STUDIES OF PLANT VIRUS INHIBITORS
FROM LEGUMOUS SEEDS

A Thesis submitted to the University of London for
the degree of Doctor of Philosophy in Botany (Plant Virology).

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Seed extracts from 18 varieties of legumes were tested for virus inhibitory activity against TNV. Unheated seed extracts fall into two categories. Those extracts which give 75-95% inhibition and includes *G. max* (Soybean), and extracts in which inhibition is between 0-60%, for example *P. vulgaris* (French bean).

Inhibition was decreased by heating some extracts such as *G. max*, whilst in other extracts, such as *P. vulgaris*, the percentage inhibition was increased by heating.

*P. vulgaris* and *G. max* seed extracts were studied in detail. *P. vulgaris* was also inhibitory to TNV, whilst *G. max* was inhibitory to TNV and FVX. Dilution experiments confirmed the presence of inhibitors and not inactivators in both extracts.

None of the inhibitors was nucleic acid. However, dialysis precipitation with alcohol or ammonium sulphate and disc electrophoresis experiments suggested that the inhibitors are composed of proteins and glycoproteins stable to a wide range of pH.

Sephadex G-100 gel filtration showed that *G. max* seed extracts inhibitors have molecular weights of about 153,500 and 17,780. On the other hand, *P. vulgaris* inhibitors have molecular weights of about 177,800 and 12,590.

Seven fractions were obtained from DEAE chromatography of each of *G. max* (Soybean) and *P. vulgaris* (French bean) seed extracts. Soybean contains three virus inhibitor fractions, one basic in nature and two acidic. None of the virus inhibitors agglutinated erythrocytes; however, the acidic inhibitors showed trypsin

\[ \text{TNV} = \text{Tobacco necrosis virus} \]

\[ \text{G. max} = \text{Glycine max} \]

\[ \text{P. vulgaris} = \text{Phaseolus vulgaris} \]
inhibition activity.

French bean contains also three virus inhibitors as well as compounds reducing the effects of the inhibitors. These are termed masking compounds. Such masking compounds were agglutinins. The basic inhibitor also showed agglutination of erythrocytes whilst only one of the two acidic virus inhibitors showed trypsin inhibition.

Plant lectins were also tested for virus inhibition and agglutination activity, and it was found that the situation is complex and although soybean and French bean seed extracts seemed to have surface effect on the susceptibility of the host, the mode of action of lectins and the virus inhibitors are different. The virus inhibitors seem either to affect the attachment of the virus to the infective centres or perhaps allow attachment but prevent entry of virus into the cells.
CONTENTS

ABSTRACT 2

CHAPTER I. INTRODUCTION 8

CHAPTER II. MATERIALS AND METHODS 40
1. Growth of assay plants 40
2. Preparation and infectivity assay of plant viruses 40
3. Seed extract preparation 43
4. Estimation of protein 45
5. Estimation of carbohydrate 45
6. Disc electrophoresis 45

CHAPTER III. EFFECT OF VARIOUS LEGUME SEED EXTRACTS ON THE INFECTION OF PHASEOLUS VULGARIS BY TNV 47

CHAPTER IV. PROPERTIES OF VIRUS INHIBITOR EXTRACTS PREPARED FROM GLYCINE MAX AND PHASEOLUS VULGARIS SEEDS 55

SECTION A. PROPERTIES OF VIRUS INHIBITOR EXTRACTS PREPARED FROM GLYCINE MAX VAR. "MEHR" SEEDS 55
(a) Effect of dilution 55
(b) Effect of pH 55
(c) Effect of dialysis 61
(d) Effect of alcohol 67
(e) Effect of ammonium sulphate 67
Discussion 71
SECTION B. PROPERTIES OF VIRUS INHIBITOR EXTRACTS
PREPARED FROM PHASEOLUS VULGARIS VAR. "THE PRINCE" SEEDS

(a) Effect of dilution
(b) Effect of pH
(c) Effect of dialysis
(d) Effect of heat
(e) Effect of alcohol
(f) Effect of ammonium sulphate

Discussion

76

78

82

82

86

86

90

CHAPTER V. NATURE OF THE VIRUS INHIBITORS

SECTION A. NATURE OF THE VIRUS INHIBITORS
EXTRACTED FROM GLYCINE MAX SEEDS

1. Gel filtration of Glycine max seed extracts

2. The analysis of Glycine max seed extracts
   by ion-exchange cellulose
   (a) Column chromatography using DE-32 cellulose
   (b) Column chromatography using DEAE-32 cellulose

Discussion

94

111

111

117

123

SECTION B. NATURE OF THE VIRUS INHIBITORS
EXTRACTED FROM PHASEOLUS VULGARIS SEEDS

1. Gel filtration of Phaseolus vulgaris seed extracts

2. Chromatography of Ext 0.2 (French bean)
   on DEAE-32 column

3. DEAE-32 chromatography of the unheated and
   the heated P. vulgaris seed extracts

Discussion

131

132

144

153

157
CHAPTER VI. COMPARISON OF G. MAX AND P. VULGARIS VIRUS INHIBITOR EXTRACTS WITH PLANT LECTINS AND TRYPsin INHIBITOR

SECTION A. HEMAGGLUTINATION AND TRYPsin INHIBITION ACTIVITY OF THE VIRUS INHIBITORS EXTRACTED FROM G. MAX SEEDS

(a) Hemagglutination activity of DEAL-52 fractions from $\text{Ext}_{93}^{A,2}$ (Soybean) 161

(b) Trypsin inhibition activity of DEAL-52 fractions from $\text{Ext}_{93}^{A,2}$ (Soybean) 162

SECTION B. HEMAGGLUTINATION AND TRYPsin INHIBITION ACTIVITY OF THE VIRUS INHIBITORS EXTRACTED FROM P. VULGARIS SEEDS

(a) Hemagglutination activity of DEAL-52 fractions from $\text{Ext}_{93}^{A,2}$ (French bean) 167

(b) Trypsin inhibition activity of DEAL-52 fractions from $\text{Ext}_{93}^{A,2}$ (French bean) 167

SECTION C. EFFECT OF LECTINS ON LOCAL Lesion PRODUCTION BY TNV

(a) Phytohemagglutinin (PHA) 167

(b) Crude soybean agglutinin (SBA) 173

(c) Effect of Con A on local lesion production by TNV 179

Discussion 179

CHAPTER VII. MODE OF ACTION OF G. MAX AND P. VULGARIS SEED EXTRACTS

SECTION A. MODE OF ACTION OF G. MAX SEED EXTRACT

1. Do G. max inhibitors act by affecting TNV? 183
2. **Effect of dipping French bean leaves in**
   *G. max* seed extract
   
3. **Effect of *G. max* seed extract on local**
   lesion production by TMV and PVX

**Discussion**

**SECTION B. MODE OF ACTION OF *P. vulgaris* SEED EXTRACT**

1. **Do *P. vulgaris* inhibitors act by affecting**
   TMV?

2. **Effect of dipping French bean leaves into**
   *P. vulgaris* seed extract
   
3. **Effect of *P. vulgaris* seed extract on local**
   lesion production by TMV and PVX

**Discussion**

**CHAPTER VIII. GENERAL DISCUSSION**

**BIBLIOGRAPHY**

**ACKNOWLEDGMENTS**

**APPENDIX (1 - 5) STATISTICAL ANALYSIS**
The presence of antimicrobial substances in higher plants has been known since ancient times. Egyptians probably used mixtures of certain vegetable oils for the preservation of mummies from protein decomposing bacteria. Greeks and Romans used the juice of green walnut shells against infectious fungal diseases of skin (Sovacs, 1964). Sharville (1960) reported that the application of plant extracts for the control of a plant disease was attempted as early as 470 B.C. by Democritus. However, it is only in recent years that intensive research has been made to discover antimicrobial substances from higher plants which could be used for the control of diseases caused by plant pathogens.

The first suggestion of a plant virus inhibitor in plant sap occurs in the work of Allard (1914, 1918). Although it is now well recognized that many plants contain potent inhibitors of virus infection, the nature of most inhibitors remains unresolved and there is still no clear understanding of the ways in which inhibitors reduce infection.

There are two schools of thought 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 that they inhibit by acting directly on the virus particles. Substances that act by inactivating the virus without affecting the host are termed inactivators (Fulton, 1943). These include certain plant extracts, milk, trypsin, normal blood serum, and other complex substances of biological origin.

Inhibitors primarily affect the susceptibility of the host used
and can be distinguished from virus inactivators by possessing the following characteristics: they are capable of instantaneous effect; their effect is dependent on the host used; they have greater effect on concentrated inocula; their effect is diminished by dilution; they have an effect when applied prior to inoculation or when applied to the undersurface of leaves (Crowley, 1955).

Bawden (1934) divided virus inhibitors into two categories: inhibitors of infection and inhibitors of virus multiplication. Inhibitors of infection are defined as "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 infecting viruses multiply".

Many plant species have been reported to contain inhibitors of plant viruses. Most work has been carried out on virus inhibitors extracted from plant sap. Some virologists noticed, for example, that crude extracts prepared from virus infected material often failed to produce 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. Of particular importance, however, is the possibility that crude inoculum may contain substances that inhibit virus infection either by influencing the virus or by acting on the plants being tested. Considerable interest has been paid to the possible presence of virus inhibitor compounds in the aerial parts of a wide range of plants. Bawden reviewed the subject of plant virus inhibitors covering the period up to 1954.
Tables 1 and 2 list plants from which inhibitor compounds have been extracted in more recent experiments. Table 1 lists plants covering 60 different families in which vegetative tissues have been tested for virus inhibitor compounds. Table 2 lists plants where fruits and seeds have been examined for inhibitors. In these tables families are listed alphabetically while genera are listed in chronological order of investigation.

Although, as Table 1 shows, virus inhibitors occur in many families of plants, it is apparent from the literature that only a few workers have investigated the chemical nature of the inhibitor compounds. Only in eight families, the AZOACEAE, CARYOPHYLLACEAE, CHENOPODIACEAE, CRASSULACEAE, LEGUMINOSAE, MYRTACEAE, ROSACEAE, and SOLANACEAE, has any substantial amount of work been carried out to establish the identity of the inhibitory compounds isolated. In the AZOACEAE, Benda (1956) found that expressed sap of New Zealand spinach (Tetragonia expansa), when mixed with tobacco ringspot virus and inoculated by rubbing on Cowpea (Vigna sinensis) leaves, caused a delay in the appearance of the primary virus symptom. 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.

By means of an ion exchange cellulose (DEAE), Simon, Swidler and Moss (1963) removed the inhibitory substance from crude extract of N. embryophytum. Attempts to establish, by proteolytic enzyme digestion, the possible proteinaceous composition of the inhibitor were only partially successful. Most of the enzymes tried were either active themselves as virus inhibitors, or were proteolytically active only at pH's low enough to degrade the inhibitors.
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Studies of the *Caryophyllaceae* made many years ago established
the occurrence of an active principle in the genus *Dianthus* that
interfered with the mechanical transmission of plant viruses. It was
shown independently in Holland and in Canada that sap from both
Carnation *D. caryophyllus* L. (Van der Want, 1951) and Sweet William
*D. barbatus* L. (Weintraub and Gilpatrick, 1952) upon mixing with
viruses such as tobacco mosaic (TMV) and tobacco ringspot, suppressed
or greatly reduced the development of symptoms on their respective
hosts. Later it was shown by Ragetli (1937) that the carnation
inhibitor probably acts via the host plant in the early stages of
virus establishment and that it is active against combinations of at
least 14 viruses and 20 different plant species. Ragetli and Weintraub
(1962a) described the isolation and purification of a potent virus
inhibitor from carnation *D. caryophyllus*. They passed carnation sap
through an ion exchange column (DEAE) and then through a cation
exchange column (CM-cellulose). A number of inhibitor-containing
fractions resulting from the second CM-cellulose treatment were
recombined and re fractionated on a hydroxylapatite column. The
fractions were tested for inhibitor 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. The inhibitor activity however
was unchanged after incubation with four proteolytic enzymes.

Nart (1972) confirmed the Ragetli and Weintraub results by
showing that, on five different local lesion hosts, sap from carnation
*D. caryophyllus* L. inhibited the number of local lesions induced by
three different viruses, namely tobacco mosaic (TMV), alfalfa mosaic
(AMV) and barley stripe mosaic virus (BSV). An attempt was made to
isolate and identify the inhibitory substance. A rough separation
was obtained, by low speed centrifugation, phenol extraction and ethanol precipitation of the sap. The inhibitor was found to be a proteinaceous material.

Many members of the Chenopodiaceae have been examined for virus inhibitors. Huntz and Walker (1947) described two inhibitors in spinach juice (*Spinacia oleracea* L.), one which was absorbed in charcoal and which was considered to be a high molecular weight protein; the other was an oxalate and was removed by adding calcium chloride. These results are at variance with those of Benda (1956) where oxalates from *Tetragonia expansa* (Aizoaceae) were thought to enhance virus activity.

More work was carried out on sap from *S. oleracea* by Chiba and Tominaga (1952). They pressed the juice from spinach and partitioned it by centrifugation into "chloroplast suspension" and "cytoplasmic suspension". Using TMV they showed the presence of a certain heat sensitive inhibitory substance or substances in "Chloroplast suspension". In contrast the "cytoplasmic suspension" did not show such inhibitory effects.

Ibrahim-Nesbat (1971), also investigating the inhibitory effect of spinach sap, isolated and partially purified an inhibitor fraction. Furthermore, he studied the mode of action of the inhibitor 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. Later it was shown by Ibrahim-Nesbat (1972) that the inhibitors resembled proteins.

Sap from Chenopodiales (an order including some members of the Chenopodiaceae and the Amaranthaceae) has also been studied by many workers. Kiramia (1969) examined a crude extract from the leaves of *Chenopodium amaranthicolor* and found it inhibited the infection of
French Bean leaves by TNV. Fractionation of the crude extract indicated that the inhibitory activity resided in the KCl portion.

Other studies of leaf extracts from Chenopodiales suggested that inhibitor compounds are proteinaceous (Kuntz and Walker, 1947; Thomson and Peddie, 1965; Yoshizaki and Murayama, 1966; Yoshii and Sako, 1967). Smokler (1971) examined more members of the Chenopodiales and found that leaf extracts from 29 species inhibited the infection of Phaseolus vulgaris by TNV. Furthermore, he described the nature and properties of inhibitors from extracts of Chenopodium fremontii, C. album, Atriplex nitens and Amaranthus caudatus. Ion-exchange column chromatography on CM-Sephadex C-25 showed that most of the protein fractions which were eluted by 0.4 M sodium acetate buffer were inhibitory against TNV.

Recently Taniguchi, Nakajima, Ymagachi and Naganawa (1974) isolated two fractions from leaf extract of Chenopodium album L. by KIESOLA cellulose column chromatography. Both fractions showed inhibitory activity against TNV. The inhibitors consisted of particles of various sizes, and their activity was destroyed by phenol but not by RNase, pronase or chloroform.

Simons, Swidler and Moss (1963) discovered that the family CHASSELACEAE is an extremely rich source of tobacco mosaic virus inhibitors. Out of 13 species tested, Simons et al. tried to isolate the virus inhibitors from crude extracts of Aeonium arboreum, Kalanchoe somaliensis and Sedum maschmannianum. The inhibitors were eluted by dilute sulphuric acid (I N) from an ion-exchange cellulose column. Attempts to establish the possible proteinaceous composition of the inhibitors were only partially successful.

Several members of the LEGUMINOSAE have been examined for virus inhibitors. El-Kendegy and Wilcoxson (1966) studied the effect of
Red clover (Trifolium pratense) flower extract on infection of Gomphrena globosa by Red Clover vein mosaic virus. The extract contained 9% glucose, 5% galactose, and 3% xylose, each of which inhibited infection.

In 1972, Nart attempted to isolate and identify the inhibitory substance from Phaseolus vulgaris (French bean) sap. A rough separation was obtained and the inhibitor was identified as a protein.

Taniguchi (1974) also studied some properties of tobacco mosaic virus inhibitor extracted from P. vulgaris. The inhibitory substance was found to be a relatively low molecular weight compound, diffusible in sepharose 2B gel. Gel filtration chromatography indicated that the partially purified inhibitor contained compounds of several molecular sizes.

The first suggestion of an inhibitor in plant sap occurs in the work of Allard (1914, 1918) who worked on a mosaic disease of pokeweed, Phyto1acea decandra, the type genus of the PHYTOACCAEAE. 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 the pokeweed mosaic virus.

Although many workers subsequently tested pokeweed juice against viruses, none of them tried to isolate the inhibitor. Marchoux (1967, 1970) however, proved that extracts of P. decandra had ribonuclease activity (RNase) which suggests that proteins may be involved in the inhibitory activity of P. decandra.

Kassanis and Kleczkowski (1943) isolated an inhibitor from the sap of P. esculenta, which was identified as a glycoprotein containing 14 to 15% nitrogen and 8 to 12% carbohydrate. Gupta (1964) used the method of Kassanis and Kleczkowski for isolating an inhibitor from
Phytolacca acinosa. The inhibitor proved to be a glycoprotein containing glucose.

Byatt and Shepherd (1969) extended the procedure developed by Kasasnis and Kleczkowski for the isolation of an inhibitor from Phytolacca americana. Following purification by column chromatography, using carboxymethyl sephadex, the inhibitor was reported to be a protein of 116 amino acid residues giving a molecular weight of 13,000. In 1973, Owens, Bruening and Shepherd reported a possible mechanism for the inhibition of plant viruses by a peptide from Phytolacca americana. The inhibitor was tested for its ability to inhibit in vitro polypeptide synthesis in systems employing ribosomes of wheat, cowpea and pokeweed. Only the pokeweed ribosome system was resistant to inhibition. This suggests that the inhibitor acts in vivo by blocking the messenger function of a potentially infective virus RNA.

Bawden and Kleczkowski (1975) reported that extracts from leaves, stems or roots of Fragaria vesca L. (strawberry), a member of the Rosaceae, contained tannins, which even at the 1% concentration, were sufficient to precipitate TMV and prevent it from infecting Nicotiana glutinosa. Van der Want (1951) suggested that tannins prevent infection of all plants, and such substances should be called "absolute inhibitors" to distinguish them from "relative inhibitors" whose action depends on the species of plant to which inoculations are made. Tannins from F. vesca L. would appear, therefore, to act more like virus inactivators than inhibitors.

A considerable volume of work has been carried out to investigate the possible occurrence of inhibitory substances in plants from the Solanaceae. Kaitlin and Siegel (1963) isolated an inhibitor of TMV infection from Nicotiana tabacum L. var. "Turkish Samsun". The inhibitor was a protein having a molecular weight greater than 40,000.
Volffgang (1970) fractionated leaf extracts of *Nicotiana tabacum* var. "Turkish Samsun" by column chromatography into high and low molecular weight fractions, both having inhibitory effects. Chiba and Tominga (1932) reported an inhibitor of TNV in the "chloroplast suspension" obtained from leaf extracts of *Nicotiana glutinosa*, *N. tabacum* and *Capsicum annuum*.

The inhibitory effect of *N. glutinosa* was also studied by Palm (1967). A salt-soluble fraction prepared from leaves of *N. glutinosa* caused 60% inhibition when mixed in equal volumes with TNV. Basic chemical and physical tests indicated that the inhibitor was a protein of the globulin type. On the other hand, two years later Kimmins (1969) fractionated crude extracts from *N. glutinosa* into protein and RNA, and suggested that the inhibitory activity was retained in the RNA portion.

Marchoux (1967, 1970) showed that the inhibitory extract of *Capsicum* sp. contained proteins with ribonuclease activity (RNase). Apablaza and Bernier (1972), however, separated the inhibitory activity of *Capsicum frutescens* into two fractions: one with a molecular weight greater than 50,000, and one with a molecular weight range of 1,000 - 50,000. Later, Fischer and Niehauo (1973) related the inhibitory effect of *Capsicum annuum* extract to a protein and a phenolic substance. The phenolic substance was identified as either a flavone, an isoflavone or a flavonone compound.

Inhibitors of FVX have been isolated from *Solanum tuberosum* (potato) (Jermoljiev and Albrechtova, 1965). The proteinaceous inhibitor was separated from other proteins by ultracentrifugation followed by subsequent gel filtration of the supernatant on a Sephadex G-50 column. Albrechtova (1968) in further work on potato leaf sap, and using DEAE cellulose columns, isolated an electro-
phoretically homogeneous protein which was eluted at 0.15-0.3 M NaCl concentration, and which inhibited FNX multiplication.

This review shows that inhibitors from vegetative parts of plants have been variously identified as oxalates, tannins, RNA, proteins and glycoproteins. Whether such compounds act in preventing or diminishing virus multiplication in vivo is not understood.

Inhibitors in vivo may act by restricting virus infection either to the production of local lesion or by preventing virus multiplication completely. Inhibitors may well be important in restricting the spread of infection throughout the plant, for example preventing the entry of viruses into reproductive structures. Such a phenomenon may be important in limiting the transmission of viruses by seeds.

Little work has been done on virus inhibitors extracted from seeds, although the problem as to why many highly infectious viruses are not transmitted via seeds is still not entirely understood and may involve inhibitors.

Failure to transmit a virus via seeds may be due to (a) failure of virus to enter seed tissue, (b) some form of inactivation or inhibition of the virus after entering into the seed.

Bennett (1963) has reviewed the work on seed transmission of viruses and although there is evidence that a virus fail to enter seed tissues, the picture is not altogether complete. Some workers have shown that seeds and fruits contain compounds inhibitory to plant viruses (Table 2). Thus, dealing with these in alphabetical order of families, in the ANACARDIACEAE Sharma and Chohan (1973) reported the inhibition of Cucumis virus I (vegetable marrow mosaic virus - WMV) by seed extract of Mangifera indica (Mango). The percentage inhibition was 77%.

CHENOPODIACEAE have been considered in rather more detail.
<table>
<thead>
<tr>
<th>SOURCE OF INHIBITOR</th>
<th>VIRUS</th>
<th>REFERENCE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>FAMILY</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ANACARDIACEAE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mangifera indica</td>
<td>Cucumis virus I</td>
<td>Sharma et al. (1973)</td>
</tr>
<tr>
<td><strong>CHENOPODIACEAE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Beta vulgaris (beet)</td>
<td>TNV</td>
<td>Szirmai (1963)</td>
</tr>
<tr>
<td>Beta vulgaris (beet)</td>
<td>TNV</td>
<td></td>
</tr>
<tr>
<td>Beta vulgaris (sugar-beet)</td>
<td>TNV</td>
<td>Stevens (1970)</td>
</tr>
<tr>
<td><strong>COMPOSITAE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Lactuca sativa</td>
<td>TNV</td>
<td>Stevens (1970)</td>
</tr>
<tr>
<td><strong>CRUCIFERAE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Brassica napus</td>
<td>TNV</td>
<td>Stevens (1970)</td>
</tr>
<tr>
<td>B. alba</td>
<td>TNV</td>
<td></td>
</tr>
<tr>
<td>Cheiranthus cheiri</td>
<td>TNV</td>
<td></td>
</tr>
<tr>
<td>Raphanus sativus</td>
<td>TNV</td>
<td></td>
</tr>
<tr>
<td><strong>CUCURBITACEAE</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cucumis sativa</td>
<td>Cucumber mosaic</td>
<td>Sill et al. (1951, 1952)</td>
</tr>
<tr>
<td>C. sativa</td>
<td>TNV</td>
<td>Crowley (1955)</td>
</tr>
<tr>
<td>Echinocystus lobata</td>
<td>TNV</td>
<td></td>
</tr>
<tr>
<td><strong>GRAMINACEAE</strong></td>
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<td></td>
</tr>
<tr>
<td>Oryza sativa</td>
<td>TNV</td>
<td>Allen et al. (1957)</td>
</tr>
<tr>
<td>O. sativa</td>
<td>TNV</td>
<td>Kohn et al. (1960)</td>
</tr>
<tr>
<td>Triticum sativum</td>
<td>TNV</td>
<td>Verma et al. (1965)</td>
</tr>
<tr>
<td><strong>LEGUMINOSAE</strong></td>
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<tr>
<td>Phaseolus vulgaris</td>
<td>Southern bean mosaic</td>
<td>Cheo (1955)</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td>Bean virus 1</td>
<td>Crispin et al. (1961)</td>
</tr>
<tr>
<td>Vicia faba</td>
<td>TNV</td>
<td>Stevens (1970)</td>
</tr>
<tr>
<td>/Contd…..</td>
<td></td>
<td></td>
</tr>
<tr>
<td>FAMILY</td>
<td>PLANT</td>
<td>VIRUS</td>
</tr>
<tr>
<td>------------------------</td>
<td>-----------------</td>
<td>-------------</td>
</tr>
<tr>
<td>LUPINOSAE (contd.)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phaseolus vulgaris</td>
<td></td>
<td>TNV</td>
</tr>
<tr>
<td>&quot;The Prince&quot;</td>
<td></td>
<td>TNV</td>
</tr>
<tr>
<td>P. aureus</td>
<td></td>
<td></td>
</tr>
<tr>
<td>LOGANIACEAE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strychnos nux-vosica</td>
<td>IVX</td>
<td></td>
</tr>
<tr>
<td>HY Marionaceae</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Syzgium cucini</td>
<td>Cucumis virus I</td>
<td></td>
</tr>
<tr>
<td>Callistemon citrinus</td>
<td>Cucumis virus I</td>
<td></td>
</tr>
<tr>
<td>PALMACEAE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ilexis spp.</td>
<td>TNV</td>
<td></td>
</tr>
<tr>
<td>PAPAVINACEAE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Popaver orientale</td>
<td>TNV</td>
<td></td>
</tr>
<tr>
<td>SOLANACEAE</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nicotiana spp.</td>
<td>TNV</td>
<td></td>
</tr>
<tr>
<td>N. spp.</td>
<td>TNV</td>
<td></td>
</tr>
<tr>
<td>N. glaminosa</td>
<td>TNV</td>
<td></td>
</tr>
<tr>
<td>Lycopersicon esculentum</td>
<td>TNV</td>
<td></td>
</tr>
</tbody>
</table>
Szirmai (1963) tested the inhibitory effect of beet "seed" pericarp on a number of viruses. It inhibited TNV by 90.6%, TNV by 86.8%, and beet mosaic virus by 64%. The inhibitory effect was also found when the dust of the pericarp, removed by mechanical treating of the beet clusters, was examined. The pericarp of the abraded seeds were found also to contain inhibitor substance but in lower quantities.

Stevens (1970) studied the effect of seed extracts of Beta vulgaris L. (Beet), Beta vulgaris var. Rapa, Dusort (Sugar-beet) and Chenopodium amaranticolor Coste and Reyn, on the local lesion production by TNV. Inhibition was most marked in the case of beet and sugar-beet seed extract, whereas C. amaranticolor gave 67% inhibition.

Stevens (1970) also prepared an extract from seeds of Lactuca sativa (lettuce), a member of the COMPOSITAE, and tested it against TNV. The extract yielded an activity quotient greater than one, suggesting enhancement of virus activity. Enhancement or augmentation of virus activity has not previously been described for seed extracts although such a phenomenon has been observed when plant saps were mixed with virus suspension (Benda, 1956; Blaszczyk, Frank Ross and Larson, 1959).

With plants of the CRUCIFERAE, Stevens (1970) showed that extracts from the seeds of Brassica napus L. caused complete inhibition of lesion formation by TNV. However, seed extracts from B. alba Rabenh. and Cheiranthus cheiri gave 74% inhibition. Erucastrum sativum L. seed extract contained a moderate inhibitor which resulted in only 20% inhibition.

Sill (1951) and Sill and Walker (1952) found that seeds from a member of the CUCURBITACEAE (Cucuris sativa) contained a stable inhibitor effective against cucumber mosaic virus when inoculated on
cowpea (*Vigna sinensis* Savi). Similarly, Crowley (1955) suggested the presence of a virus inhibitor in seeds of *C. sativa* (cucumber) and *Echinocystis lobata* (wild cucumber).

TMV inhibition by rice (*Oryza sativa*, a member of the *Gramineae*) was studied by Allen and Kahn (1957). They extracted an inhibitor from rice kernels. In 1960, Kahn, Allen and Zaumeyer tested the effect of grain extracts from 12 rice varieties on the reaction of Pinto bean to infection by TMV. All the varieties tested were sources of inhibitors. Verma and Verma (1965) reported the presence of an inhibitor in wheat (*Triticum sativum*) seed extract. Inhibition was concluded when wheat seed extract was added to TMV inoculum and then assayed on *Nicotiana glutinosa* leaves.

Some studies have been made of possible inhibitors from seeds of the *Leguminosae*. The effect of extract from *P. vulgaris* seed on the infectivity of southern bean mosaic virus (SBMV) was investigated by Cheo (1953). He found that the virus to which the bean seed extract was added produced 97.5 fewer local lesions on Kentucky Wonder bean leaves than did untreated virus of the same dilution. Extracts from mature or germinated seeds caused greater inhibition than extracts from immature seeds. Cheo postulated that the inhibition was due to a virus inhibitor that was formed as seed matured. This explained why, in serological tests, the virus titer for SBMV from embryo tissue decreased and seed transmission of the disease fell from 80 to 2% as the seeds approached maturity.

Tests were conducted for inhibitors to BV1 (bean virus 1) or BV2 (bean virus 2) in mature bean (*Phaseolus vulgaris*) seeds (Crispin and Grogan, 1961). Bean seeds from healthy plants were dissected to separate embryos from seed coats, and extracts from each were prepared. The extracts were mixed with BV1 and BV2, and were then inoculated on
leaves of Small White and Sutton Pink varieties. All inoculated plants became infected, whether or not the inoculum contained the extracts, indicating that inhibitors of BV1 or BV2 were not present. It seems unlikely, therefore, that the difference in seed transmissibility of the two viruses is due to inhibitors in the mature seeds, as was reported earlier by Cheo (1955). Stevens (1970), tested Phaseolus vulgaris L. var. "The Prince" and P. aureus, Kozb. on local lesion formation by TNV. These extracts yielded activity quotients greater than one, suggesting enhancement of virus activity.

Verma, Raychaudhuri and Khan (1970) studied the effect of medicinal plant extracts on the infectivity of PVX. They reported that seed extracts of Strychnos nux-vomica L. (Loganiaceae) gave 30 to 55% inhibition.

An attempt was made by Sharma and Chohan (1973) to study the inhibition of Cucumis virus I (WCMV) by seed extracts of various plants. Seed extracts of Syzygium cumini (Myrtaceae) showed complete inhibition of WCMV as indicated by the absence of any local lesion on leaves of Chenopodium amaranticolor. However, extract from seeds of Callistemon citrinus inhibited the infectivity of the virus by 63%.

Lucardie (1951) tested the effect of kernel extract from Illicia sp. (Illiciaceae) on local lesion formation by TNV. Inhibition of lesion number was marked on Nicotiana glutinosa plants.

Only one member of the Papaveraceae has been studied for virus inhibitors. Stevens (1970) found an inhibitive principle in seed extract of Papaver orientale L. It gave 40% inhibition when inoculated with TNV on Phaseolus vulgaris L. var. "The Prince".

In an attempt to explain the lack of seed transmission of TNV, Duggar (1939) suggested that the virus was inactivated by some "specific protein or other specific material" in the seeds.
Experimental evidence in support of this theory was published by Kausche (1940) who showed that the addition of aqueous extracts of tobacco (*Nicotiana tabacum*) seed to purified tobacco mosaic inoculum could reduce its infectivity by as much as 50%. Crowley (1955) also demonstrated the presence of a virus inhibitor in the seeds of tobacco. The addition of tobacco seed extract to TMV inoculum consistently resulted in a significant reduction in the number of lesions produced; but the extracts used by Crowley were four times as concentrated as those reported by Kausche to produce a similar effect. This is attributed to the fact that Kausche used a different variety of tobacco seed.

Stevens (1970), found that although seed extracts of *Nicotiana glutinosa* were not inhibitory to TMV, seed extracts of *Lycopersicon esculentum* Mill. gave 67% inhibition.

Table 2 summarizes the details given above for the 12 families of plants covering 23 species, where seeds have been investigated for anti-viral activity. Clearly, seeds do contain substances influencing the infection of plants by viruses. As pointed out earlier, such influences might come about by effects either on virus uptake or on virus multiplication. Inhibition could, for example, come about through compounds that act on cell surfaces, so interfering with virus uptake or in some way disrupt cell metabolism, so leading to lower yields of virus.

Legume seed extracts, for example, are known to have a wide range of biological activity in both animals and plants, including effects on cell surfaces and on cell metabolism (Naspitz and Richter, 1963).

The agglutination of blood by effects on erythrocytes surfaces was one of the first biological activities described for legume seed
extracts, and such extracts are commonly referred to as phyto-
hemagglutinin or phytoagglutinins. However, as cell-agglutinating
proteins also occur in organisms other than plants, the term
"lectins" proposed by Boyd (1970), appears to be more suitable.
Prominent examples of lectins are concanavalin A (Con A) from
jack bean, soybean agglutinin from Glycine max and phytohemagglutinin
(PHA) from Phaseolus vulgaris.

Some lectins are specific in their reaction with human blood
groups (ABO) and have therefore been used in blood typing and in
investigations of the chemical basis of blood group specificity
(Bird, 1959; Boyd, 1970; Watkins and Morgan, 1952). This
specificity is thought to arise as a result of the binding of
lectins to particular carbohydrate moieties on the erythrocyte
membrane. Further examination and purification of the crude lectins
(PHA) from P. vulgaris has shown the presence of glycoproteins which
agglutinate lymphocytes. Agglutination is brought about by the
attachment of the lectin to the lymphocyte surface (Hirschhorn,
Kolodny, Hashem and Bach, 1963). The interaction of lectins with
cells can, in many instances, be inhibited specifically by simple
sugars, polysaccharides and glycoprotein (Mekala, 1957; Goldstein,
Hollerman and Smith 1967). This inhibition is used to interpret
the nature of the binding sites.

Activity of another type was first demonstrated by Nowell (1960)
who showed that lectins are mitogenic in that they can stimulate the
transformation of lymphocytes from small "resting" cells into large
blast-like cells which may ultimately undergo mitotic division.
Cooper and Rubin (1965) demonstrated that, within 30 minutes of the
addition of PHA, for example, to lymphocyte cultures, there was a
rapid breakdown of existing cellular ribonucleic acid (RNA) followed
by the synthesis of increased amounts of new cellular RNA. This newly synthesized RNA was thought to regulate the transition of the lymphocyte from a resting to an active state.

Few studies have been made of the effect of lectins on plants. Nagl (1972) reported that HIA stimulated germination and early seedling growth in P. coccineus, but not in P. vulgaris, the species from which the compound is extracted. Nagl also found that root growth in Allium cepa was enhanced by HIA during the first 12 days of treatment, but only at temperatures below 20°C. Bangerth (1965) used HIA for the induction of fruit setting in a pollen-sterile mutant of tomato. In 1972, Bangerth, Gotz and Buchloh also found that HIA induced parthenocarpic fruit set in a male sterile mutant of tomato and also in Bartlett pear. Although it is not clear how HIA is bringing about these effects in plants, such results may reflect changes in nucleic acid metabolism induced by the lectin.

The lectin from Ricinus communis has been shown to have effect on plant organelle surfaces since Uhlenbruck and Ladunz (1972) found that ricin agglutinated chloroplasts and thylakoid fragments.

Lectins appear therefore to act in two ways: one through reactions involving combination with the membranes, and another by effects on metabolism, particularly RNA synthesis. It seems reasonable to suggest that if legume seed extracts influence the multiplications of plant viruses they might do so either by influencing virus uptake through a cell surface effect, and/or through biochemical effects mediated through changes in the synthesis of RNA.

Lectins derived from legume seeds are known to influence animal virus activity (Nicolson, 1974) although, as far as is known, no studies of the effects of such lectins on plant viruses has been
published. Few, if any, of the reports of virus inhibitors from seeds contain information regarding the identity of the compounds involved or the mode of action of such compounds.

In view of this paucity of information, legume seeds have been selected for study as a possible sources of plant virus inhibitors because

(a) they are large and likely to yield detectible quantities of inhibitor compounds capable of identification and analysis;

(b) some information is already available suggesting that they may influence virus infection;

(c) they are known, as outlined above, to contain compounds (lectins) with considerable biological activity.

The work described in this thesis is designed to:

(1) Examine a selection of legume seeds for inhibition activity against plant viruses.

(2) Establish some details of the properties of the compounds responsible for inhibition, using selected examples.

(3) Study the nature of the inhibitor fractions with a view to establishing their chemical identity and possible relationships with lectins.

(4) Gain some idea of the possible mode of action of compounds from seeds in bringing about effects on virus infection of plants.
CHAPTER II
MATERIALS AND METHODS

1. GROWTH OF ASSAY PLANTS

Greenhouse Conditions

All test plants were grown in the greenhouse under natural light supplemented with illumination from mercury vapour lamps to give 16 light hours in every day. The temperature was maintained between 20-25°C during the winter months; however, in summer it was difficult to keep the temperature at that range. High temperature was counteracted by the use of a fan, and by watering the plants twice daily.

Growth of Phaseolus vulgaris L. var. "The Prince"

Seeds of P. vulgaris var. "The Prince" were sown in 12 cm plastic pots containing John Innes compost No.2 (JI2). Five plants were grown in each pot. Only obviously healthy plants of uniform appearance were used.

Growth of Nicotiana tabacum var. "Xanthi"

Seeds of N. tabacum var. "Xanthi" were initially sown in "Jiffy pots" containing compost (JI2). After about three weeks individual healthy seedlings were transferred to 6 cm plastic pots containing JI2.

Growth of Convolvulus globosa

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

2. PREPARATION AND INFECTIVITY ASSAY OF PLANT VIRUSES

Tobacco Necrosis Virus

Deep frozen leaves of French bean infected with tobacco
necrosis virus (TNV) strain D were used as the source of virus. These leaves were ground using a pestle and mortar with a little acid washed sand and 0.06 M phosphate buffer pH 7 or water (1:1 w/v). The bulk of the leaf debris and sand was removed by squeezing the pulp through four layers of muslin. The extract was centrifuged at 4,000 r.p.m. for 15 minutes, and the supernatant decanted and kept at room temperature overnight to precipitate any proteinaceous virus inhibitor present in the leaf sap (Bawden, 1954). Finally, the solution was cleared by spinning down at 4,000 r.p.m. for 5 minutes. Aliquots (15 ml) of the supernatant were stored in glass vials at -25°C in a deep-freezer. When inoculum was required, the contents of the vial were thawed and diluted with 0.06 M phosphate buffer pH 7 or water to obtain the concentration necessary to produce approximately 40-100 local lesions per leaf.

P. vulgaris var. "The Prince" was used as the local-lesion host to quantitatively measure the activity of TNV(D). Direct measurement was made by local lesion counts. The inherent variability among leaves may be reduced by pre-inoculation removal of the growing tip and all leaves except those to be inoculated (Youden and Beale, 1954).

For virus assay comparisons, one of the primary leaves of 12-13-days old French beans was inoculated with test solution and virus and the opposite leaf with control inoculum. The control inoculum was a mixture of TNV in water or buffer and distilled water. Carborundum was used as an abrasive and inoculation was made by dipping the forefinger in inoculum and rubbing once over the leaf surface. Leaves were not rinsed after inoculation because, as Table 3 shows, there was no difference between washed and unwashed leaves. The plants were placed in the greenhouse
until local lesions appeared four or five days later.

TABLE 3

EFFECT OF WASHING FRENCH BLAN LEAVES AFTER INOCULATION BY TNV

<table>
<thead>
<tr>
<th>Source of seed extract</th>
<th>Mean number of lesions *</th>
<th>Activity quotient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + water</td>
<td>TNV + seed extract</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(unwashed)</td>
<td>102</td>
<td>65.1</td>
</tr>
<tr>
<td>P. vulgaris</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(washed)</td>
<td>123</td>
<td>87.9</td>
</tr>
<tr>
<td>G. max</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(unwashed)</td>
<td>54.14</td>
<td>5.36</td>
</tr>
<tr>
<td>G. max</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(washed)</td>
<td>53.47</td>
<td>5.60</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.

The activity quotients were calculated for each treatment as described by Benda (1956).

Activity quotient: \[ \frac{\text{Number of lesions on treated leaves}}{\text{Number of lesions on control leaves}} \]

The relative percentage inhibition of the extract was estimated according to the following calculations (Smookler, 1971):

Percentage inhibition:

\[ \frac{100 - \frac{\text{number of lesions produced by inoculum containing inhibitor}}{\text{number of lesions produced by control inoculum}}}{100} \times 100 \]

The difference between control and test treatment was analysed statistically using the "t" test.

Details of the statistical method, together with worked examples, are given in the Appendix.
Tobacco Mosaic Virus

Tobacco mosaic virus (TMV) was maintained in P. vulgaris var. "The Prince". Three to four weeks after inoculation, systemically infected leaves showing marked symptoms were triturated in a mortar with 0.06 M phosphate buffer pH 7 (1:1 w/v). TMV was extracted and stored using the same procedures as for TNV. Infectivity was assayed on the younger five expanded leaves of 7-9-weeks old N. tabacum var. "Xanthi" plants. The older leaves and the growing points were removed before inoculation.

A latin square design was employed to randomise treatments among half-leaves of test plants. (see Appendix)

Potato Virus X

Potato virus X (PVX) was prepared from N. tubacum var. "Xanthi". It was extracted and stored using the same procedure as for TNV. Infectivity of PVX was measured by the local lesion assay method on the youngest four fully expanded leaves of G. glabra L. plants.

3. SEED Viability Assay

Germination Test

Seeds used in all experiments were estimated for viability using a test based on that of Bennett and Loosli (1949). The test solution contained a 0.05% 2,3,5 triphenyltetrazolium chloride. Ten seeds of each legume variety were soaked in water overnight and were then cut carefully with a sharp razor blade through the embryo axis and transferred to the test solution for two hours at 30°C. Viable seeds showed a deep red stain by the 2,3,5 triphenyltetrazolium chloride solution over the surface of the embryo. Non-viable seeds remained unstained.
Preparation of Crude Seed Extracts

Dry seeds of 18 varieties of legumes from local health stores were ground to a flour in a mechanical homogenizer. Seed extract was prepared by mixing 1 g of seed flour with 10 ml distilled water. The mixture was allowed to stand at room temperature for 10 minutes, after which it was centrifuged at 4,000 r.p.m. for 15 minutes. The supernatant was decanted and used as test solution.

Seed extracts of French bean (P. vulgaris var. "The Prince") and soybean (Glycine max var. "Herr") were examined in more detail. In order to achieve uniform samples, larger volumes of extracts were prepared from these seeds and were lyophilized (freeze dried).

Table 4 shows that freeze drying of the crude seed extract of P. vulgaris and G. max has no effect on their inhibitory activity.

TABLE 4

COMPARISON OF THE EFFECTS OF CRUDE AND LYOPHILIZED SEED EXTRACT ON LOCAL LESION PRODUCTIVITY BY TNV

<table>
<thead>
<tr>
<th>Source of seed extract</th>
<th>Mean number of lesions *</th>
<th>Activity quotient</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + H&lt;sub&gt;2&lt;/sub&gt;O</td>
<td>TNV + seed extract</td>
</tr>
<tr>
<td>P. vulgaris &lt;br&gt;(crude)</td>
<td>67</td>
<td>47.4</td>
</tr>
<tr>
<td>P. vulgaris &lt;br&gt;(lyophilized)</td>
<td>80.8</td>
<td>56.7</td>
</tr>
<tr>
<td>G. max &lt;br&gt;(crude)</td>
<td>36.73</td>
<td>2.64</td>
</tr>
<tr>
<td>G. max &lt;br&gt;(lyophilized)</td>
<td>65.3</td>
<td>- 4.5</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications
Preparation of lyophilized seed extract

Dry seeds of *P. vulgaris* and *G. max* were each ground to a flour using a mechanical homogenizer. 20 g of seed flour were mixed with 200 ml distilled water and stirred by magnetic stirrer for 30 minutes. The mixture was then squeezed through four layers of muslin, and the cell debris discarded. The extract was centrifuged at 4,000 r.p.m. for 15 minutes, and the supernatant lyophilized overnight. The yield of lyophilized material was weighed and the quantity required to make up a concentration identical to that of fresh seed extract was calculated. The extracts were stored at 4°C in screw-topped glass bottles.

4. ESTIMATION OF PROTEIN

The method of Lowry, Rosebrough, Farr and Randall (1951) was used to determine the protein concentration. The samples were read at 660 nm in a spectrophotometer. A calibration curve was obtained using Bovine serum albumin (Sigma Chemical Company, U.S.A.).

5. ESTIMATION OF CARBOHYDRATE

The method of Dubois, Gilles, Hamilton, Rebers and Smith (1956) was used. Carbohydrate concentration was read at 490 nm by reference to a standard curve prepared using glucose.

6. DISC ELECTROPHORESIS

Polyacrylamide gel electrophoresis has proved to be an excellent tool for the separation of proteins and glycoproteins from both plant and animal sources.

Polyacrylamide gel electrophoresis was performed according
to the method of Ornstein and Davis (1964) utilizing tris-glycine buffer pH 8.3 and an acrylamide concentration of 7%. The samples were carefully layered on to the tops of acrylamide gels made in siliconized glass tubes. A trace of bromophenol blue dye was added as a marker. A constant current of 5 mA was applied for each tube until the sample had completely entered the upper part of the gel. The current was then reduced to 2 mA. Following the run the gels were removed from the siliconized glass tubes by rinsing and pulling with the aid of stainless steel needles. Gels were then immersed in the appropriate staining solution.

Proteins were stained by immersing the gel for at least four hours in 1% Coomassie Brilliant Blue R in 12.5% trichloroacetic acid. This was followed by destaining in 10% trichloroacetic acid.

Protein-bound carbohydrates were located with Periodic Acid-Schiff (PAS) reagent. Gels were first oxidized by immersion for an hour in 1% periodic acid dissolved in 3% acetic acid and were then thoroughly leached with water for one hour. Upon subsequent immersion in Schiff's reagent, sharp red bands developed within an hour, indicating the position of glycoproteins. Finished gels were stored in 1% sodium metabisulfite (Zacharius, Zell, Morrison and Woodlock, 1969). The relative mobility of protein and glycoprotein bands were expressed where appropriate as E values which were determined from the position of each band with reference to the bromophenol blue marker.

Details of special techniques, for example hemagglutination assay, trypsin-inhibiting activity assay, and crude agglutinin extraction methods, are given in their appropriate section.
CHAPTER III

EFFECT OF VALENCE LEGUMES AND EXTRACTS ON THE INFECTION OF PHASEOLUS VULGARIS BY TNN

Initial experiments were undertaken in which fresh seed extracts from 15 varieties of legume were investigated for virus inhibitor activity. In these experiments 1 ml of extract, prepared as described earlier, was mixed with an equal volume of TNN solution and inoculated onto P. vulgaris. Control leaves were inoculated with 1 ml of water and an equal volume of TNN solution.

The effect of fresh seed extracts on TNN are presented in terms of local lesion numbers, activity quotient, and percentage inhibition in Tables 5, 6 and 7. The seeds are listed according to their effectiveness in inhibiting TNN infection.

The results show that the inhibitory activity of legume seeds appeared to fall into two categories:

(i) Seed extracts in which the percentage inhibition is between 75-95

(ii) Seed extracts in which the percentage inhibition is between 0-60. *Mungus vulgaris var. "The Prince" seed extract varied in inhibition between 0-70%.

The effect of heat on the inhibitory activity of each of the seed extracts was also studied. Each seed extract was heated on a water bath at 100°C for 10 minutes and cooled to room temperature before testing. One ml of heated seed extract was mixed with equal volume of TNN solution and inoculated onto P. vulgaris leaves. Control consisted of 1 ml of water and an equal amount of TNN solution.

The results are reported in Tables 8, 9 and 10 in terms of local
lesion numbers, activity quotient, and percentage inhibition. The results again fall into two main groups:

(i) Those for extracts in which percentage inhibition is decreased by heating. This group includes most seed extracts tested, among them *Glycine max* var. "Herr" (soybean).

(ii) Those seed extracts in which the percentage inhibition is increased by heating. This group includes *Phaseolus vulgaris* (French bean), *P. limensis* (Butter bean), *P. coccineus* (Runner bean), and *P. vulgaris* (Caricot bean).

All the seed extracts tested were inhibitory against TNV; however, because of limited time a detailed study was made only on two seed extracts, *P. vulgaris* var. "The Prince" and *G. max* var. "Herr".

*P. vulgaris* var. "The Prince" seed extract showed slight inhibition which can be increased on heating, and in contrast *G. max* seed extract showed strong inhibitory activity which is reduced on heating. These differences may be due to different activities of inhibitor compounds, or *P. vulgaris* may contain in addition augmenters which counteract the effects of inhibitors (Stevens, 1970).

Both of these legumes are also known to contain lectins with cell surface activity, and there is a possibility that they might act on plant cell surfaces, so influencing the initial events in the uptake and multiplication of viruses. The sections of this thesis that follow, describe attempts to identify the properties, chemical nature and, to some extent, the mode of action of inhibitors from these two legume species.
A table showing the effect of various legume seed extracts on the injection of sunflower aphids by TMY. The table includes columns for source of extract, the number of tests, and percentage inhibition (treated and control) with respective control numbers and seed extracts. The table also includes a column for mean yellow testa and other legume testa.
of ten separate plants

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

<table>
<thead>
<tr>
<th>Source of Extract</th>
<th>Activity (control)</th>
<th>Activity (seed extract)</th>
<th>Activity (seed extract + water)</th>
<th>Activity (seed extract + inoculum)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phacelia tanacetifolia</td>
<td>0.15</td>
<td>0.30</td>
<td>0.45</td>
<td>0.30</td>
</tr>
<tr>
<td>Acanthospermum</td>
<td>0.10</td>
<td>0.20</td>
<td>0.25</td>
<td>0.20</td>
</tr>
<tr>
<td>Eucalyptus globulus</td>
<td>0.12</td>
<td>0.24</td>
<td>0.28</td>
<td>0.24</td>
</tr>
<tr>
<td>Phacelia tanacetifolia</td>
<td>0.17</td>
<td>0.35</td>
<td>0.40</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Mean number of lesions

Effect of various extracts on the inhibition of Phacelia tanacetifolia by TMY
<table>
<thead>
<tr>
<th>Source of Extract</th>
<th>Activity Content (4°/0)</th>
<th>Percentile Implantation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control + Water</td>
<td>32°.9</td>
<td>30.0</td>
</tr>
<tr>
<td>Control + Seed Extract</td>
<td>32°.5</td>
<td>31.2</td>
</tr>
<tr>
<td>Treated + Seed Extract</td>
<td>32°.8</td>
<td>32.2</td>
</tr>
<tr>
<td>Treated + Water</td>
<td>32°.9</td>
<td>31.0</td>
</tr>
<tr>
<td>Treated + Seed Extract</td>
<td>32°.8</td>
<td>32.0</td>
</tr>
<tr>
<td>Treated + Water</td>
<td>32°.9</td>
<td>31.0</td>
</tr>
</tbody>
</table>

Each figure represents the mean number of lesions for ten replications on single leaves.

TABLE 1

Effect of various extracts seed extract on the injection of *Thecosoma Vulgare* by TVA

of ten separate plants
<table>
<thead>
<tr>
<th></th>
<th>Activity Inhibition (trans)</th>
<th>Activity Inhibition (control)</th>
<th>Variety</th>
<th>Source of Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>61</td>
<td>0.39</td>
<td>6.6</td>
<td>17.1</td>
<td>White raica (var. arundinacea)</td>
</tr>
<tr>
<td>69</td>
<td>0.35</td>
<td>7.7</td>
<td>43.7</td>
<td>Insecticide focus</td>
</tr>
<tr>
<td>68</td>
<td>0.34</td>
<td>6.9</td>
<td>43.8</td>
<td>Insecticide focus</td>
</tr>
<tr>
<td>69</td>
<td>0.35</td>
<td>6.1</td>
<td>43.2</td>
<td>Insecticide focus</td>
</tr>
<tr>
<td>70</td>
<td>0.24</td>
<td>6.6</td>
<td>27.9</td>
<td>Chlorine max (sorbom)</td>
</tr>
<tr>
<td>76</td>
<td>0.33</td>
<td>14.7</td>
<td>63.9</td>
<td>Chlorine max (sorbom)</td>
</tr>
</tbody>
</table>

The data represent the mean number of lesions for ten replications on single leaves.

**Table:**

<table>
<thead>
<tr>
<th>Variety</th>
<th>Source of Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td>White raica (var. arundinacea)</td>
<td>Insecticide focus</td>
</tr>
<tr>
<td>Insecticide focus</td>
<td>Insecticide focus</td>
</tr>
<tr>
<td>Chlorine max (sorbom)</td>
<td>Chlorine max (sorbom)</td>
</tr>
</tbody>
</table>

**Legend:**

- **Activity Inhibition (trans):** The activity inhibition of the trans extract.
- **Activity Inhibition (control):** The activity inhibition of the control extract.
- **Variety:** The variety of the plant used.
- **Source of Extract:** The source of the extract used in the experiment.
<table>
<thead>
<tr>
<th>Source of extract</th>
<th>Activity factor (treated)</th>
<th>Activity factor (control)</th>
<th>Mean number of lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>(Treated)</td>
<td>0.10</td>
<td>0.10</td>
<td>10.0</td>
</tr>
<tr>
<td>(Control)</td>
<td>0.10</td>
<td>0.10</td>
<td>20.0</td>
</tr>
</tbody>
</table>

**Table 9**

Effect of selected seed extracts from various varieties of rice on the incidence of yellow leaf (TNY).
<table>
<thead>
<tr>
<th>Plantattitude</th>
<th>7.4</th>
<th>6.6</th>
<th>3.8</th>
<th>2.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. verticillata</td>
<td>0.79</td>
<td>0.62</td>
<td>0.49</td>
<td>0.35</td>
</tr>
<tr>
<td>C. ericifolia</td>
<td>0.69</td>
<td>0.57</td>
<td>0.46</td>
<td>0.33</td>
</tr>
</tbody>
</table>

### Table 10

Effect of hexad seed extracts from various varieties of leaves on the Infection of

- **Source of Infection**
  - (Extracted)  
  - (Control)  
  - 100% water  
  - 100% seed extract

<table>
<thead>
<tr>
<th>Variety</th>
<th>Activity Inhibition</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>C. verticillata</td>
<td>0.79</td>
<td>0.69</td>
</tr>
<tr>
<td>C. ericifolia</td>
<td>0.69</td>
<td>0.69</td>
</tr>
</tbody>
</table>
CHAPTER IV

PROPERTIES OF VIRUS INHIBITOR EXTRACTS PREPARED FROM GLYCINE MAX AND PHASEOLUS VULGARIS SEEDS

Studies were made of the effects of dilution, pH, dialysis, heat, alcohol precipitation and ammonium sulphate precipitation to establish some idea of the properties of the inhibitor compounds in seeds of P. vulgaris and G. max extracts with a view to the eventual identification of the active agents and their mode of action. For convenience the results for each seed species is described and discussed in two separate sections, A and B.

SECTION A

PROPERTIES OF VIRUS INHIBITOR EXTRACTS PREPARED FROM GLYCINE MAX VAR. "HER" SEEDS

(a) Effect of dilution

G. max var. "Her" (soybean, yellow testa) seed extract was diluted with distilled water in 10-fold dilution series.

Results in Table 11 show that inhibition end-point was about $10^{-5}$. This leads to the conclusion that G. max seed extracts act as inhibitors and not by inactivation of the virus. Therefore, experiments were undertaken in order to gain more information about the properties of G. max virus inhibitor.

(b) Effect of pH

Seed extracts, it might be argued, may influence local lesion number by changing the pH of the inoculum; similarly, the effect of seed extracts, in bringing about inhibition, might be pH sensitive.

In order to examine the possible effects of pH, 0.4 gram samples of lyophilized G. max seed extract were dissolved in 10 ml of
**TABLE 11**

Effect of various dilutions of *G. max* seed extracts on the susceptibility of *P. vulgaris* to infection by TNV

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + water</td>
<td>TNV + seed</td>
<td></td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>79.6</td>
<td>30.90</td>
<td>0.46</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>92.9</td>
<td>58.4</td>
<td>0.63</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>80.8</td>
<td>49.9</td>
<td>0.62</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>80.0</td>
<td>54.5</td>
<td>0.63</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>59.0</td>
<td>63.0</td>
<td>1.02</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications
Sorenson's phosphate buffer at pH 5.4, 6.0, 7.0 and 8.0 and inoculated together with TNV onto French bean leaves. Controls consisted of 1 ml of TNV solutions and 1 ml of corresponding phosphate buffer.

Results in Table 12 show that lesion numbers increased with increase in pH in the control experiments, although there was little change between pH 7.0 and 8.0. When *G. max* seed extract was mixed with phosphate buffer pH 5.4, lesion numbers were reduced by 37%. At higher pH values greater inhibition was obtained, although there was only a 10% difference between pH 6.0 (71%) and pH 8.0 (81%).

In previous experiments where 93% inhibition had been obtained, distilled water at pH 5.3 was used without any buffer. The final concentration of the mixture was measured as pH 5.6. The use of phosphate buffer at about the same pH has a marked effect since the percentage inhibition has been reduced to 37. This may be due to the so-called phosphate effect (Yarwood, 1952) in which phosphate increases the response of plants to viruses.

The effect of pH was studied further using McIlvaine's buffer which, although containing phosphate, extended the pH range. Sodium acid maleate buffer which eliminated any possible phosphate effect was also tested.

Results in Table 13 show that the McIlvaine's buffer in the control had no effect on the number of lesions. However, the number of lesions were decreased in the presence of *G. max* seed extract to a greater extent than with Sorenson's phosphate buffer, giving a higher percentage inhibition.

Results in Table 14 show that sodium acid maleate buffer over the pH range 5.2 to 6.8 had little effect in both control and treated leaves on the number of lesions produced. The percentage inhibition (92% - 94%) was again similar to that found in the absence of buffer.
TABLE 12

Effect of Sorenson's phosphate buffer on the inhibitory activity of *G. max* seed extract

<table>
<thead>
<tr>
<th>pH</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + buffer</td>
<td>TNV + buffered seed extract</td>
<td></td>
</tr>
<tr>
<td>5.4</td>
<td>37.6</td>
<td>23.5</td>
<td>0.63</td>
</tr>
<tr>
<td>6.0</td>
<td>50.4</td>
<td>14.6</td>
<td>0.29</td>
</tr>
<tr>
<td>7.0</td>
<td>60.8</td>
<td>12.9</td>
<td>0.21</td>
</tr>
<tr>
<td>8.0</td>
<td>61.8</td>
<td>11.9</td>
<td>0.19</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications
### Table 13

Effect of McIlvaine-buffer on the inhibitory activity of *G. max* seed extract

<table>
<thead>
<tr>
<th>pH</th>
<th>Mean number of lesions *</th>
<th>Activity quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + buffer</td>
<td>TNV + buffered seed extract</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>46.5</td>
<td>2.1</td>
<td>0.05</td>
</tr>
<tr>
<td>4.0</td>
<td>42.9</td>
<td>7.5</td>
<td>0.17</td>
</tr>
<tr>
<td>5.0</td>
<td>43.3</td>
<td>3.6</td>
<td>0.03</td>
</tr>
<tr>
<td>6.0</td>
<td>47.9</td>
<td>2.8</td>
<td>0.06</td>
</tr>
<tr>
<td>7.0</td>
<td>43.5</td>
<td>3.2</td>
<td>0.07</td>
</tr>
<tr>
<td>8.0</td>
<td>45.5</td>
<td>3.7</td>
<td>0.03</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.
Effect of sodium acid maleate buffer on the inhibitory activity of *G. max* seed extract

<table>
<thead>
<tr>
<th>pH</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + buffer</td>
<td>TNV + buffered seed extract</td>
<td></td>
</tr>
<tr>
<td>4.2</td>
<td>83.7</td>
<td>5.6</td>
<td>0.07</td>
</tr>
<tr>
<td>5.3</td>
<td>82.5</td>
<td>6.0</td>
<td>0.07</td>
</tr>
<tr>
<td>6.0</td>
<td>85.0</td>
<td>5.7</td>
<td>0.07</td>
</tr>
<tr>
<td>6.8</td>
<td>85.3</td>
<td>6.5</td>
<td>0.08</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications
These results substantiated the idea that buffers containing phosphate increase in some way the number of local lesions produced by viruses.

These experiments show that there is little advantage in using buffered solution, particularly as the usual phosphate buffers tended to minimise the inhibitory activity of seed extracts. Although there appears to be some advantage in preparing purified virus using buffer solution, the subsequent preparation of inoculum was performed without the use of buffers.

(c) Effect of dialysis

In order to gain some information regarding the types and sizes of molecules involved in inhibition, seed extracts of G. max were dialysed against water. In preliminary experiments 10 ml samples were dialysed against running water, and the dialysate (non-dialysable part) gave marked inhibition (94%) similar to that of whole seed extracts (Table 15). In subsequent experiments both the dialysate and the dialysable part (DP) were examined for effects against virus to determine whether there might be any small molecular weight compound lost from the seed extracts by dialysis. In order to achieve this, dialysis in 100 ml volumes of water were undertaken at room temperature. The volume of the DP was reduced to 10 ml by rotary evaporation at 40°C.

The effectiveness of both the DP and the dialysate of the seed extracts were examined against TNV. Results in Table 15 show that the inhibitory components of the extracts were retained in the "visking tubing" since it gave an activity quotient of 0.06, suggesting that the inhibitors are large molecular weight compounds unable to pass through the tube membrane. The dialysable part of the seed extract was not inhibitory and it showed a slight augmentation.

It was found from the protein determination (Table 16) that the
<table>
<thead>
<tr>
<th>Extract</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sap dialysed against running water</td>
<td>52.9</td>
<td>0.12</td>
<td>83</td>
</tr>
<tr>
<td>(non-dialysable part)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sap dialysed against 100 ml distilled water</td>
<td>62.5</td>
<td>0.96</td>
<td>94</td>
</tr>
<tr>
<td>(non-dialysable part)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sap dialysed against 100 ml distilled water</td>
<td>63.8</td>
<td>1.19</td>
<td>-19</td>
</tr>
<tr>
<td>(dialysable part)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications
TABLE 16

Protein and carbohydrate contents of the dialysable and the non-dialysable part of *Glycine max* seed extract

<table>
<thead>
<tr>
<th>Source of extract</th>
<th>mg protein/ml</th>
<th>mg carbohydrate/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-dialysable part</td>
<td>50</td>
<td>3.5</td>
</tr>
<tr>
<td>dialysable part</td>
<td>2</td>
<td>20.0</td>
</tr>
<tr>
<td>whole seed extract</td>
<td>75</td>
<td>25.5</td>
</tr>
</tbody>
</table>
dialysate of the seed extract contained 50 mg/ml protein, whereas the dialysable part (DP) contained only 2 mg/ml. On the other hand, using the Dubois carbohydrate test, the DP was found to contain a considerable amount of carbohydrates, 20 mg/ml, compared to 3.5 mg of carbohydrate in the dialysate.

Further analyses 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 DP, dialysate and, for comparative purposes, on the whole seed extract. Electrophoresis of whole seed extracts produced nine fairly discrete protein bands and three broad bands staining with PAS reagent. The dialysate gave an almost identical pattern of bands. From these staining patterns (Fig. 1) it can be concluded that at least some of the proteinaceous material in the dialysate 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 DP did not stain in the electrophoresis experiment although it gave positive protein and carbohydrate tests as indicated earlier. The compounds in the DP, 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. Therefore, an attempt was made to determine the constituents of the DP since it consists presumably of small molecules capable of passing through the dialysis tubing. The presence of amino acids and small carbohydrate molecules was tested using paper chromatography.

Amino acids were separated using a solvent composed of butanol-acetic acid-water (4:1:5 v/v). The amino acids were identified by comparison with known compounds after dipping the papers in 0.15
Fig. 1. Disc electrophoresis of the proteins and glycoproteins from G. max seed extracts

Whole seed extract

- proteins

+ glycoproteins

Non-dialysable part

- proteins

+ glycoproteins

Dialysable part

No bands detected of either proteins or glycoproteins

Position of marker

Shading indicates intensity of band staining
solution of ninhydrin in acetone, drying and heating in an oven at
100°C for five minutes. Results showed that the DP of G. max seed
eextract contained amino acids identified as aspartic acid, glutamic
acid, alanine, tyrosine and leucine.

Carbohydrates were separated by paper chromatography using a
solvent of ethyl acetate-acetic acid-formic acid-water (18:3:1:4 v/v).
Compounds were located by dipping the chromatograms in a solution
consisting of 5 ml analine, 6 g diphenylamine in 100 ml acetic acid,
100 ml acetone and 100 ml phosphoric acid (85%). The papers were
air-dried and then heated in an oven at 95°C for ten minutes.
Carbohydrates were identified by comparison with known standards.
The extracts were found to contain sucrose and galactose with trace
amounts of glucose and melibiose.

The presence in the DP of these sugars might explain the slight
augmentative effects of the DP since sucrose has been reported to
enhance virus activity (Kongsvick and Santilli, 1970). On the other
hand, amino acids have been reported to inhibit lesion production by

These dialysis experiments suggest that the inhibitory fraction
from G. max seeds is largely proteinaceous in nature. This result
is substantiated by results described previously in which it has been
shown that at least part of the inhibitory activity of the seed
extract is thermolabile; thus, heating extracts for ten minutes at
100°C reduced inhibition from 93% of control down to 60% (Table 9).

Bearing in mind reports that plant RNA (ribonucleic acid) may
act as virus inhibitors (Sela et al., 1966; Kimmis, 1969) the seed
extracts were examined for nucleic acids by ultraviolet spectrophotometry. Following clarification by centrifugation and by using a
Unicam SP 800 recording spectrophotometer, maximum absorptions were
obtained at 275 μμ and 231 μμ corresponding to proteins and glycoproteins (Smith, 1970). These results confirm the absence of the nucleic acids in the extracts and give further support to the idea that proteins are responsible for the inhibitor activity of the seed extracts.

To test further whether the inhibitor from G. max seed extract was proteinaceous in nature, the effects of alcohol and ammonium sulfate precipitates on the inhibitory activity were studied. Experiment on alcohol precipitate will be described first.

(d) Effect of alcohol

Absolute alcohol was mixed with 10 ml G. max seed extract, to give a final concentration of 80%. The precipitate formed was removed by centrifugation at 4,000 r.p.m. (3,000 g) for 15 minutes, and was dissolved in 10 ml distilled water. Alcohol was removed from the supernatant fluid by rotary evaporation at 40°C and the volume reduced to 10 ml. One ml of the dissolved precipitate or the supernatant fraction was mixed with an equal volume of TNV and inoculated onto P. vulgaris leaves. Control inoculum consisted of TNV and water.

The results in Table 17 show that the alcohol precipitate is inhibitory to virus infection reducing infection by 53%, although this inhibition is not as high as the 93% found with whole seed extracts. The supernatant was also inhibitory, but the percentage inhibition (36%) was less than that of the precipitated fraction. Those results support the contention that some of the inhibitory activity of G. max seed extracts resides in compounds probably proteinaceous in nature.

(e) Effect of ammonium sulphate

In further experiments to test the idea that proteins are
**TABLE 17**

**Effect of alcohol on the inhibitory activity of G. max seed extract**

<table>
<thead>
<tr>
<th>Extract</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + water</td>
<td>TNV + extract</td>
<td></td>
</tr>
<tr>
<td>supernatant</td>
<td>59.7</td>
<td>33.4</td>
<td>0.64</td>
</tr>
<tr>
<td>precipitate</td>
<td>51.6</td>
<td>24.0</td>
<td>0.47</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.
responsible for virus inhibition, extracts of *G. max* seeds were precipitated with \((\text{NH}_4)_2 \text{SO}_4\). Ten grams of *G. max* seed flour were dissolved in 100 ml distilled water. The mixture was stirred at room temperature for 30 minutes and then centrifuged for 15 minutes at 4,000 r.p.m. (3,000 g) to remove insoluble matter. The supernatant was decanted from the residue and brought to 10% saturation with solid ammonium sulphate added slowly with stirring. After standing for 15 minutes at room temperature the precipitate was removed by centrifugation at 3,000 g for 15 minutes and the supernatant retained. The pelleted precipitate was dissolved in 100 ml distilled water. Using the same procedure, the supernatant fluids were in turn brought to 20, 30, 40, 50, and 60% saturation with solid ammonium sulphate. The resuspended precipitates and the supernatant were each dialysed against three changes of one liter distilled water with stirring at 10°C for 48 hours to remove ammonium sulphate. The various dialysates and the final supernatant were then assayed for inhibitory activity against TNV, and for protein concentration.

Results in Table 13 show that precipitates 4, 5 and 6 obtained at 40, 50 and 60% ammonium sulphate saturation respectively, showed marked inhibitory activity against TNV infection, whereas precipitates 1, 2 and 3 were less effective. The protein precipitated giving most inhibitory activity (54%) was obtained at 40% saturation. It also contained the maximum protein concentration (6 mg/ml). Precipitate 3 (30% saturation ammonium sulphate) showed no inhibition, although it contained more protein (1.95 mg/ml) than precipitate 5 which gave 38% inhibition. The protein-free supernatant showed no significant effect on local lesion production.

Polyacrylamide electrophoresis experiments were carried out on the ammonium sulphate fractions with a view to establishing whether
TABLE 13 Effect of ammonium sulphate fractions from G. max seed extract on the susceptibility of
P. vulgaris to TNV

<table>
<thead>
<tr>
<th>Sample</th>
<th>Saturation ammonium sulphate (%)</th>
<th>Mean number of lesions</th>
<th>Activity cytokine</th>
<th>Percentage Inhibition</th>
<th>mg protein/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TNV + water</td>
<td>TNV + sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitate 1</td>
<td>10</td>
<td>25.0</td>
<td>29.7</td>
<td>0.83</td>
<td>17</td>
</tr>
<tr>
<td>Precipitate 2</td>
<td>20</td>
<td>19.9</td>
<td>10.7</td>
<td>0.84</td>
<td>16</td>
</tr>
<tr>
<td>Precipitate 3</td>
<td>30</td>
<td>15.2</td>
<td>16.7</td>
<td>1.13</td>
<td>-13</td>
</tr>
<tr>
<td>Precipitate 4</td>
<td>40</td>
<td>16.6</td>
<td>7.6</td>
<td>0.66</td>
<td>54</td>
</tr>
<tr>
<td>Precipitate 5</td>
<td>50</td>
<td>14.6</td>
<td>9.1</td>
<td>1.02</td>
<td>38</td>
</tr>
<tr>
<td>Precipitate 6</td>
<td>60</td>
<td>15.9</td>
<td>15.3</td>
<td>5.03</td>
<td>35</td>
</tr>
<tr>
<td>Supernatant</td>
<td>70</td>
<td>10.6</td>
<td>12.6</td>
<td>1.13</td>
<td>-13</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications
the inhibitor consisted of more than one protein fraction and to see
if, like lectins, a glycoprotein might be found. The results shown
in Figs. 2 and 3 show that precipitate 4, the most active in terms
of virus inhibition, can be resolved into nine distinct proteins.
These bands have been numbered 1 to 9. Protein band 1 consisted of
material at the surface of the gel, whilst band 9 consisted of the
most mobile material having moved over half-way down the gel. Bands
2, 3 and 4 each stained with PAS reagent, showing that they contained
glycoprotein. Precipitate 5, which also showed inhibitor activity
(33%), contained two weak protein bands and only one glycoprotein band.
Precipitate 6 with inhibitor activity similar to that of precipitate 5,
contained three bands of protein and two glycoprotein bands.

Precipitates 2 and 3 which were not very effective inhibitors,
contained a variety of proteins and gave only weak glycoprotein
staining. These glycoproteins appear, from their position in the
gels, to be quite distinct from those found in the inhibitory fractions.
Both the 10% sample and the supernatant which had no inhibitory
activity, were free of proteins and glycoproteins.

DISCUSSION

As pointed out in the introduction, little work, if any, has been
done on the properties of virus inhibitors extracted from seeds
(Stevens, 1970; Crowley, 1955; Verma and Verma, 1965). None of
them tested G. max seed extract against virus infection. However,
some work on G. max leaf extract has been described by Simons,
Swidler and Moss (1965). The leaf extract was inhibitory as it
gave 55% inhibition when tested against TNV infection.

In this investigation G. max seed extract was tested against TNV
infection, and it showed 89-93½% inhibition. The percentage inhibition
Fig. 2. Disc electrophoresis of (NH₄)₂SO₄ fractions from G. max

Proteins (No bands were detected in precipitate 1 and the supernatant)

\[ \text{Precipitate} \quad | \quad \text{Position of marker} \]

\[ \text{Saturation (%)} \quad 20 \quad 30 \quad 40 \quad 50 \quad 60 \]

Shading indicates intensity of band staining
Fig. 3. Disc electrophoresis of \((NH_4)_2SO_4\) fractions from G. max

Glycoproteins (No bands were detected in precipitate 1 and the supernatant)

<table>
<thead>
<tr>
<th>Precipitate</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturation</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
</tbody>
</table>

← Position of marker  
{ Shading indicates intensity of band staining }
was decreased on dilution, showing that the seed extract contained an inhibitor and not an inactivator (Dawden, 1954).

pH seemed to have little influence on this inhibitory activity. This agrees with the results found by Yoshizaki and Murayama (1966) who found that the inhibitory agent contained in Chenopodium album was stable in acid solutions between pH 3.0 and pH 10.0, giving consistent degrees of inhibition over this pH range. Similar conclusions were reported by Gupta and Raychaudhuri (1971) who showed that the inhibitory property of Acacia arabica leaf extract was not affected over the pH range of 4.0 - 10.0.

The inhibitory principle of G. max seed extract was partially heat labile when it was boiled for ten minutes. Similar results were obtained by Blaszczak et al. (1959) when they tested the inhibitory activity of heated juices of Conopodium frutescens and Chenopodium ambrosioides. Franck (1964) also found that boiling partially destroyed the inhibition produced by Nicotiana glutinosa leaf extracts. On the other hand, some juices retain their inhibitory activity after boiling such as Pelargonium fortunum (Blaszczak et al. 1959), and Spinacia oleracea (Kunz and Walker, 1947). Other plant juices such as Chenopodium album lose their inhibitory activity when boiled (Smookler, 1971). Such loss of activity is generally interpreted as indicating the proteinaceous nature of the compounds involved.

Dialysis had no effect on the inhibitors present in G. max seed extract, indicating that they consist of high molecular weight compounds. This agreed with results of Crowley (1955) from experiments on cucumber and tobacco seed extracts. However, it contrasts with the results found by Verma and Verma (1965) who reported the presence of a dialysable inhibitor in wheat seed extracts. The dialysable part of G. max seed extract was shown to
contain small molecular weight sugars and amino acid compounds known, as pointed out earlier, to enhance and inhibit respectively the infection of plants by viruses. The enhancement of local lesion production when the dialysable part of the extract was mixed with TNV, might well result from the effects of sugars in the extracts. Detailed investigations of the effects of the dialysable fractions were not undertaken since this investigation was limited mainly to studies of virus inhibitors. Such inhibitor activity was found in the non-dialysable part of the extract, and particular attention was therefore paid to this fraction.

The inhibitory principle of the seed extract was precipitated by 80% alcohol and with 40-60% ammonium sulphate, concentrations known to precipitate protein. Other workers have used precipitation techniques to assess the proteinaceous nature of virus inhibitors. 50% ammonium sulphate concentration was found to precipitate a protein inhibitor fraction from juices of Chenopodium album (Yoshizaki and Narayama, 1966) and Nicotiana glutinosa (Palm, 1967). Furthermore, 50% ethanol was found by Smookler (1971) to precipitate the inhibitors extracted from leaves of Chenopodium ambrosioides, C. album, Atriplex nitens, Amaranthus caudatus. However, Mckeen (1959) found that 95% ethanol was needed to precipitate a virus inhibitor from the juice of Convolvus frutescens.

Examination of the ultraviolet absorption characteristics of the extracts confirmed that compounds such as RNA were not the inhibitor. In those studies where RNA has been implicated as an inhibitor, Sela and Applebaum (1962) and Kimmins (1969) worked on juices extracted from virus-infected plants with a view to testing immunity in areas of systemic-induced resistance.

Preliminary examinations of the whole seed extract, the non-
dialysable part and the ammonium sulphate precipitates by gel electrophoresis suggest that the inhibitory activity is due to a complex of proteins, some of which appear to be glycoproteins. Glycoproteins have been found to be responsible for the inhibitory activity of extracts from *Phytolacca esculenta* (Kassanis and Kleczkowski, 1964). Such glycoproteins were heat labile and precipitated by alcohol and were found to contain 8-12% carbohydrate.

In more recent studies, Gupta (1964) has confirmed the virus inhibitor activity of glycoproteins, using *P. aconitifolia*.

Therefore, at this stage, evidence from a number of experiments, using a variety of techniques, indicate that the *G. max* inhibitor is proteinaceous in nature. Evidence from gel electrophoresis studies suggest that the inhibitor fractions contain glycoprotein. Studies, described in later sections, were undertaken to elucidate further details of the inhibitor from these seeds.

**SECTION III**

**PROPERTIES OF VIRUS INHIBITOR EXTRACTS PREPARED FROM**

**PHASCOLUS VULGARIS VAR. "THE PRINCE" SEEDS**

Studies were made of the effects of dilution, pH, dialysis, heat, alcohol precipitation and ammonium sulphate precipitation on *P. vulgaris* seed extract. The properties of the virus inhibitor were investigated in the same way as that of *G. max* seed extract, therefore only the results will be described in this section.

(a) **Effect of dilution**

*P. vulgaris* var. "The Prince" (French bean) seed extract was diluted with distilled water in 10-fold dilutions.

Results in Table 19 show that there was no significant difference in the number of lesions between the control and the
TABLE 19

Effect of various dilutions of *P. vulgaris* seed extract on the susceptibility of
*P. vulgaris* to infection by TNV

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + water</td>
<td>TNV + seed extract</td>
<td></td>
</tr>
<tr>
<td>Neat</td>
<td>85.36</td>
<td>91.0</td>
<td>1.07</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>72.2</td>
<td>77.3</td>
<td>1.07</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>103.8</td>
<td>96.7</td>
<td>0.93</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>91.0</td>
<td>87.1</td>
<td>0.96</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>87.8</td>
<td>83.6</td>
<td>0.95</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>103.2</td>
<td>72.0</td>
<td>0.70</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>86.0</td>
<td>90.0</td>
<td>1.05</td>
</tr>
</tbody>
</table>

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

Full statistical treatment for this table is given in the appendix.
weaker concentration of seed extracts up to $10^{-4}$ dilution, as $10^{-5}$
dilution gave 30% inhibition.

The results might be interpreted as showing that the extract
may contain more than one active component influencing virus
multiplication. In dilutions up to $10^{-4}$ the effects of inhibitors
are balanced or masked by effects of other compounds. Further
dilution of the extracts results in the inhibitor becoming more
obvious in its effect, because those compounds responsible for
masking have been diluted below their effective concentration.
Whether such masking compounds are augments as described previously
(Stevens, 1970) is not clear.

(b) Effect of pH

When P. vulgaris seed extract was dissolved in Sorensens phosphate
buffer at pH 5.4, 6.0, 7.0 and 8.0, there was an increase in lesion
numbers on the control leaves over the pH range 6.0 to 8.0 (Table 20).
However, pH 5.4 reduced the lesion number from a mean value of 35.8
per leaf compared to 53.9 at pH 8.0. On seed extract treated leaves,
the number of lesions did not change at pH 6.0, 7.0 or 8.0, giving
activity quotients between 0.91 and 0.93. At pH 5.4 the activity
quotient was greater than one (A.Q. = 1.35) suggesting enhancement
of virus activity. This enhancement is probably due to the phosphate
effect previously described for G. max seed extract.

No appreciable change was noticed in the activity quotients when
P. vulgaris seed extracts were dissolved in McIlvaines buffer at
pH 3.0, 5.0, 6.0, 7.0 and 8.0. The inhibitory activity was completely
lost at pH 4.0, giving activity quotient of 1.04 (Table 21).
Sodium acid maleate buffer also had no effect on the activity
quotient over the pH range 5.2 to 6.3 (Table 22).

These results are similar to those recorded for G. max seed
Effect of Sorensens phosphate buffer on the inhibitory activity of *P. vulgaris* seed extract

<table>
<thead>
<tr>
<th>pH</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + buffer</td>
<td>TNV + buffered seed extract</td>
<td></td>
</tr>
<tr>
<td>5.4</td>
<td>35.8</td>
<td>48.5</td>
<td>1.35</td>
</tr>
<tr>
<td>6.0</td>
<td>59.1</td>
<td>57.8</td>
<td>0.98</td>
</tr>
<tr>
<td>7.0</td>
<td>62.9</td>
<td>59.3</td>
<td>0.94</td>
</tr>
<tr>
<td>8.0</td>
<td>56.9</td>
<td>53.8</td>
<td>0.91</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications*
### TABLE 21

**Effect of Helvaines buffer on the inhibitory activity of *F. vulgaris* seed extract**

<table>
<thead>
<tr>
<th>pH</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + buffer</td>
<td>TNV + buffered seed extract</td>
<td></td>
</tr>
<tr>
<td>3.0</td>
<td>46.3</td>
<td>40.0</td>
<td>0.85</td>
</tr>
<tr>
<td>4.0</td>
<td>47.5</td>
<td>49.2</td>
<td>1.04</td>
</tr>
<tr>
<td>5.0</td>
<td>48.6</td>
<td>43.3</td>
<td>0.89</td>
</tr>
<tr>
<td>6.0</td>
<td>47.9</td>
<td>40.8</td>
<td>0.83</td>
</tr>
<tr>
<td>7.0</td>
<td>43.3</td>
<td>41.0</td>
<td>0.85</td>
</tr>
<tr>
<td>8.0</td>
<td>43.8</td>
<td>41.2</td>
<td>0.84</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.

Full statistical treatment for this table is given in the appendix.
<table>
<thead>
<tr>
<th>pH</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + buffer</td>
<td>TNV + buffered seed extract</td>
<td></td>
</tr>
<tr>
<td>5.2</td>
<td>87.5</td>
<td>84.8</td>
<td>0.97</td>
</tr>
<tr>
<td>5.8</td>
<td>84.3</td>
<td>85.3</td>
<td>1.02</td>
</tr>
<tr>
<td>6.0</td>
<td>92.4</td>
<td>92.1</td>
<td>1.00</td>
</tr>
<tr>
<td>6.8</td>
<td>92.0</td>
<td>92.0</td>
<td>1.02</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications
extracts in showing that pH has little effect on inhibitor activity.

(c) Effect of dialysis

Table 23 shows that dialysis of whole *P. vulgaris* seed extracts against running water had no significant effect on the ability of the extracts to influence local lesion production by TNV. Furthermore, when the dialysis was performed against 100 ml of distilled water, the dialysate gave an A.Q. of 0.94. The dialysable part (DP) was not inhibitory, giving an A.Q. of 1.15.

Disc electrophoresis experiments (Fig. 4) showed that whole *P. vulgaris* seed extract was similar to the dialysate consisting of seven protein bands and five bands of glycoprotein. The DP did not give any staining in the electrophoresis experiment, although it did give positive protein (1.5 mg/ml) and carbohydrate (12.75 mg/ml) tests (Table 24). DP was then studied in another method, using paper chromatography, for the identification of the proteins and the carbohydrates. Results were similar to those found for the DP of *G. max* seed extract. The carbohydrates were sucrose, galactose and traces of glucose and melibiose. Amino acids included aspartic acid, glutamic acid, alanine, tyrosine and leucine.

(d) Effect of heat

Although the unheated *P. vulgaris* seed extract gave an activity quotient (A.Q.) of 1.02, heating the seed extract reduced the A.Q. to 0.54 (Table 9). Therefore, heat revealed the presence of an inhibitor which is stable to boiling at 100°C for ten minutes. In contrast, it seems that the compounds making inhibitor activity are heat labile.

This experiment lends support to the idea forwarded earlier as a result of dilution experiments that *P. vulgaris* seed extracts contain compounds tending to "mask" the effects of inhibitors. Experiments described later support this idea further. The nature
<table>
<thead>
<tr>
<th>Extract</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sap dialysed against running water (non-dialysable part)</td>
<td>96.4</td>
<td>109.7</td>
<td>1.14</td>
</tr>
<tr>
<td>Sap dialysed against 100 ml distilled water (non-dialysable part)</td>
<td>92.7</td>
<td>87.1</td>
<td>0.94</td>
</tr>
<tr>
<td>Sap dialysed against 100 ml distilled water (dialysable part)</td>
<td>92.4</td>
<td>104.2</td>
<td>1.13</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications
Fig. 4. Disc electrophoresis of the proteins and glycoproteins from P. vulgaris seed extracts

Whole seed extract

Non-dialysable part

Dialysable part

No bands detected of either proteins or glycoproteins

{← Position of marker
  
  Shading indicates intensity of band staining}
### TABLE 24

Protein and carbohydrate contents of the dialysable and non-dialysable part of *P. vulgaris* seed extract

<table>
<thead>
<tr>
<th>Source of extract</th>
<th>mg protein/ml</th>
<th>mg carbohydrate/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>non-dialysable part</td>
<td>62.50</td>
<td>3.75</td>
</tr>
<tr>
<td>dialysable part</td>
<td>1.50</td>
<td>12.75</td>
</tr>
<tr>
<td>whole seed extract</td>
<td>65.50</td>
<td>17.00</td>
</tr>
</tbody>
</table>

### TABLE 25

Effect of alcohol on the inhibitory activity of *P. vulgaris* seed extract

<table>
<thead>
<tr>
<th>Extract</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + water</td>
<td>TNV + extract</td>
<td></td>
</tr>
<tr>
<td>supernatant</td>
<td>75.9</td>
<td>53.9</td>
<td>0.71</td>
</tr>
<tr>
<td>precipitate</td>
<td>85.0</td>
<td>112.6</td>
<td>1.33</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.
of the inhibitor will also be studied later; however, the probability that the inhibitor or the masking compound in *P. vulgaris* seed extract belongs to nucleic acids can be eliminated at this stage. Ultraviolet absorption of the extract was recorded and the maximum absorptions were obtained at 270 μm and 230 μm corresponding to protein and glycoprotein.

(e) **Effect of alcohol**

The precipitate produced when *P. vulgaris* seed extract was treated with 80% alcohol gave an activity quotient of 1.33. Statistical comparison of the number of lesions produced by TNV in the presence and absence of the precipitate material shows significant differences (*P = 0.02*), suggesting the presence of augmenters in the extract. The alcohol-free supernatant was inhibitory and it gave 29% inhibition (Table 23).

(f) **Effect of ammonium sulphate**

Using the same procedure as for *G. max*, it was found that precipitates 2*, 3*, 4*, 5* and 6* obtained at 20%, 30%, 40%, 50% and 60% ammonium sulphate saturation respectively, inhibited TNV (Table 26). The 10% (1*) precipitate and the supernatant had no significant effect on lesion production. The inhibitory precipitates were prepared in such a way that their concentration was equivalent to that found in the untreated seed extracts. Thus, precipitate 4* produced at 40% (NH₄)₂ SO₄ saturation contained only 0.5 mg protein per ml but gave 35% inhibition, whereas precipitate 6*, which gave a similar degree of inhibition (39%), contained 6.75 mg of protein (Table 26).

Disc electrophoresis experiments (Figs. 5 and 6) showed that precipitate 2* contained four proteins with one intense band moving midway down the gel. Four glycoprotein bands were detected.
<table>
<thead>
<tr>
<th>Sample</th>
<th>Saturation ammonium sulphate (°)</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
<th>mg protein/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TNV + water</td>
<td>TNV + sample</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Precipitate 1°</td>
<td>10</td>
<td>95.9</td>
<td>67.0</td>
<td>0.91</td>
<td>9</td>
</tr>
<tr>
<td>Precipitate 2°</td>
<td>20</td>
<td>72.1</td>
<td>55.3</td>
<td>0.77</td>
<td>23</td>
</tr>
<tr>
<td>Precipitate 3°</td>
<td>30</td>
<td>91.7</td>
<td>56.5</td>
<td>0.62</td>
<td>38</td>
</tr>
<tr>
<td>Precipitate 4°</td>
<td>40</td>
<td>113.3</td>
<td>73.2</td>
<td>0.65</td>
<td>35</td>
</tr>
<tr>
<td>Precipitate 5°</td>
<td>50</td>
<td>110.6</td>
<td>71.8</td>
<td>0.65</td>
<td>35</td>
</tr>
<tr>
<td>Precipitate 6°</td>
<td>60</td>
<td>108.2</td>
<td>60.4</td>
<td>0.61</td>
<td>39</td>
</tr>
<tr>
<td>Supernatant</td>
<td>70</td>
<td>81.3</td>
<td>77.5</td>
<td>0.95</td>
<td>5</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.
Fig. 5. Disc electrophoresis of (NH₄)₂SO₄ fractions from P. vulgaris

Proteins (No bands were detected in the supernatant)

<table>
<thead>
<tr>
<th>Precipitate</th>
<th>1'</th>
<th>2'</th>
<th>3'</th>
<th>4'</th>
<th>5'</th>
<th>6'</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saturation (%)</td>
<td>10</td>
<td>20</td>
<td>30</td>
<td>40</td>
<td>50</td>
<td>60</td>
</tr>
</tbody>
</table>

- Position of marker
- Shading indicates intensity of band staining
Fig. 6. Disc electrophoresis of \((\text{NH}_4)_2\text{SO}_4\) fractions from \text{P. vulgaris}

\text{Glycoproteins (No bands were detected in the supernatant)}

\begin{align*}
\text{precipitate} & \quad 1' & 2' & 3' & 4' & 5' & 6' \\
\text{saturation (\%)} & 10 & 20 & 30 & 40 & 50 & 60 \\
\end{align*}

\{ \text{Position of marker} \}
\{ \text{Shading indicates intensity of band staining} \}
Precipitate 3 consisted of six protein bands with two intense bands moved behind the marker dye. Three distinctive bands and one faint band of glycoprotein were detected. Precipitate 4 contained four fairly discrete bands of protein and three bands of glycoprotein. Precipitate 5 contained five obvious bands of protein and two sharp bands of glycoprotein as well as one diffuse area of glycoprotein. Precipitate 6 contained five distinct bands of protein, the most mobile having moved only half-way through the gel (Rf 0.65). Seven bands of glycoprotein were also detected. No protein or glycoprotein bands were noticed in the inhibitory supernatant; however, two weak protein bands and one glycoprotein band were stained in precipitate 1'.

DISCUSSION

A number of workers have tested juices extracted from the aerial parts of P. vulgaris for effects against plant viruses. Thus, using aerial parts of P. vulgaris, Cheo (1955) found such extracted juices inhibitory against Southern bean mosaic virus, and Elaszczak et al. (1959) described the inhibition of PVX by similar extracts. In 1969 Singh showed that P. vulgaris juice inhibited watermelon mosaic virus. P. vulgaris juice was also inhibitory when it was inoculated together with TGN onto test plants (Nart, 1972; Taniguchi, 1974).

There appears to be general agreement that juices from the aerial parts of P. vulgaris are inhibitory to plant viruses. The situation regarding the effects of seed extracts from these plants is more confused. Cheo (1955), for example, reported that extracts from P. vulgaris seeds produced 95% inhibition when tested against Southern bean mosaic virus. However, Crispin and Grogan (1961) showed that similar seed extracts had no effect on bean mosaic virus 1 and yellow bean mosaic virus. More recently, Stevens (1970) examined the effect
of *P. vulgaris* seed extracts and found them to enhance the local
lesion production by TNV, giving activity quotients greater than one.
The different results obtained by Stevens, Cheo, Crispin and Grogan
might be due to the different viruses used in testing the seed
extracts.

The results described in this investigation showed that the
undiluted *P. vulgaris* seed extract gave an activity quotient of 1.02;
however, when it was diluted by 10^{-5} the extract behaved as an
inhibitor giving 30\% inhibition.

The active fractions of *P. vulgaris* seed extract remained in the
visking tubing after dialysis which suggests that they are high
molecular weight compounds. The dialysable part showed no significant
effect on local lesion production.

When *P. vulgaris* seed extract was treated with 80\% alcohol, the
presence of two active fractions was revealed. One of the fractions
was an augmenter precipitated by alcohol, while the other fraction was
an inhibitor which remained in the supernatant.

*P. vulgaris* seed extract inhibitor was not nucleic acid as shown
by examination of the ultraviolet absorption spectra. Thus the
inhibitor appears to be different from that described by Kiggins (1969)
and induced in *P. vulgaris* by inoculation with TNV.

The inhibitory agents on the other hand, were precipitated at
20-60\% ammonium sulphate saturation, concentrations known to precipitate
proteinaceous virus inhibitors (Yoshizaki and Kurayama, 1966).

One interesting feature of inhibition by these seed extracts is
their insensitivity to pH. Phosphate buffer at pH 6.0, 7.0 and 8.0
had no effect on inhibition by *P. vulgaris* seed extracts. When the
seed extract was dissolved in phosphate buffer pH 5.4, however, it
gave enhancement. Using buffers free of phosphate, this enhancement
appears to be due to the phosphate effect (Yarwood, 1952). The insensitivity of inhibitors to pH, reported in this work, is similar to that described by Singh and Gupta (1970) who found that the inhibitor from Patience gunatana bark was not affected over the range of pH 4.0 - 10.5.

The presence of inhibitors and compounds which mask in some way their activity was noticed when the seed extracts were heated at 100°C for ten minutes. The heated seed extracts gave 40% inhibition, although the unheated seed extract was not inhibitory. Therefore, it can be concluded that, in the unheated P. vulgcaris seed extract, some compounds neutralise or mask the effects of inhibitors. However, on heating, such masking compounds are destroyed or inactivated in some way. These observations suggest that the masking compounds are heat labile while the inhibitor is heat stable. Hart (1972) found however, that the inhibitory effect of P. vulgcaris leaf sap was destroyed by heating at 50°C for ten minutes. This result suggests that the inhibitor extracted from aerial parts is different in composition to that of seeds. Further evidence for the presence of masking compounds can be deduced from those experiments where extracts were diluted. Progressive dilution of seed extracts produced samples with powerful inhibitory properties suggesting that the compounds masking the effect of inhibitors are more susceptible to dilution than the inhibitors.

Few, if any, reports of compounds masking the effects of virus inhibitors from plants have been described previously. Compounds enhancing or augmenting virus multiplication have been reported by a number of workers. Thus, Elasyczak et al. (1959) suggested the presence of augmenters in the diluted juices of Nicotiana cobsyvi, N. glutinosa, Lycopersicon esculentum, Cucurbit vesica and Conchone fimbria. In addition, Simons et al. (1963) described augmenters in
leaf juices of *Kleinia cylindrica*, *Echinochloa echinocarpa*, *Aloe* sp.,
*Alnus crenata*, *Castorion* sp., *Evaorthia oblita*, and *Phumnum tenax*.

Stevens (1970) found that unheated seed extracts from *P. vuliaria*,
*Vicia faba*, *P. serius* enhanced local lesion production by TNV.

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 observations of Benda
(1956) who found that New Zealand spinach leaf extracts contained two
active fractions, a virus inhibitor and an augmenter.

In the experiments described in this thesis the high activity
quotient values quoted by Stevens (1970) were not observed. At the
same time some experiments lend support to the idea that certain
compounds in seed extracts enhance virus multiplication. It is not
clear, however, whether they act in this way in vivo and are
responsible for neutralising or masking the effects of the inhibitors.

In view of the doubtful nature of augmenters from *P. vuliaria*,
attention was concentrated on the inhibitor fractions. Evidence,
accumulated from experiments described in this section, support
strongly the idea that inhibition resides in proteinaceous material.
This is further supported by disc electrophoresis of whole seed
extracts, dialysate and ammonium sulphate fractions. Results showed
that each sample analysed contained more than one protein and also
glycoproteins. An attempt was made, therefore, to purify the
inhibitor so as to ascertain which protein or glycoprotein is
responsible for the inhibitory activity. These experiments are
described in a separate section.
CHAPTER V

NATURE OF THE VIRUS INHIBITORS

Following the studies of the properties of *G. max* and *P. vulgaris* seed extracts which have shown that virus inhibitor fractions from these seeds consist of a complex of proteins and glycoproteins, more experiments were undertaken to analyse these inhibitor extracts. Gel filtration and ion exchange chromatography procedures were used to identify more precisely which parts of the seed extracts contained inhibitory agents and consequently to gain information regarding their molecular weights and other properties. For the sake of simplicity, *G. max* and *P. vulgaris* results will be described in separate sections, A and B respectively.

SECTION A

NATURE OF THE VIRUS INHIBITORS EXTRACTED FROM GLYCINE MAX SEEDS

Much work has been done on *G. max* seed extracts. Interest has been concentrated on growth depression factors in soybean meal fed to animals (Stead, Nuelenaere, and Quicke, 1966) and also in the trypsin inhibiting property of the seed proteins. Considerable attention has also been paid to *G. max* hemagglutination activity. Little work has been published showing the effects of these extracts on viruses.

Meisel and Bocker (1933) were the first to publish investigations on the isolation and fractionation of soybean proteins. In 1898 this work was continued by Gobone and Campbell who used salt extraction and precipitation methods to separate and identify four different proteins from soybeans. Recent investigations of soybeans, using more refined techniques, have shown the seeds to contain a large number of different proteins with a variety of biochemical activities. Some of
these proteins have been characterised in detail; for example, Ku
and Scheraga (1962) isolated a proteinaceous trypsin inhibitor from
soybean extract and described it as having a molecular weight of
21,000. The glycoprotein responsible for the hemagglutinating
activity in soybean seed extracts was first isolated in purified
form and characterized by Liener and his co-workers (1952, 1958).
This glycoprotein was named as soybean agglutinin (SBi) (Lis, Sela,
Sachs and Sharon, 1970), and is reported to have a molecular weight
of 110,000. This latter glycoprotein is of some interest since it
can be shown to influence animal viruses. Recently, Poste, Alexander,
Reeve and Newlett (1974) found that SBA inhibited virus release from
primary chick embryo cells and baby hamster kidney cells which were
infected by Newcastle disease virus (NDV). This activity, like that
of other hemagglutinins presumably operates by effects on membrane
surfaces, so perhaps influencing the attachment of viruses.

Bearing in mind these reports of soybean seed extract activity
it seems clear that various protein fractions may promote widely
different functions. It seems likely, in view of the experiments
described earlier, that plant virus inhibition may be a further
property of soybean seed proteins. In order to discover the nature
of the plant virus inhibitors in G. max seed extracts, gel filtration
experiments were performed, so giving a more detailed idea not only
of the constituents of the extract, but also of the molecular weights
of the inhibitors. In further experiments, ion exchange chromatography
of the extracts were allowed even further analysis and this was
followed by experiments designed to test the fractions for trypsin
inhibition and also for hemagglutinating activity. Each fraction
was tested for its inhibitory activity against TTV. In this way the
plant virus inhibitors could be compared with protein fractions of
known biological activity from soybean seed extracts.

1. GEL FILTRATION OF GLYCINE MAX SEED EXTRACTS

(a) Column chromatography of G. max seed extract on Sephadex G-100

The Sephadex G-100 gel filtration medium was prepared and packed into a glass column according to method of Andrews (1964, 1965). The Sephadex G-100 was swollen by boiling for four hours in distilled water. The water and the small particles of the gel were removed by decantation and the gel was then mixed with 0.03 M phosphate buffer pH 7. After two hours the buffer was decanted and the gel suspension was deaerated under reduced pressure. The gel suspension was packed into a glass column 1.5 x 40 cm by pouring a small amount of the gel into the column which was already filled with phosphate buffer pH 7. Excess liquid was allowed to pass through the growing gel-bed and the gel was poured into the column until a bed-height of 35-40 cm had been reached. Phosphate buffer pH 7 was allowed to pass through the column at a flow rate of 12 ml per hour for two days at 10°C. A peristaltic pump was used to maintain a constant flow rate. The buffer reservoir, 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 one ml mixture of blue dextran, yellow dextran and Vitamin B₁₂. When not in use, the column was continuously eluted with phosphate pH 7.

Lyophilized G. max seed extract (0.06 gm) was dissolved in 2 ml of 0.03 M phosphate buffer pH 7. This solution was then layered on the column and 1 ml fractions were collected using a fraction collector.

For the purpose of determining the elution volumes, effluent fractions were collected immediately as the sample had entered the
column. Ultraviolet absorption measurements were made at 280 nm.

Elution profiles of the seed extract (Fig. 7) showed the presence of three peaks with elution volumes \( (V_e) \) of 17 ml (peak I), 35 ml (peak II), and 55 ml (peak III). Effluent fractions from each peak were assayed for inhibitory activity against TNV.

(b) **Effect of Sephadex G-100 column fractions from *G. max* seed extract on local lesion production by TNV**

One ml from each of peaks I, II and III was mixed with TNV and tested for inhibitory activity on French bean leaves. Control samples consisted of one ml of TNV plus one ml of phosphate buffer pH 7.

Results in Table 27 show that peaks I and II had marked inhibitory activity giving 55\% and 52\% inhibition of TNV respectively. Peak III, however, was not inhibitory and it gave an activity quotient (A.Q.) of 1.21, but with no significant difference between mean numbers of lesions in controls and treated samples.

Each peak was examined initially for protein, carbohydrate and then examined more precisely by disc electrophoresis.

(c) **Protein and carbohydrate estimation for the Sephadex G-100 fractions from *G. max* seed extract**

Peak I consisted of high concentrations of protein (2.03 mg/ml) and relatively small amounts of carbohydrate (0.04 mg/ml). Peak II contained lower protein (0.29 mg/ml) and carbohydrate (0.01 mg/ml) concentrations. Peak III showed the highest carbohydrate concentration (2.03 mg/ml); however, it contained only 1.13 mg/ml of protein (Table 28).

(d) **Disc electrophoresis of the Sephadex G-100 fractions from *G. max* seed extract**

0.2 ml of each of peaks I, II and III were layered on top gels prepared as described previously. Fig. 8 shows that peak I contained
Fig. 7  Sephadex G-100 column chromatography of G. max seed extract
### TABLE 27

**Effect of Sephadex G-100 column fractions from *G. max* seed extract on local lesion production by TNV**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + PO₄ buffer pH 7</td>
<td>TNV + Peak</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>101.8</td>
<td>45.8</td>
<td>0.43</td>
</tr>
<tr>
<td>II</td>
<td>103.0</td>
<td>49.3</td>
<td>0.43</td>
</tr>
<tr>
<td>III</td>
<td>102.3</td>
<td>124.0</td>
<td>1.21</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.

### TABLE 23

**Protein and carbohydrate estimation for the Sephadex G-100 fractions from *G. max* seed extract**

<table>
<thead>
<tr>
<th>Peak</th>
<th>mg protein/ml</th>
<th>mg carbohydrate/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>2.03</td>
<td>0.04</td>
</tr>
<tr>
<td>II</td>
<td>0.29</td>
<td>0.01</td>
</tr>
<tr>
<td>III</td>
<td>1.13</td>
<td>2.83</td>
</tr>
</tbody>
</table>
Fig. 8  Disc electrophoresis of the Sephadex G-100 fractions from *G. max* seed extracts

Peak I

Protein

Glycoprotein

Peak II

Protein

Glycoprotein

← position of marker

Shading indicates intensity of band staining
four bands of proteins and three bands of glycoproteins. Bands in peak I were located at the upper half of the gel with IEs of 0.04, whereas the protein and the glycoprotein bands of peak II were located in the middle and the lower part of the gel with IEs of between 0.46 and 0.73. Peak III did not give any protein or glycoprotein staining on the gels, although it was previously shown to give positive protein and carbohydrate reactions in the Lowry and Dubois test respectively. The explanation for this becomes clear in later experiments.

(e) Molecular weight determination of Sephadex G-100 fractions from G. max seed extract

It has been shown by Whitaker (1963) and Andrews (1964, 1965) that a correlation exists between elution volume on Sephadex G-100 and the molecular weight of globular protein. It was decided therefore to plot the Ve/Vo ratios of a number of pure globular proteins against their logarithmic molecular weight in order to calibrate the G-100 column and determine the molecular weights of the peaks material present in G. max seed extract.

The following proteins were used as standards: bovine serum albumin ($M_w = 67,000$), horse heart cytochrome C ($M_w = 12,400$), peroxidase ($M_w = 40,000$) and Y globulin ($M_w = 100,000$). Each protein was dissolved in 2 ml of phosphate buffer pH 7 and layered on the G-100 column. The standard proteins were run and eluted as previously. Details of the elution volumes of the standard proteins are given in Table 29.

The Ve/Vo readings were plotted against their logarithmic molecular weight (Fig. 9), and they were found to fit directly on a straight line.

The plotting of Ve/Vo ratios for the three peaks obtained from G. max seed extract shows that the molecular weights of the inhibitors
TABLE 29  
Ve/Vo ratios and molecular weights of standard compounds

<table>
<thead>
<tr>
<th>Standard compounds</th>
<th>Ye</th>
<th>Ve/Vo</th>
<th>logmolecular weight (log M)</th>
<th>molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>IgG</td>
<td>19</td>
<td>0.32</td>
<td>5.00</td>
<td>100,000</td>
</tr>
<tr>
<td>Bovine serum albumin</td>
<td>21</td>
<td>1.04</td>
<td>4.63</td>
<td>67,000</td>
</tr>
<tr>
<td>Peroxidase</td>
<td>23</td>
<td>1.17</td>
<td>4.60</td>
<td>49,000</td>
</tr>
<tr>
<td>Cytochrome C</td>
<td>37</td>
<td>1.00</td>
<td>4.09</td>
<td>12,400</td>
</tr>
<tr>
<td>Vit. B12</td>
<td>56</td>
<td>2.43</td>
<td>3.10</td>
<td>1.557</td>
</tr>
</tbody>
</table>
Fig. 9  Ve/Vo ratios and log molecular weights of standard compounds

![Graph showing Ve/Vo ratios and log molecular weights of standard compounds with points labeled Vitamin B₁₂, Cytochrome C, Peroxidase, Bovine serum albumin, and γ globulin. The graph has a linear trend line.]
ranged between 153,500 (peak I) and 17,760 (peak II). Peak III which was not inhibitory, had a molecular weight of about 1,583 (Table 30).

TABLE 30

<table>
<thead>
<tr>
<th>Peak</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>153,500</td>
</tr>
<tr>
<td>II</td>
<td>17,760</td>
</tr>
<tr>
<td>III</td>
<td>1,583</td>
</tr>
</tbody>
</table>

In previous sections it has been shown that the effects of heat, dialysis and alcohol indicate that *G. max* extracts consist of complex materials and that the inhibitor is proteinaceous in nature. In experiments where extracts were heated for example, the inhibitory activity was reduced but not eliminated. Similarly, dialysis of seed extracts showed that the inhibitor was non-dialysable.

In order to find out how these various treatments affected the extracts, treated samples were analysed by passing 2 ml of each through the G-100 column.

(f) *Sephadex G-100 column chromatography of G. max seed extracts following various treatments*

(i) **Heated seed extract**

Although boiling partially reduced the inhibitory activity of the extract, three peaks were obtained which are similar to the unheated seed extract (Fig. 10).

(ii) **Dialysed seed extracts**

The dialysate and the dialysable part of *G. max* seed extracts
Fig. 10  
Sephadex G-100 column chromatography of heated \textit{G. max} seed extract
were passed through Sephadex G-100. The dialysate which had previously been shown to retain all the inhibitory activity of the extract, consisted of peaks I and II, both of which showed inhibition of TNV. (Fig. 11). The dialysable part, which was not inhibitory, contained only peak III. (Fig. 12). The Sephadex column experiments showed that peaks I and II contain compounds with molecular weights about 153,500 and 17,700. In Chapter IV it has been shown that the dialysable part of G. max seed extracts did not stain in the electrophoresis experiments, and paper chromatography showed it to contain small molecular weight carbohydrates as well as amino acids. It seems likely therefore, that peak III consists of a number of small molecular weight compounds, none of which can be detected by gel electrophoresis. Furthermore, these compounds have little, if any, inhibitory effect on TNV.

(iii) Alcohol treated extracts

Alcohol treatment of the seed extract precipitated most of peak I, all peak II and part of peak III. (Fig. 13). The supernatant consisted partly of peak I and most of peak III. (Fig. 14). This result supports the idea that in these seeds the inhibitors are proteinaceous and found in peaks I and II, whilst the material in peak III is non-proteinaceous.

The evidence produced by using Sephadex columns has emphasized the proteinaceous nature of the inhibitor. It seems that inhibition is centred on materials with molecular weights in the region of 153,500 (peak I) and 17,700 (peak II). However, to establish more precisely the identity of the fractions responsible for inhibition more refined techniques of protein separation are required. For this purpose the seed extracts were further analysed, using ion exchange
Fig. 11  Sephadex G-100 column chromatography of the non-dialysable part of *G. max* seed extract

(For comparative purposes the elution profile of the dialysable part has been dotted in)
Fig. 12. Sephadex G-100 column chromatography of the dialysable part of G. max seed extract.
Fig. 15  Sephadex G-100 column chromatography of precipitate from alcohol treated G. max seed extract

(For comparative purposes the elution profile of the supernatant fraction has been dotted in)
Fig. 14
Sephadex G-100 column chromatography of supernatant from alcohol treated G. max seed extract

---

Transmission (290 mp)
chromatography carboxymethyl cellulose (CM-52) and diethylaminoethyl cellulose (DEAE-52).

2. THE ANALYSIS OF G. max SEED EXTRACTS BY ION EXCHANGE CELLULOSE

The range of Whatman advanced ion exchange cellulose has been developed specifically for the efficient separation of compounds such as proteins, enzymes and nucleic acids. A number of cellulosic ion exchangers are now available; however, the cation exchanger carboxymethyl cellulose CM-52 and the anion exchanger diethylaminoethyl cellulose DEAE-52 are the most widely used ion exchangers. (Peterson and Sober, 1962).

(a) Column chromatography using CM-52 cellulose

Column chromatography of G. max seed extract was performed by using CM-52 cellulose (Sober, Nordman and Grasbeck, 1967). A suspension of CM-52 cellulose was prepared according to the manufacturer's instructions (Whatman). The gel suspension was packed into a glass column 1.5 x 40 cm. The column was then connected to a flask of 0.06 M KH₂PO₄ buffer pH 4.5, and the buffer was allowed to pass through the column at a constant flow rate of 60 ml per hour in the cold room.

Two ml of seed extract were applied to the column and elution was achieved by the stepwise addition of the following buffers:

(a) 0.06 M KH₂PO₄, pH 4.5
(b) 0.06 M KH₂PO₄ Na₂HPO₄, pH 6.0
(c) 0.06 M KH₂PO₄ Na₂HPO₄, pH 8.0

Monitoring of absorption at 260 nm revealed four peaks labelled A, B, C and D. (Fig. 15). Peak A was eluted at pH 4.5, peak B at pH 6.0, peaks C and D at pH 8.0. The fractions were dialysed for two days against distilled water in the cold room and they were then...
Fig. 15  
CM-52 column chromatography of *G. max* seed extract

<table>
<thead>
<tr>
<th>pH</th>
<th>Buffers</th>
</tr>
</thead>
<tbody>
<tr>
<td>4.5</td>
<td>0.06 M KH$_2$PO$_4$</td>
</tr>
<tr>
<td>6.0</td>
<td>0.06 M KH$_2$PO$_4$ - NH$_2$PO$_4$</td>
</tr>
<tr>
<td>8.0</td>
<td>0.06 M KH$_2$PO$_4$ - NH$_2$PO$_4$</td>
</tr>
</tbody>
</table>

Transmission (280 nm)

Effluent volume (ml)
tested for inhibitory activity against TNV.

(1) **Effect of CM-52 fractions from G. max seed extract on local lesion production by TNV**

Results in Table 31 show that peak A was not inhibitory; however, peaks B, C and D were inhibitory to local lesion production, giving 30%, 16% and 19% inhibition respectively.

After testing each of the three peaks against TNV, the samples were analysed for protein and also examined by disc electrophoresis.

(ii) **Protein estimation of CM-52 fractions from G. max seed extract**

The proteinaceous nature of the four peaks A, B, C and D was confirmed by the Lowry method. Peak D contained the highest protein concentration (0.25 mg/ml). Peak B, which was the most inhibitory, contained the lowest protein concentration (0.02 mg/ml). Peak A contained nearly the same protein concentration (0.025 mg/ml) as peak B, but showed no inhibitory activity.

Peak C, which gave 16% inhibition, contained 0.05 mg/ml protein. (Table 32).

Disc electrophoresis experiments were performed for each of the four peaks to gain some idea about the protein and glycoprotein components of each.

(iii) **Disc electrophoresis of the CM-52 fractions from G. max seed extract**

Peak A consisted of two bands of protein and one band of glycoprotein, all with Rf greater than 0.5. Peak B consisted of four bands of protein and two bands of glycoprotein. Peak C consisted of three bands of protein and one band of glycoprotein. Peak D consisted of five bands of protein and three bands of glycoprotein. (Figs. 16 and 17).
### TABLE 31

**Effect of C4-52 fractions of G. max seed extract on local lesion production by TNV**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + water</td>
<td>TNV + Peak</td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>49.9</td>
<td>43.9</td>
<td>0.98</td>
</tr>
<tr>
<td>B</td>
<td>33.9</td>
<td>27.3</td>
<td>0.70</td>
</tr>
<tr>
<td>C</td>
<td>46.2</td>
<td>33.9</td>
<td>0.84</td>
</tr>
<tr>
<td>D</td>
<td>41.1</td>
<td>33.4</td>
<td>0.81</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.

### TABLE 32

**Protein estimation of C4-52 fractions from G. max seed extract**

<table>
<thead>
<tr>
<th>Peak</th>
<th>mg protein/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.025</td>
</tr>
<tr>
<td>B</td>
<td>0.023</td>
</tr>
<tr>
<td>C</td>
<td>0.050</td>
</tr>
<tr>
<td>D</td>
<td>0.250</td>
</tr>
</tbody>
</table>
Fig. 16

Disc electrophoresis of the CM-52 fractions from G. max seed extracts

Protein

position of marker

Shading indicates intensity of band staining

A
B
C
D
Fig. 17
Disc electrophoresis of the CM-52 fractions from 5 max seed extracts

Glycoprotein

position of marker
Shading indicates intensity of band staining

A
B
C
D
Therefore it can be concluded at this stage that Sephadex G-100 proved useful in separating the non-protein material from the seed extracts. During the use of CH-52 cellulose, dialysis will have removed this type of non-proteinaceous material so that CH-52 has shown that the remaining fractions contain proteins with varying ability to inhibit viruses. The gel electrophoresis studies of these fractions suggest however, that CH-52 does not separate all protein. To analyse the extracts more intensively, further analysis was undertaken, using the method of Stead et al. (1966), with subsequent analysis on DEAE-52 cellulose.

(b) Column chromatography using DEAE-52 cellulose

Stead et al. isolated the protein from soybean seed extract at pH 4.2 and with 93% ammonium sulphate. Such extracts he termed Ext 4.2 (Soybean). Experiments described in this section have been produced in the same way and the same nomenclature is used.

1. Preparation of Ext 4.2 (Soybean)

400 gm of raw bean meal were suspended in 4 liters of water. The pH was adjusted to 4.2 with 5 N HCl. After thoroughly mixing for two hours it was allowed to settle overnight. Insoluble matter was removed by centrifugation at 275 g for 20 minutes. The supernatant fraction was adjusted to pH 6.8 with 5 N NaOH. Solid ammonium sulphate was added to the supernatant fraction to give a final saturation of 93%, the pH being checked and adjusted to 6.8 throughout the addition. The suspension was stored at 2°C overnight, and then centrifuged at 1,000 g for 20 minutes. The precipitate was redissolved in a minimal volume of distilled water and dialysed against distilled water for 12 hours. The dialysed solution was chilled to 2°C, cold acetone was added to a final concentration of 70% and the precipitate was allowed to
aggregate with intermittent stirring. The white precipitate was removed by centrifugation and washed twice with an equal volume of cold ether. The washed material was dried and ground to a powder. Fractions prepared in this manner are referred to as Ext$_{93}^{4.2}$ (Soybean) (Steal et al., 1966).

One gram Ext$_{93}^{4.2}$ (Soybean) was homogenized in 30 ml of 0.01 M phosphate buffer pH 7.6 and dialyzed overnight against an identical buffer at 2°C. Insoluble matter was removed by centrifugation and the supernatant fraction was diluted with distilled water by 10-fold dilutions and then tested against TNV. Results in Table 33 show that Ext$_{93}^{4.2}$ (Soybean) is inhibitory against TNV, giving 70% inhibition. Dilution seemed to reduce the percentage inhibition, and this substantiates the previous suggestion that soybean seed extract contains plant virus inhibitors and not inactivators. Ext$_{93}^{4.2}$ (Soybean) was then fractionated on DEAE-52 cellulose in order to identify which proteins are involved in the plant virus inhibition.

(ii) Column chromatography of Ext$_{93}^{4.2}$ (Soybean) on DEAE-52 cellulose

Whatman diethylaminoethyl cellulose (DEAE-52) anion exchanger was washed before using the method of Peterson and Sober (1962). The washed adsorbent was suspended in two volumes of potassium phosphate buffer (0.01 M, pH 7.6) and the suspension was poured into a 2 x 60 cm glass column and allowed to settle. At the completion of packing, one liter of buffer was forced through the column at a flow rate of 36 ml per hour. The adsorbent was subsequently left to equilibrate overnight, and a further 500 ml of buffer was run through the column before use.

One gram Ext$_{93}^{4.2}$ (Soybean) was homogenized in 30 ml of 0.01 M
TABLE 33

Effect of various dilutions of \textit{Ext}_{93}^{4.2} (soybean) on
local lesion production by TNV

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + water</td>
<td>TNV + extract</td>
<td></td>
</tr>
<tr>
<td>Neat</td>
<td>29.4</td>
<td>8.8</td>
<td>0.30</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>67.7</td>
<td>41.5</td>
<td>0.61</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>49.0</td>
<td>35.3</td>
<td>0.72</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.
phosphate buffer pH 7.6 and dialysed overnight against an identical buffer at 2°C. Insoluble matter was removed by centrifugation and the supernatant fraction was applied to the column. Elution was accomplished by a stepwise technique similar to that described by Lackie, Sammes, Anderson and Smith (1959) using six different NaCl concentrations (Fig. 18) in 0.01 M phosphate buffer pH 7.6. The small deviations from pH 7.6 which resulted from the inoculum of NaCl into the buffer system, were corrected by addition of 5 M NaCl. The final eluant consisted of unbuffered 2 M NaCl in order to remove all residual protein from the column (Peterson and Sober, 1962).

Elution profile monitored for absorption at 250 nm revealed the presence of seven peaks. (Fig. 18). Tubes corresponding to each peak were pooled, care being taken to discard at least two tubes on the leading and trailing edge of each peak. The pooled fractions were dialysed for 24 hours with continuous agitation against tap water in the cold room, followed by a further 24 hours' dialysis against distilled water. The fractions were then tested for TNV inhibition.

(iii) Effect of HN-52 fractions of Ext^4.2 (soybean) on local lesion production by TNV

One ml of each of the seven peaks was mixed with TNV and inoculated on French bean leaves. Control consisted of TNV and water.

Results in Table 34 show that although Ext^4.2 (Soybean) gave 70% inhibition, not all the seven peaks were inhibitory. Inhibition was noticed in peak 1, the most mobile, and also in peaks 5 and 6, the percentage inhibition being 16%, 12% and 37% respectively. Therefore, marked inhibition was obtained in peak 6. The difference between inhibition obtained by using Ext^4.2 (Soybean)
Fig. 18  Chromatography of Ext-93 (Soybean) on DEAE-52 column

<table>
<thead>
<tr>
<th>NaCl concentration</th>
<th>Effluent volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.015 M NaCl</td>
<td>60</td>
</tr>
<tr>
<td>0.025 M NaCl</td>
<td>180</td>
</tr>
<tr>
<td>0.060 M NaCl</td>
<td>240</td>
</tr>
<tr>
<td>0.130 M NaCl</td>
<td>300</td>
</tr>
<tr>
<td>0.170 M NaCl</td>
<td>420</td>
</tr>
<tr>
<td>0.180 M NaCl</td>
<td>480</td>
</tr>
<tr>
<td>0.250 M NaCl</td>
<td>540</td>
</tr>
<tr>
<td>2.0 M NaCl</td>
<td>600</td>
</tr>
</tbody>
</table>

Transmission (260 nm)
<table>
<thead>
<tr>
<th>Sodium chloride eluant concentration (m)</th>
<th>Peak</th>
<th>Mean number of lesions</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TVV + water</td>
<td>TVV + Peak</td>
<td></td>
</tr>
<tr>
<td>0.015</td>
<td>1</td>
<td>94.0</td>
<td>77.1</td>
<td>0.82</td>
</tr>
<tr>
<td>0.025</td>
<td>2</td>
<td>90.0</td>
<td>95.5</td>
<td>0.92</td>
</tr>
<tr>
<td>0.060</td>
<td>3</td>
<td>79.3</td>
<td>67.7</td>
<td>0.96</td>
</tr>
<tr>
<td>0.130</td>
<td>4</td>
<td>60.2</td>
<td>72.9</td>
<td>1.05</td>
</tr>
<tr>
<td>0.170</td>
<td>5</td>
<td>95.3</td>
<td>85.7</td>
<td>0.88</td>
</tr>
<tr>
<td>0.250</td>
<td>6</td>
<td>137.9</td>
<td>86.2</td>
<td>0.63</td>
</tr>
<tr>
<td>2.000 **</td>
<td>7</td>
<td>127.1</td>
<td>132.5</td>
<td>1.04</td>
</tr>
</tbody>
</table>

* Each sodium chloride solution was buffered with pH 7.6 phosphate buffer.

** Unbuffered eluant.

*** Each figure represents the mean number of lesions for ten replications.
and peak 6 was due to the dilution of the inhibitor after elution through DIAE-52 cellulose.

Peaks 2, 3, 4 and 7 showed no inhibitory effect against TNV, giving an activity quotient of 0.99, 0.95, 1.03 and 1.04 respectively.

The seven peaks were also examined for protein by using the Lowry method.

(iv) Protein estimation of DIAE-52 fractions from Ext$^{4.2}$ (Soybean)

The seven peaks showed positive protein test (Table 3). Peak 3 (1 mg/ml), peak 4 (1.5 mg/ml) and peak 5 (1.25 mg/ml) showed high protein concentration, but of these only peak 5 was slightly inhibitory. Peak 1 (0.8 mg/ml) and peak 6 (0.6 mg/ml) contained low protein concentrations, and were inhibitory. Peak 2 contained a similar concentration of protein (0.7 mg/ml) but it was not inhibitory. Marked inhibition, 37%, was found when peak 6 was mixed with TNV. Peak 7 contained the lowest protein concentration and was not inhibitory. Thus inhibitory activity was confined to peaks 1, 5 and 6.

(v) Disc electrophoresis of DIAE-52 fractions from Ext$^{4.2}$ (Soybean)

Electrophoresis experiments for Ext$^{4.2}$ (Soybean) showed that it consisted of nine bands of protein and three wide bands of glycoprotein. (Fig. 19). After fractionation of this Ext$^{4.2}$ (Soybean) on a DIAE-52 column, each peak was examined by disc electrophoresis. (Figs. 20 and 21).

Peak 1, which was inhibitory (13%), consisted only of one band of protein ($R_f = 0.27$) and one band of glycoprotein ($R_f = 0.21$).

Peak 2, which was not inhibitory, consisted of two bands of glycoprotein ($R_f = 0.42 - 0.67$), and two bands of protein.

Peak 3, like peak 2, was not inhibitory and it consisted of four
TABLE 33

Protein estimation of HAD-52 fractions from Ext.$^{2,2}$ (soybean)

<table>
<thead>
<tr>
<th>Peak</th>
<th>mg protein/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.80</td>
</tr>
<tr>
<td>2</td>
<td>0.70</td>
</tr>
<tr>
<td>3</td>
<td>1.00</td>
</tr>
<tr>
<td>4</td>
<td>1.30</td>
</tr>
<tr>
<td>5</td>
<td>1.23</td>
</tr>
<tr>
<td>6</td>
<td>0.60</td>
</tr>
<tr>
<td>7</td>
<td>0.10</td>
</tr>
</tbody>
</table>
Fig. 19  Disc electrophoresis of Ext$^{4.2}_{93}$ (Soybean)

$\text{Ext}^{4.2}_{93}$ (Soybean)

- Protein

+ Glycoprotein

← position of marker

Shading indicates intensity of band staining
Fig. 20  Disc electrophoresis of DEAE-52 fractions from Ext^{4.2}_{93} (Soybean)

Protein

1  2  3  4  5  6  7

position of marker
Shading indicates intensity of band staining
Fig. 21
Disc electrophoresis of DEAE-52 fractions from Ext$^{4-2}$ (Soybean)

Glycoprotein

Position of marker
Shading indicates intensity of band staining
bands of protein and two bands of glycoprotein. Peak 4, however, although showing no inhibition, consisted of six bands of protein but only one band of glycoprotein. Peak 5, which gave 12% inhibition, consisted of only one band of protein (RF = 0.46) and one band of glycoprotein (RF = 0.44). The markedly inhibitory peak 6 (57%) consisted of three bands of protein (RF = 0.45 - 0.73) and three bands of glycoprotein (RF = 0.43 - 0.70), which were located in the middle part of the gel, whereas peak 7 consisted of one band of protein (RF = 0.55) and one band of glycoprotein (RF = 0.69) and again showed no inhibitory activity.

It was noticeable that in the inhibitory peaks (1, 5 and 6) the RF values of protein corresponded closely to that of the glycoprotein. It seems reasonable to suggest, therefore, that in peaks 1 and 5 the inhibitor is a glycoprotein. In peak 6, three bands were observed, suggesting that three different proteins or glycoproteins were involved in inhibition.

**DISCUSSION**

The nature of *G. max* seed extract was studied by using various techniques, some of them, but not all, being effective in purifying and characterizing the plant virus inhibitors.

Gel filtration (Sephadex G-100) of *G. max* seed extract showed the presence of three peaks. Only more rapidly eluted materials of peaks I and II were inhibitory and had molecular weights of about 158,500 and 17,730 respectively. Peak III, with a molecular weight of 1,585, was not inhibitory.

Although, as listed in the introduction of this thesis, many workers have studied plant virus inhibitors, very few molecular weight
determinations for inhibitor compounds have been made. Bagotli (1957) reported that the carnation (Dianthus caryophyllus) inhibitor has a molecular weight of 10,000. In 1960 Wyatt and Shepherd found that Phytolacca americana juice contains a protein virus inhibitor with a molecular weight of 15,000. Recently, Schrödler (1971) studied the properties of inhibitors from extracts of Chenopodium amaranticolor, C. album, Atriplex nitens and Acanthium campestris. He found that gel filtration with Sephadex G-200 separated inhibitors having molecular weights of 25,000 - 38,000.

Sephadex G-100 proved useful in indicating the molecular weights of inhibitor fractions and also in allowing the separation of low molecular weight non-inhibitor compounds. Sephadex gel filtration did not, however, separate the materials precisely enough as indicated by disc electrophoresis, since the latter technique revealed several protein and glycoprotein bands in each of the two Sephadex peaks. Therefore, ion exchange chromatography experiments were performed. Chromatography of G. max seed extract on CI-52 produced four peaks (A, B, C and D), three of which (B, C and D) were inhibitory against TNV. The most inhibitory peak (B) was eluted with phosphate buffer at pH 6, whereas the other two inhibitors were eluted at pH 3. This suggests that soybean extract contains three plant virus inhibitors, one (peak B) which is weakly attached to the cation exchanger CI-52 column, and is thus acidic in nature; on the other hand, peaks C and D were strongly attached to the column (CI-52) and this proves their more basic nature. Protein estimation and disc electrophoresis experiments showed again that each of the virus inhibitors isolated consisted of more than one band of protein and glycoprotein. For example, peak D consisted of five protein bands and three bands of glycoproteins. It seemed therefore, that CI-52 chromatography was
not completely effective in purifying the virus inhibitors and bringing about good protein separation, although Wyatt and Shepherd (1969) prepared a highly purified virus inhibitor from *Phytolacca americana* by chromatography on a similar column of carboxymethyl Sephadex.

Better separation of soybean proteins was obtained by using DEAE-52 chromatography. The extract was prepared using the Stead et al. (1966) method, and it was given the same nomenclature ext\textsuperscript{4,2} (soybean). This extract contains a highly active virus inhibitor. Seven proteinaceous peaks were obtained from this extract and only peaks 1, 5 and 6 were inhibitory against TNV. Peak 1 was eluted from DEAE-52 column by 0.015 M NaCl in 0.01 M phosphate buffer pH 7.6 which suggests its basic nature since it did not attach strongly to the anion exchange DEAE-52 cellulose column. Disc electrophoresis showed that peak 1 contained one band of protein and one band of glycoprotein on the upper part of the gel. The close Rf value suggests that this fraction consisted of a single molecular species. Peak 5, which was also inhibitory against TNV and was eluted at 0.170 M NaCl, consisted of one band of protein and a glycoprotein band, and, again, may represent only one molecular species.

Marked inhibition was obtained in peak 6 which was eluted at 0.250 M NaCl and it consisted of three bands of protein and three glycoprotein bands.

The virus inhibitors isolated in both peak 5 and peak 6 were apparently strongly attached to the DEAE-52 anion exchanger and required a high concentration of NaCl to elute them. This suggests the acidic nature of the virus inhibitors in these peaks.

Peaks 2, 3, 4 and 7 did not show any inhibition against TNV, giving an activity quotient of 0.99, 0.96, 1.05 and 1.34 respectively.

Therefore, reviewing the results obtained from the ion exchange
experiments it can be concluded that the separation of soybean proteins, and consequently virus inhibitors by DEAE-52 chromatography, is more suitable and successful than using CM-52 chromatography. Albrechtova (1963) found similar results when he applied DEAE Sephadex A-25 chromatography to separate TMX virus inhibitors from potato leaf sap. The inhibitor was eluted at the concentration 0.15 - 0.3 M NaCl and was electrophoretically homogeneous. In 1971 Smokler used a different ion-exchanger (CN-Sephadex C-25) to purify the inhibitors prepared from leaves of Chenopodium amaranticolor, C. album, Atriplex riten and Acroanthes campestris. Most of the inhibitory activity was eluted by 0.4 M sodium acetate buffer, suggesting that the inhibitors were basic in nature.

At this stage it can be concluded that gel filtration and DEAE-52 chromatography are useful in adding more information to the properties of G. max virus inhibitors by giving an idea about the molecular weights of the constituents and properties of the proteins isolated from the extract. Therefore, it was decided to use these two successful techniques in the experiments on P. vulgaris seed extract which will be described in section B of this chapter.

SECTION B

NATURE OF THE VIRUS INHIBITORS EXTRACTED FROM PLANT VULGARIS

Although P. vulgaris seed extract has been tested against plant viruses (Choo, 1955; Crispin and Grogan, 1961; Stevens, 1970), no researchers have tried to isolate and characterise the plant virus inhibitors. In 1966 Stead et al. separated seven proteins from P. vulgaris seed extract; however their interest lay not in the plant virus inhibitor activity, but in isolation of proteins for
hemagglutinating activity, trypsin-inhibition activity, and animal growth toxicity studies. Since the method of Stead et al. (1966) gave satisfactory results when it was used to study the nature of the virus inhibitors extracted from G. max seed extract, it was decided to use this method in the examination of P. vulgaris seed extracts. The same nomenclature was used and extracts are labelled Ext$^{4.2}$ (French bean).

Initially gel filtration was also performed for crude P. vulgaris in the same way as was done for G. max seed extract and this will be described first in this section.

1. **GEL FILTRATION OF Phaseolus vulgaris SEED EXTRACTS**

   (a) **Column chromatography of P. vulgaris seed extract on Sephadex G-100**

   By using the same Sephadex G-100 column as used in G. max experiments, column chromatography of P. vulgaris seed extracts were undertaken. Lyophilized seed extract (0.04 g) was dissolved in 2 ml 0.03 M phosphate buffer pH 7. The solution was layered on the top of the column and fractions were collected on a volume basis (1 ml each five minutes) using a fraction collector.

   Elution profiles showed the presence of three peaks, I, II and III, with elution volumes of 16 ml, 38 ml and 49 ml respectively. (Fig. 22). Each of the peaks was tested against TNV.

   (b) **Effect of Sephadex G-100 column fractions from P. vulgaris seed extract on local lesion production by TNV**

   One ml of each of peaks I, II and III was mixed with TNV and was tested for inhibitory activity on French bean leaves. Control consisted of one ml of TNV and one ml of the eluting phosphate buffer pH 7. Results in Table 36 show that peaks I and II were inhibitory giving 33% and 12% inhibition respectively. Peak III was not inhibitory
Fig. 22 Sephadex G-100 column chromatography of *P. vulgaris* seed extract
TABLE 35

Effect of Sephadex G-100 column fractions from *P. vulgaris* seed extract on local lesion production by TNV

<table>
<thead>
<tr>
<th>Peak</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + F04 buffer pH 7</td>
<td>TNV + Peak</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>61.0</td>
<td>32.0</td>
<td>0.62</td>
</tr>
<tr>
<td>II</td>
<td>73.9</td>
<td>65.0</td>
<td>0.83</td>
</tr>
<tr>
<td>III</td>
<td>75.9</td>
<td>91.9</td>
<td>1.25</td>
</tr>
</tbody>
</table>

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

TABLE 37

Protein and carbohydrate estimation for the Sephadex G-100 fractions from *P. vulgaris* seed extract

<table>
<thead>
<tr>
<th>Peak</th>
<th>mg protein/ml</th>
<th>mg carbohydrate/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>0.06</td>
<td>0.33</td>
</tr>
<tr>
<td>II</td>
<td>0.07</td>
<td>0.003</td>
</tr>
<tr>
<td>III</td>
<td>0.02</td>
<td>2.840</td>
</tr>
</tbody>
</table>
and it gave an activity quotient of 1.2.

The three peaks were also tested for the presence of protein and carbohydrate.

(c) Protoplast enzyme protein extraction from the peak II fractions from the Sephadex G-100 extract.

Peak I contained the highest protein concentration (2.66 mg/ml) and only 0.05 mg/ml carbohydrate. Peak II contained 0.57 mg/ml protein and 0.07 mg/ml carbohydrate. The highest carbohydrate concentration was obtained in peak III (2.56 mg/ml); however, it contained only 0.02 mg/ml protein. (Table 3).

In order to gain more information about the Sephadex G-100 fractions from the reducer seed extract, each of the peaks was subjected to disc electrophoresis.

(d) Disc electrophoresis of the Sephadex G-100 fractions from the reducer seed extract.

Results in Fig. 23 show that peak I contained seven bands of protein and seven bands of glycoprotein. Peak II contained four bands of protein and one of glycoprotein staining. Peak III did not stain in the electrophoresis experiment and may consist of low molecular weight compounds as described in the following section.

(e) Molecular weight determination of the Sephadex G-100 fractions from the reducer seed extract.

By plotting V/W ratios for peaks I, II and III of Fig. 3, the molecular weights were determined. The inhibitory peaks I and II had molecular weights of about 177,000 and 12,350 respectively. Peak III, which showed no inhibition when it was tested against I and II, showed no positive results in electrophoresis studies, had a molecular weight of about 5,162. (Table 3a).

Using the same Sephadex G-100 column, samples treated by boiling,
Fig. 23

Disc electrophoresis of the Sephadex G-100 fractions from *P. vulgaris* seed extracts

**Peak I**

- Protein
- Glycoprotein

**Peak II**

- Protein
- Glycoprotein

→ position of marker

Shading indicates intensity of band staining
**TABLE 37a**

Molecular weight determination of the Sephadex G-100 fractions from *P. vulgaris* seed extract

<table>
<thead>
<tr>
<th>Peak</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>177,800</td>
</tr>
<tr>
<td>II</td>
<td>12,590</td>
</tr>
<tr>
<td>III</td>
<td>3,182</td>
</tr>
</tbody>
</table>
dialysis, or with alcohol were, as described previously, also analysed by gel filtration. This was done in order to find out how the various treatments affected the extracts, since, for example, heating the extract changed non-inhibitory extracts to inhibitory extracts.

(i) **Sephadex G-100 column chromatography of P. vulgaria seed extracts following various treatments**

(i) **Heated seed extract**

Although heat revealed the presence of inhibitors and masking compounds in *P. vulgaria* seed extract, the Sephadex G-100 profile showed the presence of the same three peaks found in the unheated seed extract. However, peak I in the heated extract was smaller than that in the unheated seed extract (Fig. 24), suggesting the destruction of protein constituents of this peak. This point will be examined in more detail later.

(ii) **Dialysate seed extracts**

The dialysate and the dialysable part of the seed extracts were passed through Sephadex G-100. The dialysate contained only peaks I and II; (Fig. 25); however, the dialysable part contained only peak III (Fig. 26), substantiating earlier evidence that peak III consisted of low molecular weight compounds.

(iii) **Alcohol treated extracts**

As previously described, alcohol treatment of the extract precipitated compounds which showed augmentation, leaving the inhibitor in the supernatant. The alcohol precipitate contained most of peak I, all of peak II and part of peak III (Fig. 27). The supernatant contained part of peaks I and III (Fig. 28). Therefore it can be concluded at this stage that Sephadex G-100 chromatography has confirmed the presence of high molecular weight plant virus inhibitors in *P. vulgaria* seed extracts.
Fig. 24  Sephadex G-100 column chromatography of heated *P. vulgaris* seed extract
Fig. 25  Sephadex G-100 column chromatography of the non-dialysable part of *P. vulgaris* seed extract

(For comparative purposes the elution profile of the dialysable part has been dotted in)
Fig. 26 Sephadex G-100 column chromatography of the dialysable part of *P. vulgaris* seed extract
Fig. 27  Sephadex G-100 column chromatography of precipitate from alcohol treated P. vulgaris seed extract

(For comparative purposes the elution profile of the supernatant fraction has been dotted in)
Fig. 28  Sephadex G-100 column chromatography of supernatant from alcohol treated *P. vulgaris* seed extract
However, to establish more precisely the identity of the fractions responsible for inhibition, more refined techniques of protein separation were performed. For this purpose the seed extracts were further analysed in the same way as that of *G. max* seed extract, using DEAE-52 column chromatography.

2. CHROMATOGRAPHY OF EXT$^{4.2}$ (FRENCH BEAN) ON DEAE-52 COLUMN

Ext$^{4.2}$ (French bean) was prepared from 400 grams of *P. vulgaris* seed flour using the same procedure which was used for the preparation of Ext$^{4.2}$ (Soybean).

One gram Ext$^{4.2}$ (French bean) was homogenized in 30 ml of 0.01 M phosphate buffer pH 7.6 and dialysed overnight against this buffer at 2°C. Insoluble matter was removed by centrifugation, and the supernatant fraction was diluted in a 10-fold dilution series using distilled water. Each dilution was tested against TNV. Ext$^{4.2}$ (French bean) was inhibitory, giving 73% inhibition (Table 33). The inhibition was reduced by diluting the extract which confirms the presence of plant virus inhibitors, rather than virus inactivators, in the extracts.

Since Ext$^{4.2}$ (French bean) proved to be markedly inhibitory against plant viruses, another one gram sample was homogenized as described above. Insoluble matter was removed by centrifugation and the supernatant fraction applied to a DEAE-52 cellulose column. As in the case of Ext$^{4.2}$ (Soybean), seven peaks were obtained (Fig. 29). Each peak was tested for its effect on TNV infection.

(a) Effect of DEAE-52 fractions of Ext$^{4.2}$ (French bean) on local lesion production by TNV

One ml of each of the seven peaks was tested against TNV infection. Results in Table 39 show that only three peaks of the seven are
TABLE 33

Effect of various dilutions of $\text{Ext}^{1.2}_{3}$ (French bean) on local lesion production by TNV

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + water</td>
<td>TNV + extract</td>
<td></td>
</tr>
<tr>
<td>Neat</td>
<td>44.2</td>
<td>12.0</td>
<td>0.27</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>49.0</td>
<td>15.2</td>
<td>0.33</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>49.7</td>
<td>33.1</td>
<td>0.63</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications
Fig. 29  Chromatography of Ext$_{93}^{4.2}$ (French bean) on DEAE-52 column

<table>
<thead>
<tr>
<th>0.015 M</th>
<th>0.025 M</th>
<th>0.060 M</th>
<th>0.130 M</th>
<th>0.170 M</th>
<th>0.250 M</th>
<th>2.0 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaCl</td>
<td>NaCl</td>
<td>NaCl</td>
<td>NaCl</td>
<td>NaCl</td>
<td>NaCl</td>
<td>NaCl</td>
</tr>
</tbody>
</table>

Transmission (280 ml)

Effluent volume (ml)
TABLE 39  
Effect of DEAE-52 fractions of Ext*4.2 (French bean) on local lesion production by TNV

<table>
<thead>
<tr>
<th>Sodium chloride eluant concentration (M)*</th>
<th>Peak</th>
<th>Mean number of lesions ***</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>TNV + water</td>
<td>TNV + Peak</td>
<td></td>
</tr>
<tr>
<td>0.015</td>
<td>1</td>
<td>74.2</td>
<td>78.6</td>
<td>0.93</td>
</tr>
<tr>
<td>0.025</td>
<td>2</td>
<td>72.2</td>
<td>53.3</td>
<td>0.81</td>
</tr>
<tr>
<td>0.060</td>
<td>3</td>
<td>79.4</td>
<td>100.5</td>
<td>1.27</td>
</tr>
<tr>
<td>0.130</td>
<td>4</td>
<td>75.9</td>
<td>79.2</td>
<td>0.92</td>
</tr>
<tr>
<td>0.170</td>
<td>5</td>
<td>76.9</td>
<td>77.5</td>
<td>1.01</td>
</tr>
<tr>
<td>0.250</td>
<td>6</td>
<td>50.5</td>
<td>40.1</td>
<td>0.79</td>
</tr>
<tr>
<td>2.000 **</td>
<td>7</td>
<td>61.6</td>
<td>33.7</td>
<td>0.63</td>
</tr>
</tbody>
</table>

* Each sodium chloride solution was buffered with pH 7.6 phosphate buffer

** Unbuffered eluant

*** Each figure represents the mean number of lesions for ten replications
inhibitory. Peak 7, the least mobile, was the most inhibitory peak giving 37% inhibition, while peaks 2 and 6 were less inhibitory giving 19% and 21% inhibition respectively.

Peaks 1 and 5 were not inhibitory, and peak 3 showed slight augmentation, giving an activity quotient of 1.27.

The seven peaks were also tested for the presence of protein using the Lowry method of estimation.

(b) Protein estimation of DEAE-52 fractions from Ext* (French bean)

A high protein concentration (0.5 mg/ml) was obtained in each of the non-inhibitory peaks 3 and 4. Peaks 1, 2, 5 and 6 contained similar protein concentration (0.1 mg/ml), but only peaks 2 and 6 were inhibitory. Peak 7 contained low concentration of protein (0.05 mg/ml), but it showed the maximum inhibitory activity (Table 40).

(c) Disc electrophoresis of DEAE-52 fractions from Ext* (French bean)

Disc electrophoresis experiments were performed on complete Ext* (French bean) and also on the material from each of the seven peaks following column chromatography. The complete extract contained seven bands of proteins and six of glycoproteins (Fig. 30). Peak 1 contained one band of protein which appeared to give a glycoprotein stain. (Figs. 31 and 32). This proteinaceous material was found in the surface layers of the gel. Peak 2 contained one broad band of protein (RF = 0.36) and one band of glycoprotein (RF = 0.37). Peak 3 contained four bands of protein, one of which was very concentrated (RF = 0.33). The gels also stained in this region for glycoprotein. A protein band running at RF 0.80 did not stain for glycoprotein.

Peak 4 contained one band of protein (RF = 0.88) and one band of glycoprotein (RF = 0.90) at the lower end of the gel. Peak 5 contained one band of protein (RF = 0.43) and one of glycoprotein (RF = 0.38). Peaks 6 and 7 each contained one band of protein with
TABLE 40

Protein estimation of DLAE-52 fractions from Ext93 (French bean)

<table>
<thead>
<tr>
<th>Peak</th>
<th>mg protein/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.10</td>
</tr>
<tr>
<td>2</td>
<td>0.10</td>
</tr>
<tr>
<td>3</td>
<td>0.30</td>
</tr>
<tr>
<td>4</td>
<td>0.30</td>
</tr>
<tr>
<td>5</td>
<td>0.10</td>
</tr>
<tr>
<td>6</td>
<td>0.10</td>
</tr>
<tr>
<td>7</td>
<td>0.05</td>
</tr>
</tbody>
</table>
Fig. 30  Disc electrophoresis of $\text{Ext}_{93}^{4.2}$ (French bean)

$\text{Ext}_{93}^{4.2}$ (French bean)

- Protein

+ Glycoprotein

← position of marker

Shading indicates intensity of band staining
Fig. 31  Disc electrophoresis of DEAE-52 fractions from Ext⁴.²⁹³⁻¹ (French bean)

Protein

<p>| | | | | | | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
</tr>
</tbody>
</table>

position of marker

Shading indicates intensity of band staining
Fig. 32
Disc electrophoresis of DEAE-52 fractions from Ext.95 (French bean)

Glycoprotein

Shading indicates intensity of band staining

d +

position of marker

1 2 3 4 5 6 7
Jif values of about 0.65 and 0.64 respectively. These bands also gave positive reactions when tested for glycoprotein. Both protein and glycoprotein staining reactions were stronger from peak 6 material than from peak 7.

It seems, therefore, that disc electrophoresis experiments, as well as DEAE-52 cellulose chromatography of Ltv$_{4.2}^{y_5}$ (French bean) seed extract, are effective techniques for isolating and identifying the P. vulgaris virus inhibitors. However, there are still some points, mentioned when the properties of the inhibitors were studied in the previous section, which need to be explained. Thus, how is it that heat converts uninhibitory French bean seed extracts to inhibitory extracts? If masking compounds are involved as suggested earlier, what are they? In order to answer these questions the heated and the unheated samples of the crude French bean seed extracts (prepared as in Chapter II) were studied once more, using DEAE-52 cellulose chromatography.

3. **DEAE-52 CHROMATOGRAPHY OF THE UNHEATED AND THE HEATED P. VULGARIS SEED EXTRACTS**

Unheated and the heated extracts were each passed through a DEAE-52 cellulose column. The unheated seed extract gave seven peaks similar to those found in examination of Ltv$_{4.2}^{y_5}$ (French bean) (Fig. 33). However, in the whole extract, two peaks (peak 2 and 2') were eluted by buffered 0.025 N NaCl, but only one peak (peak 2) was eluted in the case of Ltv$_{4.2}^{y_5}$ (French bean).

The seven peaks obtained from the unheated extract were each tested against TNV. Only peaks 2, 6 and 7 were inhibitory, giving 20%, 25% and 40% inhibition respectively (Table 41). Peaks 1, 2', 3, 4 and 5 were not inhibitory; however, peak 3 gave slight augmentation
Fig. 33  DEAE-52 column chromatography of the unheated *P. vulgaris* seed extract

<table>
<thead>
<tr>
<th>Transmission (280 nm)</th>
<th>60</th>
<th>120</th>
<th>180</th>
<th>240</th>
<th>300</th>
<th>360</th>
<th>420</th>
<th>480</th>
<th>540</th>
<th>600</th>
</tr>
</thead>
<tbody>
<tr>
<td>Effluent volume (ml)</td>
<td>1</td>
<td>2</td>
<td>3</td>
<td>4</td>
<td>5</td>
<td>6</td>
<td>7</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Concentration (M)</th>
<th>0.015 M</th>
<th>0.025 M</th>
<th>0.060 M</th>
<th>0.130 M</th>
<th>0.170 M</th>
<th>0.250 M</th>
<th>2.0 M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Salt</td>
<td>NaCl</td>
<td>NaCl</td>
<td>NaCl</td>
<td>NaCl</td>
<td>NaCl</td>
<td>NaCl</td>
<td>NaCl</td>
</tr>
</tbody>
</table>
**TABLE 41**

**Effect of DEAE-52 fractions of unheated *P. vulgaris* seed extract on local lesion production by TNV**

<table>
<thead>
<tr>
<th>Peak</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
<th>Probability (P)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + water</td>
<td>TNV + peak</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>97.7</td>
<td>95.3</td>
<td>0.98</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>95.2</td>
<td>76.5</td>
<td>0.80</td>
<td>29</td>
</tr>
<tr>
<td>2'</td>
<td>96.4</td>
<td>97.4</td>
<td>1.01</td>
<td>-1</td>
</tr>
<tr>
<td>3</td>
<td>100.0</td>
<td>123.9</td>
<td>1.25</td>
<td>-25</td>
</tr>
<tr>
<td>4</td>
<td>93.1</td>
<td>99.3</td>
<td>0.97</td>
<td>3</td>
</tr>
<tr>
<td>5</td>
<td>99.5</td>
<td>102.0</td>
<td>1.03</td>
<td>-3</td>
</tr>
<tr>
<td>6</td>
<td>101.0</td>
<td>76.9</td>
<td>0.75</td>
<td>25</td>
</tr>
<tr>
<td>7</td>
<td>95.5</td>
<td>56.1</td>
<td>0.69</td>
<td>40</td>
</tr>
<tr>
<td>3 + 2</td>
<td>98.3</td>
<td>97.7</td>
<td>0.99</td>
<td>1</td>
</tr>
<tr>
<td>3 + 6</td>
<td>96.2</td>
<td>96.8</td>
<td>1.01</td>
<td>-1</td>
</tr>
<tr>
<td>3 + 7</td>
<td>102.0</td>
<td>103.0</td>
<td>1.06</td>
<td>-6</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications
Fig. 54
DEAE-52 column chromatography of the heated P. vulgaris seed extract

<table>
<thead>
<tr>
<th>Transmission (280 nm)</th>
<th>Effluent volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>600</td>
</tr>
<tr>
<td>60</td>
<td>540</td>
</tr>
<tr>
<td>70</td>
<td>480</td>
</tr>
<tr>
<td>80</td>
<td>420</td>
</tr>
<tr>
<td>90</td>
<td>360</td>
</tr>
<tr>
<td>100</td>
<td>300</td>
</tr>
<tr>
<td></td>
<td>240</td>
</tr>
<tr>
<td></td>
<td>180</td>
</tr>
<tr>
<td></td>
<td>120</td>
</tr>
<tr>
<td></td>
<td>60</td>
</tr>
</tbody>
</table>

[Graph showing chromatography peaks and elution profile]
with an activity quotient of 1.25. These results are important in
that they emphasise the similarity between the crude unheated extract
and the \( \text{Ext} \frac{b}{2} \text{Fp} \) (French bean) in that the same number of peaks are
detected, the peaks are eluted at the same NaCl concentrations, and
each show similar activity against TNV.

The same procedure was performed for the heated seed extract,
but only six peaks were obtained (Fig. 31). Peak 3 was lacking, and
it seems likely that it contains the masking compounds since such
heated extract gives inhibition. An experiment was designed therefore,
to test whether peak 3 masked the inhibitory activity of peaks 2, 6
and 7 in the whole seed extract. One ml of peak 3 was mixed with 1 ml
of each of the three inhibitory peaks before testing against TNV.

Results in Table 41 show that peak 3 eliminated their inhibitory
activity and, therefore, it seems reasonable to suggest that peak 3
contains the masking compounds.

DISCUSSION

The nature of the plant virus inhibitors found in \( P. \text{vulgaris} \)
seed extracts was studied in the same way as that of \( G. \text{max} \) seed
extract.

Gel filtration, using Sephadex G-100, revealed the presence of
three peaks in the extract (I, II and III). Only peaks I and II
were inhibitory against TNV, having molecular weights of about
177,800 and 12,590 respectively. Peak III with a molecular weight of
3,162, was not inhibitory. Disc electrophoresis showed these peaks
to consist of proteins and glycoproteins.

Molecular weight determination of glycoproteins extracted from
\( P. \text{vulgaris} \) has been reported before, but not for the plant virus
inhibitors. Allen et al. (1969) purified a glycoprotein with
lymphocyte mitogenic properties from *P. vulgaris* seed extract which showed it has a molecular weight of 115,000. Allan and Crumpton (1971) reported that *P. vulgaris* seed extract contains a hemagglutinating glycoprotein with a molecular weight of 130,000.

Sephadex G-100 proved useful in determining the magnitude of the molecular weights of the virus inhibitors in French bean seed extracts and also in the separation of low molecular weight non-inhibitory compounds. This technique did not, however, separate the proteins and glycoproteins precisely enough to identify the compounds involved in plant virus inhibition. Therefore, ion-exchange chromatography of the extract was performed using the method of Steal et al. (1966). The extract was prepared in the same way as Int.2 (Soybean) and the same nomenclature was used. Int.2 (French bean) contained strong plant virus inhibitors giving 73% inhibition. Seven peaks were obtained from this extract all of which reacted positively in protein tests. Only peaks 2, 6 and 7 were inhibitory against TFV. Peak 2 was eluted from the DEAE-52 column by 0.025 M NaCl in 0.01 M phosphate buffer pH 7.6, indicating its basic nature, since it did not attach strongly to the anion exchanger column. Disc electrophoresis showed that peak 2 contained one band of protein and one of glycoprotein with close If values, suggesting that this inhibitor is a single glycoprotein.

Peak 6, which gave 21% inhibition, was eluted by buffered 0.25 M NaCl and also consisted of one band of protein and one of glycoprotein. Their If values suggest that this fraction of the extract consists of a single molecular species.

Strong inhibition (37%) was obtained with peak 7 which was eluted by unbuffered 2 M NaCl. Disc electrophoresis showed that this peak consisted of a glycoprotein.
The virus inhibitors in both peaks 6 and 7 were strongly attached to the DEAE-52 anion exchange column, and required a high concentration of NaCl to elute them. This attachment suggests the acidic nature of the virus inhibitors in these peaks.

Peaks 1, 4 and 5 did not show significant inhibition against TNV giving activity quotients of 0.93, 0.92 and 1.01 respectively.

Peak 3, which was eluted by buffered 0.06 M NaCl, showed no inhibition, but it gave an activity quotient of 1.27 which suggests slight augmentation or enhancement of virus activity. Peak 3 consisted of four bands of protein and three of glycoprotein of similar Rf values to the upper three bands. This peak seemed to affect the inhibitors found in the whole extract of P. vulgaris. This idea was confirmed when DEAE-52 chromatography was performed on the non-inhibitory unheated extract, and the heated extract, which gave 46% inhibition. Seven peaks were obtained from the unheated extract which proved to be similar to those obtained from Ext 4.2 (French bean) since they were eluted at the same NaCl concentration and showed similar activity against TNV. However, the heated one contained only six peaks, where peak 3 seemed to be destroyed. This suggests that peak 3 contains heat labile masking compounds. This was proved when a special experiment was carried out in which one ml of peak 3 was mixed with an equal volume of each of the inhibitory peaks. The mixtures were tested against TNV, and it was found that peak 3 masked the inhibitory activity of peaks 2, 6 and 7.

Therefore, P. vulgaris seed extract is a complicated extract which contains at least three virus inhibitors, one of which is basic in nature and two which are acidic. The three inhibitors are each homogeneous glycoproteins. The extracts contain also complex masking compounds which appear to consist, from disc electrophoresis studies, of three glycoproteins and one protein.
Some workers have tried to purify and isolate virus inhibitors from *P. vulgaris* sap. Thus, Mart (1972) obtained a rough separation of the proteinaceous inhibitory principle from *P. vulgaris* sap by using centrifugation, phenol extraction and ethanol precipitation techniques. Recently, using different procedures, Taniguchi (1974) reported the presence of two relatively low molecular weight inhibitory substances in French bean leaf sap which were diffusable in sepharose 2B gel; however, their chemical nature was not studied. No detailed work on the extraction of plant virus inhibitors from *P. vulgaris* seeds has been reported. However, as mentioned previously, some considerable interest has been shown in *P. vulgaris* seed extracts since they have been shown to contain glycoproteins with hemagglutination and mitogenic activity. Such glycoproteins are called phytohemagglutinins and, together with similar glycoproteins from legumes and other seeds, are named lectins (Haapiz and Richter, 1968).

Bearing in mind the properties described in this thesis for *G. max* and *P. vulgaris* seed extracts, it might be suggested that glycoproteins found in both extracts are involved in plant virus inhibition. It would seem appropriate, therefore, to test the virus inhibitor fractions from both *G. max* and *P. vulgaris* for trypsin inhibition, hemagglutination activity, and also to examine lectins against plant viruses. Results of these experiments will be described in detail in the next chapter.
CHAPTER VI

COMPARISON OF G. MAX AND P. VULGARIS VITAE EXTRACTS WITH PLANT LECTINS AND TRYPsin INHIBITOR

DEAE-52 fractions of Ext \(_{93}^{4.2}\) (Soybean) and Ext \(_{93}^{4.2}\) (French bean) described in the previous chapter, were compared for hemagglutination and trypsin inhibition activity with commercially available soybean trypsin inhibitor, and lectins extracted from seeds of P. vulgaris (PHA), G. max (CBA) and Canavalia ensiformis (Con A).

SECTION A

HEMAGGLUTINATION AND TRYPSIN INHIBITION ACTIVITY OF THE VITAE EXTRACTS EXTRACTED FROM G. MAX SEEDS

P. vulgaris (Rigas and Cagood, 1954) and G. max (Lis et al., 1970) are among the legume seeds which possess glycoproteins called agglutinins with remarkable ability to agglutinate erythrocytes. This phenomenon is called hemagglutination and such agglutinins act by combining to specific receptor sites on the surface of the erythrocytes. P. vulgaris agglutinin combined with N-acetyl-D-galactosamine (D-Gal NAc) residues (Borberg et al., 1965); on the other hand soybean agglutinin combined with D-Gal NAc and D-galactose-like residues (Lis et al., 1970).

(a) Hemagglutination activity of DEAE-52 fractions from Ext \(_{93}^{4.2}\)

(1) Preparation of standard erythrocyte suspension

Fresh venous whole rabbit blood was added to an equal volume of Alsever's solution containing 1/30 volume of anticoagulant (sodium citrate 3 g, formaldehyde 37.5, saline 100 ml). The erythrocytes were collected by centrifugation at 200 g for
three minutes and were washed three times with PBS (phosphate-buffered saline). The washed erythrocytes were added to PBS to give a suspension with an absorbance of 2 at 620 nm. The diluted suspension was referred to as standard erythrocyte suspension (Lien, 1955).

(ii) Hemagglutination activity assay

The hemagglutination reaction was demonstrated in standard hemagglutination trays. Samples of each of the seven peaks were diluted with PBS in a two-fold dilution series. Each well contained 0.5 ml of the seed extract peaks and to these were added 0.5 ml of standard erythrocyte suspension. Control wells contained PBS and erythrocytes only (Waterson, 1963). The hemagglutination activity (HA) was measured from the hemagglutinating titre. Fig. 55 shows that only peaks 2, 3 and 4, none of which inhibited INV infection, gave hemagglutination. The HA for these peaks was 1/2, 1/04 and 1/4 respectively. No hemagglutination was noticed in the inhibitory peaks 1, 5 and 6, nor in the non-inhibitory peak 7. Therefore, it can be concluded that the plant virus inhibitors are not the agglutinin of the seed extract. The seven peaks were also tested for trypsin inhibition activity.

(b) Trypsin inhibition activity of fDEX-52 fractions from Ext93

Trypsin inhibition activity was assayed for the seven peaks obtained from Ext93 (soybean) by a modification of the Serac Laboratories method (1963), based on the original method of Schwert and Takenaka (1955) in which the synthetic substrate N-benzoyl-L-arginine ethyl ester (BAEE) is used. The reaction consisted of BAEE, 2.5 x 10^-4 M in borate buffer pH 9; trypsin 40 μg/ml in a
solution of 0.001 N HCl and trypsin inhibitor 0.2 mg/ml in borate buffer pH 9.

The change in optical density (ΔE) was measured at 253 με at zero time (t₀) and then after five minutes (t₅ min). The difference between the amount of BAPN hydrolyzed by the control (trypsin plus water) and experimental (trypsin plus trypsin inhibitor) systems gave a measure of the residual trypsin activity.

Trypsin inhibition activity was expressed as micrograms of trypsin inhibited per microgram of test material and is referred to as the specific activity of the inhibitor.

Only peaks 5 and 6 showed trypsin inhibition activity (Table 42). It was decided therefore to test the commercial soybean trypsin inhibitor (Sigma Chemical Company, U.S.A.) at two different concentrations (1 mg/ml and 0.2 mg/ml) on TNV infection. Results in Table 43 show that at high concentration (1 mg/ml) the trypsin inhibitor gave 37.3% inhibition of local lesion production by TNV on French bean leaves. At low concentration, however, the trypsin inhibitor had no effect on TNV infection. This experiment shows that a compound with trypsin inhibitory property can also inhibit plant virus infection. However, the commercial soybean trypsin inhibitor is not identical with the virus inhibitor extracted in these experiments because the virus inhibitors have been described earlier (Chapter 5) to be mainly glycoproteins, while the trypsin inhibitor is thought to be a pure protein (Wu and Scheraga, 1962). Furthermore, peak 1 was inhibitory against TNV but showed no trypsin inhibition activity.
Fig. 35  Homagglutination activity assay of DEAE-52 fractions from Ext$^{4.2}_9$ (Soybean)

<table>
<thead>
<tr>
<th>Peak No.</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>512</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
TABLE 42

Trypsin inhibition activity of BLAb-52 fractions from Ext_{93} (Soybean)

<table>
<thead>
<tr>
<th>Peak</th>
<th>Optical density</th>
<th>$\Delta E_{253}$</th>
<th>BAE unit/ml</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_2$</td>
<td>$t_0$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.36</td>
<td>0.10</td>
<td>0.26</td>
<td>5,200</td>
</tr>
<tr>
<td>1</td>
<td>0.33</td>
<td>0.11</td>
<td>0.27</td>
<td>5,200</td>
</tr>
<tr>
<td>2</td>
<td>0.36</td>
<td>0.09</td>
<td>0.27</td>
<td>5,400</td>
</tr>
<tr>
<td>3</td>
<td>0.31</td>
<td>0.04</td>
<td>0.27</td>
<td>5,100</td>
</tr>
<tr>
<td>4</td>
<td>0.30</td>
<td>0.02</td>
<td>0.23</td>
<td>5,600</td>
</tr>
<tr>
<td>5</td>
<td>0.12</td>
<td>0</td>
<td>0.12</td>
<td>2,400</td>
</tr>
<tr>
<td>6</td>
<td>0.32</td>
<td>0.03</td>
<td>0.24</td>
<td>4,600</td>
</tr>
<tr>
<td>7</td>
<td>0.30</td>
<td>0.34</td>
<td>0.25</td>
<td>5,200</td>
</tr>
</tbody>
</table>

* No trypsin inhibition
TABLE 15

Effect of soybean trypsin inhibitor on local lesion production by TNV

<table>
<thead>
<tr>
<th>Trypsin inhibitor (concentration)</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + water</td>
<td>TNV + trypsin inhibitor</td>
<td></td>
</tr>
<tr>
<td>1 mg/ml</td>
<td>33.99</td>
<td>21.2</td>
<td>0.63</td>
</tr>
<tr>
<td>0.2 mg/ml</td>
<td>39.00</td>
<td>39.1</td>
<td>1.00</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications
SECTION B
Hemagglutination and Trypsin Inhibition Activity of the Virus
Inhibitors Extracted from M. vulgaris Seeds

(a) Hemagglutination activity of D1AE-52 fractions from Ext 4.2

(French bean)

The hemagglutinating activity (HA) was assayed in the same way as for Ext 4.2 (Soybean) fractions. Results in Fig. 36 show that only peaks 2, 3, 4 and 5 gave hemagglutination and the HA was 1/16 for the inhibitory peak 2 and 1/32, 1/2 and 1/4 respectively for the other non-inhibitory peaks. No agglutination was obtained in the non-inhibitory peak 1 and the inhibitory peaks 6 and 7. Therefore, it can be concluded that the virus inhibitor in peak 2 has some hemagglutinating activity while the virus inhibitors in peaks 6 and 7 do not agglutinate the erythrocytes.

The seven peaks were also tested for trypsin inhibition activity.

(b) Trypsin inhibition activity of D1AE-52 fractions from Ext 4.2

(French bean)

By testing the D1AE-52 fractions of Ext 4.2 (French bean), only peaks 4, 5 and 6 gave trypsin inhibition activity. Maximum trypsin inhibition was found in the virus inhibitory peak 6, giving a specific activity of 4. Peaks 4 and 5 gave much lower specific activities, 0.67 and 2 respectively (Table 44).

SECTION C
Effect of Lectins on Local Lesion Protection by TNV

(a) Phytococcum lectin (PHA)

PHA is an aqueous extract from seeds of the red kidney bean (Phaseolus vulgaris) (Li and Osgood, 1949). A number of PHA preparation are available commercially, and it is not always clear
Fig. 36. Hemagglutination activity assay of DEAE-52 fractions from $\text{Ext}^{4+2}_{95}$ (French bean).

Dilution:

<table>
<thead>
<tr>
<th></th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>512</th>
</tr>
</thead>
</table>

Peak No.

Control
# TABLE 44

## Trypsin inhibition of DEAE-52 fractions

from Ext<sup>4.2</sup> (French bean)

<table>
<thead>
<tr>
<th>Peak</th>
<th>Optical density</th>
<th>$\Delta E_{253}$</th>
<th>BALE units/ml</th>
<th>Specific activity</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$t_5$</td>
<td>$t_0$</td>
<td>5,600</td>
<td>5,600</td>
</tr>
<tr>
<td>1</td>
<td>0.36</td>
<td>0.08</td>
<td>0.23</td>
<td>5,600</td>
</tr>
<tr>
<td>2</td>
<td>0.37</td>
<td>0.03</td>
<td>0.21</td>
<td>5,600</td>
</tr>
<tr>
<td>3</td>
<td>0.30</td>
<td>0.02</td>
<td>0.29</td>
<td>5,600</td>
</tr>
<tr>
<td>4</td>
<td>0.32</td>
<td>0.05</td>
<td>0.27</td>
<td>5,400</td>
</tr>
<tr>
<td>5</td>
<td>0.35</td>
<td>0.03</td>
<td>0.27</td>
<td>5,400</td>
</tr>
<tr>
<td>6</td>
<td>0.31</td>
<td>0.05</td>
<td>0.26</td>
<td>5,200</td>
</tr>
<tr>
<td>7</td>
<td>0.33</td>
<td>0.10</td>
<td>0.28</td>
<td>5,600</td>
</tr>
</tbody>
</table>

* No trypsin inhibition
how such preparations are made. It seemed prudent therefore to test
a variety of commercially produced FIA for effects on TNV.

(i) Effect of FIA (Wellcome), FIA(P) and FIA(M) on local lesion
production by TNV.

One mg each of FIA (Wellcome), a freeze-dried sample obtained
from Wellcome Laboratories, FIA(P) and FIA(M) preparations
produced by Difco Ltd., were dissolved in 10 ml of water and
tested against TNV. Controls consisted of 1 ml of water and 1 ml
of TNV. Inoculations were randomised on French bean leaves.
Results in Table 45 show that FIA (Wellcome) has no inhibitory
effect against TNV infection. However, FIA(P) and FIA(M) were
inhibitory, giving 23% and 29% inhibition respectively.

Disc electrophoresis experiments were also performed on the FIA
samples to gain some knowledge of their constituents.

(ii) Disc electrophoresis of FIA (Wellcome), FIA(P) and FIA(M).

Fig. 37 shows that FIA (Wellcome) consisted only of one large band
of protein and one of glycoprotein which were located on the upper
part of the gel (Rf = 0.44). FIA(P) contained four bands of
protein (Rf = 0.42, 0.63, 0.77 and 0.92) and two bands of glyco-
protein (Rf = 0.41 and 0.62). FIA(M) contained three bands of
protein (Rf = 0.52, 0.64 and 0.86) and four bands of glycoprotein
(Rf = 0.12, 0.49, 0.63 and 0.90). Therefore, it seems likely
that the bands found to be involved in plant virus inhibition
with Rf 0.43 (peak 6) and 0.64 (peak 7) are found in FIA(P) and
FIA(M) respectively. This might explain why FIA(P) and FIA(M)
are inhibitory against TNV.

Since FIA extracted from P. vulgaris seeds are famous in
agglutinating erythrocytes, it was decided to assay FIA (Wellcome),
FIA(P) and FIA(M) for hemagglutination activity so as to compare
TABLE 45

Effect of PHI on local lesion production
by TNV

<table>
<thead>
<tr>
<th>PHI</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHI (wellcome)</td>
<td>117</td>
<td>119</td>
<td>1.02</td>
</tr>
<tr>
<td>PHI (P)</td>
<td>126</td>
<td>76.3</td>
<td>0.72</td>
</tr>
<tr>
<td>PHI (N)</td>
<td>126</td>
<td>09.7</td>
<td>0.71</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.
Disc electrophoresis of PHA samples

Protein

Glycoprotein

Position of marker

Shading indicates intensity of band staining
it with the activity obtained from the peaks (2, 3, 4 and 5) isolated from Ext_{93}^{4.2} (French bean).

(iii) MIA(Welcombe), MIA(P) and MIA(H) hemagglutination activity assay

Maximum hemagglutination was obtained in MIA(Welcombe) giving a titre of 1/123. MIA(P) gave a hemagglutination titre of 1/32. Minimum hemagglutination was obtained when MIA(H) was mixed with the rabbit erythrocytes and the titre was 1/4 (Fig. 38). Hemagglutination test was also made to crude soybean agglutinin, and the results will be described in the following section.

(b) Crude soybean agglutinin (SBA)

At the time of this investigation, unlike MIA, pure forms of SBA were difficult to obtain. It was considered valuable, however, to test SBA against virus even though the hemagglutinating extracts from Ext_{93}^{4.2} (Soybean) had proved non-inhibitory. Soybean agglutinin was prepared in this study and its hemagglutinating activity compared with that of fractions extracted from Ext_{93}^{4.2} (Soybean).

(i) Preparation of crude soybean agglutinin (SBA)

Using the method of Lis et al. (1966), crude SBA was prepared from 500 gm of seed flour. Crude SBA was then tested for its inhibitory activity against TNV and for its hemagglutinating activity.

(ii) Effect of crude SBA on local lesion production by TNV

One ml of crude SBA was mixed with TNV and inoculated onto P. vulgaris leaves. Control consisted of TNV and water. The extract was very inhibitory, giving 95% inhibition (Table 46). Dilution reduced inhibition, suggesting that crude SBA contained an inhibitor and not a virus inactivator.

Crude SBA was next tested for hemagglutination activity.
Hemagglutination activity assay of PHA samples

<table>
<thead>
<tr>
<th>Dilution</th>
<th>2</th>
<th>4</th>
<th>8</th>
<th>16</th>
<th>32</th>
<th>64</th>
<th>128</th>
<th>256</th>
<th>512</th>
</tr>
</thead>
<tbody>
<tr>
<td>PHA(Wellcome)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHA(M)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PHA(P)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
**TABLE 46**

**Effect of various concentrations of crude SBA on local lesion production by TNV**

<table>
<thead>
<tr>
<th>Dilution (SBA)</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + water</td>
<td>TNV + SBA</td>
<td></td>
</tr>
<tr>
<td>Neat</td>
<td>15.8</td>
<td>0.8</td>
<td>0.05</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>17.2</td>
<td>9.9</td>
<td>0.38</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>21.0</td>
<td>15.3</td>
<td>0.73</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>25.6</td>
<td>19.4</td>
<td>0.76</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>25.5</td>
<td>22.4</td>
<td>0.83</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>39.7</td>
<td>34.3</td>
<td>0.36</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>23.0</td>
<td>23.3</td>
<td>1.01</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.
(iii) SBA hemagglutination activity assay

Hemagglutination activity was assayed as described previously. Crude SBA was diluted in a two-fold series with buffered saline to a 1/512 dilution. Controls consisted of buffered saline and standard erythrocytes suspensions. Results in Fig. 39 show that crude SBA has an agglutination titre of 1/256.

Crude SBA showed not only inhibition against TNV, but also hemagglutinating activity. On the other hand, the three peaks (2, 3 and 4) fractionated from Ext\(^{4.2}\) (Soybean) and showing hemagglutination activity, were not inhibitory against TNV.

Conversely, peaks 1, 5 and 6, which showed no hemagglutination activity, inhibited TNV. It would seem therefore, that crude SBA, as the name suggests, contains plant virus inhibitors as well as agglutinins. To analyse this situation further, disc electrophoresis experiments were undertaken on crude SBA.

(iv) Disc electrophoresis of crude SBA

Fig. 40 shows that crude SBA contains four bands of material giving positive reaction in protein tests (Rf = 0.27, 0.44, 0.66 and 0.83) and four bands reacting in glycoprotein tests (Rf = 0.39, 0.44, 0.67 and 0.84). It would appear, therefore, because of the similarity of Rf values between compounds reacting in these two tests, that SBA contains four glycoproteins. SBA has been known by other workers to consist of glycoprotein (Lis and Sharon, 1973).

Glycoproteins of Rf value 0.27 and 0.43 were also found in the virus inhibitory peaks 1 and 6 as separated by DEAE-52 chromatography of Ext\(^{4.2}\) (Soybean). On the other hand, peak 3 of this extract, which was non-inhibitory to virus but which gave maximum hemagglutination, also contained glycoproteins, two of which with
Fig. 39  Hemagglutinating activity of crude soybean agglutinin (SBA), Ext\textsuperscript{4.2} \textsubscript{93} (French bean) and Ext\textsuperscript{4.2} \textsubscript{93} (Soybean)

<table>
<thead>
<tr>
<th>Dilution</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
</tr>
</tbody>
</table>

Ext\textsuperscript{4.2} \textsubscript{93} (French bean)

Ext\textsuperscript{4.2} \textsubscript{93} (Soybean)

Crude SBA

Control
Fig. 40

Disc electrophoresis of crude soybean agglutinin (SBA)

Crude SBA

- [Protein]  [Glycoprotein]

- position of marker

Shading indicates intensity of band staining
If values of 0.67 and 0.80 corresponded to similar molecules (If values 0.66 and 0.83) from SBA.

These results indicate that crude SBA contains virus inhibitors which are distinct from the agglutinating compounds.

To examine further the effect of agglutinins on plant viruses the pure agglutinin (Con A) from Canavalia ensiformis seeds was also tested against TNV.

(c) Effect of Con A on local lesion production by TNV

Although C. ensiformis (Jack bean) seed extract gave 73% inhibition when it was tested against virus, as mentioned in chapter 3, Con A at various concentrations showed no inhibitory effect (Table 47). It seems that during the preparation and purification of the agglutinin Con A, only fractions which agglutinate erythrocytes are isolated.

DISCUSSION

A detailed discussion of the implications of these results will be given in the final chapter. It seems appropriate, however, at this stage to summarize briefly the results of this chapter and to make some comparison between the two species examined.

The work described in this chapter has established that in the case of soybean, the crude seed extract contains hemagglutinating glycoproteins similar to those in the SBA and that such extracts also inhibit virus. The virus inhibitor glycoproteins are not, however, identical to the hemagglutinins.

Crude G. max seed extracts were also shown to contain glycoprotein with trypsin inhibition activity. These same extracts possessed the ability to inhibit virus. Commercially available trypsin inhibitors also inhibited virus. However, trypsin inhibitor in crude G. max seed extract appears to differ from that commercially available
### Table 47

**Effect of various concentrations of Con A on local lesion production by TNV**

<table>
<thead>
<tr>
<th>Dilution</th>
<th>Mean number of lesions **</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>TNV + water</td>
<td>TNV + Con A</td>
<td></td>
</tr>
<tr>
<td>Neat *</td>
<td>78.5</td>
<td>75.0</td>
<td>0.96</td>
</tr>
<tr>
<td>$10^{-1}$</td>
<td>69.5</td>
<td>69.1</td>
<td>0.99</td>
</tr>
<tr>
<td>$10^{-2}$</td>
<td>70.4</td>
<td>68.6</td>
<td>0.97</td>
</tr>
<tr>
<td>$10^{-3}$</td>
<td>69.3</td>
<td>72.6</td>
<td>1.05</td>
</tr>
<tr>
<td>$10^{-4}$</td>
<td>69.1</td>
<td>83.6</td>
<td>0.99</td>
</tr>
<tr>
<td>$10^{-5}$</td>
<td>73.3</td>
<td>69.8</td>
<td>0.95</td>
</tr>
<tr>
<td>$10^{-6}$</td>
<td>66.3</td>
<td>66.5</td>
<td>1.06</td>
</tr>
</tbody>
</table>

* 0.1 mg/ml

** Each figure represents the mean number of lesions for ten replications
since the former consisted of glycoprotein and the latter is proteinaceous in nature.

Crude extracts of *P. vulgaris* contain some glycoproteins with hemagglutinating activity and others with trypsin inhibition activity. One glycoprotein, showing hemagglutination activity, also inhibited plant viruses. A second virus inhibitory peak showed trypsin inhibition activity, but a third peak which inhibited virus showed neither trypsin inhibitor activity nor hemagglutination activity.

HLA from commercial sources varies in its properties. This was demonstrated when pure HLA from different commercial sources were tested against virus. Thus, the more homogeneous HLA (Wellcome) gave no virus inhibition, but heterogeneous HLA (P) and HLA (H) each inhibited virus.
It is well known that in order to infect plants mechanically with virus, host tissue must be wounded, usually by abrasion. The act of wounding creates or exposes infectible sites which are converted to infective centres upon interaction with virus particles (Siegel, 1966). When the inoculum contains virus inhibitor, no infection, or a low percentage only, is obtained. The reason might be explained in more than one way, and there is controversy about how plant virus inhibitors act. Some workers believe that they act by altering the susceptibility of the host plant, probably by blocking the infectible sites on the leaf surface. Among such inhibitors are leaf extracts of Beta vulgaris, Capsicum annuum and Datura stramonium (Paliwal and Nariani, 1965).* El-Sandelgy and Willcoxson (1966) suggest that the inhibitor extracted from Trifolium pratense flowers altered the host cells physiologically and therefore they are no longer susceptible to the virus. These results agree with those of Simons et al. (1963) who studied the mode of action of virus inhibition by 75 succulent plant species. Yoshii (1969), who studied the effects of the Chenopodium sap inhibitor against TNV and cucumber mosaic virus, showed that the effect of the inhibitor was due to interference in the formation of a virus-receptor complex at the susceptible site. Lal et al. (1973) reported that inhibitors from Datura metel acted by affecting the host susceptibility to TNV.

The other school of thought believes that plant virus inhibitors form a non-infectious complex with the virus, rather than by acting on the host plant. This idea is supported by Fulton (1943) using leaf extracts of Phytolacca decandra. Paliwal and Nariani (1965)
attributed the inhibitory activity of Carica papaya latex to its ability to bind to sunnhemp mosaic virus in an unbreakable virus-inhibitor complex. Palm (1967) described a plant virus inactivator system from the leaves of Nicotiana glutinosa. Recently, Ibrahim-Nesbat (1971) described the mode of action of TNV inhibitors extracted from spinach leaves (Spinacia olereacea). By electron microscopy he showed that inhibition is caused by aggregation of virus particles.

Therefore, it seems that plant virus inhibitors fall into two categories, one including inhibitors affecting the virus, and the other in which the host plant is influenced. In this chapter the mode of action of seed extracts of G. max (Section A) and P. vulgaris (Section B) will be studied.

SECTION A

MODE OF ACTION OF G. MAX SEED EXTRACT

1. DO G. MAX INHIBITORS ACT BY AFFECTING TNV?

In order to answer this question an experiment was designed in which TNV was incubated with the seed extract for 15 minutes. The virus was then recovered by ultra-centrifugation, and its infectivity tested and compared with untreated TNV. In this way any possible change in the virus, induced by the seed extract, could be detected. Prior to undertaking this experiment the opaque crude seed extract required clarification by ultra-centrifugation and this experiment is described first.

Lyophilized G. max seed extract prepared as described previously, was spun at 11,000 g for 30 minutes to obtain a clear extract. Both the precipitate and the supernatant were tested against TNV. The precipitate gave no inhibitory effect; however, the supernatant gave 53% inhibition, which was nearly the same as the whole seed extract
(69% inhibition) tested at this time. This level of inhibition is lower than that described earlier (Chapter II) when 92% inhibition was obtained. This reduction in the percentage inhibition was thought to be due to seasonal effects on the susceptibility of French bean leaves to infection, since samples of the same seed extract tested some weeks before and after this experiment each produced 89-93% inhibition.

Having established that the inhibitors extracted from G. max seeds were still in the clarified supernatant, 10 ml of this supernatant was mixed with 10 ml of TNV. The mixture was kept at room temperature for 15 minutes. Control mixture consisted of an equal volume of TNV and water. Treated and control mixtures were then spun at 100,000 g for two hours. The pellet of virus was washed with water to remove any residual inhibitor. Finally, the pellets in both the treated and control tubes were resuspended in 5 ml of water. Two ml of each were inoculated on French bean leaves. Results in Table 48 show that similar numbers of lesions were obtained for both. Statistical analysis also proved that there was no inhibition.

Therefore, it can be concluded that G. max inhibitors do not act by affecting virus particles, and it seems likely that they affect the susceptibility of French bean leaves to infection by TNV. To examine this, further experiments were undertaken to test the effects of seed extracts on French bean leaf surfaces.

2. **EFFECT OF DIPPING FRENCH BEAN LEAVES IN G. MAX SEED EXTRACT**

(a) **Effect of dipping French bean leaves in G. max seed extract one hour before inoculation by TNV**

(1) Dipping the whole leaf

This experiment was performed on ten intact French bean plants.
TABLE 43

Effect of G. max seed extract on TNV

<table>
<thead>
<tr>
<th>Treatment of TNV</th>
<th>Mean number of lesions * following ultra-centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ H₂O</td>
<td>177</td>
</tr>
<tr>
<td>+ Seed extract</td>
<td>163</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.

TABLE 49

Effect of dipping French bean leaves in G. max seed extract one hour before inoculation with TNV

<table>
<thead>
<tr>
<th>Surface dipped</th>
<th>Mean number of lesions * for leaves dipped into:</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>water</td>
<td>seed extract</td>
<td></td>
</tr>
<tr>
<td>whole leaf</td>
<td>44.3</td>
<td>3.80</td>
<td>0.09</td>
</tr>
<tr>
<td>upper</td>
<td>46.2</td>
<td>4.13</td>
<td>0.09</td>
</tr>
<tr>
<td>lower</td>
<td>51.0</td>
<td>55.00</td>
<td>1.03</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.
One leaf of each of the ten plants was dipped for two minutes into a petri dish containing 20 ml of G. max seed extract. The opposite control leaf was dipped into 20 ml distilled water. After drying in air at room temperature for one hour, all the leaves were inoculated with TNV. Hands were washed after inoculating each leaf so as to prevent any G. max seed extract being transferred to the inoculum. Results in Table 49 show that G. max seed extract was inhibitory, giving 91% inhibition.

(ii) Dipping the upper surface of the leaf
Using the same procedure, only the upper surfaces of the leaves were dipped in the seed extract or water. Results in Table 49 show that the inhibition was again 91%.

(iii) Dipping the lower surface of the leaf
When the lower surfaces of the French bean leaves were dipped in G. max seed extract, similar numbers of lesions were obtained on treated and control leaves.

In these experiments the seed extract inhibits when applied to the upper surface but has no effect if applied from below (Table 49). These results suggest that the extract either alters the leaf surface and affects the entry of the virus into the leaf, or the extract enters the leaf with TNV and then affects the virus multiplication by affecting cell metabolism in some way.

(b) Effect of dipping French bean leaves into G. max seed extract followed by rinsing with water before TNV inoculation

This experiment was performed in order to gain some idea as to whether G. max seed extract enters into French bean leaves through the upper surfaces, thus affecting TNV after entering the leaves. Ten French bean leaves were treated in the same way as when the whole leaves were dipped in the seed extract as described above. Control
leaves were dipped in water. After one hour, the leaves were rinsed with tap water and left to dry for 15 minutes. TNV was then inoculated on the treated and control leaves. Results in Table 50 show that rinsing the treated leaves with tap water had removed the extract, and the number of lesions produced on treated leaves were much the same as that produced on the control leaves, giving an activity quotient of 1.02. Therefore it can be concluded that the extract alone does not enter and modify the leaf in any way. It was also proved previously in this section that the extract does not act by affecting the virus itself. Another possible mode of action is that the extract affects the entry of TNV through the leaf surface. Alternatively, the seed extract might need to enter the leaf along with the virus and so influence the initial attachment of virus to infectible sites on cells. Experiments described later in this section will help to elucidate this problem further.

(c) Effect of dipping French bean leaves into G. max seed extract at different time intervals before and after inoculation by TNV

(i) Effect of dipping 1, 3, 6, 9 and 24 hours before inoculation

Since dipping French bean leaves in G. max seed extract one hour before inoculation with TNV gave inhibition, it was decided to dip French bean leaves at different time intervals before inoculation in order to find out how long seed extracts remain effective in producing inhibition.

One primary leaf of each of 50 French bean plants was dipped into seed extract. The opposite control leaf was dipped into water. After one hour, ten plants were inoculated with TNV. The remaining plants were divided into batches of ten and inoculated 3, 6, 9 and 24 hours after dipping.

Results in Table 51 show that G. max seed extract was inhibitory
### TABLE 50

Effect of dipping French bean leaves into *G. max* seed extract followed by rinsing with water before TNV infection

<table>
<thead>
<tr>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>Treated</td>
<td></td>
</tr>
<tr>
<td>32.5</td>
<td>33.2</td>
<td>1.02</td>
</tr>
</tbody>
</table>

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

### TABLE 51

Effect of dipping French bean leaves in *G. max* seed extract at different time intervals before inoculation by TNV

<table>
<thead>
<tr>
<th>Hours before inoculation</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>50.0</td>
<td>6.0</td>
<td>0.12</td>
</tr>
<tr>
<td>3</td>
<td>54.0</td>
<td>27.0</td>
<td>0.50</td>
</tr>
<tr>
<td>6</td>
<td>46.3</td>
<td>27.0</td>
<td>0.58</td>
</tr>
<tr>
<td>9</td>
<td>46.0</td>
<td>29.7</td>
<td>0.65</td>
</tr>
<tr>
<td>24</td>
<td>63.9</td>
<td>66.2</td>
<td>1.04</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications
when applied up to 9 hours before inoculation by TNV. There is a slow loss of inhibitory power with complete loss after 24 hours. Presumably the inhibitors become inactivated in some way. In this experiment virus inhibitors, which remain active on leaf surfaces, presumably enter the leaf together with the virus and bring about their inhibitory effects within the leaf.

(ii) Effect of dipping 1, 3, 6, 9 and 24 hours after inoculation

The leaves of 50 French bean plants were first inoculated with TNV and then after 1, 3, 6, 9 and 24 hours, batches of ten plants were treated by dipping one leaf into seed extract and the opposite control leaf into water.

Results in Table 52 show that G. max seed extract had no inhibitory effect on local lesion production if applied after inoculation of TNV. This supports the earlier suggestion that G. max seed extract does not change the leaf surface and is only effective when accompanying the virus during entry into the leaf. Although it seems that the inhibitors from G. max have no effect on the virus itself, it was thought useful to test two other viruses against the inhibitors. For these experiments two rod-shaped viruses, THV and PVX, were selected.

3. EFFECT OF G. MAX SEED EXTRACTS ON LOCAL LESION PRODUCTION BY TNV AND PVX

(a) TNV

One ml of the seed extract was mixed with 1 ml of partially purified TNV (legume strain) and inoculated onto ten half leaves of Nicotiana tabacum var. Xanthi. Control consisted of an equal volume of TNV and water. Results in Table 53 show that the seed extract was inhibitory, giving 64% inhibition.
TABLE 52

Effect of dipping French bean leaves in G. max seed extract at different time intervals after inoculation by TMV

<table>
<thead>
<tr>
<th>Hours after inoculation</th>
<th>Mean number of lesions</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>67.2</td>
<td>67.9</td>
<td>1.01</td>
</tr>
<tr>
<td>3</td>
<td>53.6</td>
<td>53.4</td>
<td>1.09</td>
</tr>
<tr>
<td>6</td>
<td>69.4</td>
<td>72.3</td>
<td>1.04</td>
</tr>
<tr>
<td>9</td>
<td>55.7</td>
<td>59.6</td>
<td>1.07</td>
</tr>
<tr>
<td>24</td>
<td>54.5</td>
<td>56.7</td>
<td>1.04</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.

TABLE 53

Effect of G. max seed extract on local lesion production by TMV and PVX

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean number of lesions</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>seed extract</td>
<td></td>
</tr>
<tr>
<td>TMV *</td>
<td>72.3</td>
<td>26.1</td>
<td>0.36</td>
</tr>
<tr>
<td>PVX **</td>
<td>7.9</td>
<td>1.2</td>
<td>0.13</td>
</tr>
</tbody>
</table>

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

** PVX tested on ten leaves of Gomphrena globosa
One ml of G. max seed extract was mixed with 1 ml of partially purified PVX and inoculated on Gomphrena globosa leaves. Control consisted of PVX and water. Results in Table 53 show that G. max was inhibitory, giving 85% inhibition.

DISCUSSION

Experiments have been employed to determine whether G. max seed extract reduces infectivity by affecting the virus (TNV) or the host (French bean leaves). Previous experiments on dilution indicated that the extract contains virus inhibitors and not inactivators, since the percentage inhibition was reduced as the extract was diluted up to 10^{-5} dilution.

In this section of the thesis, experiments have been described which show that virus recovered from treatment with inhibitor appeared to be unchanged. These results seem to agree with those obtained by Zaitlin and Siegel (1963) who used similar techniques and found that TNV virus inhibitor extracted from Nicotiana tabacum leaves, reduced the infectivity by affecting the host and not the virus. Crowley (1955) reported similar results when he studied the mode of action of the inhibitory extracts from Nicotiana species and Cucurbita sativa seeds on the infection of tobacco mosaic and cucumber mosaic virus respectively.

Many inhibitors of plant virus infection affect the host plants and they act by destroying or blocking the infectible sites. The work of Ragetli (1957) suggested that the inhibitor extracted from Dianthus caryophyllus (carnation) sap acts by blocking virus receptors on the surface of the leaf.

Van Hammen et al. (1961) supported this hypothesis by examining
the relationship between the number of local lesions on leaves of Nicotiana glutinosa and TN concentration, and the effect of the concentration of the inhibitor on this relationship. Their results showed that the action of the inhibitor was on the receptor sites in the leaf. Van Kammen et al. used the dipping method of the leaves for applying the virus inhibitor since it avoids the danger of the second rubbing where new wounds would be created or existing susceptible sites destroyed. Dipping was preferred as a method in the present work for studying the mode of action of G. max seed extract.

High percentage inhibition (91%) was obtained when the whole and the upper surface of the leaves were dipped into the extracts. This agrees with results obtained by Sharma and Raychaudhuri (1956) who found that extracts of chilli (Capsicum annuum), spinach (Spinacia oleracea) and strawberry (Fragaria vesca) were inhibitory when sprayed on leaf surfaces half an hour before PVX inoculation. In the experiments described here, on the other hand, no inhibition was obtained when the lower surfaces of French bean leaves were dipped in G. max seed extract. Similar results were obtained by Nart (1972), when the inhibitory P. vulgaris leaves extract showed no significant virus inhibition when it was applied to the lower surface of Nicotiana glutinosa leaves and TNV was applied to the upper surface. Different results were reported by Halm et al. (1960) who found that the inhibitory Cucurbita maxima seed extract showed virus inhibition when it was applied to the lower surface of Phaseolus vulgaris leaves.

Crowley (1955) also found that the inhibitor from Cucurbita maxima seeds affects local lesion production when applied to the underside of cowpea leaves.

The results obtained in this thesis suggest that the inhibitors
extracted from *G. max* seed extract act either by altering the susceptibility of the host cells for virus attachment, or by entering the cells and thereby creating conditions within the cells unsuitable for the replication of the virus. In experiments where French bean leaves were dipped into seed extract and then rinsed after one hour with water before inoculation by TMV, it was shown that similar numbers of lesions developed on the treated and control leaves. This confirms the idea that the inhibitors act by altering the susceptibility of the cells for virus attachment since no inhibitor was present to enter the leaves during subsequent abrasion during virus inoculation. This idea was substantiated by results obtained when the *G. max* seed extract was applied at various time intervals before and after virus inoculation. The extract was inhibitory when applied up to 9 hours before inoculation, but no inhibition was obtained when the extract was applied after inoculation. Similar results were obtained by Verma et al. (1965). They found that wheat seed extract (*Triticum aestivum*) is inhibitory when applied before the inoculation by TMV virus but is not so when applied after inoculation. This might be due to blockage of virus receptor sites as suggested by Gupta and Price (1950, 1952). Palm (1967) however, found no significant inhibition of infection when the inhibitory fractions from *Nicotiana glutinosa* were applied to *N. tabacum* leaves prior to, or after, TMV inoculation. He interpreted these results as supporting the concept that the inhibitor acts by its effect on the virus rather than on the host.

The fact that *G. max* seed extract inhibits local lesion production of both TMV and PVX shows that the inhibitor works not only on receptor sites for spherical viruses, but also for rod-shaped viruses.
These results are also valuable in that they demonstrate that the inhibitor works in various host plants other than French bean.

SECTION E

MODE OF ACTION OF P. VULGARIS SEED EXTRACT

The mode of action of *P. vulgaris* seed extract was studied in the same way as for *G. max* seed extract; therefore, only the results will be described in this section.

1. DO *P. VULGARIS* INHIBITORS ACT BY AFFECTING TNV?

Seed extract was incubated with TNV at room temperature for 15 minutes and the mixture spun at 100,000 g for two hours to recover the virus. Controls consisting of TNV and water were treated in the same way. Results in Table 54 show that similar numbers of lesions were obtained when the treated virus and the control were each tested on French bean leaves, suggesting that the inhibitors did not affect the virus and therefore act by affecting the host.

2. EFFECT OF DIPPING FRENCH BEAN LEAVES INTO *P. VULGARIS* SEED EXTRACT

(a) Effect of dipping for various lengths of time before inoculation with TNV

Fifty French bean leaves were dipped into *P. vulgaris* seed extract and after 1, 3, 6, 9 and 24 hours, ten leaves were inoculated with TNV. Control leaves were dipped into water. Results in Table 55 show that *P. vulgaris* seed extract was not inhibitory when applied up to 24 hours before inoculation by TNV.

In a further experiment, in which the upper and lower surfaces of French bean leaves were separately treated with seed extract one hour before inoculation, no inhibition of local lesion production by
### TABLE 54

**Effect of *P. vulgaris* seed extract on TNV**

<table>
<thead>
<tr>
<th>Treatment of TNV</th>
<th>Mean number of lesions * following ultra-centrifugation</th>
</tr>
</thead>
<tbody>
<tr>
<td>+ H₂O</td>
<td>91.9</td>
</tr>
<tr>
<td>+ Seed extract</td>
<td>93.3</td>
</tr>
</tbody>
</table>

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

### TABLE 55

**Effect of dipping French bean leaves in *P. vulgaris* seed extract at different time intervals before inoculation by TNV**

<table>
<thead>
<tr>
<th>Hours before inoculation</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>76.6</td>
<td>75.1</td>
<td>0.98</td>
</tr>
<tr>
<td>3</td>
<td>76.7</td>
<td>67.2</td>
<td>0.95</td>
</tr>
<tr>
<td>6</td>
<td>76.4</td>
<td>76.6</td>
<td>1.03</td>
</tr>
<tr>
<td>9</td>
<td>76.4</td>
<td>73.7</td>
<td>0.94</td>
</tr>
<tr>
<td>24</td>
<td>64.7</td>
<td>63.3</td>
<td>1.06</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications
TNV could be detected. (Table 56). Similar results were obtained when French bean leaves were rinsed with tap water before inoculation by TNV (Table 57).

(b) **Effect of dipping for various lengths of time after inoculation with TNV**

French bean leaves were first inoculated by TNV and then, after 1, 3, 6, 9 and 24 hours dipped into *P. vulgaris* seed extract. Control leaves were dipped into water. Results in Table 58 show that *P. vulgaris* seed extract had no inhibitory effect when applied to the leaves after inoculation by TNV.

3. **EFFECT OF *P. VULGARIS* SEED EXTRACTS ON LOCAL LESION PRODUCTION BY TNV AND PVX**

(a) **TNV**

Lyophilized *P. vulgaris* seed extract was tested against TNV on half leaves of *Nicotiana tabacum* var. Xanthi. Control consisted of an equal volume of TNV and water. Results in Table 59 show that the seed extract was slightly inhibitory, giving 15% inhibition.

(b) **PVX**

One ml of *P. vulgaris* seed extract was mixed with 1 ml of PVX and inoculated onto *Gossypium hirsutum* leaves. Control consisted of 1 ml of PVX and 1 ml of water. Results in Table 59 show that the seed extract was not inhibitory against PVX, giving an activity quotient of 1.04.

**DISCUSSION**

In previous chapters it has been shown that crude *G. max* seed extract was consistently inhibitory (A.Q. = 0.07), whereas crude *P. vulgaris* seed extract varied from being non-inhibitory (A.Q. = 1.02)
### TABLE 56

**Effect of dipping French bean leaves in P. vulgaris seed extract one hour before inoculation by TNV**

<table>
<thead>
<tr>
<th>Surface dipped</th>
<th>Mean number of lesions * for leaves dipped into:</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>water</td>
<td>seed extract</td>
<td></td>
</tr>
<tr>
<td>whole leaf</td>
<td>13.9</td>
<td>13.1</td>
<td>0.96</td>
</tr>
<tr>
<td>upper surface</td>
<td>20.4</td>
<td>20.6</td>
<td>1.01</td>
</tr>
<tr>
<td>lower surface</td>
<td>17.3</td>
<td>16.9</td>
<td>0.93</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.

---

### TABLE 57

**Effect of dipping French bean leaves in P. vulgaris seed extract followed by rinsing with water before TNV infection**

<table>
<thead>
<tr>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>23.1</td>
<td></td>
</tr>
<tr>
<td>Treated</td>
<td>20.4</td>
<td>1.05</td>
</tr>
</tbody>
</table>

* Each figure represents the mean number of lesions for ten replications.
TABLE 58

Effect of dipping French bean leaves in *P. vulgaris* seed extract at different time intervals after inoculation by TMV

<table>
<thead>
<tr>
<th>Hours after inoculation</th>
<th>Mean number of lesions *</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Treated</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>76.1</td>
<td>79.9</td>
<td>1.05</td>
</tr>
<tr>
<td>3</td>
<td>69.9</td>
<td>69.2</td>
<td>0.99</td>
</tr>
<tr>
<td>6</td>
<td>80.3</td>
<td>82.7</td>
<td>1.03</td>
</tr>
<tr>
<td>9</td>
<td>73.1</td>
<td>70.2</td>
<td>0.96</td>
</tr>
<tr>
<td>24</td>
<td>66.0</td>
<td>63.4</td>
<td>0.96</td>
</tr>
</tbody>
</table>

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

TABLE 59

Effect of *P. vulgaris* seed extracts on local lesion production by TMV and PVX

<table>
<thead>
<tr>
<th>Virus</th>
<th>Mean number of lesions</th>
<th>Activity Quotient</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Water</td>
<td>Seed extract</td>
<td></td>
</tr>
<tr>
<td>TMV *</td>
<td>83.1</td>
<td>70.6</td>
<td>0.35</td>
</tr>
<tr>
<td>PVX **</td>
<td>8.1</td>
<td>8.4</td>
<td>1.04</td>
</tr>
</tbody>
</table>

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

** PVX tested on ten leaves of *Gomphrena globosa*
to being slightly inhibitory \((A.Q. = 0.70)\). \textit{P. vulgaris} extract treated with heat, or alcohol, and analysed by DIAE-52 cellulose chromatography was found, however, to contain virus inhibitors. Dilution experiments confirmed the presence of inhibitors as well as compounds masking the inhibitory activity in these seed extracts. In this section experiments have shown that seed extracts influence the host plant rather than the virus. In contrast, Lal et al. (1973) studied the mode of action of inhibitors of TMV from \textit{P. vulgaris} leaf extracts and suggested that inhibitors act by forming a loose complex with the virus.

\textit{P. vulgaris} seed extract was not inhibitory when applied either to upper or lower surfaces of test plant leaves, or when applied at various time intervals before or after inoculation of virus. However, in studying the inhibitor extracted from \textit{P. vulgaris} leaves, Nart (1972) found that the extract was inhibitory only when applied up to 48 hours before TMV was inoculated on to test plants of \textit{Nicotiana glutinosa}. No inhibition was obtained when the extract was applied after TMV inoculation. On the other hand, Lal et al. (1973) reported that, although \textit{P. vulgaris} 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.

These results can be interpreted as showing that inhibitors in leaf extracts of \textit{P. vulgaris} are different from those found in seed extracts. This interpretation is supported by the further observation of Lal et al. (1973) who found that \textit{P. vulgaris} leaf extract was not inhibitory against TMV on \textit{Nicotiana tabacum} var. Xanthi. In experiments described in this section, \textit{P. vulgaris} seed extracts showed some inhibition against TMV when inoculated on to \textit{N. tabacum} var. Xanthi.
CHAPTER VIII
GENERAL DISCUSSION

Although some discussion of the results has been made in each chapter of this thesis a more general discussion will be given here.

The experiments described in this thesis have produced considerable information regarding virus inhibitor fractions from legume seeds. In the survey of such seeds, for example, it was of particular interest to notice that they were all, for the most part, inhibitory to virus, and furthermore, heating these extracts either decreased inhibition as in the case of G. max, or increased inhibition as in the case of P. vulgaris.

Dilution studies of these two legumes inhibitor extracts have established that true inhibition and not virus inactivation is brought about by these seed extracts.

Careful preliminary studies of inhibitor fractions by techniques including dialysis, precipitation with alcohol or ammonium sulphate, as well as electrophoresis have indicated that they consist of high molecular weight compounds identifiable as proteins or glycoproteins.

In the introduction (Chapter I) attention has been drawn to the large volume of work carried out on plant virus inhibitors. Many workers have attributed inhibition to proteins and one of the earliest studies, identified an active virus inhibitor from leaves of Phytolacca esculenta to be a glycoprotein (Kassanis and Kleczkowski, 1963). Furthermore, Kart (1972) has shown that P. vulgaris leaf extracts contain proteinaceous virus inhibitors. Although these latter studies were not of seed extracts, it is not unreasonable to expect that protein/glycoprotein from seeds may also
act as inhibitors since the main nitrogen storage material in some legume seeds can be identified as glycoprotein (Derbyshire et al., 1975; Pusztai and Watt, 1970; Racuse and Foote, 1971).

The situation is likely to be complex, however, since *P. vulgaris* and *G. max* seed extracts contain a wide variety of compounds with varying effects on viruses. This was confirmed, using Sephadex when low molecular weight compounds with no inhibitory effect could be separated from inhibitors.

The complex nature of extracts demands more sophisticated methods of extraction and separation of proteins, although Sephadex gel filtration and disc electrophoresis proved valuable in confirming the protein/glycoprotein nature of inhibitors as well as establishing information regarding their molecular weights.

More detailed information was obtained from chromatography on DEAE columns which proved particularly valuable in identifying those components that determined the differences in activity between *G. max* and *P. vulgaris*. Thus, as discussed earlier (Chapter IV), *P. vulgaris*, unlike *G. max*, appears to contain compounds which in some way eliminate the effects of inhibitors. Such compounds are thought to mask the activity of the inhibitors; however, the mechanism of their action is not clear. They might act either by combining with the inhibitors or they might influence the leaf surface, thus affecting its response to virus inhibitors. Masking compounds have not been described before although Benda (1956) described compounds called augmenters in the inhibitory extracts from *Tetraponia expansa* which increased the activity of viruses. Recently Stevens (1970) reported the presence of augmenters in a number of crude seed extracts including *P. vulgaris*. In the experiments described in this thesis, *P. vulgaris* seed extract did
not give augmentation; however, fractionation of the extract by DEAE column chromatography revealed the presence of substances increasing virus activity which tended to mask the effects of inhibitors. It seems that the overall effect of P. vulgaris seed extract against TNV depends upon the ratio of masking compounds to inhibitor compounds. Thus the seed samples examined by Stevens (1970) might well have contained a predominance of masking or augmenter compounds and relatively small amounts of inhibitors.

In chapter VII, it is shown that inhibition in both G. max and P. vulgaris can be attributed to effects on leaf surfaces rather than on virus.

Initially, events in virus multiplication involve
(a) attachment of virus to some receptors;
(b) entry of virus or at least its nucleic acid into host cells.

The nature of the receptors for virus attachment is unknown but recently Miyamoto and Asamiya (1972) suggested that they might be ectodesmata (structures considered as connections of the protoplast up to the leaf cuticle (Brants, 1965)) and plasmodesmata (bridges of protoplasm which join cells of plant tissue (Franke, 1961)), particularly in the hair cell bases.

Inhibitors affecting host cells could operate by
(a) interfering with the initial attachment of the virus to the receptors;
(b) perhaps allowing attachment but preventing entry of virus or its nucleic acid into cells, or
(c) preventing nucleic acid multiplication and the synthesis of viral protein, so stopping the assembly of new virus particles.
Dipping experiments described in chapter VII would suggest that with seed extracts, mechanisms (a) or (b) are most likely to operate. The question therefore arises as to identity of those compounds present in seed extracts which could have some effect on cell surfaces and therefore influence virus receptors sites.

Extracts of P. vulgaris and G. max seeds are known to contain lectins (chapter I) which influence cells chiefly by:

(a) surface effects on membranes resulting for example in agglutination of erythrocytes due to the binding of lectins to saccharides on the surface of the cells, and

(b) effect at the nucleic acid level in inducing mitosis.

In view of these major biological activities of lectins, it seemed pertinent to establish whether lectins are responsible for virus inhibition (chapter VI).

The term lectin, however, is not very precise since, in the case of MLA, for example, a number of types are available. MLA(M), a mucoprotein, and MLA(P) predominantly a protein, both of which were inhibitory against TNV were found to consist of a number of components. On the other hand, MLA(Wellcome) consisted of a single large glycoprotein band with no inhibitory properties. Electrophoretic studies showed that with MLA(M) and (P) some of the many bands caused erythrocyte agglutination. Such bands were distinct from those causing virus inhibition with exception of one band of Rf (0.36) which gave both activities.

Crude soybean agglutinin (SBA) was also inhibitory against TNV, and electrophoresis showed that inhibitor glycoproteins were distinct from those causing erythrocyte agglutination.

Another lectin prepared from Jack bean seeds and available as a pure protein, Con A, was not inhibitory against TNV, although crude
extracts prepared from Jack bean seeds were highly inhibitory to virus. These observations suggest that, during the preparation of Con A and SBA, plant virus inhibitors are discarded with the non-agglutinating fractions.

Clearly, virus inhibiting and agglutinating properties of seed extract reside in different proteins. This conclusion is supported by the findings of Wyatt and Shepherd (1969) who isolated from *Phylloclaca americana* leaf extracts a virus inhibitor of molecular weight 15,000 with high lysine constituents. The glycoprotein with lectin-like activity extracted from *P. americana* root extracts (Reisfeld et al., 1967) had a molecular weight of 32,000, homogenous in nature and contained large amounts of cystine.

Recently, Finkelstein and McWilliams (1976) tested the effect of a range of plant lectins on a number of animal viruses and found varying degrees of inhibition.

The mechanism of protection afforded by the lectins is not clear. It is suggested that it might be due to effects of lectins on the virus or due to the direct alteration of host cell membranes making them unable to manifest or contribute to the destructive changes produced by the viruses.

Although the surface effect is a mechanism which looks to be common between lectins and virus inhibitors extracted from seeds, it seems unlikely that both act in the same way. Lectins, for example, combine with specific receptors on the membranes of erythrocytes (Borberg et al., 1966; Lis et al., 1970) with the resultant of agglutination of the cells. For example, phytohemagglutinin (PHA) combined with N-acetyl-D-galactosamine; soybean agglutinin (SBA) combined with D-galactose and N-acetyl-D-galactosamine; and Con A combined with α-D-glucopyranosyl,
α-D-mannopyranosyl and β-D-fructofuranosyl residues (Poste et al., 1974).

However, the plant virus inhibitors do not agglutinate erythrocytes suggesting that they cannot combine with the same membrane sites, and may well act by a different mechanism.

Despite the enormous volume of work on lectins, for the most part the isolation and purification of the biologically active components has not been achieved. Most lectins preparation appear to be heterogeneous and it is not clear whether different activities are due to different fractions. It is not surprising, therefore, that the relatively crude extracts of seeds proved to be even more complicated and difficult to analyse, since they contain amongst other things, fractions with agglutination, trypsin inhibition and virus inhibition activity. The work described in this thesis has helped to indicate the complex nature of these extracts and to elucidate some details of their antiviral activity.

CONCLUSIONS

The following general conclusions can be drawn from this work:

1. All the 18 varieties of legume seed extracts were inhibitory against TNV.
2. The unheated extracts fall into two categories: those which gave 75-95% inhibition and includes G. max (Soybean), and extracts in which the percentage inhibition ranged between 0-60%, for example P. vulgaris (French bean).
3. Inhibition was decreased by heating some extracts such as G. max. However, in other extracts such as P. vulgaris, the percentage inhibition was increased by heating.
4. P. vulgaris and G. max seed extracts were studied in detail.
5. Dilution of French bean and soybean seed extracts confirmed the
presence of plant virus inhibitors and not inactivators.

6. Both extracts were inhibitory against TNV; however, only soybean showed inhibition against IVN.

7. Spectrophotometric absorption showed that none of the inhibitors in French bean and soybean is nucleic acid.

8. Dialysis, precipitation with alcohol or ammonium sulphate and disc electrophoresis experiments suggested that the inhibitors in both extracts are composed of proteins and glycoproteins.

9. Sephadex G-100 gel filtration proved useful in indicating the molecular weights of the inhibitor fractions and also in allowing the separation of low molecular weight non-inhibitory compounds.

10. Seven fractions were obtained from DEAE-52 chromatography of soybean seed extract. Three of the fractions were inhibitory against TNV. One of the inhibitory fractions is basic in nature while the other two are acidic. None of the virus inhibitors agglutinated erythrocytes; however, the acidic inhibitors showed trypsin inhibition activity.

11. Seven fractions were also obtained from DEAE-52 chromatography of French bean seed extract. Three of the fractions were inhibitory against TNV. One of the inhibitory fractions is basic in nature while the other two are acidic.

12. French bean, unlike soybean, in addition to the inhibitors yielded one fraction containing compounds which reduced the activity of the inhibitors; such compounds are termed masking compounds. Such masking compounds are heat labile and their presence explains the different heat responses between soybean and French bean.

13. The basic glycoprotein virus inhibitor and the masking compounds
each showed agglutination of erythrocytes; however, only one of the acidic virus inhibitors showed trypsin inhibition activity.

14. Commercially available soybean trypsin inhibitors showed inhibition against TNV. However, the glycoprotein virus inhibitors obtained in this investigation from soybean seed extracts are not identical with trypsin inhibitor since the latter is known to be proteinaceous in nature.

15. When plant lectins were tested against TNV, phytohemagglutinin (PHA) Wellcome and Con A showed no inhibition. However, PHA(P), PHA(H) and soybean agglutinin (SBA) were inhibitory. Electrophoresis experiments showed that these inhibitory lectins contain proteins and glycoproteins with both virus inhibitory properties and some with the ability to agglutinate erythrocytes.

16. Studies of the mode of action of the seed extracts confirmed the presence of inhibitors and not inactivators and showed that inhibition is brought about by effects on leaf surfaces rather than on the virus particles.


BANGERTH, F., GOTZ, G. and BUDELON, G. (1972). Effects of phytohemagglutinin sprays upon parthenocarpic fruit set of the Bartlett pear (William's) and a male sterile mutant of the tomato. Z. Pflanzenphysiol. Bd. 66 S., 375.


IN VIVO INHIBITORY EFFECT OF THE INHIBITOR FOUND IN VARIOUS LEAF SEQUENCES OF \textit{Chenopodium quinoa}.

Custe and Rey, on tobacco mosaic virus infection.


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APPENDIX 1

Randomisation of treatments on Tobacco plants

Inoculation arrangement for two treatments each of

ten replications on half leaves of Nicotiana tabacum var. Xanthi

<table>
<thead>
<tr>
<th>Plant 1</th>
<th>Plant 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>leaf number</td>
<td>L</td>
</tr>
<tr>
<td>1</td>
<td>A*</td>
</tr>
<tr>
<td>2</td>
<td>B</td>
</tr>
<tr>
<td>3</td>
<td>A</td>
</tr>
<tr>
<td>4</td>
<td>B</td>
</tr>
<tr>
<td>5</td>
<td>A</td>
</tr>
</tbody>
</table>

* treatment

L = leaf half leaf

R = right half leaf
Statistical Analysis

Local lesion counts were statistically analysed using the student 't' test as described by Bishop* (1963).

The analysis involved the calculation of:

(i) The mean number of lesions for each treatment ($\bar{x}$)

(ii) The deviation ($d$) for each number of lesions ($x$) given by the equation

$$d = x - \bar{x}$$

(iii) Frequency of each deviation ($f$)

(iv) The sum of squares of deviations ($\sum df^2$)

(v) Variance ($\sigma$) of the mean

$$\sigma^2 = \frac{\sum df^2}{E_f}$$

$E_f = \text{sum of frequencies}$

$n = \text{number of leaves in every treatment}$

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

$$\sigma^2_d = \frac{\sigma^2_1}{n_1} + \frac{\sigma^2_2}{n_2}$$

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

(vii) Standard deviation of the difference of means ($\sigma_d$)

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

(ix) Calculated 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 19 and Table 21.

Specimen calculation of the data from Table 19 showing the effect of neat P. vulgaris seed extract on the susceptibility of P. vulgaris to infection by TNV

<table>
<thead>
<tr>
<th>Local lesion Nos.</th>
<th>f</th>
<th>d</th>
<th>( f d^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>(TNV + Water)</td>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>Control treated</td>
<td></td>
<td></td>
<td>treated</td>
</tr>
<tr>
<td>(TNV + seed extract) treated</td>
<td></td>
<td></td>
<td>Control</td>
</tr>
<tr>
<td>treated</td>
<td></td>
<td></td>
<td>treated</td>
</tr>
<tr>
<td>130</td>
<td>102</td>
<td>1</td>
<td>44.7</td>
</tr>
<tr>
<td>71</td>
<td>76</td>
<td>1</td>
<td>11.3</td>
</tr>
<tr>
<td>105</td>
<td>134</td>
<td>1</td>
<td>19.7</td>
</tr>
<tr>
<td>46</td>
<td>53</td>
<td>1</td>
<td>33.3</td>
</tr>
<tr>
<td>111</td>
<td>93</td>
<td>1</td>
<td>25.7</td>
</tr>
<tr>
<td>79</td>
<td>84</td>
<td>1</td>
<td>9.0</td>
</tr>
<tr>
<td>102</td>
<td>61</td>
<td>1</td>
<td>16.7</td>
</tr>
<tr>
<td>114</td>
<td>131</td>
<td>1</td>
<td>24.7</td>
</tr>
<tr>
<td>35</td>
<td>32</td>
<td>1</td>
<td>50.3</td>
</tr>
<tr>
<td>57</td>
<td>49</td>
<td>1</td>
<td>28.3</td>
</tr>
<tr>
<td>833</td>
<td>910</td>
<td>10</td>
<td></td>
</tr>
</tbody>
</table>

\[ \bar{x} \text{ (control)} = \frac{823}{10} = 82.3 \]

\[ \bar{x} \text{ (treated)} = \frac{913}{10} = 91 \]

\[ \sigma^2 = \frac{2138.41}{10} = 918.41 \]

\[ \sigma^2 = \frac{1654.2}{10} = 165.42 \]

\[ \sigma^2 = \frac{913.94}{10} + \frac{1654.2}{10} = 257.304 \]

\[ \sigma_d = \sqrt{257.304} = 16.04 \]

\[ t = \frac{91 - 85.3}{16.04} = 0.36 \]

\( P > 0.1 \) (difference not significant)
**APPENDIX 4**

Details of the statistical analysis of the remaining data from Table 19, showing the effects of dilutions of *P. vulgaris* seed extracts on the susceptibility of *P. vulgaris* to INV infection

<table>
<thead>
<tr>
<th>Dilution</th>
<th>10^-1</th>
<th>10^-2</th>
<th>10^-3</th>
<th>10^-4</th>
<th>10^-5</th>
<th>10^-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Treatment</td>
<td>Control</td>
<td>treated</td>
<td>Control</td>
<td>treated</td>
<td>Control</td>
<td>treated</td>
</tr>
<tr>
<td>( \bar{x} )</td>
<td>72.20</td>
<td>77.39</td>
<td>103.80</td>
<td>96.70</td>
<td>91.00</td>
<td>87.10</td>
</tr>
<tr>
<td>( \Sigma )</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>( \Sigma f^2 )</td>
<td>6403.60</td>
<td>17822.1</td>
<td>9402.60</td>
<td>15269.8</td>
<td>14796.0</td>
<td>9646.90</td>
</tr>
<tr>
<td>( \sigma^2_1 )</td>
<td>640.36</td>
<td>178.21</td>
<td>949.16</td>
<td>15269.8</td>
<td>14796.0</td>
<td>9646.90</td>
</tr>
<tr>
<td>( \sigma^2_2 )</td>
<td>242.257</td>
<td>247.614</td>
<td>244.429</td>
<td>199.420</td>
<td>233.678</td>
<td>245.320</td>
</tr>
<tr>
<td>( \sigma_d )</td>
<td>15.56</td>
<td>15.74</td>
<td>15.63</td>
<td>14.12</td>
<td>16.84</td>
<td>15.66</td>
</tr>
<tr>
<td>( t )</td>
<td>0.33</td>
<td>0.45</td>
<td>0.25</td>
<td>0.30</td>
<td>1.85</td>
<td>0.26</td>
</tr>
<tr>
<td>( P )</td>
<td>&gt; 0.1</td>
<td>&gt; 0.1</td>
<td>&gt; 0.1</td>
<td>&gt; 0.1</td>
<td>&lt; 0.1</td>
<td></td>
</tr>
</tbody>
</table>

*difference is significant*
APPENDIX B

Statistical analysis of the data for treated samples from Table 21 showing the effects of McIlvaines buffer on the inhibitory activity of P. vulgaris seed extract

<table>
<thead>
<tr>
<th>pH</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\bar{x}$</td>
<td>40.00</td>
<td>49.29</td>
<td>43.30</td>
<td>40.80</td>
<td>41.00</td>
<td>41.20</td>
</tr>
<tr>
<td>$\Sigma f$</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
<td>10</td>
</tr>
<tr>
<td>$\Sigma f d^2$</td>
<td>184.00</td>
<td>471.60</td>
<td>458.10</td>
<td>187.60</td>
<td>216.00</td>
<td>231.60</td>
</tr>
<tr>
<td>$\sigma^2$</td>
<td>13.50</td>
<td>47.16</td>
<td>45.81</td>
<td>18.76</td>
<td>21.60</td>
<td>23.16</td>
</tr>
<tr>
<td>$\sigma$</td>
<td>4.29</td>
<td>6.87</td>
<td>6.79</td>
<td>4.33</td>
<td>4.65</td>
<td>4.81</td>
</tr>
<tr>
<td>$se^2$</td>
<td>1.36</td>
<td>2.17</td>
<td>2.14</td>
<td>1.37</td>
<td>1.47</td>
<td>1.52</td>
</tr>
<tr>
<td>$t x se**$</td>
<td>$\pm 3.07$</td>
<td>$\pm 4.90$</td>
<td>$\pm 4.84$</td>
<td>$\pm 3.10$</td>
<td>$\pm 3.32$</td>
<td>$\pm 3.44$</td>
</tr>
</tbody>
</table>

* $se = standard error = \frac{\sqrt{6.0}}{\sqrt{n}}$

** 95% confidence limits of the means