

PROTEIN SYNTHESIS AND ENZYME ACTIVITIES IN LOCALISED

PLANT VIRUS INFECTIONS

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Protein Synthesis and Enzyme Activities in Localised Plant Virus Infections

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ABSTRACT

Both cowpea and cucumber plants respond hypersensitively to TNV_D infection with localisation of the virus in necrotic lesions. In cowpea, necrosis is not responsible for localisation of virus or viral RNA, and two major protein fractions termed α and β appear in soluble protein extracts as evidenced by discontinuous polyacrylamide gel electrophoresis. In cucumber, one fraction designated γ is detectable in all cultivars investigated apart from Explorer which contained two fractions, γ and γ_A .

The three fractions, α , β and γ , are dissimilar with regard to chemical nature, molecular weight and inducibility, and each is composed of several proteins. Both α and β fractions contain "shoulder" proteins on gel scans and all are strictly proteinaceous. The γ fraction consists of at least three comigrating proteins, a glycoprotein, a RNase isozyme(s), and another (other) protein(s) which constitutes the bulk of the fraction. The γ -comigrating RNase is a constitutional enzyme and its subcellular location is different from the rest of the γ -band.

The function(s) of these proteins is (are) unclear, but none of the α , the β and the γ fractions is associated with an isozyme of either peroxidase or polyphenoloxidase. However, the γ fraction is associated with the RNase isozyme mentioned before. These enzymes are not directly involved in necrosis, but rather their increases are consequences of it. The γ -fraction is not a by-product of necrosis (as neither are α , β and γ_A), although it can be induced by biotic (e.g. TMV, Colletotrichum lagenarium) or abiotic (e.g. $CuSO_4$, hypertonic mannitol solutions) means. The proteins are host-specific and, although, neither active nor novel synthesis of the α or the β fractions has been proven, γ production is dependent on host-DNA transcription and not on viral RNA translation.

The ability of mannitol-induced osmotic stress to immitate virus infection in inducing similar proteins is a newly discovered phenomenon. A "path-osmotic" stress is suspected of inducing either synthesis or generation of the proteins as well as several other biochemical alterations which accompany the hypersensitive reaction.

The investigated proteins have no apparent role in either localisation or induced resistance.

LIST OF ABBREVIATIONS AND SYMBOLS

AH	= Apparently healthy tissue = Tissue of an uninoculated half leaf adjacent to the midrib of a virus-infected, necrotic half
AmD	= Actinomycin D
AMV	= Alfalfa mosaic virus
BPB	= m = Bromo-phenol blue (an electrophoresis marker)
BSA	= Bovine serum albumin
$^{\circ}\text{C}$	= Centigrade degree(s)
^{14}C	= Radio-active carbon
CAH	= Cinnamic acid-4-hydroxylase
Car.MV.	= Carnation mosaic virus
Cat.	= Catalase
Ch.Ph.	= Chloramphenicol
cm.	= Centimeter(s)
CMV	= Cucumber mosaic virus
CMV_w	= Cucumber mosaic virus (the Worcestershire strain)
Cont.-PAGE	= Continuous-polyacrylamide gel electrophoresis
c.p.m.	= Counts per minute
CRSV	= Carnation ringspot virus
Cyt.C.	= Cytochrome C
d.	= dalton
D.B.	= Disruption buffer
Disc-PAGE	= Discontinuous polyacrylamide gel electrophoresis
DNA	= Deoxy-ribonucleic acid
E_1	= An alkaline method of extracting soluble proteins at high pH value (8.3)
E_2	= An acidic method of extracting soluble proteins at low pH value (2.8)

$E_{260, 0.1\%}^{1 \text{ cm}}$	= Specific extinction coefficient
	= The absorbance value of a 0.1% virus solution in a cuvette with an optical path length of light of 1 cm, and measured at 260 nm wavelength
EDTA	= Ethylene diamine tetra-acetic acid
Fig.	= Figure
g	= Gram(s)
h	= Hour(s)
H	= Healthy
^3H	= Tritium (a radioactive hydrogen)
HMP	= Hexose mono-phosphate
HPVP	= Highly purified virus preparation
IAA	= Indolacetic acid
IN	= Infected
K	= Kilo- = 10^3
L	= Litre(s)
Lux	= 1×10^{-4} phot., 0.0929 foot-candle = 1 lumen per square metre
m	= BPB = Bromo-phenol blue (an electrophoresis marker)
M	= Molar concentration
mA	= Milliampere
Mann.	= Mannitol
mg	= Milligram(s) = 10^{-3} gram
min	= Minute(s)
ml	= Millilitre(s) = 10^{-3} Litre
mm	= Millimetre = 10^{-3} metre
mM	= Millimolar concentration = 10^{-3} molar concentration
MM	= Marker mixture = A standard protein mixture
N	= Normal concentration
nm	= Nanometre = 10^{-9} metre (formerly mμ)

no.	= No. = Number
OMT	= Caffeic acid-o-methyltransferase
O.D.	= Optical density
PA	= Polyacrylic acid
PAG	= Polyacrylamide gel
PAGE	= Polyacrylamide gel electrophoresis
PAL	= Phenylalanine ammonia-lyase
PAS	= Periodic acid Schiff's reagent
PEG	= Polyethylene glycol
pH	= Hydrogen ion exponent (a unit symbol for the degree of alkalinity or acidity)
PNG	= Polynucleotide gel = Polyuridylic acid gel (Poly-U)
PNGE	= Polynucleotide gel electrophoresis
PO	= Peroxidase
Poly-U	= Polyuridylic acid
POPOP	= 1,4-bis [2-(5-Phenyloxazolyl)] benzene; Phenyloxazolyl-phenyloxazolyl-phenyl)
ppm	= Part(s) per million
PPO	= Polyphenol oxidase
PPO	= The organic scintillator 2,5-diphenyloxazole
PPVP	= Partially purified virus preparation
PR-proteins	= Pathogenesis-related proteins
PVX	= Potato virus X
PVY	= Potato virus Y
Rf	= A value, ranges from 0.0 — 1.0, denoting the ratio of the distance migrated by a protein to the distance migrated by the bromo-phenol blue marker
Rm	= A value, ranges from 0.0 — 1.0, denoting the ratio of the distance migrated by a protein to the distance migrated by cytochrome C

RNA	=	Ribonucleic acid
RNAse	=	Ribonuclease
rpm	=	Revolutions per minute
SBMV	=	Southern bean mosaic virus
SDS	=	Sodium dodecyl sulphate = Sodium lauryl sulphate
SE	=	Standard error
sec	=	Second(s)
sh	=	Shoulder, a small peak on a major one on a densitometer scan
SRP	=	Stress-related proteins
SR-proteins	=	Stress-related proteins
STNV	=	Satellite tobacco necrosis virus
TBSV	=	Tomato bushy stunt virus
TCA	=	Trichloro-acetic acid
TMV	=	Tobacco mosaic virus
TMV-U ₁	=	Tobacco mosaic virus (type strain)
TNV	=	Tobacco necrosis virus
TNV _A	=	Tobacco necrosis virus (Strain A)
TNV _D	=	Tobacco necrosis virus (strain D)
Tom. RSV	=	Tomato ring spot virus
TRSV	=	Tobacco ring spot virus
TSWV	=	Tomato spotted wilt virus
Tu.MV	=	Turnip mosaic virus
v	=	Volume
vol	=	Volume
v-p	=	Viral particles
v-RNA	=	Viral ribonucleic acid
w	=	Weight
W	=	Watt
x	=	Times

$\frac{1}{2}H$	= An uninoculated half leaf adjacent to the midrib of a phosphate buffer inoculated half
$\frac{1}{2}IN$	= A virus-infected half leaf
α_m	= Alfa monomer
β_m	= Beta monomer
$\gamma-H$	= Human gamma globulin (heavy chain)
$\gamma-L$	= Human gamma globulin (light chain)
μ	= Micro- = 10^{-6}
μg	= Microgram(s) = 10^{-6} gram
μl	= Microlitre(s) = 10^{-6} litre
μm	= Micrometre(s) = 10^{-6} metre
μCi	= Microcurie = 10^{-6} Curie (The curie is defined as the quantity of radioactive material in which the number of nuclear disintegrations per second is the same as that in 1 g of radium, namely 3.7×10^{10} ($3.7 \times 10^{10} \text{ sec}^{-1}$))
%	= Percent

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INTRODUCTION

Localisation of a virus is one of the most efficient defence mechanisms in plants, as it actively serves to protect the plant from the effects of systemic virus infection. In this phenomenon the virus is restricted to a relatively small area and prevented from any further invasion of the plant. In hypersensitive hosts the process of localisation is invariably associated with a strong response culminating in the death of the originally infected cells at the centre of the localised areas giving rise to necrotic local lesions. The virus in this case is arrested in necrotic areas and narrow zones of tissues surrounding them (Hayashi and Matsui, 1965; Martin and Gallet, 1966; Milne, 1966). The virus is prevented from further movement by presumably a resistant zone of cells which develop at the periphery of local lesions (Yarwood, 1960) providing either a physical barrier to cell-to-cell movement of the virus (see Section II. 1. A. c) or an unfavourable environment for virus multiplication (Weintraub et al., 1960).

The hypersensitive reaction elicited by TMV in hypersensitive reacting tobacco varieties is probably the most intensively investigated situation. However, an acceptable explanation of the basic biochemical mechanism(s) underlying the reaction has eluded investigators and the cause(s) for the localisation, i.e. the inability of the virus to move from the local lesion and the surrounding area is (are) still obscure (Loebenstein, 1972). The problem is complicated by the fact that the hypersensitive reaction with its two features of localisation and necrogenesis, is associated with several reproducibly recorded physiological and biochemical alterations, many of which may well be consequences of necrotisation and not the cause of localisation.

This unspecific reaction (hypersensitive reaction) is physiologically and biochemically characterized by the stimulation of some respiratory

enzymes, particularly those involved in the hexose mono-phosphate (HMP) pathway (Section II. 1. A. d). Enzymes of the phenylpropanoid pathway (Section II. 1. A. e), peroxidase (and sometimes polyphenoloxidase and/or catalase) activity (Section II. 1. A. e), ribonuclease activity (Section II. 1. A. f), ethylene emanation (Section II. 1. A. j) are also stimulated and the accumulation of metabolites (e.g. pentoses) (Section II. 1. A. g), phenolic compounds and phytoalexins (Section II. 1. A. h) are all characteristic of hypersensitive reactions. The induction (or stimulation) of 'novel' (constitutional) soluble proteins (Section II. 1. A. i) and many other increases and sometimes decreases in enzyme activity, hormone levels and other constituents have also been reported in infected (and sometimes uninoculated) tissue of hypersensitive reacting hosts.

Following the occurrence of the hypersensitive reaction in one part of the plant, an induced (acquired) resistance appears in healthy tissue of the same plant, not only in the tissue surrounding the local lesions, i.e. localised induced resistance (Yarwood, 1960) but also in tissue far remote from the infected parts, i.e. systemic induced resistance (Gilpatrick and Weintraub, 1952; Yarwood, 1953; Ross, 1961a and b).

The possible involvement of soluble proteins associated with the hypersensitive reaction, necrogenesis, localisation and/or induced resistance has gained a deal of attention in the past ten years since the discovery by Gianinazzi et al. (1970) and Van Loon and Van Kammen (1970) of four apparently novel proteins in the soluble fractions of tobacco leaves reacting hypersensitively to TMV-infection. The appearance of these proteins in uninoculated, symptomless, non-virus containing parts of the plant (Van Loon and Van Kammen, 1970) and the ability to induce them with other viruses (Van Loon and Van Kammen, 1970) or abiotic means (Gianinazzi and Kassanis, 1974; Van Loon, 1977; White, 1979) along with the inhibitory effect of actinomycin D on their synthesis (Van Loon and Van Kammen, 1970; Kassanis and White, 1974) suggested that the proteins are actively synthesised by

the host and may be concerned with a protective mechanism(s) interfering with virus replication and/or preventing systemic spread of the virus following a challenge inoculation. Hence, the proteins have been postulated as being responsible for both localisation of virus and induced resistance phenomena in plants and are thought to represent a kind of plant interferon (Kassanis and White, 1974).

Other novel proteins in the soluble fraction of leaves have subsequently been reported in different virus/host combinations. These proteins were not linked (Tas and Peters, 1977) or only indirectly linked (Coutts, 1978) with localisation and/or induced resistance.

The notion that in hypersensitive hosts the production of 'novel' proteins by de-repression process has been propounded (Loebenstein, 1972). Accordingly, the mechanism of localisation and induced resistance requires transcription of cellular DNA to RNA and subsequent protein synthesis, i.e.

Host-DNA transcription → m-RNA translation → novel proteins

The novel proteins may act directly as an 'interferon' type compound or indirectly as an enzyme(s) to catalyse a biochemical reaction(s), whose final product(s) is (are) an active antiviral factor(s) or a chemical substance(s) which renders cells unfavourable for virus multiplication. The proteins, or their product(s) may block the cell-to-cell passage of virus, thus, preventing further spread. But other functions are not excluded.

From the foregoing, the study of the synthesis and changes in soluble proteins in plants following virus infection appears to be a useful method of studying these phenomena.

The work presented in this thesis aimed principally at studying the possible involvement of 'novel' soluble proteins in both localisation and induced resistance using different virus/host combinations from those commonly studied previously (i.e. TNV/cucumber, cowpea and tobacco plants

as opposed to TMV/hypersensitive tobacco varieties) to test the universal nature of the above proposals. This thesis has, thus, been devoted to studying and comparing the synthesis and/or the changes in soluble proteins using polyacrylamide gel electrophoresis (PAGE), radio-active labelling techniques and enzyme assays.

The study attempted to characterize the 'novel' proteins found in the soluble fraction of hypersensitive reacting cucumber tissue and cowpea primary leaves following tobacco necrosis virus (TNV) infection. Attempts at inducing similar proteins by other biotic and abiotic treatments were also performed in an effort to establish any basic similarities in the mechanism(s) of induction, between any of these treatments and the hypersensitive reaction. Searches were made for potential roles of the 'novel' fractions in both systems, and whether they act in virus localisation and/or induced resistance or another capacity.

The thesis reports a newly discovered phenomenon, and offers new suggestions on what might 'trigger' the induction of the 'novel' proteins with regard to the hypersensitive reaction and virus localisation.

II. REVIEW OF LITERATURE

Disease resistance to viral infections in plants

Although all higher plants are exposed to many viral pathogens, often only a limited number cause infection. The plant is said to be susceptible to these viruses and immune to the rest. The host-pathogen interaction between viruses and plant cells may vary between and include the two extremes of complete susceptibility and total resistance (unsusceptibility = immunity). Between these extremes various defence phenomena resulting in different levels of resistance are recognised.

Although these resistance phenomena are well known and documented, the physical and/or biochemical basis of their operation is not yet fully understood. In certain instances resistance to a particular virus may be due to a passive defence mechanism(s) where pre-formed physical barriers define the resistance, and interfere with infection; e.g. presence of fewer ectodesmata in the epidermis of tobacco T.I. 245 (Thomas and Fulton, 1968) or the lack of compatible infection sites or other physical barriers. In other instances resistance is said to be due to an active defence mechanism(s) activated only after infection (Nachman et al., 1971), i.e. the resistance mechanism(s) requires the active metabolism of host cells.

Localization of the virus is one of the most efficient active resistance mechanisms. In localised virus infections, virus particles replicate in the initially infected cell(s) and are transported as virus or nucleic acid from those cells through plasmodesmata to infect juxtapositioning cells. Only a relatively small number of cells around the focus of infection become infected without further invasion of the plant. In hypersensitivity, this process of localisation is often associated with a strong reaction by the host culminating in the death of the originally infected cells at the centre of the localised areas, producing conspicuous

necrotic local lesions. Here the virus is restricted to necrotic lesions and to some living cells surrounding them (Hayashi and Matsui, 1965; Martin and Gallet, 1966). Occasionally, the localised areas may lose chlorophyll and other pigments, giving rise to chlorotic local lesions, as seen in Chenopodium quinoa infected with carnation ringspot virus, CRSV (Hollings and Stone, 1965).

In other instances, the process of localisation takes place without any sign of necrosis or chlorosis and the infected leaves are indistinguishable from healthy tissue (e.g. cucumber cotyledons infected with TMV). The localised areas of virus replication can, however, be visualized by staining for starch lesions (Lindner et al., 1959). In some cases, the infection may be restricted to a very small number of cells without showing any symptoms and the localised areas cannot be visualized by any light microscopic means. Such infections are termed subliminal or symptomless e.g. tobacco mosaic virus (TMV) infected cotton cotyledons (Cheo, 1970).

The localisation of viruses is a genetically controlled biological process. Holmes (1954) in a genetical study on inheritance of resistance to viral diseases in plants mentioned that the genetic mechanism that controls localisation is determined in many cases by one gene. Hence, the dominant N gene in Nicotiana glutinosa and the L gene in Tobasco peppers, confer hypersensitivity to infection by TMV. Similarly, a single dominant gene in beans controls local lesion formation by Southern bean mosaic virus (SBMV).

Nagaich et al. (1968) claimed that cytoplasmic control was involved in inheritance of the local lesion response to Potato virus X (PVX) in Capsicum spp. since the inheritance was dependent on the female parent in crosses between two species of peppers. However, this has not been confirmed.

The mechanism(s) of localisation, whether associated with the necrotic reaction or not is unknown. Similarly, the nature of the possible virus localising agent(s), whether physical or biochemical, which restrict virus movement from local lesions to adjacent tissues has not been adequately described.

The hypersensitive reaction, with its two features of necrogenesis and localisation, is accompanied by the appearance of an induced (acquired) resistance response in healthy tissues on the same plant, not only in the tissue surrounding the local lesions, "Localised induced resistance", (Yarwood, 1960 and Ross, 1961a) but also in uninoculated virus-free parts of the plant, remote from the inoculated leaves, "Systemic induced resistance" (Gilpatrick and Weintraub, 1952; Yarwood, 1953; Ross, 1961b).

The leaf tissue surrounding necrotic lesions in hypersensitive hosts is resistant to further inoculation and differs from control, healthy leaves both morphologically (Israel and Ross, 1967; Simons and Ross, 1971a and b; Spencer and Kimmins, 1971; Favali et al., 1978) and metabolically (Yamaguchi and Hirai, 1959; Weintraub et al., 1960; Solymosy and Farkas, 1963).

The mechanism(s) of localisation and the acquisition of induced resistance may be related but how is not known. Localised and systemic induced resistance may operate through a single mechanism or two different ones (Ross, 1961b). Indeed Ross proposed that the concept of a mechanism common to both types of resistance provides a simple explanation for the characteristically small size of lesions in leaves possessing systemic resistance. When a localised infection is initiated in a leaf already possessing a high degree of resistance, additional resistance induced by a new infection is additive and results in the early development of enhanced resistance adjacent to the infection sites sufficient to arrest cell-to-cell movement of the virus (Ross, 1961b).

An elicited diffusible substance moving rapidly through the plant

may also operate in causing both the local and systemic induced resistance (Ross, 1961b). However, the 'sharpness' of the outer boundaries of the resistant zones around necrotic lesions (Ross, 1961a) is not compatible with this concept and suggests that two mechanisms are involved, one dependent on a readily diffusible material and the other on a substance capable of moving only short distances through plant tissue and probably being bound to a cell constituent. Ross (1961b) added that if two separate mechanisms are involved, then the small lesion size in the systemically resistant leaves could be explained on the basis that the two mechanisms are additive in their effects.

Many publications on the localisation of viruses in necrotic local lesions appear in the literature but only those relevant to the studies carried out in this work, either directly or indirectly, are reviewed.

1. Localisation of viruses in necrotic local lesions

A. Morphological and biochemical changes associated with hypersensitivity and localisation

a) Development of local lesion and distribution of virus particles

Local lesions are of two types, they may be self limiting (e.g. local lesions elicited by TMV in Datura stramonium), where lesions grow to reach their maximum size 3 days after inoculation, or a non-limited, spreading type (e.g. local lesions elicited by most strains of tobacco necrosis virus, TNV, in legumes) where lesions continue to grow indefinitely. Rappaport and Wildman (1957) made a kinetic study on local lesion growth and found that the radius of local lesions incited by three strains of TMV in

N. glutinosa, at 25°C, was a function of time. Although, the general rule is that lesion size is correlated with extractable infectious virus content (Loebenstein, 1972) a decrease in infectivity was associated with larger lesion areas induced by U8 strain of TMV in N. glutinosa (Rappaport and Wildman, 1957).

It is, however, clear that TMV particles are not all localised within necrotic lesions but are present in cells bordering lesions (Hayashi and Matsui, 1965). Milne (1966a) in electron microscopic studies on developing necrotic local lesions caused by TMV in Chenopodium amaranticolor found that new virus particles were formed in a few palisade cells 30 hours after heavy inoculation with TMV, though no necrosis was visible until 15 hours later. Whereas, 50 hours post-inoculation, crystalline arrays of viral particles were found in approximately one-third of the cells, interspersed with other cells containing few or no virus particles. In similar studies on development of local lesions in TMV-infected N. glutinosa (Milne, 1966b) a similar pattern was observed with fewer cells (approximately 2%) containing virus particles and far fewer particles per infected cell. Milne suggested that this uneven distribution and the apparent resistance of many cells to virus multiplication (though no necrosis was seen) may be connected with the confinement of the infection to the lesion.

This discontinuous distribution of accumulated virus in hypersensitive hosts is distinct from the more uniform distribution found in systemic hosts (Shalla, 1964).

Milne (1966b) estimated that 10^3 TMV particles/cell of a N. glutinosa leaf was 2 - 4 orders of magnitude lower than that in a comparable systemic infection. Carroll and Shalla (1965) found that the numbers of TMV particles/cell in tissue peripheral to local

lesions in D. stramonium was apparently much higher and formed relatively larger aggregates.

Edwardson et al. (1966) carried out similar studies using TNV in N. tabacum var. NN Turkish and SBMV in Phaseolus vulgaris var. Kentucky Wonder leaves. TNV was detectable only in necrotic areas whilst SBMV was found in both necrotic tissue and adjacent cells. Particles of both viruses were present as crystalline arrays and were not associated with enveloping membranes.

b) Cytopathic changes

Matsui and Yamaguchi (1966) and Ragetli(1967) have reviewed the cytopathic changes leading to collapse and necrosis of cells in a local lesion. In N. glutinosa infected with TMV, cytopathic changes start with a swelling of starch grains and distortion of the grana, 8 hours after inoculation. Some 16 hours later, disintegration of cytoplasmic membranes, accompanied by a two to threefold increase in the number of mitochondria was observed. These changes were followed by disintegration of chloroplasts prior to necrosis. The integrity of the nucleus was still preserved at this time. Approximately 78 h after inoculation the cells collapsed and their walls became detached from adjacent cells (Weintraub and Ragetli, 1964). Ragetli(1967) suggested that this disorganisation was indicative of an uncontrolled activity of hydrolytic enzymes (e.g. structurally bound latent phosphatases in chloroplasts). Conversely, the disruption of cell organelles may have resulted in the release of hydrolytic enzymes (Loebenstein, 1972).

Israel and Ross (1967) studied the fine structure of local lesions in the hypersensitive reacting Samsun NN tobacco following inoculation with TMV. They found a zone of metabolically active cells that stained darkly around the necrotic area in the

local lesion. Seven days after inoculation, those cells had cytological features indicative of high metabolic activity. As compared to healthy cells, these cells had smaller vacuoles, denser cytoplasm with more ribosomes, and a more marked endoplasmic reticulum system. The chloroplasts in this zone were more amoeboid and had more ribosomes, lipid globules, and starch grains than those in corresponding chloroplasts in normal cells. In a later study, crystal-containing spherosomes termed peroxisomes were identified in these cells (Ross and Israel, 1970). These cytopathic changes were most marked in cells nearest the lesion edge, but were also detectable in a zone of 10 cell diameter beyond the lesion, graduating into normal-appearing cells.

Israel and Ross (1967) visualised this zone in Samsun NN tobacco, as a cylinder of metabolically active cells, encircling a network of collapsed and disorganised cells concerned with the mechanism of localisation. Hayashi and Matsui (1965) previously illustrated a similar phenomenon in N. glutinosa infected with TMV, and stressed that the zone was intermingled with healthy cells, and such cells contained a considerable number of mitochondria.

Spencer and Kimmins (1971) reported membrane-bound vesicular bodies between the plasma membrane and cell wall of the uninfected zone surrounding local lesions developed on Pinto leaves following TMV-infection. Plasmodesmata in the cell walls adjacent to these paramural bodies were also broken.

Scanning electron microscopy of developing local lesions in hypersensitive reacting leaves revealed that small depressions on the leaf surface (24 h after inoculation) were due to collapse of the epidermal and palisade cells, and that in the area of the local lesion, five days after inoculation the thickness of the leaf was reduced by half (Favali et al., 1977).

The hypersensitive reaction has time-dependent and selective effect on membranes. Da Graça and Martin (1975) observed that chloroplasts became swollen and eventually burst with the tonoplast rupturing. Mitochondria and nuclei remained intact even in severely disorganized cells, whilst nuclei became granular in appearance with enlarged nucleoli and the ribosomes present in collapsing cells were often in high concentration. It was concluded from these studies that the initial event(s) in cells undergoing necrosis, i.e. the swelling and bursting of the chloroplasts may be caused by osmotic stress.

Favali et al. (1978) studied the modification in the vascular bundles in the resistant zone surrounding TMV-induced necrotic lesions in N. glutinosa and found that at a distance of about 1 mm from the lesions, the sieve plate pores and the plasmodesmata connecting the sieve elements with their companion cells were occluded by a callose-like substance. Nearer to the lesions, tracheids were partially or totally occluded by plugs of electron-dense material of possibly phenolic compounds, and the xylem parenchyma cells produced thickenings in their walls.

Recently, Pennazio et al. (1978) have studied the ultra-structure and histochemistry of the resistant tissue surrounding lesions of tomato bushy stunt virus (TBSV) in Gomphrena globosa leaves. This region of tissue (2 mm wide) surrounding the local necrotic lesions had localised acquired resistance with characteristic changes in chloroplasts structure. These changes were observed in older but mature, healthy G. globosa leaves, which react to TBSV as resistant tissue does. It was, therefore, suggested that the induction of localized resistance could be regarded as pre-mature senescence in this instance.

c) Cell-wall thickening and deposition of new materials

It has been proposed that paramural bodies and associated cell wall thickenings (and deposition) occurring in the cells bordering necrotic local lesions may lead to virus localisation (Rappaport and Wildman, 1957; Weintraub and Ragetli, 1961; Wu et al., 1969; Wu and Dimitman, 1970; Spencer and Kimmins, 1971; Tu and Hiruki, 1971; Hiruki and Tu, 1972; Brown and Kimmins, 1973; Wu, 1973; Allison and Shalla, 1974; Favali et al., 1974; Faulkner and Kimmins, 1975 and 1978; Schuster and Flemming, 1976).

Weintraub and Ragetli (1961) observed deposition of calcium in the walls of cells surrounding a TMV lesion for a radius of about 50 cells in N. glutinosa leaves. These cells contained predominantly insoluble calcium pectate in the middle lamella, in contrast to healthy tissue which contained mainly pectic acid. The deposition of calcium and formation of calcium pectate were thought to be responsible for localisation of the virus.

Not only was calcium pectate but also other materials such as callose, lignin and sub^eerin were found deposited in the cell walls of cells bordering necrotic local lesions. Both Wu et al. (1969) and Wu and Dimitman (1970) observed deposition of callose around TMV (U_2) necrotic local lesions in Pinto bean following staining with aniline blue. Callose was deposited in the walls of living epidermal and spongy chlorenchyma cells adjacent to the necrotic cells, but not in the palisade chlorenchyma. In a similar study in N. glutinosa, Rappaport and Wildman (1957) demonstrated little callose deposition and that was in only a small number of cells. It was suggested that callose formed a barrier sealing the plasmodesmata between infected and non-infected cells, thereby preventing virus movement.

However, Loebenstein et al. (1970) found no callose deposition in TMV-infected cucumber cotyledons, where infection is localized without necrosis. Callose deposition may be only a response to necrotization and not necessarily responsible for localization. In such studies, the specific staining properties of aniline blue for callose make these results debatable.

Favali et al. (1974) found that in N.glutinosa infected with TMV (U_5), lignification occurs in the tissues surrounding the necrotic lesions. Lignin was deposited at the border of the necrotic lesion and in the lumen of the nearby tracheids.

However, Faulkner and Kimmins (1975) observed deposition of callose, lignin and suberin in cell walls of cells bordering local lesions induced in bean leaves by either TMV or TNV and non-viral lesions induced chemically or mechanically. The timing of the response differed between treatments and the deposition of these compounds was detectable earlier in non-viral lesions. In view of this they could not correlate these changes with virus localisation.

In recent work it has been shown that more lignin was present in TNV-infected dwarf bean leaves and abraded leaves than in untreated controls (Kimmins and Wuddah, 1977). It was concluded that lignin was a constituent of secondary cell wall thickening induced by wounding and postulated lignin to be a barrier to the spread of the virus.

Brown and Kimmins (1973) extracted two protein-polysaccharide complexes from the primary leaves of Pinto bean infected with TNV. One complex (glycoprotein I) contained $\beta(1-4)$ - and $\beta(1-3)$ -linked D-glucopyranose units; the second (glycoprotein II) was shown to contain $\beta(1-4)$ -linked but not $\beta(1-3)$ -linked glucose units. In a further study (Kimmins and Brown, 1973), no relationship between these glycoproteins and localization of the virus was found.

d) Changes in respiration

Merrett and Bayley (1969) mentioned that the appearance of necrotic local lesions following virus infection is fairly consistently accompanied by an enhancement of host respiration, confirming earlier observations by Solymosy (1961) on increased activity of dehydrogenases in hypersensitive reacting leaves, and Parish et al. (1965) who demonstrated that oxygen uptake by infected leaves was proportional to lesion number on the leaves, and related to local lesion development, and not to virus increase.

Also Weintraub et al. (1960) found that the green tissue between necrotic local lesions in hypersensitive hosts respire at a greater rate than healthy controls, but necrosis induced by CuSO_4 did not result in increased respiration.

The metabolic activity of the yellow narrow area surrounding necrotic local lesions in N. tabacum was studied at the enzymatic level (Solymosy and Farkas, 1963). An increased activity of a number of respiratory enzymes in this area was found when comparisons were made to normal green areas on the same leaves. Greatest increases in activity were found in enzymes involved in the hexose monophosphate (HMP) shunt and some other enzymes, NADH- and NADPH-dependent quinone reductases, polyphenoloxidase, aconitase (Krebs' cycle) and cytochrome oxidase (cytochrome system). Whereas several enzymes of the glycolytic pathway as well as malic dehydrogenase (Krebs' cycle) showed no increase in activity. Thus it was concluded that the increase in respiratory metabolism in tissues where necrotic local lesions develop was at least partly due to the activation of the HMP shunt.

Comparison of C_6/C_1 ratios in healthy and infected tissues partially confirmed this hypothesis. Both Loebenstein and Linsey (1963) working with TMV/Datura stramonium system and Bell (1964) working with SBMV/Phaseolus vulgaris system showed that the C_6/C_1 ratio was markedly lower in infected leaves than in healthy tissue.

Conversely, Merrett and Sunderland (1967) found no change in the C_6/C_1 ratio in hypersensitively reacting tobacco following infection with TMV, although the overall glucose metabolism increased.

- e) Involvement of some enzymes of the phenylpropanoid pathway (e.g. phenylalanine ammonia-lyase, PAL) and some oxidative enzymes (polyphenol oxidase, PPO, peroxidase, PO and catalase, Cat.)

It is well known that viral infection modifies both phenol metabolism and oxidative reactions in hypersensitive reacting plants (Farkas et al., 1960; Van Kammen and Brouwer, 1964; Kosuge, 1969; Van Loon and Geelen, 1971; Faccioli, 1979). The importance of such modifications with reference to localised infections has widely been investigated, and a deal of attention has been given to certain enzymes (e.g. PAL, PO and PPO).

Since the discovery of PAL by Koukol and Conn in 1961, the enzyme has attracted considerable interest among plant pathologists. PAL is involved in aromatic biosynthesis and in the formation of lignins and appears to be a good candidate for explaining increased aromatic biosynthesis in virus-infected or injured tissue (Farkas and Szirmai, 1969). In virus-infected plants an increased PAL activity prior to and concurrent with the development of necrotic lesions was observed (Farkas and Szirmai, 1969; Simons and Ross, 1971b). This stimulation of PAL activity is restricted to a narrow zone of tissue surrounding necrotic areas, and is related not only to the number of local lesions (Fritig et al., 1973), but also to the lignification process which takes place during the primary stages of lesion formation (Conti et al., 1974a and 1974b; Favali et al., 1974). The increase in PAL activity in TMV-infected hypersensitive tobacco leaves has been unequivocally demonstrated, using density labelling

techniques, to be due to an increase in the enzyme pool size caused by an increased rate of synthesis (de novo synthesis) mediated by the hypersensitive reaction (Duchesne et al., 1977).

Legrand et al. (1976) demonstrated that not only PAL but also other enzymes of the phenylpropanoid pathway (e.g. cinnamic acid-4-hydroxylase (CAH) and caffeic acid-o-methyltransferase (OMT)) increase in activity following infection of Samsun NN tobacco leaves with TMV. Necrosis and the appearance of these enzymes were coincident and increases in activity were proportional to the number of developing necrotic lesions.

PO and PPO are two other enzymes involved in the hypersensitive reaction and have been implicated in localisation mechanism in plants following virus infection.

Many different virus-host combinations have been studied in this context not only involving necrotic examples, but also plants showing chlorotic lesions, starch lesions and systemic infections. The catalogue of published examples is excellently reviewed by Loebenstein (1972) and the role of these enzymes in both localization and necrotic reaction remains obscure as further exemplified in recent publications (Conti et al., 1974b; Weststeijn, 1976; Faccioli, 1979).

It has been suggested that the oxidation of phenols to quinones may be involved in necrogenesis and could be mediated by PO and PPO. Such an accumulation of quinones to toxic levels might cause necrosis. It was originally proposed that this reaction may constitute an active defence mechanism (Solymosy et al., 1959; Farkas et al., 1960).

In work more pertinent to this study, Farkas et al. (1960) found remarkable increases in PPO activity in cowpea (Vigna sinensis) leaves infected with cucumber mosaic virus (CMV) and N. glutinosa

infected with TNV, and the decreases in o-dihydric phenols, particularly chlorogenic acid and increases in accumulated quinones were thought to be responsible for necrosis and cell death, but Faccioli (1979) using TNV and Chenopodium amaranticolor disagreed with these hypotheses. Ascorbic acid which maintains phenols in a reduced state and prevents necrosis does not result in systemic invasion of plants by virus (Farkas et al., 1960; Parish et al., 1965). Moreover, alterations in ascorbate concentration in Pinto beans reacting hypersensitively to TMV occur after localisation of the virus in necrotic lesions (Milo and Santilli, 1967). This work and similar experiments have convinced pathologists that increases in PPO enzymes, even though qualitative changes in isoenzymes may occur, are a consequence not a cause of necrosis and have little to do with resistance to either virus spread or the elicitation of acquired resistance (Van Loon and Geelen, 1971; Loebenstein, 1972; Conti et al., 1974b; Faccioli, 1979).

The biological roles of the multi-isozymic peroxidases in plants are largely unknown, and their function in hypersensitivity and virus localisation is still one of debate. Peroxidases have been suggested as oxidative enzymes that may promote oxidation of reduced nicotinamide adenine dinucleotide (Rubin and Ivanova, 1963), remove H_2O_2 produced in systems where the terminal oxidase is a flavoprotein (Rubin et al., 1955), catalyse the oxidation of many mono and diphenols, and aromatic amines to quinones in the presence of H_2O_2 (Bonner, 1950), oxidize aromatic compounds produced through the HMP pathway (Loebenstein and Linsey, 1963) or oxidize indol-acetic acid (IAA) promoting plant senescence and participate in the process of lignification (Van Fleet, 1962; Endo, 1968; Hoyle, 1977). However, their involvement in necrotic reactions could be mediated through many pathways including the killing of infected cells to

constitute a definite barrier to further virus spread (Simons and Ross, 1971a).

Whilst, there is little doubt that necrosis in hypersensitive reacting hosts is associated with large increases in PO activity (Simons and Ross, 1970, 1971a; Loebenstein, 1972; Veggetti et al., 1975; Weststeijn, 1976; Sheen and Diachun, 1978a), similar alterations may also occur in systemically infected tissue and are related to the severity of chlorosis (Loebenstein and Linsey, 1961 and 1966; Wood, 1971). The choice of the experimental virus-host combination appears to decide whether the alterations in PO reflect quantitative or qualitative changes in isozymes with both being seen in some instances (e.g. Pinto beans with SBMV, Farkas and Stahmann, 1966; N. glutinosa tobacco with TMV, Chant and Bates, 1970; Samsun NN tobacco with TMV Van Loon and Geelen, 1971) and only quantitative alterations in other systems (e.g. French bean var. The Prince and cowpea var. Dr. Saunder's Upright with TNV_D, Bates and Chant, 1970).

Increases in both PO and PPO activity in hypersensitive reacting hosts are concurrent with the development of necrotic local lesions. Little or no increase in activity of either enzyme is detectable prior to lesion formation. These results have led most workers to conclude that the activity of these enzymes is a consequence and not a cause of cell death.

However, whilst the cause and effect relationship between PPO, localisation of the virus and/or the hypersensitive reaction has been rejected by many workers (Bozarth and Ross, 1964; Van Kammen and Brouwer, 1964; Jockusch, 1966; Conti et al., 1974b; Veggetti et al., 1975; Faccioli, 1979), the relationship involving PO is debatable and should not be totally discounted (Simons and Ross, 1970 and 1971a; Van Loon and Geelen, 1971; Sheen and Diachun, 1978a and 1978b). Indeed some recent work has re-opened the debate on PO, but

is against any direct involvement in necrosis (Weststeijn, 1976; Van Loon, 1976).

Catalase is a ubiquitous enzyme and increases in its activity during the development of necrotic lesions have been given some attention since the enzyme could compete with PO for substrate(s) and may act in a similar fashion to PO (Van Loon and Geelen, 1971). However, Faccioli (1979) found no significant difference between the activity of catalase before and after local lesion appearance in Chenopodium amaranticolor infected with TMV, and concluded that catalase seems totally unrelated to local lesion formation.

Recently, Kato and Misawa (1975 and 1976) claimed that the hypersensitive reaction caused by CMV infection of cowpea leaves is caused by the activation of lipoxygenase activity which causes peroxidation of unsaturated fatty acids in the plasma membrane and other cell membranes leading to a disruption of membrane lipid-protein integrity and finally resulting in cell death. Moreover, Kato (1977) showed that free radicals produced during peroxidation of unsaturated fatty acids (e.g. Linolenic acid) are responsible for inhibiting CMV infectivity.

f) Changes in ribonucleases (RNases)

Increase in the level of RNase activity has been shown to occur in leaf tissues following hypersensitive virus infections, but their increases are not associated with virus multiplication per se, but are due to cellular injury (Diener, 1961; Wyen et al., 1972).

However, Wyen et al. (1972) did find an increase in the level of the relatively purine specific RNase in TMV-infected Xanthi-nc tobacco leaves as compared to mechanically abraded leaves, but no correlation between this increase and virus replication was observed.

g) Accumulation of metabolites

Thrower (1965) visualised the maturing lesions of TMV in both N. glutinosa and D. stramonium leaves as metabolic "sinks" since leaves fed with $^{14}\text{CO}_2$ were highly radioactive as autoradiography of infected leaves had radioactive spots significantly larger than the necrotic lesions themselves at least in the case of D. stramonium.

Solymosy and Farkas (1963) reported that the tissues surrounding local lesions in N. tabacum var. White Burley infected with the para strain of TMV were characterized by a massive accumulation of sugars, probably pentoses. Such an accumulation is explainable if the later stages of the HMP shunt could not cope with the highly activated first two steps of the cycle.

h) Accumulation of phenolic compounds and phytoalexins

Production of phenolic compounds and phytoalexins in plant tissues following fungal, bacterial, or viral infections resulting in a hypersensitive reaction is well documented. The role of phytoalexins in resistance towards plant pathogens is, however, not clear. Accumulation of fluorescent substances in and around necrotic local lesions and the ability of viruses to elicit phytoalexins is well known. Indeed, Best (1936 and 1944) reported that 6-methoxy-7-hydroxycoumarin (scopoletin), a normal constituent of many plants, accumulates in N. tabacum infected by tomato spotted wilt virus (TSWV). Subsequently, other investigators have found different phenolics and phytoalexins associated with hypersensitive viral infections (Gill, 1965; Bailey and Ingham, 1971; Tanguy and Martin, 1972; Bailey and Burden, 1973; Bailey et al., 1976).

Bailey and Ingham (1971) reported that TNV induced the phytoalexin, phaseollin in leaves of Phaseolus vulgaris, and suggested

that its formation is accompanied by cellular browning in either necrotic cells or cells adjoining them. In a further publication, Bailey and Burden (1973) isolated and identified four phytoalexins, viz. phaseollin, phaseollidin, phaseollinisoflavan and kievitone from etiolated bean (P. vulgaris) hypocotyls, after infection with TNV. The last three phytoalexins representing new compounds. It was suggested that these phytoalexins may play a role in protecting plants from subsequent fungal attack, but no evidence was offered for any role in resistance to plant viruses.

Tanguy and Martin (1972) found that a hypersensitive reaction in N. tabacum cv. Xanthi.nc leaves after TMV-infection resulted in the accumulation of many phenolic compounds (i.e. flavonol glycosides, caffeoylquinic, feruloylquinic and p-coumaroylquinic acids, glucose esters and glucosides of cinnamic and benzoic acids). An increase in temperature inhibited the hypersensitive reaction with no effect on the appearance of the phenolics.

i) Alterations in protein synthesis

Several studies have implicated proteinaceous factor(s) being involved in localising virus in plant tissue but with an unknown mechanism(s) (Cheo and Lindner, 1964; Gianinazzi et al., 1970; Van Loon and Van Kammen, 1970; Gianinazzi and Kassanis, 1974; Kassanis and White, 1974; Rohloff and Lerch, 1977; Coutts, 1978b).

It was reported that the tissue surrounding TMV-elicited local lesions in hypersensitive reacting tobacco leaves had a slight increased soluble protein content (Solymosy and Farkas, 1963). But the subject attracted more interest when both Gianinazzi et al. (1970) and Van Loon and Van Kammen (1970) reported the appearance of four novel proteins (b) in hypersensitively reacting tobacco varieties following infection with TMV. Both observations were

followed by numerous reports on the appearance of these proteins in hypersensitive reacting tobacco (Gianinazzi and Kassanis, 1974; Kassanis and White, 1974; Rohloff and Lerch, 1977; Coutts, 1978b). These four proteins have been implicated in the acquired resistance phenomenon in plants and will be reviewed in more detail later.

Tas and Peters (1977) reported a novel glycoprotein component designated as (E_1) in the soluble protein fraction of cucumber cotyledons infected with TSWV. A similar protein was found following inoculation with the yellow strain of CMV which caused systemic infection. Thus they concluded that the E_1 protein has no apparent function in localising the virus in this system.

Coutts (1978b) detected five new proteins in cowpea primary leaves following infection with TNV using PAGE techniques. Two proteins (termed b and d) were present in large quantities with the other three appearing as shoulders on the main peaks. Symptom severity (i.e. necrosis) appeared to determine the quantity of the novel proteins present in leaves.

j) Ethylene and abscisic acid production

Ethylene is the only known gaseous plant hormone and has been termed "a hormone of plant senescence". However, whilst it is evolved from both injured and infected tissues, little information is available on its biosynthesis (Demorest and Stahmann, 1971; Kato, 1977).

It has been reported by many workers (Balázs et al., 1969; Nakagaki et al., 1970; Gáborjányi et al., 1971; Pritchard and Ross, 1975) that increased ethylene emanation from TMV-infected Samsun NN tobacco is one of the earliest measurable physiological events occurring prior to lesion appearance and is associated with lesion development.

Nakagaki et al. (1970) found that enhanced ethylene production in detached tobacco leaves infected with TMV was concurrent with necrotic local lesion development, but not with systemic infections. Increases in ethylene were directly proportional to the number and size of necrotic lesions (i.e. the degree of necrosis). When ethylene was exogenously introduced it caused a small increase of TMV multiplication in tobacco and lesion numbers on beans, although peroxidase activity was slightly inhibited. This was confirmed by Gáborjányi et al. (1971) with TNV-infected Pinto bean leaves and TMV-infected Xanthi-nc. tobacco leaves, whilst in a systemic infection (e.g. CMV and Xanthi-nc) the rate of ethylene production remained unchanged.

Pritchard and Ross (1975) found that treatments which stimulated ethylene production during lesion development also stimulated lesion growth, while those treatments which reduced ethylene levels had the opposite effect. Additionally ethylene synthesis was increased approximately 3 h prior to visual lesion appearance in these leaves which did not show induced resistance to challenge inoculation, and was maximal 4 days post-inoculation. In those leaves which did show induced resistance, ethylene levels increased 8 h prior to lesion appearance and were maximal at 3 days post-inoculation. Moreover, twice as much ethylene was found in those leaves demonstrating induced resistance and further results suggested that ethylene contributed to continued lesion growth and did not restrict virus movement.

Bailiss et al. (1977) found that Xanthi-nc tobacco leaves pre-treated with either ethrel or abscisic acid senesced and when inoculated with TMV developed more local lesions than controls. Moreover they found that the endogenous abscisic acid levels increased progressively as TMV lesions developed.

Kato (1977) reported that ethylene was evolved from cowpea primary leaves infected with CMV 8 h post-inoculation and preceded lesion appearance. However, large increases were only detectable at the onset of tissue necrosis. This period of increased ethylene production prior to lesion appearance was coincident with an active period of lipid peroxidation in infected tissue as reported earlier (Kato and Misawa, 1976). Moreover, Kato (1977) suggested that peroxidation of unsaturated fatty acids (e.g. linoleic acid) was related to the formation of ethylene, and he considered that the precursors of ethylene in vivo and the pathways of ethylene biosynthesis were altered before and after the appearance of local lesions. Production of ethylene is thought to be via conversion of some of linolenate peroxidation products by a non-enzymatic radical reaction. Conversely, after lesion appearance, ethylene production appeared to be mediated by EDTA-sensitive enzymes (e.g. peroxidase).

Van Loon (1977) elicited local lesions similar to those induced by TMV in Samsun NN tobacco by pricking leaves with needles moistened with the ethylene releasing compound, 2-chloroethylphosphonic acid (ethephon). The physiological consequences of this treatment will be reviewed later (Section II. 2. C. a. ii).

k) Loss of the selective permeability of the plasma membrane

The loss of selective permeability and the integrity of plasma membranes in hypersensitive reacting tissue is a well known phenomenon. In virus-infected leaves the leakage of cell constituents from necrosed tissue has been described in several virus-host combinations. This increase in permeability is thought to be an early event in the hypersensitive reaction (Ohashi and Shimomura, 1976; Weststeijn, 1978; Pennazio et al., 1979).

It has been suggested that in virus-induced hypersensitive reactions, multiplication of the virus leads to alterations in the permeability of the cell membranes, measurable as increases in electrolyte leakage. These permeability changes result in changes in cellular regulation processes, culminating in increased ethylene production, membrane disruption and necrosis.

Pennazio et al. (1979) suggested that the loss of integrity of the plasma membrane and resultant electrolyte leakage could be the sole cause of necrosis, and might be a consequence of other interactions between viral particles and the plasma membrane. However, it was confirmed that necrogenesis was a rapid process starting with plasmolysis and a rupturing of the plasma membrane.

B. Suppression of localisation and/or necrosis

Attempts have been made to suppress the mechanism of localisation of virus and/or necrosis in several virus-host combinations, and whilst several treatments have proved successful in halting the necrotic reaction, partially or totally, they have not always resulted in enhancing virus replication and/or the invasion of tissues outside the necrotic local lesions by virus. These studies have provided additional information on the mechanism of localisation, and some of the pertinent results are reviewed in the following sections.

a) Heat treatments

Samuel (1931) first reported that necrotic lesions of TMV on N. glutinosa increased in size, with increasing temperature, and that above 28°C, lesions tended to coalesce. Above 35°C TMV invaded the plant systemically with no visible necrosis, but the transfer of these plants back to 21°C (temperature shift) caused a total necrosis and a

rapid death of the plant. Helms (1965) suggested that micro-lesions, not normally visible were heat-activated in hypersensitively reacting tobacco leaves, and Gáborjányi and El-Hammady (1969) reported that the increased temperature also increased virus multiplication. The sensitivity of TMV elicited local lesions on Samsun NN plants to temperature shift (21 to 32°C) was maximal 48 h post-inoculation, during the period of intense virus multiplication, without lesion development. Temperature shift later than 48 h post-inoculation only resulted in slight increases in lesion size (Ross and Israel, 1970). Recently, Gianinazzi et al. (1977) noted that a temperature shift downwards (25 to 11°C) of systemically infected Samsun NN leaves, infected with TMV resulted in necrosis on these leaves, and that the effect was most apparent when the virus was actively multiplying. The effect of higher temperature (above 35°C) on lesion development and hypersensitivity is difficult to test in whole plants, during a prolonged incubation. Hence most reports on this phenomenon have illustrated experiments using individual leaves, exposed to higher temperature by water dipping. In several virus-host combinations immersion of leaves into hot water for periods of 40 - 50 sec. increased the size of lesions, the amount of extractable virus and decreased callose deposition on the walls of cells adjacent to necrosis (e.g. TMV in cucumber cotyledons, Yarwood, 1958; TMV in Pinto bean leaves, Wu et al., 1969). Balázs et al. (1977) elicited local necrosis on systemically TMV-infected Xanthi leaves by similar leaf immersion (50°C for 40 sec.) 2 to 5 days post-inoculation. PO activity increased quantitatively during necrogenesis, but neither the heat-induced necrosis nor the associated increased PO activity arrested a systemic spread of the virus. These results suggest that neither tissue necrosis nor increased PO is responsible for virus localization, i.e. host resistance.

Other reports on the effect of high temperature treatment on lesion development indicate that no increases in lesion size may be a feature of the timing of treatment, whilst in some cases decreases in lesion size were noted, e.g. SBMV on bean leaves (Ross, 1966). Resconich (1961) pointed out that the magnitude of activation energy required to increase necrotic lesion size in TMV-infected bean leaves by pre-inoculation hot water treatment was of the same order of magnitude as that required for denaturation of proteins.

b) Light intensity

Low light intensity promotes symptom expression in virus-infected plants and shading is routinely used to increase the susceptibility of experimental plants (Bawden and Roberts, 1947).

TNV causes complete necrosis on legumes in the U.K. during Winter, but only relatively few lesions are produced during Summer months. Necrosis is correlated with virus yield, and Winter plants produce ten times more virus than Autumn or Spring-grown plants. Shading leaves during the Summer months sensitizes plants, so that more and larger lesions are produced. Similarly the infectious virus content is also increased (Bawden and Roberts, 1947). Kimmins (1967) reported that the susceptibility of French bean, P. vulgaris, to TNV infection is increased by keeping plants for 24 h prior to inoculation at light intensities below 250 lumen/Ft², and maximum susceptibility was obtained in plants kept in complete darkness.

However, post-inoculation shading treatment gave conflicting results (Huguelet and Hooker, 1966; Hofferek, 1967; Desjardins, 1969).

It was suggested that a product(s) of photosynthesis interferes with virus multiplication (Bawden and Roberts, 1947; Huguelet and Hooker, 1966). Loebenstein (1972) extended this theory

to state that shaded plants are deficient in high-energy compounds, produced by photosynthesis, which are required for the operation of localising mechanism.

c) Leaf detachment

Simply detaching leaves off hypersensitive reacting plants after inoculation with virus has been found to interfere with both the necrotic reaction and resultant virus content.

Most workers reported an increase in the size of lesions and associated infectivity (e.g. Weintraub et al., 1967 with TMV on N. glutinosa; Nakagaki and Hirai, 1971 with TMV on bean leaves). However, such is not always the case and the converse has been observed (Coutts, 1978a with TNV on cowpea leaves).

Loebenstein (1972) suggested that under conditions of moderate senescence (e.g. detachment), where compounds required for localisation are depleted, the localising mechanism, but not virus synthesis is suppressed and subsequent advance of cell degeneration interferes with virus production. Such changes could be mediated through hormones.

d) Abolishing cell-to-cell contact in leaves and the removal of epidermis from leaves

Coutts et al. (1972) and Otsuki et al. (1972) demonstrated that protoplasts derived from hypersensitive tobacco leaves which normally respond in vivo to TMV infection with the formation of necrotic local lesion showed no such response when inoculated in vitro and the virus replicated as well as it did in protoplasts derived from systemic hosts. The N-gene in tobacco varieties is not operative in isolated protoplasts and apparently cell-to-cell contact is necessary

for the development of the hypersensitive reaction (Coutts, 1980).

The involvement of the leaf epidermis in the replication of several viruses and subsequent necrotic lesion formation in hypersensitive hosts has been investigated in several combinations (Kontaxis, 1961; Dijkstra, 1962; Ehara and Misawa, 1967; Yamaguchi and Shimomura, 1968; Kasamo and Shimomura, 1977; Dawson, 1978; Coutts, 1980), but the results are confusing.

It appears that the time delay between the infection of leaves and the removal of the epidermis is critical in determining the fate of the virus. In some cases the virus appears to replicate in the epidermis (e.g. TMV and N. glutinosa leaves, Kasamo and Shimomura, 1977), whilst in another it does not (e.g. TNV and cowpea leaves, Coutts, 1980), and the rapidity of movement of either infectious virus or viral RNA from the epidermis to the underlying mesophyll is clearly different in these and other combinations (Coutts, 1980; Ehara and Mink, 1980).

e) Osmotic stress

Both Kalpagam et al. (1977) and Gulyás and Farkas (1978) established that mannitol-induced osmotic stress (plasmolysis) interfered with the hypersensitive reaction of Xanthi-nc tobacco leaves towards TMV, and suppressed local lesion formation. Cell-to-cell contact is necessary during the initial phases of infection, and for the hypersensitive reaction, increases in the permeability of cell membranes, characteristic of lesion formation, do not occur if hypersensitivity is inhibited by plasmolysis (Kalpagam et al., 1977).

Coutts (1978) illustrated a similar phenomenon using TNV and cowpea leaves, where floating infected cowpea leaves on hypertonic mannitol solution (0.5 M) immediately or at any time up until 24 h post-inoculation completely suppressed lesion development. Floating

infected leaves on a series of increasing mannitol concentrations from 0 to 0.5 M, immediately after inoculation, showed a progressive decrease in lesion number, size and the amounts of associated infectious virus and viral RNA, apart from at 0.2 M mannitol solution where increases were routinely seen (Coutts, 1978a).

Osmotic stress caused by mannitol in healthy uninoculated tissue has been studied and shown to be associated with somewhat similar biochemical and enzymic alterations (Dhindsa and Cleland, 1975; Tatt, 1978a and 1978c; Yi and Todd, 1979). Such studies can throw some light on the physiological state of the osmotically stressed tissue and may help in understanding the mechanism(s) by which the hypersensitive reaction is halted and virus and viral RNA are decreased.

It was clearly shown that mannitol-induced water stress, in healthy *Avena* coleoptiles, causes a major qualitative alteration in the pattern of protein synthesis, even though it reduces the rate of incorporation of leucine into protein (Dhindsa and Cleland, 1975). Later Tatt (1978c) showed that both protein and RNA synthesis were reduced during mannitol-induced water stress in excised tomato cotyledons. Such water stress was found to cause increases in large but decreases in small protein molecules in excised tomato cotyledons with the disappearance of a fast migrating PO isozyme, which was explained by spillover of the small to the large proteins by inter-molecular bondings (Tatt, 1978a). Many enzymes have been reported to be affected by mannitol-induced water stress, some being decreased in activity, e.g. beta-fructofuranosidase, and phospho-fructokinase (Tatt, 1978c) and others being increased in activity, e.g. proteases, hexokinase, glucose-6-phosphate dehydrogenase (Tatt, 1978c) and RNase (Tatt, 1978c working with excised tomato cotyledons; Yi and Todd, 1979 working with wheat seedlings).

The increase in RNase activity has been studied more than the others mentioned and it was concluded that this increase in activity is due solely to an increase in the pre-existing RNase without novel synthesis (Yi and Todd, 1979). Mannitol-induced osmotic stress (water stress) does trigger an increase in RNase activity not only in intact tissue (reviewed above) but also in protoplasts isolated from normal non-stressed tissue and incubated in hypertonic medium (0.7 M mannitol) as shown originally by Lázár et al. (1973) and Premecz et al. (1977).

f) Hormonal treatment

The alterations in hormone levels in virus-infected plants reacting either systemically or hypersensitively are undoubtedly complex, and the subject of debate and controversy (Loebenstein, 1972; Brian, 1978). Similarly, the effect of exogenously applied hormones and 'hormone-like' substances on the hypersensitive response are dependent not only upon dosage but also on the time of application after inoculation (Aldwinckle, 1975; Coutts, 1980). The physiological state of plants is also a major factor to consider in such experiments and in the following only those examples pertinent to this study are illustrated.

Cytokinins are known to inhibit the development of TMV lesions on detached leaves of N. glutinosa (Milo and Srivastava, 1969) and on leaf discs (Kasamo and Schimomura, 1977) but this effect may depend on the presence of the epidermis (Kasamo and Schimomura, 1977; Coutts, 1980). In one instance virus production in necrotic leaves was considerably stimulated as compared to treated systemically infected hosts (Milo and Srivastava, 1969), but not in others (e.g. TMV and Pinto beans, Milo and Srivastava, 1969 and Balázs et al., 1977).

Loebenstein (1972) suggested that as no correlation between virus production and chlorophyll retention was seen in N. glutinosa,

the effect was not one of senescence delay. He added that cytokinins at low concentrations affect the equilibrium between synthetic processes required for localisation and those for virus multiplication, favouring the later.

Kato and Misawa (1976) isolated a compound from CMV-infected cowpea leaves identified as traumatic acid which is thought to be a plant wound hormone. Traumatic acid inhibited the formation of local lesions elicited by CMV on cowpea leaves.

The effects of 2,4-dichlorophenoxyacetic acid (2,4-D) on lesion development and virus multiplication appear to be contradictory when the literature is reviewed, but here auxin-mediation is suspected and the physiology of treated leaves and plant is important (Van Loon, 1979). Several studies using TMV and hypersensitive tobacco plants have demonstrated marked increases in lesion size (Simons and Ross, 1965; Cheo, 1969, and Van Loon, 1979) when concentrations of 2,4-D just below phytotoxic levels are sprayed onto plants, with opposite effect using lower concentrations. With higher levels of 2,4-D, spray treatment invariably increased TMV multiplication (Krylov et al., 1960; Cheo, 1969 and Kluge et al., 1977) and enhanced subliminal infections (e.g. TMV on cotton cotyledons, Cheo, 1971) but indole acetic acid (IAA) decreased lesion size in a concentration dependent manner when sprayed on young expanding leaves (Van Loon, 1979).

g) Metabolic inhibitors (Antimetabolites)

Antimetabolites which selectively suppress host-mediated processes, but not virus multiplication are useful tools for studying the process of localisation in virus-infected plants.

Loebenstein et al. (1969) studied the effects of actinomycin D (AMD) (a protein synthesis inhibitor interfering with transcription, i.e. inhibiting DNA-dependent RNA synthesis), on the hypersensitive

reaction and virus concentration in several hosts infected with TMV. AmD partially suppressed the localising mechanism in the leaves when applied close to the time of inoculation. The average diameter of necrotic lesions in Pinto bean leaves injected with AmD prior to TMV inoculation was $2\frac{1}{2}$ times that of controls. However, in cucumber cotyledons injected with 10 µg AmD/ml 24 h post-inoculation, the number of visible starch lesions was reduced even though virus content increased 11 times as compared to controls. When AmD was applied later, effects on lesion size and virus content decreased and were negligible three days or later after inoculation.

Sela et al. (1969) carried out a similar study with chloramphenicol (Ch.Ph.) and obtained similar results. Ch.Ph. inhibits protein synthesis at the translational level and does not enter the host cell nucleus where TMV-RNA may be synthesized. Ch.Ph. increased TMV lesion size to greater than 3 times that of controls when injected into N. glutinosa leaves, 24 h post-inoculation. Extractable virus contents also increased and were proportional to the lesion diameter and similar increases (four-fold) in virus content were seen when cucumber cotyledons were injected with 200 µg/ml Ch.Ph., within 24 h of TMV-inoculation. Later injection of Ch.Ph. (after 24 h post-inoculation) was less effective.

Király et al. (1968) found that application of Ch.Ph. to Pinto beans for 6 successive days before inoculation with TMV increased lesion size significantly. Whilst Sander (1969) obtained similar results with a pretreatment of Xanthi-nc. tobacco leaf discs with AmD. In this system, Ch.Ph. treatment resulted in an early appearance of TMV necrotic lesions.

Although 2-thiouracil (which is known to interfere with host RNA metabolism) is known as a potent inhibitor to many viruses, Kuhn (1971) reported that 2-thiouracil suppressed the localisation

of cowpea chlorotic mottle virus (CCMV) and caused systemic movement from one side of a cowpea leaf to the other. When treatments were applied not later than 24 h post-inoculation, both lesion size and extractable infectivity increased markedly.

h) Ultraviolet (UV) irradiation

UV-irradiation has been used to suppress virus localisation mechanism(s) in plants and increase multiplication and spread of viruses, and its dose-dependent effect on DNA must have consequences for both host reactions and viral nucleic acid synthesis at the transcriptional levels.

Loebenstein et al. (1970) partially suppressed the localisation of TMV in N. glutinosa leaves. Short-wave UV irradiation (254 nm) significantly increased necrotic lesion size in N. glutinosa and virus multiplication in cucumber cotyledons if applied not later than 24 h post-inoculation. Long wave irradiation (366 nm) at any time after inoculation had no effect on lesion size or virus concentration in either system.

Wu and Dimitman (1970) showed that if Pinto bean leaves were irradiated one day after inoculation with TMV and incubated afterwards for 3 days in the dark larger lesions were developed as compared to control tissue. Moreover, callose deposition around the necrotic lesions was prevented in irradiated leaves.

2. Induced (acquired) resistance

A. Discovery and description of the virus-induced acquired resistance

Acquired resistance phenomenon was first demonstrated by Gilpatrick

and Weintraub (1952) using Dianthus barbatus and carnation mosaic virus (Car.MV). When the lower leaves of two clones of D. barbatus were inoculated with Car.MV, primary necrotic local lesions developed with no systemic spread of the virus. However, virus-free upper leaves were resistant to homologous challenge inoculation, developing only 5 - 25% of the lesion number found on control leaves. Interestingly this phenomenon was reproducible over a period of two and a half years and then ceased abruptly (Weintraub and Kemp, 1961). The reasons for its cessation are not clear.

Yarwood (1953) termed this phenomenon "acquired resistance", presumably to distinguish it from the similar phenomenon of cross protection which involves systemic infection and actual interference between the challenging virus and the inducing virus. In a further publication, Yarwood (1960) showed that the leaf tissue surrounding TMV necrotic lesions in beans were highly resistant to re-inoculation and called this phenomenon "localised acquired resistance".

Subsequent to these observations, numerous reports of acquired resistance in various virus-host combinations have been published. Hypersensitive reacting tobacco plants have been particularly favourable for these studies.

Resistance has been reported to develop in various hypersensitive hosts, both in tissues near the primary local lesions "localised induced (acquired) resistance", and in more distant parts of the plant "systemic induced (acquired) resistance".

Ross (1961a) demonstrated localised induced resistance in TMV-infected Samsun NN tobacco. A zone of uninfected tissue around the local lesions was resistant to a second "challenge" inoculation. In this zone either no lesions or a few minute 'micro-lesions' developed. Resistance was clearly detectable at 20 - 24°C but not demonstrable at temperatures close to 30°C (Ross, 1961a).

Further studies by Ross (1961b) showed that this resistance was

not restricted to areas around local lesions but also developed in leaves far remote from those originally inoculated. Uninoculated half leaves of Samsun NN adjacent to necrotic halves (TMV-infected) were highly resistant to challenge inoculation seven days after the 'inducing' inoculation, lesions being smaller (1/5th - 1/3rd the diameter on controls) and fewer on this tissue. Similar resistance was detected in apical sections of leaves following the inoculation of basal halves, upper leaves following inoculation of lower leaves, and vice versa. All of the challenged tissues were initially free of virus. Resistance was detectable 2 or 3 days after the 'inducing' inoculation and reached a maximum approximately 7 days post-inoculation. Resistance was highest when plants were kept at 20°C and persisted for at least 20 days.

Induced resistance to heterologous challenge, with several viruses (e.g. TNV, tobacco and tomato ring spot viruses, TRSV, Tom RSV, turnip mosaic virus, TuMV) was also shown in 'induced' Samsun NN leaves, but the resistance was not as effective as that against TMV. However, in plants infected by PVX, no resistance around TMV lesions was detected, and the level of TMV-induced systemic resistance was lower than in plants that were free of PVX. Reciprocal challenges using TNV as the 'inducing' virus and TMV as the challenge virus also gave a positive resistance.

Loebenstein (1963) confirmed the acquired resistance phenomenon using the D. stramonium/TMV and the G. globosa/PVX host/virus combinations.

Ross (1966b) also demonstrated systemic induced resistance in Pinto beans against TMV, TNV, SBMV and AMV used in reciprocal combinations for either inducing or challenge inoculation and similar results were obtained with cowpea (Vigna sinensis var. Blackeye) leaves using TNV, CMV and AMV. Resistance developed in the opposite primary leaf regardless of the order of virus inoculation. Lesion numbers were often reduced, but effects on size were more consistent.

Since these original observations, many reports on this acquired

resistance phenomenon, using a variety of host/virus combinations, have been published (Van Loon and Van Kammen, 1970; Van Loon, 1975a, 1975b and 1976; Van Loon and Dijkstra, 1976; Conti et al., 1978).

It was originally thought that the development of fewer and smaller lesions were common features of the acquired resistance phenomenon (Ross, 1961a), but recent evidence suggest that the two may be separate processes (Fraser, 1979) with the former related to leaf water potential which may restrict infectable sites (Cassels et al., 1978; Fraser, 1979) , and the later possibly concerned with protein changes in leaves (Gianinazzi et al., 1970; Van Loon and Van Kammen, 1970).

The development of resistance has so far been reported only for viruses that induce necrotic lesions, no systemic resistance was elicited in cucumber cotyledons with TMV-induced starch lesions (Loebenstein, 1972).

B. Induction of acquired resistance by factors other than infective viruses

Induced resistance is not only elicited by infective viruses but can also be elicited by inactivated virus, viral coat protein and other biotic or abiotic agents (Loebenstein, 1972).

a) Induction by heat-killed bacteria or bacterial fractions

Heat-killed cells of Pseudomonas syringae (Loebenstein and Lovrekovich, 1966) and P. fluorescence (Klement et al., 1966) when injected into the intercellular spaces of Samsun NN or Xanthi-nc tobacco leaves respectively, 0 - 7 days before inoculation with TMV, markedly reduced lesion numbers. Moreover, heat-killed bacteria suppressed lesion formation even when applied 5 - 72 h after inoculation, when infection was established and virus multiplication had begun. Injection of a bacterial preparation 24 h after inoculation or later,

resulted in a decreased effect on lesion number, but a marked decrease in lesion size was still apparent. Resistance induced by heat-killed bacteria was sensitive to actinomycin D.

Similarly, isopropanol-extracts of Bacillus uniflagellatus reduced the number of local lesions when added to the soil or sprayed on the leaves of Xanthi-nc. tobacco prior to inoculation with TMV (Mann, 1969). It is not yet known whether the above bacterial fractions activate a host resistance mechanism or alternatively, directly inhibit viral infection or multiplication.

b) Induction by pathogenic fungi, their extracts, or by-products of their metabolism

Systemic induced resistance has been elicited in several host plants towards different viruses by a range of fungal pathogens and extracts, viz. spore suspension of Peronospora tabacina injected into Xanthi-nc. tobacco stems towards TMV (Mandryk, 1963); Thielaviopsis basicola injected into hypersensitive tobacco leaves towards TMV and TNV (Hecht and Bateman, 1964; Gianinazzi et al., 1980).

The resistance in these plants may be elicited by by-products of pathogen-elicited necrosis or the pathogens themselves. Indeed, victorin, a pathotoxic product of Helminthosporium victoriae, rendered bean leaves highly resistant to TMV and AMV, at concentrations that did not cause necrosis (Wheeler and Pirone, 1969). Also, it has been reported that a complete resistance to infection of Xanthi-nc. tobacco by TMV was induced by the total or the water-soluble fraction of ultra-sonicated Nocardia asteroides (Gianinazzi and Martin, 1975). This resistance and the associated production of additional proteins were also inhibited by actinomycin D.

c) Induction by foreign and synthetic nucleic acids

Gicherman and Loebenstein (1968) found that a high degree of resistance or interference could be induced when yeast-RNA (5 mg/ml) was injected intercellularly into Samsun NN tobacco leaves. Yeast-RNA, when applied together with TMV, acted as an inhibitor of infection, but it also induced interference when injected at least 3 days before inoculation. Interference was highest when TMV was inoculated 5 - 6 days after RNA injection and remained high even after 20 days. In the resistant tissue injected with RNA, TMV lesion number decreased by 80 - 90% compared with the control, but lesion size was unaffected. Actinomycin D or puromycin inhibited this resistance when injected shortly after the RNA induction. Similar effect of yeast RNA on induced resistance in Samsun NN was reported by Van Loon (1975b). Induced resistance was also elicited in some hypersensitive hosts using heterologous and not homologous plant RNA (Cheo et al., 1968).

In 1970, Stein and Loebenstein observed that injection of the synthetic double stranded RNA, polyinosinic-polycytidilic acid (poly I-poly C), in microgram quantities, into Samsun NN leaves and D. stramonium leaves, also induced resistance to TMV.

d) Induction by organic and inorganic compounds

Several organic and inorganic compounds have been successfully used to elicit induced resistance in several hosts, e.g. polyacrylic acid (PA) with N. tabacum var. Xanthi-nc. against TMV and TNV (Gianinazzi and Kassanis, 1974); the ethylene releasing compound, 2-chloroethylphosphonic acid (ethephon) with Samsun NN tobacco against TMV (Van Loon, 1977), benzoic, salicylic and acetylsalicylic acid (aspirin) with Xanthi-nc. tobacco against TMV (White, 1979); mercuric chloride with hypersensitive tobaccos against TMV (Van Loon, 1975b; Sziraki

et al., 1980). The use and the biochemical consequences (in terms of protein synthesis) of these chemicals will be illustrated in more detail later in this section.

C. The biochemical basis of localised and systemic induced resistance

The process of localisation of viruses in necrotic local lesions and the consequent development of localised and systemic induced resistance may be features of a common reaction of plants towards viruses.

Elucidation of the mechanism(s) controlling localised and systemic acquired resistance has proved to be a recalcitrant problem for virologists, particularly regarding the complex physiological and metabolic changes that take place in plants following a hypersensitive response and their relationships to the phenomena. Reference back to the previous section is essential to understand the relevance of some of the work reviewed next, since to eliminate repetition much of it is condensed.

The mechanism of virus localisation and the phenomena of localised and systemic induced resistance have attracted a deal of attention mainly because of the rapid and reproducible physiological and enzymic alterations that occur in leaf tissue surrounding virus-induced necrotic local lesions.

Increases in the activity of a number of respiratory enzymes (particularly those involved in the HMP pathway) and an accompanying accumulation of sugars, mainly pentoses, in tissues surrounding necrotic lesions have been reported (Solymosy and Farkas, 1963). It has been suggested that this increased activity of the HMP pathway may be associated with accumulation of phenols which may be responsible for localised acquired resistance and the accumulation of sugars may be explained if the later stages of the HMP shunt could not cope with the highly activated first two steps of the pathway (Solymosy and Farkas, 1963), but this is speculative.

The PPO enzymes do not apparently play any active role in induced

systemic resistance, since although a twofold increase in PPO activity was observed in the basal halves of Samsun NN tobacco leaves, 2 - 3 days after TMV inoculation of the apical regions (Van Kammen and Brouwer, 1964), removing the inoculated portions of the leaves 7 hours after inoculation also induced increased PPO activity in the remaining tissue, with no induced resistance (Bozarth and Ross, 1964). Such a resistance normally took at least 3 days to develop (Bozarth and Ross, 1964; Simons and Ross, 1970). Indeed no increases in PPO activity were observed even in extracts of resistant leaves when a manometric (Bozarth and Ross, 1964) instead of spectrophotometric (Van Kammen and Brouwer, 1964) method was employed. Additionally, although accumulation of polyphenols is characteristic of necrotic tissue, such changes can be induced by simple chemical damage which does not induce resistance (Loebenstein, 1972).

It has now been generally accepted that PPO has no role in induced resistance (Van Kammen and Brouwer, 1964; Van Loon and Geelen, 1971; Veggetti et al., 1975; Faccioli, 1979). However, Batra and Kuhn (1975) using soybeans and cowpea chlorotic mottle virus remain unconvinced.

However, the role of PO in both virus localisation and induced resistance is still a matter of debate.

It was reported that the activity of PO and catalase increases in parallel with the development of systemic induced resistance, and remains at high levels (Simons and Ross, 1970 and 1971a). Indeed, other workers still believe that PO has an active role in induced resistance (Van Loon and Geelen, 1971; Veggetti et al., 1975). However, injection of commercial PO preparation into tobacco leaves did not induce resistance to TMV (Loebenstein, 1972) and occasionally increased lesion size (Van Loon, 1976).

Moreover, recently, Faccioli (1979) using the Chenopodium amaranticolor and TNV combination found no role for PO in induced resistance.

Certainly an inter-relationship between PO, free radicals and hormones may well have some consequence in induced resistance studies (Kato, 1977).

a) Theories to explain the mechanism(s) of localised and systemic induced resistance

i) Depletion hypothesis

This hypothesis which has largely been rejected (Loebenstein, 1972) suggests that increased metabolic activity in the initially infected cells may create a 'metabolic sink' with a consequent depletion of metabolites (e.g. sugars) occurring in adjacent uninfected tissues. Such tissues are therefore less able to support virus synthesis and become "resistant" (Silberschmidt and Caner, 1967).

ii) Production of a resistance-inducing substance(s)

It has been postulated that following initial local necrotic infections a substance(s) is synthesised in these cells and migrates to healthy cells surrounding necrotic lesions and also into tissue far remote from that originally inoculated. This substance(s) then induces resistance (Bozarth and Ross, 1964; Ross, 1966; Loebenstein et al., 1968; Gianinazzi et al., 1970; Van Loon and Van Kammen, 1970; Gianinazzi and Kassanis, 1974; Kassanis and White, 1974; Van Loon, 1975b).

This induced resistance whether localised (Loebenstein et al., 1968) or systemic (Kassanis and White, 1974) was inhibited by AmD when the drug was injected near the time of the inducing inoculation. Such results suggest that the development of induced resistance is dependent on transcription activity in cells (DNA-dependent RNA synthesis) which leads to the synthesis of resistance-inducing substance(s) (Loebenstein, 1972). This substance(s) has been considered to be proteinaceous in nature.

Induced resistance has gained renewed interest in recent years

since the discovery by Gianinazzi et al. (1970) of four new proteins, not virus-related (termed b_1 - b_4) in the leaves of Xanthi-nc. tobacco following infection with TMV. These proteins were absent in healthy leaves and were thought to be host defined.

Independently, Van Loon and Van Kammen (1970) found four similar proteins, similarly, not virus-related (termed IV-I) in the TMV-infected Samsun NN tobacco leaves and in uninoculated, symptomless, non-virus-containing leaves. These four novel proteins were not related to TMV coat protein, and were not new isoenzymes of five enzymes tested (PO, PPO, acid phosphatase, glucose-6-phosphate dehydrogenase, or 6-phosphogluconate dehydrogenase) or a series of other enzymes tested subsequently (Van Loon and Ritter, 1978).

These authors (Gianinazzi et al., 1970; Van Loon and Van Kammen, 1970) suggested that these novel proteins (induced proteins) might play an active role in suppressing a second virus infection by inhibiting either virus multiplication or virus spread to the surrounding tissues.

Rohloff and Lerch (1977) discovered a fifth protein, only present in tissue discs, 4 mm in diameter, excised from around the lesions which was not detectable in extracts of whole leaves of Xanthi-nc. infected with either TMV or TNV. Recently, Coutts (1978b) elicited the same four proteins (termed A-D) in leaves of Xanthi-nc. tobacco following infection with TNV.

The synthesis of these four proteins could be inhibited by AmD (Van Loon and Van Kammen, 1970).

Gianinazzi and Kassanis (1974) reported that when leaves of Xanthi-nc. were injected with polyacrylic acid (PA) they became completely resistant to infection by either TMV or TNV, 1-3 days after injection, but this resistance, unlike that induced by virus infection was restricted to treated leaves. As with induced (acquired)

resistance, PA-induced resistance disappeared above 30 - 32°C suggesting that similar mechanisms might have been involved. PA treatment also resulted in the appearance of at least three of the novel proteins (b_1 - b_3) in leaves confirming that these proteins are not coded for by the viral RNA.

Van Loon (1977) showed that local lesions induced by pricking lower leaves of Samsun NN tobacco plants by needles moistened with the ethylene-releasing compound, 2-chloroethylphosphonic acid (ethephon) resulted in induction of the four novel 'b' proteins in both treated and untreated upper leaves. The treatment resulted also in both quantitative and qualitative alterations in the PO isozyme patterns similar to those seen following TMV-infection. Moreover, a systemic resistance in the upper untreated leaves was induced comparable to that found following virus infection. Thus abundant local ethylene synthesis during TMV-infection appears to be a causative factor in redirecting plant metabolism during the hypersensitive reaction.

Recently, it has been shown that not only PA and ethephon but also benzoic, salicylic and acetylsalicylic (aspirin) acids are able to induce resistance to TMV and the formation of the same three proteins (b_1 - b_3) induced by PA in Xanthi-nc. tobacco leaves. The resistance induced by these chemicals is similar to that induced by PA in that it is confined to the treated leaves (White, 1979). However, spraying lower leaves of Samsun NN plants with mercuric chloride solution in a necrotizing concentration (0.1%) induced systemic resistance but did not induce any of the 'b' proteins (Van Loon, 1975b).

Kassanis and White (1974) demonstrated that both the synthesis of these proteins (either induced by PA treatment or virus infection) and induced resistance were inhibited by AmD. They then went on to suggest that these proteins are responsible for the induced

resistance and compared them with the interferon produced during viral infection of animal tissues in response to viral infection and suggested that they might represent a type of plant interferon.

The proteins are not products of the NN genes, because they are induced in Samsun plants following infection with the Holmes' ribgrass strain of TMV (Van Loon, 1975 a). Kassanis et al. (1974) also reported that acquired resistance could be developed on Xanthi-nc. tobacco leaves with a number of viruses causing systemic infections (CMV, AMV, PVX PVY and potato aucuba mosaic virus), although they admitted that competition between the two viruses in the plant, i.e. the challenging virus and the inducing one, cannot be excluded. Moreover, they showed that varying amounts of three and sometimes four of the known novel proteins were produced.

Gianinazzi et al. (1977) partially purified the 'b' proteins and demonstrated that b_2 and b_3 are respectively dimer and trimer forms of the b_1 protein (mol. wt. of the monomer, approximately 16,000 d.) whereas b_4 protein (mol. wt. of the monomer, about 29,000 d.) appeared to be unrelated.

Antoniw and Pierpoint (1978) similarly, purified the b_1 protein and showed that the mol. wt. of the monomer was 15,000 d. by electrophoresis and 14,200 d. by ultracentrifugation.

In a recent paper, Antoni et al. (1980) have succeeded in purifying b_1 , b_2 and b_3 proteins of TMV-infected Xanthi-nc. tobacco leaves and the IV, III and II proteins of TMV-infected Samsun NN tobacco leaves and shown them to be similar, if not identical. All three proteins consist of a single polypeptide chain with a similar mol.wt. of about 14,200 d. The three proteins, b_1 , b_2 and b_3 are thought to be charge isomers of one another, while the b_4 protein is dissimilar in both charge and size.

Antoniw et al. (1980) suggested a common name, pathogenesis-related (PR) proteins to be used in the future to identify novel proteins. This unified system of nomenclature groups the "b₁ - b₄" proteins of Xanthi-nc. and the "IV - I" proteins of Samsun NN together in families, each family accommodating proteins that migrate as a single band in SDS-gels with identical electrophoretic mobility and having similar mol. wt.

Although 'b' proteins can easily be detected in TMV or TNV-infected Xanthi-nc. tobacco leaves (Gianinazzi et al., 1970; Rohloff and Lerch, 1977; Coutts, 1978b), they were not detected in callus derived from Xanthi-nc. tobacco leaves, although necrotic local lesions developed on the callus, 3 days post-inoculation (Beachy and Murakishi, 1976).

Kassanis and White (1978) reported that (PA) decreased TMV yield in Xanthi-nc. tobacco protoplasts by 40 - 60% whether treated protoplasts were kept at 25°C or 32°C after inoculation, although PA-induced resistance in plants breaks down at 32°C. They suggested that the effect of PA on virus yield in protoplasts was different from that resulting in increased resistance in whole plants, either injected or sprayed with PA. Furthermore, none of the b proteins were detected in PA-treated protoplasts although small amounts were found in protoplasts obtained from Xanthi leaves, 5 days after spraying with a 20 mg/ml PA solution. Such protoplasts did not show any reduction in virus yield. It was concluded from these results that 'b' proteins produced in PA-treated leaves had no effect on virus yield in isolated protoplasts.

Cassells et al. (1978) showed that the resistance developed in Xanthi-nc. tobacco leaves following PA treatment is abolished by applying the anti-transpirant S-600. It has been suggested from these results that PA-induced resistance is only a reflection of altered

water balance in cells which in turn leads to changes in cell turgor affecting plant susceptibility.

Sziraki et al. (1980) suggested that a cause-and-effect relationship exists between increased cytokinin levels and the development of acquired resistance in upper resistant leaves. Although, they did not show any evidence as to whether the high level of cytokinin in the resistant upper leaves was associated with the presence of 'b' proteins, they discounted any active role for the 'b' proteins in systemic induced resistance because of the fact that mercuric chloride causes induced systemic resistance without producing the proteins (Van Loon, 1975b). Moreover, in spite of a reduction in the number of visible local necrotic lesions in resistant upper leaves, infectivity of ultra-centrifuged TMV extracted from this tissue did not change markedly. Thus it appeared in this system that the necrotization is only suppressed in challenge-inoculated resistant leaves, while virus production is not. Recently, Fraser (1979) arrived at a similar conclusion that "systemic acquired resistance" as measured by a reduction in lesion number is not a true resistance at all since infectious virus and viral RNA content is statistically not different from that in control tissue. However, he could not adequately explain reproducible decreases in lesion size.

III. MATERIALS AND METHODS

1. Pathogens

A. Viruses

a) Tobacco necrosis virus (TNV)

Two strains, TNV_D (lacking satellite virus) and TNV_A (with satellite virus) have been used.

- TNV_D (Babos and Kassanis, 1963) was originally supplied in French bean leaves (Phaseolus vulgaris L. cv. "The Prince") by Drs. S.R. Chant and D. McCarthy, and was propagated through single-lesion isolates in primary leaves of cowpea.
- TNV_A, provided by Mr R.F. White, was similarly propagated in primary leaves of cowpea.

b) Tobacco mosaic virus, type-strain (TMV-U1)

TMV was obtained from Dr. R.H.A. Coutts and was purified from systemically infected Nicotiana tabacum L. cv. White Burley leaves.

c) Cucumber mosaic virus (CMV)

CMV_W, the Worcestershire strain of CMV (Tomlinson et al. 1970), was also obtained from Dr. R.H.A. Coutts and was purified from inoculated and systemically infected N. tabacum L. cv. White Burley leaves by the method of Scott (1963) using three cycles of high and low speed centrifugation.

B. Fungi

a) Colletotrichum lagenarium (Pass.) Ell. and Holst.

Race 1 of C. lagenarium was originally supplied by Wye College (University of London) and was known to cause anthracnose disease on susceptible cucumber varieties (Andebrhan, 1978).

2. Host plants and conditions of growth

A. Cowpea (Vigna sinensis Endl. cv. Blackeye) seeds were obtained from Ferry-Morse Seed Company, Mountain View, California, U.S.A., and germinated in compost (Levington's All Peat) in plastic trays under conditions of high humidity, and then seedlings were grown under glasshouse conditions at $25 \pm 3^{\circ}\text{C}$ with supplementary lighting from Phillips 400 W mercury vapour lamps, providing a 14-h photoperiod of light intensity between 10 - 20 K. lux. Plants were selected for inoculation 8 - 10 days after sowing, when the primary leaves were almost fully expanded, without the first trifoliate appearing.

B. Cucumber (Cucumis sativus L. cv. Ashley, Baton-Vert, China, Chipper) seeds were obtained from Suttons and Sons Ltd., Torquay, England. The Elem and Explorer cucumber cultivars were supplied by Professor G. Loebenstein and Dr. T. Andebrhan respectively. Seeds were germinated and plants grown as above. Plants were selected for inoculation, 8 - 10 days after sowing, when the cotyledons were almost fully expanded, without the first true leaf appearing.

C. Tobacco (Nicotiana tabacum L. cv. Xanthi-nc.) seeds were obtained from Dr. R.H.A. Coutts, and scatter-sown in compost. Seedlings of approximately 2 cm. spread were pricked out into 10 cm.

plastic pots (3 seedlings per pot) and were usually inoculated when they were at the 4 - 5 leaf stage (approximately 45 to 60 days old). Conditions of growth were the same as for cowpea plants shown earlier.

3. Preparation of inocula

A. Viral inocula

Either clarified or partially purified virus preparations were used as virus inocula.

a) Clarified virus preparations

TNV-inocula were prepared from TNV-infected cowpea primary leaves showing severe necrotic lesion symptoms, while TMV- and CMV-inocula were prepared from the corresponding infected White Burley tobacco leaves showing severe mosaic symptoms. Infected tissue was homogenised at 4°C in 0.06 M sodium phosphate buffer, pH 7.0 (1 : 10, w/v) using an ice cold mortar and pestle. Normally, the grinding rate was 1g to 10 ml of buffer. Homogenates were then centrifuged (in an MSE H1-spin 21 centrifuge) at 2000 g. (5000 rpm) for 5 min. and the supernatants were used as clarified virus inocula.

b) Partially purified virus preparations

Partially purified virus preparations were used only as inocula with TNV and was prepared as outlined later (section III. 6.A). Such preparations had dilution endpoints between

10^{-5} to 10^{-4} on cowpea leaves and were diluted in 0.06 M sodium phosphate buffer, pH 7.0 before inoculation to give the desired local lesion number on leaves.

B. Fungal inoculum

Inoculum of the fungus C. lagenarium was prepared from 5 to 8 day cultures on acidic vegetable juice (V₈, Campbells Soups Ltd., Norfolk, England, U.K.) agar. Cultures were rinsed with sterile distilled water to give spore suspensions which were filtered through four layers of muslin before washing twice by centrifugation (in the MSE centrifuge) at 830 g (2000 rpm) for 10 min. Suspensions adjusted to 1×10^5 spores/ml were then used to inoculate cucumber cotyledons.

4. Methods of inoculation

A. Inoculation with viruses

Leaves to be inoculated were first dusted with carborundum (500 mesh, The Carbonundrum Company Ltd., Trafford Park, Manchester, England, U.K.) as an abrasive and viral inocula were applied using sterile cotton-tipped applicators (Johnson & Johnson Ltd., Slough, England, U.K.), or muslin pads. Excess inocula were removed by washing inoculated leaves under running tap water within 2 - 5 mins. of inoculation. Control leaves were sham-inoculated with 0.06 M sodium phosphate buffer alone.

B. Inoculation with C. lagenarium

Droplets (5 μ l each) of conidiospore suspension (1×10^5 spores/ml) were applied onto the upper surface of cucumber cotyledons at evenly dispersed points using an Agla micrometer syringe (Burroughs

Wellcome Ltd., London). Control cotyledons were only treated with sterile water.

5. Development of starch lesions in TMV-infected cucumber cotyledons

This method is essentially that of Lindner et al., 1959. Cotyledons were first decolorized by heating in 70% ethanol at 80°C until all the chlorophyll was extracted. Cotyledons were then placed in an iodine stain (1 part of 2% iodine + 6% potassium iodide to 20 parts of Lactic acid, to facilitate iodine penetration) for 15 min. Cotyledons were then extensively washed in distilled water. Starch lesions were then visible to the naked eye and examined microscopically.

6. Purification of TNV

TNV was propagated through single lesion isolates in cowpea primary leaves and using such isolates, three day infected cowpea leaves which were heavily necrosed were used for virus purification.

A. Partially purified virus preparation (PPVP)

PPVP was prepared using a method outlined previously (Bailey and Ingham, 1971). Infected tissue (50 - 100 g) was homogenised in ice cold 0.1 M sodium phosphate buffer, pH 7.0 (1 : 1, w/v) in a top drive macerator ('Omnimixer' homogeniser; Ivan Sorvall, Inc., U.S.A.) for 6 min. The resulting homogenate was filtered through three layers of muslin. The filtrate was then heated at 50°C for 10 min. in a water-bath to coagulate host proteins. The filtrate was then cooled to 4°C and centrifuged (in the MSE centrifuge) at 8000 g (10,000 rpm)

for 20 min. at 4°C. The supernatant was then divided into aliquots (usually 1 ml) and stored at -20°C in screw capped bijoux bottles. One sample was taken for assay prior to storage to estimate the end-point titre of the PPVP on cowpea primary leaves.

B. Highly purified virus preparation (HPVP)

Two methods of purification have been employed :

- a) Polyethylene glycol method (Gooding and ⁱHebert, 1967)
- b) Differential centrifugation method (Kassanis and Phillips, 1970)

a) Polyethylene glycol method

Frozen infected tissue (150 - 200 g) was homogenised in ice cold 0.5 M sodium phosphate buffer, pH 7.5 containing 1% 2-mercaptoethanol and 10 mM EDTA (the rate of homogenisation was 1 : 1, w/v). Homogenisation was carried out in Kenwood homogeniser (Kenwood MFG, Woking, Ltd., Havant, Hampshire, U.K.). The resulting homogenate was strained through three layers of muslin into a cold measuring cylinder, then 8 ml of n-butanol/100 ml of extract was added and the mixture stirred at room temperature for 30 min. The whole was then centrifuged (in the MSE centrifuge) at 8000 g (10,000 rpm) for 30 min. at 4°C. The supernatant was retained and made 7% (w/v) with respect to polyethylene glycol 6000 (PEG-6000) and 0.2 M with respect to NaCl. The mixture was then stirred for 1.5 h at room

temperature, and subsequently centrifuged (in the MSE centrifuge) at 8000 g (10,000 rpm) for 15 min. The supernatant was discarded and the pellet resuspended in 20 ml (0.1 M sodium phosphate buffer, pH 7.5 + 2 mM EDTA)/100 ml of original extract. The resuspended pellet extract was clarified by centrifugation (in the MSE centrifuge) at 8000 g (10,000 rpm) for 15 min., the pellet discarded and the supernatant re-treated with 7% PEG (w/v) and 0.2 M NaCl as above with stirring for 1 h only. The mixture was then centrifuged (in the MSE centrifuge) at 8000 g (10,000 rpm) for 15 min. and the resultant pellet resuspended in 0.6 ml H₂O/100 ml of original extract, and left to resuspend overnight at 4°C. The virus solution was clarified at 8000 g (10,000 rpm) for 15 min. and the supernatant retained as highly purified virus. Purity was assessed by measuring the optical density of the HPVP at both 260 and 280 nm on a spectrophotometer (Beckman, model 35; Beckman Instruments, Inc., Fullerton, California, U.S.A.) and the virus content was estimated using the extinction coefficient $E_{260}^{1 \text{ cm}}$, 0.1% = 5.5 (Babos and Kassanis, 1963).

b) Differential centrifugation method

Frozen infected tissue (150 - 200 g) was homogenised in ice cold 0.05 M sodium phosphate buffer, pH 7.5 containing 1% 2-mercaptoethanol (the rate of homogenisation was 1 : 1, w/v) in the Kenwood homogeniser. The homogenate was strained through three layers of muslin into a measuring cylinder. The filtrate was treated with n-butanol (8 ml/100 ml of original extract) and left overnight with stirring at 4°C. This solution was then centrifuged (in the MSE centrifuge) at 10,000 g (11,000 rpm)

for 30 min. The supernatant was retained and passed through 3 layers of muslin to remove any coagulated host proteins and chloroplasts. The filtrate was then centrifuged (in the MSE centrifuge) at 30,000 g (19,000 rpm) for 30 min. The supernatant was retained and centrifuged (Type 50 rotor, in a Beckman L1 Spinco ultracentrifuge) at 105,000 g (41,000 rpm) for 3h. Pellets were resuspended in H₂O (20 ml/100 ml of original extract) and left overnight at 4°C. The virus solution was then clarified (in the MSE centrifuge) at 30,000 g (19,000 rpm) for 20 min. Virus was then re-pelleted from this solution by centrifugation (in the Beckman ultracentrifuge) at 105,000 g (41,000 rpm) for 3 h. The virus pellet was then resuspended in H₂O (0.9 ml/100 ml of the original extract) and then clarified (in the MSE centrifuge) at 30,000 g (19,000 rpm) for 20 min. The supernatant was retained as highly purified virus. The purity of the preparation and concentration of the virus were determined as in the previous section.

7. Extraction of infectious virus (v-p) and viral RNA (v-RNA)
from cowpea leaf tissue

Fully expanded cowpea primary leaves were chosen for these experiments because individual lesions were well separated from one another, to allow for the sampling of leaf discs and rings surrounding individual local lesions without any overlap. Virus inocula were prepared to elicit 3 or 4 lesions on a whole leaf, and when lesions were fully developed, 3 days post-inoculation, leaf discs and concentric rings were removed at different distances, 0 - 1.5, 1.5 - 3, 3 - 5 and 5 - 8 mm, from the local lesion epicentre, using a range of sterilized cork-borers.

When whole virus and viral RNA were extracted from uninoculated leaf strips adjacent to the mid-rib of completely necrotic halves, virus inocula were prepared to elicit total necrosis on one half leaf. When necrosis was complete (3 days after inoculation) a sequential series of leaf strips were removed aseptically from the uninoculated halves at different distances (0 - 3, 3 - 6, 6 - 9 and 9 - 12 mm) away from the mid-rib using a sterilized scalpel blade (Surgical Blades BS 2982, Swann Morton (Sales) Ltd., Sheffield 6, England).

A. Extraction of infectious virus

Virus was extracted from leaf discs, rings, and leaf strips as outlined previously (McCarthy et al., 1972; Coutts, 1978a). Tissue was ground in a mortar with 0.06 M sodium phosphate buffer (pH 7.0) at 4°C (the rate of extraction differed according to the suspected virus content of the tissue). Extracts were clarified by centrifugation (in the MSE centrifuge) at 10,000 g (11,000 rpm) for 10 min. Clarified extracts were assayed on cowpea primary leaves and numbers of local lesions were adjusted for dilution differences.

B. Extraction of viral RNA

Viral RNA from similar tissues to those above was extracted by homogenisation as in the previous section with 5 vol. of water-saturated phenol and 2.5 vol. of 0.06 M sodium phosphate buffer (pH 7.0) at 4°C. The amounts of phenol and buffer used were proportionally reduced with tissue expected to contain small amounts of viral RNA. The emulsion was broken by centrifugation (in the MSE centrifuge) at 10,000 g (11,000 rpm) for 10 min., and the aqueous layer decanted, excess phenol being removed by partition against

diethyl ether. Excess ether was then removed by applying a continuous stream of air. All samples were assayed on cowpea primary laves as before.

8. Infectivity assay

Infectivity of both virus (nucleoprotein) and viral RNA preparations was determined on cowpea primary leaves using a design in which inocula (four tested preparations) were inoculated onto leaves of 6 plants so that each inoculum was compared with every other inoculum on adjacent half-leaves an equal number of times and tested on an equal number of 'right' and 'left' half-leaves. Each comparison was, thus, made once at each leaf position on the plants, and each inoculum occurred once on each test plant (Gibbs and Harrison, 1976). Local lesions were counted 3 days after inoculation and adjusted for any dilution differences.

9. Determination of a suitable concentration of CuSO_4 to cause necrosis

A serial dilution of CuSO_4 solutions (from 50 to 5000 ppm) was prepared in distilled water. A drop of each (approximately 5 μl) was applied to the upper surface of either cowpea primary leaves or Ashley cucumber cotyledons at known positions. One day later the effect of each dilution was noted and a slightly higher concentration than that which resulted in necrosis penetrating the lower leaf surface chosen for further investigations.

10. Mannitol-induced osmotic stress

Leaves were subjected to mannitol-induced osmotic stress by

plasmolysis on 0.5 M mannitol solution (Coutts, 1978a). Leaves were detached from plants, washed in running tap water and finally distilled water, blotted dry and floated, lower surface down, on 100 ml of 0.5 M mannitol solution or distilled water for controls in closed transparent plastic boxes (Coutts, 1978a) and maintained in growth cabinets at $25 \pm 2^{\circ}\text{C}$ with supplementary lighting from warm white fluorescent tubes (Philips, 5 FT 65/80 W, Reflectalite 35) providing a 14 h photoperiod of 2.2 K. lux. Various times after floatation, leaves were removed from both water and mannitol floated batches, blotted dry on Kleenex tissue and either extracted for immediate disc-polyacrylamide gel electrophoresis (disc-PAGE) of soluble proteins or frozen at -20°C until all could be analysed together.

11. Quantification of the γ protein in the soluble protein fraction of Ashley cucumber cotyledons subjected to mannitol-induced osmotic stress

The amount of γ protein was quantified as a percentage of the total amount of extracted soluble proteins. Soluble protein extracts from Ashley cucumber cotyledons subjected to mannitol-induced osmotic stress for different periods of time were electrophoresed in 10% PAG, stained with Coomassie blue, destained, and scanned at 280 nm. (see Section III. 16). The areas under the γ protein peak on densitometer scans were cut out, weighed and divided by the weight of the total area under the whole trace and expressed as a percentage taking the lowest value of absorbancy in the gel as a base line.

$$\frac{\text{Area underneath } \gamma \text{ protein peak}}{\text{Total area underneath the whole trace}} \times 100 = \begin{array}{l} \% \text{ age of } \gamma \text{ protein} \\ \text{in the soluble protein} \\ \text{fraction} \end{array}$$

12. Isolation of protoplasts

Protoplasts were isolated from both cowpea primary leaves and Ashley cucumber cotyledons as before (Coutts and Wood, 1977), following the various treatments outlined above.

A. Isolation of protoplasts from cowpea primary leaves

Protoplasts were isolated from cowpea leaves which had previously been floated on water, or osmotically stressed on 0.5 M mannitol for 2 days. All operations were carried out in a sterilized laminar flow cabinet (Pathfinder Cabinets, Havant, Hampshire, U.K.).

The procedure of protoplast isolation was similar to that of Coutts and Wood (1977).

a) Leaves were surface sterilized by immersion into 5% 'Domestos' bleach, a sodium hypochlorite solution (Lever Brothers, Ltd., Surrey, England, U.K.) with 10% available chlorine. The leaves were then washed in three changes of sterilized 0.5 M mannitol and finally floated on 0.5 M mannitol solution prior to epidermal stripping.

b) The lower epidermis was manually detached from surface sterilized leaves using jeweller's forceps (5 INOX, Idealtek, Switzerland) and placed peeled surface down onto the enzyme mixture in sterilized petri dishes. The enzyme mixture consisted of 0.1% (w/v) macerozyme R10 (Yakult Manufacturing Co., Japan), 0.5% (w/v) cellulase Onozuka R/0 (Yakult Manufacturing Co., Japan), and 0.1% (w/v) bovine serum albumin (BSA) (DBH Chemicals, Ltd., Poole, England, U.K.) in a 0.5 M mannitol solution,

pH adjusted to 5.5 (with 0.1 N NaOH). The enzyme mixture was ~~previously~~ sterilized by filtration through a 0.22 μ m cellulose acetate filter (Millipore (U.K.) Ltd., London) before use. Petri dishes were then sealed with parafilm.

c) Petri dishes were incubated in the dark at 30°C on a reciprocal shaking water bath (25 excursions/min.) for approximately 4 h or until the release of protoplasts was almost complete. Drawing undigested leaf pieces through a wide mouthed plastic pasteur pipette assisted in the release of protoplasts.

d) The protoplasts + leaf debris were then filtered through 77 μ m nylon screen mesh (Henry Simon Ltd., Stockport, Cheshire, U.K.). Protoplasts were collected in sterilized glass centrifuge tubes (2.5 id. x 11 cm, MSE. HR).

e) Protoplast suspensions were then washed with three changes of 0.5 M mannitol and sedimentation in a low-speed centrifugation (in a BTL bench centrifuge) at 200 g for 2 min. Protoplasts were then examined with a light microscope and their viability assessed.

f) Finally, protoplasts were then collected by centrifugation (in the bench centrifuge) at 200 g for 3 min. and the pellets kept at -20°C prior to extraction for disc-PAGE, of the soluble proteins.

B. Isolation of protoplasts from Ashley cucumber cotyledons

Protoplasts were isolated from cucumber cotyledons floated on 0.5 M mannitol, leaf strips from uninoculated apparently healthy half

cotyledons adjacent to the midrib of completely necrotic half leaves and from the corresponding controls. The procedure of isolation was exactly the same as that used in the last section except that the enzyme concentrations were increased to ensure adequate tissue digestion viz. 1% (w/v) macerozyme R10, 2% (w/v) cellulase Onozuka R10 and 1% (w/v) BSA in 0.5 M mannitol solution at pH 5.5. The digestion time was also increased to 5 - 6 h. Leaf strips were surface sterilized prior to protoplast isolation in 70% ethanol for 5 sec, before hypochlorite treatment.

13. Application of actinomycin D

Tissue to be treated with actinomycin D (AmD) was either injected using a hypodermic syringe or immersed into solutions of AmD.

A. Leaf tissue infiltration technique

Tissue was injected with a solution (5 µg/ml) of AmD (Sigma Chemicals, London) using a hypodermic syringe and needle (30G x $\frac{1}{2}$ "; Arnold R. Horwell Ltd., London, U.K.) until the tissue was soaked with the solution.

B. Leaf tissue immersion technique

This technique was used in radioactive labelling experiments (see later, Section III. 21). Leaf discs (6 mm in diameter and a total weight of 0.5 g) were impaled on sterile hypodermic needles (25 x 0.6 mm; Sherwood Medical Industries Ltd., U.K.) and placed into 3.0 ml of AmD solution (10 µg/ml) in bijoux bottles and then covered with aluminium foil to stop photodegradation of the drug. Bijoux bottles were then put

on a rotating stage (T.C. Drum Apparatus Laboratories, and Electrical Engineering Co., Nottingham) and incubated for 4 h at 25°C in a phytotron. Following incubation, leaf discs were washed thoroughly in distilled water and then dried on filter paper. Discs were then ready for radioactive labelling. Control discs were treated in distilled water in exactly the same fashion.

14. Determination of total soluble protein

The Folin-Lowry method, as described by Layne (1957), was used for protein estimation. Reagents prepared were as follows :

- A - 2% (w/v) Na_2CO_3 in 0.1 N NaOH
- B - 1% (w/v) $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ solution and 2% (w/v) potassium-sodium tartarate solution, mixed in equal quantities on the day of use
- C - Alkaline copper reagent : 50 parts of A to 1 part of B
- D - Folin-Ciocalteu reagent diluted 1 : 2 with distilled water

1 ml protein sample and 5 ml reagent C were mixed and kept at room temperature for 30 min. 0.5 ml reagent D was then added and mixed. After a further 30 min. the absorbance of the solution was estimated at 750 nm against a reagent blank. A calibration curve was prepared using crystalline bovine serum albumin solutions (0 - 500 ppm; Koch-Light, Ltd., England, U.K.) each time estimations were made.

15. Extraction of soluble proteins

Soluble proteins were extracted by one of two methods (Van Loon,

1976; Gianinazzi et al., 1977; Coutts, 1978b).

A. High pH method (E_1 , pH 8.3)

In this method, leaf samples were homogenised in an ice cold mortar and pestle at 4°C with 0.1 M Tris buffer (pH 8.3), containing 0.5 M sucrose and 0.3% (v/v) 2-mercaptoethanol. The rate of extraction was 1 g tissue. 1 ml⁻¹ buffer. Homogenates were then centrifuged (in the MSE centrifuge) at 45,000 g (18,000 rpm) for 30 min. and the supernatant (18 K-supernatant) was used as a soluble protein extract (Gianinazzi et al., 1977; Coutts, 1978b).

B. Low pH method (E_2 , pH 2.8)

In this method, tissue was homogenised in an ice cold mortar and pestle at 4°C with MacIlvaine buffer, 0.1 M phosphate-citrate buffer (pH 2.8), containing sucrose and 2-mercaptoethanol as in the previous section. The extraction rate was the same. Homogenates were centrifuged as in high pH method to obtain the soluble protein fraction (Van Loon, 1976; Gianinazzi et al., 1977; Coutts, 1978b).

E_1 and E_2 -soluble protein extracts were either used immediately for disc-PAGE or frozen at -20°C for later analysis.

16. Disc-polyacrylamide gel electrophoresis (Disc-PAGE)

The soluble protein components contained in either E_1 or E_2 extracts were separated by the Disc-PAGE technique of Davis (1964) using a 3% acrylamide spacer-gel containing Tris-HCl buffer (pH 6.7). The resolving sample gel for protein separation contained 10% acrylamide. Gels were cast in glass tubes (7.5 x 0.5 cm. id.), the

large pore spacer-gel being polymerised on top of the small pore resolving sample gel. The running (electrophoresis) buffer was 0.05 M. Tris-Glycine buffer (pH 8.3). 10 μ l of bromo-phenol blue (pH 2.8 - 4.6) solution (0.12% in running buffer) and containing 0.5 M sucrose was first loaded on the spacer-gel as an electrophoresis marker. Aliquots of 50 - 150 μ l of either E₁ or E₂-soluble protein fractions were applied to gels. No addition of sucrose was necessary since the proteins were buffered and contained 0.5 M (i.e. 17%, w/v) sucrose. Electrophoresis was carried out at 4°C using a Shandon Kit (Shandon Scientific Co. Ltd., London, U.K.). A constant current of 2 mA/tube (Shandon Vokam 2541 or LKB 2103 power supply) was applied for about 20 min., thereafter increasing to 4 mA/tube until the bromophenol blue marker was a few millimeters from the end of the gel. After electrophoresis, gels were extruded and treated in one of the following ways :

- A - Stained overnight with 0.03% (w/v) Coomassie blue or Kenacid blue stain in a mixture of methanol : acetic acid : water (5 : 1 : 5, v/v/v), destained in 10% (v/v) acetic acid, and scanned at 280 nm to visualize soluble protein bands.
- B - Stained for carbohydrates using the periodic acid Schiff's reagent (PAS) method, destained and scanned at 550 nm to visualize glycoprotein bands (Section III. 17).
- C - Developed for zymograms of either PO or PPO (Section III. 18).
- D - Scanned at 280 nm, immediately without staining and sliced at places of interest to isolate separate bands for molecular weight determinations by sodium dodecyl sulphate-continuous-PAGE (SDS-cont.-PAGE) as described by

Maizel (1971) and outlined in Section III . 20.

Gels were scanned at the desired wavelength using a Gilford recording spectrophotometer fitted with a gel-scanning attachment and the positions of protein peaks were expressed in Rf. values by relating the distance migrated by the proteins of interest to the distance migrated by bromo-phenol blue band (Rf.) (Van Loon and Van Kammen, 1970).

17. Staining gels for glycoproteins

Following electrophoresis of soluble protein fractions by disc-PAGE, gels were stained to visualize glycoprotein bands using the periodic acid, Schiff's (PAS) reagent method, as modified for gels by Zacharius et al. (1969). Gels were immersed in 12.5% (w/v) trichloroacetic acid (TCA), using 25 ml/gel for 30 min. and then rinsed in distilled water. Gels were then immersed in fuchsin-sulfite stain in the dark for 50 min. and then washed (x 3) with fresh 0.5% (w/v) potassium-metabisulfite for 10 min. each. The gels were then washed overnight in frequent changes of distilled water and stored in 7% (v/v) acetic acid for later scanning at 550 nm. The fuchsin sulfite stain was prepared by mixing 2 litres of distilled water, 16 g of potassium metabisulfite and 21 ml of concentrated HCl and when a solution was attained, 8 g basic fuchsin was added and the mixture stirred for 2 h at room temperature. At that time the solution had a faint colouration. The solution was left to stand for a further 2 h and a small amount of Darca charcoal grade G60 added and the solution filtered within 15 min. The resultant colourless filtrate (Schiff's reagent) was kept at 4°C for several months.

18. Development of peroxidase (PO) and polyphenol oxidase (PPO)
zymograms in polyacrylamide gels

Following electrophoresis of soluble protein fractions by disc-PAGE, bands of protein exhibiting peroxidases and/or polyphenol oxidase activity in gels were detected as follows :

A. Development of PO-zymograms

Gels were immersed in a 1% (w/v) solution of pyrogallol in distilled water for 5 min. This solution was replaced with 0.5% (v/v) hydrogen peroxide (H_2O_2) solution for 3 min. Zones of enzyme activity (PO-isozymes) were visible as yellowish brown bands. Gels were scanned immediately at 520 nm. (Hall, 1967).

B. Development of PPO-zymograms

Gels were immersed vertically in a solution of 0.01 M. L-3,4-dihydroxyphenylalanine (L-Dopa) in 0.1 M sodium phosphate buffer (pH 7.0) in large glass tubes (15 x 2.5 cm. id.), and the solution rapidly aerated with an air-line for 30 min. Zones of enzyme activity (PPO-isozymes) were seen as grey black bands (Van Loon and Van Kammen, 1970).

19. Development of ribonuclease (RNase) zymograms in polynucleotide gels

RNase zymograms were developed in polynucleotide gels using the method described by Van Loon (1975c). Polynucleotide gels (PNG) were prepared by incorporating the potassium salt of polyuridylic acid (poly-U; A grade, Calbiochem-Behring Corp., C.P. Laboratories,

Bishops Stortford, Herts., England, U.K.) into the gel matrix at a final concentration of 0.05% (w/v). The poly-U was dissolved in the aqueous component during preparation of the resolving sample gel of 10% PAG (Poly-U gels = PNG). After electrophoresis, gels were stained overnight in a solution of 1% (w/v) pyronin Y in 7% (v/v) acetic acid, and destained by washing with several changes of 7% (v/v) acetic acid. Finally gels were stored in 7% (v/v) acetic acid until scanning at 550 nm. Control gels were heated at 100°C for 10 min. in 7% (v/v) acetic acid immediately after electrophoresis to inactivate the enzymes.

20. Determination of molecular weight of polypeptide subunits (monomers) of the α , β , and γ fractions

Determination of molecular weight was carried out using the technique of sodium dodecyl sulphate - continuous - polyacrylamide gel electrophoresis (SDS-cont.-PAGE), as described by Maizel (1971), and involved constructing a standard curve using a mixture of standard proteins with known molecular weights.

A. Preparation of the standard protein mixture (marker mixture = M.M.)

The following standard proteins (molecular weight in daltons is indicated between the brackets) were used, cytochrome C, Cyt. C. (12,384); satellite tobacco necrosis virus coat protein subunit, STNV (22,700); tobacco necrosis virus-strain D, TNV_D (30,000); tobacco necrosis virus, strain A, TNV_A (30,000); ovalbumin, OA (47,000); human gamma globulins, light (γ -L) and heavy (γ -H) chains (23,500 and 55,000 respectively); bovine serum albumin, BSA (67,000). A solution of the standard protein mixture was made up in a disruption buffer (DB), 0.01 M sodium phosphate buffer (pH 7.2) containing 1% (v/v) 2-mercaptoethanol, 1% (w/v) sodium dodecyl

sulphate (SDS) and 0.5 M sucrose. 0.12% (w/v) bromo-phenol blue (BPB) was added as a running marker for subsequent electrophoresis. The concentration of the individual standard protein in the final MM - solution was 50 µg/ml each. The MM-solution was heated in a water-bath at 80°C for 2 min. and cooled to room temperature prior to electrophoresis.

B. Extraction of the α , β , and γ fractions for molecular weight determination

The α and the β fractions of cowpea and the γ fraction of cucumber were obtained in relatively large quantities from E_1 -soluble protein extracts of 6-day TNV-infected cowpea primary leaves and 5-day TNV-infected Ashley cucumber cotyledons respectively, by overloading gels with 150 µl of each of the E_1 -extracts during a normal native disc-PAGE run. After electrophoresis, gels were excised at the regions corresponding to α , β and γ fractions which were determined by immediate scanning at 280 nm. Slices containing α , β , or γ fraction were kept at 4°C until the remainder of each gel was stained with Coomassie blue stain for 3 h, destained and examined to ascertain that the correct proteins had been removed and were not contaminated by neighbouring proteins. Similar gel slices were then pooled separately in bijoux bottles and macerated in DB (100 µl/gel slice) using a Teflon, Potters-Elversheim bulb arrangement. Gel pieces were removed by centrifugation (in the MSE centrifuge) at 1,000 g (4,000 rpm) for 10 min. The supernatants containing α , β , or γ fraction monomers were heated in a water-bath at 80°C for 2 min. to ensure complete disruption of the quaternary structure and cooled to room temperature prior to electrophoresis.

C. SDS-cont.-PAGE for molecular weight determination (Maizel, 1971)

The proteins to be analysed by SDS-cont.-PAGE whether standard

protein mixture or α , β , and γ fractions were denatured by the SDS/heat treatment as in the previous section.

Aliquots (50 μ l/gel) of either MM-solution or the unknown molecular weight samples, α , β , or γ were subjected to SDS-cont.-PAGE, using different concentrations of acrylamide (10, 11, 12 and 13%, w/v). 10 μ l of cytochrome C solution (250 μ g/ml DB in which 0.12% (w/v) BPB was dissolved) or 50 μ l MM-solution per gel was applied as a double marker with samples other than MM-solutions whenever necessary. No spacer-gel was used in these systems. 0.1 M sodium phosphate buffer (pH 7.2) containing 0.1% (w/v) SDS was used as a running buffer. Electrophoresis was carried out at 4 mA/gel for approximately 1 h, thereafter increasing to 8 mA/gel until the bromo-phenol blue left the gel tubes. After electrophoresis, gels were extruded and proteins stained overnight with 0.25% (w/v) Coomassie blue in a mixture of methanol : 7% (v/v) acetic acid (1 : 1, v/v), and stored in 7% (v/v) solution of acetic acid until scanning at 280 nm. In these experiments, migration rates were expressed as the ratio of distance migrated by protein bands, whether standard or unknown, to distance migrated by cytochrome C, i.e. relative mobility (Rm.).

21. Radio-active labelling of soluble protein fractions

Leaf discs (6 mm) taken from tissue adjacent to either necrotic half-leaf tissue (AH) or phosphate buffer sham-inoculated half leaves (H), were removed in a parallel line along the mid-rib. A total weight of 0.5 g representing approximately 60 leaf discs was normally taken from both tissue types. The leaf discs were then impaled on sterile hypodermic needles (25 x 0.6 mm; Sherwood Medical Industries Ltd., U.K.) and placed into radio-active solutions in bijoux bottles. Radio-active solutions were made in distilled water, with 3.0 ml of solution per sample. Final concentrations of ^3H or ^{14}C L-leucine were

10 $\mu\text{Ci. ml}^{-1}$ and 1 $\mu\text{Ci. ml}^{-1}$ respectively.

The discs were labelled for 4 h on a rotating stage (T.C. Drum Apparatus Laboratories, and Electrical Engineering Co., Nottingham) at 25°C, with constant illumination of 3 K.lux as in Section III. 13. B. Following labelling, leaf discs were washed extensively in distilled water, dried on filter papers and homogenised in 1.5 ml of cold E_1 -buffer (pH 8.3) at 4°C and centrifuged (in the MSE centrifuge) at 45,000 g (18,000 rpm) for 30 min. at 4°C. The resulting supernatant contained the radio-actively labelled products.

A. Determination of the incorporation of radio-active labelled precursors into total soluble proteins

To determine the incorporation of radio-active precursors into total soluble proteins, the radio activities in acid-insoluble fractions divided by total uptake were calculated, and the ratios between AH and H tissue, determined from these figures.

To determine incorporation into acid-insoluble material, 300 μl of the supernatant was added to 500 μl of 10% (w/v) cold trichloroacetic acid (TCA), left overnight at 4°C, and the precipitates collected on Millipore (HA. 0.45 μm) on glass fibre filters (Whatman, GF/C) were washed with 3 x 5 ml of 5% (w/v) TCA, 2 x 3 ml of 80% (v/v) ethanol, and finally 5 ml of diethyl ether. The filters were dried and then dissolved in 8 ml of scintillant (666 ml toluene, 333 ml absolute ethanol, 4 g PPO, 0.1 g POPOP).

To measure total radio-activity uptake, 50 μl of the supernatant was dissolved in 8 ml of the above scintillant (Tani and Yamamoto, 1978) and 300 μl of soluene 100, plus 10 μl of acetic acid was added to stop the quenching effects of photoluminescence and chemoluminescence caused by soluene (Paus, 1970). All samples from the above were taken in duplicate. Radio-activity (c.p.m.) was counted with a Tracerlab

Coruspectromatic 200.

B. Determination of the specific incorporation of radio-active labelled precursors into protein components in soluble protein fraction by disc-PAGE

a) Single-labelling experiments

Samples from the radio-actively labelled soluble protein fractions were analysed immediately by disc-PAGE (see Section III, 16) on 10% PAG using 150 μ l of extract/gel. Following staining, destaining and scanning, gels were sliced in the areas of interest, using an apparatus similar to the one described by Iandolo (1970).

b) Double-labelling experiments

Samples from the radio-actively labelled soluble protein fractions were similarly analysed by disc-PAGE (see Section III, 16) on 10% PAG using 150 μ l extract (containing 75 μ l of the AH extracts previously labelled with 10 μ Ci. ml^{-1} ^3H L-leucine, and 75 μ l of the H extracts labelled with 1 μ Ci. ml^{-1} ^{14}C L-leucine). Following staining, destaining, and scanning, entire gels were sliced using a commercial apparatus (The Mickle Laboratory Engineering Co., Gomshall, Surrey, England, U.K.).

In both single and double-labelling experiments, gel slices were dissolved in 0.3 ml of soluene overnight at room temperature (Coutts et al., 1975), and then 8 ml of scintillant was added and the whole counted as before. In single-labelling experiments, the ratios of radio-activity between corresponding individual gel slices of both AH and H samples were calculated and compared to densitometer scans. In the case of the double-labelling experiments,

corrections were made for cross-over of counts from the ^{14}C -channel into the ^3H -channel, before results were graphed.

22. Enzyme assays

A. Peroxidase assay

a) Enzyme extraction

Tissue samples were collected at the desired times and frozen at -20°C until all could be assayed together. Tissue was ground in a mortar with ice cold 0.01 M sodium phosphate buffer (pH 7.0) using 1 g tissue / 6 ml buffer. The homogenates were then centrifuged (in the MSE centrifuge) at 45,000 g (18,000 rpm) for 30 min. The resultant supernatants were used as enzyme preparations for the assay of peroxidase activity.

b) Reagents

i) Pyrogallol reagent (made up on the day of use) was prepared by mixing 10 ml of pyrogallol stock solution (0.5 M), 12.5 ml of 0.66 M sodium phosphate buffer (pH 6.0) and 77.5 ml of distilled water.

ii) 1% solution of H_2O_2

c) Procedure

Peroxidase activity was measured spectrophotometrically in a Beckman, model 35 Spectrophotometer (Beckman Instruments, Inc., Fullerton, California, U.S.A.) using a method similar to that described by Loebenstein and Linsey (1961). 1.25 ml of pyrogallol

reagent was placed in a silica glass cuvette and 0.025 - 0.125 ml of enzyme preparation added. The volume of enzyme added was dependent on suspected peroxidase activity. The cuvette was inverted to mix the contents and adjusted to compensate for a reagent blank, minus enzyme. 0.125 ml of 1% H_2O_2 was added rapidly to both samples in the cuvettes, both were inverted twice and immediately replaced in the spectrophotometer. The rise in optical density between 0.2 - 0.4, or 0.6 - 1.0 was then timed, the range selected being dependent on the linearity of the reaction. In such conditions, the activity of peroxidase can be considered inversely proportional to the time required for the change in optical density (i.e. the higher the activity, the less time required for the reaction to alter the optical density). Absolute peroxidase activity values were expressed as $(\text{sec./mg dry weight})^{-1}$ or sometimes $(\text{sec./mg fresh weight})^{-1}$ in 1 ml of the final reaction mixture. The absolute values were then multiplied by a factor for ease of graphical presentation.

B. Polyphenol oxidase assay

a) Enzyme extraction

Tissue samples were harvested at the desired times and frozen at -20°C until all could be assayed together. Tissues were ground in a mortar in ice cold 0.1 M phosphate buffer (pH 6.1) using 1 g tissue/8 ml buffer. The homogenates were then centrifuged (in the MSE centrifuge) at 45,000 g (18,000 rpm) for 30 min. and supernatants were dialysed for 6 h against the same buffer at 4°C in a cold room. The dialysed supernatants were then used as enzyme preparations for the assay of polyphenol oxidase activity.

b) Reagents

A chlorogenic acid solution (2.666 mg/100 ml of 0.1 M sodium phosphate buffer, pH 6.1) was prepared suitable to give 0.02 mg chlorogenic acid/ml in the final 2 ml reaction mixture.

c) Procedure

Polyphenol oxidase activity was measured spectrophotometrically in a Beckman, model 35, Spectrophotometer, using a method similar to that outlined by Van Kammen and Brouwer (1964). 1.5 ml chlorogenic acid solution was mixed in a silica glass cuvette with 0.5 ml enzyme preparation to give 0.02 mg chlorogenic acid per ml in the final 2 ml reaction mixture. Immediately after mixing, the absorbance at 330 nm was measured against a blank containing 1.5 ml buffer (0.1 M sodium phosphate, pH 6.1) and 0.5 ml enzyme preparation without chlorogenic acid. The decrease in the optical density of chlorogenic acid at 330 nm due to polyphenol oxidase activity, was monitored every 30 sec. starting 30 sec. after zero time. The change in the optical density between 30 and 60 sec. was taken as an indication of enzyme activity. Absolute polyphenol oxidase activity values were expressed as (Δ O.D. at 330 nm between 30 - 60 sec./mg dry weight or sometimes fresh weight) in 1 ml of the final reaction mixture.

C. Ribonuclease assay

a) Enzyme extraction

Tissue samples were collected at the desired time and frozen at -20°C as in the previous sections. Tissues were ground

in a mortar with ice cold 0.05 M Tris buffer (pH 7.5) using 1 g tissue/ 4 ml buffer. The homogenates were then centrifuged (in the MSE centrifuge) at 45,000 g (18,000 rpm) for 30 min. and the resultant supernatants used as enzyme preparations for the assay of RNase.

b) Reagents

i) RNA-stock solution : 78.95 mg purified yeast RNA (DBH Chemicals, Ltd., Poole, England, U.K.) was dissolved first in few drops of 0.5 N. NaOH and then 80 ml of 0.05 M sodium-acetate buffer (pH 5.5) added and pH adjusted to normality (pH 5.5) with glacial acetic acid. The solution was then made up to 100 ml, to give the correct concentration, with further addition of buffer.

ii) Reaction termination solution

A solution of 0.3% (w/v) lanthanum nitrate, $\text{La}(\text{NO}_3)_3$, in 2.5% (w/v) trichloroacetic acid was prepared for terminating reactions.

c) Procedure

RNase activity was assayed using a method similar to that described by Udvardy et al. (1969), where the increase in absorption at 260 nm of the $\text{La}(\text{NO}_3)_3$ -trichloroacetic acid (TCA) soluble digestion products of purified yeast RNA following incubation for 30 min. with an enzyme preparation was monitored.

1.9 ml RNA-stock solution was mixed with 0.1 ml enzyme preparation (so that the 2 ml enzyme mixture contained 1.5 mg purified RNA, 100 μ moles of sodium-acetate buffer (pH 5.5), and 0.1 ml of enzyme). The reaction mixture was then incubated

at 37°C for 30 min. and stopped by the addition of 2.0 ml of the reaction termination solution. The resultant mixture was allowed to stand overnight in a cold room at 4°C. It was then centrifuged (in the MSE centrifuge) at 8,000 g (10,000 rpm) for 10 min. and the supernatant diluted (1 : 10) in distilled water. The absorbance of the supernatant was measured at 260 nm., in the spectrophotometer, against a zero-time blank. Absolute RNase activity values were expressed as (O.D. units at 260 nm/mg dry weight or sometimes fresh weight in 1 ml of the final reaction mixture).

23. Dry weight determinations

Representative tissue samples of known weight were dried in an oven (A Gallenkamp & Co., Ltd., London, U.K.) at 60°C until no change in the weight could be detected.

24. Induced (acquired) resistance experiments

Induced resistance caused by TNV against TNV(homologous challenge) was investigated in both cowpea and Ashley cucumber plants. Plants were grown as described in Sections III. 2.(A and B) and at least 8 replicates of each treatment carried out. The inoculum used in these experiments was a clarified virus preparation prepared as outlined in Section III. 3. A. a). The first inoculation is referred to as the 'inducing' inoculation and the second inoculation is referred to as the 'challenge' inoculation.

A. Half leaf experiments

Cowpea and cucumber plants were selected for use before the appearance of the first trifoliate leaves in the former and first true leaf in the later in these experiments. One half of each leaf or cotyledon

of the test plants was inoculated with sufficient virus to induce numerous lesions without any extensive coalescence or collapse of the leaf (inducing inoculation). Control plants were sham-inoculated with carborundum and phosphate buffer alone. Six days after the 'inducing' inoculation, the uninoculated half leaves or half cotyledons in both test and control plants were challenge inoculated with a similar inoculum of TNV (homologous challenge). Some time later, local lesions were counted on each challenged half leaf (3 days) or half cotyledon (5 days) and the sizes of 50 random lesions per treatment were measured using a stereoscopic Orthoplan microscope equipped with an ocular micrometer. Any differences between the means of either local lesion number or size in both test and control plants were compared statistically using the student-t test.

B. Opposite leaf experiments

The experiments with opposite leaves were carried out as described in the above section, the only difference being that test plants were given the 'inducing' inoculation on one whole leaf of the two primary leaves of cowpea or cotyledon in the case of cucumber. Control plants were sham-inoculated with carborundum and phosphate buffer alone. 6 days after the inducing inoculation, the opposite leaves or cotyledons were challenge inoculated with a similar inoculum as in the previous section. Local lesion number and size on the challenged leaves and cotyledons were counted and measured and the results analysed statistically.

C. Upper leaf experiments

In these experiments, cowpea and cucumber plants were selected for inoculation when the respective first trifoliate and first true leaf were beginning to appear on plants. Both whole primary leaves of cowpea and cotyledons of cucumber were then inoculated with a clarified virus

preparation. Control plants were sham-inoculated with carborundum and phosphate buffer alone. Trifoliates of cowpea or first true leaves of cucumber were challenge inoculated 15 days and 9 days respectively after the 'inducing' inoculation. 5 days later, local lesion number and size were estimated and results were analysed statistically as before.

25. Partial Purification of α and β Fractions by Sephadex Gel Filtration

Polymerized dextran gel 'Sephadex' G-50 medium, (Pharmacia, Uppsala, Sweden) was used as the gel matrix in a borosilicate glass column (70 x 30 cm.) fitted at the bottom with an endpiece and a nylon bed supporting net. G-50 medium Sephadex (water regain value 5.0 ± 0.3 ml/g. dry Sephadex; bed volume 9 - 11 ml/g. dry Sephadex; dry particles diameter 50 to 150 μ and with a fractionation range for peptides and globular proteins from 1,500 to 30,000d molecular weight) was swollen in 0.1M TRIS-HCl buffer, pH 8 in a boiling water bath for 1 hour and the slurry (cooled to room temperature) poured carefully into the column. The gel was allowed to settle to a column height of 63 cm. (id. 2.6 cm) to obtain an even sedimentation flow, elution was carried out after the column was filled using 0.1M TRIS-HCl buffer (pH 8.0). The flow rate was adjusted to ≈ 30 drops/min.

The Sephadex column was calibrated by running 7 mls (2% of the column volume) of a mixture of two markers with different molecular weights, Blue Dextran (B.D.), (2×10^6 d.), and 2,4-Dinitrophenol (2,4-D), (184 d.) which gave the probable range for the α and the β fractions. Fractions were collected by an automatic fraction collector (LKB ULTRORAC, Sweden) (10 ml/tube) and the optical density (O.D.) of the fractions was recorded at two wavelengths, 625 nm for (B.D.) and 400 nm for (2,4-D). Ve_1 , the elution volume of B.D. was calculated as 120 ml and Ve_2 , the elution volume of 2,4-D as 390 ml. Thus, the inclusion volume (Vi) was 270 ml ($Ve_2 - Ve_1$).

Seven mls of centrifuged E_2 -extracts of both healthy and 6-day

infected cowpea primary leaves (1 ml E_2 /g. tissue) were applied separately to the column using a pipette and an applicator fitted with a permeable membrane to avoid undue disturbance of column surface and also to prevent any deposition of cell debris. Samples were eluted with 0.1M TRIS-HCl buffer (pH 8.0). The first 120 mls (V_{e1}) was discarded and the second 270 mls (V_i) was collected containing the α and the β fractions. The 270 mls was dialysed over-night against D/water with continuous stirring at 4°C to remove the buffer. The dialysed sample was then lyophilized to concentrate proteins on a lyophilizer (Edwards High Vacuum, Manor Royal Crawley, West Sussex RH10 2LW, England). The resultant powder was dissolved in a small amount of D/water, pooled into a single flask, and re-lyophilized. The final powder was redissolved in 2 mls of E_2 -buffer and kept at -20°C for later studies.

IV. EXPERIMENTAL RESULTS

1. Symptomology and histology of virus-host combinations

The virus-host combinations used in this study were TNV/Xanthi-nc. tobacco, TNV/cowpea, TNV/cucumber, TMV/cucumber and CMV/cucumber. These combinations were chosen to represent three different examples of virus-host interactions.

In the first group, the interaction resulted in a systemic infection, e.g. the CMV/cucumber combination where the virus caused a systemic infection with typical mosaic symptoms on the first and second leaves following inoculation of both cotyledons.

In the second group, the interaction resulted in localised infections associated with a hypersensitive reaction (i.e. necrotic local lesions), e.g. TNV/cowpea (Figs. 1 and 4); cucumber (Figs. 27 and 30) and tobacco. In these combinations the interaction resulted in a localised infection where virus replicates in the initially infected cell(s) and spreads to infect neighbouring cells possibly through plasmodesmata. This involves a limited number of cells juxtapositioning that originally infected at the lesion epicentre. In these hypersensitive hosts the localisation process was associated with a severe response culminating in the death of tissue at the centre of the localised areas producing necrotic local lesions which varied in colour, e.g. dull red in cowpea primary leaves (Figs. 1 and 4) to yellowish grey with a white centre and a narrow yellow halo in cucumber cotyledons (Figs. 27 and 30) or white on Xanthi-nc. tobacco leaves.

In the third group, the interaction resulted in localised infection without necrosis (e.g. TMV/cucumber). In this combination the process of localisation takes place without any visible sign of necrotisation on intact cotyledons. The infected cotyledons were indistinguishable from



Fig. 1. TNV-infection on a cowpea primary leaf showing characteristic necrotic local lesions, 3 days post-inoculation.

controls on visual inspection (see Fig. 38, H and 1N). However, when cotyledons were decolorised and stained with iodine, starch lesions limiting the areas of the localisation were seen on the infected cotyledons not present on controls (see Fig. 38, H and 1N). Examination of starch lesions by light microscopy revealed that starch grains were deposited within the mesophyll cells particularly in those cells at the border of lesions (Figs. 39 and 40).

2. Purification of TNV_D

TNV_D was purified from severely infected cowpea primary leaves, 3 days post-inoculation (Section III, 6). Two methods of purification were utilised.

A. Partial purification

This purification method is described in Section III. 6. A, and the partially purified virus preparation (PPVP) obtained was assayed for infectivity on cowpea primary leaves (Section III, 8). Virus titre varied from time to time but most preparations were highly infectious with dilution endpoints between 10^{-5} - 10^{-4} . PPVP was used for routine inoculations in this study.

B. Further purification

Two methods of purification were employed.

- a) Polyethylene glycol method (Section III. 6. B. a).
- b) Differential centrifugation method (Section III. 6. B. b).

Virus content in the highly purified virus preparation (HPVP) was assayed spectrophotometrically. The polyethylene glycol method of

purification did not give good yields of virus (0.2135 mg/ml) whereas the second method of differential centrifugation gave reproducibly satisfactory yields (4.0 mg/ml) (Fig. 2). HPVP was used for the isolation of viral coat protein and was used for molecular weight determinations by electrophoresis.

3. Distribution of viral particles (v-p) and viral RNA (v-RNA)
in and around TNV local lesions on cowpea leaves

In order to establish whether v-p and/or v-RNA were confined to lesion centres and/or the necrosing cells at the periphery of lesions, infectivity assays were performed. Attempts were made to ascertain the presence or absence of both v-p or v-RNA in the apparently healthy tissue outside of the local lesions. Infectivities of TNV (v-p) and its nucleic acid (v-RNA) extracted from tissue discs and rings at different distances from the centres of the local lesions were assayed on primary leaves of cowpea as described in Sections III. 7 (A, B) and 8.

The results presented in Fig. 3 show that whilst v-p and v-RNA could be found outside of the local lesions, infectivity decreased with increasing distance away from the lesion centres. Infectivity of v-RNA was much less than that of v-p in all samples tested (i.e. 28.8, 13.1, 14.9 and 20% of v-p infectivity respectively at 1.5, 3, 5 and 8 mm distances away from the lesion centres).

4. Searches for infectious viral particles (v-p) and viral RNA (v-RNA)
in uninoculated leaf tissue adjacent to the mid-rib of necrotic
half leaves

Virus inoculum was prepared to elicit total necrosis on half leaves (Fig. 4, AH) and when the necrosis was complete (3 days after inoculation), a sequential series of leaf strips were removed aseptically

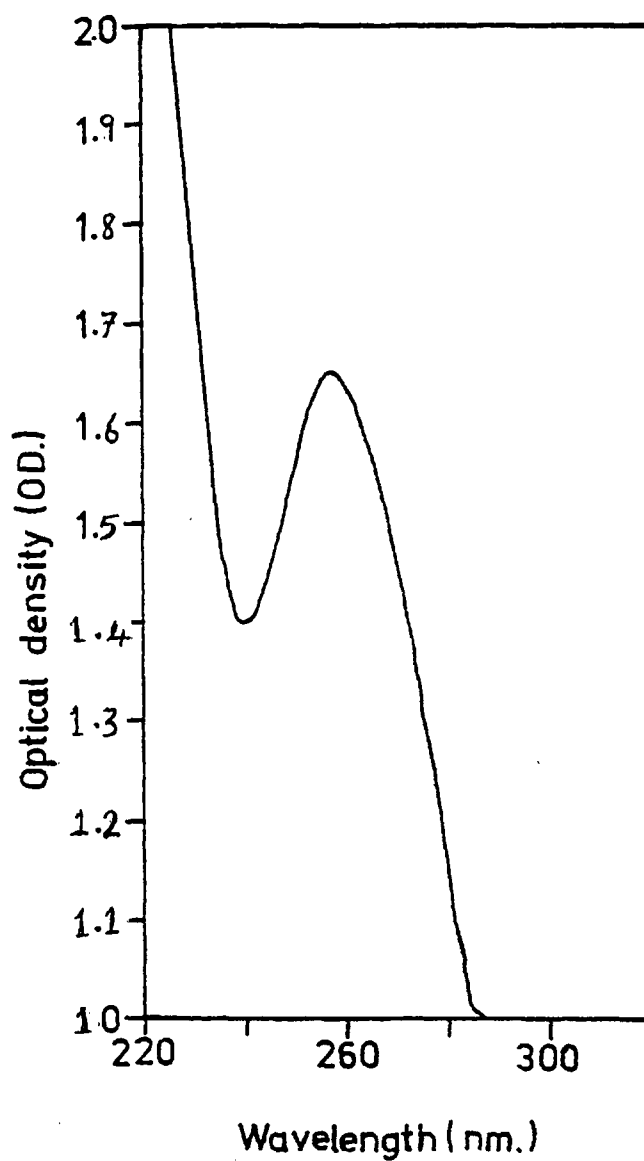


Fig. 2. Ultraviolet-absorption spectrum of a differentially centrifuged purified TNV_D preparation. Virus concentration was found to be 4.0 mg/ml using the extinction coefficient $E_{260}^{1\text{ cm}}, 0.1\% = 5.5$ (Babos and Kassanis, 1963).

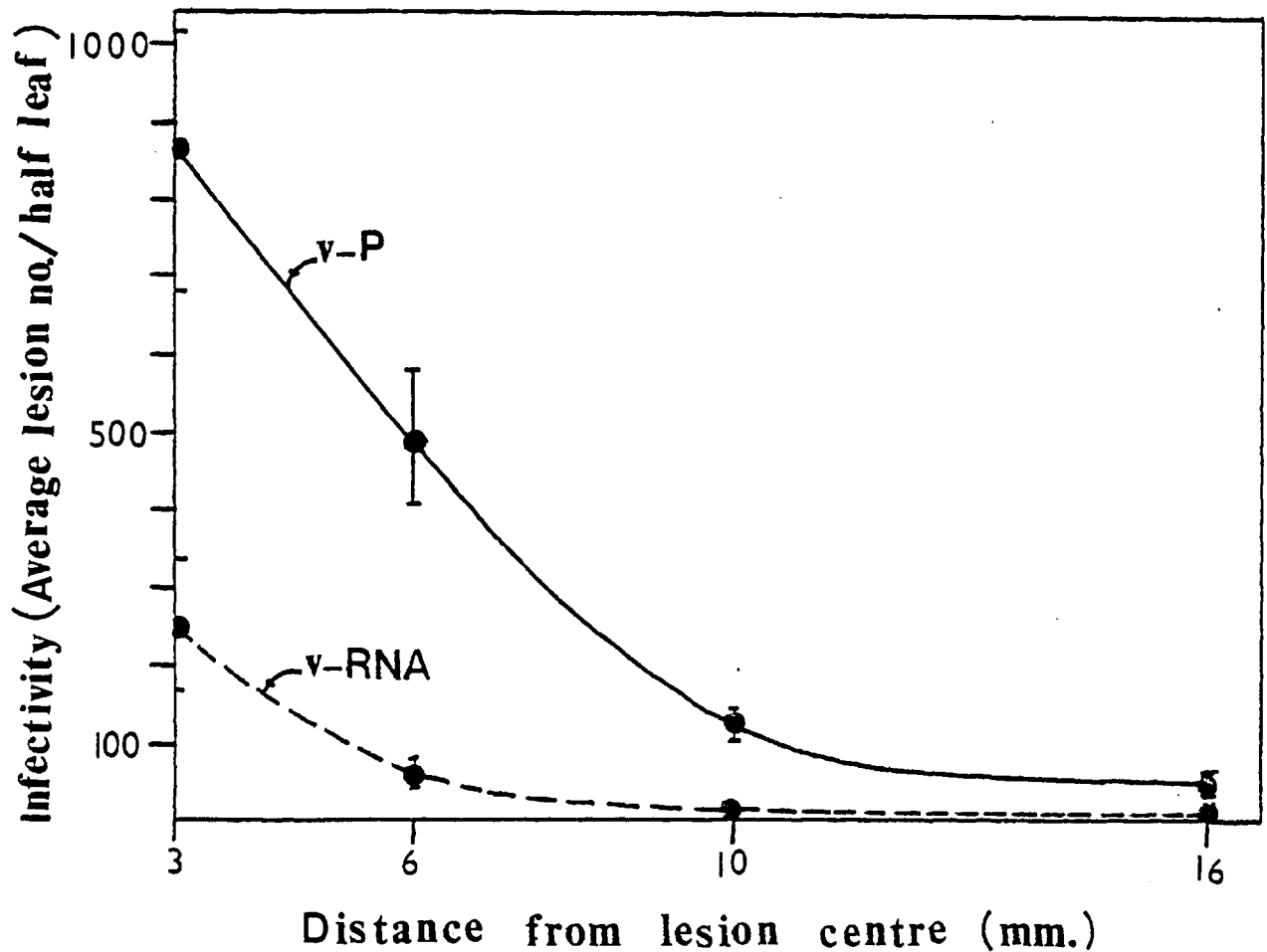


Fig. 3. Infectivities of viral particles (v-p) and viral-ribonucleic acid (v-RNA) in TNV-infected cowpea leaves. The v-p and the v-RNA were assayed in tissue-discs and rings at increasing distances away from single lesion epicentres, 3 days after inoculation.

from the uninoculated halves at different distances (0-3, 3-6, 6-9 and 9-12 mm) away from the mid-rib. The infectivity of both v-p and v-RNA in the strips was assayed as described earlier (III, 7, A, B and 8).

No virus or viral RNA was detectable even in the innermost leaf strips directly adjacent to the mid-rib in the uninoculated half leaves next to totally necrotic half leaf tissue.

5. Electrophoretic studies of soluble protein fractions of healthy and TNV-infected cowpea primary leaves

Soluble protein extracts from both sham-inoculated healthy (Fig. 4, H) and virus-infected (Fig. 4, 1N) cowpea leaves were prepared in either E_1 or E_2 buffers as indicated in Section III, 15, A and B. These extracts were then subjected to native disc-PAGE, and gels stained and scanned as indicated in Section III, 16.

The results showed that two major protein fractions, the α and the β proteins were detectable in both E_1 and E_2 extracts of the infected leaves which were apparently absent from healthy tissue. These proteins had R_f values of 0.87 and 0.74 respectively using the E_2 -extraction method and 0.89 and 0.75 using the E_1 extraction procedure (Fig. 5 and Table 1).

Table 1. R_f values of the α and the β fractions using different methods of extraction

Extraction buffer	Protein fraction	
	α	β
E_1 (pH 8.3)	0.89	0.75
E_2 (pH 2.8)	0.87	0.74

The R_f values indicated in this table represent averages from over 200 estimations



Fig. 4. Cowpea primary leaves, sham-inoculated and TNV-infected, 6 days post-inoculation.

H = phosphate buffer, sham-inoculated, control;

AH = apparently healthy half leaf adjacent to TNV-infected half;

IN = TNV-infected primary leaf with well developed local
necrotic lesions.

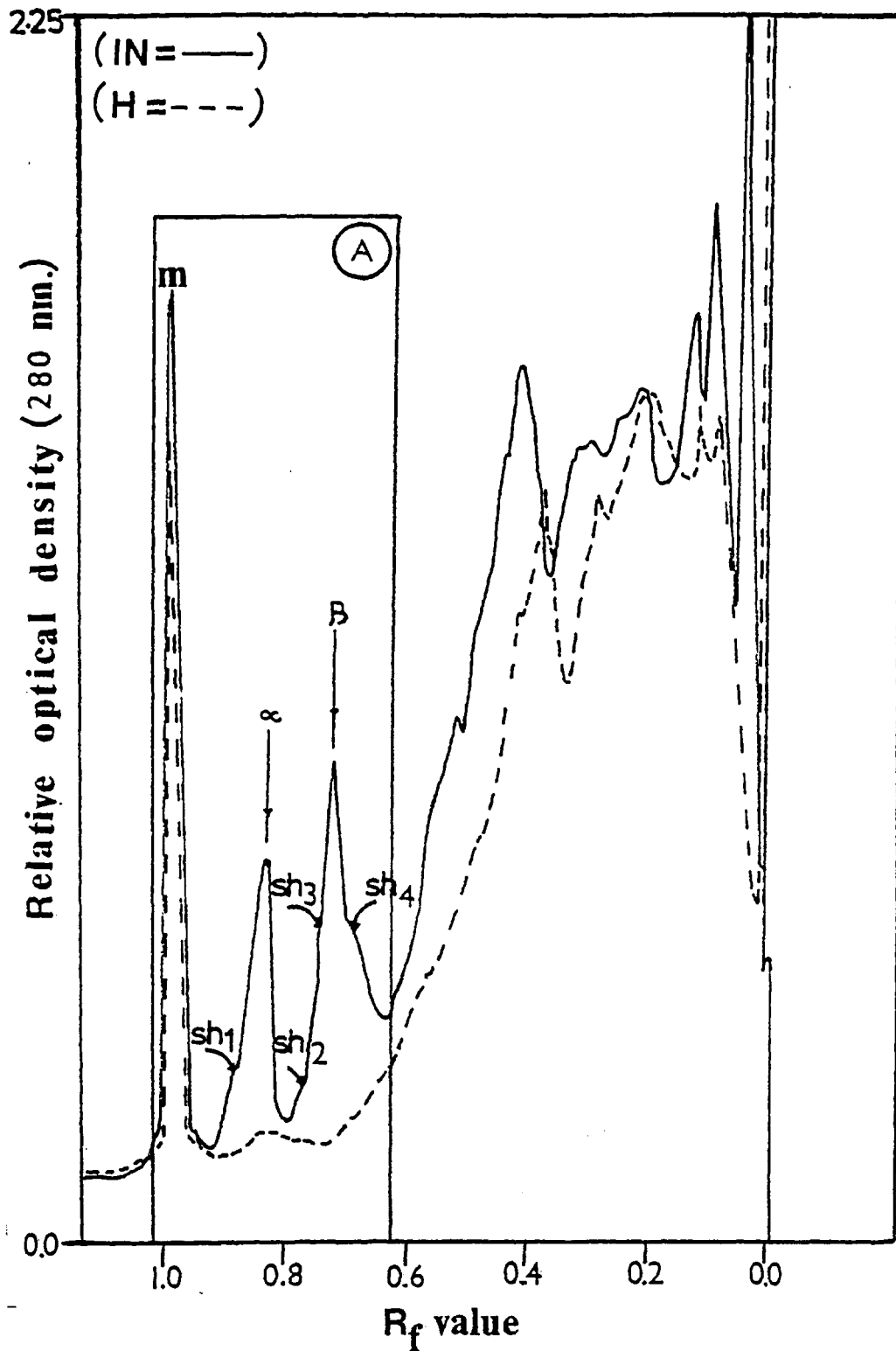


Fig. 5. Densitometer scans of the electrophoretic pattern of E_1 -soluble protein extracts on 10% PAG following staining with Coomassie blue, destaining, and scanning at 280 nm. (---), healthy, sham-inoculated, and (—), TNV-infected cowpea primary leaves, 6 days after inoculation. m, indicates the position of the bromophenol blue marker. Inset A is also shown in Fig. 8 for comparison purposes.

In addition to the two major fractions (α and β), there were four other peaks, some minor, of possibly 'novel' proteins (sh_1, sh_2, sh_3 and sh_4) with respective Rf values of 0.93, 0.83, 0.78 and 0.72 in E_1 -extracts. The same four proteins were also detected in E_2 -extracts at Rf values 0.91, 0.81, 0.76 and 0.70.

It should be noted here that the α and the β proteins were present in large quantities and represented the two major bands of soluble proteins in TNV-infected cowpea primary leaves. The other proteins illustrated above appeared as 'shoulders' (sh) on the α and the β fractions, and were not always found reproducible as evidenced by densitometry.

6. Time course of α and β protein synthesis in TNV-infected cowpea primary leaves

A time course on the production of the α and the β fractions in virus-infected cowpea primary leaves as compared to healthy (sham-inoculated) leaves was determined. Samples taken at 0, 2, 4 and 6 days post-inoculation were extracted in E_2 -buffer, and analysed by native disc-PAGE as indicated in Section III, 16.

From Fig. 6, the results can be summarized as follows :

At 0 (zero) time neither the α nor the β protein was detectable in either healthy or infected leaves (Fig. 6, A). Two days later two main peaks of 'novel' proteins, α and β proteins, were detectable in extracts from infected leaves which were absent from control tissue. These proteins had Rf values of 0.87 and 0.74 respectively (Fig. 6, B). Four days after inoculation these two major peaks were reinforced (Fig. 6, C). At six days post-inoculation the quantities of the α and the β fractions increased and were maximal at this time. Both fractions were associated with shoulder proteins, as mentioned in the previous Section (IV. 5), clearly seen on densitometer scans (Fig. 6, D). No comparable bands to

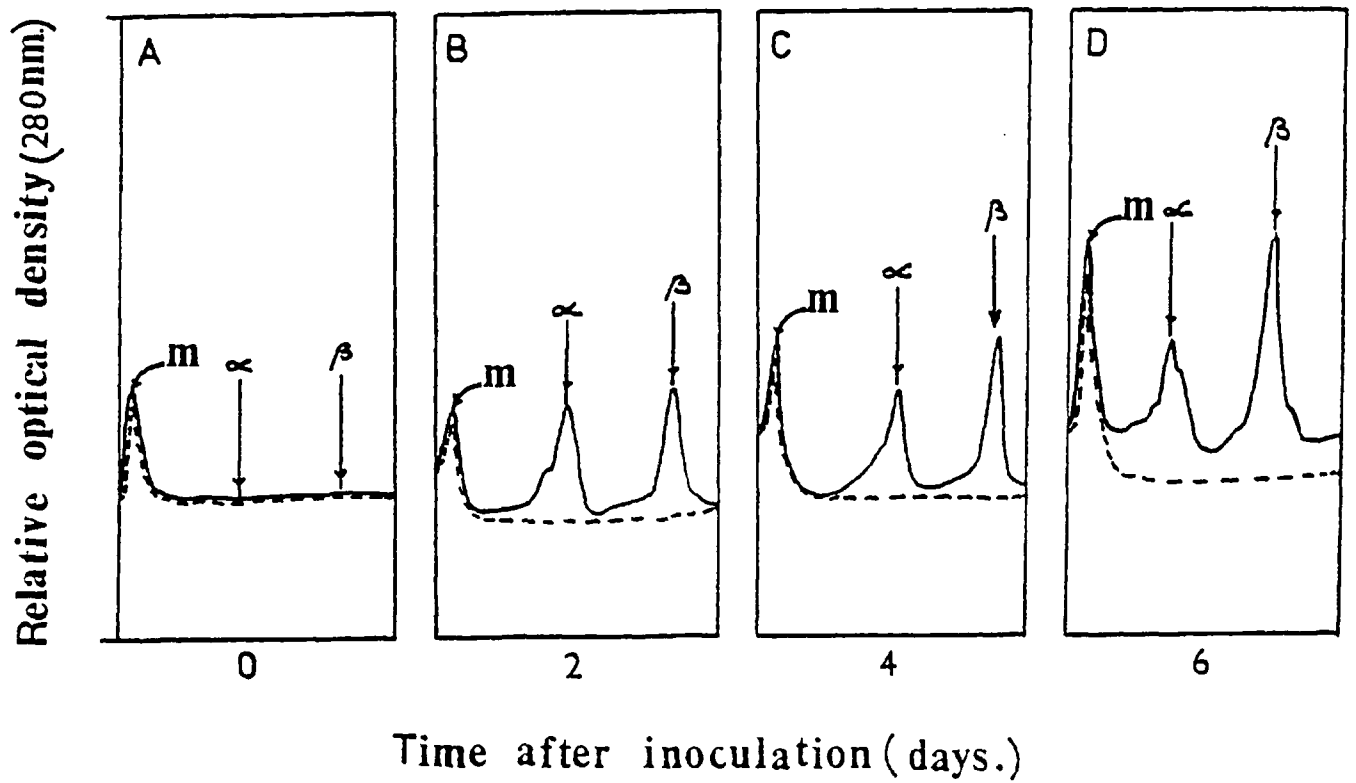


Fig. 6. Changes in the amounts of the α and the β fractions with time after inoculation. Densitometer scans of polyacrylamide gels loaded with 50 μ l of E_2 -soluble protein extracts prepared from either TNV-infected (—) or phosphate buffer, sham-inoculated healthy (---) cowpea primary leaves, at different times post-inoculation.

either the α or the β fractions were observed in extracts from control, buffer inoculated plants (Fig. 6, D).

7. Distribution of the α and the β proteins in and around individual local lesions

Virus inoculum was diluted to elicit only 3 - 5 separate local lesions per cowpea primary leaf. Soluble protein extracts were prepared from a series of concentric rings of tissue, surrounding individual lesions, at increasing distances away from the lesion epicentres. The distribution of the α and the β protein fractions in these tissues was determined as outlined in Section III, 7 with extraction and analysis of soluble proteins by native disc-PAGE (Sections III. 15. A and 16).

The results obtained by both visual inspection and densitometry of gels showed that the α and the β proteins were not equally distributed in and around the local lesions, but their concentration decreased rapidly with increasing distance from the local lesion and this distribution was dependent on the age and size of individual local lesions.

Fig. 7 (a, b, c and d) shows that three days after inoculation the α and the β bands were detected only in the local lesions and the area directly surrounding them (up to 5 mm from the centre, 10 mm total diameter). The fractions were detectable in the innermost discs (3 mm in diameter, including the local lesion), but not in 3 - 6 or 6 - 10 mm rings where they could only be detected by densitometry with difficulty. Notably, they were accompanied by the sh_2 protein (at $R_f=0.82$) at all distances up to 10 mm rings. The α and the β fractions were absent from 10 - 16 mm rings.

Fig. 7 (A, B, C and D) shows similar experiments carried out six days after inoculation, here the α and the β fractions were detected at further distances away from the local lesions (i.e. up to 8 mm from the lesion centre, 16 mm total diameter), but the concentration was lower than

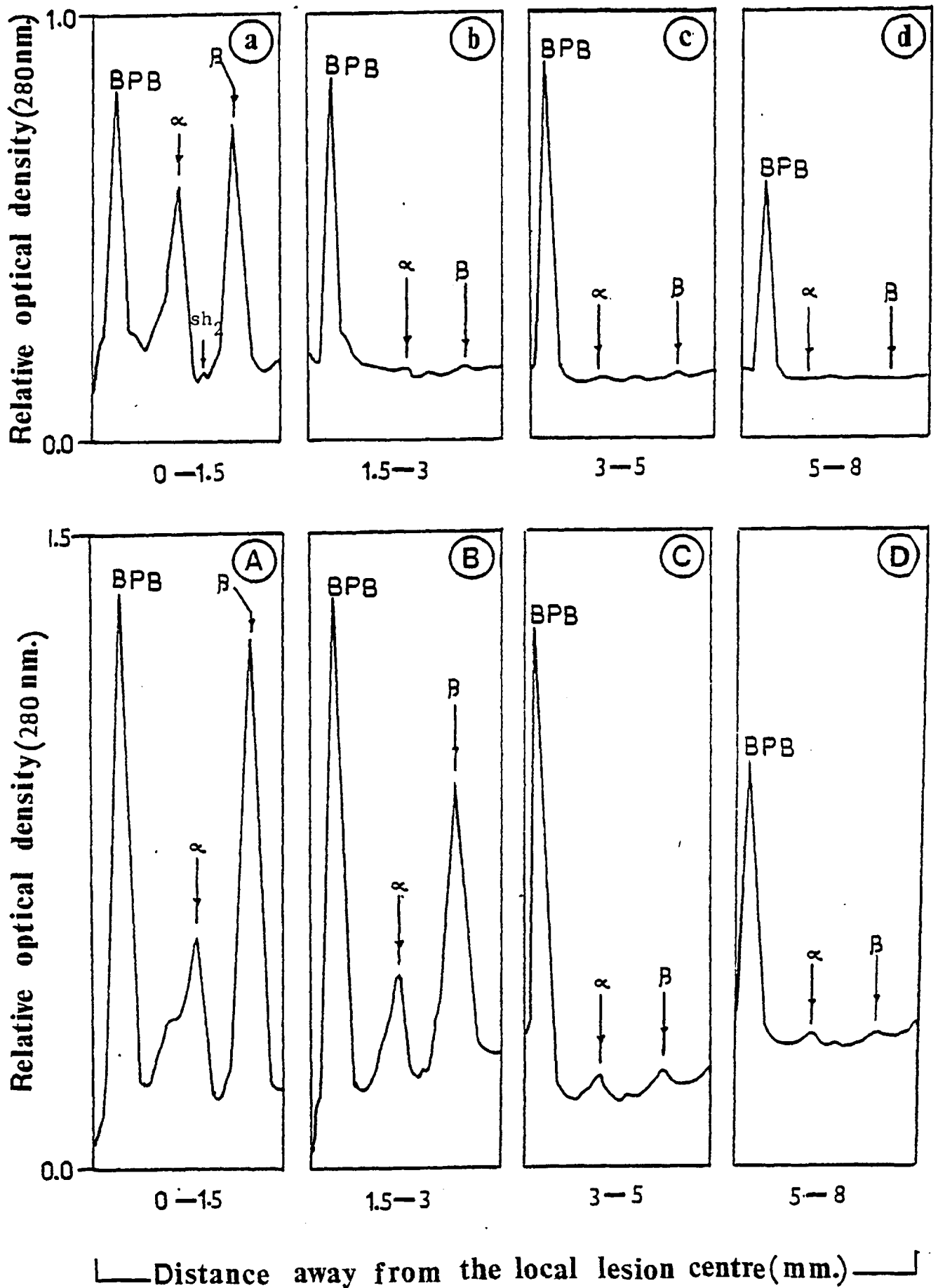


Fig. 7. Densitometer scans of electrophoretic patterns of 50 μ l E_1 -extract of soluble proteins from tissue discs and rings at different distances (0 - 1.5, 1.5 - 3, 3 - 5, and 5 - 8 mm) away from local lesion centres, 3 days post-inoculation (upper series, a - d) and 6 days post-inoculation (lower series, A - D).

that at the actual lesion sites. Again the α and the β proteins were accompanied by the sh₂ protein as before.

8. Searches for the α and the β proteins in uninoculated tissue adjacent and remote from inoculated parts

Extensive searches for the α and the β proteins were made throughout the plant, in uninoculated half leaves adjacent to inoculated halves, in uninoculated leaves opposite to inoculated ones, and in the first trifoliate leaves developed on plants whose primary leaves had previously been inoculated as described in Section III. 4. A.

A. Searches for the α and the β proteins in uninoculated half leaves adjacent to TNV-infected halves

Following the inoculation of half leaves of cowpea primary leaves with either phosphate buffer (sham-inoculated) or TNV, soluble proteins were extracted from a sequential series of leaf strips, taken from the adjacent uninoculated half leaves at increasing distances (0 - 3, 3 - 6, 6 - 9 and 9 - 12 mm) away from the mid-rib as described in Section III. 7 and at different times after inoculation. This experiment attempted to establish a time course of synthesis and distribution of the α and the β proteins in the uninoculated half leaf. All extracts were analysed by disc-PAGE as described in Section III. 16. A.

The results showed that the α and the β fractions were not detectable in measurable quantities in any of the uninoculated apparently healthy (AH) leaf strips (including those directly adjacent to the mid-rib until 4 days post-inoculation of the adjacent half leaf. Thereafter the fractions could be detected in the AH tissue up to 12 mm away from the mid-rib, but decreasing in concentration with increasing distance away from the mid-rib (Fig. 8). Soluble protein extracts contained not

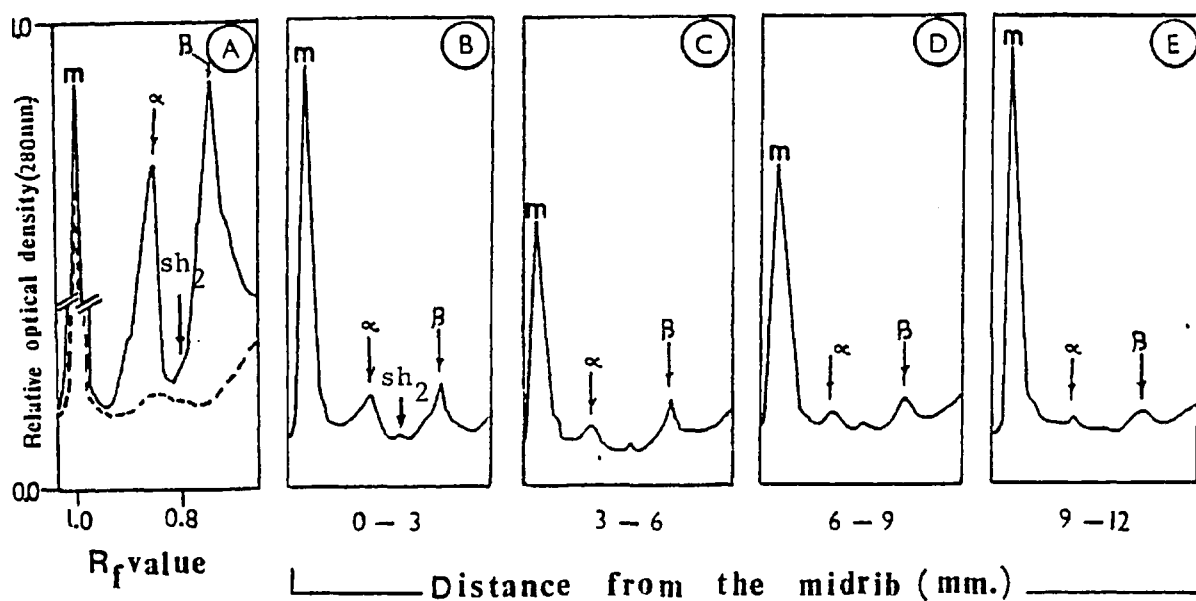


Fig. 8. Densitometer scans of electrophoretic patterns of E_2 -extracts of soluble proteins from leaf strips of uninoculated half leaves at different distances (0 - 3, 3 - 6, 6 - 9, and 9 - 12 mm) away from the mid-rib, 6-days post-inoculation. A is an inset taken from Fig. 5 for comparison purposes.

only the α and the β proteins but also the (sh_2) protein (at Rf 0.82) at least up to 9 mm away from the mid-rib (Fig. 8, B, C and D).

B. Searches for the α and the β proteins in uninoculated leaves opposite to TNV-infected ones and in the first trifoliolate developed on plants with TNV-infected primary leaves

All attempts to find the α and/or the β proteins in tissue remote from the originally inoculated sites at different times (up to 21 days) post-inoculation gave negative results. Even using gels overloaded (150 μ l per gel) with soluble protein extracts prepared from opposite primary leaves, or trifoliolate leaves as above. Negative results were obtained using both densitometry and visual inspection of gels in both cases.

9. Preliminary studies on the chemical nature of the α and the β fractions (the glycoprotein nature)

Soluble protein extracts were prepared from both healthy and TNV-infected cowpea primary leaves, 6 days post-inoculation, and subjected to native disc-PAGE as described in Section III, 16. Sister gels were either stained for total proteins, with Coomassie blue, or carbohydrates by the PAS method as indicated in Section III, 17, and compared by densitometry. Neither the α nor the β fraction or their associated 'shoulder' fractions, stained positively in PAS-stained gels (Fig. 9), and are not glycoproteins. However, several higher molecular weight glycoproteins were seen in cowpeas, quantitatively increased in virus infected tissue (Fig. 9).

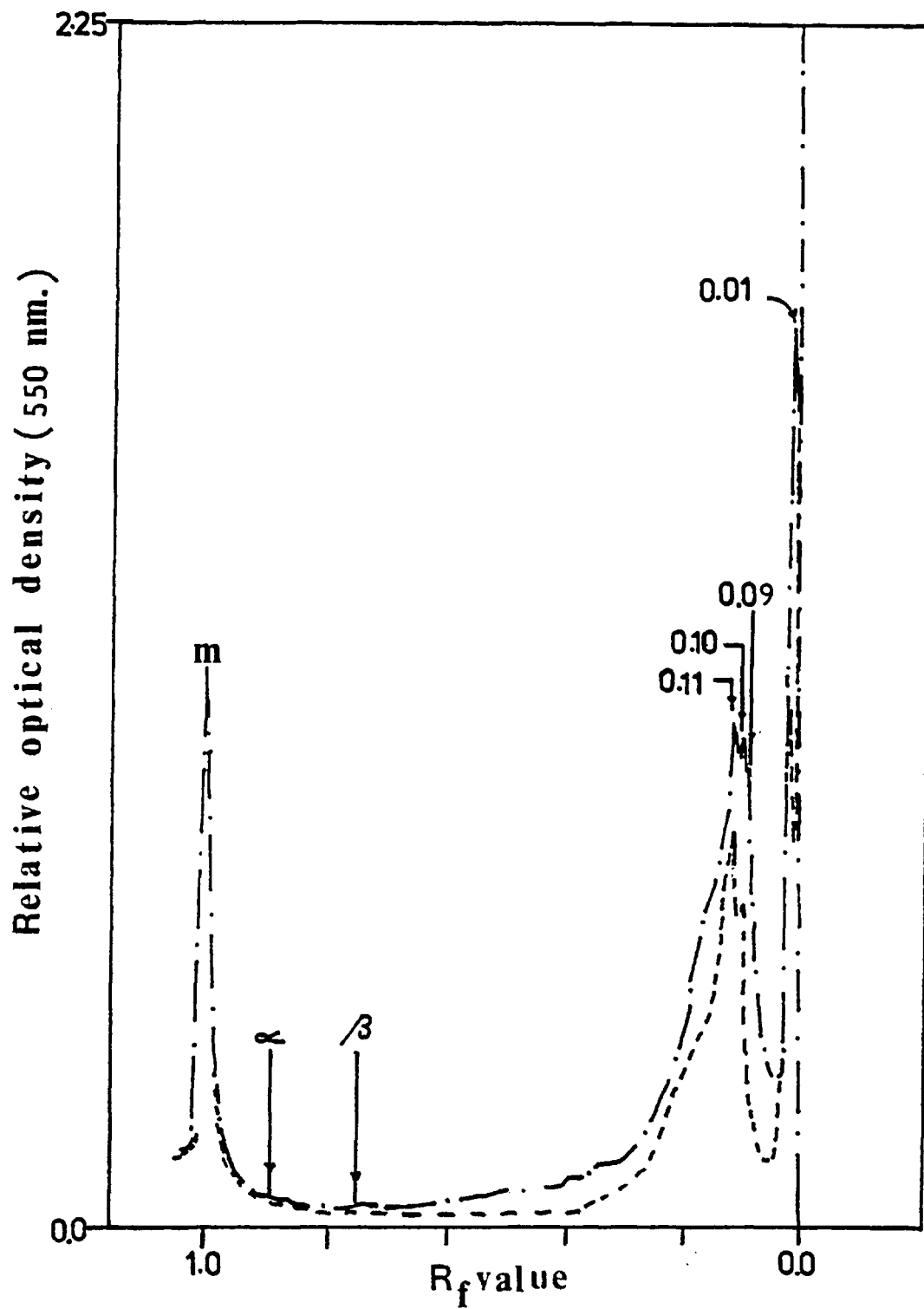


Fig. 9. Comparison of soluble glycoprotein patterns in 10% polyacrylamide gels loaded with 100 μ l of E₁-soluble protein extracts from 6 day TNV-infected (— . —) and sham-inoculated (----) cowpea primary leaves after PAS-staining.

10. Determination of molecular weights of polypeptide subunits (monomers) of both the α and the β protein fractions

Molecular weights were assessed using the sodium dodecyl sulphate (SDS)-continuous (cont.) - PAGE method as outlined in Section III. 20.

A. Construction of a standard curve for molecular weight determinations, using proteins of known molecular weight

The mixture of proteins of known molecular weight mentioned in Section III. 20. A was prepared and subjected to SDS-cont. - PAGE on a series of gels of different acrylamide strength.

Preparation of the protein mixture solution, SDS-PAGE, staining, destaining, and scanning were performed as outlined in Section III, 20. In these experiments, migration rates were expressed as the ratio of distance of protein migration to the distance of cytochrome C migration i.e. relative mobility (Rm.).

When logarithms of the relative mobilities of the polypeptides were plotted against the acrylamide concentrations (as a percentage), the slopes of the lines produced were proportional to molecular size (Fig. 11). A standard, calibration, curve was constructed from the results presented in Fig. 11 and used for further molecular weight estimations (Fig. 12).

B. Determination of molecular weights of the α and the β polypeptide subunits (monomers)

Sephadex gel purification of the α and the β proteins from the soluble protein fraction of TNV-infected cowpea did not satisfactorily separate the fractions. Native gels known to contain large quantities of the α and the β fractions, following the overloading of gels with infected leaf extracts were used as the eventual source of the α and the β fractions. Gel slices known to contain only the α or the β protein

fractions were extracted (checked by staining the remainder of the gels to ascertain the purity of α and β fractions) in a suitable buffer and denatured prior to electrophoresis as described in Section III. 20. B. Samples were then subjected to SDS-cont. - PAGE with cytochrome C or mixed protein markers and analysed as shown in Section III. 20. C.

In the different acrylamide gel concentration, the α protein separated into three bands, one single dense band and two fainter bands. The denser band (α monomer, αm_1 , with Rf value 0.88 on 12% acrylamide gels, Fig. 10, A), and the fainter bands at Rf values 0.22 and 0.27 on 12% acrylamide gels were well separated from one another. In similar gel systems the β protein separated into five bands, two dense bands (β monomers, βm_1 and βm_3 , with Rf values 0.87 and 0.48 respectively on 12% acrylamide gels, Fig. 10, B) were distinct from three fainter bands at Rf values 0.23, 0.27 and 0.64 on 12% acrylamide gels. When the relative mobilities (R_m) of these bands were plotted against the gel concentration (Fig. 11) the αm_1 and the βm_1 gave a single common straight line, whilst the βm_3 gave a straight line with a different slope. The other fainter staining bands, outlined above apart from the one with an Rf value 0.64 (βm_2) on 12% acrylamide gels did not give straight lines, and were discounted as anomalous.

Using the calibration curve in Fig. 12, the estimated molecular weights of αm_1 and βm_1 were the same, 10,000 d. and the molecular weight of βm_3 was 36,000 d. The estimated molecular weight of the band accompanying βm_1 and βm_3 (at Rf 0.64 on 12% acrylamide gel), βm_2 was 25,500 d. (Table 2).

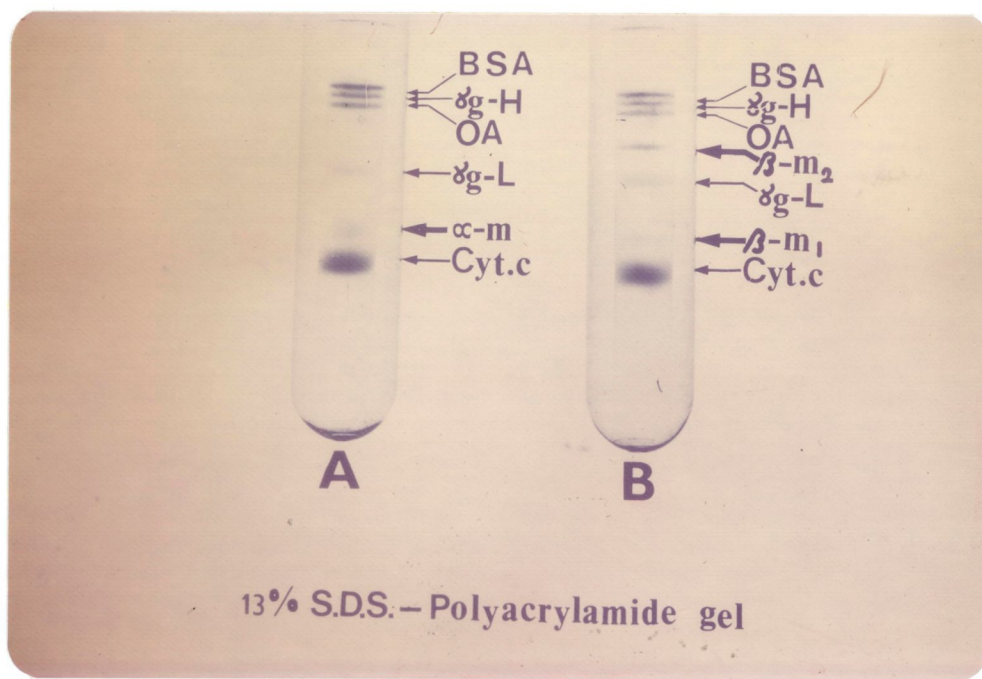


Fig. 10. Determination of the molecular weight of the α and the β polypeptide monomers by SDS-cont.-PAGE. The results using 13% acrylamide gels are presented above. A, was a gel loaded with 50 μ l of a mixture of known molecular weight proteins (cytochrome c, Cyt.c; human gamma globulin, light (γ - L) and heavy (γ - H) chains; ovalalbumin, OA; bovine serum albumin, BSA) + 50 μ l of SDS-denatured α protein fraction; B, was a gel loaded with 50 μ l of the protein mixture as in A + 50 μ l of SDS-denatured β protein fraction. $\alpha m_1 = \alpha \text{ monomer}_1$; $\beta m_1 = \beta \text{ monomer}_1$, and $\beta m_3 = \beta \text{ monomer}_3$. βm_3 is not visibly detectable in the photograph but was resolved by densitometry.

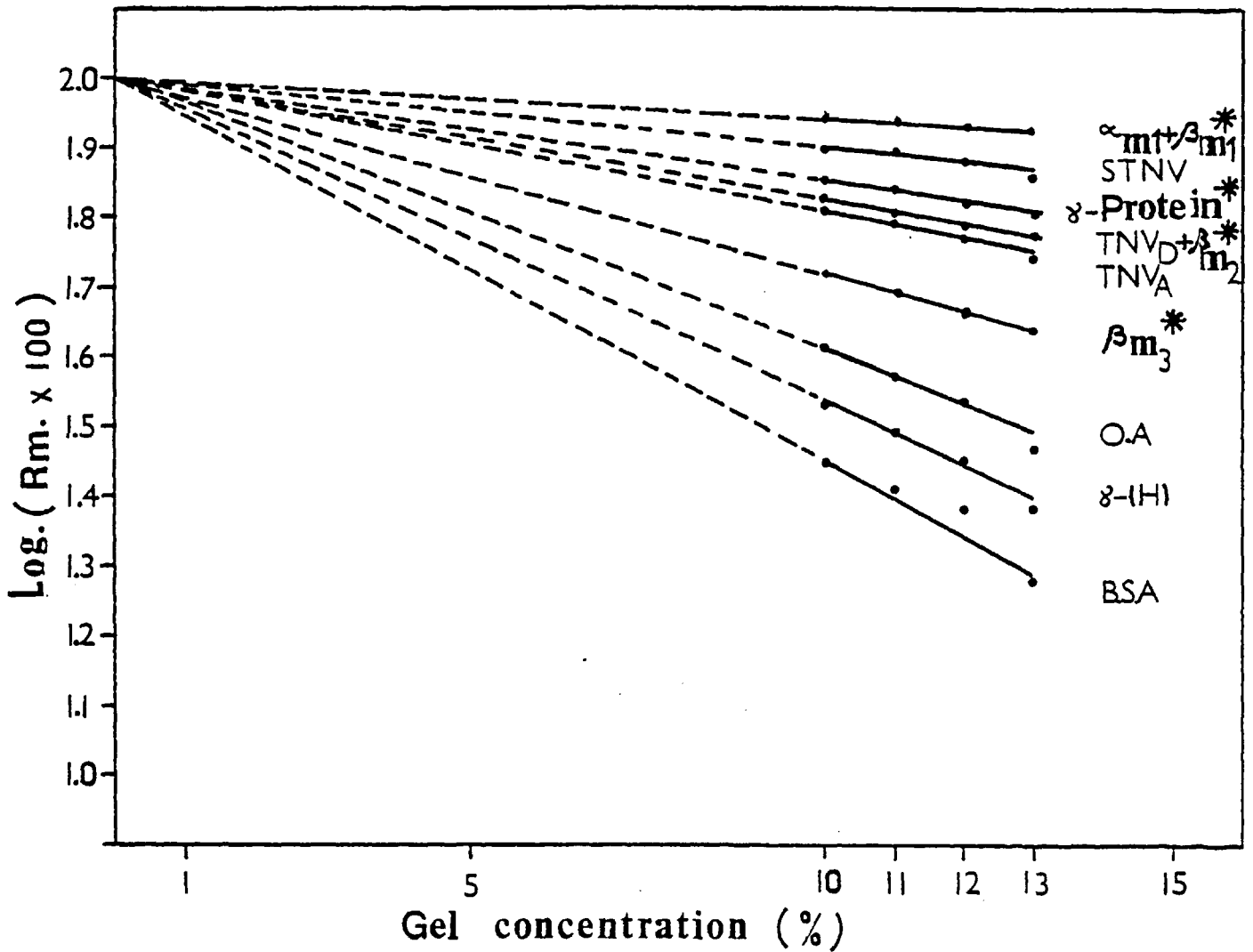


Fig. 11. Shows the relationship between polyacrylamide gel concentration and relative mobilities (Rm) of standard proteins of known molecular weight, bovine serum albumin, BSA; heavy chain human gamma globulin (γ -H), ovalalbumin, OA; light chain human gamma globulin (γ -L) and cytochrome c (Cyt. c). Rm, is the relative mobility compared to Cyt. c. on the same gel concentration. Broken lines represent extrapolated values and Asterisks indicate unknown polypeptides. The γ protein is from TNV-infected cucumber cotyledons to be detailed later in Section IV, 25.

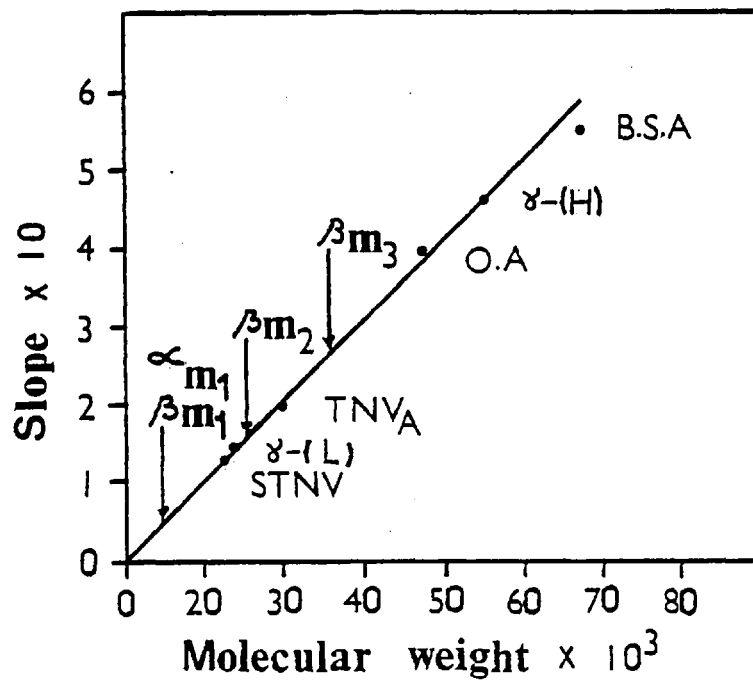


Fig. 12. A calibration curve constructed from the results shown in Fig. 11 where the slopes of the lines are proportional to the molecular weights. Slopes are expressed in arbitrary units and plotted against molecular weights. αm_1 and βm_1 are the α -and β - monomers; βm_2 and βm_3 are the β monomers 2 and 3.

Table 2. Molecular weights of the major 'novel' proteins, α and β , produced in cowpea primary leaves following TNV-infection

Induced proteins	Code for monomer	Molecular weight (daltons) from SDS-gel estimation
α	αm_1	10,000
β	βm_1	10,000
	βm_2	25,500
	βm_3	36,000

11. Quantitative alterations in total soluble protein content in cowpea primary leaves

Investigations were made on the quantitative changes in total soluble leaf proteins in TNV-infected leaves (IN), TNV-infected half leaves ($\frac{1}{2}$ IN) and uninoculated apparently healthy half leaves (AH), adjacent to the ($\frac{1}{2}$ IN) leaves at various times after inoculation. Comparisons were made with healthy cowpea primary leaves, all being extracted in E_2 buffer as described in Section III. 15. B.

Fig. 13 summarizes the results and shows that both the ratios of total soluble protein in whole leaves, infected with TNV compared to healthy tissue (IN/H) and those of infected half leaves to healthy tissue ($\frac{1}{2}$ IN/H) to be similar over the time course studied. Two days post-inoculation both the (IN/H) and the ($\frac{1}{2}$ IN/H) ratios decreased from 112.04% at 0 time to 94.71% for (IN/H) and to 95.36% for ($\frac{1}{2}$ IN/H). Four days post-inoculation ratios increased slightly to similar values of

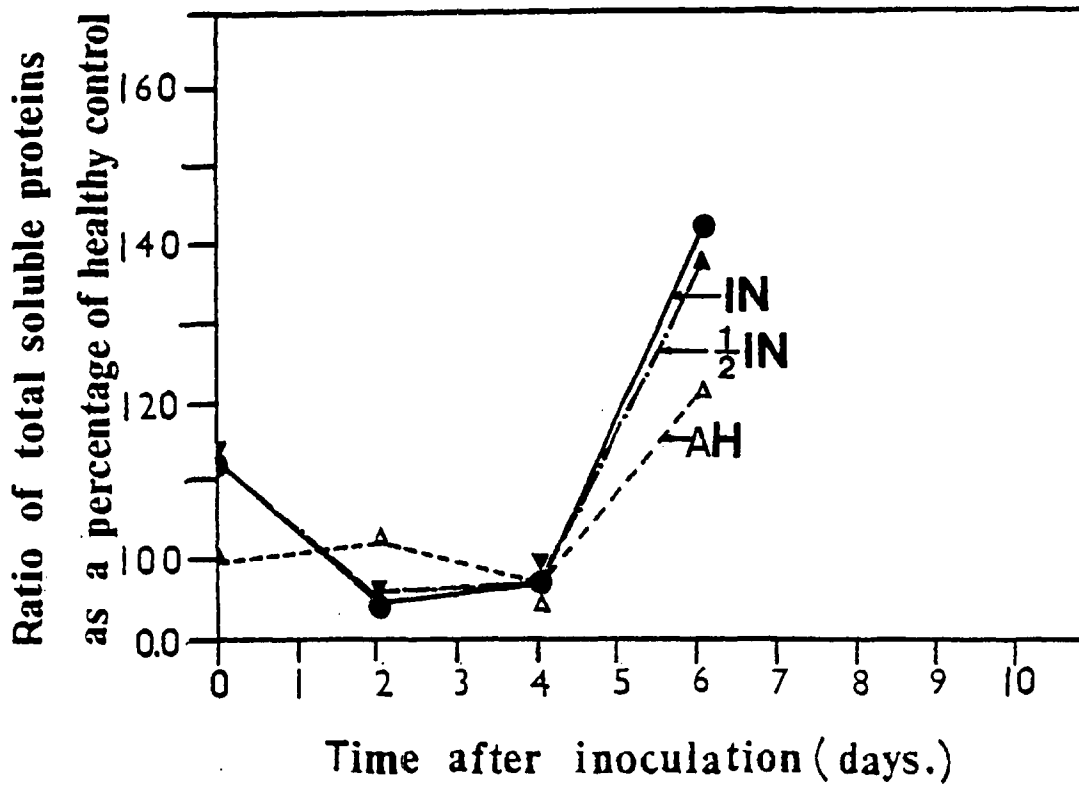


Fig. 13. Changes in total soluble protein content in TNV-infected cowpea primary leaf tissue, (IN = infected whole leaves; 1/2 IN = infected half leaves; AH = uninoculated, apparently healthy half leaves adjacent to completely necrotic halves. Changes are expressed as percentages of the soluble protein content in control healthy tissue at different times 0, 2, 4 and 6 days post-inoculation.

96.29% and 97.43% respectively in the (IN/H) and ($\frac{1}{2}$ IN/H) combinations. Six days post-inoculation both ratios increased to their maximal values of 140.9% for the (IN/H) combination and 137.64% for the ($\frac{1}{2}$ IN/H) combination.

When alterations in the soluble protein content of the (AH) tissue were monitored by comparing ratios of the protein content of (H) tissue, only a slight increase in the ratio was seen 2 days post-inoculation (103.44%). This was not significantly different from the ratio determined 2 days later (96.98%). The only significant difference was seen six days post-inoculation of the adjacent half leaf, when the (AH/H) ratio increased to 120.80%.

12. Radio-labelling the soluble protein fraction of cowpea primary leaves

Attempts were made to measure protein synthesis in the apparently healthy tissue (AH) adjacent to TNV-infected cowpea half leaves at various times after inoculation, by monitoring the incorporation of radio-labelled precursors of protein (^3H or ^{14}C L-leucine) into TCA-insoluble products by methods outlined in Section III. 21. A. Concurrently, the soluble proteins in this tissue were analysed by disc-PAGE in an effort to determine whether the precursors were being specifically incorporated into the α and/or the β protein fractions as described in Section III. 21. B. a.

A. Alterations in protein synthesis in uninoculated half leaves (AH) adjacent to completely necrotic halves

Alterations in the synthesis of proteins in AH tissue were calculated by determining ratios of incorporation, as compared to controls.

Ratios were calculated as outlined in Section III. 21. A.

Results presented in Fig. 14 show that whilst the ratios of ^{14}C L-leucine incorporation in (AH) tissue increased 4 to 7 days after adjacent half leaf inoculation, those changes were not significantly different from earlier samples. Ten days after inoculation, significantly less protein synthesis was routinely observed in AH tissue.

B. Attempted single radio-active labelling of soluble proteins in apparently healthy (AH) cowpea half leaves adjacent to necrotic halves, as analysed by disc-PAGE

Leaf discs from both (AH) and control tissue were radioactively labelled with either ^3H or ^{14}C L-leucine 6 or 7 days post-inoculation of the adjacent half leaf with TNV as described in Section III, 21. Labelled soluble proteins were extracted from this and control tissue and analysed by native-disc-PAGE. Gels were then sliced and the radio-activity in slices determined and compared.

The results in Fig. 15 illustrate that the AH tissue seven days post-inoculation of the adjacent half leaf contained detectable amounts of both the α and the β proteins, as adjudged by normal protein staining of native gels. The α and the β fractions were absent from control tissue. Gel slicing showed that both ^3H and ^{14}C L-leucine are preferentially incorporated into fractions corresponding to both the α and the β fractions (Fig. 15, A, only ^3H incorporation shown), there was also incorporation into other proteins (Fig. 15, A) which have not been previously mentioned in this study.

C. Single radio-active labelling of soluble proteins in AH tissue, following actinomycin D treatment

Leaf discs from AH tissue were taken as in the above section and

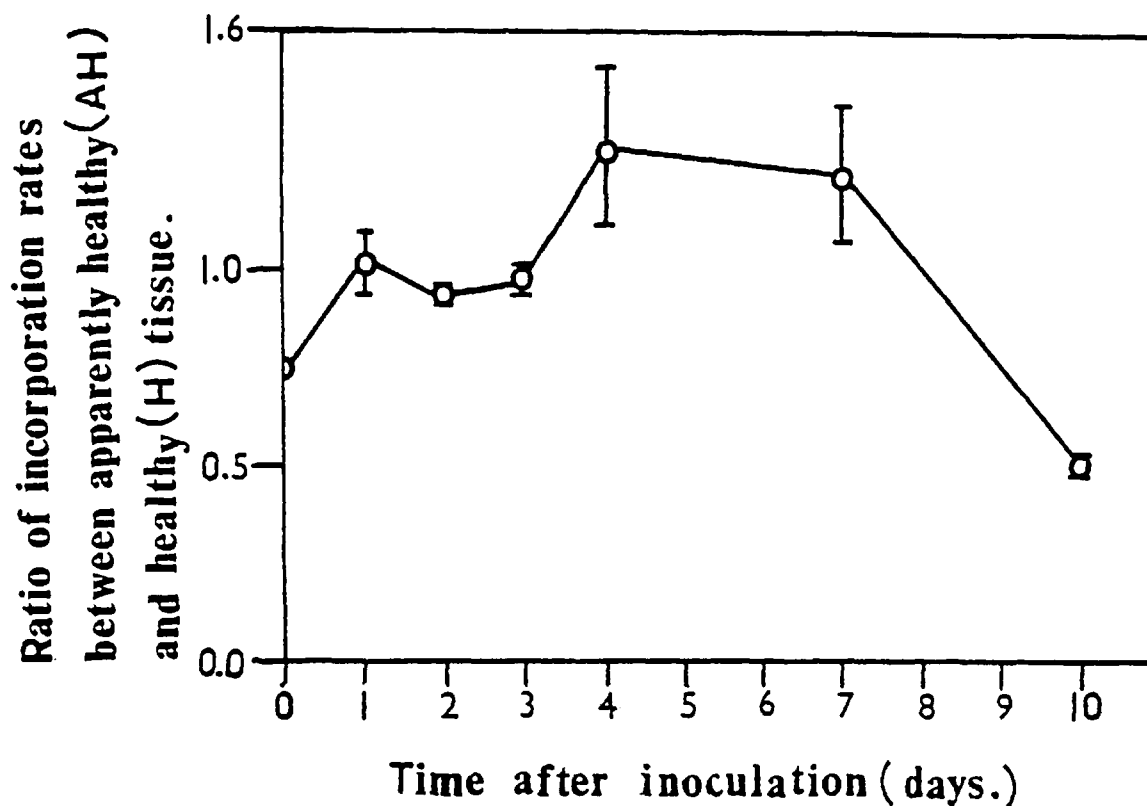


Fig. 14. Protein synthesis in the apparently healthy tissue (AH) adjacent to TNV-infected cowpea half leaves at various times after inoculation. Protein synthesis was monitored by measuring the rate of incorporation of ^{14}C L-leucine into acid-insoluble fractions of AH tissue related to that in H-tissue. Data are the means of five experiments and are expressed as a ratio between acid-insoluble and total uptake counts, calculated as in Section III, 12. A. Error bars indicate standard errors of counts.

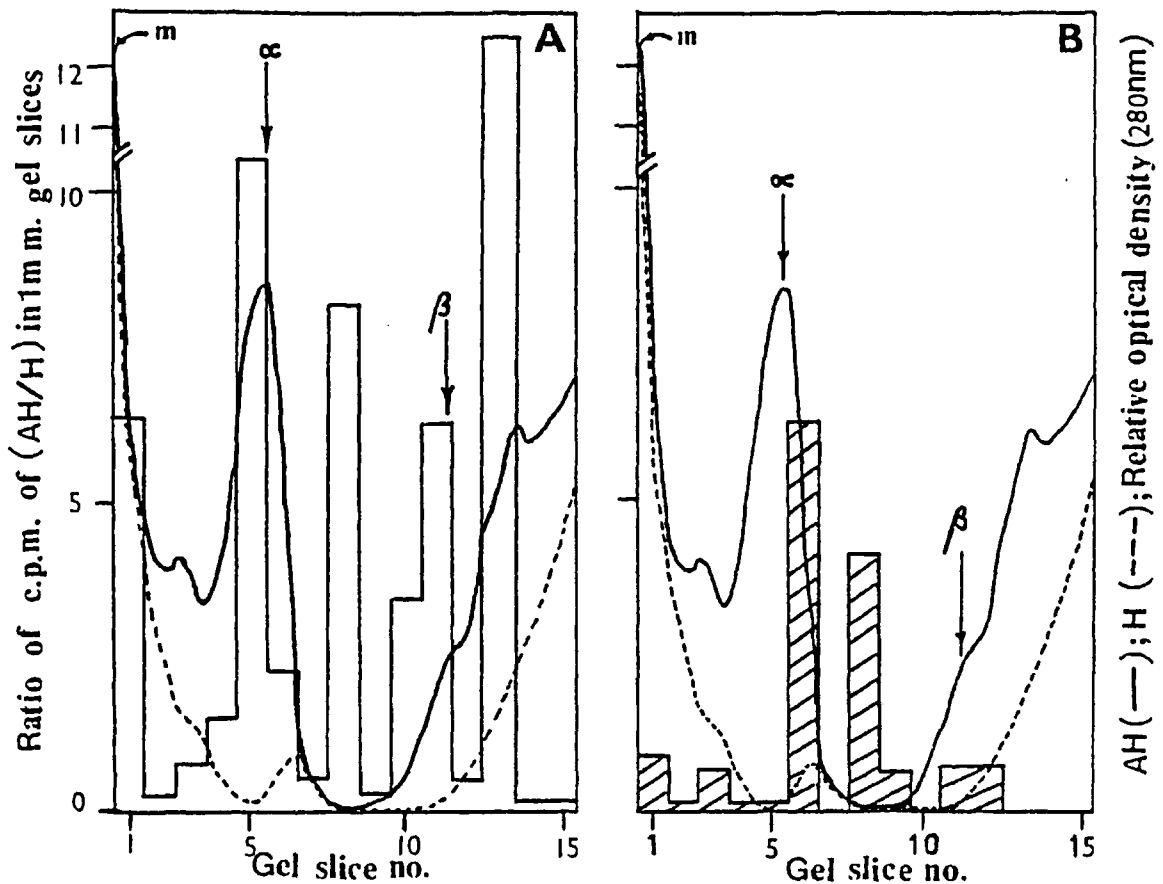


Fig. 15. Radio-labelling of the α and the β proteins as analysed by densitometry and gel slicing of 10% PAG, using soluble protein extracts from healthy (H) and apparently healthy (AH) leaf discs adjacent to the mid-rib of necrotic half leaves. Solid lines (—) indicate densitometer traces of gels loaded with AH tissue extract, whilst the stippled lines (---) refer to H tissue. White histograms (A) are c.p.m. ratios, between AH/H counts in individual 1 mm gel slices. Cross-hatched histograms (B) are similar ratios for extracts from similar tissues treated with actinomycin D for 4 h before labelling.

prior to labelling with either ^3H or ^{14}C L-leucine were treated with actinomycin D (AmD) as described in Section III. 13. B. Soluble proteins were then extracted and analysed by native disc-PAGE as outlined in Sections III. 16 and 21, B. a.

The results in Fig. 15 (B) show that whilst the total amounts of the α and the β fractions were unchanged as adjudged by total protein staining of gels, some AmD sensitivity of the proteins was observed during radiolabelling. The β protein fraction was completely AmD sensitive, while the α fraction was not. Indeed, one protein to the right of the main α peak was actually enhanced by AmD treatment, the other labelled proteins mentioned in the previous section showed both sensitivity and insensitivity (Fig. 15, B). However, these results were not reproducible particularly using ^{14}C L-leucine labelling (results not shown).

D. Attempted double radio-active labelling of soluble proteins in the AH cowpea tissue as compared to H tissue

As in the previous section leaf discs from both AH and control tissue were removed and differentially labelled with either ^3H or ^{14}C L-leucine as described in Section III. 21. B. b. Labelled soluble proteins from each were extracted and analysed by co-electrophoresis in native disc-PAGE (Section III. 16). Gels were then sliced and the radioactivity in individual slices determined (Section III. 21. B. b).

The results presented in Fig. 16 are ratio plots of radioactivity between ^3H and ^{14}C L-leucine counts in AH and H tissue respectively. Where the ratio is unity a similar rate of synthesis of a particular protein in both tissue types is suspected. Ratios above unity indicate a higher rate of synthesis of a protein in AH tissue, whilst the converse is true when the ratio falls below unity.

Fig. 16 (A) shows that in the AH tissue there was a general enhancement of protein synthesis particularly in those proteins found in

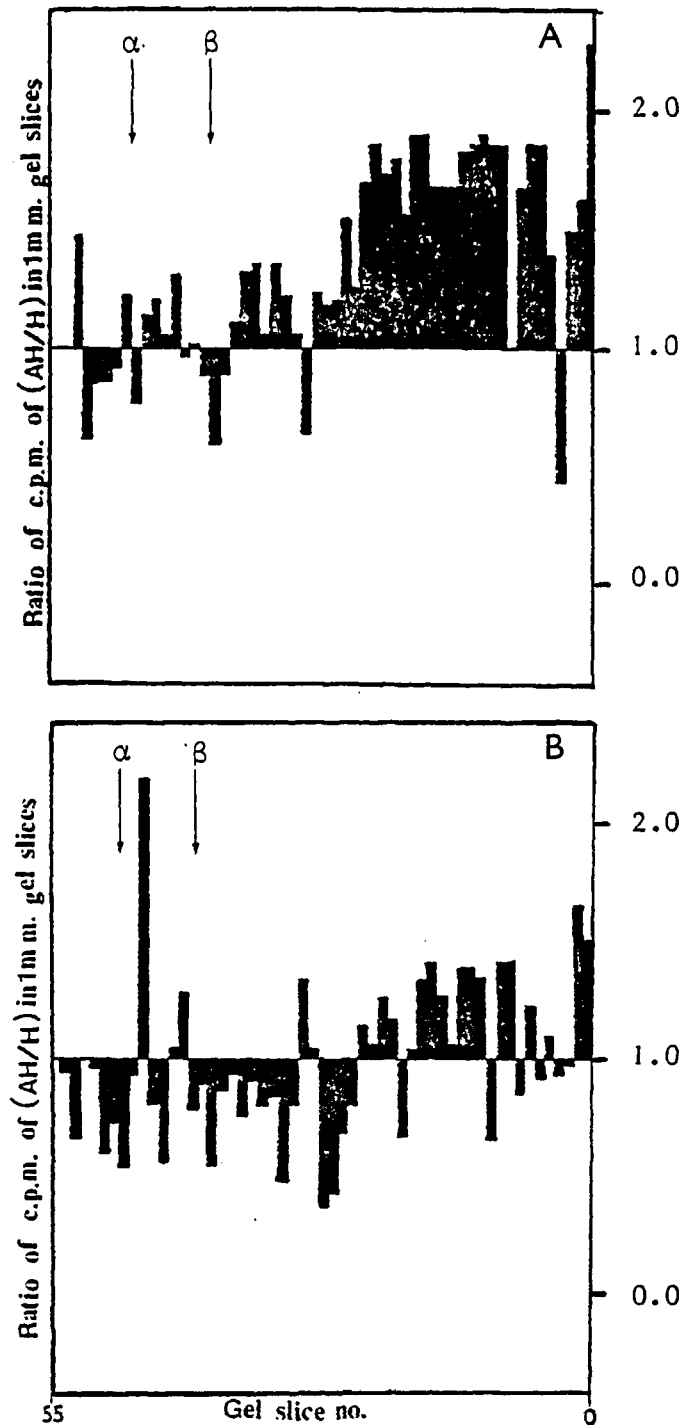


Fig. 16. Double labelling experimental data from AH and H leaf discs. In A, AH leaf discs were labelled with 10 $\mu\text{Ci/ml}$ ^3H L-leucine, whereas H leaf discs were labelled with 1 $\mu\text{Ci/ml}$ ^{14}C L-leucine. B is similar to A but both AH and H leaf discs were treated with 10 $\mu\text{g/ml}$ actinomycin D for 4 h prior to labelling. Histograms are c.p.m. ratios, between AH/H counts in individual 1 mm gel slices. Horizontal lines indicate a ratio of unity.

the first half of the gel. In the other half of the gel both increases and decreases in different gel slices were noted. There was no apparent enhanced synthesis of either the α or the β fractions in the AH tissue, at least using a 4 h pulse, and tissue 6 days post-inoculation. Conversely, decreases in the synthesis of proteins at the α and the β fraction positions were observed in AH tissue.

E. Double radio-active labelling of soluble proteins in AH cowpea tissue as compared to H tissue following actinomycin D treatment

Similar experiments to those above were also performed where the leaf discs were exposed to AmD prior to labelling and subsequent disc-PAGE, gel slicing analysis (Sections III. 13. B and 21. B. b).

The results presented in Fig.16 (B) show that prior treatment of the tissue with AmD inhibited the synthesis of those proteins fractionated in the first half of the gel. A similar inhibition was seen in the other half of the gel, but at least one protein (at gel slice no. 46) was apparently enhanced by AmD, this protein may well have been identified earlier in the 'single'-labelling experiments (see earlier, Fig. 15). No evidence of synthesis of either α or β protein fractions was found in the AH samples, treated with AmD prior to labelling. Moreover, the converse appears to be true.

13. Induction of the α and the β proteins in cowpea primary leaves by abiotic agents

Attempts were made to induce the α and/or β proteins by agents other than TNV. Two methods of induction were attempted, using either copper sulphate (CuSO_4) or 0.5M mannitol treatment.

A. Treatment with CuSO_4

CuSO_4 is known to imitate the hypersensitive reaction in plants by producing necrosis when solutions are applied to leaves. Suitable concentrations of a CuSO_4 solution which elicited the above response on cowpea leaves were determined as outlined in Section III. 9.

Droplets of CuSO_4 (500 ppm concentration) were then applied to leaves, using water droplets as controls and the leaves maintained for 3 days after treatment. Soluble protein extracts were prepared from the above leaves and subjected to disc-PAGE analysis as before (Sections III, 15, A and 16).

When densitometer scans of gels loaded with soluble protein extract from water or CuSO_4 -treated leaves were compared, no differences were noted (Fig. 17) and it was clear that neither α nor β was elicited by CuSO_4 treatment.

B. Plasmolysis in mannitol (0.5 M)

Healthy cowpea primary leaves were detached and floated on either distilled water or 0.5 M mannitol solution for 3 days as outlined in Section III. 10. Soluble protein extracts were prepared from the above leaves and subjected to disc-PAGE analysis as before (Section III. 16).

Fig. 18 summarizes the results and shows that mannitol-induced osmotic stress apparently enhanced the overall soluble protein content of leaves with several novel proteins at R_f values 0.75, 0.80 and 0.84 being found. None of these bands was found to be coincident with the α protein whilst the band at R_f value 0.75 may be similar to the β protein fraction.

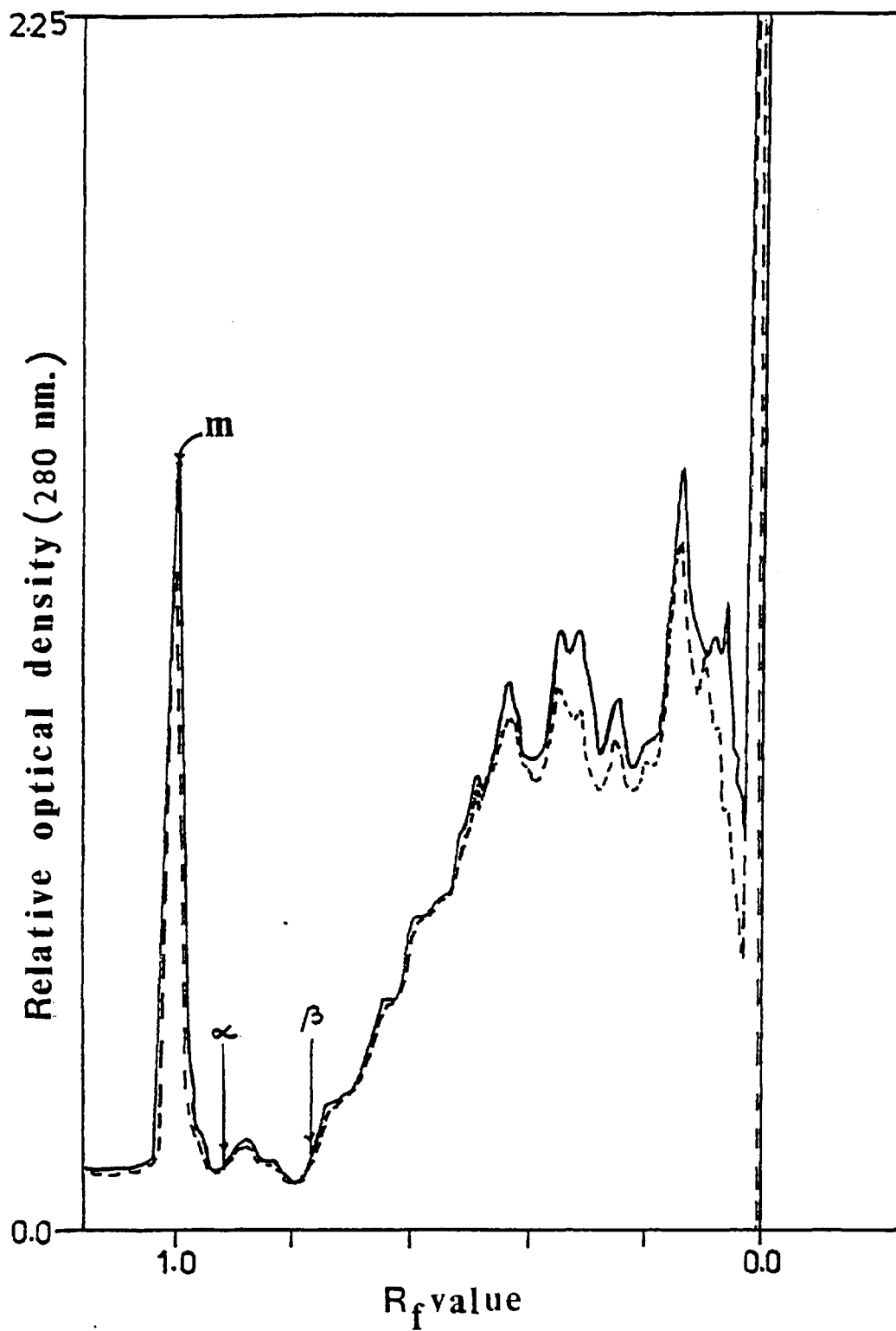


Fig. 17. Comparison of densitometer scans of 10% polyacrylamide gels loaded with 100 μ l of E₁-soluble protein extract prepared from 500 ppm Cu SO₄ treated cowpea primary leaves and water-treated controls, 3 days after treatment. m represents the bromo-phenol blue marker.

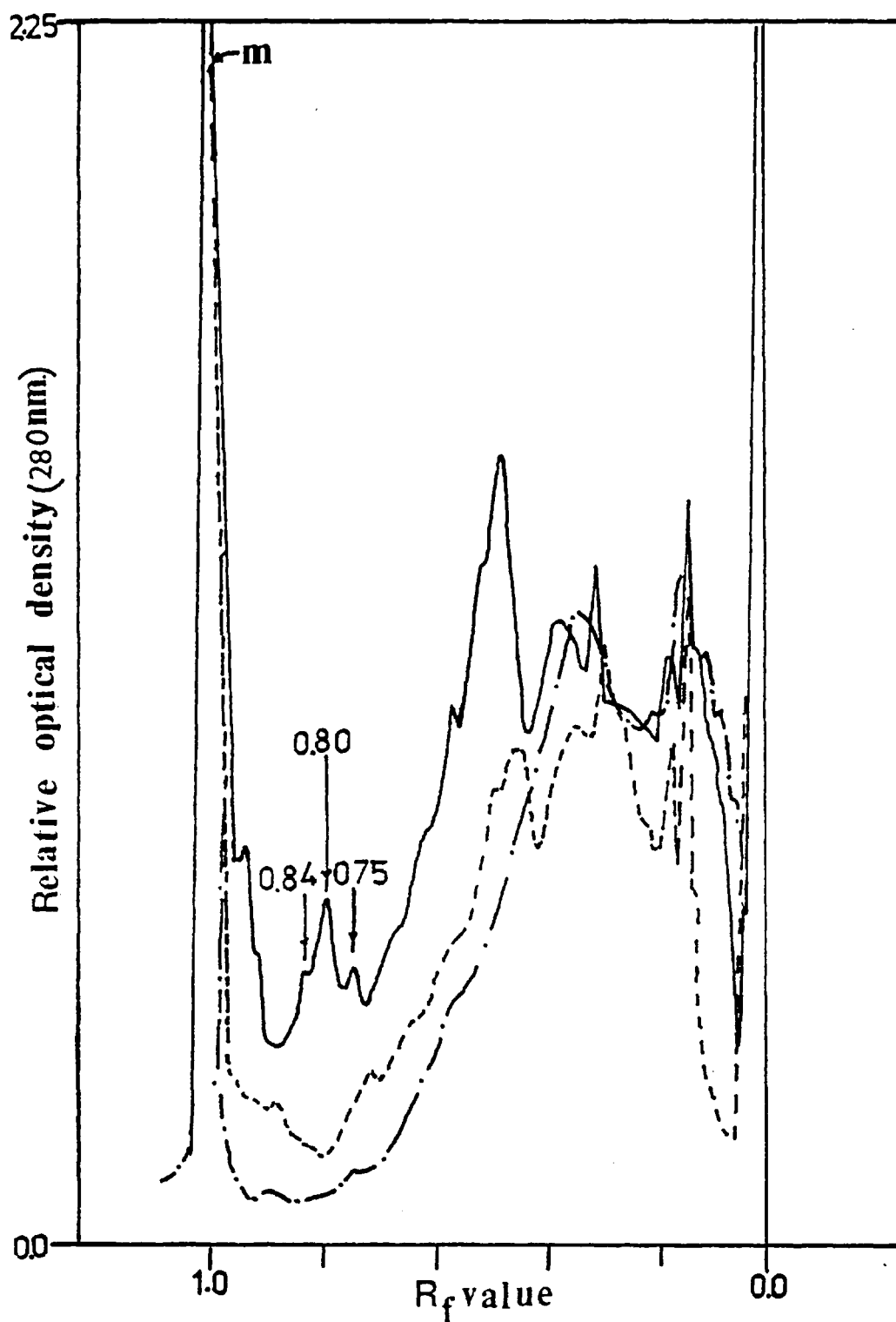


Fig. 18. Densitometer scans of soluble protein profiles of 10% PAG loaded with E₁-extracts from cowpea leaves floated on 0.5M mannitol for 2 days and the lower epidermis then detached (—), a corresponding control of leaves floated on water for 2 days (---) and protoplasts isolated from cowpea leaves, following floatation for 2 days on 0.5M mannitol (— . —). m represents the bromo-phenol blue marker.

14. Analysis of the soluble protein fraction of protoplasts isolated from plasmolysed cowpea primary leaves

Protoplasts were isolated from cowpea leaves osmotically stressed on 0.5 M mannitol for 2 days to stimulate the soluble proteins outlined in the previous section. Soluble proteins from protoplasts were extracted and analysed by disc-PAGE technique as outlined in Sections III, 15. A and 16.

The results presented in Fig. 18 show that apart from trace quantities of the protein at Rf 0.74, all of the proteins induced in cowpea leaf tissue, by plasmolysis were absent from the protoplasts isolated from that tissue.

15. Identification of peroxidase and polyphenol oxidase isozymes in soluble protein extracts from uninoculated, phosphate-(sham)-inoculated healthy and TNV-infected cowpea primary leaves, and their potential relationship to the α and the β proteins

Both peroxidase (PO) and polyphenol oxidase (PPO) activities are known to increase during hypersensitive reactions in most cases (Section II.1.A.e). This section investigates the effect of mechanical injury (abrasion) on PO and PPO isozyme pattern and whether the α and/or the β protein fractions found after viral infection are associated with isozymes of either. Soluble protein extracts from uninoculated phosphate-(sham)-inoculated healthy, and virus-infected cowpea leaves were prepared as in Section III. 15. A. Disc-PAGE of soluble proteins was carried out as previously on a series of gels (Section III. 16) which were then either stained for total proteins with Coomassie blue or developed as zymograms for either PO or PPO isozymes as outlined in Section III. 18. Results are presented as densitometer scans which have been partitioned into three zones to make comparisons easier. Zone III represents Rf 0.00 to 0.19,

zone II, Rf 0.20 to Rf 0.59, and zone I, Rf 0.60 to Rf 1.00.

The following is a summary of the results obtained.

A. Peroxidase zymograms

The results presented in Fig. 19 show that following either abrasion (Fig. 19, b) or virus infection (Fig. 19, c), quantitative and qualitative alterations in PO isozymes were similar, but virus infection had a more pronounced effect. Only alterations in zones II and III were routinely observed, there were no PO isozymes in zone I, in any of the zymograms. In uninoculated (unabraded) healthy tissue (Fig. 19, a), three major PO isozyme bands at Rf 0.07 in zone III and at Rf values 0.25 and 0.54 in zone II were found. In sham-inoculated (abraded) leaves a similar pattern was observed with one new isozyme at Rf 0.49 in zone II present, possibly elicited by mechanical injury. In virus infected leaves (Fig. 19, c) the overall increased PO activity in zones II and III masked other isozymes and made interpretation difficult, but one isozyme was resolved at Rf 0.29, away from two broader zones. The two broad zones occupied most of zone III, between Rf 0.00 and Rf 0.22 and zone II, between Rf 0.39 and 0.52. No isozymes were found to be coincident with either the α or the β fraction .

B. Polyphenol oxidase zymograms

The results presented in Fig. 20 show that following mechanical injury (abrasion, Fig. 20, b) there were only quantitative increases in isozymes whereas after virus infection (Fig. 20, c) there were both quantitative and qualitative changes. Alterations in zymograms for either sham-inoculated (abraded) or virus infected leaves were restricted to zones II and III as no PPO isozymes were found in zone I.

After sham-inoculation (Fig. 20, b) there were overall quantitative

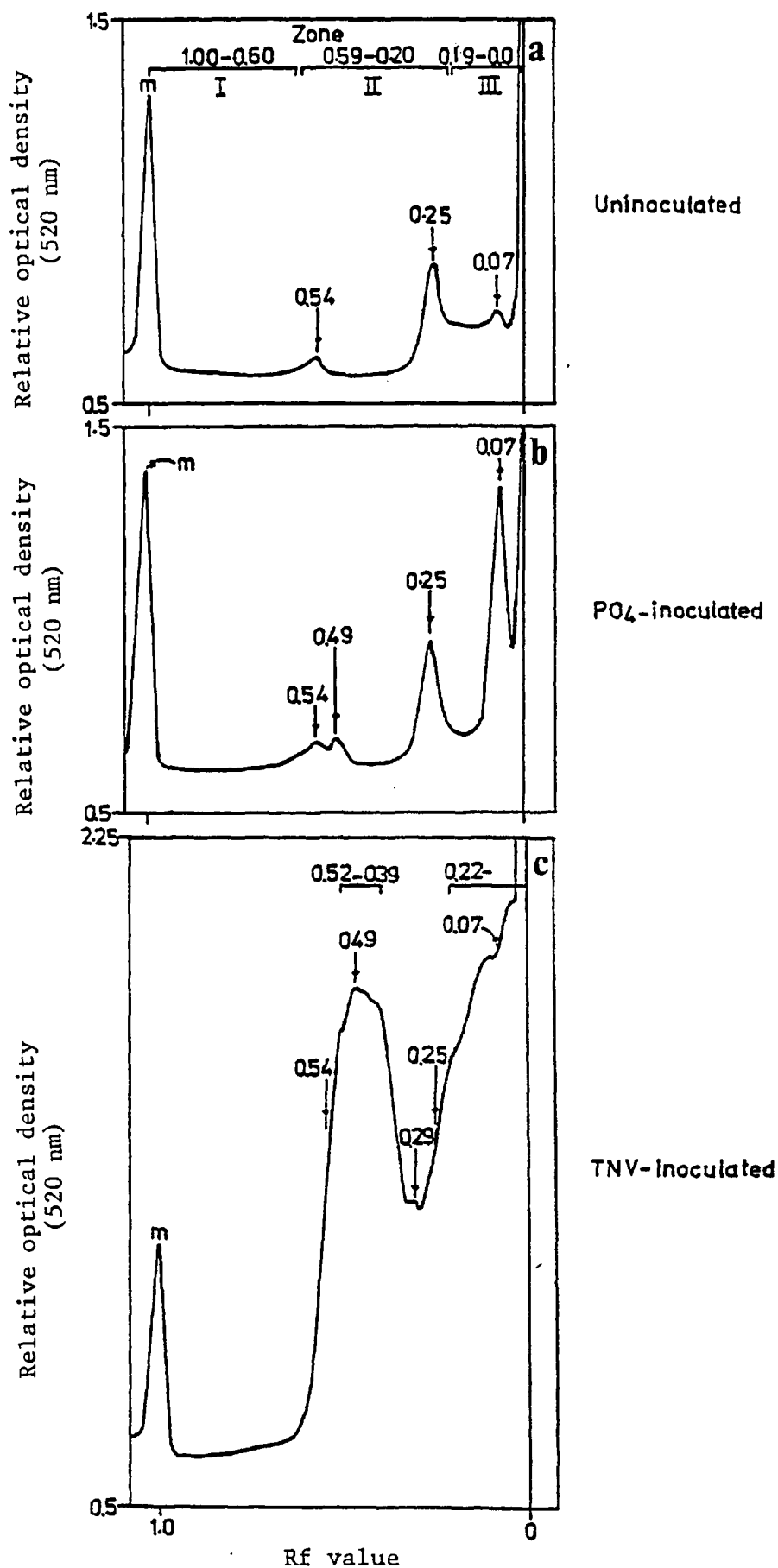


Fig. 19. PO zymograms developed in 10% polyacrylamide gels loaded with 100 μ l of E₁-soluble protein extracts prepared from uninoculated (unabraded) healthy (a), carborundum-phosphate buffer inoculated (abraded) healthy (b), and TNV-infected (c), cowpea primary leaves 3 days post-inoculation. m is the bromo-phenol blue marker.

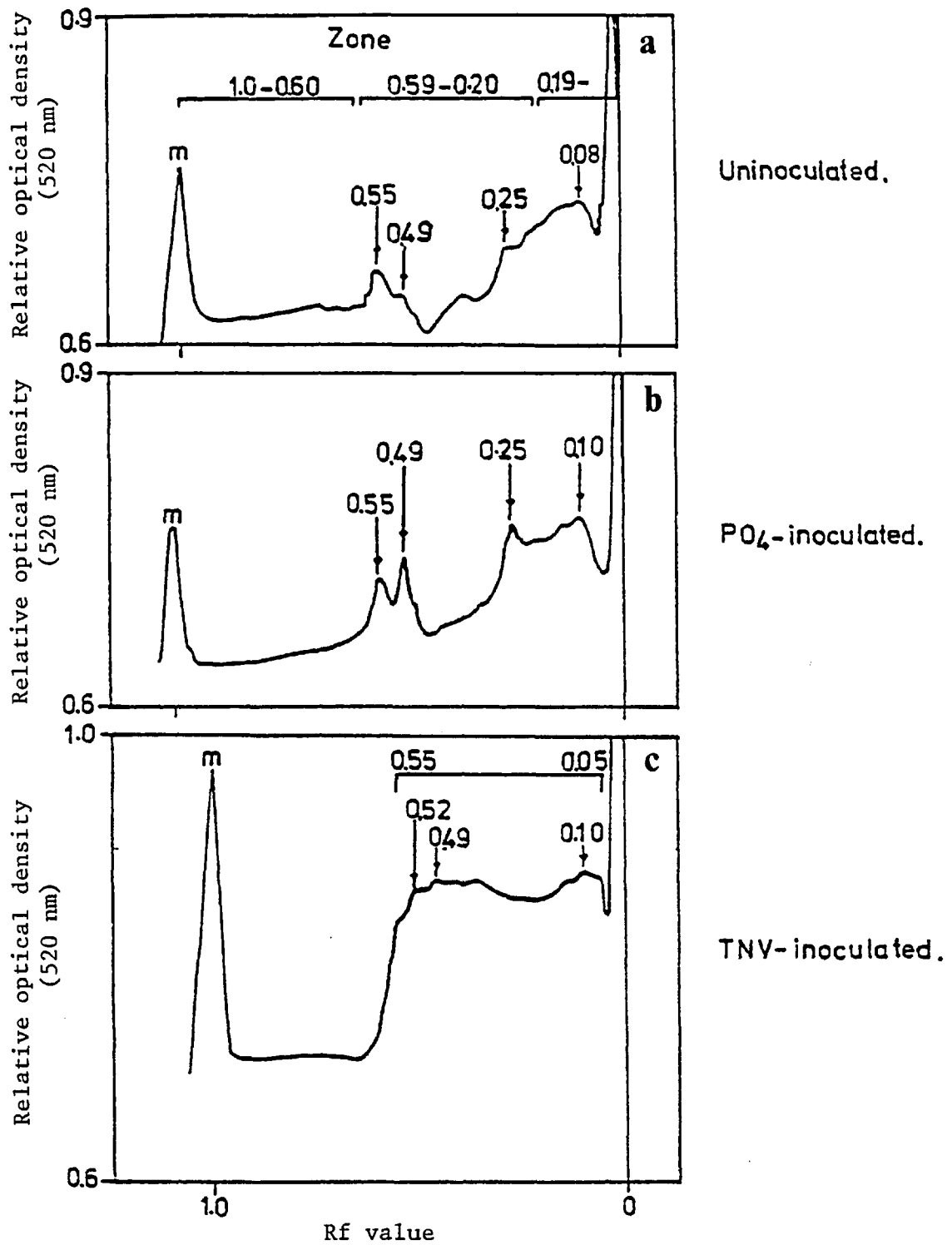


Fig. 20. PPO zymograms developed in 10% polyacrylamide gels loaded with 100 μ l of E₂-soluble protein extracts prepared from uninoculated (unabraded) healthy (a), carborundum-phosphate buffer inoculated (abraded) healthy (b), and TNV-infected (c), cowpea primary leaves, 3 days post-inoculation. The bromo-phenol blue marker is indicated as m throughout.

increases in PPO isozyme activity as compared to uninoculated leaves (Fig. 20, a) with particular enhancement to an isozyme at Rf 0.49. Following viral infection there were similar overall alterations with a broad band of activity between Rf 0.05 to 0.55 masking most of the isozymes in Fig. 20, b. This broad band was resolved into several isozymes, some of which were novel (e.g. isozymes of Rf values of 0.05 and 0.52). No isozymes were found to be coincident with either the α or the β protein

16. Analysis of PO and PPO isozymes in the soluble proteins of healthy cowpea leaves floated on either H₂O or mannitol

This section analyses the effects of plasmolysis (mannitol-induced osmotic stress) on the PO and PPO isozyme patterns and compares those effects with the effects of abrasion and virus infection.

Healthy cowpea leaves were floated on either distilled water or 0.5 M mannitol for three days prior to E₁-buffer extraction of soluble proteins, disc-PAGE and zymogram development as outlined in previous sections (III. 10, 15. A, 16 and 18). Immediate scanning of gels facilitated the detection of qualitative changes.

The following summarizes the results :

A. Peroxidase zymograms

PO zymograms for water floated leaves (Fig. 21, B) had similar isozyme patterns to those of healthy untreated leaves (Fig. 21, A) with only two new isozymes at Rf values 0.45 and 0.49. The isozyme at Rf 0.49 is thought to be the same as that specifically enhanced by mechanical injury (Fig. 21, A). The PO zymogram for mannitol floated leaves (Fig. 21, B) showed a pronounced enhancement of isozymes particularly at Rf values 0.12, 0.16, 0.45 and 0.49 with possibly three new isozymes at

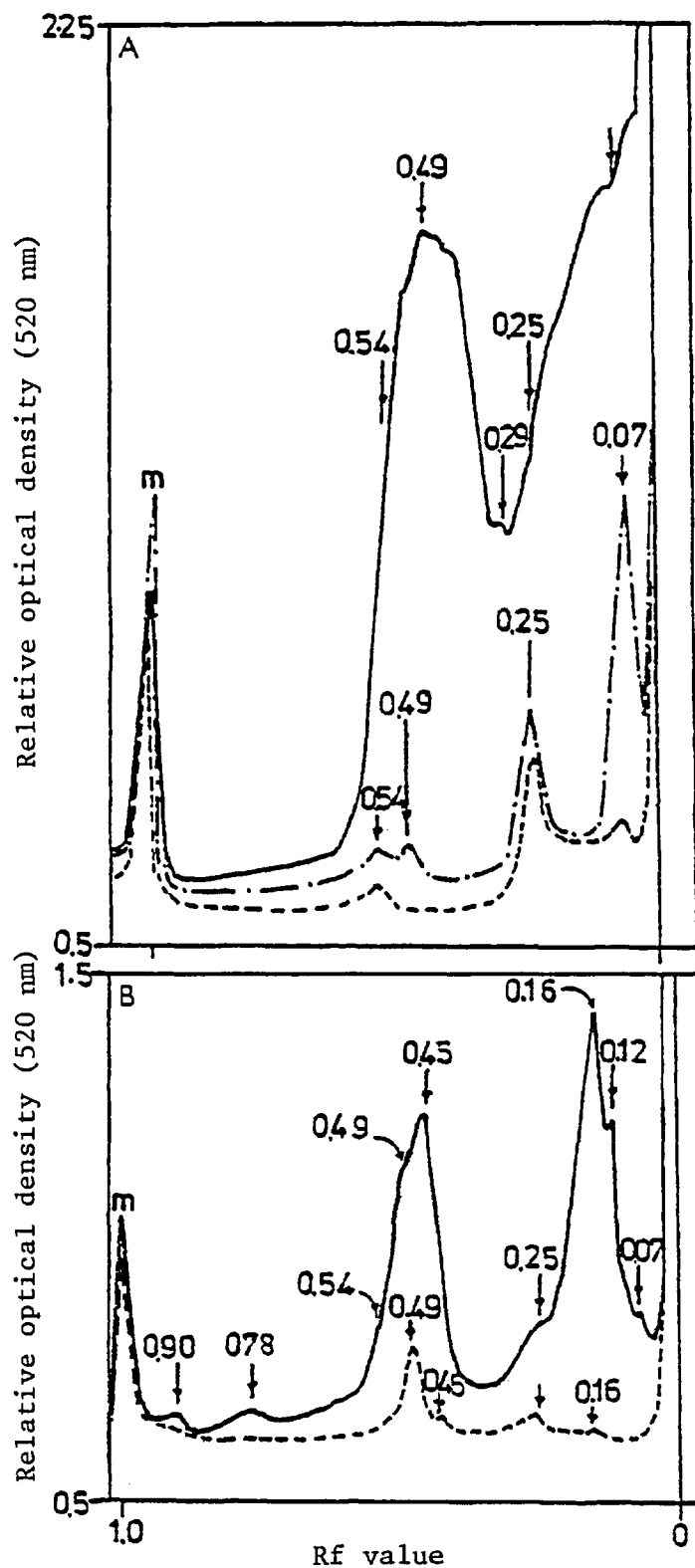


Fig. 21. PO zymograms developed in 10% PAG loaded with 100 μ l E_1 -soluble protein extracts prepared from cowpea primary leaves, 3 days after treatment. A (above) shows superimposed three scans (taken from Fig. 19 for comparison purposes) representing zymograms developed for TNV-infected (—), abraded healthy (— · —) and uninoculated healthy (---) leaves. B (below) shows zymograms developed for mannitol-floated (—), and water-floated (---) leaves. The bromo-phenol blue is indicated as m in both figures.

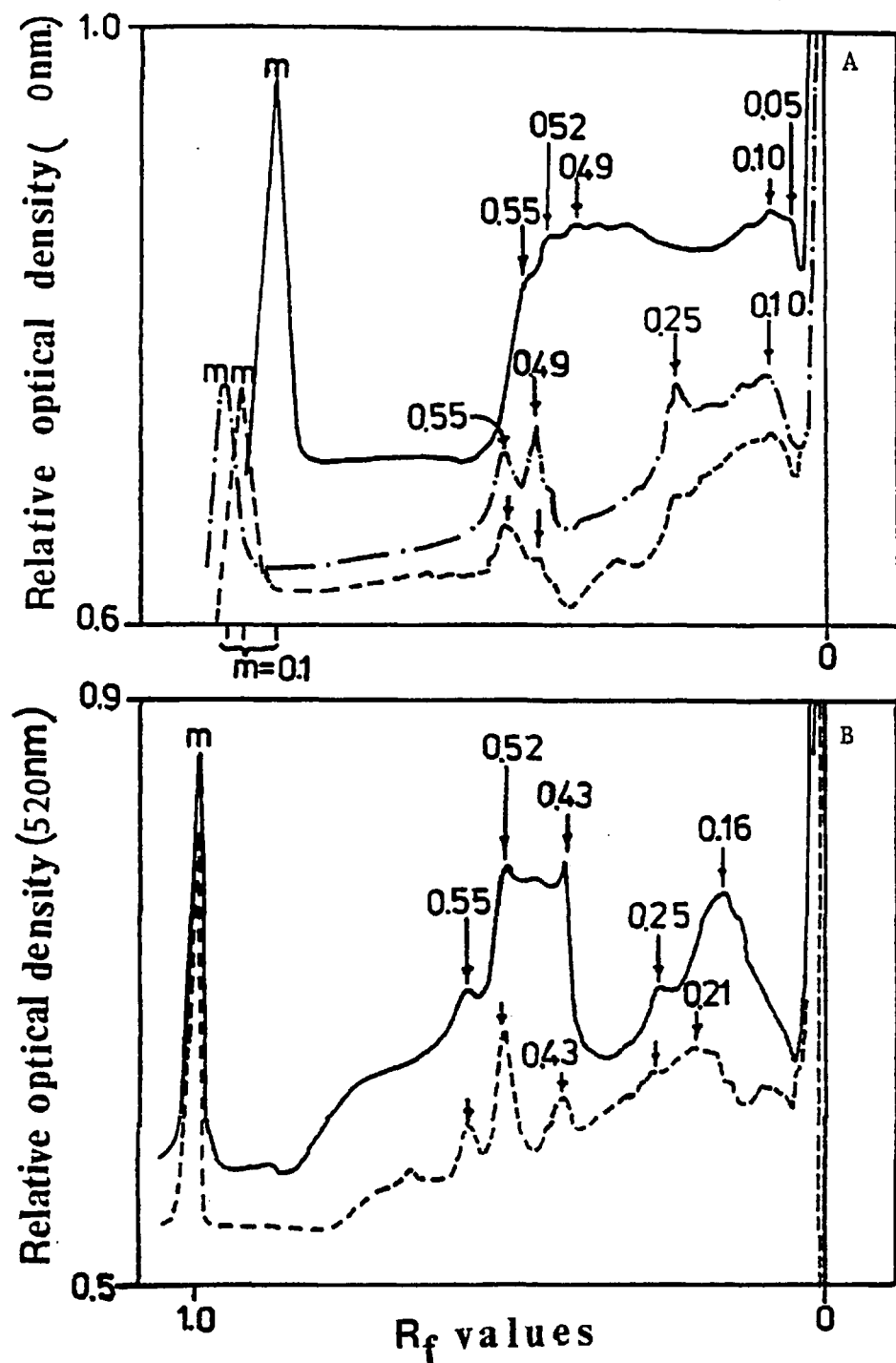


Fig. 22 PPO zymograms developed in 10% PAG loaded with 100 µl E_1 -soluble protein extracts prepared from cowpea primary leaves, 3 days after treatment. A (above) shows superimposed three scans (taken from Fig. 20 for comparison purposes) representing zymograms developed for TNV-infected (—), abraded healthy (---), and uninoculated healthy (----) leaves. B (below) shows zymograms developed for mannitol-floated (—) and water-floated (----) leaves. The bromo-phenol blue is indicated as m in both figures.

Rf values 0.12, 0.78, and 0.90. Plasmolysing leaves with mannitol resulted in PO isozyme patterns very similar to those observed following TNV infection (compare Fig. 21, B with A).

B. Polyphenol oxidase zymograms

Fig. 22 summarizes the results of PPO zymogram analysis of the various soluble protein extracts from cowpea leaves as described in Section III. 18. B.

The zymograms for water floated (Fig. 22, B), sham-phosphate buffer inoculated (Fig. 22, A), and healthy unabraded leaves (Fig. 22, A), were essentially similar, with only one novel PPO isozyme at Rf 0.43 in the extract from water floated leaves. In the zymograms for mannitol floated leaves both quantitative and qualitative alterations were noted when compared to untreated tissue, the alterations are seen in Fig. 22 (B) which show that osmotic stress caused a general enhancement of isozymes found in the water floated leaves with the same novel isozyme at Rf 0.43. The PPO zymogram for virus infected leaves (Fig. 22, A) was dissimilar to that of plasmolysed tissue but in neither case was any of the PPO isozymes at similar Rf value to the α or the β protein fraction.

17. Identification of ribonuclease (RNase) isozymes in soluble protein extracts from healthy and TNV-infected leaves and their potential relationship to the α and the β protein fractions

RNase has been shown to be increased following both abrasion and virus infection of leaves. This section investigates whether the α or the β protein fraction is associated with such activity. Soluble protein extracts from both sham-phosphate buffer inoculated (healthy), and virus-infected cowpea leaves, 3 days post-inoculation were subjected to both disc-PAGE, and disc-PNGE, as described in Section III. 16 and 19.

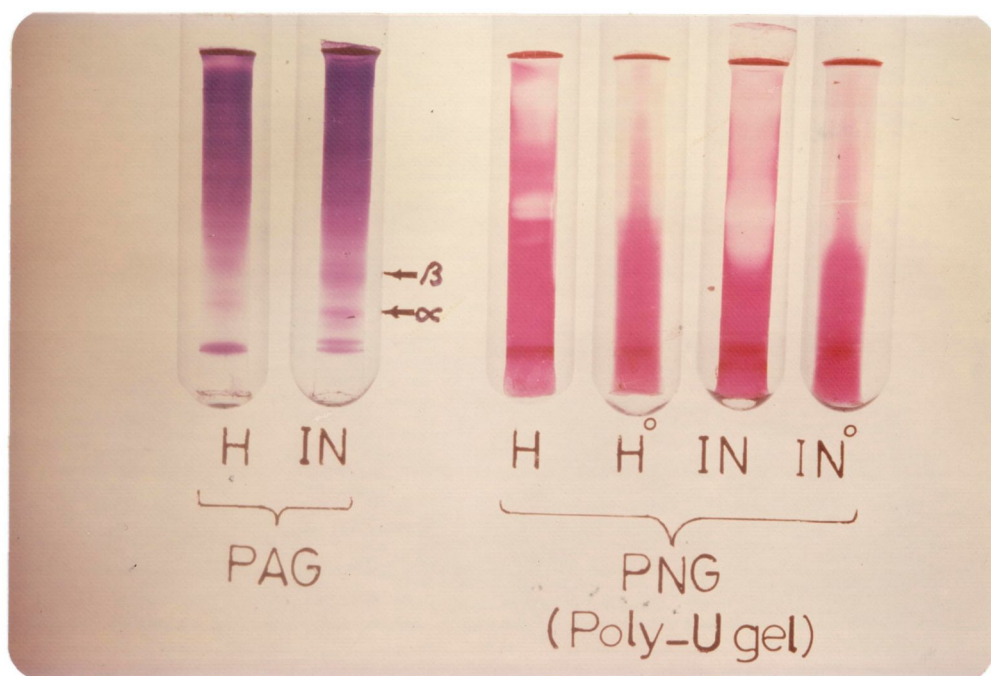


Fig. 23. Comparison of the disc-electrophoretic patterns of the soluble proteins, from healthy and TNV-infected cowpea primary leaves in 10% PAG, and RNase isozyme patterns in 10% PAG containing 0.05% polyuridylic acid (PNG = Poly - U), stained with pyronin Y.

H = Healthy;

IN = TNV-infected;

H°, IN° = The same as the corresponding H and IN in the PNG group but heated to 100°C for 10 min. in 7% acetic acid to inactivate enzymes (control).

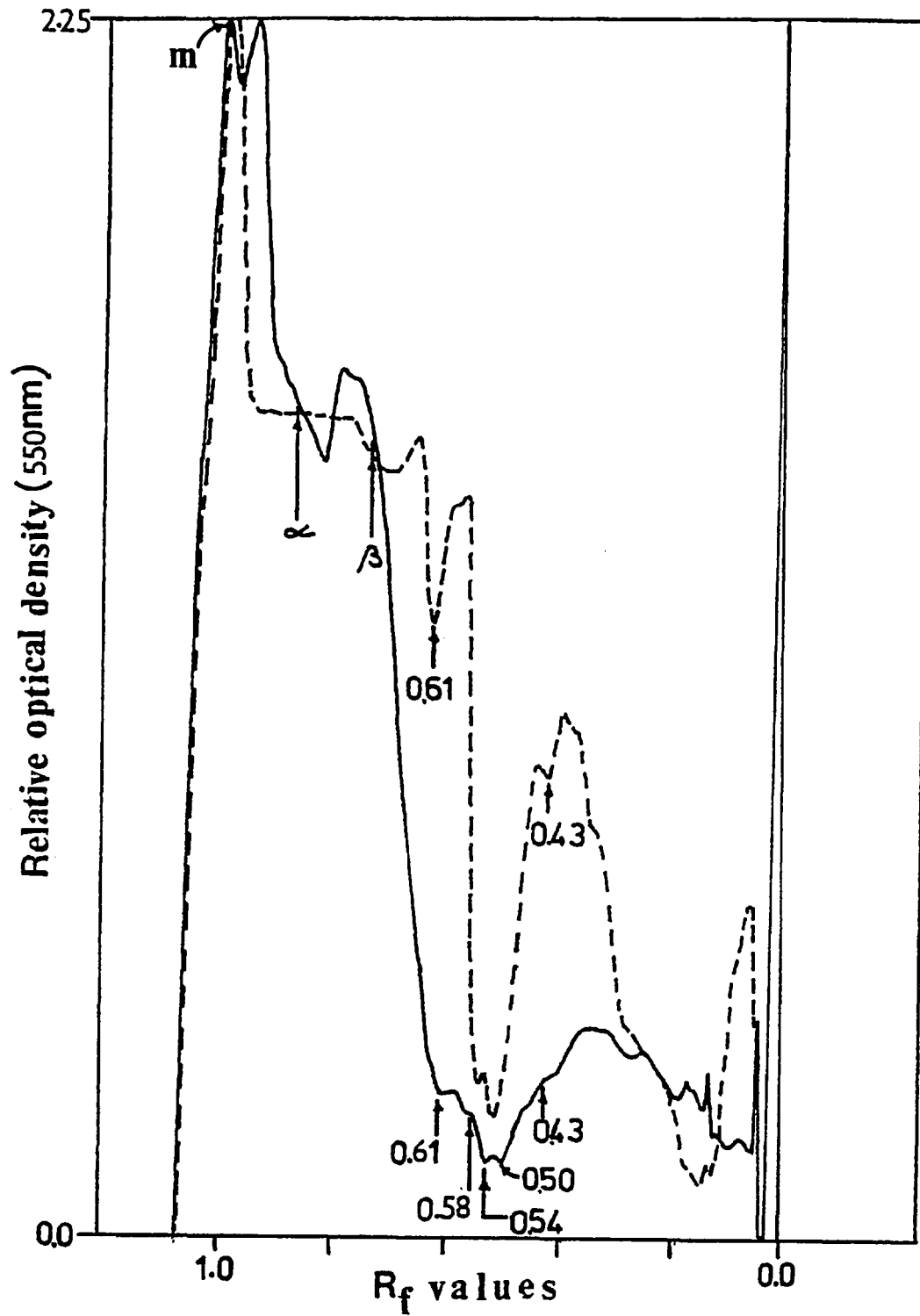


Fig. 24. RNase zymograms on 10% poly-U gels stained with pyronin Y and scanned at 550 nm for TNV-infected (—) and sham-inoculated healthy (---) cowpea primary leaves, 3 days post-inoculation.

The results shown in Figs. 23 and 24 indicated that neither the α nor the β protein was an RNase isozyme and that most of the RNase isozyme activity occupied the upper portion of native gels in both control and infected tissue.

Using TNV-infected leaf extracts, overall quantitative increases in RNase isozyme activity were seen mainly in the upper portion of the gel (Fig. 23, 1N and Fig. 24) which reinforced isozyme peaks already seen in healthy extracts (Figs. 23, H and Fig. 24). At least one novel RNase isozyme at Rf value 0.50 (Fig. 24) was identified.

18. Determination of the quantitative increases in PO, PPO and RNase in healthy, TNV-infected, water-floated and mannitol-floated cowpea primary leaves

Zymogram analysis illustrates specific changes in isozymes and only gives a comparative estimate of enzymic activity, a separate study on the quantification of this activity was carried out. Soluble protein extracts for enzyme assays were prepared and enzyme activities assayed by spectrophotometry as outlined in Section III. 22.

A. Peroxidase (PO) determinations

Cowpea leaf tissue, 3 days after treatment was harvested, extracted and PO activity determined as described in Section III. 22. A.

The results shown in Fig. 25 (A) indicate that the lowest level of PO activity was found in unabraded healthy leaves, and that following rubbing with carborundum-phosphate buffer (mechanical injury), PO activity was almost doubled. The most pronounced increase in PO activity was found in TNV-infected tissue. The activity in uninoculated, water-floated leaves was higher than that in both untreated and sham-inoculated tissue but floating leaves on 0.5 M mannitol caused considerable increases.

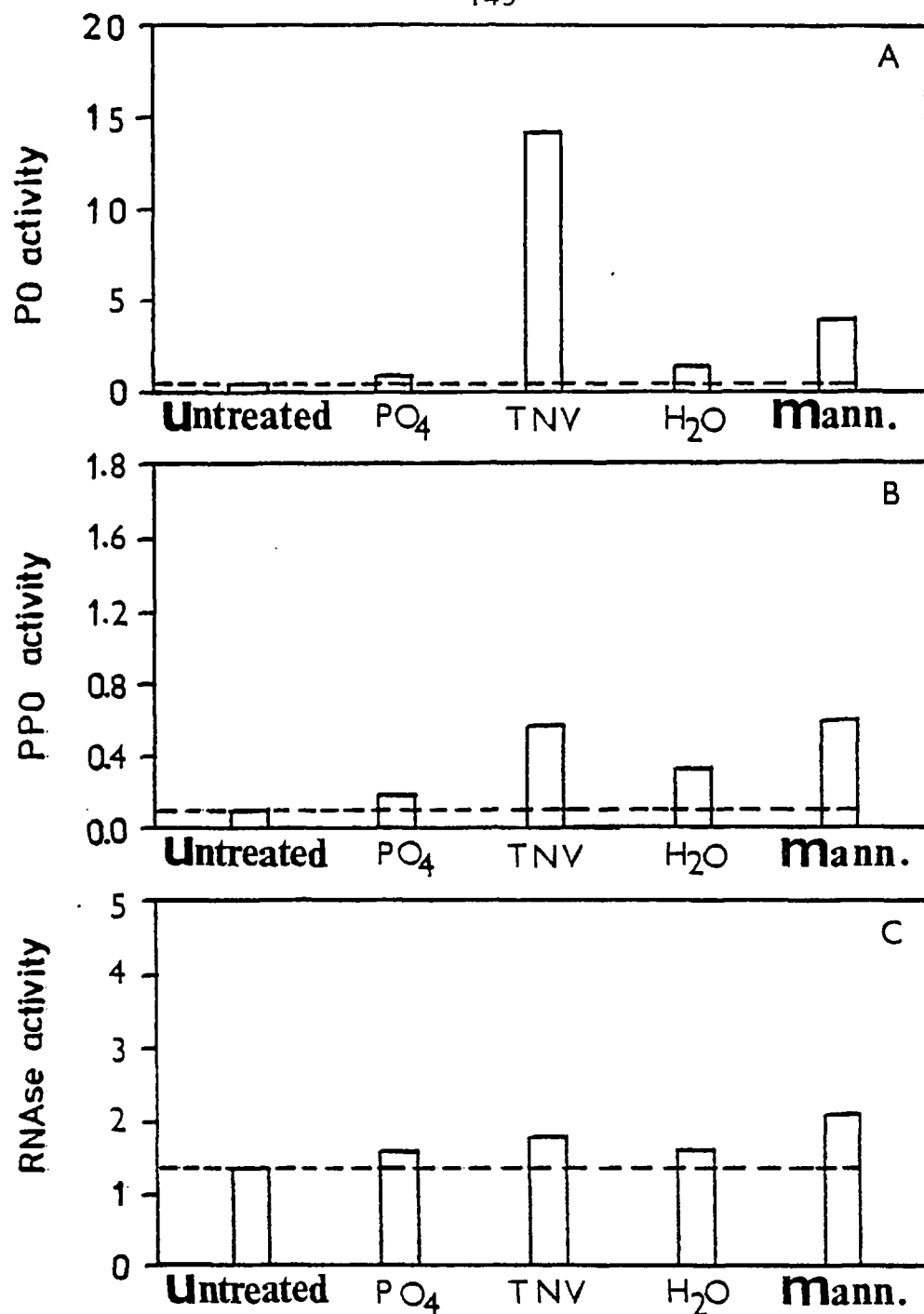


Fig. 25. The level of PO (A), PPO (B) and RNase (C) activity in uninoculated healthy (untreated), carborundum-phosphate buffer inoculated (PO_4), virus-infected (TNV), water-floated (H_2O) and mannitol-floated (Mann.) cowpea primary leaves, 3 days after treatment. PO activity is expressed as reciprocal of (Δ time for O.D. (520 nm) to change from 0.2 $\rightarrow$ 0.4/1 ml final reaction mixture/1g dry weight); PPO activity is expressed as Δ O.D. (330 nm) between 0.5 - 1 min/1 ml final reaction mixture/1 g. dry weight; RNase activity is expressed as O.D. (260 nm)/1 ml final reaction mixture/1 mg. dry weight.

However, this later activity was only a third of that found in the virus-infected tissue.

B. Polyphenol oxidase (PPO) determinations

Cowpea leaf tissue, 3 days after treatment, was harvested, extracted and PPO activity determined as described in Section III. 22. B.

The results are summarized in Fig. 25 (B) which showed that the pattern of activities in all treatments was similar to that seen for PO activity (Fig. 25, A). The only difference being that PPO activity in mannitol-floated leaves reached the same level as that found in the TNV-infected tissue, at least after three days of treatment.

C. Ribonuclease (RNase) determinations

As in the previous sections (IV. 18, A and B), cowpea leaf tissue, 3 days after treatment, was harvested, extracted and RNase activity determined as outlined in Section III. 22. C.

The results presented in Fig. 25 (C) show that the untreated (unabraded) control healthy leaves had the lowest RNase activity, but following carborundum-phosphate buffer inoculation (mechanical injury), activity increased slightly. Virus-infected leaves had RNase activities higher than those found in abraded leaves.

While water-floated leaves had a similar RNase activity to that found in abraded leaves, mannitol-floated leaves (osmotically-stressed) had the highest overall activity of RNase, almost one and a half times that found in untreated leaves.

19. Alterations in the activities of PO, PPO and RNase in TNV-infected, AH, and corresponding healthy controls, at different times after inoculation

Cowpea leaf tissue, either sham-inoculated healthy (H), virus-infected (IN) or from (AH) or ($\frac{1}{2}$ H) control leaves, was extracted various times post-inoculation and assayed for the above enzymes as indicated in Section III, 22.

A. Peroxidase (PO) alterations

Fig. 26 (PO) illustrates the alterations in PO activity in the above tissue various times after inoculation. In apparently healthy tissue (AH) whilst the PO activity, 6 days after inoculation of the adjacent half leaf was doubled as compared to zero time samples, the increases in (IN) leaves were one hundred fold that found at zero time. Four to 6 days post-inoculation of the (IN) leaves, necrosis was almost complete and PO activity appeared to increase dramatically.

B. Polyphenol oxidase (PPO) alterations

Fig. 26 (PPO) summarizes the results and show that PPO activity in (H) leaves did not alter significantly from the zero time value, over the studied six day time course. The pattern of activity was similar in both ($\frac{1}{2}$ H) and (AH) tissue. In (IN) leaves at the start of lesion formation (3 days post-inoculation) increases in PPO activity were routinely observed, which although not maintained a day later, were clear in samples taken 2 days later when necrosis was complete.

C. Ribonuclease (RNase) alterations

The results summarized in Fig. 26 (RNase) show clearly that cowpea leaf tissue, responds initially to the effects of mechanical abrasion by producing RNase as a wound response, but this enzyme activity is not maintained. In unabraded tissue (i.e. $\frac{1}{2}$ H tissue), RNase levels dropped steadily after an initial small rise in activity 1 day post-

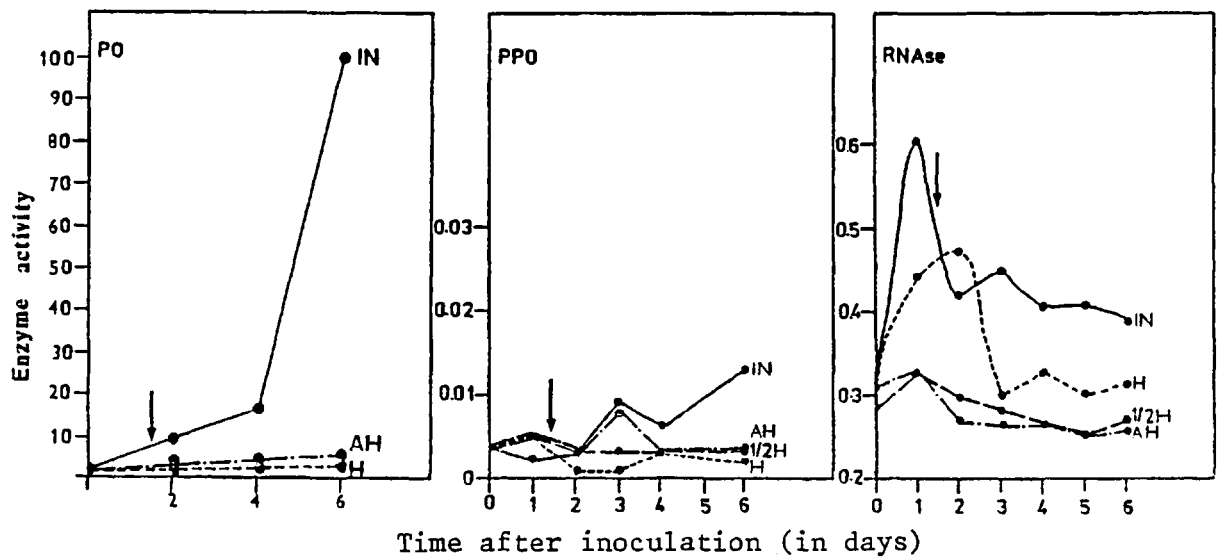


Fig. 26. Alterations in PO, PPO and RNase activity with time in tissue taken from TNV-infected, IN (o—o), phosphate buffer-inoculated, H (o----o) cowpea primary leaves, and uninoculated half leaves adjacent to either TNV-infected, AH (—.—) or phosphate buffer-inoculated, 1/2H (----). Arrows indicate the time of local lesion appearance.

inoculation the levels were almost the same as those found at zero time. RNase activity in (AH) tissue followed almost the same pattern as that found in ($\frac{1}{2}$ H) tissue. Large increases in RNase activity in both abraded (H) and (IN) leaves one day after inoculation were not maintained in (H) tissue although at 6 days post-inoculation levels were still higher than those found in (AH) and ($\frac{1}{2}$ H) leaves. The activity of RNase in (IN) leaves were the highest observed in all of the samples tested, one day after inoculation, and although dropping to a lower value a day later reproducibly increased again when local lesions matured (3 days post-inoculation). After this time the level of activity declined to a value still higher than that in any of the other samples, at 6 days post-inoculation.

20. Symptomology of TNV-infection of cucumber cotyledons

The interaction between TNV and all cultivars of cucumber cotyledons tested results in localised infections. In cucumber as in other hypersensitive hosts, the localization process was associated with a severe reaction. This culminated in the death of the tissue at the centre of the localised areas forming conspicuous necrotic local lesions. Lesions were visible 3 days post-inoculation as small white 'pin-pricks' and matured 3 - 4 days later. These lesions were yellowish grey in colour with white centres and narrow yellow haloes (Fig. 27 and 30).

21. Electrophoretic analysis of soluble protein fractions of healthy and TNV-infected cucumber cotyledons

Disc-PAGE was used to analyse the soluble protein fraction obtained from both healthy (Fig. 30, H) and TNV-infected (Fig. 30, IN) cucumber cotyledons, 6 days post-inoculation as described in Section III, 16. Soluble proteins were extracted in either alkaline (E_1 -method) or acidic buffer (E_2 -method) as described in Section III, 15. The loading



Fig. 27. Ashley cucumber cotyledon infected with TNV showing characteristic necrotic local lesions, 6 days post-inoculation.

volumes for gels were either 50 or 100 μ l/gel tube. Following electrophoresis, gels were stained with Coomassie blue, destained and scanned at 280 nm (see Section III. 16). The several cultivars used in this study viz. Ashley, Baton-Vert, China, Chipper and Elem, all had similar protein profiles, following TNV-infection. The profile for the Explorer cultivar was, however, somewhat different (see later). A single new major protein band (γ) at Rf value 0.70 ± 0.02 on 10% PAG was reproducibly seen following electrophoresis of extracts from infected cotyledons (Fig. 28).

Fig. 29 (A and B) show densitometer scans of 10% polyacrylamide gels loaded with extracts from healthy or infected cotyledons of either the Baton-Vert (Fig. 29, A) or the Ashley (Fig. 29, B) cultivars.

Only the two varieties are illustrated here since apart from the Explorer cultivar, the protein profiles of the other cultivars mentioned above were almost identical.

It is clear from Fig. 29 (A and B) that the soluble proteins from infected tissue of both Baton-Vert or Ashley cotyledons contain the γ band at Rf value 0.70 ± 0.02 . This band was absent from gels loaded with extracts from healthy cotyledons by visual inspection but discernable by densitometry as a small peak at the same Rf value.

In the Explorer cultivar, two bands, (γ) and (γ A), (Fig. 28 and 29, C) were reproducibly detected in infected cotyledon extracts at Rf values 0.70 ± 0.02 and 0.74 ± 0.02 respectively. These proteins were absent in gels loaded with healthy extracts, when inspected visually, but the γ band (Rf. 0.70) was detectable by densitometry as in the previous section. The appearance of this small peak in the profiles of healthy soluble protein extracts at a corresponding position to γ in all cultivars studied indicates one of two things. Either the γ protein is present in healthy extracts in trace quantities or a novel protein (i.e. the γ protein) migrates to a similar position on gels as a normal host component does, and therefore reinforces the peak.

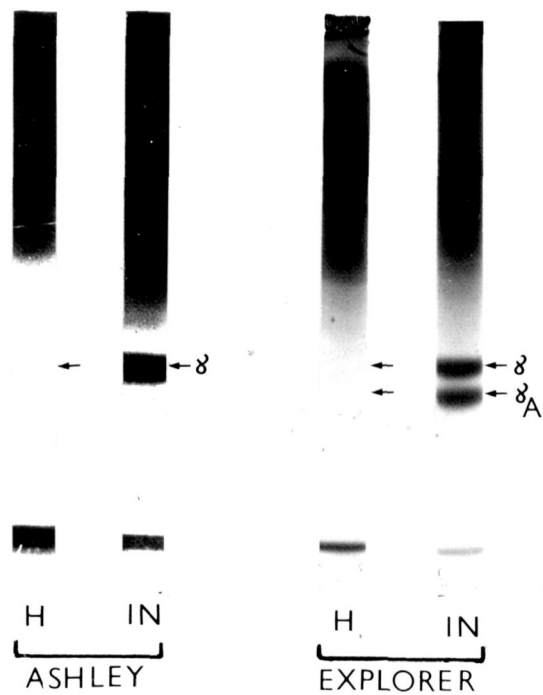


Fig. 28 Comparison of the electrophoretic patterns of 100 μ l soluble protein extract from healthy (H) and TNV-infected (IN) tissue of Ashley and Explorer cucumber cultivars on 10% PAG.

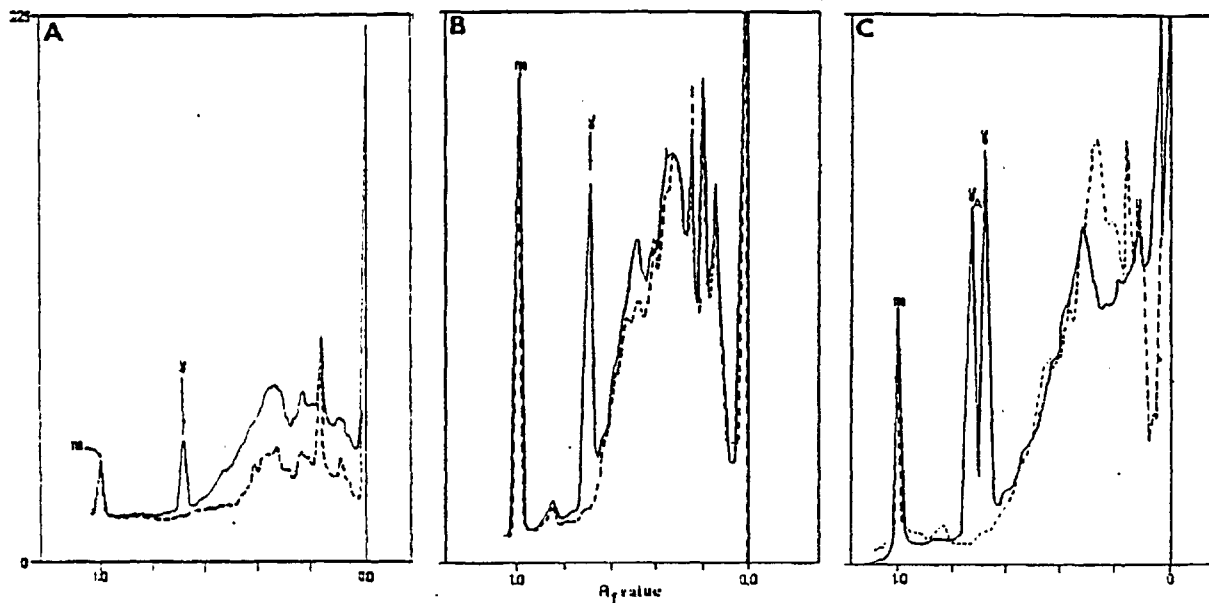


Fig. 29. Densitometer scans of the electrophoretic pattern of 50 μ l (A) or 100 μ l (B and C) E_1 -soluble protein extracts in 10% PAG. (—), TNV-infected, and (---), healthy, sham-inoculated Baton Vert (A), Ashley (B) and Explorer (C) cucumber cotyledons, 5 days after inoculation. m, indicates the position of the bromo-phenol blue marker throughout.

Co-electrophoresis of extracts from infected cotyledons of all the cultivars tested in different combinations indicated that γ was a common protein, as a single band at Rf 0.70 was reproducibly seen. However, when extracts from the Explorer cultivar were co-electrophoresed with extracts from any of the other cultivars, two bands were seen, one enhanced at Rf 0.70 (γ), the other at the same level as that found following the electrophoresis of the Explorer extracts alone at Rf 0.74 (γ A). This indicates that the γ protein is a common protein amongst all cucumber cultivars tested in this study and γ A is an Explorer-specific protein component at least as far as the electrophoretic migration is concerned.

22. Searches for the γ protein in uninoculated parts of the plant

Following the inoculation of half cotyledons (Fig. 30, AH) whole cotyledons (Fig. 30, IN) or first true leaves of the Baton-Vert cucumber cultivar with TNV, extensive searches for the γ protein in uninoculated parts of the plants were made. Soluble protein fractions were prepared from uninoculated half cotyledons adjacent to TNV-infected halves, 6-days post-inoculation and from first true leaves, 6 days and 16 days post-inoculation of both cotyledons. Second true leaf tissue was similarly analysed either 16 days after inoculating cotyledons or 7 days after inoculating first true leaves with TNV. Plants inoculated with phosphate buffer instead of TNV served as controls. The soluble protein fractions were subjected to disc-PAGE and analysed on 10% gels as described earlier (Section III, 16).

Densitometer scans revealed that the γ protein was only detectable in apparently healthy tissue in uninoculated half cotyledons adjacent to TNV-infected halves (Fig. 31), but was not detectable in any other uninoculated parts of the plants following inoculation of the other tissue with TNV as described in the last paragraph.



Fig. 30. Ashley cucumber cotyledons, sham-inoculated and TNV-infected, 6 days post-inoculation.

H = phosphate buffer, sham-inoculated control cotyledon.

AH = apparently healthy half cotyledon adjacent to TNV-infected half.

IN = TNV-infected cotyledon with well developed necrotic local lesion.

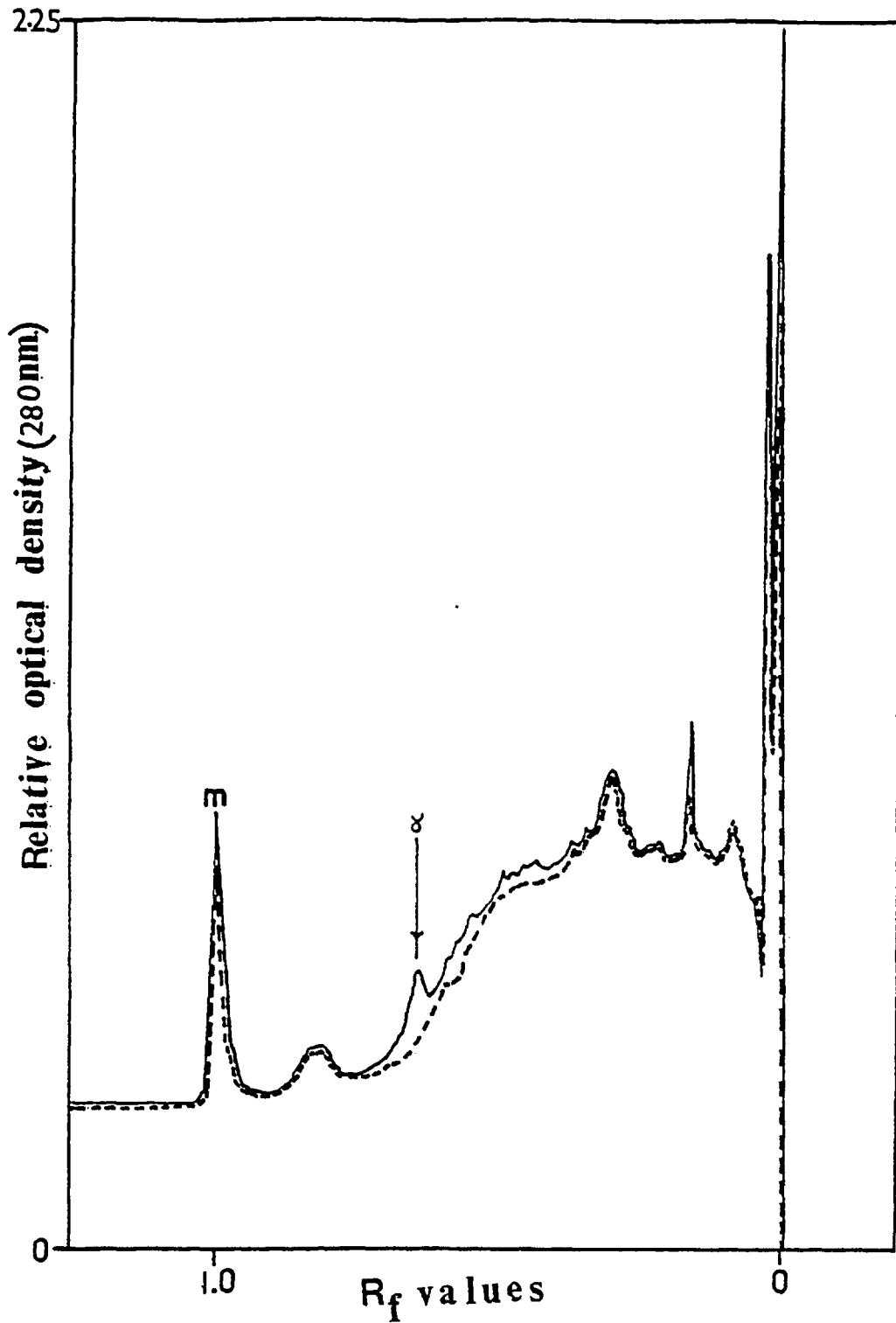


Fig. 31. Densitometer scans of electrophoretic pattern of soluble proteins in 10% polyacrylamide gels loaded with 100 μ l of E₁-extract from apparently healthy uninoculated half Ashley cotyledons (AH) adjacent to the mid-rib of necrotic halves (—) and healthy uninoculated half cotyledons adjacent to phosphate buffer inoculated halves (----), 6 days post-inoculation. m is the bromo-phenol blue marker.

23. Preliminary chemical characterization of the γ and γA proteins
(the glycoprotein nature)

Soluble protein extracts in either E_1 or E_2 buffers of both healthy and 5-day infected cucumber cotyledons of the Baton-Vert, Ashley and Explorer cultivars were subjected to native disc-PAGE on 10% gels. Following electrophoresis fresh gels were either stained for proteins with Coomassie blue or stained for glycoproteins using the PAS method (Sections III, 16 and 17).

When PAS-stained gels were compared with Coomassie blue stained sister gels, a positive reaction was seen at the corresponding position to the γ protein in both the TNV-infected Baton-Vert (Fig. 32, A) and Ashley (Fig. 32, B) cultivars. Other higher molecular weight soluble proteins, also stained positively. No such reaction was seen in gels loaded with healthy extracts from either cultivar (Fig. 32, D and E). In the Explorer cultivar two bands with identical mobilities to γ and γA stained positively with the PAS reagent in gels loaded with extracts from infected tissue (Fig. 32, C). Moreover, the same two positive bands stained positively with PAS were seen in gels loaded with healthy, sham-inoculated Explorer cotyledon extracts (Fig. 32, F). Thus, at least in the Explorer cultivar PAS stained gels revealed that the γ and γA proteins were present in healthy control tissue (in both abraded and untreated).

24. Comparison of the glycoprotein pattern in the soluble protein
fraction of healthy and TNV-infected Baton-Vert, Ashley and
Explorer tissues

PAS-stained gels of protein extracts from TNV-infected and control tissue from the above three cultivars revealed not only changes at the γ and γA positions but also other qualitative and quantitative alterations in other parts of gels.

The following is a description of the alteration seen in the three

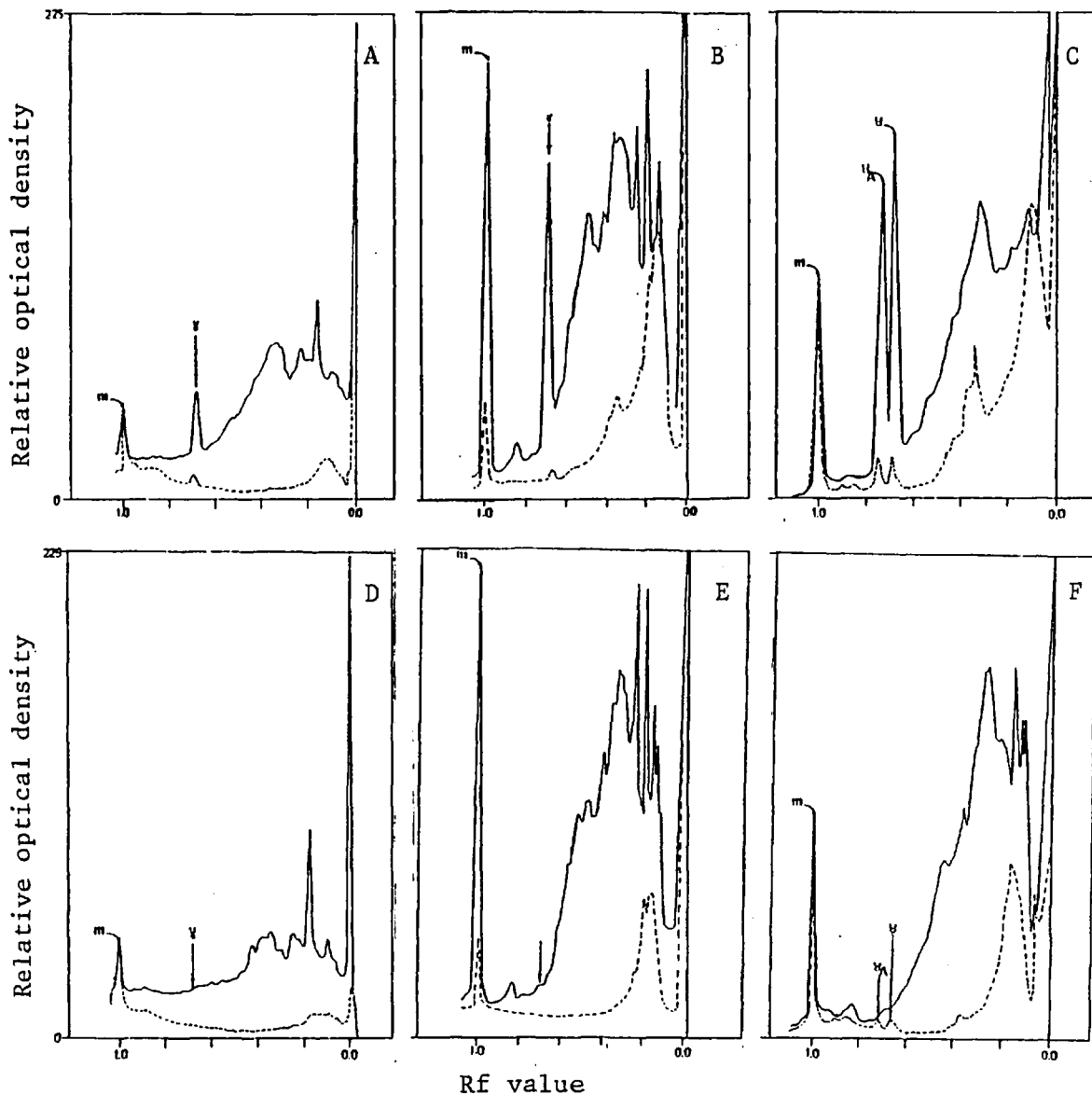


Fig. 32. Densitometer scans of 10% polyacrylamide gels loaded with 50 μ l E_2 (A and D) or 100 μ l E_1 (B, C, E and F) soluble protein extracts from infected Baton-Vert (A), Ashley (B) and Explorer (C) cucumber cotyledons, 5 days after inoculation and corresponding controls (D, E and F). (—) are densitometer scans of soluble protein patterns in gels stained with Coomassie blue, destained in 10% acetic acid and scanned at 280 nm. (---) are densitometer scans of sister gels stained with PAS-method, destained and scanned at 550 nm. m is the bromo-phenol blue marker throughout.

cultivars in turn.

A. Baton-Vert

The results presented (Fig. 33, A) show that the only detectable glycoprotein bands in healthy tissue were at Rf values 0.08, 0.11, 0.20 and 0.89. Following TNV-infection, there were mainly quantitative alterations in these proteins. The only qualitative change was the appearance of a novel glycoprotein band at γ position (Rf 0.70).

B. Ashley

The results shown (Fig. 33, B) illustrate that the healthy cotyledons contained a few glycoproteins which banded in the top third of the gel at Rf values 0.13, 0.17, 0.20 and 0.25. Following virus-infection, alterations in the glycoprotein pattern were characteristic for the Ashley variety alone. All bands with Rf value equal to 0.37 or higher (i.e. 0.37, 0.41, 0.58 and 0.70) were adjudged novel. The band at the Rf value 0.70 corresponded to the γ fraction.

C. Explorer

The results illustrated (Fig. 33, C) show that healthy cotyledons of Explorer contained more glycoproteins than healthy Ashley cotyledons (comparison between Explorer and Baton-Vert cannot be made because of different method of extraction). These glycoproteins were distributed throughout the gel at Rf values 0.02, 0.12, 0.40, 0.45, 0.70, 0.74, 0.87 and 0.91. Two notable bands in healthy scans were at Rf values 0.70 and 0.74 which corresponded to similar glycoproteins (γ and γA) in infected tissue (Fig. 33, C). Apart from the mainly quantitative alterations in glycoproteins following TNV-infection, one novel protein at Rf 0.38 was observed.

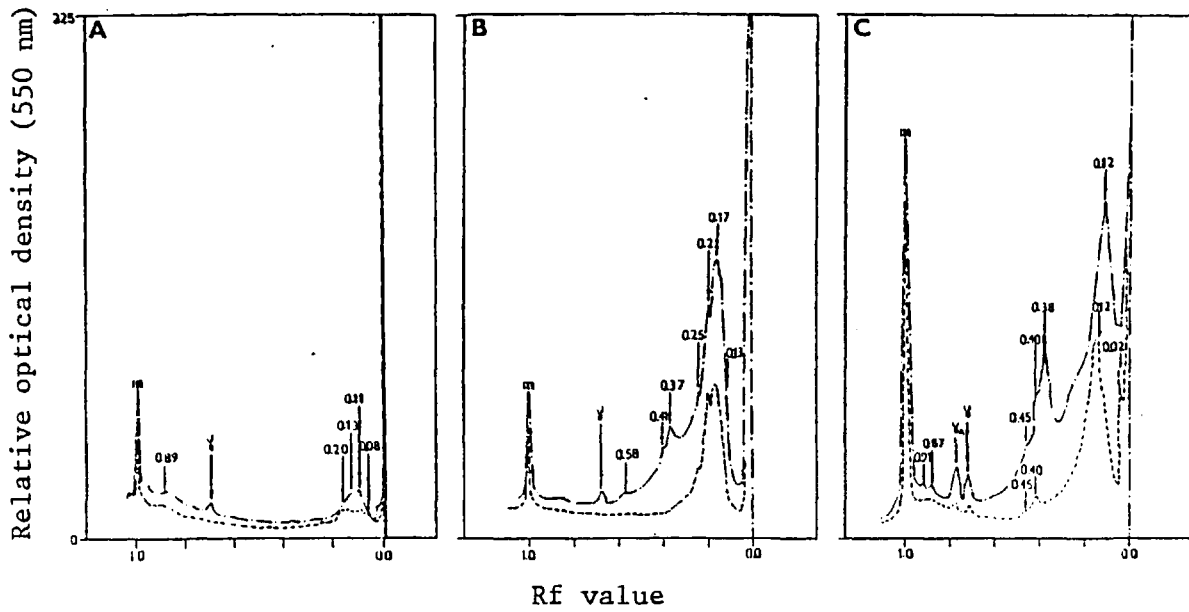


Fig. 33. Comparison of soluble glycoprotein patterns in 10% PAG loaded with 50 μ l E_2 (A) or 100 μ l E_1 (B and C) soluble protein extracts from Baton-Vert (A), Ashley (B) and Explorer (C). (—) are TNV-infected scans, and (---) are healthy control scans. m represents the bromo-phenol blue marker.

25. Determination of molecular weight(s) of γ protein subunits

The determination of the molecular weight of the γ protein fraction was carried out using SDS-cont.-PAGE and involved constructing a standard (calibration) curve (Fig. 35) as in a previous section.

Soluble proteins were extracted in E_1 -buffer from 5-days TNV-infected cotyledons as outlined in Section III. 15. A. Disc-PAGE of proteins was carried out as in Section III. 16, and both healthy and infected extracts were investigated using overloaded gels (150 μ l extract/gel). At the position in the gels where γ is known to occur (determined by rapid densitometry at 280 nm), single 2 mm slices were excised from a series of identical gels. The remainder of each gel were stained separately with Coomassie blue to ensure no contamination of the γ fraction with other proteins. The slices were then pooled and extracted as in Section III. 20. B. SDS-cont.-PAGE, staining, destaining, storage and scanning were carried out as outlined in Section III. 20. C.

The results showed that the γ protein preparation contained a single polypeptide on all gel concentrations investigated (Fig. 34, Ds). The slope of the line relating gel concentration to R_m of this band (Fig. 11) was calculated, and using the calibration curve (Fig. 35) the estimated molecular weight of γ protein monomer was 24,500 d.

26. Radio-active labelling of the γ protein

Since the γ protein was detectable in AH tissue (Fig. 31), attempts of labelling the γ protein in this tissue with radioactive precursors were performed. Leaf discs taken from the AH-tissue, 8-days post-inoculation, of the adjacent half leaf, were labelled with 10 μ Ci/ml of ^{14} C L-leucine for 4 h as described in Section III. 21. Discs taken from uninoculated half leaves ($\frac{1}{2}$ H) adjacent to the mid-rib of phosphate inoculated halves served as controls. Relative protein synthesis was

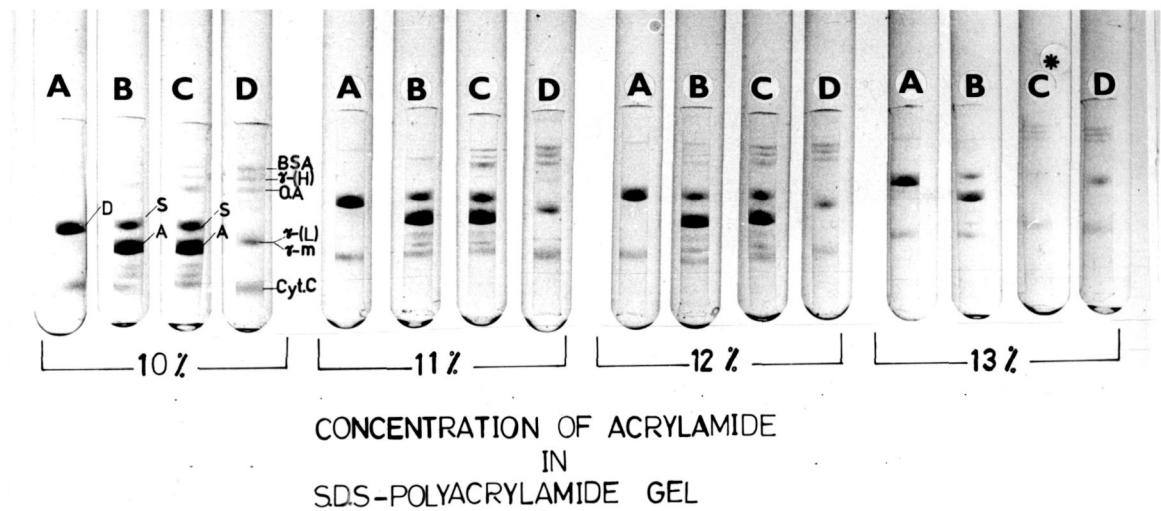


Fig. 34. Determination of the molecular weight of the γ protein by SDS-cont.-PAGE, on a range of acrylamide concentrations. All A gels were loaded with 50 μ l of TNV_D coat protein and 10 μ l of Cyt. c. All B gels were loaded with 50 μ l of (TNV_A + STNV coat proteins) and 10 μ l of Cyt. c. All C gels were loaded with 50 μ l of (TNV_A + $STNV_A$ coat proteins) and 50 μ l of standard protein mixture. All D gels were loaded with 50 μ l of γ protein and 50 μ l of standard protein mixture. The *C gel was loaded with 50 μ l of the standard protein mixture alone. Scanning of these and other similar gels made it possible to construct the curves in Fig. 11 to determine the proposed molecular weight of the γ protein monomer.

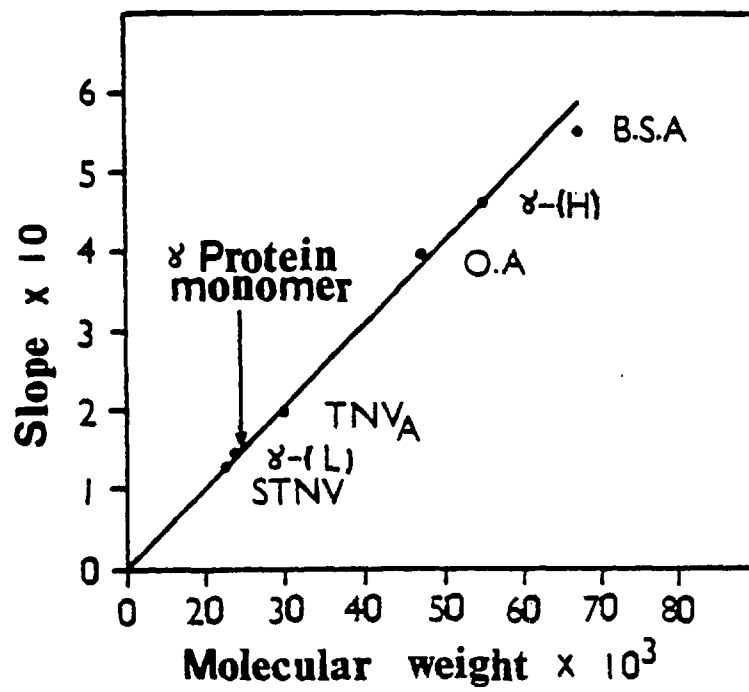


Fig. 35. A calibration curve constructed from the results shown in Fig. 11 where the slopes of the lines are proportional to the molecular weights. Slopes are expressed in arbitrary units and plotted against molecular weights.

determined as described in Section III. 21 (A and B . a). The specific incorporation (c.p.m.) of ^{14}C L-leucine was determined in 2 mm gel slices taken at the known position of the γ protein(s). Slices were taken from native gels loaded with soluble proteins from either (AH) or ($\frac{1}{2}\text{H}$) tissue immediately after electrophoresis.

The results illustrated in Fig. 36 show that total protein synthesis was greater in AH-tissue than that in $\frac{1}{2}\text{H}$ control tissue by 15.0% and that specific incorporation of ^{14}C L-leucine into protein(s) at the γ position was greater by 17.92%, when AH and $\frac{1}{2}\text{H}$ tissues were compared.

27. Induction of the γ and the γA proteins in vivo

Attempts were made to induce γ and γA in healthy cucumber cotyledons by both, biotic means (biological induction) other than TNV, and abiotic means (chemical and/or physical induction) as described in Sections III. 9 and 10.

A. Biological induction

Apart from TNV, other pathogens investigated were TMV and CMV and the fungus C. lagenarium.

a) Induction of the γ protein by C. lagenarium

C. lagenarium is the causal agent of cucumber anthracnose.

Inoculated cotyledons and leaves react hypersensitively by producing necrosis. Inoculation of Baton-Vert cotyledons was performed as described in Section III. 4 . B, and 7 to 8 days later when necrosis was complete, soluble proteins were extracted and subjected to native disc-PAGE as before (Sections III, 15. B and 16).

The results are illustrated in Fig. 37 (A and C) which show that following fungal infection the only qualitative change was the

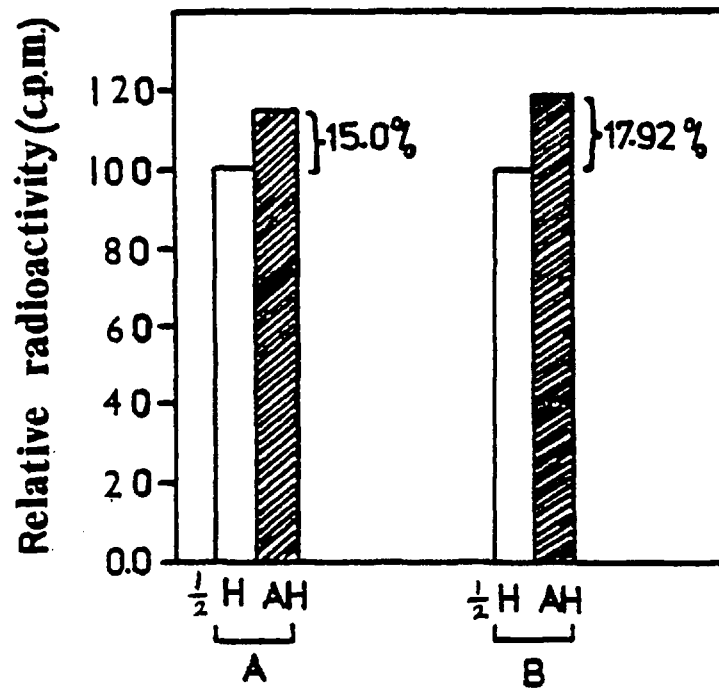


Fig. 36. Radio-active labelling of γ protein. A. Relative incorporation of ^{14}C L-leucine into soluble proteins (c.p.m. of TCA-insoluble material/c.p.m. of total uptake); B. Relative incorporation (c.p.m.) of ^{14}C L-leucine in 2 mm gel slices taken at the known R_f of γ in gels following disc-PAGE of radioactively labelled $\frac{1}{2}\text{H}$ and AH soluble protein extracts.

appearance of the γ protein at Rf 0.70 (Fig. 37, C). Co-electrophoresis of soluble proteins from TNV- and C. lagenarium-infected cotyledons gave a single band at Rf 0.70 (γ -protein).

i) Glycoprotein nature of the γ -protein induced by C. lagenarium

The γ -protein produced following fungal infection was shown to be a glycoprotein by PAS staining of sister gels (Fig. 37, A and C) as in Section III. 17. Two other glycoprotein bands were seen at Rf values 0.38 and 0.41 (Fig. 37, C). These proteins were suspected of being fungal constituents since they were absent in extracts from virus-infected tissue (Fig. 37, B).

ii) Inhibition of the γ -protein induced by C. lagenarium using actinomycin D (AmD)

Cucumber cotyledons were injected with a solution of AmD (5 μ g/ml) and inoculated 24 h later with a suspension of C. lagenarium spores (1×10^5 spores/ml) control cotyledons were injected with distilled water in place of AmD as described in Section III. 13. A. Soluble protein fractions from both sets of infected cotyledons were analysed by disc-PAGE as before (Section III. 16).

The results presented in Fig. 37 (C and D) illustrate that AmD treated tissue contained less of the γ protein than did extracts from control tissue. AmD treated tissue also contained a protein which banded at Rf 0.79 (γ_1 ; Fig. 37, D). Apart from the γ protein other glycoproteins at Rf values 0.38 to 0.41 were more abundant in fungus infected cotyledons after AmD treatment. Increased lesion size in

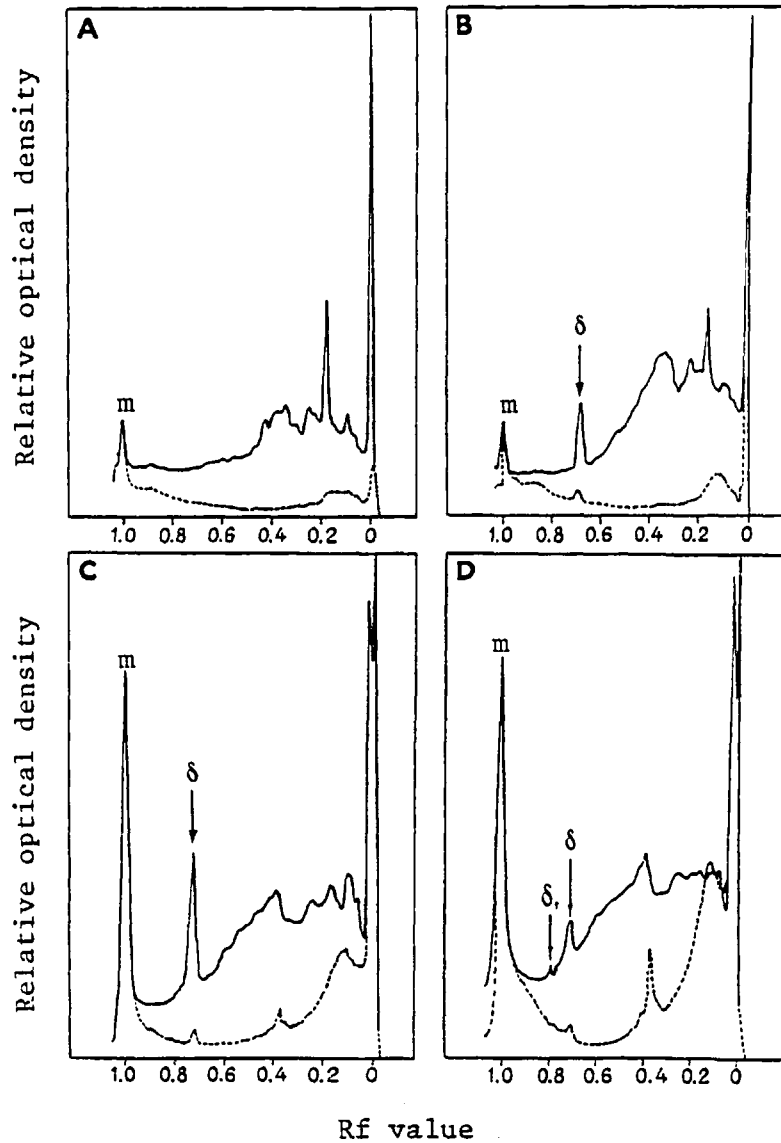


Fig. 37. Densitometer scans of 10% PAG loaded with 50 μ l E_2 soluble protein extracts from sham-inoculated healthy (A), TNV-infected (B) and C. lagenarium-infected (C and D) Baton-Vert cucumber cotyledons, 5 days post-inoculation. C is an extract from normally infected cotyledons. D is an extract of cotyledons pre-treated with AmD 24 h before inoculation with the fungus. (—) is the profiles of gels stained with Coomassie blue and scanned at 280 nm. (---) is the profiles of gels after staining with PAS and scanned at 550 nm. The bromo-phenol blue marker is indicated as m throughout.

this tissue suggested the fungal origin mentioned earlier to be true.

b) Induction of the γ -protein by TMV

Cucumber cotyledons do not react hypersensitively to infection with TMV, the virus is localised in small areas without any sign of necrosis. Instead, excess starch deposition is observed in characteristic starch lesions (Figs. 38, 39 and 40).

Following inoculation of Ashley cotyledons with a crude suspension of TMV, as described in Sections III. 3 (A. a) and 4 (A) five days after inoculation no visible symptoms of infection were noticeable when compared to controls (cf. Fig. 38 IN with H). However, when starch lesions were developed by the method outlined in Section III. 5, they were clearly visible on TMV-infected cotyledons and absent on controls (cf. Fig. 38, IN with $\bar{H}$).

Disc-PAGE of soluble proteins extracted from virus-infected and control tissue was carried out as described in Section III. 16 and the γ fraction was detected in TMV-infected cotyledons and absent from controls (Fig. 43, TMV and Fig. 41). However, the amounts found in TMV-infected tissue were less than those observed in TNV-infected tissue (cf. Fig. 43, TMV with TNV and Fig. 41 with Fig. 29, B).

c) Induction of the γ -protein by CMV

CMV_w, a strain of cucumber mosaic virus causes a systemic infection of cucumber plants. Seven days post-inoculation of cotyledons with either CMV_w or phosphate buffer, soluble protein fractions were extracted and analysed by disc-PAGE as in Sections III, 15. A and 16.

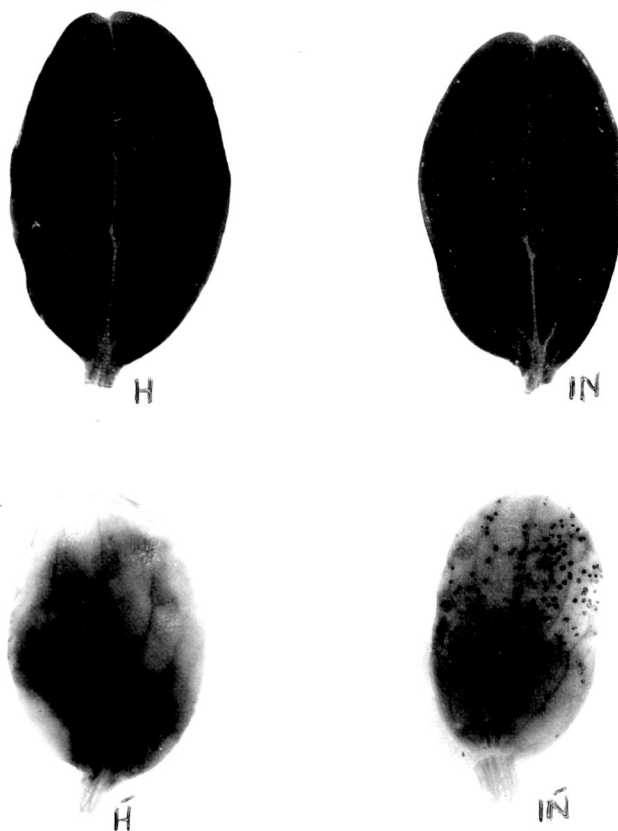


Fig. 38. Ashley cucumber cotyledons, sham and TMV-infected, 6 days post-inoculation.

H = phosphate buffer, sham-inoculated, control;

IN = TMV-inoculated cotyledon.

H and IN are similar cotyledons to the above, both decolorised and stained for starch deposition, note starch lesions in the IN sample.

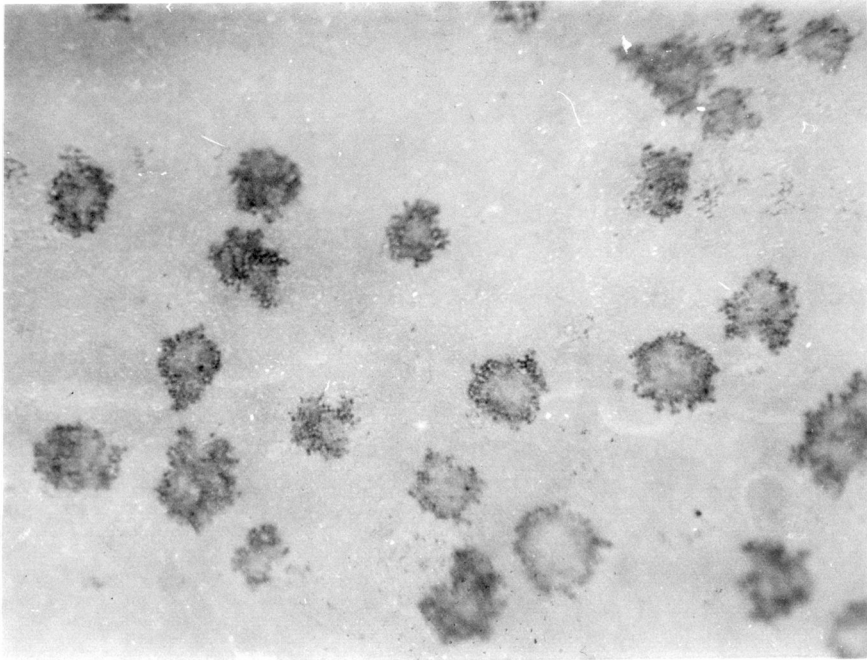


Fig. 39. Low power microscopic view of a portion of a decolorized, iodine stained, TMV-infected Ashley cucumber cotyledon showing a group of starch lesions following infection with TMV, 6 days after inoculation.

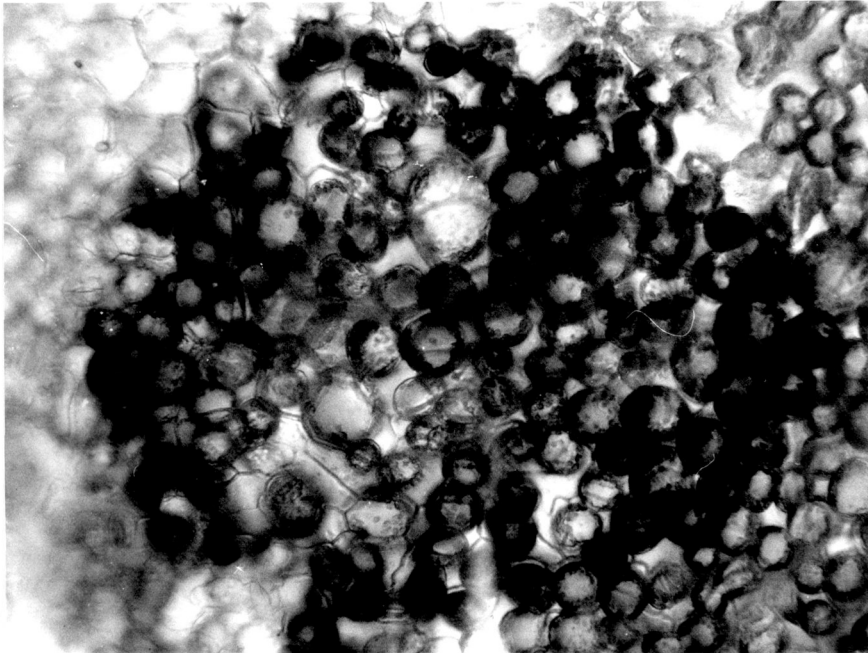


Fig. 40. High magnification of a single starch lesion showing accumulation of starch grains within mesophyll cells particularly those at the periphery of the lesion as seen by light microscopy.

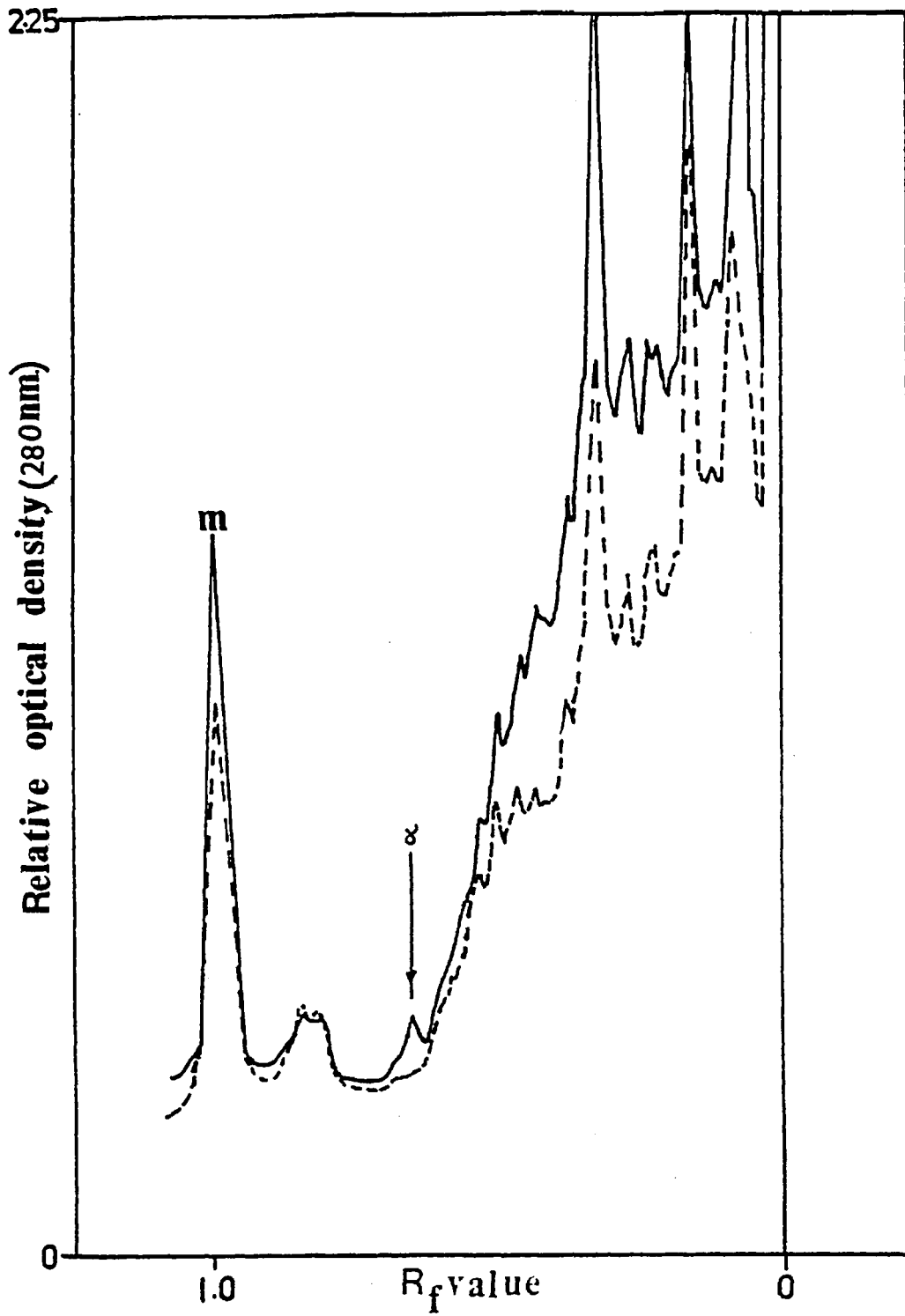


Fig. 41. Densitometer scans of soluble protein profiles in 10% PAG loaded with 100 μ l of 18 K supernatant of E_1 -extract from Ashley cotyledons, infected with TMV (—) or inoculated with phosphate buffer, pH 7.0 (---), 6 days post-inoculation. m represents the bromo-phenol blue marker.

The γ protein was not detectable in gels loaded with 100 μ l of a soluble protein extract prepared from CMV-infected cotyledons (Fig. 43, CMV).

B. Chemical and/or physical induction

Two abiotic means were used in an attempt to induce γ and/or γ A proteins in healthy cucumber cotyledons, anhydrous copper sulphate, CuSO_4 , solutions and plasmolysis on 0.5 M mannitol (see Sections III. 9 and 10).

a) Induction of the γ and the γ A proteins by CuSO_4

Copper sulphate solutions are known to imitate the hypersensitive reaction by causing necrosis when applied to plant leaves (Section III. 9). A 1000 ppm CuSO_4 solution caused a severe local necrosis that penetrated through to the lower epidermis when small droplets of the chemical were applied evenly over the upper surface of cotyledons of two cultivars of cucumber, Baton-Vert and Explorer. Three days after treatment the soluble proteins of both CuSO_4 - and water-(control) treated cotyledons were extracted and analysed by disc-PAGE (Sections III, 15. A and 16). Gels were then stained and destained as indicated in Section III. 16 and densitometer scans were compared.

The results can be summarised as follows.

i) Baton-Vert

The γ protein was produced in healthy Baton-Vert cotyledons following treatment with CuSO_4 as a band at R_f 0.70 which appeared on the densitometer scans of treated cotyledons but was absent from water-treated controls (Fig. 42, A).

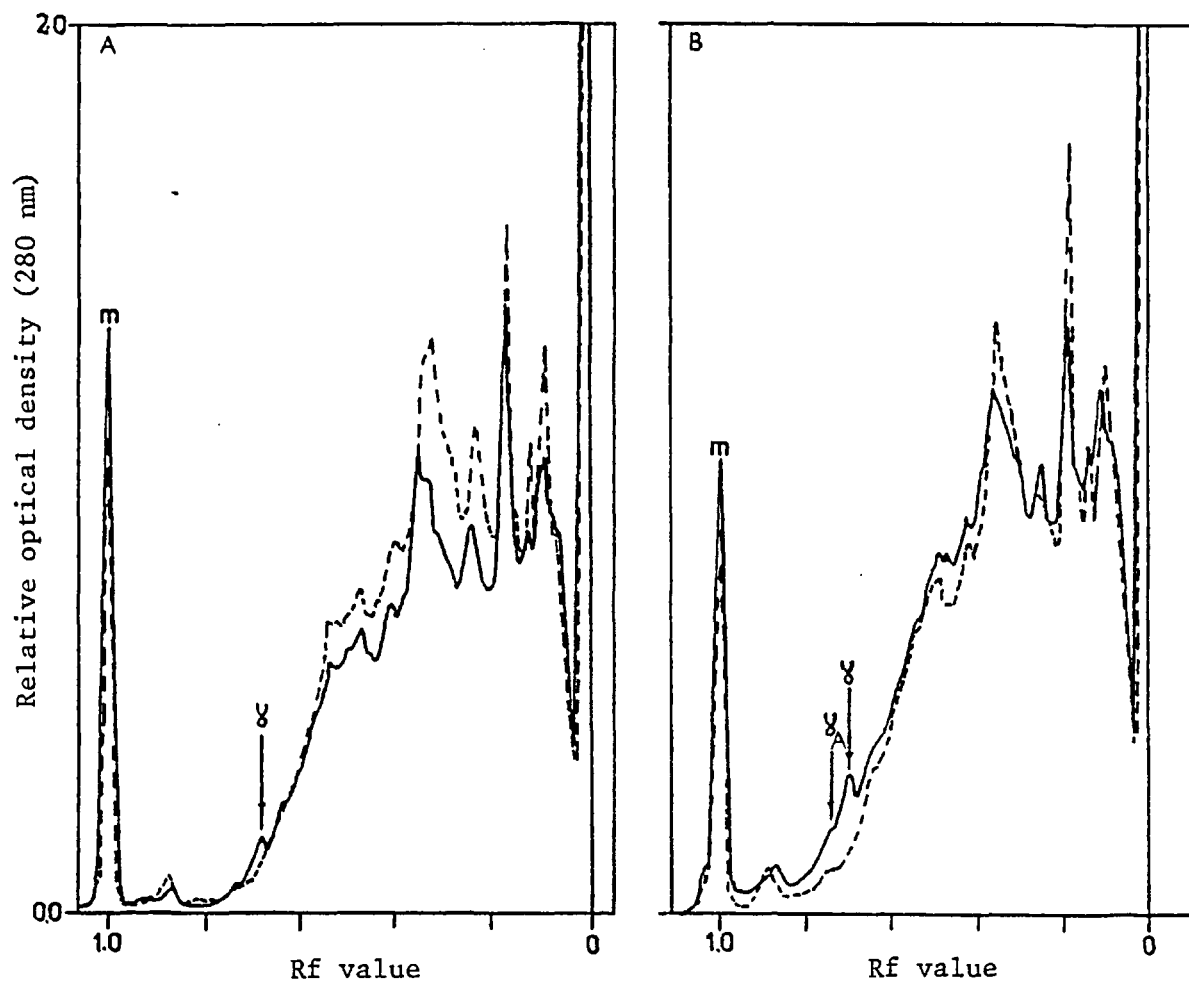


Fig. 42. Comparison of densitometer scans of 10% PAG loaded with 100 μl E_1 -soluble protein extracts from Baton-Vert (A) or Explorer (B) cucumber cotyledons treated with either 1000 ppm CuSO_4 (—) or water (---), 3 days after treatment. m indicates the position of bromo-phenol blue marker.

ii) Explorer

In the Explorer cultivar, only the γ protein (Rf 0.70) was produced in healthy cotyledons following the CuSO_4 treatment. The γA protein (Rf 0.74) was not stimulated (Fig. 42, B).

b) Induction of the γ and the γA proteins by plasmolysis on mannitol solutions

Healthy cucumber cotyledons of the Ashley, Baton-Vert and Explorer cultivars were detached and floated on either distilled water or 0.5 M mannitol solution for 3 days as described in Section III, 10. Soluble proteins were extracted in E_1 buffer and analysed by disc-PAGE as before (Sections III, 15. A and 16). Densitometer scans of gel profiles were then compared.

The results are summarised in the following.

i) Ashley

In the Ashley cultivar, plasmolysis on 0.5 M mannitol caused an overall enhancement of soluble protein content. The only qualitative alteration was the appearance of one 'novel' band at Rf 0.70, γ (Fig. 43, Mann., and Fig. 44, A).

ii) Baton-Vert

In Baton-Vert cultivar, quantitative increases were not as marked as in the above Ashley variety, but a similar qualitative alteration was the appearance of one 'novel' band at Rf 0.70, γ (Fig. 44, B).

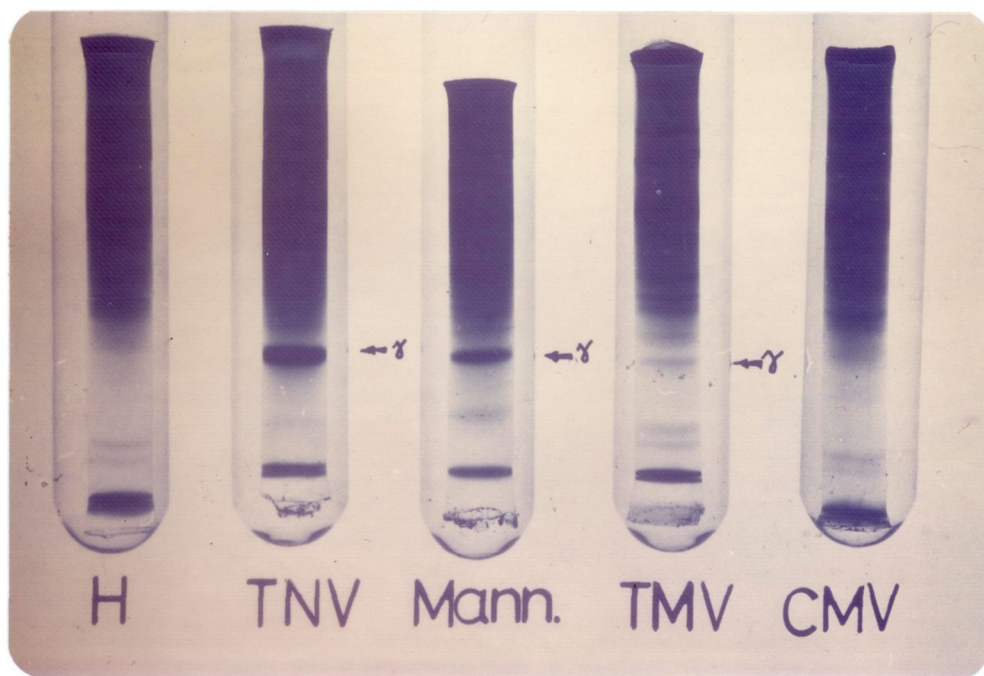


Fig. 43. Comparison of the disc.-electrophoretic patterns of 100 μ l of E_1 soluble protein extracts in 10% PAG, stained with Coomassie blue, for a) phosphate buffer-inoculated healthy (H), b) 6 days TNV-infected (TNV), c) 3 days mannitol-floated (Mann.), d) 5 days TMV-infected (TMV) and e) 7 days CMV-infected (CMV) Ashley cucumber cotyledons.

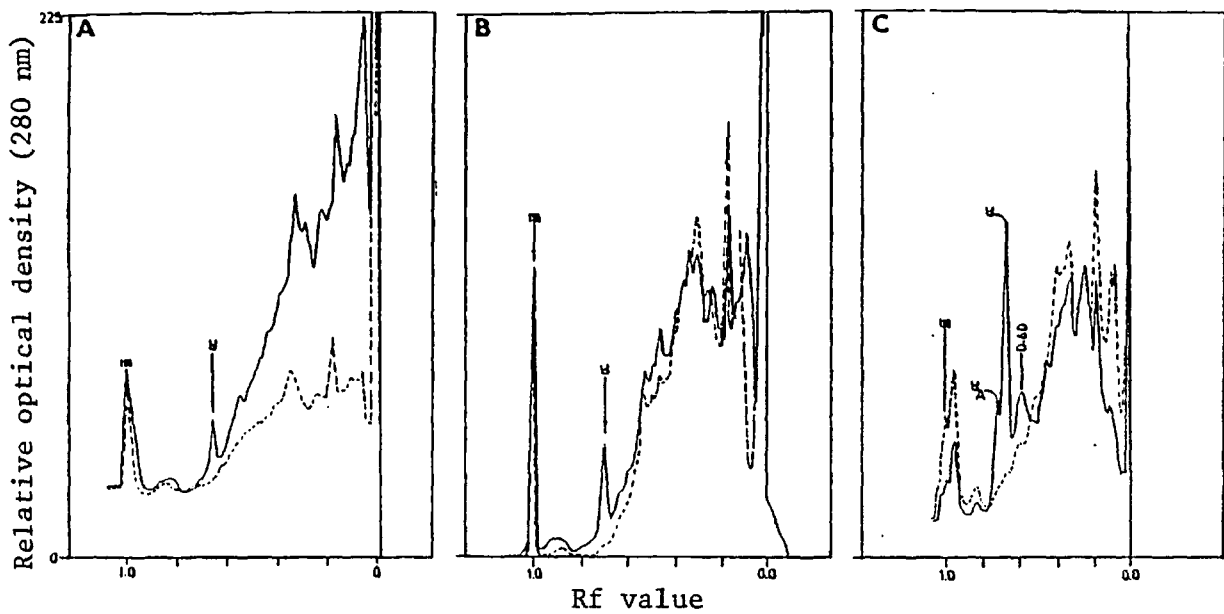


Fig. 44. Electrophoretic pattern of 100 μ l E_1 -soluble protein extracts prepared from Ashley (A), Baton-Vert (B) and Explorer (C) cucumber cotyledons floated on 0.5 M mannitol (—) or distilled water (---) for 3 days. m is the bromo-phenol blue marker.

iii) Explorer

In the Explorer cultivar, following plasmolysis, the major changes in the electrophoretic pattern were the appearance of two bands, γ (Rf 0.70) and γA (Rf 0.74). However, γA was not as strongly stimulated as γ was (Fig. 44, C).

In addition to γ and γA there was a rather reproducible protein band at Rf 0.60.

28. Time course of γ production in Ashley cucumber cotyledons following mannitol-induced osmotic stress

Following the previous observations that osmotic stress induces the γ protein in healthy cucumber cotyledons (Section IV, 27. B. b, and Fig. 43, Mann.), further studies on a time course of its appearance were made by disc-PAGE of soluble proteins. Quantification of the amount(s) of γ protein present were made by area measurements of the densitometer peaks as outlined in Section III, 11.

The results are presented in Fig. 45 which show that at zero time no γ band (Rf 0.70) was visible in gels but a trace amount was detectable by densitometry, which constituted the small peak appearing reproducibly on densitometer scans of soluble proteins from healthy cotyledons. After 12 h of exposure to mannitol, no reproducible alterations in the amount of the γ protein were distinguishable by densitometry apart from the constituent γ , mentioned above. After this time (12 h after initial plasmolysis) the level of γ protein increased and carried on increasing through 1 and 2 days of treatment reaching a plateau level, 3 days after initiation of the osmotic stress.

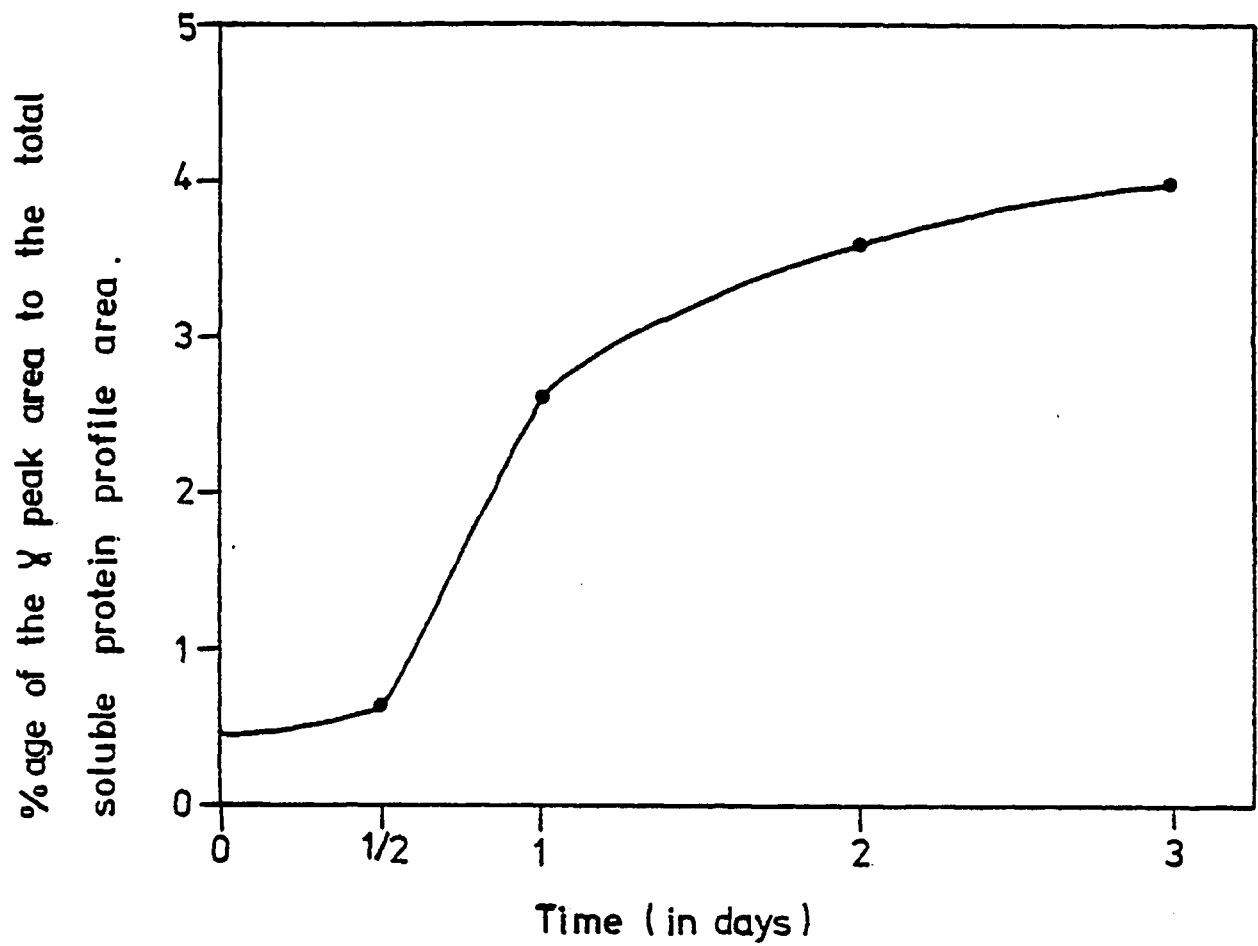


Fig. 45. Time course of γ production in Ashley cucumber cotyledons exposed to osmotic stress exerted by 0.5 M mannitol solutions.

29. Quantitative changes in the amounts of the γ and the γ A proteins in Explorer cotyledons following either virus infection and floatation on water or plasmolysis of healthy cotyledons on 0.5 M mannitol : the effect of chloramphenicol

Detached cotyledons, TNV or phosphate buffer inoculated, were floated on either distilled water or 0.5 M mannitol (Section III. 10). Chloramphenicol (Ch. Ph.) was included at 750 μ g/ml and omitted from the controls.

The results showed that simply detaching virus-infected cotyledons and floating them on water, apparently reduced the amounts of the γ and γ A proteins in infected tissue (cf. Fig. 46, A with 29, C).

The inclusion of the antibiotic (Ch. Ph.) into the solutions on which the cotyledons were floated further reduced the peak sizes of γ and γ A induced by either virus infection (cf. Fig. 46, A with C) or osmotic stress (cf. Fig. 46, B with D).

30. Separation of peroxidase (PO) and polyphenol oxidase (PPO) isozymes in the soluble protein fractions from healthy and virus-infected cotyledons and investigating their potential relationship(s) to the γ and the γ A proteins

Disc-PAGE of soluble proteins from virus-infected and control cotyledons of both the Baton-Vert and Explorer cultivars was carried out as described in Section III. 16. Gels were then either stained for total proteins with Coomassie blue or developed as zymograms for either PO or PPO as outlined in Sections III. 16 and 18.

Results are presented in the form of densitometer scans (Fig. 47, A, B, C and D). The densitometer scans were partitioned into three zones for easier analysis. Zone III represents Rf 0.00 to 0.19, zone II, Rf 0.20 to 0.59 and zone I, Rf 0.60 to Rf 1.00. Reference to these zones is recommended for illustrating specific Rf alterations, and comparing them to Coomassie blue stained gels.

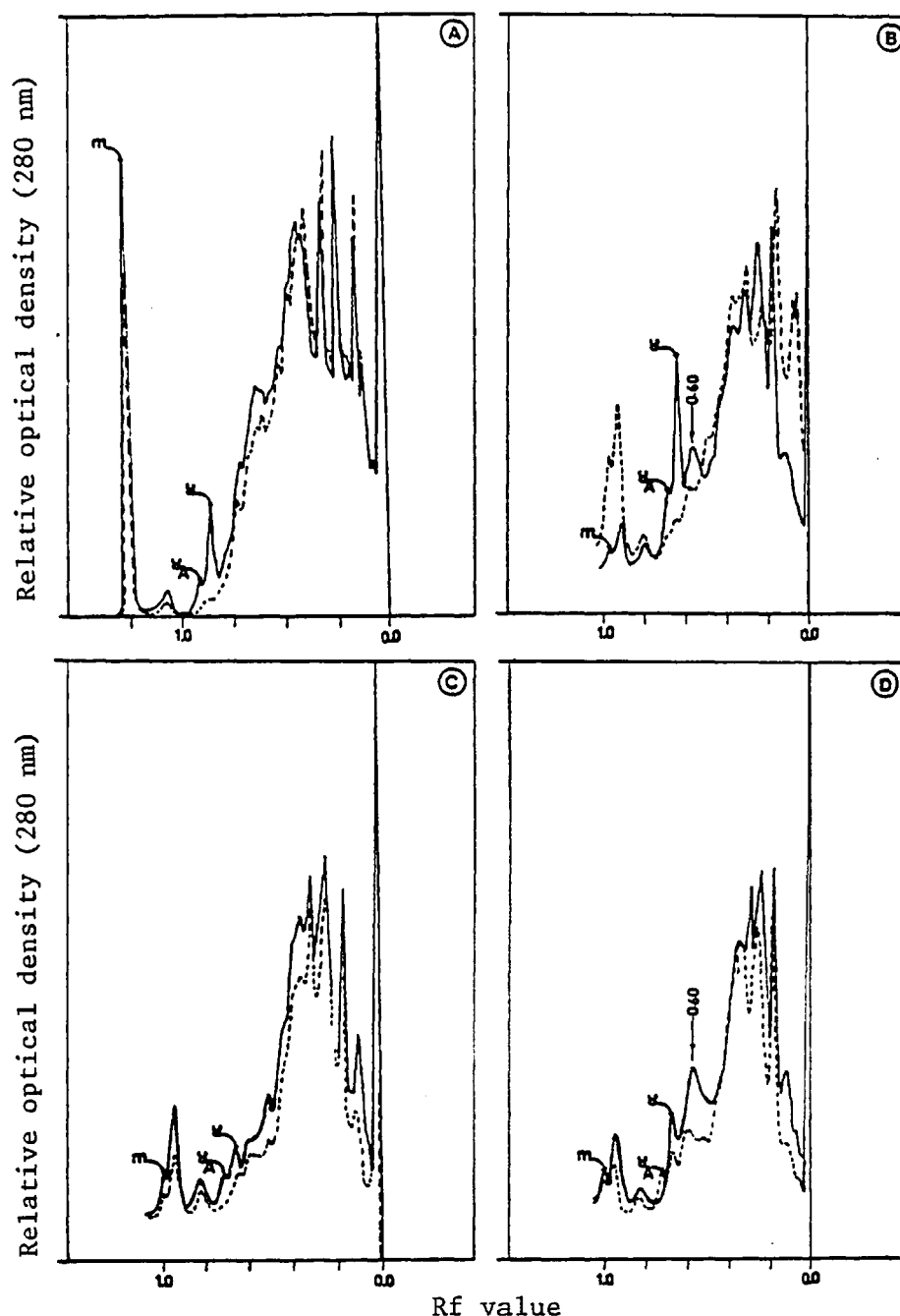


Fig. 46. Densitometer scans of soluble protein profiles in 10% PAG loaded with 50 μ l of E_1 soluble protein extracts from healthy, virus-infected and plasmolysed Explorer cucumber cotyledons. A is a comparison of virus-infected (—) to healthy (---) cotyledons floated on water for 5 days post-inoculation. B is a comparison of healthy cotyledons floated on 0.5 M mannitol (—) to water-floated cotyledons (---) 3 days after treatment. C is the same as A with Ch. Ph. included in the solution at 750 μ g/ml. D is the same as B with Ch. Ph. included as above. All scans represent Coomassie blue stained gels scanned at 280 nm. The bromo-phenol blue marker is indicated as m throughout.

The results indicated that, reproducible quantitative and variable qualitative alterations occurred in both cultivars following viral infection. In the following, the results obtained following analysis of PO and PPO zymograms are considered for each cultivar.

A. Baton-Vert

a) PO-zymogram analysis

Fig. 47 (A) illustrates that in healthy tissue, most isozymes were found in zone II with Rf peak(s) indicated (e.g. Rf 0.42), slightly lower activity was found in zone III with a major peak at Rf 0.17, whilst no detectable isozymes were found in zone I. Following virus infection the overall effect was to cause enhancement of isoperoxidase activity with a possible new isozyme at Rf 0.72 present in minor quantities. The increases in PO activity were clear in both zones II and III. No isozyme was found to be coincident with γ band at Rf 0.70.

b) PPO-zymogram analysis

Fig. 47 (B) illustrates that in healthy tissue most PPO activity is confined in zone II, with a peak at Rf 0.38 with less activity found in zone III. Following virus infection dramatic increases in activity were seen in zone II with a pronounced peak at Rf 0.29. This peak virtually obscured all other quantitative and/or qualitative changes and was shouldered by other peaks in zone II and III. Little activity was seen in zone I, but no PPO isozymes were coincident with the γ -band at Rf 0.70.

B. Explorer

a) PO-zymogram analysis

Fig. 47 (C) illustrates that in healthy tissue the isozyme pattern was similar to that in healthy Baton-Vert in that most of the isozymes were found in zone II with maximum activity at Rf value 0.36. Slightly lower activity was found in zone III with two main peaks at Rf 0.10 and 0.18. Following virus infection the overall effect was to cause a general enhancement of isozyme activity particularly in zone II between Rf value 0.39 - 0.54. Little PO activity was found in zone I with only a small shoulder peak at Rf 0.72. However, no PO isozymes were found to be coincident with either the γ or the γ_A protein.

b) PPO-zymogram analysis

Fig. 47 (D) shows that most PPO isozymes were found in zones II and III with some smaller activity in zone I. Following virus infection dramatic increases in activity were seen in zone II with a peak at Rf 0.31 masking all other quantitative and qualitative changes as in the Baton-Vert cultivar. This peak was shouldered by other peaks in zones II and III. No PPO isozymes were found at the same Rf values corresponding to either the γ or the γ_A protein.

31. Comparison of PO and PPO zymograms of uninoculated, phosphate buffer-inoculated, and TNV-infected Ashley cotyledons, 3 days following inoculation

For later comparison with the effect of plasmolysis on the isozyme and levels of PO and PPO in tissue, a comparative study of the effect of the above was made, viz. uninoculated (unabraded), phosphate-buffer, sham-

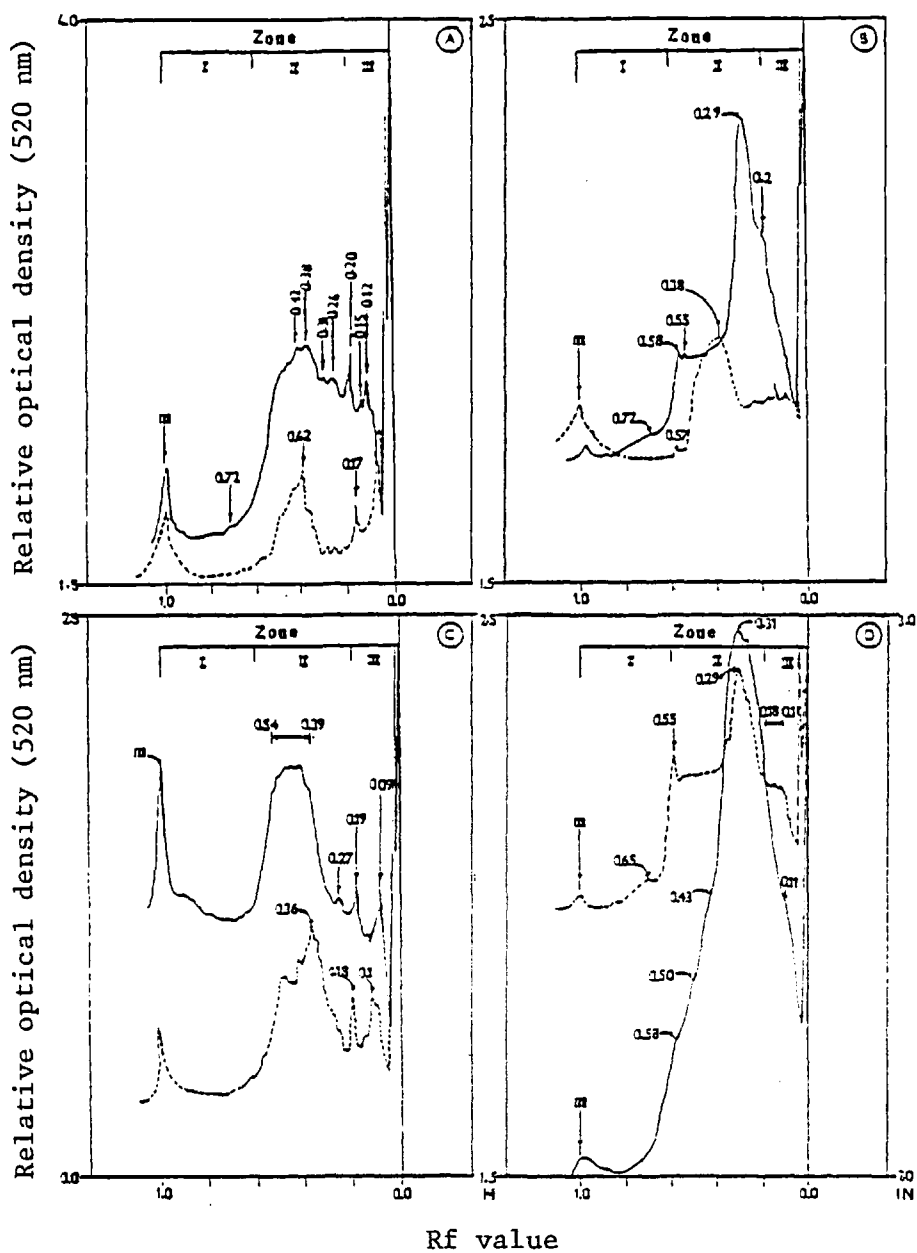


Fig. 47. PO (A and C) and PPO (B and D) zymograms developed in 10% PAG loaded with 50 μ l E_2 -soluble protein extracts prepared from TNV-infected (—) and phosphate buffer-inoculated healthy (----) Baton-Vert (A and B) and Explorer (C and D) cucumber cotyledons, 5 days after inoculation. Arrows indicate isoforms of interest. m is the bromo-phenol blue marker.

inoculated (abraded), and TNV-infected (virus-infected), cucumber Ashley cotyledons. Disc-PAGE of soluble proteins and zymogram analysis was carried out as in the previous section. No zonation was made in this section as comparisons were possible without partitioning zymograms.

The results can be summarised as follows.

A. PO-zymogram analysis

Fig. 48 (A) shows that the main PO isozymes in the uninoculated healthy cotyledons were at Rf values 0.03, 0.06 and in a broad band resolved by densitometry into two isozymes at Rf 0.36 and 0.44. Following mechanical injury (abrasion), only quantitative increases in the isozymes at Rf 0.36 and 0.44 were seen. After TNV-infection the changes in the isozyme pattern were more dramatic but similar, overall to the effects of mechanical injury. Moreover, the broad band in the TNV-infected cotyledons, illustrated above, was resolved by densitometry into four peaks at Rf values 0.36, 0.40, 0.44 and 0.48. At least one of these peaks (at Rf value 0.48) is thought to represent a novel isozyme.

B. PPO-zymogram analysis

Fig. 48 (B) shows that the isozyme pattern of the unabraded healthy cotyledons occupied the first half of the gel in a broad band (between Rf values 0.1 - 0.5) which was resolved by densitometry into several component peaks, the most active being at Rf values 0.32 and 0.43. Following mechanical injury there were some increases and decreases in the activity of PPO isozymes with a broad band of activity between Rf values 0.32 and 0.50. However, following TNV-infection there were dramatic increases in the PPO isozyme activity peaking at Rf 0.27. A possible novel PPO isozyme band was seen at Rf 0.55.

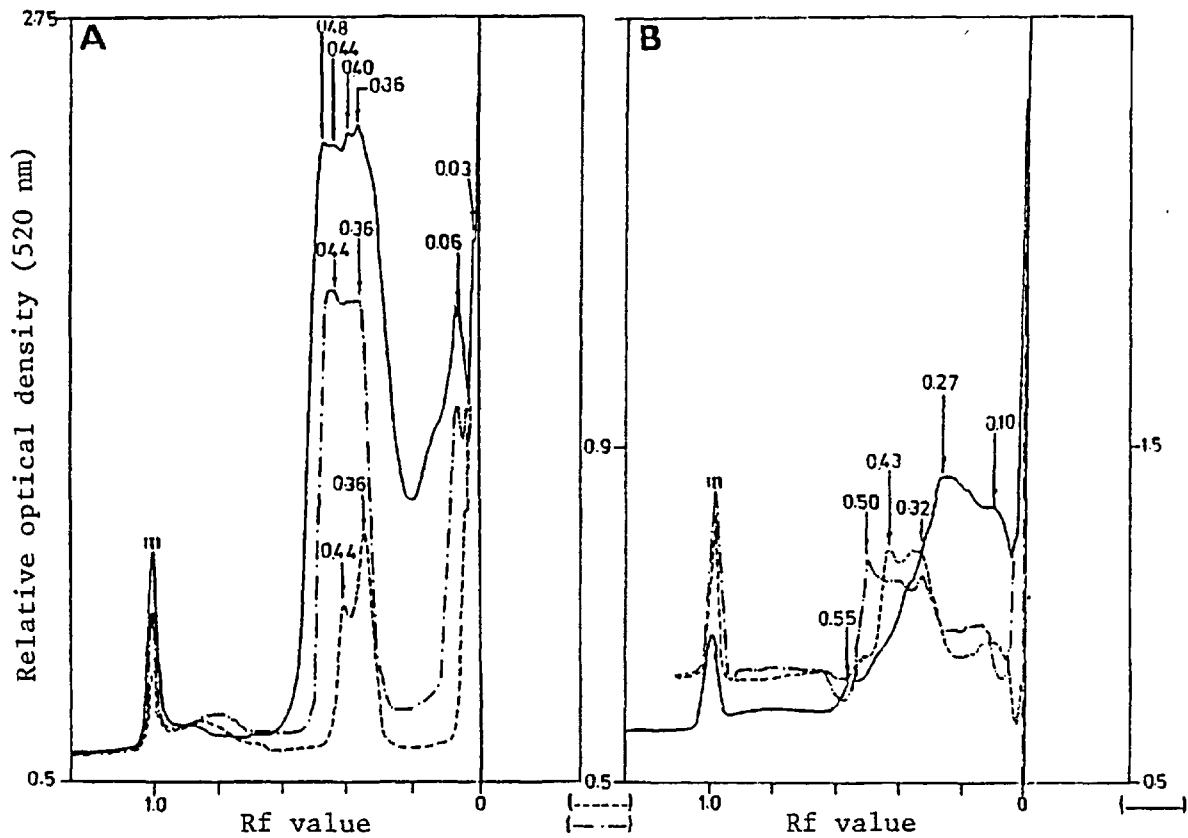


Fig. 48. PO (A) and PPO (B) zymograms developed in 10% PAG loaded with 100 μ l of E_1 soluble protein extracts prepared from unabraded healthy (----), abraded healthy (— · —) and TNV-infected (——) Ashley cucumber cotyledons, 3 days post-inoculation. Arrows indicate Rf values of interest. m is the bromophenol blue marker.

32. Analysis of PO and PPO zymograms of healthy Ashley cucumber cotyledons floated on either H₂O or mannitol

Since mannitol-induced osmotic stress imitated TNV-infection in healthy cucumber cotyledons by stimulating (or inducing) the γ protein, it was of interest to study the effects of osmotic stress on the patterns of both PO and PPO isozymes.

The experiments were carried out using healthy Ashley cotyledons as outlined in Sections III. 10 and 18.

A. PO-zymogram analysis

Following mannitol-induced osmotic stress, both quantitative and qualitative changes in the PO isozyme pattern were seen. The soluble proteins from healthy cotyledons floated on water (Fig. 49, A) contained PO isozymes which occupied the first half of the gel with the major activity seen as a broad peak at Rf 0.35 shouldered by two other peaks at Rf values 0.38 and 0.41. Minor peaks are indicated on the densitometer scans.

Subjecting cotyledons to mannitol-induced osmotic stress for 3 days resulted in isozyme pattern dissimilar to that seen following TNV-infection (cf. Fig. 49, A with Fig. 48, A). In plasmolysed tissue the activity was 'shifted' to the top of the gel, i.e. to the right hand side of the broad peak seen in water floated cotyledons with some possible new isozymes at Rf values 0.24, 0.29 and 0.32. However, no PO isozyme was coincident with the γ protein at Rf 0.70.

B. PPO-zymogram analysis

Plasmolysis, caused by mannitol, resulted in both quantitative and qualitative changes in the PPO isozyme pattern somewhat similar to those caused by TNV-infection.

In extracts from healthy cotyledons floated on water (Fig. 49; B)

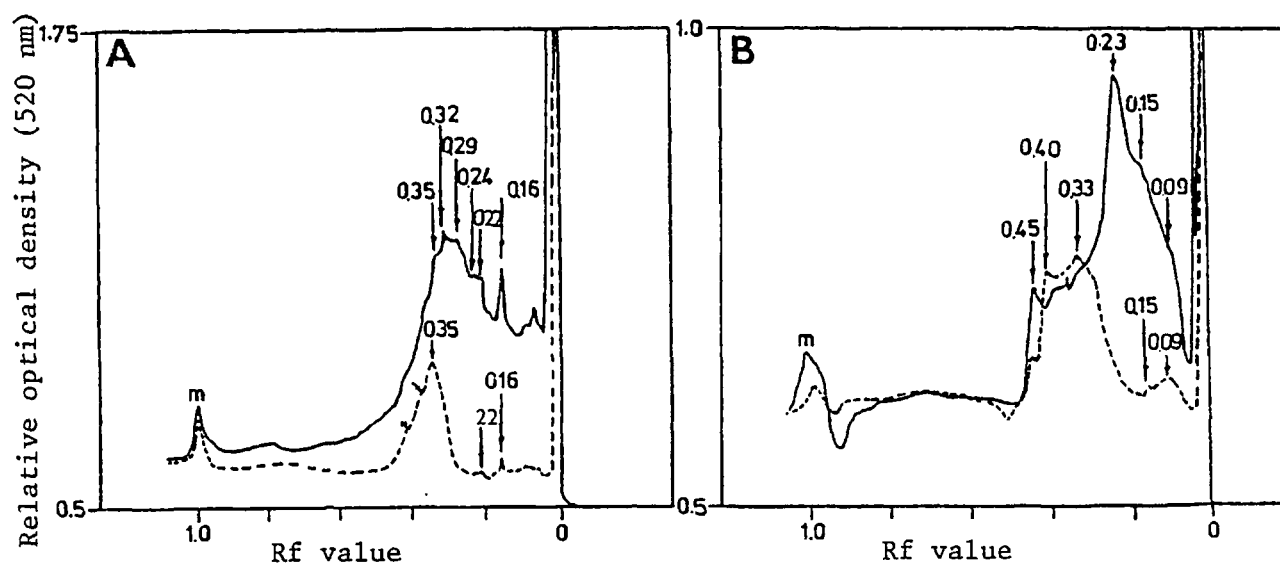


Fig. 49. Comparison of PO (A) and PPO (B) zymograms developed in 10% PAG loaded with 100 μ l E_1 -soluble protein extracts prepared from healthy Ashley cucumber cotyledons floated on 0.5 M mannitol (—) or distilled water (----) for 3 days. m is the bromo-phenol blue marker.

the activity of the PPO isozymes occupied the first half of the gel with major activity seen as a broad band which was resolved into two bands at Rf values 0.33 and 0.40. This broad band was headed by a faster migrating isozyme at Rf 0.45 which appeared as a shoulder protein.

Following a 3 days osmotic stress, alterations in PPO isozyme pattern was similar to those seen following TNV-infection (cf. Fig. 49, B with Fig. 48, B) in that the peak activity was shifted to the top of the gel, i.e. to the right hand side of the broad band seen in water-floated cotyledons reaching a maximum at Rf 0.23. This peak activity at Rf 0.23 may be a novel PPO isozyme which was shouldered with other isozymes on the side near to the top of the gel. As before, no PPO isozyme was found to be coincident with the γ protein at Rf 0.70 in plasmolysed tissue.

33. Separation of RNase isozymes present in soluble protein fractions of healthy and TNV-infected cucumber cotyledons and determination of their potential relationship with the γ protein

Disc-PAGE of soluble proteins from virus-infected and control cotyledons of the Ashley variety was carried out as described in Section III. 19. Gels were either stained for total proteins, or undegraded polynucleotide as outlined in Sections III. 16 and 19 and densitometer scans were compared.

The results summarised in Figs. 50 and 51 showed that in TNV-infected cotyledons, 6 days post-inoculation, one RNase isozyme was seen at the Rf value 0.70 which was coincident with the γ protein (Fig. 50, PNG, IN and Fig. 51). Interestingly, the same isozyme was found in gels loaded with healthy fractions (Fig. 50, PNG, H and Fig. 51). The activity of this RNase isozyme was almost completely inhibited in sister gels heated at 100°C for 10 mins. (Fig. 50, PNG, H⁰ and IN⁰).

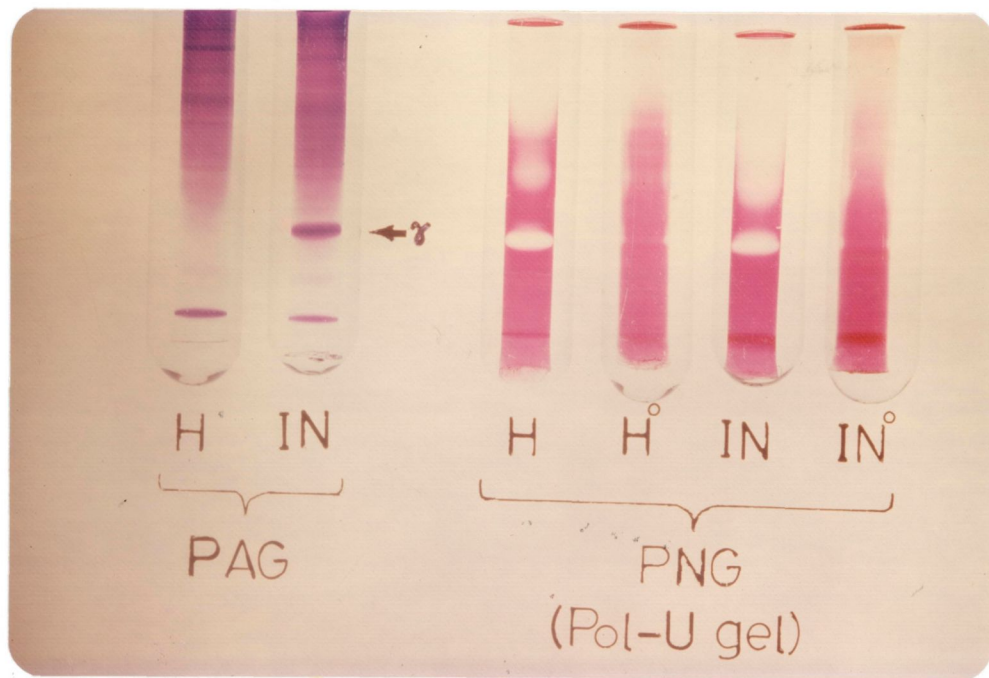


Fig. 50. Comparison of the disc-electrophoretic patterns of soluble proteins, stained with Coomassie blue from both healthy and TNV-infected Ashley cucumber cotyledon in 10% PAG (PAG) and RNase isozymes patterns in 10% PAG containing 0.05% polyuridylic acid (PNG = Poly-U), stained with pyronin Y.

H = Healthy.

IN = TNV-infected.

H^o, IN^o = The same as the corresponding H and IN in the PNG group but heated to 100°C for 10 min in 7% acetic acid to inactivate enzymes (control).

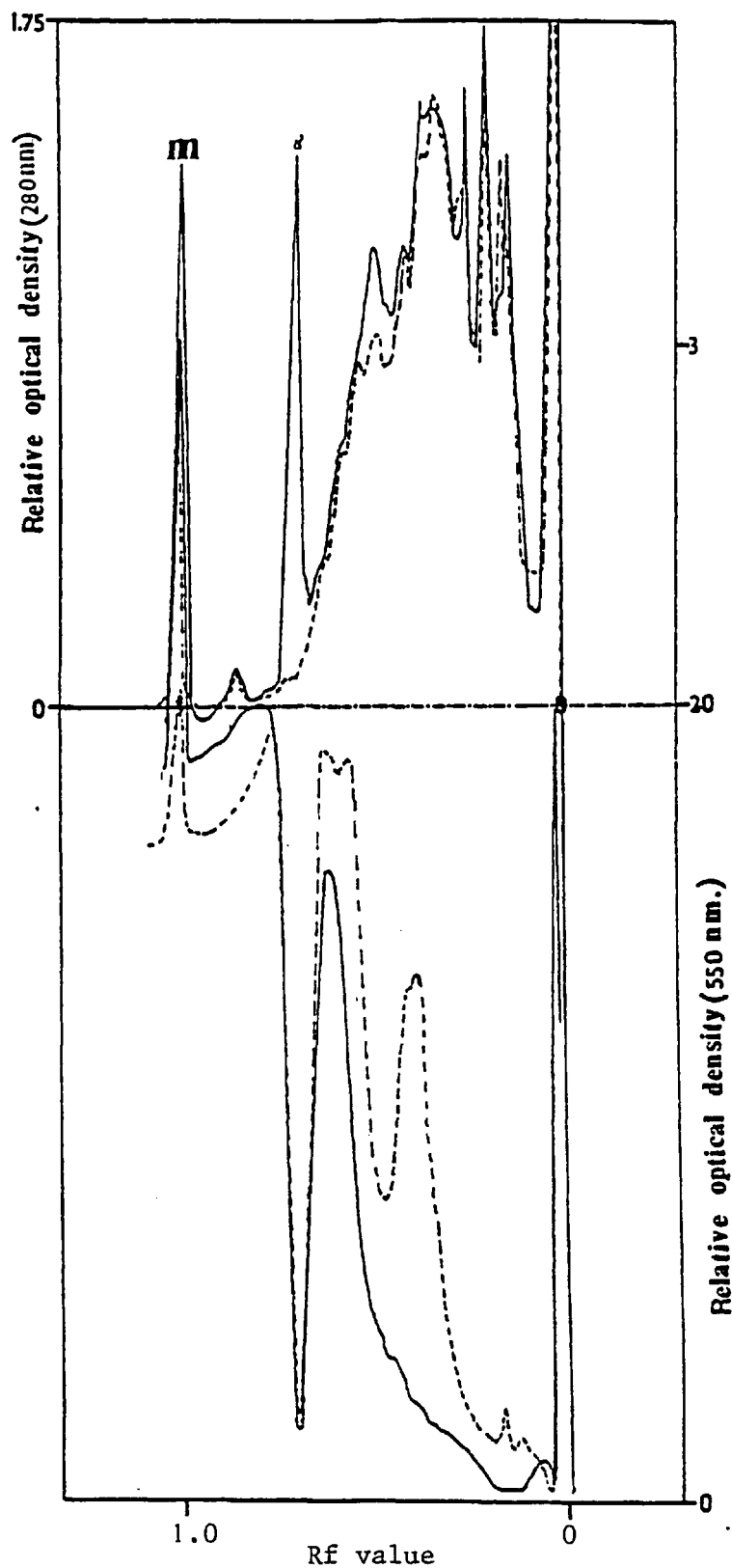


Fig. 51. A mirror image comparison of electrophoretic pattern of 100 μ l soluble protein fraction of TNV-infected (—) and phosphate-inoculated healthy (----) Ashley cucumber cotyledons in 10% PAG stained with Coomassie blue (above) and a RNase zymogram in 10% poly-U gels stained with pyronin Y (below). Infected (—) and healthy (----) scans were obtained by scanning gels similar to H and IN gels presented in Fig. 50 . m is the bromo-phenol blue marker.

34. Comparison of the RNase zymograms developed from soluble protein fractions of uninoculated healthy, mechanically injured, TNV-infected, water-floated, and mannitol-plasmolysed Ashley cucumber cotyledons

As with the PO and PPO isozymes a comparative study of the effects of the above treatments was made using PIGE technique (Section III. 19) with gels developed for RNase zymograms.

The results are presented in Figs. 52 and 53, a, b, c, d and e.

Major RNase isozymes were seen at Rf 0.65 in osmotically stressed cotyledons (Fig. 52, Mann. and 53, E), and at Rf 0.70 in all other tissue, including uninoculated (untreated) controls, the isozyme at Rf 0.65 appeared on these later zymograms as a shoulder peak (Fig. 53, a, b and c). In water-floated cotyledons, the isozyme shoulder at Rf 0.65 was difficult to resolve by densitometry (Fig. 53, d). In virus infected cotyledons, in an earlier section, this shoulder peak at Rf 0.65 may not have been detectable after Coomassie blue staining of gels, since it may have been masked by the main isozyme band at Rf 0.70, at least in tissue taken 6 days post-inoculation.

Apart from those isozymes of RNase, near to and at the position of the γ protein, other RNase activities were confined to the upper portion of the gels, in a broad region extending from Rf 0.04 to Rf 0.50. This broad region of activity was partially resolved into a series of isozyme peaks and sub-divided areas (Fig. 53, a, b, c, d and e) after the various above initial treatments of the cotyledons. In untreated (Fig. 53, a) and mechanically injured (Fig. 53, b) healthy tissue, two sub-divided areas of activity from Rf values 0.04 to 0.15, and Rf values 0.19 to 0.44 (0.41 in mechanically injured tissue) were seen. Both extracts contained an isozyme which had peak activity at Rf 0.50. Similarly, water-floated tissue had this isozyme peak with its sub-divisions of activity split between Rf values 0.04 to 0.15 and Rf values 0.19 to 0.36 (Fig. 53, d). Both virus-infected and osmotically stressed cotyledons contained much enhanced levels

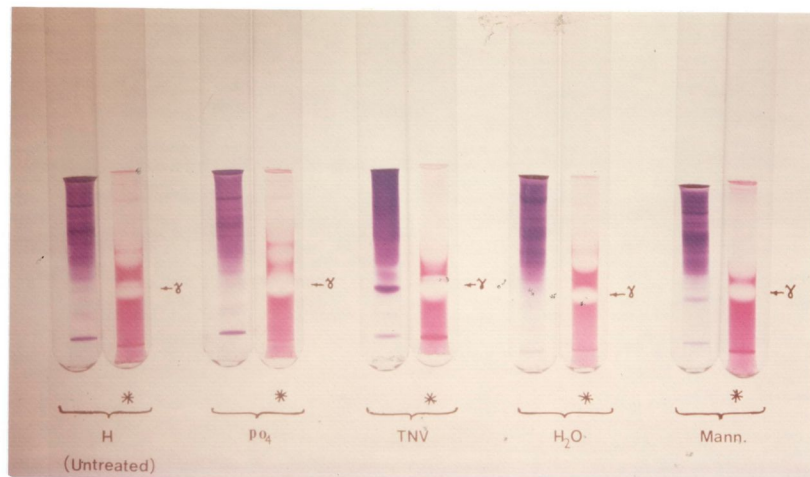


Fig. 52. Comparison of the disc-electrophoretic patterns of 100 μ l E_1 -soluble protein fractions of healthy uninoculated (untreated), phosphate-carborundum inoculated (PO_4), TNV-infected (TNV), water-floated (H_2O), and mannitol-floated (Mann.) Ashley cucumber cotyledons, 3 days after treatment, in 10% PAG stained with Coomassie blue and RNAse isozyme patterns in 10% PAG containing 0.05% polyuridylic acid (PNG=Poly-U gels), and stained with pyronin Y. (PNGs are marked with Asterisks).

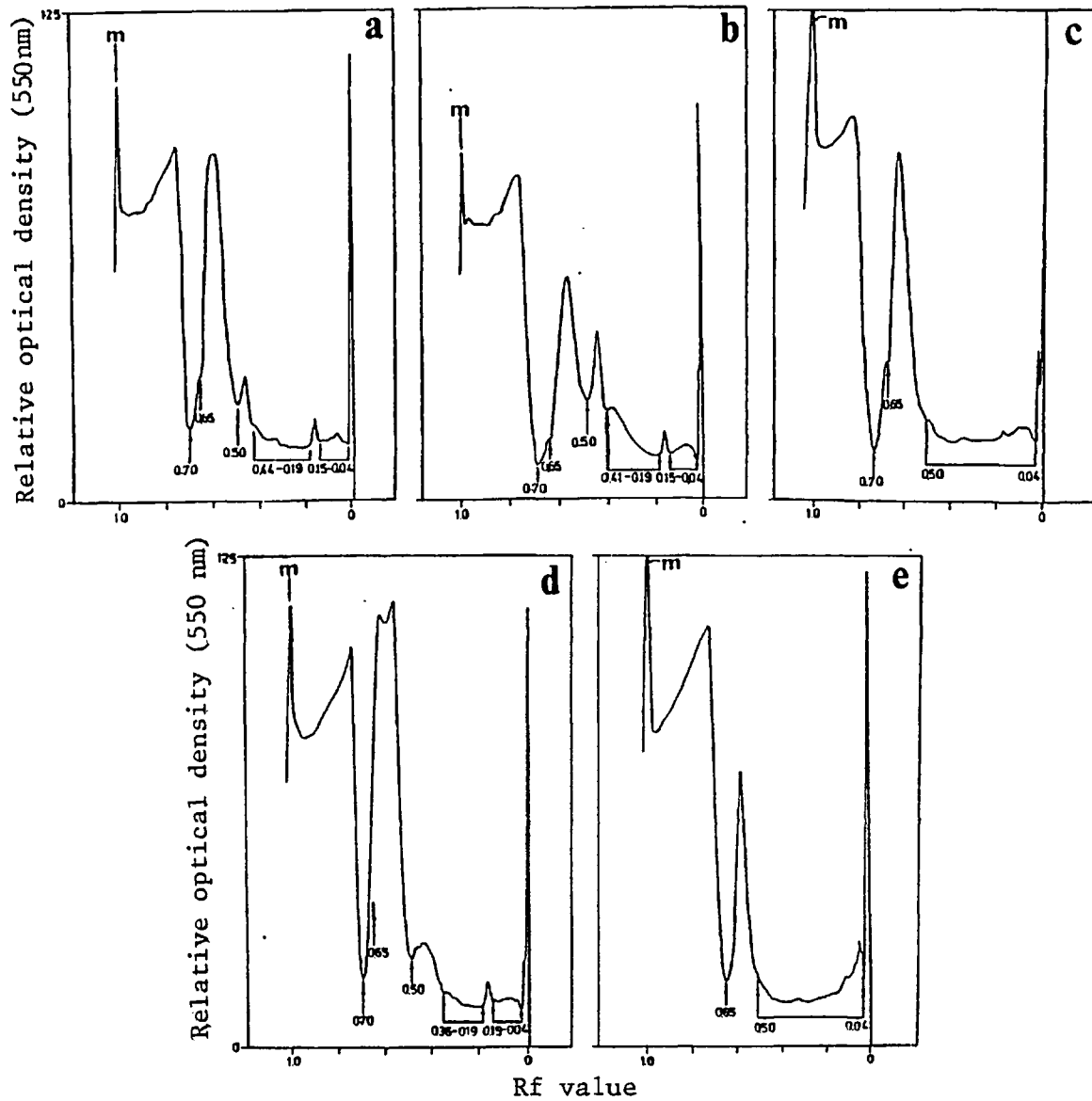


Fig. 53. RNAse zymograms developed in 10% PNGs loaded with 100 μ l E_1 -soluble protein extracts prepared from healthy uninoculated (untreated) (a), carborundum-phosphate buffer inoculated (b); TNV-infected (c), water-floated (d) and mannitol-floated (e) Ashley cucumber cotyledons, 3 days after treatment. m indicates the bromo-phenol blue marker. These densitometer scans were obtained by scanning the gels marked with Asterisks (PNGs) in Fig. 52.

of RNase activity, and the sub-divisions of the broad region mentioned earlier fused into one region from Rf 0.04 to 0.50 (Fig. 53, c and e).

Increases in RNase activity in cucumber cotyledons after the various illustrated treatments, can be ranked in descending order thus,

Mannitol > TNV > Phosphate (mechanical injury) > H₂O > No treatment

These results are, however, based on visual inspection of gels and comparison of densitometer scans, and more precise quantitative assays are shown later.

35. Analysis of the soluble protein fraction of protoplasts isolated from 1) - Mannitol-plasmolysed cotyledons and 2) - Apparently healthy (AH) half cotyledons adjacent to necrotic halves

Protoplasts were isolated from 1) - Ashley cotyledons floated on 0.5M mannitol for 2 days to stimulate (or induce) the γ protein production (Fig. 54, C) and 2) - 0.5 mm leaf strips of AH tissue directly adjacent to the midrib of completely necrotic half cotyledons, 6 days post-inoculation, where the γ protein was known to be present.

Protoplasts were isolated as described in Section III. 12. B, and the soluble proteins were extracted and analysed by disc-PAGE as outlined in Sections III. 15 (A) and 16.

The results showed that γ protein was absent from protoplasts isolated from both mannitol-plasmolysed cotyledons (Figs. 55 and 56), and AH tissue (Figs. 57 and 58).

36. Analysis of the RNase isozyme pattern in the soluble protein fraction prepared from protoplasts devoid of the γ protein on isolation

Soluble protein extracts from protoplasts, known to have lost the γ protein (see earlier section), were further analysed by PNGE in an effort

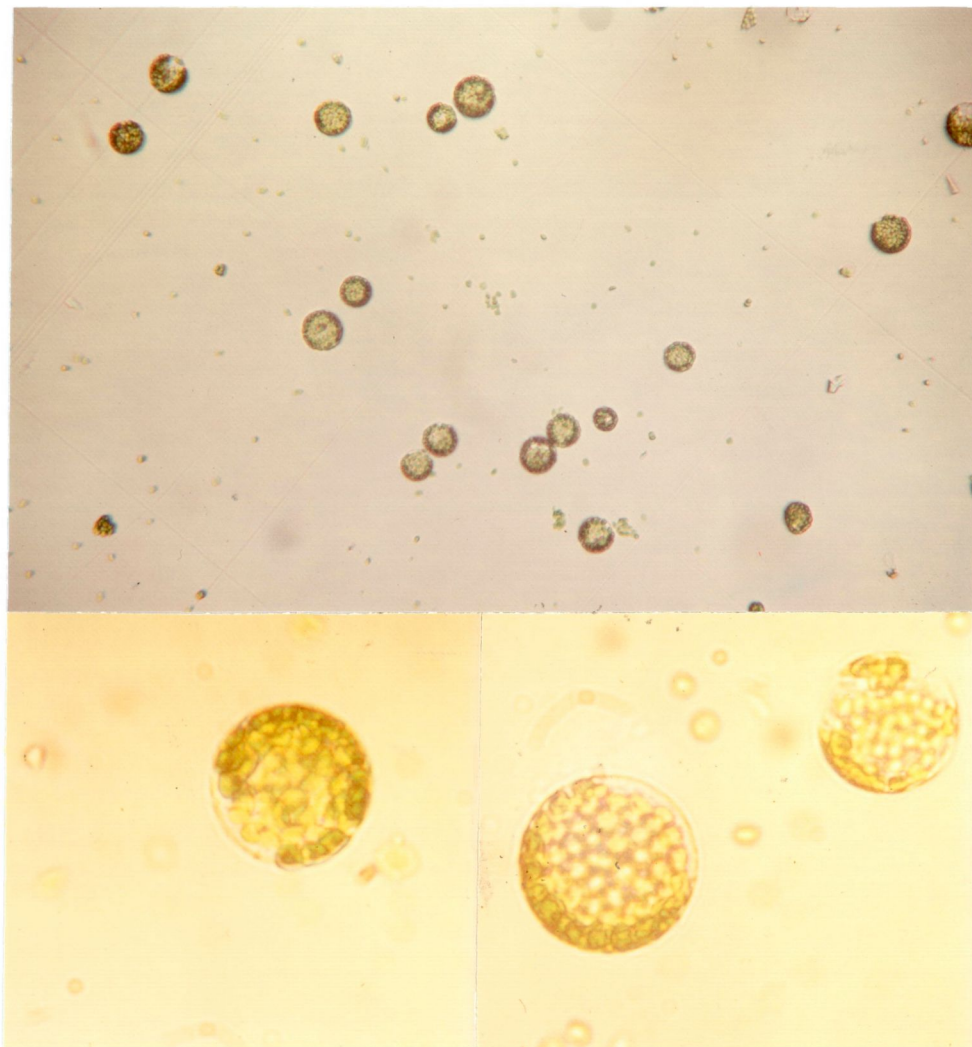


Fig. 54. Light microscope photographs of Ashley cucumber cotyledon protoplasts. A) Protoplasts freshly isolated from water-floated healthy, control, cotyledons. B) High power view of a protoplast from preparation A. C) High power view of protoplasts isolated from cotyledons subjected to osmotic stress (0.5 M mannitol) for 2 days.

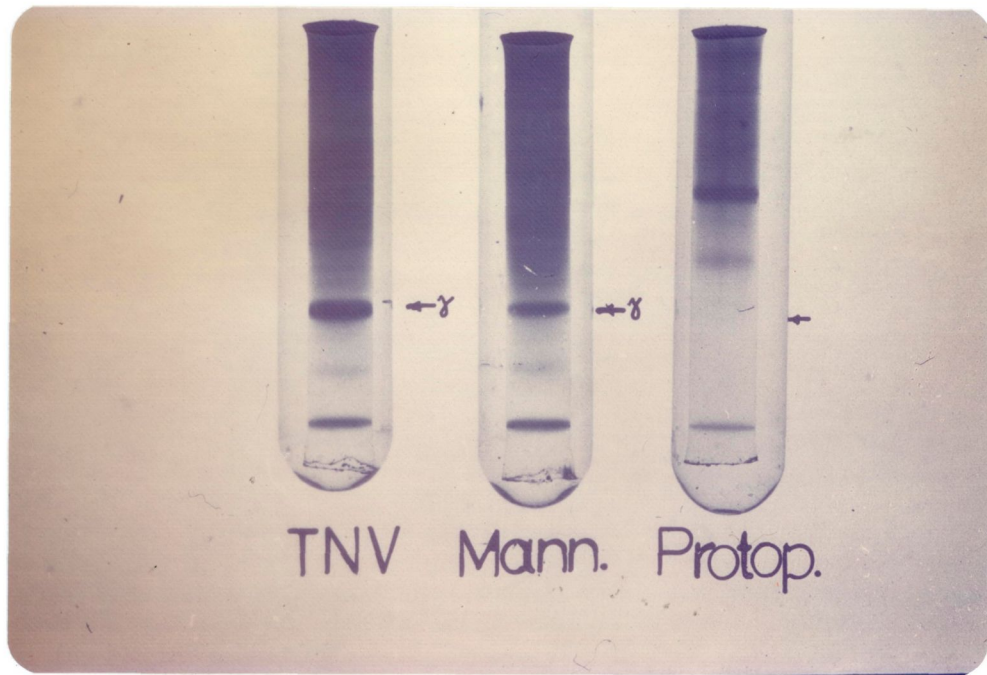


Fig. 55. Comparison of the disc-electrophoretic patterns of E₁-soluble protein fractions in 10% PAG stained with Coomassie blue, for 5 day TNV-infected (TNV), 2 day mannitol osmotically stressed (Mann.) and protoplasts derived from the 2 day mannitol-stressed Ashley cucumber cotyledons (Protop.).

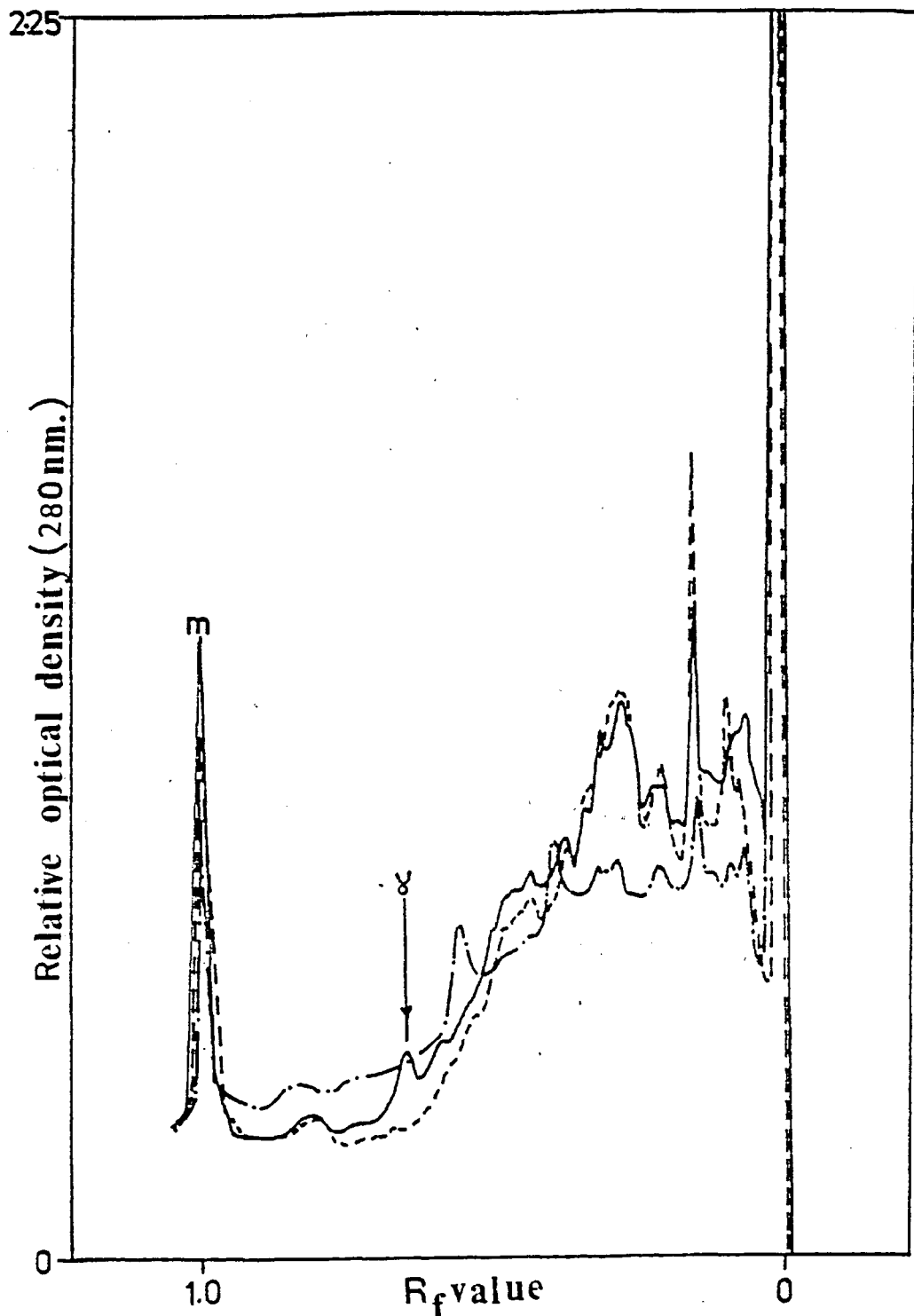


Fig. 56. Densitometer scans of E_1 -soluble protein profiles in 10% PAG loaded with 100 μ l E_1 -soluble protein fractions from Ashley cotyledons floated on 0.5M mannitol for 2 days and the lower epidermis then detached (—), a corresponding control cotyledons floated on water for 2 days (---), and protoplasts isolated from Ashley cotyledons, following floatation for 2 days on 0.5M mannitol (-.-). m indicates the position of bromo-phenol blue marker.

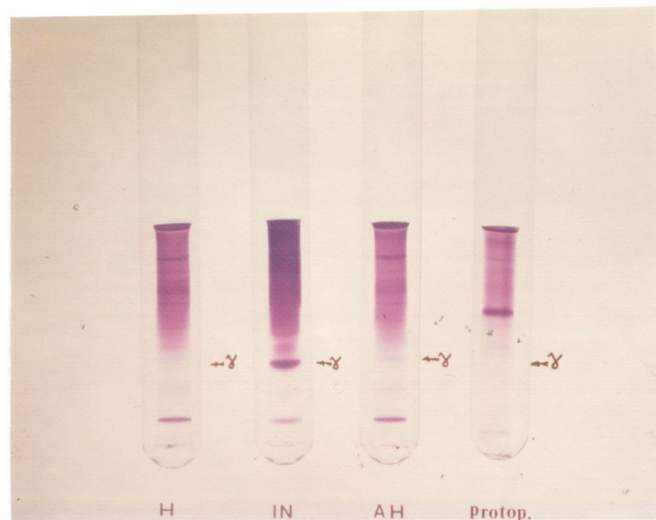


Fig. 57. Comparison of the disc-electrophoretic patterns of 100 μ l E_1 -soluble protein fractions in 10% PAG stained with Coomassie blue, for phosphate-inoculated healthy (H) and 5-day TNV-infected (IN) Ashley cucumber cotyledons and apparently healthy 0.5 mm. leaf strips adjacent to the midrib of uninoculated half cotyledons opposite to completely necrotic halves, 5 days post-inoculation (AH) and protoplasts derived from such tissue (Protop.).

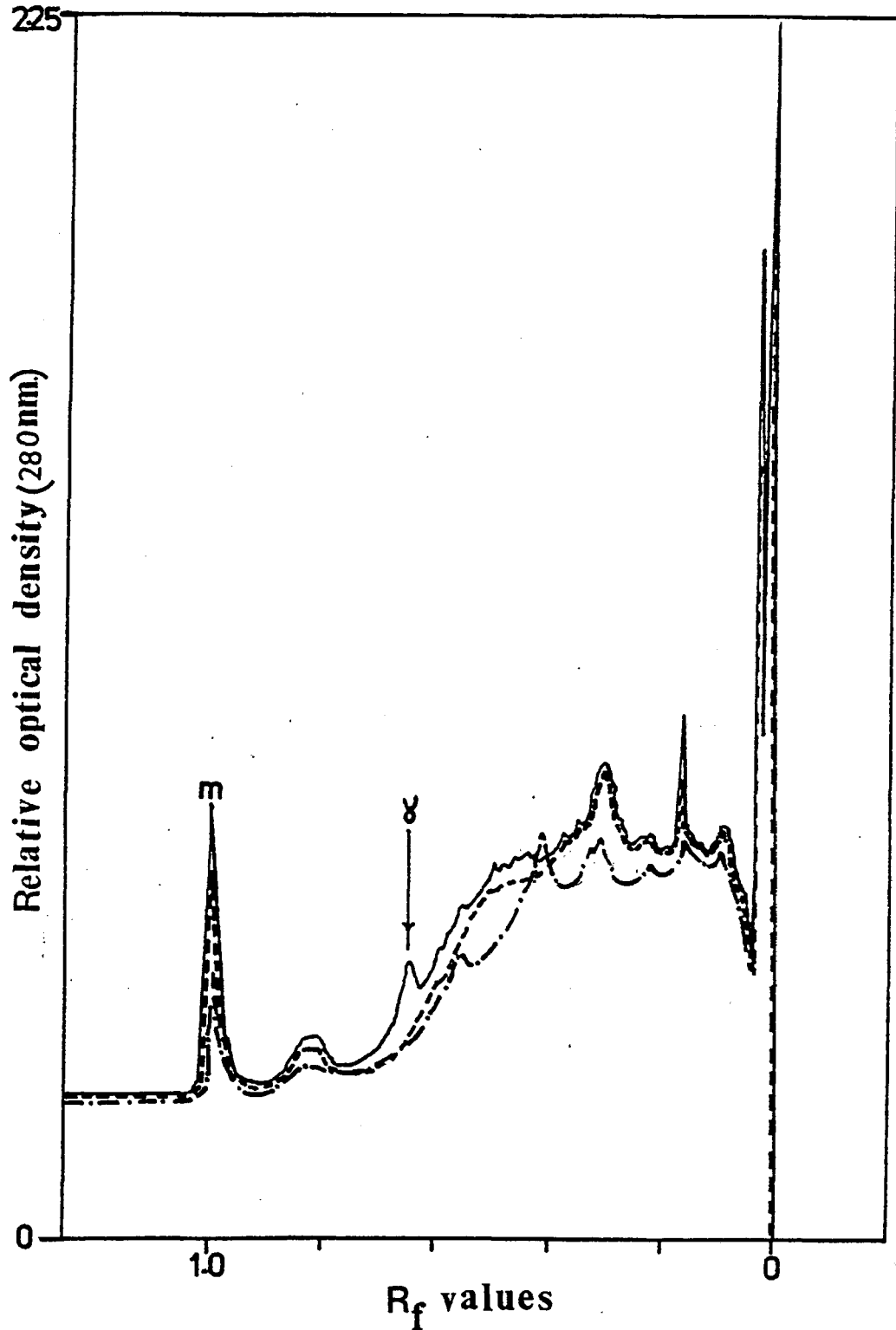


Fig. 58. Densitometer scans of electrophoretic patterns of 10% PAGs loaded with 100 μ l E₁-soluble protein extracts prepared from 0.5 mm AH leaf strips directly adjacent to the midrib of completely necrotic halves, 6 days post-inoculation, and the lower epidermis then detached (—), and corresponding control leaf strips adjacent to the midrib of phosphate-inoculated half cotyledons (---) and protoplasts isolated from AH leaf strips. m indicates the bromo-phenol blue marker.

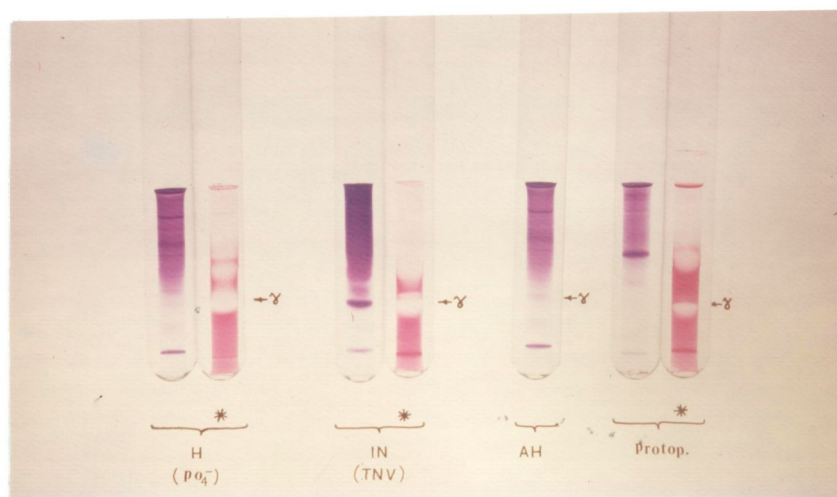


Fig. 59. Comparison of disc-electrophoretic patterns of soluble protein fractions of phosphate buffer-inoculated healthy, H (PO_4), TNV-infected, IN (TNV) Ashley cucumber cotyledons, apparently healthy leaf strips adjacent to the midrib of completely necrotic halves (AH), 6 days post-inoculation and protoplasts derived from AH leaf strips (Protop.) in 10% PAG stained with Coomassie blue and RNase isozyme patterns in 10% PNG (Poly-U gels). Gels marked with Asterisks represent PNGs.

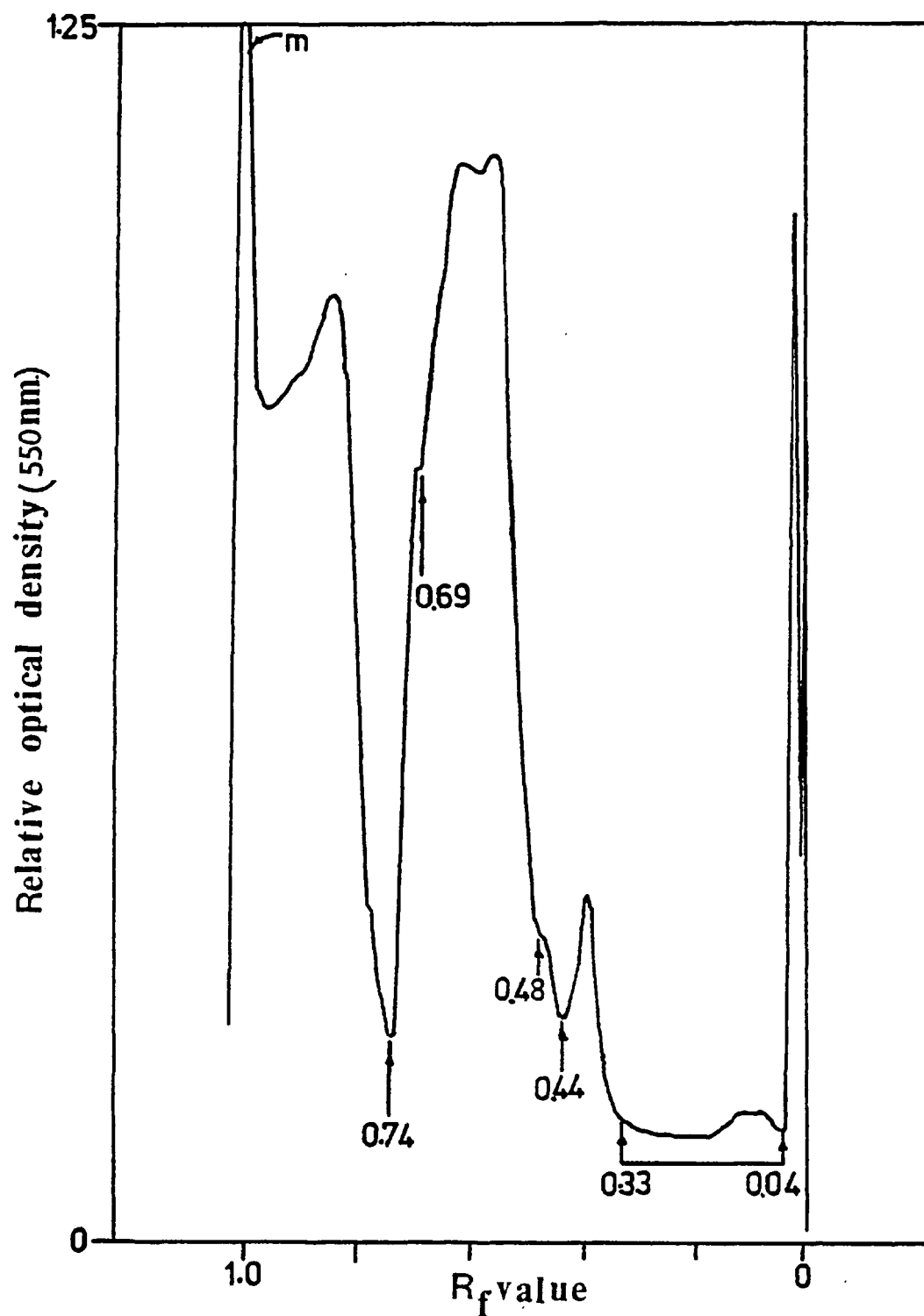


Fig. 60. RNase zymogram developed in 10% PNG loaded with 100 μ l E₁-soluble protein extract prepared from Ashley cucumber protoplasts devoid of γ protein on isolation. The protoplasts were freshly isolated from AH-tissue adjacent to completely necrotic half cotyledons, 5 days post-inoculation.

to determine if the RNase isozyme activity, known to be associated with the γ protein was also lost.

The results illustrated in Fig. 59 show that although protoplasts derived from AH-tissue had lost the γ protein they retained the RNase isozyme activity at the γ position. However, the RNase isozymes known to be associated with the γ protein at Rf values 0.65 and 0.70 had slightly higher migration rates at Rf values 0.69 and 0.74 when zymograms were developed for the protoplast extracts (Figs. 59 and 60) but the profiles were similar.

37. Determination of the quantitative alterations in PO, PPO and RNase activity in uninoculated healthy, phosphate buffer sham-inoculated healthy, TNV-infected, water-floated, and mannitol-osmotically stressed Ashley cucumber cotyledons

Having analysed the specific quantitative and qualitative alterations in the isozymes of the above enzymes in variously treated cotyledonary tissue by disc-PAGE, a further study on the quantitative alterations in these enzymes by spectrophotometric assay is shown in this section. Enzymes were extracted from cotyledons and assayed as described in Section III. 22. Tissue analysed was derived from the sources mentioned above, 3 days after the treatments.

The results were similar to those obtained following similar treatments of cowpea primary leaves (Section IV. 18) and can be summarized as follows :-

A. Determination of PO activity

The results presented in Fig. 61 (A) indicate that the lowest level of PO activity was found in uninoculated (untreated) cotyledons, and abrasion with carborundum and phosphate buffer (mechanical injury) increased PO activity by almost one and a half times. The most increased activity of PO

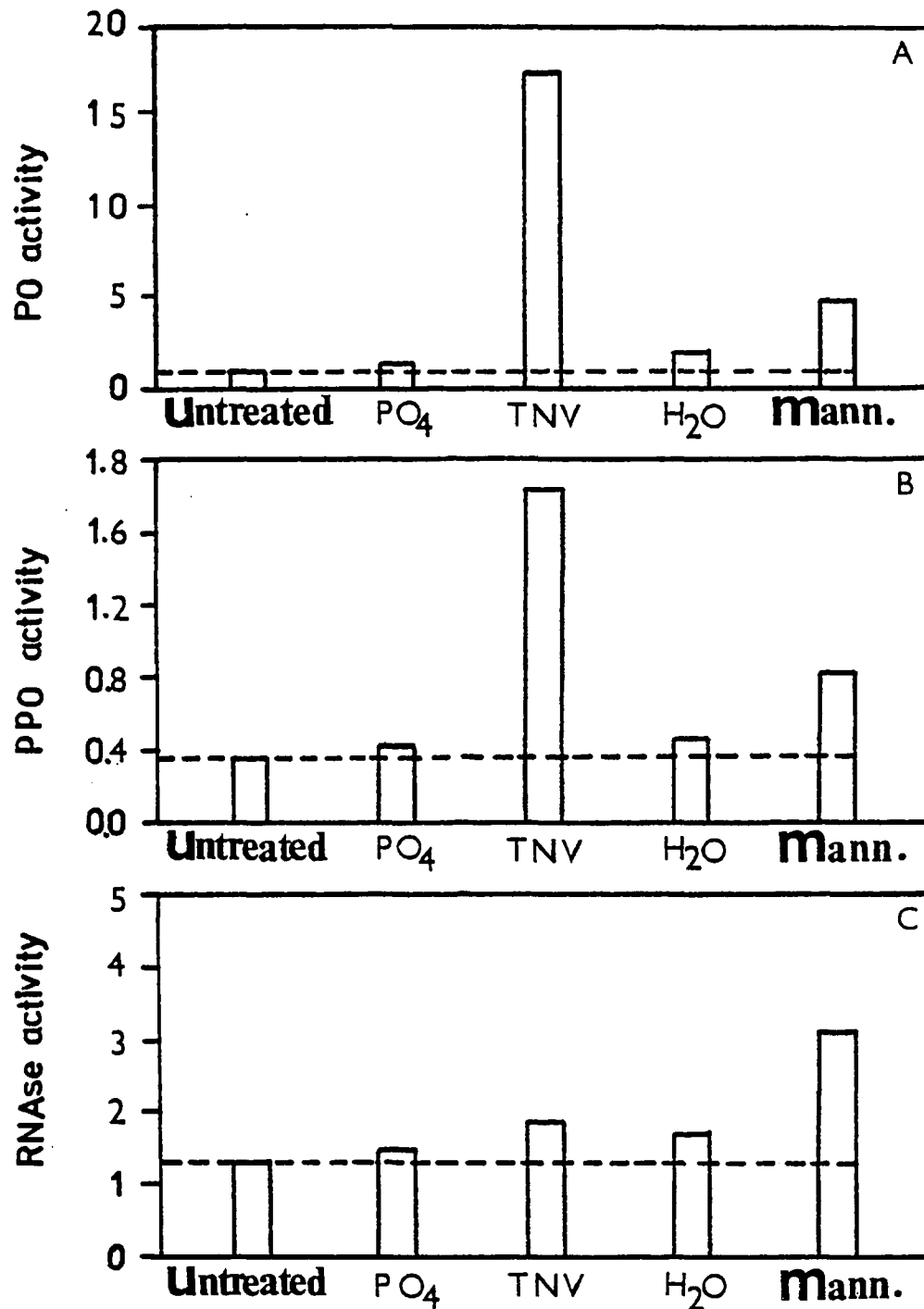


Fig. 61. The level of PO (A), PPO (B), and RNase (C) activity in uninoculated healthy (untreated), carborundum-phosphate buffer-inoculated (PO₄), virus-infected (TNV), water-floated (H₂O), and mannitol-floated (Mann.) Ashley cucumber cotyledons, 3 days after treatment. PO activity is expressed as reciprocal of (Δ time for O.D. (520 nm) to change from 0.2 $\rightarrow$ 0.4/1 ml final reaction mixture/lg. dry weight); PPO activity is expressed as Δ O.D. (330 nm) between 0.5 - 1 min/1 ml final reaction mixture/lg. dry weight; RNase activity is expressed as O.D. (260 nm)/1 ml final reaction mixture/1 mg. dry weight.

was found following TNV-infection. PO activity in uninoculated water-floated cotyledons was higher than that in both the uninoculated and abraded cotyledons. Mannitol floated cotyledons, however, showed a considerable increased PO activity but not to the same level as that found in TNV-infected tissue.

B. Determination of PPO activity

The results are illustrated in Fig. 61 (B) which shows that the pattern of activities in all treatments were generally the same as that seen for PO enzyme (Fig. 61, A).

C. Determination of RNase activity

The results are presented in Fig. 61 (C) and show that the uninoculated cotyledons had the lowest RNase activity and abrasion of the tissue with carborundum and phosphate buffer (mechanical injury) increased activity only slightly, as did water-floatation. Following TNV-infection the activity of RNase was, however, higher than that in abraded tissue.

Comparison of the activities of RNase (Fig. 61, C) with PO (Fig. 61, A) and PPO (Fig. 61, B) shows that unlike the later two enzymes, only fractional increases in RNase activity were caused by either abrasion, virus-infection or water-floatation. However, RNase activity in mannitol-floated cotyledons was almost one and a half times that found in the uninoculated leaves and even higher than that found in virus-infected material (Fig. 61, C).

38. Quantitative changes, with time, of PO, PPO and RNase activity in healthy (H), uninoculated apparently healthy half cotyledons adjacent to TNV-infected halves (AH), and TNV-infected whole cotyledons (IN)

Cotyledonary tissue from Ashley cucumber inoculated with either TNV (IN) or phosphate buffer (controls) on either half or whole leaves was

prepared and harvested various times after inoculation for enzyme assays. Methods of inoculation and enzyme assays are outlined in Sections III. 4. A and 22.

(H) tissue is sham-inoculated with phosphate buffer alone, (AH) tissue is apparently healthy half cotyledons, adjacent to TNV-infected halves, ($\frac{1}{2}$ H) tissue is half cotyledonary tissue, adjacent to sham-inoculated halves, (IN) tissue is infected with TNV.

The results obtained can be summarized as follows :-

A. Quantitative changes in PO activity

The results illustrated in Fig. 62 (PO) show that in (H) cotyledons the PO activity remained almost constant over the time course tested. Similar activities were also found in $\frac{1}{2}$ H tissue. In the IN cotyledons the activity of PO increased slightly, 1 day post-inoculation and then rose sharply over the next 2 days in an almost linear fashion. The rate of increase flattened but continued to rise up until 6 days post-inoculation. In AH tissue the activity of PO increased stepwise but was always higher than that in the controls up until 4 days post-inoculation when the activity was approximately twice that of zero time samples. Six days post-inoculation, PO activity rose sharply in AH tissue.

B. Quantitative changes in PPO activity

The overall cyclic changes and differences in PPO activity illustrated in Fig. 62 (PPO) between H, $\frac{1}{2}$ H, and AH tissue are not considered to be significantly different from the zero time values and no overall changes in PPO activity were seen in this tissue over the time-course investigated. In IN cotyledons, PPO activity decreased slightly, but not significantly 1 day post-inoculation, and thereafter increased in a 'staggered' fashion, over the time course, slight falls in activity 3 and

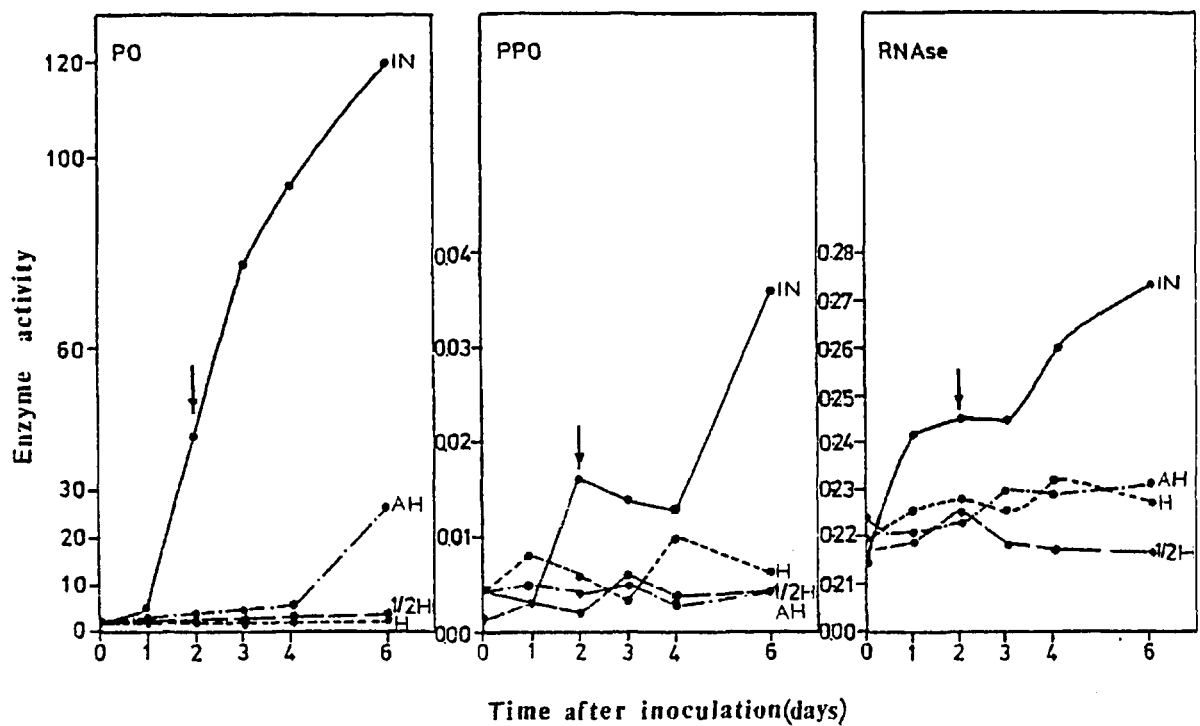


Fig. 62. Alterations in PO, PPO, and RNase activity with time in tissue taken from TNV-infected, IN, (o—o), phosphate buffer-inoculated, H, (o---o) Ashley cucumber cotyledons, and uninoculated half cotyledons adjacent to either TNV-infected, AH, (o—.—o) or phosphate buffer-inoculated, $\frac{1}{2}$ H, (o— —o).

4 days post-inoculation, were not significantly different from the activities measured 2 days post-inoculation. However, the dramatic increase in activity 6 days post-inoculation was always reproducible.

C. Quantitative changes in RNase activity

The results presented in Fig. 62(RNase) show that in H cotyledons, RNase activity increased slightly on the first and second day post-inoculation with a slight significant decrease on the third day. Activity increased slightly on the fourth day post-inoculation and dropped to a lower value 2 days later. In IN-cotyledons the RNase activity increased dramatically one day post-inoculation and the level of activity remained similar up until 3 days post-inoculation. Activity increased again on the day 4 post-inoculation (when lesions expanded) and further increases were observed two days later. In $\frac{1}{2}$ H-tissue the activity of RNase remained almost constant over the time course with only slight insignificant increases in activity two days post-inoculation. In AH-tissue, RNase activity was similar to that in $\frac{1}{2}$ H-tissue up until 2 days post-inoculation when the activity increased, and this increase was maintained throughout the time course matching that found in H-tissue.

39. Electrophoretic analysis of E_2 -soluble protein extract of healthy and TNV-infected Xanthi-nc. tobacco leaves

Soluble proteins from healthy and virus-infected Xanthi-nc. tobacco leaves were extracted in E_2 -buffer and analysed by disc-PAGE as described in Sections III. 15 (B) and 16.

The results presented in Fig. 63 show that four 'novel' proteins (A, B, C and D) were present in the soluble protein extracts of TNV-infected leaves at Rf values (0.95, 0.77, 0.68 and 0.63 respectively). These proteins were absent from healthy leaves. Similar proteins to these have been

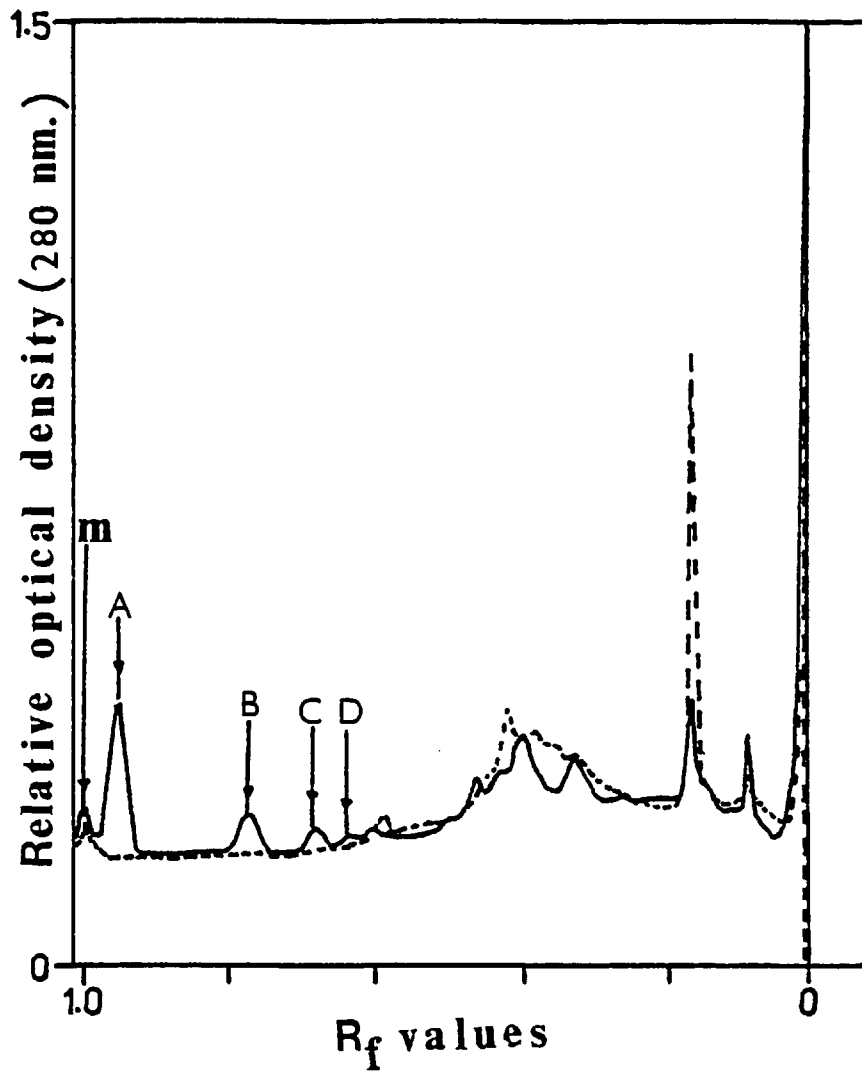


Fig. 63. Densitometer scans of electrophoretic patterns of 50 μ l E_2 -soluble protein extracts from TNV-infected (—), and healthy, sham-inoculated (---) Xanthi-nc. tobacco leaves on 10% PAG. m indicates the position of bromo-phenol blue marker.

reported previously (Rf 0.93, 0.74, 0.65 and 0.61 respectively, Coutts, 1978b).

40. Co-electrophoresis of E₂-soluble protein extracts of cucumber, cowpea, and tobacco

The co-electrophoresis of E₂-extracts of Baton-Vert cucumber (Cuc.), cowpea (Cowp.) and tobacco (Tobac.) was carried out using different combinations of two samples, viz. (Cuc. + Cowp.), (Cuc. + Tobac.) and (Cowp. + Tobac.) to determine if any of the 'novel' proteins were common proteins to these different hosts (i.e. demonstrating inter-host specificity).

The results are summarized as follows :-

A. Co-electrophoresis of (Cuc. + Cowp.)

No relationship was found between the single 'novel' protein band, γ , of infected cucumber, Baton-Vert, cotyledons and the α or the β fractions of cowpea (Fig. 64, A, and Table 3).

Table 3.

Replicate	Rf values of (Cuc. + Cowp.) proteins		
	α	β	γ
1	0.89	0.75	0.71
2	0.89	0.75	0.72
Mean	0.89	0.75	0.715

B. Co-electrophoresis of (Cuc. + Tobac.)

Although the C and D proteins of tobacco were masked by the broad base of the γ protein, their positions were determined by comparing the known Rf values on single gels (Fig. 64, B) with their Rf values when

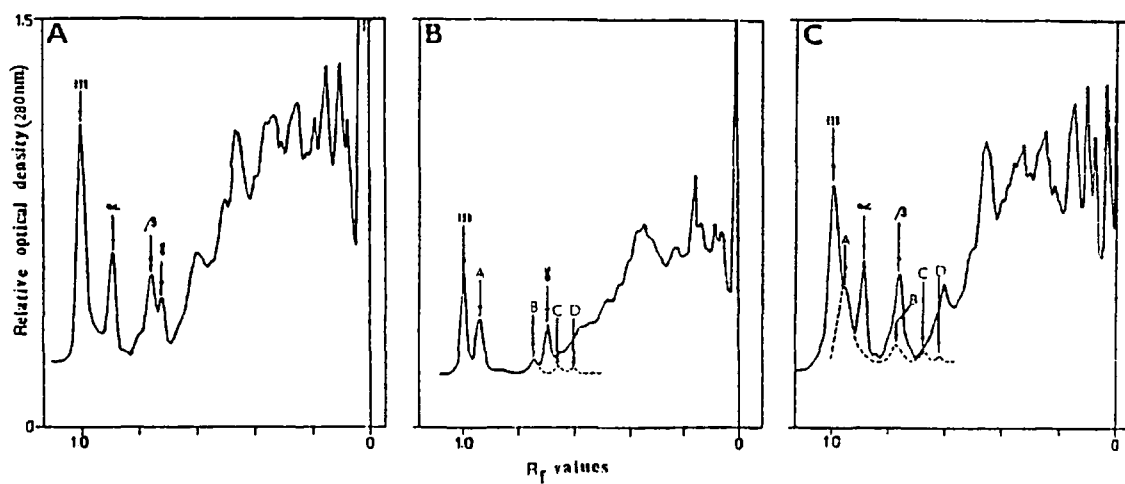


Fig. 64. Densitometer scans of co-electrophoretic pattern in 10% PAG loaded with 100 μ l E₂-soluble protein extracts from the combinations (Cuc. + Cowp., A), (Cuc. + Tobac., B), and (Cowp. + Tobac., C) infected with TNV. Cucumber and cowpea tissues were extracted 6 days post-inoculation and tobacco tissue 10 days post-inoculation. 50 μ l of each tissue extract was used. Proteins are indicated by arrows. m is the bromo-phenol blue.

co-electrophoresed, to determine any possible shifts. No relationship was found between the γ protein and either of the four proteins, A, B, C and D, of tobacco. It seems that the γ -band occupies a position at Rf value 0.695 between the B and C bands of tobacco (Fig. 64, B and Table 4).

Table 4.

Replicate	Rf values of (Cuc. + Tobac.) proteins				
	A	B	γ	C	D
1	0.94	0.76	0.70	-	-
2	0.93	0.75	0.69	-	-
Mean	0.935	0.755	0.695	Masked	Masked

C. Co-electrophoresis of (Cowp. + Tobac.)

The α band of cowpea occupies a position at 0.885 between the A and the B bands of tobacco and the α band of cowpea is apparently unrelated to any of A, B, C and D proteins of tobacco. Whereas, the β band of cowpea seems to be similar to B band of tobacco (at least in charge and possibly size) as it migrates to the same Rf on 10% PAG (Fig. 64, D and Table 5). But because of the broad base of the β band which masks the B, C and D bands on co-electrophoresis, it is somewhat difficult to make categorical statements on any similarity without further studies.

Table 5.

Replicate	Rf values of (Cowp.+ Tobac.) proteins				
	A	α	β /B	C	D
1	0.95	0.89	0.76	-	-
2	0.95	0.88	0.76	-	-
Mean	0.95	0.885	0.76	masked	masked

41. Induced (acquired) resistance experiments

Homologous challenge experiments (i.e. TNV against TNV) were carried out using both cowpea and Ashley cucumber plants. Induced resistance was tested in uninoculated tissue various times after inoculation of other plant parts.

A. Induced resistance in cowpea

Induced resistance was tested in uninoculated half or whole primary leaves adjacent to inoculated halves or opposite to inoculated whole primary leaves respectively. Induced resistance in trifoliate leaves developed on plants whose primary leaves had been inoculated, some 15 days earlier, with either TNV or phosphate buffer was also investigated. Inducing inoculations were carried out using either TNV or phosphate buffer (control) as inocula and challenge inoculations were performed, 6 - 7 days later, using TNV-inocula. Reduction in lesion number and/or size was considered as a demonstration of induced resistance. Differences between means were analysed statistically by the t-test.

The results shown in Table 6 show that systemic induced resistance could not be demonstrated in any uninoculated tissue of cowpea. However, in uninoculated half leaves adjacent to TNV-infected halves, two experiments

out of the three tested did show reductions in lesion numbers of between 47 - 66%, of those developed on control leaves.

B. Induced resistance in Ashley cucumber

Induced resistance was tested in uninoculated half or whole cotyledons adjacent to inoculated halves or opposite to inoculated whole cotyledons. Similarly resistance was tested in first true leaves developed on plants whose two cotyledons had previously been inoculated (9 days earlier) with either TNV or phosphate buffer. Lesions were assessed and data were statistically analysed as in the previous section.

The results presented in Table 7 show that no induced resistance could be demonstrated in whole cotyledons, 6 days after inoculation of the opposite cotyledons with TNV, whereas reductions in lesion number but not size were found on half cotyledons adjacent to TNV-infected halves. In first true leaves there was a reduction in both lesion number and size when compared with control leaves and a systemic induced resistance was routinely demonstrated.

Table 6. HOMOLOGOUS CHALLENGE EXPERIMENTS WITH TNV ON COWPEA (VIGNA SINENSIS CV. BLACKEYE NO. 5) PRIMARY AND TRIFOLIATE LEAVES

Inoculated tissue	Challenged tissue	Experiment number	Time of challenge (days post- inoculation)	Average lesion number		Average lesion size		Novel protein presence in challenge tissue prior to challenge
				(per cm ² for trifoliolate)		(mm)		
				$\bar{X} \pm$ S.E.	$\bar{X} \pm$ S.E.	$\bar{X} \pm$ S.E.	$\bar{X} \pm$ S.E.	
				Control (a)	Challenge	Control	Challenge	
Half primary leaf	Adjacent half primary leaf	1	6	105.75 $\pm$ 19.69	92.88 $\pm$ 15.01	1.15 $\pm$ 0.13	0.77 $\pm$ 0.27	+
		2 ^(b)	6	177.70 $\pm$ 26.30	60.80 $\pm$ 7.08*	1.54 $\pm$ 0.27	1.74 $\pm$ 0.35	+
		3	6	150.50 $\pm$ 30.10	80.12 $\pm$ 10.15*	1.31 $\pm$ 0.21	1.35 $\pm$ 0.22	+
Whole primary leaf	Opposite primary leaf	1	6	35.12 $\pm$ 5.22	36.20 $\pm$ 6.19	1.45 $\pm$ 0.12	1.49 $\pm$ 0.10	-
		2	6	41.63 $\pm$ 6.54	31.29 $\pm$ 6.81	1.51 $\pm$ 0.14	1.42 $\pm$ 0.14	-
		3	6	45.55 $\pm$ 3.32	44.61 $\pm$ 3.11	1.52 $\pm$ 0.16	1.48 $\pm$ 0.13	-
Both primary leaves	Upper trifoliolate leaves	1	15	3.28	3.74	1.05 $\pm$ 0.35	0.91 $\pm$ 0.26	-
		2	15	3.80	3.71	1.27 $\pm$ 0.23	1.25 $\pm$ 0.20	-
		3	15	3.10	3.60	1.31 $\pm$ 0.15	0.86 $\pm$ 0.05*	-
		4	15	3.30	3.25	0.99 $\pm$ 0.21	1.10 $\pm$ 0.15	-

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(a) Control tissue inoculated with phosphate buffer alone. (b) Tissue highly chlorotic at time of challenge.

* Significantly smaller at the 95% confidence level.

The (+) and (-) in the final column, indicates respectively presence or absence of the α and β fractions in the corresponding tissue.

N.B. Standard errors are not shown with the means of local lesion numbers for upper trifoliolate leaves as number of local lesions in all replicates were summed and divided by the total measured leaf area.

Table 7. HOMOLOGOUS CHALLENGE EXPERIMENTS WITH TNV ON CUCUMBER (CUCUMIS SATIVUS CV. ASHLEY) COTYLEDONS AND LEAVES

Inoculated tissue	Challenged tissue	Experiment number	Time of challenge (days post- inoculation	Average lesion number (per cm ² for leaves)		Average lesion size (mm)		Novel protein presence in challenge tissue prior to challenge
				$\bar{X} \pm$ S.E.		$\bar{X} \pm$ S.E.		
				Control (a)	Challenge	Control	Challenge	
Half cotyledon	Adjacent half cotyledon	1	7	8.66 $\pm$ 1.37	0.33 $\pm$ 0.14*	0.52 $\pm$ 0.13	0.43 $\pm$ 0.08	+
		2	6	10.30 $\pm$ 2.11	7.12 $\pm$ 0.13*	0.92 $\pm$ 0.23	0.89 $\pm$ 0.11	+
		3	6	16.83 $\pm$ 2.21	6.33 $\pm$ 1.20*	1.21 $\pm$ 0.20	0.89 $\pm$ 0.17	+
Whole cotyledon	Opposite cotyledon	1	6	15.31 $\pm$ 3.33	14.22 $\pm$ 2.31	0.99 $\pm$ 0.31	0.88 $\pm$ 0.26	-
		2	6	19.11 $\pm$ 3.75	21.22 $\pm$ 2.52	1.01 $\pm$ 0.35	0.91 $\pm$ 0.12	-
Both cotyledons	First true leaf	1	9	3.83	1.52	0.61 $\pm$ 0.13	0.32 $\pm$ 0.03*	-
		2	9	3.50	2.10	0.90 $\pm$ 0.12	0.60 $\pm$ 0.05*	-
		3	9	2.35	2.05	1.24 $\pm$ 0.07	1.09 $\pm$ 0.04*	-

(a) Control tissue inoculated with phosphate buffer alone. * Significantly smaller at the 95% confidence level.
The (+) and (-) in the final column, indicates respectively presence or absence of the γ fraction in the corresponding tissue.
N.B. Standard errors are not shown with the means of local lesion numbers for upper first true leaves as number of local lesions in all replicates were summed and divided by the total measured leaf area.

V. DISCUSSION

The hypersensitive reaction of plants to virus infection is characterized by the localisation of virus in necrotic lesions and to narrow zones of cells surrounding the lesions (Loebenstein, 1972). Such a reaction is observed in both TNV-infected cowpea leaves and cucumber cotyledons (Figs. 1 and 27). The detection of both, viral particles and RNA outside the necrotic areas of individual necrotic local lesions on cowpea primary leaves (Fig. 3) illustrates that necrosis is not the sole cause of localisation, and other factors may be involved. Indeed the presence of virus particles outside of necrotic areas has previously been shown in several other virus-host combinations (Hayashi and Matsui, 1965; Milne, 1966b). However, in the TNV/cowpea combination, these results have to be reviewed cautiously as virus may have moved through invisible necrotic microveins around the local lesions.

Simultaneous with lesion formation, two major protein fractions, α and β , were detectable in soluble protein extracts of TNV-infected cowpea leaves with four other minor protein components appearing as 'shoulders' on the main peaks as evidenced by the densitometry of stained gels (Fig. 5).

The fact that these proteins were detectable at some distance away from necrotic tissue, i.e. in tissue around individual necrotic lesions (Fig. 7) and in virus-free AH-tissue taken from half leaves adjacent to necrotic halves (Fig. 8) re-inforces the notion that these proteins are host-specific as illustrated in other hypersensitive situations (Van Loon and Van Kammen, 1970). The α and the β fractions reported in this study and previously demonstrated by Coutts (1978b) as the b and d proteins were characterized further. Both fractions were purely proteinaceous in nature (Fig. 9) and had a common polypeptide monomer, α_{m1} and β_{m1} of 10,000d. mol. wt. (Fig. 10). The β protein fraction had two additional monomers of 25,500d. and 36,000d. mol. wt., termed β_{m2} and β_{m3} respectively. Often

other faint bands accompanied the α and the β monomers on SDS-PAGE, but these were ignored as artifactual.

However, it is not clear whether these proteins are totally representative of the α and the β fractions alone, as the 'shoulder' proteins were also included in gel slices from which the proteins were extracted for SDS-PAGE. Other methods of macro-molecular separation for isolation of the individual proteins (e.g. isotachopheresis, or affinity chromatography) have to be sought to clarify this point.

The α and the β fractions were found throughout AH-tissue (up to 12 mm away from the mid-rib adjacent to completely necrotic half leaves), (Fig. 8), but neither fraction was found in leaves remote from those originally inoculated (Section IV. 8. B). However, in comparable situations in tobacco plants which react hypersensitively to virus infection, the b_1 - b_4 (IV - I) proteins were found in leaves distant from necrotic tissue (Van Loon and Van Kammen, 1970).

Concomitant with the appearance of the α and the β fractions in AH-tissue, was an increase in the total protein content (Fig. 13), which may represent an accumulation of the α and the β proteins while necrosis was developing in the adjacent lesioned tissue, and/or activated enzymes (e.g. peroxidase; Fig. 26). Since induction of phenolic compounds by TNV is restricted to necrotic tissue (Bailey and Burden, 1973) the assay of AH-tissue was unaffected by the virus-elicited phenolics. Similarly, the rates of incorporation of ^{14}C L-leucine into TCA-insoluble fractions was found to be higher at 4 - 7 days post-inoculation in AH-tissue as compared to control tissue (Fig. 14), but this was not always significant.

Neither the single nor the double labelling of proteins (in AH- and H-control tissue) accompanied by disc-PAGE and gel-slicing, gave any indications as to the novelty of synthesis of the α or the β fraction (Figs. 15, A and 16, A). Actinomycin D (AmD) pre-treatment of tissue gave similar refractive results (Figs. 15, B and 16, B). It was not possible

to demonstrate that the α and the β fractions could be specifically labelled with radio-active isotopes and novel synthesis is not proven. Previous studies (Jones and Reichman, 1973; Duchesne et al., 1977), have illustrated similar problems in radio-labelling macromolecules in necrotic and adjacent tissue. It is possible that the message(s) for the α and the β protein synthesis had appeared some time earlier than that chosen for labelling (i.e. 6 - 7 days post-inoculation of the adjacent half leaf). Alternatively the proteins may have been synthesised in the TNV-infected half leaves and diffused into the AH-tissue of the adjacent halves.

Converse to synthesis, since the proteins appear late in AH-tissue when necrosis is complete in adjacent tissue, they may represent "breakdown products" of larger protein molecules. The 'novel' proteins in hypersensitive tobacco were insensitive to trypsin and chymotrypsin treatment (Van Loon and Ritter, 1978), and may be similar in this aspect representing end-products' of digestion or cleavage. It should be possible to illustrate this in cowpea by sequential 'pulse-labelling' of soluble proteins in AH-tissue during a ten day time course after inoculation of the adjacent half leaf. However, if cleavage of proteins as well as synthesis occurs, interpretation of the radio-active patterns in gels will be difficult. The number of counts recovered from the soluble proteins in the experiments reported here represented only a fraction of those supplied to the leaf discs (Fig. 15). It is likely that most of the radio-active isotope was diluted out in large precursor 'pools'. the size of which is unknown in this tissue. Similarly, possible differences in the incorporation rates of precursors between the two compared samples, makes interpretation of the results a problem.

Trials to induce the α and/or the β fractions abiotically with either CuSO_4 or 0.5M mannitol showed that necrotizing concentrations of CuSO_4 caused no such induction in healthy leaves, 3 days after treatment (Fig. 17), eliminating the possibility that these proteins were by-products of

necrosis. Moreover, these results strengthen the idea that production of these proteins in cowpea leaves is rather an active process which requires cells to be under a continuous stress exerted by a slowly developing necrosis as opposed to a shock chemically induced necrosis. Indeed, floatation of leaves on hypertonic mannitol solutions (0.5M) induced some low molecular weight proteins in the soluble fraction, one of which had the same Rf as the β protein in native gels (Fig. 18), suggesting that the β fraction might be stress related proteins.

Almost all of these mannitol-induced proteins were absent from protoplasts isolated from plasmolysed tissue previously exposed to mannitol treatment for 2 days (Fig. 18). Trace quantities of the band coincident with the β protein were retained, but these were not easily demonstrated by densitometry (Fig. 18). These results point to a cell wall, plasma membrane, or cellular free-space location of these proteins in whole tissue.

When zymograms of peroxidase (PO), polyphenol oxidase (PPO) developed from unabraded, abraded, TNV-infected, water-floated, and mannitol-floated cowpea leaves were compared some similarities in overall quantitative increases and qualitative alterations were seen (Figs. 19, 20, 21 and 22). The changes in the PPO isozyme pattern following abrasion of leaves were however only quantitative (Fig. 20, b). No isozymes of either PO or PPO were coincident with either the α or the β fraction, or those proteins induced by mannitol treatment (Fig. 21, A and B). Similarly, ribonuclease zymograms developed from TNV-infected leaves showed both quantitative and possible qualitative changes, but none of the isozymes was coincident with either the α or the β fractions (Figs. 23 and 24). These results suggest that the α and/or the β fractions may be representative of as yet uninvestigated enzymes, but three commonly investigated in hypersensitive reactions, i.e. PO (Solymosy et al., 1959; Farkas et al., 1960; Loebenstein and Linsley, 1966; Suseno and Hampton, 1966; Bates and Chant, 1970; Simons and Ross, 1970 and 1971a; Van Loon and Geelen, 1971; Veggetti et al., 1975; Sheen and Diachun, 1978a and 1978b; Weststeijn, 1976; Faccioli, 1979).

PPO (Farkas et al., 1960; Bozarth and Ross, 1964; Van Kammen and Brouwer, 1964; Jockusch, 1966; Cabanne et al., 1971; Van Loon and Geelen, 1971; Simons and Ross, 1971a), and RNase (Wyen et al., 1972) have been eliminated and the role of the α and the β protein fractions remains obscure.

The absence of these proteins in tissue remote from inoculated leaves is accompanied by an inability to demonstrate systemic induced resistance in this tissue following a homologous challenge (Table 6). A reduction in lesion number in AH challenged tissue was noted (Table 6) and may be correlated with the presence of the α and the β fractions (Fig. 8). However, this is unlikely as a concentration gradient for the α and the β fractions in AH-tissue away from the mid-rib was found (Fig. 8), while the distribution of local lesions in this tissue was uniform. It would have been expected to find increases in local lesion inhibition towards the mid-rib if the proteins were directly involved in halting the necrotic reaction. However, an indirect involvement cannot be eliminated. Reduction in lesion number may be caused by an alteration in the water balance of the leaves, as previously illustrated in tobacco (Casells et al., 1978) and not the presence of 'novel' proteins.

The inability to demonstrate systemic induced resistance in cowpea tissue far remote from inoculated leaves is in disagreement with a previous report by Ross (1964). But clearly if water stress is involved in this phenomenon, then all challenge experiments may be inherently irreproducible.

Alterations in the soluble proteins of the several cucumber cultivars studied after TNV-infection are somewhat easier to discuss than the foregoing, because the alterations observed were often clearer, and limited to a few changes.

As with cowpea leaves, infection of cucumber (Cucumis sativus L. cv. Ashley, Baton-Vert, China, Chipper, Elem and Explorer) cotyledons with TNV_D was followed by localisation of the virus in necrotic local lesions (Figs. 27 and 30). Simultaneously, an apparently 'novel' protein (γ ,

Figs. 28 and 29) of mol. wt. 24,500d (Fig. 35) appeared in the soluble protein fraction of all infected cultivars tested, with an additional protein (γ_A , Figs. 28 and 29) in the Explorer cultivar. Both the γ and the γ_A protein fractions are glycoproteins (Fig. 33). Other studies have reported a similar protein to the γ fraction in other cucumber cultivars following infection with both systemic (Ziemiecki and Wood, 1975; Tas and Peters, 1977) and local lesion-inducing viruses (Tas and Peters, 1977). The molecular weight of the E_1 protein demonstrated by Tas and Peters (1977) in native-PAG was estimated at 22,000d and the γ and the E_1 fractions are probably identical. These results suggest that the γ fraction is probably monomeric at the quaternary structure level, and the differences in the estimated molecular weights (viz. 22,000d, for E_1 and 24,500d., for γ) may be due to experimental variation or interference caused by carbohydrate moieties with E_1 . However, the γ_A fraction in the Explorer cultivar (Figs. 28 and 29) which is produced in similar quantities to γ in TNV-infected cotyledons, has not been reported previously. The γ fraction was common to all cultivars studied as co-electrophoresis of mixtures of extracts from Explorer and any other cultivar gave a re-inforced peak characteristic for γ , whereas the γ_A fraction was specific to the Explorer cultivar only.

The γ fraction was shown to be host-specific because it could be induced by other pathogens, i.e. the starch lesion-inducing virus, TMV (Figs. 41 and 43) and the necrosis-inducing fungal pathogen, Colletotrichum lagenarium (Fig. 37), the causative agent of anthracnose. Although, induction of γ_A by these pathogens has not been attempted, it is believed that the γ_A is also a host-specific fraction and not related to the virus. The evidence for that can be inferred indirectly because if γ_A was virus-related, it would have been seen in other cucumber cotyledons, infected with TNV, regardless of which cultivar was used. Furthermore, the γ fraction was induced in healthy tissue following CuSO_4 treatment (Fig. 42), but the γ_A fraction was not. However, the hypothesis that these fractions are by products of the necrotic

reaction can be rejected for the following reasons :

- 1) The γ and the γ_A glycoproteins in Explorer were found in healthy tissue in trace quantities following PAS-staining of gels loaded with soluble protein extracts (Fig. 33, C). However, the γ fraction was not so easily detectable in other cultivars by this method (Fig. 33, A and B).
- 2) The γ fraction was induced by infecting Ashley cotyledons with TMV, which is known to cause starch lesions with no necrosis (Figs. 41 and 43).
- 3) The γ fraction of the Ashley cultivar, and the γ and the γ_A fractions of Explorer could be induced in healthy tissue by subjecting tissue to mannitol-induced osmotic stress, by floating cotyledons on 0.5M mannitol solutions for a period not less than 1 day (Figs. 43 and 44). However, the γ_A fraction was not as strongly stimulated by osmotic stress as was the γ band (Fig. 44).

These observations exclude the possibility that the γ and the γ_A fractions are by-products of necrosis, and suggest that the stress effects of necrosis caused by pathogens, elicited by abiotic means, e.g. CuSO_4 treatment or mannitol-induced osmotic stress, may have a similar fundamental trigger(s) for stimulating protein synthesis (or degradation). However, the quantities of proteins produced in either cultivar when localisation was accompanied by necrosis were greater than those when it was not (Fig. 43).

External chemical stimuli (or inducers) have been used previously to stimulate (or induce) 'novel' protein production in the soluble fraction of tobacco leaves. Polyacrylic acid (Gianinazzi and Kassanis, 1974), 2-chloroethylphosphonic acid, ethephon (an ethylene-releasing compound; Van Loon, 1977), benzoic, salicylic and acetyl salicylic acids (White, 1979) have all been successful in this context. It is conjectural as to how these stimuli, along with the CuSO_4 and mannitol-induced plasmolysis detailed

here actually stimulate protein synthesis (or degradation) and whether this effect is due to a simulation of virus infection, or an imitation of the stress effects of necrosis. Indeed all stimuli (or inducers), pathogenic or abiotic could through hormone mediation have significant effects on host protein metabolism.

The striking effect of mannitol-induced osmotic stress in imitating virus infection by stimulating similar proteins in both cucumber (Figs. 43 and 44) and to a lesser extent in cowpea tissue (Fig. 18) is without precedent. This phenomenon shows that gene expression in healthy tissue can apparently be altered directly or indirectly by either virus-infection, mannitol-induced osmotic stress or other stress conditions. However, the signal(s) for γ production, whether it (they) acts (act) for induction, amplification, or degradation is (are) stronger in necrotic tissue than in plasmolysed material as evidenced by the peak sizes on densitometer scans (Fig. 43; cf. Fig. 29 with 44). How such control is mediated is unknown but de-repression of a normal host gene(s) is possible as synthesis of a normal protein in cucumber has not been disproved.

The overall enhancement of soluble protein content caused by mannitol-induced osmotic stress in both cucumber cotyledons (Fig. 44) and cowpea leaves (Fig. 18) along with the appearance of the γ and the γ_A fractions in cucumber cotyledons (Fig. 44) and the fast migrating low molecular weight proteins in cowpea leaves (Fig. 18) in large quantities were not associated with any decreases in the faster migrating proteins observed in control (water-floated) tissue (Figs. 18 and 44). This observation is in disagreement at the notion that mannitol-induced water stress (osmotic stress) causes a 'spill-over' of small protein molecules into larger fractions caused by an aggregation of the small proteins by inter-molecular bonding as suggested by Tatt (1978a).

It is tempting to suggest that a micro-environmental osmotic stress created by the virus in localized areas, in cases where localisation is

either accompanied (e.g. TNV) or not accompanied (e.g. TMV) with necrotization, may be similar to the effects of mannitol-induced osmotic stress in inducing or amplifying γ production. This idea is strengthened by the fact that this protein or a similar one was absent from cucumber cotyledons systemically infected with CMV_w (Fig. 43) and a mild strain of CMV (Ziemicki and Wood, 1975) but was present in small quantities in cotyledons infected with a more severe CMV strain (Tas and Peters, 1977).

It is, therefore, suggested that a "path-osmotic" stress developed in the initial stages of virus infection and maintained during and after local lesion formation may be responsible for some of the biochemical changes detectable during the process of localisation. These alterations are more pronounced with the development of necrosis, and may have a direct or indirect role in localisation.

It could be argued that even though all cells in healthy tissue floated on hypertonic mannitol solutions are continuously subjected to osmotic stress, the amounts of γ produced are slightly less than that found in TNV-infected tissue (Fig. 43). This is partially invalidated by the fact that tissues subjected to osmotic stress are not as efficient in synthesizing proteins (Dhindsa and Cleland, 1975) as apparently healthy tissue surrounding local lesions is. Progressive increases in osmotic pressure around developing local lesions as opposed to the osmotic shocking of tissue (e.g. that caused by a sudden exposure to a hypertonic mannitol solution) may be more efficient in inducing such proteins.

The proposed effect of mannitol-induced osmotic stress in the production of the γ fraction, and to a lesser extent the γ_A fraction in cucumber is partly supported by other observations on the early stages of hypersensitive reactions and associated cytopathic alterations in several virus-host combinations. These changes indicate that high osmotic pressure develops in the areas of localisation causing plasmolysis of cells and rupturing of cellular membranes (Ragetti, 1967; DaGraça and Martin, 1975)

with obvious changes in the permeability of the plasma membrane (Ohashi and Shimomura, 1976; Weststeijn, 1978; Pennazio et al., 1979). Such pathological changes along with the early report that tissue surrounding local lesions are characterized by a massive accumulation of sugars, most probably pentoses (Solymosy and Farkas, 1963) suggest that hypertonic conditions developing in areas of localisation create a micro-environmental osmotic stress which in some fashion might trigger protein synthesis.

The γ fraction was only detectable in apparently healthy tissue (AH) directly adjacent to the mid-ribs of completely necrotic half cotyledons (Fig. 31) but could not be recovered from tissue far remote from that originally inoculated (Section IV. 22).

The demonstration of the γ fraction in AH-tissue made it feasible to radioactively label the γ fraction in AH-tissue discs with ^{14}C L-leucine (Fig. 36). Total incorporation of the radio-active precursor into the soluble protein fraction of AH-tissue was greater than that in control tissue by 15.0% and that specific incorporation into the γ band was nearly 18% more than that in control tissue, as measured by the radioactivity incorporated into gel slices taken at the γ position (Fig. 36).

The incorporation of ^{14}C L-leucine into the γ fraction (band) in AH-tissue may indicate an active synthesis rather than accumulation of the protein(s) by passive movement (diffusion) from the necrotic halves of cotyledons. This observation may reflect amplification (or induction) of host genome activity in synthesising proteins. It is, however, possible that this fraction(s) is (are) a degradation product(s) either generated during extraction, or present in vivo.

The observation that the amounts of the γ fraction in Baton-Vert cotyledons, infected with C. lagenarium can be partially reduced by prior AmD treatment (Fig. 37) suggests that the information for the γ protein synthesis is coded for by the host-DNA rather than the viral RNA and is mediated by DNA-dependent host-RNA synthesis involving novel mRNA forms. Worth

mentioning in this context is an additional protein at Rf 0.79 (γ_1) appears in the soluble protein fractions of C. lagenarium infected cotyledons following AmD treatment (Fig. 37, D). This protein might be a substrate for a degrading enzyme(s), whose synthesis has partially been inhibited by AmD. Alternatively, it could be a minor fungal constituent only detectable by PAGE following the drug treatment.

Concomitant chloramphenicol (Ch.Ph.) treatment and plasmolysis reduced the amounts of the γ and the γ_A proteins found in osmotically stressed Explorer cotyledons, while another protein at Rf 0.60 was stimulated by the treatment (Fig. 46). Each of the above antibiotic interferes with protein synthesis differently, AmD at the transcriptional, Ch.Ph. at the translational level, but both increase TNV-replication in other virus-host combinations (McCarthy et al., 1972; Faccioli and Rubies-Autonell, 1976) and this may suggest the involvement of these proteins in the localisation mechanism, but this is not unequivocally proven.

The γ protein in Ashley and Baton-Vert and γ and γ_A in Explorer are apparently not isozymes of either PO or PPO (Fig. 47). But the γ protein in Ashley is associated with an isozyme of RNase (Figs. 50 and 51). However, the same isozyme is found in healthy tissue (Figs. 50 and 51) and may be the same co-migrating protein reproducibly seen in minute quantity at the same Rf value in healthy gel scans (Fig. 29,B).

The relative increase in the quantity of the glycoprotein band at the γ position, in both Ashley and Explorer cotyledons following TNV-infection, as compared to the band already present in healthy tissue (Fig. 33) is not proportional to the amounts of protein found by Coomassie blue staining (Fig. 29). This imbalance along with the associated RNase activity suggests that γ band may be a family of proteins co-migrating to the same position in gels. The family may have three members, a glycoprotein, an RNase and another protein, thought to constitute the bulk of the γ band. Two dimensional gel electrophoresis and/or iso-electric focussing would resolve this point.

Analysis of the soluble protein extracts from protoplasts freshly isolated from Ashley cotyledons where the γ fraction had previously been induced by a 2 day exposure to mannitol-induced osmotic stress and from AH-tissue (5 days post-inoculation of the adjacent half leaves), where the γ fraction is known to be present (Fig. 31) showed that the γ fraction (band) disappears totally from protoplasts (Figs. 55, 56, 57 and 58). However, when the RNase zymograms were developed for these extracts, the RNase isozyme normally accompanying γ was retained (Fig. 59 and 60). The loss of the γ protein, and the retention of the associated RNase activity in the protoplasts suggest an inter-relationship may exist between the γ protein, cell wall, plasma membrane and/or the cellular free space, and confirms the familial nature of the γ band.

Following the homologous challenge of cucumber cotyledons, induced resistance has been demonstrated in half leaves adjacent to necrotic tissue, by a reduction in lesion number (Table 7). In first true leaves, a reduction in both lesion number and size was seen after challenge following prior inoculation of both cotyledons (Table 7). The absence of the γ fraction in the partially resistant upper leaves make a direct link between γ and acquired resistance unlikely, however, this does not completely rule out the existence of an indirect relationship (Andebrhan *et al.* 1980).

In other hypersensitive situations involving tobacco, low molecular weight proteins have been found in the soluble fraction of leaves remote from those originally inoculated. These leaves show a degree of induced resistance to challenge inoculations (Ross, 1961b and 1966; Van Loon, 1975b; Kassanis *et al.*, 1974), and similarities to animal interferons have been suggested for the novel proteins (Kassanis and White, 1974). However, this is apparently not the case with either cowpea or cucumber systems.

Similar to cowpea, when the effects of abrasion, TNV-infection, floatation on water and floatation on 0.5M mannitol on PO and PPO isozyme

patterns in Ashley cucumber cotyledons were compared 3 days after treatment, only quantitative changes were observed in abraded and water-floated tissue (Figs. 48 and 49), while both quantitative and qualitative changes were seen following TNV-infection and mannitol treatment (Figs. 48 and 49). However, the pattern of isozymes from the last two treatments was somewhat similar for PPO (Figs. 48, B and 49,B) but different for PO (Figs. 48,A and 49,A).

TNV-infection caused similar alterations in the PO and PPO isozyme patterns in both Baton-Vert and Ashley cotyledons with both quantitative and qualitative changes (cf. Fig. 47,A and B with 48). In Explorer (Fig. 47,C and D), alterations in both enzymes were only quantitative reflecting the rather peculiar physiological response of Explorer to different stimuli (or inducers) as compared to the other cultivars.

When RNase zymograms were developed from Ashley cotyledons subjected to various treatments (Figs. 52 and 53), similar patterns were observed and only quantitative changes were noted. The effects of the treatments on the total enhancement of RNase isozyme patterns (Figs. 52 and 53) can be ranked as follows :

Mannitol-induced osmotic stress > TNV-infection > mechanical injury (abrasion) > floatation on H₂O > no treatment (control).

Mannitol-induced osmotic stress, thus, stimulated RNase activity the most (Figs. 52 and 53,e). This ranking was based on visual inspection of gels (Fig. 52) and comparison of densitometer scans (Fig. 53). A similar order of magnitude was seen when the total RNase activities were assessed spectrophotometrically (Fig. 61, C; see later).

The RNase isozyme found coincident with the γ band in gels loaded with extracts from TNV-infected Ashley cotyledons was also found in the unabraded tissue (Fig. 52) eliminating the possibility that this particular isozyme was the result of a wound response.

Because of the similarities between the results obtained on total activities of PO, PPO or RNase following different treatments, and the alterations in enzyme activity, with time, following virus infection, in both cowpea and cucumber, the following part of the discussion will be concerned with the results from both systems.

The total activities of PO, PPO, and RNase in both cowpea leaves and Ashley cucumber cotyledons, subjected to the same treatments as mentioned earlier, were quantitatively assessed 3 days after treatment. Determination of the stress effect of these treatments on the enzyme levels as compared to healthy unabraded (untreated) controls was made (Figs. 25 and 61).

A summary of the comparative effects of the above stress conditions on these enzymes in cowpea tissue is given below.

(PO)	TNV	>	Mann.	>	H ₂ O	>	PO ₄	>	untreated
(PPO)	TNV	=	Mann.	>	H ₂ O	>	PO ₄	>	untreated
(RNase)	Mann.	>	TNV	>	H ₂ O	=	PO ₄	>	untreated

The comparative effects of the above stress condition on these three enzymes in Ashley cucumber cotyledons were similar and summarized below for comparison.

(PO)	TNV	>	Mann.	>	H ₂ O	>	PO ₄	>	untreated
(PPO)	TNV	>	Mann.	>	H ₂ O	>	PO ₄	>	untreated
(RNase)	Mann.	>	TNV	>	H ₂ O	>	PO ₄	>	untreated

One difference between the two systems was that TNV-infection resulted in an increase in PPO activity to a level higher than that induced by plasmolysis (Fig. 61, B), (but this may reflect a difference in the severity of infection). Another difference was that water-floated cotyledons had a higher activity of RNase than that in abraded cotyledons, but mannitol

induced osmotic stress consistently increased the activity of RNase to a level much higher than that caused by TNV (Fig. 61, C).

The presence of such high levels of RNase activity in mannitol osmotically stressed cowpea leaves (Fig. 25, C) and Ashley cotyledons (Fig. 61, C) without any visible necrosis supports the contention that some RNase isozymes are stress-stimulated (or induced) enzymes and are unlikely to be directly involved in necrogenesis.

Although the effect of osmotic stress on the quantitative increase in RNase activity has been illustrated previously in other systems (Diener, 1961; Wyen et al., 1972), none included a combination of treatments where a comparative stress could be employed to stimulate RNase activity to a level higher than that found in necrotic tissue with no necrosis.

Although the effects of mannitol-induced osmotic stress on PO activity in both cowpea (Fig. 25, A) and cucumber (Fig. 61, A) were consistently less effective than those of TNV-infection in stimulating enzyme activity, the results presented here are persuasive enough to eliminate these enzymes as being involved directly in the hypersensitive reaction. This supports previous conclusions drawn by Faccioli (1979) for the two enzymes, PO and PPO and Wyen et al. (1972) for RNase.

Similar conclusions could be drawn when the quantitative alterations in PO, PPO, and RNase activities with time following TNV-infection of both systems were compared (cf. cowpea, Fig. 26 and cucumber Fig. 62). Here TNV-infected leaves or cotyledons, uninoculated half leaves or cotyledons adjacent to TNV-infected halves, and the respective controls were compared. It was found that increases in PPO in cowpea leaves and PO and PPO in Ashley cucumber cotyledons were detectable in TNV-infected tissue and coincident with the appearance of visible necrotic lesions. Enzyme activity continued to increase as the necrotic reaction proceeded up until 6 days post-inoculation, but no increases were found prior to lesion appearance (Fig. 26 and 62). Similar results have been observed in other studies on

both PO and PPO enzymes where significant increases in PO activity were only detectable at the time of appearance of the local lesions (Farkas et al., 1960; Loebenstein and Linsley, 1966; Chant and Bates, 1970; Simon and Ross, 1970 and 1971a; Cabanne et al., 1971; Van Loon and Geelen, 1971; Veggetti et al., 1975). Whereas for PPO activity either no detectable increases, before, during, or even after local lesion formation have been reported (Veggetti et al., 1975) or increases were only found at the time of lesion appearance which increased in parallel with local lesion maturation (Van Kammen and Brouwer, 1964; Jockusch, 1966; Cabanne et al., 1971; Van Loon and Geelen, 1971). The results presented in this study along with the above observations support the current notion that increases in both PO and PPO activity accompanying the hypersensitive reaction are not the cause of necrosis but rather a consequence of it. It may be that the PO increases are induced totally independent of necrosis as suggested by Faccioli (1979) who showed that even though AMD increased local lesion size it did not result in an increase in PO activity before lesion appearance. The results reported here for PO alterations in cowpea leaves after infection with TNV (Fig. 26) cannot be compared to the former study, because the first leaf sample taken was that 48 hours after inoculation when local lesions were already visible. However, the results with TNV-infected cucumber (Fig. 62) are convincing enough.

With RNase a peak of activity was recorded in both systems, much clearer in cowpea leaves (Fig. 26,C) than in cucumber cotyledons (Fig. 62,C), one day after inoculation. This increase in RNase activity preceded local lesion appearance and was coincident with similar increases (quantitatively less) in abraded tissue (Figs. 26, C and 62, C). The increase in RNase activity in TNV-infected tissue represented only a wound response and is not involved in the hypersensitive reaction. This conclusion is supported by the previously described results on the effects of osmotic stress on RNase activity in cucumber cotyledons (see earlier).

The only enzyme that was detectable in AH-tissue in both systems was PO which was almost doubled in activity, 6 days after inoculation in cowpea (Fig. 26, A) with considerably higher activity in cucumber (Fig. 62, A) as compared to the controls ($\frac{1}{2}$ H). Whilst increased activity of either PPO and RNase were not routinely detectable in the AH-tissue of cowpea (Fig. 26, B and C), a slight increase in RNase activity was recorded in similar cucumber cotyledonary tissue and was as high as that in abraded cotyledons (Fig. 62, C). This alteration in enzyme activity may reflect physiological changes characteristic of a progressive senescence in the cotyledonary tissue.

Comparisons of the soluble protein profiles of TNV-infected tissue of both cowpea leaves and cucumber cotyledons and hypersensitive tobacco leaves (Gianinazzi et al., 1970; Van Loon and Van Kammen, 1970; Rohloff and Lerch, 1977; Coutts, 1978b) were also made. Analysis of the soluble protein extracts obtained from TNV-infected Xanthi-nc. tobacco illustrated four 'novel' proteins, A, B, C and D (Fig. 63) as previously (Coutts, 1978b).

In order to establish whether a common protein(s) existed in the three systems following TNV-infection, co-electrophoresis of soluble proteins from different combinations of soluble proteins was made. The results revealed that the γ fraction of cucumber is apparently cucumber-specific, whereas the β protein of cowpea is similar to the B protein of tobacco at least in terms of electrophoretic mobility. However, at the quaternary structure level the two may differ as the β fraction of cowpea was found to dissociate into three components, β_{m1} , β_{m2} and β_{m3} with respective mol. wts. of 10,000, 25,500 and 36,000d. (Table 2 and Fig. 12), while the B protein of tobacco gave a single band of mol. wt. 14,200d. (Antoniw et al., 1980).

The conclusion that the γ protein of cucumber (Baton-Vert) is a cucumber-specific protein is based on both native - and SDS-PAGE studies. This conclusion is in contrast to the suggestion that the similar E_1 protein

(Tas and Peters, 1977) may be identical to B protein (b_2 or III in the nomenclature of Gianinazzi et al. or Van Loon and Van Kammen respectively) in tobacco. This suggestion is not supported by experimental data. The γ and the β fractions band at different positions on native-PAGE (Fig. 64, B) and have different mol. wts. when denatured (cf. γ , 24,500d. with B, 14,200d. Antoniow et al., 1980). Also the γ fraction stains positively for glycoproteins (Fig. 32, A and B) while the B band does not.

From the foregoing, the suggestion that the 'b' proteins of tobacco leaves and similar proteins induced in different hosts following a hypersensitive reaction should be termed pathogenesis-related (PR) proteins (Antoniow et al., 1980) has to be rejected. The term itself implies that the proteins are strictly related to pathogenesis, even though, the same authors (Van Loon, 1977; Antoniow et al., 1980 and Antoniow, personal communication) and others (Gianinazzi and Kassanis, 1974; White, 1979) have illustrated that several non-pathogenic, abiotic, agents could induce similar proteins in healthy leaf tissue. It may be more realistic to call such proteins "Stress related proteins" (SRP) until their role(s) in pathogenesis is established. However, the defined system of nomenclature suggested by the same authors (Antoniow et al., 1980) is quite acceptable.

The suggestion proposed by Van Loon (1980, personal communication) that through stimulation of aromatic biosynthesis, ethylene produced during the hypersensitive reaction leads to the synthesis of a, presumably, aromatic compound that mimics the action of salicylic acid in inducing pathogenesis-related (PR) proteins (b-proteins of tobacco) and acquired resistance is rather dubious. The reason being that this proposal strongly links the hypersensitive reaction (necrosis) and the production of the PR-proteins via ethylene, even though, other workers (Gianinazzi and Kassanis, 1974) have clearly shown that treating Xanthi-nc. tobacco leaves with a low concentration of polyacrylic acid, which does not induce a hypersensitive reaction (i.e. does not cause necrosis) results in production of at least

three of the four 'b' proteins (PR-proteins). Moreover, although polyacrylic acid, unlike salicylic acid is aliphatic and not aromatic, it also induces the "PR" proteins.

The ability of both polyacrylic acid and salicylic acid, in spite of the difference in chemical structure, to induce the 'b' proteins of tobacco is difficult to interpret in this way unless it is unequivocally shown that polyacrylic acid treatment, or any other 'b' protein-inducing treatment, either results in an increase in ethylene production or acts as a precursor (or inducer) for the supposed (b) protein-inducing aromatic compound.

CONCLUSIONS

Infection of cowpea (Vigna sinensis Endl. var. Blackeye) primary leaves and cucumber (Cucumis sativus L. cv Ashley, Baton-Vert, China, Chipper, Elem, and Explorer) cotyledons with TNV_D is followed by localisation of the virus in necrotic local lesions and narrow areas of tissue surrounding them.

The necrotic reaction, due to the hypersensitive response in both cowpea and cucumber following TNV_D infection is apparently not responsible for complete localisation of the virus in the necrotic local lesions, as both virus particles and viral RNA were detected outside local lesions.

Concurrent with lesion formation, reproducible alterations occur in the soluble protein fractions of these tissues as evidenced by disc-PAGE. In cowpea two major fractions, α and β , are formed in association with four other proteins which appear as 'shoulders' (sh_1 , sh_2 , sh_3 and sh_4) on the two major peaks. In cucumber tissue, one apparently common 'novel' protein band (γ) appears in the soluble protein fraction of all cultivars tested, with two proteins (γ and γ_A) in the Explorer cultivar. The γ fraction is common to all the cucumber cultivars investigated in this study including Explorer as evidenced by co-electrophoresis of soluble protein extracts, whereas the γ_A fraction appears to be an Explorer-specific fraction. Although a similar protein to the γ fraction has been reported before in cucumber cotyledons infected with other viruses (viz E_1 protein, Tas and Peters, 1977), the Explorer-specific protein fraction (γ_A) is reported here for the first time.

The protein fractions from both plant species are dissimilar regarding chemical nature, molecular weight and inducibility. The α and the β fractions in cowpea are strictly proteinaceous, whilst the γ and the γ_A fractions in cucumber, are glycoproteins. The α and the β fractions have a common polypeptide of 10,000d. mol. wt. and the β fraction possesses two

additional polypeptides of 25,000d. and 36,000d. mol . wt., whilst the γ fraction is a monomeric protein and has a molecular weight of 24,500d. Both CuSO_4 treatment and mannitol-induced osmotic stress induced the γ fraction in cucumber and the γ_A fraction to a lesser extent. Mannitol-induced osmotic stress induced a group of low molecular weight proteins in cowpea, one of which was coincident with the β fraction, but CuSO_4 treatment had no effect on the soluble protein profile.

The novel proteins in both TNV-infected cowpea and cucumber are detectable only in necrotic tissue and in uninoculated apparently healthy (AH) half leaf tissue, adjacent to completely necrotic halves. The proteins decrease quantitatively away from the mid-rib in the former. Neither active nor novel synthesis of the α or the β fractions in AH-tissue of cowpea is proven. However, the production of the γ fraction in cucumber is dependent on host-DNA transcription and not on viral RNA translation.

The function(s) of these proteins is (are) obscure, but neither the α nor the β fraction is associated with peroxidase (PO), polyphenol oxidase (PPO), or ribonuclease (RNase) activity. Similarly neither the γ nor the γ_A fraction is associated with either PO or PPO activity, but a normal, constitutional, RNase isozyme is coincident with the γ band. This RNase isozyme is not stimulated by a wound response, and its subcellular location appears to be different from the rest of the γ band which is thought to have an inter-relationship with cell wall, plasma membrane, and/or the cellular free space.

The γ band is thus considered to be a family of at least 3 co-migrating proteins, on native gels, a glycoprotein, an RNase isoenzyme(s), and another (other) protein band(s), possibly "novel" which constitutes the bulk of the γ band.

The γ fraction is produced in large quantities where localisation is associated with necrosis (e.g. TNV or C. lagenarium) and in smaller quantities where it is not (e.g. TMV). The γ fraction was not detectable in tissue

systemically infected with virus (e.g. CMV). Neither the γ nor the γ_A fraction is a by-product of necrosis, but their stimulation (or induction) appears to be different.

The ability of mannitol-induced osmotic stress to imitate virus infection in inducing similar soluble proteins is a newly discovered phenomenon. This observation suggests that a similar osmotic stress condition might operate in virus-infected tissue where a "path-osmotic" stress developing in the areas of virus localisation is maintained during and after local lesion formation. Such a stress may be responsible for the recognisable biochemical alterations, detectable during the localisation process, including the generation of 'novel' soluble proteins. The path-osmotic stress may have a similar fundamental trigger(s) for stimulating or inducing protein synthesis to that of CuSO_4 treatment or mannitol-induced osmotic stress.

Neither in cowpea nor in cucumber has the presence of the 'novel' proteins been absolutely correlated with induced resistance, particularly in cucumber where the γ protein is absent from partially resistant upper leaves. Thus, the described 'novel' proteins have no apparent direct role(s) in induced resistance, but indirect ones cannot be excluded. The proteins may well be involved in as yet uninvestigated enzymic, hormonal, or structural alterations in both inoculated and other leaves.

The effects of different stress conditions on the quantitative levels of PO, PPO, and RNase in both cowpea leaves and cucumber cotyledons were similar with TNV-infection being the most effective stress factor in stimulating PO and PPO, followed by mannitol-induced osmotic stress, water floatation and mechanical injury. Mannitol-induced osmotic stress was the most effective stress condition in stimulating RNase activity in healthy tissue to a level higher than that recorded in TNV-infected tissue in both systems.

The presence of such high levels of RNase activity in mannitol-

osmotically stressed cowpea leaves and cucumber cotyledons with no visible necrosis supports the contention that RNase is not directly involved in necrogenesis, but rather that it is a consequence of the stress condition.

Studies made on the quantitative alterations of PO, PPO and RNase activities, with time after virus-infection, of both cowpea leaves and cucumber cotyledons proposed that these enzymes are not directly involved in necrosis but are consequences of it.

The increase in PO activity in the AH-tissue of both cowpea and cucumber may reflect progressive senescence of leaves.

Infection of Xanthi-nc. tobacco leaves with TNV resulted in induction of the four known 'novel' proteins, A, B, C, and D in soluble protein extracts. Co-electrophoresis of these extracts with those derived from cowpea, and cucumber showed that the γ protein fraction of cucumber is a cucumber-specific band, whereas the β fraction of cowpea has some similarity to the B protein of tobacco but only with regard to electrophoretic mobility. Also the molecularweights of the proteins on the SDS-PAGE were different for either fraction (see earlier and Antoniwi et al., 1980).

The suggestion proposed by Antoniwi et al. (1980) that the four soluble proteins, b_1 , b_2 , b_3 , and b_4 , known to be produced in tobacco tissue following virus-infection, and any other similar proteins in different hosts (e.g. α , β , γ and γ_A reported in this study) should be called pathogenesis-related (PR) proteins is rejected. An alternative name is proposed, that of "stress-related proteins" (SRP) until a definite function for these proteins in pathogenesis is described.

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