Viroids and Viroid-Like Pathogens

Editor

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PREFACE

At the outset I would like to presume the position of at least the caring, if not always humble, "midwife" at the birth of viroid research. Without further claims of any closer lineage, that position in itself has permitted me to bear direct witness to not only the pains and anxieties normally associated with such creative activities but also the fleeting yet sublime exhilaration that is born of such wondrous moments. It is my view that viroids because of their humble beginnings in the Plant Kingdom have not as yet reached a rightful position of full acceptance in the biological world. This is in part due to absence of a direct bloodline to relatives affecting members of the Animal Kingdom. From the perspective of an advocate of the existence of great unifying principles of biology, I retain the vision that this linkage will yet be made. For this reason, I have coerced Prof. R. Marsh to contribute a brief chapter on the most analogous agents in animal systems for which research data are available, namely the scrapie disease.

Once the viroids were recognized as discrete biochemical entities in the early 1970s, the science of a biological phenomenon seemingly advanced to a period mirroring the energies of adolescence. This period of viroid research development was reflective of some of the tribulations resident to such a stage in which hypotheses, propositions, and even personal intuitions were presented in spirited dialogue; and, as with teenage romance, it became too serious, too soon, only to fade too quickly.

But the undeniable forces of maturation to which we are all inevitably held captive had been primed. Substantive physical measurements established a legitimate position for viroids at least as an interesting class of unique RNAs from which structural and conformational information might be derived. We are presently heir to that treasure of information as presented in the chapter contributred by Dr. Paul Keese and Prof. Symons.

And yet without the further investment of these resources, we would be soon left with but a dwindling fortune to will to the succeeding generation of true believers in the basic importance of viroids in the grand scheme of biology. And so when I was approached with the siren and at the same time ominous proposition by CRC Press for this volume, I immediately enlisted the active participation of Robert, Hugh, and Stephen to comprise yet another set of "four evangelists." In this way it was hoped that a truth of purpose might emerge from a consensus position. My only directive was to encourage not simply another review, but a personal perspective of interpretive data and a willingness to offer explanation and even speculation on the significance of viroid synthesis and biological interactions. Viroid replication viewed as an RNA processing event has been contributed by Prof. Robertson and Dr. Andrea Branch. We have chosen to package the responses of plants to viroids with the broader agricultural insights of Dr. Garnsey and Dr. Randles, and from a more cellular position, where it has been my pleasure to interact once again with my unique friend Prof. Conejero.

If we had hoped for one unifying theme among these chapters, it would be contained in the belief that the future of viroid research resides in the basic biochemical interactions with the plant cell that result in processes which alter growth and development, some of which may be labelled as pathological reactions. And that these expressions, which we hope are made more relevant by this volume, offer even more exciting challenges and resident rewards than even the initial conception of viroids as entities would be our wish.

We would simply entreat the reader along with the fair Ophelia to not only "let all 'our' sins be remembered" but also forgiven.

2 May in 1986
Riverside, California

THE EDITOR

Joseph S. Semancik, Ph.D., is Professor of Plant Pathology and a member of the Cell Interaction Research Group at the University of California, Riverside.

Professor Semancik received the A.B. degree (cum laude) in biology in 1960 from Western Reserve University, Cleveland, Ohio. He received the M.S. (1962) and Ph.D. (1964) from Purdue University, W. Lafayette, Indiana, working under the supervision of Professor J. B. Bancroft, in part, as a NIH predoctoral fellow. In 1964, he joined the faculty of the University of California as Assistant Professor of Plant Pathology and, except for the period of 1969-71, when he held the position of Associate Professor at the University of Nebraska-Lincoln, he has continued his affiliation with the University of California, Riverside.

The research interests of Professor Semancik evolved from his early biochemical and biophysical studies of the basis for electrophoretic and centrifugal heterogeneity of plant viruses to detection and properties of free-RNA forms of plant virus infection. His current program is directed exclusively to viroids, with particular emphasis on detection, synthesis, and viroid-cell interactions. Since 1968, he, along with students and colleagues in his laboratory, have contributed 45 published papers specifically to the field of viroid research.

Professor Semancik received the Spengler Award for Excellence in Botany from Western Reserve University (1960). In 1977-78, he was named a Guggenheim Fellow and a NATO Senior Scientist in support of studies in the Department of Biochemistry, State University, Leiden, The Netherlands. In 1984 he participated in a NATO Collaborative Research Project in Valencia, Spain, investigating the viroids of the region.

The American Phytopathological Society designated Professor Semancik a Fellow in 1979. In 1975, Professor Semancik shared with Dr. T. O. Diener the Alexander von Humboldt Award for "research in the recognition and establishment of viroids, a hitherto unknown group of pathogens and for their biological and chemical characterization."

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TABLE OF CONTENTS

Chapter 1

THE STRUCTURE OF VIROIDS AND VIRUSOIDS

P. Keese and R. H. Symons

TABLE OF CONTENTS

I. INTRODUCTION

A. Viroids

Viroids (defined in the Glossary at the end of this chapter and listed in Table 1) are the smallest known pathogens of flowering plants. Their history is brief but dramatic, both in appreciation of their ecomonic impact and in the growth of knowledge concerning their unique characteristics. Viroid-induced diseases were first recognized only recently with the earliest reports dating back to 1922 for potato spindle tuber disease (see References 19 and 22 for a detailed account of the history of viroid diseases). Nevertheless, viroids have been responsible for several economically serious diseases such as chrysanthemum stunt diseases in the U.S. from 1945 to 1947[23] and cadang cadang disease of coconuts which has resulted in the death of more than 30 million palms in the Philippines since its discovery in 1930.[24] Cadang cadang disease remains uncontrolled and continues to spread (see Chapter 4).

Table 1
LIST OF VIROIDS

Viroid[a]	Abbreviation	No. of nucleotides	Ref.
Avocado sunblotch viroid	ASBV	247	3,4
Chrysanthemum stunt viroid	CSV	354,356	5,6
Citron variable viroid	CVaV	N.D.[e]	7
Citrus exocortis viroid	CEV	370—375	6,8-10
Coconut cadang cadang viroid[b]	CCCV	246,247	11
Coconut tiananga ja viroid	CTiV	N.D.[e]	20,21
Columnea viroid	CV	N.D.[e]	12
Cucumber pale fruit viroid[c]	CPFV	303	13
Hop stunt viroid	HSV	297	14
Potato spindle tuber viroid	PSTV	359	15,16
Tomato apical stunt viroid[d]	TASV	360	17
Tomato planta macho viroid	TPMV	360	17

[a] The disease agent of Burdock stunt has been considered to share some affinities with viroids.[18] The disease agent of chrysanthemum chlorotic mottle disease has often been listed as a viroid.[19] However, since these disease agents have never been isolated, they are not included in this table.

[b] CCCV infections produce four major RNA components, all derived from the infectious monomeric small form (D-O of 246 or 247 nucleotides). These include monomers and dimers of both the D-O form and any of a set of larger forms (D-41, D-50, or D-55) which contain a duplication involving 41, 50, or 55 nucleotides. The causative agent (CTiV) of another disease of coconuts, tinangaja[20] is approximately the same size as, and shows partial sequence homology with, CCCV.[20,21]

[c] CPFV is a sequence variant of HSV since the two viroids share 95% sequence homology.[13,14]

[d] In early reports, TASV was referred to as tomato bunchy top viroid.[19]

[e] N.D., not determined.

Although viroid diseases were initially linked to viruses due to the virus-like symptoms (see Chapter 3) and the small size of the causative agent, the distinctive features of potato spindle tuber "virus" were made apparent by Diener in 1971,[22] leading to the term viroid. Similar properties were reported for the citrus exocortis and chrysanthemum stunt agents.[25-28] Since then, eight additional viroids have been recognized (Table 1). Furthermore, there has been a rapid accumulation of information regarding viroid structure, replicaton, and pathogenesis.

This chapter will present the most recent findings and postulations which attempt to relate directly viroid structure to function. For a more general introduction to viroids, the reader is referred to the excellent reviews of Riesner et al.,[29] Sänger,[30] Diener,[31] Riesner and Gross,[32] and the book by Diener.[19]

Summarizing the present state of knowledge, viroids can be distinguished from viruses by:

1. **Lack of mRNA activity.** This has two important consequences. First, there is no viroid-coded protein coat; a virus-coded protein coat has normally been associated with virus survival and spread. Second, viroids rely completely on host factors for their replication in contrast to viruses which have been shown to encode a viral-specific polymerase in all cases where definitive results have been obtained.

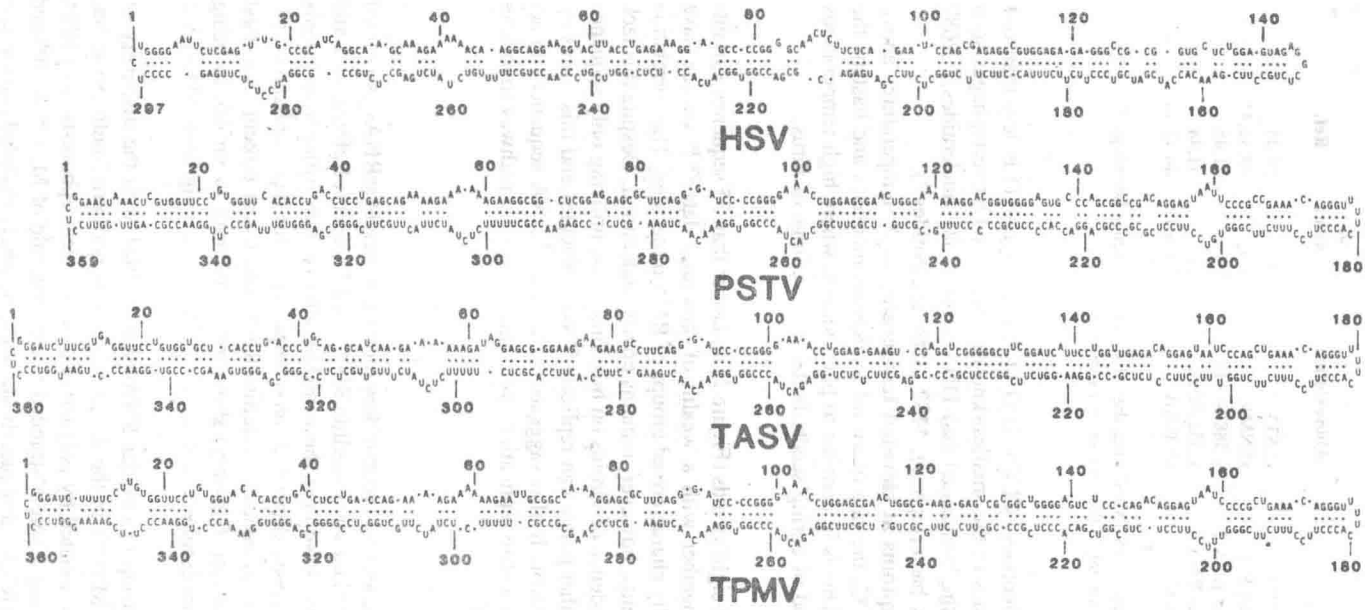

FIGURE 1. Sequences and proposed secondary structures of ASBV,[4] CCCV D-O (the basic 246 nucleotide species),[11] CEV-A,[8] CSV,[5] HSV,[14] PSTV,[15] TASV,[17] and TPMV.[17] CPFV[13] (not given) is a sequence variant of HSV (Table 1).

Table 2
LIST OF VIRUSOIDS

Virusoid	Abbreviation	No. of nucleotides	Ref.
Lucerne transient streak virus RNA 2	vLSTV	324	39,45
Solanum nodiflorum mottle virus RNA 2	vSNMV[a]	378	40,43,44
Subterranean clover mottle virus RNA 2	v(388)SCMoV[b]	388	41,48
Subterranean clover mottle virus RNA 2′	v(332)SCMoV[b]	332	41,48
Velvet tobacco mottle virus RNA 2	vVTMoV[a]	366,367	40,42,44

[a] vSNMV is a sequence variant of vVTMoV since they share 93% sequence homology.[40]
[b] Natural isolates of SCMoV contain either one or two virusoids.[48]

2. **Small size.** With 246 nucleotides, CCCV D-O (Table 1, Figure 1) is less than one tenth the size of the genomes of the smallest known viruses and bacteriophages such as maize streak virus, a single-stranded (ss) DNA virus (2681 nucleotides,[33] 2687 nucleotides[34]), or a ssRNA bacteriophage, MS2 (3569 nucleotides[35]).
3. **Increased yields and symptoms at elevated temperatures.** At temperatures above 20°C and at least up to 35°C, the rate of viroid replication increases and hastens the onset of symptoms.[30,36,37] This is in contrast to plant viruses where high temperatures have been used as a method of curing seeds, bulbs, and cuttings of virus.

With the complete sequence of eight viroids (Figure 1) and more than 35 sequence variants (see Glossary for definition), together with a wealth of structural data,[29,38] viroids have become one of the best structurally characterized groups of RNA molecules. The correlation of structure to the biology of viroids, however, remains poorly understood. Despite this lack of understanding, the total dependence of viroids on host functions, utilizing only sequence and structural signals, indicates that plants can replicate RNA molecules and this opens up the potential for biochemical control. It also suggests the ability of RNA sequences to act directly as control elements in the entire replication cycle and pathogenic pathway involving host range and symptom expression.

B. Virusoids

In addition to viroids, there is a second group of low molecular weight ssRNAs associated with plant diseases; these are the plant virus satellite RNAs (see Glossary; References 1 and 2). Although some of these RNAs also do not show mRNA activity, they differ from viroids in two significant aspects: (1) they are dependent on a helper virus for their replication; (2) they are encapsidated by either viral-coded or satellite RNA-coded coat protein. Amongst the satellite RNAs, the virusoids (see Glossary) show most similarity to viroids in being covalently closed circular RNA molecules in the same size range, varying from 324 to 388 nucleotides (Table 2, Figure 2).

The first report of this new group of circular RNAs was in 1981 with the discovery of velvet tobacco mottle virus (VTMoV[42]) in the desert regions of northern South Australia. When total virion RNA was fractionated by polyacrylamide gel electrophoresis, two RNA components were found, one being a single-stranded linear molecule of M_r 1.5×10^6 and the other a low molecular weight RNA subsequently shown to be single-stranded and circular with 355 or 356 nucleotides.[40,42] Re-investigation of two previously described viruses, solanum nodiflorum mottle virus (SNMV[40,43,44]) and lucerne transient streak virus (LTSV[39,45])

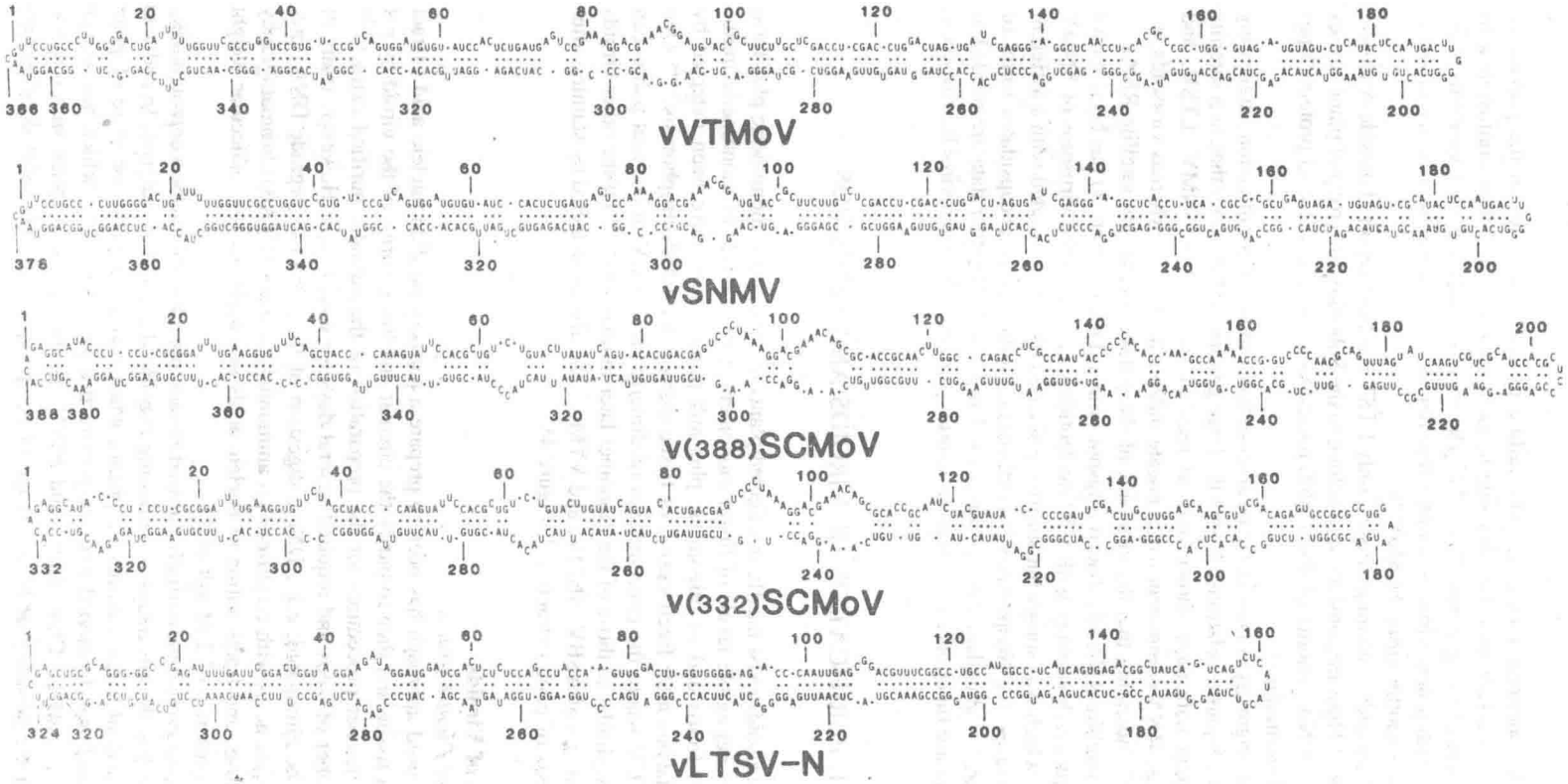

FIGURE 2. Sequences and proposed secondary structures of virusoids. References and further details are in Table 2.

showed that each contained a virusoid. The failure to detect virusoids in the previously reported SNMV and LTSV was probably due to their small size and their confusion with breakdown products of the genomic RNA.[46,47] The most recently reported virusoids were found associated with a new virus infecting subterranean clover *(Trifolium subterraneum)*, subterranean clover mottle virus (SCMoV[48]).

Of these four virusoid-containing viruses, only LTSV has been reported outside Australia and New Zealand.[49] They are considered to belong to the Sobemovirus group of plant viruses with a unipartite ssRNA genome of about 4400 nucleotides with a small viral protein (Vpg) attached at the 5′-terminus.[50]

Virusoids were originally considered to be an essential component for infection, suggesting that they acted in a bipartite relationship with the large genomic RNA rather than as a satellite RNA.[51] Subsequent data have shown that, at least in the cases of SNMV, LTSV, and VTMoV, the large RNA component can replicate independently,[52-54] whereas virusoids are not infectious,[51-53] indicating that the virusoids of these three viruses are satellite RNAs.

Recently, the satellite RNA of tobacco ringspot virus (sTRSV; Figure 3) has been shown to have high sequence homology with a 50 nucleotide sequence in the virusoids of SNMV and VTMoV[55-57] which was somewhat surprising since sTRSV is associated with a different group of plant viruses, the Neopviruses.[50] Furthermore, although the encapsidated forms of sTRSV are linear,[58,59] abundant circular forms exist in vivo.[59,60] Further data are needed to determine if there are functional as well as structural similarities between viroids, virusoids, and sTRSV.

II. PURIFICATION OF VIROIDS AND VIRUSOIDS

Viroids and virusoids exist mostly in infected plants in the circular monomeric plus form with only low or negligible levels of linear monomers. Lower levels of monomeric minus (complementary) forms and of oligomeric plus and minus forms have been detected by hybridization analysis after fractionation of tissue extracts by gel electrophoresis.[61-66] One exception is CCCV where the concentration of dimeric plus CCCV in coconut palms can increase to levels similar to those of the monomer later in infection.[67,68] Of the other viroids and virusoids, dimers of ASBV, SNMV, and VTMoV can also be detected by staining after gel electrophoresis of plant extracts[69,70] (Figure 4).

A. Purification of Viroids

1. Preparation of Plant Extracts

A commonly used approach has been to prepare a partially purified nucleic acid extract of infected plant tissue and then to purify the circular and linear forms of the viroid by gel electrophoresis. Earlier procedures for the preparation of the partially purified extract[71-73] contained a number of steps and required several days for completion. However, many of these steps can be eliminated; e.g., DNase digestion of the extract to degrade DNA, precipitation of nucleic acids with cetyltrimethyl ammonium bromide (CTAB) chromatography on CF-11 cellulose, and fractionation of nucleic acids into high and low molecular weight fractions in the presence of 2 *M* salt at 0°C.

During the preparation of the partially purified extract, phenol is still the best deproteinizing agent and it also has the advantage of removing considerable colored material. In order to circumvent the use of large volumes of phenol when 500 g quantities or more of plant material were used, we developed a two-step extraction procedure[69,74,75] which has proven very effective for ASBV, CSV, CEV, and PSTV. In the first step, plant material was homogenized in the presence of SDS a range of compounds to inhibit color development

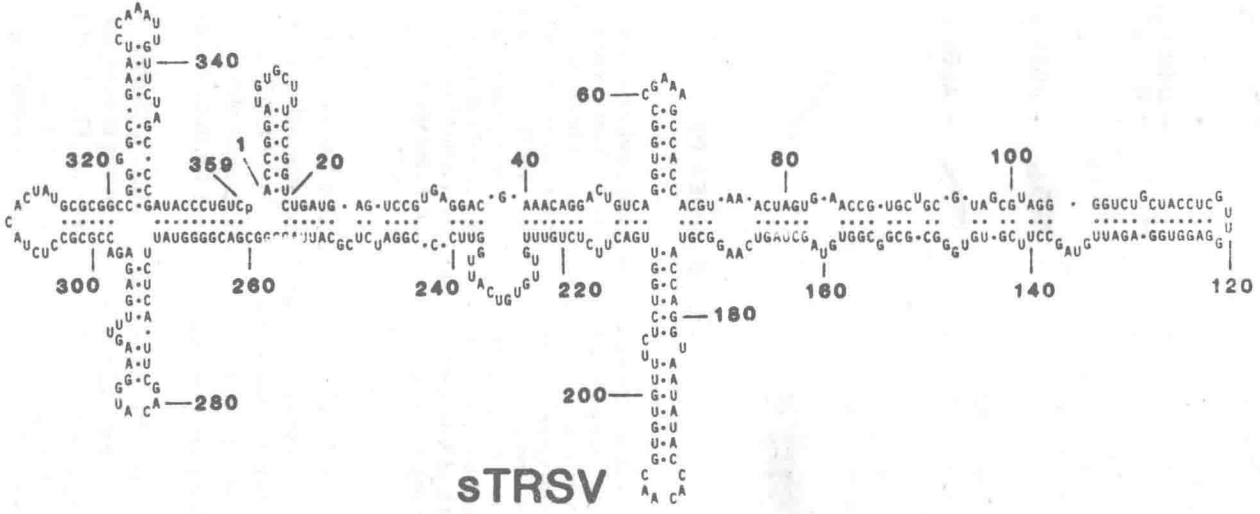

FIGURE 3. Sequence and proposed secondary structure of satellite RNA of tobacco ringspot virus (sTRSV).[55]

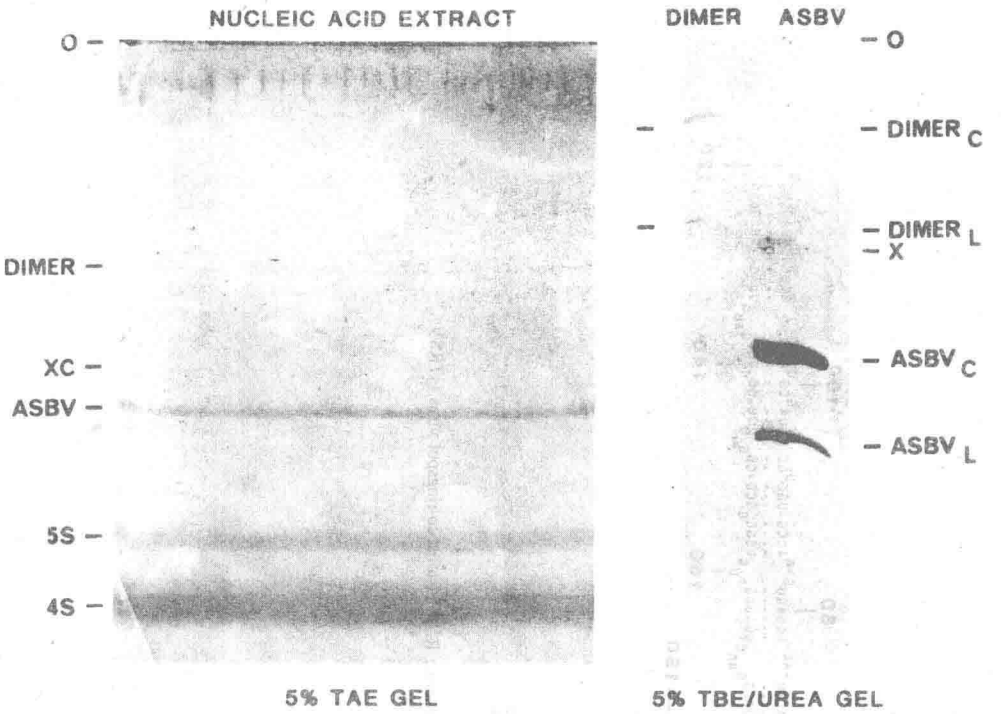

FIGURE 4. Purification of ASBV from extracts of infected avocado leaves.[69] (A) Partially purified nucleic acid extract from 35 g leaves was electrophoresed on a 16 × 16 × 0.3 cm 5% polyacrylamide gel in 40 m*M* Tris-acetate, 20 m*M* sodium acetate, 2 m*M* EDTA, pH 7.5, at 25 mA for 16 hr. The gel was stained in 0.02% toluidine blue and destained in water. The positions of the dimer ASBV, xylene cyanol FF marker dye (xc), ASBV, and 5S and 4S RNA are shown. (B) Dimer ASBV and ASBV bands were eluted and run on a 90 m*M* Tris-borate, 2 m*M* EDTA, 7*M* urea, 5% polyacrylamide gel (20 × 40 × 0.05 cm) at 20 mA for 1.8 hr. The gel was stained with 10 μg ethidium bromide/mℓ for 30 min, destained in water, and photographed under UV light. The positions of the circular (C) and linear (L) forms of the dimer ASBV, ASBV, and an unidentified band X are given. The mobility of circular ASBV was 0.4 relative to the xylene cyanol FF marker dye. (From Bruening, G., Gould, A. R., Murphy, P. J., and Symons, R. H., *FEBS Lett.*, 148, 71, 1982. With permission.)

and $MgCl_2$ to inhibit the solubilization of pectins. Addition of NaCl to 0.5 *M* caused the precipitation of proteins as a SDS complex.[76] The nucleic acids in the supernatant were concentrated by ethanol precipitation and further deproteinized with phenol-$CHCl_3$ in a volume about 10% of the original aqueous extract. After dialysis of the aqueous phase and a further ethanol precipitation step, the partially purified extract was ready for fractionation by gel electrophoresis.

A variation of the final dialysis step was found to be very useful where a number of smaller samples of about 25 g of plant material were being extracted.[77] In this method, the aqueous supernatant after phenol-$CHCl_3$ deproteinization was made to 6 *M* LiCl by the addition of an equal volume of 12 *M* LiCl; the final concentration of 6 *M* LiCl was necessary to ensure precipitation of low molecular weight RNAs. After 2 hr at 0°C, the precipitate was collected by centrifugation, redissolved, and the nucleic acids precipitated with ethanol prior to gel electrophoresis. We have also used this procedure extensively for the preparation of nucleic acid extracts of avocado leaves for the indexing of ASBV by the dot-blot procedure using ^{32}P-cDNA probes.[77]

Where only a few grams of plant material were to be extracted, a procedure modified