BIOCHEMICAL CHANGES IN COMPATIBLE AND INCOMPATIBLE FLAX/FLAX RUST INTERACTIONS

by

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Frontispiece: Flax Seedlings One Week After Seeding.

ABSTRACT

The possible biochemical mechanisms of varietal and species specificity of obligate fungal parasites are considered in relation to the established genetics and biochemistry of host-parasite systems. The two general theories of specificity which have been put forward are identified. One invokes the induction of successful pathogenesis as a primary determinant in specificity while the other predicts that recognition of avirulent pathogens by the host leads to induced resistance which acts as the determinant in specificity. The former theory is supported by evidence for the appearance of novel host enzymes during disease development. The latter is supported by observations of host gene derepression and phytoalexin accumulation which occur in resistance responses at times prior to any significant response in susceptible combinations.

These theories are examined experimentally in the flax/flax rust system. The molecular origin of ribonuclease with altered catalytic properties, which arises during disease development, is examined. Rigorous purification reveals that the altered catalytic properties can be

accounted for by altered proportions of ribonuclease I isozymes. These changes are similar during resistant and susceptible reactions until sporulation occurs.

Induced resistance is characterized and compared to primary changes during susceptibility through a detailed study of RNA and protein synthesis. Enhanced RNA synthesis occurs in the resistant reaction at times prior to any measurable response in the susceptible combination, although both combinations exhibit higher rates of RNA synthesis at later times. RNA was fractionated by electrophoresis and affinity chromatography and messenger activity assessed by *in vitro* translation. Enhanced RNA synthesis is characterized by decreased messenger polyadenylation. However, polyadenylated and non-polyadenylated messenger RNA were shown to encode many common polypeptides; this provides an explanation for the fact that few changes in *in vivo* protein synthesis can be detected by one or two dimensional electrophoresis. The only marked changes in protein synthesis occur in the susceptible combination and include a dramatic decline in the synthesis of the large subunit of ribulose-1,5-bisphosphate carboxylase.

It is concluded that incompatibility of an avirulent race of rust with the flax variety Bombay is determined by induced resistance resulting from recognition of the avirulent pathogen prior to the initiation of pathogenesis. However, specific biochemical events in the host, particularly those involving chloroplast function, may be necessary for successful pathogen development.

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LIST OF ABBREVIATIONS

A^+	Polyadenylated RNA
A^-	Non-polyadenylated RNA
PMSF	Phenylmethylsulfonyl fluoride
kD	Kilodalton
K_m	Michaelis constant
LHC	Light harvesting complex
MAK	Methylated albumin Kieselguhr
^{32}P	Phosphorus-32 (radioactive isotope)
Rf	Migration, ratio to solvent front
RNA	Ribonucleic acid
mRNA	Messenger RNA
rRNA	Ribosomal RNA
tRNA	Transfer RNA
tcRNA	Translational control RNA
RNase	Ribonuclease
RuBPCase	Ribulose bisphosphate carboxylase
S	Svedberg, unit of sedimentation
^{35}S	Sulphur-35 (radioactive isotope)
SDS	Sodium dodecyl sulphate
TCA	Trichloro-acetic acid
U^+	Poly- or oligouridylate containing RNA
U^-	Poly- or oligouridylate lacking RNA

GLOSSARY OF TERMS

Compatible combination	Host and pathogen genotypes which enable pathogenesis.
Haustorium	Fungal infection structure which is found within the host cell wall and in aposition to the host cell membrane.
Horizontal resistance	Resistance which is constant regardless of the race (or genotype) of pathogen against which it is tested. Often inherited polygenically.
Hypersensitivity	An active resistance mechanism in which the rapid death of host cells around the point of infection prevents colonization.
Incompatible combination	Host and pathogen genotypes which lead to resistance.
Non-host resistance	The quality of a plant which completely prevents pathogenesis with all races of a pathogen. It is, by definition, a particular type of horizontal resistance in which the resistance is absolute among all genotypes of the plant group (usually refers to a species) and the plants are therefore considered non-hosts.
Pathogenesis	The development of a pathogen at the expense of a host producing an accompanying disease syndrome.
Phytoalexin	Fungitoxic compound which accumulates in a plant in response to infection by a pathogen.
Resistance	The ability of a host to limit the development of a pathogen. Opposite is susceptibility.

Vertical resistance	Resistance in which there is a differential interaction between a variety of host genotypes and a variety of races of pathogen. This differential interaction is determined by major genes and is never polygenically inherited.
Virulence	Ability of a pathogen to overcome one or more major resistance genes. Opposite is avirulence. The term has also been used elsewhere in relation to horizontal resistance but is restricted to vertical resistance here.

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CHAPTER I

INTRODUCTION

The rust fungi, Uredinales, represent one of the largest and most economically important groups of plant pathogens. Among the most studied of the diseases which they cause are the rusts of cereal crops and of flax. Aside from their agricultural significance these diseases hold great promise as tools for elucidating the mechanisms of pathogenesis and resistance in all obligate biotrophic fungal parasites, of which they are representative. Such parasites have three important features. Firstly, they are dependent on living host cells for their development under natural circumstances. Secondly, they have a restricted host range, each *formae speciales* normally being restricted to a single host species. Thirdly, varietal resistance is characterized by a highly specific gene-for-gene system in which complementary alleles of host and parasite interact to determine resistance or susceptibility. These features are central to any understanding of the mechanisms involved in pathogenesis and resistance.

The gene-for-gene theory of varietal resistance postulated for flax by Flor (1956) has the following features: plant resistance genes are dominant and rust virulence genes are recessive; all resistance genes must be

overcome by their corresponding virulence genes in order for successful disease development to occur. Thus the expression of resistance is conditional upon the presence of the corresponding avirulence and resistance alleles. This is illustrated in Figure 1, below.

		HOST GENOTYPES	
		RR/Rr	rr
Rust Genotypes	AA/Aa	-	+
	aa	+	+

FIGURE 1. The outcome of host/rust combinations with complementary genotypes. A = avirulence allele, a = virulence allele; R = resistance allele, r = susceptibility allele. A susceptible combination is denoted '+' and a resistant one '-'. For simplicity the homozygous-dominant and heterozygous genotypes are not distinguished.

The flax/flax rust interactions exemplify this system perhaps better than any other host-parasite interaction. Resistance is expressed at five loci (K, L, M, N, P) with 1, 11, 6, 3 and 4 alleles (respectively) and tends to be an all or nothing response (Flor 1971). These factors have been influential in choosing the flax/flax rust system for the research presented in later chapters.

Two general theories exist for the molecular basis of specificity in gene-for-gene interactions: (1) that complementary alleles of host and pathogen interact to form

proteins with novel properties which enable pathogen development (Chakravorty and Shaw 1977a, Vanderplank 1978); (2) that the products of avirulence and resistance genes interact to elicit an active host resistance response (Albersheim and Anderson-Prouty 1975). The former may be thought of as the induction of susceptibility and the latter as the induction of resistance.

The objective of this thesis is to attempt to resolve which of the two general theories listed above may explain the molecular basis of varietal specificity in which gene-for-gene interactions are involved. These theories are assessed in relation to the established genetics of the flax/flax rust system and are also investigated experimentally. The purification of host ribonuclease with altered catalytic properties from infected plants has represented a key piece of evidence for the idea that the induction of novel proteins is necessary for disease development (Chakravorty et al. 1974c). Thus a detailed examination of ribonuclease has been carried out in order to determine the molecular basis of its altered properties. Research on host RNA and protein synthesis following inoculation has also been carried out in order to determine their role in the resistance response. In this way it is hoped that it may be possible to gain insight into the induction of the resistance response.

CHAPTER 2

REVIEW OF LITERATURE

The first two sections of this review are intended to provide a general overview and interpretation of the subject matter. They are not intended as an exhaustive review of all the original research papers as many such reviews have been written recently and are referred to here.

2.1 A Theoretical Consideration of the Genetics and Molecular Basis of Specificity

The usefulness of an understanding of gene-for-gene systems must be assessed in regard to their general occurrence. Person (1959) has suggested that such a relationship should occur as a general rule in host-parasite systems. Since that time gene-for-gene relationships have been demonstrated in nine further plant parasite systems and suggested in seventeen others (Sidhu, 1975). Gene-for-gene systems are clearly important agriculturally. However, students of pathology should be cognizant of other types of resistance such as horizontal resistance and also of the behaviour of genetic populations within an ecosystem or agricultural system. Plant pathology at this level has been called the pathosystem, a concept which is developed in a very provocative book by Robinson (1976).

The objective of the thesis is to determine the biochemical basis of specificity in gene-for-gene interactions. Briefly, what determines whether so called near isogenic lines of a plant allow successful development of a given isolate of the pathogen? Conversely, why do different isolates succeed or fail to develop on a single plant variety? Additionally the biochemical basis of obligate parasitism and restricted host range are considered.

It is generally accepted that specific changes in host metabolism are necessary for pathogen development. However, there is no genetic framework on which to build an hypothesis of the way in which such changes are induced. Many of these metabolic changes have been studied and are discussed in Section 2.2. The hypotheses presented below are confined to a consideration of differences between plant varieties and rust races. Resistance in such systems has been called varietal or vertical resistance and is known to be inherited by single genes. Polygenically inherited resistance, also referred to as horizontal resistance, is not directly discussed; similarly, non-host resistance is not considered. This approach appears rational because the inheritance of vertical resistance is well understood.

The author has concluded from his consideration of the literature reviewed in this chapter that the various

theories put forward to explain varietal specificity may be categorized within three general hypotheses:

(1) The pre-existing nutritional status of the host determines whether suitable specific metabolites are available for fungal development (Section 2.2.2).

(2) Susceptibility is an induced phenomenon which relies upon complementary gene products of host and pathogen interacting to bring about metabolic changes in the host cell which enable successful pathogen development. (Section 2.2.3).

(3) Resistance is an induced phenomenon in which a gene product of the pathogen interacts (or is recognized) by a gene product of the host and thus a resistance response is initiated (Section 2.2.4).

The first or so-called nutritional hypothesis seems unlikely to be important in varietal specificity as many rusts have now been grown on simple media (Scott 1976; and see Section 2.2). The other hypotheses must clearly be scrutinized in relation to the gene-for-gene theory.

Hypothesis 2 requires that an inducer gene product from the pathogen interacts with a gene or its product in the host in order to bring about susceptibility. Such an hypothesis is very hard to explain in terms of the gene-for-gene theory. The requirement for every resistance

gene to be overcome by a corresponding virulence gene would seem to preclude this type of mechanism. This is true even if avirulence and resistance genes are assumed to be repressors of pathogen inducers and host receptors respectively. Furthermore it can be seen that the virulence allele is not functional in pathogenesis; indeed the AA genotype (Fig. 1) still leads to a susceptible reaction in the absence of the corresponding R allele.

Hypothesis 3 can readily be explained in terms of the gene-for-gene theory. The scheme outlined in Figure 2 shows how avirulence gene products may be responsible for inducing a resistance response through interaction with a corresponding resistance allele in the host. Clearly some kind of recognition phenomenon is implied; for the sake of simplicity the mechanism of this recognition will not be discussed in this Section. In such a system avirulence genes must be seen as constitutive and hence as having a functional role in the pathogen; otherwise they would be expected to have been lost during evolution because they would confer no selective advantage. This is supported firstly by their occurrence at many loci (Flor 1971) and secondly by the fact that virulence alleles confer a disadvantage to the pathogen in the absence of the corresponding resistance alleles (Day 1974). So it can be seen that the theory of induced resistance provides a strong mechanistic interpretation of

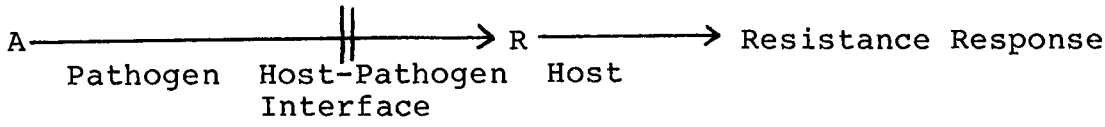


FIGURE 2. Hypothetical host-pathogen interaction leading to induced resistance; the product of the avirulence allele (A) is seen as interacting with the resistance gene (R) or its product in order to induce the resistance response. The location of this interaction in relation to the host-pathogen interface is unknown but may occur, for example, at the surfaces of the haustoria (fungal infection structures which invaginate the host cell membrane).

the gene-for-gene theory on purely logical grounds, a conclusion reached by Person and Mayo (1974).

A body of evidence as to the way in which such induced resistance occurs is beginning to emerge. Some of the earliest biochemical events in resistant reactions involve enhanced host RNA and protein synthesis. Examples of such events which are specific to resistance rather than susceptibility support the proposition that resistance is induced. For this reason these changes in host gene expression are reviewed in Section 2.4.

Clearly physiological changes are induced in the host as a result of infection with a virulent pathogen (Section 2.2). Some of these changes may be essential for pathogen development. The successful induction of these changes determines whether the plant is a host or a non-host for the pathogen. However, as explained above, this induction is unlikely to be controlled by gene-for-gene interactions of the type described by Flor (1971). Therefore two separate interactions can be identified for biotrophic fungal parasites.

a) The induction of successful disease development, which may be regarded as the breakdown of non-host resistance.

b) The breakdown or expression of vertical resistance, involving gene-for-gene interactions.

In evolutionary terms, once non-host resistance is overcome selective pressure will almost inevitably lead to gene-for-gene interactions. Person (1959) has suggested that gene-for-gene systems are a predictable evolutionary outcome of selection pressure for and against resistance genes.

The metabolic changes associated with these levels of interactions are reviewed in the following section.

2.2 Metabolism and Disease

Much of the literature which outlines metabolic changes during disease is concerned with changes that occur during disease development in susceptible combinations. In many cases it is strongly implied that these changes are specific to disease and cannot be induced by other factors. Evidence that different types of events occur during susceptible reactions in closely related race and varietal combinations is non-existent. Indeed, as we have seen, one would not expect the basis of varietal specificity to reside in the susceptible response.

2.2.1 Non-Host Resistance

Many workers have suggested that the induction of a suitable metabolic environment in the host is the basis of host species specificity (reviewed by Heath 1980). However, any such assumption should be questioned because in most instances non-host resistance is expressed prior to haustorial formation (Heath 1980). Haustorial structures, which invaginate the host cells, are the morphological basis through which a metabolic relationship is established. Heath (1981a) has summarized information which indicates that fungal suppression of non-host resistance mechanisms may be the primary determinant of host range. Extracts of bean rust infected French beans are capable of promoting haustorial formation of both cowpea rust on beans and bean rust on cowpeas. Such promotion was not effected by extracts from healthy bean plants (Heath 1980). Furthermore the increased haustorial formation was associated with a suppression of silicon deposition which occurs during non-host resistance (Heath 1981b).

2.2.2 Host Physiology and Pathogen Nutrition

There is still much research to be done before a clear understanding of non-host resistance is reached. For example, there is a possibility that the metabolic association between host and parasite is to some degree established prior to haustorial formation. In view of the long historical basis of data concerned with the significance of host metabolism during susceptibility, the possibility of its

involvement in host species specificity must still be considered.

In characterizing the essential elements of symptom development it is useful to speculate what metabolic environment favours the pathogen's development. In recent years there have been many reports on the nutritional requirements of rust fungi in axenic culture (Scott 1976), which might be expected to shed light on this matter. The nutritional requirements are, however, rather aspecific; in general, minerals, a sugar carbon source, an amino acid nitrogen source and a sulphur containing amino acid are required. A wide range of sugars will support growth as will several amino acids, although, in flax rust, aspartate or glutamate are particularly effective. Interestingly, rust fungi cannot utilize sulphate, being deficient in sulphate reduction, but incorporate sulphite which may be taken up through the y-glutamyl cycle (Scott 1972). Thus, it can be appreciated that the nutritional requirements of rust fungi could, at least potentially, be supplied by any plant. However, the availability of the appropriate nutrient balance in living host tissues could be subject to the correct metabolic environment. At present there are insufficient data relating to localized in situ carbohydrate, amino acid and mineral balance in host-parasite interactions to assess this.

All the changes in host metabolism seen during the development of obligate parasites are in essence biotrophic; enhanced metabolism is the rule. This is not so for necrotrophic parasites which should therefore be regarded separately, both physiologically and genetically. Large increases are seen in respiration, which appears qualitatively different from respiration in healthy tissue (Shaw 1963). Much of the increase can be accounted for in terms of increased activity of the pentose phosphate pathway. Concurrent increases in biosynthetic activity are also characteristic (including RNA and protein synthesis). This information and the apparent link between the respiratory rise and chloroplast collapse have prompted the idea that the pentose phosphate pathway is stimulated by higher NADP concentrations (Scott 1972). Together with the increased utilization of NADH and ATP this evidence is consistent with general enhanced metabolic and biosynthetic activity. One point which does not seem clear is the involvement of photosynthesis. Photosynthesis normally declines during disease but its maintenance is associated with resistance. Infection is not established in leaves lacking chlorophyll unless there is an exogenous sucrose supply. Thus it appears that photosynthate is drained to the infection sites and is essential for pathogen nutrition.

There are various pieces of evidence which may provide clues to the significance of these metabolic changes

to pathogen nutrition. Shaw (1963) cites increases in free amino acids as well as protein in rust infected wheat. Increases in certain amino acids were noted as early as 2 days after inoculation. Enzymes involved in the synthesis and utilization of glutamate and in the synthesis of glutamine have also been shown to increase in various rust infected plants. Such changes do not occur in closely related resistant plants. This early work has been extended by Sadler and Shaw (1979a) in the flax/flax-rust system. These workers have shown that highly purified glutamate dehydrogenase exhibits changes in catalytic properties during disease. The glutamate dehydrogenases were examined from healthy, 1 day infected and 7 day infected cotyledons; the enzymes differed from one another in the nature of inhibition by ATP and pyridoxal phosphate. Subsequent isotope pulse-chase experiments demonstrated that the glutamate synthase-glutamine synthase pathway is the major pathway of nitrogen assimilation (Sadler and Shaw 1979b). Isotope trapping experiments were also performed to elucidate the role of glutamate dehydrogenase (GDH). GDH appeared to be an enzyme of glutamate degradation in 1 day-infected cotyledons but played a minor role in glutamate synthesis in 7-day infected cotyledons. The latter might be accounted for by the elevated level of ammonium ions in these tissues. These results are interesting in the light of altered properties of GDH in infected tissue which may be significant in its altered function in glutamate metabolism. The relevance of

these findings to fungal nutrition is as yet unclear but the specific induction of novel GDH enzymes by the fungus cannot be ruled out.

2.2.3 Induced Susceptibility

There is a large body of evidence for enhanced metabolism in diseased plants at least some of which can be rationalized as directly beneficial to pathogen nutrition. The central question remains: to what extent are these changes specifically induced by the pathogen? One most obvious way in which to test the existence of induced susceptibility is to infect a non-host plant which has undergone prior inoculation with an aggressive pathogen. Clearly, there are inherent problems with this approach. Firstly, important induced metabolic changes may be very localized and secondly induction by one pathogen may not generate a suitable cellular environment for another. Also, as has already been mentioned, induction may merely represent the suppression of non-host resistance. However, a few examples of such induced susceptibility do exist. Preinoculation of barley with powdery mildew of barley (Erisyphe graminis) allows melon mildew (Sphaerotheca fuligena) limited development on this, normally non-host, plant species (Bell 1981). Other examples of induced susceptibility involve the breakdown of vertical resistance and are as such a separate issue. Many studies have involved the use of RNA and protein synthesis inhibitors in destroying varietal resistance (reviewed by Bell 1981). Clearly no active induction of susceptibility takes place

in this case; this information will be discussed in relation to vertical resistance (Section 2.4).

Chakravorty and Shaw (1977a) have reviewed possible ways in which susceptibility may be induced at the molecular level. These authors suggest that the large body of evidence for the appearance of enzymes with altered properties during susceptible reactions indicates specific induction either at the genomic level or mediated through complementation of host and parasite DNA, RNA and polypeptides. Qualitative changes in ribonuclease (RNase) seen in many rust and powdery mildew diseases provide support for this idea. Increases in RNase and other enzymes are paralleled in susceptible and resistant combinations early after infection. That other metabolic events such as the respiratory rise occur concurrently in both combinations at these early stages is well known (Shaw 1963, Scott 1972). Such evidence prompted Chakravorty and Shaw (1977a) to suggest that resistance is not determined during these events which they have referred to as 'stage 1' interactions but that susceptibility or resistance is determined by the qualitative changes brought about during later events termed 'stage 2' interactions. That such 'stage 2' events are obligatory for successful pathogenesis is a plausible and important concept.

Vanderplank (1978) has proposed that co-polymerization of host and pathogen proteins is responsible for susceptibility. Taken as such, this idea is rather similar to that of Chakravorty and Shaw (1977a), being complementation of a particular type. However, he invokes

the host and pathogen proteins as being the products of susceptibility and avirulence alleles respectively. He suggests that in this way the host protein is prevented from functioning in autoregulation. The result would be that abnormally large amounts of this protein would be synthesized and he surmizes that this extra protein provides an ideal food for the pathogen. By this theory when the corresponding resistance allele is present there is no longer copolymerization, the pathogen protein is then recognized by the host and a resistance response is elicited. In support of his theory Vanderplank cites the enhanced RNA and protein synthesis observed during disease and the existence of antigens common to host and pathogen of susceptible combinations. He also claims that temperature sensitive resistance alleles become ineffective at higher temperatures because the proposed hydrophobic copolymerization is more stable at higher temperatures. Vanderplank's theory has an inconsistency which is worth mentioning here. It is useful in this regard to consider a single resistance locus and its corresponding avirulence locus (Fig. 3).

	Host Genotypes		
	RR	Rr	rr
Pathogen Genotypes AA	-	-	+
Aa	-	-	+
aa	+	+	+

Figure 3. Reaction types in a gene-for-gene interaction; +, susceptible; -, resistant.

Firstly consider the interaction between the heterozygous genotypes (Aa/Rr). In this case one must assume that the non-matching alleles 'A' and 'R' produce a resistance response in spite of the copolymerization of the products of 'A' and 'r' and 'R' and 'a'. In other words resistance is dominant. However, in the interaction between the two homozygous genotypes 'aa' and 'rr' a susceptible response results, and hence one must assume that the product of 'a' is also capable of copolymerizing with the 'r' gene product. Thus it can be seen that for this hypothesis to be workable (on purely genetic grounds) certain elements of specificity between gene products must be overlooked. As has been pointed out in Section 2.1, the gene-for-gene theory can only be rationalized effectively if the virulence allele is assumed to be non-functional in the induction of susceptibility.

2.2.4 Induced Resistance

Thus far I have dealt with the evidence relating to successful disease development. I will now deal with that which is concerned solely with resistance mechanisms in gene-for-gene type interactions. The genetic theory which predicts that such resistance is induced as the result of the interaction of single genes has been presented in Section 2.1.

On a histological level race-specific varietal resistance is characterized by rapid necrosis (or hypersensitive cell death) of the approached or penetrated

host cells accompanied by the accumulation of phytoalexins and the inhibition of fungal growth (Heath 1980). A few studies have shown that protein synthesis and RNA synthesis (including mRNA) are enhanced prior to this stage in incompatible but not in compatible combinations (Heath 1980, Bell 1981, see also Section 2.4). Such results have been demonstrated in the flax/flax rust, oat/crown rust and soybean/Phytophthora megasperma var. sojae systems (reviewed by Heath 1980, Bell 1981). Thus there is an increasing body of evidence demonstrating induced resistance. The important question remains as to how this resistance is induced at the molecular level, and how the products of resistance and avirulence genes are involved.

The most conceptually attractive hypothesis for the mechanism of induction was put forward by Albersheim and Anderson-Prouty (1975). These workers postulate that cell surfaces are the primary site of recognition which leads to specific induction of the resistance response. They propose that pathogenic avirulence genes encode glycotransferases that give rise to race specific fungal wall-bound carbohydrate residues. Host resistance genes are seen as coding for specific membrane proteins which are capable of binding (recognizing) these residues. The resulting complex may then trigger a resistance response. Lack of the specific carbohydrate residue in the pathogen would enable it to escape recognition and hence would confer virulence. This might explain why virulence is a recessive character and furthermore why each resistance gene must be overcome by a

related virulence gene in order for susceptibility to occur. The evidence which is reviewed by Albersheim and Anderson-Prouty (1975) and supports fungal wall sugar residues as specific determinants comes from many sources including other recognition phenomena such as mating types in yeast. Additionally it has been shown that fungal walls have sufficient structural variability to fulfill this role. Plant cell walls, however, do not meet this criterion. In plant cells, membrane proteins represent a better candidate for involvement in recognition. Such proteins are likely to be lectins (glycoproteins involved in recognition); much of the evidence for this is reviewed by Callow (1977) and by Sequeira (1978).

Day (1974) has discussed a scheme by which resistance responses may be induced at the molecular level following recognition. He proposes that host resistance genes are analogous to sensor genes (equivalent to regulator genes known to exist in bacteria) capable of recognizing secondary messengers from membrane receptors. One or more integrator genes adjacent to each sensor are capable of producing an activator (perhaps RNA). Each activator may in turn induce an operon: a transcriptive unit controlled by a receptor gene sensitive to the activator. In this way a complex series of biochemical changes could be initiated at the genomic level. Many of Day's ideas might be reinterpreted in the light of recent advances in the understanding of the control of gene expression in eucaryotes, in particular the role of post-transcriptional control. These advances will be detailed in Section 2.4.

Whatever the precise mechanism of the induction phenomenon in resistance many workers feel that phytoalexin synthesis is the eventual outcome (Bell 1981, Heath 1980). Thus phytoalexin accumulation and, in some cases, hypersensitivity have been used to gauge the resistance response.

There are several examples showing that phytoalexins accumulate more rapidly and to a greater extent in incompatible interactions (Bell 1981, Heath 1980). Phytoalexin production can be elicited by a number of chemical agents, including heavy metals, and also by physical agents, such as ultra-violet light and drought stress. However, there are a few examples of highly active elicitors that can be extracted from fungal mycelia. Only one clear instance of a race-specific fungal phytoalexin elicitor exists. In the Phytophthora megasperma var. sojae/soybean system, Keen and Legrand (1980) have shown that glycoproteins isolated from fungal walls elicit glyceollin production to a greater extent in resistant varieties of soybean than in susceptible ones. The elicitor activity appears to reside in a high molecular weight glycoprotein fraction of which the carbohydrate moiety is obligatory for activity. The glycoprotein fraction was the only wall constituent bound by concanavalin A. Fluorescein-labelled concanavalin A was found to bind to living hyphal walls but not to those from which the glycoprotein had been extracted. Thus, it seems that the elicitor is borne on hyphal surfaces.

Wade and Albersheim (1979) have shown that extracellular glycoprotein (ECGP) from P. megasperma var. sojae is capable of protecting incompatible soybean seedlings from infection following subsequent inoculation with compatible races. Treatment of seedlings with ECGP from virulent races did not lead to susceptibility following inoculation with incompatible races, nor were compatible combinations protected by prior treatment with ECGP from virulent races. Surprisingly, ECGP had very low phytoalexin elicitor activity and that which did exist was not race specific. Thus it appears that the production of phytoalexins, although associated with resistance responses, is not a primary determinant of the outcome of the host-parasite interaction.

These results appear to conflict with those of Keen and Legrand in regard to the location and elicitor activity of the glycoprotein. Whether the same glycoprotein is involved in both instances and whether other factors act in conjunction with it to initiate phytoalexin synthesis are questions which await further research in order to be answered.

An elicitor of phytoalexin production in potato has been isolated from hyphal walls of Phytophthora infestans. The elicitor is not race specific but has nonetheless been the subject of several recent papers (see Kurantz and Zacharius 1981). Doke and Tomiyama (1980a) have reported that hyphal wall components cause rapid aggregation and lysis

of potato protoplasts. Protoplast response was not correlated with the presence of major resistance genes but was associated with the degree of field resistance (horizontal resistance). However soluble fungal glucans were capable of suppressing the hypersensitive response of protoplasts and this effect was partially correlated with compatible combinations of vertical virulence and resistance alleles (Doke and Tomiyama 1980b). These results are particularly interesting because they are at odds with our understanding of the mechanisms underlying gene- for-gene systems. However, they are far from complete and may merely serve to illustrate the problems of studying the effects of resistance genes in plants with greatly differing genetic backgrounds. Hence, a correlation with major gene resistance may be misinterpreted in terms of the true cause and effect relationship. Furthermore, it is clear that elicitors of phytoalexins and hypersensitivity are not necessarily specific triggers of the resistant response.

The Phytophthora systems are well studied and illustrate the directions for future research. They undoubtedly have significance for workers studying flax and cereal rust diseases. However, Phytophthora differs significantly in that it is a hemibiotroph, causing tissue necrosis fairly early in successful disease development.

Littlefield and Aronson (1969) have undertaken a detailed histological study of resistance mechanisms in flax

following inoculation with flax rust. Fungal growth in susceptible and resistant reactions was similar up to 36 to 48 hours following inoculation. After this time resistance was characterized by a reduction in length and number of hyphae and number of haustoria per infection site as compared to susceptibility. Littlefield (1973) later showed that 'L' and 'N' locus genes conditioned the most rapid and localized resistance response. At 169 hours after inoculation 'M' and 'P' genes conditioned a hypersensitive response over a larger area and the 'K' gene conveyed moderate resistance which was accompanied by extensive necrosis. However necrotic lesions in the fast-acting resistance conferred by the 'L' and 'N' genes were larger than with the slower-acting 'M' and 'P' genes at 24 hours after inoculation. Later work by Keen and Littlefield (1979) showed that two antifungal compounds, coniferyl alcohol and coniferyl aldehyde, accumulate more rapidly in incompatible reactions. Furthermore, these compounds accumulate more rapidly and to a greater extent in resistance responses conditioned by fast-acting resistance genes. These observations provide an explanation for the partial resistance to virulent races which is observed following prior inoculation with an avirulent race (Littlefield 1969).

In summary, the evidence for induced varietal resistance is now very substantial. The important areas to

be elucidated are, firstly, the involvement of avirulence and resistance genes in triggering the response and secondly the mechanisms by which this trigger brings about the response. The latter is likely to have its basis in the control of gene expression. There is already evidence which shows that RNA and protein synthesis are enhanced during resistance at times prior to hypersensitive cell death and phytoalexin accumulation. Furthermore, it has been suggested that the hypersensitive response is not the cause of resistance but merely a consequence of it. Kiraly et al. (1972) have presented evidence that hypersensitive responses are induced in compatible interactions if the fungal mycelium is killed by treatment of the infected tissue with specific inhibitors. Hypersensitivity was not observed when plants were treated with the inhibitors alone nor when they were infected in the absence of inhibitors. These workers came to the conclusion that events in host metabolism prior to the onset of hypersensitivity were responsible for fungal inhibition during resistance. Once inhibition has occurred they postulate that the damaged fungal mycelium induces hypersensitivity. For these reasons events in host gene expression which occur prior to hypersensitivity are reviewed in a following section.

2.3 Ribonuclease and Disease

Rohringer et al. (1961) were the first researchers to investigate the influence of rust infection on ribonuclease (RNase) activity. Earlier work had shown that RNA synthesis increased markedly in response to infection and it was therefore of interest to gain an insight into RNA degradation (turnover). It was revealed that a bimodal increase in RNase activity occurred, peaking at 1 to 2 and 6 to 7 days after inoculation in a susceptible wheat/leaf rust combination. In a resistant combination similar changes occurred but to a lesser degree.

Several later reports from Shaw's group showed that similar bimodal increases in RNase activity could be demonstrated in other host/rust combinations. Where both resistant and susceptible combinations were studied, the later increase occurred only in susceptible plants and its onset coincided with flecking and the initiation of sporulation. This correlation could be conveniently studied using near isogenic lines of wheat carrying the dominant or recessive alleles of the Sr6 gene which is a temperature sensitive gene for resistance to wheat stem rust (Puccinia graminis tritici) (Chakravorty et al. 1974a). Utilizing races of rust carrying the avirulence allele corresponding to

the Sr6 allele, it was shown that the later increase in RNase occurred only at temperatures permissive to rust development. Furthermore plants previously inoculated with these races and held at a non-permissive temperature only showed the late increase in RNase following transfer to the permissive temperature. Thus sporulation and increased RNase activity could be delayed up to 14 days after inoculation at the non-permissive temperature, but once initiated, occurred in a similar fashion to that in plants carrying the recessive (sr6) allele or in those infected with a race virulent on Sr6. In this and other papers emanating from Shaw's group catalytic properties of RNase were also studied. In the four host rust combinations studied the early increase in RNase activity, referred to as 'early RNase', was not always accompanied by changes in the properties of the enzyme whereas the late increase, 'late RNase', was accompanied by a variety of changes. These findings are summarized in Table I.

The changes in the catalytic properties of RNase were shown not to be due to fungal contribution, as the substrate preferences for the mycelial enzymes were determined in each case. The effects of wounding were also assessed as it is known that wounding induces large increases in RNase activity (Dove 1973). However, the catalytic properties of RNase from wounded plants appeared similar to that from healthy plants

TABLE I

CHANGES IN RNase ACTIVITY FOLLOWING RUST INFECTION

HOST	RUST	PHASE OF RUST CYCLE	INFECTION	SUBSTRATE[a] PREFERENCE	OTHER CHANGES	REFERENCES
Ribes nigrum	Cronartium ribicola (Blister rust)	Diploid	Healthy 16 days p.i.[b] (Late RNase)	pA>pU>pI>pC pA>pU>pI≡pC	Heat stability diethylpyrocarbonate sensitivity	Harvey et al. 1974
Pine tissue culture	Cronartium ribicola (Blister rust)	Haploid	Healthy 64 days p.i. (Late RNase)	pU>pA>pC pA≡pC	Heat stability, diethylpryocarbonate sensitivity	Harvey et al. 1974
Flax (cotyledons)	Melampsora lini (Flax rust)	Diploid	Healthy 8 days p.i. (Late RNase)	pU>pA>RNA>pC RNA>pC>pU>pA	Heat stability. Detailed study of differences in purified enzymes	Scrubb et al. 1972 Chakravorty et al. 1974c
Wheat (Sr6, sr6)	Puccinis graminis tritici (Wheat stem rust)	Diploid	Healthy 3 days p.i. (Early RNase) 10 days p.i. (Late RNase)	pU>pA>pC pC>pA≡pU pC>pA≡pU	Heat stability and substrate preference differ between early and late RNase	Chakravorty et al. 1974a

[a]Substrate preference is determined with respect to the relative rates of hydrolysis of the ribonucleotide homopolymers polyadenylate (pA), polycytidylate (pC), polyuridylate (pU), and polyinosinate (pI) by RNase from the host/rust combinations shown.

[b]p.i. post-inoculation.

in each case. These findings implied that novel RNase molecules had arisen specifically in response to infection; furthermore, it was possible that these novel molecules were essential for successful disease development.

These ideas led Chakravorty et al. (1974c) to attempt purification of infected flax RNase to homogeneity in order to determine whether a novel enzyme protein had arisen. Purification showed that the majority of the soluble RNase activity in flax cotyledons eluted as a single peak with a molecular weight of approximately 40,000 during gel filtration. The enzymes thus purified from healthy or 8-day infected cotyledons had the same molecular weight but markedly different catalytic properties. Among the properties which differed were Michaelis Constant (K_m), pH optimum, temperature stability and the ability to degrade various nucleotide polymers. Other studies on RNase from the flax rust mycelium grown in isolaton from the host showed the changes in catalytic properties during disease could not be accounted for in terms of fungal contribution (Chakravorty et al. 1974b). The only fungal enzyme with a molecular weight similar to that of the enzyme from flax had properties quite unlike the enzyme from infected flax.

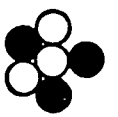
Chakravorty et al. (1974c) also undertook a detailed study of the aggregation and dissociation products which

were observed after freezing and thawing of the purified preparations from flax. Freezing and thawing yielded three peaks of RNase activity by gel filtration with apparent molecular weights of 90,000, 39,000 and 27,000 daltons. These aggregation and dissociation products were consistently formed from purified extracts of healthy or eight-day infected cotyledons. However, the substrate preference of each was not only different from the native enzyme but also depended upon whether the enzymes were derived from healthy or infected cotyledons. This led Chakravorty et al. (1974b) to suggest that the altered properties of the RNase during disease were brought about by the appearance of a novel subunit in the apparently oligomeric enzyme. This hypothesis is summarized in Figure 4.

This work also provided a large part of the evidence for an hypothesis concerning the molecular basis of obligate host-pathogen interactions. Chakravorty and Shaw (1977a) proposed that enzymes with novel catalytic properties are essential for pathogen development. Hence, it was suggested that the induction of such enzymes was the molecular basis for specificity determining resistance or susceptibility. Thus, these authors suggested that the hypothetical novel subunit in Figure 4 could arise in four possible ways:

1) The transfer of cistrons (operational units of DNA) from pathogen to host,

A summary of the molecular weights, substrate preference and hypothetical structure of the native RNase molecules and their artificially produced variants from healthy and rust-infected flax cotyledons

Native RNases (before freezing and thawing)					Artificially produced variants (after freezing and thawing)				
Enzyme	Molecular weight	Substrate preference	Hypothetical[a] structure		Artifactual variant	Molecular weight	Substrate preference	Hypothetical structure	
Healthy native RNase	40 000	p(U) > p(A) > RNA > p(C)		(a)	P-1	90 000	RNA > p(A) ≡ p(U) > p(C)		(c)
					P-2	39 000	RNA > p(A)p(C)		(d)
					P-3	27 000	RNA ≡ p(C)		(e)
Infected native RNase	40 000	RNA > p(C) > p(U) > p(A)		(b)	P-1	90 000	RNA > p(C) > p(A) > p(U)		(f)
					P-2	39 000	p(C) > p(A) > RNA		(g)
					P-3	27 000	RNA > p(A)		(h)

[a] Of the several possible combinations of monomers in (c) to (h) only one is shown, for the sake of simplicity. The number of monomers assigned to the native and the derived RNases is also arbitrary.

FIGURE 4. The subunit complementation hypothesis for flax RNase (reproduced from Chakravorty, A.K., Shaw, M. and Scrubb, L.A. 1974).

2) The transfer of inducer substances from pathogen to host, or vice versa in the case of novel pathogen enzymes, leading to derepression of an inducible gene.
3) The transfer of mRNA coding for the subunit from pathogen to host.
4) Direct transfer of polypeptides from pathogen to host.

The possibility of transfer of macromolecules (in 1, 3 and 4) from host to pathogen was considered unlikely in view of the fact that rust fungi can develop successfully on simple chemically defined media.

Chakravorty and Scott (1979) investigated RNase activity during development of powdery mildew (Erysiphe graminis f. sp. hordei) on barley. Both quantitative and qualitative changes occurred in the major soluble RNase activity during the later stages of development. Additionally, a minor RNase component which is insoluble at pH 5 was examined. In all earlier studies on RNase during rust infection this fraction had been discarded during purification. Thus, it was thought important to determine whether changes in this fraction had gone unnoticed. Indeed it was shown that changes in the substrate preference of this enzyme could be detected as early as one day after inoculation. This work has therefore demonstrated that changes in RNase activity may be characteristic of powdery mildew infection as well as rust infection and that pH 5

insoluble RNase activity should be re-examined. In particular, quantitative changes in RNase activity which occur very soon after inoculation might be considered both more significant in the establishment of the host-parasite interaction and also less likely the result of a general non-specific disruption in host metabolism.

If indeed the appearance of novel RNase molecules is an important component of disease development, then clearly it would be of interest to know firstly, whether such changes occur in other plant responses and secondly, what the function of the RNase activity is. In answering the first of these questions it is useful to consider how RNase activity responds to wounding and senescence which may be regarded as parallels to some of the events in disease. As has already been mentioned, at least in the instances where wounding has been studied, although large increases in RNase activity occur, the properties of this activity are quite different from those of RNase from infected plants. Novel RNase activity has been shown to appear in senescing leaves but it is accompanied by a corresponding decrease in RNA levels (Dove 1973). This is quite uncharacteristic of infected plants, which show increased levels of RNA (Quick and Shaw 1964, Hamilton 1969). This evidence suggests that the novel RNase activity of infected plants has a role which is specific to the metabolism of infected plants.

The possible function of the RNase studied during rust and mildew infection can be assessed with a knowledge of the known functions of RNases with similar catalytic properties and intracellular localizations. Additionally, it would be of great value to know what natural substrates these enzymes can hydrolyze. The major soluble RNase in higher plants, RNase I, has a pH optimum between 5.0 and 6.0 and brings about preferential release of purine 2'-3'cyclic nucleotides by endonucleolytic cleavage (Wilson 1975). The purified RNase from healthy or infected flax (Chakravorty et al. 1974c) represents the major soluble fraction, has a pH optimum of 5.5 and an endonucleolytic mode of action. Although the nucleotides produced during hydrolysis have not been analyzed it appears likely that this enzyme is of the RNase I type. RNase I enzymes are thought to be involved in the breakdown and reutilization of RNA (Wilson 1975). Simpson et al. (1980) have examined the action of pH 5 soluble and pH 5 insoluble enzymes from barley on barley polysomal RNA under limited digestion conditions. The pH 5 soluble enzyme, analogous to the fraction studied in rust-infected plants, was incapable of hydrolyzing messenger RNA. The insoluble enzyme, however, hydrolyzed mRNA to generate monosomes and lower order polysomes. Furthermore, this enzyme preferentially degraded chloroplast polysomes when the enzyme was derived from susceptible plants infected

with powdery mildew (Chakravorty et al. 1980). Both enzymes were capable of hydrolyzing ribosomal RNA within intact monosomes but the monosomes remained effective in protein synthesis despite extensive degradation of their RNA. Chakravorty and Scott (1979) have attempted to assign an intracellular location to these enzymes using differential centrifugation. The pH 5 soluble RNase appears within the soluble fraction whereas the pH 5 insoluble RNase is microsomal (which would include ribosomes).

As rigorous proof is lacking, any statements concerning the function of ribonucleases in plants are tentative at best. The evidence for the cytoplasmic location of RNase I (Wilson 1975) although suggestive does not constitute proof. Cell fractionation in the isolation of RNase (Chakravorty & Scott 1979, Wilson 1975) has often been crude and thus there has almost certainly been extensive nuclear disruption in such experiments. Chakravorty and Shaw (1977b) have suggested a role for flax RNase in the processing of RNA. This idea is based upon the rate of synthesis and content of RNA, and upon the specific activity of RNase following rust infection. These data show that RNA accumulates in spite of levels of RNase which are more greatly elevated than the rate of RNA synthesis. Although it would appear logical to draw the conclusion that this RNase is not involved in RNA degradation (substrate reutilization), this view may be too simplistic. Such a conclusion requires

the assumption that RNA turnover in the living cell is regulated merely by the concentration of RNase. In reality, localization of enzyme and substrate as well as the association of ribonucleoproteins are important factors.

In summary, rust and mildew infection cause marked increases in RNase activity which is catalytically different from that found in healthy plants. The novel RNase is of host origin and in the case of flax appears to be the result of an altered enzyme molecule. The function of this enzyme is at present unknown. Clearly, analysis of the enzyme's hydrolysis products and determination of its intracellular localization would be helpful in assigning a function. Whether subunit complementation actually occurs to bring about novel enzymatic proteins is also not established. Only rigorous purification and the preparation of subunits will enable proof of this to be obtained.

2.4 RNA and Protein Synthesis

Enhanced RNA synthesis has been observed in many host-parasite interactions. Heitefuss and Wolf (1976) have reviewed many studies which have demonstrated stimulation of cytoplasmic rRNA during disease development. This is usually accompanied by a continuous decline in chloroplast rRNA (Bennett and Scott 1971) and chloroplast polysome content (Dyer and Scott 1972). Most early studies involving extraction and fractionation of *in vivo* ^{32}P-labelled RNA were unable to provide conclusive evidence that concurrent increases in mRNA synthesis also occurred. This was due to labelling periods which were too long to detect the rapidly turned over messenger fraction and also to the rather ambiguous results which can be obtained from fractionation on methylated albumin kieselguhr (MAK) columns.

It might seem reasonable to assume that the synthesis of rRNA, mRNA and soluble RNA (tRNA and 5S rRNA) responds concurrently to infection as they all participate in protein synthesis. Tani *et al.* (1971) demonstrated that incorporation of ^{32}P into RNA fractions bound tenaciously to MAK columns (putative mRNA) increased markedly after crown rust infection of both susceptible and resistant oats. A subsequent investigation failed to demonstrate any increase

in the template activity of RNA extracted from infected oats by *in vitro* translation (Tani *et al*. 1973). However, since this is a measure of mRNA mass whereas the former results are a measure of synthesis, this merely indicates rapid mRNA turnover. This clearly does not preclude the possibility that the mRNA fraction encodes proteins specifically associated with infection. Novel size classes of polypeptides appear to be encoded by polysomes from rust infected wheat (Pure *et al*. 1979, 1980). These polypeptides are detected by *in vitro* translation and appear to be synthesized on cytoplasmic ribosomes on the basis of their insensitivity to chloramphenicol (Pure *et al*. 1980).

Chakravorty and Shaw (1971) examined the incorporation of ^{32}P into two RNA fractions from flax cotyledons following inoculation with flax rust. Both the salt-insoluble (containing 25S and 18S ribosomal RNA) and the salt-soluble (containing predominantly 5S rRNA and 4S transfer RNA) exhibited higher specific radioactivity following inoculation. Specific activity increased to more than two fold over that found in the healthy cotyledons in both fractions at 2 days after inoculation. This early increase was followed by lower levels of incorporation at 4 and 6 days after inoculation but still higher than in healthy cotyledons. Since it has been demonstrated that there are

significant increases in the amount of RNA in infected cotyledons from 6 days post-inoculation (Hamilton 1969), this represents a substantial increase in overall RNA synthesis in the later stages of disease. Analysis of the salt insoluble fraction by density gradient centrifugation revealed that both the 25S and 18S RNA components were more heavily labelled with ^{32}P following inoculation. The base composition of both labelled fractions was also determined at 2 and 6 days after inoculation. It was shown that the salt-soluble RNA had an altered base composition; that of the ribosomal RNA (salt-insoluble) remained unchanged.

Thus it has been shown that soluble RNA is synthesized more rapidly during rust infection. This is in general agreement with other studies, with the exception of a study by Wolf (see Heitefuss and Wolf 1976) who found no significant difference in the ^{32}P specific activity of soluble RNA in wheat leaves infected with stem rust. Whether the increased synthesis of RNA in the salt-soluble fraction from flax is also representative of increased mRNA synthesis is, as yet, unknown. The observation that changes in the base composition of the RNA synthesized in infected plants are not due to fungal contribution and, furthermore, could not be induced by treatment with the auxin indoleacetic acid suggest that gene expression may have been altered

specifically in response to infection (Chakravorty et al. 1971). A number of experiments with inhibitors of RNA synthesis imply that early increases in host RNA synthesis are critical to pathogen development. Development of bean rust was greatly impaired by application of 5-fluorouracil to bean leaves (see Heitefuss & Wolf 1976). The suppression was only effective if the inhibitor was applied within 48 hr of inoculation. Furthermore the increase in RNA synthesis normally observed was largely eliminated. Similar results have been observed with Puccinia graminis tritici on wheat and P. coronata on oats (see Heitefuss and Wolf 1976). Inhibition of flax rust on flax occurs if actinomycin D is applied within five to six hours after inoculation (Shaw 1967). Although direct inhibition of fungal growth can not be ruled out, it has been demonstrated in many cases that the levels of inhibitors used have no significant effect on spore germination. Early changes in host RNA synthesis are therefore a promising area of study. Valuable information of this type has been gained by microscopy.

Bhattacharya, Shaw and co-workers carried out a series of cytophotometric studies on the nuclei of rust infected wheat. In the susceptible reaction of wheat leaves (variety Little Club) infected with race 15B of wheat stem rust, RNA levels in the host nuclei increased markedly from two days after inoculation (Bhattacharya et al. 1965). This

was accompanied by a decrease in histones and an increase in the level of other nuclear proteins. These data and the observation that the nuclei swelled dramatically are all indicative of gene derepression and increased RNA synthesis. This is supported by the enhanced incorporation of tritiated cytidine and uridine into RNA in the nuclei of six-day infected plants, determined by microautoradiography (Bhattacharya and Shaw 1967). Concurrent increases in the incorporation of tritiated leucine into nuclear protein were also observed. Later studies on the nucleoli showed that nucleolar volume increased very dramatically, being four and eight times that in healthy parts of the leaves at two and four days after inoculation respectively (Bhattacharya et al. 1968). Thereafter nucleolar volume declined gradually but remained greater than that in healthy leaves beyond thirteen days after inoculation. Similar trends were observed with respect to the elevated levels of RNA and protein in the nucleoli. Nucleoli are involved in ribosomal RNA synthesis and ribosome assembly. Thus, it is indicated that ribosome production is greatly stimulated as soon as two days after inoculation, which may account for the relationship observed between RNA and protein content.

Bhattacharya and Shaw (1968) also studied the levels of RNA, histone and total protein in the nuclei of infected

Khapli wheat leaves (resistant). RNA and protein levels were already elevated and histones had decreased at 3 days after inoculation. These initial changes were followed by declining levels of all three substances which culminated in a general nuclear collapse at the infection sites at 12 days after inoculation. These results are of particular interest because they indicate the active nature of the resistance response which precedes localized cell death. It is unfortunate that nuclei in the resistant reaction were not examined prior to three days after inoculation, as it is likely that the outcome of the host-parasite interaction is determined within one or two days after inoculation (Shaw 1967). Additionally, Khapli and Little Club wheats are of widely differing genetic background.

It is clear that these histological studies provide a strong basis for detailed biochemical work. One problem is apparent however; the nuclei of cells more than 100 µm away from infection sites in susceptible leaves appear unaffected (Bhattacharya and Shaw 1967). Thus, in order to detect appreciable changes in plant extracts it may be necessary to use very high inoculation densities.

Tani and co-workers have carried out a number of studies on both compatible and incompatible combinations of oat and crown rust. These studies have shown that RNA and

protein synthesis are enhanced between 12 and 16 hours and 14 and 20 h after inoculation respectively in an incompatible combination (Tani and Yamamoto 1978). Similar changes were not observed following inoculation of the same oat variety with a compatible race of crown rust. Furthermore it was demonstrated that cordycepin, an inhibitor of RNA synthesis, and puromycin, an inhibitor of protein synthesis, could suppress resistance. This suppression was fully expressed when the inhibitors were supplied within 10 hours after inoculation, in the case of cordycepin and within 12 hours in the case of puromycin. In another study similar results were observed with respect to protein synthesis and the effects of the inhibitor blasticidin S (Yamamoto et al. 1976). In these experiments peroxidase isozymes and phenylalanine ammonia lyase activity were also examined. The latter increased markedly in response to infection with the incompatible race of crown rust. However, blasticidin S was not effective in supressing this increased activity. This is interesting in the light of a report by Yoshikawa et al. (1977) who have indicated that increased synthesis of polyadenylated messenger RNA is associated with glyceollin production and resistance in soybeans infected with an incompatible race of Phytophthora megasperma var. sojae. Phenylalanine ammonia lyase is one of the key enzymes in the synthesis of glyceollin

and other phenolic compounds. Mayama et al. (1981) have recently isolated antifungal compounds from oats, which accumulate specifically in response to infection with avirulent races of crown rust. Yamamoto et al. (1975) have presented evidence that a novel protein accumulates in the incompatible oat/crown rust combination. Immunological comparisons between soluble proteins of healthy and 35 hour infected leaves and those from the fungus revealed a protein which was unique to infected leaves. Unfortunately, proteins from compatible combinations were not studied so it is impossible to say whether the novel protein is associated with resistance.

Von Broembsen and Hadwiger (1972) have also presented evidence that early increases in protein synthesis are associated with the resistance response in the flax/flax rust system. These workers fed healthy or infected plants with either ^{3}H-leucine or ^{14}C-leucine at various times between 6 and 18 hours after inoculation. In this way it was possible to analyze differently labelled extracts from healthy and infected plants simultaneously by gel filtration. Of the four incompatible combinations studied incorporation increased into various size classes of protein. In the two compatible combinations either no change or a slight decrease in synthesis was noted.

These reports of enhanced RNA and protein synthesis are evocative of a specific trigger associated with resistance and imply that altered patterns of protein synthesis might be characteristic of events early in resistance responses. In this regard it is important to consider how eukaryotic gene expression is controlled. There are four possible levels of control which have identified:

1) Transcriptional activity, which is dependent on chromatin organization and RNA polymerase activity (in association with regulatory proteins)

2) Post-transcriptional RNA processing, involving removal of intervening sequences, splicing and modification (including the addition of polyadenylate to the 3' end of mRNA).

3) Translational control

4) Post-translational processing of newly synthesized protein.

The current understanding of these processes in plants has recently been reviewed (Marcus 1981).

CHAPTER 3*

CHANGES IN RIBONUCLEASE ISOZYMES DURING RUST INFECTION OF FLAX

3.1 Introduction

Scrubb et al. (1972) demonstrated that major quantitative and qualitative changes in the RNase activity of flax cotyledons occur after inoculation with uredospores of flax rust (Melampsora lini (Ehrenb.) Lev., race 3). A bimodal increase in the specific activity of a cell free extract was demonstrated in susceptible flax (Linum usitatissimum L. cv. Bison). Only the early increase (2 to 4 days after inoculation) occurs in the resistant variety of flax (L. usitatissimum L. cv. Bombay). The major RNase component from healthy and inoculated cotyledons of Bison flax was purified (Chakravorty et al. 1974c) to homogeneity with respect to Sephadex G100 gel filtration during the late phase (8 days after inoculation). Qualitative changes were demonstrated in this fraction in accordance with changes observed in the crude extracts at this stage. Among these were differences in substrate specificity, pH response, thermal stability, K_m and V_{max}. Such changes in properties could not be accounted for on the basis of contribution from the rust mycelium; mycelial RNases had been characterized previously (Chakravorty et al. 1974b).

*Chapter 3 has been published in a form close to that presented here (Sutton and Shaw 1982).

The initial objective of the present study was to determine the molecular basis of these changes.

3.2 Materials and Methods

3.2.1 Plant material

Flax plants of variety Bison and Bombay (susceptible and resistant to Melampsora lini race 3, respectively) were grown in growth chambers at an irradiance of 230 μE $m^{-2}s^{-1}$ for 18 h daily at 25°C with 20°C nights (see frontispiece). One week after the seeds were sown, plants were sprayed with a dilute suspension of uredospores in water (approximately 50 mg/100 ml). The plants were placed in a growth chamber at 10°C in the dark for 18 h in a humid atmosphere. The control plants were sprayed with water alone but otherwise treated identically. This procedure gave rise to about 10 pustules per cotyledon scored 8 days after inoculation.

3.2.2 Extraction and purification of RNase

Approximately 50 g of cotyledons were harvested at various stages after inoculation; thereafter all operations were carried out at 0 to 4°C. The cotyledons were homogenized in an Osterizer with 50 g polyvinylpoly-pyrrolidone (Sigma) and 350 ml 40 mM potassium phosphate buffer pH 6.7. The extract was then purified by the following steps, adapted from Chakravorty et al. (1974c).

I. Centrifugation: The homogenate was centrifuged at 10,000 g for 30 min to remove cell debris.

II. Acidification to pH 5.0: The supernatant from step I was adjusted to pH 5.0 with 1 N HCl and allowed to stand for 2 h. The resulting suspension was centrifuged as before and the supernatant saved for further purification.

III. Ammonium sulphate fractionation: The solution from step II was brought to 50% saturation by adding solid $(NH_4)_2SO_4$ slowly with stirring and was allowed to stand for 30 min. The precipitate formed was collected by centrifugation, dissolved in a small volume of 5 mM phosphate buffer pH 6.7 and dialyzed overnight against 1 l of the same buffer.

IV. Hydroxylapatite chromatography: A small volume of solution from step III, containing about 5 mg protein, was applied to a 3 x 2 cm column packed with hydroxylapatite (Biorad BioGel HTP) equilibrated with 5 mM phosphate buffer pH 6.7. Elution was routinely carried out in a stepwise manner with two column volumes of 5, 60 and 120 mM phosphate buffer consecutively. This procedure was developed on the basis of gradient elution studies.

V. G100 Sephadex gel filtration: For detailed studies of properties, the enzymes which were recovered with 60 mM and 120 mM buffer in step IV (termed P1 and P2 respectively)

were further purified by gel filtration. A small volume (about 3 ml) of enzyme solution was layered onto a 1.5 x 30 cm G100 Sephadex column equilibrated with 40 mM phosphate buffer pH 6.7; elution was carried out with the same buffer at a flow rate of 0.5 ml/min and 3 ml fractions were collected.

3.2.3 Electrophoretic analysis

The various ribonucleases present at each stage of purification were visualized by polynucleotide-acrylamide gel electrophoresis according to Van Loon (1975). Aliquots of fractions containing 2-100 µg protein were applied to 7.5% polyacrylamide gels containing 0.05% polyuridylic acid (poly U; Calbiochem, K salt, A grade) which was included within the gel matrix. After electrophoresis gels were stained overnight with 1% pyronin Y in 7% acetic acid. In this way enzymes capable of hydrolyzing poly U are visualized as clear bands after destaining in 7% acetic acid. Comparative experiments were run without poly U but instead by incubating gels with RNA according to the method of Wilson (1971).

3.2.4 Column isoelectric focusing

The homogeneity of purified preparations was assessed by column isoelectric focusing in an LKB 110 ml column containing 1% Ampholine (LKB 8100) pH 2.5-6.0 by the method outlined in the Ampholine LKB instruction manual (also see Appendix).

3.2.5 Enzyme assays

RNase was assayed routinely with yeast RNA (BDH, Poole, England) which had been re-precipitated in 3 vol 95% ethanol, washed and dialyzed exhaustively against EDTA and subsequently against distilled water. Otherwise, the RNase assay was carried out as described by Chakravorty et al. (1974c). One unit of activity is defined as the amount of enzyme yielding an increase of 1.0 absorbance unit at 260 nm (A^{260}) of acid soluble product in 30 min at 37°C. Substrate preference was determined using tritiated homoribopolymers of uridylic, adenylic and cytidylic acid (poly U, poly A, poly C, respectively, Miles Laboratories Inc., Elkhart, Indiana) as described previously (Scrubb et al. 1972).

DNase and phosphodiesterase were determined using heat-denatured calf thymus DNA and Ca bis-p-nitrophenyl phosphate respectively as substrates (Scrubb et al. 1972). Relevant details are also given in the Appendix.

3.2.6 Analysis of hydrolysis products

The mononucleotides released in time course studies under standard assay conditions were analyzed by thin layer chromatography by the method outlined by Tang and Maretzki (1970). Incubation mixtures were applied to cellulose thin-layer plates 0.2 mm thickness) which were run in two separate solvents: Solvent I, $(NH_4)_2SO_4$ (45 g), water (20 ml), isopropanol (2 ml); Solvent II, $(NH_4)_2CO_3$ (0.96 g), water (25 ml), isopropanol (75 ml). Spots were observed under U.V. light and compared with 2'-3' cyclic ribonucleotide standards (2'-3' cUMP, 2'-3' cAMP, 2'-3' cCMP, 2'-3' cGMP; Sigma).

3.2.7 Protein estimation

Protein concentration was estimated by the method of Lowry *et al*. (1951) using bovine serum albumin as a standard.

3.3 Results

3.3.1 Purification of RNase from flax cotyledons

3.3.1.1 Specific activity and yields:

Purification was similar to that obtained previously by Chakravorty et al. (1974c). Total units and hence specific activity were lower from all sources than those obtained by the earlier workers. This is likely to be due to the different source of yeast RNA used in this present work. Typical purifications for the variety Bison are presented in Table II. Purification and yield for the variety Bombay were similar. The higher specific activity in the older plants was largely due to apparent differences in protein, the total enzyme units on a fresh weight basis being similar. Infection did not significantly affect total protein however.

The consistently lower yield after pH 5 precipitation (step II, Table II) in younger plants was investigated by Sephadex G100 gel filtration of cell free homogenates. The patterns from 1 and 2 week old flax (var. Bison) are presented in Fig. 5. These results show that an RNase component which elutes with the void volume (Fractions 16 to 24) is more prevalent in the younger plants. Gel filtration of the pH 5 supernatant demonstrates that most of this component is lost during step II. Unpublished data have

revealed that when pH 5 precipitated material is subjected to G100 Sephadex gel filtration at pH 6.7 the RNase activity now elutes as a major peak within the included volume (Fractions 30 to 44). Thus, it is indicated that some RNase activity becomes weakly bound to large molecules or particulate matter during pH 5 precipitation; this phenomenon is enhanced in extracts from younger cotyledons.

3.3.1.2 Hydroxylapatite chromatography:

Gradient elution studies using gradients from 5 to 200 mM phosphate buffer pH 6.7 revealed that a small amount of RNase activity was eluted at 5 mM; two major components were eluted at 35 and 110 mM (termed P1 and P2 respectively). The amount of activity eluted at 5 mM was highly inconsistent and this component appeared to be very unstable. The relative amounts of activity eluted at 60 (P1) and 120 (P2) mM in routine stepwise fractionations of partially purified extracts from susceptible (var. Bison) and resistant (var. Bombay) combinations are illustrated in Fig. 6. These results show the following: a) healthy plants contain the same relative amounts of P1 and P2 regardless of the variety or age of the plants during the experimental period; b) the early increases in RNase are characterized by an increase in

TABLE II. Purifications of RNase from Healthy (H) and Rust-Infected (I) Flax (Var. Bison) Cotyledons

Step	Fraction	RNase (Units)		Protein (mg)		Specific Activity (Units/mg)		Yield (%)	
		H	I	H	I	H	I	H	I
3 days after inoculation									
I	Homogenate	2650	3830	1300	1500	2.0	2.5	100	100
II	pH 5 Supernatant	1590	2220	530	554	3.0	4.0	60	58
III	50-80% $(NH_4)_2SO_4$	1060	1600	53	57	20	28	40	42
IV	Hydroxylapatite P1	183	214	18	18	10	12	30	35
	Hydroxylapatite P2	605	1111	6.5	7.3	92	152		
8 days after inoculation									
I	Homogenate	3140	6900	950	945	3.3	7.3	100	100
II	pH 5 Supernatant	2420	5730	480	440	5	13	77	83
III	50-80% $(NH_4)_2SO_4$	1950	4480	65	58	30	78	62	65
IV	Hydroxylapatite P1	425	2077	13	14	33	139	41	43
	Hydroxylapatite P2	849	890	3.3	3.5	255	263		

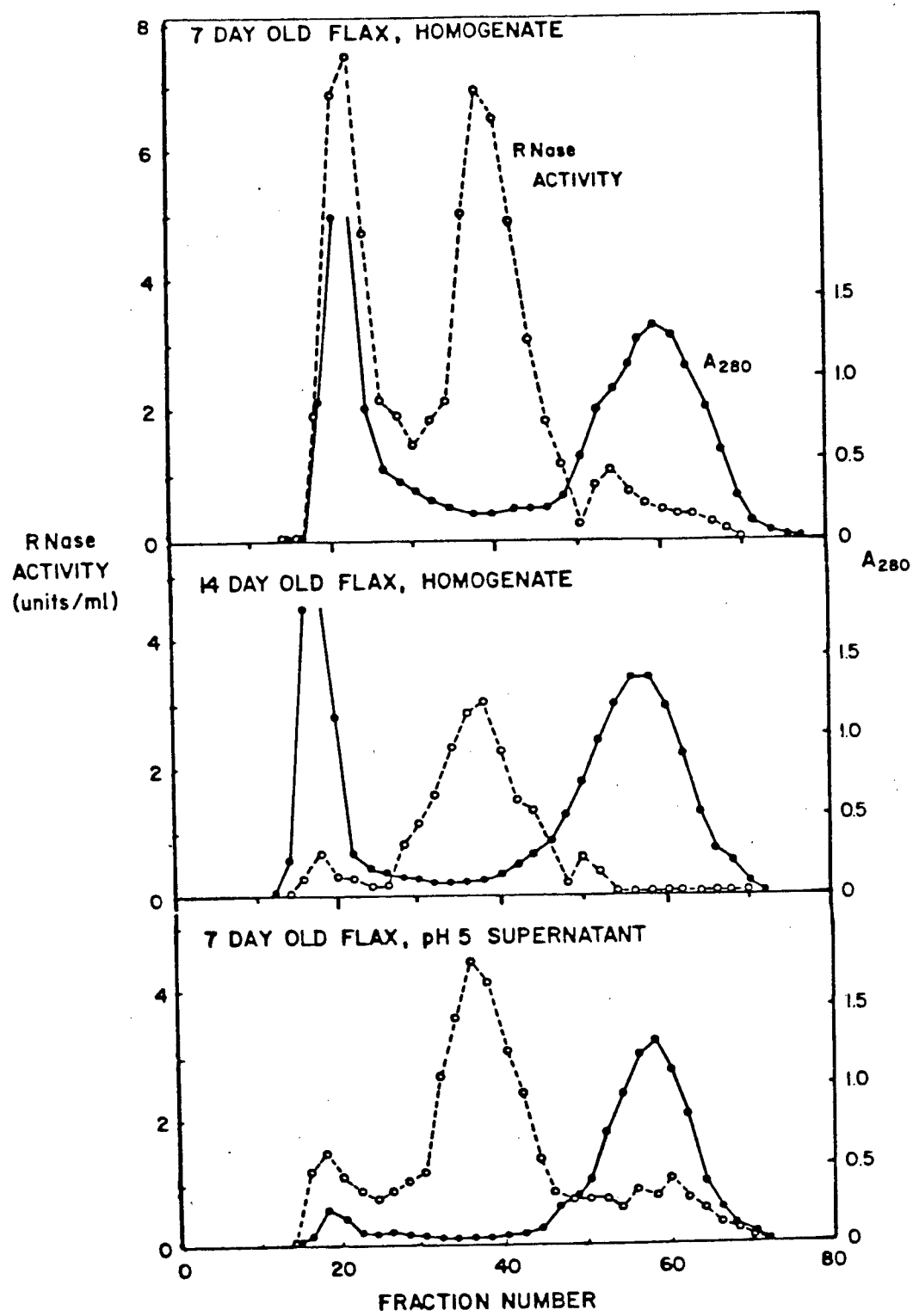


Figure 5. G100 Sephadex gel filtration profiles (2.5 x 45 cm column) of homogenates after centrifugation (Step I) from one and two week old Bison flax and following pH 5 precipitation (Step II). Considerably more RNase activity elutes with the void volume in the case of younger cotyledons, this activity is selectively precipitated at pH 5.

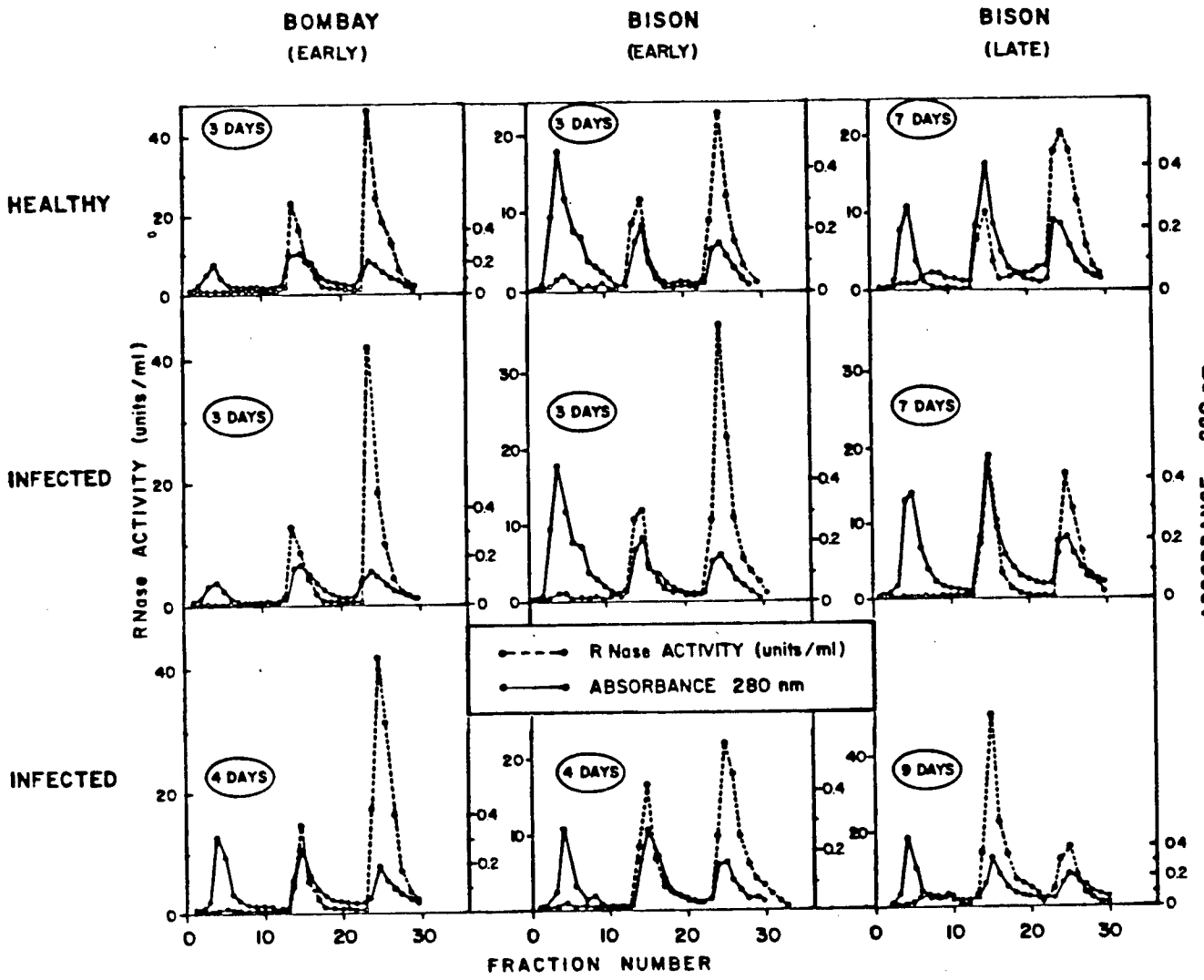


Figure 6. Hydroxylapatite (2 x 3 cm column) eluograms of RNase in step IV, elution was with 5mM, 60 mM and 120 mM potassium phosphate buffer pH 6.7 (fractions 1 to 10, 11 to 20, 21 to 30, respectively). Fractions 11 to 20 correspond to P1 and fractions 21 to 30 correspond to P2. The results presented are representative of three experiments performed at each time period for each host-pathogen combination.

the proportion of P2 and this change persists at four days after inoculation in resistant but not in susceptible plants; c) the late increase in RNase, found only in the susceptible combination, is characterized by an increased proportion of P1.

The absorbance at 280 nm indicates not only the amount of protein present but also the amount of contaminating material, the absorbance maximum being at about 270 nm. It is possible that such impurities may interfere with protein estimation by the Lowry method. However, the amount of contaminating material is not influenced by infection and thus absorbance at 280 nm is used in Fig. 6 to provide a standard by which to compare eluted RNase activity from healthy and infected plants.

3.3.1.3 Electrophoretic analysis:

Electrophoresis shows that the crude homogenates from healthy flax and infected flax contain the same nucleases capable of hydrolyzing polyuridylate regardless of the variety. Five components are visualized (Fig. 7); the two major components are selected during purification and separated by hydroxylapatite chromatography. The faster moving component increased at 7 days after inoculation

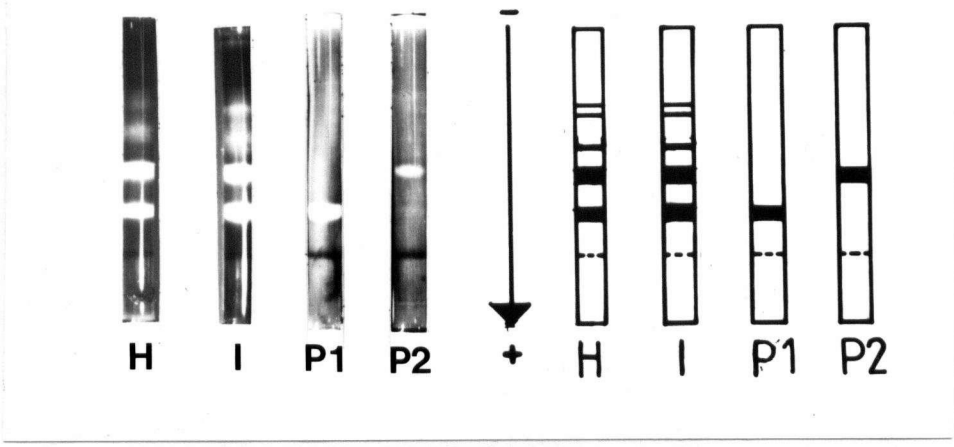


Figure 7. Polynucleotide polyacrylamide gel electrophoresis of homogenates from healthy (H) and infected (I) Bison flax 7 days after inoculation. Purified enzymes P1 and P2 from hydroxylapatite chromatography are also shown; with respect to the purified enzymes, only the gels run with enzymes from healthy plants are shown for convenience.

Gels contained 7.5% (w/v) acrylamide, 0.05% (w/v) polyuridylate and were stained with 1% (w/v) pyronin Y.

although no attempt was made to quantify this. Electrophoresis also confirmed that purification of the 50-80% $(NH_4)_2SO_4$ fraction by gel filtration (after Chakravorty et al. 1974c) gave rise to these two components. Thus the RNase fraction isolated by these workers has been resolved into two fractions by hydroxylapatite chromatography. These two components were also evident by incubation with RNA after electrophoresis but this technique appeared to be far less sensitive.

3.3.1.4 Sephadex G100 gel filtration:

The fractions P1 and P2 from hydroxylapatite were analyzed by gel filtration. A comparison with results yielded by the 50-80% $(NH_4)_2SO_4$ fraction reveals that the apparent molecular weight of the enzymes remains unchanged before and after separation (Fig. 8). Calibration studies on a 2.5 x 45 cm G100 Sephadex column using proteins of known molecular weight revealed a molecular weight of 40,000 for the RNases P1 and P2 from flax.

Gel filtration gave rise to specific activities of 900 and 4,000 units/mg protein for P1 and P2 respectively from 7 days infected Bison flax. These highly purified fractions had identical substrate specificities to fractions P1 and P2 prior to gel filtration indicating that the properties of the enzyme are unchanged by further

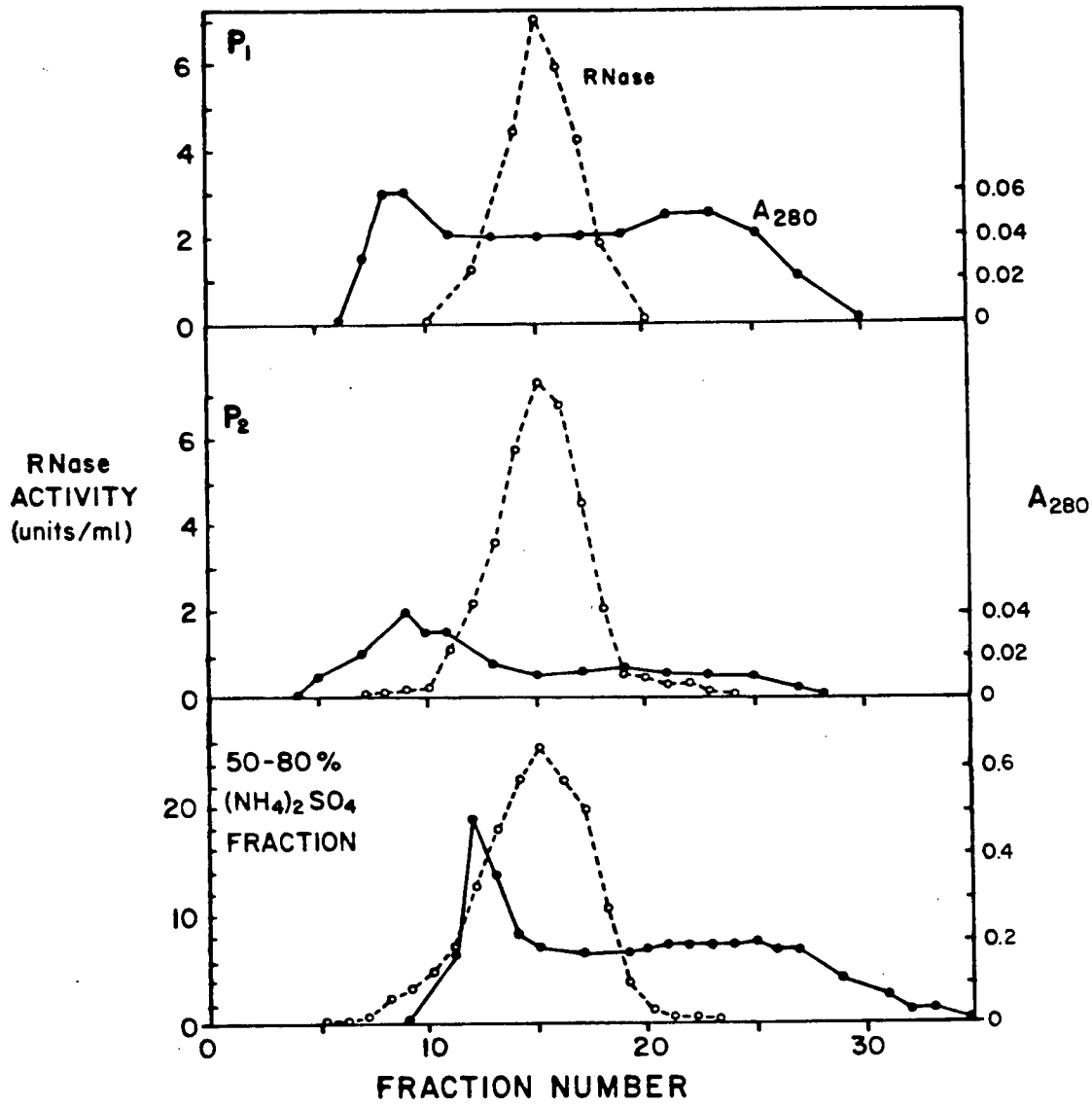


Figure 8. G100 Sephadex gel filtration profiles (1.5 x 30 cm column) of RNases P1 and P2 and fraction III from Bison flax prior to separation by hydroxylapatite chromatography.

purification. The fractions from Sephadex G100 were used in detailed studies of the properties of these enzymes.

3.3.2 The Influence of Rust Infection Upon the Specific Activity of RNases P1 and P2

The specific activity ratios (infected/healthy) for the susceptible and resistant reactions at various times after inoculation are illustrated for RNases P1 and P2 in Figure 9. In both combinations 'early RNase', described previously (Scrubb et al. 1972), is characterized by a predominance of P2. 'Late RNase' which only occurs in the susceptible reaction is characterized by large increases in P1, amounting to approximately 6-fold at 9 days after inoculation. A notable and reproducible difference between susceptible and resistant reactions is the persistence of early RNase at 4 days after inoculation in the resistant reaction. There is a notable decline in P2 activity in the susceptible reaction at this time.

The properties of the RNases P1 and P2 were monitored with respect to substrate specificity, K_m value and pH profile at each stage of infection. No differences were found between healthy and infected cotyledons or between the two varieties. This information and the low infection density in these experiments would seem to make it unlikely

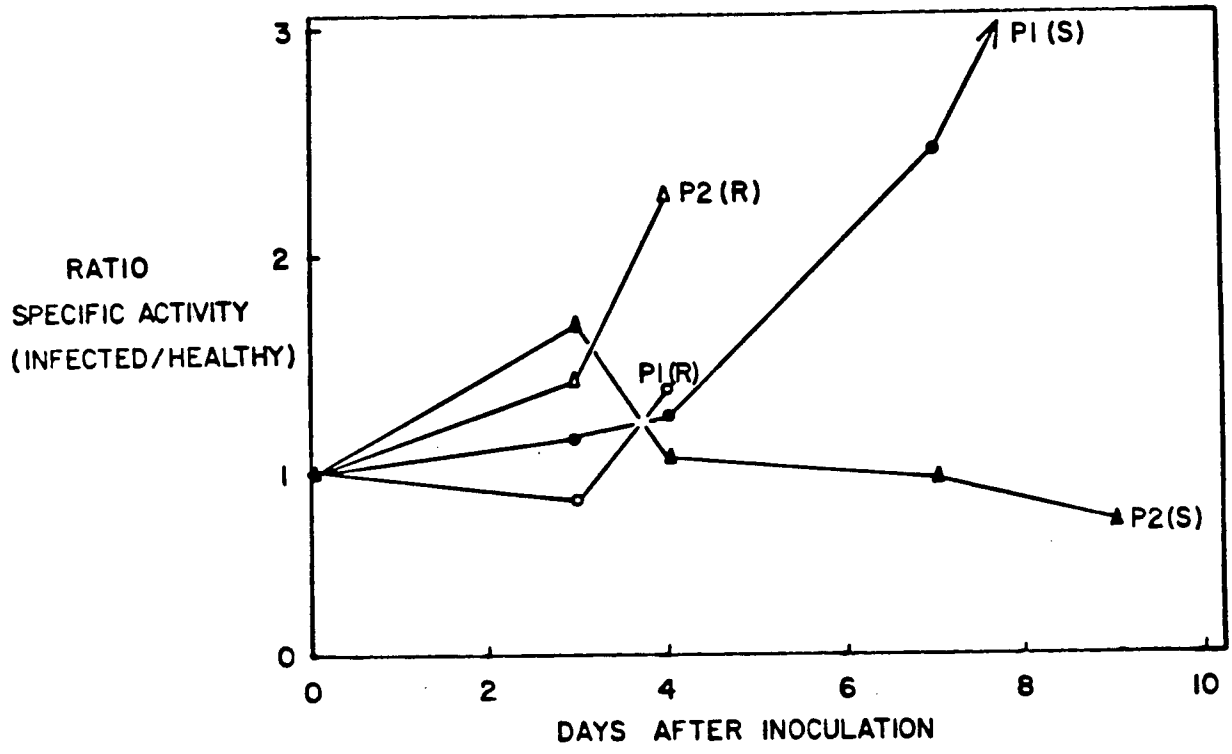


Figure 9. The specific activity ratios of RNases P1 and P2 following inoculation of Bison (susceptible-S) and Bombay (resistant-R) flax cotyledons with flax rust race no. 3. Data presented are averages for three separate experiments.

that the increases in P1 can be accounted for by fungal contribution. Although our results indicate that substrate specificity may not be a reliable indicator of catalytic properties, it would be surprising if K_m and pH optimum remained unaltered by fungal contribution. Chakravorty et al. (1974b) have characterized mycelial RNases extensively. The mycelial enzyme MII, which has the same molecular weight as P1 and P2, has a pH optimum of 6.2 as opposed to that of pH 5.5 for the plant RNases.

3.3.3 Properties of RNases

Although both enzymes appear to be of the RNase I type, as defined by Wilson (1975), they are clearly quite different from one another in their catalytic and kinetic properties (Table III). That P1 and P2 are not artifactual variants and do exist in vivo is supported by the following observations: (a) Incubation of the crude extracts does not change the relative amounts of P1 and P2, negating changes due to protease activity; (b) separated fractions of P1 and P2 are not interconvertible with long period of storage.

TABLE III. Properties of Ribonucleases from Flax

Property	Enzyme	
	P1	P2
Molecular weight	40,000	40,000
K_m Value µg/ml RNA	105	200
Electrophoretic Mobility (at pH 8.3, towards positive electrode)	0.85	0.66
pH Optimum	5.5	5.5
pH Profile	Broader	Narrower
Isoelectric Point	4.0	4.75
Substrate Specificity Poly U; A; C (relative)	100, 62, 60	86, 62, 20
Hydrolysis Products Mononucleotides released from RNA at 80 min.	2' - 3' cGMP Some non-cyclic nucleotides	2' - 3' cGMP Some non-cyclic nucleotides
Mode of Action	Endonuclease specific for RNA	Endonuclease specific for RNA

3.3.3.1 Substrate specificity:

Substrate specificities are given in terms of the relative rates of hydrolysis of tritiated poly U, poly A and poly C measured in counts per minute solubilized under standard assay conditions. Neither these results nor those for fractions containing both enzymes corresponded closely with those obtained previously with respect to poly C hydrolysis. However, the trends were similar, showing a tendency for increased hydrolysis of poly C in the later stages of a susceptible reaction. The reason for the apparent discrepancy is unclear but similar results were obtained using several batch numbers of tritiated polynucleotides.

3.3.3.2 K_m Values:

K_m values were obtained from Lineweaver-Burk plots (See Appendix). The lower K_m value for P1 is consistent with earlier observations that K_m decreases in the later stages of infection.

3.3.3.3 Endonuclease activity:

Endonuclease activity was demonstrated for both enzymes by time course assays with different precipitating reagents after Chakravorty et al. (1974c). Consistently more

A^{260} material soluble in 10% (w/v) perchloric acid was hydrolyzed as compared with material soluble in the standard precipitating reagent. Perchloric acid solubilizes short oligonucleotides whereas only mononucleotides are soluble in the standard precipitating reagent. Therefore, the results indicate that endonucleolytic cleavage takes place.

3.3.3.4 Other nuclease activities:

No deoxyribonuclease or phosphodiesterase activity was detectable in the purified fractions.

3.3.3.5 Hydrolysis products:

2'-3' cyclic GMP was the only cyclic nucleotide detected after 80 min of incubation with yeast RNA but a much lesser amount of non-cyclic nucleotide was also detected. The latter was evident as a single spot (R_f 0.05) in solvent II, in which cyclic nucleotides have an R_f greater than 0.25.

3.3.3.6 Isoelectric focusing:

Column isoelectric focusing revealed P1 and P2 to be homogeneous with respect to isoelectric point but clearly different from one another. Yield of enzyme units during this procedure was low (approximately 40%) due to a

tendency for protein to precipitate. However, the properties of the enzymes remained unchanged by the process suggesting that separate components had not been lost selectively.

3.3.4 Effects of Wounding

Excised cotyledons wounded by sectioning were incubated in the growth chamber on moist filter paper in petri dishes for 6 h. Six- to 7-fold increases in the specific activity of a component eluting with P1 from hydroxylapatite were observed. However, the properties of this enzyme were quite different from P1 or P2. This RNase component exhibited a pH optimum of 5.0, K_m value of 1000 µg/ml and a substrate preference poly C > poly U = poly A. It could be partially separated from P1 by Sephadex G100 gel filtration, had an apparent molecular weight of 25,000 and demonstrated a relatively unchanged proportion of P1. Thus the effects of wounding appear to be quite different from those of rust infection.

3.4 Discussion

This work clearly demonstrates that the qualitative changes in RNase observed in previous work can be accounted for by the observed selective enhancement of one of the two components resolved here. Detailed characteristics of the enzymes P1 and P2 categorize them as RNase I. RNase I has been identified as the major soluble RNase in many plants (see Section 2.3). The general features of RNase I are a pH optimum between 5.0 and 6.0 and preferential release of 2'-3' cGMP during hydrolysis of RNA, conditions fulfilled by the enzymes from flax.

The increases in RNase closely parallel quantitative and qualitative changes in RNA synthesis and the accumulation of RNA in later stages of disease development (see Section 2.3). This information should be viewed in relation to the function of RNase I, which is thought to exist predominantly in the cytoplasm and to be responsible for substrate reutilization (rather than processing, for example). It is of interest that early RNase persists at 4 days after inoculation in the resistant variety Bombay whereas it does not in the susceptible variety Bison. This coincides with the first appearance of symptoms and with the initiation of uredial development in the fungus. Tani et al. (1975) have

indicated that this stage of development is accompanied by specific changes in host RNA metabolism in crown rust infected oats.

The subunit complementation hypothesis described by Chakravorty and Shaw (1977a) to explain the changed properties of enzymes in rust-infected plants is not supported by the results presented here. The hypothesis is based on changes in the catalytic properties of a single RNase species. Here we have shown that no such change takes place. Rather changes in the relative amounts of two catalytically different RNase isozymes provide an explanation for the qualitative changes observed previously.

Observations utilizing polynucleotide acrylamide gel electrophoresis (Van Loon 1975 and unpublished) suggest that a multiplicity of ribonucleases is present in all plants as in Escherichia coli (Datta et al. 1974). Changes in the relative amounts of such enzymes may well account for the qualitative changes observed in many rust and mildew infected plants (see Section 2.3). Sachse et al. (1971) have given evidence for preferential enhancement of three of ten nuclease fractions following infection of wheat with Puccinia graminis tritici.

CHAPTER 4

RNA AND PROTEIN SYNTHESIS FOLLOWING INOCULATION WITH THE FLAX RUST FUNGUS

4.1 Introduction

Several reports (Yamamoto et al. 1976, Tani and Yamamoto 1978, Yoshikawa et al. 1977, Von Broembsen and Hadwiger 1972) have indicated that enhanced levels of RNA and protein synthesis are important in determining resistance in the gene-for-gene interactions of rust and downy mildew diseases. These workers have generally shown that elevated levels of synthesis occur in incompatible combinations at times when those in compatible combinations are either unchanged or show a decline.

In this research a detailed analysis of gene expression during these early stages of disease development has been carried out in the flax/flax rust system (Linum usitatissimum L./Melampsora lini (Ehrenb) Lev.). In these studies a single variety (Bombay) of flax carrying the N resistance gene has been utilized to generate resistant or susceptible combinations with race 3 or race 41 of flax rust, respectively. In this way changes in RNA or protein which are unique to either combination can be assigned to alterations in host gene expression, as both races of fungus

grow in the same way during the experimental period (Littlefield and Aronson 1969).

4.2 Materials and Methods

4.2.1 Inoculation and labelling of plant material

Flax was grown as described in Section 3.2.1. Seedlings (var. Bombay) were heavily inoculated (2 mg spores/ml) one week after seeding with either race 3 (resistant reaction) or race 41 (susceptible reaction) of flax rust. Healthy control plants were sprayed with water alone but otherwise treated identically. The plants were then placed in the dark at 17°C until 30 minutes prior to the feeding period when they were returned to the light (230 μE $m^{-2}s^{-1}$) at 25°C. For detailed studies on RNA synthesis 10 g seedlings were fed via the stems with 500 μCi/ml Na[^{32}P] orthho- phosphate (Amersham, carrier free) and 6 μg/ml gramicidin D for three hours. No significant incorporation into RNA was found if tritiated uridine was fed in this way.

For studies on protein synthesis replicates of three seedlings each were fed in the same way with [^{35}S]-L-methionine (Amersham or New England Nuclear, specific activity $>$ 1000 Ci/mmole) at a concentration of 1 μCi/μl for one hour.

4.2.2 Estimation of RNA synthesis in crude extracts

Gross rates of RNA synthesis were estimated by determining cold TCA insoluble [^{32}P] phosphate. After 3 h feeding with radioactive phosphate (about 5 µCi taken up by three seedlings), cotyledons were ground in liquid nitrogen and extracted in RNA extraction buffer (0.1 M Tris succinate, 1% SDS, 0.6% diethylpyrocarbonate pH 7.8). Particulate material was removed by centrifugation and 5 µl aliquots of supernatant spotted onto filter paper (Whatman 3 MM). Filter paper discs were placed in 10% TCA at 4°C for 10 min and then washed with 10% TCA followed by 95% ethanol. After drying the filter paper was placed in a scintillation vial and 10 ml of Econofluor (Amersham) was added. Radioactivity was measured by scintillation counting. Total uptake was estimated in the same way but without TCA precipitation and washing. The proportion of ^{32}P contained in RNA was determined by digestion with RNase buffer (50 µg/ml RNase A in 0.1 M Tris HCl, 5 mM $MgCl_2$) for 1 h. An aliquot of extract was added to 3 vol. of 95% ethanol and placed at -20°C overnight. The resulting precipitate was collected by centrifugation and suspended in RNase buffer.

4.2.3 Extraction and Purification of RNA

The levels of radioactivity necessary to characterize various fractions of RNA made it undesirable to separate cotyledons and apices from seedlings after feeding with ^{32}P. Thus, the stems were removed and the rest of the seedlings used in RNA extraction.

The seedlings were frozen in liquid nitrogen and ground with a mortar and pestle. RNA was then extracted with phenol essentially as described by Chakravorty and Shaw (1971; also see Appendix). However, the following modifications were made for routine purifications of small amounts of RNA: ethanol and cetyltrimethylammonium bromide precipitates were collected and washed on glass fiber filters (GFA Whatman) and subsequently redissolved by passing aqueous buffers through the ether dried precipitates; small molecules were removed from the purified RNA by applying to G25 Sephadex (PD10 column, Pharmacia) and eluting with sterile distilled water. Concentration and purity of RNA were assessed spectrophotometrically; the A_{260}/A_{280} ratio was typically 2.3.

A putative nuclear RNA fraction was prepared by the hot phenol SDS method at pH 5.5, adapted from Atherton and Darby (1974). Following extraction of the phenol phase with 2 volumes Tris succinate, 1% SDS at pH 7.8, an equal volume of 0.05 M NH_4 acetate pH 5.0, 1% SDS was added and the mixture stirred vigorously for 5 min at 60°C. The aqueous phase was collected after centrigfugation at 4°C and purified in the same way as the cytoplasmic RNA.

4.2.4 Poly U and Poly A Sepharose Chromatography

Ethanol precipitated RNA was redissolved in 500 µl of formamide and heated at 60°C for 3 min to minimize aggregation. An appropriate volume of the RNA solution was then added immediately to a slurry of 0.2 g poly U or poly A Sepharose 4B in 5 ml binding buffer (25% formamide, 0.7 M NaCl, 50 mM Tris HCl, 10 mM EDTA, 0.2% lauroylsarcosine, pH 7.5). After gentle stirring for 15 min the slurry was collected and washed with 25 ml of binding buffer on a glass fiber filter (GFA). The bound RNA was then eluted with elution buffer (90% formamide, 10 mM potassium phosphate, 10 mM EDTA, 0.2% lauroylsarcosine, pH 7.5). RNA binding to poly U Sepharose or poly A Sepharose is termed A^+ and U^+ RNA

respectively; RNA eluted with binding buffer is termed A^- and U^-RNA. The percentage of labelled A^+ and U^+ RNA was assessed by scintillation counting and fractions were precipitated with 0.1 vol of 2.5 M NH_4 acetate, 50 µg of cold yeast RNA and 3 vol of ethanol for electrophoretic analysis.

4.2.5 Electrophoresis of RNA

Electrophoresis was routinely carried out in a non-aqueous system using formamide as a denaturing solvent, essentially as described by Sphor et al. (1976). Tube gels (7 mm x 150 mm) containing 4% acrylamide were electrophoresed for 12 hr at 100 V and scanned directly at 265 nm in a quartz cuvette using a Gilford 2400S spectrophotometer with linear transport attachment. Alternatively slab gels were run in the same way and placed in contact with No-Screen X-ray film (Kodak) to estimate the distribution of ^{32}P following electrophoresis.

For the analysis of small RNA species RNA was dissolved in 98% formamide (deionized) containing 20 mM sodium phosphate (pH 7.6), 20 mM EDTA, 0.1% bromophenol blue. The samples were then run either on 7.5 to 22% exponential gradient polyacrylamide SDS gels (Section 4.2.8) or on urea gels. Urea gels contained a 2.5% to 20% exponential gradient polyacrylamide (acrylamide: bis-acrylamide 20:1), 7M urea,

90 mM tris borate, 4 mM EDTA, pH 8.3; the electrode buffer did not contain urea.

4.2.6 In vitro translation

Both A^+ and A^- RNA fractions eluted from poly U Sepharose were precipitated with 2.5 M ammonium acetate, dried and redissolved in sterile distilled water. The solutions were then passed through Sephadex G25 equilibrated with sterile distilled water and the eluates lyophilized. The fractions were reprecipitated in ethanol as before, dried and taken up in a small volume of 100 mM potassium acetate, 2.5 mM magnesium acetate.

Translation was carried out in the micrococcal nuclease treated rabbit reticulocyte system (Amersham or New England Nuclear). Assays contained 30 µl translation cocktail, 33-40 µCi [^{35}S]-L-methionine (specific activity >1000 Ci/mmol), and either 3 µg A^+ RNA or 16 µg A^- RNA in a final volume of 35 µl. Incubation was for 60 min at 30°C, after which SDS electrophoresis sample buffer was added and the samples heated at 100°C for 2 min. Protein synthesis was determined from hot TCA insoluble radioactivity as described in section 4.2.7.

4.2.7 Extraction of protein

Three pairs of cotyledons were ground in liquid nitrogen and extracted in 500 µl 10 mM Tris glycine pH 8.3, 1% 2-mercaptoethanol, 1 mM phenylmethyl-sulfonylfluoride (PMSF, Sigma). Extracts were centrifuged at 25,000 g for 30 min at 4°C. The supernatant and pellet were designated soluble and insoluble protein respectively. Incorporation of [^{35}S] methionine into protein was assessed on the basis of hot TCA insoluble counts. Two to 5 µl of extract was spotted on a filter paper disc (Whatmann 3 MM) and placed in cold 10% TCA, 1 mg/ml methionine for 10 min. The discs were then placed in 10% TCA at 100°C for 10 min and subsequently washed with 10% TCA and ethanol. After drying the protein was solubilized from each disc with 0.5 ml of Protosol (New England Nuclear) for 30 min at 60°C in a capped scintillation vial. The radioactivity in the vials was then counted after the addition of 50 µl glacial acetic acid and 10 ml Econofluor (Amersham).

4.2.8 One- and 2-Dimensional Electrophoresis of Protein

One-dimensional sodium dodecyl sulphate (SDS) gel electrophoresis was carried out by the method of Laemmli (1970) using slab gels (180 x 200 x 0.7 mm) with an exponential gradient separating gel from 7.5 to 22%

polyacrylamide. Proteins (5 µg each) of known molecular weight were electrophoresed alongside the samples; these were phosphorylase b, bovine serum albumin, ovalbumin, chymotrypsinogen, RNase A and cytochrome c (Pharmacia).

Two dimensional gel electrophoresis was carried out using non-equilibrium pH gradient gel electrophoresis in the first dimension as described by O'Farrell et al. (1977). Proteins were subjected to electrophoresis from the positive, acidic, electrode to the negative, basic, electrode for 2000 Vhr. The tube gels were then applied to 7.5 - 22% exponential gradient SDS acrylamide slab gels for the second dimension and run as described for one dimensional gels.

Staining was carried out in 50% methanol, 7% acetic acid and 0.5% Coomassie Brilliant Blue G250, followed by diffusion destaining in 20% methanol, 7% acetic acid. Radioactive polypeptides were detected by fluorography according to Bonner and Laskey (1974) using preflashed X-OMAT film (Kodak) enabling quantitation of radioactive polypeptides by subsequent scanning of the film (Laskey and Mills 1975). Scanning was carried out at 550 nm with a Gilford 2400-S spectrophotometer with linear transport attachment. Quantification was carried out using gels fixed without stain in order to prevent quenching of radioactive bands during fluorography.

4.3 Results

4.3.1 Rates of RNA Synthesis

When gross rates of RNA synthesis were followed at various time periods after inoculation from estimations of the fraction of TCA insoluble [^{32}P] phosphate in crude homogenates of cotyledons, a high degree of replication was possible in each experiment. Three replicates were performed for each treatment at each time period after inoculation. Each replicate consisted of three seedlings, the cotyledons of which were extracted together. The ratios of TCA precipitable counts to total uptake are presented for the feeding period 13 to 16 hours after inoculation in Table IV. A highly significant

Table IV. Gross rates of RNA synthesis at 13 to 16 hr post inoculation

	Ratio TCA insoluble: total uptake ^{32}P			
Treatment	Replicates			Average
Healthy	0.0211	0.0202	0.0186	0.0200[a]
Resistant	0.0225	0.0231	0.0225	0.0227[b]
Susceptible	0.0202	0.0191	0.0185	0.0193[a]

F value for treatments = 12.122

[a]Values marked with the same letter are not significantly different by analysis of variance.

[b]Significant increase in resistant at the 1% level by least square difference.

increase in the rate of synthesis was found in the resistant combination at this time when the data were assessed by analysis of variance. No significant increase was found in the susceptible combination at this time by this statistical criterion. However, by 18 to 21 hr after inoculation both combinations showed greatly enhanced synthesis (not significantly different from one another). Resistant plants showed a small but statistically insignificant increase in synthesis as early as 5 to 8 hr after inoculation.

The relative rates of RNA synthesis in the resistant and susceptible cotyledons at various times after inoculation are illustrated in Fig. 10. It is interesting that a reduction in labelled A^+ RNA occurs concurrent with the increases in RNA synthesis (Fig. 10 and Section 4.3.3).

The relative rates of RNA synthesis at 18 to 21 hr after inoculation were also estimated on the basis of ^{32}P incorporated per A_{260} unit of purified RNA from both apices and cotyledons. These results were in close agreement with those obtained by measurement of TCA precipitable radioactivity. Treatment with RNase revealed that about 10% (depending on sample treated) of the TCA precipitable counts were resistant to RNase treatment. The ratio of these counts to total uptake did not respond to infection.

The incorporation of ^{32}P into TCA insoluble counts was also estimated for the apices alone in each host-pathogen combination. The total amount of incorporation

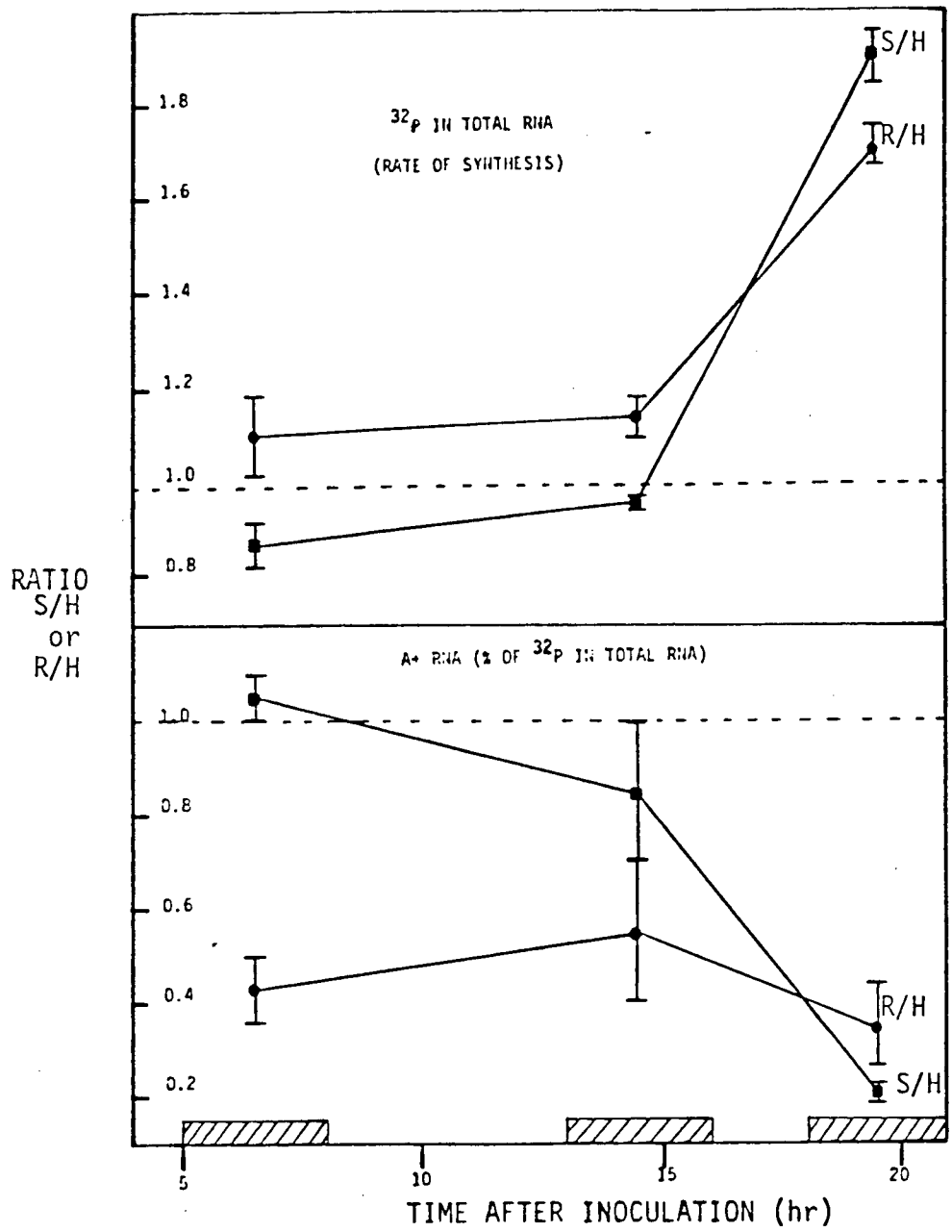


Figure 10. The response of RNA synthesis and the synthesis of A^+RNA to infection with flax rust, race 41 (susceptible combination, S) or race 3 (resistant combination, R). RNA synthesis was estimated from crude extracts of cotyledons by TCA precipitation. The synthesis of A^+RNA was assessed as a percentage of purified labelled RNA from both cotyledons and apices which bound to poly U Sepharose. Both values are expressed for resistant and susceptible combinations as a ratio of the values determined for healthy (H) plants. Bars represent standard errors for ratios. Hatched boxes represent feeding period.

within the apices was about 10% of that within cotyledons and did not appear to be influenced by inoculation (results not shown). Thus it appears that the apex of the seedling does not exhibit a greater rate of RNA synthesis in response to infection during the first 21 hours following inoculation. It has not been possible to determine whether A^+RNA synthesis (Section 4.3.3) in the apex is influenced by inoculation because both apices and cotyledons have been used in RNA purification in order to avoid the radiation exposure which the experimenter would receive while excising the apices. However, as the RNA synthesized within the apices represents only less than 10% of the labelled RNA purified from the seedlings, it is considered that changes observed in purified RNA largely reflect changes within the cotyledons. This is further supported by the fact that the first leaves were seldom infected as they had not emerged from the apical rosette at the time of inoculation.

4.3.2 Characterization of purified RNA by Electrophoresis

Purified RNA from uninoculated seedlings was analyzed by formamide electrophoresis (Fig. 11 and Table V). The identity of various absorption peaks was elucidated from molecular weight calibrations using a small amount of ^{14}C-labelled *E. coli* 16S and 23S ribosomal RNA which was electrophoresed with the sample. The identity of the 25S, 18S, 5.8S and 5S cytoplasmic rRNA and the 16S chloroplast

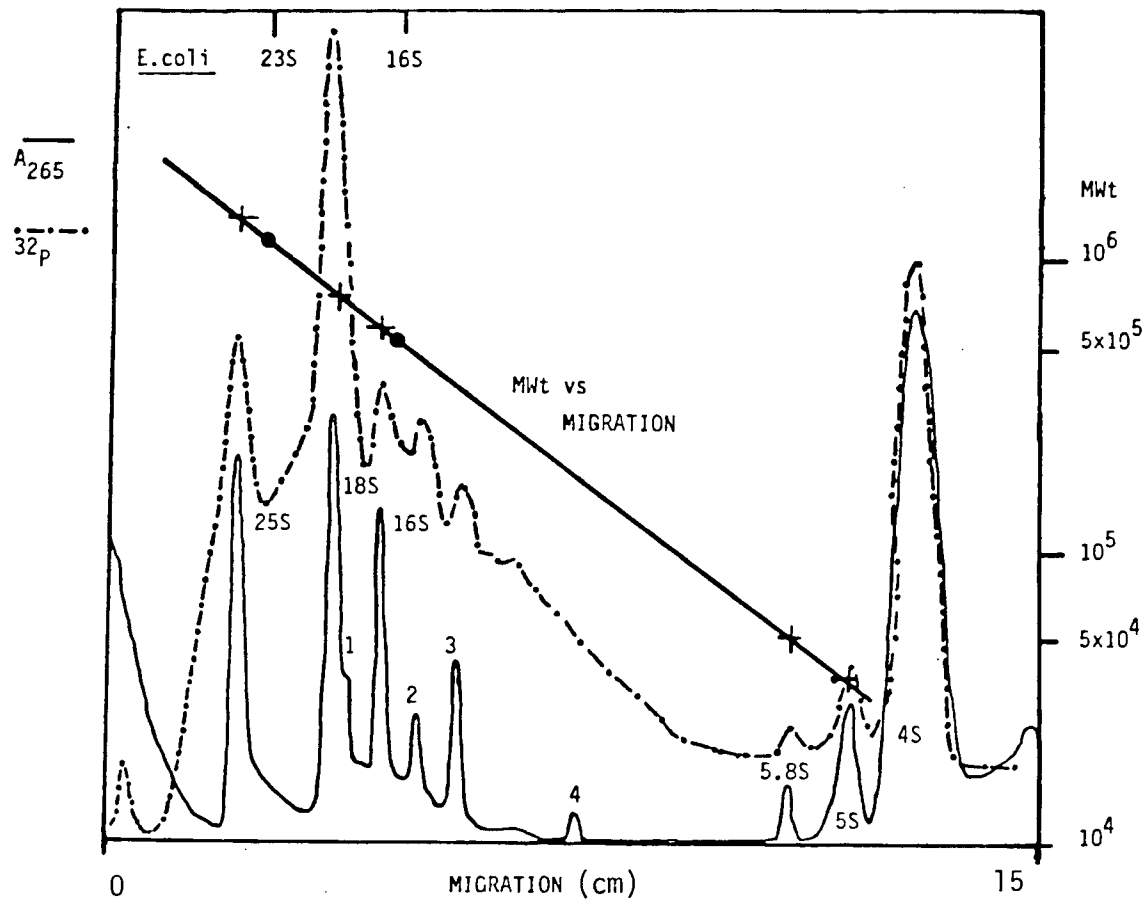


Figure 11. Electrophoresis of [^{32}P] RNA from uninoculated seedlings in 4% polyacrylamide formamide gels. Absorbance curve obtained by direct scanning of tube gel; radioactivity by autoradiography and scanning from slab gels run in the same way; 23S and 16S positions determined with ^{14}C *E. coli* ribosomal RNA. 1, 2, 3 and 4, absorbance peaks of unknown identity. Solid circles represent positions of 23S and 16S *E. coli* RNA; crosses represent positions of 25, 18, 16, 5.8 and 5S flax RNA.

rRNA was determined by fitting their published molecular weights (Leaver 1979) to the molecular weight calibration curve (Fig. 11). The 23 S chloroplast rRNA was notably absent. This is a common feature of RNA preparations run under denaturing conditions (Leaver 1979) and is a result of hidden breaks which occur *in vivo*. Four unidentified high molecular weight RNA species are also seen (Table V and Fig. 11).

TABLE V. RNA species found in purified flax RNA by formamide electrophoresis.

	Species	M.Wt.[a]
Cytoplasmic:	25S	1.3×10^6
	18S	0.7×10^6
	5.8S	5.04×10^4
	5S	3.75×10^4
Chloroplast	23S	1.1×10^6 (not observed)
	16S	0.56×10^6
Unknown: 1	18S Shoulder	0.66×10^6
2		0.42×10^6
3		0.35×10^6
4		0.18×10^6

[a]Values given for known species are those published previously (Leaver 1979) and are in agreement with molecular weights determined here.

It is noteworthy that 0.66, 0.41 and 0.17 x 10^6 dalton (D) fragments of 23S chloroplast rRNA have been described previously (Leaver 1979). The sum of the 0.66 x 10^6 D fragment and the unidentified species of 0.42 x 10^6 D found here is 1.08 x 10^6 D, very close to the published value for the 23S chloroplast ribosomal RNA. Thus it seems that both these species may be fragments of the 23S chloroplast rRNA resulting from a single cleavage of the intact molecule. Whether or not the observed pattern of ^{32}P incorporation is representative of labelling of these fragments is uncertain (Fig. 11, 2, 3 and 4). It should be realized that rapidly labelled mRNA in this region is likely to contribute to the overall pattern of ^{32}P incorporation. In particular, the large subunit of ribulose-bisphoshophate carboxylase (RuBPCase) is known to be rapidly synthesized and have a molecular weight of approximately 0.5 x 10^6 D (Steinback 1981). If indeed, the 23S rRNA fragments are heavily labelled this would imply rapid in vivo breakdown of the large chloroplast rRNA (in the apparent absence of general degradation during extraction). This has not been reported in the literature however.

Labelled RNA has been characterized for the host-pathogen combinations at 18 to 21 hours after inoculation. Total purified ^{32}P labelled RNA from healthy or infected plants was applied to slab gels containing 4% polyacrylamide

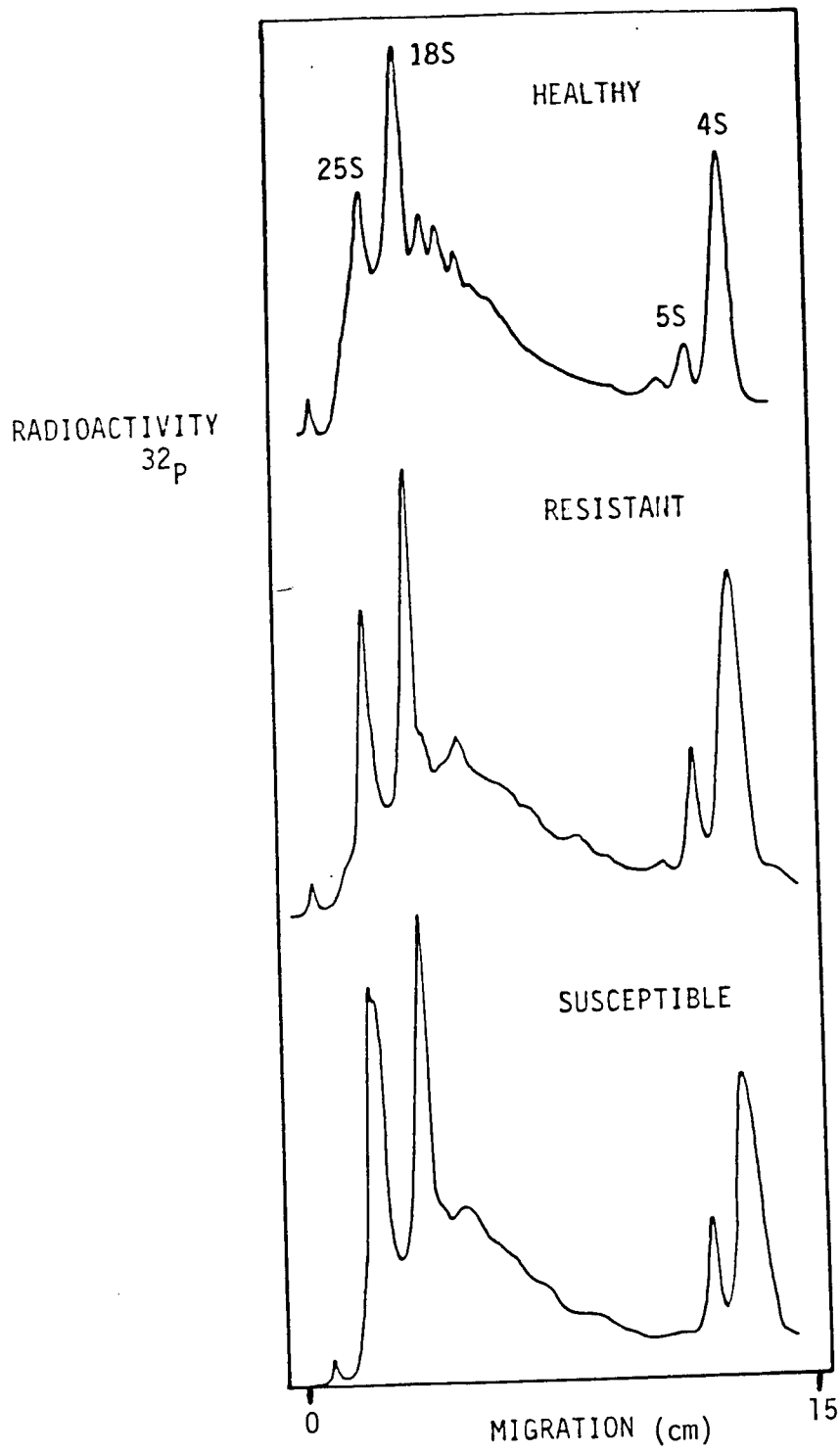


Figure 12. Electrophoretic analysis of RNA synthesized 18 to 21 hr after inoculation. Gels contained 4% polyacrylamide in formamide.

in formamide. Following electrophoresis and autoradiography the X-ray film was scanned in order to assess the distribution of ^{32}P label. The results of one such experiment are presented in Figure 12 and show that the 25S, 18S and 5S rRNA species are selectively enhanced in both resistant and susceptible combinations. This result is representative of a total of four such experiments performed. Minor RNA species were not always successfully resolved in formamide slab gels. Thus, it has not been possible to determine with certainty whether the changes seen in species migrating faster than 18S rRNA (Fig. 12) are a consistent characteristic of diseased plants. It is possible more information about the effects of disease upon the synthesis of various RNA species might be obtained by using a more reliable electrophoretic technique.

4.3.3 Poly U binding RNA (A^+RNA)

Polyadenylated (A^+) RNA was assessed as a percentage of ^{32}P labelled RNA binding to poly U Sepharose. This fraction was characterized by a high specific radioactivity, representing 0.5% of the total RNA mass and 7.3% $\pm$ 1.3 of the radioactivity. It has also been characterized electrophoretically (Fig. 13) and shows enrichment in heterogeneous high molecular weight species with a size distribution characteristic of mRNA and a small amount of rRNA contamination.

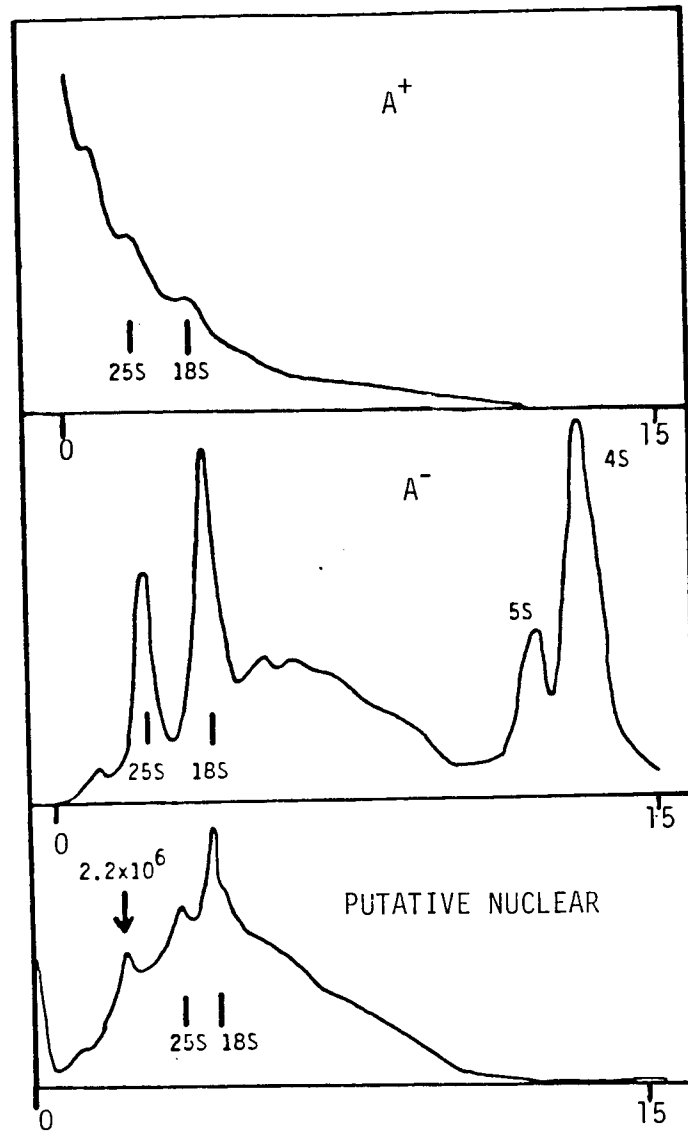


Figure 13. Electrophoretic characterization of fractionated RNA on formamide gels. 25S and 18S positions determined with total RNA run on the same slab gel.

Infection led to marked decreases in the proportion of A^+ RNA synthesized (Fig. 10). This decrease was correlated with the general increase in total RNA synthesis and occurred at earlier times after inoculation in the resistant combination than in the susceptible combination.

4.3.4 Poly A binding (U^+) RNA and small RNA

RNA containing poly or oligo U sequences and binding to poly A Sepharose constituted about 1% of the total radioactivity. Attempts to purify measurable quantities (on an absorbance basis) of this fraction were unsuccessful as it represents less than 0.05% of the total RNA mass. Electrophoretic analysis (Fig. 14) showed that this fraction was enriched in low molecular mass RNA with an average molecular mass of 15 kilodaltons (kD). Electrophoresis on SDS or urea gels (7.5 - 22% or 2.5 - 20% exponential polyacrylamide gradients respectively, see "Materials and Methods") further resolved total RNA in this region into three species (labelled a, b, and c in Fig. 14). These species were absent from both A^+ and U^- RNA and would therefore appear to be selectively bound by poly A Sepharose. Molecular masses were calculated from urea gels, as 7M urea would be expected to yield RNA with conformation-independent migration during electrophoresis. The molecular mass curve was constructed from the known values for 5.8S, 5S and 4S flax RNA (see Section 4.3.2) and extrapolated to give

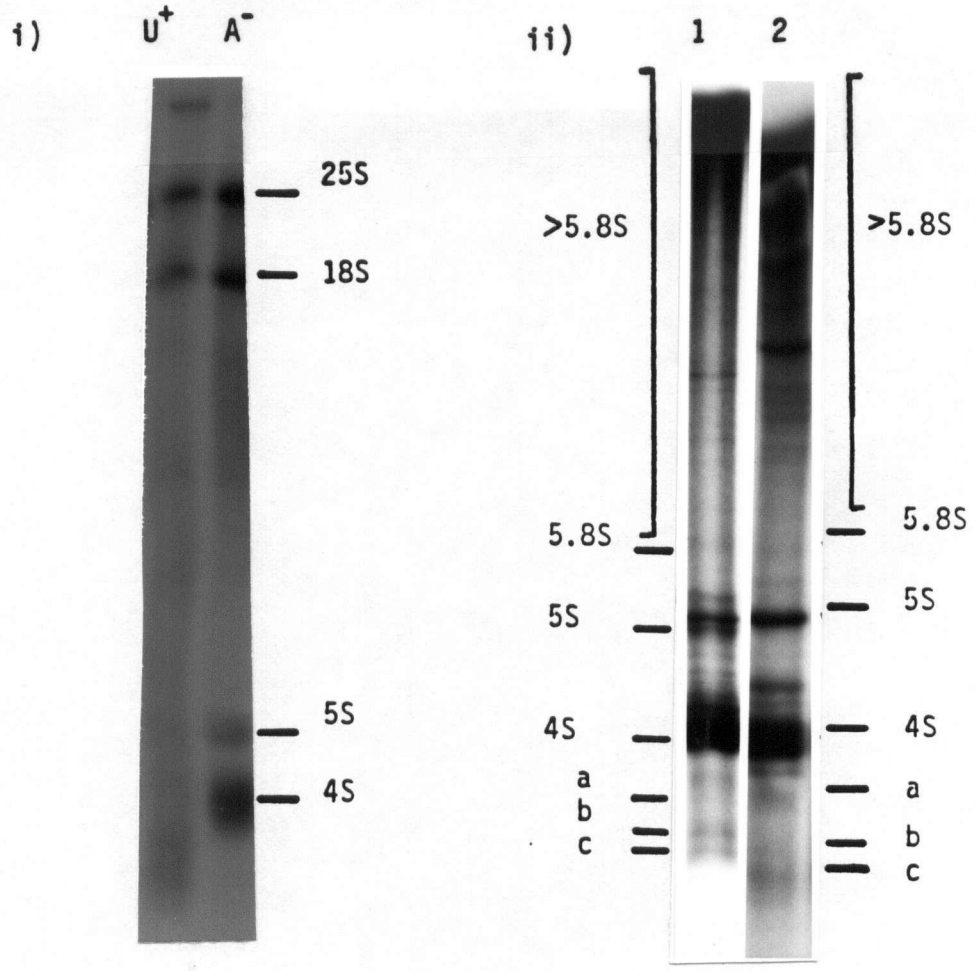


Figure 14. Analysis of U^+RNA from uninfected plants by electrophoresis and autoradiography.
i) Labelled U^+ and A^- fractions separated on 4% polyacrylamide formamide gels. 25S, 18S, 5S and 4S species identified as described in Section 4.3.2.
ii) Resolution of small RNA species within total labelled RNA:1, on SDS gels (7.5 to 22% exponential polyacrylamide gradient) and 2, on urea gels (2.5 to 20% exponential polyacrylamide gradient). The positions of the 5.8S, 5S and 4S species were observed after staining with methylene blue. Species marked a, b and c and >5.8S are discussed in the text.

estimated molecular masses for a, b, and c of 14.5, 12.5 and 10.0 kD respectively. When migration on SDS gels was used to calculate molecular masses for a, b and c in the same way values of 14.0, 11.7 and 10.5 kD were obtained. Various species of RNA larger than 5.8S rRNA were also found (>5.8S in Fig. 14). These had estimated molecular masses ranging from 75 kD to 180 kD.

The influence of infection upon both the small U^+ RNA species and those larger than 5.8S RNA has been investigated between 5 and 21 hr after inoculation. These experiments showed no significant differences following inoculation. The percentage of U^+ RNA (as a fraction of the total labelled RNA) was not significantly altered by infection at 18 to 21 hr after inoculation.

4.3.5 Significance of the altered patterns of RNA synthesis in disease

The decline in A^+ RNA as a percentage of the total RNA is in fact representative of a decline in the amount of A^+ RNA synthesized. This follows from the demonstration that the percentage decline is greater than the corresponding increase in total RNA synthesis, as shown in Table VI. It would be surprising if this represented an almost total cessation of mRNA synthesis as it has been shown that only in the susceptible combination is there any appreciable decline

in in vivo protein synthesis and this can largely be accounted for by decreased synthesis of the A^- encoded large subunit of RuBPCase (Section 4.3.7). Furthermore, in vitro translation (Section 4.3.6) has shown that about 65% of the total mRNA activity in healthy plants is contained in the A^- fraction. Thus the decline in A^+ RNA could be due to: a) decreased nuclear polyadenylaton of precursor mRNA or b) enhanced poly A removal in the cytoplasm (or possibly a decline in cytoplasmic polyadenylation) (Hall 1979).

In order to gain insight into this, nuclear RNA from healthy and diseased plants has been compared at 18 to 21 hours after inoculation (Table VI). The putative nuclear fraction has been assessed in relation to the expected composition of authentic nuclear RNA by formamide electrophoresis (Fig. 13). It is enriched in high molecular weight RNA, lacks tRNA and contains enhanced incorporation into a 2.2×10^6 dalton precursor rRNA. Furthermore, it is indicated that the decline in A^+ mRNA during disease is due to decreased nuclear polyadenylation of precursor mRNA in the nucleus.

TABLE VI. The Relative Rates of A^+RNA Synthesis in Healthy and Infected Seedlings at 18 to 21 hr after Inoculation.

	Healthy (H)	Resistant (R)	Susceptible (S)
Total RNA:			
Incorporation (cpm/A_{260} unit)	96,900	163,800	149,100
Ratio, R/H or S/H	1.0	1.7	1.5
% Poly A^+	9.8	2.5	2.3
Ratio, R/H or S/H	1.0	0.26	0.23
Relative[a] Amount of Poly A^+ Synthesis	100%	43%	35%
Putative nuclear RNA:			
% Poly A^+	5.1	1.4	1.3
Ratio, R/H or S/H	1.0	0.27	0.25

[a]Relative amount of poly A^+ synthesis = $\frac{\%\ \text{Poly } A^+ \times \text{Ratio of incorporation}}{\%\ \text{Poly } A^+ \text{ in Healthy}} \times 100$

4.3.6 In vitro translation of RNA from uninfected plants

4.3.6.1 Translational activity:

The translational activity of A^+ and A^- RNA fractions purified from healthy seedlings (apices and cotyledons) were assessed in the rabbit reticulocyte system. Maximal incorporation of [^{35}S] methionine into hot TCA insoluble counts was seven and fifteen times background for the A^- and A^+ fractions respectively. Incorporation increased linearly with respect to increasing RNA concentration over the range 0 to 1.5 µg/µl for A^- RNA and over the range 0 to 0.1 µg/ul for A^+. Translational activity was assessed from incorporation in the linear range. In this way an estimate of the total translational activity was derived (Table VII). However, upon analysis of A^- encoded polypeptides by electrophoresis it was found that about half the incorporated radioactivity was not visualized as labelled polypeptides; approximately twice as much radioactivity had to be applied to the gel in order to obtain a similar density on autoradiograms to the A^+ encoded fraction under standard conditions of exposure. Similar stimulation of non-specific incorporation was observed upon the addition of tRNA (E. coli) to translation assays and it is therefore concluded that the high concentration of non-messenger RNA in the A^- fraction is responsible for the

hot TCA precipitable radioactivity which does not appear in labelled polypeptides. For this reason a revised estimate of 66% was determined for the translational activity of the A^- fraction (Table VII). A remarkably similar distribution of activity was obtained for A^+ and A^- RNA from pea seedlings (Gray and Cashmore 1976).

TABLE VII. Translational Activity of RNA Fractions Separated by Poly U Sepharose Chromatography

	RNA Fraction	
	A^+	A^-
Incorporation (dpm $^{35}S/\mu g$/RNA)	2.22×10^6	6.0×10^4
Incorporation corrected for non-specific counts (dpm$^{35}S/\mu g$ RNA)	2.22×10^6	3.0×10^4
% of total RNA mass	0.67	99.33
% of total mRNA activity	34	66

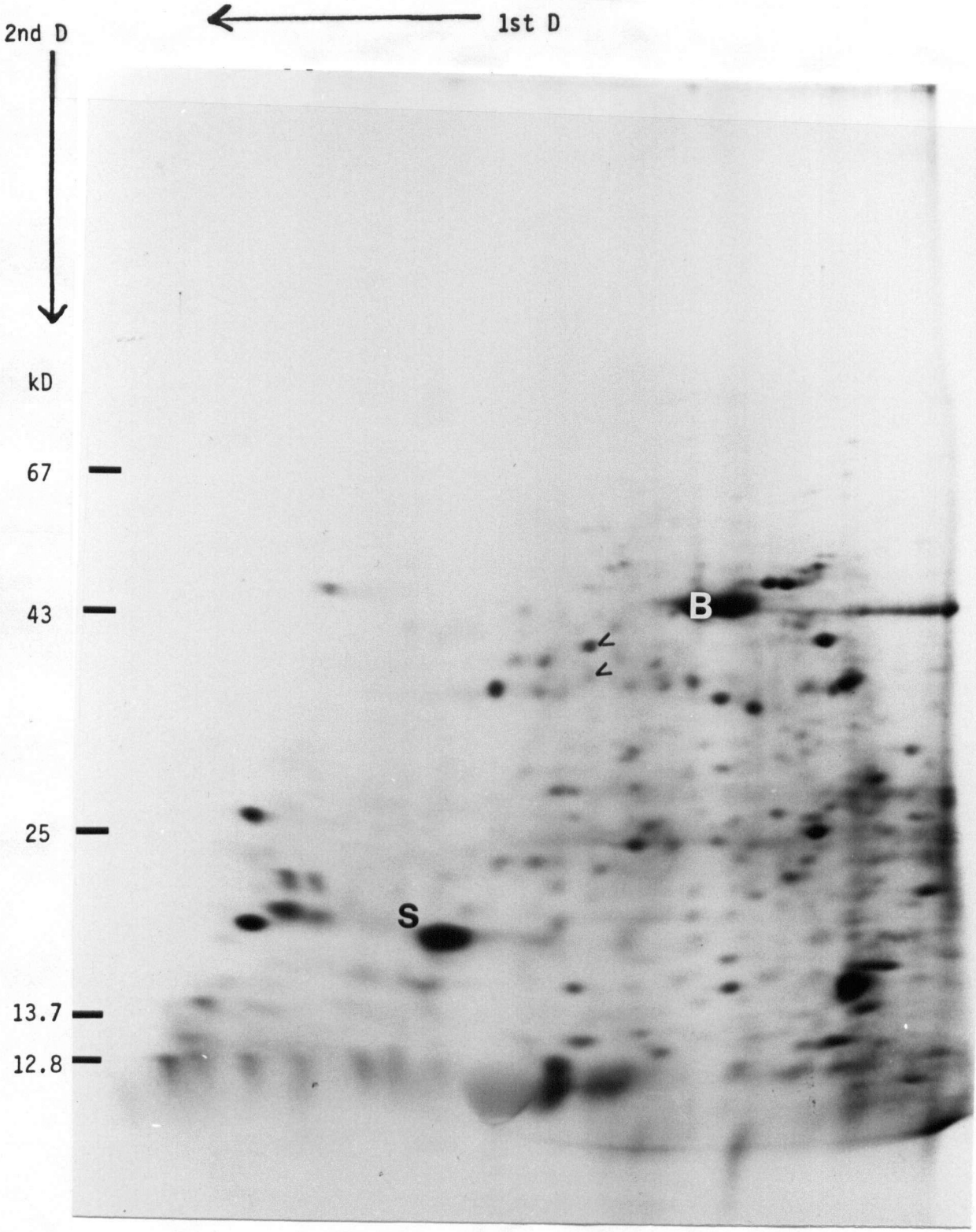
mRNA activity = (incorporation/μg RNA) multiplied by μg RNA in A^+ or A^- fraction

4.3.6.2: Two-dimensional analysis of translation products

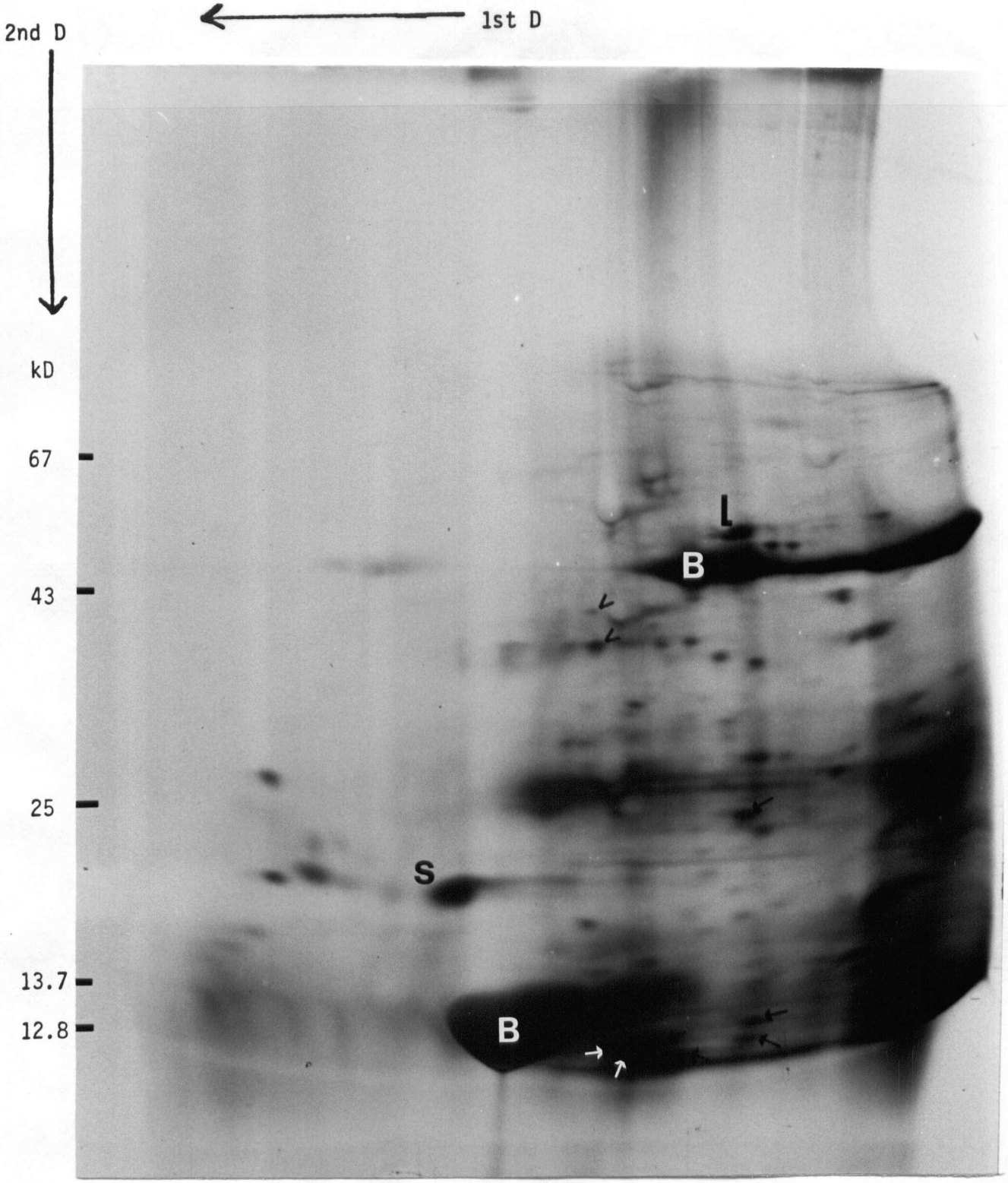
The products of translation assays containing A^+ or A^- RNA were compared on non-equilibrium two dimensional gels (Fig. 15). It can be seen that although several polypeptides are unique to the A^+ fraction very few are encoded only by poly A lacking mRNA (A^-RNA). Clearly chloroplast mRNA would be expected to be contained within the A^- fraction (Steinback 1981). This is demonstrated by the occurrence of a major polypeptide with a molecular mass of 55 kD (L in Fig. 15.2) which appears in the same position as the major *in vivo* stained protein in this size range (Fig. 15.4). This polypeptide is probably the large subunit of ribulose bisphosphate carboxylase (RuBPCase) and is unique to the A^- fraction. A few other polypeptides are unique to the A^- fraction (Fig. 15.2), however the vast majority are also found in the A^+ fraction. Included among this group is a major polypeptide with a molecular mass of 19 kD (S in Fig. 15.1 and 15.2) which on the basis of its molecular weight and basic isoelectric point is probably the precursor to the small subunit of RuBPCase (Ishiye *et al.* 1981).

These results indicate that many of the cytoplasmic mRNAs (which comprise the greatest degree of mRNA complexity) appear as polyadenylated and non-polyadenylated species. Assuming equal efficiency of translation of A^+ and A^-

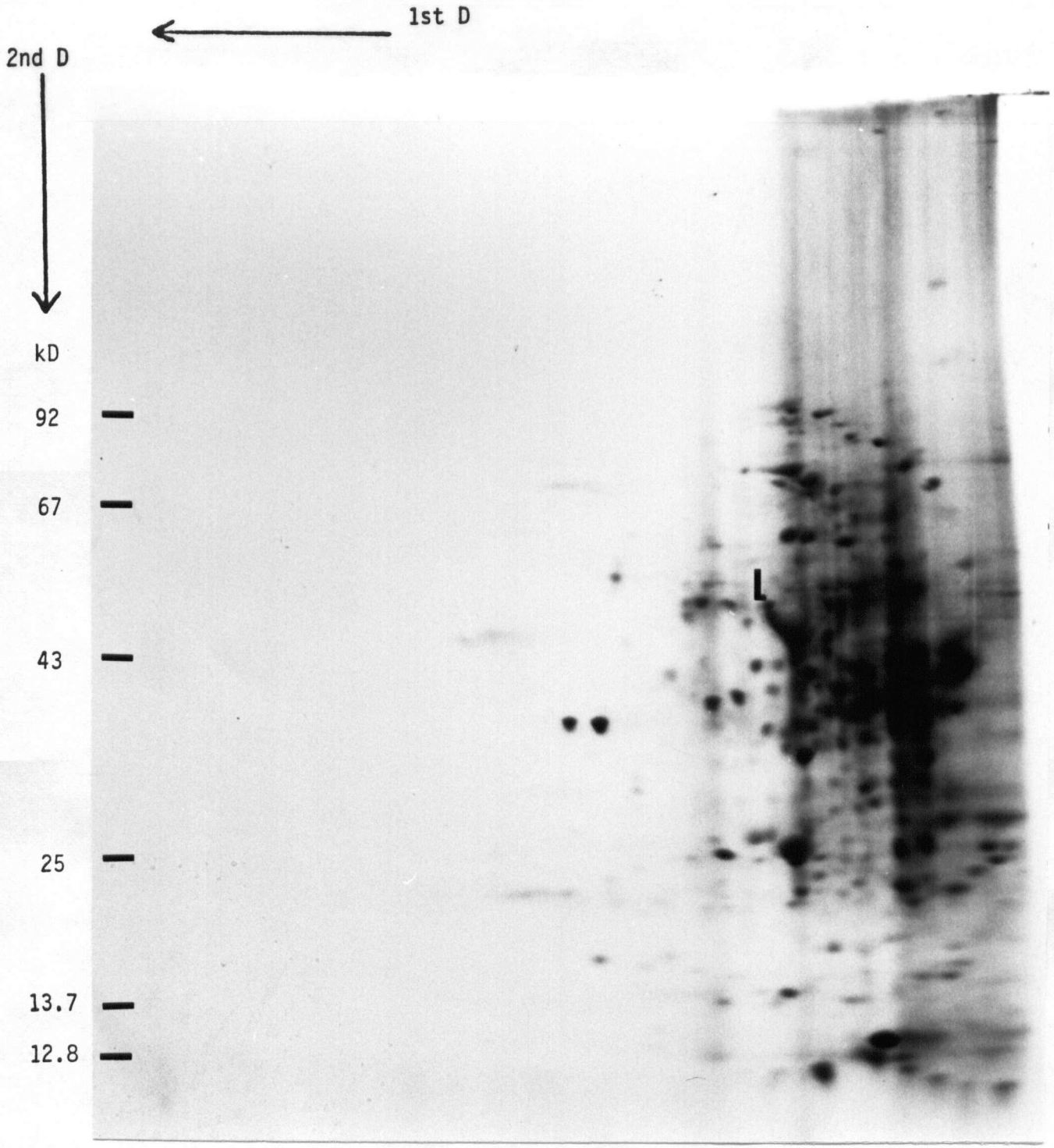
Figure 15.1, 15.2, 15.3 and 15.4.
Two dimensional electrophoresis of polypeptides. First dimension is non-equilibrium pH gradient electrophoresis, second dimension SDS electrophoresis (7.5 to 22% exponential gradient). L, large subunit of RuBPCase; S, putative precursor to the small subunit of RuBPCase; B, labelled material found in translation blank (no added RNA). Molecular weight markers, phosphorylase b 92 kD, bovine serum albumin 67 kD, ovalbumin 43 kD, chymotrypsinogen 25 kD, RNase A 13.7 kD, cytochrome c 12.8 kD.



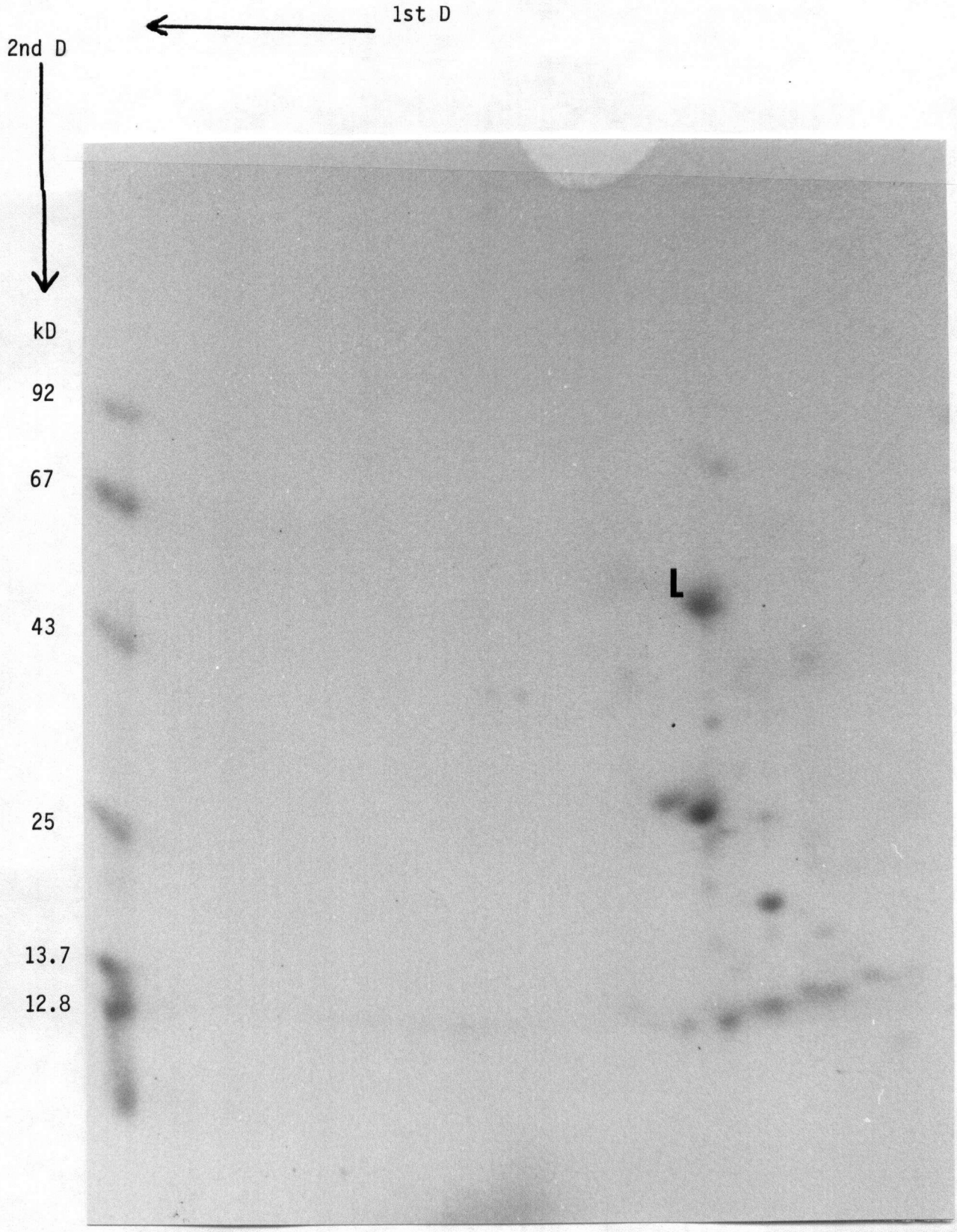
15.1 In vitro products of A^+RNA. Polypeptides marked 'V' are also encoded by A^-RNA but in different relative amounts.



15.2 In vitro products of A⁻RNA. Polypeptides marked with arrows are not found in the A⁺ fraction; those marked 'V' are examples of those also found in the A⁺ fraction but in different relative amounts.



15.3 In vivo labelled soluble polypeptides from cotyledons. Note the large subunit of RuBPCase (L) is quenched due to staining.



15.4 Stained soluble polypeptides from cotyledons.

messages,the mRNA that codes for the small subunit RuBPCase is about equally distributed between the two fractions.

4.3.7 Protein Synthesis in vivo following inoculation

Radiolabelled proteins from healthy and diseased cotyledons were examined by SDS electrophoresis and 2-dimensional electrophoresis at 8 and 18 hours after inoculation. No differences in the pattern of synthesis were found by these criteria until 18 hours after inoculation. At this time a marked decline in the gross rate of synthesis of soluble protein in the susceptible combination was apparent. Gross rates of synthesis could be reproducibly determined from the ratio of hot TCA insoluble radioactivity to total uptake after a 1 hour feeding with [^{35}S] methionine (Table VIII). The decline observed in the susceptible combination could be accounted for by a decline in the synthesis of the large subunit of RuBPCase; this appeared as the major stained and labelled soluble polypeptide with a molecular mass of 55 kD (Fig. 16.1 and 16.2). This major change was accompanied by increased synthesis of an unknown polypeptide of 50 kD (LSu+1 in Fig. 16.2), and a decline in a 30 kD polypeptide (Fig. 16.1 and 16.2).

The results of three additional experiments in which soluble in vivo labelled proteins have been analyzed have

TABLE VIII. Rates of Protein Synthesis at 18 Hours After Inoculation

Replicates	Ratio of Hot TCA insoluble ^{35}S To Total ^{35}S Uptake		
	Healthy	Resistant	Susceptible
1	0.173	0.183	0.160
2	0.189	0.179	0.120
3	0.165	0.176	0.135
4	0.175	0.187	0.133
Average	0.1755[a]	0.1813[a]	0.1370[b]

F treatments = 16.9

[a]Averages marked with the same letter, not significantly different.

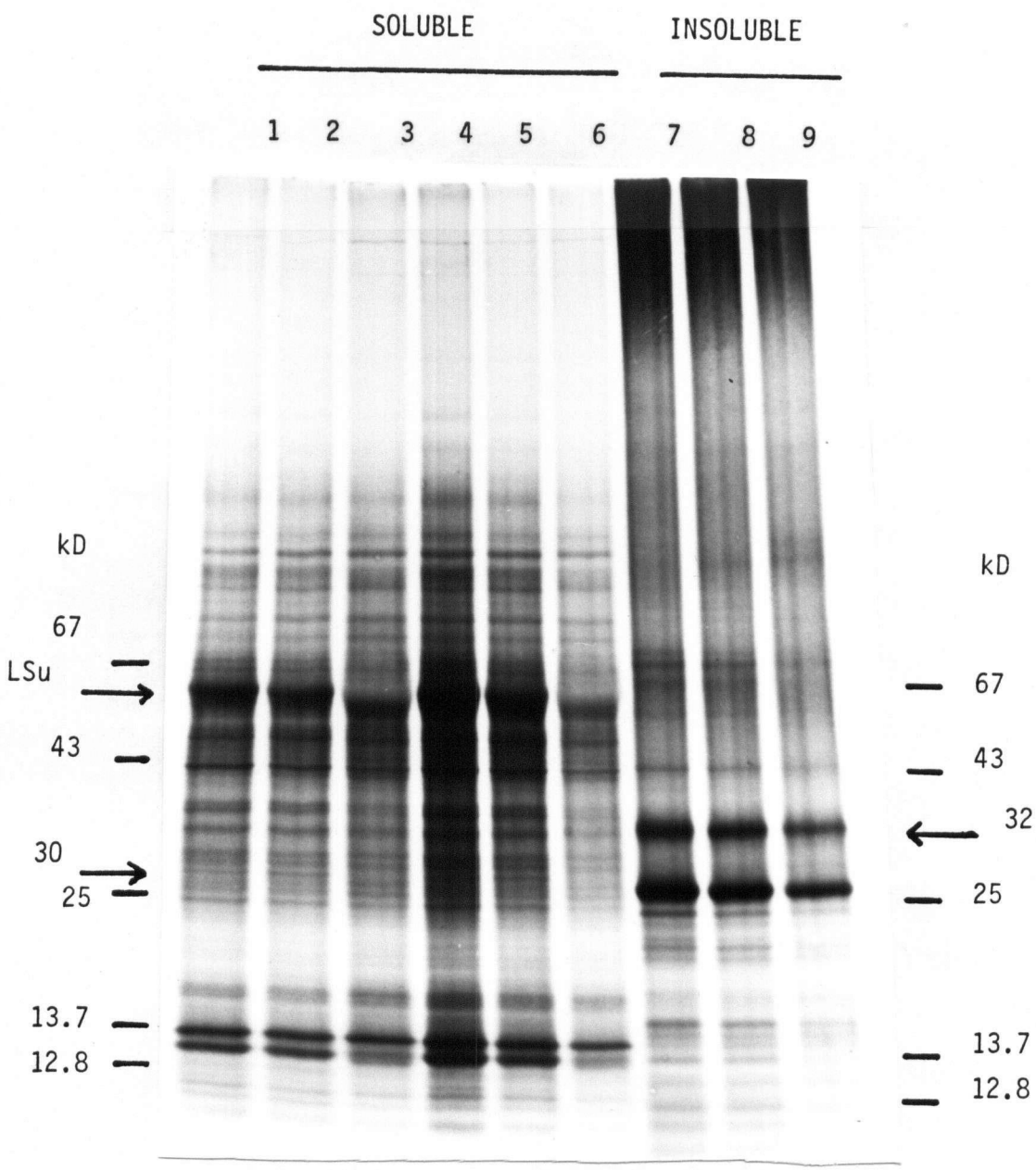
[b]Significantly different at 0.01 level by least square difference.

shown reproducible increases in both the RuBPCase large subunit (Fig. 16.2, inset) and the unknown 30 kD polypeptide (Fig. 16.2) in the resistant combination. These increases are small (10% in the case of the large subunit) but are interesting because they represent a converse reaction to the susceptible response.

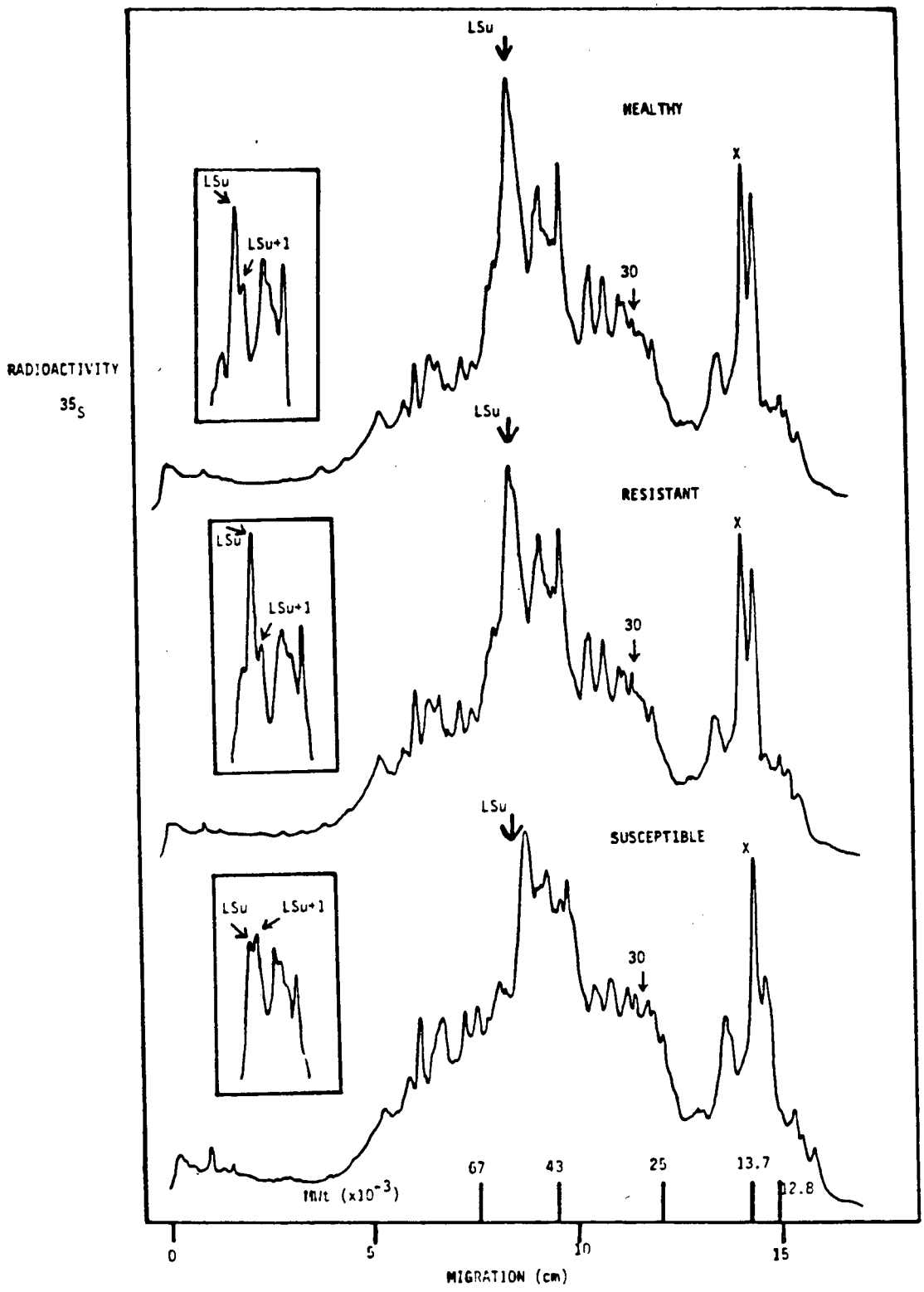
Attempts to identify positively the small subunit of RuBPCase were frustrated by the presence of two irreproducibly stained bands in the 13 kD region. However, the major radioactive polypeptide in this region coincided with the larger of these two stained polypeptides. An inconsistently resolved series of multiple radioactive bands corresponded with the smaller stained polypeptide. This may indicate that the larger consistently resolved band (labelled X in Fig. 16.2) is the authentic small subunit. If this is the case then it is implied that there is no decline in small subunit synthesis in the susceptible combination. No reproducible decrease occurred in the smaller polypeptide.

The major membrane bound chloroplast proteins, are clearly represented in the insoluble fraction (Fig. 16.3). These are the 32 kD thylakoid membrane protein and the major apoprotein of the light harvesting complex (LHC) with a molecular weight of 25 kD. It is of interest that the synthesis of neither of these polypeptides decreases significantly in the susceptible combination, particularly

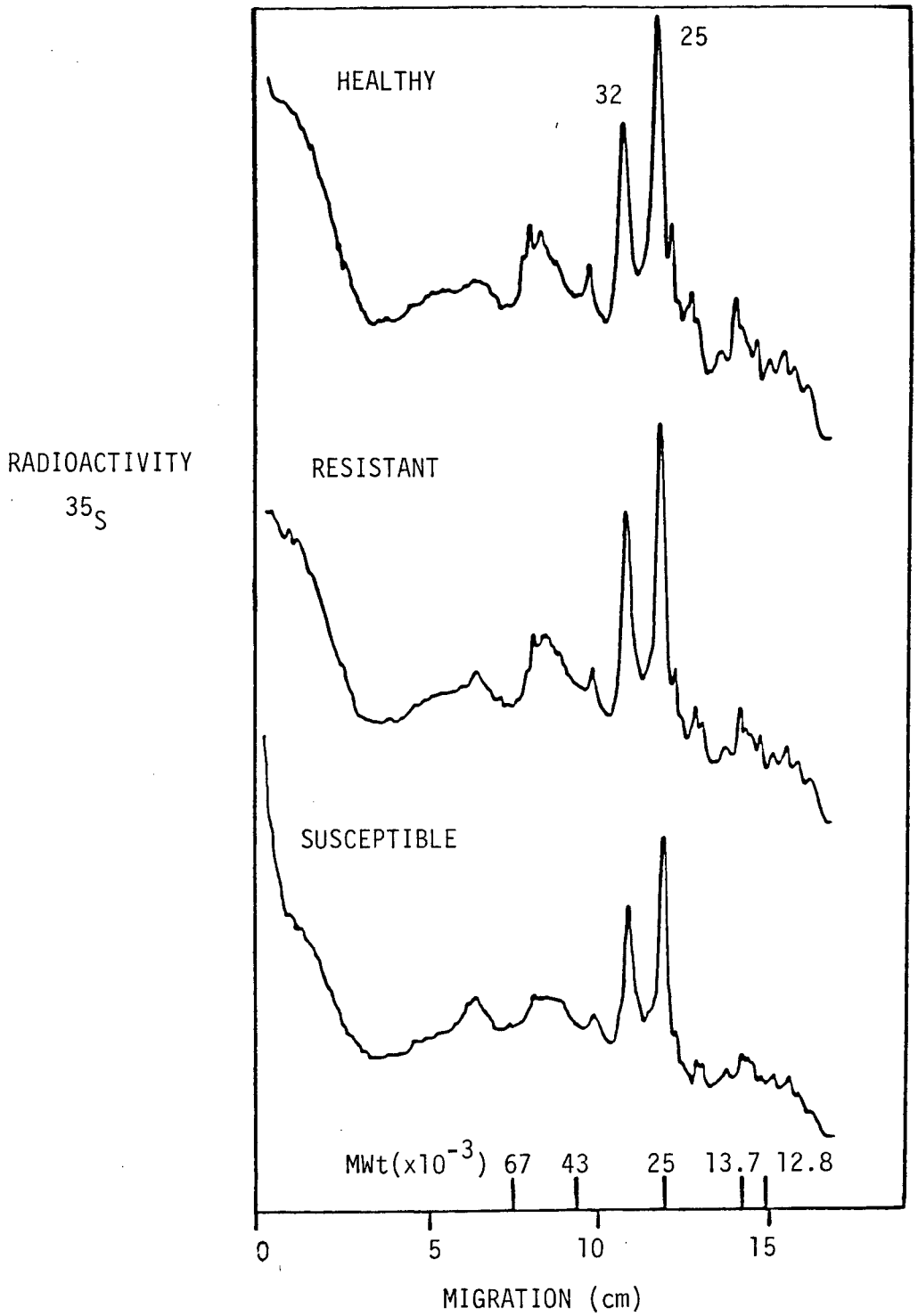
Figures 16, 16.1, 16.2 and 16.3.
Analysis of *in vivo* labelled polypeptides by SDS electrophoresis, 18 hr after inouclation. Polypeptides of known molecular mass were bovine serum albumin, 67 kD, ovalbumin, 43 kD, chymotrypsinogen, 25 kD, RNase A, 13.7 kD, cytochrome c, 12.8 kD. Other values refer to labelled flax polypeptides discussed in the text; LSu, large subunit of RuBPCase.



16.1 Fluorogram of labelled polypeptides. Tracks 1, 2 and 3: equal amounts of radioactivity in soluble proteins from healthy, resistant and susceptible cotyledons; tracks 4, 5 and 6: soluble proteins from healthy, resistant and susceptible cotyledons, each track containing equal amounts of protein; tracks 7, 8 and 9 insoluble proteins from healthy resistant and susceptible cotyledons (equal amounts of radioactivity).



16.2 Densitometric scans of tracks 1, 2 and 3 from Fig. 16.1 (soluble protein). Inset; part of a scan in the 60 to 40 kD region from another experiment showing resolution of the large subunit of RuBPCase (LSu) and an unknown polypeptide (LSu+1) with a molecular mass of 50 kD. X, is the putative small subunit of RuBPCase. The peak marked 30 is discussed in the text.



16.3 Densitometric scans of tracks 7, 8 and 9 from Fig. 16.1 (insoluble protein). Peaks marked 32 and 25 are discussed in the text.

as the former is encoded in the chloroplast and the latter in the nucleus (Steinback 1981).

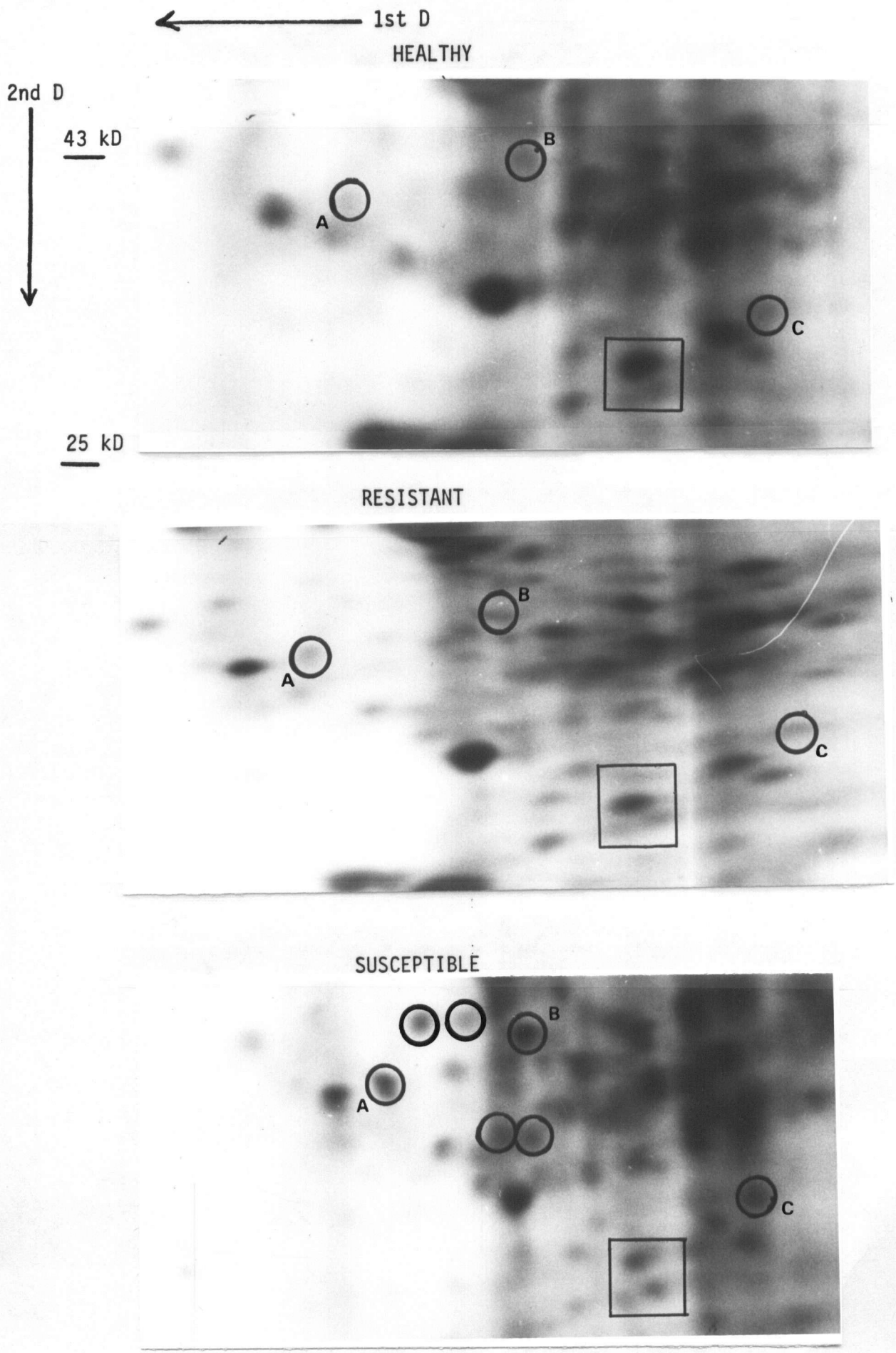
4.3.8 Two-dimensional analysis of in vivo labelled soluble protein following inoculation

In vivo labelled soluble extracts were analyzed by two-dimensional electrophoresis (non-equilibrium pH gradient) at 8 and 18 hours after inoculation. No changes in the pattern of labelled polypeptides was observed until 18 hr after inoculation. At this time several polypeptides showed enhanced synthesis in the susceptible combination (Fig. 17). Four of these were not discernable on gels of extracts from the healthy or resistant combinations. Three (A,B and C in Fig. 17) were also present in healthy and resistant combinations but at a lower level.

No differences were observed between the healthy and resistant combinations throughout the experimental period.

Other experiments utilizing pH gradients of 3.5 to 7.0 in the first dimension (adapted from O'Farrell 1975) have enabled a high degree of resolution in the acidic region. However, these experiments failed to reveal any additional information with regard to the effects of disease.

Figure 17. Two-dimensional electrophoresis of *in vivo* labelled soluble polypeptides 18 hr after inoculation. Part of the fluorograms is shown here. Circled spots are enhanced in the susceptible combination. The corresponding spots are shown in the other combinations when present(A, B, C). Spots within the box exhibit altered relative incorporation in the susceptible combination.


1st D
HEALTHY
2nd D
43 kD
A
B
C
25 kD
RESISTANT
A
B
C
SUSCEPTIBLE
A
B
C

4.4 Discussion

The results presented here support the notion of early host recognition of the avirulent pathogen. This is characterized by increased RNA synthesis and a probable shift in mRNA synthesis to predominantly non-polyadenylated species. These changes occur as early as 8 hr after inoculation and at a time when no significant response is seen in the susceptible combination. It seems that this race specific recognition occurs prior to haustorial development (Littlefield and Aronson 1969) and it is therefore necessary to invoke the involvement of a diffusible substance of fungal origin.

Among the first observable changes in protein synthesis is a marked decrease in the synthesis of the large subunit of RuBPCase in the susceptible combination. This is paralleled by a slight increase in the resistant combination. It is hard to imagine that these phenomena have any effect on RuBPCase activity in view of the large excess of this enzyme (Wildner 1981). Furthermore no significant change in the amount of enzyme protein was observed electrophoretically during the two days following inoculation. However, it is well known that photosynthesis declines during successful disease development (Daly 1976, Scott 1972). A recent

report by Montalbini *et al.* (1981) has shown that *in vitro* reduction of NADP by chloroplast membranes decreased from 1 day after inoculation in extracts derived from rust infected *Vicia faba*. Clearly this information implies a primary role for chloroplast function in determining the outcome of the host-parasite interaction.

Of the various chloroplast polypeptides that can be identified, the large subunit of RuBPCase is the only one exhibiting a dramatic decline in the incorporation of ^{35}S-methionine in the susceptible combination. This implies a fairly specific effect on the synthesis of this polypeptide and argues against the possibility that the decline is a result either of reduced uptake of radio-label by the chloroplasts or of a general disruption of chloroplast protein synthesis. The latter is further supported by the relatively unchanged proportion of the thylakoid membrane protein and the LHC apoprotein, in view of their different sites of synthesis (Steinback 1981). Another striking feature of the altered pattern of incorporation in the soluble fraction is the inverse relationship between the large subunit and the 50 kD polypeptide (Fig. 16.2, LSu+1). This is illustrated in results from other experiments where the two polypeptides were more clearly resolved (Fig. 16.2 - inset).

These results indicate a possible precursor-product relationship between the two polypeptides; however, pulse-chase and peptide mapping experiments would be required to confirm this.

The relationship between the large subunit of RuBPCase and the 50 kD polypeptide (LSu+1) should be viewed in light of a recent report by Miller and Huffaker (1982). These workers have isolated endopeptidases from senescing barley leaves which cleave RuBPCase to generate discrete fragments. Among fragments from the large subunit are 54.5 and 49 kD polypeptides. A fragment smaller than the small subunit is also observed which is derived from the small subunit by endopeptidase-3 activity which copurifies with RuBPCase and is insensitive to 1 mM PMSF. This may explain the presence of two major bands in the 13 kD region here. In flax cotyledons the apparent shift of radiolabel to LSu+1 was not accompanied by a shift in stained protein. Thus, if indeed this phenomenon is the result of protease activity, it appears to be selective for newly synthesized polypeptides. A possible explanation for this would be that only non-assembled subunits are available for proteolysis.

Two dimensional analysis of <u>in vivo</u> labelled proteins from flax has shown that although an altered pattern of synthesis occurs in the susceptible combination at 18 hr after inoculation little change is observed in the resistant

combination in spite of the marked alteration in poly A^+ mRNA synthesis. This may be explained by the fact that *in vitro* translation has revealed many polypeptides encoded both by poly A^+ and by poly A^- mRNA. Therefore, it is unnecessary to invoke the appearance of novel mRNA species in the resistant combination but merely decreased polyadenylation of precursor mRNA in the nucleus. It has been suggested that 3'poly A tails have a role in determining mRNA stability (Hall 1979). It may be that this is representative of a generalized stress response and results in rapid mRNA turnover. Wilting seedlings have also been observed to show a marked reduction in A^+ mRNA (Author, unpublished). This would enable greater flexibility in gene expression.

A further observation of interest is that both stained and *in vivo* labelled proteins from flax cotyledons (Fig. 15.3 and 15.4) show a far more acidic distribution than those translated *in vitro*. The most obvious explanation is that newly translated precursor polypeptides contain basic amino acids which are subsequently cleaved to yield functional polypeptides. The precursor to the small subunit of RuBPCase is a well known example of such a polypeptide (Highfield and Ellis 1978). Thus the observations presented here indicate that such post-translational processing is a common feature of many polypeptides. Roberts and Lord (1981)

have shown that post-translational processing is associated with transport across membranes. It is possible that basic precursors are required for successful passage through membranes.

The small RNA species described in Section 4.3.4 are of considerable interest as they have no known physiological function. A number of small RNA species from maize tissue have been described by Link and Benecke (1980); these have molecular weights in the range of those from flax which are larger than 5.8S. Similar species have been found in Hela cells (Eliceiri 1974). Oligo U and poly U containing RNA have been isolated from wheat embryos (Dobzanska et al. 1980). These RNA fractions contained up to 87 mol % UMP, lacked poly A and were capable of acting as a template for in vitro translation. Their size distribution was heterogeneous and similar to that of mRNA. No enrichment of RNA smaller than 4S was observed.

As far as can be determined, the small U^+ RNA of the type observed in flax has not been described in plants previously. A similar RNA species has been isolated from the myosin messenger ribonucleoprotein of embryonic chick muscle (Heywood and Kennedy 1976). This oligo U-containing RNA species has a molecular mass of 10 kD and reacts stoichiometrically (1:1) with myosin mRNA resulting in total inhibition of translational activity. This RNA has therefore

been described as translational control RNA (tcRNA) and is thought to exert its effect partly through duplex formation of the oligo U moiety, with the 3' poly A tract of the myosin mRNA (Bester et al. 1975). It can be appreciated that flax U^+ RNA bears a close resemblance to chick myosin tcRNA. Attempts to assay the effect of flax U^+RNA on the translational activity flax mRNA were frustrated by the technical difficulties of purifying the small amounts of U^+ RNA present. Established protocols have been developed for the assay of tcRNA (Heywood et al. 1979) and it is therefore considered that further work on flax U^+RNA would be warranted.

CHAPTER 5

GENERAL DISCUSSION

The research presented here has clearly demonstrated a primary role for changes in host RNA and protein synthesis in determining the outcome of the host-parasite interactions studied. Analysis of the major ribonuclease fraction from flax cotyledons has revealed that a novel enzyme does not arise during pathogenesis. Furthermore the enhancement of the isozyme P2 occurs in the 'early' phase of both the resistant and susceptible interaction. Only at the onset of pustule development do differences between the resistant and the susceptible combination manifest themselves. The qualitative changes observed by Chakravorty et al. (1974c) at this stage can be accounted by the selective enhancement of the isozyme P1. The ribonuclease isozymes have similar properties. Both have a pH optimum of 5.5 and showed preferential release of 2'-3' cGMP and are therefore of the RNase I type. As RNase I is generally thought to be involved in substrate re-utilization, it is now inappropriate to invoke them as having a central role in gene expression. It was suggested previously (Chakravorty and Shaw 1977b) that the major flax RNase is involved in post-transcriptional processing.

As far as the author is aware, isozymes of RNase I have not been described previously. It is of great interest,

therefore, to speculate what significance the different catalytic properties of these isozymes may have. Although they both release 2'-3' cGMP preferentially, they clearly have different affinities for macromolecular RNA; this is undoubtedly due to the fact that affinity for macromolecular RNA is dependent on secondary structure which is known to depend on RNA sequence. It is therefore reasonable to suggest that they may be involved in hydrolyzing different types of RNA. Both resistant and susceptible combinations exhibit a marked increase in RNA synthesis in the early stages of disease. In the absence of an accumulation of RNA during these early stages (Hamilton 1969), there is clearly rapid RNA turnover in which RNase I isozymes have a central role. The later stages of the susceptible reaction exhibit both elevated RNA synthesis and enhanced levels of RNase P1 which do not occur in the resistant reaction. It is possible that this enzyme may be involved in the hydrolysis of specific types of host RNA associated with fungal sporulation. This phenomenon appears to be distinct from either wounding or senescence (see Sections 2.3 and 3.3.4). It may therefore be an important element of disease development but not, however, the molecular basis of specificity.

A further question which should be addressed is the possible involvement of the pH 5 insoluble RNase fraction (see Section 2.3). Gel filtration of this fraction revealed that most of the activity was loosely associated with high molecular weight material, and was composed of predominantly RNase P2. The substrate specificity of this fraction was consistent with this observation and did not respond to infection. The possibility that a minor component within the pH 5 insoluble fraction responds to infection cannot be ruled out, however.

Although race specific induction of the resistance response is manifested in an altered pattern of RNA synthesis within a few hours after inoculation, no associated novel proteins have been identified. Increased synthesis of the RuBPCase large subunit has been observed, however. In addition, the synthesis of a 30 kD soluble polypeptide is stimulated. Clearly the increased synthesis of the chloroplast encoded large subunit is unlikely to be a primary event in gene expression associated with the resistance response as this is expected to be mediated by Mendelian type resistance genes located in the nucleus. Whether the 30 kD polypeptide is of cytoplasmic or organellar origin is

unknown. If the former is the case, then it does represent a candidate for involvement as a primary trigger in the resistance response. Other minor proteins may undergo quantitative changes in synthesis. These are hard to observe by two dimensional electrophoresis in the absence of a suitable scanning device. It should be realized that only prevalent or moderately prevalent mRNA encoded polypeptides can be observed by two dimensional electrophoresis. Polypeptides encoded by this class of mRNA represent a complexity of 10^3 kilobases (kB) of a total mRNA complexity of 10^4 to 10^5 kB in mammalian cells (Davidson and Britten 1979).

In conclusion, one might expect that subtle changes in gene expression are involved in the initiation of the resistance response. Von Broembsen and Hadwiger (1972) observed fairly marked increases in the synthesis of certain size classes of protein. This is not in agreement with the findings presented here. These workers did not study responses involving the 'N' locus (resistance locus in Bombay flax), however, responses involving the 'L' locus, which were studied, are similar (Littlefield 1973) and this is therefore probably not a suitable explanation for the apparent discrepancy here. One further possibility is that their results were the consequence of isotope effects across

intracellular membranes which would undoubtedly influence the results obtained with their dual labelling technique (see Section 2.4).

The changes in RNA synthesis in the resistance response are observed prior to haustorial formation (Littlefield and Aronson 1969) and as much as 24 hours before significant phytoalexin accumulation (Keen and Littlefield 1979). Whether phytoalexin accumulation is responsible for cessation of fungal growth is unclear as both occur simultaneously at about 36 hours after inoculation. However, staining with acid fuchsin indicates host cell necrosis and accompanying haustorial necrosis as early as 24 hours after inoculation (Littlefield 1973). Thus, hypersensitivity in the case of flax may in fact be a cause rather than a consequence of fungal inhibition as suggested by Prusky et al. (1980) for the oat/crown rust system. It seems therefore that the resistance response in flax is characterized by derepression of the genome leading to an altered pattern of RNA synthesis, subtle changes in protein synthesis followed by host cell and haustorial necrosis, culminating in the inhibition of fungal growth. A very important point is that necrosis is localized to a few cells around infection sites unless it occurs in the vicinity of veins which are capable of transmitting necrosing substances (Littlefield 1973).

On the other hand, as increased RNA synthesis can be observed in extracts and at times prior to haustorial formation, it is implied that there is a generalized preconditioning which enables rapid necrosis simultaneous with haustorial formation. This would provide an explanation for the cross protection observed by Littlefield (1969). This scheme of events is summarized in Table IX.

The observations (Section 4.3.8) of altered RuBPCase synthesis might be interpreted as demonstrating the importance of functional chloroplast integrity in the initiation of the resistance response. This implies that chloroplast disruption is an important early event in pathogenesis (see Section 2.2.2).

Some additional work with the flax/flax rust system is likely to yield important results. The increased RNA synthesis associated with the resistance response provides a convenient bioassay system for fungal elicitors of the response. The results presented here have shown that estimation of acid insoluble ^{32}P radioactivity provides highly significant results with three treatment replicates and increases in incorporation of as little as 10% can be

TABLE IX. Summary of the main early events in resistance and susceptibility during rust infection of flax

Time after Inoculation(hrs)	Combination	
	Resistant	Susceptible
8	Increased RNA synthesis Shift to A^- mRNA	
18	Greatly increased RNA synthesis. Enhanced synthesis of large subunit RuBPcase.	Greatly increased RNA synthesis Marked decline in synthesis of large subunit RuBPCase
24[a]	Haustorial formation Host cell and haustorial necrosis	Haustorial formation
36[a]	Inhibition of fungal growth. Phytoalexin accumulation.	Continued fungal growth.

Events described for the resistant reaction are those involving the 'N' gene in Bombay flax.

[a]Data from Littlefield (1973).

detected. In view of the possible existence of diffusible elicitors it may be possible to identify these by feeding plants with exudates from germinating uredospores. Such experiments are simply performed and may provide a more specific measure of elicitation than phytoalexin accumulation (see Section 2.2.4).

SUMMARY

Purification of ribonuclease (RNase) from flax has shown that the qualitative changes which are observed in the later stages of disease can be accounted for by altered proportions of two RNase I isozymes. The RNase isozymes differ from one another with respect to substrate specificity, Michaelis constant, pH response and isoelectric point. The properties of these isozymes remain unchanged during rust infection in both resistant and susceptible combinations of flax and flax rust. Early increases (3 days after inoculation) in RNase activity following inoculation are characterized by increased activity of one isozyme, a feature common to both combinations. Late increases (8 days after inoculation) are characterized by enhancement of the other isozyme and are unique to compatible combinations.

Studies on RNA synthesis within the first 21 hours following inoculation show that the resistant reaction is characterized by enhanced RNA synthesis which occurs prior to any measurable response in the susceptible combination. However by 18 to 21 hours after inoculation both combinations show greatly enhanced rates of synthesis. Enhanced synthesis of RNA is also accompanied by decreased polyadenylation of messenger RNA which occurs as early as 8 hours after inoculation in the resistant combination. In vitro

translation of RNA from uninfected plants has shown that polyadenylated and non-polyadenylated RNA encode many common polypeptides. One- and two-dimensional electrophoresis of *in vivo* labelled polypeptides has failed to show any changes in protein synthesis prior to 18 hours after inoculation. At this time several polypeptides are synthesized at altered rates in the susceptible combination; no marked changes were detected in the resistant combination, however. The most pronounced change in protein synthesis in the susceptible combination was a greatly reduced rate of synthesis of the large subunit of ribulose bisphosphate carboxylase. This polypeptide exhibits a slightly higher rate of synthesis in the resistant combination.

BIBLIOGRAPHY

Albersheim P, AJ Anderson-Prouty 1975 Carbohydrates, proteins, cell surfaces, and the biochemistry of pathogenesis. Ann Rev Plant Physiol 26: 31-52.

Atherton KT, G Darby 1974 Extraction of RNA from mammalian tissue cultures cells. Anal Biochem 57: 403-412.

Bell AA 1981 Mechanisms of disease resistance. Ann Rev Plant Physiol 32: 21-81.

Bennett J, KJ Scott 1971 Ribosome metabolism in mildew-infected barley leaves. FEBS Lett 16: 93-95.

Bester AJ, DS Kennedy, SM Heywood 1975 Two classes of translational control RNA: Their role in the regulation of protein synthesis. Proc Nat Acad Sci USA 74: 1523-1527.

Bhattacharya PK, JM Naylor, M Shaw 1965 Nucleic acid and protein changes in wheat leaf nuclei during rust infection. Science 150: 1605-1607.

Bhattacharya PK, M Shaw 1967 The physiology of host-parasite relations. XVIII. Distribution of tritium labelled cytidine, uridine and leucine in wheat leaves infected with the stem rust fungus. Can J Bot 45: 555-563.

Bhattacharya PK, M Shaw 1968 The effect of rust infection on DNA, RNA and protein in nuclei of Khapli wheat leaves. Can J Bot 46: 96-99.

Bhattacharya PK, M Shaw, JM Naylor 1968. The physiology of host-parasite relations. XIX. Further observations on nucleoprotein changes in wheat leaf nuclei during rust infection. Can J Bot 46: 11-16.

Bonner WM, RA Laskey 1974 A film detection method for tritium-labelled proteins and nucleic acids in polyacrylamide gels. Eur J Biochem 46: 83-88.

Callow JA 1977 Recognition, resistance and the role of plant lectins in host-parasite interactions. Adv Bot Res 4: 1-49.

Chakravorty AK, KJ Scott 1979 Changes in two barley leaf ribonuclease fractions during infection by the powdery mildew fungus. Physiol Plant Pathol 14: 95-97.

Chakravorty AK, M Shaw 1971 Changes in the transcription pattern of flax cotyledons after inoculation with flax rust. Biochem J 123: 551-557.

Chakravorty AK, M Shaw, LA Scrubb 1974a Ribonuclease activity of wheat leaves and rust infection. Nature 247: 577-580.

Chakravorty AK, M Shaw, LA Scrubb 1974b Changes in ribonuclease activity during rust infection. I. Characterization of multiple molecular forms of ribonuclease from flax rust grown in host-free media. Physiol Plant Pathol 4: 313-334.

Chakravorty AK, M Shaw, LA Scrubb 1974c Changes in ribonuclease activity during rust infection. II. Purification and properties of ribonuclease from healthy and infected flax cotyledons. Physiol Plant Pathol 4: 335-358.

Chakravorty AK, M Shaw 1977a A possible molecular basis for obligate host pathogen interactions. Biol Rev 52: 147-179.

Chakravorty AK, M Shaw 1977b The role of RNA in host-parasite specificity. Ann Rev Phytopathol 15: 135-151.

Chakravorty AK, RS Simpson, KJ Scott 1980 Messenger and ribosomal RNA hydrolysis by ribonucleases II. Changes in ribonuclease activities and ribosomes of barley leaves during the early stages of powdery mildew infection. Plant Cell Physiol 21: 425-432.

Datta AK, SK Niyogi 1974 Biochemistry and physiology of bacterial ribonucleases. Prog Nuc Acid Res Mol Biol 17: 271-308.

Davidson EH, RJ Britten 1979 Regulation of gene expression: Possible role of repetitive sequences. Science 204: 1052-1059.

Day PR 1974 Genetics of host-parasite interaction. (WH Freeman and Co., San Francisco).

Dobranska M, M Tomaszewski, J Buchowicz 1980 Oligo(U)- and poly (U)-containing RNA of wheat embryo. Biochim Biophys Acta 609: 425-434.

Doke N, K Tomiyama 1980a Effect of hyphal wall components from Phytophthora infestans on protoplasts of potato tuber tissues. Physiol Plant Pathol 16: 169-176.

Doke, N, K Tomiyama 1980b Suppression of the hypersensitive response of potato tuber protoplasts to hyphal wall components by water soluble glucans isolated from Phytophthora infestans. Physiol Plant Pathol 16: 177-186.

Dove LD 1973 Ribonucleases in vascular plants: cellular distribution and changes during development. Phytochemistry 12: 2561-2570.

Dyer TA, KJ Scott 1972 Decrease in chloroplast polysome content of barley leaves infected with powdery mildew. Nature 236: 237-238.

Eliceiri G 1974 Short-lived small RNAs in the cytoplasm of HeLa cells. Cell 3: 11-14.

Flor HH 1956 The complementary genetic systems in flax and flax rust. Advances in Genet 8: 29-54.

Flor HH 1971 Current status of the gene-for-gene concept. Ann Rev Phytopathol 9: 275-296.

Gray, RE, AR Cashmore 1976 RNA synthesis in plant leave tissue: The characterization of messenger RNA species lacking and containing polyadenylic acid. J Mol Biol 108: 595-608.

Hall TC 1979 Plant messenger RNA. In Nucleic Acids in Plants Eds. Hall and Davies. V. 1: 217-254. (CRC Press Inc.).

Hamilton WE 1969 RNA levels in healthy and rust-infected flax cotyledons. MA thesis. University of Saskatchewan, Saskatoon, Saskatchewan, Canada.

Harvey AE, AK Chakravorty, M Shaw, LA Scrubb 1974 Changes in ribonuclease activity in Ribes leaves and pine tissue culture infected with blister rust, Cronartium ribicola. Physiol Plant Pathol 4: 359-371.

Heath MC 1980 Reactions of nonsuscepts to fungal pathogens. Ann Rev Phytopathol 18: 211-236.

Heath MC 1981a Resistance of plants to rust infection. Phytopathology 71: 971-974.

Heath MC 1981b The suppression of the development of silicon-containing deposits in French bean leaves by exudates of the bean rust fungus and extracts from bean rust infected tissue. Physiol Plant Pathol 18: 149-155.

Heitefuss R, G Wolf 1976 Nucleic acids in host-parasite interactions. In Encyclopedia of Plant Physiology New Series V.4 Physiological Plant Pathology. Eds. Heitefuss and Williams (Springer Verlag).

Heywood SM, DS Kennedy 1976 Purification of myosin translational control RNA and its interactions with myosin messenger RNA. Biochemistry 15: 3314-3319.

Heywood SM, DS Kennedy, AJ Bester 1979 Isolation and assay for translational control RNA (tcRNA). Methods in Enzymology 50 pp. 541-549.

Highfield PE, RJ Ellis 1978 Synthesis and transport of the small subunit of chloroplast ribulose biphosphate carboxylase. Nature(London) 271: 420-424.

Ishiye M, H Goto, Y Sasaki, T Kamikubo 1981 The isoelectric point of the precursor of the small subunit of ribulose-1,5-biphosphate carboxylase and a possible role for the transit peptide. FEBS Lett 124: 233-236.

Keen NT, M Legrand 1980 Surface glycoproteins: evidence that they may function as the race specific phytoalexin elicitors of Phytophthora megasperma f. sp. glycinea. Physiol Plant Pathol 17: 175-192.

Keen NT, LJ Littlefield 1979 The possible association of phytoalexins with resistance gene expression in flax to Melampsora lini. Physiol Plant Pathol 14: 265-280.

Kiraly Z, B Barna, T Ersek 1972 Hypersensitivity as a consequence, not the cause, of plant resistance to infection. Nature 239: 456-458.

Kurantz MJ, RM Zacharius 1981 Hypersensitive response in potato tuber: elicitation by combination of non-eliciting components from Phytophthora infestans. Physiol Plant Pathol 18: 67-77.

Laemmli UK 1970 Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227: 680-685.

Laskey RA, AD Mills 1975 Quantitative film detection of ^{3}H and ^{14}C in polyacrylamide gels by fluorography. Eur J Biochem 56: 335-341.

Leaver CJ 1979 Ribosomal RNA of plants. In Nucleic Acids in Plants, Eds. Hall and Davies V.1: pp.193-216. (CRC Press Inc.).

Link G, B-J Benecke 1980 Small RNA species in maize tissue. Planta 150: 406-411.

Littlefield LJ 1969 Flax rust resistance induced by prior inoculation with an avirulent race of Melampsora lini. Phytopathol 59: 1323-1328.

Littlefield LJ 1973 Histological evidence for diverse mechanisms of resistance to flax rust, Melampsora lini (Ehrenb.) Lev. Physiol Plant Pathol 3: 241-247.

Littlefield LJ, SJ Aronson 1969 Histological studies of Melampsora lini resistance in flax. Can J Bot 47: 1713-1717.

Lowry OH, NJ Rosebrough, AL Farr, RJ Randall 1951 Protein measurement with the Folin phenol reagent. J Biol Chem 193: 265-275.

Marcus A 1981 Proteins and nucleic acids. The Biochemistry of plants, a comprehensive treatise. V.6. Eds. Stumpf and Conn. (Springer Verlag).

Mayama S, T Tani, Y Matsumura, T Ueno, H Fukami 1981 The production of phytoalexins by oat in response to crown rust, Puccinia coronata f. sp. avenae. Physiol Plant Pathol 19: 217-226.

Miller BL, RC Huffaker 1982 Hydrolysis of ribulose-1,5-bisphosphate carboxylase by endopeptidases from senescing barley leaves. Plant Physiol 69: 58-62.

Montalbini P, BB Buchanan, SW Hutcheson 1981 Effect of rust infection on rates of photochemical polyphenol oxidation and latent polyphenol oxidase activity of Vicia faba chloroplast membranes. Physiol Plant Pathol 18: 51-57.

O'Farrell PH 1975 High resolution two-dimensional electrophoresis of proteins. J Biol Chem 250: 4007-4021.

O'Farrell PZ, HM Goodman, PH O'Farrell 1977 High resolution two-dimensional electrophoresis of basic as well as acidic proteins. Cell 12: 1133-1142.

Person CO 1959 Gene-for-gene relationships in host:parasite systems. Can J Bot 37: 1101-1130.

Person CO, GME Mayo 1974 Genetic limitations on models of specific interactions between a host and its parasite. Can J Bot 52: 1339-1347.

Prusky D, A Dinoor, B Jacoby 1980 The sequence of death of haustoria and host cells during the hypersensitive reaction of oat to crown rust. Physiol Plant Pathol 17: 33-40.

Pure GA, AK Chakravorty, KJ Scott 1979 Cell-free translation of polysomal messenger RNA isolated from healthy and rust-infected wheat leaves. Physiol Plant Pathol 15: 201-209.

Pure GA, AK Chakravorty, KJ Scott 1980 Changes in wheat leaf polysomal messenger RNA populations during the early stages of rust infection. Effects of chloramphenicol and lincomycin on cell-free translation by polysomes from healthy and infected leaves. Plant Physiol 66: 520-524.

Quick WA, M Shaw 1964 The physiology of host-parasite relations. XIV. The effect of rust infection on the nucleic acid content of wheat leaves. Can J Bot 42: 1531-1540.

Roberts LM, JM Lord 1981 Synthesis and posttranslational segregation of glyoxysomal isocitrate lyase from castor bean endosperm. Eur J Biochem 119: 43-49.

Robinson RA 1976 Plant pathosystems. Advanced series in agricultural sciences V.3. (Springer-Verlag)

Rohringer R, DJ Samborski, CO Person 1961 Ribonuclease activity in rusted wheat leaves. Can J Bot 39: 775-784.

Sachse B, G Wolf, WH Fuchs 1971 Nubleinsaure abbauenda Enzyme in Blattern von Triticum aestivum nach Infektion mit Puccinia graminis tritici. Acta Phytopathol Acad Sci Hung 6: 39-49.

Sadler R, M Shaw 1979a Studies on glutamate dehydrogenase from rust infected flax cotyledons. Z Pflanzenphysiol 91: 211-224.

Sadler R, M Shaw 1979b Pathways of nitrogen assimilation in rust infected flax cotyledons. Z Pflanzenphysiol 93: 105-115.

Scott KT 1972 Obligate parasitism by phytopathogenic fungi. Biol Rev 47: 537-572.

Scott KJ 1976 Growth of biotrophic parasites in axenic culture; in Encyclopedia of Plant Physiology, New Series V.4. Physiological Plant Pathology. Eds. Heitefuss R, PH Williams (Springer Verlag).

Scrubb LA, AK Chakravorty, M Shaw 1972 Changes in the ribonuclease activity of flax cotyledons following inoculation with flax rust. Plant Physiol 50: 73-79.

Sequeira L 1978 Lectins and their role in host-pathogen specificity. Ann Rev Phytopathol 16: 453-481.

Shaw M 1963 The physiology and host-parasite relations of the rusts. Ann Rev Phytopathol 1: 259-283.

Shaw M 1967 Cell biological aspects of host-parasite relations of obligate fungal parasites. Can J Bot 45: 1205-1220.

Sidhu GS 1975 Gene-for-gene relationships in plant parasitic systems. Sci Prog Oxf 62: 467-485.

Simpson RS, AK Chakravorty, KJ Scott 1980 Messenger and ribosomal RNA hydrolysis by ribonucleases I. The action of two barley leaf ribonucleases on the messenger and ribosomal RNA of isolated polysomes. Plant Cell Physiol 21: 413-423.

Sphor G, M-E Mirault, T Imaizumi, K Scherrer 1976 Molecular-weight determination of animal-cell RNA by electrophoresis in formamide under fully denaturing conditions on exponential polyacrylamide gels. Eur J Biochem 62: 313-332.

Steinback KE 1981 Proteins of the chloroplast. In The Biochemistry of Plants, a comprehensive treatise. Eds. Stumpf and Conn. pp. 303-319.

Sutton BCS, M Shaw 1982 Changes in two ribonuclease isozymes during rust infection of flax cotyledons. Plant Physiol 69: 205-209.

Tang WJ A Maretzki 1970 Purification and properties of leaf ribonuclease from sugar cane. Biochim Biophys Acta 212: 300-307.

Tani T, H Yamamoto 1978 Nucleic acid and protein synthesis in association with the resistance of oat leaves to crown rust. Physiol Plant Pathol 12: 113-121.

Tani T, M Yoshikawa, M Naito 1971 Changes in ^{32}P-ribonucleic acids in oat leaves associated with susceptible and resistant reactions to Puccinia coronata. Ann Phytopathol Soc Japan 37: 43-51.

Tani T, M Yoshikawa, N Naio 1973 Template activity of ribonucleic acid extracted from oat leaves infected by Puccinia coronata. Ann Phytopath Soc Japan 39: 7-13.

Tani T, M Yoshikawa, M Naito 1975 Selective enhancement of ribosomal ribonucleic acid synthesis of crown rust-infected oat leaves by stem excision. Physiol Plant Pathol 5: 193-199.

Taylor JM 1979 The isolation of eukaryotic messenger RNA. Ann Rev Biochem 48: 681-717.

Vanderplank JE 1978 Genetic and molecular basis of plant pathogenesis. Advanced series in agricultural sciences V.6. (Springer Verlag).

Van Loon LS 1975 Polynucleotide-acrylamide gel electrophoresis of soluble nucleases from tobacco leaves. FEBS Lett 51: 266-269.

Von Broembsen SL, LA Hadwiger 1972 Characterization of disease resistance responses in certain gene-for-gene interactions between flax and Melampsora lini. Physiol Plant Pathol 2: 207-215.

Wade M, P Albersheim 1979 Race-specific molecules that protect soybeans from Phytophthora megasperma var. sojae. Proc Nat Acad Sci USA 76: 4433-4437.

Wildner GF 1981 Ribulose-1,5-bisphosphate carboxylase-oxygenase: Aspects and prospects. Physiol Plant 52: 385-389.

Wilson CM 1971 Plant nucleases III. Polyacrylamide electrophoresis of corn ribonuclease isoenzymes. Plant Physiol 48: 64-68.

Wilson CM 1975 Plant nucleases. Ann Rev Plant Physiol 26: 187-208.

Yoshikawa M, H Masago, WT Keen 1977 Activated synthesis of poly(A)-containing messenger RNA in soybean hypocotyls inoculated with Phytophthora megasperma var. sojae. Physiol Plant Pathol 10: 125-138.

Yamamoto H, T Tani, N Naito 1975 Changes in protein contents of oat leaves during the resistant reaction against Puccinia coronata avenae. Phytopath Z 82: 138-145.

APPENDIX. Methodological Details and Precautions

RIBONUCLEASE

Ribonuclease extraction

This study was designed to explore the mechanisms underlying the qualitative changes in RNase which are observed during rust infection. As this study and those of other workers suggest that a multiplicity of RNase isozymes may be present particular attention is paid to the yield of total enzyme units at each stage of purification. Usual precautions, such as low temperature and the avoidance of aerating enzyme solutions, have been taken to maintain high yield. In addition, the isozymic composition and properties of various fractions have been examined throughout purification. In this way the relationship between individual isozymes and the total soluble extract can be assessed.

ENZYME ASSAYS

Ribonuclease assay

The reaction mixture (final volume 1 ml) contained 50 umoles of sodium acetate buffer pH 5.5, 0.5 mg of yeast RNA (BDH, Poole England) which had been reprecipitated with 3 vol. 95% ethanol, washed and dialysed exhaustively against EDTA and subsequently against distilled water. Varying amounts of enzyme solution were added and samples were incubated for 30 min. at 37°C. Precipitating reagent - 2 ml (1 MHCl, 0.5% $LaCl_3$ in 76% ethanol) was added and the tubes allowed to stand at 4°C for 30 min. Reaction blanks were identical except they were not incubated; this gave the most realistic blank as it accounted for increased RNA precipitation which results from the inclusion of protein. After removal of precipitates by centrifugation at 4°C the clear supernatant was read at 260 nm in a SP800 spectrophotometer; this enabled the spectral identity of the product to be checked.

Deoxyribonuclease (DNase) assay

The incubation mixture (final volume, 2.0 ml) contained: 50 µmoles sodium acetate buffer pH 5.8; 10 µmoles $MgCl_2$; 0.5 mg native or heat denatured DNA (calf thymus DNA, Calbiochem). Denaturation was carried out by heating to 100 °C for 10 min and cooling rapidly on ice. The incubation and measurement of DNase activity were carried out as described for RNase.

Phosphodiesterase assay

The reaction mixture (final volume 2.5 ml) contained: 30 μmoles K_2HPO_4; 1.75 mg calcium bis (p-nitrophenyl) phosphate with equivalent amounts of enzyme solution to those used in RNase and DNase assays. After incubation for 1 hr at 37°C, the tubes were read at 410 nm against a reagent blank.

Michaelis constant

The constant K_m was calculated from double reciprocal plots of the form:

$$\frac{1}{\text{Reaction velocity}} \text{ against } \frac{1}{\text{Substrate concentration}},$$

1/v vs 1/[S] (see Figure). Assays for RNase were set up as described previously but with different amounts of RNA varying from 25 μg to 150 μg. Before precipitation additional RNA was added to give a final concentration of 200 μg in 1.2 ml. This procedure was essential to give consistent precipitation.

Substrate preference

Substrate preference was determined by substituting tritiated homoribopolymers of polycytidylic, polyadenylic and polycytidylic acid (20 μCi/mmole P) (poly U, poly A, poly C, Miles Laboratories Inc., Elkhart, Indiana) for RNA under standard assay conditions. Assays were carried out under conditions of limiting substrate concentration so that 0.125, 0.250 and 0.500 μCi produced linearly increasing reaction velocities. After incubation 0.5 mg of cold RNA were added to aid precipitation. After removal of the precipitate by centrifugation 1 ml of the supernatant was added to 10 ml of toluene scintillation fluid (Aquasol, New England Nuclear) and counted for radioactivity in a liquid scintillation system.

OTHER PROPERTIES OF RIBONUCLEASE

pH Profile

RNase assays were run at various pH values. The following buffers were used: pH 3.5-4.5 Na_2HPO_4 - citric acid 40 mM; pH 5.0-6.0 Na acetate 40 mM; pH 6.5-8.0 KH_2PO_4 - NaOH 40 mM.

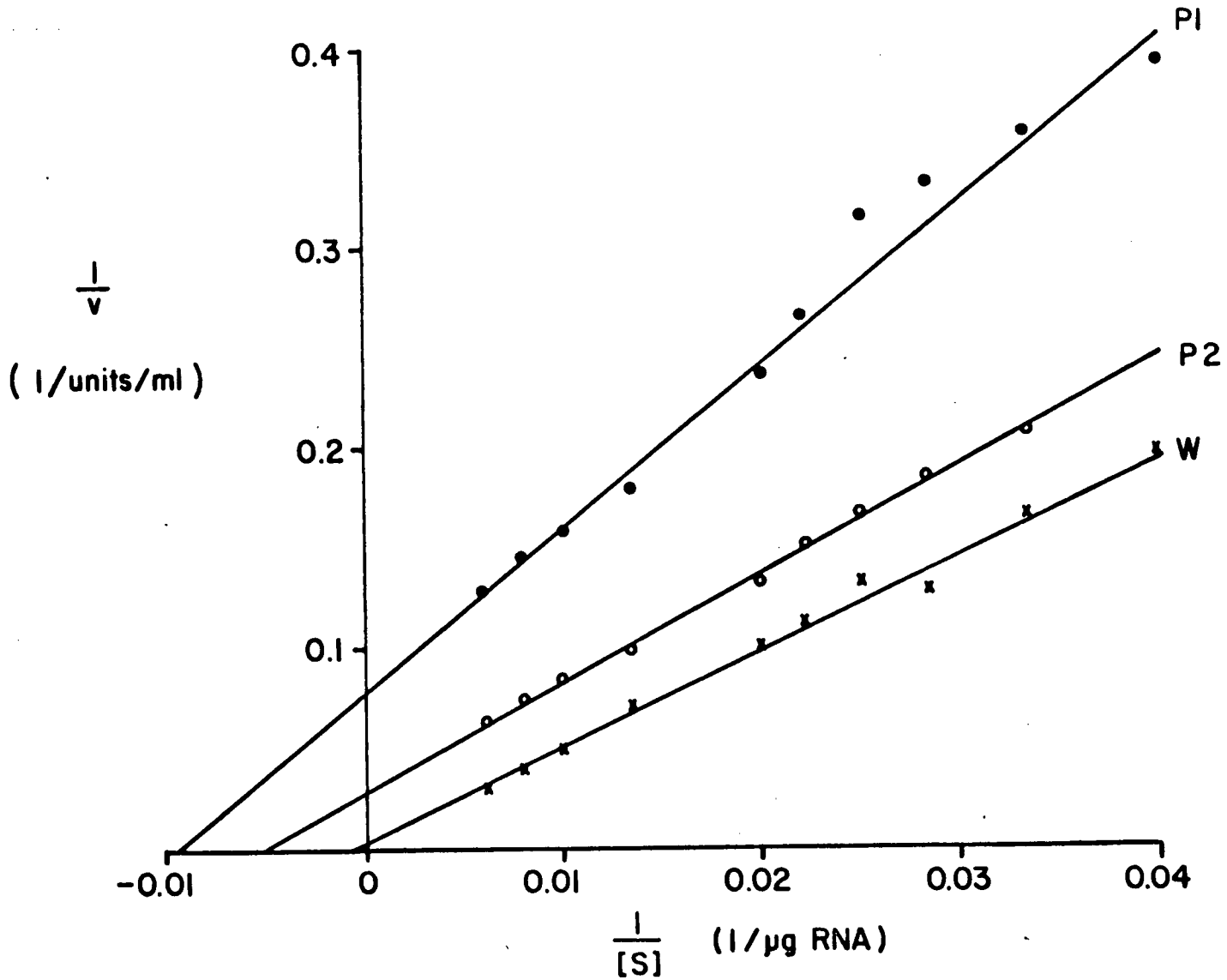


Figure: Lineweaver-Burk plots for RNases P1 and P2, and partially purified RNase from wounded cotyledons (W).

Molecular weight determination

Molecular weight was determined by Sephadex G100 gel filtration on a 2.5 x 40 cm column. The void volume (Vo) was assessed with Blue Dextran and the included volume (Vi) was assessed with 2,4-dinitrophenol: Eluted volumes (Ve) were assessed for the following proteins of known molecular weight: Pancreatic RNase A; 13,700, Myoglobin, 17,800; Chymotoypsinogen A, 25,000; Ovalbumin, 43,000; Bovine Serum Albumin, 67,000. K_{av} values were calculated from the equation: $K_{av} = \frac{Ve - Vo}{Vi - Vo}$ and a calibration curve K_{av} against log molecular weight, constructed.

Column isoelectric focusing

Preparative column electrofocusing was performed in 110 ml (LKB 810) Ampholine column.

The column contained:

1) Anode solution at bottom:

Phosphoric acid	0.2 ml
H_2O	14 ml
Sucrose	12 g

2) Separation gradient:

0 - 50% sucrose linear gradient	110 ml
Ampholine pH 2.5-6.0	1 %
Enzyme preparation	1 mg protein

3) Cathode solution at top:

Ethanolamine	0.8 ml
H_2O	40 ml

Focusing was carried out for 36 hr at 300 v. During this time the current decreased and reached a constant value indicating that the ampholytes were focused. The column was maintained at 8°C throughout the run by cooling with tap water. The power did not exceed 2.4 Watts and thus convection was minimized. The anode was placed at the bottom of the column in order to reduce interference from proteins precipitating through the gradient as it was considered that more precipitation would occur at the acidic end of the pH gradient.

RNA SYNTHESIS

RNA extraction

The RNA extraction method used in this research involves an initial phenol treatment of the aqueous extract. Since it has been suggested polyadenylated RNA is selectively absorbed to the phenol and interphase layers during extraction in the absence of chloroform (Taylor 1979), the effect of chloroform addition has been examined. A sample of flax seedlings was fed with [^{32}P] Na phosphate. Half of this sample was extracted with phenol in the organic phase and half was extracted with phenol:chloroform, 1:1. The percentage of radioactivity which bound to poly U Sepharose within the purified RNA derived from each extract was assessed. The percentages were 6.6% and 5.3% for RNA derived from phenol or phenol/chloroform extracts respectively. Thus it appears that the inclusion of chloroform does not enhance the recovery of polyadenylated RNA.