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BIOCHEMICAL ASPECTS OF PLANT VIRUS INFECTION OF CUCUMBERS

A STUDY OF THE VIRAL-INDUCED PROTEIN AND RNA SPECIES, AND THE RNA-DEPENDENT RNA POLYMERASE

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of

Master of Science

by

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from

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STATEMENT

This thesis contains no material previously submitted for any other degree or diploma in any other University. To the best of my knowledge and belief, this thesis does not contain any material previously published or written by another person, except where due reference is made in the text.

K.W.C. PEDEN

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ABBREVIATIONS

TEMED	N,N,N,N tetramethylethylenediamine
SDS	Sodium Dodecyl Sulphate
NDS	Naphthalene-2,7-disulphonic acid
DOC	Deoxycholate
BMV	Bromegrass Mosaic Virus
BBMV	Broadbean Mottle Virus
CMV	Cucumber Mosaic Virus
HRG	Holmes Ribgrass Virus
PVX	Potato Virus X
STNV	Satellite Tobacco Necrosis Virus
TMV	Tobacco Mosaic Virus
TNV	Tobacco Necrosis Virus
TRSV	Tobacco Ringspot Virus
TYMV	Turnip Yellow Mosaic Virus

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SUMMARY

An investigation has been carried out on the biochemical aspects of the infection of cucumbers by two plant viruses - Cucumber Mosaic Virus (CMV) and Tobacco Ringspot Virus (TRSV).

1. Attempts were made to characterise the TRSV-induced RNA species (replicative form, replicative intermediate and viral RNA genome); RNA was extracted from cucumber cotyledons using the phenol method and analysed on 2.4% polyacrylamide gels. No differences in gel patterns between healthy and infected plants could be found in either single labelling studies (plants labelled through the roots with ^{32}P-orthophosphate) or with double-labelling studies (plants labelled through the roots with either ^{32}P-orthophosphate or ^{3}H-uridine); actinomycin D, although completely abolishing ribosomal RNA synthesis, did not show up any differences between healthy and TRSV-infected cucumber RNA extracts.

2. Attempts were also made to characterise the TRSV-induced protein species. As radioactive amino acids could not be incorporated into cucumber proteins by absorption through the roots, ^{35}S-sulphate was used as the label. No differences could be found between healthy and infected protein extracts analysed by polyacrylamide gel electrophoresis. Further attempts to incorporate radioactive amino acids using tissue slices

and other methods were also unsuccessful. An alternative approach of differentially labelling healthy and infected plant proteins after extraction by reduction and carboxymethylation with either ^{3}H or ^{14}C-iodoacetic acid produced variable and unreliable radioactive protein profiles on polyacrylamide gels.

3. A comparison of the properties was made between the CMV-induced and the TRSV-induced RNA polymerases, both soluble and particulate enzymes; these enzymes were undetectable in healthy plants. Both the soluble and particulate RNA polymerases from TRSV-infected cucumbers were detectable two days after infection and declined rapidly after four days, whereas the CMV-induced enzymes appear after about five days, reach a plateau level at 10 days and remained at this level for several more days. The mol. wt. of the soluble CMV-induced RNA polymerase was calculated by sucrose gradient centrifugation to be 123,000 daltons while the soluble TRSV-induced enzyme sedimented over a wide range of 120-180,000 daltons. Solubilising the particulate CMV-induced RNA polymerase could be accomplished by incubating with 50-100mM $MgSO_4$ or by freezing and thawing, but these methods did not release the particulate TRSV-induced enzyme. All other properties tested were similar in both cases. This provided circumstantial evidence for the differences being

due to viral-coded functions in the RNA polymerase molecules.

4. Several purification methods have been tried in an attempt to purify the CMV-induced RNA polymerase. Those methods that gave reasonable recoveries were protamine sulphate precipitation, phosphocellulose chromatography (stepwise elution with either KCl or $(NH_4)_2SO_4$) and poly C-sepharose chromatography (stepwise elution with $MgSO_4$). A possible method of purification is outlined.

INTRODUCTION

INTRODUCTION

The overall aim of this project was to carry out an investigation of the replication of CMV in cucumber cotyledons. Work presented in the first part of this thesis has been carried out in an attempt to characterise the plant virus-induced (CMV and TRSV) RNA and protein species present in infected cucumber cotyledons. The second part of the thesis is concerned with the viral-induced RNA-dependent RNA polymerase, the enzyme presumably responsible for the replication of virus RNA; some of its properties and its partial purification are presented. This Introduction is first concerned with the work done so far in various plant virus systems on the viral coded RNA and protein functions, after which a summary of the properties of the various viral-induced RNA-dependent RNA polymerases isolated so far is given.

SECTION 1. ISOLATION OF PLANT VIRAL-INDUCED RNA

Two forms of RNA with a highly ordered secondary structure can be extracted from cells infected with RNA bacteriophages or animal RNA viruses. These are firstly a double-stranded RNA (the replicative form, RF) which consists of a full length viral RNA strand (plus strand) and a full length complementary strand (minus strand) (Weissmann *et al.*, 1964; Weissmann and Ochoa, 1967; Francke and Hofschneider, 1966; Baltimore, 1966; Bishop and Koch

1967) and secondly, a multistranded RNA which has been called the replicative intermediate (RI). This RNA is heterogeneous and consists of a double-stranded backbone of constant size with attached single-stranded tails of different lengths as shown diagrammatically in Figure 1 (Erikson et al., 1964; Baltimore and Girard, 1966; Baltimore, 1968; for review see Stavis and August, 1970). Experiments with the Qβ RNA polymerase (Pace et al., 1968) and crude poliovirus RNA polymerase (Girard, 1969) have shown that the RI is the immediate precursor to viral RNA for these two viruses.

In the plant virus system, several reports on the properties of double-stranded plant viral RNA isolated with the aid of ribonuclease, have appeared (Burdon et al., 1964; Bockstahler, 1967; Wolstenholme and Bockstahler, 1967; Shipp and Haselkorn, 1964; Ralph et al., 1965). Wolstenholme and Bockstahler (1967) studied by electron microscopy double-stranded RNA induced by TYMV and TMV and observed branched molecules. On extraction in the presence of ribonuclease they found that the fequency of branching was dependent on the concentration of RNA in their preparations, a finding to be expected since their extraction was done with a fixed concentration of ribonuclease. The authors concluded that they were extracting the RI, the single strands of which were being degraded by ribonuclease. Pinck et al. (1968) isolated from alfalfa mosaic virus infected plants, without the use of

ribonuclease, an RNA which seemed to be a mixture of both RF and RI. According to Ralph et al. (1965), short term labelling of TYMV-infected chinese cabbage leaves with ^{32}P-inorganic phosphate resulted in asymmetric labelling of the RF, while long term labelling gave equal radioactivity in both strands, a finding compatible with an intermediary function of this RNA. The asymmetrical synthesis of segments of viral plus strands in a cell free extract from TYMV-infected chinese cabbage leaves was demonstrated by Bové et al. (1968). These plus strands were associated with minus strands in a double-stranded form.

Recently several groups of workers have reported the isolation of the RF and RI from TMV-infected tobaccos and the RF from STNV and TNV-infected tobaccos. A discussion of their work follows.

(a) Nilsson-Tillgren Method.

One of the difficulties found in plant virus research is that synchronous and homogeneous infection of cells could not be achieved. However, Nilsson-Tillgren et al. (1969) overcame this problem to a certain extent by using a method developed by Zech (1952) in which only those leaves which were almost fully expanded were used for inoculation with TMV. When veinclearing symptoms occurred, electron microscipal analysis showed that these leaves contained a majority of cells at the same stage of infection.

Nilsson-Tillgren (1969) labelled leaves through their petioles with ^{14}C-uridine for 1.5 hr at various times after TMV infection, extracted the nucleic acids with phenol and chromatographed them on MAK columns. Fractions were collected and the optical density at 260 mμ, the acid-insoluble radioactivity and the ribonuclease-resistant radioactivity determined. It was found that at the outbreak of visible systemic symptoms (veinclearing) there was preferential incorporation of the label into an RNA identified as TMV RNA. Also at this time, a small peak appeared between the DNA and ribosomal RNA peaks; this peak was found to be ribonuclease-resistant and due also to its elution position, it was concluded that it was double-stranded RNA. At later stages of infection an increased synthesis of this RNA was found. However, when shorter labelling periods were used (2-5 min) proportionately more label was found in the double-stranded RNA than in the TMV RNA, but this decreased as the labelling time was increased from 3.5 min to 30 min. This was consistent with the idea that the double-stranded RNA was a precursor of TMV RNA. Sucrose gradient centrifugation put the size of this RNA at slightly less than 16S.

Nilsson-Tillgren (1969) concluded that what he had isolated was in fact the RF of TMV replication. He then further characterised this double-stranded RNA (Nilsson-Tillgren, 1970). By labelling TMV-infected plants with ^{3}H-uridine for 1 hr at the period of maximum production of viruses, extracting the nucleic acids and centrifuging

them through a sucrose gradient, he was able to show that the ribonuclease-resistant peak at approximately 16S had a heterogeneous shoulder sedimenting on the heavy side. This shoulder was partially ribonuclease-resistant and was probably the RI. Separation of the RF and RI was possible due to the fact that the RF, together with tRNA and residual DNA, were soluble in 1M NaCl whereas the RI, together with single-stranded viral RNA and ribosomal RNA were precipitated (Bishop and Koch, 1967; 1969). Purification by cellulose chromatography (Franklin, 1966; Bishop and Koch, 1969) resulted in an increased specific activity in those fractions resistant to ribonuclease digestion. The RF was found to be over 90% resistant to ribonuclease when tested in SSC, while comparable preparations of RI showed only 42% resistance under the same conditions. This difference between the RF and RI was compatible with the idea that RI molecules consist of both single and double-stranded regions. When, however, both were treated with ribonuclease under conditions which changed the secondary structure of the RNA molecules (0.1 x SSC or after thermal denaturation), both were digested. This differential behaviour of the RF and RI towards ribonuclease suggested that there are two kinds of double-stranded RNA molecules present in TMV-infected leaves, one purely double-stranded (RF) and the other partially double-stranded but with single-stranded branches (RI).

Sucrose gradient centrifugation of both RF and

RI showed that the RF was homogeneous and sedimented between 15 and 16S. The RI, however, sedimented heterogeneously between 15 and approximately 30S. If before centrifugation the RI was first digested with ribonuclease, then the sedimentation profile resembled that of the RF except that it sedimented slightly more slowly than did the RF. From this Nilsson-Tillgren (1970) concluded that the RI had a double-stranded backbone which was similar to the RF. Melting of the RI released single strands of various sizes, some of which sedimented with TMV RNA.

From these results Nilsson-Tillgren (1970) concluded that he had in fact isolated the RF and the RI of TMV infection and that both were involved in the native replication of TMV in host cells in a similar manner to the RNA bacteriophages and poliovirus (Fenwick et al., 1964; Erikson et al., 1966; Franklin, 1966; Baltimore and Girard, 1966; Baltimore, 1968; Bishop and Koch, 1969).

(b) Jackson, Mitchell, Siegel Method.

Jackson et al. (1971) infected expanded and expanding tobacco leaves with TMV in phosphate buffer containing Celite and 6-7 days after inoculation, extracted the nucleic acids by the phenol method, purified the double-stranded RNA by cellulose chromatography and characterised it by isopycnic centrifugation in Cs_2SO_4 with poly G (ρ = 1.712 g/cc) as marker. Ultraviolet absorption photographs revealed a peak at a density of 1.615 g/cc with a shoulder of greater density (ρ = 1.630 g/cc). If before

sedimentation the double-stranded RNA preparation was treated with ribonuclease, then this shoulder disappeared leaving a single peak of density 1.613 g/cc. These results suggested that two species of double-stranded RNA were present; the ρ = 1.615 and the ρ = 1.630 g/cc components corresponded respectively to the RF and the RI found in other RNA virus-infected hosts (Ralph, 1969, for review).

By using the differential precipitation method mentioned above to separate the RF from the RI, they showed that the RI had a density of 1.630 g/cc which changed to 1.613 g/cc on treatment with ribonuclease. These results further suggested that a partially single-stranded, partially double-stranded RNA (RI) was present in TMV-infected tissue and that this RNA was converted to a structure similar to the RF by ribonuclease digestion of the single strands.

To determine whether the double-stranded RNA extracted from TMV-infected leaves consisted of TMV RNA and its complementary strand, heat denatured double-stranded RNA was incubated in the presence of ^{3}H-TMV RNA under annealing conditions, followed by ribonuclease digestion. Material resistant to digestion was collected on filters and counted. There was a significant number of counts above the controls (where no double-stranded RNA was added, or double-stranded RNA was annealed to ^{3}H-rRNA) indicating that RNA complementary to TMV RNA existed in the double-stranded RNA.

By heat-denaturing double-stranded RNA and electrophoresing in 1.6% acrylamide and 0.5% agarose, polyacrylamide gels, Jackson *et al*. (1971) showed that this double-stranded RNA was composed, at least in part, of single strands of about the same size as TMV RNA. Taken together, these results indicated that Jackson *et. al*. had in fact isolated the RF and the RI of the TMV replication cycle. Further evidence came from Bradley and Zaitlin (1971) who, using a TMV-induced RNA-dependent RNA polymerase, synthesised *in vitro* double-stranded labelled material of mol. wt. 4.10^6 daltons which agreed with that quoted by Jackson *et al*. (1971) for their RF. Bradley and Zaitlin (1971) also found a ribonuclease-sensitive higher mol. wt. product of about 5.10^6 daltons and this was considered to be the RI.

(c) Double-stranded RNA from TNV and STNV Doubly-infected Tobacco Leaves.

Klein and Reichmann (1970), using the phenol method described by Bockstahler (1967), extracted two double-stranded RNA species from tobacco leaves, which had been infected with both TNV and STNV. The lower mol. wt. single-stranded RNA species were separated from the double-stranded species by chromatography on Sepharose 4B. This high mol. wt. RNA was digested with KOH and the base ratios determined. It was found that A = U and G = C as expected in double-stranded RNA. Electrophoresis on 2.4%

polyacrylamide gels revealed two species of double-stranded RNA - one corresponding to the RF of TNV and the other to the RF of STNV. The identity of these species was determined by melting both double-stranded RNA species, quick-cooling them and re-electrophoresing. A shift in their mobility to that of single-stranded TNV RNA and STNV RNA occurred.

Only preliminary annealing studies have been attempted as yet but they indicate that approximately 10% of the STNV genome was incorporated into ribonuclease-resistant material. Thus Klein and Reichmann (1970) appear to have isolated the RF of both TNV and STNV.

From the foregoing it appears that the replication of single-stranded RNA plant viruses TMV, TYMV, TNV and STNV is similar to the RNA bacteriophage systems of Q_β and MS2 in that both commence with the viral (plus) strand on which is synthesised a complementary minus strand to give the RF. From the minus strand of this RF, in the semi-conservative model, is synthesised many plus strands giving the RI; this is represented in Figure 1. For reviews of this process see Ralph (1969) and Stavis and August (1970).

SECTION 2. ISOLATION OF PLANT VIRAL-INDUCED PROTEINS.

Although there have been reports of the isolation of bacteriophage-induced proteins (e.g., Nathans *et al.*, 1966; Burgess and Denhardt, 1969; Garwes *et al.*, 1969) and animal viral-induced proteins (e.g., Summers *et al.*, 1965; Maizel, 1966; Jacobson and Baltimore, 1968; Maizel and Summers, 1968; Shapiro and Maizel, 1969; Jacobson *et al.*, 1970) there have been few corresponding reports for plant virus-induced proteins. Two recent papers are both concerned with TMV infection of tobacco plants.

(a) Identification of Non-labelled TMV-induced Proteins.

Van Loon and van Kammen (1970) extracted the soluble proteins from two different strains of tobacco plants after infection with TMV and subjected them to electrophoresis on either 5%, 7.5% or 10% polyacrylamide gels. Gels were stained and the protein bands recorded by densitometry. The two varieties of tobacco plants used, Samsun and Samsun NN, showed different types of symptoms after infection with TMV. Samsun reacted with systemic mosaic symptoms on the young developing leaves 10-14 days after inoculation, while in Samsun NN, local lesions appeared on the inoculated leaves within a few days. Also, only in Samsun NN plants was glucose catabolism via the pentose shunt pathway activated. These differences in reaction type between the two varieties is based on the presence in the Samsun NN

variety of the dominant gene N derived from Nicotiana glutinosa L. (Holmes, 1938). This gene determines the hypersensitive reaction upon TMV infection. There were no differences in the soluble leaf proteins from uninfected Samsun and Samsun NN as determined by electrophoresis of purified extracts on polyacrylamide gels (van Loon and van Kammen, 1968). In Samsun NN, the N gene product, whose presence could have explained the differences in response to TMV infection, was not identified. In Samsun NN plants four new bands appeared one week after infection with TMV. These bands were not related to the TMV coat protein and were not new isoenzymes of peroxidase, polyphenoloxidase, acid phosphatase, glucose-6-phosphate dehydrogenase or 6-phosphogluconate dehydrogenase, enzyme activities found to increase at different times after inoculation (Martin, 1958; Solymosy and Farkas, 1963; van Kammen and Brouwer, 1964; Loebenstein and Linsey, 1966; Suseno and Hampton, 1966). The bands first appeared at the onset of necrosis and increased in intensity with time. In Samsun plants only one new band was present four weeks after infection and this was characterised as the TMV coat protein by co-electrophoresis and serology.

Since the four bands appearing in Samsun NN plants after TMV infection were not present in TMV-infected Samsun plants in which the virus multiplies abundantly, and also that these same bands were found in uninoculated parts of the plant, the authors concluded that these proteins were not virus-specific functions,

but host-specific. Further evidence for this conclusion was obtained from actinomycin D studies. This antibiotic, which inhibits DNA-dependent RNA synthesis but allows TMV multiplication (Sanger and Knight, 1963), was found to reduce the quantity of the four bands, although never completely eliminating them, suggesting that it was the host protein synthesising system that was being used for their synthesis. Thus it seemed that van Loon and van Kammen (1970) had only been able to characterise one viral-specific function induced upon TMV infection. Identifying proteins solely by staining is an insensitive method of characterising proteins which may only be present in small quantities. Thus it would be expected that they could only successfully identify the coat protein which is presumably present in larger amounts than other viral-coded functions.

(b) Identification of Labelled TMV-induced Proteins.

Zaitlin and Hariharasubramanian (1970) succeeded in labelling healthy tobacco proteins with ^{14}C-leu and TMV-infected tobacco proteins with ^{3}H-leu. This was done by cutting leaves into thin slices, vacuum infiltrating them in phosphate buffer with actinomycin D (60 µg/ml) followed by incubation for $3^{1}/2$ hr at 28° in the dark in a water bath shaker. ^{3}H-leu (10µCi) was then added to the four-day infected leaves, ^{14}C-leu (10µCi) to the healthy leaves and the flasks incubated for a further $1^{1}/2$

hr. Proteins from the 20,000 g pellet, the 'mitochondrial' fraction were extracted, equal quantities of ^{14}C-labelled healthy extracts and ^{3}H-labelled infected extracts were combined and co-electrophoresed on SDS-polyacrylamide gels. By plotting the $^{3}H/^{14}C$ ratio (infected to healthy) it was found that five polypeptide components differed consistently; one component was depressed in diseased tissues whereas four new components appeared. In an attempt to characterise these four new peaks, ^{14}C-labelled TMV coat protein was co-electrophoresed with ^{3}H-leu labelled TMV-infected plant extracts. The fastest moving component in ^{14}C-labelled virus is the coat protein monomer and this component migrated to the exact position of the fifth peak in TMV-infected extracts. This characterised peak 5 then as the coat protein monomer. However, with the type of analysis done by Zaitlin and Hariharasubramanian (1970), it was not possible for them to decide if the other three new peaks were due to a stimulation of the synthesis of proteins normally found in uninfected plants or from viral-coded *de novo* synthesis. The authors speculated that one might be the TMV replicase protein or a viral-coded subunit. Evidence existed that the crude 'mitochondrial' fraction used by Zaitlin and Hariharasubramanian was involved in viral RNA replication. Ralph and Wojcik (1969) found replicase activity and also double-stranded RNA in this extract, and also Jackson *et al*. (1971) have found the RF and RI of TMV replication in this fraction (see Section 1). At the moment, however, no further characterisation

of these peaks has been published.

SECTION 3. PLANT VIRAL-INDUCED RNA-DEPENDENT RNA POLYMERASES.

In a number of plant virus systems an RNA-dependent RNA polymerase activity has been reported; in plants infected with TMV (Kim and Wildman, 1962; Hudson *et al.*, 1963; Karasek and Schramm, 1962; Ralph and Wojcik, 1969; Brishammar, 1970; Sela, 1970; Bradley and Zaitlin, 1971); with BMV (Semal and Hamilton, 1968; Semal and Kummert, 1970, 1971); with CMV (Gilliland and Symons, 1968; May *et al.*, 1969, 1970; May and Symons, 1971); with BBMV (Semal, 1969, 1970); and with TYMV (Astier-Manifacier and Cornuet, 1965; Bové *et al.*, 1968). Of these most have been isolated from particulate fractions (Hudson et al., 1963; Bové *et al.*, 1968; May *et al.*, 1970; Ralph and Wojcik, 1969; Ralph *et al.*, 1971; Semal, 1970; Bradley and Zaitlin, 1971) but the CMV-induced RNA polymerase has been found also in soluble extracts (Gilliland and Symons, 1968; May *et al.*, 1969). This CMV-induced enzyme is unique in that actinomycin D is not required in the assay system to eliminate DNA-dependent RNA synthesis which has not been detected in cucumber preparations. Also the other systems appear to be insensitive to added RNA templates while the CMV-induced RNA polymerase copies added RNA (May and Symons, 1971). None of the plant virus-induced enzymes have been purified to anywhere near the extent of the Qβ replicase (Kondo *et al.*, 1970; Kamen, 1970).

(a) Properties of the RNA Replicase.

(i) Dependence on nucleoside triphosphates.

In the TMV, BMV and CMV systems, the incorporation of labelled nucleoside triphosphates into an acid-precipitable product by a cell-free system was almost completely dependent upon the presence of all four nucleoside triphosphates (Hudson *et al.*, 1962; Brishammer, 1970; Bradley and Zaitlin, 1971; Semal and Hamilton, 1968; Gilliland and Symons, 1968). To avoid the possibility that the incorporation was due to DNA-dependent RNA synthesis and not to the viral-induced RNA-dependent RNA synthesis, either deoxyribonuclease (ribonuclease-free) or actinomycin D was added to the reaction mixture. By including a triphosphate regenerating system also in the reaction mixture, the possibility of erroneous results due to an RNA phosphorylase was eliminated.

(ii) The nature of the products.

In most of the systems studied and mentioned above, the viral-induced RNA replicases synthesised some double-stranded RNA, a necessary result since the isolation of RF and RI RNA from certain viral-infected tissues has indicated that double-stranded RNA was indeed involved in the normal replication cycle of the virus (see Section 1). Thus if these RNA-dependent RNA polymerases are to be considered as the enzymes that replicate viral RNA *in vivo*, then they should synthesise double-stranded RNA *in vitro*.

Brishammer (1970) found that his TMV-induced RNA replicase synthesised some double-stranded RNA during the first 20 min of assay. Bradley and Zaitlin (1971) actually phenol extracted the product of their reaction and found that 65% of it was insensitive to pancreatic ribonuclease but completely disgested with KOH. Further to this, they extracted from TMV-infected tissue the TMV-RF according to the method of Jackson _et al._ (1971) and co-electrophoresed it with the product of the _in vitro_ RNA replicase reaction on 1.6% polyacrylamide-0.5% agarose gels. They were found to correspond exactly thus showing that the RNA replicase isolated by Bradley and Zaitlin (1971) from TMV-infected tobacco leaves can synthesise _in vitro_ the TMV-RF and thus could possibly be the enzyme responsible for TMV replication in tobaccos. It should be noted, however, that in all work done so far, no-one has shown that the viral-induced RNA replicase makes a complete viral RNA strand. Most likely what is occurring is that partially made strands existing in the crude extracts are completed.

The CMV-induced RNA replicase, extracted either from soluble extracts or particulate extracts of cucumbers, synthesised a product which was ribonuclease-resistant (May _et al._, 1969, 1970). Nearest neighbour analysis and a melting profile indicated further that this RNA was double-stranded. The product of the soluble RNA polymerase assayed with yeast RNA as template, had a sedimentation coefficient of 5S and when assayed with CMV RNA, 6S.

Semal and Kummert (1971) isolated an RNA replicase from BMV-infected barley leaves that on a short pulse of ^{3}H-UTP produced a product that was mostly resistant to ribonuclease digestion and sedimented in sucrose gradients at 14S. This was presumed to be double-stranded RNA. If a longer pulse was given, however, the label ended up in material which became increasingly ribonuclease-sensitive. This suggested that the first formed product of the reaction was a double-stranded RNA but this then was converted into single-stranded RNA. This chasing of the label from the double-stranded to the single-stranded form could only be accomplished in the presence of all four nucleoside triphosphates; controls in which only ^{3}H-UTP was present or without any nucleotides did not show this effect. However, no complete BMV genome could be found amongst the single-stranded RNA products and the authors suggested that this could have been due to the presence of contaminating nucleases in the reaction mixture. The double-stranded RNA product isolated did not have the properties of an RI as isolated from other systems (Plagemann and Swim, 1968; Ehrenfeld _et al._, 1970) or from TMV-infected tobaccos [Nilsson-Tillgren, 1970; Jackson _et al._, 1971), but this, the authors claim, could also be due to the nucleases. Despite this, however, since the BMV-induced RNA replicase firstly synthesised a double-stranded RNA product which was converted to single-stranded products suggested that this enzyme could be involved in the _in vivo_ replication

of BMV.

(iii) Template specificity of the RNA replicase

As mentioned above, most RNA replicases isolated did not copy added RNA templates, which could be due to the extracted enzyme still having the original viral template attached. Two RNA replicases have been found to copy added RNA but neither was completely specific for their RNA. The CMV-induced enzyme was found not to be specific for CMV RNA but copied a number of templates by complementary base pairing (CMV, TMV, TRSV, E. coli, poly C; May and Symons, 1971). Brishammer (1970) isolated a TMV-induced RNA replicase which showed some specificity towards TMV RNA as template. Taking the value with TMV RNA as template to be 100% activity, TYMV RNA gave 47% activity, HRG RNA gave 23% activity while yeast rRNA would not serve as template at all. Thus this partially purified TMV-induced RNA replicase showed some specificity towards TMV RNA, and this specificity may be enhanced by further purification of the enzyme as was found with the phage systems MS2 and Q β (Haruna et al., 1963; Haruna and Spiegelman, 1965).

Sela (1970) carried out an interesting experiment to show that an RNA replicase synthesised by a cell-free protein synthesising system using TMV RNA as the mRNA exhibited considerable specificity for TMV RNA. Sela extracted a cell-free protein system from tobacco leaves,

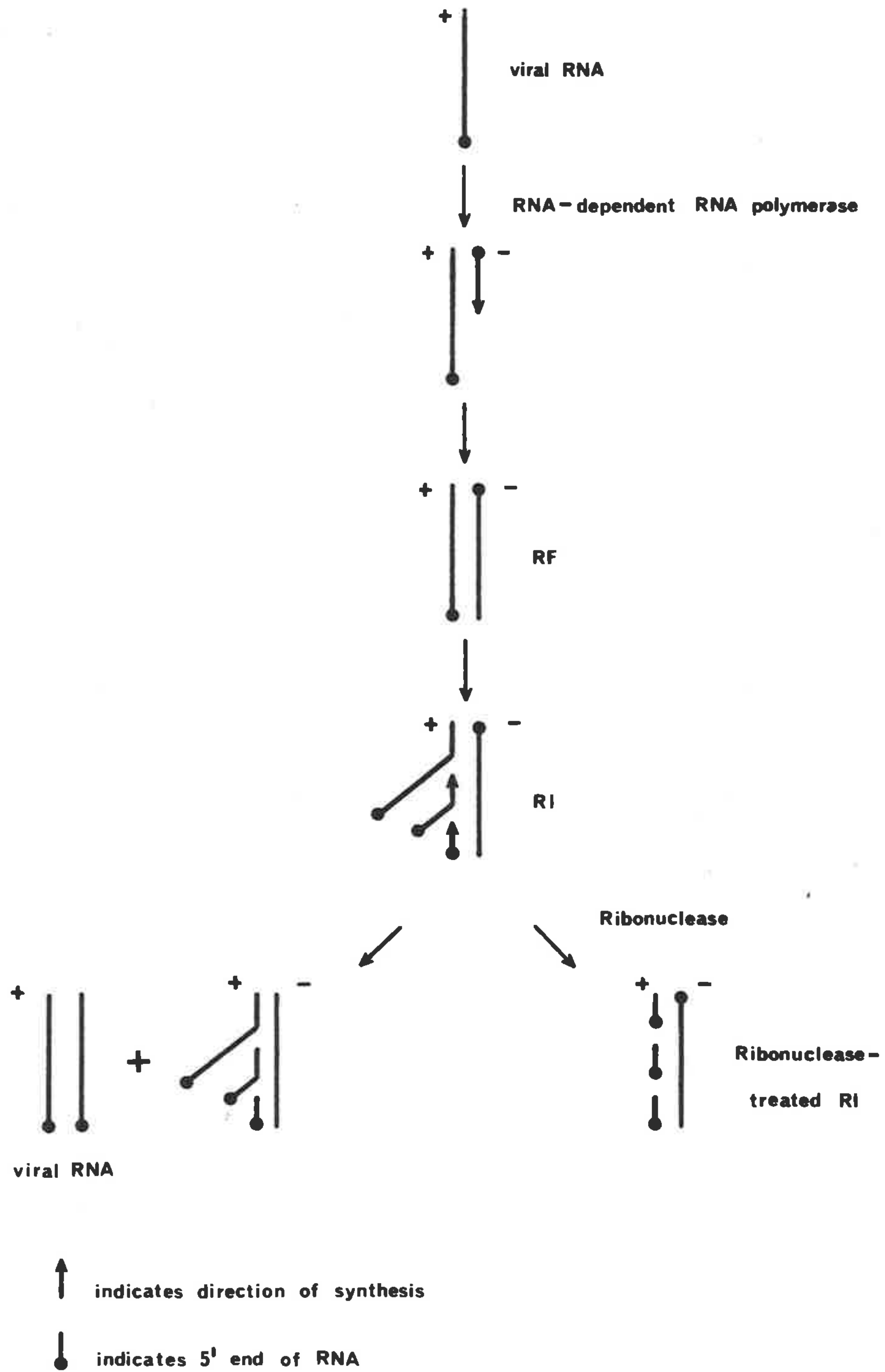
added TMV RNA and incubated the system in the usual way to allow protein synthesis to occur. The products of this incubation were electrophoresed on 7.5% polyacrylamide gels in which highly polymerised RNA will not move. Gels were polymerised in the presence of TMV RNA, partially purified samples from the *in vitro* protein synthesising system electrophoresed and after 45 min, a solution containing ATP, GTP, CTP and ^{3}H-UTP was applied to the gel and electrophoresis continued for an additional 50 min. On slicing the gels and counting the slices one large peak of ^{3}H-UTP incorporation was found whether TMV RNA was present in the original protein synthesising system or not, but a smaller peak appeared only if the system was primed with TMV RNA. This smaller peak was thought to be due to the TMV-coded RNA replicase while the larger one was probably due to a tobacco enzyme. Sela (1970) then tested the presumed RNA replicase for specificity towards TMV RNA by polymerising different nucleic acids in the polyacrylamide gels and looking for the appearance of the smaller peak. The nucleic acid samples tried were TMV RNA, TMV-infected tobacco RNA, polyoma DNA, commercial DNA, PVX RNA, ribosomal RNA, healthy tobacco RNA and no RNA. Results were clear; only when TMV RNA was present, either as pure TMV RNA or the RNA extracted from TMV-infected tobaccos, did the small peak of the presumed TMV RNA replicase appear. These results indicate that Sela has managed to synthesise *in vitro* the enzyme, or part of it, responsible for TMV RNA replication.

SECTION 4. CONCLUDING REMARKS.

In this Introduction, a brief survey of the work on plant viral-induced RNA and protein species and the viral-induced RNA-dependent RNA polymerase has been presented. A difficulty with plant virus systems has been to obtain a high proportion of cells infected with the virus. A possible way to overcome this wuld be to use single cell suspensions which could be treated in a similar manner to bacterial suspensions. Two groups have achieved single cell suspensions from tobacco plants. Takebe *et al*. (1968) obtained a suspension of tobacco mesophyll protoplasts while Jensen *et al*. (1971) made a suspension of tobacco leaf cells either from healthy plants or from those infected with TMV. The tobacco mesophyll protoplasts could be infected with TMV and TMV RNA (Otsuki and Takebe, 1969; Takebe and Otsuki, 1969; Aoki and Takebe, 1969). Perhaps use of these systems will facilitate the study of plant virus replication.

In this thesis further work on TRSV and CMV-induced RNA species, protein species and the RNA-dependent RNA polymerase is described.

FIGURE 1 SEMICONSERVATIVE SCHEME OF PHAGE RNA REPLICATION



CHAPTER ONE

GENERAL MATERIALS AND METHODS

GENERAL MATERIALS AND METHODS

In order to avoid unnecessary repetition, those materials and methods used more than once in subsequent chapters of this thesis are described below.

SECTION 1. MATERIALS.

Unlabelled nucleotides, pyruvate kinase (suspension of 10 mg/ml in 2M $(NH_4)_2SO_4$), pancreatic ribonuclease-A, pancreatic deoxyribonuclease (ribonuclease-free), haemoglobin (Type 1), polycytidylic acid (poly C) and sodium dodecyl sulphate were purchased from Sigma Chemical Co., St. Louis, Missouri. Poly C has also been obtained from Miles Laboratories, Elkhart, Indiana. Pronase (B grade) was purchased from Calbiochem, California, 5'-GMP with a specific activity of 2 mCi/µmole was prepared as described by Symons (1966, 1968) and was converted to the triphosphate as described by Symons (1969). ^{32}P-Orthophosphoric acid in dilute HCl, carrier-free, was obtained from the Australian Atomic Energy Commission, Lucas Heights, N.S.W., ^{35}S-sulphate, carrier-free, ^{3}H-uridine, ^{14}C- and ^{3}H-iodoacetic acid from the Radiochemical Centre, Amersham, England, ^{14}C-reconstituted protein hydrolysate from Schwarz Bioresearch Inc., Orangeburg, New York. Bentonite was kindly supplied by the Department of Pharmacy, University of Adelaide and washed according to Fraenkel-Conrat (1966). Dextran sulphate was

purchased from Pharmacia, Uppsala, Sweden; naphthalene disulphonic acid and 8-hydroxyquinoline from British Drug Houses, England; bis-acrylamide from Eastman Organic Chemicals, New York (recrystallised from acetone) and ethylene diacrylate from Borden Inc., Philadelphia, Pennsylvania; acrylamide from Merck A.G. Darmstadt, Germany (recrystallised from chloroform). CMV and TRSV from infected cucumber plants were purified as described by Scott (1963). Baker's yeast ribosomal RNA was prepared by the method of Crestfield et al. (1955). Phosphoenol-pyruvate (PEP; cyclohexylammonium salt) was prepared and ion exchanged to the potassium salt by the method of Clarke and Kirby (1966). Actinomycin D was kindly donated by Merck, Sharp and Dohme, Rahway, New Jersey; puromycin was purchased from Nutritional Biochemicals Corporation, Cleveland, Ohio; Penicillin-G (sodium salt) was obtained from Evans Medical Australia (Pty.) Ltd., Melbourne, Australia. Sucrose (analytical reagent) was obtained from Mann Research Laboratories, New York, New York (special enzyme grade). Protamine sulphate was bought from Calbiochem, California. Cellulose BW40, Solka-Floc, was purchased from Brown Co., Boston, Massachussetts; cellulose (CF-11), DEAE-cellulose (DE-11), CM-cellulose (CM-70), phosphocellulose (P-11) were obtained from Whatman, England; Sepharose 4B and DEAE-sephadex were obtained from Pharmacia, Uppsala, Sweden. All other chemicals were either analytical or laboratory reagent grade.

SECTION 2. VIRUSES AND PLANTS.

All virus strains and plants were kindly donated by Dr. R.I.B. Francki, University of Adelaide, South Australia. Viruses used throughout this work were CMV (Q strain) and TRSV. Cucumber seedlings (cucumis sativus L. var 'Polaris') were germinated in a glasshouse. Seven to ten days after planting, the cotyledons were lightly dusted with Carborundum powder and infected by an inoculum (serial inoculation) prepared by grinding infected plants with a small amount of water in a mortar and pestle. At intervals of 3-4 months, purified virus strains (Q-CMV and TRSV) were used as inoculum. Control plants were rubbed with an homogenate prepared from healthy plants; the results obtained from these controls, however, were identical to those obtained from untreated healthy plants. Plants were grown either under glasshouse conditions or in a constant temperature room (24-28°) using fluorescent lighting at an intensity of 100-150 ft-c for a 12 hr day. For assay of CMV-induced RNA polymerase, infected plants were harvested 7-13 days after infection, and for TRSV-induced RNA polymerase, 2-4 days after infection. Under our conditions distinct symptoms of CMV infection appeared 7-9 days after infection with CMV, and for TRSV, symptoms appeared 3-4 days after infection. The fact that the coincidence of appearance of both symptoms on the primary leaves of cucumber plants and RNA polymerase activity for both CMV and TRSV infection, allowed the

selection of the best tissue for enzyme extraction.

SECTION 3. BUFFERS USED FOR PREPARATION OF ENZYME EXTRACTS AND ASSAYS.

Buffer A. 10mM Tris-HCl, pH 8.0; 4mM $MgSO_4$; 20mM 2-mercaptoethanol.

Buffer B. Buffer A + 10% sucrose.

Buffer C. 100mM Tris-HCl, pH 8.0; 10mM $MgSO_4$; 1mM EDTA; 20mM 2-mercaptoethanol.

Buffer D. 10% SDS; 0.5M urea; 10mM Tris-HCl, pH 8.0; 4mM $MgSO_4$; 20mM 2-mercaptoethanol.

Buffer E. 50% $(NH_4)_2SO_4$; 50mM Tris-HCl, pH 8.5; 100mM NH_4Cl; 90mM 2-mercaptoethanol.

Buffer F. Buffer E minus 50% ammonium sulphate.

Buffer G. 0.3M Sucrose (10.2% w/v); 50mM Tris-HCl, pH 8.5; 100mM NH_4Cl; 90mM 2-mercaptoethanol.

Buffer H. 10% Glycerol; 10mM Tris-HCl, pH 8.5; 10mM 2-mercaptoethanol.

SECTION 4. POLYACRYLAMIDE GEL ELECTROPHORESIS TECHNIQUES.

(a) For Electrophoresis of RNA Samples.

Two types of polyacrylamide gels were used:-

(i) Gels cross-linked with bis-acrylamide.

Polyacrylamide gels were prepared using a modification of the Loening (1967) method according to Dr. D. Spencer, C.S.I.R.O., Division of Plant Industry, Canberra,

Australia. 4 ml of 15% acrylamide and 0.75% bis-acrylamide, 8.34 ml of gel buffer (see below) and 12.4 ml of water were mixed in quick succession, degassed with a water pump for 15 sec after which TEMED (0.02 ml) and 0.2 ml of freshly prepared 10% ammonium persulphate were added with as little aeration as possible. This mixture was then pipetted into eight perspex tubes (7 mm internal diameter) to a height of 9 cm. The gels were allowed 60 min at room temperature to set. Immediately before use the water extruded from the gel matrix on setting was removed. 0.1 ml samples in approximately 20% sucrose were loaded onto each gel and electrophoresed at 5 ma/gel for 5 hr.

1. Stock buffer for normal gels:-

   1M Tris base (120 ml)
   Glacial acetic acid (6 ml)
   3M sodium acetate (20 ml)
   0.2M EDTA (disodium salt) (30 ml)
   The overall pH was 7.2

2. Gel buffer was 5-fold dilution of the stock buffer.

3. Running (reservoir) buffer was a 15-fold dilution of the stock buffer.

(ii) Gels cross-linked with ethylene diacrylate.

Procedure for polymerising polyacrylamide gels was identical to that described above except that the acrylamide solution contained 15% acrylamide and 3.0%

ethylene diacrylate.

(iii) Staining procedures.

Immediately after electrophoresis, the gels were immersed in methylene blue (0.2% (w/v) in 0.2M sodium acetate and 0.2M acetic acid) for 1 hr with occasional gentle shaking. The gels were then destained in water overnight. RNA bands stained were scanned using a densitometer (Photovolt Corp., New York, U.S.A.) with the 610 mμ filter.

(iv) Procedure for slicing and counting gels.

Stained gels were immersed in 50% glycerol for 15 min then frozen in an aluminium trough surrounded by powdered dry ice. The solid gel was then removed from this trough, placed on a wet strip of filter paper attached to the Mickle Gel Slicer platform and covered with finely powdered dry ice which froze the water around the gel, thus fixing the gel firmly to the platform. Finally the gel was cut into 1 mm slices which were placed into separate scintillation vials. If the gels were made from bis-acrylamide, 0.5 ml of 30% H_2O_2 (v/v) was added to each vial, the vials incubated at a temperature of about 80° for 2 hr (or until complete digestion of the slices had occurred), a Whatman GF/C filter (2.5 cm) added to soak up the residual liquid and then finally dried completely (2-3 hr) at 80°. Only ^{32}P and ^{35}S isotopes could be counted using

bis-acrylamide gels (see Chapter II). For ethylene diacrylate cross-linked gels, 0.5 ml of 1M NH_4OH was used to digest the gel slices, a period of about 2 hr at 80° generally being required to obtain complete digestion of the slices. Addition of a GF/C filter and drying completely followed. Radioisotopes ^{14}C and ^{3}H had to be counted using this method (Chapter II). To the dried GF/C filters, scintillation fluid was added and the vials counted in a Packard Liquid Scintillation Spectrometer. Efficiencies of counting were about 20% for ^{3}H samples and about 60% for ^{14}C samples.

(b) <u>For Electrophoresis of Protein Samples</u>.

Again two types of polyacrylamide gels were used:-

(i) <u>Standard high pH gels, cross-linked with bis-acrylamide</u>

These were prepared as described by Davis (1964). Samples were loaded on in 10-20% sucrose and electrophoresed at 3 ma/gel for 2 hr.

(ii) <u>Ethylene diacrylate cross-linked gels, pH 7.0</u>

These gels were prepared based on the method first described by Choules and Zimm (1965) using in solution C, 15% acrylamide and 1.6 ml of ethylene diacrylate. Electrophoresis of these gels was carried out at pH 7.0 according to the method described by Williams and Reisfeld (1964) using diethylbarbituric acid in the electrophoresis buffer.

(iii) Staining procedure.

Following electrophoresis, gels were stained for 1 hr in 5% amido black (w/v) after which they were destained overnight in approximately 1% acetic acid (v/v). Protein bands could be scanned using a densitometer with a filter of approximately 610 mμ.

(iv) Procedure for slicing and counting.

This was identical to that method used for RNA gels described above except that immersion in 50% glycerol was not necessary.

SECTION 5. LABELLING OF PLANTS.

The most common method used (other methods used are described in Chapter III) was to label each plant by allowing it to take up the label through the roots. Cucumber plants (6-10 days old) were carefully removed from the soil, their roots washed clean of soil with distilled water and placed in small tubes containing about 0.2 ml of radioisotope. After the isotope had been absorbed (about 3 hr) the tubes were filled with distilled water and the plant left for a further 21 hr to incorporate the label.

As ^{32}P-orthophosphate was stored in 0.1M HCl, it was necessary to neutralise the solution before labelling plants. Small quantities of solid sodium carbonate were added until the solution was neutral according to universal indicator paper. All other isotopes used were added

without modification.

SECTION 6. RNA EXTRACTION PROCEDURE.

One gram of cucumber cotyledons was ground with a mortar and pestle, 3 ml of a solution of 2% SDS, 1% NDS, 0.001% dextran sulphate, 0.01% bentonite, 0.1M Tris-HCl pH 8.5, 0.01M $MgCl_2$ and 1mM EDTA (disodium salt) was added and the tissue ground further. The mixture was shaken for 15 min at 4° with an equal volume of water-saturated phenol (78%) containing 0.001% 8-hydroxyquinoline after which time the phenol and aqueous layers were separated by centrifugation at 1000 g for 5 min. Removal of the phenol layer (lower green layer) left the interphase (membranous material and denatured proteins) and the aqueous layer. An equal volume of the phenol mixture was then added followed by shaking for 5 min at 4°, centrifugation and separation of the aqueous layer which was re-extracted once more with an equal volume of the phenol mixture. The final aqueous layer was washed free of phenol in an equal volume of ether (4 washings were necessary). This solution was then made 0.01M EDTA, 0.2% SDS and 500 μg/ml of pronase (predigested for 2 hr at 37° in 0.01M EDTA to remove any contaminating ribonuclease) added. Digestion at 37° for 30 min was followed by one extraction at 4° for 5 min with an equal volume of the phenol mixture. To the resulting aqueous layer was added 2 volumes of ethanol (98%) to precipitate the nucleic acids;

precipitation was allowed to continue overnight at -15°. The precipitate was spun down and dissolved in electrophoresis buffer (0.3 ml) containing 0.2% SDS and 10% sucrose. Samples (0.05 ml) were layered onto gel surfaces, electrophoresis buffer carefully layered into the remainder of the tube and electrophoresis carried out using a current of 5 ma/gel.

SECTION 7. ASSAY OF RNA POLYMERASE ACTIVITY.

Enzyme assays were based on the incorporation of α-^{32}P-GTP into acid-insoluble material by the method described by May and Symons (1971). The standard assay medium contained in a final volume of 0.375 ml:-

45 mμmole of α-^{32}P-GTP (5-8 x 10^5 cpm/assay)
240 mμmole of each of the three unlabelled nucleoside triphosphates (neutralised to pH 8.0 with NaOH before addition to the assay medium)
2.5 μg actinomycin D
0.5 μmole PEP (K^+)
25 μg pyruvate kinase
500 μg bovine serum albumin
5.0 μmole KCl
5.0 μmole $MgSO_4$ (13mM)
9.0 μmole 2-mercaptoethanol
10.0 μmole NH_4Cl
12.0 μmole Tris-HCl buffer pH 8.5

1.0 mg yeast RNA (unless otherwise stated)

Plant protein (0.1 ml)

Assays were incubated at 37° for 30 min (unless otherwise stated) and the reaction was stopped by chilling in ice. All assays were carried out in duplicate. To each assay was added 5.0 ml of an ice-cold solution containing 5% trichloroacetic acid, 2% NaH_2PO_4 and 2% $Na_4P_2O_7$, and the acid-insoluble material filtered onto a glass fibre disc (Whatman GF/A or GF/C, 2.5 cm in diameter) in a Millipore apparatus where it was washed once with 5 ml of the ice-cold acid solution. The filters were then washed for at least one hour in the acid solution at room temperature (10 ml for every filter) with constant stirring and rinsed with 5 ml of ether on the Millipore apparatus. In assay mixtures that contained added pancreatic ribonuclease or low levels of added RNA (less than 1.0 mg/assay), 1.0 mg of yeast RNA was added immediately before acid precipitation to ensure complete recovery of the RNA product.

The ether washed discs were dried in an oven (100° for 5-10 min) followed by counting in a Packard Liquid Scintillation Spectrometer. Zero time incubation mixtures were treated in the same manner for the estimation of blank values which were normally 30-60 cpm per assay.

SECTION 8. EXTRACTION OF RNA POLYMERASE.

The following two methods were used regularly for the extraction of RNA polymerase activity (May *et al.*, 1969; 1970).

(a) Preparation of Soluble Enzyme Extracts.

All operations were performed at 4°, the preparation being kept in ice at all times. Homogenates were prepared by grinding healthy or infected plants (7-13 days after infection with CMV or 2-4 days after infection with TRSV) in a cold mortar and pestle; for each gram fresh weight of tissue, 2.0 ml of extraction buffer E was used before grinding. This homogenate was squeezed through a fine nylon cloth and centrifuged at 25,000 g for 10 min. The pellet was then resuspended (using an homogenising rod with a teflon tip) in the original volume of extraction buffer E and centrifuged again at 25,000 *g* for 10 min. Resuspension of the pellet in the original volume of extraction buffer F followed by centrifugation at 25,000 *g* for 10 min produced a supernatant which was used as the source of RNA polymerase activity; it contained negligible amounts of plant ribonuclease activity and 1.0 - 1.8 mg of protein/ml.

(b) Preparation of Particulate Enzyme Extracts.

Again all operations were carried out at 4°. Homogenates were prepared by grinding healthy or infected plants in a mortar and pestle with buffer G, 2 ml for every gram

fresh weight of tissue. This homogenate was squeezed through fine nylon cloth, centrifuged at 150 *g* for 5 min and the pellet discarded. Centrifugation of the supernatant at 16,000 *g* for 10 min produced a pellet which was washed twice by resuspension in twice the original volume of buffer G and centrifugation at 16,000 *g* for 10 min. This final pellet (the 16,000-P fraction) was resuspended in twice the original volume of buffer F. The 16,000-P fraction contained 3.5 - 4.0 mg of protein/ml and negligible plant ribonuclease activity.

SECTION 9. PROTEIN ESTIMATIONS.

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

SECTION 10. SCINTILLATION FLUID.

This was a solution of 0.3% (w/v) 2,5-diphenoloxazole (PPO) and 0.03% (w/v) 1,4-bis-2-(4-methyl 5-phenyloxazolyl benzene) (dimethyl POPOP) in toluene. Both PPO and POPOP were scintillation grade and were obtained from Packard Instrument Co., La Grange, Illinois, U.S.A.

SECTION 11. ENZYME UNITS.

A unit of RNA polymerase activity is defined as that amount which incorporated 1 µmole of α-^{32}P-GTP into acid-insoluble material per minute under the specified

assay conditions. Specific enzymatic activity is defined as units of enzyme activity per mg of plant protein.

CHAPTER TWO

ATTEMPTED ISOLATION OF VIRAL REPLICATIVE FORM REPLICATIVE INTERMEDIATE AND WHOLE VIRUS GENOME

ATTEMPTED ISOLATION OF VIRAL REPLICATIVE FORM REPLICATIVE INTERMEDIATE AND WHOLE VIRUS GENOME

Earlier work in our laboratory by P. Nelson (1970) was carried out in an attempt to isolate the so-called RF, RI and whole viral genome from infected plants. The method used was to label infected plants with ^{32}P-orthophosphate, leave for several days, extract the RNA by the phenol method and analyse on 2.4% polyacrylamide gels as described in Materials and Methods. Initially CMV was used as the virus, but to complete an experiment using this virus took between 6 and 8 days from the time of infection. Another disadvantage with the CMV system was that the RNA, with a mol. wt. of $1.1.10^6$ (Nelson, 1970; Kaper *et al.*, 1965) would run just behind the 28S ribosomal RNA peak on polyacrylamide gel electrophoresis thus making its detection difficult. TRSV replication in cucumbers (Francki *et al.*, 1964) was chosen as an alternative since lesions appear on the leaves much sooner than with CMV and also the largest TRSV RNA species has a mol. wt. of 2.10^6 (Chambers *et al.*, 1965) and would run clear of the 28S ribosomal RNA band.

With TRSV the method became that described in Materials and Methods. Using a modification of the phenol extraction procedure (Nilsson-Tillgren, 1969) incorporating a pronase digestion of the extracted RNA (Pace *et al.*, 1968),

Nelson (1970) was able to show the appearance of a small peak, not present in healthy plants, running where TRSV RNA would be expected to run (Figure 2). However, the counts were too low to allow extraction and characterisation of it.

In order to try to increase the sensitivity of the method it was decided to use two different isotopes to label the healthy plant RNA species and the infected plant RNA species. If this could be done then perhaps the RF and RI might be identified and the small difference noticed by Nelson (1970) between healthy and infected RNA extracts might be enhanced.

SECTION 1. DOUBLE-LABELLING WITH ^{3}H-URIDINE AND ^{32}P-ORTHO-PHOSPHATE.

Nelson (1970) had labelled plants with ^{3}H-uridine through the roots and electrophoresed the extracted RNA on 2.4% polyacrylamide gels. Although ^{3}H-uridine had been incorporated into acid-insoluble material, Nelson, on electrophoresing samples, slicing the gels and digesting the slices with 30% H_2O_2, could not find any radioactivity in the gels. This was undoubtedly due to the phenomenon described by Tishler and Epstein (1968) in which H_2O_2 oxidizes ^{3}H-labelled compounds to ^{3}H-water and ^{14}C-labelled compounds to ^{14}C-carbon dioxide at temperatures greater than 50°. Nelson did not do any further work on this aspect.

The alkali-soluble cross-linking agent, ethylene diacrylate was then used in place of bis-acrylamide (Choules and Zimm, 1965) to overcome this problem. Choules and Zimm originally used ethylene diacrylate in standard 7.5% gels but for electrophoresing RNA samples, 2.4% acrylamide had to be used. To polymerise these gels, the proportion of ethylene diacrylate to acrylamide had to be double that used by Choules and Zimm for their 7.5% gels.

(a) Electrophoresis of Healthy Plant RNA on Ethylene Diacrylate Cross-linked Polyacrylamide Gels.

Healthy, ^{3}H-labelled plant RNA was extracted and analysed on ethylene diacrylate cross-linked, 2.4% polyacrylamide gels; the pattern is shown in Figure 3. As can be seen the resolution was not quite as good as with the corresponding bis-acrylamide gels (cf. Figure 2) since the 25S and 23S, and the 18S and 16S RNA species were not well separated. Another feature of these gels, although not shown in Figure 3, was that when ^{32}P-orthophosphate was used to label the nucleic acids, no DNA band appeared on the gels; the reason for this is unknown. It is possible that double-stranded RNA may also be excluded from the gel. Despite these differences it was decided to use this method for the double-labelling work. Attempts were made at improving the resolution by changing both the acrylamide concentration (to 2.3% and 2.5%) and

the ethylene diacrylate to acrylamide ratio but with no success.

(b) Electrophoresis of Healthy and Infected RNA on Ethylene Diacrylate Cross-linked Polyacrylamide Gels.

Healthy plants were labelled with 50 μCi of ^{32}P-orthophosphate per plant and two day TRSV-infected plants were labelled with 10 μCi of ^{3}H-uridine per plant. Both healthy and infected plants were left for 24 hr to incorporate the labels after which time the RNA was extracted using the Nilsson-Tillgren (1969) method and pronase digestion. The RNA was electrophoresed for 5 hr at 5 ma/gel, and the gels sliced and counted as described in Materials and Methods. Results from this experiment are shown in Figure 4. As can be seen no significant differences between the two profiles exist.

A similar pattern was obtained when ^{32}P was used to label infected plants and ^{3}H healthy plants, thus removing the possibility of differential incorporation of isotopes.

Up until now the experiments with the two isotopes have been done on separate plants, but with no success. It was hoped that by labelling the one plant with both isotopes,one before infection and one after, a difference between healthy and TRSV-infected plants would be seen. Healthy plants were labelled with ^{32}P-orthophosphate (50 μCi/plant) and left for 24 hr to incorporate the isotope.

Inoculation with TRSV was followed 24 hr later with the addition of ^{3}H-uridine (10μCi/plant) together with actinomycin D (3 μg/plant) which Nelson (1970) had shown to completely terminate host ribosomal RNA synthesis. However, all the plants carried through the above procedure wilted and died; infecting plants when they have been removed from the soil always caused them to wilt and these plants were unable to further take up any label.

This problem of plant survival has not yet been overcome and seems unlikely to be until another method of infection is found. Tissue slices have been attempted (Nelson, 1970, and see Chapter III) to see if they incorporated ^{32}P-orthophosphate into RNA. They did but at the moment no method is known whereby TRSV or CMV can infect cucumber tissue slices. Perhaps a single cell suspension of cucumber cells, as has been accomplished with tobacco cells (Jensen et al., 1971) and tobacco mesophyll protoplasts (Takebe et al., 1968) would be more amenable to infection than leaf slices. Tobacco mesophyll protoplasts have been infected with TMV (Otsuki and Takebe, 1969; Takebe and Otsuki, 1969) and TMV RNA (Aoki and Takebe, 1969). Francki et al. (1971) have shown that their single cell suspension from tobacco plants took up amino acids, uracil and uridine and if a similar system could be derived from cucumber plants, then double-labelling studies on TRSV- and CMV-induced RNA species might be possible.

SECTION 2. DISCUSSION.

Although Nelson (1970) had shown a small difference in the labelling patterns of the RNA extracted from healthy plants to that extracted from TRSV-infected plants, this difference could not be reproducibly found using his method of ^{32}P-orthophosphate incorporation or using the double-isotopic labelling method described.

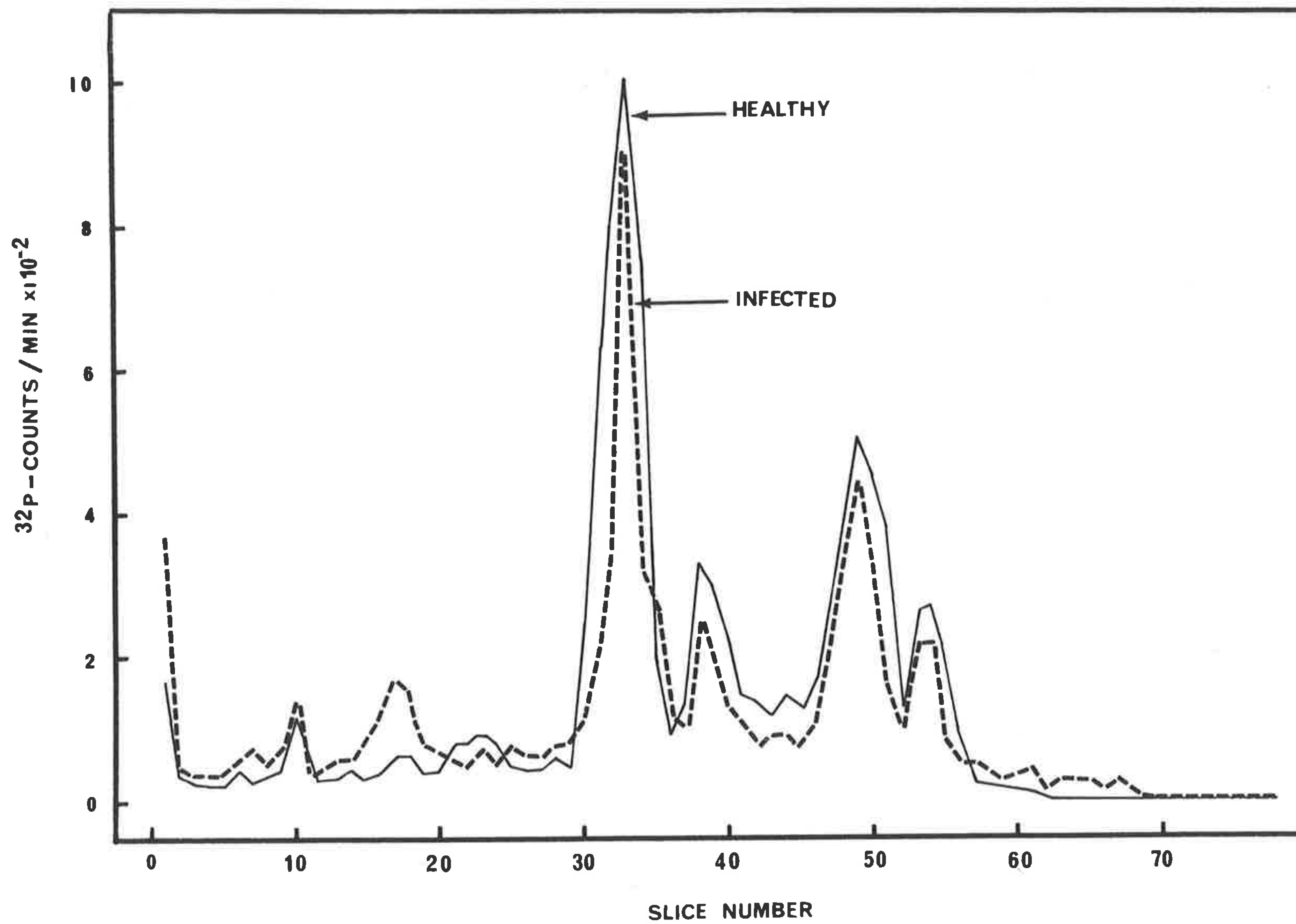
Possible reasons for the lack of success using the described systems are:-

1. We may not in fact be extracting the viral RNA species.
2. The viral RNA species if extracted might be hydrolysed during extraction.
3. Not enough label was being incorporated into viral-induced RNA relative to host RNA, and thus could not be distinguished from it.
4. Not enough viral-induced RNA is present in infected leaves to be detected.

For the practical reasons discussed above, it seemed impossible to progress any further with the isolation of TRSV- or CMV-induced RNA and so the work was left at this stage.

FIGURE 2. POLYACRYLAMIDE GEL ELECTROPHORESIS PATTERN OF ^{32}P-RNA AFTER DIGESTION WITH PRONASE.

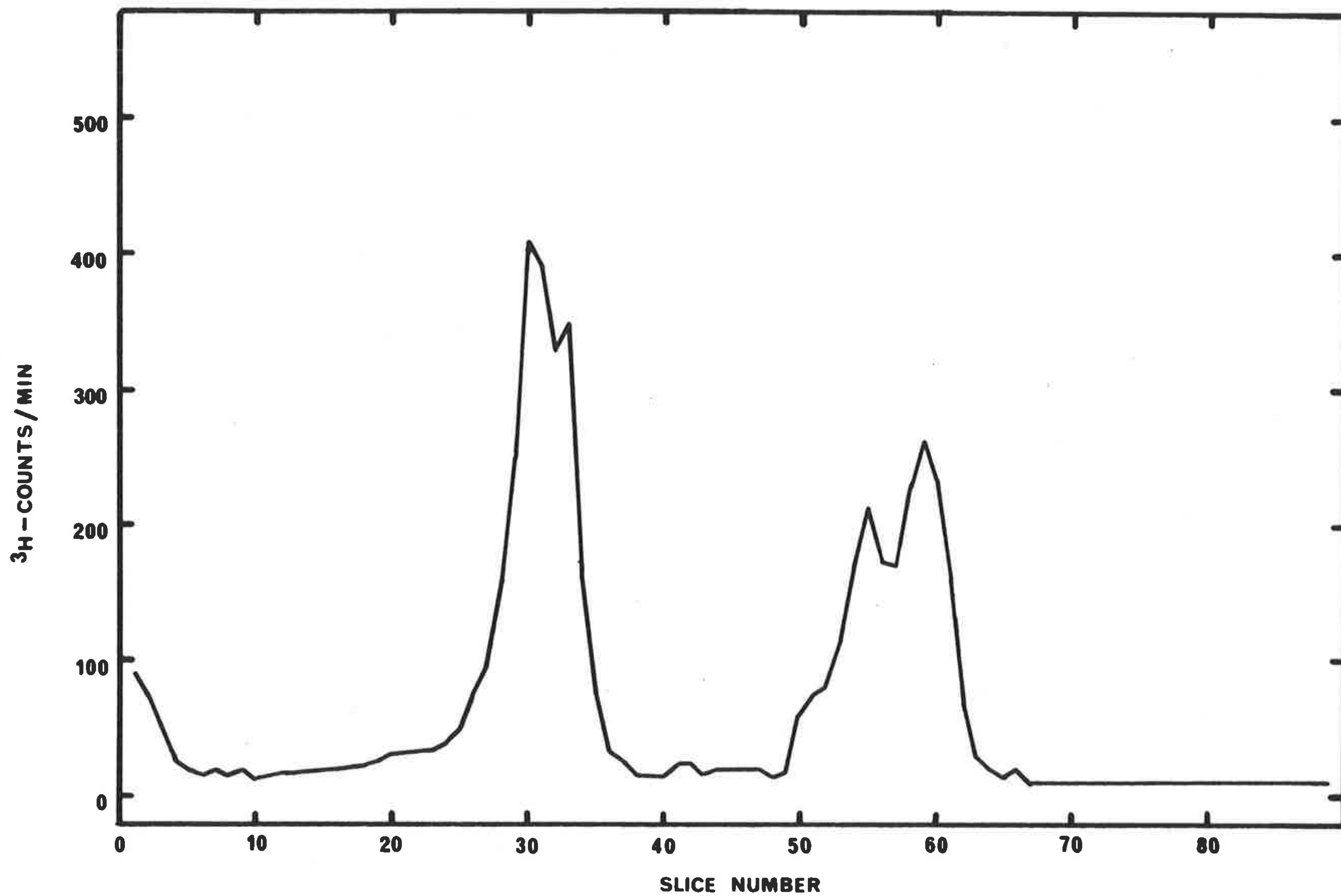
The final aqueous layer of the RNA extraction (see Materials and Methods) was made 0.01M EDTA and 0.2% SDS. Predigested pronase (500 μg/ml) was added and digested for 30 min at 37°. The digest was re-extracted once with phenol, followed by ethanol precipitation and dissolving in electrophoresis buffer. Electrophoresis was for 4 hr.



HEALTHY
INFECTED
^{32}P-COUNTS / MIN x10^{-2}
SLICE NUMBER
0
2
4
6
8
10
0
10
20
30
40
50
60
70

FIGURE 3. ELECTROPHORESIS OF ^{3}H-URIDINE LABELLED HEALTHY PLANT RNA ON ETHYLENE DIACRYLATE CROSS-LINKED POLYACRYLAMIDE GELS.

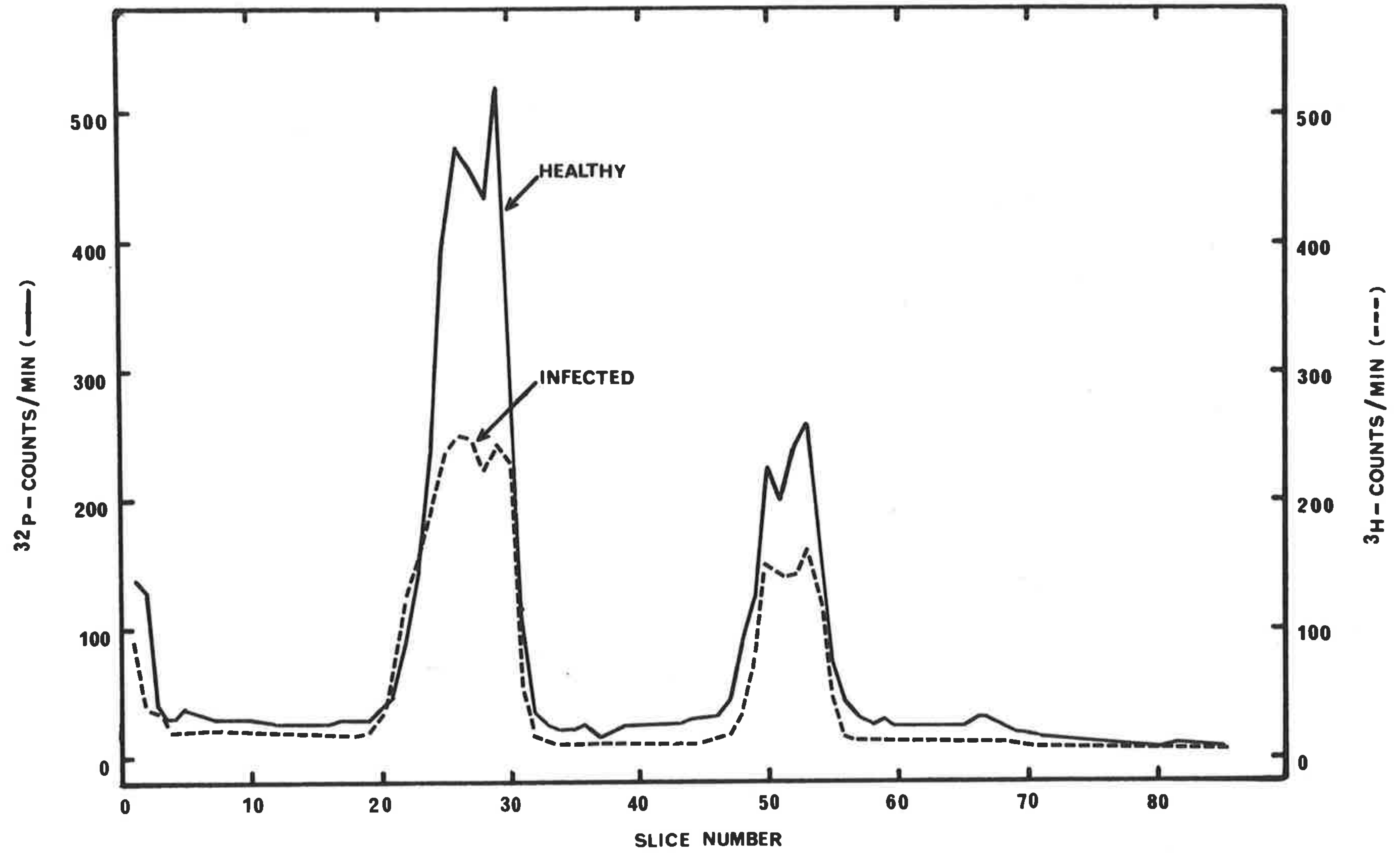
Plants were labelled for 24 hr with 10 µCi of ^{3}H-uridine per plant, the RNA phenol extracted and electrophoresed on 2.4% ethylene diacrylate cross-linked gels for 4 hr as described in Materials and Methods.



500
400
300
200
100
0
3H-COUNTS/MIN
0
10
20
30
40
50
60
70
80
SLICE NUMBER

FIGURE 4. CO-ELECTROPHORESIS OF ^{32}P-ORTHOPHOSPHATE LABELLED HEALTHY PLANT RNA AND ^{3}H-URIDINE LABELLED TRSV-INFECTED PLANT RNA ON ETHYLENE DIACRYLATE CROSS-LINKED POLYACRYLAMIDE GELS.

Healthy plants were labelled with 50 μCi of ^{32}P-orthophosphate per plant for 24 hr and 2 day TRSV-infected plants with 10 μCi of ^{3}H-uridine per plant the RNA phenol extracted and equal quantities from each co-electrophoresed on ethylene diacrylate cross-linked gels for 4 hr as described in Materials and Methods.


HEALTHY
INFECTED
32P – COUNTS/MIN (——)
3H – COUNTS/MIN (---)
SLICE NUMBER
0
100
200
300
400
500
0
10
20
30
40
50
60
70
80

CHAPTER THREE

ATTEMPTED ISOLATION OF VIRAL-INDUCED PROTEINS FROM TRSV-INFECTED CUCUMBERS

ATTEMPTED ISOLATION OF VIRAL-INDUCED PROTEINS FROM TRSV-INFECTED CUCUMBERS

CMV with a genome of 1.10^6 daltons could code for between 3 and 5 polypeptide chains each of about 30,000 daltons; similarly for TRSV with a mol. wt. of 2.10^6 daltons. Evidence for a genome heterogeneity has been found in BMV (Lane and Kaesberg, 1971), the effect of which is to increase the coding potential of the BMV genome; for example, only component 3 of the four components contains the coat protein gene. It is possible that the CMV and TRSV genomes display similar heterogeneity. A preliminary report by Kaper (1971) suggests that the CMV (strain S) genome actually consists of 5 species. If this is the case then more viral-coded polypeptide components might be present in the host cells.

SECTION 1. SOLUBLE PLANT PROTEINS.

Before a search for the viral-induced proteins could be made, a method of radioactively labelling plant proteins had to be found. Due to their ease of extraction, the soluble plant proteins were used for this purpose.

(a) Labelling of Plant Proteins.

(i) ^{14}C-Amino acids.

With the bacteriophage systems, radioactive amino

acids have been used for the incorporation into phage-induced proteins (e.g., MS2, Nathans et al., 1966; Vinuela et al., 1967; Qβ, Garwes et al., 1969). Plants 4 to 6 days after infection with CMV and 2 days after infection with TRSV were given 10 μCi of ^{14}C-algal hydrolysate through the roots, left for 24 hr, ground in a mortar and pestle in buffer A (1 g of plants to 2 ml of buffer), centrifuged at 16,000 g for 10 min and 0.05 ml of the resulting supernatant electrophoresed on 7.5% polyacrylamide gels.

The pattern of protein bands was expectedly complex. Gels were sliced and counted as described in Materials and Methods, but no radioactivity was found on them. By acid-precipitating a sample of the supernatant, it was concluded that no incorporation of radioactive amino acids into protein had occurred. This could be due to either of two reasons:-

1. Amino acids are not getting into the plant cells, either because the roots are unable to absorb them, or because the cell membranes themselves are impermeable to them.

2. Since we are only extracting the soluble proteins, any incorporation that may have occurred into the particulate proteins would not be seen.

The possibility that incorporation had occurred into particulate proteins was checked by solubilising the membranes with SDS and urea. Plants were labelled with

10 μCi of ^{14}C-algal hydrolysate as before and extracted in a buffer containing 1.0% SDS, 5M urea, 20mM 2-mercaptoethanol, 4mM $MgSO_4$, 10mM Tris-HCl, pH 8.0 with a mortar and pestle at room temperature for 10 min. Centrifiguation at 16,000 g for 10 min produced a supernatant and a pellet. To the supernatant was added cold acetone to 60% saturation, centrifuged at 30,000 g for 10 min, the pellet washed once with acetone and allowed to dry. This pellet was then redissolved in extraction buffer, samples spotted onto glass fibre filters, dried and counted. This method, although not releasing all the particulate proteins, was found to produce samples of higher protein concentration (2 fold) than the original extraction procedure and these extra proteins were assumed to be derived from the particulate material. However, results clearly showed that still no incorporation had occurred.

It did not seem necessary at this stage to determine whether the barrier to amino acid incorporation was at the root absorption stage or at the leaf cell membrane. The above experiments had clearly shown that incorporation of a radioactive label into plant proteins could not be accomplished by absorbing ^{14}C-labelled amino acids through the root system so another method was tried.

(ii) ^{35}S-Carrier-free sulphate.

It had been known for some time that the coat protein of CMV virions could be labelled with ^{35}S-sulphate

administered through the roots (Francki, 1964) and so this method was tried. 50 µCi of ^{35}S-carrier-free sulphate was administered to healthy cucumbers as described in Materials and Methods and left overnight to incorporate the label. The following morning soluble extracts were prepared as described above and 0.05 ml and 0.1 ml samples electrophoresed on 7.5% polyacrylamide gels at pH 8.9 (3 ma/gel). Standard high pH gels using bis-acrylamide as cross-linking agent could be used since the H_2O_2 digest of the gel slices did not cause oxidation of the ^{35}S-labelled proteins to ^{35}S-sulphur dioxide. Each gel was both stained and its radioactivity profile determined. The result of such an experiment is shown in Figure 5.

As can be seen from the radioactivity profile, ^{35}S incorporation occurred into proteins distributed throughout the gel. Thus the use of ^{35}S-carrier-free sulphate seemed a reasonable method with which to attempt the search for TRSV-induced proteins.

(b) Labelling of Virus-induced Soluble Proteins.

Ten of each of one-day TRSV-infected cucumber plants and healthy control plants were labelled with 50 µCi of ^{35}S-sulphate per plant and left overnight to incorporate the label. The next morning and for the following four days, two infected plants and two healthy plants were removed and soluble extracts prepared as described previously. Samples (0.05 ml and 0.1 ml were

loaded onto standard 7.5% polyacrylamide gels, electrophoresed at 3 ma/gel for 2 hr, stained and destained and the radioactivity profile determined.

Figure 5 shows the result of one day infected plants extracted after the 24 hr labelling period; as can be seen, no significant differences exist between the TRSV-infected plants and the healthy control plants. Similarly for days 2, 3, 4 and 5 after infection no differences were found in either the radioactive profiles or the protein stained profiles of healthy and infected cucumbers. A difference in the protein stained profile was not expected since the patterns were too complex to detect any viral-induced changes. Possible reasons for not detecting viral-induced proteins are:-

1. Most of the virus-induced proteins were synthesised after 2 days of infection and by that time most of the label added had been used by host protein synthesis (it is important to note here that the method used only measured the average state of the virus replication cycle existing in the cucumber leaves; this meant that a time after infection had to be found when average viral protein synthesis was maximal).

2. Viral-induced proteins were being syntehsised and labelled but the labelling of the host proteins was masking their expression.

3. ^{35}S label was not being incorporated into viral-induced

proteins although it can be into host cell proteins.

4. Virus-induced proteins did incorporate the ^{35}S label but these proteins were not present in the soluble extracts, perhaps being membrane-bound or in membrane structures as in the case the single-stranded RNA animal virus, poliovirus (Phillips et al., 1968; Caliguiri and Tamm, 1970a and b).

These possibilities were then investigated.

(i) Labelling at different times after infection.

To find out whether overall viral protein synthesis is at a maximum later than at 2 days after infection, a similar experiment to that described above was done except that the infected plants were not removed from the soil until the label was to be given. In this way each plant, whether 1 day or 5 day infected, was labelled for 24 hr just before harvesting and extracting the proteins. However, this still failed to show any differences between healthy and TRSV-infected plants. The converse argument that the ^{35}S label did not have time to be converted into sulphur-containing amino acids before viral proteins were synthesised did not seem to be valid for two reasons. Firstly, host proteins could be labelled within 24 hr, and secondly, in the first experiment, the label was present for up to 3 days in the plant before the final protein extractions were made. If, however, this argument is correct and also maximal viral protein synthesis occurs during the first 24 hr

of infection, then it would be unlikely that any viral-induced proteins would be seen. At the moment, as plants cannot be infected while growing in small tubes this possibility cannot be tested.

(ii) Fractionation of the soluble plant extract.

If viral-induced proteins are present in the soluble protein extract, but are present in small amounts, then their expression could possibly be masked by normal host proteins. One possible way to determine whether this was the case would be to fractionate the extracted proteins before electrophoresis. Two methods were tried - ammonium sulphate fractionation and acetone fractionation.

Ammonium sulphate fractions of 0-20%, 20-40%, 40-60%, and 60-80% were prepared by adding a saturated solution of ammonium sulphate to the soluble plant extract at 4°. Buffer A was used to redissolve the precipitates (0.5 ml for every gram of plants extracted) collected by centrifugation at 30,000 g for 10 min. Samples (0.1 ml) were loaded onto standard high pH, 7.5% polyacrylamide gels and electrophoresed at 5 ma/gel for 3 hr. The longer time and higher current was required since the samples were not dialysed to lower the ionic strength. Gels were stained and counted in the usual way.

A similar method was used to obtain acetone fractions of 0-20%, 20-40%, 40-60% and 60-80% except that acetone was added at -15° to the soluble plant

extract and allowed to stand for 10 min at 0° before centrifugation at 30,000 g for 10 min. Electrophoresis was for 2 hr at 3 ma/gel.

Results from these two types of experiments showed no peaks of radioactivity unique to either the healthy or infected protein fractions. It did not seem worthwhile at this stage to try to fractionate the proteins further, e.g., on small Sephadex columns, since at least some small differences should have been observed.

(iii) Attempted elimination of host protein synthesis.

Since fractionation proved unsatisfactory in exposing possible viral-induced proteins present amongst the host proteins, preventing host protein synthesis might be more successful. Two antibiotics were tried - actinomycin D and puromycin.

Actinomycin D.

As reported in Chapter II, Nelson (1970) showed that actinomycin D absorbed through the roots at levels of 3 μg/plant was able to completely stop host ribosomal RNA synthesis. It seemed likely that mRNA synthesis could also be affected at this or higher doses of the antibiotic and this might well be a useful method of removing much of the background host protein synthesis.

Healthy cucumber plants were removed from the soil, their roots washed with distilled water and placed

in aluminium foil wrapped glass tubes. Actinomycin D (3 μg) was given in a solution (100 μg/ml) to each plant which absorbed the antibiotic. Water was then added and the plants left overnight. The following morning ^{35}S-sulphate (50 μCi) was given to each plant and allowed to incorporate for 24 hr. This was followed by preparing soluble extracts in buffer A, electrophoresing, staining and counting in the usual way. Control plants where no actinomycin was administered were carried through the same procedure simultaneously.

Comparison of the two radioactivity profiles revealed that actinomycin D had no perceptible effect on host cell protein synthesis (Figure 6). Since the antibiotic does inhibit ribosomal RNA it must be entering the cell in an active form and so the reasons for it not affecting protein synthesis could be that the dose is too low to affect mRNA synthesis or that the proteins that are present are synthesised from stable messengers. To eliminate the latter possibility a longer period of incubation with actinomycin D would be necessary.

To try to overcome these possibilities experiments were done in which the dosage of actinomycin D given to each plant was increased, (5, 10, 15 and 25 μg) and also the period of incubation was increased (24, 36 and 48 hr). It was found that any higher dosage than 25 μg of actinomycin D/plant caused death, and even 25 μg killed the plant if left for 48 hr. However, none of these modifications caused the incorporation of ^{35}S-sulphate

to cease and thus presumably did not significantly inhibit host protein synthesis. It seemed that at any sub-lethal dose of actinomycin D protein synthesis would continue and so this antibiotic was unsuitable.

Puromycin.

Since actinomycin D, which inhibits DNA-dependent RNA synthesis and so indirectly inhibits protein synthesis, was unsatisfactory, an antibiotic which inhibits protein synthesis directly was tried. A similar type of experiment to that described above for actinomycin D was done for puromycin except that 10 μg was used per plant only; incubations for 24, 36 and 48 hr were tried. Results, however, were the same as for actinomycin D - puromycin did not stop host protein synthesis. This could mean that puromycin was not entering the cell, either because the roots could not absorb it or the leaf cell membrane was impermeable to it, or once in the cell it was inactive for some reason or the dose was too low. Further work with puromycin was not attempted.

SECTION 2. PARTICULATE PLANT PROTEINS.

At the present time it is not known precisely where in the cell plant viruses replicate (for a review, see Schlegel and Smith, 1967). Since most viral-induced RNA polymerases, including the CMV- and TRSV-induced enzymes, have been found in particulate extracts (see

Introduction and Chapter V) and also poliovirus proteins have been found associated with membranous structures (see above), it was decided to attempt to solubilise the particulate proteins to see if they contained any viral-induced proteins.

(a) Initial Solubilisation of Particulate Proteins

Healthy cucumbers were labelled through the roots for 24 hr with ^{35}S-sulphate (50 μCi/plant) as described previously. Plants (2 g) were ground in a mortar and pestle with buffer C (4 ml), centrifuged at 16,000 *g* for 10 min and the supernatant decanted. To the pellet was added 2 ml of buffer A containing 0.1% SDS and 0.5M urea and this mixture was homogenised and left at room temperature for 1 hr. The tube was then centrifuged at 16,000 *g* for 10 min to remove any undissolved membranous material, and the supernatant decanted and divided into two tubes. From one tube 0.1 ml samples were loaded onto standard high pH, 7.5% polyacrylamide gels, electrophoresed at 3 ma/gel, stained and counted. To the other tube was added acetone at -15° to a final concentration of 70% (v/v), left for 10 min at 0°, centrifuged at 16,000 *g* for 10 min and the pellet dissolved in the SDS-urea buffer (1 ml). Samples (0.1 ml) were electrophoresed, stained and counted as for the other samples. This acetone precipitation was done in an effort to remove most, if not all, of the chlorophyll pigment without losing any

of the proteins.

Radioactivity profiles from a number of such experiments gave variable results whether the samples were electrophoresed on normal, 0.1% SDS or 6M urea polyacrylamide gels. Figure 7 shows the type of variability in radioactivity profile found in two experiments. Obviously if slight differences in labelling pattern is being sought, then the method must be extremely reproducible. It was in this light that different extraction methods were tried.

(b) Improving the Reproducibility of the Extraction Method.

It was decided firstly to try different concentrations of SDS and urea and at different temperatures. Plants were labelled with ^{35}S-sulphate for 24 hr, extracted in buffer C, centrifuged at 16,000 *g* for 10 min to give a pellet which was extracted in the following ways (1 g of buffer for every gram of plants extracted).

Method I.

0.1% SDS
0.5M urea
4mM $MgSO_4$
20mM 2-mercaptoethanol
10mM Tris-HCl, pH 8.0

Incubated for 60 min at room temperature.

Method II. Buffer I, but incubated for 60 min at 60°.

Method III. Buffer I but with 1.0% SDS
Incubated for 60 min at room temperature.

Method IV. Buffer III but with 5M urea
Incubated for 60 min at room temperature.

Method V. Buffer III + 1/10 volume acetic acid
Incubated for 60 min at room temperature.

After incubation each mixture was centrifuged at 16,000 g for 10 min and 0.1 ml aliquots of the supernatant electrophoresed on normal, 0.1% SDS and 6M urea, 7.5% polyacrylamide gels, pH 8.9. Of the methods tried, the one that gave consistently more reproducible patterns was method III. It seems that a 1.0% SDS concentration is necessary but increasing the concentration of urea from 0.5M to 5M made no difference to the pattern indicated. This extraction buffer is now called buffer D and was used for the remainder of the work on membrane-bound proteins..

Figure 8 shows duplicate experiments using method III for solubilising the particulate proteins. Again the pattern is complex.

(c) Extraction of Particulate Proteins from TRSV-infected Plants.

TRSV-infected plants, one day after infection, were labelled and the particulate proteins extracted as

described above. A number of experiments did not produce any significant differences in the radioactivity profiles of healthy and infected proteins, the patterns being similar to those shown in Figure 9. This could again be due to the high background of host protein synthesis masking the expression of the viral-induced ones.

Fractionation of the particulate protein extract by the two methods used with the soluble extracts, ammonium sulphate and acetone precipitations, were tried in an attempt to enhance the viral-induced proteins in one of the fractions. After incubation in buffer D, the healthy and infected mixtures were centrifuged at 16,000 g for 10 min and their supernatants divided into two. To one a saturated solution of ammonium sulphate was added at 0° and fractions 0-20%, 20-40%, 40-60% and 60-80% (v/v) were collected by centrifugation at 16,000 g for 10 min. To the other was added acetone at -15° to collect similar fractions. The pellets were redissolved in 0.5 ml of buffer D and 0.1 ml samples electrophoresed, stained and counted as before.

Results showed no differences at all in any of the fractions from either acetone fractionation or ammonium sulphate fractionation. Taking plants 2, 3, 4 or 5 days after infection did not make any difference to the patterns. It was decided that other methods of release of membrane bound proteins should be tried in preference to fractionating the SDS-urea released proteins any further.

(d) Release of Particulate Proteins with $MgSO_4$ and Detergents from the 16,000 *g* Pellet.

(i) $MgSO_4$ release.

Our laboratory has previously reported that a viral-induced RNA-dependent RNA polymerase present in the 16,000 *g* pellet can be released into the soluble phase by simply incubating this pellet with 50-100mM $MgSO_4$ at 37° (May *et al*., 1970, and see Chapters IV and V). This enzyme is not present in healthy plants (Gilliland and Symons, 1968) and even although most of the enzyme components are host derived at least one component might be viral-coded as is the case for Qβ phage RNA polymerase (Kondo *et al*., 1970; Kamen, 1970). Thus providing this subunit possesses a sulphur-containing amino acid, it should be possible to detect this polypeptide amongst the $MgSO_4$ released proteins.

Cucumber plants were infected with TRSV. At one day after inoculation they were labelled with ^{35}S-sulphate (50 μCi) for 24 hr as usual and the 16,000 *g* pellet obtained. This was homogenised and incubated at 37° for 30 min with a 10mM Tris-HCl, pH 8.0, 90mM 2-mercaptoethanol buffer containing various concentrations of $MgSO_4$ - 66mM, 100mM, 200mM and also 500mM KCl to check release by raising the ionic strength. After incubation, the mixtures were centrifuged at 16,000 *g* for 10 min and 0.1 ml samples electrophoresed on standard high pH polyacrylamide gels at 3 ma/gel. Figure

10 shows the result of the 100mM incubation; similar profiles were found for the others except that the peak labelled α was absent. This peak, running just behind the bromophenol blue, must be small mol. wt. material. However, since it was not reproducibly found it was considered an artifact.

(ii) Release with detergents.

One observation that was made during the course of these experiments was that more radioactive material was left at the origin in infected samples relative to those of the control healthy plants. In other words it seemed that there was more material too large to enter the gel present in infected samples which could mean that the viral-induced proteins were still bound to membrane fragments preventing their entry into the gels. Further experiments were carried out in an attempt to break down these fragments to even smaller pieces.

These experiments consisted of treating the 16,000 *g* pellet in a variety of ways - 5% and 10% Triton X100, 5% DOC, 1% SDS + 0.5M urea, 1% SDS + 100mM $MgSO_4$ all in buffer C. Incubations were carried out at both 37°, and 60° for 1 hr after which centrifugation at 16,000 *g* for 15 min produced a supernatant of which 0.1 ml was electrophoresed and counted as usual. However, although the material on the origin had been substantially reduced, no new significant peaks appeared on the gels. Figure 11, the 1% SDS and 100mM $MgSO_4$ extraction, shows

a slight difference in the region marked β but the extent of this difference varied in a number of experiments from no difference to the difference shown here. It could not be shown whether this difference was a real variation that could be attributed to TRSV infection, or just due to slight differences in the extraction procedure.

In view of the results obtained from all the foregoing experiments it was concluded that using a single isotope to label both healthy and infected proteins was not sensitive enough in this system to detect viral-induced species. For this reason work using ^{35}S-sulphate was discontinued at this stage.

SECTION 3. DOUBLE LABELLING OF CUCUMBER PROTEINS.

It seemed that now the necessary extraction techniques had been worked out and it was only necessary to find a way of incorporating two radioisotopes into cucumber proteins. Since amino acids could not be incorporated by absorption through the roots, then another method for their uptake was sought.

(a) Methods of Labelling Cucumber Proteins *in vivo*.

(i) Through cut stems.

In this method a new razor blade was used to sever the plant from its roots just above the soil and the plant with cut stem was then placed in a small tube of distilled

water. About half of these plants did not survive and so were left for several hours before administering any label. Those surviving plants were given 10μCi of ^{14}C-algal hydrolysate, left for 3-4 hr to take up the label and left overnight in distilled water to incorporate. Soluble extracts were prepared as described earlier and acid-insoluble radioactivity determined. It was found that there was very little incorporation of the ^{14}C label into the soluble proteins, and so this method was not considered any further.

(ii) Direct introduction of the label into a secondary vein.

A small slit in the secondary vein on the underside of the leaf was made and a drop of liquid containing approximately 10μCi of ^{14}C-labelled amino acids was placed over it. It was hoped that the leaf would use this liquid in preference to, or as well as, the usual water flowing through the veins and in so doing the amino acids may enter the cell. However, on extraction of the soluble proteins and determination of the acid-insoluble radioactivity revealed that no incorporation had occurred. It was decided not to persevere with this method either.

(iii) Use of cotton thread.

This was based on the method described by Hirai and Wildman (1967) who obtained rapid and non-selective incorporation into tobacco leaves. A moistened cotton

thread with the aid of a fine needle was passed through the main stem of a young cucumber plant. The end of this thread dipped into a small glass tube containing a solution of 10μCi of ^{14}C-algal hydrolysate and this tube was taped to the stem of the plant. Again it was hoped that the plant would take up some of the label with the normal passage of water through the xylem. Most of the plants subjected to this treatment survived for several days and so this technique would be useful if incorporation occurred. Unfortunately, as with the other two techniques, no incorporation into the soluble proteins occurred, the label had travelled up the cotton but no further. This method was also abandoned.

(iv) Leaf slices.

Other workers (e.g., Zaitlin and Hariharasubramanian, 1970) have used leaf slices in phosphate buffer medium to incorporate ^{14}C-amino acids into tobacco leaves. To find out if this technique could be applied to cucumbers, leaves were cut into 0.5 mm strips with a razor blade and 500 mg were placed in 3 ml of 0.01M KH_2PO_4. To introduce 10μCi of ^{14}C-amino acids vacuum infiltration was used. This involved attaching the incubation flask to a water aspiration pump, applying the vacuum for 15 sec and repeating twice, after which the flask was incubated at 30° in the dark with continuous shaking in a water bath for 4 hr. The liquid in the flask was then decanted, the leaf slices rinsed with water to remove the excess label

and the soluble plant proteins extracted. Samples were both analysed for acid-insoluble radioactivity and electrophoresed on ethylene diacrylate cross-linked, 7.5% polyacrylamide gels.

This technique was simultaneously being applied to an investigation of RNA synthesis using ^{32}P-orthophosphate by Nelson (1970). Thus ^{32}P-orthophosphate incorporation was used as an indication of whether or not the leaf slices were still capable of RNA synthesis. Nelson found that using the above method, no incorporation of ^{32}P occurred and so it was not surprising to find that ^{14}C-amino acids were not incorporated either. Observation of the leaf slices during incubation revealed that they were curled up and flaccid, and also the medium had turned turbid suggesting bacterial growth had occurred.

Although it was adequate for tobacco leaf slices (Zaitlin and Hariharasubramanian, 1970), 0.01M KH_2PO_4 was inadequate for cucumber slices. A more isotonic medium was used which consisted of 10% sucrose, 0.05M Tris-HCl pH 7.0, 0.001M $MgSO_4$, 0.001M KCl, 0.01M 2-mercapto-ethanol. To overcome the problem of bacterial growth, 1000 units of penicillin (final concentration approx. 300 units/ml) were added to each flask. With this medium, ^{32}P-orthophosphate incorporation into RNA occurred as normal, the leaf slices remained turgid and no bacterial growth occurred.

Using this medium the incorporation of ^{14}C-amino acids into proteins was attempted. Again 10 μCi of ^{14}C-algal

hydrolysate was vacuum infiltrated and left for up to 24 hr on the water bath shaker at 30° in the dark. Still, however, no incorporation into soluble proteins was found. This could mean either that the cell membrane was impermeable to amino acids or that, although RNA synthesis can occur protein synthesis in tissue slices did not.

In an effort to distinguish between the two, leaf slices were vacuum infiltrated with 50μCi of ^{35}S-sulphate and incubated for 4 and 24 hr. The soluble proteins were extracted, electrophoresed and counted. Figure 12 shows that the incorporation was minimal (250 cpm in fraction I protein) even for the 24 hr incubation. Actinomycin D (20 μg/plant) stopped what little incorporation occurred. These results suggested that protein synthesis was not occurring as efficiently in leaf slices as RNA synthesis which seemed at a comparable level to whole plants.

Disappointingly it seemed that vacuum infiltration of amino acids into tissue slices was not going to work as a method of obtaining double-labelled proteins.

(b) Labelling of Cucumber Proteins *in vitro*.

Up until now all methods described in the literature for double labelling of host and viral-induced proteins have been by *in vivo* incorporation experiments. It was not possible yet to label cucumber proteins with radioactive amino acids; perhaps an alternative method would be to label the proteins after extraction instead of before. A

precedent already existed in the literature for differentially labelling proteins after extraction. Fox and Kennedy (1965) showed the existence of the M protein of the lac operon by labelling the non-IPTG-induced *E. coli* cells with ^{3}H-N-ethylmaleimide (NEM) and the IPTG-induced cells with ^{14}C-NEM. By mixing equal quantities of both induced and non-induced cells, fractionating the various cell components and measuring the $^{14}C/^{3}H$ ratio in the different fractions, they were able to show the enhanced presence of the M protein in their particulate fraction.

Iodoacetic acid (IAA) was chosen as the compound to differentially label host and viral-induced proteins. IAA reacts with the ε-amino group of lys (Szewczuk and Connell, 1965; Kravchenko *et al.*, 1963; Heinrikson, 1966), and with carboxyl groups (Szewczuk and Connell, 1965; Takahashi *et al.*, 1967a, 1967b), and with sulphydryl groups (Cole *et al.*, 1958; Sela *et al.*, 1959). It was decided to reduce the extracted soluble proteins and alkylate the sulphydryl groups with ^{3}H- or ^{14}C-labelled IAA. The procedure used was a modification of the method described by Sela *et al.* (1959).

About 40 mg of soluble proteins were extracted as described previously from healthy plants and obtained as a precipitate by addition of acetone at -15° to a final concentration of 70% (v/v) and centrifugation at 16,000 *g* for 10 min. This precipitate was washed once with cold acetone and twice with ether. These proteins were dissolved in 2 ml of 8M urea, the pH being adjusted to 8.5 by the

addition of trimethylamine (TMA). To this solution was added 0.5 ml of an 8M urea solution at pH 8.5 containing 0.1 ml of thioglycollic acid. The reaction was allowed to continue for 4 hr at room temperature in a stoppered flask through which nitrogen had been bubbled. By addition of 15 ml of ice-cold acid-acetone (acetone 39: 1M HCl 1), the reduced proteins were precipitated and after centrifugation at 16,000 g for 10 min, the pellet was resuspended in a second 15 ml of cold acid-acetone to remove most of the thioglycollic acid.

For alkylation this pellet was dissolved in 0.1 ml of 8M urea, the solution adjusted to pH 8.2 with ethanolamine and then in quick succession were added 5 mg of solid IAA and 0.5 ml of ^{14}C-IAA (5 μCi). The contents of the reaction tube were mixed and left at room temperature for 3 hr in the dark. At the end of this time the protein was precipitated with 10 ml of cold acid-acetone, centrifuged at 16,000 g for 10 min and washed twice with 5 ml of cold acid-acetone to remove excess ^{14}C-IAA. The proteins were redissolved in 0.2 ml of buffer A and 0.05 ml samples electrophoresed at pH 7.0 on ethylene diacrylate cross-linked 7.5% polyacrylamide gels at 3 ma/gel for 2 hr. Gels were stained and counted as described in Materials and Methods.

Results showed great variations between triplicate reactions and the amount of reaction of ^{14}C-IAA was too low for this to be a practical method to use in double labelling studies. The following modifications were made in an

attempt to get more complete reaction - more carefully controlled pH conditions, doubling the quantity of thioglycollic acid used and leaving the reduction to continue for 6 hr instead of 4 hr. However, these modifications still did not give reproducible protein profiles.

It became necessary to check the method on a well-defined protein to see if the method in my hands was working correctly. Many of the original references quoted above were worked out using ribonuclease. Reduction and alkylation of ribonuclease was carried out exactly as described above and electrophoresed. Two major radioactive bands appeared on the gels in a number of experiments and from this it was concluded that the method was reproducible, at least for single protein species.

Again the reduction and alkylation of extracted soluble plant proteins was tried, but again no reproducible pattern could be found. This was most likely due to the impurity of the protein samples, the method probably only working with purified proteins.

After it was decided not to continue with the search for the viral-induced proteins, a communication by Rice and Means (1971) appeared in which they applied a similar technique to the radioactive labelling of some whole viruses.

(c) Future Proposals.

Another method of introducing two labels into plant proteins *in vivo* would be to use ^{3}H-water and ^{14}C-bicarbonate instead of radioactive amino acids. These compounds could be introduced by adsorption through the roots. A possible objection to using two different compounds could be that one label may preferentially be incorporated into one type of protein which would appear as a new peak on polyacrylamide gels when co-electrophoresed. However, this problem could be overcome simply by reversing the labels so that the other compound is used to label the infected plants.

SECTION 4. DISCUSSION.

Initially ^{35}S-sulphate was used to label cucumber proteins, both soluble and particulate, and differences in the protein constitution of both healthy and infected plants had to be ascertained by comparing two separate polyacrylamide gel patterns from two separate extractions. In this way any differences in extraction procedure and electrophoresis could produce artifactual results. Probably because of the large quantity of host proteins present relative to the viral-induced components, the technique was too insensitive to detect the presence of the latter. This suggested that two labels must be used, one to label the host proteins, the other the host plus viral-induced ones. Radioactive amino acids were used since these should

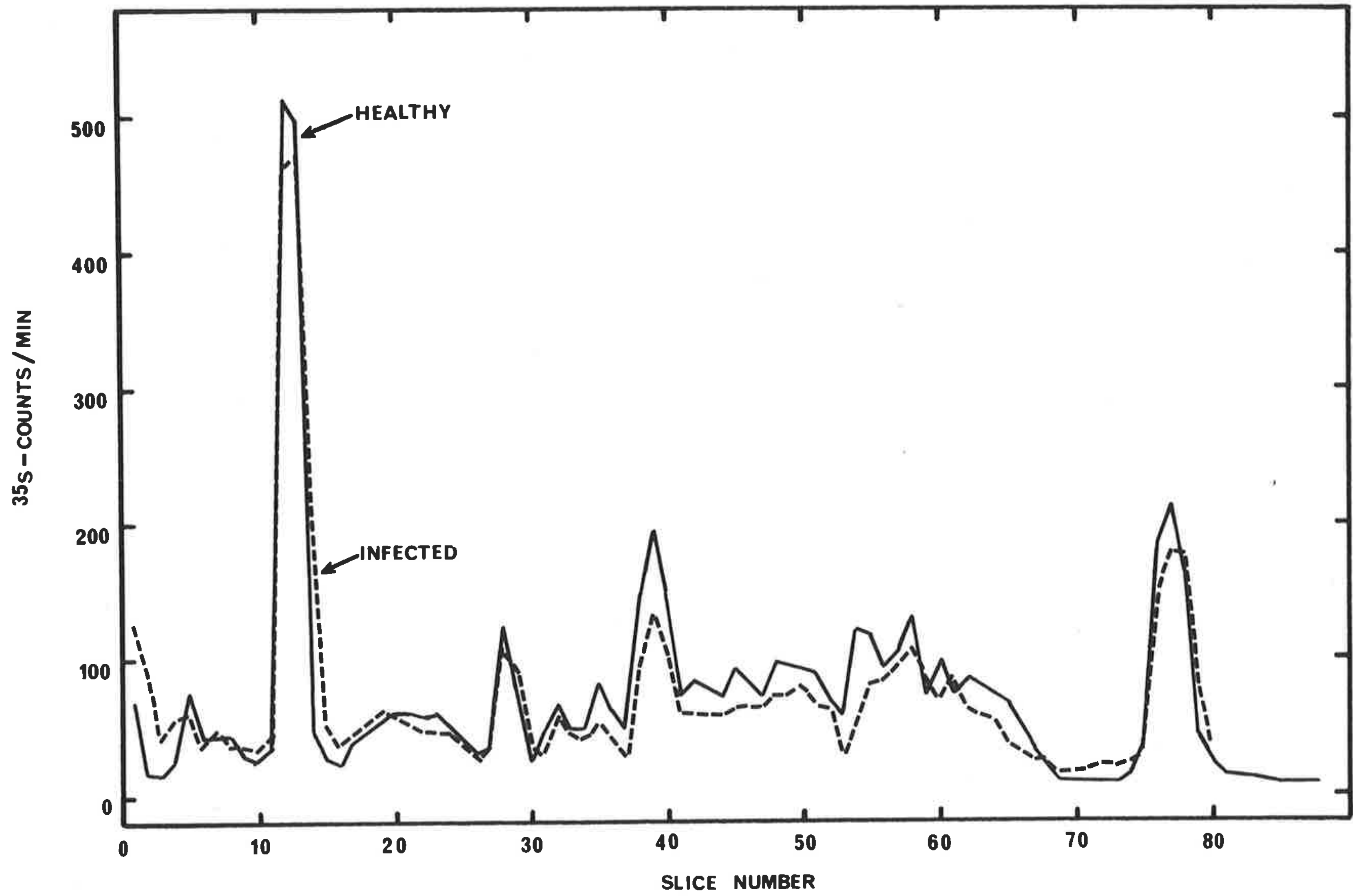
be directly incorporated into proteins; regrettably, by none of the labelling methods was label incorporated into cucumber proteins. Of those methods tried, leaf tissue slices, since they could incorporate ^{32}P-orthophosphate into nucleic acids, seemed the most promising. Perhaps using more complex media developed by White (1934) and Gautheret (1955), protein synthesis may occur.

As mentioned in Chapter I1, perhaps the only way that CMV- and TRSV-induced protein and RNA species are going to be found is by preparing single cell suspensions of cucumber cells, healthy and infected. It may even be possible, as has been accomplished with tobacco mesophyll protoplasts and TMV (Takebe and Otsuki, 1969) to infect these cultures with CMV or TRSV.

For the practical reasons discussed it was decided to investigate the properties of one viral-induced protein, the RNA-dependent RNA polymerase, more thoroughly and not to continue with this work.

FIGURE 5. ELECTROPHORESIS OF ^{35}S-LABELLED PROTEINS FROM HEALTHY AND TRSV-INFECTED PLANTS ON POLYACRYLAMIDE GELS.

Both healthy and one-day TRSV-infected plants were labelled for 24 hr with 50 µCi of ^{35}S-sulphate per plant by absorption through the roots. The soluble proteins were extracted from each and electrophoresed on separate high pH 7.5% polyacrylamide gels, the gels sliced and counted and the profiles compared.



HEALTHY
INFECTED
35S-COUNTS/MIN
SLICE NUMBER
0
100
200
300
400
500
0
10
20
30
40
50
60
70
80

FIGURE 6. EFFECT OF ACTINOMYCIN D ON THE INCORPORATION OF ^{35}S-SULPHATE INTO SOLUBLE PLANT PROTEINS.

Actinomycin D (3 μg/plant) was administered through the roots and left for 24 hr. Proteins were then labelled with ^{35}S-sulphate (50 μCi/plant) and the soluble proteins extracted, electrophoresed and the radioactivity determined as for Figure 5.

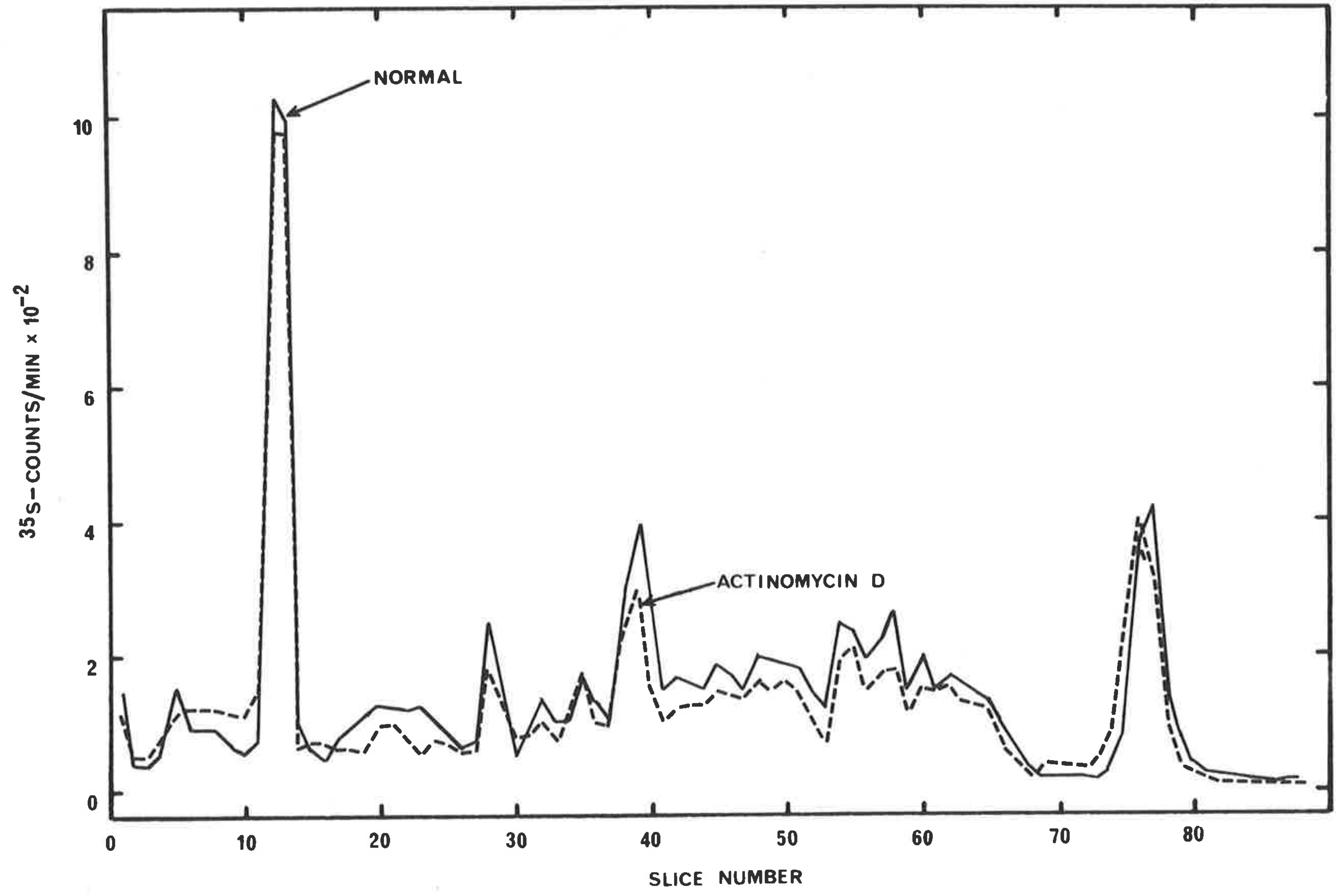
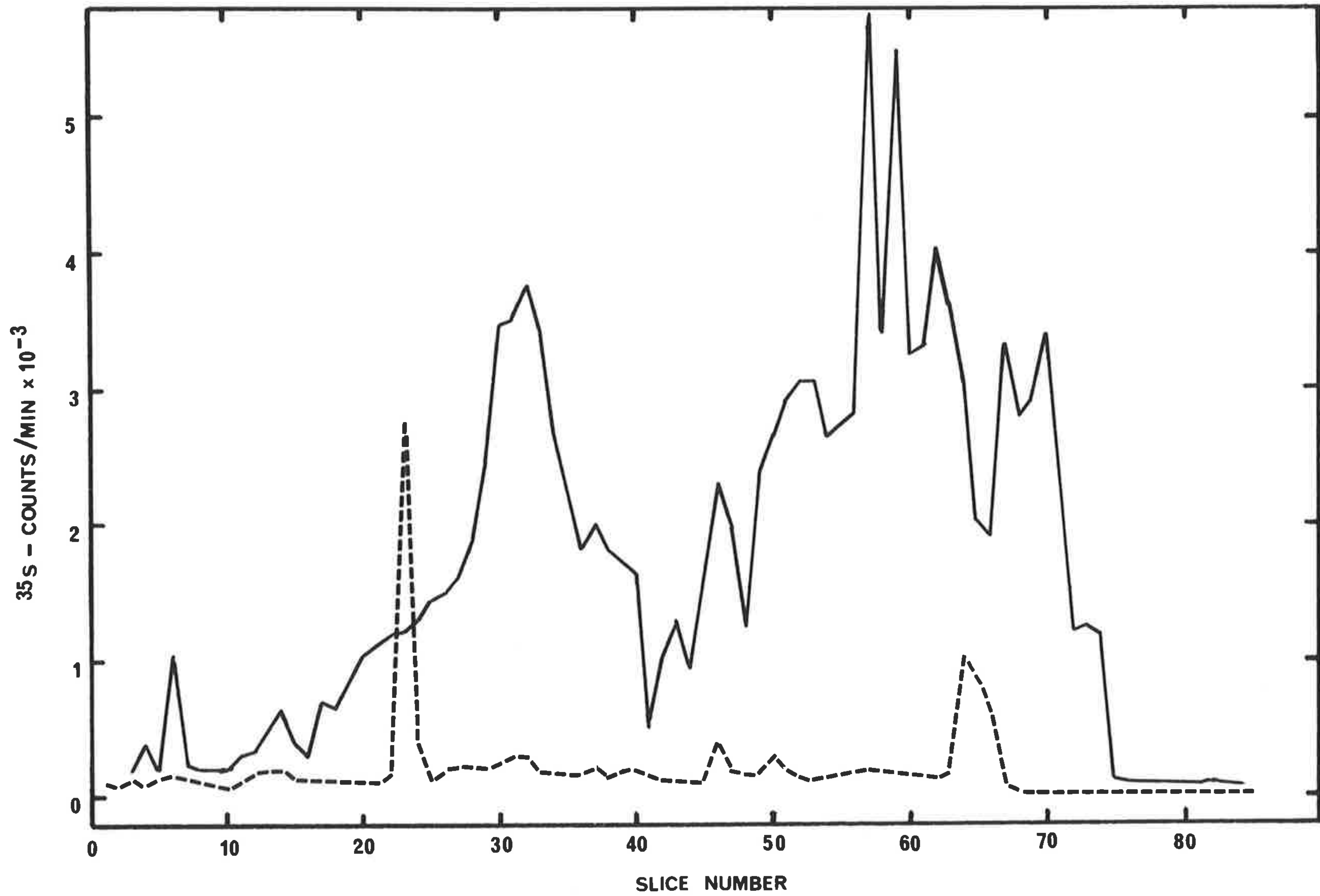

NORMAL
ACTINOMYCIN D
^{35}S-COUNTS/MIN × 10^{-2}
0
2
4
6
8
10
0
10
20
30
40
50
60
70
80
SLICE NUMBER

FIGURE 7. TWO SEPARATE SOLUBILISATIONS OF THE ^{35}S-LABELLED PROTEINS FROM THE 16,000 *g* PELLET USING 0.1% SDS AND 0.5M UREA.

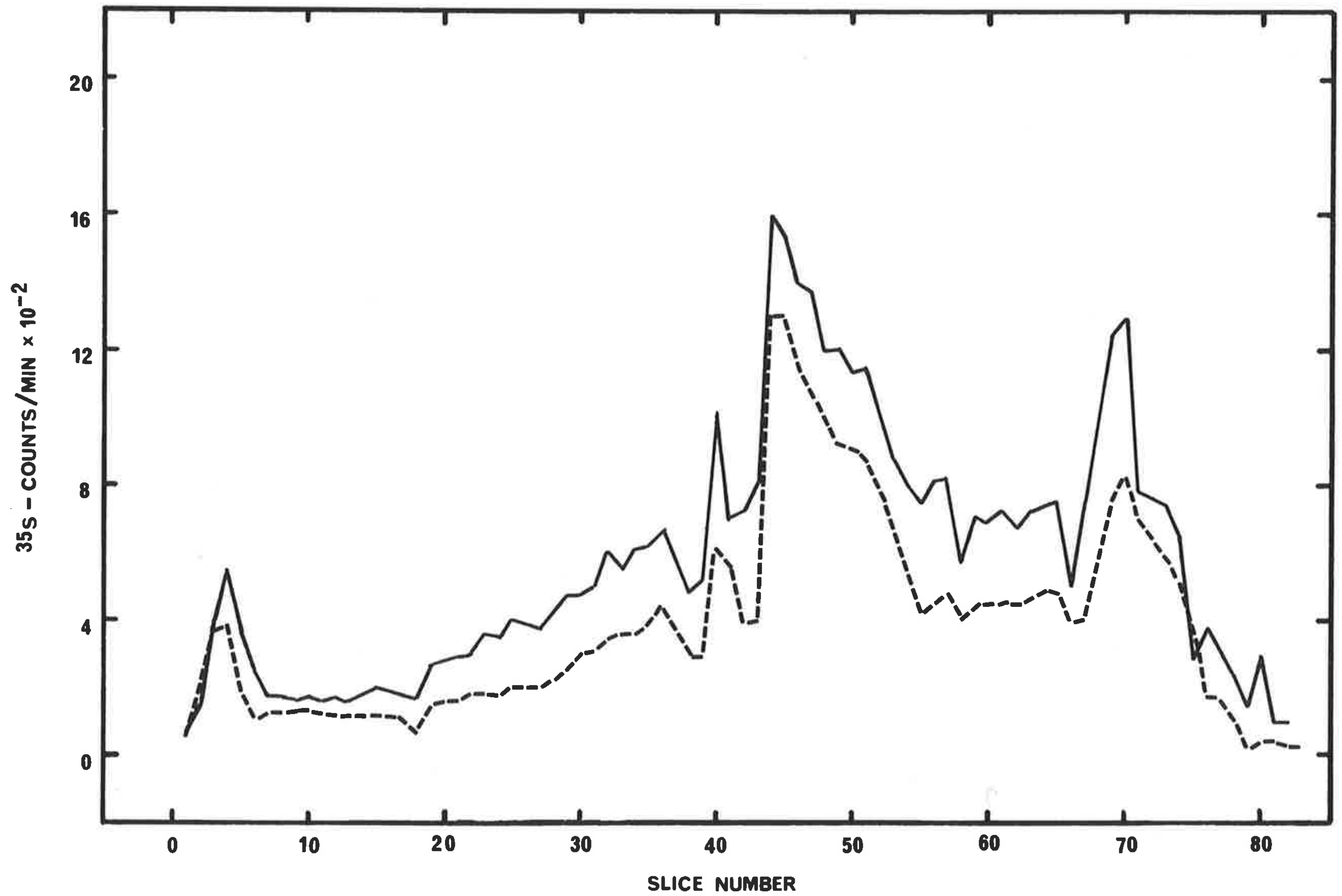
Healthy plants were labelled as before with 50 μCi of ^{35}S-sulphate and 16,000 *g* pellet obtained (see text). This pellet was homogenised with 0.1% SDS and 0.5M urea in buffer A and left at room temperature for 1 hr. After centrifugation at 16,000 *g* for 10 min, 0.1 ml samples of the supernatants were electrophoresed and the radioactivity determined as before.



^{35}S – COUNTS/MIN × 10^{-3}
0
1
2
3
4
5
0
10
20
30
40
50
60
70
80
SLICE NUMBER

FIGURE 8. TWO SEPARATE SOLUBILISATIONS OF THE ^{35}S-LABELLED PROTEINS FROM THE 16,000 *g* PELLET USING 1.0% SDS AND 0.5M UREA.

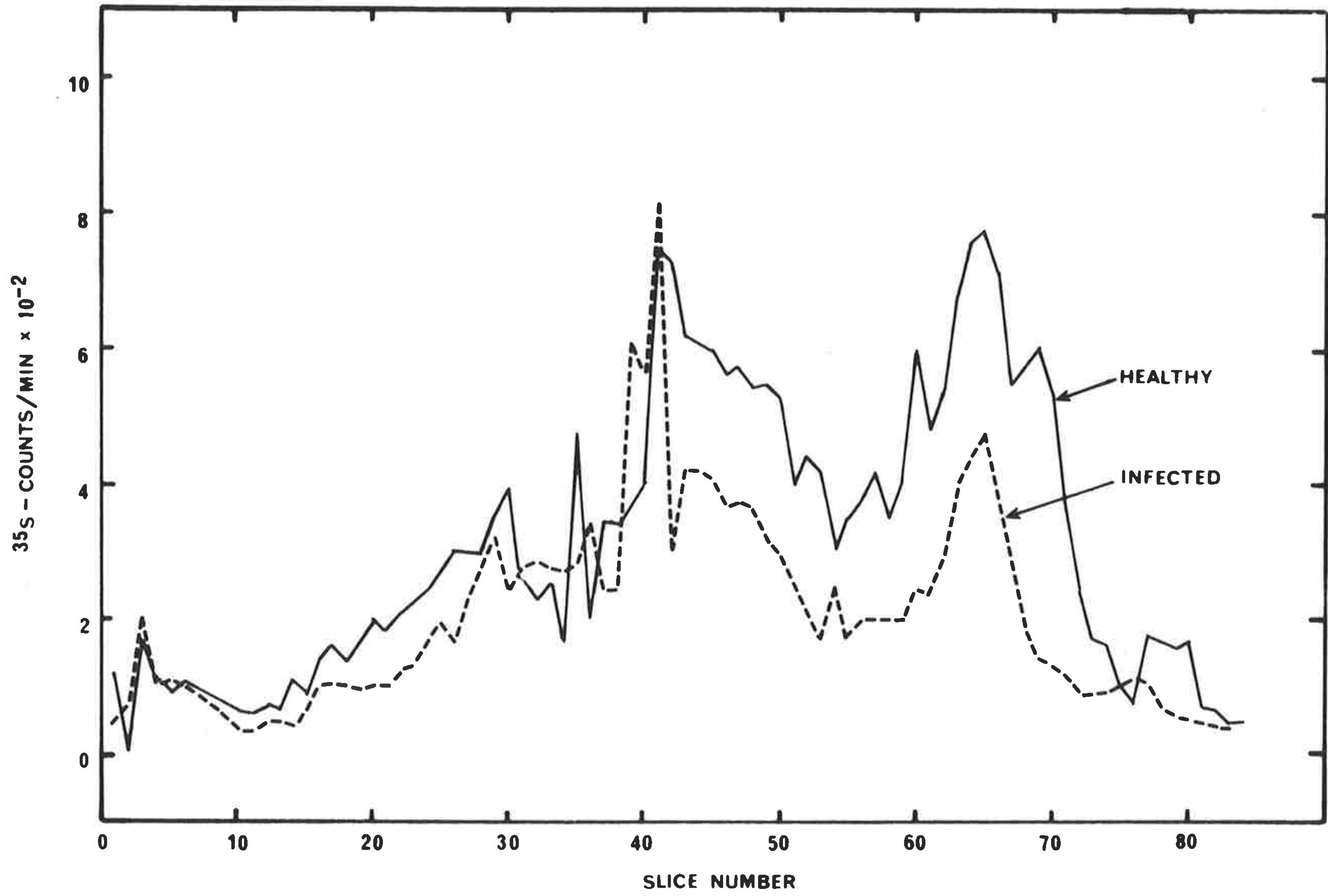
Conditions were the same as for Figure 7 except that 1.0% SDS was used instead of 0.1%.



^{35}S - COUNTS/MIN x 10^{-2}
20
16
12
8
4
0
0
10
20
30
40
50
60
70
80
SLICE NUMBER

FIGURE 9. A COMPARISON OF THE 1.0% SDS AND 0.5M SOLUBILISED PROTEINS FROM HEALTHY AND TRSV-INFECTED PLANTS.

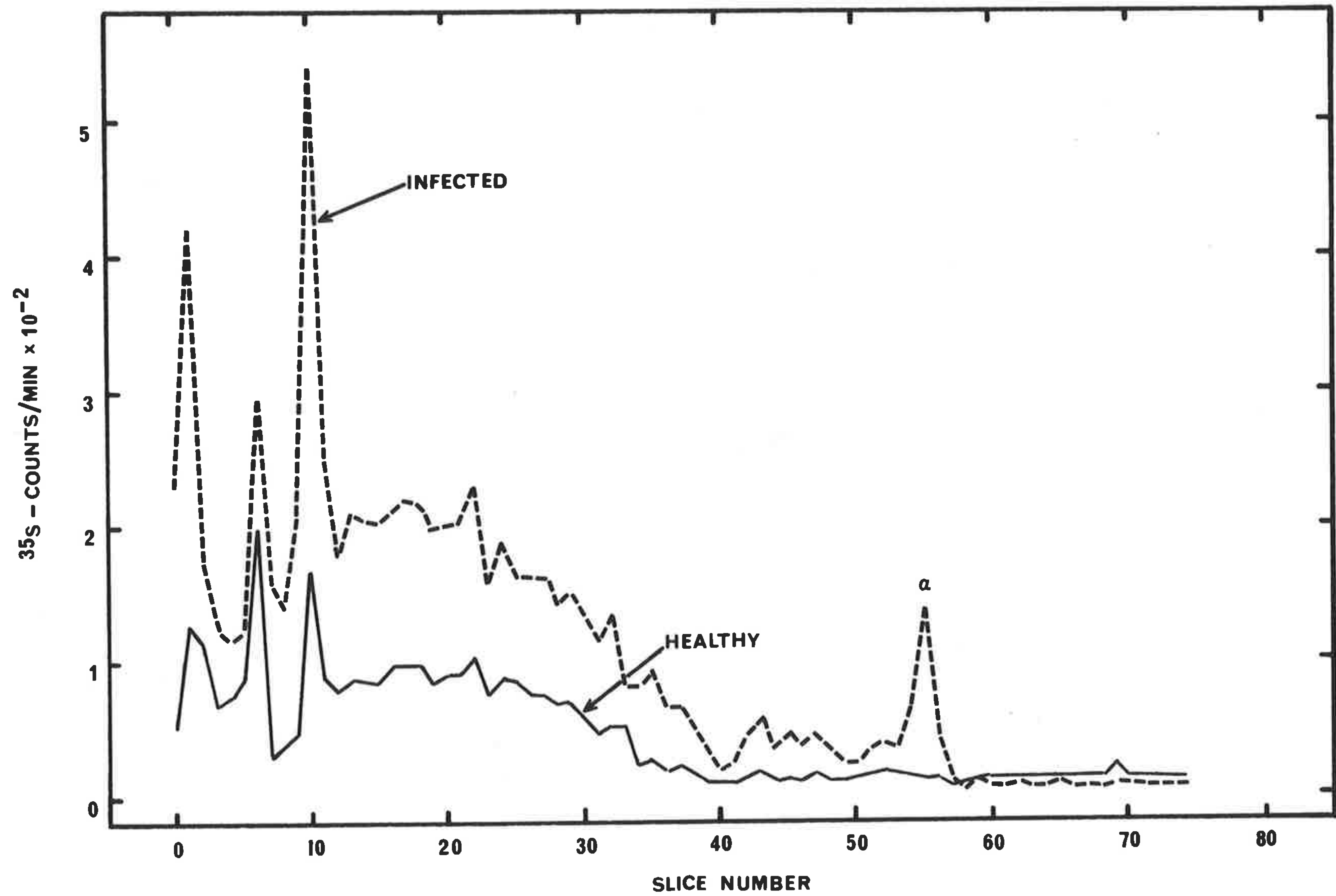
Conditions were the same as for Figure 7, healthy and one day TRSV-infected plants being used.



^{35}S - COUNTS/MIN × 10^{-2}
10
8
6
4
2
0
0
10
20
30
40
50
60
70
80
SLICE NUMBER
HEALTHY
INFECTED

FIGURE 10. A COMPARISON OF THE $MgSO_4$ SOLUBILISED ^{35}S-LABELLED PROTEINS FROM HEALTHY AND TRSV-INFECTED PLANTS.

The 16,000 *g* pellets obtained from both healthy and one day TRSV-infected plants, were incubated with 66mM $MgSO_4$ at 37° for 30 min. Centrifugation at 16,000 *g* for 10 min produced a supernatant from which 0.1 ml samples were analysed on polyacrylamide gels as before.



INFECTED
HEALTHY
a
35S - COUNTS/MIN × 10⁻²
SLICE NUMBER
0
1
2
3
4
5
0
10
20
30
40
50
60
70
80

FIGURE 11. A COMPARISON OF THE 100mM $MgSO_4$ + 1% SDS SOLUBILISED ^{35}S-LABELLED PROTEINS FROM HEALTHY AND TRSV-INFECTED PLANTS.

Conditions were the same as for Figure 10 except that the 16,000 g pellets were solubilised with 100mM $MgSO_4$ + 1% SDS at 37° for 60 min.

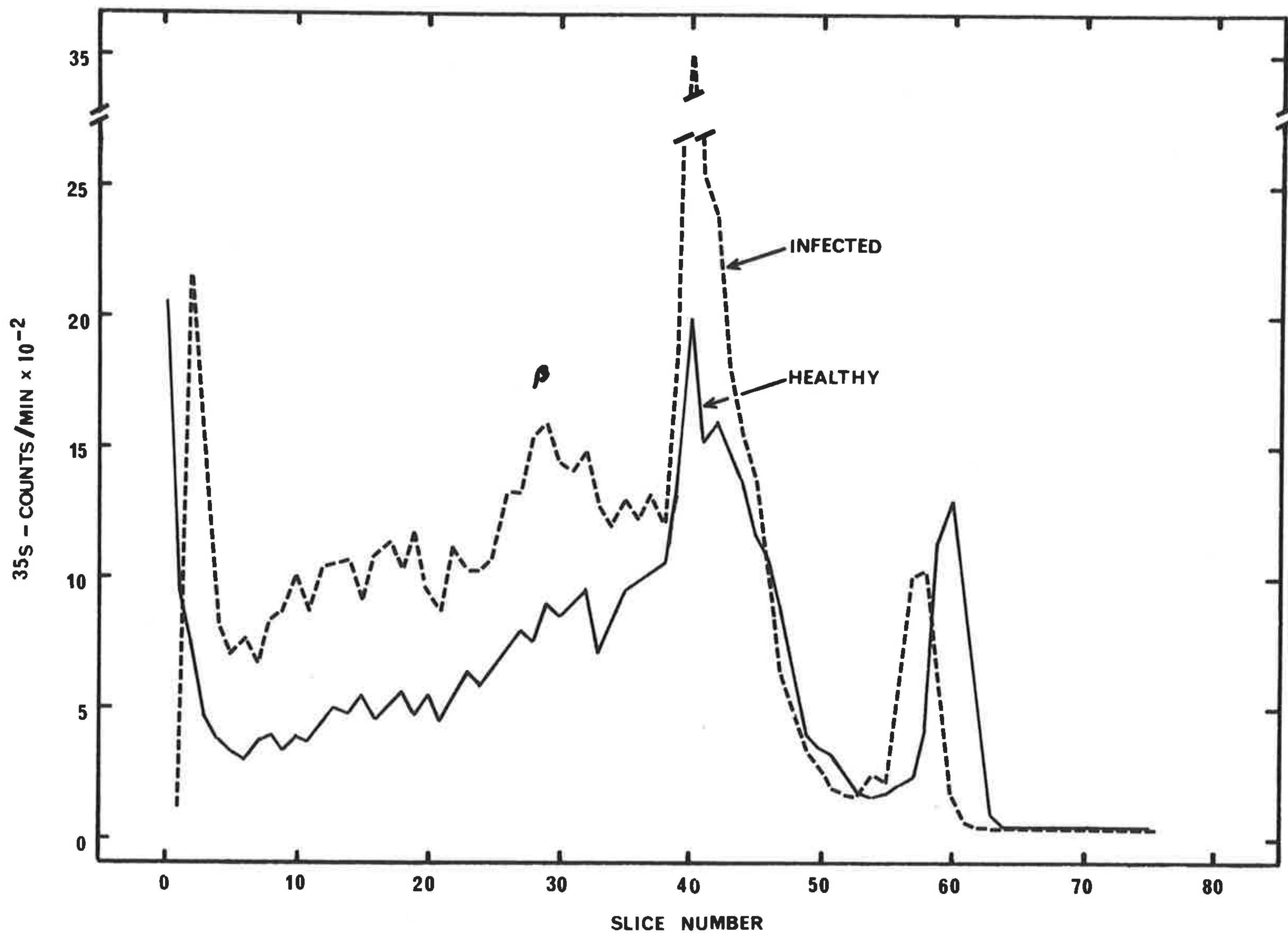
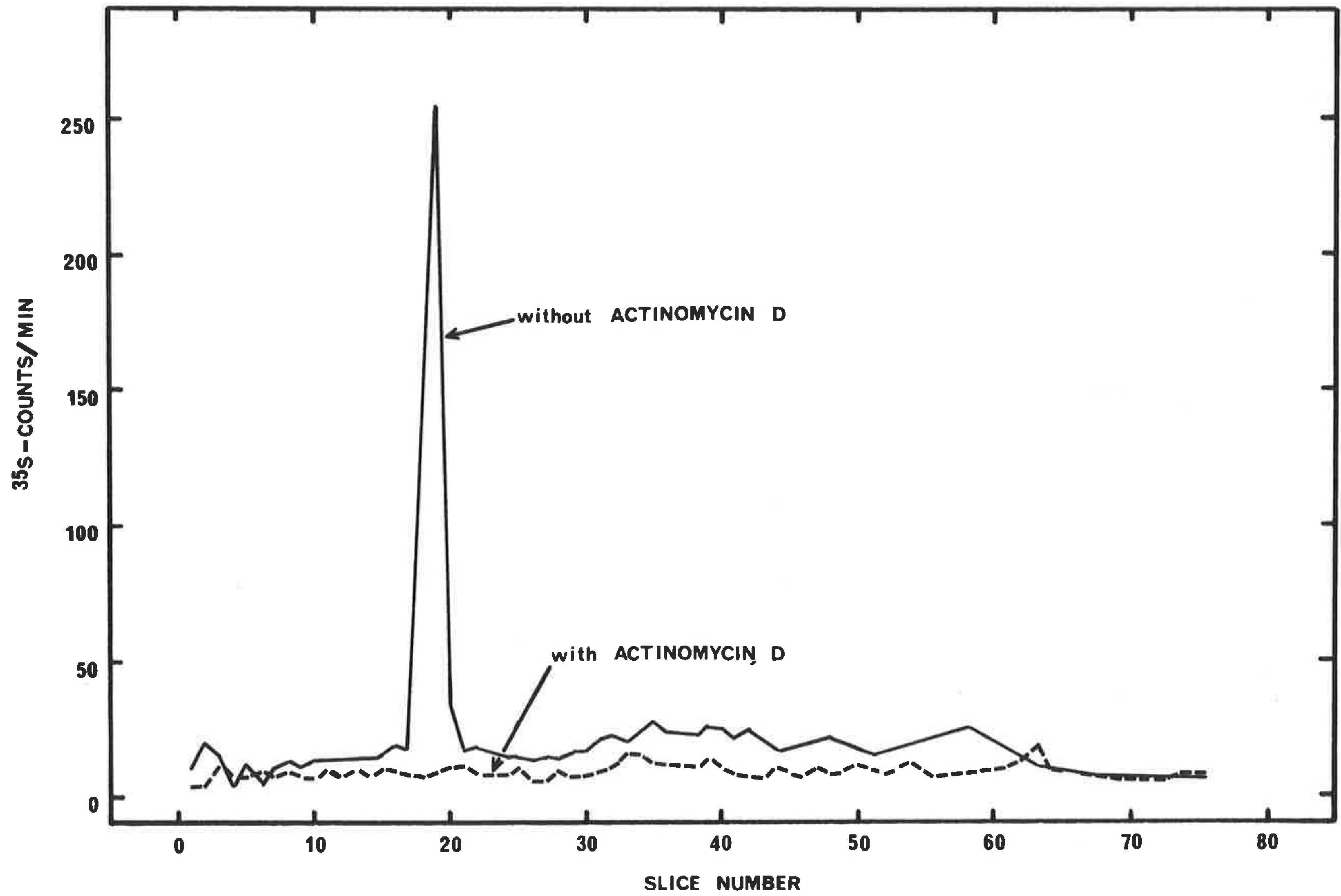

35
25
20
15
10
5
0
^{35}S - COUNTS/MIN x 10^{-2}
INFECTED
HEALTHY
β
0
10
20
30
40
50
60
70
80
SLICE NUMBER

FIGURE 12. INCORPORATION OF ^{35}S-SULPHATE INTO THE SOLUBLE PROTEINS IN LEAF SLICES WITH AND WITHOUT ACTINOMYCIN D.

Healthy leaf slices were prepared as described in the text and vacuum infiltrated with 50 μCi of ^{35}S-sulphate and incubated for 24 hr at 30° in the dark. Actinomycin D was vacuum infiltrated 24 hr before the label.



250
200
150
100
50
0
35S-COUNTS/MIN
without ACTINOMYCIN D
with ACTINOMYCIN D
0
10
20
30
40
50
60
70
80
SLICE NUMBER

CHAPTER FOUR

A COMPARISON OF TWO PLANT VIRUS-INDUCED RNA POLYMERASES

A COMPARISON OF TWO PLANT VIRUS-INDUCED RNA POLYMERASES

Our laboratory previously reported the properties of a CMV-induced RNA-dependent RNA polymerase present in both soluble and particulate fractions of infected cucumber cotyledons (Gilliland and Symons, 1968; May *et al.*, 1969; May *et al.*, 1970; May and Symons, 1971). In order to provide evidence that the CMV-induced enzyme is virus specific and not simply due to activation of a latent host enzyme, a comparison of the properties of the partly purified RNA polymerases induced after infection by CMV and the unrelated TRSV has been made. The RNA content of the TRSV virion is twice that of CMV (Randles and Francki, 1965; Stace-Smith *et al.*, 1965). Any differences found would provide circumstantial evidence that the virus specifies at least part of the RNA polymerase since if the RNA polymerase activity found was due to an activation of an already present but latent host enzyme, then it might be expected that infection with any plant virus would activate the same enzyme. However, it is quite possible that, if the viral RNA replicating system of CMV and TRSV-infected plants is similar to that of Qβ phage, then it would consist of one viral-coded polypeptide and several host-coded polypetpides (Kondo *et al.*, 1970; Kamen, 1970). Hence the differences expected in partly purified enzyme preparations would most likely be small.

SECTION 1. PROPERTIES OF THE SOLUBLE TRSV-INDUCED RNA POLYMERASE.

The method for the preparation of the partly purified soluble RNA polymerase from TRSV-infected cucumber cotyledons was the same as that used for the CMV-induced RNA polymerase (May *et al.*, 1969) except that plants were harvested 2-4 days after infection, and is described in Materials and Methods. RNA polymerase activity was assayed using α-^{32}P-GTP as the labelled substrate in the presence of 13mM $MgSO_4$ as described in Materials and Methods.

(a) Effect of Temperature, Time of Incubation and pH of the Assay Medium.

It was found that the optimum temperature for TRSV-induced RNA polymerase activity was 37° and at this temperature the incorporation of labelled substrate into RNA product was linear for at least 60 min. The optimum pH for the reaction was between 8.0 and 8.5. These results compare almost exactly to those reported for CMV-induced RNA polymerase activity (May *et al.*, 1969).

(b) Nucleoside Triphosphate Requirements of the Assay Medium.

The dependence on the presence of all four nucleoside triphosphates in the measurement of soluble RNA polymerase activity was investigated using α-^{32}P-GTP as

labelled substrate and omitting either one or all three of the other triphosphates from the assay medium. Table 1 shows that there seems to be an absolute requirement for all four triphosphates for TRSV-infected plants, and these results agree with the previous finding of May *et al.*, (1969) for CMV-infected cucumber cotyledons (shown for comparison).

(c) Effect of Magnesium Ion Concentration on the Assay Medium.

By varying the concentration of Mg^{++} ions in the assay medium from 0-50mM it was found that the optimum concentration for RNA polymerase activity was between 6 and 30mM when yeast RNA was used as template. This agreed almost exactly with the Mg^{++} optimum profile for CMV-induced RNA polymerase. When poly C was used as template the Mg^{++} optimum was around 10mM compared to the CMV-induced enzyme activity at 6-10mM.

(d) Effect of Adding Various RNA Templates.

As with the CMV-induced soluble RNA polymerase, the TRSV-induced enzyme showed activity with or without an added RNA template (see May *et al.*, 1969; May and Symons, 1971). This presumably reflected the presence of an RNA in the partly purified enzyme preparations. It was found that this activity present when no RNA was added could be competed out by addition of a template (May *et al.*, 1969;

May and Symons, 1971). The following RNA samples were tested in the assay mixture to see if the TRSV-induced RNA polymerase would use them - 250 μg of either yeast RNA, TRSV RNA, CMV RNA, TMV RNA and also poly C. Results showed that each of these templates were used and thus no specificity was shown towards TRSV RNA, a similar situation to that found for the CMV-induced polymerase where no specificity was shown by that enzyme for CMV RNA.

(e) Resistance of the RNA Polymerase Activity to Preincubation with Pancreatic Deoxyribonuclease, Actinomycin D or Phosphate.

Incubation of the TRSV-induced RNA polymerase with pancreatic deoxyribonuclease (10 μg/ml at 37° for 30 min), actinomycin D or orthophosphate had no effect on the incorporation of α-^{32}P-GTP. This was similarly found for the CMV-induced enzyme.

(f) Inhibition of the RNA Polymerase Activity by Preincubation with Ribonuclease or Pyrophosphate.

Incubation with pancreatic ribonuclease (5 μg/ml at 37° for 30 min) or pyrophosphate caused an inhibition of the incorporation of α-^{32}P-GTP, a situation also found with the CMV-induced RNA polymerase.

(g) Time Course of Appearance of the TRSV-induced RNA Polymerase.

Figure 13 shows the appearance of the soluble virus-induced RNA polymerase in cucumber cotyledons as a function of time after infection with CMV and TRSV and with yeast RNA as template. The TRSV-induced RNA polymerase rose rapidly after 1 day to a peak of activity on the third day and then decayed rapidly. By contrast the CMV-induced RNA polymerase activity rose to a plateau level after 10 days and remained there for a further 6 days (see also Gilliland and Symons, 1968; May et al., 1970). The same time course of activity was found whether the polymerase was assayed in the presence or absence of added RNA or poly C, or when particulate fractions were used (see Section 2). No RNA polymerase activity was detected in either soluble or particulate fractions of uninfected plants prepared under identical conditions or in extracts prepared as described by Astier-Manifacier and Cornuet (1971) for Chinese cabbage except that the agarose column chromatography step was omitted.

(h) Sedimentation Analysis of the RNA Polymerase.

The RNA polymerase from CMV-infected cucumber cotyledons was centrifuged through a 5-20% sucrose gradient (Figure 14) with haemoglobin as marker. Using the equation of Martin and Ames (1961) and taking the mol. wt. of haemoglobin to be 68,000 daltons, the mol. wt. of the

CMV-induced RNA polymerase was calculated to be 123,000 daltons, confirming the value obtained by May *et al*. (1969). In contrast to the sharp peak of CMV-induced RNA polymerase activity, the TRSV-induced enzyme activity showed a wide activity profile with a mol. wt. range of 120,000 to 180,000 daltons. Identical results were obtained when poly C (100 μg/assay) replaced yeast RNA as template. Since these sedimentation profiles were reproducible (6 experiments) it was concluded that the mol. wt. differences reflected real differences in the structure of the soluble enzymes and not just variations in the extraction technique.

(i) Properties of the Product of the Soluble RNA Polymerases.

The product of the RNA polymerase reaction was isolated as follows. After incubation, the reaction mixture containing the radioactive product (5-20 times the normal assay size and about 2.5 x 10^6 cpm of labelled substrate/ assay) was shaken with SDS (final concentration 1%) and Chelex resin (Na^+, 50 mg/ml) (Feix *et al*., 1967). An equal volume of 78% aqueous phenol was added and the mixture shaken vigorously for 10 min. After centrifugation at 5,000 *g* for 10 min the aqueous layer was collected and most of the residual phenol removed by shaking with an equal volume of ether. The ether-water mixture was centrifuged at 5,000 *g* for 10 min and the aqueous layer removed, the RNA precipitated by the addition of two

volumes of ice-cold ethanol and the precipitate dissolved in 0.15M NaCl, 0.015M sodium citrate, pH 7.0 (SSC) and used for studies on the nature of the RNA product.

When either TRSV RNA or yeast RNA was used as template for the TRSV-induced RNA polymerase, more than 80% of the product was found to be resistant to digestion with pancreatic ribonuclease (5.0- 10 μg/ml at 37° for 30 min) either before or after phenol deproteinization of the mixture. This resistance to ribonuclease indicated the presence of a double-stranded RNA structure. Similar results were found for the product of the CMV-induced RNA polymerase (May *et al.*, 1969).

SECTION 2. PROPERTIES OF THE PARTICULATE TRSV-INDUCED RNA POLYMERASE.

As with the soluble enzyme, the method of extraction of the particulate TRSV-induced RNA polymerase from cucumber cotyledons was the same as that described for the particulate CMV-induced enzyme (May *et al.*, 1970) and is described in Materials and Methods. Here again plants were harvested between 2 and 4 days after infection. RNA polymerase activity was assayed as before with linearity lasting for about 60 min (CMV-induced enzyme was linear for at least 30 min).

(a) General Properties.

The particulate TRSV-induced RNA polymerase had an absolute requirement for all four nucleoside triphosphates,

an optimum pH at about 8.5, an optimum temperature at 37° and an optimum Mg^{++} ion concentration of between 20 and 30mM with yeast RNA as template. Again more than 80% of the phenol extracted product was resistant to pancreatic ribonuclease digestion (5 - 10 µg/ml at 37° for 30 min).

(b) Solubilisation of the Particulate RNA Polymerase Activity.

May *et al*. (1970) found that the CMV-induced particulate RNA polymerase activity, dependent on an added RNA template, could be released into the soluble phase by two techniques; this solubilised enzyme had similar general properties to the soluble CMV-induced RNA polymerase. It was decided to see if the particulate TRSV-induced RNA polymerase could be solubilised by the same techniques.

(i) $MgSO_4$ solubilisation.

Particulate RNA polymerase extracts (16,000-P) were prepared as described in Materials and Methods and made 70mM with respect to $MgSO_4$, incubated at 37° for 15 min, cooled in ice and centrifuged at 25,000 *g* for 10 min. The supernatant was collected and used as the '$MgSO_4$ solubilised RNA polymerase'.

For the CMV-induced particulate RNA polymerase May *et al*. (1970) found that 90% of the activity could be solubilised by incubating with 70mM $MgSO_4$. Also during the normal assay of particulate polymerase with yeast RNA as

template about 90% of the activity was released into the supernatant.

With the TRSV-induced particulate RNA polymerase approximately 10% of the activity assayed with yeast RNA as template was released by $MgSO_4$ incubation and this was always low and variable. During a normal assay none of the activity was released into the medium and the recovery was only 35% when assayed with yeast RNA.

(ii) Freezing and thawing solubilisation.

Particulate RNA polymerase extracts (16,000-P) prepared as described in Materials and Methods were twice frozen in an ethanol-dry ice mixture followed by thawing in a water bath at 37° (liquid temperature did not rise about 4°). The preparation was centrifuged at 25,000 *g* for 10 min and the supernatant used as the 'frozen and thawed solubilised RNA polymerase'.

Freezing and thawing the particulate CMV-induced RNA polymerase caused the release of 50% of the activity assayed with yeast RNA as template. For the TRSV-induced particulate enzyme very low (approx. 5%) and variable release was found by freezing and thawing.

SECTION 3. DISCUSSION.

The differences found in the size of the soluble CMV and TRSV-induced RNA polymerases and in the solubilisation of these enzymes from particulate fractions therefore provide

circumstantial evidence for a viral-coded function in the RNA polymerases studied. The marked differences in the rate of appearance and decay of the virus-induced RNA polymerases after infection most likely only reflect differences in the life cycle of the two viruses, but they emphasize that the CMV-induced enzyme system is much more amenable to further characterisation since the activity in the plants lasts for a longer period of time.

TABLE 1. NUCLEOTIDE TRIPHOSPHATE REQUIREMENTS OF THE ASSAY SYSTEM USED FOR THE MEASUREMENT OF SOLUBLE RNA POLYMERASE ACTIVITY IN PARTLY PURIFIED EXTRACTS OF HEALTHY, CMV AND TRSV-INFECTED CUCUMBER COTYLEDONS.

	Specific Enzymatic Activity		
	Healthy Plants	CMV-Infected*	TRSV-Infected
Complete	0.2	19.4	26.1
- ATP	0.0	0.4	0.2
- CTP	0.3	0.3	0.4
- UTP	0.0	1.4	2.1
- ATP,CTP,UTP	0.2	0.2	0.3

*from J. May, Ph.D. Thesis, University of Adelaide, 1971.

Assays were carried out as described in Materials and Methods with 1.0 mg of yeast RNA per assay.

FIGURE 13. EFFECT OF TIME AFTER VIRUS INFECTION ON THE RNA POLYMERASE ACTIVITY IN SOLUBLE EXTRACTS OF CUCUMBER COTYLEDONS INFECTED WITH CMV OR TRSV.

Specific activity: pmoles of α-^{32}P-GTP incorporated into acid-insoluble material per min per mg protein with added yeast RNA as template.

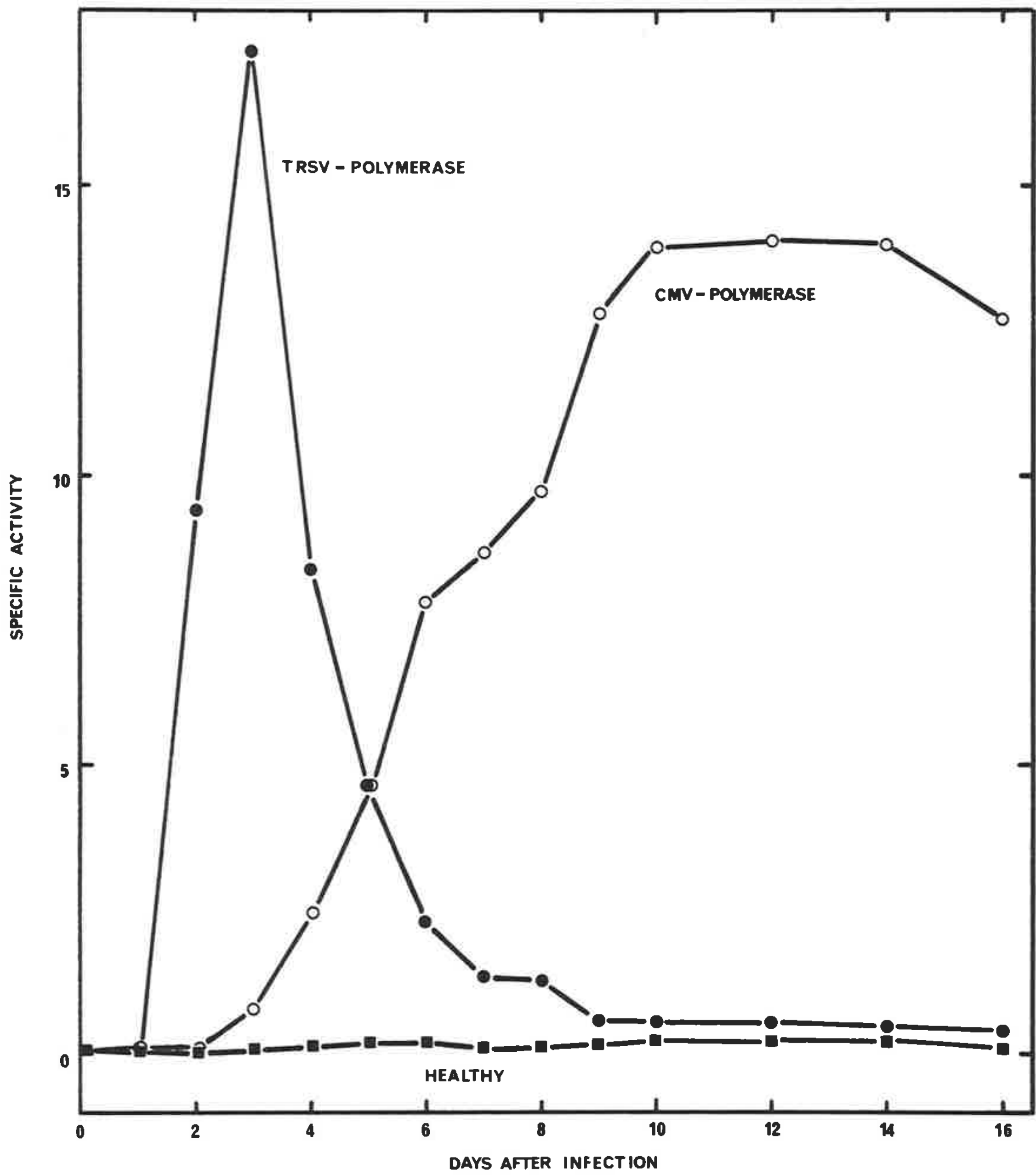
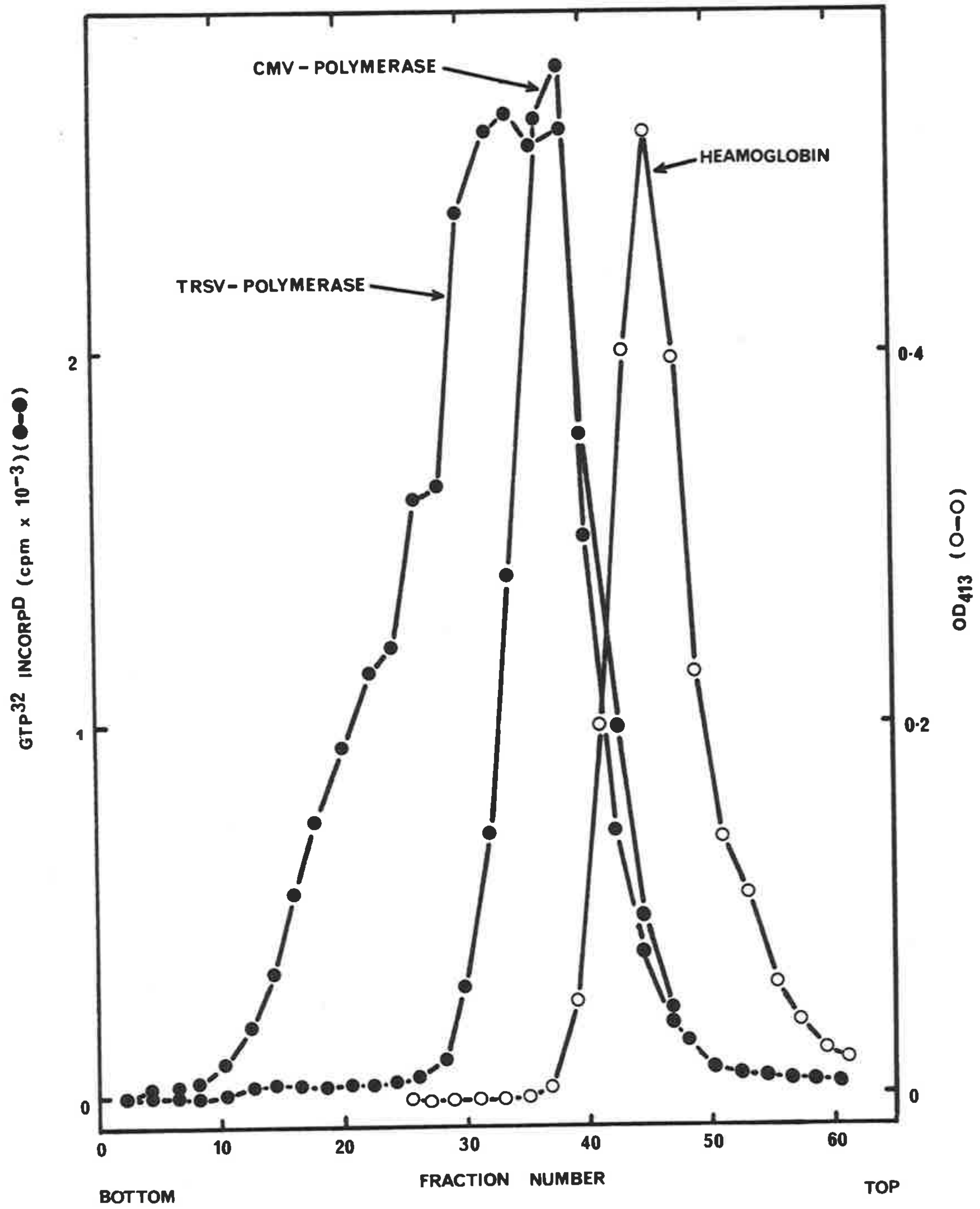

TRSV - POLYMERASE
CMV - POLYMERASE
HEALTHY
SPECIFIC ACTIVITY
DAYS AFTER INFECTION
0
5
10
15
0
2
4
6
8
10
12
14
16

FIGURE 14. SEDIMENTATION PROFILE OF THE SOLUBLE VIRUS-INDUCED RNA POLYMERASES.

The partly purified TRSV-induced RNA polymerase (2 mg of protein in 0.6 ml; prepared from plants 3 days after infection) and haemoglobin marker (2 mg) were centrifuged through an 11.6 ml gradient of 5-20% sucrose in 0.1M NH_4Cl, 90mM 2-mercaptoethanol, 50mM Tris-HCl buffer, pH 8.5, in a Beckman SW41 rotor at 36,000 rpm for 17 hours. Fractions (4 drop, 0.19 ml) were collected from the bottom of the tube and alternate fractions were assayed for RNA polymerase activity with 1.0 mg of yeast RNA per assay (see Materials and Methods) or the optical density read at 413 mμ. The partly purified CMV-induced RNA polymerase (2 mg of protein in 0.6 ml; prepared from plants 9 days after infection) plus haemoglobin (2 mg) were centrifuged at the same time under the same conditions in a separate tube. Recovery of RNA polymerase activity was 95% for both peaks.



CMV - POLYMERASE
HEAMOGLOBIN
TRSV - POLYMERASE
GTP32 INCORPD (cpm x 10-3)
OD413
2
1
0
0·4
0·2
0
0
10
20
30
40
50
60
FRACTION NUMBER
BOTTOM
TOP

CHAPTER FIVE

PARTIAL PURIFICATION OF THE CMV-INDUCED RNA POLYMERASE

PARTIAL PURIFICATION OF THE CMV-INDUCED RNA POLYMERASE

Having obtained circumstantial evidence for a viral-coded function in the CMV-induced and TRSV-induced RNA-dependent RNA polymerase (Chapter IV), more precise proof was sought. To show this definitely it would be necessary to examine in detail the structure of the enzyme. Obviously, to do this means that the enzyme must be pure. Another longer term advantage of having a purified enzyme would be that it might be possible to obtain antibodies to it. Attachment of a fluorescent or radioactive group to this antibody, labelling sections of infected cells and examining with the electron microscope where these antibodies end up could provide good evidence to where viral RNA is made in the host cell. Knowing where viral RNA is being made would complement an investigation of viral-induced protein and RNA species.

Initially the purification of only the soluble RNA polymerase was attempted.

SECTION 1. SIMPLE PRECIPITATION STEPS.

The attempted purification was carried out on the soluble enzyme, extracted in buffer E as described in Materials and Methods, 1 g of plants in 2 ml of buffer. This ammonium sulphate precipitate was dissolved in $^{1}/4$ of the original volume of extraction buffer (i.e., 1 g of plants in 0.5 ml of buffer F) for all purification

work. All operations were carried out at 4° unless otherwise stated.

(a) Protamine Sulphate Precipitation.

This was tried in an attempt to remove most of the green coloured material present in partly purified ammonium sulphate extracts. Various solutions of protamine sulphate were made - 0.1%, 0.5%, 1.0%, 1.5%, 2.0% (w/v) in water. Division of the ammonium sulphate prepared enzyme into five tubes was followed by the addition of one of the protamine sulphate solutions to each (0.05 ml for every ml of enzyme extract) with constant stirring. After centrifugation at 25,000 g for 10 min, the supenatants were assayed for RNA polymerase activity with yeast RNA as template. The following recoveries were found:

% Final Concentration of Protamine Sulphate	% Recoveries of RNA Polymerase Activity
0.005	109
0.025	94
0.05	95
0.075	81
0.1	80

Most of the green-coloured material was found to be

precipitated at protamine sulphate concentrations of 0.05% and higher. Thus it was decided to use a protamine sulphate precipitation step (final concentration 0.05%; 0.05 ml of a 1.0% solution per ml of enzyme extract) in the purifcation procedure.

(b) Acetone Fractionation.

In the purification procedure a concentration step was required. It was known that ammonium sulphate could be used to precipitate the RNA polymerase but this had a disadvantage in that the enzyme must be desalted before ion-exchange chromatography. Acetone precipitation was therefore tried.

To the ammonium sulphate prepared enzyme was added acetone at -15° with continuous stirring to a final concentration of 20% (v/v); fractions of 0-20%, 20-40%, 40-50%, 50-70% were collected by centrifugation at 25,000 *g* for 10 min and the pellets redissolved in 1/4 the original volume in buffer F. These dissolved pellets were assayed for RNA polymerase activity with yeast RNA as template. Results obtained were as follows:-

Acetone Fraction	% Recoveries
0-20%	3
20-40%	15
40-50%	14
50-70%	5
0-50%	30

As these results were discouraging a 0-50% cut was tried, but only 30% recovery could be achieved. It was decided not to persevere with acetone precipitation.

(c) Ethanol Fractionation.

The technique here was similar to that used for the acetone fractionation except ethanol was added at 0°. Fractions 0-20%, 20-30% and 30-40% were prepared and the pellets redissolved in $^{1}/4$ of the original volume of buffer E. Results were as follows:-

Ethanol Fraction	% Recoveries
0-20%	<1
20-30%	10
30-40%	4

These results showed extremely low recoveries from ethanol precipitation and it did not seem worthwhile to continue with it. It was decided not to spend any more time on precipitation procedures but to attempt to purify the RNA polymerase further by column chromatography. Of the precipitation steps tried only the protamine sulphate one was of any practical use.

SECTION 2. COLUMN CHROMATOGRAPHY PROCEDURES.

Initially small columns of the ion exchange resin were used. The enzyme preparation used for all this work was the partly purified soluble CMV-induced RNA polymerase prepared from a 50% ammonium sulphate precipitation (as described in Materials and Methods) followed by protamine sulphate precipitation (as described in Section 1 of this chapter) and dialysis for 2 hr in buffer H (10% glycerol) with two changes of buffer. For the remainder of this chapter this enzyme preparation will be referred to as the 'partly purified enzyme extract'.

(a) DEAE-cellulose.

(i) Elution with KCl buffer.

Washed DEAE-cellulose (Cl^-) was equilibrated overnight with buffer H before pouring a small column 4 cm x 1 cm. The partly purified enzyme extract was loaded onto it and run very slowly through (1 ml in 15 min). No more than 2 ml was ever loaded onto a column of this size. The column was treated in the following ways and RNA polymerase activity determined in each fraction.

Buffers Applied to Column (3 ml of each)	% Activity Recovered in Eluate
1. Run through and wash with buffer H	19
2. Washed with 0.15M KCl in buffer H	9
3. Eluted with 0.5M KCl in buffer H	20
4. Eluted with 0.5M KCl in buffer H	5

It was found that approximately 80% of the RNA polymerase bound to the DEAE-cellulose (this figure varied from 75-90% in different experiments; this presumably reflected the ionic strength of the enzyme preparations caused by differing efficiency of dialysis). Of this 80%, some activity was recovered after elution (25% of the original activity loaded onto the column) but this was too low for a purification step. Enzyme extracts containing more than 0.2M KCl would not bind to the DEAE-cellulose column.

(ii) Elution with K_2SO_4 buffer.

A similar experiment to that just described was conducted except that the column was washed with 0.15M K_2SO_4 and eluted with 0.5M K_2SO_4 in buffer H. Now 50% of the activity was eluted by 0.5M K_2SO_4 and protein estimations showed a three-fold purification.

(b) DEAE-sephadex.

After equilibrating washed DEAE-sephadex overnight in buffer H, a small (6 cm x 1 cm) column was poured and washed with 10 ml of buffer H. The partly purified enzyme extract was loaded on and run slowly into it as before (1 ml in 15 min). Two types of elutions were then carried out:-

Buffers Applied to Column (3 ml of each)	% Original Activity Recovered in Eluate
Experiment 1.	
1. Run through and wash with buffer H	11
2. Washed with 0.15M KCl in buffer H	2
3. Eluted with 0.5M KCl in buffer H	20
4. Eluted with 0.5M KCl in buffer H	15
5. Washed with 1.0M KCl in buffer H	14
Experiment 2.	
1. Run through and wash with buffer H	10
2. Washed with 0.15M KCl in buffer H	2
3. Eluted with 0.75M KCl in buffer H	50
4. Eluted with 0.75M KCl in buffer H	10

As can be seen from the results, it was necessary to use a higher concentration of KCl than 0.5M for elution

from DEAE-sephadex. Using 0.75M KCl, yields of about 60% could be obtained and it was decided to attempt the elution with a linear gradient on a larger column (10 cm x 2 cm). 4 ml of the partly purified enzyme extract was loaded onto the column and washed with 10 ml of buffer H. Elution was carried out with a linear KCl gradient of 0-0.75M in buffer H, the flow rate being 0.3 ml/min and 6 ml fractions collected. Fractions were assayed for RNA polymerase activity with yeast RNA as template and refractometry used to determine KCl concentration in each tube.

Regrettably no activity could be recovered from the column in any of three experiments. Since the RNA polymerase was stable at 4° overnight, it was concluded that activity was being lost by long exposure to the ion-exchange resin.

(c) Phospho-cellulose.

Phospho-cellulose has been used in the purification of other RNA polymerases (e.g., DNA-dependent RNA polymerase from *E. coli* (Burgess, 1969) and phage Qβ RNA replicase (Kamen, 1970)). The resin was washed according to Burgess (1969), equilibrated overnight in buffer H and a small column (4 cm x 1 cm) poured. Partly purified enzyme extract (2 ml) was loaded on and treated in the following way:-

Buffers Applied to Column (3 ml of each)	% Activity Recovered
1. Run through and wash with buffer H	10
2. Washed with 0.15M KCl in buffer H	3
3. Eluted with 2.0M KCl in buffer H	58
4. Eluted with 2.0M KCl in buffer H	10

It was found that 90% of the enzyme activity bound to the column and about 70% could be recovered. It was decided to try another salt for elution in attempt to increase recovery of the activity. Ammonium sulphate was chosen since the RNA polymerase was stable to this salt during extraction. An experiment similar to that described above was done except that the elution was carried out with 1.0M ammonium sulphate in buffer H instead of the 2.0M KCl. It was found that recoveries were as high as 80% relative to the initial activity loaded onto the column. Thus it seemed that phospho-cellulose would be useful in the purification procedure.

(d) CM-cellulose.

Washed CM-cellulose was equilibrated overnight as before, a small column poured and a similar experiment carried out as described for DEAE-cellulose with elution with 0.5M KCl. Results were conclusive - no RNA

polymerase activity would bind to the column, and most other proteins would not either. Hence this resin was of no use in the purification procedure.

(e) RNA-cellulose.

This resin was tried since RNA, being a substrate for the enzyme, may help to stabilise it. The method used to prepare RNA-cellulose was that described by Fedoroff and Zinder (1971). However, after three unsuccessful attempts at its preparation, further attempts were discontinued.

(f) Poly C-sepharose.

This was made by Dr. J.T. May in our laboratory according to the method of Poonian *et al*. (1971) and stored moist at 4°; before use it was equilibrated overnight with buffer H. A very small column was poured (3 cm x 0.5 cm) and through this was run slowly 1 ml of partly purified enzyme extract and treated with the following buffers:-

Buffers Applied to Column (3 ml of each)	% Activity Recovered
1. Run through and wash with buffer H	9[a,b]
2. Eluted with 100mM $MgSO_4$ in buffer H	75[a]
	85[b]

[a], measured with yeast RNA as template
[b], measured with polyC as template.

About 90% of the RNA polymerase activity bound to the poly C-sepharose. Using yeast RNA as template there was a three-fold increase in specific activity after poly C-sepharose chromatography.

SECTION 3. DISCUSSION.

Although a purified enzyme has not been achieved, certain purification steps have been tried and found successful. These are protamine sulphate precipitation of the original ammonium sulphate prepared soluble enzyme, stepwise elution from a DEAE-sephadex column with 0.75M KCl, stepwise elution from a phospho-cellulose column with 1.0M ammonium sulphate and a stepwise elution from a poly C-sepharose column with 100mM $MgSO_4$. Only stepwise elutions seem to be possible due to the instability of the RNA polymerase once subjected to prolonged periods of ion-exchange chromatography; linear gradient elution may be possible with phospho-cellulose. This could reflect the fact that the enzyme is made up of subunits, as is suspected, and during chromatography these subunits are being torn from the enzyme producing inactive fragments. May (1971) found that the enzyme was stable in 50% glycerol for several weeks. Perhaps by running the columns in higher glycerol (40-50%) or high sucrose concentrations (60%), activity can be preserved during chromatography.

Another possible way of increasing the purity

of the enzyme would be to run it through various columns under conditions in which no RNA polymerase binds but some other proteins do. For example, by loading the RNA polymerase onto a DEAE-cellulose column in 0.3M KCl, the RNA polymerase will not bind to the column but some other proteins and all the free RNA of mol. wt. of tRNA and above will.

Thus the suggested way to purify the CMV-induced RNA polymerase is to add protamine sulphate (0.05% final concentration) to the ammonium sulphate prepared enzyme, run it through a small DEAE-cellulose column in 0.3M KCl, and then subject the enzyme to phospho-cellulose chromatography with either stepwise or gradient elution. Affinity chromatography with RNA cellulose or poly C-sepharose could be used to further purify the RNA polymerase. Unfortunately, lack of time did not permit this procedure to be tried.

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