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MOLECULAR
BIOLOGY
OF VIROIDS

by

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Thesis submitted to the University of Adelaide in fulfillment of the requirements for the degree of Doctor of Philosophy.

February, 1980.

Awarded 12th Sept. 1980

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SUMMARY

This thesis is concerned with various aspects of the molecular biology and/or pathology of chrysanthemum stunt viroid (CSV), the two RNAs associated with the cadang-cadang disease of coconuts: cadang-cadang RNA-1 (ccRNA-1) and cadang-cadang RNA-2 (ccRNA-2), and the avocado sun-blotch viroid (ASBV).

1. A procedure was developed for the rapid extraction of low molecular weight nucleic acids from plants, as well as a protocol for the purification of CSV. A bio-assay system for CSV was developed using *Gynura aurantiaca* as a host.

2. Two forms of CSV, a linear and a circular form, were characterized by electron microscopy, thermal denaturation, nucleic acid hybridization and infectivity; both forms of CSV were shown to be equally infectious in *G. aurantiaca*. Linear CSV formed *in vitro* by the magnesium-ion catalyzed cleavage of circular CSV was found to be as infectious as circular CSV or "naturally occurring" linear CSV; 63% of the latter had a 5'-phosphate and could not have arisen as the result of metal-ion catalyzed cleavage of circular CSV *in vivo* or during the extraction process.

3. A method for the synthesis of complementary DNA (cDNA) to viroids was developed using a viroid associated with the cadang-cadang disease of coconuts, ccRNA-1. The method involves the partial cleavage of circular ccRNA-1 with nuclease S_1, the enzymatic addition of a poly(A) tract to 3'-ends so produced, and the synthesis of cDNA with

reverse transcriptase using polyadenylated ccRNA-1 as a template and oligo$(dT)_{10}$ as a primer. The cDNA probe to ccRNA-1 was characterized and shown to be specific for sequences present in both ccRNA-1 and ccRNA-2.

4. The above method was slightly modified to synthesize cDNA to CSV. The CSV cDNA was shown to be specific for CSV RNA and sequences present in nucleic acid extracts from CSV-infected but not healthy plants. The CSV cDNA did not hybridize to citrus exocortis viroid (CEV) suggesting little-if-any sequence homology between these two viroids.

5. The high degree of secondary structure characteristic of viroids was shown to interfere with the hybridization reaction between CSV cDNA and CSV RNA, under conditions used for hybridization of normal single-stranded RNA molecules. This resulted in the observation of different rates of hybridization for circular CSV, linear CSV and fragmented CSV molecules. Under hybridization conditions that ensured the denaturation of all forms of CSV, circular, linear and fragmented CSV molecules all hybridized at identical rates. These conditions were used to determine the level of CSV in partially purified nucleic acid extracts from CSV-infected chrysanthemums and *G. aurantiaca*; the level of CSV in chrysanthemums was approximately 17 times higher than in *G. aurantiaca*.

6. Analysis of chrysanthemum and *G. aurantiaca* genomic DNA failed to reveal the presence of any sequences complementary to CSV; however, the large genome size of the chrysanthemum plants may have precluded the detection of CSV sequences

at the single-copy-or-less/genome level.

7. A low molecular weight RNA was found in avocado leaves infected by sunblotch disease, but not in healthy avocado leaves. The RNA was shown to be a viroid; i.e., an infectious, single-stranded, covalently-closed, circular RNA with a high degree of secondary structure.

8. cDNA was prepared against ASBV using the method developed with ccRNA-1 and CSV. The cDNA to ASBV was shown to be specific for ASBV sequences and did not hybridize to either CSV, CEV or ccRNA-1 and ccRNA-2. Hybridization of ASBV cDNA to nucleic acid extracts from biologically indexed healthy and sunblotch infected (symptomed or symptomless carrier) avocado trees showed (1) the viroid was always present in infected trees; (2) the viroid was absent from almost all healthy trees; two biologically-indexed healthy trees had levels of ASBV at approximately 10^{-5}% of the total nucleic acid extract; (3) the level of ASBV fluctuated widely between different trees and during different seasons of the year; and (4) there was no correlation between high ASBV levels and the symptomless carrier state vs. the symptomed state; all four possible combinations were observed.

9. ASBV was inoculated onto chrysanthemum, tomato, avocado, *G. aurantiaca* and *G. sarmentosa*; no symptoms of a disease were observed on any of the above plants; however, replication of ASBV was observed in avocado and to a very slight extent, in *G. aurantiaca*. It has yet to be shown that the viroid is the causative agent of sunblotch disease.

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.

PETER PALUKAITIS.

ACKNOWLEDGEMENTS

I wish to thank Prof. W.H. Elliott for permission to undertake these studies in the Department.

I also wish to thank my supervisor, Dr. R.H. Symons, for advice, discussions, experimental assistance and for the preparation of some of the materials used during the couse of this work.

In addition, I wish to express my appreciation to the following people:

Dr. R.N. Allen, for sending avocado leaf samples, for the extraction of some of these samples and for helpful discussions;

Mr. D.McE. Alexander, for sending avocados, avocado leaf samples, and for providing us with his unpublished biological data;

Dr. R.I.B. Francki, for the use of glasshouse facilities and for providing us with our isolate of CSV (originally obtained from T.C. Lee, Adelaide Botanic Gardens);

Dr. T. Hatta for help with the electron microscopy analysis of viroids and for providing electron micrographs of RNAs for measuring purposes;

Mr. A.G. Rakowski, for his assistance with the extraction of avocado samples and hybridization analysis of these samples;

Dr. J.W. Randles for his assistance and for providing materials relevant to the preparation of a cDNA probe to coconut cadang-cadang viroid;

Miss J.L. Rosey, for technical assistance, the preparation of diagrams and the preparation of photographs for this thesis;

Drs. A. Branch, E. Dickson, C. Niblett and M. Zaitlin for their correspondence and for providing me with their unpublished data;

the other numerous "Symons' slaves" (both past and present) who have provided me with entertainment, refreshments, and distractions, and on occasions helpful discussions;

Mrs J. Brooker for her excellent typing of this (epi)tome;

and finally my parents (to whom I dedicate this thesis) for their continued support, both financial and otherwise.

This work was also supported by a Commonwealth Postgraduate Research Award.

ABBREVIATIONS.

ASBV	avocado sunblotch viroid
AMV	alfalfa mosaic virus
BMV	brome mosaic virus
ccRNA-1	cadang-cadang associated RNA-1
ccRNA-2	cadang-cadang associated RNA-2
cDNA	complementary DNA
CEV	citrus exocortis viroid
ChCMV	chrysanthemum chlorotic mottle viroid
CMV	cucumber mosaic virus
Cot	Initial DNA concentration x time (sec)
CPFV	cucumber pale fruit viroid
cRNA	complementary RNA
CSV	chrysanthemum stunt viroid
CTAB	cetyltrimethylammonium bromide
DEPC	diethylpyrocarbonate
DIECA	diethyldithiocarbamate
DNAse/DNase	deoxyribonuclease
Dot	Initial cDNA concentration x time (sec)
$d(pT_8A)$	5'-p-dT-dT-dT-dT-dT-dT-dT-dT-dA-3'
$d(pT_8C)$	5'-p-dT-dT-dT-dT-dT-dT-dT-dT-dC-3'
$d(pT_8G)$	5'-p-dT-dT-dT-dT-dT-dT-dT-dT-dG-3'
DTT	dithiothreitol
E. coli	*Escherichia coli*
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid
HSV	hop stunt viroid
$I.D._{50}$	dose at which 50% of the plants become infected
M_r	molecular weight

NS-ChCMV	nonsymptomatic strain of ChCMV
PEG	Polyethylene glycol (6000)
P.I.	post inoculation
POPOP	1,4-bis-[2-(5-phenyloxazolyl)]-benzene
PPO	2,5-diphenyloxazole
PSTV	potato spindle tuber viroid
PVP	polyvinylpyrrolidone
RNAse/RNAase	ribonuclease
Rot	Initial RNA concentration x time (sec)
SAT-CMV	satellite RNA of cucumber moasic virus
SDS	sodium dodecyl sulfate
SSC	standard saline citrate
S-TNV	satellite virus of tobacco necrosis virus
TCA	trichloroacetic acid
T_h	temperature of hybridization
T_m	melting temperature
TMV	tobacco mosaic virus
TRSV	tobacco ringspot virus

CHAPTER I

GENERAL INTRODUCTION

A. THE STRUCTURE, REPLICATION AND PATHOLOGY OF VIROIDS.

Viroids are low molecular weight RNAs pathogenic to some plants (Diener, 1979). They are characterized by a single-stranded, covalently-closed, circular structure with a high degree of base pairing (Sänger *et al.*, 1976). Unlike conventional viruses, viroid RNAs appear to be naked with no associated protein (Diener, 1971a; Semancik and Weathers, 1972a).

As a class of pathogenic agents, viroids are a fairly recent discovery. The viroid concept was first recognized when the infectious agents (thought to be viruses) of the spindle tuber disease of potato (Diener and Raymer, 1967) and the exocortis disease of citrus (Semancik and Weathers, 1968) were shown to have unusual properties for viruses: (1) phenol or other organic solvents had no effect upon the infectivity of buffered extracts from infected plants; (2) no virus particles could be isolated or demonstrated by electron microscopy; (3) the infectious agents displayed a resistance to nucleases or an elution profile off cellulose columns similar to double-stranded RNA; and (4) the infectious agents were always present in high speed supernatants; the sedimentation co-efficient was 10 - 15S. The level of these RNAs in plant tissue extracts was very low and the presence of the agent could only be determined by bio-assay and not spectrophotometrically.

As more detailed knowledge of the approximate molecular weights and physico-chemical properties of these two disease agents became available (Raymer and Diener,

1969; Diener and Raymer, 1969; Semancik and Weathers, 1970; Diener, 1971a; Semancik and Weathers, 1972a), it was suggested that these two agents were the first of a new class of infectious nucleic acids (Diener, 1971b; Semancik and Weathers, 1972b; Sänger, 1972): the term viroid was proposed (Diener, 1971b) and these causal agents were re-named potato spingle tuber viroid (PSTV) and citrus exocortis viroid (CEV).

There are at present eight known viroids; the first two, PSTV and CEV, have already been described.

Chrysanthemum stunt viroid (CSV) was shown to have a lower molecular weight, different host range and greater sensitivity to digestion by ribonuclease, than PSTV (Diener and Lawson, 1973).

Cucumber pale fruit disease was suggested as being caused by a low molecular weight RNA (van Dorst and Peters, 1974); electron microscopy (Sänger *et al.*, 1976) and physico-chemical studies (Henco *et al.*, 1977; Klump *et al.*, 1978; Langowski *et al.*, 1978) have shown this RNA to be a viroid: cucumber pale fruit viroid (CPFV).

The cadang-cadang disease of coconuts in the Philippines was shown to have two low molecular weight RNAs associated with the disease, cadang-cadang RNA-1 (ccRNA-1) and cadang-cadang RNA-2 (ccRNA-2; Randles, 1975). Although the evidence that one or both of these RNAs is the causal agent of a disease leading to the death of the coconut tree ("cadang-cadang" means "dying-dying" in a native Philippine dialect) is not yet available, the size, the high degree of secondary structure (Randles *et*

al., 1976), the association of ccRNA-1 and ccRNA-2 with diseased and not healthy plants, the demonstration of replication of ccRNA-1 in inoculated palms (Randles *et al.*, 1977) and the circularity of both ccRNA-1 and ccRNA-2 (Randles and Hatta, 1979) may be considered as evidence for a viroid etiology.

Chrysanthemum chlorotic mottle viroid (ChCMV) was shown to be pathologically distinct from CSV (Romaine and Horst, 1975). A non-symptomatic infectious form of ChCMV (NS-ChCMV) has also been discovered in cultivars of *Chrysanthemum morifolium* (Horst, 1975); the NS-ChCMV was not identified, but was shown to cross-protect against infection by ChCMV. Physico-chemical studies on ChCMV (Langowski *et al.*, 1978: Henco *et al.*, 1979) suggested ChCMV has a similar secondary structure to other viroids. The host range of ChCMV appears to be limited to a few *Chrysanthemum* species (Niblett, personal communication).

Hop stunt disease was recently shown to be caused by a slowly sedimenting RNA with a number of properties similar to viroids (Sasaki and Shikata, 1977a). Symptoms induced by hop stunt viroid (HSV) on cucumber plants are identical to those induced by CPFV on cucumbers (Sasaki and Shikata, 1977b; Takahashi and Takusari, 1979), prompting the suggestion that HSV may be related to CPFV.

A low molecular weight RNA present in *Columnea* plants was shown to replicate in tomatoes (Owens *et al.*, 1978). PSTV migrated more slowly than the *Columnea* RNA upon polyacrylamide gel electrophoresis and shared 33 - 41% (allowing for mis-matching) of its sequences in common with the

Columnea RNA, indicating that this RNA is a (*Columnea*) viroid related to but distinct from PSTV (Owens *et al.*, 1978).

1. Viroid Structure.

The high degree of secondary structure characteristic of viroids was first suggested by the resistance of PSTV to degradation by ribonuclease in high molarity salt buffers (Diener and Raymer, 1967) and the partial resistance to inactivation by diethylpyrocarbonate of CEV relative to single-stranded viral RNAs (Semancik and Weathers, 1972a). When photometrically detectable amounts of both viroids became available, thermal denaturation studies revealed the presence of a highly ordered secondary structure in both PSTV (Diener, 1972) and CEV (Semancik *et al.*, 1975). The viroid structure produced a co-operative melting process, similar to that observed upon denaturation of double-stranded RNA; however, the Tm of the melting curves of both PSTV and CEV was approximately 30°C lower than the Tm of the melting curve of double-stranded RNA in the same buffer (0.1 x SSC). The overall hyperchromicity of viroids was 22 - 24%, compared with 32% for double-stranded RNA (Diener, 1972; Semancik *et al.*, 1975). Thus viroids were considered to have hairpin-like structures with alternating single- and double-stranded regions (Diener and Smith, 1973; Semancik *et al.*, 1973; Sogo *et al.*, 1973).

The possibility that viroids may be circular was

first considered by Semancik and Weathers (1970), Diener (1971c) and Singh and Clark (1971), based on the resistance or partial resistance of PSTV and CEV (as determined by infectivity) to digestion by exonucleases. However, both Singh and Clark (1971) and Semancik and Weathers (1972a) considered this partial resistance insufficient to postulate a circular structure for PSTV or CEV, respectively.

Electron microscopy of PSTV under partly denaturing conditions (Sogo *et al.*, 1973) revealed the presence of only short, double-stranded, rod-like structures. Electron microscopy of CPFV (Sänger *et al.*, 1976), PSTV (McClements and Kaesberg, 1977) and ccRNA-1 and ccRNA-2 (Randles and Hatta, 1979) under non-denaturing conditions also revealed the presence of only rod-like structures; however, under denaturing conditions the presence of circular as well as linear molecules were observed. Sänger *et al.* (1976) claimed that CPFV, CEV and PSTV were all circular RNAs (although only evidence for CPFV was presented) with very few linear molecules being present; however, McClements and Kaesberg (1977) and Owens *et al.*, (1977) have shown the existence of two forms of PSTV, one linear and one circular.

The results of physico-chemical studies of several viroids (Henco *et al.*, 1977; Klump *et al.*, 1978; Langowski *et al.*, 1978; Henco *et al.*, 1979; Riesner *et al.*, 1979) have been combined with knowledge of the sequence of PSTV (Gross *et al.*, 1978) to produce a secondary structure

model for PSTV (Gross *et al.*, 1978; Henco *et al.*, 1979). Since all viroids show very similar physico-chemical properties to PSTV (Henco *et al.*, 1979), they are considered to share a similar secondary structure (Henco *et al.*, 1979), viroids apparently have no tertiary structure folding (Riesner *et al.*, 1979). Some care, however, must be taken in interpreting physico-chemical data which relies upon comparisons relative to some so-called standard values. Initially, thermodynamic and kinetic studies (Henco *et al.*, 1977) suggested the existence of an uninterrupted double helix of 52 base pairs. This latter postulate was clearly untenable, since a region of 52 uninterrupted base pairs would confer properties of double-stranded RNA on viroids; e.g. (1) a Tm of denaturation 30°C higher than experimentally observed (Vizard *et al.*, 1978); (2) render the RNA cleavable by double-strand-specific *E. coli* RNAse III (which requires 25 or more base-pairs in an uninterrupted segment; Robertson and Hunter, 1975). Since neither PSTV nor ^{125}I-CEV (Dickson, 1976) were cleaved by RNAse III, nor was the infectivity of PSTV reduced by RNAse III (Diener, 1977), a region of 52 uninterrupted base-pairs could not exist. A re-examination of the thermodynamic parameters of five different viroids using measured Tm values and enthalpies (Langowski *et al.*, 1978) suggested that "in their native conformation, viroids exist as an extended rodlike structure characterized by a series of double helical sections and internal loops."

The sequence of PSTV was recently determined, and a secondary structure model based on chemical as well as physico-chemical properties was proposed (Gross *et al.*, 1978). In the absence of tertiary folding (Riesner *et al.*, 1979) the three dimensional structure of PSTV is one of a rod-like, mis-matched, double-helical circular molecule with loops at both ends.

2. Viroid Replication

Subcellular fractionation of viroid-infected plant tissue followed by infectivity tests on these fractions, showed PSTV and CEV to be mostly associated with nuclei, or more specifically, chromatin fractions (Diener, 1971a; Sänger, 1972). Sub-cellular fractionation of CEV-infected *Gynura aurantiaca (Gynura)* by others (Semancik *et al.*, 1976) suggested the association of CEV with nuclei and plasma-membrane like components; the latter association has been suggested as being due to contamination by nuclear membranes and/or chloroplast fragments (Diener and Hadidi, 1977).

Viroid replication in PSTV-infected tomato leaf strips was demonstrated and shown to be inhibited by actinomycin D (Diener and Smith, 1975). A slow rate of viroid synthesis was also observed in isolated nuclei from viroid infected plants (Takahashi and Diener, 1975); this viroid synthesis was also inhibited by actinomycin D. Low levels of viroid replication were observed in protoplasts prepared from CEV-infected tomato (Mühlbach *et al.*, 1977a), and protoplasts inoculated with CPFV (Mühlbach and Sänger, 1977), PSTV or CEV (Mühlbach and Sänger, 1977;

Mühlbach *et al.*, 1977b). CEV replication was also observed in neoplastic tomato cell cultures (Semancik *et al.*, 1978).

The involvement of host messenger RNA (mRNA) and protein synthesis in viroid replication has been suggested by the inhibition of viroid replication by actinomycin D (inhibiting DNA-dependent RNA synthesis; Diener and Smith, 1975; Takahashi and Diener, 1975; Mühlbach and Sänger, 1979), α-amanitin (inhibiting RNA polymerase II, and thus mRNA synthesis; Mühlbach and Sänger, 1979) and cyclo-heximide (inhibiting cytoplasmic protein synthesis and RNA polymerase I, and thus ribosomal RNA synthesis, Mühlbach and Sänger, 1979). Given the limited coding potential for viroids (see below), the involvement of numerous host synthetic functions in viroid bio-synthesis is not surprising.

Complementary RNA (cRNA) to CEV, presumedly involved in its replication, was found to be associated with nuclei and cytoplasmic fractions (Grill and Semancik, 1978), while infectivity was associated only with nuclei and plasma-membrane fractions (Semancik *et al.*, 1976); cRNA to PSTV has also been detected (Dickson and Zaitlin, personal communication). Thus, the presence of viroid RNA and cRNA in nuclei, the demonstration of viroid replication to similar levels in leaf strips, protoplasts and isolated nuclei and the involvement of a nuclear DNA-dependent RNA polymerase (II) all suggest viroid replication occurs in nuclei.

Low levels of activity of RNA-dependent RNA polymerases have been observed in healthy plants by researchers

studying RNA virus-stimulated RNA polymerases (Bol *et al.*, 1976; Fraenkel-Conrat, 1976; Romaine and Zaitlin, 1978; Ikegami and Fraenkel-Conrat, 1978; 1979; Duda, 1979). These RNA-dependent RNA polymerases may be involved in viroid replication, since these enzymes seem to be cytoplasmic and cRNA to CEV was found in cytoplasmic fractions (Grill and Semancik, 1978). Furthermore, some viral-induced RNA polymerases have been found to be membrane bound (May *et al.*, 1970; Hardy *et al.*, 1979; Zabel *et al.*, 1979), suggesting a membrane-associated function in virus and possibly viroid bio-synthesis; CEV was also found to be associated with membrane fractions (Semancik *et al.*, 1976). No clear picture has emerged with any plant viral RNA-induced polymerase as to whether the same RNA polymerase (i.e. same single protein or arrangement of protein subunits) is involved in both minus RNA (cRNA) strand synthesis and plus RNA strand synthesis. Perhaps both nuclear and cytoplasmic or plasma-membrane bound RNA polymerases are involved in various aspects of viroid bio-synthesis.

Whatever the mechanism of viroid bio-synthesis, the end product is a linear viroid that must be ligated to a curcular form; RNA ligases requiring either a 3'-phosphate and a 5'-hydroxyl (Wolfe, 1979) or a 5'-phosphate and a 3'-hydroxyl (Silber *et al.*, 1972) have been identified. *In vivo* labelling of PSTV with ^{32}P-orthophosphate has revealed the initial rapid appearance of circular PSTV followed by a slow increase in the level of circular PSTV concomitant with a faster increase in the level of

linear PSTV (Hadidi and Diener, 1978) suggesting a conversion of circular molecules to linear molecules as a function of time. However, the different types of PSTV synthesis observed could have occurred in different tissues or different parts of the same cell with widely different nucleotide pools; only a "pulse-chase", which is not possible in whole plants, would have shown that linear molecules accumulated as the result of breakdown of circular molecules.

Circularity of the viroid is not a pre-requisite for replication, since circular PSTV molecules were shown to be as infectious as linear PSTV molecules (Owens *et al.*, 1977), although this may depend on the method of isolation of linear viroid molecules. For example, Morris (1979) isolated an RNA with a similar base-composition to PSTV, but a different mobility on denaturing gels and showed this RNA to be essentially non-infectious; neither electron microscopic evidence to indicate linearity of this RNA, nor any other data to identify this RNA as PSTV was presented. Linear PSTV molecules generated by magnesium-catalyzed cleavage of circular PSTV (Sänger *et al.*, 1979) were claimed to be 10^3 - 10^4 fold less infectious than circular molecules.

Attempts to translate PSTV *in vitro* (Davies *et al.*, 1974) or CEV *in vitro* (Hall *et al.*, 1974) or *in vivo* (Semancik *et al.*, 1977) using extracts from wheat germ, wheat embryo, *E. coli*, *Pseudomonas aeruginosa*, *Bacillus stearothermophilus* (at 60°C) or in frog oocytes, have been unsuccessful. Comparisons of (a) proteins isolated

from PSTV-infected tomato with those isolated from healthy tomato tissue (Zaitlin and Hariharasubramanian, 1972), or (b) proteins isolated from CEV-*Gynura* with those isolated from healthy *Gynura* (Conejero and Semancik, 1977), have revealed no evidence of viroid-coded protein synthesis. Conejero and Semancik (1977) observed the stimultion of two host proteins in CEV-infected *Gynura*; the molecular weights of the two proteins induced by CEV in *Gynura* and *Citrus medica* were the same, but different from the molecular weights of two proteins induced by CEV in tomato and potato (Conejero *et al.*, 1979). Furthermore, the two "CEV-induced" *Gynura* proteins were also induced during the senescence of healthy *Gynura* (Conejero *et al.*, 1979), lending support to the suggestion of the host-specific nature of viroid-induced proteins. Thus, there is not evidence for the role of a viroid-coded replicase or replicase subunit.

Semancik *et al.* (1978) have observed viroid synthesis and the presence of cRNA to CEV primarily in meristem tissue, tumor tissue and isolated neoplastic cells, suggesting replication occurs predominantly in metabolically active dividing tissue. This is supported by increased levels of viroids in plants grown at higher temperatures (Sänger and Ramm, 1975; Morris and Smith, 1977). The low levels of RNA-dependent RNA polymerases which are stimulated by cell division (White and Murakishi, 1977) and the slow rate of viroid bio-synthesis in non-dividing cells, protoplasts or nuclei, all reflect the need to use dividing tissue or protoplasts in order to observe sign-

ificant viroid replication or to study the effects of viroids on/in host cells.

3. Viroid Pathology

Symptoms induced by viroid infections have been described in detail by Diener and Hadidi (1977) and Diener (1979). The most common symptom is the shortening of the internodal distance resulting in the stunting of the infected plant. Leaf size may be reduced and leaves may display chlorotic or necrotic lesions, mottles or streaks. Leaves are often curled and the plant may have a bushy appearance. The symptoms resulting from viroid infection have been compared to abnormal cell development produced as the result of hormonal malfunctions (Conejero and Semancik, 1977).

The CEV-stimulated *Gynura* protein, CEV-P_1, has no binding activity for the phytohormones gibberellin GA_1/GA_3 or β-indolacetic acid (Flores *et al.*, 1978); no change in abscissic acid levels or auxin-like substances with the properties of indolacetic acid were detected in CEV-infected *Gynura*, although a new auxin-like substance was formed and decreases in the levels of endogenous gibberellins GA_3 and/or GA_1 were detected (Rodriĝuez *et al.*, 1978).

The association of viroids with nuclei (Diener, 1971a; Sänger, 1972; Semancik *et al.*, 1976), the stimulation of the synthesis of a histone-like protein in CEV-infected *Gynura* (Conejero and Semancik, 1977) and the replication of viroids in mitotic tissue, all suggest that viroids alter host development by causing some permanent change at the level of host genetic expression. This process

probably occurs in cells of the meristem early in the formation of the leaf. As the leaf emerges from the shoot tip, the various types of cells and viroid symptoms, and thus the pathological effects of viroid infection, are already present. Leaf growth and maturation from this point on consist of division of the various cell types and cellular elongation. These latter processes seem to be influenced by viroid infection. In CSV or CEV-infected chrysanthemums, leaf size is greatly reduced; in CSV or CEV-infected *Gynura* leaf size is partially reduced and differential growth between upper and lower leaf surfaces results in leaf curl.

Pathological effects of viroid infection at the (sub-) cellular level appear to depend upon both the viroid and the host: PSTV-infected tomatoes showed a marked hypertrophy of nuclei when compared with healthy tomatoes (Diener, 1971c). Electron microscopy of CEV-infected tomatoes showed the presence of large dense bodies within chloroplasts (Wahn *et al.*, 1978). CEV-infection of *Gynura* resulted in the appearance of plasmalemmasomes in cells of symptom-bearing tissue but rarely or never in healthy or symptomless leaves (Semancik and Vanderwoude, 1976). The appearance of these paramural bodies or boundary formations at the cell wall-plasma membrane interface as well as malformations of the cell wall's middle lamellae has also been observed by others (Wahn *et al.*, 1978) in CEV-infected *Gynura*. The appearance of electron-dense inclusions (thought to be phytoferritin) was observed in chloroplasts of chlorotic tissue in both CSV-infected and

healthy chrysanthemums (Lawson and Hearon, 1971); inclusions were also observed in chloroplasts of palisade and mesophyll cells from green tissue of CSV-infected but not healthy plants.

The cytopathological changes in infected plants have not been correlated with a change in host protein synthesis; CEV-P_1 appears to be in a post-ribosomal supernatant fractions (Conejero and Semancik, 1977); i.e., in the cytoplasm. How viroids induce changes in chloroplasts or plasma-membranes or how they alter the genetic expression of a host plant is unknown.

A number of theories have been proposed as to how viroids may act as genetic regulators (Diener, 1971b; Semancik and Weathers, 1972b; Conejero and Semancik, 1977; Diener, 1977), as well as to how some of the slow virus diseases of animals may be caused by (DNA) viroids (Diener, 1972b; Sänger, 1972; Semancik *et al.*, 1976).

Hopefully, studies on viroid replication, alteration of host protein metabolism and viroid cross-protection (Niblett *et al.*, 1978) will elucidate the mechanisms of viroid replication and pathology. These studies will be greatly assisted by the use of complementary DNA probes for viroid sequences in nucleic acid hybridization experiments.

B. THE APPLICATION OF MOLECULAR HYBRIDIZATION TO THE STUDY OF VIROIDS

1. Background and General Applications

Molecular hybridization as an experimental technique involves the determination of the rate and the extent of the formation of base-paired nucleic acid duplexes from their component strands. There are several classes of hybridization reactions, such as: (1) the reassociation of denatured double-stranded DNA; (2) hybridization of a nucleic acid species immobilized on a filter to complementary strands in solution (either RNA or DNA); and (3) the hybridization of complementary DNA (cDNA) transcribed *in vitro* from an RNA template, to the template RNA or other RNA species. It is the third class of hybridization reaction that will be applied to viroids in this thesis. There are several techniques available for the estimation of hybrid formation (Gillespie *et al.*, 1975), such as the use of single-strand specific nuclease S_1 (Ando, 1966; Sutton, 1971), hydroxylapatite chromatography and binding to nitrocellulose.

Analysis of the kinetics of hybrid formation will not be discussed in detail here; this subject has been thoroughly covered in the literature (e.g. Wetmur and Davidson, 1968; Bishop, 1972). However, there are two important points which are relevant to the work described in this thesis: (1) the rate of a hybridization reaction is inversely proportional to the analytical complexity of the reacting species [the analytical complexity is the minimum number of nucleotides (or their molecular weight)

which comprises all the non-identical nucleotide sequences of the nucleic acid species]; (2) the rate of a hybridization reaction is directly proportional to the (molar) concentration of the reacting species.

An estimation of the analytical complexity of the concentration of an RNA or DNA species can be achieved by studying the rate of hybridization of, for example, an RNA species to an appropriate cDNA (see below), since, as mentioned above, the rate of the reaction is inversely proportional to the complexity. In the second case, if the complexity is known, the concentration of the RNA in question can be determined from the rate. Thus, viral RNA in infected cells was quantitated by Taylor *et al.*. (1977) using this approach to study transcription and replication of influenza virus RNA.

2. Synthesis Of Complementary DNA

Complementary DNA is usually synthesized using the DNA-dependent RNA polymerase (reverse transcriptase) of retroviruses (Green and Gerard, 1974). This enzyme, like all other known DNA polymerases, requires a primer which is base-paired to the template (RNA) and which has a free 3'-hydroxyl group. For eukaryotic messenger RNAs which have a 3'-poly(A) 'tail', oligo(dT) can be used as a primer (Green and Gerard, 1974). The method of Taylor *et al.* (1976) can be used with most other RNAs; this utilizes the random binding of oligodeoxyribonucleotides, generated by DNAse I digestion of eukaryotic DNA, to the template RNA to provide primers. The cDNA can be made highly radioactive by including radio-labelled

dNTP in the cDNA synthesis reaction mixture.

3. The Use Of Molecular Hybridization In Virology

Molecular hybridization has been widely used in the study of animal viruses, particularly in work on retroviruses (Gillespie *et al.*, 1975). With plant viruses, the random-primed synthesis of cDNA has permitted an examination of the structure of the cucumber mosaic virus (Gould and Symons, 1977) and the alfalfa mosaic virus genomes (Gould and Symons, 1978). This technique has also enabled comparisons between members of the cucumovirus family (Gonda and Symons, 1978) and tymovirus family (Kummert *et al.*, 1978) to be made. cDNA to a viroid, PSTV, (Owens, 1978) was prepared using the random-primer technique and was used to compare PSTV with several other viroids (Owens *et al.*, 1978).

Molecular hybridization of cDNA to RNA has several advantages over the system which uses competitive hybridization, in which the complementary strand is supplied by double-stranded RNA, to estimate sequence homology between the genomes of RNA plant viruses (Bol *et al.*, 1975; Vandewalle and Siegel, 1976; Zaitlin *et al.*, 1977). These advantages include analytical complexity analysis, estimates of contamination by other (viral) RNAs and determination of the stability of the hybrids formed and have been covered in detail elsewhere (Gonda and Symons, 1978; Gonda, 1979). Aspects of cDNA and cRNA synthesis to viroids will be described in Chapter IV.

The study of the replication of plant viruses has been greatly facilitated by the use of protoplasts (see

reviews by Zaitlin and Beachy, 1974 and Takebe, 1975). Optimization of conditions for the infection of protoplasts by viruses or viral RNAs is usually determined by fluorescent antibody staining (Otsuki and Takebe, 1969), wherein antibodies prepared against viruses (coat proteins) are coupled to a fluorescent dye; the percentage of fluorescent cells provides an indication of the number of infected protoplasts. Since viroids have no coat protein, this approach cannot be used to optimize the conditions for infection of protoplasts by viroids. *In situ* hybridization experiments (Pardue and Gall, 1975) involving tritiated or iodinated viroid cDNA offer a feasible alternative to determining the percentage of viroid-infected protoplasts, as well as determining the sites of viroid bio-synthesis and accumulation. The use of radio-labelled viroid in *in situ* hybridization reactions offers a means of determining the time and place of viroid cRNA synthesis.

Viroid cDNA and radio-labelled viroid can also be used to determine the levels of viroid and viroid cRNA, respectively, as a function of time post inoculation of protoplasts. This latter approach has been used to quantitate the four cucumber mosaic virus (CMV) RNAs in CMV-infected cowpea protoplasts as a function of time post inoculation (Gonda and Symons, 1979; Gonda, 1979).

Molecular hybridization of high specific activity viroid cDNA to nucleic acid extracts of whole plants can be used to determine the absolute level of the viroid RNA in the nucleic acid extract. Thus, estimations of the

presence of low or high levels of viroid RNA in different plants can be made, as well as the types of tissue or growth conditions that best support viroid replication. This would enable the rapid indexing of plants for viroid or viral diseases and could replace or supplement time consuming and expensive biological indexation. Thus, there is a strong need for developing methods of synthesizing and using cDNA probes to viroids.

C. THE AIMS OF THIS PROJECT

The work described in this thesis has three main aims.

1. The establishment of a rapid extraction procedure for viroids using chrysanthemums infected with CSV and the purification and characterization of CSV.

2. The development of a procedure for the synthesis of cDNA probes to viroids, the characterization of the cDNA probes, the use of cDNA probes to characterize viroids, and the establishment of a hybridization procedure for viroids that would enable the level of viroid RNA in partially purified nucleic acid extracts to be determined.

3. To establish a viroid etiology for the sunblotch disease of avocado, to characterize the avocado sunblotch viroid (ASBV) and to develop a rapid indexing procedure for ASBV using cDNA to ASBV in molecular hybridization reactions.

CHAPTER II

EXTRACTION, PURIFICATION AND BIO-ASSAY OF CHRYSANTHEMUM STUNT VIROID

INTRODUCTION

Fundamental to the study of the nature of viroid structure, replication and pathology are (1) the establishment of a sensitive and reliable bio-assay procedure for detecting the presence of sub-microgram quantities of viroid, and (2) the procurement of sub-milligram quantities of purified viroid.

This Chapter will be concerned with the development of (1) a procedure for biologically indexing the presence of chrysanthemum stunt viroid; (2) a rapid extraction procedure for the preparation of low molecular weight plant nucleic acids; and (3) a polyacrylamide gel electrophoresis and elution protocol for the preparation of purified viroid on a large scale.

MATERIALS

Healthy and chrysanthemum stunt viroid infected chrysanthemums (Charm type) were kindly provided by T.C. Lee, Adelaide Botanic Gardens, via Dr. R.I.B. Francki, University of Adelaide.

The following reagents were used directly as provided by their respective manufacturers, without any further purification: polyvinylpyrrolidone (Sigma PVP-40T), sodium azide (BDH or Ajax), sodium diethyldithiocarbamate (Sigma), 2-mercaptoethanol (Sigma), mercaptobenzothiazole (Eastman Organic Chemicals), sodium dodecyl sulfate (Sigma or BDH), DNAse I (Sigma), 4-amino-salicylate (Searle), 8-hydroxy-guinoline (BDH) and diethylpyrocarbonate (Sigma).

The following chemicals were either re-crystallized or re-distilled:phenol (M & B or Ajax; both local suppliers), acrylamide (Sigma) and N,N'-methylenebisacrylamide (Sigma).

METHODS

A. Plants and Growth Conditions

Chrysanthemums (*Chrysanthemum* sp. Charm type) and *Gynura* (*Gynura aurantiaca*) were vegetatively propagated in 15 cm diameter pots in vermiculite and "University of California" soil mix, respectively. After 3 to 4 weeks, chrysanthemum cuttings were transferred to soil in 25 cm dia. pots. Cineraria (*Senecio cruentus* cv. Kemp; cv. Springtime) was grown in 10 cm dia. pots in soil.

Plants were grown under one of four sets of conditions.

1. Glasshouse. The average temperature was 20 - 25°C; supplementary heating and cooling were provided in winter and summer, respectively, but in extremes of weather the temperature was often outside this range.

2. Room A. Temperatures ranged from 20 - 28°C. Lighting was provided by fluorescent and incandescent lamps to approximately 6 - 8,000 lux at plant apex height, during a 14 h photoperiod.

3. Room B. Similar to Room A, except (1) temperatures ranged from 26 - 31°C, and (2) light intensity varied from 8 - 12,000 lux at plant apex height.

4. Room C. The day and night temperatures were controlled at 28°C and 22°C, respectively. Lighting

during the 14 h photoperiod was provided by banks of 15 x 65 W fluorescent tubes and 2 - 4 x 60W incandescent lamps, and the intensity varied between 10 - 20,000 lux. Relative humidity (not controlled) usually only varied between 60 - 65% (day) and 80 - 85% (night).

B. Extraction Procedures.

1. Procedure A; Phenol/SDS/EDTA/High Salt

Extraction Buffer A: 100 mM Tris-HCl, pH 8.5, 500 mM NaCl, 10 mM EDTA, 0.5% (w/v) SDS.

TEN Buffer: 20 mM Tris-HCl, pH 8.5, 1 mM EDTA, 100 mM NaCl.

Extraction and Partial Purification of Chrysanthemum Nucleic Acids: Shoot tips and leaves from healthy or CSV-infected chrysanthemums or *Gynura* were frozen in liquid nitrogen or powdered dry ice and were pulverized. Various amounts of freshly frozen or frozen and stored (at -15°C) leaf tissue were extracted as described below for 100 g of tissue.

One hundred grams of pulverized chrysanthemum tissue was homogenized at room temperature with 200 ml of extraction buffer A, 2 ml of 2-mercaptoethanol, 150 ml of water-saturated phenol and 50 ml of chloroform in a 5 litre Waring Commercial Blendor in short bursts for a total period of 3 min. The homogenate was stirred at room temperature for 15 - 60 min followed by centrifugation at 15,000 g for 10 - 15 min at 5 - 10°C; the aqueous phase was combined with an equal volume of (3:1) water-saturated phenol:chloroform, mixed in a Waring Blendor for 2 min and stirred for 10 - 30 min. After centrifugation

(as described above), the aqueous phase was combined with 2 volumes of cold ethanol and incubated at -15°C for 3 - 4 h or overnight. The precipitated nucleic acids were collected by centrifugation at 15,000 g for 10 - 15 min at - 5 to -10°C, resuspended in 25 ml of either 1 mM EDTA or TEN buffer (either with or without 1% 2-mercapto-ethanol), combined with an equal volume of 4 M LiCl and left overnight at 0°C. Following centrifugation at 15,000 g for 15 min at 0°C, the supernatant was combined with 2 volumes of cold ethanol, stored at -15°C for 3 h or overnight and the nucleic acids were collected by centrifugation at 15,000 g for 15 min at -10°C. The nucleic acid pellet was dried *in vacuo* and resuspended in 4 - 5 ml of 1 mM EDTA, pH 7.

Digestion of Partially Purified Nucleic Acid Extracts with DNAse I: Most preparations of LiCl-soluble nucleic acids were incubated at 25°C for 1 - 1.5 h with 10 μg/ml of DNAse I in 5 - 20 mM sodium acetate, pH 5.0, 10 mM magnesium acetate. The reaction was terminated by the addition of 1 - 2 volumes of extraction buffer A, 2 - 5 volumes of water and 1 - 7 volumes of water-saturated phenol:chloroform (3:1). After stirring for 20 - 30 min, the phases were separated by low speed centrifugation at room temperature; the aqueous phase was extracted one-to-three times with an equal volume of diethyl ether, the sodium ion concentration was adjusted to 0.25 - 0.3 M with 4 M NaCl, and two volumes of cold ethanol were added to the aqueous phase. After incubation overnight at -15°C, the nucleic acid

precipitate was collected by centrifugation at 8 - 12,000 g for 10 min at -10°C. The nucleic acid pellet was dried *in vacuo* and resuspended in 1/10th the original volume of 0.1 mM EDTA, pH 7.

Several modifications were made to extraction procedure A to inhibit polyphenol oxidase activity and increase the yield of extractable nucleic acid.

1) Additions to extraction buffer A: 0.1 - 0.5% (w/v) diethyldithiocarbamate (DIECA), 1% (w/v) polyvinylpyrrolidone (PVP). 5% (w/v) 4-amino-salicylate, 1% (w/v) sodium azide and 0.1% (w/v) 8-hydroxyquinoline in various combinations.

2) Blending plants with extraction buffer A and all the additional components described in (1) above, followed by phenol:chloroform (2:1) extraction and stirring for 1 h at room temperature. After centrifugation, the phenol:chloroform extraction was repeated twice more prior to ethanol precipitation of the aqueous phase.

2. Procedure B: DEPC/SDS/Mg^{2+} (Solymosy *et al.*, 1970)

Extraction Buffer B: 100 mM Tris-HCl, pH 7.6, 2% (w/v) SDS, 10 mM magnesium acetate, 7% (w/v) PVP.

Solution A: 15% (w/v) DIECA, 3% (w/v) sodium azide, 15 mM mercaptobenzothiazole.

One hundred grams of fresh chrysanthemum leaves and tips was homogenized at 4°C with 250 ml of extraction buffer B and 6 ml of diethylpyrocarbonate (DEPC) in a 1 litre Waring Blendor for 2 - 5 min. Ten millilitres of solution A was added, the brei was incubated at 37°C for 10 min with occasional stirring, cooled on ice for 10 min

and centrifuged at 17,000 g for 10 min at 4°C. The supernatant was made 10% with respect to sodium chloride by the addition of solid sodium chloride, stirred at 37°C for 10 min, cooled on ice for 10 min and centrifuged at 10,000 g for 20 min at 4°C. The resulting supernatant was combined with 2 volumes of cold ethanol, left at -15°C for 3 h, subjected to centrifugation at 10,000 g for 10 min at -10°C and the nucleic acid was resuspended in 20 ml of 0.1 mM EDTA, pH 7.

The suspension was combined with 20 ml of (1:1) water-saturated phenol (containing 0.1% (w/v) 8-hydroxyquinoline):chloroform, shaken for 30 min at room temperature and subjected to centrifugation at 2,000 g for 5 min at room temperature. The aqueous phase was dialyzed overnight against 1 change of 1 mM NaCl; the dialyzate was clarified by low speed centrifugation, made 0.2 M with 5 M sodium acetate, pH 6.0, and combined with 2 volumes of cold ethanol. After at least 24 h incubation at -15°C, the nucleic acid was collected by low speed centrifugation at 4°C, the pellet dried *in vacuo* and resuspended in 0.1 mM EDTA, pH 7.

3. Procedure C: SDS/Mg^{2+}/High Salt

Solution A: 0.2 M Tris-HCl, pH 8.5 (at room temperature), 1 M NaCl, 1% (w/v) SDS.

Solution B: 25 mM magnesium acetate, 5% (w/v) PVP, 2% (w/v) sodium azide, 1% (w/v) DIECA, 2% (v/v) 2-mercaptoethanol, 1 mM mercaptobenzothiazole. Solution B was prepared just prior to use by mixing 20 ml of 1 M magnesium acetate, 400 ml of 10% (w/v) PVP plus 4% (w/v)

sodium azide (stock solution stored at 4°C), 8 g of DIECA, 16 ml of 2-mercaptoethanol, 8 ml of 0.1 M mercaptobenzothiazole in ethanol, and water to 800 ml.

Solution C: 0.1 M Tris-HCl, pH 8.5, 0.5 M NaCl, 0.5% (w/v) SDS, 1% (v/v) 2-mercaptoethanol.

Extraction and Partial Purification of Chrysanthemum Nucleic Acids: The procedure described used 500 g of chrysanthemum shoots, 5 - 10 cm long; either fresh material or shoots frozen in liquid nitrogen and stored at -80°C were used. Plant tissue was homogenized at room temperature with 750 ml each of Solution A and of Solution B in a 5 litre Waring Commercial Blendor in short bursts for a total period of 4 min. The homogenate was stirred at room temperature for 15 min, 200 g NaCl was then stirred in over 15 min followed by a further 15 min stirring. After cooling on ice for 30 min, the homogenate was centrifuged at 20,000 g for 20 min at 4°C and the nucleic acids precipitated from the supernatant by the addition of 2.0 vol. of cold (-15°C) ethanol and standing at -15°C for one hour. The precipitate was collected by centrifugation at 20,000 g for 10 min at -5°C and the pellets dried *in vacuo* with an oil pump for about one hour. The dried pellets were well suspended in 80 ml Solution C and the suspension centrifuged at 1,000 g for 5 min at room temperature to give a bulky green precipitate (discarded) and a green supernatant to which was added 20 ml water-saturated phenol (containing 0.1% 8-hydroxyquinoline) plus 20 ml chloroform. The mixture was stirred for 20 min then centrifuged at 1,000 g

for 5 min.

The aqueous phase was dialysed at 4°C for 16 h against 4 litres of 1 mM NaCl, 0.1 mM EDTA, pH 7 - 8, with one change of dialysis solution. Sodium acetate (5.0 M, pH 6.0) or sodium chloride (5.0 M) was added to 0.2 M followed by 2.0 volumes of cold ethanol and storage at -15°C for three hours. The precipitated nucleic acid was collected by centrifugation at 10,000 g for 10 min at -5°C, dried *in vacuo* and resuspended in 10 ml of 0.1 mM EDTA, pH 7. The yield of nucleic acid averaged 250 mg/kg of chrysanthemum material, based on A_{260} nm of 20 for 1.0 mg/ml.

Nucleic acids were extracted from healthy, CSV-infected and CEV-infected *Gynura aurantiaca* in the same way.

C. Polyacrylamide Gel Electrophoresis (PAGE)

1. Analytical PAGE: Conditions for analytical PAGE, buffers, percent acrylamide and bis-acrylamide, and staining procedures are described in individual Figure legends.

2. Preparative PAGE and Electrophoretic Elution: Nucleic acids extracted by procedure C from 500 g of infected chrysanthemum plants were then fractionated by polyacrylamide slab gel electrophoresis (De Wachter and Fiers, 1971; Symons, 1978) on four 16 x 16 x 0.6 cm 5% acrylamide, 0.25% methylene bisacrylamide slab gels using a buffer of 40 mM Tris-acetate, pH 8.1, 20 mM sodium acetate, 2 mM EDTA (Loening, 1967). Electrophoresis was

at 45 mA/gel for 15 - 17 hours. The single bands of CSV (circular plus linear) and host 7S RNA were located by brief staining (4 min) in 0.05% toluidine blue and destaining (1 - 2 h) in water. The stained bands were cut out and the RNA electrophoretically eluted as described by Symons (1978) except that a larger elution apparatus was used; each elution barrel was 8.0 cm long with an internal diameter of 2.8 cm while each elution chamber was 3.0 cm long with an internal diameter of 1.9 cm. Electroelution was at 80 mA/elution tube for 5 h.

Each partly purified RNA was further purified on either a 16 x 16 x 0.5 cm 5% acrylamide, 0.25% methylene bisacrylamide slab gel in the buffer described above (electrophoresis at 40 mA/gel for 14 - 20 h) or on a 16 x 16 x 0.3 cm 5% acrylamide, 0.17% methylene bis-acrylamide slab gel in 7 M urea, 90 mM Tris-borate, 3 mM EDTA, pH 8.3 (Air *et al.*, 1976). Before loading, each RNA sample in 0.1 mM EDTA, pH 7, was heated at 75°C for 4 min. Electrophoresis was at 65 - 75 mA/gel for 4.5 h, RNA bands were located by staining and the RNA electro-phoretically eluted (Symons, 1978).

D. Bio-assay Of CSV.

Senecio cruentus (Cineraria): Half-leaves of cineraria plants were incolated by placing a drop on one side of a carborundum-dusted leaf and rubbing the drop over half the leaf surface with a rubber finger-stall. One half of each leaf was inoculated with water, and the other half with a test solution; a crude nucleic acid extract from healthy or CSV-infected chrysanthemum. After inoculation,

the leaves were washed to remove carborundum and the plants were maintained in either a glasshouse or plant room A.

As the expected symptoms, consisting of distinct chlorotic spotting, were never observed, the starch-lesion assay for CSV infection (Lawson, 1968) was performed 3 - 5 weeks post inoculation. Plants were placed in the dark for 24 h; leaves were removed, boiled in ethanol for 2 - 5 min, stained in 2% KI/o.5% I_2 and destained in water. Starch-lesions were seen as black spots on a light green-white transparent background.

Gynura aurantiaca: All except the terminal leaves of rooted cuttings of *Gynura aurantiaca* were removed and the stem inoculated by needle puncture (Sänger, 1972) using a set of 20 fine needles set closely together in a piece of Perspex, or by stem slashing with a No. 22 scalpel blade (Semancik and Weathers, 1970). Plants were maintained under all the growth conditions previously described. Symptoms consisted of stunting and severe epinasty and appeared from 17 days post inoculation. Shoot tips from symptomless plants were cut back at 5 weeks post inoculation and new shoots were examined three weeks later for symptom development. Unless otherwise stated, all plants were scored at 8 weeks post inoculation.

RESULTS

A. Development of An Extraction Procedure

1. Procedure A: Phenol/SDS/EDTA/High Salt

This method is similar to most other procedures used to extract viroids (Diener *et al.*, 1977; Semancik and Weathers, 1972b; Singh and Sänger, 1976), except that several steps were modified or omitted to shorten the extraction procedure. The plant tissue was solubilized in the presence of a high concentration of salt, chelating agents and SDS in an alkaline buffer in the presence of phenol and chloroform. The plant debris and phenol:chloroform phase were removed by centrifugation; the aqueous phase was re-extracted one-to-two more times with phenol to remove proteins; nucleic acids were concentrated by ethanol precipitation and subjected to lithium chloride partitioning.

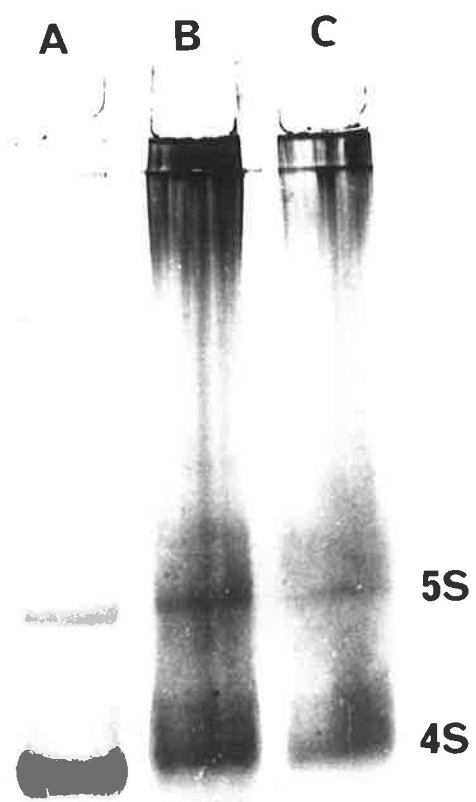
Semancik and Weathers (1968) and Raymer and Diener (1969) had shown that viroids require high salt for their extraction from plant tissue; Semancik and Weathers (1970) and Singh and Clark (1971) showed that viroids are LiCl soluble.

After removal of the LiCl, DNA was removed from the remaining nucleic acid via treatment with DNAse I and the nucleic acids were analyzed by polyacrylamide gel electrophoresis. Figure II-1 shows that only 4S and 5S RNAs as well as some darkly staining material at the top of the gel were visible; no bands in the viroid region could be seen.

FIGURE II-1

Polyacrylamide slab gel electrophoresis of (A) *E. coli* tRNA, (B) CSV-infected and (C) healthy chrysanthemum nucleic acids prepared by extraction procedure A.

Nucleic acid samples (0.4 mg, 3.5 mg and 2.1 mg, respectively) were heated at 80°C for 5 min, cooled on ice and subjected to electrophoresis on a 16 x 16 x 0.3 cm 10% acrylamide, 0.25% bis-acrylamide slab gel (Symons, 1978) in the buffer of Loening (1967) at 60 mA for 9 h. The gel was stained for 10 min in 0.05% (w/v) toluidine blue and destained overnight in water.


A
B
C
5S
4S

The nucleic acid extract was highly coloured (dark brown-to-black) as was previously reported by Diener and Lawson (1973), due to polyphenol oxidase activity. Various inhibitors (Loomis, 1974) of polyphenol oxidases were used (Table II-1) as well as the chelating agent and detergent 4-amino-salicylate: separately they had no effect on colour formation; together, they severely inhibited the browning reaction at this early stage. However, during the remainder of the extraction procedure, the material became darker. Therefore, two forms of oxidation seemed to be occurring: (1) enzyme-mediated oxidation (by oxidases) of polyphenols that could be partially inhibited in the initial extraction procedure; and (2) aerobic oxidation of polyphenols that occurred slowly throughout the procedure. Thus, 4-amino-salicylate, sodium diethyldithiocarbamate, sodium azide, polyvinyl-pyrrolidone and 8-hydroxyquinoline were added to inhibit enzymic oxidation and 2-mercaptoethanol was included in the extraction buffer to reduce aerobic oxidation.

Carbohydrates were also extracted from the chrysanthemums by this procedure; one of the steps used by Diener *et al.* (1977) but omitted here was the use of methoxy-ethanol and cetyltrimethylammonium bromide (CTAB) to remove carbohydrates (Bellamy and Ralph, 1968). This is a long and laborious procedure and does not remove most plant polysaccharides (Singh and Sänger, 1976). The solution of partially purified nucleic acids was highly viscous, predominantly due to the presence of pectins.

Polyphenols could be partially removed by gel filtration on G-75 Sephadex; however, polyphenols and/or

TABLE II-1

EFFECT OF INHIBITORS OF POLYPHENOL OXIDASES ON THE OXIDATION OF POLYPHENOLS DURING THE EXTRACTION AND PURIFICATION OF NUCLEIC ACIDS

Inhibitors of polyphenol oxidases	Colour of initial extract[a]	Colour of 2 M LiCl supernatant
0.1% (w/v) DIECA	Brown	Black
1% (w/v) PVP	Brown	Black
1% (w/v) sodium azide	Brown	Black
1% PVP/0.5% DIECA	Yellow-Light green	Brown
1% PVP/0.5% DIECA/1% sodium azide	Yellow-White	Brown

a All extractions were performed on 4 - 10 g of CSV-infected plant material as described in Materials and Methods using extraction procedure A (with 5% (w/v) 4-amino-salicylate). "Initial extract" refers to the aqueous phase after the first centrifugation.

pectins could not be removed by gel filtration on G-50 Sephadex, chromatography on a diethylaminoethyl-cellulose column, dialysis, adsorption to activated charcoal, the use of bentonite in the extraction buffer or CTAB precipitation (results not shown).

Although the modifications (listed above) to the extraction procedure and the use of gel filtration allowed stainable amounts of an extra nucleic acid species present only in CSV-infected chrysanthemum nucleic acid extracts to be detected (Figure II-2) on polyacrylamide gels, the addition of the above extra steps resulted in the breakdown and contamination of the extra nucleic acid species by host RNA; the "background haze" could be reduced by treatment with RNAse but not DNAse (results not shown).

An increase in solution viscosity to a gel-state was also observed. This may have been due to a increase in the level of extractable pectin from the vegetatively propagated chrysanthemums as a function of time of growth under artificial conditions; also observed was a decrease in stainable distinct nucleic acid species and an increase in the stainable background. Rather than introduce a series of extra steps to remove carbohydrates and polyphenols and reduce RNA breakdown, a different extraction procedure was adopted.

2. Procedure B: DEPC/SDS/Mg^{2+}

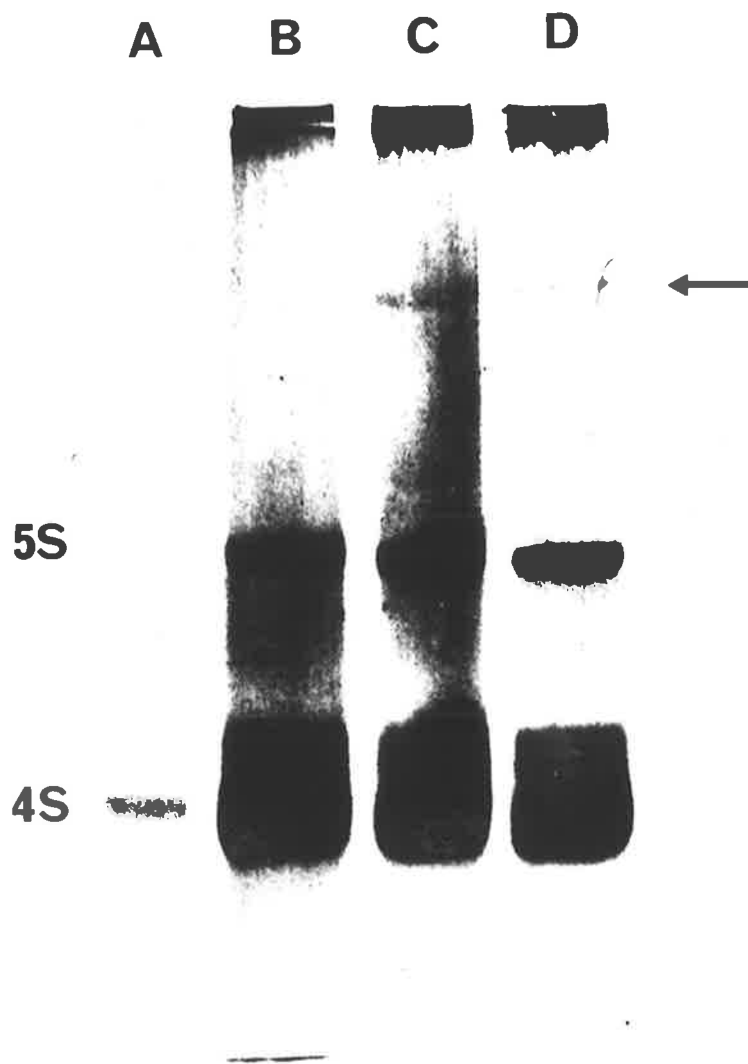
The extraction procedure of Solymosy *et al.* (1970) was modified for use on fresh chrysanthemum leaves. The plants were homogenized in a buffer containing

FIGURE II-2

Polyacrylamide slab gel electrophoresis of (A) *E. coli* tRNA, (B) healthy chrysanthemum nucleic acid extract, (C) and (D) CSV-infected chrysanthemum nucleic acid extract. Chrysanthemum plants in (B) and (D) were grown in plant room B; chrysanthemum plants in (C) were grown in plant room A. All plants were extracted using the "modified" procedure A.

The positions marked are 4S and 5S RNAs and an extra band found only in (C) and (D) (arrow).

Nucleic acid samples (20 µg, 2.0 mg, 2.5 mg, and 1.3 mg, respectively) were heated at 80°C for 5 min, cooled on ice and subjected to electrophoresis on 16 x 16 x 0.4 cm 12% acrylamide, 0.6% methylene bis-acryalmide slab gels (Symons, 1978) in the buffer of Loening (1967) at 40 mA for 18 h. The gel was stained for 10 min in 0.05% (w/v) toluidine blue and destained overnight in water.


A
B
C
D
5S
4S

SDS to dissociate cells and to denature proteins, magnesium to stabilize RNA and diethylpyrocarbonate (DEPC), to inhibit nucleases. The extraction buffer was modified to include polyvinylpyrrolidone to inhibit polyphenol oxidation.

After the initial homogenization and during the subsequent steps, i.e., the incubation of the homogenate at 37°C, salting out and removing proteins by centrifugation, the homogenate blackened. The addition of DIECA, mercaptobenzothiazole and sodium azide, all potent oxidase inhibitors (Dawson and Magee, 1955; Loomis, 1974), to the homogenate just after the initial blending had little-to-no-effect on subsequent blackening, suggesting the oxidation was aerobic rather than enzymic; 2-mercaptoethanol could not be added at any step during the initial extraction because it would inactivate the DEPC.

After removal of salted out proteins by centrifugation, the crude nucleic acid preparation was concentrated by ethanol precipitation, resuspended in dilute EDTA and residual proteins were removed by phenol:chloroform extraction. The nucleic acid in the aqueous phase could not be concentrated for polyacrylamide gel analysis by ethanol precipitation, because of the presence of saturating levels of sodium chloride (which co-precipitated with nucleic acids). Therefore, the aqueous phase was dialyzed prior to collection of the nucleic acids by ethanol precipitation and centrifugation.

The extraction of chrysanthemum nucleic acid by this procedure resulted in (1) very little ribosomal RNA in

the final nucleic acid preparation and (2) a low viscosity of the nucleic acid solution due to the absence of pectins (see Discussion).

Analysis of the nucleic acid extract by polyacrylamide gel electrophoresis revealed the presence of 4S and 5S RNA and a darkly coloured smear throughout the gel; no bands were observed in the region of the gel where the viroid was expected (results not shown).

Certain positive elements obtained from extraction procedures A and B were combined and modified to form extraction procedure C.

3. Procedure C: SDS/Mg/High Salt

The initial extraction medium contained polyvinylpyrrolidone, sodium azide, sodium diethyl-dithiocarbamate, 2-mercaptoethanol and mercaptobenzo-thiazole to inhibit darkening of the initial extract (Dawson and Magee, 1955; Loomis, 1974). Magnesium acetate was added to inhibit the solubilization of pectins (Thornber and Northcote, 1961; Cook and Stoddart, 1973). Sodium dodecyl sulphate was added to inhibit plant nucleases, to dissociate cells and to allow the precipitation of proteins at 0°C in the presence of a high concetration of NaCl (Kay *et al.*, 1952). Much of the higher molecular weight RNA was also removed during this deproteinization step (Crestfield *et al.*, 1955). Nucleic acids in the supernatant were then concentrated by ethanol precipitation prior to further deproteinization with phenol:chloroform. This extraction and partial purification procedure has obviated the need for large volumes of phenol since the

phenol extraction was done at the second step with small volumes and not on the large initial extract (Singh and Sänger, 1976; Diener *et al.*, 1977) as in extraction procedure A.

After phenol:chloroform extraction, the aqueous phase was dialyzed to remove salt and the nucleic acids were concentrated by ethanol precipitation.

A comparison of the spectra obtained from partially purified nucleic acids extracted via procedure C compared with nucleic acids extracted via procedures A or B is shown in Figure II-3. A 260:230 nm ratio of 1.9 - 2.3:1 was obtained using extraction procedure C and there was less light-scattering due to carbohydrate observed at greater than 300 nm; the colour of the final nucleic acid solution was usually light-green to yellow, but was sometimes white.

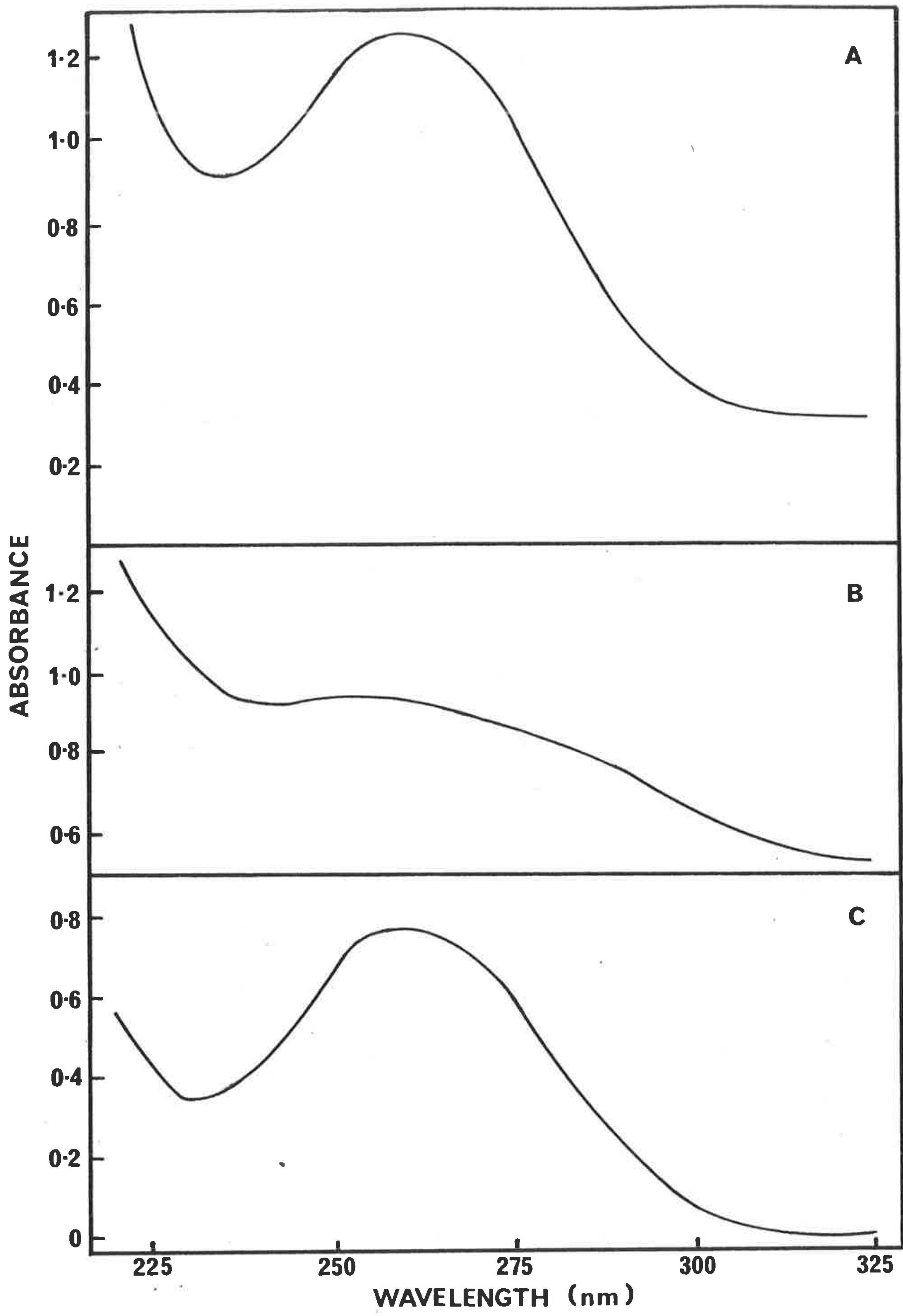
Polyacrylamide Gel Analysis of Nucleic Acid Extracts.

When nucleic acids extracted from healthy and CSV-infected chrysanthemums by procedure C were compared by polyacrylamide tube gel electrophoresis under non-denaturing conditions, the infected plant extract (Figure II-4B) contained an extra band (CSV RNA, as shown below) not found in the healthy plant extract (Figure II-4A). Nucleic acids extracted in the same way from *Gynura aurantiaca* inoculated three weeks earlier with extracts from CSV-infected chrysanthemums also contained an extra band of CSV RNA (Figure II-4C) not found in extracts from healthy plants (Figure II-4D), although it was always at a much lower level than found in extracts from

FIGURE II-3

Continuous UV spectra of nucleic acids extracted from CSV-infected chrysanthemums by (A) extraction procedure A, (B) extraction procedure B, and (C) extraction procedure C.

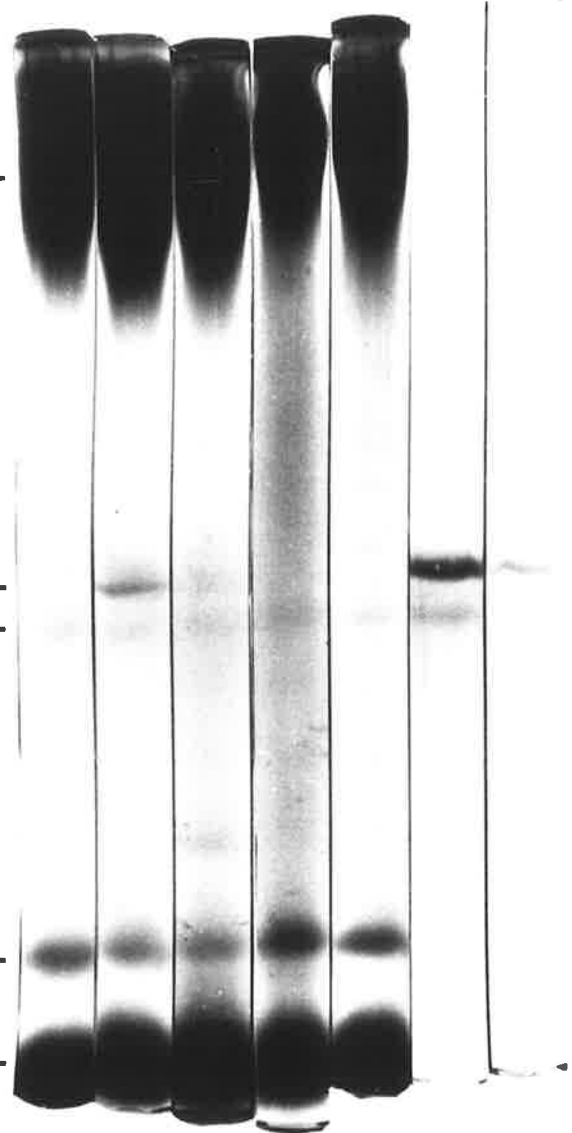
The 260:230 nm ratios were 1.6:1, 1:1 and 2.2:1, respectively.



A
B
C
ABSORBANCE
1·2
1·0
0·8
0·6
0·4
0·2
0
225
250
275
300
325
WAVELENGTH (nm)

FIGURE II-4

Polyacryalmide tube gel electrophoresis under non-denaturing conditions of nucleic acids from various sources. Partially purified nucleic acid extract (160 - 240 μg), prepared as described in Materials and Methods using extraction procedure C, from (A) healthy chrysanthemum; (B) CSV-infected chrysanthemum; (C) CSV-infected *Gynura*; (D) healthy *Gynura*; and (E) CEV-infected *Gynura*. (F) and (G) CSV band after electrophoresis on one and two non-denaturing polyacrylamide slab gels, respectively (see Text). Nucleic acid samples were run on 5% acrylamide, 0.125% bis-acrylamide tube gels (0.6 x 10 cm) in the tris-acetate-EDTA buffer of Loening (1967) at 5 mA/gel for 3.5 h. Gels were stained with 0.05% toluidine blue overnight and destained in water for three days. The positions of host DNA, 4S RNA and 5S RNA are given.


A
B
C
D
E
F
G
DNA
CSV
7S
5S
4S
CEV

infected chrysanthemums. Nucleic acids extracted from CEV-infected *Gynura* also showed the presence of a weak extra band (Figure II-4E), but with a different mobility than observed with CSV RNA. All five samples also contained a host RNA running just faster than CSV, which has been designated 7S RNA (Singh and Sänger, 1976), as well as a minor band between 5S and 7S (Figure II-4). DNA present at the top of the gel, clearly did not interfere with polyacrylamide gel analysis of the samples and thus no attempt was made to remove the DNA.

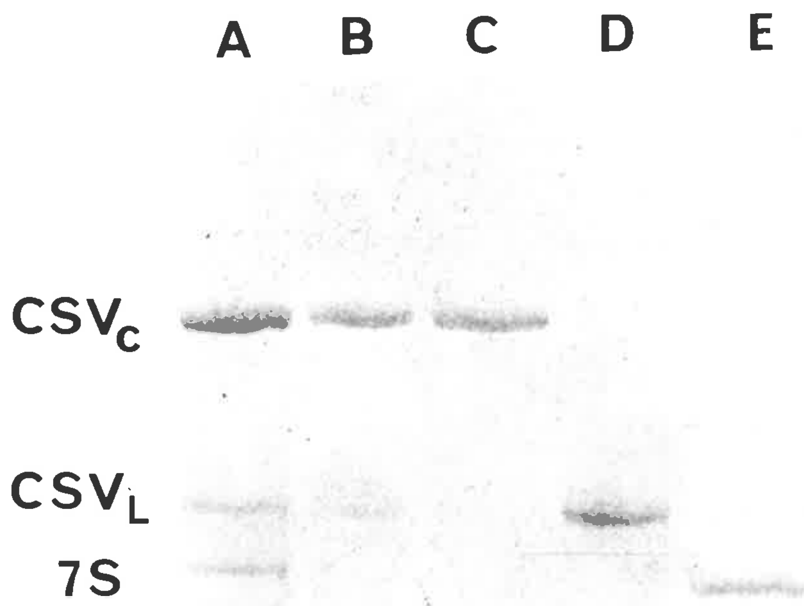
B. Purification Of CSV

Extraction of this extra band from CSV-infected chrysanthemum nucleic acids subjected to polyacrylamide slab gel electrophoresis run under non-denaturing conditions and re-electrophoresis gave the pattern of Figure II-4F, which shows contamination of the main CSV RNA band by host 7S RNA. Elution and re-electrophoresis of the main band gave a single RNA band (Figure II-4G).

Analysis of the RNAs used in Figure II-4F and II-4G on a polyacrylamide slab gel containing 7 M urea gave three and two RNA bands, respectively (Figure II-5A and II-5B). The major, slowest moving band was subsequently shown to be covalently closed circular CSV RNA, the middle band to be linear CSV RNA while the fastest band was host 7S RNA. (The full characterization of these three RNAs is described in Chapter III.) These results formed the basis of the large scale slab gel purification of

FIGURE II-5

Polyacrylamide slab gel electrophoresis under denaturing conditions of various nucleic acid samples. (A) CSV band after electrophoresis on one non-denaturing polyacrylamide slab gel (same sample as in Figure IV-4F) (B) CSV band after electrophoresis on two non-denaturing polyacrylamide slab gels (same sample as in Figure IV-4G); (C), (D), and (E) samples of circular CSV (CSV_C) linear CSV (CSV_L) and chrysanthemum 7S RNA, respectively, obtained after electro-elution of the three RNA bands found after the second polyacrylamide slab gel electrophoresis step described in Materials and Methods [pattern was the same as in (A)]. RNA samples (2 - 24 µg) were run on a 5% polyacrylamide urea slab gel as described in Materials and Methods except that gel thickness was 0.2 cm. Electrophoresis was at 35 mA for 2.3 h. The gel was stained with 0.05% toluidine blue in water for 1.0 h and destained in water.


A
B
C
D
E
CSV$_C$
CSV$_L$
7S

circular and linear CSV RNA described in Materials and Methods in which the partly purified, deproteinized nucleic acid extract of infected chrysanthemums was first fractionated by slab gel electrophoresis under non-denaturing conditions. The single viroid band was isolated and re-run on a denaturing slab gel in the presence of 7 M urea to separate the circular and linear forms of CSV RNA. Analysis of the final purified RNAs on a polyacrylamide slab gel in the presence of 7 M urea showed them to be electrophoretically pure (Figure II-5C and II-5D). Host 7S RNA purified in the same way is shown to Figure II-5E.

In the second gel step, the circular and linear forms of CSV were well separated. The yield of circular CSV was 200 μg/kg and linear CSV 35 μg/kg, based on an A_{260} nm of 20 for 1.0 mg/ml. Both forms of CSV gave a typical nucleic acid spectrum from 220 to 320 nm with an A_{260} nm/ A_{230} nm ratio of 2.5:1.

C. Bio-assay Of CSV

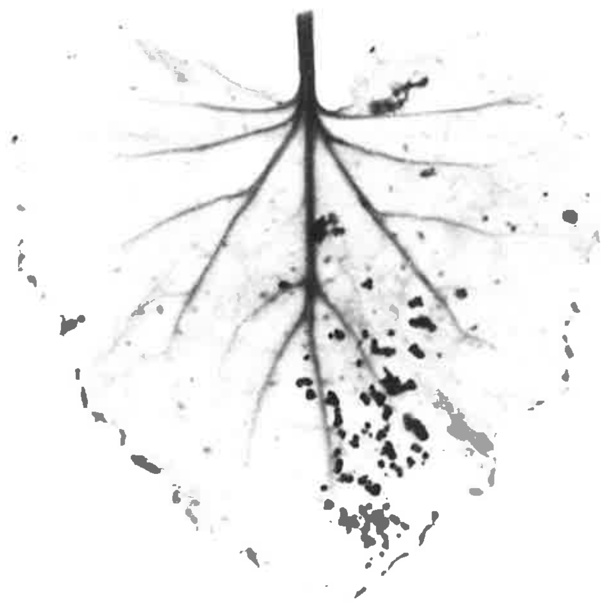
1. Cinerarias

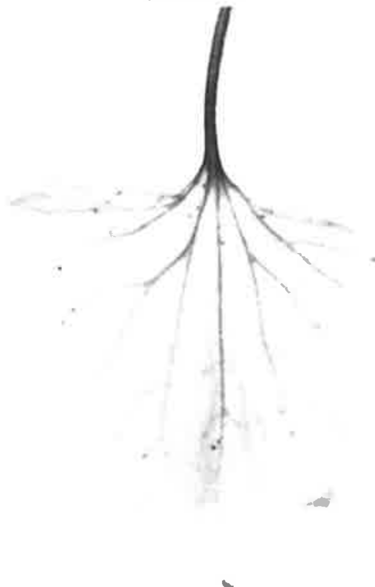
Attempts to demonstrate CSV-induced chlorosis in cinerarias failed. Therefore, the starch-lesion assay of Lawson (1968) was used to indicate the presence of CSV. Two different local varieties of cinerarias and a number of preparations of partially purified nucleic acid extracts (prepared via procedure A) resulted in the observation of starch-lesions with only sporatic frequency (Figure II-6).

FIGURE II-6

Cineraria leaves stained with iodine to detect starch lesions induced by infection by CSV as described in Materials and Methods.

One half-leaf (right-half) was inoculated with a nucleic acid extract from CSV-infected chrysanthemum (A) or healthy chrysanthemum (B); the other half-leaf (left-half) was inoculated with water. Plants were maintained as described in Materials and Methods.


CSV
A


HEALTHY
B

2. *Gynura*

Gynura inoculated with partially purified nucleic acid extracts of CSV-infected chrysanthemums (prepared via procedure A) developed symptoms in the glass-house only during the summer months; in plant room B with a frequency of 50 - 75%; in plant room C with a frequency of 66 - 100%; but not at all in plant room A. Therefore, the optimal conditions for symptom development seemed to involve higher light intensities (present in all but room A), and not just higher temperatures (present only in room B and the glasshouse) (Sänger and Ramm, 1975).

Symptoms consisted of stunting and severe epinasty, and sometimes rugosity (Figure II-7), but were less severe than those induced by CEV.

Inoculating plants by needle puncture resulted in a greater number of plants showing symptoms than inoculating by razor slashing (results not shown). Therefore, the needle-puncture method of inoculation was used throughout all further work.

Treatment of partially purified nucleic acid extracts from CSV-infected chrysanthemums (via procedure A) with RNAse destroyed all infectivity, whereas treatment with DNAse had no effect on infectivity (Table II-2). This strongly suggests that the infectious agent is an RNA.

Nucleic acid extracts prepared by procedure C also contained infectious material. Purification of CSV and 5S and 7S RNAs from CSV-infected chrysanthemums by two cycles of non-denaturing polyacrylamide slab gel electro-phoresis (with CSV as a mixture of linear and circular

FIGURE II-7

Top: *Gynura* plants inoculated with nucleic acids extracted from CSV-infected chrysanthemums or CEV-infected *Gynura*, or with water (healthy). Plants were maintained in plant room C for 6 - 8 weeks.

Bottom: CSV-infected *Gynura* showing epinasty and rugosity.

CSV
HEALTHY
CEV

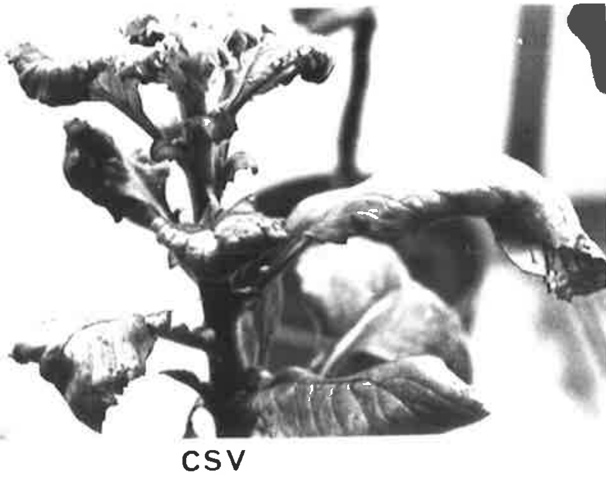

CSV

TABLE II-2

EFFECT OF NUCLEASES ON INFECTIVITY IN *GYNURA AURANTIACA* OF PARTIALLY PURIFIED NUCLEIC ACID EXTRACTS OF CSV-INFECTED CHRYSANTHEMUMS.

Treatment	Number of Plants Showing Symptoms over number of plants inoculated
None	2/3
RNAse[a]	0/3
DNAse[b]	3/3
	2/2[c]

a Nucleic acid, partially purified via extraction procedure A was incubated with 50 μg/ml of pancreatic RNAse A and 300 units/ml of RNAse T_1 for 2 h at 25°C. Samples were deproteinized by phenol extraction and concentrated by ethanol precipitation. Plants were inoculated and maintained as described in Materials and Methods. All plants were scored 8 weeks post inoculation.

b As for "a", except that the sample was treated with 100 μg/ml of pancreatic DNAse I in the presence of 10 mM Mg^{2+} instead of RNAses A and T_1.

c Sample "b" diluted 8-fold prior to inoculation onto plants.

molecules; Figure II-4G and Figure II-5B) and bio-assay on *Gynura*, showed the infectivity to be associated only with the CSV RNA (Table II-3). From a number of preparations of CSV (linear plus circular) the dilution end-point of CSV was between 200 pg/ml and 2.0 ng/ml (Table II-4); the presence of carrier RNA appeared to increase the specific infectivity.

DISCUSSION

A. Extraction Of CSV

The usual extraction procedures for viroids (Diener *et al.*, 1977; Semancik and Weathers, 1972b) involve the homogenization of plant material followed by numerous steps to selectively remove different components (e.g., lipids, carbohydrates, high molecular weight RNA, DNA and coloured impurities). The viroid is then separated from low molecular weight RNA by polyacrylamide gel electrophoresis.

Using this approach, Diener and Lawson (1973) were able to extract and detect CSV, but only by infectivity across a gel and not photometrically. In attempting to shorten the extraction procedure to avoid possible losses of CSV, two problems arose: (1) chrysanthemums have highly active polyphenol oxidases, which makes difficult any subsequent detection of CSV by polyacrylamide gel electrophoresis and staining; (2) chrysanthemums have high levels of pectins which form gels when nucleic acid (plus pectin) solutions are concentrated prior to polyacrylamide gel analysis. The usual solution to these

TABLE II-3

INFECTIVITY COMPARISON OF CSV AND CHRYSANTHEMUM 5S AND 7S RNAS ON *GYNURA AURANTIACA*

Expt. No.	RNA tested[a]	Number of plants showing symptoms over number of plants inoculated at an RNA concentration (μg/ml) of[b]				
		2	1	0.1	0.02	0.01
1.	5S	0/2	-	-	-	-
	7S	0/3	-	-	0/3	-
	CSV	3/3	-	-	1/3	-
2.	7S	-	0/3	0/3	-	0/3
	CSV	-	2/3	2/3	-	0/3

a RNAs were extracted via extraction procedure C and subjected to two cycles of non-denaturing polyacrylamide gel electrophoresis and elution as described in Materials and Methods.

b Infectivity scored at 4 weeks post infection. Separate preparations of RNA were used in each experiment.

TABLE II-4

INFECTIVITY ANALYSIS OF CSV: DETERMINATION OF DILUTION END-POINT ON *GYNURA AURANTIACA*

Expt. No.	RNA tested[a]	Number of plants showing symptoms over number of plants inoculated at RNA concentration (μg/ml) of[b]					
		10	2	0.2	0.02	0.002	0.0002
1.	*E. coli* tRNA	0/3	-	-	-	-	-
	CSV	-	2/3	-	1/3	-	-
	CSV[c]	3/3	2/3	-	2/3	-	-
2.	CSV[d]	-	-	-	3/3	1/3	1/3
3.	CSV	-	-	5/5	3/5	0/5	0/5

a CSV RNA was extracted via extraction procedure C and subjected to two cycles of non-denaturing polyacrylamide gel electrophoresis and elution as described in Materials and Methods.

b Infectivity scored at 4, 7 and 4 weeks post-inoculation in experiments 1, 2 and 3, respectively.

c CSV at the concentrations stated in the presence of 10 μg/ml carrier *E. coli* tRNA.

d CSV at the concentrations stated in the presence of 100 μg/ml carrier *E. coli* tRNA.

problems is the introduction of extra steps in the extraction procedure, which probably also results in severe losses of viroid and increases the degree of host nucleic acid breakdown, resulting in contamination of viroid RNA on polyacrylamide gels by host nucleic acid.

Owens *et al.* (1978) have recently extracted low molecular weight nucleic acids from CSV-infected chrysanthemums using the lengthy procedure of Diener *et al.* (1977) on a larger amount of plant material than was used by Diener and Lawson (1973); Owens *et al.* (1978) were able to detect a band on a polyacrylamide gel that they designated CSV.

The extraction proceudre of Solymosy *et al.* (1970), which utilizes DEPC to inhibit nucleases, resulted in the presence of very little ribosomal RNA in the final extract, thus obviating the need for LiCl fractionation of nucleic acids. The presence of magnesium in the initial extract, rather than EDTA, resulted in pectins remaining in their normally insoluble state in the plant debris (Cook and Stoddart, 1973) and allowed the nucleic acid sample to be concentrated without gel formation. Unfortunately, as DEPC and 2-mercaptoethanol are highly reactive towards each other, they could not be present simultaneously, and the extracts subsequently blackened as the result of aerobic oxidation of polyphenols. This rendered polyacrylamide gel analysis for CSV in nucleic acid extracts impossible.

An extraction procedure was designed around the

benefits accrued from the Solymosy *et al.* (1970) procedure; i.e., removal of ribosomal RNA during the early steps as a result of the salting out of proteins and not solubilizing pectins, thus avoiding the problem of their subsequent removal. DEPC was replaced by the addition of a number of polyphenol oxidase inhibitors and 2-mercaptoethanol to reduce aerobic oxidation of polyphenols. Under these conditions, partially purified nucleic acid extracts containing detectable levels of CSV could reproducibly be obtained.

Yields of chrysanthemum nucleic acid from the three extraction procedures were 175 mg, 20 mg and 250 mg/kg of plants, using procedures A, B and C, respectively. The yield of *Gynura* nucleic acid using procedure C was 190 mg/kg. Clearly, procedure C is superior in terms of the quality of the nucleic acids, the virtual absence of polyphenol oxidation and the absence of pectins.

B. Purification Of CSV

The method described here for the purification of the circular and linear forms of CSV has proven reliable for the handling of 500 to 1,000 g quantities of plant material. In spite of the marked symptom development on CSV-infected *Gynura aurantiaca*, the level of viroid was appreciably lower than that found in infected chrysanthemums (see also Chapter V) which have therefore been used for routine purification of viroid. The final purification step involving the polyacrylamide slab gel electrophoresis of RNAs in the presence of 7 M urea gave a good separation of the circular and linear forms of CSV. At this stage,

the linear CSV represented about 15% of the total CSV; the circular and linear forms (characterized in Chapter III) comigrated during the previous slab gel electrophoresis step run under non-denaturing conditions.

Our yields of purified circular CSV of 200 μg/kg of infected chrysanthemum are similar to the yields reported for purified PSTV, CEV and CPFV of 80 to 220 μg/kg of tomatoes (Mühlhach and Sänger, 1977). Diener (1972) obtained a yield of purified PSTV from tomatoes of 15 - 20 μg/kg while Semancik *et al.* (1975) obtained purified CEV from *Gynura* in a yield of 20 - 40 μg/kg. Estimates of viroid concentration in partially purified extracts of nucleic acids by polyacrylamide tube gel electrophoresis followed by scanning of the stained or unstained gels (e.g., Morris and Smith, 1977) cannot be considered as yields since they do not represent the final purified viroid.

C. Biological Properties Of CSV

Infectivity experiments performed with partially purified nucleic acid extracts obtained via procedure A were not reproducibly capable of inducing starch-lesions in cineraria. As this response is highly dependent on the variety of cineraria used (Lawson, 1968) and we did not have access to the varieties most susceptible to CSV, this result was not surprising.

Gynura aurantiaca was found to be a susceptible host for CSV, contrary to a previous report by Hollings and Stone (1973). The symptoms (epinasty and stunting) were very much dependent upon growth conditions and this may

explain the difference between the results presented here and those of Hollings and Stone (1973).

Infectivity experiments performed on *Gynura* showed that (1) the infectious agent in nucleic acid extracts of CSV-infected chrysanthemums was RNA. This is supported by the data of Diener and Lawson (1973) who also found the chrysanthemum stunt disease to be caused by an RNA; and (2) the dilution end point of a mixture of linear and circular CSV was 0.2 - 2 ng/ml. This will be compared with infectivity data on other viroids and isolated linear and circular CSV in Chapter III.

Whether or not the specific activity of CSV was actually increased by the presence of other nucleic acids, cannot be ascertained from the data in Table II-4, since plants were scored positive for symptoms at different times in different experiments. However, either non-specific aggregation of viroid to other RNAs or protection of viroids against host RNAses by the presence of an excess of RNA could explain the high infectivity titres of nucleic acid extracts of PSTV-infected tomato (Raymer and Diener, 1969) and CEV-infected *Gynura* (Semancik and Weathers, 1972a), compared with purified PSTV (Diener *et al.*, 1974; Mühlbach *et al.*, 1977).

CHAPTER III

CHARACTERIZATION OF CHRYSANTHEMUM STUNT VIROID

INTRODUCTION

The development of an extraction and purification protocal for CSV (see Chapter II) has allowed the routine purification of 100 - 200 μg of viroid/kg of infected tissue; enough RNA to physically, biochemically and biologically characterize CSV.

This Chapter describes the identification and characterization of two forms of CSV (one circular and one linear) as well as the 7S RNA of chrysanthemum, using the combined techniques of electron microscopy, thermal denaturation, nucleic acid hybridization, 5'-end-labelling and analysis, *in vitro* translation and bio-assay. The infectivity of circular CSV, linear CSV and linear CSV generated by magnesium-ion catalyzed cleavage of circular CSV is compared.

The spreading of RNAs for electron microscopy was carried out with the generous help of Dr. T. Hatta, Dept. of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide.

MATERIALS

Wheat germ S_{30} was prepared as described by Roberts and Paterson (1973) and generously donated by Dr. J.F.B. Mercer and Dr. R. O'Conner of this Department.

[^{3}H]-leucine, 105 Ci/mmole, 1 mCi/ml, was obtained from Amersham. Purified cucumber mosaic virus RNAs were prepared as described by Symons (1978) and generously donated by Dr. R.H. Symons of this Department.

γ-^{32}P-ATP was prepared by the method of Maxam and

Gilbert (1977) and donated by Mr. R. Richards of this Department.

Calf intestinal phosphatase (Sigma) was further purified as described by Efstradiatis *et al.* (1977) and donated by Dr. R.H. Symons.

High voltage paper electrophoresis was carried out on an apparatus described by Symons (1975) and obtained from Paton Industries, Adelaide.

Penicillium citrinum P_1 nuclease, creatine phosphate and creatine phosphokinase were all obtained from Sigma and spermidine-HCl from Calbiochem. All other materials were from sources described in Chapters II, IV and VI.

METHODS

A. Electron Microscopy Of RNA

RNAs were spread for electron microscopy by the method of Randles and Hatta (1979). RNA (0.6 μg/ml) in 98% (v/v) formamide, 10 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.003% (w/v) cytochrome *c*, at 60°C was spread onto a hypophase containing 60% (v/v) formamide, 1.5 mM Tris-HCl, pH 8.5, 0.15 mM EDTA, at 45°C. Samples were picked up on parlodion covered grids, shadowed at 5 - 6° with platinum-palladium and covered with evaporated carbon. Grids were examined in a Siemens Model 102 electron microscope at an operation magnification of 10,000 x or a Jeol electron microscope (100 cx) at an operational magnification of 20,000 x.

B. Thermal Denaturation Of RNA

RNAs were subjected to thermal denaturation in 0.1 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0). The temperature was increased at the rate of 1.0°C/min using the apparatus described by Randles *et al.* (1976).

C. Nucleic Acid Hybridization

[^{32}P]-cDNA to the circular form of CSV was prepared as described in Chapter IV. Hybridization of this cDNA to RNAs and the assay of cDNA:RNA hybrids were as described in Chapter IV. Hybridizations were carried out in hybridization Buffer B of Chapter V (10 mM Tris-HCl, pH 7.0, 180 mM NaCl, 2 mM EDTA, 40% (v/v) deionized formamide) at 50°C.

D. Infectivity Of RNA

Infectivity analyses of purified RNAs were carried out as described in Chapter II. Plants were maintained in plant room C (Chapter II).

E. 5'-End Labelling And Determination Of The 5'-Terminal Residue

Partial digestion of circular CSV by nuclease S_1 was as described in Chapter IV. RNA samples (2 μg) from which the 5'-phosphoryl groups had or had not been removed by treatment with purified calf intestinal phosphatase (Efstradiatis *et al.*, 1977) were labelled at their 5'-termini using γ-^{32}P-ATP and polynucleotide kinase as described by Efstradiatis *et al.* (1977). The two samples of labelled linear CSV and two samples of

labelled 7S RNA were then electrophoresed on a thin 6% polyacrylamide, 7 M urea gel (Sanger and Coulson, 1978) at 20 mA for 2 hours. The single RNA bands (linear CSV or 7S) were detected by staining with 0.05% toluidine blue for 10 min and destaining in water; the gels were then autoradiographed. The radioactive bands, which corresponded exactly to the stained bands, were cut out and counted by Cerenkov radiation. RNA in each band was hydrolysed to 5'-mononucleotides by incubation with 150 μg/ml of nuclease P_1 in 0.03 M sodium acetate, pH 5, at 37°C overnight. After centrifugation to remove acrylamide, the mononucleotides in the supernatant were fractionated by high voltage paper electrophoresis at pH 4.1 (Symons, 1975) and the electrophoretogram autoradiographed. The labelled bands, which corresponded exactly to the marker 5'-mononucleotides, were cut out and counted to give the relative amounts of the 5'-terminal residues. Nuclease S_1-digested circular CSV after 5'-labelling was separated fron unused γ-^{32}P-ATP by passage over Sephadex G50 (Gould and Symons, 1977). Samples were digested with 150 μg/ml of nuclease P_1 at pH 5 for 2 hours at 37°C and the relative amounts of the four 5'-residues determined as described above.

F. Magnesium-Ion Catalyzed Cleavage Of Circular CSV And The Purification Of Magnesium Cleavage Generated Linear CSV.

The magnesium-ion catalyzed cleavage of circular CSV to a linear form of CSV was carried out as described by Sänger *et al.* (1979). Twenty micrograms of circular CSV was incubated for 2.5 hours at 37°C in 5 mM magnesium

acetate, 50 mM glycine-NaOH, pH 9.0. The reaction was stopped by the addition of EDTA, to a final concentration of 5.6 mM, and freezing.

The magnesium cleavage generated linear CSV was separated from the uncleaved circular CSV by electrophoresis on a 15 x 15 x 0.2 cm 5% acrylamide, 0.17% methylene bisacrylamide slab gel in 7 M urea, 90 mM Tris-borate, 3 mM EDTA, pH 8.3 (Air *et al.*, 1976). The 20 μg of RNA treated with magnesium acetate at pH 9.0, was loaded onto a 4 x 0.2 cm slot and 10 μg of linear CSV, isolated as described in Chapter II, was run in parallel on a similar sized slot; electrophoresis was at 55 mA for 3 hours. RNA bands were located by staining and the RNA electrophoretically eluted as described in Chapter II.

The mobility of the "naturally occuring" linear CSV and the "magnesium-ion generated" linear CSV coincided.

Yields of recovered RNAs were 50% for both linear CSVs and 75% for circular CSV.

G. *In Vitro* Translation Of RNAs.

The translation of purified RNAs was carried out in the wheat germ cell free translation system described by Roberts and Paterson (1973). Each reaction mixture in a volume of 50 μl, contained: 26 mM HEPES-KOH, pH 7.5, 2 mM DTT, 1 mM ATP, 0.2 mM GTP, 8 mM creatine phosphate, 20 μM of each amino acid except leucine, 10 μCi of 105 Ci/mmole [^{3}H]-leucine, 20 μg/ml creatine phosphokinase, 24 mM KCl, 66 mM potassium acetate, 3 mM magnesium acetate, 0.12 mM spermidine-HCl, 1 - 2 μg of RNA and 10 μl

of wheat germ S_{30}. The mixture was incubated for 90 min at 26°C; 5 μl samples were spotted onto Whatman GF/A filters, washed in 200 ml of cold 5% (w/v) TCA/1% (w/v) casamino acids at 4°C for 20 min, washed twice more in 5% TCA/1% casamino acids for 15min/wash and washed for 10 min in cold (0°C) ether. The filters were dried at 105°C for 30 min; 3 ml of toluene scintillation fluid (3.5 g PPO, 0.35 g POPOP per litre of toluene) was added to each filter in a vial and the filters were counted in a Packard Tricarb Scintillation Spectrometer at a counting efficiency of 25%.

RESULTS

A. Electron Microscopic Analysis Of Purified RNAs

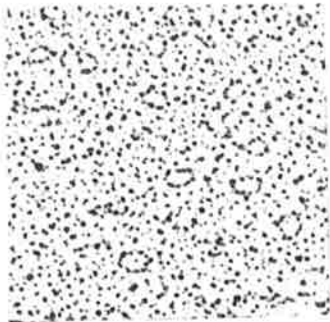
When the CSV RNA of Figure II-5C was spread and visualized by electron microscopy, mostly denatured circular molecules were seen together with some linear molecules (Figure III-1A). Some of the latter (not shown) were much thicker than the circular molecules and were taken to be undenatured circular molecules. The longer, thinner molecules were presumably denatured linear molecules formed by the nicking of circular molecules during the 15 - 20 min incubation in 90% formamide at 60°C needed for the spreading.

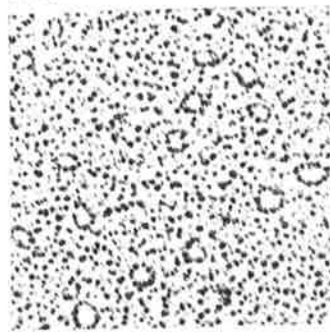
The faster moving CSV RNA of Figure II-5D and the host 7S RNA of Figure II-5E consisted almost exclusively of linear molecules (Figure III-1B and III-1C) although a few circular-like molecules were seen on scanning the

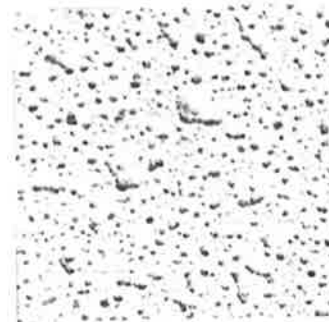
FIGURE III-1

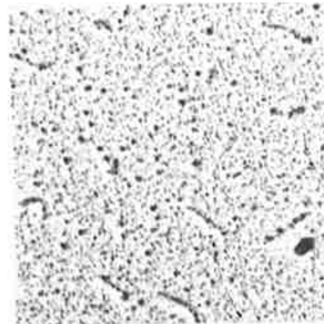
Electron micrographs of (A), circular CSV; (B), linear CSV; and (C), chrysanthemum 7S RNA. Samples were prepared for electron microscopy as described by Randles and Hatta (1979). RNA (0.6 μg/ml) in 98% (v/v) formamide, 10 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.003% cytochrome *c*, at 60°C was spread onto a hypophase at 45°C of 60% (v/v) formamide, 1.5 mM Tris-HCl, pH 8.5, 0.15 mM EDTA. Samples were picked up on parlodion covered grids, shadowed at 5 - 6° with platinum palladium and covered with evaporated carbon. The bar represents 150 nm.

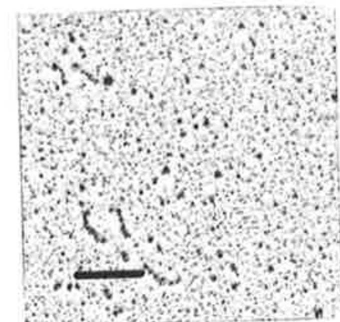
A B C











grids (not shown).

Contour length measurements of circular CSV, linear CSV and chrysanthemum 7S RNA were made. The mean length and standard deviation of the mean for circular CSV was 136 ± 19 nm (Figure III-2). The linear CSV and 7S RNA molecules showed a much broader distribution of molecular lengths (Figure III-2) suggesting the presence of RNA aggregates. Since both linear CSV and 7S RNA were taken as defined size classes from a denaturing (7 M urea) gel, the above aggregation suggests that the electron microscopy spreading conditions may not have been completely denaturing.

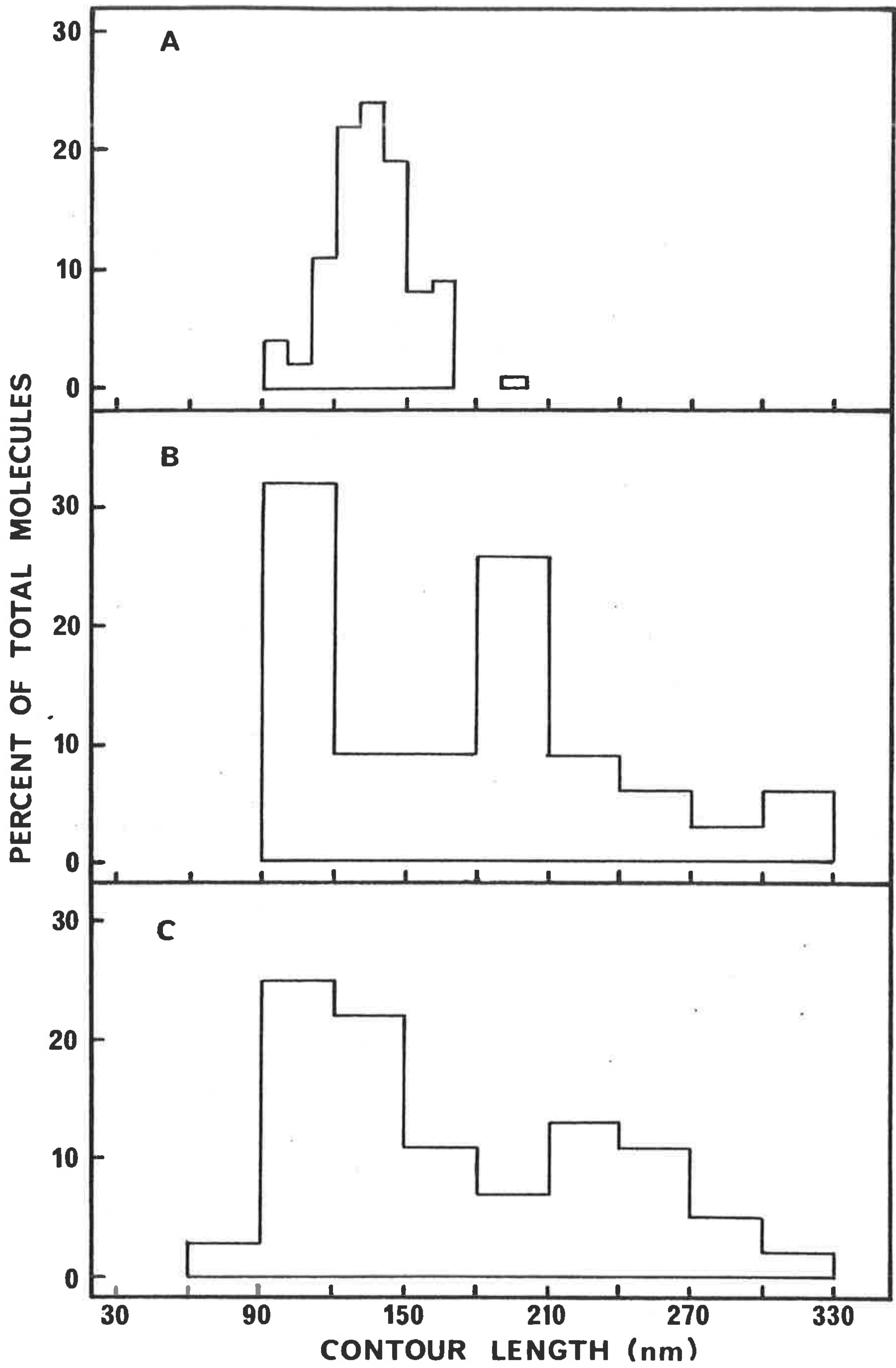
B. Thermal Denaturation Of Purified RNAs

Circular CSV RNA showed a sharp temperature transition with a T_m of 47°C and a hyperchromicity of 21% under the conditions used (Figure III-3A). The shape of the melting profile was similar to that usually observed with viroid RNA (Diener and Hadidi, 1977; Randles *et al.*, 1976; Semancik *et al.*, 1975). The 7S RNA (Figure III-3C) showed a broad temperature transition with a T_m of 42°C and a hyperchromicity of 15%, consistent with normal melting profile parameters observed with single-stranded RNAs (Semancik *et al.*, 1975; Mossop and Francki, 1979). Linear CSV RNA displayed a mixed profile with elements of both broad and sharp temperature transitions being present (Figure III-3B); the T_m was 46°C and the hyperchromicity was 18%.

FIGURE III-2

Length measurements of (A) circular CSV, (B) linear CSV and (C) chrysanthemum 7S RNA molecules. The number of molecules measured was: 85 circular CSV, 34 linear CSV and 87 7S RNA molecules. The data were experssed as percentage of total molecules vs. contour length.

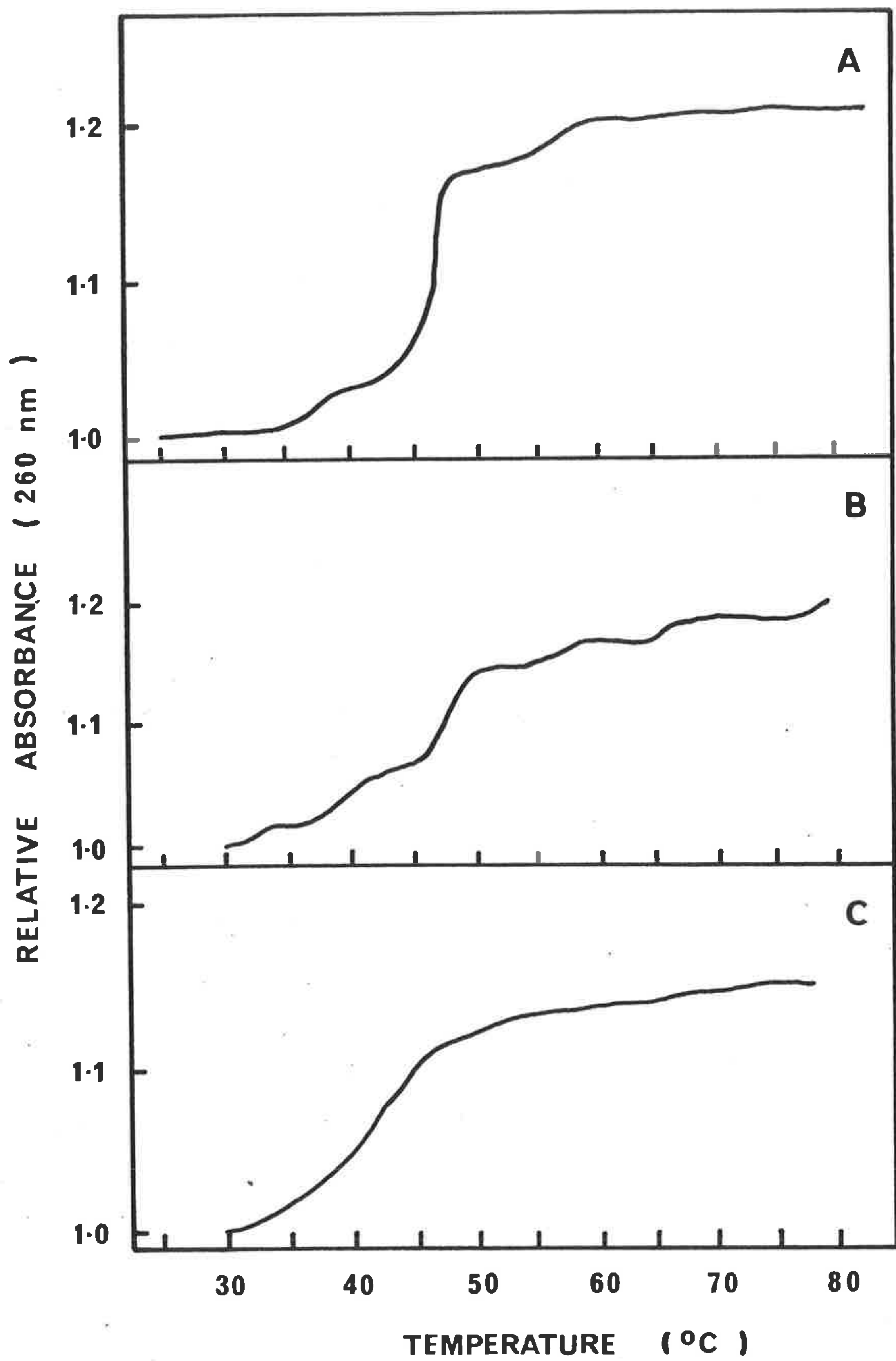
RNA molecules were spread under denaturing conditions as described in Materials and Methods. Electron micrographs at an instrument (JEM 100 cx) magnification of 20,000 were calibrated with a carbon grating replica (2160 lines/mm; Ladd Research Industries Inc.). Negatives were enlarged 10 times and molecular lengths were measured with a Numonics electronic graphics calculator at a resolution of 0.25 mm.



A
B
C
PERCENT OF TOTAL MOLECULES
30
20
10
0
30
90
150
210
270
330
CONTOUR LENGTH (nm)

FIGURE III-3

Thermal denaturation profiles of (A), circular CSV; (B), linear CSV; and (C), chrysanthemum 7S RNA in 0.1 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M tri-sodium citrate, pH 7.0). The temperature was increased at the rate of 1.0°C/min as described by Randles *et al.* (1976).



A
B
C
1·2
1·1
1·0
RELATIVE ABSORBANCE (260 nm)
30
40
50
60
70
80
TEMPERATURE (°C)

C. Hybridization Analysis Of Purified RNAs

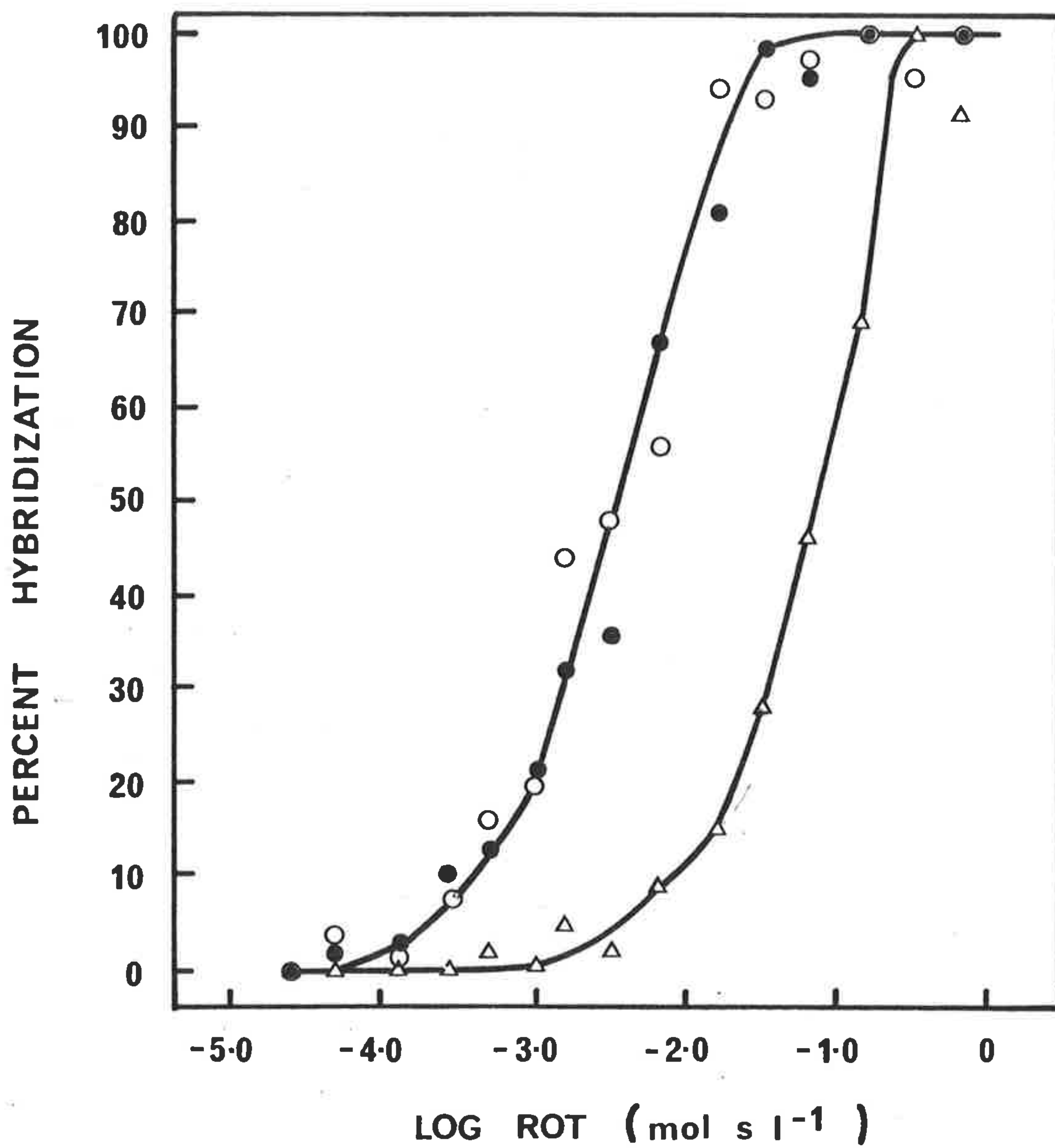
The sequence homology between the CSV circular and linear RNAs was shown by preparing a [^{32}P]-cDNA probe (as described in Chapter IV) to CSV circular RNA and hybridizing it to both the circular and linear forms. The two RNAs hybridized at identical rates with the same $Rot_{\frac{1}{2}}$ of 3.2×10^{-3} mol sec litre^{-1} (Figure III-4). The much slower rate of hybridization of the [^{32}P]-cDNA to 7S RNA showed that there was no sequence homology between the viroid and 7S RNAs while the $Rot_{\frac{1}{2}}$ of 7.5×10^{-2} mol sec litre^{-1} indicated that the 7S RNA was contaminated with viroid sequences to the extent of 4% ($3.2 \times 10^{-3}/7.5 \times 10^{-2} \times 100\%$) (see Gould and Symons, 1977, 1978; Gould *et al.*, 1978).

D. 5'-Terminal Labelling And Determination Of The 5'-Terminal Residue Of Purified RNAs

In view of the recent report on the conversion of purified circular PSTV to the linear form by treatment for at least one hour with 5 mM Mg^{2+} at 37°C and pH 9 (Sänger *et al.*, 1979), it was feasible that some of the linear CSV found in the present work was due to Mg^{2+} catalysed hydrolysis of circular CSV since Mg^{2+} was present during the initial extraction of plant material. Such cleaved RNA would possess a 5'-hydroxyl and a 2'(3')-phosphate (Huff *et al.*, 1964), as would any RNA cleaved by plant ribonucleases with a specificity similar to pancreatic, T_1 or T_2 ribonucleases. Thus, any linear CSV molecules with a 5'-phosphate that were derived from circular molecules must have arisen by a mechanism other

FIGURE III-4

Kinetics of hybridization of [^{32}P]-cDNA prepared on circular CSV with circular (○), linear CSV (●), and chrysanthemum 7S RNA (△). Hybridization and S_1 nuclease assays were carried out as described in Materials and Methods of Chapter IV. The Rot curves were normalized to a maximum of 100% to facilitate comparison. The actual plateau values were 49%, 64%, and 52% for circular CSV, linear CSV, and 7S RNA, respectively.


100
90
80
70
60
50
40
30
20
10
0
PERCENT HYBRIDIZATION
-5·0
-4·0
-3·0
-2·0
-1·0
0
LOG ROT (mol s l⁻¹)

than metal ion-catalyzed hydrolysis.

The 5'-terminal residues were labelled by phosphorylation using γ-^{32}P-ATP and polynucleotide kinase, either with or without prior phosphatase treatment to remove any 5'-phosphate. In the case of linear CSV, there was 2.7 times (1.0/0.37) as much labelling of the 5'-residues with prior phosphatase treatment as without (Table III-1). This indicates that linear CSV contained 37% of its 5'-ends as 5'-hydroxyls and 63% as 5'-phosphates; the latter figure is a minimum estimate due to the exchange reaction between γ-^{32}P-ATP and a 5'-phosphate catalysed by the bacteriophage T4 polynucleotide kinase (van de Sande *et al.*, 1973). A measure of the extent of this exchange under the conditions used was obtained by the phosphorylation of circular CSV after partial digestion with nuclease S_1 which cleaves single-strand nucleic acids to give a 5'-phosphate and a 3'-hydroxyl (Ando, 1966). This partially digested CSV contained a mixture of RNA fragments varying in size from full length linear molecules to fragments about 100 residues long (Figure IV-6) and 6.7 times (10./0.15) as much phosphorylation occurred after removal of the 5'-phosphate (Table III-1). This result indicates that 15% of the 5'-labelling occurred by exchange but the conclusion is complicated by the possibility that some 5'-hydroxyls may have been produced during the S_1 nuclease treatment which requires 1 mM Zn^{2+} at pH 4.6 for activity. In spite of these difficulties, the results indicate that roughly 72% (63% plus 9% correction (0.15 x 0.63) for exchange) of linear molecules had a 5'-phosphate and therefore could not

TABLE III-1

LABELLING OF 5'-TERMINI OF LINEAR CSV, NUCLEASE S_1-TREATED CSV AND CHRYSANTHEMUM 7S RNA WITH γ-^{32}P-ATP AND POLYNUCLEOTIDE KINASE AND THE EFFECT OF PRIOR PHOSPHATASE TREATMENT[a]

RNA	Phosphatase Treatment	Relative ^{32}P Incorporation	5'-Terminal Residue (moles %)			
			A	C	G	U
Linear CSV	-	0.37	35	16	18	31
	+	1.0	34	17	17	32
Nuclease S_1 treated circular CSV	-	0.15	36	19	17	28
	+	1.0	42	19	16	23
7S	-	0.67	56	8	16	20
	+	1.0	58	7	17	18

a Experimental details are given in Materials and Methods

have arisen from circular molecules by metal ion-catalysed hydrolysis.

Chrysanthemum 7S RNA is a complex mixture of RNAs (see Figure V-6). Table III-1 shows that at least 43% (37% plus 6% correction (0.15 x 0.37) for exchange) of 7S RNA molecules have a phosphate at their 5'-termini and could not have arisen as the result of metal-ion catalyzed hydrolysis of higher molecular weight RNAs.

The nucleotides present at the 5'-termini of the four types of RNA were determined by complete digestion of the 5'-^{32}P-RNA with nuclease P_1 to the 5'-mononucleotides which were then separated by high voltage paper electrophoresis and counted. All four nucleotides were found at the 5'-termini while the remarkably consistent base composition (Table III-1) indicates that there is little specificity at the site of nicking of the linear molecules. Although the base composition of CSV is not known, the high proportion of A + U residues relative to G + C residues for the two types of CSV suggests that linear molecules were produced from circulars by nicking in a poorly base paired region.

The nucleotides present at the 5'-termini of chrysanthemum 7S RNA consisted mostly (56 - 58%) of adenosine residues (Table III-1), irrespective of the presence or absence of a phosphate at the 5'-termini.

E. Infectivity Analysis Of Purified RNAs

Two separate preparations of circular and linear CSV RNA were tested for infectivity on *Gynura aurantiaca*. The results of Table III-2 show clearly that both circular

TABLE III-2

INFECTIVITY ANALYSIS OF CIRCULAR AND LINEAR CSV RNAs ON *GYNURA AURANTIACA*

Expt. No.	RNA Tested	Number of plants showing symptoms over number of plants inoculated at an RNA concentration (μg/ml) of[a]						
		5	1	0.5	0.1	0.05	0.01	0.005
1.	CSV circular	4/4	-	4/4	-	3/4	-	1/6
	CSV linear	6/6	-	6/6	-	5/6	-	-
	Chrysanthemum 7S	6/6	-	0/6	-	-	-	-
2.	CSV circular	-	4/5	-	3/5	-	1/6	-
	CSV linear	-	5/5	-	3/6	-	1/6	-

[a] Infectivity scored at 8 weeks post infection. A separate preparation of CSV RNAs was used in each experiment.

and linear forms were equally infectious while the purity of the RNAs used (Figure II-5) rules out the possibility that the infectivity of the linear CSV RNA was due to contaminating circular RNA. The chrysanthemum 7S RNA was infectious at 5 μg/ml but not 0.5 μg/ml; since this RNA contained 4% CSV RNA sequences (Figure III-4), equivalent to 0.2 μg/ml CSV RNA, this infectivity is not surprising.

Linear CSV molecules were also generated by treatment with magnesium ions at high pH (Sänger *et al.*, 1979). These were separated from intact circular CSV molecules by denaturing polyacrylamide slab gel electrophoresis. These linear molecules were called "magnesium-ion cleavage generated" linear CSV, to differentiate them from the "natural" linear CSV molecules described earlier. Infectivity tests on both types of linear RNAs showed them to be equally infectious (Table III-3). Both linears were as infectious as the circular CSV molecules remaining intact after incubation at high pH with magnesium ions. Extraction of symptom-bearing *Gynura* plants and analysis of the nucleic acid on a denaturing (7 M urea) gel revealed the presence of linear and circular CSV in *Gynura* inoculated with either circular CSV, linear CSV or magnesium-ion cleavage generated linear CSV; regardless of the type of CSV used in the inoculum, at least 90% of the CSV in the inoculated *Gynura* was circular (results not shown).

Contamination of the linear molecules by circular molecules appears unlikely in view of the following.

(1) Extensive destaining of the gel track containing

TABLE III-3

INFECTIVITY ANALYSIS OF CIRCULAR, LINEAR AND MAGNESIUM-ION CLEAVAGE GENERATED LINEAR CSV RNAs ON *GYNURA AURANTIACA*

Expt. No.	RNA Tested[a]	Number of plants showing symptoms over number of plants inoculated at an RNA concentration (μg/ml) of[b]				
		5	0.5	0.05	0.005	0.0005
1.	CSV linear	5/5	2/5	0/6	1/6	1/6
	CSV-Mg-linear	4/5	4/5	4/6	2/6	0/6
2.	CSV linear	-	4/5	1/5	2/5	-
	CSV-Mg-linear	-	4/5	4/5	3/5	-
3.	CSV circular	-	5/5	4/5	3/5	-
	CSV linear	-	5/5	3/5	1/5	-
	CSV-Mg-linear	-	5/5	4/5	1/5	-

a RNAs purified as descibed in Methods. CSV-Mg-linear = circular CSV treated with Mg^{2+} to generate linear molecules. CSV linear = 'naturally occurring' linear CSV RNA.

b Infectivity scored at 8 weeks post inoculation. The same preparation of CSV RNAs was used in all three experiments.

10 μg of "natural" linear CSV molecules did not reveal any trace of circular CSV; conditions under which 0.5 μg (5% contamination) could easily have been detected.

(2) Circular CSV and "magnesium-ion cleavage generated" linear CSV were separated by 5.0 cm on the slab gel.

(3) "Natural" linear CSV was as infectious after re-electrophoresis on a second urea gel (Table III-3) as after electrophoresis on only one urea gel (Table III-2); i.e., the urea gel used to initially separate "natural" linear CSV from circular CSV (Chapter II, Materials and Methods and Figure II-5).

F. *In Vitro* Translation Of Purified RNAs

Attempts at translating linear CSV, circular CSV and chrysanthemum 7S RNA into proteins in the wheat germ cell free system were unsuccessful (Table III-4). The conditions chosen for the translation reaction were found to be near optimal for animal mRNAs (Crawford *et al.*, 1979; Powell and Rogers, 1980), bacterial mRNAs (O'Conner, 1978) and plant viral RNAs (Schwinghamer and Symons, 1977; Palukaitis, results not shown). Under these conditions, neither circular CSV, linear CSV, chrysanthemum 7S RNA nor avocado sunblotch viroid (ASBV; see Chapter VI) were capable of stimulating the incorporation of [^{3}H]-leucine into TCA precipitable cpm above the background endogenous level (which was not due to protein synthesis, but was due to the precipitation of [^{3}H]-leu-tRNA, since no steps were taken to remove or degrade the aminoacyl-tRNAs; results not shown).

Under the same translation reaction conditions, the coat protein mRNAs (RNA 4) of three strains of cucumber

TABLE III-4

AMINO ACID INCORPORATION IN WHEAT GERM EXTRACTS[a]

RNA		Incorporation of $[^3H]$-leucine		
		Gross cpm[b]	Net cpm[c]	% Activity[d]
None		12,700	0	0
CSV linear	1.0 μg	14,850	2,150	1.69
CSV circular	1.1 μg	13,500	800	0.63
ASBV circular	1.1 μg	12,900	200	0.16
Chrysanthemum				
7S	1.0 μg	15,200	2,500	1.96
SAT-CMV	1.0 μg	33,750	21,050	16.5
Q-CMV_3	1.6 μg	135,800	123,100	96.62
M-CMV_4	0.8 μg	98,550	85,850	67.39
P-CMV_4	0.8 μg	129,500	116,800	91.68
Q-CMV_4	1.2 μg	140,100	127,400	100.00

a RNAs incubated in the wheat germ cell-free extract as described in Methods with 10 μCi of $[^3H]$-leucine (105 Ci/mmole) in 50 μl.

b "Gross cpm" is the cpm obtained per 5 μl of sample acid precipitated.

c "Net cpm" is the radio-activity above the control without viral RNA.

d "% Activity" is the percentage of incorporation due to Q-CMV RNA 4.

mosaic virus (Q-, P- and M-CMV), RNA 3 of the Q-strain of CMV and a satellite RNA of CMV (SAT-CMV; Gould *et al.*, 1978) were capable of stimulating the incorporation of [^{3}H]-leu into acid precipitable cpm (Table III-4).

Analysis of the protein synthesis reaction mixtures of circular CSV, linear CSV and chrysanthemum 7S RNA by electrophoresis on a discontinuous 20% acrylamide, 0.12% bis-acrylamide slab gel containing 0.2% SDS (Laemmli, 1970) and fluorography at -80°C (Laskey and Mills, 1975) revealed no products (bands or smears) for any of the above RNAs, whereas the viral RNAs all produced the expected products (Schwinghamer and Symons, 1977; results not shown).

DISCUSSION

A. Physical And Biochemical Characterization Of CSV

The two RNAs obtained after polyacrylamide gel electrophoresis of CSV in the presence of urea (Figure II-5) were characterized and were shown to be two distinct forms of the same RNA.

The identification of the electrophoretically faster migrating RNA on urea gels as the linear form of CSV was made by electron microscopy and molecular hybridization. The latter procedure confirmed the identity of sequence between the slower migrating circular and the faster migrating linear forms and removed the possibility that the linear form was a second infectious agent unrelated to CSV.

The size of the circular form of CSV (136 $\pm$ 19 nm) is

similar to the size of PSTV (140 ± 10 nm, McClements and Kaesberg, 1977; 125 ± 13 nm, Owens *et al.*, 1977) as determined by electron microscopy. Since the three size estimations were made under completely different spreading conditions, a direct comparison to establish the molecular weight of CSV relative to PSTV is not possible. The broad distribution of the lengths of linear viroid molecules (Figure III-2B) was also observed by Owens *et al.* (1977) for linear PSTV and was thought to be due to different degrees of renaturation of linear molecules.

The sharp melting profile found here (Figure III-3) for circular CSV is similar to that reported by Langowski *et al.* (1978) for CSV and four other circular viroids. These authors concluded, on the basis of physical measurements, that circular viroids exist as an extended rod-like structure characterized by a series of double-helical sections and internal loops. The appreciable loss in sharpness of the melting profile in going from the circular to the linear form of CSV (Figure III-3), indicates a reduction in the co-operative melting process; it can be predicted that the exact change would be dependent on the position of strand cleavage. Thermal denaturation of linear PSTV generated by magnesium-ion catalysed cleavage of circular PSTV (Sänger *et al.*, 1979) produced a similar change in melting profile. The appearance of an extra, lower temperature melting component in repeated thermal denaturations of circular RNA preparations of several viroids has been attributed to the generation of nicked molecules (Klump *et al.*, 1978).

The inability of linear or circular CSV or circular ASBV to act as messenger RNAs in an *in vitro* translation system, is consistent with the inability of CEV (Hall *et al.*, 1974) or PSTV (Davies *et al.*, 1974) to be translated *in vitro* in a number of eukaryotic or prokaryotic cell free systems; CEV was also non-translatable *in vivo* in frog oocytes (Semancik *et al.*, 1977). Furthermore, the sequence of PSTV (Gross *et al.*, 1978) contained no AUG initiation codons, but did contain multiple termination codons in all three reading frames making it unlikely that PSTV could function as a mRNA. To determine whether or not viroids are negative strand viruses, as has been recently suggested (Mathews, 1979), will require the isolation of the negative strand of a viroid and its subsequent translation. Negative strands for viroids have been found for both CEV (Grill and Semancik, 1978) and PSTV (Dickson and Zaitlin, personal communication).

Analysis of *in vivo* or *in situ* radio-labelled proteins from infected and non-infected plants has not revealed the presence of any new low molecular weight viroid-encoded proteins for PSTV (Zaitlin and Hariharasubramanian, 1972) or CEV (Conejero and Semancik, 1977; Conejero *et al.*, 1979).

The chrysanthemum 7S RNA, used as a control RNA in the course of this work, was shown to be linear, single-stranded, non-infectious, non-translatable and unrelated to CSV by sequence. This RNA co-migrates with an RNA from *Gynura* (Figure II-4C-E); the latter has been shown to have a sedimentation co-efficient of 7S (Sänger *et al.*,

1976). The origin of this RNA and its role in the infectious process, if any, remain unknown.

B. Biological Characterization Of CSV

Using *Gynura aurantiaca* as an assay host the relative infectivities of circular and linear CSV were compared. Both linear and circular CSV gave an ID_{50} of about 0.05 μg/ml in this host; no other data on the infectivity of purified CSV has been published. In fact, the only infectivity data published for any purified viroid is for PSTV on tomato and the results have shown enormous variation. The statement (no data) by Sänger *et al.* (1976) that 50 - 100 molecules of PSTV are needed to produce symptoms in 10% of inoculated tomato seedlings is a factor of about 10^9 lower than the ID_{50} of between 2 μg/ml and 0.2 μg/ml for purified PSTV on tomato reported from the same laboratory (Mühlbach *et al.*, 1977b). Likewise, Morris and Smith (1977) reported an ID_{50} of between 1.6 and 160 fg/ml (9.6×10^4 - 9.6×10^5 molecules/ml) for purified PSTV on tomato but the data of Morris (1979) gives an ID_{50} of about 0.1 ng/ml, or a factor of about 10^4 higher, and no infectivity at 1 pg/ml (6×10^6 molecules/ml). Diener *et al.* (1974) reported an ID_{50} of 0.2 - 2.0 ng/ml for purified PSTV on tomato and 10 - 20% of the plants were infected at 20 pg/ml.

The dilution end-point for circular CSV was below 5 ng/ml (Table III-3) and the end-point for linear CSV was 0.5 - 5 ng/ml (Table III-3). This compares with a mixture of circular and linear CSV (Chapter II) where the dilution

end-point was 0.2 - 2 ng/ml. These small differences may be due to the normal variations inherent in bio-assays.

The infectivity of the linear as well as the circular form of CSV (Table III-2) is intriguing as a similar result has been obtained for PSTV by Owens *et al.* (1977). Morris (1979) found only one infectious species in his preparations of PSTV but this was not characterized so that it is difficult to compare his results with those of Owens *et al.* (1977). Further, it is also difficult to interpret the infectivity of linear PSTV produced by Mg^{2+}-catalysed hydrolysis (Sänger *et al.*, 1979) since infectivity of control circular PSTV was presumably not done at the same time). If the circular form represents the true viroid, then the linear form is presumably ligated to the circular form *in vivo* after infection. Further, whatever mechanism of viroid replication is proposed, the end product would be linear and circular ligation must occur. If an RNA ligase exists in plants with a comparable specificity to the bacteriophage T4 RNA ligase (Silber *et al.*, 1972), then ligation of nicked circular CSV molecules with a 5'-phosphate and a 3'-hydroxyl would most likely occur and the nature of the residues on either side of this nick would not be important. Hence, on the basis of these considerations, the infectivity of linear CSV reported here and of linear PSTV (Owens *et al.*, 1977) would be expected.

The fact that "natural" linear CSV was as infectious

as "magnesium-ion cleavage generated" linear CSV may indicate that *Gynura aurantiaca* has a greater ability to repair nicked viroid RNA than does *Lycopersicon esculentum*. This is supported by the presence of predominantly circular CSV in symptom-bearing *Gynura* inoculated with either of the two types of linear CSV or with circular CSV.

CHAPTER IV

SYNTHESIS AND CHARACTERIZATION OF COMPLEMENTARY DNA PROBES TO COCONUT CADANG-CADANG VIROID AND TO CHRYSANTHEMUM STUNT VIROID.

INTRODUCTION

Questions related to the time, place, rate and mode of viroid replication can be answered by the use of complementary RNA or DNA probes to viroid sequence in liquid, filter or *in situ* hybridizations.

An RNA probe to PSTV was synthesized by Owens and Diener (1977) using the replicase of the RNA phage Qβ. Although this probe was specific for PSTV sequences, there was no apparently simple method of separating the cDNA from the template; a necessary step if the probe is to be used to detect viroid sequences in plant tissue nucleic acid extracts.

The synthesis of a complementary DNA probe to PSTV was reported by Hadidi *et al.* (1977); however, this cDNA may have been made to a plant nucleic acid contaminant in the PSTV, since it hybridized to preparations of the satellite RNA of tobacco ringspot virus (TRSV) to the same extent as it did to PSTV.

More recently, Owens (1978) synthesized a cDNA probe to PSTV that was shown to be specific for PSTV and did not hybridize to healthy plant nucleic acids or to the satellite RNA of TRSV.

In this Chapter, I will described (1) attempts at synthesizing cDNA probes to two viroid [coconut cadang-cadang associated RNA-1 (ccRNA-1) and CSV] by the technique of Taylor *et al.* (1976) which uses random oligodeoxyribonucleotides to prime cDNA synthesis and which has been successfully applied to make cDNA to high molecular weight RNAs (Gould and Symons, 1977; 1978) but not to low molecular weight viral RNAs (Gould *et al.*, 1978) or 5S rRNA (Taylor *et*

al., 1976); this technique was not successful with CSV, ccRNA-1 or ASBV (see Chapter VI) as the template, but was successfully applied to the synthesis of PSTV cDNA (Owens, 1978); (2) an alternative approach developed with ccRNA-1 and applied to CSV and ASBV, which was to (a) cleave the circular viroid with the single-strand specific nuclease S_1; (b) polyadenylate the S_1-cleaved RNA; and (c) use the polyadenylated, S_1-cleaved viroid RNA as a template and oligo$(dT)_{10}$ as a primer to synthesize cDNA to these viroids; and (3) the characterization of the viroid probes to ccRNA-1 and CSV.

All work involving the cadang-cadang viroid was carried out in collaboration with Dr. J.W. Randles, Department of Plant Pathology, Waite Agricultural Research Institute, University of Adelaide.

MATERIALS

[α-^{32}P]ATP (80 Ci/mmole), [α-^{32}P]dCTP (30 Ci/mmole), and [α-^{32}P]dATP (20 Ci/mmole) were provided by Dr. R.H. Symons and synthesized by the method of Symons (1977). [5-^{3}H]dCTP (15, 5 or 25 Ci/mmole), provided by Dr. J.W. Randles, and [2,8-^{3}H]ATP (26 Ci/mmole) were obtained from Amersham. Actinomycin D was a generous gift from Merck, Sharp and Dohme Research Lab., Rahway, N.J.
Salmon sperm DNA was obtained from Sigma.

Single-strand specific nuclease S_1 from *Aspergillus oryzae* was prepared by the method of Vogt (1973) up to and including the DEAE-cellulose chromatography step, starting with a Takadiastase powder. The S_1 units used were as

described by Vogt (1973).
Coconut nucleic acids, ccRNA-1 and ccRNA-2 were obtained from Dr. J.W. Randles and were prepared as described by Randles *et al.* (1976).
Poly(A) polymerase from *E. coli* B was prepared as described by Sippel (1973).

Avian myeloblastosis virus reverse transcriptase was kindly provided by the Office of Program Resources and Logistics, Viral Cancer Program, Division of Cancer Cause and Prevention, National Cancer Institute, Bethesda, MD.

A CEV-inoculum was provided by Dr. R. van Velsen, South Australian Department of Agriculture, and the viroid maintained in *Gynura aurantiaca*. CEV was transmitted to *Gynura* by needle puncture using crude nucleic acid extracts as described for CSV in Chapter II.

All other materials were obtained as described in preceeding Chapters.

METHODS

A. Partial Digestion of Viroid RNA by Nuclease S_1

1. Nuclease S_1 Digestion of CSV

One to two micrograms of CSV was dried down, resuspended in 18 µl 0.03 M sodium acetate, 0.05 M NaCl, 0.001 M $ZnSO_4$, 5% (v/v) glycerol, pH 4.6, combined with 6 µl of nuclease S_1 (3.9 units/µl) in 0.02 M Tris-HCl, pH 7.5, 0.2 M NaCl, 1×10^{-4} M $ZnSO_4$, 5% (v/v) glycerol, and incubated in the dark at 25°C for 2 hours. The reaction was terminated by the addition of EDTA to a final concentration of 17 mM and the RNA was purified by one phenol:chloroform

(1:1) extraction, three ether extractions and one ethanol precipitation. The RNA was collected by centrifugation at 11,000 g for 10 min at room temperature or at 15,000 g for 10 min at -5°C, dried and dissolved in 50 μl of sterile water.

2. Nuclease S_1 Digestion of ccRNA-1

ccRNA-1 (2 μg) was dissolved in 10 μl of 0.03 M sodium acetate, 0.05 M NaCl, 1 mM $ZnSO_4$, 5% (v/v) glycerol, pH 4.6. Two units of nuclease S_1 (1 μl) were added and the mixture was incubated at 45°C for 30 min before adding 1 μl of 0.2 M EDTA and 3 volumes of ethanol to terminate the reaction. After standing overnight at -15°C, the ethanol precipitate was collected by centrifugation, washed once with ethanol, dried and resuspended in 40 - 50 μl of sterile water.

B. Polyadenylation of RNA

Nuclease S_1 treated viroid RNAs (in 40 - 50 μl of water) were heated at 80°C for 3 min or 100°C for 1 min, chilled and used directly in the polyadenylation reaction.

RNAs were polyadenylated by a modification of the procedure described by Devos *et al.* (1976). RNA (1 - 3 μg) was incubated at 37°C for 40 min in 100 μl of 50 mM Tris-HCl, pH 7.9, 1 mM dithiothreitol, 10 mM magnesium acetate, 2.5 mM $MnCl_2$, 0.3 M NaCl, 0.1 mM [α-^{32}P]ATP (50 mCi/mmole) or 0.1 mM [^{3}H]ATP (100 mCi/mmole), and 13 μg of the peak phosphocellulose fraction of the poly(A) polymerase. The polyadenylated RNA was separated from unreacted ATP by gel filtration on a Sephadex G-50 column (1.5 x 15 cm). The RNA was eluted with either 10 μM EDTA, pH 7, or water;

30-drop fractions were collected and samples of each fraction were counted. The peak fractions of polyadenylated RNA were then lyophilized.

C. Oligo(dT)-Primed Synthesis of cDNA

Complementary DNA to each RNA, polyadenylated as described above, was synthesized by the method described by Kemp (1975). Reaction mixtures of 50 µl contained 50 mM Tris-HCl, pH 8.3, 8 mM dithiothreitol, 8 mM magnesium chloride, three non-radioactive dNTPs, each at 0.67 mM and one radioactively labelled dNTP (either [α-^{32}P]dATP (20 Ci/mmole), [5-^{3}H]dCTP (15.5 or 25 Ci/mmole) or [α-^{32}P]dCTP (30 Ci/mole)), 100 µg/ml actinomycin D, 1 - 2 µg of RNA, 1 - 2 µg of oligo(dT)$_{10}$, and 10 units of avian myeloblastosis virus reverse transcriptase. Incubation was at 37°C for 2 hours. The reaction was stopped by the addition of 50 µl water, 100 µl 1% (w/v) SDS, and 300 µl 0.5 N NaOH. After incubation for 3 hours at 37°C or overnight at room temperature to hydrolyze the RNA template, the cDNA was separated from the low molecular weight material by passage through a 1.5 x 15 cm column of Sephadex G-50 in 0.1 M NH_4HCO_3. The [^{32}P]cDNA (or [^{3}H]cDNA) was recovered by freeze-drying after the addition of excess triethylamine. cDNA was either dissolved in hybridization buffer (A or B; see Chapter V), or water.

D. Random-Primed Synthesis of cDNA

Complementary DNA to each RNA was prepared essentially by the methods of Taylor *et al.* (1976) and Kemp (1975) as described by Gould and Symons (1977). This

procedure was as described above for oligo(dT)-primed cDNA synthesis, except that salmon sperm primer DNA (Taylor *et al.*, 1976) at 2.5 mg/ml was used instead of oligo$(dT)_{10}$.

E. Length Determination of cDNA Probes

The size distribution of $[^{32}P]$cDNA was estimated by electrophoresis in 5% acrylamide, 0.6% bisacrylamide - 99% (v/v) formamide tube gels (Staynov *et al.*, 1972; Gould and Symons, 1977). The molecular weight markers were *E. coli* tRNA (2.2 - 2.9 x 10^4), CSV (1 x 10^5), the satellite RNA of CMV (1.15 x 10^5), CMV RNA 4 (3.5 x 10^5) and 16S rRNA (5.5 x 10^5). RNAs were located by staining with 0.05% toluidine blue for 10 min and destaining in water. The gel was cut up into 2 mm slices and counted by Cerenkov radiation.

F. Nucleic Acid Hybridization

One microlitre of $[^{32}P]$- or $[^{3}H]$cDNA at 2000 cpm/µl was added to 40 µl of appropriately diluted RNA or crude plant nucleic acid extracts (partially purified as described in Chapter II) in hybridization Buffer A (0.18 M NaCl, 10 mM Tris-HCl, pH 7.0, 0.05% (w/v) SDS, 1 mM EDTA) in a siliconized test tube. After thorough mixing, the solution was transferred to a siliconized capillary tube (100 µl), sealed at both ends, boiled for 3 - 5 min and incubated at 60° (for CSV cDNA hybridizations) or 65°C (for ccRNA-1, cDNA hybridizations) for the appropriate length of time. Hybridization was terminated by chilling the capillaries on ice.

The sealed capillaries were opened and the contents added

to 420 μl of either nuclease S_1 assay buffer A (30 mM sodium acetate, pH 4.6, 50 mM NaCl, 1 mM $ZnSO_4$, 5% (v/v) glycerol containing 40 μg/ml denatured calf thymus DNA) or nuclease S_1 assay buffer B (30 mM sodium acetate, pH 4.6, 300 mM NaCl, 1 mM $ZnSO_4$, 5% (v/v) glycerol containing 40 μg/ml denatured calf thymus DNA). Two samples, each of 200 μl, were taken and to one was added 2 or 10 units of nuclease S_1; the other 200 μl served as a control. Both samples were then incubated for 30 min at 45°C or 45 min at 37°C. Digestion was terminated by the addition of 1.0 ml 10% (w/v) TCA; bovine serum albumin (100 μg) was added as as carrier. After 30 min at 0°C, the TCA-precipitates were collected onto (Whatman) GF/A filters, washed four times with 5 ml 5% TCA and twice with 5 ml ether. The cDNA retained by the filters was counted by liquid scintillation spectrometry. The hybrid formation was calculated from a comparison of the duplicates incubated either in the presence or absence of nuclease S_1.

G. Thermal Denaturation of cDNA:RNA Hybrids

The melting profiles of cDNA:RNA hybrids were determined as follows. Homologous hybridization reactions were set up as previously described and the reactions allowed to proceed to a Rot of 1.0 or greater to ensure complete hybrid formation. Capillary tubes containing the hybrid mixtures were then heated for 5 min at the designated temperature (range: 60 - 100°C), chilled on ice and the percent hybrid formation was determined as described above.

RESULTS

A. cDNA Probe to ccRNA-1

1. Random-Primed cDNA Synthesis

cDNA made to ccRNA-1 by the random-primer method of Taylor *et al.* (1976) showed a nuclease S_1 resistance of 29 - 55% and saturation hybridization values to ccRNA-1 of only 32 - 36% after correction for this background nuclease S_1 resistance (results not shown). Furthermore, transcription of cDNA was inefficient with approximatley 0.6 ng cDNA synthesized/μg of untreated ccRNA-1 template (Table IV-1).

2. Oligo(dT)-Primed cDNA Synthesis

a. Nuclease S_1 cleavage of ccRNA-1:

Preliminary attempts at synthesizing cDNA using purified ccRNA-1 as a template by the polyadenylation-reverse transcription procedure of Gould *et al.* (1978) were largely unsuccessful. The circularity (Randles and Hatta, 1979) and unusual molecular conformation of ccRNA-1 (Randles *et al.*, 1976) may have prevented or strongly inhibited the attachment of either poly(A) polymerase or reverse transcriptase to initiation sites. Thus, nuclease S_1 was used to cleave ccRNA-1 at its presumed single-stranded regions (Randles *et al.*, 1976). Nuclease S_1 has been shown to cleave single-stranded regions of tRNA (Harada and Dahlberg, 1975; Rushizky and Mozejko, 1977).

It has already been shown that ccRNA-1 was partially digested by nuclease S_1 in the presence of 0.15 M NaCl (Randles *et al.*, 1976). Figure IV-1 shows that in 0.3 M

TABLE IV-1

[a] Hybridization to ccRNA-1 in hybridization Buffer A at 65°C.

[b] No pre-treatment; no polyadenylation; random-primed cDNA synthesis

[c] No pre-treatment; polyadenylation is as Figure IV-2A; oligo(dT)$_{10}$-primed cDNA synthesis

[d] ccRNA-1 heated in water at 100°C for 3 min and polyadenylated at 54°C as in Figure IV-2B; oligo(dT)$_{10}$-primed cDNA synthesis

[e] Nuclease S_1 cleaved ccRNA-1, polyadenylated as in Figure IV-2C; oligo(dT)$_{10}$-primed cDNA synthesis

[f] Nuclease S_1 cleaved ccRNA-1, heated in water at 100°C for 1 min and polyadenylated as in Figure IV-2D; oligo(dT)$_{10}$-primed cDNA synthesis

[g] Oligo(dT)$_{10}$-primed cDNA synthesis by the method of Kacian and Myers (1976)

[h] Nuclease S_1 cleaved ccRNA-1, heated in water at 80°C for 3 min and polyadenylated as described in Materials and Methods; oligo(dT)$_{10}$-primed cDNA synthesis.

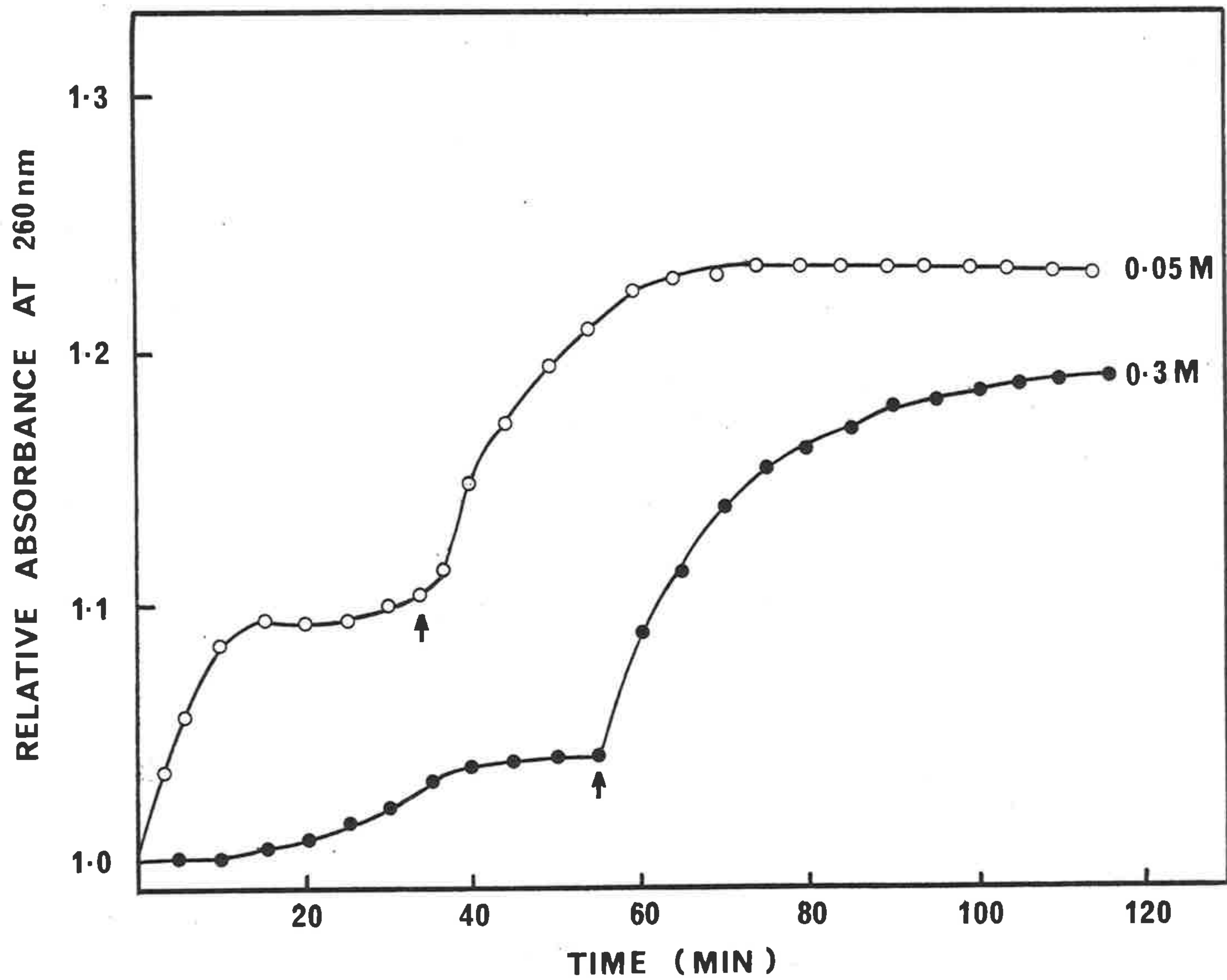
TABLE IV-1

THE EFFECTS OF PRE-TREATMENTS OF ccRNA-1 ON THE EXTENT OF POLYADENYLATION, cDNA SYNTHESIS AND $Rot_{\frac{1}{2}}$ OF HYBRIDIZATION OF THE cDNA

PRE-TREATMENT OF ccRNA-1	POLYADENYLATION TEMPERATURE (°C)	PERCENT (%) POLYADENYLATION	PERCENT (%) cDNA SYNTHESIS	YIELD OF cDNA (ng/1 μg RNA)	$Rot_{\frac{1}{2}}$ (mol s l^{-1})[a]
None[b]	-	-	0.01[b]	0.6	2×10^{-3}
None[c]	37	4.5	0.024	1.4	1×10^{-1}
Melted[d]	54	2.8	-	-	-
S_1-cleaved[e]	37	8.5	0.011	0.6	-
S_1-cleaved and melted[f]	37	7	0.031	1.8	1×10^{-3}
			0.155[g]	9[g]	1×10^{-3}[g]
S_1-cleaved and melted[h]	37	13-15	0.5-1.0	19-58	$1\text{-}2 \times 10^{-3}$
E. coli tRNA (No pre-treatment)	37	68.5	-	-	-

FIGURE IV-1

Effect of salt concentration on the kinetics of the sequential digestion of ccRNA-1 with nuclease S_1 and RNAse A. Substrate was at a concentration of 1.5 μg/ml in nuclease S_1 assay buffer A (with 0.05 M NaCl) or nuclease S_1 assay buffer B (with 0.3 M NaCl). Nuclease S_1 (5.5 units/ml) was added at zero time; RNAse A (1.4 μg/ml) was added at the time shown by the arrow.


1·3
1·2
1·1
1·0
RELATIVE ABSORBANCE AT 260 nm
20
40
60
80
100
120
TIME (MIN)
0·05 M
0·3 M

NaCl, ccRNA-1 was more resistant to nuclease S_1 digestion that in 0.05 M NaCl, probably reflecting the influence of secondary structure on the availability to the enzyme of single-stranded regions. Incomplete hydrolysis occurred at hoth salt concentrations, as shown by the residual hyperchromicity obtained upon the addition of ribonuclease A (Figure IV-1).

The time course of digestion of ccRNA-1 in an unfractionated coconut nucleic acid extract in nuclease S_1 assay buffer A analyzed by polyacrylamide gel electrophoresis, showed the digestion was complete by 30 min (results not shown).

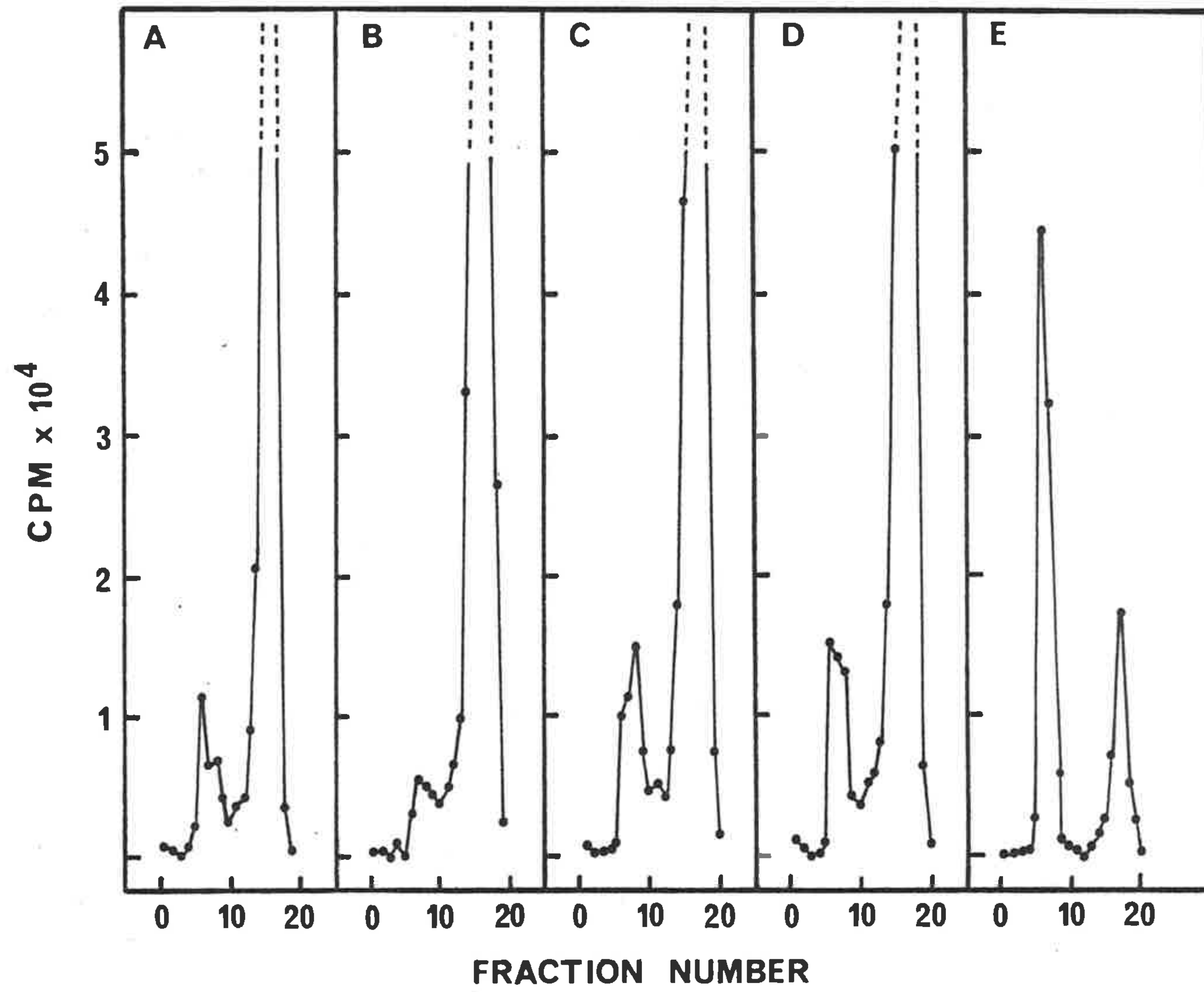
b. <u>Polyadenylation of ccRNA-1:</u>

A comparison was made of the extent of polyadenylation of ccRNA-1 after it had been subjected to various pretreatments. Polyadenylation was compared on the basis of the proportion of [α-^{32}P]ATP in the reaction mixture incorporated into the RNA.

Table IV-1 and Figure IV-2 show the effects of various treatments of ccRNA-1 on the extent of polyadenylation. Untreated ccRNA-1 (Figure IV-2A) and ccRNA-1 melted and chilled immediately before adding the reaction mixture and incubating at 54°C (Figure IV-2B) gave 4.5% and 2.8% incorporation, respectively (Table IV-1). Nuclease S_1 treated ccRNA-1 (Figure IV-2C) and nuclease S_1 treated, then melted and chilled ccRNA-1 (Figure IV-2D) gave 8.5% and 7% incorporation, respectively (Table IV-1), in this experiment. Polyadenylation was far less efficient than for *E. coli* tRNA compared in the same experiment (68.5%; Figure

FIGURE IV-2

Elution profiles of polyadenylated ccRNA-1 on G50 Sephadex columns, showing the relative sizes of the peaks containing polyadenylated RNA (leading peak) and the unreacted [α-^{32}P]ATP precursor. Substrates were: (A) 2 µg untreated ccRNA-1, polyadenylated at 37°C as described in Materials and Methods; (B) 2 µg ccRNA-1 (in 40 µl water), heated to 100°C for 3 min and polyadenylated at 54°C as described in Materials and Methods; (C) 2 µg ccRNA-1 digested with nuclease S_1, ethanol precipitated and (D) as for (C), except that after ethanol precipitation, the RNA was heated (in 40 µl water) to 100°C for 1 min, chilled and polyadenylated at 37°C; (E) 4 µg *E. coli* tRNA, polyadenylated at 37°C as described in Materials and Methods.



A
B
C
D
E
CPM x 10^4
5
4
3
2
1
0
10
20
FRACTION NUMBER

IV-2E; Table IV-1).

c. cDNA synthesis to ccRNA-1:

The ccRNA-1's polyadenylated as described in Figure IV-2 were used as templates in the cDNA synthesis reaction. The efficiency of cDNA synthesis was again compared on the basis of the proportion of the radioactive substrate incorporated into cDNA (Table IV-1). The kinetics of hybridization to untreated ccRNA-1 were assayed for each cDNA, plotting percentage hybridization against the Rot (moles of ribonucleotide/litre x time of hybridization in seconds) value for a series of samples diluted four-fold with respect to ccRNA-1 concentration (results not shown).

When untreated, polyadenylated ccRNA-1 (Figure IV-2A) was used as a template, 0.024% of the imput radioactivity was incorporated into cDNA (Table IV-1). Hybridization kinetics showed a Rot½ (midpoint of inflection of the Rot curve) above 0.1 mol sec litre^{-1}; approximately 100-fold higher than expected for an RNA the size of ccRNA-1 (Gould *et al.*, 1978). Using S_1-pretreated, polyadenylated ccRNA-1 (Figure IV-2C), 0.011% of the imput radioactivity was incorporated into cDNA (Table IV-1). The hybridization kinetics did not show a sigmoidal pattern and no Rot½ value was obtainable. S_1-pretreated ccRNA-1, which had been melted and rapidly chilled before polyadenylation (Figure IV-2D), stimulated the incorporation of 0.031% of the radio-label and showed a Rot½ of about 1 x 10^{-3} mol sec litre^{-1} (Table IV-1). For all the studies described below, cDNA was synthesized by the procedure and its hybridization kinetics characterized before use.

The maximum yield for cDNA produced, when S_1-cleaved RNA heated to 100°C for 1 min and rapidly chilled prior to polyadenylation was used as the template (Figure IV-2D; 7% polyadenylation; 1.8 ng of cDNA/μg of RNA), was increased at least 10-fold when S_1-cleaved RNA was instead heated to 80°C for 3 min and rapidly chilled prior to polyadenylation (13 - 15% polyadenylation results not shown; Table IV-1); the yield of cDNA was 19 - 58 ng/μg of RNA (Table IV-1).

cDNA was also synthesized to nuclease S_1 treated ccRNA-1, polyadenylated as in Figure IV-2D, using the method of Kacian and Myers (1976), which contains no actinomycin D in the reaction mixture. The efficiency of cDNA synthesis was increased (9 ng cDNA/μg RNA) and upon hybridization, the Rot½ value was 1 x 10^{-3} mol sec litre^{-1}, but the nuclease S_1 resistance of the cDNA was high (45 - 55%) and maximum hybridization values of only 16 - 23% were obtained. The high nuclease S_1 resistance was apparently due to 'snap back' annealing of the cDNA, as melting and rapidly chilling the cDNA had no effect on subsequent nuclease S_1 resistance.

3. Characterization of cDNA to ccRNA-1

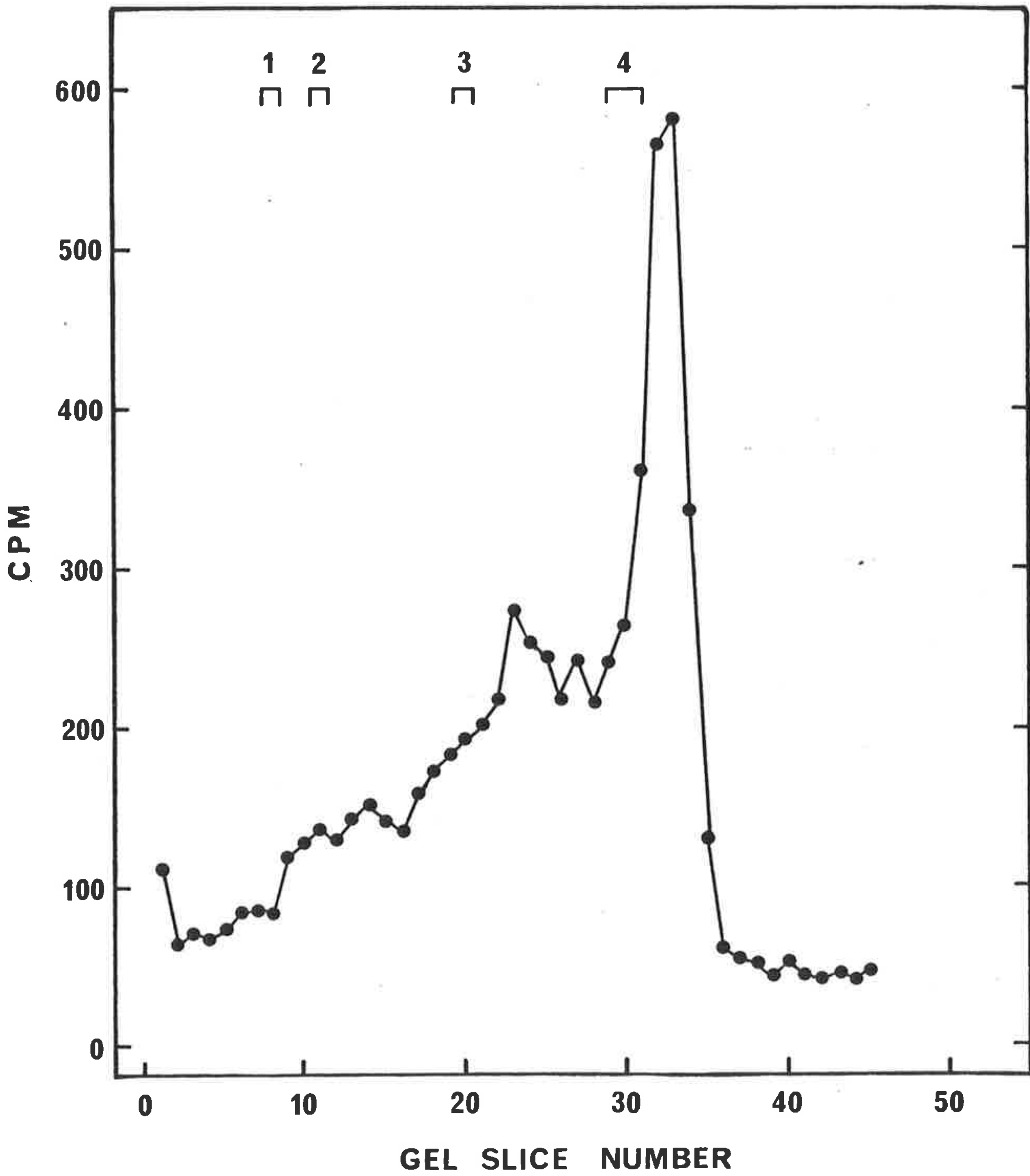
a. Size of the cDNA probe:

The size distribution of cDNA-ccRNA-1 was determined on denaturing polyacrylamide-formamide gels. The majority of the cDNA fell into the 4S size class (Figure IV-3); some of the cDNA was full length. The size distribution is similar to that of CSV-cDNA (see below) and PSTV-cDNA (Owens, 1978). Although the molecular weight of ccRNA-1 is similar to that of the satellite-RNA of CMV, it has not been

FIGURE IV-3

Size distribution of [^{32}P]cDNA on a denaturing 5% acrylamide-99% formamide gel, shown in relation to marker RNA species (1) 16S rRNA (5.5×10^5); (2) CMV RNA 4 (3.5×10^5); (3) satellite RNA of CMV (1.15×10^5); (4) *E. coli* tRNA ($2.2 - 2.9 \times 10^4$).

The gel was cut into 2 mm slices that were counted by Cerenkov radiation.



1
2
3
4
600
500
400
300
200
100
0
CPM
0
10
20
30
40
50
GEL SLICE NUMBER

accurately determined and thus was not included as a molecular weight marker with the other RNAs.

b. Specificity of the cDNA probe:

That the cDNA probe was specific for ccRNA-1 and ccRNA-2 sequences is shown in Table IV-2. Other small RNA species (including CSV) viral RNAs and nucleic acids extracted from healthy coconut palms (Table IV-3) show no significant homology with the cDNA when hybridized to Rot values at least twenty times greater than the Rot value for homologous hybridization. Although nuclease S_1 digests ccRNA-2 (Randles, personal communication), S_1-resistance sequences remain which hybridize to a maximum value of around 36% (Table IV-2). ccRNA-1 can be detected in unfractionated nucleic acids from diseased palms (Table IV-3), prepared either by the 'PEG procedure' (Randles *et al.*, 1976) or by the 'total nucleic acid extraction procedure' (Randles *et al.*, 1976).

The presence of sequences complementary to PSTV in host DNA has been shown by Hadidi *et al.* (1976). In this study, the absence of homology with DNA from healthy or diseased palms at this Cot value (Table IV-3) precludes the possibility that any presumptive high frequency ccRNA-1 copies in the DNA might interfere with the detection of ccRNA-1 or -2 in total nucleic acid extracts. Whether such sequences do in fact exist in the host DNA at low copy numbers, remains to be determined.

c. Thermal stability of cDNA-ccRNA-1:ccRNA-1 hybrids:

Hybrids formed between cDNA-ccRNA-1 and

TABLE IV-2

HYBRIDIZATION SPECIFICITY OF cDNA TO ccRNA-1

Nucleic Acid	Concentration (μg/ml)	Rot	% Hybrid-ization[a]
None	-	-	6.7
ccRNA-1	0.9	0.2	43.9
ccRNA-1 (S_1 treated)[b]	1	0.2	46.0
ccRNA-2	1	0.7	51.9
ccRNA-2 (S_1 treated)[b]	1	0.7	35.7
Chrysanthemum stunt viroid	1	0.2	5.2
Cucumber mosaic virus RNA	1	0.2	3.6
CMV satellite RNA	1	0.2	5.5
Tobacco ringspot virus RNA	5	1.0	5.4
Tobacco mosaic virus RNA	14.4	3.0	4.1
Yeast tRNA	20	4.1	3.6

a Hybridization was carried out at 65°C in hybridization Buffer A (see Materials and Methods). Nuclease S_1 assay of hybrids formed was carried out at 45°C for 30 min as described in Materials and Methods in nuclease S_1 assay Buffer B.

b Treated with nuclease S_1 as described in Materials and Methods; concentration is that of RNA before S_1 treatment.

TABLE IV-3

HYBRIDIZATION OF cDNA TO ccRNA-1 WITH NUCLEIC ACIDS OF DISEASED AND HEALTHY PALMS

Palm	Nucleic Acid Preparation	% Hybridization[a]
Diseased A	Purified ccRNA-1[b]	46
Diseased A	PEG procedure, unfractionated[c]	56
Healthy	PEG procedure, unfractionated[c]	10
Diseased A	Total, phenol extracted[d]	45
Diseased B	Total, phenol extracted[d]	46
Diseased C	Total, phenol extracted[d]	31
Diseased D	Total, phenol extracted[d]	61
Healthy	Total, phenol extracted[d]	3
Diseased A	Total DNA[e]	0
Healthy	Total DNA[e]	0

[a] Hybridizations and nuclease S_1 assays as in Table IV-2. Hybridization percentages corrected for self-hybridization of cDNA (7%).

[b] Heterologous ccRNA-1, Rot = 0.48 mol sec $litre^{-1}$.

[c] Nucleic acids from 1 g of leaf tissue; hybridization time 15.5 hours. 'PEG procedure' is as described by Randles *et al.* (1976).

[d] Nucleic acids from 0.1 g of leaf tissue; hybridization time 68 hours. 'Total phenol extraction procedure' is as described by Randles *et al.* (1976).

[e] Cot = 185 mol sec $litre^{-1}$.

ccRNA-1 melted sharply in hybridization Buffer A with a T_m of 87°C (Figure IV-4). The melting profile closely resembles that obtained from other homologous hybrids formed in the same buffer (Gould and Symons, 1977; Gould *et al.*, 1978), and hybrid formation was therefore apparently specific with no evidence of mismatching. The thermal stability of cDNA-ccRNA-1:ccRNA-2 hybrids was not determined.

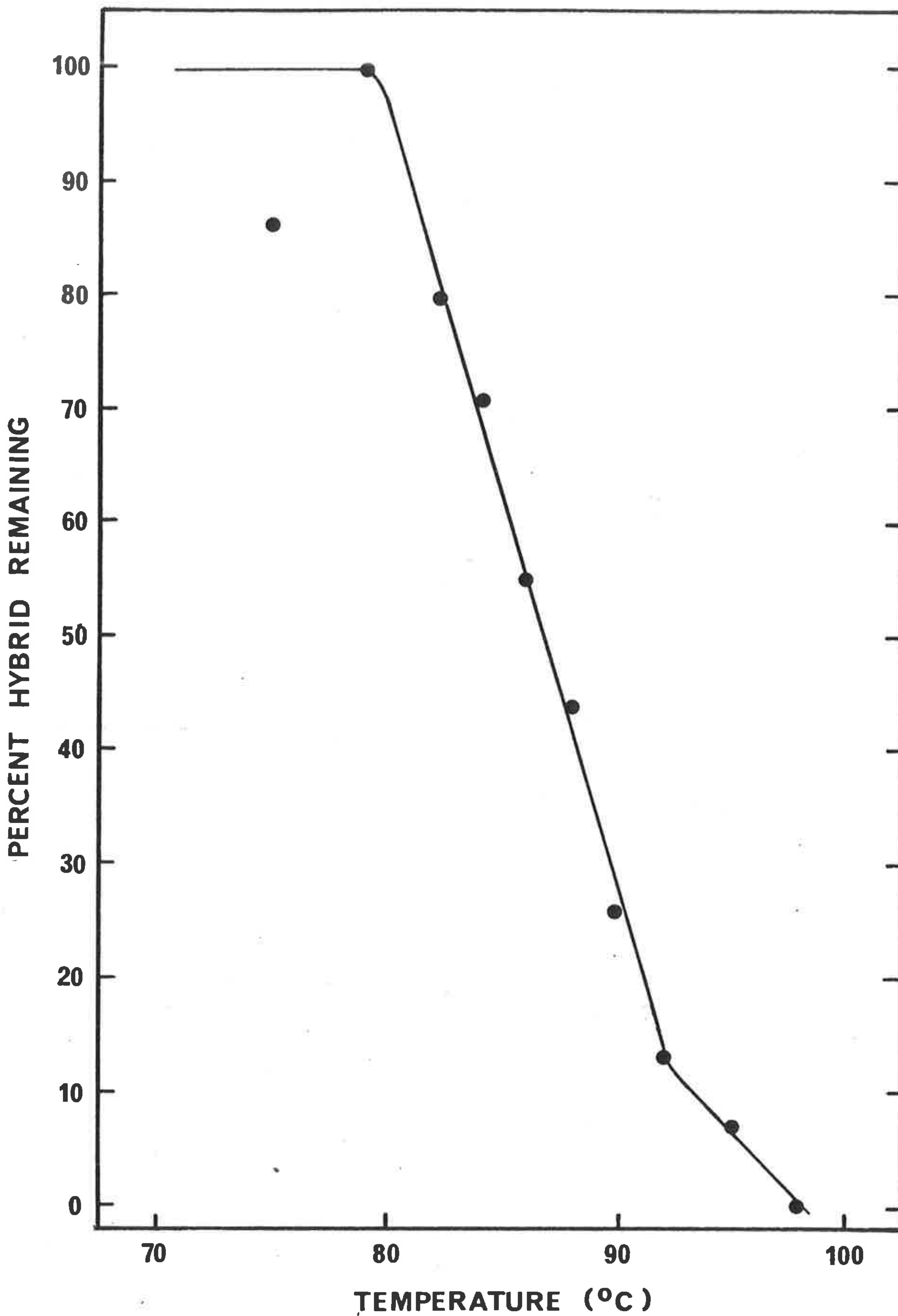
4. Hybridization Analysis of ccRNA-1 and ccRNA-2.

Figure IV-5A shows that kinetics of hybridization of cDNA-ccRNA-1 against the homologous ccRNA-1. The curve showed the expected sigmoid shape, but maximum hybridization values rarely exceeded 50%. This low maximum may have been due to ccRNA-1 retaining considerable secondary structure under these hybridization conditions; however, varying the temperature and salt concentration and including formamide in the hybridization buffer failed to increase the maximum hybridization values obtained, making this an unlikely explanation (results not shown). The $Rot_{\frac{1}{2}}$ value of $1 - 1.5 \times 10^{-3}$ mol sec $litre^{-1}$ for the hybridization of the cDNA with ccRNA-1 is close to that obtained by Gould *et al.* (1978) for the satellite RNA of CMV (1×10^{-3} mol sec $litre^{-1}$; $M_r = 1.15 \times 10^5$) but slower than that obtained for the same RNA in Chapter V (7×10^{-4} mol sec $litre^{-1}$). ccRNA-1 therefore has a nucleotide complexity similar to that of the unique RNA species, satellite RNA of CMV, and close to its estimated molecular weight of approximately 1×10^5 (Randles, 1975).

The hybridization kinetics of cDNA with the homologous ccRNA-1 preparation from which it was transcribed (Figure IV-

FIGURE IV-4

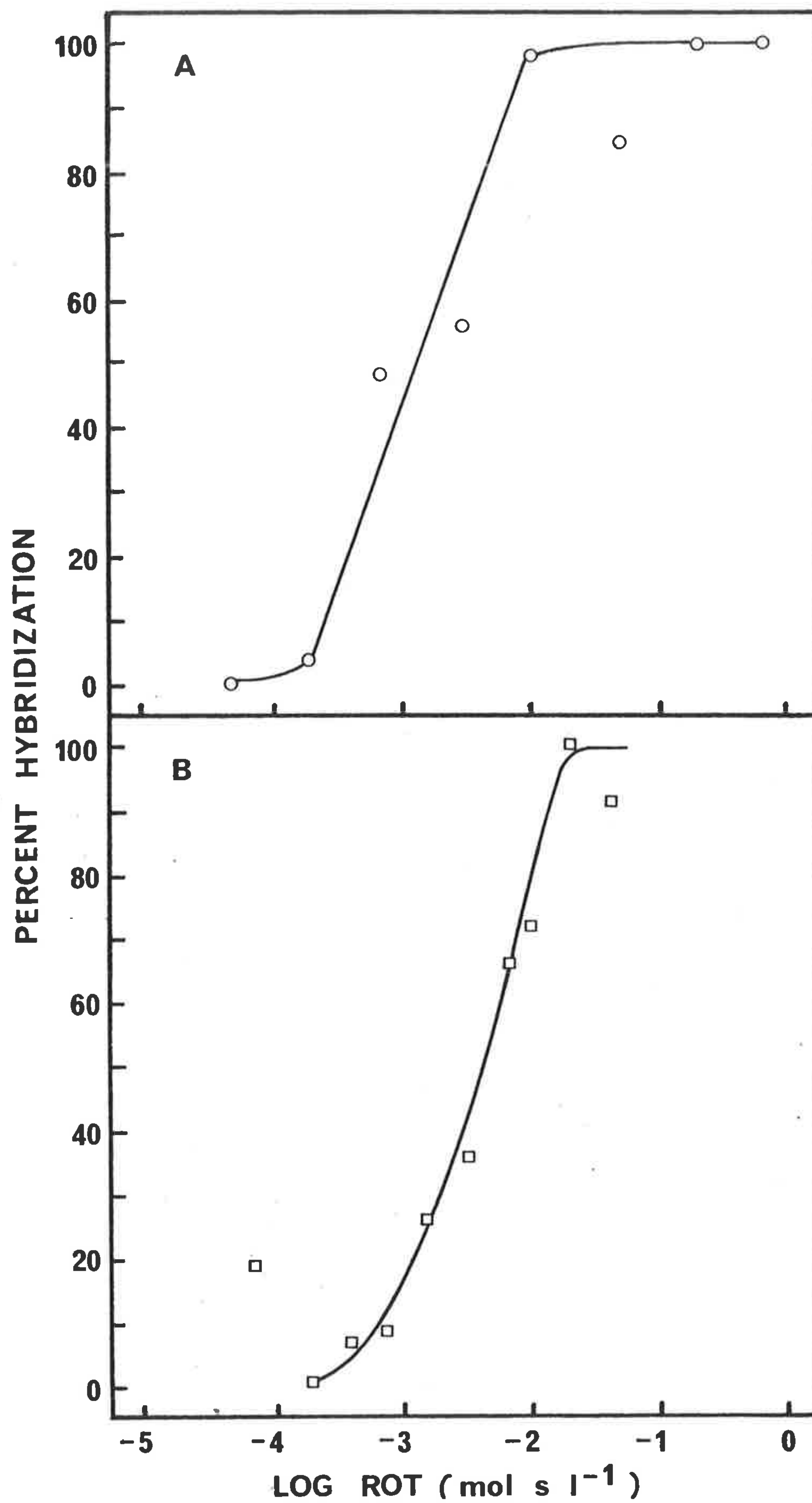
Thermal denaturation of the cDNA-ccRNA-1:ccRNA-1 hybrid in hybridization Buffer A, performed as described in Materials and Methods.



PERCENT HYBRID REMAINING
100
90
80
70
60
50
40
30
20
10
0
70
80
90
100
TEMPERATURE (°C)

FIGURE IV-5

Kinetics of hybridization of [^{32}P]cDNA-ccRNA-1 to (A) homologous ccRNA-1 and (B) ccRNA-2 extracted from another diseased palm. The Rot curves have been normalized to 100% to facilitate comparison of the Rot½'s. The plateau values for (A) and (B) were 53% and 54%, respectively.



A
B
100
80
60
40
20
0
PERCENT HYBRIDIZATION
−5
−4
−3
−2
−1
0
LOG ROT (mol s l⁻¹)

5A) was compared with that obtained when the cDNA was hybridized with ccRNA-2 (Figure IV-5B). The similarities of the shapes of the curves, the rates of hybridization and the maximum hybridization values indicate that there is a high degree of homology between these RNAs.

The Rot½ of the cDNA-ccRNA-1:ccRNA-2 hybridization was approximately 4 - 4.5 x 10^{-3} mol sec $litre^{-1}$. It seems improbable that contamination of ccRNA-2 with ccRNA-1 could account for such homology, because more than 33% contamination would be required to give such a Rot½ value. Re-analysis of ccRNA-2 on polyacrylamide gels both with and without prior treatment in 4 M urea at 60°C for 15 min and staining in both ethidium bromide and toluidine blue, showed no detectable ccRNA-1 at a threshold of detection of around 5% of the ccRNA-2 concentration (Randles, personal communication).

Since the hybridization assay has the limitation that a maximum of approximately 50% of the cDNA has hybridized in all cases, it cannot be concluded that ccRNA-1 and -2 have identical nucleotide sequences. Nevertheless, ccRNA-1 sequences to which the cDNA hybridizes are found in both ccRNA-1 and -2.

B. cDNA Probe to CSV

1. Oligo(dT)-Primed cDNA Synthesis

The approach used to synthesize a cDNA probe to CSV was: (1) partial cleavage of CSV by nuclease S_1; (2) enzymic addition of a poly(A) tract to the 3'-end of the S_1-treated CSV; and (3) synthesis of cDNA with reverse transcriptase, using polyadenylated, S_1-treated

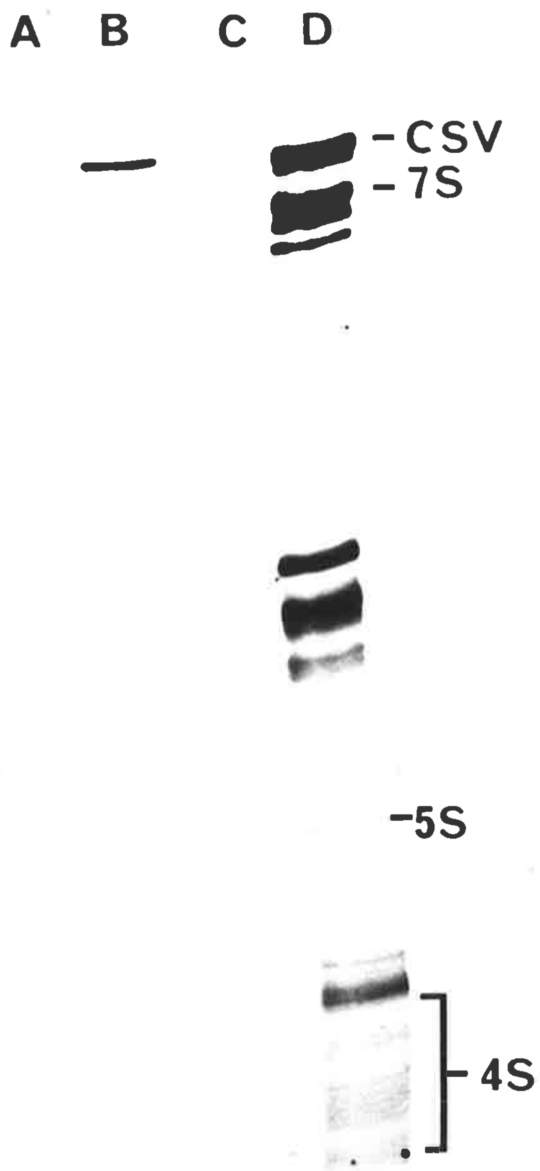
CSV as a template and oligo(dT)$_{10}$ as a primer.

This is the same approach used for the synthesis of cDNA to ccRNA-1 (see above); however, the conditions for S_1-cleavage were changed. The conditions had to take account of the lower T_m of CSV relative to ccRNA-1 (Randles *et al.*, 1976; Chapter III Results), but also had to ensure sufficient nicking of the RNA. To accomplish this, the temperature of the digestion was decreased, but the concentration of nuclease S_1 and the time of incubation were increased.

5'-end labelled S_1-cleaved CSV (with and without prior treatment with calf intestinal phosphatase) subjected to electrophoresis on a denaturing (7 M urea) polyacrylamide gel revealed a number of minor fragments in the range of 100 - 340 residues (Figure IV-6C and D); the latter values is the approximate size of the linear CSV molecule when compared to the satellite RNA of CMV (335 residues; Richards *et al.*, 1978) by electrophoresis on denaturing (7 M urea) polyacrylamide gels (results not shown). A large proportion of the label was associated with full length linear CSV (Figure IV-6A and B) suggesting that some of the RNA is only nicked once. The nick is probably specific and the product contains a 5'-phosphate, as would be expected after cleavage by nuclease S_1 (Ando, 1966). Radio-active bands appearing in the absence of phosphatase treatment (Figure IV-6C) probably reflect random breakdown (possibly Zn^{2+} catalyzed) of the RNA during the two hour nuclease S_1 digestion, or they could reflect digestion by a contaminating nuclease in the S_1.

FIGURE IV-6

Denaturing polyacrylamide gel electrophoresis and autoradiography of RNAs labelled at the 5'-end by [γ-^{32}P]ATP and polynucleotide kinase. Samples: (A) and (B) linear CSV; (C) and (D) S_1-treated circular CSV. Samples (B) and (D) were pre-treated with calf intestinal phosphatase prior to labelling with polynucleotide kinase and [γ-^{32}P]ATP. Phosphatase and kinase treatments and denaturing (7 M urea) thin gel electrophoresis (Sanger and Coulson, 1978) were as described in Chapter III Materials and Methods. Positions of marker RNAs (4S, 5S, 7S, linear CSV) are given. A number of faint bands were visible in (C) on the original autoradiogram (not visible here), but there was no band corresponding to full length, linear CSV.


A
B
C
D
CSV
7S
5S
4S

After treatment with nuclease S_1, the extent of polyadenylation of CSV RNA increased such that up to 70% of the imput ATP was incorporated into poly(A) tails, compared with 5% obtained without nuclease S_1 treatment (results not shown).

Table IV-4 shows that only the addition of a poly(A) tail and an oligo$(dT)_{10}$ primer resulted in any appreciable cDNA synthesis. S_1-cleaved CSV was not capable of self-priming cDNA synthesis and did not appear to contain any natural oligo(A) or oligo(U) sequences capable of binding to the oligo(dT) or poly(A) tails, respectively, which could then prime cDNA synthesis. cDNA yields were increase above the levels shown in Table IV-4 (about 5 - 10 fold) by heating the S_1-cleaved RNA at 80°C for 3 min and rapidly chilling prior to polyadenylation.

cDNA could also be made to polyadenylated linear CSV that had not been pre-treated with nuclease S_1; however, the yield of cDNA was low (5% of that obtained with prior nuclease S_1 treatment) and this cDNA proved to be unsuitable for hybridization purposes (results not shown).
cDNA to CSV could not be made using the random primer method of Taylor *et al.* (1976) (results not shown). This is similar to the situation with ccRNA-1 (this Chapter) and ASBV (Chapter VI), but different from PSTV (Owens, 1978).

2. Characterization of cDNA to CSV

a. Size of the cDNA probe:

The cDNA to CSV was heterogeneous in size (Figure IV-7) with most of the cDNA in the 20 - 100,000 molecular weight range. Only a small proportion of the cDNA

TABLE IV-4

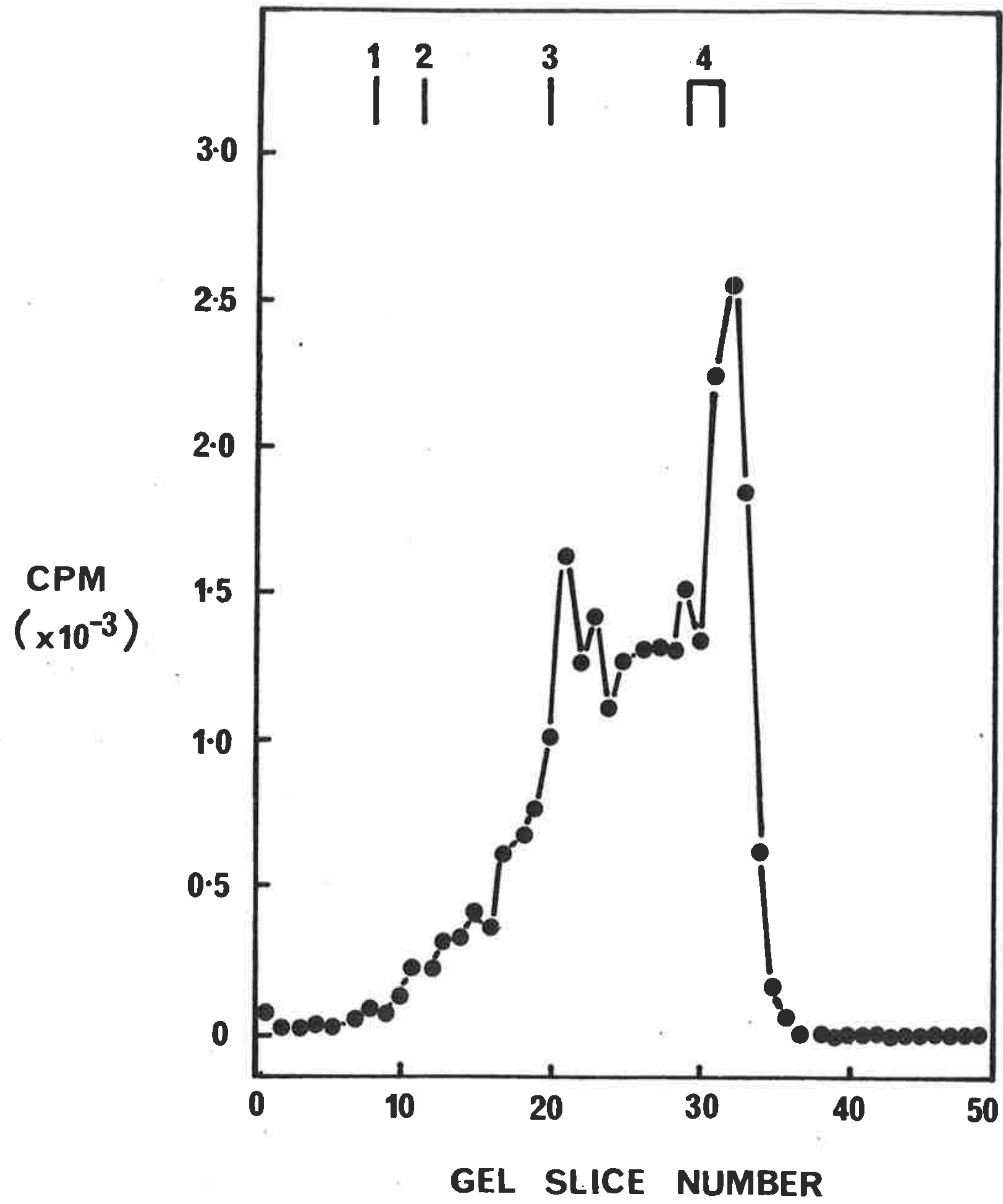
CONDITIONS FOR CSV cDNA SYNTHESIS

RNA Template	Oligo(dT)$_{10}$-primer	ng cDNA[a]
S_1-treated CSV	-	0
	+	0.23
Polyadenylated,	-	0.71
S_1-treated CSV	+	7.02

[a] S_1-treated CSV was either used directly for cDNA synthesis, or was first polyadenylated; both as described in Materials and Methods. The RNA (0.8 μg) was divided into two fractions, to one of which was added 2 μg oligo(dT)$_{10}$. cDNA yield is expressed in ng of cDNA synthesized/400 ng CSV RNA template.

FIGURE IV-7

Size estimation of [^{32}P]cDNA to CSV by electrophoresis on 99% formamide:5% acrylamide tube gels as described by Gould and Symons (1977). The gel was cut up into 2 mm slices and counted by Cerenkov radiation. RNA markers were (1) 16S rRNA (5.5×10^5); (2) CMV RNA 4 (3.5×10^5); (3) CSV (approximately 1.1×10^5); and (4) *E. coli* tRNA ($2.2 - 2.9 \times 10^4$).



1
2
3
4
3·0
2·5
2·0
1·5
1·0
0·5
0
CPM
(x10⁻³)
0
10
20
30
40
50
GEL SLICE NUMBER

was full length (100 - 120,000). This distribution of fragments of cDNA to CSV is similar to that observed for ccRNA-1 (earlier this Chapter) and PSTV (Owens, 1978) and may reflect difficulties in synthesizing cDNA to RNAs containing appreciable secondary structure.

b. Specificity of the cDNA probe

The cDNA specifically hybridized only to CSV RNA (Table IV-5) and not to any of the plant viral RNAs tested or to *E. coli* tRNA. Therefore, not only was the cDNA probe specific for CSV, but CSV appears to have no sequence homology with the RNAs of AMV, BMV, CMV or TMV. The specificity of the probe was further demonstrated by the ability of the cDNA to find complementary sequences only in partially purified nucleic acids extracted from CSV-infected chrysanthemums and *Gynura*, but not from healthy chrysanthemums or *Gynura* or from CEV-infected *Gynura* (Table IV-5).

c. Thermal stability of cDNA-CSV:CSV RNA hybrids:

The low maximum level of hybridization (50 - 64%) could be due to the secondary structure of the RNA (as was proposed for ccRNA-1) or could be due to the short size of some of the cDNA (15 - 30,000 mol. wt. range). cDNA of shorter size and/or lower G:C content forms less stable hybrids; both factors lower than T_m of a hybrid (Gillespie *et al.*, 1975). The latter possibility was supported by the thermal denaturation profile of the hybrid (Figure IV-8) wherein the curve obtained was broader than one obtained with the satellite RNA of CMV and its cDNA (Gould *et al.*, 1978) and the T_m was lower (81°C cf. 87°C). However, the shape of the melting curve was not as broad

TABLE IV-5

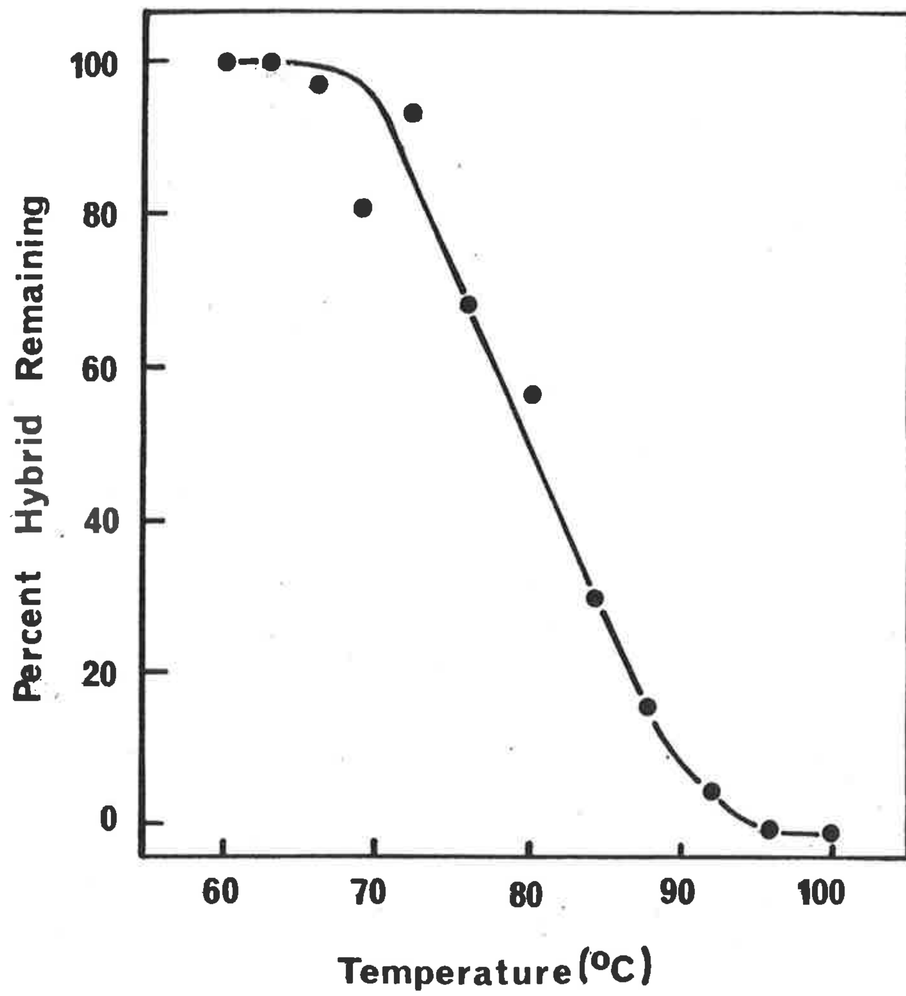
SPECIFICITY OF CSV cDNA

Nucleic Acid	Concentration (mg/ml)	Rot (mol sec litre^{-1})	% Hybridization[a]
CSV	0.01	2.5	64.7
None	–	–	6.6
E. coli tRNA	1.25	320	6.8
Alfalfa mosaic virus RNA	1.25	320	6.8
Brome masaic virus RNA	1.25	320	8.2
Cucumber mosaic virus RNA	1.25	320	9.3
Tobacco mosaic virus RNA	1.25	320	12.3
Healthy chrysanthemum	2.0	100	12.3
CSV-infected chrysanthemum	2.0	100	48.9
Healthy - *Gynura*	2.0	100	10.6
CSV-infected *Gynura*	2.0	100	61.2
CEV-infected *Gynura*	2.0	100	12.8

[a] Hybrid formation and nuclease S_1 assay of hybrids formed was as described in Materials and Methods using nuclease S_1 assay buffer B at 37°C for 45 min with 10 units S_1/assay.

FIGURE IV-8

Thermal denaturation of [^{32}P]cDNA to CSV:CSV RNA hybrids in hybridization Buffer A, performed as described in Materials and Methods.



Percent Hybrid Remaining
100
80
60
40
20
0
60
70
80
90
100
Temperature (°C)

as the melting profiles obtained from hybrids that were greatly mis-matched (Gillespie *et al.*, 1975; Gonda and Symons, 1978), indicating little if any mis-matching.

DISCUSSION

Attempts at making cDNA to three different viroids (CSV, ccRNA-1 and ASBV) via the random primer technique of Taylor *et al.* (1976) were unsuccessful. An alternative technique for the synthesis of cDNA to viroids was developed, based on the following. (1) Gould *et al.* (1978) had shown that oligo(dT)$_{10}$-primed cDNA could be synthesized to non-poly(A)-containing, low molecular weight RNA after *in vitro* polyadenylation. This reaction, however, is dependent on the presence of a 3'-hydroxyl on the RNA, and the circular structure of viroids prevents polyadenylation of the RNA. (2) Randles *et al.* (1976) demonstrated the resistance of PSTV and ccRNA-1 to total digestion by the single-strand specific nuclease S_1, and digestion of CSV by nuclease S_1 produced a number of fragments including full length linear viroid (Figure IV-6), suggesting that the high degree of secondary structure protects viroids from being degraded even under vigorous digestion conditions. Furthermore, the RNA fragments produced by nuclease S_1 digestion have 3'-hydroxyls (Ando, 1966). Therefore, the approach developed with ccRNA-1 and applied to CSV was to (1) partially cleave viroid RNA with nuclease S_1; (2) polyadenylate the S_1-cleaved RNA fragments; and (3) using polyadenylated, S_1-cleaved RNA as a template and oligo(dT)$_{10}$ as a primer, synthesize cDNA to the viroids.

cDNA synthesized to CSV was shown to be dependent on both the polyadenylation of S_1-cleaved RNA and the presence of oligo(dT) as a primer. The cDNA synthesized was specific for the viroid to which it was made; the cDNA hybridized to neither healthy host nucleic acids nor to the other viral RNAs tested.

The cDNA made to ccRNA-1 showed that the two RNAs associated with the cadang-cadang disease of coconuts, ccRNA-1 and ccRNA-2, are related by sequence and that ccRNA-1 is unrelated to CSV.

The CSV-cDNA probe showed that CSV sequences were present only in the RNA of infected and not healthy plants and that CSV is unrelated by sequence to CEV.

The possibility of the existence of some small sequence relationship (homology) between these viroid cannot be ruled out, as there is no real estimate of the 'representivity' of the probes; i.e., whether all the viroid sequences were copied into cDNA and whether all fragments were copied to the same extent.

cDNA could also be synthesized to polyadenylated, S_1-treated CSV using all three specific primers, d(pT_8G), d(pT_8C) and d(pT_8A), albeit with different efficiencies, (results not shown) suggesting initiation of cDNA synthesis was occurring on a number of different S_1-digestion fragments. Some of these fragments were full length, as was some of the cDNA. The secondary structure present in long CSV fragments may have interfered with cDNA synthesis by reverse transcriptase, resulting in the low yields of cDNA often obtained.

The size distribution of the cDNA to CSV was similar to

that of the cDNAs to ccRNA-1, the satellite RNA of CMV (Gould *et al.*, 1978) and PSTV (Owens, 1978); all highly base-paired RNAs. Only approximately 50% of the cDNA (for PSTV, ccRNA-1 and CSV) was capable of hybridizing. Owens, (1978) showed that the majority of the cDNA incapable of hybridizing was less than 25,000 in molecular weight; a large proportion of all three cDNAs (CSV, ccRNA-1 and PSTV) was 25,000 of below in molecular weight. A discussion on the rates of hybridization of viroids will be presented in Chapter V, where the secondary structure characteristic of viroids will be shown to have an effect on the rate of hybridization.

CHAPTER V

HYBRIDIZATION ANALYSIS, QUANTITATION OF CSV LEVELS IN EXTRACTS OF INFECTED PLANTS AND ANALYSIS OF PLANT DNA FOR VIROID SEQUENCE HOMOLOGY

INTRODUCTION

Hybridization analysis of labelled complementary DNA (cDNA) to RNA can be used to determine the molecular complexity and the concentration of a particular RNA in a nucleic acid extract. This reaction is dependent upon the ability of bases in the cDNA to form hydrogen bonds with complementary bases in the RNA; i.e., the two strands must be complementary and the bases must not be involved in other stronger hydrogen bond formations. In the case of single-stranded RNAs, hybridization conditions are chosen that enable duplex formation to occur and remain stable, but do not permit stable intramolecular hydrogen bonding of small, randomly oriented complementary sequences.

Under conditions used for the hybridization of normal, single-stranded RNA molecules (e.g., those of Gould and Symons, 1977; Taylor *et al.*, 1976), viroid RNA molecules, with their high degree of ordered secondary structure may not be fully denatured and thus not show the expected kinetics of hybridization. If viroid RNA is not fully denatured during the hybridization reaction with its cDNA, then the cDNA may (1) not hybridize to the RNA; (2) hybridize at a much slower rate by hybridizing with partially denatured molecules; or (3) hybridize only to fragments of viroid RNA with no secondary structure. Therefore, attempts to quantitate the levels of viroid RNA in plant nucleic acid extracts by hybridization, must be carried out using conditions under which no viroid secondary structure remains and the rate of hybridization is dependent on only the total concentration of viroid sequences and not on the physical

nature of the RNA; i.e., circular, linear, or fragmented.

In this Chapter, the rate of hybridization of CSV under different conditions is investigated, with the development of a method that permits the quantitation of viroid sequences regardless of the physical nature of the RNA in plant nucleic acid extracts.

PSTV was shown to have some sequence homology with CSV (Owens *et al.*, 1978) as well as with several plant genomes (Hadidi *et al.*, 1976). In order to determine whether CSV sequences are also present in host DNA, hybridization studies involving the chrysanthemum and *Gynura aurantiaca* genomes were initiated. The analysis of these two CSV host genomes for the presence of sequences complementary to CSV is described in this Chapter as well as a comparison of my results with those of Hadidi *et al.* (1976).

MATERIALS

All materials used have previously been described in Chapters II - IV.

METHODS

A. Conditions of Hybridization

Hybridizations were carried out in either of two buffers; Buffer A: 0.18 M NaCl, 10 mM Tris-HCl, 0.05% sodium dodecyl sulphate, 1 mM EDTA, pH 7.0; Buffer B: 0.18 M NaCl, 10 mM Tris-HCl, pH 7.0, 2 mM EDTA, 40% (v/v) formamide. The nuclease S_1 assay of [^{32}P]cDNA:RNA hybrids was carried out as described in Chapter IV.

B. Thermal Denaturation of CSV

Viroid RNA was melted in either 1 x SSC (0.15 M Na Cl, 0.015 M trisodium citrate, pH 7.0) or in 1 x SSC containing 40% (v/v) formamide as described in Chapter III.

C. Thermal Denaturation of cDNA:RNA Hybrids

The thermal denaturation of cDNA:RNA hybrids was carried out as described in Chapter IV. Hybrids formed in hybridization Buffer A were denatured over the temperature range 60 - 100°C; those formed in Buffer B were denatured over the range 55 - 95%. Nuclease S_1 assays of hybrids were carried out as described in Chapter IV.

D. Preparation of Chrysanthemum and *Gynura aurantiaca* DNA.

Partially purified nucleic acids extracted via procedure C (Chapter II) from healthy or CSV-infected, chrysanthemums or *Gynura*, were sonicated, treated with 0.3 N NaOH at 37°C for 24 hours to digest RNA, neutralized with acetic acid, dialyzed overnight against 1 mM EDTA, pH 7, at 4°C to remove low molecular weight material and concentrated by ethanol precipitation. The DNA was collected by centrifugation and dissolved in water or hybridization Buffer A.

E. Preparation of cDNA to Chrysanthemum DNA

[^{32}P]cDNA to healthy chrysanthemum DNA was prepared by the method of Taylor *et al.* (1976) as described by Gould and Symons (1977), except that denatured, sonicated chrysanthemum DNA was the template, and actinomycin D was left out

of the cDNA synthesis reaction mixture.

F. Hybridization of cDNA to Genomic DNA

Hybridization of [^{32}P]cDNA to CSV, chrysanthemum 7S RNA or healthy chrysanthemum DNA against healthy or CSV-infected chrysanthemum or *Gynura* DNA was carried out in hybridization Buffer A at 60°C as described in Chapter IV; Materials and Methods.

RESULTS

A. Analysis of Hybridization Conditions

1. Factors Relevant to the Hybridization Analysis of CSV using Labelled cDNA

a. Hybridization buffers used:

Two buffers have been used, one (Buffer A) with a monovalent cation concentration of 0.19 M which we have used extensively for work with plant viral RNAs (Gould and Symons, 1977, 1978; Gould *et al.*, 1978), and another (Buffer B) which was essentially the same as Buffer A but also contained 40% (v/v) formamide, a concentration found suitable for other hybridization work (Friedrich and Feix, 1972; Hutton, 1977). In terms of cation concentration, Buffer A and Buffer B are essentially equivalent to 1 x SSC (0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0).

b. Melting profiles and T_m values:

The melting profiles of circular and linear CSV in 1 x SSC and circular CSV in 1 x SSC containing 40% (v/v) formamide are given in Figure V-1A to V-1C, respectively. Circular CSV showed a co-operative melting

FIGURE V-1

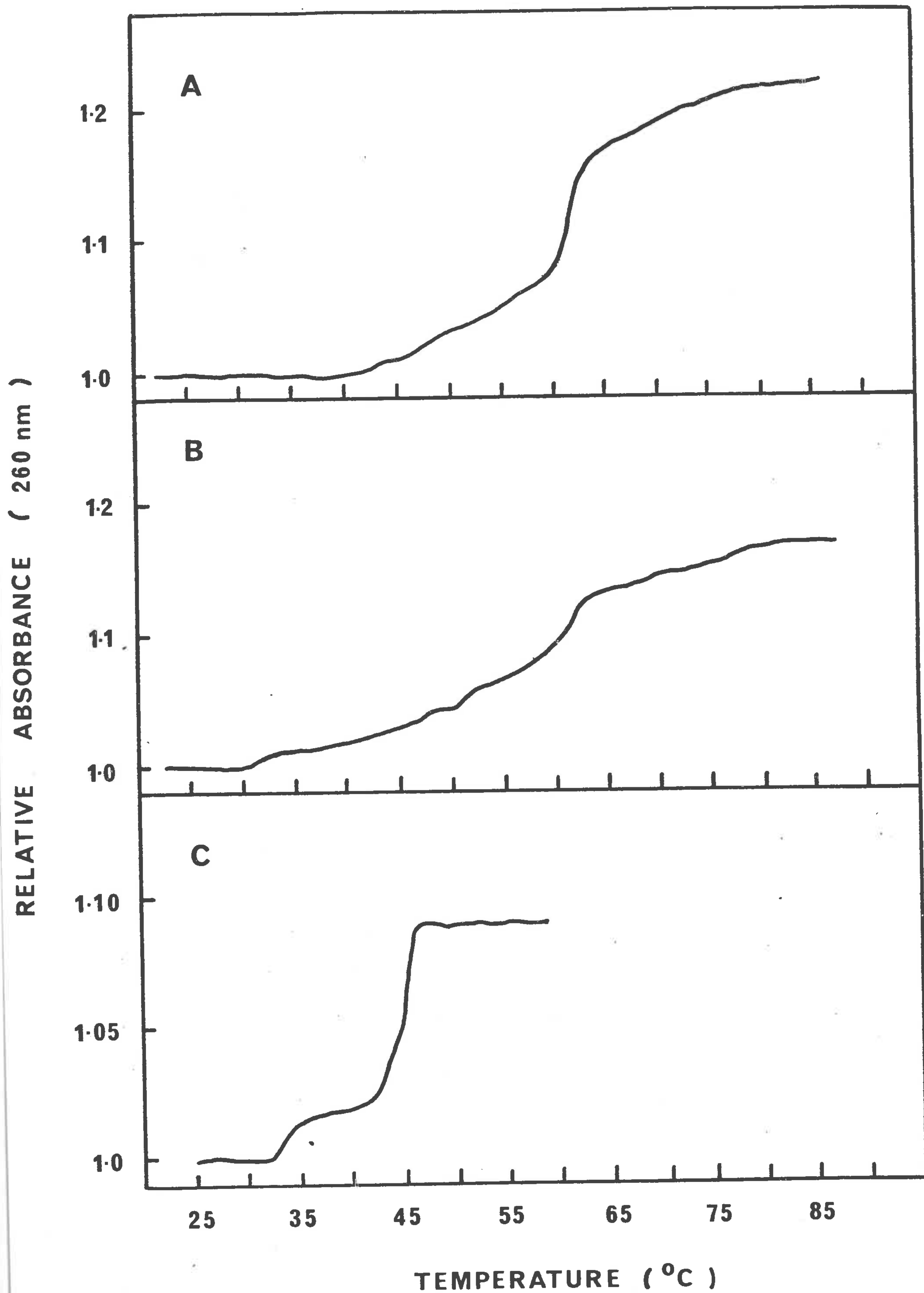
Thermal denaturation profiles of circular and linear CSV.

(A) Circular CSV in 1 x SSC;

(B) Linear CSV in 1 x SSC;

(C) Circular CSV in 1 x SSC containing 40% (v/v) formamide.

The temperature was increased at the rate of 1.0°C/min as described in Materials and Methods (Chapter III).


A
B
C
1·2
1·1
1·0
1·10
1·05
RELATIVE ABSORBANCE (260 nm)
25
35
45
55
65
75
85
TEMPERATURE (°C)

profile (Langowski *et al.*, 1978) in both solvents with T_m values of 62°C and 44°C, respectively (Table V-1). On the other hand, linear CSV, which is assumed to be the complete CSV molecule with a single nick since it is as equally infectious as the circular form (see Chapter III, Results), gave a broad melting profile in 1 x SSC with only a small region of co-operative melting and a T_m of approximately 57°C (Figure V-1B, Table V-1).

The melting profiles of circular CSV:[^{32}P]cDNA hybrids in Buffers A and B are given in Figure V-2. Sharp melting profiles were obtained in each case with T_m values of 81°C and 60°C, respectively (Table V-1).

In view of these results, the usual procedure of carrying out hybridizations at a temperature of T_m - 25°C (Wetmur and Davidson, 1968; Hutton, 1977) was considered unsuitable for the formation of CSV:[^{32}P]cDNA hybrids since this temperature would then be below the T_m of the CSV in both Buffers A and B (Table V-1). Hence, in most experiments described below, hybridizations in Buffer A were carried out at 60°C as these were conditions already used extensively in this laboratory (Gould and Symons, 1977, 1978; Gould *et al.*, 1978), while hybridizations in Buffer B were carried out at 50°C.

c. Preparation of labelled cDNA:

This was by the previously described method (Chapter IV) which involves the partial cleavage of circular CSV with nuclease S_1, the enzymic addition of a poly(A) tract to 3'-ends so produced, and the synthesis of cDNA with reverse transcriptase using the polyadenylated

TABLE V-1

T_m VALUES OF VARIOUS FORMS OF CSV AND OF CSV:[^{32}P]cDNA HYBRIDS[a]

Expt. No.	Nucleic Acid	Buffer[b]	T_m (°C)
1.	Circular CSV	1 x SSC	62
	Linear CSV	1 x SSC	57
	Circular CSV	1 x SSC in 40% (v/v) formamide	44
2.	CSV:[^{32}P]cDNA hybrid[c]	Buffer A	81
	CSV:[^{32}P]cDNA hybrid	Buffer B	60

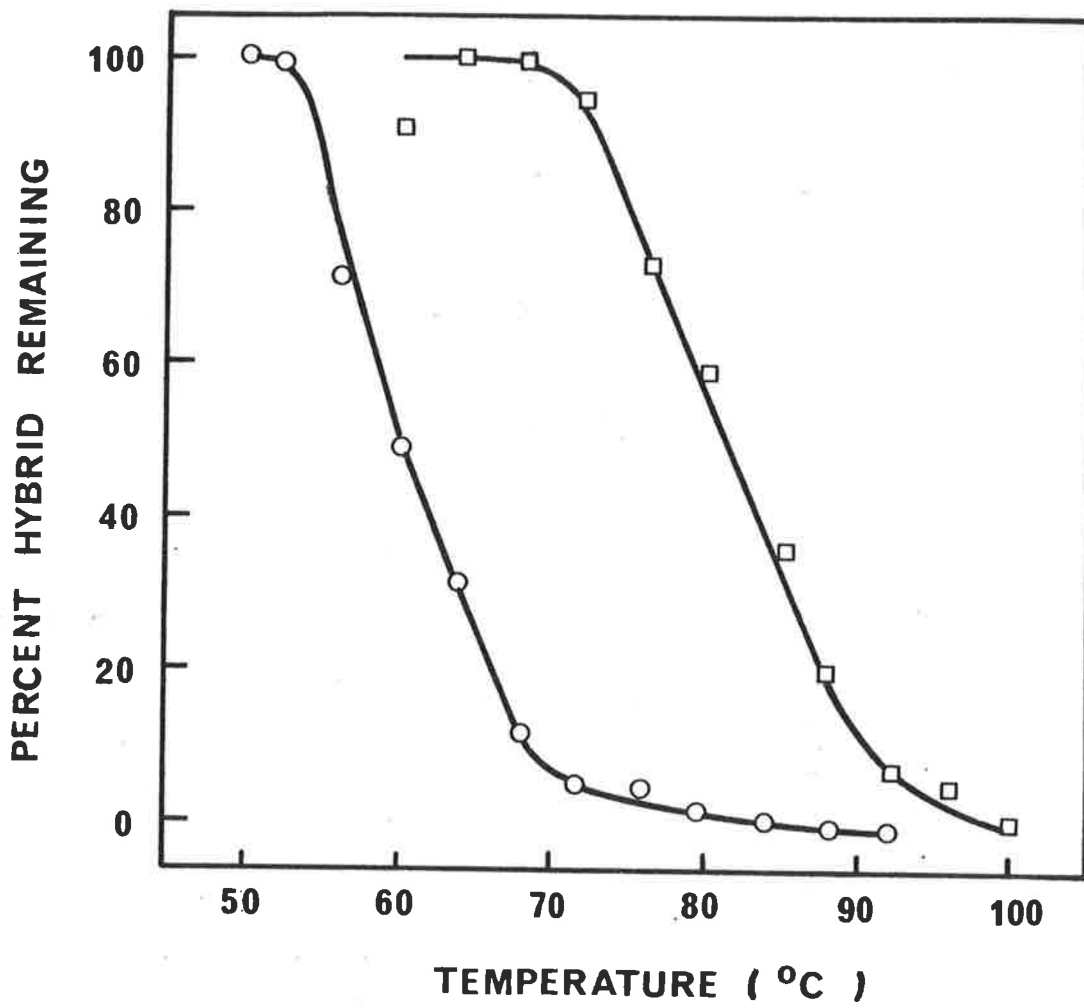
a T_m values taken from Figure V-1 for Expt. 1 and from Figure V-2 for Expt. 2.

b SSC, 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0; Buffer A, 0.18 M NaCl, 10 mM Tris-HCl, 0.05% sodium dodecyl sulphate, 1 mM EDTA, pH 7.0; Buffer B, 0.18 M NaCl, 10 mM Tris-HCl, pH 7.0, 2 mM EDTA, 40% (v/v) formamide.

c Circular CSV:[^{32}P]cDNA hybrids were formed as described in Figure V-2.

FIGURE V-2

Thermal denaturation profiles of circular CSV: [^{32}P]cDNA hybrids. CSV (1.5 μg/ml) was hybridized with 3 ng/ml [^{32}P]cDNA to a Rot of 4 x 10^{-1} mol sec $litre^{-1}$ in Buffer A at 60°C (□) or in a Buffer B at 50°C (○). The thermal denaturation of the hybrids in the same buffers and nuclease S_1 assay of the percentage hybrid remaining were carried out as described in Materials and Methods.


100
80
60
40
20
0
PERCENT HYBRID REMAINING
50
60
70
80
90
100
TEMPERATURE (^{o}C)

CSV as template and oligo$(dT)_{10}$ as a primer. This method was successful because of the substantial resistance of CSV to nuclease S_1 and it provided cDNA with high specificity probably because any contaminating host RNA sequences were degraded during the nuclease S_1 treatment. It is important to appreciate that the cDNA contained a mixture of linear DNA fragments of varying sizes and that it had a relatively low degree of secondary structure as indicated by its low resistance to the nuclease S_1 treatment used during the assay for DNA:RNA hybrids (Chapter IV).

2. Comparison of Hybridization Rates of Different RNAs in Two Hybridization Buffers

Initial experiments to test the hybridization of $[^{32}P]$cDNA to RNA in Buffers A and B at 60°C and 50°C, respectively, were carried out using *E. coli* 23S RNA (M_r 1.07×10^6; Stanley and Bock, 1965) and CMV satellite RNA (M_r 1.15×10^5; Richards *et al.*, 1978; Gould *et al.*, 1978), both of which had been polyadenylated at their 3'-ends *in vitro* prior to the oligo(dT)-primed synthesis of $[^{32}P]$-cDNA using reverse transcriptase. Identical hybridization curves were obtained for each RNA (Figure V-3A) in the two buffer systems with a Rot½ of 7×10^{-4} mol sec litre^{-1} for CMV satellite RNA and of 1.5×10^{-2} mol sec litre^{-1} for *E. coli* 23S RNA (Table V-2). It was hoped, therefore, that CMV satellite RNA could be used as a molecular weight standard to determine the molecular complexity of CSV RNA by hybridization analysis.

However, hybridization analysis of CSV $[^{32}P]$cDNA to the circular and linear forms of CSV RNA in the two buffer

FIGURE V-3

Rot curves of the hybridization of [^{32}P]cDNAs to their homologous RNAs in Buffer A at 60°C and in Buffer B at 50°C. Hybrids were formed and assayed with nuclease S_1 as described in Materials and Methods (Chapter IV).

(A) cDNA:*E. coli* 23S RNA in Buffer A (○) and in Buffer B (●); cDNA:CMV satellite RNA in Buffer A (□) and in Buffer B (■);

(B) cDNA to circular CSV:circular CSV in Buffer A (□) and in Buffer B (■); cDNA to circular CSV:linear CSV in Buffer A (○) and in Buffer B (●);

(C) cDNA to circular CSV:nuclease S_1-treated CSV in Buffer A (○) and in Buffer B (●).

The Rot curves were normalized to 100% hybrid formation to facilitate comparison. The actual plateau levels were:

(A) (○) 79%, (●) 89%, (□) 82%, (■) 79%;

(B) (○) 74%, (●) 64%, (□) 64%, (■) 50%;

(C) (○) 45%, (●) 45%.

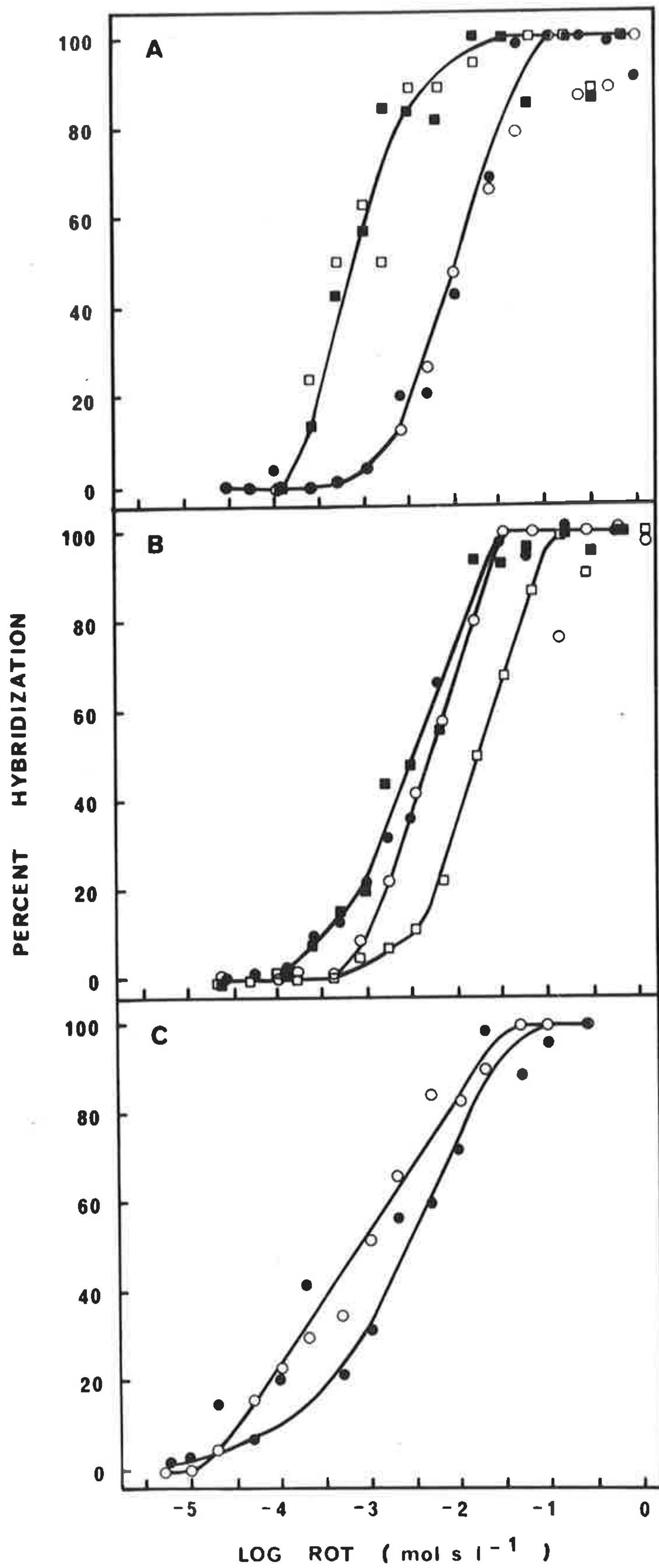

A
B
C
PERCENT HYBRIDIZATION
100
80
60
40
20
0
-5
-4
-3
-2
-1
0
LOG ROT (mol s l^{-1})

TABLE V-2

Rot½ VALUES FOR THE FORMATION OF VARIOUS RNA:[^{32}P]cDNA HYBRIDS[a]

Expt. No.	Source of RNA	RNA from which [^{32}P]cDNA prepared	Rot½ (mol s l^{-1})	
			Buffer A (60°C)	Buffer B (50°C)
1.	CMV satellite RNA	CMV satellite RNA	7.0×10^{-4}	7.0×10^{-4}
	E. coli 23S rRNA	*E. coli* 23S RNA	1.5×10^{-2}	1.5×10^{-2}
2.	Circular CSV	Circular CSV	2.0×10^{-2}	3.3×10^{-3}
	Linear CSV	Circular CSV	5.5×10^{-3}	3.3×10^{-3}
	Nuclease S_1-treated circular CSV	Circular CSV	8.0×10^{-4}	2.3×10^{-3}

[a] Rot½ values taken from data of Figure V-3A for Expt. 1 and from Figure V-3B and V-3C for Expt. 2. Buffers A and B were as in Table V-1.

systems gave the curves of Figure V-3B. The same Rot½ of 3.3 x 10^{-3} mol sec $litre^{-1}$ was obtained for both circular and linear CSV in Buffer B but two higher and appreciably different Rot½ values were obtained in Buffer A (Table V-2). By comparison with the Rot½ of CMV satellite RNA, which has essentially the same molecular weight as CSV (Richards *et al.*, 1978; my results not shown), these CSV Rot½ values were 5 to 30 times greater. It would appear, therefore, that residual secondary structure in the viroid had an appreciable inhibitory effect on the rate of hybridization, especially in the case of Buffer A at 60°C.

When the hybridization analysis was carried out against circular CSV RNA partially cleaved with nuclease S_1 (the same material from which the [^{32}P]cDNA was prepared), the hybridization curves of Figure V-3C were obtained. In Buffer A, a Rot½ value of 8 x 10^{-4} mol sec $litre^{-1}$ was obtained, and in Buffer B, a Rot½ of 2.3 x 10^{-3} mol sec $litre^{-1}$ (Table V-2). Since the Rot½ value obtained for nuclease S_1-treated CSV RNA in Buffer A is essentially the same as the obtained for CMV satellite RNA (Table V-2), it appears that the molecular complexity of CSV is equivalent to its molecular weight, a conclusion consistent with the fingerprint data on CSV (Gross *et al.*, 1977). There is at present no explanation for the 3-fold slower hybridization rate in the formamide-containing Buffer B as compared to Buffer A (Figure V-3C, Table V-2); CMV satellite RNA did not show this difference (Figure V-3A, Table V-2).

3. Rate of Hybridization as a Function of Temperature of Hybridization

The results of Figure V-3 were obtained with hybridization in Buffer A at 60°C and in Buffer B at 50°C and the variation in Rot½ values strongly suggested that the residual secondary structure of CSV was responsible for variations observed in the rate of hybridization. This was tested by carrying out hybridizations at 5 different temperatures in both buffer systems, the maximum temperature of hybridization selected being governed by the T_m of the DNA:RNA hybrids formed in that system (Figure V-2, Table V-1). Temperatures chosen ranged from 5°C to 25°C below the T_m of the hybrids. The results from these hybridization curves (Figure V-4) have been plotted as the log Rot½ vs $T_m - T_h$, where T_h is the temperature of hybridization (Figure V-5A); a linear relationship for both buffer systems as the hybridization temperature increased from 25°C to 10°C below the T_m was obtained. There was no further change in going from 10°C to 5°C below the T_m in the case of Buffer B, but there was a further increase in rate (lower Rot½) for Buffer A. By contrast, when the same experiment was carried out with CMV satellite RNA (Figure V-6B) and chrysanthemum 7S RNA (Figure V-6A), the more usual bell-shaped curves (Hutton, 1977; Wetmur and Davidson, 1968) were obtained (Figure V-5B and -5C). Further, there was only about a 3-fold change in Rot½ for these two latter RNAs over the temperature range used compared with a 50 - 100-fold change for CSV. The marked difference in results between CSV and the two other RNAs clearly indicates the importance of secondary structure

FIGURE V-4

Rot curves of the hybridization of [^{32}P]cDNA to circular CSV:circular CSV at different temperatures in Buffer A (A) or Buffer B (B). Hybrids were formed and assayed with nuclease S_1 as described in Materials and Methods (Chapter IV). The T_m's of the hybrids formed in Buffer A and Buffer B were (A) 81°C and (B) 60°C, respectively. The temperatures of hybridization were :

(A) (●) 76°C, (■) 71°C, (○) 66°C, (△) 61°C, (□) 56°C;

(B) (●) 55°C, (■) 50°C, (○) 45°C, (△) 40°C, (□) 35°C.

The Rot curves were normalized to 100% hybrid formation to facilitate comparison. The actual plateau levels were:

(A) (●) 62°, (■) 64%, (○) 69%, (△) 68%, (□) 68%;

(B) (●) 51%, (■) 54%, (○) 61%, (△) 66%, (□) 60%.

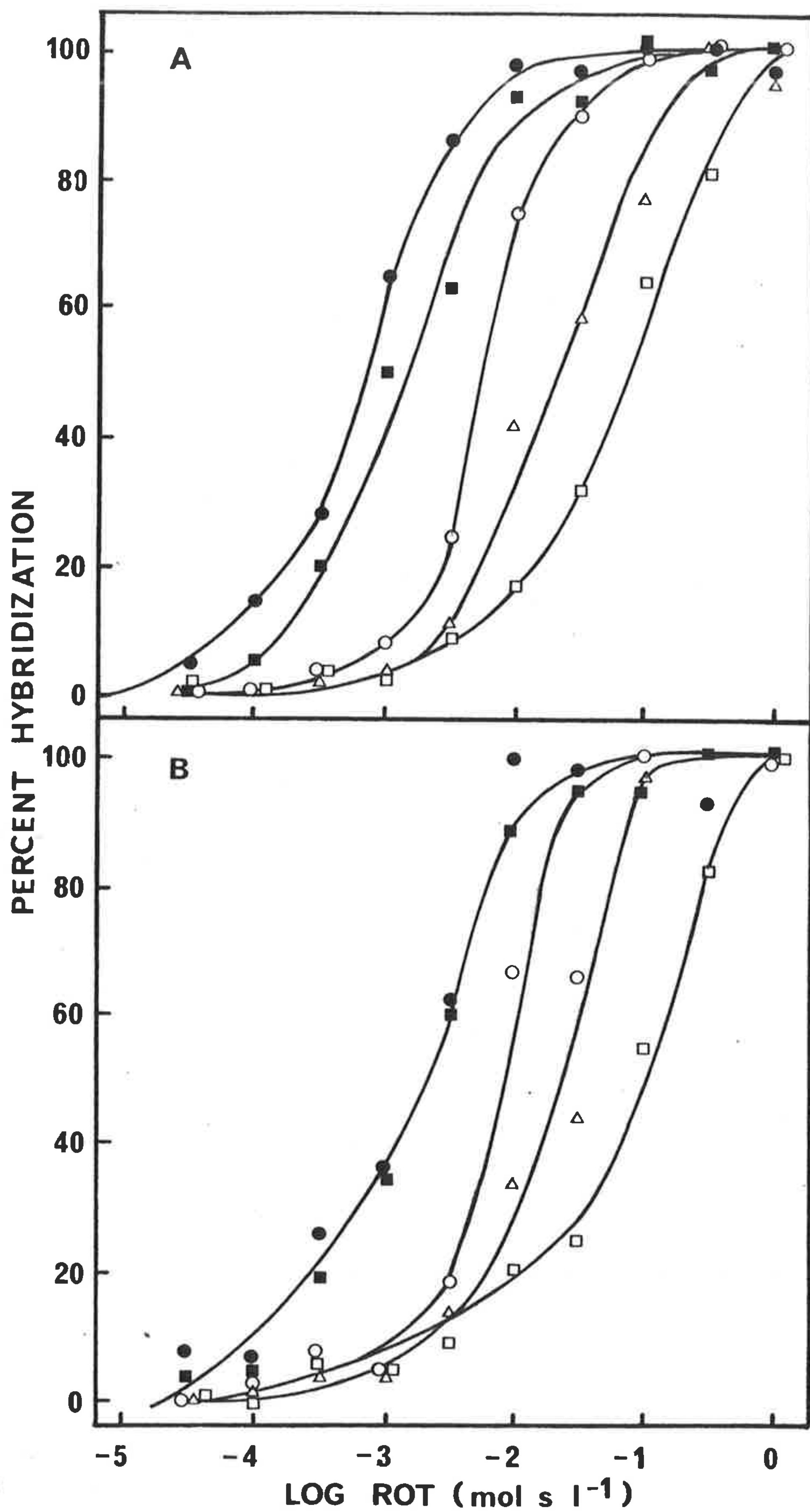

A
B
PERCENT HYBRIDIZATION
100
80
60
40
20
0
-5
-4
-3
-2
-1
0
LOG ROT (mol s l^{-1})

FIGURE V-5

The effect of temperature of hybridization of $[^{32}P]$cDNA to RNA on the rate of hybridization as measured by Rot½. Hybridizations were carried out in either Buffer A or Buffer B at five temperatures ranging from 5 to 25°C below the T_m of the hybrids formed in that buffer system (see Table V-1 and Figures V-4 and V-6). The Rot½ of each Rot curve (Figure V-4 and V-6) was plotted on a log scale as a function of the $T_m - T_h$° where T_h was the temperature of hybridization.

(A) cDNA to circular CSV:circular CSV hybridized in Buffer A (○) or Buffer B (●);

(B) cDNA to chrysanthemum 7S RNA:chrysanthemum 7S RNA hybridized in Buffer B (■);

(C) cDNA to CMV satellite RNA:CMV satellite RNA in Buffer A(△).

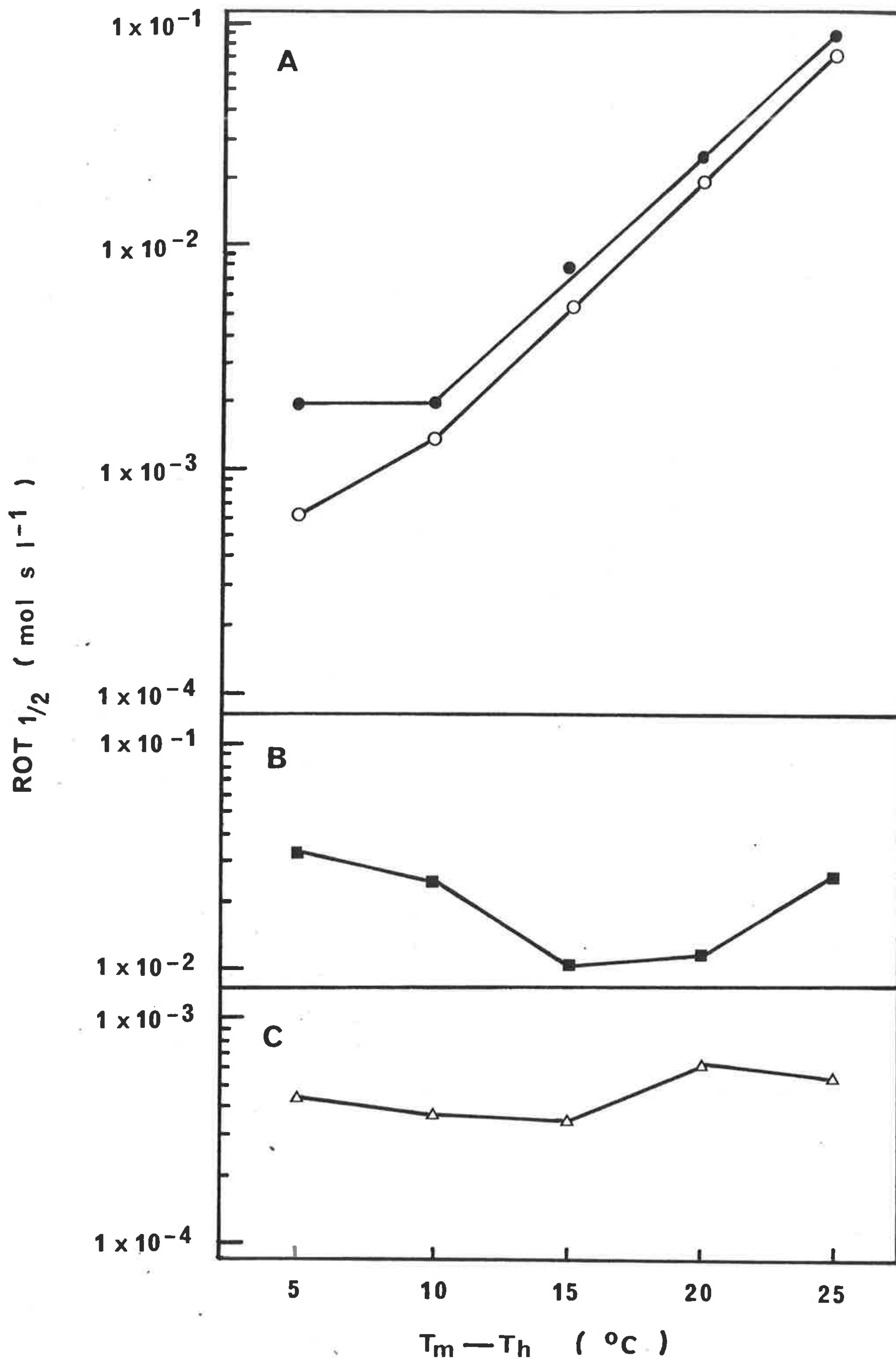

A
B
C
1 x 10^-1
1 x 10^-2
1 x 10^-3
1 x 10^-4
1 x 10^-1
1 x 10^-2
1 x 10^-3
1 x 10^-4
ROT 1/2 (mol s l^-1)
5
10
15
20
25
Tm — Th (°C)

FIGURE V-6

Rot curves of the hybridization of

(A) [^{32}P]cDNA to chrysanthemum 7S RNA:chrysanthemum 7S RNA in Buffer B and

(B) [^{32}P]cDNA to CMV satellite RNA:CMV satellite RNA in Buffer A.

Hybrids were formed and assayed with nuclease S_1 as described in Materials and Methods (Chapter IV). The T_m of the [^{32}P]cDNA to chrysanthemum 7S RNA:chrysanthemum 7S RNA hybrids in Buffer B was 64°C (results not shown); the T_m of the [^{32}P]cDNA to CMV satellite RNA:CMV satellite RNA hybrids in Buffer A was 88°C (Gould *et al.*, 1978). The temperatures of hybridization were:

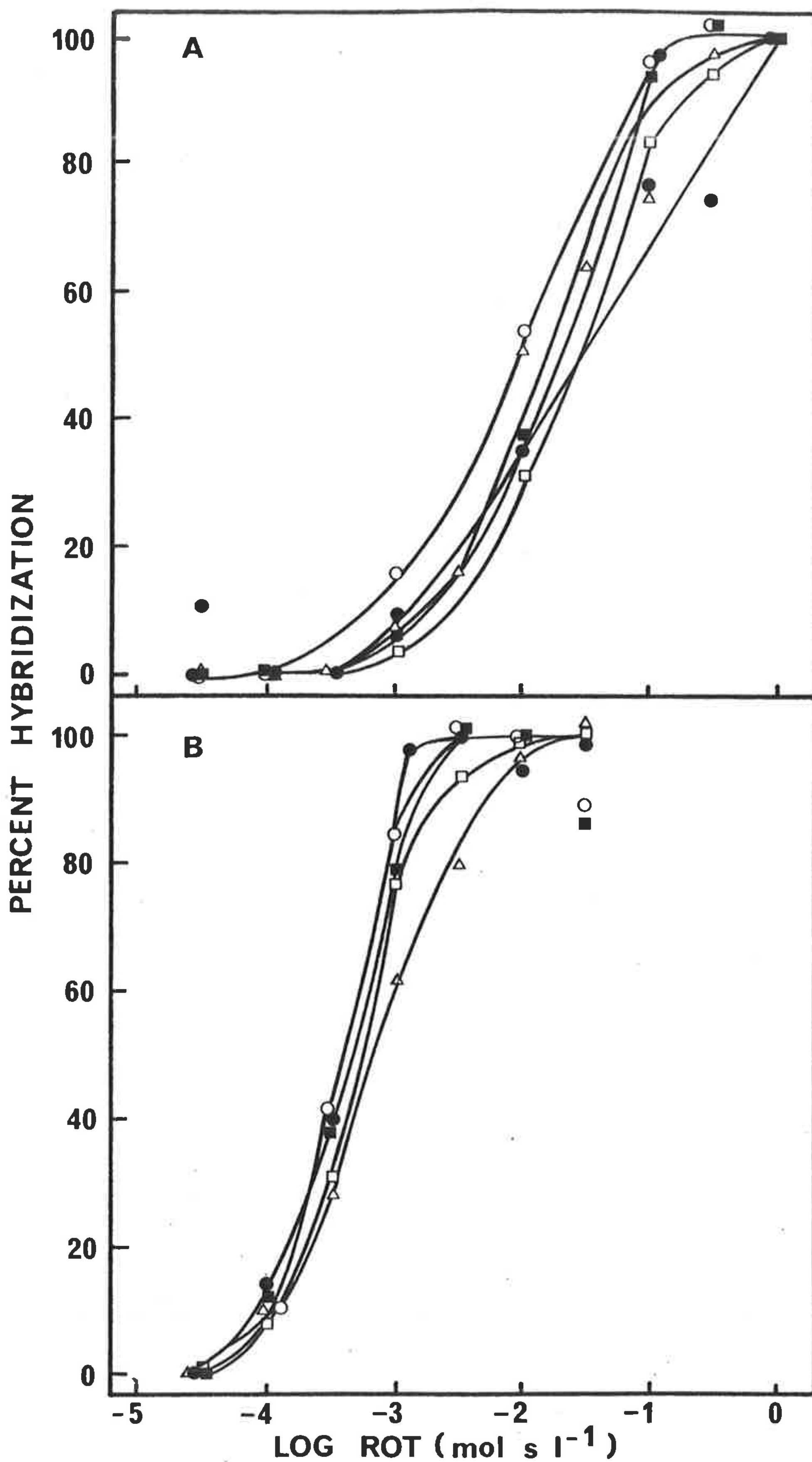
(A) (●) 59°C, (■) 54°C, (○) 49°C, (△) 44°C, (□) 39°C;

(B) (●) 83°C, (■) 78°C, (○) 73°C, (△) 68°C, (□) 63°C.

The Rot curves were normalized to 100% hybrid formation to facilitate comparison. The actual plateau levels were:

(A) (●) 50%, (■) 69%, (○) 52%, (△) 80%, (□) 67%;

(B) (●) 47%, (■) 84%, (○) 70%, (△) 65%, (□) 82%.


A
B
PERCENT HYBRIDIZATION
100
80
60
40
20
0
-5
-4
-3
-2
-1
0
LOG ROT (mol s l⁻¹)

in these hybridization reactions.

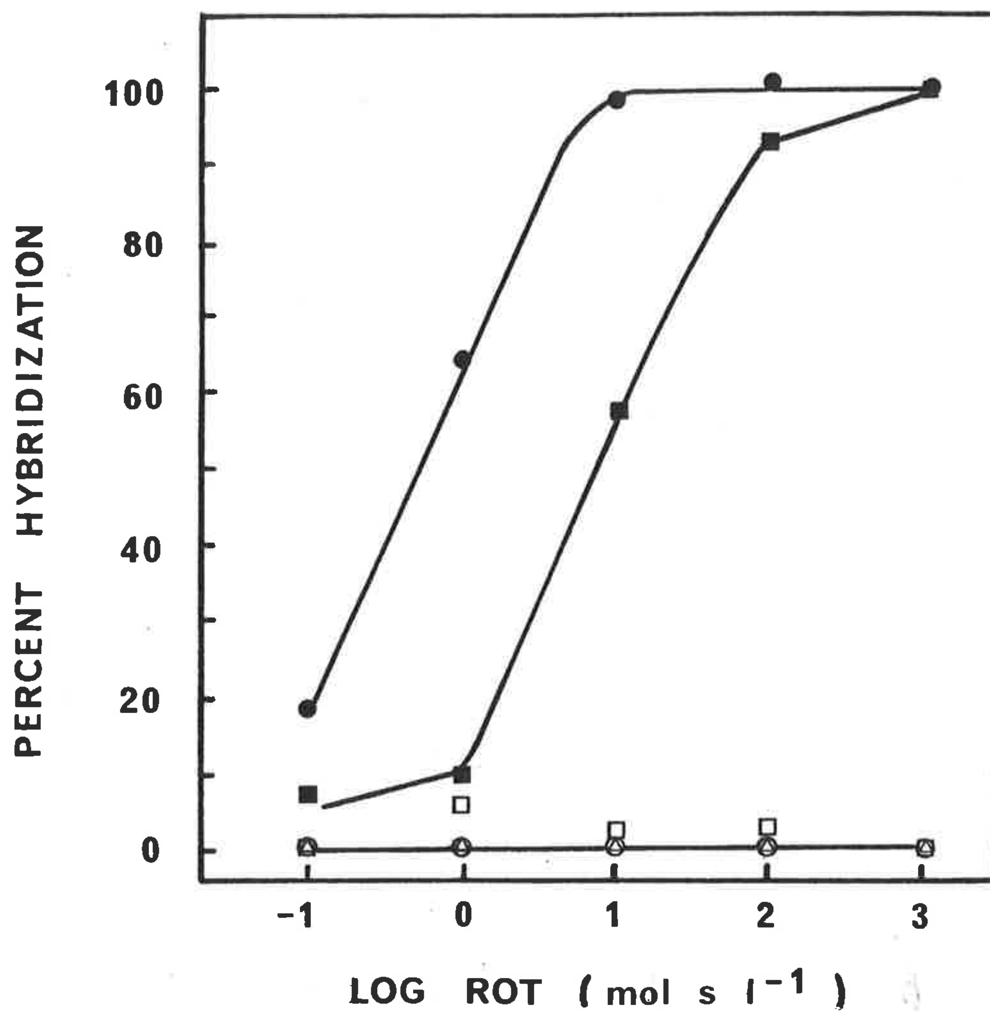
B. Quantitation of CSV Levels in Plant Nucleic Acid Extracts

In view of the preceding results, hybridization analyses using [^{32}P]cDNA to determine the level of CSV sequences in partially purified nucleic acid extracts were carried out in the formamide-containing Buffer B at T_m - 10°C (50°C) since, at this temperature, the maximum rate of hybridization was obtained (Figure V-5A) and the Rot½ values for the circular, linear and partial S_1 nuclease-cleaved RNAs of CSV were essentially the same (Table V-2). In addition, a temperature of 50°C in Buffer B rather than a temperature of 76°C in Buffer A (T_m - 5°C) was considered better suited to prolonged incubations as less RNA breakdown would be expected (Friedrich and Feix, 1972; Hutton, 1977).

Figure V-7 shows the Rot curves obtained when the [^{32}P]cDNA probe was used to determine the levels of CSV RNA in partially purified nucleic acid extracts of CSV-infected chrysanthemums and *Gynura*; Rot½ values of 5 x 10^{-1} and 7 mol sec litre^{-1}, respectively, were obtained. When these values were compared to the Rot½ value of 3.3 x 10^{-3} mol sec litre^{-1} for the purified viroid determined under the same conditions (Table V-2), it was calculated that CSV RNA constituted 0.66% by weight (3.3 x 10^{-3}/5 x 10^{-1}, x 100%) of the CSV-infected chrysanthemum nucleic acid extract, and 0.05% (3.3 x 10^{-3}/7, x 100%) of the CSV-infected *Gynura* nucleic acid extract. The 13-fold difference in the

FIGURE V-7

Hybridization at 50°C in Buffer B of [^{32}P]cDNA to circular CSV alone (△), or against partially purified nucleic acid extracts (Chapter II, Materials and Methods, extraction procedure C) from healthy chrysanthemums (○), CSV-infected chrsyanthemums (●), healthy *Gynura* (□), or CSV-infected *Gynura* (■). Hybrids were formed and assayed with nuclease S_1 as described in Materials and Methods (Chapter IV), except that the nuclease S_1 concentration was 50 units/ml. Rot curves were normalized to 100% hybrid formation to facilitate comparison. The actual values for CSV-infected chrysanthemums and CSV-infected *Gynura* were 36% and 40%, respectively.



PERCENT HYBRIDIZATION
100
80
60
40
20
0
-1
0
1
2
3
LOG ROT (mol s l^{-1})

level of CSV in the two extracts is consistent with the qualitative difference already noted (Chapter II) when these extracts were analysed by polyacrylamide gel electrophoresis. Since we routinely obtain 250 mg of nucleic acid (DNA and low molecular weight RNA) in the partially purified nucleic acid extract from chrysanthemums and 190 mg of nucleic acid from *Gynura* per kg of plant material using the procedure described in Chapter II (procedure C), these values represent 1.65 mg and 0.095 mg of CSV per kg, respectively.

When the CSV [^{32}P]cDNA was hybridized against partially purified nucleic acid extracts prepared from healthy chrysanthemums and *Gynura*, no hybridization was found up to a Rot of 10^3 mol sec litre^{-1} (Figure V-7). This result not only indicates the purity of the cDNA probe by the virtual absence of sequences complementary to host RNA, but it also provides an upper estimate of the presence of CSV sequences in the partially purified nucleic acid extract of healthy plants. If such sequences were present, then they must represent appreciably less than 0.00033% by weight ($3.3 \times 10^{-3}/1 \times 10^3$, x 100%) of the nucleic acid extract.

C. Analysis of Plant DNA for the Presence of CSV Sequences

1. Hybridization of CSV cDNA to Chrysanthemum and *Gynura* DNA

[^{32}P]cDNA, prepared against the circular form of CSV, was hybridized to sonicated, denatured healthy and CSV-infected chrysanthemum and *Gynura* DNA in hybridization Buffer A at 60°C (under these hybridization conditions, any

linear viroid DNA sequences present in the host genome would not be expected to exhibit any secondary structure that could affect the rate of hybridization). As can be seen in Figure V-8A, no-to-negligible (6%) hybridization occurred against all four sources of DNA even at a Cot (concentration of host DNA in moles of deoxyribonucleotide per litre x time of hybridization in seconds) value of 1×10^4 mol sec litre^{-1}. Under these same conditions of hybridization, [^{32}P]cDNA to chrysanthemum 7S RNA hybridized to CSV-infected chrysanthemum DNA with a Cot½ of 30 mol sec litre^{-1} (Figure V-8A).

2. Determination of the Size of the Chrysanthemum Genome

Whether the above hybridization reactions of CSV cDNA to host DNA were carried out in cDNA excess or single-copy gene excess over the cDNA, could not be determined without knowing the size of the chrysanthemum or *Gynura* genome. Since the DNA content/diploid cell varies enormously between chrysanthemum species (Baetcke *et al.*, 1967), the size of the "Charm type" chrysanthemum genome was determined from reassociation kinetics of chrysanthemum DNA by comparison with similar data from the tomato genome where the DNA content/diploid cell was known. The re-association curve of chrysanthemum DNA (Figure V-8B; using [^{32}P] random-primed chrysanthemum DNA as a "tracer" of the hybridization reaction between the two complementary chrysanthemum DNA strands) was triphasic; the Cot½ values 10 - 15, 250 and 1.3×10^4 mol sec litre^{-1} probably reflected the reassociation of highly repeated, intermediate repeated

FIGURE V-8

Hybridization to genomic DNA.

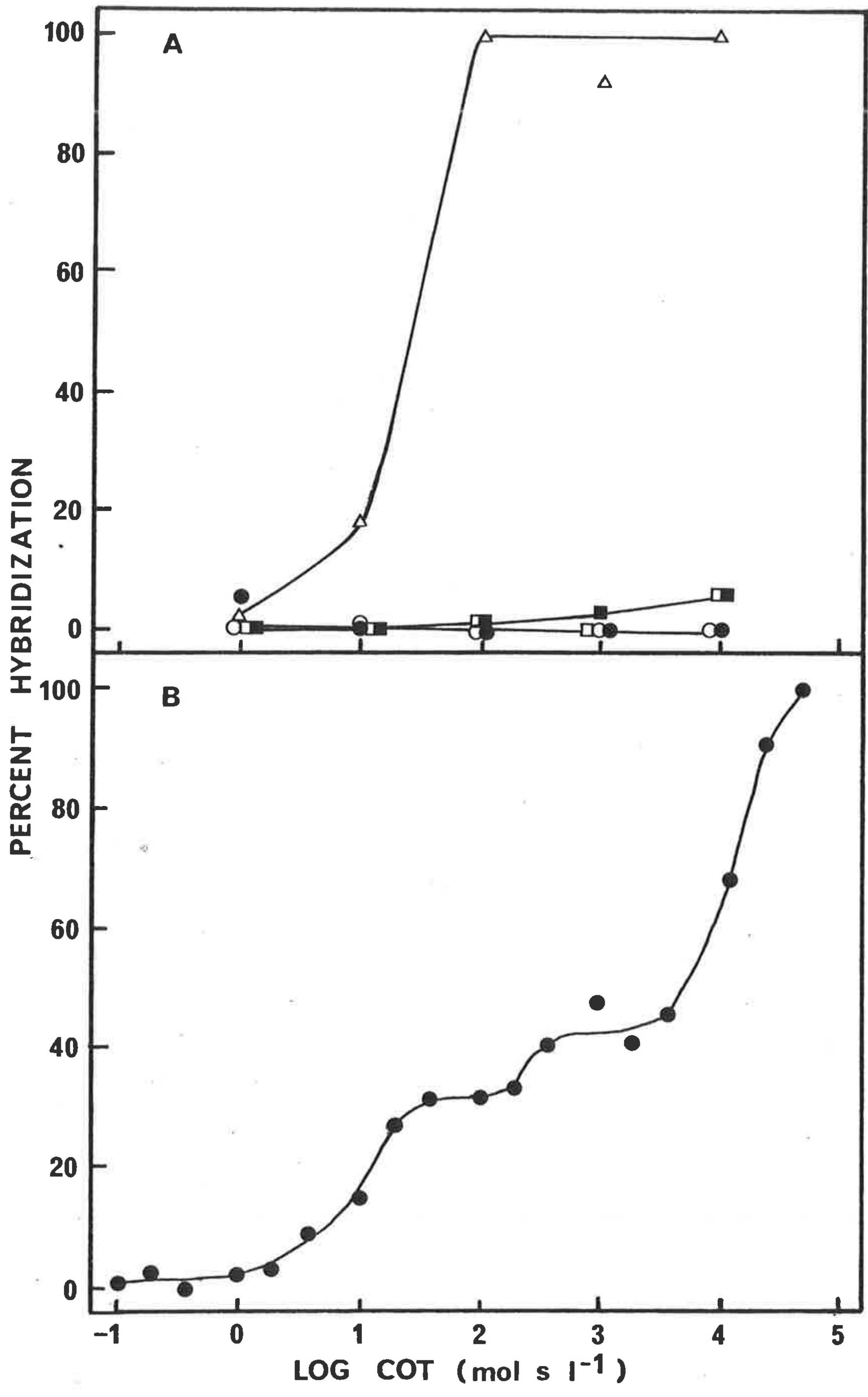
(A) Hybridization of [^{32}P]cDNA to CSV against healthy chrysanthemum DNA (□), CSV-infected chrysanthemum DNA (■), healthy *Gynura* DNA (○), CSV-infected *Gynura* DNA (●) and [^{32}P]cDNA to chrysanthemum 7S RNA against CSV-infected chrysanthemum DNA (△).

(B) Re-association of chrysanthemum DNA and [^{32}P] chrysanthemum DNA (●).

Hybridizations and nuclease S_1-assays were carried out as described in Materials and Methods. Cot curves for [^{32}P]cDNA-7S RNA:chrysanthemum DNA and [^{32}P]cDNA-chrysanthemum DNA:chrysanthemum DNA were normalized to 100% hybridization; Cot curves for [^{32}P]cDNA-CSV:host DNA were normalized relative to [^{32}P]cDNA-7S:chrysanthemum DNA hybrids (i.e., the same correction factors were applied). The actual plateau or maximum hybridization values were:

(A) (□) 6%, (■) 6%, (○) 4%, (●) 6%, (△) 38%.

(B) (●) 61%.



A
B
100
80
60
40
20
0
PERCENT HYBRIDIZATION
-1
0
1
2
3
4
5
LOG COT (mol s l-1)

and unique (single-copy) sequences, respectively. A comparison of the Cot½'s of the unique sequence DNA from chrysanthemum (1.3×10^4 mol sec litre^{-1}) and tomato (2×10^3 mol sec litre^{-1}; Hadidi *et al.*, 1976) with the DNA content/diploid cell of the tomato genome (3.9 pg; Rees and Jones, 1972) suggested the DNA content/diploid cell of the chrysanthemum genome was 25 pg ($1.3 \times 10^4 \times 3.9 \div 2 \times 10^3$) and the molecular weight of the chrysanthemum genome was 1.5×10^{13} ($6.023 \times 10^{23} \times 2.5 \times 10^{-11}$). The proportion of haploid, single-copy, double-stranded DNA of molecular weight 1×10^5 is 2.7×10^{-8} [(2 strands/n $\times$ 1×10^5) $\div$ ($1.5 \times 10^{13} \div 2n$)] and thus the amount of single-copy, single-stranded DNA of molecular weight 1×10^5 present in the 200 µg of DNA used/hybridization point was 2.7 pg (200 µg diploid DNA = 100 µg haploid DNA $\times$ 2.7×10^{-8}). Therefore, the CSV cDNA (22 pg) was in an 8-fold excess over the amount of single-copy sequence/haploid genomic DNA and the hybridization was carried out under cDNA excess conditions.

DISCUSSION

A. Optimization of Hybridization Conditions

The ability of residual viroid secondary structure to decrease the rate of hybridization of viroid RNA to its cDNA was demonstrated here with CSV; this effect was not observed by Owens (1978) in the hybridization of PSTV-cDNA to PSTV. However, as will be shown below, analysis of the data of Owens (1978) does indeed show an effect by residual PSTV secondary structure on the rate of hybridization of

PSTV-cDNA to PSTV.

Rot analysis of cDNA-PSTV:PSTV (Owens, 1978) produced a Rot½ of 6×10^{-4} mol sec litre^{-1}. Owens (1978) claimed that this Rot½ was consistent with the expected complexity of PSTV (M_r 1.2×10^5; Gross *et al.*, 1978) based on a comparison of the Rot½ and molecular weight of PSTV with the Rot½ (1.7×10^{-3} mol sec litre^{-1}) and molecular weight (4×10^5) of the satellite RNA of tobacco necrosis virus (S-TNV RNA); the Rot½ of S-TNV RNA was determined by Taylor *et al.* (1976) in the same hybridization buffer used by Owens (1978). On this basis of this comparison, the expected Rot½ of PSTV was 5.1×10^{-4} mol sec litre^{-1} ($1.2 \times 10^5 \times 1.7 \times 10^{-3} \div 4 \times 10^5$). This value was considered to be in good agreement with the observed Rot½ of 6×10^{-4} mol sec litre^{-1}.

However, Rot values in Table III of Taylor *et al.* (1976) had all been corrected for salt concentration (0.6 M Na^+ to 0.18 M Na^+); i.e., "a correction factor of 5.0 for salt concentration was used" (p. 326 of Taylor *et al.*, 1976). Therefore, the actual Rot½ of S-TNV RNA in the hybridization buffer of Taylor *et al.* (1976) containing 0.6 M Na^+ was 3.4×10^{-4} mol sec litre^{-1} ($1.7 \times 10^{-3} \div 5$) and the expected Rot½ of PSTV would have been 1.0×10^{-4} mol sec litre^{-1} ($1.2 \times 10^5 \times 3.4 \times 10^{-4} \div 4 \times 10^5$) (Table V-3). Thus, PSTV was hybridizing to its cDNA at a rate 6 times ($6 \times 10^{-4} \div 1 \times 10^{-4}$) slower than expected.

When PSTV-cDNA was hybridized to PSTV in a buffer containing 0.15 M Na^+ at the same temperature as above (68°C), a two-fold decrease in the Rot½ was observed (Owens,

TABLE V-3

a Observed Rot½

b Expected Rot½

c Expected T_m of PSTV

d T_h = Temperature of hybridization = 68°C

e value from Gross *et al.* (1978)

f values from Taylor *et al.* (1978)

g values from Owens (1978)

h Derived as described in Discussion (Chapter V)

TABLE V-3

A COMPARISON OF THE T_m OF PSTV AND THE Rot½ OF cDNA-PSTV:PSTV HYBRIDS AT TWO DIFFERENT $[Na^+]$: COMPLEXITY ESTIMATES OF PSTV RELATIVE TO S-TNV RNA

RNA	M_r	$[Na^+]$	Obs. Rot½[a] (mol sec $litre^{-1}$)	Exp. Rot½[b] (mol sec $litre^{-1}$)	Obs. Rot½ / Exp. Rot½	Exp. T_m[c] (°C)	$T_m - T_h$[d]
PSTV	1.2×10^5[e]	0.15 M	1.2×10^{-3}[g]	3.2×10^{-4}[h]	1.7	66[h]	-2
		0.6 M	6.0×10^{-4}[g]	1.0×10^{-4}[h]	6.0	75[h]	+7
S-TNV	4×10^5[f]	0.15 M	-	2.4×10^{-3}[h]			
		0.6 M	3.4×10^{-4}[f]	-			

1978). Under these conditions, the expected Rot½ for S-TNV RNA would have been 2.4×10^{-3} mol sec litre^{-1} [3.4×10^{-4} x 7-fold correction factor (Britten and Smith, 1970)]. Under these conditions, the Rot½ of PSTV was 1.7 times [($6 \times 10^{-4} \times 2$) ÷ ($1.2 \times 10^5 \times 2.4 \times 10^{-3} \div 4 \times 10^5$)] higher than expected (Table V-3).

That the above results were caused by the effect of residual secondary structure present in PSTV on the rate of hybridization, can be shown (see below) by determining the T_m of PSTV in 0.15 M Na^+ and 0.6 M Na^+, and comparing these T_m's to the temperature of hybridization (68°C).

The determination of the T_m of PSTV at different salt concentrations, is based on the following relationships.

(1) Cordeiro-Stone and Lee (1976) demonstrated the existence of a linear relationship between the T_m of satellite (double-stranded) DNA and the log of the ionic strength ([Na^+]) of a buffer. This relationship is expressed by the equation

$$T_{m_1} - T_{m_2} = 18.7 \times (\log \mu_1 - \log \mu_2), \qquad \text{Equation I}$$

where μ_1 and μ_2 are the ionic strengths which produce T_{m_1} and T_{m_2}, respectively. The slope value, 18.7 is true for double-stranded DNA.

(2) The slope for double-stranded RNA can be calculated from values in Figure I of Friedrich and Feix (1972); the slope is 13.6 (derivations not shown).

(3) The slope for viroids can be calculated from a comparison of the T_m's of circular CSV in 1 x SSC (T_m = 62°C) and in 0.1 x SSC (T_m = 47°C). The slope derived from the T_m's of CSV is 15 [(62°C - 47°C) ÷ (log 0.195 - log 0.0195)].

(4) The slope for viroids can also be calculated from Figure 3 of Langowski *et al.* (1978), where a linear relationship between the T_m of CPFV and the log of the sodium ion concentration was demonstrated. The slope derived from values in Figure 3 (Langowski *et al.*, 1978) is 13. Therefore, the average viroid slope (and the one used in the calculations below) is 14 and is similar to the slope for double-stranded RNA.

(5) Since the T_m of PSTV in 0.1 x SSC (0.0195 M Na^+) is 54°C (Diener and Hadidi, 1977), the T_m of PSTV in 0.6 M NA^+ should be 75°C [54°C + 14 (log 0.6 - log 0.0195) = 54°C + 14 (-0.22 - -1.71)] and the T_m of PSTV in 0.15 M Na^+ should be 66°C [54°C + 14 (log 0.15 - log 0.0195)]. Thus, as can be seen from Table V-3, in a hybridization buffer containing 0.6 M Na^+ the T_m of PSTV is 7°C above the temperature of hybridization (68°C) and in a hybridization buffer containing 0.15 M Na^+ the T_m of PSTV is 2°C below the temperature of hybridization. Therefore, under the former set of conditions, PSTV would be expected to hybridize to its cDNA at a rate much slower (observed rate = 6 times slower) than expected, and under the latter set of conditions, PSTV would be expected to hybridize to its cDNA at a rate only slight slower (observed rate = 1.7 times slower) than expected, since the RNA is in a more denatured state. Hence, the hybridization of PSTV cDNA to PSTV (Owens, 1978) and the demonstration of increasing levels of PSTV in PSTV-infected tomatoes as a function of time after inoculation (Owens *et al.*, 1978) were not carried out under optimized hybridization conditions. This is in contrast to the situation

with CSV-cDNA hybridized to partially purified nucleic acid extracts from CSV-infected chrysanthemums and *Gynura* (see below), where the rate of hybridization was optimized.

The above calculations for PSTV were based on an assumption that most of the RNA was the circular form of PSTV; the results of Owens *et al.* (1977) suggest that only 18% of the RNA is the circular form. This may affect the above calculations to the extent that the T_m of PSTV in 0.6 M Na^+ will be lower by a few degrees (cf. circular CSV vs linear CSV).

B. Quantitation of CSV Levels in Plant Nucleic Acid Extracts

A comparison of the level of CSV in partially purified nucleic acid extracts (1.65 mg CSV/kg chrysanthemums) with the yield of purified CSV (100 - 200 μg/kg chrysanthemums) suggests a recovery of 6 - 12% of the CSV present in the partially purified nucleic acid extract. As the efficiency of electro-elution of CSV was 50 - 75%/gel (see Materials and Methods, Chapter III), the expected yield of CSV was 400 - 900 μg/kg chrysanthemums. The reason for the observed low recovery is not known, but it is feasible that fragmented CSV may account for some of the RNA to which the [^{32}P]cDNA hybridized; this fragmented RNA would be removed during the purification process. The 13-fold lower level of CSV in *Gynura* cf. chrysanthemum could not be correlated with a yield of purified CSV from CSV-infected *Gynura*, since CSV was never purified from this host.

In determining the levels of CSV in partially purified nucleic acid extracts, it must be remembered that the values

quoted are minimal estimates, since the efficiency of nucleic acid extraction is not known. It should be feasible, however, to determine the efficiency of extraction by re-extracting all phases (e.g., plant debris) by either the same or different extraction procedures and quantitating the level of CSV in them.

In summary, the hybridization kinetics of [^{32}P]cDNA to CSV have been investigated and the results have led to the development of a suitable method for the accurate and specific estimation of CSV sequences in nucleic acid extracts of infected plants. The approach and methodology used here should be applicable to other viroids.

C. Analyzing Plant Genomes for Viroid Sequences

No sequence homology between CSV and either the (healthy or CSV-infected) chrysanthemum or *Gynura* genomes was observed. This is similar to the results of Branch, Dickson and Zaitlin (personal communications), who observed no sequence homology between PSTV and tomato DNA, but is in contrast to the result of Hadidi *et al.* (1976) wherein sequence homology between PSTV and DNA from several plants including non-hosts of PSTV was observed.

In the case of CSV cDNA hybridized to chrysanthemum DNA, the hybridization reaction was carried out in cDNA excess over a single-copy gene of M_r 1 x 10^5 (as calculated from the specific activity of the cDNA; see Results). The Dot value (Dot = concentration of cDNA in moles of dNMP/litre x time of hybridization in seconds) for the hybridization was 1.1 x 10^{-3} mol sec litre^{-1} (22 pg/40 µl x 7 days). At this Dot value (comparable to the Rot value of S_1-cleaved

CSV RNA against CSV cDNA in the same hybridization buffer (Figure V-3C), since the T_m of cDNA is lower than the T_m of RNA), approximately 60 - 70 % of the maximum amount of possible hybridization could have occurred. As 12% of the cDNA (2.7 pg single-copy DNA ÷ 22 pg cDNA CSV) is the maximum amount that could have been protected, then the observed percent hybridization at this Dot value should have been 7 - 8% (12% x 60 - 70%). This value is not significantly above the variable background of the cDNA alone (2 - 5%) to be able to draw any conclusions from the 6% hybridization of CSV cDNA to chrysanthemum DNA observed in Figure V-8A. Hence, in order to increase the level of hybridization to a significant percentage value (10 - 20% or more), it would be necessary to reach higher Dot values. However, this could only be carried by increasing the time of hybridization and/or decreasing the volume of the reaction; increasing the amount of cDNA would result in a lower maximum level of possible hybridization to single-copy genes (see above).

The above calculations assume CSV and other viroids are represented by only one-copy/haploid genome. From Figure 1 of Hadidi *et al.* (1976), the excess of RNA ([^{125}I]-PSTV) over a single-copy gene in the DNA can be calculated (Table V-4) for both the tomato and the barley genomes. The Rot value for PSTV under the conditions described by Hadidi *et al.* (1976) would have been 8 x 10^{-3} mol sec litre^{-1} (20 pg [^{125}I]PSTV/10 μl x 336 hours). From Table V-4, it can be seen that the amount of [^{125}I]PSTV hybridized exceeds the amount expected if only one copy of PSTV existed/haploid genome. These values are further complicated by the observation

TABLE V-4

[a] Values derived from Results and Discussion, Chapter V.

[b] Values derived from Hadidi *et al.* (1976)

[c] DNA content/diploid cell

[d] Value obtained from Flavell *et al.* (1974)

[e] M_r of diploid genome = DNA content/2C x 6.023×10^{23}.

[f] Proportion of single-copy DNA of M_r 1×10^5 = single-stranded M_r x 2 strands/haploid genome ÷ M_r of haploid genome.

[g] Amount of single-copy DNA/hybridization = amount of haploid DNA/hybridization X proportion of single-copy DNA of M_r 1×10^5.

[h] Assuming 1 copy of a gene with an M_r of 1×10^5/ haploid genome = g x amount of diploid DNA/hybridization ÷ 2.

[i] Cot value (for genomic DNA) or Rot and Dot value (for [^{125}I]PSTV and [^{32}P]cDNA-CSV, respectively) giving rise to the "Observed Percent Hybridization".

TABLE V-4

A COMPARISON OF VALUES RELEVANT TO THE HYBRIDIZATION OF VIROID PROBES AGAINST PLANT GENOMIC DNA

Relevant Value	DNA Source		
	Chrysanthemum[a]	Tomato[b]	Barley[b]
DNA content/2C[c]	2.5×10^{-11}g	3.9×10^{-12}g	1.3×10^{-11}g[d]
M_r of 2C genome[e]	1.5×10^{13}	2.3×10^{12}	8.0×10^{12}
Proportion of single-copy DNA[f]	2.7×10^{-8}	1.7×10^{-7}	5.0×10^{-8}
Amount of diploid DNA/hybridization	2×10^{-4}g	5×10^{-5}g	5×10^{-5}g
Amount of single-copy DNA/hybridization[g]	2.67×10^{-12}g	4.25×10^{-12}g	1.25×10^{-12}g
Amount of probe/hybridization	2.2×10^{-11}g	2.0×10^{-11}g	2.0×10^{-11}g
Percent Hybridization Expected[h]	12%	~~23%~~ 21%	6%
Percent Hybridization Observed	6%	35%	13%
Cot value (mol sec litre^{-1})[i]	1×10^{4}	2×10^{4}	2×10^{4}
Rot or Dot value (mol sec litre^{-1})[i]	1.1×10^{-3}	8.1×10^{-3}	8.1×10^{-3}

that only 17% and 60% of the [^{125}I]PSTV could hybridize to the barley and tomato genomes, respectively, at an infinite DNA/RNA ratio (Hadidi *et al.*, 1976). Furthermore, it is difficult to understand how PSTV could be represented by single-to-a-few-copies/haploid genome, when a hybridization reaction (performed to determine the thermal stability of PSTV:genomic DNA hybrids; Figure 3 of Hadidi *et al.*, 1976) containing 500 μg of genomic tomato DNA and 5 ng of [^{125}I]-PSTV was in a 200-fold RNA (PSTV) excess over single-copy genes [5×10^{-9} g PSTV ÷ (5×10^{-4} g diploid DNA ÷ 2 n, x 1.7×10^{-7} x 60% maximum hybridization), i.e., 0.5% of the [^{125}I]PSTV should have hybridized/single-copy gene (2.5×10^{-11} g/5×10^{-9} g, x 100%)] and the observed percent hybridization was 46 - 48% (Table 1, Hadidi *et al.*, 1976).

Clearly, there are discrepancies in the data supplied by Hadidi *et al.* (1976) and the possibility that the above results may be due to the hybridization of contaminating host RNAs, present in the PSTV preparations, to the genomic DNAs should be examined. Such a possibility is further strengthened by reports of the non-hybridization of PSTV to tomato DNA, but the hybridization of contaminating host RNAs, present in some PSTV preparations, to tomato DNA (Semancik, 1979; Branch and Dickson, personal communication).

CHAPTER VI

AVOCADO SUNBLOTCH VIROID

INTRODUCTION

The avocado (*Persea americana*) is a fruit-tree of New World origin. At present, it is a tree of economic importance in a number of sub-tropical countries (Gustafson, 1976) subject to two main disease: (1) avocado root rot, caused by the fungus *Phytophthora cinnamomi*; and (2) a "viral" disease called avocado sunblotch disease.

The avocado (from the Aztec: Ahuacaquahuitl, or its contraction, Ahuacatl) was first described in "western literature" during the conquest of Mexico by Cortez (de Enciso, 1519). As a fruit tree, it was well known to Aztec, Mayan, Incan and Pre-Incan civilizations (Gustafson, 1976), but it was not cultivated by these people. The avocado was introduced into the U.S. in either 1848 (Gustafson, 1976) or 1858 (Poole and Poole, 1967) and not grown commercially until 1900 (Poole and Poole, 1967).

Sunblotch, as a disease (or syndrome) affecting avocados was first recorded in 1914 (Whitsell, 1952) in Southern California. The syndrome was also observed in Palestine in 1924 (Whitsell, 1952) in avocado trees of Californian origin. Coit (1928) first described the disease (deformation of limbs and some fruit) and attributed the symptoms he observed to sunburn of the bark. Horne and Parker (1931), however, showed the disease to be transmissible and suggested a virus was the causal agent of the disease and not the environment.

The sunblotch disease of avocado has since been observed in Florida (Stevens, 1939), Mexico (Trask, 1948), Chile (Magdahl, 1958), Peru (Zentmyer, 1959), South Africa (Loest and Stofberg, 1959), Israel (Comelli, 1960), West Indies (as

Azteca disease; Olson, 1963), Australia (Trochoulias and Allen, 1970) and Venezuela (Rondon and Figueroa, 1976).

Symptoms of the sunblotch disease have been described on the leaves and the trunk of infected avocados (Horne and Parker, 1931; Horne *et al.*, 1941; Whitsell, 1952); however, there was no consistent leaf symptom (i.e., (1) leaves show white, yellow or grey-green variegation; a mottled condition varying from faint light spotting to large spotted areas sometimes causing abnormal leaf development and resulting in a ruffled or distorted appearance; (2) leaves show a warped, curled, or crinkled appearance) and symptoms on the bark (described by Horne *et al.*, 1974) of the trunk occur only late, if at all, in the development of the disease.

Sunblotch symptoms on the fruit consist of long and narrow, shallow, longitudinal grooves or depressed, smooth streaks near the stem end of the fruit. The colour of the fruit streak (or blotch; hence, the name "sunblotch") is white-to-yellow in green fruit and red-to-purple-red in purple fruit (Horne and Parker, 1931). However, the percentage of infected fruit on sunblotch infected trees is highly variable (Horne *et al.*, 1941).

The only consistent sunblotch symptoms are on the green stem (Whitsell, 1952), where yellow or light-coloured (pink) streaks develop on the bark of the younger green limbs and shoots. On young seedlings, yellow or red streaking on the green bark of the trunk may appear. The streaking may be depressed in the wood, sometimes just under the leaf stem.

Infected avocado trees are generally less productive

than healthy trees and the infected trees grow into a low flattened shape with the limbs bending toward the ground (Wallace, 1958). Some infected trees set fewer fruit, reducing the yield, and streaking of the fruit further reduces the marketable yield.

Until recently, the only known means of spread of the sunblotch disease was by grafting healthy buds or budwood onto infected rootstock (or infected buds onto healthy rootstock) or seed transmission (Wallace and Drake, 1962). It had been suggested that transmission of sunblotch also occurred by natural root grafting (Whitsell, 1952); however, the evidence was highly circumstantial. Insect transmission of sunblotch was ruled out on the basis of the poor, natural spread of the disease in the field (Whitsell, 1952). However, it has recently been demonstrated that sunblotch disease is pollen transmissible (Desjardins *et al.*, 1979) and that bees are involved in the pollination of avocado trees (Papademetriou, 1976; Desjardins *et al.*, 1979).

Sunblotch-infected avocado trees are known to exist in two separate states: (1) the "symptom-bearing" state and (2) the "symptomless carrier" state (Wallace and Drake, 1962).

In the symptom-bearing state, seeds from sunblotch-infected trees (the trees displaying the symptoms described above) produce two kinds of seedlings: healthy and sunblotch infected. The rate of this type of seed transmission is very low (0 - 5.5%). However, there is a high rate of graft transmission when buds or budwood from healthy trees are grafted onto infected rootstock (Wallace and Drake, 1962).

In the symptomless carrier state, the rate of seed

transmission is high (80 - 100%), yet seedlings never display symptoms, even after three successive generations (Wallace and Drake, 1962). The symptomless carrier seeds, when grown into (symptomless) seedlings and grafted to healthy avocado material (buds, budwood or rootstock), cause the healthy material to become infected and develop sunblotch symptoms; i.e., enter the symptom-bearing state. Sunblotch disease symptoms cannot be induced in symptomless carrier seedlings by grafting to sunblotch-infected (symptom bearing) avocado material, i.e., there is complete protection by the sunblotch agent already present in the symptomless carriers against symptom development (Wallace and Drake, 1962).

The causal agent of the sunblotch disease (symptom bearing or symptomless) has not been identified; however, the absence of any detectable viruses and the slow rate of symptom development (2 mo - 4 yr; Wallace and Drake, 1962) suggested to us the possibility that the sunblotch disease of avocado was of viroid etiology.

In this Chapter, I will describe the analysis of low molecular weight nucleic acid extracts from healthy and sunblotch-infected avocados; the purification of, characterization of and cDNA synthesis to a low molecular weight, viroid-like RNA; and the association of this RNA with sunblotch-infected, but not healthy avocados.

Although direct evidence that this viroid-like RNA is the causal agent of the sunblotch disease has not yet been obtained, it will be referred to as avocado sunblotch viroid (ASBV).

Some of the work described here was carried out in collaboration with Mr. A. Rakowski of this Department, Dr. R.N. Allen of the New South Wales Department of Agriculture, and Mr..D.McE. Alexander of the C.S.I.R.O. Division of Horticultural Research.

MATERIALS.

Avocado Isolates

The avocado isolates were obtained from three main sources:

1) "ex Merbein"; i.e., provided by Mr D. McE. Alexander, C.S.I.R.O., Division of Horticultural Research, Merbein, Victoria.
2) "ex Wollongbar"; i.e., provided by Dr. R.N. Allen, Agricultural Research Centre, Wollongbar, New South Wales.
3) "ex Northfield"; i.e., provided by Dr. R. van Velsen, South Australian Department of Agriculture, Northfield, South Australia.

Other avocado sources are given below.

Isolate 1: A Mexican seedling field tree (Av 1); biologically indexed sunblotch negative; ex Merbein.

Isolate 2: A Duke (7) avocado; biologically indexed sunblotch negative; ex Woolongbar.

Isolate 3: A Hass field tree; biologically indexed sunblotch negative; ex Wollongbar.

Isolate 4: A Maoz seedling; biologically indexed sunblotch negative (by Dr. Ben-Yakov, Volcani Institute); ex Northfield.

Isolate 5: A Zutano (L-1) seedling; biologically indexed sunblotch negative; ex Northfield.

Isolate 6: A Todd, symptomless carrier seedling; biologically indexed sunblotch positive; ex Merbein. The parent of Isolate 6 was Isolate 18, ex Wollongbar.

Isolate 7: A Mexican seedling (Av 1) graft-inoculated with Hass sunblotch (Hass SB-1) material; biologically indexed sunblotch positive; ex Merbein.

Isolate 8: A mixed lot of Mexican seedlings graft-inoculated with Hass SB-1 (see Isolate 7); biologically indexed sunblotch positive; ex Merbein.

Isolate 9: A Zutano (Av 35) field tree; biologically indexed sunblotch positive; ex Merbein (Coomealla farm; Row 4, Tree 6).

Isolate 10: A Hass (Av 45/Av 1) field tree; biologically indexed sunblotch positive; ex Merbein.

Isolate 11: A Zutano (Av 119) seedling; biologically indexed sunblotch negative; ex Merbein.

Isolate 12: A Hass (Av 130) seedling; biologically indexed sunblotch negative; ex Merbein.

Isolate 13: A Fuerte (Av 118) seedling; biologically indexed sunblotch negative; ex Merbein.

Isolate 14: An Edranol seedling; biologically indexed sunblotch negative; ex Wollongbar.

Isolate 15: A graft-inoculated Ganter seedling; expected to become sunblotch positive, but at present unindexed; ex Merbein. This was the only biologically non-indexed isolate of 37 non-indexed isolates that was ASBV-positive by hybridization (see Results).

<u>Isolate 16</u>: A Bacon (Av 57) tree; biologically non-indexed, ex Merbein.

<u>Isolate 17</u>: A symptom-bearing seedling graft-inoculated with a Todd symptomless carrier (Isolate 18); ex Wollongbar.

<u>Isolate 18</u>: A Todd symptomless carrier; biologically indexed sunblotch positive; ex Wollongbar; the parent of Isolate 6.

<u>Isolate 19</u>: An avocado seedling originally obtained from Merbein and graft-inoculated at Merbein with SB-1 (see Isolate 8); biologically indexed sunblotch positive; ex Wollongbar.

<u>Isolate 20</u>: A Zutano field tree (Av 35/Av 2); biologically indexed sunblotch positive; ex Merbein (Coomealla farm; Row 8, Tree 9).

<u>Isolate 21</u>: A Fuerte seedling obtained from the C.S.I.R.O. Division of Horticultural Research, Glen Osmond, South Australia; apparently sunblotch negative.

<u>Isolates 22, 25, 27 and 28</u>: These isolates were seedlings, the same as Isolate 1; biologically indexed sunblotch negative; ex Merbein.

<u>Isolate 23</u>: An unclassified seedling obtained as a seed from Mr. R.H. Lloyd of Paringi, N.S.W., and grown by Dr. R.H. Symons; biologically non-indexed.

<u>Isolate 24</u>: A Hass seedling obtained from the source of Isolate 21.

<u>Isolate 26</u>: A Rincon seedling obtained from the source of Isolate 21.

Isolates 29 and 30: Mexican seedlings; the same as Isolate 7, but biologically non-indexed; both isolates had been graft-inoculated with SB-1 in Merbein and were brought to Adelaide by Dr. R.H. Symons.

All other materials were obtained from sources described in preceding Chapters.

METHODS

A. Extraction and Purification of ASBV

Nucleic acids were extracted and partially purified from healthy and sunblotch-infected avocados by Extraction Procedure C of Chapter II, Materials and Methods, with the following modifications: (1) The volume (of solution A + solution B) to weight of plant material ratio was increased from 3:1 to 4:1; (2) the ratio of 80 ml of solution C to nucleic acids from 500 g of plant material was not maintained for smaller amounts of plant material; e.g., 10 ml of solution C/10 - 25 g of plant material and 20 ml of solution C/25 - 50 g of plant material, was used.

Circular ASBV was purified from partially purified nucleic acid extracts of sunblotch-infected avocados by one cycle of non-denaturing polyacrylamide gel electrophoresis and electroelution, and one cycle of denaturing (7 M urea) polyacrylamide gel electrophoresis and electroelution, exactly as described for the purification of circular CSV in Chapter II, Materials and Methods.

B. Electron Microscopy

The electron microscopic analysis of ASBV was carried

out in collaboration with Dr. T. Hatta, Department of Plant Pathology, Waite Agricultural Institute, University of Adelaide.

RNAs were spread for electron microscopy by the method of Randles and Hatta (1979) as described in Chapter II, Materials and Methods.

C. Thermal Denaturation

RNA was subjected to thermal denaturation in 0.1 x SSC. The temperature was increased at the rate of 1.0°C/min using the apparatus described by Randles *et al.* (1976).

D. cDNA Synthesis, Hybridization and the Thermal Stability of Hybrids.

Random-primed cDNA synthesis to ASBV was carried out by the method of Taylor *et al.* (1976) as described for ccRNA-1 in Chapter IV, Materials and Methods, with the following modification: RNA (ASBV or CSV) was heated at 70°C for 1 min with the random primer and allowed to cool to room temperature before the other components of the cDNA synthesis reaction mixture were added.

Oligo$(dT)_{10}$-primed cDNA synthesis to ASBV was carried out essentially as described in Chapter IV, Materials and Methods for the preparation of oligo$(dT)_{10}$-primed cDNA to CSV, except that (1) the preliminary nuclease S_1 cleavage of ASBV was carried out with either 250 units/ml of nuclease S_1 for 1 hour at 25°C or 1000 units/ml of nuclease S_1 for 2 hours at 25°C, and (2) the radioactive triphosphate was either [α-^{32}P]dCTP (100 - 340 Ci/mmole) or [α-^{32}P]dGTP (180 - 350 Ci/mmole).

The formation of [^{32}P]cDNA:RNA hybrids in hybridization Buffer A at 60°C, the determination of the thermal stability of [^{32}P]cDNA:RNA hybrids and the assay of the extent of hybrid formation by the resistance of [^{32}P]cDNA to digestion by nuclease S_1 were as described in Chapter IV, Materials and Methods with the following modifications: (1) the nuclease S_1 digestion was at 37°C for 45 min in nuclease S_1 assay buffer B (Chapter IV, Materials and Methods); (2) nuclease S_1 was used at a concentration of 100 units/ml with 1 or 2 mg/ml of nucleic acid in the hybridization solutions, at 200 units/ml for 5 mg/ml, and at 50 units/ml for purified viroid or no added viroid.

Hybridizations, for the purpose of indexing nucleic acid samples for the presence of ASBV, were carried out in hybridization Buffer A at 60°C for 72 hours with partially purified nucleic acid samples at 5 mg/ml and the [^{32}P]cDNA-ASBV at 150 pg/ml.

E. Plant Inoculation Procedure

Lycopersicon esculentum (tomato): Tomato plants (cv. Rutgers) were inoculated at the two-leaf stage of development. Twenty-five microlitres of either water or 1 μg/ml of purified ASBV was placed on a carborundum-dusted cotyledon and rubbed over the surface of the entire cotyledon with the aid of a rubber finger-stall. Both cotyledons on each tomato plant were inoculated in this manner.

The plants were maintained in plant room C (Chapter II, Materials and Methods) and harvested 8 weeks post inoculation (P.I.). The individual ASBV-inoculated tomato plants were combined, as were the individual water-inoculated tomato

plants, for extraction purposes.

Gynura: *Gynura aurantiaca* and *Gynura sarmentosa* were inoculated with 30 µl/plant of either water or 1 µg/ml of purified ASBV as described in Chapter II, Materials and Methods.

Gynura aurantiaca plants were maintained in either plant room B or plant room C and *Gynura sarmentosa* plants were maintained in plant room C (Chapter II, Materials and Methods). All plants were harvested at 8 weeks P.I.; the ASBV-inoculated *G. aurantiaca* plants were combined, as were the ASBV-inoculated *G. sarmentosa* plants, for extraction purposes.

Chrysanthemum: Chrysanthemums (Charm type) were inoculated with 1 µg/ml of purified ASBV by the procedure of Niblett *et al*. (1978). Plants were maintained in plant room B and harvested at 8 weeks P.I. and combined for extraction.

Persea americana (avocado): Avocados (var. Hass, Fuerte, Rincon, Mexican seedlings) were inoculated by removing 5 - 6 leaves just below the growing tip of young seedlings at the stem-petiole axis, placing 20 - 30 µl in several drops/plant on the stem at the former stem-petiole juncture and the stem inoculated by needle puncture as described for the inoculation of *G. aurantiaca* in Chapter II, Materials and Methods.

The plants were maintained in plant rooms A, B or C and the terminal leaves were harvested at 3 - 4 months P.I.

Graft inoculations of sunblotch-infected budwood to healthy avocado seedlings were carried out by Mr. D. McE.

Alexander of the Division of Horticultural Research, C.S.I.R.O., Merbein, Victoria. The graft-inoculated seedlings were brought to Adelaide by Dr. R.H. Symons and were maintained in either plant room B or plant room C.

RESULTS

A. Analysis of Partially Purified Nucleic Acid Extracts by Polyacrylamide Gel Electrophoresis

When examined by electrophoresis on 5% polyacrylamide tube gels, a partially purified nucleic acid extract from leaves of one isolate of sunblotch-infected avocado contained an extra band (Figure VI-1D) not found in similar extracts from either healthy avocado leaves (Figure VI-1C) or another sunblotch-infected avocado plant (Figure VI-1B). This extra band was shown to be a viroid (see below) and will be referred to as avocado sunblotch viroid (ASBV). Its mobility was appreciably greater than that of CSV present in a nucleic acid extract of infected chrysanthemum (Figure VI-1A) which indicates that it most likely has a lower molecular weight.

ASBV was extracted and purified by the two-step acrylamide gel procedure (Chapter II, Materials and Methods) used for CSV and obtained in a yield of 32 μg from 250 g of infected avocado leaves. The two-step procedure involved one cycle of non-denaturing and one cycle of denaturing (7 M urea) polyacrylamide slab gel electrophoresis. The purified viroid gave a single band on tube gel electrophoresis (Figure VI-1E).

FIGURE VI-1

Polyacrylamide tube gel electrophoresis under non-denaturing conditions of nucleic acids from various sources. Partially purified nucleic acid extract (Materials and Methods) from (A), CSV-infected chrysanthemum (230 μg); (B), ASBV-infected avocado (Isolate 9) (325 μg); (C), healthy avocado (Isolate 1) (318 μg); (D), ASBV-infected avocado (Isolate 8) (150 μg). (E), purified ASBV (1.6 μg) after the two-step polyacrylamide gel electrophoresis purification procedure described in Chapter II, Materials and Methods.

Nucleic acid samples were run on 5% acrylamide, 0.125% bisacrylamide tube gels (0.6 x 10 cm) in the Tris-acetate-EDTA buffer of Loening (1967) at 5 mA/gel for 2.5 hours. Gels were stained in 0.05% toluidine blue for 1.5 hours and destained in water overnight.

A
B
C
D
E
CSV
ASBV

B. Physical Characterization of ASBV

Thermal denaturation of ASBV in 0.1 x SSC (SSC, 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0) produced an absorbance-temperature profile with a sharp transition typical of viroids (Langowski *et al.*, 1978), a hyperchromicity of 21% and a T_m of 38°C (Figure VI-2B and Figure III-3A) and implies that ASBV can be denatured under less extreme conditions than CSV.

Examination of ASBV and CSV by electron microscopy under partly denaturing conditions showed a mixture of circles and rod-like structures for ASBV (Figure VI-3A) and almost exclusively rod-like structures for CSV (Figure VI-3B). When spread under completely denaturing conditions, mostly circular molecules were seen in both cases (Figure VI-3C and D). The better spreading of ASBV circles under the partly denaturing conditions is consistent with its lower thermal stability (Figure VI-2). That ASBV consisted of RNA was shown by spreading ASBV and CSV in the presence of the single-strand circular DNA of phage ϕX174 after treatment with DNase I (Figure VI-3E and F) or with pancreatic RNase (Figure VI-3G and H). DNase treatment degraded the phage DNA but had no effect on the viroids while the reverse occurred with the RNase treatment.

Length measurements of 100 molecules of ASBV and CSV spread under the completely denaturing conditions of Figure VI-3C and D (results not shown) showed that the average length of ASBV was 0.80 that of CSV; this is consistent with its faster electrophoretic mobility (Figure VI-1).

Partially purified nucleic acid extracts of CSV-infected

FIGURE VI-2

Thermal denaturation profiles of (A), circular ASBV; and (B), circular CSV, in 0.1 x SSC (1 x SSC = 0.15 M NaCl, 0.015 M trisodium citrate, pH 7.0). The temperature was increased at the rate of 1°C/min. The profile for CSV (B) was taken from Figure III-3.

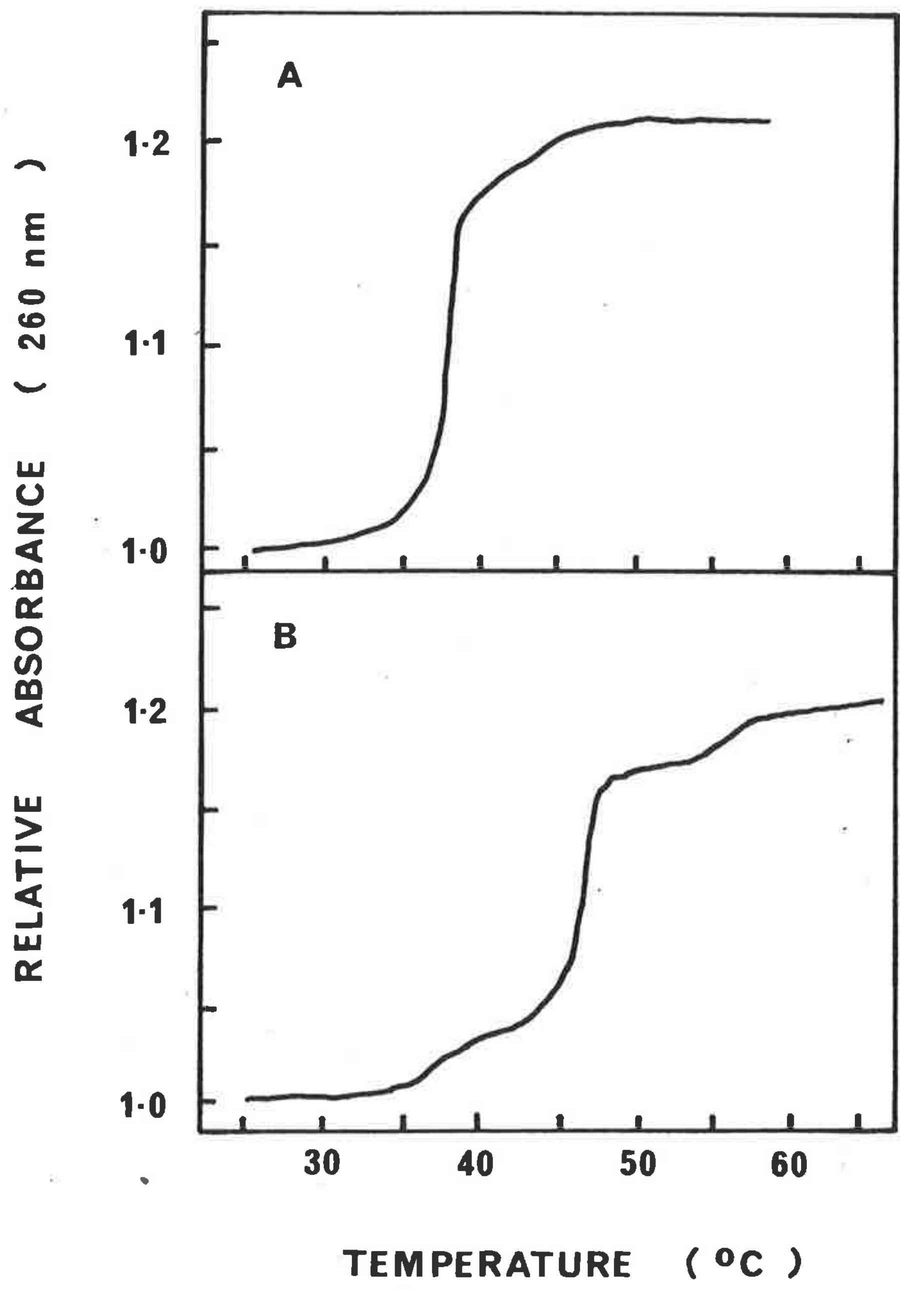
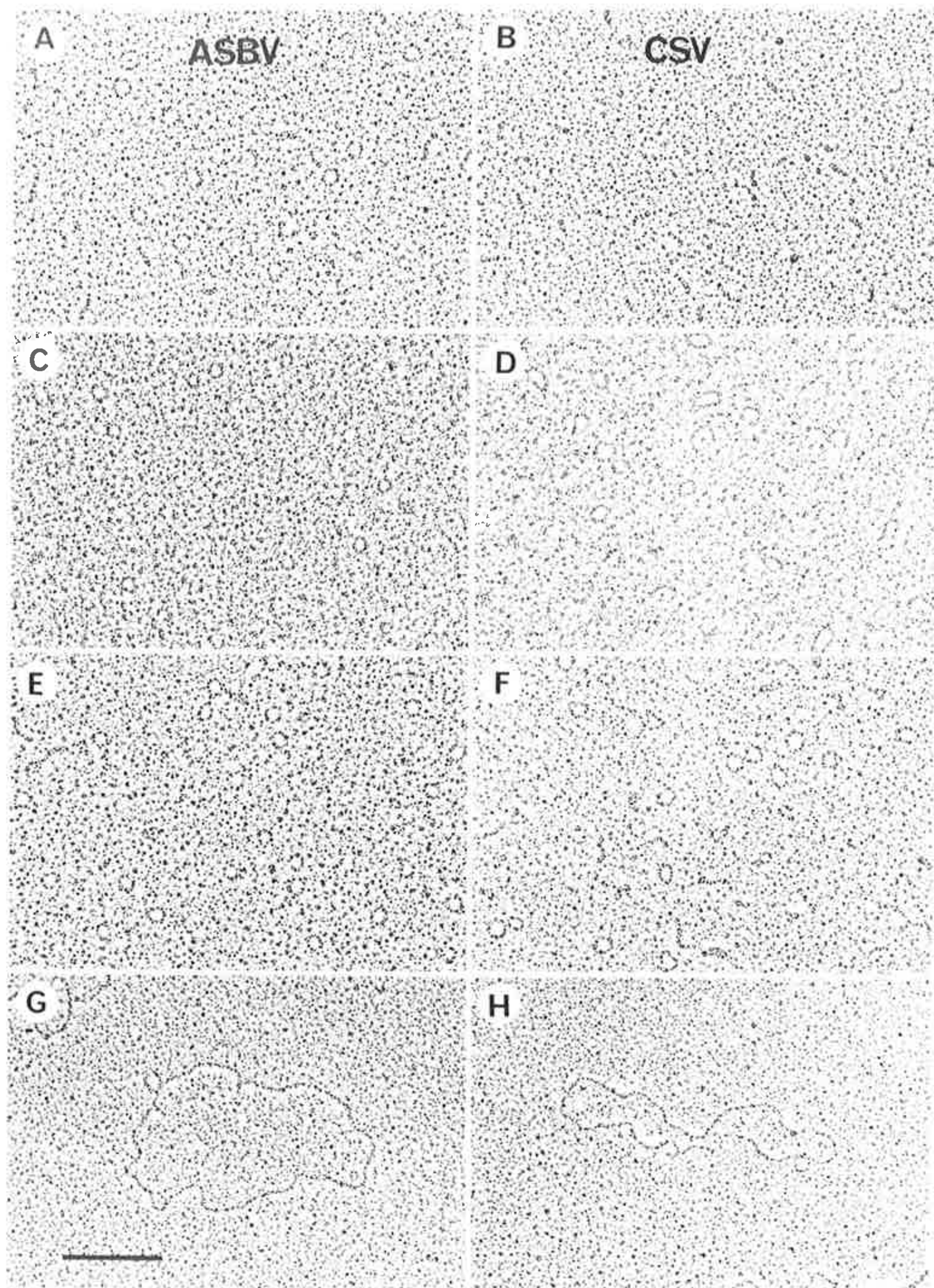

A
B
1·2
1·1
1·0
RELATIVE ABSORBANCE (260 nm)
30
40
50
60
TEMPERATURE (°C)

FIGURE VI-3

Electron micrographs of ASBV and circular CSV, prepared essentially as described by Randles and Hatta (1979). (A) and (B), ASBV and CSV spread under partially denaturing conditions. RNA (0.6 μg/ml) in 40% (v/v) formamide, 10 mM Tris-HCl, pH 8.5, 1 mM EDTA, 0.003% cytochrome c at room temperature was spread onto a hypophase at room temperature of 10% (v/v) formamide, 1.5 mM Tris-HCl, pH 8.5, 0.15 mM EDTA. Samples were picked up on parlodion covered grids, shadowed at 5 - 6° with platinum palladium and covered with evaporated carbon. (C) and (D), ASBV and CSV spread under fully denaturing conditions as for (A) and (B) except that the hyperphase contained 98% (v/v) formamide at 60°C and the hypophase contained 60% (v/v) formamide at 45°C. (E) and (F), ASBV and CSV were mixed with 0.1 μg/ml of phage φX174 single-strand circular DNA (a gift from Dr. J.B. Egan) and treated with 50 μg/ml of DNase I for 15 min at 20°C in the presence of Mg^{2+} before spreading as in (C) and (D). (G) and (H), as for (E) and (F) except that the DNase treatment was replaced by treatment with 1 μg/ml of RNase A for 5 min at 20°C prior to spreading. The bar represents 250 nm.



A
ASBV
B
CSV
C
D
E
F
G
H

chrysanthemum were shown to contain linear (about 10 - 15% of total) as well as circular viroid molecules (Chapter III, Results). The possibility that linear ASBV molecules were present in partially purified nucleic acid extracts of avocado leaves was not investigated, since no band with the expected mobility of the linear form of ASBV was visible after staining a urea gel to detect (and purify) circular ASBV.

C. cDNA Synthesis to ASBV

As previously stated, attempts at synthesizing cDNA to ccRNA-1 and CSV by the random-primer method of Taylor *et al.* (1976) were largely unsuccessful (Chapter IV). When the random-primer method was applied to ASBV, 6.7 ng of cDNA was synthesized/μg of RNA. This is similar to the amount of cDNA synthesized by the same technique with CSV as the template. cDNA was also synthesized to ASBV by the three step procedure [(1) partial nuclease S_1 cleavage of the RNA; (2) polyadenylation of the S_1-cleaved RNA; and (3) oligo(dT)-primed cDNA synthesis to the polyadenylated, S_1-cleaved RNA] described in Chapter IV for the synthesis of cDNA to ccRNA-1 and CSV. The yield of cDNA to ASBV by this method was 18 ng/μg of RNA. This was about one-half the yield of cDNA to CSV synthesized under the same conditions (results not shown) and two-to-three-fold more than the yield of random-primed cDNA synthesized to ASBV (see above).

Hybridization kinetics of the random-primed cDNA synthesized to ASBV were not examined; all hybridization analyses were carried out with oligo(dT)-primed cDNA to

ASBV (see below).

D. Hybridization Analysis with [^{32}P]cDNA to ASBV

1. Rot Analysis

Hybridization of ASBV [^{32}P]cDNA against ASBV and of CSV [^{32}P]cDNA against CSV produced the Rot curves shown in Figure VI-4. For ASBV, the Rot½ was 7.0×10^{-4} mol sec litre^{-1} (Figure VI-4A) and the Rot curve was appreciably sharper than that obtained for CSV (Figure VI-4B) with a Rot½ of 8.0×10^{-4} mol sec litre^{-1}; the latter Rot½ required the use of CSV partially cleaved with nuclease S_1 (Chapter IV). These Rot½ values were similar to those obtained under the same conditions for the satellite RNA of cucumber mosaic virus and its cDNA (Rot½ 7.0×10^{-4} mol sec litre^{-1}; M_r 1.2×10^5; Chapter V, Results). These results indicate that ASBV is a single species of RNA rather than a population of molecules of similar size.

2. Thermal Stability of cDNA:RNA Hybrids

Hybrids formed between cDNA-ASBV and ASBV melted sharply with a T_m of 72°C (Figure VI-5A). The melting profile was similar to that obtained for cDNA-CSV: CSV hybrids (Figure VI-5B), but the T_m of the cDNA-ASBV: ASBV hybrids was 9°C lower than the T_m of cDNA-CSV:CSV hybrids (81°C; Table V-1). The lower T_m of ASBV-cDNA:ASBV hybrids cf. CSV-cDNA:CSV hybrids is consistent with the lower T_m of ASBV in 0.1 x SSC (38°C; Figure VI-2A) compared with CSV in 0.1 x SSC (47°C; Figure VI-2B); i.e., a difference of 9°C. Therefore, in spite of the lower T_m value for ASBV-

FIGURE VI-4

Rot curves of the hybridization of ASBV (A) and CSV (B) to their homologous [^{32}P]cDNAs. The hybridization buffer was 0.18 M NaCl, 10 mM Tris-HCl, 0.05% sodium dodecyl sulphate, 1 mM EDTA, pH 7.0, and incubation was at 60°C. Hybrid formation was assayed with nuclease S_1 as in Chapter IV, Materials and Methods. The data for CSV (B) was taken from Figure V-3C. The Rot curves were normalized to 100% hybrid formation to facilitate comparison. The actual plateau levels were 70% for ASBV (A) and 45% for CSV (B).

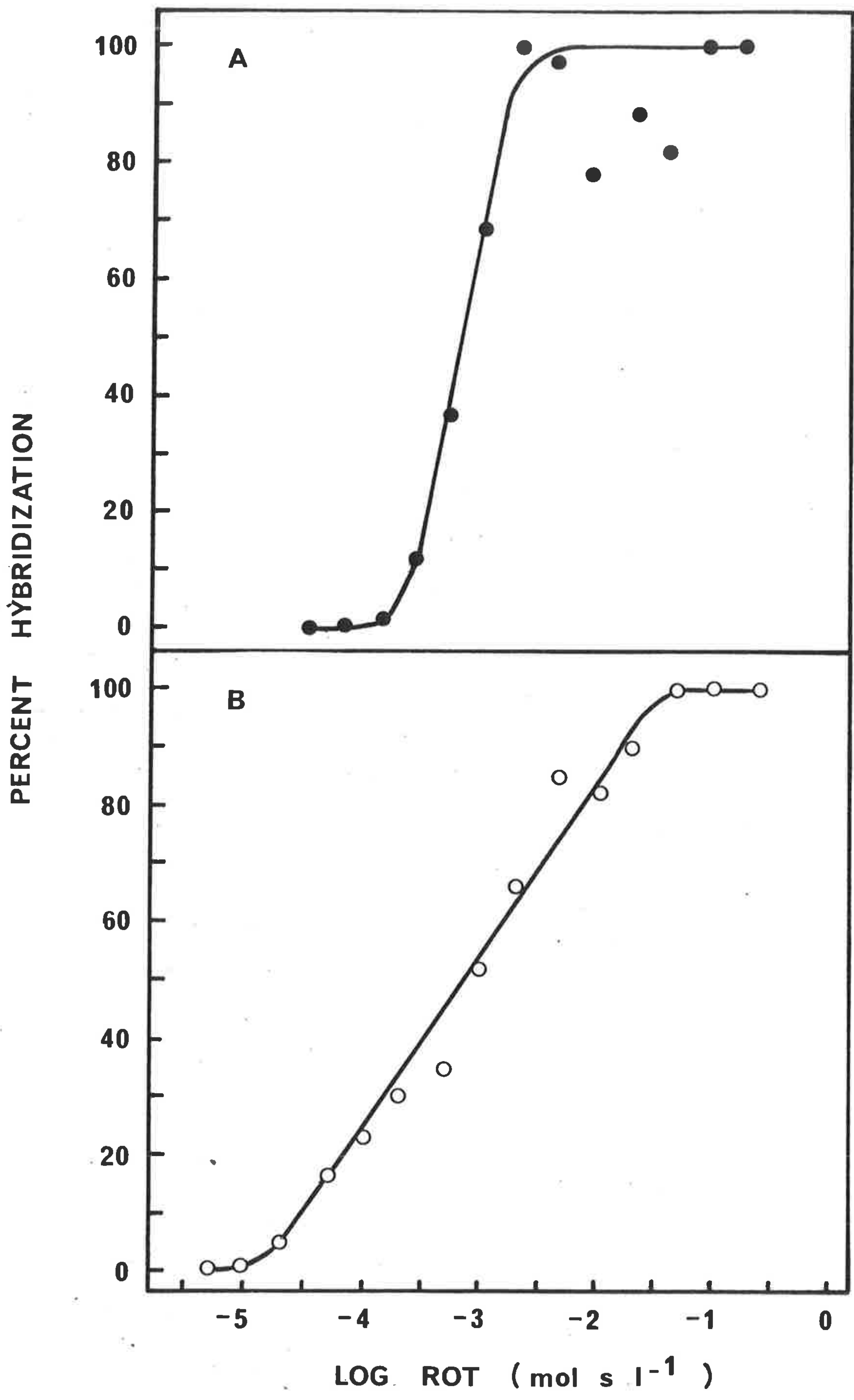
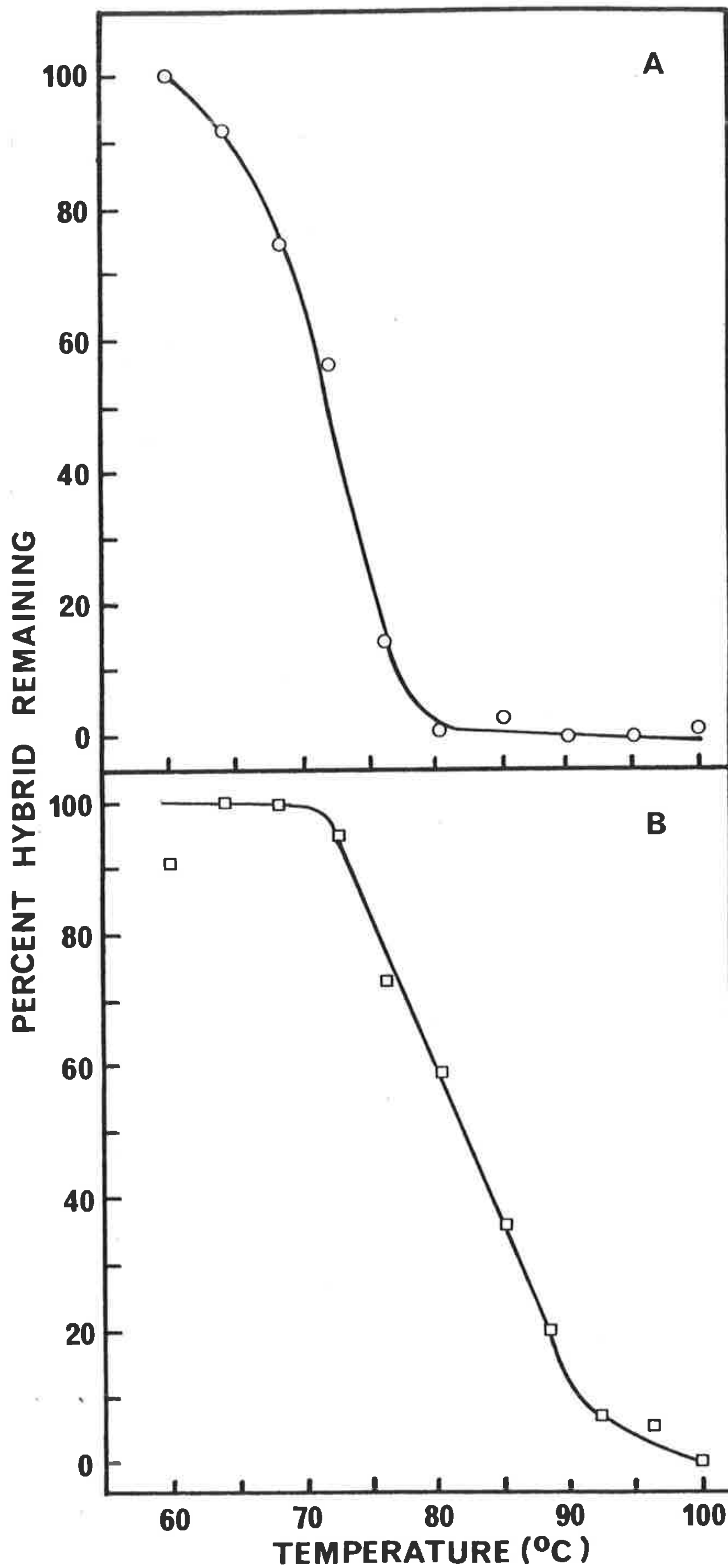

A
B
PERCENT HYBRIDIZATION
100
80
60
40
20
0
−5
−4
−3
−2
−1
0
LOG ROT (mol s l^{-1})

FIGURE VI-5

Thermal denaturation profiles of circular ASBV:[^{32}P]cDNA-ASBV hybrids (A) and circular CSV:[^{32}P]cDNA-CSV hybrids (B). ASBV (0.5 μg/ml) was hybridized with 0.5 ng/ml [^{32}P]cDNA-ASBV to a Rot of 1 x 10^{-1} mol sec litre^{-1} in Buffer A at 60°C. CSV (1.5 μg/ml) was hybridized with 3 ng/ml [^{32}P]cDNA-CSV to a Rot of 4 x 10^{-1} mol sec litre^{-1} in Buffer A at 60°C. The thermal denaturation of the hybrids in the same buffer and nuclease S_1 assay of the percentage hybrid remaining were carried out as described in Chapter IV, Materials and Methods.


A
B
100
80
60
40
20
0
PERCENT HYBRID REMAINING
60
70
80
90
100
TEMPERATURE (°C)

cDNA:ASBV hybrids (which may merely indicate a lower G:C content in ASBV; the size distribution of ASBV-cDNA (results not shown) was similar to that of CSV-cDNA), the hybrid formation was apparently specific with no evidence of mismatching.

3. <u>The Specificity of cDNA-ASBV: The Use of cDNA for Indexing</u>

Since [^{32}P]cDNA to ASBV hybridized to ASBV with the expected Rot½ for an RNA of M_r approx. 1×10^5 (Figure VI-4A) in hybridization Buffer A at 60°C, this implies that under the above conditions there was no secondary structure present in ASBV that was interfering with the hybridization of cDNA-ASBV to ASBV. This was unlike the situation observed with CSV (Chapter V), but was consistent with the lower T_m of ASBV compared with CSV (see above). Therefore, the use of a hybridization buffer containing formamide to ensure denaturation of ASBV was not required, and all hybridizations involving ASBV-cDNA were carried out in hybridization Buffer A at 60°C.

The specificity of the ASBV [^{32}P]cDNA was determined by hybridization against purified ASBV in the presence and absence of *E. coli* ribosomal RNA, and against CSV and the RNAs of alfalfa mosaic virus, cucumber mosaic virus and tomato aspermy virus (Table VI-1). Significant hybridization was obtained only against ASBV.

The hybridization of ASBV [^{32}P]cDNA against partially purified nucleic acid extracts of healthy and CSV-infected chrysanthemum and healthy and CEV-infected *Gynura aurantiaca* gave 5% or less hybridization (Table VI-2). Hence, there

TABLE VI-1

SPECIFICITY OF HYBRIDIZATION OF [^{32}P]cDNA OF ASBV AGAINST ASBV, CSV, AND PLANT VIRAL RNAs[a]

Nucleic acid	Concentration of viroid or viral RNA during hybridization	Rot of assay (mol s l^{-1})	Extent of hybridization of [^{32}P]cDNA (%)
None	-	(24 hours)	0.5
ASBV	1 μg/ml	2.7×10^{-1}	55
ASBV plus 1 mg/ml *E. coli* ribosomal RNA	1 μg/ml	2.7×10^{2}	66
1 mg/ml *E. coli* ribosomal RNA	-	2.7×10^{2}	0.9
CSV (circular)	5 μg/ml	1.4	0.3
Alfalfa mosaic virus RNA	50 μg/ml	13.5	0.5
Cucumber mosaic virus RNA	50 μg/ml	13.5	0.9
Tomato aspermy virus RNA	50 μg/ml	13.5	1.1

[a] Experimental details are given in Materials and Methods.

TABLE VI-2

HYBRIDIZATION OF [^{32}P]cDNA OF ASBV AGAINST PURIFIED ASBV AND PARTIALLY PURIFIED NUCLEIC ACIDS FROM AVOCADO PLANTS INDEXED AS NEGATIVE OR POSITIVE FOR SUNBLOTCH DISEASE, HEALTHY AND CSV-INFECTED CHRYSANTHEMUM AND HEALTHY AND CEV-INFECTED *GYNURA AURANTIACA*[a]

Nucleic acid - ASBV or partially purified nucleic acid extract[b]	Concentration of nucleic acid during hybridization	Rot of assay (mol s l^{-1})	Extent of hybridization (%)	Viroid band visible on tube gel.
None	-	(24 hours)	2	-
None	-	(72 hours)	8	-
Purified ASBV	230 ng/ml	6.1×10^{-2}	72	-
Healthy avocados				
Isolate 1	2 mg/ml	1.6×10^{3}	5	No
Isolate 1	5 mg/ml	4.0×10^{3}	11	No
Isolate 2	5 mg/ml	4.0×10^{3}	9	No
Isolate 3	5 mg/ml	4.0×10^{3}	12	No
Isolate 4	5 mg/ml	4.0×10^{3}	11	No
Isolate 5	5 mg/ml	4.0×10^{3}	9	No
Sunblotch infected avocados				
Isolate 6	3 mg/ml	2.0×10^{3}	69	No
Isolate 7	1 mg/ml	8.1×10^{2}	69	Yes
Isolate 8	1 mg/ml	8.1×10^{2}	89	Yes

TABLE VI-2 cont..

Isolate 9	2 mg/ml	1.6×10^3	16	No
Isolate 9	5 mg/ml	4.0×10^3	73	No
Isolate 10	5 mg/ml	4.0×10^3	89	No
Healthy chrysanthemum	2 mg/ml	1.6×10^3	1	No
CSV-infected chrysanthemum	2 mg/ml	1.6×10^3	1	Yes
Healthy *Gynura aurantiaca*	2 mg/ml	1.6×10^3	5	No
CEV-infected *Gynura aurantiaca*	2 mg/ml	1.6×10^3	3	Yes

[a] Experimental details are given in Materials and Methods, Chapter IV and this Chapter.

[b] Avocado isolates are described in Materials of this Chapter.

appears to be no detectable sequence homology between ASBV and either CSV or CEV; this agrees with the lack of homology with purified CSV in Table VI-1.

When ASBV [^{32}P]cDNA was hybridized against various partially purified nucleic acid extracts from healthy and infected avocados, the results of Table VI-2 were obtained. From five isolates of avocado which had been indexed (Wallace and Drake, 1962; Burns *et al.*, 1969) as negative for sunblotch disease, 5 - 12% hybridization to a nuclease S_1-resistant form was found and this was taken as the background level under the conditions used; it is not known why this level was higher than the maximum of 1.1% obtained in a different experiment (Table VI-1) using the same conditions. Of five avocado isolates indexed positive for sunblotch disease (four were showing positive symptoms; one was a symptomless carrier), a viroid band was visible on polyacrylamide tube gel electrophoresis of the nucleic acid extract of two of the isolates and a high level of hybridization was obtained (Table VI-2). In the three isolates where a viroid band was not visible, the presence of the viroid was shown using a high concentration of nucleic acid (3 - 5 mg/ml) and hybridizing for 72 hours to give Rot values of 2.4 - 4 x 10^3 mol sec litre^{-1} (Table VI-2).

The [^{32}P]cDNA to ASBV was further tested by hybridization to seven more avocado isolates biologically indexed sunblotch negative, seven more avocado isolates biologically indexed sunblotch positive, and 37 biologically non-indexed isolates (Table VI-4 and results not shown); in the latter instance, the hybridization to [^{32}P]cDNA was being used as a method of

indexing for sunblotch; i.e., cDNA-indexing. A comparison of the cDNA-indexing results with the biologically indexing results (Table VI-3) revealed the following.

(1) All twelve biologically indexed sunblotch-infected isolates were ASBV-positive by hybridization.

(2) Of twelve biologically indexed sunblotch-free isolates, 8 were indexed ASBV negative by hybridization (results not shown); one was indexed sunblotch positive (35 - 81% hybridization, Isolate 11, Materials); and three isolates (Isolates 12, 13 and 14, Materials) showed hybridization plateau values of 16 - 30%, 12 - 32%, and 26 - 28%, respectively, for a number of hybridizations involving three separate extractions of Isolates 12 and 13 and one extraction of Isolate 14. Therefore, these last three isolates remain uncertain viz. cDNA indexing.

(3) Only one of 37 biologically non-indexed isolates was found to be ASBV positive by hybridization (93%, Isolate 15, Materials; results not shown); one isolate (16, Materials) showed 23% hybridization and thus remains uncertain (i.e., non-indexed); and 35 isolates were clearly ASBV negative by hybridization (Table VI-3, results not shown). Not one of the 37 non-indexed isolates was showing sunblotch symptoms.

4. Quantitation of ASBV Levels in Partially Purified Nucleic Acid Extracts

Since polyacryalmide gel electrophoresis of partially purified nucleic acids extracted from sunblotch positive avocados showed the presence of a stainable band with the mobility of ASBV in 7 out of 12 isolates (Figure VI-1 and results not shown), all 12 of which were ASBV positive

TABLE VI-3

A COMPARISON OF THE RESULTS OF BIOLOGICAL INDEXATION FOR AVOCADO SUNBLOTCH DISEASE WITH THE RESULTS OF cDNA INDEXATION FOR THE PRESENCE OF ASBV

Biological Indexation[b] for Sunblotch		cDNA Indexation for ASBV[a]		
		+ASBV	-ASBV	Unknown
Sunblotch infected	(+)	12	0	0
Sunblotch free	(-)	1	8	3
Non-indexed	(?)	1	35	1

[a] cDNA Indexation by hybridization as described in Methods.

[b] Biological Indexation as described by Wallace and Drake (1962) and Burns *et al.* (1969).

by hybridization, the level of ASBV present in partially purified nucleic acids was obviously variable. The extent of this variability could be determined by quantitating the level of ASBV in nucleic acids extracted from a number of sunblotch-infected avocados.

Quantitation of ASBV levels in partially purified nucleic acid extracts was carried out by Rot analysis as described for the quantitation of CSV levels in partially purified nucleic acid extracts (Chapter V, Results); i.e., by determining the Rot½ of the hybridization of [^{32}P]cDNA-ASBV against infected-plant nucleic acids and comparing this to the Rot½ of [^{32}P]cDNA-ASBV against purified ASBV (7×10^{-4} mol sec litre^{-1}). The value obtained is expressed as the percent of the infected-plant nucleic acids consisting of ASBV sequences. Table VI-4 shows the values obtained from the Rot curves (not shown here) expressed as the percent of ASBV in the total nucleic acid extract. The results can be summarized as follows.

(1) The level of ASBV is highly variable. The percent ASBV ranges from 0.2 to 2.3×10^{-5}% of the nucleic acid extract for partially purified nucleic acid extracts from different isolates taken from different locations at different times of the year. Part of the variability may be due to sample selection (see below).

(2) There was a 25-fold difference in the level of ASBV (expressed as the amount of ASBV per weight of leaf nucleic acid and not per leaf weight) between young leaves and old leaves of Isolate 20, whereas young and old leaves of Isolate 9 taken during the winter showed similar levels of

TABLE VI-4

a Hybridizations were carried out as described in Methods. The percent hybridization is at the plateau of the Rot curves for all isolates except Isolates 12 and 13 where no plateau was obtained.

b Percent of ASBV in total nucleic acid extract.

c No symptoms; biologically indexed sunblotch free.

d No symptoms; symptomless carrier.

e Old leaves of Isolate 9 harvested in summer (Jan. 1979).

f Old leaves of Isolate 9 harvested in winter (Aug. 1979).

g Young leaves of Isolate 9 harvested in winter (Aug. 1979).

h Old leaves of Isolate 20 harvested in autumn (Mar. 1979).

i Young leaves of Isolate 20 harvested in autumn (Mar. 1979).

j Approximated percent, since a plateau of hybridization was not reached. Percent hybridization values represent the maximum observed percent in the Rot curve.

TABLE VI-4

QUANTITATION OF ASBV LEVELS IN PARTIALLY PURIFIED EXTRACTS OF SUNBLOTCH-INFECTED AVOCADOS BY Rot ANALYSIS: HYBRIDIZATION TO [^{32}P]cDNA-ASBV

Avocado isolate number	Symptoms of sunblotch disease	Viroid band visible on tube gel	Percent Hybridization[a]	Concentration of ASBV (% by weight)[b]
8	Yes	Yes	89%	0.2%
9	Yes	No	73%[e]	2.3×10^{-5}%
		No	30%[f]	2.2×10^{-4}%
		No	44%[g]	2.2×10^{-4}%
10	Yes	No	89%	3.5×10^{-3}%
11	No[c]	No	35%	1×10^{-4}%
12	No[c]	No	23%	$<1 \times 10^{-5}$%[j]
13	No[c]	No	32%	$<1 \times 10^{-5}$%[j]
17	Yes	No	43%	1.8×10^{-3}%
18	No[d]	Yes	52%	2.2×10^{-2}%
19	Yes	Yes	57%	0.2%
20	Yes	No	85%[h]	1.5×10^{-3}%
		No	65%[i]	6×10^{-5}%

ASBV; the level of ASBV in old leaves of Isolate 9 was 10-fold less during the summer than during the winter. However, to draw any conclusions about the effect of temperature or season or the effect of leaf age on ASBV levels would require a much larger sample selection than presented here. Furthermore, it is not known if all leaves in a particular sample are equally infected; i.e., have similar levels of ASBV. Fluctuations in the level of ASBV in different leaves, in different parts of the tree, during different seasons, etc., may explain the variability observed in (1) and above.

(3) One sunblotch positive symptomless carrier isolate was obtained from two sources (Isolates 6 and 18) and the level of ASBV in the two isolates was dissimilar. No ASBV could be seen as a band on a gel with Isolate 6 (result not shown), but could be seen with Isolate 18 (Table VI-4). Thus, symptomless carriers show similar variations to those observed with symptom-bearing plants in (2) above; possibly for similar reasons.

(4) Of three isolates (11, 12 and 13) biologically indexed sunblotch negative, but showing either high levels (Isolate 11) or low levels (Isolates 12 and 13) of hybridization, only one (Isolate 11) showed a plateau of hybridization upon Rot analysis (Rot½ = 7×10^{2} mol sec litre^{-1}) for which a level of ASBV could be determined ($7 \times 10^{-4}/7 \times 10^{2}$, x 100% = 1×10^{-4}%; Table VI-4). No plateau of hybridization was observed for Isolates 12 and 13, and if these isolates were indeed sunblotch infected, then the level of ASBV must be less than 1×10^{-5}%. This value represents the limit

of detectability of ASBV sequences by hybridization under the conditions described in Chapter IV and this Chapter, Materials and Methods, since 5 mg/ml of partially purified nucleic acid containing ASBV at 1 x 10^{-5}% hybridized for 72 hours would show a Rot value for ASBV of 4.0 x 10^{-4} mol sec $litre^{-1}$ (5 mg/ml x 1 x 10^{-5}% x 72 hours); i.e., below the Rot½ for ASBV (7 x 10^{-4} mol sec $litre^{-1}$).

(5) Since the average yield of nucleic acid from sun-blotch-infected avocados was 100 mg/kg, the range of ASBV concentrations (0.2% to 2.3 x 10^{-5}%) in the partially purified nucleic acid extracts would represent 200 µg to 0.02 µg of ASBV/kg of avocado leaves.

E. Transmission Studies with ASBV: Replication of ASBV

Purified ASBV, mechanically inoculated onto tomato, chrysanthemum, *Gynura aurantiaca*, *G. sarmentosa* and avocado, did not induce the development of any disease symptoms; all ASBV-inoculated plants resembled water-inoculated (control) plants.

Extraction of nucleic acids from the above ASBV-inoculated plants and hybridization to ASBV-cDNA (Table VI-5) showed the presence of ASBV in *G. aurantiaca* and two avocados. There was no replication of ASBV in tomatoes, chrysanthemums, or *G. sarmentosa*. Quantitation of the ASBV levels in *G. aurantiaca* and the two ASBV positive avocados showed the presence of a very low level of ASBV in one inoculated avocado and the inoculated *G. aurantiaca*, and the presence of a higher level of ASBV in the second ASBV positive avocado (Table VI-5); however, this high level of

TABLE VI-5

HYBRIDIZATION ANALYSIS OF PARTIALLY PURIFIED NUCLEIC ACID EXTRACTS FROM PLANTS INOCULATED WITH ASBV.

Partially purified nucleic acid extract.	Viroid band visible on tube gel	Extent of hybridization[a]	Approximate level of ASBV (% by weight) in nucleic acid extract[b]
Healthy chrysanthemum	No	4%	
ASBV-inoculated chrysanthemum	No	6%	
Healthy tomato	No	6%	
ASBV-inoculated tomato	No	6%	
Healthy *G. aurantiaca*	No	8%	
ASBV-infected *G. aurantiaca*	No	35%[c]	2×10^{-4}%[c]
Healthy *G. sarmentosa*	No	19%	
ASBV-infected *G. sarmentosa*	No	15%	
ASBV-inoculated Avocado seedlings[d]			
Isolate 21	No	15%	
Isolate 22	No	72%	1.4×10^{-2}%

TABLE VI-5 cont..

Isolate 23	No	8%	
Isolate 24	No	8%	
Isolate 25	No	32%	2×10^{-5}%
Isolate 26	No	9%	
Isolate 27	No	9%	
Isolate 28	No	8%	
Sunblotch (graft) inoculated Avocado seedlings[d]			
Isolate 29	Yes	77%	2.8×10^{-1}%
Isolate 30	No	25%	

[a] Plateau of hybridization of Rot curve or maximum level of hybridization at a Rot of 4×10^{3} mol sec litre^{-1}.

[b] Determined by dividing the Rot½ of the Rot curve (of [^{32}P]-cDNA-ASBV against partially purified plant nucleic acid) by the Rot½ of ASBV (7×10^{-4} mol sec litre^{-1}) and multiplying by 100%.

[c] Hybridization value at Rot of 3×10^{2} mol sec litre^{-1}; % ASBV is based on a Rot½ of 3×10^{2} mol sec litre^{-1}.

[d] Isolates described in Materials.

ASBV was still below the limit of detectability as a band on a gel.

The two avocado seedlings inoculated with ASBV and showing detectable levels of ASBV were maintained in plant room B (Chapter II, Materials); i.e., at 30°C. Thus, five of the ASBV-inoculated avocado seedlings with no detectable levels of ASBV were re-inoculated with ASBV and maintained in plant room B (at 30°C). After 4 months, nucleic acids were extracted from each of the five re-inoculated seedlings and analyzed for the presence of ASBV by hybridization to [^{32}P]cDNA-ASBV; no ASBV was detected in any of the five inoculated (and symptomless) seedlings (results not shown).

Thus, while there is evidence for the replication of ASBV in *G. aurantiaca* and two avocado seedlings, albeit to very low levels, no symptoms of the sunblotch disease have been observed on ASBV-inoculated seedlings even after 7 months post inoculation [a graft-inoculated seedling developed symptoms on the stem (yellow streaks) during the same time period (4 months in plant room B; this seedling (Isolate 29) showed a high level of ASBV (Table VI-5)]. Therefore, it yet remains to be shown that ASBV is the causal agent of the sunblotch disease of avocados.

DISCUSSION

A low molecular weight RNA was found to be associated with some isolates of avocados infected by the sunblotch disease. This RNA was absent from other isolates of sunblotch-infected avocados and from healthy avocados (Figure VI-1).

The low molecular weight RNA was purified and shown to be circular and to possess a high degree of secondary structure; these are the physical properties characteristic of a viroid (Diener and Hadidi, 1977), and suggest that the sunblotch disease of avocados is caused by a viroid:avocado sunblotch viroid (ASBV).

The suggestion that the sunblotch disease of avocados is caused by a viroid has also been independently proposed by two other groups (Dale and Allen, 1979; Thomas and Mohamed, 1979), who have reported a low molecular weight RNA, present in partially purified nucleic acid extracts of sunblotch-infected avocados, but absent in similar extracts of healthy avocados. However, neither of these two groups purified the RNA or characterized it.

A cDNA probe was (1) prepared to ASBV, (2) shown to be specific for ASBV and for ASBV sequences present in sunblotch-infected but not healthy avocado plants, (3) used to quantitate ASBV levels in sunblotch-infected avocados, and (4) used to index avocados for the presence of ASBV.

Results of the hybridization analysis of nucleic acids extracted from sunblotch-infected and healthy avocados, showed the following.

(1) The level of ASBV in nucleic acid extracts, and probably the plants used as the nucleic acid source, varied widely (10,000-fold); some of this variation may have been due to sample selection (e.g., tissue type and age of tissue, avocado variety) or seasonal variation.

(2) Nucleic acids extracted from symptom-bearing plants were of two types: those containing an ASBV band on a gel and

those without a visible ASBV band. However, all types of nucleic acids from symptom-bearing plants showed high levels of hybridization.

(3) Symptomless carrier plants produced the same results as symptom-bearing plants; i.e., ASBV levels were present at both high and low levels in nucleic acid extracts of symptomless carrier plants. This is in contrast to the results of (a) Thomas and Mohamed (1979), who suggested that detectable levels of ASBV by gel analysis of nucleic acid extracts could not be obtained from symptom-bearing leaves, but could be obtained from symptomless carrier leaves (implying that symptomless carriers have a much higher level of ASBV than symptom-bearing plants), and (b) Wallace and Drake (1962), who suggested that the level of sunblotch was higher in symptom-bearing plants than in symptomless carriers. My results suggest that both situations can occur.

(4) There was a complete correlation between the presence of ASBV and the presence of sunblotch disease (Table VI-3).

(5) Point (4) made it feasible to test apparently healthy, but biologically non-indexed, glasshouse and field avocados for the presence of sunblotch. Of 37 such isolates, one (Isolate 15) was ASBV-positive (a sunblotch graft-inoculated recipient), one was showing low levels of hybridization to the ASBV-cDNA and remains non-indexed, and 35 isolates were indexed ASBV-negative.

Therefore, if the cDNA-indexing procedure was to be used to index for the presence of sunblotch, the time required to

test a prospective candidate could be reduced from a minimum of 2 yr (Wallace and Drake, 1962; Burns *et al.*, 1969) to 5 days (two days for the extraction and three days for the hybridization). A time-saving of this nature could be of great economic importance.

In order to replace the present biological indexing technique with a cDNA-indexing technique, it is necessary to conclusively establish proof that ASBV is the causal agent of the sunblotch disease. Such proof requires the inoculation of ASBV onto avocados with the subsequent development of the characteristic sunblotch symptoms. Such an experiment is underway with the expectation that symptoms may take at least one year to develop (Wallace and Drake, 1962; Burns *et al.*, 1969).

That the sunblotch agent is a nucleic acid and is mechanically transmissible has been shown by Mohamed and Thomas (Mohamed, personal communication), who were able to transmit sunblotch disease to 4 out of 10 avocado (Hass) seedlings in 4 mo by using a total nucleic acid extract from sunblotch-infected avocados.

The results of attempting to transmit sunblotch to avocados by inoculating avocados with purified ASBV (shown here in Results) have so far shown the following.

(1) After 7 mo post inoculation, not one of 8 inoculated avocados has shown the symptoms of sunblotch disease.

(2) However, low levels of replication of ASBV have been detected (by hybridization analysis) in two avocado seedlings and in *Gynura aurantiaca*, a host-plant of a number of viroids (Diener and Hadidi, 1977; Niblett, personal comm-

unication; Results, Chapter II).

(3) Neither symptoms nor the replication of ASBV was observed in several other plants that are known viroid host-plants; i.e., tomatoes, chrysanthemums and *Gynura sarmentosa* (Table VI-5). At present, the only known hosts of avocado sunblotch disease are the avocado (*Persea sp.*) and the cinnamon plant (*Cinnamomun zeylanicum*; da Graca, 1979); there are no known herbaceous hosts of sunblotch disease (or ASBV).

In summary, establishment of proof that ASBV is the causal agent of the sunblotch disease of avocados will enable the cDNA-indexing procedure to replace the costly, laborious, and time consuming biological indexing procedure for sunblotch disease. Eventually, the cDNA-indexing technique should replace biological indexing for a large number of viroid-induced (e.g., PSTV in potatoes and CEV in citrus) and viral-induced (e.g., potyviruses and luteoviruses) plant diseases.

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