VIROIDS:

NATURE'S LITTLEST KILLERS

These naked bits of RNA are the smallest known agents of infectious disease

By GARDINER MORSE

Theodor Diener's healthy looking lipstick vine was not a suspect. But he enlisted the locally purchased ornamental in an experiment at the United States Department of Agriculture's Plant Virology Laboratory in Beltsville, Md., and made "an accidental discovery which caused a little concern to the plant quarantine people here," he says. The vine was infected with viroids.

Viroids are not, as the name suggests, "virus-like." Even Diener, who coined the term in 1971, admits it's a misnomer. "At the time when the viroid concept was advanced, viroids could reasonably be regarded as relatives of conventional viruses," he wrote in the 1982 ANNUAL REVIEW OF MICROBIOLOGY. But recent findings make that notion "increasingly less likely." So what *are* viroids? One of the few things that's certain about them is that they're the smallest infectious agents known, naked RNA molecules smaller and simpler than any virus. But their size belies their virulence—they've killed over 10 million coconut trees in the Philippines and nearly destroyed the United States' chrysanthemum industry in the early 1950s.

These disease-causing molecules, which so far have only been seen in plants, have been called "novel," "improbable" and "selfish." At the very least, they're mysterious: It's not clear, where they came from, how they work, or even if they're alive. (That's "a question of semantics," says Diener.) But in several labs, efforts to figure function from structure may lead to explanations of how viroids cause a range of devastating diseases, and may offer new insights into the workings of life's most basic genetic machinery.

No known living thing lacks nucleic acids, either DNA or RNA, as the storehouse of its genetic information. Usually these genetic instructions are contained, protected by a shell as simple as a virus's protein capsid or as complex as the membranes of a mammalian cell. But viroids, bare strings of nucleotides, are "life" at its most streamlined.

The dozen or so known viroids ("It depends how you count," Diener notes) range in size, but potato spindle-tuber viroid (PSTV), which he co-discovered, is one of the biggest—359 bases long. That's one-tenth the genetic material of even the simplest virus. For comparison, imagine that subunits, or bases, of DNA and RNA are spaced a millimeter apart. If that were the case, PSTV would be just over a foot long. The DNA of the bacterial virus T2 would run the length of two football fields, and the DNA in a human sperm, connected into a single strand, would stretch over 1,800 miles.

With only a few hundred bases, viroids are too small to encode the information needed to construct even a small enzyme, and attempts to use viroids as "messages" in test-tube syntheses of proteins have failed. If they don't encode proteins, how do they wreak such havoc in cells?

Three theories are popular: 1) they commandeer cellular enzymes for their own replication (hence are "selfish RNAs") and thus disrupt the host's metabolism, 2) they mimic normal molecules that control gene regulation and 3) Diener's theory, that they hamper the normal editing, or "splicing," of the cell's messenger RNA, molecules that carry information from DNA to the structures that make protein in the cell's cytoplasm.

"One objection to most of these theories, including my own," Diener admits, "is that they don't account for the fact that in many plant species, viroids can replicate just as efficiently [without causing disease] as in those where they do. The mere replication of a viroid in a host cell does not necessarily cause disturbances." Diener's lipstick vine, which seemed untroubled by the viroid it harbored, is a case in point. That viroid, Diener found, caused a severe disease when he infected potatoes with it.

"It was very puzzling how these diseases often occurred suddenly out

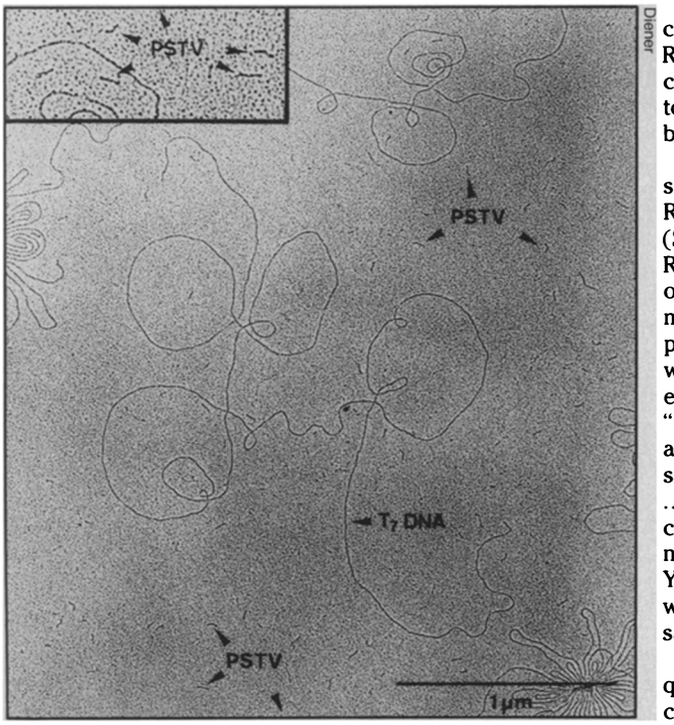
Diener

The tomato plant on the right is stunted by infection with the planta macho viroid. On the left, a healthy control plant.

of nowhere," Diener recalls. But 10 or 15 years ago, he says, Mexican researchers discovered that a viroid which caused disease in local tomato plants was multiplying inconspicuously in at least four species of wild plants nearby. What this and other findings suggest is that viroids evolved in wild plants and only recently have spread into crops where they're pathogenic. "In many cases where you have coevolution of a pathogen and host, you usually have very mild if any symptoms," Diener explains. But when the viroid gets into "a different genetic milieu," such as a cultivated plant, its potential for troublemaking is somehow unleashed, he says. The movement of viroids from wild to cultivated plants "is an area we know absolutely nothing about," says Diener, who is just now starting to study that process.

In recent years, one of the most active lines of viroid research has been sequence studies, the meticulous mapping and comparison of viroid base sequences with each other and other RNAs and DNAs. The assumption is that the viroid's secret lies hidden in the order of its bases. Just as you might take apart a clock to figure how it works, researchers hope that taking apart viroids will explain them. What's been found is tantalizing. What it means is anyone's guess.

The base sequences determined so far are filled with homologies — base orders that are identical or complementary — both within individual viroids and between different "species." (RNA's four bases, A,G,C and U, like DNA's, pair with each other in specific ways: A always pairs with U, and G always pairs with C, and sometimes with U as well.) Each viroid's base sequence allows the linear, single-stranded molecule to fold over on itself and form a double-stranded "hairpin" or rod. No one knows why viroids form hairpins, but one idea is that this rod shape protects them from attack by enzymes in the cells they infect.



The long spaghetti-like strand is DNA from the bacterial virus T7. The tiny threads that look like they're from lint on the negative are actually complete PSTV viroids.

Another structural curiosity is "boxes" of bases in precise order that don't vary from viroid to viroid and which researchers are now finding in RNAs that occur naturally in a variety of cell types. These "central conserved regions" presumably serve some function. When sequences remain unchanged through time, it's likely the order is kept, or conserved, for a reason (SN: 7/14/84, p. 21).

It may be that viroids need those bases for reproduction. "When you think about the kinds of forces that would be constraining the molecule, and preventing it from mutating, replication is certainly the function that comes to mind," says Andrea D. Branch of Rockefeller University in New York, who studies how viroids reproduce.

If these boxes of bases do figure into the viroid's replication scheme, what are similar boxes doing in the RNAs that are normal constituents of many cells? Gail Dinter-Gottlieb at the University of Colorado in Boulder, for instance, has recently discovered a 19-base stretch in an RNA of Tetrahymena, a single-celled protozoan, that's identical to part of the viroid's conserved base sequences.

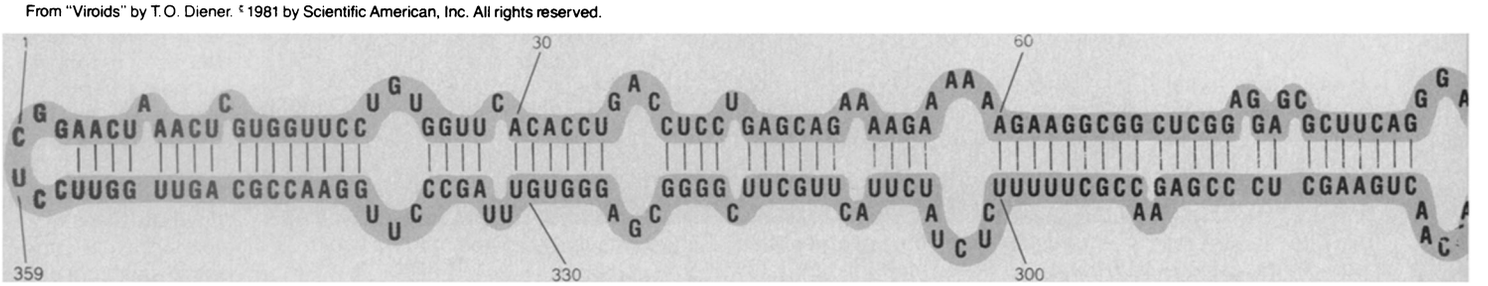
What makes the similarity striking is that Dinter-Gottlieb's RNA is a self-splicing "intron" (SN: 11/27/82, p. 342), a piece of RNA that is removed, or "spliced out" of a longer RNA strand as it matures. This, it turns out, supports a theory Diener proposed when RNA splicing was discovered in 1976 — that viroids are "escaped introns" that have adopted an independent lifestyle. "When I sent him this work ... showing that there are these conserved sequences, he wrote a nice letter back saying, 'Ah ha! You're supporting my theory,' which it does," Dinter-Gottlieb says.

Similarities between base sequences don't prove that one nucleic acid evolved from another, however. "Homologies have been found between viroids and so many RNAs and there aren't any good rules for figuring out what it means," says Branch. But she adds that "you might want to look for some necessary connection," such as related function, in RNAs that share sequences.

Perhaps viroids and introns "tap into the same RNA replication machinery," Branch speculates. Plants do have such machinery, enzymes called endogenous RNA replicases, and viroids may appropriate these enzymes for their own reproduction. While such machinery would obviously benefit viroids, what it accomplishes for plants is a mystery. But that these enzymes exist implies that plants "have RNAs that are their [the enzyme's] natural template, and it's quite easy to imagine that viroids are their *unnatural* templates," Branch says. Thus viroids may be telling us that they are part of "a molecular species that's more widespread," Branch suggests — a species whose members could include introns and as yet undiscovered RNAs that are important in functions of healthy cells.

Animals are one place to look for viroids and viroid-like (viroidoid?) nucleic acids, but despite an enormous amount of interest and effort, no one has been able to isolate an animal viroid. Nonetheless, viroids, or something like them, may cause a num-

The potato spindle-tuber viroid is 359 bases long. Its four bases, adenine (A), uracil (U), guanine (G) and cytosine (C), are arranged in such a way that extensive base-pairing can occur and the molecule folds over on itself to form a "hairpin" structure that may protect it from being degraded in the cells it infects. This model assumes maximum base pairing.



DNA screens to keep viroids out of plant propagation programs

Prevention of plant diseases is particularly crucial because, in general, plants do not recover from an infection. One important strategy for avoiding disease is the use of pathogen-free material for commercial plant propagation. But that requires effective screening tests. A method for diagnosing the presence of at least one viroid has been developed.

The viroid under investigation causes potato spindle-tuber disease. Some symptoms of the disease are stunted growth, curled leaves and elongated, spindle-shaped potatoes. The disease causes a loss of about $3.5 million worth of potatoes in the United States each year. And its toll is expected to increase as potato growing is extended into the tropics. "Once the viroid is in a crop or breeding program, it is difficult to get out," says Robert Owens of the Beltsville (Md.) Agricultural Research Center.

Two methods are currently in use for detecting viroid infection. One is to inoculate tomatoes with a potato extract and after two to six weeks look for stunted growth. Besides the slowness of this technique, it is poor at picking up mild forms of the disease. The other technique in use is faster — it can be done in a day — but more difficult to perform and less sensitive. The viroid shows up in gel electrophoresis as a characteristic band in DNA taken from plants and distributed by size and charge. But it is difficult to extract plant DNA to use in this test, Owens says.

Owens and colleagues have developed a new method for detecting the potato spindle-tuber viroid (PSTV) that is approximately a thousand times more sensitive than the electrophoresis method, says Owens. The test uses a radioactive DNA probe and takes two to three days to obtain results. Its sensitivity may make the difference between a pathogen-free and an infected line of potatoes being used for propagation.

A DNA probe is a string of nucleotides that are complementary to, and therefore bind to, the genetic material being sought. The binding depends on the same forces that hold together the strands of double-stranded DNA. The probe for PSTV is a DNA strand about 360 nucleotides long, complementary to the entire length of the viroid. To allow detection, it is labeled with radioactivity.

In a recent screening of different potatoes being used to develop new varieties by the International Potato Center in Lima, Peru, gel electrophoresis identified the viroid in only one of 10 samples. In contrast, with the DNA probe technique, Owens found that at least seven samples were infected with the viroid. Infection was confirmed with the tomato test. The DNA probe technique can detect strains of PSTV that cause mild, as well as severe, disease. These viroids differ by a few nucleotides.

The DNA probe can detect a few picograms of viroid RNA. Owens says that although some non-PSTV viroids share 70 to 80 percent of their nucleotide sequence with that of the PSTV, the PSTV probe binds almost exclusively to the PSTV RNA.

A "red flag" or broad spectrum test for viroids would be useful, but in most cases so far examined a probe detects only one specific viroid. Owens has developed probes to several viroids besides PSTV. He is now trying to find a probe that will bind to many or all viroids, and has found a stretch of 19 nucleotides that is similar in many viroids examined. A probe complementary to this stretch binds to at least five of the viroids related to PSTV, he reports.

In the fight against crop disease, Owens expects the utility of DNA probes to extend beyond the viroids. In a variety of laboratories, work has already begun on probes for plant viruses. Probes may eventually be also developed for mycoplasmas, tiny single-celled intracellular parasites. —*Julie Ann Miller*

ber of animal diseases, among them one type of arthritis and certain encephalopathies (brain diseases). Joseph Coggin Jr. at the University of South Alabama in Mobile and his colleagues report "epidemics" of malignant lymphoma in hamster colonies (and it now appears the infectious disease strikes mice as well) that seem to be caused by something that "behaves like a mammalian viroid," Coggin says. The agent seems to be a naked DNA, not RNA, but Coggin is quick to point out that "viroids don't have to be RNA just because the plant ones are."

To pacify those who think that true viroids must be made of RNA, Coggin says his group "now uses the term 'sub-viral' [instead of 'viroid'] to keep people from getting upset." Diener maintains, "I still haven't seen convincing evidence" of an animal viroid, but encourages searches for viroids in animals that appear to have viral disease but where no virus has been found.

While the search continues for new viroids, and molecular biologists have their hands full trying to learn how the known ones work, much of what is known about these weird pathogens is already being put to use.

Robert Owens, one of Diener's colleagues at the Agricultural Research Center in Beltsville, has developed a sensitive probe to screen plants for viroid infection. The probe should help check the spread of some viroid-caused diseases (see box).

In Israel, citrus trees are intentionally infected with a mild viroid strain to protect them from the severe form — something like inoculating a child with a weakened virus to ward off the virulent strain. Diener suggests "there's no reason why you couldn't attenuate viroids artificially" in the lab for use in "cross protection" of crops. Concern that these mild viroids might mutate into more dangerous forms, or threaten other crops, has inhibited testing in the United States, he says.

And in one counterintuitive scheme, growers actually infect plants with pathogenic viroids to deform them, Branch says. The citrus exocortis viroid stunts citrus trees, and this can increase their yield while decreasing their water requirement. "So in some arid regions, they might be more well adapted," she says.

Diener speculates that DNA copies of viroids could be put to work as vehicles to carry foreign genes into plants. Viruses are already used for gene transfers (SN: 4/28/84, p. 264).

Not all viroid research offers immediate practical applications, but that shouldn't discourage it, says Branch. "To me, basic science studies of all kinds have certain features in common ... [and when] you have a particularly focused and dedicated band of people trying to get a more highly developed technology so they can push through their scientific experiment," practical applications result "from just doing new things." □