STUDIES ON RIBONUCLEIC ACIDS ASSOCIATED WITH THE REPLICATION OF TOBACCO RINGSPOT VIRUS

by

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STATEMENT

This thesis has not previously been submitted for an academic award at this or any other university, and is the original work of the author, except where due reference is made in the text.

M.A. Rezaian

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CONTENTS

SUMMARY

The concentration of tobacco ringspot virus (TRSV) in the cotyledons of cucumber and primary leaves of French bean increased linearly between two and five days after inoculation and decreased thereafter when the plants were grown at 25° under constant illumination. The proportion of middle (M) to bottom (B) component in TRSV preparations purified from cucumber cotyledons did not vary significantly between three and twelve days after inoculation. In some virus preparations, dimers and trimers of B component were observed. TRSV-RNA species of molecular weight about 1.4×10^6 (RNA_1) and 2.3×10^6 (RNA_2) daltons were isolated from TRSV preparations and it was confirmed that M component particles contained one molecule of RNA_1 whereas B particles contained either one molecule of RNA_2 or two molecules of RNA_1.

High molecular weight double-stranded RNA (ds-RNA) with the expected properties of either replicative form or replicative intermediate was not detected in TRSV-infected plants. However, a polydisperse ds-RNA with nucleotide sequence complementary to TRSV-RNA was isolated which had a mean molecular weight of 50,000 daltons and a mean sedimentation coefficient of 6S. This TRSV-specific ds-RNA was

unaffected by deoxyribonuclease or ribonuclease A (RNase) in high salt containing buffers, but was digested by RNase in media with low salt concentration or after heat denaturation. It had a buoyant density of 1.60 gm/cm^3 in caesium sulphate, a sharp-heat denaturation curve with a Tm of 91.5 in 1 x SSC and reacted with formaldehyde at 75° but not at 37°. However, its chromatographic behaviour on MAK, cellulose and hydroxyapatite columns was unlike that expected for a ds-RNA, and it did not react with an antiserum to poly inosinic : poly cytidilic acid. The denaturation of TRSV-specific ds-RNA did not change its electrophoretic mobility significantly; this, together with its RNase resistance and thermo-denaturation profiles, suggested that the ds-RNA molecules must have a regularly base-paired "hair-pin" structure.

The saturation hybridization of TRSV-specific ds-RNA with ^{14}C-labelled TRSV-RNA indicated that the ds-RNA contained nucleotide sequences complementary to all the TRSV-RNA base sequences and it was estimated that about 150 ds-RNA molecules are required to anneal to the full length of TRSV-RNA_1 and RNA_2.

The ds-RNA and the RNA-dependent RNA polymerase activity were found to be associated with the 17,000g supernatant of the extracts of TRSV-infected cucumber cotyledons. Fractions containing chloroplasts and nuclei had neither significant RNA-dependent RNA polymerase activity

nor contained TRSV-specific ds-RNA.

Although the function of TRSV-specific ds-RNA remains obscure, it appears to be involved in the synthesis of TRSV because: (a) it could be isolated in high yields only during the period of rapid virus multiplication, and (b) its concentration in cucumber cotyledons was positively correlated with the activity of virus-induced RNA-dependent RNA polymerase.

Using the virus-specific ds-RNA, the nucleotide composition of TRSV-RNAs were compared by competition hybridization techniques. The results indicated that RNA_1 isolated from either M or B component particles of TRSV have indistinguishable nucleotide sequences. However, the sequences of RNA_2 are distinct from those of RNA_1 although these RNAs may have sequences of 900 nucleotides in common. The ds-RNA appears to contain two populations of RNA; one containing the nucleotides complementary to the sequences of RNA_1 and the other to those of RNA_2. The proportion of these two populations was similar to that of RNA_1 and RNA_2 in TRSV-RNA preparations.

CHAPTER 1

GENERAL INTRODUCTION

I. Tobacco Ringspot Virus and Viruses with Divided Genome

Viruses with polyhedral particles of about 30 nm, consisting of a protein capsid and single-stranded (ss-) RNA are often referred to as small RNA viruses. These viruses infect plants and a wide range of other organisms (Brown and Hull, 1973). Recent findings have shown that the genome of many of the small plant viruses is carried in more than one particle. Viruses with such functional heterogeneity have been referred to as viruses with divided genomes (van Kammen, 1972). An example of such a system is brome mosaic virus (BMV) whose genome consists of four species of RNA, probably carried in three distinct particles. These component particles are thought to contain about the same net amount of RNA and since their protein composition is the same, they are not separable by the conventional method of analysis (normally rate zonal centrifugation in sucrose) (Lane and Kaesberg, 1971). Although the function of different RNA species in most plant viruses with divided genomes is not understood, recently Shih and Kaesberg (1973), using the wheat

emryo protein synthesizing system directed by BMV-RNA species, have shown that the two smallest viral RNA species each contain the viral coat protein cistron. Cucumber mosaic virus with many properties similar to those of BMV has also been identified as a virus with a functionally divided genome (Peden and Symons, 1973).

There are some other RNA viruses with divided genomes which can be readily separated into component particles. Examples are cowpea mosaic virus, alfalfa mosaic virus (see van Kammen, 1972), and, as has been suggested by Harrison *et al.* (1972a) and Murant *et al.* (1973), viruses of the nepovirus group, of which tobacco ringspot virus (TRSV) is a type member.

Preparations of cowpea mosaic virus consist of three centrifugal components which are somewhat similar to those of nepoviruses. These component particles sediment at 58S, 95S and 118S and contain 0%, 24% and 34% RNA respectively (van Kammen, 1972). Van Kammen (1968) demonstrated that when the components of cowpea mosaic virus were isolated in pure form, none of them could initiate infection and both RNA containing particles were required for virus replication.

Nepoviruses have polyhedral particles of about 27.5 nm and are transmitted by nematodes (Cadman, 1963; Harrison *et al.*, 1971). Another member of this group which has been extensively studied is raspberry ringspot virus (RRV) (Murant *et al.*, 1972; Harrison *et al.*, 1972a; 1972b). Isometric particles of nepoviruses have apparently

identical protein capsids but sediment at three different rates; about 58S (top component or T), 94S (middle component or M) and 128S (bottom component or B) (Randles and Francki, 1965; Stace-Smith *et al.*, 1965). While T component particles are empty protein shells, each particle of M contains one RNA molecule of MW ≃ 1.4 x 10^6 daltons (RNA_1) and each of B contains either one RNA molecule of MW ≃ 2.3 x 10^6 daltons (RNA_2) or two molecules of RNA_1 (Diener and Schneider, 1966; Murant *et al.*, 1972).

It has been shown that only B component particles of TRSV and RRV are infectious (Diener and Schneider, 1966; Francki, 1972; Harrison, 1972a). However, the observation that the infectivity of RNA_2 of TRSV, RRV and tomato plack ring virus can be increased by the addition of homologous RNA_1 (Harrison *et al.*, 1972a; Murant *et al.*, 1973) indicates that the nepoviruses have divided genomes. Using RNA_1 and RNA_2 from different strains of (RRV), it was shown that RNA_1 determines the serological specificity and symptom expression of the hybrid virus progeny (Harrison *et al.*, 1972b).

A few satellite virus isolates have been identified which require the presence of TRSV for their own multiplication (Schneider, 1971; Schneider *et al.*, 1972a). One of the satellites of TRSV has a protein composition identical to that of TRSV but contains 12 - 25 molecules of an homogeneous RNA of MW = 86,000 daltons (Schneider *et al.*, 1972b).

II. Double-stranded Forms of Viral RNA

Independence of viral RNA replication from host DNA, and the finding that double-stranded (ds-) DNA is involved in the replication of ss-DNA containing phage Qx174 led to the discovery of ds-RNA in encephalomyocarditis virus infected cells (Montaignier and Sanders, 1963). Since then, two distinct classes of viral RNA other than the viral genome have been identified and isolated from virus-infected plants and other organisms; a completely double-stranded RNA designated as replicative form (RF) and a partly double-stranded replicative intermediate (RI) (Anman *et al.*, 1964; Fenwick *et al.*, 1964; Pinck *et al.*, 1968; Nilson-Tillgren, 1970; Jackson *et al.*, 1971; see also reviews by Ralph, 1969; Bishop and Levintow, 1971; Stavis and August, 1970). Moreover, a number of low molecular weight RNA species have also been detected in association with some virus infections but their functions are not understood (Reich *et al.*, 1966; Banergee *et al.*, 1969; Marcaud *et al.*, 1971).

In virus infected bacterial or animal cells, RF comprises as little as 1-5% of the virus specific RNA (Bishop and Levintow, 1971) and its concentration in TYMV infected tissue is estimated to be about 2 µg per gm fresh tissue (Matthews, 1970). RF in high NaCl concentrations is relatively resistant to ribonuclease (RNase) but it can be hydrolysed by long incubations with high concentrations of RNase (Bishop and Levintow, 1971). A few reports have described

the isolation of RNase III which digests ds-RNA (Libonati and Floridi, 1969; Robertson and Mathews, 1973); RNase III from *E. coli* is specific for ds-RNA and does not hydrolyse single-stranded RNA (Robertson *et al.*, 1968). RNase A in the dimeric form is also active in hydrolysis of ds-RNA (Alessio *et al.*, 1972). Some other characteristics of RF can be summarized as follows:

(a) Equal amounts of complementary nucleotides (A = U and G = C), therefore RF has about twice the molecular weight of the corresponding ss-RNA (viral RNA or plus strand);

(b) solubility in high salt solution (1M NaCl or higher), and

(c) heat denaturation resulting in separation of complementary strands (at about 100°) and reannealing to form double-stranded structures on slow cooling (see Ralph, 1969; Bishop and Levintow, 1971).

RI, on the other hand, is only partly resistant to RNase and is known to consist of a double-stranded core which is resistant to RNase, with single- stranded branches. The exact structure of RI is not known; however, on RNase treatment it assumes the properties of RF. RI is insoluble in 1M NaCl and has a higher sedimentation coefficient than the corresponding RF. The buoyant density of RI in Cs_2SO_4 is less than that of single-stranded RNA but it bands at a higher density than RF.

There is considerable evidence that RI is an intermediate in

viral RNA replication (Bishop and Levintow, 1971). *In vivo* and *in vitro* experiments with bacteriophage and animal virus systems have shown that in a short exposure of radioactive precursor (pulse), RI is labelled and on addition of excess cold precursor (chase) the radioactivity is detected in progeny viral RNA (Noble and Levintow, 1970; Girard, 1969; Pace *et al.*, 1968; Baltimore and Girard, 1966; Kelly *et al.*, 1965). Results of recent studies with plant virus systems support the general view that the replication of viral RNA proceeds via RI. Jackson *et al.* (1972) carried out pulse-chase experiments with separated leaf cells from TMV infected plants and found that the radioactive precursor was first incorporated mainly into TMV-RF and RI. The radioactivity could be chased completely from RI and partially from RF into TMV-RNA. In their studies of RNA synthesis *in vitro*, Semal and Kummert (1971) utilized the pulse-chase procedure and found that ds-RNA and ss-RNA appeared sequentially in cell free extracts of BMV infected tissue. They later (Kummert and Semal, 1972) found that after a 2 min radioactive pulse, both 2M LiCl-soluble (a property of RF) and LiCl-insoluble (a property of RI) fractions of BMV induced RNA-polymerase product were largely RNase resistant in high NaCl concentrations. The latter fraction had sedimentation properties of RI. After a 2 min chase, the LiCl-insoluble fraction was RNase sensitive and sedimented with the three BMV-RNA species. Jacquemin (1972) also reported an essentially similar finding while studying the *in vitro* product of an RNA

polymerase induced by broadbean mottle virus. These investigations all lead to the conclusion that the synthesis of ss-RNA proceeds via RI.

The role of RF in the viral RNA replication is not certain, mainly because its formation and accumulation *in vivo* is not correlated with either RI or ss-RNA and its pattern of appearance suggests that it may be an end product of viral RNA replication (Noble and Levintow, 1970; see Reddi, 1972). However, a contrary indication is that *in vivo*, parental RNA first appears in the form of RNase resistant ds-RNA (Pfefferkorn *et al.*, 1967).

Although RI and RF are the central topics in discussions on the replication of viral RNA, they may not represent the *in vivo* structures thought to be functional in viral RNA replication. Investigations on Qβ bacteriophage (Weissmann *et al.*, 1968) and poliovirus (Öberg and Philipson, 1971) indicate that the extensive base pairing in the double stranded forms of viral RNA may be the result of deproteinization during the RNA extraction procedure. However, contrary indications (see Bishop and Levintow, 1971) leave the *in vivo* states of RF and RI uncertain.

III. Scope of the Present Work

The origin and function of TRSV components and their nucleic acids are not understood. Investigations by Diener and Schneider

(1966) led to the suggestion that TRSV nucleic acid may be synthesized in the form of two pieces, which may or may not be equal in size and later join to make an infectious unit. However, they could not rule out that RNA_1 was a degradation product. On the other hand, Harrison *et al.* (1972a) showed that the infectivity of TRSV-RNA_2 can be increased by the addition of RNA_1, suggesting that the latter is functional.

One approach to the problem of understanding the origin of TRSV-RNA species is a comparison of the nucleotide sequences of viral RNAs by means of hybridization with the virus-specific ds-RNA. Such techniques have been used for study of sequence homology between RNA species of influenza virus (Content and Duesberg, 1971), cowpea mosaic virus (van Kammen, 1971) and tobacco rattle virus (Minson and Darby, 1973). Therefore, the first objective of this work was to isolate TRSV-RF from virus-infected plants. Although structures with the expected properties of TRSV-RF RNA could not be detected, a low molecular weight ds-RNA was isolated. Because of its unusual properties, the virus-specific ds-RNA was characterized in some details. Since it appeared that this low molecular weight ds-RNA contained all complementary nucleotide sequences of TRSV-RNAs, it was used in hybridization experiments and the nucleotide sequences of TRSV-RNA species were compared.

CHAPTER 2

GENERAL MATERIALS AND METHODS

In order to avoid repetition, some of the methods employed routinely and the materials used during the course of this investigation are described below.

1. Materials

Unlabelled nucleoside triphosphates, pyruvate kinase, phosphoenol pyruvic acid (tri-sodium salt), ribonuclease (RNase A, pancreatic), deoxyribonuclease (DN-EP, electrophoretically pure), pronase (type VI fungal protease) were obtained from Sigma Chemical Co., St. Louis, U.S.A. Polyinosinic : polycytidylic acid (poly (I) : poly (C)) double-stranded polynucleotide was purchased from Nutritional Biochemical Corporation, Cleveland, Ohio. ^{14}C-bicarbonate (sodium salt), ^{3}H- and ^{14}C-uridine were obtained from the Radio- chemical Centre, Amersham, and NCS from Amersham/Searle, Illinois. Methylated albumin was kindly provided by Dr. J.W. Randles of the University of Adelaide; yeast RNA, cellulose (Whatman standard grade) and ^{32}P-GTP were generous gifts from Dr. R.H. Symons

of the same University; CF11 cellulose was that used by Jackson *et al.* (1971) and was kindly provided by Dr. A.O. Jackson of Purdue University. High specific activity ^{32}P-orthophosphate was supplied by Australian Atomic Energy Commission and hydroxyapatite by Clarkson Chemical Co., Williamsport, U.S.A. Antiserum to synthetic poly (I) : poly (C) was a gift from Dr. R.I.B. Francki of this University. t-RNA was prepared from cucumber cotyledons by G-200 Sephadex chromatography (see Chapter 5). Other chemicals were of laboratory grade or analytical reagent grade.

2. Plants and Viruses

TRSV originally isolated from *Gladiolus* (Randles and Francki, 1965) was maintained either on cucumber (*Cucumis sativus* L. cv. Polaris) seedlings or French bean (*Phaseolus vulgaris* L. cv. Hawkesbury Wonder). A tobacco mosaic virus (TMV) strain of unknown origin (Crowley *et al.*, 1969) was maintained on French beans. Plants were raised in a glasshouse; 7-10 days after sowing, both surfaces of the cucumber cotyledons or primary leaves of French beans were dusted with 500-mesh carborundum and then inoculated with virus by rubbing a plant extract on the leaves with the forefinger. Excess inoculum was washed off with tap-water and the plants were maintained in a constant temperature room at 25 $\pm$ 2° under fluorescent lights providing continuous illumination of 350-450 f.c. TRSV infected cucumber cotyledons were

sometimes kept at -15° for up to three months and used as a source of inoculum, without any obvious loss of infectivity.

3. Purification of TRSV and TMV

All centrifugations were carried out at 4°. TRSV was purified by a combination of polyethylene glycol (PEG) precipitation and ultracentrifugation (Atchison, 1971). Cucumber cotyledons infected for 5 days were homogenized at room temperature with 1 ml 0.1M phosphate buffer, pH 7, and 1 ml chloroform per 1 gm of leaf tissue and centrifuged at 10,000g for 30 min. The buffer phase was recovered, PEG (4000) and NaCl were added to a final concentration of 6% and 0.3M respectively and the extract was left at 0° for 30 min. The precipitate was pelleted by centrifugation at 10,000g for 10 min, resuspended in 0.1M phosphate buffer, pH 7, and clarified by repeating the centrifugation. After addition of 0.01M EDTA and storage at 4° overnight, virus was sedimented by ultracentrifugation at 160,000g for 50 min, resuspended in 0.1M phosphate buffer, pH 7, and clarified by centrifugation at 10,000g for 10 min. Yield of virus was usually about 20 mg/100 gm of leaf tissue.

TMV was also purified by the PEG precipitation technique (Hariharasubramanian *et al.*, 1970). French bean leaves were harvested five days after inoculation with virus and ground in 2 volumes of 0.066M phosphate buffer, pH 7.0, containing 0.005M 2-mercaptoethanol.

The ground tissue was filtered through cheesecloth, heat coagulated at 50° for 15 min, and centrifuged at 10,000g for 15 min. The pellet was discarded; sodium chloride was added to 0.1M to the supernatant, followed by 4 gm of PEG (6,000) for every 100 ml. Immediately after stirring, the precipitate was collected by centrifugation at 10,000g for 10 min, resuspended in grinding buffer and centrifuged at 10,000g for 15 min. The supernatant was recovered, treated with sodium chloride and PEG and centrifuged as before. The pellet was taken up in the grinding buffer containing 1% Triton X-100 and the virus was pelleted after addition of salt and PEG as before. The pellet was resuspended in plain grinding buffer and clarified by centrifugation at 10,000g for 15 min. The supernatant was ultracentrifuged at 105,000g for 60 min and the virus pellet was taken up in 0.02M phosphate buffer, subjected to one more cycle of low speed and high speed centrifugation and the final pellet was resuspended in 0.02M phosphate buffer. Virus yield was about 400 mg per 100 gm of leaf tissue.

4. Preparation of Viral RNA

Purified preparations of TRSV and TMV were dissociated into protein and RNA by the single-phase phenol-sodium dodecyl sulphate (SDS) method (Diener and Schneider, 1968). Phenol-SDS reagent was prepared by adding 1 volume of 0.02M Na-phosphate buffer, pH 7, containing 3%

SDS and 10 mM EDTA, to 0.4 volume of water saturated phenol at 4°. Equal volumes of a virus preparation in 0.02M Na-phosphate buffer, pH 7, and the above reagent were mixed and left at 25° for 15 min. The protein was then removed by extracting twice with phenol; $\frac{1}{3}$ volume of water saturated phenol was added and mixed with a vortex for 1 min. After centrifugation at 5,000g for 10 min at 4°, the aqueous phase was extracted again with phenol. RNA was then precipitated by addition of two volumes of chilled ethanol and was left at -15° for at least 3 hr. The precipitate was recovered by slow speed centrifugation, washed with either cold ethanol or acetone and then with ether. It was dried under vacuum and resuspended in the required buffer. The RNA was divided into small portions and stored at -15°.

In the experiments described in Chapter 3, the dissociated virus was directly precipitated with ethanol and processed as described above.

5. Preparation of ^{14}C-labelled TRSV and TMV-RNA

Plants were transferred to a large desiccator immediately after inoculation with virus. $^{14}CO_2$ was released in the desiccator by introducing H_2SO_4 into a small beaker containing 2.5 mc of ^{14}C-labelled sodium bicarbonate. Five days later the plants were harvested, virus was purified and the RNA isolated as described above. The specific

activity of viral RNA was usually about 1,400 cpm/µg.

6. Density-gradient Centrifugation

Sucrose solutions were prepared in either 0.02M phosphate buffer, pH 7 (for analysis of virus and TRSV-RNA), or 1 x SSC buffer (for nucleic acids). Purified virus preparations were centrifuged on 5-25% linear gradients at either 25,000 rpm for 3 hr or at 50,000 rpm for 30 min, using SW25.1 or SW50 rotors respectively. RNA was centrifuged in similar gradients for 3 hr at 50,000 rpm. Optical density profiles of the gradients were determined at 254 nm using an ISCO U.V. analyzer.

7. Determination of TRSV Concentration in Tissue

Virus was precipitated with PEG from a known weight of leaf material processed as described above, resuspended in 0.1M phosphate buffer, pH 7, and subjected to sucrose density gradient centrifugation using a SW50 rotor. The gradients were analyzed with an ISCO density gradient analyzer and the virus concentration was determined from the peak areas recorded with the aid of a planimeter (Ott Kempton Bayern).

8. Polyacrylamide-gel Electrophoresis of Nucleic Acids

Polyacrylamide or composite polyacrylamide and 0.5% agarose gels were prepared in plexiglass tubes with 6 or 10 mm internal diameter as described by Loening (1969). The ratio of acrylamide to

bis-acrylamide monomers for different gel concentrations was determined according to the equation

$$100R = 6.5 - 0.3C,$$

where C = total monomer concentration and R = weight fraction of bis in monomer mixture (Adesnik, 1971).

Up to 200 µl of RNA preparations containing 20-200 µg of nucleic acids in 5% sucrose was loaded onto each gel. The maximum amount of RNA applied to gels with 6 mm diameter was 50 µg. Ten µl of 0.2% solution of bromophenol blue was layered onto a separate gel as a marker. The stock electrophoresis buffer contained 0.3M NaH_2PO_4, 0.36M Tris, 0.01M EDTA and 0.2% SDS. After electrophoresis at room temperature, at 80 volts for the required time, the gels were rinsed in distilled water and scanned at 265 nm in a Joyce Loebl Chromoscan densitometer. When it was required to locate RNA bands by staining, the gels were stained with 0.02% toluedene blue in 40% 2-methoxyethanol overnight, and destained with 30% 2-methoxyethanol (Clark and Lister, 1971).

9. RNA-RNA Hybridization Techniques

The ds-RNA preparation to be hybridized, suspended in 150 to 250 µl of SSC (0.15M NaCl and 0.015M sodium citrate) containing 2 µg of pronase, was pre-incubated for 30 min at 37° and then denatured by

heating for 10 min at 100°. The radioactive labelled ss-RNA was added and the samples transferred to a water bath at 85°, which was then allowed to cool to 37° over about 8 hr. When previously denatured ds-RNA was used for the hybridization, it was heated at 100° for only 3 min before adding ss-RNA. After reannealing, RNase was added to a concentration of 50 µg/ml and the samples were incubated at 37° for 30 min. The preparations were spotted on discs of Whatman No. 3 filter paper, excess water was evaporated by blowing air until the discs were moist and then they were extracted three times in 5% trichloroacetic acid (TCA), twice in 80% ethanol, once in ether (Byfield and Scherbaum, 1966), and then transferred to scintillation vials and dried. To each vial 10 ml of toluene based scintillation liquid containing 0.5% PPO and 0.01% POPOP was added for radioactivity determination in a Packard Scintillation Spectrometer.

In competition hybridization experiments, mixtures of ds-RNA and the required amounts of cold and ^{14}C-labelled viral RNA species were prepared in a volume of 0.25 ml and annealing was carried out as described above.

Fractions of sucrose density-gradients were directly used for hybridization (sucrose at a concentration of 25% had no significant effect on the annealing efficiency).

10. Heat Denaturation of ds-RNA

Samples of RNA in either 0.01 x SSC buffer or 1 mM EDTA,

pH 7.0, were heated at 100° for 10 min and immediately transferred to an ice-ethanol bath.

11. Precautions Against RNase

All buffers and solutions coming in contact with RNA were autoclaved at 15 psi for 15 min. Glassware was either autoclaved or heated at 130° for at least 2 hr. Plastic tubing, ISCO flow cell and gradient markers were rinsed with a detergent containing 10% KOH and 90% ethanol, and then washed with autoclaved distilled water. Dialysis membranes were soaked in a solution of 0.1% diethyl pyrocarbonate for 15 min and washed with distilled water.

12. Spectrophotometry

Virus and RNA concentrations were determined using extinction coefficients ($E^{0.1\%}_{1\ cm}$) at 260 nm of 25 for RNA, 7.0 for purified TRSV (Murant *et al.*, 1972) and 3 for TMV. Optical densities were determined using either a Unicam SP800 or SP1800 spectrophotometer.

Heat denaturation profiles were determined using a Unicam SP1800 spectrophotometer, coupled with a temperature programme controller and a Philips X-Y recorder. RNA suspensions in 1 ml cuvettes were degassed under vacuum, the cuvettes were stoppered before heating at a rate of 0.5°/min.

13. Centrifugation

All low speed centrifugations were carried out in an MSE Magnum centrifuge with a swing-out head or in an MSE high speed 18 machine. For ultracentrifugation either a Beckman Model L or L2-65 ultracentrifuge, with Beckman rotors, was used.

CHAPTER 3

SYNTHESIS OF TRSV IN CUCUMBER COTYLEDONS

INTRODUCTION

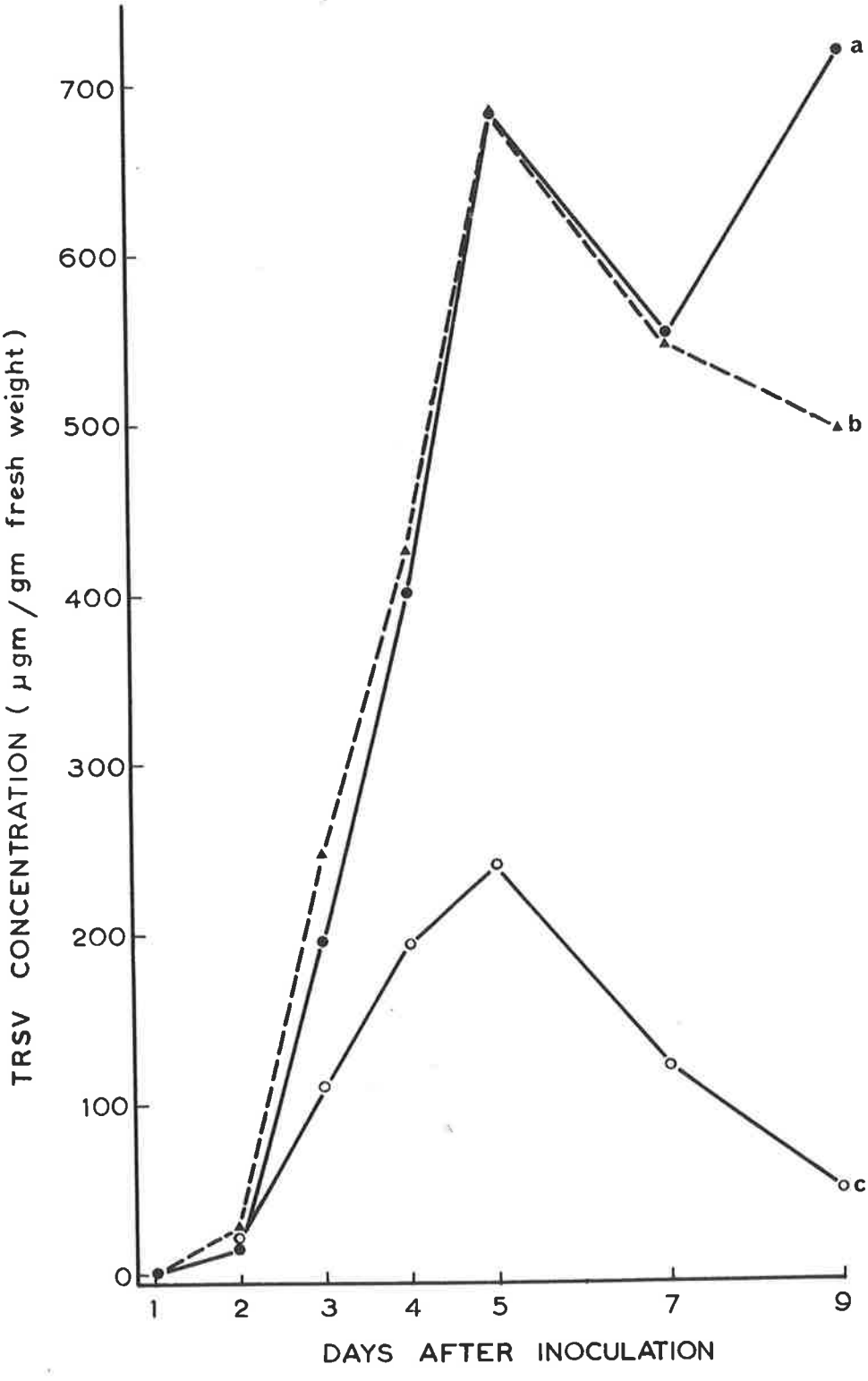
In planning the initial experiments for the detection of virus specific ds-RNA it seemed essential to have some information on the properties and the rate of multiplication of this isolate of TRSV in cucumber cotyledons. Therefore, the experiments described in this chapter were conducted, the results of which point to some differences in the mode of synthesis of TRSV components from those previously published (Schneider and Diener, 1966; 1968).

RESULTS

I. TRSV Multiplication in Cucumber Cotyledons

TRSV was readily detected by sucrose density-gradient centrifugation in cotyledons of cucumber plants grown at 25° under continuous illumination two days after inoculation and thereafter virus increase was linear until the fifth day after inoculation (Fig. 1). In most experiments the amount of virus in the cotyledons declined after reaching a maximum (Fig. 1). Chlorotic lesions on

Fig. 1. Multiplication of TRSV in cotyledons of cucumber plants. Partially purified virus from a known amount of cotyledon tissue was layered on 5-25% sucrose gradients in 0.02 M phosphate buffer, pH 7, centrifuged at 50,000 rpm for 30 min and analysed by an ISCO fractionator and recorded at 254 nm. Areas under the peaks of M and B components were measured and virus concentrations calculated from areas obtained in experiments using a known amount of virus. Curves a, b and c are from three independent experiments.

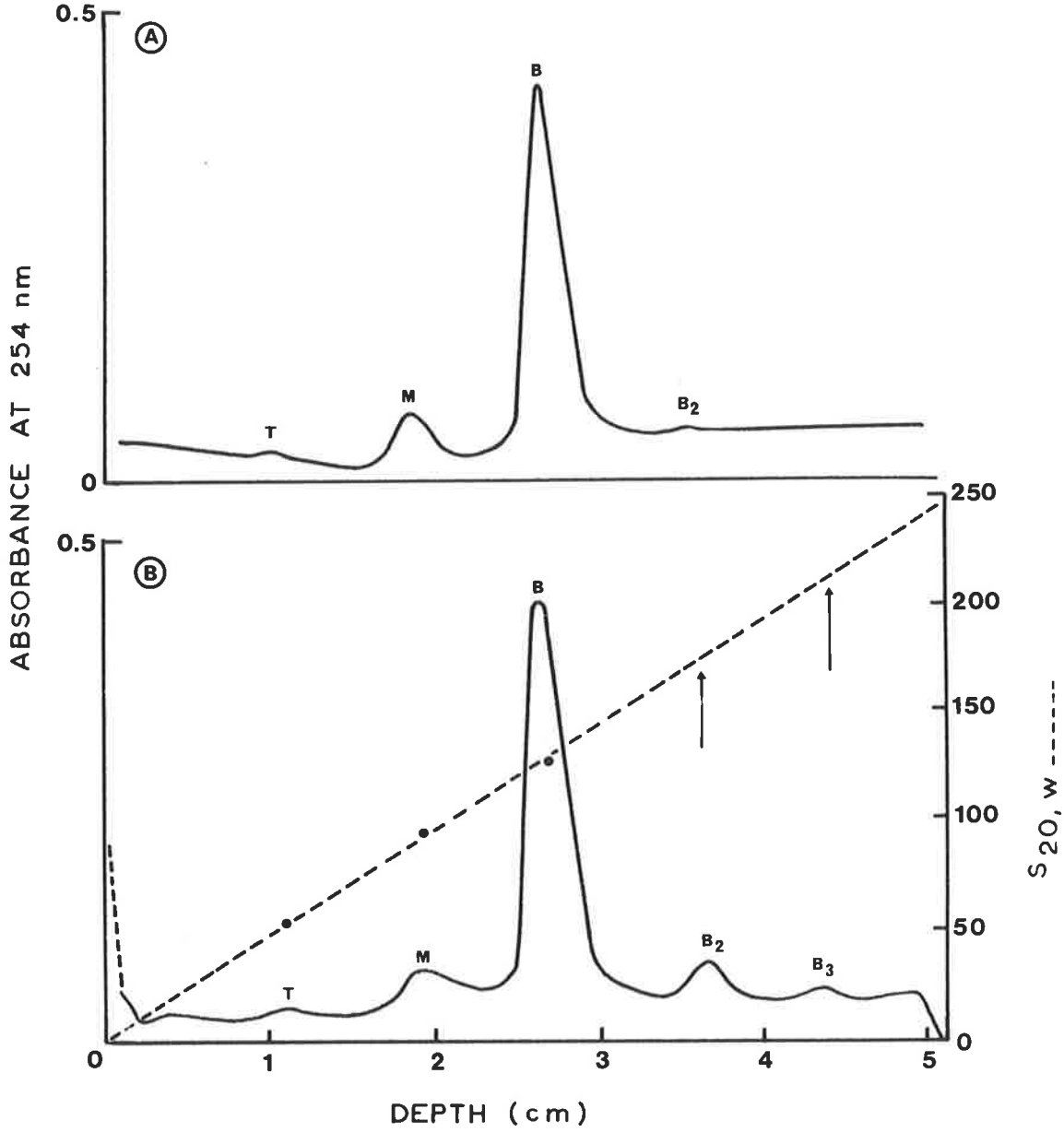

TRSV CONCENTRATION (µgm / gm fresh weight)
700
600
500
400
300
200
100
0
1
2
3
4
5
7
9
DAYS AFTER INOCULATION
a
b
c

the inoculated cotyledons were first observed on the third day after inoculation and were readily visible on the fourth day (Fig. 2); thereafter the growth rate of the plants decreased considerably.

Invariably all three TRSV components, T, M and B, were detected in virus preparations from cotyledons harvested at various times after infection (Fig. 3A). However, in some preparations, two additional components, B_2 and B_3 sedimenting ahead of B, were also detected (Fig. 3B). The presence of B_2 in TRSV preparations has also been reported by Ladipo and de Zoeten (1972), who found that it was infectious. In my experiments, a preparation of B_2 particles with an optical density of 0.25 at 260 nm produced a mean of 89 lesions per half-leaf, whereas a preparation of B particles of the same optical density produced 156 lesions per half-leaf when inoculated to 12 leaves of cowpea plants. Both preparations had ultraviolet absorption spectra characteristic of nucleoproteins but the 260/280 nm ratio of the B_2 preparation was 1.55 and that of B preparation 1.62. Identical polyhedral particles characteristic of TRSV (Chambers *et al.*, 1965) were observed in negatively stained preparations of both B_2 and B examined in the electron microscope. RNA isolated from TRSV preparations containing B_2 and subjected to sucrose density-gradient centrifugation and polyacrylamide-gel electrophoresis contained two species of RNA with indistinguishable sedimentation rates and mobilities from those of RNA isolated from virus preparations devoid of B_2 and B_3.

Fig. 2. Symptoms of TRSV on cucumber cotyledons, 4 days after inoculation. Plants were kept at $25^{\circ} \pm 2$ under fluorescent lights with continuous illumination.

Fig. 3. Sucrose density-gradient centrifugation of TRSV preparations purified from cucumber cotyledons. Virus preparations containing only a trace of component B_2 (A) and one containing relatively high concentrations of B_2 and B_3 components (B). Virus preparations were obtained in two independent experiments. One hundred µg virus was layered on each tube and centrifugation was carried out as in Fig. 1.


0.5
A
B
M
T
B_2
0
ABSORBANCE AT 254 nm
0.5
B
250
200
150
100
50
0
$S_{20,w}$
B
M
T
B_2
B_3
0
1
2
3
4
5
DEPTH (cm)

Taking the S_{20w} of T, M and B to be 53S, 94S and 128S respectively (Stace-Smith *et al.*, 1965), it was calculated that the S_{20w} of B_2 and B_3 was 180S and 210S respectively (Fig. 3B). These values are very close to the expected S_{20w} of the dimer and trimer of B which are 1.4 and 1.7 times those of the monomers respectively (Markham, 1962). All these observations support the view that B_2 and B_3 are aggregates of B component particles. The reason why such aggregates should be present in TRSV preparations isolated in some experiments but not in others remains unknown (Fig. 3).

Schneider and Diener (1966) reported that in TRSV-infected bean plants the proportion of M to B components was higher at early stages of infection. However, in the experiments with TRSV-infected cucumber cotyledons, the ratio of M to B components appears to be constant during all stages of infection (Table 1). It is also noteworthy that in some experiments the proportion of B_2 component was almost as high as that of M (Table 1).

II. Properties of RNA Isolated from TRSV Preparations

Sucrose density-gradient profiles of RNA isolated from TRSV preparations show the presence of two species of RNA (Fig. 4). The ratio of the amount of the slower sedimenting species (RNA_1) to that of the faster sedimenting one (RNA_2) was remarkably constant in preparations from virus isolated at various times after inoculation (Fig. 4). The amount of RNA_1 was always greater than that of RNA_2

TABLE 1

PROPORTIONS OF VARIOUS TRSV COMPONENTS IN PURIFIED VIRUS PREPARATIONS FROM CUCUMBER COTYLEDONS HARVESTED AT VARIOUS TIME INTERVALS AFTER INFECTION

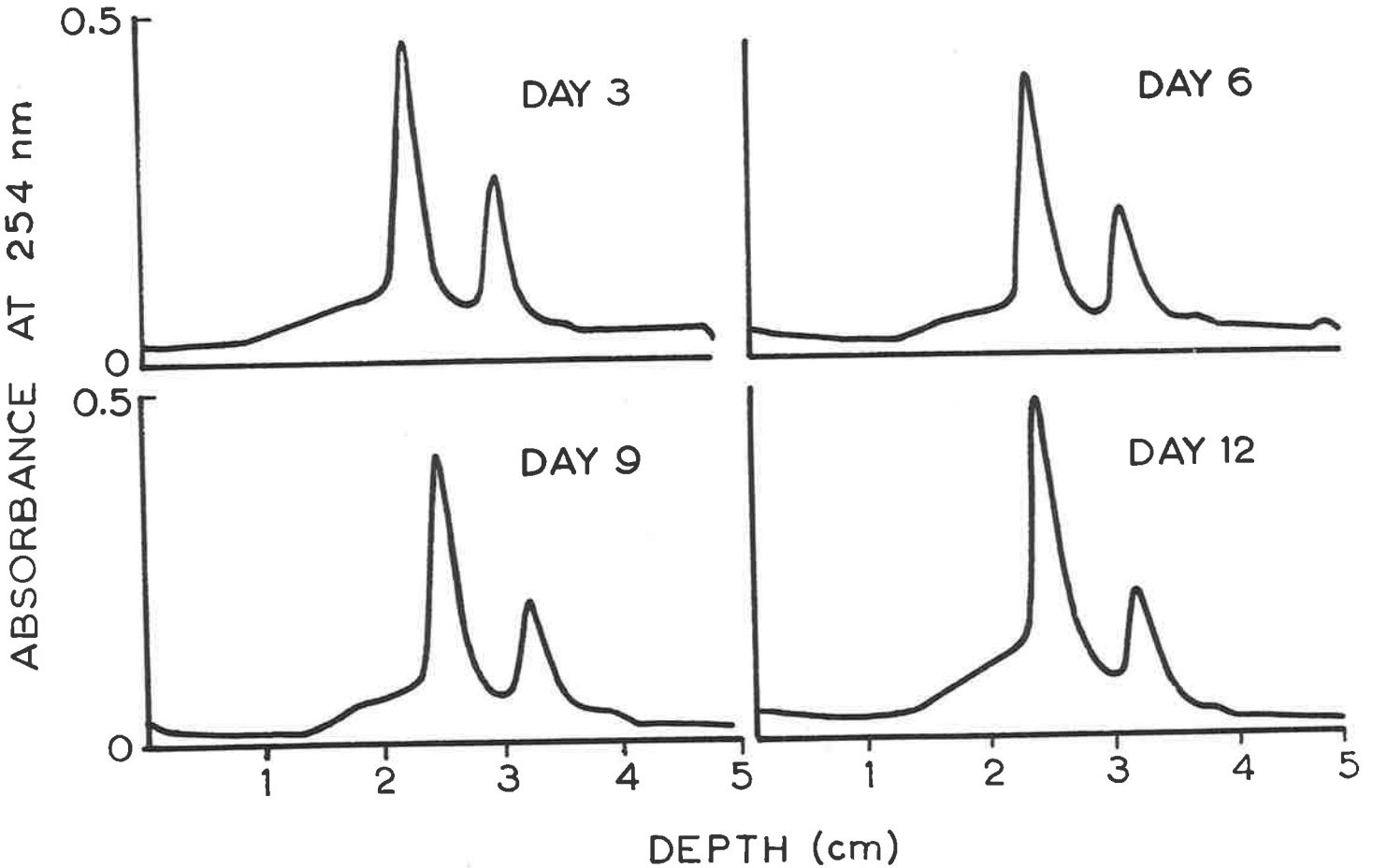
Day after inoculation	% of each component[a]					
	Experiment 1			Experiment 2		
	M	B	B_2	M	B	B_2
1	0	0	0	-[c]	-	-
2	13.2	79.9	6.9	-	-	-
3	11.0	83.5	5.5	12.6	75.1	12.4
4	10.6	84.5	4.9	-	-	-
5	11.6	83.4	5.0	-	-	-
6	-	-	-	11.8	73.7	14.5
7	11.0	83.5	5.5	-	-	-
9	9.9	84.8	5.3	11.1	77.8	11.1
12	-	-	-	12.6	77.2	10.3

[a] Calculated on the basis of areas under the peaks recorded at 254 nm with the ISCO apparatus.

[b] 0 = not detectable.

[c] - = not determined.

Fig. 4. Sucrose density-gradient centrifugation of TRSV-RNA. RNA was prepared by the single-phase phenol-SDS procedure and resuspended in 0.02 M phosphate buffer, pH 7. RNA from 100 µg virus was layered on 5-25% sucrose gradient in 0.02 M phosphate buffer, pH 7, and centrifuged at 50,000 rpm for 3 hr at 1°. All samples of virus were harvested from the same batch of virus-infected plants.


0.5
0
DAY 3
DAY 6
0.5
0
DAY 9
DAY 12
ABSORBANCE AT 254 nm
1
2
3
4
5
1
2
3
4
5
DEPTH (cm)

in RNA from all virus preparations examined, which invariably contained a higher proportion of B than M components (cf. Figs. 3 and 4, also see Table 1). This, together with the fact that both RNA_1 and RNA_2 were isolated from preparations of purified B component, confirms the previous conclusion (Diener and Schneider, 1966) that M component particles contain one RNA_1 molecule each, whereas B component particles each contain either one molecule of RNA_2 or two molecules of RNA_1.

In my experiments, both RNA_1 and RNA_2 sedimented as homogeneous peaks in sucrose density-gradients (Fig. 4). No obvious signs of degradation were detected in RNA preparations isolated from virus obtained from leaves at late stages of infection as observed in virus from bean plants (Schneider and Diener, 1968). Schneider and Diener (1968) also observed that TRSV-RNA showed increased degradation when isolated from virus preparations stored *in vitro* at 27° for one week. In our experiments, TRSV-RNA preparations failed to show significant degradation when isolated from virus stored at 4° for periods as long as 80 days (results not shown).

Diener and Schneider (1966) reported that RNA_1 and RNA_2 from their isolate of TRSV have molecular weights of 1.2×10^6 and 2.2×10^6 daltons, respectively, whereas Murant *et al.* (1972) estimated values of 1.4×10^6 and 2.3×10^6 daltons for the two RNA species prepared from

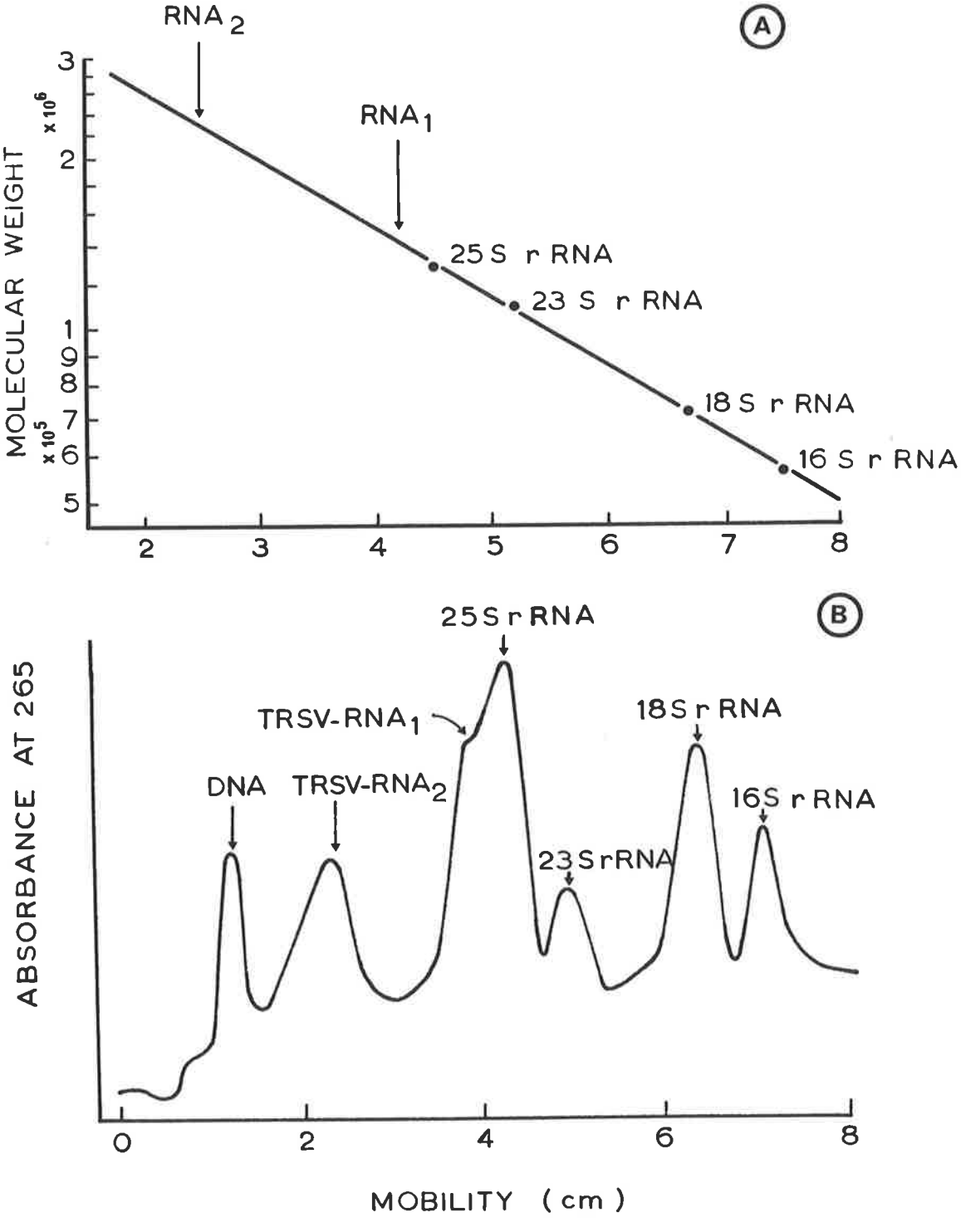
the isolate of TRSV they used. Polyacrylamide-gel electrophoretic analyses of RNA_1 and RNA_2 from our isolate of TRSV, using tobacco leaf r-RNA species as markers, revealed values of 1.4×10^6 and 2.3×10^6 daltons respectively (Fig. 5A). In all experiments RNA_1 invariably migrated more slowly than the 25S tobacco leaf r-RNA, which would have a molecular weight similar to that of 25S r-RNA from other plants (1.29×10^6 daltons; Loening, 1968) (Fig. 5B).

CONCLUSIONS

Multiplication of TRSV was investigated in cucumber cotyledons grown under continuous illumination at 25°. Results of the experiments demonstrated that: (1) the rapid multiplication of TRSV was confined to a period between two and five days after inoculation (Fig. 1); (2) in some virus preparations dimers and trimers of B component particles were observed (Fig. 3); and (3) the TRSV isolate used in these investigations showed some characteristics distinct from those reported previously (Schneider and Diener, 1966; 1968) in that: (a) the proportion of B component to M component did not vary significantly between three and twelve days after inoculation (Table 1); (b) no significant sign of degradation appeared in the RNA_1 and RNA_2 when TRSV-RNA was isolated from virus preparations stored for periods as long as 80 days at 4°; and (c) virus preparations from plants infected for up to 12 days contained two homogeneous RNA species (Fig. 4).

Fig. 5. A) Estimation of the molecular weights of TRSV-RNA species by polyacrylamide-gel electrophoresis. Electrophoresis was carried out in 11 cm long, composite 2.4% acrylamide, 0.5% agarose gels, at room temperature for 5 hr at 8 v/cm. Tobacco leaf r-RNA was used as markers.

B) Coelectrophoresis of TRSV-RNA and tobacco leaf RNA as described in A.


A
RNA 2
RNA 1
25 S r RNA
23 S r RNA
18 S r RNA
16 S r RNA
MOLECULAR WEIGHT
x 10^6
x 10^5
3
2
1
9
8
7
6
5
2
3
4
5
6
7
8
B
25S r RNA
TRSV-RNA 1
DNA
TRSV-RNA 2
18S r RNA
16S r RNA
23S rRNA
ABSORBANCE AT 265
0
2
4
6
8
MOBILITY (cm)

CHAPTER 4

DETECTION OF A LOW MOLECULAR WEIGHT DOUBLE-STRANDED RNA FROM TRSV INFECTED PLANTS

INTRODUCTION

The only reported work on an attempt to detect TRSV-RF is that by Atchison (1971) in which RF could not be detected in RNase treated ^{32}P-labelled nucleic acids when analyzed on methylated albumin coated keisilghuar columns. This chapter describes the results of experiments designed to detect RF and/or RI from TRSV infected plants. Although such structures could not be detected, a low molecular weight ds-RNA was isolated.

METHODS

1. Extraction of Total Leaf Nucleic Acids

Up to 100 g of leaf material was used to extract nucleic acids from healthy or virus-infected plants. In earlier experiments freshly harvested tissue was homogenized with TNE buffer (0.1M NaCl, 0.1M Tris-HCl, 0.01M EDTA, pH 7) (Jackson *et al.*, 1971) and TNE-saturated phenol containing 0.1% 8-hydroxyquinoline (2 ml of each reagent/gm leaf

tissue). In later experiments the leaves were frozen in liquid nitrogen prior to extraction with TNE buffer containing 1% SDS and phenol containing 0.1% 8-hydroxyquinoline. The slurry was shaken for 45 min, centrifuged at 5,000g for 10 min and the buffer phase was re-extracted twice more with half a volume of the phenol reagent. The RNA was precipitated by addition of two volumes of chilled ethanol and kept at -15° for at least 3 hr. The precipitated RNA was sedimented by centrifugation at 5,000g for 10 min, washed with acetone followed by ether, dried under vacuum and resuspended in STE buffer (0.1M NaCl, 0.05M Tris-HCl, 0.001M EDTA, pH 6.85) (Jackson *et al.*, 1971), or in SSC buffer if the RNA was to be used for analysis on sucrose density-gradients at this stage of purification.

2. Salt Fractionation of Nucleic Acid Preparations

NaCl was added to nucleic acid preparations in STE buffer to a final concentration of 1.5M and kept at -15° for at least 3 hr (Bishop and Koch, 1967). After slow thawing, the preparation was centrifuged at 5,000g for 20 min at 4° and the salt insoluble RNA species were recovered in the pellet. Two volumes of ethanol were added to the supernatant to precipitate the salt soluble nucleic acids (DNA, transfer (t-RNA), 5S ribosomal (r-RNA) and in the case of preparations from virus-infected tissues, ds-RNA).

Where it was required to remove DNA from a nucleic acid

preparation, 10 µg/ml of DNase free from pancreatic ribonuclease (RNase) were added to a buffer containing 0.01M $MgCl_2$ (usually STMg buffer, 0.2M NaCl, 0.01M Tris, 0.01M $MgCl_2$, pH 7.4) and the mixture was incubated at 37° for 30 min. To remove ss-RNA the preparation was incubated with 2 µg/ml (or 10 µg/ml if ss-RNA had not been removed by NaCl precipitation) of RNase in buffer containing not less than 0.2M NaCl and 0.01M EDTA and incubated at 37° for 30 min. Following enzyme treatment, nucleic acid preparations were incubated with 10 µg/ml of pronase for 30 min at 37° and extracted with one-half volume of phenol reagent, and the remaining nucleic acids were precipitated with ethanol and washed as described above.

3. Purification of Leaf Nucleic Acids

After salt fractionation and/or nuclease digestion, the preparations contained polysaccharides which in some experiments were removed as described by Ralph and Bergquist (1967). Nucleic acid preparations in 0.15M NaCl, 0.01M Tris-HCl, pH 7.4, were mixed with an equal volume each of 2-methoxyethanol and 2.5M phosphate buffer, pH 8.1, at 4°. The mixture was centrifuged at 5,000g for 5 min and the upper phase was added to an equal volume of 0.2M sodium acetate and half a volume of 1% cetyltrimethylammonium bromide (CTA). After 5 min on ice the CTA nucleate was isolated by centrifugation at 5,000g for 10 min and converted to the sodium salt by washing three times with cold 70%

ethanol containing 0.1M sodium acetate. The precipitate was further washed with acetone, then ether, dried under vacuum and resuspended in STMg or SSC buffer. By this method it was possible to concentrate nuclease-treated preparations obtained from 100 gm tissue to a volume less than 100 µl.

4. Labelling of Cucumber Cotyledon RNA

a) With radioactive uridine

Five excised cucumber cotyledons, healthy or infected with TRSV for 3 days, were placed in small petri dishes. Incisions were made about 2 mm apart, parallel to the veins and starting from the mid-rib (Zaitlin *et al.*, 1968); 0.2 ml of water containing 2.4 µCi ^{14}C-uridine was applied to each healthy cotyledon and 24 µCi ^{3}H-uridine to each virus-infected cotyledon. After 4 hr the cotyledons were rinsed several times with distilled water, total nucleic acids were extracted as described above and these were taken up in STE buffer. The nucleic acids were again precipitated with ethanol, the precipitate was then washed with acetone and ether and resuspended in a small volume of electrophoresis buffer.

b) With ^{32}P-orthophosphate

Healthy and infected (for 3 days) cucumber plants (4 of each) were removed from the soil. Roots were gently washed with water,

rinsed with distilled water and blotted. The plants were placed in two small containers and 250 μCi ^{32}P-orthophosphate was applied to the roots of each plant. After 30 min, 10 ml of water were added to the roots and 3.5 hr later the cotyledons were harvested and nucleic acids extracted as described above. The RNA was then purified by 2-methoxyethanol extraction and CTA precipitation as described before and resuspended in a small volume of electrophoresis buffer. The CTA purification step was essential in removing the non-specific ^{32}P counts.

5. Analysis of Nucleic Acids by Polyacrylamide-gel Electrophoresis

Composite 2.4% or 1.8% polyacrylamide gels containing 0.5% agarose, 7.5 cm and 11 cm long, respectively, were prepared as outlined in Chapter 2. In double-labelling experiments, samples of RNA from healthy and infected plants were mixed to give $^{3}H/^{14}C$ ratio of approximately 3 : 1 and applied to the gels (see Chapter 2 for other details). After electrophoresis for the indicated time, the radioactivity was located by slicing the gels with a multi-bladed apparatus into sections 1.4 mm thick (Bradley and Zaitlin, 1971). The 1.8% gels were sliced immediately but 2.4% gels were sliced after freezing at -15°. The individual slices were placed in glass scintillation vials with a small spatula and 0.5 ml of 9 : 1 NCS : water was added to each vial. After incubation overnight at

45° radioactivity was determined as described in Chapter 2, Section 9.

When it was required to recover RNA from the gels, each gel slice was pulverized in 0.5 ml of SSC buffer, containing 5 µg pronase, in a 10 x 1 cm test tube, using a fine glass rod. After incubation at room temperature for 5 hr the gel fragments were removed by slow speed centrifugation and the eluted RNA was withdrawn with a micro-syringe.

6. RNA-dependent RNA Polymerase Assay

Partially purified enzyme from a known weight of cucumber cotyledons was prepared and the enzyme was assayed as described by May and Symons (1971). Tissue homogenates were prepared by grinding (at 4°) healthy or infected cucumber cotyledons at various times after inoculation, with a pestle and mortar in 2 volumes (W/V) of the following extraction buffer: 50% saturated $(NH_4)_2SO_5$, 0.01M NH_4Cl, 90 mM 2-mercaptoethanol, 50 mM Tris-HCl buffer, pH 8.5. The homogenate was squeezed through two layers of cheese cloth and the filtrate was centrifuged at 20,000g for 10 min. The pellet was resuspended in twice the original volume of the extraction buffer and centrifuged as before. The pellet was then resuspended in half the original volume of extraction buffer without $(NH_4)_2SO_4$ and centrifuged at 20,000g for 10 min. The supernatant was used for assay of polymerase.

Each assay tube contained in a final volume of 0.350 ml: 45 nmoles of GTP-α-^{32}P (about 4 x 10^5 CPM), 240 nmoles of each of the three other nucleoside triphosphates, 2.5 µg actinomycin D, 0.5 µmole pyruvic acid (sodium salt), 25 µg pyruvate kinase, 500 µg bovine serum albumin, 5 µmoles of each KCl and $MgSO_4$, 9 µmoles of 2-mercaptoethanol, 10 µmoles of NH_4Cl, 12 µmoles of Tris-HCl, pH 8.5, 250 µg yeast RNA and 0.1 ml aliquot of the enzyme preparation.

The radioactivity of polymerase product was determined after 30 min incubation at 37°, as described by Gilliland and Symons (1968). Extracts from healthy control plants incorporated about 40 cpm of GTP; zero time samples had a background of about 60 cpm.

RESULTS

I. Detection of Virus-specific ds-RNA in TRSV-infected Cucumber Cotyledons

In preliminary experiments, ^{14}C-labelled TRSV-RNA was used to detect ds-RNA with base sequences complementary to viral RNA by annealing TRSV-RNA with nuclease-treated nucleic acid preparations from virus-infected cucumber cotyledons. In one such experiment, for instance, 1,966 cpm TRSV-RNA annealed to a preparation from infected plants and only 19 cpm to a similar nucleic acid preparation from healthy tissue.

Although such ds-RNA was detected in all nucleic acid preparations from infected cotyledons, the amounts varied from experiment to experiment. However, no significant amounts of complementary sequences to those of TRSV-RNA were ever detected in nucleic acid preparations from cotyledons of healthy plants. The reason for the variable amounts of virus-specific ds-RNA detected in TRSV-infected cotyledons became apparent from the results of an experiment in which salt soluble and nuclease-treated ds-RNA was assayed at daily intervals following inoculation (Table 2). In a subsequent experiment at each time of sampling, the amount of virus, the virus-specific ds-RNA and the RNA-dependent RNA polymerase activity was determined (Fig. 6). Results of this experiment indicated that there was a rapid increase in both the amount of virus-specific ds-RNA and polymerase activity just prior to, and during the rapid synthesis of virus. However, as soon as virus synthesis was arrested, the level of the polymerase activity decreased very rapidly as previously observed by Peden *et al.* (1972) and this was soon followed by a similar decline in the level of the ds-RNA. In all subsequent experiments, TRSV-specific ds-RNA preparations were obtained from cucumber cotyledons three days after inoculation with virus, to ensure a high yield of the ds-RNA.

It was found that the virus-specific ds-RNA, together with 5S r-RNA and 4S t-RNA, was soluble in 1.5M NaCl. The ds-RNA suspended in

TABLE 2

DETECTION OF TRSV SPECIFIC ds-RNA IN CUCUMBER COTYLEDONS AT DIFFERENT TIMES AFTER INOCULATION[a]

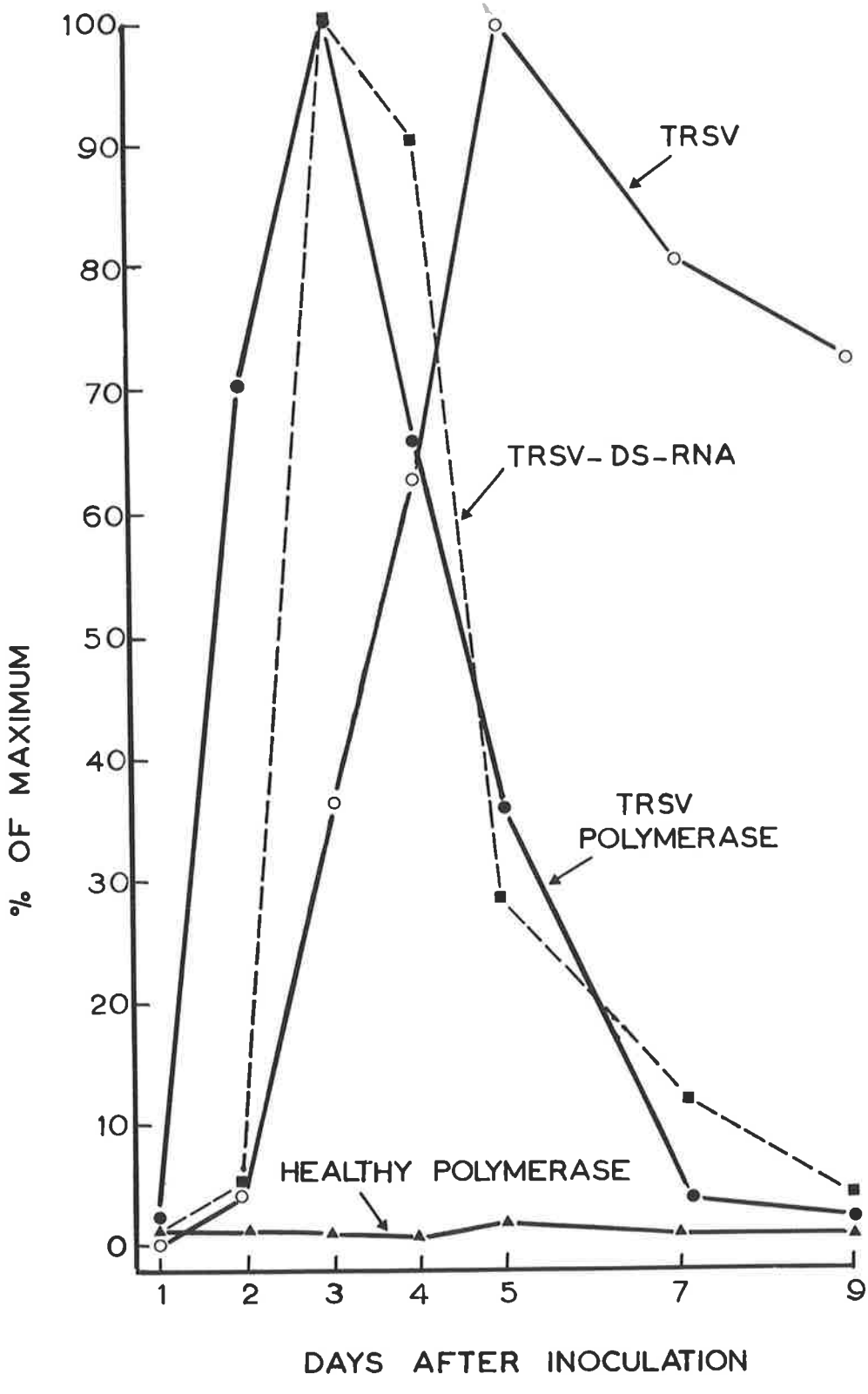
Day after inoculation	Cpm annealed[b]	Corrected[c] Cpm	ds-RNA % of maximum[c]
2	908	653	39
3	1676	1676	100
4	1218	988	59
5	1049	787	47
7	235	134	8
9	116	67	4
Healthy extract 1	34	-	-
" " 2	0	-	-

a Salt soluble, RNase and DNase treated nucleic acid preparations were purified by the CTA precipitation procedure (see Methods, Chapter 4) and were used for annealing to ^{14}C-TRSV ss-RNA.

b 3,600 cpm TRSV ss-RNA was used in each hybridization tube.

c As determined by the equation $\frac{\text{ds-RNA}}{\text{ss-RNA}} = \frac{2a}{100-a}$ (see Results for details).

Fig. 6. Correlation of TRSV multiplication with RNA-dependent RNA polymerase activity and virus specific ds-RNA concentration in cucumber cotyledons. Virus concentration was estimated as described in Fig. 1; polymerase activity was assayed as described in Methods (Chapter 4); and TRSV specific ds-RNA by annealing of ^{14}C-labelled viral-RNA (4,300 cpm per assay) with the salt soluble, RNase and DNase treated nucleic acid preparations extracted from infected cotyledon tissue. The amount of annealing was determined using the equation $\text{ds-RNA} = \frac{2a \times \text{ss-RNA}}{100 - a}$ (see Results for details). All results are expressed as percentage of the maximum recorded during the course of the experiment (on day 5 TRSV concentration in cotyledon tissue was 681 µg/gm fresh weight; on day 3 ds-RNA concentration was 54 µg/gm).


100
90
80
70
60
50
40
30
20
10
0
% OF MAXIMUM
TRSV
TRSV- DS-RNA
TRSV
POLYMERASE
HEALTHY POLYMERASE
1
2
3
4
5
7
9
DAYS AFTER INOCULATION

1 x SSC buffer was unaffected by RNase or DNase but became sensitive to RNase after heat denaturation or on suspension in 0.01 x SSC buffer (Table 3). These observations confirm that the virus-specific ds-RNA was indeed a double-stranded polyribonucleotide.

II. Sedimentation and Electrophoretic Properties of TRSV-specific ds-RNA

Cells infected by many RNA viruses, whose mode of multiplication has been adequately investigated, have been shown to contain species of ds-RNA defined as replicative form (RF) and replicative intermediate (RI) (Bishop and Levintow, 1971; see Chapter 1). In all cases investigated, the RF appears to be a duplex of the viral RNA and hence has a molecular weight twice that of the RNA (Bishop and Levintow, 1971). In planning the initial investigations of the TRSV-specific ds-RNA detected in virus-infected cucumber cotyledons it was expected that it would have properties characteristic of an RF. It was also considered that there could be two species of RF in TRSV-infected tissues, one corresponding to the viral RNA_1 and one to RNA_2. This has been demonstrated in plants infected with cowpea mosaic virus, a multi-component virus with T, M and B particles somewhat similar to TRSV (van Griensven and van Kammen, 1969). However, experiments designed to detect ds-RNA species of molecular weights approximately 4.6×10^6 (RF corresponding to RNA_2) and 2.8×10^6 daltons (RF

TABLE 3

EFFECT OF NUCLEASES ON THE TRSV-SPECIFIC ds-RNA

Treatment of ds-RNA[b] before annealing	^{14}C-labelled TRSV-RNA annealed to ds-RNA[a]			
	Experiment 1		Experiment 2	
	Cpm	% of control	Cpm	% of Control
Untreated (control)	372	100	870	100
RNase in 1 x SSC	352	94.2	850	97.7
RNase in 0.01 x SSC	9	2.4	17	2.0
Heat denatured	369	99.2	848	97.5
Heat denatured + RNase in 1 x SSC	79	21.2	215	24.7
DNase	356	95.7	865	99.4

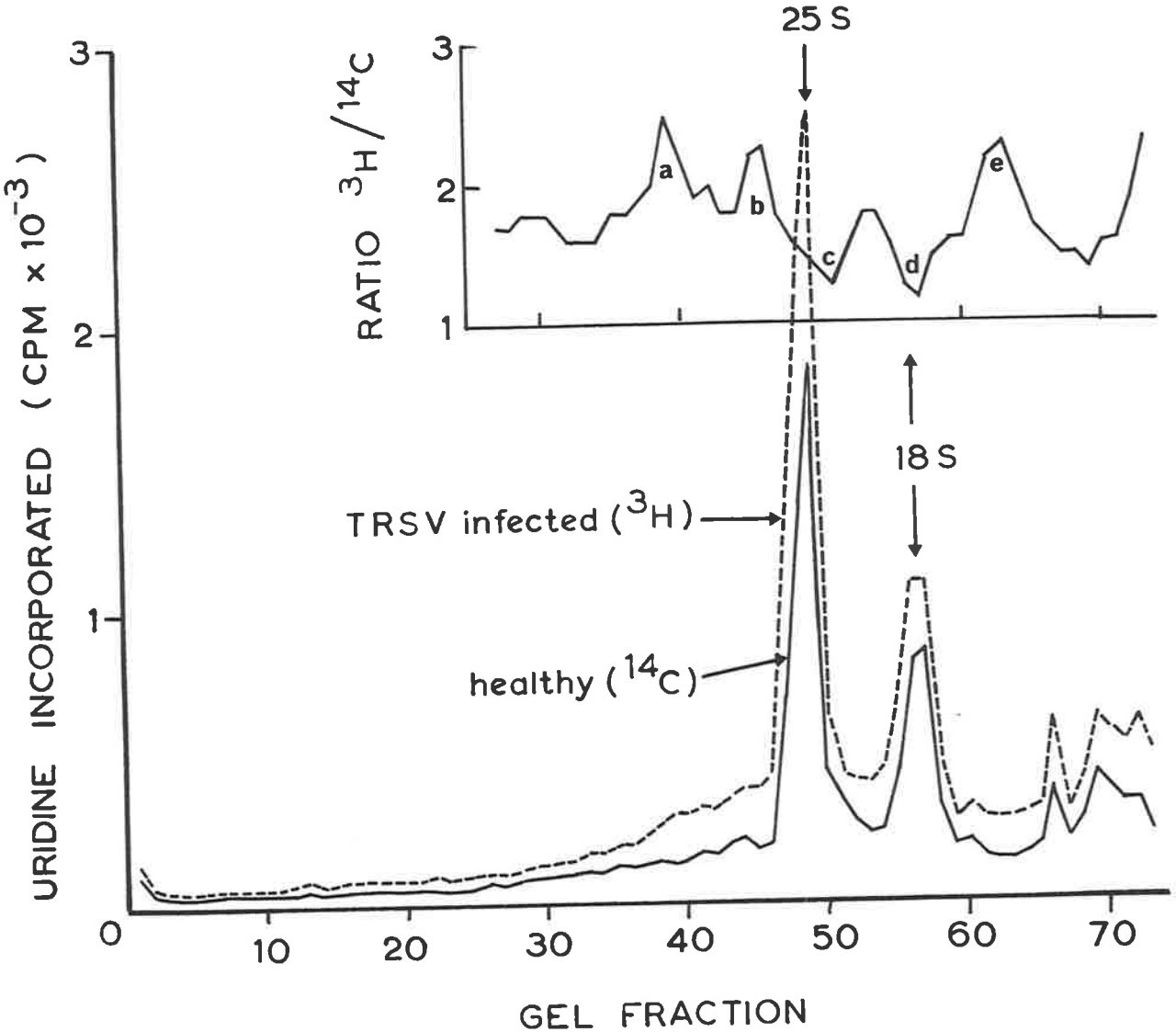
a Annealing procedure as described in Materials and Methods.

b Following RNase (20 µg/ml) or DNase (50 µg/ml) treatment of unlabelled salt soluble leaf nucleic acid preparations, RNase was added to all samples which had not been treated and a phenol extraction was carried out immediately. The buffer phase was recovered, nucleic acid precipitated with ethanol, resuspended in 1 x SSC and annealed to ^{14}C-labelled TRSV-RNA.

corresponding to RNA_1) in TRSV-infected cucumber cotyledons of plants labelled with ^{32}P-orthophosphate or ^{14}C-uridine were unsuccessful.

The most sensitive method for detecting incorporation of label into TRSV-specific RNA species during short exposures was by labelling healthy cucumber cotyledons with ^{14}C-uridine, TRSV-infected ones with ^{3}H-uridine and then co-electrophoresing the isolated RNA's in polyacrylamide gels. Results of such an experiment using 1.8% polyacrylamide-gel (Fig. 7) show that virtually no label was incorporated into RNA species with molecular weights in excess of 2.8×10^6 daltons (Fractions 1-30 in Fig. 7). Incorporation of ^{14}C-uridine into 25S and 18S r-RNA of healthy cotyledons and ^{3}H-uridine into those of diseased ones was readily detected (Fig. 7). In addition, by plotting the ratio of ^{3}H-uridine/^{14}C-uridine detected along the polyacrylamide-gels, it was possible to detect the incorporation of label into three species of TRSV-specific RNA (peaks a, b and e in the inset to Fig. 7). In a subsequent experiment, by co-electrophoresing RNA from purified TRSV with RNA from healthy cucumber cotyledons, it was found that peaks a and b were in the expected positions of the viral RNA's, RNA_2 and RNA_1 respectively (see Fig. 5B). The troughs c and d in Fig. 7 are in positions where 23S and 16S r-RNA from chloroplasts should migrate. The troughs suggest that TRSV infection reduces the rate of synthesis of chloroplast r-RNA as has been shown to occur in plants infected with several other

Fig. 7. Polyacrylamide-gel electrophoresis of total nucleic acids from healthy and TRSV-infected cucumber cotyledons, labelled with ^{14}C- and ^{3}H-uridine respectively. Excised cotyledons infected 60 hr previously and healthy controls (5 of each) were labelled for 4 hr as described in Methods (Chapter 4). Samples of RNA from healthy and diseased plants were mixed and coelectrophoresed in 1.8% polyacrylamide, 0.5% agarose gel (11 cm long) at room temperature and 8 v/cm for 4 hr. Radioactivity of ^{3}H and ^{14}C were determined in two counting channels and corrected for ^{14}C cross-over (6.2%). The inset shows ratios of ^{3}H to ^{14}C. (Ratios were not calculated when ^{3}H samples counted at less than 100 cpm).


25 S
RATIO $^{3}H/^{14}C$
a
b
c
d
e
18 S
TRSV infected (^{3}H)
healthy (^{14}C)
URIDINE INCORPORATED (CPM x 10^{-3})
GEL FRACTION
0
10
20
30
40
50
60
70
1
2
3

viruses (Hirai and Wildman, 1969; Randles and Coleman, 1970; Mohamed and Randles, 1972).

The TRSV-specific RNA species migrating as peak a in Fig. 7 was even more readily detected when the RNA preparations were co-electrophoresed in a 2.4% polyacrylamide gel (Fig. 8). This RNA species has also been detected on polyacrylamide-gels by direct staining with toluidine blue (see Chapter 5). Its salt solubility and ability to anneal with ^{14}C-labelled TRSV-RNA is illustrated in Fig. 9.

When nucleic acid preparations from TRSV-infected cucumber cotyledons were subjected to sucrose density-gradient centrifugation under conditions in which 18S r-RNA sedimented about half-way down the tube, only fractions near the meniscus contained material which annealed with ^{14}C-labelled RNA (Fig. 10). It was also shown that the electrophoretic mobility of the virus-specific RNA was not significantly altered by RNase treatment in 1 x SSC. Similar RNA preparations from healthy cucumber cotyledons failed to anneal to ^{14}C-labelled TRSV-RNA either after RNase treatment or after the removal of ss-RNA species by salt precipitation. It is concluded from these experiments that the virus-specific RNA detected as peak e in Figs. 7 and 8 is double-stranded and consists of molecules very much smaller than those expected for RF.

It seems unlikely that the ds-RNA detected in TRSV-infected

Fig. 8. Coelectrophoresis of uridine labelled total nucleic acids from healthy and TRSV infected cotyledons in 2.4% polyacrylamide, 0.5% agarose gel. Electrophoresis was carried out in 7.5 cm long gels for 3 hr; other details as described in Fig. 7.

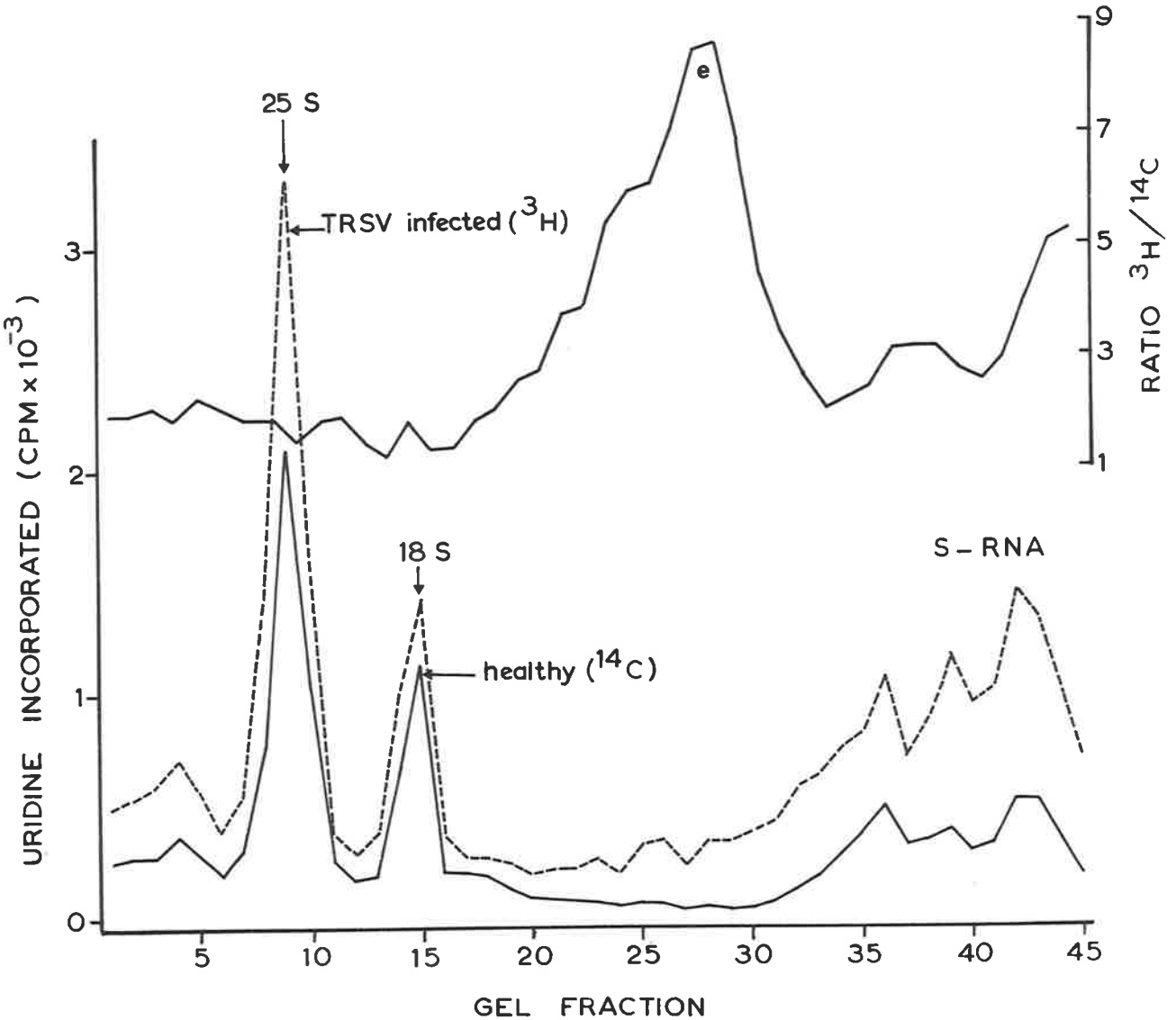

25 S
TRSV infected (^{3}H)
e
18 S
healthy (^{14}C)
S – RNA
URIDINE INCORPORATED (CPM x 10^{-3})
RATIO ^{3}H/^{14}C
0
1
2
3
1
3
5
7
9
5
10
15
20
25
30
35
40
45
GEL FRACTION

Fig. 9. Electrophoretic mobility of TRSV-specific ds-RNA as determined by annealing to ^{14}C-labelled TRSV-RNA (1,000 cpm/assay). Salt soluble nucleic acid fraction from unlabelled virus-infected cucumber cotyledons (2 gm fresh weight) was prepared and electrophoresis was carried out as described in Fig. 8. The gel was removed, sliced and each slice was eluted in 1 x SSC and the eluate used for annealing to ^{14}C-labelled TRSV-RNA as outlined in Chapter 2. Nucleic acid fractions from uninfected plants failed to anneal to ^{14}C-labelled TRSV-RNA (see Table 3 and 4 for results of similar experiments).

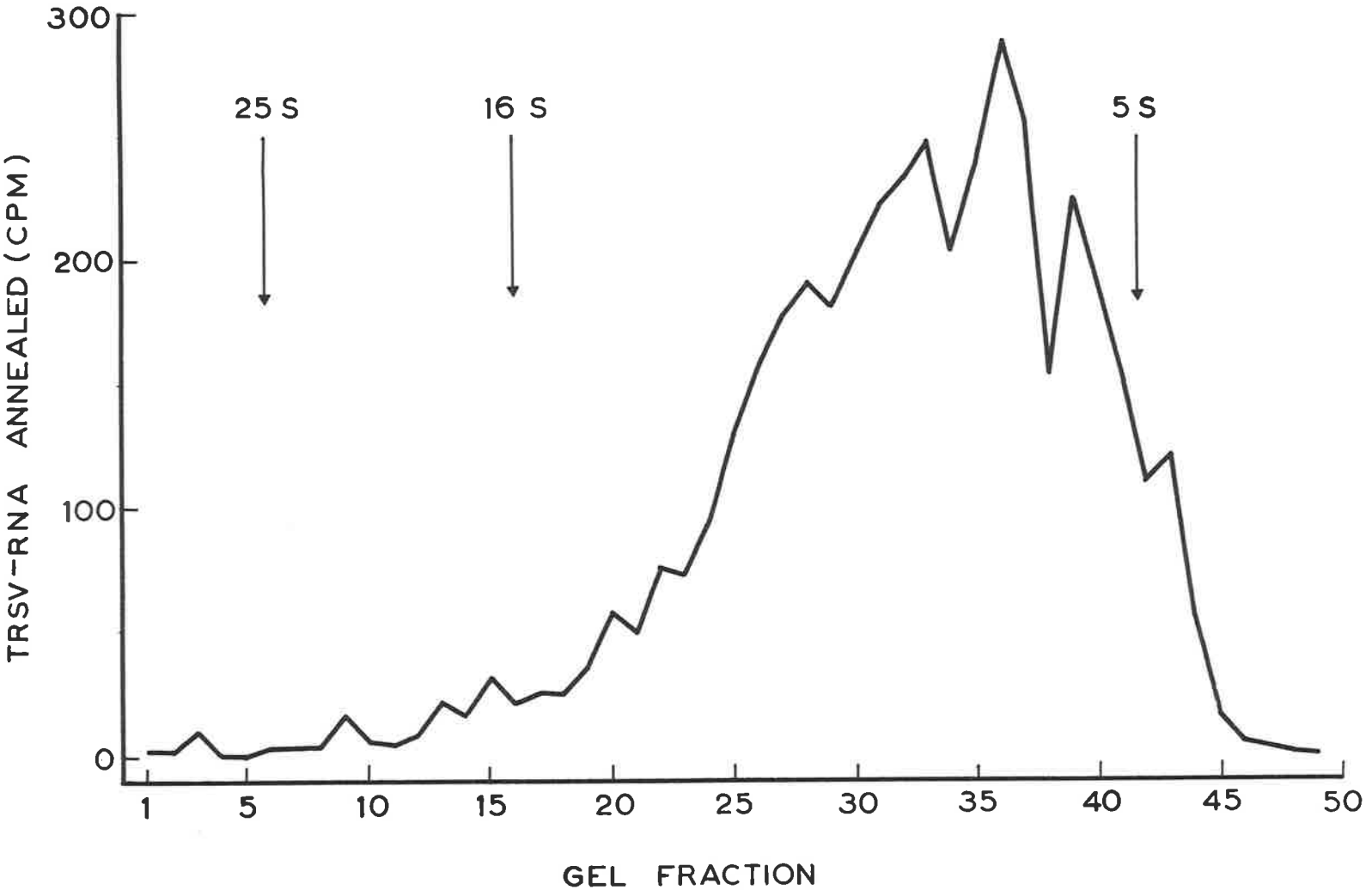
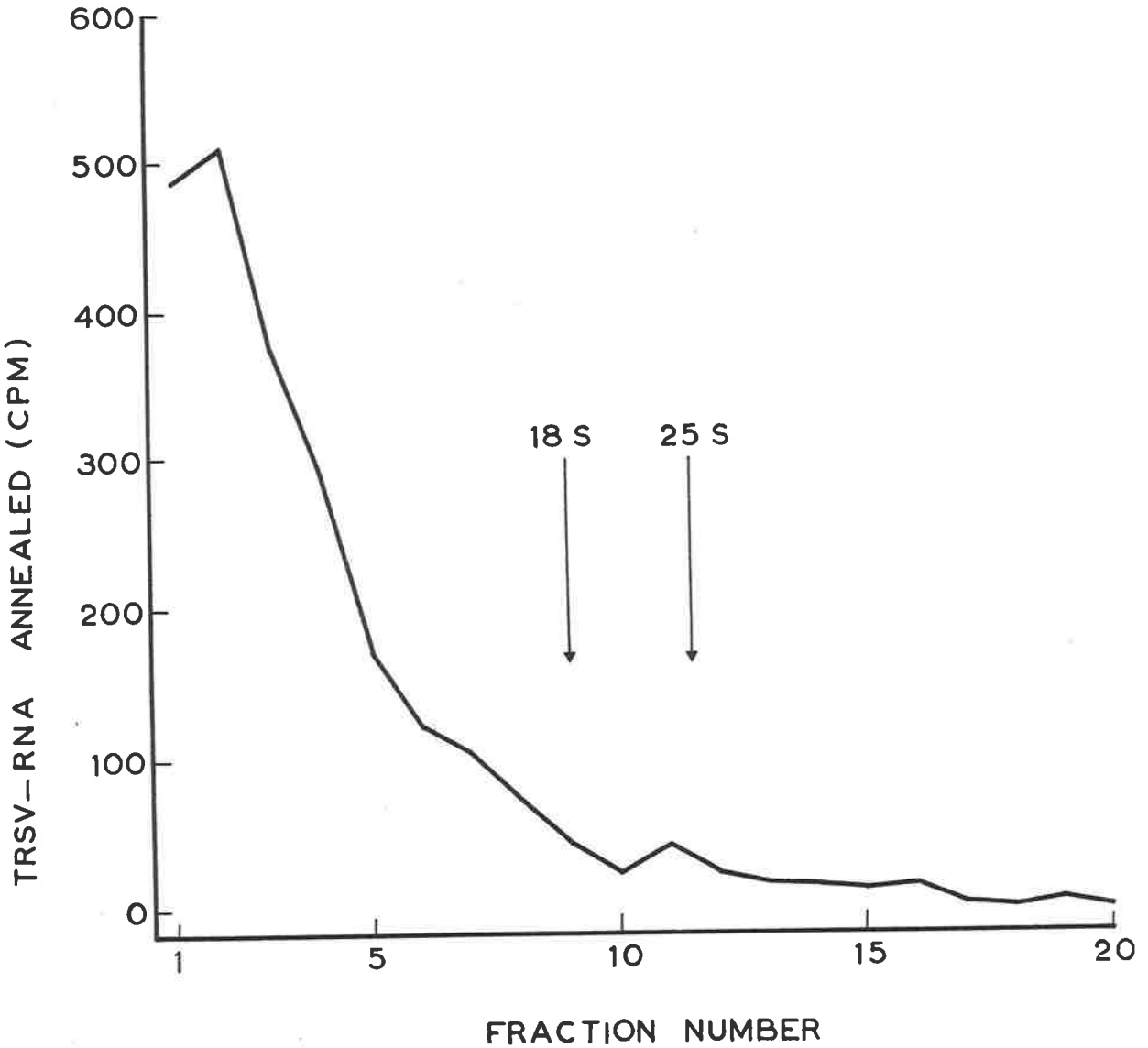

300
200
100
0
25 S
16 S
5 S
TRSV-RNA ANNEALED (CPM)
1
5
10
15
20
25
30
35
40
45
50
GEL FRACTION

Fig. 10. Sucrose density-gradient centrifugation of TRSV specific ds-RNA as determined by annealing to ^{14}C-labelled TRSV-RNA. Total nucleic acids from virus-infected cucumber cotyledons (2 gm fresh weight) were prepared as described in Methods (Chapter 4). RNA in 1 x SSC was layered on a 5-25% sucrose density-gradient in the same buffer and centrifuged as described in Fig. 4. Fractions (250 µl) were collected and each fraction was annealed to 1,500 cpm of ^{14}C-labelled TRSV-RNA (similar results were obtained when the salt soluble fraction of a nucleic acid preparation from TRSV-infected cotyledons were subjected to sucrose density-gradient centrifugation).


600
500
400
300
200
100
0
TRSV-RNA ANNEALED (CPM)
18 S
25 S
1
5
10
15
20
FRACTION NUMBER

cucumber cotyledons was a degradation product of larger molecules as most of the nucleic acid preparations were not subjected to enzymatic treatments (Figs. 7-10). Furthermore, when the extraction procedure was modified, ds-RNA with virtually identical properties was detected. Neither the addition of 1% diethyl pyrocarbonate (Solymosy *et al.*, 1968) nor 0.1% bentonite (Fraenkel-Conrat *et al.*, 1961) to the extraction buffer changed the properties of the virus-specific ds-RNA isolated (results not shown). In another experiment the time of the initial phenol-SDS extraction was reduced to only 10 min and another extraction buffer, containing 0.1M Tris-HCl, 0.05M NaCl, 0.005M EDTA, 0.05M sodium tetraborate, 1% ascorbic acid, 1% SDS and 0.1% bentonite, pH 7.6 (Bockstahler, 1967), was used; but again, the properties of the isolated virus-specific ds-RNA were similar to those already reported in Fig. 10 (results not shown).

III. Comparison of Virus-specific ds-RNA in Beans Infected with TRSV and TMV

TRSV and a bean strain of TMV have been shown to multiply well in primary leaves of French bean (Crowley *et al.*, 1969). Since the RF of TMV has been characterized in some detail and shown to consist of a molecule of MW approximately 4 x 10^6 daltons (Jackson *et al.*, 1971), it was intended to determine if undegraded TMV-RF could be isolated from infected bean leaves by the same techniques

used for the isolation of the virus-specific ds-RNA from TRSV-infected tissue.

The multiplication curve of TRSV in primary leaves of beans (Fig. 11) was very similar to that in cucumber cotyledons (cf. Fig. 1) and virus-specific ds-RNA was readily detected in nucleic acid preparations from infected bean leaves (Table 4). After centrifugation in sucrose density-gradients, the distribution of TRSV-specific ds-RNA isolated from infected bean leaves was similar to that isolated from cucumber cotyledons (Fig. 12). However, TMV-specific ds-RNA from infected bean leaves prepared by the same techniques, sedimented as a peak between 12S and 13S, which is approximately that observed for TMV-RF and that expected for a ds-RNA molecule of approximately 4 x 10^6 daltons (Burdon *et al.*, 1964). The broadness of the peak is mainly due to the non-linear increase in the amount of ^{14}C-labelled TMV-RNA hybridized, with the increase in the amount of RF (see next section for explanation).

It has recently been reported that residual amounts of RNase in leaf nucleic acids cannot be removed by repeated phenol extractions (Atchison *et al.*, 1974). Although using the RNase inhibitors such as bentonite and diethyl pyrocarbonate during extraction did not change the properties of TRSV-specific ds-RNA, the presence of an enzyme capable of degrading RNA in the ds-RNA preparations was tested in a

Fig. 11. Multiplication of TRSV in primary leaves of French bean plants. Experimental details as in Fig. 1.

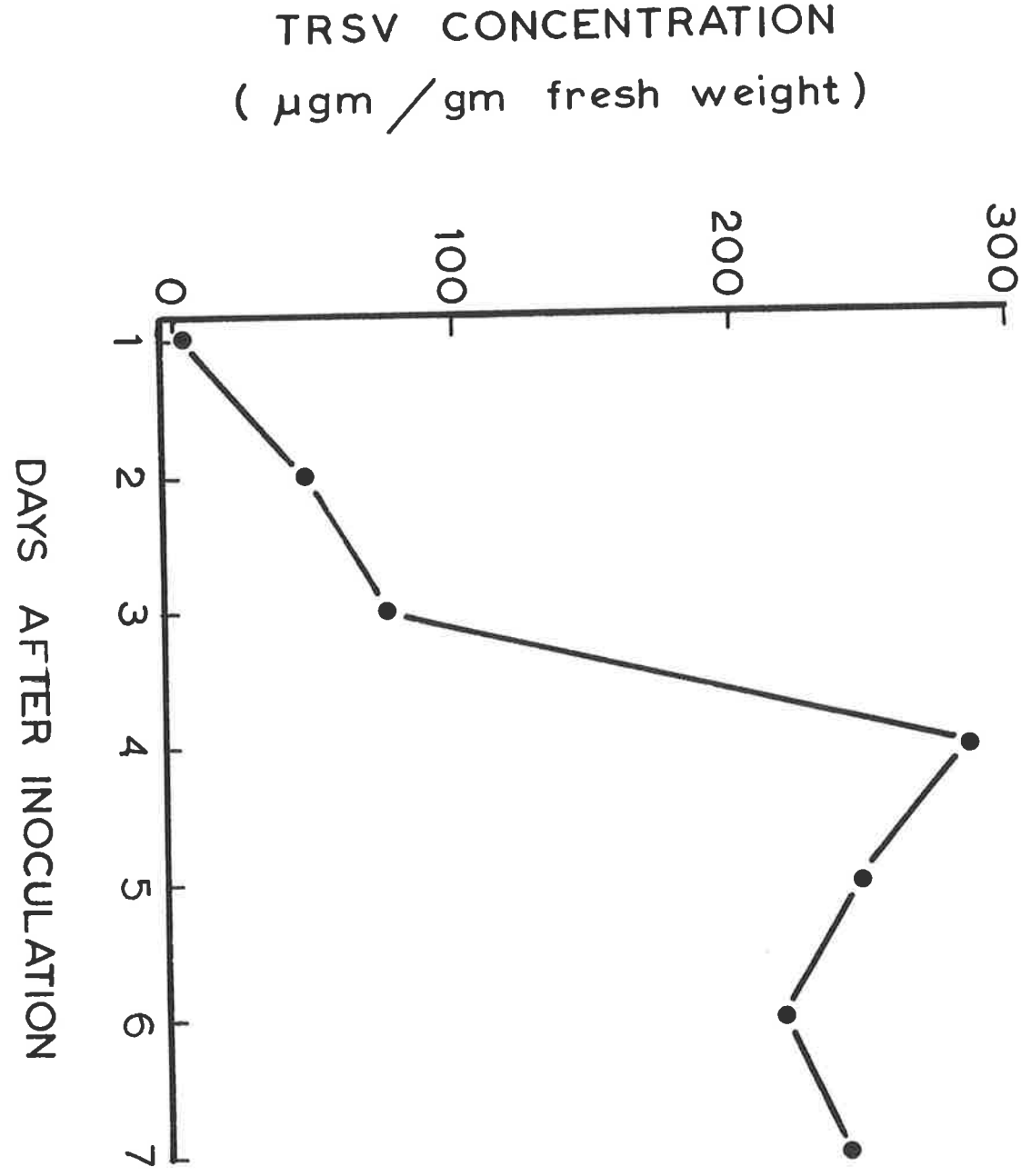
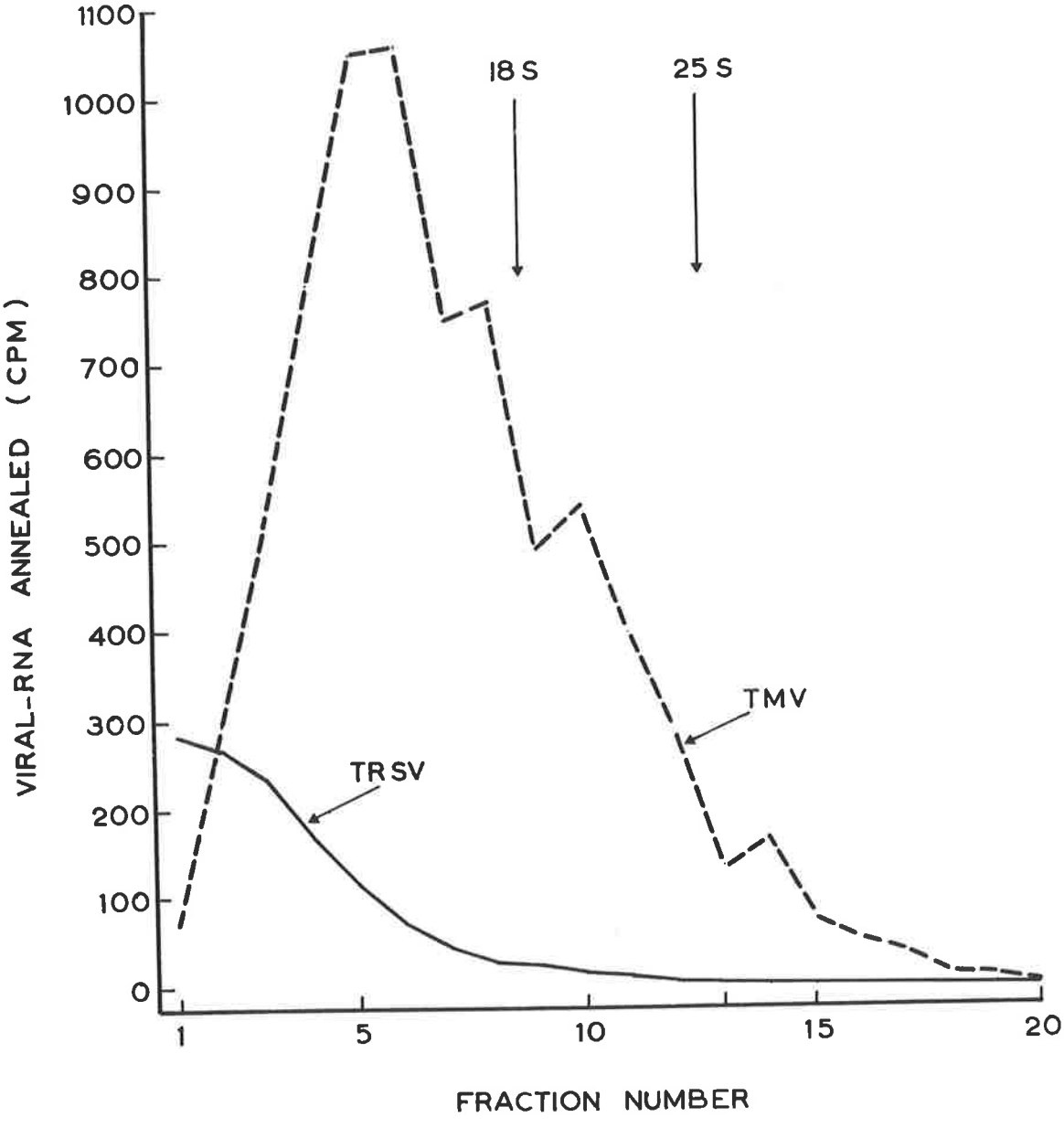

TRSV CONCENTRATION
(µgm / gm fresh weight)
0
100
200
300
1
2
3
4
5
6
7
DAYS AFTER INOCULATION

Fig. 12. Sucrose density-gradient centrifugation of TRSV and TMV specific ds-RNA's isolated from primary leaves of French beans as determined by annealing to ^{14}C-labelled homologous RNA. The salt soluble nucleic acid fractions from 15 gm fresh weight of leaves infected with each virus were further purified by 2-methoxyethanol extraction and CTA precipitation (see Methods) and subjected to sucrose density-gradient centrifugation. Fractions from gradients were annealed to the respective ^{14}C-labelled viral RNA's as described in Fig. 10.


1100
1000
900
800
700
600
500
400
300
200
100
0
VIRAL-RNA ANNEALED (CPM)
18 S
25 S
TMV
TRSV
1
5
10
15
20
FRACTION NUMBER

subsequent experiment. TMV specific ds-RNA was added to TRSV-infected cotyledons and the nucleic acids were extracted by the, usual procedure. TMV ds-RNA was then detected in the nucleic acid after sucrose density-gradient centrifugation, with no significant change in its sedimentation properties (cf. Figs. 12 and 13).

IV. Estimations of Virus-specific ds-RNA Concentrations in Infected Leaves

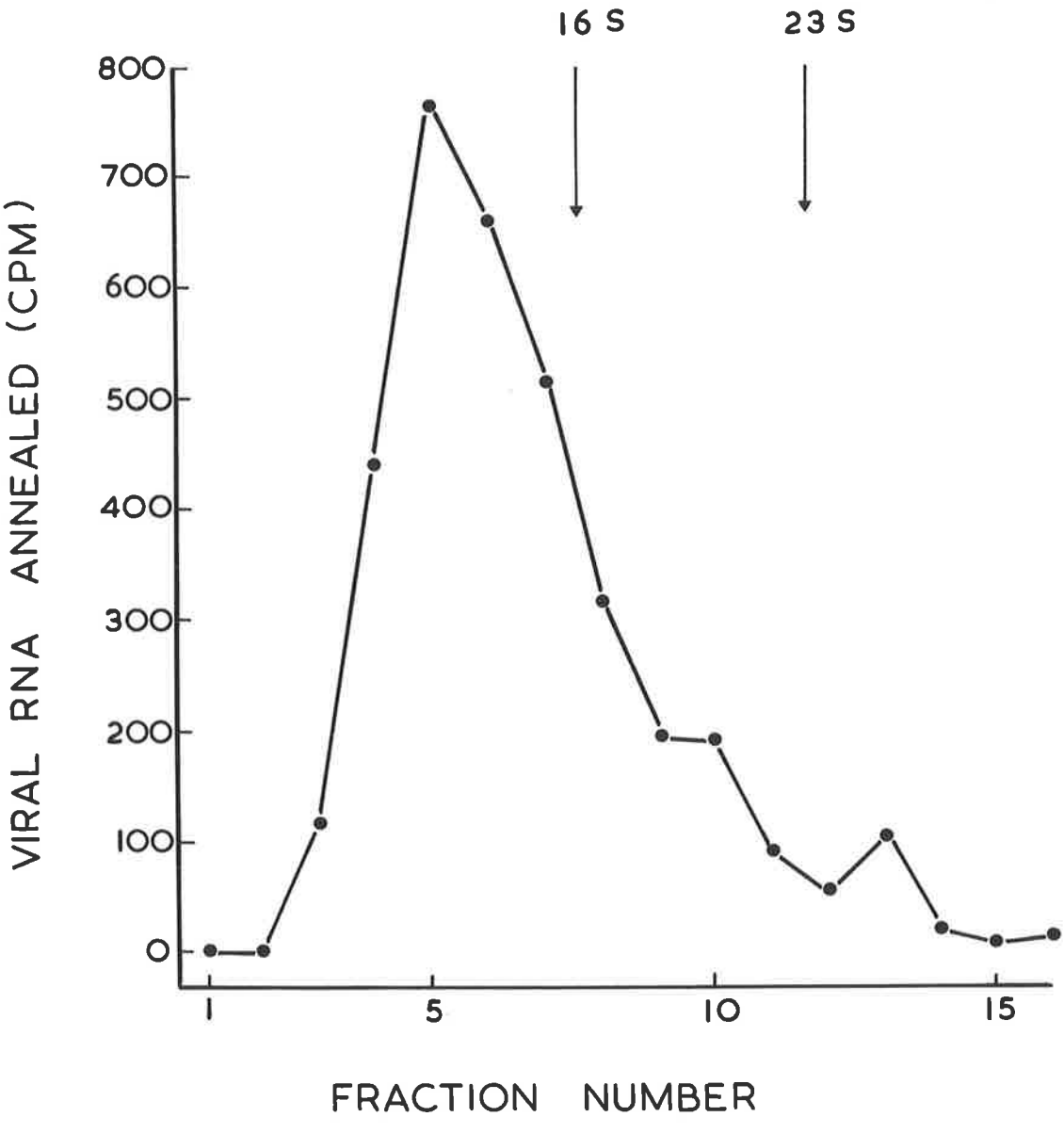
From theoretical considerations it can be deduced that when a given weight of labelled viral ss-RNA is exposed to annealing conditions with double the weight of unlabelled virus-specific ds-RNA, 50% of the labelled ss-RNA will anneal into double-stranded structures. Thus, if a known weight of labelled ss-RNA is added to an unknown weight of ds-RNA and the mixture melted and reannealed to equilibrium, the weight of ds-RNA in the mixture can be calculated from the equation

$$\text{ds - RNA} = \frac{2a \times \text{ss-RNA}}{100 - a},$$

where a is the percentage of ss-RNA annealing. Since it is unlikely that RNA melting and reannealing is 100% efficient, calculations of ds-RNA concentrations by this method probably result in under-estimations; however, they can serve as an approximation.

Using the above equation, the approximate concentrations of

Fig. 13. Sucrose density-gradient centrifugation of TMV specific ds-RNA. The same amount of TMV ds-RNA as used in Fig. 12 was added to 1 gm cucumber cotyledons, infected with TRSV for 3 days; total tissue nucleic acids were then extracted as described in Methods. The nucleic acids were resuspended in 1 x SSC buffer and centrifuged as outlined in Fig. 12. TMV specific ds-RNA was detected in the fractions by annealing to 6,220 cpm of ^{14}C-labelled TMV-RNA as described in Chapter 2.


16 S
23 S
800
700
600
500
400
300
200
100
0
1
5
10
15
VIRAL RNA ANNEALED (CPM)
FRACTION NUMBER

TRSV-specific ds-RNA in virus-infected leaves have been calculated. The concentration of virus-specific ds-RNA in cucumber cotyledons when it reaches its maximum (Table 2 and Fig. 6), three days after inoculation, was found to range from 8 µg per gm (Expt c, Fig. 1) to 54 µg per gm (Expt b, Fig. 1) fresh weight of leaf material. Similar calculations derived from data on beans infected with TRSV and TMV are summarised in Table 4, which indicates the presence of lower concentrations of virus-specific ds-RNA in infected French beans.

CONCLUSIONS

Although, in experiments described in this chapter, TRSV-RF and RI were not detectable, a polydisperse low molecular weight ds-RNA was shown to be present in the nucleic acids from TRSV-infected plants. This RNA is unlikely to be a degradation product of TRSV high molecular weight ds-RNA for the following reasons: (1) several methods of RNA extraction resulted in the isolation of ds-RNA with essentially identical properties, (2) the same techniques used for extraction of RNA from TMV-infected plants resulted in the isolation of RF (Fig. 12), and (3) since the added TMV-RF was recovered from TRSV-infected tissue by the usual extraction procedure (Fig. 13), it does not appear that there is any evidence for a TRSV-induced nuclease capable of degrading

TABLE 4

DETECTION AND ESTIMATION OF ds-RNA IN TRSV AND TMV INFECTED PLANTS WITH NUCLEOTIDE SEQUENCES COMPLEMENTARY TO VIRAL RNA

ds-RNA isolated from[a]	Homologous ss-viral RNA added to each annealing assay		Viral RNA[b] annealed (cpm) to		Calculated[c] concentration of ds-RNA per gm infected (D) tissue (µg)
	cpm	g	H	D	
0.5 gm TRSV-infected cucumber cotyledons	4300	3.8	56	3800	54
10 gm TRSV-infected primary bean leaves	1600	1.3	118	884	0.33
10 gm TMV-infected primary bean leaves	2250	1.6	54	1493	0.64

a ds-RNA in salt soluble fraction of leaf nucleic acid preparations was further purified by the 2-methoxyethanol and CTA procedure (see Methods).

b Preparations from healthy plants (H) and TRSV-infected (D) were used.

c Calculations made using the equation $\text{ds-RNA} = \frac{2a \times \text{ss-RNA}}{100-a}$ (see Results for details).

TRSV-RF or RI during extraction.

It appears that the presence of TRSV-specific ds-RNA in infected cucumber cotyledons is associated with virus synthesis because: (1) the appearance and increase of ds-RNA concentration in leaves follows closely the rise and fall in the concentration of the virus-induced RNA-dependent RNA polymerase (Fig, 6) which is very similar to that reported by Peden *et al.* (1972), and (2) increase in ds-RNA concentration precedes the rapid accumulation of TRSV in infected cotyledons and its concentration drops very rapidly as soon as the virus concentration reaches a maximum (Fig. 6).

The equation used for estimation of ds-RNA, was found useful for correcting data obtained from annealing experiments in this and following chapters, like those in Table 2. As the relationship between % annealing and the ratio of $\frac{ds}{ss}$RNA is not linear when using extreme ratios of $\frac{ds}{ss}$RNA in the annealing mixture, experimental errors can lead to erroneous estimations; however, the relationship is close to linear when "a" is between 30 and 70% (see Fig. 31B).

From the estimation of TRSV ds-RNA concentration in cucumber cotyledons, isolation of large quantities of ds-RNA for further experiments seemed possible, and this is the subject of the following chapter.

CHAPTER 5

CHARACTERIZATION OF LOW MOLECULAR WEIGHT TRSV-SPECIFIC ds-RNA

INTRODUCTION

There have been several reports on the detection of low molecular weight RNA species in association with bacteriophage infections (Banergee *et al.*, 1969; Marcaud *et al.*, 1971), animal virus infection (Reich *et al.*, 1966) and in healthy animal cells (Patnaik and Taylor, 1973; Monckton and Naora, 1974). Viroids - the causal agents of some plant diseases - have also been identified as low molecular weight RNA (Diener, 1972).

The proposed structures of these RNA species differ from each other. Investigations of Prives and Silverman (1972) indicated that the 6S-RNA molecules associated with Qβ phage infection consist of two RNA chains held together by an extensive region of hydrogen-bonded base pairs which has single-stranded configuration at one or both termini. The nucleotide sequences of a 6.5S RNA associated with adenovirus infections has been determined, from which it has been concluded that this RNA is a unique species of nucleic acid with large regions of

intra-molecular base pairs (Ohe and Weissman, 1970, 1971). Based on the physical properties of the pathogenic RNA from citrus exocortis disease Semancik *et al.* (1973) suggested that this viroid has a t-RNA type, hair-pin structure with non-complementary single stranded regions.

In the previous chapter detection of a polydisperse low molecular weight ds-RNA from plants infected with TRSV was reported. High concentration of this ds-RNA produced in infected tissue allowed its purification and further characterization. The properties of TRSV-specific ds-RNA described in this chapter have provided suggestions on its possible structure.

METHODS

1. Polyacrylamide-gel Electrophoresis

Analysis of RNA preparations was carried out in 7.5 or 15% polyacrylamide gels (Peacock and Dingman, 1968) using a flat-sheet apparatus (Reid and Bieleski, 1968). Gels were made at room temperature and allowed to polymerise for at least 1 hr before use. The stock electrophoresis buffer, pH 8.3, contained 108 gm Tris, 55 gm Boric acid and 9.3 gm of EDTA per litre of solution. Up to 80 µl of RNA preparations in 1 x SSC buffer, containing 5% sucrose

was loaded in each well of the polyacrylamide gel and subjected to electrophoresis at 20 mA (100 volts) for the required time at 4°. At the conclusion of electrophoresis the gels were fixed in 5% TCA for 15 min, washed with distilled water and stained with a 0.02% aqueous solution of toluidine blue (Randles and Coleman, 1970). After destaining in water the gels were scanned at 620 nm using a Joyce-Loebl densitometer.

The flat-sheet apparatus was also used for preparative electrophoresis of TRSV-specific ds-RNA in 7.5% polyacrylamide gels. Samples of RNA in a volume of 1-1.5 ml were loaded onto a well spanning the entire width of the polyacrylamide sheet (110 x 90 x 3 mm) and electrophoresed for 2½ hr. At the conclusion of electrophoresis, three 5 mm wide strips of the gels were cut from both ends and the middle of the sheet. These were stained with toluidine blue as described above and used for locating the position of the ds-RNA in the gel sheet. The area of the gel containing the ds-RNA with an electrophoretic mobility slower than the 5S r-RNA was cut with a razor blade into pieces of about 30 mm^3. The gel cubes were mixed with 5 ml of 1M NaCl containing 1% SDS and 1 ml of water-saturated phenol containing 0.1% 8-hydroxyquinoline and left at 25° for 24 hr with slow shaking. The liquid phase was withdrawn with a Pasteur pipette and kept at -15°. Another volume of the salt-SDS solution and phenol were added to the gel cubes for further

extraction of the ds-RNA as before. The combined extracts were mixed with another 3 ml of water-saturated phenol, shaken for 1 min and centrifuged at 5,000g for 10 min to isolate the buffer phase. The RNA was precipitated with 2 volumes of chilled ethanol and left at -15° for at least 3 hr. The precipitate was collected by centrifugation at 5,000g for 10 min, resuspended in 0.5 ml of either 1 x SSC or 1 mM EDTA, pH 7, dialysed against the same solution for 6 hr at 4° and was then washed twice with ether. The residual ether was evaporated under vacuum. About $O.D._{260nm}$ units of TRSV-specific ds-RNA were recovered from each gel to which the salt soluble nucleic acid from 10 gm infected tissue was applied.

2. Isopycnic Ultracentrifugation

Nucleic acid preparations in 5 mM Tris-HCl, pH 7.5 (Bishop and Koch, 1967), were mixed with solutions of caesium sulphate in the same buffer to give a density of 1.6 gm/cm^3. These preparations were centrifuged at 44,000 rpm for 50 hr in a SW50 rotor at 5°. After centrifugation 0.25 ml fractions of the gradients were collected from the bottoms of the tubes, the density of every third fraction was determined gravimetrically and the absorption at 260 nm of each fraction was determined after appropriate dilution. To determine the distribution of TRSV-specific ds-RNA in the gradients, consecutive pairs of fractions were pooled, dialysed against three changes of 1 x SSC

buffer for 24 hr at 4° and after the addition of 100 µg of leaf RNA from healthy cucumber plants to each sample, the nucleic acids were precipitated with ethanol, resuspended in 1 x SSC and used for annealing with ^{14}C-labelled TRSV-RNA as described in Chapter 2.

3. Gel Filtration Through Sephadex Columns

Sephadex G-200 was washed and soaked in 0.2M NaCl, degassed and packed into a 1 x 150 cm or 1 x 100 cm column. The flow rates of the longer columns was increased by application of pressure to the reservoir. The salt-soluble fraction of a nucleic acid preparation (prepared as described in Chapter 4) from up to 40 gm of TRSV-infected cucumber cotyledons in a final volume of about 2 ml was applied to a column and was eluted with 0.2M NaCl at room temperature. The absorbancy of the effluent was monitored at 254 nm with an ISCO analyzer and 4 ml fractions were collected.

4. Chromatography on Methylated Albumin Kieselguhr (MAK) Columns

MAK columns were prepared as described by Sueoka and Cheng (1962). Celite (14 gm) was mixed with 70 ml 0.1M NaCl in 0.05M phosphate buffer, pH 7.6, boiled to expel air, and the flask sealed with a rubber bung. After cooling, 5 ml of water containing 35 mg of methylated serum albumin was added, the mixture was shaken

vigorously, and then stored at 4°. Compressed air was used to pack this material into columns of 0.7 x 7 cm. About 100 µg of salt soluble nucleic acid was applied to the column at room temperature and low molecular weight material washed with 0.2M NaCl in 0.01M Tris-HCl, pH 7.8, until no ultraviolet absorbing material was detected in the effluent. Nucleic acids were then eluted with 30 ml of a linear 0.4 - 1.0M NaCl gradient buffered with 0.01M Tris, pH 7.8.

5. Chromatography of Hydroxyapatite

Columns of 1 x 1.5 cm were packed with commercially prepared hydroxyapatite in 1 mM phosphate buffer, pH 6.8 (Bernardi, 1969), and were equilibrated with 0.05M potassium phosphate buffer, pH 6.8 (KP buffer), at room temperature. Nucleic acids in either 0.2M NaCl or 0.05M KP buffer were applied to the column and washed with 0.05M KP buffer. Elution was then carried out with a 50-60 ml linear gradient of 0.05 - 0.3M KP buffer.

6. Chromatography on Cellulose Powder

Columns of 1 x 15 cm were prepared as described by Franklin (1966). Cellulose powder was washed with STE buffer (0.05M Tris, 0.1M NaCl, 0.001M EDTA, pH 6.85) and fine particles were removed by decantation. Columns were packed and washed with STE containing 10 mM EDTA and 1% 2-mercaptoethanol. They were then washed with 35%

ethanol in STE buffer. Salt soluble nucleic acids isolated from 1-4 gm of TRSV-infected cucumber cotyledons in 0.2 ml of ethanol : STE 35 : 65 were applied to the column and eluted step-wise with 35% ethanol in STE, 15% ethanol in STE and finally STE buffer alone. RNA obtained after each elution step was ethanol precipitated and resuspended in 1 x SSC buffer for determining the amount of TRSV-specific ds-RNA by annealing to ^{14}C-TRSV RNA or for analysis by polyacrylamide-gel electrophoresis.

7. Formaldehyde Treatment of RNA

To samples of RNA (20-40 µg/ml) was added NaCl to 0.1M (Miura, 1966), and HCHO to 1.1M (Boedtker, 1967). Reaction with formaldehyde was carried out either at 37^{o} for 30 min or at 75^{o} for 15 min, followed by quick coding in an ethanol-ice bath. Ultra-violet absorption spectra were determined as described in Chapter 2.

RESULTS

I. Detection of TRSV-specific ds-RNA in Polyacrylamide-gels by Staining Technique

When the salt soluble fractions of RNA preparations from TRSV infected and healthy cucumber cotyledons were subjected to electro-phoresis in 7.5% polyacrylamide gels, a broad band of nucleic acid

migrated behind 5S r-RNA in the preparations from infected cotyledons (Fig. 14a), but not in that from healthy tissue (Fig. 14b). Both the RNase resistance and electrophoretic mobility of this RNA (Fig. 15) indicate that it is the same virus specific ds-RNA which was described in the previous chapter. Using 4S t-RNA and 5S r-RNA as markers (Bishop *et al.*, 1967) (Fig. 15), the molecular weight of the ds-RNA was estimated to be between 35,000 and 70,000 daltons.

II. Effect of Denaturation of TRSV-specific ds-RNA on its Electrophoretic and Sedimentation Properties

A preparation of ds-RNA purified by polyacrylamide-gel electrophoresis was divided into two parts and one was denatured by heat. Results of an experiment in which native and denatured ds-RNA were subjected to polyacrylamide-gel electrophoresis are summarized in Fig. 16, showing that denaturation causes the RNA to migrate as a broader band without significantly changing its rate of movement (Fig. 16A), but makes it sensitive to RNase (Fig. 16B). In a subsequent experiment it was demonstrated that denaturing the ds-RNA by incubation at 20° for 10 min in 90% dimethyl sulphoxide (DMSO) (Pinck and Hirth, 1973) or by heating at 75° with 1.1M formaldehyde (Prives and Silverman, 1972) failed to change the position of the ds-RNA band in polyacrylamide gels (Fig. 17). On formaldehyde treatment t-RNA and 5S r-RNA migrated at much slower rates (Fig. 17C).

Fig. 14. Gel electrophoresis of the salt soluble nucleic acid isolated from TRSV-infected (a) and healthy (b) cucumber cotyledons. Electrophoresis was carried out in 7.5% polyacrylamide at 20 mAmp (90-100 volts) for 2.5 hr at 4^{o} as described in Methods (Chapter 5). The procedure for preparation of the salt soluble nucleic acid was described in Chapter 4 (Methods).

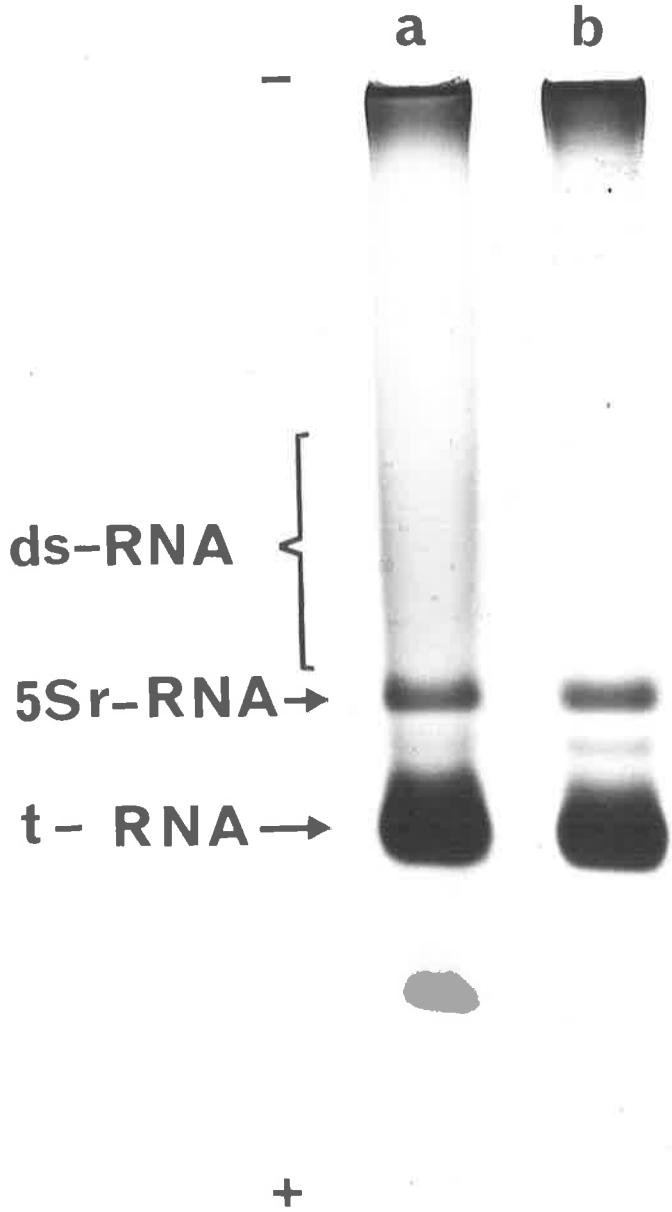

a
b
–
ds-RNA
5Sr-RNA
t- RNA
+

Fig. 15. Polyacrylamide-gel electrophoresis of the salt soluble nucleic acid from TRSV-infected tissue before (a) and after (b) RNase treatment. RNase was added (10 µg/ml) to the nucleic acid sample resuspended in 1 x SSC and incubated at 37° for 30 min, before phenol re-extraction. Electrophoresis was carried out as described in Fig. 14.

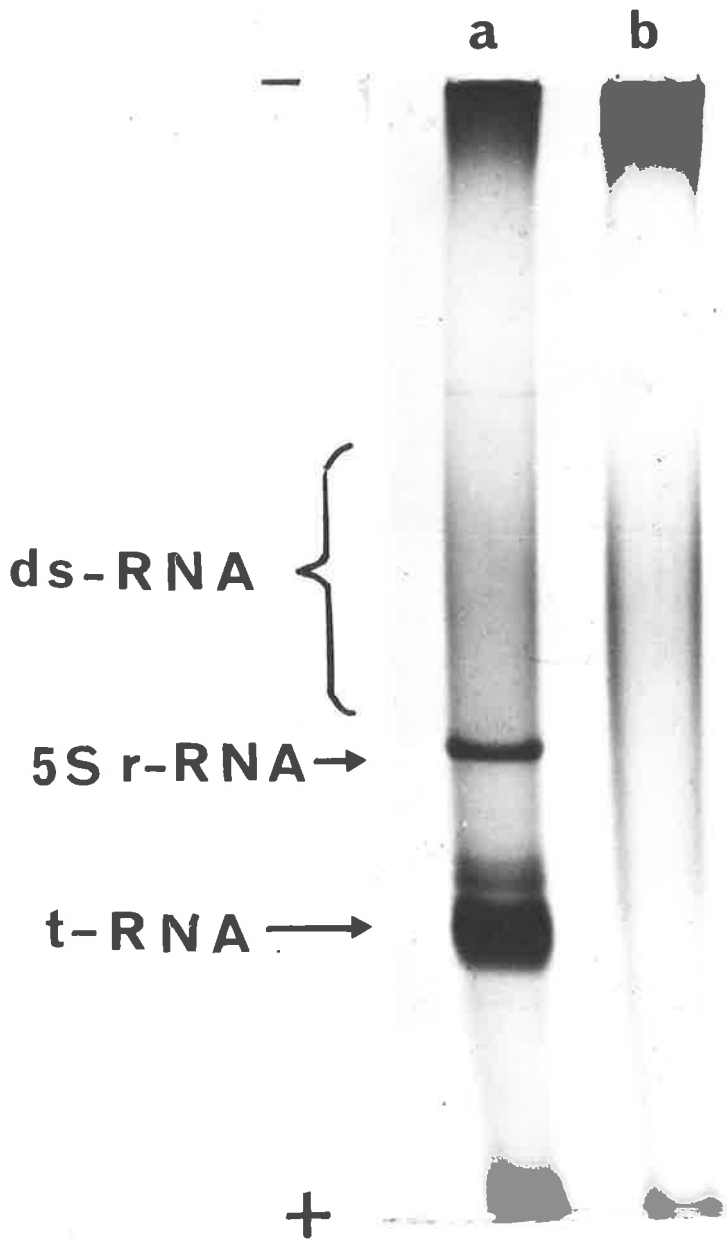

a
b
–
ds-RNA
5S r-RNA
t-RNA
+

Fig. 16. Polyacrylamide-gel electrophoresis of native and heat denatured TRSV-specific ds-RNA before and after treatment with RNase. The ds-RNA was prepared electrophoretically as described in Methods (Chapter 5). Denaturation of ds-RNA was carried out in 1 mM EDTA, pH 7, by heating at 100° for 10 min followed by rapid cooling at about -8°. Conditions for RNase digestion as described in Fig. 15. Electrophoresis was carried out for 1.5 hr as outlined in Fig. 14.

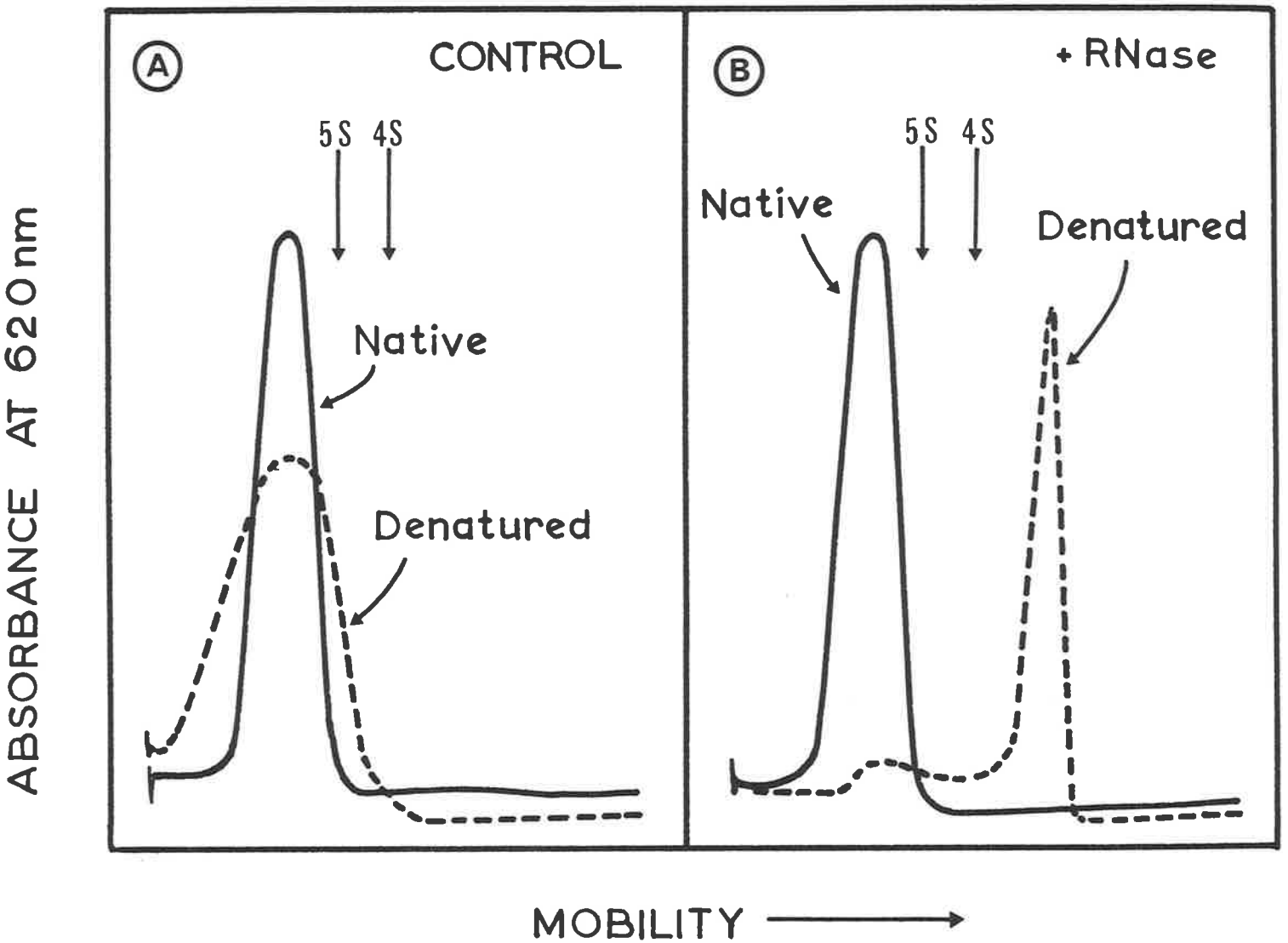
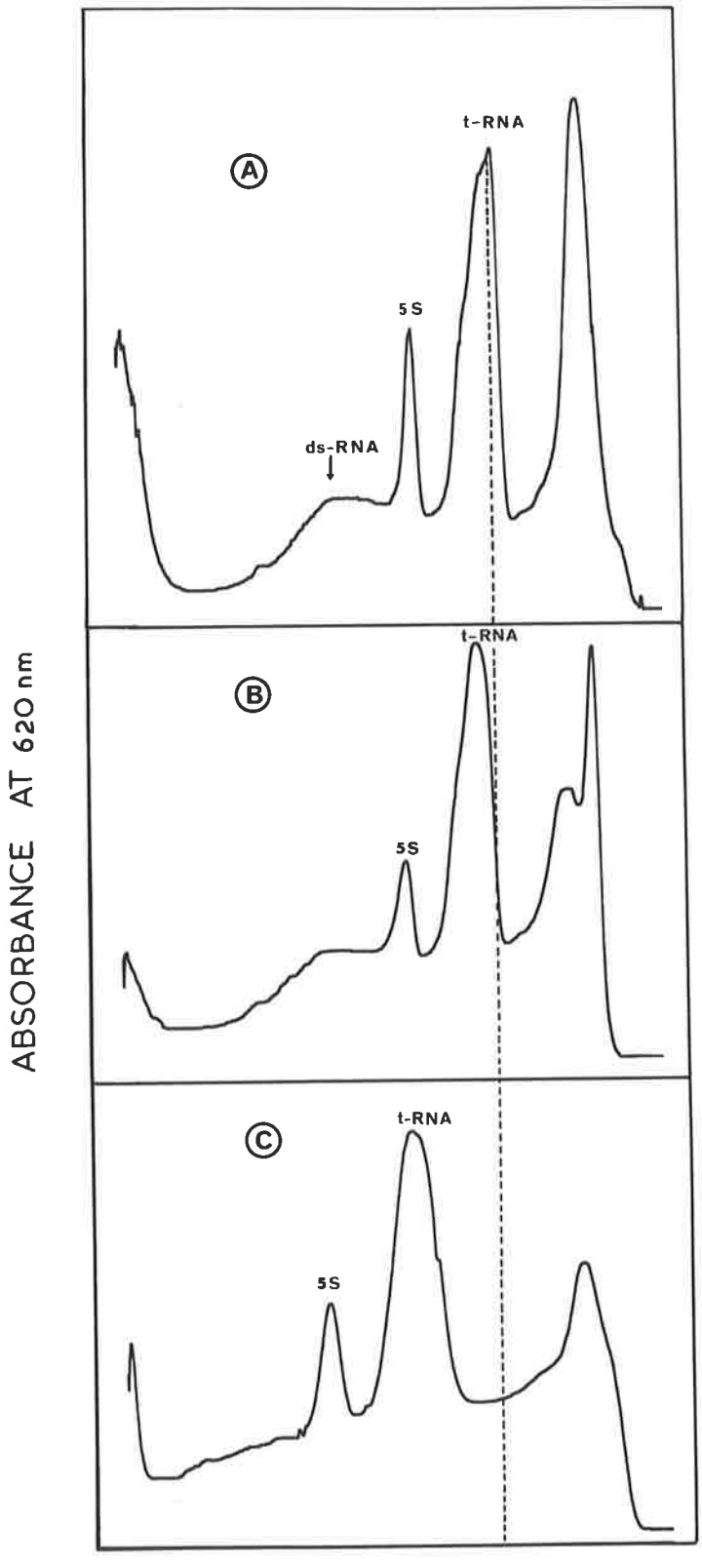

A
CONTROL
5S
4S
Native
Denatured
B
+ RNase
5S
4S
Native
Denatured
ABSORBANCE AT 620nm
MOBILITY

Fig. 17. Polyacrylamide-gel electrophoresis of the salt soluble nucleic acid from TRSV-infected cucumber cotyledons before (A) and after denaturation with DMSO (B) and formaldehyde (C). Nine volumes of DMSO were added to the RNA preparation and incubated at 20° for 10 min. Formaldehyde treatment was carried out at 85° as described in Methods (Chapter 5). Both denatured nucleic acid samples were ethanol precipitated and taken up in 1 x SSC before polyacrylamide gel electrophoresis for 1.5 hr, as described in Fig. 14. The RNA bands were located by scanning of the stained gel in a Joyce-Loebl densitometer.


A
t-RNA
5S
ds-RNA
B
t-RNA
5S
C
t-RNA
5S
ABSORBANCE AT 620 nm
MOBILITY

Experiments in which native and heat denatured ds-RNA were sedimented in rate-zonal sucrose density-gradients demonstrate that denaturation did not have a significant effect on the sedimentation rate of the RNA (Fig. 18). By using 16S and 23S *E. coli* r-RNA and yeast 4S t-RNA as markers, the mean sedimentation coefficient of ds-RNA was estimated to be approximately 6S (results not shown).

III. Isopycnic Ultracentrifugation of TRSV-specific ds-RNA

When the salt soluble fraction of a nucleic acid preparation from TRSV-infected cucumber cotyledons was digested with RNase and subjected to isopycnic centrifugation in caesium sulphate, two bands with densities of 1.41 gm/cm^3 and 1.60 gm/cm^3 were detected (curve a, Fig. 19). Annealing of the fractions from the gradient with ^{14}C-labelled TRSV-RNA demonstrated that the material with density of 1.60 gm/cm^3 contained TRSV-specific ds-RNA (curve b, Fig. 19). In the same experiment, a partially purified preparation of ds-RNA recovered from a Sephadex G-200 column (Fig. 20, peak a) and not treated with RNase at any time, was also subjected to isopycnic ultracentrifugation. Again, two bands with densities of 1.41 gm/cm^3 and 1.60 gm/cm^3 were detected (curve c, Fig. 19). These data indicate that TRSV-specific ds-RNA has a buoyant density of 1.60 gm/cm^3 in Cs_2SO_4 which is characteristic of ds-RNA (Ralph, 1969).

Fig. 18. Sucrose density-gradient centrifugation of a purified preparation of TRSV-specific ds-RNA before (A) and after (B) heat denaturation as described in Fig. 16. A mixture of the native and the heat denatured RNA was also analysed (C). Approximately 20 µg of RNA was layered on 5-25% linear sucrose density gradients, buffered with 1 x SSC and centrifuged at 50,000 rpm for 10 hr as described in Chapter 2.

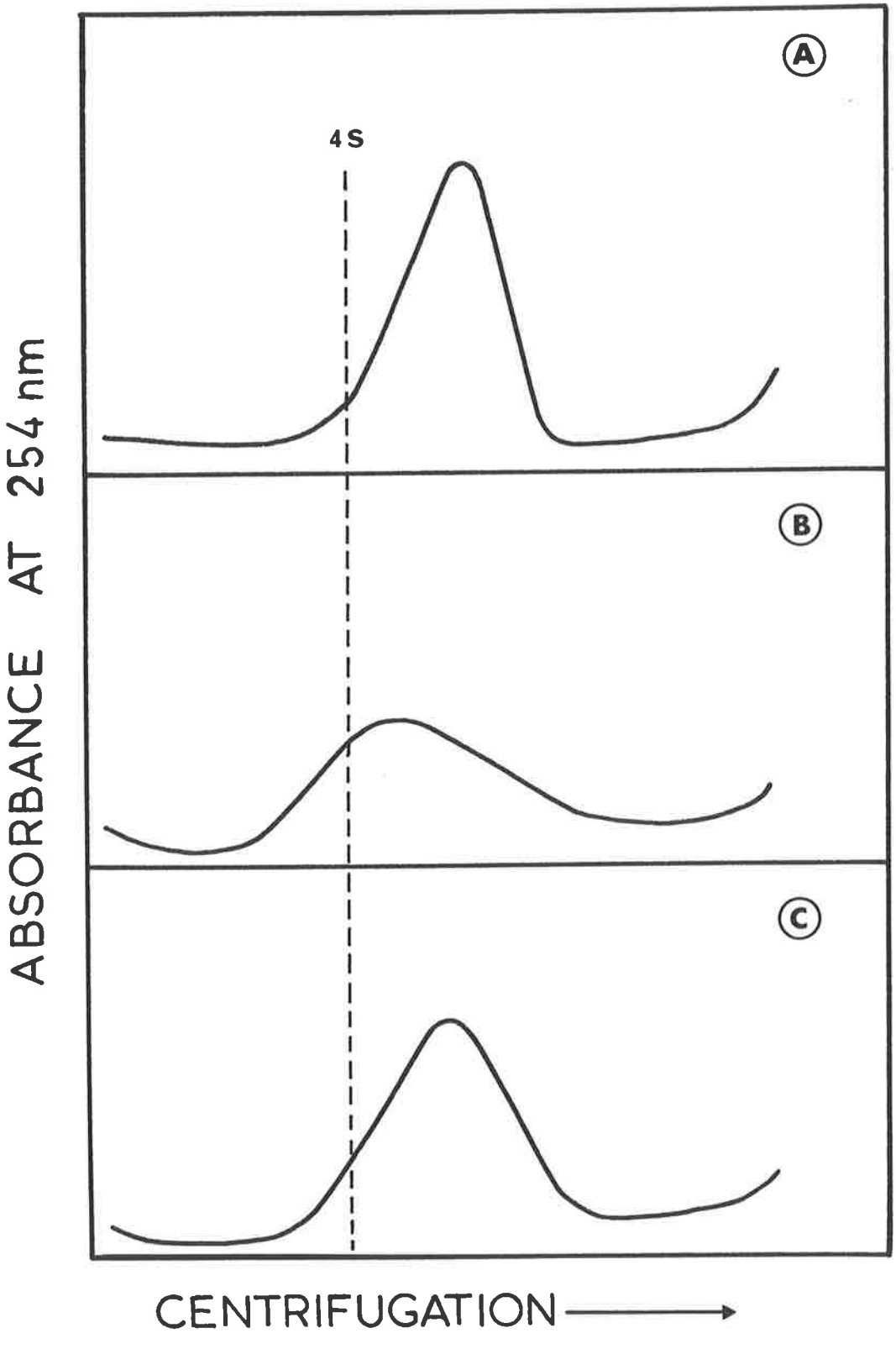
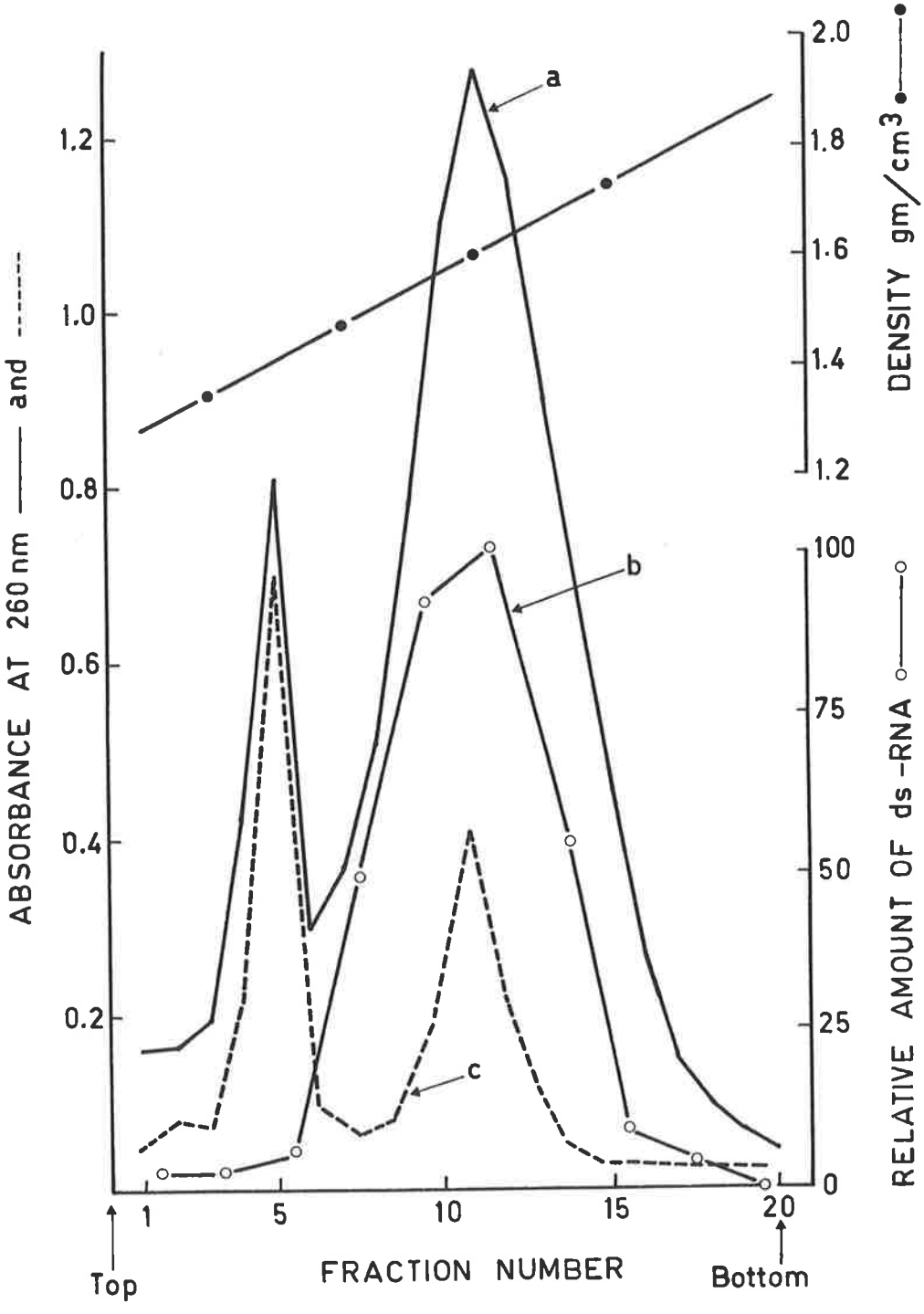

A
4S
B
C
ABSORBANCE AT 254 nm
CENTRIFUGATION

Fig. 19. Isopycnic centrifugation of the nucleic acids containing TRSV-specific ds-RNA in caesium sulphate. RNA samples were mixed with solutions of Cs_2SO_4 and centrifuged at 44,000 rpm for 50 hr in a Beckman SW50 rotor (see Methods, Chapter 5). Fractions of 0.25 ml were collected and used for determination of ultraviolet absorbance and for annealing with ^{14}C-labelled TRSV-RNA as described in Methods (Chapter 2).

Curve a: a salt soluble fraction of nucleic acids from cucumber cotyledons infected with TRSV for 3 days was digested with RNase as described in Fig. 15.

Curve b: distribution of the virus-specific ds-RNA in the curve a as determined by annealing of fractions with labelled TRSV-RNA; the percentage radioactive annealing "a" was used in the equation $\frac{\text{ds-RNA}}{\text{ss-RNA}} = \frac{a}{100-a}$ (see Chapter 4, Results) to estimate the relative amount of ds-RNA.

Curve c: a partially purified preparation of ds-RNA not treated with nucleases (peak a, Fig. 20).


ABSORBANCE AT 260 nm —— and ------
DENSITY gm/cm³ ●——●
RELATIVE AMOUNT OF ds-RNA ○——○
a
b
c
1.2
1.0
0.8
0.6
0.4
0.2
2.0
1.8
1.6
1.4
1.2
100
75
50
25
0
1
5
10
15
20
Top
FRACTION NUMBER
Bottom

IV. Gel Filtration of TRSV-specific ds-RNA through Sephadex

When the salt soluble fraction of a nucleic acid preparation from TRSV-infected cucumber cotyledons was chromatographed on a column of G-200 Sephadex, three distinct ultraviolet absorbing peaks were eluted (Fig. 20).

Annealing material from the fractions of the eluate to ^{14}C-labelled TRSV-RNA, it was shown that the virus specific ds-RNA eluted as a broad peak which did not exactly coincide with any of the peaks detected by monitoring absorbancy at 254 nm (Fig. 20). Polyacrylamide-gel electrophoresis (Fig. 21A) and isopycnic density-gradient centrifugation (curve c, Fig. 19) of the material recovered from peak a in Fig. 20 showed that it contained both DNA and ds-RNA, whereas that from peak b in Fig. 20 contained t-RNA (Fig. 21B). The material eluted as peak c (Fig. 20) did not have an absorption spectrum typical of nucleic acids and most of the absorption could be accounted for by light scattering: it may have contained polysaccharides.

In a subsequent experiment the salt soluble fraction of nucleic acid from infected cotyledons was digested with DNase, followed by RNase and then subjected to Sephadex column filtration (Fig. 22A). An ultraviolet absorbing peak was eluted in a similar position to that of peak a in Fig. 20. This material had an ultraviolet absorption spectrum typical of nucleic acids with a 260/280 nm ratio of 2.0 and was shown to consist of only ds-RNA on analysis by polyacrylamide-gel electrophoresis (Fig. 22B).

Fig. 20. Gel filtration of the salt soluble nucleic acid preparation from TRSV-infected cucumber cotyledons through a column of G-200 Sephadex (1 x 150 cm). Elution was carried out with 0.2M NaCl at room temperature and 4 ml fractions were collected. Aliquots of 0.15 ml from the fractions were used for annealing with labelled viral RNA and relative amounts of ds-RNA were estimated as described in Fig. 19.

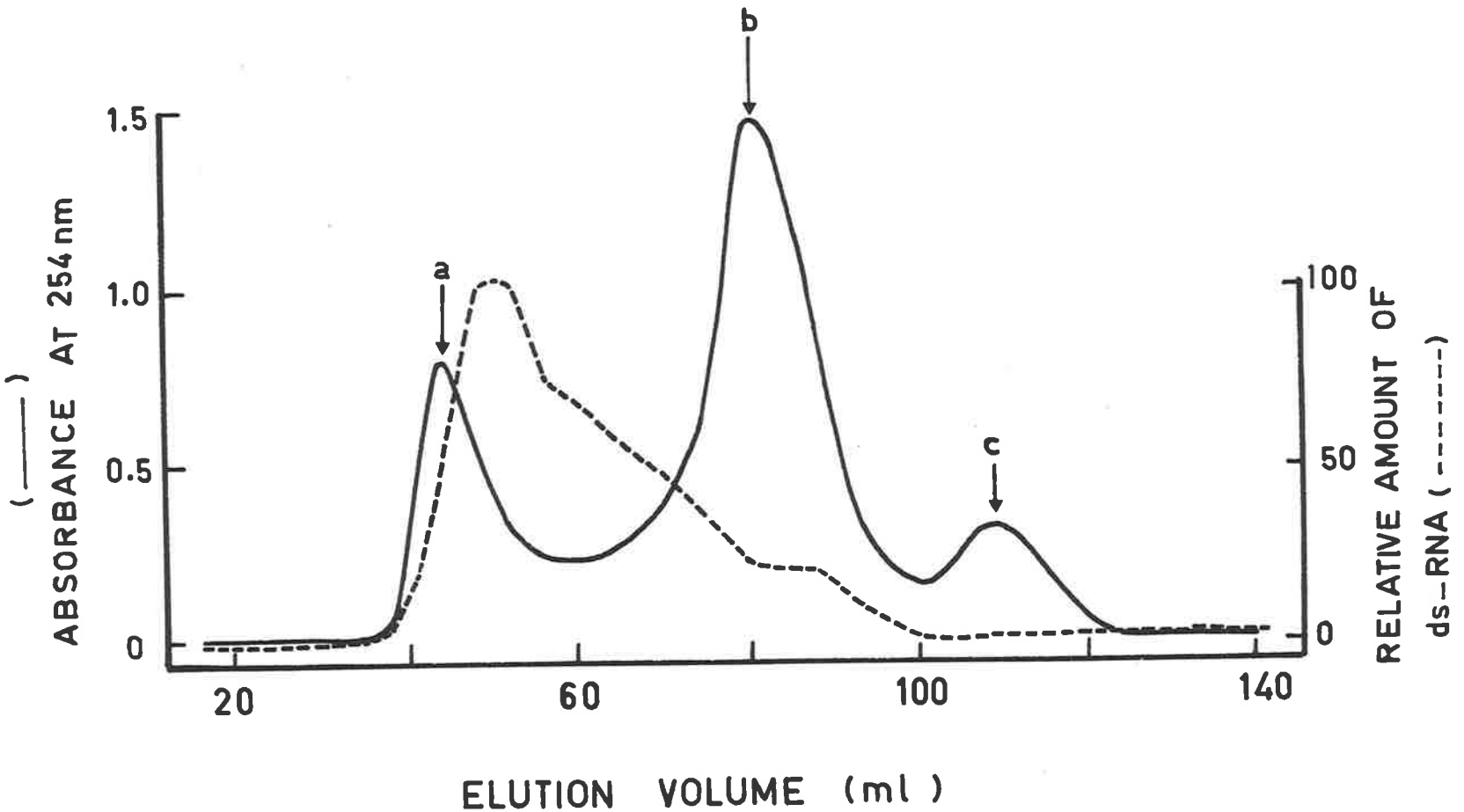

b
a
c
1.5
1.0
0.5
0
100
50
0
20
60
100
140
ABSORBANCE AT 254 nm (——)
RELATIVE AMOUNT OF ds-RNA (- - - - - -)
ELUTION VOLUME (ml)

Fig. 21. Electrophoresis of the nucleic acid eluting (A) as the first peak and (B) as the second peak from the Sephadex column (peak a and peak b, Fig. 20) in 15% polyacrylamide. The nucleic acids in each ultraviolet absorbing peak of the Sephadex column (Fig. 20) was reconcentrated by ethanol precipitation and subjected to electrophoresis for 5 hr as described in Fig. 14. After staining the gel with toluidine blue, the positions of the bands were recorded by the use of a Joyce-Loebl densitometer.

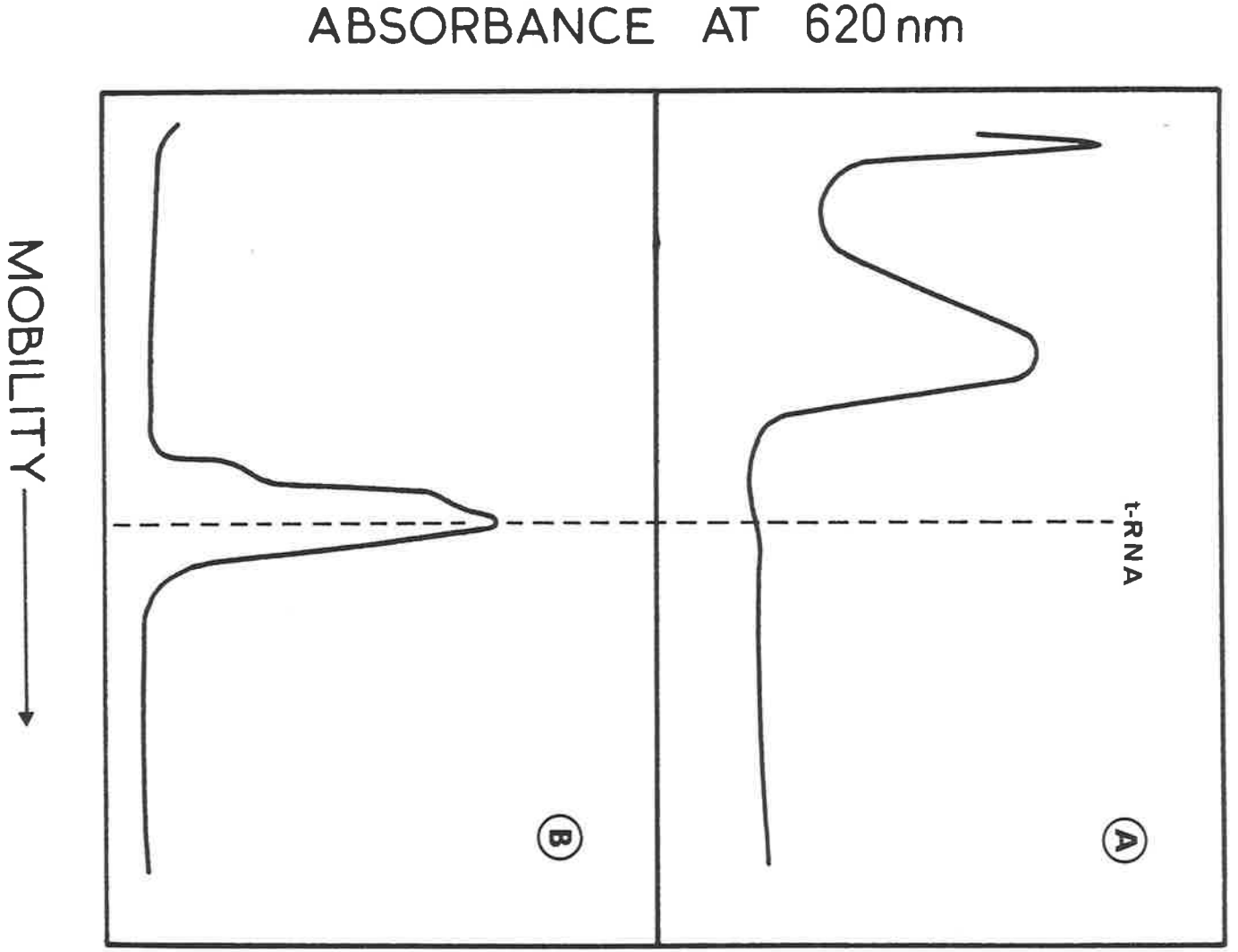
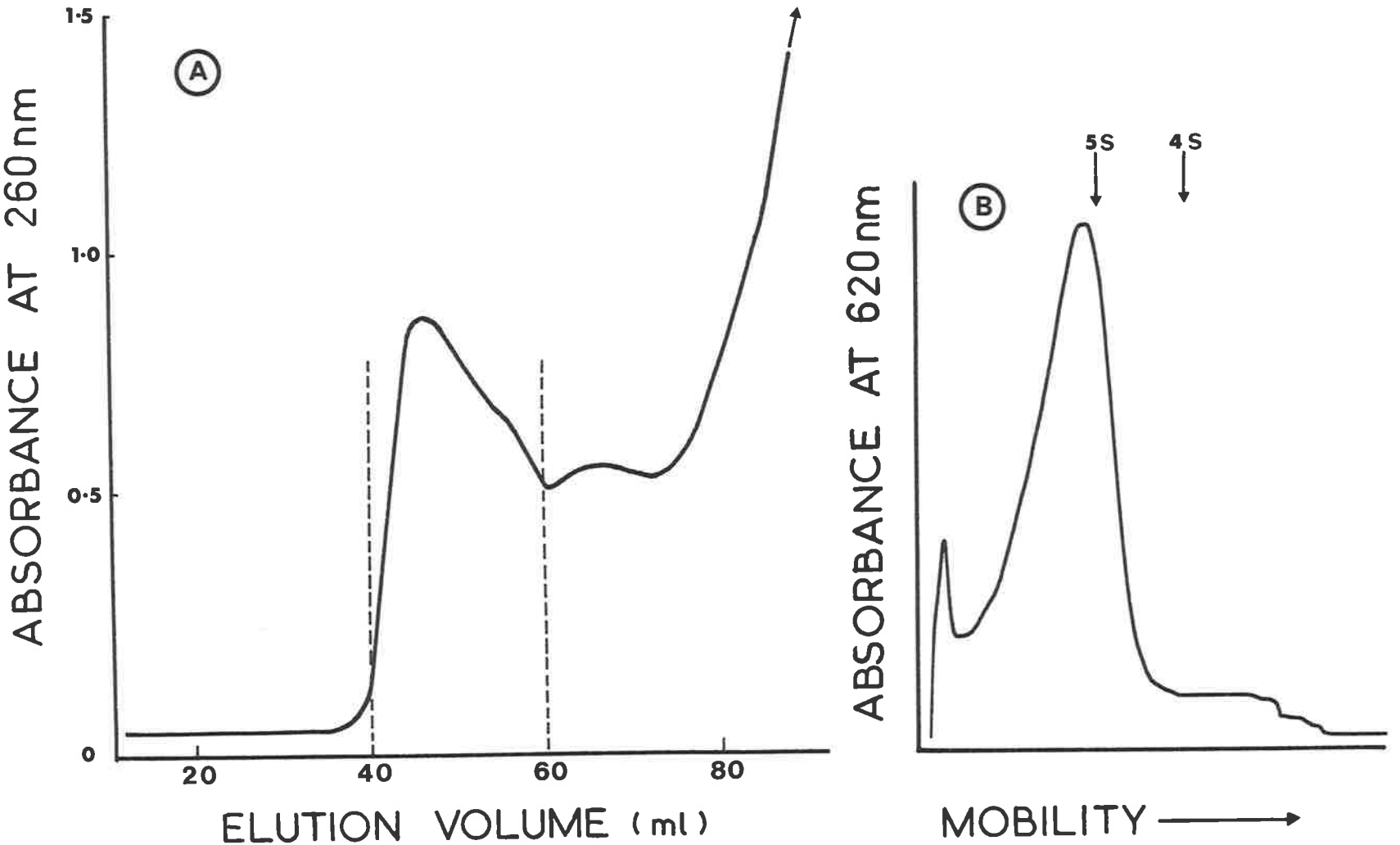

ABSORBANCE AT 620 nm
MOBILITY
t-RNA
B
A

Fig. 22. (A) Gel filtration of the salt soluble nucleic acid preparation from infected cucumber cotyledons after treatment with nucleases. The nucleic acid resuspended in STMg buffer (0.2M NaCl, 0.005M Tris, 0.01M $MgCl_2$, pH 7.6), was incubated with DNase (100 µg/ml at 30°) for 90 min, then with RNase (10 µg/ml at 37°) for 30 min. After phenol re-extraction of the nucleic acid, RNA was applied to a Sephadex G-200 column of 1 x 100 cm as described in Fig. 20. The material eluting as the first ultraviolet absorbing peak was concentrated by chromatography on a hydroxyapatite column eluted with 0.2M KP buffer (see Methods, Chapter 5), dialysed to reduce phosphate concentration and ethanol precipitated. The precipitate was resuspended in 1 x SSC and subjected to electrophoresis in 15% polyacrylamide (B).


A
1·5
1·0
0·5
0
ABSORBANCE AT 260nm
20
40
60
80
ELUTION VOLUME (ml)
B
5S
4S
ABSORBANCE AT 620nm
MOBILITY

V. Chromatography of TRSV-specific ds-RNA on MAK Columns

Purified virus specific ds-RNA, prepared by nuclease treatment and Sephadex gel filtration as described above, eluted as a sharp peak with 0.4M NaCl, which was the initial molarity of the eluate (Fig. 23A). Similarly, when the salt soluble fraction of the nucleic acids extracted from TRSV-infected cucumber cotyledons without any nuclease digestion steps was chromatographed on a MAK column, material annealing with ^{14}C-labelled TRSV-RNA eluted with 0.4M NaCl (Fig. 23B).

These data indicate that TRSV-specific ds-RNA elutes from MAK columns under conditions somewhat similar to that of t-RNA. However, it is possible that an even lower NaCl concentration than 0.4M can release the ds-RNA from MAK columns.

VI. Chromatography of TRSV-specific ds-RNA on Hydroxyapatite Columns

The salt soluble fraction of a nucleic acid preparation from TRSV-infected cucumber cotyledons eluted as a broad band of ultra-violet absorbing material partially separated into two peaks, a and b (Fig. 24A). Peak a eluted with approximately 0.13M KP buffer which is the phosphate concentration required for the elution of t-RNA (Bernardi, 1969). Virus specific ds-RNA prepared by nuclease

Fig. 23. Chromatography of purified TRSV-specific ds-RNA (A) and the salt soluble nucleic acid preparation from virus-infected cucumber cotyledons (B) on MAK columns. Samples of RNA were applied to columns of 0.7 x 7 cm and washed with 0.2M NaCl in 0.01M Tris, pH 7.8, until the ultraviolet absorbance of the effluent was the same as that of the salt solution. A linear gradient of NaCl in the same buffer was applied and the absorbance was recorded at 254 nm using an ISCO apparatus. Fractions of 1 ml were collected and used for annealing with ^{14}C-labelled TRSV-RNA. The relative amount of ds-RNA was estimated as described in Fig. 19.

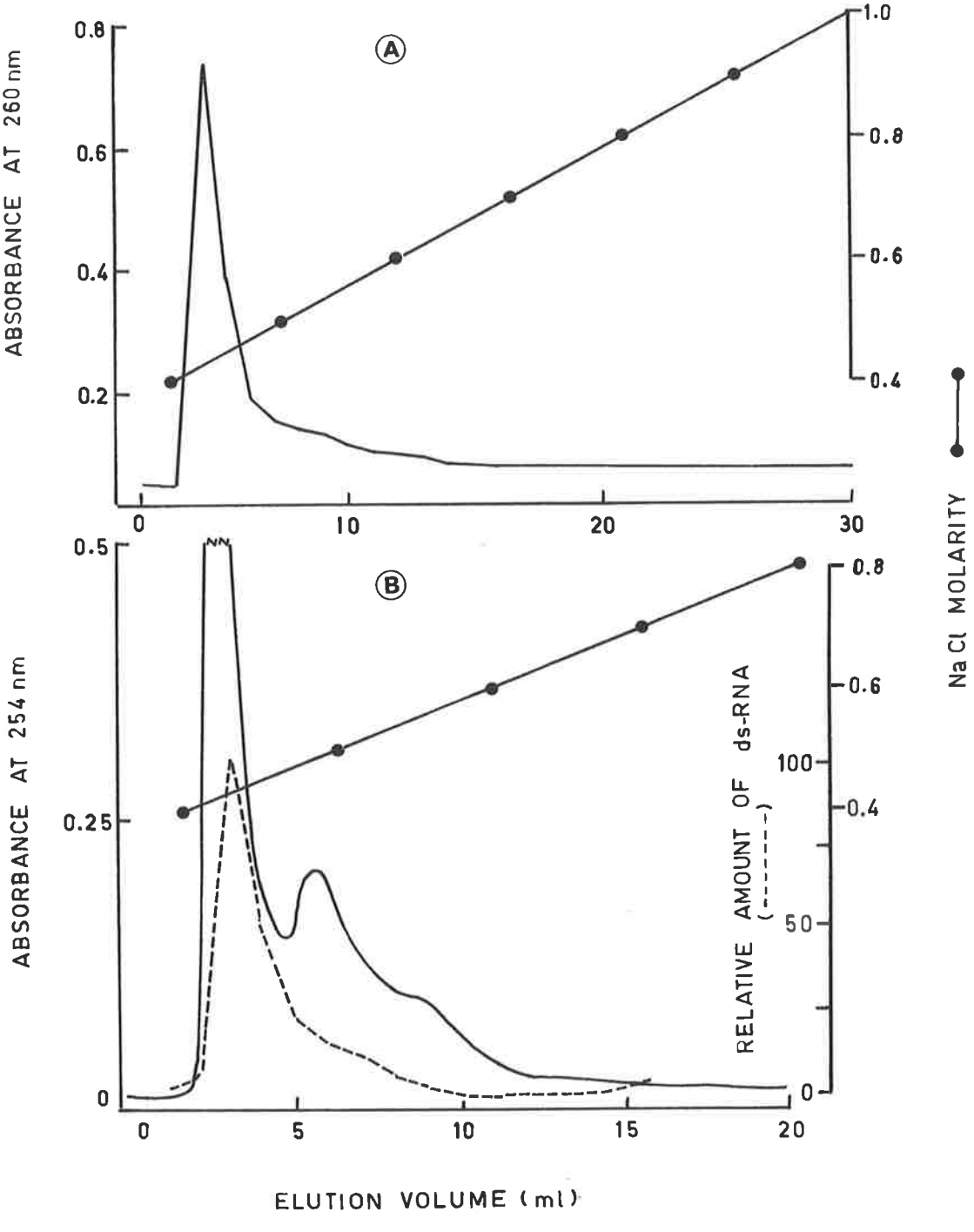
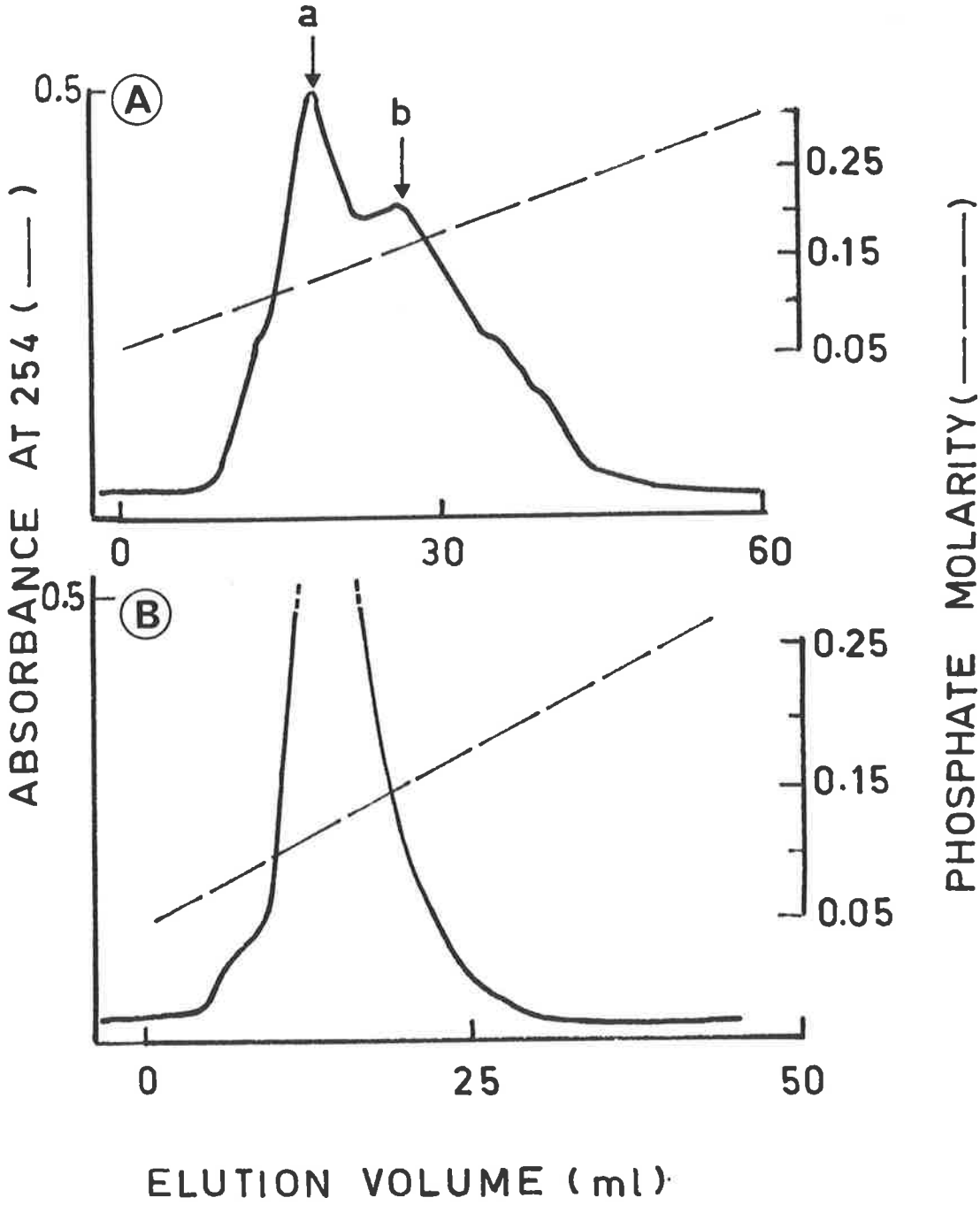

ABSORBANCE AT 260 nm
0.8
0.6
0.4
0.2
A
1.0
0.8
0.6
0.4
0
10
20
30
ABSORBANCE AT 254 nm
0.5
0.25
0
B
0.8
0.6
0.4
100
50
0
RELATIVE AMOUNT OF ds-RNA
(- - - - - -)
0
5
10
15
20
ELUTION VOLUME (ml)
NaCl MOLARITY

Fig. 24. Chromatography of the salt soluble fraction of nucleic acids from TRSV-infected cucumber cotyledons (A) and the purified virus specific ds-RNA (B) on columns of hydroxyapatite. Columns of 1 x 1.5 cm were prepared and eluted with a linear gradient of potassium phosphate buffer, pH 6.8, as described in Methods (Chapter 5). (Calf thymus DNA eluted from this column with 0.22M phosphate buffer.)


a
b
0.5
A
0.25
0.15
0.05
0
30
60
0.5
B
0.25
0.15
0.05
0
25
50
ABSORBANCE AT 254 (——)
PHOSPHATE MOLARITY (— — —)
ELUTION VOLUME (ml)

digestion and gel filtration as described above, eluted from a similar column (Fig. 24B) in a position corresponding to peak a in Fig. 24A. These results demonstrate that TRSV-specific ds-RNA does not elute from hydroxyapatite with phosphate concentrations characteristic of double-stranded nucleic acids (0.22M, Bernardi, 1969).

VII. Chromatography of TRSV-specific ds-RNA on Cellulose Columns

The salt soluble fraction of a nucleic acid preparation from TRSV-infected cucumber cotyledons was chromatographed on a cellulose column and the fractions eluting after each STE buffer-ethanol elution step were pooled (Fig. 25). By annealing each of these pooled fractions with ^{14}C-labelled TRSV-RNA it was demonstrated that most of the ds-RNA eluted after the 15% ethanol in STE buffer elution step (Fig. 25). Each of the three pooled fractions recovered from the column (Fig. 26A) were concentrated by ethanol precipitation and analyzed by polyacrylamide-gel electrophoresis. It was found that t-RNA eluted after the 35% ethanol elution step, whereas both ds-RNA and 5S r-RNA eluted after the 15% ethanol step (Fig. 26B-D). These data indicate that TRSV-specific ds-RNA elutes from cellulose columns neither like the t-RNA nor like typical ds-RNA, the latter eluting with STE buffer (Franklin, 1966).

Fig. 25. Chromatography of the salt soluble fraction of nucleic acids from TRSV-infected cucumber cotyledons on a cellulose column (1 x 15 cm), eluted step-wise with STE : ethanol mixtures. The nucleic acids eluted at each step were reconcentrated by ethanol precipitation and used for annealing with ^{14}C-labelled TRSV-RNA. Relative amounts of ds-RNA were estimated from the annealing data as described in Fig. 19.

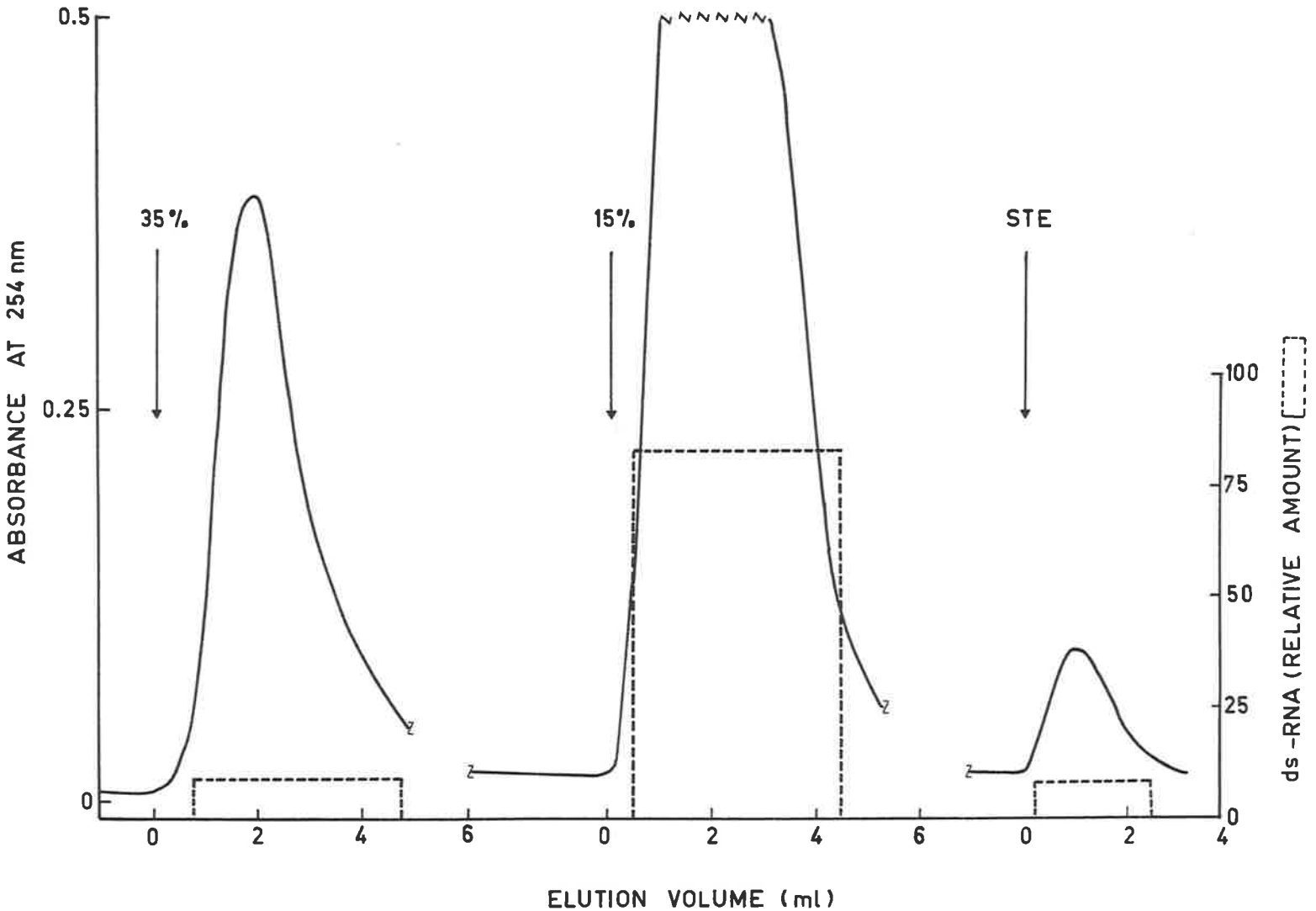
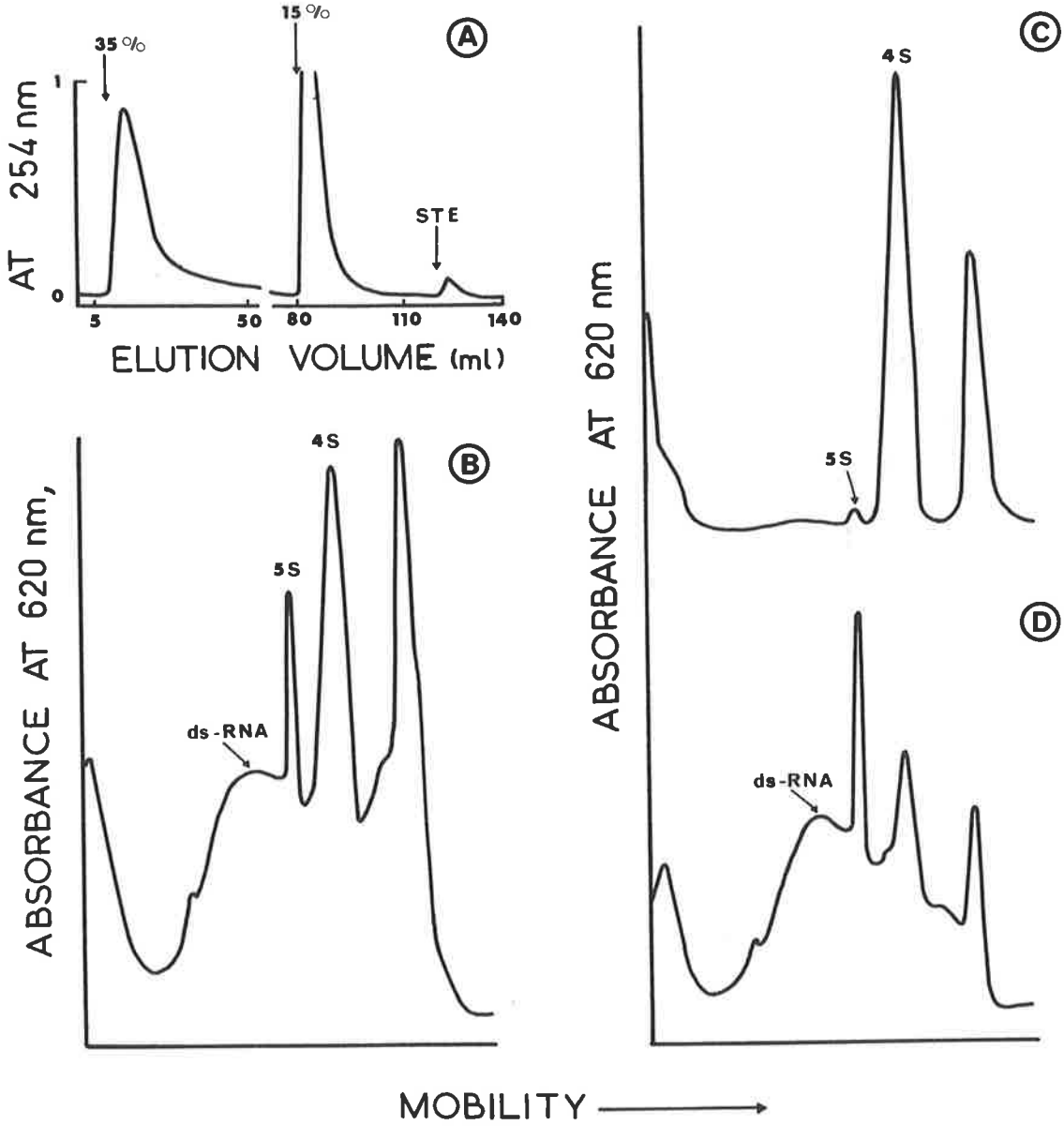

0.5
0.25
0
ABSORBANCE AT 254 nm
35%
15%
STE
100
75
50
25
0
ds -RNA (RELATIVE AMOUNT)
0
2
4
6
0
2
4
6
0
2
4
ELUTION VOLUME (ml)

Fig. 26. (A) Chromatography of the salt soluble nucleic acids on CF11 cellulose as described in Fig. 25. The same nucleic acid preparation was analyzed in a 7.5% polyacrylamide-gel before cellulose chromatography (B) and after elution with 35% ethanol in STE (E) and 15% ethanol in STE (D).


A
35 %
15 %
STE
AT 254 nm
1
0
5
50
80
110
140
ELUTION VOLUME (ml)
B
4S
5S
ds-RNA
ABSORBANCE AT 620 nm,
C
4S
5S
D
ds-RNA
ABSORBANCE AT 620 nm
MOBILITY

VIII. Serological Tests with Antiserum to Synthetic ds-RNA

A preparation of TRSV-specific ds-RNA prepared by nuclease digestion and gel filtration was used in immunodiffusion tests against an antiserum to poly (I) : poly (C). Preparations of the ds-RNA with absorbance at 260 nm between 0.78 and 6.25 failed to produce visible immunodiffusion bands. However, the same antiserum reacted with the homologous antigen with absorbance at 260 nm between 0.07 and 0.55.

It is worth mentioning that antiserum to synthetic poly (I) : poly (C) double-stranded polynucleotide was used as an additional tool in attempts at detecting ds-RNA in nuclease treated nucleic acids from infected tissue. Initially, the preparations obtained from 100 gm tissue (see Methods, Chapter 4) and suspended in about 1 ml buffer failed to react with the antiserum in double diffusion tests. However, a reaction was discernible with nucleic acids from healthy and infected tissue, when concentrated preparations were used, after purification and precipitation with CTA (see Methods, Chapter 4). This reaction could be eliminated by dialysing the preparations against STMg buffer, but not by heating at 100° in the presence of RNase followed by rapid cooling, and therefore it was a non-specific reaction involving CTA-purification.

IX. Thermal Denaturation of TRSV-specific ds-RNA

A preparation of virus-specific ds-RNA, purified by nuclease

digestion and Sephadex gel-filtration suspended in 1 x SSC buffer had a sharp heat denaturation curve with a Tm of approximately 91.5° (Fig. 27A). On slow cooling of the denatured ds-RNA its absorbance at 260 nm decreased to about 10% above that of the original absorbance indicating a renaturation of about 90% (Fig. 27A). When the ds-RNA was suspended in 0.1 x SSC the Tm was 80° and in 1 x SSC containing 1.1M formaldehyde it was 77° (Fig. 27B). The maximum increase in absorbance at 260 nm on heating the ds-RNA in either 1 x SSC or 0.1 x SSC was 37.5%. In contrast to the ds-RNA, when a preparation of cucumber cotyledon t-RNA purified by Sephadex-gel filtration (Fig. 20, peak b) was suspended in 0.1 x SSC and heated, it showed a gradual increase in absorbance at 260 nm (Fig. 27B).

A preparation of TRSV-specific ds-RNA purified without the use of nucleases, by polyacrylamide-gel electrophoresis was subjected to heat denaturation in 0.1 x SSC. Both the shape of the melting curve and the Tm of this preparation (Fig. 28) were indistinguishable from those of the preparation purified by nuclease treatment and filtration through Sephadex (Fig. 27B). These data indicate that the thermal denaturation of TRSV-specific ds-RNA is not effected by treatment with nucleases and that the ds-RNA has thermal denaturation properties typical of double-stranded polyribonucleotides (Bocksthaler, 1967; Bishop and Koch, 1967).

Fig. 27. Heat denaturation profiles of TRSV-specific ds-RNA and cucumber cotyledon t-RNA. RNA preparations (0.5-1 O.D. unit) were dialysed for 6 hr at 4° against the resuspending solutions and then degassed in 1 ml cuvettes under vacuum. The cuvettes were stoppered and heated gradually in a spectrophotometer while recording the absorbance at 260 nm. Formaldehyde (when required) was added to 1.1M. The Tm's shown by arrows on the abscissa were determined graphically. No corrections were made for volume changes during the heating. (Spectrophotometry method is described in Chapter 2.)

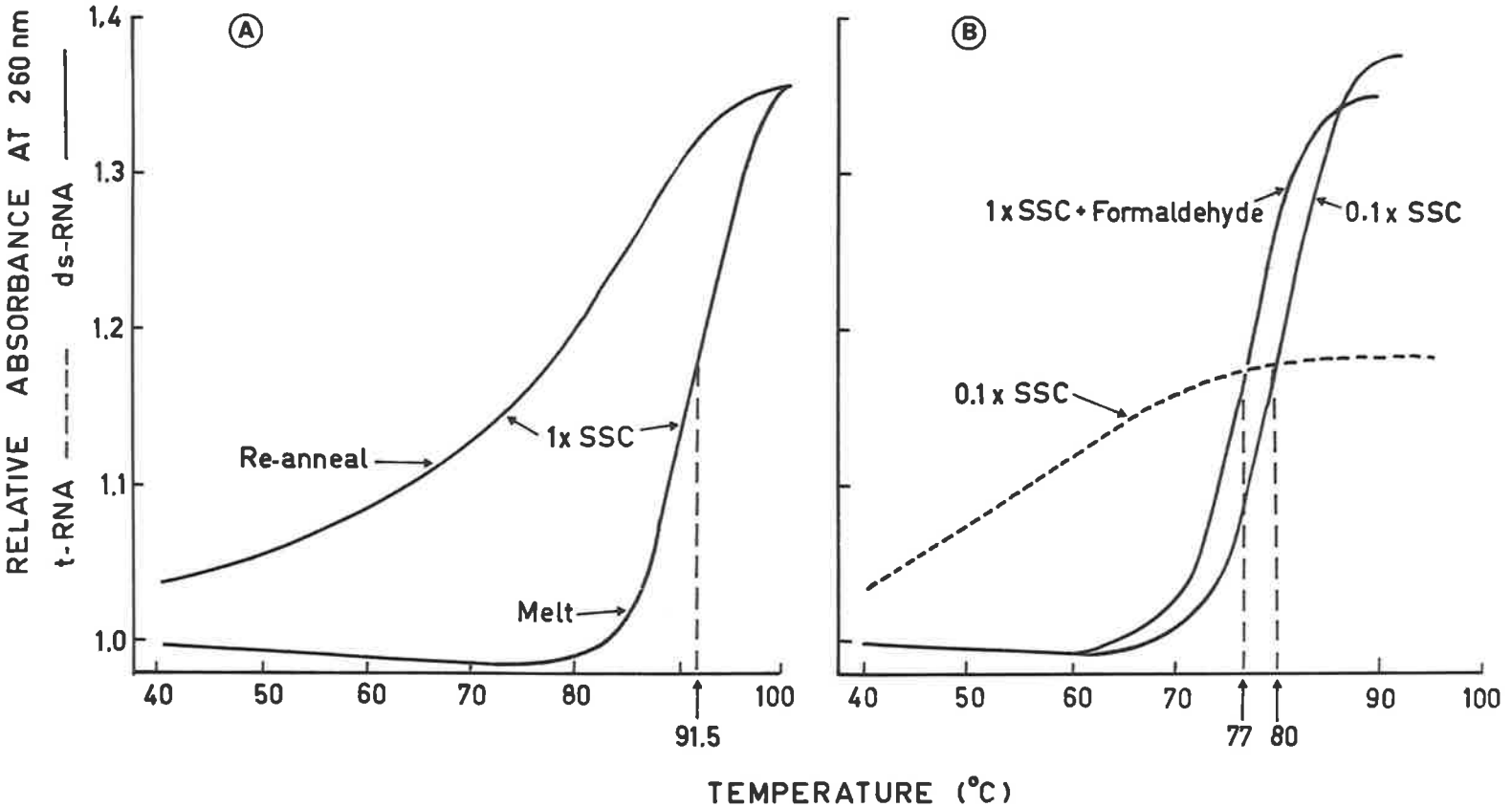
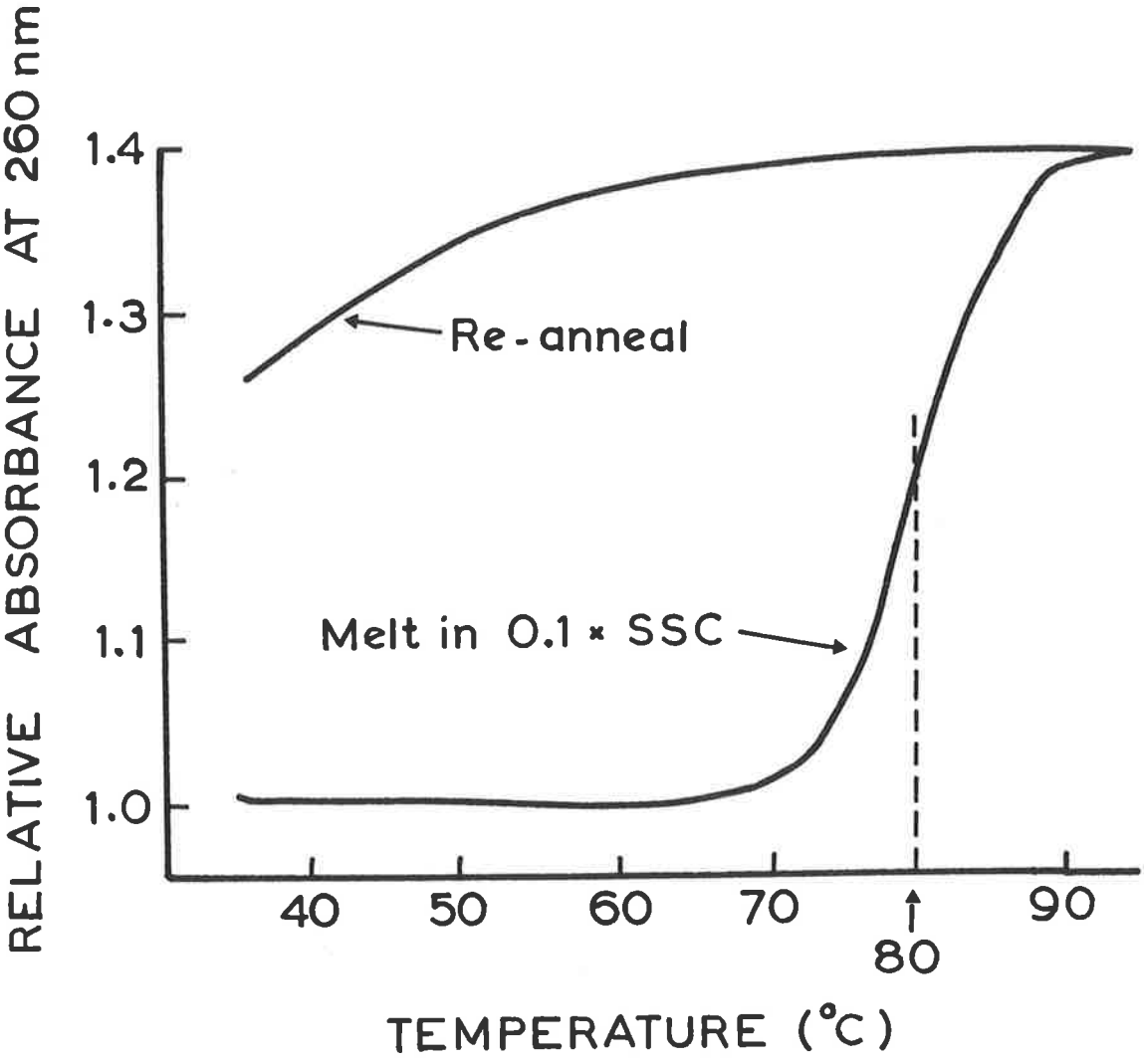

RELATIVE ABSORBANCE AT 260 nm
t-RNA ----- ds-RNA ——
1.4
1.3
1.2
1.1
1.0
A
Re-anneal
1x SSC
Melt
40
50
60
70
80
91.5
100
B
1x SSC + Formaldehyde
0.1 x SSC
0.1 x SSC
40
50
60
70
77
80
90
100
TEMPERATURE (°C)

Fig. 28. Heat denaturation profile of an electrophoretically purified preparation of ds-RNA, not subjected to nucleases at any stage, in 0.1 x SSC. Experimental details as described in Fig. 27.


RELATIVE ABSORBANCE AT 260 nm
1.4
1.3
1.2
1.1
1.0
Re-anneal
Melt in 0.1 × SSC
40
50
60
70
80
90
TEMPERATURE (°C)

X. Spectral Changes of TRSV-specific ds-RNA Treated with Formaldehyde

When TRSV-RNA, cucumber cotyledon t-RNA and TRSV-specific ds-RNA were treated with 1.1M formaldehyde at 37°, the maximum absorption of TRSV-RNA and t-RNA increased by 17 and 18% respectively, but there was no significant change in spectrum of the ds-RNA (Fig. 29). However, when the formaldehyde treatment was carried out at 75°, the maximum absorptions of TRSV-RNA, t-RNA and ds-RNA increased by 39, 18 and 38% respectively. The maximum absorptions also were shifted slightly towards a higher wavelength (Fig. 30). The maximum absorptions of the RNA samples heated at 75° for 15 min in the absence of formaldehyde followed by rapid cooling, did not change significantly. These data indicate that the reaction of TRSV-specific ds-RNA with formaldehyde is characteristic of a double-stranded polynucleotide (Miura, *et al.*, 1966; Prives and Silverman, 1972).

CONCLUSIONS

The TRSV-specific ds-RNA was purified from infected cucumber cotyledons by two methods: (a) by nuclease digestion followed by chromatography on G-200 Sephadex column, and (b) by polyacrylamide-gel electrophoresis. The virus specific ds-RNA migrates as a polydisperse

Fig. 29. Ultraviolet absorption spectra of native and formaldehyde treated RNA (indicated by F). Formaldehyde treatment of the RNAs was carried out at 37° as described in Methods (Chapter 5). Ultraviolet spectra were determined as outlined in Chapter 2, using a reference cuvette containing a solution with identical composition to that in the sample cuvette except that the RNA was omitted.

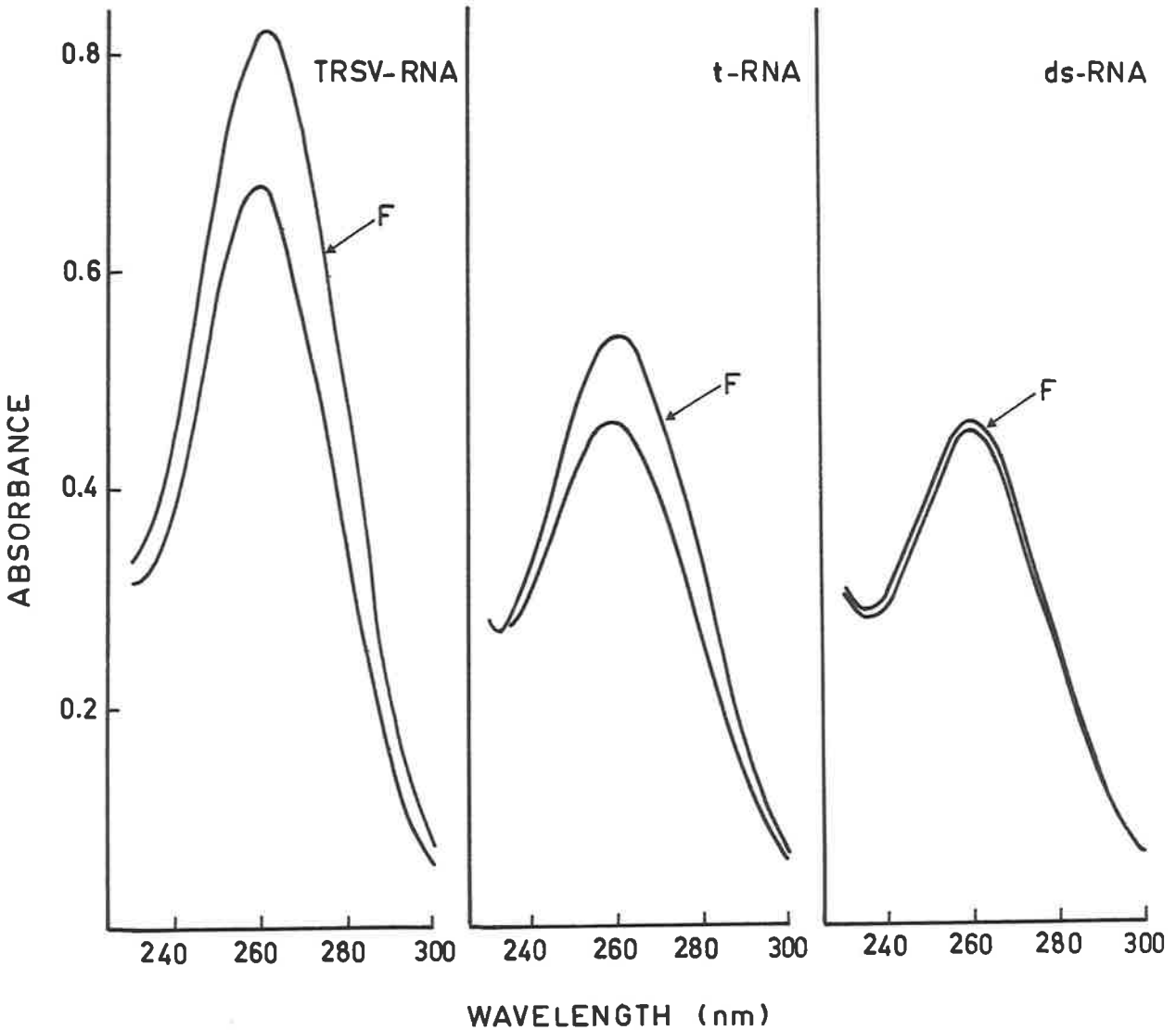
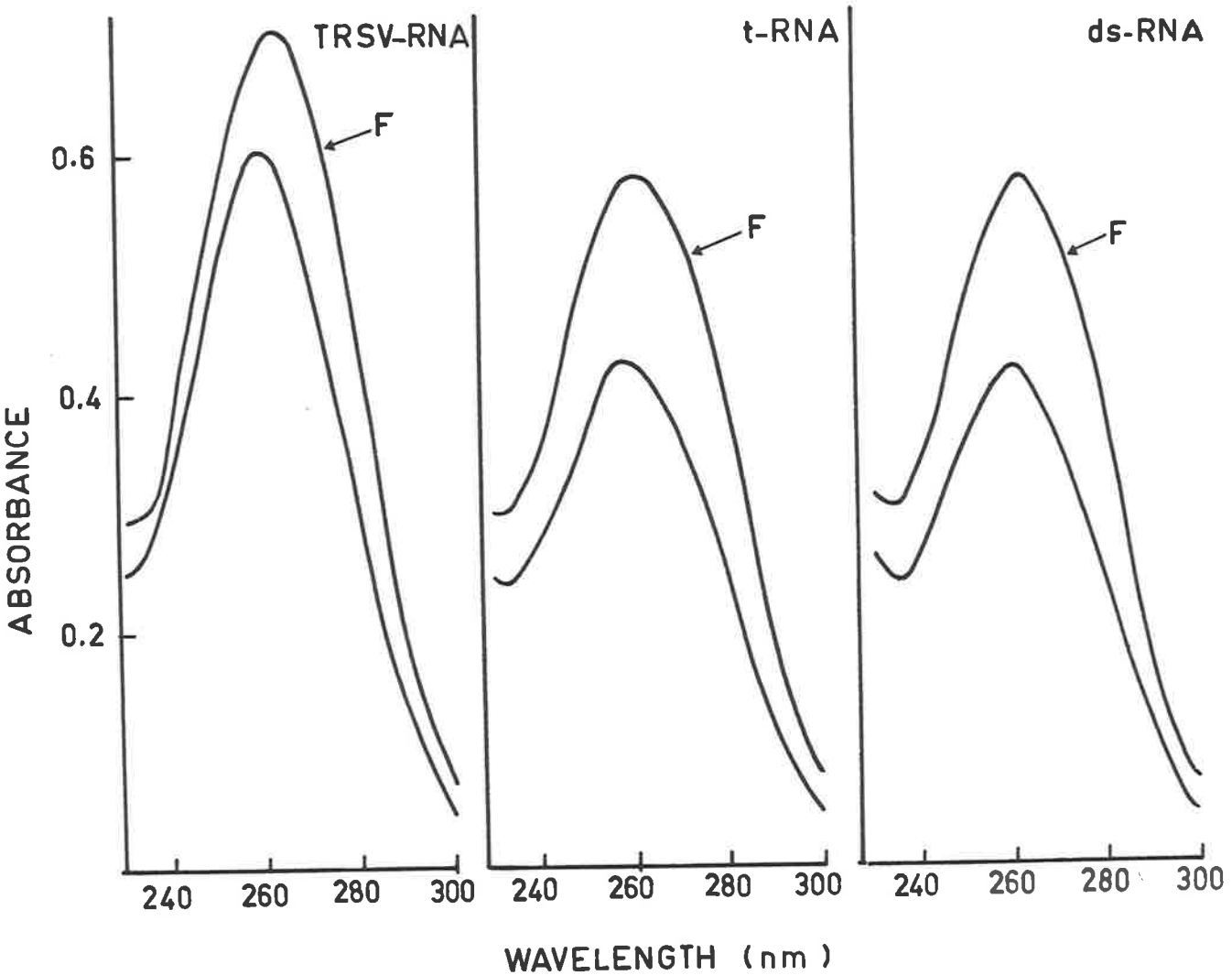

TRSV-RNA
t-RNA
ds-RNA
F
F
F
0.8
0.6
0.4
0.2
ABSORBANCE
240
260
280
300
240
260
280
300
240
260
280
300
WAVELENGTH (nm)

Fig. 30. Ultraviolet absorption spectra of native and formaldehyde treated (shown by arrows) RNA. Formaldehyde treatment was carried out for 75°. Other experimental details as in Fig. 29.


TRSV-RNA
t-RNA
ds-RNA
F
F
F
0.6
0.4
0.2
ABSORBANCE
240
260
280
300
240
260
280
300
240
260
280
300
WAVELENGTH (nm)

band when electrophoresed in polyacrylamide-gels and centrifuged in sucrose density gradient. It has a mean molecular weight of about 50,000 daltons and a sedimentation coefficient of approximately 6S. This RNA has properties typical of ds-RNA in that: (1) its electrophoretic mobility is unaffected by RNase in buffers containing 0.15M NaCl; (2) it has a density of 1.60 gm/cm^3 in caesium sulphate; (3) its ultraviolet absorption increases on treatment with formaldehyde at 75° but not at 37°; and (4) its thermal denaturation curve is typical of ds-RNA. However, it was shown that TRSV-specific ds-RNA behaved unlike a ds-RNA in that: (1) it eluted unlike ds-RNA from MAK, hydroxyapatite and cellulose columns; (2) it did not react with an antiserum specific to ds-RNA; and (3) its electrophoretic mobility did not increase significantly following denaturation. This latter characteristic indicates that the ds-RNA has a hair-pin structure.

CHAPTER 6

LOCALIZATION OF TRSV INDUCED ds-RNA AND RNA POLYMERASE IN SUBCELLULAR FRACTIONS OF CUCUMBER COTYLEDONS

INTRODUCTION

As a general phenomenon, membranes appear to be the sites of synthesis of animal viral RNA (Bishop and Levintow, 1971; Baltimore, 1969). Similar results have also been reported for bacteriophage MS2 RNA replication (Haywood *et al.*, 1969). However, there are conflicting opinions as to the site of synthesis of plant viral RNA (Matthews, 1970). While Reddi (1972) supports the view that the nucleus is the site of TMV-RNA synthesis, Ralph and Wojcik (1969) found that TMV specific ds-RNA and also synthesis of viral ds-RNA in cell free extracts were associated with cytoplasmic structures. Cytoplasmic membranes were later suggested as a possible site of TMV-RNA replication (Ralph *et al.*, 1971a). Some reports indicate that chloroplasts are involved in viral RNA synthesis. Turnip yellow mosaic virus (TYMV) ds-RNA was isolated from fractions rich in chlorophyll but low in DNA (Ralph *et al.*, 1971b; Ralph and Clark, 1966), and the peripheral vesicles of chloroplasts have been suggested

to be the sites of TYMV ds-RNA synthesis (Ushiyama and Matthews, 1970). Assink *et al.* (1973) detected cowpea mosaic virus ds-RNA mainly in the chloroplast fraction which also contained vesiculated membrane structures and amorphous material.

It seems that the site of plant viral RNA replication may vary from one virus infection system to another. In order to obtain some evidence on the site of synthesis of TRSV-RNA, two TRSV induced macromolecules associated with virus replication, i.e. TRSV-specific ds-RNA and RNA dependent RNA polymerase were located in subcellular fractions of cucumber cotyledons. A positive correlation between the concentration of TRSV ds-RNA and RNA polymerase activity during infection has already been established (Chapter 4).

METHODS

1. Preparation of Cell Fractions for Polymerase Assay

TRSV infected cucumber cotyledons, inoculated 3 days previously and corresponding healthy control tissues were washed with distilled water, blotted and placed in a small petri dish. All operations hereafter were performed at 2°. Tissue was minced by chopping with a razor blade, in 2 volumes (W/V) of an isolation medium containing 50 mM Tris-HCl, pH 8.5, 90 mM 2-mercaptoethanol, 0.1M NH_4Cl and 0.5M sucrose (May and Symons, 1971). The homogenate was strained through

two layers of 250 mesh nylon cloth and centrifuged at 500g for 5 min. The 500g pellet (which will be referred to as "chloroplast and nuclear fraction") was suspended in half the original volume of the same medium. The 500g supernatant was centrifuged at 17,000g for 10 min; the pellet was taken up in half the original volume of isolation medium. The 17,000g supernatant and pellet are classed as "cytoplasmic" and "mitochondrial" fractions respectively. 0.5 ml aliquots of each cellular fraction were mixed with 4 volumes of acetone and after slow speed centrifugation for 5 min, the concentration of chlorophyll was estimated by measuring the optical density of the supernatants at 652 nm (Bruinsma, 1961).

When it was required to remove RNase from the cellular preparations, a 95% saturated solution of ammonium sulphate containing all the ingredients of the isolation medium except sucrose, was added to each cellular fraction to bring the concentration of ammonium sulphate to 50% saturation (May and Symons, 1971). After centrifugation at 20,000g for 10 min, the pellets were resuspended in a small volume of isolation medium.

RNA polymerase activity was determined in the presence of about 800,000 cpm of ^{32}P-GTP, 0.5 mg of yeast RNA and other required materials, as outlined in Chapter 4, using 0.1 ml aliquots of each cellular fraction per assay tube. Healthy tissue homogenates were strained and used as controls. Assay was carried out at 37^{o} for 1 hr. TCA insoluble

radioactive counts were corrected for the original volumes of leaf extracts.

2. Preparation of TRSV-specific ds-RNA from Cell Fractions

Cell fractions in isolation media, prepared (from 10g tissue) as described for the polymerase assay, were mixed with an equal volume of TNE buffer (0.1M NaCl, 0.1M Tris-HCl and 0.01M EDTA, pH 7) (Jackson *et al.*, 1971) containing 2% SDS. RNA was extracted with $\frac{1}{3}$ volume phenol and precipitated with ethanol as outlined in Chapter 4. Nucleic acids were resuspended in 1 x SSC and incubated with RNase A, then with pronase, and extracted with phenol as described in Chapter 4. After ethanol precipitation, using 250 mg yeast RNA as carrier, the nucleic acids were washed with cold ethanol, then with ether and were finally taken up in 0.5 ml 1 x SSC buffer. TRSV-specific ds-RNA was assayed by the hybridization techniques described in Chapter 2. A healthy tissue homogenate was filtered through nylon cloth and used as a control.

RESULTS

I. Distribution of Polymerase Activity in Cellular Fractions

"Cytoplasmic" fractions of homogenates from cucumber cotyledons contained 62.6 - 80% of virus induced RNA polymerase activity but only 1.1 - 2.8% of the total chlorophyll (Table 5). On the other hand,

TABLE 5

DISTRIBUTION OF TRSV-INDUCED RNA-DEPENDENT RNA POLYMERASE IN FRACTIONS OF CUCUMBER COTYLEDON HOMOGENATES[a]

Fractions	Experiment 1			Experiment 2				
	Chlorophyll %	Polymerase activity		Chlorophyll %	Polymerase activity			
		Cpm	% of total		Cpm	% of total	After $(NH_4)_2SO_4$ precipitation	
							Cpm	%
Healthy tissue extract		41			108			
Infected tissue extract					1102			
500g supernatant	[b] 12.5	330	86	7.7	1120	91		
500g pellet (chloroplast and nuclear fraction)	87.5	54	14 8.7	92.3	108	9 9.3	100	12.4
500-17,000g pellet (mitochondrial fraction)	9.7	178	28.7	6.6	125	10.7	204	25.2
17,000g supernatant (cytoplasmic fraction)	2.8	387	62.6	1.1	933	80	505	62.4

[a] Cellular fractions were prepared and RNA polymerase was assayed as described in Methods.

[b] Fractions used in the calculation of the percentage.

the "chloroplast and nuclear fraction" contained 87.5 - 92.3% of the chlorophyll, but 8.7 - 9.3% of the polymerase activity (Table 5). However, a low but significant amount of RNA polymerase (10.7 - 28.7%) was found to sediment with "mitochondrial" fraction at 17,000g/10 min. Table 5 (experiment 2) shows that after $(NH_4)_2SO_4$ precipitation of polymerase from different fractions the distribution of polymerase activity did not change significantly. This was probably due to the presence of 0.5 mg yeast RNA in assay mixture which protected the polymerase activity before ammonium sulphate precipitation.

These data indicate that the virus induced RNA dependent polymerase is associated with the cytoplasmic fractions of infected cucumber cotyledons.

II. Detection of TRSV-specific ds-RNA in Cellular Fractions

Results of an experiment in which the concentration of TRSV ds-RNA was related to the chlorophyll content in fractions of cucumber cotyledon homogenates are summarized in Table 6. Annealing experiments revealed that 90% of the ds-RNA was associated with the "cytoplasmic" fraction of the cells which contained only 1.9% of the total chlorophyll. The "chloroplast and nuclear fraction" and "mitochondrial" fraction contained respectively 85.1% and 13% of the total chlorophyll but their respective ds-RNA content was estimated to be only 4.6% and 5.3%. Nucleic acids from healthy plants failed

TABLE 6

DETECTION OF TRSV-SPECIFIC ds-RNA IN SUBCELLULAR FRACTIONS OF CUCUMBER COTYLEDONS[a]

Fraction	cpm		TRSV ds-RNA		% chlorophyll
	annealed[b]	corrected[c]	µg/gm tissue[b]	%	
Healthy total extract	0	0	0	-	-
500 g pellet (chloroplast and nuclear fraction)	1024	184	1.0	4.6	85.1
500 - 17,000 g pellet (mitochondrial fraction)	1145	221	1.2	5.3	13.0
17,000 g supernatant (cytoplasmic fraction)	3669	3669	19.9	90.1	1.9

a Nucleic acids were prepared from cellular fractions as described in Methods and annealed with 3.2 µg (4260 cpm) of ^{14}C-labelled TRSV-RNA.

b Number of counts of blank tubes without added leaf RNA (419 cpm) was subtracted from all other counts.

c Corrections and estimations were made using the equation described in Chapter 4.

to anneal with ^{14}C-TRSV-RNA. Examination of the 'chloroplast and nuclear fraction" using a phase contrast microscope revealed that it contained few nuclei and many intact chloroplasts. The low content of nuclei in this fraction was probably due to the extraction procedure employed because solvent pretreatment which is apparently necessary for release of nuclei (Hamilton *et al.*, 1972) was not carried out.

CONCLUSIONS

The simple fractionation procedure employed, proved sufficient for the purpose of this study and no further purification of cell organelles was required. The results obtained demonstrate that the occurrence of both virus induced RNA-dependent RNA polymerase and TRSV-specific ds-RNA are in fractions devoid of chlorophyll (cf. Tables 5 and 6). It appears that chloroplasts and nuclei are unlikely to be the sites of TRSV-RNA replication. The data summarized in Tables 5 and 6 demonstrate that both RNA dependent RNA polymerase activity and TRSV-specific ds-RNA are associated with cytoplasmic fractions of tissue homogenates from infected cucumber cotyledons.

CHAPTER 7

NUCLEOTIDE SEQUENCE HOMOLOGY BETWEEN TRSV-RNA SPECIES

INTRODUCTION

As it was mentioned in Chapter 1 purified preparations of TRSV contain four types of particles; T component contains no RNA, each particle of M component contains one molecule of RNA_1 (RNA_{1M}), one type of B component particles each contain a single molecule of RNA_2, whereas another type of B component each contain two molecules of RNA_1 (RNA_{1B}) (Stace-Smith, 1965; Diener and Schneider, 1966; see Chapter 3). It was not known whether RNA_{1M} and RNA_{1B} have identical base sequences and if they have any homology with RNA_2.

This chapter describes how TRSV specific low molecular weight ds-RNA was used in competition hybridization experiments to determine the base sequence homology of the various TRSV-RNA species.

METHODS

1. Preparation of TRSV Components

Separated components of TRSV were prepared by sucrose density-

gradient centrifugation (see Chapter 2). Fractions from the gradients containing separated M and B components were pooled and the virus was concentrated by ultracentrifugation at 160,000g for 50 min. After another cycle of density-gradient centrifugation each virus component was recovered as above and resuspended in small volumes of 0.02M phosphate buffer.

2. Preparation of TRSV-specific ds-RNA

Total nucleic acid from infected cucumber cotyledons was fractionated by 1.5M NaCl precipitation (see Chapter 4). The salt soluble nucleic acids containing TRSV ds-RNA were recovered by ethanol precipitation and taken up in 1 x SSC buffer.

3. Preparation of Viral RNAs

RNA was isolated from M component and unfractionated TRSV preparations by phenol-SDS extraction procedure described in Chapter 2. M component yielded RNA which did not require further purification whereas RNA from unfractionated virus yielded a mixture of RNA_{1M}, RNA_{1B} and RNA_2. RNA_2 was separated from RNA_{1M} and RNA_{1B} by electrophoresis in 2.3% polyacrylamide-gels, 7.5 cm long and 1 cm in diameter. Samples containing 200 µg of RNA were applied onto each gel and electrophoresis was carried out as described in Chapter 2, for 5 hr. The bands of RNA located by staining for 30 min in

toluidine blue (see Chapter 2) were excised and washed with distilled water at 0° for 1 hr. RNA was recovered from the gel slices by homogenization in 1 x SSC buffer containing 1% SDS (0.5 ml per gel slice). Fine homogenization was achieved by forcing the slurry in turn through 18, 20, 22, 24 and finally 26 gauge hypodermic needles and the homogenates were shaken with water saturated phenol containing 0.1%, 8-hydroxyquinoline (0.25 ml per gel slice) for 1 hr. The mixture was centrifuged at 5,000g for 15 min and the aqueous phase was recovered. The phenol and gel fragments were re-extracted with 1 x SSC (0.25 ml per gel slice), centrifuged as before and the buffer layer recovered. RNA in the combined buffer phases was precipitated with 2 volumes of cold 95% ethanol and left at -15° for at least 3 hr. The precipitate was collected by centrifugation at 5,000g for 10 min, washed with cold ethanol and resuspended in 1 x SSC buffer. The RNA was washed three times with three volumes of ether and excess ether was evaporated under vacuum. The final RNA preparations were not free of traces of polyacrylamide but showed typical ultraviolet absorption spectra of RNA and were found satisfactory for hybridization studies.

RESULTS

I. Saturation Hybridization of TRSV-RNA with Virus-specific ds-RNA

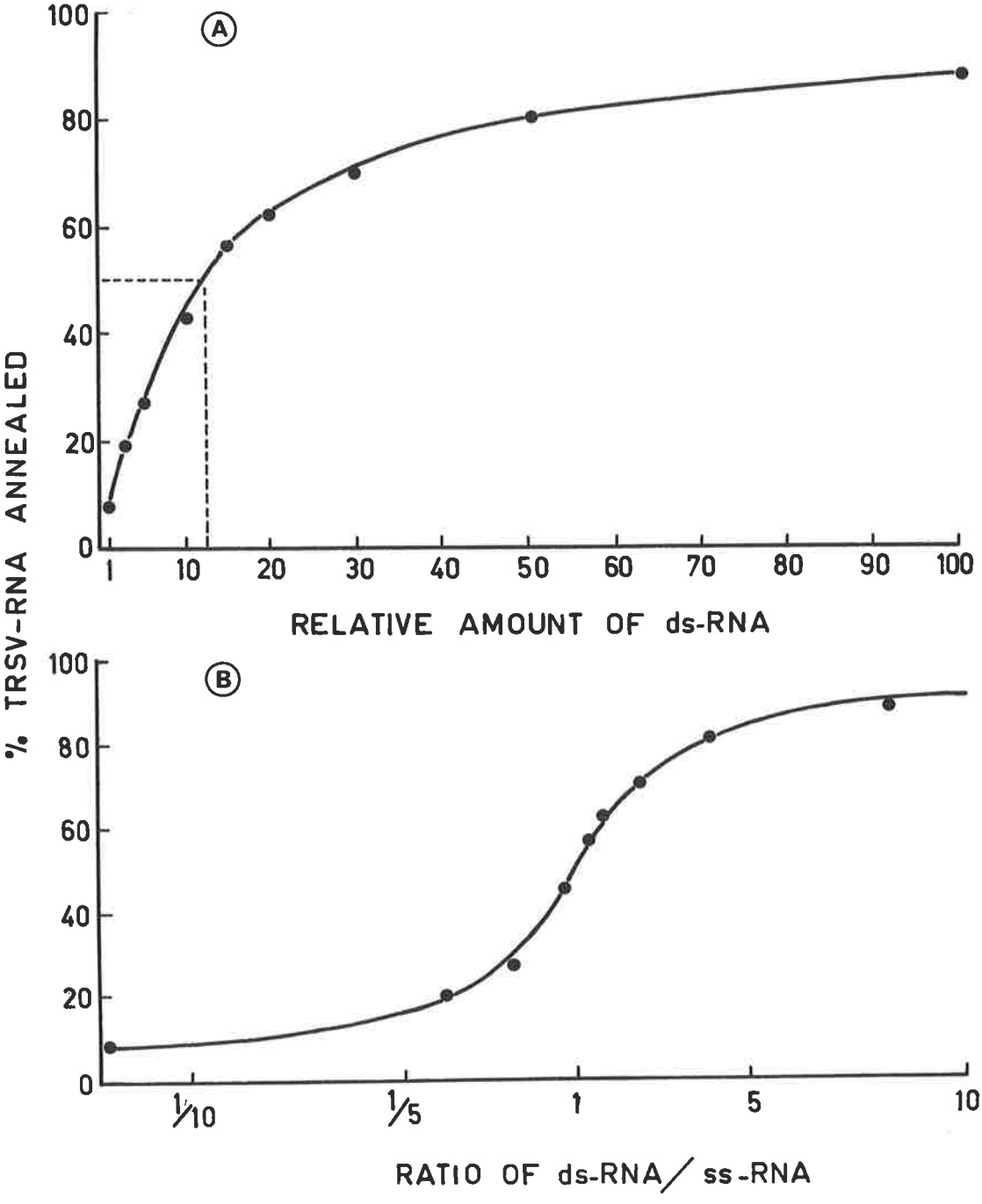
Although in Chapter 4 it was demonstrated that ^{14}C-labelled TRSV-RNA would anneal to virus-specific ds-RNA, it was not estimated

what proportion of the nucleotide sequences of the viral RNA could find complementary sequences on the ds-RNA. Before the ds-RNA could be used for comparing the sequences of the various RNAs from TRSV, it was essential to show what proportion of the TRSV-RNA nucleotides were represented in the ds-RNA.

Results of an experiment in which increasing amounts of a preparation of ds-RNA were annealed with standard samples of ^{14}C-labelled TRSV are summarised in Fig. 31A. The highest amount of ds-RNA used for annealing in this experiment rendered about 88% of the viral RNA resistant to RNase and the saturation curve (Fig. 31A) appears similar to a typical one where a ds-RNA is annealed to its homologous single-stranded RNA (ss-RNA). The data presented in Fig. 31A have also been replotted as the percentage of TRSV-RNA annealed against the molar ratio of ds-RNA/ss-RNA (Fig. 31B). The ratios were determined graphically from Fig. 31A by assuming that 50% annealing of TRSV-RNA corresponds to a ds-RNA/ss-RNA ratio of 1. The theoretical curve for 100% nucleotide sequence homology was derived from the equation $\frac{\text{ds-RNA}}{\text{ss-RNA}} = \frac{a}{100-a}$, where a is the percentage of the ss-RNA annealing (see Chapter 4). Data summarised in Fig. 31B show that the experimentally determined points fit the theoretical curve very well, indicating that the TRSV-specific ds-RNA preparations contain all the nucleotide sequences.

Fig. 31. (A) Saturation hybridization of TRSV-specific ds-RNA with viral RNA. Increasing amounts of ds-RNA were added to samples each containing 1,210 cpm of ^{14}C-labelled TRSV-RNA and subjected to annealing conditions as described in Chapter 2.

(B) Results of the same experiment (individual points) as compared with a theoretical saturation curve (solid line) (see text for explanation).


A
100
80
60
40
20
0
1
10
20
30
40
50
60
70
80
90
100
RELATIVE AMOUNT OF ds-RNA
% TRSV-RNA ANNEALED
B
100
80
60
40
20
0
1/10
1/5
1
5
10
RATIO OF ds-RNA / ss-RNA

II. Preparation of Viral RNA Species

RNA_{1M} was prepared from a purified preparation of viral M component (Fig. 32) but RNA_1 and RNA_2 were separated from each other by polyacrylamide-gel electrophoresis (Fig. 33A). From the ultraviolet traces of the polyacrylamide-gels (Fig. 33A) it was estimated that the maximum contamination of RNA_1 and RNA_2 by the other species would be less than 2%.

Mayo *et al.* (1973) have recently shown that ultraviolet irradiation of raspberry ringspot virus (RRV) preparations for a short period of time results in cross-linking of the two RNA_1 molecules in B particles. Since the level of ultraviolet irradiation entering the ISCO apparatus flow cell was well in the range used by May *et al.* (1973), it was considered that TRSV-RNA_{1B} may be affected in the same way as RRS, during fractionation by the ISCO apparatus. On one occasion RNA_2 of an isolated B component appeared as a double band in polyacrylamide gels (Fig. 33B). Therefore separated B component was not used in this work to avoid possible complications.

III. Competition Hybridization Experiments

In an initial experiment, increasing amounts of unlabelled viral RNAs were added to annealing mixtures of TRSV-specific ds-RNA and ^{14}C-labelled unfractionated TRSV-RNA. Results of the experiment summarised in Fig. 34 demonstrate that the maximum interference with the annealing of ^{14}C-labelled RNA was produced by unfractionated

Fig. 32. Sucrose density-gradient of isolated M component of TRSV. Three $O.D._{260}$ nm units of virus was layered on each gradient and centrifugation was carried out in SW25 tubes at 25,000 rpm for 3 hr, as described in Chapter 2. The indicated fraction was used for the isolation of RNA_{1M}.

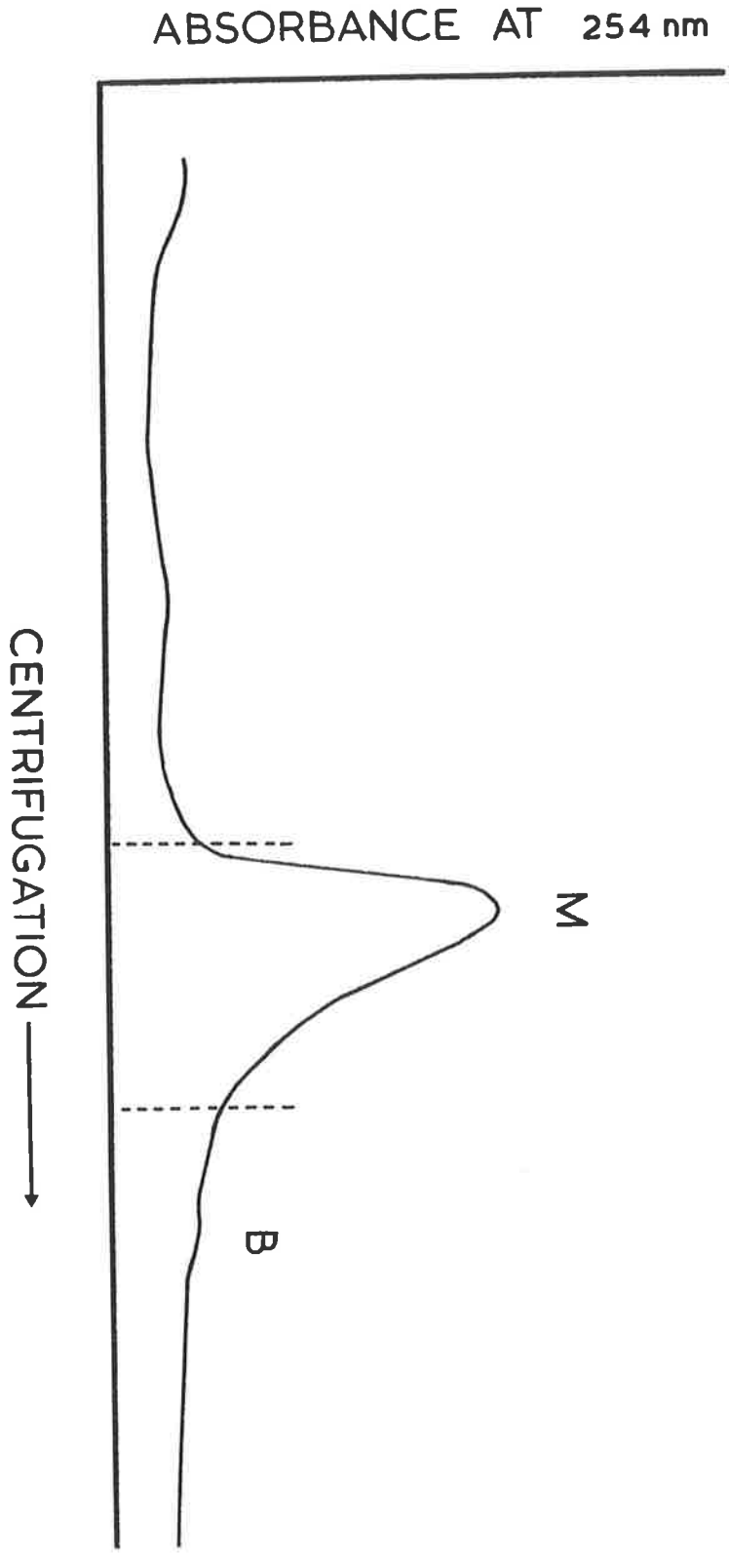
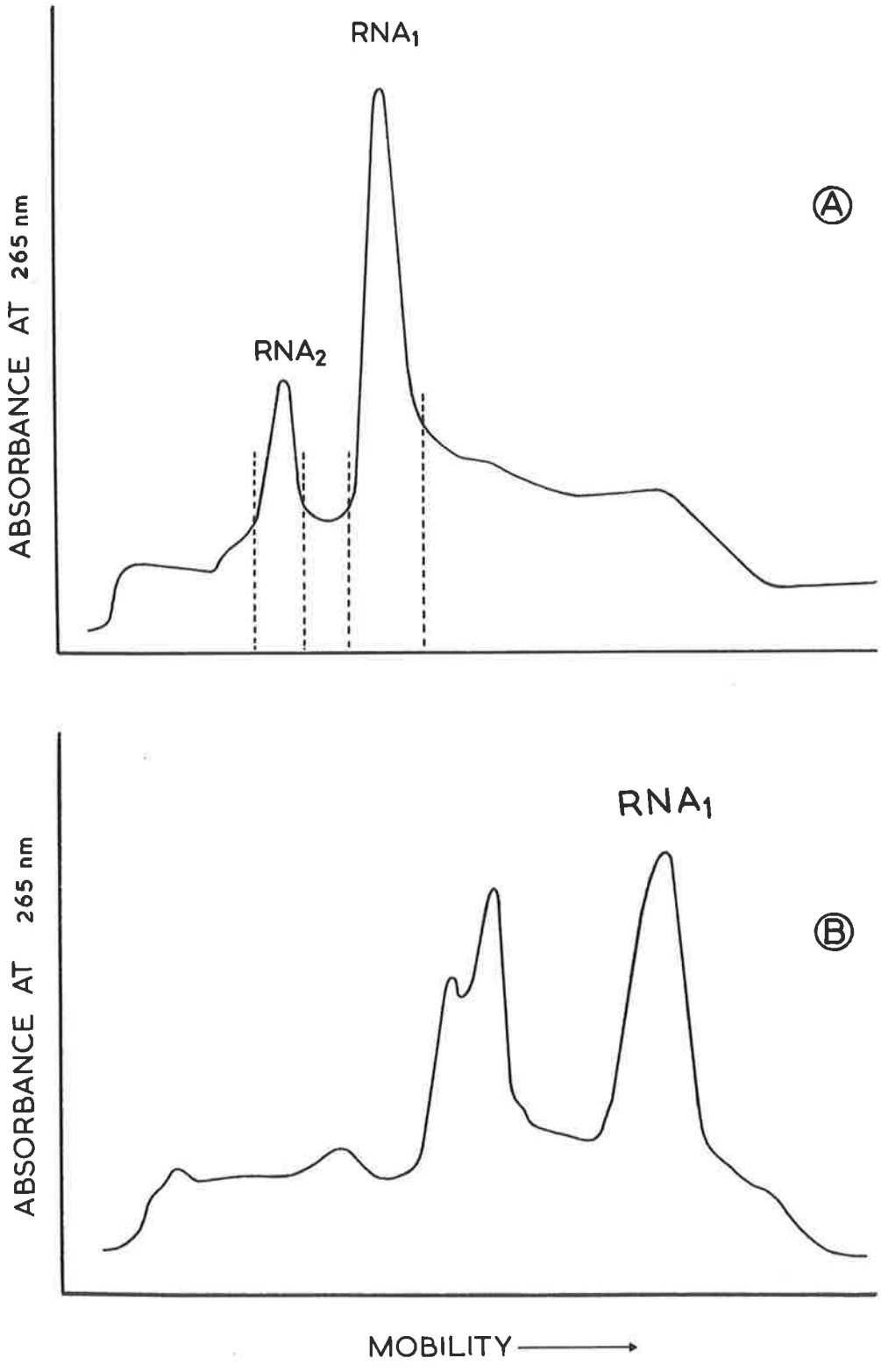

ABSORBANCE AT 254 nm
CENTRIFUGATION
M
B

Fig. 33. (A) Polyacrylamide-gel electrophoresis of TRSV-RNA. Electrophoresis was carried out in 2.3% polyacrylamide at 8 v/cm for 5 hr as described in Chapter 2. The indicated sections of the gel were used for extraction of TRSV-RNA_1 and RNA_2.

(B) Polyacrylamide-gel electrophoresis of RNA from an isolated B component preparation of TRSV. Electrophoresis was carried out for 7 hr as in A.


RNA1
RNA2
A
ABSORBANCE AT 265 nm
RNA1
B
ABSORBANCE AT 265 nm
MOBILITY

TRSV-RNA (homologous reaction). When purified RNA_2 was used as the competitor, interference was only slight. The level of interference by both RNA_1 (containing RNA_{1M} and RNA_{1B}) and RNA_{1M} was the same and was intermediate between that of RNA_2 and TRSV-RNA (Fig. 34). These results indicate that in TRSV-specific ds-RNA preparations there is a population of molecules possessing nucleotide sequences complementary to RNA_1 and a distinct population with sequences complementary to RNA_2; it also follows that sequences in RNA_{1M} are the same as those in RNA_{1B}. From the data presented in Fig. 34 it can be deduced that about one third of the ds-RNA molecules contain complementary sequences to RNA_2 and about two thirds to RNA_1. It can also be calculated from data previously reported (Table 1 and Fig. 4) that preparations of TRSV contain about 72% RNA_1 (including 12% RNA_{1M} and 60% RNA_{1B}) and about 28% RNA_2. Thus it would appear that the ds-RNA molecules with nucleotide sequences complementary to RNA_1 and those to RNA_2 in infected cells occur in roughly similar proportions to those of RNA_1 and RNA_2 in unfractionated TRSV preparations.

In another experiment, increasing amounts of unlabelled RNA_2, RNA_{1M} (isolated from purified M capsids) and RNA_1 (isolated from unfractionated TRSV and therefore containing both RNA_{1M} and RNA_{1B}) were added to annealing mixtures of ^{14}C-labelled RNA_1 and TRSV-specific ds-RNA. Results of the experiment summarised in Fig. 35A demonstrate

Fig. 34. Hybridization of TRSV-specific ds-RNA with ^{14}C-labelled TRSV-RNA (1,400 cpm per sample) in the presence of increasing amounts of individual viral RNA species.

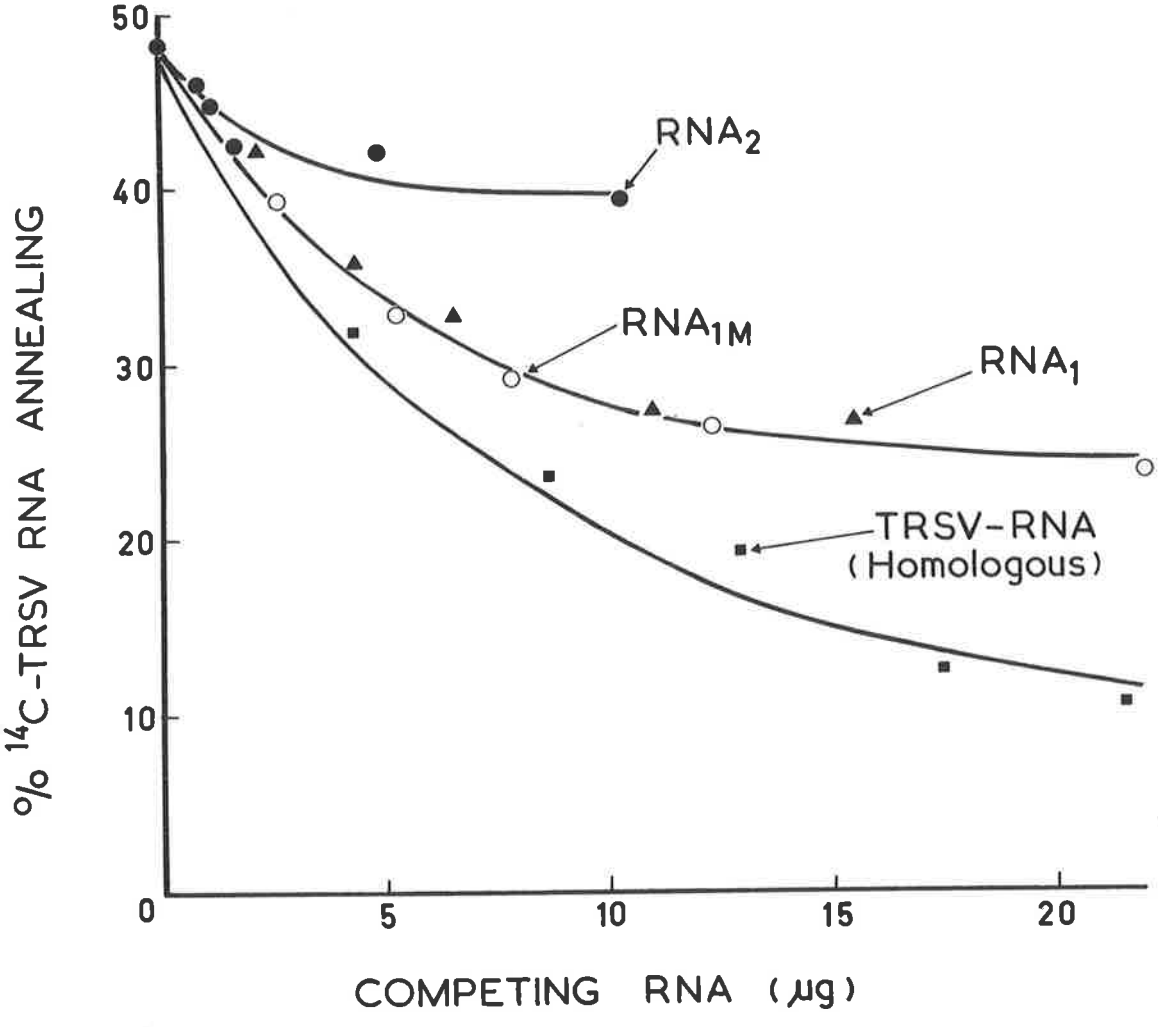
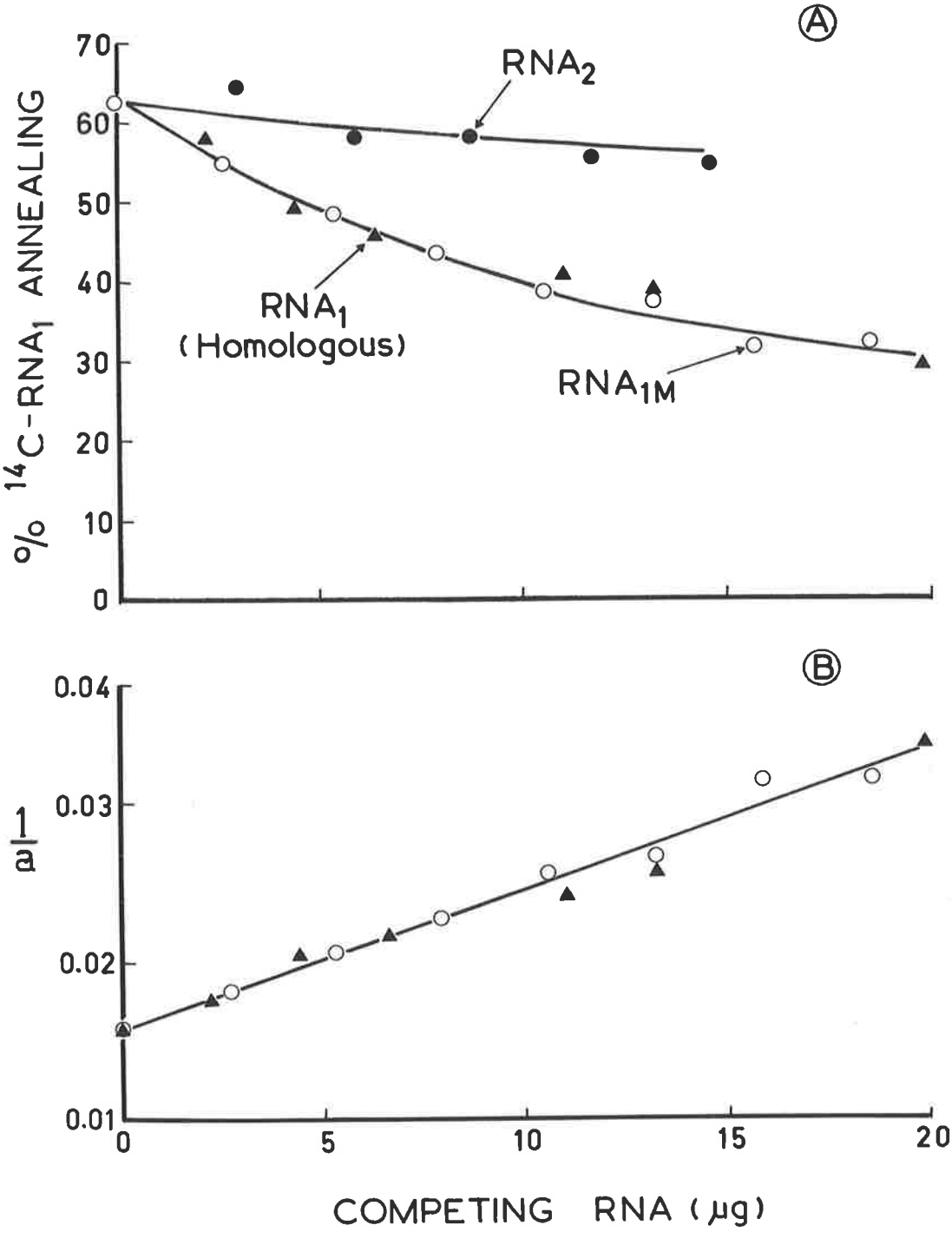

50
40
30
20
10
0
5
10
15
20
% ^{14}C-TRSV RNA ANNEALING
COMPETING RNA (µg)
RNA$_2$
RNA$_{1M}$
RNA$_1$
TRSV-RNA
(Homologous)

Fig. 35. (A) Competition hybridization of TRSV-specific ds-RNA with isolated ^{14}C-labelled TRSV-RNA_1 (1,440 cpm per sample) in the presence of either cold RNA_{1M}, RNA_2 or the homologous RNA.

(B) Linear relationship between the reciprocal of % annealing and the amount of competing RNA_1 and RNA_{1M} used in A.


A
70
60
50
40
30
20
10
0
% ^{14}C-RNA$_1$ ANNEALING
RNA$_2$
RNA$_1$
(Homologous)
RNA$_{1M}$
B
0.04
0.03
0.02
0.01
1/ω
0
5
10
15
20
COMPETING RNA (μg)

that both RNA_{1M} and RNA_1 interfered to the same degree with the annealing of ^{14}C-labelled RNA_1 to ds-RNA, confirming the results obtained in the previous experiment (Fig. 34).

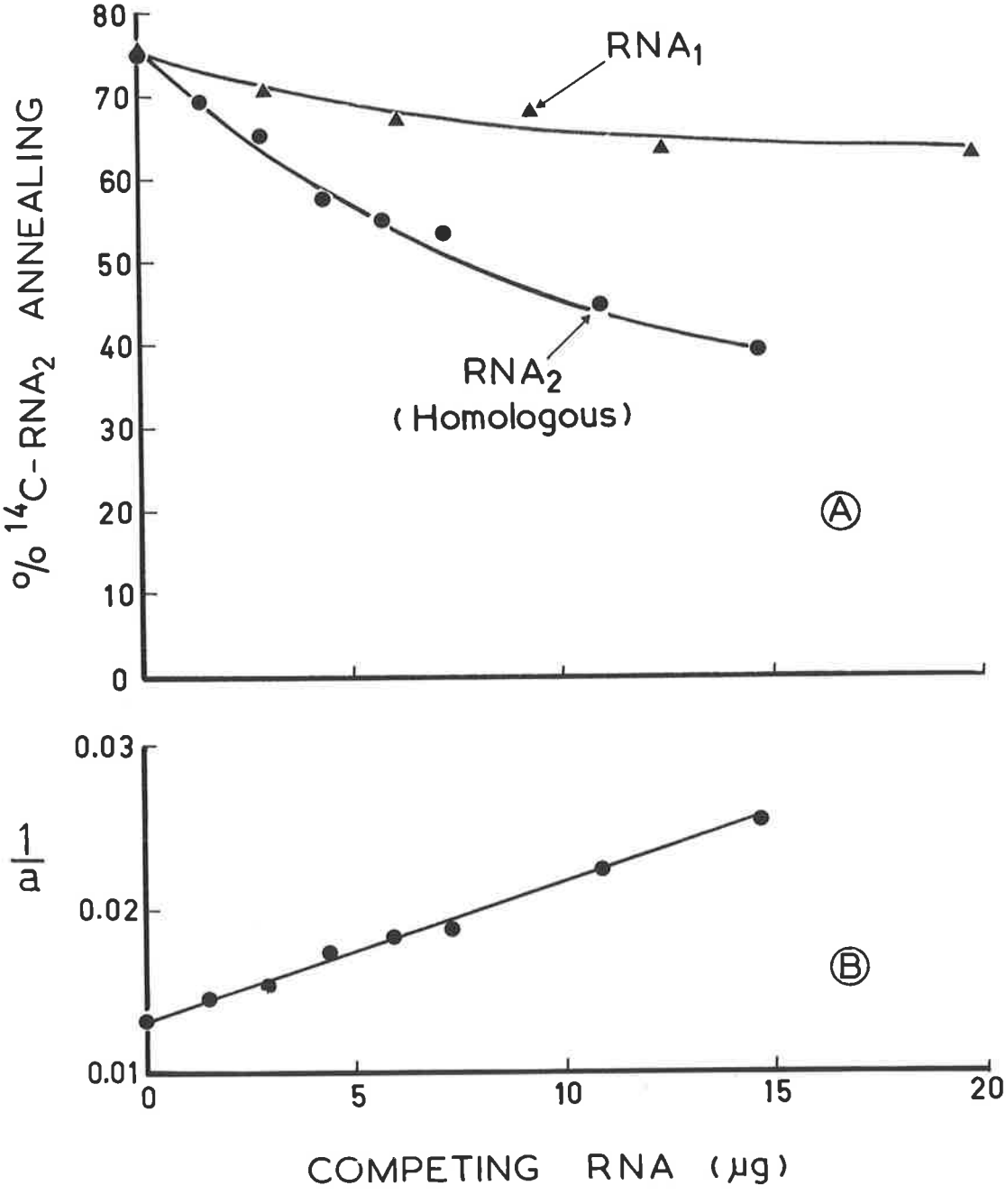
The theoretical relationship between the concentration of a competing ss-RNA and the proportion of the labelled ss-RNA annealed in homologous competition can be derived from the equation $\frac{\text{ds-RNA}}{\text{ss-RNA}} = \frac{a}{100-a}$. Since the concentration of ds-RNA is constant in a competition experiment, the relationship of $\frac{1}{a}$ $(= \frac{1}{100} + \frac{\text{ss-RNA}}{100 \times \text{ds-RNA}})$ against the amount of ss-RNA used as competitor must be linear. The results plotted in Fig. 35B show that the determined points from the experiment using RNA_1 (homologous) as competitor fitted a linear point very well. It can also be observed (Fig. 35B), that when RNA_{1M} was used as the competitor, its behaviour was not significantly different from that of RNA_1. This confirms that RNA_{1M} and RNA_1, and hence RNA_{1M} and RNA_{1B}, are indistinguishable.

When RNA_2 was used as a competitor in the same experiment (Fig. 35A), it was determined graphically that the RNA_2 was about 14% as efficient as RNA_1 in interfering with the annealing of ^{14}C-labelled RNA_1 with ds-RNA.

The last experiment was carried out to check the results already obtained,but by using RNA_1 and RNA_2 as competitors of annealing between ^{14}C-labelled RNA_2 and ds-RNA. The results (Fig. 36) confirm those deduced from Fig. 35. When RNA_1 was used as the competitor, it

Fig. 36. (A) Hybridization of TRSV-specific ds-RNA with isolated ^{14}C-labelled RNA_2 (1,300 cpm per sample) in the presence of competing RNA_1 and RNA_2.

(B) Linear relationship between the reciprocal of % annealing and the amount of competing RNA_2 used in A.


80
70
60
50
40
30
20
10
0
% ^{14}C-RNA$_2$ ANNEALING
RNA$_1$
RNA$_2$
(Homologous)
Ⓐ
0.03
0.02
0.01
a|-1
Ⓑ
0
5
10
15
20
COMPETING RNA (µg)

was again shown to be about 14% as efficient as the homologous competitor (Fig. 36A) and that a linear relationship exists between $\frac{1}{a}$ and the amount of ss-RNA competitor used (Fig. 36B).

CONCLUSIONS

The data presented in this chapter (Fig. 31) indicate that TRSV-specific ds-RNA contains all complementary nucleotide sequences to those of viral RNA species. Competition hybridization experiments revealed that (Figs. 34-36) the proportion of ds-RNA populations annealing to TRSV RNA_1 and RNA_2 resembles the ratio of these two RNA species in the viral RNA preparations. RNA_1 isolated from either middle or bottom component particles of TRSV have indistinguishable nucleotide sequences. However, the sequences of RNA_2 are distinct from those of RNA_1 although these RNAs may have sequences of about 900 nucleotides (14% of RNA_2) in common. This apparent homology between RNA_1 and RNA_2, on the other hand, is possible to be the reflection of RNA_2 contamination (maximum of 14%) by RNA_1 in the form of dimers.

CHAPTER 8

DISCUSSION

I. *In vivo* Synthesis of TRSV

Under the experimental conditions of continuous light at 25°, the multiplication of TRSV in both inoculated cucumber cotyledons (Fig. 1) and primary leaves of French bean (Fig. 11) is very rapid. Virus increase is confined to only two to three days, and five days after inoculation there is no further increase in the net virus concentration of inoculated leaves; thereafter TRSV concentration appears to decrease. Similar TRSV multiplication curves have been reported by Ladipo and de Zoeten (1972) when virus was assayed by infectivity. Reasons for the decrease of virus concentration in inoculated leaves remain unknown but one possibility is that some virus is translocated out of the leaves to other parts of the plant. Data suggesting that TRSV is translocated into bean root-tips from other parts of bean plants have recently been reported by Atchison and Francki (1972).

From their studies of two TRSV strains in French beans, Schneider and Diener (1966) concluded that although M and B components

were synthesized concurrently, M component accumulated more rapidly than B during early stages of infection and later this trend was reversed. In the present studies the ratio of M to B component remained constant both during the period of virus synthesis and after the net increase in virus concentration had ceased (Table 1). The reason why in some of the experiments dimers and trimers of virus particles were detected remains unknown (Fig. 3). Ladipo and de Zoeten (1972) concluded that the presence of B_2 component was seasonal but this can hardly explain the results of the present study as all experiments were carried out under the same controlled conditions.

Differences in properties of TRSV as shown in these experiments and those of others (Schneider and Diener, 1966; 1968) may well be a reflection of strain differences.

II. TRSV-specific Low Molecular Weight ds-RNA

Results of the experiments described in Chapters 4 and 5 demonstrated that a low molecular weight virus specific RNA was readily detected in nucleic acid preparations from TRSV-infected plants by means of isotopic labelling, RNA-RNA hybridization and ultraviolet absorption. However, these experiments failed to detect any high molecular weight RNA such as RF and RI, or to demonstrate the degradation of high molecular weight ds-RNA during the extraction procedure.

While the TRSV-specific low molecular weight RNA has several properties typical of ds-RNA it has some characteristics which are unlike other ds-RNAs which have been characterized (Ralph, 1969; Bishop and Levintow, 1971). Its resistance to RNase and DNase (Table 3 and Fig. 16), sensitivity to RNase in low salt concentration (Table 3), salt solubility, buoyant density in caesium sulphate (Fig. 19), heat denaturation profile (Fig. 27) and reaction with formaldehyde (Fig. 30) are all characteristics of ds-RNA. However, its behaviour on columns of MAK (Fig. 23), hydroxyapatite (Fig. 24) and cellulose are not typical of ds-RNA. Its failure to react with anti poly (I) : poly (C) serum is also atypical of ds-RNA as the same serum produced positive reactions with ds-RNAs from several sources (Francki and Jackson, 1972; Moffit and Lister, 1973; Monkton and Naora, 1974). It seems reasonably clear, from its properties when electrophoresed in polyacrylamide-gel after heat denaturation (Fig. 16), that TRSV-specific ds-RNA is not a double-stranded structure consisting of two regularly base paired single-stranded molecules. The electrophoretic mobility of a conventional ds-RNA would have increased very considerably on denaturation (see Pinck and Hirth, 1972; Prives and Silverman, 1972), whereas that of TRSV-specific ds-RNA changed only in that it became more polydisperse (Fig. 16). These data suggest that the ds-RNA molecules have a "hair-pin" structure. The heat denaturation kinetics of TRSV-specific ds-RNA (Fig. 27) and its resistance to RNase in high salt buffers (Fig. 16) lead to the conclusion that the "hair-pins"

must be regularly base paired polynucleotide chains devoid of any significant single-stranded regions.

Since the data presented in this work indicate that all TRSV-RNA nucleotide sequences anneal to the low molecular weight ds-RNA (Chapter 7), it is concluded that on annealing, each species of TRSV-RNA must accept complementary sequences from a number of molecules of ds-RNA. It therefore follows that preparations of ds-RNA contain a population of molecules with distinct nucleotide sequences. The observation that after TRSV-RNA is subjected to annealing condition only about 8% of the viral RNA resists RNase A digestion precludes the possibility that TRSV-specific ds-RNA is a product of self-annealing of viral RNA fragments.

An interesting characteristic of TRSV-specific ds-RNA is its heterogeneity and polydispersity which makes it distinct from other low molecular weight RNAs found in association with viral infections (Banergee *et al.*, 1969; Prives and Silverman, 1972; Marcaud *et al.*, 1971; Ohe, 1972) which are discrete RNA species. The ds-RNA molecules appear to range in size between 35,000 and 70,000 daltons whereas the viral genome consists of two single-stranded molecules of about 1.4×10^6 and 2.3×10^6 daltons (Fig. 5, Murant *et al.*, 1972). Since the mean molecular weight of the ds-RNA is approximately 50,000 daltons, therefore there must exist about 150 distinct species of ds-RNA which together, contain the nucleotide sequences of the entire

TRSV genome. The apparent existence of such a large number of distinct ds-RNA species would explain why it was not possible to resolve distinct species by electrophoresis even in 15% polyacrylamide gels and why only a broad band of ds-RNA was observed.

Purification of TRSV-specific ds-RNA by nuclease treatment followed by Sephadex gel filtration exposes the ds-RNA to RNase which could modify it by digestion of any single-stranded regions that may be present. The existence of single-stranded regions seems unlikely since the electrophoretic mobility of ds-RNA appears not to be affected by RNase. The second method of purification involves the recovery of ds-RNA from polyacrylamide-gels migrating behind 5S r-RNA. As some of the ds-RNA migrates ahead of the 5S r-RNA, it means that a small fraction of the ds-RNA will not be recovered in preparations recovered by this method. However, in all the experiments in which the properties of ds-RNA purified by both methods were examined, the results have been similar. Thus it is concluded that the ds-RNA molecules of relatively low molecular weight, which are eliminated during purification on polyacrylamide-gels, have similar properties to those of higher molecular weight which are recovered.

At present it is not possible to make any concrete suggestions as to the function of TRSV-specific ds-RNA in the infection process, although it appears to be involved in virus synthesis. The concentration of the ds-RNA in TRSV-infected cucumber cotyledons was shown to be as

high as 54 µg/gm fresh weight of tissue and high concentrations were only detected in tissue supporting rapid virus synthesis (Chapter 4). The concentration appears to be positively correlated with the activity of virus induced RNA-dependent RNA polymerase and both TRSV-specific ds-RNA and the RNA polymerase were detected in the same subcellular fraction. The almost complete disappearance of ds-RNA from infected tissue soon after virus accumulation ceases suggests that the ds-RNA is unlikely to be a "dead-end" product. Despite these indications, it is not easy to envisage the TRSV-specific ds-RNA as an intermediate in the synthesis of viral RNA because of its low molecular weight in comparison to that of TRSV-RNA.

Several double-stranded RNA species have been shown to be involved in some biological processes of the cell. Apart from interferon induction (Kleinschmidt, 1972), ds-RNA has been recently reported to inhibit protein synthesis in cell-free protein synthesizing systems (Robertson and Mathews, 1973; Kaempfer and Kaufman, 1973). In Ehrlich ascites tumour cells infected with a picora virus, addition of non-infectious viral ds-RNA inhibited the synthesis of host protein without affecting the viral protein synthesis (Cordel-Stewart and Taylor, 1973). It is conceivable that TRSV-specific ds-RNA may have a cell regulatory function.

The detection of both TRSV-specific ds-RNA and virus induced RNA-dependent RNA polymerase activity of the extracts from infected

cucumber cotyledons in the "cytoplasmic" fraction points to the possibility that the replication of TRSV-RNA may occur in the cytoplasm of infected cells. However, a considerable amount of polymerase activity could be sedimented at 17,000g for 10 min, suggesting its attachment to some cytoplasmic structure(s). This is probably the particulate RNA polymerase activity reported previously (Peden *et al.*, 1972).

III. The Genome of TRSV

Diener and Schneider (1966) were the first to recognise the existence of RNA_1 and RNA_2 in TRSV and correlated infectivity with RNA_2. From their experiments they found support for the hypothesis that TRSV-RNA was synthesized in the form of two non-infectious pieces (RNA_1) which were later joined to form infectious RNA_2. Data presented in Chapter 7 demonstrate that the majority of the nucleotide sequences of RNA_1 and RNA_2 differ, and this precludes the possibility that RNA_1 is a precursor of RNA_2.

The competition hybridization experiments indicate that a maximum of about 14% of the nucleotide sequences of RNA_2 are similar to those of RNA_1. This would represent some 900 nucleotides and a coding capacity of about 300 amino acids. If this is correct then we could expect RNA_1 and RNA_2 each to code for one common protein. Some common sequences required as recognition sites for replication and

translation may also be present. It must also be considered that the partial homology between RNA_1 and RNA_2 may be due to contamination of RNA_1 and RNA_2 preparations used in the annealing experiments. Although cross-contamination of RNA_1 and RNA_2 used in the experiments were shown to be less than 2% which would produce up to 4% apparent homology, it is also possible that RNA_2 may have been contaminated by dimers of RNA_1. If such dimers existed, then they would migrate together with RNA_2 during electrophoresis in polyacrylamide-gels. Recently, Mayo *et al.* (1973) have demonstrated that ultraviolet irradiation of B component particles of raspberry ringspot virus (RRV) containing two RNA_1 molecules can cross-link them to produce an RNA component with electrophoretic mobility similar to that of RNA_2. RRV is similar to TRSV in many respects (Harrison *et al.*, 1972a) and both viruses are included in the Nepovirus group (Harrison *et al.*, 1971). It may be that cross-linking of RNA inside B particles is not confined to RRV and that it may occur under some conditions in the absence of ultraviolet irradiation. The finding that after sucrose density gradient centrifugation of TRSV-RNA, infectivity of the fractions coincides with RNA_2 only (Diener and Schneider, 1966; Francki, private communication) is compatible with the view that his fraction may contain RNA_1 molecules in the form of dimers (Harrison *et al.*, 1972a).

Although the nucleotide sequences of RNA_2 are distinct from

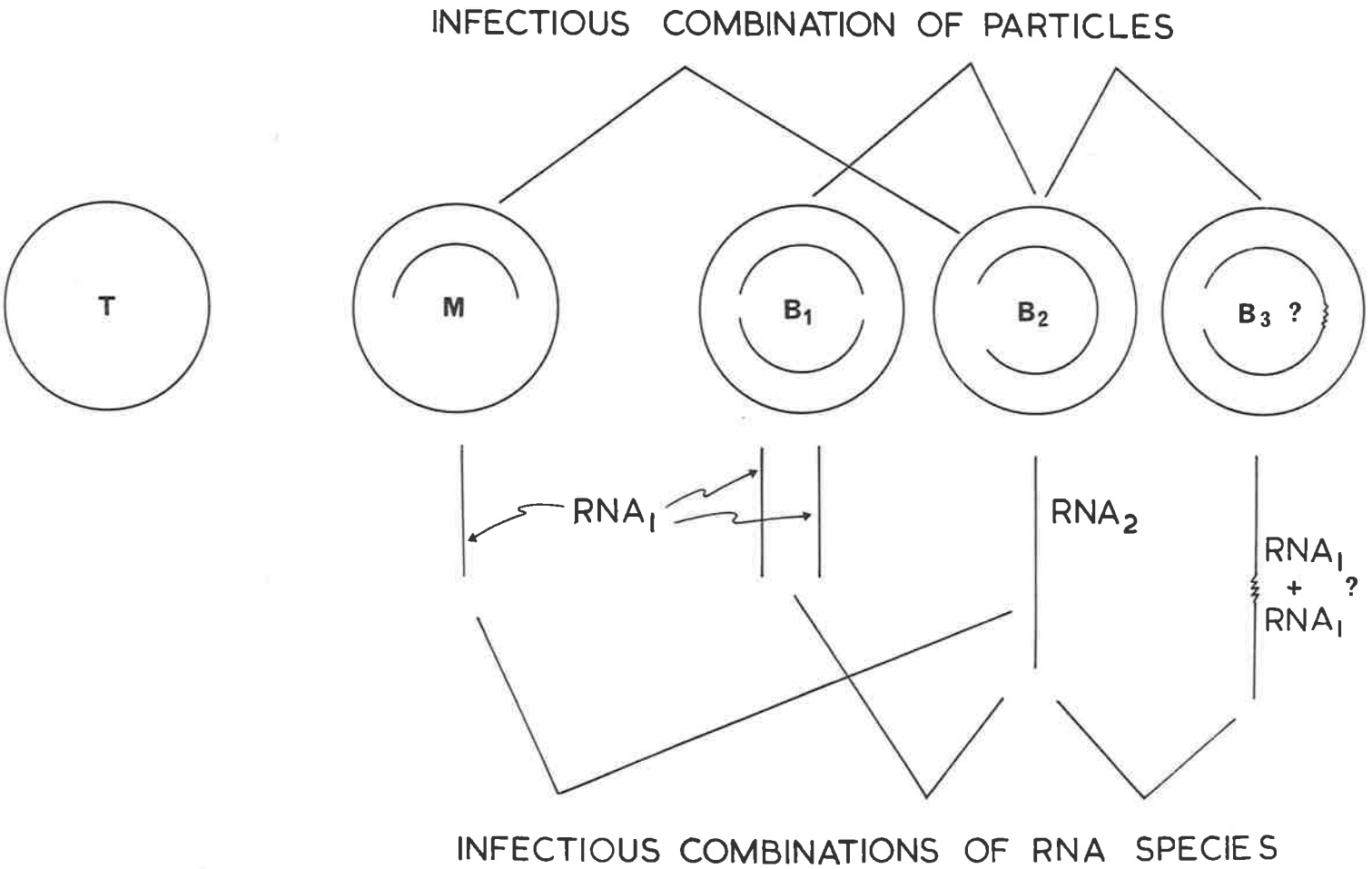
those of RNA_1, those of RNA_{1M} and RNA_{1B} appear to be identical. From the results of experiments in which preparations of TRSV and TRSV-RNA were subjected to analysis by density-gradient centrifugation (Table 1 and Fig. 4), it would appear that during virus replication and assembly about 85-90% of the encapsidated RNA_1 finds its way into B particles whereas only 10-15% becomes enclosed in M particles. This apparent preference of RNA_1 molecules for being encapsidated in pairs is at present obscure.

The fact that RNA_1 and RNA_2 of TRSV have largely distinct nucleotide sequences suggests that the virus has a divided genome although, at present, there is no conclusive evidence that both RNAs are functional. However, Harrison *et al.* (1972a) working with both RRV and TRSV, and Francki (private communication) have shown that the infectivity of RNA_2 can be increased several-fold by the addition of RNA_1, suggesting that both RNAs are required for infectivity. In the case of RRV, it has been shown that some of the properties of the virus are determined by RNA_1 (Harrison *et al.*, 1972b).

Taking into account the possibility that the dimers of RNA_1 may occur in some TRSV B-particles together with the findings that the addition of RNA_1 to RNA_2 increases the infectivity of the latter (Harrison *et al.*, 1972a) and that these RNAs are distinct species (Chapter 7), a possible model for TRSV component particles and their infectious combinations is illustrated in Fig. 37. Although evidence

Fig. 37. Possible infection combinations of TRSV components. T (top), M (middle), B_1, B_2 and B_3 (bottom) are the component particles of TRSV. The presence of B_3 and dimers of RNA_1 in TRSV preparations have not been demonstrated.

A POSSIBLE MODEL FOR TRSV COMPONENT PARTICLES



for the presence of empty capsid component (T), middle component (M) and two types of bottom component (B_1 and B_2) is convincing (Stace-Smith *et al.*, 1965; Diener and Schneider, 1966; also see Chapter 3), the existence of B_3 particles containing dimers of RNA_1 has yet to be proven experimentally. Considering estimates of 1.4×10^6 and 2.3×10^6 daltons for RNA_1 and RNA_2, respectively (Murant *et al.*, 1972; Chapter 3), the RNA content of B_2 particles is 0.5×10^6 daltons less than those of each B_1 and B_3 particle. This difference appears to be sufficient to isolate B_2 from B_1 and B_3 particles (Fig. 37) by isopycnic centrifugation and to test for the dependence of infectivity of B_2 on RNA_1-containing particles. However, attempts at separation of the B component into two bands in both caesium chloride and sulphate isopycnic gradients have been unsuccessful (Francki, private communication), which may indicate that the molecular weight estimates of RNA_1 and RNA_2 may be erroneous. These estimates are similar to those made by sedimentation techniques (Diener and Schneider, 1966; Kaper and Waterworth, 1973), by polyacrylamide-gel electrophoresis (Murant *et al.*, 1972) and by polyacrylamide-gel electrophoresis in formamide system (Habili, private communication). However, using polyacrylamide-gel electrophoresis, Kaper and Waterworth (1973) have estimated the molecular weights of TRSV-RNA_1 and RNA_2 as 1.24 and 1.83, respectively. The molecular weight of RNA_2 obtained in this study appears considerably less than those in other reports.

APPENDIX

PAPERS PUBLISHED OR SUBMITTED

Rezaian, M.A., and Francki, R.I.B. (1973).
Replication of Tobacco Ringspot Virus. I. Detection of a low molecular weight double-stranded RNA from infected plants.
Virology 56, 238-249.

Rezaian, M.A., and Francki, R.I.B. (1974).
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