STUDIES OF PLANT VIRUS INHIBITORS FROM LEGUME SEEDS

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

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May, 1976

ABSTRACT

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 TMV, whilst *G. max* was inhibitory to TMV and PVX. 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 158,500 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

TNV = Tobacco necrosis virus

G. max = *Glycine max*   *P. vulgaris* = *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.

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CHAPTER I

INTRODUCTION

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 (Kovacs, 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 (1954) 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 *AIZOACEAE*, *CARYOPHYLLACEAE*, *CHENOPODIACEAE*, *CRASSULACEAE*, *LEGUMINOSAE*, *PHYTOLACCACEAE*, *ROSACEAE*, and *SOLANACEAE*, has any substantial amount of work been carried out to establish the identity of the inhibitory compounds isolated. In the *AIZOACEAE*, 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), Simons, Swidler and Moss (1963) removed the inhibitory substance from crude extract of *Mesembryanthemum caprohetum*. 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.

TABLE 1

PLANTS TESTED FOR VIRUS INHIBITOR ACTIVITY (excluding tests on seeds)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
AIZOACEAE		
Tetragonia expansa	Tobacco ringspot	Benda (1956)
Cheiridopsis aspera	Tobacco mosaic (TMV)	Simons et al. (1963)
Mesembryanthemum caprohetum	TMV	
AMARANTHACEAE		
Gomphrena globosa L.	TMV	Yoshii et al. (1954)
G. globosa L.	Potato virus X (PVX)	Blaszczak et al. (1959)
AMARYLLIDACEAE		
Agapanthus africanus	TMV	Simons et al. (1963)
Agave americana L.	TMV	
Sanseveria anthispica	TMV	
Allium cepa	TMV	Dhaliwal (1971)
A. sativum	TMV	
ANACARDIACEAE		
Schinus molle L.	TMV	Simons et al. (1963)
S. terebinthifolius	TMV	
Mangifera indica L.	TMV	Singh (1969)
M. indica L.	PVX	Singh (1971)
M. indica L.	Cucumis virus I	Sharma (1973)
APOCYNACEAE		
Nerium oleander L.	TMV	Simons et al. (1963)
Plumeria rubra L.	TMV	Singh (1969)
Vinca rosea L.	PVX	Verma et al. (1970)
Plumeria rubra L.	PVX	Singh (1971)
Nerium indicum Mill.	TMV	Lal et al. (1974)
ARACEAE		
Monsterra deliciosa	TMV	Simons et al. (1963)
Dieffenbachia seguine	TMV	
Acorus calamus L.	PVX	Verma et al. (1970)
ARALIACEAE		
Hedera canariensis	TMV	Simons et al. (1963)

/Contd......

TABLE 1 (contd.)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
ASCLEPIADACEAE		
Calatropis gigantea	TMV	Lal et al. (1974)
BOMBACACEAE		
Bombax ceiba L.	PVX	Singh (1971)
BUXACEAE		
Simmondsia californica	TMV	Simons et al. (1963)
CACTACEAE		
Echinocactus sp.	TMV	Simons et al. (1963)
Opuntia robusta	TMV	
CAMPANULACEAE		
Campanula biononiensis	TMV	Blaszczak et al. (1969)
CANNACEAE		
Canna generalis	TMV	Simons et al. (1963)
CAPPARIDACEAE		
Gynandropsis pentaphylla	Sunnhemp mosaic	Paliwal et al. (1965)
Cleome viscosa L.	TMV	Thakur et al. (1971)
Gynandropsis gynandra	TMV	
CAPRIFOLIACEAE		
Lonicera sp.	TMV	Simons et al. (1963)
CARYOPHYLLACEAE		
Dianthus caryophyllus	TMV	Van der Want (1951)
D. barbatu	Tobacco ringspot	Weintraub et al. (1952)
	TMV	
D. caryophyllus	TMV	Van Kammen et al. (1961)
D. caryophyllus	TMV	Ragetli et al. (1962)
D. caryophyllus	TMV	Simons et al. (1963)
D. caryophyllus	Sunnhemp mosaic	Paliwal et al. (1965)
D. caryophyllus	TMV	Blaszczak et al. (1969)

/Contd....

TABLE 1 (contd.)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
CARYOPHYLLACEAE (contd.)		
Dianthus caryophyllus	TMV	Nart (1972)
	Alfalfa mosaic	
	Barley stripe mosaic	
D. caryophyllus	Papaya leaf reduction	Singh (1972)
CHENOPODIACEAE		
Spinacia oleracea L.	TMV	Grant (1934)
S. oleracea L.	TMV	Kuntz et al. (1947)
	Cucumber mosaic	
	Cabbage mosaic	
	Tobacco ringspot	
	Necrotic potato ringspot	
Beta vulgaris L.	TMV	Kuntz et al. (1947)
	Cabbage mosaic	
Chenopodium album L.	TMV	Manil (1949)
Beta vulgaris L.	Cucumber mosaic	Bhargava (1951)
Spinacia oleracea L.	TMV	Chiba et al. (1952)
S. oleracea L.	Bottle-gourd mosaic	Vasudeva et al. (1952)
Chenopodium album L.	Tobacco etch	Greenleaf (1953)
Beta vulgaris L.	Cucumber mosaic	Gendron et al. (1954)
	Tobacco necrosis (TNV)	
B. vulgaris L.	TMV	Bartels (1955)
Spinacia oleracea L.	PVX	Sharma et al. (1956)
Chenopodium album L.	PVX	Blaszczak et al. (1959)
C. amaranticolor	PVX	
Spinacia oleracea	PVX	
S. oleracea L.	PVX	Raychaudhuri (1961, 1963)
	Radish mosaic	
	Zinnia mosaic	
Beta vulgaris L.	Yellow virus	Jermoljev et al. (1964)
Chenopodium amaranticolor	Cucumber mosaic	Francki (1964)
C. quinoa	Cucumber mosaic	
Spinacia oleracea L.	Radish mosaic	Raychaudhuri et al. (1965)
S. oleracea L.	PVX	Sharma et al. (1965)
Chenopodium amaranticolor	TMV	Bhullor (1965)
C. album L.	TMV	
C. album L.	TMV	Thomson et al.(1965)
C. amaranticolor	TMV	
C. album L.	Sunnhemp mosaic	Paliwal et al.(1965)

/Contd......

TABLE 1 (contd.)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
CHENOPODIACEAE (contd.)		
Beta vulgaris L.	Sunnhemp mosaic	Paliwal et al.(1965)
Spinacia oleracea L.	Sunnhemp mosaic	
Chenopodium album L.	TMV	Yoshizaki et al. (1966)
C. album L.	Turnip mosaic	Yoshii et al. (1967)
Beta vulgaris	Cucumber mosaic	Ruppel (1967)
Chenopodium album L.	Watermelon mosaic	Demski (1968)
C. amaranticolor	Watermelon mosaic	
C. amaranticolor	TMV	Kimmins (1969)
Chenopodium album L.	Watermelon mosaic	Singh (1969)
C. amaranticolor	Watermelon mosaic	
Spinacia oleracea L.	Watermelon mosaic	
Chenopodium quinoa	Apple chlorotic leafspot	Saksena (1969)
Chenopodium sp.	Cucumber mosaic Daikon mosaic TMV	Yoshii (1969)
Chenopodiales (29 spp)	TNV	Smookler (1971)
Spinacia oleracea L.	TMV	Ebrahim-Nesbat (1971)
Beta vulgaris L.	TMV	Ebrahim-Nesbat (1972)
Spinacia oleracea L.	TMV	
Chenopodium amaranticolor	TMV	Horvath (1973)
C. album L.	TMV	Taniguchi et al. (1974)
COMBRETACEAE		
Terminalia chebula	PVX	Verma et al. (1969)
T. belerica	PVX	Verma et al. (1970)
COMPOSITAE		
Kleinia cylindrica	TMV	Simons et al. (1963)
K. tomentosa	TMV	
CONVOLVULACEAE		
Convolvulus batatas L.	TMV	Duggar et al. (1925)
Cuscuta sp.	Tomato spotted Wilt Lucerne mosaic Cabbage black ringspot Potato virus Y (PVY) PVX Cucumber mosaic Potato rattle Tomato bushy stunt	Schmelzer (1956)

/Contd.....

TABLE 1 (contd.)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
CRASSULACEAE		
13 spp.	TMV	Simons et al. (1963)
CRUCIFERAE		
Brassica oleracea	TMV	Chiba et al. (1952)
Senebiera didyma	Sunnhemp mosaic	Paliwal et al.(1965)
Raphanus sativus	Sunnhemp mosaic	
CUCURBITACEAE		
Cucurbita melopepo	TMV	Duggar et al. (1925)
Cucumis sativus	Cucumber mosaic	Sill et al. (1952)
C. sativus	Stone-fruit	Weintraub et al. (1953)
Cucurbita medullosa	Stone-fruit	
C. pepo	Stone-fruit	
Cucumis sativus	PVX	Blaszczak et al. (1959)
C. sativus	Cucumber mosaic	Francki (1964)
C. sativus	Sunnhemp mosaic	Paliwal et al.(1965)
Lagenaria siceraria	Sunnhemp mosaic	
Cucumis sativus	Watermelon mosaic	Singh (1969)
CUPRESSACEAE		
Chamaecyparis lawsoniana	TMV	Simons et al. (1963)
Juniperus communis	PVY	
Thuja orientalis	TMV	
CYPERACEAE		
Cyperus papyrus	TMV	Simons et al. (1963)
EUPHORBIACEAE		
Euphorbia schimperi	TMV	Simons et al. (1963)
Ricinus communis	TMV	
Euphorbia geniculata	Sunnhemp mosaic	Paliwal et al.(1965)
Croton roxburghii	PVX	Singh (1971)
Euphorbia milii	TMV	Lal et al. (1974)
E. pulcherrima	TMV Cucumis mosaic	
FUMARIACEAE		
Fumaria parviflora	PVX	Verma et al. (1970)

/Contd.....

TABLE 1 (contd.)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
GERANIACEAE		
Pelargonium sp.	TMV	Duggar et al. (1925)
P. hortorum	PVX	Blaszczak et al. (1959)
P. hortorum	TMV	Simons et al. (1963)
P. hortorum	TMV	Blaszczak et al. (1969)
P. hybridum	TMV	
P. hortorum	TMV	Apablaza et al. (1972)
GINGIBERACEAE		
Gingiber officinale	Sunnhemp mosaic	Paliwal et al. (1965)
GRAMINEAE		
Oryza sativa	TMV	Allen et al. (1957)
Triticum sativum	Wheat streak mosaic	Brakke (1958)
Oryza sativa	TMV	Kahn et al. (1960)
O. sativa	Sunnhemp mosaic	Paliwal et al. (1965)
LABIATAE		
Mentha sylvestris	Sunnhemp mosaic	Paliwal et al. (1965)
Ocimum sanctum	Sunnhemp mosaic	
O. sanctum	Papaya leaf reduction	Singh (1972)
LAURACEAE		
Cinnamomum camphorum	TMV	Simons et al. (1963)
Persea americana	TMV	
LEGUMINOSAE		
Vicia faba	TMV	Yoshii et al. (1954)
Arachis hypongaea	TMV	
Robinia pseudoacacia	TMV	
Medicago denticulata	TMV	
Phaseolus vulgaris	Southern bean mosaic	Cheo (1955)
P. vulgaris	PVX	Blaszczak et al. (1959)
Trifolium pratense	PVX	
Vicia faba	PVX	
Vigna sinensis	PVX	

/Contd....

TABLE 1 (contd.)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
LEGUMINOSAE (contd.)		
Glycine max	TMV	Simons et al. (1963)
Prosopis juliflora	Sunnhemp mosaic	Paliwal et al.(1965)
Trifolium pratense	Red clover vein	El-Kandelgy et al. (1966)
Phaseolus vulgaris	TNV	Kimmins (1969)
P. vulgaris	Watermelon mosaic	Singh (1969)
Vicia faba	Watermelon mosaic	
Vigna sinensis	Watermelon mosaic	
Tamarindus indica	TMV	Singh (1969)
Glycyrrhiza glabra	PVX	Verma et al. (1970)
Cassia occidentalis	PVX TMV	Thakur et al. (1971)
Acacia arabica	PVY	Gupta et al. (1971)
Tamarindus indica	PVX	Singh (1971)
Bauhinia variegata	PVX	
Phaseolus vulgaris	TMV Alfalfa mosaic Barley stripe mosaic	Nart (1972)
Medicago sativa	Alfalfa mosaic	Nasil et al. (1972)
Phaseolus vulgaris	TMV	Taniguchi (1974)
LILIACEAE		
Allium cepa	TMV	Simons et al. (1963)
Aloe sp.	TMV	
Gasteria sp.	TMV	
Haworthia chalivinia	TMV	
Phormium tenax	TMV	
MALVACEAE		
Gossypium sp.	TMV	Duggar et al. (1925)
Malva sylvestris	PVX	Verma et al. (1970)
MORACEAE		
Morus alba	TMV	Simons et al. (1963)
Cannabis sativa	Sunnhemp mosaic	Paliwal et al.(1965)
Artocarpus lakoocha	TMV	Singh (1969)
Ficus bengalensis	TMV	
F. elastica	TMV	
F. rumphii	TMV	
Artocarpus heterophyllus	TMV	
Morus alba	TMV	
Artocarpus heterophyllus	PVX	Singh (1971)

/Contd...

TABLE 1 (contd.)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
MORACEAE (contd.)		
Ficus bengalensis	PVX	Singh (1971)
F. racemosa	PVX	
F. elastica	PVX	
F. hispida	PVX	
F. semicordata	PVX	
F. rumphii	PVX	
F. elastica	PVX	Singh et al. (1973)
MUSACEAE		
Strelitzia reginae	TMV	Simons et al. (1963)
MYRTACEAE		
Eugenia paniculata	TMV	Simons et al. (1963)
Eucalyptus globulus	TMV	
Psidium guajava	TMV	
Callistemon lanceolatus	TMV	Singh (1969)
Psidium guajava	TMV	
Syzygium jambolanum	TMV	
Psidium guajava	TMV	Singh et al. (1970)
Callistemon lanceolatus	PVY	Gupta et al. (1971)
Syzygium cuminii	PVY	
Callistemon lanceolatus	PVY	Gupta et al. (1972)
Syzygium cuminii	PVY	
S. cuminii	Cucumis virus I	Sharma et al. (1973)
Callistemon citrinus	Cucumis virus I	
NYCTAGINACEAE		
Bougainvillea spectabilis	TMV	Thakur et al. (1971)
Mirabilis jalapa	TMV	
PALMACEAE		
Cocos nucifera	TMV	Weeraratne et al. (1961)
C. nucifera	Sunnhemp mosaic	Paliwal et al. (1965)

/Contd.....

TABLE 1 (contd.)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
PAPAVERACEAE		
Papaver somniferum	Sunnhemp mosaic	Paliwal et al.(1963)
PASSIFLORACEAE		
Carica papaya	Sunnhemp mosaic	Paliwal et al.(1963)
PHYTOLACCACEAE		
Phytolacca decandra	Pokeweed mosaic	Allard (1914, 1918)
P. decandra	TMV	Duggar et al. (1925)
P. decandra	TMV	Fulton (1943)
P. esculenta	TMV	Kassanis et al. (1948)
P. decandra	Cucumber mosaic	Diachun (1952)
P. acinosa	TMV	Gupta (1964)
P. americana	Southern bean mosaic	Wyatt et al. (1969)
P. decandra	Cucumber mosaic	Marchoux (1970)
P. americana	TMV	Owens et al. (1973)
PINACEAE		
Pinus thunbergii	TMV	Yoshii et al. (1954)
Cedrus deodara	TMV	Simons et al. (1963)
Pinus contorta	TMV	
Cedrus deodara	Capsicum mosaic	Raychaudhuri et al. (1965)
Pinus thunbergii	Turnip mosaic	Tamura (1969)
PIPERACEAE		
Piper cubeba	PVX	Verma et al. (1970)
P. nigrum	PVX	
PITTOSPORACEAE		
Pittosporum tenuifolium	TMV	Simons et al. (1963)
POLYPODIACEAE		
Cyrtomium flacerum	TMV	Simons et al. (1963)
Polypodium pruinatum	TMV	
Pteris vittata	TMV	
Asplenium nidus	TMV	
Microlepia strigosa	TMV	

/Contd....

TABLE 1 (contd.)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
PORTULACACEAE		
Portulacaria afra	TMV	Simons et al. (1963)
PRIMULACEAE		
Anagallis arvensis	PVX	Verma et al. (1970)
PROTEACEAE		
Grevillea robusta	PVX	Singh (1971)
PUNICACEAE		
Punica granatum	Sunnhemp mosaic	Paliwal et al. (1965)
RANUNCULACEAE		
Aconitum ferox	PVX	Verma et al. (1970)
A. heterophyllum	PVX	
ROSACEAE		
Malus sp.	TMV	Duggar et al. (1925)
Fragaria vesca L.	TMV	Bawden et al. (1945)
F. vesca L.	PVX	Sharma et al. (1956)
F. vesca L.	PVX	Raychaudhuri et al. (1961)
	Radish mosaic	
	Zinnia mosaic	
F. vesca L.	Sunnhemp mosaic	Paliwal et al. (1965)
F. vesca L.	PVX	Sharma et al. (1965)
Eriobotrya japonica	TMV	Singh (1969)
Pyrus communis	TMV	
P. communis	PVX	Singh (1971)
Prunus persica	PVX	
RUBIACEAE		
Cinchona ledgeriana	PVX	Verma et al. (1969)
C. ledgeriana	PVX	Verma et al. (1970)
C. officinalis	PVX	
C. robusta	PVX	
C. succirubra	PVX	
RUTACEAE		
Citrus limon	TMV	Simons et al. (1963)

/Contd.......

TABLE 1 (contd.)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
SCROPHULARIACEAE		
Antirrhinum majus	Watermelon mosaic	Singh (1969)
Digitalis purpurea	PVX	Verma et al. (1970)
SOLANACEAE		
Datura stramonium	TMV	Duggar et al. (1925)
Nicotiana sp.	TMV	Melchers et al. (1940)
Capsicum sp.	TMV	Hirai (1949)
Datura stramonium	Bottle-gourd mosaic	Vasudeva et al. (1952)
Capsicum annuum	Bottle-gourd mosaic	
Solanum nigrum	Bottle-gourd mosaic	
Lycopersicon esculentum	Bottle-gourd mosaic	
Nicotiana glutinosa	TMV	Chiba et al. (1952)
N. tabacum	TMV	
Capsicum annuum	TMV	
Nicotiana sp.	TMV	Weathers et al. (1954)
Capsicum frutescens	Cucumber mosaic	McKeen (1956)
C. annuum	PVX	Sharma et al. (1956)
Nicotiana sp.	TMV	Bawden et al. (1957)
Capsicum frutescens	PVX	Blaszczak et al. (1959)
Datura stramonium	PVX	
D. metel	PVX	
Solanum integrifolium	PVX	
S. tuberosum	PVX	
Physalis floridana	PVX	
Petunia hybrida	PVX	
Nicandra physalodes	PVX	
N. tabacum	PVX	
N. debneyi	PVX	
N. glutinosa	PVX	
Lycopersicon esculentum	PVX	
Capsicum annuum	PVX	Raychaudhuri (1961)
	Radish modaic	
	Zinnia mosaic	
Nicotiana tabacum	TMV	Zaitlin et al. (1963)
Capsicum annuum	TMV	Simons et al. (1963)
Solandra longiflora	TMV	
Nicotiana glutinosa	Cucumber mosaic	Francki (1964)
Capsicum annuum	Radish mosaic	Raychaudhuri et al. (1965)
Datura stramonium	Radish mosaic	

/Contd.......

TABLE 1 (contd.)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
SOLANACEAE (contd.)		
Capsicum annuum	PVX	Sharma et al. (1965)
C. annuum	PVX	Rao et al. (1965)
Datura stramonium	PVX	
D. stramonium	Sunnhemp mosaic	Paliwal et al.(1965)
Nicotiana glutinosa	Sunnhemp mosaic	Paliwal et al.(1965)
Withania somnifera	Sunnhemp mosaic	
Physalis peruviana	Sunnhemp mosaic	Paliwal et al.(1965)
Solanum tuberosum	PVX	Jermoljev et al. (1965)
S. tuberosum	PVX PVY	
S. tuberosum	PVX	Jermoljev (1966)
Capsicum annuum	Cucumber mosaic Alfalfa mosaic Tobacco mosaic	Marchoux (1967)
Nicotiana glutinosa	TMV	Palm (1967)
Solanum tuberosum	PVX	Albrechtova (1968)
Nicotiana glutinosa	TNV	Kimmins (1969)
N. tabacum	PVY	Chod et al. (1969)
Datura stramonium	Watermelon mosaic	Singh (1969)
Lycopersicon esculentum	Watermelon mosaic	
Hyoscyamus niger	PVX	Verma et al. (1970)
Withania somnifera	PVX	
Nicotiana tabacum	TMV	Wolffgang (1970)
Capsicum annuum	Cucumber mosaic	Marchoux (1970)
C. frutescens	TMV	Apablaza et al. (1972)
Datura stramonium	TMV	
Capsicum annuum	Papaya leaf reduction	Singh (1972)
Petunia hybrida	Papaya leaf reduction	
Capsicum annuum	TMV Alfalfa mosaic Cucumber mosaic PVX PVY	Fischer et al. (1973)
Datura metel	TMV	Lal et al. (1973)
Solanum melongena	TMV	
TAXODIACEAE		
Sequoia sempervirens	TMV	Simons et al. (1963)

/Contd.....

TABLE 1 (contd.)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
THEACEAE		
Camellia sinensis	Sunnhemp mosaic	Paliwal et al.(1965)
Thea sinensis	Cucumber mosaic TMV	Okada (1971)
ZINGIBERACEAE		
Curcuma longa	PVX	Verma et al. (1970)

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 (1957) 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 refractionated 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 (BSMV). 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. Kuntz 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. Kimmins (1969) examined a crude extract from the leaves of *Chenopodium amaranticolor* 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 RNA 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). Smookler (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 amaranticolor*, *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, Yamaguchi and Naganava (1974) isolated two fractions from leaf extract of *Chenopodium album* L. by ECTEOLA 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 *CRASSULACEAE* 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 nussbaumerianum*. The inhibitors were eluted by dilute sulphuric acid (1N) 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-Kandelgy 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, Phytolacca decandra, the type genus of the PHYTOLACCACEAE. 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 (1948) 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.

Wyatt and Shepherd (1969) extended the procedure developed by Kassanis 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 (1945) 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*. Zaitlin 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.

Wolffgang (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 (1952) reported an inhibitor of TMV 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 TMV. 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 Nienhaus (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 PVX have been isolated from *Solanum tuberosum* (potato) (Jermoljev 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 electrophoretically homogeneous protein which was eluted at 0.15-0.3 M NaCl concentration, and which inhibited PVX 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 (1969) 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 - VMMV) by seed extract of *Mangifera indica* (Mango). The percentage inhibition was 77%.

CHENOPODIACEAE have been considered in rather more detail.

TABLE 2

FRUITS AND SEEDS TESTED FOR VIRUS INHIBITOR ACTIVITY

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
ANACARDIACEAE		
Mangifera indica	Cucumis virus I	Sharma et al. (1973)
CHENOPODIACEAE		
Beta vulgaris (beet)	TMV TNV Beet mosaic	Szirmai (1963)
B. vulgaris (beet)	TNV	Stevens (1970)
B. vulgaris (sugar-beet)	TNV	
COMPOSITAE		
Lactuca sativa	TNV	Stevens (1970)
CRUCIFERAE		
Brassica napus	TNV	Stevens (1970)
B. alba	TNV	
Cheiranthus cheiri	TNV	
Raphanus sativus	TNV	
CUCURBITACEAE		
Cucumis sativa	Cucumber mosaic	Sill et al. (1951, 1952)
C. sativa	TMV	Crowley (1955)
Echinocystus lobata	TMV	
GRAMINEAE		
Oryza sativa	TMV	Allen et al. (1957)
O. sativa	TMV	Kahn et al. (1960)
Triticum sativum	TMV	Verma et al. (1965)
LEGUMINOSAE		
Phaseolus vulgaris	Southern bean mosaic	Cheo (1955)
P. vulgaris	Bean virus 1	Crispin et al. (1961)
	Bean virus 2	
Vicia faba	TNV	Stevens (1970)

/Contd.....

TABLE 2 (contd.)

SOURCE OF INHIBITOR	VIRUS	REFERENCE
FAMILY		
LEGUMINOSAE (contd.)		
Phaseolus vulgaris "The Prince"	TNV	Stevens (1970)
P. aureus	TNV	
LOGANIACEAE		
Strychnos nux-vomica	PVX	Verma et al. (1970)
MYRTACEAE		
Syzigium cumini	Cucumis virus I	Sharma et al. (1973)
Callistemon citrinus	Cucumis virus I	
PALMACEAE		
Elaeis spp.	TMV	Lucardie (1951)
PAPAVERACEAE		
Papaver orientale	TNV	Stevens (1970)
SOLANACEAE		
Nicotiana spp.	TMV	Duggar (1930)
N. spp.	TMV	Kausche (1940)
N. spp.	TMV	Crowley (1955)
N. glutinosa	TNV	Stevens (1970)
Lycopersicon esculentum	TNV	

Szirmai (1963) tested the inhibitory effect of beet "seed" pericarp on a number of viruses. It inhibited TMV 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 abrased 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, Dumort (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; Blaszczak, 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. *Raphanus sativus* 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* (*Cucumis 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 *Echinocystus 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 (1955). He found that the virus to which the bean seed extract was added produced 95% 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*, Roxb. 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 (WMV) by seed extracts of various plants. Seed extracts of *Syzigium cumini* (*MYRTACEAE*) showed complete inhibition of WMV 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 *Elaeis* sp. (*PALMACEAE*) on local lesion formation by TMV. 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 TMV, Duggar (1930) 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 (*SOLANACEAE*)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 summarises 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, 1968).

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 phytohemagglutinin 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 PHA 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 PHA during the first 12 days of treatment, but only at temperatures below 20°C. Bangerth (1965) used PHA for the induction of fruit setting in a pollen-sterile mutant of tomato. In 1972, Bangerth, Gotz and Buchloh also found that PHA induced parthenocarpic fruit set in a male sterile mutant of tomato and also in Bartlett pear. Although it is not clear how PHA 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 18 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 *Gomphrena globosa*

Seeds of *G. 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. parts. 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, 1934).

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 BEAN LEAVES AFTER INOCULATION BY TNV

Source of seed extract	Mean number of lesions *		Activity quotient
	TNV + water	TNV + seed extract	
P. vulgaris (unwashed)	102	65.1	0.64
P. vulgaris (washed)	123	87.9	0.71
G. max (unwashed)	54.14	5.36	0.09
G. max (washed)	53.47	5.60	0.10

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

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

$$\text{Activity quotient:} \quad \frac{\text{Number of lesions on treated leaves}}{\text{Number of lesions on control leaves}}$$

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

Percentage inhibition:

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

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. tabacum* 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. globosa* L. plants.

3. SEED EXTRACT PREPARATION

Germination Test

Seeds used in all experiments were estimated for viability using a test based on that of Bennett and Loomis (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 gm 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. "Merr") 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

Source of seed extract	Mean number of lesions *		Activity quotient
	TNV + H_2O	TNV + seed extract	
P. vulgaris (crude)	67	47.4	0.70
P. vulgaris (lyophilized)	80.8	56.7	0.70
G. max (crude)	36.73	2.64	0.07
G. max (lyophilized)	63.8	4.5	0.07

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

/Contd....

Preparation of Lyophilized Seed Extract

Dry seeds of P. vulgaris and G. max were each ground to a flour using a mechanical homogenizer. 20 gm 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 mμ 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 mμ 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 rimming 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 Rf 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 VARIOUS LEGUME SEED EXTRACTS ON THE INFECTION OF PHASEOLUS VULGARIS BY TNV

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 TNV solution and inoculated onto *P. vulgaris*. Control leaves were inoculated with 1 ml of water and an equal volume of TNV solution.

The effect of fresh seed extracts on TNV 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 TNV 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. *Phaseolus 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 TNV solution and inoculated onto *P. vulgaris* leaves. Control consisted of 1 ml of water and an equal amount of TNV 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. "Merr" (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* (Haricot 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. "Merr".

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.

TABLE 5 Effect of various legume seed extracts on the infection of *Phaseolus vulgaris* by TNV

Source of Extract	Mean number of lesions *		Activity Quotient (A.Q.)	Percentage Inhibition
	TNV + water (Control)	TNV + seed extract (Treated)		
Vicia faba (var. Giant)	26.0	1.6	0.06	94
Glycine max (Soybean, black testa)	55.9	4.5	0.08	92
Vicia faba (var. Aquadulce)	35.7	3.0	0.08	92
Lens esculenta	34.6	3.0	0.09	91
Robinia hispida	44.5	3.8	0.09	91
Glycine max (Soybean, yellow testa)	19.8	2.2	0.11	89

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

TABLE 6 Effect of various legume seed extracts on the infection of <u>Phaseolus vulgaris</u> by TNV

Source of Extract	Mean number of lesions *		Activity Quotient (A.Q.)	Percentage Inhibition
	TNV + water (Control)	TNV + seed extract (Treated)		
Pisum sativum	10.5	1.2	0.11	89
Phaseolus mungo	59.7	8.0	0.13	87
Vigna sinensis	19.3	3.0	0.16	84
Canavalia ensiformis	21.2	3.6	0.17	83
Lotus tetragonolobus	21.7	4.7	0.22	78
Phaseolus aureus	11.8	3.0	0.25	75

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

TABLE 7 Effect of various legume seed extracts on the infection of *Phaseolus vulgaris* by TNV

Source of Extract	Mean number of lesions *		Activity Quotient (A.Q.)	Percentage Inhibition
	TNV + water (Control)	TNV + seed extract (Treated)		
Securigera securidaca	40.3	18.5	0.46	54
Cicer arietinum	14.1	7.4	0.52	48
Phaseolus coccineus	34.9	22.5	0.64	36
Phaseolus limensis	8.0	5.4	0.68	32
Phaseolus vulgaris (Haricot)	34.1	23.2	0.68	32
Phaseolus vulgaris (French bean)	32.3	32.8	1.02	- 2

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

TABLE 8 Effect of heated seed extracts from various varieties of legume on the infection of *Phaseolus vulgaris* by TNV

Source of Extract	Mean number of lesions *		Activity Quotient (A.Q.)	Percentage Inhibition
	TNV + water (Control)	TNV + seed extract (Treated)		
Glycine max (Soybean, black testa)	63.8	14.3	0.22	78
Vicia faba (var. Giant)	27.8	6.6	0.24	76
Phaseolus vulgaris (Haricot bean)	28.2	9.0	0.32	68
Phaseolus coccineus	28.8	9.9	0.34	66
Phaseolus mungo	43.7	15.7	0.36	64
Vicia faba (var. Aquadulce)	17.1	6.6	0.39	61

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

TABLE 9

Effect of heated seed extracts from various varieties of legume on the infection of *Phaseolus vulgaris* by TNV

Source of Extract	Mean number of lesions *		Activity Quotient (A.Q.)	Percentage Inhibition
	TNV + water (Control)	TNV + seed extract (Treated)		
Robinia hispida	49.0	19.8	0.40	60
Glycine max (Soybean, yellow testa)	25.0	10.0	0.40	60
Vigna sinensis	16.6	7.3	0.44	56
Phaseolus limensis	10.8	5.1	0.47	53
Phaseolus aureus	10.9	5.7	0.52	48
Phaseolus vulgaris (French bean)	23.4	12.6	0.54	46

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

TABLE 10 Effect of heated seed extracts from various varieties of legume on the infection of *Phaseolus vulgaris* by TNV

Source of Extract	Mean number of lesions *		Activity Quotient (A.Q.)	Percentage Inhibition
	TNV + water (Control)	TNV + seed extract (Treated)		
Lens esculenta	19.7	11.1	0.56	44
Securigera securidaca	34.2	22.3	0.65	35
Cicer arietinum	8.0	6.3	0.79	21
Canavalia ensiformis	27.9	24.7	0.89	11
Lotus tetragonolobus	30.3	28.6	0.94	6
Pisum sativum	7.4	8.6	1.16	- 16

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

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. "MERR" SEEDS

(a) Effect of dilution

G. max var. "Merr" (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 <u>G. max</u> seed extracts on the susceptibility of <u>P. vulgaris</u> to infection by TNV

Dilution	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + water	TNV + seed		
10^{-1}	79.6	36.90	0.46	54
10^{-2}	92.6	58.4	0.63	37
10^{-3}	80.8	49.9	0.62	38
10^{-4}	80.0	54.5	0.68	32
10^{-5}	59.0	60.0	1.02	- 2

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

Sorensons phosphate buffer at pHs 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 McIlvaines 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 McIlvaines 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 Sorensons 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 Sorensons phosphate buffer on the inhibitory activity of G. max seed extract

pH	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + buffer	TNV + buffered seed extract		
5.4	37.6	23.5	0.63	37
6.0	50.4	14.6	0.29	71
7.0	60.8	12.9	0.21	79
8.0	61.8	11.9	0.19	81

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

TABLE 13

Effect of McIlvaines buffer on the inhibitory activity of G. max seed extract

pH	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + buffer	TNV + buffered seed extract		
3.0	46.5	2.1	0.05	95
4.0	42.9	7.5	0.17	83
5.0	43.3	3.6	0.08	92
6.0	47.9	2.8	0.06	94
7.0	43.5	3.2	0.07	93
8.0	45.5	3.7	0.08	92

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

TABLE 14

Effect of sodium acid maleate buffer on the inhibitory activity of _G. max_ seed extract

pH	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + buffer	TNV + buffered seed extract		
5.2	80.7	5.6	0.07	93
5.8	82.5	6.0	0.07	93
6.0	85.0	5.7	0.07	93
6.8	85.3	6.5	0.08	92

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

Extract	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + water	TNV + extract		
Sap dialysed against running water (non-dialysable part)	52.9	6.1	0.12	88
Sap dialysed against 100 ml distilled water (non-dialysable part)	62.5	4.0	0.06	94
Sap dialysed against 100 ml distilled water (dialysable part)	63.8	75.7	1.19	- 19

* 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

Source of extract	mg protein/ml	mg carbohydrate/ml
non-dialysable part	50	3.5
dialysable part	2	20.0
whole seed extract	75	25.5

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.

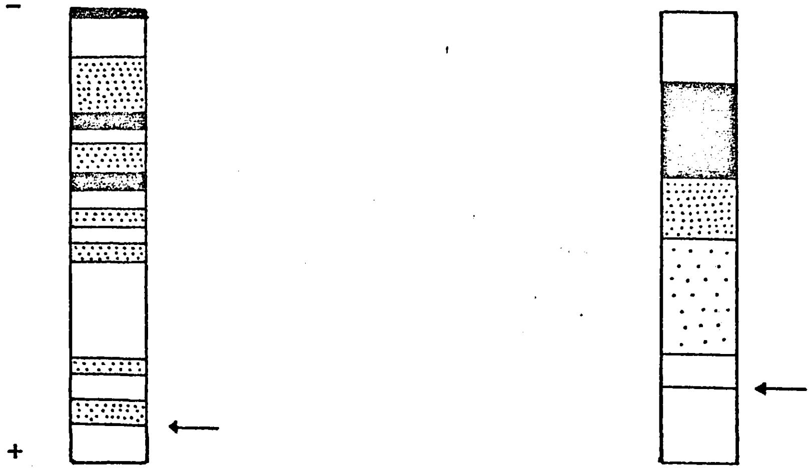
Further analyses of the fractions were made in order to establish whether any of the carbohydrates might be bound to protein in the form of glycoprotein. Polyacrylamide electrophoresis was carried out, as described earlier, on the DP, dialysate and, for comparative purposes, on the whole seed extract. 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 0.1%

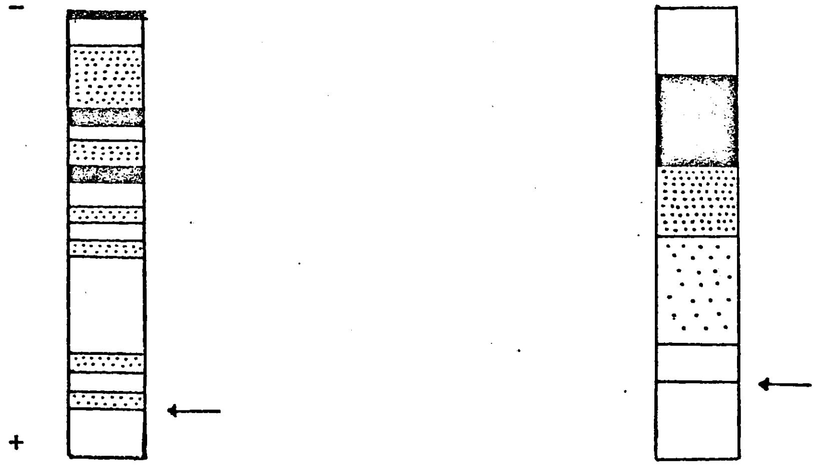
Fig. 1. Disc electrophoresis of the proteins and glycoproteins from G. max seed extracts

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 extract 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 TMV (Lal, Verma and Verma, 1973).

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; Kimmins, 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 mμ and 231 mμ 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 sulphate 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. These 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

Extract	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + water	TNV + extract		
supernatant	59.7	38.4	0.64	36
precipitate	51.6	24.0	0.47	53

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

responsible for virus inhibition, extracts of G. max seeds were precipitated with $(NH_4)_2 SO_4$. 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 18 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 18 Effect of ammonium sulphate fractions from *G. max* seed extract on the susceptibility of *P. vulgaris* to TNV

Sample	Saturation ammonium sulphate (%)	Mean number of lesions *		Activity Quotient	Percentage Inhibition	mg protein/ml
		TNV + water	TNV + sample			
Precipitate 1	10	25.0	20.7	0.83	17	0
Precipitate 2	20	19.9	16.7	0.84	16	2.25
Precipitate 3	30	15.2	16.7	1.10	- 10	1.95
Precipitate 4	40	16.6	7.6	0.46	54	0.80
Precipitate 5	50	14.6	9.1	0.62	38	1.50
Precipitate 6	60	15.9	10.3	0.65	35	2.75
Supernatant	70	10.6	12.6	1.19	- 19	0

* 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 (38%), 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.

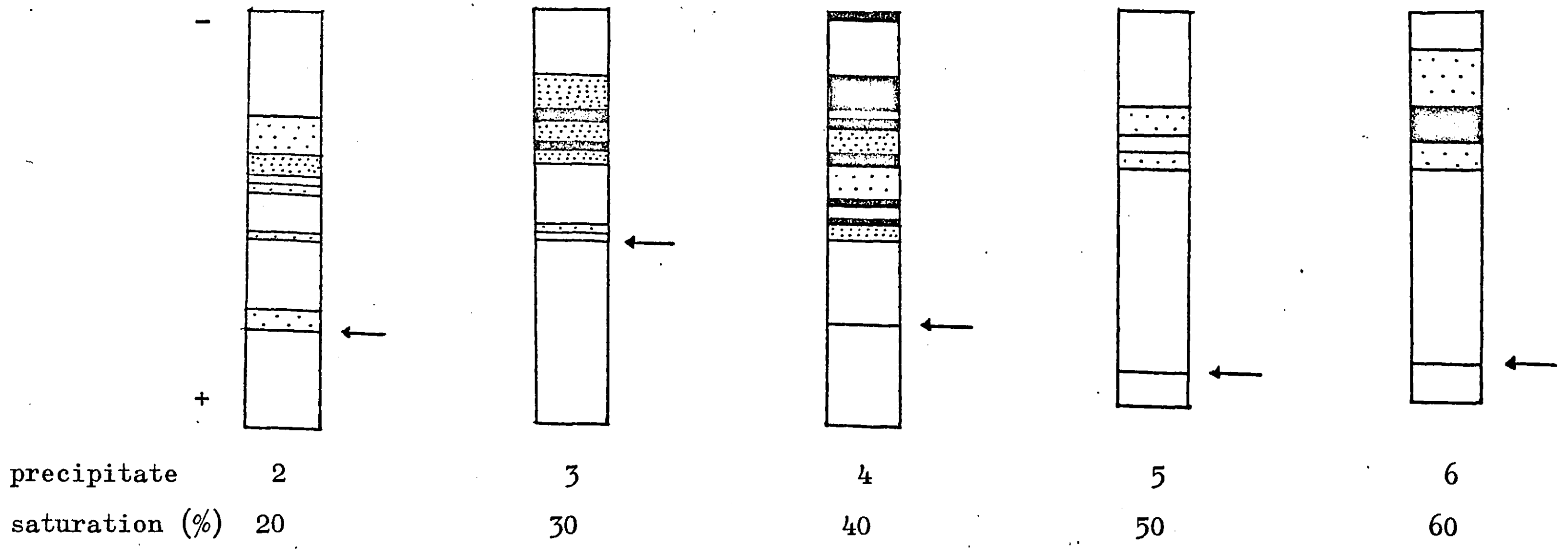
DISCUSSION

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, 1963). 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 (1963). The leaf extract was inhibitory as it gave 55% inhibition when tested against TMV infection.

In this investigation G. max seed extract was tested against TNV infection, and it showed 89-93% inhibition. The percentage inhibition

Fig. 2. Disc electrophoresis of $(NH_4)_2SO_4$ fractions from G. max

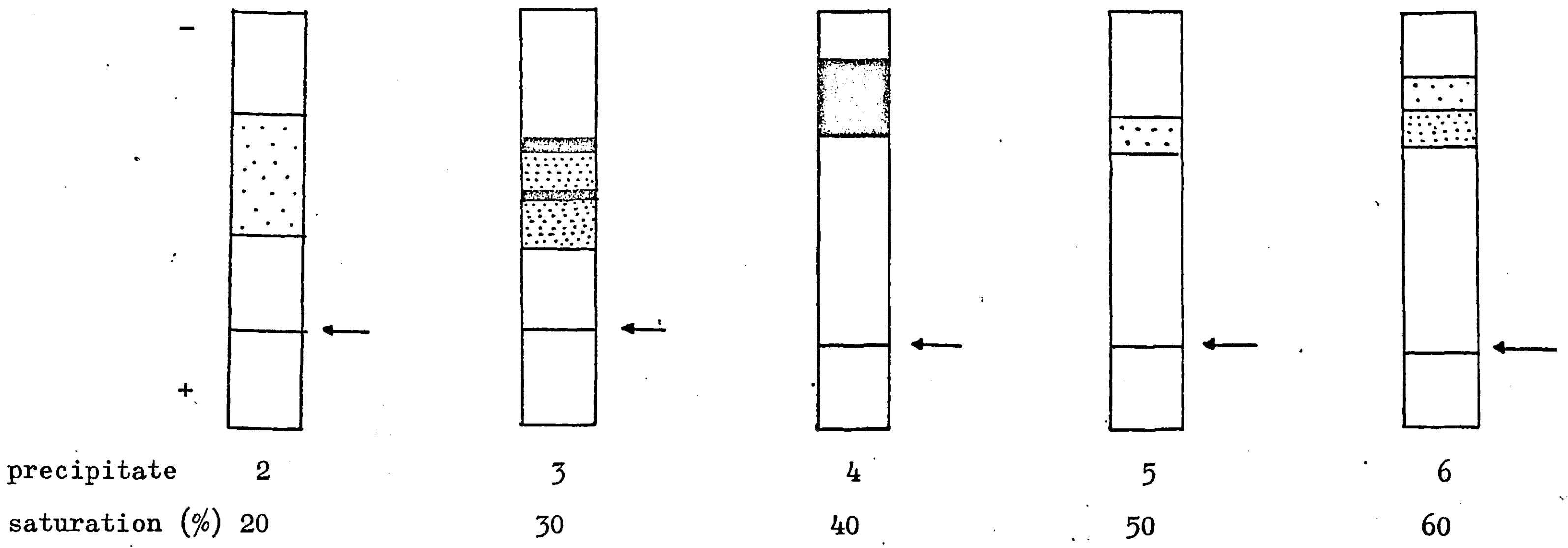
<u>Proteins</u> (No bands were detected in precipitate 1 and the supernatant)



(← Position of marker
(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)



(← 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 (Bawden, 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 heatlabile 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 *Capsicum frutescens* and *Chenopodium amaranticolor*. Francki (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 fortorum* (Blaszczak et al. (1959), and *Spinacia oleracea* (Kuntz 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 (1963) 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 Murayama, 1966) and *Nicotiana glutinosa* (Palm, 1967). Furthermore, 50% ethanol was found by Smookler (1971) to precipitate the inhibitors extracted from leaves of *Chenopodium amaranticolor*, *C. album*, *Atriplex nitens*, *Amaranthus caudatus*. However, McKeen (1956) found that 95% ethanol was needed to precipitate a virus inhibitor from the juice of *Capsicum 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, 1948). 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. acinosa.

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 D

PROPERTIES OF VIRUS INHIBITOR EXTRACTS PREPARED FROM PHASEOLUS 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

Dilution	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + water	TNV + seed extract		
Neat	85.36	91.0	1.07	- 7
10^{-1}	72.2	77.3	1.07	- 7
10^{-2}	103.8	96.7	0.93	7
10^{-3}	91.0	87.1	0.96	4
10^{-4}	87.8	83.6	0.95	5
10^{-5}	103.2	72.0	0.70	30
10^{-6}	86.0	90.0	1.05	- 5

* 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 augmenters as described previously (Stevens, 1970) is not clear.

(b) Effect of pH

When *P. vulgaris* seed extract was dissolved in Sorensons 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 58.9 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 0.98. 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 6.8 (Table 22).

These results are similar to those recorded for *G. max* seed

TABLE 20

Effect of Sorensons phosphate buffer on the inhibitory activity of P. vulgaris seed extract

pH	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + buffer	TNV + buffered seed extract		
5.4	35.8	48.5	1.35	- 35
6.0	59.1	57.8	0.98	2
7.0	62.9	59.3	0.94	6
8.0	58.9	53.6	0.91	9

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

TABLE 21

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

pH	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TMV + buffer	TMV + buffered seed extract		
3.0	46.3	40.0	0.86	14
4.0	47.5	49.2	1.04	- 4
5.0	48.6	43.3	0.89	11
6.0	47.9	40.8	0.85	15
7.0	48.3	41.0	0.85	15
8.0	48.8	41.2	0.84	16

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

Full statistical treatment for this table is given in the appendix.

TABLE 22 Effect of sodium acid maleate buffer on the inhibitory activity of *P. vulgaris* seed extract

pH	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + buffer	TNV + buffered seed extract		
5.2	87.5	84.8	0.97	3
5.8	84.3	85.8	1.02	- 2
6.0	92.4	92.1	1.00	0
6.8	90.0	92.0	1.02	- 2

* 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 1.13.

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 mellibiose. 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 masking 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

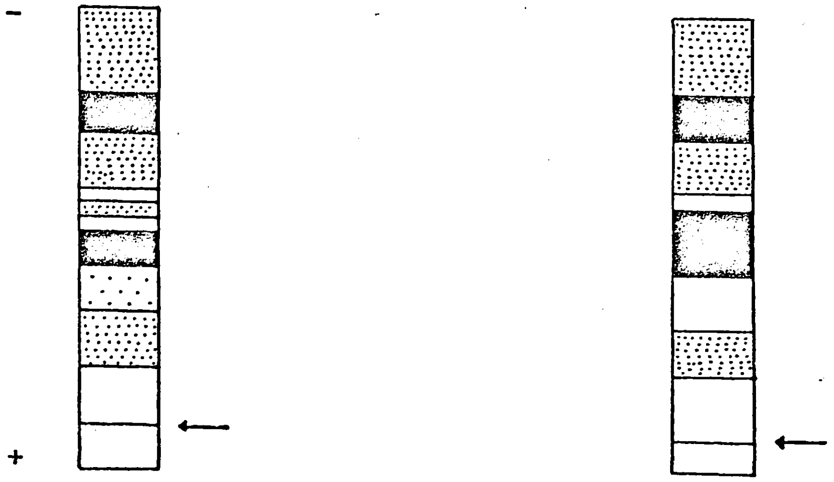
TABLE 23 Effect of dialysis on the inhibitory activity of *P. vulgaris* seed extract

Extract	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + water	TNV + extract		
Sap dialysed against running water (non-dialysable part)	96.4	109.7	1.14	- 14
Sap dialysed against 100 ml distilled water (non-dialysable part)	92.7	87.1	0.94	6
Sap dialysed against 100 ml distilled water (dialysable part)	92.4	104.2	1.13	- 13

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

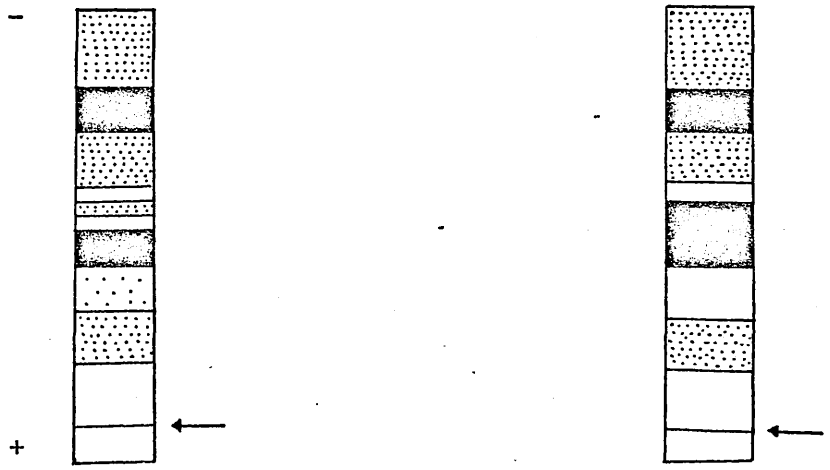
Fig. 4. Disc electrophoresis of the proteins and glycoproteins from P. vulgaris seed extracts

Whole seed extract



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)

TABLE 24

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

Source of extract	mg protein/ml	mg carbohydrate/ml
non-dialysable part	62.50	3.75
dialysable part	1.50	12.75
whole seed extract	65.50	17.00

TABLE 25

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

Extract	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + water	TNV + extract		
supernatant	75.9	53.9	0.71	29
precipitate	85.0	112.6	1.33	- 33

* 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 mμ and 230 mμ 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 25).

(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% $(NH_4)_2SO_4$ saturation contained only 0.5 mg protein per ml but gave 33% 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 26 **Effect of ammonium sulphate fractions of *P. vulgaris* seed extract on the susceptibility of *P. vulgaris* to TNV**

Sample	Saturation ammonium sulphate (%)	Mean number of lesions *		Activity Quotient	Percentage Inhibition	mg protein/ml
		TNV + water	TNV + sample			
Precipitate 1'	10	95.9	87.0	0.91	9	0.15
Precipitate 2'	20	72.1	55.3	0.77	23	3.50
Precipitate 3'	30	91.7	56.5	0.62	38	3.60
Precipitate 4'	40	113.3	73.2	0.65	35	0.50
Precipitate 5'	50	110.6	71.8	0.65	35	2.00
Precipitate 6'	60	108.2	66.4	0.61	39	6.75
Supernatant	70	81.3	77.5	0.95	5	0

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

Fig. 5. Disc electrophoresis of $(NH_4)_2SO_4$ fractions from P. vulgaris

Proteins (No bands were detected in the supernatant)

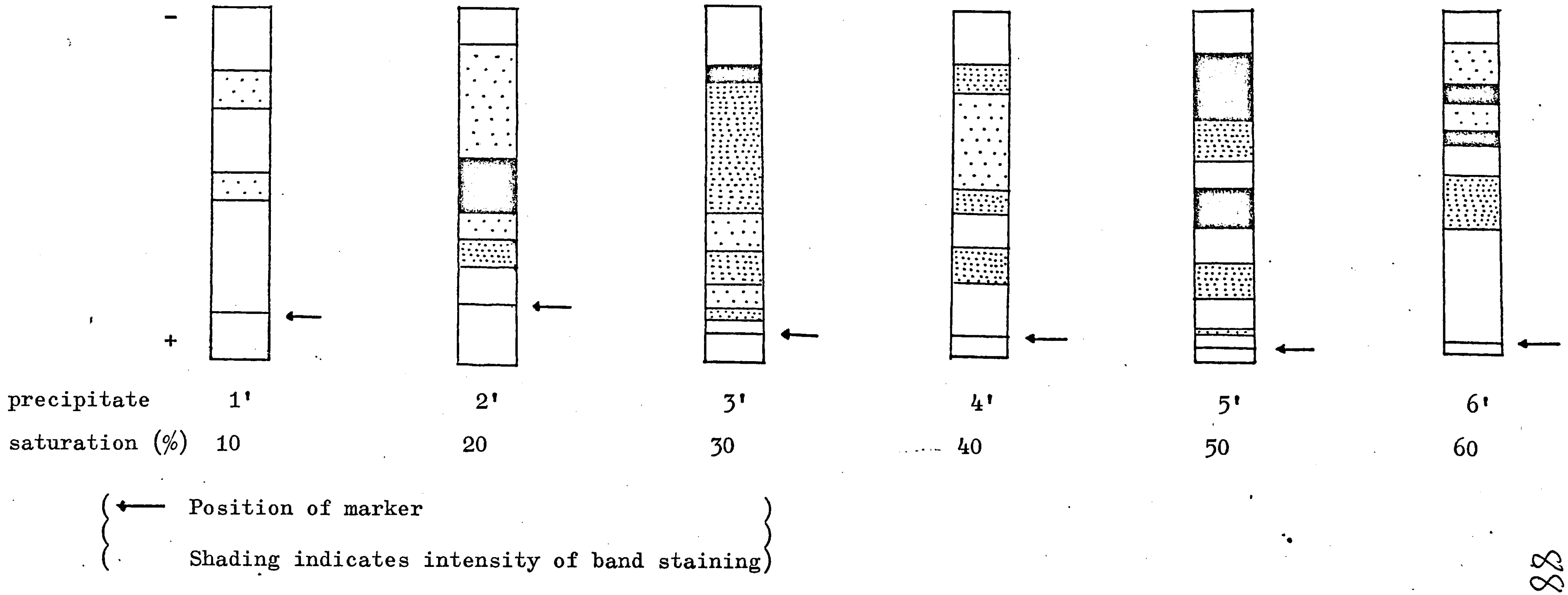
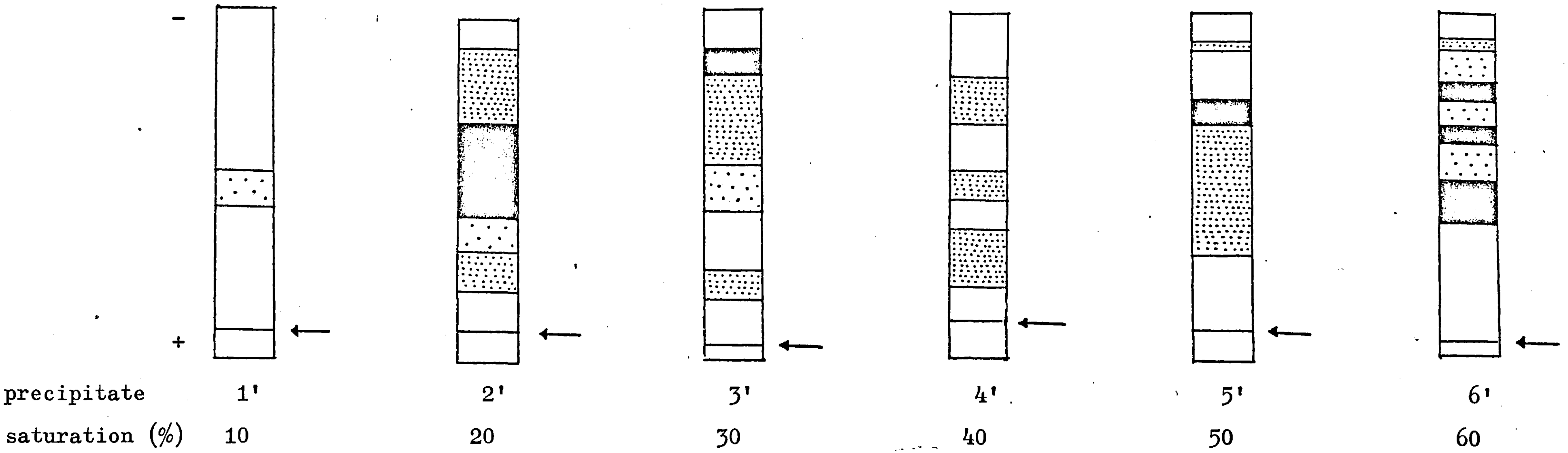


Fig. 6. Disc electrophoresis of $(NH_4)_2SO_4$ fractions from P. vulgaris

Glycoproteins (No bands were detected in the supernatant)



(← Position of marker)
(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 unhibitory 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 Blaszczak 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 TMV 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 Kimmins (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 Murayama, 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 *Psidium guajava* 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. vulgaris* 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. Nart (1972) found however, that the inhibitory effect of *P. vulgaris* 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, Blaszczak et al. (1959) suggested the presence of augmenters in the diluted juices of *Nicotiana debneyi*, *N. glutinosa*, *Lycopersicon esculentum*, *Cucumis sativus* and *Gomphrena globosa*. In addition, Simons et al. (1963) described augmenters in leaf juices of *Kleinia cylindrica*, *Euphorbia schimperi*, *Aloe* sp., *Alum cepa*, *Gasteria* sp., *Haworthia chalivinia* and *Phormium tenax*. Stevens (1970) found that unheated seed extracts from *P. vulgaris*, *Vicia faba*, *P. aureus* enhanced local lesion production by TNV.

Enhancement might come about by effectson 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. vulgaris*, 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, Muelenaere, 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.

Meissl and Bocker (1883) were the first to publish investigations on the isolation and fractionation of soybean proteins. In 1898 this work was continued by Osborne and Campbell who used salt extraction and precipitation methods to separate and identify four different proteins from soybeans. Recent investigationsof 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, Wu 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 (SBA) (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 Hewlett (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 TMV. 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 B_{12}. When not in use, the column was continously 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 mμ.

Elution profiles of the seed extract (Fig. 7) showed the presence of three peaks with elution volumes (Ve) 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 II 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 (2.83 mg/ml); however, it contained only 1.18 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

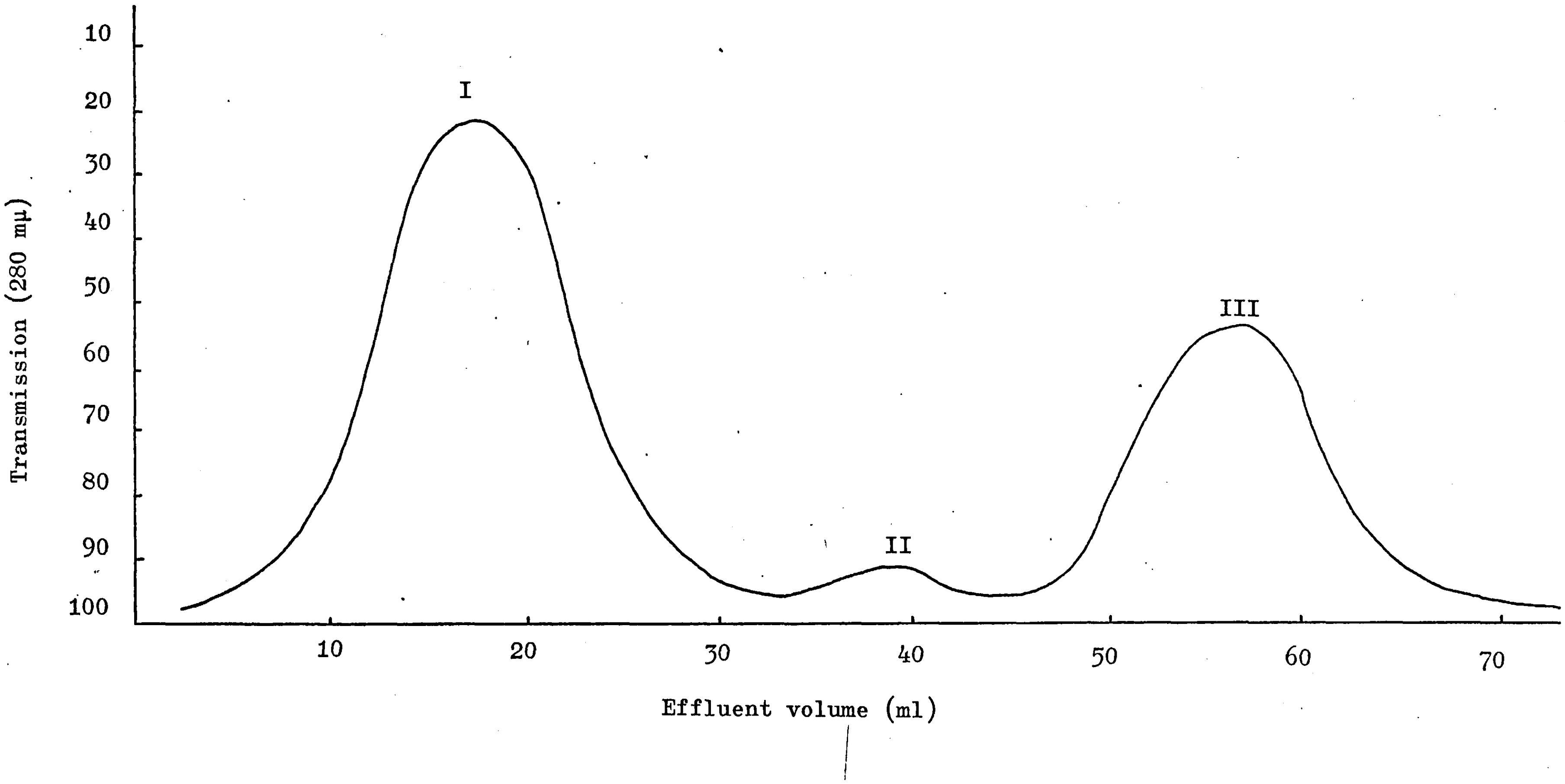


TABLE 27

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

Peak	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + PO_4 buffer pH 7	TNV + Peak		
I	101.8	45.8	0.45	55
II	100.0	48.3	0.48	52
III	102.3	124.0	1.21	- 21

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

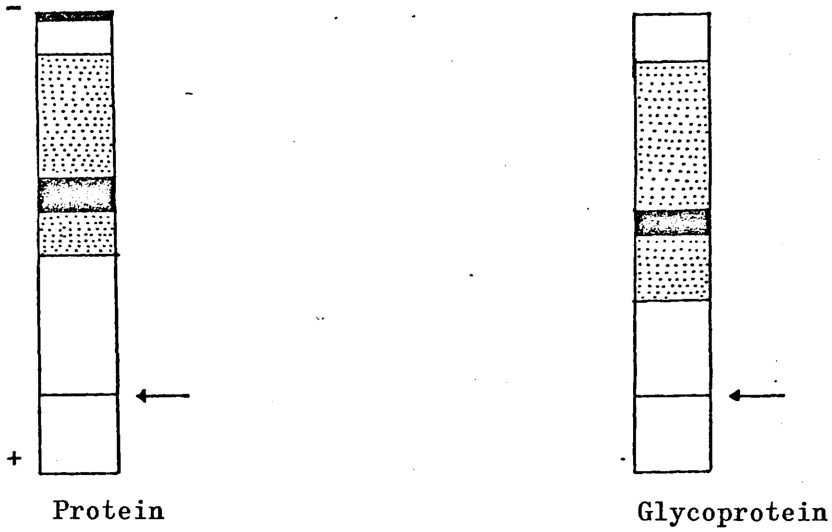
TABLE 28

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

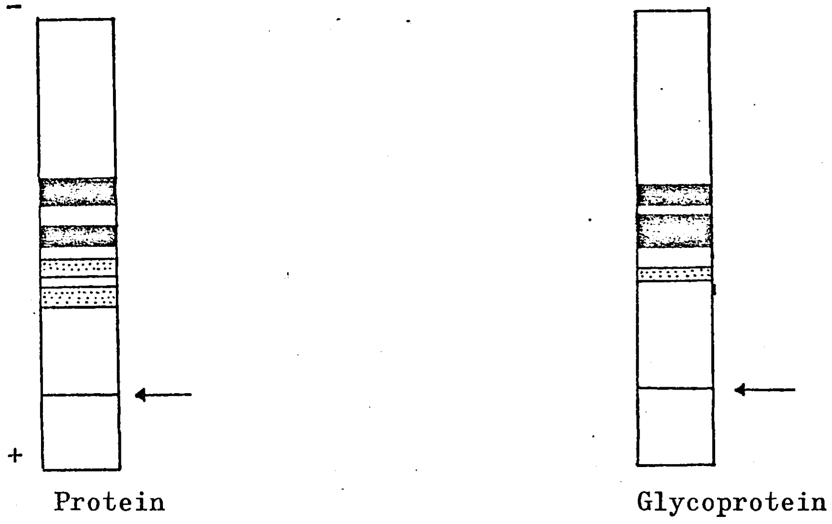
Peak	mg protein/ml	mg carbohydrate/ml
I	2.03	0.04
II	0.29	0.01
III	1.18	2.83

Fig. 8 Disc electrophoresis of the Sephadex G-100 fractions from G. max seed extracts

Peak I



Peak II



← 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 Rf 0.64, whereas the protein and the glycoprotein bands of peak II were located in the middle and the lower part of the gel with Rf's of between 0.46 and 0.78. 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 (MW = 67,000), horse heart cytochrome C (MW = 12,400), peroxidase (MW = 40,000) and γ globulin (MW = 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.

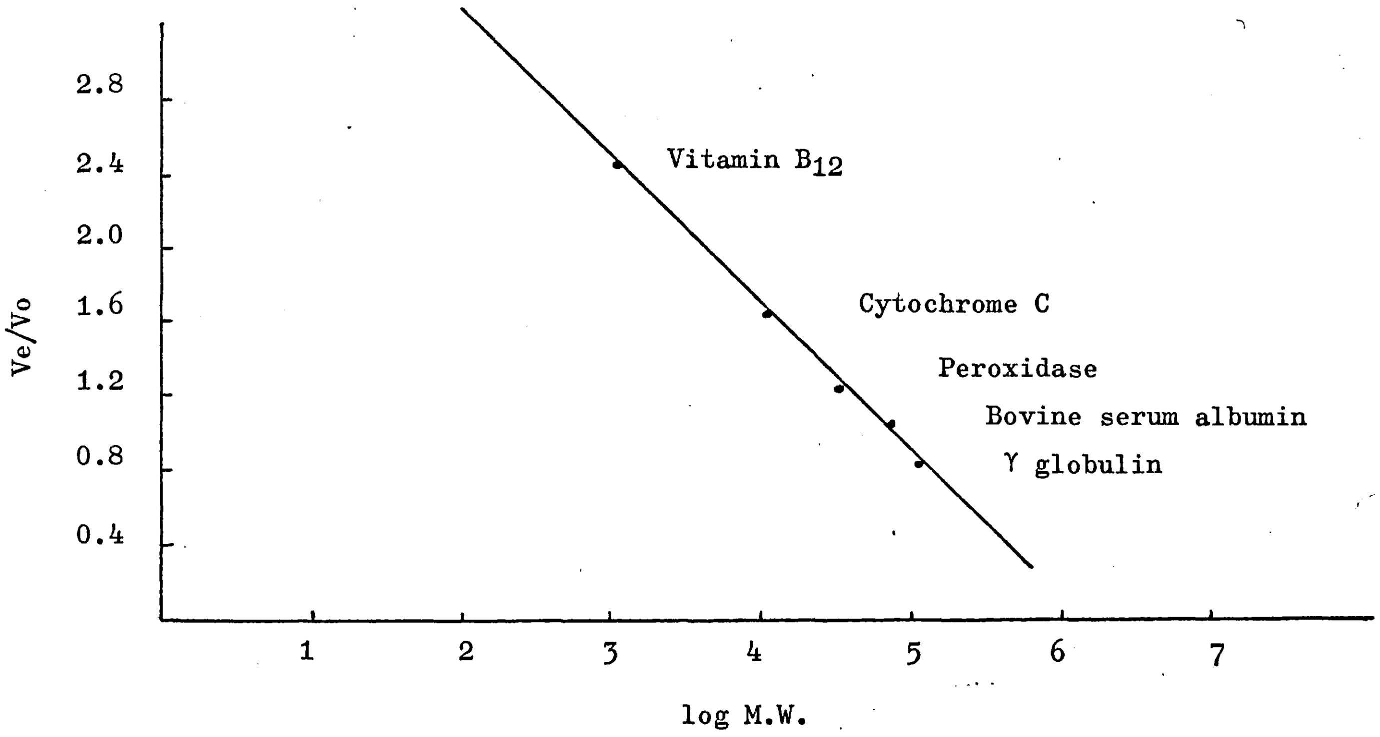
The Ve/Vo readings were plotted against their logarithmic molecular weight (Fig. 9), and they were found to fit directly on a straight line.

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**

Standard compounds	Ve	Ve/Vo	log molecular weight (log M.W.)	molecular weight
γ globulin	19	0.82	5.00	100,000
Bovine serum albumin	24	1.04	4.83	67,000
Peroxidase	28	1.17	4.60	40,000
Cytochrome C	37	1.60	4.09	12,400
Vit. B_{12}	56	2.43	3.10	1,357

Fig. 9 Ve/Vo ratios and log molecular weights of standard compounds



ranged between 158,500 (peak I) and 17.780 (peak II). Peak III which was not inhibitory, had a molecular weight of about 1,585 (Table 30).

TABLE 30

Molecular weight determination of Sephadex G-100
fractions from *G. max* seed extract

Peak	Molecular weight
I	158,500
II	17,780
III	1,585

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

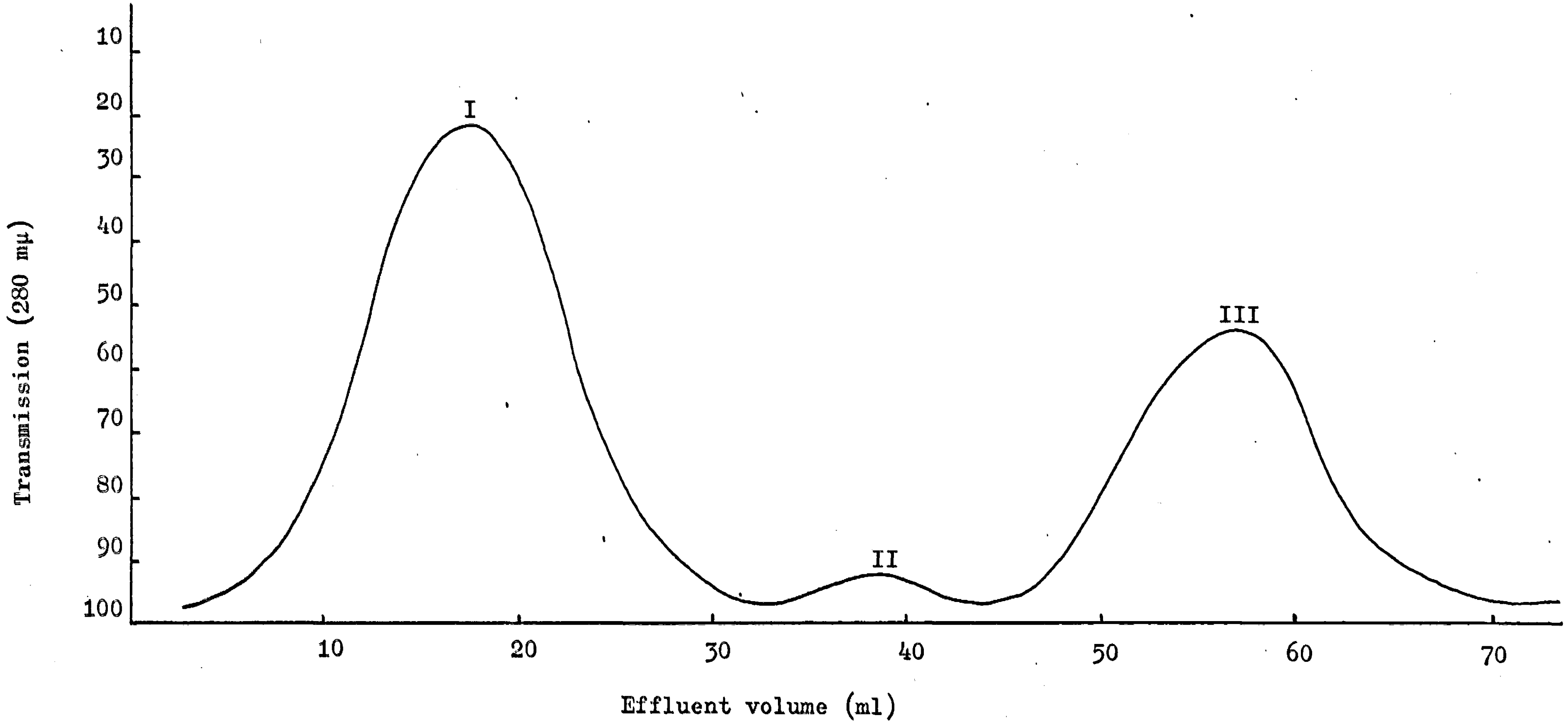
(i) Heated seed extract

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 *G. max* seed extract



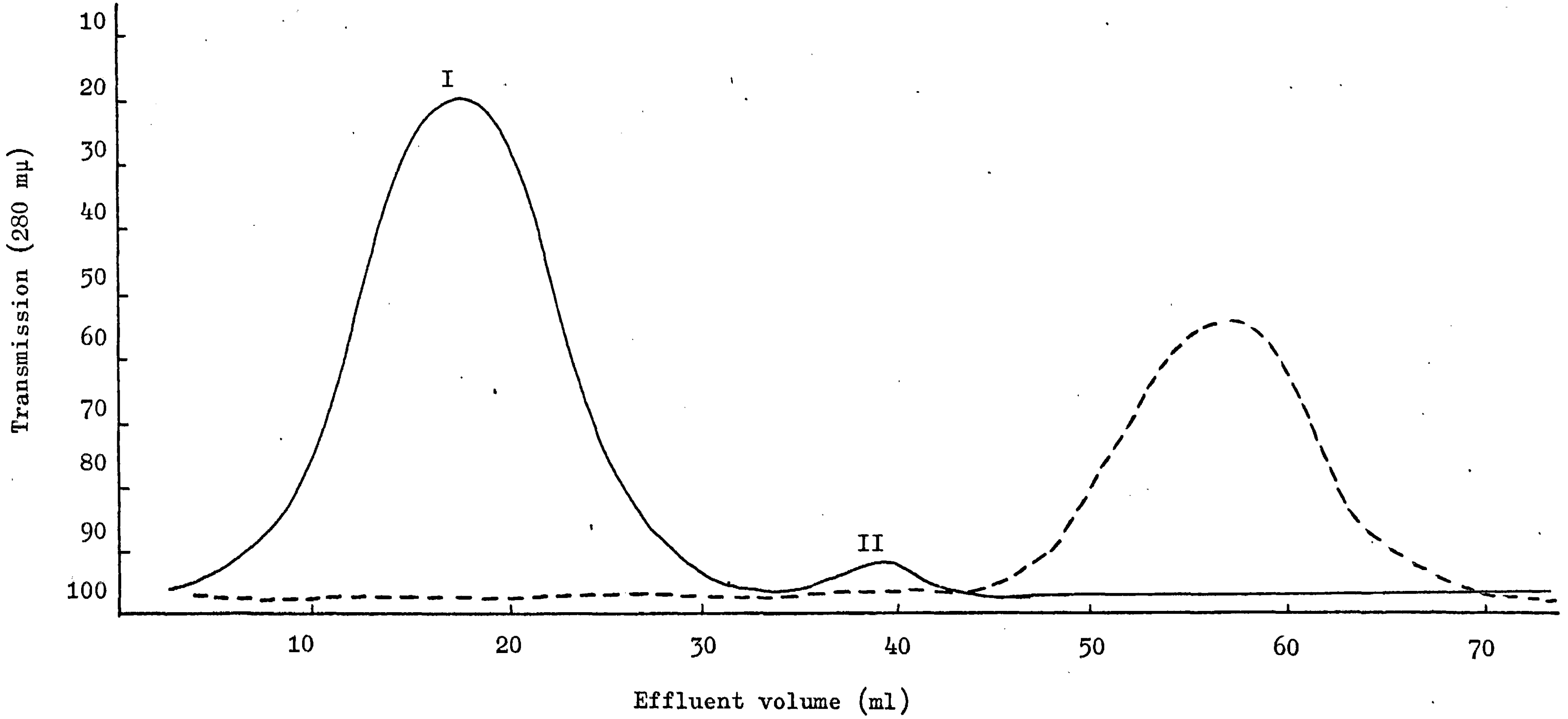
were passed through Sephadex G-100. The dialysate which had previously been shown to retain all the inhibitory activity of the extract, consisted of peaks I and II, both of which showed inhibition of TNV. (Fig. 11). The dialysable part, which was not inhibitory, contained only peak III. (Fig. 12). The Sephadex column experiments showed that peaks I and II contain compounds with molecular weights about 158,500 and 17.780. 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 158,500 (peak I) and 17.780 (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 <u>G. max</u> seed extract

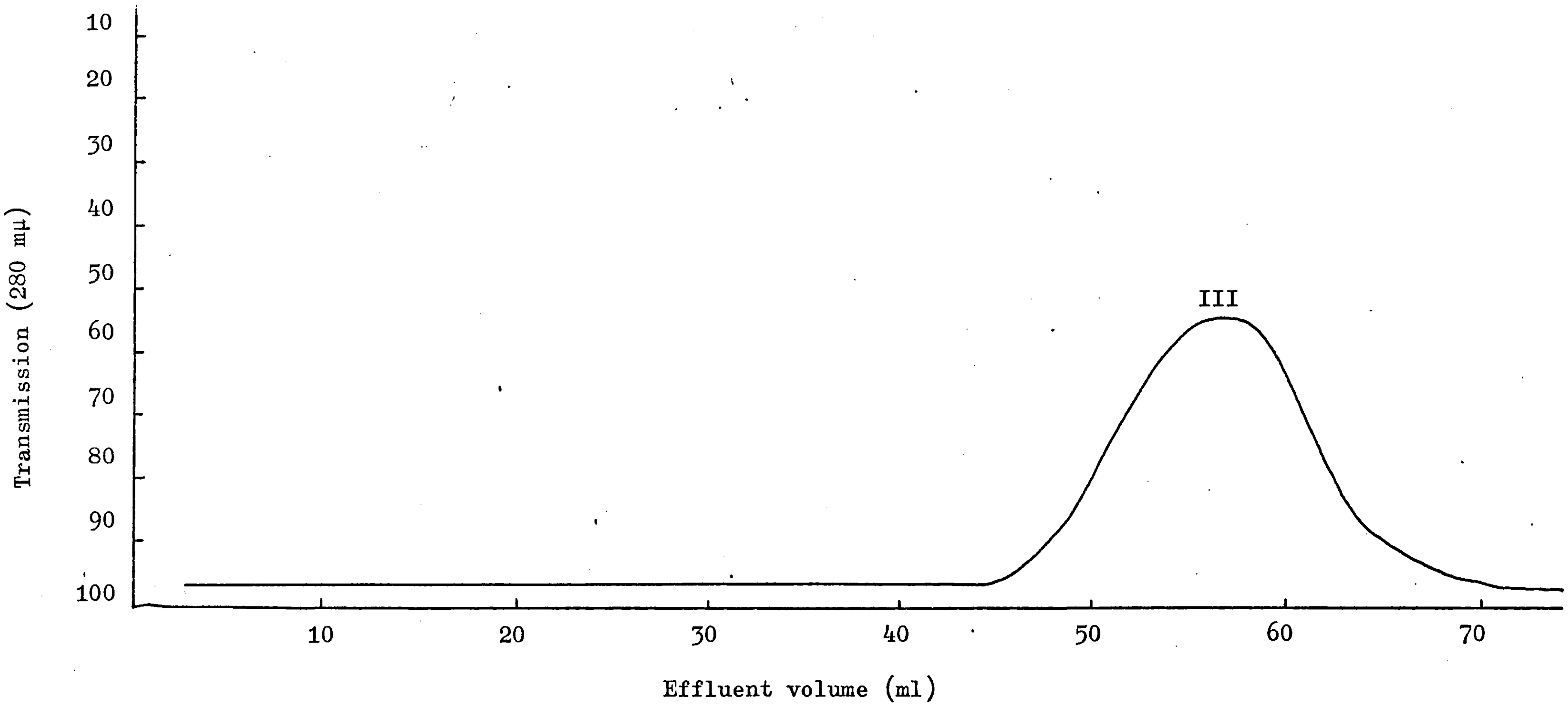
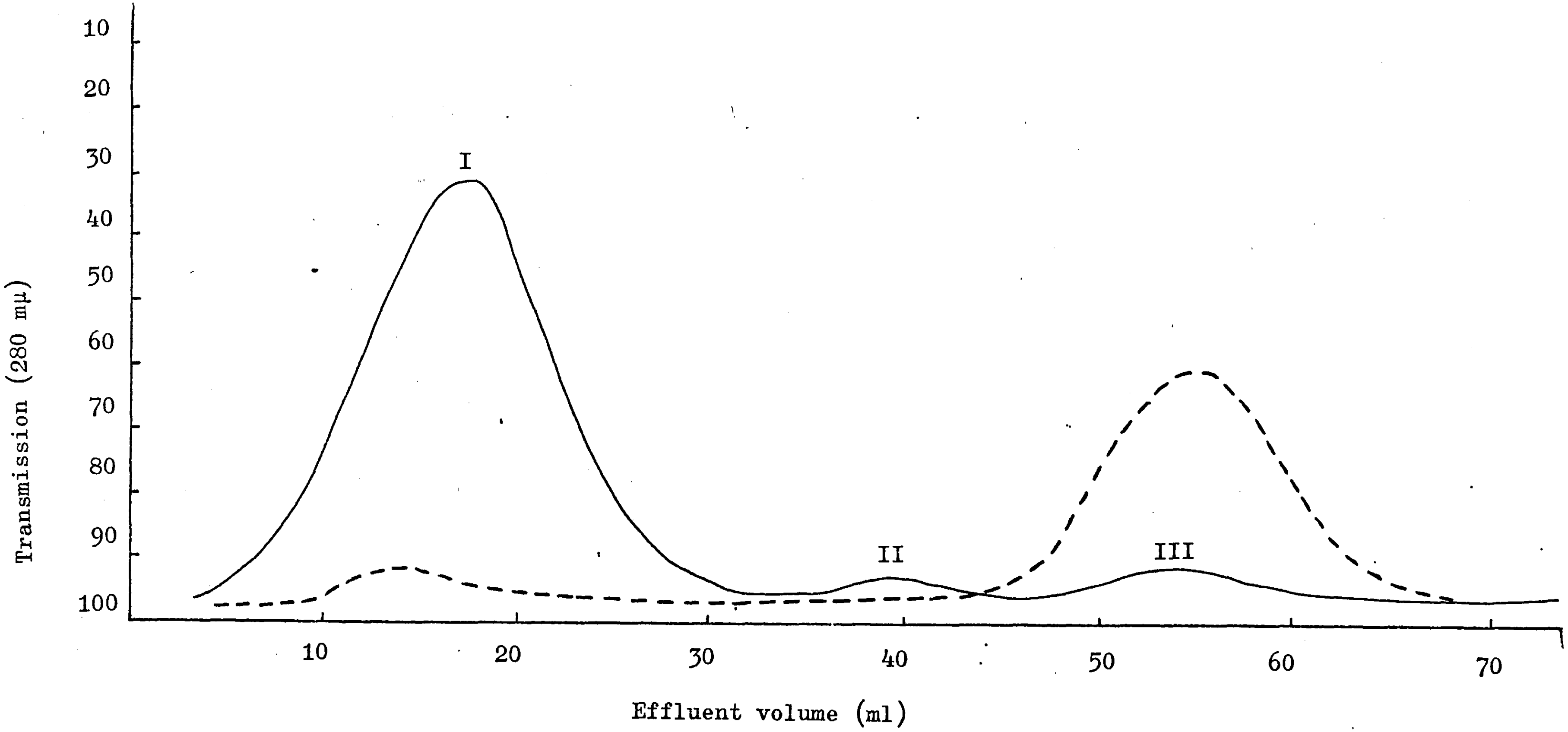
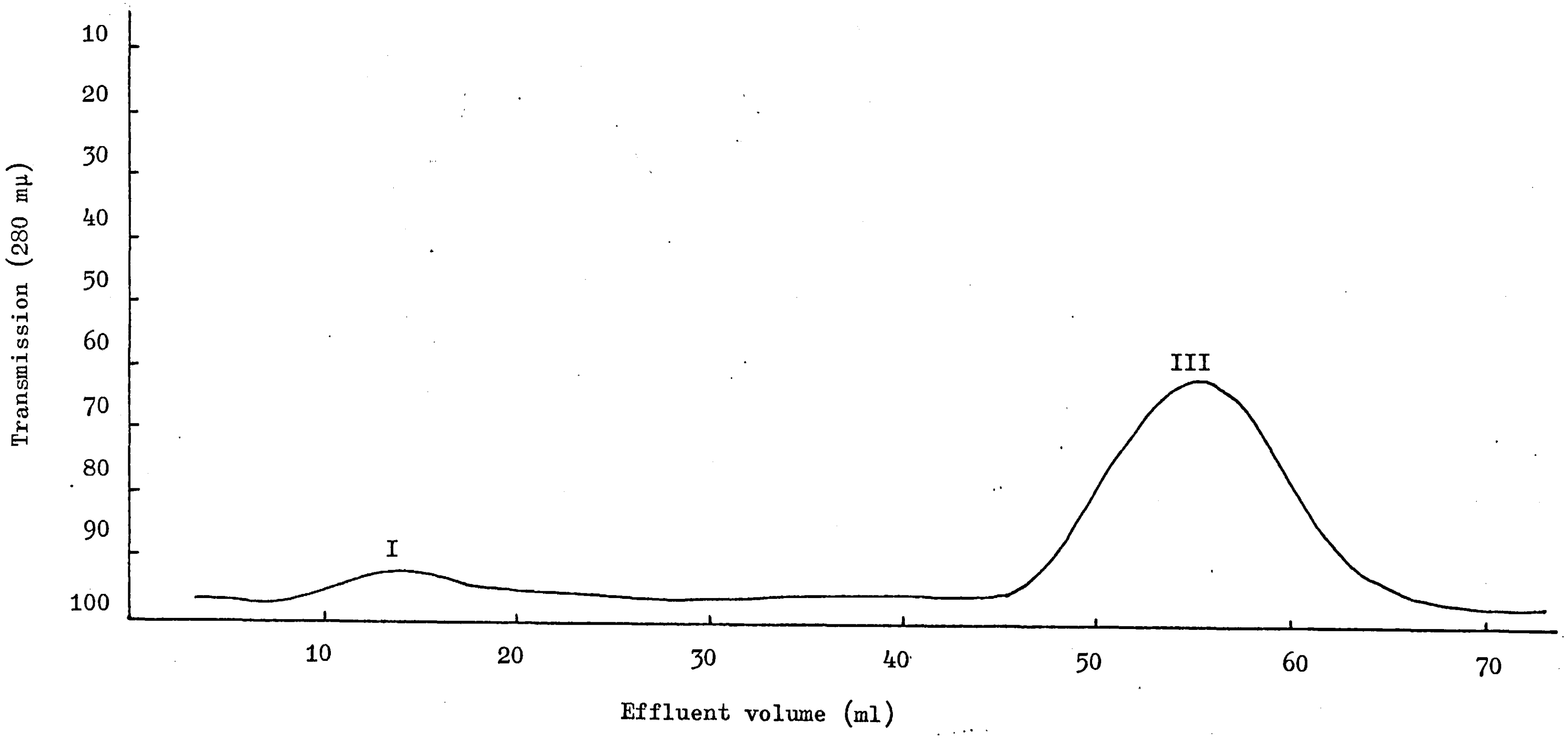


Fig. 13 Sephadex G-100 column chromatography of precipitate from alcohol treated <u>G. max</u> seed extract



(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 <u>G. max</u> seed extract



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

2. THE ANALYSIS OF GLYCINE 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 (Weber, Nordman and Grasbeck, 1967). A suspension of CM-52 cellulose was prepared according to the manufacturers' 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 KH_2PO_4 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:

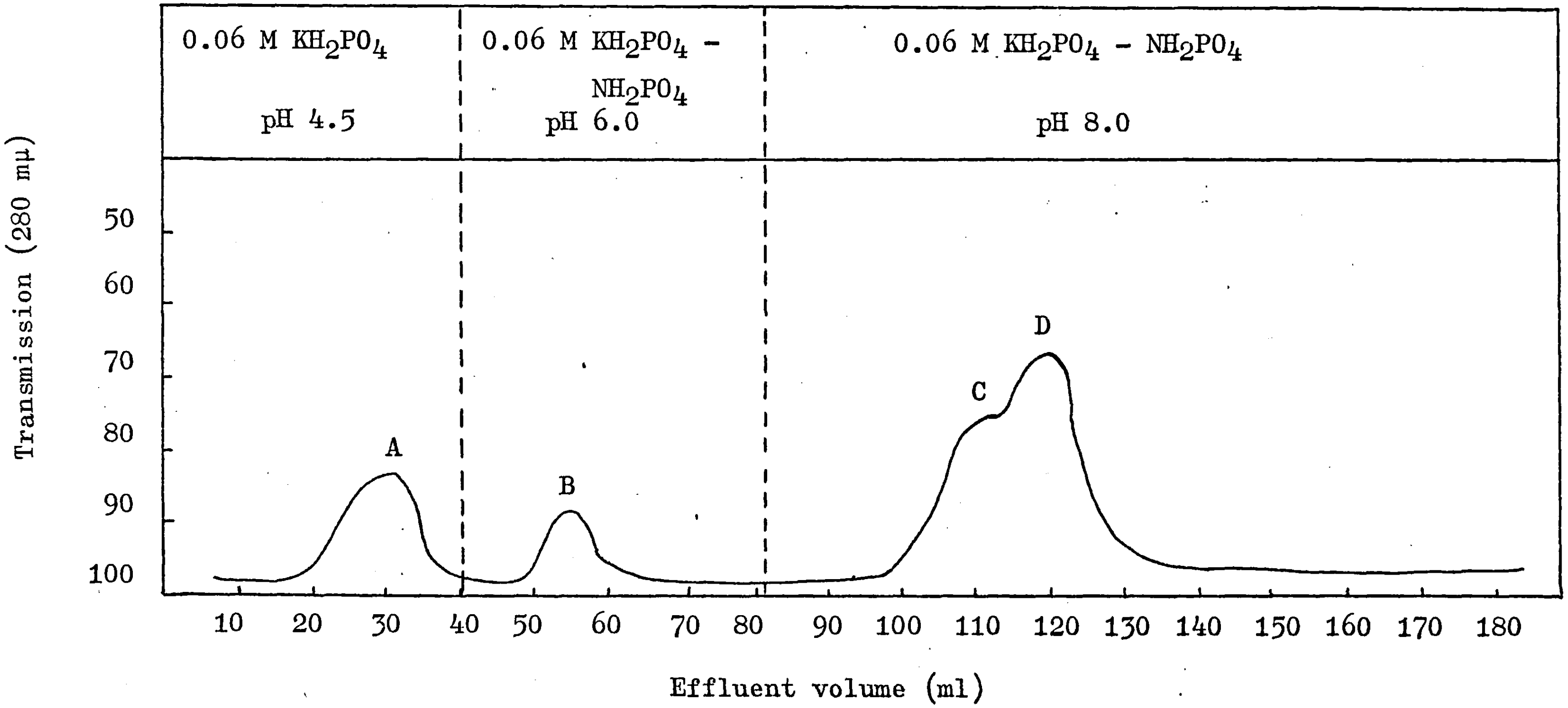
(a) 0.06 M KH_2PO_4, pH 4.5

(b) 0.06 M KH_2PO_4 Na_2HPO_4, pH 6.0

(c) 0.06 M KH_2PO_4 Na_2HPO_4, pH 8.0

Monitoring of absorption at 280 mμ 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 <u>G. max</u> seed extract



tested for inhibitory activity against TNV.

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

Peak	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + water	TNV + Peak		
A	49.9	43.9	0.98	2
B	38.9	27.3	0.70	30
C	46.2	38.9	0.84	16
D	41.1	33.4	0.81	19

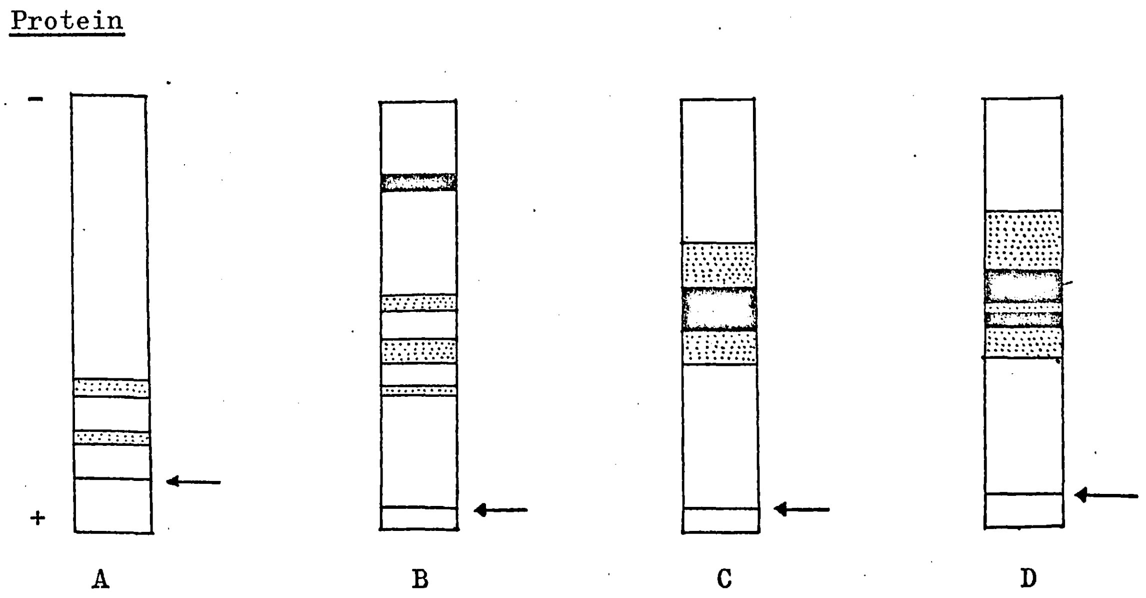
* Each figure represents the mean number of lesions for ten replications

TABLE 32

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

Peak	mg protein/ml
A	0.025
B	0.020
C	0.050
D	0.250

Fig. 16 Disc electrophoresis of the CM-52 fractions from <u>G. max</u> seed extracts

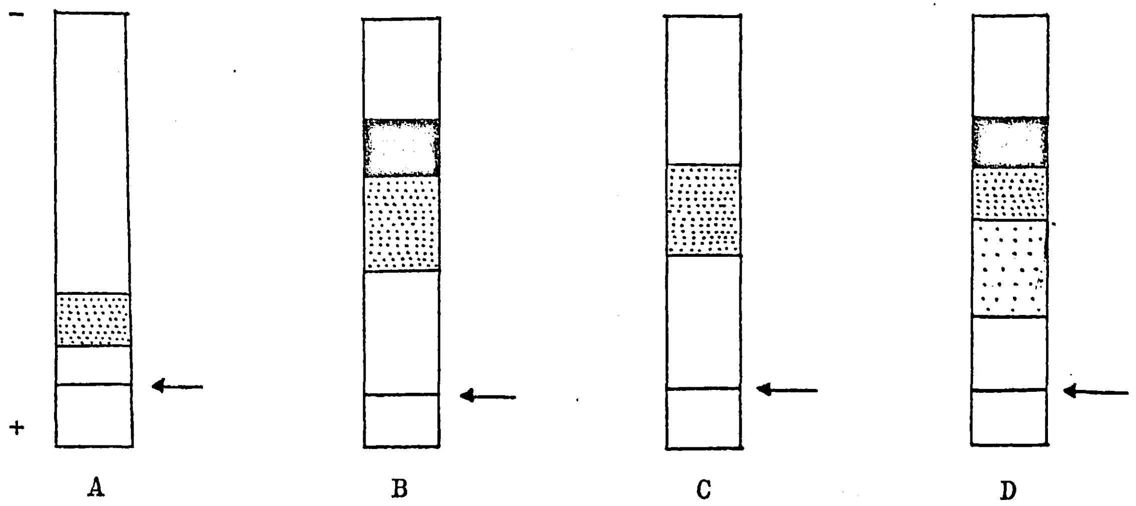


← position of marker

Shading indicates intensity of band staining

Fig. 17 Disc electrophoresis of the CM-52 fractions from G. max seed extracts

Glycoprotein



← position of marker

Shading indicates intensity of band staining

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 CM-52 cellulose, dialysis will have removed this type of non-proteinaceous material so that CM-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 CM-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 $\text{Ext}_{93}^{4.2}$ (Soybean). Experiments described in this section have been produced in the same way and the same nomenclature is used.

(i) Preparation of $\text{Ext}_{93}^{4.2}$ (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) (Stead 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 dialysed 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 equilibriate 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 $Ext_{93}^{4.2}$ (soybean) on local lesion production by TNV

Dilution	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + water	TNV + extract		
Neat	29.4	8.8	0.30	70
10^{-1}	67.7	41.5	0.61	39
10^{-2}	49.0	35.3	0.72	28

* 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 Mackis, Sasame, 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 N NaOH. The final elutant 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 280 mμ 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 DEAE-52 fractions of Ext$^{4.2}_{93}$ (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$^{4.2}_{93}$ (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 18%, 12% and 37% respectively. Therefore, marked inhibition was obtained in peak 6. The difference between inhibition obtained by using Ext$^{4.2}_{93}$ (Soybean)

Fig. 18 Chromatography of Ext$_{93}^{4.2}$ (Soybean) on DEAE-52 column

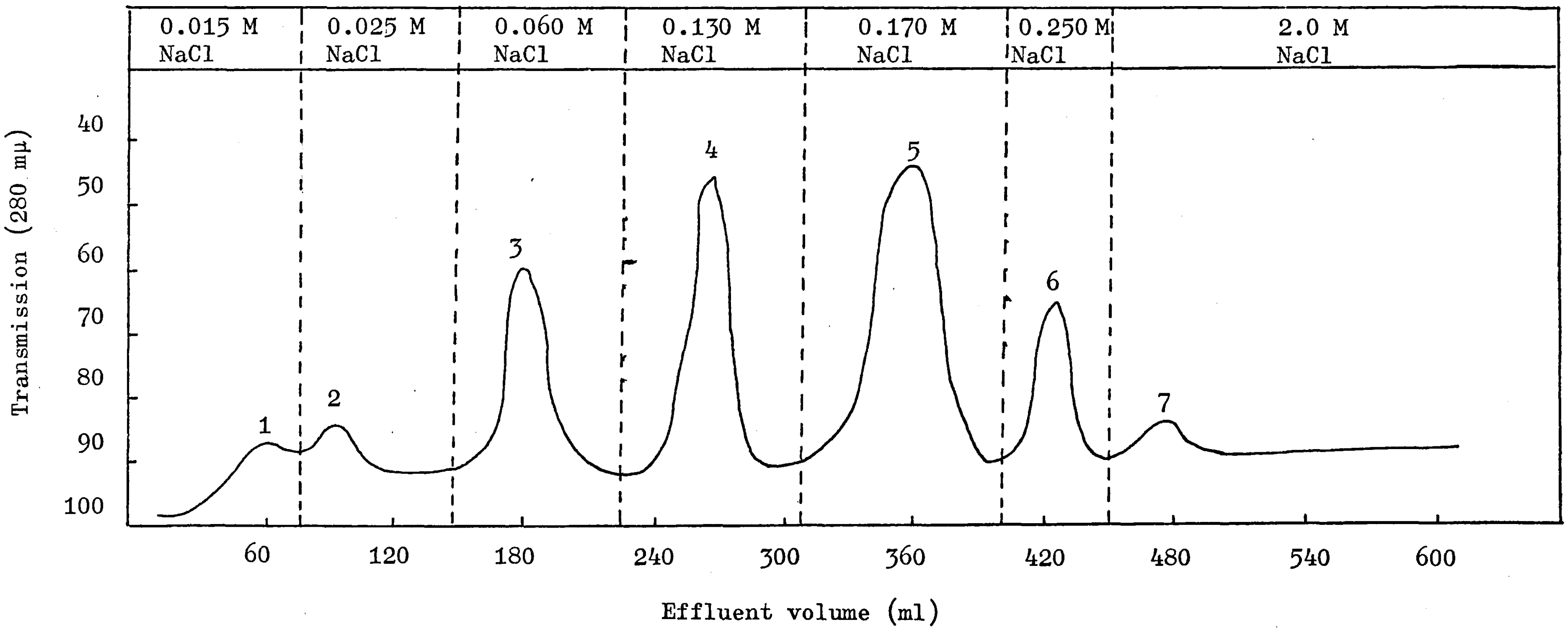


TABLE 34 Effect of DEAE-52 fractions of Kal$^{4.2}_{93}$ (soybean) on local lesion production by TNV

Sodium chloride elutant concentration (M)*	Peak	Mean number of lesions ***		Activity Quotient	Percentage Inhibition
		TNV + water	TNV + Peak		
0.015	1	94.0	77.1	0.82	18
0.025	2	96.0	95.5	0.99	1
0.060	3	70.3	67.7	0.96	4
0.130	4	69.2	72.9	1.05	- 5
0.170	5	95.3	85.7	0.88	12
0.250	6	137.9	86.2	0.63	37
2.000 **	7	127.1	132.5	1.04	- 4

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

** Unbuffered Elutant

*** 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 DEAE-52 cellulose.

Peaks 2, 3, 4 and 7 showed no inhibitory effect against TNV, giving an activity quotient of 0.99, 0.96, 1.03 and 1.04 respectively.

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

(iv) Protein estimation of DEAE-52 fractions from $\text{Ext}_{93}^{4.2}$ (Soybean)

The seven peaks showed positive protein test (Table 33). 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 DEAE-52 fractions from $\text{Ext}_{93}^{4.2}$ (Soybean)

Electrophoresis experiments for $\text{Ext}_{93}^{4.2}$ (Soybean) showed that it consisted of nine bands of protein and three wide bands of glycoprotein. (Fig. 19). After fractionation of this $\text{Ext}_{93}^{4.2}$ (Soybean) on a DEAE-52 column, each peak was examined by disc electrophoresis. (Figs. 20 and 21).

Peak 1, which was inhibitory (18%), consisted only of one band of protein (Rf = 0.27) and one band of glycoprotein (Rf = 0.21).

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

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

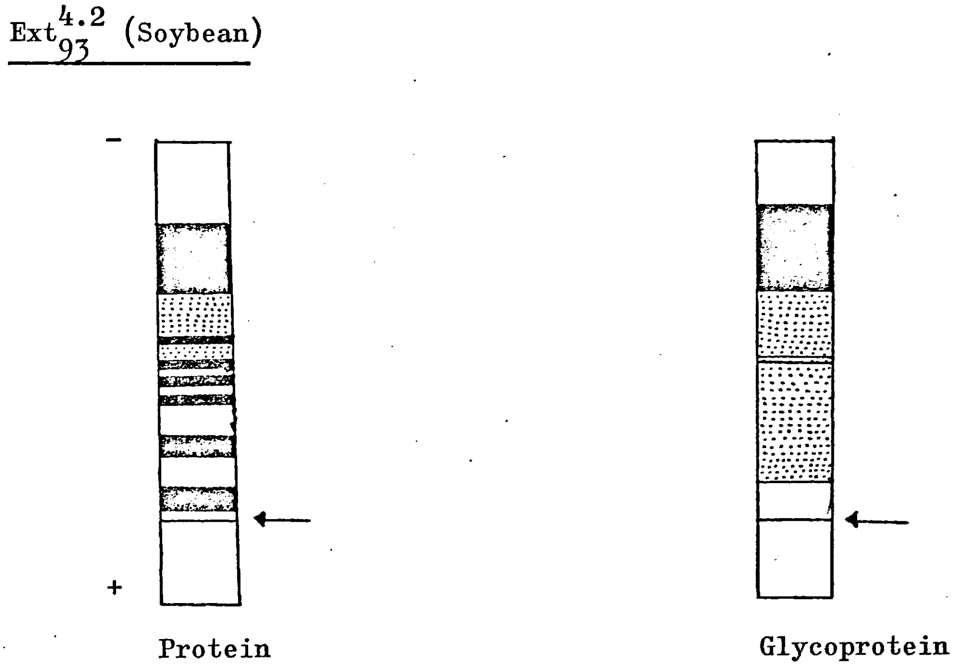
TABLE 35

Protein estimation of DEAE-52 fractions from

$Ext_{93}^{4.2}$ (Soybean)

Peak	mg protein/ml
1	0.80
2	0.70
3	1.00
4	1.50
5	1.25
6	0.60
7	0.10

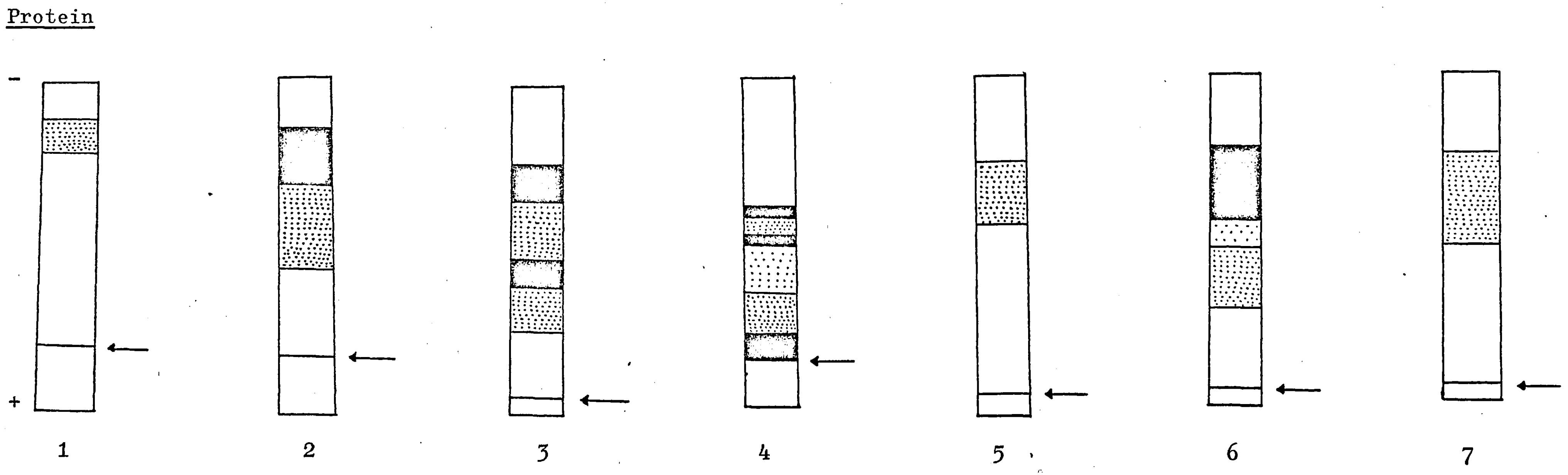
Fig. 19 Disc electrophoresis of $\text{Ext}_{93}^{4.2}$ (Soybean)



← position of marker

Shading indicates intensity of band staining

Fig. 20 Disc electrophoresis of DEAE-52 fractions from $\mathrm{Ext}_{93}^{4.2}$ (Soybean)

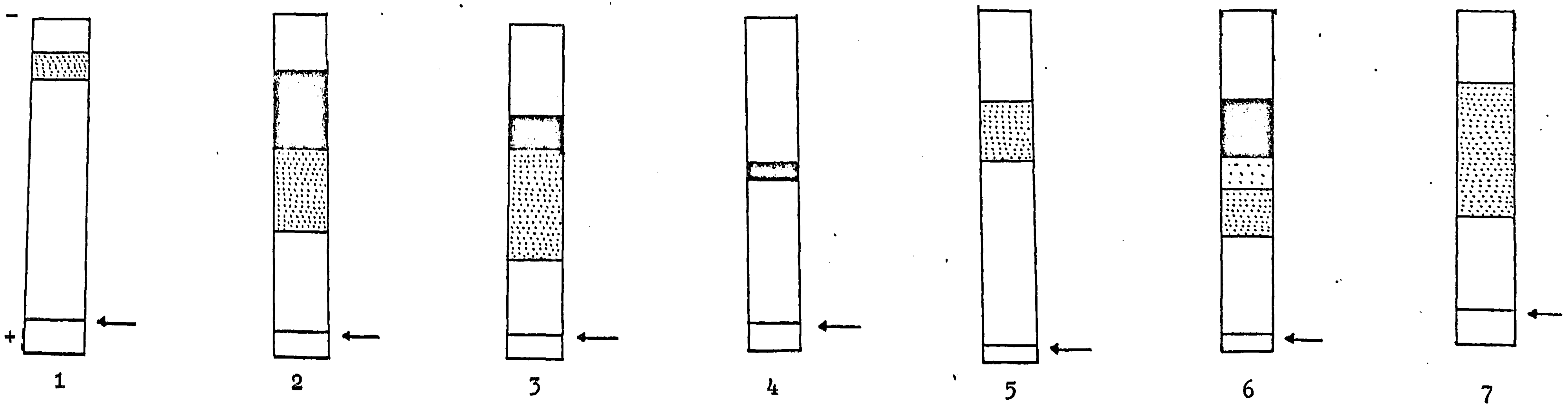


← position of marker

Shading indicates intensity of band staining

Fig. 21 Disc electrophoresis of DEAE-52 fractions from $Ext_{93}^{4.2}$ (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 (37%) 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 seem 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 17,750 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. Ragetli (1957) reported that the carnation (*Dianthus caryophyllus*) inhibitor has a molecular weight of 10,000. In 1969 Wyatt and Shepherd found that *Phytolacca americana* juice contains a protein virus inhibitor with a molecular weight of 13,000. Recently, Smookler (1971) studied the properties of inhibitors from extracts of *Chenopodium amaranticolor*, *C. album*, *Atriplex nitens* and *Amaranthus caudatus*. 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 CM-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 8. This suggests that soybean extract contains three plant virus inhibitors, one (peak B) which is weakly attached to the cation exchanger CM-52 column, and is thus acidic in nature; on the other hand, peaks C and D were strongly attached to the column (CM-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 CM-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 $Ext_{93}^{4.2}$ (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 1.04 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 (1968) found similar results when he applied DEAE Sephadex A-50 chromatography to separate PVX virus inhibitors from potato leaf sap. The inhibitor was eluted at the concentration 0.15 - 0.3 M NaCl and was electrophoretically homogeneous. In 1971 Smookler used a different ion-exchanger (CM-Sephadex C-25) to purify the inhibitors prepared from leaves of *Chenopodium amaranticolor*, *C. album*, *Atriplex nitens* and *Amaranthus caudatus*. 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 those 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 PHASEOLUS VULGARIS SEEDS

Although *P. vulgaris* seed extract has been tested against plant viruses (Cheo, 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 $\text{Ext}_{93}^{4.2}$ (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 38% and 12% inhibition respectively. Peak III was not inhibitory

Fig. 22 Sephadex G-100 column chromatography of <u>P. vulgaris</u> seed extract

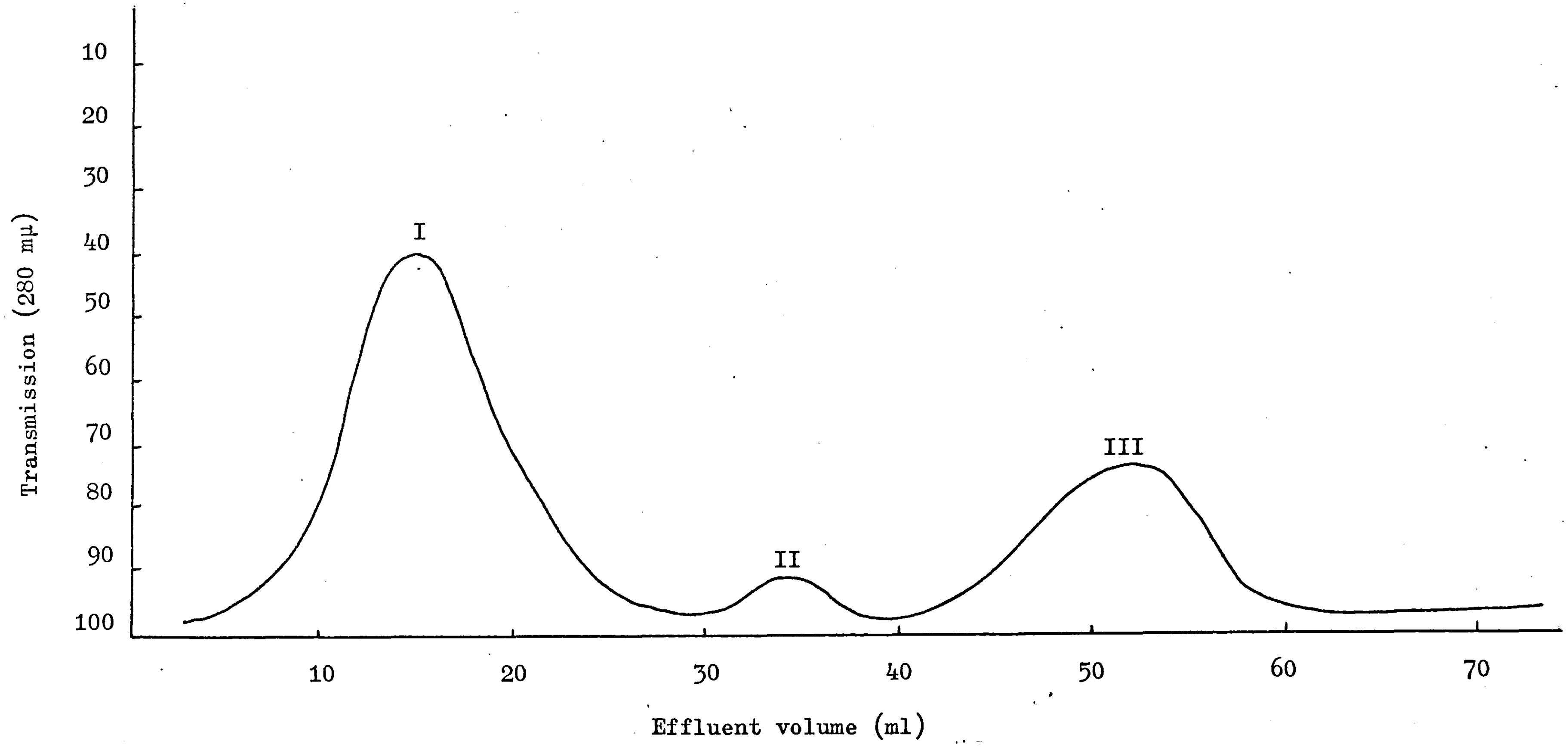


TABLE 36

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

Peak	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + PO_4 buffer pH 7	TNV + Peak		
I	61.0	38.0	0.62	38
II	73.9	65.0	0.88	12
III	73.5	91.9	1.25	- 25

* 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

Peak	mg protein/ml	mg carbohydrate/ml
I	0.66	0.030
II	0.07	0.003
III	0.02	2.840

and it gave an activity quotient of 1.23

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

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

Peak I contained the highest protein concentration (3.66 mg/ml) and only 0.03 mg/ml carbohydrate. Peak II contained 0.57 mg/ml protein and 0.093 mg/ml carbohydrate. The highest carbohydrate concentration was obtained in peak III (2.34 mg/ml); however, it contained only 0.02 mg/ml protein. (Table 37).

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

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

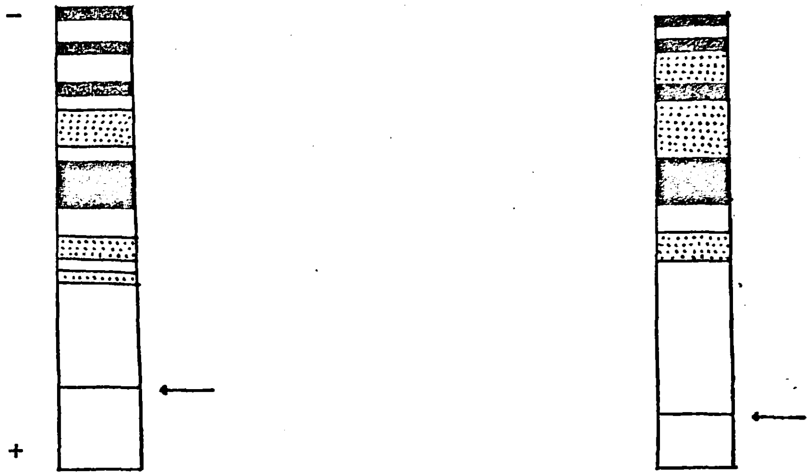
By plotting Ve/Vo ratios for peaks I, II and III on Fig. 3, the molecular weights were determined. The inhibitory peaks I and II had molecular weights of about 177,800 and 12,500 respectively. Peak III, which showed no inhibition when it was tested against [illegible], and showed no positive results in electrophoresis studies, had a molecular weight of about 3,162. (Table 37a).

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

Fig. 23

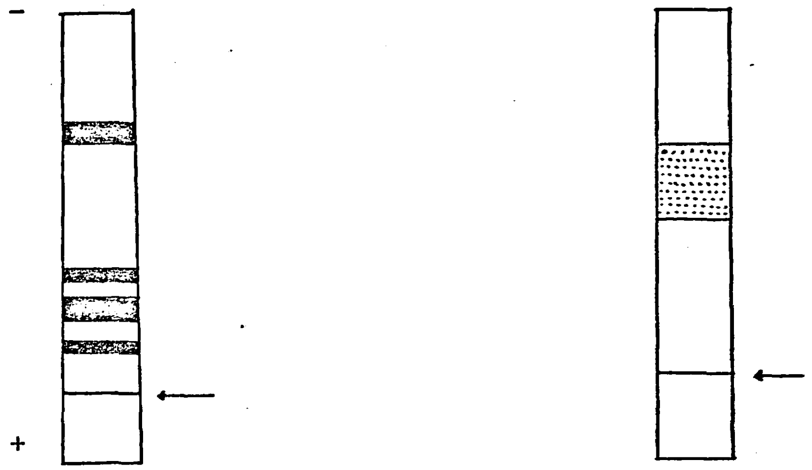
Disc electrophoresis of the Sephadex G-100 fractions from *P. vulgaris* seed extracts

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

Peak	Molecular weight
I	177,800
II	12,590
III	3,162

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.

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

(i) Heated seed extract

Although heat revealed the presence of inhibitors and masking compounds in *P. vulgaris* 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) Dialysed seed extract

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. vulgaris* seed extracts.

Fig. 24 Sephadex G-100 column chromatography of heated *P. vulgaris* seed extract

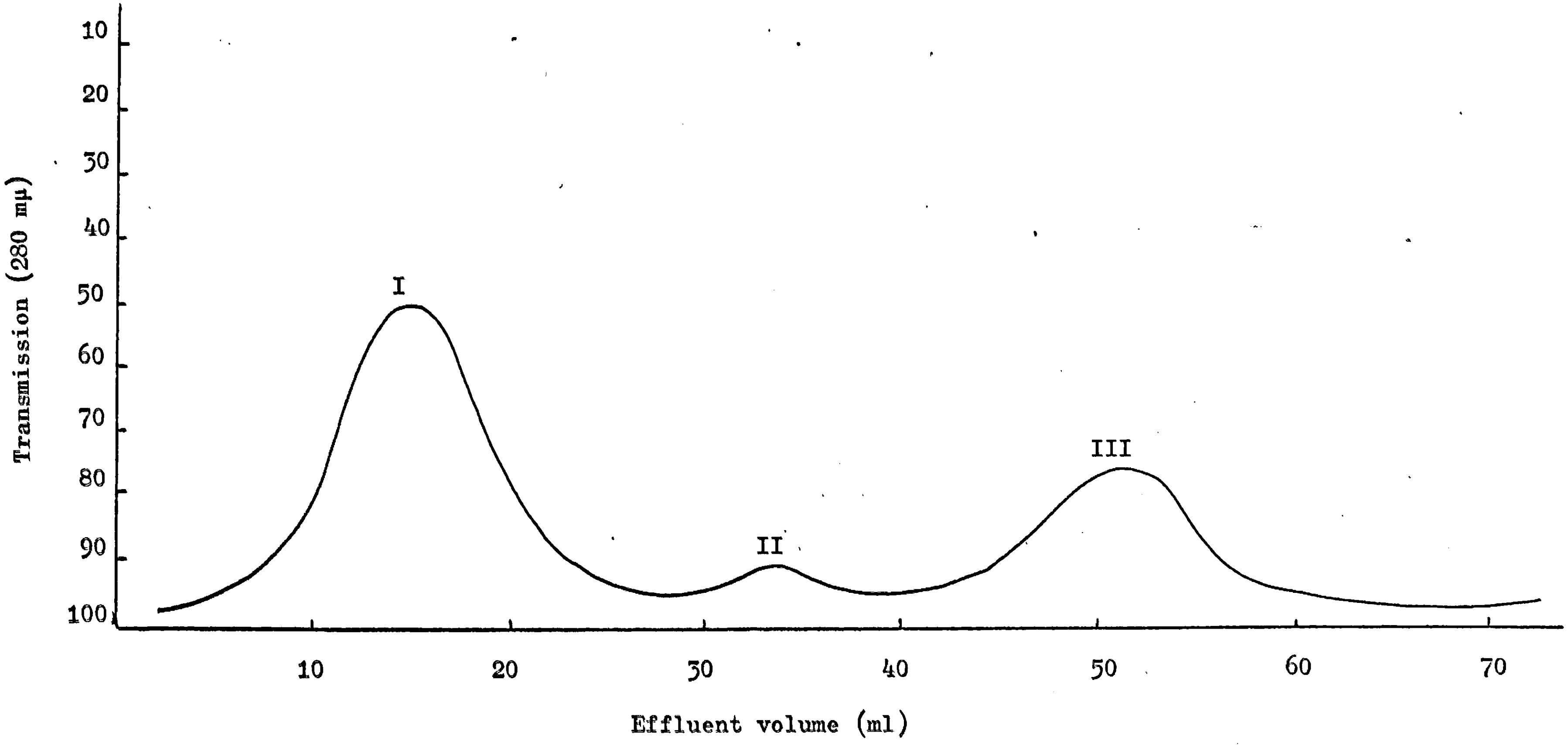
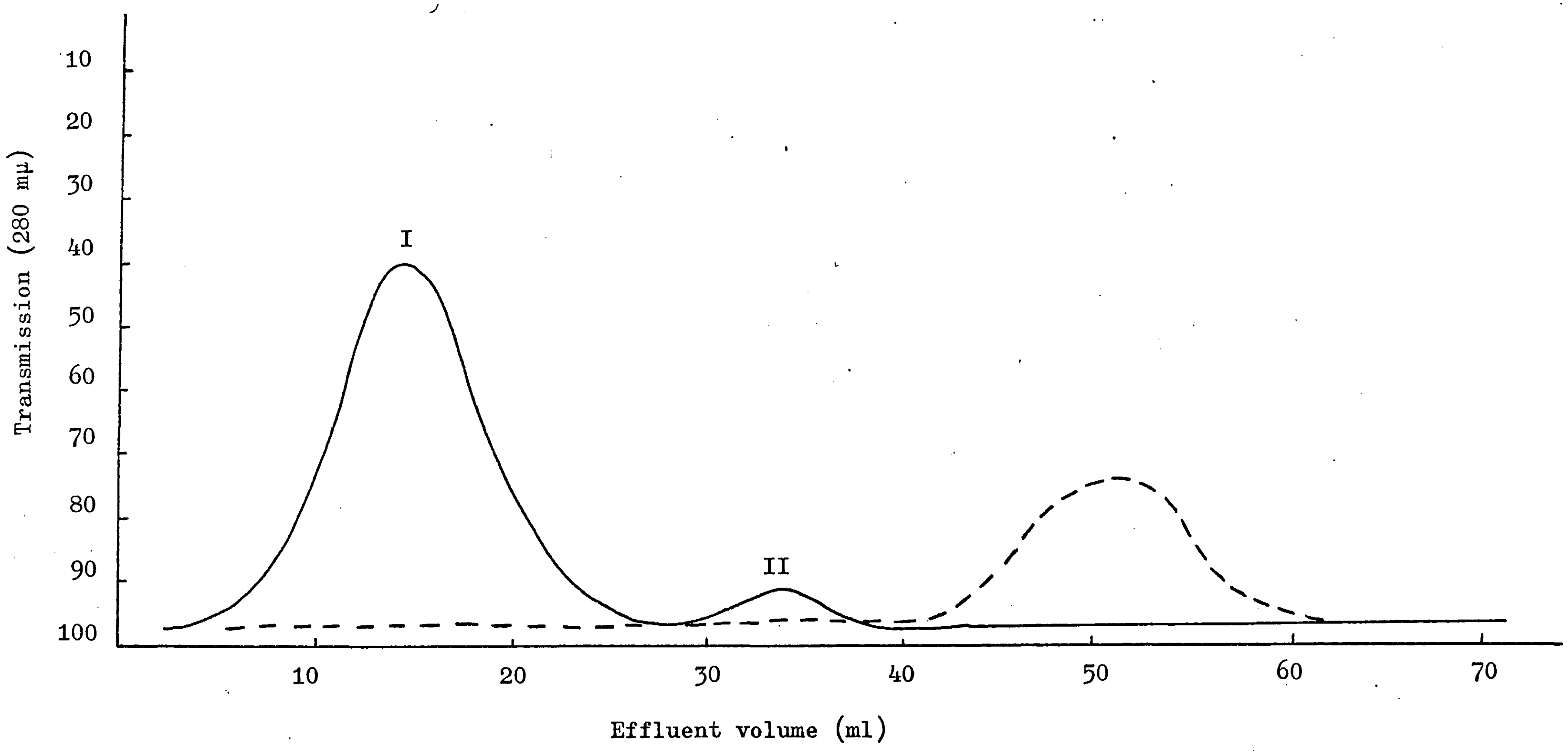


Fig. 25 Sephadex G-100 column chromatography of the non-dialysable part of <u>P. vulgaris</u> seed extract



(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 <u>P. vulgaris</u> seed extract

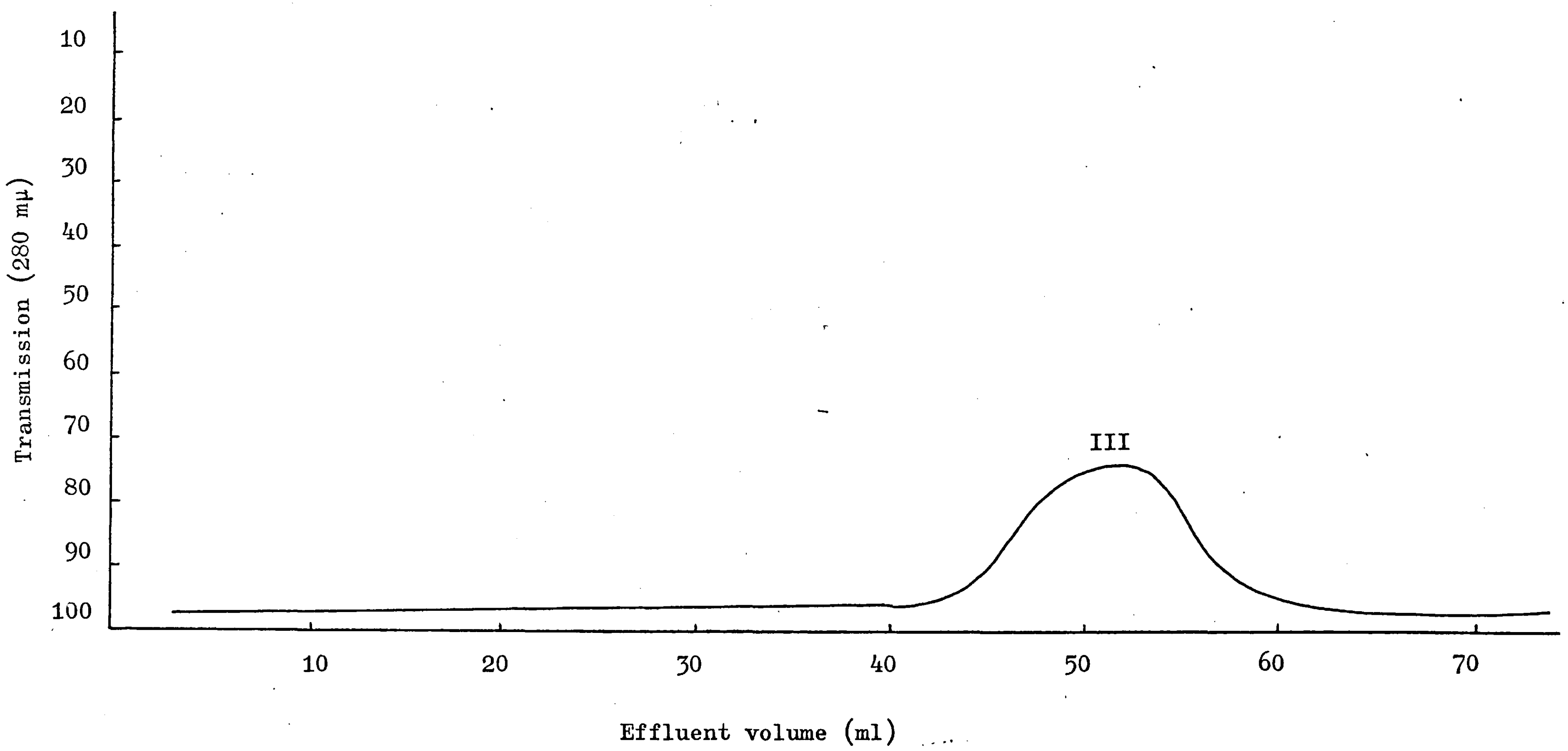
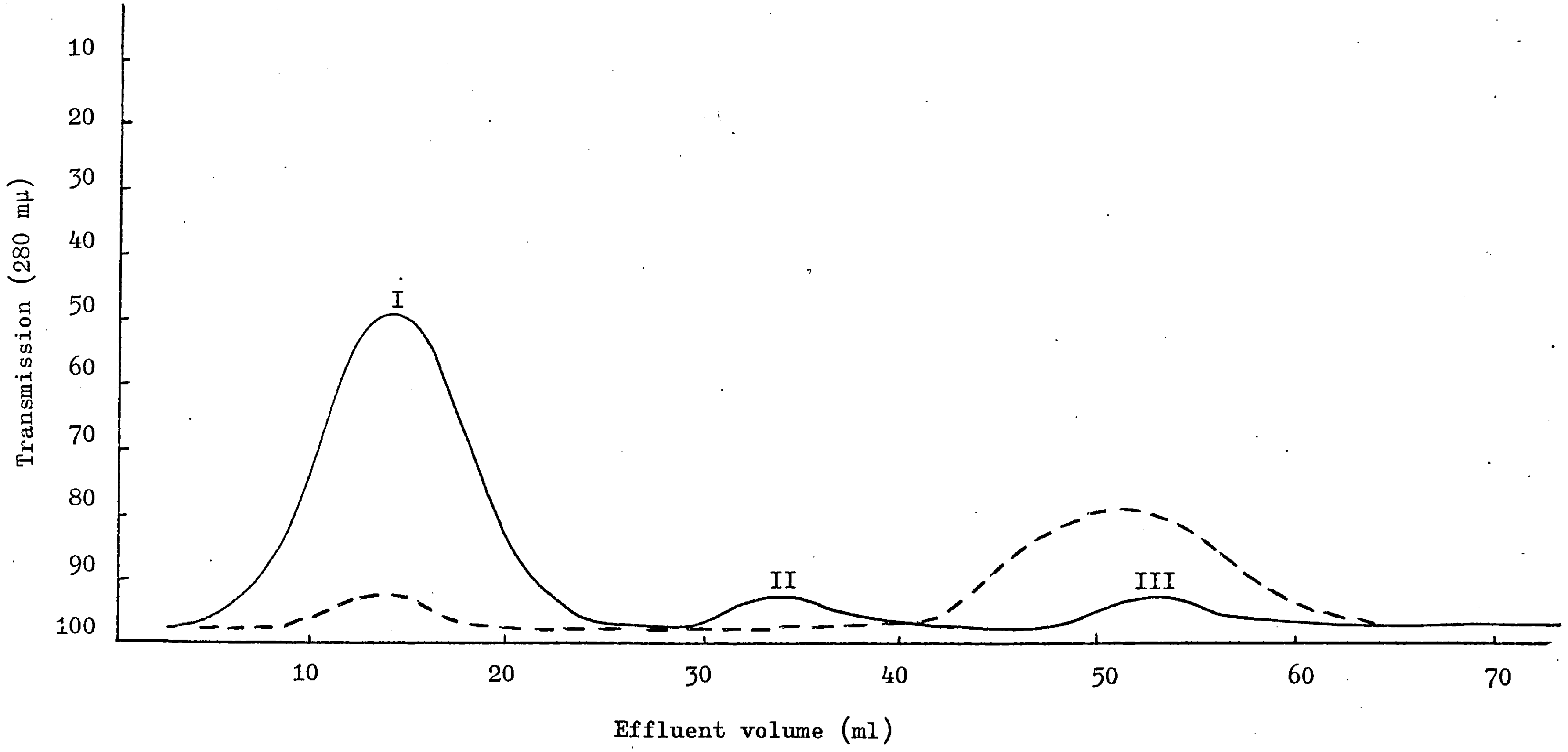
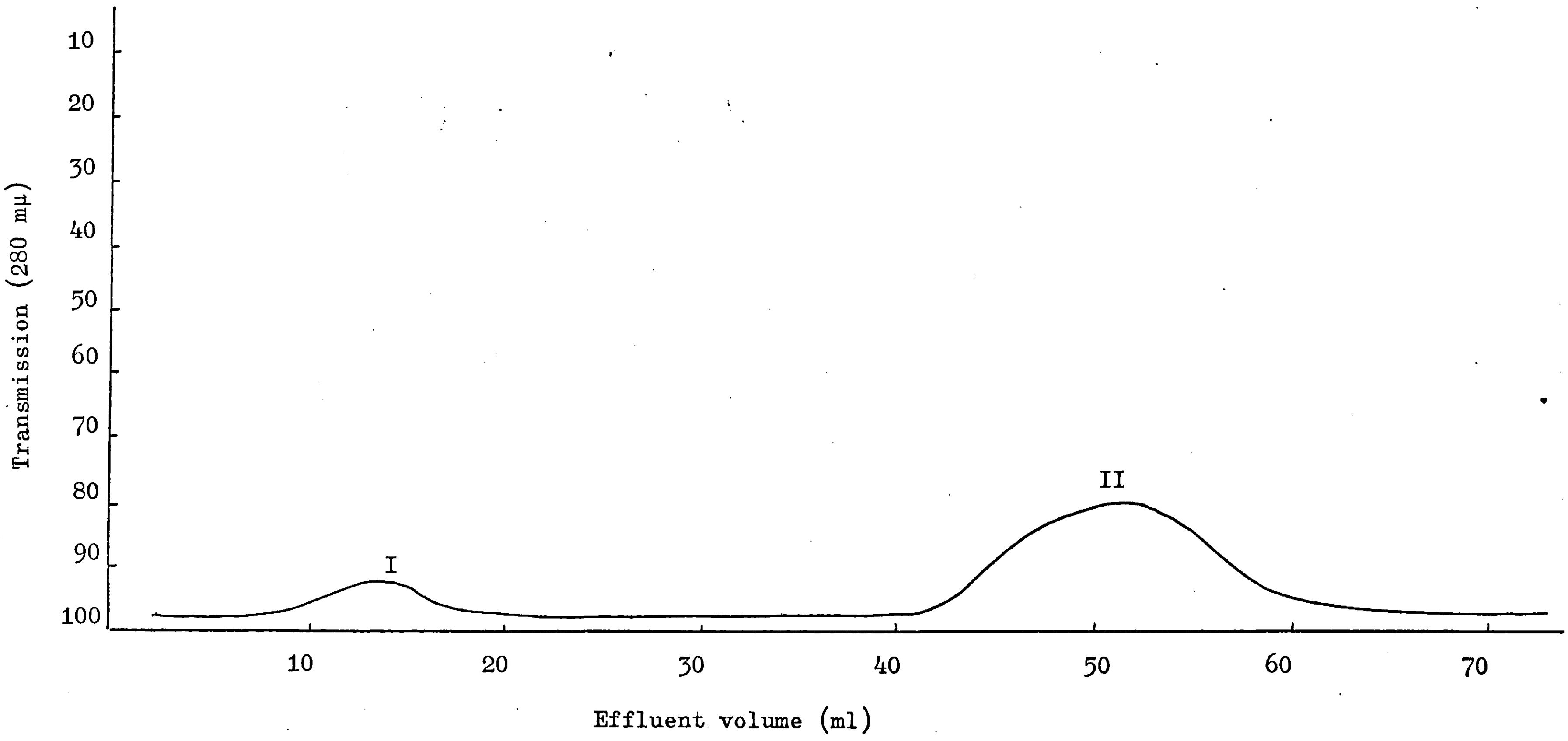


Fig. 27 Sephadex G-100 column chromatography of precipitate from alcohol treated *P. vulgaris* seed extract



(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$_{93}^{4.2}$ (FRENCH BEAN) ON DEAE-52 COLUMN

Ext$_{93}^{4.2}$ (French bean) was prepared from 400 grams of *P. vulgaris* seed flour using the same procedure which was used for the preparation of Ext$_{93}^{4.2}$ (Soybean).

One gram Ext$_{93}^{4.2}$ (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$_{93}^{4.2}$ (French bean) was inhibitory, giving 73% inhibition (Table 38). 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$_{93}^{4.2}$ (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$_{93}^{4.2}$ (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$_{93}^{4.2}$ (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 38

Effect of various dilutions of $Ext_{93}^{4.2}$ (French bean) on local lesion production by TNV

Dilution	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + water	TNV + extract		
Neat	44.2	12.0	0.27	73
10^{-1}	40.0	15.2	0.38	62
10^{-2}	48.7	33.1	0.68	32

* 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

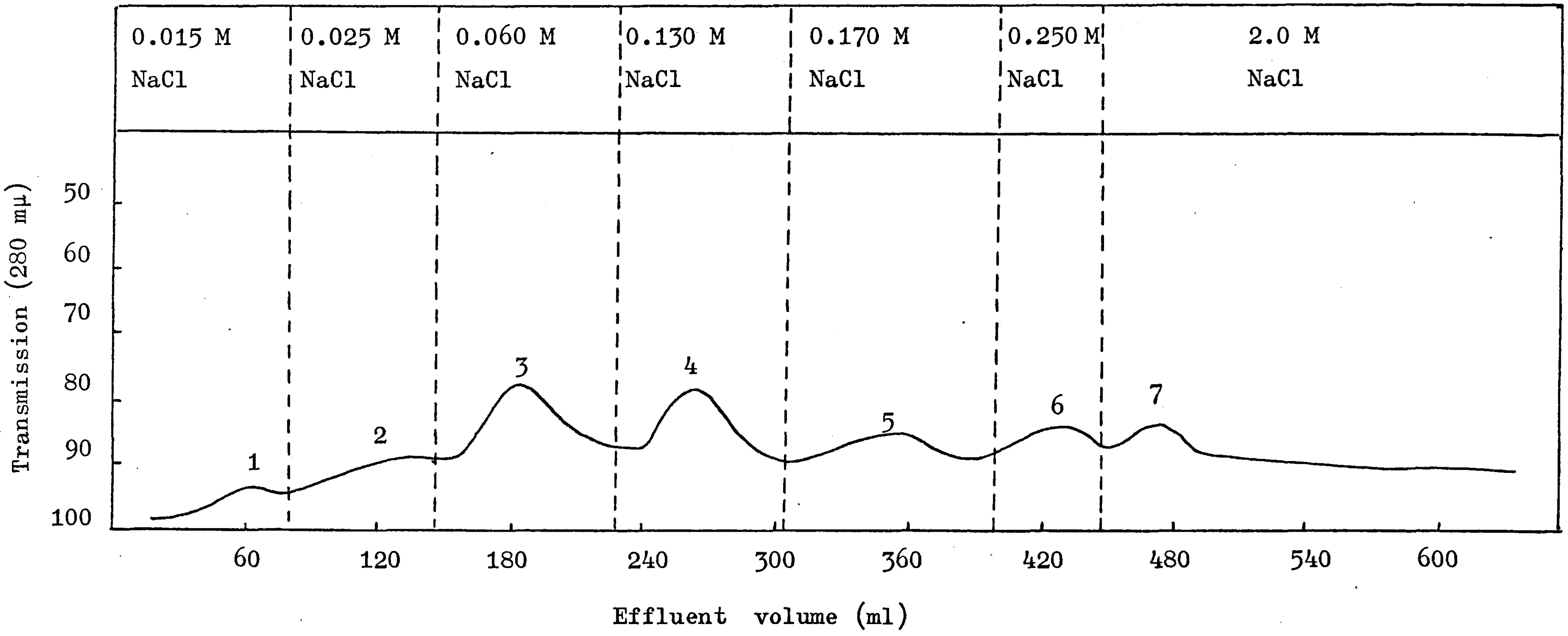


TABLE 39 Effect of DEAE-52 fractions of $Ext_{93}^{4.2}$ (French bean) on local lesion production by TNV

Sodium chloride elutant concentration (M)*	Peak	Mean number of lesions ***		Activity Quotient	Percentage Inhibition
		TNV + water	TNV + Peak		
0.015	1	84.2	78.6	0.93	7
0.025	2	72.2	58.3	0.81	19
0.060	3	79.4	100.5	1.27	- 27
0.130	4	75.9	70.2	0.92	8
0.170	5	76.9	77.5	1.01	- 1
0.250	6	50.5	40.1	0.79	21
2.000 **	7	61.6	38.7	0.63	37

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

** Unbuffered elutant

*** 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 $Ext_{93}^{4.2}$ (French bean)

A high protein concentration (0.3 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 $Ext_{93}^{4.2}$ (French bean)

Disc electrophoresis experiments were performed on complete $Ext_{93}^{4.2}$ (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

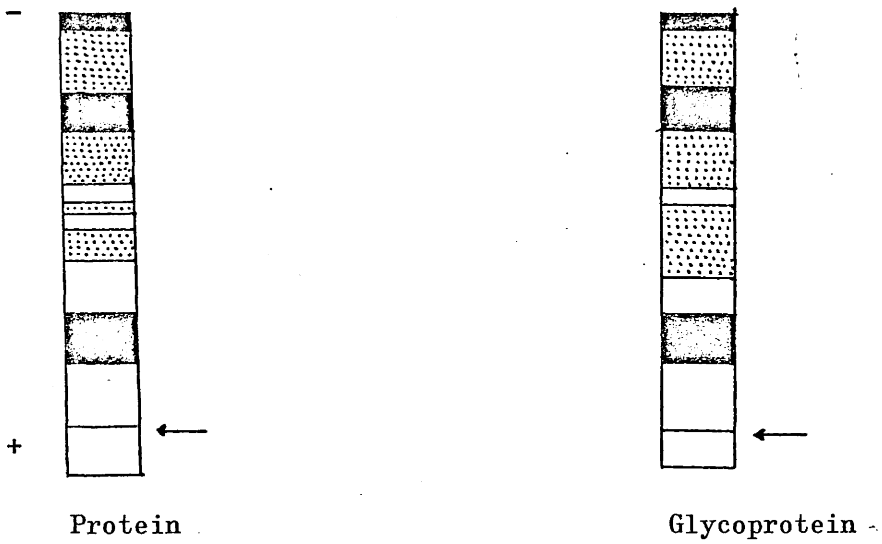
TABLE 40

Protein estimation of DEAE-52 fractions from $Ext_{93}^{4.2}$ (French bean)

Peak	mg protein/ml
1	0.10
2	0.10
3	0.30
4	0.30
5	0.10
6	0.10
7	0.05

Fig. 30 Disc electrophoresis of $Ext_{93}^{4.2}$ (French bean)

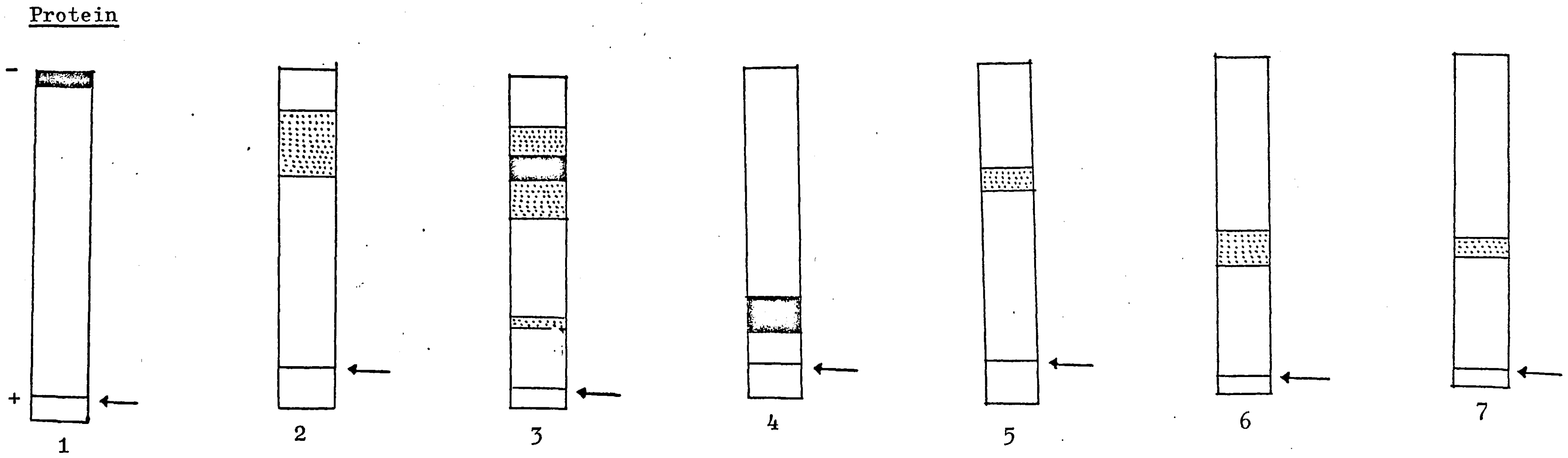
$Ext_{93}^{4.2}$ (French bean)



← position of marker

Shading indicates intensity of band staining

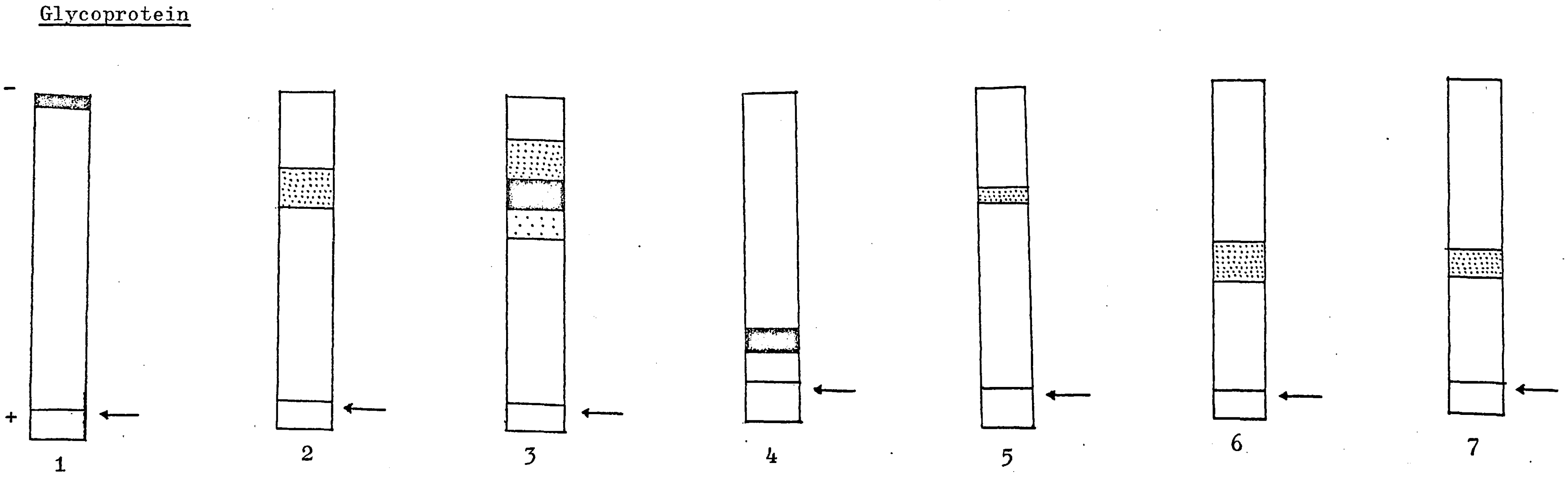
Fig. 31 Disc electrophoresis of DEAE-52 fractions from $\mathrm{Ext}_{93}^{4.2}$ (French bean)



← position of marker

Shading indicates intensity of band staining

Fig. 32 Disc electrophoresis of DEAE-52 fractions from $\mathrm{Ext}_{93}^{4.2}$ (French bean)



← position of marker

Shading indicates intensity of band staining

Rf 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 $\mathrm{Ext}^{4.2}_{93}$ (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 $\mathrm{Ext}^{4.2}_{93}$ (French bean)(Fig. 33). However, in the whole extract, two peaks (peak 2 and 2') were eluted by buffered 0.025 M NaCl, but only one peak (peak 2) was eluted in the case of $\mathrm{Ext}^{4.2}_{93}$ (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

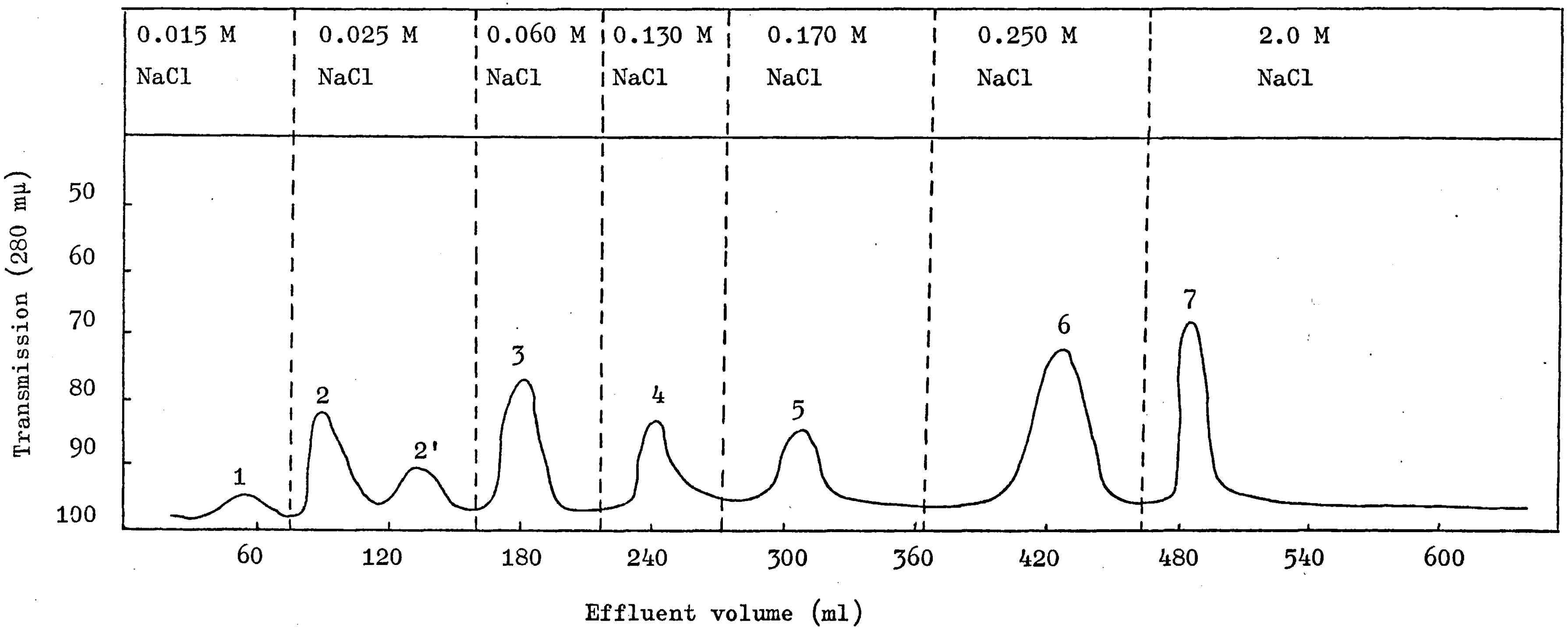


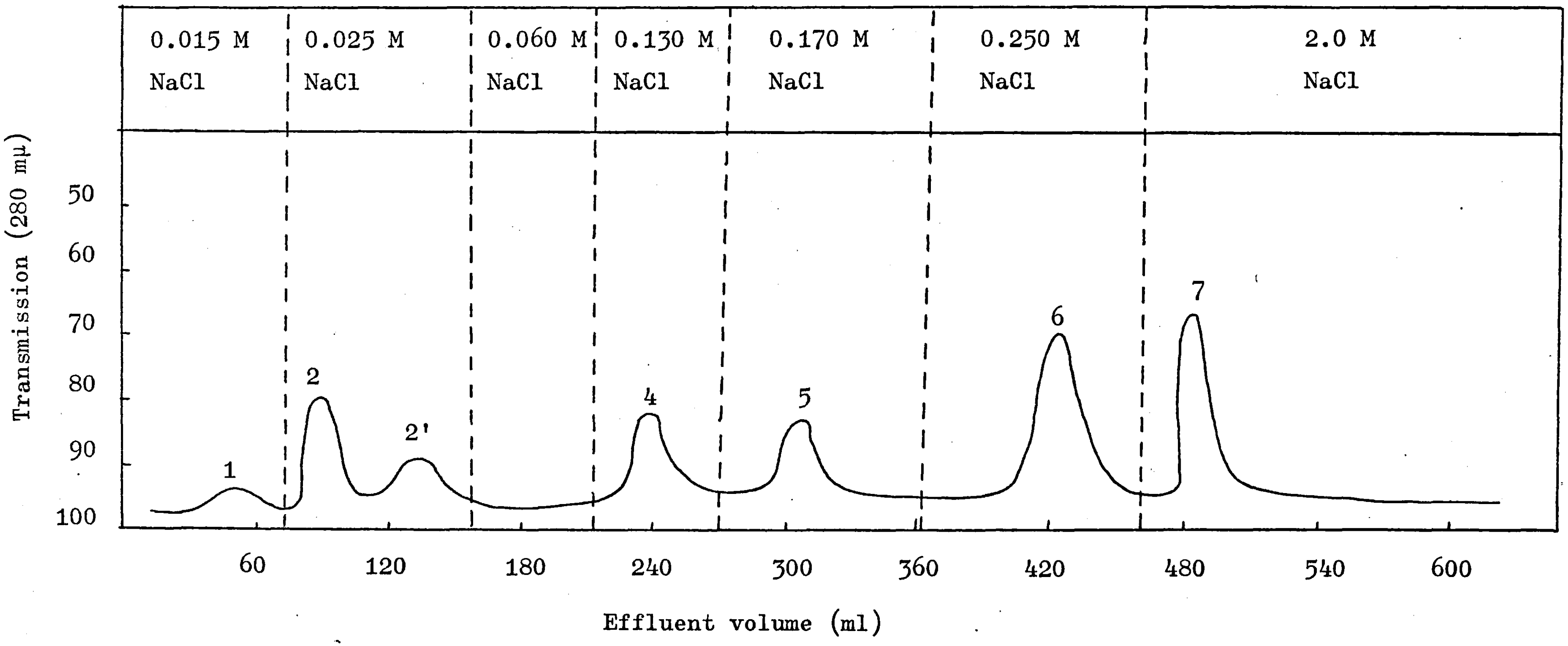
TABLE 41

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

Peak	Mean number of lesions *		Activity Quotient	Percentage Inhibition	Probability (P)
	TNV + water	TNV + peak			
1	97.7	95.8	0.98	2	> 0.100
2	95.2	76.5	0.80	20	< 0.001
2'	96.4	97.4	1.01	- 1	> 0.100
3	100.0	125.0	1.25	- 25	< 0.001
4	93.1	90.3	0.97	3	> 0.100
5	99.5	102.0	1.03	- 3	> 0.100
6	101.0	76.0	0.75	25	< 0.001
7	93.5	56.1	0.60	40	< 0.001
3 + 2	98.3	97.7	0.99	1	> 0.100
3 + 6	96.2	96.8	1.01	- 1	> 0.100
3 + 7	102.0	108.0	1.06	- 6	> 0.100

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

Fig. 34 DEAE-52 column chromatography of the heated P. vulgaris seed extract



with an activity quotient of 1.25. These results are important in that they emphasise the similarity between the crude unheated extract and the $\text{Ext}_{93}^{4.2}$ (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. 34). 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. vulgaris* seed extracts was studied in the same way as that of *G. 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. 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 138,000.

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 $\text{Ext}^{4.2}_{93}$ (Soybean) and the same nomenclature was used. $\text{Ext}^{4.2}_{93}$ (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 TMV. 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 Rf 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 Rf 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 effect 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 $\text{Ext}^{4.2}_{93}$ (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, Nart (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 diffusible 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 (Naspitz 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 VIRUS INHIBITOR EXTRACTS WITH PLANT LECTINS AND TRYPSIN INHIBITOR

DEAE-52 fractions of $\text{Ext}_{93}^{4.2}$ (Soybean) and $\text{Ext}_{93}^{4.2}$ (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* (SBA) and *Canavalis ensiformis* (Con A).

SECTION A

HEMAGGLUTINATION AND TRYPSIN INHIBITION ACTIVITY OF THE VIRUS INHIBITORS EXTRACTED FROM G. MAX SEEDS

P. vulgaris (Rigas and Osgood, 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., 1966); 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 $\text{Ext}_{93}^{4.2}$ (Soybean)

(i) 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 8 g, formaldehyde 37%, 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 mμ. The diluted suspension was referred to as standard erythrocyte suspension (Liener, 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, 1968). The hemagglutination activity (HA) was measured from the hemagglutinating titre. Fig. 35 shows that only peaks 2, 3 and 4, none of which inhibited TNV infection, gave hemagglutination. The HA for these peaks was 1/2, 1/64 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 DEAE-52 fractions from Ext$^{4.2}_{93}$ (Soybean)

Trypsin inhibition activity was assayed for the seven peaks obtained from Ext$^{4.2}_{93}$ (Soybean) by a modification of the Seravac 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, 2.5×10^{-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 ($\triangle E$) was measured at 253 mμ at zero time (t_o) and then after five minutes ($t_{5\ min}$). The difference between the amount of BAEE 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% 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 Hemagglutination activity assay of DEAE-52 fractions from $\mathrm{Ext}_{93}^{4.2}$ (Soybean)

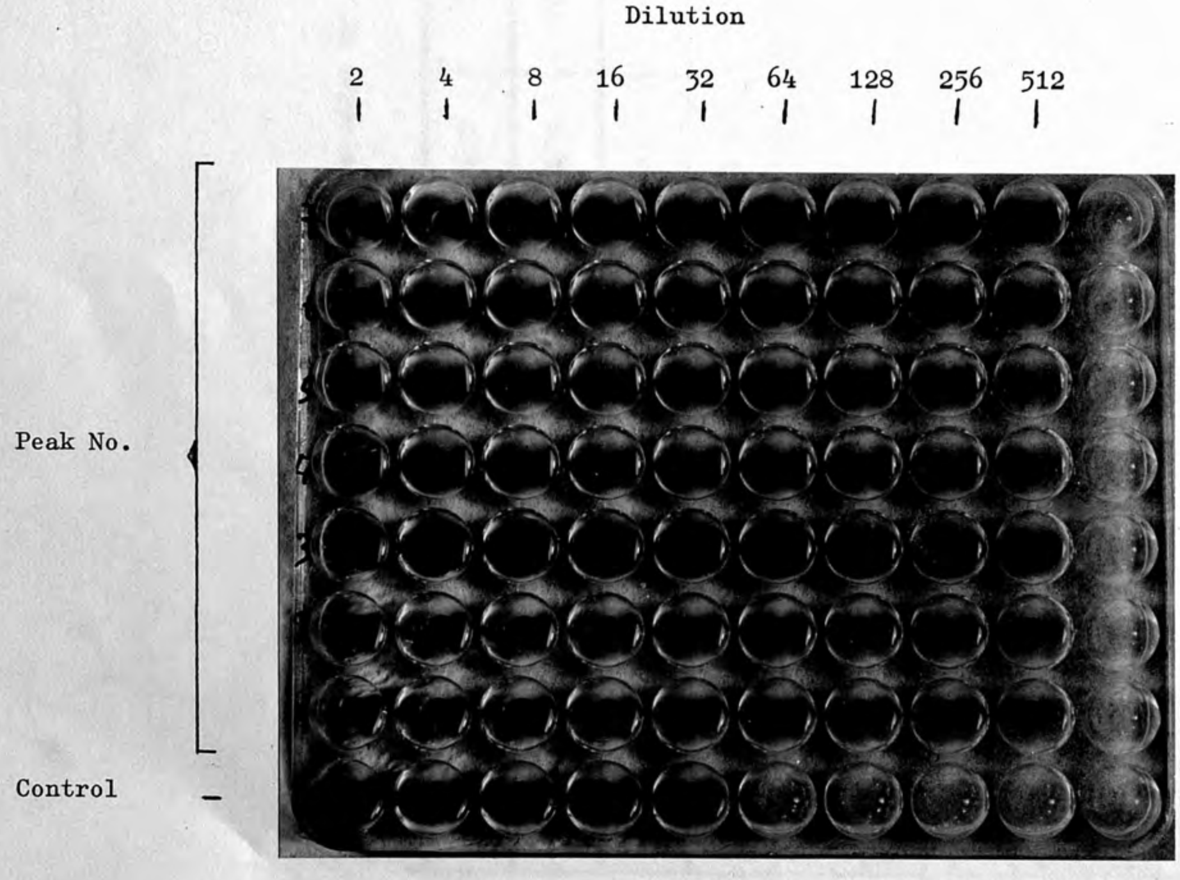


TABLE 42

Trypsin inhibition activity of DEAE-52 fractions from $Ext_{93}^{4.2}$ (Soybean)

Peak	Optical density		ΔE_{253}	BAEE unit/ml	Specific activity
	t_5	t_0			
Control	0.36	0.10	0.26	5,200	
1	0.38	0.11	0.27	5,400	- *
2	0.36	0.09	0.27	5,400	-
3	0.31	0.04	0.27	5,400	-
4	0.30	0.02	0.28	5,600	-
5	0.12	0	0.12	2,400	0.24
6	0.32	0.08	0.24	4,800	0.67
7	0.30	0.04	0.26	5,200	-

* No trypsin inhibition

TABLE 43

Effect of Soybean trypsin inhibitor on local lesion production by TNV

Trypsin inhibitor (concentration)	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + water	TNV + trypsin inhibitor		
1 mg/ml	33.90	21.2	0.63	37
0.2 mg/ml	30.00	30.1	1.00	0

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

SECTION B

HEMAGGLUTINATION AND TRYPSIN INHIBITION ACTIVITY OF THE VIRUS INHIBITORS EXTRACTED FROM P. VULGARIS SEEDS

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

The hemagglutinating activity (HA) was assayed in the same way as for $\text{Ext}_{93}^{4.2}$ (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 1/64, 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 DEAE-52 fractions from $\text{Ext}_{93}^{4.2}$ (French bean)

By testing the DEAE-52 fractions of $\text{Ext}_{93}^{4.2}$ (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 PRODUCTION BY TNV

(a) Phytohemagglutinin (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 $\mathrm{Ext}_{93}^{4.2}$ (French bean)

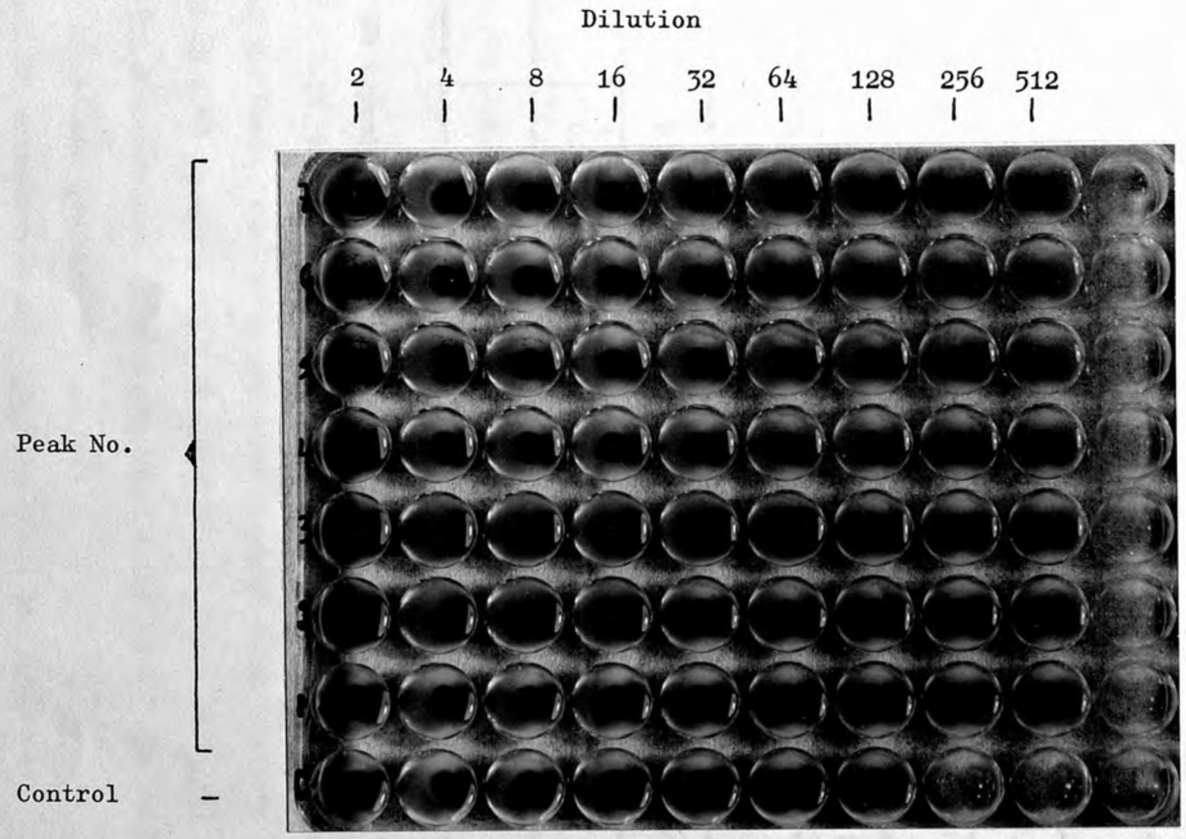


TABLE 44

Trypsin inhibition of DEAE-52 fractions from $Ext^{4.2}_{93}$ (French bean)

Peak	Optical density		ΔE_{253}	BALE units/ml	Specific activity
	t_5	t_o			
Control	0.38	0.10	0.28	5,600	
1	0.36	0.08	0.28	5,600	- *
2	0.37	0.08	0.29	5,800	-
3	0.30	0.02	0.28	5,600	-
4	0.32	0.05	0.27	5,400	0.67
5	0.35	0.08	0.27	5,400	2
6	0.31	0.05	0.26	5,200	4
7	0.38	0.10	0.28	5,600	-

* No trypsin inhibition

how such preparations are made. It seemed prudent therefore to test a variety of commercially produced PHA for effects on TNV.

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

One mg each of PHA (Wellcome), a freeze-dried sample obtained from Wellcome Laboratories, PHA(P) and PHA(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 PHA (Wellcome) has no inhibitory effect against TNV infection. However, PHA(P) and PHA(M) were inhibitory, giving 23% and 29% inhibition respectively. Disc electrophoresis experiments were also performed on the PHA samples to gain some knowledge of their constituents.

(ii) Disc electrophoresis of PHA (Wellcome), PHA(P) and PHA(M)

Fig. 37 shows that PHA (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). PHA(P) contained four bands of protein (Rf = 0.42, 0.63, 0.77 and 0.92) and two bands of glycoprotein (Rf = 0.41 and 0.62). PHA(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 PHA(P) and PHA(M) respectively. This might explain why PHA(P) and PHA(M) are inhibitory against TNV.

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

TABLE 45

Effect of PHA on local lesion production by TNV

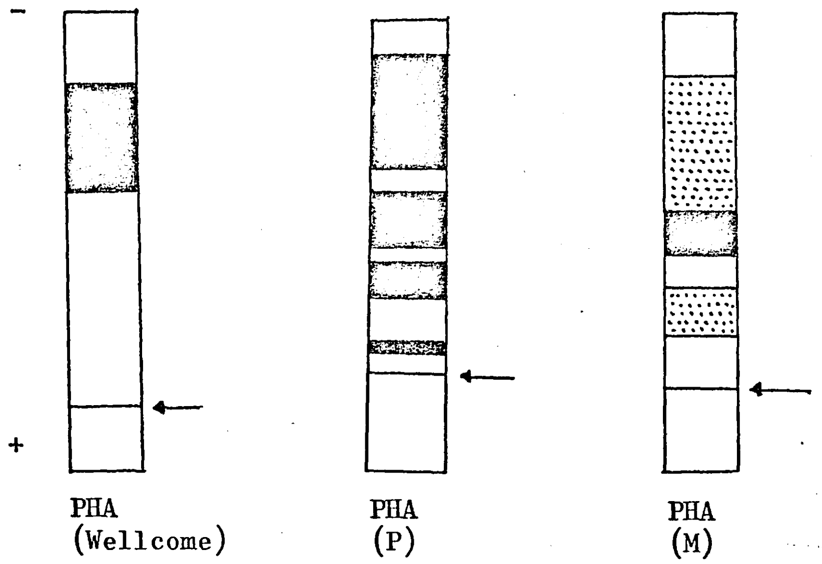
PHA	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + water	TNV + PHA		
PHA(wellcome)	117	119	1.02	- 2
PHA(P)	106	76.3	0.72	28
PHA(M)	126	89.7	0.71	29

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

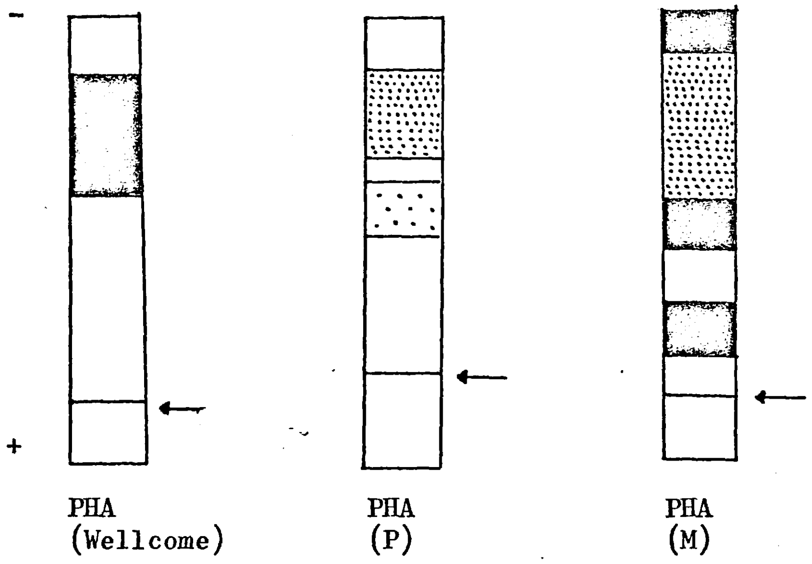
Fig. 37

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

(iii) PHA(Wellcome), PHA(P) and PHA(M) hemagglutination activity assay

Maximum hemagglutination was obtained in PHA(Wellcome) giving a titre of 1/128. PHA(P) gave a hemagglutination titre of 1/32. Minimum hemagglutination was obtained when PHA(M) 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 PHA, pure forms of SBA were difficult to obtain. It was considered valuable, however, to test SBA against virus even though the hemagglutinating extracts from $\text{Ext}_{93}^{4.2}$ (Soybean) had proved non-inhibitory. Soybean agglutinin was prepared in this study and its hemagglutinating activity compared with that of fractions extracted from $\text{Ext}_{93}^{4.2}$ (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.

Fig. 38 Hemagglutination activity assay of PHA samples

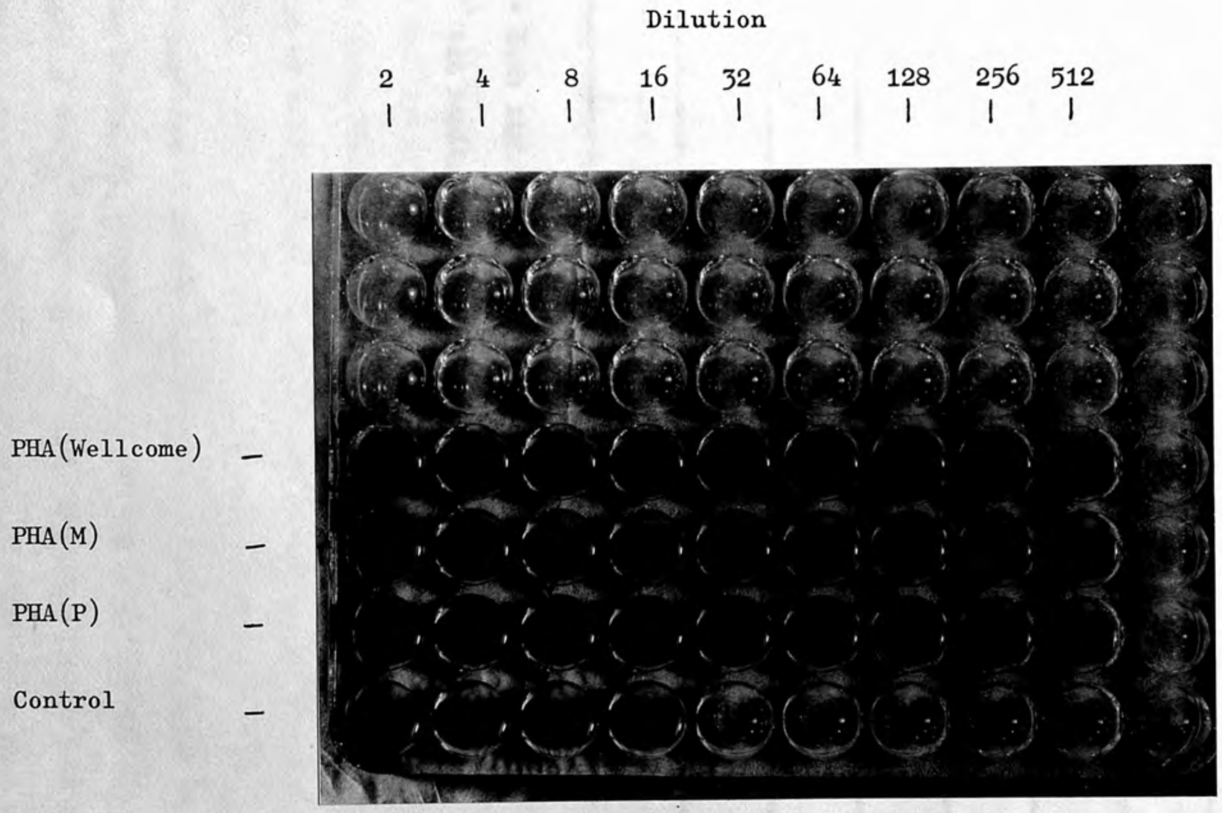


TABLE 46

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

Dilution (SBA)	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	TNV + water	TNV + SBA		
Neat	15.8	0.8	0.05	95
10^{-1}	17.2	9.9	0.58	42
10^{-2}	21.0	15.3	0.73	27
10^{-3}	25.6	19.4	0.76	24
10^{-4}	25.5	22.4	0.88	12
10^{-5}	39.7	34.3	0.86	14
10^{-6}	23.0	23.3	1.01	- 1

* 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 $\mathrm{Ext}_{93}^{4.2}$ (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 = 0.30, 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 $\mathrm{Ext}_{93}^{4.2}$ (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), $Ext_{93}^{4.2}$ (French bean) and $Ext_{93}^{4.2}$ (Soybean)

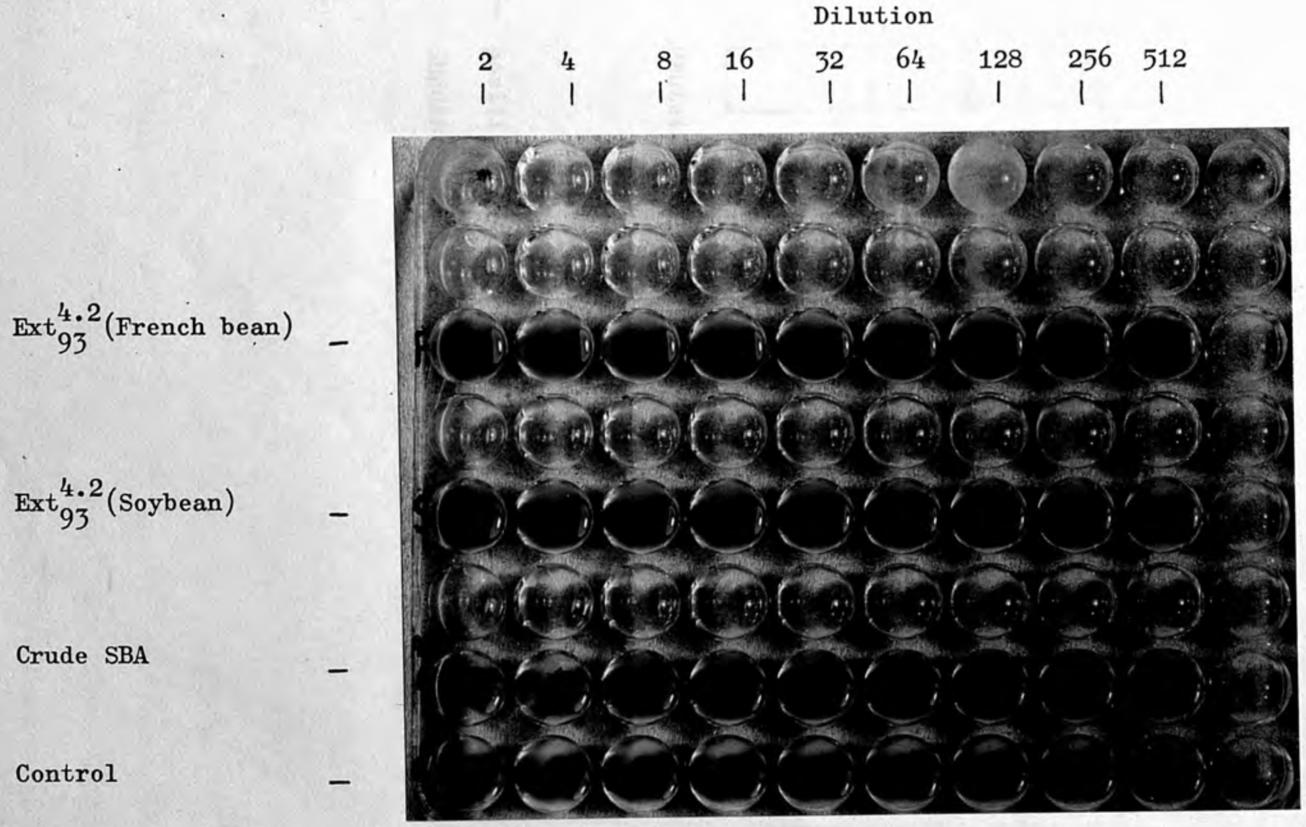
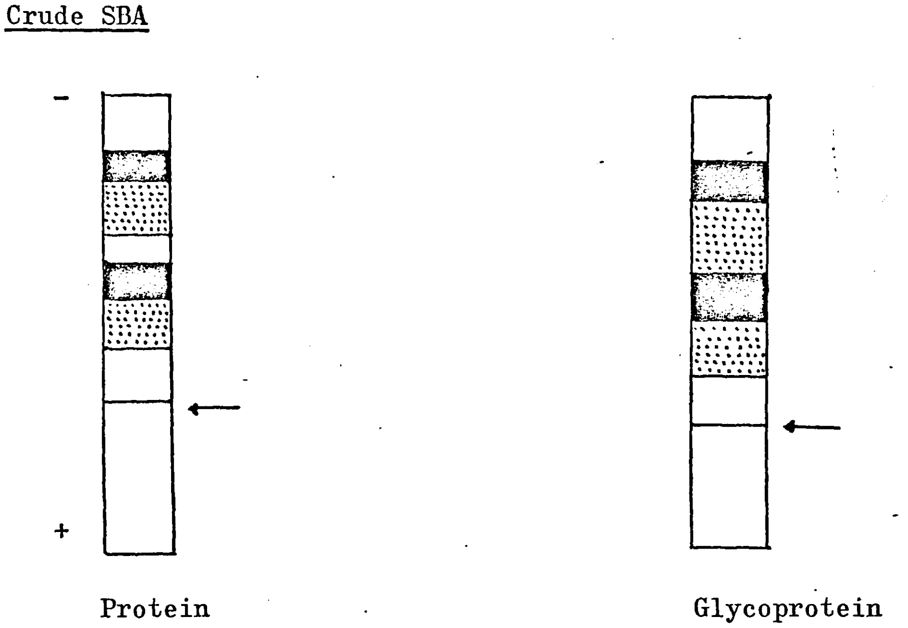


Fig. 40

Disc electrophoresis of crude soybean agglutinin (SBA)



← position of marker

Shading indicates intensity of band staining

Rf values of 0.67 and 0.80 corresponded to similar molecules (Rf 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 Canavalis 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

Dilution	Mean number of lesions **		Activity Quotient	Percentage Inhibition
	TNV + water	TNV + Con A		
Neat *	78.5	75.0	0.96	4
10^{-1}	69.5	69.1	0.99	1
10^{-2}	70.4	68.6	0.97	3
10^{-3}	69.3	72.6	1.05	- 5
10^{-4}	89.1	88.6	0.99	1
10^{-5}	73.3	69.8	0.95	5
10^{-6}	66.3	66.5	1.00	0

* 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.

PHA from commercial sources varies in its properties. This was demonstrated when pure PHA from different commercial sources were tested against virus. Thus, the more homogeneous PHA(Wellcome) gave no virus inhibition, but heterogeneous PHA(P) and PHA(M) each inhibited virus.

CHAPTER VII

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

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-Kandelgy and Wilcoxson(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 TMV.

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 TMV inhibitors extracted from spinach leaves (*Spinacia oleracea*). 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 58% inhibition, which was nearly the same as the whole seed extract

(68% inhibition) tested at this time. This level of inhibition is lower than that described earlier (Chapter II) when 93% 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

(i) Dipping the whole leaf

This experiment was performed on ten intact French bean plants.

TABLE 48

Effect of G. max seed extract on TNV

Treatment of TNV	Mean number of lesions * following ultra-centrifugation
+ H_2O	177
+ Seed extract	168

* 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

Surface dipped	Mean number of lesions * for leaves dipped into:		Activity Quotient	Percentage Inhibition
	water	seed extract		
whole leaf	44.3	3.80	0.09	91
upper	46.2	4.13	0.09	91
lower	51.0	55.00	1.08	- 8

* 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

Mean number of lesions *		Activity Quotient	Percentage Inhibition
Control	Treated		
32.5	33.2	1.02	- 2

* 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

Hours before inoculation	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	Control	Treated		
1	50.0	6.0	0.12	88
3	54.0	27.0	0.50	50
6	46.3	27.0	0.58	42
9	46.0	29.7	0.65	35
24	63.9	66.2	1.04	- 4

* 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, TMV and PVX, were selected.

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

(a) TMV

One ml of the seed extract was mixed with 1 ml of partially purified TMV (legume strain) and inoculated onto ten half leaves of *Nicotiana tabacum* var. Xanthi. Control consisted of an equal volume of TMV 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 TNV

Hours after inoculation	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	Control	Treated		
1	67.2	67.9	1.01	- 1
3	53.6	58.4	1.09	- 9
6	69.4	72.3	1.04	- 4
9	55.7	59.6	1.07	- 7
24	54.5	56.7	1.04	- 4

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

TABLE 53

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

Virus	Mean number of lesions		Activity Quotient	Percentage Inhibition
	Water	seed extract		
TMV *	72.3	26.1	0.36	64
PVX **	7.9	1.2	0.15	85

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

** PVX tested on ten leaves of *Gomphrena globosa*

(b) PVX

One ml 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 (TMV) 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 till 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 TMV 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 Cucumis 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 Kammen et al. (1961) supported this hypothesis by examining the relationship between the number of local lesions on leaves of *Nicotiana glutinosa* and TMV 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 TMV was applied to the upper surface. Different results were reported by Kahn et al. (1960) who found that the inhibitory *Oryza sativa* 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 *Cucumis sativa* seeds affects local lesion production when applied to the undersurface of cowpea leaves.

The results obtained in this thesis suggests 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 TNV, 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 sativum) 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 B

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

Treatment of TNV	Mean number of lesions * following ultra-centrifugation
+ H_2O	91.9
+ Seed extract	95.3

* 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

Hours before inoculation	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	Control	Treated		
1	76.6	75.1	0.98	2
3	70.7	67.2	0.95	5
6	74.4	76.6	1.03	- 3
9	78.4	73.7	0.94	6
24	64.7	68.3	1.06	- 6

* 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 TMV AND PVX

(a) TMV

Lyophilized *P. vulgaris* seed extract was tested against TMV on half leaves of *Nicotiana tabacum* var. Xanthi. Control consisted of an equal volume of TMV 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 *Gomphrena globosa* 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 <u>P. vulgaris</u> seed extract one hour before inoculation by TNV

Surface dipped	Mean number of lesions * for leaves dipped into:		Activity Quotient	Percentage Inhibition
	water	seed extract		
whole leaf	18.9	18.1	0.96	4
upper surface	20.4	20.6	1.01	- 1
lower surface	17.3	16.9	0.98	2

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

TABLE 57

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

Mean number of lesions *		Activity Quotient	Percentage Inhibition
Control	Treated		
25.1	26.4	1.05	- 5

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

TABLE 58

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

Hours after inoculation	Mean number of lesions *		Activity Quotient	Percentage Inhibition
	Control	Treated		
1	76.1	79.9	1.05	- 5
3	69.9	69.2	0.99	1
6	80.3	82.7	1.03	- 3
9	73.1	70.2	0.96	4
24	66.0	63.4	0.96	4

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

TABLE 59

Effect of <u>P. vulgaris</u> seed extracts on local lesion production by TMV and PVX

Virus	Mean number of lesions		Activity Quotient	Percentage Inhibition
	Water	Seed extract		
TMV *	83.1	70.6	0.85	15
PVX **	8.1	8.4	1.04	- 4

* TMV tested on ten half leaves of <u>Nicotiana tabacum</u> var. Xanthi

** PVX tested on ten leaves of <u>Gomphrena globosa</u>

to being slightly inhibitory (A.Q. = 0.70). *P. vulgaris* extract treated with heat, or alcohol, and analysed by DEAE-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 *P. vulgaris* leaf extracts and suggested that inhibitors act by forming a loose complex with the virus.

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 *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 *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 *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 *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 *P. vulgaris* leaf extract was not inhibitory against TMV on *Nicotiana tabacum* var. Xanthi. In experiments described in this section, *P. vulgaris* seed extracts showed some inhibition against TMV when inoculated on to *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, 1948). Furthermore, Hart (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; Racusen 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 virus. 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 Tetragonia 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 Amemiya (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 PHA, for example, a number of types are available. PHA(M), a mucoprotein, and PHA(P) predominantly a protein, both of which were inhibitory against TNV were found to consist of a number of components. On the other hand, PHA(Wellcome) consisted of a single large glycoprotein band with no inhibitory properties. Electrophoretic studies showed that with PHA(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 Phytolacca americana leaf extracts a virus inhibitor of molecular weight 13,000 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.Σ 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.

Inspite of 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 TMV; however, only soybean showed inhibition against PVX.
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(M) 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.

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ACKNOWLEDGEMENTS

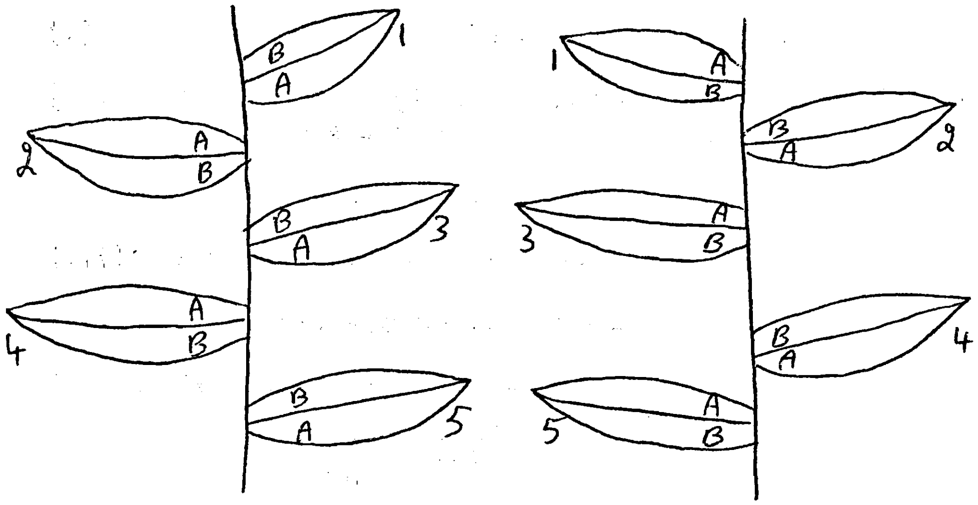
I wish to express my thanks to Professor K. Wilson and all members of the Botany Department at Royal Holloway College; in particular Dr. W.A. Stevens for his supervision, advice, encouragement and help, Mrs. I. Judd for typing the thesis, Miss C. Spurdon for technical and Mr. D. Ward for photographic assistance.

I would also like to thank the Lebanese National Council for Scientific Research for financial support.

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



Plant 1				Plant 2		
leaf number	L	R		leaf number	L	R
1	A*	B		1	B	A
2	B	A		2	A	B
3	A	B		3	B	A
4	B	A		4	A	B
5	A	B		5	B	A

* treatment

L = leaf half leaf

R = right half leaf

APPENDIX 2

Statistical Analysis

Local lesion counts were statistically analysed using the student 't' test as described by Bishop* (1968).

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 (Σfd^2)

(v) Variance (σ) of the mean

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

Σf = sum of frequencies

n = number of leaves in every treatment

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

$$\sigma_d^2 = \frac{\sigma_1^2}{n_1} + \frac{\sigma_2^2}{n_2}$$

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

(vii) Standard deviation of the difference of means (σ_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.

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

APPENDIX 3

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

Local lesion Nos.		f	d		fd^2	
(TNV + Water) Control	(TNV + seed extract) treated		Control	treated	Control	treated
130	102	1	44.7	11	1998.09	121
74	76	1	11.3	15	127.69	225
105	134	1	19.7	43	388.09	1849
46	93	1	39.3	7	1544.49	49
111	93	1	25.7	2	660.49	4
79	84	1	6.0	7	36.00	49
102	61	1	16.7	30	278.89	900
114	181	1	28.7	90	823.69	8100
35	32	1	50.3	59	2530.09	3481
57	49	1	28.3	42	800.89	1764
853	910	10			9188.41	16542
$= \Sigma x$	$= \Sigma x$	$= \Sigma f$			$= \Sigma fd^2$	$= \Sigma fd^2$

$$\bar{x}\ (\text{control}) = \frac{853}{10} = 85.3$$

$$\bar{x}\ (\text{treated}) = \frac{910}{10} = 91$$

$$\sigma_1^2 = \frac{9188.41}{10} = 918.84$$

$$\sigma_2^2 = \frac{16542}{10} = 1654.2$$

$$\sigma_d^2 = \frac{918.84}{10} + \frac{1654.2}{10} = 257.304$$

$$\sigma_d = \sqrt{257.304} = 16.04$$

$$t = \frac{91 - 85.3}{16.04} = 0.36$$

$P > 0.1$ (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 TNV infection

Dilution	10^{-1}		10^{-2}		10^{-3}		10^{-4}		10^{-5}		10^{-6}	
Treatment	Control	treated	Control	treated	Control	treated	Control	treated	Control	treated	Control	treated
$\bar{x}$	72.20	77.30	103.80	96.70	91.00	87.10	87.80	83.60	103.20	72.00	86.00	90.00
Σf	10	10	10	10	10	10	10	10	10	10	10	10
Σfd^2	6403.60	17822.1	9492.60	15269.8	14796.0	9646.90	6759.60	13182.4	8247.80	2012.0	8968.0	15564.0
σ_1^2	640.36	1782.21	949.16	1526.98	1479.6	964.69	675.96	1318.24	824.78	201.20	896.8	1556.4
σ_2^2	242.257		247.614		244.429		199.420		283.678		245.320	
σ_d	15.56		15.74		15.63		14.12		16.84		15.66	
t	0.33		0.45		0.25		0.30		1.85		0.26	
P	> 0.1		> 0.1		> 0.1		> 0.1		< 0.1 difference is significant		> 0.1	

APPENDIX 5

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

pH	3	4	5	6	7	8
$\bar{x}$	40.00	49.20	43.30	40.80	41.00	41.20
Σf	10	10	10	10	10	10
Σfd^2	184.00	471.60	458.10	187.60	216.00	231.60
σ^2	18.40	47.16	45.81	18.76	21.60	23.16
σ	4.29	6.87	6.79	4.33	4.65	4.81
Se^*	1.36	2.17	2.14	1.37	1.47	1.52
$txSe^{**}$	± 3.07	± 4.90	± 4.84	± 3.10	± 3.32	± 3.44

* Se = standard error = $\frac{\sqrt{\sigma^2}}{\sqrt{n}}$

** 95% confidence limits of the means