

VIROIDS: MINIMAL GENETIC SYSTEMS

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SUMMARY

Viroids are nucleic acid species of relatively low molecular weight and unique structure that cause several important diseases of cultivated plants. Viroids are the smallest known agents of infectious disease. Unlike viral nucleic acids, viroids are not encapsidated. Despite their small size, viroids replicate autonomously in cells of susceptible plant species. Known viroids are single-stranded, covalently closed circular, as well as linear, RNA molecules with extensive regions of intramolecular complementarity; they exist in their native state as highly base-paired rods. The biological properties of viroids are determined by their primary structures; viroids thus constitute genetic systems of minimal complexity.

INTRODUCTION

Viroids are distinct nucleic acid species of relatively low molecular weight (1.1 to 1.3×10^5) that can be isolated from certain organisms afflicted with specific maladies. They are not detectable in healthy individuals of the same species, but, when introduced into such individuals, they replicate autonomously despite their small size and cause the appearance of the characteristic disease syndrome (DIENER, 1979). In certain other species, however, some viroids replicate without producing obvious disease symptoms. Unlike viral nucleic acids, viroids are not encapsidated; that is, no viruslike nucleoprotein particles occur in infected tissue (DIENER, 1971a). Viroids are the smallest known agents of infectious disease (DIENER, 1974); so far, they are definitely known to exist only in higher plants and to consist of RNA (DIENER & HADIDI, 1977).

The first viroid was discovered in attempts to purify and characterize the causative agent of the potato spindle

tuber disease, which for many years had been assumed to be of viral etiology (DIENER & RAYMER, 1971). DIENER and RAYMER reported in 1967 that the transmissible agent of this disease is a free RNA and that no viral nucleoprotein particles (virions) are detectable in infected tissue. Later, in 1971, sedimentation and gel electrophoretic analyses conclusively demonstrated that the infectious RNA is far smaller than the smallest genomes of autonomously replicating viruses (DIENER, 1971b). No evidence could be found for the presence of a helper virus in uninfected tissue; it thus appeared that, despite its small size, the RNA was able to replicate autonomously in susceptible cells. Because of the basic differences between this infectious agent and conventional viruses, the term *viroid* was introduced to denote pathogenic nucleic acids with properties similar to those of the potato spindle tuber agent (PSTV) (DIENER, 1971b).

Five additional plant diseases--citrus exocortis (SEMANCIK & WEATHERS, 1972), chrysanthemum stunt (DIENER & LAWSON, 1973), cucumber pale fruit (VAN DORST & PETERS, 1974), chrysanthemum chlorotic mottle (ROMAINE & HORST, 1975), and hop stunt (SASAKI & SHIKATA, 1977)--are now known to be caused by viroids and two additional diseases--coconut cadang-cadang (RANGLES *et al.*, 1977) and avocado sunblotch (PALUKAITIS *et al.*, 1979)--probably also are of viroid etiology.

Furthermore, a viroid has been isolated from apparently healthy *Columnnea erythrophae* plants that causes symptoms similar to, if not identical with, those of PSTV in tomato and potato, yet is distinct from PSTV in its primary structure (OWENS *et al.*, 1978).

In this article, recent advances in our knowledge of the physical-chemical properties of viroids, and of their interactions with susceptible host cells are summarized. Also, possible mechanisms of viroid replication are discussed and the question is raised as to whether viroids exist in life forms other than higher plants.

PHYSICAL-CHEMICAL PROPERTIES

PURIFICATION

Although viroids constitute only a small portion of the RNA extractable from infected tissue, large-scale extraction of leaves and improvements in the methods of viroid separation and purification made it possible to obtain viroid preparations essentially free of contaminating host nucleic acids (DIENER, 1972a), thereby allowing purification of PSTV and other viroids in amounts sufficient for biophysical and biochemical characterization (DIENER *et al.*, 1977).

MOLECULAR STRUCTURE

NATIVE MOLECULES. Electron microscopy of purified

PSTV revealed a uniform population of rods (about 50 nanometers long) with widths similar to that of double-stranded DNA (SOGO *et al.*, 1973). Although these observations suggested that PSTV is double-stranded, other results were not compatible with this concept. Thus, from hydroxyapatite columns the viroid mostly elutes at a lower phosphate buffer concentration than does double-stranded RNA (DIENER, 1971c), and its elution pattern from CF11 cellulose is consistent with the presence of both single- and double-stranded infectious molecules (DIENER & RAYMER, 1969). Similar results have been reported for the citrus exocortis viroid (CEV) (SINGH & SANGER, 1976).

The thermal denaturation properties of PSTV (DIENER, 1972a) and CEV (SEMANCIK *et al.*, 1975) are also not compatible with those of double-stranded RNA. PSTV melts at lower temperatures than does double-stranded RNA, but denaturation occurs over a narrower temperature range than denaturation of single-stranded RNA's. Values for T_m (the temperature at which 50 percent of the molecules are denatured) of 50° to 58°C and total hyperchromicities of 10 to 24 percent have been measured for various viroids under ionic conditions equivalent to 0.01 to 0.1 x SSC (SSC is 0.15 M sodium chloride and 0.015 M sodium citrate at pH 7.0) (DIENER, 1972a; SEMANCIK *et al.*, 1975; RANGLES, *et al.*, 1976; SANGER *et al.*, 1976). These results suggest that native viroids are single-stranded RNA molecules with hairpin-like configurations and extensive regions of intramolecular base-pairing (DIENER, 1972a; SOGO *et al.*, 1973).

After denaturation of PSTV by heating in the presence of formaldehyde, the RNA reproducibly separates into two distinct bands upon electrophoresis in high concentration polyacrylamide gels under denaturing conditions (DIENER & SMITH, 1973). Results obtained by electron microscopy of denatured viroids explained this previously puzzling observation.

DENATURED MOLECULES. McCLEMENTS and KAESBERG (1975, 1977) first demonstrated the presence of covalently closed circular RNA molecules in purified viroid preparations by electron microscopy. To eliminate secondary structure, PSTV was treated with formaldehyde at 63°C, and the formylated RNA spread at a formamide concentration of 45 percent (McCLEMENTS & KAESBERG, 1977). Three structures were evident in such preparations: seemingly double-stranded rods, with a mean length of 50 nm; single-stranded circular molecules with a mean length of 140 nm; and single-stranded linear molecules with a mean length of 110 nm. In contrast to partially denatured preparations, no hairpin structures were detected in any preparations of formylated RNA. The presence of circles and the complete absence of hairpin structures suggested to the authors that the circular molecules are covalently closed rather than held together by invisibly short base-paired segments. In two independent isolates of PSTV, the proportion of circular to linear

molecules was nearly the same: 20 percent of the molecules were circular, 70 percent were linear, and 10 percent remained in their native conformation even after formylation (McCLEMENTS & KAESBERG, 1977).

CIRCULAR VS. LINEAR MOLECULES

Results of RNA oligonucleotide mapping (fingerprinting) (DICKSON *et al.*, 1975), done with a mixture of the two components, make it unlikely that the circular and linear PSTV structures are two distinct RNA species. More likely, the two structures represent two stages of maturity of PSTV. In the presence of formamide and urea, PSTV can be separated by gel electrophoresis into two fractions (OWENS *et al.*, 1977). One fraction contains predominantly circular molecules; the second fraction contains exclusively linear molecules. Bioassay on tomato demonstrated that both circular and linear molecules are infectious.

These results conflict with those of SÄNGER *et al.* (1976) who consider viroids to be exclusively covalently closed circular RNA molecules and linear molecules to be rare (0.5 to 1.0 percent) and to arise solely by "nicking" of circular molecules during viroid isolation and purification. Indeed, exposure of viroid preparations in alkaline buffers to elevated temperatures in the presence of Mg^{2+} has been shown to lead to a conversion of circular viroid molecules to linear strands, as well as to further fragmentation of the linear molecules (SÄNGER *et al.*, 1979). Because some purification procedures (DIENER & HADIDI, 1977; DIENER *et al.*, 1977) subject viroids to comparable conditions (although not at elevated temperatures), the presence of linear molecules in viroid preparations has been attributed to Mg^{2+} -catalyzed cleavage of viroid circles (SÄNGER *et al.*, 1979). However, in contrast to the linear molecules present in preparations obtained by these purification procedures [which have specific infectivities comparable to those of circular molecules (OWENS *et al.*, 1977)], linear viroid molecules obtained by Mg^{2+} -catalyzed cleavage of circles exhibit little, if any, infectivity (SÄNGER *et al.*, 1977). It would appear, therefore, that the two types of linear viroid molecules are not identical, but further work is evidently required to clarify these observations and to determine the biological significance, if any, of linear viroid molecules.

Viroids may be radioactively labeled *in vivo* by introduction of phosphorus-32 through roots of infected plants (SÄNGER & RAMM, 1975; HADIDI & DIENER, 1977). Kinetic studies of the *in vivo* labeling of circular and linear PSTV molecules with varying times of ^{32}P incorporation suggest that linear PSTV arises *in vivo* by cleavage of circular PSTV molecules (HADIDI & DIENER, 1978).

MOLECULAR WEIGHT

Early attempts to determine the molecular weight of viroids had to depend on the detection of the RNA by infectivity tests. Combined sedimentation and gel electrophoretic analyses of native PSTV resulted in a molecular weight estimate of 5×10^4 (DIENER, 1971b). The low molecular weight of PSTV was confirmed by its ability to penetrate small pore size polyacrylamide gels that exclude high-molecular-weight RNA's (DIENER & SMITH, 1971). In another study, similar molecular weight values were obtained for PSTV and CEV (SÄNGER, 1972), whereas other workers reported an estimate of 1.25×10^5 daltons for CEV (SEMANCIK & WEATHERS, 1972). Later, when purified PSTV had become available, gel electrophoresis of formylated RNA resulted in an estimate of 7.5 to 8.5×10^4 daltons (DIENER & SMITH, 1973).

Although these and other determinations conclusively demonstrated that viroids are far smaller than the genomes of conventional viruses, it is now clear that unambiguous molecular weight determinations by such comparative methods were not feasible because of the then unknown molecular structure of viroids, which rendered all RNA standards of known molecular weight inappropriate.

Sensitivity to irradiation with both ultraviolet light and ionizing radiation has also been used to estimate viroid molecular weights (target volumes). Thus, PSTV has been shown to require 70 to 90 times the dose of 254-nm ultraviolet light for inactivation than does tobacco ringspot virus (DIENER *et al.*, 1974); and a molecular weight of 1.1×10^5 has been calculated for CEV on the basis of its relative sensitivity to ionizing radiation (SEMANCIK *et al.*, 1973).

Determination of the molecular weights of three viroids by equilibrium sedimentation under denaturing conditions resulted in values of $119,000 \pm 4,000$, $127,000 \pm 4,000$, and $110,000 \pm 5,000$ for CEV, PSTV, and cucumber pale fruit viroid (CPFV), respectively (SÄNGER *et al.*, 1976). As calculated from its primary sequence, the sodium salt of PSTV has a molecular weight of 123,337 (GROSS *et al.*, 1978).

PRIMARY STRUCTURE

Despite their apparent homogeneity, purified viroid preparations might conceivably be composed of a population of several RNA species of about equal length but different nucleotide sequence which together might constitute a viral genome of more or less conventional size. Early experiments showed, however, that the infectivity dilution curve of PSTV is of the single-hit type, suggesting that for successful infection only one type of particle is required at each infection site (DIENER, 1972c). More decisive evidence has been obtained by two-dimensional

fingerprinting of oligonucleotides derived from PSTV or CEV by digestion with either pancreatic or T₁ ribonucleases (DICKSON *et al.*, 1975). With each viroid, the complexity of the resulting oligonucleotide pattern was compatible with an RNA of 250 to 300 nucleotides and, because this number agreed fairly well with the molecular weight estimates of the viroids, these viroid preparations appeared to contain only one specific RNA species and not a mixture of several different species. These results also clearly demonstrated that PSTV and CEV do not have the same primary sequence. Similar experiments demonstrated that chrysanthemum stunt viroid and the coconut cadang-cadang disease-associated RNA also are distinct RNA species with defined primary structures (DICKSON 1976); and with somewhat different methodology, GROSS *et al.* (1977) have confirmed these conclusions for PSTV, CEV, and chrysanthemum stunt viroid.

With PSTV, the existence of strains that differ in the severity of symptom expression in potato or tomato has long been recognized (DIENER, 1979); and it has been demonstrated that mild, moderate, and severe strains of PSTV have minor differences in their nucleotide sequences (DICKSON *et al.*, 1979).

Evidently, viroids constitute genetic systems whose properties are encoded in the nucleotide sequences of their RNA's. Consistent with this conclusion is the observation that the nucleotide sequences of individual "species" or viroids, such as PSTV, CEV, or chrysanthemum stunt viroid, differ extensively from each other, whereas the sequences of strains of one "species" differ only slightly.

The complete primary sequence of PSTV has been determined (GROSS *et al.*, 1978) by a combination of procedures, consisting of conventional digestions (complete and partial) by pancreatic and T₁ ribonucleases and newer base-specific cleavage of long 5'-terminal-labeled oligonucleotides, followed by polyacrylamide gel fractionation. The RNA consists of 359 ribonucleotides (73 adenosine, 77 uridine, 101 guanosine, and 108 cytidine monophosphate molecules); no modified nucleotides were found at the 5' terminus of any fragment sequenced. One unusual feature of the PSTV sequence is a stretch of 18 purines, mainly adenines, in positions 48 to 65. Similar sequences have also been identified in other viroids (GROSS *et al.*, 1978).

SECONDARY STRUCTURE

On the basis of quantitative thermodynamic and kinetic studies of their thermal denaturation, HENCO *et al.* (1977) concluded that viroids contain an uninterrupted double helix of 52 base pairs, as well as several short double-helical stretches, and they proposed a tentative model for the secondary structure

of viroids.

The presence of a long, uninterrupted double helix in PSTV or CEV is incompatible with the results of experiments in which treatment of unlabeled PSTV or iodine-125-labeled CEV preparations with double-strand-specific *Escherichia coli* ribonuclease III affected neither the electrophoretic mobility of PSTV or ¹²⁵I-labeled CEV (DICKSON, 1976) nor the infectivity of PSTV (DIENER, unpublished). For RNA to be cleaved by ribonuclease III, it must contain either an extended region of perfect double-stranded RNA (25 or more base pairs) or a highly specialized RNA sequence (ROBERTSON & HUNTER, 1975); the lack of effect implies that neither viroid contains such regions.

In a further study (LANGOWSKI *et al.*, 1978), a refinement of the earlier model was proposed: Viroids exist in their native conformation as extended rodlike structures characterized by a series of double-helical sections and internal loops. Between 250 and 300 nucleotides are needed to interpret the thermodynamic properties of the molecules (LANGOWSKI *et al.*, 1978). In contrast to the earlier model, the refined model proposes that, on the average, each helical sequence of four to five base pairs is followed by a defect in the form of an internal loop of two bases.

The nucleotide sequence of PSTV (GROSS *et al.*, 1978) precludes a perfect intramolecular base complementarity within the viroid molecule. Thus, the rigid, rodlike structure of PSTV must be based on a defective rather than a homogeneous RNA helix (DOMDEY *et al.*, 1978) in confirmation of conclusions arrived at earlier by different techniques.

A secondary structure model of PSTV has been proposed on the basis of its known nucleotide sequence (GROSS *et al.*, 1978). In this model, the primary sequence has been arranged to maximize the number of base pairs, as well as to reflect the location of sites of preferential nuclease cleavage and the sensitivity of cytidine residues to modification by sodium bisulfite (DOMDEY *et al.*, 1978). According to this model, a total of 122 base pairs (73 guanine-cytosine, 38 adenine-uracil, and 11 guanine-uracil pairs) are involved.

Present knowledge clearly indicates that viroids have a novel structure. Covalently closed circular single-stranded RNA molecules have not been reported previously. Also, the very high degree of intramolecular complementarity, which results in collapsed circles and hairpin structures with the appearance of double-stranded RNA molecules, is unusual. It is difficult to believe that this structure of viroids does not have important biological significance.

VIROID-HOST CELL INTERACTIONS

When viroids are introduced into susceptible cells, they are capable of autonomous replication; that is, replication without the assistance of a helper virus (DIENER, 1971b). This basic biological fact raises a number of intriguing questions. Foremost among these are the following. (i) By what mechanisms are viroids replicated? (ii) By what mechanisms do viroids incite diseases in certain hosts, yet replicate in many other susceptible plant species without discernible damage to the host? (iii) How did viroids originate?

In contrast to the extensive knowledge of viroid structure that has been achieved, it is not yet possible to answer these questions.

SUBCELLULAR LOCATION

Bioassays of subcellular fractions prepared from PSTV-infected tomato tissue demonstrated that only the tissue debris and nuclear fractions contain appreciable infectivity (DIENER, 1971a). The chloroplast, mitochondrial, ribosomal, and supernatant fractions contain only traces of infectivity. Most infectivity is chromatin-associated and can be extracted as free RNA with phosphate buffer. The significant infectivity associated with the tissue debris probably is due to incomplete extraction of nuclei. The CEV is also located primarily in the nuclear fraction in close association with the chromatin (SÄNGER, 1972). Comparison of nuclei isolated from uninoculated and PSTV-infected tomato leaf tissue showed that nuclei from infected tissue are larger and have increased RNA/DNA and protein/DNA ratios (TAKAHASHI & DIENER, 1975). It appears, therefore, that the infecting viroid migrates to the nucleus (by an unknown mechanism) and is replicated there. The absence of significant amounts of PSTV in the cytoplasmic fraction of infected cells suggests that most of the progeny viroid remains in the nucleus.

TRANSLATION

Viroids are of sufficient chain length to code for a polypeptide of about 10,000 daltons, although, with circular PSTV, the uneven number of 359 nucleotides theoretically permits three rounds of translation (GROSS *et al.*, 1978).

Testing *in vitro* for messenger RNA function of PSTV and CEV in cell-free protein-synthesizing systems from wheat germ, wheat embryo, *E. coli* and *Pseudomonas aeruginosa* showed that neither viroid has this function (DAVIES *et al.*, 1974; HALL *et al.*, 1974). Incubation at 60°C with extracts from the thermophilic *Bacillus stearothermophilus* and denaturation with dimethyl sulfoxide were tried in the hope that these procedures might permit ribosome recognition of RNA regions normally inaccessible *in vitro*, but no viroid messenger activity was induced. Furthermore, CEV is not translated in *Xenopus laevis* oocytes, even after polyadenylation *in vitro*, and does not interfere with the translation of endogenous messenger RNA's (mRNA's)

(SEMANCIK *et al.*, 1977).

Although viroids thus do not act as mRNA's in protein-synthesizing systems *in vitro*, they might be translated *in vivo*. Preexisting host enzymes might accept the infecting viroid as a template and synthesize a complementary strand which might then act as an mRNA. RNA sequences complementary to viroids have been identified in infected tissue (GRILL & SEMANCIK, 1978) and, with PSTV, the complementary strand, as constructed from the nucleotide sequence of the viroid, could theoretically serve as an mRNA (MATTHEWS, 1978). Neither PSTV nor its complement contain adenine-uracil-guanine initiation triplets, but the complementary strand contains four guanine-uracil-guanine triplets and six possible termination triplets that could theoretically result in four polypeptides containing 108, 79, 43, or 28 amino acids. With CEV, complementary RNA strands are present in both the cytoplasmic and nuclear fractions after cell fractionation, whereas infectious CEV is primarily found in the nucleus. Also, complementary sequences located in the cytoplasmic fraction are mostly insoluble in 2 M lithium chloride; that is, they appear to be essentially single-stranded molecules (GRILL and SEMANCIK, 1978).

These properties of the complementary strands would *a priori* render them more suitable to act as mRNA's than is the case with the infectious viroid molecules. Whether they do act in this capacity has not been determined but, if they do, novel, viroid-specific proteins should be detectable in protein preparations from infected host tissue. Comparisons of protein species in healthy and PSTV-infected tomato (ZAITLIN & HARIHARASUBRAMANIAN, 1972) and healthy and CEV-infected *Gynura aurantiaca* (CONEJERO & SEMANCIK, 1977) did not, however, reveal qualitative differences between healthy and infected plants. In both studies, synthesis of certain polypeptides was enhanced in infected as compared with healthy tissue, but, presumably, these are host- and not viroid-specific polypeptides.

These results, however, must be regarded with caution, because the one-dimensional SDS (sodium dodecyl sulfate)-polyacrylamide gel electrophoresis systems used in both studies may not be adequate to separate any viroid-specific low-molecular-weight polypeptides from host-specific polypeptides of similar size.

On the basis of present knowledge, it appears unlikely that the infectious viroid strand serves as an mRNA. Thus, the complementary RNA (cRNA) sequences detectable in infected tissue are probably synthesized by a preexisting host-specific enzyme and not by a viroid-coded one. Whether or not complementary viroid strands are translated is still an unsettled question, but even if they are, involvement of any resulting polypeptides in viroid replication would appear questionable.

POSSIBLE MECHANISMS OF REPLICATION

Whether the mechanism of viroid replication involves transcription from RNA or DNA templates is not known. Theoretically, an RNA-directed mechanism requires the presence of RNA sequences complementary to the entire viroid in infected tissue, as well as a preexisting host enzyme with the specificity of an RNA-directed RNA polymerase.

A DNA-directed mechanism requires the presence of DNA sequences complementary to the entire viroid. These DNA sequences might already be present, in repressed form, in uninfected hosts or they might be synthesized as a consequence of infection with viroids. In the latter case, a preexisting host enzyme with the specificity of an RNA-directed DNA polymerase (reverse transcriptase) would also be required. To distinguish between RNA- or DNA-directed mechanisms, the effects of certain antibiotic compounds on viroid replication have been investigated.

Actinomycin D is widely used as a specific inhibitor of DNA-directed RNA synthesis; it neither binds to double-stranded RNA (HASELKORN, 1964) nor inhibits tobacco mosaic virus replication, once the infection has been established (DAWSON and SCHLEGEL, 1976).

In a study of the effect of actinomycin D on subsequent PSTV synthesis, leaf strips from healthy and PSTV-infected plants were first incubated with actinomycin D or water and then in solutions containing tritiated uracil. Extraction of nucleic acids and analysis by polyacrylamide gel electrophoresis revealed ^3H incorporation into a component with electrophoretic mobility identical to that of PSTV in extracts from infected leaves, but ^3H incorporation into PSTV could not be detected when leaf strips were treated with actinomycin D under conditions that do not seriously inhibit the replication of several plant viral RNA's (DIENER & SMITH, 1975). Similar results were obtained with an RNA synthesizing system *in vitro* in which purified cell nuclei from healthy or PSTV-infected tomato leaves were used as an enzyme source (TAKAHASHI & DIENER, 1975). Isolation of low-molecular-weight RNA's from the reaction mixtures *in vitro* and analysis by gel electrophoresis showed that, in this system also, PSTV replication is sensitive to actinomycin D.

Sensitivity of viroid replication to actinomycin D has been confirmed recently in a study of CPFV synthesis in protoplasts isolated from tomato leaves (MÜHLBACH & SANGER, 1979). In the same study, the effect of α -amanitine on viroid synthesis has been investigated. Intracellular concentrations (10^{-8}M) of α -amanitine sufficient to inhibit tomato DNA-directed RNA polymerase II inhibited CPFV replication, but did not inhibit either tobacco mosaic virus RNA replication or the RNA polymerase III-directed synthesis of host 5S and transfer RNA.

All of these studies suggest that DNA-directed RNA synthesis may be involved in viroid replication and, in view of the results with α -amanitine, it appears that this involvement may be mediated by RNA polymerase II.

The following results of molecular hybridization experiments between labeled viroids and DNA isolated from host plants might possibly also be interpreted as favoring DNA-directed replication of viroids.

SEMANCIK and GEELLEN (1975) originally reported that ^{125}I -labeled CEV hybridizes specifically with "DNA-rich preparations" from CEV-infected tomato or *Gynura aurantiaca*, but not with "DNA-rich preparations" from uninfected tobacco, cowpea, tomato, or *Gynura*. The experimental results, however, are ambiguous, because RNA sequences complementary to CEV in the "DNA-rich preparations" would have permitted the formation of RNA-CEV duplexes as well as of DNA-CEV hybrids. In a later report from the same laboratory (GRILL & SEMANCIK, 1978), the existence of RNA sequences complementary to CEV has indeed been documented, but the relation between these RNA sequences and the previously reported DNA sequences has not been clarified.

In another investigation of DNA sequences complementary to viroids (HADIDI *et al.*, 1976), rigorously purified DNA preparations were used in molecular hybridization experiments, thus eliminating the possibility of RNA-viroid duplex formation. These studies demonstrated that infrequent, if not unique, sequences complementary to PSTV occur in the DNA's of several uninfected solanaceous plant species in which PSTV is capable of replication. DNA titration experiments indicated that the DNA's of uninfected tomato, potato, and *Physalis peruviana* contain sequences complementary to at least 60 percent of the PSTV molecule, whereas the DNA's of Chinese cabbage or barley, plants not known to be hosts of PSTV, contain sequences complementary to at most a small portion of PSTV or no complementary sequences. Other experiments showed that infection with PSTV has no detectable effect on the hybridization kinetics between PSTV and host DNA, indicating that no new DNA sequences are synthesized as a consequence of infection (HADIDI *et al.*, 1976). Whether or not these DNA sequences are used in PSTV replication is not known.

Experimental evidence that seems to favor RNA-directed viroid replication has also been obtained. Thus, it appears to have been established beyond reasonable doubt that uninfected cells of a number of plant species contain enzymes that are able to transcribe RNA from RNA templates. Such enzymes were first isolated from Chinese cabbage and tobacco leaves (ASTIER-MANIFACIER & CORNUET, 1971; DUDA *et al.*, 1973). These enzymes require the presence of a divalent metal ion and of four ribonucleotide triphosphates for activity. The enzymes show no template specificity;

apparently they will accept any single-stranded RNA. The reaction product is double-stranded RNA.

An enzyme with similar properties has been detected in healthy tomato leaves; enzymatic activity is greatly enhanced by the addition of PSTV, as well as by several other RNA species, but not by addition of DNA (HADIDI, unpublished). As has been pointed out (DIENER & HADIDI, 1977), such preexisting RNA-directed RNA polymerases may well be involved in viroid replication, but whether this is, indeed, the case, is not known.

Another indication that viroid replication may be RNA-directed consists of the demonstration of RNA sequences complementary to viroids in infected plants. Molecular hybridization between ^{125}I -labeled CEV and various nucleic acid fractions from healthy and CEV-infected *Gynura* showed that significant RNA sequences complementary to CEV were detectable only with nucleic acid fractions from infected tissue (GRILL & SEMANCIK, 1978). Treatment of nucleic acid preparations with pancreatic ribonuclease or with NaOH, but not treatment with deoxyribonuclease, abolished their ability to hybridize with ^{125}I -labeled CEV. The high T_m values, sharp melting profiles, and insensitivity to ribonuclease H of these hybrids indicate that they are well matched RNA-RNA duplexes.

Involvement of these complementary RNA sequences in viroid replication requires that the entire viroid sequence is represented. So far, this has not been demonstrated.

If viroids were transcribed from preexisting DNA templates, one might expect that viroids propagated in different hosts would have somewhat different primary structures, because the information for this structure would reside primarily with host DNA and not with the infecting viroid. Theoretically, the viroid genome could undergo major alterations upon replication in different hosts. If, however, viroid replication were DNA-independent, that is, if viroids were transcribed from complementary RNA strands produced after infection with the incoming viroid serving as template, such major alterations in the primary structure evidently could not occur. Thus, determination, in different hosts, of the primary structure of progeny viroids should give clues to the mode of viroid replication.

Two different approaches to this problem have yielded results indicating that the primary structure of viroids is faithfully maintained, regardless of the host in which the RNA is propagated.

In one study, PSTV and CEV were each propagated in both tomato and *Gynura*; and the progeny viroids were analyzed by fingerprinting of ribonuclease T_1 digests (DICKSON et al., 1978). The results clearly indicated that the primary sequence of each viroid is maintained, irre-

spective of the host in which it is replicated.

In a second approach, hybridization reactions between DNA complementary to PSTV (cDNA) prepared *in vitro* (OWENS, 1978) and PSTV propagated either in tomato, tobacco, *Gynura*, or chrysanthemum did not disclose any differences in the extent of hybridization, thus indicating that the viroid does not undergo significant sequence alterations if propagated in any of these hosts (OWENS *et al.*, 1978). Evidently, this sequence is determined by the infecting viroid and not by the host.

In summary, indications for both DNA- and RNA-directed viroid replication exist, but the latter possibility appears more likely. It is possible that the incoming viroid is transcribed by host RNA-directed RNA polymerase into a complementary RNA strand, from which, in turn, progeny viroid molecules are transcribed. If so, the demonstrated sensitivity of viroid replication to treatment with actinomycin D and α -amanitine might be explained by a complete dependence of viroid replication on the continued synthesis of a short-lived host RNA. As has been suggested earlier (DIENER, 1979), such a host RNA might most plausibly serve as a primer for RNA-directed viroid replication.

Such a scheme would be analogous to that operative in influenza virus RNA replication. Replication of influenza virus *in vivo* is inhibited by actinomycin D and α -amanitine (BISHOP, 1977), but RNA synthesis *in vitro* is resistant to these inhibitors. Recently, it has been shown (BOULOY *et al.*, 1978) that synthesis of influenza "plus" strands *in vitro* by the virion-associated polymerase requires host mRNA as primer. Because this mRNA is synthesized by DNA-directed RNA polymerase II, synthesis is blocked by low concentrations of α -amanitine.

Despite the plausibility of such a scheme as applied to viroid replication, it must be stressed that present information does not rule out other modes of replication.

POSSIBLE MECHANISMS OF PATHOGENESIS

It is not known how viroids interfere with their host's metabolism to produce the characteristic macroscopic symptoms observed in certain species. The nuclear location and replication of viroids and their apparent inability to act as mRNA's suggest, however, that these symptoms may be caused by interference with gene regulation in the infected host cells.

The PSTV has an extended host range, particularly among solanaceous plant species, but in most host species no damage resulting from viroid replication is discernible (DIENER, 1979). Thus, in these symptomless hosts, viroid-induced metabolic aberrations do not occur, or, if they occur, they must be harmless in the particular genetic

milieu of the host.

With both PSTV (ZAITLIN & HARIHARASUBRAMANIAN, 1972) and CEV (CONEJERO & SEMANCIK, 1977), certain host proteins occur in larger amounts in infected than in healthy tissue. Possibly these aberrations in host protein synthesis are related to the pathogenic properties of viroids.

POSSIBLE ORIGIN OF VIROIDS

The finding of sequences complementary to PSTV in the DNA's from several uninfected host species (HADIDI *et al.*, 1976) suggests that PSTV originated from host genetic material. Among the species tested, several solanaceous species have DNA's with the highest affinity to PSTV; the more phylogenetically distant a plant species is from solanaceous plants, the fewer PSTV-related sequences are generally found in the DNA. This supports the idea that PSTV originated from genes normally present in certain solanaceous plant species.

With the discovery of split genes and RNA splicing (CRICK, 1979), it has become apparent that viroids might have originated by circularization of spliced-out intervening sequences (introns). Although introns are generally regarded as "nonsense" sequences and are believed to be rapidly degraded, one might speculate that if such excised sequences would permit extensive regions of intramolecular basepairing (as do viroids), they might become stabilized and thus escape degradation.

DO VIROIDS EXIST IN ANIMALS?

Although viroids are definitely known to occur only in higher plants, similar agents may exist in other forms of life. It appears reasonable to search for viroids in the many instances in which viral etiology of an infectious disease has been assumed, but in which no causative agent has been identified.

One case in point is a group of animal and human diseases, the subacute spongiform encephalopathies (GAJDUSEK, 1977). On the basis of comparisons of known properties of PSTV with those of the agent of one of these, scrapie, the hypothesis has been advanced that the latter may be a viroid (DIENER, 1972b). Efforts to isolate infectious nucleic acid from brain preparations of scrapie-infected animals were fruitless (MARSH *et al.*, 1974; WARD *et al.*, 1974), but some evidence suggests that disease-specific low-molecular-weight DNA (ADAMS, 1972; HUNTER *et al.*, 1973) occurs in infected tissue. Also, a DNA component essential for the expression of scrapie infectivity has been identified recently (MARSH *et al.*, 1978) and this infectious DNA appears to be of relatively low molecular weight (MALONE *et al.*, 1979).

Although further characterization of this DNA and

confirmation of the findings are required, present results suggest that viroid-like nucleic acids may exist in organisms other than higher plants and that such entities may be responsible for certain infectious diseases of animals as well as of plants.

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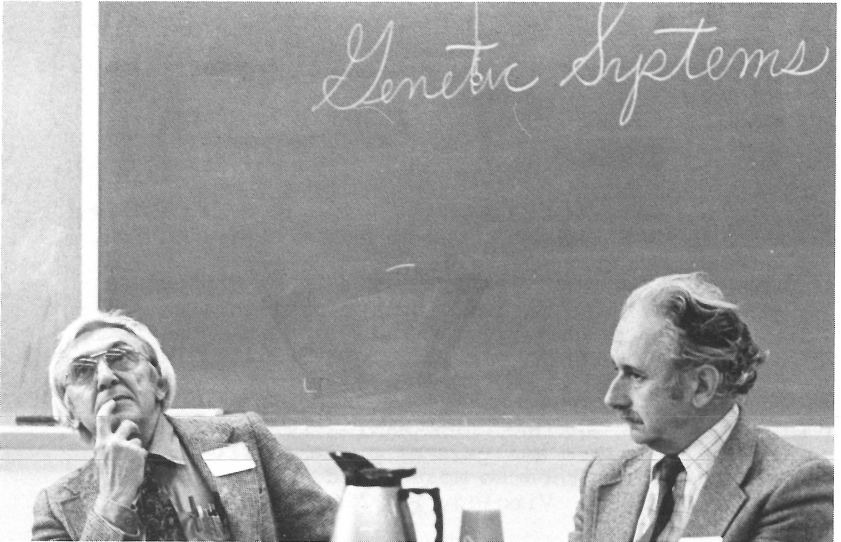
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Drs. Maurice Green and T. O. Diener at the symposium.