Experimental procedure

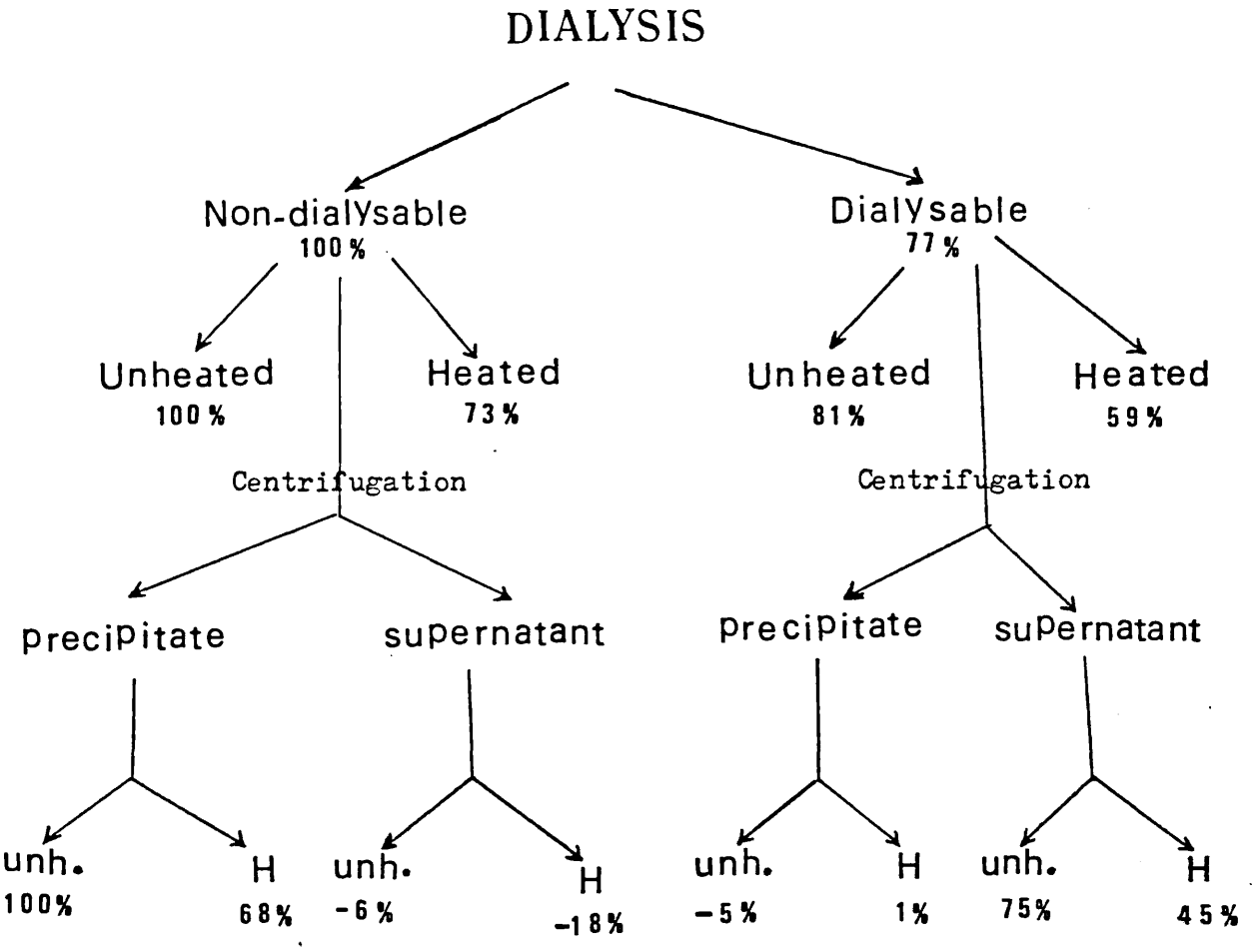
Samples of Gp extracts (10 ml) prepared from lyophilized preparations were dialysed using Visking tubing (size 1-8/32"). The dialysis sacs were subjected to dialysis against one liter of distilled water at 4 °C . The solution was agitated by means of a magnetic stirrer.

In order to test whether the samples contained active small molecular weight compounds, the volume of dialysing water containing the dialysable material was reduced to 10 ml by rotary evaporation at 40 °C. Two ml of each of the dialysable and non-dialysable parts were mixed with 2 ml of TNV inoculum and applied to P.vulgaris. Controls consist of 2 ml of water added to 2 ml of TNV. In subsequent experiments, both dialysable and non-dialysable fractions were centrifuged at 2,000 x g for 15 minutes and the effectiveness of both the supernatant and precipitate were examined against TNV. The effect of heat on dialysable and non-dialysable fractions and also on the supernatants and precipitates produced by centrifugation of these extracts were studied as shown in Figure 12.

Results

The percentage inhibition produced by each sample is shown in Figure 12. Substances passing through the Visking tubing referred to as small, or low molecular weight molecules. Substances retained

Figure 12 : Scheme of the investigation of the properties of dialysed Gypsophila paniculata extracts.



% values refer to virus inhibition

-% values refer to virus enhancement

within the dialysis sac are non-dialysable and referred to as large, or high molecular weight molecules.

Results in Table 17 and Figure 12 show that the large molecules gave total inhibition (100 %), heating this fraction of the extracts produced a slight reduction in the inhibitory activity giving 73 % inhibition. On the other hand, the small molecule fraction was inhibitory,but with less effect than the large molecules, giving 77 % inhibition. Heating this fraction slightly reduced its inhibitory activity to 59 %. After centrifugation to separate the large molecules into precipitate and supernatant, inhibition was found to be present in the precipitate, which gave 100 % inhibition. On heating this was reduced to 68 % inhibition. The supernatant of non-dialysable fraction had no inhibitory activity, it showed a slight enhancement of virus activity both before and after heating. Centrifugation of the dialysable fraction showed that the supernatant retained the inhibitory activity giving 75 % inhibition, this percentage was slightly reduced on heating. The precipitate, however, either heated or unheated had no inhibitory effects.

It was found from the protein determination that the whole extract contained 30 mg/ml protein, the non-dialysable fraction contained 18.3 mg/ml protein; whereas the dialysable fraction contained 9.4 mg/ml protein. Further analysis of the fractions were made in order to establish whether any of the carbohydrates might be bound to protein in the form of glycoprotein.

Polyacrylamide electrophoresis was carried out, as described earlier, on the non-dialysable, dialysable, and for comparative purposes, on the

Table 17 : Effect of heat and dialysis on the virus inhibitory activity of G.paniculata extracts

G.paniculata extract		Unheated extract		% Inhibition	Heated extract		% Inhibition
		Mean number of lesions*			Mean number of lesions*		
		TNV + water	TNV + extract		TNV + water	TNV + extract	
Non-dialysable part	Non-dialysable	121.5	0	100	121.5	32.5	73
	Supernatant	61.7	65.3	-6	61.7	72.6	-18
	Precipitate	105.0	0	100	105.0	33.5	68
Dialysable part	Dialysable	123.3	27.8	77	123.3	50.0	59
	Supernatant	63.8	15.9	75	63.8	35.0	45
	Precipitate	75.2	78.9	-5	75.2	74.8	1

* Each figure represents the mean number of lesions for ten leaves

whole extract. Electrophoresis of the whole extract produced seven protein bands and 4 bands of glycoprotein. The non-dialysable fraction gave an almost identical pattern of bands (Figure 13). It can be concluded that at least some of the proteinaceous material in the non-dialysable fraction is glycoprotein. In repeated experiments it proved impossible to narrow the glycoprotein bands so as to know more precisely to which of the protein bands each corresponded.

The dialysable fraction did not stain in the electrophoresis experiment although it gave positive protein and carbohydrate tests. The compounds in dialysable fraction staining for protein, might be amino acids since the Lowry et al. method of protein determination is known to give positive reaction with some amino acids. The presence of amino acids and small carbohydrate molecules in the extracts was tested using ninhydrin reagent and Molish's test respectively.

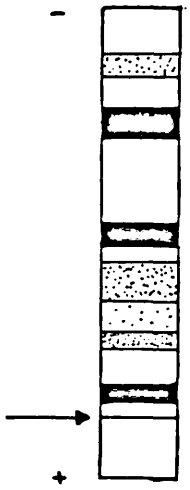
Bearing in mind reports that plant RNA (Ribonucleic acid) may act as virus inhibitors (Sela et al., 1966; Kimmins, 1969) the Gp extracts were examined for nucleic acids by ultraviolet spectrophotometry. Following clarification by centrifugation and by using a Unicam SP 800 recording spectrophotometer, maximum absorption were obtained at 275 nm and 231 nm corresponding to proteins and glycoproteins (Smith, 1970). These results confirm the absence of nucleic acids in the extracts and give further support to the idea that proteins and other small molecular weight compounds are responsible for the inhibitory activity of the extracts.

Finally, it can be concluded from dialysis experiments that:

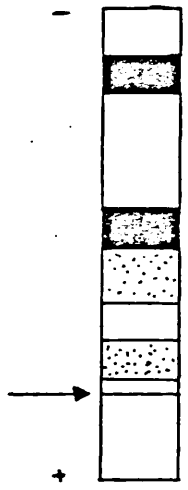
a) The inhibitory activity of G.paniculata extracts is found in both

Figure 13 : Disc electrophoresis of the proteins and glycoprotein from G.paniculata extracts

Whole Gp extract

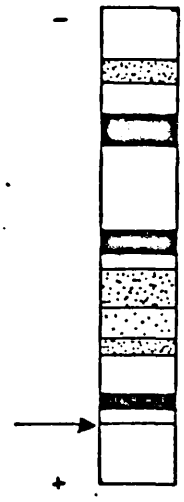


Proteins

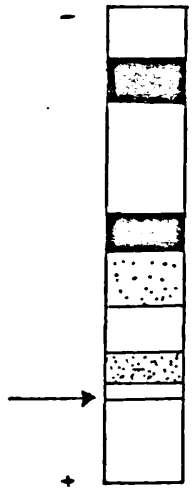


Glycoproteins

Non-dialysable fraction



Proteins



Glycoproteins

Dialysable fraction

No bands detected of either proteins or glycoproteins

⟶ Position of marker

Shading indicates intensity of band staining

large and small molecular weight compounds.

b) Large molecular weight compounds are slightly sensitive to heat

c) Small molecular weight compounds are also responsible for the inhibitory activity of Gp extracts, and are slightly affected by heating.

d) Both the large and small inhibitory molecular weight fractions, it is suggested, consist of mixtures of active compounds some thermolabile and some thermostable.

Discussion

Extracts that reduce local lesion production may do so by either destroying or inactivating the virus particles, or alternatively may prevent or inhibit virus replication. Caldwell (1935) suggests that substances inhibiting plant virus infection may be distinguished from inactivators by their differential reaction to dilution as mentioned above. Extracts from Gp showed the highest reduction of lesion numbers with concentrated inocula, suggesting that the extracts act as inhibitors rather than inactivators.

Dilution experiments also proved useful in that they revealed the complex nature of the compounds involved in influencing virus symptom production. Thus, although crude extracts, and dilution up to 10^{-3} caused inhibition, further dilutions produced samples that enhanced virus activity. As pointed out earlier other workers have studied the effects of dilution on inhibitor plant extracts (Kuntz and Walker, 1947; Thomson and Peddie, 1965; Marchoux, 1967; Noronha et al., 1980). None of these investigators, however, diluted their extracts up to or beyond the inhibition dilution end-point. In experiments described here 10^{-4} and 10^{-5} dilutions revealed the presence of augmenter compounds.

Enhancement of virus activity by diluted plant extracts has been reported previously by Benda, 1956; Blaszczak et al., 1959; Stevens, 1970.

Clearly extracts contain a variety of materials some which interfere with virus replication and other that favour viruses. The remaining experiments described in this section emphasize the complex nature of the extracts under examination. For example, although extracts remain active for 2 months or more at room temperature, they are sensitive, in part, to heat. Stability of plant inhibitor extracts at room temperature has been demonstrated in other laboratories, but without any comment as to the nature of the possible compounds involved. The presence in extracts of proteinaceous inhibitors might be infered if extracts have a short "shelf life". Longevity for several weeks suggest compounds other than proteins being the active ingredients. However, 21 % of the inhibitor activity can be destroyed in 10 minutes exposure to 100 °C, a result in support of the idea that at least part of the inhibitory activity resides in proteins. Proteinaceous virus inhibitors are well known from plants including Phytolacca esculenta (Kassanis and Kleczkowski, 1948), Capsicum frutescens (McKeen, 1956), Dianthus caryophyllus (Ragetli and Weintraub, 1962b), Phytolacca americana (Wyatt and Shepherd, 1969).

Blaszczak et al. (1959) found that sap from Chenopodium amaranticolor, C.frutescens, Spinacia oleacera lost a slight amount of their inhibitory activity after boiling. Apablaza and Bernier (1972) also reported that the extracts from pepper and geranium were not be completely heat stable. Where part at least of the inhibitory activity of extracts was thermolabile, this would suggest that, here too, some proteins are

responsible for virus inhibition.

Additional support for the complex nature of Gp extracts came from those experiments where variously diluted extracts were heated. Heating the progressive dilutions of extract revealed the presence of augmenter compounds with all dilutions used, these suggest that these compounds enhancing virus activity are thermostable. Extracts may, therefore, contain at least three fractions as follows: 1- thermostable inhibitor, 2- thermostable augmenter, 3- thermolabile inhibitor.

An interesting feature of the Gp extracts was their stability over a wide pH range. This feature is not however unique to Gp inhibitor extracts as demonstrated by Gupta and Raychaudhuri (1972); Yoshizaki and Murayama (1966); and Demski and Chalkley (1977).

In experiments designed to partially fractionate the complex inhibitor extracts by dialysis, a number of interesting results were obtained. It reasonable to assume that large molecular weight compounds will remain in the dialysis sac and that only the smaller compounds will dialyse. Dialysis of extracts revealed the presence both large and small molecular weight inhibitors. Similar results were obtained by Verma and Verma (1965) who reported the presence of a dialysable inhibitor in wheat seed extracts. Apablaza and Bernier (1972) also found inhibitory activity in both residue and filt rate after separation of extracts prepared from pepper and geranium into two different molecular weight fractions. More recently, Takagi and Sugimura (1977) reported that the inhibitor activity from Lentinus edodes was diminished by dialysation to give 25 % inhibition and the activity was detected in external water of dialyzing bag to give 75 % inhibition.

However, the results reported here showed the large molecular weight compounds were more effective than the small molecular weight compounds. The possible explanation of these results is that both large and small molecular weight fractions in Gp extracts have a role in the overall inhibitory activity of the extracts.

Evidence collected from experiments described in this section support further the idea that Gp inhibitors are complex mixture of compounds. The major part of the inhibitor might be proteinaceous in nature, even though partially insensitive to heat and pH. The other part comprises small molecular weight compounds which also reduced markedly local lesion production by TNV.

In the following section an attempt was carried out to study the proteinaceous nature of the Gp inhibitor extracts.

SECTION B

PROTEINACEOUS PROPERTIES

Extracts were examined after treatment with either ethanol or ammonium sulphate followed by electrophoresis separation of the precipitated materials. The possible proteinaceous nature of the inhibitors was also tested using proteolytic enzymes.

1. Effect of protein precipitants

a) Effect of ethanol

A number of workers have used precipitation techniques to confirm the proteinaceous nature of plant virus inhibitors. Kassanis and Kleczkowski (1948) precipitated a virus inhibitor from Phytolacca esculenta sap with ethanol. Crowley (1955) found that the inhibitor from Cucumis sativus embryo seeds was precipitated only by 60 % ethanol. Similarly, Kuntz and Walker (1947) found that 50 % ethanol precipitated the inhibitor of TMV from spinach sap, while the inhibitor from cabbage mosaic virus infection was unaffected. However, this latter inhibitor was completely or almost completely precipitated by KCl. Furthermore, 50 % ethanol was found by Smookler (1971) to precipitate the inhibitors extracted from Chenopodium amaranticolor, C.album, Atriplex nitens and Amaranthus caudatus, and by Verma and Awasthi (1979),who reported that 50 % ethanol precipitated the inhibitor from Boerhaavia diffusa roots.

McKeen (1956) found that 95 % ethanol was needed to precipitate a virus inhibitor from the juice of Capsicum frutescens. Demski and Chalkley(1977) reported that inhibitory activity of watermelon leaf sap treated with chloroform, butanol, ethanol, acetone, ether, or hydrated calcium phosphate at concentrations of 8, 20, and 50 % was

significantly reduced at any of the concentrations based on studies using tobacco ring spot virus infection of cowpea.

Experimental procedure

Lyophilized extract of Gp was dissolved in distilled water, then 2 ml of reconstituted extract was mixed with 8 ml of absolute alcohol to give a final concentration of 80 %. The mixture was shaken for 2 min. and then kept at room temperature for 20 min. The precipitate formed was removed by centrifugation at 2000 x *g* for 15 min. and then redissolved in 2 ml of distilled water. Ethanol was removed from the supernatant fluid by rotary evaporation at 40 °C, and the volume reduced to 2 ml. The dissolved precipitate and the supernatant fractions were each mixed with an equal volume of TNV and inoculated onto *P.vulgaris* leaves. Controls consisted of TNV and water. The non-dialysable and dialysable fractions of Gp extract were also treated with ethanol in the same way.

Results

The results in Table 18 show that the ethanol precipitates of whole and non-dialysable part, totally eliminated local lesion production by TNV giving 100 % inhibition. Although most of the inhibitory activity of the dialysable fraction (82 %) remains behind in the supernatant after mixing with ethanol, a small amount (12 %) is associated with the precipitate. The supernatant from the whole and non-dialysable fraction also showed inhibitory effects giving 80 & 73 % inhibition respectively. This was not due to residual alcohol as this was all removed by rotary evaporation.

It seems reasonable to suggest from these results that Gp extracts contain a number of inhibitor compounds, part in the compounds precipi-

Table 18 : Effect of ethanol on virus inhibitory activity of *G.paniculata* extracts

G.paniculata fractions		Mean number of lesions *		% Inhibition
		TNV + Water (control)	TNV + Extract (treated)	
Whole extract	Precipitate**	74.9	0	100
	Supernatant	108.7	21.6	80
Non-dialysable fraction	Precipitate**	93.5	0	100
	Supernatant	75.3	20.1	73
Dialysable fraction	Precipitate**	52.5	46.3	12
	Supernatant	105.0	18.4	82

* Each figure represents the mean number of lesions for ten leaves of french bean

** Precipitates produced with 80 % ethanol

tated by ethanol, and likely therefore to be proteinaceous in nature. Other small molecular weight compounds appear to have a significant role in the powerful inhibitory activity of Gp extracts, substantiating the results described in section A.

b) Effect of ammonium sulphate

To confirm that part of the Gp inhibitor is proteinaceous and also to partially fractionate the extracts, ammonium sulphate was used at different concentrations. Some workers have precipitated virus inhibitors from plant extracts using ammonium sulphate. Crowley (1955) for example, found that the inhibitor from Blue prior tobacco seeds was precipitated by 75 % saturated ammonium sulphate. However, Varma (1973) showed that the protein fractions obtained after 75 % ammonium sulphate precipitate of the leaf extract of cabbage did not inhibit TMV infection when assayed on Nicotiana Xanthi and N.glutinosa, while the protein free solution showed appreciable inhibition.

Smookler (1971) found that most of the inhibitory activity of some species of the Chenopodiales could be precipitated from extracts by 70 - 90 % saturated ammonium sulphate. The same concentration was found by Kimmins (1969) to precipitate the inhibitory factors present in fractions of Datura stramonium, N.glutinosa, P.vulgaris and C.amaranticolor. Verma and Awasthi (1979) also showed 90 % ammonium sulphate precipitated the inhibitor from B.diffusa roots.

Other plant inhibitors have been precipitated using 50 % ammonium sulphate, including those from juices of C.album (Yoshizaki and Murayama, 1966), N.glutinosa (Palm, 1967), and N.tabacum var. Turkish Samsun (Zaitlin and Siegel, 1963). However, McKeen (1956) observed

that the inhibitor from pepper sap began to be precipitated with 50 % saturation ammonium sulphate, but a progressively heavier inhibitory precipitate was found as the concentration of the salt increased to 95 %.

Experimental procedure

Ten ml of lyophilized extract in distilled water was centrifuged at 4000 rpm for 30 min. to remove any insoluble matter. The precipitate was discarded and the clear supernatant was brought to 10 % saturation with solid ammonium sulphate added slowly with stirring for 15 minutes (Chaykin, 1966). After a further 20 min. at room temperature the precipitate was removed by centrifugation at 2000 x g for 15 min. and resuspended in 10 ml distilled water. Using the same procedure, the clear supernatant fluids were in turn brought to 20, 30, 40, 50, & 60 % saturation with solid ammonium sulphate. The resuspended precipitates and the remaining supernatant were each dialysed with stirring at 4 °C for 3 days against 3 changes of one liter distilled water to remove ammonium sulphate. The protein concentration of each sample was measured. Two ml of these various dialysates and the final supernatant were mixed with equal volumes of TNV, then assayed on P.vulgaris leaves. Suitable controls consisted of distilled water and TNV.

Results

Table 19 shows that precipitates 1, 2, & 3 produced at 10, 20 and 30 % ammonium sulphate saturation respectively, were each inhibitory (54, 66 & 81 % inhibition), but were less effective than precipitates 4, 5 and 6 produced with 40, 50 & 60 % ammonium sulphate saturation. These latter precipitates inhibited lesion production by 95, 99 & 97 % respectively. The final supernatant left after addition of ammonium

Table 19 : Effect of ammonium sulphate fractions of G.paniculata extracts on the susceptibility of P.vulgaris to TNV

Sample	Ammonium sulphate saturation (%)	Mean number of lesions *		% Inhibition	Protein concentration (mg/ml)
		TNV + water (control)	TNV + sample (treated)		
Precipitate 1	10	78.2	35.8	54	1.48
Precipitate 2	20	79.5	26.8	66	1.12
Precipitate 3	30	90.8	17.2	81	2.25
Precipitate 4	40	67.3	3.3	95	1.23
Precipitate 5	50	108.2	1.2	99	0.83
Precipitate 6	60	50.8	1.5	97	0.42
Supernatant	0	88.7	36.3	59	0.15

* Each figure represents the mean number of lesions for ten leaves

sulphate showed a significant reduction in lesion numbers giving 59 % inhibition.

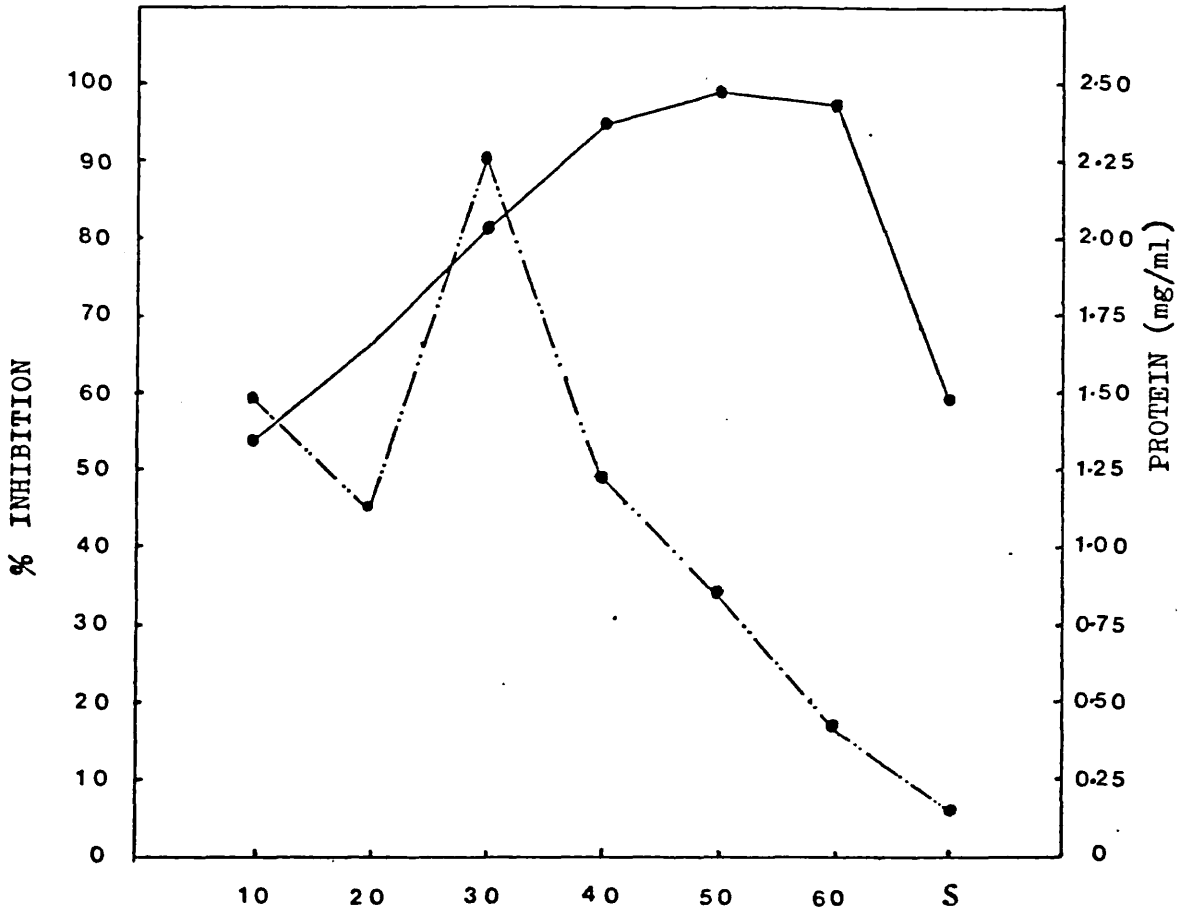
The maximum inhibitory activity was not directly correlated with maximum protein concentration as shown in Figure 14. The precipitate giving most inhibitory activity (99 %) was obtained at 50 % saturation, and contained only 0.83 mg/ml protein; whereas the precipitates obtained at 10 and 30 % saturation ammonium sulphate contained most protein, but showed less inhibitory activity producing 54 and 76 % inhibition respectively. In fact, the 10 % (precipitate 1) gave least inhibition (54 %) and was second only to the 30 % (precipitate 3) in protein concentration containing 1.48 mg protein/ml. On the other hand, precipitate 6, gave 97 % inhibition, but contained the least amount of protein (0.42 mg/ml).

It can be concluded from these results that: 1- the main parts of the inhibitory activity were precipitated at 40-60 % ammonium sulphate saturation, these concentrations were shown by other workers to precipitate proteinaceous virus inhibitors 2- there is no direct correlation between maximum inhibitory activity and maximum protein concentration.

To examine whether the Gp inhibitors consisted of more than one protein fraction and to see if any glycoprotein might be found, the ammonium sulphate precipitates were examined by polyacrylamide electrophoresis using the methods described earlier.

Results in Figures 15 & 16 show that precipitates 1 & 2 which were relatively weak inhibitors contained only protein; whereas precipitates 3, 4, 5 & 6 which strongly inhibited lesion production, contained detectable levels of glycoprotein. For example, precipitate 5, which gave the highest inhibitory activity (99 %) contained two bands of

Figure 14 : Inhibitory activity (●—●) and amount of protein (●---●) associated with different ammonium sulphate saturation precipitates of lyophilized G.paniculata extracts



S = The supernatant

Figure 15 : Disc electrophoresis of ammonium sulphate fractions from G.paniculata

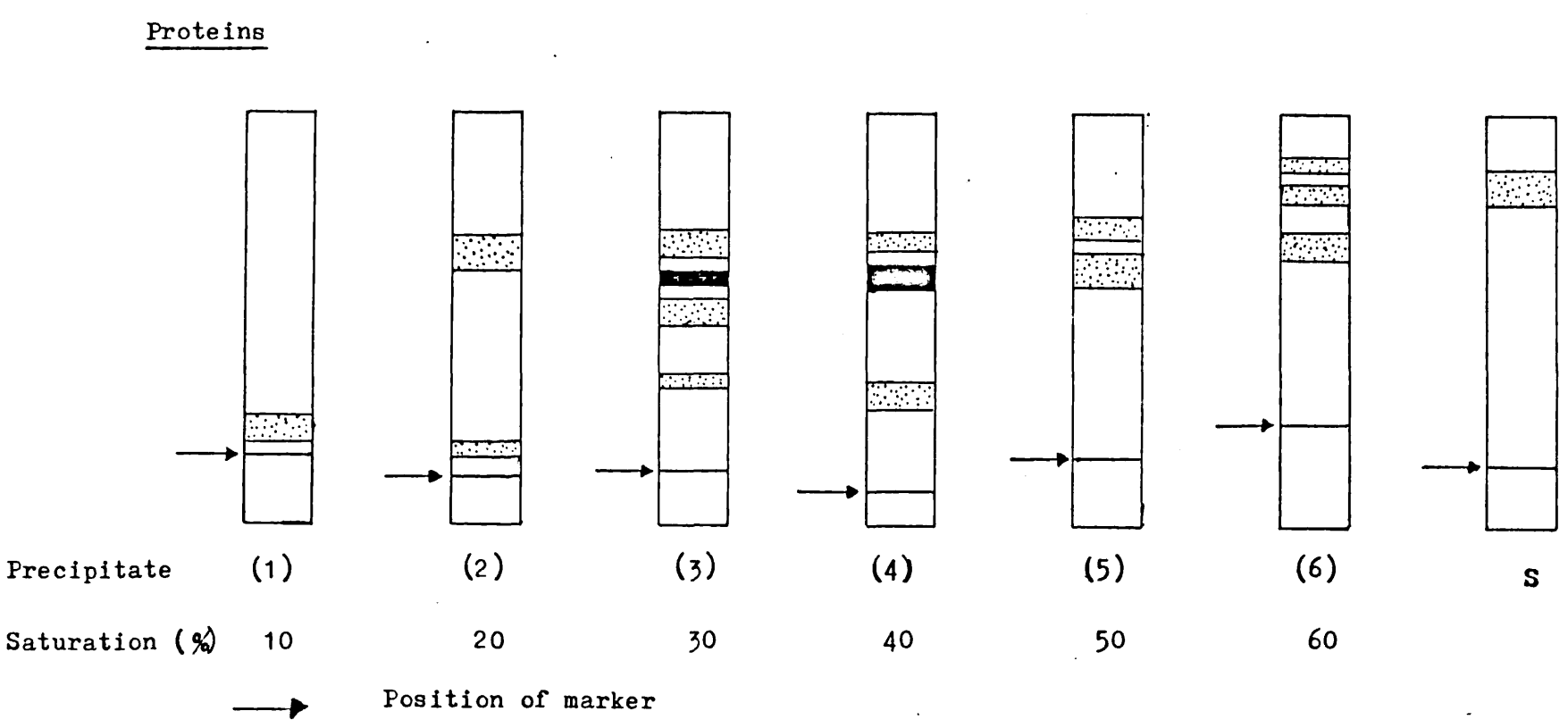
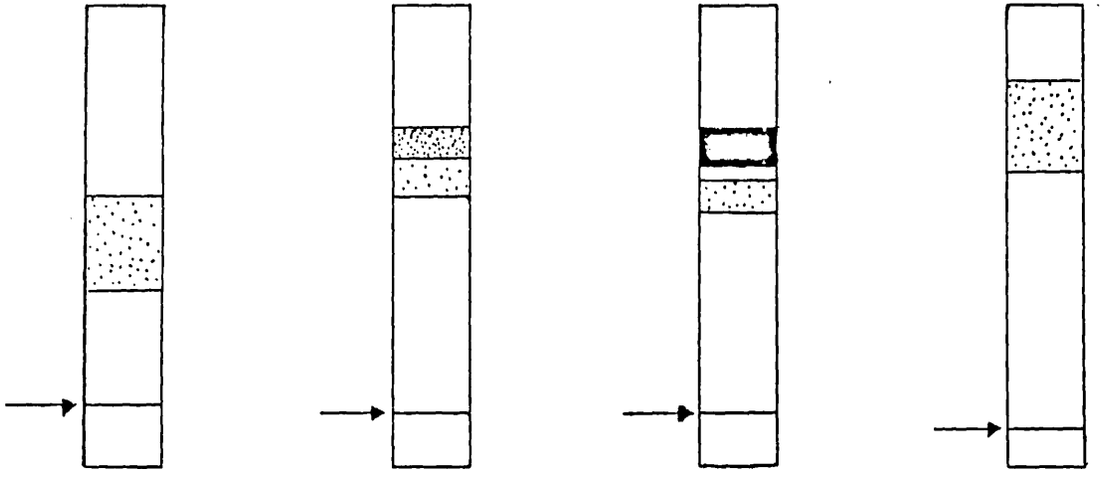


Figure 16: Disc electrophoresis of ammonium sulphate fractions from G.paniculata

Glycoproteins(No bands were detected in precipitates 1, 2 and the supernatant)



Precipitate	(3)	(4)	(5)	(6)
Saturation (%)	30	40	50	60

→ Position of marker

Shading indicates intensity of band staining

protein and two bands of glycoprotein with similar Rf values. Precipitate 3, which showed maximum concentration of protein (2.25 mg/ml), consisted of 4 protein bands and only one wide band of glycoprotein. Precipitate 4, which showed 95 % inhibition, contained 3 bands of protein and 2 bands of glycoprotein. Precipitate 6, contained 3 bands of protein and only one diffuse area of glycoprotein. Precipitate 1, which showed least inhibition, contained one weak mobile protein band, but no glycoprotein was detected. Precipitate 2, which gave 66 % inhibition, consisted of 2 bands of protein and no glycoprotein. The supernatant, which gave 59 % inhibition, showed one band of protein but was free of glycoprotein. Precipitates 1, 2 and the supernatant which were not strong effective inhibitors, contained weak proteins and were free of glycoprotein. The association of inhibitory activity with fractions containing glycoproteins is of some interest and will be discussed later.

2. Effect of proteinase enzymes

If inhibitory activity of G.paniculata extracts is due to proteinaceous substances, it is reasonable to suggest that they will be sensitive to treatment with proteolytic enzymes. To test this suggestion, Gp extracts were exposed to hydrolysation by three different proteolytic enzymes.

Exprimental procedure

The enzymes trypsin (Hog pancreatic, Sigma), pepsin (BDH) and pronase (Exstreptomyces griseus, Koch-light) were prepared at 0.4 mg/ml in 0.2 M phosphate buffer pH 7.2, 0.05 M acetate - HCl buffer pH 2.2 and 0.01 M tris - HCl buffer pH 7.7 respectively. Each enzyme was mixed with an equal volume of Gp inhibitor prepared from lyophilized

extracts and dissolved in the appropriate buffer. Control solutions contained enzymes in buffers and distilled water instead of Gp inhibitor. The various enzyme-inhibitor mixtures and controls were incubated at 25 °C for 6, 24 and 48 hours. All the samples, treated and controls, were then dialysed against neutral phosphate buffer for 24 hours, since the acetate and the tris buffers gave unsatisfactory lesions counts. Two ml of treated and control samples were each mixed with 2 ml of TNV and inoculated onto P.vulgaris leaves. The non-dialysable fraction was also treated with enzymes and tested in the same manner.

Results

The results in Tables 20, 21 & 22 show that none of the three proteolytic enzymes tested had any effect on the inhibitory activity of the whole and non-dialysable fraction of Gp extracts. The percentage inhibition remained unchanged (100 %) after incubation for 6, 24 and 48 hours. The results indicate, in general, that the activity of Gp inhibitors is not influenced by any of the proteinase enzymes tested.

It might be argued that the amino acids liberated by hydrolysis of the protein would themselves inhibit virus. This seems very unlikely, however, since the reaction mixtures have been dialysed to remove interfering buffer reagents which will also remove small molecular weight amino acids. Other workers have observed the insensitivity of virus inhibitor protein to proteolytic enzymes. Kassanis and Kleczkowski (1948) found that the inhibitor activity from Phytolacca esculenta was not diminished after treatment with pepsin and trypsin. Similar results were reported by Ragetli and Weintraub (1962b) who found that the inhibitory activity of carnation extracts was unchanged

Figure 20 : Effect of trypsin (0.4 mg/ml) on the virus inhibitor properties of the whole and non-dialysable fraction of G.paniculata

G.paniculata fractions	Mean number of lesions*, following incubation of extracts for:						% Inhibition		
	6 hrs		24 hrs		48 hrs				
	Control	Treated	Control	Treated	Control	Treated	6 hrs	24 hrs	48 hrs
Whole extract	29.1	0	36.4	0	42.6	0	100	100	100
Non-dialysable fraction	23.6	0	45.1	0	44.6	0	100	100	100

* Each figure represents the mean number of lesions for ten leaves

Control = enzyme + water + TNV

Treated = enzyme + Gp extract + TNV

Figure 21 : Effect of pronase (0.4 mg/ml) on the virus inhibitor properties of the whole and non-dialysable fraction of G.paniculata

G.paniculata fractions	Mean number of lesions*, following incubation of extracts for:						% Inhibition		
	6 hrs		24 hrs		48 hrs				
	Control	Treated	Control	Treated	Control	Treated	6 hrs	24 hrs	48 hrs
Whole extract	36.7	0	43.4	0	45.2	0	100	100	100
Non-dialysable fraction	62.1	0	45.3	0	53.8	0	100	100	100

* Each figure represents the mean number of lesions for ten leaves

Control = enzyme + water + TNV

Treated = enzyme + Gp extract + TNV

Figure 22 : Effect of pepsin (0.4 mg/ml) on the virus inhibitor properties of the whole and non-dialysable fraction of G.paniculata

G.paniculata fractions	Mean number of lesions*, following incubation of extracts for:						% Inhibition		
	6 hrs		24 hrs		48 hrs				
	Control	Treated	Control	Treated	Control	Treated	6 hrs	24 hrs	48 hrs
Whole extract	104.8	0	135.5	0	98.9	0	100	100	100
Non-dialysable fraction	85.7	0	108.0	0	76.3	0	100	100	100

* Each figure represents the mean number of lesions for ten leaves

Control = enzyme + water + TNV

Treated = enzyme + Gp extract + TNV

after incubation at room temperature for 4, 9, 12 and 24 hours with each of four proteolytic enzymes (papain, trypsin, leucine amino-peptidase and carboxypeptidase). They suggested that the inhibitor was not affected due to the cyclic structure of the inhibitor molecule, since aminopeptidase and carboxypeptidase require free ∝- amino and ∝- carboxyl end group, respectively. Furthermore, experiments carried out by Fukaya and Taniguchi (1979) showed that the activity of inhibitors extracted from Phytolacca americana was diminished only by pepsin, but not by trypsin and protease.

Zaitlin and Siegel (1963) found that enzymes; chemotrypsin, papain, pangestin and trypsin destroyed the inhibitory activity of tobacco leaf tissue when treated at high concentration (100 µg/ml). Alpha-amylase and lipase were also effective at higher concentration. However, the crystalline trypsin and chemotrypsin although effective, worked at much lower concentration (2.0 µg/ml). On the other hand, Alberghina (1976) noticed that the antiviral activity of Chenopodium amaranticolor extracts was not reduced when the extracts was mixed with with pronase (4 µg/ml) at 38 °C for 72 hours. Similar results were obtained when the extract was treated with trypsin for 20 minutes (Sela et al., 1966).

The insensitivity of Gp extracts to proteolytic enzymes is not therefore altogether unusual and leads to the ideas:
1- The inhibitor proteins must be special in some way making it resistant to hydrolysis. 2- This may arise because the inhibitor is a glycoprotein, and the sugar radicals protect the molecule from enzymatic degradation.

In none of the previous accounts of inhibitor resistance to hydro-

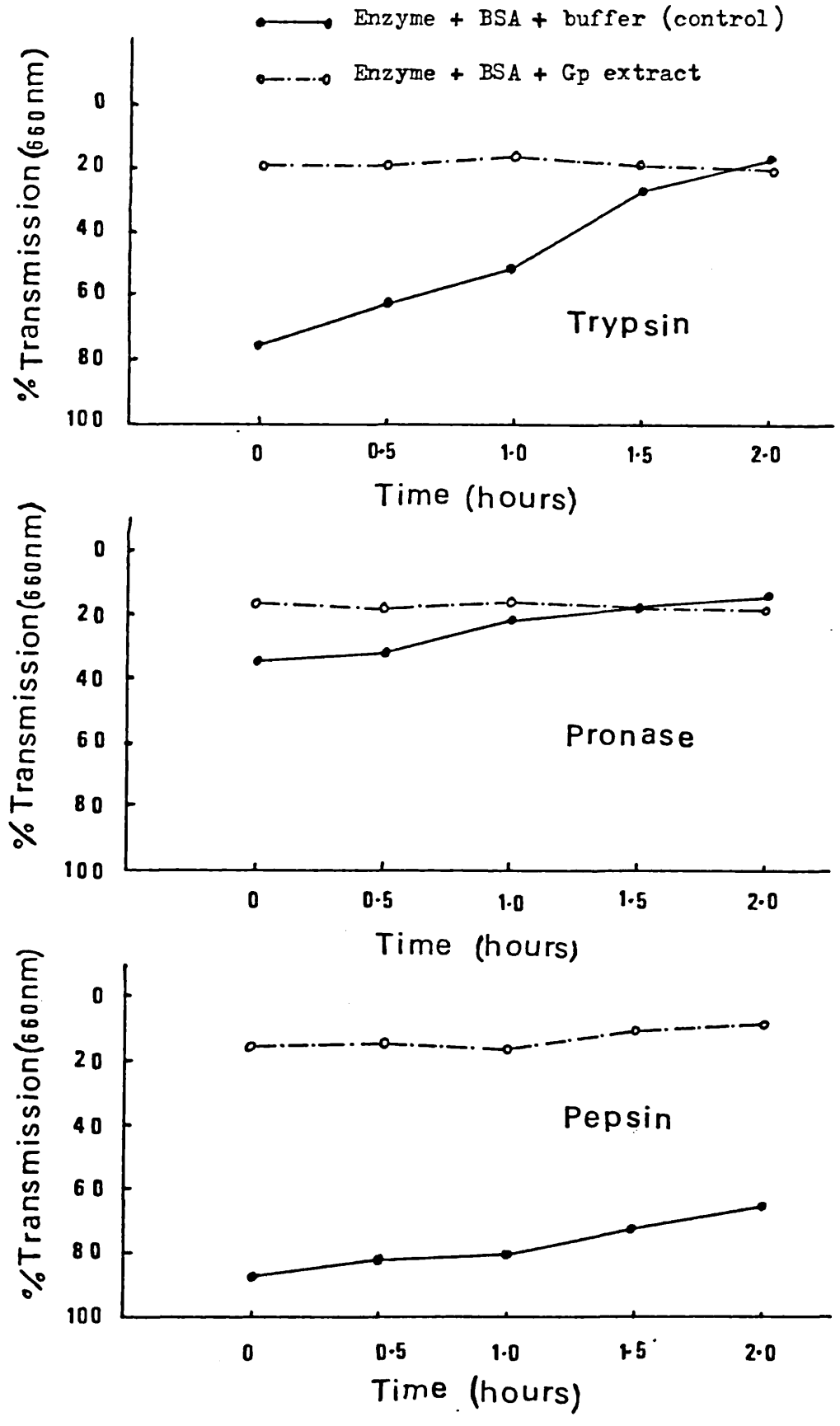
lysis have the authors tested the effects of these extracts on the activity of the proteinases being used. In view of this deficiency the following experiment was undertaken.

a) Effect of Gp virus-inhibitor on proteinases

0.4 mg/ml of each of the three proteolytic enzymes (trypsin, pronase and pepsin) were prepared in their appropriate buffers as described above. Two ml of each enzyme solution were mixed with 2 ml of bovine serum albumin (BSA) as substrate at a concentration of 1 mg/ml, and then 2 ml of Gp extracts prepared from lyophilized extracts were added to see if it inhibited enzyme activity. Control samples consisted of proteolytic enzyme, BSA and 2 ml of the appropriate buffer instead of the Gp extract. The various enzyme-Gp mixtures and control samples were incubated at 25 °C. At 0, 0.5, 1.0, 1.5 and 2.0 hours, 1 ml of each sample was mixed with 1 ml of absolute alcohol to stop the reaction. The samples were centrifuged at 4,000 rpm for 10 minutes to precipitate any proteinacous materials. One ml of the clear supernatant of each sample was tested for liberated amino acids by treating with 4 ml of 0.25 % ninhydrin. A reference sample consisted of 1 ml of water and 4 ml of ninhydrin. All the samples and reference were heated in a water bath at 100 °C for 15 minutes. The percentage of transmission of each sample was measured at 660 nm.

The results in Figure 17 show that the Gp extracts inhibited markedly the activity of trypsin. There was a considerable production of free amino acids when BSA was treated with trypsin in the absence of Gp extracts. The colour in the sample containing Gp extracts was initially high due to amino acids in the Gp extract itself, however,

Figure 17 : Effects of Gp-virus inhibitor extracts on the activity of proteinase enzymes



there was a little change in the colour intensity showing that no amino acids were produced by enzymic hydrolysation of the BSA. Gypsophila extracts seem to have a similar inhibitory effect against pronase and pepsin (Figure 17), although the enzymic activity of these latter enzymes was less than for trypsin. A high initial amino acid contents of pronase possible explains the intense colour shown in the initial samples using this enzyme system. It can be concluded from these interesting results that Gp extracts which showed a potent virus inhibition, can also inhibit the activity of proteinase enzymes.

Discussion

It appears when G.paniculata extracts were treated with ethanol (final concentration 80 %) the inhibitory principle of the extracts was precipitated. On the other hand, the supernatants produced after treatment of the whole and non-dialysable fraction with ethanol contained active inhibitor compounds giving 80 and 73 % inhibition respectively. Similar results have been reported with Amaranthus candatus inhibitor extracts which remain in the supernatant producing 32 % inhibition and a small amount (14 %) associated with the precipitate (Smookler, 1971). He suggested that 50 % ethanol may be insufficient to precipitate the inhibitor from the sap. Our results support the contention that inhibitory activity of Gp extracts resides both in large molecules probably proteinaceous in nature, as well as, in other small molecules likely to be non-protein substances.

Evidence for the proteinaceous nature of part of the extracts came from experiments using ammonium sulphate. Ammonium sulphate acts by a phenomenon called "salting out". Such salting out occurs with

proteins, electrolytes, uncharged molecules and gases (White et al., 1968). The mechanism of this phenomenon might be due to displacement of the solvent by the salt (Keller and Block, 1960), and results in a decreased interaction between the solute and solvent or to the removal of water molecules from the solute by the salt and a subsequent loss in the solute solubility.

The most inhibitory part of Gp extracts was precipitated at 40 - 60 % ammonium sulphate saturation. However, other precipitates produced by lower levels of ammonium sulphate also showed considerable inhibition but were less effective. There is no correlation between protein concentration of the ammonium sulphate fractions and their inhibitory activity.

Disc electrophoresis of ammonium sulphate precipitates suggests that the inhibitory activity found in large molecular weight compounds is due to a complex of proteins, some of which appear to be glycoproteins. Glycoproteins have been found to be responsable for the inhibitory activity of extracts from Phytolacca esculenta (Kassanis and Kleczkowski, 1948). Such glycoproteins were slightly heat labile, precipitated by ethanol and were found to contain 8-12 % carbohydrate. Phytolacca acinosa (Gupta, 1964) and materials extracted from virus infected plants (Mozes et al., 1978) were also found to contain glycoprotein virus inhibitors.

Evidence accumulated from a number of experiments using a variety of techniques indicate that the Gp extracts consist of: 1- proteinaceous inhibitors of large molecular weight. This conclusion is supported by experiments including dialysis, ammonium sulphate precipitates and disc

electrophoresis. The results also showed that most ammonium sulphate precipitates contained more than one protein, some of which are glycoproteins. 2- non-protein inhibitors of small molecular weight. Similar fractions have been described by Bawden and Freedman (1952), Hirai and Shimomura (1965), El-Kandelgy and Wilcoxon (1966), and Apablaza and Bernier (1972).

Attempts were made to confirm the proteinaceous nature of the large molecular weight fraction of the Gp extracts using three proteinases. Trypsin which is active toward positively charged substrates and it is used in peptide sequence analysis because it will hydrolyse proteins at lysyl and arginyl residues to give a limited number of peptides. Pronase which has a wide specificity toward carboxylic acid derivatives and its active sites are cystine residues, essential for catalysis. Pepsin which can hydrolyse peptide of L-amino acids, as well as, catalyse the hydrolysis of organic sulphate esters. All these proteolytic enzymes had no effect on the inhibitory activity of the whole and non-dialysable part of Gp virus inhibitors even after incubation for 48 hours. Similar results were obtained by a number of workers as discussed earlier. Ragetli and Weintraub (1962b) found that the inhibitory activity of the protein-virus inhibitor extracted from Dianthus caryophyllus was unchanged after incubation with each of four proteolytic enzymes (see earlier), and related this observation to the possible cyclic structure of the protein. Furthermore, Dianthus inhibitor was not affected by trypsin, despite the presence of lysine in the molecule, this they suggested, indicates that disulphide groups were not involved in maintaining the structural integrity of the molecule.

Alberghina (1976) found that trypsin and pronase did not reduce the antiviral activity of C.amaranticolor extracts, and suggested that the inhibitor is not a protein, but some other high molecular weight compound.

The proteinaceous nature of the virus inhibitors from P.esculenta (Kassanis and Kleczkowski, 1948), P.americana (Fukaya and Taniguchi, 1979) has been established. However, both inhibitors were unaffected by pronase and trypsin. The latter extract was affected by proteinases when the reaction mixture was diluted. Furthermore, Zaitlin and Siegel (1963) described the inhibitor in the homogenate of tobacco leaf tissue as a heat stable protein. When this extract was mixed with several enzymes, it was effected only at higher enzyme concentrations (0.01 mg/ml). They suggest that the sensitivity of inhibitor to low concentration of trypsin and chemotrypsin was due to the presence of peptide bonds necessary for its activity as a virus inhibitor.

If proteinases act on Gp-virus inhibitor proteins, the amino acids liberated might be responsible for virus inhibition and this too might explain the observations of other workers. However, as explained earlier, our extracts contain no free amino acids because the samples have been dialysed. The apparent insensitivity of the extracts to proteolytic enzyme degradation comes about because the Gp extracts act as or contain trypsin inhibitor, which also influences pronase and pepsin. Trypsin inhibitor (TI) has been isolated from animals but more importantly from a number of plant materials. That from ox-pancrease obtained by Kunitz and Northrop (1936), is a small protein with molecular weight about 9,000. In the pH range 4-8 the inhibitor reacts with trypsin to form an active complex, which can be crystallized.

Trypsin inhibitors are also present in human placenta (Scevola, 1956), and in Ascaris (Green, 1957).

Soybean TI has been crystallized from soybean by Kunitz (1947, 1948), and from Lima bean by Tauber et al. (1949). Moreover, Stead et al. (1966) found with soybeans that the peaks eluted from DEAE-cellulose up to 0.17 M NaCl had residual trypsin-inhibitory activity, those eluted after 0.17 M NaCl had higher activity. However, Rackis et al. (1959) reported maximal trypsin-inhibitor activity after 0.17 M NaCl, but no activity at all in the earlier peaks of their separation.

Trypsin inhibitors react with other enzymes. For example, plasmin is inhibited by the soybean TI (Tagnon and Sulier, 1946), the ox-lung TI (Astrup and Stage, 1956), and the plasma inhibitor I from ox-blood (Wu and Laskowski, 1960). It is not surprising therefore that pronase and pepsin are also inhibited by the TI from Gp extracts. The question remains, is this TI also the virus inhibitor?. Hajj (1976) found that crude extracts prepared from soybean and french bean contained glycoproteins with trypsin-inhibitor activity. These extracts also possessed the ability to inhibit virus. Hajj, showed that the commerical soybean trypsin-inhibitor was not identical with the virus inhibitors extracted from these two species. The TI is thought to be a pure protein (Wu and Scheraga, 1962), whereas virus inhibitor a glycoprotein (Hajj, 1976).

It is probable that the virus inhibitor proteins (glycoproteins) are separate compounds from TI and that the latter protects the virus inhibitor from enzymatic degradation so making it look likes a proteinase resistance protein with unusual properties.

This investigation suggests that G.paniculata inhibitors may not

be a single substance, but rather, a group of closely related substances having various properties. More details concerning the chemical nature of the extracts will be given in the following chapter.

CHAPTER VI

CHEMICAL NATURE OF GYPSOPHILA PANICULATA EXTRACTS

Previous studies on the properties of G.paniculata extracts have shown that virus inhibitor fractions from this plant consist of a complex mixture of compounds, some of which are proteinaceous in nature and others,of low molecular weight,non-proteinaceous compounds. Gel filtration and ion exchange chromatography techniques have been used to identify more precisely the nature of the inhibitory compounds to gain information regarding molecular weight and other properties of the fractions. Amino acids and sugars in the low molecular weight fractions have also been examined.

For simplicity, results will be described in two separate sections A and B. In section A, studies carried out on the proteinaceous fractions are described. In section B, the results of investigations of the low molecular weight fractions are given.

SECTION A

STUDIES OF THE PROTEINACEOUS PORTION

The proteinaceous nature of some plant virus inhibitors has been widely reported by various researchers. The first report was made by Osborne and Campbell (1898) who used salt extraction and precipitation methods to separate and identify four different virus inhibitor proteins from soybeans. Recent investigations have shown some plant inhibitor extracts contain a large number of different proteins with a variety of biochemical activities. Wu and Scheraga (1962) isolated a protein with virus and trypsin inhibitor activity from soybean extracts and

described it as having a molecular weight of 21,000. Smookler (1971) using gel-filtration (Sephadex G-200) found virus inhibitor proteins of molecular weights of 25,000 - 38,000 in extracts from Chenopodium amaranticolor, Chenopodium album, Atriplex nitens and Amaranthus caudatus. Taniguchi (1974) who also used Sephadex G-200, G-100 and G-50 gel filtration, found that the partially purified inhibitor from crude extracts of french bean leaves contained compounds of several molecular sizes. Recently, Taniguchi and Goto (1979) purified a virus inhibitor protein from C.amaranticolor leaves by Sephadex G-100, G-50 and DEAE - Sephadex ion exchange chromatography, they found the inhibitor to have a molecular weight of 32,000.

1. Gel filtration of G.paniculata extracts

a) Column chromatography of Gp extracts on Sephadex G-100

Sephadex G-100 gel filtration medium was prepared and packed into a glass column 1.5 x 50 cm by the method of Andrews (1964; 1965). The Sephadex G-100 was swollen at room temperature in distilled water for 3 days. The water and the small gel particles were removed by decantation and the gel was then mixed with 0.06 M phosphate buffer pH 7.0. Three hours later, the buffer was decanted and the gel suspension was degassed under pressure. The gel suspension was packed into a glass column by pouring a small amount of the gel into the column which was already filled with phosphate buffer pH 7.0. Excess liquid was allowed to pass through the growing gel-bed and the gel was poured into the column to a bed-height of 35-40 cm. Phosphate buffer pH 7.0 was allowed to pass through the column at a constant flow rate of 10 ml per hour for 2 days at 4 °C. The buffer resevoir, the inlet and outlet tubes

to the column were arranged to produce a 30 cm operating pressure. The column was checked for irregularities by passing through it a 1 ml mixture of blue dextran, yellow dextran and vitamin B12 to observe if any skewness in the bands developed. When not in use, the column was continuously eluted with phosphate buffer pH 7.0.

Lyophilized Gypsophila extracts (50 mg) were dissolved in 2 ml of 0.06 M phosphate buffer pH 7.0 and layered onto the column. The sample was eluted with phosphate buffer and fractions collected on a volume bases (1 ml each 5 minutes) using a fraction collector. To determine the elution volumes, effluent fractions were collected immediately as the sample had entered the column. Ultraviolet absorption measurments were made at 280 nm.

The elution profiles of Gp extracts is shown in Figure 18. Three peaks with elution volume (Ve) of 19 ml (peak I), 24 ml (peak II) and 54 ml (peak III) are obvious, with a shoulder at about (Ve) 75 ml.

Effluent fractions from each peak were assayed for inhibitory activity against TNV. For this purpose, 2 ml from each peak were mixed with an equal volume of TNV and tested for inhibitory activity on french bean leaves. Control samples consisted of 2 ml of TNV and 2 ml of phosphate buffer. Measurements were also made on the protein content of the peaks by Lowry method. Results in Figure 18 show that peak II contained most of the inhibitory activity giving 81 % inhibition, and the highest protein concentration (188 µg/ml). Peaks I & III were also inhibitory to local lesion production by TNV giving 28 and 43 % inhibition respectively. However, Peak I, contained the lowest protein concentration (98 µg/ml), peak III, contained nearly the same protein concentra-

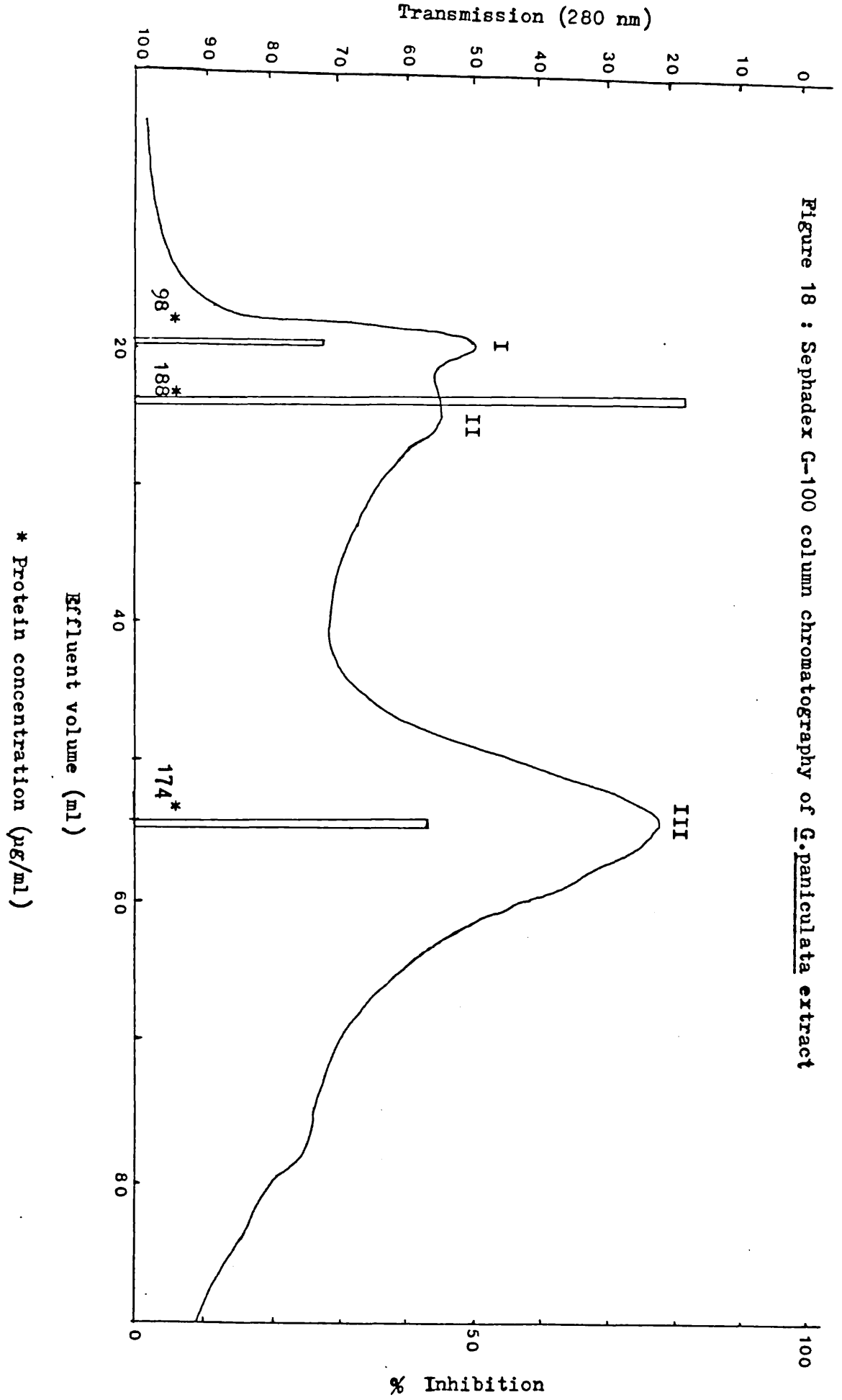


Figure 18 : Sephadex G-100 column chromatography of G.paniculata extract

tion as peak II (174 µg/ml), but showed less inhibition.

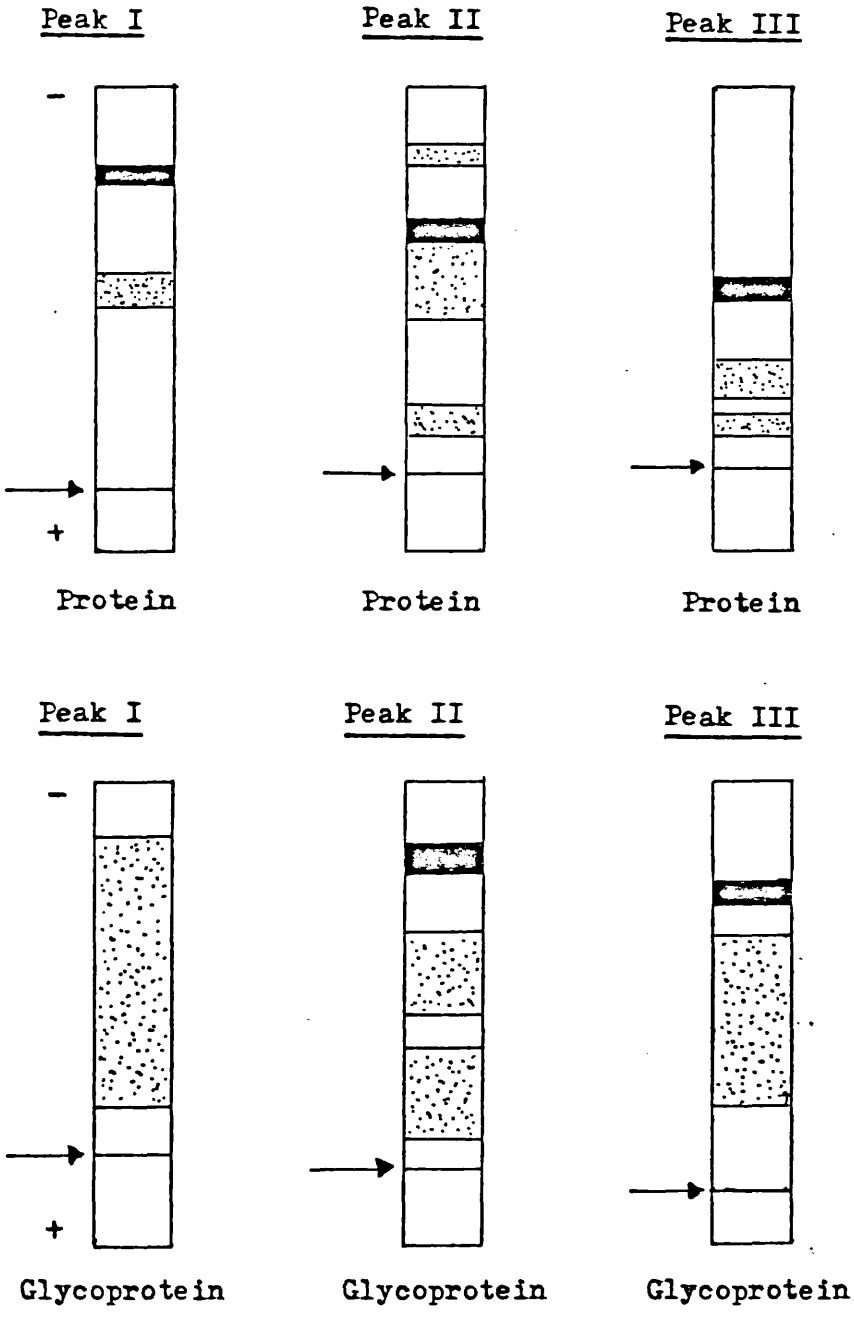
b) Disc electrophoresis of the Sephadex G-100 fractions from Gp extracts

0.2 ml of each peak (I, II & III) were layered on gels prepared as described previously. Figure 19 shows that Peak I contained two protein bands, located at the upper half of the gel at Rf = 0.56-0.25, and a very wide diffuse band of glycoprotein. Peak II contained 4 bands of protein, two were located in the middle (Rf = 0.40-0.60), one in the upper (Rf = 0.21) and one in the lower part of the gel (Rf = 0.92). The 3 glycoprotein bands of Peak II were located in the upper, middle, and the lower part of the gel with Rf's values 0.24, 0.60 and 0.90. Peak III consisted of 3 bands of protein (Rf = 0.55, 0.81 and 0.92), and 2 bands of glycoprotein (Rf = 0.30 & 0.79). It was observed that the Rf values of protein in peak II corresponded closely to that of the glycoprotein. It seems reasonable to suggest, therefore, that the inhibitor in peak II is a glycoprotein at least in part.

c) Molecular weight determination of Gp extract components by Sephadex G-100

Whitaker (1963) and Andrews (1964; 1965) have shown that a correlation exists between elution volume on Sephadex G-100 and the molecular weight of globular proteins. In order to calibrate the G-100 column and determine the molecular weights of the peak materials present in Gp extracts, it was necessary to plot Ve/Vo ratios of a number of globular proteins against their logarithmic molecular weight. Ve is the effluent volume containing the maximum concentration of protein estimated to the nearest 1 ml from the elution diagrams, and Vo is the void volume measured by finding the elution volume of blue dextran 2000.

Figure 19 : Disc electrophoresis of the Sephadex G-100 fractions from G.paniculata extracts



——→ Position of marker

Shading indicates intensity of band staining

Proteins used as standards, their elution volumes and molecular weights are shown in Table 23. Readings of Ve/Vo, plotted against their logarithmic molecular weight were found to give a linear relationship (Fig. 20).

Ve/Vo ratios for the three peaks obtained from G.paniculata extracts showed that the molecular weights of the inhibitors ranged between 27,500 (peak I), 12,600 (peak II) and 2,000 (peak III).

d) Sephadex G-100 column chromatography of Gp extracts following various treatments

In chapter V, it has been shown that the effects of various treatments (heat, dialysis and ethanol) indicate that Gp extracts consist of complex materials, but the major part of the inhibitor is proteinaceous in nature. To investigate how these various treatments affect the extracts, 2 ml of each treated sample was analysed by passing through the G-100 column.

i. Heated extract

Although heat had only slight effects on the inhibitory activity of Gp extracts, the Sephadex G-100 profile showed that heating removed peaks I & II (Figure 21), suggesting the destruction of protein constituents of these two peaks. Peak III,which showed 43 % inhibition in unheated extracts, fractionated into two peaks (H-III-a & H-III-b). Peak H-III-a showed higher concentration of protein (168 µg/ml) and produced 36 % inhibition; whereas peak H-III-b contained less protein (75 µg/ml), but produced greater inhibition (52 %).

ii. Dialysed extract

It has been previously shown that the inhibitory activity of Gp extracts was found in both non-dialysable (ND) and dialysable (D)

Table 23: Ve/Vo ratios and molecular weights of standard proteins

Standard proteins	Ve	Ve/Vo	Log molecular weight (log M.W.)	Molecular weight
γ-globulin	20	0.82	5.000	100,000
Bovine serum albumin	25	1.04	4.83	67,000
Peroxidase	28	1.17	4.60	40,000
Cytochrome C	38	1.60	4.09	12,400
Vitamin B12	58	2.43	3.10	1,357

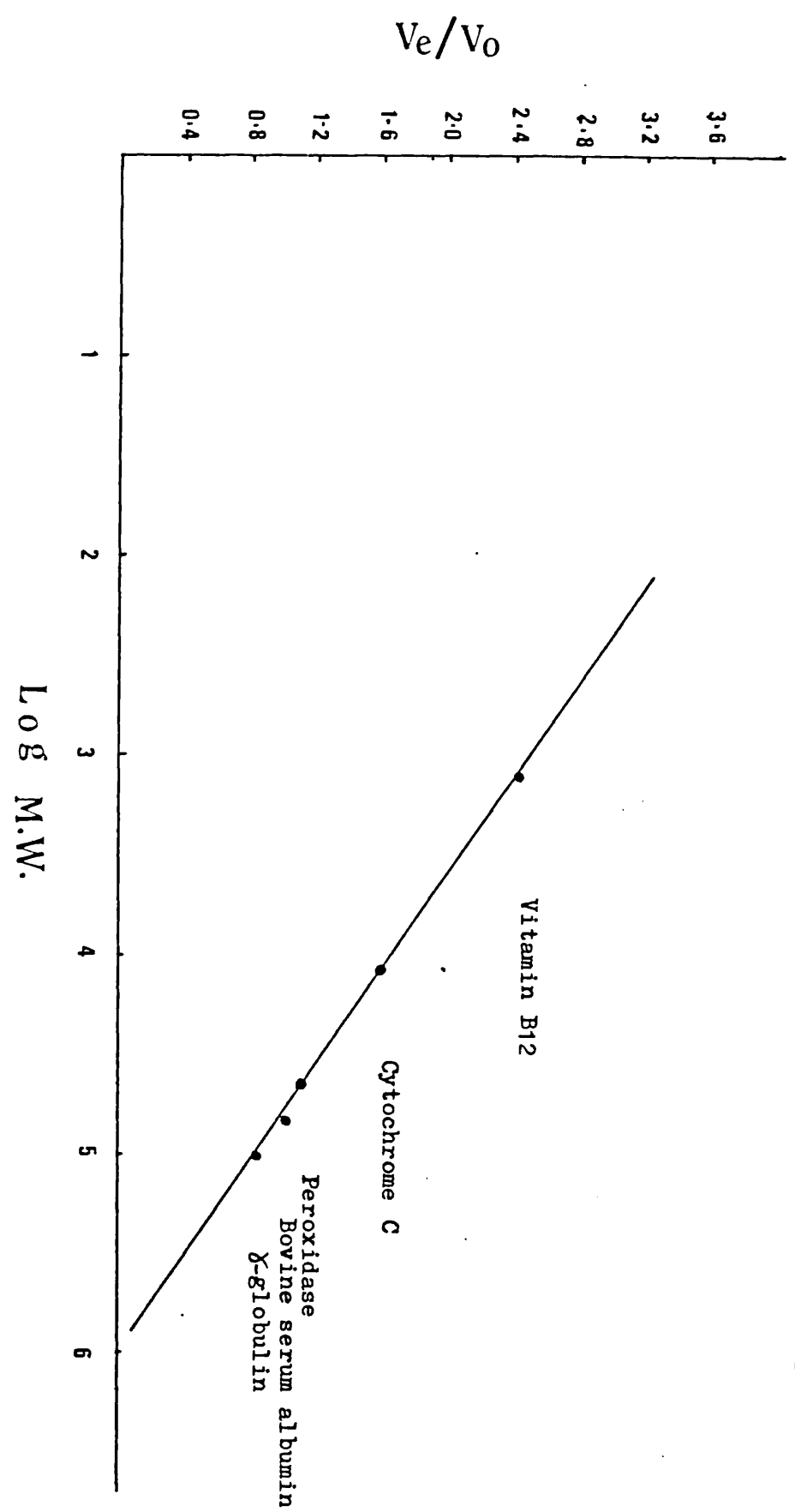


Figure 20 : Ve/Vo ratios and log molecular weights of standard proteins

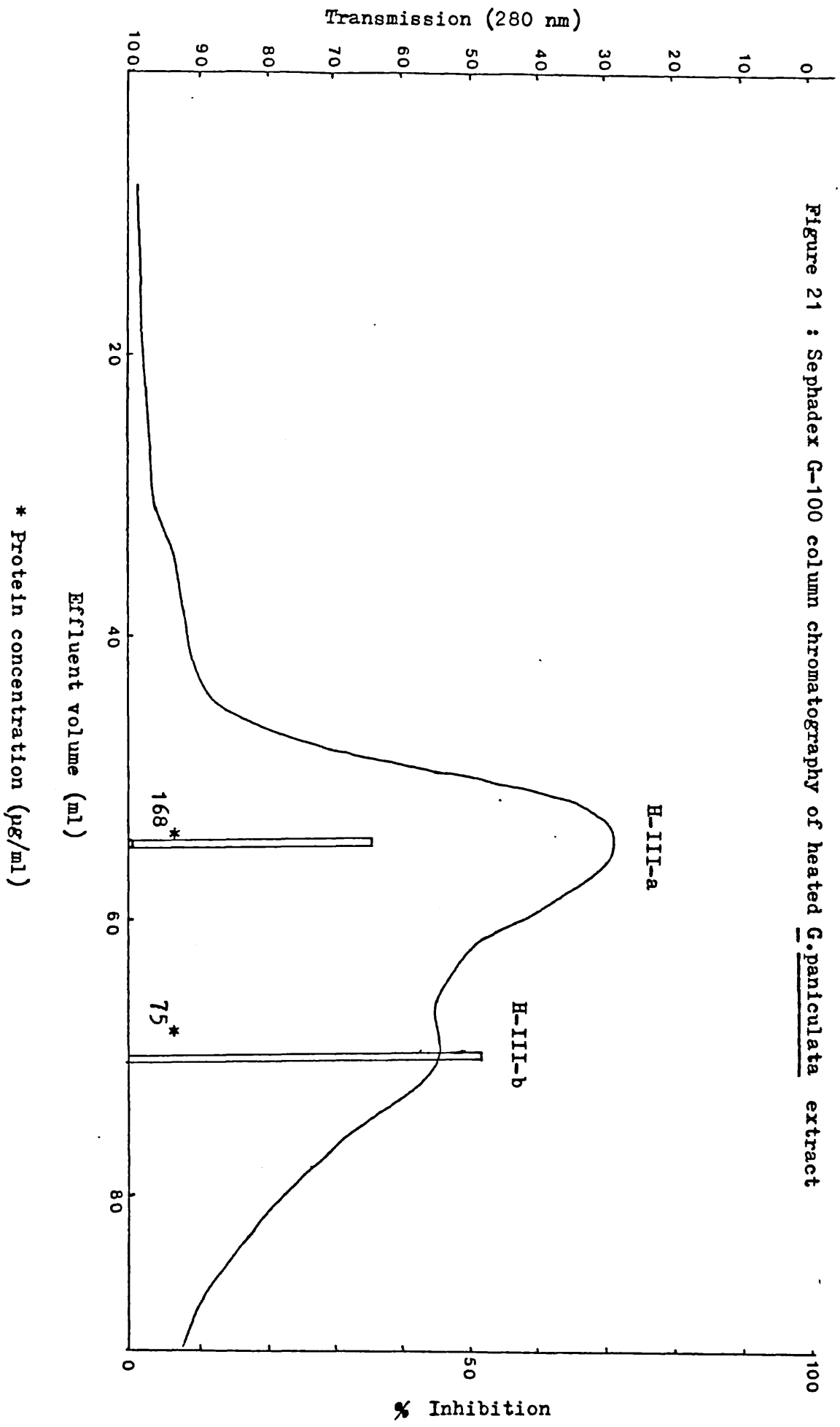


Figure 21 : Sephadex G-100 column chromatography of heated G.paniculata extract

* Protein concentration (µg/ml)

fractions. When the fractions were passed through Sephadex G-100, it was found that the non-dialysable fraction contained 3 peaks (NDI, NDII & NDIII) with protein concentration of 64, 150 and 38 µg/ml respectively. Each peak inhibited TNV. The non-dialysable fraction contained most of peaks I & II and part of peak III present in the lyophilized Gp extracts. Peaks NDII & NDIII showed the highest level of activity giving 85 % and 78 % inhibition respectively. Peak NDI, however, showed only 37 % inhibition (Figure 22).

The dialysable fraction which had previously been shown to retain some of the inhibitory activity of the whole extracts, but did not stain in the electrophoresis experiments (chapter V), contained only part of peak III and is referred to as DIII. The compounds in peak DIII (Fig. 23) showed 40 % inhibition and contained high levels of protein (193 µg/ml). It seems likely, therefore, that peak DIII consists in part, of a number of low molecular weight substances likely to be amino acids and carbohydrates, since the dialysable fraction gave positive reaction with amino acid and carbohydrate tests as described earlier. The apparently high levels of protein in peak DIII is probably attributable to the tyrosine and tryptophan contents. These two free amino acids are known to react strongly and produced colour with the Lowry reagent.

Studies described in the later section were undertaken to elucidate further details concerning the small molecular weight compounds in Gp extracts particularly in the dialysable fraction.

iii. Ethanol treated extract

As previously described, 2 ml of Gp extracts were mixed with 8 ml ethanol. The precipitate produced was removed by centrifugation and

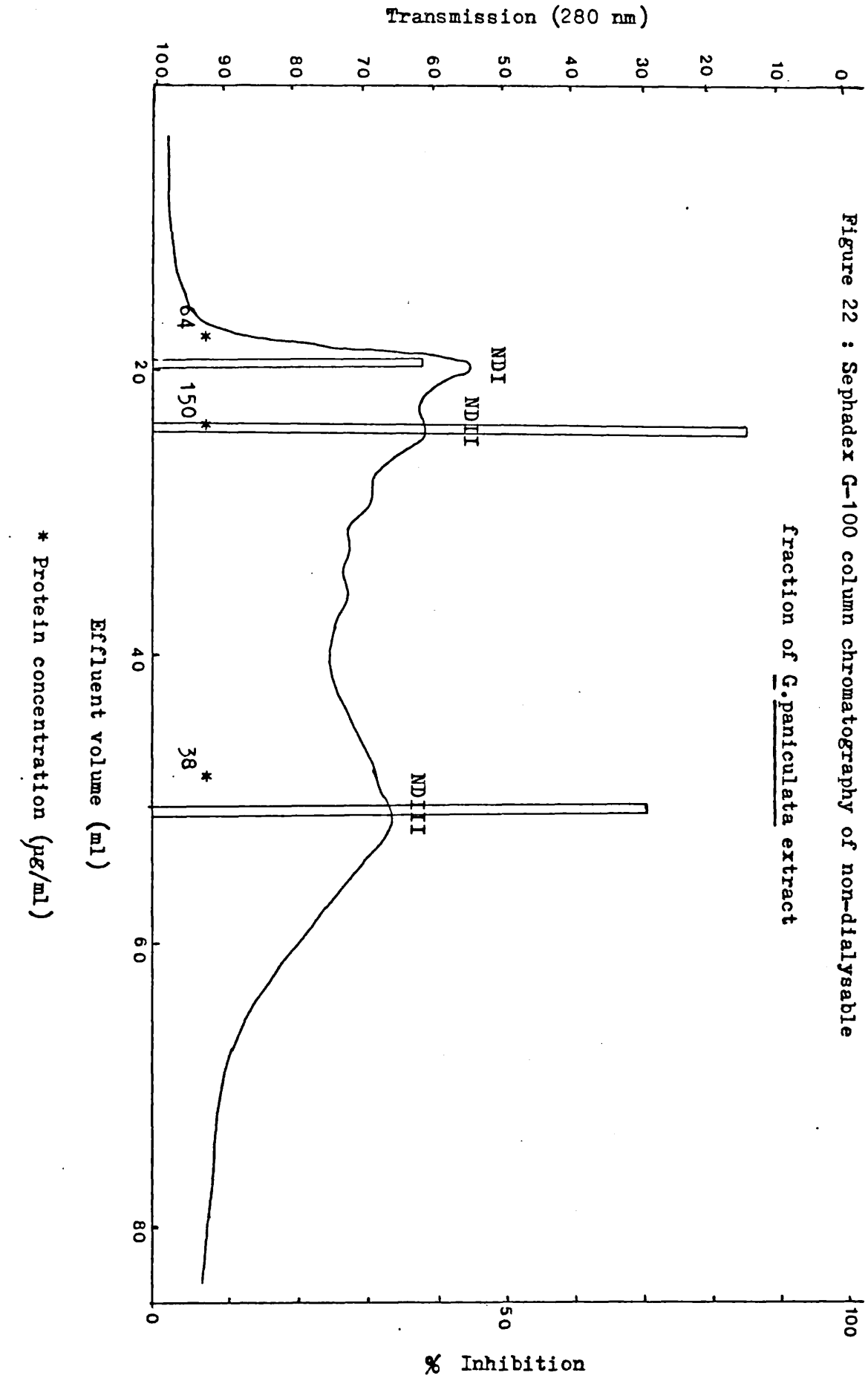


Figure 22 : Sephadex G-100 column chromatography of non-dialysable fraction of G.paniculata extract

* Protein concentration (µg/ml)

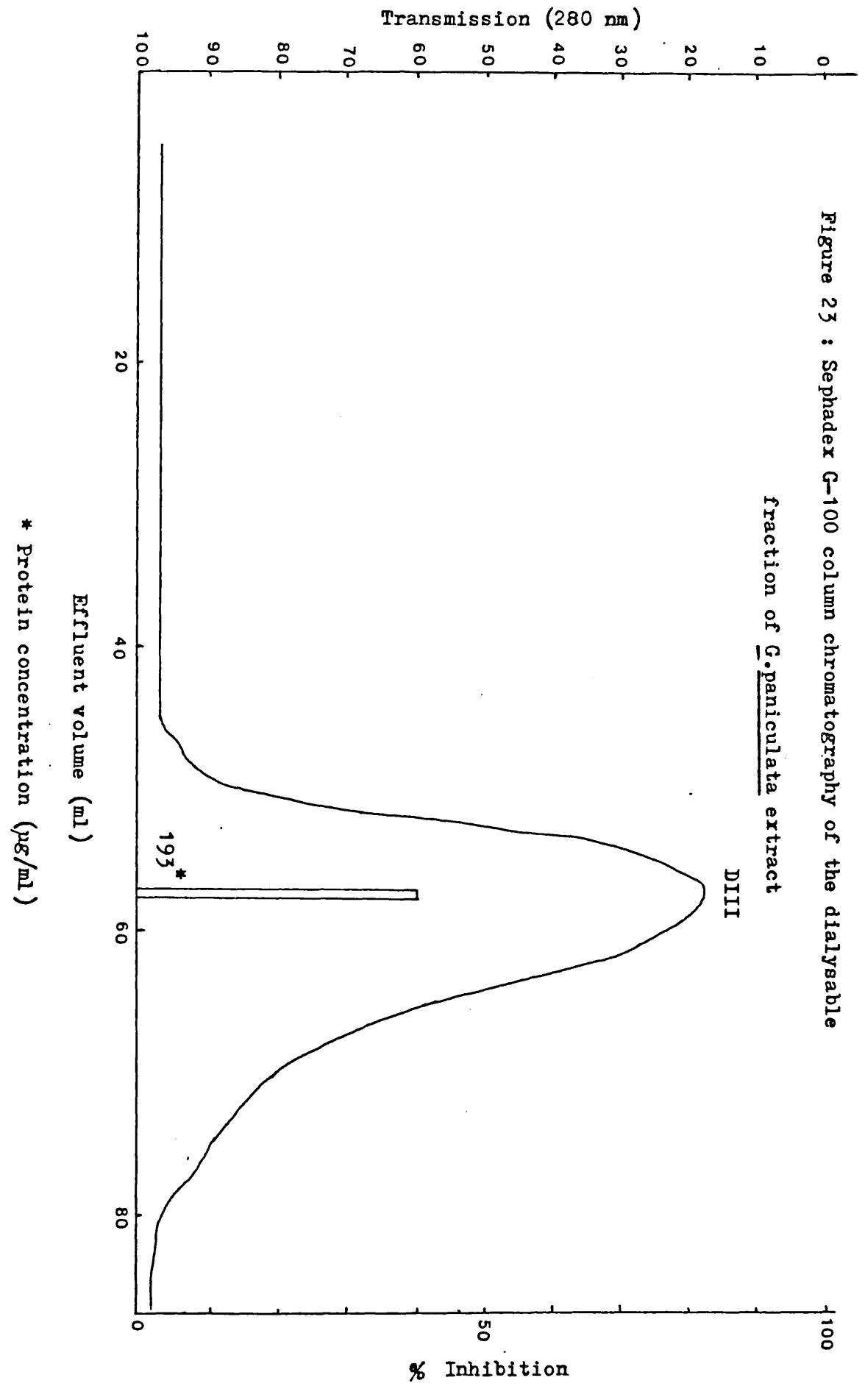


Figure 23 : Sephadex G-100 column chromatography of the dialysable fraction of G.paniculata extract

* Protein concentration (µg/ml)

dissolved in 2 ml of o.06 M phosphate buffer pH 7.0, then layered onto the column. It was found that ethanol treatment of Gp extracts precipitated all compounds in peaks I & II giving one large peak (EPI) as shown in Figure 24. This peak showed 87 % inhibition and high a concentration of protein (261 µg/ml). The supernatant consisted of peak III which is referred to as ESIII. Peak ESIII produced 44 % inhibition and contained 156 µg/ml of protein (Figure 25).

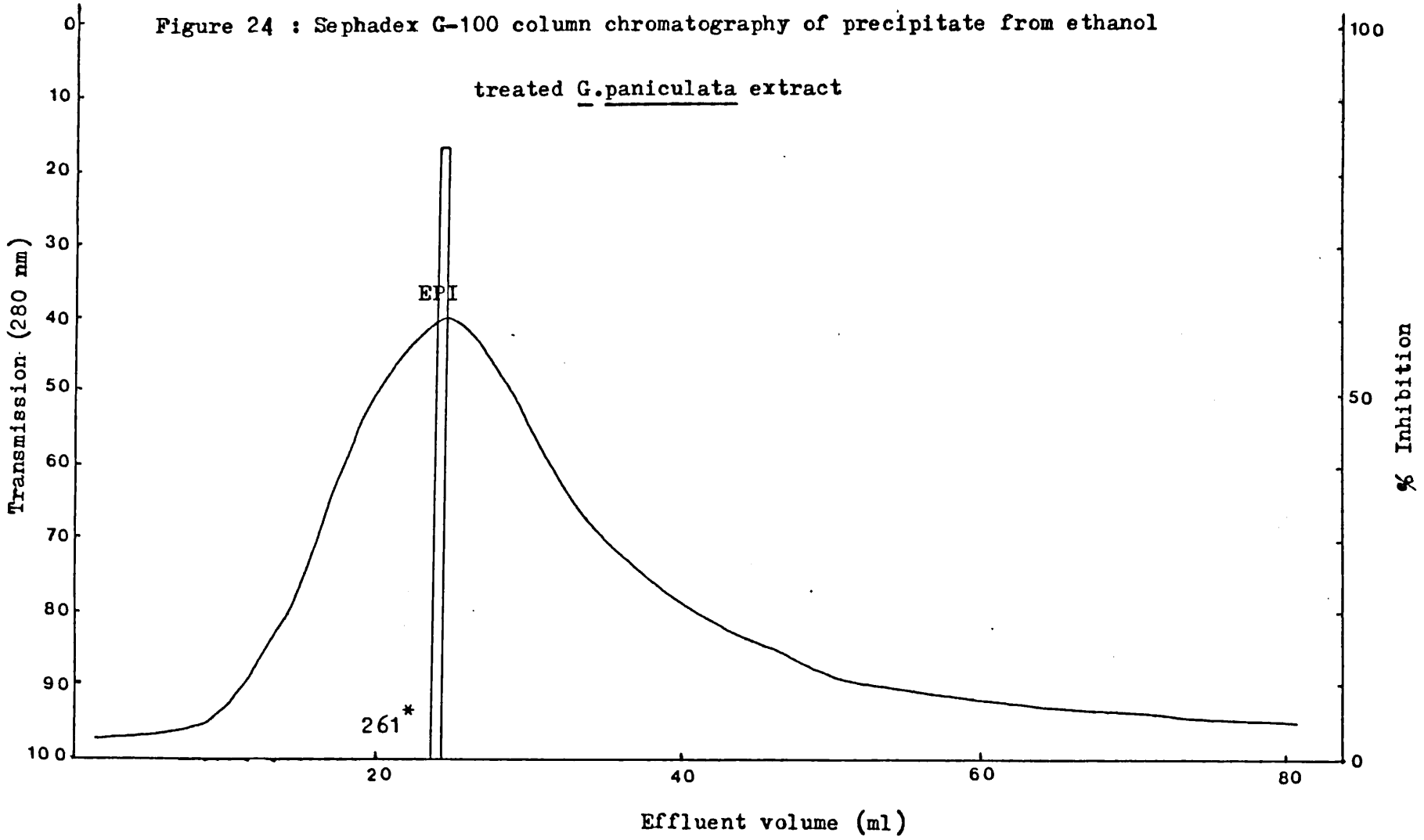
These results support the idea that in this extract the main part of the inhibitor is proteinaceous in nature and found in peaks I & II (Figure 18). On the other hand, the materials in peak III contain proteinaceous and non-proteinaceous substances. The non-proteinaceous fraction is unaffected by heat, not precipitated by ethanol and of small molecular weight, but nevertheless inhibits TNV. Sephadex column chromatography has confirmed that G.paniculata inhibitors are complex mixtures of compounds, the major part being proteinaceous in nature with molecular weights ranging between 12,600 - 27,500.

2. Ion exchange chromatography

Several compounds such as proteins, hormones, nucleic acids and carbohydrates have been efficiently separated using ion exchange cellulose. A number of cellulosic ion exchangers are now available; however, the cation exchangers carboxymethyl cellulose (CM-52), and the anion exchangers N,N.diethylaminoethyl cellulose (DEAE-52) are those most widely used (Peterson and Sober, 1962).

Some researchers have used ion exchangers in the purification and characterization of plant virus inhibitors. Ragetli and Weintraub (1962a) purified the inhibitor from Dianthus caryophyllus by adsorption

Figure 24 : Sephadex G-100 column chromatography of precipitate from ethanol treated G.paniculata extract



* Protein concentration (µg/ml)

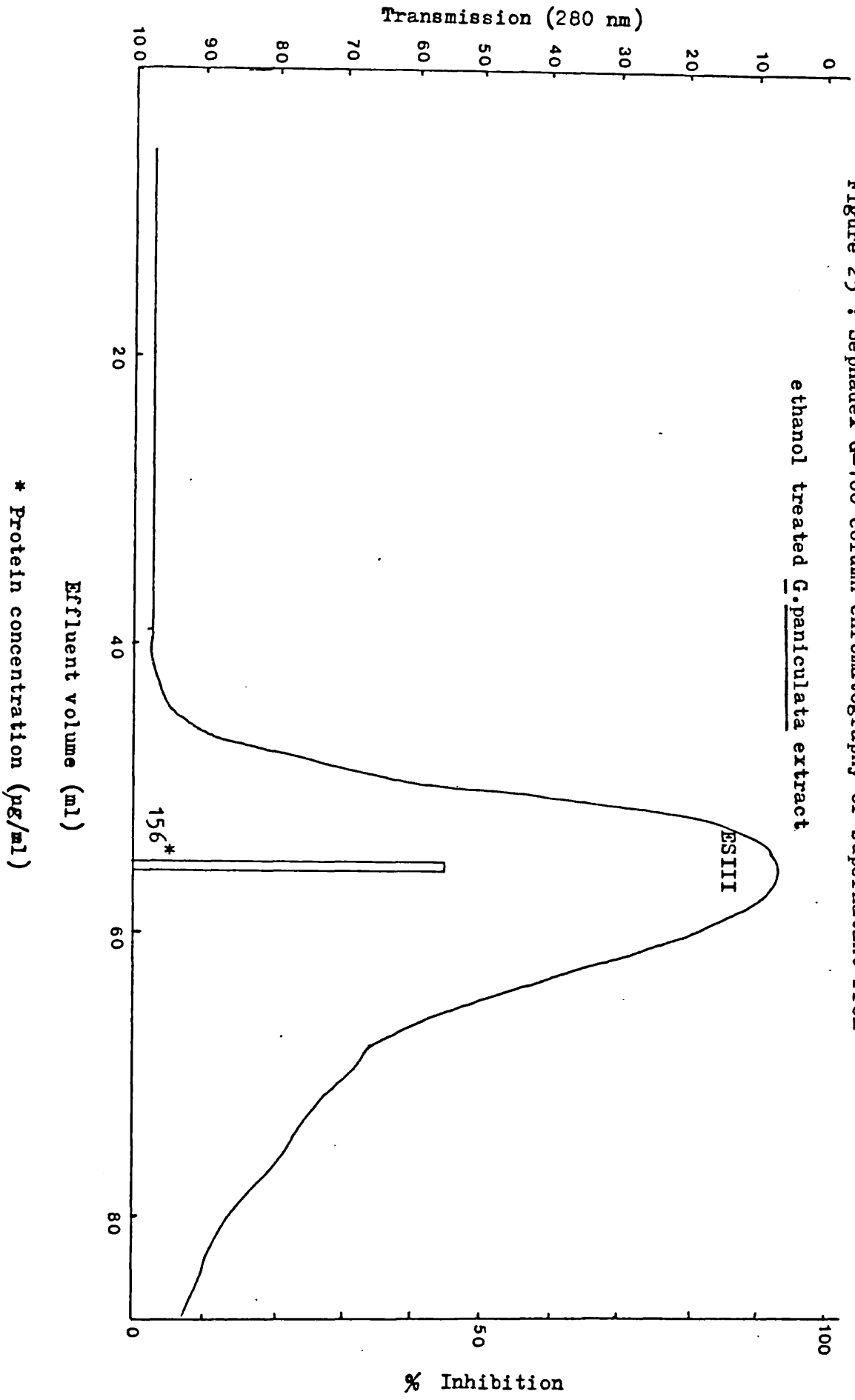


Figure 25 : Sephadex G-100 column chromatography of supernatant from ethanol treated G.paniculata extract

onto CM-cellulose at pH 6.0. However, the inhibitor was not adsorbed at pH 7.5 by DEAE-cellulose. Sela et al. (1964) separated an antiviral factor which occurred in Nicotiana glutinosa infected with TMV by using DEAE and CM-cellulose at pH 7.6 and pH 4.0 respectively. Recently, Stirpe et al. (1981) used CM-cellulose (CM-52) in the purification, from carnation leaves, of two proteins each with protein synthesis and virus inhibitor properties. Fukaya and Taniguchi (1979) also purified an inhibitor of plant virus infection occurring in the leaves of P.americana by using ion-exchange columns (DEAE & CM-cellulose).

a) Column chromatography using CM-52 cellulose

A suspension of Whatman CM-52 cellulose was prepared according to the manufacturers instructions. The suspension was packed into a glass column 1.5 x 40 cm. The buffer, 0.03 M citric acid-phosphate pH 4.0, was allowed to pass through the column at a constant flow rate of 50 ml per hour in the cold room for 24 hours. Two ml of standard citric acid-phosphate buffer pH 4.0 containing 5 mg of lyophilized Gp extracts were applied to the column. Elution was carried out using a continuous linear salt gradient up to 0.5 M NaCl in 0.03 M citric acid-phosphate buffer.

The elution profiles of Gp extracts revealed 4 peaks (A, B, C & D). Peak A was eluted by 0.13 M NaCl, peak B by 0.20 M NaCl; whereas peaks C & D eluted with 0.22 and 0.25 M NaCl respectively (Figure 26). The fractions were dialysed for two days against 3 changes of 500 ml distilled water in a cold room at 10 ^{o}C to remove the NaCl, and they were then tested 1- for inhibitory activity against TNV 2- for protein content by the Lowry et al. method, and 3- examined by disc electrophoresis using polyacrylamide gel.

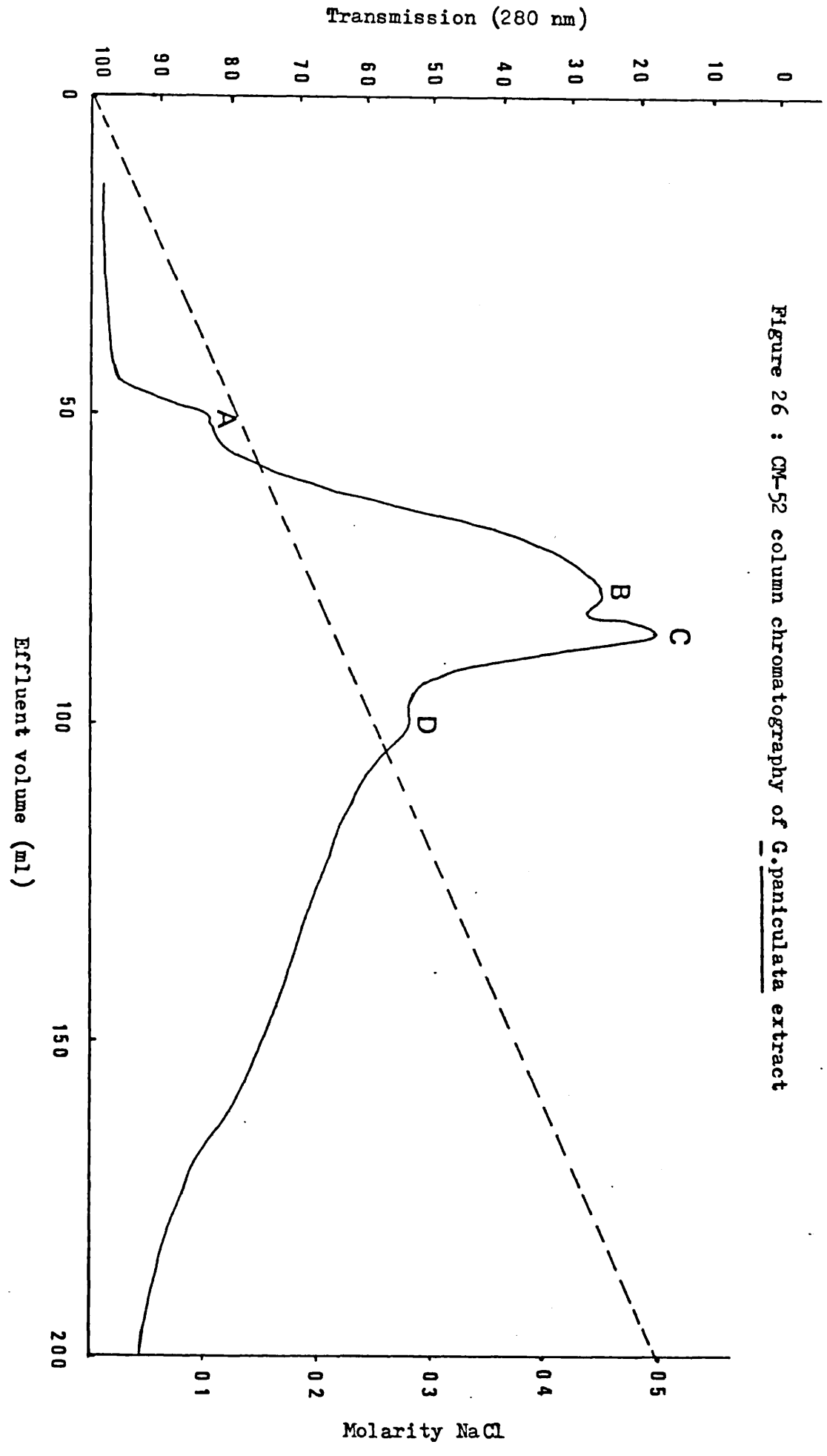


Figure 26 : CM-52 column chromatography of G.paniculata extract

Results in Table 24 show that peak B was not inhibitory, but contained a protein concentration of 110 µg/ml. Peak A showed some inhibition (16 %) and contained the lowest concentration of protein (43 µg/ml). Peak C, showed the highest inhibitory activity giving 41 % inhibition and the highest protein concentration (125 µg/ml). Peak D, which gave 39 % inhibition contained nearly the same protein concentration (113 µg/ml) as peak C.

Disc electrophoresis in Figures 27 and 28 show that peak A, which was inhibitory (16 %), consisted only of one band of protein (Rf = 0.31), and one wide band of glycoprotein located at the middle of the gel (Rf = 0.60). Peak C, which produced the highest inhibition (41 %), consisted of 3 bands of protein (Rf = 0.79, 0.64 & 0.23) and 3 bands of glycoprotein (Rf = 0.80, 0.64 & 0.24). The band at Rf = 0.23 gave strong reaction for both protein and glycoprotein. Peak D, which produced similar inhibition as peak C, consisted of 2 bands of protein (Rf = 0.66 & 0.52), and 2 bands of glycoprotein (Rf = 0.68 & 0.54). It was clearly observed that the Rf values of protein in peaks C & D corresponded closely to that of the glycoprotein. This suggests that the inhibitor in peaks C & D is a glycoprotein. In peak A, the protein and the glycoprotein have different Rf values, either compound may be involved in inhibition. Peak B, which was not inhibitory, contained 3 protein bands (Rf = 0.80, 0.66 & 0.46), however, no bands of glycoprotein were detected in the gel.

b) Column chromatography using DEAE-52 cellulose

Although using CM-52 was a suitable technique for the purification of virus inhibitors and brought about a relatively good protein

Table 24 : Effects of CM-52 fractions of G.paniculata extract (see Fig. 26) on local lesion production by TNV

Peak (see Fig.26)	Mean number of lesions *		% Inhibition	Protein content (µg/ml)
	TNV + Water	TNV + Peak		
A	113.5	95.5	16	43
B	75.7	79.9	-6	110
C	86.3	51.0	41**	125
D	63.6	39.1	39**	113

* Each figure represents the mean number of lesions for ten leaves

Negative values of % inhibition = enhancement

** Significant inhibition (P = 0.001 & 0.01 for peaks C & D respectively)

Figure 27 : Disc electrophoresis of the CM-52 fractions from G.paniculata extracts

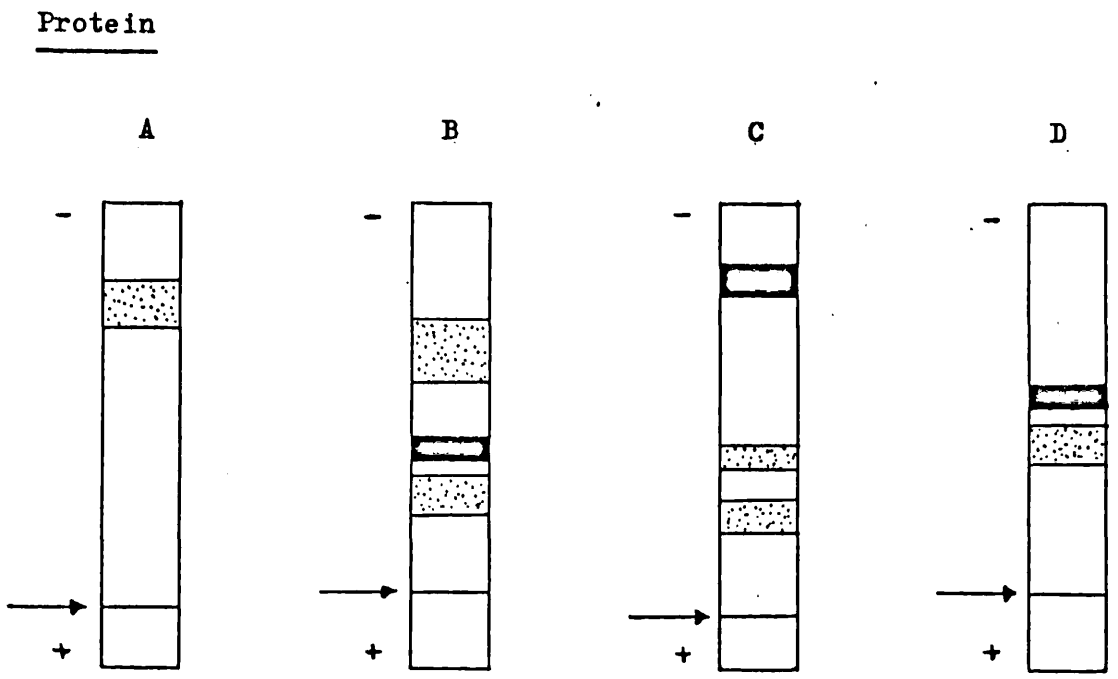
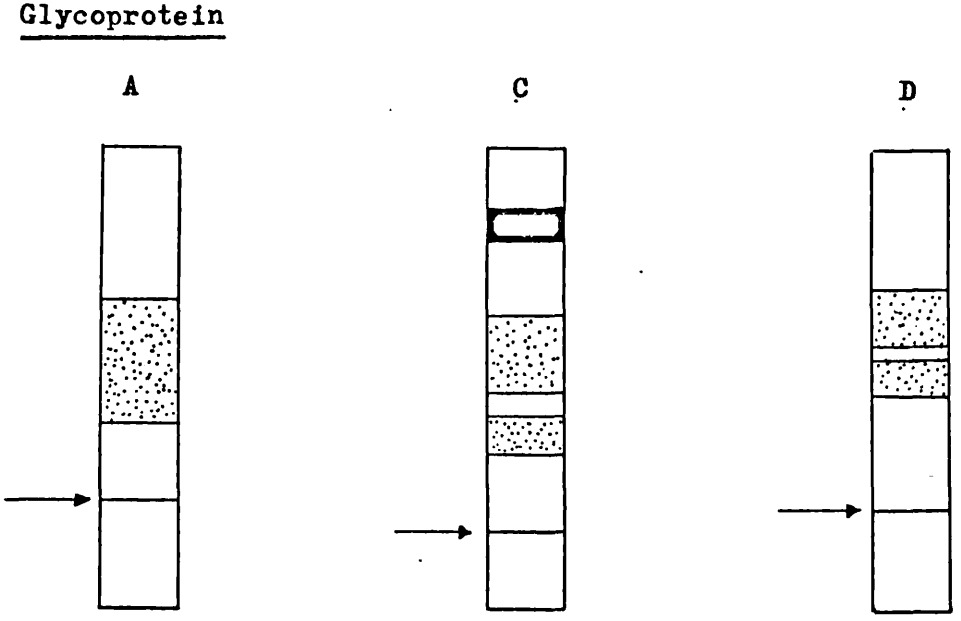


Figure 28 : Disc electrophoresis of the CM-52 fractions from G.paniculata extracts



⟶ Position of marker

Shading indicates intensity of the band staining

Peak B, showed no staining reaction for glycoprotein

separation, several trials were made to produce better resolution of the Gp proteins using DEAE-52 chromatography, but unfortunately with little success. It was noticeable that the Gp extracts strongly attached to the DEAE-anion exchange but could not be eluted using high NaCl concentrations.

Discussion

Although numerous higher plant extracts are known to contain potent inhibitors of plant viruses, the nature of most of the inhibitors is still controversial, and little is known about the ways in which they reduce infection.

Molecular weight determinations, for inhibitor compounds, in plant extracts have been studied by a number of workers and a wide size range of compounds implicated as virus inhibitors. Allen et al. (1969) found that Phaseolus vulgaris seed extract contains a glycoprotein inhibitor with a molecular weight of 115,000. Results similar to those reported by Hajj and Stevens (1979). Most workers, however, report lower molecular weight inhibitors. Ragetli (1957), for example, found that the carnation (Dianthus caryophyllus) inhibitor has a molecular weight of 10,000. Stirpe et al. (1981) isolated two proteins from carnation (dianthin 30, and dianthin 32) with molecular weights of 29,000 and 31,000 respectively. Wyatt and Shepherd (1969) reported that juice from Phytolacca americana contains a basic peptide with a molecular weight of 13,000. However, Irvin (1975) and Fukaya and Taniguchi (1979) found that the protein from this species consisted of a single polypeptide of molecular weight 27,000. Inhibitors from Chenopodiales species have been reported with molecular weights of 25,000 - 38,000

(Smookler, 1971). These latter figures correspond closely to the results given in this thesis.

Sephadex G-100 columns chromatography of G.paniculata extracts revealed the presence of rapidly eluted inhibitory materials with molecular weights of between 27,500 - 12,600. Some materials which eluted slowly (peak III) showed inhibitory activity giving 43 % inhibition, and had a molecular weight of 2,000.

The complexity of the extracts was demonstrated using CM-52 column chromatography and gel electrophoresis. Of the four peaks (A, B, C & D) found by CM-52 chromatography, three (A, B & C) were inhibitory to local lesion production by TNV. This suggests that G.paniculata extracts contain at least three plant virus inhibitors. It was noticeable that peak A was weakly attached to the cation exchanger (CM-52), indicating its neutral or slightly acidic nature. Peaks C & D, on the other hand, appeared to be more strongly attached to the columns and required relatively high concentrations of NaCl to elute them, indicating their more basic nature. Several basic proteins showing virus inhibitory activity have been reported from plants such as D.caryophyllus (Ragetli, 1957) and Phytolacca americana (Wyatt and Shepherd, 1969). Both these inhibitors were reported to attach strongly to their cation columns and required high concentrations of buffer to remove them. These workers suggest that the biological activity of the inhibitors is due to the basic nature of the proteins. In addition, Smookler (1971) who used a different ion exchangers (CM-Sephadex G-25) to purify the inhibitors from leaves of Chenopodium amaranticolor, Chenopodium album, Atriplex nitens and Amaranthus caudatus, showed that active extracts

were eluted by 0.4 M sodium acetate buffer, suggesting that the inhibitors are basic in nature.

Disc electrophoresis showed again that each of the virus inhibitors, with the exception of peak A, consisted of more than one band of proteins and glycoproteins. Both protein and glycoprotein in peak A appeared to have different Rf's values suggesting that the inhibitor may be either protein or glycoprotein. On the other hand, peaks C & D which showed the strongest activity (41 & 39 % inhibition respectively), consisted of more than one band of proteins which corresponded closely to those bands which stained for glycoproteins. This may indicate that the inhibitors in these two peaks are glycoproteins. As mentioned earlier many workers have suggested that virus inhibitors are glycoproteins. One further piece of evidence supporting this idea is the observation that peak B, which contained protein but no glycoprotein, had no inhibitory activity.

It seems clear that CM-52 chromatography was an effective technique for the purification of virus inhibitors and bring about good protein separation. Similar results were reported by Wyatt and Shepherd (1969) who prepared a highly purified virus inhibitors from P.americana by chromatography on a similar column of CM-Sephadex. However, Albrechtova (1968) applied DEAE Sephadex A-50 chromatography to separate PVX virus inhibitors from potato leaf sap. The inhibitor was eluted at the concentration 0.15 - 0.3 M NaCl and was electrophoretically homogeneous.

Gypsophila extracts also contain low molecular weight compounds which showed inhibitory activity against plant viruses. Similarly, Taniguchi (1974) reported the presence, in Phaseolus vulgaris sap, of

two relatively low molecular weight inhibitory substances which diffused in Sephadex 2 B gel, however, their chemical nature was not studied.

At this stage it can be concluded that gel filtration and CM-52 chromatography are useful techniques in adding more information about the properties of G.paniculata virus inhibitors,by giving an idea about the molecular weights of the constituents and properties of the protein isolated from the extracts.

Bearing in mind the properties of Gp extracts described in this thesis, it might be suggested that glycoproteins found in the extracts are involved in plant virus inhibition. However, it would seem appropriate to separate and identify the small molecular weight inhibitory substances in the extracts. Results of these experiments will be described in section B of this chapter.

SECTION B

STUDIES OF THE LOW MOLECULAR WEIGHT FRACTIONS

It has been established throughout this investigation that G. paniculata extracts are complex mixtures of compounds. The preliminary studies of extracts by techniques including dialysis, precipitation with ethanol or ammonium sulphate, as well as, gel filtration and disc electrophoresis have indicated that they consist mainly of high molecular weight compounds identifiable as proteins or glycoproteins. On the other hand, the evidence from several experiments described in this thesis supports strongly the idea that low molecular weight materials also have considerable inhibitory effects against plant viruses.

In chapter V, amino acids and sugars were detected as the main constituents of the low molecular weight fractions of inhibitory extracts. In view of these observations both amino acids and sugars have been examined to test their effects on local lesion production by TNV. Two approaches have been made, in the first instance Gp extracts have been examined to determine their natural content of amino acids and sugars. Secondly, using this knowledge, pure samples of amino acids and sugars have been tested for virus inhibitory properties. The results of amino acids studies will be described first followed by those of sugars.

a) AMINO ACIDS

1. Qualitative and quantitative determination of amino acids in G.paniculata extracts

Amino acids have been separated and identified from Gp extracts using: a- paper chromatography b- an automatic amino acid analyser

(a) Paper chromatography

Amino acids were separated from the whole and dialysable part of Gp extracts by one dimensional paper chromatography using a solvent composed of butanol, acetic acid and water (4:1:5 V/V). The amino acids were identified by comparison with known compounds after dipping the paper in 0.1 % solution of ninhydrin in acetone, drying and heating in an oven at 100 °C for 5 minutes.

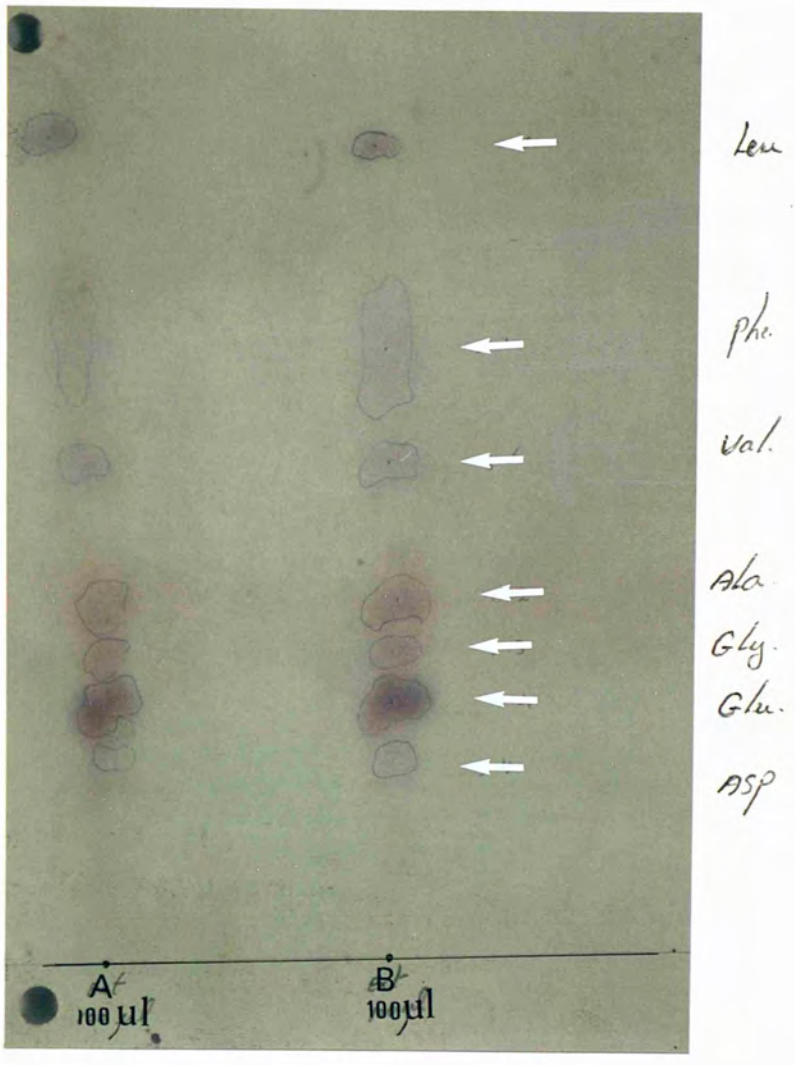
Results in Figure 29 show that the whole extract of Gp contained seven amino acids identified as aspartic acid, alanine, glutamic acid, glycine, valine, leucine and phenyl-alanine. The dialysable fraction gave an almost identical pattern of amino acids (Figure 29).

Paper chromatography proved useful in separating some of the amino acids in the extracts. The technique, however, did not separate all components. To analysis the free amino acids more precisely, the more refined technique of amino acid analysis was performed using an automatic amino acid analyser.

(b) Automatic amino acid analysis

The separation and quantitative determination of free amino acids were carried out using a Joel model JLC 6AH fully automatic amino acid analyser. In principle, this instrument automatically records the value of the ninhydrin colour of the effluent from ion exchange columns. The influent buffer is pumped at a constant rate through a column of sulfonated polystyrene resin. The effluent is met by a capillary steam of ninhydrin reagent, colour is developed by passing the mixture of reagent and effluent through a spiral capillary Teflon tube immersed in a bioling water bath. The absorbance of the resulting solution is measured continuously at 570 and 440 nm as it flows through a cylinder-

Figure 29 : Analysis of the amino acids from the whole and dialysable fraction of G.paniculata extracts using paper chromatography



A = The whole Gp extracts

B = The dialysable fraction

ical glass cell. The peaks on the recorded curves can be integrated with a precision of 100 ± 3 % for loads from 0.1 to 3.0 µmls for each amino acid (Spackman et al., 1958).

50 mg of lyophilized G.paniculata extracts were prepared for the analyser by mixing in 5 ml of 2 % phenol and 10 ml of 30 % TCA (trichloroacetic acid). The non-dialysable and dialysable fractions were treated in the same way. The mixtures were left overnight and then filtered through Whatman No. 1 filter paper. The pH of the filt rate which contained free amino acids was adjusted to pH 2 ± 0.2 before placing in the analyser. The identity of the components was found from the standard curve produced using known amino acids (Figure 30). The amount of each individual amino acid in the extracts was calculated by comparison of peak areas with those obtained using a calibration mixture as described by Eveleigh and Winter (1970).

Amino acids in the Gp extracts

The free amino acids present in the whole extract are shown in Figure 31. Seventeen free amino acids were identified and two other small peaks (A & B) remained unidentified. The dialysable fraction gave an almost identical pattern of amino acids (Figure 32). The non-dialysable fraction, however, did not show any distinct peaks of free amino acids (Figure 33).

The quantity of each individual free amino acid and the total amount of amino acids present in the whole extract are shown in Table 25. Aspartic acid, histidine and glutamic acid showed the highest concentrations, whereas lysine, alanine and arginine were present only in small amounts. The 17 amino acids were tested for virus inhibitor properties.

Figures 30, 31, 32 and 33 : Amino acids analysis using a Joel model JLC 6AH fully automatic amino acid analyser.

Photographs of traces from analysis of:

Figure 30 : Standard amino acids

Figure 31 : Crude extract of G.paniculata

Figure 32 : Dialysable part of G.paniculata extract

Figure 33 : Non-dialysable part of G.paniculata extract

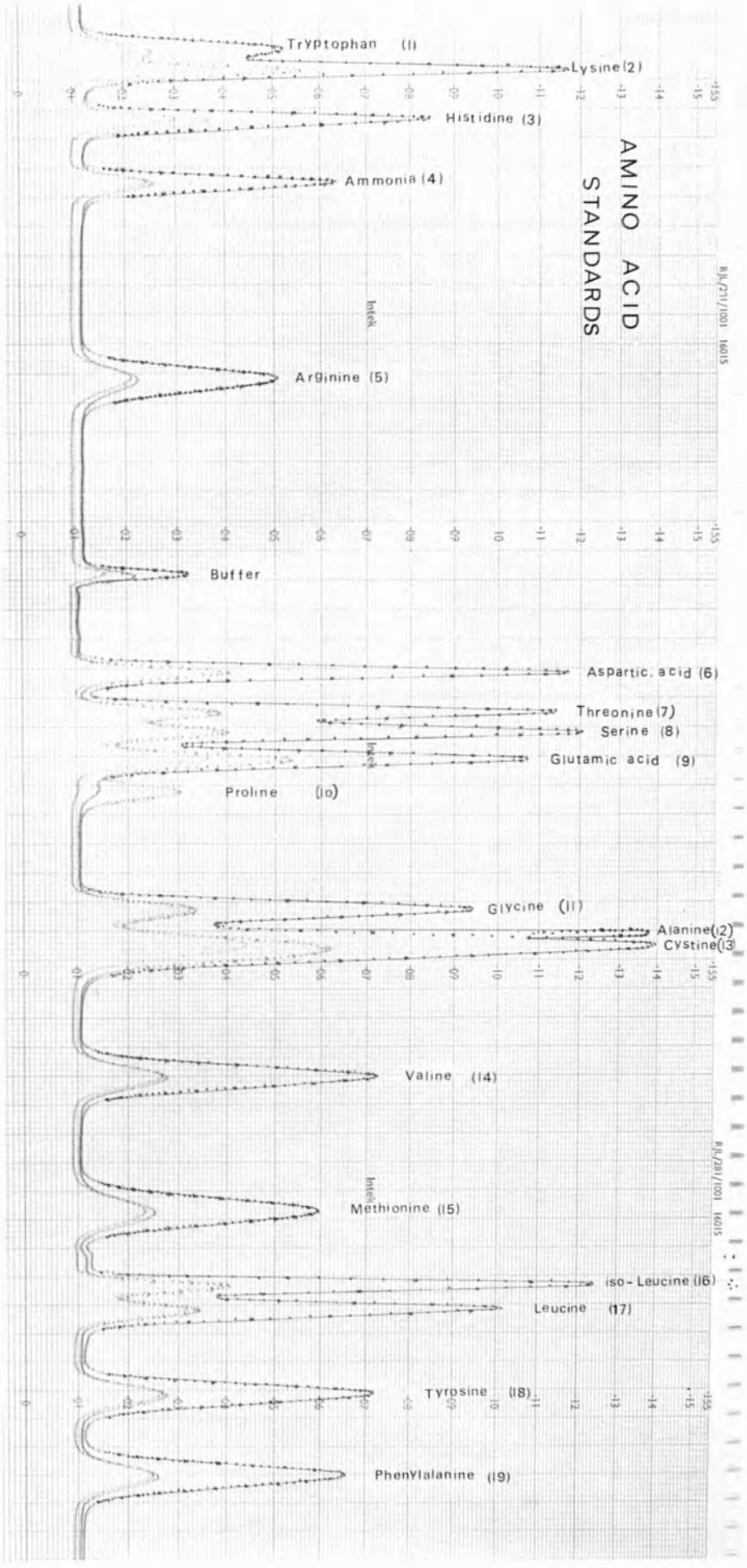
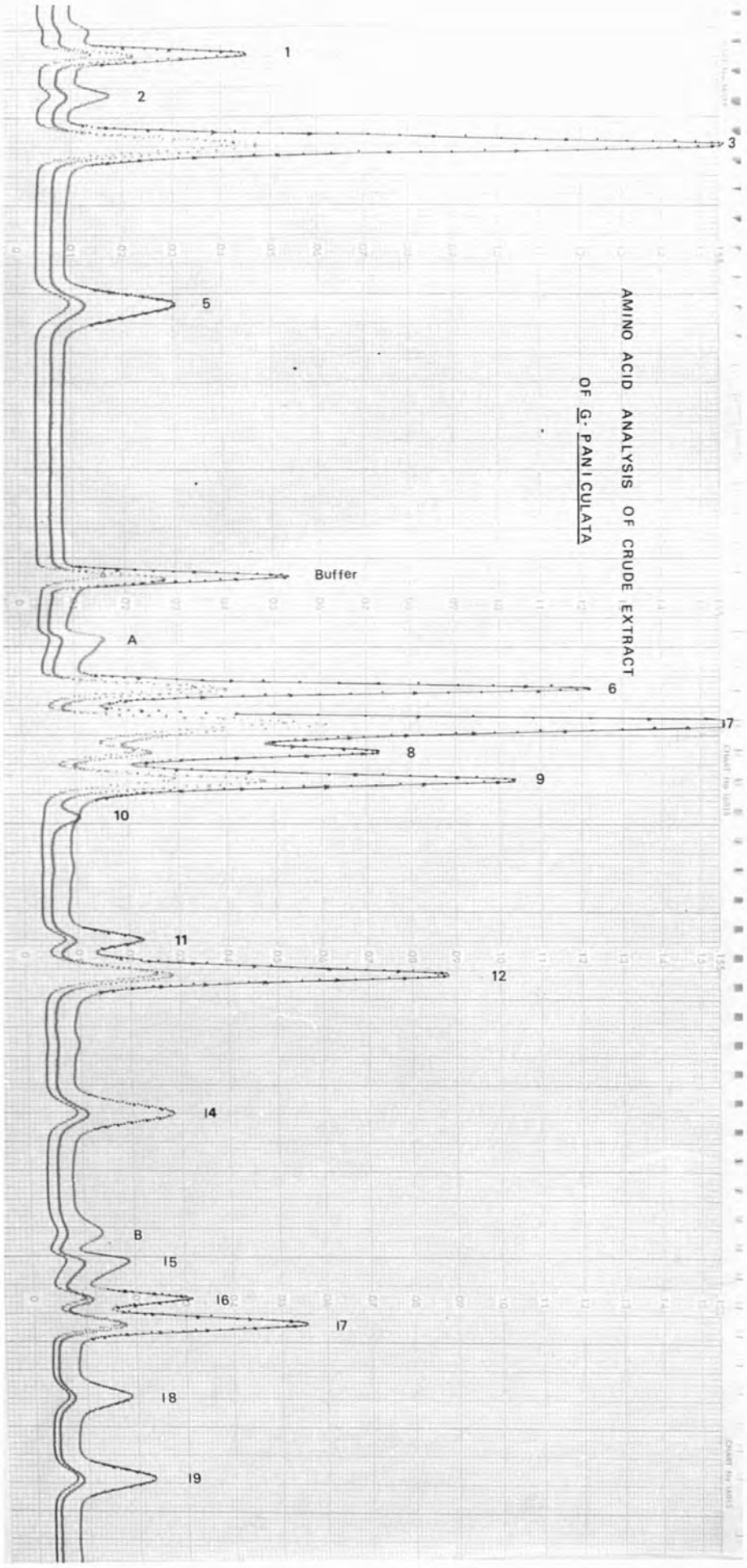

AMINO ACID
STANDARDS
Tryptophan (1)
Lysine (2)
Histidine (3)
Ammonia (4)
Arginine (5)
Buffer
Aspartic acid (6)
Threonine (7)
Serine (8)
Glutamic acid (9)
Proline (10)
Glycine (11)
Alanine (12)
Cystine (13)
Valine (14)
Methionine (15)
iso-Leucine (16)
Leucine (17)
Tyrosine (18)
Phenylalanine (19)
Intek
RJL/211/1001 16015
RJL/281/1001 16015

Figure : 30


AMINO ACID ANALYSIS OF CRUDE EXTRACT
OF G. PANICULATA
1
2
3
5
Buffer
A
6
7
8
9
10
11
12
14
B
15
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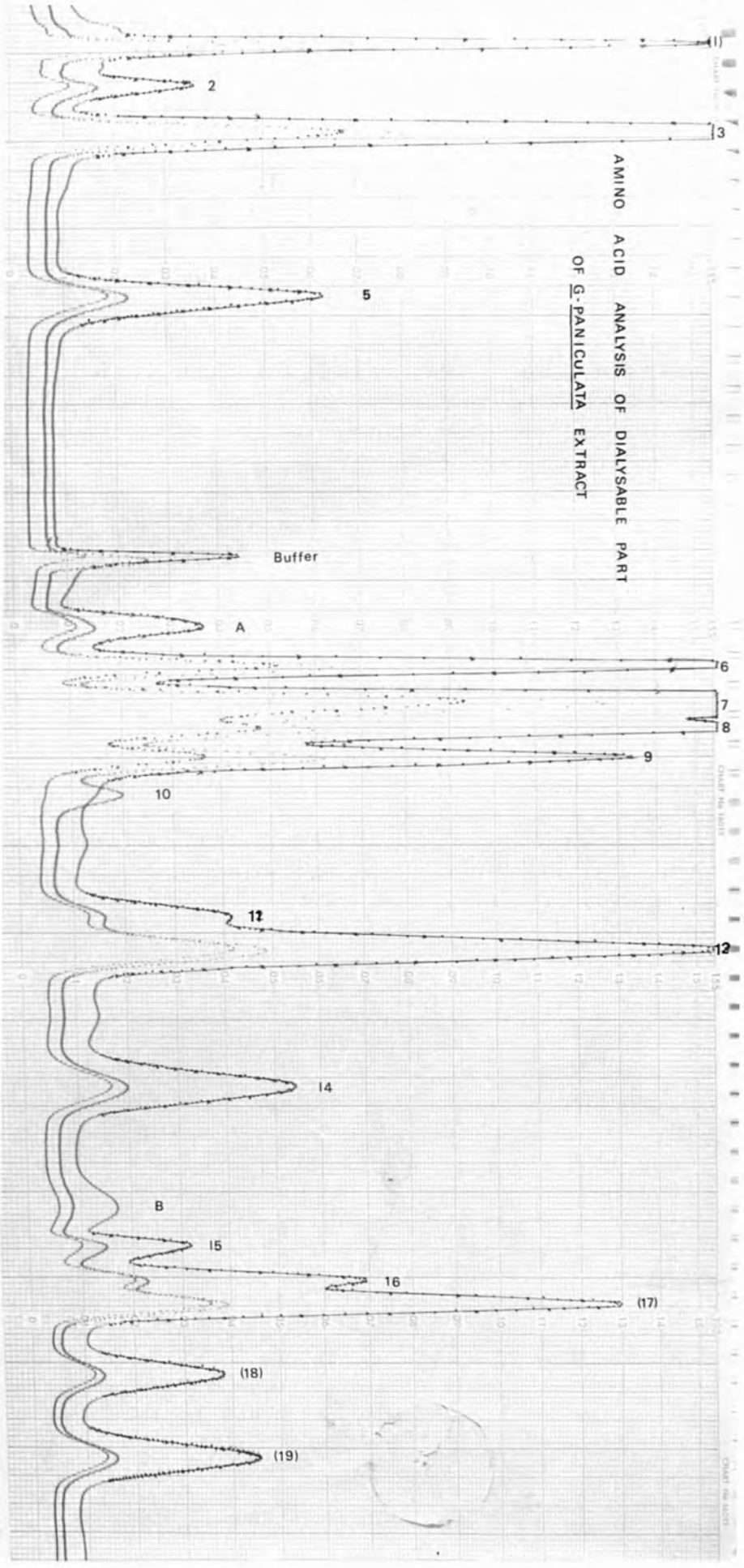
17
18
19

Figure : 31


AMINO ACID ANALYSIS OF DIALYSABLE PART
OF G. PANICULATA EXTRACT
1
2
3
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Buffer
A
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B
15
16
(17)
(18)
(19)

Figure : 32

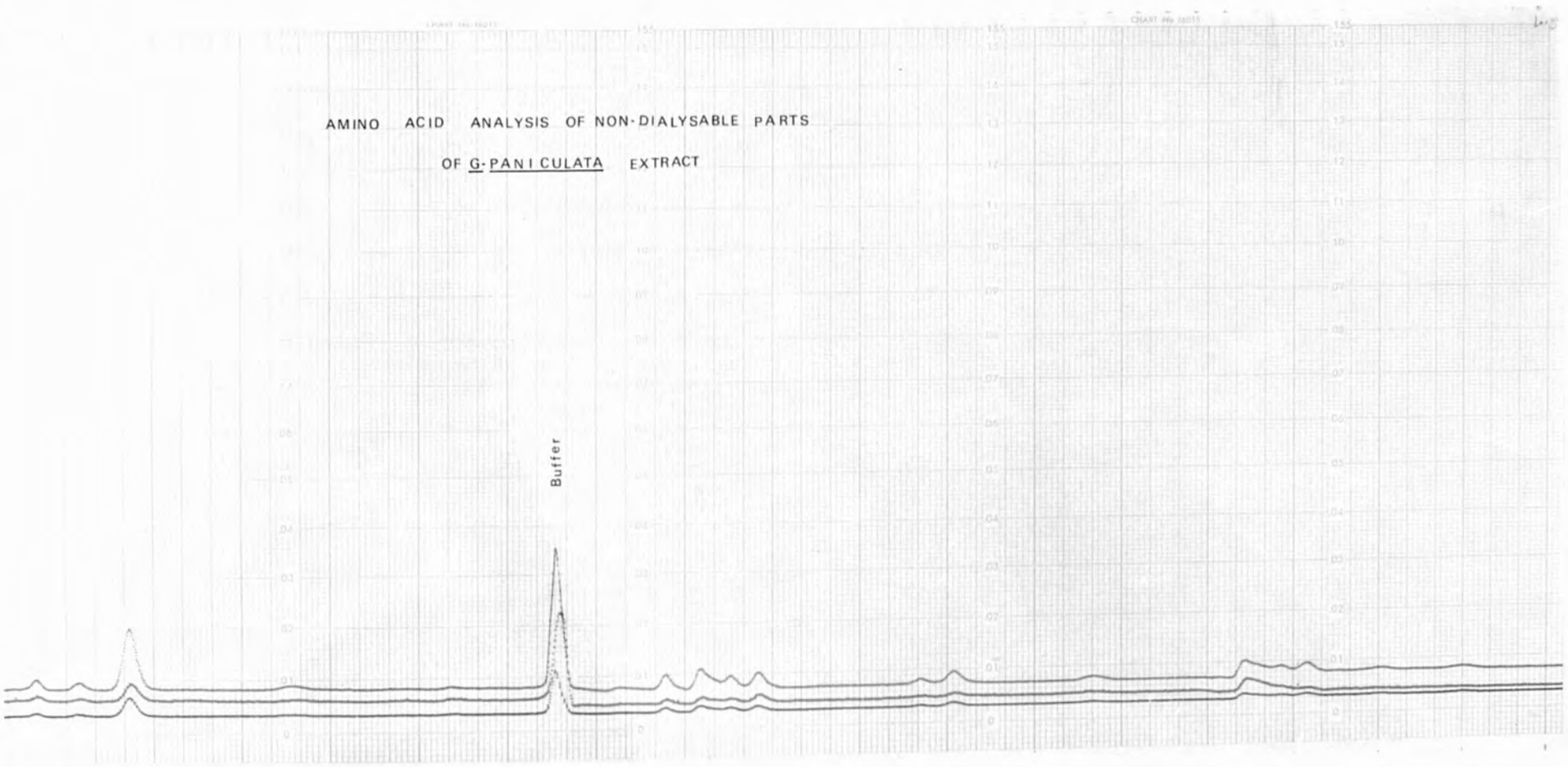

AMINO ACID ANALYSIS OF NON-DIALYSABLE PARTS
OF G. PANICULATA EXTRACT
Buffer

Figure : 33

Table 25: Free amino acids in lyophilized G.paniculata extract

Amino acid	* concentration (µg/mg)	Amino acid	* concentration (µg/mg)
Aspartic acid	48.9	Leucine	25.7
Histidine	124.4	Proline	6.2
Glutamic acid	49.1	Glycine	5.0
Phenyl-alanine	13.8	Methionine	10.2
Valine	12.9	Serine	24.3
Tyrosine	63.8	Lysine	3.8
Iso-leucine	11.1	Alanine	16.5
Tryptophan	101.9	Arginine	14.1
Threonine	18.8	Total free amino acid	550.5

* µg amino acid per mg lyophilized extract

2. Effects of free amino acids on local lesion production by TNV

Pure samples of each of the individual amino acids, identified in the whole Gp extract, were tested against TNV. All the amino acids were analytical grade and used at a concentration of 200 µg/ml, since higher concentrations can cause phytotoxicity. To do this, 400 µg of each pure amino acid was dissolved in 1 ml of distilled water, mixed with 1 ml of TNV in phosphate buffer pH 7.0 and inoculated onto french bean leaves. Control samples consisted of 1 ml of water and 1 ml of TNV.

The results in Tables 26 & 27 show that the different amino acids had varied effects on lesion production by TNV. In term of lesion numbers and percentage inhibition, the amino acids fall into 2 groups: i- those that inhibit, and ii- those that stimulate lesion production.

i. Amino acids which inhibit local lesion production by TNV

This group includes 10 free amino acids (Table 26). The most marked inhibition was shown with aspartic acid, histidine and glutamic acid giving 38, 31 and 26 % inhibition respectively. The rest of the amino acids within this group (phenyl-alanine, valine, tyrosine, iso-leucine, tryptophan, threonine and leucine) were less effective, inhibition ranging between 23 - 16 %.

ii. Amino acids which stimulate local lesion production by TNV

This group includes 7 free amino acids (Table 27). Proline, glycine, methionine and serine had no effect on lesion numbers compared with their controls. On the other hand, arginine, alanine and lysine strongly stimulated lesion production giving 60, 36 and 19 % more lesions respectively than their controls.

A mixture of the individual free amino acids, identified in the whole Gp extract, was also tested for inhibitory activity against TNV.

Table 26: Effect of pure amino acids (200 µg/ml) on local lesion produced by TNV, on French bean leaves

Amino acid	Mean number of lesions *		% Inhibition
	TNV+ Water (control)	TNV+ AA (treated)	
Aspartic acid	85.9	53.5	38
Histidine	65.7	45.3	31
Glutamic acid	76.0	56.0	26
Phenyl-alanine	81.8	63.1	23
Valine	75.0	58.5	22
Tyrosine	101.8	80.2	21
Iso-leucine	92.0	72.5	21
Tryptophan	103.3	83.6	19
Threonine	71.0	58.1	18
Leucine	102.4	85.7	16

* Each figure represents the mean number of lesions for ten leaves

AA = amino acid

Table 27: Effect of pure amino acids (200 µg/ml) on local lesion produced by TNV, on French bean leaves

Amino acid	Mean number of lesions *		% Inhibition
	TNV+ Water (control)	TNV+ AA (treated)	
Proline	66.7	68.8	-3
Glycine	51.8	53.2	-3
Methionine	85.2	88.7	-4
Serine	57.2	60.3	-5
Lysine	91.8	109.7	-19
Alanine	102.4	139.0	-36
Arginine	61.3	97.9	-60
Amino acid mixture **	93.5	68.4	27

* Each figure represents the mean number of lesions for ten leaves

** Each amino acid was at a final concentration of 200 µg/ml

AA = amino acid

Negative values of % inhibition = enhancement

Full statistical treatment for this table is givin in the appendix 1

800 µg of each of the 17 amino acids were dissolved in 2 ml of water, then mixed with 2 ml of TNV and inoculated onto french bean leaves. Controls consisted of 2 ml of water plus 2 ml of TNV. Results in Table 27 show that the amino acid mixture produced 27 % inhibition. It can be suggested from these results that inhibitory amino acids act more strongly than those with enhancing properties.

It is noticeable that those amino acids producing the highest levels of inhibition, are present at higher natural concentrations in the Gp extracts than amino acids producing enhancement. For example, aspartic acid, histidine and glutamic acid which showed 38, 31 & 26 % inhibition respectively were present at concentrations of 48.9, 124.4 & 49.1 µg/mg of lyophilized extract (Table 25), whereas lysine, alanine and arginine with augmenter activity were present only at concentrations of 3.8, 16.5 and 14.1 µg/mg lyophilized extract respectively (Table 25). Incidentally, it is interesting to note that the high levels of tryptophan and tyrosine in the small molecular weight fraction may explain the positive protein reaction given using the Lowry test as mentioned previously (p. 127).

It seems from these results that the concentration of 200 µg/ml of aspartic acid gave the maximum inhibitory effect (38 %) compared with other inhibitory acids (Table 26). In contrast, similar concentration of arginine showed the maximum virus enhancement (60 % more lesions). It was decided, therefore, to examine the effects of various concentrations of these two interesting amino acids against TNV.

a) Effect of various concentrations of aspartic acid on local lesion production by TNV

Five concentrations of aspartic acid (50, 100, 250, 500 & 1000 µg/ml)

were prepared in distilled water. Two ml of each concentration were mixed with 2 ml of TNV and then inoculated onto french bean leaves. Control samples consisted of 2 ml of water and 2 ml of TNV. Results in Table 28 and Figure 34 show that the lower concentrations (50 & 100 µg/ml) had no inhibitory activity. At higher concentrations (250, 500 and 1000 µg/ml) the percentage inhibition increased with concentration, giving 36, 41 and 48 % inhibition respectively. None of the concentrations used caused any phytotoxic effects.

b) Effect of various concentrations of arginine on local lesion production by TNV

Five concentrations of arginine were prepared and assayed on french bean leaves as for the aspartic acid. Results in Table 29 and Fig. 34 show that the higher concentrations of arginine (500 and 1000 µg/ml) slightly stimulated lesion production, while at the lower concentrations (50, 100 and 250 µg/ml) arginine produced virus enhancement giving 64, 53 and 38 % more lesions respectively than their controls. No phytotoxic effects were observed. In view of these interesting results, it was thought useful to examine the possible effect of antagonism between these two differently behaving amino acids.

c) Effect of mixing aspartic acid and arginine on local lesion production by TNV

500 µg of aspartic acid and of arginine were dissolved in 1 ml of distilled water, then mixed with 1 ml of TNV and inoculated onto french bean leaves. Controls consisted of 1 ml of water plus 1 ml of TNV. Results in Table 30 show that the net effect of these two amino acids was inhibitory giving 21 % reduction in lesion numbers. Such a result may be interpreted as showing that the effect of aspartic acid, which

Table 28 : Effect of various concentrations of aspartic acid on local lesion production by TNV on French bean leaves

Concentration (µg/ml)	Mean number of lesions *		% Inhibition
	TNV + Water (control)	TNV + Aspartic acid	
50	81.5	79.3	3
100	66.8	68.1	-2
250	97.3	62.5	36
500	117.0	68.9	41
1000	72.7	37.9	48

* Each figure represents the mean number of lesions for ten leaves

Table 29 : Effect of various concentrations of arginine on local lesion production by TNV on French bean leaves

Concentration (µg/ml)	Mean number of lesions *		% Inhibition
	TNV + Water (control)	TNV + Arginine	
50	57.3	94.0	-64
100	76.0	116.2	-53
250	71.3	98.5	-38
500	48.9	52.4	-7
1000	67.3	69.5	-3

* Each figure represents the mean number of lesions for ten leaves

Negative values of % inhibition = enhancement

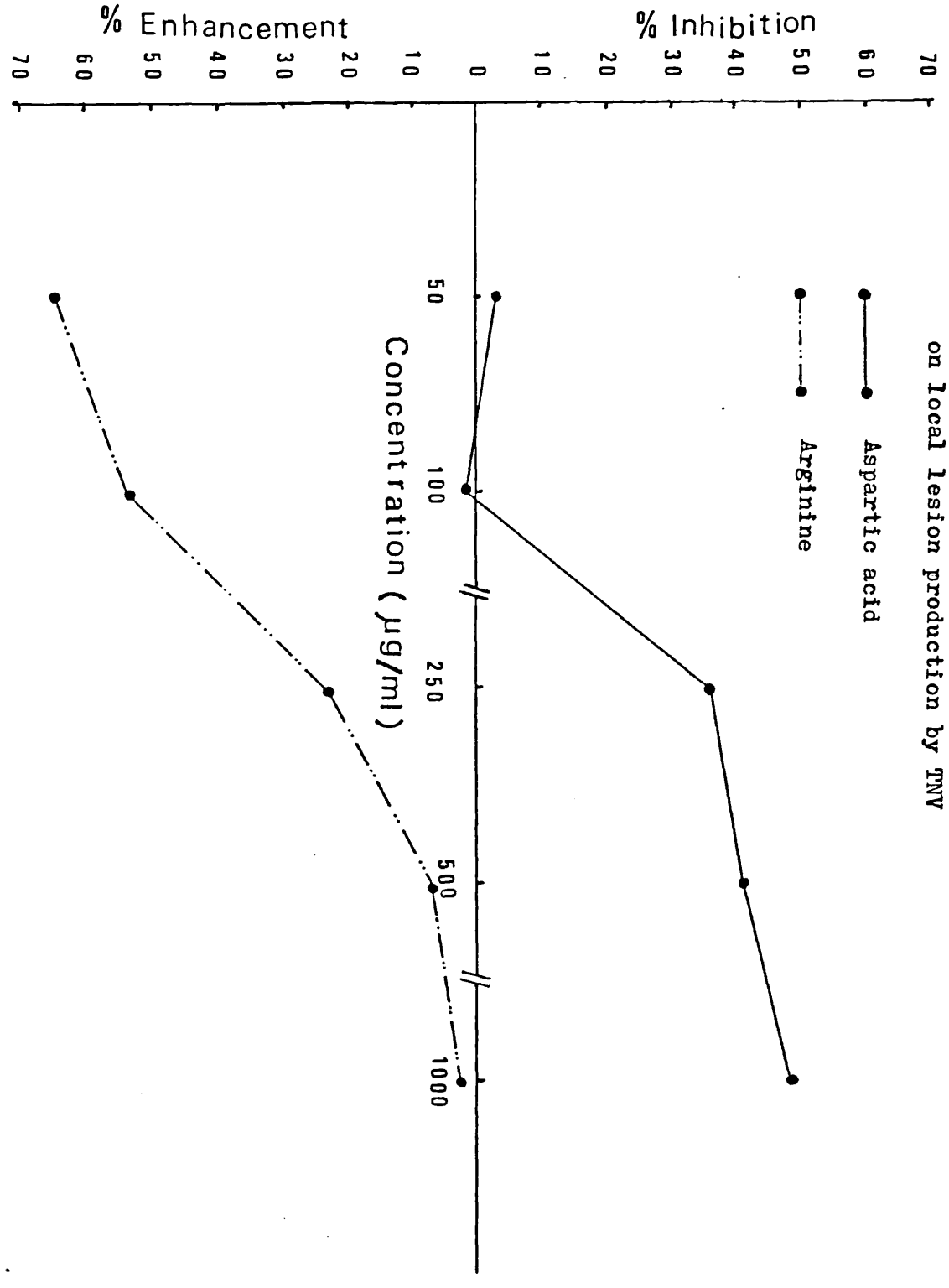


Figure 34 : Effect of various concentrations of aspartic acid and arginine on local lesion production by TNV

Table 30: Effect of aspartic acid and arginine on local lesion production by TNV

Concentration (250 µg/ml)	Mean number of lesions *		% Inhibition
	TNV + Water (control)	TNV + Amino acid	
Aspartic acid (1)	109.8	77.2	30
Arginine (2)	52.5	70.5	-34
Mixture (1) + (2)	94.7	75.1	21

* Each figure represents the mean number of lesions for ten leaves

Negative values of % inhibition = enhancement

acts as inhibitor, is strong enough to overcome the enhancing effect of arginine. An experiment concerning the possible role of aspartic acid in the induction of resistance to virus infection is described in chapter VIII.

The separation and identification of amino acids described here have added more information regarding the properties and chemical nature of the Gp virus inhibitor by giving ideas about: 1- the qualitative and quantitative nature of the free amino acids in the extracts and, 2- the effects of such free amino acids on local lesion production by TNV.

Since sugars are also important components of the Gp extracts, it was decided to determine the quality and quantity of the free sugars in such extracts and to examine their inhibitory activity against TNV.

b) SUGARS

Because the low molecular weight (dialysable fractions) of Gp extract contains carbohydrates, it was thought useful to test simple carbohydrates for their effects on plant viruses. There are only a few reports on the effects of sugars on the susceptibility of plant to virus infection. It has been reported that addition of sugars to virus inocula can inhibit virus infectivity (El-Kandelgy and Wilcoxon , 1966). Other researchers found that sugars act to enhance virus infectivity (Grant and Corbett, 1960; Whitcomb and Sinha, 1964; Davis and Whitcomb, 1967). However, Brants (1964) showed that increases in the carbohydrate content of leaves decreased their susceptibility to virus infection.

Thin layer chromatography (TLC) was used to separate and identify the free sugars from G.paniculata fractions, and to determine the quantity of each free sugar in the natural extracts. These sugars were then tested for their effects on virus infection in plants.

1. Qualitative and quantitative determination of free sugars in G.paniculata extracts

(a) Determination of free sugars using TLC

i. Samples preparation for TLC

50 mg of lyophilized Gp extracts were mixed with 10 ml of ethanol, then centrifuged at 4000 rpm for 10 minutes. The clear supernatant, approximately 9 ml, was reduced to 2 ml by rotary evaporation at 40 °C. The concentrated clear supernatant was stored in 2 ml screw-cap bottles at -25 °C. The non-dialysable and dialysable fractions, prepared as described in chapter V, were treated in the same way. Sugar standards of sucrose, glucose, fructose, maltose and xylose at 4 mg/ml were prepared.

ii. Application of sample on TLC

Plastic backed thin layer plates (20 x 20 cm), coated with a layer of 0.25 mm silica gel G, type Polygram Sil G, were supplied by Macherey-Nagel and Co. Ltd. The sugar samples were applied as bands 0.5-1.0 cm long and 2 mm wide, two cm above the lower edge of each plate. The application of 5-10 µl was found suitable for sugars, and satisfactory separation could be achieved using one dimensional chromatography.

iii. Development and colour reaction of TLC plates

The solvent system of Menzies and Mount (1975) was modified as follows: ethyl acetate (60 ml), pyridine (30 ml), glacial acetic acid (15 ml) and water (5 ml). The plates were developed twice with the same solvent system flowing right to the top of the plates. Between the runs the plates were air dried in the fume cupboard overnight to remove the pyridine completely as it inhibits many colour reactions for sugar (Menzies and Mount, 1975). Sugars were detected using a reagent

prepared by dissolving 7 gm of 4-aminobenzoic acid in 400 ml methanol and 17.5 ml orthophosphoric acid (90 %W/V). This solution was made up to 500 ml with methanol. The plates were dipped in the reagent as briefly as possible to prevent zone trailing. The colour was developed by heating the plates in an oven at 100-110 °C for 5-10 minutes. Sugars from each series of standard and sample chromatographs were cut out and scanned in a chromoscan double-beam recording and integrating densitometer (Joyce-Loebl & Co. Ltd.) with quartz iodine light source. The areas under the peaks on the recorded trace are proportional to the amount of sugar present in the sample (Christie, 1973). Individual spots were estimated using the formula:

$$\frac{\text{Peak area of standard}}{\text{Peak area of sample}} \equiv \frac{\text{Amount of sugar in standard}}{\text{Amount of sugar in sample}}$$

(b) Determination of total carbohydrates using the Dubois test

The quantitative determination of carbohydrates in the samples was carried out using a colorimetric technique (Dubois et al., 1956). In this technique disaccharide and polysaccharide are hydrolysed and the products together with free sugars give a colour reaction with phenol. Two ml of the extract to be tested were mixed with 0.5 ml of 80 % phenol in water, and 5 ml of concentrated sulphuric acid. After standing for 10 minutes, the mixture was shaken and placed for 10-20 minutes in a water bath at 25-30 °C. The absorbance of the characteristic yellow-orange colour was measured at 490 and 480 nm for hexoses and pentoses respectively. The amount of sugar in the samples was determined by reference to a standard curve prepared using xylose, mannose, galactose, glucose, sucrose, maltose and fructose.

2. Results of carbohydrate determination

Using the technique described above the following were investigated:

a- The identity of free sugars in G.paniculata fractions.

b- The quantity of individual free sugar present in the extract.

c- The total carbohydrates present in Gp fractions.

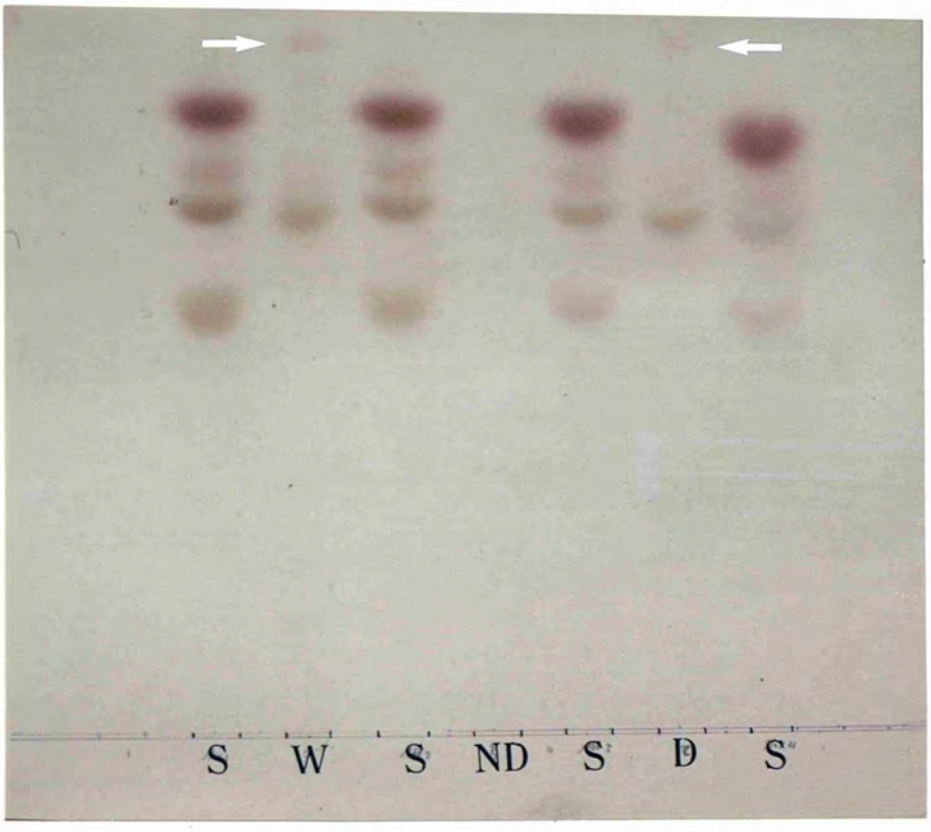
a) Free sugars from G.paniculata extracts

The free sugars of the whole Gp extract were identified as fructose, glucose, sucrose and unknown fraction near the top of the plate showed up as pink spots (Figure 35). The unknown fraction had different chromatographic properties to erythrose and D(-) 3-phosphoglyceric acid. The erythrose spot showing up below and D(-)3-phosphoglyceric acid above the unknown fraction. However, it seems that due to it proximately to these substances it could be a 3-carbon compound. The low molecular weight (dialysable fraction) showed an identical pattern of free sugars to the whole extract, whereas the non-dialysable fraction contained no free sugars (Figure 35).

b) Quantitative estimation of free sugars in Gp extracts

The amount of free sugars in each sample was found by comparing peak areas with those obtained using known amounts of glucose, fructose, sucrose, maltose and xylose. The results in Table 31 show that the total free sugars in the whole extract was 10.89 mg/ml. The dialysable fraction showed a similar amount of free sugars (10.78 mg/ml) as the whole extract, indicating that the bulk of the free sugars had been removed by dialysis. This was confirmed since no sugars could be detected in the non-dialysable fraction. Glucose showed the highest concentration (4.24 mg/ml) followed by fructose, the unknown fraction and sucrose at 3.93, 1.45 & 1.27 mg/ml respectively. The dialysable

Figure 35 : Analysis of the free sugars from the whole, non-dialysable, and dialysable fractions of G.paniculata extracts using Thin Layer Chromatography (TLC)



S = Standard mixture of sugars

W = Whole extract from G.paniculata

ND = Non-dialysable fraction

D = Dialysable fraction

Table 31 : Carbohydrate content of the G.paniculata extracts

Sugar	Carbohydrate content (mg/ml)		
	Whole extract	Dialysable fraction	Non-dialysable fraction
Glucose	4.24	4.65	0
Fructose	3.93	3.12	0
Sucrose	1.27	1.43	0
Unknown sugar	1.45	1.58	0
Total free sugars	10.89	10.78	0
Total carbohy.	13.26	12.75	2.17

fraction showed an almost identical concentrations for these free sugars.

c) Total carbohydrates

Results in Table 31 show that the whole and the dialysable fraction of Gp extracts contained a considerable amount of carbohydrate giving 13.3 and 12.8 mg/ml respectively, whereas the non-dialysable fraction contained only 2.2 mg/ml of carbohydrate. It can be concluded that Gp extracts contain some carbohydrates other than free sugars. Although some may be present in starch-like molecules, it is also possible that some is present in the whole extract as glycoprotein.

3. Effects of free sugars on local lesion production by TNV

Pure samples of each of the free sugars, identified in the Gp extracts, were prepared by dissolving 5 mg analytical grade sugars in 1 ml of water, so giving a final concentration approaching that of the extracts. After mixing with 1 ml of TNV, the samples were inoculated onto french bean leaves. Controls consisted of 1 ml of water and 1 ml of TNV. Results in Table 32 show that each of the 3 sugars tested produced a stimulatory effect on virus infectivity. Sucrose showed the optimum virus enhancement giving 123 % more lesions compared with its control. Glucose and fructose also produced a high levels of enhancement, but were less effective than sucrose, giving 62 and 75 % more lesions respectively. The mixture of these 3 sugars also acted as virus augmenters. The mean number of lesions was 156 per leaf compared with 81 lesions per leaf for control.

Sugars did not, it seems, play any role in the inhibitory activity of the small molecular weight fractions. The possible interpretation of these observations will be given in the discussion of this section.

Table 32 : Effect of sugars, identified from G.paniculata extract, on the infectivity of TNV

Sugar	Mean number of lesions *		% Inhibition
	TNV + Water (control)	TNV + Sugar (treated)	
Fructose (F)	87.8	153.9	-75
Glucose (G)	65.2	105.3	-62
Sucrose (S)	74.5	165.9	-123
Mixture (F+G+S)	81.0	155.7	-92

* Each figure represents the mean number of lesions for ten leaves

Negative values of % inhibition = enhancement

Discussion

In this study 17 free amino acids and 3 free sugars were identified in the dialysable fraction of G.paniculata extracts. Initial tests indicated that 10 out of 17 free amino acids can cause considerable inhibition of the hypersensitive local lesion reaction. The inhibitory action of some pure amino acids against plant viruses has been reported previously. For example, Varma and Verma (1967) found that alanine, serine and glutamic acid at concentration of 100 µg/ml, when sprayed on Nicotiana glutinosa leaves one week before inoculation with TMV, caused about 68, 50 and 37 % inhibition respectively of localized reaction to TMV. Moreover, Verma et al. (1970) reported that glutamic acid and aspartic acid at 1000 µg/ml concentration had profound inhibitory effects when sprayed immediately after virus inoculation, suggesting that these acids affected the virus infection process or virus multiplication in initial stages of virus development. Alanine, they reported, decreased virus multiplication at initial stages of replication.

Seven free amino acids stimulated local lesion production by TNV. Arginine, lysine (both basic in nature) and alanine showed the highest enhancement of virus infectivity (Table 27). The results agree with the observations of Bobyr (1959) who reported the stimulation of necrotic reactions by alanine. Verma et al. (1970) also found that asparagine, glycine, leucine and arginine at 0.5 - 1.0 mg/ml concentration had stimulatory effects on TMV when sprayed on N.glutinosa leaves.

The mixture of 17 pure amino acids produced a considerable reduction in lesion numbers. The mode of action of these amino acids on local lesion production can not be attributed to a pH effect, since amino acid-virus mixtures were buffered (pH 7.0) before inoculation. It has

been suggested that amino acids effects may be due to the accumulation of these compounds inside the host cell, so influencing lesion formation, either by affecting the normal chemical balance of the virus particles, or by imparing the normal cell environment (Varma and Verma, 1967).

Aspartic acid and glutamic acid (acidic) had the strongest inhibitory action, whereas arginine and lysine (basic) showed pronounced virus enhancement. Similar observations were reported by Verma et al. (1970) who found that amino acids having OH or COOH groups in addition to the $CH(NH_2)COOH$ group were generally inhibitory to TMV. On the other hand, those acids where H ions dominate over OH or COOH groups had no inhibitory influence, but could enhance virus infectivity. Richkov (1951) and Bobyr (1959) also reported inhibitory effects of glutamic and aspartic acid against TMV, and Verma et al. (1970) observed the stimulatory effect of arginine on N.glutinosa by TMV. It was noticeable that increased concentrations of aspartic acid produced increased inhibiting activity. Similar results were reported by Chowfla and Nariani (1976), who found that the inhibition of broad bean mosaic virus by alanine, tryptophan, aspartic acid and glutamic acid was directly correlated with amino acid concentration. They also showed that the highest concentration (2000 ppm) of aspartic acid produced the highest percentage inhibition (63 %) followed by alanine (43 %), glutamic acid (39 %), and tryptophan (31 %). Lower concentration (100 ppm) gave 26, 6, 11 and 8 % inhibition respectively.

In the case of sugars, it was shown that all 3 sugars, identified from Gp extracts, were capable of enhancing TNV infectivity. There are some reports that sugars and other carbohydrates can inhibit infection of plant by viruses (Bawden and Freedman, 1952; El-Kandelgy, 1964;

Brants, 1964; Whitcomb and Sinha, 1964). Many workers, however, describe enhancement of viral activity by carbohydrates (Grant and Corbett, 1960; Whitcomb and Sinha, 1964; Davis and Whitcomb, 1967; Kongsvik and Santilli, 1970).

The mechanism of enhancement of virus infectivity by sugars is not known. However, Brants (1964) suggests that enhancement results from the increased carbohydreate content of host leaves, resulting in increased respiration and a subsequent increase in susceptibility. Similar interpretations were made by Yarwood (1952b), Panzer (1957) and Lindner et al. (1959). Schlegel and Rawlins (1954) suggest that sugars can serve as an energy source for host metabolism and in this way bring about an increase in virus production. However, Kongsvik and Santilli (1970) consider that sucrose acts to enhance infectivity of TMV-RNA by affecting an early step in the infection process, probably the penetration of RNA into the cell.

It may be recalled that an amino acid mixtures similar to the plant extracts, reduced the infectivity by 21 %, whereas a sugar mixtures increased the infectivity by 93 %. However, the dialysable part of the plant extracts containing these compounds, produced about 80 % inhibition. This suggests that: 1- there are inhibitors other than amino acids in the dialysable fraction such as organic compounds (Schlegel and Rawlins , 1954), and lipid (El-Kandelgy and Wilcoxon, 1966), or 2- the amount and ratio of amino acids to sugars varied from the amounts present in the natural extracts.

Clearly the overall effect of plant extracts on local lesion production is dependent on the varying effects of the different small molecular weight constituents. Interaction between the effects of

sugars (enhancing compounds or augmenters) and other small molecular weight constituents including amino acids (inhibitors) determines the response as shown by local lesion numbers. Such interaction are further complicated by the effects of large molecular weight fraction such as proteins and glycoproteins.

CHAPTER VII

MODE OF ACTION OF GYPSOPHILA PANICULATA AND MINURATIA CAPILLACEA EXTRACTS

Introduction

Although numerous plants have been shown to contain plant virus inhibitor fractions, relatively few reports have indicated their mechanism of action. This chapter, describes attempts to study the mode of action of two Caryophyllaceae inhibitors. Barakat and Stevens (1980) have shown, as described in chapter III, that members of the family Caryophyllaceae contain powerful inhibitors of plant virus infection. Two species, Gypsophila paniculata (Gp), which showed powerful inhibitory activity and Minuratia capillacea (Mc), which showed weak inhibition, have been studied in some detail to gain information regarding their possible mode of action.

There are conflicting reports about the way in which inhibitors of infection act. Bawden (1954) has classified plant extracts as either inhibitors of infection or inhibitors of virus multiplication. Among the inhibitors of infection, there are two types: 1- those which affect virus particles either by preventing, or "blocking" their activity, or by competing with virus for receptor sites on the plant cell surface. The second type 2- act exclusively on the cytoplasm of host cells by interrupting some processes concerned with replication, so that they can no longer support virus multiplication.

Inhibitors which act by blocking virus particles include extracts prepared from yeast (Takahashi, 1942, 1946), Physarum polycephalum (Mayhew and Ford, 1971), and some leaf extracts which contain tannins

(Thresh, 1956).

Complete inactivation of virus particles by some plant virus inhibitors has been reported by Gupta and Raychaudhuri (1972), who, showed that Acacia arabica extracts inactivate virus particles completely. Partial inactivation of PVX could be brought about by treatment with Callistemon lanceolatus and Syzygium cumini. A similar effect is found when TMV is treated with geranium tannin (Cheo and Lindner,1964).

Ragetli (1957) suggested that the inhibitor extracted from Dianthus caryophyllus acts by competing with virus for receptor sites on the plant cell surface. The results of Van Kammen et al. (1961), and Ragetli and Weintraub (1962b), using similar species support this idea. Alberghina (1976) concluded that the inhibitor isolated from Chenopodium amaranticolor acts by blocking virus receptor sites situated on the surface of the leaf. Extracts strongly reduced virus infectivity when applied before or up to 30 minutes after inoculation with virus. He suggested that the binding of the virus to the susceptible sites remains reversible for a 30 minutes period after inoculation. Nart (1972) also noticed that the action of the inhibitors prepared from carnation and french bean was through the host plant rather than on the virus. He suggested that once the inhibitor was introduced to the leaf surface it blocked the virus entry points or destroyed the infectible sites.

Inhibitors which act by altering the host cells metabolism so that they are no longer susceptible to the virus, have been reported from a variety of plants including Trichothecium roseum (Bawden and Freedman, 1952), Capsicum frutescens (McKeen, 1956), New Zealand spinach juice (Benda, 1956), succulent plants (Simons et al., 1963), red clover (El-Kandelgy and Wilcoxon, 1966), Chenopodium (Yoshii, 1969), and

Datura metel (Lal et al., 1973). More recently, Verma et al. (1979) found that the mechanism by which virus infectivity is inhibited by extracts isolated from Boerhaavia diffusa is due to the interaction of inhibitor with the test plant. Similarly, Vicente et al.(1977) reported that the inhibitor extracted from C.amaranticolor leaves acts on the host plant, and inhibition of infectivity varied with the different host plant species.

Other workers believe that inhibitors do not involve directly the host plant, but they act by forming a non-infective complex with the virus. This suggestion is supported by Grasso (1977), who showed the inhibitor activity from Phytolacca americana comes about as a consequence of ionic bonding between virus and inhibitor. Verma et al. (1975) found that Lawsonia alba extracts did not reduce the multiplication or infectivity of TMV when sprayed after or prior to virus inoculation on tobacco plants. Furthermore, the extracts were not effective against TMV when its came into direct contact with the virus in vitro. They concluded that the inhibitor does not inactivate the virus particles in vitro, but it forms a non-infective reversible complex with the virus in vivo. Similar results were obtained by Fulton (1943) with leaf extracts of Phytolacca decandra, by Paliwal and Nariani (1965), with Carica papaya latex, and by Palm (1967) who described a plant virus inhibitor system from the leaves of Nicotiana glutinosa.

Electron microscopy has been used to investigate the mode of action of some plant virus inhibitors by some workers. Moraes et al. (1974) found two carbohydrate inhibitors from Physarum polycephalum and Abutilon striatum when assayed against TMV. By electron microscopy, they showed the inhibitor coats the virus particles, so inhibiting

normal protein coat stripping, when virus particles enter the host cell. Similarly, Ebrahim-Nesbat (1971) showed that there was an aggregation of virus particles when partially purified inhibitor from spinach was added to TMV. He concluded that inhibition is caused by such aggregation of virus particles.

It seems from the literature that there is no clear answer to problem as whether inhibitors affect the resistance of inoculated host plants, or act directly on virus particles. More information on more examples need to be studied. With this in mind, several experiments have been undertaken to determine whether inhibitor extracts prepared from G.paniculata and M.capillacea reduce infectivity by affecting the TNV particles, or by acting on the host plant.

Experimental procedures and results

Before testing the direct effect of Gp and Mc extracts on TNV, it was necessary to clarify both extracts by ultracentrifugation, particularly as the extracts appeared cloudy. Clarified extracts were then incubated with TNV for 15 minutes, the virus recovered by ultracentrifugation, and its infectivity tested and compared with untreated TNV.

1. The inhibitory activity of clarified Gp and Mc extracts

Lyophilized Gp and Mc extracts,prepared as described previously, were each reconstituted in 2 ml distilled water, then they were partially clarified by centrifugation at 11,000 x *g* for 30 minutes. The clear supernatants were tested against virus. The precipitates produced on centrifugation were each reconstituted in 2 ml distilled water and these also tested against TNV. Controls consisted of TNV and an equal volume of water instead of extract.

The results in Table 33 show that both the supernatant and precipitate of Gp were inhibitory giving 100 % and 84 % inhibition respectively, whereas only the supernatant of Mc reduced lesion numbers giving 80 % inhibition. The Mc precipitate had no inhibitory effect. The levels of inhibition of each clarified supernatant was the same as for the whole extracts as reported earlier (chapter III). The supernatant fractions or clarified extracts were used in the remaining experiments described below. In further experiments, it was thought useful to test these clarified extracts against two other viruses TMV and PVX. Since this permitted the evaluation of the inhibitors against: a) two further host plants, and b) a rigid rod-shaped virus (TMV), and a flexible rod-shaped virus (PVX).

2. Effect of clarified Gp and Mc extracts on local lesion production by TMV and PVX

Two ml of each of these clarified supernatants were mixed with 2 ml of partially clarified TMV (Legume strain), or PVX and inoculated onto ten leaves of their respective test plants (Nicotiana glutinosa var. Xanthi, or Gomphrena globosa). Controls consisted of an equal volume of virus and distilled water.

Results in Table 34 show that Gp and Mc partially clarified supernatants inhibited local lesion production by TMV giving 100 % and 60 % inhibition respectively. Table 34, also shows that Gp extracts gave total inhibition (100 %) with PVX, while Mc extracts showed less inhibition giving 63 % inhibition.

It seems from these results that both extracts were inhibitory against TNV, TMV and PVX, but Gp extracts showed the strongest effect.

Table 33 : Effects of supernatant and 11,000 x g precipitates from G.paniculata and M.capillacea extracts on local lesion production by TNV

Source of extract	Mean number of lesions*		% Inhibition	
	Super-natant	Preci-pitate	Super-natant	Preci-pitate
G.paniculata	0	34.3	100	84
M.capillacea	44.4	217.3	80	2
Control	220.8	0	0	0

* Each figure represents the mean number of lesions for ten leaves

Table 34: Effect of clarified supernatants of G.paniculata and M.capillacea on local lesion production by TMV and PVX

Virus	Gypsophila paniculata			Minuratia capillacea		
	Mean number of lesions		% Inhibition	Mean number of lesions		% Inhibition
	Water	Extract		Water	Extract	
TMV *	136.2	0	100	136.2	54.4	60
PVX **	74.3	0	100	36.6	13.5	63

* TMV tested on ten whole leaves of Nicotiana tabacum var. Xanthi

** PVX tested on ten whole leaves of Gomphrena globosa

3. Effect of clarified extracts on TNV

Having established that the inhibitors from each extract were still present in the clarified supernatants, the direct effects of inhibitors on virus was studied. Five ml of each clarified extracts were incubated for 15 minutes with an equal volume of TNV purified from frozen leaves of P.vulgaris by the method of Kassanis (1970). The virus was then recovered by centrifugation at 100,000 x g for 2 hours. The pelleted virus was washed by resuspending in distilled water to remove any residual inhibitor, and then repelleted by centrifugation to obtain TNV. Control samples consisted of TNV in water which were centrifuged in the same way. To be certain that ultracentrifugation at 100,000 x g for 2 hours had no effect on inhibitor extracts; extracts alone were also subjected to ultracentrifugation. Finally, the virus pellets in both the treated and control tubes were resuspended in distilled water. All samples of virus were inoculated onto P.vulgaris test plants.

Results in Table 35 show that ultracentrifuged inhibitors from both Gp and Mc were as effective as crude extracts in reducing lesion numbers induced by TNV when mixed with virus and immediately inoculated onto leaves. However, virus treated with Mc extracts for 15 minutes and precipitated by ultracentrifugation was not inhibited, suggesting that Mc extracts had no effect on the virus particles. In contrast, Gp extracts appear to have some direct effect on virus since lesions were reduced by 79 %. It may be argued however that: 1- some inhibitory materials are bound to the virus particles, or 2- some inhibitory materials may be carried free in the solution with the virus particles, so that this "residual" inhibitor accoumts for inhibition rather than virus inactivation.

Table 35: Effects of purified extracts of G.paniculata and M.capillacea on TNV virus

Extract tested	Mean number of lesions*					
	(A) TNV + Water (control)	(B) TNV + Purified extract	% Inhibition (B) compared to (A)	(C) TNV repre-cipitated from water	(D) TNV repre-cipitated from extract	% Inhibition (D) compared to (C)
G.paniculata	62.4	0	100	107.9	22.4	79
M.capillacea	62.4	13.5	76	150.0	243.0	3

* Each figure represents the mean number of lesions for ten leaves

Further experiments to study the possible effect of these extracts on host plant were performed, in order to establish whether the extracts operate either by interfering with the initial uptake of virus, or by affecting cell metabolism so interrupting some processes concerned with virus multiplication. Application of virus inhibitor by the leaf dip method was used in this investigation, since Van Kammen et al. (1961) suggested that this technique avoided the danger of the second rubbing where new wounds would be created or existing susceptible sites destroyed.

4. Effect of dipping french bean leaves in either Gp or Mc extracts one hour before TNV inoculation

a) Dipping the whole leaf

One primary leaf of each of the ten french bean plants was total immersed for 2 minutes into a petri dish containing either Gp or Mc extracts. Opposite leaves were dipped into distilled water and served as controls. All the leaves were dried in air at glass house temperature for one hour, then inoculated with TNV. Control leaves were inoculated firstly to prevent any inhibitor extracts being transferred to the inoculum. The results in Table 36 show that both Gp and Mc extracts were inhibitory giving 100 % and 83 % inhibition respectively.

b) Dipping the upper surface of the leaf

In further experiments, only the upper leaf surfaces were dipped in either Gp or Mc extracts using the same procedures as described above. Control leaves were dipped in water. Results in Table 36 show that the inhibition was the same for the dipping of whole leaves.

It is possible that inhibitor on upper leaf surfaces may be introduced into leaves together with virus in the inoculation process.

Table 36: Effects on local lesion production by TNV following dipping of P.vulgaris into inhibitor extracts from G.paniculata and M.capillacea

Surface dipped	Gypsophila paniculata			Minuratia capillacea		
	Mean number of lesions*		% Inhibition	Mean number of lesions *		% Inhibition
	Water	Extract		Water	Extract	
Upper	42.5	0	100	72.9	15.9	78
Lower	29.5	0.7	98	49.5	11.9	76
Whole leaf	35.8	0	100	23.5	4.1	83
Whole leaf washing **	79.1	2.1	97	85.3	43.5	49

* Each figure represents the mean number of lesions for ten leaves

** Leaves dipped into extracts one hour before inoculation with TNV then washed in water for 2 minutes to remove any residual inhibitor

To avoid this possibility an experiment was designed where the lower surface was treated with inhibitor and the upper surface with virus.

c) Dipping the lower surface of the leaf

On dipping the lower surfaces of P.vulgaris leaves in either Gp or Mc extracts, the percentage inhibition was found to be almost the same as for dipping the whole or upper surfaces (Table 36).

It seems from these results that there is considerable inhibition of local lesion production whether leaves are immersed in solutions, or solutions applied to a single surface. The fact that inhibition occurrs when the lower surface is treated with extracts and virus applied to the upper surface, indicates that extracts in vivo influence leaf susceptibility rather than having direct effects on virus. This conclusion was further tested in the following experiments.

5. Effect of dipping french bean leaves in either Gp or Mc extracts followed by washing with water before TNV inoculation

Ten primary french bean leaves were treated with each extract using the same procedures as for the whole leaves. Opposite control leaves were dipped in water. After one hour, the leaves were rinsed with tap water and left to dry for 15 minutes before inoculation with TNV.

Results in Table 36 show that rinsing the treated leaves with water had no effect on the inhibitory activity of Gp extracts giving 97 % inhibition. On the other hand, rinsing the leaf surface to which Mc extracts had been applied produced some reduction in the inhibitory activity giving 49 % inhibition as compared with unrinsed leaves (78 % inhibition) as shown in Table 36.

These results suggest that there is fairly strong attachment of the inhibitors to the leaf surfaces particularly with Gp extracts. Alter-

natively, it can be concluded that the Gp inhibitor has some effect on cell metabolism in some way, so influencing leaf susceptibility to virus. The results of the previous experiment (4-c) supports this idea. Another possibility is that antiviral factors are induced in the leaves which prevent local lesion production after virus application (see chapter VIII). Mc inhibitor appears to act by the same mechanism as the Gp inhibitor, but with less effect.

To examine more closely the possibility that inhibitors influence host susceptibility either by preventing early events such as virus attachment to the leaf cells, or by stopping later events, french bean leaves were dipped into inhibitors prior to and after virus inoculation.

6. Effect of dipping french bean leaves in either Gp or Mc extracts at different time intervals PRIOR to TNV application

It was shown previously that the immersing of leaves in Gp or Mc extracts one hour before inoculation with TNV produced inhibition. In further experiments, leaves were dipped into either Gp or Mc extracts for 2 minutes and thenleft for 1, 4, 6, 12, or 24 hours before inoculation with virus in order to test the effect of longer exposure of leaves to the inhibitors. Control leaves were dipped into water instead of extract.

Results as shown in Table 37 indicate that the extracts from Gypsophila are very effective as inhibitors, there being no lesions developed even when virus inoculation was made 24 hours after inhibitor application. On the other hand, Minuratia extracts were inhibitory but less effective, it showed the highest percentage inhibition (89 %) when applied 1 hour before inoculation with TNV, and the lowest inhibition (45 %) after 12 hours.

Table 37 : Effects of pretreatment of leaves of P.vulgaris with inhibitor extracts on subsequent local lesion production by TNV

Hours before inoculation	Gypsophila paniculata			Minuratia capillacea		
	Mean number of lesions *		% Inhibition	Mean number of lesions *		% Inhibition
	Control	Treated		Control	Treated	
1	17.0	0	100	109.5	11.5	89
4	53.1	0	100	60.0	23.6	61
6	32.3	0	100	89.4	40.0	55
12	27.0	0	100	44.0	24.0	45
24	77.2	0	100	171.6	49.4	71

* Each figure represents the mean number of lesions for ten leaves

These results suggest that either the inhibitors: 1-remain active on leaf surfaces, enter the leaf together with the virus and cause their inhibitory activity inside the leaf, or 2- they may alter cell metabolism in some way, so affecting virus replication.

An interesting feature of this experiment was the observation that the virus concentration required to give adequate lesion numbers in opposite control leaves treated with Gp extracts was double that normally giving 100 - 150 lesions. This observation suggests that Gp inhibitor may induce antiviral compounds which prevent local lesion production in the opposite leaves. Further experiments substantiating this claim will be described in the following chapter.

7. Effect of dipping french bean leaves in either Gp or Mc extracts at different time intervals AFTER TNV application

The above experiment was repeated with the exception that inhibitors were applied 1, 4, 6, 12 and 24 hours after inoculation with TNV.

The results in Table 38 show that no significant inhibition occurred with Gp extracts if applied one hour or more after inoculation with virus. However, there is a slight effect with Mc extracts when applied 1 or 6 hours after inoculation with TNV giving 25 % and 23 % inhibition respectively.

These results may indicate that early events in virus replication have occured during the first hour after virus application giving rise to lesion production. Inhibitor extracts to be effective must be present during these early events, since application one hour after treatment with virus is too late to bring about reduction in local lesion numbers.

Table 38 : Effects of post-treatment of leaves of P.vulgaris with inhibitor extracts on subsequent local lesion production by TNV

Hours after inoculation	Gypsophila paniculata			Minuratia capillacea		
	Mean number of lesions *		% Inhibition	Mean number of lesions *		% Inhibition
	Control	Treated		Control	Treated	
1	128.0	122.1	5	130.7	98.3	25 **
4	137.0	138.4	-1	103.4	98.4	5
6	100.9	79.9	20	94.1	72.1	23 **
12	127.3	119.8	6	119.0	113.6	4
24	110.1	111.4	-1	143.2	157.1	-10

* Each figure represents the mean number of lesions for ten leaves

Negative values of % inhibition = enhancement

** Significant inhibition (P = 0.1)

Discussion

The results of these experiments indicate that the inhibitory fractions from Gypsophila paniculata differ from those of Minuratia capillacea. On centrifugation Mc inhibitor was confined to the supernatant fraction, similar results were obtained by Thomson and Peddie (1965) with an extract from Chenopodium amaranticolor. Gp extracts, on the other hand, contain large molecular weight inhibitors which precipitate on centrifugation as well as small molecular weight inhibitors, suggesting that there is more than one inhibitor in the extracts of Gp as described earlier (chapter V). However, the inhibitor may be adsorbed to large particles in the extracts and sedimented with them. The precipitated inhibitors from Gp may well be proteinaceous, since inhibitors from related plants (Dianthus caryophyllus) have been characterized as protein (Van Kammen et al., 1961; Ragetli and Weintraub, 1962b; Stirpe et al., 1981).

Although Gp extracts seem to have some direct effect on TNV, since lesion numbers were reduced by 79 % on separation of the virus from the Gp extracts, it is possible that the inhibitor is adsorbed onto virus and influences local lesion production on entry into plant cells along with virus during the inoculation process. On the other hand, Mc extracts appear to have their effect on the host susceptibility rather than on the virus, because the virus recovered after treatment with inhibitor appeared unchanged. Similar results were reported by Crowley (1955) who found that the inhibitor extracted from species of Nicotiana and Cucumis sativa seeds had little effect on TMV and cucumber mosaic virus respectively. Vicente et al. (1977) and Thomson and Peddie

(1965) also found that the inhibitor from C.amaranticolor leaves acted on the host plant and not on the virus.

In this investigation the leaf dip method was used (Van Kammen et al., 1961) to study the mode of action of both Gp and Mc extracts. When the lower surfaces of french bean leaves were dipped in either Gp or Mc inhibitors and upper surfaces inoculated with virus, no lesions were formed with Gp inhibitor, and slight reduction in lesion numbers was produced with Mc inhibitor. Similar results were obtained by Crowley (1955) using cucumber embryo extracts, where local lesion production on upper cowpea leaf surfaces was inhibited by application of extracts to the under-surfaces. Crowley suggested that the effect of this inhibitor was to reduce the susceptibility of cowpea leaves to infection. Kahn et al. (1960) also reported that the inhibitor from Oryza sativa extracts showed virus inhibition when it was applied to the lower surface of inoculated leaves. Apablaza and Bernier (1972); Simons et al. (1963) found that the inhibitors extracted from geranium and pepper inhibited the development of local lesions when sprayed onto the underside of leaves before inoculation of the upper surface. Blaszczak et al. (1959), however, reported some inhibition of PVX when similar extracts were applied to lower surfaces of Gomphrena globosa. Different results were reported by Nart (1972), who found that the inhibitor isolated from french bean leaves showed no significant virus inhibition when it was applied to the lower surface of N.glutinosa and TMV was applied to the upper leaf surface.

The possible mechanism by which Gp extracts inhibit virus infection when applied to lower surfaces may be due to: 1- Gp extracts contain a mixture of complex compounds which can alter the metabolism of the leaf

cells, so that introduced virus particles can not multiply, 2- the extracts are translocated across the leaf to the opposite side of the leaf and directly inhibit virus replication, or 3- the extracts may induce the production by the leaf of antiviral compounds. On the other hand, Mc extracts produced little inhibition when applied to the lower surface of leaves, indicating that the inhibitor is not translocated or has a slower or weaker effects on cell metabolism, or induction of antiviral compounds.

Washing french bean leaves with tap water, one hour after treatment with inhibitor, had no effect on the biological activity of the Gp extracts. These results confirm the idea that inhibitors from Gp act by altering the susceptibility of the cells to virus attachment, or early events in virus replication rather than having direct effects on the virus. Inhibition induced by Mc extracts was partially reduced by washing, suggesting that such extracts act either by altering the susceptibility of host plant to virus attachment, or by altering the cells and interrupting some processes concerned with the replication of virus.

Further evidence about the mode of action of both extracts were obtained when each was applied at various time intervals either before or after virus inoculation. Both extracts were inhibitory when applied up to 24 hours before virus inoculation, Gp inhibitors showed the strongest effect. These results agree, in part, with those obtained by Fischer and Nienhaus (1973) who found that the inhibitor isolated from Capsicum annuum leaves was inhibitory at least 24 hours before inoculation and up to 1 hour after application of virus. Nart (1972) reported that the inhibitor from french bean was still effective in reducing the infection of TMV in N.glutinosa when it was applied to leaves 48 hours before virus inoculation, whereas like Gp extract, there was no signi-

ficant effect when applied after virus inoculation. On the other hand, Lal et al.,(1973) reported that, although french bean leaves extract was inhibitory when it was mixed and inoculated at the same time as TMV, the extract was not inhibitory when applied up to 24 hours before or after TMV inoculation.

No significant inhibition was obtained when either Gp or Mc extracts were applied after virus application. Similar results were reported by Verma and Verma (1965) who found that wheat seed extracts were inhibitory when applied before the inoculation by TMV, but not when applied after inoculation. They suggested that this effect might be due to blockage of virus receptor sites as reported by Gupta and Price (1950, 1952). However, Palm (1967) showed no significant inhibition of infection when the inhibitory fractions from N.glutinosa were applied to N.tabacum leaves prior to, or after TMV inoculation. He concluded that the inhibitor acts its effect on the virus rather than on the host.

Both Gp and Mc extracts inhibited local lesion production by two rod-shaped viruses (TMV and PVX) as well as, the spherical TNV virus. However, Gp extracts were more effective inhibitors than Mc extracts. These results indicate that both Gp and Mc inhibitors work in host plants other than Phaseolus vulgaris.

The fact that inhibitor activity is translocatable between lower and upper leaf surfaces and from one leaf to another (see chapter VIII), indicates that either inhibitors are mobile, or that they induce the production of compounds with antiviral activity. Antiviral factors induced by Gypsophila paniculata extracts will be examined in the next chapter.

CHAPTER VIII

ANTIVIRAL SUBSTANCE INDUCTION IN PLANTS BY GYPSOPHILA PANICULATA EXTRACTS

Introduction

Plants infected by virus may produce compounds which can be shown to prevent virus activity, such compounds are known as antiviral factors (AVF). Antiviral factors have been found in virus infected leaves of a number of plants and are not detected in non-infected plants. Such antiviral factors were first reported by Sela and Applebaum (1962). Since then, the presence of antiviral substances induced by virus infection in plants has been confirmed by additional investigations (Yoshizaki, 1966; Chadha and MacNeill, 1969; Kimmins, 1969; Nagaich and Singh, 1970; Antignus et al., 1971; Sakai and Takebe, 1974; Paterson and Knight, 1975; Antignus et al., 1977; Mozes et al., 1978; Devash et al., 1981). Sela et al. (1964) found that the juice from uninfected Nicotiana glutinosa leaves showed antiviral activity, but an additional new antiviral substances were produced when the plants became infected with TMV.

The production of AVF has been correlated, by a number of workers, with the appearance of new proteins not present in healthy plants. Gianinazzi et al. (1970) reported that tobacco shows varying degrees of resistance to infection by different viruses. Such resistance is associated with the appearance of 4 new bands of protein on disc electrophoresis of leaf extracts. Similar findings were reported by Van Loon and Van Kammen (1970), and Kassanis et al. (1974). Barker (1975), on the other hand, found that disc electrophoresis of soluble protein in

Nicotiana benthamiana leaf extracts failed to reveal any change in protein pattern induced by infection with raspberry ring spot virus either in symptomless leaves, or in systemically infected symptom- bearing leaves or leaves recovered from infection. He suggested that 3 of the 4 proteins previously thought to occur only in virus infected Nicotiana tabacum leaves are probably normal leaf costituents.

When a leaf is inoculated with virus it becomes resistance to attack by virus applied in subsequent inoculations. Similarly, leaves opposite to and distant from the inoculated leaf often become resistant, this is called "acquired resistance". Acquired resistance in plant infected with viruses that cause only necrotic local lesions has been reported by Ross (1961a) who observed no acquired resistance with non-necrosis (systemic) causing viruses. He suggested, therefore, that this resistance develops only if some part of the plant is infected by necrosis inducing viruses. Van Loon and Van Kammen (1970) found that resistance induced by virus infection in hypersensitive (local lesion) species acts at some distance from the sites of infection.

Other studies showed that induced resistance develops in both local and systemically infected plants. Loebenstein (1963) found systemic resistance in Datura stramonium and Gomphrena globosa after inoculation with TMV and PVX respectively. Ross (1961 b); and Bozarth and Ross (1964) reported that when lower leaves of Samsun NN tobacco were inoculated with TMV, systemic resistance was induced in the upper leaves. They suggested that development of systemic resistance was dependent upon the movement of some substance (s) from infected to uninfected regions of the plant. The chemical nature of such translocatable

substance (s) is not known.

The protection developed by plants treated with inhibitor extracts has been studied by a number of workers. McKeen (1956) found that when pepper extract was applied to one half of the lower leaf surface of cowpea, then inoculated with cucumber mosaic virus, lesion production was reduced to the same extent on both halves of the upper inoculated surfaces. He also demonstrated that application of extracts to the surface of one primary leaf reduced by half the number of lesions produced on the opposite primary leaf. Similar results were obtained by Simons et al. (1963) with geranium and pepper extracts sprayed onto the underside of N.glutinosa leaves one day before inoculation with TMV. In contrast, Blaszczak et al. (1959) observed little or no inhibition in untreated leaves, opposite to those that had recieved extracts from pepper and Chenopodium album, possibly because the extracts had been applied 5-7 days before inoculation.

Apablaza and Bernier (1972) applied extracts from geranium, pepper and jimsonweed to the lower surface of one of a pair of primary leaves of pinto bean plants, 4 and 2 hours before both primary leaf upper surfaces were inoculated with TMV. They found all the plant extracts were effective in inhibiting local lesion development on the opposite leaf, as well as, on the leaf that recieved the inhibitors. The degree of inhibition on leaves that recieved the inhibitor (65 - 85 %) was greater than that on the opposite leaves (40 - 75 %), but less than the 99-100 % inhibition obtained when each extract was mixed with the virus only. More recent examples of induced resistance by plant extracts are given by Verma and Awasthi (1979) and Noronha et al. (1980).

Induction of resistance can also be brought about by chemicals, thus Gianinazzi and Kassanis (1974) found that injection of healthy leaves of N.tabacum var. Xanthi with polyacrylic acid (PA) induced resistance to infection by TMV and TNV. Previously, Gicherman and Loebenstein (1968) found that the injection of yeast RNA into leaves of a hypersensitive tobacco caused a resistance to TMV. White (1978) showed that acetyl salicylic acid, salicylic acid and benzoic acid each induced virus resistance in leaves. Resistance induced by PA is accompanied by the production of 3 new proteins reminiscent of the production of AVF as described earlier. However, resistance induced by PA may be different to that induced by virus, since PA induced resistance in local and does not extend to distant leaves.

The occurrence of antiviral factors in some plants treated with inhibitors raises the very interesting possibility that AVF plays some part in the mechanism of production of resistance of plant tissues to viral infection. In this chapter, experiments are described that attempt to answer the following questions:-

1- Can Gp extract induce resistance in parts of plant distant from its point of application ?

2- Can Gp extracts induce AVF or AVF-like activity ?

3- Is induction of resistance associated with detectable chemical changes in the leaves ?

Experimental procedures and results

The experiments in this chapter are described in seven sections. The experimental details and results are included in each section. A discussion of the results is given at the end of the chapter.

1. Local induction of resistance in french bean leaves by Gp extracts

To test whether Gp extracts produce resistance in tissue around the point of its application, one drop (50 µl) of Gp extracts was placed on the upper surface of one primary leaf of each of sixty french bean plants. The opposite control leaves recieved 50 µl of water. Both treated and control leaves were left for 15 minutes at the glass house temperature when the droplets had completely dried. At 0.5, 1, 3, 6, 24 and 48 hours after inhibitor application, ten leaves from both treated and control plants were inoculated with TNV.

Results in Table 39 show that the inhibitory effect of Gp extracts appears to be related to the time intervals between inhibitor application and virus inoculation. The lowest level of inhibition (37 %) showed after 0.5 hour from inhibitor application, and the highest of 76 % after 24 hours, dropping slightly to 71 % after 48 hours. Interestingly the area of local lesion inhibition was spread over a much larger area than was previously occupied by the drop of inhibitor. Areas farthest away from the drop showed the least inhibition.

The results suggest that: 1- the inhibitor may be translocated at least locally through the leaf and induces localized acquired resistance in tissue near the point of inhibitor application. Or, 2- the inhibitor may induce the formation of protective or antiviral substance (s).

To examine this hypothesis further, leaves distant from those treated with the inhibitor were studied to see whether they were resistant to virus attack, such resistance would suggest that systemic acquired resistance had been induced.

Table 39 : Effect of one drop (50 µl) of Gp inhibitor on local lesion production by TNV, applied at different time intervals after inhibitor application

Time in hours after Gp application	Mean number of lesions *		% Inhibition
	Control (water)	Treated (Gp extract)	
0.5	122.0	76.8	37
1	127.0	60.0	53
3	97.6	32.3	67
6	75.9	30.8	60
24	89.5	21.5	76
48	105.0	30.0	71

* Each figure represents the mean number of lesions for ten leaves

2. Distant induction of resistance

a) Induction of resistance by Gp extracts in opposite leaves of french bean

One primary leaf of each of sixty french bean plants, previously dusted with carborundum powder, was rubbed with Gp crude extracts on its upper surface. The leaves were not washed after inoculation. Control plants were similarly rubbed with water. After different time intervals (0, 1, 3, 6, 24 & 48 hours), ten treated and ten control plants were inoculated with TNV on the leaf opposite to the Gp or water treated leaf.

Results in Table 40 show that the percentage decrease in lesion numbers increased with increasing time between application of extracts and virus inoculation. No significant reduction in the number of lesions was shown when the leaves opposite to those recieving the inhibitor were immediatly inoculated with TNV. The lesion numbers decreased by 33 % when the intervals between Gp application and virus inoculation was one hour, and by 62 % when the intervals was one day.

It seems from these results that: 1- Gp inhibitor induced resistance in the opposite leaves even one hour after application. 2- The degree of resistance depends on the time intervals between inhibitor application and virus inoculation. 3- Gp inhibitor induces the maximum inhibition to infection after 24 hours. 4- The ability of Gp extracts to inhibit when applied to plants some distance from the point of virus application suggests the involvement of mobile inhibitor(s), likely to be small molecular weight compounds. This support the idea described earlier regarding the nature of, at least part of the Gp inhibitor

Table 40 : Effect of Gp extracts on the induction of resistance in opposite untreated primary leaves of french bean

Time in hours after Gp application	Mean number of lesions		% Inhibition
	Control'	Opposite"	
0	38.5	36.2	6
1	54.3	36.3	33
3	129.0	67.0	48
6	115.4	63.4	45
24	51.4	19.7	62
48	91.7	40.5	56

* Each figure represents the mean number of lesions for ten leaves

' Leaves opposite to water treated leaves

" Leaves opposite to Gp treated leaves

complex. 5- The inhibitor may act by stimulating the production in distant tissues of antiviral substance(s) not found in healthy leaves. Further evidence for induced resistance was obtained in tobacco plants as described below.

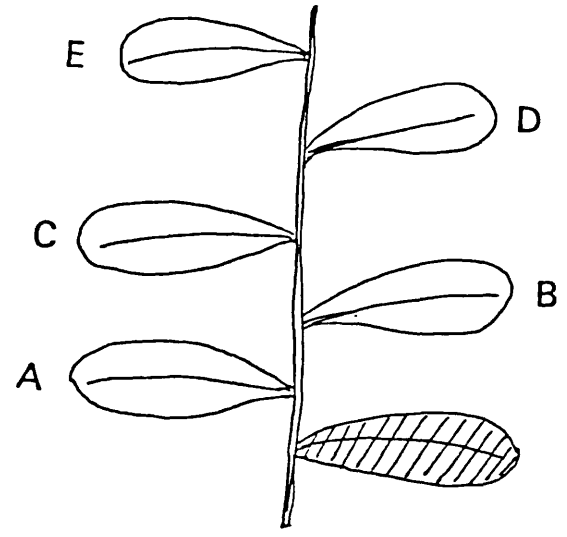
b) Induction of resistance by Gp extracts in distant leaves of N.tabacum

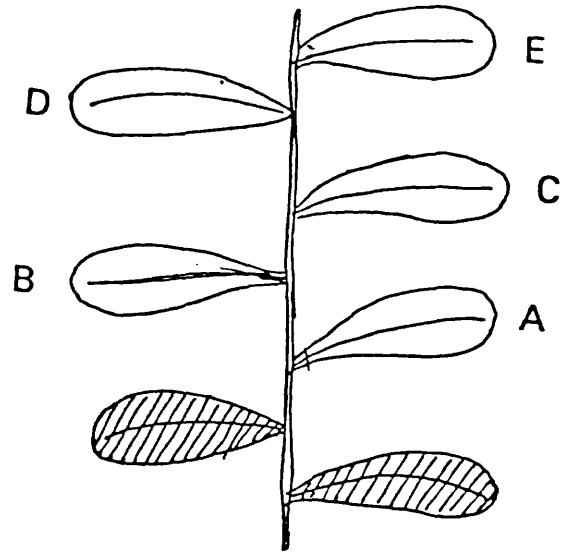
The basal leaf of each of five N.tabacum plants (6-8 weeks old) was rubbed with Gp extract mixed with carborundum. Leaves of control plants were similarly rubbed with distilled water. Twenty-four hours later, all the other fully expanded leaves A, B, C, D &.E as arranged from base to top (Figure 36) were inoculated with TMV, and local lesions counted 5 days later. Similarly, in another experiment, extracts were applied to the two lowest leaves of N.tabacum. Basal leaves of control plants were rubbed with water (Figure 36). The leaves were inoculated and sampled as before.

Results in Table 41 show that the number of lesions produced by TMV, 24 hours after inhibitor application to a single basal leaf, was reduced in all the leaves above those which recieved the inhibitor. The degree of inhibition increased from the lowest to the upper most leaves. On the other hand, the leaves (A, C & E) which were on the opposite side to the inoculated leaf showed less reaction giving 14, 31 and 34 % inhibition respectively as compared with their controls. Leaves (B & D) which were arranged immediatly above the Gp treated ones showed higher inhibition giving 47 and 56 % inhibition respectively.

When the two lowest leaves were treated with the inhibitor, inhibition increased from the lowest to upper most leaves but there was little lateral variation as shown in Table 42.

Figure 36 : Diagrams to show the position of leaves of Nicotiana tabacum var. Xanthi as used in experiments concerning induction of resistance to virus





Gypsophila extracts applied to one or two shaded leaves

TMV applied to leaves A to E (see text)

Table 41 : The induction of viral resistance in N.tabacum leaves following treatment of a single basal leaf with Gp extracts

Leaf position (see Fig. 36)	Mean number of lesions *		% Inhibition
	Control'	Treated"	
A(basal)	39.2	33.6	14
B	47.7	25.5	47
C	51.9	35.7	31
D	61.8	27.3	56
E(top)	60.4	39.6	34

* Each figure represents the mean number of lesions for ten leaves

' Basal leaf treated with water

" Basal leaf treated with Gp extracts

Full statistical analysis for this Table is given in the appendix 2

Table 42 : The induction of viral resistance in N.tabacum leaves following treatment of a pair of basal leaves with Gp extracts

Leaf position (see Fig. 36)	Mean number of lesions *		% Inhibition
	Control'	Treated"	
A(basal)	28.6	18.9	34
B	56.6	39.0	31
C	62.3	32.4	48
D	68.3	28.6	58
E(top)	41.4	22.9	45

* Each figure represents the mean number of lesions for ten leaves

' Two basal leaves treated with water

" Two basal leaves treated with Gp extracts

These results suggest that: 1- The degree of resistance depends on the age of the leaf i.e the youngest leaves develop resistance quicker than the oldest. 2- Application of the extract induces resistance in untreated regions, showing that the protection was systemic. It was also noticed that Gp extract induces resistance not only in french bean, but also in N.tabacum plants, and furthermore inhibition is induced against TMV as well as TNV.

As previously suggested such resistance may be due to the mobility of the inhibitor, or alternatively, the inhibitor induced substance(s) with antiviral activity in untreated regions. In order to gain more information regarding the induction of systemic acquired resistance by Gp extracts, the roots of french bean plants were treated with inhibitor extracts prior to and after inoculation with TNV.

3. Effects of immersing french bean roots in Gp extracts on the induction of virus resistance

a) Immersing for 0.5 hour PRIOR to virus inoculation

To facilitate application of the Gp extracts, seeds of french bean were grown initially in plastic seed trays containing damp vermiculite. After 9-10 days, healthy seedling of uniform size were selected and their roots were carfully washed with tap water to remove any attached vermiculite particles. Roots of thirty plants (60 primary leaves) were immersed in 300 ml of dilute (1:10) Gp extracts for 0.5 hour. Similar numbers of control plants were immersed in water for the same length of time. The roots of treated plants were washed with water to remove any excess extract. The treated and control plants were then divided to batches of five plants, each batch was transferred to 12 cm plastic

pots (5 plants per pot) containing standard compost. After 0, 0.5, 1, 6, 24 & 48 hours, ten leaves from treated and from control plants were inoculated with TNV. The resistance induced by the extract on the leaves calculated as the percentage reduction in the number of lesions produced as compared with their controls. The results are average numbers of lesions for ten leaves.

Results in Table 43 and Figure 37 show that there was only a slight variation in the percentage reduction in the number of lesions when the virus was applied one hour (48 %) or 48 hours (57 %) following inhibitor application. The leaves which were immediately inoculated with TNV after inhibitor application to their roots (zero time) showed no reduction in lesion numbers compared with control leaves. The first sign of lesion number reduction (29 %) appeared 0.5 hour after inhibitor application. The greatest level of inhibition (66 %) was produced after 24 hours. It seems from these results that:

1- Half an hour from the time of initial contact with the inhibitor is not sufficient for the inhibitor to move from the roots to the leaves, or to induce any protective substance(s) with antiviral activity. Contact for a total of one hour, however, produces significant inhibition.

2- These results support the idea suggested earlier (chapter VII) that Gp inhibitor acts on the early events of virus infection, since the number of lesions was not reduced compared with control, when the virus was applied at zero time after inhibitor application.

3- Since 29 % inhibition was produced when the virus was applied 0.5 hour following inhibitor treatment, it can be assumed that at least

Table 43 : The induction of viral resistance in french bean leaves following immersion of roots in dilute (1:10) Gp extracts for 0.5 hour

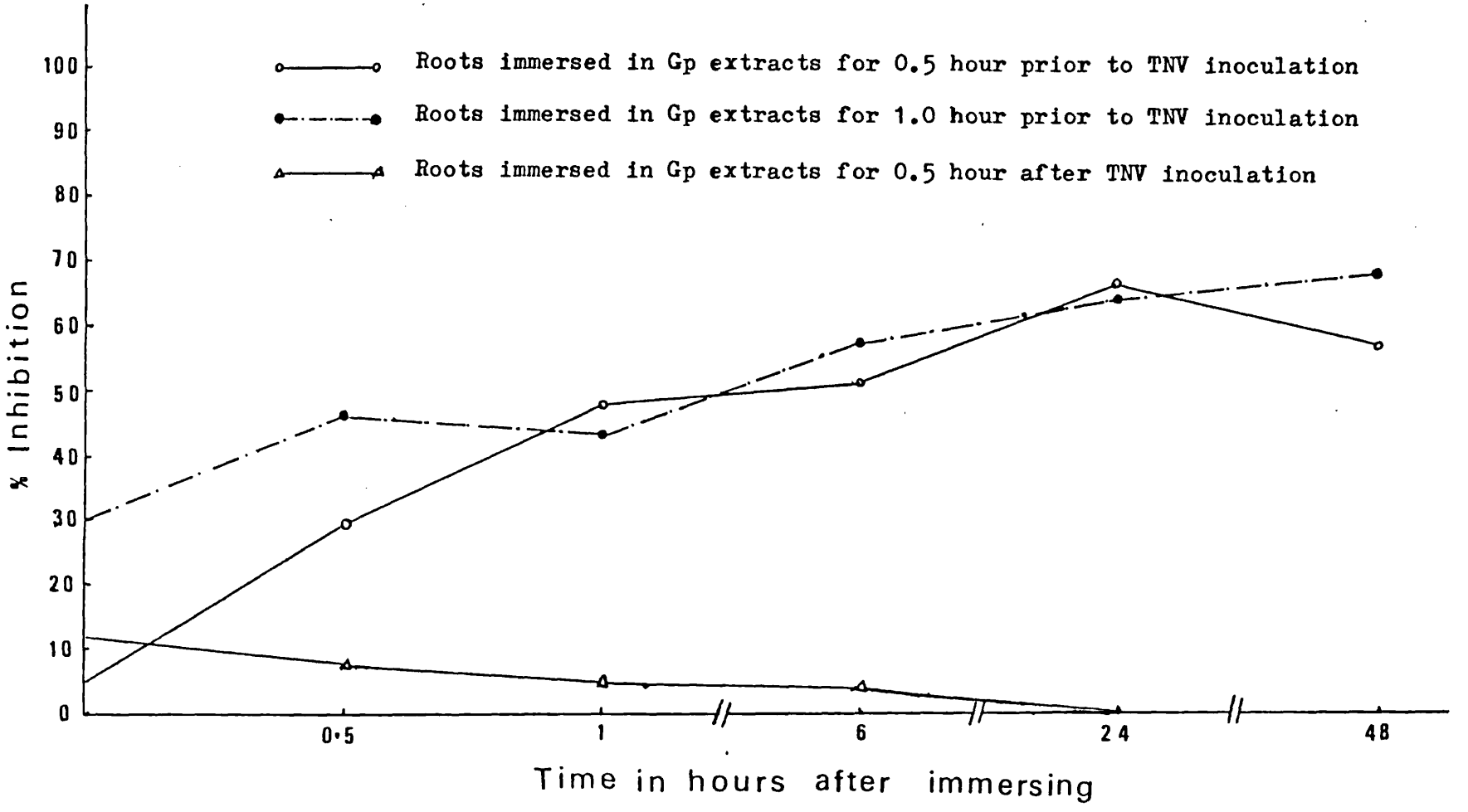
Time in hours after immersion of roots	Mean number of lesions *		% Inhibition
	Control'	Treated"	
0	70.3	66.9	5
0.5	53.8	38.2	29
1	82.8	42.9	48
6	88.3	43.0	51
24	57.6	19.3	66
48	31.5	13.4	57

* Each figure represents the mean number of lesions for ten leaves

' Roots immersed in water for 0.5 hour before virus inoculation

" Roots immersed in Gp extracts for 0.5 h. before virus inoculation

Figure 37 : Effects of immersing french bean roots in Gp extracts prior to and after inoculation with TNV on local lesion production



part of the inhibitor had moved or, the inhibitor had induced some antiviral compounds which brought about resistance in the leaves.

b) Immersing for one hour PRIOR to virus inoculation

French bean roots were immersed in dilute (1:10) Gp extracts for one hour following the procedures described above. Results in Table 44 and Figure 37 show that:

1- Zero time after inhibitor application to the roots and virus inoculation produced 30 % reduction in the number of lesions compared with controls. This suggest that the inhibitor within this time (1 hour) may have reached the leaves or, induced some protective substance(s) with antiviral activity which prevents local lesion production.

2- The percentage inhibition gradually increased with the increases in the time intervals between inhibitor application and virus inoculation. The highest levels of inhibition, or resistance, induced in the leaves of plants with roots having been immersed in the inhibitor for one hour, was shown after 24 and 48 hours giving 64 and 68 % inhibition respectively.

Having established that Gp inhibitor can induce resistance in the leaves when applied to plant roots prior to virus inoculation, experiments were carried out to determine whether such inhibitors can induce resistance when applied after inoculation with the virus.

4. Effects of immersing french bean roots in Gp extracts AFTER inoculation with TNV on the induction of virus resistance

Thirty french bean plants (60 primary leaves), grown in vermiculite as described above, were inoculated with TNV. Similar numbers of plants

Table 44 : The induction of viral resistance in french bean leaves following immersion of roots in dilute (1:10) Gp extracts for 1 hour

Time in hours after immersion of roots	Mean number of lesions*		% Inhibition
	Control'	Treated"	
0	70.0	49.1	30
0.5	100.9	54.7	46
1	65.5	37.6	43
6	63.6	27.5	57
24	92.6	33.7	64
48	51.3	16.4	68

* Each figure represents the mean number of lesions for ten leaves

' Roots immersed in water for 1 hour before virus inoculation

" Roots immersed in Gp extracts for 1 h. before virus inoculation

were also treated with the same virus to serve as controls. After 0, 0.5, 1, 6, 24 & 48 hours batches of five experimental plants and controls were taken and their roots immersed in dilute (1:10) Gp extracts for 0.5 hour. The plants were washed and transferred to plastic pots as described earlier.

Results in Table 45 and Figure 37 show that Gp extracts had no inhibitory effect on local lesion production if applied after inoculation of TNV. This supports earlier suggestion that Gp extracts to be effective, must be present during the early events of virus infection (chapter VII). Application of Gp inhibitor immediatly after inoculation was too late to bring about any significant reduction in lesion numbers.

Experiments were carried out next to see whether extractable antiviral factors could be detected in regions of the plants showing induced resistance.

5. Effects of extracts from Gp treated, from opposite untreated and from healthy french bean leaves on local lesion production by TNV

Samples of ten leaves of french bean plants were rubbed with: a- Gp extract. b- Extract from leaves taken from positions opposite to such Gp treated leaves. c- Water (controls). At 1, 2, 3, 6, 24 & 48 hours after such treatments, sap was prepared from the leaves by grinding 5 g of washed leaf lamina in distilled water (1:2 W/V). The pulp was squeezed through muslin and the fluid extracts centrifuged at 2,000 x g for 15 minutes. Two ml of each clarified supernatant were mixed with 2 ml of TNV and inoculated onto french bean leaves. For comparison, 2 ml of TNV were mixed with 2 ml of distilled water.

Results in Table 46 show that the crude extracts prepared from

Table 45 : Effect on local lesion production of immersing french bean roots in dilute (1:10) Gp extracts, for 0.5 h., at different time intervals after inoculation with TNV

Time in hours after TNV inoculation	Mean number of lesions *		% Inhibition
	Control'	Treated"	
0	39.5	35.2	11 **
0.5	36.9	34.0	8 **
1	49.0	46.5	5 **
6	55.2	53.0	4 **
24	51.7	52.8	-2 **
48	62.5	67.4	-8 **

* Each figure represents the mean number of lesions for ten leaves

' Roots immersed in water for 0.5 hour after inoculation with TNV

" Roots immersed in Gp extracts for 0.5 hour after TNV inoculation

Negative values of % inhibition = enhancement

** $P > 0.1$ (difference not significant)

Table 46 : Effects of extracts from Gp treated, from opposite untreated and from healthy french bean leaves on local lesion production by TNV

Time in hour after Gp application	Mean number of lesions *				% Inhibition	
	TNV + Water (control)	TNV + A (control)	TNV + B (treated)	TNV + C (opposite)	(B) compared to (A)	(C) compared to (A)
1	43.8	41.2	1.3	36.7	97	11
2	78.5	89.4	5.5	72.7	94	19
3	70.4	75.6	6.0	53.9	92	29
6	53.0	60.3	3.9	42.1	94	30
24	65.8	62.4	8.5	36.5	86	42
48	45.9	53.0	8.9	33.1	83	38

* Each figure represents the mean number of lesions for ten leaves

A = Extract prepared from french bean leaves rubbed with water

B = Extract prepared from french bean leaves rubbed with Gp extracts

C = Extract prepared from leaves opposite to Gp treated leaves

untreated control leaves had no inhibitory effect when mixed with TNV. If anything there was slight enhancement of the virus production. Extracts from Gp treated plants produced more than 90 % inhibition for up to 6 hours. Inhibitory activity of such extracts was slightly reduced after 24 and 48 hours from extract application. Extracts from opposite untreated leaves produced considerable inhibition in all the samples. The maximum inhibition (42 %) was shown after 24 hours from the time of extract application. For comparative purposes, the mean number of lesions per leaf after 24 hours from extract application was 8 & 36 with sap from treated and opposite untreated leaves respectively, and 62 from french bean control leaves or, 65 with distilled water. These results indicate that the inhibitor acts by formation and translocation of a protective substance(s) from treated to untreated parts of the plant.

At this stage it can be concluded that Gp extracts induced local acquired resistance in tissue around the point of its application, as well as, systemic acquired resistance in leaves opposite to and distant from that had been recieved the extracts. Resistance was also induced in the leaves of plants, the roots of which had been immersed in Gp extracts. Similarly, sap prepared from treated and opposite untreated regions contained an extractable plant virus inhibitor. In order to gain more information regarding whether such extract is likely to be translocated, or to act by inducing more inhibitor, or other antiviral substance(s), it was thought useful to determine which fraction of the Gp extracts induced resistance.

6. Effects of crude, non-dialysable, and dialysable part of Gp extracts on the induction of resistance in the opposite leaves of french bean

One leaf of each of the two primary leaves of ten french bean plants was rubbed with the whole Gp extracts. Further similar batches of plants were rubbed with non-dialysable and dialysable fraction prepared as described previously (chapter V). Control plants were rubbed with water. Twenty-four hours later, all opposite leaves of treated and control plants were inoculated with TNV.

The data presented in Table 47 shows that the whole Gp extracts produced the greatest reduction in the number of lesions on the opposite leaves giving 62 % inhibition. The dialysable part (small molecular weight fraction) produced 42 % inhibition on the opposite leaves compared with 13 % inhibition with the non-dialysable fraction.

It seems from these results that the reduction in lesion numbers may be due to the ability of small molecular weight compounds to move from treated to untreated parts of the plant, since the number of lesions was reduced by 42 % on the leaves opposite to those treated with the dialysable fraction. In contrast, the large molecular weight fraction had little effect on the induction of resistance on opposite untreated leaves. This suggests that such resistance is likely to be dependent on the ability of inhibitor molecules to move from treated to untreated parts of the plant.

In chapter VI, aspartic acid was found not only to be in high concentrations in the small molecular weight fraction but was markedly inhibitory to local lesion production by virus. This substance, like other amino acids, might be sufficiently mobile to move from treated to

Table 47 : Effects of crude, non-dialysable, and dialysable part of Gp extracts on the induction of resistance in opposite untreated leaves of french bean

G.paniculata fractions	Mean number of lesions*		% Inhibition
	Control'	opposite"	
Whole extract	48.5	18.5	62
Non-dialysable part	77.4	67.5	13
Dialysable part	71.3	41.5	42

* Each figure represents the mean number of leasions for ten leaves

' Leaves opposite to water treated leaves

" Leaves opposite to Gp treated leaves

untreated leaves and hence be responsible, at least in part, for the phenomenon of induced resistance. This was tested in the following experiment.

Induction of resistance by aspartic acid

Three concentrations of aspartic acid (500, 1000 & 2000 µg/ml) were prepared from analytical grade as described earlier (chapter VI). Two ml of each concentration were rubbed onto one of each of the two primary leaves of ten french bean plants. Control plants were rubbed with water. Twenty-four hours later, all the opposite leaves of treated and control plants were inoculated with TNV.

Results in Table 48 show that the two highest concentrations (2000 & 1000 µg/ml) induced resistance in the opposite leaves, resulting in 30 and 17 % inhibition respectively. The lower concentration (500 µg/ml) had no effect on the opposite leaves. These results support the idea that low molecular weight inhibitors can move through plants and bring about virus inhibition in area distant from their point of application. The levels of induced resistance in this experiment were relatively low.

There still remains the possibility that the Gp extracts induce the production of protective substance (s) with antiviral activity. To examine this an experiment using gel electrophoresis was undertaken.

7. Gel electrophoresis

Polyacrylamide gel electrophoresis was performed on the extracts listed below to see if any new proteins had been induced by the various treatments. The discontinuous polyacrylamide gel electrophoresis system of Laemmli (1970) was used.

Table 48: Effect of various concentrations of aspartic acid on the induction of resistance in opposite untreated primary leaves of French bean

Concentration (µg/ml)	Mean number of lesions *		% Inhibition
	Control '	Opposite "	
2000	53.1	37.2	30
1000	82.6	68.7	17
500	71.0	69.1	3

* Each figure represents the mean number of lesions for ten leaves

' Leaves opposite to water treated leaves

" Leaves opposite to aspartic acid treated leaves

a) Preparation of samples for electrophoresis

Samples were prepared from french bean plants by extracting leaves:

1- Opposite to those treated with Gp extracts

2- Opposite to those treated with water

3- Treated with Gp extracts

4- Treated with water

5- Opposite to those inoculated with TNV

6- Inoculated with TNV

7- Opposite to those inoculated with inhibitor-virus mixture

8- Treated with inhibitor-virus mixture

Samples were taken 24 hours after treatment and ground with 0.5 M Tris-HCl pH 6.8. The samples were boiled for 3 minutes in a mixture of 3 % glycerol, 10 % sodium dodecyl sulphate (SDS), β mercaptoethanol and 0.1 % bromophenol blue. Optimum resolution and detection of protein bands was obtained when 50-100 µg protein was applied per sample.

b) Stock solutions

The following stock solutions were used to prepare the 10 % separating gel and the 5 % stacking gel.

Solution A : 30 % (W/V) acrylamide, 0.9 % (W/V) bisacrylamide

" B : 0.2 M ethylenediaminetetraacetic acid di Na^+ (EDTA)

" C : 0.5 M Tris-HCl, pH 6.8, 10 % SDS

" D : 1.5 M Tris-HCl, pH 8.8, 10 % SDS

" E : 10 % ammonium sulphate freshly prepared

c) Preparation of polyacrylamide-SDS gel slabs

The gel slabs (1 mm thick) were set up to form a discontinuous system of two layers. To prepare the polymerizing mixture for each layer, the appropriate amounts of stock solutions previously chilled were added

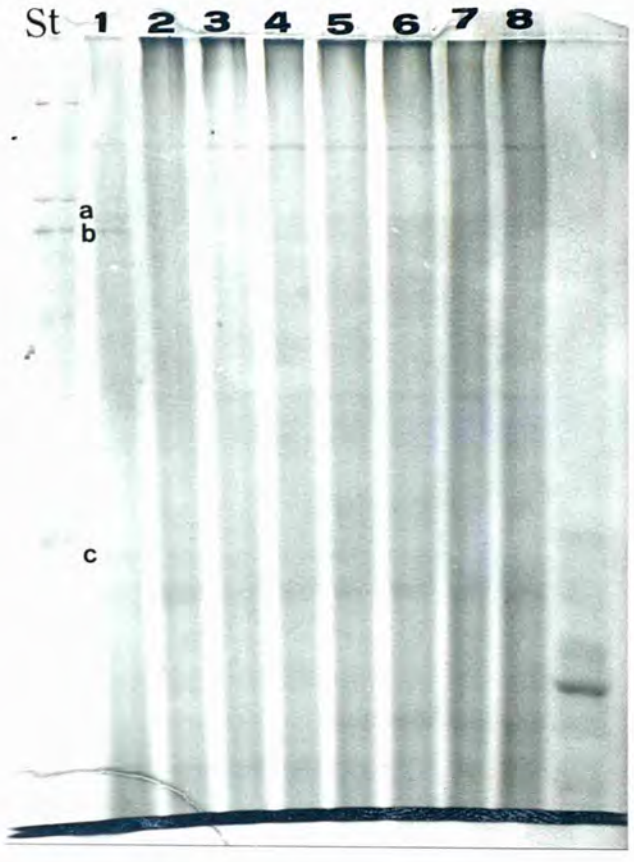
to a flask, stirred and degassed under vacuum for about 5 minutes. TEMED(N,N,N, Tetramethylmethylenediamine) was added and the final mixture stirred and poured into the glass plate frame at room temperature.

d) Electrophoresis

Electrophoresis was run in a 4 °C air cooled chamber at constant current (20 mA) for about 3.5 - 4 hours. The apparatus and electrode buffer (Tris/Glycine buffer containing EDTA and SDS) used were essentially those described by Studier (1972, 1973). A standard consisting of myosin (200,000), β-galactosidase (116,500), phosphorylase-B (94,000), bovine serum albumin (68,000), and ovalbumin (43,000) was run along side the samples in order to assess molecular weights. After electrophoresis, gels were fixed over night in 50 % trichloroacetic acid, stained for 24 hours with Coomassine brilliant blue G made up freshly in 50 % TCA. The gels were destained in solution composed of 125 ml propan-2-ol, 75 ml glacial acetic acid and 800 ml distilled water.

Results in Figure 38 show the protein patterns obtained from 100 µl of extracts from the 8 samples listed above. Of particular interest is the sample taken from leaves opposite to those treated with Gp which showed three additional protein bands with molecular weights approximately 105,000, 94,000 and 40,000. These new proteins were not detected in water treated leaves or in any other samples. It was found that the additional proteins appeared in opposite untreated leaves, one day after application of Gp extracts to the plant. As shown from previous experiments, resistance to virus infection was not present in extracts prepared from control french bean plants. It seems that there is a correlation between the appearance of the additional proteins and the resistance to infection.

Figure 38 : SDS-polyacrylamide gel electrophoresis of french bean leaf extracts. Electrophoresis was conducted at 4 °C constant current (20 mA) for 3-4 hours



French bean extracts prepared from leaves:

1- Opposite to those treated with Gp extracts

2- Opposite to those treated with water

3- Treated with Gp extracts

4- Treated with water

5- Opposite to those inoculated with TNV

6- Inoculated with TNV

7- Opposite to those inoculated with inhibitor-virus mixture

8- Treated with inhibitor-virus mixture

St = Standard protein (see text)

Discussion

In these experiments, it has been shown that resistance to virus infection can be induced by application of G.paniculata extracts. Loss of susceptibility to virus by opposite, and by distant untreated leaves, as well as, by areas immediate to the point of application of the Gp extracts, suggests that acquired resistance has been induced as described in the introduction to this chapter.

Acquired resistance has been reported in various hypersensitive hosts, both in tissues near primary local lesions, and in more distant parts of the plant. In the first case, the term localized acquired resistance (LAR) is used, whereas the resistance developing in other parts is termed systemic acquired resistance (SAR).

LAR was first demonstrated by Yarwood (1960a) when TMV was inoculated onto one half of each primary leaf of pinto beans in two strips about 8 mm apart. On the opposite half-leaf, similar strips were brushed with control solution without virus. Two days after strip inoculation the whole leaf was challenge inoculated with more of the same virus. The reduction in lesion numbers between strips varied between 40 % and 80 % of those between control strips.

SAR has been shown in virus infected plants by Ross (1960b), who showed that when half-leaves of Samsun-NN tobacco were inoculated with TMV, high levels of virus resistance were recorded in the opposite half-leaves. Similarly, resistance could be induced in the basal part of a leaf by inoculating the apical half or, in the opposite primary leaves to those treated with the virus. Furthermore, Ross (1961b) and Bozarth and Ross (1964) suggested that development of systemic resistance was dependent upon the movement of some substance(s) from virus

infected to uninfected regions of the plant.

Systemic acquired resistance has been found to be induced by plant extracts (McKeen, 1956; Apablaza and Bernier, 1972; Fischer and Nienhaus, 1973; Verma and Mukerjee, 1975; Verma and Awasthi, 1979). McKeen (1956) explained SAR as being due to a readily transportable inhibitive agent. Similarly, Fischer and Nienhaus (1973) attributed SAR to the production of a chemical effect translocated through cells to untreated parts of the plant. Recently, Verma and Awasthi (1979) suggested that the inhibitor may induce synthesis of translocatable virus inhibitory or protective substance(s) in host plant. These explanations may apply to the results discussed in this chapter, although it should be noted that LAR and SAR are being induced not by the pathogen, but by a plant extract which is itself inhibitory.

There are two hypothesis to explain acquired resistance in virus infected plants.

a) Depletion theory: Due to hypermetabolic activity of infected cells metabolites are depleted from adjacent uninfected tissues. Thus tissues adjacent to the infected area are less able to support virus synthesis and became resistant. Such a theory may explain LAR but is less satisfactory in the case of SAR.

b) Resistance-inducing substance(s) theory: Initial virus infection induces a substance(s) which confers resistance in uninfected tissues. This might explain both LAR and SAR.

When Gp extracts were applied to french bean roots at different intervals before inoculation with virus, SAR was induced in the leaves. Such extracts may act by inducing synthesis of translocatable substance(s) with antiviral activity. These results are comparable to those of

Sela and Applebaum (1962) and Loebenstein and Ross (1963), who found that TMV inoculum, mixed with extracts from resistance half-leaves of Datura, induced only 8 % as many lesions as it did when mixed with extracts from comparable control leaves. They suggested that the infection in basal leaf-halves either induced the formation in apical halves of an interferon-like agent(s) not found in healthy leaves, or stimulated the production of an inhibitor(s) normally found in Datura leaves. Kimmins (1969) also found that systemic induced resistant areas contained an extractable inhibitor of plant viruses.

Substances which confer resistance to virus infection may be induced, not only by viruses but by a variety of chemicals. For example, Gicherman and Loebenstein (1968) found that when yeast RNA was applied to plants before inoculation with virus, it induced the synthesis of proteins inhibitory to infection. Similar results were reported by Stein and Loebenstein (1970) using synthetic double stranded RNA, polyinosinic (Poly I) and polycytidilic acid (Poly C), and by Merigan and Finkelstein (1968) and Stein and Loebenstein (1972) using polyanions. Gupta et al. (1974) found that a polysaccharide in extracts of Trichothecium (T-poly) induced local and systemic resistance to infection of Nicotiana glutinosa leaves by TMV. Plant extracts can also act in a similar way, thus Verma and Mukerjee (1975, 1977) showed SAR induction with the extracts of brinjal leaves. Similarly, in the experiments described in this thesis the inducer of resistance is a plant extract.

A significant aspect of this study is the resistance and inhibition induced by the small molecular weight fractions of Gp extract. It was shown that small molecular weight fractions induced the greatest resis-

tance on the leaves opposite to those had recieved it. In contrast, the large molecular weight fractions had only a slight effect on the opposite leaf. The difference may be attributed to the different mobility of the extract components. This idea gained support when systemic acquired resistance was induced in leaves opposite to those that had been treated with aspartic acid. Other acids have been reported to induce resistance to virus infection such as (Poly I. C) as mention earlier, as well as, polyacrylic acids of various molecular weights (Gianinazzi and Kassanis, 1974), and acetyl salicylic acid, salicylic acid and benzoic acid (White, 1978).

A particularly interesting suggestion is that acquired resistance in plants may be caused by AVF which may be like interferon (Antignus et al., 1975, Sela, 1981). Interferons are virus inhibiting proteins developed in animal tissues after inoculation with active or inactive virus. Interferon, may also be induced by synthetic polynucleotides and other non-viral agents such as, nucleic acids, Poly I . C, and phytohaemagglutinin. Like interferon action, acquired resistance in plants is not specific to a single type of virus and may be induced by various agents such as, chemical and fungi, as well as, viruses (Hecht and Bateman, 1964). Viral protein such as that from TMV (Loebenstein, 1962), yeast RNA (Gicherman and Loebenstein, 1968), and plant extracts also induced acquired resistance.

Further evidence for a possible relationship between acquired resistance and the induction of AVF, or interferon-like proteins comes from the observations of by Gianinazzi et al. (1970) and Van Loon and Kammen (1970), who showed 4 new proteins appeared in TMV-infected plants, when the leaves become resistant to further infection, and

suggested a causal connection. Gianinazzi and Kassanis (1974) also suggest such a connection in plants treated with polyacrylic acid, they too suggest that these new proteins may be compared to animal interferon. These observations are similar to those described earlier in this chapter inwhich Gp extracts induced three new proteins not found in untreated healthy leaves.

Unfortunately with the techniques available it was not possible to isolate the induced protein in sufficient quantity to test directly against virus and to examine its AVF or interferon-like properties. To undertake this type of experiments, large volumes of Gp extracts will be required and/or perhaps some more refined protein separation techniques. Gianinazzi and Kassanis (1974) tested the proteins induced in tobacco by polyacrylic acid for antiviral activity, but failed to show that they acted as virus inhibitors. Further experiments are required to examine whether, for example, the proteins found by workers were introduced into suitable sites in the plant to bring about virus inhibition. Similar studies might be applied to Gp extracts.

The experiments concerning Gp inhibitors and the induction of acquired resistance described here are particularly interesting and provide a firm basis upon which to study further the reaction of plants to virus infection.

CHAPTER IX

GENERAL DISCUSSION AND CONCLUSIONS

The results of each chapter in this thesis have been discussed in detail, however a more general discussion concerning some points of wider interest will be given here.

In the survey of the Caryophyllaceae species, it was of particular interest to note that of the thirty species tested all were inhibitory to virus infection but in varying degrees. Twenty species totally eliminated local lesion production by TNV.

Loss of inhibitory activity on dilution of most of the species examined showed that they act as inhibitors of virus infection rather than as virus inactivators. Progressive dilutions of some other species revealed the presence of at least two materials in these extracts; an inhibitor which decreased, and an "augmenter" which increased the number of lesions. Such augmenters or enhancing compounds appear to eliminate in some way the effect of inhibitors. The effect of these compounds seems to become apparent on dilution and overcomes the effect of inhibitors which have been diluted beyond their effective concentration as described earlier (chapter III). Benda (1956) described compounds in the inhibitory extracts from New Zealand spinach plants which increased the activity of virus. He used the term "augmenter" for such compounds. Enhancement or augmentation of virus activity has since been observed by plant extracts (Blaszczak et al., 1959), seed extracts (Stevens, 1970; Hajj and Stevens, 1979), various sugars (Kongsvik and Santilli, 1970), alkali (Helms and Zaitlin, 1970), amino acids (Verma et al., 1967). The mechanism of action of these virus-enhancing compounds (augmenters) is not clear. However, they might act either: 1- by

combining with inhibitors, and so eliminating their effects, or, 2- by influencing leaf surface cells in some way, thus affecting their response to virus inhibitors 3- in the absence of inhibitor, augmenters presumably assist some stage of virus infection and replication.

Particularly interesting are the results obtained by heating Caryophyllaceae extracts (chapter IV). The inhibitory activity of the crude extracts of 20 species was reduced, but not completely eliminated by heating, these are thermolabile inhibitors. Crude extracts of another 6 species appeared to be unaffected by heating, these are thermostable inhibitors. Furthermore, heating progressive dilutions of thermolabile inhibitor extracts revealed the presence of virus-enhancing compounds. This suggests that these extracts contain thermolabile inhibitors and thermostable augmenters. On the other hand, dilution of some other spesies showed virus-enhancing activity which became stronger on heating, suggesting the inactivation of inhibitors in the mixture. It is likely that these extracts contain at least 3 fractions: 1- thermolabile inhibitors 2- thermostable inhibitors 3- thermostable augmenters. It can be concluded from the effects of dilution and heat that the nature of Caryophyllaceae extracts is complex, since these extracts contain a wide variety of compounds with varying effects on virus.

Turning now to dilution effects on the G.paniculata extracts, these extracts act as virus inhibitors rather than virus inactivators. Dilution also revealed that extracts from Gp contain both inhibitors and virus-enhancing compounds. Studies of the physical properties of Gp fractions by techniques including dialysis, precipitation with ammonium sulphate, and disc electrophoresis have indicated that extracts

contain a variety of materials with a complex nature. The results of dialysis experiments indicate that the inhibitory fractions from Gp resides in the small molecular weight fractions as well as fractions precipitated with ethanol or ammonium sulphate. The inhibitory compounds present in the large molecular weight fractions were identified as proteins or glycoproteins. The main constituents of the small molecular weight fractions were amino acids and sugars.

Experiments carried out in chapter V using gel filtration and disc electrophoresis proved valuable in confirming the protein/glycoprotein nature of the major part of the inhibitor fractions. Many workers have described the inhibitors from plant extracts as proteins (Kuntz and Walker, 1947; Jermoljev and Brack, 1966; Okuyama et al., 1973; Grasso and Shepherd, 1978), while others have described them as glycoproteins (Kassanis and Kleczkowski, 1948; Verma et al., 1979). Sephadex G-100 column chromatography suggests that the proteinaceous inhibitors from Gp extracts have a molecular weights ranging between 12,600 - 27,500. Similar figures have been found by other workers as mentioned earlier (chapter VI). More detailed information was obtained from chromatography on CM-52 columns which proved particularly valuable in identifying the inhibitor compounds as basic proteins. Similar basic proteins have been reported to act as inhibitors from other plants (Ragetli and Weintraub, 1962; Wyatt and Shepherd, 1969; Smookler, 1971). It seems likely that such basic proteins may be electrically charged in such a way as to bind to, or interfere with virus, perhaps at early stages of infection.

Studies of the small molecular weight fractions indicate that the inhibitor compounds in these fractions are unlikely to be sugar, since

all free sugars from Gp extracts were capable of enhancing-virus activity. On the other hand, most of the free amino acids separated from Gp extracts reduced considerably lesion numbers produced by TNV. It seems that the overall inhibitory effect of the small molecular weight fractions (dialysable part) against TNV, depends upon the ratios of sugars with augmenter activity to other inhibitor compounds such as amino acids (Bobyr, 1959; Verma et al., 1970), organic compounds (Schlegel and Rawlins, 1954), and lipid (El-Kandelgy and Wilcoxon, 1966).

In the experiments described in chapter VII, although extracts from M.capillacea were much less effective as inhibitors to lesion production by TNV, TMV and PVX, than G.paniculata, both extracts were also effective against TNV when they were applied up to 24 hours before inoculation. Similar observations were reported by other workers using other plant extracts (Yoshii and Sako, 1967; Ragetli, 1957). In contrast, post-inoculation treatment of P.vulgaris leaves with both extracts had little or no effect on virus infectivety. These results suggest that both inhibitors act on the host plant influencing the early stages of infection. Similar findings were reported by Ragetli (1957), who found that virus symptoms could be eliminated or reduced by treatment with carnation inhibitor if applied during a period up to 2.5 hours after virus inoculation. Later application had no effect on virus, indicating that this inhibitor acts on early or the "establishment phase" of virus replication. Further, the inability of foliar applied inhibitor to be taken up through the vascular system was interpreted by Ragetli as showing that the antiviral agent interes only with the primary phase of the infection process.

Gypsophila extracts seem to have some direct effect on TNV. This

does not mean that inhibitory substance is actually a virus inactivator because it is possible that the inhibitor was adsorbed onto virus and influenced local lesion production on entry into plant cells along with the virus during the inoculation process. Consideration of an analogous situation, involving inactive homologous antibody complexes, will illustrate this. Such inactive complexes are sedimented during ultracentrifugation, while fully active virus may be recovered from the suspended pellets by appropriately lowering the pH (Rappaport, 1961).

It can be concluded, in general, that inhibitors from both G. paniculata and M.capillacea suppress disease symptoms by interfering with the infection process proper, without altering or irreversibly changing the virus. They may accomplish this by interfering with either of the two phases which constitute infection; the establishment phase or the multiplication phase (Ragetli, 1957). The early events of virus establishment involve: 1- attachment of the virus to some receptors 2- entry of virus or at least its nucleic acid into host cells.

Inhibitors which act by affecting host cells may do so by: 1- interfering with the initial attachment of the virus to the receptors (blocking virus receptor sites), 2- allowing attachment but preventing entry of virus or its nucleic acid into cells, or 3- altering the physiology of the host cell by preventing nucleic acid multiplication and the synthesis of viral protein, so stopping the assembly of new virus particles.

The fact that inhibitor activity is translocatable between lower and upper leaves, and from one leaf to another, indicates that either inhibitors are mobile or that they induce the production of mobile compounds with antiviral activity. Interesting experiments described

in chapter VIII, show that the whole inhibitor extracts from Gypsophila, as well as, the small molecular weight fractions (dialysable part) can induce resistance in tissues distant from their points of application. Another interesting feature is the observation that crude extracts from resistant leaves, untreated with the inhibitor, when mixed with virus decreased the lesion numbers by about 40 %. Furthermore, SDS-polyacrylamide gel electrophoresis of such extracts from resistant leaves revealed the presence of 3 new protein bands not normally found in healthy leaves. These interesting observations may be attributed to the formation and translocation of protective substances with antiviral activity in untreated part of the plants.

Antiviral compounds have been found in virus infected leaves. Sela and Applebaum (1962) demonstrated for the first time the presence of an antiviral factor specific to TMV-infected plants. This antiviral activity was attributed to the presence of an antiviral factor (AVF) which limited virus spread. AVF from TMV-infected N.glutinosa leaves was purified (Sela et al., 1964), and identified as phosphorylated glycoprotein of a molecular weight of about 22,000 daltons (Mozes et al., 1978). Kassanis et al. (1974) also found that induced resistance of infected leaves when challenge with a second virus was correlated with the appearance of 3 proteins not present in healthy leaves.

Antiviral factors can also be induced by some chemicals such as polyacrylic acid of various molecular weights (Gianinazzi and Kassanis 1974), polyinosinic and polycytidylic acids (Poly I. Poly C) (Stein and Loebenstein, 1970; Gat-Edelbaum et al., 1983), and by acetyl salicylic acid, salicylic acid and benzoic acid (White, 1978). These same chemicals induced resistance to virus infection. Faccioli and Rubies-

Antonell (1976) using antimetabolites including actinomycin D and chloramphenicol, concluded that antiviral factors become activated on infection of Chenopodium amaranticolor with TNV. They suggested that antiviral factors probably partly prevent viral protein formation or maturation of virus particles.

Resistance induced by virus infection in local lesion host and that induced by polyacrylic acid seems to differ in that the latter only acts in the treated leaves, whereas virus infection confers resistance to distant leaves (Kassanis et al., 1974). It seems reasonable to suggest that resistance induced by our plant extracts (G.paniculata) is similar to that induced by viruses. It has not, as yet been possible to show that the induced proteins act as virus inhibitors when test on leaves. With modern methods of high performance protein chromatography it may prove possible, in the future, to isolate the induced proteins and assay them against virus. It may be that extracts of plants contain compounds, some of which act directly as virus inhibitors and others that induce the formation of antiviral factors. The former include proteins which act as protein synthesis inhibitors and operate on ribosomes derived from plant species other their own (Stevens et al., 1981; Stirpe et al., 1981). In the latter group, inducers, may include not only proteins but smaller molecules. Viruses themselves may also act as inducers.

These induced virus inhibitors or antiviral factors (AVF) share characteristics and activities reminiscent of animal interferon. AVF resembles interferon in the following features:

1- It is released in virus infected tissue and exhibits antiviral activity against a range of different viruses.

2- AVF can be induced by poly (I.C), polyacrylic acid or tRNA preparations.

3- It is a glycoprotein of molecular weight of 22,000, stable in SDS and at pH 2.0, features which are reminiscent of interferon.

4- AVF is active at the level of few molecules per cell.

5- It is capable of polymerizing ATP in the presence of ds RNA.

The difference between AVF and interferon are as follows:

1- AVF is phosphorylated, interferon appears not to be.

2- The release of AVF in virus-infected or ds RNA-treated plant tissue seems to be a process of activation rather than a new genomic expression (Sela, 1981).

3- No species specificity was found for AVF activity within the plant world.

In spite of the evidence for induction of resistance with antiviral activity in the resistant part of the plants when treated with aqueous plant extracts, the isolation and purification of the AVF has not as yet been carried out.

Extracts from Caryophyllaceae, in general, and from G.paniculata, in particular, appear to contain fractions with many different characteristics combined with an ability to inhibit virus replication. These complicated features make analysis of the extracts difficult. Gypsophila extracts, for example, contain proteinaceous virus inhibitors and virus augmenters. Some inhibitors also have trypsin activity (chapter V), and may inhibit virus at early stages of infection. It is of some interest that Dianthus caryophyllus contains virus inhibitor glycoprotein which additionally, inhibit protein synthesis (Stevens et al., 1981; Stirpe et al., 1981).

In view of these observations one may query whether virus inhibitor

activity is incidental to the major role of a number of proteins in plants. The answer may not be found until more protein fractions have been tested against virus. The answer to this question also requires a more fundamental understanding of the activity of inhibitor compounds. Further questions arise as to the role of AVF in virus control (localization of the virus in the lesion and induced resistance). Where AVF is induced by treatment of tissue with virus, this may be considered as a defence mechanism by the plant. It is not clear, however, whether the same mechanism operates when AVF is induced by chemicals or by otherwise innocuous plant extracts. It might be argued that any number of compounds which interfere with cell metabolism may, for example, redirect protein synthesis to the production of "new" proteins some or all of which control viruses. On the other hand, some compounds from plant extracts might act more directly by stopping protein synthesis (protein synthesis inhibitors), so preventing the production either of viral proteins or some protein essential to the virus replication process.

The value of the work described in this thesis lies in the fact that attention has been directed to the complexity of the problems of understanding and analysing the effects of plant extracts on virus symptom production. By concentrating attention on one species, in particular, it has proved possible to make a number of valuable observations regarding virus inhibition, and to highlight some features of inhibition of considerable interest in terms of the mode of action of these inhibitors. These findings may be summarized in the following conclusions.

Conclusions

1. All the thirty species from the family Caryophyllaceae were found to contain inhibitors of local lesion production by TNV.
2. Crude, undiluted extracts of twenty species totally eliminated lesion production.
3. In eighteen species, inhibitory activity decreased on dilution, indicating that they act as inhibitors of virus infection rather than inactivators.
4. Progressive dilutions of another 12 species revealed the presence of augmenters. This suggests that extracts contain both inhibitors and virus-enhancing or augmenter compounds.
5. Heating most of the extracts (20 species) destroyed much of their inhibitory properties (thermolabile), this suggests that at least part of the inhibitory activity resides in protein or glycoprotein. In contrast, other extracts appeared resistant to heating (thermostable).
6. Dilution of G.paniculata extracts up to 10^{-5} confirmed the presence of plant virus inhibitors and not inactivators, and also showed compounds enhancing virus activity.
7. Dialysis experiments confirmed that the inhibitory activity of Gp extracts resides in both large and small molecular weight fractions.
8. Precipitation with ethanol or ammonium sulphate, as well as, disc electrophoresis indicated that the inhibitors are composed, at least in part, of proteins and glycoproteins.
9. Sephadex G-100 gel filtration proved valuable in determining the molecular weights of the Gp protein or thermolabile inhibitor fractions - these ranged between 12,600 - 27,500.

10. Four fractions were obtained from CM-52 column chromatography, three of them were inhibitory against TNV. One of the inhibitory fraction was neutral in nature, while the other two were basic.
11. The inhibitory effect of the proteinaceous portion of Gp extracts was unchamged after treatment with each of the 3 proteolytic enzymes.
12. The virus inhibitor materials obtained from Gp extracts also showed trypsin-inhibitor activity.
13. Seventeen free amino acids and three free sugars were identified in the low molecular weight fraction of Gp extracts.
14. Ten free amino acids showed considerable reduction in lesion numbers by TNV, while the other seven free amino acids, as well as all 3 free sugars stimulated local lesion production by TNV.
15. Studies of the mode of action of G.paniculata and M.capillacea confirmed the presence of inhibitors and not inactivators, and showed that inhibition is brought about by effects on leaf surface rather than on the virus particles. However, Mc extracts were much less effective as inhibitors to local lesion production by TNV, TMV and PVX, than were Gp extracts.
16. Local and systemic resistance to virus infection was induced in untreated regions of the host plant after application of Gp extracts.
17. The small molecular weight fraction (dialysable part) was capable of inducing resistance in leaves opposite to those to which it had been applied.
18. SDS-polyacrylamide electrophoresis revealed the presence of three new protein bands from the resistant leaves not normally found in healthy leaves.

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APPENDIX 1

STATISTICAL ANALYSIS

The results obtained in this thesis have been analysed where appropriate statistically by methods taken from Bishop* (1968). A specimen student 't' test, used to compare mean number of lesions, are shown below. The following observations and calculations have been used.

(1) The mean number of lesions for each treatment ($\bar{X}$)

(2) The deviation (d) for each number of lesions (X) given by the equation $d = X - \bar{X}$

(3) Frequency of each deviation (f)

(4) The sum of squares of deviations (Σfd^2)

(5) Variance (S) of the mean

$$S_n^2 = \frac{\Sigma fd}{\Sigma f}$$

Σf = sum of frequencies

n = number of leaves in every treatment

(6) The sum of the variances of the two means (control and treated)

$$S_d^2 = \frac{S_1^2}{n_1} + \frac{S_2^2}{n_2}$$

$$\text{i.e.} = \frac{\text{variance of control}}{\text{number of leaves in control}} + \frac{\text{variance of treated}}{\text{number of leaves in treated}}$$

(7) Standard deviation of the difference of means (S_d)

(8) $$t = \frac{\text{deviation of the differences of the means from zero}}{\text{standard deviation of the difference of means}}$$

(9) Calculation values of t were compared with published figures after finding the number of degrees of freedom, using the formula $n_1 + n_2 - 2$

The probability value (P) is given in the appropriate tables

A specimen calculation and details of statistical analysis are given for Table 10 and Table 27.

An analysis of variance is given on page 266, APPENDIX 2

* Bishop, O.N. (1968)
Statistics for Biology. Longmans, Green and Co. Ltd., London.

Student 't' test

Specimen calculation of the data from Table 10 showing the effects of various dilutions of G.paniculata extracts on local lesion production by TNV.

Local lesion Nos.		f	d		fd^2	
TNV + water (control)	TNV + extract (treated)		Control	Treated	Control	Treated
117	1	1	4.2	2	17.64	4
132	4	1	19.2	1	368.64	1
100	4	1	12.8	1	163.84	1
114	2	1	1.2	1	1.44	1
119	7	1	6.2	4	38.44	16
98	3	1	14.8	0	219.04	0
83	2	1	29.8	1	888.04	1
135	4	1	22.2	1	492.84	1
87	2	1	25.8	1	665.64	1
143	1	1	30.2	2	912.04	4
1128 = ΣX	30 = ΣX	10 = Σf			3767.60	30

$$\bar{X} \text{ (control)} = \frac{112.8}{10} = 112.8$$

$$\bar{X} \text{ (treated)} = \frac{30}{10} = 3.0$$

$$S_1^2 = \frac{3767.6}{10} = 376.76$$

$$S_2^2 = \frac{30}{10} = 3.0$$

$$S_d^2 = \frac{376.76}{10} + \frac{3.0}{10} = 37.98$$

$$S_d = \sqrt{37.98} = 6.163$$

$$t = \frac{112.8 - 3.0}{6.16} = 17.82$$

$P < 0.001$ (difference highly significant)

Details of the statistical analysis of the remaining data from Table 10, showing the effects of various dilutions of G.paniculata extracts on local lesion production by TNV

Dilution	10^{-2}		10^{-3}		10^{-4}		10^{-5}	
Treatment	Control	Treated	Control	Treated	Control	Treated	Control	Treated
$\bar{X}$	102.8	24.7	75.4	58.9	84.8	113.7	127.8	227.6
Σf	10	10	10	10	10	10	10	10
Σfd	5001.6	1612.1	6594.4	7920.9	9835.6	5546.1	16036.6	55886.4
S_1^2	500.16	161.21	659.44	792.09	983.56	554.61	1603.	5588.64
S_d^2	66.14		145.15		153.82		719.23	
S_d	8.13		12.05		12.40		26.8	
t	9.6		1.36		2.33		3.8	
P	< 0.001		> 0.1 ** NS		< 0.05		< 0.01	

** NS = Difference not significant

Details of the statistical analysis of the data from Table 27, showing the effect pure amino acids (200 µg/ml) on local lesion production by TNV on french bean leaves

Amino acid	Proline		Glycine		Methionine		Serine	
Treatment	Control	Treated	Control	Treated	Control	Treated	Control	Treated
$\bar{X}$	66.7	68.8	51.8	53.2	85.2	88.7	57.2	60.3
Σf	10	10	10	10	10	10	10	10
Σfd^2	2904.1	2125.6	2256.0	7489.6	8457.6	16226.1	1423.6	1136.1
s_1^2	290.41	212.56	225.60	748.96	845.76	1622.61	142.36	113.61
s_d^2	50.30		97.45		246.84		25.59	
S_d	7.09		9.87		15.71		5.06	
t	0.30		0.14		0.22		0.61	
P	> 0.1 ** NS		> 0.1 ** NS		> 0.1 ** NS		> 0.1 ** NS	

** NS = Difference not significant

(continued on p. 265)

Details of the statistical analysis of the data from Table 27, showing the effect of pure amino acids (200 µg/ml) on local lesion production by TNV on french bean leaves

Amino acid	Lysine		Alanine		Arginine		Amino acid mixture	
Treatment	Control	Treated	Control	Treated	Control	Treated	Control	Treated
$\bar{X}$	91.8	109.7	102.4	139.0	61.3	97.9	93.5	68.4
Σf	10	10	10	10	10	10	10	10
Σfd^2	1988.66	11196.1	4322.4	16186.0	1652.07	2296.9	5546.5	9458.4
S_1^2	198.87	1119.61	432.24	1618.60	165.21	229.69	554.65	945.84
S_d^2	131.85		205.08		39.49		150.05	
S_d	11.48		14.32		6.28		12.25	
t	1.56		2.56		6.31		2.05	
P	> 0.1 ** NS		< 0.02		< 0.001		> 0.05	

** NS = Difference not significant

APPENDIX 2

Analysis of variance

A worked example for the analysis of variance of the data from Table 41 is given below. The sources of variation tested are:

(1) Between leaf position along the plant

(2) Between control and treated plants

(3) Between leaves (B & D) and leaves (C & E) see diagrams p. 203

Table 1-A: presentation of the raw data from Table 41, showing the induction of virus resistance in N.tabacum leaves following treatment of a single basal leaf with G.paniculata extracts.

Leaf Position	Local lesion numbers		Mean
	Control	Treated	
A	25, 38, 47, 42, 30, 58, 22, 25, 43, 62	35, 50, 29, 39, 43, 26, 33, 41, 31, 9	36.4
B	55, 62, 51, 35, 32, 56, 48, 51, 30, 84	36, 31, 18, 27, 23, 20, 32, 17, 23, 28	35.1
C	62, 59, 55, 37, 61, 32, 30, 61, 52, 70	41, 33, 27, 45, 29, 33, 34, 43, 26, 46	43.8
D	52, 51, 93, 60, 61, 40, 37, 80, 41, 103	21, 16, 44, 30, 35, 22, 21, 40, 25, 19	44.5
E	71, 55, 61, 72, 38, 71, 58, 63, 51, 64	33, 38, 53, 29, 50, 23, 30, 40, 48, 52	50.0
Means	52.20	32.34	

Table 2-A : Sums derived from Table 1-A in the statistical analysis of the data from Table 41.

Leaf position	Control	Treated	Total
A	392	336	728
B	477	225	702
C	519	357	876
D	618	273	891
E	604	396	1000
Total	2610	1587	4197

Details of the statistical analysis of the sources of variation:

(a) Correlation for the mean = $\frac{G^2}{N} = \frac{(4197)^2}{100} = 176148.1$

Where G = Grand total of all observations

N = Number of observations

(b) Total sum of squares of all obsevations = $\Sigma X^2 - \frac{G^2}{N}$

$= (25^2 + 38^2 + ... + 52^2) - 176148.1$

$= 211462.0 - 176148.1 = 35313.9$

d.f. = 99 (where d.f. equals degrees of freedom).

(c) Sum of squares between leaf position along the plant

$= (728)^2 + (702)^2 + ... + (1000)^2/20 - 176148.1$

$= 179202.3 - 176148.1 = 3054.2$

d.f. = 4

(d) Sum of squares between control and treated plants

$= (2610)^2 + (1587)^2 /_{50} - 176148.1$

$= 186613.4 - 176148.1 = 10465.3$

d.f. = 1

(e) Sum of squares between leaves (B & D) and leaves (C & E)

$= (1592)^2 + (1876)^2 /_{40} - \frac{(3469)^2}{80}$

$= 151346.0 - 150424.5 = 921.5$

d.f. = 1

Table 3-A:: Analysis of variance for the induction of virus resistance in N.tabacum leaves following treatment of a single basal leaf with G.paniculata extracts

Source of variation	d.f.	Sum of squares	Mean squares	Variance ratio (F)
Between leaf position	4	3054.2	763.6	3.44
Between control and treated plants	1	10465.3	10465.3	47.12
Between leaves(B&D) and leaves (C&E)	1	921.5	921.5	4.15
Error	94	20872.9	222.1	
Total	100	35313.9		

From variance ratio (F) tables, the significant of these (F) values is found to be:-

(a) For 4 - 94 d.f.

when P = 0.05 $\simeq$ 2.48

P = 0.01 $\simeq$ 3.56

P = 0.001 $\simeq$ 5.15

(b) For 1 - 94 d.f.

when P = 0.05 $\simeq$ 3.69

P = 0.01 $\simeq$ 6.95

P = 0.001 $\simeq$ 11.65

F = 3.44 with 4 - 94 d.f. is therefore significant at the 0.05 level

F = 47.12 with 1 - 94 d.f. is therefore significant at the 0.001 level

F = 4.15 with 1 - 94 d.f. is therefore significant at the 0.05 level

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Effects of extracts from species of Caryophyllaceae on local lesion production by tobacco necrosis virus

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Abstract

Thirty species from the family Caryophyllaceae were found to contain inhibitors of local lesion production by tobacco necrosis virus. Most extracts were temperature sensitive, and some appeared to contain enhancing compounds as well as inhibitors.

Introduction

Extracts from *Dianthus caryophyllus* L. and *D. barbatus* L. have been reported to inhibit the infection of plants by virus (van der Want, 1951; Ragetli and Weintraub, 1962a). Further studies provided information regarding the chemical identity and possible mode of action of the inhibitor (van Kammen *et al.*, 1961; Ragetli and Weintraub, 1962b). No other species within the family Caryophyllaceae appears to have been studied for antiviral properties in spite of the possible relationship of the *Dianthus* inhibitor to interferon (Fantes and O'Neill, 1964). In this paper we report the examination of thirty species from the Caryophyllaceae for virus inhibitor activity and compare them briefly with extracts from *D. caryophyllus*.

Materials and methods

Tobacco necrosis virus (TNV) was purified from frozen infected French bean (*Phaseolus vulgaris* var. The Prince) leaves by grinding 10 g of leaf lamina in 20 ml of 0.06 M phosphate buffer, pH 7.0, with a little washed sand. After squeezing through muslin and centrifuging at 2,000 x *g* for 15 min, the supernatant was kept at room temperature overnight to precipitate proteinaceous inhibitors (Bawden, 1954), and then clarified by a further centrifugation. The supernatant virus solution was stored at −25°C.

Plant extracts were prepared from each of thirty Caryophyllaceae species supplied by the Botanical Supply Unit, University of London. Plant shoots were ground in distilled water (10 g per 20 ml). The pulp was squeezed through muslin and the fluid centrifuged at 2,000 x *g* for 15 min. The clear supernatant was lyophilized in 2 ml aliquots and stored at −25°C. The dried material was reconstituted by adding 2 ml of distilled water.

Inhibitor activity of extracts was assayed by mixing reconstituted samples 1:1 with TNV, or with water in controls. The effects of diluting samples with distilled water were studied. The effect of heating extracts to 100° for 10 min was also measured. Virus assay was made by counting local lesion production on 10- to 12-day-old *P. vulgaris* (var. The Prince) plants using carborundum as an abrasive. Each treatment was replicated ten times on ten plants, with opposite leaves acting as controls.

Table 1 Effect of extracts of Caryophyllaceae on local lesion production by tobacco necrosis virus

Species number	Source of extract	Unheated extract: No. of local lesions*: Cont	CE	DE	% Inhibition: CE	DE	Heated extract: No. of local lesions*: Cont	CE	% Inhibn
1	*Arenaria balearica*	41.8	0	45.6	100	−9	80.7	1.9	98
2	*Cerastium biebersteinii*	88.8	29.5	63.9	67	28	88.8	42.8	52
3	*Cerastium tomentosum*	67.7	3.8	39.8	94	41	67.7	21.0	69
4	*Cucubalus baccifer*	71.8	0	72.2	100	0	71.8	6.7	91
5	*Dianthus arenarius*	131.7	0	95.5	100	27	86.0	20.2	77
6	*Dianthus caryophyllus*	110.3	0	55.1	100	50	76.9	27.8	64
7	*Dianthus campestris*	25.6	0.4	33.2	98	−30	25.6	12.7	50
8	*Dianthus gratianopolitanus*	77.3	0	212.1	100	−174**	24.0	4.8	80
9	*Dianthus knappii*	112.8	0	203.1	100	−80	116.2	148.7	−28
10	*Dianthus monspessulanus*	58.1	0	177.5	100	−206**	18.3	9.4	49
11	*Dianthus petraeus*	75.6	1.0	77.9	99	−3	48.7	43.0	12
12	*Dianthus plumarius*	105.7	0	147.5	100	−40	117.1	56.2	52
13	*Gypsophila elegans*	53.8	18.3	54.6	66	−1	53.8	40.2	25
14	*Gypsophila paniculata*	136.9	0	84.9	100	38	118.0	4.8	96

Table 1 continued

15	*Herniaria glabra*	98.1	0	115.0	100	−17	145.0	0	100
16	*Lychnis chalcedonica*	83.9	0	67.3	100	20	83.9	0	100
17	*Lychnis coronaria*	79.0	0	79.0	100	–	31.3	66.2	−112**
18	*Lychnis flos-jovis*	80.1	0	51.7	100	35	30.5	0.4	99
19	*Lychnis viscaria*	82.5	0	336.6	100	−308**	107.3	109.0	−1
20	*Minuratia capillacea*	77.0	12.3	52.9	84	31	80.2	4.3	95
21	*Petrorhagia saxifraga*	55.8	0	33.2	100	41	41.7	0	100
22	*Saponaria ocymoides*	53.3	0	23.0	100	57	53.3	0	100
23	*Silene alpestris*	35.3	10.2	13.4	71	62	126.2	53.5	48
24	*Silene coeli rosa*	90.3	39.5	49.1	56	46	90.3	75.6	16
25	*Silene dioica*	23.6	0	28.6	100	−21	23.6	11.2	53
26	*Silene maritima*	117.0	0	90.6	100	23	117.0	10.5	91
27	*Silene saxifraga*	118.0	1.7	76.3	98	32	94.2	8.2	91
28	*Silene shafta*	80.2	1.0	39.4	99	51	80.2	13.6	83
29	*Telephium imperati*	131.7	0	102.3	100	22	129.5	71.5	45
30	*Vaccaria pyramidata*	57.2	0	61.9	100	−7	47.6	4.3	91

* Mean number of lesions on ten leaves.
** Significant enhancement of lesion numbers.
Cont = control; CE = crude extract; DE = dilute extract; % Inhibn = Percentage inhibition.

Results and discussion

Table 1 shows that of the thirty species examined all inhibit to some degree local lesion production by TNV. Crude, undiluted extracts of twenty species totally eliminated lesion production. Extracts of *Gypsophila elegans, Cerastium biebersteinii* and *Silene alpestris* were least effective but still inhibited 64%, 69% and 71% respectively of the lesion production of the controls.

The most powerful inhibitor extracts included *Dianthus caryophyllus,* and a 10^{-3} dilution of this extract still produced a 50% reduction of lesion numbers compared to controls. Extracts from *Gypsophila paniculata, Lychnis flos-jovis* and *Saponaria ocymoides* were equally as effective inhibitors as *D. caryophyllus* extracts.

Heating extracts for 10 min at 100°C destroyed much of their inhibitory properties, although other extracts appeared resistant to heating. *Herniaria glabra, Lychnis chalcedonica, Pterorhagia saxifraga* and *Saponaria ocymoides* still gave total inhibition of local lesion production after heating.

Dilution of extracts produced samples enhancing virus activity in the case of *Dianthus gratianopolitanus, Dianthus monspessulanus, Lychnis viscaria.* Heating treatment converted inhibitory extracts of *Lychnis coronaria* into ones showing enhancement of the numbers of local lesions produced by TNV.

These results suggest that virus inhibitor components are present in all the plants tested but in varying quantities. The effects of heating the extracts show that at least part of the inhibitory activity resides in proteins or glycoproteins. Many workers have shown virus inhibitors to be proteinaceous in nature (see Verma, Awasthi and Saxena, 1979); some have identified inhibitors as glycoproteins (Kassanis and Kleczkowski, 1948; Mozes *et al.,* 1978).

In those extracts insensitive to heat the inhibitors may well be either polysaccharides (Takahashi, 1946; Hodgson *et al.,* 1969) or other non-protein inhibitors (see Apablaza, 1972).

In extracts showing weak inhibitory activity the effects of inhibitor compounds may either be poor, because the inhibitors are present in low concentrations, or their effect is reduced as a result of the antagonistic effects of enhancing compounds. The effect of such enhancing compounds can be seen in those extracts where inhibition is reversed by dilution or by heating. Compounds enhancing virus activity have been described previously from plants (Benda, 1956; Blaszczak *et al.,* 1959; Stevens, 1970) as well as for various sugars (Kongsvik and Santilli, 1970), and other compounds including DEAE-dextran (Hull, 1971) and alkali (Helms and Zaitlin, 1970).

The evidence produced in this paper that many plants contain inhibitors, some equally as powerful as the *D. caryophyllus* inhibitor, is important. Plants such as *Gypsophila paniculata, Lychnis flos-jovis* and *Saponaria ocymoides* may provide extractable inhibitors each with a different spectrum of antiviral activity. If these inhibitors are similar to the carnation inhibitor with interferon-like properties (Fantes and O'Neill, 1964) they may be of considerable importance in antiviral and anticancer research. Studies are in progress to ascertain the chemical composition of some of the inhibitors described here and also to determine their mode of action. Furthermore, it is hoped to test some of these compounds against animal viruses.

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Studies on the mode of action of inhibitors of local lesion production by plant viruses

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Abstract

Aqueous extracts prepared from *Gypsophila paniculata* and *Minuratia capillacea* each inhibit local lesion production by tobacco mosaic (TMV) and tobacco necrosis (TNV) viruses. The *Gypsophila* extracts have direct effects on TNV and some are precipitated on centrifugation. *Minuratia* inhibitors are not precipitated by centrifugation. Inhibition in both extracts influences host plants and induces antiviral activity at sites distant from their point of application.

Introduction

In a previous paper Barakat and Stevens (1980) have shown that members of the family Caryophyllaceae contain powerful inhibitors of plant virus infection.

Such inhibitors may operate either by inactivating the invading virus, or alternatively by effects on the host plant which might operate either by interfering with the initial uptake of virus or by interrupting some process concerned with replication.

Although numerous plants have been shown to contain plant virus inhibitor fractions, relatively few reports have indicated their mechanism of action. In this paper we report on experiments designed to give some idea of the mode of action of inhibitors from *Gypsophila paniculata* and from *Minuratia capillacea*.

Materials and methods

Crude inhibitor extracts were prepared from leaves and stems of *Gypsophila paniculata* (GP) and from *Minuratia capillacea* (MC) supplied by the Botanical Supply Unit, University of London. Tissue was ground in distilled water (1:2 w/v); the pulp was squeezed through muslin and the fluid extract centrifuged at 2,000 x *g* for 15 min. The clear supernatant was lyophilized in 2 ml aliquots and stored at −25°C.

Tobacco necrosis virus (TNV) was purified from frozen leaves of *Phaseolus vulgaris* by the method of Kassanis (1970). Tobacco mosaic virus (TMV) was purified by the method of Gooding and Herbert (1967) from frozen leaves of *Nicotiana tabacum* var White Burley. All virus samples were stored as 2 ml aliquots at −25°C in 0.06 M phosphate buffer, pH 7.0.

Test plants of *Phaseolus vulgaris* var The Prince, were used as local lesion host for TNV. These plants were grown in 15 cm plastic pots, five plants per pot, in a glasshouse under natural lighting conditions at a temperature of 20–24°C.

Plants of *N. tabacum* var Xanthi, were used as local lesion host for TMV and were initially grown from seed in trays. After three weeks, individual seedlings were transferred to 7 cm plastic pots. Only healthy plants of uniform appearance were used in virus assays.

Inoculations of samples were made by rubbing virus mixed with test solutions onto ten leaves of the appropriate test plant using carborundum as an abrasive. Suitable controls of virus in water or buffer were randomized with the test samples. Each experiment was replicated two or three times. Where appropriate, lesion numbers were compared using the Student 't' test.

Results and discussion

Lyophilized extracts of *G. paniculata* and *M. capillacea* were reconsituted in 2 ml water and partially clarified by centrifugation at 11,000 x *g* for 30 min. The clear supernatants were tested against virus. The precipitates produced on centrifugation were each resuspended in 2 ml water and these also tested against TNV. The results in Table 1 show that both the supernatant and precipitate of GP were inhibitory whereas only the supernatant of MC reduced lesion numbers.

In a further experiment it was shown that each of these supernatants inhibited local lesion production by TMV in *N. tabacum* var Xanthi (Table 2). The supernatant fraction or clarified extract was used in the remaining experiments described below.

To test the direct effect of GP and MC extracts on TNV, each clarified extract was further purified by centrifugation at 100,000 x *g* for 2 h. These purified extracts were incubated with an equal volume of purified TNV for 15 min. The virus was recovered by centrifugation at 100,000 x *g* for 2 h. The pelleted virus was washed by resuspending in distilled water and then repelleted by centrifugation. Control virus samples were treated in a similar manner with water instead of sap. All samples of virus were inoculated onto *P. vulgaris* test plants.

Table 3 shows that purified inhibitor from both GP and MC were as effective as crude extracts in reducing lesion numbers induced by TNV, when mixed with virus and immediately inoculated onto leaves. However, virus treated with MC extract for 15 min and reprecipitated by centrifugation was not inhibited suggesting that MC acts on the test plant leaf rather than on the virus. In contrast GP extract appears to have some direct effect on virus since lesions were reduced by 79%.

Experiments to study the possible effects of these extracts on host plants were performed by immersing whole leaves in inhibitory extracts for 2 min, drying in air for 1 h and then inoculating with TNV. Opposite leaves were dipped into distilled water as controls. In other experiments either the upper or lower surface was covered in extract, care being taken to exclude air so that complete contact was made between test solutions and leaf cells.

Table 1 Effects of supernatant and 11,000 x *g* precipitates from *Gypsophila* (GP) and *Minuratia* (MC) extracts on local lesion production by TNV

Source of extract	Mean number of local lesions:		% Inhibition compared to controls:	
	Supernatant	Precipitate	Supernatant	Precipitate
+ GP	0	34.3	100	84
+ MC	44.4	217.3	80	2
Control	220.8	–	–	–

Table 2 Effect of extracts of *Gypsophila* (GP) and *Minuratia* (MC) on local lesion production by TMV

Source of extract	Mean number of local lesions	% Inhibition
GP	0	100
MC	54.4	60
Control	136.2	–

The results in Table 4 show that significant inhibition of local lesion production occurred whether leaves were immersed in solutions, or solutions were applied to a single surface. The fact that inhibition occurred when the lower surface was treated with extract and virus applied to the upper surface, indicates that extract *in vivo* influence leaf susceptibility rather than having direct effects on virus. This conclusion was further tested in experiments where leaves were washed after 2 min immersion in the inhibitor extracts, dried and then inoculated. Both plant extracts produced inhibition of local lesion production although that of GP was stronger than that of MC.

An interesting feature of these experiments was the observation that the virus concentration required to give adequate lesion numbers in opposite control leaves was double that normally giving 100–150 lesions. This observation suggests that the inhibitor on treated leaves influences lesion production on opposite leaves. Further experiments substantiating this claim will be described elsewhere.

To examine more closely the possibility that inhibitors influence entry of TNV into leaf surfaces, leaves were treated with inhibitor extracts at various times before inoculation with virus. One primary leaf of each *P. vulgaris* plant was dipped into inhibitor for 2 min, the opposite control leaf being dipped into water. After 1 h ten plants were rinsed with water, dried and inoculated with virus. Further batches were inoculated at 4, 6, 12 and 24 h after immersion in inhibitor. The extract from *Gypsophila* was very effective as an inhibitor (Table 5), and no lesion developed even when inoculation was made 24 h after inhibitor application. *Minuratia* extracts were inhibitory but less effective and the results more variable.

This experiment was repeated with the exception that inhibitor was applied after inoculation with virus. The results in Table 6 show that no significant inhibition occurred with either extract if applied 1 h or more after inoculation with virus.

Table 3 Effects of purified extracts of *Gypsophila* (GP) and *Minuratia* (MC) on TNV virus

Extract tested	Number of local lesions: (A) TNV + water (control)	(B) TNV + purified extract	% Inhibition (B) compared to (A)	Number of local lesions: (C) TNV reprecipitated from water	(D) TNV reprecipitated from extract	% Inhibition compared to (D)
GP	62.4	0	100	107.9	22.4	79
MC	62.4	13.5	76	250.0	243.0	3

Table 4 Effects on local lesion production by TNV following dipping of leaves of *P. vulgaris* into inhibitor extracts

Surface dipped	*Gypsophila:* Mean number of local lesions: Extract	Water	% Inhibition	*Minuratia:* Mean number of local lesions: Extract	Water	% Inhibition
Upper	0	42.5	100	15.9	72.9	78
Lower	0.7	29.5	98	11.9	49.5	76
Whole leaf	0	35.8	100	4.1	23.5	83
Whole leaf washing*	2.1	79.1	97	40	79.1	49

* Leaves dipped into extracts then washed in water for 2 min to remove any residual inhibitor.

Table 5 Effects of pretreatment of leaves of *P. vulgaris* with inhibitor extracts on subsequent local lesion production by TNV

Hours before inoculation	*Gypsophila* source of inhibitor extract: Mean number of lesions: Control	Treated	% Inhibition	*Minuratia* source of inhibitor extract: Mean number of lesions: Control	Treated	% Inhibition
1	17.0	–	100	109.5	11.5	89
4	53.1	–	100	60.0	23.6	61
6	32.3	–	100	89.4	40.0	55
12	27.0	–	100	44.0	24.0	45
24	77.2	–	100	171.6	49.4	71

Table 6 Effects of post-treatment of leaves of *P. vulgaris* with inhibitor extracts on subsequent local lesion production by TNV

Hours after inoculation	*Gypsophila* source of inhibitor extract: Mean number of lesions: Control	Treated	% Inhibition	*Minuratia* source of inhibitor extract Mean number of lesions: Control	Treated	% Inhibition
1	128.0	122.1	5	130.7	98.3	25
4	137.0	138.4	– 1	103.4	98.4	5
6	100.9	79.9	20	94.1	72.1	23
12	127.3	119.8	6	119.0	113.6	4
24	110.1	111.4	– 1	143.2	157.1	–10

The results of these experiments indicate that the inhibitory fractions from *Gypsophila paniculata* differ from those of *Minuratia capillacea.* GP extracts contain small molecular-weight inhibitors as well as inhibitors precipitating on centrifugation, whereas MC inhibition was confined to the supernatant fraction (Apablaza and Bernier, 1972). The precipitated inhibitor from GP may well be proteinaceous since inhibitors from related plants have been characterized as proteins (Ragetli and Weintraub, 1962; Stirpe *et al.*, 1981).

The supernatant inhibitors are very likely to be small molecular weight non-protein molecules, since in a previous paper (Barakat and Stevens, 1980) crude extracts of these same species of plant remained inhibitory even after heating to 100°C for 10 min. Fukaya and Taniguchi (1979), however, found that the pokeweed inhibitors, known to be polypeptides (Irvin, 1975; Irvin, Kelly and Robertus, 1980), were insensitive to heating for 5–10 min at 100°C. Similarly the lectin-like glycoprotein from *Phaseolus vulgaris* seeds lacked heat sensitivity (Hajj and Stevens, 1979), and inhibitors from geranium and peppers retained their capacity to inhibit following heating.

Although GP extracts seem to have some direct effect on TNV it is possible that the inhibitor is adsorbed onto virus and influences local lesion production on entry into plant cells along with the virus during the inoculation process.

The induction of resistance to local lesion production when leaves were treated some time before inoculation but not after virus application suggests that inhibitors act on the host plant so influencing the early stages of infection. However, the fact that inhibitor activity is translocatable between lower and upper leaf and from one leaf to another indicates that either inhibitors are mobile or that they induce the production of compounds with antiviral activity. Antiviral compounds have been found in virus infected leaves (Sela and Applebaum, 1962; Mozes *et al.*, 1978) and identified as protein or phosphoglycoprotein. Kassanis, Gianinazzi and White (1974) found that induced resistance of infected leaves when challenged with a second virus was correlated with the appearance of three proteins not present in healthy leaves. Such proteins could also be induced by polyacrylic acids of various molecular weights (Gianinazzi and Kassanis, 1974), and by acetyl salicylic acid, salicylic acid and benzoic acid (White, 1978). These same chemicals induced resistance to virus infection. Resistance induced by virus infection in hypersensitive (local lesion) host and that induced by polyacrylic acid seem to differ in that the latter only acts in the treated leaves whereas virus infection confers resistance on distant leaves (Kassanis, Gianinazzi and White, 1974). These workers could not show that the induced protein acts as virus inhibitor when injected into leaves. Such injection techniques may have placed these proteins into intercellular spaces rather than into cells.

Faccioli and Rubies-Antonell (1976) using antimetabolites including actinomycin D and chloramphenicol concluded that antiviral factors become activated on infection of *Chenopodium amanticolor* with TNV. They suggested that antiviral factors probably partly prevent viral protein formation or maturation of virus particles. Inhibitors of protein synthesis extracted from plants can inhibit local lesion production by viruses (Stevens *et al.*, 1981; Stirpe *et al.*, 1981) and act by effects on 80S ribosomes. However, it is suggested that such protein inhibitors only act by damaging 'foreign' rather than their own ribosomes. It seems likely therefore that extracts of plants may contain compounds, some of which act directly as virus inhibitors and others that induce the formation of antiviral factors. The former include proteins

which act as protein synthesis inhibitors and operate on ribosomes different from their own. In the latter group inducers may include not only proteins but smaller molecules. Viruses, too, may act as inducers. The induced virus inhibitors or antiviral factors may resemble interferon (Gianinazzi and Kassanis, 1974; Mozes *et al.*, 1978).

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